

Universidade de Lisboa
Faculdade de Medicina de Lisboa



The role of NRARP in the pathogenesis of T-cell Acute Lymphoblastic Leukemia

Inês Garcia Brandão e Silva Pinto

Orientador: Doutora Ana Rita Matos Fragoso

Coorientador: Prof. Doutor João Taborda Barata

Dissertação elaborada para obtenção do Grau de Mestre em Oncobiologia

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Resumo

A leucemia linfoblástica aguda de células T (LLA-T) é uma neoplasia hematológica severa, resultante da expansão clonal de progenitores linfóides de células T, representando cerca de 15% dos casos de adultos e 25% dos casos pediátricos de leucemia linfoblástica aguda (LLA). Apesar do prognóstico de doentes com LLA-T ter melhorado significativamente ao longo dos anos, a taxa de recidiva é ainda elevada e o prognóstico dos doentes com doença resistente à terapia ou recidivante é muito reservado.

A via de sinalização NOTCH é fundamental para a regulação de processos de homeostasia e regeneração de tecidos e desempenha funções muito importantes na hematopoiese. O compromisso de progenitores linfóides para com a linhagem T e os passos iniciais do desenvolvimento de células T no interior do timo são processos regulados pela sinalização da via NOTCH. Desta forma, não é de estranhar que a sua desregulação seja uma característica da patogénese de LLA-T, e que mutações em *NOTCH*, levando a uma activação constitutiva da via, se verifiquem em mais de 50% dos casos. A identificação destas mutações levou ao desenvolvimento de terapias dirigidas para esta via. A inibição da via NOTCH foi alcançada essencialmente pelo uso de inibidores da γ -secretase. Estes inibem a via NOTCH ao bloquearem a clivagem final do receptor NOTCH, impedindo desta forma que a sua forma activa seja translocada para o núcleo e, conseqüentemente, que actue como factor de transcrição. No entanto, verificou-se posteriormente que a sua utilização terapêutica tem efeitos anti-leucémicos reduzidos, associados a efeitos secundários graves, a nível intestinal. Outras abordagens terapêuticas, tais como a utilização de anticorpos que têm como alvo receptores e ligandos da via NOTCH, foram desenvolvidas, mas também estas apresentam efeitos secundários severos, tais como a indução de tumores vasculares e toxicidade hepática. Desta forma, uma melhor compreensão dos mecanismos envolvidos na sinalização via NOTCH em LLA-T poderá originar terapias mais eficazes e seguras.

Recentemente, foi demonstrado que a perda de expressão do microRNA miR-181ab1 inibe o desenvolvimento de LLA-T induzido pela activação de NOTCH1, em parte devido ao aumento da expressão de *NRARP*. Este último é um alvo transcricional da via de sinalização NOTCH e um regulador negativo da mesma. É também importante realçar que a sobre-expressão deste gene em células estaminais hematopoiéticas de ratinho compromete a especificação de células na linhagem T e a sua posterior diferenciação em timócitos. Igualmente importante é o facto de ter sido demonstrado que *NRARP* está associado à via de sinalização WNT através da estabilização do factor de transcrição LEF1, capaz de induzir transformação maligna de timócitos de ratinho. Desta forma, a desregulação da função de *NRARP* pode desempenhar um papel na patogénese de LLA-T. Assim, o principal objectivo da presente dissertação é estudar o papel de *NRARP* no desenvolvimento desta doença.

Começámos por caracterizar a expressão de NRARP em células de LLA-T, quantificando para tal os níveis de expressão de mRNA e proteína de NRARP em linhas celulares e amostras primárias. Além disso, para investigar a sua função nestas células, NRARP foi sobre-expresso em algumas linhas celulares de LLA-T, por transdução lentiviral. Estas células foram usadas para avaliar os efeitos de NRARP na sinalização de NOTCH, através da quantificação da expressão da forma intracelular de NOTCH, bem como de alvos a jusante desta via. Foram ainda executados ensaios funcionais nas mesmas células, nomeadamente ensaios de proliferação e viabilidade, bem como de progressão do ciclo celular.

Verificámos que os níveis de proteína de NRARP estão significativamente mais elevados em células de LLA-T, comparando com tímócitos saudáveis. Este resultado é consistente com o facto de *NRARP* ser um alvo transcricional da via NOTCH, comumente hiper-activado num contexto de LLA-T. Por outro lado, sendo NRARP um regulador negativo da via NOTCH, estes resultados sugerem que os níveis de NRARP não são suficientes para bloquear os sinais oncogénicos produzidos por esta via. Para testar essa hipótese, procedemos à sobre-expressão de NRARP em linhas celulares humanas de LLA-T e analisámos os efeitos na via NOTCH. No geral, observámos que a sobre-expressão de NRARP suprime a via NOTCH, resultando não só numa diminuição dos níveis da forma intracelular e activa de NOTCH, bem como no decréscimo da maioria dos alvos transcricionais analisados. Contudo, é importante realçar que os níveis do oncogene *cMYC*, um importante alvo desta via, estavam diminuídos num grupo de linhas celulares, mas aumentados num outro grupo.

Curiosamente, a análise funcional da sobre-expressão de NRARP revelou que, apesar da via de sinalização NOTCH estar bloqueada, em algumas linhas celulares NRARP inibe a proliferação, mas noutras promove a sua expansão. A análise da viabilidade e do ciclo celular, demonstrou que a sobre-expressão de NRARP não tem um impacto significativo a nível da viabilidade na maioria das linhas celulares analisadas, mas afecta a progressão do ciclo celular. Estes resultados são consistentes com as observações a nível proliferativo e com as diferenças observadas na expressão de *cMYC*. Ou seja, nas linhas celulares em que a sobre-expressão de NRARP induz uma diminuição na proliferação, observámos atrasos na progressão do ciclo celular e uma diminuição da expressão de *cMYC*. Nas linhas celulares em que a sobre-expressão de NRARP resulta num aumento da proliferação, verificámos uma progressão mais rápida no ciclo celular e um aumento na expressão de *cMYC*.

Uma observação interessante é o facto de as linhas celulares cujo crescimento é diminuído por NRARP apresentarem mutações em *NOTCH*, enquanto as linhas cujo crescimento é favorecido por NRARP, não apresentarem mutações neste gene.

Com base nestes resultados, formulámos a hipótese de que a sobre-expressão de NRARP, para além de bloquear a via de sinalização NOTCH, pode afectar directa ou indirectamente outra(s) via(s).

Uma vez que NRARP regula LEF1, um factor de transcrição da via WNT, investigámos os efeitos da sobre-expressão de NRARP na via de sinalização WNT. Ainda que bastante preliminares, os nossos resultados sugerem uma diminuição na activação da via WNT nas linhas celulares com um crescimento mais lento. Curiosamente, observámos ainda nestas linhas uma ligação entre NRARP e uma isoforma de LEF1, descrita como tendo um papel negativo na via WNT. Por outro lado, nas linhas celulares com crescimento mais rápido, observámos a interacção de NRARP com outra isoforma de LEF1, possivelmente a isoforma *full-length*. Contudo, é importante ressaltar que estes resultados são preliminares e necessitam de ser validados.

Em suma, os resultados obtidos sugerem que NRARP pode desempenhar funções opostas em LLA-T. Apesar de NRARP ser capaz de bloquear a via NOTCH em todas as linhas celulares analisadas, em células que não dependam de sinais oncogénicos produzidos por NOTCH (*NOTCH* wild-type), NRARP poderá afectar outra via de sinalização, tendo um papel oncogénico. Em células dependentes dos sinais oncogénicos de NOTCH (*NOTCH* mutado), NRARP terá um papel de supressor tumoral. A importância da via WNT e o envolvimento de LEF1 nesta dualidade na função de NRARP requer estudos mais extensivos.

Palavras-chave

Leucemia Linfoblástica Aguda de Células T (LLA-T); Via de sinalização NOTCH; Via de sinalização WNT; NRARP; LEF1

Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is a severe hematologic malignancy, commonly associated with high frequency of relapse. NOTCH signaling is a hallmark of T-ALL pathogenesis and more than 50% of T-ALL cases harbor *NOTCH* activating mutations. Despite considerable clinical advances throughout the years, NOTCH signaling inhibition cannot be efficiently achieved, due to weak therapeutic effects and severe toxicity.

NRARP is a negative regulator of the NOTCH pathway and a transcriptional target of NOTCH. It is known to regulate both T-cell lineage commitment and early differentiation. Importantly it has been shown that in NOTCH-induced T-ALL, loss of the microRNA181ab1 inhibits T-ALL development, partly by de-repressing *NRARP*. NRARP further modulates WNT signaling, by stabilizing LEF1, a transcription factor acting downstream of WNT pathway, shown to induce the malignant transformation of murine thymocytes. Altogether, these findings suggest that a deregulation of NRARP function may play a role in T-ALL. Thus, the main goal of this project is to understand the role of NRARP in T-ALL pathogenesis and evaluate its therapeutic potential.

We started by determining NRARP expression in human T-ALL cells. We observed that NRARP protein levels are significantly increased in T-ALL comparing with human thymocytes. This result, although consistent with the fact that NRARP is a transcriptional target of NOTCH, suggests that NRARP is not sufficient to block NOTCH oncogenic signals. To test this hypothesis, we overexpressed NRARP in human T-ALL cell lines. Intriguingly, even though we observed a block in NOTCH signaling in all cell lines analyzed, functional studies revealed that while some T-ALL cells proliferate less upon NRARP overexpression, some proliferate more. Importantly, this suppression or promotion of cell expansion correlated with the changes observed in cell cycle progression (delayed or accelerated, respectively) and *cMYC* expression (downregulated or upregulated, respectively) in NRARP overexpressing cells and, curiously, with the mutational status of NOTCH (mutated or wild-type, respectively).

Altogether, our results suggest that NRARP is capable of blocking NOTCH signaling independently of *NOTCH* mutational status. Nonetheless, in T-ALL cells that depend on NOTCH oncogenic signals (*NOTCH* mutated cells), NRARP overexpression has a “tumor-suppressor”-like function, whereas in leukemia cells that do not depend on NOTCH oncogenic signals (*NOTCH* wild-type) it plays an oncogene-like role. Further studies are necessary to understand if WNT signaling contributes to this dual role of NRARP in T-ALL.

Keywords

T-Cell Acute Lymphoblastic Leukemia (T-ALL); NOTCH signaling; WNT signaling; NRARP; LEF1

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7AAD: 7-Aminoactinomycin D

AEBSF: 4-(2-Aminoethyl) Enzenesulfonyl Fluoride Hydrochloride

ALL: Acute Lymphoblastic Leukemia

AXIN2: Axis inhibition protein 2

BM: Bone Marrow

BSA: Bovine Serum Albumin

CB: Cord Blood

CD: Cluster of differentiation

CMJ: Cortico-medullary junction

DN: Double negative

DNA: Deoxyribonucleic Acid

dNTP: deoxyribonucleotide

DMSO: Dimethyl sulfoxide

DP: Double Positive

ECD: Extracellular domain (of NOTCH)

EDTA: Ethylenediamine tetraacetic acid

FACS: Fluorescence Activated Cell Sorting

FBS: Fetal Bovine Serum

GFP: Green Fluorescent Protein

GSI: Gamma-secretase inhibitor

HBSS: Hank's Balanced Salt Solution

HD: Heterodimerization domain (of NOTCH)

HEPES: 4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid

HES: Hairy and Enhancer of Split 1

HSC: Hematopoietic Stem Cells

JME: Juxtamembrane expansion

KD: Knockdown

LEF1: Lymphoid enhancer-binding factor-1

MYC: Myelocytomatosis Viral Oncogene Homolog

NaCl: Sodium chloride

NaOV: Sodium Orthovanadate

NICD/ICD: NOTCH Intracellular Domain/Intracellular Domain

NRARP: NOTCH-regulated ankyrin repeat protein

OE: Overexpression

PBS: Phosphate Buffered Saline

PI: Propidium iodide

PI3K-Akt: phosphoinositide-3-kinase–protein kinase B/Protein kinase B

PTEN: Phosphatase and tensin homolog

RNA: Ribonucleic Acid

RT-qPCR: Real Time Quantitative Polymerase Chain Reaction

SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SKP2: S-phase kinase-associated protein 2

T-ALL: T-cell Acute Lymphoblastic Leukemia

TAN1: Translocation-associated NOTCH homolog

TBS: Tris Buffered Saline

TCF: T Cell Factor

TCR: T Cell Receptor

TEC: Thymic epithelial cells

WB: Western Blot

WT: Wild-type

1. Introduction

Abridging T-Cell Development

Constant input from thymic microenvironment is fundamental for supporting and guiding T cell development from hematopoietic stem cells (HSC) (Petrie & Zúñiga-Pflücker, 2007). Committed hematopoietic precursors from adult bone marrow (BM) are continuously recruited to the thymus, where T-lineage specification and further development take place (Koch & Radtke, 2011).

The thymus is divided into four regions, which guide different stages of T cell maturation. T cells travel throughout the adult thymus, in a highly coordinated migration process (Figure 1.1) and can be distinguished by alterations in several cell-surface markers' expression (Shah & Zúñiga-Pflücker, 2014). Thymic epithelial cells (TEC) play key roles in T cell development and different subsets of these cells are responsible for helping different phases of T cell maturation, contributing to positive and negative selection of T cells (Klein et al., 2014).

As hematopoietic precursors migrate towards the thymus, they become more restricted in their fate options. Only a small number of precursors enter the thymus, through the cortico-medullary junction (CMJ) (Lind et al., 2001). These early thymic progenitors generate a double-negative (DN) CD4⁻ CD8⁻ population that gives rise to $\gamma\delta$ or $\alpha\beta$ TCR-expressing cells (Starr et al., 2003). DN cells development occurs over four stages, DN1 through DN4, distinguished by the differential expression of CD25, CD44 and CD117 (Koch & Radtke, 2011). DN1 cells proliferate in the CMJ (process promoted by NOTCH1-mediated signals) (Zúñiga-Pflücker, 2004) and then migrate to the cortex *en route* to the subcapsular zone where they differentiate into DN2 (Germain, 2002). These cells start the rearrangement of the *TCR* gene loci (Ciofani & Zúñiga-Pflücker, 2007) and the ones that succeed TCR β rearrangement further proceed beyond DN3 stage (β -selection) (Takahama, 2006), a process mediated by NOTCH3 signals, for example (Bellavia et al., 2002). The cells then rearrange the TCR α locus to give rise to a functional $\alpha\beta$ T cell receptor (TCR) (Starr et al., 2003). Past DN3 stage, DN4 cells migrate back towards the medulla, upregulating CD4 and CD8, and giving rise to double positive (DP) $\alpha\beta$ TCR expressing cells (CD4⁺CD8⁺) (Germain, 2002). These undergo positive and negative selection, originating ultimately mature thymocytes that proceed to circulation (Takahama, 2006).

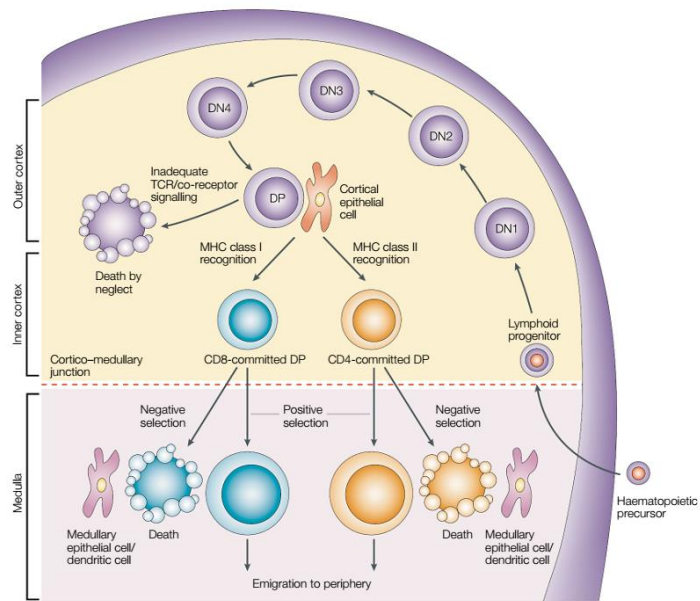


Figure 1.1 - Schematic overview of T-cell development in the thymus (from Germain *et al.*, 2002)

T-Cell Acute Lymphoblastic Leukemia

Acute Lymphoblastic Leukemia (ALL) refers to a set of malignant disorders originated from clonal expansion of lymphoid progenitors at different developmental stages. ALL is the most common leukemia among children, accounting for about 80% of childhood leukemias and 20% of adult leukemias (Durinck *et al.*, 2015; Fullmer *et al.*, 2009). It is the leading cause of death for patients under 20 years and has a peak of incidence between 3 and 5 years of age. An increased risk of ALL can be associated with genetic aspects but usually it is not related to inherited factors (Hunger & Mullighan, 2015).

When malignant transformation occurs in T-cell progenitors, it originates an arrest at the early stages of T cell development. This form of ALL is referred to as T-Cell Acute Lymphoblastic Leukemia (T-ALL). T-ALL covers 10%-15% of pediatric and 25% of adult ALL cases and patients usually manifest bone marrow infiltration with immature T cell lymphoblasts, high white blood cell counts and, frequently, infiltration of the central nervous system at time of diagnosis (Van Vlierberghe *et al.*, 2012).

T-cell malignant transformation involves the occurrence of multi-step genetic alterations that can cooperate leading to the malfunction of the normal pathways that regulate cell proliferation, survival or cell cycle during thymocyte development. In this context, there are some oncogenic hallmarks associated with T-ALL, being the most meaningful, the alterations in *NOTCH* genes. For example, *NOTCH3* is found overexpressed in almost all T-ALL cases (Bellavia *et al.*, 2002) and genetic alterations in *NOTCH1* such as translocations or mutations, which lead to the constitutive activation of NOTCH signaling, contribute to the onset and development of T-ALL (Ferrando *et al.*, 2002; Weng *et al.*, 2004).

Moreover, gene expression alterations in specific T-ALL transcription factors and their aberrant activation can lead to malignant transformation of T cells. These alterations can involve chromosomal abnormalities or abnormal genetic expression due to mutations. The chromosomal rearrangements normally place T-ALL-associated transcription factors under the control of strong TCR promoters or enhancers, resulting in their aberrant expression. Among those oncogenic factors are the basic helix-loop-helix (bHLH) family members such as TAL1, LMO family members and MYC or HOXA homeobox (HOX) genes (Ferrando et al., 2002; Van Vlierberghe et al., 2012). In addition, gain-of-function/loss-of-function mutations in oncogenes/tumor suppressor genes, respectively, can also promote T cell transformation. These comprise activating mutations in genes such as *JAK1/3* or IL7 receptor (*IL7R*) or inactivating mutations in *PTEN* or *GATA3* genes (Durinck et al., 2015).

Currently, the prevailing treatment of T-ALL involves multi-drug chemotherapy regimens, with specific sequences of dose and time intensity, promoting the reconstitution of normal hematopoiesis and preventing the emergence of resistant clones. This allowed T-ALL cure rates to improve dramatically over the last decades (Fullmer et al., 2009) with 75% of children and 50% of adult T-ALL cases (Oudot et al., 2008) now considered curable. Nonetheless, despite the advances in the development of T-ALL targeted therapies, it is associated with a high rate of relapse, often related to chemotherapy resistance, having these patients a worse prognosis (Bhojwani & Pui, 2013). Furthermore, current chemotherapy regimens can lead to long term severe complications (Roti & Stegmaier, 2014). Therefore, the development of more efficient and less toxic drugs is urgent and a deep knowledge of the molecular mechanisms involved in T-ALL pathogenesis is required.

NOTCH signaling: Central pathway in development

The NOTCH signaling pathway is critical to regulate normal embryonic development as well as adult tissue renewal and homeostasis through the regulation of cell fate decisions and cellular processes, such as differentiation, proliferation and apoptosis in different cellular contexts (D'Souza et al., 2010). The basis for NOTCH signaling activation is cell-to-cell interaction, promoting the link between the fate of one cell to its neighbor. Consequently, it must be able to generate appropriate signaling outputs in a variety of tissues (Andersson et al., 2011). Activation of the pathway can be obtained in a canonical or non-canonical manner (Guruharsha et al., 2012). For the purposes of this dissertation we will only refer to the canonical cascade (Figure 1.2).

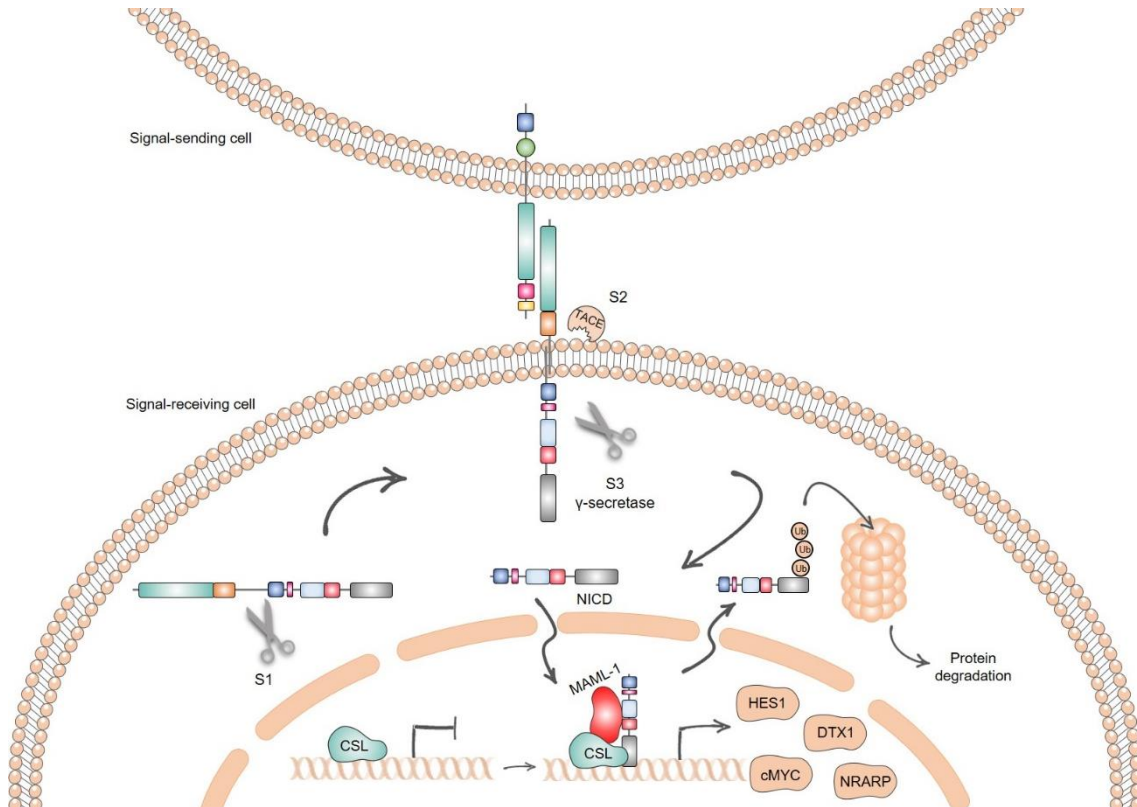


Figure 1.2 - Schematic representation of the proteolytic processing of NOTCH receptor and signal transduction of the core NOTCH pathway

The key players in NOTCH signaling are the NOTCH ligands and receptors. There are five known type I transmembrane NOTCH ligands: Jagged 1 and 2 (JAG1/2) and Delta-like 1, 3 and 4 (DII1/3/4), often referred to as DSL proteins. Jagged and Delta-like ligands differ in the presence of a C-rich domain and, in some cases, of a DOS (Delta and OSM-11) domain, which precondition the affinity to different NOTCH receptors (Kopan & Ilagan, 2009), as described in Figure 1.3B.

Regarding NOTCH receptors, the mammalian NOTCH receptor family is composed by four type I transmembrane receptors (NOTCH1-4) (Figure 1.3A) (Ranganathan et al., 2011). The precursor form of these four receptors is identical and is characterized by several domains. The extracellular domain (ECD) of the receptor integrates epidermal growth factor (EGF)-like, LIN12 and NOTCH repeats, responsible for the interaction with ligands. These repeats are followed by the heterodimerization (HD) domain, which is cleaved, leading to receptor activation (Kopan & Ilagan, 2009; Ranganathan et al., 2011). The transmembrane domain is followed by the intracellular domain (ICD), comprised of protein interaction domains, nuclear localization signals and the PEST domain, known to be the target to ubiquitination for degradation (Andersson et al., 2011). Despite the common structure of NOTCH receptors, some differences in length and composition, essentially in ECD and PEST domain of NOTCH ICD (NICD), distinguishes the 4 members of this receptor family (Kopan & Ilagan, 2009).

Before signaling initiation, NOTCH precursor proteins undergo cleavage by a furin-like convertase, in the HD domain (S1 cleavage), generating the mature receptor, comprised of ECD and ICD subunits held together by non-covalent interactions (Ranganathan et al., 2011).

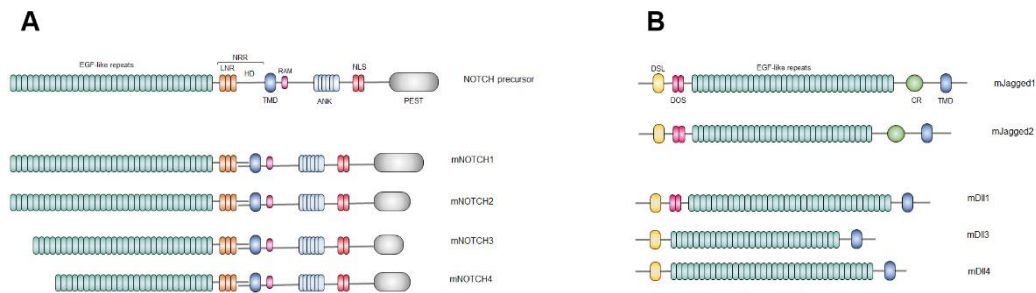


Figure 1.3 - NOTCH receptors and ligands. A | Structure of NOTCH receptor precursor and of the four NOTCH receptors. **B** | Structure of the five NOTCH ligands

NOTCH signaling initiates by the engagement of NOTCH ligand to NOTCH receptor, mediated by cell-to-cell contact. This binding promotes conformational changes in the ECD of the receptor, making it prone to cleavage by the metalloproteinase Tumor Necrosis factor- α -Converting Enzyme (TACE) (S2 cleavage). This step is followed by a third, intramembrane, cleavage (S3), mediated by the presenilin- γ -secretase complex, which allows the release of the NICD from the membrane and its translocation to the nucleus. Once in the nucleus, NICD mediates the conversion of CSL repressor complex, into a transcriptional activation complex, recruiting co-activator proteins such as MasterMind-like 1 (MAML1) (Ranganathan et al., 2011). This allows the initiation of a transcriptional cascade that involves both activation and repression of target genes related to basic cellular processes.

The best-characterized NOTCH targets are the basic-helix-loop-helix (bHLH) transcriptional repressors of the hairy enhancer of split (*HES*) family, involved in the repression of key cell fate determinants and cell cycle regulators. An also important target of NOTCH signaling is *DTX1*, which encodes an ubiquitin-ligase (DELTEX1) that guides NOTCH trafficking, acting as signaling regulator in many tissues (Bray & Bernard, 2010). NOTCH signaling is also known to control *cMYC* transcription, a positive regulator of cell proliferation processes (Palomero et al., 2006) and cyclin D1 (*CCDN1*), involved in cell cycle progression (Ronchini & Capobianco, 2001). Recently, *NRARP* gene was also identified as a target, acting as a NOTCH signaling inhibitor (Lamar et al., 2001).

Although NOTCH signaling is one of the most remarkably simple core pathways, it is tightly regulated by complex synergistic mechanisms, enabling distinct patterns of gene expression, that are tissue and context specific (Bray, 2006).

As NOTCH signaling relies on interactions between the main pathway elements, a small difference in receptor and/or ligand surface expression will have an important impact in restricting

signaling (Artavanis-Tsakonas & Muskavitch, 2010). In addition, receptor-ligand interactions *in cis* or *in trans* can regulate signaling direction (Guruharsha et al., 2012) and post transcriptional modifications (such as ubiquitination, glycosylation, phosphorylation or hydroxylation) of both NOTCH receptors and ligands have a major importance in modulating the signaling cascade. Moreover, regulation of NOTCH receptor endocytosis and trafficking, by proteins known to promote receptor degradation and regulate post endocytic events, such as DELTEX1 or Numb, is also an important step in signal transmission modulation (Andersson et al., 2011). Finally, the crosstalk between NOTCH and other signaling pathways such as WNT, TGF β (Andersson et al., 2011) or PI3K-Akt (Ranganathan et al., 2011) and the cooperation with tissue-specific activators (Bray, 2006) can contribute to the induction of NOTCH signaling in different cell types and contexts. Impairment of any of these regulation layers may have a huge impact in NOTCH signaling, leading to several diseases.

In adult mammals NOTCH has decisive roles in intestinal tissue turnover, skin self-renewal, neurogenesis and synaptic plasticity, skeletal muscle regeneration and hematopoiesis (Sato et al., 2012). Regarding the latter, the best studied role for NOTCH signaling concerns T cell lineage commitment, whose major checkpoints seem to be critically regulated by this pathway. NOTCH signaling, mainly NOTCH1/Dll4 axis, is known to play a critical role during commitment of lymphoid progenitor cells into the T cell lineage, continuing to act throughout the early stages of intrathymic T-cell development (Li & von Boehmer, 2011). Besides its role during the early stages of thymopoiesis, later on, NOTCH signaling continues to regulate T-cell development, for instance during the decision between $\alpha\beta$ or $\gamma\delta$ lineage at DN3 stage (NOTCH1-mediated) (Taghon et al., 2009) or β -selection process (NOTCH3-mediated) (Bellavia et al., 2002). The differentiation into single positive CD4⁺ or CD8⁺ T cells is also thought to be controlled by this pathway (Deftos & Bevan, 2000).

Therefore, any fluctuation in any of these T cell development regulation steps can have a significant effect in tissue homeostasis, leading to several known human diseases, such as T-ALL.

The Dark Side of NOTCH

Given the role of NOTCH signaling in T cell development, it is not surprising that deregulation of NOTCH proteins was found to be involved in T-ALL. The first evidence came from the observation of a translocation between chromosome 7 and 9, juxtaposing the ECD region of the human *NOTCH1* gene to the strong promoter/enhancer of the TCR β . This translocation originates a truncated, ligand-independent form of NOTCH1 termed Translocation-associated NOTCH homolog (TAN1), known to induce T-ALL-like disease in mice (Hales et al., 2014; Li & von Boehmer, 2011). In humans, the frequency of this translocation is <1%.

Later on, it was shown that more than 50% of T-ALL cases harbor *NOTCH1* activating mutations. These mutations cluster mainly in the HD and PEST domains. HD gain-of-function mutations lead

to conformational changes that destabilize heterodimers, facilitating the ligand-independent proteolysis of the receptor; PEST partial or complete deletion, promotes a decrease in NICD degradation, enhancing NOTCH1 signaling (Weng et al., 2004). Thus, both types of mutations lead to constitutive activation of the NOTCH signaling pathway, resulting in the transcription of genes involved in cell proliferation, survival and cell cycle. More recently, another type of mutation has been described, resulting in extracellular juxtamembrane expansions (JMEs), which increase the distance between HD complex and the membrane, promoting ligand-independent NOTCH signaling (Sulis et al., 2008).

Associated to *NOTCH1* mutations are often mutations or deletions in *FBXW7* (found in 15% of T-ALL cases). This gene encodes an F-box protein known to interact with NOTCH1, promoting its ubiquitination via the PEST domain. Mutations in this gene lead to impaired substrate recognition function, promoting the stability of NOTCH1 activated protein and, therefore, the enhanced activation of the NOTCH signaling pathway. Likewise, mutated *FBXW7* performs a defective mediation of proteasomal degradation of additional oncoproteins such as cMYC, Cyclin E and mTOR (Ferrando, 2009).

As discussed above, the constitutive activation of the NOTCH pathway leads to the increased transcription of NOTCH downstream targets, with consequences in several cellular processes. However, the way these downstream targets relate to T-ALL pathogenesis is far from being completely understood.

A large body of evidence indicates that cMYC is a critical direct target of NOTCH signaling in T-ALL context, being responsible for regulating cell growth and proliferation and promoting cell cycle progression (Li & von Boehmer, 2011). Other proteins involved in cell cycle have also been associated with NOTCH signaling in T-ALL development. Cyclin D3, encoded by the *CYCD3* gene, was identified as a direct target of NOTCH, and its expression contributes to cell-cycle progression in NOTCH-mediated T-ALL (Joshi et al., 2009). Furthermore, the protein SKP2, a subunit of the ubiquitin-ligase SCF^{SKP2}, responsible for the degradation of p27^{Kip1} and p21^{Cip1}, was identified as a direct transcriptional target of NOTCH in T-ALL. Importantly, up-regulation of SKP2 by NOTCH signaling accelerates the entry in the S phase of the cell cycle, due to increased degradation of p27 and p21 (Sarmiento et al., 2005).

Finally, the crosstalk of NOTCH aberrant signaling with other signaling pathways is key for its oncogenic role in T-ALL. In this regard, it has been shown that NOTCH1 induces PI3K-AKT activation via upregulation of *HES1* (NOTCH downstream target), which negatively controls the expression of *PTEN*, a PI3K-AKT signaling pathway negative regulator. These studies demonstrated an indirect regulation of PTEN by NOTCH signaling, which contributes to secondary oncogene addiction to PI3K-AKT signaling during T-ALL development and treatment resistance (Palomero et al., 2008).

In addition, NOTCH signals are known to positively regulate the activity of the mTOR pathway in T-ALL (Chan et al., 2007), as well as to induce cell transformation through several signaling

pathways such as NF- κ B (Vilimas et al., 2007), Ras-MAPK/ERK (Li & von Boehmer, 2011), and possibly WNT signaling (Weerkamp, van Dongen, et al., 2006).

Regarding the latter, it is known to regulate several developmental processes and it was found, similarly to NOTCH signaling, to be required for the first stages of T-cell development in the thymus (Weerkamp, Baert, et al., 2006). The oncogenic role of WNT signaling has been identified in several types of cancers. Surprisingly, despite WNT signaling role in T cell development, there are opposing evidences regarding its function in T-ALL.

Some studies point WNT signaling as having a tumor suppressive role in T-ALL. In this context, increased β -catenin-mediated WNT signaling was shown to promote the anti-leukemic effects of histone deacetylase inhibitors in T-ALL cells (Shao et al., 2012). Supporting these data, *LEF1* inactivating mutations were described in approximately 15% of T-ALL patients (Gutierrez et al., 2010). Arguing the opposite, some studies suggest WNT signaling as an oncogenic event in T-ALL. Abnormal WNT pathway activation was shown to occur in a significant fraction of human T-ALL cases, in a NOTCH-independent manner (Ng et al., 2014), with significant upregulation of WNT target genes. Supporting these data, *LEF1* was shown to be highly expressed in T-ALL patients, in association with a high mutation rate of *NOTCH1* and *JAK1*, suggesting its involvement in leukemogenesis (Guo et al., 2015).

It was further demonstrated that *TCF1* and *LEF1* might have cooperative and opposing roles in T cell malignancy. *TCF1*-deficient mice develop an aggressive T cell malignancy, similar to human T-ALL. Interestingly, *LEF1* was found highly upregulated in pre-malignant *TCF1*-deficient thymocytes and its conditional inactivation prevented disease development (Yu et al., 2012).

Targeting NOTCH signaling in T-ALL

The discovery of *NOTCH* mutations provided a rationale for therapeutic targeting of NOTCH signaling. In this regard, Weng and colleagues demonstrated that T-ALL cell lines are sensitive to γ -secretase inhibitors (GSI). These, originally developed as a therapy for Alzheimer's disease, block signaling at S3 cleavage, thus avoiding NICD release to the nucleus and, therefore, transcription activation. However, given NOTCH signaling crucial role in diverse tissues' homeostasis, the use of GSI results in severe side effects, particularly gastrointestinal toxicity (due to off-target effects) (Andersson & Lendahl, 2014). Nonetheless, combinational therapy with GSIs and glucocorticoids can improve the anti-leukemic effects of these inhibitors and reduce their toxicity, in mice (Real et al., 2009). Other approaches to target NOTCH signaling have been developed. These include the inhibition of NOTCH receptors or its ligands individually, a strategy used for example to target *NOTCH1* in leukemias (Wu et al., 2010) and NOTCH ligand *DII4* in solid tumors (Briot & Iruela-Arispe, 2015). Yet, these strategies lead to long-term off-target effects, associated with intestinal and hepatic toxicity and the development of vascular tumors (Andersson & Lendahl, 2014). Thus, improved knowledge on the oncogenic mechanisms controlled by NOTCH in T-ALL is crucial to develop more effective and less toxic therapeutic approaches. One possibility to target effectively NOTCH signaling is to explore its downstream targets and their

specific role in T-ALL pathogenesis. An interesting candidate is the recently described NOTCH target NRARP, NOTCH Regulated Ankyrin Repeat Protein. This protein is known to negatively regulate NOTCH signaling and positively regulate WNT signaling (by LEF1 protein stabilization). Moreover, it was recently found that loss of microRNA181ab1 in NOTCH-induced T-ALL inhibits leukemia development, partly by de-repressing the expression of *NRARP* (Fragoso et al., 2012). These data make NRARP into an attractive candidate to study in T-ALL context.

NRARP: A fascinating regulator of NOTCH signaling

NRARP is a gene that encodes a conserved small protein containing two ankyrin repeats. Its transcription is activated by CSL-dependent NOTCH signaling and can modulate NOTCH-mediated transcription by binding to NICD and promoting its degradation, acting, therefore, as negative feedback regulator of the pathway (Lamar et al., 2001). NRARP was first identified in *Xenopus* embryos and afterwards in mammals, namely mouse embryos. Mouse's *NRARP* was shown to share great homology with human *NRARP* (Krebs et al., 2001) and was confirmed as a direct target of NOTCH signaling, process mediated by Su(H) protein (Pirot et al., 2004).

NRARP was firstly shown to play an important role in embryogenic processes such as development of the central nervous system and somite formation (Krebs et al., 2001). During neurogenesis, *NRARP* is expressed in regions where NOTCH signaling is active, such as the neural plate. It was found that its overexpression decreases NOTCH signaling, leading to an increase in the number of primary neurons forming within the neural plate (Lahaye et al., 2001). Moreover, in mouse somitogenesis, *NRARP* is expressed in a cycling manner and contribute to severe Dll3 (NOTCH ligand) mutant phenotype, not only by NOTCH signaling regulation but also WNT pathway modulation (Sewell et al., 2009).

NRARP was confirmed as a NOTCH signaling downstream target in mouse hematopoietic stem cells (HSC) and T cells (Yun & Bevan, 2003) and in CD34⁺ human umbilical cord blood (CB) cells (Van Walle et al., 2009; Weerkamp, Luis, et al., 2006). *In vitro* studies with mouse HSC and T cells showed that *NRARP* overexpression is capable of inhibiting NOTCH-mediated activation and, *in vivo*, to specifically inhibit T cell development, by blocking T lineage commitment and progression through the DN stages (Yun & Bevan, 2003). In fact, studies with human CD34⁺ cells show that NRARP expression is high in extrathymic CB progenitors and maintained in uncommitted thymocyte, but upon T-cell commitment, its expression is sharply downregulated. This allows further progression into T-cell development, confirming NRARP role as a negative regulator of NOTCH signaling in this context (Van Walle et al., 2009).

NRARP was also shown to regulate WNT signaling. In zebrafish's embryonic development, NRARP was shown to stabilize LEF1 protein by blocking its ubiquitination and consequently to positively regulate WNT signaling (Ishitani et al., 2005). These studies are important because they revealed that NRARP independently regulates canonical NOTCH and WNT signaling (through NICD and LEF1 respectively). This role of NRARP as a molecular link between NOTCH signaling and LEF1-dependent WNT signaling was further demonstrated, in endothelial cells, in

both mice and zebrafish studies. During sprouting angiogenesis, Dll4-mediated NOTCH signaling induces *NRARP* expression in stalk cells, which will limit NOTCH signaling and promote WNT signaling (through interaction with LEF1), to maintain stability during the angiogenic process (Phng et al., 2009). These data demonstrate that NRARP can regulate both NOTCH and WNT signaling, which might be valid in other cellular contexts.

Considering the importance of NRARP in T cell lineage commitment and the involvement of NOTCH and WNT signaling in T-cell leukemogenesis, these observations suggest that deregulation of NRARP may contribute to T-ALL pathogenesis. Moreover, the discovery of miR-181ab1 regulation of NRARP in T-ALL (Fragoso et al., 2012), make it a prone candidate to be involved in disease development.

All together, these data indicate that NRARP deregulation or mutated expression may impair its function, contributing to T-ALL development. Due to the adversities in targeting NOTCH signaling with the available drugs, we believe that understanding the role of NRARP in T-ALL pathogenesis might have considerable clinical relevance. Targeting NRARP as a therapeutic approach represents an unexplored strategy to modulate NOTCH signaling, which may improve the current panorama of T-ALL targeted therapies.

Objectives

Despite the improvement on the survival of T-ALL patients over the years, patients with relapsed or resistant disease show decreased survival. This makes it imperative to find alternative therapeutic strategies, more specific and effective.

Different therapeutic NOTCH-targeting approaches have been explored but all have been associated with high toxicity and insufficient anti-leukemic effects. For that reason, a better understanding of the oncogenic mechanisms underlying NOTCH signaling in T-ALL is necessary to develop novel targeted therapies, to ultimately improve patients' outcome.

Therefore, in this project, we aimed to understand a possible role for NRARP in T-ALL pathogenesis and to investigate its therapeutic potential to target NOTCH oncogenic signaling. This project will improve our understanding of the leukemogenic process and potentially overcome the challenging inhibition of NOTCH signaling. Our main goals were to:

1. Characterize NRARP expression in human T-ALL by:
 - a. Analyzing NRARP mRNA and protein levels in T-ALL cell lines and primary cells and comparing it with total thymocytes;
 - b. Investigating a correlation between NRARP levels and NOTCH signaling activation (NICD levels).

2. Investigate the functional role of NRARP in T-ALL cells, in vitro, by:
 - a. Dissecting the effects of NRARP overexpression on NOTCH signaling;
 - b. Exploring the effects of NRARP overexpression on cell proliferation, viability and cell cycle.

To address these goals, we used several T-ALL cell lines, corresponding to different differentiation stages and presenting diverse genetic alterations, as well as human primary leukemia samples.

2. Materials and Methods

Cell culture

T-ALL cell lines, corresponding to different developmental stages and presenting distinct mutational profiles, were used in the *in vitro* studies (CEM, MOLT4, HPB-ALL, DND41, Jurkat, Loucy and TALL1). These were cultured in RPMI-1640 medium with L-glutamine (Gibco), supplemented with 10% of fetal bovine serum (FBS) (Biowest), 1% penicillin/streptomycin (Gibco) and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco) (hereafter referred to as R10 medium). Cells were kept in culture at a density of $0,5 \times 10^6$ cells/mL (with the exception of HPB-ALL cultured at a density of 1×10^6 cells/mL), at 37°C in 5% CO₂ environment.

The cell lines established during this project by the stable transduction of pCDH empty vector and pCDH cloned with NRARP ORF vector (see subchapter "Lentiviral production and Transduction of T-ALL cell lines") were cultured in the same conditions as the parental cell lines.

Lentiviral production and transduction of T-ALL cell lines

Lentiviral vectors, derived from HIV-1, are capable of infecting a wide variety of dividing and nondividing cells, and to stably integrate their RNA into the host genome, resulting in the long-term expression of a transgene. These vectors can be designed to express (constitutively or conditionally) both transgenes and shRNAs in single units or multiple combinations (Tiscornia et al., 2006).

To address the functional effects of NRARP in T-ALL we performed overexpression studies. For that purpose, we transduced T-ALL cells (CEM, MOLT4, HPB-ALL, DND41, Jurkat, Loucy and TALL1) with lentiviruses produced using the lentiviral vector pCDH-CMV-MCS-T2AcopGFP from SBI (Figure 2.1) with NRARP ORF cloned in, as described in Fragoso et al., 2012. This vector contains a Green Fluorescent Protein (GFP) tag to allow transduced cells to be detected by flow cytometry.

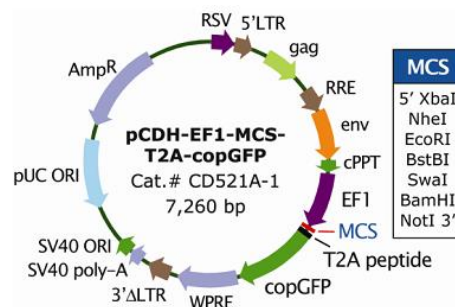


Figure 2.1 - Map of pCDH-EF1-MCS-T2A-copGFP lentiviral vector. The expression vector contains, for example, a constitutive Elongation factor 1 α (EF1) promoter (for transcription of reporter and cloned cDNA insert), a T2A peptide (to mediate protein cleavage from a single open reading frame to generate multiple proteins from a single promoter) and a copepod green fluorescent protein as a reporter for the transduced cells. (Figure from SBI Cloning and Expression Lentivectors Manual)

Cells transduced with the pCDH Empty vector will be referred to as 'Empty' and cells transduced with pCDH NRARP vector will be referred to as 'NRARP OE'.

Cell infection was performed by spinoculation (centrifugation at 2300 rpm, 120 minutes at room temperature) of 2×10^6 cells per mL of viruses, in the presence of 8 µg/mL of polybrene. Transduction efficiency was accessed by Fluorescence Activated Cell Sorting (FACS) analysis of GFP expression, using a LSRFortessa flow cytometer (FITC channel, Blue laser, 488nm), 48-72h after infection. Cells were sorted for 100% GFP-positive purity using a BD FACSAria™ cell sorter.

RNA analysis

Extraction and quantification

Harvested cells were centrifuged at 3200 rpm for 5 minutes at 4°C and the pellet resuspended in 1mL of TRIzol® Reagent (Ambion™, ThermoFisher Scientific) and stored at -80°C. RNA extraction was performed according to the TRIzol® RNA isolation protocol (ThermoFisher Scientific). RNA pellets were resuspended in DNase/RNase free H₂O and stored at -80°C. RNA quantification was performed using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific) and absorbance measurements at 230, 260 and 280nm were taken to evaluate RNA purity. Only the RNAs with an $A_{260/280}$ ratio between 1.8 and 2 and an $A_{260/230}$ ratio higher than 1.8 were used to proceed with cDNA synthesis protocol.

cDNA synthesis

cDNA synthesis was performed using 500ng of RNA (in a volume of 10 µL). Upon denaturation at 65°C for 10 minutes, 10 µL of cDNA synthesis mix was added to the RNAs (Random Primers 50ng/µL, mix dNTPs 10 mM, Dithiothreitol (DTT) 0,1M, 5x First Strand Buffer, Recombinant Ribonuclease Inhibitor (RNase Out) 400U/µL, Super Script II Reverse Transcriptase 200U/µL (Invitrogen)). Synthesis was performed in a T100™ Thermal Cycler (Bio-Rad), programmed for 10 minutes at 25°C plus 50 minutes at 42°C and 15 minutes at 70°C. cDNA was stored at -20°C.

Real-time/Quantitative PCR

In order to quantify gene expression in NRARP overexpressing T-ALL cell lines (and respective control cells), Quantitative/Real-time PCR was performed using TaqMan or SYBR Green methods. The expression of *NRARP* was quantified using TaqMan Gene Expression assays and Master Mix (Applied Biosystems), following manufacturer's instructions (Thermo Fisher Scientific). The expression of the remaining genes was quantified using Power SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies) and specific primers, as follows:

hHES1 Forward: 5' GCA GAT GAC GGC TGC GCT GA 3'

Reverse: 5' AAG CGG GTC ACC TCG TTC ATG C 3'

hDTX1 Forward: 5' CAG CTT GTG CCC TAC ATC ATC 3'

Reverse: 5' ACG ACG GGT CGT AGA AGT TG 3'

hcMYC Forward: 5' GGC TCC TGG CAA AAG GTC A 3'

Reverse: 5' CTG CGT AGT TGT GCT GAT GT 3'

hNOTCH1 Forward: 5' GAG GCG TGG CAG ACT ATG C 3'

Reverse: 5' CTT GTA CTC CGT CAG CGT GA 3'

hCCND1 Forward: 5' CAA TGA CCC CGC ACG ATT TC 3'

Reverse: 5' CAT GGA GGG CGG ATT GGA A 3'

hLEF1 Forward: 5' TGC CAA ATA TGA ATA ACG ACC CA 3'

Reverse: 5' GAG AAA AGT GCT CGT CAC TGT 3'

hAXIN2 Forward: 5' CTC CCC ACC TTG AAT GAA GA 3'

Reverse: 5' GTT TCC GTG GAC CTC ACA CT 3'

h18S Forward: 5' GGA GAG GGA GCC TGA GAA ACG 3'

Reverse: 5' CGC GGC TGC TGG CAC CAG ACT T 3'

The PCR reactions were performed using ViiA™ 7 Real-Time PCR System (ThermoFisher Scientific). Gene expression was normalized using the gene *18S*, and relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method (according to manufacturer's instructions).

Proteasome inhibitor treatment

To analyze the expression of proteins with fast turnover, we used a proteasome inhibitor in order to block protein degradation. Cells were treated with 1 μ L/mL of MG-132 inhibitor (10mM in DMSO, Calbiochem), at a cell density of 1×10^6 cells/mL and incubated for 8h at 37°C. Cells were then harvested, centrifuged at 3200 rpm for 5 minutes at 4°C and lysed as described below.

Protein analysis

Extraction and quantification

Cells were harvested and centrifuged at 3200 rpm for 5 minutes at 4°C. Cell pellet was resuspended by repetitive pipetting with Lysis Buffer (50mM Tris-Base pH 8.0, 150 mM NaCl, 5mM EDTA, 1 mM NaOV (Sigma), 10 mM NaF, 10mM Sodium Pyrophosphate (Sigma), 1% NP-40), supplemented with protease inhibitor cocktail Complete Mini (Roche) and protease inhibitor AEBSF (1 mM). Lysates were then centrifuged at 13000 rpm for 20 minutes at 4°C and supernatants were collected and stored at -20°C. Total protein quantification was accessed using Bradford reagent (Bio-Rad), at a 1:500 v/v dilution in H₂O. Quantification was obtained using GeneQuant pro (Amersham Biosciences) spectrophotometer at a 595nm wavelength (Protein595 Program).

Immunoprecipitation

Cells were harvested and washed twice with PBS (3200 rpm, 5 minutes, 4°C). Cell pellet was resuspended by repetitive pipetting with RIPA Buffer (50mM Tris-Base pH 8.0, 150 mM NaCl, 0,5% SDS, 0,5% Sodium Deoxycholate, 0,1% NP-40), supplemented with protease inhibitor cocktail Complete Mini (Roche) and protease inhibitor AEBSF (1 mM). Lysates were then centrifuged at 13000 rpm for 20 minutes at 4°C and supernatants were collected. Total protein quantification was accessed using Bradford reagent and GeneQuant pro, as described above. 250µg of total protein were diluted in RIPA buffer to a final volume of 492µL and incubated overnight at 4°C with 8µL of a specific antibody (Santa Cruz Biotechnology), with rotation. To allow antibody precipitation, beads (25µL/sample) Protein A/G (sc-2003, Santa Cruz Biotechnology) were washed in PBS, equilibrated for 1h at 4°C with rotation and added to the samples, rotating afterwards for 3h30 at 4°C. Samples were then centrifuged at 5000rpm for 5 minutes and the supernatants were collected and stored at -20°C (for later confirmation of the immunoprecipitation efficiency). The pellets were washed four times in RIPA buffer, rotating for 15 minutes in the first two. Supernatants were then discarded and pellets stored at -20°C.

Western Blot

Protein extracts were resuspended in Laemmli buffer and denatured at 90°C for 5 minutes. Samples (50µg-100µg of protein depending on the protein to be analyzed), were loaded in a SDS-PAGE gel (10%, 12%, 14% or 16% of acrylamide depending on protein molecular weights), ran at a constant voltage for variable periods of time and then transferred onto a nitrocellulose membrane for 90 minutes at 400 mA. The Ponceau S solution (Sigma) was used to evaluate the protein's content. Membranes were blocked during 1 hour with 3% w/v skimmed milk or 5% w/v Bovine Serum Albumine (BSA), diluted in Tris-Buffered Saline with 0.1% Tween 20 (TBS-T buffer). Immunoblotting was then performed using desired diluted primary antibodies: Cleaved NOTCH1 (Val1744) (1:1000), LEF1 (C18A7) (1:1000), α -Tubulin (1:10000) (Cell Signaling Technology), NRARP (E-13) (1:200), phospho- β -catenin (Ser675) (1:1000), total β -catenin (1:1000), Histone H3 (1:1000) and β -Actin (1:1000) (Santa Cruz Biotechnology). This incubation was done overnight at 4°C, under soft agitation. In the following day, membranes were washed for variable time intervals in TBS-T and incubated for 1 hour, at room temperature, with the respective horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG (1:5000, Promega), anti-mouse IgG (1:5000, Promega) or anti-goat IgG (1:5000, Santa Cruz Biotechnology)), diluted 1:5000 v/v in 3% milk/TBS-T buffer solution or TBS-T buffer alone, as adequate. Membranes washed after secondary antibody incubation were stained using Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific Inc.) and immunodetection was carried by chemiluminescence using Curix60 (AGFA HealthCare).

Densitometry was performed on scanned immunoblot images, using Photoshop CS6.

Membrane stripping

In order to re-blot the membranes, priorly incubated antibodies were removed by stripping process. Membranes were incubated in Stripping Buffer (15 mM Tris Base, 100 mM 2- β -Mercaptoethanol (Sigma), 2% SDS, pH=6.7), supplemented with 1:1000 v/v dilution of 2- β -Mercaptoethanol and incubated at 56°C for 30 minutes, with agitation. Membranes were then thoroughly washed with H₂O and TBS-T (twice, for 10 minutes each time, with agitation). After the stripping process and washing, a new immunoblot was performed, starting from the blocking step, as described above.

Cell growth assays

To access the cell growth potential of NRARP overexpressing T-ALL cell lines, expansion and proliferation assays were carried out. Expansion assays were performed by culturing a fixed number of “Empty” and “NRARP OE” cells at optimal density (see subchapter “Cell culture”) and determining cell number using a hemocytometer and trypan blue stain (for dead cells exclusion) at days 3, 6, 9, 12 and 15, only in some cases. For proliferation experiments, $1,5 \times 10^5$ cells were plated at optimal culture conditions and then harvested at 24, 48, 72 and 96h. Cell number was determined by FACS analysis (LSR Fortessa, APC channel, Red laser, 633nm) using counting beads (Beckman Coulter, 10 μ m, diluted and counted using a hemocytometer before use).

Viability analysis

Cell viability was accessed in NRARP overexpressing T-ALL cell lines at several time points: 24, 48, 72 and 96h. Apoptosis was determined using AnnexinV-APC (since transduced cells contain a GFP tag, read in FITC channel) and necrosis evaluated using the 7AAD dye. $1,5 \times 10^5$ cells were plated at optimal culture conditions and then harvested at determined time points, washed with ice-cold PBS and resuspended in 100 μ L Annexin V Buffer 1x (eBioscience). 2 μ L of 7AAD (BD Pharmingen) and 1 μ L Annexin V-APC (eBioScience) were added to cell suspension and incubated for 15 minutes at room temperature, in the dark. Afterwards, 200 μ L Annexin V Buffer were added and viability was accessed by FACS analysis (LSR Fortessa, APC channel, Red laser, 633nm).

Cell cycle analysis

To analyze cell cycle profiles of NRARP overexpressing T-ALL cell lines (as comparing with empty control vectors), 1×10^6 cells were incubated for 1h at 4°C and plated afterwards into 12-well plates at optimal density. Cells were then collected, washed, resuspended in cold PBS and fixed with equal volume of 80% ethanol in PBS (drop by drop, vortexing gently). Before staining, cells were washed twice with cold PBS and incubated with RNase A solution of 50 μ g/mL in PBS, for 30 minutes at 37°C. Propidium iodide (PI) was added at a concentration of $2,5 \times 10^{-3}$ μ g/ μ L and incubated overnight. Cell cycle profile was accessed the following day by FACS analysis (using a FACSCalibur, FL2 channel, 585/42nm).

We have further characterized the cell cycle profiles of NRARP overexpressing cells but starting with cells in the same phase of the cell cycle. To avoid chemical synchronization, we have sorted DND41 and TALL1 NRARP OE and Empty cells in G1 cell cycle phase, using for that purpose the Vybrant® DyeCycle™ Violet Stain. This is a non-fluorescent DNA dye that allows the use of the violet laser of flow cytometers to determine the DNA content in living cells. Cells were resuspended in Hank's Balanced Salt Solution (HBSS) buffer (Thermo Fisher Scientific) and Vybrant® DyeCycle™ Violet stain was added at the following concentrations: for TALL1, 19µL/mL with 19×10^6 cells and for DND41, 5µL/mL with 15×10^6 cells (adjusted based on TALL1 sorting assay). Cells stained were incubated for 30 minutes at 37°C, protected from light and finally analysed/sorted on a BD FACSAria™ Cell Sorter (containing a violet laser, 407nm), without being washed nor fixed.

Data was analyzed using FlowJo™ or ModFit LT™ software, as appropriate.

Statistics

Statistical analysis was performed using GraphPad Prism 6.01 (Windows). Differences between values were calculated using Student's t-test, One-way ANOVA or Two-way ANOVA, as appropriate. P values were considered statistically significant when lower than 0,05.

3. Results

A. Characterization of NRARP expression in human T-ALL

The first goal of this project was to characterize NRARP mRNA and protein expression in human T-ALL cells. For that purpose, we analyzed NRARP expression in several T-ALL cell lines and human primary samples (collected at patients' diagnosis) and compared it to that in normal thymocytes, collected from healthy donors. In order to analyze NRARP expression at both mRNA and protein levels we used real-time PCR and Western Blot (WB), respectively.

We started by exploring NRARP expression in T-ALL cell lines and primary cells, presenting diverse genetic alterations, in order to have a biologically relevant range of samples. Since *NRARP* is a transcriptional target of NOTCH signaling, which is aberrantly activated in T-ALL, one could expect NRARP expression to be upregulated. However, since *NRARP* is also a target of miR-181ab1, which is upregulated in T-ALL (Fragoso et al., 2012), it was possible that NRARP would be downregulated instead. We started by analyzing *NRARP* expression at the transcriptional level. We observed that *NRARP* mRNA expression is higher in T-ALL primary samples than in control thymocytes (Figure 3.1A). At the protein level, NRARP levels are significantly higher in both T-ALL cell lines and primary cells comparing with control thymocytes (Figure 3.1B). These results are consistent with the hypothesis that, as a NOTCH target, NRARP is overexpressed as a result of NOTCH signaling aberrant activation in T-ALL cells.

Afterwards, and given that NRARP is not only a transcriptional target of NOTCH1, but also a negative regulator of this pathway, we sought of determining NICD levels, in order to explore a possible correlation with NRARP levels.

As observed in Figure 3.1C, although some T-ALL samples present both high levels of *NRARP* mRNA and of NICD protein, others (for example Pt#115) present high levels of NICD protein but low levels of *NRARP* mRNA. We did not observe also an association between NRARP and NICD protein levels (Figure 3.1D). Thus, we found no statistically significant correlation between NICD protein levels and *NRARP* mRNA expression or NRARP protein levels (Figure 3.1).

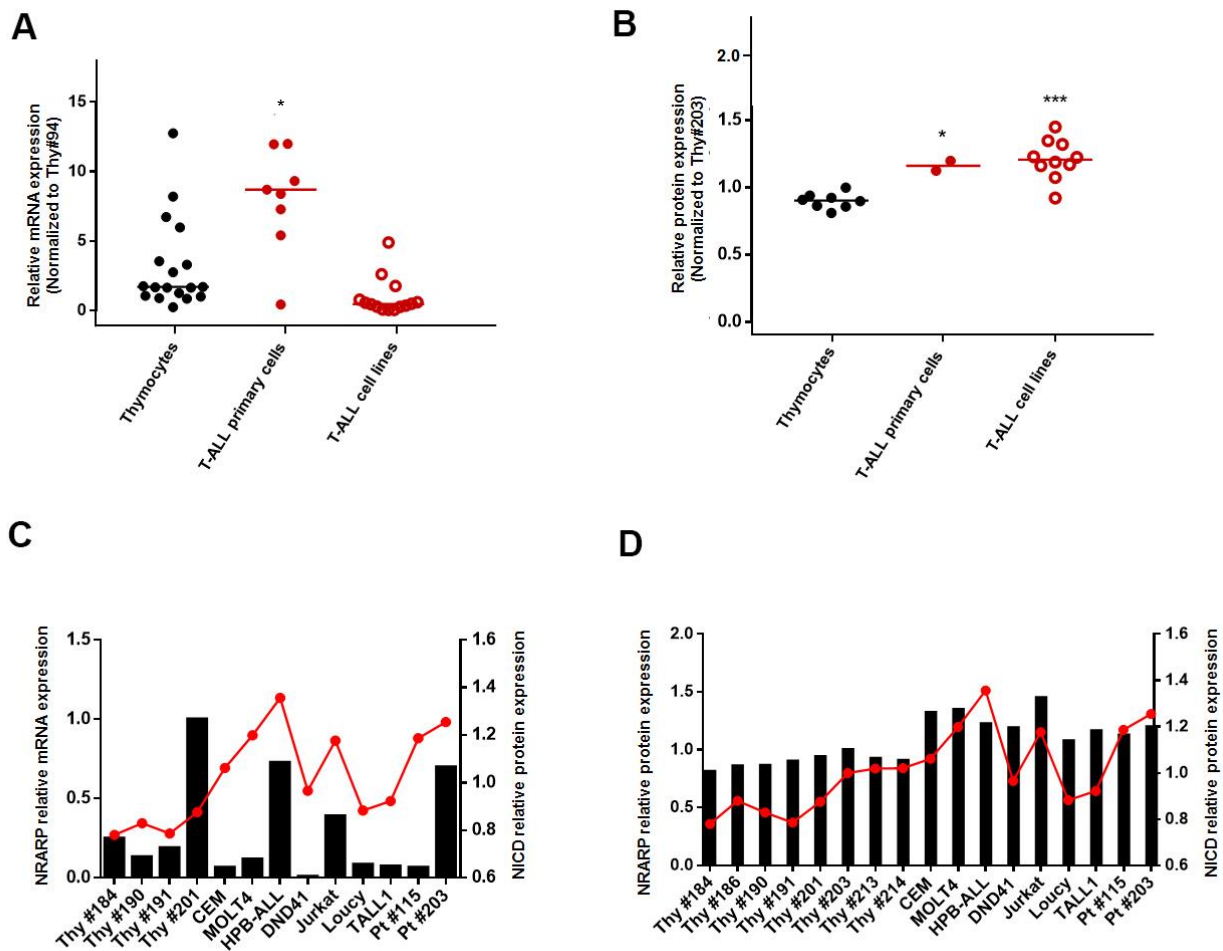


Figure 3.1 – NRARP expression in T-ALL cells. **A** | *NRARP* mRNA relative expression in T-ALL primary samples, T-ALL cell lines and human thymocytes, determined by RT-qPCR and calculated using the $2^{-\Delta\Delta Ct}$ method. Ct values were normalized against the 18S gene and *NRARP* expression was normalized against a control thymocyte sample. **B** | *NRARP* protein relative expression in T-ALL primary samples, T-ALL cell lines and human thymocytes, determined by Western Blot. Expression levels were normalized to their respective loading control and relative protein levels were obtained by normalization against a control thymocyte sample. Statistical analysis was performed using One-way ANOVA. * $p \leq 0.05$; *** $p < 0.001$. | Correlation graphs between *NRARP* mRNA (**C**) and protein relative levels (**D**) and NICD protein levels.

The observation that NRARP protein levels are high and NICD is still active, raises an important question: If NRARP levels are increased, why is it unable to block NOTCH signaling? One hypothesis, given the abnormal levels of NOTCH signaling, is that NRARP levels are not sufficient to block NOTCH oncogenic signals. Another hypothesis is that the occurrence of mutations in *NOTCH* or *NRARP* may impair their interaction and, consequently, NRARP function.

B. Functional analysis of NRARP in T-ALL

To understand if NRARP levels are not enough to downregulate NOTCH signaling in T-ALL, we overexpressed NRARP in T-ALL cells and studied its functional effects on NOTCH signaling activation and cell proliferation and viability. Thus, we generated several stable T-ALL cell lines (CEM, MOLT4, HPB-ALL, DND41, Jurkat, Loucy and TALL1) overexpressing NRARP by lentiviral transduction of pCDH NRARP vector or the Empty vector (to establish the correspondent control cell lines). Cells transduced and sorted for a 100% GFP-positive purity were used for subsequent studies. NRARP overexpression was confirmed by analysis of *NRARP* mRNA (by RT-qPCR) and protein expression (by Western Blot) (Figure 3.2). In order to obtain a clearer visualization of NRARP protein in the WB, we decided to use a proteasome inhibitor, MG-132, to block ubiquitin-mediated degradation. The detection of NRARP by WB revealed two bands at the protein level (Figure 3.2B). The higher band (full arrow) should correspond to the endogenous ubiquitin-conjugated NRARP protein and the lower sized band (dashed arrow), to the NRARP exogenous form.

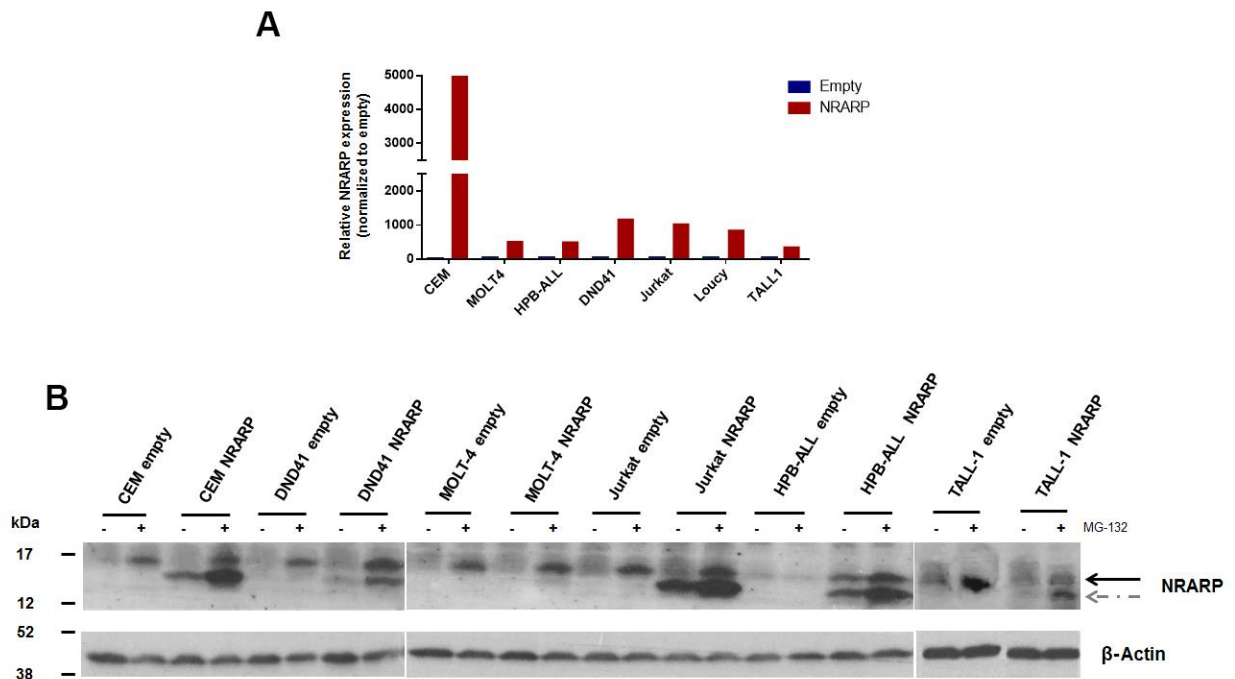


Figure 3.2 – NRARP overexpression in T-ALL cell lines by lentiviral transduction. A | *NRARP* mRNA relative expression in T-ALL cell lines after stable transduction, determined by RT-qPCR and calculated using the $2^{-\Delta\Delta Ct}$ method. Ct values were normalized against the 18S gene and *NRARP* expression was normalized against the respective empty vector control. **B** | *NRARP* protein expression in T-ALL cell lines after transduction, determined by Western Blot. Two *NRARP* forms were identified. The higher band corresponds to endogenous *NRARP* (full arrow) and the lower band might correspond to exogenous *NRARP* (dashed arrow). β -Actin was used as loading control.

After confirming NRARP overexpression in T-ALL cells we proceeded to the analysis of NOTCH signaling.

Effects of NRARP overexpression on NOTCH signaling

To analyze the functional effects of NRARP overexpression in T-ALL cells we started by analyzing its effects on NOTCH signaling, not only in what regards NICD levels but also NOTCH downstream targets.

Using NRARP overexpressing cell lines, we started by assessing NICD protein levels by Western Blot. We observed that, overall, NRARP overexpression leads to a decrease in NICD protein levels in T-ALL cell lines (Figure 3.3A). Interestingly, we noticed that in most cell lines (CEM, Jurkat, Loucy, HPB-ALL and TALL1), NRARP overexpression leads to NICD degradation via the proteasome, since inhibition of the proteasome with MG-132 inhibitor restores NICD levels. By contrast, in DND41 and MOLT4 cells, NRARP overexpression decreases NICD levels in a proteasome-independent manner (NICD levels upon NRARP overexpression are maintained low in the presence of MG-32 inhibitor) (Figure 3.3A).

We next analyzed the mRNA expression of NOTCH signaling downstream targets upon NRARP overexpression, to understand how it would affect the pathway. Evaluating mRNA levels of *HES1*, *DTX1* and *NOTCH1*, we observed that there is an overall block on NOTCH signaling. In particular, *cMYC* levels are decreased by NRARP overexpression in a group of cell lines (CEM, MOLT4, HPB-ALL and DND41), whereas in other cells (Jurkat, Loucy and TALL1) they are maintained or increased (Figure 3.3B).

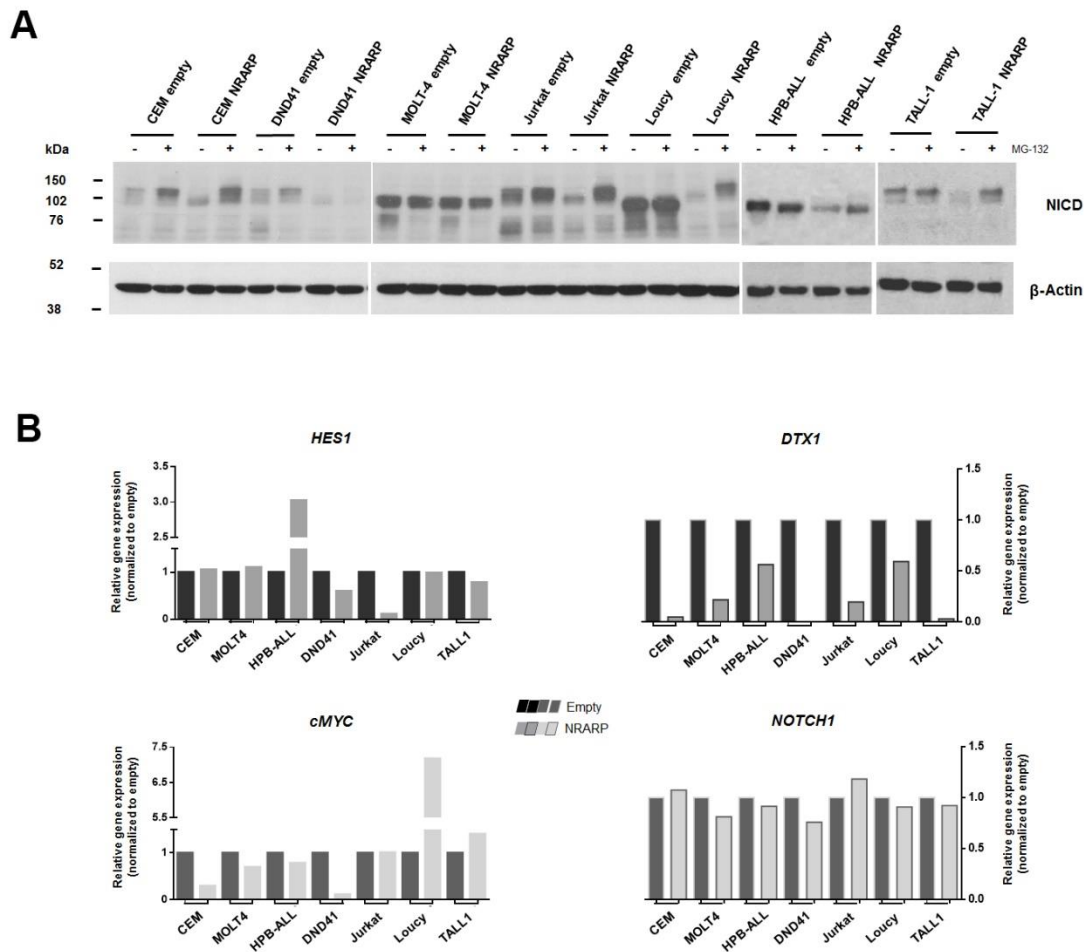


Figure 3.3 – Effect of NRARP overexpression on NOTCH signaling. A | NICD protein expression in T-ALL cell lines overexpressing NRARP, determined by Western Blot. To better analyze protein levels, samples were stimulated with proteasome inhibitor MG-132. The same cells were stimulated with DMSO, as a mocking control. β -actin was used as loading control. **B** | Human *HES1*, *DTX1*, *cMYC* and *NOTCH1* mRNA expression in T-ALL cell lines overexpressing NRARP, determined by RT-qPCR and calculated using the $2^{-\Delta\Delta Ct}$ method. Ct values were normalized against the 18S gene and gene expression was normalized against the respective empty vector control.

These results suggest that the impaired ability of NRARP to block NOTCH signaling, does not seem to stem from putative mutations in the NOTCH domain that interacts with NRARP. If that was the case, NRARP overexpression should not result in a block of NOTCH signaling transcriptional activity and/or promote NICD degradation.

NRARP effects on T-ALL cell growth, viability and cell cycle

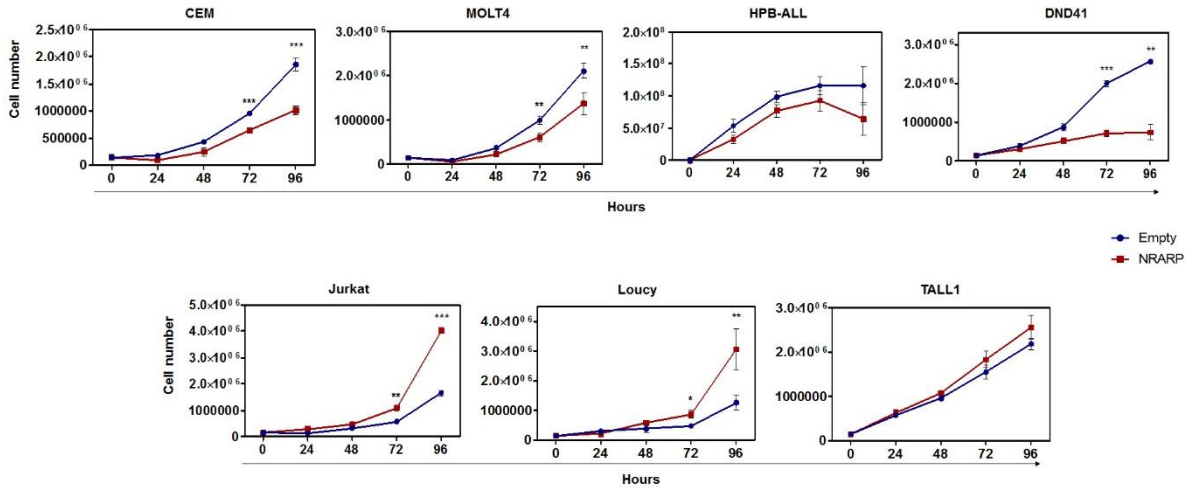
Besides exploring NRARP overexpression effect on NOTCH signaling, we also wanted to understand if NRARP overexpression has a functional impact in terms of cell proliferation, viability and cell cycle.

We started by performing proliferation assays to evaluate the effects of NRARP overexpression in T-ALL cells. Cell proliferation potential was assessed by flow cytometry using counting beads

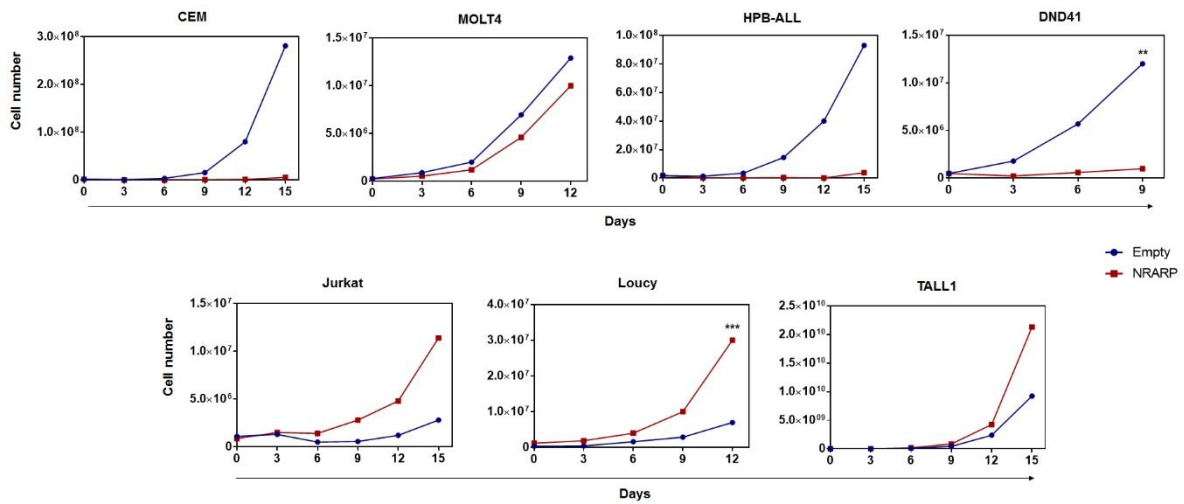
(dead cells were excluded using AnnexinV/7AAD). Interestingly, we observed that a group of cell lines (CEM, MOLT4, HPB-ALL and DND41) tend to proliferate less when NRARP is overexpressed, while the remaining cell lines (Jurkat, Loucy and TALL1) proliferate more upon NRARP overexpression (Figure 3.4A). Since the differences in proliferation seem to be more pronounced at later time points, we next performed cell expansion assays, to evaluate cell proliferation later in time. A certain number of cells were cultured and grown, and cell number was obtained at day 3, 6, 9, 12 and 15 (in some cases) (Figure 3.4B). The experiment was repeated at least two times. We observed the same growth trend in the two different groups of cell lines, as described above. Even though in some cell lines, differences in growth are not statistically significant, expansion trend is similar among cell lines of the same group (Figure 3.4C).

Despite the differences seen in proliferation/expansion, analysis of cell viability (through AnnexinV/7AAD stain) revealed no statistically significant differences, with the exception of DND41 cells, which have a lower viability when NRARP is overexpressed (Figure 3.4D).

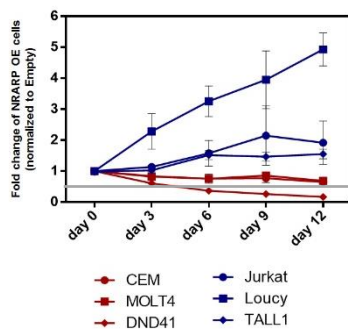
A



B



C



D

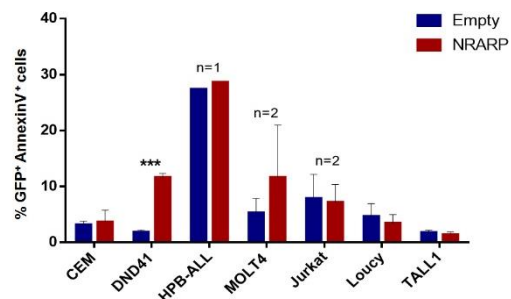


Figure 3.4 – Effects of NRARP overexpression on T-ALL cell proliferation and viability. **A** | Representative experiment of T-ALL transduced cell lines' proliferation, determined by flow cytometry, using counting beads to obtain absolute cell number. Dead cells were excluded using AnnexinV/7AAD stain. Statistical analysis was performed using Student's t-test. CEM, n=4; MOLT4, n=2; HPB-ALL, n=1; DND41, n=4; Jurkat, n=2; Loucy, n=3; TALL1, n=3. **B** | Representative experiment of T-ALL transduced cell lines' expansion. A fixed cell number was cultured for 9 to 15 days, at optimal conditions, and counted every 3 days using a hemocytometer. p value refers to the statistical analysis of three independent biological experiments combined, using Two-way ANOVA. **C** | Summary graph of independent biological expansion experiments. The graph represents fold change of cell growth in NRARP overexpressing cells, normalized to Empty cells. Lines above the grey threshold line (y=1) represent increased growth upon NRARP overexpression and lines below the threshold represent decreased growth. **D** | T-ALL cell lines viability, determined by AnnexinV/7AAD stain. *p<0.05; **p<0.01; ***p<0.001

Since we saw differences in cell proliferation/expansion but no variations in viability, we decided to assess possible alterations in cell cycle progression. We started by analyzing cell cycle in NRARP OE/Empty transduced T-ALL cells, not synchronized (Figure 3.5A). Regarding HPB-ALL and DND41 cells, slight differences were observed: NRARP overexpression increases the % of cells in G1 phase and decreases the % of cells in S-G2/M phases. In Jurkat and Loucy cells, NRARP overexpression has the opposite effect, decreasing the % of cells in G1 phase and increasing the % of cells in S-G2/M phases. Although these changes were consistent with the changes in these cell lines' proliferation, we did not observe differences in the other cell lines. Since this could result from the fact that cells were not synchronized, two cell lines (DND41 and TALL1) were expanded in culture and sorted in G1 cell cycle phase, in order to compare the cell cycle progression of cells that were initially exactly in the same phase of the cell cycle. We have also chosen to look at shorter time points to have a better idea of when cells proceed to the next phase and for how long they remain in those phases. We first analyzed cell cycle profiles obtained with ModFit LT™ software (data not shown) but since it is based on automatic algorithms there were some issues defining the correct percentages of cells in each peak. For that reason, we analyzed cell cycle using FlowJo10, where we defined peaks manually and analyzed manual gate percentages (Figure 3.5B). We observed that both analysis methods give similar results. Looking at DND41 cells isolated at G1 phase (confirmed by 100% cells in G1 at 0h), we observed that, the percentage of cells entering S phase at 8h is higher in Empty cells than in NRARP overexpressing cells. Concerning TALL1 cells, we noticed the opposite phenotype. At 8h, there is a higher percentage of NRARP overexpressing cells in S phase, whereas Empty cells seem to accumulate in G1. In summary, DND41 cells overexpressing NRARP seem to present a cell cycle delay, whereas TALL1 cells progress faster in cell cycle upon NRARP overexpression, which is consistent with cell proliferation/expansion results.

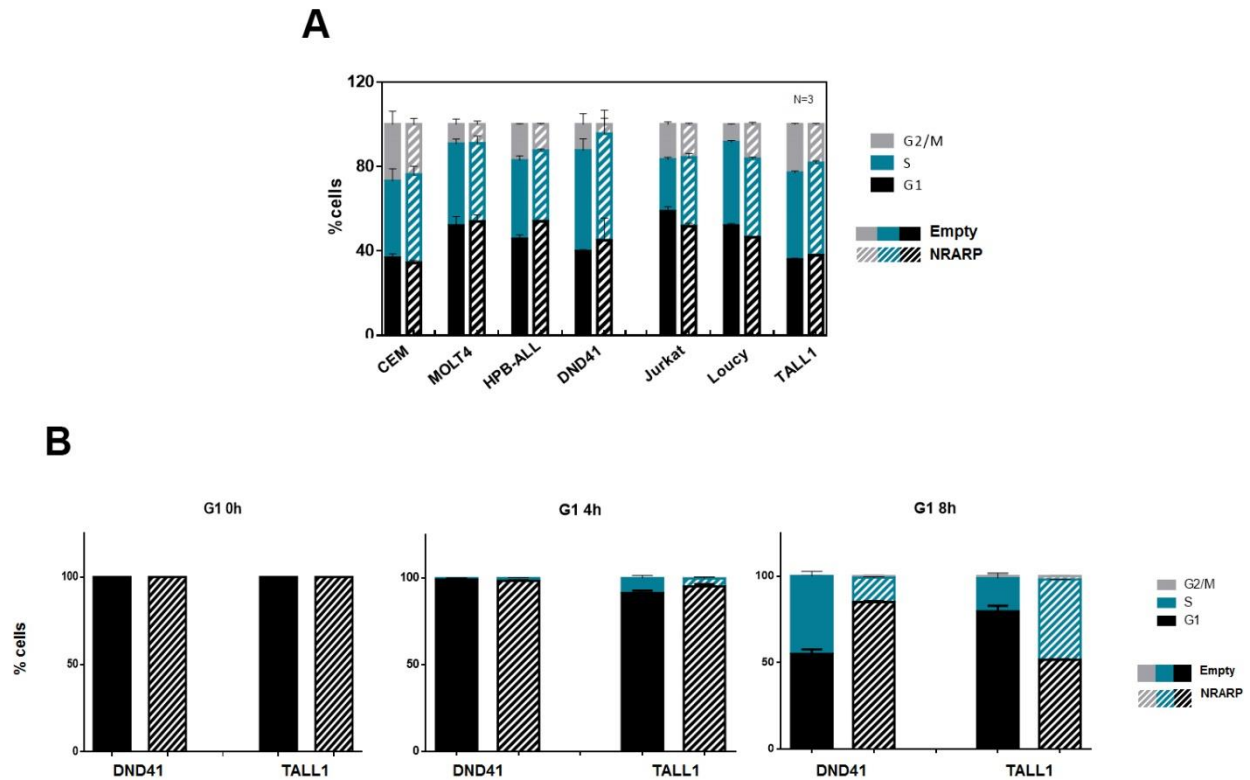


Figure 3.5 – Effects of NRARP overexpression on T-ALL cell cycle progression. A | Cell cycle profile of non-synchronized T-ALL transduced cell lines. 1×10^6 cells were cultured for 24h, at optimal conditions, and fixed at that time point. PI staining and FACS analysis was performed and percentage of cells in each cycle phase was calculated using ModFit software algorithm. **B** | Cell cycle profile of synchronized T-ALL transduced cell lines. Cells were synchronized through sorting of G₁ phase. Cells were collected immediately after sorting (0h) as control. $0.5-1 \times 10^6$ cells were cultured for 4 and 8h and fixed at those time points. PI staining and FACS analysis was performed and the percentage of cells in each cycle phase was calculated using FlowJo10 software.

Effects on WNT signaling

Our results showed that, although NRARP overexpression blocks NOTCH signaling in all cell lines, some grow more upon NRARP overexpression, a phenotype that seems to correlate with changes in cell cycle. Interestingly, it also seems to correlate with the pattern of *cMYC* mRNA expression. These observations suggest that, in these cell lines, NRARP may act on another signaling pathway. Since it has been shown that NRARP mediates WNT signaling through LEF1, we sought to understand if NRARP might play a role on WNT cascade and if that could explain the differences seen in cell proliferation.

LEF1 expression in T-ALL cell lines

First, we characterized LEF1 expression in T-ALL cell lines. There are seven isoforms of LEF1 described (Hovanes et al., 2000), produced by alternative promoter usage or alternative splicing, but only a few have been functionally described. The two major transcripts studied are the full-length isoform (48-55 kDa) (Willinger et al., 2006) and a shorter isoform that functions as dominant negative (around 35/38kDa) (Hovanes et al., 2001). We observed that LEF1 expression profiles

vary largely between T-ALL cell lines with no evident, consistent pattern common to all of them (Figure 3.6).

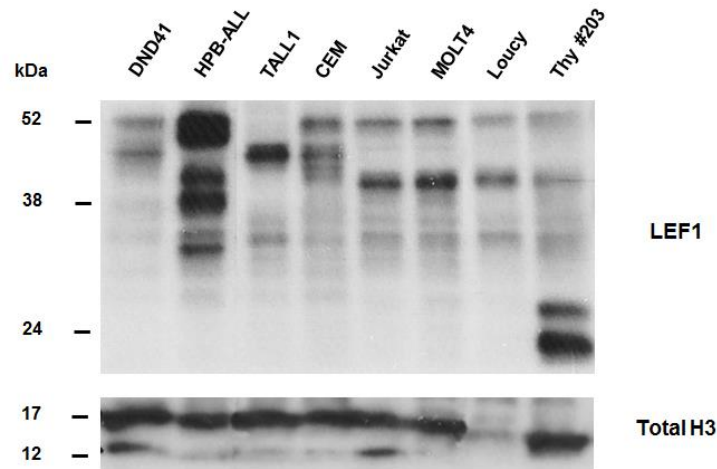


Figure 3.6 – LEF1 protein expression profiles in T-ALL cell lines. Protein profiles were accessed by Western Blot in several T-ALL cell lines and control thymocytes (#203). Total Histone3 (H3) was used as loading control.

Unraveling NRARP and LEF1 relation

To understand if there was a correlation between NRARP overexpression and LEF1 expression, we analyzed Empty/NRARP OE transduced cell lines. No major differences are induced by NRARP overexpression in most cell lines. In HPB-ALL and DND41 cells, we identified an isoform induced by NRARP overexpression (Figure 3.7, full arrows). Investigation is warranted to identify its possible biological relevance.

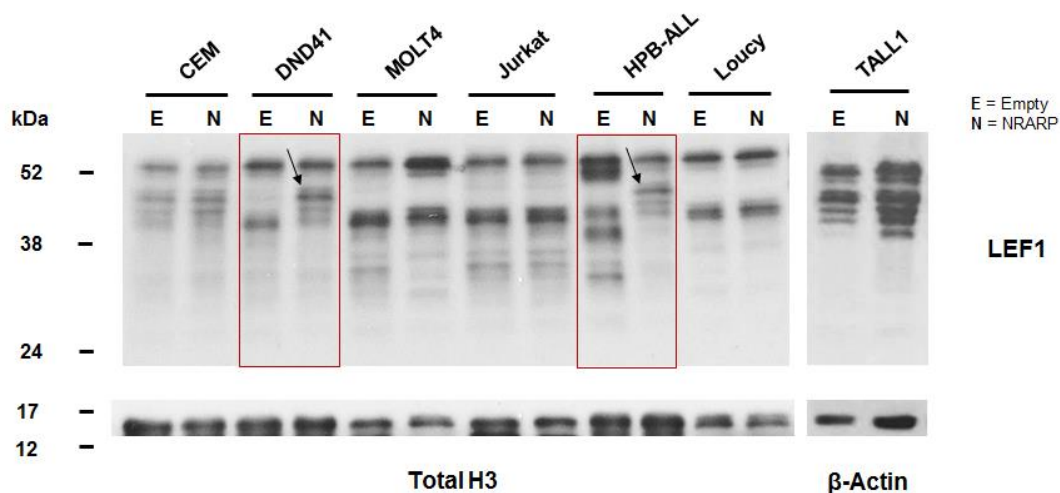


Figure 3.7 – Effects of NRARP overexpression on LEF1 protein expression. LEF1 protein expression profile in T-ALL cell lines overexpressing NRARP (comparing with Empty), determined by Western Blot. Full arrows point to specific LEF1 isoforms which expression is induced by NRARP overexpression in DND41 and HPB-ALL cell lines. Total Histone3 (H3) or β -Actin were used as loading controls.

Although we did not observe a clear effect of NRARP overexpression on LEF1 expression in most cell lines, and given that there are LEF1 isoforms with opposite functional outputs in WNT signaling, we decided to investigate the binding profile between NRARP and LEF1 by immunoprecipitation assays. This way we could determine to which isoform of LEF1 NRARP binds and correlate it with our cell proliferation results. We did not see variations between Empty and NRARP overexpressing cell lines but we could observe some interesting differences among cell lines (Figure 3.8). It appears that NRARP binds to particular isoforms in cell lines with the same growth trend. In Jurkat and Loucy cell lines, whose growth is accelerated by NRARP overexpression, we observed that NRARP binds to a long isoform (full arrow, Figure 3.8). On the other hand, in CEM, DND41 and MOLT4 cell lines, which present lower growth rate upon NRARP overexpression, NRARP binds to a shorter isoform (dashed arrow, Figure 3.8). However, these results care further validation. The experiment needs to be repeated and performed with more cell lines. Thus, further studies regarding LEF1 and NRARP interactions are required to evaluate a possible role of NRARP in WNT signaling modulation through LEF1.

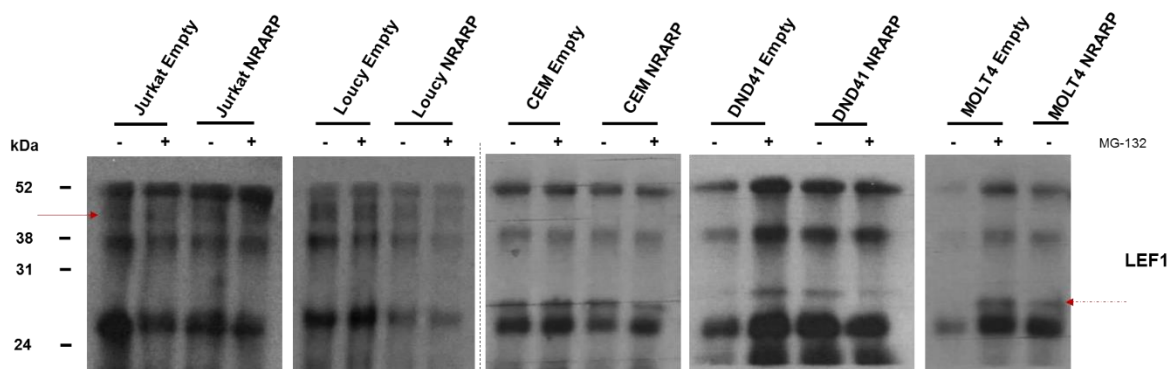


Figure 3.8 – NRARP binding to LEF1 by immunoprecipitation. After immunoprecipitation of NRARP, LEF1 isoforms were identified by Western Blot and differences among cell lines were analyzed (full or dashed arrows).

Effects of NRARP overexpression on WNT downstream players

Finally, we explored a possible effect of NRARP overexpression on WNT signaling by analyzing its effect on WNT downstream targets' expression.

Overall, the mRNA expression analysis of WNT cascade downstream targets showed no differences induced by NRARP overexpression (Figure 3.9A). Since the WNT pathway depends on β -catenin for signaling to be perpetuated, we have further explored β -catenin levels as a measure of WNT signaling activation. WNT cascade is activated when cytoplasmatic β -catenin protein accumulates, allowing its translocation to the nucleus where it contributes to target genes' transcription. In the absence of WNT proteins (signaling off), β -catenin is degraded by a destruction complex. Some proteins of the complex are capable of phosphorylating β -catenin which allows its recognition by ubiquitin-ligases and subsequent ubiquitination and proteasomal degradation (Bryan T. MacDonald; Keiko Tamai and Xi He, 2009). However, phosphorylation of

β -catenin in specific sites promotes its activation (Hino et al., 2005). Evaluating phospho- β -catenin levels at those sites is an indirect measure of β -catenin activation and consequently, WNT signaling promotion. We observed a slight decrease in phospho- β -catenin levels upon NRARP overexpression in MOLT4, HPB-ALL and DND41 cell lines whereas it seems that there are no differences in phospho- β -catenin levels in Loucy and TALL1 cell lines (Figure 3.9B). Moreover, although we are still optimizing the protocol, we investigated total β -catenin protein levels. Preliminary results suggest that in CEM cells overexpressing NRARP, total β -catenin is decreased, whereas in Jurkat cells it seems that there is no difference in expression (Figure 3.9C). Altogether, despite our efforts, our results do not allow to formally conclude whether or how NRARP overexpression affects WNT signaling. However, it appears that NRARP tends to decrease WNT signaling (as measured by β -catenin activating phosphorylation) preferentially in T-ALL cells that proliferate less upon NRARP overexpression (e.g. MOLT4 and HPB-ALL).

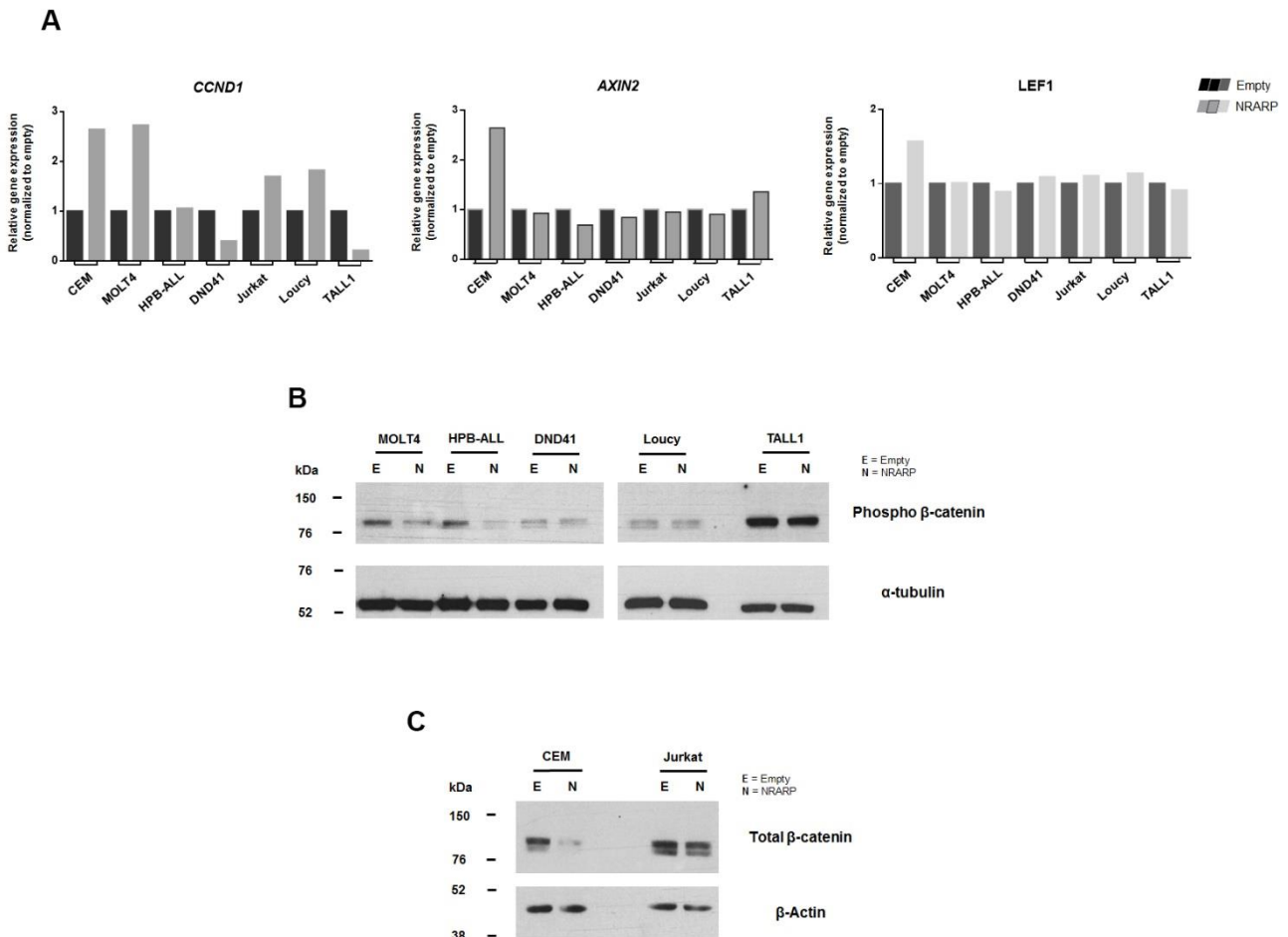


Figure 3.9 – Effects of NRARP overexpression on WNT signaling. **A** | Human *CCND1*, *AXIN2* and *LEF1* mRNA expression in T-ALL cell lines overexpressing NRARP, determined by RT-qPCR and calculated using the $2^{-\Delta\Delta Ct}$ method. Ct values were normalized against the 18S gene and gene expression was normalized against the respective empty vector control. **B** | Phospho- β -catenin protein expression in T-ALL cell lines, determined by Western Blot. α -tubulin was used as loading control. **C** | Total β -catenin protein expression in two T-ALL cell lines, determined by Western Blot. β -Actin was used as loading control.

In sum, the effects of NRARP overexpression on T-ALL cells' proliferation are consistent with the effects observed on cell cycle and coherent with *cMYC* expression levels (Table 3.1).

T-ALL cell line	Mutational status		Upon NRARP overexpression					
	NOTCH HD	NOTCH PEST	Notch signaling	cMYC expression	Proliferation	Viability	Cell cycle progression	Activated phospho- β -catenin levels
CEM	Mutated	WT	Inhibited	Downregulated	Decreased	-	ND	ND
MOLT4	Mutated	Mutated	Inhibited	Downregulated	Decreased	-	ND	Decreased
HPB-ALL	Mutated	Mutated	Inhibited	Downregulated	Decreased	-	ND	Decreased
DND41	Mutated	Mutated	Inhibited	Downregulated	Decreased	Decreased	Delayed	Decreased
Jurkat	WT	WT	Inhibited	No difference	Promoted	-	ND	ND
Loucy	WT	WT	Inhibited	Upregulated	Promoted	-	ND	No difference
TALL1	WT	WT	Inhibited	Upregulated	Promoted	-	Promoted	No difference

Table 3.1 – Summary of T-ALL cell lines *NOTCH1* mutational status and results obtained. The table summarizes the mutational status of *NOTCH* in the T-ALL cell lines studied and the functional effects of NRARP overexpression in those cells. **ND** = not determined; **-** = not affected

4. Discussion and conclusions

NOTCH signaling is crucial in T-cell development regulation, determining T-cell fate and early thymic commitment (Li & von Boehmer, 2011). Therefore, its deregulation plays a central role in T-ALL pathogenesis. *NOTCH* activating mutations were verified in more than 50% of the T-ALL cases (Weng et al., 2004) and despite the clinical advances throughout the years, clinical inhibition of NOTCH signaling with the drugs currently available was shown to induce severe toxic effects and insufficient anti-leukemic effects (Andersson & Lendahl, 2014).

NRARP is a transcriptional target of NOTCH signaling, acting also as a negative regulator of this pathway. It is expressed throughout human T cell development (Van Walle et al., 2009) and its overexpression in mouse HSC is known to inhibit T-cell lineage commitment and differentiation (Yun & Bevan, 2003). Moreover, it stabilizes the WNT signaling transcription factor LEF1 in neurogenesis (Ishitani et al., 2003). LEF1 was shown to induce malignant transformation in murine thymocytes (Yu et al., 2012). These data suggest that NRARP deregulated function may play a role in T-ALL development.

The main goal of the present thesis was to explore the role of NRARP in T-ALL pathogenesis. To evaluate it, we started by investigating NRARP levels in T-ALL cells (cell lines and primary cells). Although at the transcriptional level we only observed an increase in *NRARP* expression in T-ALL primary cells, at the protein level NRARP was increased in both T-ALL primary cells and cell lines. Low levels of NRARP observed in total thymocytes are coherent with the evidence of NRARP downregulation in committed thymocytes (Van Walle et al., 2009). The high levels of NRARP in T-ALL cells are consistent with its transcription by NOTCH1, which is constitutively activated in the context of T-ALL.

To understand if high NRARP levels correlate with NICD levels, we also analyzed NICD protein levels in T-ALL samples. In healthy thymocytes, we observed low levels of NICD protein and *NRARP* mRNA and protein (except in Thy#201 sample, at the transcriptional level). In T cell development, NOTCH signaling is extremely regulated and it is decreased during thymocyte differentiation. Therefore, it makes sense that NOTCH signaling activation is decreased in thymocyte samples, which is consistent with the low levels of NICD observed in our samples. As discussed above, NRARP low levels in thymocytes are in accordance to what is described in the literature and match the evidence of being a transcriptional target of NOTCH signaling.

Looking at disease context (T-ALL cells), we saw high NICD protein levels in most samples, which is consistent with the aberrant activation of this pathway in T-ALL. Although in most samples high levels of *NRARP* mRNA associate with high levels of NICD, some samples present high levels of NICD but low levels of *NRARP*. Considering what is known about NRARP regulation in the context of T-ALL, one hypothesis is that *NRARP* expression in T-ALL cell lines is being repressed by miR-181ab1. Nonetheless, considering that NRARP protein levels are increased in all T-ALL cells, this disparity between mRNA and protein levels may be the result of differences in mRNA stability,

decay, transcription and/or translation rates, and protein turnover in those cell lines (Vogel et al., 2010). Although NRARP levels are increased in T-ALL cells, NICD levels are high, which suggest that NRARP protein might not be functional or, at least, not enough to act as an effective negative regulator of the pathway.

This raises two hypotheses that, by themselves or in conjugation, could explain the results obtained: *NRARP* or *NOTCH* might display mutations that make NRARP ineffective in blocking NOTCH signaling or NRARP levels are not enough to block NOTCH oncogenic signals.

Our first attempt was to test the possibility of NRARP levels not being sufficient to shut down NOTCH oncogenic signals in T-ALL. To do so, we overexpressed NRARP in T-ALL cell lines and analyzed the effect on NOTCH signaling. Confirming NRARP overexpression in the cell lines used, we detected, by Western Blot, two bands with proximal molecular weights. The higher weight band should correspond to ubiquitinated endogenous NRARP since it is detected in untransduced cell lines and in empty vector cells, and it accumulates when the proteasome is inhibited by MG-132. The addition of molecular groups to the protein justifies the higher molecular weight verified. The lower weight band should correspond to the exogenous and non-ubiquitinated NRARP.

In order to understand if NRARP overexpression could have an effect on NOTCH signaling, we used cell lines overexpressing NRARP and the same cells transduced with the respective empty vector, to evaluate NICD levels as well as NOTCH downstream target genes' expression. As observed, NRARP overexpression is able to decrease NICD protein levels in all cell lines. However, we could observe that in some cell lines, inhibition of the proteasome in NRARP overexpressing cells could partially or completely restore NICD levels. This means that NRARP can degrade NICD via the proteasome (described before in Lamar et al., 2001). However, in other cell lines, the levels are maintained low despite proteasome inhibition, which suggests that NICD is being degraded in a proteasome-independent manner. Several might be the possibilities but interestingly, two studies were published recently, discussing possible proteasome-independent degradation mechanisms. Numb proteins are negative regulators of NOTCH signaling, known to reduce NICD levels through receptor-mediated endocytosis and/or proteasome-mediated degradation (Andersson et al., 2011). Tang et al. (2005) showed that the abolishment of the binding motifs for endocytic proteins or the reduction of proteasome activity does not affect the activity of Numb proteins as negative regulators of NOTCH signaling. Therefore, they discuss the possibility of a proteasome-independent pathway through which these regulators decrease NOTCH signaling but they couldn't define the nature of such mechanism. More recently, another group described autophagy (intracellular, lysosomal degradation pathway) as a route for NOTCH1 degradation, via uptake into autophagosome-precursor vesicles (Wu et al., 2016). These findings give insights on alternative degradation pathways for NICD, that may act as regulators of NOTCH signaling. However, the purpose of this dissertation was not to unravel the possible degradation pathways for NICD proteins and further studies would be required to explain our observations.

We then explored how NRARP affected NOTCH downstream signaling. We observed that *DTX1* is significantly decreased in all cell lines. *DELTEX1* can function as negative (Lehar & Bevan, 2006) or positive (Matsuno et al., 1995) regulator of NOTCH signaling and it is one of the most important targets of this pathway. Thus, it is not surprising that it could have a significant downregulation upon NOTCH signaling decrease. Monitoring *HES1* mRNA levels, we observed that these are normally maintained and even upregulated in HPB-ALL cell line. *NOTCH1* mRNA levels are maintained upon NRARP overexpression which is coherent with NRARP acting downstream of the receptor, at the intracellular active form, and not in the receptor itself. Finally, observations at *cMYC* expression show two groups of cell lines with opposite trends. In some lines (CEM, MOLT4, HPB-ALL and DND41), *cMYC* is downregulated and in others (Jurkat, Loucy and TALL1) is maintained or even upregulated. Since *cMYC* is known to be regulated by other signaling pathways, the increase in its levels might not be related to NOTCH signaling (that seems to be overall blocked) but instead, to other signaling pathways that could be activated in those cells.

Besides the NRARP effects on NOTCH signaling, we also characterized NRARP functional effects in T-ALL cells. First, we determined cells' expansion and proliferation potential and we observed an interesting growth trend. In some cell lines (CEM, MOLT4, HPB-ALL and DND41) NRARP overexpression seems to cause a delay in cell proliferation whereas in the others (Jurkat, Loucy and TALL1) it has the opposite effect (Table 3.1).

As observed, viability was, in general, not affected and so, our results might imply that the effect in cell proliferation is due mainly to cell cycle progression differences. Analyzing cell cycle profiles, we observed that DND41 cells isolated at G1 phase enter faster in S phase in the control than in NRARP overexpressing cells, and continue to progress faster in cycle. TALL1 cells, at 8h, present a higher percentage of NRARP overexpressing cells in S phase, suggesting a tendency to progress faster in cycle, whereas Empty cells accumulate in G1. These results suggest that DND41 cells have a cell cycle delay upon NRARP overexpression while TALL1 cells progressed faster in cycle.

Curiously, cell lines with the same growth tendency have similar mutational profiles regarding *NOTCH1* (Table 3.1). Cells whose expansion is delayed by NRARP overexpression harbor *NOTCH* activating mutations, whereas the ones that proliferate more with NRARP overexpression do not display *NOTCH* mutations. In addition, cells presenting downregulation of *cMYC* upon NRARP overexpression correspond to the *NOTCH* mutated ones (with decreased growth) and vice-versa. This correlation between cell proliferation and *cMYC* levels should be confirmed at the protein level.

Our observations suggest the intriguing possibility that, although NRARP is able to block NOTCH signaling regardless of cells' *NOTCH* mutational status, the latter may determine (perhaps indirectly) how NRARP impacts on leukemia cells: when *NOTCH* is mutated and cells rely on constitutive NOTCH oncogenic signals, NRARP acts as a negative regulator of leukemic cells, decreasing disease progression. However, when *NOTCH* does not display mutations and other

signals either than NOTCH might be decisive to promote disease, NRARP actually promotes T-ALL cell proliferation and, thereby, possibly contributes to leukemia advancement. The fact that, despite NOTCH signaling blockade, some cells showed increased proliferation and *cMYC* upregulation, led us to hypothesize that NRARP might be playing a more fundamental role in these cells other than blocking NOTCH signaling, ultimately leading to increased proliferation.

Our immediate candidate to explain these effects was WNT signaling. As discussed, it is known that NRARP positively regulates LEF1, a key player in WNT signaling, promoting pathway activation. Moreover, *LEF1* was found upregulated in pre-malignant TCF1-deficient thymocytes and its conditional inactivation prevented disease development (Yu et al., 2012). Interestingly, *cMYC* is also a downstream target of WNT signaling.

There are seven LEF1 isoforms identified, produced by alternative promoter usage or alternative splicing (Hovanes et al., 2000). However, only few of them have been functionally described. The two major transcripts studied are a long isoform (full-length, expected size of around 48-55 kDa) and a shorter isoform that functions as dominant negative (expected size of 35/38kDa) (Hovanes et al., 2001). The first one contains a β -catenin binding domain, allowing LEF1 to function as a transcription enhancer factor, leading to WNT signaling activation. The other one is a truncated form that cannot interact with β -catenin but still interacts with DNA, suppressing WNT signaling (Wang et al., 2005).

We started by exploring LEF1 expression profiles in untransduced T-ALL cell lines. We observed a huge variation in LEF1 expression among the different cell lines, which is not astonishing giving the variety of LEF1 isoforms. To try to understand if there was a correlation between NRARP overexpression and LEF1 expression, we explored it in transduced cell lines. However, only in HPB-ALL and DND41 we could observe a long isoform, induced by NRARP overexpression. The isoform identified is longer than the dominant negative isoform and it could correspond to the full-length LEF1. The results obtained care further investigation, to explore whether this has importance for NRARP-mediated effects or not. Alternatively, different LEF1 isoforms could interact with NRARP, leading to the cellular effects observed. That was our rationale to study NRARP binding to LEF1, through immunoprecipitation assays. We did not find differences between Empty and NRARP OE cells but we observed binding profile variations among cell lines. Our preliminary results show that in *NOTCH*WT cell lines, which have increased expansion upon NRARP overexpression, NRARP seems to bind exclusively to a long isoform. In *NOTCH*mutated cell lines, where the opposite proliferation trend is verified, NRARP binds to a shorter isoform. Further work is warranted to validate these results and to understand the biological role of the isoforms identified in the context of NRARP-mediated effects. Nonetheless, we may hypothesize that when *NOTCH* is mutated, NRARP binds to a short isoform that might lack β -catenin binding domains and act as dominant negative, decreasing WNT signaling and contributing to disease attenuation. On the other hand, in *NOTCH*WT cells, NRARP binds to a larger isoform, that may correspond to the full-length LEF1 or to an alternative transcript that is capable of binding to β -catenin and still activate WNT signaling, promoting T-ALL development. Currently we are

proceeding with LEF1 knockdown (KD) studies, with simultaneous NRARP overexpression, to understand if in cells where NRARP promotes growth, LEF1 KD can reverse those effects. Alternative approaches such as studying the functional effects of LEF1 overexpression in NRARP overexpressing cell lines whose growth is decreased, could also help elucidating the possible role of NRARP in modulating WNT signaling through LEF1.

Finally, we explored NRARP overexpression effects downstream of WNT signaling by analyzing its target genes' expression. *CCND1* levels are increased in most cell lines, which is consistent with increased WNT signaling, but may also be the consequence of modulation of other pathways. On the other hand, *AXIN2* and *LEF1* mRNA levels are, overall, maintained. Moreover, we could not correlate expression oscillations upon NRARP overexpression with the functional results obtained.

Since β -catenin is a key player in WNT signaling, its levels could constitute a more rigorous measure of the activation of this pathway. Therefore, we wanted to explore if its levels could be altered when NRARP is overexpressed. We analyzed protein levels of β -catenin phosphorylated at Ser675. This modification induces β -catenin accumulation in the nucleus, increasing its transcriptional activity (Hino et al., 2005). Interestingly, we observed a decrease in phospho- β -catenin in some cell lines (MOLT4, HPB-ALL and DND41) but no differences in others (Loucy and TALL1). In addition, our preliminary results on total β -catenin protein levels showed that it is decreased in CEM cells, upon NRARP overexpression, and no differences were verified in Jurkat cells. Overall, these results suggest that WNT signaling may be downregulated by NRARP in cells in which NRARP decreases growth, whereas it seems to have no major effect on the pathway in cells that benefit from NRARP overexpression. Exploring other targets of this pathway may give more conclusive hints about NRARP involvement in WNT signaling in T-ALL.

Putting all our results together, we find that, when *NOTCH* is mutated in T-ALL cells, NRARP overexpression decreases NOTCH signaling (and possibly also WNT signaling), delaying cell proliferation/expansion and having an anti-leukemic effect. On the other hand, in cells without *NOTCH* mutations (that do not rely on NOTCH oncogenic signals), NRARP overexpression promotes leukemia cell proliferation, despite inhibiting NOTCH (and possibly having no major effect on WNT signaling) (Table 3.1).

Thus, our studies interestingly suggest a dual role for NRARP, capable of decreasing NOTCH signaling independently of NOTCH mutational status, but likely modulating other pathways in order to act either in a tumor-suppressor-like manner (in NOTCH mutated cells), inhibiting proliferation of T-ALL cells, or in an oncogene-like manner (in NOTCH wild-type cells), promoting proliferation.

An obvious candidate to be modulated by NRARP in addition to NOTCH is WNT pathway, but further studies are required to confirm whether NRARP role promoting T-ALL is actually related to WNT signaling.

Our studies may have important therapeutic implications. Using NRARP as therapeutic tool, for example delivering recombinant NRARP protein to T-ALL patients harboring *NOTCH* mutations, could decrease disease burden, improving patients' outcome. Patients with resistance to GSIs could benefit considerably from a strategy as described above. Moreover, a therapeutic approach combining NRARP protein with GSIs could allow the reduction of GSIs' dose, lowering the severe side effects associated to this therapy and increasing the anti-leukemic effects, due to the combined action of both drugs. However, using the same strategy in T-ALL patients without *NOTCH* mutations, could promote T-ALL development, impairing patients' survival. Further studies need to be performed in order to understand NRARP therapeutic potential.

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