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ORIGINAL ARTICLE

# Identification of Notch target genes in uncommitted T-cell progenitors: no direct induction of a T-cell specific gene program

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**Deregulated Notch signaling occurs in the majority of human T-ALL. During normal lymphoid development, activation of the Notch signaling pathway poses a T-cell fate on hematopoietic progenitors. However, the transcriptional targets of the Notch pathway are largely unknown. We sought to identify Notch target genes by inducing Notch signaling in human hematopoietic progenitors using two different methods: an intracellular signal through transfection of activated Notch and a Notch-receptor dependent signal by interaction with its ligand Delta1. Gene expression profiles were generated and evaluated with respect to expression profiles of immature thymic subpopulations. We confirmed *HES1*, *NOTCH1* and *NRARP* as Notch target genes, but other reported Notch targets, including the genes for Deltex1, pre-T-cell receptor α and E2A, were not found to be differentially expressed. Remarkably, no induction of T-cell receptor gene rearrangements or transcription of known T-cell specific genes was found after activation of the Notch pathway. A number of novel Notch target genes, including the transcription factor *TCFL5* and the *HOXA* cluster, were identified and functionally tested. Apparently, Notch signaling is essential to open the T-cell pathway, but does not initiate the T-cell program itself.**
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## Introduction

One of the most important signaling cascades involved in T-cell development is the Notch pathway.[1,2] The Notch signal transduction pathway is an evolutionary conserved mechanism that regulates cell fate determination during developmental processes.[3] Four family members of the transmembrane Notch receptor are recognized, named Notch1 to 4. Signaling is initiated when the large extracellular domain of the Notch receptor binds a membrane-bound ligand on a neighboring cell. The five Notch ligands in mammals are Delta1, 3 and 4 and Jagged1 and 2. Binding of a ligand induces proteolytic cleavage of the intracellular part of the Notch protein (IC-Notch), which translocates to the nucleus and binds to the transcription factor CSL (in human referred to as CBF1, in mouse as RBP-Jκ), activating transcription of Notch target genes.[4]

T cells develop from multipotent progenitor cells that seed the thymus from the bone marrow (BM) or fetal liver.[1] The thymus is rich in expression of Notch ligands[5] and after entering the thymic microenvironment, progenitors immediately start expressing Notch target genes.[6] These newly generated thymocytes do not express CD4 and CD8 and are therefore called double negative (DN). In humans, these most immature DN thymocytes are characterized as $CD34^+CD1a^-CD38^-$ and are homologous to murine DN1 cells.[7] In the next DN stage, thymocytes are $CD34^+CD1a^-CD38^+$ (DN2). This subset still contains (at the population level) potential to develop into all hematopoietic lineages.[8] In the subsequent stage ($CD34^+CD1a^+$, DN3), thymocytes become irreversibly committed to the T-cell lineage.[7] Finally, thymocytes lose expression of CD34 and proceed into the immature single positive (ISP) stage.

The importance of Notch signaling for the induction of a T-cell fate was first demonstrated in mice in which the Notch1 gene was conditionally deleted, resulting in a complete block in T-cell development at the DN1 stage[9] and the emergence of ectopic B-cell development in the thymus.[10] Conversely, overexpression of IC-Notch in the BM instructed a T-cell fate in BM progenitors and inhibited B-cell development.[11] The same effect ensued when Delta4 was overexpressed in the BM.[12] *In vitro*, this phenomenon can be mimicked by expression of Delta1, but not Jagged1, in BM stromal cell lines. Using this co-culture system, T-cell precursors can be efficiently generated from murine and human hematopoietic progenitors.[13–16] The significance of Notch signaling is underscored by studies on T-ALL leukemogenesis. At least half of all human T-ALL carry activating mutations in the intracellular part of Notch1.[17,18]

To develop into a T cell, Notch signaling is clearly indispensable, but the downstream mechanisms by which a Notch signal is translated into a T-cell program are still largely unclear. The best known Notch target genes are Hairy-Enhancer of Split (*HES*)*1* and *HES5* and Hes-related repressor protein (*HERP*). Hes and Herp are basic-helix–loop–helix (bHLH) proteins that function as transcriptional repressors.[19,20] Indeed overexpression of Hes1 and Hes5 in the BM partly inhibits B-cell development.[21] But although proliferation of early thymocytes is severely affected by Hes1 deficiency,[22,23] thymocytes still develop in these mice. Hes1 can therefore not be the sole target of Notch signaling responsible for inducing a T-cell fate.

*PTCRA* (the gene for pre-T-cell receptor α (pTα)) was found as a Notch target using Representational Difference Analysis (RDA) in murine thymoma cell lines with or without retroviral transduction of IC-Notch.[24] The pTα promoter was demonstrated to contain a CSL-binding site and could be activated by IC-Notch *in vitro*.[25] Nevertheless, the physiological role of Notch signaling for pTα expression during T-cell development remains controversial, as pTα expression was not affected in mice in which Notch1 was conditionally deleted from the DN3 stage onwards.[26] A role for Notch1 in opening of the T-cell receptor-β (*TCRB*) locus has been shown, but only for the complete *TCRB* rearrangements (V to DJ).[26,27]

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Other Notch target genes identified in the thymoma cell line were *DTX1* (gene for Deltex1), *Ifi-202*, *Ifi-204*, *Ifi-D3*, *ADAM19* (Meltrin$\beta$).[24] A number of other genes have been reported as being Notch targets, including *NOTCH1* itself,[28] *NRARP* in *Xenopus* embryos,[29] *BCL2* in thymoma cells,[30] *CCND1* (gene for cyclin D1) in a kidney cell line,[31] *CDKN1A* (gene for cyclin-dependent kinase inhibitor 1A (p21, Cip1)) in keratinocytes[32] and *TCF3* (gene for E2A).[11,33] It is unknown whether these genes are *in vivo* targets in the earliest thymocytes and whether they function in T-cell commitment.

In this study, we investigated the downstream mechanisms of Notch signaling in human hematopoietic progenitor cells.

## Materials and methods

### *Isolation of cells*

Human umbilical cord blood (UCB) material was obtained according to the informed consent guidelines of the Medical Ethical Committee of Erasmus MC, Rotterdam. UCB mononuclear cells were isolated using Ficoll density centrifugation and frozen down until further use. For each experiment, frozen UCB cells of at least four different donors were used. $CD34^+$ progenitor cells were purified using immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the recovered subpopulations was checked by flow-cytometry (FACS Calibur, BD Biosystems, San Jose, CA, USA) and was always $>95\%$.

### *Transfection of $CD34^+$ UCB cells*

Human cDNA encoding the intracellular domain of Notch1 (amino acids 1770-2555[34]) was cloned into the multiple cloning site of eukaryotic expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA). $CD34^+$ UCB cells were cultured for 1 h in the presence of 10 ng/ml recombinant human IL-7 and 50 ng/ml recombinant human SCF (both from R&D Systems, Minneapolis, MN, USA). Subsequently, $4 \times 10^6$ cells were transfected with 4 $\mu$g pcDNA3-IC-Notch or empty pcDNA3 using a human CD34 Nucleofector kit (AMAXA) and an AMAXA Nucleofector Device, according to the manufacturers protocol. pEGFP-C1 (0.1 $\mu$g) (BD Clontech, San Jose, CA, USA) was co-transfected with each sample. After transfection, cells were cultured on confluent layers of the murine BM stromal cell line S17, in the presence of 10 ng/ml IL-7 and 50 ng/ml SCF. After 6 and 24 h, cells were carefully harvested without disturbing the S17 monolayer and RNA was isolated. An aliquot of cells was taken from each sample to monitor transfection efficiency (GFP expression) by flow-cytometry.

### *Co-culture of $CD34^+$ UCB cells on S17*

S17 BM stromal cells retrovirally transduced with either Delta1-IRES-eGFP (S17-DL) or control LZRS-IRES-eGFP (S17-GFP) were kindly provided by Dr L Parreira (Faculdade de Medicina de Lisboa, Portugal) and have been described previously.[13] S17 cells were grown in confluent monolayers in 24-wells plates. $1 \times 10^5$ $CD34^+$ UCB cells were co-cultured with S17 in the presence of 10 ng/ml IL-7 and 50 ng/ml SCF. After 3, 6 and 24 h cells were carefully harvested and processed for RNA isolation.

### *Microarray analysis*

Affymetrix microarray analysis of the samples transfected with pcDNA or pcDNA-IC-Notch was performed as described previously.[35] For the samples co-cultured on S17, an extra cycle of cDNA and cRNA synthesis was performed. The starting amount of RNA for the first round of cDNA synthesis was 30–60 ng.

For the transfected samples, Affymetrix U133A and B microarrays were used, for co-cultured samples only U133A arrays. In comparison experiments, care was taken that the 5′/3′ GAPDH ratio, scaling factor, noise, and percentage of presence calls were comparable. Scanned images were analyzed using Affymetrix Microarray Suite 5.1 software, and pair-wise comparisons were generated using the appropriate control samples (without induced Notch signaling) as baseline. Differential expression was considered significant when the gene received a 'present call' for at least one of the microarrays used for the comparison, the fold change was $>1.8$ and change-*P* value $<0.003$. Differential expression of genes after Notch induction was compared to expression profiles of $CD34^+$ UCB cells and the relevant thymocyte subsets: comparisons of $CD34^+$ UCB cells vs $CD34^+CD1a^-CD38^-$ (DN1) and $CD34^+CD1a^-CD38^+$ (DN2) (data generated previously[7]).

Probes for *DTX1* and *NRARP* were present only on the U133B array. *HES5* was not represented on the microarray used (U133). Of the Ifi family, only *Ifi16* was present on the microarrays.

### *Pharmacological inhibition of Notch signaling*

Human $CD34^+$ UCB cells ($4 \times 10^5$) were cultured on confluent layers of the S17-DL1 stromal cell line, in the presence of 10 ng/ml IL-7 and 50 ng/ml SCF. After 16 h incubation, DAPT was added to the cells to the final concentrations of 1 or 5 $\mu$M. Alternatively, medium or DMSO alone was added as control. The cells were harvested before addition of DAPT and after 24 h incubation, and processed for RNA isolation and cDNA synthesis. *TCFL5* and *HES1* expression was analyzed by real-time quantitative-polymerase chain reaction (PCR). The vehicle alone control (DMSO) did not have any effect.

### *Real-time quantitative-PCR*

The expression of several target genes was tested using TaqMan-based RQ-PCR. cRNA (1 $\mu$g) generated in the microarray analysis procedure was treated with 1 U of DNase I and subsequently reverse transcribed to cDNA with avian myeloblastosis virus-reverse transcriptase (5 U), oligo(dT) and random hexanucleotide primers.

Mixes containing the appropriate pre-designed primer/probe sets were obtained from Applied Biosystems. For *TCFL5*, *HES1*, *HoxA9* and *HoxA10* these were Assays-on-Demand, for *HoxA5* an Assay-by-Design. A 1/20 cDNA mixture was used for RQ-PCR for each primer/probe set and performed in duplicate. The RQ-PCR reaction was performed using TaqMan Universal mastermix (Applied Biosystems) and was run on a PRISM 7700 sequence detection system containing a 96-well thermal cycler (Applied Biosystems). RQ-PCR results were normalized to *GAPDH* expression (kit from Applied Biosystems) in the same sample.

### *Functional testing of TCFL5 in fetal thymic organ culture*

Full-length cDNA for human TCFL5 was obtained from Invitrogen, cloned into the retroviral vector LZRS-IRES-eGFP and high titer retrovirus was produced in the Phoenix packaging cell line. $CD34^+$ UCB cells were cultured for one day and retrovirally transduced for 2 days in the presence of 50 ng/ml SCF, 10 ng/ml recombinant human TPO and 50 ng/ml recombinant human Flt3L (all from R&D Systems). The percentage of

GFP positive cells was determined using flow cytometry and cells were transferred into irradiated murine embryonic day 14 thymic lobes. After 2 days, lobes were cultured on filters floating on medium. After an additional nine days, lobes were harvested, single cell suspensions were made and T-cell development was analyzed using a FACS Calibur flowcytometer (BD Biosystems). Monoclonal antibodies against human antigens were CD1a-RD1 (Beckman Coulter, Fullerton, CA, USA) and TCR$\gamma\delta$-PE, CD3-PerCP, CD8-PerCP, CD4-APC and CD34-APC (all from BD Pharmingen). At least 50 000 events were acquired and analyzed by flow cytometry.

### *HoxA5 mutant mice*

Mice deficient for HoxA5 function have been described in detail.[36] Hoxa5 heterozygous mice from an outbred genetic background were bred to generate homozygous, heterozygous and wild type littermates. Surviving HoxA5 homozygous mice were viable and did not display evident abnormalities.[36] In mice of 2.5, 4 and 25 weeks old, BM, spleen and thymus were analyzed for presence of different hematopoietic lineages using flow cytometry. Embryonic thymic lobes of day 16 were directly stained for flow-cytometry. Embryonic thymic lobes of day 13 and 14 were cultured on a filter for 16 days, after which T-cell development was analyzed using flow-cytometry. Fetal livers of embryonic day 13 and 14 were either directly used for flow-cytometry to analyze progenitor populations or transferred into irradiated wild type day 14 thymic lobes for FTOC experiments. Chimeric thymic lobes were cultured for 14 days on a filter, after which T-cell development was analyzed using flow-cytometry.

HoxA5-deficient BM cells were assayed for the presence of granuloctye-macrophage colony-forming units (CFU-C) and erythroid burst-forming units (BFU-E) by *in vitro* colony formation in viscous methylcellulose culture medium containing cytokines: for BFU-E 4 U/ml human erythropoietin (EPO, Behringwerke AG) and 100 ng/ml murine SCF (R&D); for CFU-C 30 ng/ml murine IL-3, 100 ng/ml SCF and colony stimulating factor (CSF, 300 $\times$ dilution of ConA adsorbed fraction of pregnant mouse uterus extract). In all, 40 000 and 100 000 cells were plated in duplicate dishes. The number of colonies was determined after 10 days of culture.

HoxA5-deficient BM cells were assayed for their ability to reconstitute the immune system of sublethally irradiated mice. 8- to 11-week-old C57Bl/6 mice harboring the CD45.1 (Ly5.1) allele as a congenic marker, received a sublethal dose of 6 Gy total body irradiation. Mice were intravenously injected with $2 \times 10^6$ thawed BM cells (CD45.2$^+$). At 10 weeks after transplantation, mice were killed and BM, spleen and thymus were isolated. In each organ, total cell numbers were determined and the percentage of CD45.2$^+$ cells and their subset distribution was assayed by flow cytometry.

Monoclonal antibodies against mouse antigens were ER-MP20-FITC (anti Ly6c, own culture), CD25-FITC, IgM-FITC, Sca1-FITC, CD45.2-FITC, CD4-PE, CD43-PE, TER119-PE, NK1.1-PE, CD127-PE, Sca-1-PE, CD3-PerCP, CD8-PerCP, Lin-PerCP (CD3-biotin, B220-PerCP, NK1.1-biotin, MAC1-PerCP, GR1-biotin, TER119-biotin, Streptavidin-PerCP), CD44-APC, B220-APC, CD19-APC, MAC1-APC and c-kit-APC (all from BD Pharmingen).

## Results

### *Induction of Notch signaling*

To identify target genes of Notch signaling, gene expression profiles were generated of human hematopoietic progenitors that did or did not undergo Notch signaling. We provided Notch signals in two different ways: by transfection with IC-Notch or by co-culturing cells on Delta1 expressing stromal cells.

It has been shown previously that human CD34$^+$ cells expressing IC-Notch develop into T/NK progenitors when co-cultured on BM stroma.[37] Human CD34$^+$ UCB cells were transfected with the *IC-NOTCH* gene, together with a separate GFP vector in order to monitor transfection efficiency. At 6 h after transfection, the percentage of GFP expressing cells was high and comparable between IC-Notch transfected and control cells (Supplementary Figure 1). At 24 h after transfection GFP expression had slightly decreased (Supplementary Figure 1).

Another way to induce the Notch signaling cascade is to allow progenitor cells to interact with their cognate ligand responsible for inducing T-cell development. To this end, human CD34$^+$ UCB cells were cultured on the BM stromal cell line S17, which was retrovirally transduced with either human Delta1 in combination with GFP (S17-DL) or with GFP only (S17-GFP).[13] Other groups have shown that CD34$^+$ UCB cells cultured in this and similar systems develop into double positive (DP) thymocytes[13,16] and also in our hands CD34$^+$ progenitor cells efficiently developed into CD7$^+$ T/NK precursors cells after 2 weeks of culturing on S17-DL (data not shown). CD34$^+$ progenitor cells were cultured on S17-DL for 3, 6 and 24 h, as kinetics of translocation of Notch-IC to the nucleus and induction of target RNA transcription are unknown.

From all samples RNA was isolated and gene expression profiles were generated using Affymetrix technology. Pair-wise comparisons were made between samples with or without Notch signaling. Numbers of significantly differentially expressed genes ($\geqslant$2 fold difference, $P<0.003$ by *t*-test[38] are listed in Table 1) (*t*-test). Both up- and downregulated genes were found, indicating that Notch functions as an activator as well as a repressor of transcription in hematopoietic progenitors.

Expression profiles of human thymocyte subsets have been previously generated and described in detail.[7] The differential expression of genes induced by Notch signaling in hematopoietic progenitors was compared to the relevant UCB and thymocyte subsets: CD34$^+$ UCB vs DN1 (CD34$^+$ CD1a$^-$CD38$^-$) and DN1 vs DN2 (CD34$^+$CD1a$^-$CD38$^+$). DN1

**Table 1** Numbers of significantly up- or downregulated genes ($\geqslant$2 fold, $P<0.003$)

| | *6 h ICN*[a] | *24 h ICN*[a] | *3 h S17-DL*[b] | *6 h S17-DL*[b] | *24 h S17-DL*[b] |
|---|---|---|---|---|---|
| U133A array | 70↑ 12↓ | 17↑ 44↓ | 10↑ 87↓ | 13↑ 14↓ | 137↑ 162↓ |
| U133B array | 35↑ 5↓ | 13↑ 0↓ | nt | nt | nt |

nt, microarray not performed.
[a]ICN: progenitor cells from human UCB transfected with pcDNA-IC-Notch versus control pcDNA.
[b]S17-DL: progenitor cells from human UCB cultured on S17-DL versus S17-GFP.

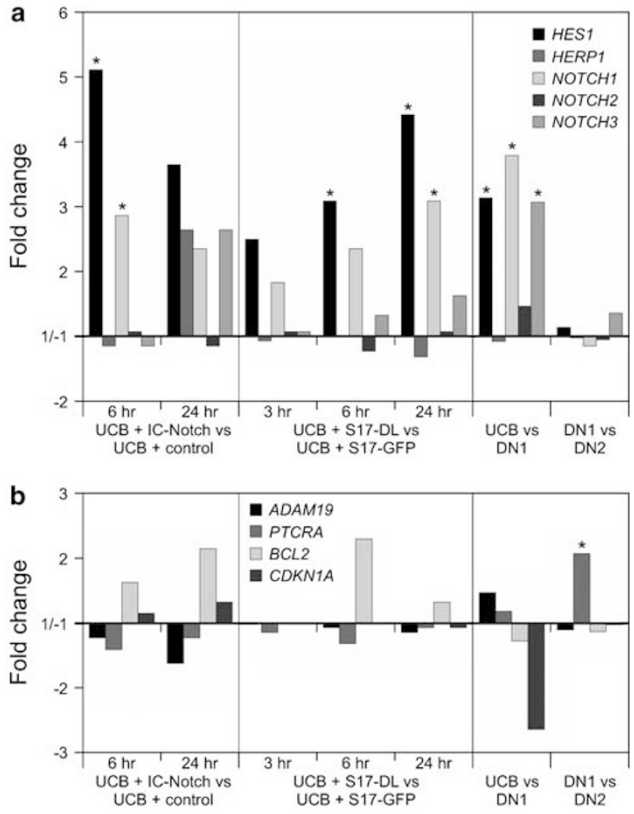


**Figure 1** Differential expression of putative Notch target genes. (**a**, **b**) Fold changes in expression levels extracted from gene expression profiles generated from hematopoietic progenitor cells with or without induced Notch signaling, as well as previously performed gene expression profiles from human UCB and thymocyte subsets.[7] ICN vs control: comparisons of progenitors transfected with pcDNA-IC-Notch vs control pcDNA. DL vs GFP: comparisons of progenitors cultured on S17-DL vs S17-GFP. CB vs DN1: comparisons of $CD34^+$ UCB cells vs $CD34^+CD1a^-CD38^-$ (DN1) thymocytes. DN1 vs DN2: comparisons of $CD34^+CD1a^-CD38^-$ (DN1) thymocytes vs $CD34^+CD1a^-CD38^+$ (DN2) thymocytes. Asterisks indicate a significant difference in expression levels (fold change $>1.8$, $P<0.003$).

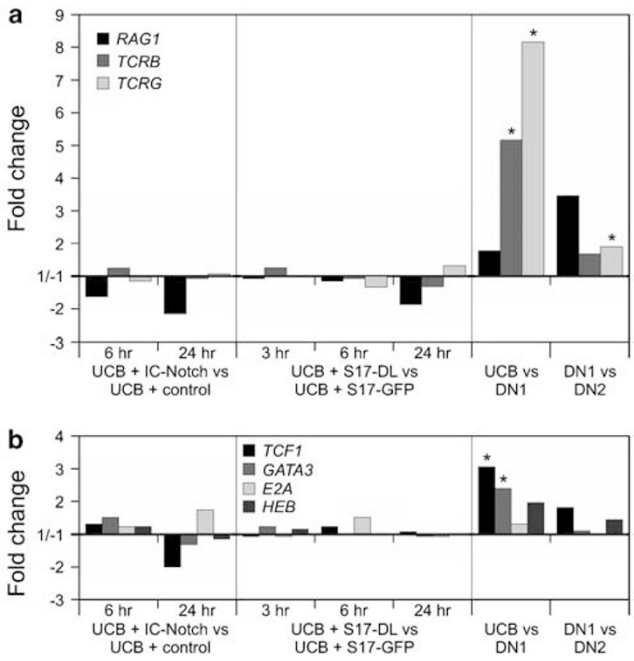


**Figure 2** Differential expression of T-cell associated genes. Determined by microarray analysis as indicated for Figure 2. (**a**) TCR gene rearrangement-associated genes. (**b**) Transcription factors involved in T-cell development.

cells have just entered the thymus and are therefore expected to upregulate Notch target genes.[6] Indeed, the development of murine early thymic progenitors (ETP) into subsequent thymic stages is strictly Notch-dependent.[39] DN2 cells should also express Notch target genes, as they have contacted Notch ligands for prolonged periods and as Notch-ligand interactions throughout the DN1 and 2 stage are necessary for irreversible T-cell commitment.[40] In addition, during the DN2 stage, commitment to the T-cell lineage is initiated and cells start rearranging T-cell receptor $\beta$ (*TCRB*) genes.[7]

### *Expression of previously reported Notch target genes*

Comparison files were mined for the expression of known Notch target genes. In all samples, transcription of *HES1*, the universal Notch target,[3] was clearly increased after Notch signaling (Figure 1a). This was most pronounced in the progenitor cells transfected with IC-Notch for 6 h, reflecting the strong Notch signal induced in this way. 24 h after transfection, *HES1* was still upregulated, but to a lesser extend. This might indicate negative feed-back mechanisms that are initiated after receiving a strong Notch signal. Alternatively it may reflect the fact that transfection was transient, consistent with the slightly diminished GFP expression found after 24 h (Supplementary Figure 1).

In the cells cultured on S17-DL, *HES1* expression was most prominent after 24 h (Figure 1a). *HES1* was also highly upregulated in the DN1 thymocytes as compared to $CD34^+$ UCB cells (Figure 1a), demonstrating that the artificially induced Notch signals in our experiments mimic the relevant processes *in vivo*.

*HERP1* and *2* were not expressed in any of the samples, neither before nor after inducing a Notch signal (Figure 1a). *NOTCH1* itself is also a Notch target[28] and was found to be significantly upregulated after 6 h of transfection and after 24 h of culturing on S17-DL (Figure 1a). This was reflected by high expression in the DN1 subset as compared to UCB (Figure 1a). Also *NOTCH3* was transcribed at high levels in the DN1 cells as compared to UCB, but Notch signaling *in vitro* induced *NOTCH3* transcription to a lesser extend and with slower kinetics than *NOTCH1* (Figure 1a). *NOTCH2* (Figure 2a) and *NOTCH4* (not shown) were not differentially expressed in any of the samples.

Of the other previously reported Notch target genes mentioned above, only *NRARP* was significantly upregulated after induction of Notch signaling (3.5-fold increased 6 h after transfection of IC-Notch (not shown)). The other genes were either not differentially expressed or not expressed at all (Figure 1b and data not shown), indicating that they are not Notch target genes in early hematopoietic progenitor cells. This finding is especially remarkable for Deltex1 and pT$\alpha$, which have been reported as Notch targets during T-cell development.[14,24,25] In the thymocyte subsets, significantly increased mRNA expression of *PTCRA* was only found at the DN2 stage, but not at the DN1 stage (Figure 1b), while genuine Notch target genes (*HES1*, *NOTCH1*) were expressed already in DN1 thymocytes (Figure 1a). PT$\alpha$ may be a Notch target gene at

later stages of T-cell development, but is not immediately upregulated when cells enter the thymus.

For *BCL2* a consistent, but not significant, trend of upregulation was observed in most comparisons, which was nevertheless not reflected in the thymocyte subsets (Figure 1b). Conversely, *CDKN1A* (p21) was significantly decreased in the DN1 subset, but did not show any differential expression after Notch activation (Figure 1b).

### *Notch does not induce a T-cell program*

The mechanisms by which Notch signaling induces or promotes a T-cell fate still warrant elucidation. To investigate whether Notch signaling can trigger T-cell commitment by directly inducing TCR gene rearrangements or transcription of 'classical' T-cell specific genes, we mined our data set for such genes.

No induction of *RAG1*, *RAG2* and *DNTT* (gene for TdT) and germline transcripts of *TCRB*, *TCRD* and *TCRG* were found after Notch signaling (Figure 2a and not shown), suggesting that Notch has no direct function in opening of the TCR loci or the initiation of TCR gene rearrangements.

In addition, we found no differential expression (mostly no expression at all) of T-cell specific genes after activation of Notch signaling, while these genes were highly upregulated in the DN1 thymocytes (including *CD7* and *CD2*) or in the DN2 subset (including *CD1a*, *LAT* and *CD3*$\gamma$) (data not shown).

Next, we investigated differential expression of a number of transcription factors that are known to be important for T-cell development.[1] Tcf1 and GATA3 are among the first transcription factors to show up when a cell initiates a T-cell program,[41] which we confirmed in our DN1 subset (Figure 2b). Although it has been suggested that Notch1 acts upstream of Tcf1 and GATA3,[27] we did not find induction of these genes after Notch signaling (Figure 2b).

Some reports have proposed that Notch inhibits B-cell development through inhibition of bHLH factor E2A,[11,33] although E2A is clearly important for T-cell development as well, both as a homodimer and as dimerization partner for HEB.[1] In our experiments, we found no change in expression of either *E2A* or *HEB* after Notch signaling (Figure 2b).

### *Novel Notch target genes in hematopoietic progenitors*

Next to the known Notch target genes *HES1* and *NOTCH1*, we discovered significant differential expression of a number of previously unknown Notch target genes. At 6 h after transfection with IC-Notch, high induction of *HOXA5*, *HOXA9*, *HOXA10* and to a lesser extend *HOXA7* mRNA was detected (Figure 3a and b). Upregulation was markedly decreased after 24 h. In S17-DL cultures, a small increase in HOXA transcripts could be detected after 24 h by RQ-PCR but not by microarray analysis (Figure 3a and b). *HOXA5*, *A9* and *A10* were previously shown to be expressed in the human thymus,[42] but we did not observe upregulation of these genes in DN1 and DN2 thymocytes (Figure 3a).

Among the highest upregulated genes 6 h after IC-Notch transfection were *TCFL5* (transcription factor like 5), *MEK5c* (mitogen-activated protein kinase kinase) and *DUSP6* (dual-specificity phosphatase 6, also termed MAP kinase phosphatase 3) (Figure 3c). TCFL5 is a transcription factor of the bHLH family, first identified in spermatocytes.[43,44] Both MEK5c and Dusp6 can influence MAP kinase activity and interactions between the Notch and MAP kinase pathways have been described.[45] Our microarray results showed limited upregulation of *MEK5c* and *TCFL5* transcripts after 24 h of culturing on S17-DL, while for unknown reasons RQ-PCR validation of *TCFL5* showed high induction in this condition as well (Figure 3d). *TCFL5* and *DUSP6* were significantly increased in the DN1 thymocytes as compared to CD34$^+$ UCB (Figure 3c), supporting a functional role of these genes in T-cell differentiation.

Lastly, we found high upregulation of a number of genes associated with vasculogenesis, including *ANGPT2* (gene for angiopoietin), *FGF22* and *FBLN* (gene for fibulin) (Figure 3e). These genes were detected only after transfection with IC-Notch and not after co-culture on S17-DL or in thymocyte subsets, suggesting that their induction is not related to thymic function, but rather to other biological processes. These vasculogenesis genes may be physiologically induced by Notch ligands other than Delta1, for instance by Jagged. Interestingly, Notch signaling has been implicated in vasculogenesis and angiogenesis.[46–48] Furthermore, hemangioblasts (bipotent progenitors for endothelial and hematopoietic cells) are present among CD34$^+$ UCB cells.[49] This intriguing finding warrants further research, but is beyond the scope of this report.

### *Pharmacological inhibition of Notch signaling*

Before starting on functional experiments to elucidate the role of TCFL5 in T-cell development, we sought to obtain other experimental evidence that TCFL5 is a Notch target gene. For this, we have induced Notch signaling in CD34$^+$ uncommitted progenitor cells and subsequently inhibited Notch signaling with a $\gamma$-secretase inhibitor (DAPT). Direct addition of DAPT to cells cultured on S17-DL1 led to less Hes-1 and TCFL5 expression in some experiments, but this was inconsistent (data not shown) presumably due to the strong Notch signal delivered by S17-DL1 and the complex competition between activating (Dl1) and inhibitory (DAPT) signals. Therefore, we chose to first have cells undergo a Notch signal, followed by inhibition of that signal by DAPT. CD34$^+$ progenitor cells were cultured over night (16 h) on S17-DL1 to induce Notch signaling. DAPT was added for the indicated periods of time, after which cells were harvested, RNA was isolated and used for RQ-PCR to determine levels of GAPDH, TCFL5 and Hes-1. CD34$^+$ UCB cells do not express significant levels of HES-1 nor TCFL5, but after inducing Notch signaling HES1 is abundantly expressed and TCFL5 moderately. Both the levels of Hes-1 and TCFL5 were reduced by 50–70% of controls (Figure 4), after inhibition of Notch signaling by the well-known Notch inhibitor DAPT. Interestingly, in absence of Delta signal, both Hes-1 and TCFL5 decreased, and further decreased by incubation with DAPT. (Figure 4). This pharmacological approach therefore provides additional evidence that TCFL5, similar to *HES1*, is a true Notch target gene showing similar kinetics of induction and pharmacological inhibition, albeit with lower absolute expression.

### *Functional validation of novel Notch target genes* TCFL5 *and* HOXA5

Among the most striking novel Notch target genes identified in our studies were *TCFL5* and the *HOXA* genes. The relevance of these genes is substantiated by the finding of putative CSL-binding sites upstream of *TCFL5* exon 1 and in the human *HOXA5-HOXA4* intergenic region (data not shown). Nevertheless, functional studies are necessary to understand the significance of TCFL5 and HoxA proteins for hematopoiesis and T-cell development in particular.

First we data-mined previously performed microarrays of all major thymocyte subsets[7] for the expression of *TCFL5*. We found a progressive increase in *TCFL5* mRNA from DN1 up to

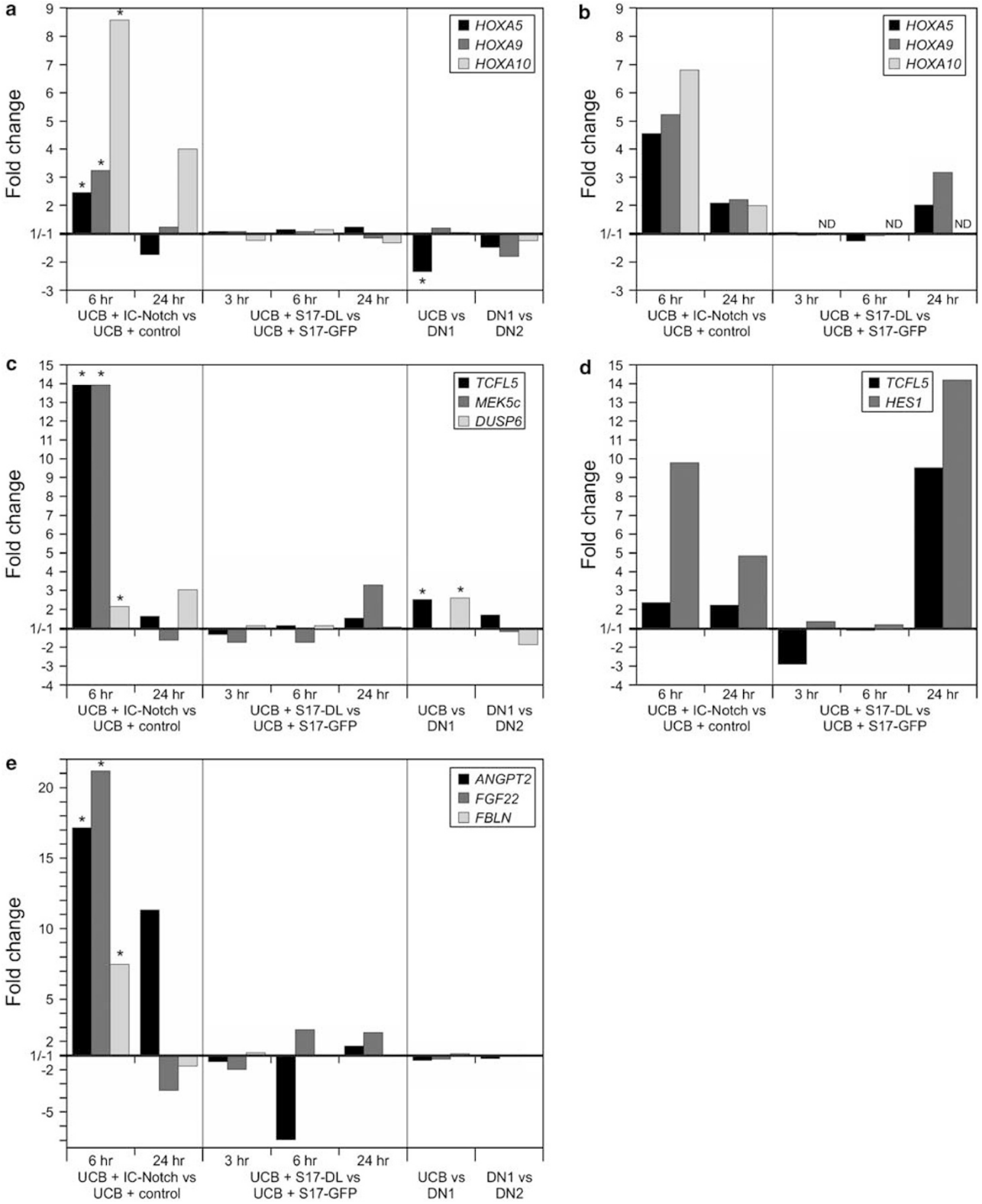


**Figure 3** Differential expression of novel Notch target genes. (**a**) *HoxA* transcripts determined by microarray analysis as indicated for Figure 2. (**b**) *HoxA* transcripts determined by RQ-PCR. ND: not detected. (**c**) *TCFL5*, *MEK5c* and *Dusp6* transcripts determined by microarray analysis. (**d**) *Hes1* and *TCFL5* transcripts determined by RQ-PCR. (**e**) Transcription of angiogenesis genes determined by microarray analysis.

the early DP stage (Figure 5a). Levels sharply dropped when thymocytes started expressing CD3, suggesting that TCFL5 functions mainly during the early stages of T-cell development (Figure 5a).

To test whether TCFL5 is functionally important during T-cell development, we retrovirally expressed the *TCFL5* gene together with the *GFP* gene in human $CD34^+$ UCB hematopoietic progenitors. As a control, $CD34^+$ cells were transduced with

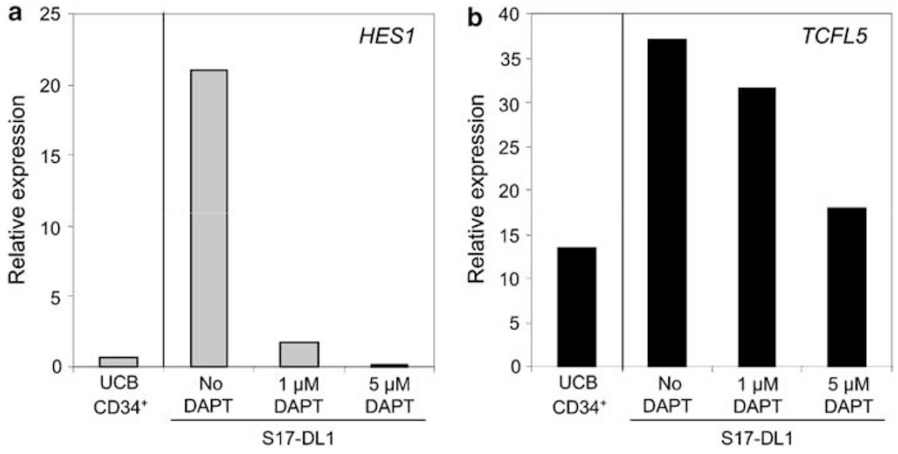


**Figure 4** Pharmacological evidence for TCFL5 as Notch target gene. CD34$^+$ cells were cultured on S17-DL1 to induce Notch signaling, as evidenced by upregulation of *HES1* (**a**) and *TCFL5* (**b**) expression. Subsequently, cells were cultured in medium and DAPT was added to inhibit Notch signaling. Expression of *HES1* and *TCFL5* was downregulated in a dose-dependent fashion. Data shown are averages of duplicate measurements by RQ-PCR. Duplicate values always varied less than one $C_t$. Experiment shown is representative out of two.

the same construct containing only the *GFP* gene. After transduction, cells were cultured in fetal thymic organ cultures (FTOC) to allow them to differentiate into T cells. After 9 days in FTOC, thymic lobes were harvested and stained for flow-cytometric analysis. The TCFL5 transduced GFP$^+$ cells showed an acceleration of T-cell development: the percentage of DP cells was almost doubled as compared to control transduced GFP$^+$ and GFP$^-$ cells and TCFL5 transduced GFP$^-$ cells (Figure 5b and data not shown). In addition, the percentage of TCR$\gamma\delta^+$ cells was markedly increased in the TCFL5 transduced GFP$^+$ cells (Figure 5c).

We found a remarkable increase in HoxA5, A9 and A10 in progenitor cells transfected with IC-Notch (Figure 4a), suggesting a role for these genes in hematopoiesis. Mice deficient for HoxA9 have previously been shown to display defects in myeloid, B-cell and T-cell development,[50,51] while on the other hand overexpression of HoxA10 inhibited lymphoid development.[52] HoxA5 mutant mice were generated some years ago,[53] but their hematopoietic system has not been studied. We now analyzed the BM, spleen and thymus of HoxA5 homozygous, heterozygous and wild-type littermates of 2.5, 4 and 25 weeks old. No consistent abnormalities were detected in populations of B, T, NK and myeloid cells in the thymus, BM and spleen of HoxA5-deficient mice (data not shown). As the thymic defects of HoxA9-deficient mice were more pronounced in fetal mice than in adults, we also analyzed HoxA5-deficient embryos. We found normal T-cell development in fetal thymic lobes of HoxA5-deficient embryos and in FTOCs of HoxA5-deficient fetal liver cells (Figure 6a). Furthermore, no differences were found in numbers of Lin$^-$Sca-1$^+$c-Kit$^+$ cells between the fetal livers of HoxA5-deficient and normal embryos (Figure 6b), indicating that hematopoietic stem cells are normally present in these mice. Colony assays were performed to determine the frequency of erythroid and myeloid progenitors in HoxA5-deficient BM: no abnormalities were detected (data not shown). To investigate whether HoxA5-deficient BM progenitors can reconstitute the immune system under competitive conditions, BM cells were transplanted into sublethally irradiated recipients. After 5, 8 and 10 weeks, the percentage of donor cells in the blood was identical between mice transplanted with wild-type or HoxA5-deficient BM cells (Figure 6c). Furthermore, all hematopoietic lineages were normally present among donor cells in thymus, BM and spleen of the recipients.

## Discussion

Despite the unmistakable role of Notch signaling for T-cell lineage specification, studies to identify downstream Notch target genes in relevant primary progenitors have not been undertaken.

In the present study, we took advantage of the recently introduced AMAXA nucleofection system, which allows transient transfection of primary hematopoietic progenitor cells with a high efficiency and relatively low mortality. Using this technique, we were able to induce a rapid and strong Notch signal, as shown by high *HES1* expression after 6 h. Furthermore, the S17-DL stromal cell co-culture system allowed us to more physiologically mimic the Notch signal encountered by progenitor cells that enter the thymus. We chose to use human CD34$^+$ UCB cells as source of progenitor cells, as these are readily available, unactivated, multipotent and efficiently develop into T-cells when cultured in murine fetal thymic lobes or on BM stroma expressing Delta1.[13,14,37] Progenitor cells start expressing Notch target genes only after entering the thymus,[6] which made them preferable over CD34$^+$ thymocytes which have already contacted Notch ligands. Using CD34$^+$ UCB cells and either IC-Notch transfection or the S17-DL co-culture system in combination with Affymetrix microarray technology, we found upregulation of the generally acknowledged Notch target genes *HES1*, *NOTCH1* and *NRARP*, which validated our procedures. The delayed upregulation of target genes in cells cultured on S17-DL as compared to transfection with IC-Notch indicates that cells need prolonged or multiple contacts with the Delta1 ligand to get accumulation of significant amounts of IC-Notch.

Our assays did not show induction of a number of genes previously reported to function as Notch targets during T-cell development, including *PTCRA*, *E2A* and *DTX1*.[11,24,33] This is especially critical for Deltex1, as many studies use expression of Deltex1 as the read-out for active Notch signaling, mostly in combination with Hes1,[14,54] but in some cases as sole evidence.[55] The main evidence for *PTCRA* and *DTX1* as Notch targets in T-cell development comes from studies by Deftos *et al.*,[24,30] in which IC-Notch was retrovirally transduced into a murine DP thymoma cell line. Apart from the fact that Notch signaling and downstream events may be dysregulated in malignantly transformed cells, retroviral transduction takes several days and identified genes may represent indirect targets

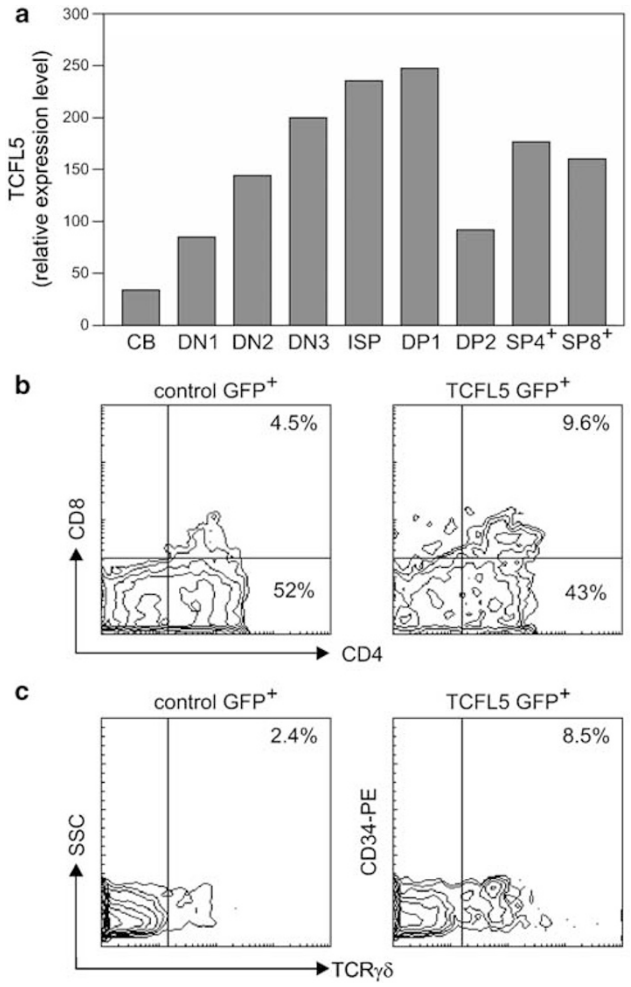


**Figure 5** Functional validation of a role for TCFL5 in T-cell development. (**a**) Relative amounts of *TCFL5* mRNA in thymocyte subpopulations. Values extracted from previously performed gene expression profiles.[7] CB: $CD34^+$ UCB, DN1: $CD34^+CD1a^-CD38^-$, DN2: $CD34^+CD1a^-CD38^+$, DN3: $CD34^+CD1a^+$, ISP: $CD4^+CD3^-$, DP1: $CD4^+CD8^+CD3^-$, DP2: $CD4^+CD8^+CD3^+$, SP4+: $CD4^+CD3^+$, SP8+: $CD8^+CD3^+$. (**b**, **c**) Human $CD34^+$ UCB cells were retrovirally transduced with a vector containing only the gene for *GFP* (left panels) or *TCFL5* and *GFP* (right panels) and allowed to develop into T-cells in FTOC. After 9 days of culture, cells were harvested and examined by flow cytometry. (**b**) Expression of CD4 and CD8 within the $GFP^+$ gate. (**b**) Expression of TCR$\gamma\delta$ within the $GFP^+$ gate.

or may have resulted from compensatory mechanisms. Therefore, Deltex expression may strongly depend on the context in which the Notch signal is given. Upregulation of genes identified in the thymoma line was confirmed in the thymi of mice transgenic for IC-Notch,[24] but may again result from indirect activation. Nevertheless, it possible that *PTCRA* is a Notch target gene in more differentiated T-cell precursors. This would fit with our finding that *PTCRA* is upregulated at the DN2 stage (Figure 2b) and with the fact that Notch signaling continues throughout the DN2 stage.[40]

We did not find any evidence for direct induction of a T-cell program by Notch signaling. This is in contrast with a recent study by Höflinger *et al*.,[27] in which a Notch signal was induced by culturing multipotent progenitor cells on OP9-DL. These cells rapidly upregulated *PTCRA, DTX1, GATA3* and *TCF1*, but not *HES1* transcripts. However, these results should be interpreted with caution, as this study used murine $Pax5^{-/-}$ pro-B cells expanded on the ST2 cell line as source of uncommitted progenitors: non-physiological cells that express many B-cell specific genes and have already started TCR gene rearrangements (V$\gamma$-J$\gamma$, V$\delta$-J$\delta$ and D$\beta$-J$\beta$).[27] It remains however possible that sustained activation of Notch signaling (for at least several days), in addition to other signals and special microenvironments is required to induce a T-cell specific gene program. We cannot exclude that some of the target genes induced by Notch in turn can activate T-cell specific genes (either alone or in concert with the right other signaling routes). Our findings are consistent with experiments of Taghon *et al*.[54] in which murine fetal liver progenitors were cultured on OP9-DL. This study showed that *GATA3*, *TCF1* and *PTCRA* began to be expressed after three days of culture on OP9-DL, while high *HES1* transcription was detected already after one day. These findings indicate that either Notch signaling induces T-cell genes in more differentiated thymocytes, or Notch signaling stimulates the expression of other transcription factors, which then in turn activate or repress lineage differentiation genes. TCFL5 is a likely candidate for such a function.

TCFL5 is a member of the group B bHLH proteins and presumably binds to the same non-canonical E-box as Hes1.[43] It was first found in spermatocytes, but was not detected in the thymus by Northern-blot analysis.[43,44] This might be explained by the fact that *TCFL5* levels decrease in the more mature stages of T-cell development (Figure 5a), which comprise the larger part of the thymus. Also during spermatogenesis, TCFL5 is expressed in a highly cell-type and stage-specific pattern.[43,44]

By overexpressing TCFL5 in FTOC, we obtained initial evidence that TCFL5 positively regulates T-cell development. The observed effects were mild, presumably because TCFL5 is expressed already during the early stages of T-cell development (Figure 5a), and enhanced expression is not likely to induce remarkable changes. We have attempted loss of function studies using siRNA for TCFL5. These have not been successful in a setting (lentiviral vector) that allows sustained siRNA expression required in T-cell developmental studies, while in transient assays downregulation of TCFL5 was readily obtained (data not shown). The gain-of-function experiments, however, give an indication that TCFL5 plays a functional role during T-cell development in the thymus. Thus, TCFL5 may be a negative regulatory bHLH factor that function in concert with or alternative to Hes1. The similar expression pattern during subsequent stages of thymic T-cell development (not shown) further suggests such a role.

The finding of the *HOXA* cluster genes as Notch targets is novel and intriguing. Notch induced *HOXA* transcripts may be important for T-cell development, as HoxA9-deficient mice have a partial block at the DN1 stage of thymocyte development.[51] Furthermore, overexpression of *HOXA* genes is observed in human T-ALL.[56] We did not find upregulation of *HOXA* genes in DN1 cells. Possibly, *HOXA* genes are induced only in a tiny subset of DN1 cells that have just contacted Delta, and are rapidly downregulated afterwards. However, we did not detect any defects in T-cell differentiation or hematopoiesis in general in HoxA5-deficient mice. The high functional redundancy of the different *HoxA* genes probably obscures the importance of HoxA5 for hematopoiesis. Other than a role in T-cell development, the Hox genes may be involved in alternative Notch-regulated processes in hematopoietic stem cells. Notch signaling is clearly important for self-renewal of hematopoietic progenitors (reviewed by Radkte *et al*.[57]). Interestingly, *HOXA5, A9* and *A10* were found to be part of the 'stem cell profile'.[58] Without doubt, the relation between Notch signaling and expression of the HoxA cluster should be studied in greater detail.

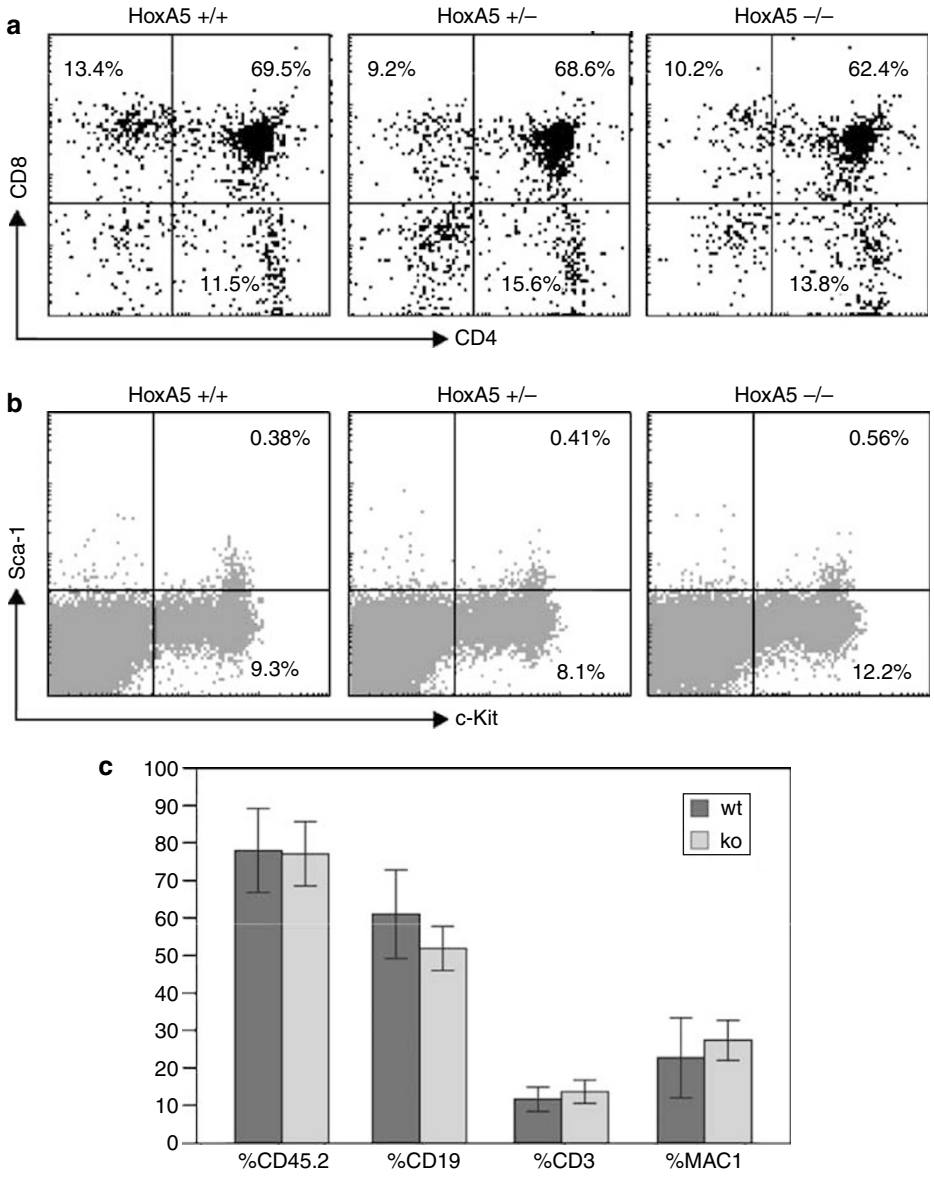


**Figure 6** Hematopoiesis in HoxA5-deficient mice. (**a**) Thymic lobes of normal (left and middle panels) and HoxA5-deficient (right) embryos of day 14 of gestation (E14) were cultured on filters. After 16 days, T-cell development in the lobes was analyzed using flow cytometry. (**b**) Hematopoietic progenitor populations were determined in fetal livers of normal (left and middle panels) and HoxA5-deficient (right) E12 embryos. Percentages of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells are shown in the upper right quadrant. (**c**) Sublethally irradiated mice were transplanted with wild-type or HoxA5-deficient BM cells. After 10 weeks, the percentage of $CD45^{+}$ donor cells and the percentage of $CD19^{+}$ B cells, $CD3^{+}$ T cells, and $MAC1^{+}$ myeloid cells within a $CD45^{+}$ gate were determined in the blood of the recipients.

The progenitor population we have used contains a very small population of apparently T-cell committed $CD34^{+}CD7^{+}$ cells, as described by Haddad *et al*.[59] It is possible that activation of the Notch pathway leads to differentiation of $CD34^{+}CD7^{-}$ progenitors into $CD34^{+}CD7^{+}$ progenitors, as activation of Notch signaling by culturing on S17-DL1 leads to upregulation of CD7 (data not shown, but see also[60]). Alternatively, Notch signaling leads to selective outgrowth of the $CD45^{hi}CD34^{+}CD7^{+}$ cells described by Haddad *et al*., but this seems less likely, as the $CD34^{+}CD7^{+}$ population expresses TCRB, whereas in our micro array experiments these were undetectable. Therefore, uncommitted $CD34^{+}$ cells (most likely $CD34^{+}CD38^{-}$ HSC) are induced to start T cell commitment, while losing myeloid potential, which is retained when cultured on S17-Jagged1[60]).

The mechanism by which Notch signaling initiates a T-lineage differentiation program in hematopoietic progenitors remains a fascinating issue. Here, we demonstrate that Notch does not directly activate a T-cell specific transcription program, nor does it directly induce TCR gene rearrangements. Apparently, other factors are needed besides Notch to induce T-cell commitment. Perhaps Wnt signaling, by delivering essential proliferative factors to immature thymocytes undergoing Notch signaling is important in this respect.[61] For sure, Hes1 executes part of the downstream Notch effects. In addition, TCFL5 and HoxA proteins may prove to be major player in Notch-regulated mechanisms during T-cell development. Furthermore, these novel Notch target genes may contribute to the pathogenesis of T-ALL with gain-of-function mutations in Notch1.

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## References

1 Staal FJ, Weerkamp F, Langerak AW, Hendriks RW, Clevers HC. Transcriptional control of t lymphocyte differentiation. *Stem Cells* 2001; **19**: 165–179.
2 Weerkamp F, Pike-Overzet K, Staal FJ. T-sing progenitors to commit. *Trends Immunol* 2006; **27**: 125–131.
3 Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science* 1999; **284**: 770–776.
4 Tamura K, Taniguchi Y, Minoguchi S, Sakai T, Tun T, Furukawa T *et al*. Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H). *Curr Biol* 1995; **5**: 1416–1423.
5 Parreira L, Neves H, Simoes S. Notch and lymphopoiesis: a view from the microenvironment. *Semin Immunol* 2003; **15**: 81–89.
6 Harman BC, Jenkinson EJ, Anderson G. Entry into the thymic microenvironment triggers Notch activation in the earliest migrant T cell progenitors. *J Immunol* 2003; **170**: 1299–1303.
7 Dik WA, Pike-Overzet K, Weerkamp F, de Ridder D, de Haas EF, Baert MR *et al*. New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. *J Exp Med* 2005; **201**: 1715–1723.
8 Weerkamp F, Baert MR, Brugman MH, Dik WA, de Haas EF, Visser TP *et al*. The human thymus contains multipotent progenitors with T/B-lymphoid, myeloid and erythroid lineage potential. *Blood* 2006; **107**: 3131–3137.
9 Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR *et al*. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* 1999; **10**: 547–558.
10 Wilson A, MacDonald HR, Radtke F. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J Exp Med* 2001; **194**: 1003–1012.
11 Pui JC, Allman D, Xu L, DeRocco S, Karnell FG, Bakkour S *et al*. Notch1 expression in early lymphopoiesis influences B vs T lineage determination. *Immunity* 1999; **11**: 299–308.
12 Dorsch M, Zheng G, Yowe D, Rao P, Wang Y, Shen Q *et al*. Ectopic expression of Delta4 impairs hematopoietic development and leads to lymphoproliferative disease. *Blood* 2002; **100**: 2046–2055.
13 Jaleco AC, Neves H, Hooijberg E, Gameiro P, Clode N, Haury M *et al*. Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *J Exp Med* 2001; **194**: 991–1002.
14 La Motte-Mohs RN, Herer E, Zuniga-Pflucker JC. Induction of T-cell development from human cord blood hematopoietic stem cells by Delta-like 1 *in vitro*. *Blood* 2005; **105**: 1431–1439.
15 Lehar SM, Dooley J, Farr AG, Bevan MJ. Notch ligands Delta 1 and Jagged1 transmit distinct signals to T-cell precursors. *Blood* 2005; **105**: 1440–1447.
16 Schmitt TM, Zuniga-Pflucker JC. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 *in vitro*. *Immunity* 2002; **17**: 749–756.
17 Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C *et al*. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* 2004; **306**: 269–271.
18 Weerkamp F, van Dongen JJ, Staal FJ. Notch and Wnt signaling in T-lymphocyte development and acute lymphoblastic leukemia. *Leukemia* 2006; **20**: 1197–1205.
19 Iso T, Sartorelli V, Chung G, Shichinohe T, Kedes L, Hamamori Y. HERP, a new primary target of Notch regulated by ligand binding. *Mol Cell Biol* 2001; **21**: 6071–6079.
20 Jarriault S, Brou C, Logeat F, Schroeter EH, Kopan R, Israel A. Signalling downstream of activated mammalian Notch. *Nature* 1995; **377**: 355–358.
21 Kawamata S, Du C, Li K, Lavau C. Overexpression of the Notch target genes Hes *in vivo* induces lymphoid and myeloid alterations. *Oncogene* 2002; **21**: 3855–3863.
22 Tomita K, Hattori M, Nakamura E, Nakanishi S, Minato N, Kageyama R. The bHLH gene Hes1 is essential for expansion of early T cell precursors. *Genes Dev* 1999; **13**: 1203–1210.
23 Kaneta M, Osawa M, Sudo K, Nakauchi H, Farr AG, Takahama Y. A role for pref-1 and HES-1 in thymocyte development. *J Immunol* 2000; **164**: 256–264.
24 Deftos ML, Huang E, Ojala EW, Forbush KA, Bevan MJ. Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes. *Immunity* 2000; **13**: 73–84.
25 Reizis B, Leder P. Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway. *Genes Dev* 2002; **16**: 295–300.
26 Wolfer A, Wilson A, Nemir M, MacDonald HR, Radtke F. Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes. *Immunity* 2002; **16**: 869–879.
27 Hoflinger S, Kesavan K, Fuxa M, Hutter C, Heavey B, Radtke F *et al*. Analysis of Notch1 function by *in vitro* T cell differentiation of Pax5 mutant lymphoid progenitors. *J Immunol* 2004; **173**: 3935–3944.
28 Kimble J, Simpson P. The LIN-12/Notch signaling pathway and its regulation. *Annu Rev Cell Dev Biol* 1997; **13**: 333–361.
29 Lamar E, Deblandre G, Wettstein D, Gawantka V, Pollet N, Niehrs C *et al*. Nrarp is a novel intracellular component of the Notch signaling pathway. *Genes Dev* 2001; **15**: 1885–1899.
30 Deftos ML, He YW, Ojala EW, Bevan MJ. Correlating notch signaling with thymocyte maturation. *Immunity* 1998; **9**: 777–786.
31 Ronchini C, Capobianco AJ. Induction of cyclin D1 transcription and CDK2 activity by Notch(ic): implication for cell cycle disruption in transformation by Notch(ic). *Mol Cell Biol* 2001; **21**: 5925–5934.
32 Rangarajan A, Talora C, Okuyama R, Nicolas M, Mammucari C, Oh H *et al*. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *EMBO J* 2001; **20**: 3427–3436.
33 Ordentlich P, Lin A, Shen CP, Blaumueller C, Matsuno K, Artavanis-Tsakonas S *et al*. Notch inhibition of E47 supports the existence of a novel signaling pathway. *Mol Cell Biol* 1998; **18**: 2230–2239.
34 Carlesso N, Aster JC, Sklar J, Scadden DT. Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics. *Blood* 1999; **93**: 838–848.
35 Staal FJ, Weerkamp F, Baert MR, van den Burg CM, van Noort M, de Haas EF *et al*. Wnt target genes identified by DNA microarrays in immature CD34+ thymocytes regulate proliferation and cell adhesion. *J Immunol* 2004; **172**: 1099–1108.
36 Jeannotte L, Lemieux M, Charron J, Poirier F, Robertson EJ. Specification of axial identity in the mouse: role of the Hoxa-5 (Hox1.3) gene. *Genes Dev* 1993; **7**: 2085–2096.
37 De Smedt M, Reynvoet K, Kerre T, Taghon T, Verhasselt B, Vandekerckhove B *et al*. Active form of Notch imposes T cell fate in human progenitor cells. *J Immunol* 2002; **169**: 3021–3029.
38 de Ridder D, Staal FJ, van Dongen JJ, Reinders MJ. Maximum significance clustering of oligonucleotide microarrays. *Bioinformatics* 2006; **22**: 326–331.
39 Sambandam A, Maillard I, Zediak VP, Xu L, Gerstein RM, Aster JC *et al*. Notch signaling controls the generation and differentiation of early T lineage progenitors. *Nat Immunol* 2005; **6**: 663–670.
40 Schmitt TM, Ciofani M, Petrie HT, Zuniga-Pflucker JC. Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med* 2004; **200**: 469–479.
41 Hattori N, Kawamoto H, Fujimoto S, Kuno K, Katsura Y. Involvement of transcription factors TCF-1 and GATA-3 in the initiation of the earliest step of T cell development in the thymus. *J Exp Med* 1996; **184**: 1137–1147.
42 Taghon T, Thys K, De Smedt M, Weerkamp F, Staal FJ, Plum J *et al*. Homeobox gene expression profile in human hematopoietic multipotent stem cells and T-cell progenitors: implications for human T-cell development. *Leukemia* 2003; **17**: 1157–1163.
43 Siep M, Sleddens-Linkels E, Mulders S, van Eenennaam H, Wassenaar E, Van Cappellen WA *et al*. Basic helix-loop-helix

transcription factor Tcfl5 interacts with the Calmegin gene promoter in mouse spermatogenesis. *Nucleic Acids Res* 2004; **32**: 6425–6436.
44 Maruyama O, Nishimori H, Katagiri T, Miki Y, Ueno A, Nakamura Y. Cloning of TCFL5 encoding a novel human basic helix-loop-helix motif protein that is specifically expressed in primary spermatocytes at the pachytene stage. *Cytogenet Cell Genet* 1998; **82**: 41–45.
45 Sharp LL, Schwarz DA, Bott CM, Marshall CJ, Hedrick SM. The influence of the MAPK pathway on T cell lineage commitment. *Immunity* 1997; **7**: 609–618.
46 Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes Dev* 2004; **18**: 901–911.
47 Liu ZJ, Shirakawa T, Li Y, Soma A, Oka M, Dotto GP *et al.* Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. *Mol Cell Biol* 2003; **23**: 14–25.
48 Limbourg FP, Takeshita K, Radtke F, Bronson RT, Chin MT, Liao JK. Essential role of endothelial Notch1 in angiogenesis. *Circulation* 2005; **111**: 1826–1832.
49 Cogle CR, Wainman DA, Jorgensen ML, Guthrie SM, Mames RN, Scott EW. Adult human hematopoietic cells provide functional hemangioblast activity. *Blood* 2004; **103**: 133–135.
50 Lawrence HJ, Helgason CD, Sauvageau G, Fong S, Izon DJ, Humphries RK *et al.* Mice bearing a targeted interruption of the homeobox gene HOXA9 have defects in myeloid, erythroid, and lymphoid hematopoiesis. *Blood* 1997; **89**: 1922–1930.
51 Izon DJ, Rozenfeld S, Fong ST, Komuves L, Largman C, Lawrence HJ. Loss of function of the homeobox gene Hoxa-9 perturbs early T-cell development and induces apoptosis in primitive thymocytes. *Blood* 1998; **92**: 383–393.
52 Taghon T, Stolz F, De Smedt M, Cnockaert M, Verhasselt B, Plum J *et al.* HOX-A10 regulates hematopoietic lineage commitment: evidence for a monocyte-specific transcription factor. *Blood* 2002; **99**: 1197–1204.
53 Aubin J, Chailler P, Menard D, Jeannotte L. Loss of Hoxa5 gene function in mice perturbs intestinal maturation. *Am J Physiol* 1999; **277**: C965–C973.
54 Taghon TN, David ES, Zuniga-Pflucker JC, Rothenberg EV. Delayed, asynchronous, and reversible T-lineage specification induced by Notch/Delta signaling. *Genes Dev* 2005; **19**: 965–978.
55 Huang EY, Gallegos AM, Richards SM, Lehar SM, Bevan MJ. Surface expression of Notch1 on thymocytes: correlation with the double-negative to double-positive transition. *J Immunol* 2003; **171**: 2296–2304.
56 Soulier J, Clappier E, Cayuela JM, Regnault A, Garcia-Peydro M, Dombret H *et al.* HOXA genes are included in genetic and biologic networks defining human acute T-cell leukemia (T-ALL). *Blood* 2005; **106**: 274–286.
57 Radtke F, Wilson A, Mancini SJ, MacDonald HR. Notch regulation of lymphocyte development and function. *Nat Immunol* 2004; **5**: 247–253.
58 Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, Lemischka IR. A stem cell molecular signature. *Science* 2002; **298**: 601–604.
59 Haddad R, Guardiola P, Izac B, Thibault C, Radich J, Delezoide AL *et al.* Molecular characterization of early human T/NK and B-lymphoid progenitor cells in umbilical cord blood. *Blood* 2004; **104**: 3918–3926.
60 Neves H, Weerkamp F, Gomes AC, Naber BA, Gameiro P, Becker JD *et al.* Effects of Delta1 and Jagged1 on early human hematopoiesis: correlation with expression of notch signaling-related genes in CD34+ cells. *Stem Cells* 2006; **24**: 1328–1337.
61 Weerkamp F, Baert MR, Naber BA, Koster EE, de Haas EF, Atkuri KR *et al.* Wnt signaling in the thymus is regulated by differential expression of intracellular signaling molecules. *Proc Natl Acad Sci USA* 2006; **103**: 3322–3326.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)