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An Invitation to T and More: Notch Signaling in Lymphopoiesis

Review

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Cell fate decisions in metazoans are regulated by Notch signals. During lymphoid development, Notch influences a series of cell fate decisions involving multipotent progenitors. This review focuses on current views and lingering uncertainties about Notch function in lymphoid cells.

Notch genes encode highly conserved cell surface receptors that regulate the development of a remarkably wide spectrum of cell types in metazoans ranging from sea urchins to humans. Notch signals influence multiple processes that govern normal morphogenesis, including lineage specification among bipotent progenitor cells, programmed cell death, cellular proliferation, and border formation. The varied and far-reaching consequences of Notch signaling are also reflected by the diverse phenotypes caused by mutations in Notch loci, ranging from the notching of wings in *Drosophila* (Mohr, 1919) to the malignant transformation of human T cells (Ellisen et al., 1991). Here, we will discuss how dissection of Notch signaling pathways at the cellular and molecular level has provided a framework to address several long-standing questions in lymphocyte development. First, we will review key studies that have provided insight into how Notch signals are produced and regulated. We will then discuss the role of Notch signals in control of mammalian hematopoiesis, focusing on recent evidence supporting a pivotal role for Notch in early B/T lymphocyte lineage determination. Finally, we will summarize studies investigating the impact of Notch signaling during later stages of T cell development, as well as the role of Notch in lymphoid transformation.

Outcomes of Notch Signaling during Invertebrate Development

Genetic studies in invertebrates have identified several highly conserved elements of the Notch signaling pathway (Table 1). These include the Delta/Serrate/LAG-2 (DSL) family of Notch ligands, transmembrane proteins that initiate Notch signaling through cell-cell interactions; CSL, a transcription factor termed CBF1/RBP-Jκ in mammals, Suppressor of Hairless [Su(H)] in *Drosophila*, and LAG-1 in *C. elegans*, which functions as a major target of Notch-mediated signal transduction (reviewed by Artavanis-Tsakonas et al., 1999); factors that initiate or regulate proteolytic processing of Notch receptors, such as presenilins (reviewed by Kopan and Goate, 2000); and regulatory factors, such as the extracellular protein Fringe and the intracellular proteins Deltex and Numb (reviewed by Greenwald, 1998).

A general theme to emerge from studies in *D. melanogaster* and *C. elegans* is that Notch signaling specifies the developmental fates of progeny derived from bipotential precursor cells (reviewed in Simpson, 1998). Interactions between cells expressing Notch receptors and ligands influence lineage specification through two general mechanisms, lateral inhibition and induction. Inductive signaling occurs when two nonequivalent cell types expressing Notch ligand or receptor, respectively, interact. In this circumstance, access of receptor-expressing cells to the appropriate ligand(s) determines lineage specification. In contrast, lateral inhibition operates within clusters of equivalent precursors that express both Notch ligand and receptor, and depends on the existence of a negative feedback loop whereby Notch signals downregulate ligand expression. Due to stochastic variation or position effects, Notch signaling is lowered within one cell, which then expresses more ligand, raising Notch signaling in neighboring cells. This reinforces and amplifies differences in Notch signaling and leads to two classes of cells (Notch high and Notch low) that adopt distinct fates. A classic example of lateral inhibition is peripheral neurogenesis in *Drosophila*, during which a cluster of proneural cells yields a single sensory organ precursor and several epidermal cells. In the absence of Notch signals, all cells adopt the "default" sensory organ precursor cell fate (a neurogenic phenotype), whereas transgenic expression of constitutively active Notch causes cells to adopt the alternative epidermal cell fate.

Notch activity can also influence developmental outcomes without directly affecting lineage choice decisions through varied effects on terminal differentiation, proliferation, or apoptosis. For example, Notch cooperates with Wingless in the developing fly wing to induce cell cycle arrest (Johnston and Edgar, 1998), whereas in *C. elegans*, enforced Notch signaling leads to the massive overgrowth of germ cell precursors by inhibiting cell cycle arrest (Berry et al., 1997). Similarly, while Notch activity regulates patterning of the developing *Drosophila* retina by inducing the death and removal of superfluous cells (Miller and Cagan, 1998), it can also promote the survival of hematopoietic cell lines following exposure to apoptotic stimuli (reviewed in Osborne and Miele, 1999; Deftos and Bevan, 2000). These remarkably pleiotropic effects indicate that the outcome of Notch activity is not stereotyped, but highly dependent upon signal strength, timing, and developmental context. Indeed, it appears that physiologic Notch signaling is precisely

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Table 1. Conservation of Notch Signaling

Component	*Drosophila*	Mammals
Receptors	Notch	Notch 1–4
Ligands	Delta	Delta-like 1,3,4
	Serrate	Jagged 1,2
Downstream Transcription Factors	Su(H)	CSL (RBP-Jκ)
Modulators	Fringe	Lunatic, Radical, & Manic Fringe
	Numb	Numb, Numb-like
	Deltex	Deltex 1–3
	Mastermind	Mastermind-like 1–3
Target Genes	Hairy/En(spl)	Hes 1,5
		HeyL
Processing Molecules	Presenilin	Presenilin 1,2
	Metallo-protease	Metallo-protease
	Furin-like protease	Furin-like protease

controlled at multiple levels and can be exploited by other signaling pathways.

Modes and Consequences of Notch Signaling

Notch receptors are large single pass transmembrane proteins composed of a series of well-defined structural motifs (Figure 1). During transit to the cell surface, Notch proteins are cleaved within the trans-Golgi network by a furin-like convertase, giving rise to mature heterodimeric receptors comprised of noncovalently associated extracellular (ECN) and transmembrane (NTM) subunits. The extracellular subunits contain 11–36 tandem epidermal growth factor (EGF)-like repeats that bind ligand, and three Notch-specific LIN12/Notch repeats that are needed to prevent inappropriate Notch receptor activation prior to ligand binding. The intracellular region contains a RAM domain that binds CSL, a series of iterated cdc10/ankyrin repeats that participate in protein-protein interactions with CSL and other polypeptides (described in more detailed below), and a C-terminal PEST sequence. There are also less highly conserved sequences lying between the ankyrin repeats and the PEST sequences that include, in some receptors such as mammalian Notch1, a strong transcriptional activation domain.

Although it is extremely difficult to detect Notch in the nuclei of normal cells, multiple lines of investigation have converged on a model in which the intracellular domain of Notch (ICN) translocates to the nucleus in a ligand-dependent fashion (reviewed in Kopan and Goate, 2000) (Figure 2). Nuclear access of ICN appears to require two successive proteolytic cleavages within the NTM subunit. The protease responsible for the first cleavage, which occurs just external to the transmembrane domain, is likely mediated by an ADAM metalloprotease. Although TNFα converting enzyme (TACE) has the capacity to cleave NTM at this site, the identity of the physiological protease awaits verification. Subsequently, a second cleavage within the transmembrane domain liberates ICN from its membrane tether. This cleavage requires the function of two different classes of transmembrane proteins, presenilins and nicastrin (Yu et al., 2000), two components of a multisubunit complex that processes a number of transmembrane proteins in addition to Notch. Significantly, mutations in Notch1 that render it resistant to presenilin-dependent cleavage, as well as drugs that inhibit γ-secretase, both cause Notch loss-of-function phenotypes.

Once in the nucleus, ICN interacts with nuclear factors that regulate transcription. Genetic studies conducted in invertebrates have identified CSL homologs, a structurally unique class of sequence-specific DNA-binding transcription factors, as the major downstream mediators of Notch signals (reviewed in Bray and Furriols, 2001). In the absence of ICN, CSL acts as a transcriptional repressor due to its ability to bind transcriptional corepressor complexes. Binding of ICN displaces corepressor complexes, thereby derepressing transcription from promoters with CSL binding elements. In addition, the ankyrin repeats and C-terminal transcriptional activation domains of ICN recruit several different transcriptional coactivators, providing an additional stimulus for

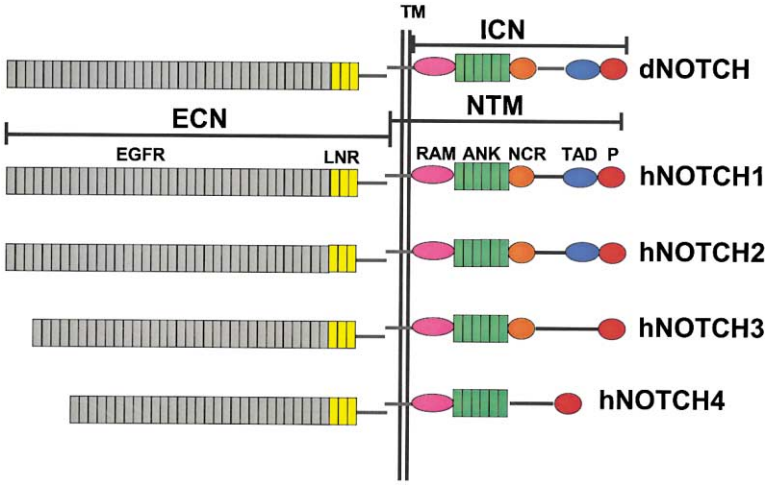

Figure 1. Notch Receptors in Flies and Humans

Diagrammatic representations of the single *Drosophila* (dNOTCH) and 4 known human Notch receptors (hNotch). The full-length proteins are expressed on the cell surface as heterodimers composed of noncovalently associated extracellular (ECN) and transmembrane subunits (CTM). All Notch receptors contain epidermal growth factor-like repeats (EGFR), Lin12 Notch repeats (LNR), a RAM23 domain (RAM), Ankyrin repeats (ANK), and PEST (P) sequences. The highest degree of homology between Notch receptors is in the ankyrin repeats, whereas the C-terminal sequences show the greatest degree of divergence. Human Notches1–3 contain sequences immediately C-terminal of the ankyrin repeats (NCR) that regulate functional activity. Further C-terminally, hNotch1 and hNotch2 contain strong and weak, respectively, C-terminal transcriptional activation domains (TAD); a similar domain is also present in dNOTCH.

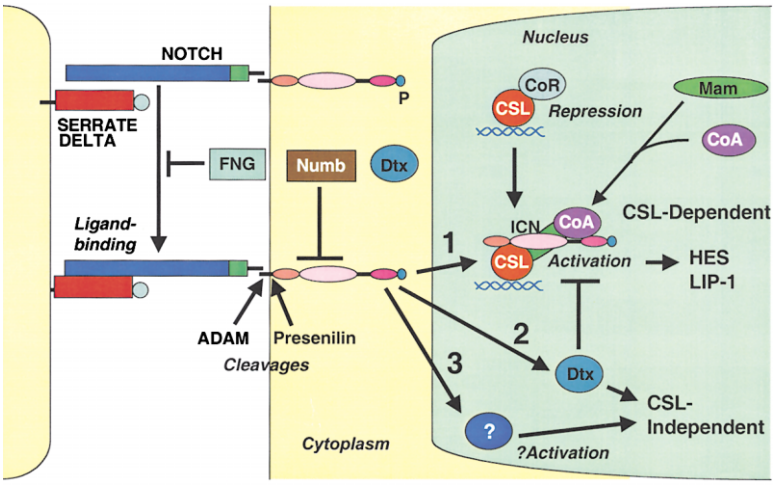

Figure 2. Ligand-Induced Notch Signaling Pathways

Binding of Notch receptors to ligands of the Serrate and Delta families result in successive cleavages, first in the extracellular domain by ADAM-type proteases, and then in the transmembrane domain by presenilin-dependent proteases, which release ICN and permit its translocation to the nucleus. The ability of Serrate-like ligands to activate Notch is antagonized by Fringe (FNG) glycosylases, which modify Notch extracellular domains. In the nucleus, ICN activates target gene expression by binding the transcription factor CSL, displacing corepressors (CoR), and recruiting coactivators (CoA), including mastermind (MAM) ("1"). Poorly characterized CSL-independent pathways also exist that may proceed through Dtx ("2") or unknown factors ("3"). Notch signals are negatively regulated by the cytoplasmic protein Numb, and may be positively or negatively regulated by deltex (Dtx) proteins.

transcription. One functionally conserved protein that may act as a Notch-specific coactivator is mastermind, an adaptor molecule that stabilizes CSL/ICN interaction and potentiates ICN stimulation of transcription from CSL-sensitive promoters. The more variable sequences C-terminal of the ankyrin repeats of Notch1 have been shown to interact with the general transcriptional coactivators p300, PCAF, and GCN5. The complexity of these interactions with positive and negative transcriptional regulators suggests that the strength of CSL-mediated signals is subject to precise tuning (Figure 3).

Targets of ICN/CSL signals in *Drosophila* include the genes *wingless* and *vestigial*, both of which are involved in wing morphogenesis. Less is known about the downstream genes in mammals, but genes of the Hairy/Enhancer of Split (HES) family, which encode bHLH-type transcription factors, constitute one conserved target. HES proteins have C-terminal WPRW sequences that recruit transcriptional corepressors of the groucho family, and thereby downregulate transcription (reviewed by Fisher and Caudy, 1998). One important target of transcriptional repression by HES proteins in the fly is the Delta locus, which completes the negative feedback loop underlying lateral inhibition. Other ICN/CSL targets that may explain the effects of Notch on cell cycle kinetics in certain contexts are cyclin D1 and p21. Upregulated expression of cyclin D1 promotes G1 progression, and has been proposed to contribute to the transformation of BHK cells (Ronchini and Capobianco, 2001). In contrast, upregulation of p21 may promote the cell cycle exit of keratinocytes during differentiation (Rangarajan et al., 2001). These opposing effects further illustrate the diverse context-specific responses induced by Notch signaling.

A variety of studies have suggested the existence of Notch signals that are independent of CSL; however, the mechanisms are largely unknown. For instance, the Notch-dependent patterning of the dorsal epidermis in the embryonic fly does not require CSL, and instead acts through a c-Jun N-terminal kinase (JNK) pathway (Zecchini et al., 1999). Likewise, unprocessed monomeric Notch receptors may, in some circumstances, be expressed on the cell surface, and following ligand binding, these receptors produce CSL-independent, but not CSL-dependent, signals (Bush et al., 2001). Further complexity was recently demonstrated in studies of

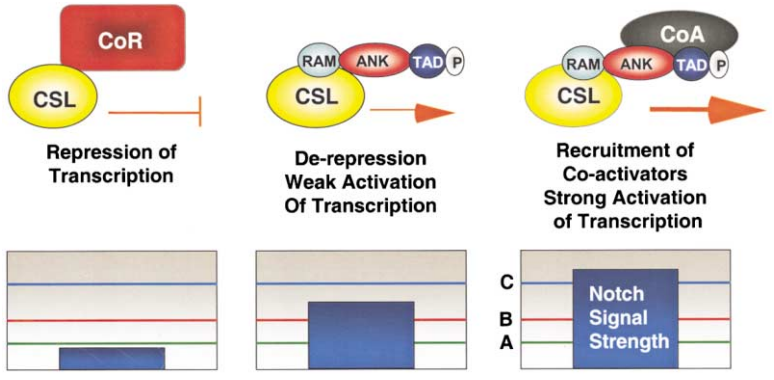

Figure 3. Molecular Basis for Differences in Notch Signal Strength

Model for graded activation of CSL by ICN through step-wise displacement of corepressors (CoR) and recruitment of coactivators (CoA). As shown, the overall "intensity" of Notch signaling is finely regulated. At its lowest level (indicated by the green line as threshold "A"), Notch activity would be insufficient to displace corepressors from CSL. In contrast, Notch signals rising to threshold "B" (red line) would lead to derepression and relatively weak transcriptional activity of target promoters. Recruitment of additional coactivators of transcription produces strong transcriptional activation that surpasses threshold "C." Some outcomes of Notch signaling may only require threshold "B" intensity, whereas others may require threshold "C."

mechanoreceptor development in *Drosophila*, where it was found that activation of CSL (and targets such as HES genes) can occur in a Notch-independent fashion (Barolo et al., 2000). Thus, while Notch and CSL most often cooperate, both can also function independently of one another.

Extracellular and Intracellular Regulators of Notch Activity in Invertebrates

Multiple steps along the central ligand-receptor-CSL signaling axis are subject to regulation by accessory proteins. Notch ligands are regulated by at least two different mechanisms. *Drosophila neuralized* encodes an E3 ubiquitin ligase that is required for receipt of Notch signals in developing peripheral neurons and sensory organs (reviewed in Kramer, 2001). It appears that *neuralized* targets Delta for degradation, and that this paradoxically enhances Notch activation. Delta can inhibit Notch when they are expressed in the same cell, suggesting that *neuralized*-dependent degradation of Delta causes a cell-autonomous increase in Notch activation. A second possibility is that *neuralized*-dependent Delta degradation is required for efficient Notch receptor endocytosis, which may be a prerequisite for signaling (reviewed in Kramer, 2001). The Fringes are a group of polypeptides that modify the responsiveness of Notch to different ligands. For example, *Drosophila* Fringe renders Notch receptors resistant to activation by Serrate, but not Delta (Panin et al., 1997). Biochemical studies indicate that Fringe is an acetyl-glucosaminyl transferase that adds O-linked fucose residues to the EGF-like repeats of the extracellular portion of Notch (Moloney et al., 2000). How this alters responsiveness to specific ligands is unknown, but the net effect may be to restrict Notch signaling to particular microenvironments.

Two distinct types of cytoplasmic Notch regulators have been described, Numb and SEL-5. Through incompletely understood mechanisms, Numb suppresses Notch signals, and may thereby influence cell fate choices. In one well-characterized instance, Numb protein is differentially distributed to one of two progeny of sensory organ precursors which adopt different fates (reviewed in Greenwald, 1998). An additional level of control in vertebrates may be provided by regulated degradation of Numb by the RING domain protein "ligand of Numb protein X" (LNXp80) (Nie et al., 2002). In contrast, SEL-5 encodes a serine/threonine kinase in *C. elegans* that functions before or during ligand-dependent release of ICN to facilitate Notch signaling in a tissue-specific manner (Fares and Greenwald, 1999). Additional modifiers of Notch activity that may function (at least in part) in the nucleus include SEL-10, Supressor of Deltex (Su(Dx)), and Deltex. SEL-10 and Su(Dx) both appear to be negative regulators that promote the degradation of Notch signaling components (Hubbard et al., 1997; Wu et al., 2001). The role of Deltex is less clear. Although initially identified as a positive regulator of Notch signaling in *Drosophila* (Matsuno et al., 1995), recent work in mammalian cells suggests that enforced Deltex expression can antagonize Notch signaling, possibly by competing with ICN for transcriptional coactivators (Sestan et al., 1999; Izon et al., 2002).

Notch-Mediated Signal Transduction in Mammals

The Notch signaling pathway is extraordinarily well conserved between invertebrates and mammals (Table 1), although many Notch signaling components have undergone several duplications during evolution. For instance, four Notch genes (Notch 1–4) and at least five Notch ligands (Delta-like1, 3, and 4, and Jagged 1 and 2) have been identified in mice and humans. While the selective pressures driving these duplications are not readily apparent, it is tempting to speculate that variation among Notch receptors permits more precise regulation of signal strength at key stages of development. In this regard, Notch1, the family member most closely related to *Drosophila* Notch, is unique among the mammalian Notch receptors in having a strong transcriptional activation domain (TAD) in its C terminus (Kurooka and Honjo, 2000). This domain likely accounts for the high basal transcriptional activation potential of ICN1 relative to ICN2-4, and contributes to some activities of Notch1 (Aster and Pear, 2001). Interestingly, all four mammalian Notch receptors bind and activate CSL (Mizutani et al., 2001), which is the one nonredundant member of the mammalian Notch signaling pathway thus far identified. In competing with one another for CSL, combinations of various ICNs may dampen or amplify overall Notch signaling intensity. Thus, the effect of mammalian CSL-dependent Notch signaling on the trancriptosome may vary depending on (1) the accessibility of CSL to specific promoters, (2) the level of various ICNs, corepressors, and coactivators, and (3) the level of signaling in other pathways that interact with Notch. The relative ability of various ICNs to signal through CSL-independent pathways is unknown.

Notch Regulation of Early Hematopoiesis in Mice and Humans

Pluripotent hematopoietic stem cells (HSCs) sit at the apex of a hierarchy of progenitors that become increasingly restricted to specific differentiation programs. The general ability of Notch activity to regulate lineage choice in other contexts suggests that Notch signaling may also function to guide HSCs toward particular lineages. However, most data to date suggest that Notch activity in HSCs promotes self-renewal rather than lineage specification (reviewed in Milner and Bigas, 1999). For instance, exposure of mouse (Varnum-Finney et al., 1998) and human (Karanu et al., 2000) HSCs to Notch ligands induces their self-renewal and expansion without markedly altering their differentiative potential. Furthermore, Bernstein and colleagues found that transduction of isolated HSCs with ICN1 resulted in the outgrowth of immortalized, cytokine-dependent cell lines with the capacity to yield lymphoid and myeloid lineage cells (Varnum-Finney et al., 2000). Unlike long-term HSCs, however, these cells did not rescue radiation chimeras, suggesting that ICN1 activity had immortalized a late-stage HSC. Normally, HSCs express higher levels of Notch2 than Notch1 (Varnum-Finney et al., 1998), raising the possibility that individual Notch family members might have distinct roles in HSC self-renewal and differentiation. The effect of deficiencies in various Notch receptors on HSCs has not yet been assessed.

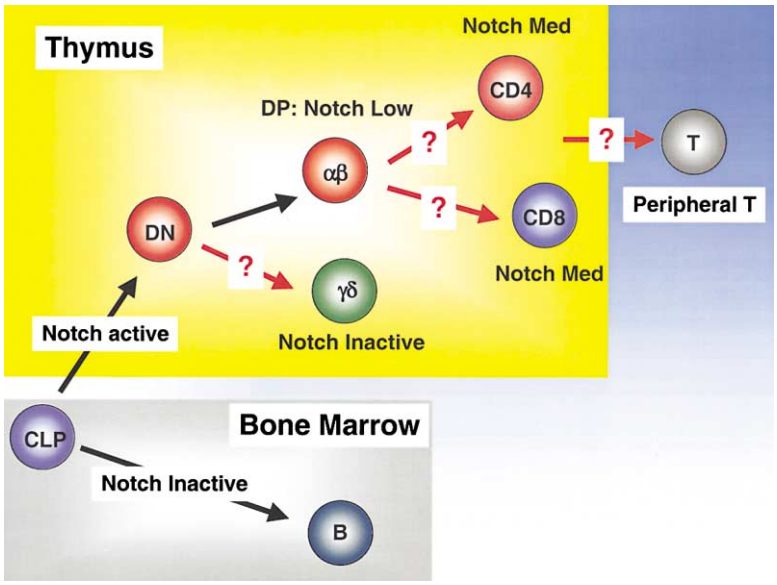

Figure 4. Impact of Notch Activity on Lymphopoiesis

Notch signaling functions in multiple cell fate decisions during lymphocyte development. Cell fate decisions for which there is strong evidence for Notch involvement are indicated by "black" arrows. "Red" arrows indicate potential functions for Notch signaling that require confirmatory experiments. Notch "low" and Notch "med" (medium) refer to the relative levels of Notch signaling at these stages of thymocyte differentiation. The highest levels of Notch signaling are observed during the $CD4^-$ $CD8^-$ stages of T cell development.

Notch Regulation of Early B/T Lineage Specification

One long-standing question in lymphocyte development is how equipotential progenitor cells become committed to different cell fates. Prior studies indicate that HSCs give rise to common lymphoid progenitors (CLPs) in the bone marrow and adult thymus (reviewed in Kondo et al., 2001). The first evidence that Notch activity regulates B/T lineage specification came from studies of mice containing conditional Notch1 knockout alleles. These mice are characterized by a very early arrest in T cell development and increased numbers of intrathymic B cells (Radtke et al., 1999) that appear to derive from thymic progenitors (Wilson et al., 2001). These experiments were complemented by adoptive transfer studies in which ICN1 expression in bone marrow progenitors promoted the thymic-independent development of immature T cells (Pui et al., 1999; Allman et al., 2001) and a block in early B cell ontogeny (Pui et al., 1999). Together, these findings suggest that Notch-derived signals modulate B/T lineage determination by targeting early lymphoid-restricted progenitors, and support a scenario in which Notch1 activity is necessary and sufficient to cause T lineage commitment in early lymphoid progenitors. It follows that the thymus provides a unique environment for the generation of Notch signals with the capacity to induce early T cell differentiation (Figure 4).

A unique role for Notch1 in regulation of T cell development was foreshadowed by earlier observations showing that, of all cell types and tissues analyzed, Notch1 is most highly expressed in immature $CD4^-$ $CD8^-$ thymocytes (Ellisen et al., 1991; Hasserjian et al., 1996). Other work has shown that the strong C-terminal transcriptional activation domain of Notch1 contributes to its ability to induce extrathymic T cell development (Aster et al., 2000). These observations suggest that T cell commitment within the thymus requires the upregulation of Notch1 expression in progenitors within a specific microenvironment that permits the receipt of strong signals. At present, the critical signals that upregulate Notch1 expression are unknown. A recent report suggested that Delta-expressing S17 stromal cells, but not their Jagged-expressing counterparts, were able to induce T cell commitment in human hematopoietic progenitors (Jaleco et al., 2001). However, Jagged ligands are more highly expressed in the thymus, whereas Delta is more highly expressed in the bone marrow and spleen (Bash et al., 1999). Deciphering the physiologic signals that result in T cell commitment will require a better description of the microenvironment in which commitment occurs, as well as a more complete characterization of the expression pattern and activation state of Notch signaling molecules in cells undergoing commitment.

A major gap in current knowledge concerns the precise cellular and molecular mechanisms that govern Notch-mediated B/T lineage determination. At the cellular level, the impact of Notch signaling on specific lymphoid progenitors, including CLPs, has not been addressed directly. However, T lineage commitment appears to occur after lymphoid-restricted progenitors enter the thymus (Koch et al., 2001; Wilson et al., 2001; Izon et al., 2002), suggesting that deregulated Notch1 activity does not promote T cell development by expanding or inducing the differentiation of preexisting T-lineage-committed progenitors in the bone marrow. Further, Notch signaling does not merely promote the survival of early T cell progenitors, since enforced expression of antiapoptotic proteins, such as Bcl-2, in bone marrow progenitors does not appreciably perturb hematopoiesis or lymphoid differentiation (Innes et al., 1999). Thus, although Notch signaling may promote survival at later stages of T cell development (Deftos et al., 1998; Jehn et al., 1999), the existing evidence is most compatible with an inductive role for Notch1 signaling in promoting early T lineage development.

How then is Notch signaling (and thus T cell differentiation) minimized among lymphoid progenitors in the bone marrow? One possibility is that signaling is silenced in marrow microniches harboring CLPs. This

could be achieved by downregulation of Notch receptors, sequestration of CLPs away from ligand-expressing cells, or upregulation of factors that inhibit signaling pre- (e.g., fringe) or post- (e.g., numb) receptor cleavage. Studies on the mechanisms governing T/B lineage determination in the thymus have provided some indirect support for the latter idea. For instance, enforced expression of Lunatic Fringe, a mammalian homolog of *Drosophila* Fringe, abrogates early T cell development while promoting thymic B cell development (Koch et al., 2001). Fringe could restrict Notch signaling in bone marrow CLPs similarly. We also recently noted that enforced expression of Deltex1 suppresses T cell development and promotes thymic B cell development, possibly by hampering the ability of Notch1 to recruit coactivators (Izon et al., 2002). This result suggests that Deltex1 might promote the differentiation of bone marrow CLPs into B cells by dampening Notch signaling.

A better appreciation of the mechanisms governing Notch-mediated T/B lineage determination will also be gained through the further identification of early lymphoid-specific target genes. Proposed target genes associated with early T cell development include the bHLH gene HES-1, the IL-2R component CD25, and pre-Tα (Deftos et al., 2000). Interestingly, while HES-1-deficient mice were originally reported to exhibit an early arrest in thymocyte development (Tomita et al., 1999), Takahama and colleagues reported hypocellularity without alterations in relative frequencies of thymocyte subpopulations in HES-1$^{-/-}$ fetal thymi (Kaneta et al., 2000). Thus, HES-1 deficiency does not phenocopy Notch1 deficiency, suggesting that it may act in combination with other Notch1-dependent transcriptional targets. A recent report supports pre-Tα as a direct Notch target (Reizis and Leder, 2002). Murine and human pre-Tα promoters contain at least one CSL binding site and are activated by Notch in tissue culture and in vivo. In thymocytes, Notch upregulation pre-Tα expression occurs during the DN2 stage, providing further support for the hypothesis that Notch signals at this specific stage enforce T cell commitment.

An understanding of the basis for Notch-mediated regulation of B/T lineage specification will also require an examination of how Notch signaling inhibits early B cell development. Notch signaling interferes with the activity of E2A (Pui et al., 1999), a bHLH transcription factor that is required for early B cell ontogeny. Intriguingly, E2A knockout mice share several phenotypes with mice that express activated Notch constructs, including a B cell deficiency, altered CD4:CD8 ratios, and the tendency to develop T cell leukemia. However, E2A deficiency also inhibits early T cell development (Bain et al., 1997), indicating that the effects of Notch on lymphoid commitment are unlikely to be accounted for purely by inhibition of E2A. Additionally, Notch signaling may prevent B cell development by inducing apoptosis and/or cell cycle arrest in early B cell progenitors. Indeed, constitutive Notch signaling induced apoptosis and a G1 cell cycle arrest in a chicken B cell line (Morimura et al., 2000). Thus, it will be interesting to determine whether Notch-induced B cell deficiency is rescued by enforced expression of genes that increase the survival of early B cell precursors.

Although Notch activity is incompatible with early B cell development, Notch signaling may nonetheless act to modulate later stages of B cell development and/or function. Supporting this possibility, Notch receptors are expressed at multiple stages of B cell development (Bertrand et al., 2000) and Notch ligands are expressed in the avian bursa (Morimura et al., 2001) and mammalian marrow and spleen (reviewed in Anderson et al., 2001a). Exposure of cocultivated B cells to cells expressing the ligand Jagged-1 leads to activation of CSL target genes, such as CD23 (Bash et al., 1999). However, enforced expression of ICN1 causes the death or growth arrest of avian B cells, and the downregulation of immunoglobulin heavy chain gene transcription in both avian and murine cells (Strobl et al., 2000; Morimura et al., 2001). In a parallel story that also suggests the importance of regulating Notch signals in B cells, multiple polypeptides encoded by the genome of the Epstein-Barr virus (EBV) interact with Notch signaling pathway components (reviewed in Zimber-Strobl and Strobl, 2001). EBNA2, which is essential for B cell transformation by EBV, binds and activates CSL, although more weakly than ICN1. Three other EBV proteins, EBNA3A, EBNA 3C, and RPMS, also bind CSL, but inhibit rather than activate Notch signaling. The net effect of these interactions on signaling is not clear, but the positive and negative effects of various proteins suggest that EBV modifies CSL-dependent signaling in B cells in a highly regulated way, rather than merely turning it on constitutively.

Notch Regulation of Downstream Developmental Branch Points in Thymocyte Development

Developing T cells pass through a series of developmental stages defined, in part, by expression of the coreceptor molecules CD4 and CD8. TCR rearrangements are initiated among CD4$^-$ CD8$^-$ cells, which give rise to either $\gamma\delta$ T cells or $\alpha\beta$ T lineage progenitors. Cells containing productive TCRβ rearrangements are selected for their ability to generate a signal through the pre-TCR, a complex of TCRβ, CD3, and pre-Tα polypeptides. Successful pre-TCR signaling commits a cell to the TCR$\alpha\beta$ lineage and results in their differentiation into CD4$^+$ CD8$^+$ (double positive, DP) thymocytes. A small proportion of DP thymocytes are subsequently selected to differentiate into mature CD4$^+$ CD8$^-$ MHC class II restricted or CD4$^-$ CD8$^+$ MHC class I restricted single positive (SP) thymocytes that subsequently migrate to peripheral lymphoid tissues.

Notch ligands are expressed on thymic epithelial cells (Anderson et al., 2001b), suggesting that Notch signaling may influence several thymocyte fate decisions, including $\alpha\beta$ versus $\gamma\delta$ and CD4$^+$ versus CD8$^+$ lineage choices. Using bone marrow chimeric mice repopulated with both Notch1$^{+/+}$ and Notch1$^{+/-}$ HSCs, Robey and colleagues found that thymocytes derived from Notch1$^{+/-}$ HSCs were skewed toward production of $\gamma\delta$ T cells (Washburn et al., 1997). Interestingly, altered ratios of $\alpha\beta$ and $\gamma\delta$ T cells were observed only when haploinsufficient HSCs were mixed with Notch1$^{+/+}$ HSCs, suggesting that lateral interactions between neighboring developing thymocytes mediate $\alpha\beta/\gamma\delta$ lineage choice. Further, enforced expression of an ICN1 transgene favors $\alpha\beta$ T cell development, even in mice bearing a $\gamma\delta$ TCR transgene or a disrupted TCRβ locus (Washburn et al., 1997), again

suggesting that stronger Notch signals favor αβ over γδ T cell development. This simple view is complicated, however, by studies showing that mice homozygous for a mutation disrupting Jagged2 ($Jag^{\Delta DSL}$) showed an ~50% decline in the numbers of fetal-derived γδ T cells, and no alteration of αβ T cell development (Jiang et al., 1998). The explanation for these discordant results is uncertain, but multiple Notch ligands are expressed in both developing thymocytes and thymic stroma. It has been suggested, for example, that Jagged 2 function is specifically required for γδ T cell development, and that some other ligand is responsible for the generation of signals that promote αβ T cell development (Jiang et al., 1998). Thus, while changes in Notch signaling can influence the αβ/γδ T cell specification, additional work is needed to understand the normal role of specific Notch receptors, ligands, and modifiers in this process.

Several groups have investigated the role of Notch in regulating the $CD4^+$ versus $CD8^+$ fate choice, but the physiological significance of Notch receptors in this context remains controversial. Studies using transgenes encoding truncated ICN1 proteins variously suggested that Notch1 signaling selectively promotes $CD8^+$ and inhibits $CD4^+$ thymocyte development (Robey et al., 1996), or promotes the development of both $CD4^+$ and $CD8^+$ cells (Deftos et al., 2000). These discordant outcomes may stem from the use of different ICN1 transgenes, variation in ICN1 expression levels, or analysis of mice of differing age. Experiments where Notch dosage is reduced have also suggested that Notch plays a role in CD4 versus CD8 development. Germain and colleagues found that inhibiting Notch1 in reaggregated thymic cultures with blocking antibodies or antisense RNA appeared to enhance development of CD4 cells (Yasutomo et al., 2000). Similarly, γ secretase inhibitors, which prevent cleavage of all Notch receptors, inhibited CD8 development and, in at least one system, shifted maturation toward the CD4 fate (Doerfler et al., 2001; Hadland et al., 2001).

Because these studies suggested a role for Notch in positive selection, it was surprising that the CD4/CD8 ratio was unaltered in thymocytes derived from mice in which Notch1 was inactivated just prior to the onset of positive selection (Wolfer et al., 2001). Although this result suggests that Notch1 signaling is dispensable for positive selection, it fails to address whether other Notch receptors that are expressed in thymocytes, such as Notch2 and Notch3, might compensate for the absence of Notch1. Consistent with the model presented in Figure 3, the level of Notch activity required for positive selection may be less than that needed for commitment to T cell fate at the T:B decision point. If true, other Notch receptors could replace Notch1 in the former but not the latter developmental process.

The outcome of positive selection depends on interactions between thymocyte TCR and MHC/peptide ligands on thymic epithelial cells. Recent data suggest that the CD4 versus CD8 fate choice may be regulated by TCR signal strength and/or duration (reviewed in Hogquist, 2001). Positive selection requires the ras/MAPK signaling pathway and the outcome of positive selection appears to be influenced by the activity of the tyrosine kinase lck (Alberola-Ila et al., 1996; Legname et al., 2000). Thymocytes receiving stronger or longer TCR signals

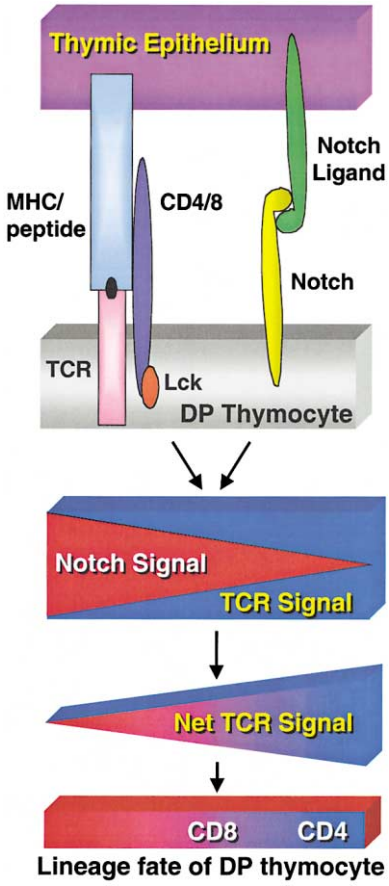

Figure 5. Notch Signaling May Modulate Positive Selection by Inhibiting TCR Signaling in a Dose-Dependent Fashion

This figure illustrates one model to explain the consequences of experimentally induced variations in Notch activity on $CD4^+CD8^+$ thymocyte development. In this model, Notch activity downregulates net TCR signaling, perhaps by manipulating the Ras/MAPK pathway, and thereby influences CD4 versus CD8 lineage choice. Thus, the highest doses of Notch inhibit net TCR-mediated positive selection signals maximally and the lowest doses of Notch have minimal effects on net TCR signaling. Stronger TCR signaling (low Notch activity) will result in a bias toward CD4 development and weaker TCR signals (high Notch activity) will favor CD8 development. The highest Notch activity would be expected to reduce TCR signaling below the threshold required for positive selection of both CD4 and CD8 T cells. It is important to note that in a physiological setting, $CD4^+$ $CD8^+$ thymocytes are unlikely to experience variable Notch activity, and instead, will integrate basal Notch activity with variable TCR signals to make a lineage decision. Finally, unlike the B/T cell fate choice (which is dependent on Notch1 function), positive selection may be influenced by signals produced by Notch1, Notch2, or Notch3.

appear to adopt the $CD4^+$ cell fate, whereas those receiving TCR signals that are weaker or of shorter duration are more likely to become $CD8^+$ T cells.

One possible explanation for the influence of Notch signaling on $CD4^+$ versus $CD8^+$ lineage commitment is that Notch may antagonize net TCR signal strength during positive selection. Consistent with this view, enforced expression of full-length ICN1 abrogates TCR signaling in $CD4^+CD8^+$ thymocytes and inhibits their differentiation into both $CD4^+$ and $CD8^+$ thymocytes (Izon et al., 2001). As illustrated in Figure 5, high levels

of Notch activity may inhibit net TCR signaling most profoundly and therefore be incompatible with positive selection. Likewise, intermediate levels of Notch activity will abrogate TCR signaling less and therefore may favor $CD8^+$ T cell development, and low levels of Notch signaling (as induced experimentally by γ secretase inhibitors) may permit optimal TCR signaling and therefore favor $CD4^+$ T cell development.

A recent clue as to how Notch may influence TCR-mediated positive selection was provided by studies investigating *C. elegans* vulval development. In vulval precursor cells, Notch-expressing cells adopt one fate, whereas Notch-ligand-expressing cells adopt the alternative cell fate via lateral inhibition. The primary difference between Notch receptor expressing cells and Notch ligand expressing cells is that MAPK signaling is suppressed in the former and enhanced in the latter. Notably, Berset et al. recently showed that a direct transcriptional target of Notch signaling in *C. elegans* vulval precursors is LIP-1, a MAPK phosphatase (Berset et al., 2001) that inhibits MAPK signaling in Notch-expressing cells. Thus, it is plausible that a similar mechanism inhibits ras/MAPK signaling to influence positive selection in developing T cells. Indeed, Notch activity inhibited an AP1 reporter in the Jurkat T cell line, following stimulation by reagents that act both proximally (anti-TCR) and distally (PMA/ionomycin) in the ras/MAPK pathway (Izon et al., 2001). An additional level of control may result from CD4 downregulation due to Notch-mediated expression of HES repressors, which bind to the CD4 silencer (Allen et al., 2001).

The NF-κB signaling pathway, which is of broad importance in lymphocyte maturation and function, may also interact with Notch signals in developing T cells. Enforced activation of NF-κB permits $Rag2^{-/-}$ thymocytes to proceed to the $CD4^+8^+$ stage of differentiation (Voll et al., 2000), suggesting that it also acts downstream of TCR signals. Conversely, inhibition of NF-κB decreases the number of $CD4^+8^+$ thymocytes and preferentially inhibits the positive selection of $CD8^+$ SP thymocytes (Hettmann and Leiden, 2000). However, Notch has been variously reported to have both positive (Cheng et al., 2001) and negative (Wang et al., 2001) effects on NF-κB signaling in mammalian cells, and there are no published reports showing strong interactions between Notch and NF-κB (e.g., dorsal) signaling in invertebrates. Thus, additional work is needed to evaluate the importance of Notch/NF-κB interactions during T cell development.

Currently, there is little information on how Notch might influence T cells once they exit the thymus. In one study, enforced expression of Jagged-1/Serrate-1 in mouse antigen presenting cells induced $CD4^+$ peripheral T cells to differentiate into regulatory cells that inhibited the primary and secondary immune response (Hoyne et al., 2000). While this finding might lead to methods to manipulate the immune response in clinical settings, whether Notch signaling truly regulates peripheral T cell function remains to be determined.

Notch and T Cell Leukemogenesis

A sometimes-ignored consequence of deregulated Notch signaling is malignant transformation. In mammals, the Notch1 gene was originally identified through its participation in a recurrent t(7;9)(q34;q34.3) chromosomal translocation found in a small fraction of human T cell acute lymphoblastic leukemias (T-ALLs) (Ellisen et al., 1991). In all t(7;9)-associated T-ALLs, the translocation fuses sequences corresponding roughly to ICN1 with the promoter/enhancer sequences of the TCRβ locus. This leads to dysregulated expression of N-terminally truncated forms of Notch1 that localize to the nucleus, where they presumably cause constitutive activation of Notch1 signaling.

Mammalian models have confirmed that transformation is a general feature of constitutive Notch signaling in T cells, as ICN1, ICN2, and ICN3 all cause T-ALLs (reviewed in Aster and Pear, 2001). The capacity of ICN1 to transform hematopoietic cells is sharply restricted to T cell progenitors with intact pre-TCR signaling. ICN1 will not transform donor HSCs from $Rag\text{-}2^{-/-}$ and $SLP\text{-}76^{-/-}$ mice, despite evidence of increased commitment of marrow progenitors to T cell fate (Allman et al., 2001). These mice do not develop neoplasms despite persistent high-level expression of ICN1 in marrow progenitors, demonstrating the Notch1 has little (if any) capacity to transform myeloid cells. The loss of transforming activity in the $Rag\text{-}2^{-/-}$ background is reconstituted by a TCRβ transgene that permits expression of a pre-Tα/TCRβ signaling complex and progression to the DP stage of differentiation (Allman et al., 2001). This synergy illustrates how interactions between Notch and other signaling pathways contribute to particular phenotypes, and emphasizes the link between Notch and TCR signaling in both normal lymphoid development and leukemia. The requirement for TCR function in Notch-induced T-ALL is distinct from certain other genes implicated in T-cell leukemia. For example, $p53^{-/-}$ mice reproducibly develop T-ALLs irrespective of TCR expression, and the incidence of T-ALL is reduced by expression of a TCRβ transgene in ATM-deficient mice (Liao et al., 1998; Liao and van Dyke, 1999). Conversely, the dependency of Notch-induced transformation on pre-TCR signaling bears some resemblance to T-ALLs arising from deregulation of the Zn finger transcriptional regulator Ikaros (Winandy et al., 1999).

Although it is likely that enforced Notch signaling both drives T cell commitment in progenitors and subsequently prevents T cell maturation beyond the DP stage, the mechanisms underlying T-lineage specific Notch-mediated leukemogenesis are unclear. In our Notch1 retroviral transduction models, thymic-independent T cell development in the bone marrow is polyclonal, whereas the Notch-induced T-ALLs are monoclonal, suggesting that additional genetic events are necessary for transformation. Consistent with this finding, both myc and E2A-PBX have been shown to synergize with Notch1 to induce murine T-ALL (reviewed in Aster and Pear, 2001). Activated Notch is unlikely to provide a proliferative advantage, as our retroviral transduction studies have shown that the Notch-induced ectopic T cells are both small and resting, similar to thymic double-positive cells (Allman et al., 2001). It is not clear if either the antiapoptotic or antidifferentiative propensities of Notch contribute to transformation. In addition to elucidating the mechanisms of Notch transformation, it will be interesting to learn whether Notch signaling

plays a role in other lymphoid tumors besides the infrequent T-ALLs containing chromosomal translocations involving Notch1.

Conclusion

Despite the overall complexity of Notch function during development, and some controversy over the role of Notch signaling in immature T cell development, a general picture has emerged regarding the impact of Notch activity in lymphopoiesis. In both invertebrates and mammals, Notch activity regulates the development of a wide spectrum of cell types. During hematopoiesis, Notch signaling may promote self-renewal rather than differentiation, whereas in early lymphoid progenitors, Notch signaling functions to induce the development of early T cell precursors and inhibit early B cell differentiation. The upstream factors that trigger Notch1 expression and signaling and the downstream Notch1-dependent transcriptional program that promotes T cell development both largely remain to be elucidated. The role of Notch signaling in later branch points in T-lineage development is more contentious. Notch signaling may influence the CD4 versus CD8 choice by modifying TCR signaling during positive selection. It is unclear if Notch upregulates transcription of the same set of genes as in T cell commitment, or if it upregulates novel genes. Most likely, the answer is a combination of the two. Transcripts that are expressed during both stages of development include HES-1, CD25, Meltrin β, and Ifi-204 (Deftos et al., 2000), and it will be interesting to learn if all of these are bona fide Notch targets, and how their collective expression affects different stages of T cell ontogeny. The causal association between dysregulated Notch signaling and T cell leukemia emphasizes the requirement for exquisite control of Notch signaling during lymphoid development.

The use of Notch signaling to influence cell fate decisions during successive stages of hematopoietic and T cell development is reminiscent of the repeated use of this pathway during *Drosophila* neurogenesis. In the latter, CSL activity is a crucial determinant of outcome at each developmental branch point, but its activity is controlled in different ways as development proceeds. Thus, specific stages are regulated by lateral inhibition, asymmetric distribution of numb between daughter cells, or even Notch-independent CSL signaling. The intensity of the Notch (or CSL) signal required for a particular activity is cell-context-dependent, indicating the existence of feedback loops that reset Notch signaling as development proceeds. The complexity of Notch signaling control and degree of integration with other pathways must be even greater in mammals than invertebrates, given the duplication and divergence of certain signaling components and the addition of others (e.g., Deltex) that do not exist in simple organisms such as *C. elegans*. Although the challenge is great, understanding the multiple mechanisms and functions of Notch signaling in lymphoid development will not only provide important new insights into lymphopoiesis, but may also lead to novel approaches to immune modulation, stem cell manipulation, and cancer treatment.

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