

# Pre-TCR signaling inactivates Notch1 transcription by antagonizing E2A

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Precise control of the timing and magnitude of Notch signaling is essential for the normal development of many tissues, but the feedback loops that regulate Notch are poorly understood. Developing T cells provide an excellent context to address this issue. Notch1 signals initiate T-cell development and increase in intensity during maturation of early T-cell progenitors (ETP) to the DN3 stage. As DN3 cells undergo  $\beta$ -selection, during which cells expressing functionally rearranged TCR $\beta$  proliferate and differentiate into CD4<sup>+</sup>CD8<sup>+</sup> progeny, Notch1 signaling is abruptly down-regulated. In this report, we investigate the mechanisms that control Notch1 expression during thymopoiesis. We show that Notch1 and E2A directly regulate *Notch1* transcription in pre- $\beta$ -selected thymocytes. Following successful  $\beta$ -selection, pre-TCR signaling rapidly inhibits *Notch1* transcription via signals that up-regulate Id3, an E2A inhibitor. Consistent with a regulatory role for Id3 in *Notch1* down-regulation, post- $\beta$ -selected Id3-deficient thymocytes maintain *Notch1* transcription, whereas enforced Id3 expression decreases *Notch1* expression and abrogates Notch1-dependent T-cell survival. These data provide new insights into *Notch1* regulation in T-cell progenitors and reveal a direct link between pre-TCR signaling and *Notch1* expression during thymocyte development. Our findings also suggest new strategies for inhibiting Notch1 signaling in pathologic conditions.

[*Keywords:* Notch; T cell; lymphocytes; leukemia]

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Notch1 controls multiple essential functions during thymocyte development (Maillard et al. 2005; Ciofani et al. 2006). Notch1 signals initiate the generation of the earliest intrathymic T cells from multipotent hematopoietic progenitors (Radtke et al. 1999; Sambandam et al. 2005). Subsequently, Notch1 is required for  $\alpha\beta$  T-cell development through  $\beta$ -selection, an important checkpoint during which immature thymocytes expressing functionally rearranged TCR $\beta$  proliferate and then differentiate into quiescent CD4<sup>+</sup>CD8<sup>+</sup> cells. Conditional inactivation of *Notch1*, *Rbpj*, or inhibition of Notch signaling by dominant-negative Mastermind-like 1 ((DNMAML) arrests T-cell development at the DN3 stage, prior to  $\beta$ -selection (Wolfer et al. 2002; Tanigaki et al. 2004; Maillard et al. 2006). In vitro studies using OP9 feeder cells have shown that both Notch1 and pre-TCR signals are required to traverse the  $\beta$ -selection

checkpoint (Ciofani et al. 2004); Notch1 provides important differentiation, survival, proliferation, and metabolic signals during this juncture in T-cell development (Ciofani and Zuniga-Pflucker 2005; Campese et al. 2006).

Following  $\beta$ -selection, Notch signaling and *Notch1* expression are abruptly down-regulated. CD27 expression can be used to separate DN3 cells into two distinct populations, DN3a and DN3b (Taghon et al. 2006). The pre- $\beta$ -selection CD27<sup>-</sup>DN3a population is Notch-dependent, whereas post- $\beta$ -selection CD27<sup>+</sup>DN3b cells do not require Notch signals for further intrathymic differentiation or survival. Significantly, *Notch1* expression is high in DN3a cells and low in DN3b cells (Taghon et al. 2006; Weng et al. 2006).

Although the mechanism of *Notch1* down-regulation in  $\beta$ -selected cells is poorly understood, high levels of Notch signaling post- $\beta$ -selection may be oncogenic. For example, expression of the Notch1 intracellular domain (ICN1) driven by either a retroviral vector (Izon et al. 2002; Li et al. 2008) or a Lck transgene (Deftos et al. 2000; Fowlkes and Robey 2002) allows sustained Notch

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activity past the DN3 stage that is associated with increased proliferation and survival, a developmental block, and acute lymphoblastic T-cell leukemia (T-ALL). These findings emphasize the importance of precise control of Notch1 signaling at the  $\beta$ -selection checkpoint.

E-proteins, which include E12, E47, E2-2, and HEB in mammals, encode a class of widely expressed basic helix-loop-helix (bHLH) transcription factors that are critical for B-cell development and play important roles in thymocyte development (Murre 2005). E12 and E47 (collectively termed E2A) are encoded by one gene, *Tcfe2a*, and are generated through alternative splicing (Murre et al. 1989), whereas E2-2 and HEB are encoded by distinct genes (Murre 2005). The primary E-protein complex in thymocytes is a E47/HEB heterodimer (Sawada and Littman 1993; Barndt et al. 2000). The functions of E-proteins in thymocyte development have been revealed through several loss-of-function approaches. E2A knockout mice exhibit an incomplete block in early T-cell development at the DN1 stage (Bain et al. 1997), whereas HEB knockout mice display reduced thymic cellularity and increased immature single positive (ISP) cells (Barndt et al. 1999). Expression of a HEB dominant-negative protein causes a more severe decline in thymocyte numbers and an earlier block in T-cell development than HEB knockout mice, as this antagonist prevents compensation by other E-proteins (Barndt et al. 2000). Enforced expression of the E-protein antagonist Inhibitor of DNA binding 3 (Id3) in human T-lineage precursor cells blocks T-cell lineage differentiation from CD34<sup>+</sup> progenitors (Heemskerk et al. 1997). Like Notch, E2A activity is dynamically regulated during thymocyte development. E2A is active prior to  $\beta$ -selection, whereupon pre-TCR signals up-regulate *Id3* expression to reduce the DNA-binding activity of E2A in DP or DN thymocytes (Bain et al. 2001; Engel et al. 2001; Xi et al. 2006).

Emerging data suggest cross-talk between E2A and Notch signals during T-cell development (Ikawa et al. 2006; Rothenberg et al. 2008). Expression of several genes that are important in T-cell development, such as Hes1 and pT $\alpha$ , are coregulated by Notch and E2A, and both *Notch1* and *Notch3* mRNA levels are decreased in E47-deficient fetal thymocytes (Takeuchi et al. 2001; Ikawa et al. 2006). Furthermore, retroviral ICN1 expression in E2A<sup>-/-</sup> fetal thymocyte progenitors rescues the developmental arrest caused by E2A deficiency (Ikawa et al. 2006). Although they provide synergistic functions, the precise nature of the interactions between Notch and E2A have not been determined.

In this study, we investigate the mechanism underlying the dynamic regulation of Notch1 during  $\beta$ -selection. We show that prior to  $\beta$ -selection, Notch1 and E2A bind the *Notch1* locus and promote *Notch1* transcription in DN3 cells. At  $\beta$ -selection, MAPK-dependent pre-TCR signals up-regulate Id3 expression, which inhibits E2A binding to the *Notch1* promoter and decreases *Notch1* expression. Consistent with this model, loss of Id3 expression enhances *Notch1* expression in post- $\beta$ -selected thymocytes, whereas loss of E2A decreases *Notch1* expression in pre- $\beta$ -selected thymocytes in a dose-dependent manner.

Furthermore, enforced Id3 expression inhibits *Notch1* expression and Notch1-dependent cell survival in Notch1-dependent T-cell lines. Together, these data reveal a direct link between pre-TCR signaling and Notch1 expression during thymocyte development and provide new strategies to disable *Notch1* expression and signaling.

## Results

### *Notch1* directly autoregulates its own transcription

To investigate the regulation of Notch1 expression during thymocyte development, we quantified *Notch1* mRNA in thymocyte subsets. A progressive increase in *Notch1* mRNA occurred from the earliest intrathymic T-cell progenitors (ETP/DN1) to the DN3 stage (Fig. 1A). This was followed by an abrupt decrease in *Notch1* mRNA after the DN3 stage, whereupon *Notch1* mRNA remained low throughout intrathymic T-cell development (Fig. 1A). These data confirm previous observations (Huang et al. 2003; Taghon et al. 2006; Weng et al. 2006).

To understand the basis for this dynamic pattern of Notch1 expression during early thymocyte development, we investigated how *Notch1* transcription is regulated. We previously found an approximately fourfold decrease in endogenous mouse *Notch1* mRNA when oncogenic Notch1 signals were blocked in T-cell leukemia cell lines (Weng et al. 2006), suggesting that Notch1 autoregulates its own transcription. To investigate this possibility, we searched for putative CSL (the protein encoded by *Rbpj*)-binding sites (CCTGGGAA or STGGGAR) using the VISTA program (Couronne et al. 2003). We found two CSL-binding sites that are conserved in mouse and human *Notch1* in intron 1 and at -5 kb, and three non-conserved CSL-binding sites at -1 kb, -6 kb, and -10 kb (Fig. 1B). In a chromatin immunoprecipitation (ChIP) assay with DN3 cells from Rag2<sup>-/-</sup> thymocytes, we observed Notch1 binding at the -6-kb, -5-kb, and intron 1 sites (Fig. 1C). Little binding was found at the -1-kb and -10-kb sites (Fig. 1C).

To determine if these sites are important for *Notch1* autoregulation, a construct containing the entire 6-kb upstream region of the *Notch1* promoter fused to luciferase was studied for its responsiveness to Notch signals (Fig. 1D). Activated Notch1 produced a dose-dependent increase in reporter activity (Fig. 1E). These data demonstrate direct binding of endogenous Notch1 to *Notch1* regulatory sequences in primary T cells and further show that Notch1 has the capacity to directly autoregulate its own promoter.

### *Notch1* mRNA expression is down-regulated by pre-TCR signals in DN3 cells

The marked decrease in *Notch1* mRNA that occurs at the  $\beta$ -selection checkpoint suggests that pre-TCR signals down-regulate *Notch1*. Rag2<sup>-/-</sup> thymocytes are blocked at the DN3 stage due to the inability to rearrange a  $\beta$ -chain to form a pre-TCR, and therefore fail to undergo  $\beta$ -selection. To determine if down-regulation of Notch1 expression at the DN3 stage was associated with pre-TCR

signals, DN3 cells from *Rag2*<sup>-/-</sup> mice were stimulated with PMA and/or ionomycin, which mimic signals generated through the pre-TCR. *Notch1* expression was reduced with PMA and/or ionomycin treatment (Fig. 2A), but PMA stimulation alone reduced *Notch1* expression by nearly the same amount as PMA/ionomycin stimulation, suggesting that signals downstream from PMA, which activates the PKC/Ras/MAPK pathways, are most important.

To further dissect *Notch1* down-regulation at the  $\beta$ -selection checkpoint, DN3 cells were stimulated with PMA for 1 h, 2 h, and 6 h. *Notch1* mRNA expression was inhibited by ~40% at 1 h, ~50% at 2 h, and ~70% at 6 h post-PMA stimulation (Fig. 2B). In contrast to the decrease in *Notch1* mRNA, Notch1 protein amounts were not significantly decreased at 2 h post-PMA treatment (Fig. 2C,D). By 6 h post-PMA treatment, however, Notch1 protein decreased by ~80% (Fig. 2C,D).

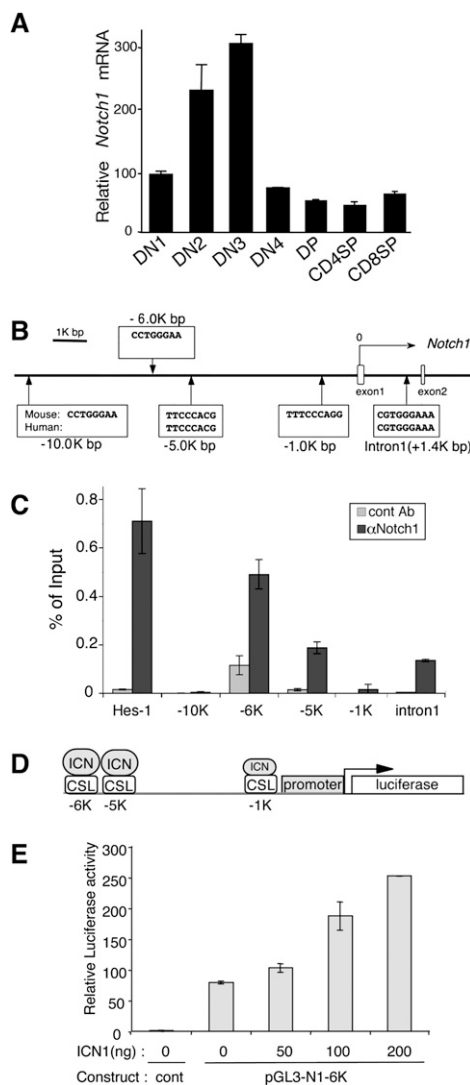
To determine whether pre-TCR signals influenced *Notch1* mRNA or steady-state protein levels, we measured these parameters in the presence of proteasome

and/or MEKK1/2 inhibitors. In the presence of the proteasome inhibitor MG132, Notch1 protein accumulated (Supplemental Fig. S1A, lanes 6–9), and *Notch1* mRNA levels markedly increased (Supplemental Fig. S1B), consistent with Notch1 autoregulation. Even in the presence of elevated levels of Notch1 protein due to MG132 treatment, PMA stimulation decreased *Notch1* mRNA levels (Supplemental Fig. S1B), showing that *Notch1* mRNA is reduced in a proteasome-independent manner. Consistent with an important role for pre-TCR signals in the regulation of *Notch1* mRNA, treatment of *Rag2*<sup>-/-</sup> DN3 cells with the MEKK1/2 inhibitor U-0126 prevented the PMA-induced decrease in *Notch1* mRNA without affecting the steady-state amount of Notch1 protein (Supplemental Fig. S1). Together with the time-course studies, our data suggest that pre-TCR signaling decreased *Notch1* mRNA expression before affecting steady-state protein levels.

To identify whether pre-TCR signals affected pre-spliced *Notch1* transcripts, primary and spliced transcripts were compared in *Rag2*<sup>-/-</sup> DN3 cells. PMA stimulation for 2 h resulted in lower levels of both mature and immature *Notch1* transcripts (Fig. 2E), a time at which Notch1 protein was not significantly decreased (Fig. 2C). Thus, our data demonstrate that pre-TCR signals down-regulate *Notch1* mRNA, which leads to decreased Notch1 protein.

#### Pre-TCR signals block Notch1 transcription via *Id3*

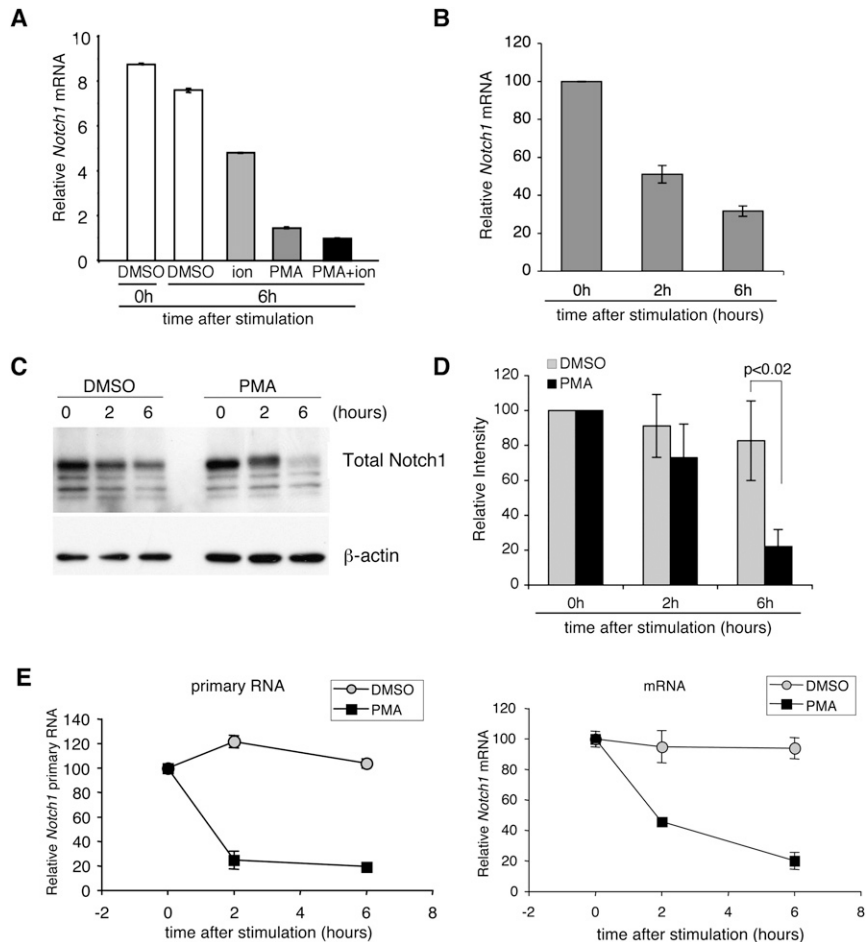
Previous work demonstrated that TCR and pre-TCR signals induce *Id3* via ERK/MAPK signaling (Bain et al. 2001; Engel et al. 2001). These studies showed that TCR ligation reduced E2A DNA-binding activity as a



**Figure 1.** Notch1 autoregulates its transcription via direct binding to CSL sites. (A) *Notch1* expression in thymocyte subsets. Each fraction was sorted from C57BL/6 thymocytes. qPCR was performed and *Notch1* expression relative to *EF1a* is shown as a mean of values from triplicate wells  $\pm$ SD. Notch1 expression in DN1 cells was set at 100, and the expression in other subsets was set relative to this. Data are representative of three independent experiments. (B) Schematic representation of the 5' region of the mouse *Notch1* locus indicating potential CSL-binding sites. The CSL-binding sequences from mouse and human are shown. (C) ChIP analysis to identify Notch1 binding to CSL sites in *Notch1*. *Rag2*<sup>-/-</sup> DN3 thymocytes were used for ChIP analysis. qPCR was performed with primers flanking each putative CSL site. The *Hes1* promoter region was the positive control. Values represent the mean of signal intensity relative to input DNA from triplicate wells  $\pm$ SD. Data are representative of three independent experiments. (D) Schematic representation of the Notch1 promoter sequences used in the luciferase assays. The CSL sites are shown. (E) ICN1 enhances *Notch1* promoter activity. NIH3T3 cells were transiently cotransfected with the *Notch1* promoter construct containing the -6K, -5K, -1K CSL-binding sites, and with either empty vector or pDNA3-ICN1. Luciferase activity relative to Renilla luciferase activity was normalized to the pGL3-basic alone. The data are shown as a mean of values from triplicate wells  $\pm$ SD. Data are representative of at least three independent experiments.

**Figure 2.** Pre-TCR signals down-regulate *Notch1* transcription. (A) *Notch1* mRNA was diminished by PMA treatment in DN3 cells. DN3 cells from *Rag2*<sup>-/-</sup> mice were treated with DMSO and/or PMA (20 ng/mL) and/or ionomycin (500 ng/mL) for 6 h, and qPCR was performed. *Notch1* expression relative to *EF1α* is shown as the mean of values from triplicate wells ±SD. *Notch1* expression in the PMA/ionomycin sample was set at 1, and the values in the other samples were normalized to this. The data are representative of three independent experiments. (B) Time course of *Notch1* mRNA expression following PMA stimulation. DN3 cells from *Rag2*<sup>-/-</sup> mice were treated with PMA (20 ng/mL) for 0 h, 2 h, or 6 h, and qPCR was performed. *Notch1* expression relative to *EF1α* is shown as the mean of values from triplicate wells ±SD. Data are relative to the 0-h sample, whose value was set at 100, and representative of three independent experiments. (C) Time course of Notch1 protein expression following PMA stimulation. *Rag2*<sup>-/-</sup> DN3 thymocytes were sorted and treated with DMSO or PMA (20 ng/mL) for 0 h, 2 h, or 6 h. Notch1 and β-actin expression were determined by Western blotting. Data are representative of three independent experiments. (D) Quantification of Notch1 protein expression after PMA stimulation. Protein amounts were measured by densitometer and quantified by Image Quant. The protein amounts were relative to unstimulated control, and error bars show SD from three independent experiments. (E) PMA-induced

*Notch1* down-regulation is regulated through transcriptional changes. Primary-transcript RT-PCR analysis was performed with DN3 cells from *Rag1*<sup>-/-</sup> mice. Cells were treated with either DMSO or PMA. Primers were designed to flank between *Notch1* exon 27 and exon 28 for mRNA and exon 27 and intron 27 for primary RNA. *Notch1* mRNA or primary RNA expression is relative to *18S rRNA* and is shown as a mean of values from triplicate wells ±SD. Data are relative to the 0-h sample, whose value was set at 100 and is representative of two independent experiments.

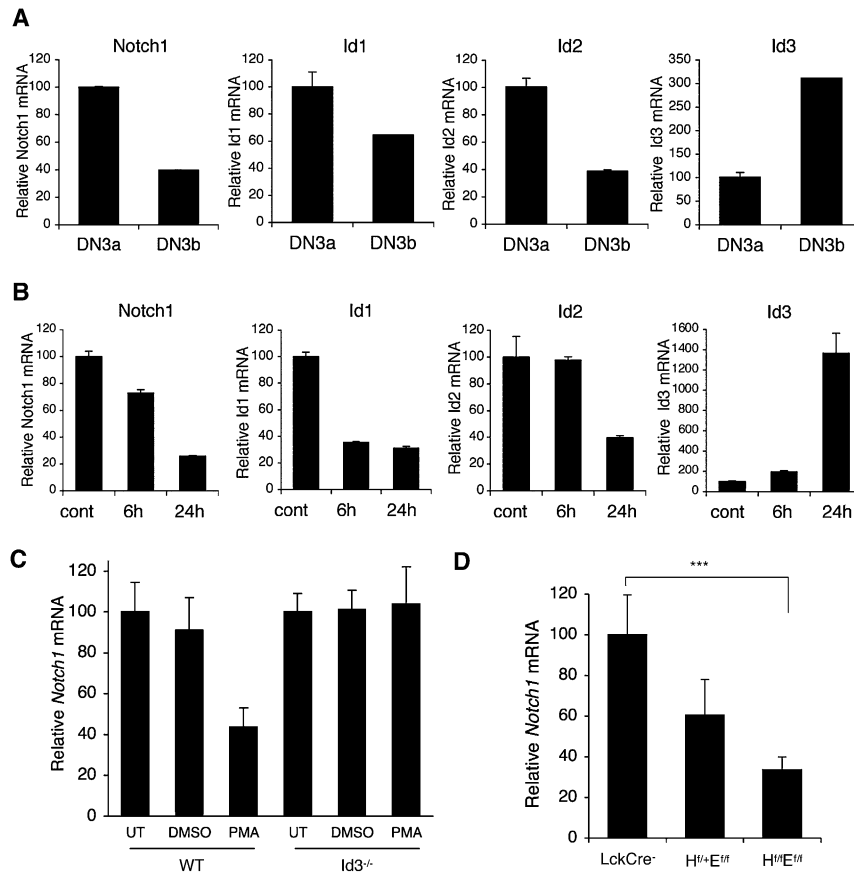


consequence of up-regulated *Id3* expression in CD4<sup>+</sup>CD8<sup>+</sup> DP thymocytes and DN thymocytes. To determine whether pre-TCR stimulation induced *Id3* expression in DN3 cells, we examined *Id3* mRNA expression in both wild-type DN3a and DN3b cells (Fig. 3A) and thymocytes from *Rag2*<sup>-/-</sup> mice stimulated with α-CD3 in vivo (Fig. 3B). Like *Notch1*, the expression of *Id1* and *Id2*, both reportedly Notch targets (Talora et al. 2003; Weng et al. 2006), decreased in DN3b cells (Fig. 3A). In contrast, *Id3* expression was highly up-regulated in post-selected DN3b cells. Consistent with these observations, α-CD3-stimulated DN3 cells showed reduced *Notch1*, *Id1*, and *Id2* expression, whereas *Id3* expression was dramatically increased by 24 h (Fig. 3B).

The inverse relationship between *Id3* and *Notch1* expression suggested that *Id3* mediates pre-TCR-induced inhibition of *Notch1* transcription. To directly test this model, we measured *Notch1* mRNA in PMA-treated *Id3*<sup>-/-</sup> DN3a thymocytes (Fig. 3C). PMA treatment of

*Id3* knockout DN3a cells failed to down-regulate *Notch1*, whereas *Notch1* expression decreased by 60% in PMA-treated DN3a cells from littermate controls. These data show that *Id3* has an important role in mediating the decrease in *Notch1* transcription that occurs at β-selection.

*Id3* functions by inhibiting E-protein-mediated transcriptional activation. Thus, if loss of *Id3* activity prevented the decrease in *Notch1* transcription, loss of E2A activity should result in decreased *Notch1* transcription. HEB and E47, encoded by the genes *Tcf12* and *Tcf2a*, form the major E2A heterodimer in developing T cells (Barndt et al. 2000). We tested the association between E2A signaling and *Notch1* transcription by measuring *Notch1* mRNA in DN3a thymocytes lacking E47 and/or HEB. DN3a thymocytes were purified from conditional knockout mice in which both *E47* and *HEB* were deleted with an Lck-Cre transgene (*Tcf12*<sup>fl/fl</sup>*Tcf2a*<sup>fl/fl</sup>LckCre<sup>+</sup>). These mice exhibit a developmental block at the DN3



**Figure 3.** Expression of *Notch1* inversely correlates with *Id3*. (A) DN3a cells and DN3b cells were sorted from thymocytes of C57BL/6 mice. *Notch1*, *Id1*, *Id2*, and *Id3* expression relative to *EF1 $\alpha$*  is shown as the mean of values from triplicate wells  $\pm$ SD. Data are representative of three independent experiments. (B) *Rag2*<sup>-/-</sup> mice were injected with  $\alpha$ -CD3 or PBS. Thy1.2<sup>+</sup> cells were sorted from the thymocytes 6 h or 24 h post-injection. The transcript quantities relative to *EF1 $\alpha$*  are shown as the mean of values from triplicate wells  $\pm$ SD. Data are representative of three independent experiments. (C) DN3a cells were sorted from C57BL/6 or *Id3*<sup>-/-</sup> thymocytes and stimulated with DMSO or PMA for 2 h. *Notch1* expression relative to *EF1 $\alpha$*  was determined by qPCR. Data are relative to untreated samples (UT) of each mouse and represent the mean of three independent experiments  $\pm$ SD. (D) Dose-dependent *Notch1* expression in E2A/HEB double-deficient thymocytes. *Notch1* expression relative to *EF1 $\alpha$*  was determined from DN3a cells of *Tcf12*<sup>fl/fl</sup>*Tcfe2a*<sup>fl/fl</sup>LckCre<sup>-</sup> (LckCre<sup>-</sup>), *Tcf12*<sup>fl/fl</sup>*Tcfe2a*<sup>fl/fl</sup>LckCre<sup>+</sup> (H<sup>fl</sup>/E<sup>fl</sup>), *Tcf12*<sup>fl/fl</sup>*Tcfe2a*<sup>fl/fl</sup>LckCre<sup>+</sup> (H<sup>fl</sup>/E<sup>fl</sup>) thymocytes. Data are from triplicates of two independent experiments ( $n = 3$ ) and relative to the LckCre<sup>-</sup> sample. (\*\*\*)  $P < 0.05$ , Student's *t*-test, two-tailed. Graphed results are means with error bars representing the standard error of the mean (SEM).

stage (Wojciechowski et al. 2007). The genotypes of the floxed mice were either *Tcf12*<sup>fl/+</sup>*Tcfe2a*<sup>fl/fl</sup>LckCre<sup>+</sup> or *Tcf12*<sup>fl/fl</sup>*Tcfe2a*<sup>fl/fl</sup>LckCre<sup>+</sup>, which provided the opportunity to compare the effects of different E-protein levels on *Notch1* expression. The amount of *Notch1* expression correlated with the amount of E2A (Fig. 3D). Thus, the complementary results provided by the *Id3* and E2A loss-of-function studies suggest that E2A is an important regulator of *Notch1* transcription.

#### E2A directly activates *Notch1* transcription

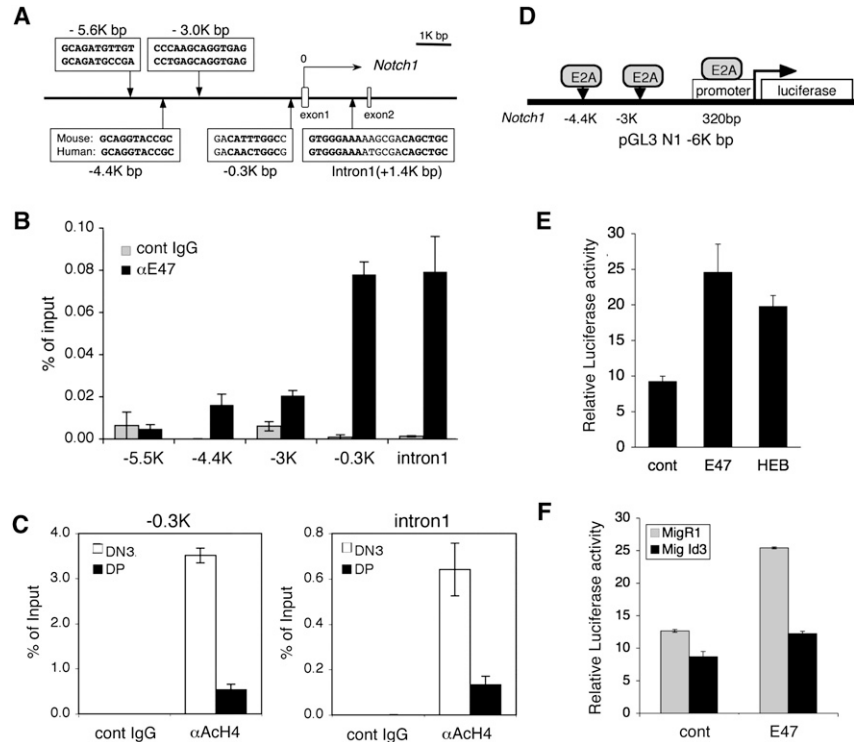
To investigate whether E2A directly regulates *Notch1* transcription, we searched for canonical E-box sites (CANNTG) in the *Notch1* locus. Five binding sites conserved between human and mouse were found in a region spanning from 6 kb upstream of the transcriptional start to intron 1 of the *Notch1* gene (Fig. 4A). We performed ChIP assays with DN3 cells from *Rag2*<sup>-/-</sup> thymocytes and observed strong E47 binding to the -0.3-kb and intron 1 sites (Fig. 4B). Binding was minimal at the -4.4-kb and -3-kb sites and absent at the -5.5-kb site of *Notch1*. Histone 4 (H4) was hyperacetylated at both the -0.3-kb and intron 1 sites in DN3 cells but not in DP cells, indicating that these are likely to be regions of open chromatin in DN3 cells but not in DP cells (Fig. 4C).

To evaluate the importance of these regions in *Notch1* promoter activity, we investigated whether E47 up-regulated luciferase activity in our *Notch1* reporter that contains ~6 kb of the *Notch1* promoter region, which includes the E-box sites (Fig. 4D). Both E47 and HEB increased *Notch1* reporter activity (Fig. 4E). Furthermore, the E47-mediated increase in *Notch1* reporter activity was abrogated by *Id3* (Fig. 4F). These data demonstrate that E47 and HEB directly activate *Notch1* transcription.

#### Pre-TCR signals inhibit binding of E47 and *Notch1* to the *Notch1* promoter

Our data demonstrate that both E2A and *Notch1* are direct positive regulators of *Notch1* transcription. At the  $\beta$ -selection checkpoint, pre-TCR signals induce *Id3*, which blocks E2A, leading to decreased *Notch1* transcription and therefore decreased *Notch1* protein resulting in a block in the positive feedback loop. Thus, we propose that pre-TCR signaling is the switch that extinguishes *Notch1* transcription (Fig. 5A). Since PMA and/or PMA/ionomycin treatment of primary DN3 cells markedly reduced *Notch1* mRNA amounts within several hours (Fig. 2A), the events proposed in our model should occur within this time frame. In order to obtain sufficient protein for these studies, we tested this hypothesis in Scid-adh cells. Scid-adh cells phenotypically resemble

**Figure 4.** E47 directly up-regulates *Notch1* transcription. (A) Schematic representation of the 5' region of the *Notch1* locus with putative E2A-binding sites. The translational start site is labeled "0." Only the first two *Notch1* exons are shown. (B) E47 binds to E2A-binding sites of *Notch1*. Rag2<sup>-/-</sup> DN3 thymocytes were subjected to ChIP analysis. qPCR was performed with primers flanking putative E2A-binding sites at 5.5, 4.4, 3, and 0.3 kb upstream of the transcriptional start site as well as intron 1 of *Notch1*. Graphs represent the mean of the ratio of the amount of immunoprecipitated DNA/input from values from triplicate wells  $\pm$ SD. Data are representative of two independent experiments. (C) Differential binding activity of acetylated histone 4 to the *Notch1* locus in DN3a and DP cells. ChIP analysis to identify acetylated Histone 4 was performed on DN3a and DP cells from wild-type B6 mice. qPCR was performed to detect E2A-binding sites at -0.3 kb and intron 1 of *Notch1*. Data are representative of two independent experiments. (D) Schematic of the *Notch1* promoter luciferase construct including the E2A-binding sites. (E) E47 and HEB transactivate the *Notch1* promoter. NIH3T3 cells were transfected with the *Notch1* promoter construct containing the indicated E2A-binding sites and with the empty vector, E47, or HEB. Luciferase activity relative to Renilla luciferase activity is shown as the mean of values from triplicate wells  $\pm$ SD. Data are representative of two independent experiments. (F) Id3 inhibits E47-induced transactivation of the *Notch1* promoter. NIH3T3 cells were transfected with either MigR1 or Id3. At 18 h, cells were transiently cotransfected with the *Notch1* promoter construct with empty vector or E47. Luciferase activity relative to Renilla luciferase activity is shown as a mean of values from triplicate wells  $\pm$ SD. Data are representative of three independent experiments.

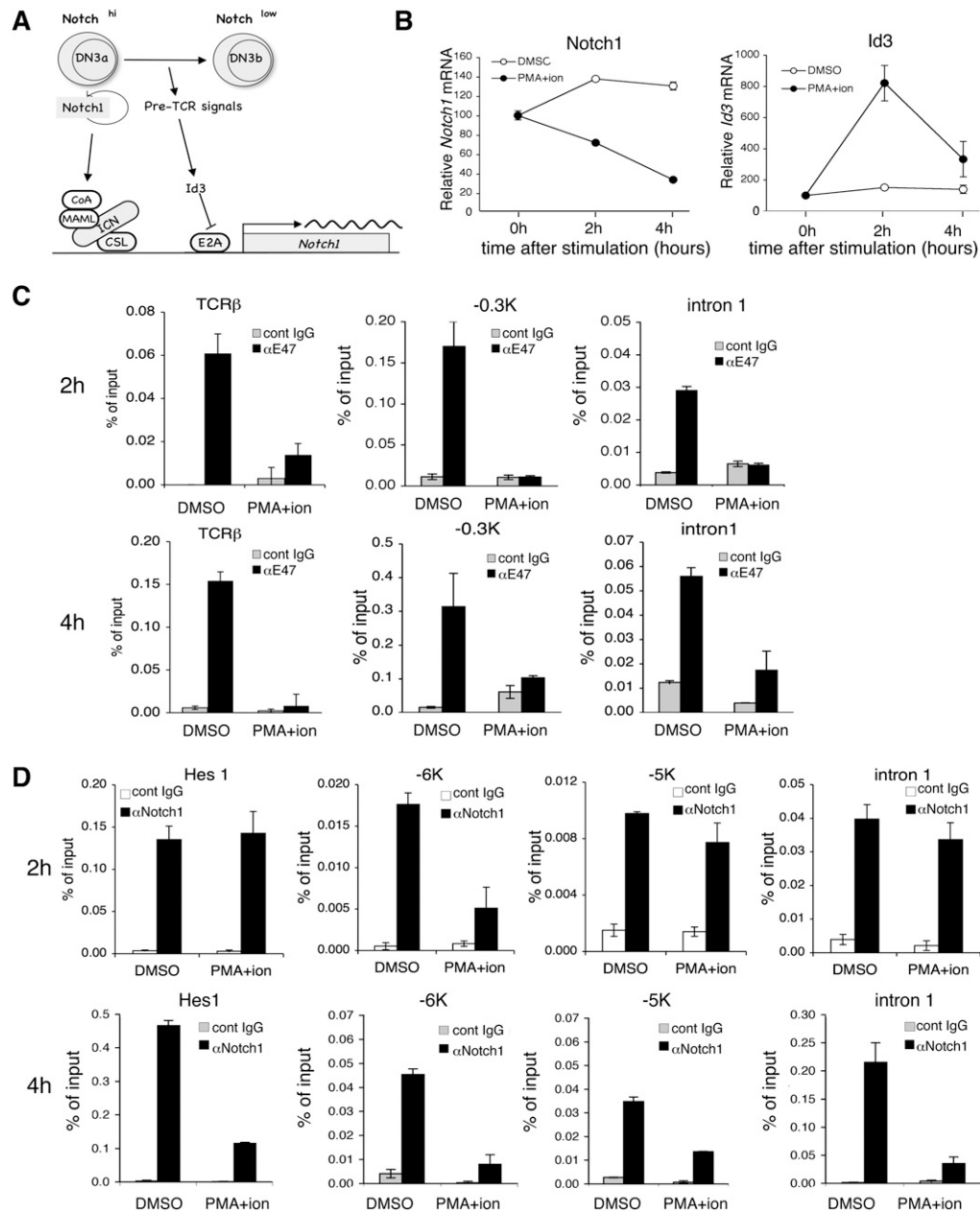


DN3 thymocytes and respond to stimuli mimicking pre-TCR signals by undergoing molecular and cellular changes resembling the DN3-to-DN4 transition (Carleton et al. 1999). Thus, these cells serve as an in vitro model to study the role of pre-TCR signaling in driving T-cell differentiation. *Notch1* mRNA amounts were reduced by 80% at 4 h post-treatment of Scid-adh cells with PMA/ionomycin (Fig. 5B). The effects on *Notch1* mRNA were independent of Scid-adh differentiation, as these cells did not show phenotypic evidence of differentiation until after 6 h of PMA treatment (Supplemental Fig. S2). PMA/ionomycin treatment of Scid-adh cells increased the amount of *Id3* mRNA by fourfold within 2 h of treatment (Fig. 5B). ChIP was performed to detect binding of E47 and Notch1 to the sites we identified in the *Notch1* promoter and intron 1 (Figs. 1B, 4A). By 2 h following PMA/ionomycin stimulation, the interaction of E47 with the previously identified E2A target gene *TCRβ* (*Vβ5.1*) (Agata et al. 2007) was significantly reduced, showing that E2A DNA-binding activity was reduced by 2 h of PMA/ionomycin stimulation. The interaction of E47 with both of the strong E2A-binding sites on *Notch1* in DN3 cells (-0.3 kb and intron 1) was also abrogated (Fig. 5C). In contrast, Notch1 binding was significantly reduced at the -6-kb binding site but was unaffected at the -5-kb and intron 1-binding sites (Fig. 5D). Furthermore,

*Notch1* binding to the *Hes1* promoter region was unaffected 2 h after treatment with PMA/ionomycin (Fig. 5D); whereas by 4 h post-PMA/ionomycin treatment when *Notch1* mRNA levels were maximally reduced, *Notch1* binding to all three binding sites in *Notch1* as well as the *Hes1* promoter was markedly reduced (Fig. 5D). E47 binding to the *Notch1* sites remained low at this time (Fig. 5B). Taken together, these data demonstrate that pre-TCR signals inhibit *Notch1* transcription via a mechanism that rapidly abrogates the ability of both E2A and Notch1 to stimulate *Notch1* transcription.

#### *Id3* down-regulates *Notch1* expression in multiple *Notch1* T-ALL cell lines

The ability of pre-TCR signaling and Id3 to inhibit *Notch1* transcription suggested that this signaling axis might inhibit *Notch1* transcription in contexts outside of the down-regulation of *Notch1* transcription that normally occurs following successful  $\beta$ -selection. We were particularly interested in *Notch1*-dependent T-ALLs, which contain activating point and insertion/deletion mutations that do not appear to affect the regulation of *Notch1* transcription. We thus asked whether ectopic Id3 expression could diminish *Notch1* expression and thereby block Notch-dependent growth of these cells. For



**Figure 5.** Id3 down-regulates *Notch1* expression. (A) Model for Notch1 and E2A transcriptional regulation of *Notch1*. Both Notch and E2A are positive regulators of *Notch1* transcription. Following  $\beta$ -selection, pre-TCR signaling induces Id3 expression. This inhibits transcriptional activation of *Notch1* by E2A leading to lower Notch1 protein, which extinguishes the initial positive autoregulatory loops. (B) Time course of *Notch1* and *Id3* mRNA down-regulation by PMA stimulation in Scid-adh cells. qPCR was performed on Scid-adh cells that were stimulated with PMA (20 ng/mL) for 0 h, 2 h, and 6 h. *Notch1* and *Id3* expression relative to *EF1 $\alpha$*  is shown as the mean of values from triplicate wells  $\pm$ SD. Data are relative to the 0-h sample, which was set at 100, and are representative of three independent experiments. (C,D) E2A binding (C) and Notch1 binding (D) to *Notch1* is inhibited by pre-TCR stimulation. Scid-adh cells were treated with DMSO or PMA/ionomycin for 2 h or 4 h and fixed for ChIP. qPCR was performed with primers flanking either the E2A-binding sites or CSL-binding sites on the *Notch1* locus. E47 and Notch1 binding were also measured at the *TCR $\beta$*  locus or at the *Hes1* locus as a control of E47 or Notch1 signaling activity. Values represent the mean of the ratio of the amount of immunoprecipitated DNA/input from values from triplicate wells  $\pm$ SD. Data are representative of two independent experiments.

these studies, we used four different Notch-dependent T-ALL cell lines: a BCR-ABL-transformed T-ALL, G4A2 (Pear et al. 1998), and three different Kras-induced leukemia lines—LCR5, LCR144, and LCR434—that have

mutations in the Notch1 PEST domains (Chiang et al. 2008). Retroviral expression of the pan-Notch inhibitor, DNAM1L, or GSI treatment blocked the growth of all four T-ALL cell lines, showing Notch dependency of

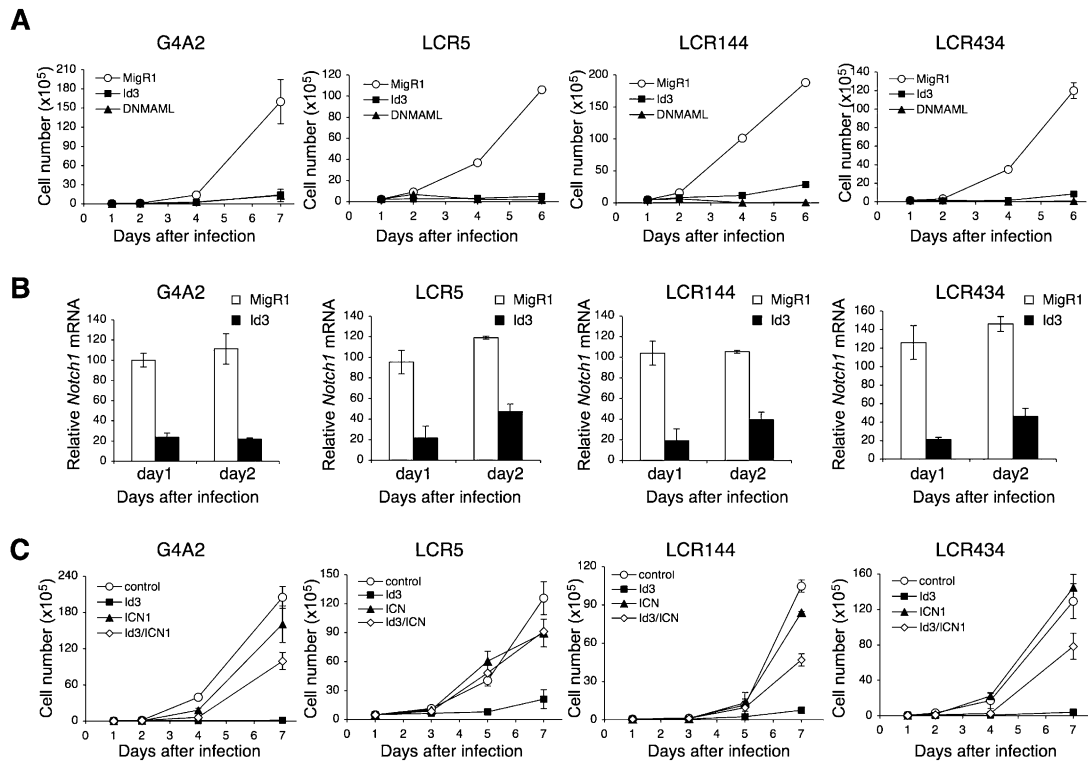
these tumor cell lines (Fig. 6A; data not shown). Retroviral expression of Id3 also blocked the growth of each of these Notch-dependent cell lines to an extent that was comparable with DNMAML expression (Fig. 6A). Id3-induced growth inhibition was accompanied by markedly decreased *Notch1* expression by day 1 following Id3 expression (Fig. 6B).

To confirm that the effects of Id3 on proliferation of the Notch-dependent cells were Notch-specific, we used retroviruses to coexpress Id3 and a constitutively active form of Notch1 (ICN1). Because retrovirally expressed ICN1 lacks the Id3-dependent regulatory sequences, we reasoned that activated Notch1 expressed in this fashion would resist the effects of Id3. Consistent with this prediction, ICN1 maintained the proliferation of Id3-sensitive cell lines in the presence of *Id3* expression (Fig. 6C), which (as expected) suppressed the expression of the endogenous murine *Notch1* alleles (Supplemental Fig. S3). Id3 suppression of *Notch1* mRNA was lower when ICN1 was coexpressed, likely due to the stimulatory effects of activated Notch on *Notch1* expression (Supple-

mental Fig. S3). We also investigated the possibility that Id3 inhibited ICN1 directly by assaying the ability of Id3 to suppress ICN1-mediated activation of a Notch1-dependent *Hes1* luciferase reporter (Supplemental Fig. S4). Id3 failed to suppress the ability of ICN1 to stimulate the *Hes1* reporter; however, it did suppress a control *E2A*-luciferase reporter in the presence of *E2A*. Together, these data show that Id3 antagonizes the growth of Notch-dependent tumor cell lines by inhibiting *Notch1* expression.

**Discussion**

Notch receptor expression is regulated by both transcriptional and post-transcriptional mechanisms. Important post-transcriptional mechanisms include the events that regulate Notch cleavage and trafficking prior to its appearance at the cell surface (Gordon et al. 2008). In contrast, the mechanisms that regulate *Notch* transcription are poorly understood. T-cell development provides a particularly good context to study *Notch* transcription



**Figure 6.** Id3 down-regulates *Notch1* expression and inhibits the growth of Notch1-dependent T-ALL cells. (A) Id3 inhibits T-ALL cell growth. G4A2, LCR5, LCR144, and LCR434 were transduced with either GFP (MigR1), Mig-DNMAML, or Mig-Id3. GFP<sup>+</sup> cells were sorted at day 1 post-transduction (day 1) and cultured for the indicated days. Extrapolated GFP<sup>+</sup> cell numbers are shown as a mean of values from triplicate wells ±SD. Data are representative of three independent experiments. (B) Id3 represses *Notch1* mRNA expression. T-ALL cells were transduced with either MigR1 or Mig-Id3. GFP-expressing cells were sorted at day 1 post-transduction and cultured for 1 d (day 2). *Notch1* expression relative to *EF1α* was determined at days 1 and 2, and is shown as a mean of values from triplicate wells ±SD. Data are representative of three independent experiments. (C) ICN1 rescues Id3-mediated growth arrest of T-ALL cells. T-ALL cells were cotransduced with the GFP (MigR1), Mig-ICN1, NGFR, and NGFR-Id3 retroviruses; and GFP<sup>+</sup>NGFR<sup>+</sup> cells were sorted at day 1 post-transduction. GFP<sup>+</sup>NGFR<sup>+</sup> cells were cultured for the indicated days. The extrapolated cell numbers of the GFP<sup>+</sup>NGFR<sup>+</sup> fraction are shown as a mean of values from triplicate wells ±SD. Data are representative of three independent experiments.



as multiple developmental stages are known and *Notch* transcription changes during development. The initial steps of thymocyte development are Notch1-dependent, and *Notch1* mRNA progressively increases during the early steps of thymocyte development up to and including the  $\beta$ -selection checkpoint. The level of *Notch1* mRNA plummets following  $\beta$ -selection, and this is associated with stages of T-cell differentiation where the requirement for Notch signaling is no longer absolute. Despite this dynamic expression of *Notch1* mRNA, very little is known about its regulation.

In this study, we describe a Notch1-dependent autoregulatory loop that is an important contributor to the progressive increase in *Notch1* expression that occurs during the earliest stages of T-cell development and further show how pre-TCR signals disrupt this loop to down-regulate *Notch1* transcription following  $\beta$ -selection. Thus, our data directly link pre-TCR signaling and *Notch1* expression. We propose that the ability of pre-TCR signals to down-regulate *Notch1* expression occurs via pre-TCR-mediated induction of the bHLH inhibitor Id3. Id3 then prevents E2A-dependent activation of *Notch1* transcription, leading to a decrease in both *Notch1* mRNA and protein. In this model, the loss of E2A-mediated transcriptional activation of *Notch1* would initiate a progressive decrease in *Notch1* transcription due to the loss of transcriptional activation by both E2A and Notch1. Our model also provides a mechanistic explanation for the observation of Murre and coworkers (Ikawa et al. 2006), who showed that ectopic E2A signals could up-regulate *Notch1* mRNA. In this context, E2A provides requisite signals for the initial *Notch1* up-regulation, which then leads to Notch1-driven positive autoregulation. In fact, our reporter studies suggest that E2A exerts a larger effect on *Notch1* transcription than Notch1 itself.

Previous studies showed that pre-TCR and TCR signals induced expression of the E-protein inhibitor Id3, thereby blocking E-protein transcriptional activation (Bain et al. 2001; Engel et al. 2001; Xi et al. 2006). We have now linked Id3 expression directly to Notch, where it caused a decrease in *Notch1* reporter activity, a decrease in *Notch1* mRNA, and blocked growth of Notch-dependent T-cell lines, which is recovered by retrovirally expressing ICN1. Thus, E2A regulation appears to be a critical link between pre-TCR signaling and *Notch1* transcriptional regulation.

Although it was known that *Notch1* mRNA expression undergoes wide fluctuations in developing T cells, the mechanism behind this dynamic regulation had not been described. We identified *Notch1* regulatory sequences that are directly controlled by Notch1 itself and E-proteins. Our data show that Notch1 directly binds autoregulatory CSL sites in the *Notch1* locus to increase its own transcription; thus providing the mechanism to explain how Notch1 influences its own transcription (Huang et al. 2004; Weng et al. 2006). It will be interesting to determine whether these CSL-binding sites are exclusive to Notch1 or may be occupied by other Notch family members. Binding of E2A and Notch1 to the *Notch1* regulatory sequences was accompanied by H4 hyper-

acetylation, showing that these interactions influence the chromatin structure of *Notch1*. This suggests that the pre-TCR-initiated down-regulation of *Notch1* transcription ultimately leads to epigenetic changes that result in low levels of *Notch1* transcription during the subsequent stages of thymocyte development.

In addition to Notch1 and E2A, the only other protein shown to directly bind and regulate *Notch1* transcription is p53, which induces *Notch1* transcription in epithelial cells (Lefort et al. 2007; Yugawa et al. 2007). The extent to which p53 regulates *Notch1* transcription in T cells remains to be determined, and current data suggest that p53 regulation of Notch1 in T cells may be post-transcriptional (Laws and Osborne 2004).

Synergistic signaling by Notch and E2A is evolutionarily conserved. The enhancer of split complex is an important mediator of *Drosophila* sensory organ development whose transcription is coregulated by Notch and bHLH activity (Bailey and Posakony 1995). Several members of this complex contain both CSL and E-box sites, which are synergistically activated by Notch and daughterless, an E2A ortholog (Bailey and Posakony 1995). Some of the enhancers of split loci, such as m8, contain paired CSL-binding sites (SPS) with an adjacent E-box (Bailey and Posakony 1995; Cave et al. 2005). Caudy and coworkers (Cave et al. 2005) suggest that the paired binding site/E-box configuration is necessary for transcriptional activation of this locus and that this activation may depend on physical interactions between Notch and daughterless. Recently, Blacklow and colleagues (Nam et al. 2007) showed that the SPS sites allow for Notch dimerization, which is critical for Notch-induced transcriptional activation at these sites. Thus, specific configurations of Notch- and E2A-binding sites may be required to activate a subset of Notch targets. Our initial analysis of the *Notch1* locus has not identified canonical SPS sites, but it is certainly possible that there are long-range interactions between proteins bound at regulatory sites distant from each other.

*Notch1*-activating mutations are frequent in human and mouse T-ALLs, and proliferation of cell lines derived from these tumors requires persistent Notch signaling (Weng et al. 2004; Aster et al. 2008). This provided the rationale for treating patients with agents that block Notch receptor activation, such as  $\gamma$  secretase inhibitors (Aster et al. 2008). Our studies show that inhibiting *Notch1* transcription with Id3 can also be used to block Notch-dependent growth of T-ALL cell lines. Ectopic Id3 expression inhibited *Notch1* transcription and blocked growth of four Notch-dependent T-ALL cell lines as effectively as dominant-negative mastermind, a potent Notch inhibitor. This effect was Notch-specific as retroviral expression of ICN1, which was resistant to Id3 inhibition, rescued growth of these cell lines. These data suggest that therapeutic strategies that block *Notch1* transcription may be beneficial in treating Notch1-dependent tumors. Even though enforced Id3 expression inhibited T-ALL growth, manipulation of E2A for therapeutic purposes will require a better understanding of the effects of inhibiting E2A. For example, inactivating E2A

in the germline of mice is frequently associated with T-ALL (Bain et al. 1997), whereas tumors are rare when E2A is conditionally inactivated in thymocytes (Pan et al. 2002).

In summary, we identified a novel regulatory mechanism for *Notch1* expression, which involves Notch1 itself and E-proteins. Our data explain the rapid diminution in *Notch1* mRNA expression that occurs after  $\beta$ -selection. In our model, E2A and Notch1 are important transcriptional regulators of Notch expression in pre- $\beta$ -selected thymocytes. With the onset of  $\beta$ -selection, pre-TCR signals lead to *Id3* expression, which inhibits E2A activity leading to decreased *Notch1* transcription and decreased Notch1 protein; thus extinguishing the positive feedback loop. Not only do these findings provide new insights into the control of Notch signaling in T-cell development, but they also suggest new strategies for inhibiting *Notch1* transcription in pathologic conditions.

## Materials and methods

### Mice

Eight-week-old to 12-wk-old C57BL/6 mice (National Cancer Institute, Frederick, MD) and 4- to 6-wk-old *Rag1*<sup>-/-</sup> or *Rag2*<sup>-/-</sup> mice (Taconic Laboratories) were purchased. *Id3*<sup>-/-</sup>, *H<sup>1h</sup>E<sup>1h</sup>* LckCre<sup>+</sup> mice were generated as described (Pan et al. 1999; Zhang et al. 1999; Wojciechowski et al. 2007). All animal experiments were performed according to guidelines from the National Institutes of Health and with an approved protocol from the University of Pennsylvania Animal Care and Use Committee.

### Isolation of thymocyte subsets

CD4<sup>-</sup>CD8<sup>-</sup> DN thymocytes were negatively selected with  $\alpha$ -CD4 and  $\alpha$ -CD8 MACs beads by MACS-LD columns (Miltenyi Biotec) from thymocytes of C57BL/6 mice and were stained with labeled antibodies against lineage markers (TCR $\beta$ , TCR $\gamma$ , CD3 $\epsilon$ , CD4, CD8, NK1.1, Gr-1, CD11b, CD11c, B220, ter119), CD25 and CD44, or c-Kit antigen (Pharmingen). DN1 (CD44<sup>hi</sup> or c-Kit<sup>hi</sup>, CD25<sup>-/lo</sup> Lin<sup>-</sup>), DN2 (CD44<sup>hi</sup> or c-Kit<sup>hi</sup>, CD25<sup>hi</sup>, Lin<sup>-</sup>), DN3 (CD44<sup>lo</sup> or c-Kit<sup>lo</sup>, CD25<sup>hi</sup>, Lin<sup>-</sup>) and DN4 (CD44<sup>lo</sup> or c-Kit<sup>lo</sup>, CD25<sup>-/lo</sup>, Lin<sup>-</sup>) subsets were purified by cell sorting on a FACS Moflo (Cytomation). DN3a (CD44<sup>lo</sup> CD25<sup>hi</sup>, Lin<sup>-</sup>, CD27<sup>lo</sup>) and DN3b (CD44<sup>lo</sup> CD25<sup>hi</sup>, Lin<sup>-</sup>, CD27<sup>hi</sup>) cells were purified by sorting after staining with  $\alpha$ -CD27 antibody (eBioscience) as described (Taghon et al. 2006). Thymocytes of C57BL/6 mice were stained with antibodies against CD4, CD8, and TCR- $\beta$  (Pharmingen) and purified by cell sorting to obtain CD4<sup>+</sup>CD8<sup>+</sup> (DP), CD4<sup>+</sup>CD8<sup>-</sup> (CD4SP), and CD4<sup>-</sup>CD8<sup>+</sup> (CD8SP) cells and CD4<sup>-</sup>CD8<sup>+</sup> TCR- $\beta$ <sup>-</sup> cells (ISP). DN3 cells from *Rag2*<sup>-/-</sup> mice were negatively selected with biotinylated antibodies against NK1.1, CD11b, CD11c, B220, Ter119, and CD44 with streptavidin beads (Miltenyi Biotec) and confirmed to be >98% pure.

### In vivo stimulation

*Rag2*<sup>-/-</sup> mice were injected with either PBS or 50  $\mu$ g of purified  $\alpha$ -CD3 (Pharmingen). Thymocytes were obtained at 6 h or 24 h, purified using Thy1.2 beads (Miltenyi Biotec), and used for quantitative real-time PCR (qPCR). Three mice were used at each time point, and data are representative of three independent experiments.

### qPCR

RNA was purified with the RNeasy Mini Kit or micro kit (Qiagen), digested with DNase I, and used for reverse transcription according to the manufacturer's instructions (SuperScript II kit; Invitrogen). Genes were validated with the primer sets indicated in Supplemental Table S1, and relative quantities were determined against *EF1 $\alpha$* , whose expression was stable in our experiments. Transcripts were amplified with Sybr Green PCR Master Mix (Applied Biosystems) on the ABI 7900HT sequence detection system (Applied Biosystems). All qPCR reactions were performed in triplicate.

### ChIP assays

ChIP was performed with the ChIP assay kit (Millipore, #17-295). DN3 cells, purified from *Rag2*<sup>-/-</sup> mice, and Scid-adh cells were treated with either DMSO or PMA (20 ng/mL) for 4 h. All procedures have been described (Weng et al. 2006). Briefly,  $2.5 \times 10^6$  cells for each immunoprecipitation sample were fixed and immunoprecipitated with control antibody (rabbit IgG; Santa Cruz Biotechnologies, Inc., #sc-3888), Notch1 TAD/PEST-specific antiserum (Aster et al. 2000),  $\alpha$ -E47 (Santa Cruz Biotechnologies, #sc-763), or anti-acetylated histone 4 (Upstate Biotechnologies, #06-866). After DNA purification, SYBR Green qPCR was performed with primers flanking the CSL-binding sites on the *Hes1* promoter (330 base pairs [bp] upstream of the first exon of *Hes1*) or the *Notch1* locus (Fig. 1A) and for E2A-binding sites on the *Notch1* locus (Fig. 5A). The DNA quantity recovered from each ChIP sample is shown as the relative value to the DNA input sample that was not immunoprecipitated. Primer sequences are provided in Supplemental Table S1. Values are from triplicate wells  $\pm$ SD. Data are representative of three independent experiments.

### Luciferase reporter assay

Approximately 6.3 kb of *Notch1* sequences upstream of the translational start site was cloned (*Notch1*-6K) into the pGL3-basic vector (Promega). The primer sequences are provided in Supplemental Table S1. *Id3*, *E47*, and *HEB* cDNAs were cloned into the MigR1 retroviral vector. NIH3T3 cells were transfected with MigR1 or *Id3*-MigR1 retroviral supernatants 18 h prior to the transfection for Figure 4F. Cells were transfected with pGL3-*Notch1*-6K (200 ng) and pRL-TK (25 ng) by Lipofectamin-2000 (Invitrogen). MigR1, ICN1-MigR1, E47-MigR1, or HEB-MigR1 (400 ng) were cotransfected as indicated. Cells were collected at 30 h post-transfection, and luciferase activity was measured with the Dual-Luciferase reporter assay kit (Promega).

### Cell preparation and Western blotting

*Rag2*<sup>-/-</sup> DN3 cells ( $5 \times 10^5$ ) were stimulated with PMA (20 ng/mL) or DMSO. Cells were collected at 0, 2, or 6 h after treatment, and 20  $\mu$ g of protein lysate was subjected to Western blotting. Antibodies against the Notch1 TAD/PEST domain and  $\beta$ -actin (Sigma) were used.

### Retroviral transduction and proliferation assay

Mig-ICN1 and Mig-DNMAML have been described (Weng et al. 2003; Maillard et al. 2004). *Id3* was also cloned into MSCV-tNGFR, which coexpresses the truncated nerve growth factor receptor (tNGFR) as a surrogate marker (Izon et al. 2002). T-ALL cells ( $2 \times 10^6$ ) were centrifuged with the appropriate amount of viral supernatant and 8  $\mu$ g/mL polybrene (Sigma) for 90 min at

2500 rpm (day 0). GFP<sup>+</sup> or NGFR<sup>+</sup> cells were purified by cell sorting on a FACS MoFlo (Cytomation). Cells were cultured for up to 7 d, and the total cell number was determined using a hemocytometer. Each experiment was carried out at least three times.

#### Real-time, primary-transcript RT-PCR

All procedures have been described (Murray 2005). Briefly, total RNA was isolated from DN3a cells from Rag1<sup>-/-</sup> mice using TRIzol (Invitrogen). RNA was resuspended at a final concentration of 0.1 µg/µL in 100 µL and treated with 10 U of RNase-free DNase I (Promega) for 30 min, then purified with acid phenol/chloroform. RNA (0.4 µg) was reverse-transcribed using SuperScript II (Invitrogen). Primers were designed to amplify the region exon 27 and exon 28 for mRNA and exon 27 and intron 27 for primary RNA on *Notch1*. All samples were processed in triplicate, and relative quantities were determined against a diluted standard of 18S rRNA.

#### Statistical analysis

Statistical analysis was done using an unpaired Student's *t*-test.

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