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Perturbations of interactome networks in acute lymphoblastic leukemia: identification of EXT-1 tumor suppressor as a Notch pathway regulator

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SUMMARY (ENGLISH)

Whole genome sequencing technologies have enabled the identification of mutations implicated in diseases including cancer. Recently, research efforts to compare and categorize mutations, genes expression and genomic characteristics helped generating literature-curation databases. A large number of databases were developed to address data integration and standardization for human cancers, such as Catalogue Of Somatic Mutations In Cancer (COSMIC), The Cancer Genome Atlas (TCGA), International Cancer Genome Consortium, Integrative Onco Genomics (IntOGen). Although the identification of these mutations highlights "cancer causative genes", it does not give a detailed explanation of molecular mechanisms leading to the development of cancer. Though, understanding mechanisms leading to cancer development and progression remains a challenge that requires further investigations.

The great majority of mutated genes are found in liquid tumors such as leukemia and lymphomas. In the first part of this study, we reasoned that leukemia associated genes could be extended to additional candidates identified using interactomic approaches. We used protein-protein interaction (PPI) mapping strategies to explore information on cancer genes frequently mutated in Acute lymphoblastic leukemia (ALL). We first extracted mutational data associated to ALL, and used interactome mapping analysis for literature-curated interactions and yeast two-hybrid experimental data in order to identify potential novel target genes associated with ALL. We highlighted mutated hub proteins interconnected in an ALL-cancer gene products network and identified novel interacting partners that link key ALL-cancer driver gene products. We identified EXT1 tumor suppressor gene as a novel common interactor for NOTCH1 and FBXW7.

In the second part of this study, we experimentally validated EXT1, as a novel player in the regulation of the Notch pathway.

Our study thus provides a proof-of-concept on how systematic interactome approaches could allow identification of novel targeted genes and pathways associated to human cancer.

RÉSUMÉ (FRANÇAIS)

Les technologies de séquençage de génomes entiers ont permis l'identification de mutations impliquées dans des maladies comme le cancer. Récemment, des efforts de recherche dans le but de comparer et de classer des mutations, l'expression des gènes et les caractéristiques génomiques ont aidé à construire des répertoires de données se basant sur la littérature. Un grand nombre de bases de données ont été développées afin d'intégrer des données sur les cancers humains, telles que COSMIC; le catalogue des mutations somatiques dans le cancer, "The Cancer Genome Atlas" (TCGA), International Cancer Genome Consortium, Integrative Genomics Onco (IntOGen). L'identification de ces mutations permet une classification des gènes responsables des cancers, par contre elle ne permet pas une compréhension détaillée des mécanismes moléculaires conduisant au développement du cancer. Ainsi, la comprehension des mécanismes menant au développement du cancer et sa progression reste un défi qui nécessite des investigations complémentaires.

La majorité des gènes mutés se trouvent dans les tumeurs liquides tels que la leucémie et les lymphomes. Dans la première partie de cette etude, nous avons soumis l'hypothèse que l'ensemble des gènes associés à la leucémie pourrait être étendu pour inclure d'autre candidats identifiés en utilisant une approches interactomique. Nous avons utilisé des stratégies de cartographie des réseaux d'interaction proteines-protéines (IPP) afin d'explorer des informations sur les gènes du cancer fréquemment mutés dans la leucémie lymphoblastique aiguë (LLA). Nous avons extrait les données de mutations associées à la LLA, par la suite nous avons analysé les réseaux des interactions extraites de la littérature ainsi que des données expérimentales provenant du double hybride en levure, ceci afin d'identifier de nouveaux gènes potentiellement associés à la LLA. Nous avons souligné des "hubs" de protéines mutées et interconnectées dans le réseau de produits des gènes de cancer liés à la LLA, et de nouveaux partenaires d'interaction qui relient les produits des gènes associés à la LLA. Nous avons identifié *EXT1*, un gène suppresseur de tumeur, comme étant un nouvel interactant commun pour NOTCH1 et FBXW7.

Dans la deuxième partie de cette étude, nous avons validé EXT1, en tant que nouvel acteur dans la régulation de la voie Notch.

Notre etude fournit ainsi une preuve de concept démontrant que l'approche interactomique pourrait permettre l'identification de nouveaux gènes et des voies de signalisation associés aux cancers.

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It has been six years since I entered the "research world" as a PhD student, I knew from the beginning that to survive and succeed this "experiment" I needed motivation, hard work and ambition but most important of all, none of this would have been possible if it weren't for the support and the help of a number of people without whom this thesis might not have been written, and to whom I am greatly indebted.

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It is to you, Mama & Baba that I dedicate this dissertation.

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LIST OF ABBREVIATIONS

AGM: aorta-gonad-mesophros

AGS: Alagille syndrome

ALL: acute lymphoblastic leukemia

AML: acute myeloid leukemia

Ank: Ankyrin repeats

AP-MS: affinity purification – mass spectrometry

B-ALL: B cell acute lymphoblastic leukemia

BD: basic domain

bHLH: basic helix-loop-helix

BiFC: biomolecular fluorescence complementation

BM: bone marrow

BRET: bioluminescence resonance energy transfer

CBF1: C-promoter binding factor1

cDNA: complementary DNA

CDK: syclin dependent kinase

CDL: Cullin dependent ligases family

Chip: chromatin immunoprecipitation

CHX: cycloheximide

CIC: cancer initiating cell

CML: chronic myeloid leukemia

DLL: delta like ligand

CNS: central nervous system

CoR: co-repressor

CPD: Cdc4 phosphodegrons

DNA: desoxyribonucleic acid

DNER: Delta/Notch like EGF related receptor

DN: double negative

DOS: Delta and OSM-11 like proteins

DP: double positive

DSL: delta-serrate-lag2 type ligands

ECM: extracellular matrix

ELR: epidermal growth factor repeat

ER: endoplasmic reticulum

ESC: embryonic stem cells

ETP: early T cell progenitor

FBW: F-box and WD40 repeats

FRET: fluorescence resonance energy transfer

GlcNAc: N-acetylglucosamine

GO: gene ontology

GPI: Glycosylphosphatidilinositol

GSI: gamma-sectretase inhibitor

HDAC1: histone deacethylase 1

HD: heterodimerization domain

HECT: homolog to the E6AP carboxy terminus domain family

HT: high throughput

HSC: hematopoietic stem cells

IP: immunoprecipitation

JAK: januse kinase

JM: juxtamembrane

Luc: luciferase

Mib: minbomb

miRNA: micro RNA

Mo: morpholino

mRNA: messenger RNA

NICD: Notch1 intracellular domain

LNR: lin12-notch repeats

MSC: mesenchymal stromal cell

N-coR: nuclear co-repressor

NEC: notch extracellular subunit

Neur: neuralized

NLR: LIN-12-Notch repeats

NLS: nuclear localization signal

NRARP: Notch-regulating ANKyrin repeats protein

NRR: negative regulatory region

NTM notch transmembrane subunit

NKT: natural killer T cell

nTR: natural regulatory T cell

ORF: open reading frame

PCA: protein complementation assay

PCR: polymerase chaine reaction

PDZL: PSD-95/Dlg/ZO-1-ligand

PEST: proline, glutamic acid, serine and threonine - rich domain

PPI: protein-protein interaction

PSM: presomitic mesoderm

qPCR: quantitative PCR

RAM: RBP-Jk associated module

RBR: ring-between-Ring family

Rluc: Renilla luciferase

RNA: ribonucleic acid

RT: reverse transcription

siRNA: small interfering RNA

SMRT: silencing mediator retinoid and thyroid receptors

TAD: transactivation domain

T-ALL: T cell acute lymphoblastic leukemia

TAN1: truncated form of Notch1

TAP: Tandem Affinity Purification

TK: tyrosine kinase

WB: western blot

WD: tryptophan-aspartic acid

Y2H: yeast two hybrid

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INTRODUCTION

1. Analyzing networks in cancer

Cancer is a complex disease in which various cellular processes, signalling pathways and environmental influences contribute to the development and the expression of cancer phenotypes. Understanding the mechanisms leading to cancer, is not fulfilled by studying individual components in isolation, but requires systems biology approach to establish interactions between genes, proteins and cellular components, and the associations of mutations and deregulations to the perturbation of cellular processes and pathways implicated in cancer [1]. It is thus important to analyze networks in cancer, where biological systems are represented and described as networks such as protein-protein interactions networks (PPIs), cell signalling pathways networks, transcriptional regulatory networks and other functional association networks.

The development of high-throughput interaction assays such as yeast two-hybrid (Y2H) and affinity purification coupled to mass spectrometry (AP-MS), and of curated databases has led to the generation of large-scale interaction networks for a considerable number of organisms [2, 3]. Constructing such networks not only sheds the light on the complexity of cellular mechanisms and processes, but also helps generating hypotheses about therapeutic targets or deregulated pathways in cancer [4].

1.1. Types of pathways and network analysis techniques

The first step to establishing network and pathway analyses for cancer mutations is defined by setting the database resources, represented by a list of genetic alterations in addition to databases for pathways and network interactions. Analysis techniques can be divided into three major approaches [4]. The first is the "fixed-gene set enrichment analysis" approach, consisting in analyzing gene sets without considering their interactions. Gene lists are gathered from literature-curated databases or experimental sources, and using different tools of enrichment analysis leads to determining pathways and cellular processes for filtered gene sets. The second

approach "*de novo* network construction and clustering" consists in analyzing a list of mutated or altered genes taking in consideration their molecular and functional interactions provided by interactomic databases such as the Biological General Repository for Interaction Datasets BioGrid [5, 6], the Molecular Interaction Database IntAct [7] and the Human Protein Reference Database HPRD [8]. An interesting advantage of this approach is that networks are expanded due to the "guilt by association" concept, which increases the complexity of interactomes and helps providing potential cancer candidate genes. The third approach is "network-based modeling", that have been applied to map signalling pathways and functional networks, which helps to predict the influences of deregulation and perturbations in cancer. An example of this approach is comparative analysis of regulatory networks in normal and disease states [9, 10]. A graphical summary of the three major approaches is represented in figure 1 indicating the goals and different tools used in each method [4].

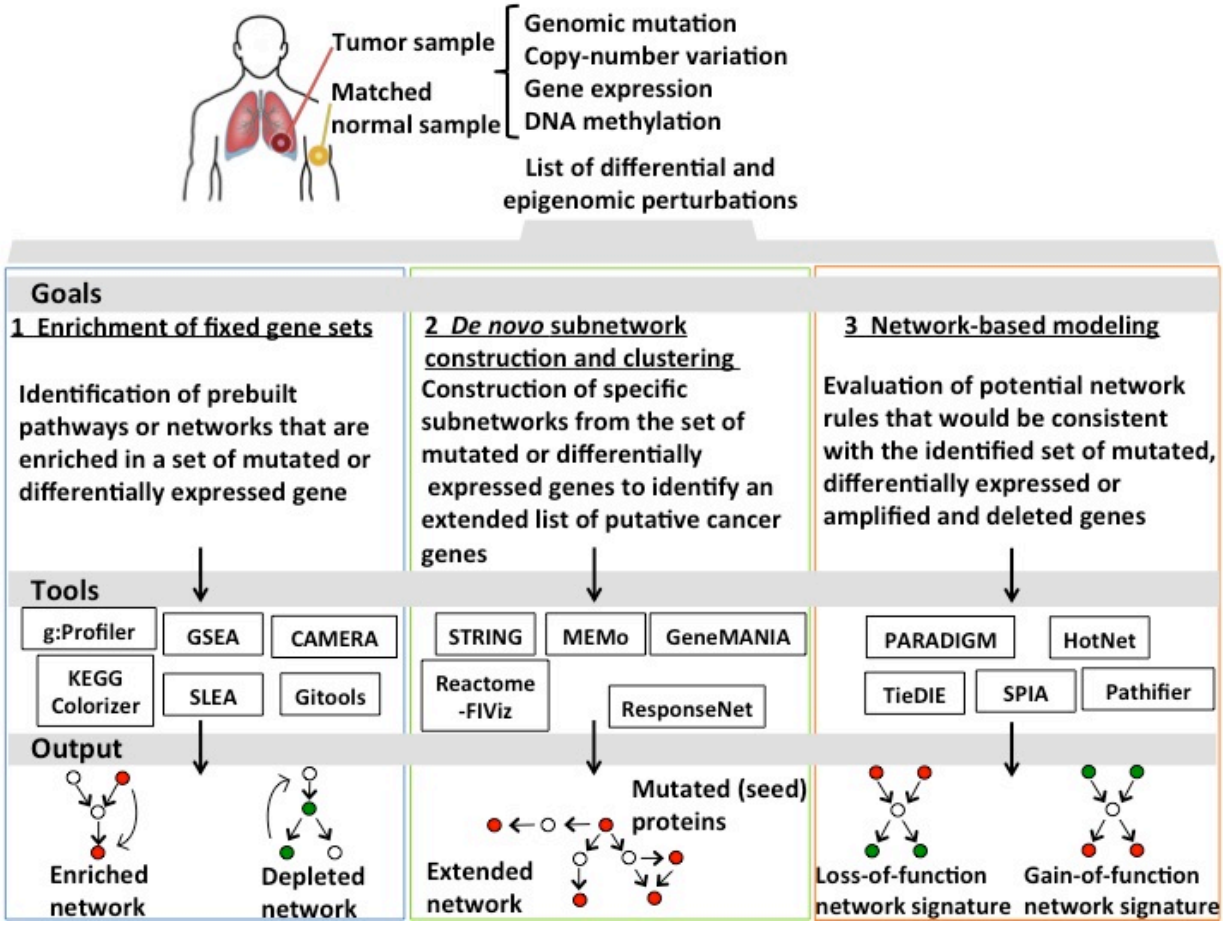


Figure 1: Major approaches to pathway and network analysis of cancer data. In the network diagrams designated by "output", red nodes represent genes whose activities are increased (first and third columns) or altered by mutations (center column). Green nodes are genes whose activities are decreased. Adapted from [4].

1.2. Protein-protein interaction detection methods

Protein-protein interaction (PPI) is one of the key topics for the development and progress of modern systems biology. In this part we will introduce essential protein-protein interaction detection methods.

PPIs detection methods can be classified into three major categories: *in vitro*, *in vivo* and *in silico* methods.

1.2.1. *In vitro* methods

Tandem affinity purification (TAP) method coupled with mass spectrometry. This method is based on tagging proteins and purifying protein complexes associated to the protein of interest. When associated with mass spectrometry analysis, it generates high throughput data for protein interactions. Analyzing AP-MS datasets in order to derive biologically meaningful information from protein interactions remains challenging. A variety of statistical models were developed to assess scoring methods for dataset testing [11].

Protein microarrays technology has also been developed to study biochemical activities of proteins and their interactions *in vitro*. Three types of protein microarrays are used: analytical microarrays, functional microarrays and reverse phase microarrays [12]. Analytical microarrays mostly use antibody microarrays while functional microarrays are composed of full-length or protein domains chips. As for the third type of microarrays, it enables the protein expression of hundreds of samples, printed on nitrocellulose slides to be interrogated simultaneously, using labeled antibodies (with fluorescent detection for example). Reverse phase protein microarrays have been developed to generate a functional patient-specific circuit "map" of the cell signalling networks based directly on cellular analysis of a biopsy specimen [13]. In other words, differential protein expression across samples in a high throughput manner, generating protein interaction and activation maps that lead to the identification of critical nodes for individualized or combinatorial target therapy [14, 15].

Using these protein-based microarrays enables the global observation of biochemical activities, where thousands of proteins can be screened for different types of interactions. These methods have important applications in disease marker identification and pharmaceutical target screening [16].

Another method for identification of protein-protein interactions is the protein-fragment complementation assay (PCA), providing a simple and direct method to studying PPIs in living cells. In this strategy, two proteins are fused to complementary fragments (fluorescent protein or an enzyme). If these proteins interact, the reporter and its activity are reconstituted [17] (figure 2). This method is also applied on a high throughput scale for PPIs detection [18].

1.2.2. *In vivo* methods

The yeast two-hybrid system is a powerful technique for studying PPIs. Proteins of interest that potentially interact are fused independently to the DNA-binding and transcriptional activations domains of the Gal4 transcription factor of yeast. This technique is easily automated for high throughput analyses of protein interactions on a genome-wide scale [19], either by screening open reading frames (ORFs) matrices such as the human ORFeome [20], or screening cDNA libraries that led to generating large amounts of interaction data. Other two-hybrid *in vivo* methods such as the mammalian protein–protein interaction trap (MAPPIT) founded on type I cytokine signal transduction [21] (figure 2).

Other used techniques, such as bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfers (FRET), and bimolecular fluorescence complementation (BiFC), require extensive instrumentation. These methods are based on visualization of protein-protein interactions via light or enzymatic excitation of fluorescent or bioluminescent proteins. These methods allow not only protein localization within the cell or its organelles but they also allow quantification of fluorescent signals and discovering weak or strong interaction partners [22].

Synthetic lethality is also a type of genetic screening, enabling the detection of functional interactions rather than physical direct interactions. It is based on producing mutations or deletions in genes, which are viable alone but when combined together can cause lethality [23].

Though Y2H and MS-based methods are the most used for interaction detection, both methods have limitations. MS is less accessible than Y2H due to higher cost of equipment, but unlike the Y2H method, AP/MS may determine components of large proteins complexes, even if these components interact indirectly. On the other hand the Y2H also has its limitations concerning PPIs detection; interactions involving

membrane proteins or proteins that require post-translational modifications are missed. Thus both methods are considered complementary in the type of interactions they detect. Recently, advance made in AP-MS technology has helped increase its sensitivity and robustness [24].

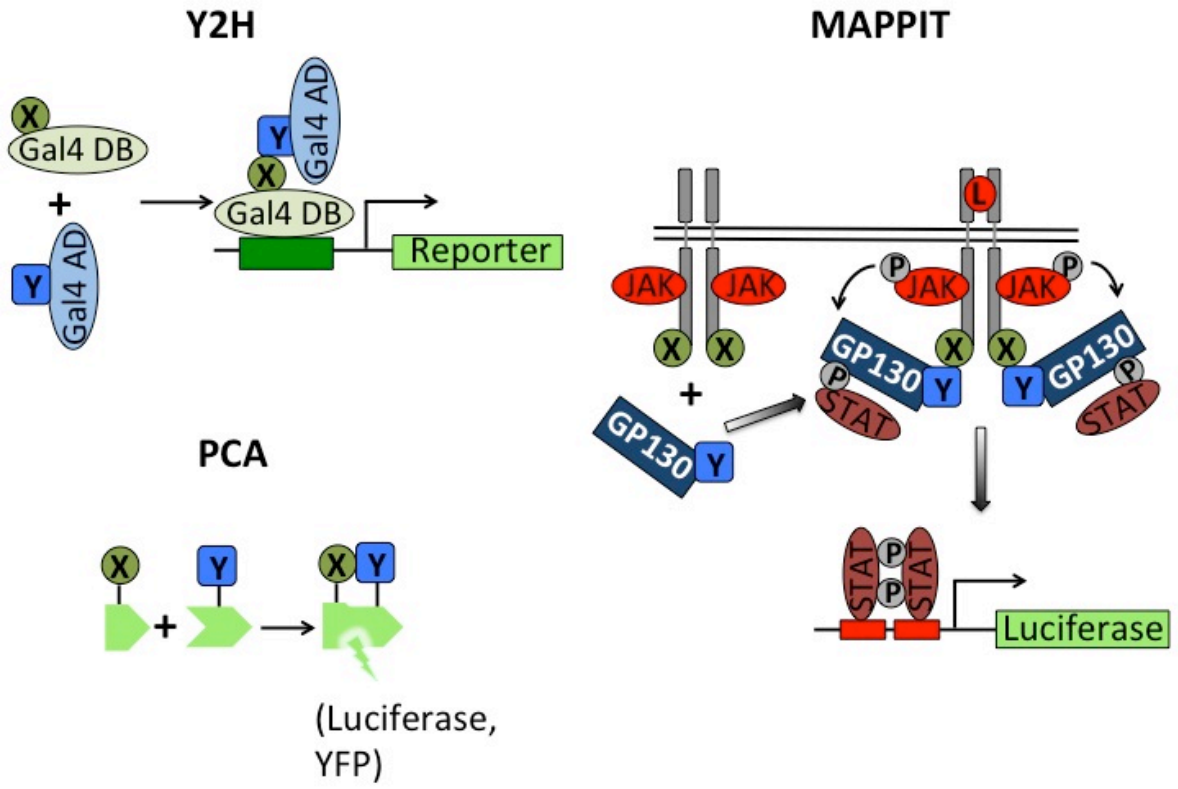


Figure 2: schematic representation of PPI detection methods. Y2H: the yeast two-hybrid system (Y2H) consisting of bait and prey proteins (represented by X and Y) are fused to the Gal4 DNA binding domain (DB) and the Gal4 activating domain (AD). When fusion proteins are produced and bait part of the first interact with prey part of the second, transcription factor of the reporter gene occurs. MAPPIT: The bait protein is a fusion with a leptin receptor (LR), which contains three Y-to-F mutations so it is unable to activate STATs spontaneously. The prey fusion contains a domain of gp130 which can recruit STATs. After interaction of the bait with the prey, Janus kinases (JAKs) phosphorylate gp130, which stimulates binding of gp130 with the STATs. The STATs themselves are phosphorylated by the JAKs, which results in the formation of a STAT complex. The STAT complex binds the rat PAP1 promoter (rPAP1p) and activates luciferase transcription. The leptin receptor is further fused with the extracellular domain of EpoR, a receptor of erythropoietin (Epo), and therefore LR complex formation, which is necessary to make the association with the JAKs, is induced by addition of Epo [21]. PCA: schematic of the protein complementation assay. X and Y are bait and prey proteins, fused to inactive fragments luciferase or YFP proteins. Interaction between bait and prey results in the reconstitution of an active form of the protein and detection of luciferase activity of YFP signal [25].

1.2.3. *In silico* methods

A variety of *in silico* methods have been developed to support the interactions that have been detected by experimental approach. The computational methods for *in silico* prediction include sequence-based approaches, structure-based approaches, chromosome proximity, gene fusion, *in silico* two-hybrid, mirror tree, phylogenetic tree, gene ontology, and gene expression-based approaches.

Structure-based method aims to predict protein-protein interaction based on homology modeling methods. Different algorithms were developed, such as processes that involve prediction of the binding interface, evaluation of the compatibility of the interface with an interface coevolution based model, and evaluation of the confidence score for the interaction [26]. As for sequence-based method, it depends on primary structure homology in order to classify potential interacting [27]. Gene fusions approach relies on complete genome sequences to identify fusion events. In this method certain protein families in given species consist of fused domains that usually are found as single full-length proteins in other species. These fusions can predict either direct or indirect functional interactions. This method can be used to predict protein-protein interaction by using information of domain arrangements in different genomes [28].

In silico two-hybrid analysis is based on previous studies of sequence correlation in multiple sequence alignments leading to the prediction of physical closeness between residue pairs of pairs individual proteins. The result from this method automatically indicates the possible physical interaction between the proteins [29].

Similarity of phylogenetic tree method is based on the analyzing the relationship between protein interactions and co-evolution histories that are represented by phylogenetic trees. The *mirror tree* approach has been used to determine potential interaction partners in large datasets of proteins and also to better understand the co-evolution and interactions in specific pairs of protein families [30, 31].

The gene expression approach predicts interactions based on the relationship between gene co-expression and protein interactions. It consists of grouping genes according to their expression in different experimental conditions, and evaluating similarities between expression profiles [32]. The concept behind this method is explained by the fact that genes belonging to common expression profiles more likely interact with each other than proteins encoded by gene from different clusters [33].

Recent technological advances have allowed the development of high throughput interaction detection methods. Despite progress made in this field, each of these experimental techniques has its own advantages and limitations. Only a combination of different approaches that necessarily includes bioinformatics tools, will eventually lead to a complete characterization of physiologically relevant protein-protein interactions in a given cell or organism.

1.3. Protein interactions databases

A number of publicly available databases collect and store protein-protein interaction data providing researches with access to these curated datasets. In order to avoid the duplication of the curation data and enable data exchange, the International Molecular Exchange (IMEx) consortium was formed. In addition Proteomics Standards Initiative - Molecular Interaction (PSI-MI) format sets data standards in order to specify a unified structure for sharing PPIs [34]. There are two types of PPIs databases based on their content; those containing data supported by experimental validation and those derived from *in silico* predictions [35].We can name several PPIs databases such as the Biological General Repository for Interaction Datasets (BIOGRID) [6], the IntAct molecular Interaction Network database (IntAct) [36], the Human Protein Reference Database (HPRD) [8], the Molecular INTeraction database (MINT) [37], which only report experimentally verified interactions. Some of the features of these PPI databases are represented table 1.

Table 1: Features of human PPI databases.

Database	Number of unique human PPIs	Number of proteins	PPI data	Unique features	Download options	PSI-MI compatibility	Download version
HPRD	41327	30047	Experimental	Protein annotations are included (e.g. PTMs, substrate information, tissue expression, disease association, protein complexes, subcellular localization). Signal transduction pathways	Yes	Yes	Release 9
MINT	26830	8762	Experimental	PPIs for other organisms, non-protein interactions	Yes	Yes	2015
IntAct	352696 (includes non-human PPIs)	89310 (includes non-human proteins)	Experimental	Protein complexes, PPIs for other organisms, non-protein interactions, provides web based applications, ProViz and Hierarch View, for visualization of interactions	Yes	Yes	Release 192
BioGrid	288982	19906	Experimental	Genetic and protein interactions curated from the primary biomedical literature for all major model organism species and humans	Yes	Yes	Release 3.4.128
DIP	7891	4615	Experimental	PPIs for other organisms, protein complexes	Yes	Yes	Jan-14

1.4. Protein-protein interaction network management

Network construction consists of establishing the links between proteins after retrieving interactomic data either experimentally and/or computationally determined. Tools such as Cytoscape [38] and MEDUSA [39], are employed to create graphical representation of the system as a network of interactomic map in which proteins are represented by nodes and their interactions by edges linking interactors. Cytoscape is considered as the most popular visualization tool. One of its features is the development of a collection of plugins enabling different functional interpretations of the constructed networks. MEDUSA on the other hand was specially designed for accessing protein interaction data from STRING database. Networks can be visualized in different layouts: circular, hierarchical, orthogonal or random… One of the ways to analyze essential nodes in a complex network is the identification of central network hubs, and ranking elements of the network according to criteria defined for each study [40]. Another approach is generation of functional modules that

can be established by functional annotation for representing biological networks, according to gene ontologies, common pathways, and disease implication [41]…

The availability of high-throughput experimental data and computational interaction prediction datasets has allowed construction of increasingly comprehensive and accurate protein-protein interaction networks. As we have seen, each method or approach has its strengths and weaknesses; therefore we cannot define a "perfect" approach. Accordingly it is essential to integrate different techniques and define criteria depending on the specificity and the aim of each study.

2. Analyzing networks in Acute Lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is a malignant disease characterized by the uncontrolled proliferation of immature lymphocytes. According to the cell type that is affected it can be divided into two subtypes: B-cell ALL and T-cell ALL. ALL is the most common leukemia in pediatrics accounting for 80% in childhood leukemia and 20% in adult leukemia. This disease is characterized by a large number of structural chromosomal abnormalities and translocations, and rearrangements (figure 3).

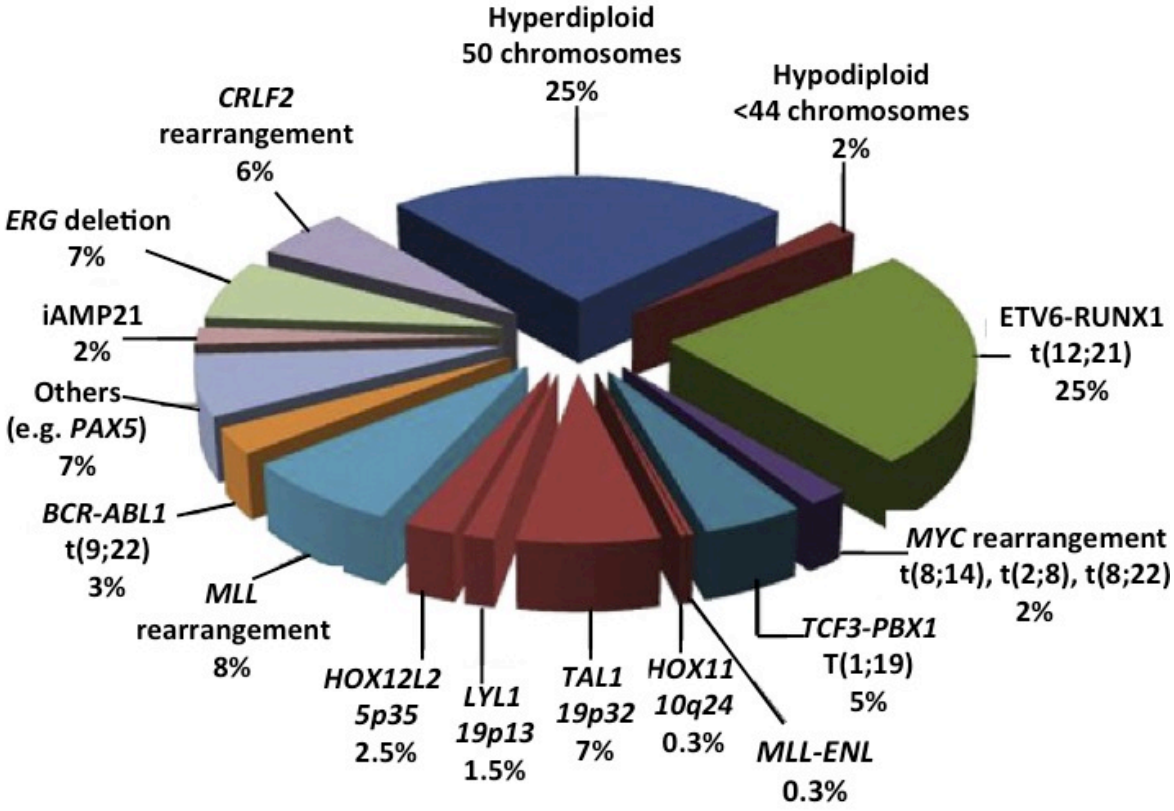


Figure 3: Spectrum of recurring chromosomal rearrangements in childhood ALL. Representation of comment recurring numerical and structural genetic alterations in childhood B-progenitor and T-lineage ALL, including approximate frequencies. Alterations specific to T-lineage ALL are shown at the bottom of the pie chart in magenta. Reviewed in Mulligan 2011 [42].

The Notch signalling pathway plays a vital role in determination of the fate of hematopoietic cells, it is essential for the generation of embryonic hematopoietic stem cells and also in controlling T cell differentiation. Numerous studies have shown that in mammals, the 4 Notch receptors are expressed in hematopoietic cells but at different stages and in different contexts of differentiation [43]. Hematopoietic development starts in two distinct phases; the first is the primitive hematopoiesis at extra-embryonic sites initiated in the yolk sac and the second is the definitive hematopoiesis in the embryo itself. The first hematopoietic stem cells (HSCs) appear in the dorsal aorta in a region called AGM (aorta-gonad-mesonephros) in adult mice and it is thought that these cells might originate from endothelial cells even if they are first detected in the yolk sac. Notch signalling promotes expansion of HSCs by activating Runx1 expression, and also arterial specification through Gata2 regulation [44].

One of the most characterized functions of Notch signalling is its role in promoting T cell differentiation in the bone marrow (BM). Active Notch signalling involving NOTCH1 receptor and Dll4 ligand in early stages of T-cell development is necessary for inhibiting the differentiation of B-cell and myeloid lineages. Notch1 is also required to promote transformation of early T lineage progenitors into progressively mature T lymphocytes (figure 4). In fact lymphoid precursors lacking NOTCH1 result in T-cell defect in the thymus.

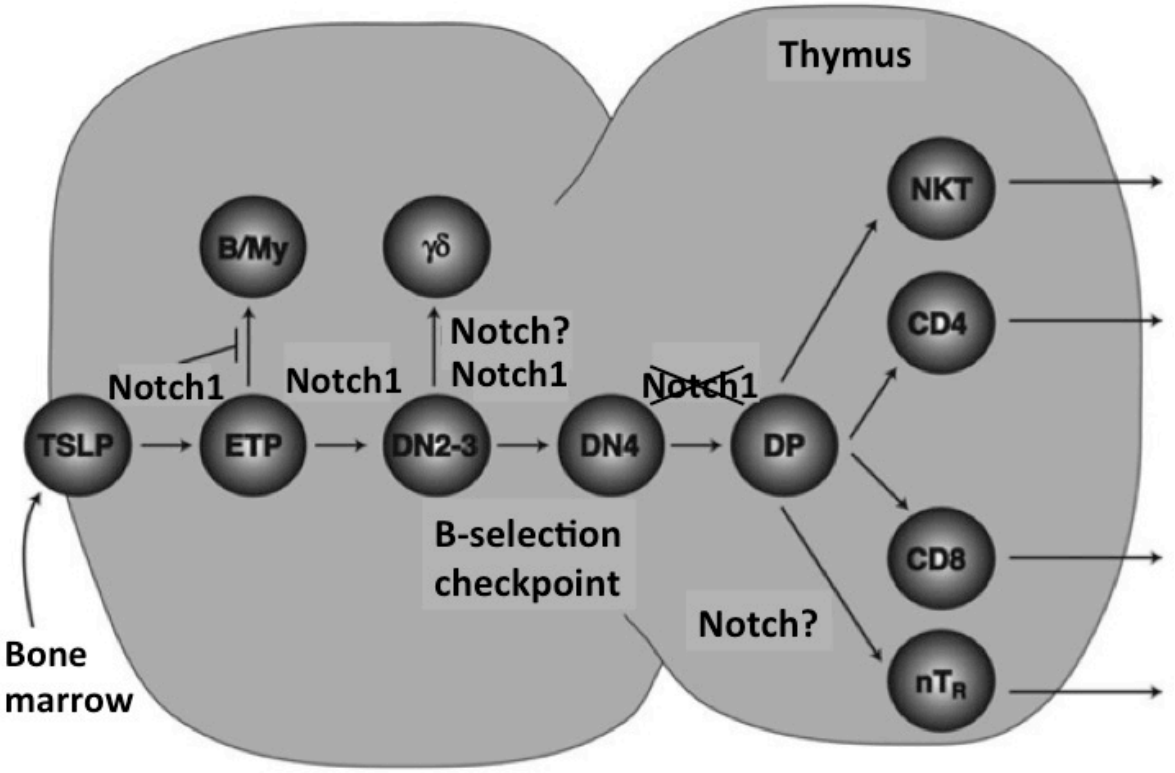


Figure 4: Notch and T-cell development. Notch1 and Dll4 interactions in the thymus are absolutely required during early stages of T-cell development. ETPs, double negative 2 (DN2) and DN3a cells experience a high intensity of Notch signalling. Active Notch signalling during early stages of T-cell development leads to inhibition of both B-cell and myeloid lineages. At the β-selection checkpoint, Notch signalling is rapidly turned off as a consequence of preTCR signalling. Hence, double positive (DP) T-cells experience a very low intensity of Notch signalling. TSLP: Thymus-Seeding Lymphoid Progenitors; ETP: Early T lineage Progenitor; DN: Double Negative; DP: Double Positive; NKT: NK T-cell; nTR: natural regulatory T-cell. Extracted from [45].

Recently, advances made in genomic techniques such as whole genome sequencing, genome wide profiling and cytogenetic methods enabled the identification of mutations and aberrations in ALL patients, which led to a better understanding of this

disease.

It became certain that Notch signalling regulates hematopoiesis at different levels, through raging from HSC formation to their differentiation and fate decision, while mutations in the receptor and aberrant Notch signalling were also linked to leukemogenesis and hematopoietic disorders such as acute lymphoblastic leukemia. Therefore it has been the subject of intensive research to better understand the mechanisms underlying tumorigenesis and studies showed increased interest in therapeutic modulation of the Notch pathway in this field.

2.1 B cell acute lymphoblastic leukemia

Chromosomal rearrangements were highly associated to B-cell ALL, with hyperdiploidy and ETV6-RUNX1 fusion representing 25% incidence for each aberration among other types of genetic alterations described in acute leukemias. Other fusions were also found with a less frequency such as MLL rearrangements, TCF3-PBX1, BCR-ABL1 translocations. Observations show that these genetic alterations modify the normal lymphoid maturation process through disrupting hematopoietic transcription factors or activate oncogenes [42]. Several studies have shown that IKFZ alterations is a hallmark of BCR-ABL1 B-ALL subtype, and usually it is associated with a poor outcome, it was also demonstrated that in almost 50% of BCR-ABL1 ALL patients, CRLF2 (encoding cytokine receptor like factor 2) expression is disrupted. CRLF2 was found to be overexpressed in ALL patients accompanied by other activating mutations in IKAROS gene, JAK1 and JAK2 (janus kinases), IL7R (IL 7 receptor), with high rates of relapse [46]. As previously mentioned, Notch signalling might play a role as a tumor suppressor or an oncogene, according to cell context and microenviromental conditions. In B-cell ALL Notch signalling induction in B-ALL cells lines leads to cell cycle arrest and apoptosis. Notch receptors and their ligands are expressed on B-ALL cell surface. Levels of expression of ligands and receptors in bone marrow mesenshymal stromal cells (MSCs) and their interactions are important in leukomegenesis of B-cell ALL and was also linked to chemoresistance to therapy in these patients [47].

2.2. T-cell acute lymphoblastic leukemia

T-cell ALL accounts for 10 to 15% of pediatric ALL and 25% of adult ALL [48]. It is characterized by diffusion of immature T-cells through the bone marrow. In contrast to B-lineage ALL, where malignant cells often have additional specific genetic abnormalities (chromosomal rearrangements and genetic fusions), which have significant impact on the clinical outcome of the disease, in T-lineage ALL few molecular abnormalities have been detected. A chromosomal translocation t(7;9)(q34;q34.3) involving *Notch* gene was found in T-ALL patients. This translocation fuses the 3' portion of the truncated form of Notch1 TAN1 on chromosome 9 to the TCRβ locus on chromosome 7, but was only found in less than 1% of T-ALL patients [49]. In murine model, transplantation of bone marrow progenitors expressing TAN1, develop T-cell neoplasm within two weeks, proving that this translocation can be causative for T-ALL [50].

Gene expression profiles using oligonucleotide microarrays was applied to T-ALL samples and cell lines in order to characterize immunologic markers as well as cytogenetic and molecular abnormalities. A study carried out on T-ALL patient samples, showed that applying hierarchical clustering on a set of differentially expressed genes (313 genes) between T-ALL patients reflect the degree of differentiation of leukemic cells. In addition gene expression profiling was also associated with response to treatment and long-term outcome of the disease[51]. Consistent with these findings, A. Ferrando and T. Look established gene expression profiles analysis in T-ALL showing that different oncogenic transcription factors define molecularly distinct groups of T-ALL, which are characterized by transcriptional patterns that involve regulators of cell growth, apoptosis, thymocyte development, and responsiveness to therapy. Using gene expression analysis in T-ALL, they identified HOX11 expression as an indicator of favorable prognosis category while TAL1 (T-cell acute lymphocytic leukemia 1 protein) and LYL1 (lymphoblastic leukemia associated hematopoiesis regulator 1) showed poorer outcome [52]. These examples along with other studies show how development of microarrays, making possible the analysis of T-ALL on a genomic scale, has helped to define the oncogenic pathways responsible for leukemic transformation in this disease.

On the other hand, mutations in different oncogenes and tumor suppressor genes that are initially known to be involved in the deregulation of mechanisms of T-cell proliferation, differentiation and thymopoiesis have been linked to T-ALL pathogenesis [53]. Among these genes and mutations, activating mutations of NOTCH1 were found in more than 50% of T-ALL patients [54]. A large number of Notch mutations were limited to specific regions of the protein, involving the heterodimerization domain and leading to a ligand-independent constitutive activation of the receptor, and also in the N-terminal PEST domain affecting NOTCH1 stability and degradation. Mutational Notch and aberrant Notch signalling in T-ALL are also accompanied by deregulation of other oncogenes such as cMYC, E2A-PBX, Ikaros and tumor suppressors like FBXW7, PTEN and PIK3CA. Therefore Notch mutations alone are not sufficient for disease development but they rather highly contribute to leukomogenesis and resistance to chemotherapeutic treatments. Gain of function mutations of Notch accompanied with mutational loss of PTEN induce resistance to Notch1 inhibition in T-cell leukemia. NOTCH1 downregulates PTEN expression through cMYC and HES1, which could mediate an upregulation of PI3K-AKT signalling pathway in both normal and leukemic T-cell lines [55]. cMYC is a Notch target gene in T-ALL. It has been shown that when overexpressed, cMYC is able to induce T-ALL in animal models. In addition Notch blockade using ϒ-secratase inhibitors GSI, lead to downregulation of cMYC expression in different T-ALL cell lines [56]. As some data show that cMYC is a downstream target of Notch in T-ALL, other studies demonstrate that cMYC and Notch1 can act through independent but yet complementary pathways to promote pre-T-cell transformation and thus expanding a pool of "high risk oncogenic" pre-T-cells [54]. A study carried out on T-ALL patients, showed that the presence of Notch activating mutations in the heteodimerization and PEST domains, lead to upregulation of HES1, cMYC, Deltex downstream Notch genes. In addition some of these patients also present mutations in the FBXW7 gene, associated with a higher transcriptional activation for NOTCH1 gene targets, and chemotherapy related genes such as Bcl-2 and MDR1 [57]. Another study also showed that in some cases FBXW7 mutations in leukemic cells mediate Notch pathway activation, and that mutant forms of FBXW7 cannot bind to the intracellular form of NOTCH1 (NICD) leading to NICD and MYC stabilization [58]. Mutations in the *FBXW7* gene are found in 15% of T-ALL cases, interfering also with NOTCH1 proteasomal degradation [58]. The essential role

that Notch plays in T-ALL, shown by several studies, *in vitro*, *in vivo* and driven by analyses made on samples from leukemic patients, highlighted the importance of Notch signalling and its effect in the regulation of downstream Notch targets and other signalling pathway, and its potential in targeted therapy against ALL.

Figure 5: The landscape of genetic alterations in T-ALL. Schematic representation of the most common genes targeted by chromosomal translocations, deletions, and mutations in T-ALL. Font size is indicative of the relative prevalence of these alterations, with highly prevalent targeted genes shown in in larger font sizes and less frequently altered loci shown in smaller font size. Adapted from [59].

2.2.1. Cross talk between Notch1, PI3K-AKT-mTOR1 signalling pathways in T-ALL

2.2.1.1. Regulation of PI3K-AKT signalling in T-ALL by Notch1

PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and facilitates AKT activation. AKT binds to PIP_3 through its pleckstrin homology domain at the membrane, enabling phosphorylation of AKT at Thr308 within its activation loop domain by

phosphoinositide-dependent kinase 1 (PDK1). Additional phosphorylation of AKT at Ser473 within its hydrophobic motif by mammalian target of rapamycin complex 2 (mTOR2 or PDK2) results in full activation of AKT, and phosphorylation of its substrates (i.e., glycogen synthase kinase 3 (GSK3), the FOXO-family of transcription factors, BAD, MDM2, and TSC2, thereby promoting cell growth, survival, and proliferation [60].

Notch1 plays an important role in regulating PI3K-AKT signalling. The first study to prove that Notch1 can activate PI3K-AKT pathway showed that Notch signals via the Delta-like 1 ligand (Dll1) interactions promoting the survival of pre T-cells through maintenance of cell size, glucose uptake and metabolism. Furthermore, the trophic effects of Notch signaling were mediated by the pathway of phosphatidylinositol-3-OH kinase and the kinase Akt, such that expression of active Akt overcame the requirement for Notch in β-selection[61].

T-ALL cells are also dependent on Notch1 and AKT signalling for proliferation and survival. The link between Notch1 and activation of PI3K-AKT signalling was established by Palomero *et al.,* they showed that NOTCH1 regulates the expression of PTEN and the activity of the PI3K-AKT signaling pathway in normal and leukemic T cells [55]. *Hes1* gene, one of Notch1 transcriptional targets, binds to *Pten* promoter and represses its activity, decreasing PTEN protein levels and increasing AKT phosphorylation and downstream signalling (figure 6). In addition, alterations of PI3K, PTEN, and AKT were reported in 47,7% of T-ALL cases from children, with PTEN mutants being most common [62]. Several studies showed that Notch could regulate PI3K-AKT-mTOR1 signalling at multiple levels, through both PTEN-dependent and independent routes. An example is the maturation of ILR7 leading to activation of JAK-STAT5 and PI3K pathways, which play important roles in normal hematopoiesis and leukemia [63] . Notch1 can also regulate IGF1R levels and PI3K-AKT activity in T-ALL, and Notch signaling is required to maintain IGF1R expression at high levels in T-ALL cells [64].

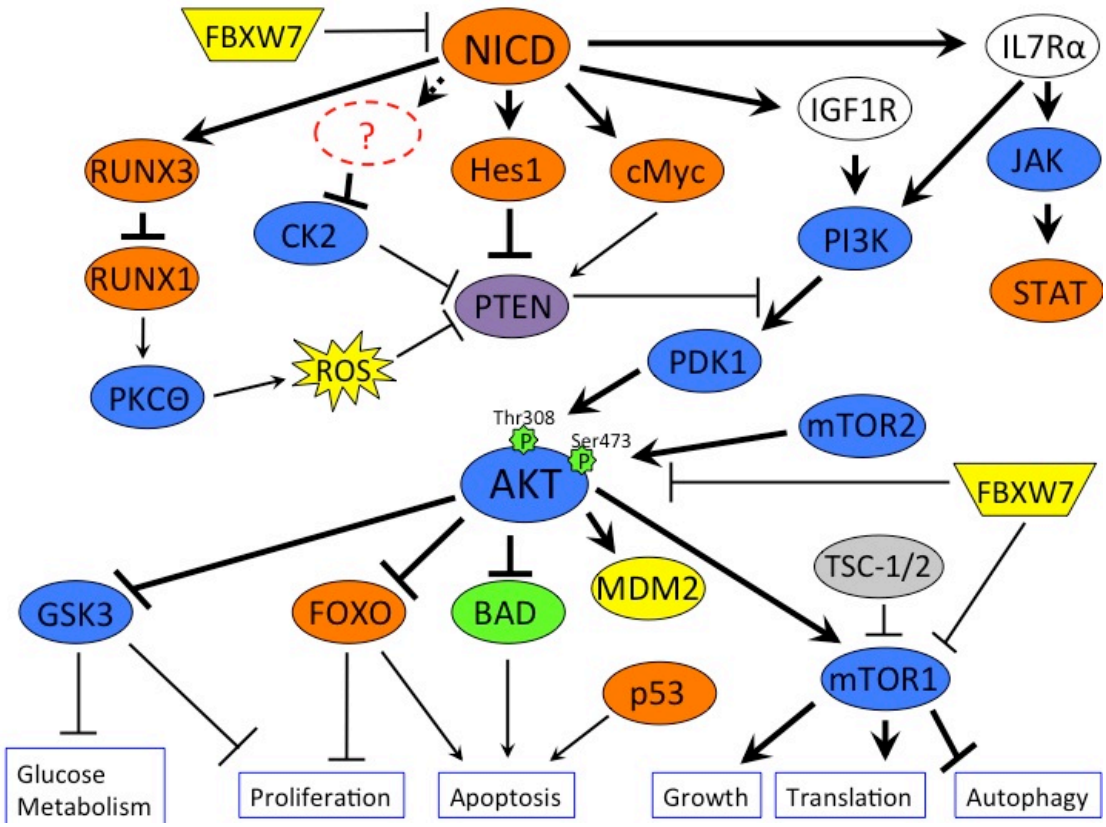


Figure 6: Regulation of PI3K–AKT signalling in T-ALL cells by Notch1. The signalling pathways downstream of Notch1 that converge on PI3K–AKT signaling in T-ALL are shown. The activated form of Notch1 (NICD) is shown and results in net activation of PI3K–AKT signaling by increasing levels of growth factor receptors [IGF1R and IL7R (IL7Rα subunit)] that recruit PI3K, or by reducing PTEN levels through transcriptional repression of the *Pten* gene by Hes1. Although cMyc can activate *Pten* transcription, Hes1 repression dominates. PI3K phosphorylates PIP2(4,5) to generate PIP3(3,4,5) that recruits AKT for phosphorylation at Thr308 by PDK1 and Ser473 by mTOR2. This results in phosphorylation of AKT substrates (GSK3, FOXO, BAD, MDM2, TSC2, and mTOR), promoting glucose metabolism, proliferation, growth, and translation, but impairing apoptosis and autophagy. PTEN dephosphorylates PIP3(3,4,5) back to PIP2(4,5) to block AKT activation. NICD was reported to decrease PKCθ and ROS levels through induction of the RUNX3 transcription factor. ROS promotes PTEN oxidation and inactivation. NICD might also contribute to regulation of PTEN inactivation since GSI treatments further increased PTEN phosphorylation, stabilization, and inactivation by CK2, which suggests that NICD may play a more active role in regulating CK2 activity, although NICD- independent pathways are also likely to contribute. Either of these pathways could influence PTEN activity through post-translational modifications. Loss of PTEN results in constitutive AKT activation and contributes to GSI-resistance. FBXW7 regulates NICD stability and signal duration but has also been suggested to decrease AKT and mTOR1 levels. FBXW7 is subjected to frequent mutations in T-ALL. Notch1 pathway activation and/or loss of PTEN result in net activation of PI3K–AKT signaling in T-ALL cells (line weights are proportional to the net effects on signaling). Arrows denote activation and blunt arrows repression. Dashed lines denote uncharacterized mechanisms. Transcription factors (orange), growth factor receptors (white), kinases (blue), phosphatases (purple), E3-ubiquitin ligases (MDM2 and FBXW7; yellow), BAD (bgreen), TSC1/2 (white/gray), and unknown negative regulatory factors (red dashed ovals) are depicted. AKT activating phosphorylations

are indicated (green stars with "P") while phosphorylations of other proteins have been omitted for simplicity. Extracted from [65] .

2.2.1.2. Notch1 affects p53, cMYC and PI3K-AKT pathways in T-ALL

Recent studies suggest that Notch1 regulates p53 levels and activation in T-ALL. Activation of mutant Notch1 in some T-ALL with constitutively high levels of PI3K-AKT signalling, secondary to loss or inactivation of PTEN, may contribute to loss of p53 via MDM2 [66]. P53 mutations are frequent in T-ALL relapse, but rarely mutated in primary T-ALL. Induction of NICD in an inducible murine lymphoma model decreased ARF and p53, which is a key mechanism underlying the initiation of T-cell lymphoma [67]. cMYC, a well-characterized Notch1 target gene, can induce T-ALL in mice and zebrafish. However Notch1 is oncogenic dominant over cMyc in T-ALL[68]. Recent studies suggest that activation of PI3K-AKT pathway downstream of Notch1 may be sufficient to drive T-ALL. PI3K-AKT can functionally replace Notch1 during β-seletion. MAP signalling pathway and GSK3β phosphorylate cMyc leading to its ubiquitination by FBXW7 and its subsequent degradation (figure 7). Recent evidence show that post-transcriptional deregulation of cMyc via PTEN is a major alternative pathway of MYC activation in T-ALL [69].

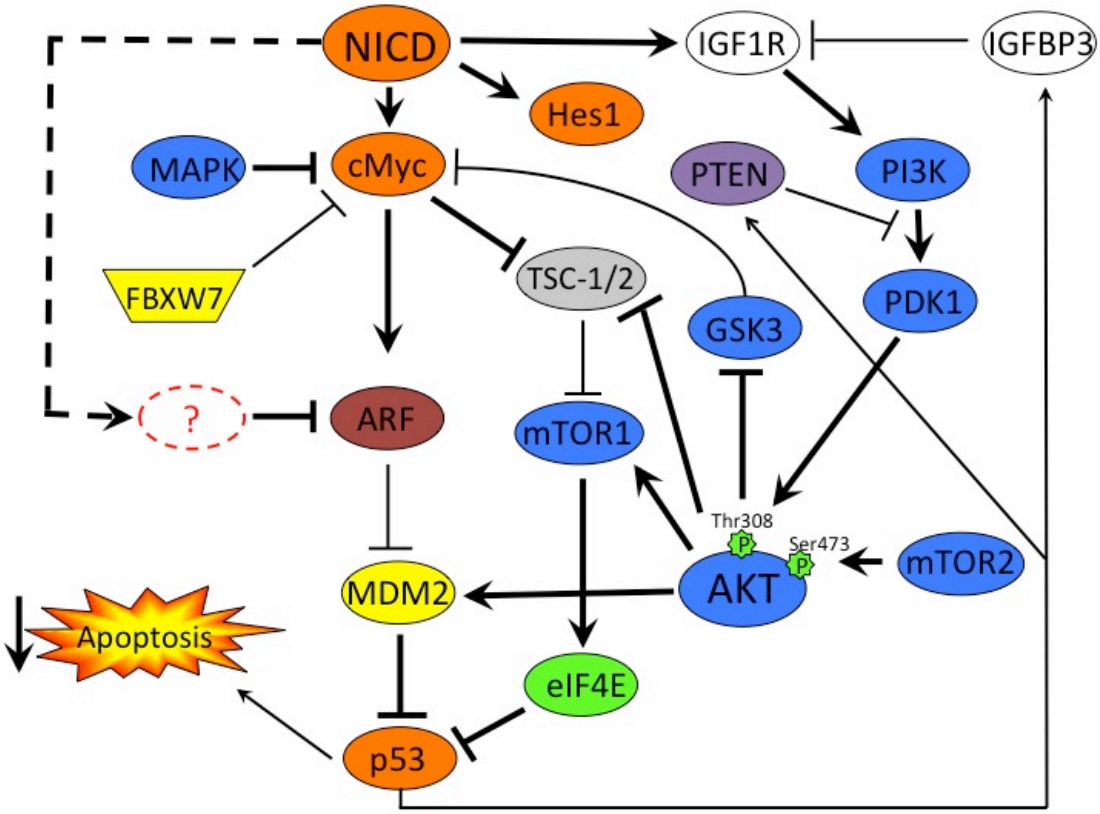


Figure 7: Notch1 orchestrates crosstalk between p53, cMyc, and PI3K–AKT pathways in T-ALL cells. ARF is a negative regulator of the E3-ubiquitin ligase MDM2 that negatively regulates p53 levels and apoptosis. Repression of MDM2 by ARF activates p53 and induces apoptosis. NICD can reduce ARF levels and p53. NICD activates AKT through growth factor receptors (i.e. IGF1R) and transcriptional repression of PTEN by Hes1. AKT promotes destruction of p53 by MDM2, while the p53 protein once activated, promotes its own stability by increasing PTEN and IGFBP3 levels to antagonize AKT activation. Loss of PTEN constitutively activates AKT to continually block p53-mediated apoptosis and may contribute to GSI-resistance. By reducing p53 levels, NICD creates an environment for activation of cMyc, which itself can induce p53-mediated apoptosis. Notch1 activates mTOR1 through both cMyc and AKT. Activation of mTOR1 can reduce p53 levels via eIF4E. PI3K–AKT–mTOR1 not only reduces p53 levels but also promotes cMyc stability by direct phosphorylation and repression of GSK3β. Phosphorylation by both GSK3β and MAPK are required to mediate cMyc degradation by FBXW7, which is frequently mutated in T-ALL. Line weights are proportional to the net effects on signaling in response to Notch1 pathway activation. Arrows denote activation and blunt arrows denote repression. Dashed lines denote uncharacterized mechanisms. Transcription factors (orange), growth factor receptors (white), kinases (blue), phosphatases (purple), E3-ubiquitin ligases (MDM2 and FBXW7; yellow), ARF (brown), TSC1/2 (white/gray), eIF4E (green), and unknown negative regulatory factors (red dashed ovals) are depicted. AKT activating phosphorylations are indicated (green stars with "P"), while phosphorylation of other proteins has been omitted for simplicity. Extracted form [65].

2.3. Therapeutic approach in ALL

Glucocorticoids were among the first drugs used in the treatment of acute lymphoblastic leukemia, and have remained essential components of therapy. Primary genetic abnormalities of leukemic cells have important prognostic significance and in some cases they are associated with drug resistant. The current chemotherapeutic approaches in ALL are adapted according to patient's age, cytogenetics and bone marrow response. Due to the differences in drug tolerability, adult and paediatric ALL treatments vary considerably between these groups. Prednisolone, vincristine, asparaginase, and daunorubicin are widely used medications in ALL treatment [70].

The gene expression profiles of leukemia cells have been used to identify genes related to the intracellular disposition of anti-leukemic agent *in vivo* and to reveal different sets of genes associated to drug resistance. An interesting gene expression patterns study identified 172 gene-probe sets as differentially expressed in primary B-lineage leukemia cells. These genes were also associated with resistance to drug treatments (Prednisolone, vincristine, asparaginase, and daunorubicin). They showed that resistance to mechanistically distinct anti-leukemic agents is associated with the expression of different functional groups of genes and support the use of combination chemotherapy for cancer treatment [71]. A similar analysis of gene expression in ALL was carried out on over 9600 genes before and after *in vivo* treatment with methoxtrexate and mercaptopurine alone or in combination. They identified 124 genes differentially expressed among these treatments. The identified set included genes related to apoptosis, mismatch repair, cell cycle control and stress response [72]. Comparisons of gene expression levels through time in B- ALL patients showed consistent differences among a set of 23 genes at least at two of the three time points evaluated and the differences in the expression levels of IL2RA, SORT1, DEFA1, and FLT3 genes in at least one of the times studied were associated with relapse and/or B-ALL-related death [73].

Exploring acute treatment-induced changes in gene expression in leukemia cells offers new insight into the differences in cellular response to individual agents and drug combinations. Identification of treatment-induced changes in gene expression can serve as a new tool for assessing the interaction of anticancer agents and may provide a basis for optimizing combination of chemotherapy.

Other agents in the early phase of clinical testing are being developed; including FLT3 inhibitors [74], γ-secretase inhibitors (GSI) [54], proteasome inhibitors and short interfering RNAs [75]. ALL treatment has advanced significantly. In addition gene profiling performed in T-ALL cell lines showed that GSI treatment induce gene expression changes in 239 genes including direct Notch1 targets such as *DELTEX* and *HES1*. Importantly, this analysis also identified c-MYC, a master regulator of multiple biosynthesis and metabolic pathways [76]. Recent findings in gene expression profiles, mutations and molecular characterization of ALL led to the development of novel targeted therapies. However, cure is often challenging and toxic. If we take for example γ-secretase inhibitors though effective in some cases but they still present high gastrointestinal toxicity [77]. Another example is bortezomib a proteasome inhibitor drug that was tested on ALL patients in both pediatric and adult patients, and showed infectious toxicity in some patients [78]. The challenge going forward will be to find safe and effective combinations and determine where in the treatment schema these agents will be most effective in ALL therapy. Studies in the field of therapy focus on the effect of drugs on prognosis and relapse rather than gene expression profiling. Therefore, comparing gene expression profiling for leukemic patient samples undergoing treatment can serve as a powerful tool to better understand the effect of these drugs.

The following chapters focus on Notch1 and FBXW7, two connected proteins frequently mutated in T-ALL.

3. NOTCH1 network and signalling

NOTCH1 was described for the first time in 1917, when Thomas Hunt Morgan an American geneticist and embryologist, described a strain of fruit flies *Drosophila melanogaster*, with notches at the margin of their wing blades [79]. The Notch homolog in human was described in the mid 1980's, as a transmembrane receptor that is essential in a highly conserved signalling pathway involved in the regulation of different processes during development and tissue homeostasis [80]. The first time NOTCH1 was linked to human cancer when the t(7; 9)(q34; q34.3) chromosomal translocation found in T-cell acute lymphoblastic leukemia (T-ALL) was sequenced

and cloned [81]. This chromosomal translocation resulted in an N-terminal truncated dominant active ligand - independent human NOTCH1 receptor (TAN1). It wasn't until years later that studies showed that TAN1 is causative for disease development in mouse models [50], and later another study showed that approximately 50% of all T-ALL patients had activating mutations in the human NOTCH1 gene [54].

3.1. The Notch protein

Notch signalling is an evolutionary conserved mechanism from *Drosophila* to humans. Notch receptors are large single-pass proteins, in *Drosophila*, there is only one Notch-encoding gene [82], in *C. elegans*, there are two genes encoding for Notch (lin-12 and glp-1) [83]. Whilst in mammals, there are four Notch genes encoding different receptors (NOTCH 1-4) [84]. The Notch receptors are composed of two functional domains, the extracellular (NEC) and transmembrane (NTM) domains. The extracellular domain contains between 29 to 36 epidermal growth factor-like repeats (ELRs) involved in ligand binding followed by three modules of LIN-12-Notch repeats (NLR) linked non-covalently by an heterodimerization domain to NTM [85]. These three cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerization domain represent a region called NRR for negative regulatory region playing a central role in preventing receptor activation in the absence of ligands. The NTM contains a RAM domain for RBP-Jk associated module, linked by a nuclear localization sequence to seven Ankyrin repeats domain. An additional nuclear localization sequence links the ANK domain to a transactivation domain (TAD) known to be different and evolutionarily divergent among Notch orthologs. TAD is followed by a PEST domain rich in proline, glutamic acid, serine, and threonine in the C-terminus of the NTM, which harbors degradation signals (degrons) regulating NICD stability.

Although there are broad variations in size amongst Notch family orthologs, especially relative to the *C. elegans* members, lin-12 and glp-1, several major structural features are conserved amongst all members (figure 8). As previously mentioned, the extracellular domain is characterized by the large number of EGF repeats, which number varies from 10 in glp-1 to 36 in *Drosophila* and some vertebrate Notch receptors. Several data suggested that the 11th and 12th ELRs in both *Drosophila* and vertebrate Notch receptors play a crucial role for being considered as primary sites of

ligand receptor interaction. On the other hand ELRs 11 and 12 equivalents in *C. elegans* extracellular domain of the receptor have not been reported previously [86]. In its intracellular domain lie several common features between Notch homologs. First is the presence of a cleavage site characterized by a conserved valine residue near the intracellular side of the transmembrane domain. All Notch receptors have RAM domain and ankyrin repeats that where described to interact with a number of different molecules including CSL group of transcription factors that are considered as one of the most important factors. The TAD domain is the least conserved domain amongst the different Notch receptors. As for the C terminal region it is well conserved in Notch homologs, harboring a PEST domain but with variable sequences and lengths.

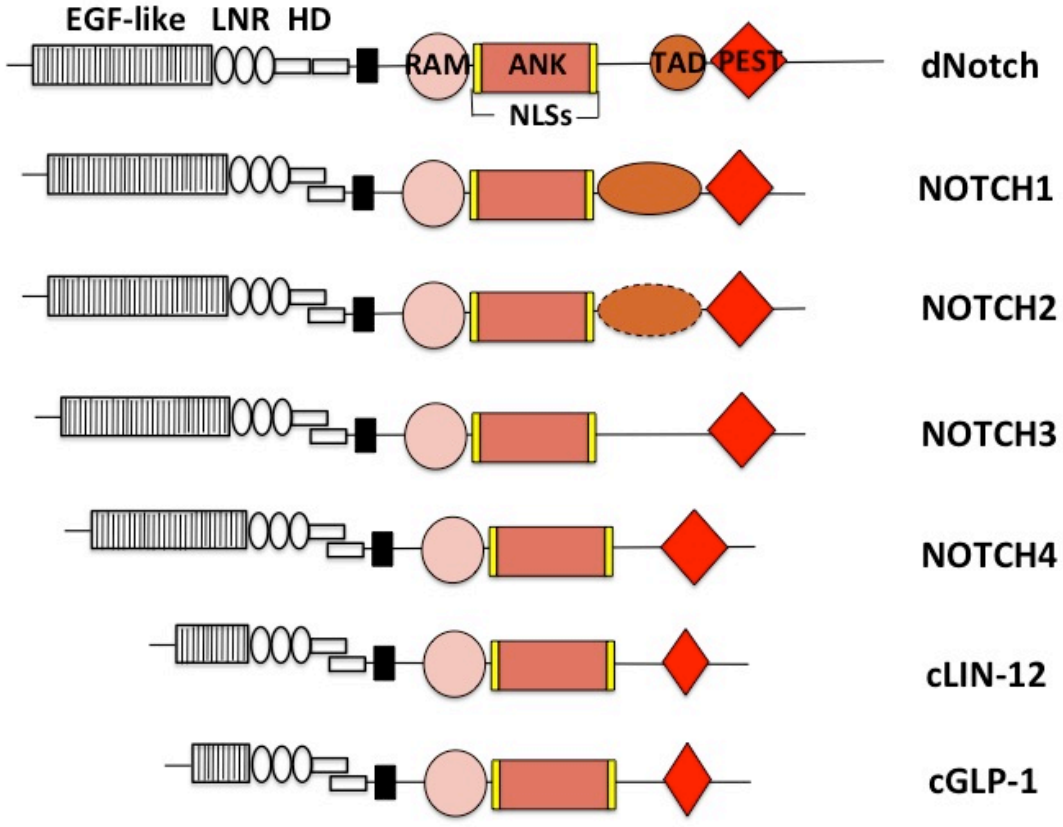


Figure 8: Structure of Notch proteins. *Drosophila* has one Notch receptor (dNotch), vertebrates have four (NOTCH1–4) and *C.elegans* have two (LIN-12 and GLP-1). The ectodomain of Notch receptors contains epidermal-growth-factor (EGF)-like repeats and a cysteine-rich Notch/Lin12 domain (LN); this is followed by a transmembrane domain, the RAM domain and six ankyrin repeats, two nuclear-localization signals (NLSs), followed by the transactivation domain (TAD) and a PEST sequence. NOTCH1 contains a strong and NOTCH2 a weak transactivation domain in the cytoplasmic part of the receptor. They differ in the number of repeats (29–36) but all are much longer than the *C. elegans* Notch proteins [84].

After being synthetized in the endoplasmic reticulum, the full length NOTCH1 is cleaved in the Golgi apparatus by a furine like convertase at the site S1 in the HD domain, resulting in two subunits (NEC and NTM) that are linked non-covalently at the HD domain all along their migration through the plasma to the cytoplasm where the receptor is represented as a heterodimere at the cell surface (figure 9).

The canonical Notch signalling pathway is initiated by the interaction between NOTCH transmembrane receptor and the transmembrane ligand present at the surface of the contacting cell [87], this binding is centered at the EGF 12 repeat containing residues that coordinate Ca^{2+} binding and O-glucosylation at serine 458 and serine 496 residues as well as O-fucosylation at the threonine 466 residue. This binding leads to two successive proteolytic cleavages of the receptor. The first cleavage at the S2 site located within the negative regulatory region approximately at 12 amino acids before the transmembrane domain, is mediated by metalloprotease of the ADAM family at the heterodimerization domain [88, 89], shedding the extracellular subunit and triggering the S3 cleavage at the transmembrane domain by the γ-secretase multiprotein enzyme complex [90-92], releasing NICD to translocate to the nucleus where it assembles into a transcriptional activation complex [93].

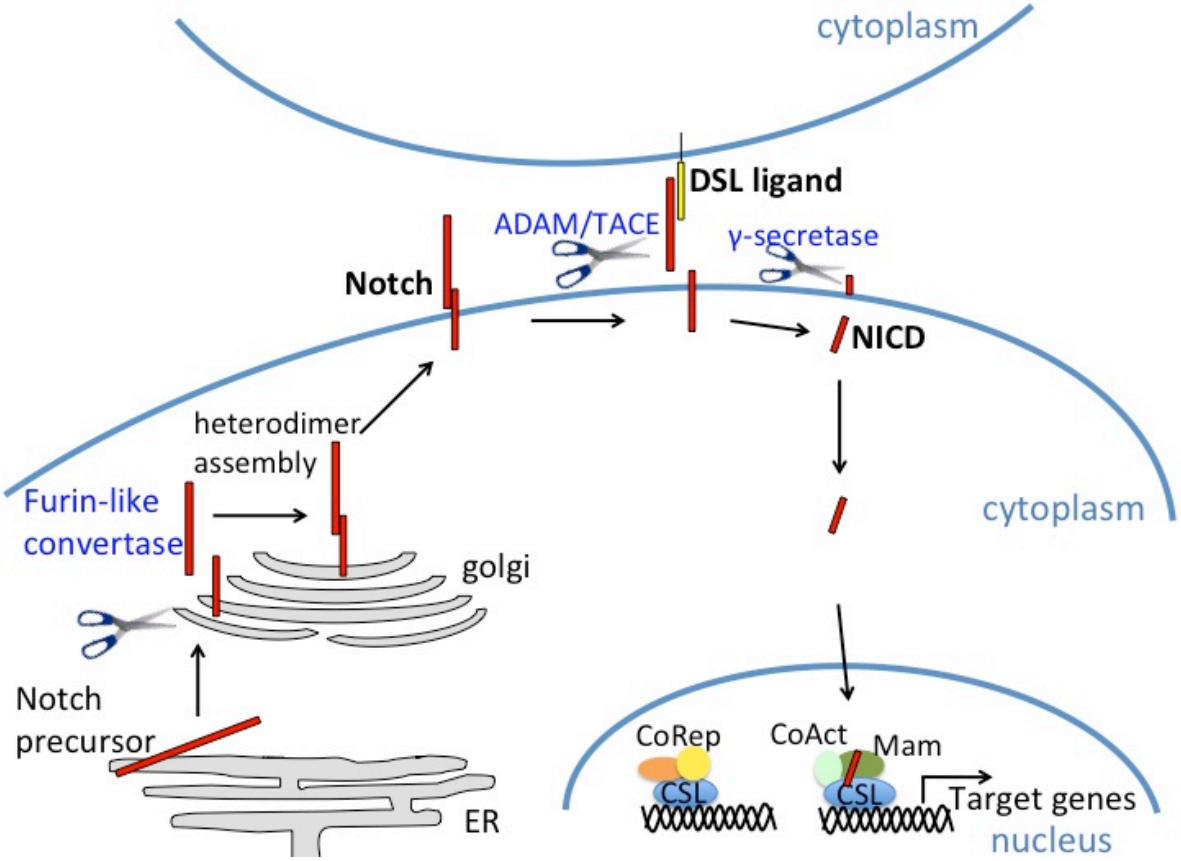


Figure 9: Canonical Notch signalling pathway. The Notch protein is syntheized as a precursor for that is cleaved by furin-like convertase (S1 cleavage) to generate the mature receptor, which is composed of two subunits that are held together by non-covalent interactions. On binding to the Notch receptor, the ligand induces a conformational change, exposing the S2 cleavage site in the extracellular domain of Notch to the metalloproteinase tumor necrosis factor-α-converting enzyme (TACE also known as ADAM). Following S2 cleavage, Notch undergoes a third cleavage (S3) that is mediated by the presenilin-γ-secratase complex. The S3 cleavage results in the release of the active NICD from the plasma membrane ad the subsequent translocation into the nucleus and activation of transcription of target genes. Adapted from [84].

3.2. Notch ligands

3.2.1. Canonical ligands

Notch ligands can be divided into several groups according to their domain composition. Based on their homology to the *Drosophila* Delta and Serrate ligands, in mammals there are five canonical ligands of the delta-serrate-lag2 type (DSL) classified as either Delta-like (Dll1, Dll3 and Dll4) or serrate-like (Jagged1 and jagged2) [94]. Notch ligands are type I transmembrane proteins, composed of an intracellular (ICD) and extracellular (ECD) domains. ECD contains DSL domain

followed by EGF repeats both calcium and non-calcium binding. The DSL domain with the first two EGF repeats and the Delta and OSM-11-like proteins (DOS) are essential sites for ligand binding to Notch receptor. While these ligands have several conserved domains features in their extracellular domain (according to their sequence alignments and function), the intracellular domain lacks notable sequence homology. Some of DSL ligands contain a carboxy-terminal PSD-95/Dlg/ZO-1-ligand (PDZL) motif, the role of this motif have been linked to interaction with the cytoskeleton rather than to the notch signalling (figure 10). It has also been shown that the cytoplasmic tail of these ligands contains several lysine residues representing potential ubiquitination sites targeted by the E3 ubiquitin ligase (Mind-Bomb 1 and 2 and Neuralized 1 and 2 in mammals) leading to the subsequent endocytosis of the ligand [95]. Canonical ligands have been well defined for activating the Notch signalling by cell-to-cell contact in *trans* as described previously. On the other hand these ligands have been described for their role in *cis* inhibition in Notch signalling. Some data indicate that *trans* activation or *cis* inhibition implies competitive mechanisms at the receptor-ligand interaction level, with a high threshold required for *cis* inhibition [96], and that the *cis* effect is more likely to prevent the shedding of Notch ecto-domain by an interaction mediated by the EGF repeats 10-12 of the receptor [97].

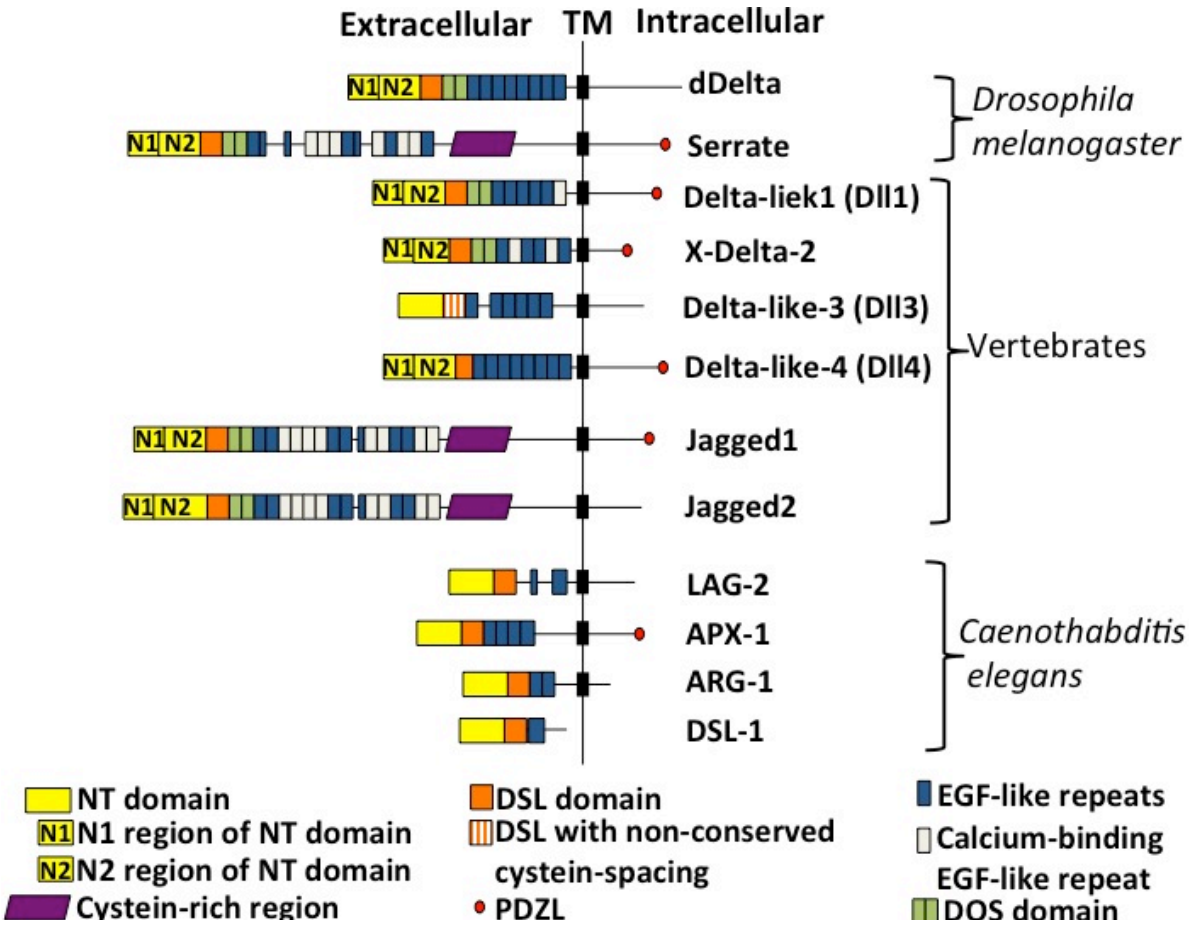


Figure 10: Structural domains of canonical ligands. The domain organization of Notch DSL-family ligands from *D.melanogaster*, vertebrates and *C.elegans* as indicated. The extracellular domains of canonical ligands are characterized by the presence of an N-terminal (NT) domain followed by a Delta/Serrate/LAG-2 (DSL) domain and multiple tandemly arranged Epidermal Growth Factor (EGF)-like repeats. The DSL domain together with the flanking NT domain and the first two EGF repeats containing the Delta and OSM-11-like proteins (DOS) motif are required for canonical ligands to bind Notch. The NT domain of vertebrate and *Drosophila* ligands is subdivided into a region containing six conserved cysteine residues, N1 and a cysteine- free region, N2. Serrate/Jagged ligands contain an additional cysteine-rich region not present in Delta-like ligands. The intracellular domains of some canonical ligands contain a carboxy-terminal PSD-95/Dlg/ZO-1-ligand (PDZL) motif that plays a role independent of Notch signaling. *C. elegans* DSL ligands lack a DOS motif. Dll3 is the most structurally divergent vertebrate DSL ligand and lacks structural features required by other DSL ligands to bind and activate Notch. Adapted from [94].

The levels of expression of Notch receptors and ligands on interacting cells define signalling polarity that is highly regulated temporally depending on cellular context and timing during development. Several studies show that deregulation of either the receptor or the ligand is associated with disease development. In humans, haploinsufficiency in Notch1 is implicated in an aortic valve disease [98], while haploinsufficiency of either Notch2 or Jagged1 is associated with Alagille syndrome

(AGS) which is a dominant, multisystem disorder defined clinically by hepatic bile duct paucity and cholestasis in association with cardiac, skeletal, and ophthalmologic manifestations. Ninety four percent of patients clinically diagnosed of AGS have mutations in Jagged1 ligand [99]. In addition to *trans* interaction between Notch and ligands that activates the signalling mechanism, *cis* interaction between ligand and receptor within the same cell also occur and limits the levels of activation by inhibiting Notch signalling through the process defined as *cis*-inhibition thus restricting Notch activation to signal-receiving cells [100]. Therefore it became important to understand the mechanism of *cis*-inhibition and the factors that contribute to the regulation of *cis* or *trans* interaction between ligand and receptor. Two different hypotheses have been proposed for this mechanism, Cordle et al. assume that interaction sites for both *cis* and *trans* inhibition overlap [101], while other define specific EGF repeats for each type of interaction. Though *cis* and *trans*-interaction binding sites with Notch might overlap, but only *trans*-ligand interactions activate Notch and induce proteolytic cleavage due to conformational changes of the receptor at the cell surface [102]. Several data support that ligand inhibition can take place as a *cis*- inhibitory effect acting by preventing a step before Notch ecto-domain shedding and involves an interaction mediated by Notch EGF repeats 10-12 [97, 101].

3.2.2. Non-canonical ligands

In addition to DSL ligands known as the canonical ligands of Notch, non-canonical ligands lacking the DSL domain have been identified. This category of Notch ligands is divided into 3 subclasses: integral membrane-bound, GPI-linked membrane bound and secreted ligands.

3.2.2.1. Membrane-bound non-canonical ligands

The first non-canonical ligand identified was delta like 1 (DLK1). This ligand represents similarities in structure with delta like ligands; DLK1 is cleaved by ADAM metalloprotease and negatively regulated by Notch. The role of DLK1 was more evident in *cis*-inhibition of Notch signalling, and it was described as an antagonist of DSL ligands for Notch binding and also decreases expression of Hes1 [103].

Delta/Notch like EGF related receptor (DNER) is another integral membrane bound Notch ligand lacking DSL domain, and similar to DLK1 it contains EGF repeats. DNER binds the Notch receptor in *trans*, and activates γ-secretase and Deltex-dependent Notch signalling thus promoting neuron-glial interaction and leading to morphological differentiation in the central nervous system (CNS) [104].

In 2007 Krictsov *et al.* identified a novel DSL ligand like protein called Jedi (for Jagged and Delta protein (Jedi)), coding for a transmembrane protein containing multiple EGF repeats, and expressed in early hematopoietic cells. They demonstrated that soluble form of Jedi inhibits Notch signalling, in a similar manner to that of soluble Jagged1, but there has been no proof that demonstrates the direct interaction between Jedi and Notch receptors [105].

3.2.2.2. Membrane –bound GPI-linked non-canonical ligands

The identified Glycosylphosphatidilinositol linked neural cell recognition molecules are the F3/contactin1 and NB3/contactin6. F3/contactin1 interacts with Notch receptor and induces its cleavage and nuclear translocation promoting maturation of oligodendrocytes. NB3/contactin6 promotes neural progenitor cell differentiation into oligodendroctes by activating the Notch signalling pathway. It has been demonstrated that both GPI-linked ligands promote Notch/DTX1 signalling pathway [106, 107].

3.2.2.3. Secreted non-canonical ligands

In *Drosophila melanogaster* two secreted ligands were identified: Scabrous (Sca) and Wingless (Wn) the fly ortholog of mammalian Wnt proteins. Sca and Wn both activate Notch signalling by *trans*-binding to the receptor. In *C.Elegans* five secreted ligands lacking DSL domain have been identified: OSM11, OSM7, DOS1, DOS2 and DOS3. They all contain DOS motif (Delta and OSM-11) that is conserved across species and found in canonical Notch ligands and overlapping the EGF motifs. Interaction between Lin12 and OSM11 was detected in yeast two-hybrid but no other evidence showed that Notch directly interacts with OSM. On the other hand, the effect of OSM11 on Notch signalling was demonstrated in *C.elegans,* during vulval development [108].

In vertebrate five putative secreted non-canonical ligands have been identified: Connective Tissue Growth Factor/cysteine rich 61/Nephroblastoma Overexpressed Gene family member, CCN3/NOV [109], the microfibril associated glycoprotein family (MAGP1 and MAGP2), thrombospondin 2 (TSP2), Y box protein 1 (YB1) and finally the EGF like domain 7 (EGFL7). These ligands interact with Notch receptors, inducing the activation of Notch signalling in different cellular contexts. CCN3, MAPG1, MAPG2 and TSP2 enhance Notch signalling induced by DSL ligands when exposed to Notch receptors or co-expressed in the same cell [110-112]. YB1, a cold shock protein binds to Notch3, activates its nuclear translocation followed by an upregulation of its target genes [113]. EGFL7 was found to be expressed in neural stem cells (NSCs) where it binds to a region in Notch involved in ligand-mediated receptor activation, thus acting as an antagonist of Notch signalling and regulating their proliferation and differentiation [114]. It has been also demonstrated for the majority of these ligands, that the interaction with the receptor activates CSL dependent reporter constructs in a γ-secretase dependent manner.

3.3. Notch transcriptional regulation

3.3.1. NICD-CSL-MAML ternary complex

Following Notch activation and cleavages, NICD translocates to the nucleus, where it activates the transcription of several target genes. NICD cannot bind directly to DNA; it acts through CSL transcription factor, enabling Notch to regulate gene expression and transcription. CSL is for CBF1 (C-promoter binding factor1), RBP-jk/Su(H)/Lag-1 in mammals/Drosophila/C. elegans), it is composed of three domains: N-terminal Rel homology domain (NTD), a central beta-trefoil domain (BTD) and a C-terminal Rel homology domain (CTD) [115]. CSL protein binds to the DNA target gene regions that was identified 5'-CGTGGGAA-3' [116]. In the absence of NICD, RBP-jk forms a complex with co-repressor proteins such as SMRT (silencing mediator retinoid and thyroid receptors), N-coR (nuclear co-repressor), CBF1-interacting co-repressor associated with histone deacethylase 1 (HDAC1), which prevents chromatin transcriptional activation. SHARP (SMRT and HDAC associated repressor protein) interacts directly with RBP-jk and with other co-repressors such as SMRT/N-coR via a highly conserved sequence in its SPOC-domain also called SHARP repression domain (SHARP-RD) at the C-terminus end of the protein [117]. SHARP plays a central role by interacting with

two different co-repressing complexes. It recruits CtIP/CtBP (C-terminal binding protein/C-terminal interacting protein) or ETO in addition to other co-repressors and histone modifying enzymes (HDAC) [117, 118] (figure 11). It was also demonstrated that SHARP recruits HDAC/SMART/SKIP containing complex [119]. SKIP (Ski-interacting protein) that was originally identified in a yeast two hybrid screen and CIR (CBF1 interacting co-repressor) are both direct RBP-j binding proteins, they bridge interactions between the transcription factor and other co-repressors. Some data also demonstrated their role in mediating the interaction between Notch and CBF1 suggesting that they might also play a role as co-ativators [120, 121]. When Notch is activated, NICD recruits co-activators, displacing co-repressors and forming a ternary protein complex SBF-NICD-MAM bound to DNA promoter region [122], where the BTD domain of CSL interacts with the RAM domain of NICD but only weakly with the ANK domain via its CTD [123], and MAM interacts directly with NICD via the ANKyrin repeats and also with other co-activators, like CBP/p300 (histone acetyltranferase) through a transactivation domain TAD1 in the C-term of Mastermind. P/CAF and GCN5, are conserved histone acetyltransferases, that were described for their role in the RBP-jk-mediated transactivation by NICD, interact through their N-terminal regions with activated Notch [124]. Other factors are recruited by this ternary complex such as SKIP which is thought to displace co-repressors, and recruits kinases that specifically phosphorylate NICD in order to be later targeted by FBXW7 for subsequent proteosomal degradation[121].

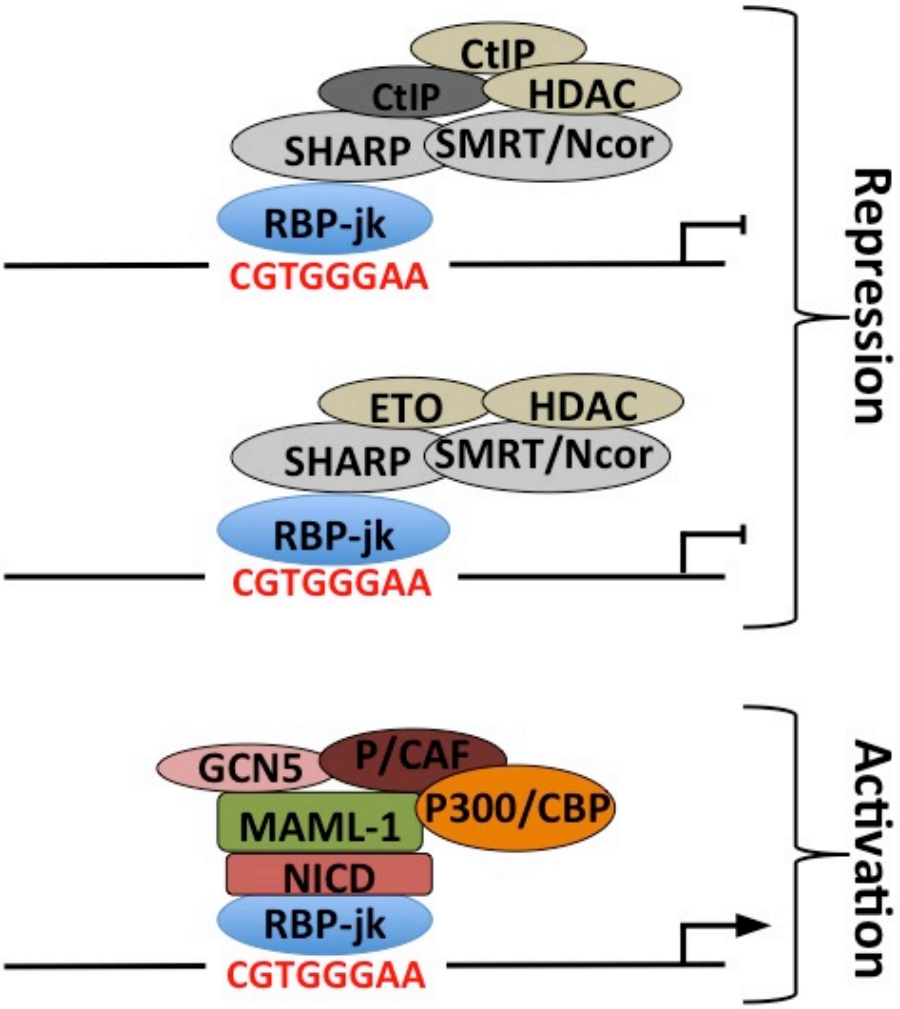


Figure 11: Activation and repression complexes regulation transcription of Notch target genes. In the absence of activated Notch signalling, the DNA-binding protein RBP-J recruits co-repressor complexes to represses transcription of Notch target genes (upper panel). Activation of Notch target genes: upon Notch ligand binding and cleavage, activated NICD interacts with RBP-J and recruits a co-activator complex composed of Mastermind (MAML-1) and other chromatin modifying transcription factors resulting in the transcriptional activation of Notch target genes (lower panel) reviewed in [125].

Crystallization of the NICD-CSL-MAML1 ternary complex bound to DNA has enabled the characterization of this complex and its structure. Structures were determined for for *C. elegans* and human orthologous proteins. In general, theses complexes show high similarity among species, which highlights the function and fold conservation throughout evolution. CSL bridges the interaction between NICD, MAML and the DNA. The structures show that Mastermind adopts a strikingly bent helical conformation; with its N-terminal helical region forming a tripartite complex with ankyrin repeats 3–7 of NICD and the CTD of CSL, while the C-terminal helix of Mastermind interacts with the NTD of CSL. With the exception of the first ankyrin repeat becoming

structurally ordered in the ternary complex, the overall structure of NICD ANK is unchanged upon complex formation. In the CSL–NICD–Mastermind ternary complex, the interactions of CSL with DNA and the overall conformation of DNA are maintained in both ternary complexes compared to the isolated structure of CSL bound to DNA, suggesting that, in general, DNA binding is not affected by ternary complex formation [126, 127] (figure 12).

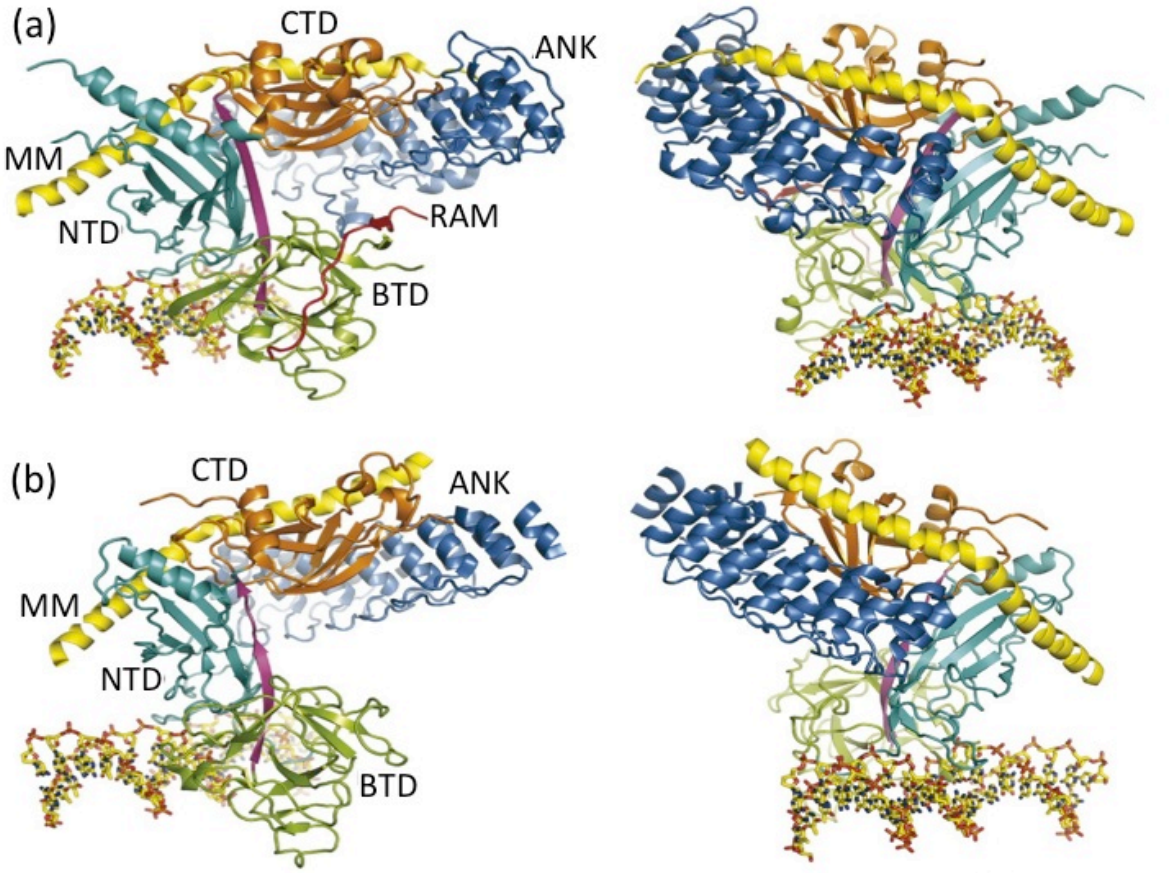


Figure 12: The CSL–NICD–Mastermind ternary complex bound to DNA. (a) Ribbon diagram of the ternary complex bound to DNA for worm components (PDB code 2FO1). CSL domains: NTD, BTD and CTD are colored cyan, green and orange, respectively. Notch ANK and RAM are colored blue and red, respectively. Mastermind (MM) is colored yellow. Approximately 1808 views are shown. (b) Ribbon diagram of CSL–NICD–Mastermind ternary complex structure bound to DNA for the human proteins (PDB code 2F8X). Domain coloring and views are similar to (a). Extracted from [126].

Crystallization have revealed some of the molecular details of this trimeric complex, but the molecular events leading to the assembly of this transcriptional activation complex requires further investigations. Data from different studies showed that NICD multimerization is an initial step in the complex assembly. Subsequently, the NICD multimer forms a complex with Skip, thus providing a docking site to recruit

Maml1 and forming a preactivation complex. The interaction between the preactivation complex and CSL results in the loading of NICD and Maml1 onto CSL to form the transcriptional activation complex on DNA. This observation does not exclude that Notch is also present as a monomer in the activation complex as described previously. In fact data suggest that Maml1 either facilitates or stabilizes the interaction between NICD and CSL, and the combination of these interactions helps converting NICD multimers to monomers in the presence of Maml1 [128] (figure 13). An important study carried out by Nam *et.al* have described a cooperative assembly of higher-order Notch complexes implicated in the activation of some Notch target genes such as *HES1* that contains in its promoter region dual "sequence-paired" binding sites called SPSs. SPS consists of two CSL-binding sites oriented head to head and typically separated by 16 or 17 nucleotides. They have demonstrated that assembled Notch transcriptional activating complexes dimerize on SPSs, and that this dimerization requires both CSL and MAML. It is depends on ANK-ANK interaction occurring through conserved residues of this domain and proper spacing and orientation of CSL-binding sites [129].

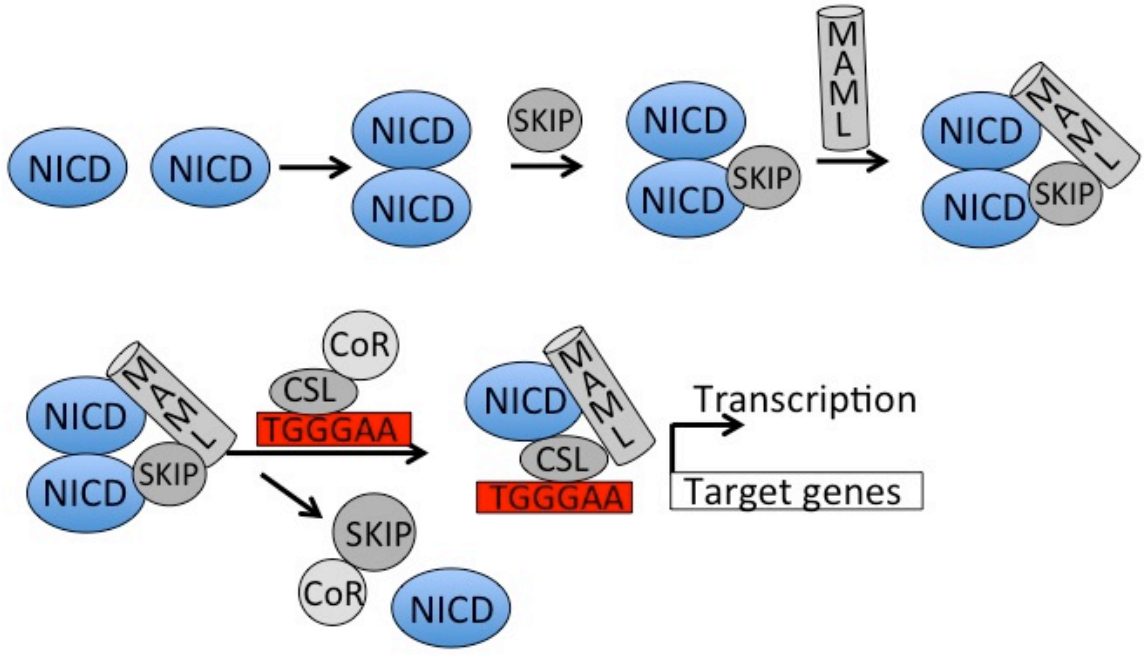


Figure 13: Model of assembly of Notch activation complex. Monomers associate to form multimers, SKIP binds to NICD dimer, and next MAML1 is recruited to the NICD-Skip complex forming the preactivation complex. The intermediate complex interacts with Co-repressors (CoR) leading to the recruitment of NICD monomer and MAML into CSL-DNA, generating therefore the Notch activation complex and releasing the unbound NICD monomer along with Skip and corepressors [128].

3.3.2. NOTCH1 target genes

3.3.2.1. HES and HERP genes

Notch regulates the transcription of several target genes, activation of Notch at the surface followed by the formation of the NICD-RBPj-k complex leads to an upregulation of the expression of primary target genes. The most known are the HES/E(spl) (for Enhancer of split in Drosophila). Several studies have provided evidence showing that these genes are direct Notch targets, including promoters of Hes1, Hes5 and Hes7 as well as Hey1, Hey2 and HeyL (subfamily of Hes, related with YRPW motif). Hes and Hey proteins are helix-loop-helix transcription factors that function as transcriptional repressors [130]. Other effectors of the Notch signalling are bHLH family that has been isolated and named as Hey/Hesr/HRT/CHF/gridlock/HERP (hereafter HERP or HES related proteins). This family also functions as transcriptional repressors, but employs different repression mechanisms than HES [131]. bHLH family are classified into several groups. Class A proteins that are transcriptional activators such as MyoD and Mash1, class B proteins are leucin zipper proteins such as Myc and Max. Class C proteins are transcriptional repressors HES and HERP proteins. HES proteins are characterized by the presence of a conserved proline residue at a specific site in their basic domain, as for the HERP family has a conserved glycine at the corresponding position. All HES family members share a WRPW motif in the C-terminus whereas HERP family has YRPW motif. Both HES and HERP families contain bHLH domain, and another domain, termed the Orange (or helix3- helix4) in the corresponding regions carboxy-terminus to bHLH region. Although both families have closely-related motifs and domains, they seem to employ different mechanisms for transcriptional repression. Three different mechanisms of repression were described for HES proteins; (1) an active repression where HES homodimer binds DNA and interacts with proteins repressors (Groucho in *Drosophila* TLE ortholog in mammals) then recruits histone deacethylase proteins that can alter chromatin structure and repress gene transcription. (2) Passive repression where HES protein can form heterodimer with other HES activating proteins (such as MyoD) disrupting the formation of functional heterodimers. (3) A mechanism mediated by the orange domain/helix3-helix4 that recruits co-repressors or stabilizes the WRPW-mediated repression [132]. HERP proteins associate with N-CoR, they can recruit other repressor complexes such as Sin3/HDAC. In addition passive

repression mechanism have also been suggested for the HERP family [133].

Regulation of HERP and HES by Notch signalling is essential for different developmental processes including angiogenesis, somitogenesis myogenesis and gliogenesis [134].

3.3.2.2. Other Notch target genes

Members of the bHLH family were identified as primary targets of Notch, but this fact does not exclude that Notch targets a large number of effectors as it has been demonstrated by several studies. Notch1 exerts its multiple effects by specifically regulating the expression of genes depending on cellular context. As an example, genome-wide transcriptome analyses of Notch1-induced genes in murine ESC under different cell extrinsic cues and in mesodermal cells revealed that Notch signalling regulates the expression of numerous genes playing key roles in cell differentiation, cell cycle control and apoptosis. Meier-Stiegen *et.al* identified specific Notch target genes using genome wide gene expression arrays, with 465 transcripts that can be differentially expressed due to Notch1 induction in different conditions. They were able to validate transcription factor Sox9, Pax6, Runx1, Myf5 and Id proteins as direct Notch1 targets that are critically involved in lineage decisions in the absence of protein synthesis [135].

Notch promotes proliferation via regulating the transcription of Cyclin-D and CDK5 and can directly control apoptosis effector genes such as bcl2. It was also demonstrated that Notch regulates the expression of Deltex, a ubiquitin ligase that regulates Notch1 trafficking and TCR in T cells [136]. NRARP (for Notch-regulating ANKyrin repeats protein) a negative regulator of Notch is also one of Notch targets regulated through DLL4/Notch signalling. NRARP is implicated in the cross talk between Notch and Wnt signalling in endothelial cells to control stability of new vessel connections in mouse and zebrafish [137].

Notch also regulates its own expression; it can directly bind autoregulatory CSL sites in Notch locus increasing its own expression [138].

ChIP on chip analysis combined with gene profiling data, enabled the identification of NOTCH1 direct target genes regulating cell growth genes and T cell transformation. In

this study c-MYC was identified as a direct target gene regulated by NOTCH1, and this interaction is part of a feed-forward-loop transcriptional regulatory motif implicated in the leukemic cell growth where some of c-MYC and NOTCH1-regulated genes overlap [56, 76].

Now it became evident that Notch directly regulates expression of genes encoding proteins that implement variable cellular functions depending on cellular context. In fact as previously described, systematic studies of Notch targets have revealed several regulatory motifs controlled by notch and linked to other signalling pathways.

3.4. Post-translational modifications in Notch signalling

Notch signalling is highly dependent of the ligand-receptor interaction related to the expression of these two components at the cell surface. Both ligand and receptor are subject to post-translational modifications such as glycosylation and ubiquitination.

3.4.1. Glycosylation

Notch receptors and ligands both undergo glycosylation, their correspondent extracellular domains are modified by a variety of glycans that can regulate aspects of Notch signalling and activation. Several mutations reported in enzymes that regulate the glycosylation mechanisms are associated with Notch signalling disruption and deregulation.

After being translated in the Golgi, Notch protein is fucosylated by a GDP fucose protein O-fucosyltransferase (Ofut-1 in Drosophila and Pofut1 in mammals) within EGF repeats of the ECD to a serine or threonine residue, facilitating the proper folding of the protein in the endoplasmic reticulum (ER). Subsequent to the O-fucosylation, the EGF O-linked fucose chain is elongated by the glycosyltransferase, Fringe, which attaches N-acetylglucosamine (GlcNAc) in a β1,3 linkage [139]. Fringe is a glycosyltransferase which transfers N-acetylglucosamine (GlcNAc) to fucose on the EGF repeats of Notch, it was first discovered in *Drosophila* [140], where it can modulate dorsal-ventral cell interactions in the developing wing by modulating the response of Notch to its ligands and later on mammalian homologues were identified: Lunatic, Manic an Radical Fringe[141]. Mammalian Fringe might have different catalytic activities,

it has been shown in different animal models (mice, zebrafish…) that they are differentially expressed according to cellular context [141, 142]. Modifications induced by Fringe contribute to the receptor-ligand binding interaction [143], it has also been demonstrated that reduced levels of O-linked fucose decrease Notch activation, which highlights their role in modulating Notch activation. On the other hand some studies showed that a normal Notch activation is also possible in the absence of Fringe proteins, supporting the hypothesis that fucosylation and glycosylation are important in modulating the signal strength but not obligatory in the notch pathway activation. Transfer of fucose residues to the extracellular domain of Notch is catalyzed by *O*-fucosyltransferase encoded by Ofut1 gene in *Drosophila* and the Pofut1 homologous gene in mammals. This enzyme transfers fucose to serine or threonine amino acid residues in the EGF repeats [144]. *O*-fucosyltransferase and Fringe also modify Notch ligands, Delta and Serrate in *Drosophila* and mammals [145].

In addition to *O*-fucose glycans, *O*-glucose glycans were also discovered. *O*-glucose glycans are catalyzed by *O*-glucosyltransferases, this enzyme in encoded by *rumi* gene [146], and elongation of *O*-glucose residues is possible by addition of xylose residues through the function specific xylostransferases, for example both Notch1 and Notch2 are extended by the addition of one or two xylose residues. In fact it has been demonstrated that Rumi is required for both *Drosophila* and mammalian Notch signaling at a step downstream of ligand binding [142].

Another modification that has been identified is the presence of *O*-GLcNAc in the intracellular domain of Notch, and surprisingly for the first time as a modification in the extracellular of Notch receptors [147]. These modifications by glycosylation occur during transit through the endoplasmic reticulum (ER) and Golgi apparatus where the glycosylation machinery adds glycans progressively by the corresponding glycosyltransferases.

Table 2: Mammalian glycosyltransferases that preferentially modify EGF repeats. Extracted from Hideyuki *et al.* [148].

Gene	Acceptor substrate	Donor substrate	Subcellular localization	Effect on Notch
POFUT1/Ofut1	EGF	GDP-Fuc	ER	Essential
Lunatic fringe	Fuc-EGF	UDP-GlcNAc	Golgi	Notch activation
Manic fringe	Fuc-EGF	UDP-GlcNAc	Golgi	Notch activation
Radical fringe	Fuc-EGF	UDP-GlcNAc	Golgi	Notch activation
POGLUT1/Rumi	EGF	UDP-Glc UDP-Xyla	ER	Essential
GXYLT1	Glc-EGF	UDP-Xyl	Unknown	Inhibitory
GXYLT2	Glc-EGF	UDP-Xyl	Unknown	Inhibitory
XXYLT1	Xyl-Glc-EGF	UDP-Xyl	ER	Inhibitory
EOGT1	EGF	UDP-GlcNAc	ER	?

Defects in Notch-related glycosylation caused by mutations found in the enzymes that regulate this mechanism, have been associated to human diseases and cancer. Mutations in POFUT1 cause Dowling-Degos disease (DDD), an autosomal-dominant genodermatosis characterized by reticular pigmented anomaly. In fact loss of function of pofut1 influences the process of melanin synthesis, and knock down of Pofut1 was shown to downregulate the expression of NOTCH1, NOTCH2 and HES1 in HaCat human keratinocyte cells [149].

In prostate cancer, elevated Notch ligand and receptor expression have been associated to an aggressive form of this tumor, and a recent study have reported a critical role for Lunatic Fringe (lfng). Lfng may function as a tumor suppressor through modulation of Notch signalling [150]. Lfng deficiency was also reported in basal-like breast cancer, promoting an accumulation of Notch intracellular domain fragments, with an increased expression of proliferation-associated Notch targets [151]. Overexpression of some glycosyltransferases was found in different types of cancers. POFUT1 is upregulated is brain tumor [152] and colorectal cancer [153] and POGLUT1 is overexpressed in T cell acute lymphoblastic leukemia [154]. The effect of changes and deregulation of glycosylation on Notch signalling and function has became more clear and evident but the mechanisms by which these mutations affect their substrate remain unclear.

3.4.2. Phosphorylation

Notch1 contains multiple conserved cyclin-dependent kinase phosphorylation sites within the TAD and PEST domains. Serine residues within either the TAD or PEST domains phosphorylated by CDK8, point mutations within these motifs prevent hyperphosphorylation by the CDK8 and lead to NICD stabilization *in vivo.* CDK8 complex associates with MAM through its catalytic domain, leading also to the phosphorylation of P300, thus suggesting that this regulation occurs when NICD activation transcriptional complex is bound to DNA and more specifically to the HES1 promoter [155]. CDK3 and CDK19 kinases also phosphorylate NICD within the consensus motif known as the Cdc4 phosphodegron, which is shared by most substrates of FBXW7 ubiquitin ligase [156]. NOTCH1 stability is also regulated by GSK3β, the serine/threonine kinase, that phosphorylates the intracellular NICD within its TAD domain [157, 158]. ILK, an integrin-linked kinase phosphorylates mouse Notch1 leading to down-regulation of NICD protein stability [159]. Phosphorylation can also occur in other domains. AkT1 can inhibit Notch1 transcriptional regulation through promoting NICD hyper-phosphorylation (ankyrin domain) and disrupting its translocation [160].The Casein Kinase 2 (CK2), a ubiquitous kinase, can target NICD for phosphorylation at serine residue (ser 1901) within its ankyrin domain. Phosphorylation of this amino acid motif generates a second phosphorylation at threonine in position 1898 resulting in a decreased binding of the Notch-Mastermind-CSL complex to DNA and therefore lower transcriptional activity [161].

Different studies show that NICD phosphorylation modification is critical for enzymatic activation (such as ubiquitination), complex formation, degradation and subcellular localization [162]. Therefore proving that this phosphorylation is essential for the regulation of NICD transcriptional activity, which requires precise and tight regulation of this mechanism.

3.4.3. Ubiquitination

3.4.3.1. Ubiquitination mechanism

Ubiquitination is a post-translational modification essential for proteasomal degradation, which is the main degradation path in eukaryotes. Ubiquitination controls several cellular processes via degradation. Ubiquitination is carried out by a three-step process that starts by an activation step, followed by conjugation and ends by the ligation step. Ubiquitin is a small protein consisting of only 76 amino acid polypeptide of 8,5 Kda, it is highly conserved from yeast to human. This modification was initially linked to protein half-life since ubiquitinated proteins undergo proteosomal ATP-dependent degradation. Ubiquitin is added from its C-terminus to a lysine residue of a substrate, due to the contribution of a set of three enzymes; an E1 ubiquitin activating enzyme that binds the ubiquitin via a thioesther bond in an ATP-dependent manner, than carries the ubiquitin to an E3 ubiquitin-conjugating enzyme. The final step is performed by the E3 ubiquitin ligase that binds both the charged E2 enzyme and the substrate facilitating their binding to the ubiquitin (figure 14). Only two E1 enzymes are found in humans, and 37 E2 enzymes while a wide range of E3 enzymes were identified accounting for more than 600 encoded by the human genome. Ubiquitination is a reversible process therefore deubiquitinases were also identified with a number of ≈85 enzymes in humans [163].

Different forms of ubiquitination were described, according to the number and the type of ubiquitin display on amino acids within the substrate. Monoubiquitination is adding one ubiquitin to a substrate, while multi-monoubiquitination is the addition of several ubiquitin moieties each on a single lysine residue of the targeted protein. An important characteristic of ubiquitin is that it contains seven lysine residues that are also subjected for subsequent ubiquitination, serving as substrates for polyubiquitination. Several studies were conducted on ubiquitin structure and complexity, more specifically on the role of lysine residues in determining the specificity towards substrate recognition and protein processing. Ubiquitination through lys48 is essential for polyubiquitination targeted by proteosomal degradation (lys63 can also act as a proteasomal signal), while monoubiquitination through lys6 is involved in DNA repair. Lysosomal degradation and protein recycling also require

ubiquitination and more precisely multi-monoubiquitination through lys29, allowing endosomal sorting of targeted proteins through ESCRT machinery (endosomal sorting complex required for transport). Interestingly, proteins involved in these processes employ highly specific ubiquitin-binding domains, which recognize specific Lysine residues and ubiquitin chain types specifically regulating different aspects of cellular biology [164].

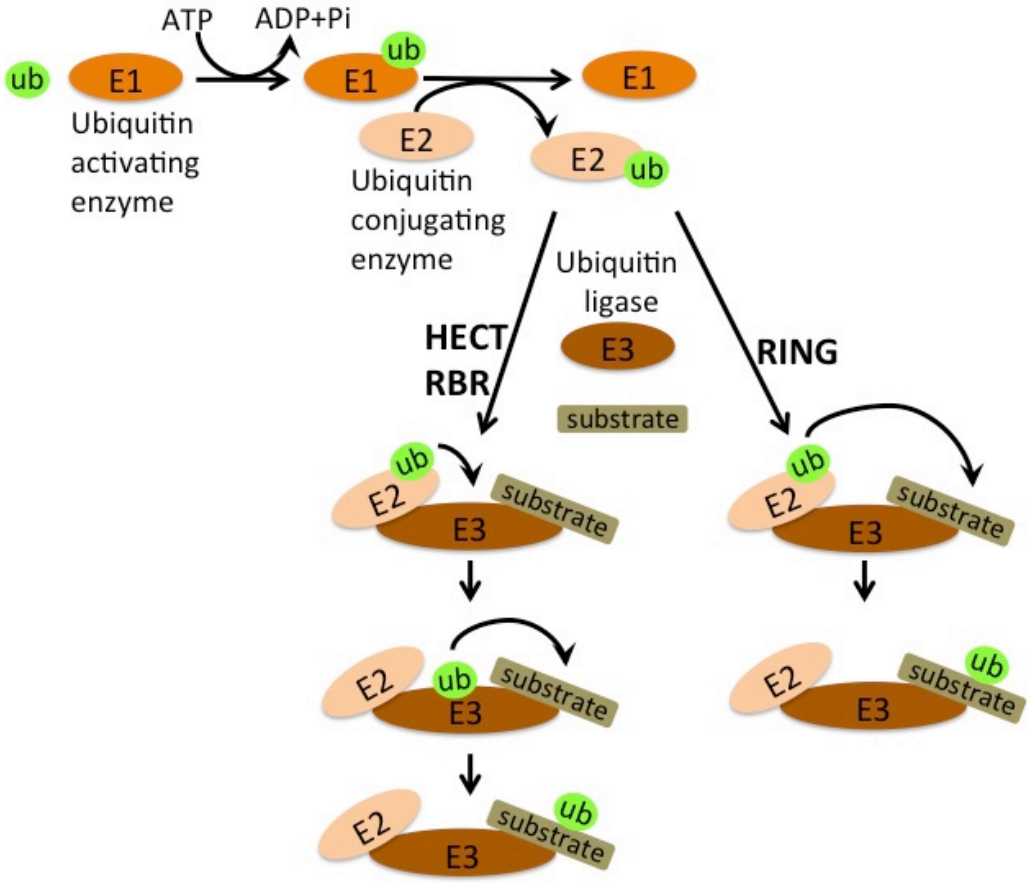


Figure 14: Ubiquitination mechanism. The ubiquitination cascade begins with ATP-dependent charging of the E1 enzyme and results in formation of a thioester bond between the ubiquitin C terminus and the E1 active site cysteine. Ubiquitin is transferred to the E2 active site cysteine in a transthioesterification reaction. An E3 ligase catalyzes transfer of the ubiquitin from the active site cysteine of the E2 to a primary amine on a lysine side chain or protein N terminus. There are three classes of E3 ligases: RING E3s, which bind to both E2~Ub thioester and substrate and catalyze attack of the substrate lysine on the thioester, and HECT and RBR E3s, which both have active site cysteines and catalyze substrate ubiquitination in a two-step reaction involving formation of a thioester with the HECT or RBR E3 followed by attack of the substrate lysine or N terminus on the E3~Ub thioester to form an isopeptide (or peptide) linkage between the ubiquitin C terminus and lysine (or the protein N terminus). Adapted from [165].

3.4.3.2. Ubiquitination in the Notch signalling pathway

Ubiquitination of Notch and its ligands plays an important role in modulating ligand endocytosis, their level of expression at the cell surface and in regulating Notch stability and degradation.

Multiple lysine residues serve as potential ubiquitination sites in DSL domain of Notch ligands, and they are targeted by E3 ubiquitin ligases Minbomb (Mb) and Neuralized (Neur) in both human and *Drosophila.* Ubiquitination is important for generating active ligand via endocytosis where several mechanisms have been described. The ubiquitinited ligand can be internalized by endocytosis in sorting or recycling endosomes to be later expressed at cell surface as active ligands. Ubiquitination may also occur after ligand binding to Notch, leading to endocytosis of both the ligand and Notch extracellular domain in a Clathrin-coated endocytic structure [166]. The pulling force resulting from this mechanism helps exposing the S2 site to metalloprotease complex, and subsequent cleavage releases NICD in the cell [85].

Ubiquitination of the receptor occurs in two different contexts; the first is during receptor endocytosis and the second in Notch proteosomal degradation.

Unliganded Notch receptor in endocytosed into rab5 and endocytic syntaxin avalanche containing compartiments, where Notch is either recycled back to cell surface or directed for lysosomal degradation. In early endosomes, full length Notch is targeted by the RING finger E3 ubiquitin ligases: Deltex (Dx) and Nedd4 and/or Cbl. Ubiquitinated Notch is sorted into multivascular bodies or lysosomes by ESCRT components (Endosomal Sorting Complex Required for Transport) for subsequent degradation. Throughout this phase, an activation of Notch can occur in endosomes where V-ATpase are thought to play a role in endosomal acidification required for γ-secretase dependent cleavage [166]. Another E3 ubiquitin ligase target Notch, it is FBXW7 (WD40-repeat containing F-Box protein component of an Skp1/Cul1/F-box protein- Rbx1-type ubiquitin ligase) promoting its proteosomal degradation (figure 15). While previously reported E3 ligase Dx and Nedd4 target NECD, FBXW7 targets NICD. It has been also demonstrated that Itch, another E3 ligase that ubiquitinates NICD, negatively regulates Notch mediated signalling [167].

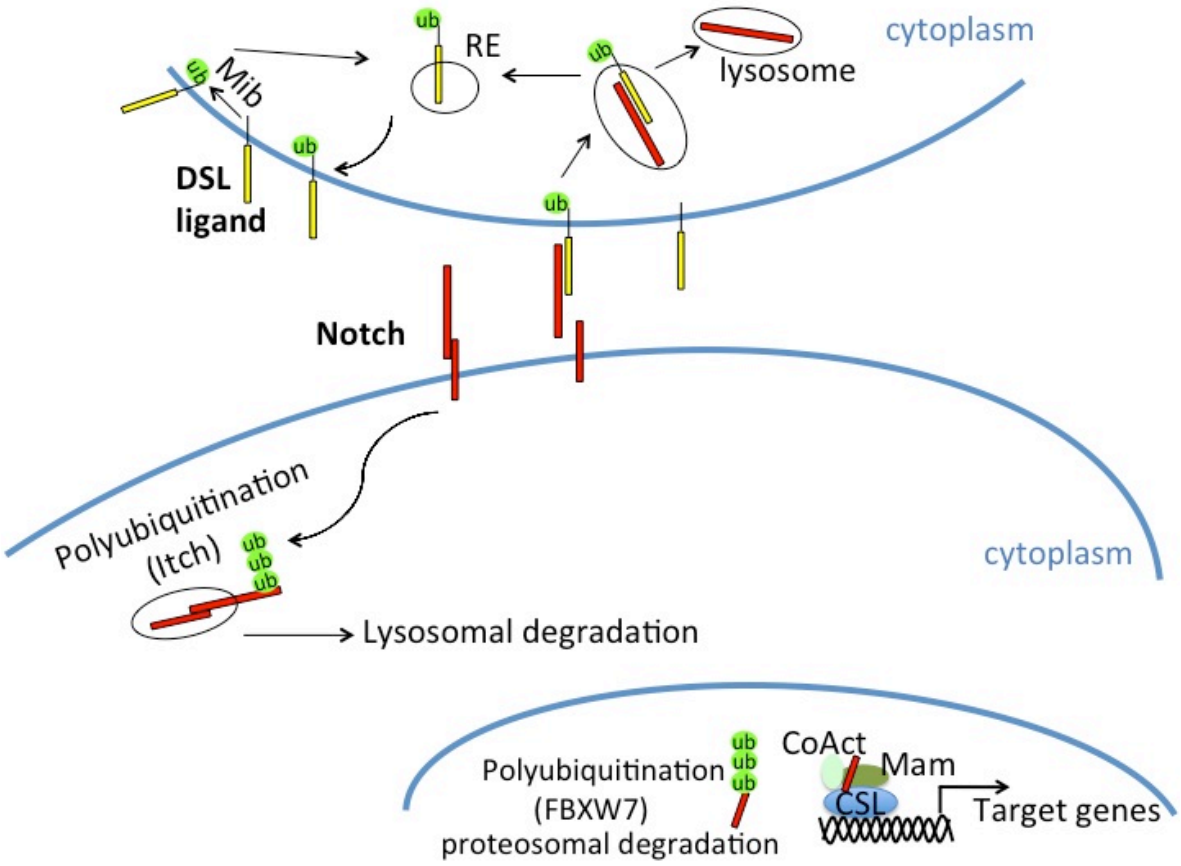


Figure 15: Ubiquitination of Notch receptor and ligands at different stages of Notch signalling. Prior to Notch engagement, ligand ubiquitination by Mib and endocytosis allows ligand to enter the recycling endosomes (RE) where it is processed into an active ligand. Alternatively ligand binding to Notch may induce ligand ubiquitination for recruitment of epsin to orchestrate the formation of a clathrin-coated endocytic structure specialized in force generation to pull the non-covalent heterodimeric Notch apart. Notch activation and signalling at the cell surface is terminated by phosphorylation of the PEST domain and in the NICD followed by ubiquitination by FBXW7 and proteasomal degradation. The non-activated Notch receptor is constitutively internalized and then ubiquitinated by Itch the E3 ubiquitin ligase to be addressed to lysosomal degradation. Adapted from [168].

3.4.3.2.1. Ubiquitination and NICD stability

Ubiquitination of Notch was first identified as a process regulating Notch stability, and levels of expression of the intracellular Notch by leading to proteasomal degradation. The PEST domain present in the C-terminus of the intracellular domain of Notch is essential for stability of the protein. This domain is subject to ubiquitination by E3 ubiquitin ligase FBXW7 in mammals [169, 170], SEL-10 in *C.elegans* and Archipelago in *Drosophila*. The CyclinC/CDK8 complex is recruited by MAML and phosphorylates Notch1 in the TAD and PEST domains. The C-terminal domain of the NICD contains

multiple conserved cyclin-dependent kinase phosphorylation sites that can be phosphorylated by CycC:CDK8 *in vitro*. A study showed that mutation of Serine residues within either the TAD or PEST domains of the NICD can stabilize the protein[155]. Nuclear Notch can also be phosphorylated by GSK3β. It was demonstrated that Notch2 interacts with GSK3β *in vitro* and *in vivo*, and phosphorylates specific threonine and serine residues in the serine threonine rich region located in the C-terminal of the ankyrin repeats, and regulates negatively NICD transcriptional activity. Though phosphorylated forms of Notch interact with FBXW7 for degradation, GSK3β mediated phosphorylation seems to regulate Notch by a different mechanism and does not lead to its subsequent degradation [157]. GSK3β is also able to phosphorylate Notch1 and enhances NICD1 stability [158], and could also up-regulate NICD transcriptional activity by enhancing its nuclear localization [171]. When FBXW7 binds the phosphorylated residues in the PEST domain of Notch, NICD is degraded via the proteasome.

3.4.3.2.2. Ubiquitination of Notch at the cell surface

At the cell surface Notch is activated by ligands and the receptor will undergo a series of proteolytic cleavages. Notch quantity is highly regulated at the cell surface; it has a limited half-life and is constantly internalized, recycled or degraded via the lysosome. Notch receptor and its ligand can also be internalized into endocytic vesicles. It can be cleaved by γ-secretase in the cytoplasm releasing NICD to the nucleus. But normally after internalization, Notch is recycled back to the membrane or degraded. Ubiquitination plays an important role in the regulation of Notch at the cell surface. Non-activated Notch receptor is constitutively internalized; Itch/AIP4 the mammalian E3 ubiquitin ligase targets Notch for ubiquitination and promotes its lysosomal degradation [172]. In Drosophila Su(dx) performs the same function as mammalian Itch and regulates endosomal sorting of Notch [173]. Itch seems to bind Notch indirectly through interacting with other protein factors such as Numb that interacts with the cytosolic HECT domain containing of this E3 ligase and acts cooperatively with Itch to promote ubiquitination of membrane-tethered Notch1 [174]. The RING finger containing E3 ligase c-Cbl also regulates the ubiquitination of membrane-bound Notch1 and has been reported to result in lysosomal degradation of Notch1 [175].

Another E3 ubiquitin ligase in the Notch signalling is Deltex, which interacts with different factors to promote Notch degradation such Itch described previously and with deubiquitinases DUB for Notch activation. The deubiquitinating enzyme eIF3f interacts with deltex, before Notch enters the nucleus in order to perform its transcriptional functions. It is more likely that eIF3f interacts with monoubiquitinated NotchΔE in the endocytic pathway thus serving as a scaffolding protein enabling Notch trafficking and modifications, and eventually Notch signalling [176] (figure 16).

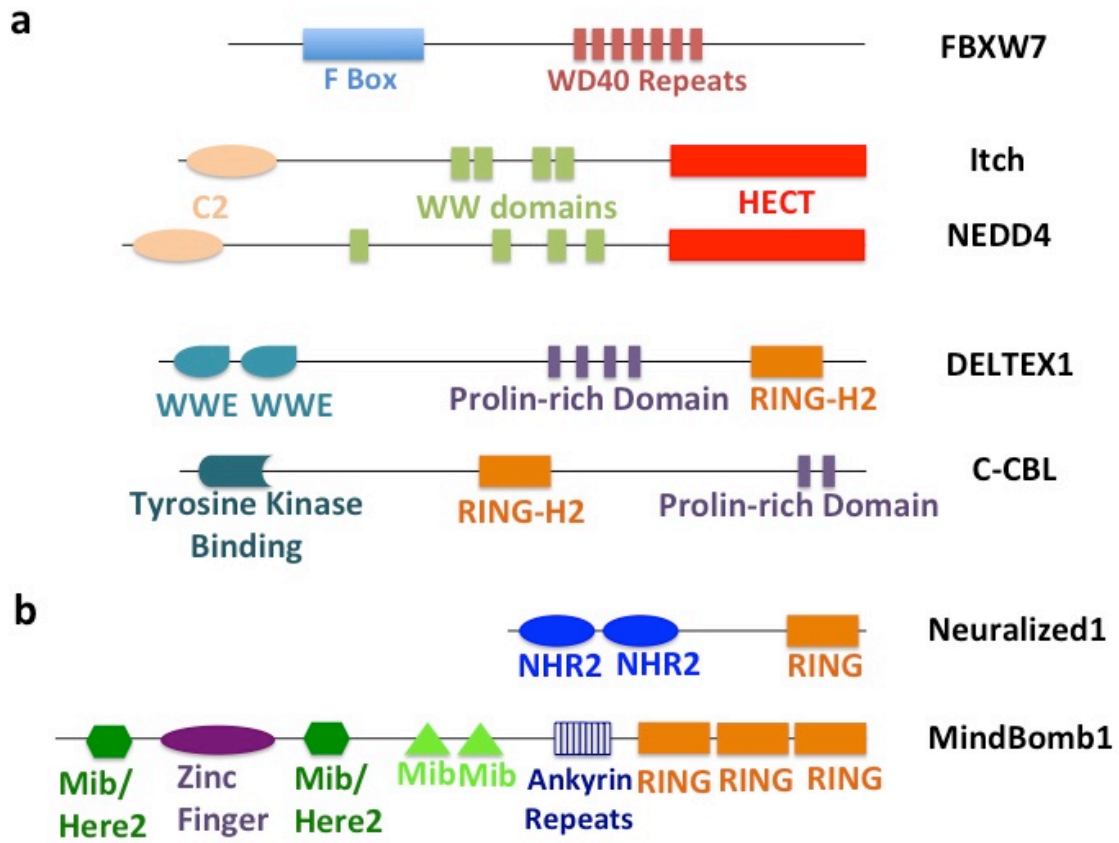


Figure 16: Structure of E3 ubiquitin ligase for Notch receptor (a) or DSL ligands (b). HECT: homologous to E6-AP Carboxy Terminus; RING: Really Interesting New Gene; NHR: Neuralized Homology Repeat; Mib: Mindbobo; Herc2: HECT domain and RCC1 domain protein 2. Adapted from [177].

3.4.3.2.3. Ligand ubiquitination

Ubiquitination process is not exclusive for Notch receptors, it also controls the regulation of expression of the ligands as well.

Two distinct RING-containing E3 ligases, Neuralized (Neur) and Mind bomb (Mib), interact with DSL ligands, promoting their ubiquitination and enhance their

endocytosis. Ubiquitination of ligands is required for transendocytosis and ligand recycling at the cell surface; two phenomena necessary for Notch activation through its ligands.

Transendocytosis and ligand recycling

Upon receptor-ligand interaction, a proteolytic cleavage is produced and Notch is released in the cytoplasm of the signal-receiving cell, while the ligand expressed on the surface of the signal-sending cell is internalized. In fact, in the absence of endocytocis, DSL ligands accumulate at the cell surface, unable to activate Notch. Moreover mechanical forces generated during DSL ligand endocytosis function to physically dissociate Notch, and that dissociation is a necessary step in Notch activation[166]. In some of the endocytic events, monoubiquitinated DSL ligands require the presence of Epsin a conserved clathrin-interacting endocytic adaptor. Epsin binds phosphatidylinositol 4,5-biphosphate (PIP2), inserts into the plasma membrane and induces membrane curvature. Then the epsin C-terminus recruits clathrin directly and also AP-2 and/or Eps15 to eventually promote clathrin-dependent ligand endocytosis [178, 179]. One of the hypotheses proposed for DSL ligand in the regulation of Notch signalling is that internalization of the ligand and their entry into coated vesicles in the signal-sending cell is an essential process leading to shedding of the extracellular Notch, thus enabling its cleavage and release in the cell [180]. Another hypothesis focuses more on events that take place after the ligands are internalized where several enzymatic processes occur in order to recycle active ligands to the cell surface [181].

In the absence of Notch, DSL ligands undergo endocytosis, it was demonstrated that delta ligand was concentrated in recycling endosomes during specific steps of cell determination in *Drosophila* [182]. Rab5 and Rab11 colocalize with internalized DSL ligands. Rab GTPases are associated with distinct cellular compartments and function as specific regulators of intracellular transport, in the endocytic pathway. Moreover loss of Rab11 induce deregulation of and loss of DSL ligand activity [182].

Recycling of the ligands before they interact with Notch is an important step for proper signalling. After their endocytosis, ligands such as Dll1 and Dll4 undergo mono or multi-ubiquitination. It was demonstrated for Dll1 that ubiquitination is essential

for Dll1 recycling and that recycling is required to acquire affinity for the receptor. Dll1 mutant (Dll1K17R) is endocytosed but is unable to signal, this observation is explained by the lack or absence of ubiquitination of this mutant. However the chimeric ligand Dll1–3, although not ubiquitinated, was able to recycle to the cell surface, indicating that an unknown signal present in the intracellular domain of Dll3 can avoid the necessity for ubiquitination. On the other hand, this molecule is unable to signal. These data prove that ubiquitination process is indeed necessary for Dll1 recycling and Notch activation [183].

Notch1 signalling and activation by the Dll4 ligand is crucial in intrathymic T cell development. Dll4 function depends on a combination of several factors that involve interaction with the ubiquitin ligase Mib1 for ubiquitination, internalization and recycling back to the cell surface for effective Notch signalling and therefore supporting T cell development and inhibiting B cell development. In contrast with Dll1, Dll4 are excluded from lipid rafts in order to be recycled [184]. Though they might differ in their specific localization or the factors contributing to their subsequent recycling and maturation, all data point at the necessity of ubiquitination during this process.

Ubiquitination events occur at different levels of Notch signalling. Recent studies have identified several new regulators in the ubiquitination and deubiquitination mechanisms, raging from degradation to regulation of membrane trafficking and affecting the developmental control of the signalling activities of both Notch receptors and their ligands.

3.4.3.2.4. E3 ubiquitin ligases

In this part, we will discuss more in detail E3 ubiquitin ligases and more specifically FBXW7 protein. As previously mentioned, E3 ligases carry out the final step in the ubiquitination cascade, catalyzing transfer of ubiquitin from an E2 enzyme to form a covalent bond with a substrate lysine. Three distinct classes of E3 ligases have been identified the family of RING finger E3s, homolog to the E6AP carboxy terminus (HECT) domain family and the Ring-between-Ring (RBR) family. The great diversity found among these ligases is due to their specificity toward their targets, therefore

each class is also characterized by their conserved domains and the mechanisms of ubiquitin transfer from E2 enzymes to the substrate [185].

Ring E3 enzymes catalyze ubiquitin transfer process, the E2 binding domain of the Ring domain binds the E2 enzyme through its N-terminus, and it also contains additional domains required for substrate recognition and its subsequent ubiquitination. Ring E3s can act as monomers, dimers or even multisubunit complexes. An interesting superfamily of the Ring E3 ligases, that function as complexes are the Cullin Ring ligases superfamily, consisting of multi-subunit proteins containing the cullin protein, a Ring-box protein, and an F-Box protein essential for binding the substrate. The RING E3 act as scaffold that brings E2 and substrate together, on the other hand it was suggested that RING finger domains can also allosterically modify and activate E2 enzymes [186]. HECT E3 ligases catalyze the transfer reaction in a two-step process of what is called a direct ubiquitination. The first step is the transfer of the ubiquitin from the E2 conjugating enzyme to the HECT-domain E3 ligase, and next this ubiquitin residue is transferred from the cysteine in the E2 active site to the lysine residue of the substrate [187]. The HECT domain consists of two lobes; one in the N-terminal lobe interacting with the E2 enzyme and the second C-terminal lobe that contains the cysteine residue that forms a catalytic intermediate with the ubiquitin moiety. During ubiquitin transfer the HECT domain undergoes conformational changes due to the flexible region separating the lobes, thus allowing the ubiquitin transfer from the E2 enzyme to the substrate [188]. The RBR (Ring-between-Ring)-domain E3 ligases contain two Ring domains (Ring1 and Ring2) separated by a conserved domain called the IBR domain (InBetween Ring), they also share features with HECT domain ubiquitination mechanism directly catalyzing ubiquitin transfer an intrinsic cysteine present in the Ring 2 domain, while Ring1 domain helps recruiting thioester-bound E2 enzymes. This class of E3 ligases is characterized by having an auto-inhibition mechanism that modulates ubiquitination activity, and by combining aspects of both Ring and HECT E3 ligases function [189]. The resemblance with HECT relies in the mechanism, by which Ring1 domain binds to E2-ubiquitin via a cysteinyl-thiol within the same E3 protein.

3.4.3.2.5. F box proteins

The SCF (SKP1-CUL1-FBP) family belongs to the Cullin dependent ligases (CDL) family that has been well characterized. In this complex, SKP1 (S-phase-kinase associated protein 1) interacts with the Cul1 proteins functioning as a scaffold protein that sorts the proteins destined for degradation. Cul1 is also bond to the RING-finger protein (Rbx1), and to the ubiquitin conjugating enzyme (UBC) and to one of the FBPs. FBPs confer to the targeted substrates. Originally Skip1 was found to be essential to for interaction with several proteins such as Cdc4 and cyclin F via their 40 amino acid domain that was later found to be a conserved domain characterizing the F-box proteins [190].

Mammalian F-box proteins are classified into three groups according to their substrate-binding domain. We recognize the FBW (F-box and WD40 repeats), the FBL (F-box and Leucine-rich repeat) and the FBX that do not have either a WD domain or a Leucine rich repeat. FBX class rather contains protein-protein interaction domains such as zinc-finger, proline –rich domain… FBPs recognition of their substrate often requires specific post-translational modifications such as phosphorylation, glycosylation, methylation, acetylation and so on. In 2004 Cardozo *et al.* reviewed the SCF ubiquitin ligases, the mechanism of this protein complex was described and compared to the mechanism known for typical enzymes. Therefore in the SCF complex, the ubiquitin is transferred instead of what is usually a small chemical group for other protein enzymes. The activated E2 conjugating enzyme can be considered like the key catalytic residue of the enzyme, whereas the entire FBP plays the role of the substrate binding domain in the enzymatic complex [191].

As previously mentioned, the F-box proteins class consists of a wide variety of proteins, due to their role as the substrate-recognizing subunits of the SCF E3 complex. In the human genome, the F-box proteins account for ≈70 proteins, the most well known among them are FBXW7, Skp2 and β-TrCP.

A single F-box protein can target several proteins, for example NOTCH1, cMYC and cJUN are targeted by FBXW7 for ubiquitination and subsequent proteasomal degradation, on the other hand a protein can be a target of different F-box proteins, for instance CyclinE can be recognized by FBXW7 and Skp2 [192].

3.4.3.2.6. FBXW7 E3 ubiquitin ligase

FBXW7 is the substrate recognition unit of the conserved SCF E3 ubiquitin ligase as previously described. FBXW7 gene was originally found in yeast, it is called Cdc4, its role was described in the control of the cell–cycle-regulated disposal of Sic1 protein; an inhibitor the cyclin-dependent kinase (CDK). Cdc4 binds specifically to phosphorylated residues of their substrates, also known as phospho-degrons. FBXW7 is the human Cdc4 orthologue, it was also demonstrated that in human, it catalyzes the phosphorylation-dependent ubiquitination of cyclin E [193]. In addition, the fly Cdc4 orthologue AGO also regulates cyclin E in *Drosophila melanogaster* [194].

In the human genome, FBWX7 gene encodes for three protein isoforms: FBW7α, FBXW7β and FBXW7γ, that are produced by alternative splicing and that localize to the nucleoplasm, cytoplasm, and nucleolus, respectively. Several studies have focused on defining exon-specific localization characteristics, and they demonstrated that FBXW7 proteins comprise a common region containing three basic domains (BDs). FBXW7α and FBXW7β both contain in their common region, localized at the N-terminus of the protein, a nuclear localization signal (NLS). FBXW7α contains a second NLS, and it was revealed that both NLSs are required for its nucleoplasmic localization. Whereas FBXW7β contains a putative hydrophobic transmembrane domain, within its exon-specific region in the N-terminus, that was associated to its cytoplasmic localization [195]. The α isoform of FBXW7 constitutes the most abundant and major isoform of cellular FBXW7, and data also suggest that FBXW7α is responsible for cMYC and Cyclin E degradation whereas Notch is targeted by the β isoform [196].

3.4.3.2.7. FBXW7 substrates

FBXW7 has pivotal roles in cell division, growth, and differentiation by targeting several proteins. Approximately 20 substrates are known to be targets of FBXW7; amongst them the most studied are the oncoproteins c-Myc, Notch1, Notch4, c-Jun, and cyclin E that are targeted by FBXW7 for degradation. FBXW7 binds substrates at specific conserved phosphorylated motifs, known as the Cdc4 phosphodegrons (CPD). It has also been demonstrated that FBXW7 acts as a dimer, and that dimerization

enables binding modes in order to establish specificity toward its substrate [197]. Data also suggest that dimerization regulates FBXW7 stability and autoubiquitylation, in fact FBXW7 is also subject to post-translational modifications, including phosphorylation at Ser205 and subsequent isomerization by Pin1 leading to autocatalytic ubiquitination of FBXW7 [198]. Though FBXW7 turnover is largely mediated by phosphorylation through autocatalytic ubiquitination, it is also controlled by deubiquitinases such as Usp28. Usp28 deubiquitinase forms a complex with FBXW7 and antagonizes substrate ubiquitination such as C-Myc, and C-Jun [199]. Usp28 antagonizes both substrate targeting and autocatalytic ubiquitination by FBXW7, and this model for dual regulation of FBXW7 was found to be a tissue specific mechanism [200].

The question whether FBXW7 isoforms recognize specifically their substrates remains unclear. Most studies used overexpression approaches in order to clarify this issue, but only few isoform-specific functions have been described. It has been demonstrated that FBXW7γ regulates of c-Myc nucleolar regulation, while FBXW7α recruits Pin1 leading to its isomerization thus promoting cyclin E degradation. Using a gene-targeting approach and specific isoform knock out in cell lines, it has also been shown FBXW7α isoform is largely responsible for Cyclin E degradation, but targeting Cyclin E can also be carried out by FBXW7γ and FBXW7β in other cellular contexts [201]. A recent study revealed that isoform-specific FBXW7 ubiquitination mediates differential regulation of PGC-1α; a member of the PPARγ (Perixosome proliferator activated receptor gamma) coactivators-1 family, that functions as transcriptional co-regulator. They observed that while FBXW7β promotes PGC-1α ubiquitination and proteasomal degradation, FBXW7α stabilizes PGC-1α [202]. Despite data showing that in different cellular contexts, FBXW7s can somehow target their substrate in an isoform-specific manner, the evidence remains controversial and requires further investigation, in order to better understand the mechanisms involved in substrate recognition and specificity for different FBXW7 isoforms.

C-MYC, a target of FBXW7, functions as transcription factor regulating transcription of several target genes, it has an important role in cell cycle progression, cellular transformation and apoptosis. Deregulation of MYC expression and gene mutations have been associated to several types of cancer such as hematopoietic tumors. C-MYC

protein's abundance are regulated by levels of degradation of the protein, and phosphorylation of c-Myc on threonine-58 by GSK3 regulates its binding to FBXW7 (like Cyclin E phosphorylation at T380), as well as FBXW7-mediated c-MYC degradation and ubiquitination [203, 204].

Another target is C-jun, a transcription factor and an oncoprotein, implicated in the regulation of many cellular processes such as proliferation, transformation and cell death. It is a component of the activator protein-1 (AP1) transcription factor complex. Deregulation of C-jun has been reported in many types of cancer. It is known to be a highly unstable oncoprotein that needs to be tightly regulated on both transcriptional and post-translational levels. C-jun can be stabilized once it is phosphorylated at the N-terminus by C-jun N-terminal kinases (JNKs) or other protein kinases. This phosphorylation decreases C-jun ubiquitination and degradation[205]. C-jun ubiquitination is carried out by FBXW7 in a GSK3 phosphorylation-dependent manner. Phosphorylation of amino acid residues at positions 239 and 243 is required for FBXW7 recognition[206]. Ubiquitin dependent degradation can also be promoted by CSK-dependent phosphorylation (CSK a protein tyrosine kinase) [207]. C-jun can also be recognized by other ubiquitin ligases such as Itch and COP1, in a phosphorylation-independent manner, inducing its ubiquitination and degradation [208].

CyclinE is also among the FBXW7 targets. CyclinE binds CDK2 the cyclin-dependent kinase subunit that is implicated in the regulation of cell cycle processes and cell division. The role of CyclinE is not limited to CDK2 activation; it is also implicated in CDK2-independent regulation. In a CDK2 dependent context, degradation of Cyclin E is mediated by the SCF FBXW7complex that recognize phosphorylated degrons upon the substrate. Cyclin E contains two CDPs: a C-terminal degron at threonine 380 and an N-terminal degron at threonine 62. These residues are phosphorylated by GSK3 and CDK2 leading to subsequent binding and ubiquitination by FBXW7 [209].

3.4.3.2.8. FBXW7 a tumor suppressor protein

FBXW7 is considered as a tumor suppressor protein due to the fact that it targets several oncoproteins, therefore regulates their levels of expression and stability via ubiquitination and subsequent degradation. Many studies have reported mutations in FBXW7 in different types of cancer. The majority of these mutations are point

mutations resulting in amino-acid substitutions within the WD40 repeats and consequent disruption of substrate binding. Nonsense mutations were also identified resulting in the production of truncated forms of FBXW7. These mutations lead to a deregulation or loss of function of FBXW7 promoting an accumulation of FBXW7 substrates[210].

FBXW7 is required for normal stem cells maintenance, for hematopoietic, neural and intestinal stem cells (HSCs, NSCs and ISCs), and for the proper regulation of cell cycle progression and balance between differentiation and self-renewal. Absence of FBXW7 in HSCs leads to an accumulation of cMYC inducing the re-entry of HSCs in the cycle. In a P53-downregulated context, this accumulation leads to the development of T-cell malignancies. On the other hand, NSCs deficient for FBXW7 accumulate Notch1, c-JUN but not cMYC and represent higher proliferation rate than normal HSCs in addition to morphological abnormality in the brain [211] (figure 17). FBXW7 deregulation is also implicated in promoting proliferation and maintenance of cancer stem cells (CSCs) also known as cancer initiating cells (CICs). CICs are characterized by their ability to self-renew and proliferate and their ability to initiate cancer as indicated by their name. After cancer therapies, CICs may be latent but can reemerge at interruption of treatment gaining also resistance to therapy [212]. FBXW7 plays a pivotal role in maintenance of quiescence in leukemia-initiating cells (LICs) of chronic myeloid leukemia. It has been demonstrated that FBXW7 ablation in LICs results in deregulated activation of c-Myc and impaired maintenance of quiescence followed by p53-dependent apoptosis. LICs were found to be more sensitive to FBXW7 deficiency than are HSCs, suggesting their potential role as therapeutic targets [213, 214].

An extensive genetic analysis of FBXW7 was carried out in primary human tumors of diverse tissue origin in order to determine FBXW7's role as a tumor suppressor. In this study they generated data from 534 primary tumor specimens tested in addition to data previously reported on FBXW7 (total of 1554 specimens) and they found that FBXW7 is mutated in a variety of human tumor types with an overall mutation frequency of 6%. The highest frequencies of mutations were observed in tumors of the bile duct (cholangio- carcinomas, 35%), blood (T-cell acute lymphocytic leukemia, 31%), colon (9%), endometrium (9%), and stomach (6%). Furthermore, they show that expression of an FBXW7 mutant corresponding to one of the major mutational hotspots in primary tumors interferes with wild-type FBXW7 function, suggesting a

potential dominant negative mechanism of FBXW7 inactivation [215].

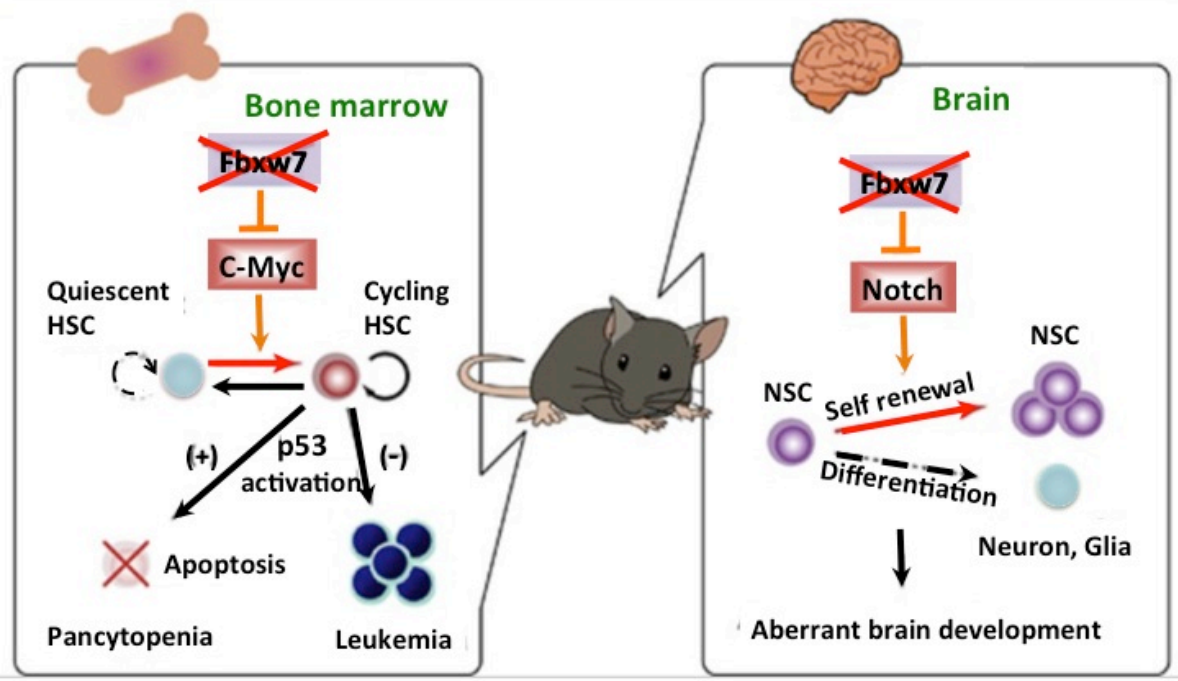


Figure 17: FBXW7 is required for maintenance of normal stem cells. Fbxw7 ablation in HSCs results in the accumulation of c-Myc and consequent re-entry of the cells into the cell cycle. The cycling HSCs are then subject to apoptosis as a result of p53 activation, leading to the development of pancytopenia. In the absence of p53 induction, the Fbxw7-deficient HSCs eventually give rise to leukemia. On the other hand, loss of Fbxw7 in NSCs results in Notch accumulation, with the consequent imbalance between self-renewal and differentiation in these cells, leading to aberrant brain development. Reviewed in Takeishi *et al.* [211].

Inactivating FBXW7 mutations were identified in a large fraction of human T-ALL lines and primary leukemias. FBXW7 mutation were identified in 40% of human T-ALL cell line and 16% of primary leukemias where FBXW7 ability to interact with its targets (NOTCH1, cMYC and cyclin E) was abolished. The majority of the FBXW7 mutations were present during relapse, and they were associated with NOTCH1 HD mutations. Interestingly, most of the T-ALL lines harboring FBXW7 mutations were resistant to γ-secretase inhibitor treatment and this resistance appeared to be related to the stabilization of the c-Myc protein [216].

Hotspot mutation sites were identified and associated to cancer phenotypes. The catalogue of somatic mutations in cancer database (COSMIC) have reported 496 mutation in FBXW7, where half of these mutations were identified as missense

mutations affecting residues in the β-propellor structure within the WD40 repeats, thus altering substrate binding to the E3 ligase. These changes affected more specifically arginine residues 465, 479 and 505 with a majority being monoallelic mutations. In a recent study, they demonstrated that an FBXW7 propellor tip mutation at the arginine residue 482 which is a site homologous the 479 amino acid residue, directly promotes intestinal tumorigenesis in mouse models [217].

Epigenetic inactivation of FBXW7 through promoter hypermethylation is an important factor that can occur during tumorigenesis. Significantly, in breast cancer, methylation of FBXW7/hCDC4-β is related to favorable prognosis despite its association with poorly differentiated tumors, and CpG-methylation correlates with loss of FBXW7/hCDC4-β expression in tumor cell lines[218]. In another context, P53-dependent loss of FBXW7 was linked to genetic instability in cancer mouse models, through a mechanism that might involve the activation of AURORA kinase, an identified substrate of FBXW7 ligase [219].

A large number of studies have focused on the role of FBXW7 in cancer and how regulation of ubiquitination and its function can be crucial in promoting cancer and tumorigenesis. Data provide evidence on FBXW7 as a critical tumor suppressor mutated and inactivated in melanoma, leukemia, colon cancer, breast cancer and other types of tumors, resulting in sustained activation and upregulation of its known substrates that are also implicated and linked to several signalling pathways such as the Notch pathway, mTOR pathway…These findings render FBXW7 targeting in cancer a promising therapeutic strategy that can be largely investigated in these contexts.

3.4.3.2.9. NOTCH1 FBXW7-dependent degradation

The link between NOTCH1 protein stability and FBXW7 is established and well characterized. It became clear that FBXW7 regulates NICD degradation through proteosomal degradation [170]. It was demonstrated that Fbxw7-mediated degradation is primarily responsible for the regulation of Notch stability during neural development [220]. Notch1 PEST domain is known to be important for NICD/FBXW7 interaction, in fact a potential phosphodegron is present within the PEST domain and contains a conserved threonine residue (T2512), which is central to the binding of

FBXW7 to NOTCH1 [221], a study showed that NOTCH T2512A mutant represents increased stability and decreased binding to FBXW7 [58].

The C-terminal domain of the NICD contains multiple conserved cyclin-dependent kinase phosphorylation sites that can be phosphorylated by CycC:CDK8. MAM interacts directly with the catalytic subunit of the CycC:CDK8 complex, and CDK8 directly targets serine residues in the PEST motif that control NICD hyperphosphorylation and turnover, mediated by FBXW7 PEST-dependent degradation [155]. Moreover NICD is phosphorylated by GSK3β that regulates its proteasomal-dependent degradation [155].

3.5. NOTCH1 protein-protein interactions

When we look through STRING database for NOTCH1 protein-protein interactions, we identify ten interconnected proteins in the NOTCH1 network (figure 18). Among these proteins RBPJ and MAML1 that are part of NICD transcriptional complex in the nucleus. ADAM17, a metalloprotease responsible for NOTCH1 cleavage at the cell surface independent of ligand induced activation. We can also add ADAM10 another enzyme required for receptor activation but in another ligand –dependent context [222]. PSEN1, the catalytic subunit of γ-secretase also interacts with notch and has been shown to play an important role in the proteolytic processing of NOTCH1 [223]. STRING list includes FBXW7 and DTX1 ubiquitin ligases that are both known for direct physical interaction with NOTCH1 and inducing its proteasome-mediated degradation [221, 224]. NUMB promotes NOTCH1 ubiquitination and proteasomal degradation of NICD, described also as an inhibitor of Notch pathway [225, 226].The polyubiquitin C protein encoded by *UBC* gene, is among the reported interactors. As we have explained previously, ubiquitination is an essential post-translational modification in the regulation of the Notch signalling, and NOTCH1 can undergo ubiquitination at different stages of its signalling process through a variety of ubiquitin enzymes [169, 227, 228]. The last two proteins listed are HEY1 and HEY2 from Hey family of bHLH transcription factors that are direct targets of Notch signaling. Hey1 and Hey2 are essential mediators of Notch functions in blood vessel morphogenesis [229]. The list provided by STRING database is limited, we have discussed in the previous parts different aspects of the Notch signalling pathway and thus a wide variety of proteins

interact with NOTCH1. Among them ligands of delta and serrate which activate the receptor at the cell surface, different enzymes involved in post-translational modifications: POFUT1, POGLUT glycosyltransferases that preferentially modify EGF repeats of NOTCH1; kinases (CDK2, CDK19, CDK8…) and ubiquitin ligases (FBXW7, DELTEX, Itch…). Yatim *et al.* established the NOTCH1 nuclear interactome in T-ALL, and showed that beyond the NICD-CSL-MAML1 activation complex, NOTCH1 associates a multifunctional complex containing DNA-binding partners, transcriptional coactivators and corepressors acting at different stages of the transcription activation process [230].

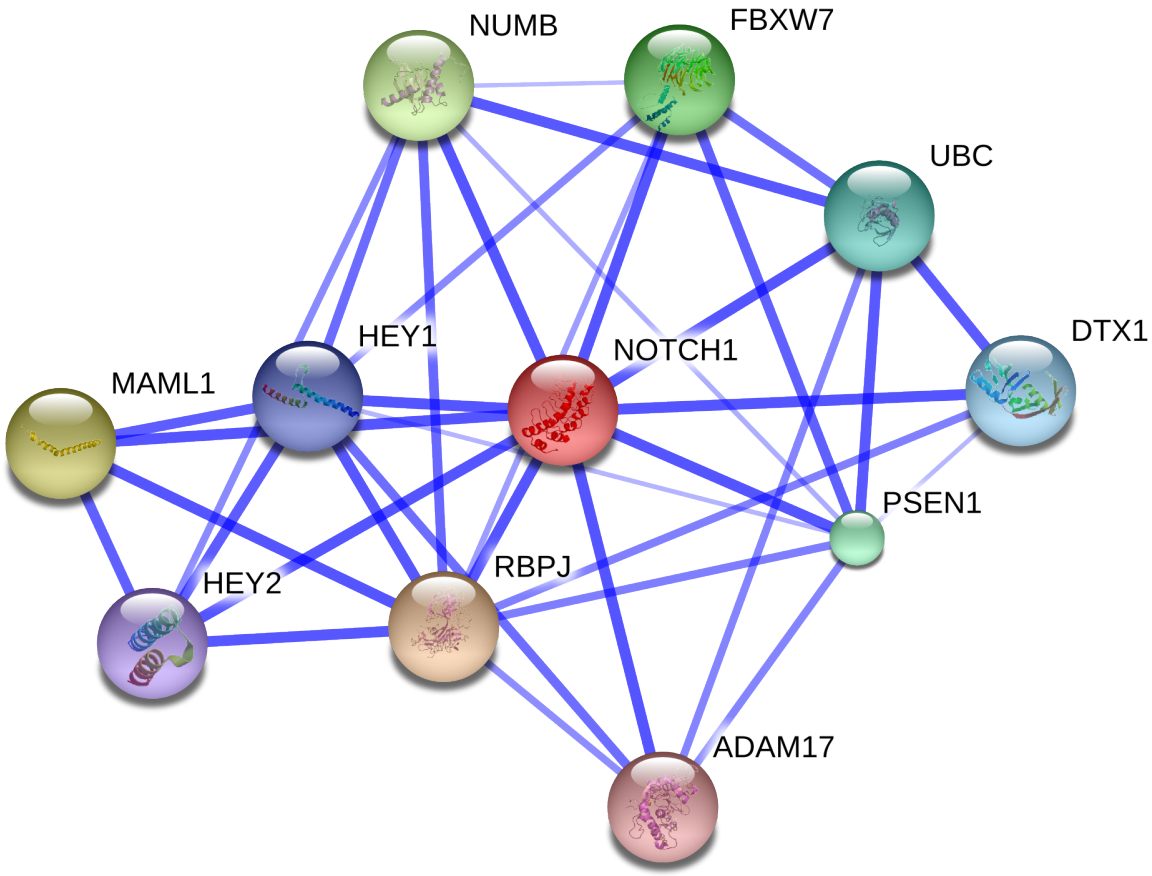


Figure 18: Notch1 protein interaction network extracted from STRING database. Colored nodes represent proteins that are directly linked to Notch1. Edges represent predicted functional links. Edge width is proportional to the number of evidence for these interactions (consisting of: databases, experiments, textmining and homology).

AIM OF THE WORK

The first aim of this thesis was to establish a comprehensive interactomic map of proteins that are associated to acute lymphoblastic leukemia (ALL) using high-throughput experimental techniques and bioinformatics methods, to identify unexplored axes to better understand ALL.

This interactomic approach enabled us to define important proteins hubs for proteins and pathways known to be linked to ALL, but also new molecular determinants that can be potentially linked to ALL.

Then, the second aim of the project was to characterize the functional interplay between EXT1, NOTCH1 and FBXW7 as a proof of our concept for combining interactome analysis and bioinformatics tools.

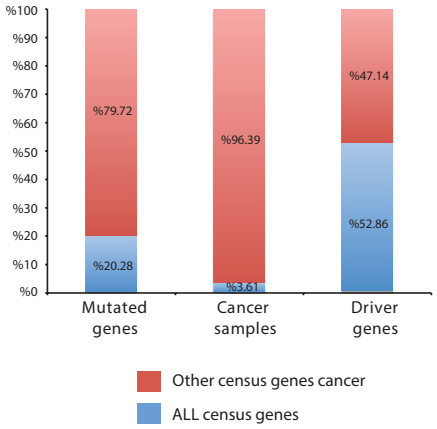
RESULTS

PART I. Interactome mapping of acute lymphoblastic leukemia gene products

1. Mutations associated to ALL in cancer gene census

In order to identify cancer genes associated with acute lymphoblastic leukemia (ALL), we searched the COSMIC database version 71 and collected all available information about mutated genes in ALL samples. COSMIC V71 contains over 1058292 tumor samples containing over 2710449 coding mutations in 28977 genes [231]. We found more than 2500 mutations in coding sequences of 366 genes that were reported in 36909 ALL samples. In the COSMIC database, a set of 572 genes whose mutations are causally linked to oncogenesis, are called human Cancer Gene Census [232]. This set includes 140 genes well accepted as "cancer driver genes" because mutations in those genes directly promote tumorigenesis [233]. In ALL samples, we found that 20% of the cancer gene census is affected by mutations in coding regions of 116 genes (Supplemental table S1A). This high number of mutated genes is not due to over representation of ALL samples in COSMIC, as ALL samples count for about 3% of tumor samples compiled in the COSMIC V71 (Figure 19 a). The "ALL-genes" set contains 74 well-known driver genes including 35 oncogenes and 39 tumor suppressor genes (TSG) (Supplemental table S1B).

(a)



(b)

Gene name	Number of samples	Proportion of samples	Rank per number of samples with mutations	Distinct mutations per gene	Rank per distinct mutations
FLT3	3656	%9.91	1	47	8
NRAS	2266	%6.14	2	23	17
JAK2	2244	%6.08	3	36	13
NOTCH1	2167	%5.87	4	595	1
IKFZ1	2093	%5.67	5	40	10
KRAS	2037	%5.52	6	22	18
PTPN11	1963	%5.32	7	37	12
CDKN2A	1545	%4.19	8	29	15
JAK1	1509	%4.09	9	36	14
FBXW7	1404	%3.80	10	42	9
PTEN	1145	%3.10	11	140	2
PAX5	1049	%2.84	12	79	5
TP53	964	%2.61	13	85	4
IL7R	955	%2.59	14	73	6
WT1	797	%2.16	15	86	3
BRAF	660	%1,79	16	7	34
IDH1	649	%1.76	17	5	43
CREBBP	619	%1.68	18	40	11
IDH2	607	%1.64	19	2	73
PHF6	556	%1.51	20	62	7

Total of samples = 36909

Figure 19: ALL census genes ranking (a) Distribution of ALL census genes and other census genes according to number of mutations and number of samples and their distribution among driver genes. Red and bleu bars represent ALL census genes and other cancer census genes respectively (b) Mutations associated to ALL in cancer gene census. Frequency of mutations in the top 20 genes (36909 ALL samples). The number and proportion of ALL samples in which gene mutations were detected are represented. Data source: COSMIC database.

For each ALL-gene we extracted the number of samples as well as the number of distinct mutations. Figure 19 b represents the top 20 frequently mutated genes among the 116 ALL-genes. Each gene has at least 2 distinct mutations observed in at least 556 different samples, from a total of 36909 ALL samples were examined. Seven genes were found mutated in more than 5% of ALL samples, including genes encoding for FLT3 (9.9%), NRAS (6.14%), JAK2 (6.08%), NOTCH1 (5.87%), IKZF1 (5.67%), KRAS (5.52%) and PTPN11 (5.32%) (Figure 19 b and figure 20 a). We also ranked ALL-genes according to the number of distinct mutations found in ALL samples (Figure 19 b and figure 20 b). The top ranked gene was NOTCH1 with 595 distinct mutations mostly found in its heterodimerization (HD) domain (63% of mutations) and in its proline, glutamic acid, serine, threonine-rich (PEST) domain (27% of mutations) (Figure 22). Mutations in the HD domain that enhance NOTCH1 cleavage and nuclear translocation of the intracellular NOTCH1 protein (ICN), and mutations in the PEST domain that result in the stabilization of NICD, are gain-of-function mutations affecting the transcriptional activation of Notch1-target genes. The majority of these activating mutations were found in human T lymphocytes ALL (T-ALL) samples, as previously reported [54]. Other highly mutated ALL-genes include

PTEN (140 distinct mutations) WT1 (86 distinct mutations), TP53 (85 mutations), PAX5 (79 mutations), and IL7R (73 mutations) (Figure 19 b).

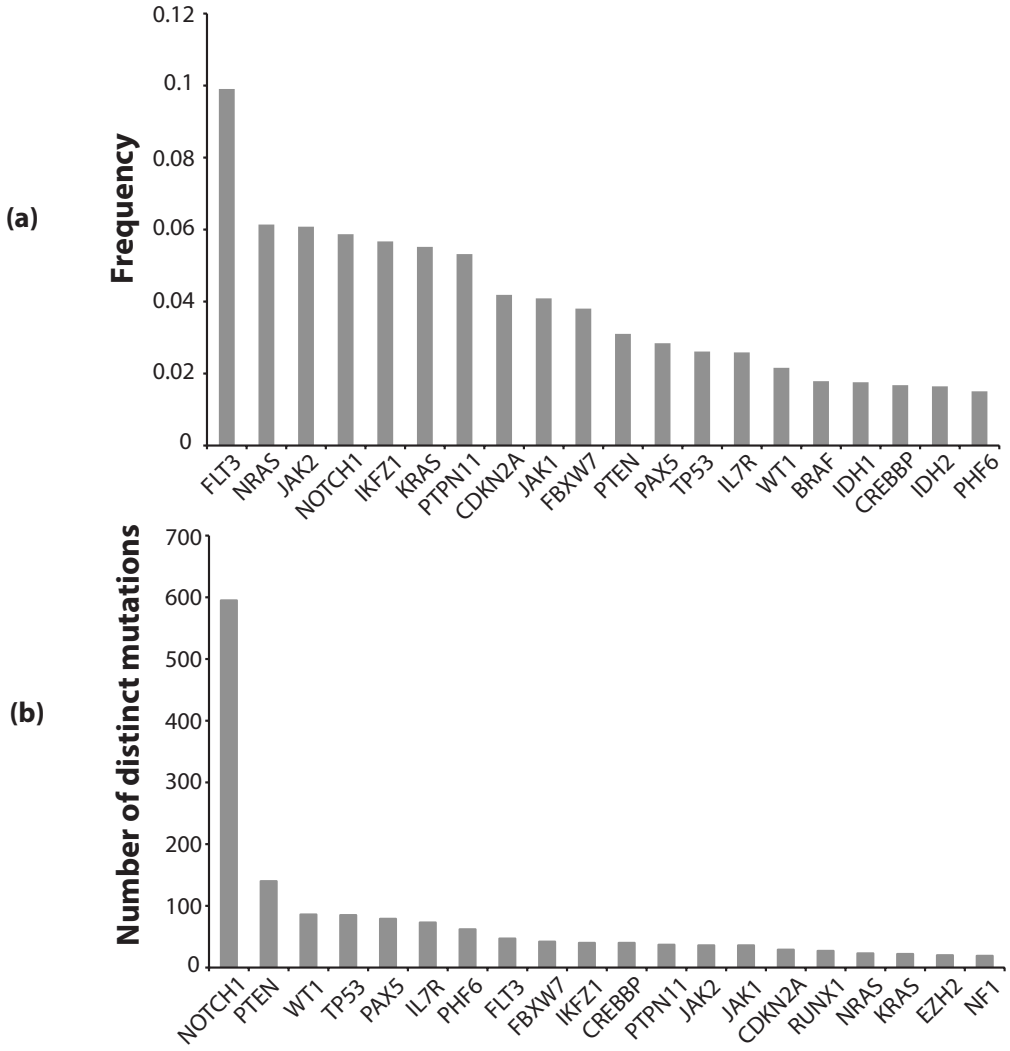


Figure 20: Top 20 ALL census genes. (a) The 20 most frequently mutated genes in ALL samples; X-axis represents the proportion of samples where mutations were reported. (b) Number of distinct mutations per genes in ALL samples; X-axis represents the number of distinct mutations found in the coding sequences and Y-axis the top 20 genes with higher number of distinct mutations.

For the majority of ALL-genes, the number of distinct mutations per gene correlated with the number of mutated samples (Figure 21, red circled), suggesting that a number of somatic mutations occurred randomly during oncogenesis, as previously observed for other types of cancers such as ovarian carcinoma or acute myeloid leukemia [234]. Another set of eight genes (*FBXW7*, *CDKN2A*, *PTPN11*, *IKZF1*, *JAK1*, *JAK2*, *KRAS* and *NRAS*) exhibit an average of 33 mutations in 1500 – 2500 examined ALL samples (Figure 21). These genes are characterized by similar mutations occurring in

distinct ALL samples, suggesting their potential roles in clonal expansion of ALL. Two genes are outliers, and display many more mutations (*NOTCH1*) or are mutated in many more samples than average (*FLT3*). These larger numbers reflect the high rate of *NOTCH1* mutations specifically in T-ALL samples (99,9% of *NOTCH1* mutations); and the involvement of FLT3 in childhood ALL, as previously described [235]. Interestingly, *NOTCH1* and *FLT3* mutations, mostly localized in two functional domains (HD and PEST for NOTCH1, juxtamembrane (JM) and tyrosine kinase (TKD) for FLT3), are found respectively in 1897 and 723 different patients, and are exclusive in examined ALL samples (Figure 22 and Figure 19 b).

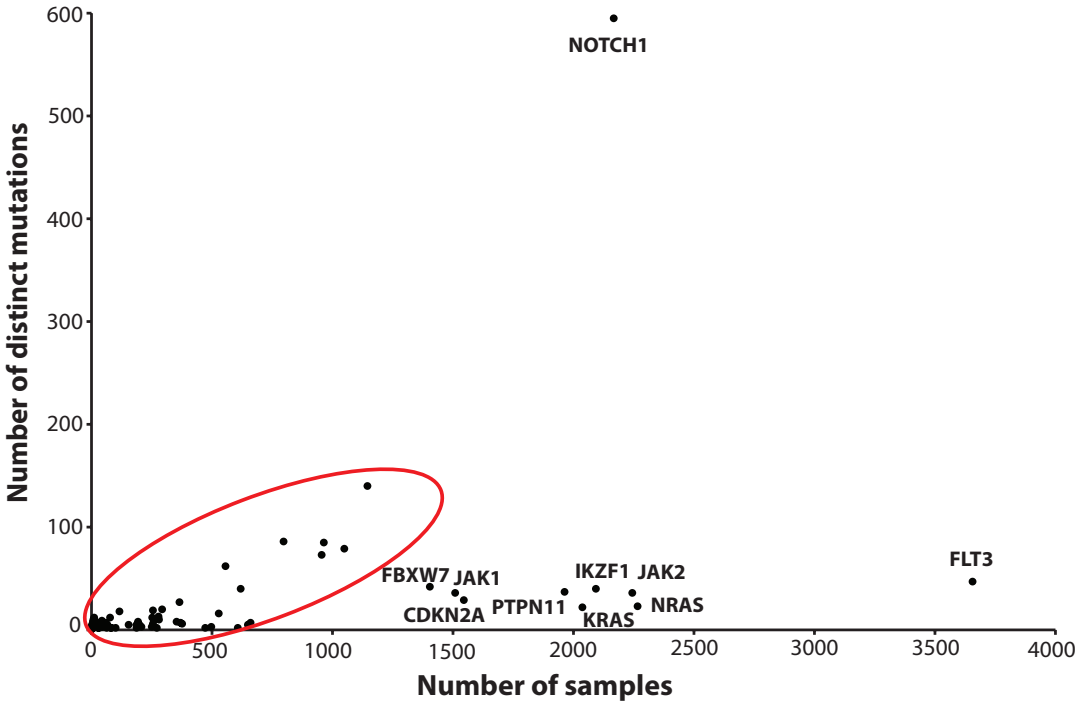


Figure 21: Occurrence of mutations per gene in ALL samples. X-axis represents the number of distinct mutations found in the coding sequences and Y-axis the number of ALL samples. Each dot represents a single gene.

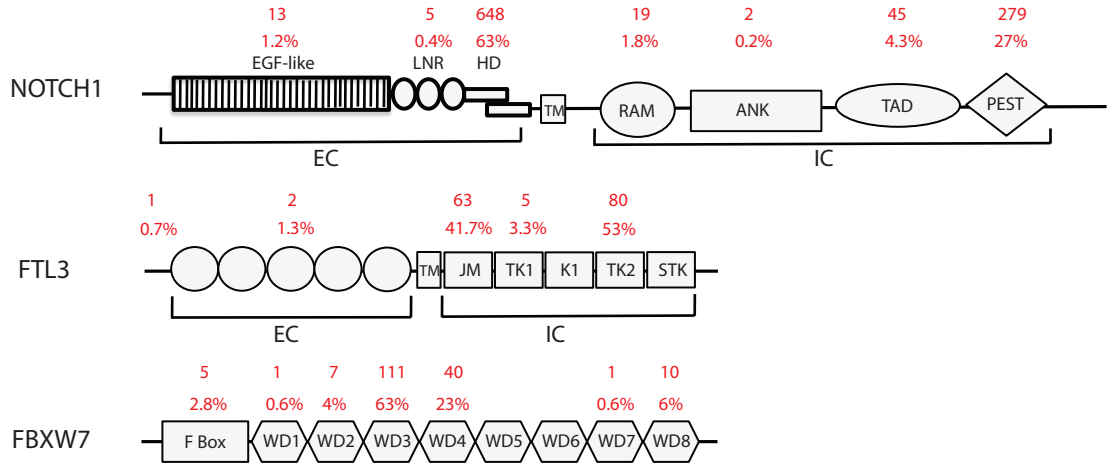


Figure 22: Distribution of mutations in ALL patients for NOTCH1, FLT3 and FBXW7. Schematic representation for each protein and its domains, the number of ALL samples with mutations localization and their percentage for each protein are represented above each domain. Notch1 domains: Epidermal growth factor repeats (EGF repeats), Lin12 and Notch repeats (LNR), heterodimerization domain (HD), transmembrane domain (TD), RBP-Jκ-associated module (RAM), ankyrin repeats (ANK), transactivation domain (TAD), proline, glutamic acid, serine, threonine-rich domain (PEST). FLT3 domains: extracellular domain (EC), transmembrane domain (TM), juxtamembrane (JM), amino-terminal and carboxy-terminal kinase domains (TK1 and TK2 respectively), kinase domain (K1), (STK). FBXW7 domains: tryptophan-aspartic acid 40 repeat (WD).

2. Interconnections between ALL-gene products

The phenotypic impact of a genetic alteration is not entirely determined by the known function of the mutated gene, but also by the functions of components with which the gene and its products interact and their interaction partners. In addition, the association of biological networks is not random but rather follows basic organizing principles in their structures. Recent network analyses show that proteins involved in the same disease have an increased tendency to interact with each other. Moreover, mutations in interacting proteins often lead to similar disease phenotype [236]. To analyze the connectivity between ALL-gene products, we collected protein-protein interactions (PPIs) data from three databases: BioGRID [5], HPRD [237] and IntAct [7] and filtered all reported interactions between the 116 ALL-gene products. Figure 23 shows that 63 out 116 ALL-gene products are interconnected. We then prioritized ALL-gene products based on their degree of interconnectivity (Supplemental table S2B). One of the top interconnected ALL-proteins is beta-catenin (encoded by

CTNNB1 gene), which is a central hub in the Wnt/β-catenin signalling pathway and plays a crucial role normal haematopoiesis [238]. It has been shown that 50 - 85% of the childhood T-ALL patients overexpress β-catenin [239], further supporting our finding that β-catenin is an important hub in ALL. Other Wnt/β-catenin signaling pathway members such as APC, TCF3 and TCF7L2 interacting with β-catenin, are part of our ALL-gene product set and were previously found differentially expressed in T-ALL patients [239].

Another example is PIK3R1 with 8 partners including PIK3CA that interacts with additional 4 ALL-gene products. PI3K members are essential effectors in the PI3K/AKT/mTOR signalling pathway, which is activated in a number of ALL samples [240]. Another example is ABL1 that interacts with 7 partners. The BCR-ABL1 fusion is the driver chromosomal rearrangement in chronic myeloid leukemia (CML) [241] and is also found in more than 20% of ALL patients [242]. Mutations in *ABL1* gene were associated to different types of cancer [243].

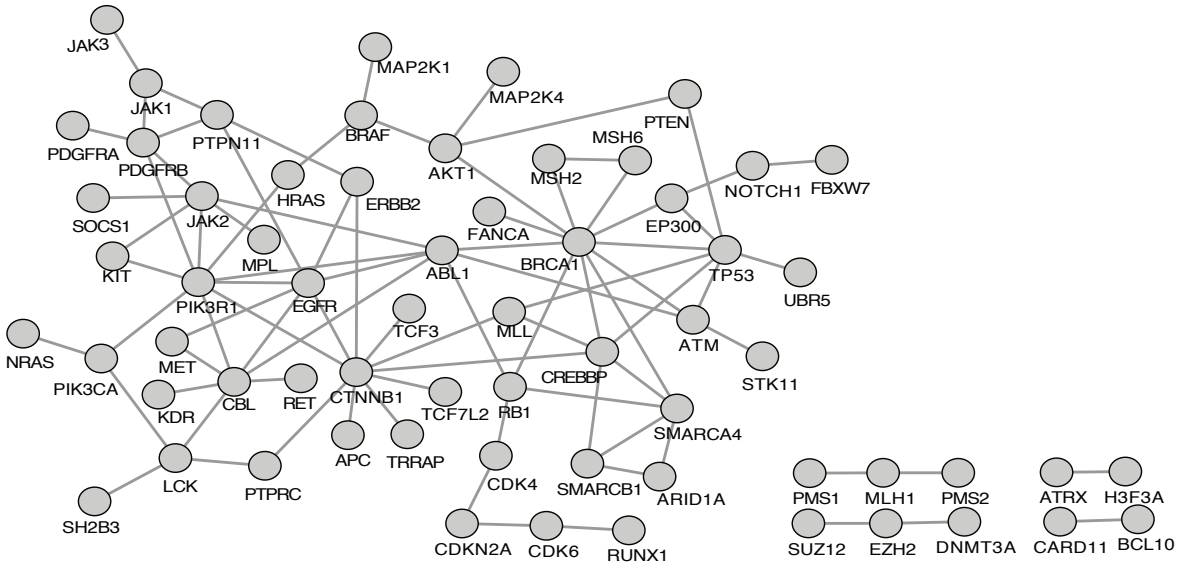


Figure 23: Interactions between proteins mutated in ALL samples. Protein-protein interactions were extracted from three databases: IntAct, HPRD and BioGRID and only interactions reported with at least two publications, and detected by two experimental methods are represented in this map.

3. Co-occurrence of mutations in ALL-genes

We then explored the relationship between interacting genes based on the occurrence of mutations in the same ALL samples. We showed that, in addition to biophysical interactions, several ALL-gene products are mutated in the same patient samples,

suggesting several ways of deregulating cancer pathways (Figure 24). As shown on figure 24, our analysis revealed that ALL samples could be classified into 4 distinct clusters of affected pathways, based on co-occurrence of mutations in important cancer driver genes: PI3K/AKT and NOTCH pathways, JAK and RAS pathways, Wnt/β-catenin and the cell cycle, and the transcriptional regulation pathways. Interestingly, protein phosphatases PTEN and PTPN11, and proteins important in genome maintenance and chromatin modification P53 and CREB binding protein are centrals and connect with deregulated pathways through different set of mutations (Supplemental table S3).

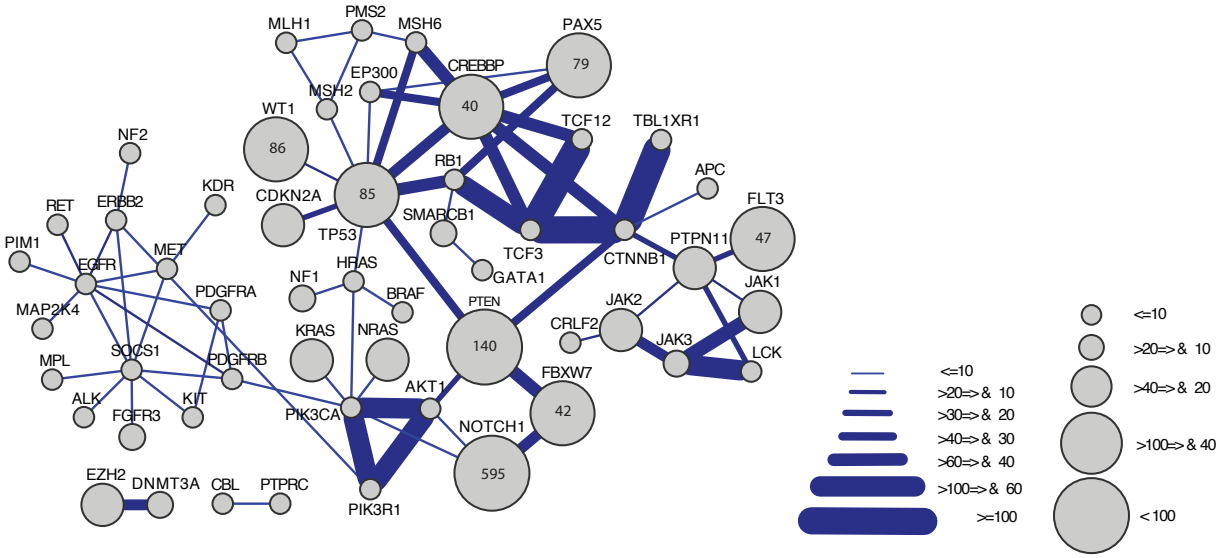


Figure 24: Co-occurrences of mutations in ALL samples. Nodes represent proteins associated with ALL, with an area proportional to the number of distinct mutations. Edges join pairs of interacting proteins for which mutations co-occur in the same samples. Edge widths are proportional to the number of samples with co-occurring mutations.

Combining all the above criteria: frequency of mutations in individual cancer genes in ALL samples, number of distinct mutations and their pattern in ALL-gene (Figures 18, 19, and 20), interconnections between ALL-gene products and co-occurrence of mutated genes in the same samples (Figures 23 and 24), we prioritized ALL-genes and suggest that *TP53*, *NOTCH1*, *CREBBP*, *PTEN*, *EGFR*, *JAK2*, *ABL1*, *PTPN11, CBL* and *EP300* are the top 10 ALL driver genes (Supplemental table S4).

4. Functional associations between ALL-gene products and their partners in the human proteome

We hypothesized that the ALL-genes set is not limited to mutated genes in ALL samples, but could be extended to functional related genes and their products. In order to identify ALL-gene products interactors, we filtered from 3 different PPI databases (BioGRID, HPRD and IntAct), proteins that interact with at least 2 of the 116 ALL-gene products (Supplemental table S5A). The obtained interactome map (Figure 25) shows that interconnected ALL-gene products have also several common partners, prioritized according to the number of interacting ALL-gene products (Supplemental table S5B). PPI stored in databases are curated from the literature and some proteins such as P53, BRCA1 or ATM heavily studied with hundreds of publications, have more PPI reported than others that are not studied with equal intensity. Previous studies suggested that unbiased PPI mapping allow characterization of overlooked PPI and identification of unknown diseases-related candidates [244].

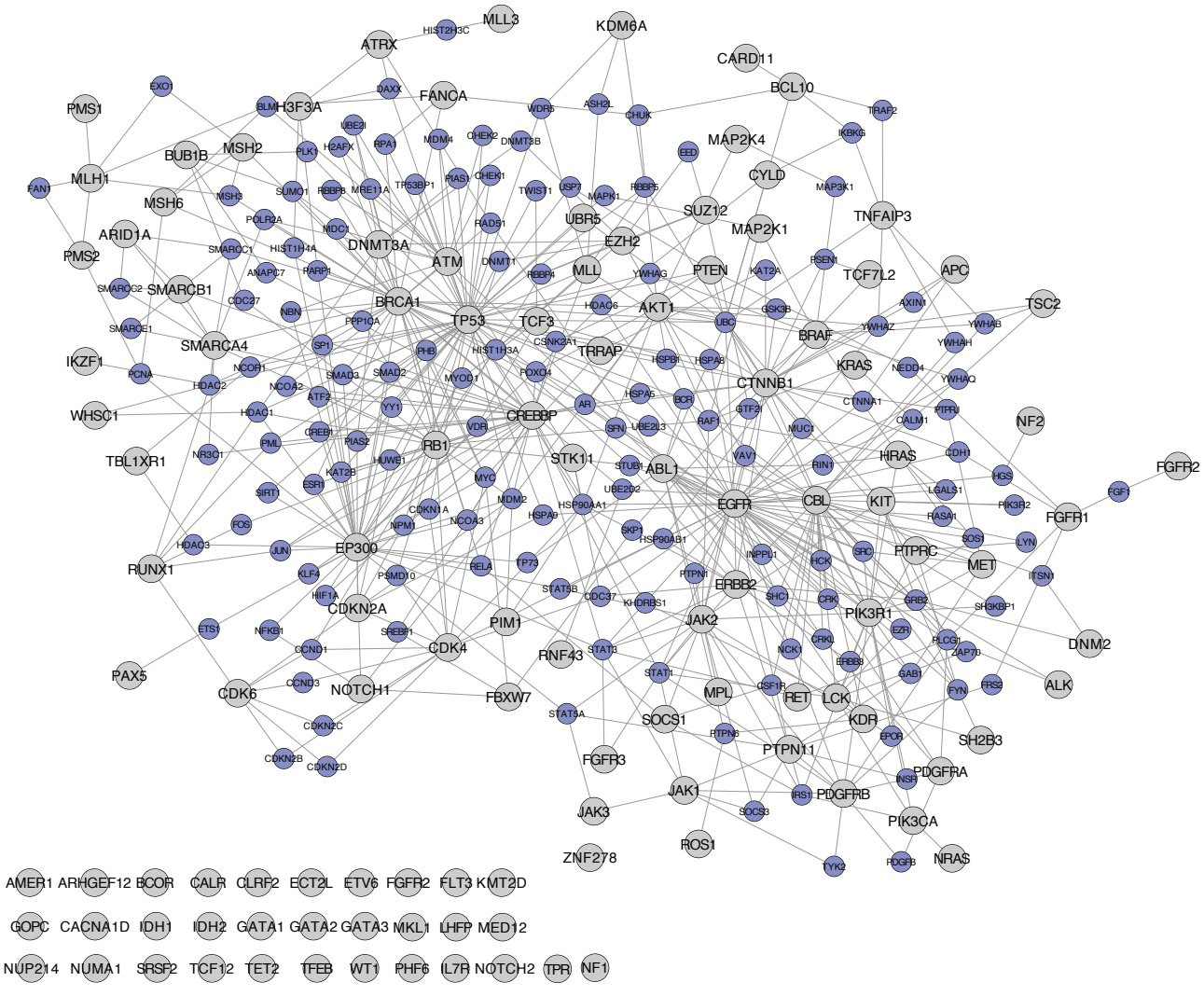


Figure 25: Literature curation of interactions between the 116 proteins mutated in ALL and their human partners. Grey nodes represent proteins mutated in ALL and the purple nodes human interactors.

We then performed a yeast-hybrid (Y2H) unbiased PPI detection assay using a set of ALL-genes and the human ORFeome collection. Out of the 116 ALL census genes, 21 were present in the human ORFeome 5.1 collection. The hORFeome 5.1 is a collection of 15483 open reading frames (ORFs) representing 12794 genes accounting for ≈ 50% of the human genome. hORFs are cloned into the pAD-dest-CYH and pDB-dest encoding the Gal4 Activating and DNA-binding domains, respectively.

We identified 193 interactions between 13 ALL gene products and 168 human partners. This experiment confirmed our observations using literature curated

interactions, that interconnected ALL-gene products are also connected through several common partners in complex macromolecules (Figure 26). We identified several novel central hubs such as GOLGA2 that interacts with ALL-gene products NOTCH1, SMARCB1, PTPN11 and WT1, and CDC33, which is a common interactor of ALL-gene products MLH1, QT1, and SMARCB1 (Figure 26, Supplemental table S4B).

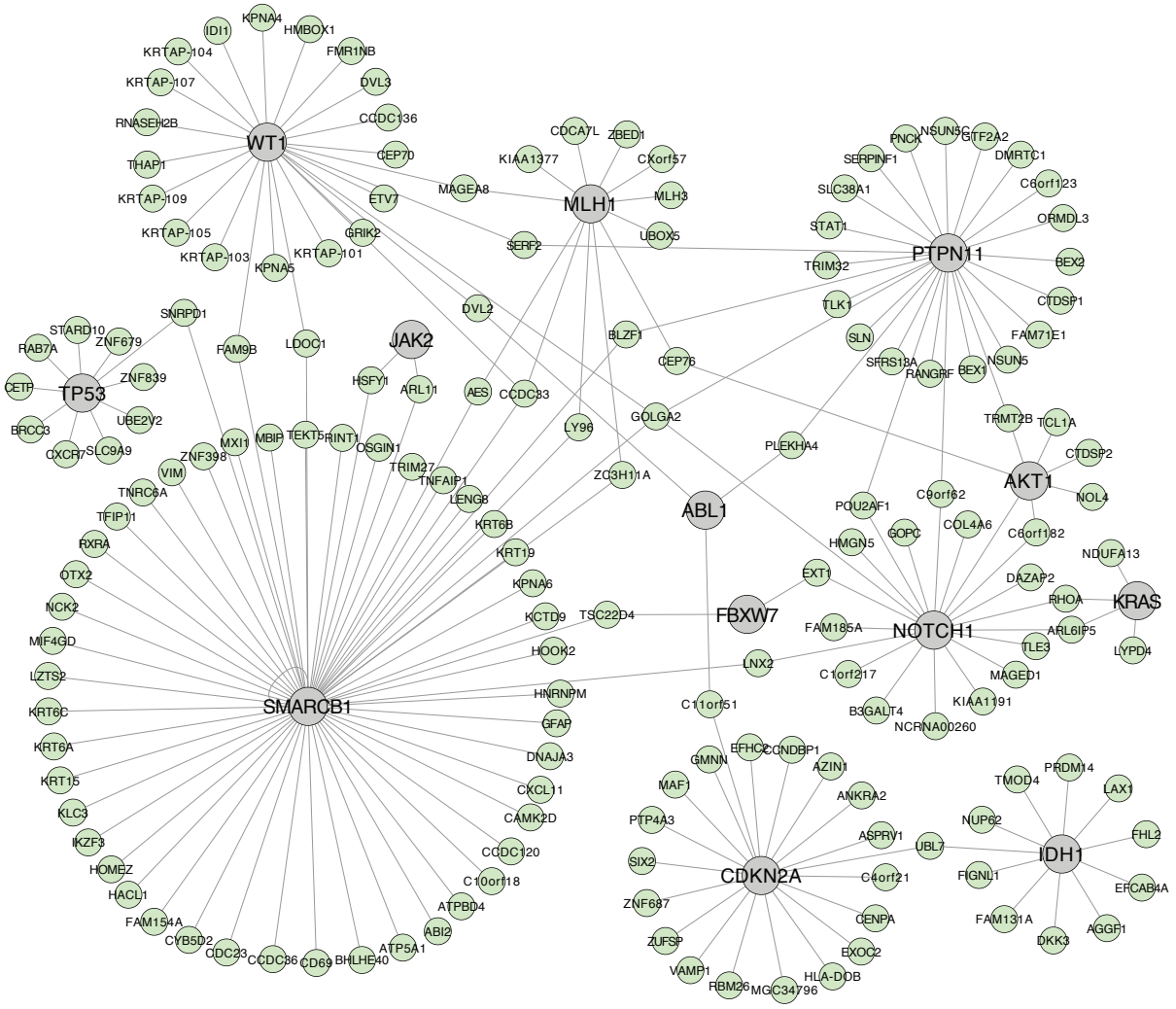


Figure 26: Interactions identified by high throughput Y2H screen. Cancer census gene encoded proteins are represented in grey and their partners in green.

As suggested by other studies, interconnected proteins are more associated with common diseases than expected by chance [245] and the same cancer driver genes are often involved in different cancer types, as evidenced by several examples [233]. To identify novel ALL-gene candidates through a "guilt-by-association" prediction, we prioritized ALL-gene products interactors using three criteria: (1) the number of ALL-

gene products partners, (2) their implications in other types of cancer and (3) their expression in 24 common ALL cell lines. In total, we identified 37 ALL-gene products interactors that could be considered as ALL-associated candidates (Figure 27 a and b).

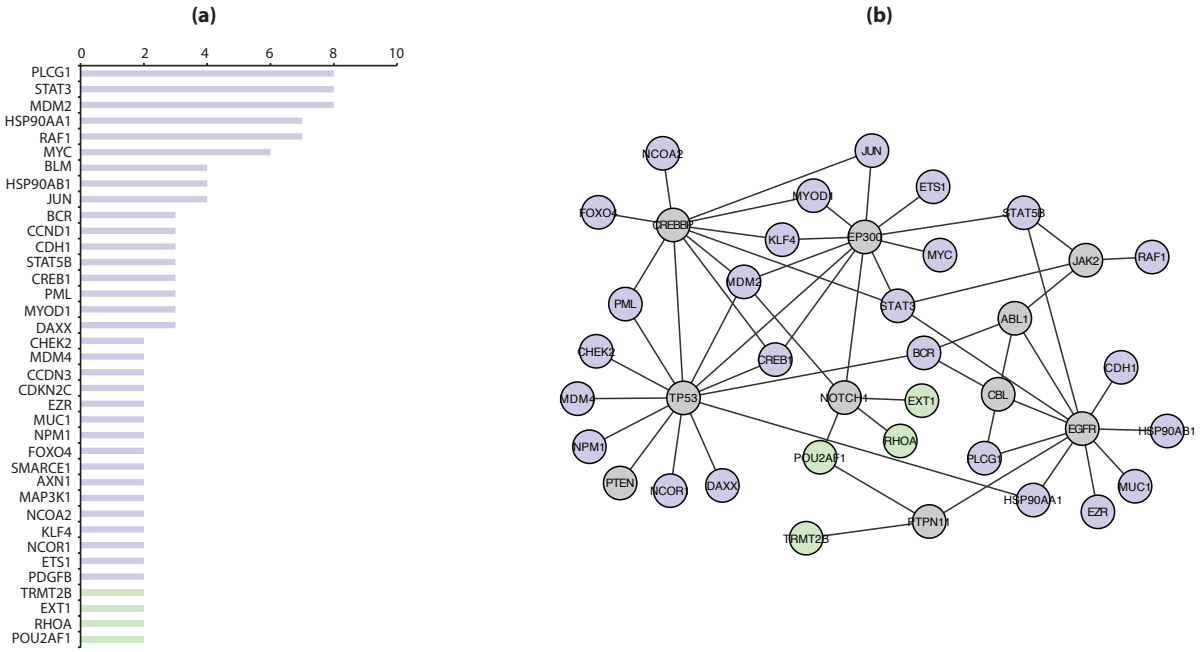


Figure 27: Prioritization of ALL-associated candidates. (a) The graph represents a ranking 37 candidates among the ALL-related partners based on the number of interactions between human proteins and ALL related proteins. In addition these genes are among the cancer gene census and they are expressed in 60% of ALL-cell lines (purple bars represent literature-curated interactors and green bars represent Y2H interactors). The X-axis represents gene symbols; the Y-axis represents the number of partners. (b) Interactions between the 37 identified ALL-candidate genes and their partners among the ALL gene census.

DISCUSSION PART I

A major and powerful approach to elucidating the molecular mechanisms underlying the complex diseases such as cancer is represented by the investigation of interactions between proteins encoded by disease genes in the human protein-protein interaction network [246]. Thus, a systematic examination of the proteins encoded by ALL cancer genes in the human interactome may help us identify new candidate genes, improve candidate gene prioritization methods, and have a deeper understanding of the genetic landscape of ALL.

Deregulated signaling is considered a major contributing factor in leukomogenesis of T-ALL, it is thus important to analyze interaction hubs illustrated by different examples in our network. Proteins interact with each other and with other cellular components forming complex networks. These networks share some features and they are part of common pathways. Diseases are not independent from each other, potential cellular network-based dependencies between human diseases has led to the generation of various global disease network maps, which link disease phenotypes together if some molecular or phenotypic relationships exist between them [247]. An interesting example elucidating "disease interdependency" is given by study that used high throughput datasets such as mRNA expression and PPI networks. They revealed significant pathological similarities between 54 human diseases, in addition to common pathways and processes implicating many proteins that are mutated and known to be therapeutic targets [248]. Studying the interconnectivity between ALL gene products enabled the identification of several ALL-gene candidates. In our map we showed that 54% of the ALL related proteins are interconnected forming one complex network. Some of these genes are highly connected; we take as an example BRCA1 gene interacting with 12 proteins in the interactome of ALL proteins. Among its partners; the family of DNA repair mismatch proteins: MSH2, MSH6 and MLH1, the transcription factor TP53 and ATM (ataxia telangiectasia mutated serine-protein kinase), that are involved in the DNA-damage repair pathway along with BCRA1 and BCRA2 proteins [249, 250]. The interactions linking these proteins to BCRA1 indicate that, in addition to PPIs, this protein hub also represents a common signalling pathway in which these proteins interact functionally and physically. BRCA1 and BRCA2 are the

most well-known genes linked to breast cancer risk [251]. In addition to being implicated in breast cancer, it was demonstrated that defects in BRCA pathway increase the risks of a subset for lymphomas and leukemias that are probably associated with gene rearrangements among them ALL [252]. Their connections to Fanconi anemia proteins (FANC), further implicate BRCA1 and BRCA2 deficits in hematological cancers [253]. Faconi anemia is a genetically and phenotypically heterogenous autosomal disorder defined by congenital malformations, progression marrow failure, and marked predisposition to malignancy [254].

As previously mentioned, the CTNNB1 (the β-catenin gene) interaction hub also drew our attention, this protein in linked to 9 partners among the ALL related proteins. The β-catenin protein encoded by CTNNB1 is essential in the WNT-signalling pathway; it binds to several proteins in the cytoplasm (such as APC) triggering its translocation to the nucleus. It interacts with other nuclear proteins (TCF, p300) leading to the transcriptional activation of target genes regulating different cellular processes such as adhesion, proliferation and development [255].CTNNB1 mutations and deregulation are linked to many epithelial-originating malignancies such as breast, ovarian, endometrial, colorectal and skin cancers [256]. We observe how CTNNB1 gene product is a central protein linked to proteins that are implicated in WNT signalling pathway (Figure 23). It is known that activation of the Wnt/β-catenin signalling pathway through loss-of-function mutations in the APC protein and axin, or gain-of-function mutations in β-catenin are linked to various hematological malignancies including ALL. In fact β-Catenin plays a crucial role as an effector of the Wnt/β-catenin pathway in normal haematopoiesis [238]. In the absence of WNT signals, a complex of proteins, including APC, axin and GSK3β, controls phosphorylation and subsequent proteasomal degradation of β-catenin. When WNT signalling is activated, β–catenin accumulates in the nucleus where it interacts with T-cell factor transcription factors (TCF) leading to the transcriptional activation of target genes. Mutations in β-catenin or APC can lead to the formation of constitutively active β-catenin/TCF complexes and altered expression of target genes, that are associated to cancer [257]. More than 85% of the childhood T-ALL patients showed upregulated β-catenin expression at the protein level compared with normal human thymocytes. A recent study using microarray analysis carried out on T-ALL patients showed that 50% of T-ALL patients represent a two-fold change in β-catenin expression. In addition, other WNT signalling member

such as TCF3 and TCF7L2, partners of CTNNB1 shown in the interactome map, were differentially expressed in T-ALL patients, and in some cases associated to β-catenin upregulation [239].

Another example of protein hubs in our map is PIK3R1 with 8 partners among them we find PIK3CA that interacts with 4 proteins. PI3K proteins are essential effectors in the PI3K/AKT/mTOR signalling pathway. This signalling cascade is activated by receptor tyrosine kinases, integrins, B and T-cell receptors and other stimuli that induce the production of phosphatidylinositol triphosphate (PIP3) by phosphoinositide 3-kinase (PI3K). This step induces the activation of AKT due to phosphorylation by the PDK1 and mTORC2 [258, 259]. AKT is a proto-oncoprotein with many substrates and effects such as apoptosis and protein synthesis. The tumor suppressor phosphate and tensin homolog PTEN, inhibits AKT activity by dephosphorylating PIP3, while inactivating mutations or deletion of PTEN leading to Akt activation occur in ALL [240].

ABL1 is connected to 7 partners, this gene constitutes the subject of a large number of studies, and mutations in this gene were associated to different types of cancer. An important gene fusion that implicates ABL1 and BCR genes has been described in leukemias (chronic myeloid leukemia; CML), the fusion protein resulting is essential and sufficient for the malignant transformation of CML [241]. In addition, BCR-ABL1 fusion is also found in other cancers such as breast and prostate cancers.

These examples of protein hubs prove that interactions between a set of proteins or interactions linked to a central protein are not randomly organized. Moreover in the context of mutations and genes associated to ALL, we show that proteins with mutations in ALL are highly interconnected to each other, they are also implicated in common signalling pathways where deregulation in one or several components show high incidence in ALL development. Mutations in one or more interconnected proteins may lead to disruption of these complexes and development of ALL, and drug resistance mechanisms. In addition, these proteins were linked to other cancers such as breast cancer and other types of leukemias. Other studies have highlighted the importance of evaluating gene mutations from a global point of view; and went deeper to assess the interconnectivity between diseases. Goh *et al.* showed that genes associated with similar disorders show both higher likelihood of physical interactions between their products and higher expression profiling similarity for their

transcripts, supporting the existence of distinct disease-specific functional modules. They established the human disorder network, showing that cancer in among the most connected disorders. This is due in part to the fact that cancer subtypes are tightly connected with each other through tumor suppressors such as *TP53* and *PTEN.* Another interesting finding was that leukemia, in contrast to most disorders, relate to a large number of disease genes [260]. This observation was also confirmed in our interactome. Disease annotation analysis using DAVID tool revealed for the ALL-related genes that they are implicated in common cancers such as colorectal cancer (*EP300*, *APC*, *TP53*, *CTNNB1, NRAS*, *FGFR3*, *AKT1*, *BRAF* and *PIK3CA)*, ovarian and breast cancer (*BRCA1*, *CTNNB1*, *MSH2, KRAS*, *AKT1* and *PIK3CA*), acute myeloid leukemia (*FLT3*, *ARHGEF12*, *NUP214*, *RUNX1*, and *KIT).*

Co-occurrence of mutations

Exploring the relationship between the ALL-gene candidates based on the co-occurrence of mutations in the same ALL samples, provided us with valuable data for potential ALL-gene candidates.

The network in figure 24 shows that in addition to the physical interactions these proteins are also found to be mutated in the same ALL samples. PIK3CA, PIK3R1 and AKT1, proteins of the PIK3-AKT pathway, are interconnected and are co-mutated in a high number of ALL samples. Mutations at the PI3K/AKT/PTEN pathway can contribute to leukemogenesis by elevating the expression of the pleiotropic anti-apoptotic AKT protein, which can stimulate antiapoptotic proteins and inhibit pro-apoptotic proteins. Inactivating PTEN mutations are known to occur in ALL and to disturb signalling of AKT/PI3K pathway. Co-occurrence of mutations in the same ALL samples can have an important significance when it comes to the clinical outcome of ALL patients. Inactivation of PTEN can be associated with poor treatment response in ALL. A study was carried out on 300 ALL patients, analyzing the incidence of PTEN point mutations, they show that inactivating PTEN mutations associated with activating NOTCH1 mutations in patients have better response to therapy [261]. Importantly the majority of *PTEN* identified mutations localize in exon 7 “hotspot” in the C2 domain of the protein, accounting for 82% of ALL samples with PTEN mutations. These mutations lead to the truncation of the C2 domain responsible of

binding to the membrane for subsequent dephosphorylation of the membrane-bound PIP3 [262]. Moreover, in T-ALL, activation of AKT has been described downstream of NOTCH1 and AKT may be activated upon transcriptional repression of PTEN by the NOTCH1-activated transcriptional repressor HES1 [55]. Notch1 the top mutated gene in ALL (Figure 20 b) interacts with both AKT1 and FBXW7 and several ALL samples exhibit mutations in genes encoding those proteins. For example, *NOTCH1* and *FBXW7* mutations co-occur in ≈ 40 samples, most of these mutations result in a higher expression and activation of NOCTH1, which is highly linked to T-cell ALL cancer development. PTEN, PI3K and AKT abnormalities showed high incidence in T cell acute lymphoblastic leukemia [62]. In fact the PTEN/PI3K/AKT pathway constitutes an important pathway that regulates the signalling of multiple processes such as apoptosis, cell proliferation and growth. Deregulation of this pathway is implicated in a number of human diseases, where activating mutations in one of these proteins were reported [263]. This subnetwork also includes NOTCH1 and FBXW7. Mutations in the HD/PEST domains of NOTCH1 and FBXW7, which leads to an over-activation of the Notch pathway, frequently occur in T-ALL patients [58]. In addition PTEN mutations also occurred with FBXW7 mutations in the same T-ALL samples. PTEN was described as an indirect NOTCH1 target (through HES1 and cMYC), resulting in constitutively active PI3K- AKT signalling [57]. In figure 24 we show that FBXW7 mutations co-occur with NOTCH1 mutations and with PTEN mutations in 30 and 37 ALL samples respectively. In fact, several studies have reported better outcome in T-ALL harboring NOTCH1 and/or FBXW7 mutations, conversely when PTEN mutations co-occurred in these patients, it associates with poor prognostic [264].

The same observation is valid for JAK proteins, a family of large tyrosine kinases (JAK1, JAK2 and JAK3). This family of genes along with STAT family (signal transducers and activators of transcription) and the CIS/SOCS family constitute the effector genes of the JAK/STAT pathway. JAKs mediate signals from a variety of cytokines and growth factors. In general, receptor dimerization or oligomerization due to ligand binding results in the juxtapositioning of the JAKs, either through homodimeric or heterodimeric interactions. The recruitment of JAKs results in their phosphorylation, either via autophosphorylation and/or transphosphorylation by other JAKs or other families of tyrosine kinases. This activation results in increased

JAK kinase activity. Activated JAKs then phosphorylate receptors on target tyrosine residues that serve as docking sites allowing the binding of STATs and their subsequent phosphorylation. Activated STATs dimerize and translocate to the nucleus leading to the regulation of target gene promoters, including the bcl-2 family genes, MYC, CyclinD…[265]. Since activating mutations in JAK2 have been linked to leukemogenesis, several studies have been focusing on the development of small molecule JAK2 inhibitiors, such as AG-490 a specific tyrosine kinase blocker. Inhibition of Jak2 with AG-490, selectively blocks leukemic cell growth *in vitro* and *in vivo* by inducing programmed cell death, with no deleterious effect on normal haematopoiesis [265, 266]. Sequencing analyses and gene expression profiles for ALL patients revealed high frequency of recurrent somatic alterations in Janus kinase signalling and also other key signalling pathways including the TP53/RB tumor suppressor pathway and Ras signalling [267].

Exploring mutations co-occurring in several different genes for the same patients enables the characterization of driver genes that are specifically mutated in ALL. The interacting proteins encoded by genes with mutations, or proteins that have common interactors that are implicated in common signalling pathways such as NOTCH1, FBXW7 and PTEN represent interesting candidates for targeted therapy development.

Functional associations between ALL-gene products and their partners in the human proteome

The identification of novel proteins linked to the proteins that are mutated in ALL, can also be associated to this disease and therefore represent novel targets or ALL candidates. Applying the "guilt-by-association" concept, with specific criteria linked to ALL such as the number of ALL-gene products partners, their implications in other types of cancer and their expression ALL cell lines, provided a valuable clue to the role of novel gene products potentially associated with ALL. In fact we find that ALL-gene products are interconnected by the mean of these proteins that were not previously linked to ALL in the human proteome (Figure 25). In figure 25, the interactions between the ALL proteins and their partners from literature are represented. The interactions were extracted from 3 different databases (BioGRID, HPRD and IntAct). Proteins that interact with at least 2 of the ALL related proteins, and interactions reported in two scientific papers and detected with two different experimental

methods are represented. This complex map shows that in addition to being directly linked to each other, the ALL related proteins are interconnected by the mean of common partners. The degree of connectivity for the common interactors is represented by the graph that shows the number of ALL proteins interacting with the identified partners from the human interactome. The proteins in grey were not previously associated with ALL, but can be considered as interesting candidates that can be studied in ALL, due to their connectivity with genes mutated in ALL. The top interconnected protein is the ubiquitin C, a polyubiquitin precursor encoded by *UBC* gene, which interacts with 13 ALL-related proteins (node in the center of CTNNB1, PTEN, AKT1 and BRAF on the upper right zone of the map in figure 25). Ubiquitin exists in the cell either covalently attached to another protein of free. Ubiquitin-conjugated proteins are targeted for degradation via the proteasome, lysosome or the endoplasmic reticulum, depending on the ubiquitinated residues. Therefore it is not surprising to find that UBC is highly connected to several ALL proteins. In fact, the ubiquitin system plays an important role in maintaining the homeostatic balance of cellular processes; therefore its deregulation can promote the development of cancer [268]. GRB2 interacts with 10 of the ALL related proteins. GRB2 is a ubiquitously expressed adaptor protein that facilitates the assembly of multiprotein signalling complexes at activated receptors and signalling proteins. GRB2 contains a single SH2 domain that allows it to bind tyrosine-phosphorylated receptors, this domain binds to the hematopoietic specific adaptor protein linker for activation of T cells (LAT) and other adaptor proteins. In addition to two SH3 domains which brings various ligands to the sites of activating signals [269]. Due to its role in propagating signalling pathways downstream of several receptors, deregulation of GRB2-mediated signalling complexes has been linked to oncogenesis. Targeting the GRB2 has been explored as an anti-cancer drug, using candidates that can associate with GRB2 and antagonize its signalling [270].GRB2 was not previously linked to ALL, but in a recent study they investigated the role of GRB2 in ETV6/FLT3 mediated leukemogenesis, they found that FLT3 mutations at specific tyrosine residues alter the GRB2 binding to FLT3/ETV6 and result in an impaired activation of STAT5, Erk1/2 and Akt in Ba/F3 cells. Their data indicate that GRB2 is a potential therapeutic target in patients with EXT6/FLT3-positive myeloid/lymphoid neoplasm with eosinophilia-patients[271]. Other examples of highly interconnected proteins are STAT1 and 5 of the JAK/STAT

pathway. MDM2 the negative regulator of p53 protein interacts with 8 ALL-related proteins, MDM2 is known to bind to p53 and inhibit its transcriptional activity, thus favoring its nuclear transport and degradation. Overexpression of MDM2 was described to favor uncontrolled cell proliferation through inhibition of p53 in various cancers. Several compounds that inhibit the p53-MDM2 pathway interaction show anti-proliferative effect in tumour cells overexpressing MDM2 [272]. Targeting MDM2 with a natural product called Triptolide, induces apoptosis in a subgroup of acute lymphoblastic leukemia cells[273]. Another example is Nilotinib the selective BCR-ABL tyrosine kinase inhibitor, widely used to treat Philadelphia-positive and Philadelphia-negative ALL, studies also demonstrated that nilotinib inhibits MDM2 by inducing its self-ubiquitination and degradation, leading to p53-independent apoptosis pathway and helping treating ALL [274]. These interconnected proteins constitute interesting example showing that even proteins that are not mutated in leukemic patients constitute potential candidates for targeted anti-cancer therapy. Thus interactomic approach and establishing the connections between proteins that are mutated in ALL and their partners in the human interactome enables the identification of key proteins that can open doors for novel targeted therapies.

Databases gathering data about interactions from scientific reports, attempt to create a complete listing of literature-curated data. It should be noted that interactomic data for genes are biased, due to the fact that some genes or proteins are more frequently studied than others. An example illustrating this theory is BRCA1 that we found in our interactome, as the most interconnected protein among the ALL-gene products. This protein was frequently studied in ovarian and breast cancer, in addition to recent findings that relate BRCA proteins to leukemias as we previously mentioned. Furthermore experimental methods used to detect interactions, largely vary among different studies. Using a high throughput PPI screening technique limits the bias resulting from the irregularity of applied methods and focusing on “well-studied” genes.

High throughput yeast two-hybrid screening

Despite these extensive curation efforts, the existing maps are considered incomplete, and the literature-based datasets, while richer in interactions, are prone to

investigative biases, containing more interactions for the more explored disease proteins [236].

High throughput Y2H screening enabled the identification of novel interactions between ALL gene products and proteins from the human interactome. The constructed network confirmed that ALL-related proteins are interconnected; they represent several common partners such as GOLGA2 interacting with NOTCH1, SMARCB1, PTPN11 and WT1, and CDC33, which interacts with MLH1, QT1, and SMARCB1. We classified the identified partners according to the degree of connectivity, which is represented by the number of ALL gene products interacting with each protein. The advantage of identifying these novel interactions is to highlight proteins that have not yet been associated with ALL, these proteins are not known to be involved in ALL and may provide new insights for research in this domain. GOLGA2 is a *cis*-Golgi matrix protein that plays a major role in the stacking of Golgi cisternae and maintenance of Golgi structure. It also participates in the glycosylation and transport of proteins and lipids in the secretory pathway [275]. Targeting GOLGA2 using specific shRNA showed to inhibit angiogenesis and induce autophagy-dependent cell death in lung cancer cells [276], but it has not been previously linked to ALL. ABI2 the abl-interactor 2 (encoded by the Abl associated gene ARG) is a non-receptor tyrosine kinase, together with c-Abl they constitute the members of the mammalian Abelson family of kinases that were first identified as oncogenes associated to human leukemias. Arg is activated by fusion with Tel transcription factor in cases of AML [277]. Among the common interactors in our Y2H map some were previously associated to cancer and have served as therapeutic targets, but not in the ALL context. It is thus important to exploit these novel partners and analyze their potential implication in ALL.

The advantage of using the high throughput approach is in the reduction of interactomic hubs constituted by proteins that are most studied. As we have previously mentioned, information represented in different literature–curation databases are biased due to the fact that repeatedly studied proteins ,are more likely to have more interactomic data than less studied proteins. Thus using a systematic high throughput interaction detection technique such as the Y2H helps minimize the observed bias. In our Y2H map we can highlight novel candidates that can be

associated to ALL, such GOLGA2 and CDC33. Another example that grabbed our attention is EXT1 that interact with 2 ALL related proteins, NOTCH1 and FBXW7 (Figure 26). EXT1 was detected in our Y2H screen as a partner for both Notch1 and FBXW7. Notch was the subject of the analysis and investigations in a huge number of publications involving its mechanism of action, regulation of the signalling pathway to its implication in diseases and its role as important and promising therapeutic target [278].

We investigated the expression of interactors in T-ALL cell lines and found that 79% of genes these are expressed in 60% of T-ALL cell lines. Applying selection criteria such as the degree of connectivity and gene expression in ALL cell lines to the interaction partners identified from both literature curated data and our Y2H interactome enabled the prioritization of 37 ALL-gene products interactors as ALL-candidates (Figure 26). Using similar approach that integrates expression profiles, mutation effects and interactome data has been recently used as an analytical tool for identifying novel driver genes in cancer [246, 279].

In summary, we extracted mutations found in ALL patients that were reported in COSMIC database, and identified 116 ALL-related genes among cancer gene census. Network analyses of protein-protein interactions show that 54% among the ALL-gene products are interconnected and represent central protein hubs such as the beta-catenin protein and PIK3R1. In addition, we found that several mutations in the ALL-genes products co-occur in the same samples, which can be classified into 4 clusters of affected pathways. ALL-related gene products are also interconnected through common partners from the human interactome. Our experimental data from Y2H screening enabled the identification of novel partners for the ALL-related proteins. Among the Y2H and literature-curated identified interactors we prioritized 37 genes as ALL-associated candidates.

The field of systems biology has evolved enormously during the recent years, due to the availability of a huge amount of genomic and proteomic data and the progress made in technologies allowing high-throughput screening and the development bioinformatics tools. Despite the great advantage of applying this approach in the first part of our analysis, represented by the identification of several novel potential cancer genes, it should be noted that interactomic and bioinformatics analyses have their

limitations. Their advantage resides in highlighting interesting candidates but these hypotheses need to be validated, in order to provide biological and molecular explanation for the role and the impact of these cancer gene products in the disease development or therapy.

Further analyses are required to clarify this point; interactions can be tested in conditions where ALL-related proteins are mutated (reported mutations in ALL samples). We expect that mutations will disrupt the network organization, leading to loss of some interactions, which can provide a more detailed explanation of the molecular basis of ALL pathogenesis and development. On another level ALL-candidate genes can be tested for their potential role in ALL, first by characterizing the links between these candidates and their ALL-related protein partners and in a second step investigating their effect in ALL using a T-ALL cell lines or ALL-specific animal models.

In the second part of our analyses we provide a proof-of-concept on how systematic interactome approaches could allow identification of novel targeted genes, by investigating EXT1-NOTCH1 novel interaction.

PART II. EXT1 is functionally associated with the Notch pathway through its interaction with NOTCH1 and FBXW7

The following example illustrates the validity of combining interactome approaches and gene mutations characterization to identify specific cancer type-related genes. Exostosin glycosyltransferase 1 (EXT1) is an endoplasmic reticulum transmembrane protein frequently mutated in multiple osteochondromas [280-282]. We identified EXT1 as a common interactor of two ALL-gene products NOTCH1 and FBXW7 (Figure 26 and 26a). We then investigated the potential functional interplay between EXT1 and the NOTCH pathway.

The exostin (EXT) family of genes encodes glycosyltransferases involved in heparan sulfate (HS) biosynthesis. Five human members of this family have been cloned to date: EXT1, EXT2, EXTL1 (EXT-like 1), EXTL2, and EXTL3. EXT1 and EXT2 form a golgi-located complex that catalyzes the chain elongation step in heparan sulfate biosynthesis. HS are sulfated glycosaminoglycans (GAGs) distributed on the cell surfaces and in the extracellular matrices of most tissues. EXT1 and EXT2 localize predominantly to the endoplasmic reticulum (ER). Together they form a complex in the golgi apparatus. EXT1/EXT2 complex is responsible for chain elongation, and the levels of the individual proteins affect the polymerization process. The level of EXTL3 also affects chain elongation, but the changes indicate that or EXTL3 must be an initiator of HS chains [283]. HS proteoglycans have been implicated in diverse number of biological processes linked to intracellular signalling, cell-cell interactions, and tissue morphogenesis [284]. Mutations in EXT1 and/or EXT2 were essentially linked to hereditary multiple exostoses (HME). Hereditary multiple exostoses (HME) is an autosomal dominant hereditary disorder with a prevalence of 1/100 000 characterized by the formation of cartilage-capped tumors, known as osteochondromas or exostoses, which develop primarily on the long bones of affected individuals from early childhood until puberty [285]. Mutations in either EXT1 or EXT2 and the resulting reduction or absence of HS in the exostosis cartilage cap has been implicated in disturbed signalling response in exostosis chondrocytes [286, 287]. Moreover, individuals with HME have a significantly higher risk than the general

population of developing malignancies such as chondrosarcomas [288, 289]. Mutational analysis of HME patients has shown that inactivating mutations in the EXT1 and EXT2 genes are responsible for the majority of familial cases: 60–70% of the cases have mutations in EXT1. The role of EXT1 as a classical tumor-suppressor gene was strongly supported by two sets of observations: the formation of cartilage-capped tumors and a higher risk of cartilaginous and bone malignancies in HME, and the presence of genomic and chromosomal abnormalities at the EXT1 locus in sporadic neoplasms [290]. An interesting study showed that epigenetic loss of EXT1 disrupts HS synthesis in cancer cells. They have demonstrated that the main malignancy type undergoing EXT1 CpG island hypermethylation is leukemia. Two mechanisms of disruption of HS levels in cancer cells are shown: the blocking of HS production by hypermethylation of EXT1 as occurs in APL and ALL, and the increase in the degradation of HS by overexpression of heparanase, as occurs in the vast majority of solid tumors, probably including chondrosarcomas and osteosarcomas [291].

1. Validation of NICD interactions

Our yeast two-hybrid screen performed for Notch intracellular domain with the human ORFeome version 5.1 enabled us to determine 19 proteins interacting with NICD. We wanted to validate the identified interactions, therefore we selected a set of literature-curated interactions for NOTCH1 (32 interactors), in addition to interactions identified by our Y2H screen for NICD (19 interactors) and we tested them using the protein complementation assay (PCA) in mammalian cells. The principle of this technique is similar to the Y2H method. In the PCA two potential interacting proteins (bait and prey) are fused to parts of the *Gaussia princeps* luciferase, and overexpressed in HEK 293 cells, if an interaction occurs between bait and prey, the luciferase is then reconstituted and luciferase activity is measured. Luciferase normalized ratio is calculated for each interaction, and according to Cassonet et al. for a calculated ratio higher than 3,5 an interaction is considered true and validated [25]. For the 51 tested interactions with NICD, we were able to validate 72% of our Y2H interactions and 75% of literature (Table 3).

Table 3: Normalized luciferase value calculated for Notch1 interactors tested in PCA.

Iteractor	NLR	Source
DTX2	2457	LCI
AKT1	551	Y2H
B3GALT4	400	Y2H
GOLGA2	714	Y2H
RHOA	224	Y2H
POFUT1	134	LCI
FBXW7	62	LCI
SMAD3	42	LCI
XRCC6	40	LCI
RELA	32	LCI
NUMB	26	LCI
MEF2C	20	LCI
SNW1	17	LCI
YY1	15	LCI
PIK3CG	14	LCI
EXT1	13	Y2H
LCK	13	LCI
COL4A6	13	Y2H
HMGN5	10	Y2H
NUMBL	6	LCI
ADAM17	6	LCI
MAGED1	6	Y2H
XRCC6	5.6	LCI
ARL6IP5	4.7	Y2H
JAG1	4.6	LCI
RBP3	4.1	LCI
GOPC	3.9	Y2H
PSEN1	3.9	LCI
LFNG	3.7	LCI
NOV	3.6	LCI
ADCK5	3.6	LCI
GSK3B	2.8	LCI
DAZAP2	2.7	Y2H
RBPJ	2.6	LCI
LNX2	2.3	Y2H
POU2AF1	2.1	Y2H
ESM1	1.7	LCI
CNTN1	1.68	LCI

DLL4	1.6	LCI
PSEN2	1.46	LCI
REL	1.4	LCI
KIAA1191	1.19	Y2H
TLE3	1.3	Y2H
PIK3CA	0.8	LCI
LEF1	0.6	LCI
FAM185A	0.5	Y2H
UBC	0.4	LCI
NFKB1	0.4	LCI
NCRNA00260	0.3	Y2H
C6orf182	0.3	Y2H
BLOC1S1	0.2	LCI

2. EXT1 affects NOTCH1 transactivation and protein level

EXT1-NICD interaction was validated by the PCA method, to determine whether EXT1 is involved in regulating the transcriptional activation of Notch1 target genes, we performed Notch-responsive luciferase reporter assays. Depletion of EXT1 using small interfering RNA in HeLa Notch1ΔE-eGFP cell line stably expressing an eGFP-tagged, transcriptionally inactive and ligand-independent Notch1 construct increased the luciferase activity for Notch transactivation with a 2-time fold compared to control condition (Figure 28 b). A similar effect was observed in HEK293 cell, after EXT1 depletion leading to a 1.5-time fold increase in Notch1 luciferase transactivation (Figure 28 c).

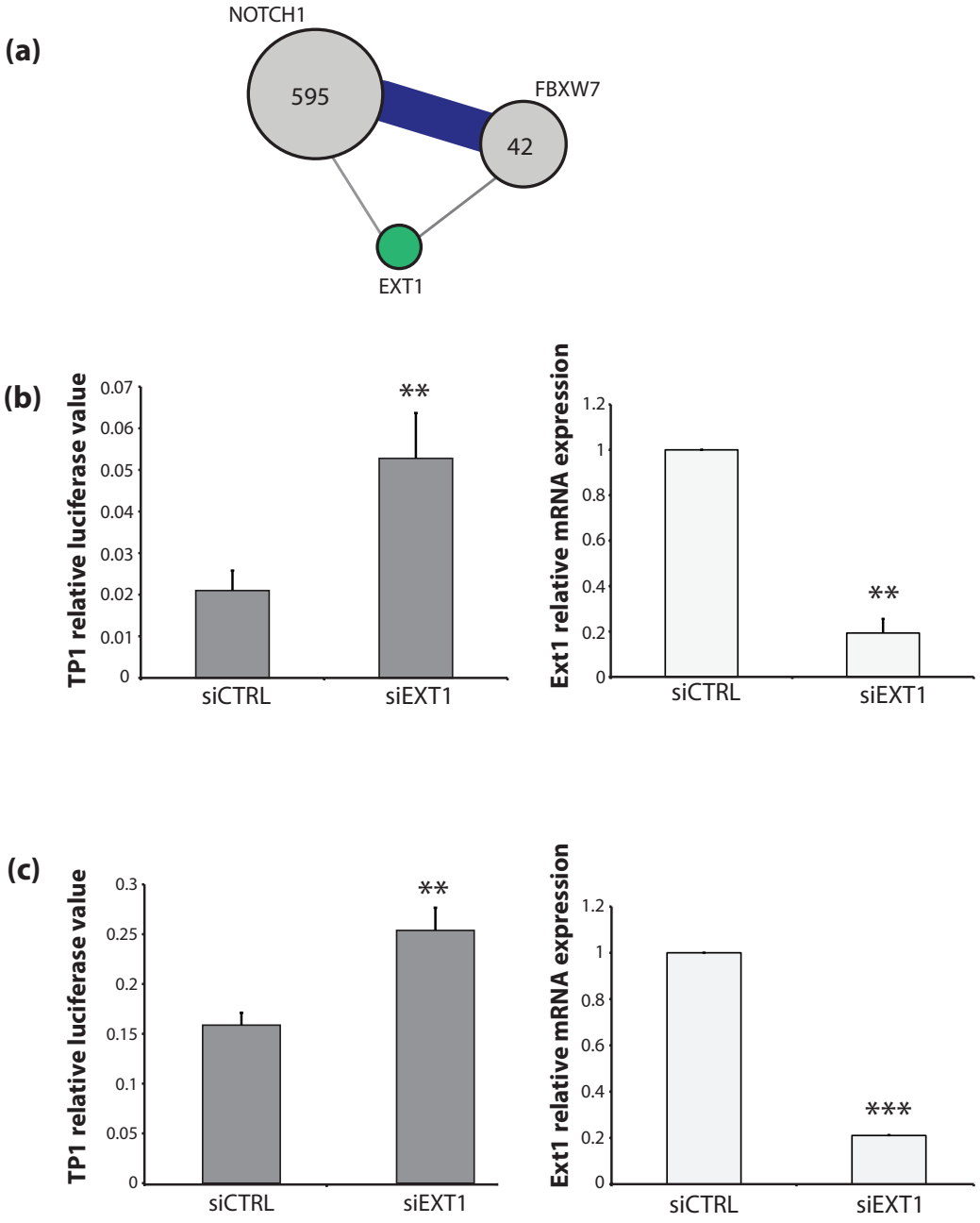


Figure 28: EXT1 depletion promotes NOTCH1 transcriptional activity. (a) Interactions between NOTCH1, EXT1 and FBXW7. Grey nodes represent proteins associated with ALL, with an area proportional to the number of distinct mutations. Bleu edges join pairs of interacting proteins for which mutations co-occur in the same samples. Edge widths are proportional to the number of samples with co-occurring mutations. The green node represent EXT1 and grey edges interactions identified in Y2H (b) and (c) Luciferase reporter assay using TP1-luciferase construct in HeLa NotchΔE-eGFP cell lines and HEK293 cells respectively. Cells were transfected with EXT1 siRNA or control siRNA as indicated. The relative luciferase values are normalized using a *Renilla* luciferase construct. Knock-down of EXT1 was analyzed by qPCR. Data represent the means ±SEs of three independent experiments. **: p-value < 0.01, ***: p-value < 0.001.

Consistent with previous finding, Notch1 induced TP1 luciferase reporter activity was inhibited by EXT1 transfection in HEK293 and HeLa Notch1ΔE-eGFP cell lines by 50% and 30% respectively (Figure 29).

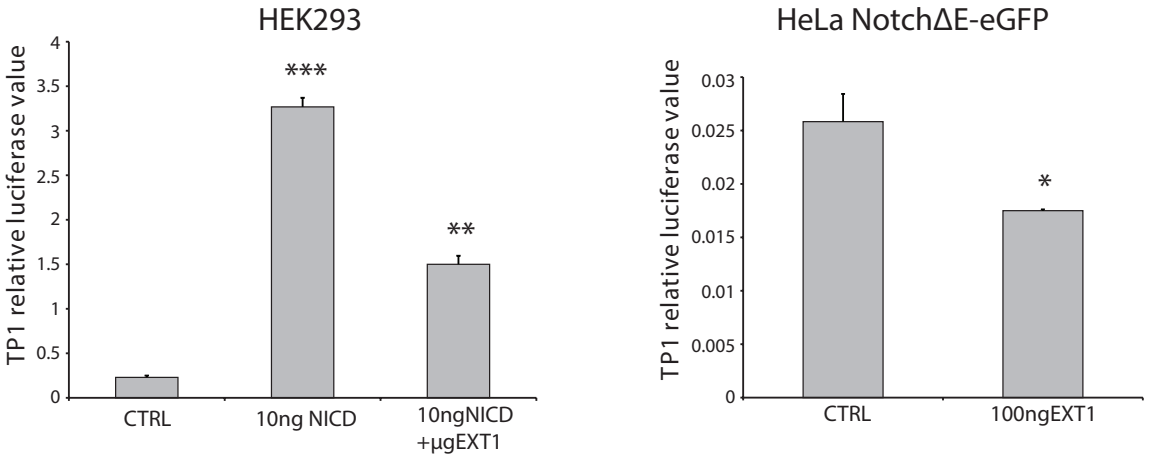


Figure 29: EXT1 inhibits Notch-1 transcriptional activation. Luciferase reporter assay using TP1-luciferase construct in HEK293 and HeLa NotchΔE-eGFP cell lines transfected with PSG5C control plasmid, NICD or EXT1 constructs as indicated. The relative luciferase values are normalized using a *Renilla* luciferase construct. Data represent the means ±SEs of three independent experiments. *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001.

Moreover, EXT1 depletion increases mRNA levels of expression of Notch1 target genes: HES1 and cMYC (Figure 30).

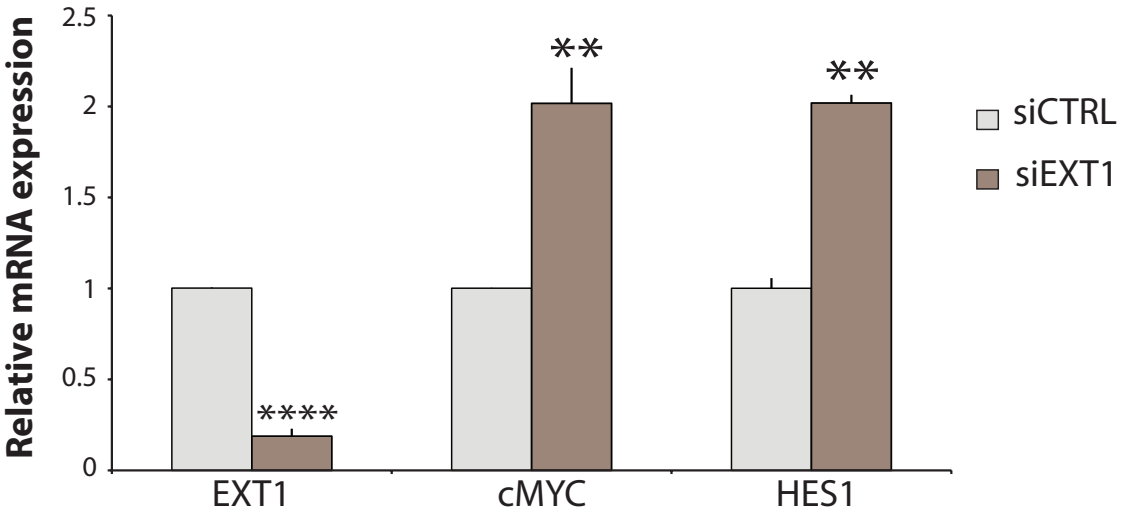


Figure 30: EXT1 depletion increases mRNA levels of NOTCH1 target genes. mRNA expression levels of Notch1 target genes; cMYC and HES1 analysed by qPCR following EXT1 Knock down. Data represent the means ±SEs of three independent experiments. **: p-value < 0.01, ****: p-value < 0.0001.

The effect of EXT1 on Notch1 transactivation was also confirmed in a zebrafish model. We performed a knockdown of EXT1 gene using EXT1 morpholino designed for specific splice blocking of *EXT1b* ortholog in transgenic zebrafish line Tg(Tp1bglob:eGFP)um13 expressing fluorescent marker eGFP under the control of a Notch-responsive element TP1, this line can report on Notch signalling activity.

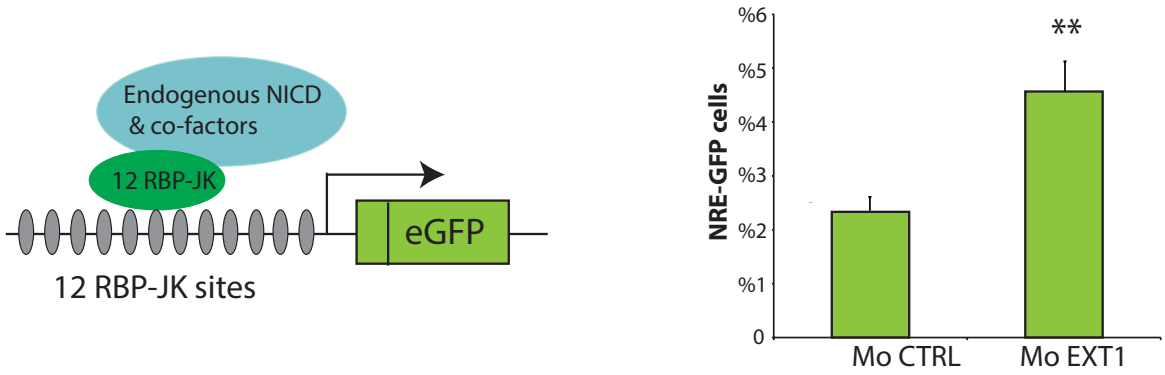


Figure 31: EXT1 depletion promotes NOTCH1 transcriptional activity in zebrafish. A zebrafish transgenic line Tg(Tp1bglob:eGFP)um13, reporter for Notch1 transcriptional activity, were treated with control or *Ext1b* ortholog-targeted morpholinos. Left panel represents *TP1 bglob:hngb1-eGFP* construct. The graph represents the percentage eGFP cells sorted by FACS. Data represent the means ±SEs of three independent experiments. **: p-value < 0.01.

The percentage of GFP cells measured in FACS was increased by 40% following EXT1 depletion, compared to condition with control morpholinos (figure 31).

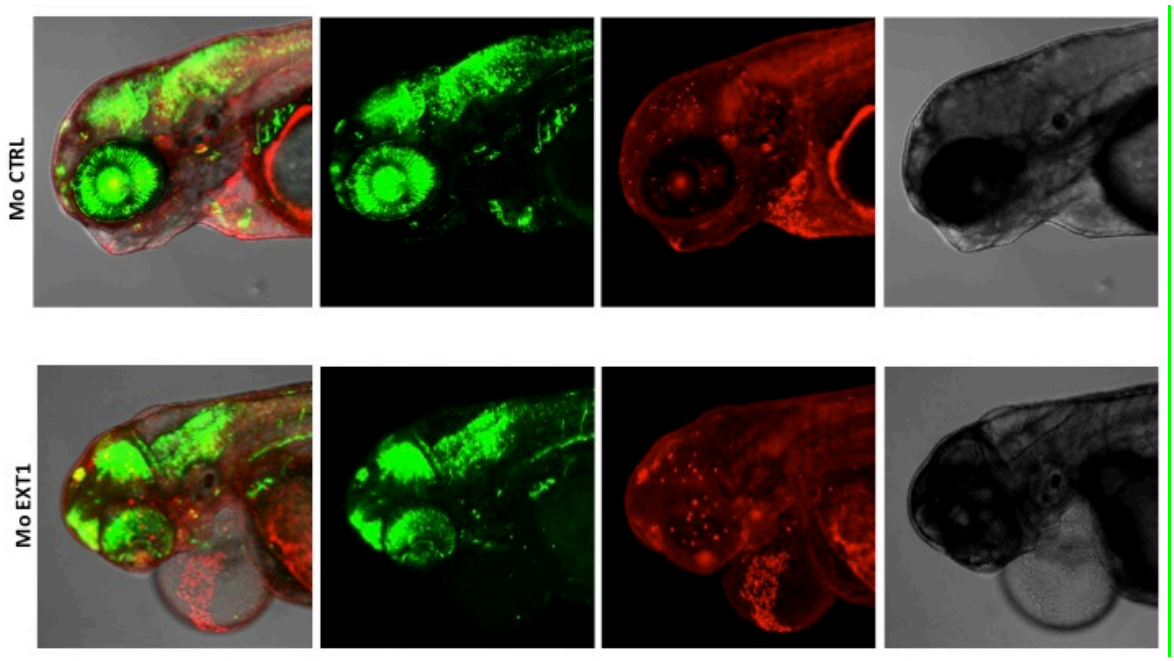


Figure 32: EXT1b knockdown in transgenic zebrafish line Tg (Tp1bglob:eGFP)^{um13}. Embryos were injected with EXT1b morpholino or control morpholino both with an mcherry expressing construct. Forty-two hours post fertilization embryos were observed under confocal microscopy. This transgenic zebrafish line can report on Notch-signalling activity, when signalling is activated eGFP is expressed (green). Red staining marks either Mocontrol or MoEXT1b in the upper and lower panel respectively. Embryos injected with MoEXT1b represent malformation in the head and heart edema.

The advantage of using HeLa N1ΔE-eGFP cell line is that we can easily determine Notch1 localization in confocal microscopy. HeLa N1ΔE-eGFP cells were transfected with mcherry tagged EXT1 plasmid and we followed EXT1 and Notch-eGFP localization in time-lapse confocal microscopy. We used Nikon A1 confocal microscope that took images of fixed areas in transfected wells every 20 minutes for 12 hours and we made a movie of eGFP cells with EXT1-mcherry. We observed that when EXT1-mcherry is produced in the cell, GFP fluorescence _representing Notch1_ is reduced over time (Figure 33, movie).

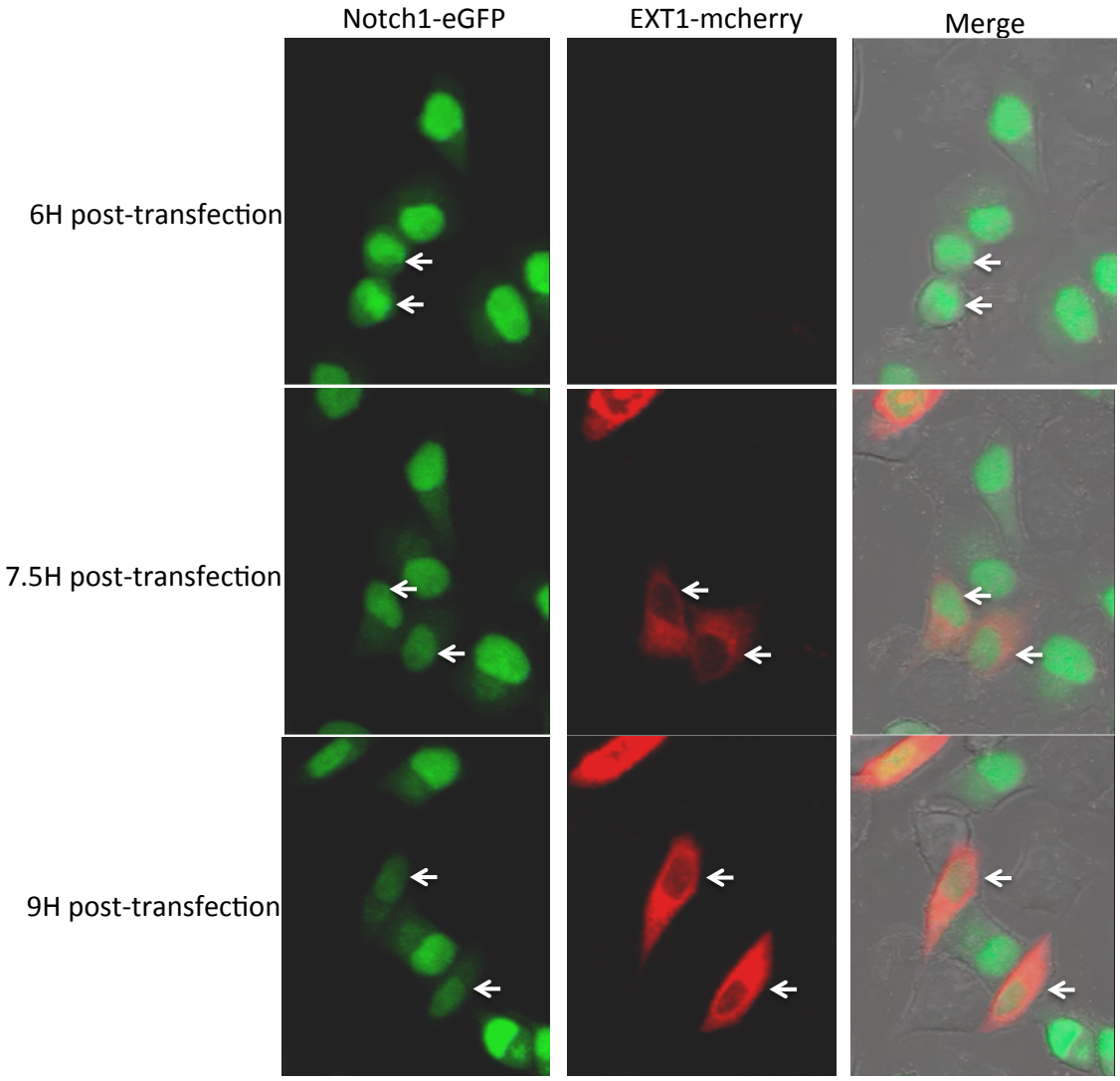


Figure 33: EXT1 reduces NOTCH1 levels in HelaNotch1ΔEeGFP cells. Confocal images of HeLaNotch1ΔE-eGFP cells transfected with EXT1-mCherry during time - lapse experiment. Green and red labeling correspond to NOTCH1-GFP and EXT1-mCherry proteins localizations, respectively. White arrows indicate the same set of cells followed during the time-lapse in which overexpression of EXT1 induced a decrease in GFP fluorescence.

We performed Notch1 reporter assay in a system that mimics the canonical activation of Notch signalling via its ligand *in vitro* with special U2OS Tet-on flp-in cell line bearing isogenic transgene encoding Notch1-Gal4 receptor co-cultured with K562 cells expressing DLL4 ligand that can activate NOTCH1. EXT1 overexpression decreased Notch transactivation in both conditions for U2OS cells co-cultured with control or DLL4 expressing cells. We calculated the activation ratio by DLL4 ligands, in presence and absence of EXT1, and observed no difference in the activation rate by

the ligand suggesting that EXT1 does not affect NOTCH1 ligand dependent activation (Figure 34).

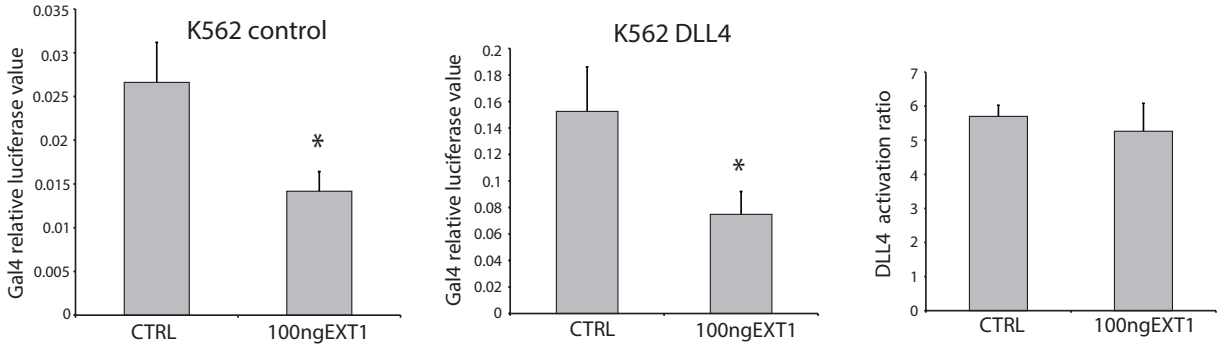


Figure 34: EXT1 inhibits Notch-1 transcriptional activation in U2OS N1-Gal4 cell line. Luciferase reporter assay using Gal4-luciferase construct in U2OS N1-Gal4 cell line transfected with PSG5C control plasmid or EXT1 construct and co-cultured with K562 control cells or K562 cells expressing Notch ligand DLL4 as indicated. Activation ratio by DLL4 expressing cells compared to control K562 cells was calculated in both control and EXT1-overexpression conditions as indicated. Data represent the means ±SEs of three independent experiments, each performed in triplicate. *: p-value < 0.05.

These observations confirm that EXT1 has an effect on Notch1 signalling at transcriptional level, inhibiting its transactivation and some of NOTCH1 target genes transcription. We also showed that this effect is independent of receptor activation by the ligand at the cell surface.

3. EXT1 regulates NOTCH1 degradation through FBXW7

One of the main reasons for selecting EXT1-NOTCH1 interaction was that EXT1 also interacts with FBXW7 that regulates NICD proteasomal degradation. The transcriptional effect observed previously does not exclude that EXT1 might play a role in the regulation of Notch1 protein levels may be through promoting its degradation.

Previously we have validated EXT1-NICD interaction in mammalian cells. We tested FBXW7-NICD interaction using PCA and we were also able to validate this interaction with a NLR ≈ 15. Overexpression of EXT1 with both NICD-GL1 and FBXW7-GL2

vectors induces 10-fold increase in calculated NLR (Figure 35).

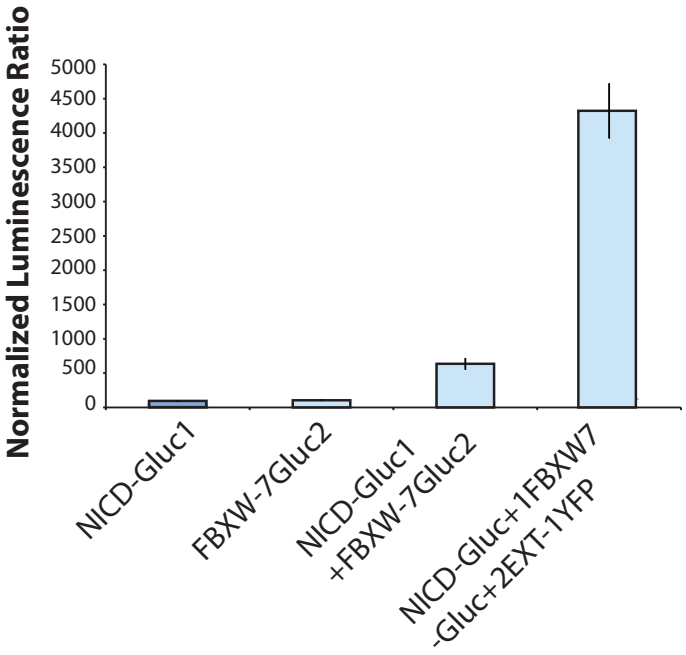


Figure 35: Validation of NICD-EXT1-FBXW7 interactions using PCA method. Protein complementation assay in HEK cells transfected with NICD-Gluc1 and/or FBXW7-Gluc2, in addition to EXT1-yfp as indicated in the X-axis, Normalized Luciferase Value (NLR) is represented by the Y-axis.

Next, we determined whether EXT1 plays a role in the regulation of NICD protein level by using western blot analysis in HEK393 cells. The cells were co-transfected with Flag-tagged NICD and YFP-tagged EXT1. We observe a downregulation of the NICD protein level with co-expression of EXT1 (figure 36 a).

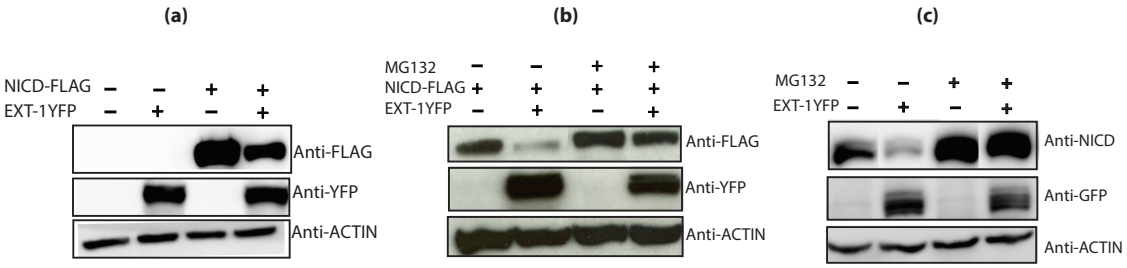


Figure 36: EXT1 regulates NOTCH1 degradation through proteasomal degradation. (a) and (b) HEK293 cells were transfected with NICD-Flag and EXT1-YFP expressing plasmids as indicated. Eighteen hours post-transfection cells were treated with proteasomal inhibitor MG132 for 6 hours and lysates analyzed by western blot using anti-Flag M2 and anti-GFP antibodies. (c) HeLaNotch1ΔE-eGFP cells were transfected with EXT1-YFP expressing plasmid as indicated. Eighteen hours post transfection, cells were treated with proteasomal inhibitor MG132 for 6 hours and lysates were analyzed by western blot using a NICD antibody.

A similar effect of was observed in HeLaNotch1ΔE-eGFP cells transfected with EXT1-yfp, showing a downregulation of NICD protein level (Figure 36 c). Moreover, EXT1 depletion using specific siRNA induced an upregulation of NICD protein levels in both HEK293 and HeLaNotch1ΔE-eGFP cell lines (Figure 37 a and b respectively).

We evaluated the involvement of EXT1 in the NICD proteasome-dependent degradation pathway by performing western blot analysis. NICD protein level was lower in HEK293 cells overexpressing EXT1 than in cells transfected with control plasmid (Figure 36 a, lanes 3 and 4 and 36 b, lanes 1 and 2), and the treatment of MG132 enhanced the NICD protein level by inhibiting proteasomal degradation (figure 36 b lanes 2 and 4). This result was also confirmed in HeLa Notch1ΔE-eGFP cell line with a similar observation (Figure 36 c). In addition, NICD protein level was higher in EXT1-depleted Hela Notch1ΔE-eGFP cells than in cells transfected with a control siRNA (Figure 37 b, lanes 1 and 2), and the treatment of MG132 enhanced the NICD protein level by inhibiting proteasomal degradation (Figure 37 b, lanes 3 and 4).

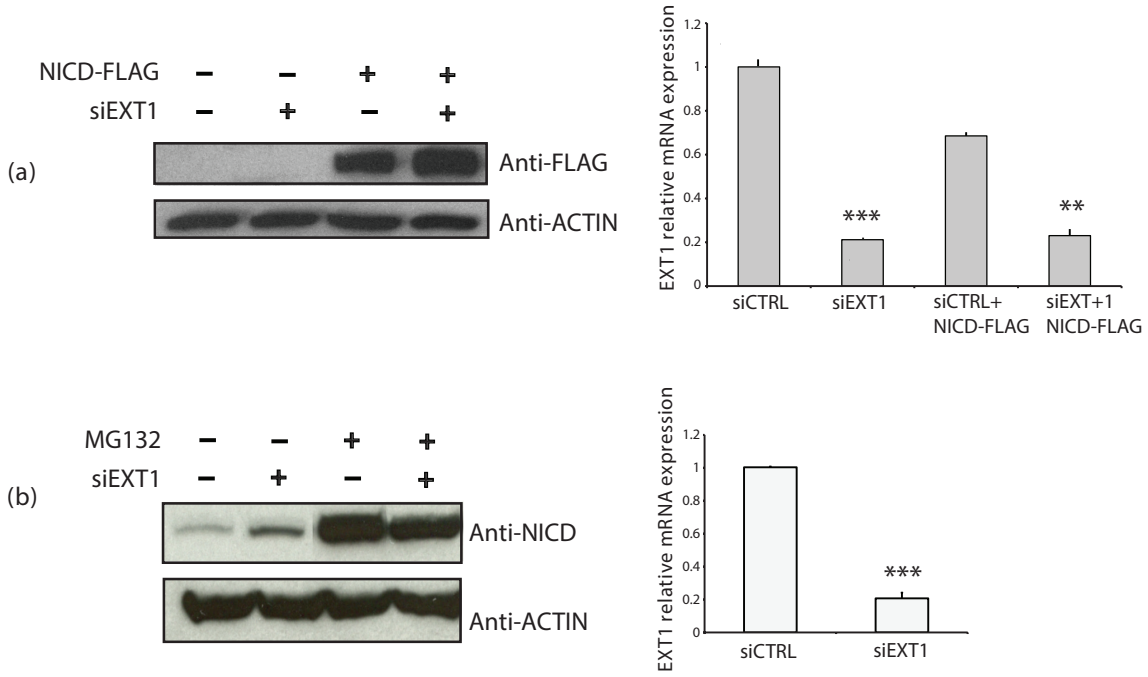


Figure 37: EXT1 depletion increases NICD preotein levels. (a) HEK293 cells were transfected with siRNA for *EXT1* or a siRNA control using calcium phosphate. Twenty-four hours post-transfection cells were transfected with NICD-Flag expressing plasmid as indicated, and after 24 hours lysates analyzed by western blot using anti-Flag M2 antibody. Knock-down of EXT1 was analyzed by qPCR. (b) HeLaNotch1ΔE-eGFP cells were transfected with EXT1 siRNA or control siRNA as indicated. Forty two hours pot-transfection, cells were treated with proteasomal inhibitor MG132 for 6 hours and lysates were analyzed by western blot using a NICD antibody. Relative mRNA expression levels of *EXT1* analyzed by qPCR. Data in the graphs represent the means ±SEs of three independent experiments, each performed in triplicate. **: p-value < 0.01, ***: p-value < 0.001.

We tested the involvement of FBXW7 in the degradation of NICD by EXT1. FBXW7 depletion in HEK293 cells using specific siRNA targeting *Fbxw7*, can partially recover and enhance NICD protein level in the presence of EXT1 (Figure 38). This result indicate that downregulation of NICD by EXT1, occurs in part, via an FBXW7-dependent mechanism.

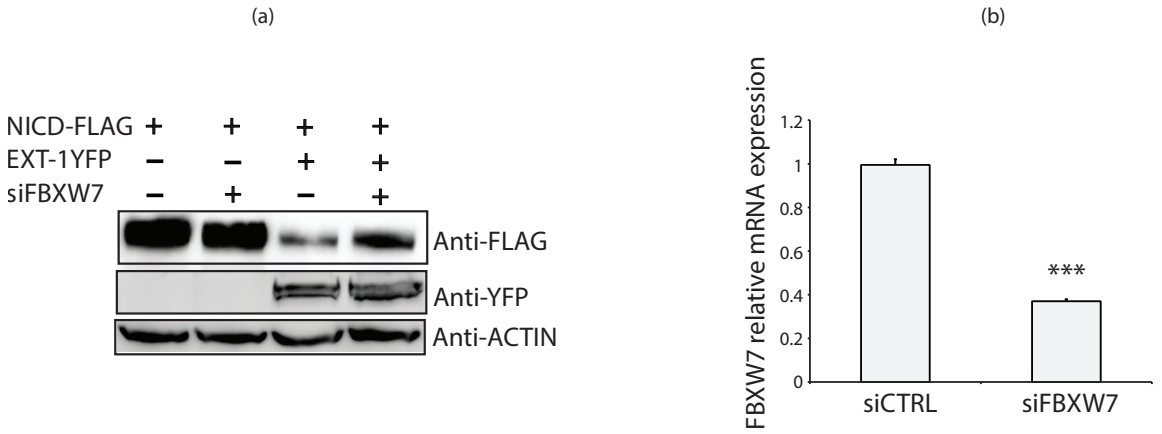


Figure 38: EXT1 regulates NOTCH1 degradation through FBXW7. (a) HEK293 cells were transfected with siRNA for FBXW7 or a siRNA control using calcium phosphate. Twenty-four hours post-transfection cells were transfected with NICD-Flag and EXT1-YFP expressing plasmids as indicated. Twenty-four hours later lysates analyzed by western blot using anti-Flag M2 and anti-GFP antibodies. (b) Relative mRNA expression levels of *FBXW7* analyzed by qPCR. Data represent the means ±SEs of three independent experiments, performed in triplicate. ***: p-value < 0.001.

4. Genes coregulated by EXT1 and FBXW7

To confirm the potential interplay between EXT1 and FBXW7 we performed RNA sequencing on total RNA extracted from HeLa Notch1ΔE-eGFP cell line in three different conditions. Cells were silenced for *EXT1* or *FBXW7* genes using specific siRNA and siRNA control for the control condition [292, 293]. We were able to identify sets of genes that are differentially expressed; up or down - regulated compared to the control condition (figure 39 a). The intersection in the Venn diagram with 479 genes represents co-regulated genes; accounting for 22% and 30% of deregulated genes for siEXT1 and siFBXW7 knockdown conditions respectively. We analyzed the set of "co-regulated" genes using ToppFun for gene list analysis functional annotation, and we were able to categorize them into 49 clusters.

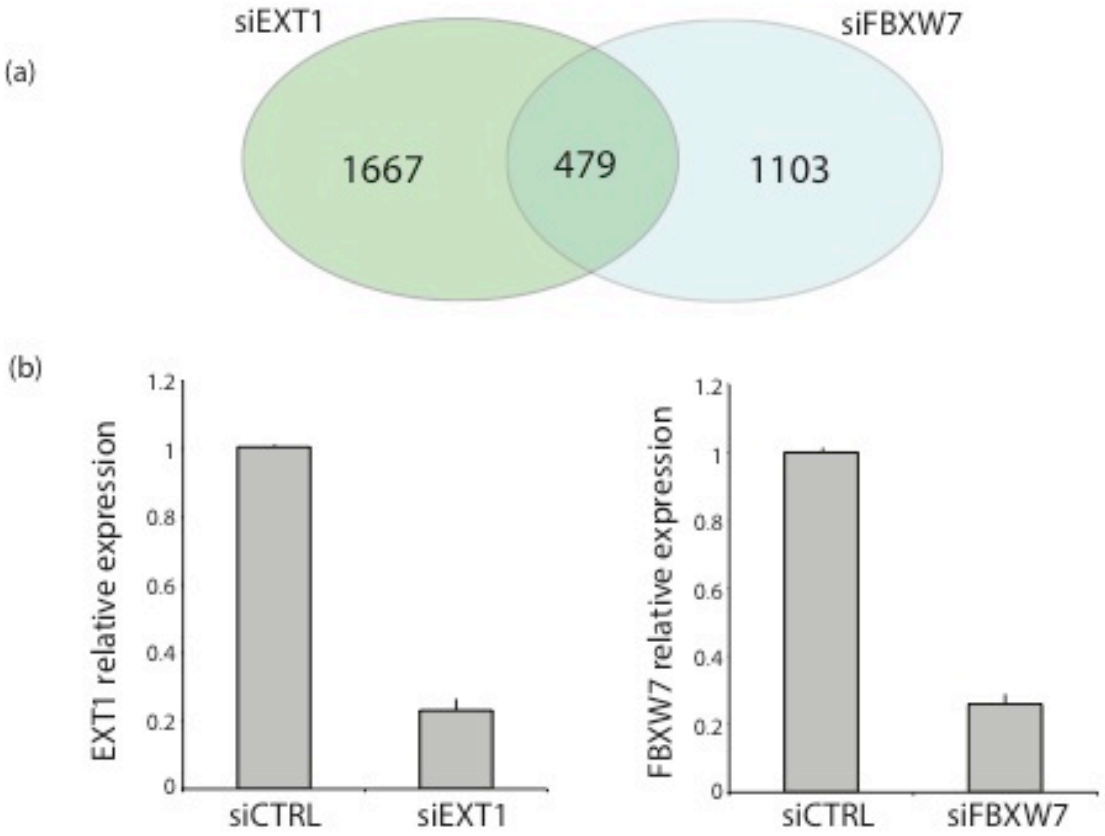


Figure 39: Genes coregulated by EXT1 and FBXW7. (a) HeLa NotchΔE-eGFP were treated siRNA for EXT1, FBXW7 or a control siRNA. Relative mRNA expression levels of *EXT1* and *FBXW7* were then analyzed by qPCR and RNA samples subjected to high throughput Illumina sequencing (RNA-seq). Ven diagrams represent a comparison between deregulated genes following knock down of EXT1 or FBXW7. (b) Relative mRNA expression levels of *EXT1* and *FBXW7* analyzed by qPCR. Data in the graphs represent the means ±SEs of three independent experiments, each performed in triplicate.

Interestingly the most relevant GO-term corresponding to the top 5 molecular functions with the lowest p-values (supplemental table S7) were linked to phosphorylation and kinases activities. Substrate recognition by FBXW7 requires phosphorylation of its substrates including c-MYC, NOTCH, c-JUN, cyclin-E and mTOR [294], suggesting that EXT1 could also play a role in FBXW7-substrates recognition.

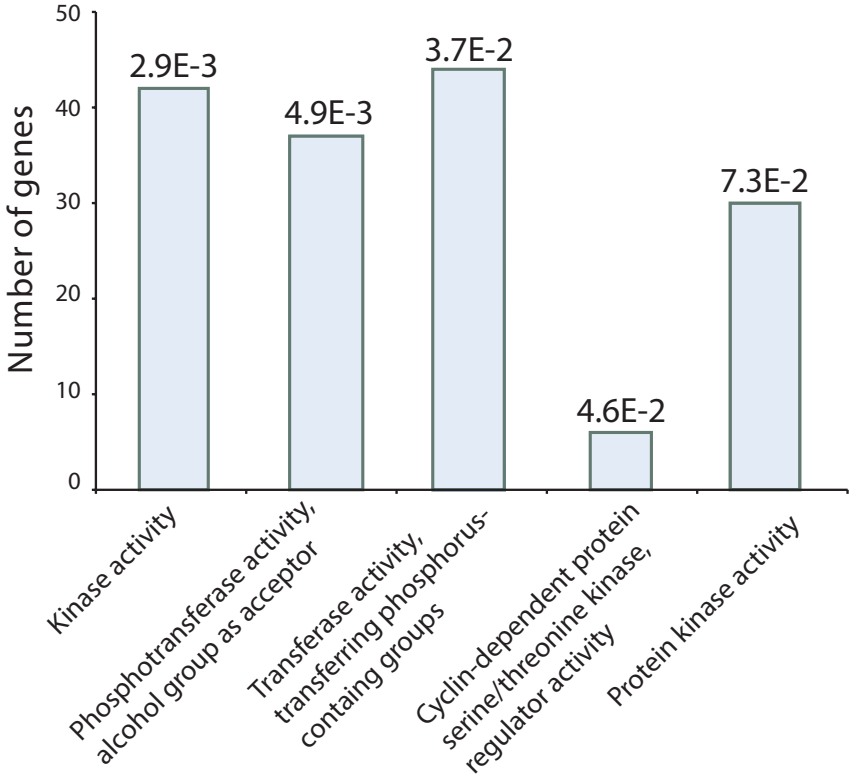


Figure 40: Gene ontology enrichment analysis of common deregulated genes. The 479 genes coregulated by EXT1 and FBXW7 were analyzed for molecular function enrichment using ToppFun for gene list analysis functional annotation. This graph represents the top 5 most relevant GO-terms corresponding to lowest p-values among GO-terms. The Y-axis represents the top 5 GO-terms, the X-axis the number of genes for contained in each GO-term. P-value corresponding to each term is represented in the graph.

DISCUSSION PART II

Several examples can illustrate the effectiveness of using the "interactomes" approach for the identification of novel cancer related genes. As we have prioritized ALL-gene products, the exostin glysosyltransferase 1 (EXT1) gene grabbed our attention for several reasons; first for being a novel interaction linked to two of the ALL-gene products: NOTCH1 and FBXW7, second for being already implicated in an other type of cancer (osteosarcoma) via inactivating mutations.

Validation of interaction in NOTCH1 interactome

Before exploring the relationship between EXT1 and NOTCH1 we analysed the Notch1 interactome and proceeded to the validation of the identified interactions.

Our yeast two-hybrid screen performed for Notch intracellular domain with the human ORFeome version 5.1 enabled us to determine 19 proteins interacting with NICD. Among these NICD partners, AKT1 has been reported previously for interacting with Notch signalling and being essential for neural development through regulation of this signalling pathway. It was demonstrated that both AKT and Notch signalling pathways interact through complex molecular interactions in development [295, 296]. Several data showed that Notch can affect AKT signalling and AKT1 also affects Notch signalling but their direct interaction was not previously demonstrated.

Among the identified NICD partners, AKT1, RhoA, LNX2 and TLE3 can be linked to Notch signalling according to data published in literature.

Recently it was demonstrated that RhoA/Rho kinase is regulated by Notch in endothelial cells, promoting senescence phenotype associated with barrier dysfunction [297]. RhoA is a member of Ras family of small GTPases that we identified in our screen as a partner of Notch1. In fact, Notch and Rho GTPase signalling were previously shown to control dendritic development [298]. Activation of RhoA in a number of different cell types appears to limit dendritic growth, whereas activation of Notch appears to restrict dendritic growth and promote dendritic branching by regulating the activation of members of Rho GTPase family. As shown for AKT1, RhoA

might be affected by Notch signalling. We are the first to show a direct interaction between Notch1 and RhoA.

LNX2 in the ligand of Numb X 2, LNX is a RING finger and PDZ domain containing protein that interacts with the cell fate determinant Numb. Numb was identified as a substrate ubiquitinated by LNX which is considered as the first example of an E3 ligase that appears to involve PDZ domains for substrate recognition [299]. It is known that proteins with PDZ domains often interact with the cytoplasmic tails of transmembrane proteins and localize to discrete sub-membranous sites. Therefore, the LNX PDZ domains may be involved in the recognition of transmembrane receptors, which may in turn be substrates for ubiquitylation by LNX. This explanation could be valid for LNX2 and NOTCH1, on the other hand Numb promotes Notch ubiquitination and degradation. Numb can interact with other factors such as Itch to promote Notch degradation. Knowing that Numb can bind LNX2, we speculate that LNX2 might be involved in Notch1 ubiquitination via Numb also. These hypothesis need to be investigated, and are not part of our analysis.

TLE family has been implicated in tumorigenesis and has been shown to interact with and modulate the Notch pathway, via phosphorylation by mitogen-activated protein kinase (MAPK) in response to epidermal growth factor receptor (EGFR) signalling [300]. TLE3 have been shown to interact with the Notch pathway for the control of epithelial differentiation [301, 302]. TLE family also act as important effectors of Notch signalling through their interactions with Hes proteins, and the deregulation of Notch signalling has been implicated in the pathogenesis of some cancers.

We represent the 19 proteins identified in the Y2H in table 4, with known functions described for each protein.

Table 4: NICD interacting protein partners identified in the Y2H screen.

Gene	Pubmed-Id	Annotation	Protein function
AKT1	207	V-akt murine thymona viral incogene homolog 1	Serine-threonine protein kinase
ARL6IP5	10550	ADP-Ribosylation-Like Factor 6 Interacting Protein 5	The encoded protein associated with the cytoskeleton
B3GALT4	8705	UDP-Gal beta GLcNAc beta 1,3-galactosyltransferase polypeptide 4	Type II glycoprotein
COL4A6	1288	Collagen type IV alpha 6	Gene encoding one of the six subunits of type IV collagen the major structural component of basement membranes
C1orf217	84719	Chromosome 1 open reading frame 217	Long intergenic non-protein coding RNA 260
C6orf182	285753	Chromosome 6 open reading frame 182	
C9orf62	157927	Chromosome 9 open reading frame 62	Non coding region
DAZAP2	9802	DAZ associated protein 2	Proline rich protein interacts with several proteins such as E3 ubiquitin ligases
EXT1	2131	Exostin glycosyltransferase 1	Endoplasmic reticulum resident type II transmembrane glycosyltransferase involved in Heparan Sulfatebiosynthesis
FAM185A	222234	Family with sequence similarity 185, member A	
GOLGA2	2801	golgin A2	Protein playing roles in the stacking of Golgi cisternae and in vesicular transport
GOPC	57120	Golgi associated PDZ and coiled-coil motif containing	
HMGN5	79366	High mobility group nucleosome binding domain 5	Nucleosomal binding and transcriptional activating protein
LNX2	222484	Ligand of numb-protein X 2	
NCRNA00260	84719	Non-protein coding RNA 260	
POU2AF1	5450	POU Classe 2 Associating Factor 1	Gene related to transcription coactivator activity and transcription cofactor activity
RHOA	387	Ras homology family member	Member of the Rho family of GTPases
TLE3	7090	Transducin-like enhancer of split 3	Transcriptional co-repressor protein-function in Notch pathway-associated

Though the Y2H is a stringent method for identifying protein-protein interactions (PPIs), it requires cross-validation to increase coverage and accuracy, which is the case for any other information provided by a certain screening methods for PPIs. Therefore it was important to test a set of NOTCH1 interactions from both literature-curated interactions and Y2H interactions. The result obtained using the protein complementation assay is very promising for the set of interactions that we extracted from our Y2H screen, with a rate comparable to the one obtained for literature interactions (table 3).

Notch1 - EXT1 a novel interaction

If we take a closer look to the NOTCH1 and FBXW7 interactomes merged together, with interactions extracted from literature (BioGRID, HPRD and IntAct databases) and from our Y2H screen we identify 2 common interactors: PSEN1 and EXT1 as it is shown in figure 41. The interactomic map highlights novel Y2H interactions for both Notch1 and FBXW7 represented in red, as for literature-curated interactions (LCI) they are represented in grey. Apart from being known to directly interact, Notch1 and its E3 ubiquitin ligase FBXW7 have a common interactor PSEN1, reported in literature. PSEN1 belongs to the family of presenilin basically known for being mutated in patients with Alzheimer's disease. Presenilins are implicated in the regulation of Notch signalling through affecting the receptors cleavage at the cell surface. PSEN1, with PSEN2 form the central components of γ-secretase complexes that are required for intra-membrane cleavage of over 70 different substrates including Notch1. Psen1 is a critical facilitator of Notch signalling and other signalling pathways [303]. On the other hand PSEN1 is a substrate of FBXW7.

Another common interactor linking NOTCH1 and FBXW7 interactomes was EXT1.

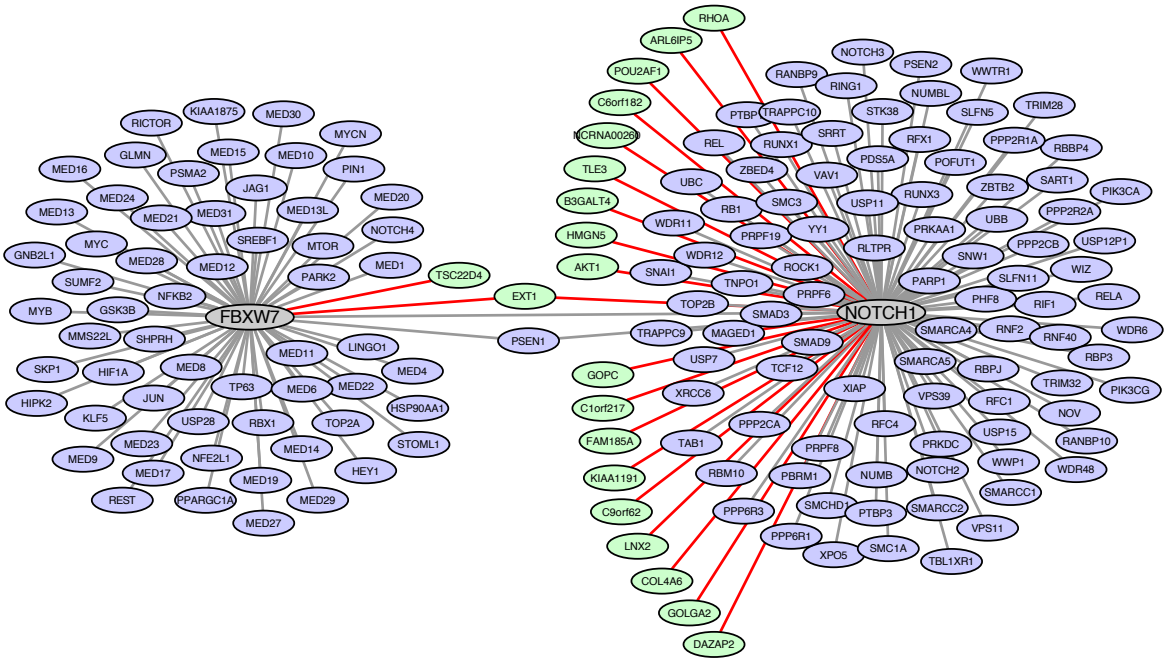


Figure 41: NOTCH1 and FBXW7 interactomes. The grey nodes represent NOTCH1 and FBXW7, while purple and green nodes represent interactors from literature and Y2H respectively.

The link between FBXW7 and Notch1 is well established, as re-visited in our introduction section. EXT1 was described in the biosynthesis of heparin sulfate (HS), and mutations in EXT1 are associated to multiple exostosis; a dominantly inherited genetic disorder characterized by multiple cartilaginous tumors. Heperan sulfate proteoglycans are expressed at the cell surface of nearly all vertebrates. One of the important function of heparan sulfate proteoglycans is to modulate signalling via mediating the formation of ligand receptor complexes [304]. Knowing that EXT1 essential function is linked to the HS, the first question we asked is whether it affects Notch1 signalling at the cell surface through HS regulation. The answer to this question was clarified when analysing the effect of activating the Notch1 signalling through DLL4 ligand in U2OS cell line. The rate of activation by the ligand was not affected by EXT1 overexpression.

Using NOTCH1 transcriptional-responsive luciferase reporter assay, we showed that depletion of *EXT1* using small interfering RNA increased NOTCH transactivation activity in different cell lines (Figure 28). We also showed that depletion of *EXT1*

increases mRNA expression levels of two important NOTCH1-target genes: *HES1* and *MYC* (Figure 30). Consistent with this finding, over-expression of EXT1 inhibits NOTCH1-transactivation in different cell lines (Figures 29 and 34) and correlates with a reduction of NICD protein levels (Figure 33 and time-lapse video). We confirmed the effect of EXT1 on NOTCH1 pathway using a zebrafish *in vivo* model. We treated transgenic zebrafish line Tg(Tp1bglob:eGFP)um13 expressing fluorescent marker eGFP under the control of a Notch-responsive element TP1, with morpholino targeting *EXT1* b zebrafish orholog. As shown on Figure 32, we observed a 40% increase cells expressing Notch activity following depletion of *EXT1* zebrafish orthologs.

FBXW7 is an E3 ubiquitin ligase regulating NOTCH1 proteasomal degradation [305]. To determine whether EXT1 interferes with FBXW7 – NOTCH1 association, we first showed that the interaction between NOTCH1 and FBXW7 was dramatically enhanced in the presence of EXT1 (Figure 35). Then, we showed that, in the presence of EXT1, the level of NOTCH1 intracellular domain (NICD) is reduced in a proteasome-dependent manner (Figure 36 b and c: compare presence and absence of MG132 proteasome inhibitor). Interestingly, we also showed that reduced levels of NICD in the presence of EXT1 are FBXW7-dependent (Figure 38). To more deeply analyze the functional relationship between EXT1 and FBXW7 in regulating cellular homeostasis, a function well known for FBXW7 [305], we performed a genome-wide analysis of the transcriptome of HeLa-NICDdeltae-GFP [306] depleted for *EXT1* or *FBXW7*. We identified 479 mRNAs co-regulated by both EXT1 and FBXW7, which represent more than 30% of FBXW7 targets (Figure 39). Functional annotation enrichment analysis of these EXT1/FBXW7 co-regulated genes finally indicated a significant enrichment in genes encoding for kinases including cyclin-dependent and MAP kinases (Figure 40).

Kinases play an essential role in cell cycle regulation, and protein phosphorylation regulates most aspects of cell life [307]. Deregulation of kinase mechanism leads to disease and cancer development, therefore Kinases are intensively studied as anti-cancer drugs through the developing inhibitor candidates that are able to arrest proliferation and induce apoptosis [308]. FBXW7 is known to regulate proteasomal degradation of a large number of proteins, in a mechanism dependent of phosphorylation activity that is mediated by kinases and cyclin-dependent kinases.

Recent studies demonstrated that GATA3 is a novel target of FBXW7, and its degradation depends on CDK2 [309] and CDK1 [310] kinases. GSK3B is also one of the kinases that are crucial for FBXW7-dependent degradation, for MYC, JUN, cyclin E and NOTCH1 proteins[195]. It is not surprising to find that FBXW7 depletion can affect a set of genes encoding proteins implicated in the kinase and phosphorylation pathway. As for EXT1, the link with kinases was described through the role of heparan sulfate for proper binding of growth factor to its kinase receptor. Among growth factors, fibroblast growth factors (FGFs) depend on heparan sulfate proteoglycans (HSPGs) for cell signalling activity, and FGFs act through tyrosine-kinase receptors. These components form a complex at cell surface leading to the phosphorylation of the receptor and therefore triggering the activation of signalling cascades. This role of EXT1 role was described in growth factor signalling and fibroblast interactions with the extracellular matrix [311, 312]. However EXT1 association with kinases activity was not previously established.

Our data suggest a novel role of EXT1. Performing RNA-seq transciptome analysis combined with enrichment analysis confirmed that EXT1 is functionally linked to FBXW7, probably through priming kinases and substrates such as Notch1 towards proteasomal degradation.

CONCLUSION AND PERSPECTIVES

The sequencing of different cancer genomes allows identification and characterization of mutated genes in cancer samples. However, the development of genome-based therapies requires greater knowledge of the specific driver genes implicated in diverse cancer types and subtypes. Prioritization of cancer-implicated genes has received growing attention, using a multitude of tools that integrate data source covering gene sequences and mutations, protein interactions, signalling pathways and function annotations. Studies nowadays tend to analyze cancer types and disease development from a global point of view where a complete spectrum of "omics" is involved, including genomics, transcriptomics, proteomics, and metabolomics in addition to their interactions and relationships with other phenotypes. In 2012, Moreau and Tranchevent published an interesting review describing computational tools for prioritizing candidate genes that were classified into 4 strategies as it is shown in figure 42.

The Filtering strategy relies on reducing the list of candidates that fulfill specific properties into a smaller list. The disadvantage of this method is higher rate of false negative due to strict filtering criteria. While ranking methods classify the candidates according to multiple criteria. Text-mining depends on defining disease-relevant keywords to identify promising candidates. Similarity profiling combines both knowledge bases and raw data in order to determine candidate genes according to their similarity to already known genes for a specific disease. Network analysis has recently become popular for gene prioritization, having the advantage of expanding the potential candidates according to relationships between nodes.

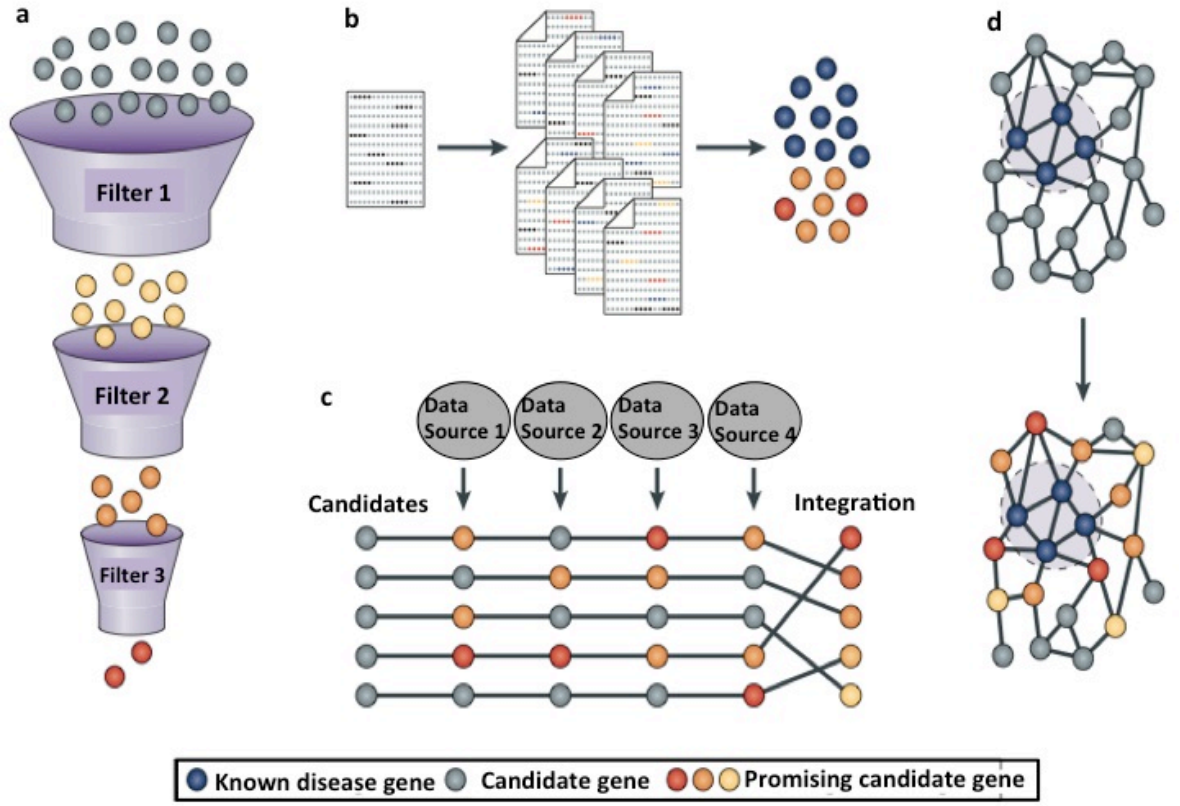


Figure 42: Computational strategies for prioritization. Prioritization methods can be classified into a filtering strategy and three ranking strategies. (a) Filtering strategy, (b) text-mining strategy, (c) Similarity profiling and data fusion strategy, (d) Network-based strategy. Adapted from [313].

The goal of data integration in network-based prioritization is to identify nodes of the network that are relevant to the disease or biological process of interest rather than infer the edges of the network. So instead of only prioritizing genes in isolation, generating hypotheses about potential interactions would increase the value of prioritizing methods leading to hypotheses that can be tested experimentally for biological validation [313]. In addition, combining multiple methods in parallel can improve the quality of predictions.

It has been observed that cancer proteins are inter-connected in systematic interactome maps than proteins with no known association with cancer. This observation can be extended to additional candidate cancer genes identified by systematic approaches such as (i) cancer genome sequencing, (ii) transposon-based screens in mice, (iii) investigation of DNA tumor virus targets, and (iv) genome-wide association studies [244]. These studies thus suggest that the human cancer genomic

landscape is not limited to the 572 relatively well-accepted genes reported in the Sanger Catalogue Of Somatic Mutations in Cancer (COSMIC).

In our PhD thesis, we focused on acute lymphoblastic leukemia (ALL) cancer genes and demonstrated the added value of systematic interactome maps to help prioritize cancer genes. We showed that, combining the frequency of mutations in samples, the number and pattern of distinct mutations, and the inter-connectivity between genes products, could help determine specific affected signalling pathways in ALL. We also showed that, novel ALL-genes candidates, such as EXT1, could be identified based on their functional association with well-known ALL-genes such as NOTCH1 and FBXW7.

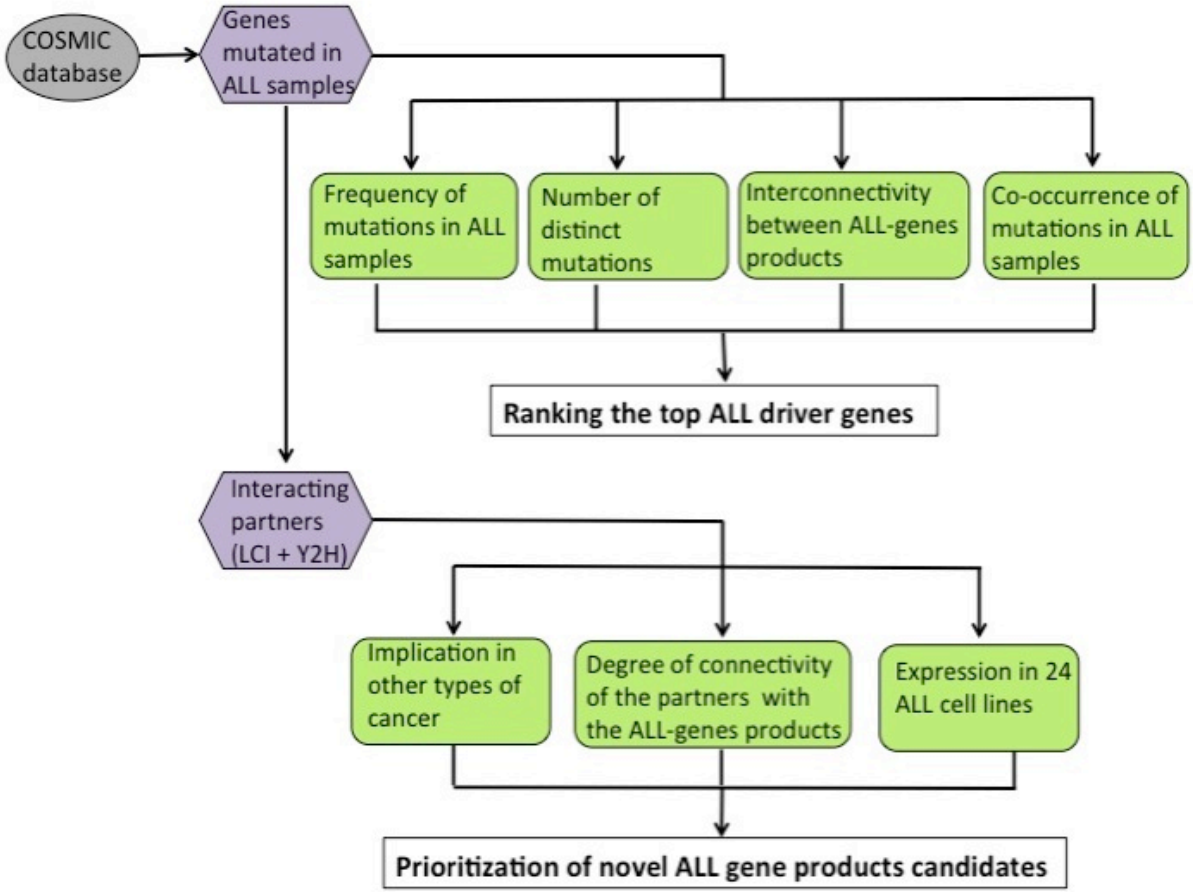


Figure 43: Prioritization workflow for ALL genes. Using COSMIC (Catalogue Of Somatic Mutations In Cancer) database, genes mutated in ALL samples were selected. Applying different ranking criteria (cited in the green boxes in the upper panel) we defined a set of the top ALL driver genes. Next we established the interactome maps of the "genes mutated in ALL samples" using literature and a Y2H screen, and we applied different criteria (cited in the green boxes in the lower panel) in order to prioritize novel ALL gene products candidates.

We then provided experimental results supporting the hypothesis that *EXT1* gene, a tumor suppressor not previously linked to ALL by mutations, is involved in the regulation of the NOTCH pathway through its dual interaction with ALL-related proteins: NOTCH1 and FBXW7. Interestingly the comparative analysis of EXT1 and FBXW7 targets revealed kinases as major regulated components, and experimental data suggest that EXT1 acts on the Notch pathway through an FBXW7-dependent mechanism. The protein level and transcriptional activity of NOTCH1 were higher when *EXT1* was depleted, and the levels of NICD increased by the blocking of the proteasome. EXT1 negatively regulated Notch1 signalling pathway both at the transcriptional level and in term of protein stability. The inhibitory mechanism we suggest functions through suppression of RBP-Jk (CSL) transactivation that we observed in our luciferase reporter assays, in response to the down regulation of NICD protein probably through promoting its degradation.

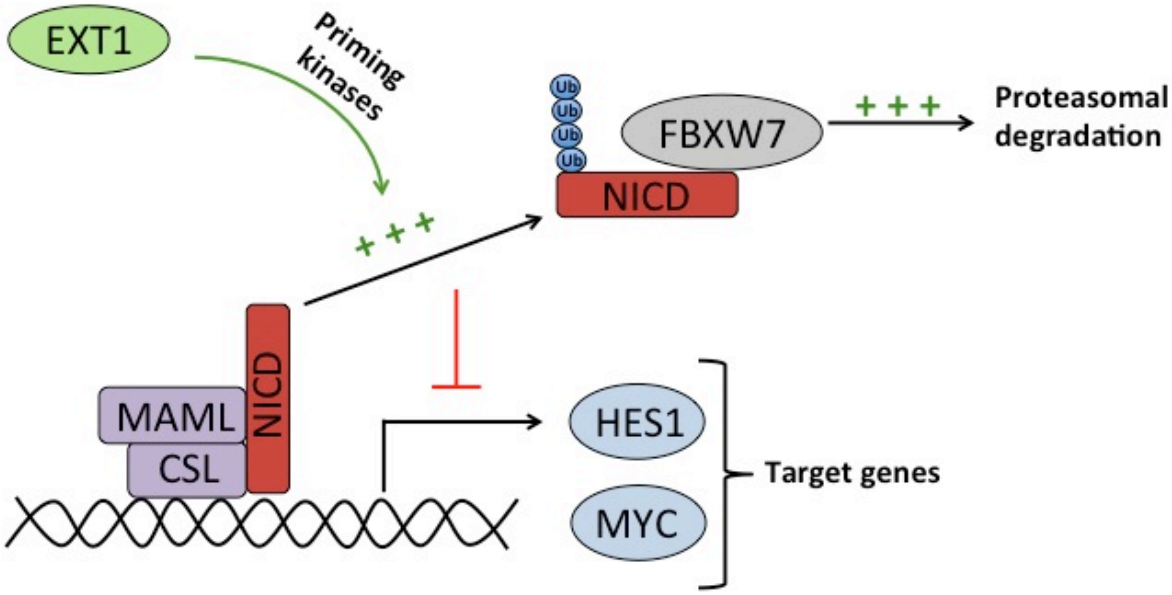


Figure 44: Graphical summary. EXT1 regulates NOTCH1 signalling through an FBXW7-dependent pathway. EXT1 inhibits NICD transactivation and decreases Notch1 target genes mRNA levels (HES1 and MYC), by promoting NICD proteasomal degradation through an FBXW7-dependent mechanism.

Understanding the impact of EXT1 on NOTCH signaling *in vivo*, is being investigated in our laboratory using conditional knock-out mice for *Ext1* and *Notch1* and xenograft model for T-ALL.

In conclusion, we believe that this work presents conceptual advances of broad interest for cancer biology and application of systems biology in the identification of novel targeted genes and pathways associated to human cancer.

MATERIALS AND METHODS

1. Plasmids

Open reading frames (ORF) encoding Notch1 partners (tested for Protein complementation assay) were obtained from human ORFeome v5.1 (center of cancer systems biology: CCSB) as pDONR223 vectors. As for human NICD plasmid, it was purchased from addgene. FBXW7 α was obtained from Dr. E. Dejardin from the laboratory of molecular immunology and signal transduction – Groupe Interdisciplinaire de Génoprotéomique Appliquée (GIGA). ORFs that were not available from the hORF V5.1 (BRAF, HRAS, ABL1, JAK2 and SMARCB1 and NOTCH1 genes), and Notch1 seperate intacellular domains were cloned by Gateway recombination technology (Invitrogen) using specific primers flanked with the following AttB1 and AttB2 Gateway sites: 5'-GGGGACAACTTTGTACAAAAAAGTTGGCATG-3' (AttB1) and 5'-GGGGACAACTTTGTACAAGAAAGTTGA-3' (AttB2). These constructions were verified by PCR and sequencing.

Inserts from pDONR223 were transferred by LR cloning (Invitrogen) into different destination vectors: pAD-destCYH and pDB-dest the Y2H expression vectors, and pDEST1899 (flag tag), pDEST491 (YFP-tag) and pDEST-mcherry.

2. Gateway cloning

Cloning was carried out using the Gateway recombination technology as mentioned previously. This system is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda to provide a rapid and highly efficient way to move DNA sequences into multiple vector systems. Genes of interest were cloned in the donor vector pDNR223 and in different expression vectors: pAD and pDB yeast expression vectors, and pN1GLuc and pN2GLuc for Gaussia luciferase complementation assay and other vectors such YFP, mCherry, Flag expression vectors. In order to clone NICD, BRAF, HRAS, ABL1, JAK2 and SMARCB1 that were not present in the hORFeome collection 5.1, we performed PCR to amplify the gene of interest with specific primers flanked by attB Gateway sequences. PCR

product was then cloned into the donor vector in BP reaction, using Gateway BP clonase (Invitrogen) (figure 45). This reaction in performed by adding 100ng of the PCR product with 150ng of the donor vector with 1µl of the BP clonase II enzyme mix in a total volume of 10µl in a 1,5ml eppendorf at 25^{o}C for an hour. To terminate the reaction 1µl of proteinase K solution was added to the mixture and incubated for 10 minutes at 37^{o}C. 2µl of each BP reaction were transformed into 40µl of Hb101 electrocompetent bacterial cells, then transferred into 1ml of LB medium and incubated at 37^{o}C for 1 hour with shaking. 100µl of each transformation were plated onto LB agar plates complemented with ampicillin antibiotic (1µg/ml) and incubated at 37^{o}C overnight. Positive colonies were selected and transferred into 5 ml of liquid LB + ampicillin medium for DNA extraction using NucleoSpin Plasmid kit (Marcherey-Nagel) according to manufacturer instructions. Extracted DNA was verified by PCR, subsequent electrophoresis and sequencing using specific primers in the donor vectors.

Next, in order to clone genes into expression vectors, LR reaction was carried out using Gateway LR clonase (Invitrogen) (Figure 45). This reaction was performed by adding 150ng of the entry clone (containing the gene of interest flanked by attL sites) and 150 ng of a destination vector (containing attR sites) with 1µl of the LR clonase II enzyme mix in a total volume of 10µl in a 1,5ml eppendorf at 25^{o}C for an hour. Next we follow the same steps mentioned above for the BP clonase reaction but spectinomycin antibiotic is used instead ampicillin antibiotic in bacterial culture. Cloned plasmids were verified by PCR and gel subsequent electrophoresis and sequencing.

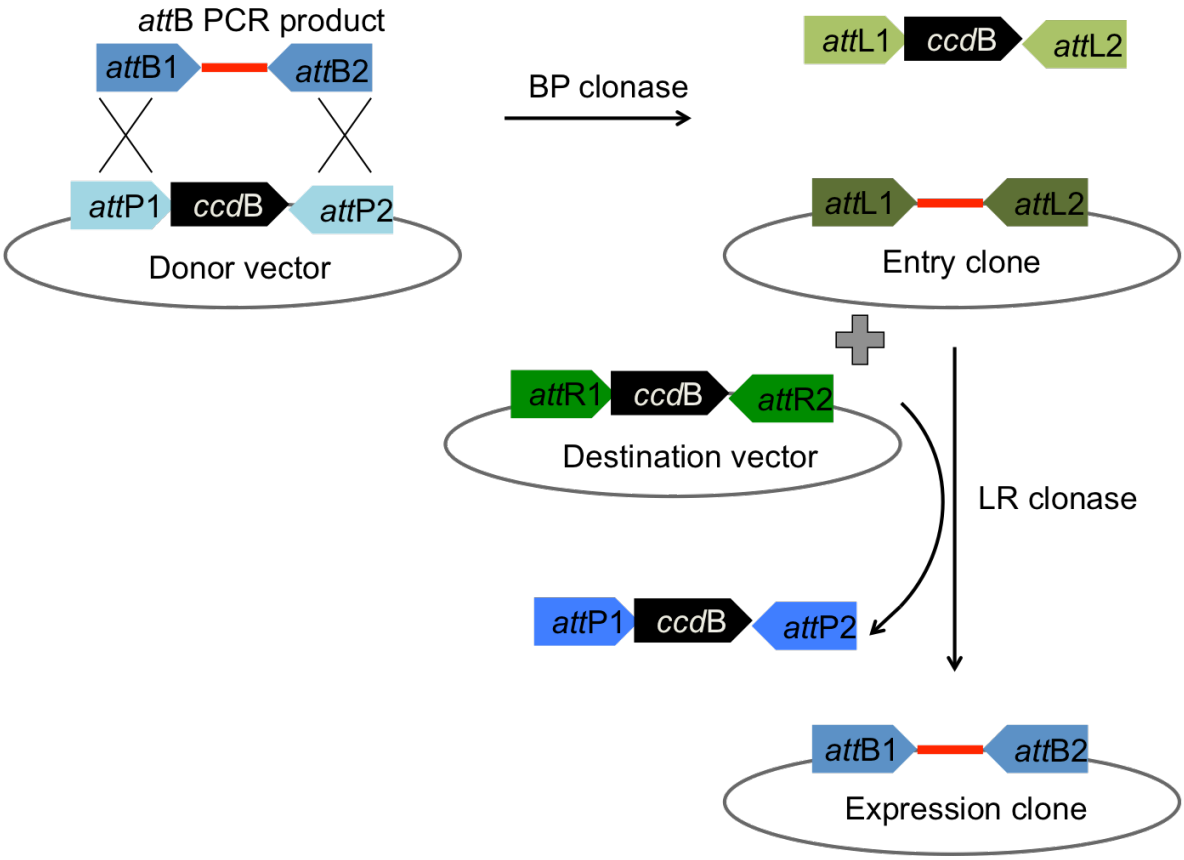


Figure 45: Gateway cloning. BP and LR reactions. The Gateway BP reaction consists of a reaction using BP clonase in addition to the PCR product with flanking *att*B sites and the donor vector containing *att*P sites. The resulting is an entry clone containing *att*L sites flanking gene of interest. The Gateway LR reaction consists of a reaction using LR clonase in addition to the entry clone and the destination vector containing *att*R sites. The resulting product is an expression clone containing *att*B sites flanking gene of interest and ready for gene expression. *att*P and *att*B are specific recombination sites in phage λ and *E.coli* respectively. *ccdB* is a lethal gene used as positive selection marker that acts by killing the background of bacterial cells with no cloned DNA.

3. Cell culture, DNA and siRNA transfection, and treatments.

In cell culture experiments, HEK293 cells, HeLa cells and HeLaN1ΔE-eGFP were grown in DMEM supplemented with 10% FBS, non-essential amino acids, sodium pyruvate, glutamate, and antibiotics: streptomycin and penicillin (Lonza), in 5% CO2 at 37ºC.

HeLaN1ΔE-eGFP cells were provided by Andreas Krämer from Leibniz Institut für Altersforschung–Fritz Lipmann Institut, Jena, Germany.

U2OS Tet-on flp-in cells bearing isogenic transgenes encoding Notch1-Gal4 and K562-Dll4 cells were provided by Inge Van De Walle from Department of Clinical Chemistry, Microbiology and Immunology, Faculty of Medicine and Health Sciences, Ghent University. K562 were grown and maintained in RPMI (Gibco) containing 15% FBS in a 5 %$C0_2$ incubator at 37°C.

13 T-ALL cell lines were grown and maintained in RMPI containing 10-20% FBS in a 5 %$C0_2$ incubator at 37°C.

T-ALL cell lines: ALL-SIL, CTV-1, JURKAT, Karpas 45, KOPT-K1, LOUCY, MOLT-13, MOLT-4, PEER, SUP-T11, SUP-T13, SUP-T7, TALL-1.

HEK293 cells were DNA–transfected with polyethylenimine (PEI) purchased from Sigma, reagent was dissolved in water at 1mg/ml and preserved at -80°C. Transfection with PEI was performed on HEK cells cultured in DMEM at 80% confluence. Medium was changed before transfection and cells were collected 24 hours post-transfection.

HeLa cells and HeLaN1ΔE-eGFP cells were DNA-transfected with lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions and collected 24 hours post-transfection.

SiRNA transfection was performed with Calcium Phosphate using ProFection Mammalian Transfection kit from Promega according to manufacturer's instructions on cells cultured in DMEM at 40-50% confluence. Medium was changed 24 hours later and cells were collected 48h post-transfection.

As for experiments involving both DNA and siRNA transfections, siRNA-transfection was performed as we previously described and 24 hours later after changing medium cells were transfected with DNA using lipofectamin 2000 reagent (Invitrogen) and cells were collected 24h post DNA-transfection.

For proteasomal degradation inhibition, cells were treated of 10µg/ml MG132 for 6 hours before being collected.

For inhibition of γ-secretase we used DAPT at 10µm/ml for 6 hours.

siRNA sequences:

siEXT1: 5′-GGAUUCCAGCGUGCACAUUtt-3′

siFBXW7: 5'- GCAUAGAUUUUAUGGUAAtt-3'

siCtrl: 5'- GGCUGCUUCUAUGAUUAUGtt-3'

4. Immunofluorescence and confocal microscopy

Cells were grown on glass slides in 24-well plates. They were washed in warm PBS three times and fixed with PBS-paraformaldehyde 4% for 15 min and washed twice with PBS. Cells were permeabilized in PBS-Triton X-100 0,5% for 20 min and blocked in blocking solution PBS-FBS 20% for 30 min and washed twice with PBS. Cells were then incubated with corresponding primary antibody diluted in the PBS-Triton X-100 0,5% for 2 hours, washed 3 times with PBS, incubated 1 hour with the corresponding Alexa-conjugated secondary antibodies (Invitrogen) diluted 1/1000 in PBS-Triton solution, washed 3 times with PBS and mounted on glass coverslips using ProLonf Gold Antifade montant with DAPI (life technologies).

Slides were examined by confocal microscopy with Leica TCS SP2 or Nikon A1R confocal microscope with one 60X oil-objective and pictures were taken with a 1024x1024 pixels resolution. Pictures were processed and assembled with Leica LAS AF Lite software.

5. Luciferase reporter assay

Cells were seeded in 24-well plates and transfected with 300ng of either TP1 luciferase reporter plasmid (TP1-luc) or CBF1 reporter plasmid (CBF1-luc) and 30ng of renilla Luciferase (R-Luc). TP1 promoter consists of 8 multimerized RBP-Jκ binding motifs upstream of luciferase. The TP1 promoter is transactivated in a RBP-Jκ dependent manner by the activated forms of the four mammalian Notch receptor and thus, has been often used to read out Notch pathway activity. CBF1 reporter also contains a firefly luciferase gene under the control of multimerized CSL responsive element upstream of a minimal promoter. 24 hours post-transfection luciferase activity was measured in cell lysates.

As for U2OS N1-Gal4 cells they were transfected 300ng of Gal4-firefly luciferase and 30ng R-Luc reporter plasmid. After 24 hours, K562 cells expressing Notch ligands DLL4 or K562 control cells were added to the transfected cells in the presence of tetracycline (2 μg/mL). After 24-hour co-culture, luciferase activity was measured in cell lysates.

Cell lysis and luciferase assays were performed in triplicate using Dual-luciferase reporter assay system from Promega. Luciferase measurements were performed in 96 well plates using DLR automated machine. Firefly luciferase values were normalized to R-luc values and calculated ratio represent luciferase activity.

6. qRT-PCR

Total RNA was extracted using GeneJET RNA Purification Kit (Thermo scientific), DNaseI-treated on the column (Thermo Scientific) and reverse-transcribed with random primers (Thermo scientific). qPCR was performed using SYBER Green detection from Roche and run on Lightcycler 480 (Roche). mRNA quantification was ensured by normalization on GAPDH housekeeping gene. Relative expression levels were calculated for each gene using the ΔCt or ΔΔCt method. Primers used in RT-qPCR are represented in table 5.

Table 5: qRT-PCR primer sequences

Target	sequence reference	Forward primer	Reverse primer
mNotch1	NM_008714	TGCAGAACAACAAGGAGGAG	AGTGGTCCAGCAACACTTTG
eGFP	pEGFP-N1 ORF frame1	TGACCCTGAAGTTCATCTGC	GAAGTCGTGCTGCTTCATGT
GAPDH	2597	TTGCCATCAATGACCCCTTCA	CGCCCCACTTGATTTTGGA
hNotch1		CAACTGCCAGAACCTTGTGC	GCACTCGTCGATCTCCTCAG
FBXW7		CCCGGAGCTGTGCAGCAA	CAGATGTAATTCGGCGTCGTT
HES1		TAGCTCGCGGCATTCCAAGC	GTGCTCAGCGCAGCCGTCATCT
HES5		GAAAAACCGACTGCGGAAGC	GGAAGTGGTACAGCAGCTTC
HEY1		CGAGGTGGAGAAGGAGAGTG	CTGGGTACCAGCCTTCTCAG
EXT1		GCTCTTGTCTCGCCCTTTTGT	TGGTGCAAGCCATTCCTACC
cMYC		CTCCTACGTTGCGGTCACAC	CCGGGTCGCAGATGAAACTC

7. FACS analyses and labeling

Heparan sulfate staining: about 2.10^5 cells were trypsinized, washed twice and resuspended in PBS. Cells were incubated for 1 hour with Heparan sulfate antibody (10E4 anti-HS from USBio) diluted 1/50 in PBS, washed three times and incubated

with Alexa-conjugated secondary antibodies (Invitrogen) diluted 1/1000 in PBS. Cells were analysis a FACSCalibur flow cytometer (BD Biosciences).

Apoptosis assay: 2.10^5 cells were washed twice with cold PBS then resuspended in 100µl of 1X binding buffer, 5µl of V450 Annexin (BD Biosciences) was added, the cells were gently vortexed and incubated for 15 min in the dark, 400µl of 1X biding solutions was added and cells were analyzed by flow cytometry.

8. High-throughput yeast-two hybrid

The yeast two-hybrid assay is a regular and well-established interaction screening method allowing binary interactions detection. In regular molecular biology laboratory settings, it is possible to either screen interactions one-by-one, or to screen libraries of Gal4-AD fusion proteins representing entire proteomes. We performed a large-scale pairwise interaction screen using specific equipment and robotics to perform most of the screenings steps in an automated way. The method described here below corresponds to a standard yeast two-hybrid interaction assay. In our screen we used the hORFeome version 5.1 a collection library of human ORFs cloned from the Mammalian Gene Collection (MGC) resource, representing a resource of ORFs that can be transferred easily to any Gateway compatible destination vectors. This collection contains 15 483 ORFs representing almost half of the human genome, that they are cloned into the pAD-dest-CYH and pDB-dest encoding the Gal4 Activating and DNA-binding domains, respectively. The resulting individual clones were transferred in MATa Y8800 (pAD) and MATα Y8930 (pDB) S. cerevisiae strains (figure 46). As for the ALL-genes ORFs that were not available in the hORF V5.1 collection, they were purchased from addgene and cloned into pAD-dest-CYH and pDB-dest.

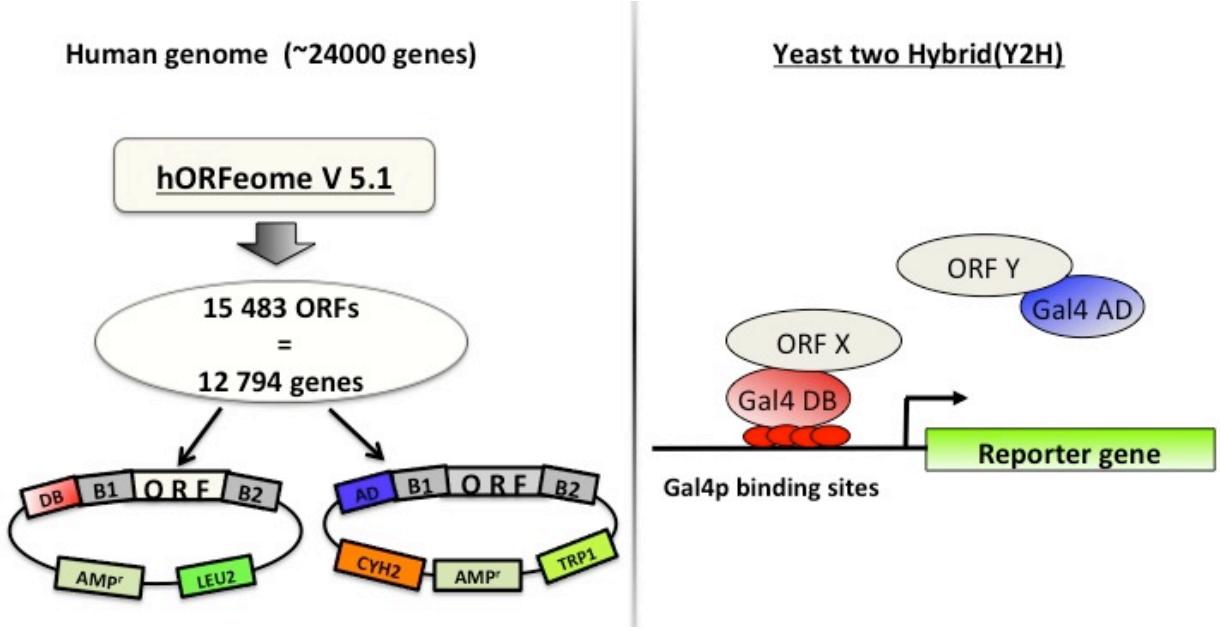


Figure 46: The human ORFeome V5.1 and the Y2H system. The human ORFeome version 5.1 is a collection of 15483 human ORFeomes representing 12794 genes cloned into AD and DB yeast vectors. On the right we represent the yeast two-hybrid system (Y2H) consisting of bait and prey proteins (represented by ORF X and ORF Y) are fused to the Gal4 DNA binding domain (DB) and the Gal4 activating domain (AD). When fusion proteins are produced and bait part of the first interact with prey part of the second, transcription factor of the reporter gene occurs.

One pool of 21 AD of selected genes-Y8800 was mated to each of the 15,483 DB-ORFs Y8930 of the hORFeome v5.1 (CCSB, Dana-Farber Cancer Institute) and each of our cloned DB-genes Y8930; and 165 pools of 94 AD-ORFs of the hORFeome v5.1 were mated to each of the 21 DB-selected genes Y8930. One Y2H screening was performed in the reciprocal orientation, as described in [3]. Colonies positive for the GAL1::HIS3 and GAL1::ADE2 selective markers but negative for autoactivation were selected for PCR-amplification (Zymolyase 20T from Seikagaku Biobusiness, and Platinum® Taq DNA Polymerase from Invitrogen) and identification of interacting proteins by sequencing of the respective AD- and DB-ORFs.

High throughput Y2H screening was performed according to the steps outlined in the pipeline below.

1. Primary screening. In this step, distinct yeast clones (MATα), containing individual DB-ORFs of each of the 21 ALL selected genes arrayed in a 96-well plate, are mated to minipools of yeast containing 100 AD-human ORFs (MATa) from the

ORFeome collection on YEPD plates media. Alternatively, each DB-human ORF from the ORFeome collection is mated to a pool of the AD-ORFs of 21 ALL-genes. After mating yeast are replicated to Sc-Leu-Trp-His selection plates and Sc-Leu-His + 3-AT + 1mg/ml of cycloheximide (CYH). Three to five days post selection, primary positives diploid cells are picked and used for secondary phenotyping.

2. Secondary phenotyping. Primary positives are grown in liquid selecting media (Sc-Leu-Trp-His) to saturation (at least 24 hours) and spotted on selection and control plates: Sc-Leu-Trp-His+1mM of 3-AT; Sc-Leu-His + 1 mM of 3-AT + 1mg/ml of CYH; Sc-Leu-Trp-Ade + 1mM of 3-AT and Sc-Leu-Ade + 1 mM of 3-AT + 1mg/ml of CYH for the Y strains. Replica cleaning is necessary to decrease the background growth and to insure that you start out with comparable amounts of cells in each spot. To determine candidate positive clones we systematically add to selection and control plates, a collection of six diploid control strains. We usually score positives colonies showing a reporter transcriptional activity level equal or higher to that of control 2 (pDEST-AD-E2F1 + pDEST-DB-CYH2-pRB).

3. Identification of interacting pairs: Positives yeast clones are grown overnight on YPD plates. A small amount of yeast cells is lysed using zymolase, an enzyme preparation from Arthrobacter luteus, which effectively lyses live yeast wall. For each positive diploid, two PCRs are then performed to individually amplify DB-ORF (primers DB and Term) and AD-ORF (primers AD and Term). PCR products are then verified on E-Gel 96 agarose 1%, and used as template in sequencing reactions to obtain identities of interacting pairs.

5. Retesting interacting pairs. Candidate Y2H pairs should were verified by two-fold independent experiments. To this end, the location of each clone is identified in the ORFeome database and corresponding fresh archival yeast stocks cultured in Sc-Leu (DB-ORFs) or Sc-Trp (AD-ORFs) liquid media, mated on YPD media plates and transferred two-fold, on two phenotyping selection (His3 and Ade2) and control media (+CYH).

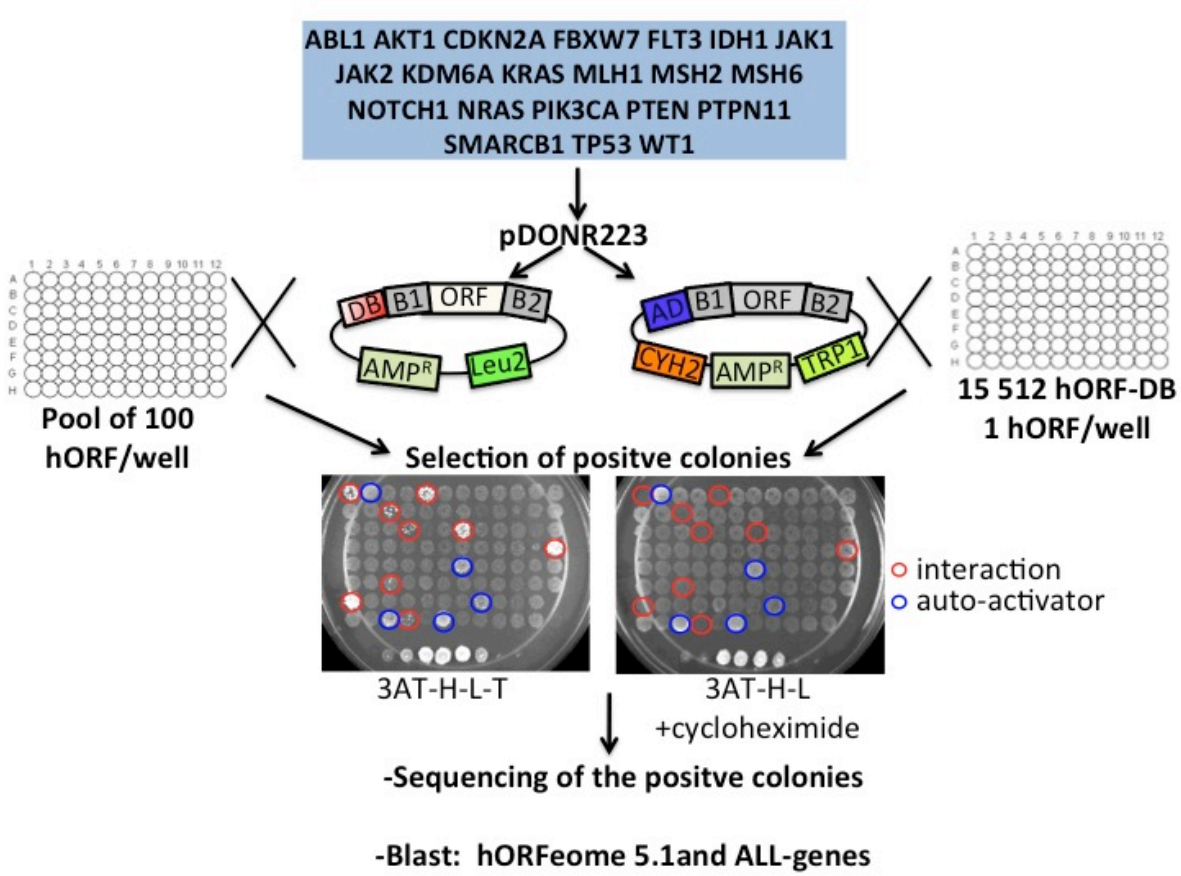


Figure 47: Overview of the Y2H screen performed for genes of interest. A simplified workflow of the Y2H screen shows that individual DB-ORFs of each of the 21 ALL selected genes arrayed in a 96-well plate, are mated to minipools of yeast containing 100 AD-human ORFs from the hORFeome V5.1 collection. Alternatively, each DB-human ORF from the ORFeome collection is mated to a pool of the AD-ORFs of 21 ALL-genes. After mating yeast are replicated to Sc-Leu-Trp-His selection plates and Sc-Leu-His + 3-AT + 1mg/ml of cycloheximide (CYH). Three to five days post selection, primary positives diploid cells are picked and used for secondary phenotyping. Extracted DNA from positive colonies is sequenced and interacting protein partners are identified using Basic Alignment Research Tool (BLAST).

9. Databases and literature PPI curation

9.1. Mutational datasets

COSMIC: is a Catalogue Of Somatic Mutations in Cancer, published by the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/genetics/CGP/cosmic/) [231, 314]. COSMIC combines data gathered from scientific literature with data from high throughput mutation detection experiments performed by the Cancer Genome Project, to identify genes causally critical to the development of different types of

human cancers. The current version of COSMIC database (v75) of somatic mutations in cancer contains information referenced in 22621 scientific reports and 23223 human genes implicated via mutation in cancer, 3702312 coding mutations and 1177397 samples. It includes precise definitions of mutations compared to reference sequences from GenBank, published articles, type of mutation, number of patients and samples affected by these mutations, etc.

Human ORFeome V5.1: (http://horfdb.dfci.harvard.edu/index.php?page=home): It is a collection of 18,414 cloned human Open Reading Frames (ORFs) representing more than 15,000 human genes. We have access to this collection through our collaboration with the Center for Cancer Systems Biology (CCSB) at the Dana Farber Cancer Institute (DFCI), Harvard Medical School, Boston, USA.

9.2. Protein-protein interactions datasets

Human PPIs were collected and verified from the different interactomics databases BioGRID [5], HPRD [237] and IntAct [7]. Only physical PPIs validated at least in two independent references or by two methods were considered as confident and maintained for the analysis.

Information about genes containing mutations in their coding regions is retrieved from the COSMIC database, evaluated; organized and selected genes were submitted for experimental analysis. To establish a catalog of genes and mutations associated with ALL, we used the version 71 of COSMIC, previously downloaded to a local server and we extracted data related to ALL. We developed and implemented a procedure that automatically collects information and check the consistency of changes with the coding sequences and find the corresponding positions on clones from the human ORFeome.

The retrieved information include details provided at either nucleotide or protein level (mutation syntax), sample id (portion of a tumour being examined for mutations), tissue from which the sample originated, histological classification of the sample and the Pubmed id of the article that published the study.

10. Network data analyses and visualization

For each interaction in IntAct, HPRD and BioGRID databases, we retrieved information corresponding to official symbols of genes in interaction (gene names), experimental system and the pubmed id. To this information, we added the entrez gene ids from Entrez gene (NCBI's repository for gene-specific information) [315]. We removed duplicate interactions (interactions found in more than one databases) and we generated a list of all interactions present in all these three databases. From this list, we built a network of interactions, in which we extracted a subnetworks of genes associated with ALL. This procedure was performed using R graph packages.
Network analyses and visualization of protein-protein interactions were carried out with Cytoscape software [38].

11. Protein complementation assay (PCA)

NICD, FBXW7 and selected interactants were cloned in Gaussia luciferase 1 and 2 (GL1 for NICD and its domains, GL2 for FBXW7 and the interactants) using the Gateway cloning technology. HEK293 cells were seeded in 24 well-plates at a concentration 5.10^4 cell/well, then transfected with GL1 or/and GL2 plasmids and 24 hours post-transfection, luciferase activity was measured on lysates transferred into 96-well plate using and automated machine DLR with Renilla luciferase substrate. Normalized luciferase ratio was calculated as follows: NLR= luciferase value GL1+GL2/ (luciferase value GL1+luciferase value GL2). An interaction is considered positive or validated when NLR≥ 3.5.
Cell lysis and luciferase assays were performed in triplicate for each condition.

12. RNA sequencing

Total RNA was extracted from HeLaN1ΔE-eGFP cells (siCTRL, siEXT1, siFBXW7 and combined siEXT1 + siFBXW7), quantified and tested for RNA quality controlled using Agilent 2100 bioanalyzer using the Eukaryote Total RNA Nano assay. Total RNA strands from were used to generate libraries and sequenced by HiSeq2000 sequencer.

Quality control and sequencing were performed by the GenoTranscriptomics platform – GIGA, Liège.

13. EXT1 silencing in zebrafish

Transgenic zebrafish line Tg (Tp1bglob:eGFP)^{um13} was provided by Pr. Parsons from McKusick-Nathans Institute for Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, 21205, USA [316]. Fish of the Tg (Tp1bglob:eGFP)um13 line were maintained according to EU regulations on laboratory animals. Knockdown experiments were performed by injecting embryos at the one- to two-cell stage with 10ng of single splice-blocking morpholino designed specifically for EXT1b.

Couplings fish and harvest embryos: the day before the injection of morpholinos, the adult zebrafish pairs are separated and placed in cages of couplings. 8 to 10 cages, each containing couplings 3 males and 3 females were considered in each experiment. The next day, the separation between the couples is removed to allow mating. As soon as the egg laying occurred, eggs are removed, put in petri dishes and the fish are returned to their original aquarium after assessing their health.

Morpholino injection: harvested zygotes injected with our morpholinos of interest Mo EXT1b by conventional techniques. About 1nL of morpholinos (10 ng/nL) were injected into zygotes with a micro injector. The injected eggs are cultured at 28 ° C in E3 medium for 48 hours. At 24 hours, the embryos are placed in the culture medium E3 + PTU (30mg/L) in order to prevent pigmentation.

Confocal microscope: between 24 and 72 hours, the embryos were observed under a confocal microscope. To do this, living embryos previously “put to sleep” (Tricaine methane sulfonate 80 mg / L for at least 2 minutes) will be mounted in a low melting agarose gel in a culture dish for microscopic observation (type transwell) and covered with culture medium E3. At the end of the experiment, the embryos will be euthanized (Tricaine methane sulfonate 500 mg / L for at least 10 minutes).

Morpholino EXT1b sequence: TATCGTTTTTTGGCCTGCATGTGTC (genetools).

14. Statistical analysis

Graph values are presented as mean +/- standard deviation, calculated on at least three independent experiments. samples were Unless stated otherwise, significance was determined using a two-tailed Student's t-test (comparison of means). P-value thresholds are depicted as follows; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ and ****: $p<0.0001$. It should be noted that standard deviation and mean values in each of the following experiments: luciferase reporter assays, relative mRNA expression determined in qPCR analysis, and the NLR values calculated in the PCA; were calculated for samples tested in triplicate in each independent experiment.

To prioritize ALL-genes, we combined the ranking from separate results (rank per number of mutation, rank per number of samples, rank per degree) by using order statistics. First, ranks are divided by the total number of ranked genes and we calculated the Q statistic [317], which represents the probability of obtaining the observed ranks r by chance, calculated using joint cumulative distribution of order as:

$$Q(r_1, r_2, \ldots, r_N) = N! V_N \qquad V_0 = 1, \qquad V_k = \sum_{i=1}^{k} (-1)^{i-1} \frac{V_{k-i}}{i!} r_{N-k+1}^{i}$$

Where ri is the rank ratio for result i, N is the number of genes used.

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PUBLICATIONS

1. **Daakour S**, Hajingabo LJ, Kerselidou D, Devresse A, Kettmann R, Simonis N, Dequiedt F, Twizere JC; Systematic interactome mapping of acute lymphoblastic leukemia cancer gene products reveals EXT-1 tumor suppressor as a Notch1 and FBWX7 common interactor. In press, BMC Cancer.
Contribution to this publication: performed all the experiments, co-wrote the manuscript.

2. Mansour MR, Reed C, Eisenberg AR, Tseng JC, Twizere JC, **Daakour S**, Yoda A, Rodig SJ, Tal N, Shochat C, Berezovskaya A, DeAngelo DJ, Sallan SE, Weinstock DM, Izraeli S, Kung AL, Kentsis A, Look AT; Targeting oncogenic interleukin-7 receptor signalling with N-acetylcysteine in T cell acute lymphoblastic leukaemia. Br J Haematol. 2015 Jan.
Contribution to this publication: performed protein complementation assays.

3.Hajingabo LJ, **Daakour S**, Martin M, Grausenburger R, Panzer-Grümayer R, Dequiedt F, Simonis N, Twizere JC; Predicting interactome networks perturbations in human cancer: application to gene fusions in acute lymphoblastic leukemia. Mol Biol Cell. 2014 Dec.
Contribution to this publication: performed luciferase assay experiments and western blot analyses.

4. Bergiers I, Lambert B, **Daakour S**, Twizere JC, Rezsohazy R; Hox protein interactions: screening and network building, Methods Mol Biol. 2014.
Contribution to this publication: co-wrote the manuscript.

5. Simonis N, Rual JF, Lemmens I, Boxus M, Hirozane-Kishikawa T, Gatot JS, Dricot A, Hao T, Vertommen D, Legros S, **Daakour S**, Klitgord N, Martin M, Willaert JF, Dequiedt F, Navratil V, Cusick ME, Burny A, Van Lint C, Hill DE, Tavernier J, Kettmann R, Vidal M, Twizere JC; Host-pathogen interactome mapping for HTLV-1 and -2 retroviruses. Retrovirology. 2012 Mar.
Contribution to this publication: performed western blot analyses.

ANNEXES

Annexes are available on the following internet references:

Table S1A.xlsx: Mutations associated to ALL in cancer gene census
Table S1B.xlsx: Driver genes affected by subtle mutations among the ALL census genes.
Tables S2A.xlsx: Interactions between ALL-related proteins identified in the literature, in 2 different scientific reports and by 2 different methods.
Table S2B.xlsx: degree of connectivity for interacting ALL-related proteins
Table S3.xlsx: Co-occurrence of mutations in ALL samples
Table S4.xlsx: Ranking ALL driver genes
Table S5A.xlsx: Interactions of ALL-related proteins identified in literature-curated data (LCI) and in our yeast two-hybrid screen (Y2H)
Table S5B.xlsx: Degree of connectivity for the LCI and Y2H interactors with ALL-related protein
Table S6A.xlsx: Genes expression from RNA sequencing analysis in siEXT1 condition
Table S6B.xlsx: Upregulated genes in siEXT1 condition
Table S6C.xlsx: Downregulated genes in siEXT1 condition
Table S6D.xlsx: Genes expression from RNA sequencing analysis in siFBXW7 condition
Table S6E.xlsx: Upregulated genes in siFBXW7 condition
Table S6F.xlsx: Downregulated genes in siFBXW7 condition
Table S6F.xlsx: Co-regulated genes by EXT1 and FBXW7
Table S7.xlsx: Molecular function enrichment for the common deregulated genes using Toppfun.
Timelapse video.avi: EXT1 reduces NOTCH1 levels in HelaNotch1ΔEeGFP cells