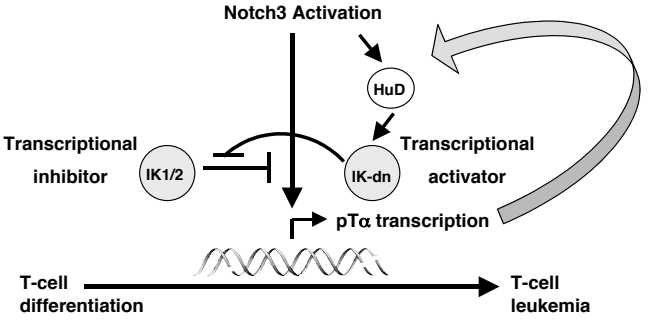
Notch3 overexpression induces an alteration in Ikaros splicing mediated by the RNA-binding protein HuD in T-ALL leukemia



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THE NOTCH GENE FAMILY

INTRODUCTION

The Notch signaling is one of the fundamental pathways that regulate metazoan development and adult tissue homeostasis, that is highly conserved in evolution throughout the animal kingdom. Thomas Hunt Morgan first described notches at the margin of wing blades of fruit flyes (*Drosophila Melanogaster*).[1] These notches were found to be the result of a partial loss of function (haplo-insufficiency) of the *Notch* gene encoding for a single transmembrane receptor that was cloned almost 70 years later as *Drosophila Notch.*[2-3] Notch receptors and their corresponding ligands play a key role in the development of multicellular organisms, as they control cell fate by regulating cell proliferation, survival, positioning in the organism, and differentiation.[4] Notch signaling is also important in adults, in which it regulates stem-cell manteinance, binary cell fate decisions, such as the T or B-lymphocyte lineage decision, and differentiation in self renewing organs.[5] *Drosophila* has one Notch receptor activated by two different transmembrane bound ligands, called Serrate and Delta, while *Caenorhabditis elegans* contains two receptors (Glp-1 and Lin-12) and two ligands (Apx-1 and Lag-2). In contrast, the mammalian Notch family consists of four highly conserved transmembrane receptors (Notch-1, 2, 3 and 4),[6-10]which can interact with at least five Notch ligands: Jagged1 and Jagged2 (homologues of Serrate) and Delta-like 1,3 and 4 (homologues of Delta).[11-15]

OVERWIEW OF NOTCH SIGNALING PATHWAY

The Notch signaling pathway is complex and involves the coordinated activities of many different molecules. Notch proteins are membrane-bound type I receptors (with a single-pass transmembrane domain), harboring a large extracellular domain involved in ligand binding, and a cytoplasmatic domain involved in signal transduction. The extracellular domain contains between 29 and 36 epidermal growth factor-like (EGF) repeats, that are critical for binding interactions with ligand DSL (Delta, Serrate, Lag-2) domains,[16-17]followed by three cysteine-rich Notch/Lin-12 (LNR) repeats, that prevent signaling in the absence of ligand. The intracellular domain of Notch receptors (NICD), which conveys the signal to the nucleus, harbors several protein-protein interaction domains such as the RAM23 domain[18] and six ankyrin/CDC10 repeats,[19]which bind downstream effector molecules, two nuclear localization signals (NLS), and a PEST sequence [rich in proline (P), glutamic acid (E), serine (S) and threonine (T)] that negatively regulates protein stability.[20] While Notch1 and Notch2 also contain a transcriptional transactivation domain (TAD), such domains have not yet been described for Notch3 and Notch4. The Notch receptors are synthesized as single precursor proteins. O-fucosyltransferase1 (OFUT1) functions as a chaperone and is required for the transport of pre-Notch from the endoplasmic reticulum (ER) to the Golgi apparatus and for fucosylation of glycosylated serine and threonine residues of the extracellular domain in the Golgi.[21]

Glycosylation of these residues is carried out by members of the Fringe family (radical, manic and lunatic), and this modification is important because it affects

sensitivity to the ligands.[22-24] A Furin-like convertase cleaves pre-Notch into the extracellular and intracellular domain (S1 cleavage), which results in a heterodimeric receptor with non-covalently associated domains that is transported to the plasma membrane.[25-26] Notch signaling initiates through ligand-receptors interactions between neighboring cells, leading to two consecutive proteolytic cleavages of the receptor (S2 and S3), which ultimately liberate NICD. After ligand binding the ubiquitin ligases mind bomb[27-28] or neuralized[29-31] interact with the ligand intracellular domain to promote its ubiquitination and internalization. Ligand endocytosis leads to a conformational change in Notch that allows ADAM (metalloprotease and disintegrin) protease TACE (tumor-necrosis factor α-converting enzime) to cleave the receptor at a second site (S2) on the extracellular side, close to the transmembrane domain.[32] The extracellular Notch domain is then transendocytosed by the ligand-expressing neighboring cell.[33] S2 cleavage determines a conformational change that allows access of the γ-secretase complex, whose key components are presenilin and nicastrin, which is responsible for the second proteolytic cleavage (S3) that occurs in the transmembrane domain and liberates the intracellular domain (NICD).[34-36] Then, NICD translocates to the nucleus where it binds through its RAM23 domain to the transcription factor CSL (CBF1 in *humans*, RBP-J_κ in *mice*, Suppressor of Hairless in *Drosophila*, Lag1 in *C. Elegans*). In the absence of Notch activity, CSL proteins bind to promoters of its target genes and recruit histone deacetylases[37] and corepressors, as SMRT/Ncor and SHARP/MINT, that inhibit transcription.[38] The NICD/CSL interaction converts CSL from a transcriptional repressor into a transcriptional activator by displacing the corepressor complex and recruiting coactivators, through the ankyrin repeats of Notch, such as Mastermind-like proteins (MAML)[39-40], which

in turn recruit additional coactivators such as histone acetyltransferase CBP/P300.[41-43]

To date only a few Notch target genes have been identified, some of which are utilized in multiple tissues while others seem to be tissue specific. The best known Notch target genes are members of the basic helix-loop-helix family such as HES1 and HES5[44], the related HERP (HES-related repressor protein) transcription factor family[45], the cell cycle regulator p21[46], Nrarp (notch-regulated ankyrin repeat protein)[47], Deltex1[48], and the pre-T cell receptor-α gene.[49] An additional level of complexity derives from the fact that Notch signaling can be regulated by several modulators that act at extracellular, cytoplasmic or nuclear levels. Besides Fringe, described above, which acts at extracellular level, examples of cytoplasmic modulators of notch signaling include Numb and Deltex. Numb suppresses Notch signaling, possibly by preventing nuclear localization and targeting NICD for degradation through the E3 ligase Itch.[50-51] Deltex is a positive regulator of Notch in *Drosophila*[52] but can inhibit Notch signaling in mammals.[53] An alternative CSL-independent pathway has been described in which Notch signaling is mediated through Deltex.[54] Examples of nuclear modulators are Nrarp and MINT(Msx2-interacting nuclear target protein) both of which seem to negatively regulate Notch signaling. It is currently unknown how Nrarp inhibits Notch signaling[55], whereas MINT has been shown to compete with NICD for binding to CSL.[56] The stability of nuclear NICD is regulated through its PEST domain. Binding of MAML to p300 and cyclin-dependent kinase 8 (CDK8) promotes hyperphosporylation of the NICD PEST domain to facilitate ubiquitylation, probably by members of the SEL1 (also known as FBW7) family of E3 ligases and the Itch E3 ligase, which target NICD to the proteasome.[57-58]

NOTCH IN HEMATOPOIETIC AND LYMPHOID DEVELOPMENT

The notch pathway is gaining increasing recognition as a key regulator of developmental choices, differentiation and function throughout the hematolymphoid system. Notch controls the generation of hematopoietic stem cells during embryonic development and may affect their subsequent homeostasis. Commitment to the T cell lineage and subsequent stages of early thymopoiesis is critically regulated by Notch. The Notch pathway is linked to early hematopoiesis during embryonic development and to the control of hematopoietic stem cell (HSC) self-renewal. Notch1 plays a critical role in the generation of the earliest embryonic HSCs required for definitive hematopoiesis.[59] Hirai's group[59] found a marked impairment in the ability of Notch1$^{-/-}$ embryos to generate hematopoietic colonies from structures including the aorta-gonad-mesonephros region, an important site of early hematopoietic activity where HSCs likely arise from hemangioblast precursors. Reconstitution of conditioned newborn recipients was defective, strongly suggesting a defect in HSC numbers or function. Importantly, Notch1 seemed critical during a narrow time window, after which the HSC pool could be maintained without Notch. The molecular mechanisms by which Notch controls the generation of embryonic HSCs is unknown, but the effect is cell autonomous.[60] Interactions with other key factors in this process, such as LMO2, GATA-2, AML1, and SCL/Tal-1, need to be explored.

Ligand-mediated Notch stimulation, or expression of constitutively active forms of Notch, can promote self-renewal of adult HSC.[61-63] Recent data suggest that

osteoblasts, a critical cell type in HSC niches, upregulate Jagged1 when activated by parathyroid hormone (PTH) or PTH-related protein, which may activate the Notch pathway in resident HSCs.[64] However, it is unclear if this translates into a physiological role for Notch in the maintenance of HSCs. To date, no genetic models of Notch inactivation have shown clearcut evidence for such a role. In particular, bone marrow (BM) from conditional Notch1 or CSL/RBP-J knockout mice can compete with wild-type BM in trilineage hematopoiesis of mixed BM chimeras, which is strong evidence against a major HSC deficiency in the absence of Notch1 or CSL/RBP-J.[65-66] More definitive answers may come from careful studies of HSC function in genetic models of Notch inactivation, including systems in which CSL/RBP-J-independent activities can be blocked. Because HSC self-renewal is controlled through multiple pathways, it is critical to understand the relative importance and interactions of Notch and other factors, such as Wnt, HoxB4, and Sonic Hedgehog.[91-92-93]

NOTCH AND T CELL DEVELOPMENT

The development of mature T cells from lymphoid progenitor cells involves a series of cell fate choices and differentiation steps that direct cells along one of several distinct developmental pathways. T cell development occurs in the thymus, a multi-lobed organ composed of cortical and medullary areas surrounded by a capsule. T cell precursors enter the subcapsular cortical areas, where they encounter networks of cortical epithelial cells, the thymic stroma, and undergo a period of proliferation. As they differentiate, they move from the cortex towards the medulla of the thymus. Hematopoietic stem cells, just arrived in the thymus, begin to express CD2, but have not yet rearranged their genes for

T cell antigen receptor (TCR), are negative for the expression of CD3, and finally they lack markers typical of mature T cells, (CD4 and CD8), for this reason they are defined double negative thymocytes (DN or $CD4^-CD8^-$), the most immature subpopulation of T cells. A small amount of double negative thymocytes, about 5%, express TCRγδ on the membrane, the majority are committed to become mature αβ T cells. Depending on the expression of two markers on the mebrane, the adhesion molecule CD44, and the α chain of the IL-2 receptor, also known as CD25, DN thymocytes can be classified in four subpopulation: $CD44^+CD25^-$(DNI), $CD44^+CD25^+$(DNII), $CD44^-CD25^+$(DNIII), $CD44^-CD25^-$ (DNIV). First, αβ T cells express CD44, then CD25. $CD44^{low}CD25^+$ double negative T cells rearrange TCRβ chain. β chain rearrangement begins with D-J joining, followed by V-DJ joining. Only DN thymocytes that have successfully rearranged β chain survive, the others undergo apoptosis and die. Productive rearrangement of β chain is followed by its expression on the T cell membrane with CD3 and surrogate α chain, pTα, forming the pre-T cell receptor complex (preTCR). Signaling through the preTCR causes the cells to stop rearranging β chain, undergo a period of intense proliferation, and begin to express both CD4 and CD8, becoming double positive T cells (DP or $CD4^+CD8^+$). Membrane CD25 is lost at this stage, and DP cells express again RAG-1 and RAG-2 to rearrange their α chain genes. At this point DP αβ TCR^{low} cells move into the cortico-medullary junction, where they undergo positive and negative selection, two processes fundamental for establishing immunity and self-tolerance, and mature into single positive T cells ($CD4^+$ or $CD8^+$).

Proliferation, cell fate specification or death of T cell progenitors is determined by intrathymic instructive and permissive signals, that are either cell

autonomous or arise from interactions with the thymic stroma and direct the thymocyte progression from immature $CD4^-CD8^-$ double negative (DN) towards $CD4^+CD8^+$ double positive (DP) phenotype and their $CD4^+CD8^-$ versus $CD4^-CD8^+$ single positive (SP) cell lineage choice. These signals include those generated by T cell specific differentiation products, i.e. pre-T cell receptor (TCR) and mature αβ TCR.[70-71]

T cell development is also controlled by general biological regulators, including Notch receptors. Indeed, an expression profile description of components of the Notch signaling pathway (Notch family gene members and their cognate ligands and target genes) has been provided for the thymus.[72] More specifically, Notch1 expression is high in early DN thymocytes, low in DP cells and intermediate in CD4 and CD8 SP cells.[53,73] Interestingly, unlike Notch1, Notch3 expression window takes place in DN thymocytes at the $CD44^-/CD25^+$ DN3 stage, and is down-regulated before the transition to DP cells to reach very low levels in mature T lymphocytes.[72,74]

The presence of multiple Notch receptors and their differential expression along the different developmental steps raise the question of whether different such receptors play distinct roles during thymocyte differentiation. In particular, Notch1 and Notch3 have been related to distinct steps of T cell differentiation program, from the initial specification of T cell lineage precursors, at an early or prethymic stage, until the final decision of thymocytes to become $CD4^+$ or $CD8^+$ lymphocytes.[75] A significant role for Notch1 has been suggested in the initial T cell lineage commitment of bone marrow-derived common lymphoid precursors[76,77] and in intrathymic differentiation of T lymphoid cell lineages, by favoring αβ versus γδ T cell lineage decision[78,79] as well as in thymocyte survival and protection against glucorticoid- and TCR-induced apoptosis.[80,81] More recently it has been shown that Notch1 is required for a correct VDJβ

rearrangement.[82] Still debated, instead is the role in the CD8 versus CD4 T cell lineage decision. The best-established function of Notch1 during lymphoid development is its role in T lineage commitment. As first shown by Radtke's group, Notch1's loss of function results in a marked decrease in the size of the thymus that lacks T cells and contains an excess of B cells.[65-66] These B cells resemble BM B cells and develop intrathymically.[83] Conversely, expression of constitutively active Notch1 in HSCs results in extrathymic T cell development and suppression of BM B cell development.[84] In culture, expression of Notch ligands of the Delta-like family (Dll-1 or Dll-4) in BM stromal cell lines is sufficient to drive multipotent hematopoietic progenitors, or even embryonic stem cells, to the T cell lineage, while suppressing B cell development.[84-87]Notch1 inactivation causes a complete block in T lineage development, indicating that other Notch family members cannot compensate for the loss of Notch1 in vivo.[66] The distinctive functions of Notch family members could be related to the activation of different downstream signals or to a variable binding efficiency with different Notch ligands.

In terms of the relevant Notch-mediated intracellular signals for T lineage commitment, conditional inactivation of CSL/RBP-J results in a developmental block similar to the block observed in Notch1-deficient mice,[65,88] indicating that relevant Notch-dependent signals are mediated by CSL/RBP-J during T cell commitment. Beyond initial T lineage commitment, developing thymocytes undergo the decision to become either αβ or γδ lineage T cells. An instructive model of αβ versus γδ development suggests that the nature of the first successful T cell receptor (TCR) gene rearrangement dictates the lineage decision, so that cells first producing a functional β chain together with pre-TCRα (pTα) commit to the αβ lineage, and cells rearranging both γ and δ genes

are instructed to the γδ lineage. A role for Notch1 in the αβ versus γδ lineage decision was first suggested by Robey's group.[78] These investigators generated mixed BM chimeras with Notch1$^{+/-}$ and Notch1$^{+/+}$ progenitors.[78] In the thymus of these mice, a decreased proportion of αβ lineage cells was derived from the Notch1$^{+/-}$ progenitors in competition with the Notch1$^{+/+}$ cells, whereas the reduction in γδ T cells was much less dramatic. These data suggest that higher levels of Notch signaling can favor the αβ lineage. It is unclear from these data if γδ development is the default cell fate and increasing Notch signaling plays an inductive role toward the αβ lineage, or if Notch controls a true binary cell fate decision from a common progenitor. Additional results from Robey's group and others indicate that Notch can probably influence γδ versus αβ lineage choice independently of the expression of pre-TCR or TCRγδ. Indeed, the thymus of lck-ICN1 transgenic mice showed an increased number of CD8$^+$ TCRγδ$^+$ cells but not CD4$^-$CD8$^-$ TCRγδ$^+$ cells. Lck-ICN1 transgenic mice crossed to a TCRγδ transgenic strain had a significant increase in CD8TCRγδ$^+$ cells but a decrease in the percentage of CD4$^-$CD8$^-$TCRγδ$^+$ cells, suggesting that the increased CD8$^+$ TCRγδ$^+$ cells may actually be αβ lineage cells selected through the expression of TCRγδ. Moreover, expression of transgenic ICN1 in a TCRβ-deficient background increased the generation of CD4$^+$CD8$^+$ cells expressing TCRγδ with functional rearrangements at the TCRδ locus, suggesting that Notch could increase the development of αβ lineage cells even when pre-TCR components are deficient and αβ lineage cells are selected through the expression of TCRγδ. Honjo's group[89] reported the phenotype of CSL/RBP-J conditional knockout mice crossed to lck-Cre, in which the CSL/RBP-J-dependent activity of all four Notch family members is abolished. The earliest studies examining the role of Notch signaling in the development of CD4 and CD8 T-cell lineages employed

transgenic mice expressing an activated form of Notch1 (NotchIC). In these mice, fewer CD4 SP and more CD8 SP thymocytes were generated. Moreover, thymocytes recognizing class II MHC could be diverted from the CD4 to the CD8 lineage, leading to the proposal that Notch plays a role in CD4/CD8 lineage commitment.[78] Notably, activated Notch could not bypass the requirement for MHC-dependent positive selection. In contrast, another group using a more transcriptionally active transgenic construct concluded that Notch activity promoted the survival and development of both CD4 and CD8 lineage T cells and could do so in the absence of MHC recognition.[48] This discrepancy could be related to a difference in propensity to develop tumors. When young mice were analyzed before tumors arose, activated Notch inhibited CD4 and promoted CD8 SP thymocyte development in both strains. Moreover, development of SP thymocytes in both strains was MHC dependent. Although these gain-of-function studies suggested that forced Notch activity could influence lineage commitment, the role of endogenous Notch in CD4/CD8 fate determination remained unresolved. Thymocytes individually deficient in Notch1, -2, or -3 show no alterations in CD4/CD8 T-cell development. There is the possibility for functional redundancy among the four Notch homologs, but analyses of compound Notch deficiencies in thymocytes have not been reported. Conversely, a specific role of Notch3 at the preTCR checkpoint has been suggested, since it has been demonstrated a preferential upregulation of Notch3 (compared to Notch1) in DN immature thymocytes prior to their transition to more mature DP and a subsequent downregulation across the DN to DP transition.[72] These events are controlled by the pre-TCR signalling pathway, and are characterized by an activated NfkB.[90-91] Moreover, Lck promoter-Notch3-IC transgenic mice display a peculiar phenotype of dysregulated early T cell development characterized by the significant expansion of $CD25^{+}$ DN

thymocyte subsets, the sustained expression of CD25 and pTα invariant chain of the preTCR and a constitutively activated NFkB in all thymocyte subsets and peripheral T cells.[92] Consistent with the different timing of Notch1 and Notch3 activity during T cell development process, in vivo experimental models characterized by constitutively active transgenes have shown significant differences between Notch1 and Notch3 in regulating and/or affecting T cell development. Indeed, Notch1 is not able to trigger the generation of DP thymocytes in the absence of a functional preTCR.[93] In contrast, Notch3 is able to rescue the blockage of the pre-TCR check-point by forcing immature DN thymocytes to progress toward a more differentiated phenotype (e.g. acquisition of CD4, CD8 and TCRα expression) in the absence of pTα/preTCR.[74] However, the role of Notch3 in T cell development will be treated in more details below.

NOTCH ACTIVATION IN THE MOLECULAR PATHOGENESIS OF T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

T-cell acute lymphoblastic leukemia (T-ALL) is a malignancy of thymocytes that develops mainly in children and adolescents but also arises in adults, which constitutes approximately 15-20% of ALL. This aggressive tumor is characterized by high peripheral blood cell counts, increased numbers of blast cells, CNS dissemination and large mediastinal masses that cause tracheal compression and respiratory distress at diagnosis. Altough T-ALL often arises in the thymus, it spreads throughout the body and, without therapy, is rapidly fatal. Current treatment for T-ALL consists mainly of multi-agent combination chemoterapy.[94] Leukaemic trasformation of developing thymocytes is caused by

multistep mutagenesis involving various genetic alterations that shift normal cells into uncontrolled growth and clonal expansion. These changes can affect cell cycle control, stem-cell manteinance and cell proliferation, differentiation and survival. Cytogenetic analyses and the molecular cloning of chromosomal translocations breakpoints led to the discovery of transcriptional regulatory proteins that are aberrantly expressed in T-ALL owing to the juxtapositioning of their respective genes next to strong T-cell receptor (TCR) gene enhancers and promoters, without alterations in protein structure (with the exception of TAN, or truncated activated Notch1). In general, these translocated proteins have important functions during normal embryogenesis, although many are not essential for the development of normal thymocytes.[95] So, many cases of T-ALL seem to result from the thymocyte specific aberrant expression of proteins that normally function primarily in non-lymphoid tissues. Aberrant Notch signaling occupates a central role in the pathogenesis of human T cell acute lymphoblastic leukemia/lymphoma. Unlike most other genes associated with human T-ALL, that are not normally expressed in developing T cells, Notch exerts its trasforming function in the same lineage in which it is most highly expressed.[96] In fact, as seen, Notch signaling mediates hematopoietic cell fate determination in the embryo and in the adult, and consequently deregulated expression of Notch pathway elements can lead to development of haematological malignancies. Historically, the first human homologue of *Drosophila* Notch, Notch1, was identified at the chromosomal breakpoint of a subset of T cell lymphoblastic leukemias/lymphomas containing a t(7;9)(q34;q34.3) chromosomal translocations.[96] The traslocation fuses the 3' portion of Notch1 to the T cell receptor Jβ locus. The molecular breakpoints in the t(7;9)-associated tumours all cluster within 100 base pairs of intron 24 of Notch1. So, most of the extracellular domain is deleted, which results in a series of Notch1 peptides of

different size, all of which are devoid of EGF repeats and Notch/LIN12 repeats. These truncated Notch1 isoforms are temporally and spatially expressed in the manner of the TCRβ locus and most of them possess constitutive, ligand-independent activity. However this initial discovery did not reveal the full oncogenic potential of the truncated version of N1ICD. A causative role of Notch1 in T cell lymphomagenesis was only shown when Pear[97] showed in a murine system that the overexpression of N1ICD using a retroviral transduction assay of hematopoietic stem cells led to development of highly aggressive immature T-cell malignancies, reinforcing an immature T cell neoplasms. These and subsequent studies[98-99] provided the initial basis for an experimental model whereby mutations in Notch1 have been analyzed for their oncogenic potential. This was shortly followed by studies with transgenic mice expressing dominant active forms of Notch1,[48,78,100] by the identification of additional Notch1 rearrangements in mice with radiation-induced thymomas,[101] and Notch1 mutations in murine transgenic models of T-ALL.[102]Despite the efficiency of Notch1 in inducing T-ALL in murine models, it became soon clear that the rare t(7;9) translocation event could account for only a minor fraction of T-ALL cases.[103] Ellisen et al.[96] originally screened 40 T cell leukemia/lymphoma patient samples and found 4 with the t(7;9) translocation. Based on this, an incidence of ~10 % was estimated. However, in subsequent studies, it appears that <1% of all human T cell leukemias or lymphomas contain this translocation.[103] However, more importantly, aberrant Notch signaling was subsequently found in several human leukemias and lymphomas that lacked genomic rearrangements,[74,104-105] signifying that upregulated Notch signaling might have a common role in human leukemogenesis. Jundt et al.[104] found high levels of NOTCH1 protein expression in twelve T cell anaplastic large cell lymphoma (ALCL) samples as compared to B cell lymphomas, and high levels of cleaved (activated) NOTCH1 were seen in

two human ALCL-derived cell lines, compared to normal T cells. Definitive evidence for a central role of Notch1 in human T-ALL came from a recent study in which somatic activating mutations in Notch1 were found in more than 50% of human T-ALL samples.[106] Moreover, when 30 human T-ALL cell lines were screened for Notch1 dependency using a γ-secretase inhibitor, five showed a G0/G1 cell-cycle arrest that could be rescued by viral expresson of the intracellular domain of notch1 (ICN1). Sequencing of Notch alleles in these cell lines identified missense mutations at conserved amino-acid positions in the N- and C-terminal heterodimerization domains, that lead to ligand independent metalloprotease cleavage at site S2,[106] and short insertions or deletions in the polypeptide enriched in proline, glutamate, serine and threonine (PEST) domain, resulting in partial or complete deletion of this domain, and consequent stabilization of ICN1, finally in cis in the same Notch1 allele. Furthermore, Screpanti's lab demonstrated that Notch3 was consistently expressed in 30 human T cell acute leukemias samples, and dramatically reduced levels were seen at clinical remission.[74] Interestingly, in T-ALL, Notch-3 is associated with the expression of its target gene, HES1, and of the gene encoding pTα.[74] Expression of these three genes is normally limited to thymocytes and none is usually expressed in normal mature peripheral T cells. Thus, a T-ALL signature, resulting from the combined expression of NOTCH3, pTα and HES1, characterizes the active and relapsing disease. N3ICD could possibly function also by activating NF-kB via its recently described effect on IKKα.[107] However, the role of Notch3 in T-ALL leukemia will be described in details in an another paragraph. These observations suggest the possibility that up- regulation of Notch may play a role in more than the small subset of lymphomas that have the t(7;9) translocation. More compelling evidence was brought about by a study

published from the laboratory of Aifantis.[108] They analyzed the gene expression profile of primary hematopoietic stem and lymphocyte progenitor cells, as these are the cell populations where random Notch1 activating mutations initially occur. The analysis revealed that a panel of significantly up-regulated genes were components of the NF-kB pathway and included target genes such as Nfkb2, Relb, Nfkbia, Bcl2a1 and Ccr7. A direct link between the Notch and NF-kB pathway was provided using N1ICD to induce the activity of NF-kB reporters. Dominant negative forms of the MAML1 and IkBa genes, respectively, could efficiently antagonize these interactions. Conclusive evidence was provided in the same study showing that NOTCH1 human T-ALL derived mutations activate the NF-kB pathway and that T-ALL cell lines have an activated NF-kB pathway. However, the precise mechanism by which Notch1 induces T-ALL is not yet fully elucidated. Several studies published recently have identified potential Notch target genes that may play a role in the oncogenic potential of Notch in the development and/or maintenance of disease, like the transcription factor E2A, the F-box protein SKP2, cyclin D3, p53 and c-myc.[109-113]

THE ROLE OF NOTCH3 IN T-CELL DEVELOPMENT AND T-ALL LEUKEMIA

The presence of multiple Notch receptors raised the question of whether such different receptors play distinct roles during thymocyte differentiation. Indeed, we observed previously that a significantly higher percentage of DN thymocytes express Notch3 relative to Notch1 and that there was a preferential up-regulation of Notch3 (compared with Notch1) in DN thymocytes prior to their transition to

the $CD4^+8^+$ double positive (DP) stage following interaction with thymic epithelial cells.[72] Most provocatively, Notch3 is down-regulated across the DN–DP transition that is controlled by the pre-TCR signaling pathway.

To assess the ability of Notch3 to influence T-cell development, Notch3 transgenic mice were generated using the *lck* proximal promoter to overexpress a hemagglutinin (HA) epitope-tagged Notch3-IC (Notch3 intracellular domain), able to trigger ligand-independent Notch3 intracellular signaling.[114]

Constitutive activation of Notch signaling in Notch3 transgenic mice is suggested by enhanced expression of specific Notch target genes, such as HES-1 and Deltex.

The thymocyte phenotype of wild-type and tg (transgenic) mice was analyzed at different ages. Until 4 weeks of age, there was a reproducible increase in the thymocyte number in tg mice ($3.0x10^8$/thymus compared with $1.8x10^8$/thymus in wild-type 3-week-old mice). Absolute numbers of cells of each subset (DN, DP and SP) were increased. Although there was no obvious impairment of thymocyte subset distribution with respect to $CD4^+$ and/or $CD8^+$ cells, most tg thymocytes, including DN thymocytes, stained brightly for CD3 and TCR. A higher percentage of tg thymocytes also stained brightly for CD69, mainly observed in the DP subsets. Thus, overexpression of Notch3-IC alters thymocyte differentiation. Further evidence of this was provided by the fact that all tg thymocyte subsets included cells expressing high levels of CD25, broadly distributed in cortex and medulla of thymus sections. This phenotype might be interpreted as a failure to down-regulate CD25 expression that occurs ordinarily in DP and SP subsets after 17–18 days post-conception.[115] To examine whether the increased number of thymocytes was attributable to changes in the earliest subsets, we examined DN thymocytes, segregated into four subsets (I–IV) based on the discrete expression of CD44 and CD25.[116]Both $CD25^+$ DN subsets (II and

III) were over-represented in tg mice, the latter consistent with a failure to down-regulate CD25. Ordinarily, the pre-TCR-regulated transition of subset III ($CD44^-/CD25^+$) cells into subset IV ($CD44^-/CD25^-$) cells is a critical point in thymocyte differentiation, as thymocytes are rescued from apoptosis and induced to expand clonally.[117] DN cells from tg versus non-tg littermates showed little increase in the proportion of cells in S + G_2-M phases of the cell cycle (29.0 versus 23.8%, respectively). A noticeably smaller percentage of tg DN cells were apoptotic compared with wild-type mice (2.4 versus 8.1%, respectively).

Two transient peaks of NF-κB activity occur in the thymus, one in $CD8^+$ SP cells and one as cells move through the late DN stages.[118-119] We and others observed that the latter subset-specific NF-κB activity provides relief from apoptosis as late DN thymocytes proliferate in response to signals from the pre-TCR.[90,119] Given the expansion of late DN thymocytes, the reduced levels of apoptosis and the sustained expression of a number of previously described NF-κB target genes, including CD25, IL-2, IFN-γ, IL-4, TNF-α and Bfl-1/A1, in tg mice[120-121] we examined NF-κB activity. Compared with wt mice, nuclear extracts from thymocytes of 2- to 4-week-old Notch3 tg mice displayed constitutive high levels of specific p50–p65 NF-κB–DNA complexes. These data demonstrate that Notch3 promotes NF-κB activation in thymocytes, consistent with improved survival and increased numbers of thymocytes in Notch3 tg mice.

One of the most intriguing features of transgenic mice is the overexpression of both pTα isoforms, a and b, and the persistence of their expression also in double positive and single positive thymocytes, and moreover in mature peripheral T cells, where they are not normally expressed. By mutliple criteria,

young tg animals appeared normal until 7 weeks of age, at which point all 14 founders and progeny developed a rapidly evolving disease, characterized by lethargy, hunched posture and distended abdomen. Eighty percent of tg animals succumbed between 10 and 12 weeks of age, and by 16 weeks 95% of tg mice had died. By 30 weeks, all mice were sacrificed. Examination of all tg mice revealed, as early as 5–6 weeks and at all ages thereafter, a significant enlargement of the upper mediastinum and a 5- to 6-fold increase in the size and weight of the spleen and peripheral (axillary, cervical and abdominal) lymph nodes, compared with wt mice. A hyperplastic thymus was also observed in some tg mice. Histological examination of the thymus, spleen and lymph nodes showed a complete disruption of the normal architecture and a massive infiltration by a monotonous lymphoblastic cell population. Tumor cell infiltration was also detected in liver, lungs, kidney, bone marrow and peripheral blood, thus indicating the existence of a leukemic phase of the neoplastic disease. All splenic or lymphonode lymphomas tested and a cell line [N3-232T, established from the thymocytes of a 6-week-old tg mouse] could be serially transplanted ($20x10^6$ cells injected subcutaneously) and elicited a lethal infiltration into the lymphoid organs of recipient nude or syngeneic mice, with latency periods of 2 and 3 weeks, respectively. Moreover, N3-232T cells shared phenotypic features of the spontaneously arising lymphomas, such as $CD3^{high}$ (>80% of cells), $CD25^{high}$ (30% of cells), pTα expression and activated NF-κB. Interestingly, N3-232T cells also displayed the DN phenotype of immature thymocytes.

Splenic lymphoma cells from three indepedent Notch3 tg lines displayed variable percentages of CD4 and CD8 expression, ranging from mainly DP to CD8 SP, suggesting their origin in heterogeneous differentiation stages. Lymphoma cells closely resembled Notch3 tg thymocytes in their expression of

CD25, increased in frequency by 10- to 20-fold compared with spleen cells of normal mice. This evident parallel between the lymphomas and the dysregulated thymocyte development seen in younger tg mice suggested that other early thymocyte markers might also be retained aberrantly in the periphery of the tg mice. Indeed, tg spleen cells showed sustained expression of both pTα isoforms, a and b,[122] not normally expressed in mature peripheral T cells.

In conclusion, in this study we demonstrated that the expression of a constitutively active form of Notch3 in transgenic mice leads to specific alterations in thymocyte development that are sustained in the outgrowth of aggressive T-cell lymphoblastic lymphomas by 6 weeks of age. The specific altered thymocyte phenotype, particularly the persistence of CD25 and pTα expression and activation of NF-κB, and their sustained expression in tumors, suggests that late DN T cells are the initial target of Notch3-induced dysregulation, and that such early dysregulation may constitute the platform for subsequent lymphomagenesis, albeit in parallel with further differentiation, and indicated a novel and particular role for Notch3 in coordinating growth and differentiation of thymocytes, across double negative to double positive transition, consistent with the normal expression pattern of Notch3. To study whether the presence of Notch3-associated dysregulation of T cell development at the preTCR stage might also occur in human T-ALL, we have investigated the expression of Notch3 and pTα in a series of T-ALL samples. Thirty cases of T-ALL were examined by RT-PCR and Northern blot for the expression of Notch3, Notch1, and both forms of pTα mRNA. Expression of Notch3 and both forms of pTα was revealed by both RT-PCR and Northern blot analysis in all cases of T-ALL investigated, irrespective of the immunophenotypic and cytogenetic subclassification of the tumor. Notch1 expression was also usually

observed in T-ALL; however, unlike Notch3, Notch1 expression was not pathognomonic for T-ALL, because Notch1 was generally detected not only in normal peripheral blood T lymphocytes but also in non-T cell leukemias.

To examine further the association of Notch3 and pTα expression in human T-ALL and the state of the disease, bone marrow samples at different stages of the disease were examined. The remission stage was distinguished from active disease by barely detectable levels or absence of both Notch3 and HES-1 expression, suggesting the activation of Notch3 signaling in active disease. pTα expression was also decreased dramatically in remission samples, and extensive analyses showed that the most reproducible distinguishing feature was the expression of the pTα b transcript, which may represent a more sensitive indicator of the gene expression.[127]

The lymphomagenic potential of Notch3 overexpression in vivo has been reported.[101] The combined expression of Notch3 and pTα in human T-ALL and their absence in remission, in normal peripheral T cells and in non-T cell lymphomas, as reported here, suggested a link between Notch3 signaling pathway activation and pTα expression in human T-ALL. To investigate the relationship between Notch3 expression, pTα expression and lymphomagenesis, two lines of Notch3-IC transgenic mice were crossed to pTα ko mice, and animals selected at the F2 generation that lacked both alleles of pTα. Unlike Notch3-IC/pTα$^{-/+}$ mice, the follow-up of Notch3-ICpTα$^{-/-}$ mice until the age of 50 weeks showed neither splenomegaly or other histological or phenotypic abnormalities of lymphoid organs nor early mortality in most of the mice, all of which are properties of Notch3-IC/pTα$^{-/+}$ mice.[74] Indeed, only half of the Notch3-IC/pTα$^{-/-}$ mice that died spontaneously after the age of 20 weeks displayed tumor development at autopsy. Therefore, Notch3-IC/pTα$^{-/-}$ mice

develop tumors, albeit with a reduced kinetics, only in about 10% of cases. In contrast, the presence of a single copy of the pTα gene allows tumor development in Notch3-IC $pT\alpha^{-/+}$. Indeed, 95% of Notch3-IC $pT\alpha^{-/+}$ mice had died by 40 weeks and all of them displayed tumor development at autopsy, although tumor appearance and subsequent mortality were delayed with respect to Notch3-IC $pT\alpha^{+/+}$ mice. These data establish that the causal role of activated Notch3 in T cell lymphomagenesis in mice depends on pTα expression.

To study whether the absence of Notch3-induced tumors in Notch3-IC/$pT\alpha^{-/-}$ mice was due to a block of T cell development, as usually observed in $pT\alpha^{-/-}$ mice,[128] we analyzed thymocyte maturation and peripheral T cell development in Notch3-IC/ $pT\alpha^{-/-}$ mice versus $pT\alpha^{-/-}$ animals. Clearly, the lack of tumors in Notch3-IC/$pT\alpha^{-/-}$ mice cannot be attributed to impaired T cell development and/or a reduction in peripheral T cells. Instead, T cell development through the β-selection stage (that facilitates subsequent generation of $CD4^+CD8^+$ thymocytes) is rescued by Notch3 activation. We observed that the distribution of thymocyte subsets and, to same extent, the cell yield and the surface expression of TCR chain are rescued in Notch3-IC/$pT\alpha^{-/-}$ mice. Moreover, an increased number of T lymphocytes expressing the TCR chain on their surface was observed in the spleen and lymphnodes of Notch3-IC/ $pT\alpha^{-/-}$ mice compared with $pT\alpha^{-/-}$. These observations suggest that activated Notch3, although able to push immature T cells through preTCR-dependent development steps in the absence of pTα, strictly requires pTα expression for sustaining its lymphomagenic potential.

THE ROLE OF THE IKAROS GENE IN LYMPHOCYTE DEVELOPMENT AND HOMEOSTASIS

PROPERTIES OF THE IKAROS GENE

In the search for transcription factors that control differentiation of the T cell lineage, the Ikaros gene was isolated,[129] which encodes a family of hemopoietic-specific zinc finger proteins, described as a central regulator of lymphocyte differentiation. During fetal development, it is required at the earliest stage of T cell and B cell specification.

Ikaros is abundantly expressed in the day-8 yolk sac, the first site of extra-embryonic hemopoiesis. Subsequently, its mRNA is detected in the day-9.5 fetal liver primordium, a subsequent site of hemopoiesis in the embryo proper.[129] At these sites, Ikaros is expressed in hemopoietic progenitors and in erythroid and myeloid precursors long before the appearance of fetal lymphocytes. Ikaros is expressed in the fetal thymus from the beginning of its colonization by hemopoietic progenitors that generate the fetal T lineages. High levels of Ikaros mRNA are detected in maturing thymocytes in the fetal as well as in the adult organ. It is also expressed in mature T and B lymphocytes and natural killer cells. Within hemopoietic progenitors, Ikaros is expressed in the Sca-1^{+}/c-kit^{+} population that is highly enriched for the pluripotent HSC.[130]It is equally expressed in multipotent progenitors with a strong erythro-myeloid (Sca-1^{-}/c-kit^{+}) and lymphoid (Sca-1^{+}/$ckit^{+}$/Sca-2^{+}) potential. Although it is also expressed in erythroid and myeloid precursors, it is turned off in most of their terminally differentiated products.[129] The highly restricted and complex pattern of Ikaros

expression in embryonic, fetal, and adult hemopoietic sites qualifies this gene as a potential regulator of cell fate in the fetal and adult hemopoietic systems. Alternative splicing of Ikaros pre-mRNA results in a family of zinc finger proteins.[131-132] The zinc finger modules comply with the Kruppel C2-H2 consensus motif and are spatially arranged in two distinct domains. All of the Ikaros proteins share a common C-terminal domain with two zinc fingers, whereas their N-terminal domains contain different combinations of one to four finger motifs. Three N-terminal zinc fingers are required for sequence-specific, high-affinity DNA interactions on a single binding site.[131] Therefore, of all the Ikaros isoforms, only three (Ik-1, Ik-2, and Ik-3) bind to sequences that share a four base pair core motif, GGGA.[131] In contrast, one of the Ikaros proteins, Ik-4, with two N-terminal zinc fingers, binds to tandem recognition sites that share this sequence. In spite of the common core consensus, the overall DNA sequence specificity and the affinity of binding varies between these isoforms. At the other end of the spectrum, Ikaros proteins with one or no N-terminal zinc fingers (Ik-5, Ik-6, Ik-7 and Ik-8) cannot engage in high-affinity DNA interactions.[131] The C-terminal zinc finger domain shared by all of the Ikaros isoforms is utilized to engage these Ikaros isoforms in stoichiometric homo- and heterodimeric complexes.[133] These protein interactions are pivotal for Ikaros activity. In fact, formation of homo- and heterodimers between isoforms with an N-terminal domain capable of binding DNA (i.e. Ik-1, Ik-2, and Ik-3) increases dramatically their affinity for DNA and activity in transcription.[133] However, heterodimers between DNA-binding- and non DNA- binding isoforms cannot bind DNA and are transcriptionally inert. As a result, Ikaros isoforms lacking a functional DNA-binding domain can interfere with the activity of DNA-binding isoforms through a dominant negative mechanism. Ik-1 and Ik-2 are the

predominant isoforms while Ik-3, Ik-4, Ik-5 and Ik-6 are present at significantly lower levels in all hemopoietic cell populations studied.

MECHANISMS OF REGULATION OF GENE EXPRESSION BY IKAROS

Commitment of hemopoietic progenitors toward lymphoid lineages is dependent on the controlled activation or repression of gene expression. Chromatin accessibility plays an important role in the transcriptional regulation of gene. Transactivation studies have shown activating and repressing activity in transient expression assays of promoters linked to Ikaros binding sites.[134] The large complexes that Ikaros proteins form with silent centromeric heterochromatin in the nucleus colocalize with several genes that become silenced during lymphocyte differentiation.[135-137] Although this mechanism explains how Ikaros might affect gene expression, the observation that some genes can be silenced without being relocated to heterochromatin suggests that control of gene repression is a more complex mechanism. Early biochemical studies with Ikaros protein showed that it can interact with itself.[133] This property underlies the pronounced accumulation of wild-type Ikaros protein in heterochromatin-associated structures and the inability of mutants in this interaction domain to do so.[137]

In lymphocytes, most Ikaros is present in a 2-MDa complex that contains 10–12 Ikaros molecules as well as several other proteins.[138] Characterization of the main Ikaros-containing complex in T cells revealed the presence of Mi-2b, an ATP-dependent chromatin remodeller (also known as Chd4), as well as the HDACs HDAC1 and HDAC2. These three proteins are components of the

nucleosome remodelling and deacetylation (NURD) complex that is active in both chromatin remodelling and histone deacetylation. The previously characterized NURD complex in epithelial cells also contains the MTA2 (metastasis-associated protein 2; also known as MTA1L1), MBD3 (methylCpG-binding domain protein 3) and Rbp48/46 (RNA-binding proteins 48/46) proteins.[139-141] With the exception of MBD3, most of these factors are also present in the Ikaros complex isolated from lymphocytes. Like its counterpart in epithelial cells, the Ikaros–NURD complex has potent chromatin remodelling activity in vitro and can deacetylate histones.[142] Although most Ikaros in mature lymphocytes exists in a NURD complex, a small amount is associated with the SWI–SNF remodelling complex and with two other corepressors (the Sin3 and CtBP proteins).[142-145] The recent finding that the SWI–SNF complex also contains the co-repressors Sin3, HDAC1 and HDAC2 provides a possible link between the minority of Ikaros protein that associates with the SWI–SNF and Sin3–HDAC components. Taken together, these biochemical studies show that Ikaros is stably associated with enzymatic machines, remodellers and HDACs, which could function during haematopoiesis by providing chromatin fluidity and establishing chromatin codes. There are other possible mechanisms by which Ikaros could affect gene expression. An important observation is that binding sites for Ikaros have been found in the promoter of several lymphoid-specific genes, suggesting that it could act as a classical transcription factor. In fact, it has been identified a core consensus motif (C/TGGGA/T), recognized by Ikaros transcription factor in the regulatory domains of the T cell receptor, i.e, TCR-α, -β, and -δ genes and the CD3δ, -ε and -γ genes. Binding sites for Ikaros proteins were also found in the promoters of CD4 and CD2, in the early pre-B cell differentiation antigen mb-1, in the IL-2Rα, in the PRDII element of the beta interferon gene, in the anti-apoptotic protein Bcl-2, in the enhancer of the H-2kb

gene and in the E-Ad promoter.[146-154] Taken together, these results suggest that, at least, one of the functions of Ikaros proteins is to act as repressor of transcription by direct binding to promoters of target genes. Therefore, the differential expression of the Ikaros isoforms during T-cell ontogeny, their overlapping binding specificities and the different transcriptional activity and nuclear localization, suggest a differential participation in distinct regulatory pathways during lymphocyte development. Synergistic interactions and/or competition between members of the Ikaros family proteins and other transcription factors may explain the complex changes in gene expression observed during lymphocyte differentiation and activation.

IKAROS FUNCTION IN VIVO: ROLE IN HEMATOPOIESIS, T LYMPHOCYTE DEVELOPMENT, THYMOCYTE SELECTION AND HOMEOSTASIS

The role of the Ikaros gene in development of the hemo-lymphoid system was addressed first by the study of phenotype of mice deleted in the DNA binding domain of the Ikaros gene, that results in a dominant negative mutation.[133]Proteins generated by the Ikaros locus in which the DNA binding domain is deleted cannot bind DNA. However, these mutant isoforms have an intact C-terminal zinc finger dimerization domain and engage readily in protein interactions. In lymphocytes heterozygous for this mutation, mutant Ikaros proteins sequester the DNA binding isoforms made by the intact wild-type allele, into transcriptionally inactive heterodimers.[133] In addition, dominant negative Ikaros mutant isoforms may interfere with the activity of other factors that share similar dimerization domains and work in concert with Ikaros during

development.[155] The phenotype of mice homozygous for the Ikaros DNA binding deletion ($DN^{-/-}$) supports this hypothesis.[156] In fact, Ikaros $DN^{-/-}$ mice display an early and complete block in the development of all lymphoid lineages, including T cells, during both fetal and adult hemopoiesis. In addition, severe effects in the development of other hemopoietic lineages are manifested. A five-to-tenfold decrease in erythroid and myeloid populations is observed in the bone marrow, which is, however, compensated for by a dramatic increase in extramedullary hemopoiesis in the spleen of these animals. Moreover, a severe block in granulocyte differentiation is seen in these mice. Myeloid cells with a granulocytic phenotype ($Mac\text{-}1^{+}/Gr\text{-}1^{lo/+}$) are absent. These more severe hemopoietic defects may, in part, reflect the complete absence of T lymphocytes that can provide necessary factors for granulocyte differentiation. They may also relate a requirement in multipotent progenitors for Ikaros that is essential for their expansion and differentiation along different arms of the myeloid pathway. Low levels of expression of cytokine receptors as well as cytokines produced by these progenitors and their progeny may interfere with their in vivo differentiation, an effect that may be overcome in vitro when hemopoietic growth factors are present in excess. Moreover, in absence of Ikaros activity a 30–40-fold reduction in HSC activity in the fetus and in the adult was observed.[157] Gene-expression studies in stem-cell and progenitor populations sorted from Ikaros-null mice revealed, in fact, the deregulation of two tyrosine kinase receptors that control the size of the HSC/progenitor pool.[157] Expression of the tyrosine kinase receptor fetal liver kinase 2 (Flk-2) was absent and c-kit levels were reduced five to ten-fold. Analysis of Ikaros mutant mice phenotype showed that Ikaros is not only required at the earliest stage of T cell specification, but also later in thymocyte selection and homeostasis. T cell development begins in the thymus of the Ikaros-null mice a few days after they

are born.[158] Thymocytes that are polyclonal in their TCR repertoire and that have a propensity to differentiate into the CD4 lineage are detected from 3 to 5 days after birth. TCR stimulation of $CD4^+$ and $CD8^+$ thymocytes in Ikaros-null mice results in excessive proliferation, compared to wild type.[158] As early as five days after the appearance of T cells in the thymus of these mutant mice, thymocytes with the same TCR specificity are found expanding in all three of the $CD4^+/CD8^+$, $CD4^+$, and $CD8^+$ compartments. In older animals, monoclonal T cell populations of similar phenotypes take over and replace the polyclonal thymocyte repertoire. These T cells are of a malignant nature and are also found in increased numbers in the periphery at later times. Deregulated T cell homeostasis is also manifested in mice heterozygous for the dominant negative DNA binding mutation in Ikaros ($DN^{+/-}$).[159] In contrast to the Ikaros-null homozygotes, $DN^{+/-}$ mice are born with "apparently normal" lymphoid compartments. However, in the $DN^{+/-}$ mice, as in the Ikaros-null, thymocytes display augmented proliferation when triggerred via the TCR complex. Changes in the composition of the thymocyte compartment are detected in the aging heterozygotes, with kinetics that are relatively delayed compared to those observed in the thymus of the Ikaros-null mice. Expansion in the intermediate double positive ($CD4^+/CD8^{int}/TCR^{int}$ and $CD4^{int}/CD8^+/TCR^{int}$) and in single positive ($CD4^+/TCR^{hi}$ and $CD8^+/TCR^{hi}$) thymocytes is detected between the second and third month. Ikaros $DN^{+/-}$ mice develop a lymphoproliferative disorder between the third and sixth month of their lives.[159] The hallmark of the disease is the dramatic accumulation of clonal lymphoblastic T cells in all peripheral lymphatic centers, accompanied by invasion of other tissues. Within a given animal, a percentage of these lymphoblastic T cells express the CD25 activation marker. This percentage varies between populations and correlates with the stage of disease. At late stages, defined by lymphoid organs that are

greatly enlarged in size and dominated by clonal lymphoblastic T cells, the great majority of these cells express the CD25 activation marker. The surface phenotype of accumulating thymocytes at the onset of the lymphoproliferative syndrome suggests that TCR engagement, followed by a series of deregulated downstream signaling events, may trigger the initial expansion of double positive thymocytes during selection. After their initial expansion, these Ikaros-mutant thymocytes can further differentiate to a single positive state. Persistence of TCR-engaging antigenic stimuli, such as superantigens from endogenous retroviruses expressed on epithelial cells of the thymus and peripheral lymphatic centers, in combination with deregulated TCR signaling, may underlie the dramatic accumulation of peripheral lymphoblastic T cells with a mature and activated phenotype ($CD25^{+}$). The highly malignant nature of Ikaros $DN^{+/-}$ lymphoblasts was established in adoptive transfer experiments. Tumors with the same cell surface phenotype and genetic makeup as the input cells are detected shortly after their transfer to nude mice. These cells invade not only peripheral lymphatic centers but also all of the host organs. The highly aggressive and malignant phenotype of these cells suggests that they have evaded growth factor requirements and proliferative signals usually supplied by the thymic, splenic, or lymph node microenvironments. Genetic analysis of the malignant T cells in the Ikaros $DN^{+/-}$ mice revealed loss of Ikaros heterozygosity. However, these malignant cells contained a normal number of chromosomes, indicating that the loss of the wild-type allele was due either to aberrant mitotic segregation of the two mutant alleles or to a gene conversion event. The majority of such cases lead to lethality or confer no selective advantage to the mutated cell. However, the segregation of chromatids with a mutation in the Ikaros gene may present cells with a growth advantage over other cells and thus establish the first stage in neoplasia. Such events have been described in the tumor progression of cells

heterozygous for mutations in tumor suppressor genes such as p53 and retinoblastoma.[160-161]

The TCR-driven hyperproliferative phenotype of thymocytes in mice homozygous for a null and heterozygous for a dominant negative Ikaros mutation strongly suggests that this gene is required for the negative regulation of TCR signaling and proliferation of immature thymocytes. The role of Ikaros in T cell differentiation was further delineated breeding Ikaros $DN^{+/-}$ with $RAG\text{-}1^{-/-}$ mice. Thymocytes from $RAG1^{-/-}$ mice can't rearrange TCR genes and consequently do not express a preTCR complex, so they arrest their development at an early precursor stage (DN $CD44^{-}CD25^{-}$). Pre-TCR signaling is required for progression to the next stage of differentiation where both CD4 and CD8 coreceptors are expressed ($CD4^{+}CD8^{+}$). It is also required for the proliferative expansion of thymocytes during ths transition. However, in the absence of Ikaros activity, thymocyte precursors can transit from the double negative to the double and CD4 single positive stages without the signaling events provided by preTCR and TCR engagement. However, this differentiation event occurs without the normally occurring proliferative expansion, resulting in a highly hypocellular thymus. Thus, lack of Ikaros activity in thymocyte precursors uncouples the process of differentiation from that of proliferation, allowing the one to occur in the absence of the other. Interestingly, the preferential development of CD4 lineage cells observed in Ikaros $null^{-/-}$ x $RAG^{-/-}$ thymi correlates with the phenotype previously reported for Ikaros $null^{-/-}$ thymi without the RAG mutation. Therefore, Ikaros plays a central role in establishing thresholds for differentiation that give rise first to double positive, and subsequently to CD4 single positive thymocytes.

IKAROS AS AN ESSENTIAL TUMOR SUPPRESSOR GENE FOR THE T LINEAGE

A stepwise decrease in Ikaros activity in developing thymocytes leads to their inappropriate selection, expansion, and finally to their transition to a neoplastic state. Differences not only in the kinetics of appearance but also in the phenotype and properties of lymphoblastic T cells generated in Ikaros-mutant mice can be related to the level of functional Ikaros in these cells. In the presence of one wild-type and one dominant negative mutant Ikaros allele, clonal expansions become apparent after one to two months of age and are concomitant with the loss of the wild-type locus.

Ikaros $DN^{+/-}$ develop leukemias and lymphomas with 100% penetrance within 3 months of life.[159] Interestingly, when a cohort of Ikaros $DN^{+/-}$ x RAG-$1^{-/-}$ mice was followed for 11 months, no lymphomagenesis was observed, whereas when mice transgenic for TCR were bred with Ikaros $DN^{+/-}$ x $RAG^{-/-}$ mice, thereby restoring T cell differentiation, the transformation phenotype could be restored.[160] Therefore, expression of a functional preTCR or TCR complex at subsequent stages of differentiation is necessary for Ikaros deficiency to have a destabilizing effect on T cell homeostasis and trigger proliferative events that led to malignancy. These studies implicate Ikaros as a critical regulator of the preTCR mediated checkpoint in T cell differentiation known as β-selection. Intriguing is the observation that, similarly to what happens in Notch3-IC transgenic mice, the development of leukemia arising in Ikaros mutant mice is depending on either pre-TCR or TCR signaling whereas DN thymocytes can differentiate to the DP stage without expression of pre-TCR.[160]Therefore, the Ikaros gene family is essential for the development of T lymphocytes and

functions as tumor suppressor in T and B cells.[156,161,159] The fact that Ikaros functioned as a tumor suppressor gene in mice suggested that Ikaros expression alteration could be involved in human hematological malignancies. Actually, it has been shown that reduction in Ikaros activity correlates with development of blast crisis in patients with chronic myelogeneous leukemia.[162-163] Bone marrow from those patients show either a reduced expression of Ikaros at the RNA level or an overexpression of dominant negative Ikaros isoforms. Therefore, reduction of Ikaros activity may be an important step in the development of blast crisis in chronic myelogenous leukemia.[162] Mutations that cause overexpression of dominant negative Ikaros isoform are also involved in human B and T cell acute lymphoblastic leukemias.[164-165] Similarly to the observation made in Ikaros DN –/+ mice, aberrant expression of a dominant negative Ikaros isoform has been reported in T acute lymphoblastic leukemia in infants,[166] and moreover, an abnormal subcellular compartimentalization of Ikaros, with exclusive cytoplasmic localization, was observed. These results indicate that post-trancriptional regulation of RNA alternative splicing is defective in leukemic cells from infants with ALL.[164-166]

Overexpression of dominant negative Ikaros isoforms might be the consequence of abnormal splicing accompanied by a 10 amino acids deletion adjacent to exon 7 or by a 60 base pair insertion following exon 2.[162]

In mice model, diminution of Ikaros expression causes hyperstimulation through antigen receptor leading to polyclonal lymphoid hyperplasia prior to tumoral transformation. Although Ikaros DN+/– mice develop T cell leukemia/lymphoma in 100% cases before the age of 6 months, transformation might be the direct consequence of the hyperproliferation state. In fact, Ikaros seems to be directly involved in cell cycle regulation throughout the regulation of expression of p27 (one of the few genes that is upregulated by Ikaros), and

cyclin D1.[168-169] An interesting observation was made by Beverly and Capobianco that, by using proviral insertion mutagenesis with moloney murine leukemia virus (MLV) demonstrated a direct link between Ikaros deficiency and Notch activation in T cell lymphomas. In fact, they observed that Ikaros locus is a common target of proviral integration in 40% of tumors arising in Notch1-IC transgenic mice. Such proviral insertion-dependent mutations lead to the production of dominant negative Ikaros isoforms that finally may sinergize with activated Notch1 during tumorigenesis, being able to inhibit the repression sustained by DNA-binding isoforms of Ikaros on Notch-dependent genes. Thus, Lck-Notch1-IC transgenic mice infected intraperitoneally with MLV increased the penetrance of leukemia from 20% to 100%.

Moreover, these authors observed that Ikaros and the Notch effector protein CSL/RBP-Jk were capable to recognize the same DNA-binding sequence *in vitro*, the core motif TGGGAA. Therefore, they suggested a model in which Ikaros may antagonize Notch-IC/CSL –induced transcriptional activation by competing with the similar consensus DNA-binding motif.[169] More recently, S. Chan's group demonstrated that re-introduction of a functional Ikaros in Ikaros mutant mice downregulates Notch target gene expression in tumor cells and inhibits the Notch-induced CSL/RBP-Jκ transcription on the target sequence in HES-1 promoter.[170] Altogether, these observations clearly suggested a connection between Ikaros deficiency and Notch activation in T cell leukemogenesis, without indicating the direct targets and/or mediators of such a connection.

THE RNA BINDING PROTEIN HUD

Transcription factors have traditionally been viewed as the main determinants of gene expression. Yet, in recent years it has become apparent that RNA-binding proteins also play a critical role in determining the levels of expression of a large number of genes. Once mRNAs are transcribed, RNA-binding proteins can control all subsequent steps in their function, from alternative splicing and translation to mRNA transport and stability. From this point of view, a good example is represented by the nervous system, in which a large number of genes are regulated post-transcriptionally via the interaction of their mRNAs with specific RNA-binding proteins. This type of regulation is particularly important in the control of the temporal and spatial pattern of gene expression during neural development.

There are a number of well-characterized RNA binding proteins that have been shown to assume specific roles in normal neuronal development and function, such as CPEB, ZBP and FMRP.[171-173]

Over the last 15 years, there has therefore been growing interest in the function and regulation of the Hu family of RBP, which is directly involved in development of paraneoplastic encephalomyelitis and sensory neuronopathy sydromes.[174-176]

The antibody produced in the above disorder was termed anti-Hu based on the name of the patient in which the antibody was discovered.[175,177] The Hu antibody was used to identify HuD and other Hu protein.[175] Upon cloning and sequencing of these proteins,[175,178] it was found that they are the human homologues of Drosophila ELAV, an RNA-binding protein whose deletion results in an embryonic lethal abnormal vision phenotype in flies.[175] Thus, to refer to this

family we will use the name of ELAV/Hu herein. There are four mammalian ELAV/Hu proteins, HuA also known as HuR, HuB also known as Hel-N1, HuC, and HuD. These proteins are encoded by separate genes and are present in the cell in multiple splice variants. In mammals, birds, and Xenopus, three proteins of this family (HuB, HuC, and HuD) are neuronal-specific, while the fourth member, HuA, is expressed in other tissues. Hu proteins are thought to be one of the earliest markers of neuronal differentiation.[179-180]All four of the Elav/Hu family members contain three RNA recognition motifs (RRMs), a highly conserved 80 amino acid region, that was first recognized in splicing factors and poly(A)-binding protein.[181]

RRM is a conserved structure of two consensus ribonucleoprotein (RNP) motifs separated by 25–35 amino acids that interact directly with the RNA. The RNP motifs, octameric RNP-1 and hexameric RNP-2, each contain three conserved aromatic residues that are implicated in RNA interactions in a number of different RBP including proteins that bind pre-mRNA, mRNA, pre-rRNA as well as small nuclear and heteronuclear RNAs.

HuD, in particular, is encoded by a relatively large gene (146 Kb) located on chromosome 1 in humans (1p34) and chromosome 4 in mice (49.5 cM). Some of the exons are separated by large intronic regions. The localization of the HuD gene in the mouse genome was based on syntenic regions between human and mouse, suggesting that the flanking genes are conserved between species.182 The gene consists of three putative non-coding exon 1 variants, termed 1a, 1b and 1c, and several common coding exons (2, 3, 4, 5 and 8).[183-184] The first and second RRM are encoded by exons 2 and 3, and exons 4 and 5, respectively, while the third RRM is encoded by exon 8. The HuD gene is also subject to alternative splicing of exons 6 and 7 which affects the length of the hinge region linking the second and third RRM. This results in the three different transcripts

and molecular forms, termed HuDpro, HuD and HuDmex[183] of which HuDpro and HuD are the major variants.[182-185] The resulting proteins have molecular masses ranging between 37 and 43 kDa and are characterized by the three RRMs and unique nuclear export signals located within the hinge region.[186]

HuD has long been known as one of the earliest markers of neural differentiation.[187] Several in vivo and in vitro experiments indicate an important role of neuron-specific HuD proteins in multiple aspects of neuronal function including the commitment and differentiation of neuronal precursors as well as synaptic remodeling in mature neurons.

HuD proteins recognize and bind specifically to well-described AU-rich elements (ARE) found within the 30 UTR of approximately 1 in 20 human genes[188-189] and directly implicated in RNA turnover.[188-191] The AREs are separated into three classes based on their sequence and structure. Class I AREs consist of one to three dispersed AUUUA motifs separated by U-rich regions while class II AREs consist of multiple clusters of AUUUA motifs. Class III AREs, although less-well defined, consist mainly of U-rich sequences and do not contain the AUUUA pentamer.[192]

Unlike many other ARE-binding proteins that principally act to destabilize transcripts, such as AUF1 family members, Hu proteins act to stabilize ARE-containing transcripts thereby significantly prolonging heir half- life.[193-195] In vitro binding studies perfor med with HuD have demonstrated that the RNA is bound primarily by the first RRM while the second RRM, which also binds the RNA, functions mostly to stabilize the RNA–protein complex.[196] The third RRM, in addition to participating in maintaining RNA–protein complex stability, binds to poly(A) tails.[197-200]

As the variety of cis-acting elements recognized by the RRM have increased, so have the number of transcripts that interact with HuD. Accordingly, HuD has

been shown to bind and stabilize several developmentally regulated transcripts including c-fos, c-myc, N-myc, p21waf1, neuroserpin and MARCKS.[201-206] Interestingly, and in agreement with its role in neuronal differentiation, many of the transcripts bound by HuD play a key role in the formation of neuronal processes such as GAP-43 and tau.

Additionally, the role of HuD in multiple aspects of a neuron's life from early differentiation to changes in mature neurons during learning paradigms and in response to injury and regeneration.[207]

More recently, HuD has also been described in the $CD34^-$ subset of bone marrow cells, suggesting a role for this RNA-binding protein also in the hematopoietic system.[208]Finally, after evidence of nuclear-cytoplasmic shuttling[209-210] and the predominant nuclear localization,[211]HuD proteins have been reported to have a nuclear function. In Drosophila, the HuD protein homologue, the embryonic lethal abnormal visual (ELAV) protein, has been shown to have a very important function in the nucleus, where regulates alternative pre-mRNA processing in neurons.[212-214] Recently, we and others have demonstrated that HuD proteins may regulate alternative splicing in thymocytes and mammalian neurons, identifying two target sequence of HuD proteins, Ikaros and the human calcitonin/calcitonin gene-related peptide (CGRP) pre-mRNA (Zhu).[168,215] The role of Hud in regulating Ikaros splicing will be treated in more details in the section reporting my activity during my phd.

EXPERIMENTAL THESIS

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).[74,159-160]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 pTα 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.[101] Interestingly, by generating double mutant mice transgenic for Notch3-IC and lacking pTa (N3-IC/pTα-/- mice), we observed that an intact pre-TCR is required for T-cell leukemogenesis,[74] although it is not required for Notch3-driven T-cell differentiation.

Indeed, whereas pTα knockout arrests T-cell development, transgenic N3-IC overcomes this block, driving immature CD4CD8 double-negative (DN) thymocytes to progress toward a more differentiated phenotype (e.g. acquisition of CD4, CD8 and TCRβ expression) despite the absence of a functional pre-TCR.[74]

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,[159] 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.160 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.[74,160] 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,[74] and activating mutations of Notch1 have been found in over 50% of these tumors.[106] A high percentage of infant B- and T-ALLs also display an increased expression of short non-DNA-binding IK isoforms.[165-166] Alternatively spliced transcripts of the ikaros gene encode at least nine protein isoforms (IK-1–9) with different DNA-binding capabilities.[131,169,216]

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.[169] 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 alterated 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 pTα 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.

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 pTα/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 pTa, 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-Ic tg,[101] pTα-/-[217] and N3-IC/pTα/ double mutant mice[74] have been described elsewhere.

FLOW CITOMETRY ANALYSIS

Freshly isolated cells from thymi and lymph nodes were prepared and stained as previously described[101] 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/pTα/ and pTα/ 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 X95%) with the FACSAria cell sorter (BD Biosciences).

RT–PCR ANALYSIS

Total RNA was extracted from unfractioned 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[218] 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 mg portion of RNA was processed for RT–PCR as previously described.[72] PCR was performed at the appropriate annealing temperature with the following primers: IK 5'-

CTCCAGATGAAGGGGATGAG-3' and 5'-CAGCAGCAGCAAGTTATCCA-3', IKexon7 5'- TGGTTATCACAGCCAGGACA-3' and 5'-AAATCAAACGCCAAACAACC-3'; HuD 5'-AGAAGGGAATGTCAGCTTTT-3' and 5'-TGAATTCCTCTTGGGTCATA-3';[219] b-actin 5'-GTGG GCCGCTCTAGGCACCAAT-3' and 5'-CTCTTTGATGTCACGCACGATTTC-3'. Human IK: 5'-CCCCCTGTAAGCGATACTCCAGAT-3' and 5'-GGCTTGGTCCATCACGTGGGGA-3'; human HUD: 5'-GAGTCTCTT CGGGAGCATTG-3' and 5' -CTTGTGGGCTTTGTTGGTTT-3' ; mouse Hes-1: 5'-ATGCCAGCTGATATAATGGAG-3' and 5'-CACGCTCGGGTCTGTGCTGAG-3'; mouse Deltex: 5'-CACTGGCCCTGTCCACCCAGCCTTGGCAG-3' and 5'-ATGCGAATT CGGGAAGGCGGGCAACTCAG-3'; mouse Notch3: 5'-CCAGGGCTGC AACACTGAGGAATG-3' and 5'- TTGTGGCCAGCAGCTATGTCCTTG-3 ; mouse pTalpha: 5'-GCGAT GCTTCTCCACGAGTGGGC-3' and 5'-GCGCTATGTCCAAATTCTGTGGG-30 ; human b-actin: 5'-CTACAATGAGCTGCGTGTGG-3' and 5'- CGGTGAGGATCTTCATGAGG; mouse Notch1 : 5'-GTGGATGCTGACT GCATGGATGTC-30 and 50 -ATGCAAAGCCGACTTGCCTAGGTC-30 ; human Notch1: 5'-CTACCTGTCA GACGTGGCCT-3' and 5'-CGCAGA GGGTTGTATTGGTT-3' ; human Notch3: 5'-TTCTTAGATCTTGGGGGCCT-3' and 5'-GGAAGAAGGAGGTCCCAGAC-3' ; human pTα: 5'-CTGCAGCTGGGTCCTGCCTC-3' and 5'-AGTCTCCGTGGCCGGGTGCA-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 b-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.[169] In brief, 50 mg 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[218]and M31 immature T-cell line.[220]

PLASMIDS

IKcDNAs were cloned from RT–PCR reactions from both wt and Notch3-IC transgenic thymi as previously suggested.[169]The fragment containing the putative pTα promoter region (pubMed sequence U27268) was amplified by PCR from genomic DNA using the primers pTa-prom-FL 5'-GCTTTGGATCTGGAGGATGA-3' and 5'-CTCTGCTAGTCTGCCTCCCA-3' and subcloned in topo TA-cloning (Invitrogen, Carlsbad, CA, USA). The pTa promoter-luciferase fusion plasmids were constructed by cleaving with XhoI and SacI the PCR products and inserted into the XhoI and SacI sites upstream of

the luciferase cDNA of pGL3-basic vector (Promega, Madison, WI, USA). The mutant constructs pTa-prom-FL CSL1MUT and pTa-prom-FL CSL2MUT and 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.[39,169] HuD was amplified by PCR by using the primers HuD 5'-GGGTCCATCTTCTGATCACA-3' and 5'-GGGTGAGAAATTCAGGATTT-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[101]and RBP-Jk,[221]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)[170] by resuspending 107 cells in 500 ml 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 ml 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; 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 mg of poly(dI-dC), 1 mg of bovine serum albumin and 10 mM ZnCl2 in 19 ml 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'; CSLMUT1: 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 ($4x10^5$ 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, $3x10^6$ 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 mantained 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 $4x10^6$ 2017 cells with or without 10 mM of g-secretase

inhibitor I (alternate name Z-LLNLe-CHO; Calbiochem, San Diego, CA, USA) and harvested after 48 h for RNA extraction.

CELL TRANSFECTIONS AND LUCIFERASE ASSAYS

Trasfection 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 ml/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 means7s.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 about 70 and 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[107]and divided into aliquots. In total, 5 mg 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 41C. 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 pTa mouse promoter including the CSL-core1 site: 5'-GCTTTGGATCTGGAGGATGA-3' and 5'-GAACTCAGGTCCCACTCCCA-3'.

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.[101] 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.[101] 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 lymphnode-derived lymphoma cells consistently displayed identical immunophenotype (Figure 2A). Similar IK isoform profiles were also observed at protein levels (Figure 2B).

Noteworthily, 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,[169] 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 pTa/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[74] and for that observed in mice with a dominant DNA- binding mutation in the IK gene.[159-160]

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/pTα/ mice, pTα/ 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/pTα/ and pTα/ cells, which lack a functional pre-TCR complex.
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/pTα double mutant mice were significantly lower than those observed in wt, N3-ICtg and pTa/ 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 $M31^{220}$and 2017^{218}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). Noteworthily, 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 expression of IK-dn isoforms in premalignant and malignant thymocytes of Notch3–IC tg mice.

Notch3 and IK act cooperatively to regulate pTα gene expression

The findings described above, together with a recent report that loss of IK function leads to increased expression of Notch3 and pTα in murine T lymphoma cells[170]suggest the possibility of direct crosstalk between Notch3 and IK that serves to regulate pTα gene transcription.

Previous studies have shown that the pTα enhancer is activated by Notch1 signaling via CSL-binding sites,[49] and we have demonstrated dose-dependent activation of the pTα promoter by Notch3.[222]To further elucidate the mechanisms underlying the effects of Notch3 on the regulatory region of pTα, we transfected non-lymphoid HEK293 cells with a luciferase reporter construct containing the entire pTα 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 pTα promoter activity about 10-fold, and additional increases were observed after the addition of RBP-Jk/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,[39,40,43] this is the first demonstration that Notch3-IC-driven activation of the pTα regulatory element is significantly enhanced by a transcriptional complex composed of RBP-Jk/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.[169] Similarly, enforced expression of the IK-1 isoform in HEK 293 cells significantly inhibited activation of the pTα 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 pTα 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 pTα. It remained to be seen how the Notch3-triggered complex interacts with the pTα promoter region and what is the basis for IK antagonism. Our analysis revealed that the pTα promoter contains a canonical consensus CSL-binding sequence, several consensus sequences that are specific for IK and three TGGGAA motifs corresponding to the RBP-Jk/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.[131,169]The three motifs were designated CSL-core 1, CSL-core 2 and CSL-core 3 in accordance with their positions in the pTa promoter (485, 200 and –149 bp, respectively) (Figure 5B). The pTα 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 pTα.

In order to study the basis for Notch3 and IK-1 antagonism on pTα transcriptional activation, we first examined the ability of both IK-1 and CSL to bind the same site of pTα regulatory sequence by using the CSL wt sequence of the pTα 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 pTα 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 pTα 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 pTα promoter, we performed chromatin immunoprecipitation (ChIP) experiments 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 pTα promoter in chromatin extracts derived from wt thymocytes. In contrast, CSL but not IK antibodies efficiently recovered the pTα 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 pTα promoter

and might explain the functional collaboration observed between Notch3 and the IK-dn isoforms in sustaining pTα 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 pTα expression.

The effects of Notch3 on the expression of IK spliced variants depend on 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 premalignant thymocytes (results currently unpublished), we discovered a striking increase (about 39-fold compared with wt controls from 2-week-old mice) of mRNA for HuD, an RNA-binding protein of the ELAV/Hu family,[184] which is reportedly capable of regulating both the stability[178,193,195], and the alternative splicing[212,215] 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/pTα/ and pTα/ 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 down-stream 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 pTα, Notch3 and Notch1, whereas M31 cells express similar levels of Notch1, but very low levels of Notch3 and undetectable pTα 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.[85,223]
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 g-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,[163,165-166] 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.[165] Figure 7A shows that this cell line, similar to the mouse 2017 cell line, constitutively expresses Notch1 and 3, HuD, both the pTa 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-dependent T-cell leukemia.[74,217] The pTα gene encodes an essential pre-TCR component and is a key target of Notch3 signaling, which strongly en- hances 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 pTa 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 splicing and translation to modulation of mRNA transport and stability.[178,193,195] HuD has long been known as one of the earliest markers of neural differentiation,[187] and more recently it has also been described in the CD34 _ subset of bone marrow cells,[208] 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 wasstrictly 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 coculturing 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$\alpha^{-/-}$ 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$\alpha^{-/-}$ 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 pTα, independently of

the overexpression of Notch3, suggests that Notch signaling alone, in the absence of pTα, 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 pTα, and an effect of this type might explain Notch3's partial reversal of the developmental arrest that characterizes thymocytes from pTα$^{-/-}$ single mutant mice.[74] IK target/binding sites have also been reported in the enhancer region of CD3 and CD4 genes.[19]

In conclusion, we provide evidence for a novel non-redundant mechanism whereby 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 pTα/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 pTa, 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.

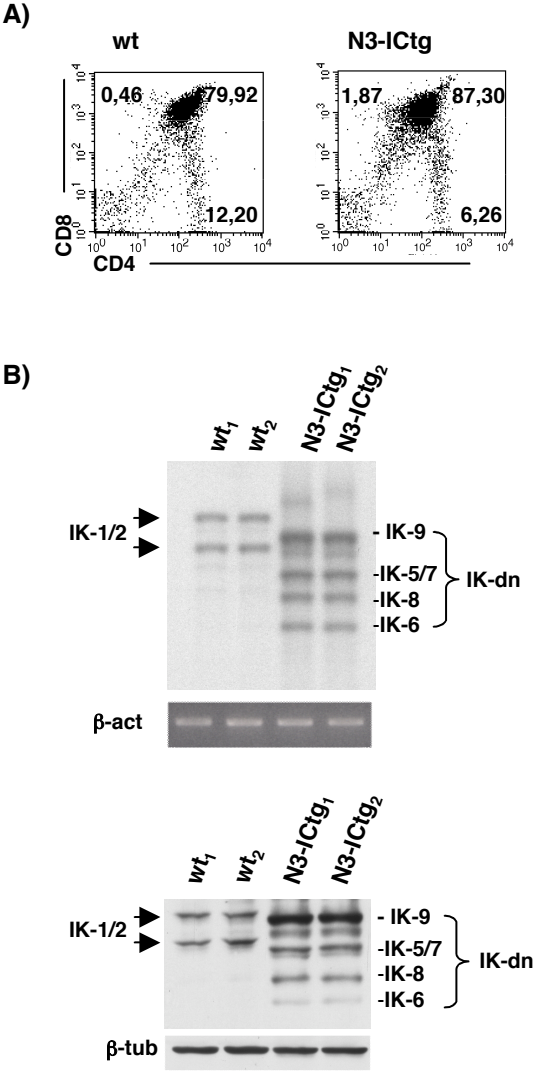

A)
wt
N3-ICtg
0,46
79,92
12,20
1,87
87,30
6,26
CD8
CD4
B)
wt1
wt2
N3-ICtg1
N3-ICtg2
IK-1/2
- IK-9
-IK-5/7
-IK-8
-IK-6
IK-dn
β-act
β-tub

FIG.1 Alternatively spliced Ikaros (IK) isoforms are overexpressed in pre-malignant thymocytes from Notch3-IC transgenic (*N3-ICtg*) mice.
(A) $CD4^+$ and $CD8^+$ subset distributions (documented by two-color FCA) of thymocytes from 2-week-old wild -type (*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. IK1 and IK2/3: DNA-binding IK isoforms; IK-dn: Dominant negative IK splice 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.

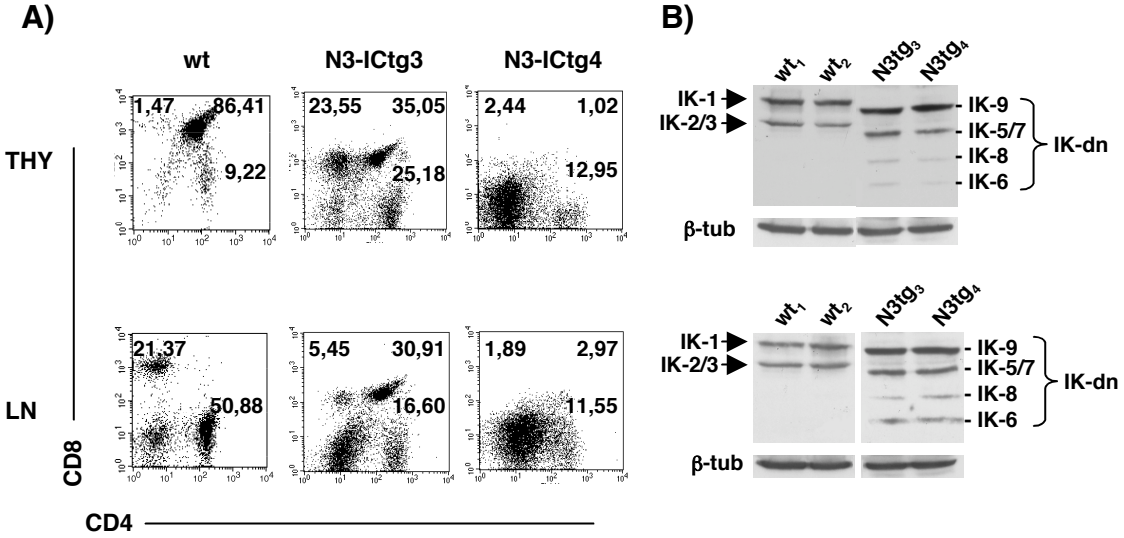
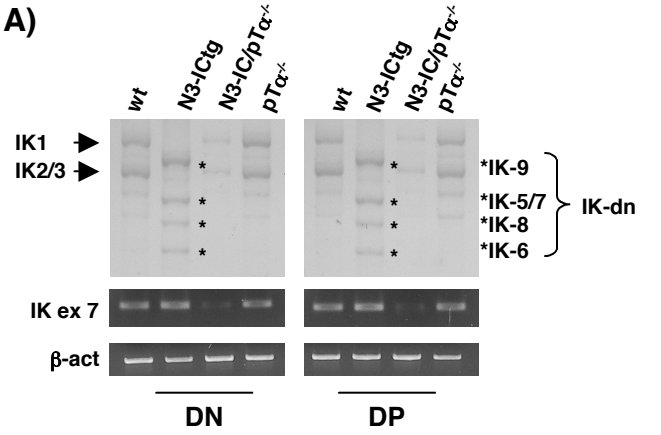


FIG.2 Ikaros (IK) isoform expression patterns are altered in T lymphoma cells from Notch3-IC transgenic (*N3-ICtg*) mice.

(A) Two-color-FCA analysis of CD4 vs CD8 expression in freshly isolated thymocytes (THY) (upper panels) and lymph-node cells (LN) (lower panels) from 5-week-old mice: two wild-type (wt_1 and wt_2) and two *N3-ICtg* ($N3\text{-}ICtg_3$ and $N3\text{-}ICtg_4$). The number in each quadrant indicates the percentage of total cells represented by the corresponding subset.

(B) (B) Immunoblots of whole-cell extracts from the cells shown in panels A. IK1 and IK2/3: DNA-binding Ikaros isoforms; IK-dn: Dominant negative Ikaros isoforms incapable of DNA-binding.


A)
wt
N3-ICtg
N3-IC/pTα-/-
pTα-/-
wt
N3-ICtg
N3-IC/pTα-/-
pTα-/-
IK1
IK2/3
*IK-9
*IK-5/7
*IK-8
*IK-6
IK-dn
IK ex 7
β-act
DN
DP

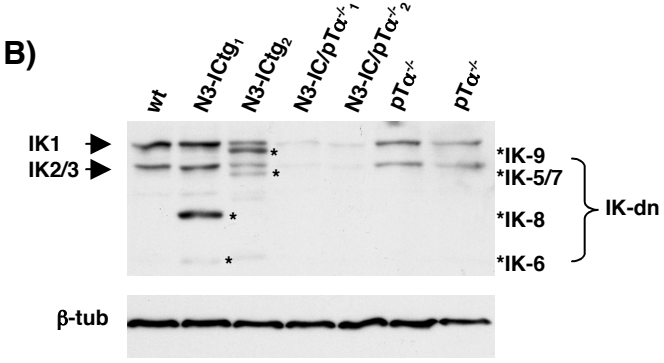

B)
wt
N3-ICtg1
N3-ICtg2
N3-IC/pTα-/-1
N3-IC/pTα-/-2
pTα-/-
pTα-/-
IK1
IK2/3
*IK-9
*IK-5/7
*IK-8
*IK-6
IK-dn
β-tub

FIG.3 Ikaros (IK) isoform expression patterns in thymocyte depend on pT*a* expression. (A) RT-PCR analysis of IK isoform expression was performed on RNA from sorted $CD4^-$ $CD8^-$ double negative (DN) and $CD4^+CD8^+$ double positive (DP) thymocytes from wild-type (*wt*), Notch3-IC transgenic (*N3-ICtg*), *Notch3-IC/pT*$\alpha^{-/-}$ and *pT*$\alpha^{-/-}$ mice. (B) Ikaros 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 Ikaros isoforms; IK-dn: Dominant negative Ikaros isoforms incapable of DNA-binding. IK ex 7: Ikaros exon 7. Results were normalized to β-tubulin (β-tub).

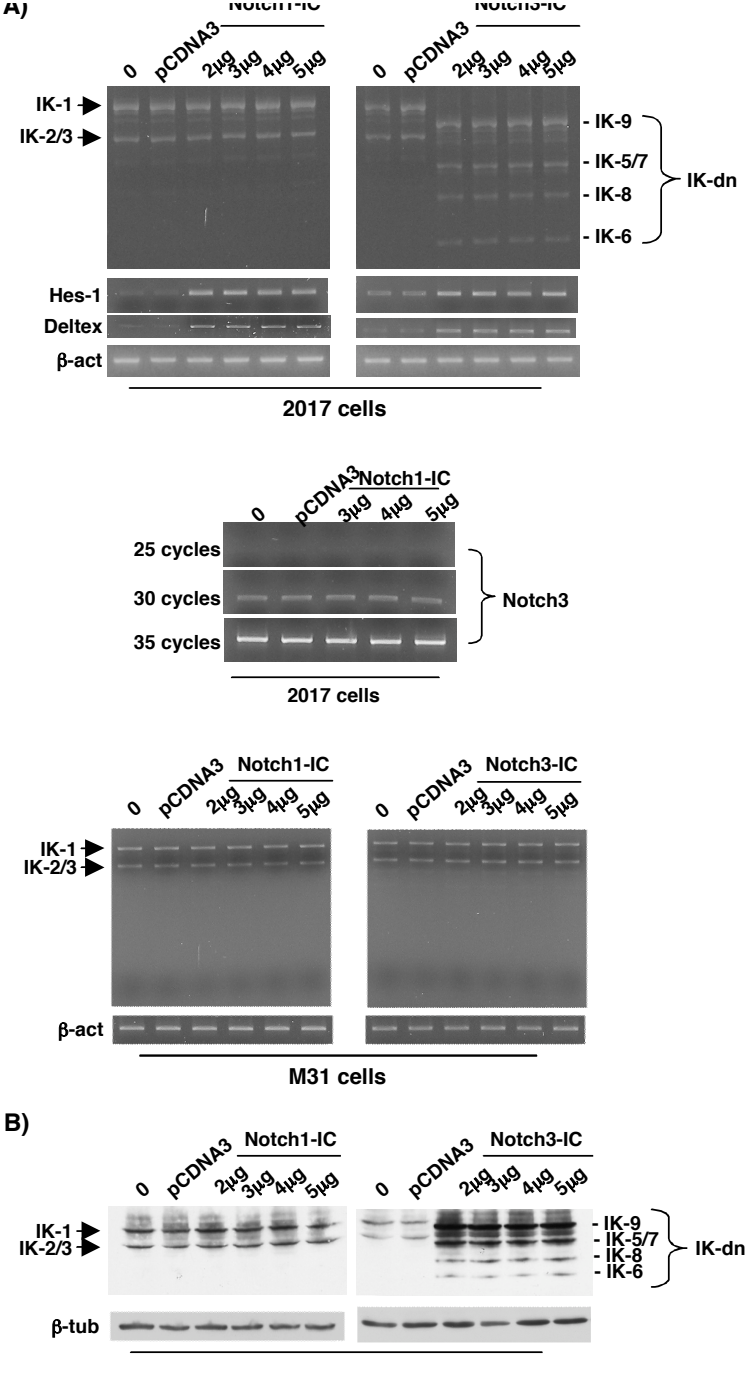

A)
Notch1-IC
Notch3-IC
0
pCDNA3
2μg
3μg
4μg
5μg
IK-1
IK-2/3
- IK-9
- IK-5/7
- IK-8
- IK-6
IK-dn
Hes-1
Deltex
β-act
2017 cells
Notch1-IC
25 cycles
30 cycles
35 cycles
Notch3
2017 cells
M31 cells
B)
β-tub

FIG.4 Notch3 regulates alternative splicing of Ikaros (IK) *in vitro*.
DN1 and DN3 thymocytes (represented respectively by the M31 and 2017 cell lines were transfected with increasing amounts of Notch3 (right upper and lower panels) or Notch1 (left upper and lower panels), and Ikaros mRNA and protein expression was assessed by RT-PCR. Hes-1 and Deltex RNA expression were used as control of correct function of Notch1-IC and Notch3-IC constructs, while Notch3 RNA expression confirms the specific role of Notch3 with respect of Notch1 in increasing expression of Notch3 (A) and Western blotting (B). 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

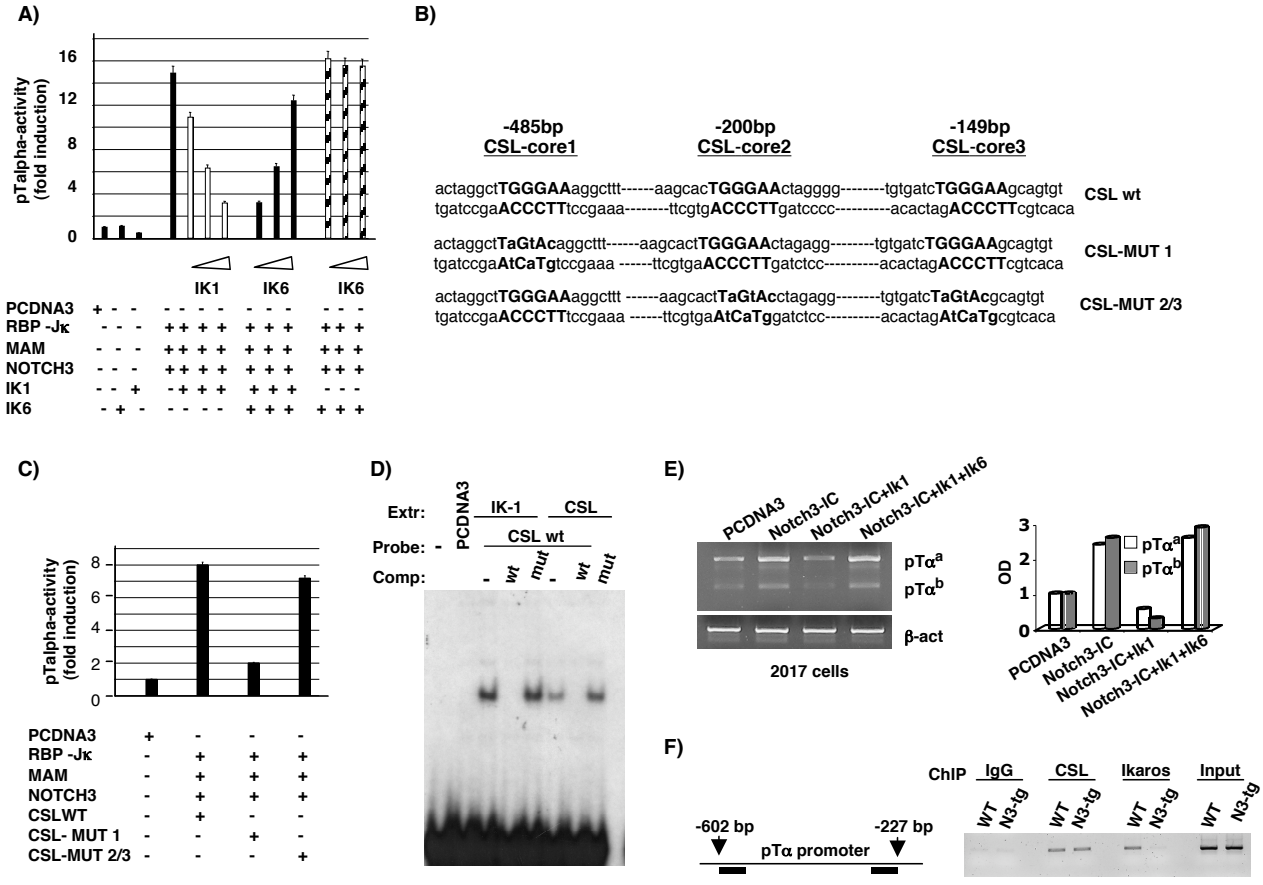


FIG.5 Transcriptional activation of the pTα promoter by Notch3 is differentially regulated by Ikaros isoforms.

HEK 293 cells were transfected with a luciferase reporter construct containing the pTα promoter together with Notch3, RBP-Jκ, and Mastermind (MAM). A) The pTα promoter is activated by the Notch3 transcription activator complex (Notch3+RBP-Jκ+MAM). Increasing amounts of IK-1 alone (200-800 ng) dose-dependently repressed Notch3-driven transcription of the pTα promoter, but the non-DNA-binding IK6-dn isoform alone (200-800ng) had no impact. When IK-1 and IK-6 were co-transfected, 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 core sequence in the pTα promoter. Wild-type and mutated sequences from the entire promoter are shown in bold-face type, and the cloned CSL wt sequence and mutant constructs, CSL1MUT and CSL2MUT+CSL3MUT, are shown. (C) Luciferase activity of wild-type and mutant pTα 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: both the Ik1 and CSL protein, obtained from nuclear extracts derived from COS cells transfected with either Ik-1 or CSL expression vectors. We used as a probe an oligo containing the wild type CSL-core1 consensus (TGGGAA) as indicated in the panel B of revised figure 5. For competition (comp), 100 fold molar excess of cold CSL-wt or CSL-MUT 1 was used. (E) 2017 cells were used in transient transfection experiments to evaluate the ability of Notch3-IC to induce increased pTalpha expression and to demonstrate that this induction is repressed by Ik-1 and derepressed by Ik-6.

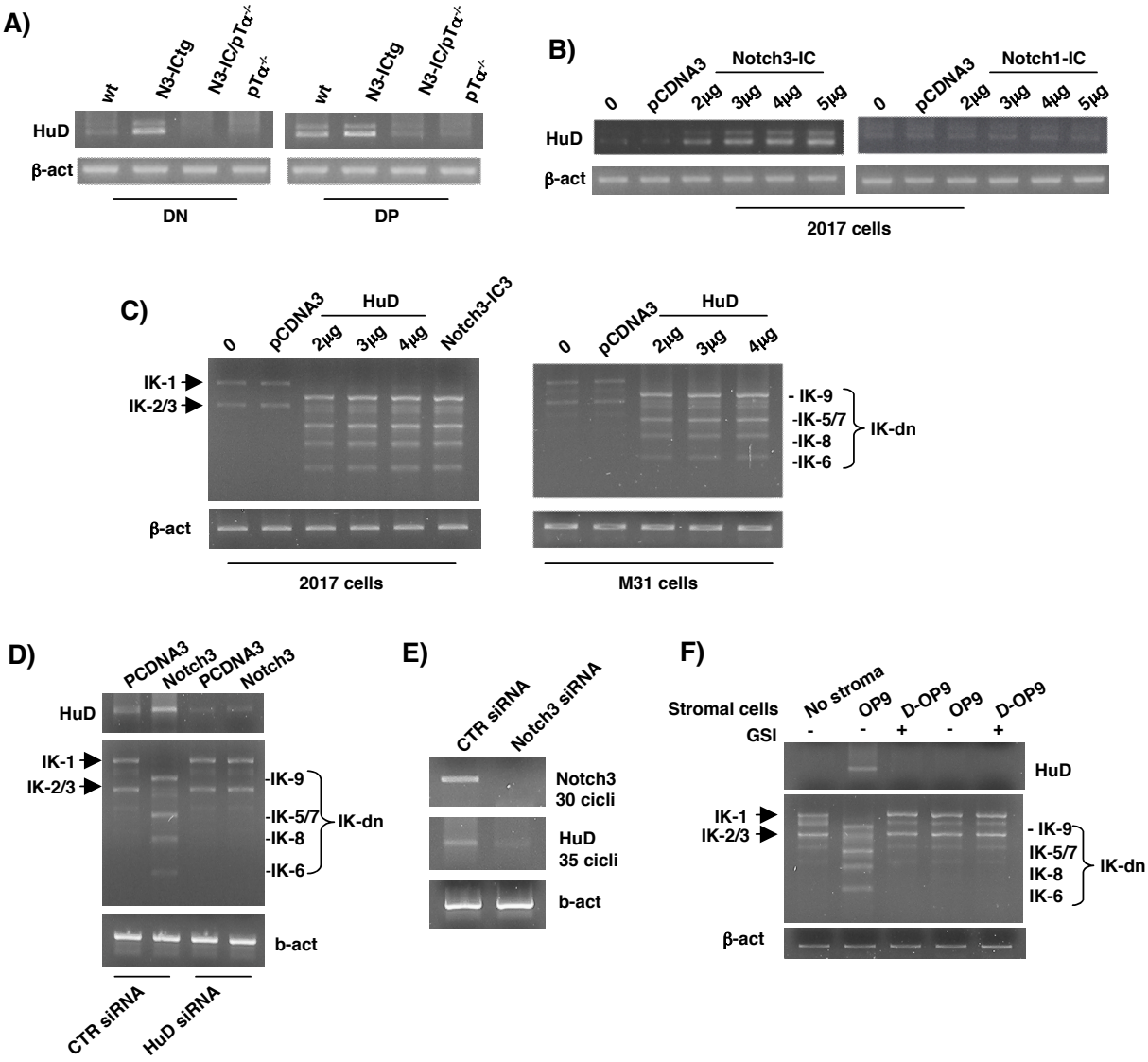

A)
wt
N3-ICtg
N3-IC/pTα-/-
pTα-/-
wt
N3-ICtg
N3-IC/pTα-/-
pTα-/-
HuD
β-act
DN
DP
B)
0
pCDNA3
Notch3-IC
2μg
3μg
4μg
5μg
0
pCDNA3
Notch1-IC
2μg
3μg
4μg
5μg
HuD
β-act
2017 cells
C)
0
pCDNA3
HuD
2μg
3μg
4μg
Notch3-IC3
IK-1
IK-2/3
β-act
2017 cells
0
pCDNA3
HuD
2μg
3μg
4μg
- IK-9
-IK-5/7
-IK-8
-IK-6
IK-dn
M31 cells
D)
PCDNA3
Notch3
PCDNA3
Notch3
HuD
IK-1
IK-2/3
-IK-9
-IK-5/7
-IK-8
-IK-6
IK-dn
b-act
CTR siRNA
HuD siRNA
E)
CTR siRNA
Notch3 siRNA
Notch3
30 cicli
HuD
35 cicli
b-act
F)
Stromal cells
No stroma
OP9
D-OP9
OP9
D-OP9
GSI
- - + - +
HuD
IK-1
IK-2/3
- IK-9
IK-5/7
IK-8
IK-6
IK-dn
β-act

FIG.6 Notch3-induced skewing of Ikaros (IK) isoform profiles depends on Notch3-dependent upregulation of the RNA-binding protein, HuD.
HuD expression was assessed by semiquantitative RT-PCR analysis of RNA from (A) sorted $CD4^-CD8^-$ double-negative (DN) and $CD4^+CD8^+$ double-positive (DP) thymocytes from *wt*, *N3-ICtg*, *N3-ICtg /pTα*$^{-/-}$ and *pTα*$^{-/-}$ mice; and (B) DN-3 thymocytes of the 2017 line transfected with increasing amounts of Notch3 (*left panel*) or Notch1 (*right panel*). (C) RT-PCR detected expression of alternatively spliced IK isoforms in DN-3 and DN-1 thymocytes (2017 and M31 cell lines, respectively) transfected directly with HuD. (0= non-transfected cells; pCDNA3=empty vector-transfected cells). (D) siRNA experiment to study the effect of HuD silencing on the Notch3-induced modulation of IK-dn isoforms. (E) siRNA of Notch3 is able to inhibit the constitutive expression of HuD in 2017 cells. (F) Coculture experiment of 2017 cells on OP9 stromal cells expressing the Notch ligand Delta-like 1 (OP9-DL1) or parental OP9 cells, expressing Notch ligands Jagged-1 and Jagged-2. GSI: γ-secretase inhibitor; 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).

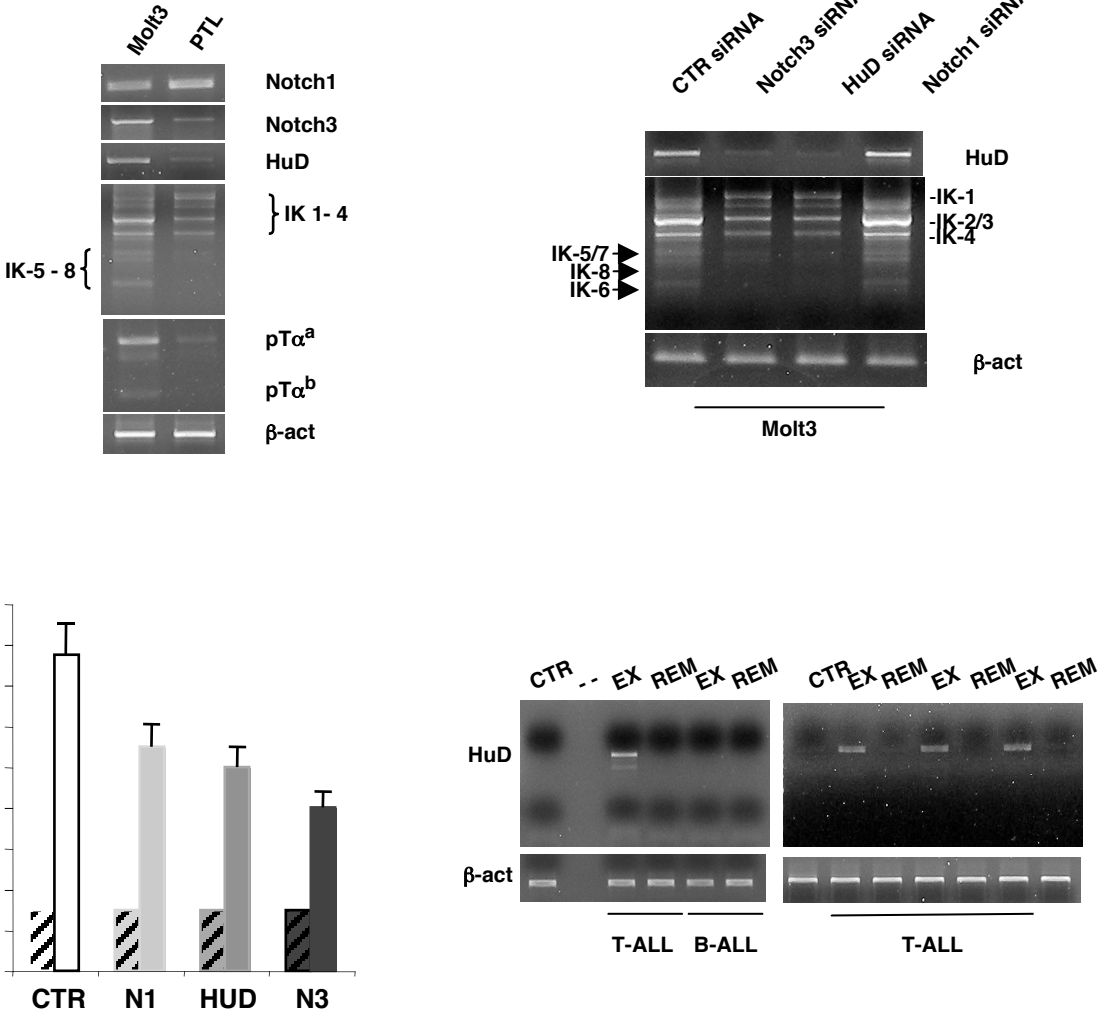


Figure 7 The RNA-binding protein HuD is expressed in human T-ALL and regulates IK

isoform profile.

(A) RT–PCR expression analysis of human Notch1, Notch3, HuD, IK and pTa mRNAs in Molt-3 cell line and peripheral T lymphocytes from one healthy donor (PTL) used as control. Results were normalized to b-actin (b-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 b-actin (b-act).

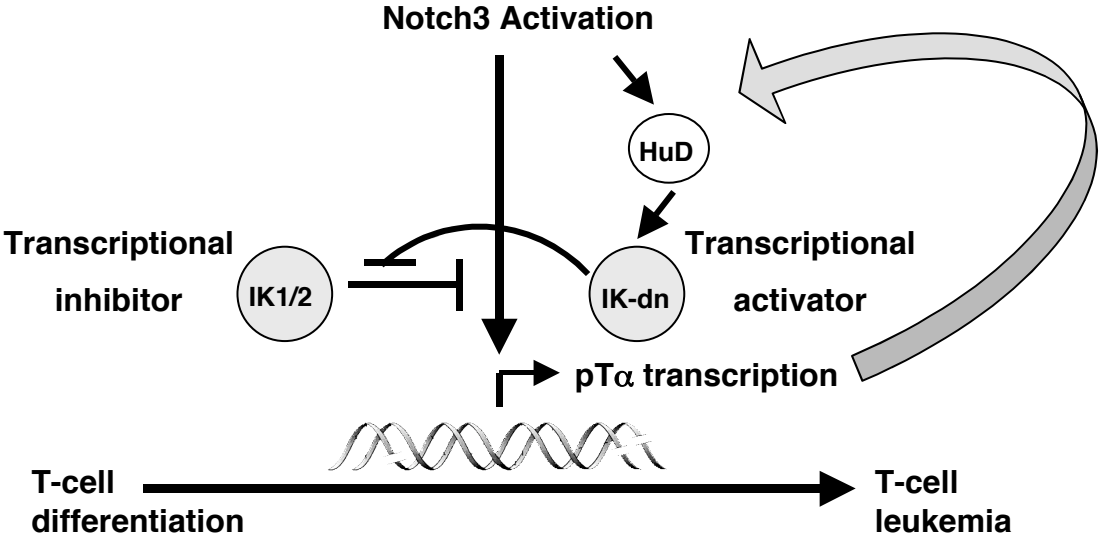


FIG. 8 Cross-talk among Notch3, Ikaros, and pre-TCR in the regulation of T-cell development and lymphomagenesis.
Overexpression of HuD by thymocytes is triggered in a pre-TCR-dependent manner by the activation of Notch3 signalling. As a result of its modulating effects on the splicing and/or stability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

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