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## Notch-Regulated Ankyrin-Repeat Protein Inhibits Notch1 Signaling: Multiple Notch1 Signaling Pathways Involved In T Cell Development<sup>1</sup> ✓

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# Notch-Regulated Ankyrin-Repeat Protein Inhibits Notch1 Signaling: Multiple Notch1 Signaling Pathways Involved In T Cell Development<sup>1</sup>

Theodore J. Yun,\* and Michael J. Bevan<sup>2†</sup>

**We have characterized the function of Notch-regulated ankyrin-repeat protein (Nrarp) in mouse cell lines and in hematopoietic stem cells (HSCs). Nrarp overexpression is able to block Notch-induced activation of CBF-1. In AKR1010 thymoma cells, Nrarp overexpression blocks CBF-1-dependent transcriptional activation of Notch-responsive genes and inhibits phenotypic changes associated with Notch activation. Enforced expression of Nrarp in mouse HSCs results in a profound block in T lineage commitment and progression through early stages of thymocyte maturation. In contrast, Deltex-1 overexpression in HSCs can also block T lineage commitment but not progression through the early double negative stages of thymocyte maturation. The different effects of Deltex-1 and Nrarp overexpression suggest that alternate Notch signaling pathways mediate T vs B lineage commitment and thymocyte maturation. *The Journal of Immunology*, 2003, 170: 5834–5841.**

The Notch family of transmembrane receptors regulates a conserved signaling pathway that is involved in determination of precursor cell fate in multiple developmental systems (reviewed in 1). Orthologous Notch proteins have been described in species as diverse as sea urchins, fruit flies, and mammals. In mice and humans, four Notch family members have been identified. The Notch homologues bind to at least five different ligands belonging to either the Jagged or the Delta-like family. The tissue distribution of the Notch receptors and ligands is widespread and overlapping. Many characteristics of Notch signal transduction appear to be conserved among species, and a general mechanism has been proposed (1). Upon productive interaction with ligand, the intracellular region of Notch (Notch-IC)<sup>3</sup> is proteolytically cleaved, likely by a presenilin family protease (2, 3). The Notch-IC domain translocates to the nucleus and interacts with the CSL (CBF-1, Suppressor of Hairless, Lag-1) family of transcription factors. In the absence of Notch signaling, CBF-1 functions as a transcriptional repressor, however upon Notch-IC binding, it is converted into a transcriptional activator. By this mechanism, activated Notch regulates the expression of multiple genes. In addition to CBF-1, Notch has been reported to regulate the activity of other transcription factors, such as the E protein family of basic helix-loop-helix transcription factors (4).

In terms of higher vertebrate immune system development, Notch1 signaling is critical in the T vs B lineage decision in the common lymphoid progenitor (CLP). This regulation of T vs B

cell fate has been demonstrated by a number of complementary genetic approaches. Retroviral overexpression of Notch1-IC in HSCs caused CLPs to commit to the T lineage at the expense of B cell differentiation (5). These transduced cells eventually developed into fatal T lineage tumors (6). Clinically, Ellisen et al. (7) observed that in a fraction of T-acute lymphoblastic leukemia tumors, chromosomal translocations produced a constitutively activated form of Notch. Finally, in a complementary approach, Radtke et al. (8) inducibly inactivated Notch1 in adult mice. The effect of conditionally deleting Notch1 in HSCs resulted in a lack of T cell development and ectopic development of CD4<sup>-</sup>CD8<sup>-</sup>B220<sup>+</sup>CD44<sup>+</sup>CD25<sup>-</sup> immature B cells in the thymus (8, 9). Collectively, these data implicate the key role of Notch1 signaling in lineage choice of CLPs by promoting T cell development and inhibiting B cell development.

Notch1 signaling has also been implicated in regulating thymocyte development during the initiation of TCR  $\beta$  locus rearrangement. Conditional deletion of Notch1 at the double negative (DN)2 to DN3 stage of thymocyte development resulted in reduced numbers of double positive (DP) thymocytes and the appearance of an aberrant population of TCR $\beta$ <sup>-</sup> thymocytes. Thus, Notch1 deletion at this stage in thymocyte development affects the ability of the TCR  $\beta$  locus to create a functionally expressed receptor. Conditional deletion of Notch1 during the CD44<sup>-</sup>CD25<sup>-</sup> stage of thymocyte differentiation had no effect on further T cell development (10); however, ectopic overexpression of Notch1-IC at this stage can drive the differentiation of DP to CD4<sup>+</sup> or CD8<sup>+</sup> single positive (SP) (11, 12).

Despite the fact that Notch1 signaling has a very specific function in the differentiation of CLPs, Notch1, 2, 3 and their ligands are expressed widely in the bone marrow (BM) and during T cell differentiation (13–15). Signals through the Notch receptors are regulated by multiple mechanisms. Molecules that modify Notch signaling have been postulated to spatially and temporally regulate Notch signaling. In terms of T cell development, ectopic overexpression of the glycosyltransferase Lunatic Fringe in immature thymocytes inhibited Notch-dependent T cell commitment in a cell nonautonomous manner (16). Similarly, when Deltex-1, a transcriptional target of Notch signaling in T cells (17), was overexpressed in HSCs, T cell development was blocked and ectopic

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<sup>3</sup> Abbreviations used in this paper: Notch-IC, intracellular region of Notch; Nrarp, Notch-regulated ankyrin-repeat protein; BM, bone marrow; CLP, common lymphoid progenitor; DN, double negative; DP, double positive; HPRT, hypoxanthine phosphoribosyltransferase; EGFP, enhanced green fluorescent protein; HSC, hematopoietic stem cell; HES, hairy enhancer of split; SP, single positive.

thymic B cell development was observed (18). In this system, enforced expression of Deltex-1 appeared to block Notch-1 signaling. However, the *Drosophila* homologue of Deltex-1 has been postulated to act as a positive regulator of the Notch pathway (19). Depending on their expression pattern, these regulators of Notch signaling potentially control the timing of the signal and modulate downstream functional effects.

We and others have described Notch-regulated ankyrin-repeat protein (Nrarp) as another transcriptional target of Notch signaling (20–22). This small protein is composed of two ankyrin domains and a unique region that bears little homology to other molecules. Nrarp is highly conserved in higher vertebrates. In mice and humans, Nrarp is widely expressed throughout many tissues, however its expression was not detectable by Northern blot analysis in lymphoid organs (21). Overexpression of Nrarp in *Xenopus* embryos modified Notch signals by forming a multimeric complex that includes Notch and CBF-1 (20, 22). It was hypothesized that Nrarp may promote Notch-IC degradation by targeting Notch-IC to a cell type-specific degradation enzyme. Depending on the cellular context, overexpression of Nrarp had a positive or negative effect. In *Xenopus* embryos, Nrarp inhibited Notch signaling, however, in cultured HeLa cells, Nrarp enhanced Notch signaling when suboptimal levels of Notch-IC are expressed or in synergy with Mastermind, a scaffold molecule for Notch-1 and CBF-1 (22).

We investigated the consequence of Nrarp overexpression in mouse HSCs and T cells. In vitro, Nrarp inhibits Notch-mediated CBF-1 activation. Using this inhibitory function of Nrarp as a tool to probe Notch-mediated CBF-1 activation in vivo, we showed that Nrarp specifically inhibited T cell development in the hematopoietic system. A small percentage of Nrarp-overexpressing HSCs were able to commit to the T lineage, but were impeded throughout DN development. Specifically, we observed a block in DN1 to DN2 transition, which has not been previously documented. Collectively, these observations demonstrate that Notch signaling is involved in all of the major stages of early T cell development, from HSCs to DN3. When compared with the ability of Deltex-1 to block T vs B lineage commitment, we found that while Nrarp and Deltex-1 block this early step, only Nrarp could block transition through the DN stages of thymocyte development. These data argue that a Deltex-1-sensitive and a Nrarp-sensitive Notch signaling pathway are involved in T lineage commitment, however, the Deltex-1-sensitive pathway is not involved in early thymocyte development.

## Materials and Methods

### RT-PCR

Thymocytes from C57BL/6 mice were sorted by standard methods, based on their expression of CD4, CD8, and CD3. Sorted cells were lysed in STAT-60 (Tel-Test, Friendswood, TX). Total RNA was isolated according to manufacturer's instructions. cDNA was prepared using SuperScript II (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. For negative amplification control (no-RT), RNA was subjected to reverse transcriptase conditions without addition of the enzyme. Five-fold serial dilutions were prepared. Using primers specific for either Nrarp (5'-CCTCG CACTTAGGAAGGGAAGGGGACGC-3'; 5'-GGACAGCCAGTGACG CTCCAGCACCTC-3') or hypoxanthine phosphoribosyltransferase (HPRT) (5'-GATACAGGCCAGACTTTGTTG-3'; 5'-GGTAGGCTGGCCTATAG GCT-3'), the presence of either transcript was determined by PCR using Advantag (Advantage cDNA PCR kit; Clontech Laboratories, Palo Alto, CA).

### Retroviral transduction

Retroviral transduction was performed as previously described (17). A detailed protocol can be found on Dr. G. Nolan's web page ([http://www.stanford.edu/group/nolan/protocols/pro\\_helper\\_dep.html](http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html)). Briefly, Phoenix amphotropic or ecotropic packaging cell lines were grown to 80% confluency in 12-cm tissue culture dishes. Retroviral construct DNA (10  $\mu$ g) was precipitated by  $\text{Ca}_2\text{PO}_4$  then added to cells. Cells were incubated

for 8–10 h at 37°C. Medium was changed and the transfected cells were incubated overnight at 32°C. AKR1010 cells were cultured in retrovirus-containing supernatant and 5  $\mu$ g/ml Polybrene (Sigma-Aldrich, St. Louis, MO) and spun at 2500 rpm for 90 min at 32°C. For BM transduction, 6-well culture dishes were coated with fibronectin (Sigma-Aldrich). BM cells were added and cultured in DMEM containing 75% retrovirus-containing supernatant, 15% FCS, 4  $\mu$ g/ml polybrene, 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), 5 mM HEPES (Invitrogen), then spun at 2500 rpm for 120 min at 37°C. After centrifugation, cells were placed at 37°C overnight. For BM cells, transduction was repeated the following day. The pMI2-Notch construct was previously described (17). The pMI5 retroviral construct is a derivative of pMX-IRES-hCD2 in which human CD5 is used in place of human CD2 (M. L. Deftos, unpublished data). The MIGR1 retroviral vector was a kind gift from Dr. W. Pear (University of Pennsylvania, Philadelphia, PA). The open reading frame for Nrarp was cloned by PCR and verified by sequence analysis (Ref. 21 and M. L. Deftos, unpublished data). Nrarp was subcloned into the multicloning sites of either pMI5 or MIGR1. The open reading frame for Deltex-1 was cloned by PCR and verified by sequence analysis (M. L. Deftos, unpublished data). Deltex-1 was subcloned into the multicloning site of MIGR1.

### BM culture and reconstitution

BM was flushed from tibias and femurs of donor mice 4 days after injection of 3 mg of 5-fluorouracil (Sigma-Aldrich). RBC were lysed and BM cells were resuspended at a concentration of  $10^6$  cells/ml in DMEM containing 15% FCS, 100 ng/ml recombinant mouse stem cell factor, 20 ng/ml recombinant human IL-6, 10 ng/ml recombinant mouse IL-3. Cells ( $10^7$ ) were plated in 12-cm tissue culture dishes and incubated for 48 h at 37°C. BM was transduced (see previous description) on day 2 and day 3. On day 4, BM cells were trypsinized and resuspended in serum free DMEM at a concentration of  $0.3\text{--}1.5 \times 10^6$  cells/ml. Cells ( $1\text{--}5 \times 10^6$ ) were i.v. injected into host mice that were lethally irradiated ( $\sim 1050$  rads) and maintained on an antibiotic containing water. Mice were sacrificed and organs of interest were harvested.

### Flow cytometry

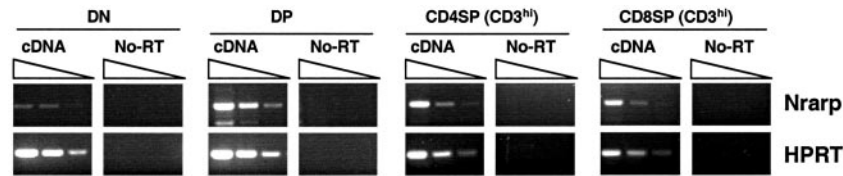
Cells were stained with mAbs using standard procedures. Biotinylated mAbs were revealed by incubation with Streptavidin-PerCP (BD Pharmingen, San Jose, CA). For some experiments, cells were fixed in PBS containing 1% paraformaldehyde. Cells were analyzed by flow cytometry using a FACSCalibur (BD Pharmingen). The mAbs used in this report were purchased from BD Pharmingen and are as follows: anti-CD3 $\epsilon$ -APC (145-2C11); anti-CD2-PE (RPA-2-10); anti-CD5-FITC (L17F12); anti-CD45R (B220) (RA3-6B2); anti-CD11b-PE (M1/70); anti-CD4-APC (RM4-5); anti-CD8 $\alpha$ -PE or -APC (53-6.7); and anti-CD44-biotin (IM7).

### Real-time PCR

Total RNA was prepared as previously noted from transduced cell lines. Total RNA was treated with DNase-free (Ambion, Austin, TX), following manufacturer's instructions. cDNA and negative amplification control (no-RT) were prepared using SuperScript II, as described. Oligonucleotide primers and TaqMan probes were designed to detect transcripts: Pre-T $\alpha$  (5'-CTGCTTCTGGGCGTCAGGT-3'; 5'-TGCCTTCCATCTACCAGCA GT-3'; 5'-CCTTCCGCTCTGGCTCCACCCA-3'); Deltex-1 (5'-TGA GGATGTGGTTCCGGAGGT-3'; 5'-CCCTCATAGCCAGATGCTGTG-3'; 5'-CGCCTGATGAGGACTGTACCATTTGCAT-3'); hairy enhancer of split (HES) (5'-TACCCAGCCAGTGTCAACA-3'; 5'-TTCTTGCCTTCG CCTCTT-3'; 5'-TGAGCACAGAAAGTCATCAAAGCCATCATGG-3'); endogenous Nrarp (5'-TGCTGTGCTCTTTTCGCCCTC-3'; 5'-ACAGC CCCTCCCTCATGG-3'; 5'-CCCTGTCCAAAGGCCCTATTATGATCTG-3'); HPRT (5'-TGGAAAGAATGTCTTGTATTGTTGAA-3'; 5'-AGCTTG CAACCTTAACCATTTT-3'; 5'-CAAACCTTGTCTTCCCTGGTTAAGC AGTACAGC-3'). For each reaction, the final concentration of amplification primers was 0.5  $\mu$ M, and the final concentration of TaqMan probe was 0.2  $\mu$ M. Real-time PCR was conducted using 2X TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA) using an ABI Prism 7700 Sequence Detector (Applied Biosystems). Data were analyzed using Sequence Detector software (Applied Biosystems). The conditions for the reaction were: 50°C, 2 min; 95°C, 10 min; and cycled 50 $\times$  at 95°C, 20 s and 60°C, 2 min.

### Luciferase assay

Six-well cultures dishes were seeded with  $1.5\text{--}2.5 \times 10^5$  3T3 cells/well for 12–18 h. Indicated amount of pEF-Notch1-IC and pMI5-Nrarp were diluted in OptiMEM I medium (Invitrogen). pGL2-8XCBF-1 (2.5  $\mu$ g; a gift



**FIGURE 1.** Nrarp transcripts are detectable in thymocyte subsets. Thymocytes from C57BL/6 mice were sorted into the indicated subsets. cDNA was prepared from each subset. Nrarp and HPRT transcripts were amplified from 5-fold serial dilutions of cDNA. For a negative amplification control (no-RT), RNA was subjected to identical reaction conditions without the addition of reverse transcriptase.

from Dr. S. D. Hayward, Johns Hopkins University, Baltimore, MD) and 0.5  $\mu$ g pRL-TK (*Renilla* luciferase) were added to DNA mixture, and the amount of DNA was brought up to  $\sim 8$   $\mu$ g with empty vector (pEF). DNA mixture was incubated with 15–20  $\mu$ l of Lipofectamine 2000 (Invitrogen) for 15–30 min at room temperature. DNA-Lipofectamine complexes were added to cells and incubated at 37°C for  $\sim 48$  h. Luciferase and *Renilla* luciferase activity was revealed using the Dual Luciferase Assay System (Promega, Madison, WI), following manufacturer's instructions. Luciferase and *Renilla* luciferase activity was quantified using a MicroLumat-Plus LB96V luminometer (Berthold Technologies, Bad Wildbad, Germany) and WinGlow software (Berthold Technologies). Relative units of luciferase activity were normalized to relative units of *Renilla* luciferase activity.

#### Proliferation assay

Proliferation assay of transduced cells in the presence of dexamethasone was previously described (17). Briefly,  $10^4$  cells of interest were cultured in the presence of varying concentrations of dexamethasone (Sigma-Aldrich). After 48 h, 1  $\mu$ Ci [ $^3$ H]thymidine (PerkinElmer Life Sciences, Foster City, CA) was added to the culture. After 12–18 h, 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 or quadruplicate cultures were averaged. Proliferation in the presence of dexamethasone was calculated relative to proliferation occurring in the absence of dexamethasone.

## Results

### Nrarp is expressed in thymocyte subsets

Notch signaling has a critical role in T cell development and Notch1 responsive genes are expressed in the thymus (23, 24). Nrarp is up-regulated upon Notch activation in T cell lines and other cell types (20–22). However, previous data suggested that Nrarp expression in thymus was undetectable by Northern blot analysis (21). Nrarp expression in thymocyte subsets was reanalyzed by RT-PCR. Thymocytes were stained for CD4, CD8, and CD3 expression, sorted into DN, DP, and SP ( $CD4^+CD8^-$  and  $CD4^-CD8^+$ ) subsets, and assayed for Nrarp expression. As shown by the data in Fig. 1, all thymocyte subsets had detectable Nrarp transcripts, with highest levels in the DP subset.

### Nrarp overexpression blocks Notch1-IC activation of CBF-1

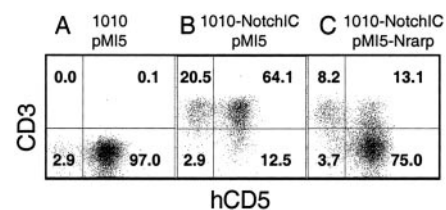
Nrarp contains two ankyrin repeat domains that probably mediate protein-protein interactions (25). In *Xenopus* embryos and HeLa cells, Nrarp has been shown to interact with Notch1-IC and CBF-1. Depending on cellular context, this multimeric complex inhibits or augments CBF-1 activation. We analyzed the effect of Nrarp on Notch1-IC signaling using the mouse AKR1010 thymoma cell line. Expression of the constitutively active Notch1-IC in this cell line causes several phenotypic changes in this cell line, including up-regulation of surface CD3 and resistance to glucocorticoid-induced apoptosis (17). AKR1010 cells were transduced with a retroviral expression vector pMI2-Notch1-IC that can co-express Notch1-IC and human CD2 from a bicistronic message so that Notch1-IC-expressing cells can be monitored using flow cytometry. As previously shown, Notch signaling in this cell line resulted in up-regulation of surface CD3 (Fig. 2, A and B). To

study the effect of Nrarp on Notch signaling, Notch1-IC-expressing AKR1010 cells were further infected with Nrarp cloned into the pMI5 retroviral vector (pMI5-Nrarp). pMI5 is a derivative of pMI2 that utilizes human CD5 as a reporter instead of human CD2. Both reporter molecules are truncation variants that lack the cytoplasmic domain. In bulk-transduced cells, overexpression of Nrarp blocked the ability of Notch1-IC to induce CD3 up-regulation (Fig. 2). The fraction of AKR1010 cells expressing surface CD3 as a result of Notch1-IC transduction was decreased in cells coexpressing Nrarp (Fig. 2C). AKR1010 cells transduced with Notch1-IC continued to express CD3 when transduced with human CD5 only (Fig. 2B).

Overexpression of Notch1-IC in AKR1010 cells also confers resistance to glucocorticoid-induced apoptosis (17). To test whether Nrarp inhibition was a general inhibitor of Notch function, AKR1010-Notch1-IC-Nrarp cells were incubated in varying doses of dexamethasone. As shown in Fig. 3A, AKR1010-Notch1-IC and AKR1010-Notch1-IC-pMI5 cells were resistant to the effects of dexamethasone. In comparison, AKR1010 cells expressing both Notch1-IC and Nrarp were more sensitive to dexamethasone (Fig. 3A). These experiments suggest that Nrarp is able to block multiple functions of Notch signaling in the AKR1010 thymoma cell line.

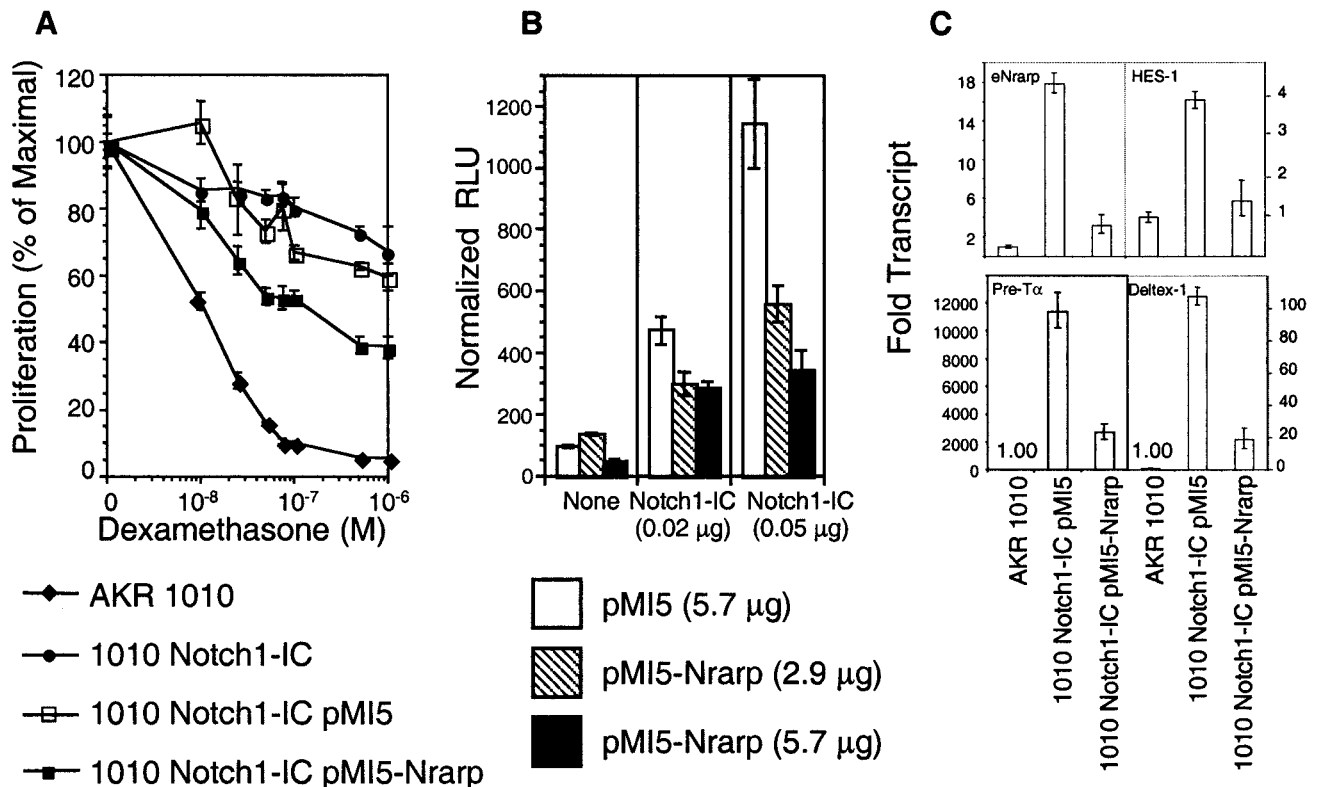
CBF-1 is a major target of activated Notch, and activation of CBF-1 can account for many phenotypic changes induced by Notch (26). To examine whether Nrarp blocked CBF-1 activation, 3T3 cells were transiently transfected with a CBF-1/luciferase reporter construct (27) along with increasing amounts of Notch1-IC and increasing amounts of Nrarp. As expected, Notch1-IC induced CBF-1 activity in a dose-dependent manner. Coexpression of Nrarp inhibited Notch1-IC-induced CBF-1 activation in a dose-dependent manner (Fig. 3B).

In AKR1010 cells, the consequence of Notch-mediated CBF-1 activation is up-regulation of HES, pre-T $\alpha$ , Deltex-1, and endogenous Nrarp transcription levels, as assayed by quantitative real-time PCR (Fig. 3C). However, in AKR1010 cells expressing both Notch1-IC and Nrarp, transcription levels of each target gene decreased up to 5-fold from the control Notch1-IC-expressing cells depending on the gene assayed (Fig. 3C). Collectively, these data argue that Nrarp overexpression inhibits Notch function by down-regulating CBF-1-mediated transcriptional activation.



**FIGURE 2.** Nrarp inhibits Notch-induced CD3 up-regulation. AKR1010 cells were transduced with pMI2-Notch1-IC and either pMI5 or pMI5-Nrarp. Cells that integrated the retrovirus were monitored by human CD5 expression. Cells were analyzed for surface expression of CD3.





**FIGURE 3.** Nrp1 inhibits Notch induction of CBF-1 activation. *A*, Nrp1 overexpression in AKR1010 cells partially blocks the ability of Notch to confer glucocorticoid resistance. AKR1010 cells were transduced with pMI2-Notch1-IC and either pMI5 (control) or pMI5-Nrarp. Cells were incubated for 48 h in the presence of varying concentrations of dexamethasone, then pulsed with [<sup>3</sup>H]thymidine. <sup>3</sup>H incorporation was quantified and calculated relative to maximal proliferation in the absence of dexamethasone. *B*, Nrp1 inhibits Notch activation of a CBF-1 reporter. 3T3 cells were transiently transfected with 8X CBF-luc reporter, *Renilla* luciferase, varying amounts of pEF-Notch1-IC, and either varying amounts of pMI5-Nrarp or pMI5 (control). Luciferase activity was determined for each transfected cell population and then normalized to the amount of *Renilla* luciferase activity. Representative data are shown from three independent experiments. *C*, Nrp1 inhibits Notch activation of CBF-1 target genes. AKR1010 cells transduced with Notch1-IC and either pMI5 (control) or pMI5-Nrarp. cDNA was prepared from each cell type. The amount of the indicated transcript was determined for each cell line and normalized to the amount of HPRT transcript. The relative amount of transcript was calculated using the parental AKR1010 cell line as a negative control for Notch signaling.

#### *Nrp1 and Deltex-1 overexpression inhibits T lineage commitment*

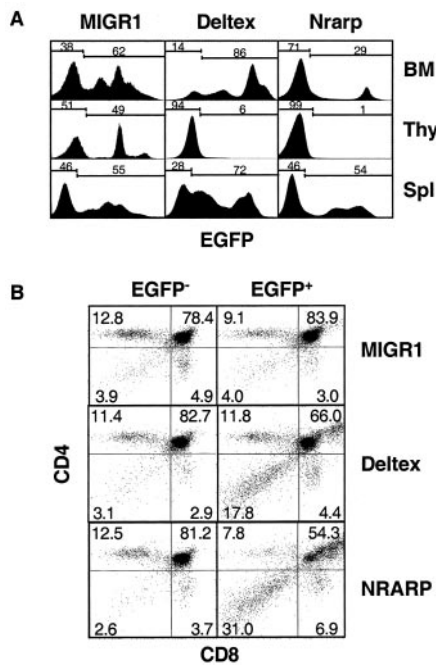
BM HSCs were transduced with the MIGR1 retroviral vector expressing the cloned gene of interest and enhanced green fluorescent protein (EGFP) from a bicistronic message (28). Lethally irradiated mice were reconstituted with the transduced cells and analyzed 4–6 wk later. As shown in Fig. 4*A* and Table I, overexpression of Nrp1 in HSCs consistently resulted in a decreased percentage of EGFP<sup>+</sup> cells in the thymus, relative to the percentage of transduced cells in the BM or spleen. In host mice receiving HSCs transduced with control EGFP-only retrovirus, the percentage of EGFP<sup>+</sup> cells in the thymus approached that in the BM (Fig. 4*A*, Table I). However, in recipients of Nrp1-overexpressing HSCs, the percentage of EGFP<sup>+</sup> thymocytes was consistently between 0.2% and 3% of that found in the BM (Fig. 4*A*). The ratio of EGFP<sup>+</sup>:EGFP<sup>-</sup> thymocytes was normalized to the ratio of EGFP<sup>+</sup>:EGFP<sup>-</sup> B220<sup>-</sup> BM cells. On average, in mice receiving empty vector transduced HSCs, the normalized ratio was close to 1, indicating that there was no change in the EGFP<sup>+</sup>:EGFP<sup>-</sup> ratio between BM cells and thymocytes. In contrast, in mice receiving Nrp1-transduced HSCs, the normalized ratio was 0.01 on average (Table I). The data suggest that overexpression of Nrp1 significantly inhibits commitment of HSCs to the T lineage.

We examined the effect of Deltex-1 because like Nrp1, Deltex-1 is a transcriptional target of Notch signaling in T cells (Refs.

17 and 21 and Fig. 3*C*). In line with previously published work, introduction of Deltex-1 into HSCs resulted in decrease of the percentage of EGFP<sup>+</sup> thymocytes compared with the percentage of EGFP<sup>+</sup> BM cells or spleen (18). The ability of Deltex-1 to block T lineage commitment was comparable to Nrp1 (Fig. 4*A*, Table I).

In splenocyte populations from recipient mice, Nrp1 overexpression in HSCs resulted in a significant decrease in EGFP<sup>+</sup>:EGFP<sup>-</sup> ratios of mature T cells. In contrast, there was no significant change in the EGFP<sup>+</sup>:EGFP<sup>-</sup> ratios of B cells or myeloid cells in Nrp1 or MIGR1 expressing BM recipients (data not shown). The effect of enforced expression of Deltex-1 on the development of splenic T cells, B cells, and myeloid cells was similar to that of Nrp1 in that Deltex-1 appeared to only perturb commitment to the T lineage (18). Analysis of EGFP<sup>+</sup> BM cells using B220, IgM, and CD43 to separate B lineage cells into developing subsets (29) revealed no perturbations in B cell development by Nrp1 or Deltex-1 overexpression (data not shown). These experiments are consistent with *in vitro* observations that overexpression of Nrp1 inhibits Notch1 signaling and that Notch1 signaling is a critical step in T cell commitment, but does not play a significant role in development of other HSC-derived lineages.

Mice that received either Nrp1- or Deltex-transduced HSCs had small percentages of EGFP<sup>+</sup> thymocytes (Fig. 4*A*). This small



**FIGURE 4.** Overexpression of Nrarp in HSCs specifically blocks T cell commitment. *A*, Deficiency of EGFP<sup>+</sup> thymocytes from mice receiving Deltex-1- or Nrarp-overexpressing HSCs. The indicated organs were harvested and then analyzed for EGFP<sup>+</sup> cells by flow cytometry. Percentages of EGFP<sup>+</sup> cells in thymus or spleen are compared with cells in BM. *B*, Thymocyte development in mice overexpressing Deltex-1 and Nrarp. Thymocytes from host mice receiving BM cells transduced with the indicated retroviral construct were stained for CD4 and CD8 expression. Cells were gated on expression of EGFP. Representative data from multiple experiments are shown.

population of cells was examined for developing thymocyte populations by staining with CD4 and CD8. Ratios of CD4<sup>+</sup> or CD8<sup>+</sup> SP subsets relative to the DP subset were comparable to the internal EGFP<sup>-</sup> negative control and vector-only transduced HSCs (Table II). Thus, Deltex-1 or Nrarp overexpression appeared to have no effect on development of SP cells from DP cells (Fig. 4*B* and Table II). However, Nrarp or Deltex-1 overexpression resulted in a significant increase in the percentage of DN thymocytes, relative to the internal EGFP<sup>-</sup> control and vector-only transduced HSCs (Fig. 4*B* and Table II) as has been previously observed.

#### *Nrarp but not Deltex overexpression blocks DN thymocyte development*

To determine whether there was a partial block in the development of the DN subsets, B220<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup> thymocytes were stained with CD44 and CD25 to segregate the DN1–4 subsets. Percentages of Deltex-1-overexpressing DN thymocyte subset populations were comparable to MIGR1 expressing thymocytes (Table II).

However, Nrarp overexpression resulted in a slight but significant increase of EGFP<sup>+</sup> cells in the DN1 and DN2 subsets compared with vector alone (Table II). The increase in the DN2 subset is consistent with Nrarp blocking the Notch signal at the DN2 to DN3 transition (30).

The significant increase in DN1 cells suggested a potential block in the DN1 to DN2 transition. To address which DN population could be blocked, Rag-2-deficient HSCs were transduced with MIGR1, Deltex-1, or Nrarp. Because these mice cannot rearrange their TCR loci, their thymocytes are arrested at the DN3 stage and cannot progress into DP. Overexpression of Nrarp or Deltex in Rag-2<sup>-/-</sup> HSCs resulted in decreased percentage of EGFP<sup>+</sup> cells in the thymus, relative to the percentage of transduced cells in the BM and the level of EGFP expression in thymocytes expressing Deltex is much lower than the level in BM (Fig. 5*A*). In host mice receiving Rag-2<sup>-/-</sup> HSCs transduced with control EGFP-only retrovirus, the percentage of EGFP<sup>+</sup> cells in the thymus approached that in the BM (Fig. 5*A*).

We analyzed the DN subsets expressing EGFP, Deltex-1, or Nrarp generated from Rag-2<sup>-/-</sup> HSC. Control MIGR1 virus had no effect on development of the Rag-2<sup>-/-</sup> DN subsets (Fig. 5*B*). The DN subset profile of Deltex-1-overexpressing thymocytes that had escaped the initial block in T cell commitment and that expressed lower levels of EGFP was similar to the DN profile of the control (Fig. 5*B*). However, in contrast to enforced expression of empty virus or Deltex-1 in Rag-2<sup>-/-</sup> HSCs, Nrarp overexpression resulted in the accumulation of DN1 and DN2 cells (Fig. 5*B*), relative to thymocytes expressing empty virus or Deltex-1. On average, we observed an 18-fold increase in the percentage of Nrarp-overexpressing DN1 cells over EGFP<sup>+</sup> MIGR1 expressing control cells. Also, the percentage of Nrarp-overexpressing DN2 cells is increased nearly 12-fold over EGFP<sup>+</sup> MIGR1 expressing control cells. This observation suggests that, in addition to its effect on T vs B lineage commitment, overexpression of Nrarp blocked thymocyte development at the DN1 to DN2 and the DN2 to DN3 transitions.

## Discussion

Overexpression of Nrarp in cultured cell lines and in BM stem cells results in inhibition of Notch1-IC signaling. In mouse AKR1010 T lineage and 3T3 fibroblast cell lines, Nrarp overexpression inhibited the ability of Notch1-IC to activate CBF-1. In mouse HSCs, the effect of Nrarp inhibition of Notch signaling was examined at various stages of T cell development. When compared with the effect of Deltex-1 overexpression that only blocked T lineage commitment, Nrarp was able to block T lineage commitment and progression through the DN stages of thymocyte development.

Nrarp is expressed in the thymus, but at a low level relative to other tissues. Because of the importance of Notch signaling in T cell development and because of the inhibitory ability of Nrarp on Notch-1 signaling, Nrarp expression may be highly regulated in

Table I. Normalized ratios of EGFP<sup>+</sup> to EGFP<sup>-</sup> cells in various tissues of reconstituted mice<sup>a</sup>

Population	MIGR1 (n = 8)	Nrarp (n = 10)		Deltex (n = 7)	
	Ratio	Ratio	p	Ratio	p
Thymocytes	1.2 ± 0.32	0.013 ± 0.0026	0.0004	0.11 ± 0.07	0.03
BM B cells (B220 <sup>+</sup> )	2.1 ± 1.3	1.3 ± 0.37	0.4	1.24 ± 0.16	0.37
Splenocytes	1.2 ± 0.42	1.0 ± 0.24	0.35	1.6 ± 0.37	0.21

<sup>a</sup> The ratio of EGFP<sup>+</sup>:EGFP<sup>-</sup> cells in the indicated populations was normalized to the ratio of EGFP<sup>+</sup>:EGFP<sup>-</sup> of B220<sup>-</sup> bone marrow cells. The average of the normalized ratios from the indicated number of mice was calculated. SE and statistical significance (*t* test) compared with MIGR1-transduced HSCs is shown.

Table II. Comparison of EGFP<sup>+</sup> thymic subpopulations<sup>a</sup>

Thymic Subpopulation	MIGR1 (n = 8)		Nrarp (n = 10)		Deltex (n = 6)	
	EGFP <sup>+</sup>		EGFP <sup>+</sup>	p	EGFP <sup>+</sup>	p
DN	3.0 ± 0.20		24.2 ± 4.0	0.0006	13.2 ± 2.8	0.02
DP	81.8 ± 1.3		62 ± 3.6	0.00004	67 ± 4.6	0.02
CD4	11.9 ± 1.3		8.9 ± 1.0	0.06	13.3 ± 2.1	0.56
CD8 (CD3 <sup>high</sup> )	2.6 ± 0.53		3.3 ± 0.52	0.36	4.6 ± 0.76	0.06
CD8 (CD3 <sup>low</sup> )	0.8 ± .13		1.5 ± 0.27	0.01	1.8 ± 0.33	0.01
DN1	7.8 ± 1.5		15 ± 3.1	0.05	9.6 ± 2.1	0.48
DN2	3.5 ± 0.86		5.4 ± 0.78	0.002	4.0 ± 1.1	0.33
DN3	38 ± 2.3		48 ± 8.5	0.64	53.5 ± 3.4	0.14
DN4	51 ± 5.1		31 ± 5.5	0.81	33 ± 3.2	0.09

<sup>a</sup> Cells were gated on expression of EGFP. The percentage of each thymic subpopulation, as defined by CD4 and CD8 or CD44 and CD25 expression, was averaged from the indicated number of mice. The percentage of CD3<sup>high</sup> and CD3<sup>low</sup> cells was determined, then used to calculate CD8 SP subpopulations. SE and statistical significance (*t* test) of the EGFP<sup>+</sup> population compared with the internal EGFP<sup>-</sup> control were calculated.

developing thymocytes. Unlike other transcriptional targets of Notch, Nrarp was higher in DP, relative to DN. This expression pattern suggests that Nrarp may be part of a negative regulatory pathway because Notch signaling is low in DP, based on the levels of CBF-1 target gene transcription (17). In addition, Nrarp expression could be controlled by other factors *in vivo*, which could account for its expression pattern in thymocytes. Supporting this hypothesis is the observation that Nrarp was detectable in many adult tissues where Notch signaling is not thought to be active (21).

We and others have used the technique of overexpression of Notch-1, Nrarp, and/or Deltex-1 as a tool to study Notch-1 signaling *in vivo* and *in vitro* (18, 22, 27). *In vivo* expression of Nrarp or Deltex-1 has been reproducibly demonstrated to mirror the effects of Notch-1 signal inhibition. Studying how these molecules could interact with the Notch-1 signaling components is facilitated using reporter gene assays in cultured cells. However, in this context overexpression of these molecules has yielded conflicting results. It is likely that in cultured cells, depending on the cellular context, enforced expression of Notch-1, Deltex-1, and/or Nrarp effect variable outcomes on CBF-1 activation. Enforced coexpression of multiple molecules at nonphysiologic levels yield data that suggest whether the molecules could interact in a common signaling pathway.

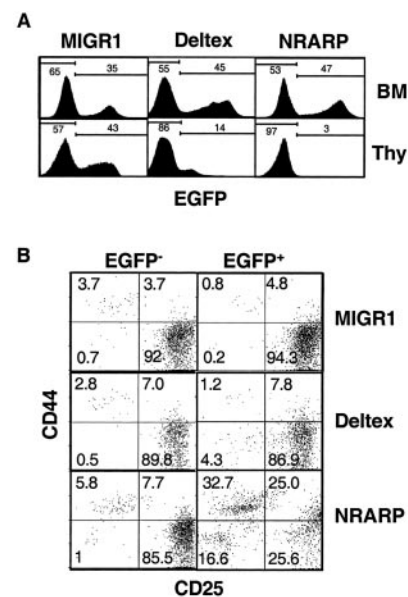
In AKR1010 cells, overexpression of Nrarp inhibited several phenotypic changes dependent on Notch1 signaling, such as surface expression of CD3, resistance to glucocorticoid-induced apoptosis, and induction of CBF-1 target genes. Transient cotransfection of 3T3 cells revealed that expression of Nrarp could block the ability of Notch1-IC to activate a CBF-1 reporter. Taken together, Nrarp overexpression is inhibitory for Notch1 function in AKR1010 and 3T3 cell lines, HSCs, and *Xenopus* embryos (22). In contrast, overexpression of Nrarp in HeLa cells synergizes with Notch1-IC to activate CBF-1 (22). Thus, the effect of Nrarp on Notch1-IC may depend on cellular context.

Nrarp overexpression did not completely abrogate Notch1 signaling. For example, resistance to glucocorticoid-induced apoptosis was partially blocked and transcription of CBF-1 target genes was reduced, but neither was reduced to the level of control cells lacking Notch1-IC. Because a small percentage of Nrarp-overexpressing cells escaped the block at the T vs B decision, we were able to investigate the effect of enforced expression of Nrarp throughout thymocyte differentiation, in contrast to conditional deletion of Notch1 that causes an absolute loss of signal at one stage. Overexpression of Nrarp in HSCs inhibited T lineage commitment and impeded progression through the DN stages. Because Nrarp affects Notch1 activation of CBF-1, these observations suggest that

CBF-1 is required for commitment from the HSCs to the T lineage and for development of the DN stages of thymocyte development.

Previous studies using conditional deletion of Notch1 (8) or inhibition of Notch signaling (16, 18) reported an accumulation of CD44<sup>+</sup>CD25<sup>-</sup> cells in the thymus. However, because these cells were also B220<sup>+</sup>, they were not DN1 thymocytes. In line with previous reports, Nrarp or Deltex-1 overexpression in HSCs resulted in ectopic thymic B cell development in some mice receiving Nrarp-transduced or Deltex-1-transduced BM. Because our interest was to investigate the effect of Nrarp or Deltex-1 overexpression on thymocyte development, we focused on the EGFP<sup>+</sup>B220<sup>-</sup> thymic populations.

Enforced expression of Nrarp in B6 and Rag-2<sup>-/-</sup> cells caused an increase of DN1 thymocytes. This observation suggests that



**FIGURE 5.** Nrarp, but not Deltex-1, impedes development of Rag-2<sup>-/-</sup> thymocytes. BM cells were isolated from Rag-2<sup>-/-</sup> donor mice, transduced with the indicated retroviral construct, and transplanted into irradiated, B6 host mice. Four week after transplant, organs were harvested from host mice and analyzed by flow cytometry. Representative data from multiple experiments are shown. *A*, Deficiency of EGFP<sup>+</sup> Rag-2<sup>-/-</sup> thymocytes from mice receiving Deltex-1- or Nrarp-overexpressing Rag-2<sup>-/-</sup> HSCs. *B*, Thymocytes from host mice were stained for expression of CD4, CD8, B220, CD44, and CD25. CD4<sup>-</sup>CD8<sup>-</sup>B220<sup>-</sup> cells were gated on DN1–3 populations. Percentages of thymocytes in each population of EGFP<sup>-</sup> and EGFP<sup>+</sup> fractions were calculated.



Notch1 signaling is important for progression of DN1 to DN2, in addition to the DN2 to DN3 transition. This requirement for a Notch1 signal at this stage is a developmental stage earlier than that observed by conditional deletion of Notch using an *lck-cre* transgene (30). In that report, Notch1 signaling was eliminated during thymocyte development from DN2 to DN3 to DP. Taken together, the evidence supports the hypothesis that Notch signaling is required for maintenance of T lineage commitment from the CLP through the DN stages, until rearrangement of TCR  $\beta$  locus can produce a functional TCR  $\beta$ -chain. Our data do not rule out the formal possibilities that Deltex-1 or Nrarp overexpression affect processes independent of Notch-1 signaling. For example, overexpression of either molecule could affect the ability of progenitors to migrate to the thymus or could inhibit other aspects of thymocyte maturation. However, our data are consistent with the observed effects of conditional deletion of Notch-1 during thymocyte maturation.

Nrarp inhibition of Notch signaling phenotypically resembles CBF-1<sup>-/-</sup> and HES-1<sup>-/-</sup> thymocytes (31, 32). Hes-1 is a basic helix-loop-helix transcription factor that is transcriptionally regulated by Notch (Refs. 1 and 27 and Fig. 3C). In their report, Tomita et al. (32) observed thymocyte development arrested either at DN1 or DN3 stage. Inducible deletion of CBF-1 resulted in a B220<sup>-</sup> thymocyte population arrested at DN1 (31). Collectively, these studies support the argument that Nrarp blocks Notch1 activation of CBF-1 in vivo.

Confirming the results of Izon et al. (18), overexpression of Deltex-1 in HSCs inhibited T lineage commitment. Despite the fact that Deltex-1 is up-regulated in DN thymocytes (17, 18), Deltex-1 overexpression did not have any functional consequence past commitment to the T lineage, particularly at DN1 through DN3 stages of thymocyte development, where enforced Nrarp expression can impede development. Although the molecular mechanism by which Deltex-1 affects Notch signaling has not been clearly defined, Izon et al. (18), demonstrated that Deltex-1 could inhibit Notch1 signaling by disrupting assembly of specific coactivators to the transactivation domain of Notch1. One possible explanation for the difference between the effects of Deltex and Nrarp overexpression is that the Notch signal that mediates T lineage commitment is more sensitive to perturbation than the Notch signal required for maintenance of thymocyte development. In this model, Deltex-1 may be a "weak" inhibitor and Nrarp may be a "strong" inhibitor of Notch1 signaling.

However, evidence to date suggests that Deltex-1 appears to modify a different Notch1 signaling pathway (4, 18). Although Nrarp clearly inhibits CBF-1 (22), in our hands, Deltex-1 did not have a similar effect in vitro. Although studies of Notch1 signaling have predominantly focused on the ability of activated Notch1 to regulate the transcriptional activity of CBF-1, there is accumulating evidence for the existence of another Notch signaling pathway that is independent of CBF-1 activation (4, 33–35). Several groups have implicated Deltex-1 as a regulator of a CBF-1 independent pathway that affects multiple developmental systems, such as myogenesis and *Drosophila* eye development (4, 36, 37). Furthermore, in overexpression systems, Deltex-1 has been demonstrated to affect activity of the E protein transcription factors (4, 18, 36, 37). Thus, another possible model is that T vs B lineage choice is dependent on two Notch signaling pathways. One pathway involving E protein activity is modulated by Deltex-1 overexpression and Nrarp inhibits the other, probably by blocking CBF-1 activation. It is also possible that in HSCs, components of the Deltex-1 sensitive pathway can be transcriptionally controlled by CBF-1. Further work is necessary to determine whether the Deltex-1 sensitive

pathway signals parallel and independent from the CBF-1 pathway during T vs B lineage choice.

Because Notch signaling is critical in the T vs B decision of the CLP and in early DN development, understanding the regulation of Notch signaling is important in studying thymocyte maturation. Our data argue that differentiation of HSCs to the T lineage is mediated by multiple Notch signaling pathways and that Deltex-1 and Nrarp could affect Notch signaling by modifying the function of distinct molecules in HSCs. However, the Deltex-1 sensitive Notch signaling pathway has minimal affect on early thymocyte development.

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