UNDERSTANDING THE MOLECULAR MECHANISMS INVOLVED IN NOTCH1 INDUCED T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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“E’ il tempo che tu hai perduto per la tua rosa che ha fatto la tua rosa così importante”

Antoine De Saint-Exupéry, Le Petite Prince

E la mia Rosa, è per te.

Agraïments

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Abstract

T-cell acute lymphoblastic leukemias (T-ALLs) are hematologic tumors affecting T-cells. Notch pathway is essential during T-cell development and its misregulation is related to T-ALL, nonetheless the downstream events that support Notch-dependent leukemia are not completely characterized. We previously demonstrated that Notch-Hes1-CYLD-NFkB axis is crucial for T-ALL. Still, the contribution of each one of those elements wasn't assessed. Here, we demonstrate that Hes1 activation and CYLD repression are needed events in Notch-dependent leukemogenesis. Notch and β-catenin pathways collaborate in several systems; thus we evaluated the contribution of B-catenin in T-ALLs. Notch1 is unable to develop leukemia in absence of β-catenin. C-Myc is an important Notch1 effector in T-ALL, and β-catenin target. We found that Notch1 regulation of C-Myc is impaired in β-catenin deficient cells, and that both Notch1 and β-catenin regulate its promoter. Nonetheless, c-Myc over-expression is not sufficient to restore the ability of β-catenin null cells to develop leukemia; conversely β-catenin is needed for the activation of a subset of Notch1 target genes.

Resum

les leucèmies limfoblastiques agudes T (LLA-T) son tumors de la sang, que afecten les cèl·lules T. la via de senyalització de Notch te un paper essencial en el desenvolupament de les cèl·lules T i la seva desregulació es relaciona amb les LLA-T, tot i així els factors que regulen les leucèmies induïdes per Notch1 no s'han caracteritzar completament. El nostre grup va demostrar anteriorment que la cascada Notch1-Hes1-CYLD-NfkB es crucial per les LLA-T, encara que la contribució de cada element no es va definir. En aquest treball demostrem que l'activació de Hes1 i la repressió de CYLD son passos essencials en les leucèmies que depenen de Notch1. Les vies de senyalització de Notch i de β-catenin col·laboren en molts sistemes, per això vam decidir estudiar una possible contribució de β-catenin en les LLA-T. Notch1 no es capaç de desenvolupar leucèmia en absència de β-catenin. C-Myc en un element important per les leucèmies que depenen de Notch1, i també es una diana transcripcional de β-catenin. Hem trobat que la regulació de c-Myc per part de Notch1 es troba desregulada en les cèl·lules que no tenen β-catenin, i que els dos factors poden actuar en la seva regió de promotor. No obstant, la sobre expressió de c-Myc no es suficient per restaurar la capacitat de les cèl·lules sense β-catenin de desenvolupar leucèmies. Per altra banda, β-catenin resulta necessària per l'activació de tot un grup de gens dianes de Notch1.

Prologue

The work presented in this thesis has been supervised by Dr. Anna Bigas and Dr. Lluís Espinosa; It has been performed in Institute Hospital del Mar d'Investigacions Mèdiques IMIM, in Barcelona, where I have been working as Master and PhD student between March 2008 and June 2013.

During those 5 years that I spent in Dr. Anna Bigas and Dr. Lluis Espinosa laboratory I have been working on several projects. They were all addressing different questions of the leukemia process in specific types. I consider that all of them have been good opportunities for me to learn about different aspects of this disease, as well as to acquire experience in a wide variety of techniques. Nonetheless, I decided to focus this manuscript only on two specific questions, and thus only on part of the work that I performed. I have chosen to do this, to avoid mixing different subjects and present preliminary un-related data. Thus, the work I presented here has been performed mainly between 2010 and 2013.

T-cell Acute Lymphoblastic Leukemia (T-ALLs) are aggressive hematological tumors with still poor outcome, thus improvement of therapies for this disease is nowadays imperative. Notch pathway is aberrantly activated in the majority of T-ALL cases, but current available therapies targeting Notch present high toxicity and low efficiency. In this background, a better understanding of the molecular mechanisms involved in Notch1 induced T-ALL is a

crucial issue. In this work we demonstrate the importance of the Hes1 – CYLD - NfκB axis downstream of Notch1 during T-ALL generation. Furthermore, we prove the involvement of β-catenin in T-cell leukemia.

During the development of this work we have collaborated with Pr. Iannis Aifantis (New York University School of Medicine in NY, USA) and Pr. Stephen Blacklow, (Dana Farber Cancer Institute in Boston, MA, USA).

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1. INTRODUCTION

1. INTRODUCTION

1.1 The Hematopoietic system

The hematopoietic system is composed by several cell types. They are maintained in a tightly regulated equilibrium and generated by a process called Hematopoiesis (from the ancient greek: αιμα, haima=blood and ποιεω, poieo= to make). Deregulation of the hematopoietic system can lead to different pathologies including Leukemia.

1.1.1 Hematopoietic Stem Cells and their regulation

More than 50 years ago was firstly demonstrated that blood cells derive from a common ancestor called Hematopoietic Stem Cell (HSC) (McCulloch and Till, 1960). In adult mammalian organisms HSCs reside in the bone marrow, nonetheless they are generated during embryonic development (Muller et al., 1994), reviewed in (Bigas et al., 2012). Thus, adult mammalian hematopoiesis relies on the HSC population generated during embryonic life.

HSCs are characterized by the functional ability of maintain the whole hematopoietic system. Several laboratories around the world have contributed to the characterization of HSCs. For example,

thanks to the identification of cell-surface markers we can isolate subpopulations of cells that are enriched of HSCs. The lineage markers (CD3, B220, Mac-1, Ter-119, Gr-1) that identify the differentiated cells have been used to select the lineage negative (lin-) population, which contains hematopoietic progenitors and HSCs. In the mouse, the expression of surface molecules such as c-kit and Sca1, within the lin- population, identify the LSK cells (for Lin- sca1+kit+) highly enriched in HSCs (Okada et al., 1992). Furthermore the use of CD150 and CD48 markers has allowed the purification of cells with increased capacity for long-term repopulation of the hematopoietic system (CD150+CD48-) (Goodell et al., 1996). In the bone marrow HSCs co-exist with their hematopoietic progeny, mesenchymal cells, endothelial cells and osteoblasts (Zhang et al., 2003), and reviewed in (Isern and Mendez-Ferrer, 2011). All these cells together constitute the niche for HSCs that regulates their behavior (Lemischka, 1997), (Quesenberry et al., 1994) by cell-cell contact and by secreted factors. The importance of the microenvironment in regulating HSCs was first proposed by Schofield in 1978 (Schofield, 1978), but nowadays many questions still remain. One example of niche-HSC interaction is the secretion of Stem Cell Factor (SCF) by the osteoblasts. SCF binds to and activates the tyrosine kinase receptor KIT (also referred as CD117 and used as an HSC marker, as previously mentioned) expressed on the surface of HSCs and progenitors and regulates their functions (Zsebo et al., 1990) and reviewed in (Kent et al., 2008). Many cell-niche interactions are important for the HSC regulation such as the Ang-1/Tie2 signaling

pathway which plays a crucial role in the maintenance of quiescent state (Arai et al., 2004). Other HSC regulators are the N-cadherin pathway, B1 integrins as well as Notch and Wnt pathways.

1.1.2 Hematopoietic differentiation

The blood is composed by many different cell types (Figure 1) generated from the HSCs.

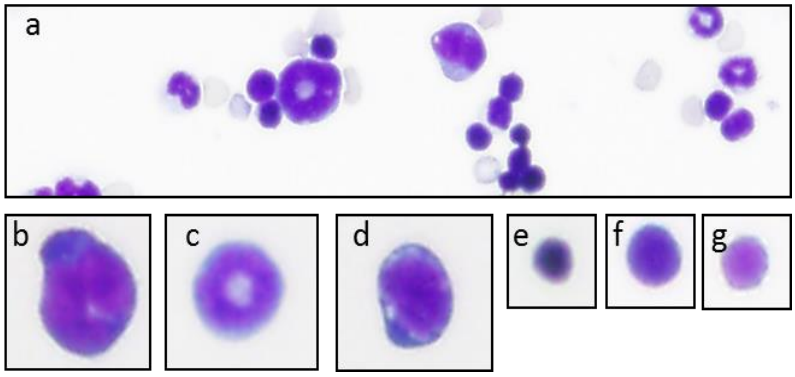


Figure1. **Giemsa staining of murine bone marrow cells.** Panel (a) shows total bone marrow, while panel from (b) to (g) represent respectively a megakaryocyte, macrophage, erythroid precursors (d) and (e), a B-lymphocyte, and T-lymphocyte.

During many years the prevalent model to explain hematopoietic differentiation has been the classical myeloid–lymphoid dichotomy differentiation model (see Figure 2, panel a). According to this model there is a common progenitor of all lineages, the MPP, that differentiates into a MECP (Myeloid-erythroid common progenitor) or a CLP (Common Lymphoid progenitor) that generatesT and B lymphocytes. The base of this model comes from the identification

by Weissman and colleagues in the 1997 of a cell being Lin(-)IL-7R(+)Thy-1(-)Sca-1loc-Kit(lo) that was able of generate both T and B lymphocytes, but unable to give rise to myeloid cells (Kondo et al., 1997).

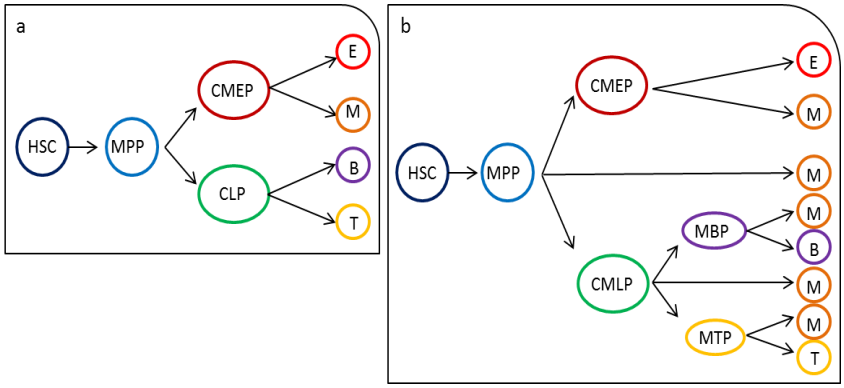


Figure2. Hematopoietic differentiation models. The classical model (a) postulates that early during the differentiation MPP differentiates towards the lymphoid or either the myeloid lineage. According to the myeloid-based model (b), the myeloid potential is then maintained in the T and B progenitors even after they diverge.

Recently, many evidences are in contrast with this classical model. In 1997, Kawamoto and coworkers studied the hematopoietic progenitors in the fetal liver, and they failed to identify any cell similar to CLP, that was able to generate B and T cells (Kawamoto et al., 1997). In 2005 a similar result was obtained in Jacobsen's laboratory when they identified the common myeloid-lymphoid progenitor (CMLP) from bone marrow cells (Adolfsson et al., 2005). The observation that T cell precursor in the thymus still retain myeloid potential but not B-cell potential (Porritt et al., 2004; Lu et al., 2005; Wada et al., 2008) also support the alternative model for the differentiation of the hematopoietic progenitor cells

(HPC). Nonetheless, this T-cell precursor only can be re-directed to other lineages *in vitro*, but not under physiological conditions. According to this so called myeloid-based model (Figure2 panel (b)), CMEP and CMLP are first generated from MPP, and some myeloid potential is maintained in the T and B progenitors; reviewed in (Kawamoto et al., 2010).

1.2 Leukemia

Leukemia is the hematological cancer. The term comes from the ancient greek λευκός leukos "white", and αιμα haima "blood", and means literally “white blood”. Leukemia is generally characterized by the amplification of a clone of transformed hematopoietic cells that overgrow and disrupt the homeostasis of the hematopoietic system. Cell transformation is a multi-step process in which different genetic alterations cooperate to impair the mechanisms that control cell growth, proliferation, survival, and differentiation during cell development.

Nowadays approximately 250,000 children and adults around the world develop leukemia every year and 200,000 die from it. Leukemia is the tenth most frequently occurring type of cancer. Leukemia incidence is among 7.4 and 13.3 per 100,000 USA citizens; (Data from WHO website and the American Cancer Society).

1.2.1 Classification

Leukemia is subdivided into different groups depending on their clinical and pathological features, such as their growth rate:

- Acute Leukemia is characterized by a rapid increase of immature blood cells.
- Chronic leukemia is characterized by a slower but excessive buildup of relatively mature, still abnormal, white blood cells.

Further subdivisions are made according to the lineages affected, which distinguish two main types: lymphoid and myeloid leukemia, affecting the lymphoid and myeloid cells respectively. Additionally, leukemia is classified depending on the age of the patient at the moment of the diagnosis. Thus, childhood leukemias are those diagnosed before 15 years of age and adult leukemias those identified thereafter. Recently, a new category has started to be identified: the Infant Leukemias, those diagnosed before the first year of age, which have special pathological and clinical features.
Using this classification, the four main categories that include the majority of the leukemias are specified below:

- **Acute lymphoblastic leukemia** (ALL) is the most common type of leukemia in young children, but also affects adults. The group includes B and T leukemia. 6,050 patients are diagnosed for this pathology is USA in 2012.

- **Chronic lymphocytic leukemia** (CLL) mainly affects male adults over the age of 55. 16,060 patients are diagnosed in USA in 2012.
- **Acute myeloid leukemia** (AML) occurs more commonly in male adults than in women or children. 13,780 patients are diagnosed in USA in 2012.
- **Chronic myeloid leukemia** (CML) occurs mainly in adults and small number of children. 5,430 patients are diagnosed in USA in 2012.

Prevalence data are from "Cancer Facts & Figures 2012", American Cancer Society, and "Facts, spring 2013", Leukemia and Lymphoma society.

1.2.2 Acute Leukemias: Lymphoblastic and Myeloblastic

Acute myeloid leukemia. AML is the most common form of acute leukemia among adults and accounts for the largest number of annual deaths from leukemia in the United States. It accounts for close to 14,000 diagnosed cases per year and 10,200 patients are estimated to die of the disease in 2013 (Siegel et al., 2012). The median age of diagnosis is 67 years, with 54% of patients diagnosed at 65 years or older. Thus, as the population ages, the incidence of AML along with myelodysplasia seems to be rising. Environmental factors that have long been established to increase the risks of myelodysplastic syndromes (MDS) and AML include prolonged

exposure to petrochemicals, solvents such as benzene, pesticides and ionizing radiation (Smith et al., 2004). Recent reports suggest that therapy-related MDS/AML may account for 5% to 20% of patients with MDS/AML (Leone et al., 2007). Most initial treatment decisions for AML are based on age, history of prior myelodysplasia or cytotoxic therapy, and performance status. Standard treatments are appropriate for patients younger than age 60 years. These regimens are based on a backbone of cytarabine and an anthracycline, and have an overall survival in the 60% to 70% range, still it variates consistently depending on the cytogenetic of the tumor (O'Donnell et al., 2012).

Acute lymphoblastic leukemia. ALL occur in both children and adults but its incidence peaks between 2 and 5 years of age. An estimated 6000 new cases of acute lymphoblastic leukemia are diagnosed yearly in the USA (Siegel et al., 2012). Relapse is the most important complication related to this disease. Cytogenetic and genomic profiling of samples collected at diagnosis and relapse shows substantial change, in most cases relapse often arises from a minor subclone with genetic alterations distinct from those of the predominant clone at diagnosis (Inaba et al., 2013). Treatment typically spans 2–2,5 years and comprises three phases: induction of remission, intensification (or consolidation), and continuation (or maintenance). Most of the drugs used were developed before 1970. Chemotherapy generally includes a glucocorticoid, vincristine, and asparaginase, with or without anthracycline. After remission, Intensification therapy is given to eradicate residual leukemic cells

(Pui et al., 2008). Continuation therapy typically lasts 2 years or longer and has the objective of avoiding relapse (Inaba et al., 2013).

1.2.3 Alterations causing Leukemia

Different types of alterations can be found in leukemia: point mutations, deletions, amplifications; however the most common genetic abnormality found in these tumors are chromosomal translocations. Their existence and their correlation with leukemic malignancy has been known for a long time (Stern, 1950; Nowell and Hungerford, 1960). Chromosomal translocations are rearrangements involving exchange of DNA between two non-homologous chromosomes; reviewed in (Nambiar et al., 2008). Two main kinds of translocations exist, resulting in different outcomes. One involves the juxtaposition of the coding region of one gene close to the promoter/enhancer region of another gene, hence leading to aberrant expression of the former. An example is the IgH-BCL2 translocation where BCL2 is the target gene whose expression level increases due to its delocalization near the immunoglobulin heavy chain (IgH) gene which is actively transcribed in B cells (Rabbitts, 1991). The second type occurs when two codifying regions are rearranged to generate a unique chimeric gene that codifies for a fusion protein. For example, the t(9;22) translocation results in the production of a fusion protein with aberrant functions and regulation composed by a part of the ABL gene on chromosome 9 and the other of the BCR gene on

chromosome 22 (Rowley, 1973). A broad range of genes has been found involved in this type of rearrangements, and more than 500 recurrent chromosomal aberrations are reported in hematologic malignancies. There are genes that are more commonly translocated and they can be cell type or leukemia type specific. One example of a translocation found in different leukemia subtypes is the MLL gene, which is rearranged in the 5-10 % of acute myeloid leukemia (AML), close to 85% of infant leukemia (ALL and AML), and 33% of therapy-related AML cases (Liu et al., 2009). The mechanistic generation of chromosomal rearrangements is mostly unclear; however it is well known that double-strand DNA breaks (DSBs) are essential intermediaries. The mechanism can be RAG-dependent, involving a wrong V(D)J recombination process (Lieber et al., 2006); when this is the case, the rearrangements are characterized by a clustered breakpoint distribution. Alternatively, an aberrant activity of the Topoisomerase II enzyme during DNA repair can be the cause, this mechanism requires the presence of a functional cleavage site and this type of rearrangements typically show a more diffusely clustered breakpoint distribution (Adachi et al., 2003). A less frequent mechanism involves the activity of apoptotic endonucleases, which "cut and paste" large DNA loops in open chromatin region; reviewed in (Nambiar et al., 2008).

1.2.4 Leukemic Initiating Cell

The existence of a specific population of leukemic cells that is called Leukemic Initiating cells (LIC) has been postulated for many years and different experimental evidences support their existence; however their relevance in human tumors or leukemia is still controversial. The term Leukemic Stem Cell was proposed from the studies of Bonnet and Dick in which they showed that only few human AML cells were able to undergo self-renewal and recapitulate the disease (Bonnet and Dick, 1997). Those results suggested that only a subset of the leukemic cells retained the key features of normal stem cells and were biologically distinct from the bulk of the leukemia. Other studies examined the cell cycle status of these LICs and found that both AML and CML LICs contain a quiescent cell population (Guan et al., 2003), which resemble the normal HSCs. The identification of LICs with specific markers has been the focus of different laboratories. In human AML, the LIC immunophenotype has been defined as CD34+, CD38–, CD71–, CD90–, CD117–, and CD123+ (Hope et al., 2003). Similarly, CML LICs are included in the CD34+, CD38– population (Holyoake et al., 2002). Research in mouse models has greatly contributed to our understanding of LICs, including the identification of the signaling pathways important for the generation and maintenance of LICs and leukemia. Also, serial transplantation experiments of leukemic cells carrying specific mutations have led to the identification of signals that are required for LIC self-renewal. Some examples include: β-catenin (Zhao et al., 2007; Wang et al., 2010), Hedgehog pathway

(Zhao et al., 2009) or PML (Ito et al., 2008). In addition, mechanisms controlling the cell cycle and DNA damage repair are important for LIC maintenance (Viale et al., 2009).

Since leukemia treatments mainly target dividing cells, the existence of quiescent LICs could be at the base of treatment failure and disease relapse after treatment. Thus, a better understanding of human LIC would likely provide new therapeutic opportunities for leukemia patients. Such approaches should be tailored to the unique biological properties of the LIC, while protecting the critical functions of normal HSC. Reviewed in (Gilliland et al., 2004).

1.3 T-cell development and T-ALL

1.3.1 T-cell development overview

T cell precursors originated in the BM colonize the thymus where they mature before being delivered into the blood circulation. Comparable to other hematopoietic lineages, their developmental stages are identified based on the expression of proteins present on the cell surface (Figure3). During their development, T-cells are exposed to different signals, being Notch one of the most important. After reaching the thymus, the Early thymic Precursor (ETP) CD3- CD44+ cKit+ is further specified following Notch signaling activation (Radtke et al., 1999); this cell is already primed towards T-cell fate, but still retains the potential for other lineages under

appropriate stimuli (Allman et al., 2003; Bell and Bhandoola, 2008; Ikawa et al., 2010). In the thymus, ETPs proliferate and go through different developmental stages in which rearrangement of T-cell Receptor (TCR) genes occurs (Rothenberg et al., 2008). Before the expression of the CD4 and CD8 molecules, T-cell progenitors are classified as double negative and several stages are distinguished by the acquisition of CD25 and CD44 antigens with the following sequence: DN1(CD44+ CD25-), DN2(CD44+ CD25+), DN3(CD44- CD25+), DN4(CD44- CD25-). By the DN3 stage, thymocytes are fully committed to the T-cell lineage and their further differentiation requires the expression of the pre-T-cell receptor (TCR). At this point they have to pass a critical developmental checkpoint termed β-selection (Michie and Zuniga-Pflucker, 2002) that consists in selecting cells that have been able to assemble a functional TCR.

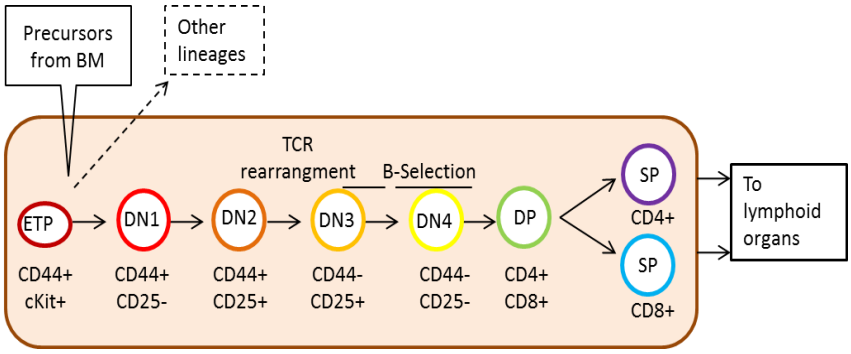


Figure3. T-cell development. T-cell precursors coming from the BM progenitors colonize the thymus, where they mature before being delivered to the lymphoid organs. The markers that define developmental stages are indicate below each cell type.

When successful recombination occurs, DN3 receive signals to survive and proliferate; reviewed in (Taghon and Rothenberg, 2008). After β-selection DN3 lose the CD25 marker and become DN4, which give rise to CD4+ CD8+ double positive (DP) thymocytes, which rearrange the TCR-α locus. They are then positively selected on the base of the affinity of their αβ TCR with MHC proteins presented on the thymic epithelium; this is followed by negative selection of cells that recognize self-peptides. The remaining T cells then proliferate and generate mature single positive (SP) CD4+ or CD8+ lymphocytes that are then directed to lymphoid organs; reviewed in (Yashiro-Ohtani et al., 2010). γδ T cells also develop from DN1 to DN3 stages, but do not undergo selection checkpoint in the same manner then αβ thymocytes (Hayes et al., 2005).

3.2.4 T-Acute Lymphoblastic Leukemia

T cell acute lymphoblastic leukemias (T-ALLs) are aggressive hematologic tumors affecting the T-cell lineage. They are characterized by infiltration of the bone marrow with immature T-lymphoblasts, which results in high white blood cell counts (Figure4), and the frequent infiltration of the central nervous system (CNS). T-ALL accounts for 10%–15% of pediatric and 25% of adult ALL cases (Pui and Evans, 2006); Nowadays, chemotherapy results in an overall survival rate of 75% for children and 40-50% for adults (Pui and Evans, 2006; Pui et al., 2008); nonetheless, T-

ALL patients with primary resistance or with relapsed leukemia still have a poor outcome (Goldberg et al., 2003).

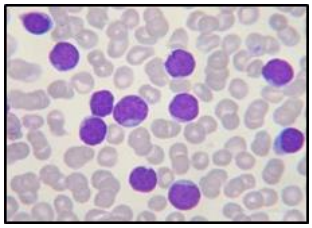


Figure4. Giemsa staining of T-ALL blast cells in blood.

Clinical treatments for T-ALL have been previously detailed and the choice of doses and timing protocols is based on age, leucocyte count and cytogenetic analysis. Special CNS-directed treatment is also useful to prevent relapse due to surreptitious leukemic cells (Inaba et al., 2013). T-cell transformation is a multistep oncogenic process in which multiple lesions involving different oncogenes and tumor suppressor genes cooperate to disrupt the normal circuitry that controls cell proliferation, differentiation and survival during T-cell development. T-ALLs are very heterogeneous and several kinds of lesions can be found with different frequencies. Table 1 includes the most common alterations found in T-ALL and their frequency, grouped by category. T-ALLs often present chromosomal translocations and intra-chromosomal rearrangements. These abnormalities typically juxtapose strong promoter and enhancer elements responsible for the high levels of expression of TCR genes close to genes that codify for transcription factors, resulting in their aberrant expression in the developing thymocytes (Kagan et al., 1989). Some of these genes include *HOX11/TLX1 (Dube et al., 1991), TAL1/SCL (Aplan et al., 1992), TAL2 (Xia et al., 1991),*

LYL1(Mellentin et al., 1989), *BHLHB1(Wang et al., 2000)*, *LMO1*, and *LMO2(Royer-Pokora et al., 1991)* . Deletions of CDKN2 that lead to a miss-regulated cell-cycle are also present in over 70% of T-ALL patients (Hebert et al., 1994). Other genes deleted in T-ALL encode for transcription factors that are important for T-cell development and/or act as tumor suppressors such as ETV6 (Van Vlierberghe et al., 2011), BCL11B (Wakabayashi et al., 2003), RUNX1 (Della Gatta et al., 2012) and PTEN (Palomero et al., 2007). Activating mutations can also occur in T-ALL, as those found in K-Ras or N-Ras genes (Bar-Eli et al., 1989) and in the Notch1 gene (this subject is extensively described in the next section). Genes involved in chromatin remodeling can also be altered in T-ALL: loss of- function mutations and deletions of EZH2 and SUZ12 genes, which encode two critical components of the Polycomb Repressive Complex 2 (PRC2) have been identified in up to 25% of T-ALLs (Ntziachristos et al., 2012).

Category	**Gene target**	**Alteration type**	**frequency**
bHLH Family members	TAL1 TAL2 LYL1 BHLHB1	TR t(1;14), t(1;7) / DEL TR t(7;9) TR t(7;19) TR t(14;21)	6%/16%-20% 1% 1% 1%
LMO Family members	LMO1 LMO2 LMO3	TR t(11;14), t(7:11) TR t(11;14), t(7:11) / DEL TR t(7;12)	1% 6% /3% <1%
Homeobox family members	TLX1 TLX3 HOXA NKX2.1 NKX2.2	TR t(11;14) TR t(11;14) TR t(7;7), t(10;11), t(11;19), DEL, INV TR t(7;14), INV TR t(14;20),	10%-30% 5%-20% 1%-5%/ 3% /3% 5% 1%
Proto-	c-MYB	TR t(6;7)	3%

oncogene			
Notch pathway	NOTCH1 FBWX7	TR/AM IM	<1% /> 50% 8%-30%
Cell cycle	CDKN2A/2B CCND2 RB1 CDKN1B	DEL TR t(7;12),t(12;14) DEL DEL	70% 1% 4% 1%
Transcription factors and tumor suppressors	MYC LEF1 ETV6 BCL11B RUNX1 GATA3	TR t(8;14) IM/DEL IM/DEL IM/DEL IM/DEL IM/DEL	1% 10%-15% 13% 10% 10%-20% 5%
Signal transduction	PTEN NUP214-ABL1 EML1-ABL1 ETV6-ABL1 BCR-ABL1 NRAS NF1 JAK1 ETV6-JAK2 JAK3 FLT3 IL7R	IM/DEL AMPL TR t(9;14) TR t(9;12) TR t(9;22) AM IM/DEL AM TR t(9;12) AM AM AM	10%/20% 4% <1% <1% <1% 5%-10% 3% 4%-18% <1% 5% 2%-4% 10%
Chromatin remodelling	EZH2 SUZ12 EED PHF6	IM/DEL IM/DEL IM/DEL IM/DEL	10%-15% 10% 10% 20-40%

Table1. Common lesions found in T-ALL, grouped by category. The frequency and type of each lesion is indicated. TR= translocation, DEL=deletion, AMPL= amplification, IM= inactivating mutation, AM= activating mutation. Adapted from (Van Vlierberghe and Ferrando, 2012).

3.3 The Notch pathway

The Notch locus was first identified and named by Morgan in Drosophila Melanogaster as responsible for a mutation that resulted in notches in the wing margin of the fly. In 1983 this gene was cloned and sequenced by Dr. Artavanis-Tsakonas (Artavanis-Tsakonas et al., 1983) in Drosophila . The first mammalian Notch (Notch1) was identified in 1991, when it was cloned from a chromosomal translocation present in a patient with T-lymphoblastic leukemia (T-ALL) (Ellisen et al., 1991). These authors characterized the expression of Notch1 in human and mouse tissues and found that is was abundantly expressed in lymphoid cells. Consequently, they proposed that this gene might be important for normal lymphocyte function and that its alteration could play a role in the pathogenesis of T cell neoplasms. Further studies proved this prediction to be right and demonstrated that Notch activity is essential for T-cell development but also plays a pivotal role in T-cell transformation.

1.4.1 Notch receptors

Mammals have four paralog genes codifying for four Notch receptors (Notch1-Notch4) with partially redundant functions. They are large single-pass trans-membrane receptors composed by an extracellular (ECD), a transmembrane (TMD) and an intracellular domain (ICD). Figure 5 shows the structure of Notch receptors.

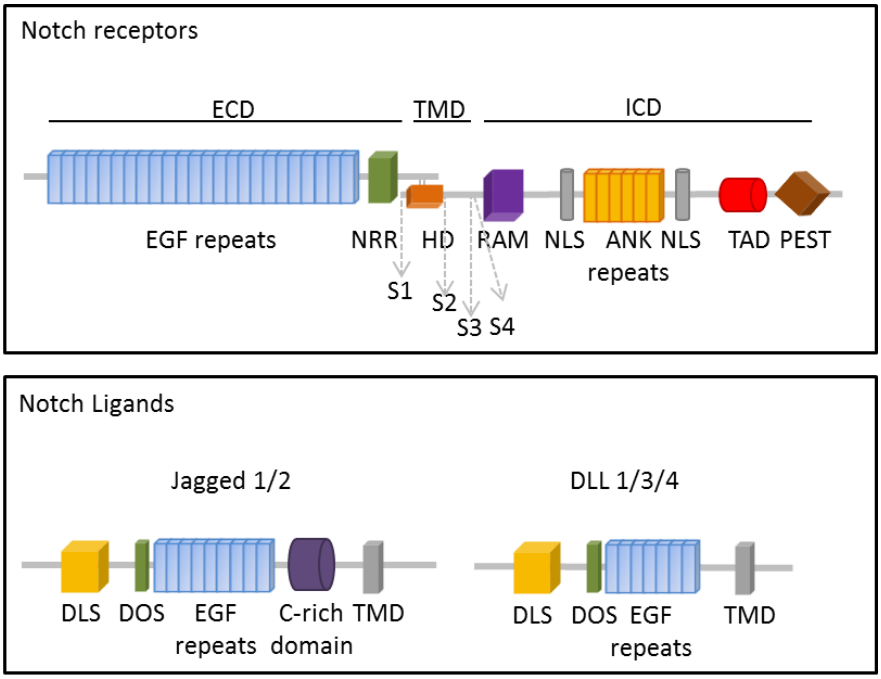


Figure5. Upper panel shows the structure of Notch receptors. They are composed by an extracellular (ECD), a transmembrane (TMD) and an intracellular domain (ICD). S1-S4 indicate the position of protolithic cleavages that the receptors suffer to be activated. Boxes represent different domains: NRR (negative regulating Region), HD (Heterodimerization), RAM (RBP-association-module), (NLS) Nuclear locatilization signal, (ANK) Ankirin repeats, (TAD) Transcription Activating domain, (PEST) proline/glutamic acid/serine/threonine-rich motifs.
Lower panel shows the structure of Notch ligands. Boxes represent different domains: DSL (Delta/Serrate/LAG-2), DOS domain (Delta and OSM-11-like proteins), Cystein-rich domain (CRD).

To become functional, the receptor is first cleaved in the Golgi to generate two fragments that remain attached through disulfide bridges. The C-terminal part includes the extracellular domain of this “hetero-dimeric” receptor whereas the N-terminal part contains the TMD and the ICD. The extracellular domain of Notch contains 29-36 EGF repeats in tandem that regulate ligand-receptor interaction. Several EGF repeats bind to calcium ions, which play

an important role in determining the affinity of receptor-ligand binding (Cordle et al., 2008). Tandem EGF repeats are followed by a unique NRR (negative regulating Region), which prevents ligand-independent activation of the receptor. The TMD ends with a "stop translocation" signal composed by 3–4 arginine/lysine residues, which is required for stopping the translocation of the receptor to the membrane. The ICD is composed by the RBP-association-module (the RAM domain), two Nuclear localization signals (NLS), seven ankyrin repeats, a poorly conserved Transcription Activating domain (TAD) and a conserved proline/glutamic acid/serine/threonine-rich (OPA/PEST) motif in the C-terminus end. OPA/PEST is a conserved domain which harbor degradation signals that regulate ICD stability.

1.4.2 Notch receptor modifications

In addition to their proteolytic cleavage, Notch receptors suffer different post-translational modifications in the Golgi apparatus and the endoplasmatic reticulum before being exposed on the cell surface. EGF repeats can be fucosylated by the O-fucosyltransferase Pofut1, a modification that is not essential for proper folding of the receptor (Stanley, 2007; Okajima et al., 2008) but is needed for the subsequent Fringe glycosyltransferase activity. Fringe enzymes extend the O-fucose glycosylation with additional sugar moieties. These modifications can modulate the affinity of the Notch receptors to different ligands. For example, the Fringe-mediated addition of a single N-acetyl-glucosamine in EGF repeat 12 of the

Drosophila Notch enhances receptor binding to Delta but reduces its binding to Serrate (Xu et al., 2005). Similarly, work done in Dr. Guidos' laboratory showed that Fringe-dependent modification of murine Notch receptor enhances the Delta-to-Notch signaling in T cells and limits Jagged-to-Notch signaling (Visan et al., 2006). The presence of three mammalian Fringe paralogs (Lfng, Lunatic fringe; Mfng, Manic fringe; and Rfng, Radical fringe) has complicated the elucidation of their specific functions. Another Notch modifier, the glycosyltransferase RUMI, has been recently identified in Drosophila (Acar et al., 2008) and in mammals (Fernandez-Valdivia et al., 2011). RUMI adds O-glucose to serine residues in the EGF repeats of Notch carrying the consensus sequence C1-X-S-X-P-C2; its deficiency results in embryonic lethality at E9.5 in mice, suggesting impaired Notch signaling.

1.4.3 Ligands

Notch ligands are trans-membrane proteins that contain a large extracellular domain, involved in receptor binding, and a much shorter intracellular domain with still unknown function; reviewed in (D'Souza et al., 2008). There are five Notch ligands in mammals: JAG1 and JAG2, containing a Cystein-rich domain (CRD) and DLL1, DLL3 and DLL4 lacking the CRD. They conserved structural motifs: an N-terminal DSL (Delta/Serrate/LAG-2) motif, the DOS domain (Delta and OSM-11-like proteins) containing tandem EGF repeats and EGF-like repeats (Komatsu et al., 2008). Both the DSL and DOS domains are extracellular and are involved

in receptor binding. The trans-membrane and intracellular domains are located at the C-terminus (Figure 5, lower panel).

1.4.4 Regulation of Notch ligand-receptor interaction

Physical association between Notch and its ligands is strictly regulated. On one hand, availability of both ligands and receptors at the cell surface of adjacent cells, which can be spatially and temporally restricted, is a key element in controlling Notch activation. The amount of receptors and ligands on the cell surface greatly depends on their trafficking and endocytosis. Thus, ubiquitination of Notch, which is mainly regulated by Deltex, Nedd4 and Su(Dx) can drive Notch receptor trafficking toward lysosomal degradation or recycling, thereby impacting receptor half-life; reviewed in (Le Borgne et al., 2005). On the other hand, ligand endocytosis that is triggered by the monoubiquitination catalyzed by the E3 ubiquitin ligases Neuralized and Mindbomb, is absolutely required for the generation of a functional ligand, although this process is poorly characterized (Koo et al., 2005; Koutelou et al., 2008). Also, and as mentioned before, Notch receptor modification by fringe is a crucial mechanism that regulates the specificity of Notch ligand-Receptor interactions.

1.4.5 Signaling activation

The first step for the activation of the Notch signaling is the binding of the receptor to the ligand present in the membrane of a

neighboring cell (Figure 6), which induces a conformational change that exposes the S2 site to the ADAM metalloproteases and permits its cleavage (Brou et al., 2000) (Mumm et al., 2000). Ligand independent Notch signaling occurs when mutations or deletions that affect the NRR generate a receptor that is permanently sensitive to γ-secretase activity (Shah et al., 2005). Notch cleavage by the γ-secretase complex involving site 3 (S3) and 4 (S4) results in the release the ICD. The product of S4 cleavage, starting at the Val1744 of NICD is the most abundant species of cleaved Notch1, (Schroeter et al., 1998) and it is also called active Notch1. The γ-secretase complex is composed of four Proteins: Presenilin, NCT (Nicastrin), Pen2, and Aph1 in a 1:1:1:1 stoichiometry (Sato et al., 2007). Those proteins exist in different isoforms in mammals and their function and regulation is largely unknown. Once NICD is released to the cytoplasm, it can translocate into the nucleus; there, NICD cannot directly bind DNA and it requires the DNA binding protein CSL (also called RBPj or CBF1). NICD interacts with CSL through a conserved WxP motif in the RAM domain. Despite some sequence divergence, all four mammalian NICD RAM domains interact with CSL with a similar affinity of 200 nM (Lubman et al., 2007). In the absence of Notch, CSL can function as a transcriptional repressor interacting with co-repressors as SMRT and HDAC-1 (Kao et al., 1998). In the presence of Notch, the repressors are displaced, and other co-activators are recruited to the Notch-CSL complex, such as Mastermind (MAML) that stabilizes the Notch-CSL interaction and also enhances target genes

expression by recruiting Histone acetyl transferases, co-activators and remodeling enzymes (Fryer et al., 2004).

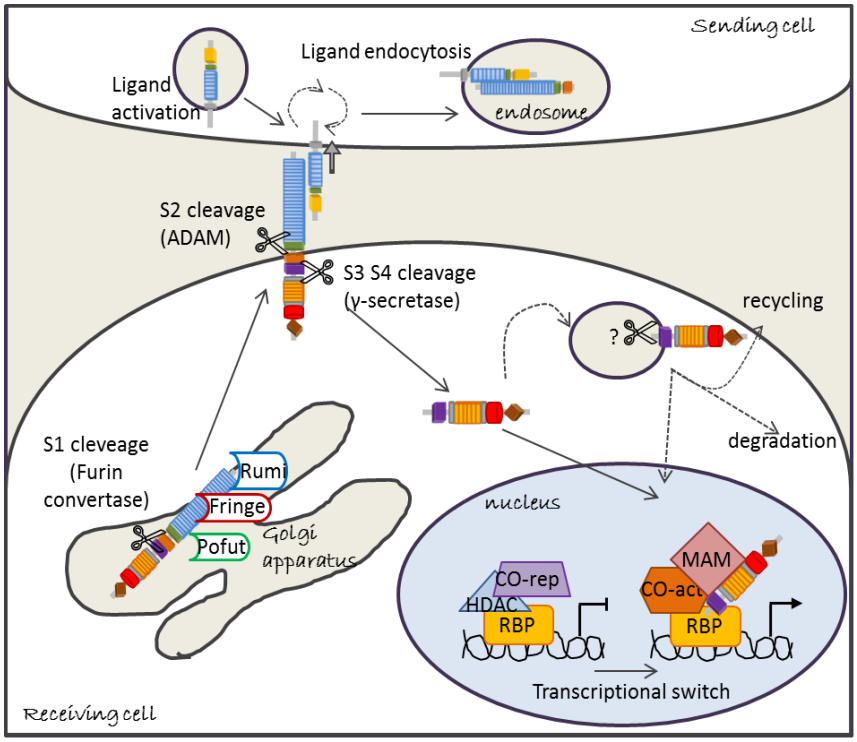


Figure 6. The Notch pathway. Notch receptors are processed in the Golgi apparatus before being exposed on the membrane. After the binding of the Notch receptor to the ligand, the receptor suffers sequential proteolitic cleavages and the ICD is released inside of the cell. ICD is then translocated into the nucleus where interacts with CSL, displacing co-repressors and thus activating transcription.

1.4.6 Notch target genes

The best-characterized and conserved Notch targets are the Hair Enhancer of Split (HES) and Hes-related genes (HRT). They include two families of helix-loop-helix (HLH) transcription factors. The Hes family comprises 7 members, from Hes1 to Hes7,

being Hes1, Hes3, Hes5 and Hes7 Notch target genes (Jarriault et al., 1995; Ohtsuka et al., 1999; Iso et al., 2003). HRT family comprises three members, HEY1, HEY2 and HEY3. Both, Hes and HRT proteins are transcriptional repressors that function through their HLH domain (Iso et al., 2003). There seems to be two kinds of Notch target genes: the Hes/Hrt family which is activated in most cells and tissues . In contrast, there is growing evidences indicating the existence of a second group of context-dependent Notch-targets genes that participate in specific cell responses. Some examples of the latter are: Gata2 (Robert-Moreno et al., 2005; Guiu et al., 2013), Gata3 (Amsen et al., 2007), IL7R (Reizis and Leder, 2002), c-Myc (Weng et al., 2006), cdkn1a (Rangarajan et al., 2001). Recently, our group demonstrated that Gata2 is regulated in the embryonic aorta through an incoherent feed forward loop in which Notch1 activates its transcription while Hes1, which is also downstream of Notch1, promotes its transcriptional repression. The fine regulation obtained with this mechanism is essential for HSC generation during mice development (Guiu et al., 2013).

1.4.7 Notch dimerization

The DNA-binding protein CSL recognizes the consensus sequence GTGGGAA in the DNA (Tun et al., 1994) which characterizes Notch-target genes. Several Notch targets contain more than one CSL binding site on their regulatory region. The presence of those sequences at a certain distance in a head to head position promotes the formation of higher-order CSL/ ICN/MAML complexes. In

1995, this mechanism was first observed in different Drosophila genes (Bailey and Posakony, 1995). Further studies performed by Dr. Blacklow's group showed that the cooperative binding between two CSL/NICD/MAM complexes is mediated by direct interaction between the ANK domains of the Notch proteins (Nam et al., 2007). This fact explains the importance of the proper orientation and spacing (15-22 bp) between adjacent CSL binding sites (Cave et al., 2005).

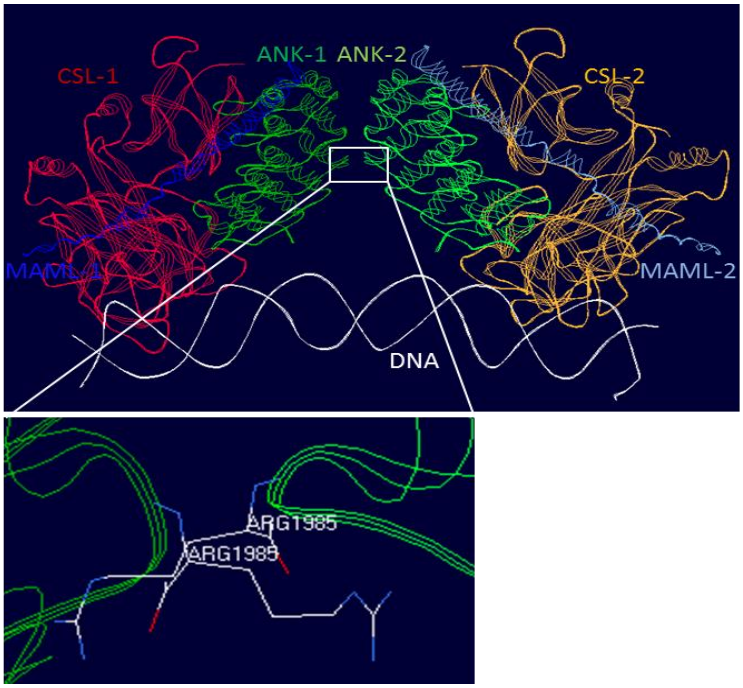


Figure7. Image of the crystal stucture of dimeric Notch complex on the DNA. Green chains are the ANK domain of Notch1. Red and Orange chains represent CSL, while the light and dark blue chains represent a portion of MAMl. Lower panel is a magnification of the region of interaction of the two ANK, and the ARG1985 aminoacid is shown. The figure has been made with the use of the Deep-viewer program.

In 2010, Kelly Arnett in Dr. Blacklow's laboratory crystallized and described the molecular structure of the dimeric CSL/ICN/MAML complex bound to the DNA (Arnett et al., 2010) (Figure 7). In this same work they identified the R1985 as crucial for maintaining the ANK/ANK interaction, as shown in figure7; mutation of this Arginine in Alanine (R1985A) impairs dimerization of the Complex. Interestingly, this R1985A Notch mutant protein was still able to drive T-cell development, but unable to induce T-ALL (Liu et al., 2010). The molecular explanation is that mutant Notch can still activate transcription of genes involved in T cell development such as CD25 or Hey1, but not of others such as c-Myc, pTα and Hes1. As predicted, the pTα promoter does contain coupled head to head and 16 bp spaced CSL binding sites however a comparable architecture has not been identified on the c-myc promoter, being its regulation still puzzling.

1.5 The Notch pathway in the Hematopoietic system and T-ALL

1.5.1 Notch pathway in HSCs regulation

Notch is required for the generation of the HSC during embryonic development (Kumano et al., 2003; Burns et al., 2005; Robert-Moreno et al., 2005; Robert-Moreno et al., 2008). However, whether the Notch pathway regulates the adult HSC compartment is

still under investigation, and published results are controversial. In fact, most of the data from loss of function mutants show that Notch is dispensable for maintaining HSCs and the integrity of the hematopoietic system, except for the T-cell lineage. For example, this is the result of conditionally deleting Jagged1, Notch1 alone or in combination with Notch2 (Radtke et al., 1999) (Mancini et al., 2005). To exclude that redundancy between receptors or ligands was responsible for the lack of phenotype observed in the analyzed mutants, Dr. Pear and colleagues took advantage of a dominant negative form of MAML, proved to be a potent inhibitor of canonical Notch transcriptional activity. They found that mice expressing dnMam do not show any significant hematopoietic abnormality indicating that Notch signaling is not required for adult HSC and HPs function (Maillard et al., 2008). This result is confirmed by studies using the Mindbomb knockout mouse (Kim et al., 2008) or the CSL/RBPj knockouts (Maillard et al., 2008). Nevertheless, gain-of-function experiments involving exposure of HSCs and progenitors to Notch ligands both *in vivo* and *in vitro* indicated that Notch activity could promote the expansion of hematopoietic progenitors (Stier et al., 2002), (Varnum-Finney et al., 2003). Moreover, recent experiments also showed that some fine-tuned regulation of Notch activity may be required to maintain the adult HSCs: the expression of weak Notch alleles amplifies, but then exhausts, the HSC compartment (Chiang et al., 2013).

1.5.2 Notch pathway in Commitment and differentiation

Although Notch seems to be dispensable for HSC functions, the Notch pathway is involved at various stages of hematopoietic lineage specification in the adult. The best-characterized hematopoietic function for Notch occurs during T-cell development, but several evidences suggest its involvement in the regulation of other lineages.

1.5.2.1 The role of Notch in T-cell development

The thymus contains the specialized environment needed for T-cell development; reviewed in (Maillard et al., 2005).

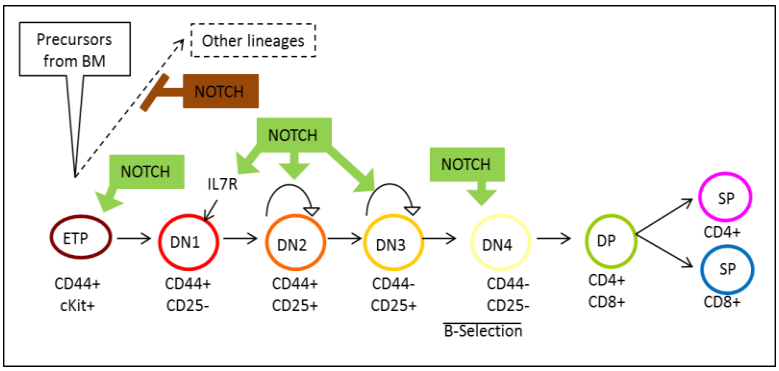


Figure8. The role of Notch signaling during T cell development. Boxes and arrows indicate requirement of the pathway in the different steps of the process. Markers that define developmental stages are indicated below each cell type. (Adapted from Sara Gozalez-Garcia, 2012).

Early stages of that process require Notch1 activation, which is delivered by the interaction of the receptors expressed in T-cell precursors with the ligands exposed on thymic stromal cells

(Hozumi et al., 2008b). The demonstration that Notch is required for T-cell development is supported by many different experiments and mouse models (Radtke et al., 1999) (Wilson et al., 2001) (Han et al., 2002).

Notch signaling is required at multiple stages of T cell development for proliferation, survival and differentiation (Figure 8).

Down-regulation of Notch signaling prior to β-selection results in the death of developing thymocytes (Ciofani et al., 2004) (Maillard et al., 2006). By co-culture experiments of thymocytes and stromal cells that express specific Notch ligands, Schmitt and co-workers demonstrated that the expression of Dll1 in the thymic cortex is needed for the correct development of DN2 (Schmitt et al., 2004). Using a similar approach, Dr. Zuñiga-Pflucker's laboratory discloses a role for Notch pathway both in αβ and γδ T Lineage specification (Ciofani et al., 2006). Notch-1, Notch-2 and Notch-3 receptors are all expressed in the thymocytes (Felli et al., 1999), being the Notch-1 the most expressed and the most differentially modulated throughout T cell development (Hasserjian et al., 1996). Different ligands are also expressed in the thymus (Hozumi et al., 2008a), although conditional deletion of Dll4 in the thymic stroma have provided conclusive prove that the Notch1/Dll4 interaction mediates the Notch signaling that is required for T-cell development (Wilson et al., 2001) (Koch et al., 2008a). In 2002, Dr. Zuñiga-Pflucker's group described a system that supports T-cells differentiation from undifferentiated BM progenitors (Schmitt and Zuniga-Pflucker, 2002). The establishment of this method, based on the co-culture of HPs on a Dll1-over-expressing OP9 stromal cell

line, was a breakthrough in the field and is still used to develop and to study T-cells *in vitro*.

Despite the importance of the Notch signal for T-cell development, the downstream effectors in this process are not completely identified yet. Hes-1 is an important Notch target for T-cell development; Hes-1 deficient embryos cannot form the thymus and Hes-1 deficient hematopoietic progenitors have impaired ability to make thymic T cells when transplanted into RAG-deficient mice (Tomita et al., 1999). Moreover, Dr. M. Luisa Toribio and collaborators demonstrated that Notch1 is responsible for IL7-Rα activation in *in vitro* developing T cells, and that Ectopic IL-7Rα expression can rescues defective proliferation due to Notch down-regulation, but cannot compensate Notch deficiency at the β-selection checkpoint (Gonzalez-Garcia et al., 2009). Other authors demonstrated that Notch1 activates transcription of the *pre-TCR α* chain gene (*pTa*) in developing thymocytes, in a CSL-dependent manner (Deftos et al., 2000; Reizis and Leder, 2002). The Akt pathway is also associated with the effects of Notch-1 at DN3, although the expression of active Akt (myr-Akt) cannot completely overcome the requirement for Notch in β-selection (Ciofani and Zuniga-Pflucker, 2005). Several groups have shown that *c-Myc* is a direct target of Notch in T-ALL (Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006). As c-Myc regulates proliferation, survival and metabolism in a wide variety of processes, it has been proposed that c-Myc could also participate of normal T-cell development downstream of Notch1. In agreement with this possibility, C-Myc transcription decreases simultaneously to

Notch1 as cells develop from the DN3a stage into the DN3b stage (Weng et al., 2006).

Myeloid cells. Work from different authors demonstrates that Notch pathway negatively regulates myelopoiesis both in vivo and in vitro (Stier et al., 2002). Exposure of HSCs and HPs to stromal cells overexpressing notch ligands impairs granulocyte generation (de Pooter et al., 2006); concordantly, excessive granulopoiesis has been observed in mice harboring a mutation that globally impairs fucosylation, (Zhou, 2012). Those data suggest an involvement of Notch in the generation of the GMP progenitors from their ancestors CMP. More recently, work from Iannis Aifantis' laboratory demonstrated that different Notch-loss of function mouse mutants also display an increase of GMP, but not of CMP, which results in a myeloproliferative syndrome (Klinakis et al., 2011). They identify different genes important for granulocyte/monocyte differentiation that are silenced by Hes1, downstream of Notch.

Dendritic cells. Dendritic cells (DCs) are also regulated by Notch activity. DLL1-mediated Notch signaling promotes the development of DCs at the expense of myeloid cells (Olivier et al., 2006). Moeover, CSL deletion in DC decreased the number of splenic and cytokine-secreting DC subset, a subpopulation that is localized adjacent to cells expressing the Notch ligand Delta-like 1 in the marginal zone (MZ) of the spleen (Caton et al., 2007).

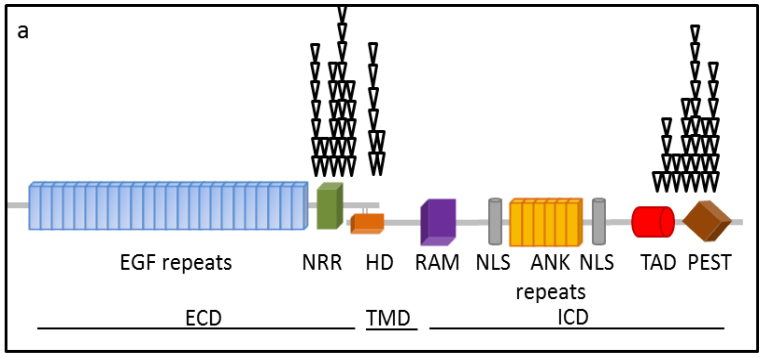
B cells. Generation of specialized MZ B cells in the MZ of the spleen requires the interaction between Notch2 and Dll1 (Tanigaki et al., 2002; Hozumi et al., 2004). Also, it has been proposed that Notch contributes to B cell activation (Santos et al., 2007). In this study, Santos and coworkers found increased frequency of antibody-secreting cells (ASC) in cultures of splenic B cells when exogenous Notch signalling was provided.

1.5.3 The Notch pathway in T-ALL

Consistent with the essential role of the Notch pathway during T-cell development and specification, constitutive Notch activation promotes malignant extra-thymic T-cells production and T-ALL (Pear et al., 1996). The cloning of human Notch from a translocation with the TCR from a T-ALL patient, (Ellisen et al., 1991), as mentioned in section4, pioneered a large plethora of studies to understand the role of Notch in T-cell leukemia.

1.5.4 Aberrant activation of the pathway

The first identification of aberrant Notch expression in human leukemia comes from 1991 (Ellisen et al., 1991), nonetheless the translocation of Notch with TCR described in that study or with other genes is extremely rare. In 2004 Jon Aster's group identified mutations on the Notch1 gene in 54 out of 96 primary TALL samples analyzed (56,2%) (Weng et al., 2004).



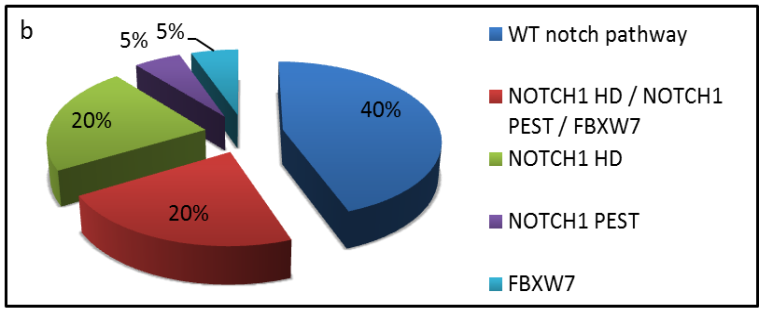


Figure9. (a) Mutations on the Notch1 gene identified in primary TALL samples. Triangles represent single mutation, and their position indicate the affected domain. (Figure adapted from Weng et al, 2004). **Many sample carry more then one mutation (panel b).** (Figure adapted from Van Vlierberghe et al, 2012).

Those mutations cluster in the HD and PEST domains (Figure9). Mutations in the HD domain (26% of the cases) generate a ligand-independent activating receptor or enhance γ-secretase cleavage and in both cases increase the production of ICN1. Mutations in the PEST domain (12,5 of the cases) generate a shorter protein with increased half-life. 17,7% of samples showed mutation in both HD and PEST domains with synergistic effects on Notch activity. This discovery was a breakthrough in the field and opened a new exploratory way to understand the T-cell disease.

Additional mutations can also contribute to achieve aberrant activation of the pathway. For example, mutations in FBXW7, an E3 ubiquitin-ligase that targets nuclear Notch1 to the degradation, are found in 15% of primary T-ALL patient samples sequenced (Thompson et al., 2007). Mutated FBW7 is unable to bind Notch1 and to induce its degradation, thus leading to the stabilization of the N1ICD. Around 20% of the T-ALL samples carry mutations both on Notch1 and on FBW7 genes (O'Neil et al., 2007). Although mutations on FBW7 gene can affect the stability of other important factors such as c-Myc (Welcker et al., 2004) and cycline E (Koepp et al., 2001), these findings further highlighted the importance of Notch pathway activation in T-ALL development.

The identification of Notch1 mutation in the neonatal blood spot of young T-ALL patients showed that these mutations can occur prenatally as an initiating event (Eguchi-Ishimae et al., 2008). On the other hand, Notch1 mutations are frequently found at T-ALL diagnosis but patients that relapse can contain different Notch1 mutant clones (Mansour et al., 2007), suggesting that Notch1 can function both as initiating and as secondary event in T-ALL progression.

1.5.5 Mouse models for Notch1 induction of T-ALL

In 1996 Warren Pear and collaborators firstly demonstrated that ectopic expression of an active form of Notch1 (N1IC) in murine hematopoietic progenitors was sufficient to drive cell transformation (Pear et al., 1996); when transduced cells were

transplanted to immunodepleted recipient mice, extra-thymic CD4+/CD8+ malignant T-precursors developed. This pioneer work preceded many transgenic mouse models that, together with the protocol that they describe, are used by many laboratories to study T-ALL.

Despite the robustness of the mouse N1IC models, different naturally occurring human Notch1 gain-of-function mutations have marked differences in their ability to induce T-cell transformation in murine cells (Chiang et al., 2008). Whereas, few *NOTCH1* alleles initiated a strong downstream signals that promote ectopic T cell development and induce leukemia, the more represented ones failed to efficiently initiate leukemia development. However, these weak active *NOTCH1* alleles accelerated the onset of leukemia initiated by active K-ras, and gave rise to tumors that were sensitive to Notch inhibition. These results indicate that Notch1 mutations differently contribute to T-ALL development. This assumption is also supported by data obtained by Dr. Dupuy's laboratory showing that human forms of full-length Notch1 with two frequent mutations, L1601P on the HD, and S2493X on the PEST domain, have a poor capacity to develop leukemia in mice, and only when the both mutations are present together (Berquam-Vrieze et al., 2012). Apart from mutations, other mechanisms have been found to induce Notch1 activation leading to T-ALL. Recently, it was decribed that Ikaros1 deficient mice expresses a ligand-independent Notch1 truncated form due to the alternative usage of an intragenic promoter, and thus develop T-cell leukemia (Gomez-del Arco et al., 2010). Moreover, intragenic deletions resulting in expression of 5'

truncated alternatively spliced forms of NOTCH1 have also been reported (Tsuji et al., 2003; Ashworth et al., 2010; Jeannet et al., 2010).

More recently, various mouse models have also been use to study the LIC activity in Notch1 induced leukemia. As exposed in section 2, Leukemic Initiating Cells (LICs) are proposed to be a subset of cells that propagate the leukemia. Recently, Dr. Pear's group purified different populations of cells from the BM of leukemic mice and transplanted these cells into secondary recipient animals; With this strategy they found that although most of the leukemic cells were CD4+CD8+, LIC activity was enriched in the CD8+CD4- HSA^{hi} T-cell subset (Chiang et al., 2013).

1.5.6 Downstream effectors

Although there is a general agreement for the role of Notch1 in inducing T-ALL, the required downstream events and effectors are still under investigation. Weng and collaborators firstly demonstrated that Notch1 induces c-Myc expression in T-ALL cell lines (Weng et al., 2006) and showed the direct binding of Notch1 on c-MYC promoter by Chromatin Immuno-precipitation (CHIP) and EMSA. This result was further confirmed by using gene expression array and ChIP-on-chip in T-ALL cell lines (Palomero et al., 2006). This study also showed that active Notch1 in T-ALL induces multiple anabolic genes involved in ribosome byosintesis, protein translation and metabolism. Notably c-Myc downstream of Notch1, promotes the expression of anabolic genes, and both Notch

and MYC dependent transcriptomes display a strong overlap (Palomero et al., 2006; Margolin et al., 2009). Although these results were obtained in cell lines, conditional deletion of c-Myc by CD4-Cre impaired the ability of N1IC to induce tumors in mice; thus confirming *in vivo* the requirement of Myc at the DP stage for N1IC-induced leukemia (Li et al., 2008). N1IC directly induces proliferation-related genes such as CCDN3, CDK4, CDK6 (Joshi et al., 2009). Concordantly, inhibition of Notch1 in T-ALL cell lines induces up-regulation of the cycline-dependent kinase inhibitors CDKN2D and CDKN1B (Rao et al., 2009). In addition, Notch1 activates the PI3K-AKT pathway in T-cell leukemia through the Hes1-mediated repression of PTEN, whereas mutational deletion of PTEN causes GSI-resistance in T-ALL cell lines (Palomero et al., 2007). Consistent with the importance of Hes1 as a downstream target of Notch in T-ALL, conditional deletion of Hes1 by Mx-Cre impairs Notch1-induced leukemia (Wendorff et al., 2010). In those experiments, Hes1 is shown to be required for both the initiation and the maintenance of the oncogenic program induced by N1IC. On the other hand, Hes1 sustains the activation of the NFkB survival pathway through Cyld repression. The mechanism of this activation is explained in detail in section 9.

1.5.7 Targeting Notch1 in T-ALL

The relevance of Notch1 activation in T-ALL has generated a great interest in the development of anti-Notch1 targeted therapies against

this disease. The effectiveness of γ-secretase inhibitors (GSI) on blocking proliferation of T-ALL cell lines (Palomero et al., 2006) (Weng et al., 2004), and primary T-ALL cells (Armstrong et al., 2009) (Tatarek et al., 2011) carrying activating mutation of Notch1, put forward the idea of testing the efficiency of γ-secretase inhibitors as a therapeutic agent. In this sense, a clinical trial was performed at the Dana Farber Cancer Institute to test the activity of MK-0752, an oral GSI, in T-ALL patients; in this trial, GSI treatment resulted in the down-regulation of Notch1 target genes in the T-lymphoblasts, however a manifest toxicity in the gut was observed, which was attributed to the inhibition of Notch signaling; furthermore patients did not get objective response to the treatment (DeAngelo et al., 2006). These disappointing results highlighted some of the challenges facing the development of anti-Notch1 targeted therapies for T-ALL: obtaining a more efficient inhibition of lymphoblasts proliferation and avoiding gut-related effects. In this sense, it has been shown that combination of GSI with glucorticoids could provide interesting results, as glucorticoids seem to induce down-regulation of Notch1 protein stability (Mo et al., 2011) and reduce intestinal toxicity (Real et al., 2009). Peptides acting as dominant negative of MAML1 can inhibit Notch signaling (Weng et al., 2003); a synthetic, cell-permeable and stabilized α-helical peptide of MAML1 dominant negative has been developed (Moellering et al., 2009); This peptide strongly inhibits Notch1 in human T-ALL cell lines, and in a mouse model of T-ALL. However, small peptides against MAML1 would still have the inconvenience of inhibiting signaling from all four Notch receptors.

Since gut toxicity by GSI is mostly due to the essential role of Notch pathway in regulating its homeostasis, it could be anticipated that therapies inhibit Notch1, but not all Notch receptors, or that block the specific ligands that activate Notch in the intestine might result in reduced side effects. Specific anti-Notch1 antibodies capable of blocking *in vitro* the leukemia-associated variants of the receptor have been recently developed, still their efficiency in vivo has not been tested (Aste-Amezaga et al., 2010).

1.6 Notch and β-catenin pathways interaction

The Wnt/β-catenin pathway (see section 7) participates in many cell fate decisions in which Notch plays an essential role, and there is evidence that both pathways interact at different levels. For example, in the hair Follicle β-catenin is upstream of the Notch pathway through the trascriptional activation of the Notch ligand Jagged1. Most important, generation of new hair follicles in the adult epidermis induced by β-catenin is prevented by pharmacological inhibition of Notch signaling or following *Jag1* deletion (Estrach et al., 2006). Similarly, results obtained by Veronica Rodilla in our laboratory demonstrated that Jagged1 expression was also induced by β-catenin in CRC and genetic deletion of Jagged1 in the APC^{min} background (a mouse model of intestinal cancer), strongly reduced tumor formation (Rodilla et al.,

2009). Conversely, cyclin/CDK inhibitor $p21^{WAF1/Cip1}$ is a direct transcriptional target of Notch1 in keratinocytes and down-regulates *Wnt4* gene expression, thus Notch acts a negative regulator of the Wnt signaling (Devgan et al., 2005).

Recently, Kwon and coworkers demonstrated that membrane-bound Notch physically associates with un-phosphorylated (active) β-Catenin and negatively regulates stability of the protein. (Kwon et al., 2011). This work was a follow-up from Martinez Arias' work in drosophila, which reported that full-lenght Notch regulates the activity and the amount of the active form of β-catenin independently of Notch cleavage and RBP-mediated transcription (Hayward et al., 2005).

Convergence of Notch and Wnt pathways has also been found in the arterial-venous specification (Yamamizu et al., 2010). In this system, a protein complex formed by RBP-J, ICN1, and β-catenin binds the RBP-J binding sites of the regulatory regions of several genes including Hes1, Dll4 and CXCR4. Unpublished results from our laboratory demonstrate that Notch1 and β-catenin collaborate to maintain the intestinal Stem Cells by triggering the transcriptional activation of Bmi1, c-Myc and EphrinB2 genes. Simultaneous requirement of Notch and β-catenin signaling for intestinal Stem Cells have been described in Embryonic Stem Cells (Ogaki et al., 2013).

Other interactions between Notch and Wnt include the existence of common regulators for both pathways. One example is our previous observation that GSK3β kinase, which is mainly responsible to

regulate β-catenin levels, was able to phosphorylate Notch2, leading to reduced Notch activity (Espinosa et al., 2003a).

system	Upstream element	Downstream element	Regulation	reference
hair Follicle	B-catenin	Notch	Positive. β-catenin activates the transcription of Jagged1.	(Estrach et al., 2006)
Colorectal Cancer	B-catenin	Notch	Positive. β -catenin activates the transcription of Jagged1.	(Rodilla et al., 2009)
	Notch	B-catenin	Negative. Notch1 post-translationally inhibits β -catenin.	(Kwon et al., 2011)
Skin	Notch	B-catenin	Negative. Notch1 activates the transcription of a negative regulator of Wnt4.	(Devgan et al., 2005)
Drosophila	Notch	B-catenin	Negative. Notch1 post-translationally inhibits β -catenin.	(Hayward et al., 2005)
arterial-venous specification	-	-	Convergence. Both are needed simultaneously for target genes transcription	(Yamamizu et al., 2010)

Table2. Summary of the publications about Notch and β-catenin pathways crosstalk.

In summary, Notch and Wnt pathways cooperate in different systems to achieve not just induction or inhibition of specific genes, but also a fine regulation of cell-fate decision and tissue homeostasis.

1.7 Wnt /β-catenin signaling pathway

Wnt/β-catenin pathway regulates multiple developmental processes such as embryonic axis formation, organogenesis, stem cell generation and maintenance, among others; most of these functions are conserved from flies to mammals. Consistent with this, aberrant activation of the pathway is strongly associated with different oncogenic processes.

1.7.1 Wnt ligands

WNTs are evolutionarily conserved secreted Cys-rich glycoproteins, and in mammals there are 19 genes that encode for different WNTs. They are characterized by a Signal peptide on the N-terminus that regulates their secretion and a Frizzled binding domain on the C-terminus (Nusse and Varmus, 1992). Despite being secreted, they are relatively insoluble and for this reason they only activate the receptor of neighboring cells (Willert et al., 2003). Specific WNTs preferentially activate either β-catenin-dependent or

β-catenin-independent pathways, and it has been demonstrated that WNT1, WNT3A and WNT8 are more commonly encountered in β-catenin-dependent signaling, whether WNT5A and WNT11 are predominantly involved in β-catenin-independent signaling (Kikuchi et al., 2011). However, the use of each of these downstream pathways also depends on the cellular context and the receptor used. Cellular secretion of WNTs requires the activity of Wls and Evi proteins, which are located in the Golgi apparatus. In absence of Wls/Evi, WNTs cannot be secreted and are retained inside the producing cell (Banziger et al., 2006; Bartscherer et al., 2006) .

1.7.2 Wnt receptors

Frizzled proteins (Fz) are seven-pass trans-membrane receptors that act as common receptors for both the β-catenin-dependent and β-catenin-independent pathways. Mammalians have ten genes encoding for Frizzled with different capacity for being activated by Wnts. On the other hand, the use of the LDL receptor-related proteins 5 or 6, (LRP5 and LRP6) lead to activation of the canonical pathway (He et al., 2004; Tamai et al., 2004), while ROR1 and ROR2 determine the alternative pathway activation (van Amerongen et al., 2008).

1.7.3 Canonical Wnt pathway: β-catenin-dependent signaling.

Canonical Wnt pathway is triggered by the interaction of WNT with Frizzled and LRP5 or LRP6. In absence of Wnt ligands, cytoplasmic β-catenin is phosphorylated by a complex composed by glycogen synthase kinase 3 (GSK3), adenomatosis polyposis coli (APC), Axin and casein kinase Iα (CKIα) (Lee et al., 2003) called destruction complex. CKI and GSK3 β phosphorylation induces ubiquitination-mediated degradation of β-catenin by the proteasome (Price, 2006). Upon Wnt ligand stimulation, the dimerization of the Fz receptor with a specific LRP co-receptor occurs, which results in the recruitment of Axin to the Fz receptor and causes the disassembly of the destruction complex (Lee et al., 2003). Recent data from Clevers' laboratory highlighted the possibility of triggering the canonical Wnt signal without the disassembly of the complex (Li et al., 2012). In the absence of a functional destruction complex, non-phosphorylated β-catenin is stabilized in the cytoplasm and can enter in the nucleus. The mechanism controlling this nuclear transfer is not clear, still it seems to be NLS-independent (Fagotto et al., 1998). Once in the nucleus, β-catenin does not bind the DNA directly, but associates with transcription factors such as TCF (T cell factor) and LEF (lymphoid enhancer-binding factor) to regulate the transcription of target genes (Behrens et al., 1996). Post-activation repression of the pathway requires that β-catenin is exported back to the cytoplasm, which also depends on

Axin (Cong and Varmus, 2004) and APC (Rosin-Arbesfeld et al., 2000).

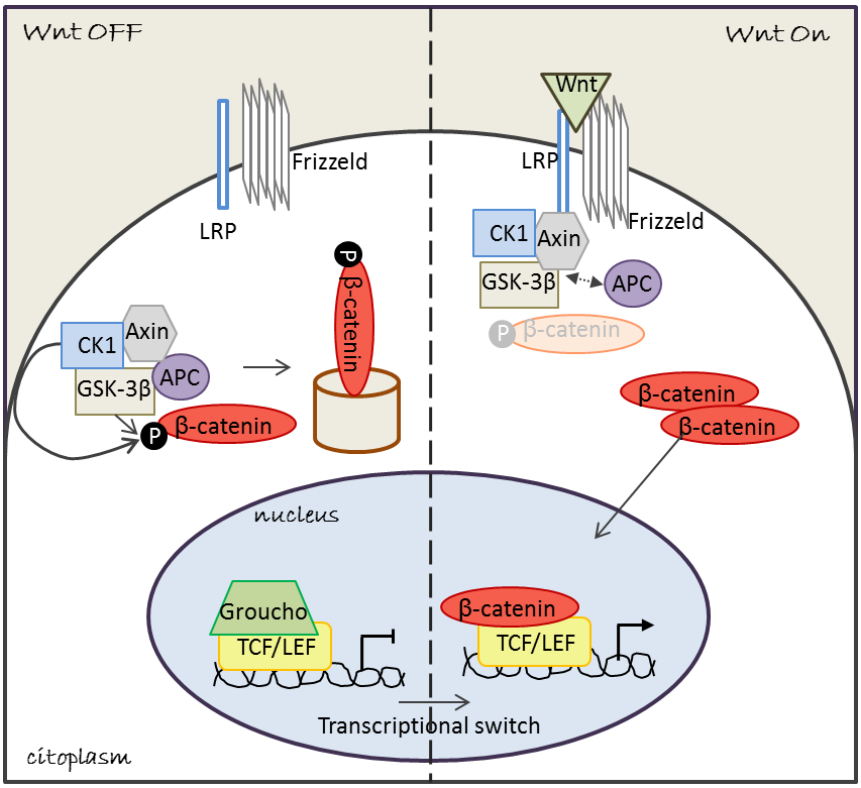


Figure10. Wnt/β-catenin pathway. In absence of Wnt ligands, β-catenin is phosphorylated by a destruction complex composed by glycogen synthase kinase 3 (GSK3), adenomatosis polyposis coli (APC), Axin and casein kinase Iα (CKIα). the phosphorylation induces degradation of β-catenin by the proteasome. Upon Wnt ligand stimulation, non-phosphorilated β-catenin is stabilized and translocated into the nucleus. Once in the nucleus, β-catenin associates with transcription factors TCF (T cell factor) or LEF (lymphoid enhancer-binding factor), to regulate transcription.

1.7.3.1 β-catenin

Before being described as mediator of Wnt canonical pathway, β-catenin was originally identified in the cell adherent junctions where it functions to bridge the cytoplasmic domain of cadherins to α-catenin and the actin cytoskeleton (McCrea et al., 1991; Hulsken et al., 1994). The most evolutionary conserved part of β-catenin protein is the Armadillo-repeats (ARM) domain (Figure 11 left panel), an approximately 40 amino acid long tandem repeat sequence that was first identified in the Armadillo gene, the Drosophila homologous of mammalian β-catenin (Huber et al., 1997; Graham et al., 2000). Most of the described β-catenin partners interact through the ARM domain (figure11, left panel) and many of them are critical for cell adhesion and Wnt signaling (Huber et al., 1997). C- and N-terminal domains of β-catenin are less conserved than the ARM domain. The N-terminus contains the phosphorylation residues recognized by GSK3 and CKIα kinases, while the Transcription Activation Domain (TAD) is in the C-terminus (Xing et al., 2008). A crystal structure of the full length β-catenin/Armadillo protein has only been obtained from the zebrafish β-catenin ortholog (Xing et al., 2008) (Figure 12) , but some crystalized fragments of the mouse and human protein can be found in the Protein Data Bank. Recently, the dimer β-catenin ARM-domain/LEF1 N-ter has been also crystallized (Sun and Weis, 2011).

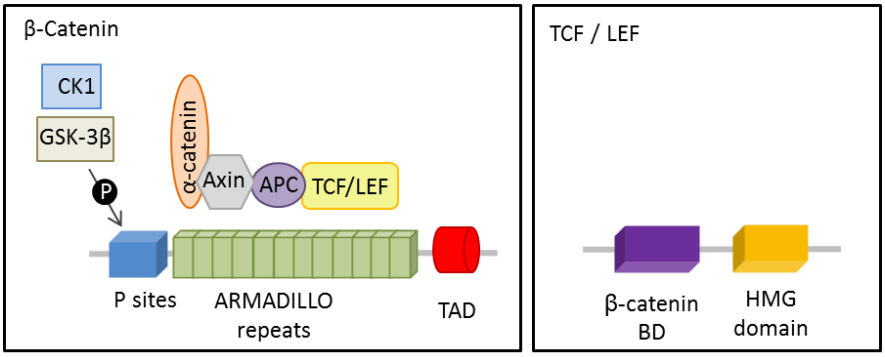


Figure11. (left panel) **β-catenin structure**. Boxes represent different domains: Phosphorilation sites (Psites), Armadillo-repeats (ARM), Transcription Activating domain (TAD). The position of the protein above indicates their interaction region on β-catenin structure. (right panel) **TCF/LEF proteins structure**. B-catenin binding domain (β-catenin BD), high-mobility group (HMG).

1.7.3.2 TCF/LEF proteins

TCF/LEF proteins belong to a family of high-mobility group (HMG) proteins, which utilize their HMG domain for binding the DNA on a specific consensus sequence. There are four TCF/LEF proteins in vertebrates: TCF1 (the product of the gene Tcf7), LEF1, TCF3 and TCF4. They contain a virtually identical HMG motif, and almost identical sequence-specificity. At the N-terminus they all include a β-catenin binding domain (B-catenin BD) (Figure 11, right panel). In the absence of Wnt signals, TCF/LEF transcription factors exist in a repressor complex with Groucho, which is susceptible to be displaced by β-catenin following activation (Cavallo et al., 1998). At least eight isoforms of TCF1 have been

identified, some of them lacking the β-catenin BD, which act as natural repressors of the pathway (van de Wetering et al., 1992).

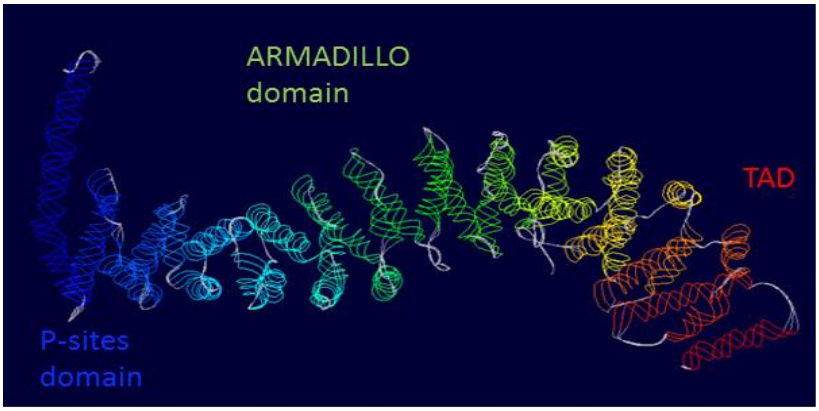


Figure 12. Image of the crystal structure of full length zebrafish β-catenin. The N-terminus is blue colored, and the C-terminus is the red part. the Armadillo repeats have a helix-loop-helix structure and represent the biggest domain of the protein.

1.7.3.3 Target genes

Some potential β-catenin transcriptional target genes can be predicted by the presence of TCF/LEF binding sites on their promoter region. A growing number of Wnt responsive genes has been identified, most of them being cell-type and context-dependent; reviewed in (Clevers, 2006). Axin2 is a direct β-catenin target gene in many systems (Jho et al., 2002). In 1998 c-Mys was shown to be regulated by canonical Wnt pathway in colorectal cancer (He et al., 1998) but also in the normal Intestinal Stem Cell compartment (Pinto et al., 2003). Given the prominent role of Wnt/β-catenin in this disease, many studies have focused on the

identification of β-catenin target genes in intestinal cells. Our group identified the Notch ligand Jagged1 as one of the pathologic links between Notch and Wnt pathways in Colorectal Cancer (Rodilla et al., 2009).

1.7.4 Alternative Wnt pathway: β-catenin-independent signaling.

Alternative WNT signaling pathways comprises those that do not use the β-catenin–TCF/LEF module. The two alternative Wnt cascades are the Planar Cell Polarity (PCP) and the calcium (WNT–Ca2+) pathway. In PCP signaling Frizzled receptors activate a cascade that involves the GTPases RAC1 and RHOA and the JUN-N-terminal kinase (JNK). Activation of this pathway results in changes in cytoskeleton and cell polarity, reviewed in (Simons and Mlodzik, 2008). In the WNT–Ca2+ pathway, WNTs trigger Frizzled-mediated activation of hetero-trimeric G-proteins. This leads to Ca2+ release from intracellular storage; the process involves the activation of phospholipase C (PLC), and of effectors such as Ca2+ and calmodulin-dependent kinase II (CAMKII). This cascade is involved in cancer, inflammation and neurodegeneration, reviewed in (De, 2011).

1.7.5 Wnt/β-catenin in the hematopoietic system and leukemia

1.7.5.1 Wnt/β-catenin in HSC regulation

Wnt/β-catenin activity is transiently required in the AGM region to generate long-term HSCs during embryonic development (Goessling et al., 2009; Ruiz-Herguido et al., 2012). The first evidence for WNT in regulating proliferation and differentiation of adult hematopoietic progenitors dates back to 1997 (Austin et al., 1997) (Van Den Berg et al., 1998). Thereafter, Dr. Weissman's group used a TCF-GFP reporter to demonstrate that Wnt/β-catenin-TCF/LEF signaling is active in HSC (Reya et al., 2003) . These investigators also showed that over-activation of β-catenin in murine HSC led to an increase in their number and enhanced their ability to reconstitute lethally irradiated recipient mice. Concordantly, over- expression of Axin (Wnt signaling inhibitor) in the same system led to reduced reconstitution (Reya et al., 2003). Those results strongly supported a functional role for the Wnt/β-catenin pathway in maintaining and expanding HSCs. Kincade's laboratory showed that expression of constitutively active β-catenin could confer MPP properties to committed lymphoid and myeloid hematopoietic progenitors, thus inducing *in vitro* the regression of cell differentiation (Baba et al., 2005; Baba et al., 2006). However, mouse models with constitutive activation of β-catenin in the hematopoietic system demonstrated that deregulated β-catenin

activity impaired multi-lineage differentiation and resulted in the exhaustion of the HSC pool (Kirstetter et al., 2006; Scheller et al., 2006). in contrast to the above described results, conditional deletion of β-catenin using the interferon-inducible Mx-Cre had no obvious effect neither on HSC nor on progenitor differentiation (Cobas et al., 2004). Possible redundant and compensatory function of gamma-catenin was also examined by deleting both genes by Mx-Cre and resulted in normal HSC functionality (Koch et al., 2008b). Analysis of the hematopoietic activity in the fetal liver of Wnt3a null embryos showed functionally impaired HSCs (Luis et al., 2009). Similarly, β-catenin deletion in the hematopoietic compartment (under the control of Vav-Cre) showed a decrease in HSC frequency of around 5-fold when tested in limiting dilution and competitive BM transplantation experiments (Zhao et al., 2007).

These apparent contradictory results may be due to the different experimental strategies and models used in the different laboratories, but they also may highlight the possibility of dose-related effects of β-catenin activation in HSC and progenitors. In fact, a recent work published by Staal's group explored the dosage-related implication of Wnt/B-catenin pathway in the hematopoietic system: taking advantage of mice models carrying different mutation on the Apc gene, they show that different levels of Wnt/β-catenin signaling specifically affect hematopoietic lineages, and they propose different dose-requirement of the pathway at different stages of hematopoietic commitment (Luis et al., 2011). Similarly, our group recently demonstrated that specific doses of β-catenin

signaling are required for HSCs specification in the AGM during embryonic development (Ruiz-Herguido, Guiu et al. 2012).

1.7.5.2 Wnt/β-catenin in hematopoietic cell differentiation

Most studies about the role of β-catenin in hematopoietic differentiation focus on T-cells development but there is also evidence on the effect of β-catenin in other lineages. For example, the receptor Frizzled-9 is required for B-cell function and differentiation. Mice deficient for this receptor show a defect in the clonal expansion of pre-B cells during development and they display an accumulation of plasma cells in the lymph-node when they age (Ranheim et al., 2005). In addition, activation of β-catenin increase myeloid progenitors although terminal differentiation is not affected (Luis et al., 2011).

1.7.5.3 Wnt/β-catenin in T-cell development

The role of canonical Wnt pathway in T-cell development is still matter of intense investigation. One of the first observations described in Tcf1- and Lef1-deficient mice referred to multiple defects in T-cell development, both at DN stages and immature SP. (Verbeek et al., 1995; Okamura et al., 1998; Schilham et al., 1998). Some of these defects could be partially dependent of the Wnt/β-catenin pathway, however many questions still remain.

Several authors have described T-cell defects dependent on β-catenin. Sen and colleagues observed that T cell-specific deletion of

β-catenin using Lck-Cre impaired maturation of DN3 cells to the DN4 stage and decreased the numbers of splenic T cells in β-catenin null Lck-Cre+ compared to Lck-Cre- animals (Xu et al., 2003). Gounari and colleagues showed that high levels of β-catenin expression in the developing thymocytes bypassed pre-TCR signals and were able to rescue RAG2-immunodeficiency (Gounari et al., 2001). Furthermore, β-catenin through TCF-1 ensures thymocyte survival (Ioannidis et al., 2001) (Xie et al., 2005), and partially controls the expression of the CD4 gene (Huang et al., 2006). In addition, activation of β-catenin in DP thymocytes freezes the transition to the single-positive stage and predisposed thymocytes to malignant transformation (Guo et al., 2007).

	Model	**phenotype**	**reference**
Gain of function	Ctnnb Δex3 // CD4-Cre	Impaired maturation from DP to SP	(Guo et al., 2007)
	Ctnnb Δex3 // Lck-Cre	altered thymocyte development and impaired β-selection	(Guo et al., 2007) (Gounari et al., 2001)
Loss of function	Ctnnb fl/fl // Mx-Cre	Normal T lymphopoiesis and Tcells	(Cobas et al., 2004)
	Ctnnb fl/fl // Mx-Cre // Ctnnγ-/-	Normal T lymphopoiesis and T cells	(Koch et al., 2008b)
	Ctnnb fl/fl // Vav-Cre	Normal mature T cells	(Zhao et al., 2007; Ruiz-Herguido et al., 2012).
	Ctnnb fl/fl // Lck-Cre	impaired maturation of DN3 cells, reduced numbers of splenic T cells	(Xu et al., 2003)

Table3. Studies in genetic mouse models addressing the involvement of β-catenin in T-cell development.

In contrast to the previous described results, the deletion of β-catenin and/or ϒ-catenin in the hematopoietic system (Cobas et al., 2004) (Koch et al., 2008b) did not result in any T-cell defect. In these studies, T-cell stages (DN1-DN4, DP, SP) were analyzed in detail and differences between the WT and β-catenin-deficient thymocytes were not detected. Comparable, normal T cell development is observed in animals in which β-catenin has been specifically deleted in the hematopoietic system by using Vav-Cre (Zhao et al., 2007; Ruiz-Herguido et al., 2012).

1.7.5.4 Wnt/β-catenin pathway in hematopoietic malignancies

The Wnt/β-catenin pathway participates in many oncogenic processes such as colon, breast and prostate cancer, in addition to leukemia (Reya and Clevers, 2005). Carson's laboratory in 2004 first described β-catenin activation in B-CLL (Lu et al., 2004). In 2007, Reya's group demonstrated that β-catenin is required for the self-renewal of Chronic Myeloid Leukemia initiating cells in a mouse model. Specifically, mice transplanted with β-catenin-deficient BM cells expressing the BCR-ABL fusion gene showed an increased survival compared to the mice that received β-catenin WT leukemic (BCR-ABL+) cells (Zhao et al., 2007). Similar results were obtained by HU and coworkers using a retroviral-expressed BCR-ABL gene (Hu et al., 2009). In Acute Myeloid Leukemia, cells induced by MLL-AF9 contain Wnt/β-catenin activity, which is

also required for self-renewal of LICs (Wang et al., 2010). In fact, these authors propose β-catenin as a putative therapeutic target for this disease. In a mouse model for Multiple Myeloma (MM), treatment with the β-catenin inhibitor PKF-115-584 increased the survival of sick animals (Sukhdeo et al., 2007). In Chronic Lymphocytic leukemia (B-CLL) β-catenin contributes to the extended survival of CLL cells in vivo by inhibition of apoptosis (Lu et al., 2009; Filipovich et al., 2010).

Not much is known about a putative role of β-catenin in malignancies of the T-cell lineage. Work performed in Fotini Gounari's laboratory indicated that expression of a constitutive active form of β-catenin (Ctnnb Δex3) during thymic development induced an accumulation of DP T cells and impaired the progression to SP stage, however these cells were not fully transformed until they acquired secondary mutations. When transplanted at late stages, cells carrying active β-catenin generate invasive secondary lymphomas in recipient animals. This transformation event is Notch-independent but c-Myc-dependent (Guo et al., 2007).

1.8 The NF-κB pathway

The NF-κB pathway is a key regulator of the immune system and the inflammation processes. It is also implicated in the control of cell proliferation and apoptosis, reviewed in (DiDonato et al.,

2012). NF-κB signaling can be activated by both extra-cellular (like cytokines, among others) and intra-cellular stimuli, (like DNA damage). In un-stimulated condition, NF-κB factors are sequestered in the cytoplasm by the inhibitor IκB; the activation of canonical NF-κB pathway is produced through phosphorylation of IκB by the IKK complex of kinases. Phosphorylated IκB is then ubiquitinated and degraded via proteasome leading to the release of the NF-κB complex that enters the nucleus to activate transcription of genes containing the consensus κB sites; reviewed in (Vallabhapurapu and Karin, 2009). IκBα is one the main transcriptional targets of NF-κB activation, which results in a very effective negative feedback loop that prevents the pathway from chronic activation.

1.8.1 NFkB pathway in hematopoietic diseases

It is now well demonstrated that inflammation favors oncogenesis and NF-**κ**B activity is required for this process ; reviewed in (Ben-Neriah and Karin, 2011). Chromosomal amplification, overexpression and rearrangement of genes coding for NF-**κ**B factors and regulators have been found in many human hematopoietic and solid tumors (Franzoso et al., 1992; Cabanes et al., 1999; Gilmore, 2003). Furthermore, oncogenic NF-**κ**B activity can be due to mutations in other components of the pathway or to the exposure to inflammatory cytokines from the tumor microenvironment and inflammatory cells (Karin et al., 2002). In

hematological malignancies, NF-κB activation is detected in lymphomas and associated to mutations and chromosomal translocations that increase expression of the adaptors Bcl-10 and MALT1 (Willis et al., 1999; Uren et al., 2000). For example, in diffuse B-cell lymphomas constitutive activation of NF-κB results increased proliferation and survival of B lymphocytes (Lenz et al., 2008). In addition, mutations in members of the pathway (NIK, TRAF3, CYLD, BIRC2/BIRC3, CD40, NFKB1 or NFKB2) have been associated with Multiple myeloma (Annunziata et al., 2007) (Keats et al., 2007). The NF-κB pathway is also involved in T-ALL. Aifantis' group first described that NFkB activation was found in T-ALL and it was dependent on Notch1 activity (Vilimas et al., 2007). In 2010, our group in collaboration with Aifantis' group demonstrated the requirement for IKK and NFkB activity to develop Notch-dependent leukemia and identified the mechanism that supports this interaction (Espinosa et al., 2010). In brief, we demonstrated that Hes-1 was directly repressing the deubiquitinase enzyme CYLD, a negative regulator of the IKK complex; thus NF-κB is constitutively activated and promotes cell. The function of CYLD and Hes1 in Notch-dependent T-ALL has been further studied in this thesis.

2. OBJECTIVES

2. OBJECTIVES

The role of Notch in T-ALL is still not well understood. On one hand T-cell neoplasms cannot develop without Notch activity, but on the other hand the naturally occurring Notch mutations seem to be not sufficient to induce this leukemia in mouse, thus being other elements/pathways required.

In this scenario, our work has focused on a general aim:

Understanding how other pathways are simultaneously activated or activated downstream of Notch in T-ALL.

The specific objectives covered in this work are:

- Revealing the downstream mechanisms that activate NFκB in Notch1-dependent T-cell leukemia.
- Exploring the involvement of the β-catenin pathway in Notch1- induced T-ALL.

3. RESULTS

3. RESULTS

3.1 PART 1: NFκB activation downstream of Notch1

Notch activation is a recurrent event in T-ALL but at the beginning of this work little was known about the downstream elements required for Notch-dependent T-cell leukemia. The NFκB pathway had been reported to be activated in T-ALL (Vilimas et al., 2007) and our group had published before several cross-talks between NFκB and the Notch pathway (Espinosa et al., 2002) (Espinosa et al., 2003). Thus, we focused in studying whether different Notch elements contribute to regulate NFκB during T-cell leukemia generation.

In previous experiments we had found that cells carrying active Notch displayed an increased NFκB activity associated with reduced levels of its negative regulator CYLD; we found that Hes1 inhibits CYLD transcription. Based on this, we aimed to study the role of Hes1 and CYLD downstream of Notch1 in T-ALL. We first used an *in vitro* clonogenic assay that is based on serial plating of hematopoietic cells on methylcellulose, a semi-solid media where proliferating cells generate colonies. In general, normal hematopoietic progenitors grow during two/three platings and then are exhausted, while cells with leukemic properties acquire the

ability to grow virtually "infinitely". For these experiments, we obtained BM cells from C57B6/J mice flushing the femurs with PBS, and performed a Lineage-depletion by using a cocktail of antibodies that stain most of the hematopoietic differentiated cells: this is composed by the markers CD3, B220, Mac-1, Gr-1, Ter-119; with this strategy, we obtain an enriched fraction of undifferentiated hematopoietic cells, also called Lin-.

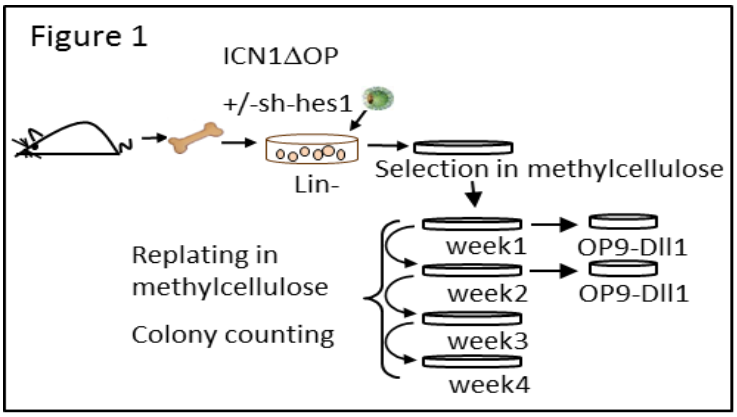


Figure1. Experimental design: Lin-BM cells transduced with the indicated viral constructs were serially plated in methylcellulose. Number of colonies was counted every passage. After the first and second plating cells were plated on OP9-Dll1 to test their ability to generate T-cells.

Those cells were kept in liquid culture in presence of SCF, Flt3L, IL6, IL7, and next transduced with a vector carrying the active form of Notch1 (pLXSH-N1ICΔOP) or the empty vector, both having Hygromycin resistance. The day after, we infected again the cells with a vector carrying the ShRNA against Hes1 (pLKO-ShHes1-puro) or the corresponding empty vector. 48 hours later, infected cells were plated into methylcellulose in the presence of

Hygromycin and Puromycin to select double infected cells. After 1 week, selected cells were extracted from the methylcellulose by pipetting with media; 1000 cells per condition were then re-plated into fresh methylcellulose. Every seven days colonies were counted, disrupted and cells were re-plated again for a total of 4 weeks. A scheme of this experiment is shown in figure 1.

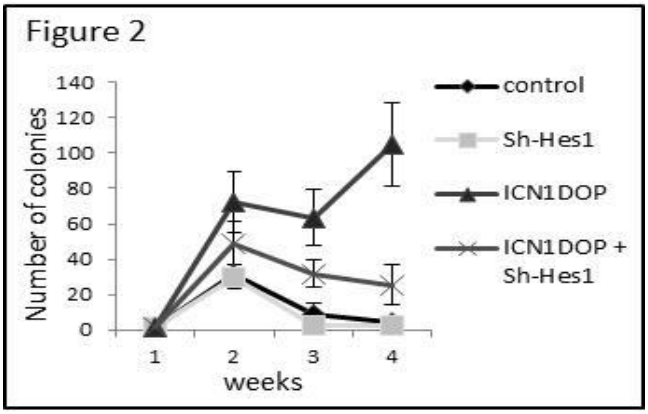


Figure2. Clonogenic assay. The number of colonies (Y axis) obtained every week (X axis) from BM Lin- cells transduced the different plasmids is shown. Each point represents the average of 4 independent experiments, and error bars indicate SEM.

We found that cells infected with control vectors generated few colonies, and died around passage three. In contrast, cells infected with active Notch1 increased their clonogenic ability and gave rise to around 100 colonies at passage 4. Down-regulation of Hes1 in this condition decreased the number of colonies in the active-Notch1 background about 5 fold (figure2).

In the conditions used most of the cells differentiate into the myeloid lineage. To check whether colony-generating cells (in the

different conditions) still retain the T-cell lineage potential, we used an *in vitro* assay in which cells are plated on an OP9 stroma that over-expresses the Notch ligand Dll1 (Schmitt and Zuniga-Pflucker, 2002). After 5-7 days, and at different passages, cells were analyzed by FACS for the presence of the T-cell marker CD3 and the pan-hematopoietic marker CD45 (see scheme of figure1). We found that the percentage of T cells generated in this cultures increased in the presence of active Notch1 but decreased when Hes1 was simultaneously down-regulated (figure 3).

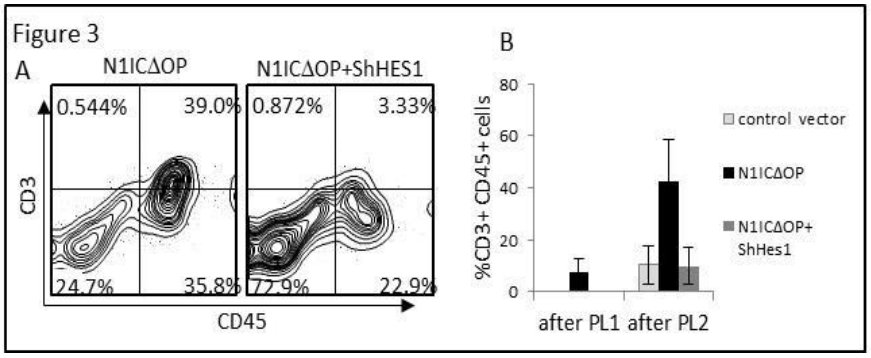


Figure3. FACS analysis of cells plated on OP9-Dll1. (A) Representative plot of CD3 and CD45 expression. PL1= plating 1; PL2= plating 2. (B) Average of 2 experiment. Bars indicate SEM.

Next we investigated the requirement of Hes1 *in vivo* during T-cell leukemia generation using the same strategy as before but using the retroviral pMIG-ICN1-ires-GFP to induce and trace Notch activation. BM cells transduced with active Notch1 or the empty vector were subsequently infected with the vector carrying the shRNA against Hes1 and, 48hours later transplanted into 8 Gy irradiated recipient mice. This experimental strategy is illustrated in figure 4, panel A.

Peripheral blood (PB) of the transplanted animals was analyzed at different time points for the appearance of circulating CD4+CD8+ double positive (DP) cells which is the hallmark of this disease.

We found that N1IC transduced cells generated T-cell leukemia after 1 month. In contrast, we could not detect leukemic DP CD4+CD8+ cells in the animals that received the double N1IC/hes1-knockdown cells. In those mice, DP CD4+/CD8+ cells were not found neither in PB (Figure 4, panel B) nor in BM (Figure 5) 3 months post-transplantation.

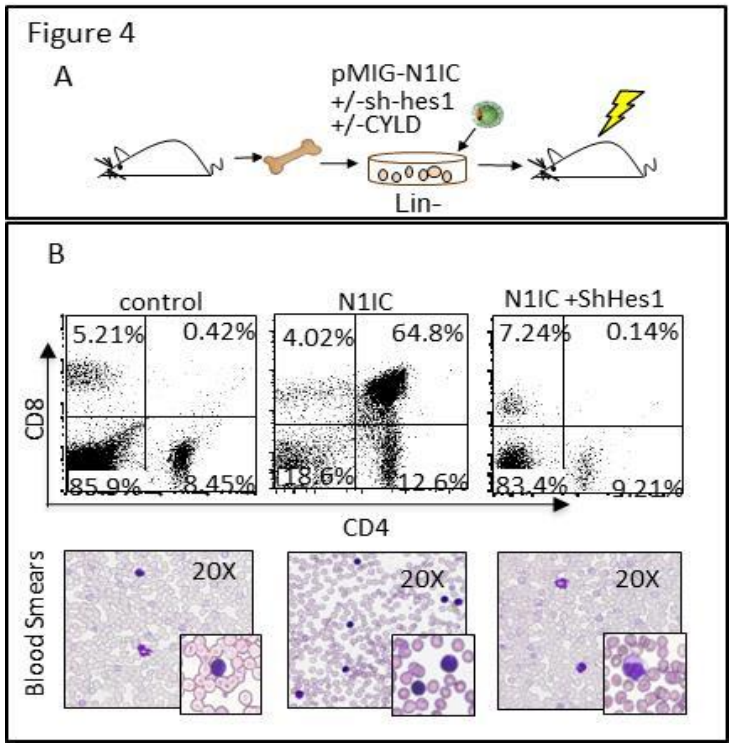


Figure4. Transplantation assay. (A) Experimental design: Lin- BM cells infected with the indicated viral constructs were transplanted into 8 Gy irradiated recipient animals. (B) 3 month analysis of recipient animals. FACS analysis of CD4 and CD8 markers in peripheral blood of mice transplanted with the indicated vectors. Giemsa staining of blood smears.

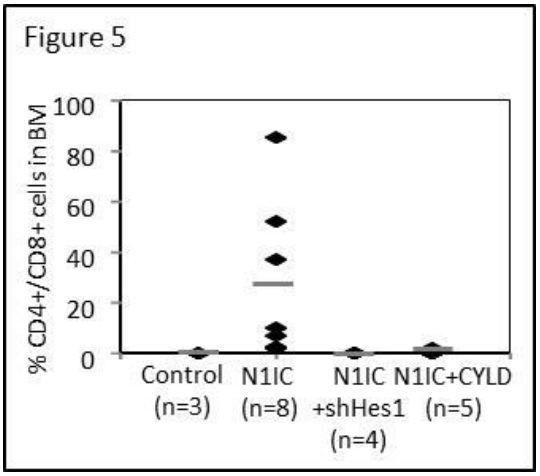


Figure5. analysis of recipient animals Bone marrow analysis of transplanted animals. Percentage of DP CD4+/CD8+ cells is shown.

Since one of the functions of Hes1 was to repress the NFkB modulator CYLD, we next tested the relevance of this deubiquitinase in the development of T-cell leukemias. We took advantage of the same experimental strategy, but inducing ectopic CYLD in the cells carrying N1IC.

In the clonogenic assay, overexpression of CYLD resulted in a strong decrease in Notch1-induced colony formation (figure 6, panel A). In the OP9 co-cultures, cells with CYLD overexpression showed reduced T-cell potential, which was comparable to that observed after knocking down Hes1 (Figure 6, panel B). Finally, we compared the capacity of these double infected cells to induce T-cell leukemia in transplanted mice compared with N1IC-expressing cells (see the experimental design in figure 4). We found that

ectopic CYLD expression strongly reduced the ability of N1IC to induce T-ALL in the transplanted mice (figure 6 and 9).

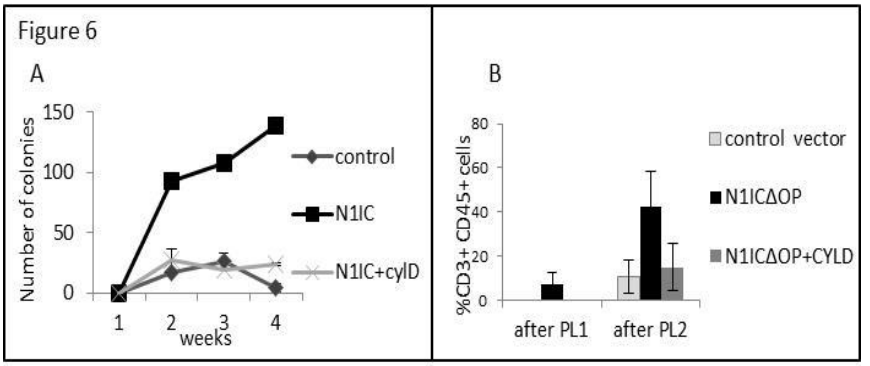


Figure6. (A) **Clonogenic assay**. Cells transduced with the indicated constructs have been serially re-plated in methylcellulose. The number of colonies obtained every week in the different conditions is shown. (B) FACS analysis of cells plated on OP9-Dll1. the average of 2 experiments is shown. PL1= plating 1; PL2= plating 2. Error bars indicate SEM.

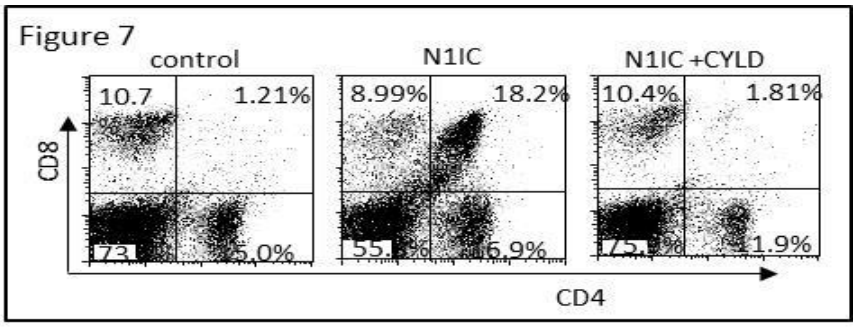


Figure7. Analysis of recipient animals. FACS analysis of CD4 and CD8 markers in peripheral blood of mice transplanted with the indicated vectors.

To control the transduction and expression of the different viral vectors, we selected the transduced cells during 1 week in puromycin and hygromycin, and checked the expression levels by qPCR. In these experiments we confirmed the induction of Notch1 expression upon infection with N1IC, the down-regulation of Hes1

after shHes1 transduction and the up-regulation of CYLD. Also, we found that endogenous Hes1 was upregulated in the N1IC infected cells concomitant with a decrease in CYLD expression. Conversely, Hes1 Knockdown reverted the inhibition of CYLD induced by N1IC, as expected (Figure8).

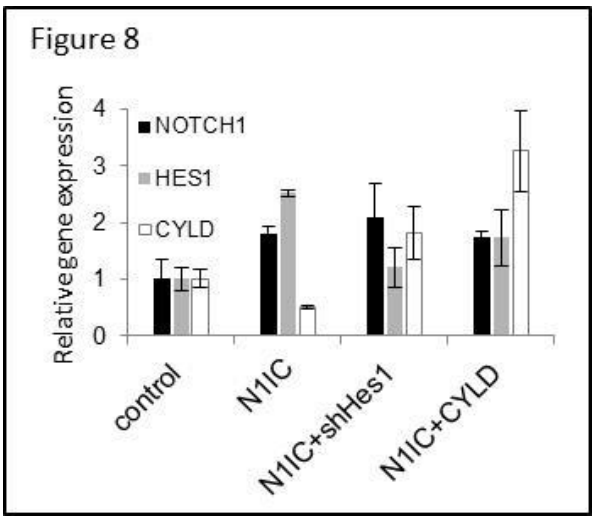


Figure8. Quantitative PCR of transduced cells. Gene expression has been normalized to cell infected with control vector.

These results have been published in (Espinosa et al., 2010) and (D'Altri et al., 2011).

3.2 PART 2: Notch1 and β-catenin crosstalk in T-ALL

3.2.1 Coexistance of β-catenin and Notch activity in T-ALL cells

As described in the introduction, the Wnt/β-catenin pathway functions closely associated to the Notch pathway in the regulation of many cell fate decisions (Estrach et al., 2006) (Yamamizu et al., 2010) (Devgan et al., 2005) but also under pathological conditions (Rodilla et al., 2009).

Since the involvement of Wnt/β-catenin in T-ALL has never been addressed, we checked the expression of β-catenin in the T-ALL cell lines carrying activating Notch mutations, compared to leukemic cell lines from other lineages. We found that β-catenin protein levels were increased in T-ALL cell lines compared to B-cell or myeloid cell lines (figure 9, panel A) being more prominent in RPMI8402 and DND41.

As expected, most T-ALL cell lines contained active Notch1 (including truncated proteins), while the rest of the cell lines did not. We next obtained the nuclear and cytoplasmic fractions of DND41 and RPMI8402 cell lines and analyzed them by Western blot. We found that both T-ALL cell lines contained nuclear β-catenin, suggestive of its activation (figure 9, panel B). Concordantly with those findings, the growth of these two cell lines was affected not only by DAPT (Notch/ γ-secretase inhibitor) as previously described (Weng et al., 2006; Palomero et al., 2007), but also by the β-catenin inhibitor PKF 115-584 (that impairs the

binding of β-catenin to the DNA binding protein TCF/LEF) (figure 10, panel A).

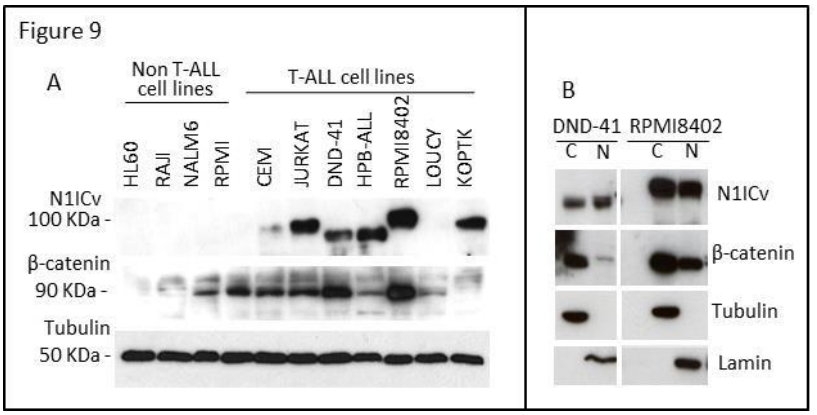


Figure9. Notch1 and β-catenin protein levels in T-ALL and non T-ALL cell lines. (A) Protein extracts from the indicated cell lines were blotted with Cleaved-Notch and β-catenin antibodies. Tubulin was used to normalize the amount of total proteins. (B) Notch1 and β-catenin protein levels in cytoplasmic and nuclear fraction of DND41 and RPMI8402 cell lines. Tubulin and Lamin are used to normalize the amount of protein.

After testing different doses of the inhibitors (not shown), T-ALL cell lines were treated during 4 days with 5 μM DAPT, 0.1 nM PKF-115-584 or a combination of both. The number of cells in the cultures was evaluated at the end of the treatments. We found that PKF-115-584 drastically decreased the number of cells, whereas DAPT showed a slight effect in these conditions. Importantly, the combined treatment was more efficient in inhibiting the growth of these T-ALL cell lines than either treatment alone (figure 10 A). As a control, we determined the levels of active Notch1 and β-catenin in treated cells. As expected, 5 μM DAPT resulted in a complete inhibition of Notch1 cleavage, while no changes in total β-catenin

levels were observed in any of the treated cultures (figure 10 B); these data indicate that inhibiting Notch does not affect β-catenin levels and are also consistent with the mechanism of action of PKF-115-584 as inhibitor of the β-catenin-TCF/LEF binding.

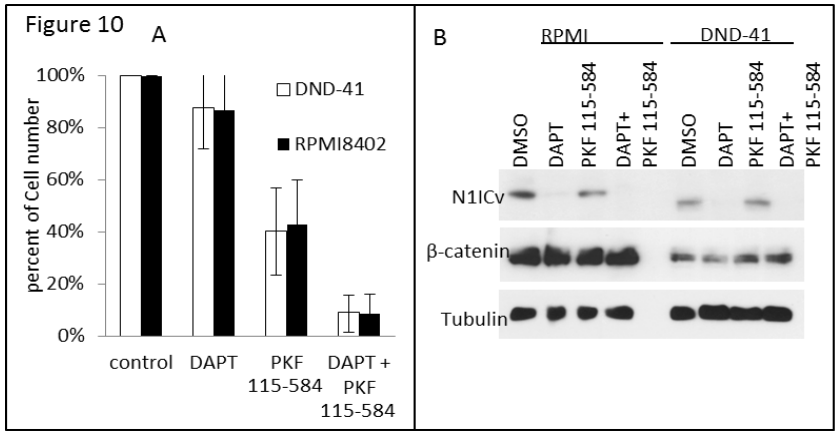


Figure10. DND41 and RPMI8402 treatment with DAPT and/or PKF 115-584. (A) Percent of cell number relative to cell treated with DMSO. (B) Western Blot of Notch1 and β-catenin protein levels after treatment with 5 μM DAPT and/or 0,1 nM PKF 115-584.

Altogether, those results indicate that both Notch1 and β-catenin are active in T-ALL cell lines.

3.2.2 β-catenin is required for Notch-induced T-cell neoplasm generation in mice

Ectopic activation of Notch1 in hematopoietic progenitors is a well-established model to develop T-ALL in mice (Pear et al., 1996). Thus, we took advantage of this model to assess the role of β-catenin in Notch1-dependent T-cell leukemia. We obtained WT and

β-catenin KO BM cells (derived from Ctnnb+/+ Vav-cre+ in the case of the β-catenin WT and Ctnnb fl/fl Vav-cre+ for β-catenin KO; see table R1).

Genotype	β -catenin locus	Vav-Cre locus
β-catenin WT	Ctnnb +/+	Vav-Cre+
β-catenin KO	Ctnnb fl/fl	Vav-Cre+

Table R1. Genotype of the animal models used.

We purified the lineage-negative population and transduced these cells with a retrovirus codifying for the active form of Notch1 (pMIG-N1IC-ires-GFP) or the empty vector. Then we transplanted these cells into 8 Gy irradiated recipient animals. The protocol that we followed is the same exposed in the section 1 of the results, and it is shown in the scheme of figure 11.

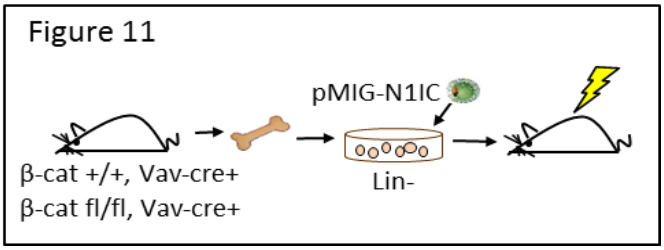


Figure11. Experimental design: Lin- BM cells from the indicate donor animals were infected with viral constructs and transplanted into irradiated recipient animals.

Transplanted animals were regularly analyzed for the appearance of the disease, which is determined by the presence of CD4+CD8+ blasts in the peripheral blood. Two weeks after the transplant, mice

that received β-catenin WT BM cells infected with N1IC already showed about 2-3% of GFP+, DP CD4+/CD8+ cells in the blood circulation, while those that received β-catenin KO cells contained less than 1% of GFP+ CD4+/CD8+ DP (figure12). One month after transplant, mice that received N1IC-transduced β-catenin WT BM cells showed different amounts of DP CD4+/CD8+ cells in the peripheral blood. Interestingly, mice that received N1IC-transduced β-catenin KO cells did not contain detectable amounts of DP CD4+ /CD8+ (figure 13) or any other sign of disease.

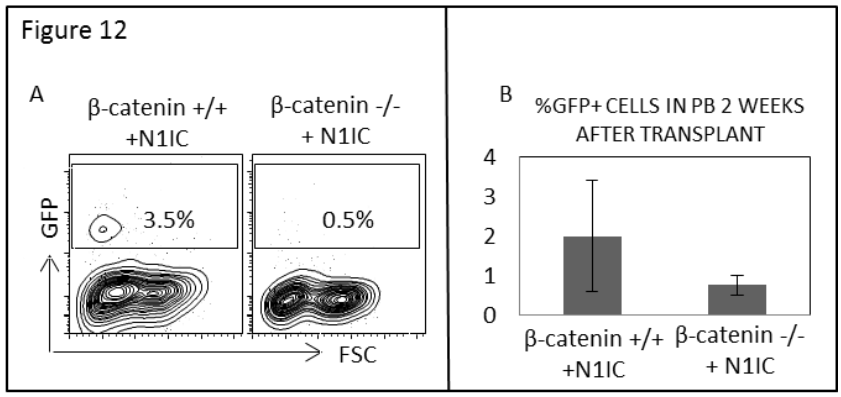


Figure12. Two weeks blood FACS analysis of transplanted mice. (A) Dot plot is a representative experiment showing GFP expression; histograms (B) represent the average of 3 analyzed animals.

Concordantly with these blood analyses, mice transplanted with β-catenin WT cells transduced with N1IC were moribund 2-3 months after transplant and had to be euthanized. In contrast, mice that received β-catenin KO cells did not show any sign of disease even after 6 months post-transplantation. Survival curve of transplanted animals is shown in figure 14.

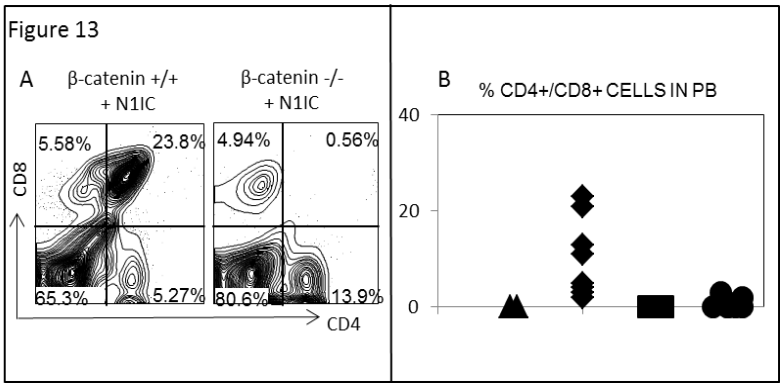


Figure 13. One month blood FACS analysis of transplanted mice. (A) Dot plot is from one representative experiment, percentage of CD4+/CD8+ cells in peripheral blood is shown. (B) summary of the percentage of CD4+/CD8+ cells in animals transplanted with indicated genotypes.

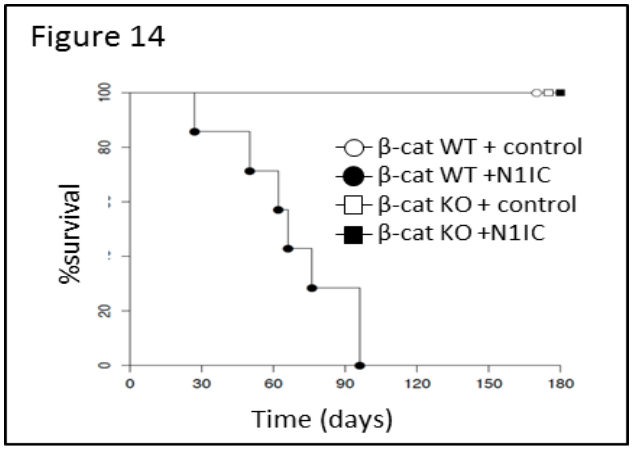


Figure14. Kaplan-Meier curve of transplanted animals. Percentage of alive animals (y axis) is shown for every time point (x axis).

3.2.3 β-catenin KO and WT BM cells are similarly transduced with N1IC and contribute to the hematopoiesis of the transplanted animals.

One possibility to explain that β-catenin KO cells are refractory to the leukemogenesis induced by ICN1 may be that they are not transduced at the same extent as the WT cells. To test this, we determined the percentage of cells transduced 48 hours after infection in both genotypes. Since our retroviral vector contains an Internal Ribosomal Entry Site (IRES) followed by the GFP cDNA (pMIG-N1IC-ires-GFP), we could test the infection efficiency by flow cytometry.

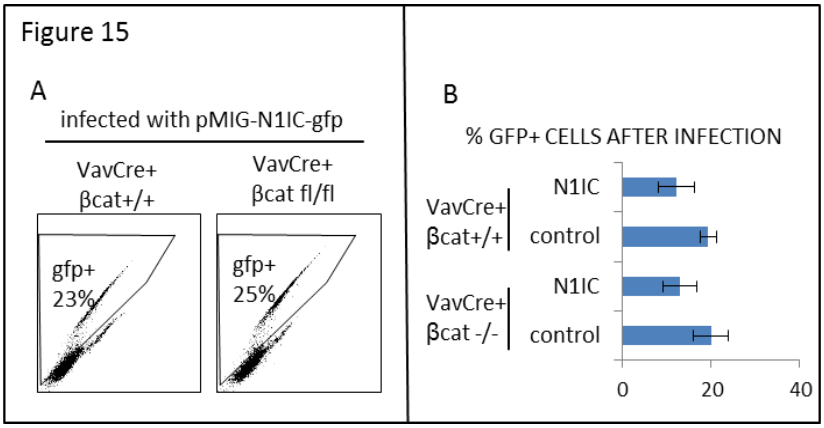


Figure15. FACS analysis of Lin- BM cells from mice with the indicated genotypes transduced with the control or pMIG-N1IC-ires-GFP vectors. Cells were analyzed for the presence of GFP 48 hours after infection. (A) shows the dot plot from a representative experiment and (B) represent the average GFP expression in 4 experiments.

We found that a similar percentage of β-catenin WT and β-catenin KO cells expressed GFP (figure 15). This result indicates that the

different leukemogenic capacity of WT or β-catenin KO cells is not the result of different transduction efficiencies.

As explained in the introduction, β-catenin KO BM cells show a normal capacity to engraft in transplantation experiments (Cobas et al., 2004) (Koch et al., 2008b), However β-catenin fl/fl vav-cre+ mice contain a slightly reduced number of LT-HSC when these cells are transplanted under limiting dilution conditions (Zhao et al., 2007). To investigate whether β-catenin KO N1IC-transduced cells were able to engraft in irradiated recipients, we used as donors BM cells from mice containing a ROSA26-loxP-stop-loxP-YFP reporter (that labels the cells that expressed the Cre recombinase) in combination with the β-catenin floxed allele (Table R2).

Genotype	**β-catenin locus**	**Vav-Cre locus**	**Rosa26-YFP locus**
β-catenin WT	Ctnnb +/+	Vav-Cre+	YFP FL/FL
β-catenin KO	Ctnnb FL/FL	Vav-Cre+	YFP FL/FL

Table R2. Genotype of the animal models used.

Using this strategy, we found that mice transplanted with N1IC-transduced WT (YFP fl/fl vav-cre+) cells contained a 10-20 % of YFP+ cells in circulation at 1 month after the transplant. Similar numbers of YFP+ cells were present in mice that received N1IC-transduced β-catenin KO cells, indicating that β-catenin deficiency

does not affect engraftment in these conditions; still they are not able to generate T-cell leukemia (figure 16).

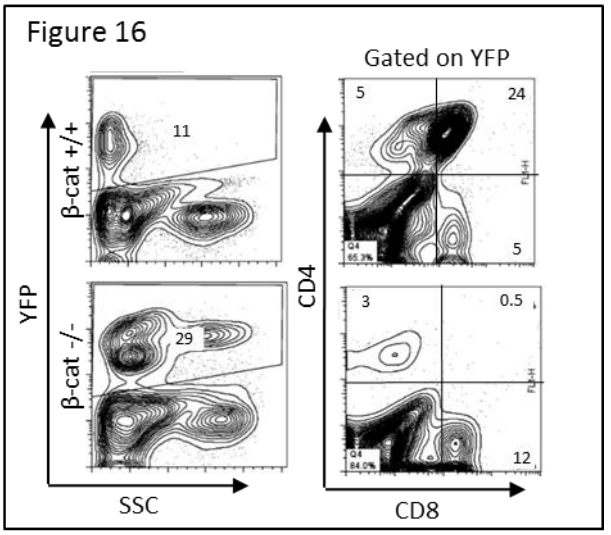


Figure16. PB FACS analysis of mice transplanted with β-cat+/+ VavCre+ YFPfl/fl or β-cat fl/fl VavCre+ YFPfl/fl Lin+ BM cells. YFP and CD4/CD8 expression from transplanted animals are shown**.**

As mentioned in the introduction, the role of β-catenin in T-cell development is controversial (Gounari et al., 2001; Guo et al., 2007) (Cobas et al., 2004) (Koch et al., 2008) (Zhao et al., 2007; Ruiz-Herguido et al., 2012) (Xu et al., 2003). Thus we tested whether T-cell production was affected in our β-catenin mutant mice. FACS analysis of the thymus of β-catenin+/+ Vav-Cre+, β-catenin+/fl VavCre+ or β-catenin fl/fl Vav-Cre+ showed similar percentage of DP CD4+/CD8+ cells (figure 17, panel A) in all genotypes. Splenic and circulating CD3 T-cells were also not affected by β-catenin

deletion (figure 17, panel B). These analyses demonstrate that T-cell development is not affected in our mice model.

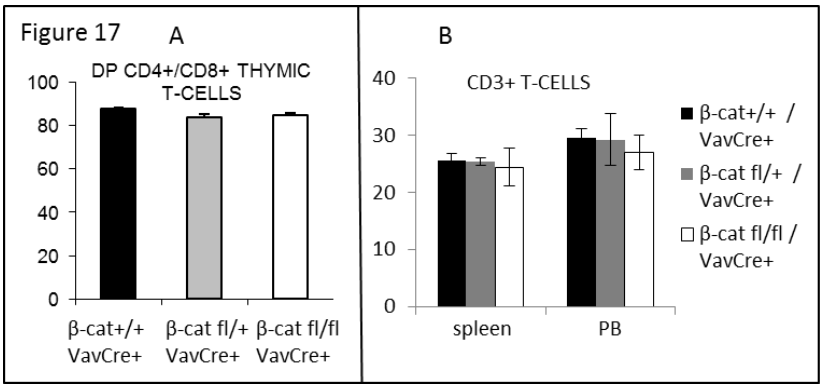


Figure17. FACS analysis of β-cat+/+ VavCre+ YFPfl/fl and β-cat fl/fl VavCre+ YFPfl/fl. (A) Percentage of DP CD4+/CD8+ cells in the thymus of the indicated genotype is shown. (B) PB and Spleen FACS analysis of CD3+ T cells.

We next analyzed the contribution of N1IC-transduced β-catenin KO cells to the different T-cell subpopulations in the thymus of the transplanted animals. At 2 months after transplant, we found that YFP+ cells were present in the DP, CD4+ and CD8+ populations with a comparable contribution of β-catenin KO and β-catenin WT cells (figure 18).

We then tested the possibility that YFP+ engrafted cells from β-catenin KO-transplanted animals were not transduced with N1IC, thus explaining the absence of leukemic cells in these animals. We sorted total YFP+ and DP cells from the thymus of transplanted animals that were used for extraction of genomic DNA and RNA (figure 19, panel A). By PCR we determined the presence of the pMIG-N1IC-ires-GFP vector integrated in the genomic DNA (see figure19, panel B). We found the presence of the integrated N1IC

retroviral vector in cells from both WT and β-catenin KO transplanted animals, indicating that transduced cells were engrafted in both cases (figure 19, panel C).

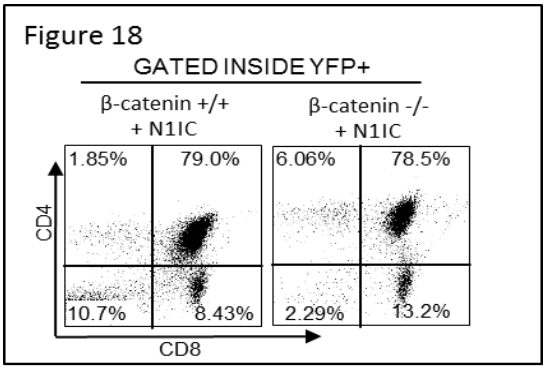


Figure18. Thymus FACS analysis of mice transplanted with β-cat+/+ VavCre+ YFPfl/fl or β-cat fl/fl VavCre+ YFPfl/fl Lin- BM cells. CD4/CD8 expression in YFP gated cells is shown**.**

Next we analyzed the expression of Notch1 and Notch1-target genes in the sorted YFP+ DP subpopulation by qPCR. We found that expression of Notch1 was higher in T-cells from the thymus of the leukemic animals transplanted with N1IC-infected β-catenin WT BM cells compared to those that received β-catenin-KO cells. Nonetheless, expression of the IL7R, a N1IC-target gene in T-cells (Gonzalez-Garcia et al., 2009), was comparable in the N1IC-transduced leukemic and the non-leukemic β-catenin KO DP cells (figure 19, panel D).

Altogether these results indicate that β-catenin KO cells are efficiently transduced with the N1IC retrovirus; they are able to engraft in recipient mice and they develop normally into T-cells. Furthermore once transduced with N1IC they are able to

differentiate into DP T-cells in the thymus of transplanted animals without developing leukemia.

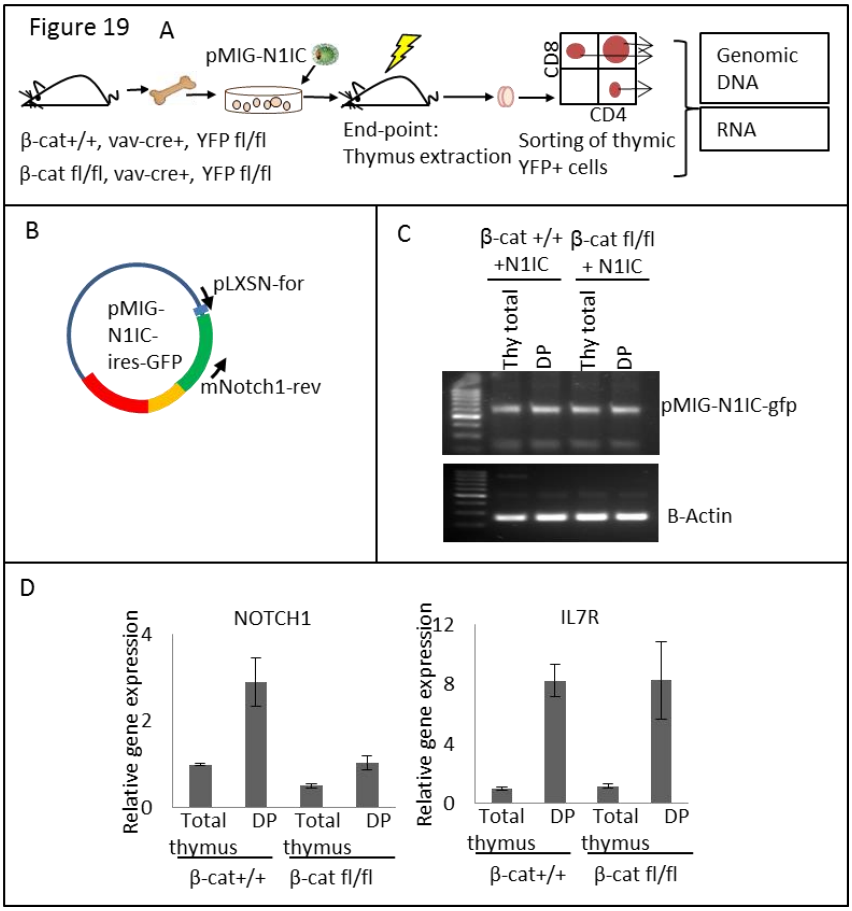


Figure19. Thymus expression analysis of mice transplanted with β-cat+/+ VavCre+ YFPfl/fl or β-cat fl/fl VavCre+ YFPfl/fl Lin- BM cells. (A) Experimental design: YFP+ thymocytes from the indicated transplanted animals have been sorted and genomic DNA and RNA have been extracted. (B) Scheme of the pMIG-N1IC-ires-GFP and the primers used for detecting its integration in transduced cells. The vector backbone is blue, the green box represent the N1IC cDNA, the yellow box represents the IRES and the red part is the GFP cDNA. (C) Genomic PCR of the vector and β-Actin. (D) Quantification of Notch1 and IL7R gene expression in sorted thymocytes.

3.2.4 C-MYC expression is impaired in β-catenin KO BM cells

To understand which is the contribution of β-catenin to the development of Notch1-induced leukemia, we analyzed the transcriptional program induced by active Notch1 in both WT and β-catenin KO cells. As shown in figure 20, panel A, WT or β-catenin KO BM cells were transduced with pMIG-N1IC-ires-GFP and GFP+ cells were sorted 48 hours after infection. Then, we extracted the RNA, obtained cDNA and performed qPCR to measure the expression of Notch1 and several known Notch-target genes. We found that Notch1 transcription is increased about 10 fold in both WT and β-catenin KO cells after transduction with N1IC compared to the vector-transduced cells (figure 20, B).

Moreover, the Notch-target Hes1 was activated of 4-8 folds in BM cells from both genotypes compared to their basal controls. Still expression levels of both Notch1 and hes1 were lower in β-catenin KO cells, both in the control and ICN1-transduced condition (figure 20, panel C). We also tested the expression levels of c-Myc, a Notch target in T-ALL (Weng et al., 2006) and a known β-catenin target in other tissues (He et al., 1998). We found that c-myc expression was increased about 2.5 fold after N1IC infection in WT cells, but not in β-catenin KO cells. This lack of activation was observed also by comparing β-catenin KO cells transduced with N1IC with cells transduced with the control vector (figure 20, panels B and C).

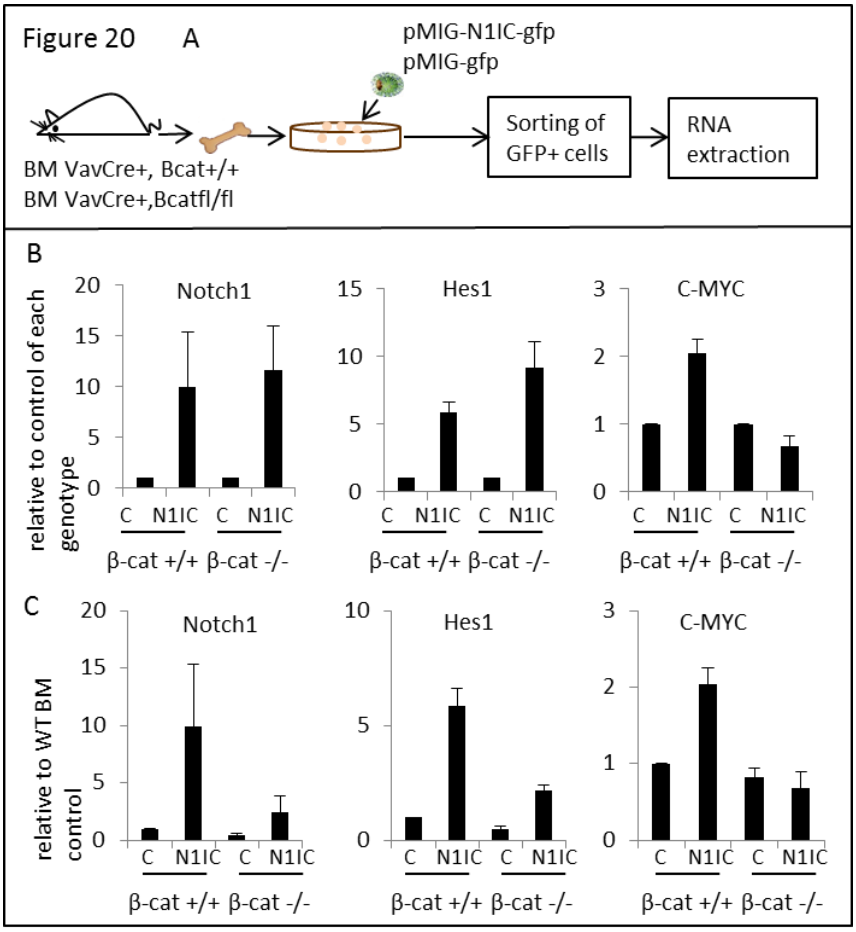


Figure20. Gene expression in Lin- BM cells infected with pMIG-N1IC-iresGFP or pMIG-GFP. (A) Scheme of the experiment: BM from mice with the indicated genotypes has been extracted and lineage-depleted. Cells were infected with pMIG-N1IC-iresGFP or pMIG-GFP and sorted for GFP expression. RNA was extracted from sorted cells. (B-C) Quantification of Notch1, Hes1 and c-Myc in the infected cells. C=Control(pMIG-ires-GFP), N1IC= pMIG-N1IC-ires-GFP. The average of 2 independent experiments is shown and bars represent SEM. Expression has been normalized to the basal level of each genotype (B) or to the control-infected β-cat +/+ BM cells (C).

These results suggest a requirement of β-catenin for the activation of c-myc downstream of active Notch1.

3.2.5 β-catenin and Notch1 co-regulate c-Myc transcription

To investigate whether β-catenin and Notch1 are directly co-regulating c-Myc transcription in T-ALL, we first searched for TCF/LEF and RBPj/CSL DNA binding motifs in the c-myc promoter with the Genomatix Mathinspector software (http://www.genomatix.de). The conserved consensus core sequence for RBPj/CSL binding is GTGGGAA (Tun et al., 1994), the consensus for TCF/LEF binding is CCTTTGA (van de Wetering et al., 1991). We found 6 TCF/LEF and 2 RBPj/CSL putative binding sites (BS) in the human promoter and 4 TCF/LEF and 3 RBPj/CSL in the murine promoter. A scheme of both human and murine c-myc promoter sequence is shown in figure 21. It is of particular interest that both promoters contain two paired TCF/LEF and CSL DNA motifs. Table R3 and R4 indicate the sequences and positions of the paired binding sites, in both human and murine promoters. To investigate whether Notch1 and/or β-catenin can bind to the c-Myc promoter, we performed Chromatin Immuno-precipitation (CHIP) in the RPMI8402 T-cell leukemic line and test the enrichment of different c-Myc promoter regions in the precipitated chromatin by PCR. In 3 independent experiments, we detected enrichment of the region corresponding to the distal paired site (RBP BS -3081 / TCF-LEF BS -3114; table R4, line1) in both Notch1 and β-catenin precipitates, compared to the Ig control precipitates (Figure 21).

paired BS	RBP Binding Site			TCF/LEF Binding Site			Distance
	Seq	Core	S	Seq	Core	S	
D	gtcgTGA Gaatct	-2454	+	gctagCAAA gaatag	-2468	+	14 bp
P	aaacTGG Gaaatt	-1310	+	cagatcAAA Gtaacg	-1232	-	78 bp

Table R3. Mouse c-Myc promoter. Details of paired CSL and TCF/LEF BS. D= distal; P= proximal; Seq= sequence; Core= Core position of the sequence fro the TSS; S= strand. Distance indicate the bp fro core to core of the two sequence.

paired BS	RBP Binding Site			TCF/LEF Binding Site			Distance
	Seq	Core	S	Seq	Core	S	
D	ttccTGG Gaaatc	-3081	+	agctTCAAtt agagt	-3114	+	33 bp
P	agcgTG GGatgtt	-511	-	ttgatCAAAg cgcgg	-447	+	64 bp

Table R4. Human c-Myc promoter. Details of paired CSL and TCF/LEF BS. D= distal; P= proximal; Seq= sequence; Core= Core position of the sequence fro the TSS; S= strand. Distance indicate the bp fro core to core of the two sequence.

These results indicate that both Notch1 and β-catenin bind to the region -3081 -3114 of the c-myc promoter in T-ALL.

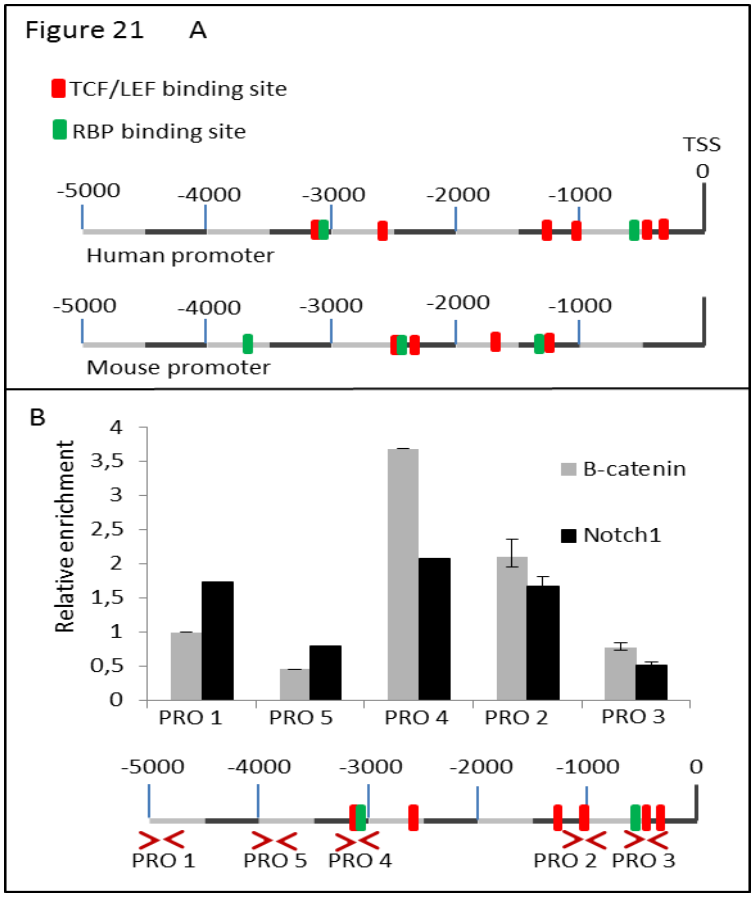


Figure21. c-Myc promoter analysis. (A) Scheme of human and mouse promoter sequence. 5Kb upstream of the TSS are shown. Green boxes indicate RBP binding sites, and Red boxes indicate TCF/LEF binding sites. (B) CHIP assay performed in RPMI8402 cell line. Arrows indicate the position of primers used for quantitative PCR. Enrichment of N1ic and B-catenin binding on human MYC promoter (relative to Ig) is rapresented. One representative experiment is shown and bars represent SD.

To assess the functionality of the Notch1 and β-catenin binding in the c-Myc promoter, we cloned different regions of the murine c-myc promoter in a luciferase-driving vector. Using this strategy, we

can measure the transcriptional activity of different C-Myc reporters in response to Notch, β-catenin or specific inhibitors.

The constructs used are represented in figure 22 and they include:

- **3000-Mycp-luc** contains the region from the +389 to -2971 bp to the TSS;
- **500-Mycp-luc** contains the region surrounding the distal paired BS, from – 2421 to -2971;
- **35-Mycp-luc** only contains the 35 bp around this same distal paired BS, from -2446 to – 2481.

First, we assayed the transcriptional activity of the 3000-Mycp-luc construct in the presence of the Notch inhibitor (DAPT) and β-catenin inhibitor (PKF-115-584). We transfected 293T cells with this reporter and the constitutive β-galactosidase vector. Because of the different mechanisms of action of each inhibitor, for the DAPT treatments 293T cells were treated for 48 hours before transfection and 48 additional hours after transfection, while PKF115-584 was added only after transfection. We found that 3000-Mycp-luc was efficiently inhibited by PKF115-584 but not by DAPT. The combination of both inhibitors had a greater effect (but not statistically different) than PKF alone (figure 22, B). This suggested that β-catenin and Notch cooperate in the activation of c-Myc, however we consistently failed to induce the 3000-Mycp-luc reporter by ectopic expression of active β-catenin S37I or Notch. Only when cotransfected with the Notch co-activator MamL, both β-catenin S37I and N1IC were able to activate the

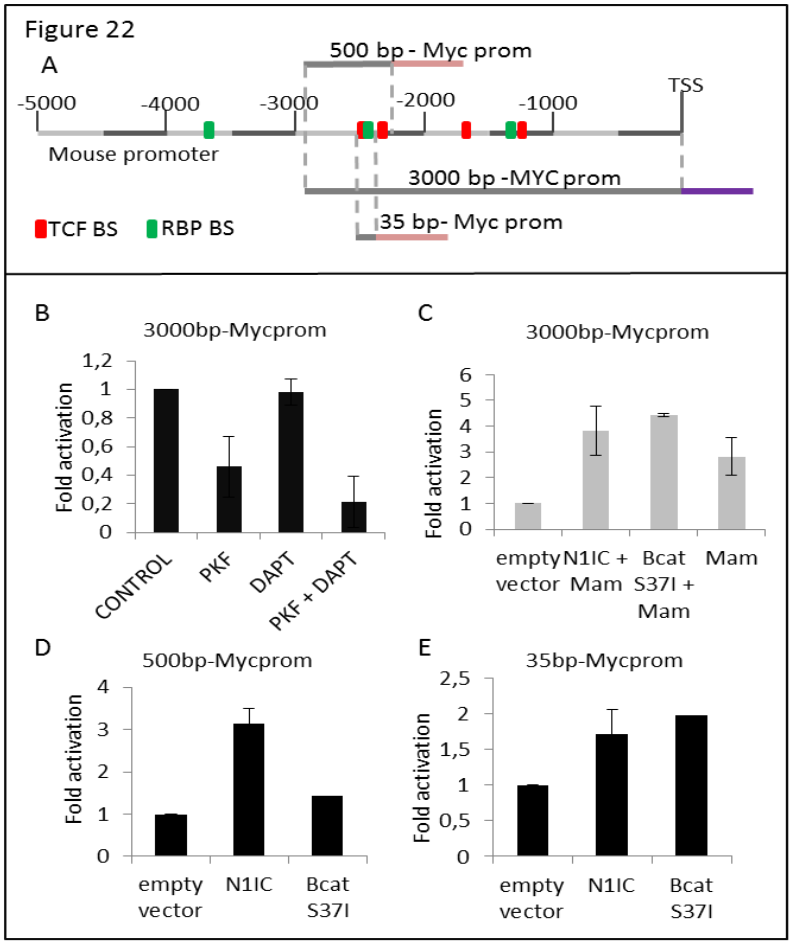


Figure22. Luciferase assay. (A) scheme of the constructs of c-Myc promoter used for luciferase assays. 3000-Mycp-luc contains the region from the +389 to -2971 bp respect to the TSS; 500-Mycp-luc contains the region surrounding the distal paired BS, from – 2421 to -2971; 35-Mycp-luc only contains the 35 bp around this same distal paired BS, from -2446 to –2481. (B- E) Luciferase assay performed with the indicated promoter constructs. Fold activation are relative to control, which have been normalized to 1. Graphs represent the average of at least 3 experiments, and bars represent SEM. (B)Assay performed in 293T cell line with drug treatments. (C)Assay performed in 293T cell line with transfection of N1IC, MamL and β-catenin S37I. (D-E)Assay performed in U2OS cell line with transfection of N1IC and β-catenin S37I.

promoter (figure 22, C), suggesting that Mam was a limiting factor for c-Myc expression. In these conditions, the simultaneous presence of Notch1 or β-catenin did not show any further effect on the promoter activation (data not shown). Similar, but not identical, results were obtained by using the 500-Mycp-luc and the 35-Mycp-luc contructs, which can be activated by N1IC and β-catenin S37I at different levels in both 293T (not shown) and U2OS cells (figure 22 D and E).

To further explore the binding of Notch1 and β-catenin to the c-Myc promoter, we performed Electrophoretic Mobility Shift Assay (EMSA) using a double strand DNA probe that contained the sequence of the 35-Mycp-luc contruct. This region includes the distal paired RBP-TCF/LEF BS of the murine promoter (table R3). The probe was radioactively labeled at the protruding ends by Klenow polymerase reaction. We also produced recombinant proteins corresponding to the Notch binding domain of CSL, the RAM and Ankyrin domain of Notch1, and the Notch binding domain of MamL. These proteins have previously been proved to form a ternary complex on a CSL consensus DNA probe (Nam et al., 2007) and this complex has been crystallized (Arnett et al., 2010). Different combinations of these recombinant proteins were incubated with the probe during 30 minutes at 30C, and then electrophoresed in polyacrylamide gel and analyzed by autoradiography. We observed a complex formed by CSL, Notch1 and Mam on the c-Myc probe (figure 23, lines 2-3-4).

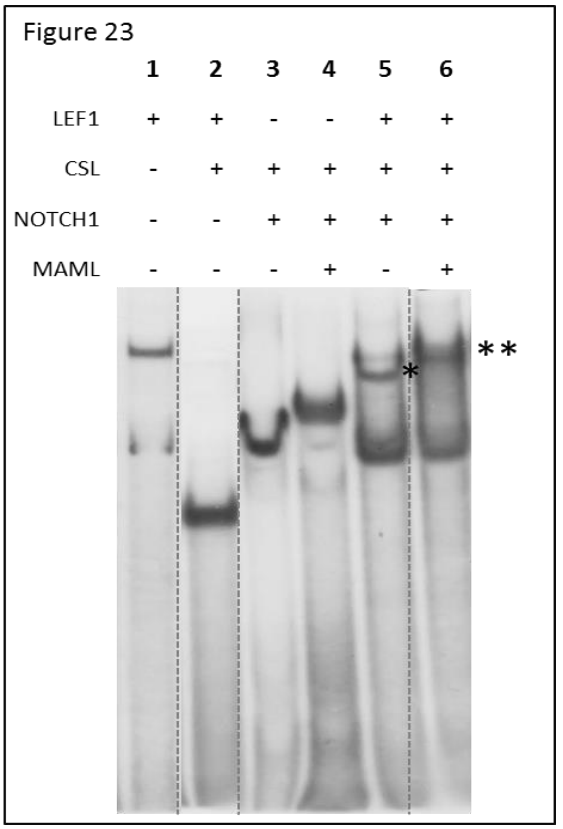


Figure 23. EMSA performed with c-Myc promoter radioactively labeled probe. **N**umbers are used to identify the lines, and + and – indicate the recombinant proteins added to the probe.

The Lef1 protein was used in the full-length form and it was difficult to produce it in similar concentration as the other proteins. We found that Lef1 can efficiently bind our probe (figure 23, line 1). However, depending on the amount of protein loaded, two different retardation bands can be observed, which could suggest the presence of Lef1 homodimers. When we mixed CSL and Notch1 together with Lef1, we found that a higher complex was formed (labeled with the asterisk) (line 5), which was shifted up in

the presence of MamL (line6, double asterisk). These results indicate that the Notch1 complex (CSL-Notch1-Mam) and Lef1 can simultaneously bind and form a complex on the c-myc promoter probe. We next, tested whether recombinant β-catenin bound to this complex; however we have not been able to determine this possibility due to different technical problems (not shown). Nonetheless, the binding of Lef1 suggest that β-catenin may also be recruited to this complex.

3.2.6 c-Myc is required for Notch1 induced leukemia but not sufficient for inducing N1-dependent leukemia in β-catenin deficient BM cells

In order to elucidate the requirement of c-Myc for Notch1 induced leukemia in our *in vivo* system, we used the strategy of Knocking down c-Myc by shRNA that has been previously proved to down-regulate myc (Uribesalgo et al., 2011). As shown in figure 24A, we transduced WT BM Lineage negative cells with the c-myc-specific shRNA together with pMIG-N1IC-gfp. We transplanted these cells in irradiated mice and analyzed the presence of leukemic cells after 1 month. We did not find any signs of leukemic disease as determined by the presence of CD4+CD8+ DP cells in blood circulation (figure 24, panel B).

After 6 months transplanted animals were sacrificed and we did not observe any sign of leukemia (figure 26). Noteworthy, the animals transplanted with the cells transduced with N1IC and myc-shRNA

were reconstituted with YFP+ donor cells (from 15 to 40 % of YFP+ cells), indicating that those cells were able to engraft.

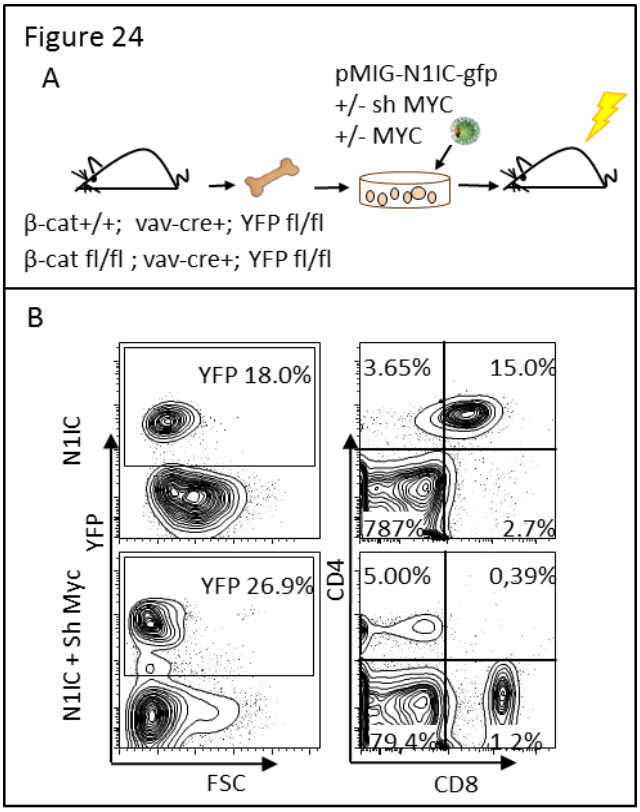


Figure24. Down-regulation of c-Myc. (A) Scheme of the experiment. BM from mice with the indicated genotype has been extracted and lineage-depleted. Cells were infected with the indicated viruses and transplanted in irradiated recipient animals. (B) PB FACS analysis of recipient animals 1 month after transplant. Percentage of CD4+/CD8+ cells is shown.

These results suggest that c-myc is required for Notch1-induced leukemia, which is in agreement with results obtained by genetic deletion of c-myc at the DP stage of T-cell development (Li et al., 2008).

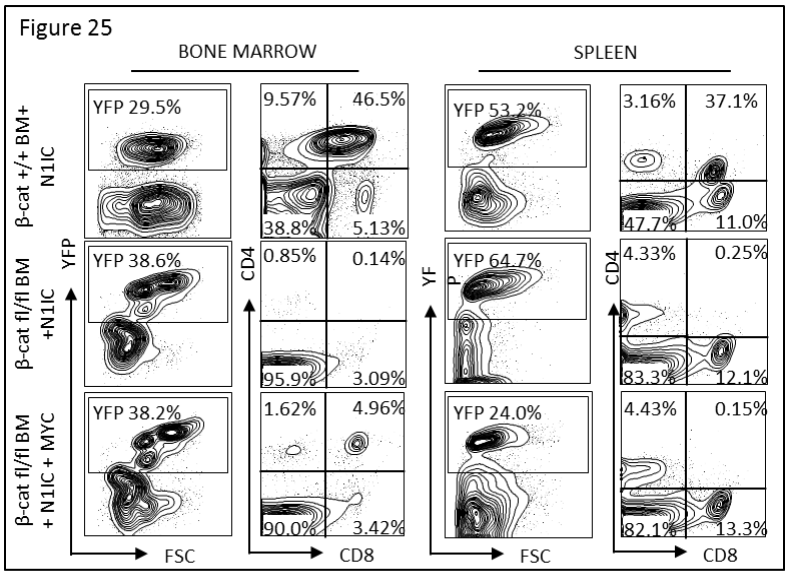


Figure25. End-point analysis of animals transplanted transplanted with Lin- BM cell with the indicated genotypes. YFP and CD4+/CD8+ FACS analysis of spleen and Bone marrow is shown.

Next, we investigated whether restoration of c-myc expression was sufficient to produce Notch1-dependent T-cell leukemias in the absence of β-catenin. As shown in figure 24 A, we performed a transplantation assay with β-catenin KO Lin-BM cells, co-transduced with pMIG-N1IC-ires-GFP and a c-Myc retroviral vector. Mice transplanted with β-catenin KO cells co-expressing c-Myc and N1IC did not develop T-cell leukemia. Interestingly, we detected a small amount (1%-5%) of DP CD4+CD8+ cells in blood circulation in 2 out of 6 mice 1 month after the transplant, which persisted after 6 months but neither increased nor invaded the spleen (figure 25). Similar to previous experiments, mice transplanted with N1IC-transduced β-catenin WT BM cells showed

severe signs of disease and had to be euthanized about 2-3 month after transplant. These animals presented leukemic CD4+CD8+ DP cells in PB, BM and spleen while those that received β-catenin KO BM cells did not (figure 25).

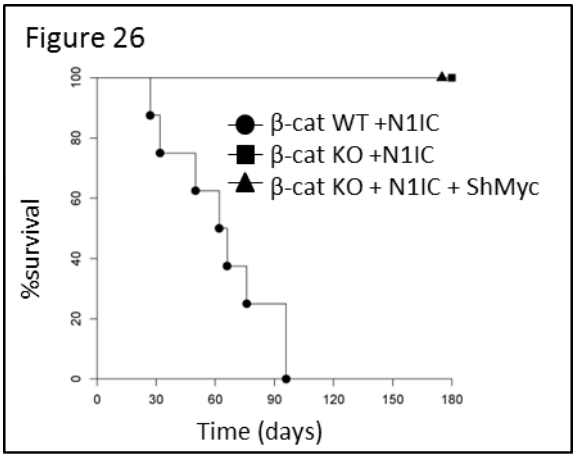


Figure26. Kaplan-Meier curve of transplanted animals. Percentage of alive animals (y axis) is shown for every time point (x

These results suggest that the re-expression of c-myc is not enough for Notch1 to generate leukemia in the absence of β-catenin.

3.2.7 β-catenin is needed for the activation of a subset of Notch1 target genes in BM cells.

Since ectopic c-myc expression was not enough to generate N1IC-induced leukemias in the absence of β-catenin, we hypothesized that other genes that depend on both Notch1 and β-catenin are required. To identify putative candidate genes, we performed an expression

microarray following the protocol shown in figure 20A. In brief, β-catenin WT or β-catenin KO Lin- BM cells were transduced with the control or the N1IC vector and after 48 hours GFP+ cells were sorted. RNA was extracted, retro-transcribed in cDNA, amplified and then hybridized on an Affymetrix Array. In figure 27 some selected genes are shown, red color indicates positive expression while green color indicates negative expression.

We have found that some known Notch target genes were activated by N1IC both in β-catenin WT and β-catenin KO BM cells, such as DTX1, DTX2, JAG1, HES5, HEY1, DLL1, GATA3, NOTCH2 and NOTCH1 itself. However, c-myc activation was too low to reach significance in these experiments, and is not present in this gene list.

An important observation was that the general basal intensity of expression obtained in the hybridization of the RNA from β-catenin KO cells was lower. Consequently, we needed to normalize the total intensity of each set of samples to compare the gene activation induced by Notch1 in both β-catenin WT and KO cells.

By performing hierarchical clustering on the data set we could visualize four main groups of genes (figure 27):

1) Genes activated by Notch1 and requiring β-catenin (activated in WT but not in β-catenin KO cells).

2) Genes activated by Notch1 independently of β-catenin (activated in both β-catenin WT and KO cells).

3) Genes activated by β-catenin independently of Notch1 (down-regulated in β-catenin KO cells respect to WT cells, independently on N1IC).

4) Genes down-regulated in the presence of N1IC (not included in this thesis).

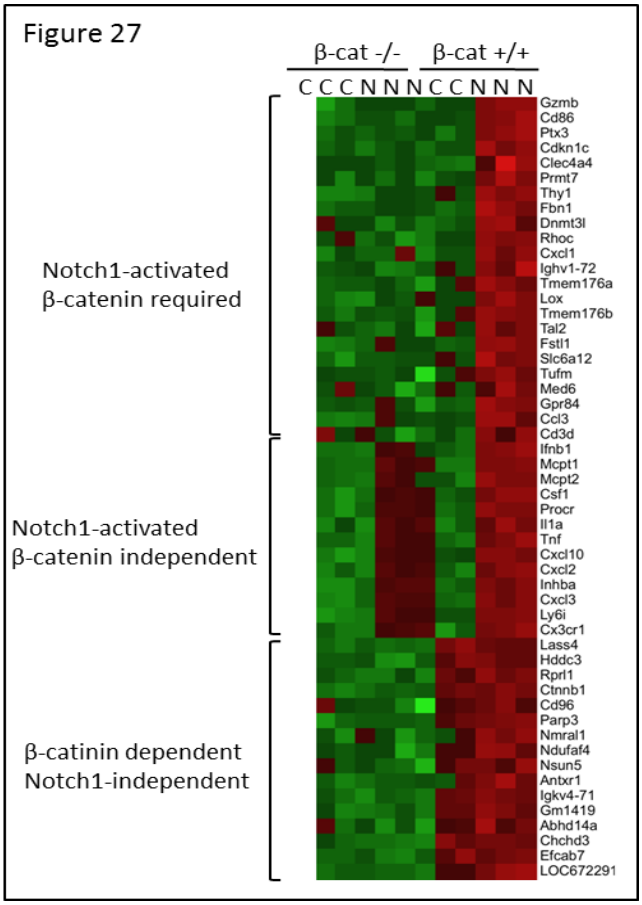


Figure27. Expression microarray. WT or β-catenin KO BM cells were transduced with the control and N1IC vector. RNA was extracted from GFP+ sorted cells and then hybridized on an Affymetrix array. Red color indicates positive expression while green color indicates negative expression.

The obtained gene lists were subjected to gene set enrichment analysis (GSEA) (http://www.broadinstitute.org/gsea) for analysis of significant overlaps with published gene sets and gene ontology (GO) categories.

Figure 28

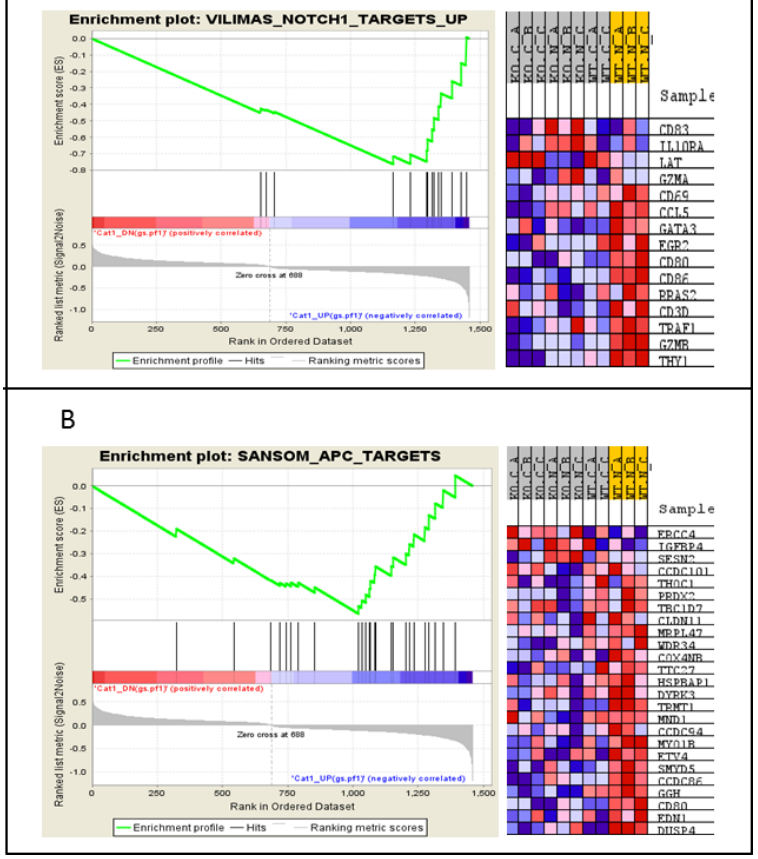


Figure28. GSEA analysis. Enrichment plots of genes overlapping with the indicated published microarray data set. Red color indicate higher expression and blues indicates lower expression. The green curve plots the running enrichment score. (A) Enrichment of the gene list published in (Vilimas et al., 2007). (B) Enrichment of the gene list published in (Sansom et al., 2007).

Analyzing the Group 1 as input genes list we have found an overlapping with the data from the microarray published in (Vilimas et al., 2007) (Figure 28, panel A). In this publication from Iannis Aifantis' laboratory they demonstrated that over-expression of N1IC in BM cells increases the expression of NfκB target genes (as exposed in section 9 of the introduction). This correlates with the fact that many NfκB target genes (IL1, TNF, CXCL1, CXCL3, CXCL10), as well as the significant enrichment of GO category "inflammatory response", are enriched in this gene cluster. Furthermore, Group 1 genes also overlapped with the data from the microarray published in (Sansom et al., 2007) (figure 28, panel B), which showed that c-Myc deletion could rescue Apc deficiency (that causes β-catenin over-activation) in the intestine. Together, these results suggest that β-catenin is required for the activation of a subset of Notch1-target genes in BM cells during the early events of T-cell transformation. In addition, some of those β-catenin dependent genes (Group 1) are related to NfκB activation, which is a crucial event in this process; and moreover they are related to c-Myc, which supports the idea of its regulation being impaired in absence of β-catenin.

These results suggest that β-catenin is required for the activation of a subset of Notch1-target genes in the early events of T-cell transformation. Moreover, some of the genes in group 1 might be directly induced by Notch1 and β-catenin similarly to c-myc.

4. MATERIALS AND METHODS

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Cell lines culture. T-ALL cell lines (CEM, JURKAT, DND-41, HPB-ALL, RPMI8402, LOUCT, KOPT) and cell lines from other lineages (RPMI, RAJI, HLl-60, NALM6) have been cultured in RPMI media (Gibco, ref.12633-012).

Cell treatment. Cells were treated with 5 μM DAPT (SIGMA-ALDRICH, D5942) and 0.2 nM PKF-115-584 (Novartis) during 4 days. Cell counting has been done in the Neubauer chamber with Trypan-blue exclusion criteria.

Western blotting. Whole cell lysates were obtained by incubating for 20min at 4C in PBS plus 0.5% Triton X-100, 1mM EDTA, 100 mM Na-orthovanadate, and complete protease inhibitor cocktail (Roche). Lysates were electrophoresed in SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), transferred to Immobilon-P transfer membranes (Millipore) for western blotting and incubated with the primary antibodies (listed below) at 4C overnight. The membranes were then incubated with the corresponding secondary HRP-conjugated antibody (DACO, ref.P0448 and P0260) during 1 hour at RT. After washing, they were exposed to EZ-ECL mixed reagents A and B (BI, ref. 20-500-500A/ 20-500-500B), and developed in a radiography machine.

Antibodies

- Monoclonal anti-αTubulin (SIGMA-ALDRICH, ref.T6074)
- Anti-Cleaved Notch1 (Cell Signaling, ref.4147)
- Anti-β-catenin (SIGMA-ALDRICH, ref.C22069)
- Polyclonal anti-Lamin-B (SANTA CRUZ BIOTECHNOLOGY, ref.sc-6216)

Cell fractionation. Nuclei were isolated in 0.1% NP-40 in PBS for 5 min on ice, followed by centrifugation at 1900 rpm and then lysed in 50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Nonidet P-40, 5mM EGTA, 5mM EDTA, 20mM NaF and complete protease inhibitor cocktail (Roche). Supernatants were recovered as the cytoplasmic fraction.

Mice. C57/BL6 mice were kept in specific pathogen free conditions (SPF). All the procedures have been approved by the PRBB ethic committee. Ctnnb+/+ Vav-cre+ were used to obtain β-catenin WT BM and Ctnnb f/f Vav-cre+ for β-catenin KO BM.

Clonogenic assay in methylcellulose: BM cells were obtained from C57B6/J mice flushing the femurs with PBS. Cells were Lineage-depleted by using a cocktail of antibodies (CD3, B220, Mac-1, Gr-1, Ter-119) that stain most of the hematopoietic differentiated cells; Those cells were kept in liquid culture in presence of SCF, Flt3L, IL6, IL7, and next transduced with a vector carrying the active form of Notch1 (pLXSH-N1ICΔOP) or the

empty vector, both having Hygromycin resistance. The day after, cells were infected again with a vector carrying the shRNA against Hes1 (pLKO-ShHes1-puro), an expression lentivirus for CYLD or the corresponding empty vector. 48 hours later, infected cells were plated into methylcellulose (StemCell technologies, REF 03434). After 1 week cells were extracted from the methylcellulose by pipetting with media; 1000 cells per condition were then re-plated into fresh methylcellulose. Colonies were counted weekly, disrupted and cells were re-plated again during 4 weeks.

Hematopoietic transplantation for leukemia induction. Bone marrow from Ctnnb1 fl/fl; vav1-Cre+ or Ctnnb1+/+; Vav-Cre+ were obtained by flushing the femurs. BM cells were then lineage-depleted using the Biotinylated mouse Lineage panel of antibodies (CD3, B220, Mac-1, Gr-1, Ter-119, BD Pharmingen) and the anti-biotin micro-beads on the Midi-MACS separator (Miltenyi). Lin- cells were cultured during 24 hours with 10%FCS, 1% Penicillin/Streptomycin, 55μM β-Mercaptoethanol, SCF 50ng/ml, Flt3L 50 ng/ml, IL6 10 ng/ml, IL7 10 ng/ml and then infected with pMIG-N1IC-iresGFP or p-MIG-GFP. After 48 hours, infected cells were harvested in 300 μl of PBS and transplanted in recipient animals by tail vein injection, together with 250000 nucleated WT bone marrow cells for radio-protection. Recipient mice were previously irradiated with two doses of 4 Gy, separated by an interval of two hours, receiving a total dose of 8Gy from a 137Cs source.

Mice analysis. As soon as 2 weeks after the transplant mice were observed for detecting signs of disease and 100µl of blood obtained from the tail vein was analyzed by FACS. At the end-point, mice were euthanized by cervical dislocation; hematopoietic organs such as Spleen, PB, BM, and thymus were also collected and analyzed by FACS.

Giemsa staining. Blood from animals has been smeared onto slides and incubated during 30 seconds with the quick panoptic reagents (QCA, ref 990091) reproducing the Wright-Giemsa staining pattern. Slides have been deeply washed and dried.

Flow Citometry. For FACS analysis, cells were collected in FBS10%PBS, blocked in rat serum 20%PBS and stained 30 minutes in FBS10%PBS with BD Pharmingen antibodies: PE-Cy7 Rat Anti-Mouse CD4, PE Rat Anti-Mouse CD8a. Peripheral blood was erithrocyte-depleted with ACK lysis buffer (Lonza, ref10-548E). Data were analysed with the Flowjo software.

Genomic DNA extraction. Cells were incubated during 1 hour at 90C with 75 µl of (25 mM NaOH / 0,2 mM EDTA), then 75 µl of 40 mM Tris HCl pH 5.5 were added. Supernatant was harvested after centrifugation at 26000 rpm during 2 hours at 4C.

Quantitative RT-PCR. Total RNA was obtained using the RNeasy kit from Qiagen. RNA quality was assessed on agarose gels and quantified by Nano-Drop1000 (Nano-Drop, Wimington, DE).

cDNA was synthesized with the RT-First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech) following manufacture instructions. Real-time polymerase chain reaction (PCR) was performed in triplicates on the Light Cycler 480 (Roche) and SYBR-Green (Roche) was used to detect gene expression. Used primers are listed here on. GAPDH and β2microglobulin were used as housekeeping genes for normalization.

Target gene	**Forward Primer**	**Reverse Primer**
Notch1	CAATCAGGGCACCT GTGAGCCCACAT	TAGAGCGCTTGATTGG GTGCTTGCGC
hes1	CGGCATTCCAAGCTA GAGAAGG	GGTAGGTCATGGCGTT GATCTG
c-MYC	GCTGGAGATGATGA CCGAGT	AACCGCTCCACATACA GTCC
GAPDH	TGTTCCTACCCCCAA TGTGT	TGTGAGGGAGATGCT CAGTG
CYLD	GCCTGGCTTTTCTTT GACAG	GAAGGGCCATCATCA AAAGA
β2-microglobulin	CTGACCGGCCTGTAT GCTAT	CAGTCTCAGTGGGGGT GAAT
β-Actin	GTGGGCCGCCCTAG GCACCAG	CTCTTTGATGTCACGC ACGATTTC
IL7R	CCCAGGATGGAGAC CTAGAA	ACTCGTAAAAGAGCC CCACA

Viral production. Recombinant lentivirus and retrovirus were produced by transient transfection of HEK-293T cells according to Tronolab protocols (http://tronolab.epfl.ch/page58122.html). Briefly, subconfluent HEK-293T were cotransfected with 20 μg of transfer vector, 15 μg of packaging plasmid (psPAX2), and 6μg of envelope plasmid (pMD2.G). After 3 days, supernatant was ultracentrifuged in Beckman L-70 at 26000rpm for 2 hours at 4C and viral pellet resuspended in 100 μl of PBS. 20μl of fresh viral suspension was used per infection. Plasmids used for virus production are: pMIG-N1IC-ires-GFP (obtained from Dr.Iannis Aifantis); pLKO-shHes1 (Sigma, ref. TRCN0000028854), LV-rrCMV-HA-CYLD (obtained from Dr. Pfeifer), pRetrosuper-ShMYC (obtained from Dr.Di Croce), pBABE-puro-HA-MYC (obtained from Dr.Di Croce).

Bioinformatics promoter analysis. The sequence of 5 Kb DNA region up-stream of the TSS has been analysed for murine and human c-Myc gene. We performed the analysis by using the Genomatix Mathinspector software (http://www.genomatix.de).

Luciferase assays. Luciferase reporter assays were performed in HEK-293T cells and U2OS. Cells were seeded in 12-well plates at a density of 5 × 104 cells per well. Equal amounts (200ng) of the different reporter constructs, indicated amounts of the different expresion vectors and 150 ng CMV-β-Galactosidase plasmids were transfected in triplicate wells. Cells were transfected using Polyethylenimine (PEI) (Polysciences, Inc.). Luciferase was

measured after 48h of transfection following manufacturer instructions (Luciferase Assay System; Promega). Luciferase values were normalized according to β-Galactosidase levels. Fold activation values are referred to the controls, which have been normalized to 1. Plasmids used for the luciferase assays are: pCMV-β-galactosidase, pGL3-3000bp-Mycprom, pGL3 enhancer-500 bp-Mycprom, pGL3 enhancer-35 bp-Mycprom, pCDNA3-N1IC, pCDNA3-β-catenin S37I.

Chromatin immunoprecipitation assay. Cross-linked chromatin from RPMI8402 cell lines was sonicated for 10 minutes, medium-power, 0.5 interval with a Bioruptor (Diagenode). Equivalent parts of chromatin were precipitated with anti-ICN1 (Abcam), anti-β-catenin (BD Bioscience) or an irrelevant Ig. After cross-linkage reversal, precipitated DNA was used as a template for quantitative PCR.

EMSA. Double-stranded 32P-labeled DNA was prepared by filling of annealed oligonucleotides with Klenow fragment in the presence of 32P-labeled dCTP. Each duplex was designed to incorporate at least three labeled cytosine nucleotides. Labeled DNA was incubated with indicated recombinant proteins for 30 min at 30 C in a 15 μl volume containing HEPES buffer (20 mM, pH 7.9), KCl (60 mM), MgCl2 (5 mM), DTT (10 mM), BSA (0.2 mg/ml), dGdC (200 nM) and 10% glycerol. Samples were electrophoresed at 180 V on 10% Tris-glycine-EDTA gels. Gels were dried on whatman paper and analyzed by autoradiography.

Microarray hybridization and analysis. β-catenin WT or β-catenin KO Lin- BM cells were transduced with the control or the N1IC vector and after 48 hours GFP+ cells were sorted. RNA was extracted with Quiagen RNAsy kit (QUIAGEN), retro-transcribed in cDNA using RT-First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech), amplified and then hybridized on the Affymetrix Array AFM 4.0. The obtained gene lists were subjected to gene set enrichment analysis (GSEA) (http://www.broadinstitute.org/gsea).

5. CONCLUSIONS

5. CONCLUSIONS

1. Hes1 is a crucial element for the development of Notch-1-induced T-ALL.
2. Down-regulation of CYLD by Hes1 is required for the persistence of NFkB activatity and maintenance of Notch-1-induced T-ALL
3. T-ALL cell lines contain detectable levels of nuclear β-catenin
4. β-catenin pathway inhibitors reduce the growth of T-ALL cell lines
5. Active Notch1 is not able to develop leukemia in β-catenin deficient BM cells.
6. Notch1 induced C-MYC transcription is impaired in β-catenin deficient BM cells.
7. C-MYC promoter has functional CSL and TCF/LEF binding sites and is regulated by Notch1 and β-catenin
8. Knocking down c-Myc prevents Notch1 induced leukemia *in vivo.*
9. C-Myc over-expression is not sufficient for restoring the ability of β-catenin deficient BM cells to develop leukemia upon Notch1 activation
10. β-catenin is needed for the activation of a subset of genes in N1IC-transduced Lin- BM cells.

6. DISCUSSION

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6.1 NfκB activation downstream of Notch1

The central role of the Notch pathway in T-ALL ontogenesis is well established (Pear et al., 1996; Weng et al., 2004). Still, the downstream events that participate in the Notch-related oncogenic program are not understood. This work has been performed with the aim of better understanding the Notch1-induced downstream events that are relevant in T-ALL. Our group has demonstrated that the Notch-Hes1-CYLD-NFkB axis is crucial for the development of T-ALL. Our data show that Hes-1, downstream of Notch1, directly represses the deubiquitinase enzyme CYLD, a negative regulator of the IKK complex; NfKb is then constitutively active. In addition, the work presented in this thesis, directly assessed the *in vivo* contribution of each protein involved in this cascade.

Our results demonstrate that the down-regulation of Hes1 is sufficient to abolish the ability of Notch1 to generate leukemia in our mouse model. In our experiments we down-regulated Hes1 infecting BM cells with a lentiviral construct carrying a shRNA against Hes1. These results have further been confirmed by a work published by Dr Radtke's group where deleting Hes1 they abolished the generation of ICN1-induced leukemias (Wendorff et al., 2010). In this work they used Hes1fl/fl- Mx-Cre mice to conditionally delete Hes1 in the hematopoietic system. They induced the deletion

concomitantly to Notch1 activation, or following leukemia establishment, thus proving the requirement of Hes1 downstream of Notch1 both during the initiation and the maintenance of the disease.

The bHLH transcription factor Hes1 is one of the best-accepted direct Notch target genes. It functions as a transcriptional repressor of genes that require bHLH factors for their transcription. Which particular Notch dependent functions are mediated by Hes1 has not been completely elucidated. Importantly, Dr Radtke's work revealed the role of Hes1 during normal T cell development: Hes1-deficient progenitors have a clear disadvantage to generate thymocytes compared to WT cells. Nevertheless, accumulation of immature B or myeloid cells was not observed in thymi of Hes1 mutant mice. Once the T cell lineage has been specified, thymocyte maturation was not measurably perturbed in the absence of Hes1 (Wendorff et al., 2010). Overall, Dr. Radtke's publication demonstrates that Hes1 is a major component of Notch1-induced signaling during T cell lineage commitment as well as during T-cell leukemia generation, concordantly to our results.

The over-expression of CYLD was carried out also infecting the cells with CYLD lentivirus the day after of N1IC activation. In this set of experiments leukemia development was strongly reduced, still some DP CD4+/CD8+ appeared in transplanted animals; those DP cells never increased in number neither infiltrated in the BM. These results demonstrate that CYLD down-regulation, which in turn maintains NfkB activation, is a crucial event downstream of Notch1 and Hes1 in T-ALL. These results proved a crucial role for

NfκB activity in the establishment/ initiation of leukemia in this mouse model. Results published in our previous work (Espinosa et al., 2010), demonstrated that NfkB activity is needed as well for the maintenance of the disease, as its repression (performed inducing the deletion the regulatory subunit of the IKK complex, IKKγ) following to T-ALL insurgence caused regression of the disease. All together these data highlight the multi-step crucial role of NfkB survival pathway in Notch1-induced leukemia. A recent report highlighted that the miR-19 (that inhibits CYLD expression) is up-regulated in human T-ALL samples, thus further confirming the importance of down-regulating this factor for triggering Nf-kB activation in T-ALL (Ye et al., 2012). NfκB pathway is implicated in many oncogenic processes in mammalian systems, lymphomas and leukemia among others. Deciphering the molecular cascade of events that lead to its constitutive activation in a system is an important issue for the development of targeted therapies.

6.2 Notch1 and β-catenin cross-talk in T-ALL

Notch1 and Wnt/β-catenin pathway crosstalks have been decribed in many systems ((Estrach et al., 2006) (Yamamizu et al., 2010) (Devgan et al., 2005) (Rodilla et al., 2009)and introduction section 6). However, a possible involvement of Wnt/β-catenin in T-ALL has never been addressed.

6.2.1 T-ALL cell lines express β-catenin and are sensitive to β-catenin pathway inhibitors

T-ALL cell lines are a good model for studying the alterations that lead to leukemic transformation and maintenance. For example, most of the characterized T-ALL cell lines are known to contain mutations in the Notch receptor that result in the persistence of the activated truncated Notch1 protein. We have found that many T-ALL cell lines contain high levels of β-catenin, which is an indirect indication of activation of the Wnt pathway and stabilization of β-catenin protein and also a required step previous to its nuclear translocation. Moreover, in some cell lines, we have also specifically tested the subcellular localization of β-catenin and found a nuclear fraction of this protein in DND41 and RPMI8402. This finding suggested that T-ALL cells could be dependent on the Wnt/ β-catenin pathway for their growth. Thus, we tested the effect of a previously described β-catenin transcriptional inhibitor on these cells. The growth of the T-ALL cell lines DND41 and RPMI8402 treated with the β-catenin inhibitor PKF-115-584 was significantly reduced. As exposed in the results section, PKF-115-584 inhibits β-catenin transcriptional activity impairing its binding to TCF and LEF DNA-binding protein.

In a work published by Dr.Carrasco's group, they tested In Vivo Efficacy of PKF115–584 in a murine xenograft model of human multiple Myeloma (Sukhdeo et al., 2007). In those experiments PKF treatment significantly prolonged survival of treated animals compared with controls; furthermore it decreased the formation of

intestinal metastasis. Nonetheless BM hematopoiesis was adversely affected in those PKF115–584-treated mice. Because of the important role of β-catenin signaling for intestinal homeostasis, gut toxicity could also be a problem for the use of this compound in patients. Thus, our results of cell lines treatment may suggest the possibility of treating T-ALL mouse model with PKF115-584.

6.2.2 Active Notch1 is not able to develop leukemia in β-catenin deficient BM cells.

The results of our *in vivo* experiments reveal that β-catenin is needed for the development of Notch1 induced leukemia. Differently from the experiments of part1 in which we down-regulated Hes1, in this set of experiments the deletion of β-catenin is previous to the activation of Notch1. As exposed in the results section, we carried out β-catenin deletion crossing Ctnnb fl/fl with Vav-Cre mice; since the Vav1 gene is expressed from the day E12,5-E13,5 of embryonic development (Bustelo et al., 1993), β-catenin deletion is occurring just after hematopoietic cells are determined. For this reason, in our experimental approach, the BM cells lack β-catenin before being transduced with N1IC. In fact, we have previously confirmed that deletion of β-catenin in Ctnnb fl/fl - Vav-Cre fetal liver is almost complete (Ruiz-Herguido et al., 2012). The deletion of β-catenin done concomitantly to the transduction of the active form of Notch1 could give rise to the same or either a different result. With this strategy we cannot distinguish between

the involvements of β-catenin during the initiation (establishment of the oncogenic program that defines a leukemic cell) or the maintenance of the disease. To understand weather β-catenin is needed for the maintenance of the disease we should abolish its activity after T-ALL establishment. A strategy for doing this would imply the use of an inducible Cre that allows deleting the gene at a certain time point, like has been done for proving the role of Hes1 and NfκB in the maintenance of T-ALL. In Dr. Radtke work (Wendorff et al., 2010), they infected Hes1fl/fl Mx-Cre+ with pMIG-N1IC-GFP retrovirus and after establishment of the leukemia, they induced Cre expression by injecting pI-pC to transplanted animals and deleted Hes1; this caused disappearing of DP CD4/CD8 cells and the regression of the leukemia. The same strategy has been used by Dr. Aifantis laboratory for prove the role of NfκB in the maintenance of T-ALL (Espinosa et al., 2010).

To exclude the possibility that β-catenin WT and KO cells had different capacity of transduction with the pmig-N1IC-gfp retrovirus we tested the transduction efficiency of both cell types. Our results however ruled out this possibility since the percentage of β-catenin WT and KO transduced GFP+ cells before the transplantation was totally equivalent.

Still, questions remain about their fate after the transplant. Results of figure 23 show that cells with integration of the pMIG-N1IC plasmid are present in the thymus of transplanted animals two month after the transplant. However, it is still possible that the retroviral vector is silenced in the β-catenin deficient cells. We found that those cells express different levels of Notch1 mRNA, but

this quantification includes both the endogenous and the exogenous Notch1. Thus, we decided to test the Notch1 target IL7R, to measure the Notch1 activity present in the β-catenin WT or KO; we found that IL7R is similarly expressed, suggesting that overall levels of the pathway are not very different in the two conditions.

Transplantation of β-catenin null cells containing a ROSA26-YFP reporter (donor cells are detected by YFP expression) shown that those cells are able to engraft and survive in the recipient animals. These data exclude the possibility that the lack of leukemia development in our model was due to a failure in the engraftment of β-catenin KO cells. Furthermore, β-catenin fl/fl /VavCre+ mice have normal T-lymphopoiesis, tested by the percentages of thymic, splenic and circulating T-cells (figure 20). This excludes the possibility that the lack of T-cell leukemia formation is due to a lack of T-cell development. As exposed in the introduction, the role of β-catenin during T-lymphopoiesis is not completely clear: the study of different mouse models in different laboratories generated discrepant results. The deletion of β-catenin in thymic cells performed with Lck-Cre causes a block at the DN3 stage of T-cell development , and thus a decrease of splenic T cells (Xu et al., 2003). On the other hand, β-catenin deletion done with Mx-Cre and Vav-Cre do not give rise to any T-cell defect (Cobas et al., 2004; Zhao et al., 2007; Koch et al., 2008; Ruiz-Herguido et al., 2012). Thus, we considered important to specifically check T-cell formation in the model we used.

The relevance of Notch1 activation in T-ALL has generated a great interest in the development of anti-Notch1 targeted therapies for

this disease. Clinical trials aiming to test the efficiency of γ-secretase inhibitors in T-ALL patients have been performed (DeAngelo et al., 2006); still toxicity attributed to the inhibition of Notch1 signaling in the gut and no objective response to the treatment frustrated the expectations (DeAngelo et al., 2006). As exposed in the introduction, the possibility of treating those leukemia with others types of Notch inhibitors, like small peptides (Moellering et al., 2009) or anti-Notch1 antibodies (Aste-Amezaga et al., 2010) is now a very promising idea. Our results highlight a role for β-catenin signaling in Notch1 induced leukemia. Thus, further studies may put forward the possibility of using β-catenin as target for developing new T-ALL therapies. As we have shown before, β-catenin inhibitor PKF 115-854 inhibit the growth of T-ALL cell lines, still we have not tried it *in vivo* on sick animals.

6.2.3 Notch1 and β-catenin cooperate in regulate c-myc transcription

Our data pointed out an impaired regulation of c-Myc in β-catenin null BM cells (figure 26). C-Myc activation is an important Notch-downstream effector that is absolutely required for Notch1 to induce leukemogenesis (Palomero et al., 2006; Weng et al., 2006) (Li et al., 2008). The fact that Notch1 was not able to activate c-myc in the absence of β-catenin could suggest that myc may be a direct target of β-catenin downstream of Notch1. Myc has been shown to be a β-catenin target in the intestine (He et al., 1998),

however in T-ALL myc is well described as Notch target, thus, our data indicate that myc regulation was dependent on both factors.
The observed impairment of its activation could represent a possible explanation for inability to develop Notch1 induced leukemia.
We have now analyzed the C-Myc promoter region and found that contains multiple RBP and TCF/LEF binding sites, as shown in figure 27 and exposed in table R3 and R4. Our results from CHIP, luciferase, and EMSA collectively suggest that the c-myc gene can be regulated both by Notch1 and β-catenin in T-ALL: by ChiP analysis we showed that Notch1 and β-catenin bind to the region corresponding to a paired BS. In addition, EMSA confirmed the binding *in vitro* of Notch complex together with LEF1 to this same region. We tested the activity of the promoter in response to Notch and β-catenin inhibitors by Luciferase assays and they also showed the possibility of co-regulation of this promoter by Notch and β-catenin.
Furthermore, unpublished results obtained in our laboratory prove that Notch1 and β-catenin pathways collaborate to maintain intestinal Stem Cells by triggering transcriptional activation of BMI, c-Myc and ephrinB2, among other common target genes. Concordantly to our findings, c-Myc gene simultaneous recruits both Notch1 and β-catenin on its promoter region as tested by sequential ChIP assay on intestinal cells. In addition to those data, Notch and β-catenin pathways collaboration has been reported in many systems and with several different outputs. Specifically, convergence of both pathways for the regulation of common target genes has been proposed in vascular progenitors (Yamamizu et al.,

2010), ECS (Ogaki et al., 2013) and colon rectal cancer (Rodilla et al., 2009).

Thus, collaboration of Notch and β-catenin pathways has already been described, still had never been reported in T-ALL. Due to the importance of Notch1 transcriptional program in this disease, the identification of its partners in that process is a crucial issue.

6.2.4 C-Myc is needed for Notch1 induced leukemia but it is not sufficient for restoring the ability of β-catenin deficient BM cells to develop leukemia

C-Myc deficiency in BM cells completely abrogates the ability of Notch1 to develop leukemia as demonstrated by Von Boehmer's group (Li et al., 2008). In these experiments, c-Myc was genetically deleted with a CD4-Cre prior to N1IC activation and animals transplanted with those cells did not develop leukemia. Thus, we wanted to test the effect of down-regulating Myc on the BM lineage negative N1IC transduced cells. For this reason, we used a ShRNA against c-Myc and found that it abrogates the ability of N1IC to develop leukemia. This is in agreement with Von Bohemer's data and also other reports that indicate a crucial role for c-Myc downstream of Notch1 in T-ALL (Weng et al., 2006) (Palomero et al., 2006).

Next, we asked whether this factor could be the unique/main target of Notch1- β-catenin interaction during T-ALL generation in mice. In case this was true, we would expect that restoring c-Myc could

rescue the lack of β-catenin to form T-ALL. However, our experiments suggest that C-Myc over-expression is not sufficient for restoring the ability of β-catenin deficient BM cells to develop leukemia upon Notch1 activation. It is important to underlie that in this experimental design β-catenin deletion occurs at a prior time point respect to C-Myc over-expression, which is concomitant to Notch1 activation. Whether c-Myc re-introduction at the same moment of β-catenin deletion could give rise to a different outcome has not been assessed in this work.

C-Myc is widely involved in the regulation of proliferation and metabolism (Gordan et al., 2007; Eilers and Eisenman, 2008). The c-Myc oncogene is overexpressed in the majority of human cancers and can contribute to at least 40% of tumors; reviewed in (Dang et al., 2009). It can be amplified or mutated, but more commonly its expression is induced by oncogenic pathways, which is the case of Notch in T-ALL, and its role in neoplastic transformation has been widely demonstrated. However, the role of this factor in tumor generation can be complex: the oncogenic properties of c-Myc are counterbalanced by its ability to induce apoptosis (Pelengaris et al., 2002a; Pelengaris et al., 2002b); this dichotomy may explain why c-Myc is not commonly the driving oncogene in early tumors; reviewed in (Dang et al., 2009). Furthermore, c-Myc protein levels are tightly regulated both in normal and malignant cells, likely due to its very short half-life: ~30 minutes, (Hann and Eisenman, 1984) and phosphorylation- dependent degradation (Yada et al., 2004).

The fact that ectopic expression of c-myc in β-catenin KO cells cannot reproduce the leukemic phenotype, suggests that myc is not

sufficient for this function. However, it is important to take in account that ectopic c-Myc is under the control of a retroviral promoter and cannot be properly regulated at least at a transcriptional level. We are currently evaluating whether the levels are too high and induce apoptosis or they are too low for inducing transformation. Alternatively, c-myc may be needed at a different time-point during the oncogenic process downstream of Notch1, which β-catenin KO cells may not be able to reach. In any case, we cannot exclude that some technical details may be the reason why T-ALL cannot be restored by ectopically expressing c-Myc in β-catenin KO cells.

6.2.5 β-catenin is needed for the activation of a subset of Notch1 target genes in BM cells

On the other hand, the result obtained with ectopic expression of C-Myc, in β-catenin KO cells, suggested that other miss-regulated genes could be responsible for the inability of β-catenin deficient BM cells to develop leukemia upon Notch1 activation. We took a high throughput experimental approach and performed an expression microarray in the different conditions. Our analysis highlighted the existence of β-catenin dependent as well as β-catenin independent Notch1 transcriptional targets. Among the β-catenin dependent Notch1 targets there are genes codifying for cytokines, like CCL3 and CXCL1, genes involved in cell-cycle regulation, like CDKN1c, and alsoTAL2, a member of the bHLH

Family of transcription factor mutated in some cases of T-ALL *(Xia et al., 1991)*. Thus, β-catenin appears to be required for the activation of a subset of Notch1 target genes in BM cells. The correct regulation of all or some of those factors could be needed for the generation of Notch1 induced T-ALL. Furthermore, some of the genes that are differentially expressed in the two conditions (β-catenin WT and β-catenin KO) could regulate other downstream genetic programs, also involved in leukemogenesis. Noteworthy, BM cells were lysated for RNA extraction 48H after N1IC infection, the same time-point used for the transplantation experiments. This procedure allowed us to identify genes activated during T-ALL initiation; however, it is not possible to exclude that other factors also important for leukemia generation are regulated at a later time point, and thus did not come out in our gene list. In fact, c-Myc itself does not appear in the list of Notch and β -catenin dependent. The reason of this is that the activation folds of c-Myc upon Notch1 induction may be low (around 2 folds) and not sufficient to reach the significance in this experiment. Still, raw data from the expression microarray show a tendency of c-myc gene to get more activated in WT then in β-catenin KO cells, in agreement to the quantitative PCR results (figure 39). To our understanding, the fact that c-Myc gets poorly activated does not necessarily disagree with its importance in the process: its different level of expression could be crucial for the oncogenic program initiated by Notch1. GSEA analysis interestingly showed overlapping of our microarray data with those from Iannis Aifantis' report (Vilimas et al., 2007), thus suggesting that some of the genes in group 1 (β-

catenin dependent) could also be involved in activating NfκB downstream of Notch1 activation. This may provide an interesting starting-point to further studies linking NfκB activation and β-catenin requirement in Notch1 induced T-ALL. Furthermore, the overlapping found with (Sansom et al., 2007), and the enrichment of c-Myc related genes in the group 1 of genes, further support our data about c-Myc regulation by N1IC being impaired in absence of β-catenin.

The results obtained with this microarray analysis suggest that many other factors downstream of Notch1 and β-catenin could be important for T-ALL development, and candidates as possible new therapeutical targets.

Collectively, the work presented in this thesis provided important data for a better understanding of the molecular mechanisms involved in Notch1 induced leukemia. Specifically, in the first part of results we demonstrated the importance of Hes1 expression and CYLD down-regulation to maintain NFκB activation downstream of Notch1 during T-ALL. In the second part we proved the involvement of β-catenin in T-cell leukemia; our results indicate the existence of a crosstalk between β-catenin and Notch1 in this disease, and reveal c-Myc as an important target, but not the only one, of this crosstalk.

7. BIBLIOGRAPHY

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8. ABBREVIATION

AGM: Aorta Gonad Mesonephros

ALL: Acute lymphoblastic leukemia

AML: Acute myeloid leukemia

ARM: Armadillo-repeats

bHLH: basic helix-loop-helix

BM: Bone Marrow

BS: binding site

BSA: Bovine Serum Albumin

Ca+2: Calcium

CHIP: Chromatin immune-precipitation

CKI: Caseine kinase I

CLL: Chronic Lymphocytic leukemia

CLP: Common Lymphoid progenitor

CML: Chronic myeloid leukemia

CMLP common myeloid lymphoid progenitor

CMP: Common Myeloid progenitor

CNS: central nervous system

CRC: Colorectal Cancer

CRD: Cystein-rich domain

CSC: Cancer stem cell

DAPI: 4',6-diamidino-2-phenylindole

DC: Dendritic cell

DLL: Delta-like protein

DMEM: Dulbecco's Modified Eagle's Medium

DN: double negative

DOC: Deoxycholate

DOS: Delta and OSM-11-like

DP: Double positive

DSL: Delta/Serrate/LAG-2

ECD: Extracellular domain

EMSA: Electrophoretic Mobility Shift Assay

ETP: Early thymic precursor

FACS: Fluorescence Activated Cell Sorting

FBS: Fetal Bovine Serum

FZ: Frizzled

GFP: Green Fluorescent Protein

GSI: γ secretase inhibitor

GOF: gain-of-function

GSK3β: glycogen synthase kinase 3 beta

GST: Gluthathione-S-Transferase

HCP: hematopoietic cell progenitors

HES1: Hair Enhancer of Split

HLH: helix-loop-helix

HRT: Hes-related gens

HSC: Hematopoietic Stem Cell

IB: immunoblot

ICD: intracellular domain

ICN1: intracellular Notch1

IL: Interleukin

IL7R: interleukin 7 receptor

IP: Immuniprecipitation

JAG: Jagged ligand

Kb: Kilobase

KO: Knock out

LEF1: Lymphoid Enhancer Factor

LRP: Lipo-protein receptor

LSC: Leukemic Stem Cell

MAML: Mastermind-like

MECP: Myeloid-erythroid common progenitor

MET: Mesenchymal-to-Epithelial Transition

MPP: multipotent progenitors

Mr: Molecular weight

MZ: marginal zone

NLS: Nuclear localization signal,

NRR: Negative regulatory Region

ON: overnight

PB: Peripheral blood

PBS: Phosphate Buffered Saline

PCP: Planar Cell Polarity

PEI: Polyethylenimine, cationic polymer transfection reagent

PEST: Proline (P), Glutamic acid (E), Serine (S), Threonine (T)-rich motifs

RAM: RBPJκ association module

RT: Room temperature

SCF: Stem Cell Factor

SD: Standard Deviation

SP: Single positive

TAD: Trans-activation domain

TAD: Transcription activation domain

T-ALL: T-cell Acute lymphoblastic leukemia

TBS-T: Tris-Buffered Saline Tween-20

TCF: T-Cell Factor

TMD: trans-membrane domain

TMD: Trans-membrane Domain

TSS: Transcription Start Site

WB: Western Blot

WT: wild type