

Notch3 and the Notch3-upregulated RNA-binding protein HuD regulate Ikaros alternative splicing

Diana Bellavia¹, Marco Mecarozzi¹,
Antonio F Campese¹, Paola Grazioli¹,
Claudio Talora¹, Luigi Frati², Alberto
Gulino^{1,2} and Isabella Screpanti^{1,3,*}

¹Department of Experimental Medicine, University 'La Sapienza', Roma, Italy, ²Neuromed Institute, Pozzilli, Italy and ³Istituto Pasteur-Fondazione Cenci Bolognetti, University 'La Sapienza', Roma, Italy

Constitutive activation of the transmembrane receptor, Notch3, and loss of function of the hematopoietic transcription repressor, Ikaros (IK), play direct roles in T-cell differentiation and leukemogenesis that are dependent on pre-T-cell receptor (pre-TCR) signaling. We demonstrate the occurrence of crosstalk between Notch3 and IK that results in transcriptional regulation of the gene encoding the pT α chain of the pre-TCR. We also show that, in the presence of the pre-TCR, constitutive activation of Notch3 in thymocytes causes increased expression of dominant-negative non-DNA-binding IK isoforms, which are able to restrain the IK inhibition of Notch3's transcriptional activation of pT α . This effect appears to be mediated by Notch3's pre-TCR-dependent upregulation of the RNA-binding protein, HuD. Notch3 signaling thus appears to play a critical role in the diminished IK activity described in several lymphoid leukemias. By exerting transcription-activating and transcription-repressing effects on the pT α promoter, Notch3 and IK cooperate in the fine-tuning of pre-TCR expression and function, which has important implications for the regulation of thymocyte differentiation and proliferation.

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Introduction

The process of T-cell leukemogenesis is regulated by some of the same mechanisms that control T-cell differentiation, and the final outcome depends on fine balancing. Both processes are known to be affected by aberrantly activated Notch signaling and by the loss of function of the transcription factor, Ikaros (IK), depending on the presence of a functional pre-T-cell receptor (pre-TCR) (Winandy *et al*, 1995, 1999; Allman *et al*, 2001; Bellavia *et al*, 2002).

*Corresponding author. Laboratory of Molecular Pathology, Dipartimento di Medicina Sperimentale, University 'La Sapienza', Viale Regina Elena 324, Roma 00161, Italy.
Tel.: +39 06 44700816; Fax: +39 06 4464129;
E-mail: isabella.screpanti@uniroma1.it

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Our previous studies in transgenic mice expressing the intracellular domain (IC) of the Notch3 receptor (N3-IC) have shown that constitutive Notch3 signaling leads to the persistent overexpression in thymocytes of the pT α chain, an essential component of the pre-TCR. This overexpression is a hallmark of the T-cell leukemia/lymphoma that ultimately develops in these animals (Bellavia *et al*, 2000). Interestingly, by generating double mutant mice transgenic for Notch3-IC and lacking pT α (N3-IC/pT α ^{-/-} mice), we observed that an intact pre-TCR is required for T-cell leukemogenesis (Bellavia *et al*, 2002), although it is not required for Notch3-driven T-cell differentiation. Indeed, whereas pT α knockout arrests T-cell development, transgenic N3-IC overcomes this block, driving immature CD4⁻CD8⁻ double-negative (DN) thymocytes to progress toward a more differentiated phenotype (e.g. acquisition of CD4, CD8 and TCR β expression) despite the absence of a functional pre-TCR (Bellavia *et al*, 2002).

An intriguingly similar picture emerges when we analyze the effects of the loss of IK activity. Dominant mutation that eliminates the DNA-binding activity of this transcription repressor leads to rapid development of murine leukemia and lymphoma, which is strictly dependent on either pre-TCR or TCR signaling (Winandy *et al*, 1995), and yet IK-deficient DN thymocytes can differentiate to the CD4⁺CD8⁺ double-positive (DP) and CD4⁺ single-positive stages in the absence of a pre-TCR complex (Winandy *et al*, 1999). Therefore, like constitutive activation of Notch3, loss of IK function is characterized by pre-TCR-independent thymocyte differentiation from the DN to the DP stage and pre-TCR-dependent expansion of immature thymocyte populations (Winandy *et al*, 1999; Bellavia *et al*, 2002).

The roles of Notch and IK in human leukemogenesis are supported by several reports. Notch3 overexpression has been observed in virtually 100% of human T-cell acute lymphoblastic leukemia/lymphomas (ALL), including tumors from all major molecular and immunophenotypic subtypes (Bellavia *et al*, 2002), and activating mutations of Notch1 have been found in over 50% of these tumors (Weng *et al*, 2004). A high percentage of infant B- and T-ALLs also display an increased expression of short non-DNA-binding IK isoforms (Sun *et al*, 1999a, b). Alternatively spliced transcripts of the *ikaros* gene encode at least nine protein isoforms (IK-1–9) with different DNA-binding capabilities (Hahm *et al*, 1994; Molnar and Georgopoulos, 1994; Beverly and Capobianco, 2003). Isoforms IK-1–3 are characterized by at least three N-terminal zinc-finger motifs that allow efficient DNA binding. Shorter isoforms, lacking one or more of these DNA-binding motifs, form heterodimers with full-length isoforms and exert dominant-negative effects that can decrease or even suppress normal IK activity. It has recently been suggested that increased expression of dominant-negative IK isoforms (IK-dn) and constitutively activated Notch play cooperative roles in leukemogenesis, involving effects that may converge in the transcriptional regulation of one or more key genes (Beverly and

Capobianco, 2003). However, the identity of these putative common targets is still obscure, and thus far there has been no demonstration of a direct link between aberrant Notch signaling and altered IK isoform expression.

In the present paper, we show that Notch3 activation upregulates the expression in thymocytes of the RNA-binding protein HuD. This effect results in the increased expression of IK-dn isoforms, which diminishes the DNA-binding activity of IK and, consequently, its ability to inhibit Notch3-induced increase of pT α expression. These events could be expected to lead to a persistent activation of pre-TCR signaling, which is responsible for the triggering of a number of oncogenic pathways.

Results

Notch3-IC transgenic mice display increased expression of alternatively spliced IK isoforms

To better define the relationships between IK and Notch3 in the processes of T-cell differentiation and leukemogenesis, we first analyzed IK isoform expression in premalignant thymocytes from young (2-week-old), wild-type (wt) and N3-ICtg mice. We define the line between preleukemia and leukemia by the finding of CD4⁺CD8⁺ DP cells in the spleen of transgenic mice. The two groups of cells were similar in terms of thymocyte subset distributions (Figure 1A), but the N3-ICtg cells displayed increased expression of IK-dn isoforms at both the RNA and protein levels (Figure 1B, upper and lower panels, respectively). We then investigated IK protein expression in adult N3-ICtg mice that had already developed unequivocal signs of leukemia/lymphoma (Bellavia *et al*, 2000). Thymocytes (Figure 2A, upper panels) and peripheral T-cells (i.e. lymph node cells) (Figure 2A, lower panels) from these animals exhibited significantly increased expression of the IK-dn isoforms (compared with wt littermates). The IK isoform profiles were unrelated to the immunophenotype of the lymphoma cell, which can vary from animal to animal (Bellavia *et al*, 2000). In fact, similar profiles were observed in cells with different immunophenotypes (e.g. N3-ICtg3 and N3-ICtg4 in Figure 2A and B), and those with identical phenotypes sometimes had different IK expression profiles (although the increase in IK-dn isoforms was a constant) (data not shown). It is interesting to note that in each tumor-bearing mouse, thymus- and lymph node-derived lymphoma cells consistently displayed identical immunophenotype (Figure 2A). Similar IK isoform profiles were also observed at protein levels (Figure 2B).

Noteworthy, the IK-9 isoform, of intermediate size with respect to IK-1 and IK-2/3, appears mainly represented in all the samples obtained from N3-ICtg cells. This isoform, previously described by Beverly and Capobianco (2003), includes all the exons except exon 4, which encodes the critical DNA-binding zinc-fingers, and thus behaves as dominant negative. We cloned and sequenced IK isoforms from thymocytes of both Notch3-IC and wt mice, and Supplementary Figure 1 schematically represents the bands more represented, including the distribution of zinc-finger motifs and the size range of RNA.

The pT α /pre-TCR is required for Notch3-induced redistribution of IK isoforms

Previous work has shown that the presence of the pre-TCR is necessary for Notch3-induced leukemogenesis (Bellavia *et al*,

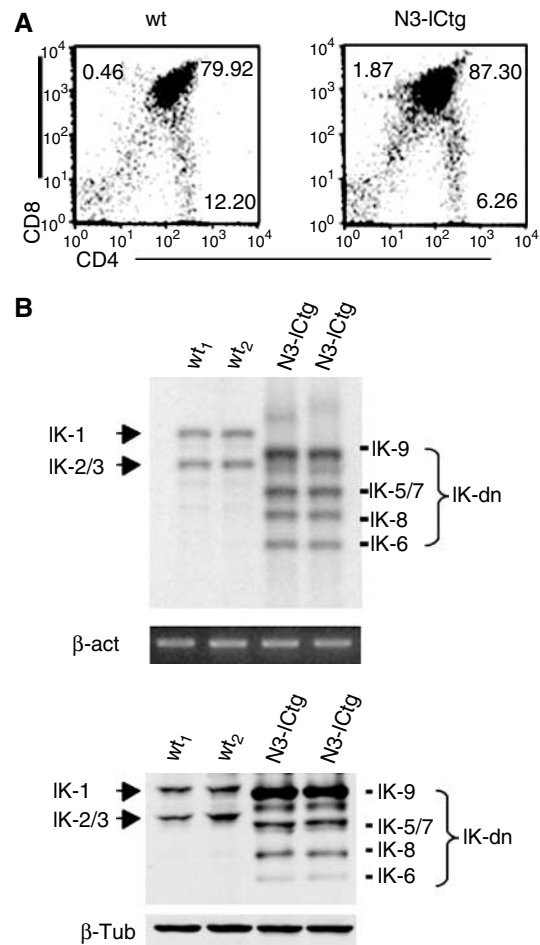


Figure 1 Alternatively spliced IK isoforms are overexpressed in premalignant thymocytes from Notch3-IC transgenic (N3-ICtg) mice. (A) CD4⁺ and/or CD8⁺ subset distributions (documented by two-color FCA) of thymocytes from 2-week-old wt and N3-ICtg mice. The number in each quadrant indicates the percentage of total cells represented by the corresponding subset. (B) IK isoform expression profiles assessed by RT-PCR (upper panel) and Western blot (lower panel) analysis of whole thymocyte lysates from two wt and two N3-ICtg mice. IK-1 and IK2/3: DNA-binding IK isoforms; IK-dn: dominant-negative IK spliced variants incapable of DNA binding. PCR products were analyzed by Southern blot using IK-6 cDNA as probe to detect the specific sequences. mRNA expression was monitored along the exponential phase of amplification and normalized to β-actin (β-act). In Western blot assay, results were normalized to β-tubulin (β-tub). The data are representative of three similar experiments.

2002) and for that observed in mice with a dominant DNA-binding mutation in the IK gene (Winandy *et al*, 1995, 1999). We hypothesized that increased expression of IK-dn isoforms is characteristic of Notch3-dependent leukemia and may also contribute to its development. To investigate this possibility, we compared the IK isoform expression profiles of thymocytes from N3-ICtg mice, double mutant N3-ICtg/pT α ^{-/-} mice, pT α ^{-/-} mice and wt controls. DN (CD4⁻CD8⁻) and DP (CD4⁺CD8⁺) thymocytes were sorted and analyzed separately to minimize the effects of any strain-related differences in thymocyte subset distributions. As shown in Figure 3A, the shift toward IK-dn expression observed in N3-ICtg thymocytes was absent in both N3-ICtg/pT α ^{-/-} and pT α ^{-/-} cells, which lack a functional pre-TCR complex.

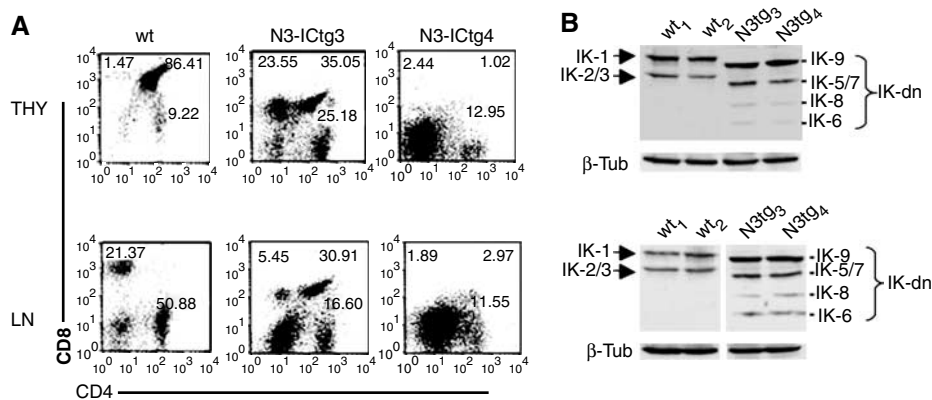


Figure 2 IK isoform expression patterns are altered in T lymphoma cells from Notch3-IC transgenic (N3-ICtg) mice. (A) Two-color FCA analysis of CD4 versus CD8 expression in freshly isolated thymocytes (THY) (upper panels) and lymph node cells (LN) (lower panels) from 5-week-old mice: two wild-type (wt₁ and wt₂) and two N3-ICtg (N3-ICtg3 and N3-ICtg4). The number in each quadrant indicates the percentage of total cells represented by the corresponding subset. (B) Immunoblots of whole-cell extracts from the cells shown in panel A. IK1 and IK2/3: DNA-binding IK isoforms; IK-dn: dominant-negative IK isoforms incapable of DNA binding.

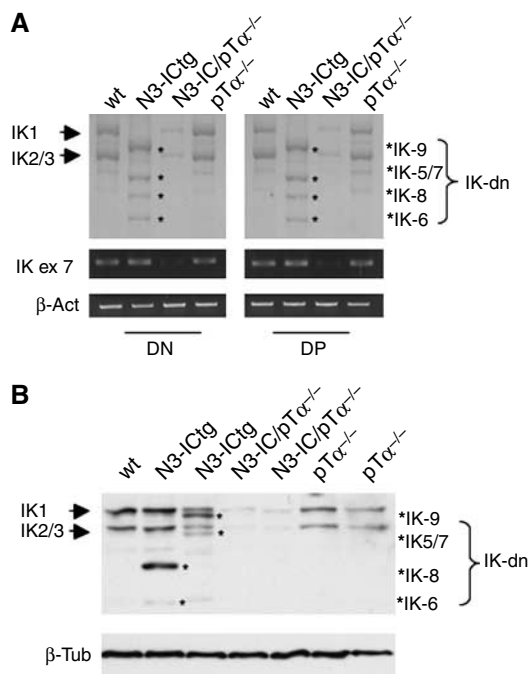


Figure 3 IK isoform expression patterns in thymocytes depend on pT α expression. (A) RT-PCR analysis of IK isoform expression was performed on RNA from sorted CD4⁺CD8⁻ DN and CD4⁺CD8⁺ DP thymocytes from wild-type (wt), Notch3-IC transgenic (N3-ICtg), Notch3-IC/pT α ^{-/-} and pT α ^{-/-} mice. (B) IK immunoblot of whole-cell lysates of unfractionated thymocytes from one wt mouse and two mice of each of the three mutant genotypes listed above. IK1 and IK2/3: DNA-binding IK isoforms; IK-dn: dominant-negative IK isoforms incapable of DNA binding; IK ex 7: IK exon 7. Results were normalized to β -tubulin (β -tub).

Intriguingly, semiquantitative RT-PCR amplification of IK exon 7 (present in all of the isoforms) revealed that total IK transcript levels in DN and DP thymocytes from N3-ICtg/pT α ^{-/-} double mutant mice were significantly lower than those observed in wt, N3-ICtg and pT α ^{-/-} cells (Figure 3A, middle panels). These findings were confirmed at the protein expression level by Western blot analysis of total cell extracts from unfractionated thymocytes (Figure 3B).

Notch3 modulates expression patterns of IK mRNAs and proteins *in vitro*

Our next step was to determine whether Notch3 signaling directly affects IK isoform expression. The *in vivo* findings presented above indicated that such an effect is probably dependent on the presence of an intact pre-TCR. Based on CD25 and CD44 expression, DN thymocytes can be divided into four subsets, DN1-4, and functional pre-TCR expression is first observed at the DN3 (CD25⁺CD44⁻) stage. Therefore, we compared Notch3-induced IK splice variant profiles in DN1 and DN3 cells represented respectively by the M31 (Primi *et al*, 1988) and 2017 (Spolski *et al*, 1988) cell lines (Supplementary Figure 2). The two cell lines (both of which constitutively express full-length DNA-binding IK isoforms) were transiently transfected with increasing amounts of N3-IC, and IK isoform profiles were investigated 48 h later by semiquantitative RT-PCR and Western blotting. N3-IC-transfected 2017 cells displayed significantly increased expression of IK-dn isoforms (compared with untransfected or empty vector-transfected controls) at both the mRNA (Figure 4A, upper right panel) and protein (Figure 4B, right panel) levels, but there was no evidence of these isoforms in transfected M31 cells (Figure 4A, lower panels). The shift toward IK-dn expression seems to be a Notch3-specific effect as it was not observed after transient transfection of 2017 or M31 cells with Notch1-IC (Figure 4, left panels). Noteworthy, Notch1-IC was able to increase the expression of Hes-1 and Deltex in transfected 2017 cells (Figure 4A, upper left panels). Moreover, it was able to increase the Hes-1 promoter activity in a luciferase reporter assay to a greater extent than Notch3-IC in both HEK293 and M31 cells (Supplementary Figure 3). We finally addressed the possible effect of exogenous Notch1-IC on the expression of Notch3 in 2017 cells. The middle panel of Figure 4A shows that enforced expression of exogenous Notch1-IC does not modify the endogenous Notch3 expression of 2017 cells.

Collectively, the findings presented thus far indicate that activated Notch3 signaling in the presence of pre-TCR expression can modulate IK isoform expression profiles *in vivo* and *in vitro*. This effect, which appears to be Notch3-specific, could thus be responsible (at least in part) for the abnormal

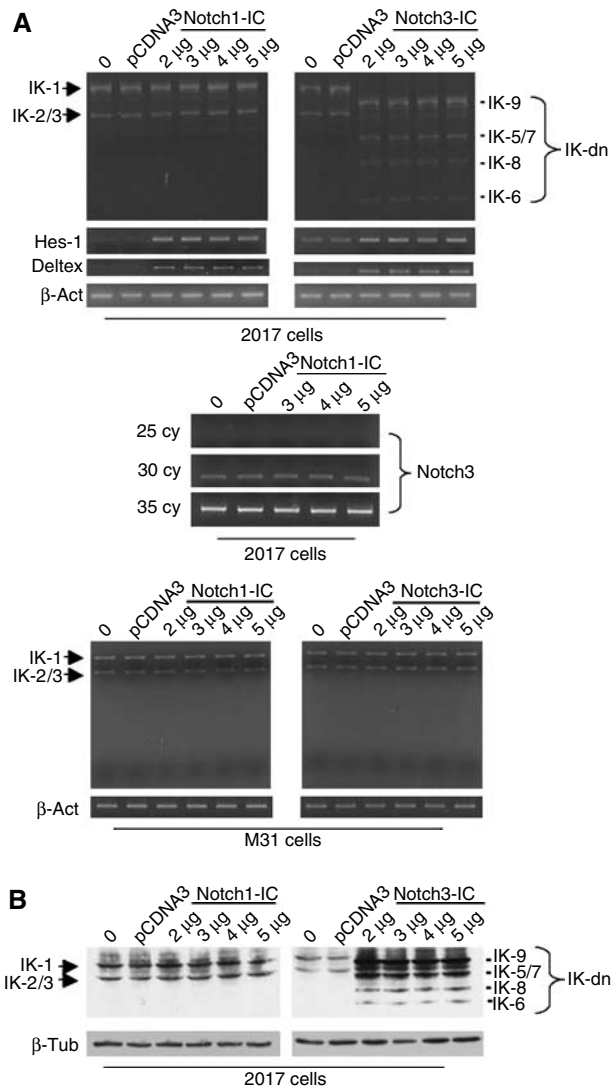


Figure 4 Notch3 regulates alternative splicing of IK (IK) *in vitro*. DN1 and DN3 thymocytes (represented respectively by the M31 and 2017 cell lines) were transfected with increasing amounts of Notch3 (right panels) or Notch1 (left panels), and IK mRNA and protein expression was assessed by RT-PCR (A) and Western blotting (B). In the upper panels (A), Hes-1 (30 cycles for Notch1 and 35 cycles for Notch3) and Deltex RNA expressions were used as control of correct function of Notch1-IC and Notch3-IC constructs. The middle panel (A) shows the inability of Notch1-IC to increase the constitutive expression of Notch3 by 2017 cells at any cycles considered. IK1 and IK2/3: DNA-binding IK isoforms; IK-dn: dominant-negative IK isoforms incapable of DNA binding. β -Actin (β -act) and β -tubulin (β -tub) are shown as controls of equivalent loading of each sample.

expression of IK-dn isoforms in premalignant and malignant thymocytes of N3-ICtg mice.

Notch3 and IK act cooperatively to regulate pT α gene expression

The findings described above, together with a recent report that loss of IK function leads to increased expression of Notch3 and pT α in murine T lymphoma cells (Dumortier *et al*, 2006), suggest the possibility of direct crosstalk between Notch3 and IK that serves to regulate pT α gene transcription. Previous studies have shown that the pT α enhancer is activated by Notch1 signaling via CSL-binding sites (Reizis and

Leder, 2002), and we have demonstrated dose-dependent activation of the pT α promoter by Notch3 (Talora *et al*, 2003). To further elucidate the mechanisms underlying the effects of Notch3 on the regulatory region of pT α , we transfected non-lymphoid HEK293 cells with a luciferase reporter construct containing the entire pT α promoter sequence (as reported in GenBank, accession number U27268). Enforced coexpression in these cells of N3-IC and Mastermind (MAM), the positive regulator of Notch activity, increased pT α promoter activity \sim 10-fold, and additional increases were observed after the addition of RBP-J κ /CSL (Figure 5 and not shown). Although the ability of Notch-IC to activate transcription is known to be potentiated by its interaction with these coactivators (Wu *et al*, 2000; Fryer *et al*, 2002; Jeffries *et al*, 2002; Lin *et al*, 2002), this is the first demonstration that Notch3-IC-driven activation of the pT α regulatory element is significantly enhanced by a transcriptional complex composed of RBP-J κ /CSL and MAM.

Full-length DNA-binding IK isoforms have been shown to repress CSL-dependent Notch-triggered transcriptional activity, and this effect is attenuated by the coexpression of alternatively spliced IK-dn isoforms (Beverly and Capobianco, 2003). Similarly, enforced expression of the IK-1 isoform in HEK 293 cells significantly inhibited activation of the pT α promoter triggered by the Notch3 and coactivator complex (Figure 5A), but there was no evidence of transcriptional repression in cells transfected with the dominant-negative IK-6 alone (Figure 5A). In the presence of a constant level of coexpressed IK-1, the increasing presence of IK-6 progressively diminished and ultimately overcame the transcriptional repression induced by the full-length isoform, restoring pT α promoter activation to levels similar to those produced by the Notch3-dependent transcriptional complex in the absence of IK (Figure 5A). This body of evidence strongly suggests that Notch3 regulates the alternative splicing of IK and that, in so doing, it modulates its own ability to induce transcriptional activation of pT α .

It remained to be seen how the Notch3-triggered complex interacts with the pT α promoter region and what is the basis for IK antagonism. Our analysis revealed that the pT α promoter contains a canonical consensus CSL-binding sequence, several consensus sequences that are specific for IK and three TGGGAA motifs corresponding to the RBP-J κ /CSL core sequence. The latter finding was particularly interesting as this same core sequence is also present in the consensus DNA-binding sequences of IK (Molnar and Georgopoulos, 1994; Beverly and Capobianco, 2003). The three motifs were designated CSL-core 1, CSL-core 2 and CSL-core 3 in accordance with their positions in the pT α promoter (-485 , -200 and -149 bp, respectively) (Figure 5B). The pT α promoter region containing the three motifs was cloned and to evaluate their possible roles in Notch3-dependent transcriptional activation, we generated two luciferase reporter constructs, one (CSL-Mut1) with a specific mutation in CSL-core1 and the second (CSL-Mut2/3) with mutations in CSL-core2 and CSL-core3 (Figure 5B), and transfected them, together with N3-IC and its coactivators, into HEK293 cells. In cells expressing the CSL-Mut2/3, luciferase activity was similar to that observed with the wt sequence, but CSL-Mut1 expression was associated with drastically reduced activity (Figure 5C). These findings indicate that the TGGGAA sequence at -485 bp is necessary for Notch3-dependent transcriptional activation of pT α .

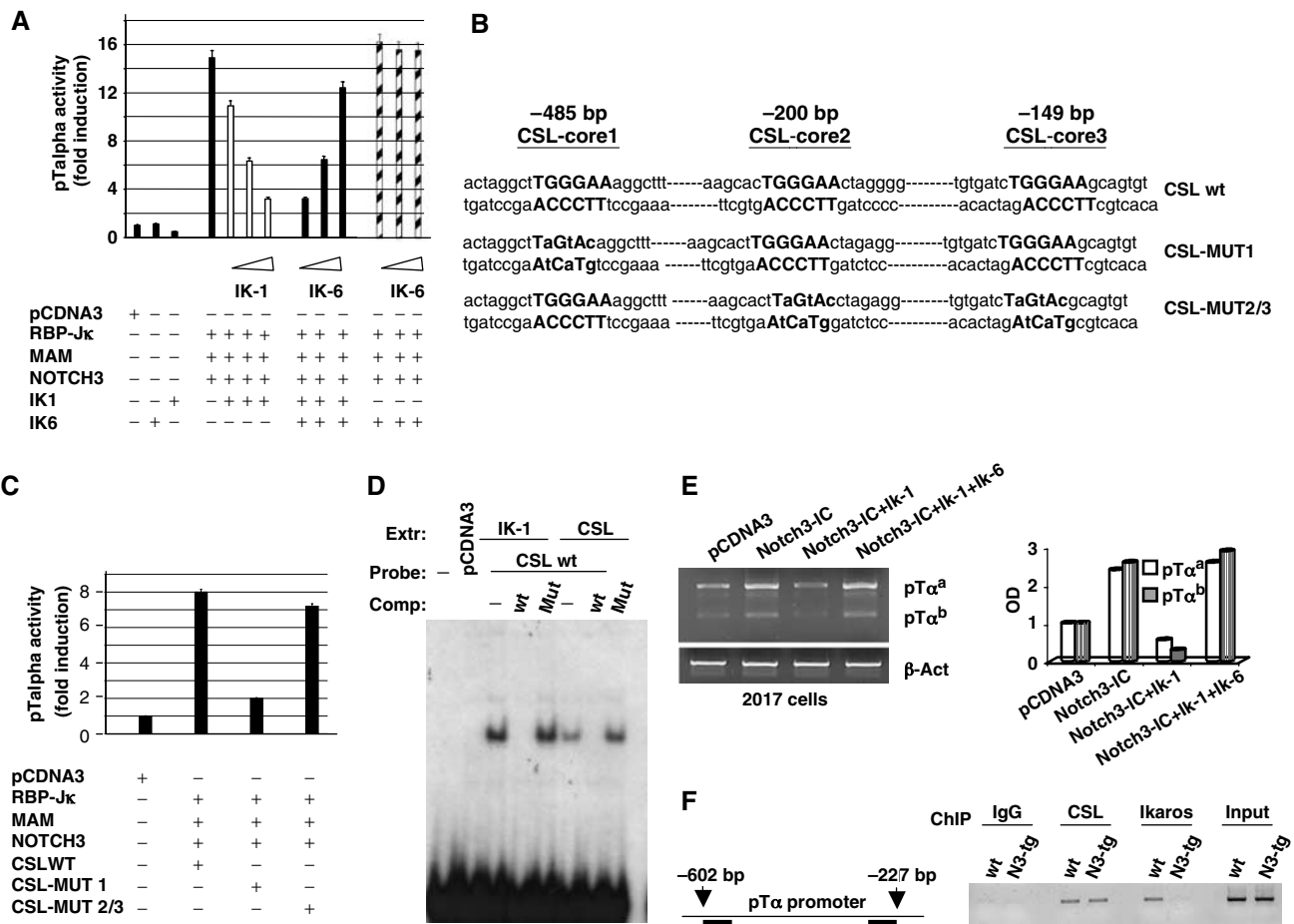


Figure 5 Transcriptional activation of the pT α promoter by Notch3 is differentially regulated by IK isoforms. (A) HEK 293 cells were transfected with a luciferase reporter construct containing the pT α promoter together with Notch3, RBP-J κ , and Mastermind (MAM). The pT α promoter is activated by the Notch3 transcription activator complex (Notch3 + RBP-J κ + MAM). Increasing amounts of IK-1 alone (200–800 ng) dose dependently repressed Notch3-driven activation of the pT α promoter, but the non-DNA-binding IK6-dn isoform alone (200–800 ng) had no impact. When IK-1 and IK-6 were cotransfected, increasing amounts of IK-6 progressively diminished the transcription repression induced by fixed amount of IK-1. DNA contents in the different transfection assays were normalized with empty vector (pcDNA3). The data shown were collected from three independent experiments; vertical bars indicate standard deviation. (B) Mutational analysis of the TGGGAA sequence in the pT α promoter. Wild-type and mutated sequences from the entire promoter are shown in boldface type and the cloned CSL wt sequence and mutant constructs, CSL-MUT1 and CSL-MUT2/3, are shown. (C) Luciferase activity of wild-type and mutant pT α promoter constructs. pCDNA3: empty vector. The histograms represent mean results of three independent transfection experiments. Vertical bars indicate the range of standard errors. (D) EMSA: nuclear extracts derived from COS cells transfected with either IK-1 (IK1) or CSL (CSL) expression vectors were probed with an oligo containing the wild-type CSL-core1 consensus (TGGGAA) (CSL-wt), as indicated in (B). For competition (comp), 100-fold molar excess of cold CSL-wt or CSL-MUT 1 was used. (E) RT-PCR expression analysis of pT α^a and pT α^b isoform mRNAs in 2017 cells. The cells were transiently transfected with Notch3-IC alone to induce increased pT α expression or together with Ik-1 or Ik-1 + Ik-6 to demonstrate that Notch3-dependent induction is repressed by Ik-1 and derepressed by Ik-6. pT α mRNA levels were normalized to β -actin (β -act) (left panel) and optical densities relatively to empty vector-transfected cells (pCDNA3) are shown (right panel). (F) Schematic representation of pT α promoter region, assessed by ChIP is indicated (left panel). Primary thymocytes derived from wt and Notch3-IC transgenic mice (N3-tg) were processed for chromatin preparation and then subjected to immunoprecipitation with antibodies against RBP-J κ (CSL), IK or IgG as control. Immunoprecipitated DNA was analyzed by PCR with pT α promoter specific primers (right panel). The results are representative of three similar experiments.

In order to study the basis for Notch3 and IK-1 antagonism on pT α transcriptional activation, we first examined the ability of both IK-1 and CSL to bind the same site of pT α regulatory sequence by using the CSL wt sequence of the pT α promoter in an electrophoretic mobility shift assay (EMSA). We observed that both the IK-1 and CSL proteins, obtained from nuclear extracts derived from COS cells transfected with either IK-1 or CSL expression vectors, were able to bind the same site. We used as probe an oligo containing the wt CSL-core1 consensus (TGGGAA) of the pT α promoter, as indicated in Figure 5B. The interactions of IK-1 and CSL with the oligo were specific, as they were able to be competed with 100-fold

molar excess of the unlabeled CSL-wt, but not with 100-fold molar excess of unlabeled mutant oligo CSL-MUT 1 (Figure 5D).

Moreover, the luciferase reporter and EMSA assays were confirmed in Notch3-IC transiently transfected 2017 cells by evaluating the ability of Notch3-IC to induce an increase in endogenous pT α expression and demonstrating that this induction is repressed by IK-1 and derepressed by IK-6 (Figure 5E).

Finally, to address whether IK and CSL truly compete *in vivo* for the same candidate site in the pT α promoter, we performed chromatin immunoprecipitation (ChIP)

experiments (Saccani and Natoli, 2002) using primary thymocytes derived from wt and N3-ICtg mice. Figure 5F (right panel) shows that both CSL and IK proteins are associated with the pT α promoter in chromatin extracts derived from wt thymocytes. In contrast, CSL but not IK antibodies efficiently recovered the pT α promoter fragment using chromatin extracts derived from N3-ICtg mice. These results demonstrate that CSL and IK directly associate at the same binding site in the pT α promoter and might explain the functional collaboration observed between Notch3 and the IK-dn isoforms in sustaining pT α expression, finally supporting the hypothesis that the increased amount of IK-dn isoforms in thymocytes of N3-ICtg mice prevents the DNA binding of IK, thus allowing the derepression of pT α expression.

The effects of Notch3 on the expression of IK spliced variants depend on its pre-TCR-dependent upregulation of the RNA-binding protein HuD

Next, we attempted to identify mechanisms specifically triggered by Notch3 that would account for the presence of alternatively spliced IK isoforms. In a recent Affymetrix MAS 5.0 microarray analysis of the global gene expression profile of N3-ICtg pre-malignant thymocytes (results currently unpublished), we discovered a striking increase (~ 39 -fold compared with wt controls from 2-week-old mice) of mRNA for HuD, an RNA-binding protein of the ELAV/Hu family (Inman *et al*, 1998), which is reportedly capable of regulating both the stability (Levine *et al*, 1993; Mobarak *et al*, 2000; Deschenes-Furry *et al*, 2003; Ratti *et al*, 2006) and the alternative splicing (Lisbin *et al*, 2001; Zhu *et al*, 2006) of RNA. In the present study, we confirmed, by means of semiquantitative RT-PCR the presence of significantly increased HuD mRNA levels in DN and DP thymocytes from N3-ICtg mice (Figure 6A). Even more interesting was our finding that Notch3-induced enhancement of HuD expression is pre-TCR dependent. In fact, in contrast to wt and N3-ICtg thymocytes, cells from both Notch3-IC/pT α ^{-/-} and pT α ^{-/-} mice displayed HuD expression levels that were virtually undetectable in both DN and DP cells (Figure 6A). In addition, HuD expression was dose-dependently upregulated in the pre-T cell line 2017 after transient transfection with increasing amounts of Notch3-IC (Figure 6B, left panel), but no increases were observed in transfected M31 cells (not shown). The negative results observed when transfections were carried out with Notch1-IC (Figure 6B, right panel) indicate that the upregulation of HuD is a Notch3-specific effect.

Collectively, these results strongly suggest that the Notch3-induced upregulation of HuD expression, which is pre-TCR-dependent, may be related to the increased generation and/or enhanced stability of IK-dn isoforms. As a putative downstream effector of pre-TCR signaling, HuD expression alone would be expected to allow the shift toward IK-dn isoform expression even in the absence of pre-TCR. This hypothesis was confirmed when we transiently transfected 2017 and M31 cells with a pcDNA3 expression vector containing cloned HuD. In both cell lines, increasing amounts of transfected HuD elicited progressive increases in short IK-dn isoforms, an effect that was unrelated to the differentiation stage or to pre-TCR expression (Figure 6C).

Together, the above results suggest that HuD protein mediates the effect of Notch3 on the differential expression of IK isoforms. In order to directly address this issue, we

performed siRNA experiment to study the effect of HuD silencing on the Notch3-induced modulation of IK-dn isoforms. Figure 6D shows that the silencing of HuD abrogates the effect of Notch3 on IK isoform modulation. We also show the reverse by silencing endogenous Notch3. Indeed, Figure 6E shows that the siRNA of Notch3 is able to inhibit the constitutive expression of HuD in 2017 cells.

It remained to be seen whether the HuD-increased expression and the shift toward IK-dn isoforms were induced by endogenous Notch signaling. As shown in Supplementary Figure 2, 2017 cells constitutively express endogenous pT α , Notch3 and Notch1, whereas M31 cells express similar levels of Notch1, but very low levels of Notch3 and undetectable pT α when compared with 2017. Thus, we performed coculture experiments of 2017 cells on OP9 stromal cells that have been described to constitutively express the Notch ligand Jagged-1 at levels similar to those of normal thymic epithelial cells and to be able to trigger endogenous Notch signaling (Schmitt and Zúñiga-Pflücker, 2002; Lehar *et al*, 2005). We observed that the coculture with OP9 cells induced the same effect on HuD and IK-dn isoform expression as the transfection of Notch3-IC, and when the γ -secretase inhibitor I was added to the culture medium the effect was inhibited, thus demonstrating that it was due to the triggering of endogenous Notch signaling (Figure 6F).

The RNA-binding protein HuD is expressed in human T-ALL

Whereas the expression of alternatively spliced IK isoforms has been previously reported in several human leukemias (Sun *et al*, 1999a-c; Olivero *et al*, 2000), HuD expression was not addressed before. The observations reported above prompted us to analyze HuD mRNA expression in human T-ALL, as well as the possible role of HuD and Notch3 in regulating the IK isoform expression pattern. We utilized the previously described human T-ALL cell line Molt-3 (Sun *et al*, 1999a). Figure 7A shows that this cell line, similar to the mouse 2017 cell line, constitutively expresses Notch1 and 3, HuD, both the pT α isoforms a and b and an altered IK isoform profile. In order to address directly the relationships between Notch, HuD and IK isoforms in human T-ALL, we performed siRNA experiments in Molt-3 cells. Figure 7B shows that knockdown of Notch3 and HuD but not of Notch1 is able to alter the IK isoform profile displayed by Molt-3 cells. Interestingly, besides specifically affecting the pattern of IK isoform expression, the knockdown of Notch3 results in a higher inhibition of proliferation of Molt-3 cells when compared with the knockdown of Notch1 (49 versus 30%) (Figure 7C).

Finally, we studied four different primary human T-ALLs and one primary B-ALL utilizing bone marrow samples from patients at different stages of disease (exordium and remission) and one control patient. We observed expression of HuD in all of the T-ALL exordium samples; in contrast, HuD expression was undetectable in all the samples of the same patients in remission stage, in the sample from the B-ALL-bearing patient and in the control (Figure 7D).

Discussion

Pre-TCR expression is indispensable for the early steps of T-cell differentiation and for progression of Notch3-

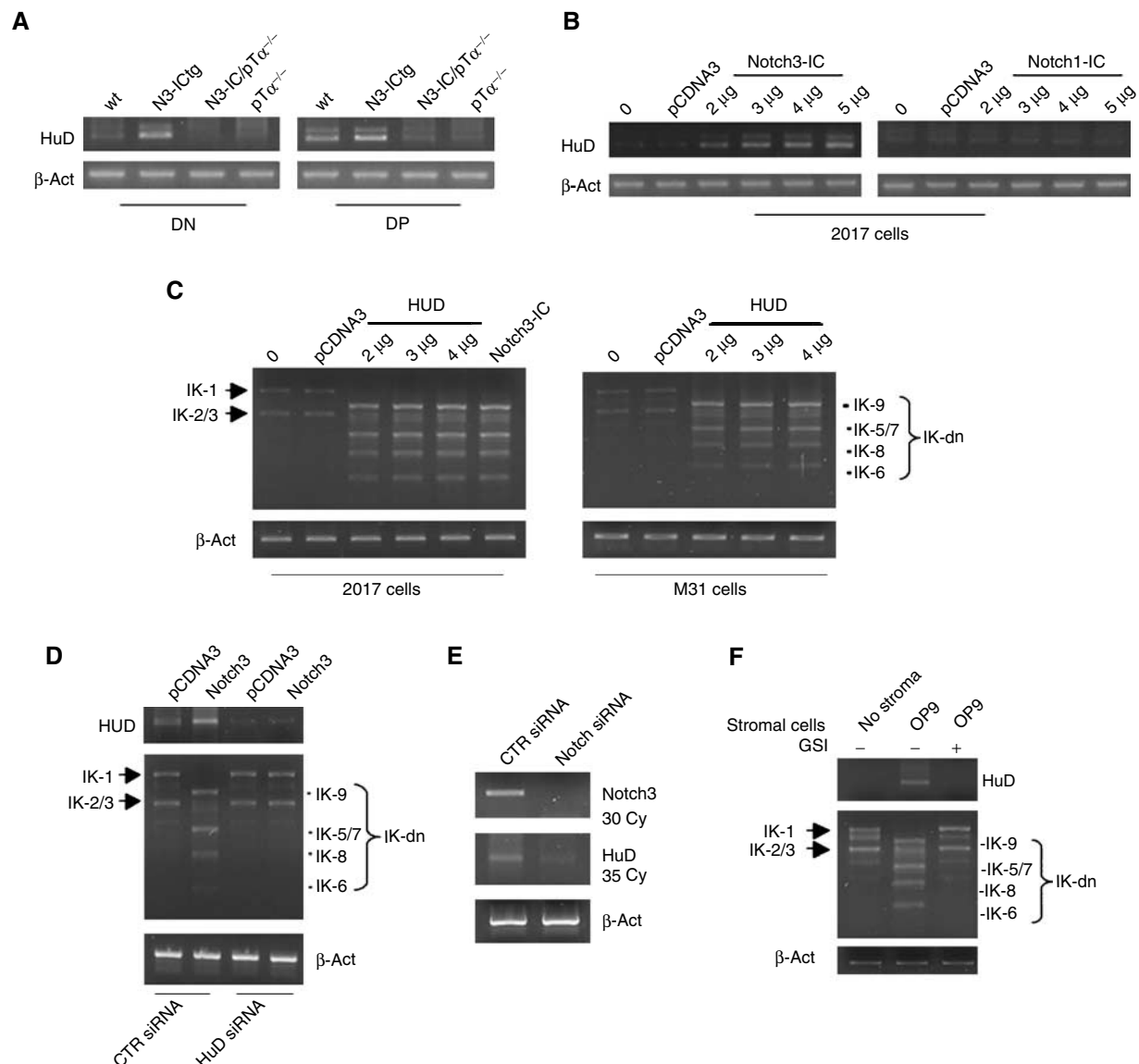


Figure 6 Notch3-induced skewing of Ikaros (IK) isoform profiles is mediated by Notch3-dependent upregulation of the RNA-binding protein, HuD. HuD expression was assessed *in vivo* by semiquantitative RT-PCR analysis of RNA in (A) sorted CD4⁻CD8⁻ DN and CD4⁺CD8⁺ DP thymocytes from wt, N3-ICtg, N3-ICtg/pTα^{-/-} and pTα^{-/-} mice and (B) *in vitro* in 2017 cells transfected with increasing amounts of Notch3 (left panel) or Notch1 (right panel). (C) RT-PCR detected expression of alternatively spliced IK isoforms in 2017 and M31 cells transfected directly with HuD. (0 = non-transfected cells; pCDNA3 = empty vector-transfected cells.) (D) HuD siRNA assay showing that knockdown of endogenous HuD prevents the Notch3-induced modulation of IK-dn isoforms. HuD and IK mRNA expression profile as assessed by RT-PCR in 2017 cell line, transfected with pCDNA3 or Notch3 expression vectors, after HuD or scrambled (CTR) siRNA is shown. (E) siRNA of Notch3 is able to inhibit the constitutive expression of HuD in 2017 cells. RT-PCR for HuD and Notch3 mRNAs in 2017 cell line, after HuD or scrambled (CTR) siRNA is shown. (F) Coculture experiment of 2017 cells on OP9 stromal cells. RT-PCR for HuD (30 cycles) and IK mRNAs is shown. GSI: γ-secretase inhibitor I; IK-1 and IK-2/3: DNA-binding IK isoforms; IK-dn: dominant-negative IK isoforms incapable of DNA binding. mRNA expression was monitored along the exponential phase of amplification and normalized to β-actin (β-act).

dependent T-cell leukemia (Fehling *et al*, 1997; Bellavia *et al*, 2002). The pTα gene encodes an essential pre-TCR component and is a key target of Notch3 signaling, which strongly enhances pTα promoter transcription through the formation of an activator complex composed of N3-IC, CSL and MAM. Our findings argue that the hematopoietic cell-specific transcription repressor, IK, is an important regulator of Notch3's effects on pTα. The pTα promoter region contains three consensus DNA-binding TGGGAA sequences that are recognized by both IK and the Notch effector protein CSL, and one of these was shown to be essential for Notch3-induced activation of pTα. This activation is inhibited by DNA-binding IK isoforms, but our findings

indicate that, in the presence of a functional pre-TCR complex, Notch3 can eliminate this restraint by increasing the expression of IK-dn splice variants that are incapable of DNA binding. It appears, therefore, that, in addition to the direct effect exerted by its activator complex, Notch3 signaling also enhances pTα transcription indirectly by diminishing the transcription-repressing activity of IK.

The effect of Notch3 on IK isoform expression seems to be mediated, at least in part, by the RNA-binding protein, HuD. The highly conserved Hu proteins (and HuD in particular) play critical roles in the post-transcriptional modulation of gene expression, ranging from regulation of alternative

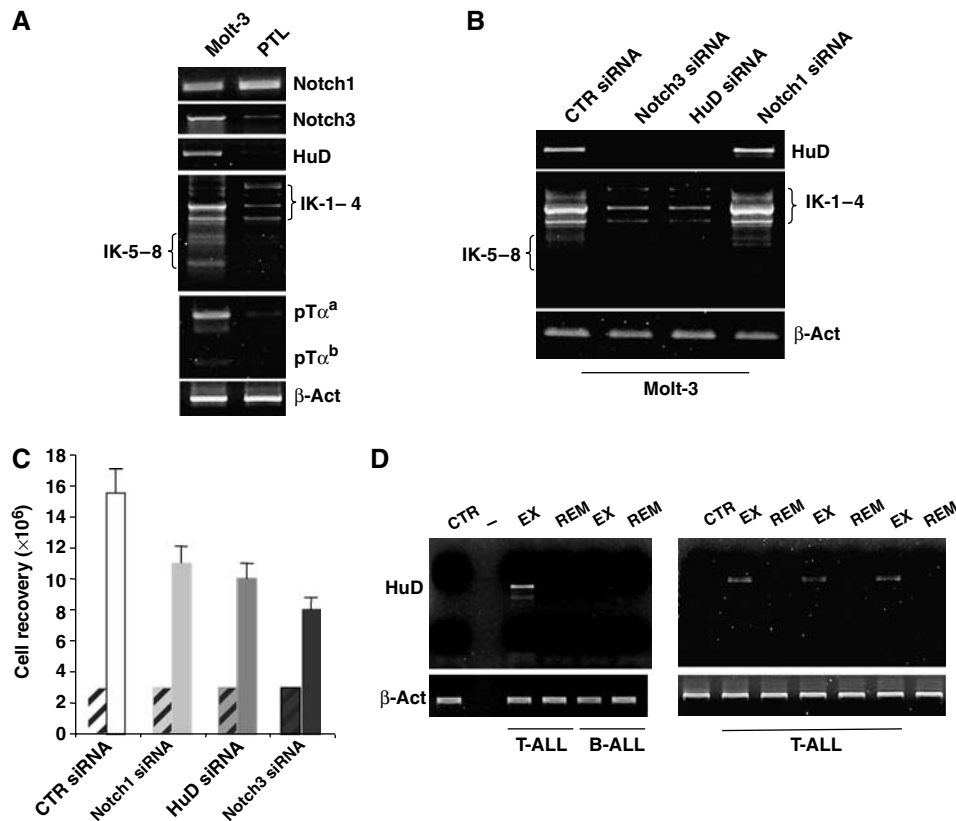


Figure 7 The RNA-binding protein HuD is expressed in human T-ALL and regulates IK isoform profile. (A) RT-PCR expression analysis of human Notch1, Notch3, HuD, IK and pT α mRNAs in Molt-3 cell line and peripheral T lymphocytes from one healthy donor (PTL) used as control. Results were normalized to β -actin (β -act). (B) siRNA assay showing that HuD and Notch3, but not Notch1, are able to regulate IK mRNA expression profile in Molt-3 cells, as assessed by RT-PCR; CTR siRNA: scrambled siRNA. (C) Inhibition of proliferation of Molt-3 cells. Recovery of Molt-3 cells after Notch3, HuD, Notch1 or scrambled siRNA transfection, compared to the cell number at the starting point (hatched bars). The data shown represent the average of three independent experiments; vertical bars indicate standard deviation. (D) HuD expression was assessed by semiquantitative RT-PCR analysis of RNA in bone marrow samples from four different primary human T-ALLs and one primary B-ALL at different stages of disease (exordium, EX; and remission, REM) and one control patient (CTR). Results were normalized to β -actin (β -act).

splicing and translation to modulation of mRNA transport and stability (Levine *et al*, 1993; Mobarak *et al*, 2000; Lisbin *et al*, 2001; Deschenes-Furry *et al*, 2003; Ratti *et al*, 2006; Zhu *et al*, 2006). HuD has long been known as one of the earliest markers of neural differentiation (Perrone-Bizzozero and Bolognani, 2002), and more recently it has also been described in the CD34⁺ subset of bone marrow cells (Goolsby *et al*, 2003), but our findings are the first evidence of its expression by mouse thymocytes and, more importantly, in human T-ALL. Whereas low levels were observed in wt DP thymocytes, DN and DP thymocytes from N3-ICtg mice display high levels of HuD expression. Moreover, N3-IC transfection resulted in significantly increased dose-dependent expression of HuD. Even more intriguing was the correlation observed between this upregulation of HuD (which was strictly pre-TCR-dependent *in vivo* and *in vitro*) and the shift toward increased generation of IK-dn isoforms in cells. The fact that the same shift was also observed following transfection of HuD alone, independently of pre-TCR presence, being instead inhibited by siRNA-induced silencing of endogenous HuD, adds further support to the view that this protein plays a direct role in Notch3's modulation of IK isoform expression patterns. Moreover, the decreased expression of HuD following the knockdown of endogenous Notch3 by siRNA and its increased expression observed after cocul-

turing 2017 cells on Jagged-1-expressing OP9 cells, inhibited by the treatment with GSI, suggest that HuD expression is induced by and depends on the triggering of endogenous Notch signaling.

The virtual absence of IK-dn isoforms in N3-IC/pT α ^{-/-} double mutant thymocytes was accompanied by a decrease in IK-1 and IK-2/3 transcripts as well (with respect to levels found in N3-ICtg, pT α ^{-/-} and wt cells). This finding suggests that the absence of HuD might also affect IK expression at a pre-mRNA level. More intriguingly, the observation that HuD is not expressed in the absence of pT α , independently of the overexpression of Notch3, suggests that Notch signaling alone, in the absence of pT α , is not sufficient in sustaining HuD expression.

Reduced expression of IK-1 and 2/3, by decreasing the inhibitory role of IK DNA-binding isoforms, could conceivably potentiate Notch3 activity and favor the activation of Notch target genes other than pT α , and an effect of this type might explain Notch3's partial reversal of the developmental arrest that characterizes thymocytes from pT α ^{-/-} single mutant mice (Bellavia *et al*, 2002). IK target/binding sites have also been reported in the enhancer region of *CD3* and *CD4* genes (Molnar and Georgopoulos, 1994).

In conclusion, we provide evidence for a novel non-redundant mechanism whereby the transmembrane receptor

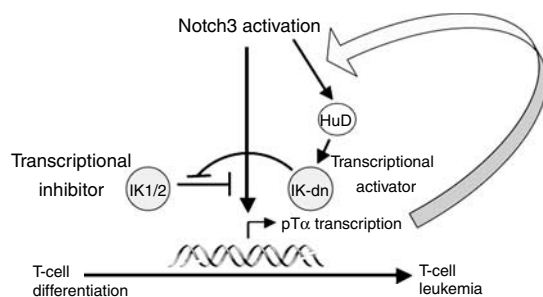


Figure 8 Crosstalk among Notch3, IK and pre-TCR in the regulation of T cell development and lymphomagenesis. Overexpression of HuD by thymocytes is triggered in a pre-TCR-dependent manner by the activation of Notch3 signaling. As a result of its modulating effects on the splicing and/or stability of IK mRNA, HuD promotes the preferential expression of IK-dn isoforms, which diminish IK-induced transcriptional repression and further enhance the upregulation of pT α gene expression induced directly by the Notch3-CSL transcription activator complex.

Notch3 and the hematopoietic nuclear factor IK cooperate in the regulation of T-cell development and lymphomagenesis. The molecular model portrayed by our findings (Figure 8) is characterized by crosstalk among Notch3, IK and the pT α /pre-TCR signaling activity that is mediated in part by the RNA-binding protein HuD. Its pre-TCR-dependent upregulation, specifically induced by Notch3, diminishes the IK-induced repression of transcriptional activation of pT α , an effect that appears to be related to regulation of the differential splicing and/or the stability of IK mRNAs. This is the first evidence that the RNA-binding protein HuD plays a role in the complex scenarios of T-cell differentiation and tumorigenesis.

Materials and methods

Mice

The generation and typing of N3-ICtg (Bellavia *et al*, 2000), pT α ^{-/-} (Fehling *et al*, 1995) and N3-IC/pT α ^{-/-} double mutant mice (Bellavia *et al*, 2002) have been described elsewhere.

Flow cytometry analysis

Freshly isolated cells from thymi and lymph nodes were prepared and stained as previously described (Bellavia *et al*, 2000) and analyzed on a FACScan (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences). Forward and side scatter gatings were used to exclude dead cells from the analysis. Cells were stained with anti-CD4-FITC and anti-CD8-PE antibodies (BD PharMingen). PE- and FITC-conjugated rat IgG (BD PharMingen) were used as a control for immunofluorescence.

Cell sorting

Thymocyte suspensions from wt, Notch3-IC tg, Notch3-IC/pT α ^{-/-} and pT α ^{-/-} mice were prepared and stained with anti-CD4-FITC and anti-CD8-PE antibodies as described above. CD4⁻CD8⁻ DN and CD4⁺CD8⁺ DP subsets were then separated (purity level $\geq 95\%$) with the FACSaria cell sorter (BD Biosciences).

RT-PCR analysis

Total RNA was extracted from unfractionated T lymphocytes (both from thymi and lymph nodes), from CD4⁻CD8⁻ and CD4⁺CD8⁺ sorted thymocytes, from untransfected and transfected M31, pre-T-cell line 2017 (Spolski *et al*, 1988) and human Molt-3 cell line, and from different samples of human ALLs and relative controls, using Trizol (Gibco) following the manufacturer's protocol. A 1 μ g portion of RNA was processed for RT-PCR as previously described (Felli *et al*, 1999). PCR was performed at the appropriate annealing temperature with the following primers: IK 5'-CTCCAGATGAAGGG

GATGAG-3' and 5'-CAGCAGCAGCAAGTTATCCA-3', IK exon 7 5'-TGGTTATCACAGCCAGGACA-3' and 5'-AAATCAAACGCCAAAC AACC-3'; HuD 5'-AGAAGGGAATGTCAGCTTTT-3' and 5'-TGAA TTCTTCTGGGTCATA-3' (Akamatsu *et al*, 2005); β -actin 5'-GTGG GCCGCTTAGGCACCAAT-3' and 5'-CTCTTTGATGTCACGCACGA TTTC-3'. Human IK: 5'-CCCCTGTAAGCGATACTCCAGAT-3' and 5'-GGCTTGGTCCATCACGTGGGGA-3'; human HUD: 5'-GAGTCTCTT CGGGAGCATTG-3' and 5'-CTTGTGGGCTTTGTGG TTT-3'; mouse Hes-1: 5'-ATGCCAGCTGATATAATGGAG-3' and 5'-CAGCTCCGGG TCTGTGCTGAG-3'; mouse Deltex: 5'-CACTGGCCCTGTCCACCAG CCTTGGCAG-3' and 5'-ATGCGAATT CGGGAAGCGGGCAACTCAG-3'; mouse Notch3: 5'-CCAGGGCTGC AACACTGAGGAATG-3' and 5'-TTGTGGCCAGCAGTATGTCCTTG-3'; mouse pT α : 5'-GGCAT GCTTCCACGAGTGGGC-3' and 5'-CGCTATGTCCAAATCTGTG GGG-3'; human β -actin: 5'-CTACAATG AGCTGCGTGTGG-3' and 5'-CGGTGAGGATCTTCATGAGG; mouse Notch1: 5'-GTGGATGCTGACT GCATGGATGTC-3' and 5'-ATGCAAAGCCGACTTGCCTAGGTC-3'; human Notch1: 5'-CTACCTGTCA GACGTGGCCT-3' and 5'-CGCAGA GGGTTGTATTGGTT-3'; human Notch3: 5'-TTCTTAGATCTTGGGGG CCT-3' and 5'-GGAAGAAGG AGGTCCCAGAC-3'; human pT α 5'-CTGCAGCTGGGTCTGCCTC-3' and 5'-AGTCTCCGTGGCCGGGTG CA-3'. To quantify transcript expression levels, PCR was carried out in the linear exponential phase of amplification through 20–35 cycles. Sample loading was monitored by a β -actin transcript that was subjected to the same treatment. IK PCR products were analyzed by agarose gel electrophoresis followed by Southern blotting and hybridization with a probe specific to the amplified sequences.

Western blot analysis

Whole-cell extracts were prepared as described previously (Beverly and Capobianco, 2003). In brief, 50 μ g of protein extraction from each sample was subjected to SDS/PAGE, transferred to nitrocellulose membrane and probed with the following antibodies: anti-IK (SC-9861, Santa Cruz Biotechnology Inc.) and anti-tubulin (SC-8035, Santa Cruz Biotechnology Inc). Bound antibodies were detected with enhanced chemiluminescence (ECL kit, Amersham).

Cell lines

Transient transfection experiments were performed using the pre-T-cell line 2017 (Spolski *et al*, 1988) and M31 immature T-cell line (Primi *et al*, 1988).

Plasmids

IKcDNAs were cloned from RT-PCR reactions from both wt and Notch3-IC transgenic thymi as previously suggested (Beverly and Capobianco, 2003). The fragment containing the putative pT α promoter region (pubMed sequence U27268) was amplified by PCR from genomic DNA using the primers pT α -prom-FL 5'-GCTTTGGATCTGGAGGATGA-3' and 5'-CTCTGCTAGTGCCTCC CA-3' and subcloned in topo TA-cloning (Invitrogen, Carlsbad, CA, USA). The pT α promoter-luciferase fusion plasmids were constructed by cleaving with *Xho*I and *Sac*I the PCR products and inserted into the *Xho*I and *Sac*I sites upstream of the luciferase cDNA of pGL3-basic vector (Promega, Madison, WI, USA). The mutant constructs pT α -prom-FL CSL1MUT and pT α -prom-FL CSL2MUT+CSL3MUT were prepared using QuikChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the instructions of the manufacturer. MAM was cloned in pFLAG-CMV2 as previously suggested (Wu *et al*, 2000; Beverly and Capobianco, 2003). HuD was amplified by PCR by using the primers HuD 5'-GGGTCCATCTTCTGATCACA-3' and 5'-GGGTGAGAAATT CAGGATTT-3' and cloned in pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA, USA) expression vector. All the correct clones were confirmed by sequencing. The expression vectors for Notch3-IC (Bellavia *et al*, 2000) and RBP- κ (Talora *et al*, 2002), were previously described.

Electrophoretic mobility shift assay

IK-1 and CSL proteins were produced in transfected Cos cells using expression vectors containing IK-1 or CSL cDNA. Nuclear extracts were prepared (according to Dumortier *et al*, 2006) by resuspending 10⁷ cells in 500 μ l of lysis buffer (10 mM HEPES, pH 7.9; 1.5 mM MgCl₂; 10 mM KCl; and 0.5 mM dithiothreitol (DTT)). After 10 min, cells were vortexed and nuclei were pelleted and resuspended in 50 μ l of the following buffer: 20 mM HEPES, pH 7.9; 25% glycerol; 420 mM NaCl; 1.5 mM MgCl₂; 0.2 mM EDTA; 0.5 mM DTT; and

protease inhibitor cocktail. Lysates were kept on ice for 20 min and vortexed thoroughly. After centrifugation, supernatants (nuclear extracts) were quantified using the Bradford colorimetric assay. Three micrograms of nuclear extract was used for each sample. Samples were incubated for 25 min at room temperature with 2 µg of poly(dI-dC), 1 µg of bovine serum albumin and 10 µM ZnCl₂ in 19 µl of HGDE buffer (20 mM HEPES, pH 7.9; 0.2 mM EDTA; 20% glycerol; 100 mM KCl; and 1 mM DTT). End-labeled, double-stranded probe was then added to each reaction mixture and the mixture was incubated for 20 min at room temperature. Protein-DNA complexes were resolved on a 5% polyacrylamide gel, dried and analyzed by autoradiography. The following probes were used: CSL: 5'-AGGAACTAGGCTTGGGAAAGGCTTTGAGAAT-3'; CSL MUT1: 5'-AGGAACTAGGCTTAGTACAGGCTTTGAGAAT-3'.

siRNA silencing

Mouse and human Notch3, HuD and control and human Notch1 siRNA (Dharmacon, Lafayette, CO, USA) were incubated in OPTI-MEM (Gibco, Gaithersburg, MD, USA) for 10 min at room temperature with Hy-perfect transfection reagent (Qiagen, Hilden, GE) at a final siRNA concentration of 5 nM. Complexes thus formed were added dropwise onto the cells (4×10^5 per well in a 24-well plate in 0.5 ml of RPMI medium containing FBS and antibiotics) and incubated for 72 h after transfection. To study Molt-3 cell survival/proliferation after siRNA, 3×10^6 cells/well were plated in a six-well plate; 72 h later, total cell recovery was evaluated.

Coculture experiments

OP9 stromal cells, kindly provided by Dr A Rolink, were maintained in ISCOVE'S modified supplemented medium, supplemented with 20% fetal bovine serum, 2% penicillin, 1% L-glutamine and 2% sodium pyruvate, and plated 1 day before use to achieve a confluent monolayer of cells. Cocultures were initiated with 4×10^6 2017 cells with or without 10 µM of γ -secretase inhibitor I (alternate name Z-LLNLe-CHO; Calbiochem, San Diego, CA, USA) and harvested after 48 h for RNA extraction.

Cell transfection and luciferase assays

Transfection of the pre-T-cell line 2017 and M31 cell line was performed using the lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. *Renilla* luciferase reporter vector pTK-*Renilla*-Luc (0.5 ng) was also incorporated into each transfection (Promega, Madison, WI, USA)

for normalization. The total amount of transfected DNA was kept constant by adding empty vector. At 48 h post-transfection, the cells were lysed in a reporter lysis buffer (Passive Lysis Buffer; Promega, Madison, WI, USA) at 120 µl/well. Firefly- and pRL-TK-derived *Renilla* luciferase activities were measured in each sample with the Dual luciferase Assay System (Promega, Madison, WI, USA) using a Model TD-20/30 luminometer (Turner Designs). Light emission was measured for 10 s after injection. The specific luciferase activity of different transfections was determined in triplicate samples and was normalized on the *Renilla* luciferase activity. Data were expressed as means \pm s.d. of at least three independent experiments. Transient transfection efficiency in M31 and 2017, as assessed by expression of a GFP expression vector added in trace amounts, was \sim 70 and \sim 40%, respectively.

ChIP assay

Protein complexes were crosslinked to DNA in living nuclei of freshly isolated thymocytes to a final concentration of 1%. Crosslinking cells were processed, as previously described (Vacca *et al*, 2006) and divided into aliquots. In total, 5 µg of antibody (anti-RBP-Jk sc-28713X, anti-IK sc-13039, rabbit IgG sc-2027, Santa Cruz Biotechnology Inc.) was added to each aliquot of chromatin and incubated on a rotating platform for 12–16 h at 4°C. Antibody-protein-DNA complexes were isolated by immunoprecipitation with salmon sperm DNA/Protein A agarose (#157, Upstate Biotechnology). Following extensive washing, bound DNA fragments were eluted and analyzed by subsequent PCR using the following primers specific for the pT α mouse promoter including the CSL-core 1 site: 5'-GCTTTGGATCTGGAGGATGA-3' and 5'-GAAGCTCAGGTCCCACTCCCA-3'.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

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