Mechanisms of Notch signaling specificity in lymphocytes and their leukemic counterparts
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Chapter 4

Human T cell development and T-ALL do not require Notch dimerization

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Abstract
As a major oncogene, especially in T cell acute lymphoblastic leukemia (T-ALL), Notch is an attractive therapeutic target. However, general inhibition of Notch signaling causes unacceptable side effects, due to the many critical physiological functions of Notch. Recent studies identified a mechanism allowing biochemical separation of Notch functions. In a subset of target genes, responsiveness to Notch requires homodimerization of the Notch transcriptional complex (NTC) on sequence-paired sites (SPS). In mice, such dimerization controls development of T cells, leukemic transformation and growth of T-ALL. Drugs targeting the dimerization interface might therefore disarm the oncogenic potential of Notch with fewer side effects. Here, we addressed the requirement for NOTCH1 homodimerization in human T cell development and T-ALL. We found that homodimerization is required for optimal expansion of human thymic CD34+ progenitors and differentiation of type 2 innate lymphocytes. However, homodimerization is not required for development of human T cells, due to architectural differences between the human and murine pre-T cell receptor alpha (pTα) enhancer. Finally, we demonstrate that growth of human and many murine T-ALL lines does not require homodimerization. Therefore, Notch homodimerization is mechanistically conserved in humans, but drugs preventing dimerization of Notch are unlikely to possess potent therapeutic activity.

Keywords
Notch, dimerization, T-ALL, T cell, ILC2, pre-TCR alpha
Introduction

The evolutionarily conserved Notch signaling cascade operates in developing as well as adult organisms to orchestrate multiple functions in many organ systems, including the central nervous system, the hematopoietic system, the gastrointestinal system, muscle and skin. Notch is well known for its involvement in (binary) cell fate decisions, but also regulates basic cellular processes such as metabolism, proliferation and survival. Consequently, Notch is a major oncogene, although it can also act as a tumor suppressor in certain cell types.

The Notch pathway plays a pivotal role in multiple steps of T cell development. Notch signals are essential for commitment of multipotent progenitors to the T cell lineage, as well as for passage through the β-selection checkpoint, during which cells are selected for productive rearrangement of a functional TCRβ chain gene. Its critical involvement in development of T cells is reflected by the oncogenic role of NOTCH1 in T-ALL, a malignancy caused by uncontrolled expansion of immature T cells. Constitutive activation of NOTCH1 induces rapid and aggressive T-ALL in bone marrow transfer models in mice. More than 50% of murine and human T-ALL cases harbor activating mutations in NOTCH1 and growth of many T-ALLs can be blocked by inhibition of Notch signaling. The prognosis for T-ALL patients displaying treatment failure is currently dismal, necessitating the development of novel therapeutic options. Clearly, the prominent oncogenic role of Notch in T-ALL makes it an attractive therapeutic target for the treatment of this disease. Its appeal as such is diminished, however, by the fact that Notch regulates so many important physiological functions. Indeed, general inhibition of Notch signaling was found to result in severe acute side effects on gastrointestinal health in patients. Although specific targeting of individual Notch receptors or combination with glucocorticoids could mitigate some of these side effects, it is likely that long term treatment, even with those types of modifications, will yield additional complications, given the plethora of physiological functions of Notch. Ideally, therapies targeting Notch would selectively interfere with its oncogenic functions, while leaving all its other functions intact.

The desire to specifically target selective functions of Notch seems at odds with the apparent simplicity of the pathway. Canonical Notch signaling is activated upon binding of ligands of the Delta like (Dll1, Dll4) and Jagged families (Jag1, Jag2) to the Notch receptors (NOTCH1-4 in mammals), inducing a proteolytic cascade. The final cleavage step, which can be efficiently blocked by small molecule inhibitors referred to as γ-secretase inhibitors (GSI), results in the release of the Notch intracellular domain (NICD) from the membrane. Cleaved NICD translocates to the nucleus, where it functions as a switch to induce target gene expression: NICD associates with the DNA binding protein CSL (CBF-1 (mammals), Su(H) (Drosophila) and Lag-1 (C. elegans)) and this complex subsequently recruits co-activators in exchange for previously bound repressors. The core Notch
transcriptional complex (hereafter called NTC) consists of the NICD, CSL and a co-activator of the Mastermind-like (MAML) family.

Despite the apparent simplicity of Notch signaling, activation of Notch can have very diverse and sometimes even opposite effects. For instance, Notch induces several distinct T helper cell fates from the same naïve CD4+ T cell precursor and can induce either development of αβ T cells or type 2 innate lymphocytes from the same CD34+CD1a+ thymic progenitors. Mechanisms must therefore exist, which confer plasticity to the pathway. Understanding the molecular details of these mechanisms may yield opportunities for more specific targeting of Notch. In this light, recent biochemical studies have revealed one mechanism determining Notch target gene selectivity. Some target promoters contain so-called sequence-paired sites (SPS), which consist of two CSL binding sites oriented in a head-to-head fashion and separated by a spacer of 15-22 base pair length. This specific promoter architecture allows for cooperative assembly of dimeric NTCs, mediated by homotypic interactions between the Ankyrin (ANK) domains of neighboring NICD1 molecules. Strikingly, point mutants deficient for homodimerization fail to transactivate SPS containing targets, but are fully capable of activating genes with single CSL binding sites. Several studies have now revealed that Notch target genes differ in their sensitivity to dimerization: While relatively few genes like hes5 strictly depend on dimerization, most others are completely independent of dimeric Notch signaling, like hey1, hey2, HEYL and CD25. Expression of a third group of target genes (including HES1) is activated by input from dimeric as well as monomeric Notch signals.

The biological relevance of dimeric Notch1 signaling has been addressed in a mouse study, which demonstrated that dimerization is crucial for β-selection and, importantly, the induction of T-ALL as well as continued growth of T-ALL lines. The same study also identified c-myc and ptcra (the gene encoding the pre-T cell receptor alpha chain, pTα) as the responsible target genes in these processes. Since most targets of Notch do not depend on homodimerization, these findings offer the exciting possibility that drugs preventing Notch homodimerization may exhibit potent anti tumor activity without adverse on-target effects elicited by pan-Notch inhibitors.

To determine its therapeutic potential, we studied the requirement for dimeric Notch signaling for growth of human T-ALL as well as development of human T cells and ILC2. We found that effective development of ILC2 requires homodimerization of NOTCH1. However, even though expansion of early human thymocytes was partially promoted by homodimerization, their differentiation along the T cell lineage occurs independently of dimeric signaling. We show that, unlike the murine locus, the human PTCRA enhancer does respond to monomeric Notch signaling, providing a rationale for why human T cell development does not require dimerization. Finally, we demonstrate that sustained growth of a panel of human, but also multiple murine T-ALL lines, is independent of NOTCH1 homodimerization. We
conclude that dimeric Notch signaling is conserved between mice and humans, although critical differences exist in the functions that depend on this mechanism. Unfortunately, our data do not support the notion that drugs targeting NOTCH1 homodimers can be expected to possess potent anti leukemic activity.

Results

R1985A mutation specifically abrogates NOTCH1 homodimerization but does not affect the formation of functional monomers and general signal strength.

A critical residue mediating NOTCH1 homodimerization via the ANK domain is an Arginine at position 1985, as revealed by mutation to an Alanine. To confirm that this mutation indeed specifically affects homodimerization, we measured activation of different Notch responsive luciferase reporter genes: HES1-luciferase contains a classical SPS motif comprised of two CSL binding sites in head-to-head orientation and separated by a spacer of 16 nucleotides, while four single CSL binding sites oriented in head-to-tail fashion control CBF1-luciferase. Expression of the R1985A mutant failed to activate HES1-luciferase (Figure 1A, left), but activated the CBF1 reporter equally well as wild-type NICD1 (Figure 1A, right), confirming earlier studies.

It is conceivable that the apparent specificity of the R1985A mutant stems from a quantitative rather than a qualitative defect in transactivation capacity. CBF1-luciferase might have a low threshold for activation and thus, even a hypothetically weak signal induced by R1985A might cause similar activation as wild-type NICD1. This would, however, not be sufficient to activate promoters with higher thresholds, such as the HES1 promoter. To test this, we titrated wild-type or R1985A NICD1. While the CBF1 reporter was activated to the same extent by wild-type and R1985A NICD1 at all concentrations in a dosage-dependent manner (Figure 1A, right), the R1985A mutant failed to activate the HES1 reporter even at concentrations 10fold higher than wild-type NICD1 (Figure 1A, left).

We further investigated potential differences in the overall signal strength by using proteins N-terminally fused to a mutated Estrogen receptor binding domain (mER-NICD1). Transcriptional activation by mER-NICD1 can be regulated quantitatively by Tamoxifen. While mER-R1985A and mER-wild-type NICD1 activated the CBF1 reporter equally well at all concentrations of Tamoxifen (Figure 1B, right), mER-R1985A failed to activate the HES1 promoter even at the highest concentration (Figure 1B, left). Hence, the defect of the R1985A dimerization mutant is not due to induction of an overall weaker Notch signal, but specific to dimerization dependent promoter architectures.
Finally, the R1985A dimerization mutant not only lost the capacity to transactivate the HES1 reporter, but also interferes with transactivation by wild-type NICD1 on this promoter upon co-transfection (Figure 1C, left). In contrast, co-transfection of R1985A and wild-type NICD1 resulted in additive induction of CBF1-luciferase (Figure 1C, right). Taken together, these results show that the R1985A mutation selectively abrogates the capacity of NOTCH1 to homodimerize, while formation of a fully functional, monomeric NTC and general Notch signal strength are not perturbed. Therefore, the R1985A mutant represents a versatile tool to study the requirements for dimeric NOTCH1 signaling, also in the context of endogenous Notch signaling.
Growth of human and murine T-ALL lines does not depend on NOTCH1 homodimerization.

Leukemic transformation and continued growth of murine T-ALL lines critically depend on NOTCH1 homodimerization\(^{38}\). To examine whether NOTCH1 homodimerization represents a potential therapeutic target for the human disease, we investigated the requirement for this process in several human T-ALL lines. These lines are highly dependent on Notch, as inhibition of γ-secretase induces growth arrest and apoptosis\(^{20–22,46,47}\). Since NICD acts downstream of γ-secretase mediated cleavage, ectopic expression of NICD can be used to rescue T-ALL cells from GSI treatment\(^{21,22,38}\). Inhibition of γ-secretase markedly reduced the size of DND41 cells and this was rescued by constitutive expression of wild-type NICD1 (Figure 2A). Relative differences in cell size indicate changes in translational and metabolic activity\(^{48}\), which have been implicated in Notch-driven T-ALL and T cell differentiation\(^6\). To assess proliferation directly, we measured dilution of a proliferation dye (Figure 2B). Cells transduced with an empty vector, but not wild-type NICD1, underwent slightly fewer divisions in the presence of the γ-secretase inhibitor DAPT. To our surprise, however, the dimerization R1985A rescued both GSI induced reduction of cell size (Figure 2A) and proliferation (Figure 2B) to the same extent as wild-type NICD1. Accordingly, total expansion of T-ALL cells transduced with NICD1, but not empty vector, was rescued from GSI mediated growth inhibition (Figure 2C). Interestingly, we typically observed that NICD1 transduced cells grew better in the presence of GSI (Figure 2C, left) than without (Figure 2C, right).

These findings are in line with the observation that (strong) NICD1 transgenes cannot be stably expressed in T-ALL lines, unless endogenous Notch signaling is inhibited by GSI (RG and DA, unpublished data), indicating that there might be a threshold of the maximum Notch signal strength tolerated by T-ALL cells.
Chapter 4

A

Mock

DAPT

events [% of max]

FSC

empty vector

wild-type NICD1

R1985A NICD1

B

events [% of max]

CellTrace Violet

undivided

empty vector

wild-type NICD1

R1985A NICD1

C

Mock

DAPT

# transduced [fold of d0]

[weeks]

DND41

empty vector

wild-type NICD1

R1985A NICD1

HPBALL

TALL1
As two murine T-ALL lines were previously shown to require homodimerization of NOTCH1 for their growth\(^{38}\), we considered the possibility that DND41 might be an exceptional line, which happens to have found a way around this requirement. We therefore examined the requirements for Notch homodimerization of two other human T-ALL lines. To our surprise, expression of dimerization mutant R1985A NICD1 fully overcomes GSI mediated growth inhibition in all human cell lines tested, including DND41 (Figure 2C, top) HPBALL (Figure 2C, middle) and TALL1 (Figure 2C, bottom) and two other T-ALL lines (SupT1, MOLT4; RG and DA, unpublished data).

To address whether species differences account for the striking discrepancy between these results and previously published work\(^{38}\), we tested the dimerization dependence in a set of murine T-ALL lines. The murine T6E T-ALL line was previously shown to require homodimerization of NICD1 for its growth\(^{38}\). Indeed, we also found that expression of R1985A NICD1 failed to rescue this cell line from GSI mediated cell cycle arrest, whereas expression of wild-type NICD1 rendered the cells insensitive to GSI (Figures 3A and 3B). The dependence on homodimerization of Notch is so pronounced in this cell line that expression of R1985A NICD1 even blocks cell cycle progression and expansion of these cells in the absence of GSI (Figures 3A and 3B). In sharp contrast, a panel of different murine T-ALL cells was fully rescued by dimerization mutant NICD1 (Figure 3C; RG and DA, unpublished results). These highly GSI sensitive lines were derived from mice specifically lacking PTEN in (pre-) T cells caused by conditional deletion of a floxed \(Pten\) allele by Lck-Cre\(^{40,49}\). In mice, \(c-myc\) was identified as a critical target of NOTCH1 homodimers\(^{36}\). As the \(c-myc\) gene is frequently activated in T-ALL due to translocations, we also examined the requirement for Notch homodimerization in T-ALL lines obtained from \(Pten^{flox/flox}\times Lck-Cre\)
mice also deficient for Rag2 (Figure 3C, bottom), in which such translocations are much less frequent. However, none of the lines tested from these mice showed dependence on homodimerization of Notch (Figure 3; RG and DA, unpublished results). Therefore, the requirement for homodimerization of Notch is not a general property of murine T-ALL.

**Proliferation of early human thymocytes partially depends on homodimerization of NOTCH1.**

The prominent role of Notch in T-ALL reflects its physiological function in expansion of T cell precursors in the thymus. Because monomeric Notch is able to drive growth of human T-ALL, we asked whether proliferation of its physiological counterpart is similarly independent of NOTCH1 homodimerization. To test this, we transduced CD34+CD1a- early human T cell progenitors with wild-type NICD1 or R1985A NICD1 and co-cultured these cells with OP9 stromal cells, a widely used system to study early human T cell differentiation. To exclude that any possible differences between results obtained upon expression of wild-type NICD1 or R1985A NICD1 are caused by quantitative signaling differences (for instance due to unequal expression), we examined induction of CD7 by these constructs. We recently showed that surface expression of CD7 serves as a sensitive readout for Notch signal strength in this system. After three days of co-culture with OP9 cells, thymocytes transduced with the wild-type and R1985A NICD1 showed comparable elevated CD7 expression compared to empty vector control cells (Figure 4A, left). This was also the case when thymocytes were cultured with OP9 cells expressing the Notch ligand Dll1 (Figure 4A, right), demonstrating that R1985A does not inhibit general endogenous Notch signaling in primary human thymocytes.

As described above, differences in cell size correlate with changes in cellular metabolism and translational activity. Indeed, expression of wild-type NICD1 or stimulation with Dll1 resulted in a marked increase in the size of CD34+CD1a- thymic precursors cultured with OP9 control stromal cells (Figure 4A). Expression of R1985A NICD1 elicited a similar increase in cell size as expression of wild-type NICD1 (Figure 4B). Furthermore, expression of R1985A NICD1 did not interfere with the increase in cell size induced by endogenous Notch signaling in thymocyte co-cultures with OP9 Dll1 (Figure 4B, right).

Marked proliferation of human CD34+CD1a- thymocytes was observed upon expression of wild-type NICD1 (Figure 4C, left) or co-culture with OP9 Dll1 (Figure 4C, right). R1985A NICD1 also stimulated proliferation of human thymocytes, albeit weaker than wild-type NICD1 (Figure 4C, left). Strikingly, however, R1985A, but not wild-type NICD1, inhibited proliferation induced by OP9 Dll1 in a dominant-negative fashion (Figure 4C, right).

Consistent with the stimulatory effects on proliferation, expression of wild-type NICD1 resulted in gradual enrichment of transduced (Thy1.1+) cells in
Figure 3: Dimerization mutant NICD1 inhibits growth of T6E cells, but rescues other murine T-ALL lines from γ-secretase inhibition. Rescue of murine T-ALL lines from γ-secretase inhibition. Cells were retrovirally transduced with MigRI plasmids encoding GFP alone (empty vector, grey) or GFP and wild-type (black) or R1985A NICD (dark grey), respectively, and cultured for up to five days in the presence of DAPT (10µM) or solvent (DMSO) only (Mock). (A) Cell cycle distribution of T6E cells was analyzed after three days of culture by staining with the DNA dye Draq5 (Biostatus). (B, C) Numbers of transduced T-ALL cells were calculated based on the percentage of transduced cells as determined by flow cytometry and absolute viable cell counts. Data are displayed as fold
expansion of numbers at the start of the cultures and represent at least three independent experiments performed in triplicates (mean + SD). (B) T6E cells. (C) T-ALL lines established from malignant Pten<sup>flox/flox</sup> x Lck-Cre thymocytes of wild-type (top) and Rag2<sup>−/−</sup> (bottom) mice.

unsorted, mixed populations. Enrichment (Figure 4D, left) and expansion (Figure 4E, left) of R1985A expressing thymocytes were weaker than those of cells transduced with wild-type NICD1, although this difference did not reach statistical significance. Furthermore, thymocytes expressing wild-type NICD1 were also slightly enriched (Figure 4D, right) and expanded better than empty vector transduced control cells when cultured with OP9 Dll1 cells (Figure 4E, right). In sharp contrast, however, thymocytes ectopically expressing R1985A NICD1 were selected against during co-culture with OP9 Dll1 (Figure 4D, right) and hardly expanded (Figure 4E, right), consistent with the dominant-negative effect observed on proliferation (Figure 4C). Collectively, these data demonstrate that induction of thymocyte expansion is partially dependent on homodimerization of NOTCH1.

Human T cell development does not require NOTCH1 homodimerization.

Notch has particularly well studied functions during multiple stages of T cell development, including commitment to the T cell lineage and progression to the CD4<sup>+</sup>CD8<sup>+</sup> stage during β-selection<sup>50,51</sup>. In mice, commitment to the T lineage from multipotent progenitors occurs independently of dimeric NOTCH1 signaling, while homodimerization is absolutely required for DN3 cells to successfully complete β-selection<sup>38</sup>. To study the role of dimeric NOTCH1 signaling in human T cell development, we initiated experiments with the earliest subset of human thymocytes. This CD34<sup>+</sup>CD1a<sup>−</sup> population<sup>57,58</sup> has not yet committed to the T cell lineage<sup>16,59</sup>. Many studies have shown that hematopoietic progenitors give rise to phenotypically and functionally mature T cells upon Notch activation during co-culture with OP9 cells<sup>52-56</sup>. In addition, this system can be used to study differentiation of ILC2. As we showed recently, constitutive expression of NICD1 induced prominent ILC2 differentiation in thymic progenitors, while weaker Notch signals preferentially induced T cell development<sup>32</sup>. Strikingly, substantially fewer CRTH2<sup>+</sup>IL-7-Rα<sup>+</sup> ILC2 were found upon expression of R1985A than after expression of wild-type NICD1 (Figure 5). Thus, homodimerization of NOTCH1 is required for optimal induction of ILC2 development.

Leakiness of the mER-NICD1 transgenes in the absence of Tamoxifen elicits low levels of Notch activity (see Figure 1B, left), which drive differentiation of T cells<sup>32</sup>. In humans, commitment to the T cell lineage is marked by expression of CD1a<sup>+</sup><sup>38</sup>, followed by expression of CD4<sup>+</sup>. Ectopic expression of
NOTCH1 homodimerization in human T cell development and T-ALL

A

OP9

OP9 Dll1

CD7

events [% of max]

empty vector

wild-type NICD1

R1985A NICD1

B

events [% of max]

empty vector

wild-type NICD1

R1985A NICD1

FSC

CellTrace Violet

C

events [% of max]

undivided

empty vector

wild-type NICD1

R1985A NICD1

D

OP9

OP9 Dll1

% transduced [fold of do]

1 week

2 weeks

empty vector

wild-type NICD1

R1985A NICD1

E

# transduced [fold of do]

1 week

2 weeks

empty vector

wild-type NICD1

R1985A NICD1
both wild-type and dimerization mutant mER-NICD1 resulted in appearance of a comparable CD4⁺CD1a⁺ population after one week of OP9 co-culture in the absence of Tamoxifen (Figure 6A). We conclude that, like in mice, Notch mediated T cell lineage specification from early thymocytes occurs independently of dimerization in humans.

Next, we addressed whether successful β-selection in humans depends on NOTCH1 homodimerization, a process which requires expression of a pre-TCR comprised of the TCRβ chain and a substitute α chain, known as the pre-T cell receptor α (pTα). After two weeks of OP9 co-culture in T cell promoting conditions, thymocytes transduced with mER-wild-type NICD1, but not empty vector transduced control cells, co-expressed TCRβ chains and CD3 intracellularly, a phenotype consistent with β-selected immature T cells (Figure 6B). Surprisingly, the same phenotype was observed in thymocytes ectopically expressing mER-R1985A. Furthermore, equal populations of CD4⁺CD8α⁺ DP T cells were obtained from thymocytes expressing mER-wild-type NICD1 or the dimerization mutant version, but not empty vector control cells (Figure 6C, 6D), demonstrating that Notch mediates human T cell development up to the early DP stage independently of dimerization.

Monomeric NOTCH1 induces sufficient levels of PTCRA to allow for successful β-selection.

A critical dimerization-dependent target gene of Notch in murine T cell development is ptcra, which encodes the α-chain of the pre TCR. As we found human β-selection not to be dependent on homodimerization of NOTCH1, we examined the human PTCRA locus. Expression of ptcra, a prerequisite to successful β-selection, is directly regulated by Notch via an
A NOTCH1 homodimerization in human T cell development and T-ALL

**Figure 5: Homodimerization is involved in NOTCH1 mediated ILC2 differentiation.** (A) Flow cytometric analysis of ILC2 differentiation from CD34+CD1a– thymocytes transduced with MSCV retroviruses encoding Thy1.1 alone (empty vector, left), or in combination with wild-type (middle) or R1985A NICD1 (right). Thymocytes were cultured on OP9 cells in the presence of IL-7 for one week. Cells were pre-gated as transduced (Thy1.1+) CD45-linearage (CD1a CD3-CD4 CD8 CD11c CD123 TCRαβ TCRγδ BDC2 FcεRI) and further analyzed for the expression of CRTH2 and CD127 (IL-7-Rα). ILC2 were defined as IL-7-Rα+CRTH2+. Percentages of cells within this gate are shown. Data are representative of four independent experiments. (B) Quantification of the fraction of ILC2 derived from thymocytes expressing empty vector Thy1.1 only (light grey bars) wild-type NICD1 (black bars) or R1985A NICD1 (dark grey bars). ILC2 were defined as in (A). Data were obtained from four experiments and displayed as mean ± SD. Statistical significance was analyzed using the Mann-Whitney test. *p<0.05.

upstream enhancer, which is necessary and sufficient for expression of a functional pTα chain. The core enhancer region contains a consensus CSL binding site and a divergent low-affinity binding site in reverse orientation, which is separated from the forward site by a spacer of 16 base pair length (Figure 7A). As such, the ptcra enhancer contains a bona fide SPS motif, which has been confirmed biochemically. In line with this, R1985A fails to induce ptcra expression in mouse DN3 thymocytes, and cells expressing this mutant are blocked at the β-selection checkpoint.
Figure 6: Human T cell development does not require NOTCH1 dimerization. Human CD34+CD1a- thymic progenitors were transduced as indicated and co-cultured with OP9 cells in the presence of IL-7 and Flt3l for up to two weeks. (A) Thymocytes expressing Thy1.1 alone (empty vector, left), or in combination with mER-wild-type (middle) or mER-R1985A NICD1 (right) were analyzed for the expression of CD4 and CD1a by flow cytometry after one week of co-culture. Percentages of the CD4+CD1a+ populations are displayed. Data are representative of two individual experiments. (B) Flow cytometric analysis of intracellular expression of CD3 and TCRβ by thymocytes transduced with the indicated retroviruses and cultured for two weeks. Numbers correspond to icCD3+icTCRβ+ cells. One representative of two experiments is shown. (C, D) Analysis of the surface expression of CD4 and CD8α after two weeks of co-culture. (C) FACS plots from one representative of three individual experiments. Percentages of CD4+CD8α+ cells are shown. (D) Quantification of the fraction of CD4+CD8α+ DP T cells obtained from co-cultures initiated with Thy1.1+ thymocytes expressing mER-wild-type NICD1 (black), mER-R1985A NICD1 (dark grey) or Thy1.1 only (light grey). Data are shown as mean + SD and were analyzed for statistical significance using the Mann-Whitney test. n = 3. *p<0.05.
Importantly, however, the human PTCRA enhancer contains an additional consensus CSL site which is located downstream of the SPS motif and is lacking in the murine enhancer\cite{43,62} (Figure 7A).

![Figure 7A](image)

**Figure 7:** Monomeric NOTCH1 sufficiently induces expression of PTCRA to allow for successful β-selection. (A) Sequence of the human pTα enhancer. The SPS motif as described by Liu and colleagues\cite{38} is shown in blue, the additional downstream CSL binding site specific for humans is shown in red. Sequences were retrieved from the UCSC genome browser\cite{62,66}. (B) Simulation of simultaneous binding of a dimeric and a monomeric NTC to a model of a human pTα enhancer element. The high-resolution structure of dimeric NTC bound to the HES1 promoter sequence was used as a template (PDB ID: 3nbn)\cite{37}. An additional CSL binding site (shown in red) was introduced in 4bp distance to the SPS motif (blue). A monomeric NTC was structurally aligned to the introduced downstream CSL site. PyMol software (Schrödinger) was used for molecular modeling. Orange: CSL, light-orange: NOTCH1 ANK, light-yellow: MAML1, grey: DNA. (C) Activation of a luciferase reporter under control of the human pTα enhancer. The reporter constructs used contained either the wild-type sequence (left) or an enhancer where both forward CSL binding sites were mutated (right)\cite{43}. Reporter assays were performed as described in Figure 1. Induction by wild-type (black) or R1985A NICD1 (dark grey) is shown as fold of empty vector controls (mean ± SD) and representative of three experiments.
This suggests that, unlike previously proposed, human \textit{PTCRA} could in fact be activated by a combination of dimeric and monomeric NTCs. Notably, the CSL binding site specific to the human enhancer was not included in earlier biochemical analyses\textsuperscript{38}. This additional CSL binding site is only 4 base pairs downstream of the SPS motif (Figure 7A). To analyze whether this close proximity allows for simultaneous binding of a dimeric and monomeric NTC, we modeled loading of these complexes on a synthetic DNA element (Figure 7B). Our model was based on the high-resolution structure of a dimeric NTC bound to the SPS motif of the HES1 promoter\textsuperscript{37} (PDB ID: 3nbn). We extended the DNA fragment by 13 base pairs to introduce the downstream CSL binding site found in the human \textit{PTCRA} promoter. We then structurally aligned an additional, monomeric NTC to this CSL site. The model predicted that this monomeric NTC faces away from the SPS motif. Thus, the SPS motif and the additional CSL binding site can presumably be simultaneously occupied by dimeric and monomeric complexes, respectively, allowing for contribution of both signals to activation of human \textit{PTCRA}.

Consequently, induction of human \textit{PTCRA} by Notch might not exclusively depend on homodimerization. We further addressed this experimentally by revisiting the capacity of dimerization mutant NICD1 to activate a human pTα-luciferase reporter\textsuperscript{43}, which contains the additional CSL binding site. As predicted by our structural model, R1985A NICD1 was capable of activating the human pTα reporter, albeit somewhat weaker than wild-type NICD1 (Figure 7C, left). Activation of the pTα reporter by wild-type and R1985A NICD1 was abrogated when both high-affinity CSL binding sites were mutated\textsuperscript{43} (Figure 7C, right). Thus, monomeric Notch signaling indeed functionally contributes to pTα induction. These findings strongly support a model in which activation of human \textit{PTCRA} by Notch does not strictly depend on dimerization, and thus provide a rationale why human T cell development does not depend on dimerization.

\section*{Discussion}

Notch is a major oncogene and therefore an attractive therapeutic target, especially for the treatment of T-ALL\textsuperscript{27,63–65}. However, the abundance of its physiological functions limits its therapeutic usefulness, due to the severe side effects of interfering with general Notch signaling\textsuperscript{23,24}. The pathway would be much more amenable to therapeutic exploitation if oncogenic functions of Notch could be separated biochemically from (most) general functions. The recent identification of Notch homodimerization as an important property for oncogenesis and growth of murine T-ALL thus represented a major opportunity\textsuperscript{38}. For this reason, we examined whether the requirement for homodimerization of Notch is a conserved feature in humans and whether this property also controls growth of human T-ALL. We found that homodimerization of Notch serves important functions during human
hematopoiesis: optimal induction of ILC2 differentiation and proliferation of CD34^+CD1a^- thymic progenitors both require homodimerization. On the other hand, differentiation along the T cell lineage appears to be much less dependent on this signaling mode than its murine equivalent. Importantly, we failed to document a requirement for Notch homodimerization in expansion of T-ALL. The fact that an NICD1 mutant incapable of dimerization was fully able to promote the growth of all tested human T-ALL and additional murine lines demonstrates that T-ALL dependence on dimerization is an exception rather than the rule. Having only used established T-ALL lines for our studies, we cannot exclude the possibility that a requirement for homodimerization of Notch would be much more prominent in primary T-ALL \textit{in vivo}. Nonetheless, the widespread independence in these established lines suggests that mechanisms to escape reliance on homodimerization can rather easily be mobilized. On the basis of these data, drugs preventing homodimerization of Notch cannot be expected to possess durable therapeutic potency, unfortunately, at least for the treatment of T-ALL.

Our results do not exclude a role for homodimerization in initial transformation of human T-ALL. In contrast to wild-type NICD1, a dimerization mutant NICD1 only weakly stimulates expansion of thymic progenitors. Importantly, this mutant actually inhibits expansion of such cells when combined with endogenous Notch activation (elicited by stimulation with a Notch ligand), arguably the physiological context for oncogenic transformation by Notch. In mice, \textit{c-myc} and \textit{ptcra} were identified as important dimerization dependent Notch targets required for expansion\textsuperscript{38}. The residual ability of NICD monomers to transactivate the human PTRCA enhancer may explain why dimerization mutant NICD still weakly promotes expansion of human progenitors. However, inability to activate transcription of such growth promoting factors seems unlikely to also explain growth inhibition by the mutant in the context of endogenous Notch signaling (see Figure 4D). One possible explanation invokes the existence of mutually antagonistic pathways downstream of Notch as well as a role for Notch signal strength. Indeed, our results are consistent with the notion that strong Notch signaling, which almost by definition reflects oncogenic activation, activates growth inhibitory mechanisms (RG and DA, unpublished observations). We propose that the growth inhibitory effects of high NICD levels predominantly stem from monomers, whereas dimers activate mechanisms that counteract these growth inhibitory mechanisms. It stands to reason that simultaneous expression of dimerization mutant NICD1 and stimulation with Dll1 results in greater levels of NICD monomers than those induced by either manipulation alone. We hypothesize that those levels exceed the threshold for activation of the growth suppressive program. On the other hand, the ability of dimerization mutant NICD to inhibit dimerization by endogenous Notch would block induction of the (dimer dependent) positive program. The fact that most T-ALL lines (in which endogenous Notch is also active) have developed independence of dimeric Notch complexes may underscore the importance of homodimerization targets for transformation. Further
studies to determine the identity of these targets is therefore of great interest, especially in the light of possible therapeutic exploitation of this signaling event.

Material and Methods

Cell lines, culture conditions and GSI treatment. The human T-ALL lines DND41 and HBPALL were purchased from the DSMZ (German Collection of Microorganisms and Cell Cultures). TALL1 was provided by Jon Aster. PTEN deficient murine T-ALL lines were derived from malignant $Pten^{flx/flx}$ x $Lck-Cre$ thymocytes from wild-type, $Rag2^{-/-}$ or $γc^{-/-}$ mice\(^4\). Murine T6E cells were a gift from Warren Pear. T-ALL lines were cultured in RPMI supplemented with FCS (10%), GlutaMax (2mM), penicillin (100 U/ml), streptomycin (100 µg/ml), Sodium Pyruvate (1mM) and non-essential amino acids (for T6E, all Gibco). The viral packaging cell lines Phoenix GALV and PlatE were cultured in IMDM supplemented with FCS (10%), GlutaMax (2mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) (all Gibco). OP9 cells were grown in MEMα (Gibco) containing FCS (20%, FetalClone I, HyClone) and GlutaMax (2mM, Gibco).

T-ALL cells were incubated with the GSI DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, Sigma) at the indicated concentrations. The solvent DMSO (Dimethyl sulfoxide, Sigma) served as a negative control (Mock). Cultures were refreshed every 3 to 4 days. A Casy Counter (Innovatis, Roche) was used to determine viable cell counts for cell lines and thymocytes.

Retroviral expression constructs. The human NICD1-IRES-Thy1.1-MSCV\(^{29}\) and mER-NICD1-IRES-Thy1.1-MSCV\(^{32}\) have been described before. R1985A NICD1-IRES-Thy1.1-MSCV was generated by exchanging a HincII flanked fragment in wild-type NICD1-IRES-Thy1.1-MSCV (spanning nucleotides 5597 to 6066 of human NOTCH1) for the corresponding fragment from a construct encoding the R1985A mutant RAMANK domain of NOTCH1 (R1985A RAMANK-pGEX-4T1\(^{36}\), a kind gift from Stephen Blacklow). mER-R1985A NICD1-IRES-Thy1.1-MSCV was created as described for wild-type\(^32\). Briefly, an N-terminal mER domain was PCR amplified and cloned into pBluescript (pBS) to generate mER-pBS. R1985A NICD1 lacking a translation initiation signal was PCR amplified using R1985A NICD1-IRES-Thy1.1-MSCV as template. The product was subsequently ligated into mER-pBS. The mER-R1985A NICD1 fusion insert was then cloned into IRES-Thy1.1-MSCV. In our constructs, NICD1 refers to residues R1758 to K2555 of human NOTCH1.

Virus production and transduction. Transient transfection of the Phoenix GALV or PlatE packaging cell lines was used to produce high-titer viruses.
for infection of human or murine cells, respectively. Transfections were
done using the FuGene HD (Promega) transfection reagent according to the
manufacturer’s instructions. Virus containing supernatants were harvested
48 hours after transfection, snap frozen on dry ice and stored at -80°C until
use. For transduction, primary human thymocytes were incubated with virus
supernatant in plates coated with Retronectin (30μg/ml, Takara Biomedicals)
for 6 to 8 hours at 37°C. T-ALL lines were transduced by spin infection for 90
minutes at 32°C and 2200 rpm in a table-top centrifuge (Hettich), followed
by an additional incubation of 4.5 to 6.5 hours at 37°C. Transductions of T-
ALL cells were performed in the presence of 8μg/ml Polybrene (Sigma).
Transduction efficiencies were measured by flow-cytometry 48 hours post
transduction.

Flow Cytometry. Viable cells were distinguished from dead cells based
on stainings with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride,
Invitrogen), 7-AAD (7-Aminoactinomycin D, eBiosciences) or fixable live/
dead dyes (Invitrogen). Cell surface stainings were performed at 4°C for 20-30
minutes. For intracellular stainings, the Fix & Perm kit (Life Technologies) was
used as instructed by the manufacturer. As a readout for cell cycle progression,
the DNA dye Draq5 (Biostatus) was at a final concentration of 10μM. To
determine cell divisions directly, cells were labeled with a CellTrace Violet
dye (Invitrogen) according to the manufacturer’s instructions. Single cell
suspensions were stained with antibodies directly labeled with Fluorescein
Isothiocyanate (FITC), Phycoerythrin (PE), Phycoerythrin-Cyanine 5 (PE-
Cy5), PE-Cy5.5, PE-Cy7, PerCP-Cy5.5, Allophycocyanin (APC)/Alexa Fluor
647, APC-Cy7, AF700 (BD Bioscience, Biolegend or MACS Miltenyi), Horizon
V500 (HV500, BD Bioscience), Brilliant Violet (BV) 421, BV711 and BV785 (all
Biolegend). Antibodies specific for the following human antigens were used:
CD1a, CD3, CD4, CD7, CD8α, CD11c, CD14, CD19, CD25, CD34, CD45,
CD94, CD123, CD127 (IL-7R-α), CD294 (CRTH2), CD303 (BDCA2), TCRαβ,
TCRγδ and FcεR1. Cells successfully transduced with MSCV-IRESThy1.1
retroviruses were detected by staining with anti-mouse CD90.1 (Thy1.1) -
FITC, -PE or -APC-eFluor 780 (eBioscience) antibodies. Data were acquired
on LSR Fortessa or CantoII flow cytometers (BD Bioscience) and analyzed
with FlowJo software (TreeStar).

Isolation of thymic hematopoietic progenitors. Human postnatal thymus
(PNT) specimens were obtained from children undergoing open heart surgery
at the LUMC (Leiden, the Netherlands). Experimental use was approved
by the AMC ethical committee according to the declaration of Helsinki.
Tissues were mechanically disrupted using the Stomacher 80 Biomaster
(Seward) to obtain cell suspensions, which were incubated overnight at 4°C.
Viable cells were isolated from a Ficoll-Hypaque (Lymphoprep; Nycomed
Pharma) density gradient. Thymocytes were enriched for CD34+ cells by
MACS (Miltenyi Biotec), stained with fluorescently labeled antibodies and
subsequently sorted by flow cytometry as CD34+CD1a CD3 CD56 BDCA2+
(referred to in this study as CD34+CD1a) to > 99% purity. Cell sorts were
performed on a FACS Aria instrument (BD Bioscience).

**In vitro differentiation of thymic progenitors.** After sort, thymic progenitors were cultured overnight in Yssel’s medium containing 5% normal human serum, SCF (20ng/ml) and IL-7 (10ng/ml, both PeproTech). OP9 cells were seeded at a density of 5x10^3/cm^2 one day before initiation of co-cultures. Thymocytes were added to pre-seeded OP9 cells following transduction. Co-cultures of thymocytes and OP9 cells were performed in MEMα (Invitrogen) containing FCS (20%, FetalClone I, Hyclone) and IL-7 (5ng/ml) or IL-7 and Flt3l (5ng/ml, PeproTech). Cultures were refreshed every 3 to 4 days. Cells were harvested by forceful pipetting, passed through 70μm nylon mesh filters (Spectrum Labs) and analyzed at different time points. For longer incubation periods, cells were transferred to fresh OP9 cells every week.

**Reporter gene assays.** Transient transfection of U2OS cells was performed with the FuGene HD transfection reagent (Promega). Cells were co-transfected with a Notch responsive reporter construct and retroviral plasmids encoding different versions of wild-type and/or R1985A NICD1 as indicated in the figures and text. Transfections were performed in triplicate. Differences in transfection efficiency were corrected for by co-transfection of the pRL-CMV control vector, from which Renilla luciferase is expressed constitutively. Relative Firefly luciferase activities were normalized to the respective empty vector controls and all data are displayed as fold of these controls. Where applicable, 4-Hydroxy-Tamoxifen (Sigma) was added after overnight incubation to induce nuclear translocation of mER-NICD1. Cells were lysed 48h post transfection and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) on a Synergy HT microplate reader (Syntek). The Notch responsive reporter constructs HES1-luciferase\(^{41}\), CBF1-luciferase\(^{42}\) and human pTα-luciferase (wild-type and with mutated CSL binding sites)\(^{43}\) have been described before.

**Molecular modeling.** Simultaneous loading of a dimeric and monomeric NTC onto a DNA fragment resembling the human pTα enhancer was modeled using PyMol software (De Lano Scientific). The structure of a dimeric NTC on a DNA fragment from the HES1 promoter (PDB ID: 3nbn)\(^{37}\) was used as template. The model was built in four steps: The initial thymidine was deleted from the 5’ end of the DNA due to geometric distortions. The DNA structure was manually extrapolated by adding a copy of a truncated DNA molecule to the model. To ensure correct geometry and connectivity of the newly created linker region a structure idealization tool was used (Refmac, CCP4i software package\(^{44}\)). The resulting DNA sequence in our model is 5’-ACTGTGGGAAGAAGATTTGGAATTTCCAGAGctttggaaaga-3’.
(bold: CSL binding regions, capital letters: original sequence present in 3nbn, minuscle: additional nucleotides introduced in our model). Finally, the NTC binding to the forward site of the SPS motif was copied and structurally aligned to the introduced downstream CSL binding site.
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