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Oncogenic role of miR-223 in Notch3 induced

T Cell Acute Lymphoblastic Leukemia

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Abstract

Notch Signaling is one of the important signaling pathway in the development of T-Lymphocytes . Its role as transcriptional regulator during the development of T-cells has been well established. Also, it has been well established that miRNAs are responsible for the regulation of many important cellular functions, but the transcriptional regulators of miRNAs have been poorly understood. In order to identify microRNAs regulated by oncogenic Notch3 signaling, we performed microarray-based miRNA profiling of T-cell acute lymphoblastic leukemia (T-ALL) models, differentially expressing Notch3. We identified 7 miRNAs to be regulated by Notch3 modulation in different models. Among them, miR-223 putative promoter analysis revealed a conserved CSL/RBPJ κ binding site which was nested to p65. Luciferase & ChIP assays on wild type and mutated promoter of miR-223 showed direct regulation by NF κ B (p65) and indirect regulation by Notch3 transcriptional complex. We also demonstrated that tumor suppressor FBXW7 is regulated by miR-223, and analysed its oncogenic effects on cell growth and cell cycle by overexpression or knockdown of miR-223. Moreover we saw an increase in miR-223 expression and NF κ B activation in GSI resistant T-ALL cell lines treated with GSI, without affecting their proliferation rate. The same cell lines deleted for NF κ B pathway, showed less expression of miR-223 when compared with wild type and lost their ability to resist GSI treatment, suggesting the involvement of miR-223 in the mechanism of GSI resistivity. Thus we conclude that miR-223 has an oncogenic role in Notch3 induced T-Cell Acute Lymphoblastic Leukemia and could play a significant role in GSI resistivity.

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1 Introduction

1.1 Notch Signaling pathway.

1.1.1 General

Notch signaling is one of the fundamental signaling mechanisms essential for proper embryonic development, cell fate specification, and stem cell maintenance. The Notch gene was discovered almost 90 years ago by Morgan and colleagues in *Drosophila*, who observed that X-linked dominant mutations in *Drosophila* caused irregular Notches at the wing margin. The study of the embryonic lethal phenotype caused by complete lack of Notch function [1], and its complex allelic series and genetic interactions [2] brought Notch to the forefront, so that in the mid-1980s the *Drosophila* Notch gene product was identified [3] [4]. Notch is a local signaling mechanism that is evolutionarily conserved throughout the animal kingdom. In *Drosophila*, Notch encodes a receptor that is activated by two different membrane bound ligands called Delta and Serrate. Mammals have four Notches (Notch1-4) (Figure 1) and five ligands: Delta-like-1, Delta-like-3, Delta-like-4, Jagged-1 and Jagged-2 (Figure 2)

1.1.2 Components of Notch signaling Pathway

A large number of studies, mainly conducted on *Drosophila*, *Caenorhabditis* and vertebrates, have characterized the molecular properties and functions of the main components and auxiliary factors of the Notch pathway. These are strongly conserved in bilaterians . Both, the Notch receptors and its ligands are type I transmembrane proteins with a modular architecture. The core components include: i) metalloproteases ADAM 10 and 17 (involved in the second cleavage (S2) after ligand binding) [5] [6] [7] ii) γ -secretase complex (Presenilin-Nicastrin-APH1-PEN2) (involved in the final cleavage (S3)) and iii) Co-repressor complex composed of CBF1, Su(H), Lag-1)/Ncor/SMRT/Histone Deacetylase (HDAC) . In addition to these core components of the Notch pathway, several other proteins are involved in the Notch signalling regulation in several cellular contexts, and act either on the Notch receptor or on the ligand DSL. Some of these regulators modulate the amount of receptor available for signalling [8]. Numb, the NEDD4/Su(dx) E3 ubiquitin ligases, and Notchless are important negative regulators, while Deltex is considered to antagonize NEDD4/Su(dx) and therefore to be an activator of Notch signaling [9]. Strawberry Notch (Sno), another modulator of the pathway whose role is still unclear, seems to be active downstream and disrupts the CSL repression complex [10]. Regulation may also occur at the level of ligand activity via the E3 ubiquitin ligases Neuralized and Mindbomb [11, 12]. The list of the components of Notch signaling pathway has been mentioned in Table1 for references.

Table .1

Different Components involved in Notch Signalling Pathway

Description	PDB ID code	Species	Method	Reference
Extracellular Notch				
LNR-A domain (NOTCH1)	1pb5	Human	NMR	(Vardar et al., 2003)
Ligand-binding, EGF-like repeats 11-13 (NOTCH1)	1toz	Human	NMR	(Hambleton et al., 2004)
LNR-HD (NOTCH2)	2oo4	Human	X-ray	(Gordon et al., 2007)
Ligand-binding, EGF-like repeats 11-13 (NOTCH1)	2vj3	Human	X-ray	(Cordle et al., 2008a)
Notch ligands				
JAGGED1, DSL domain and EGF-like repeats 1-3	2vj2	Human	X-ray	(Cordle et al., 2008a)
Intracellular Notch				
ANK (Notch)	1ot8	Fly	X-ray	(Zweifel et al., 2003)
CSL(LAG-1)-DNA	1ttu	Worm	X-ray	(Kovall and Hendrickson, 2004)
Ankyrin repeats 3-7 (Notch1)	1ymp	Mouse	X-ray	(Lubman et al., 2005)
ANK (NOTCH1)	1yyh	Human	X-ray	(Ehebauer et al., 2005)
ANK (NOTCH1)	2f8x	Human	X-ray	(Nam et al., 2006)
ANK(NOTCH1)-MAML1-CSL(RBP-J)-DNA	2f8y	Human	X-ray	(Nam et al., 2006)
RAMANK(LIN-12)-MAM(SEL-8)-CSL(LAG-1)-DNA	2fo1	Worm	X-ray	(Wilson and Kovall, 2006)
CSL(Rbp-j)-DNA	-	Fly	EM	(Kelly et al., 2007)
Hydroxylated ANK (Notch1)	2qc9	Mouse	X-ray	(Coleman et al., 2007)
RAM(LIN-12)-CSL(LAG-1)-DNA	3brd	Worm	X-ray	(Friedmann et al., 2008)
RAM(LIN-12)-CSL(LAG-1)-DNA	3brf	Worm	X-ray	(Friedmann et al., 2008)
CSL(RBP-J)-DNA	3brg	Mouse	X-ray	(Friedmann et al., 2008)
Extracellular Notch-associated molecules				
TACE	1bkc	Human	X-ray	(Maskos et al., 1998)
Maniac fringe	2j0a	Mouse	X-ray	(Jinek et al., 2006)
Maniac fringe + UDP/Mn	2j0b	Mouse	X-ray	(Jinek et al., 2006)
γ -Secretase	-	Human	EM	(Ogura et al., 2006)
γ -Secretase	-	Human	EM	(Lazarov et al., 2006)
Intracellular Notch-associated molecules				
Suppressor of Deltex [Su(dx)] WW domains 3-4	1tk7	Fly	NMR	(Fedoroff et al., 2004)
Deltex ring-H2 finger	1v87	Mouse	NMR	(Miyamoto et al., 2004)
Deltex WWE domain	2a90	Fly	X-ray	(Zweifel et al., 2005)
Su(dx) WW domain 4/phosphorylated Notch peptide	2jmf	Fly	NMR	(Jennings et al., 2007)
S-phase kinase-associated protein 1 (SKP1)/F-box and WD repeat domain-containing 7 (FBW7)	2ovp	Human	X-ray	(Hao et al., 2007)
SKP1/FBW7/cyclin-E C-terminal degron	2ovq	Human	X-ray	(Hao et al., 2007)
SKP1/FBW7/cyclin-E N-terminal degron	2ovr	Human	X-ray	(Hao et al., 2007)
Factor Inhibiting HIF-1 (FIH)/Notch1 peptide	N/A	Mouse	X-ray	(Coleman et al., 2007)
FIH/Notch1 peptide	N/A	Mouse	X-ray	(Coleman et al., 2007)

PDB, protein data bank. Note that, in this table, ANK refers to the ankyrin domain.

1.1.3 Biogenesis of Notch receptor

The Notch receptors are synthesized as single precursor proteins that are proteolytically cleaved in the Golgi (at site S1) during their transport to the cell surface by a furin-like protease. This cleavage generates a heterodimeric receptor consisting of a Notch Extracellular subunit (N^{EC}) that is noncovalently linked to a second subunit containing the extracellular heterodimerization domain and the transmembrane domain followed by the cytoplasmic region of the Notch receptor. The extracellular part of the receptors contains 29-36 epidermal growth factor (EGF) like repeats involved in ligand binding, followed by three cysteine-rich LIN12 repeats that prevent ligand-independent activation and a hydrophobic stretch of amino acids mediating heterodimerization between N^{EC} and Notch Intracellular Domain (N^{ICD}). The cytoplasmic tail of the receptor also known as N^{ICD} harbors multiple conserved elements including nuclear localization signals, as well as protein-protein interaction and transactivation domains. N^{ICD} contains a RAM23 domain [13] six ankyrin/cdc10 repeats involved in protein-protein interactions [14], two nuclear localization signals (N1 and N2), a transcriptional activation domain (TAD) that differs among the four receptors, and a PEST sequence [rich in proline (P), glutamic acid (E), serine (S) and threonine (T)] that negatively regulates protein stability [15]

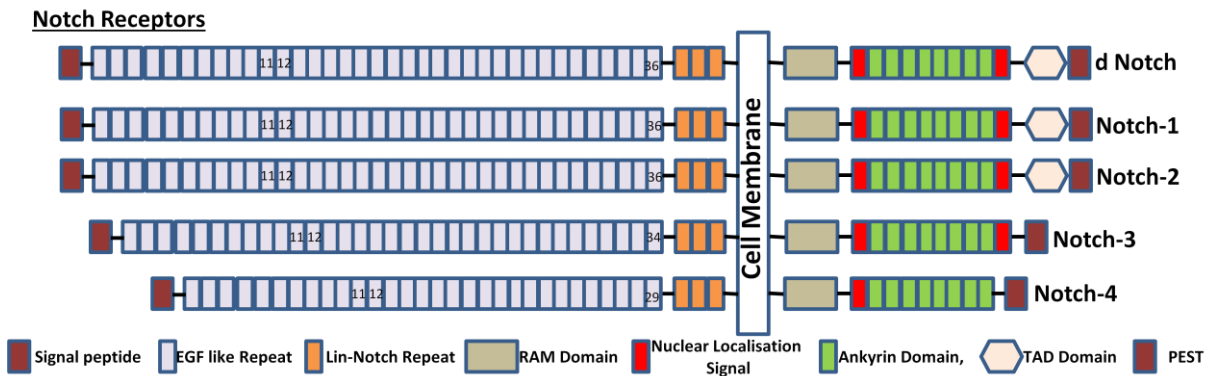


Figure 1: Structure of Notch receptors in *Drosophila* and mammals. Diagrammatic representation of the *Drosophila* Notch (dNotch) receptor and the four known mammalian receptors. The Notch proteins are expressed on the cell surface as heterodimers composed of a large extracellular domain non-covalently linked to the intracellular domain. The extracellular domain of all Notch receptors contains epidermal growth-factor-like repeats (EGFLR) and three LIN Notch (LNR) repeats. The intracellular domain contains the RAM23 domain and seven Ankyrin/CDC10 repeats (ANK), necessary for protein- protein interactions. In addition, Notch receptors 1-3 contain two nuclear localization signals (NLS) compared to one NLS in Notch4. The NSL is necessary to target the intracellular domain to the nucleus where the transcriptional activation domain (TAD) activates downstream events. All four Notch receptors contain a C-terminal Pro Glu Ser Thr (PEST) sequence for degradation.

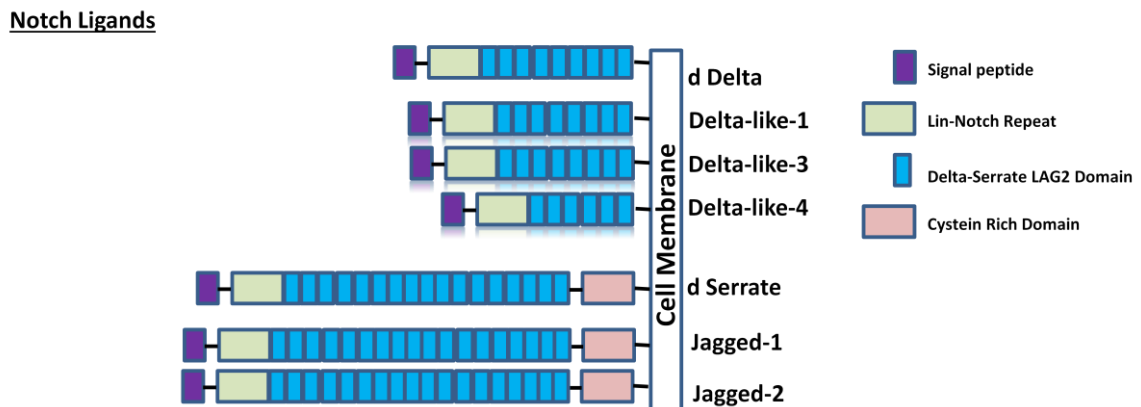


Figure 2: Structure of the DSL ligands in *Drosophila* and mammals. Mammals have five DSL family members. Delta-like 1, 3 and 4 are homologs of *Drosophila* Delta (dDelta), while Jagged1 and 2 are homologous to *Drosophila* Serrate (dSerrate). DSL ligands are transmembrane proteins of which the extracellular domain contains a characteristic number of EGF-like repeats and a cysteine rich N-terminal DSL domain. The DSL domain is a conserved motif found in all DSL ligands and required for their interaction with Notch. Serrate, Jagged1, and Jagged2 contain an additional cysteine rich domain (CRD).

1.1.4 Mechanism of Notch Signalling

Notch signaling is initiated by ligand-receptor interaction between neighboring cells (Figure 3), leading to two successive proteolytic cleavages of the receptor. The first is mediated by metalloproteases of the ADAM family, which cleave the receptors 12–13 amino acids external to the transmembrane domain. The second cleavage is mediated by γ -secretase, a complex that contains presenilin, nicastrin, PEN2 and APH1 [8]. The shedded extracellular domain is endocytosed by the ligand-expressing cell, a process that is dependent on monoubiquitinylation of the cytoplasmic tail of the ligands by E3-ubiquitin. N^{ICD} then translocates to the nucleus and acts as a transcriptional co-activator. N^{ICD} cannot bind directly to DNA, but heterodimerizes with the DNA binding protein RBPJ κ (recombination signal sequence-binding protein J κ , also called CSL, CBF1, Su(H) and LAG-1) and activates transcription of genes containing RBPJ κ binding sites. Interestingly, RBPJ κ was originally identified as a repressor of transcription by Vales and colleagues [16]. The RBPJ κ mediated repression could be relieved by the recruitment of distinct transcription factor such as Notch. Thus the RBPJ κ activator/repressor paradox was resolved with the realization that repression and activation via RBPJ κ involves the recruitment of specific protein complexes, which influence the transcription of target genes in a positive or negative fashion. In the absence of ligand, hence without nuclear N^{ICD} , RBPJ κ is retained at the gene regulatory elements of Notch target genes repressing their transcription, through the recruitment of corepressor complexes. Transcriptional repression seems to be mediated by different mechanisms. Based on biochemical experiments, it was proposed that RBPJ κ can interact directly with TFIID, or can recruit histone deacetylase-containing complexes. Previously, at least three different interactions between RBPJ κ and corepressor complexes have been described: i) a complex containing SMRT/mSin3A/ HDAC-1 (SMRT, Silencing Mediator for Retinoic acid and Thyroid hormone receptor; HDAC-1, histone deacetylase-1) or ii) NCoR/mSin3A/HDAC-1 complex and a iii) CIR/SAP30/HDAC-2 complex. So far, the functional relevance of these

biochemical findings still remains to be seen. Recently it was characterized that the RBPJ κ -associated repressor complex is composed of corepressors RBPJ κ , SHARP (SMRT and HDAC associated repressor protein), CtBP (C-terminal binding protein) and CtIP (CtBP interacting protein). N^{ICD} binding to RBPJ κ is crucial for the switch from repressed to activated state. N^{ICD} first displaces corepressors from RBPJ κ , resulting in derepression of promoters and subsequently recruits a coactivator complex to activate the transcription of Notch target genes. In the initial phase of transcriptional activation complex assembly, N^{ICD} forms multimers. Subsequently, the N^{ICD} multimer forms a complex with Skip, which then provides a docking site to recruit Maml1 and forms a pre-activation complex. The interaction between the pre-activation complex and CSL results in formation of the transcriptional activation complex on DNA. Mastermind is a glutamine-rich transcriptional co-activator protein that is localized to the nucleus [17], [18]. A short, approximately 75-residue, N-terminal domain of Mastermind is required for binding to the CSL- N^{ICD} complex, which additionally requires the three conserved domains of CSL (NTD, BTD, and CTD) and the ANK domain of N^{ICD}. Mastermind has dual roles of both activating Notch target gene transcription through the direct binding of CBP/p300 and promoting hyperphosphorylation and degradation of N^{ICD}.

Mechanism of Notch Signalling (Canonical)

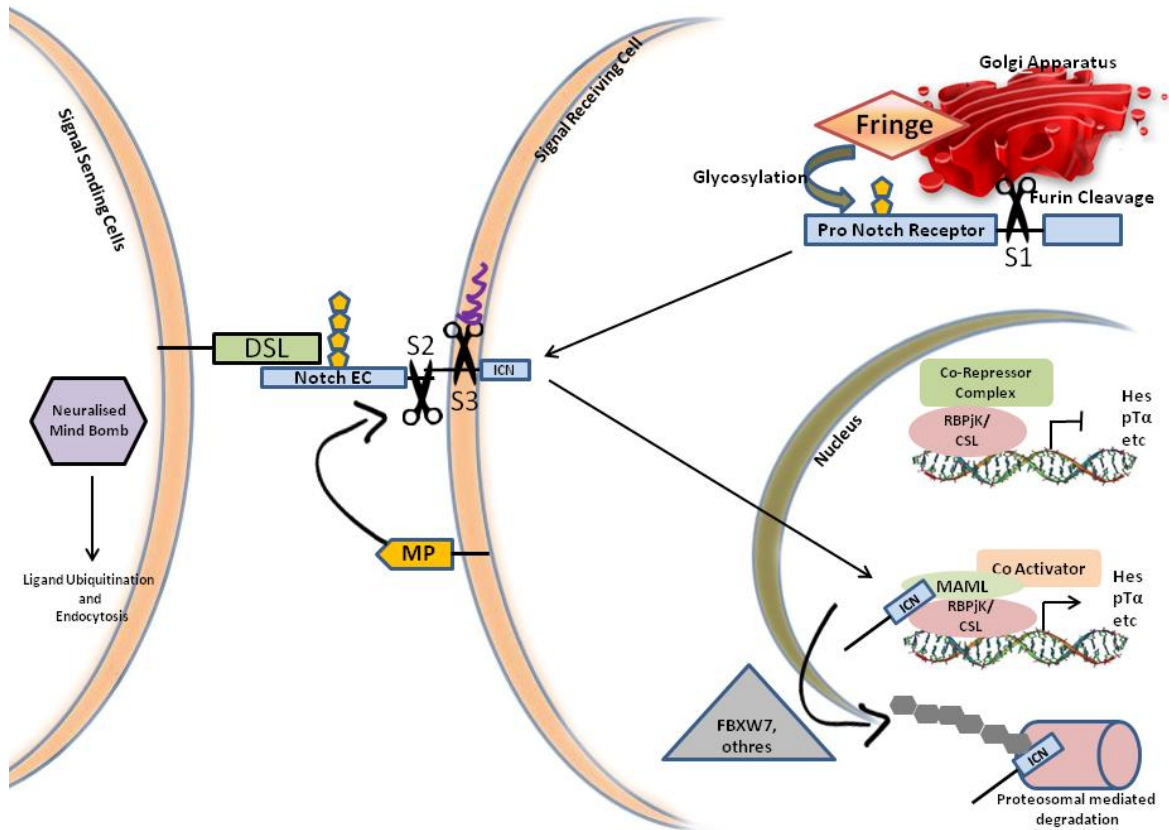


Figure 3: Summary of the main features of Notch signalling. Three proteolytic cleavage steps are required for canonical Notch receptor signalling. The first proteolytic cleavage step (S1 cleavage) is mediated by Furin, occurs in the *trans*-Golgi and produces a heterodimer composed of a ligand-binding Notch extracellular domain (N^{ECD}) and a single-pass transmembrane signalling domain referred to as the Notch intracellular domain (N^{ICD}). The functional importance of this cleavage is still somewhat unclear. The association of N^{ECD} and the transmembrane portion of the receptor heterodimer is dependent on non-covalent interactions. Pathway activation occurs when the N^{ECD} binds to Delta-Serrate-LAG2 (DSL) ligands that are expressed on the membrane of neighbouring cells. This *trans* interaction results in the second proteolytic event (S2 cleavage) of the Notch receptor, which clears most of the N^{ECD} from the outer portion of the membrane, a process mediated by the TACE (also known as ADAM17) metalloproteinase. The N^{ECD} is subsequently released and internalized through endocytosis by the ligand-expressing cell, where it undergoes lysosomal degradation. Subsequently, γ -secretase cleaves the tethered receptor near the inner leaflet of the membrane (S3 cleavage) in the Notch-expressing cell, producing the transcriptionally active N^{ICD} , which translocates to the nucleus through a poorly understood process. In the nucleus, the N^{ICD} interacts with *Drosophila melanogaster* Suppressor of Hairless (SU(H)) and the transcriptional co-activator, Mastermind (MAM) — the mammalian orthologues of which are CBF1-SU(H)-LAG1 (CSL) and Mastermind-like (MAML) proteins, respectively — thereby inducing transcription of target genes, by converting CSL into a transcription activator through the exchange of co-repressors for co-activators. Many Notch target genes encode transcriptional regulators, which influence cell-fate decisions through the regulation of basic helix-loop-helix hairy and enhancer of split (HES) proteins: Hairy and Enhancer of split (E(SPL)) in *D. melanogaster* and their mammalian orthologues HES1 and HES5. The HES proteins

subsequently regulate the expression of genes involved in Notch-dependent cell-fate determination, such as apoptosis, proliferation or differentiation. By contrast, expression of ligands and the Notch receptor on the same cells results in *cis* inhibition of Notch signals and receptor degradation. Recycling the receptor through the endocytic pathway has been shown to be important for receptor and ligand maturation, non-canonical signalling and degradation (BOX 2). Notch activity is regulated by ubiquitylation of nuclear N^{ICD} by the E3 ubiquitin ligases, SEL-10 in *Caenorhabditis elegans* and Suppressor of Deltex (SU(DX)) in *D. melanogaster*, leading to N^{ICD} degradation, thus allowing the cell to become ligand-competent once again. The ubiquitylation status of the receptor (by Kurtz, Deltex and Shrub) in the multi-vesicular bodies can also determine whether Notch continues to signal or undergoes proteasomal degradation. Additionally, signal attenuation is achieved through lysosomal degradation of the N^{ICD}.

1.1.5 Notch Signalling in Multipotent Haematopoietic Cells

The first definitive hematopoietic stem cells (HSCs) capable of generating adult-type erythrocytes, myeloid and lymphoid cells arise in murine embryos at around embryonic day 9.5 and express Notch1, Notch2, and Notch 4 [19]. However, Notch1, but not Notch2, is required to generate definitive HSCs during embryonic development. The *Scl*, *Gata2* and *Runx1* genes, which encode transcription factors required for definitive hematopoiesis, are down-regulated in *Notch1*^{-/-} [19] and *CSL*^{-/-} [20] embryonic HSCs, suggesting that Notch1/CSL-dependent signaling regulates their induction. Functions of Notch signaling in postnatal HSC self-renewal and maintenance have been less clear. Notch ligands in vitro, or overexpressing both the constitutively active Notch alleles [21], [22] [23], or the Notch downstream targets such as *Hes1* [24], [25], have suggested that Notch increases self-renewal and decreases differentiation of hematopoietic progenitors. Moreover, genetic manipulations that appear to increase *Jagged1* expression in bone marrow stem cell niches enhance self-renewal of adult HSCs [26]. However, the notion that Notch signaling critically regulates HSC self-renewal in vivo has not been clearly supported by loss-of-function data. For example, conditional ablation of *Notch1* or *Jagged1* did not reveal defects in HSC maintenance even in competitive reconstitution assays [27] , but potential functional redundancy with other Notch ligands and receptors could not be excluded. Nonetheless, HSCs lacking CSL, which therefore lack all canonical Notch signaling, generate normal numbers of short-lived myeloid cells [28], suggesting that HSC maintenance is not compromised by loss of Notch signaling. Globally inactivating canonical Notch signaling in HSCs by expressing a mutant version of the co-activator MAML, which binds N^{ICD} to dominantly inhibit CSL-dependent Notch activation, revealed that canonical Notch signaling is dispensable in maintaining adult HSCs, contradicting an earlier study that concluded that CSL-dependent Notch activation enhances adult HSC differentiation at the expense of self-renewal [29].

Although dispensable for HSC maintenance, recent *in vitro* and *in vivo* data strongly implicate Notch signaling in early stages of myeloerythroid differentiation. HSCs cultured with OP9 bone marrow stromal cells expressing Dll1 underwent CSL-dependent megakaryocyte specification [30]. Fresh *ex vivo* megakaryocyte-erythrocyte precursors expressed Notch4 and several direct Notch target genes, indicating, Notch4 as a possible mediator of Notch-induced megakaryocyte development. However, neither the Notch receptor(s) nor ligand(s) that drives this hematopoietic outcome *in vivo* was identified. Interestingly, recent evidence implicates dysregulated activation of CSL-dependent Notch signaling in acute megakaryoblastic leukemia [31]. In multipotent progenitors that have lost erythro-megakaryocytic potential, maintenance of Notch1 expression is part of a lymphoid specification program induced by transcription factors such as Ikaros, PU.1, E2A, and Mef2c [32], [33], [34] [35]. Notch1 expression in such lymphoid-primed multipotent progenitors (LMPPs) specifically depends on E2A [34], [36], and perhaps also on Ikaros [37]. Current evidence indicates that thymus-seeding progenitors reside within a Flt3⁺ and CCR9⁺ subset of early lymphoid-biased progenitors in the bone marrow [38], [39]. As discussed further, Notch1 is specifically required to generate intrathymic T cell precursors, but is dispensable for B lymphoid and myeloid cell development in bone marrow. Therefore, Notch1 expression in pre-thymic T cell progenitors such as LMPPs likely represents one of the earliest events in specification of the T cell lineage. Given that several Notch ligands are expressed in the bone marrow [40], there must be some mechanisms to prevent LMPPs from activating Notch1 and generating T cells prior to thymic seeding. Indeed, Notch1 activation and T cell development in bone marrow progenitors are actively repressed by the lymphoma-related transcriptional repressor [41]. This repression can apparently be overcome by retroviral expression of Dll4 in bone marrow cells, which induces ectopic T cell development up to the CD4/CD8 double-positive (DP) stage in the bone marrow [42], [43]. Importantly, these and previous studies [44], [45], [46] demonstrated that with the exception of failing to promote robust Notch1

activation, the bone marrow provides a suitable microenvironment for supporting T lymphopoiesis. Interestingly, Notch/CSL signaling promotes T cell development up to the DP stage in the spleen and lymph nodes of irradiated mice, but this process appears to be suppressed in the absence of lymphopenia [47]. This extrathymic T cell development may have therapeutic relevance in a bone marrow transplantation setting. Additional studies are needed to identify the Notch receptors and ligands involved in this process and will likely identify additional regulatory mechanisms that prevent Notch-induced extrathymic T cell development.

1.1.6 T-Cell Development and Notch Signalling

T lymphocytes are part of adaptive immune system that recognizes and eliminates specific foreign antigens. T lymphocytes arise in the bone marrow and migrates to the Thymus Gland to mature into CD4 or CD8 cells. Mature T cells express a unique antigen binding molecules, the T cell receptor (TCR) on their membrane, and can only recognize antigen that is bound to cell membrane protein called major histocompatibility complex (MHC) molecules. T cell that recognize self MHC molecules are selected for survival during positive selection [48]. However, T cells that react too strongly with self-MHC are eliminated through negative selection [48]. Maturation of T cells consists of six major steps (Figure 4). Thymocytes early in development lack detectable CD4 and CD8, and are referred to as double negative (DN). DN T Cells can be sub divided into 4 subsets (DN1-4) characterized by the presence or absence of cells surface molecules in addition to CD4 and CD8, such as CD44, an adhesion molecules, and CD25, the α chain of the IL-2 receptor. The cells that enter the thymus, DN1, are capable of giving rise to all subsets of T cells, and are phenotypically CD44^{hi} and CD 25⁻. Once DN1 cells encounter the thymic environment, they begin to proliferate and express

CD25, becoming CD44^{low}, and CD25⁺, they are called DN2 cells, where rearrangement of genes for the TCR chains begins. As cells progress to DN3 cells, the expression of CD44 is turned off and cells stop proliferating to start TCR β chain rearrangement. Upon its completion, the DN3 cells quickly progress to DN4 where the level of CD 25 decreases. Both CD4 and CD8 receptor are expressed in the Double positive (DP) stage, where rapid cell division increases the diversity of the T cells repertoire. After the rapid proliferation, TCR α chain rearrangement starts, which is then followed by positive and negative selection. Cells that fail to make productive TCR gene arrangement or thymic selection are eliminated by apoptosis. The cells that survive will develop into immature Single Positive (SP) CD4 or CD8 thymocytes. These Single Positive thymocytes undergo additional negative selection and migrate to the medulla, where they pass from the thymus to the circulatory system. During the development of the T Lymphocytes, Notch expression and thus signaling plays an important role. Notch1 expression is high in early DN thymocytes, low in DP cells, and intermediate in CD4 and CD8 SP cells [49]. Conversely, when compared to Notch1, Notch3 expression levels are significantly higher in DN and DP thymocytes (Figure 5), although they are specifically downregulated past the DN to DP transition and stay at very low to undetectable levels in mature T lymphocytes [50], [51]. Moreover, a significant role for Notch1 has been suggested in the initial T cell lineage commitment of bone marrow-derived common lymphoid precursors [52], [53] and in intrathymic T cell lineage choices, by favoring the CD8 versus CD4 and $\alpha\beta$ versus $\gamma\delta$ T cell lineage decision [54], [55], [56] as well as its requirement for a correct VDJ κ rearrangement [57]. Conversely, a specific role of Notch3 at the pre-TCR checkpoint has been suggested. Indeed, Notch3 expression has been demonstrated to be preferentially upregulated by thymic stromal cell-derived signals in DN immature thymocytes prior to their transition to more mature DP cells and to be subsequently downregulated across the DN to DP transition [58].

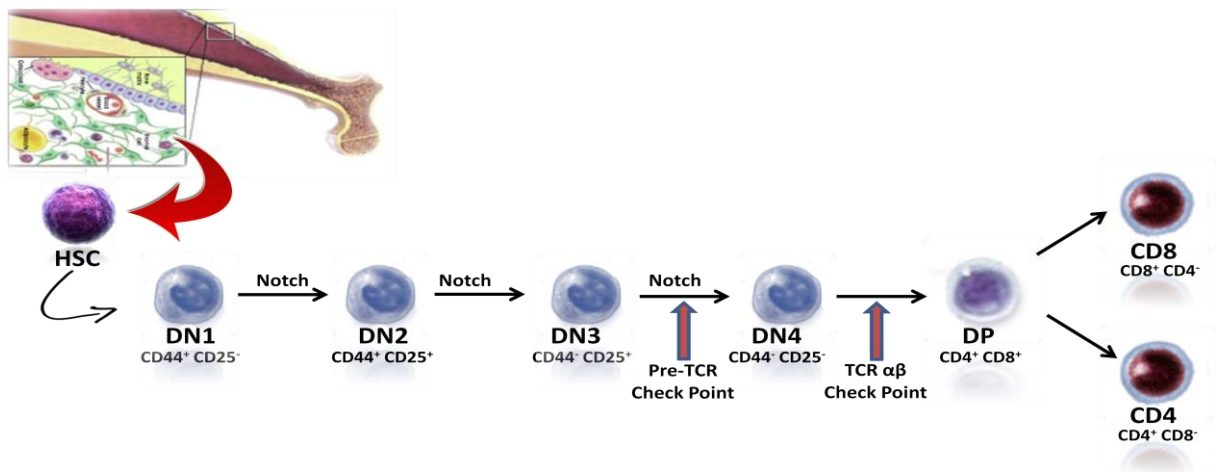


Figure 4 : Notch in T-Cell Development : Notch signalling is required for commitment of a subset of double-negative 1 (DN1) cells to early thymocyte progenitors (ETPs) and for T-cell maturation up to the double-positive (DP) stage; the CD4 versus CD8 lineage commitment seems largely unaffected by Notch 1. T-cell precursors form in the bone marrow and migrate to the thymus to become double-negative (DN) thymocytes. The Proliferation and differentiation of DN thymocytes occurs in the thymus. Rearrangement of T-cell receptor- β (TCR β) genes (usually together with TCRG and TCRD genes) in such thymocytes is one of the first significant steps in differentiation. If $\gamma\delta$ rearrangement succeeds first, the cell is identified as a $\gamma\delta$ T-cell. If the TCR β gene is rearranged first, the cell becomes an $\alpha\beta$ T-cell. β - and α -chain surrogates are then expressed on the cell surface together with CD4 and CD8, making the cell a double-positive thymocyte. In addition to the Delta-like 1 (DLL1) or DLL4–Notch-1 interactions (D1) that are required for T-cell development, the differentiation of late DN T-cell precursors to DP T-cell precursors also requires (pre-) TCR signalling.

1.1.7 Role of Notch signaling in Leukemogenesis

The role of Notch in the development of leukemia was from the observation that in rare cases of human T cell acute lymphoblastic leukaemia (T-ALL), Notch1 was truncated by $t(7;9)(q34;q34.3)$ chromosomal translocation, leading to the production of a dysregulated constitutively active N1^{ICD} [59]. Subsequently, several research groups have generated in vitro and in vivo experimental models, involving constitutively active Notch receptors or other components of its transduction pathway, in an attempt to understand the cellular and molecular events involved in the pathogenesis of the human disease. The different in vivo experimental systems were all based on a gain-of-function approach, in most cases using T cell-specific Lck promoter-dependent expression of the constitutively active N1^{ICD} or N3^{ICD}, in transgenic mice [60] [61] [54]. Alternatively, retroviral vector-driven N^{ICD} constructs were utilized to transduce bone marrow precursors, in order to reconstitute in lethally irradiated mice [62] [63]. The outcome of the different experimental systems appears to be quite different. Indeed, when experimental systems involving Notch1 are considered, it appears that mice injected with retrovirally-transduced bone marrow precursors develop a T cell leukemia with a significantly higher penetrance than Lck promoter N1^{ICD} transgenic mice, even when comparable Notch constructs were used [60] [61] [62] [63]. In this regard it is interesting to note that the retroviral-driven N1^{ICD} expressing bone marrow precursors have been shown to first repopulate the bone marrow (Days 22 postinjection) and later on the lymphoid organs, including the thymus, (after Day 40 postinjection) of irradiated recipient mice and between Days 65 and 110 all of the injected mice developed T cell leukemia [52]. Moreover, it was recently shown that N1^{ICD} transduced bone marrow precursors were not able to induce T cell leukaemic transformation in the absence of a functional pre-TCR [64]. Together, these observations suggest that the constitutive activation of Notch1 signalling in pre-thymic precursors is not sufficient to trigger the development of T cell leukemia before the transit in the thymus. A different oncogenic potential is observed when different N1^{ICD} constructs are

used to generate transgenic mice. Indeed, while transgenic mice carrying N1^{ICD} with an incomplete TAD only occasionally develop thymomas in older age [54], a low but significant percentage of N1^{ICD} transgenic mice carrying a complete TAD region develops T cell leukaemia [60]. The discrepancy between the 100% incidence of T cell leukaemia in mice injected with bone marrow precursors retrovirally transduced with N1^{ICD} and the occasional to low percentage of Lck- N1^{ICD} transgenic mice developing T cell leukaemia suggests that the main Notch1-dependent oncogenic event resides in the bone marrow, being possibly related to the ability of Notch1 to selectively induce the early T cell lineage commitment at the level of common lymphoid precursors [52]. In contrast to Lck-N1^{ICD} transgenic mice, Lck-N3^{ICD} transgenic mice, overexpressing a N3^{ICD} domain, that ordinarily lacks a TAD region, develop an aggressive T cell leukaemia, early in age [61], suggesting that the Notch3-dependent oncogenic event takes place inside the thymus. Interestingly, lymphoma cells of Lck-N3^{ICD} transgenic mice retain the same phenotypic features as pre-leukaemic thymocytes (i.e. sustained expression of CD25 and pT α chain and a constitutively activated NF κ B [61] . Ligand-independent pre-TCR signalling is known to result in NF κ B activation, and may be responsible for anti-apoptotic and proliferation signals, thereby promoting neoplastic transformation. Therefore, the sustained expression of pT α observed in Lck-N3^{ICD} transgenic mice could be responsible, at least in part, for the constitutive activation of NF κ B, which mediates an anti-apoptotic response in leukaemic cells, suggesting possible relationships between constitutively activated survival and/or proliferation related transduction pathways and T cell neoplastic transformation.

Pre and post-receptor conserved components of the Notch signalling pathway are also directly or indirectly involved in T cell leukemogenesis. Retroviral-mediated overexpression of the Notch ligand Dll4 in bone marrow cells induces T cell leukaemia [42]. As for post-receptor events, the DNA-binding protein RBPJ κ (CBF1), after physical interaction with N1^{ICD}, activates the transcription of target genes including the HES family of basic helix–loop–helix

(bHLH) transcription factors (reviewed in [65]). Among HES family members, HES-1 has been suggested to have a potential role in T cell leukemogenesis, because it is overexpressed in murine T lymphoma cells carrying a murine leukaemia provirus insertion in one of the Notch1 alleles, which leads to a constitutively active truncated Notch1 protein [66]. Transcriptional activation of HES-1 has also been shown to correlate with leukaemogenesis induced in mice transplanted with bone marrow cells retrovirally transduced with Notch1 and in Notch3 transgenic mice. The expression of Deltex, is up-regulated in both N1^{ICD} and N3^{ICD} transgenic mice and was found at high levels in a number of murine thymomas.

Together these results suggest a crucial role for HES1 and Deltex in mediating Notch activity in leukaemogenesis. However, enforced expression of each of them is not sufficient to induce leukaemia [49], suggesting that the leukaemogenic process needs the collaboration of different Notch-dependent transduction pathways. The generality of involvement of dysregulated Notch signalling in stemming from the above described experimental models, has been supported recently by its occurrence in spontaneous human T-ALL. Indeed, combined misexpression of Notch3, HES1 and Deltex has been described to be pathognomonic of T-ALL and to correlate with either remission or relapsing stages of the disease. A critical candidate check point for neoplastic transformation appears to be represented by the DN to DP transition, and is dependent on pre-TCR signalling since abrogation of pre-TCR signals prevents Notch3-induced leukaemogenesis [51]. Indeed, this step recapitulates a number of pro-survival and proliferation signals mainly represented by the activation of ligand-independent pre-TCR signalling and the subsequent activation of NFκB and inhibition of E2A activity, which are possibly related to each other. This step is also characterized by the transient up-regulation of Notch3, followed by its decreased expression in DP cells. Hampering the Notch3 down-regulated expression past the DN to DP transition, through transgenic expression of N3^{ICD}, has been shown to be able to increase pTα expression

and NF κ B activation, resulting in T cell leukaemia. Notch1 may share some of these features with Notch3. Indeed, up-regulation of pT α by Notch1 has been reported [60]. Moreover, Notch1 has been shown to control NF κ B activity, although controversial results have been described. Indeed, Notch1, by binding to p50 subunit, displays both stimulatory and inhibitory activity upon NF κ B-mediated transactivation. The constitutive activation of NF κ B activity has been observed in both transiently transfected T cells with N3^{ICD} and thymocytes from Lck-N3^{ICD} transgenic mice. The leukemogenic potential of NF κ B has been reported. Indeed, the development of aggressive T cell lymphoma/leukemia has been demonstrated in transgenic mice expressing v-Rel under the control of a T cell-specific promoter. NF κ B has also been shown to be involved in HTLV1-induced lymphomagenesis via Tax induction of the degradation of I κ B α , thereby activating NF κ B. By different criteria, the Notch3-induced T cell malignancies strikingly resemble HTLV-1-associated T cell leukaemia [61]. A further mechanism leading to the leukemogenic process relates to the sustained downregulation of E2A activity as a consequence of dysregulated pre-TCR and/or Notch signalling. It is also worthwhile noting that the activity of E2A-encoded E47 and E12 transcription factors may be negatively regulated by the HLH transcription factors Tal-1, Tal-2 and Lyl-1, which are reported to be overexpressed or to harbour genetic abnormalities in human TALL. Indeed, Tal-1 transgenic mice develop T cell leukaemia. Finally, NF κ B is strongly activated in thymocytes from Tal-1 transgenic mice, further supporting a relationship between T cell leukaemogenesis, activation of NF κ B and inhibition of E2A activity.

Together, these results suggest possible relationships between Notch receptors, HLH transcription factors and leukaemogenesis. Consistent with this hypothesis there is the observation that a constitutively active Notch1 accelerates the development of T cell tumours in E2A-PBX1 transgenic mice, suggesting a possible collaborative relationship between Notch signalling and the oncogenic fusion protein involving the *E2A* gene [67].

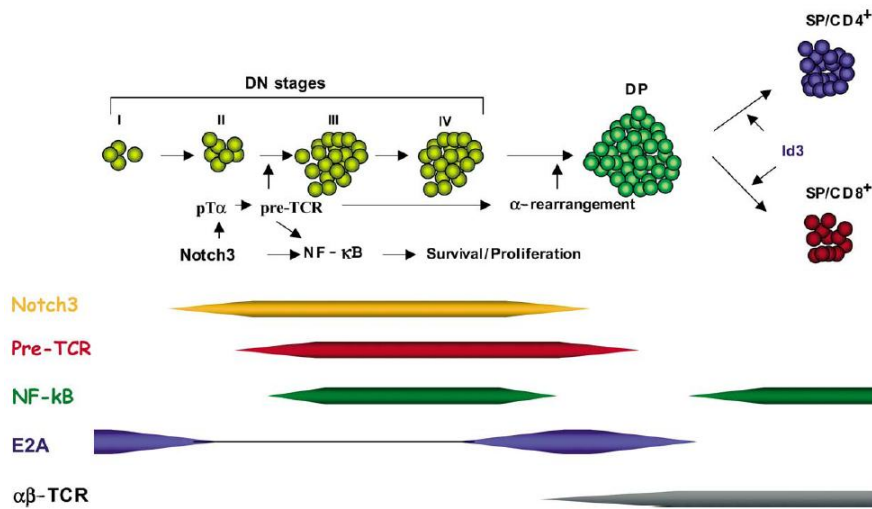


Figure 5 : Schematic diagram of intrathymic T cell development. The sequence of developmental stages and the main signaling pathways involved are shown. DN, CD4⁻CD8⁻ double negative thymocytes; DP, CD4⁺CD8⁺ double positive thymocytes; SP, CD4⁺ and CD8⁺ thymocytes. The model illustrates the possible role of Notch3 in regulating pre-TCR checkpoint and its interaction with main survival/proliferation-triggering signals (E2A, pre-TCR, NFκB)

1.2 microRNAs

1.2.1 General

Cells contain a variety of non coding RNAs, including components of the machinery of gene expression, such as tRNAs and rRNAs, and regulatory RNAs that influence the expression of other genes [68]. It has become increasingly apparent that non coding RNAs are impressively diverse, and that a significant fraction of the genes of all organisms do not encode proteins. One of the small noncoding RNAs - the microRNAs (miRNAs) has been recognized when in 1993 the regulation of gene regulation of the gene *lin-14* by a small RNA, *lin-4*, was reported in *Caenorhabditis elegans* [69]. It was not until seven years later that a second small RNA, *let-7*, was identified [70]. Since then, miRNAs have been discovered in virtually all plant and animal species, and their biological functions and mechanisms of action have become subjects of intense research. miRNAs are small , evolutionary conserved non-coding RNAs of 18-25 nucleotides in length, that are often encoded in clusters in the genome. Members of miRNA families have homologous sequences, but are not necessarily transcribed from the same genomic region. However, there are many cases in which miRNA families are found in clusters and their transcription is co-regulated. According to the latest version of the miRNA database miRBase, released in August 2012, approximately 2,042 mature miRNAs have been experimentally identified so far in human, and approximately 1,281 in mouse [71].

2.2 Biogenesis of miRNAs

Early annotation of the genomic position of miRNAs indicated that most miRNAs are located in the intergenic regions (>1 kb away from the annotated / predicted genes), although a sizeable minority was found in the intronic regions of known genes in the sense or antisense orientation (Figure 6) [72] [73]. Thus most miRNA genes are transcribed as autonomous transcription units. Another interesting observation was that these clustered miRNAs might be transcribed from a single polycistronic transcription unit. miRNA genes encoded in the genome are transcribed into long primary miRNAs (pri-miRNAs) by RNA polymerase II or, in some cases, by RNA polymerase III. [74] [75] [76]. Typically, animal pri-miRNAs display a 33 bp stem and a terminal loop structure with flanking segments [77]. Many pri-miRNAs originate from clusters; loci of miRNAs positioned closely together. Primary miRNA processing begins in the nucleus where an RNase III enzyme, Drosha, removes the flanking segments and a 11 bp stem region, thereby catalyzing conversion of pri-miRNAs into precursor miRNAs (pre-miRNAs) [74] [78]. Pre-miRNAs are generally 60–70 nt long hairpin RNAs with 2-nt overhangs at the 3' end. Drosha typically acts together with the DiGeorge syndrome critical region 8 protein (DGCR8) for efficiency and precision [79] [80] [81]. However, a Drosha / DGCR8- independent processing pathway can also produce pre-miRNAs. In this pathway, the nuclear splicing machinery provides pre-miRNA from introns. miRNAs produced by this pathway are appropriately called mirtrons [82] [83]. Mirtrons encompass a small group of miRNAs but are found in many organisms. Pre-miRNAs are exported from the nucleus to the cytoplasm by the exportin-5 / RanGTP heterocomplex ([84] [85] [86] and processed by RNase III enzyme Dicer [87]. Dicer is thought to act with dsRBDs containing partner proteins HIV TAR RNA-binding protein (TRBP) [88] [89] and / or PKR activating protein (PACT) [89] [90]. Dicer cleaves pre-miRNAs into 21–25 nt long miRNA / miRNA* duplexes, each strand of which bears 5' monophosphate, 3' hydroxyl group and a 3' 2-nt overhang. Of a miRNA / miRNA* duplex, only one strand, designated the miRNA strand,

is selected as the guide of mature RISC, whereas the other strand, the miRNA* strand, is discarded during RISC assembly. Such biased strand selection depends on the balance of at least three properties of a miRNA/miRNA* duplex: i) the structure; ii) the 5' nucleotide identity; and iii) the thermodynamic asymmetry [91]. The core component of RISC is a member of Argonaute (Ago) subfamily proteins, of which there are four paralogs (Ago1–4) in humans. RISC assembly follows a multi-step pathway. The first well-characterized event of RISC assembly is RISC loading. During RISC loading, miRNA/miRNA* duplexes are incorporated into Ago proteins. Ago1–4 disfavor duplexes bearing only non-central mismatches, but can incorporate siRNA-like perfectly complementary duplexes. RISC loading is not a simple binding of miRNA/miRNA* duplexes and Ago proteins, but rather an ATP-dependent active process [92]. Structural analyses of *Thermus thermophilus* Ago protein suggest that a generic miRNA/ miRNA* duplex is too bulky to fit directly [93]. To enable loading, drastic conformational opening of Ago protein appears necessary; an energetic reaction likely powered by ATP hydrolysis [91]. Recently it was reported that Hsc70/Hsp90 chaperone machinery mediates such conformational opening [94]. After RISC loading, the duplex is unwound and the miRNA* strand is discarded from Ago protein. Surprisingly, unwinding does not require ATP; presumably unwinding is linked to the release of the structural opening incurred upon Ago proteins during RISC loading [91]. Unwinding can be further classified into slicer-dependent unwinding and slicer independent unwinding. In humans, only Ago2 retains the cleavage activity [95] and thus can facilitate unwinding by slicing the miRNA* strand [92] [96]. However, passenger strand cleavage occurs only if the duplex is extensively base-paired, especially around position 10–11 of the guide. Most miRNA/ miRNA* duplexes bear central mismatches and are therefore unwound by a slicer-independent mechanism. Mismatches in the seed region (guide position 2–8) and / or the middle of the 3' region (guide position 12–16) greatly enhance the unwinding efficiency in all four Ago proteins [92].

MicroRNA target sites often lie in the 3' untranslated region (UTR) rather than in the 5' UTR or ORF, probably because translation (i.e. movement of ribosomes) will counteract RISC binding [97] [98]. Typically, a target mRNA bears multiple binding sites of the same miRNA (e.g. let-7 and HMGA2) [99] and/or several different miRNAs (e.g. miR-375, miR-124, and let-7b and Mtpn) [100]. Importantly, not all nucleotides of a miRNA contribute equally to RISC target recognition. This target recognition is largely determined by base-pairing of nucleotides in the seed region and is enhanced by additional base-pairing in the middle of the 3' region. [97] [100] [101] [102]. How RISC acts upon target mRNA is determined by both the character of the Ago protein in which the miRNA is incorporated and complementarity between the miRNA strand and the target mRNA. Ago2 is capable of RNA cleavage, (Liu J [95], Science 2004; 305: 1437–41) but this reaction requires extensive base-pairing between the miRNA strand and mRNA target. Some miRNAs silence target mRNAs through cleavage, just as siRNAs do. [103] In contrast to this example, the complementarity between the miRNA strand and target mRNA is typically limited, [97] [100] [101] [102] which renders RISC incapable of target cleavage. In such a case, Ago protein provides a platform to recruit factors, including GW182 proteins (TNRC6A–C in humans), required for translational repression and mRNA deadenylation/degradation of target mRNAs. Such slicer independent silencing can be reconstituted by artificial tethering of an Ago protein or a GW182 protein to an mRNA, independent of the presence of a miRNA [104] [105]. This contrasts with target mRNA cleavage, where the presence of a guiding small RNA is a de facto requirement.

Mechanism of Translation repression:-

At least six models of translational repression have been proposed :

- i) RISC induces deadenylation which causes decrease of translational efficiency by blocking target mRNA circularization; [106] [107] [108].
- ii) RISC blocks cap function by interacting with either the cap or eIF4E; [109] [110] [111] [112] [113].
- iii) RISC blocks a late step in initiation of translation such as recruitment of 60S ribosomal subunit[114]
- iv) RISC blocks a post-initiation step such as elongation and / or ribosome dropoff [115]
- v) RISC induces proteolysis of nascent peptides during translation [116].
- vi) RISC recruits target mRNAs to processing bodies, in which mRNA is degraded and/or stored in a translationally inactive state [117] [118].

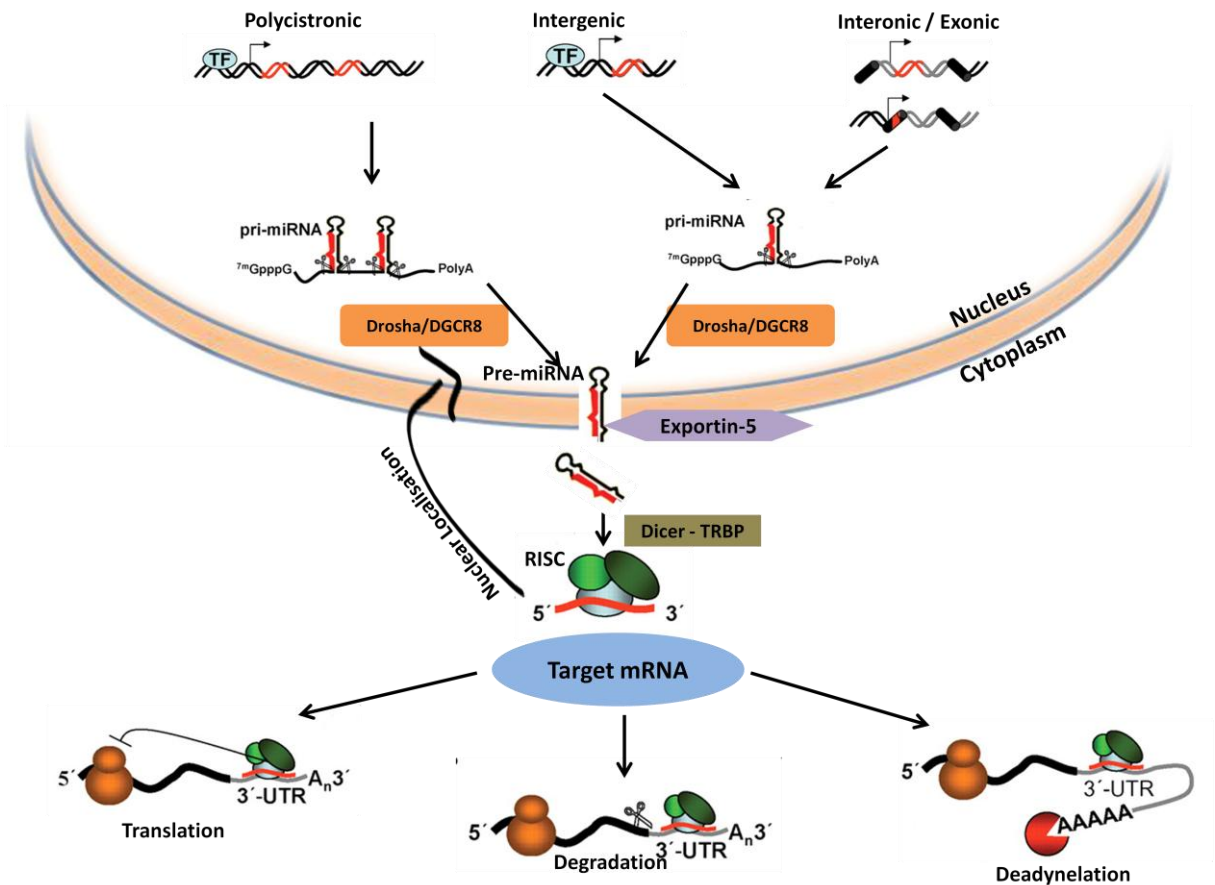


Figure 6 : MicroRNA (miRNA) genomic organization, biogenesis and function. Genomic distribution of miRNA genes. The sequence encoding miRNA is shown in red. TF: transcription factor. (A) Clusters throughout the genome transcribed as polycistronic primary transcripts and subsequently cleaved into multiple miRNAs; (B) intergenic regions transcribed as independent transcriptional units; (C) intronic sequences (in grey) of protein-coding or -non-coding transcription units or exonic sequences (black cylinders) of non-coding genes. Primary miRNAs (pri-miRNAs) are transcribed and transiently receive a 7-methylguanosine (7^mGpppG) cap and a poly(A) tail. The pri-miRNA is processed into a precursor miRNA (pre-miRNA) stem-loop of ~60 nucleotides (nt) in length by the nuclear RNase III enzyme Drossha and its partner DiGeorge syndrome critical region gene 8 (DGCR8). Exportin-5 actively transports pre-miRNA into the cytosol, where it is processed by the Dicer RNaseIII enzyme, together with its partner TAR (HIV) RNA binding protein (TRBP), into mature, 22 nt-long double strand miRNAs. The RNA strand (in red) is recruited as a single-stranded molecule into the RNA-induced silencing (RISC) effector complex and assembled through processes that are dependent on Dicer and other double strand RNA binding domain proteins, as well as on members of the Argonaute family. Mature miRNAs then guide the RISC complex to the 3' untranslated regions (3'-UTR) of the complementary messenger RNA (mRNA) targets and repress their expression by several mechanisms: repression of mRNA translation, destabilization of mRNA transcripts through cleavage, de-adenylation, and localization in the processing body (P-body), where the miRNA-targeted mRNA can be sequestered from the translational machinery and degraded or stored for subsequent use. Nuclear localization of mature miRNAs has been described as a novel mechanism of action for miRNAs. Scissors indicate the cleavage on pri-miRNA or mRNA.

1.2.3 miRNAs in Haematopoiesis.

Haematopoietic stem cells (HSCs) reside mainly in the bone marrow and give rise to all blood cell lineages, including cells that constitute the immune system [119]. HSCs must maintain a precise balance between self renewal and differentiation into multipotent progenitors, which subsequently give rise to both the lymphoid and myeloid branches of the haematopoietic system. Thus, Hematopoiesis represents an elegant developmental model to observe normal changes in miRNAs expression and to test the effects of miRNA levels on differentiation and tumorigenesis. Although miRNAs have been studied in other stem cell types, such as embryonic stem cells, there are currently limited data on the role of miRNAs in HSCs [120]. Several groups have carried out global miRNAs expression profiling of human CD34⁺ stem and progenitor cells and have identified certain miRNAs expressed by this cell population [121] [122]. Mice deficient in ARS2, which contributes to pri-miRNA processing, have bone marrow failure possibly owing to defective HSC function [123]. These studies provide initial evidence that the miRNA pathway is important in HSC function.

Individual miRNAs have also been implicated in HSC biology. Homeobox (HOX) genes have important roles in regulating HSC homeostasis, and miRNAs from the miR-196 and miR-10 families were found to be located in the HOX loci; both could directly repress HOX family expression [124] [125] [126] [103] [127] [128]. miR-196b is expressed specifically in mouse short-term HSCs, regulated by the HSC transcription factor family mixed lineage leukaemia (Mll), and has a functional role in modulating HSC homeostasis and lineage commitment, possibly through the regulation of expression of certain HOX genes³³. miR-126 has also been shown to regulate expression of HOXA9 [128] and the tumour suppressor polo-like kinase 2 (PIK2) [129], through which miR-126 is thought to mediate its biological effects. Functional studies of bone marrow progenitors showed that miR-126 increased colony formation *in vitro*, suggesting that it may promote the production of downstream progenitors by HSCs [129]. miR-221 and miR-222 were also shown to inhibit KIT expression in stem and progenitor

cells, leading to impaired cell proliferation and engraftment potential [130]. Although these studies suggest a role for specific miRNAs in HSC biology, additional work is needed to directly assess the influence of these and other miRNAs on the function of carefully sorted HSC populations regarding long-term, multilineage engraftment *in vivo*.

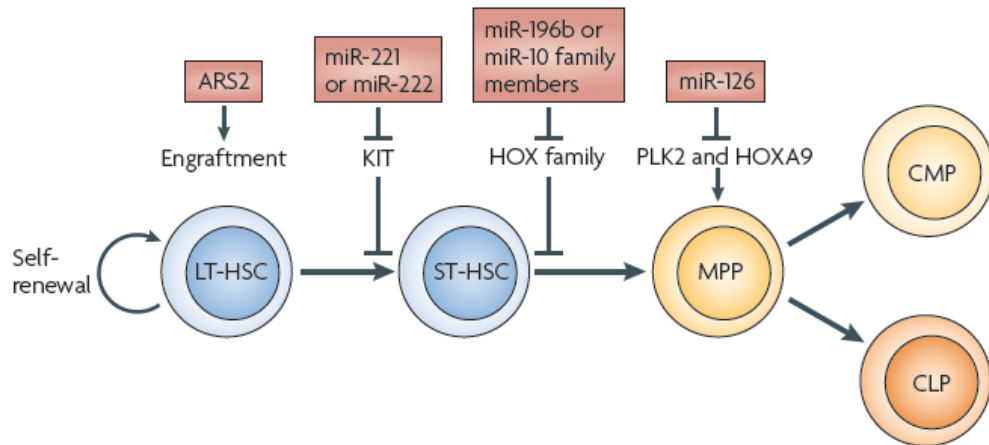


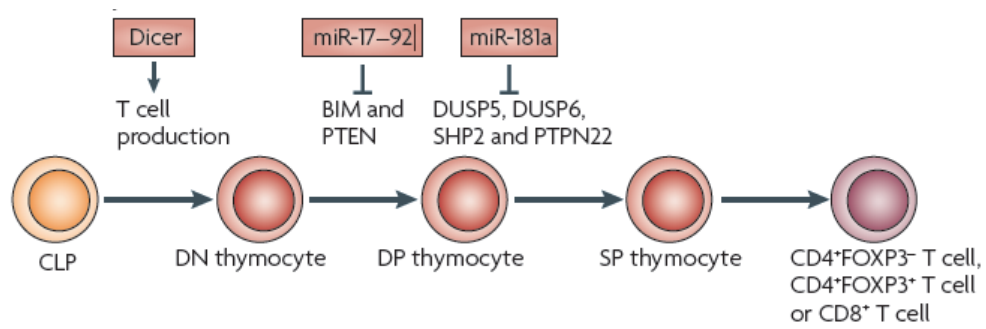
Figure 7: miRNA-mediated regulation of early haematopoietic cell development. miRNA pathway, which involves arsenate resistance protein 2 (ARS2), has a general role in haematopoietic stem cell (HSC) engraftment, which probably influences the reconstitution potential of long-term HSCs (LT-HSCs). Individual miRNAs have been shown to repress the expression of HSC-relevant genes and affect the production of haematopoietic progenitor and lineage-positive cells. Potential points of miRNA function during early haematopoiesis are indicated. miR-221 or miR-222 regulate KIT expression, which is thought to affect stem cell homeostasis. miR-196b is specifically expressed by short-term HSCs (ST-HSCs) and regulates mRNAs encoding the homeobox (Hox) family, in cooperation with miR-10 family members. The expression of miR-10 family members by HSCs is not as clearly defined. miR-126 represses the mRNAs encoding polo like kinase 2 (PLK2) and HoxA9, and has been shown to promote expansion of progenitor cells. CLP, common lymphoid progenitor; CMP, common myeloid progenitor; MPP, multipotent progenitor.

1.2.4 miRNAs functions in T Cells Development

Similar to the development of innate immune cells, the development of T cells in the thymus and their activation in the periphery are also controlled by complex protein signalling networks that are subjected to regulation by miRNAs. Expression profiling of T cells has identified a broad range of expressed miRNA species and found that the expression patterns vary between T cell subsets and stages of development [131] [132] [133]. Adding to this complexity, several variants of a given miRNAs species can be found in T cells, with the mature miRNAs varying in length at either the 3' or 5' end or containing mutated sequences [133]. Furthermore, proliferating T cells express genes with shorter 3' UTRs than those in resting T cells [134], rendering these mRNAs less susceptible to regulation by miRNAs owing to the loss of miRNA binding sites. These findings suggest that miRNA-mediated regulation of mRNA targets in T cells, and probably other immune cells, is a dynamic process that is influenced by a broad range of factors. T cell-specific deletion of *Dicer* has revealed a requirement for the miRNA pathway in the development of mature T cells, as the total numbers of which are lower in the mutant mice than wild-type mice [135] [136]. To date, two specific miRNAs have been implicated in T cell development, and probably account for some of the phenotype of *Dicer* deficiency in T cells. The miR-17–92 cluster impairs the expression of mRNAs encoding pro-apoptotic proteins, including BCL-2-interacting mediator of cell death (BIM; also known as BCL2L11) and phosphatase and tensin homologue (PTEN). This miRNA cluster is thought to increase T cell survival during development and is expressed during the DN2 stage of thymopoiesis [137]. Furthermore, the strength of TCR signaling influences whether thymocytes are positively or negatively selected during thymic development, and specific miRNAs have been implicated in this process. miR-181a, which is increased during early T cell development, enhances TCR signalling strength by directly targeting a group of protein phosphatases, including dual specificity protein phosphatase 5 (*DUSP5*), *DUSP6*, SH2-domain-containing protein tyrosine phosphatase 2 (*SHP2*; also

known as *PTPN11*) and protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*) [138]. Recent data have also indicated a role for miRNAs in the differentiation of T cells into distinct effector T helper cell subsets. This is best exemplified by mice deficient in miR-155, in which T cells are biased towards T helper 2 (TH2)-cell differentiation, indicating that miR-155 promotes differentiation into TH1 cells [139]. Certain miRNAs, such as the miR-17-92 cluster, might also be involved in the development and function of T follicular helper (Tfh) cells, which are specialized T cells that are dedicated to supporting B cells in germinal centres and facilitating antibody affinity maturation and class switching [140] [141]. TH17 cells have been identified as important mediators of inflammatory disease. A recent study found that miR-326 promotes TH17 cell development both *in vitro* and *in vivo* by targeting [142]. The generation of mice with a conditional deletion of *Dicer* or *Drosha* in regulatory T (TReg) cells has shown a requirement for the miRNA pathway in forkhead box P3 (fOXP3)+ TReg cells. [143] [144]. These mice develop a lethal autoimmune inflammatory disease, consistent with impaired development or function of TReg cells. Specifically, it was shown that miR-155 is important for TReg cell homeostasis and overall survival, and this is thought to involve the direct targeting of *Socs1* [145]. However, because the absence of miR-155 did not reproduce the severe disease that occurs in mice with a conditional deletion of *Dicer*, additional miRNAs are probably involved in TReg cell biology. Of note, the expression of miR-142-3p was recently shown to be repressed by FOXP3, leading to increased production of cyclic AMP and suppressor function of TReg cells [146]. Several other miRNAs are expressed by TReg cells and await functional assessment [147].

A) miRNAs in T-Cell Development



B) miRNAs in T-Cell Function

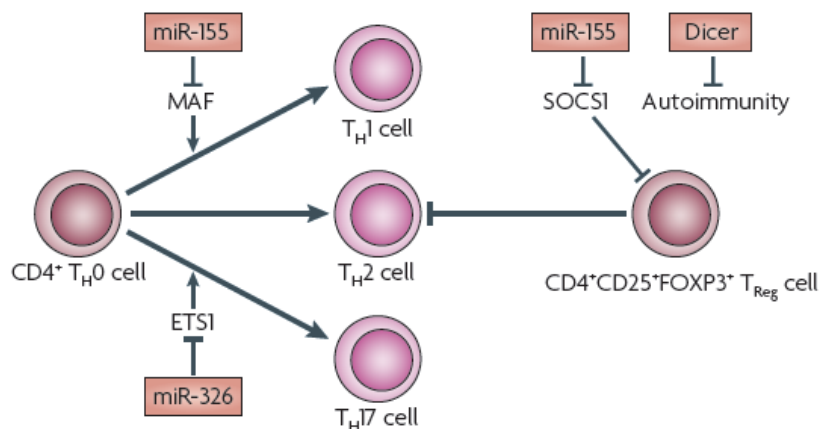


Figure 8 : miRNA-mediated regulation of T cell development and function. **A)** The production of miRNAs by Dicer is required for efficient T cell development *in vivo*. T cells go through a stepwise developmental programme in the thymus. During this process, T cell survival and selection is influenced by the miR-17-92 cluster (which targets the mRNAs encoding BCL-2 interacting mediator of cell death (BIM), and phosphatase and tensin homologue (PTEN)) and miR 181a (which targets mRNAs encoding several phosphatases, including dual-specificity protein phosphatase 5 (DUSP5), DUSP6, SH2-domain-containing protein tyrosine phosphatase 2 (SHP2) and protein tyrosine phosphatase, non-receptor type 22 (PTPN22)). **B)** In the periphery, mature T cell differentiation is modulated by miRNAs, including miR-155, which promotes skewing towards T helper 1 (TH1) cells through macrophage-activating factor (MAF) repression, and miR-326, which promotes skewing towards TH17 cells by targeting the mRNA encoding ETS1. Regulatory T (TReg) cells also depend on miRNAs to maintain immune tolerance to self tissues, thereby preventing autoimmunity. miR-155 repression of suppressor of cytokine signalling 1 (SoCS1) expression has been implicated in TReg cell survival. CLP, common lymphoid progenitor; DN, double negative; DP, double positive; FoxP3, forkhead box P3; SP, single positive.

1.2.5 Notch and miRNAs

miRNAs play critical roles in Notch signaling pathway. Several miRNAs have been shown to cross-talk with Notch pathway. However, the role of miRNAs in the Notch pathway remains unclear.

miR-1 belongs to tumor suppressor group which are generally down-regulated in cancer such as primary human hepatocellular carcinoma (HCC), prostate cancer, head and neck, and lung cancers. [148] [149] [150] [151] [152]. Ectopic expression of miR-1 has been shown to downmodulate these tumours by downregulating its target genes exportin-6 and protein tyrosine kinase 9 [148] MET, Pim-1, FoxP1, and HDAC-4. Recently, it has been reported that miR-1 regulates Notch signaling pathway by directly targeting the Notch ligand Delta-1 (Dll-1) in *Drosophila* (Figure 9) [153]. It has also been found that Dll-1 protein levels are negatively regulated by miR-1 in mouse embryonic stem cells [154]. These results suggest that miR-1 could regulate the Notch signaling pathway; however further in-depth research is needed in order to fully understanding how miR-1 regulate the Notch pathway.

miR-34, which has been found to participate in p53 and Notch pathways regulation is consistent with its tumor suppressor activity [155]. The expression of miR-34a was reported to be lower or undetectable in pancreatic cancer, osteosarcoma, breast cancer and non-small cell lung cancer [156] [157] [158], and also the inactivation of miR-34a was identified in cell lines derived from some tumors including lung, breast, colon, kidney, bladder, pancreas and melanoma [159]. miR-34a overexpression in glioma cells down-regulated the protein level of Notch-1, Notch-2, and CDK6 [160]. Moreover miR-34 restoration in human gastric cancer cells reduced the expression of target gene Notch and down-regulated Notch1 and Notch2 in the pancreatic cancer cells [161] [155]. Consistently, pancreatic cancer stem cells which are enriched with tumor-initiating cells or cancer stem cells show high levels of Notch-1/2 and loss of miR-34. Thus, these evidence suggest that miR-34 may be involved in pancreatic cancer stem cell self-renewal, potentially via the direct modulation Notch signaling[155].

The miR-146 was previously reported to function as novel negative regulators that help to fine-tune the immune response. It has been demonstrated a decrease in miR-146b in adult T-cell Leukemia cells. The decrease in miR-146b may lead to increased inflammation and decreased TReg functions, resulting in leukemia [162]. Transduction of miR-146a or miR-146b into breast cancer cells i) decreased the expression of epidermal growth factor receptor ii) down-regulated NFκB activity iii) inhibited migration and invasion in vitro and iv) suppressed lung metastasis in experimental xenograft models [163] [164]. Interestingly, miR-146a in C2C12 cells was found to regulate Numb [165], which is known to induce the ubiquitin mediated Notch protein degradation. Indeed, Notch activation and the loss of Numb expression were found in a large proportion of breast [166] [167]. It has been reported that over-expression of Notch1 stimulates NFκB activity in several cancer cell lines [168] and since miR-146 also regulate NFκB activity, it clearly suggest that miR-146 could regulate NFκB through Notch mediated signaling pathway. However, the role of miR-146 in Notch signaling pathway need further innovative investigations.

miR-199a was observed to be down-modulated in ovarian cancer [169], and in hepatocellular cancer. Moreover, it was found that over expression of miR- 199a can introduce cell cycle arrest in G2/M phase [170]. Very recently, miR- 199b-5p was seen to be a regulator of the Notch pathway targeting the transcription factor Hes-1, thus negatively regulating the Medulloblastoma (MB) tumors cell growth. Moreover, over-expression of miR-199b-5p decreased the MB stem-like cells (CD133⁺) and also blocked expression of several cancer stem-cell genes. Further, the expression of miR-199b-5p in the non-metastatic cases was significantly higher than in the metastatic cases. The patients with high levels of miR-199b expression showed a better overall survival [171]. These results clearly suggest that miR-199 family could be very important in the regulation of multiple signaling pathways including Notch, and thus further in-depth studies are needed in order to clarify the biological

significance and mechanisms on how miR-199 can regulate the Notch signaling pathway in human cancers.

The miR-200c was down-regulated in benign or malignant hepatocellular tumors [172]. miR-200 family regulates epithelial–mesenchymal transition (EMT) by targeting zinc-finger E-box binding homeobox 1 (ZEB1) and [173] [174] [175] [176]. miR-200 family regulates the expression of ZEB1, Slug, E-cadherin, and vimentin, and thus the re-expression of miR-200 could be useful for the reversal of EMT phenotype to mesenchymal- to-epithelial transition [177]. It has been found that the expression of both mRNA and protein levels of Notch1 to -4, Dll-1, Dll-3, Dll-4, Jagged-2 as well as Notch downstream targets, such as Hes and Hey, were significantly higher in PC3 PDGF-D cells (unpublished data). Importantly, Notch-1 could be one of miR-200b targets, because over-expression of miR-200b significantly inhibited Notch-1 expression (unpublished data).

Recently, miR-451 and miR-709, were suggested as potent tumor suppressors in Notch1-induced mouse T-ALL which expressions are progressively down-regulated during T-ALL transformation from benign polyclonal cells to malignant monoclonal cell and when co-expressed in a Notch1-induced model of TALL induce the block of the tumor cell growth. [178]. Forced expression of miR-150 reduces Notch3 levels in T-cell lines and has adverse effects on their proliferation and survival suggesting that control of the Notch pathway through miR-150 may have an important impact on T-cell development and physiology [179]. Also miR-206 was shown as a pro-apoptotic activator of cell death, which was associated with its inhibition of Notch3 signaling and tumor formation [180]. Although, certain miRNAs have been established clear to regulate Notch pathway in both development and progression in different malignancies [181], the cross-talk between miRNAs and Notch pathway in T-ALL remains to be better elucidate.

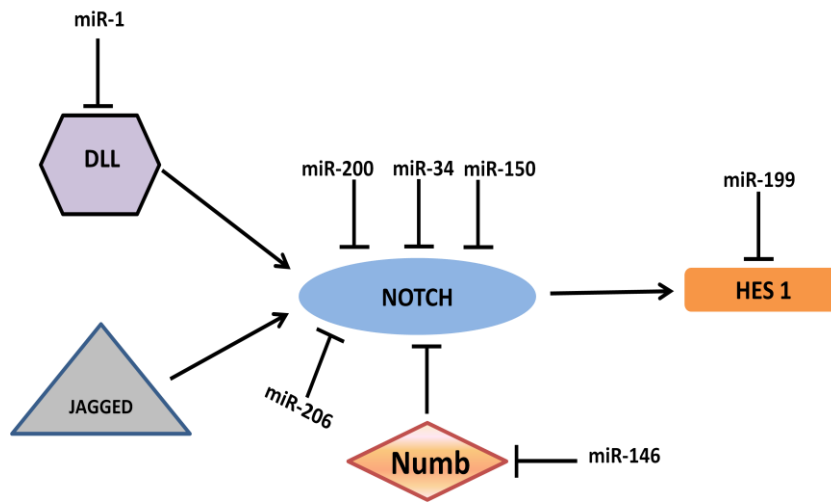


Figure 9 : Diagrammatic representation of involvement of miRNAs in regulating Notch Signalling Pathway

2. Aim of the Work

Despite the considerable progress in the study of the miRNAs biology and their role in various diseases and in human tumors [182] [183] it is still not fully understood the mechanism and signalling involved in the regulation of their expression in different contexts. In the last few years, it has been demonstrated that the miRNA pathway plays a critical roles in the Notch signaling [181] [184] [178], and a growing number of works revealed Notch as post-transcriptional target of several miRNAs [185] [186] [187] [179] [180], suggesting that the study of the cross talk between the two pathways may be a new point of view to better understand the biology and the causes of tumors associated with Notch deregulation, such as T-ALL leukaemia. In this regard, it was demonstrated that in a mouse model of T-ALL the constitutively activation of Notch1 induces the repression of miR-451 expression that acts as tumor suppressor in T cell leukemogenesis. [178]. Recently, the comparison of microarray miRNA profiling of GSI treated versus the vehicle alone treated T-ALL cell lines suggested that Notch1 signaling repress the expression of miR-223 and, on the other hand, a different study identified miR-223 as one of five onco-miRNAs that accelerates leukemogenesis in a Notch1-driven mouse T-ALL model [188]. Given the oncogenic role of Notch3 in T-ALL [61] [51] and that the activation of its signaling was linked to the regulation of the transcription of different genes [51] [189] [190], in this work, we evaluated the set of miRNAs which are regulated by the activation of Notch3 signaling and that cooperate to its oncogenic activity in T-ALL leukemia context.

3) *Materials and Methods* :

3.1) *Mice*

The generation and typing of N3^{LC} tg mice have been described (Bellavia et al., 2000). The studies involving animals have been conducted following the Italian National Guidelines for Animal Care established in Decree number 116 of 27 January 1992, in accord to the directive CEE 86/609, as well as in Circular number 8 of the Italian Ministry of Health, 23 April 1994.

3.2) *Cell Lines*

Jurkat cell lines were purchased from (DSMZ) , Jurkat stably deleted for IKK γ were obtained from Prof Tuosto L, and Molt3 cell lines were kindly provided by Prof Indraccolo S. Human T-lymphoblastic cell lines JURKAT, Jurkat IKK γ ^{-/-} & Molt3 were kept in culture in RPMI 1640 (Gibco) supplemented with 10% FBS (Gibco) and 1 mmol/L L-glutamine at 37°C in 5% CO₂. HEK 293T & M31cells lines were kept in culture in D-MEM (Gibco) supplemented with 10% FBS (Gibco) and 1 mmol/L L-glutamine at 37°C in 5% CO₂.

3.3) *Antibodies, Drugs and Treatments*

Primary antibodies used for Western Blot : Notch3 #2889 (Cell Signalling), c-Myc – (9E10)-SC-40, Cleaved Notch1(Val1744) #4147 (Cell Signalling), p-p65(Ser536) (93H1) #3033 (Cell Signalling), Ikb α - #9242 (Cell Signalling), NF κ B(p65) – (H-286) sc-7151, Cyclin E (HE12) sc-247, Aurora A/AIK (IG4) #4718 (Cell Signalling), FBxw7 – (H-300) sc-33196, β -actin (C4) sc-47778, Notch1 (C-20) sc-6014-R, α -tubulin (TU-02) sc-803, Primary antibodies used for ChIP assays : NF κ B(p65) (relA) Cat # 17-10060 (Milipore), ChIP Anti-RBPJK antibody Cat – ab25949 (Abcam). Whenever required, γ -secretase inhibitor IX (GSI) (Cat nos 565770) (Calbiochem) was added at the concentration of 10 μ M to the growth medium of human T-lymphoblastic cell lines JURKAT and MOLT3 for mentioned time. 12-O-

tetradecanoyl phorbol-13-acetate (TPA) P-8139 (Sigma) were used at 50ng/ml wherever required. BAY 11-7082 (Calbiochem) was used at concentration of 10 nM.

3.4) RNA isolation

RNA was isolated from cells using Trizol (Invitrogen) or TriReagent (Ambion) according to the manufacturer's instructions. In briefly, TRIzol or TRIreagent was added to each sample and incubated at room temperature for 5 min, then 200 μ l of chloroform per ml of TRIzol (100 μ l of 1-Bromo-3-Chloropropane (BCP) per ml of TRIreagent) was added to each sample. The samples were then vortexed for 30 sec, incubated for a further 5-10 mins. Samples were then centrifuged at 13,000 x g for 15 min at 4°C. The upper aqueous phase was carefully removed and equal volume of Isopropyl alcohol was added and vortex for 30 sec, incubated for 10 mins at Room Temperature. The sample was centrifuged at 13000 RPM for 15 mins. The supernatant was discarded out and 1 ml of 70% ethanol was added. The sample was centrifuged at 8000RPM for 5 mins. Supernatant was discarded. The samples were dried and RNA was re-suspended in RNase Free water.

3.5) RT-PCR

The mRNA were reverse transcribed using Superscript II reagents (Invitrogen). In briefly, oligo-dT, dNTP (1mM) were added to mRNA to be reverse transcribed, heated for 5 mins at 65°CcDNA, followed by incubation on ice for 1 mins. Then 5X first strand buffer, 0.1M DTT and RNase were added , mixed and incubated for 2 mins at 42°C. Superscript II Reverse transcriptase was added and incubated at 42°C for 60 mins. The reaction was inactivated by incubating at 70°C for 15 mins. 20 ng of the products were subjected to RT-PCR . Results were analyzed using the $\Delta \Delta$ Ct method with normalization against β -Actin expression for

mRNA, respectively. The primer used for RT-PCR were bought from Applied Biosystem and their codes are as follows:

	Human		Mouse
Notch1	Hs01062014_m1	Notch1	Mm00435249_m1
Notch 3	Hs00166432_m1	Notch 3	Mm00435270_m1
Hes1	Hs00172878_m1	Hes1	Mm01342805_m1
cMyc	Hs00905030_m1	cMyc	Mm00487803_m1
B-actin	Hs99999903_m1	pTα	Mm00478361_m1
Tubulin	Hs00258236_m1	B-actin	Mm00607939_s1

3.6) miRNA profiling by Megaplex quantitative real-time PCR.

The expression of genomic miRNAs in cells were profiled using stem-loop RT-PCR based 384 well Taqman Low-density Array (TLDA) cards (Applied Biosystems). Briefly, for megaplex RT reaction, five hundred nanograms of total RNA was reversed transcribed, using multiplexed specific looped miRNA primers for pool A & B (both human and mouse). The thermal-cycling condition used were : 40 cycles consisting of incubation at 16°C for 2 mins, 42°C for 1 min, 50°C for 1 sec, then incubation for 5 mins at 85°C and hold at 4°C. The product of the megaplex reaction was mixed with TaqMan Universal PCR Master Mix, No AmpErase UNG, 2X. Then, 100 µl of the reaction mix were dispensed into each port of TaqMan MicroRNA Array (TLDA). Then, it was centrifuged and sealed, and were subjected to the microfluidic RT-PCR technology which was performed by 7900HT System, Applied Biosystems (Foster City, CA, USA).

3.7) Analysis of TLDA data

Raw data files from TLDA obtained from mice samples were processed using RealTime StatMiner™ v3 software (Integromics™ SL, Spain) . The normalization was done using mouse snoU6. The statistical analysis for the regulated miRNA was carried out with Mann-Whitney (Wilcoxon) Test. For the raw data which were obtained from M31, Jurkat, Molt3 samples, RQ

Manager Software were used which calculates the Cycle Threshold (Ct). The default value of 0.2 was used. The miRNA which were having the Ct value of more than 35 was deleted manually. Normalization of the Ct values in each sample was done with U6 snRNA control: $[\Delta Ct = Ct(miR) - Ct(U6\ snRNA)]$. The fold-change for each miRNA probe was calculated: $Fold\ change = 2^{-((mean\Delta Ct(experimental) - mean\Delta Ct(control))}$. The heat map was generated using Spotfire Software.

3.8) Individual miRNA RT-PCR

Individual Taqman miRNA assays (Applied Biosystems) were performed according to manufacturer's instructions. Briefly, 5 μ l of total RNA (10ng) isolated from cells was converted to cDNA using the microRNA reverse transcriptase Kit (Applied Biosystems) with 3 μ l of specific miRNA assay RT primer in a reaction volume of 15 μ l. 1.33 μ l of cDNA was used with 10 μ l of Taqman Gene Expression Mastermix in a final volume of 20 μ l. Plates were then run on a StepOnePlus qRT-PCR instrument (Applied Biosystems) using the manufacturer's recommended cycling conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with data collection at the end of each cycle. Ct values > 35 were below detection limit and excluded. Data was analysed by the $\Delta\Delta Ct$ method and U6 snRNA (Code nos - 001973) was used to normalize the expression levels of miRNAs. Specific Taqman miRNA used in this study was miR-223 (Code Nos – 002295).

3.9) Plasmids

Expression vectors used were as follows: Flag- N3^{ICD} (Palermo et al., 2012), HA- N3^{ICD} (Bellavia et al., 2000), RBPJk and N1^{ICD} was kindly provided by Prof. Talora. MAM (Bellavia et al., 2007) were previously described. p65 & I κ B α -sr was kindly provided by Prof. Enrico De Smaele; CEBP α (provided by Prof Gianluca Canettieri).

3.10) Cell transfections

Transfection of the HEK 293T and M31 cell line was performed using the lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transient transfection efficiency in M31, as assessed by expression of a GFP expression vector added in trace amounts, was ~70 respectively. For Luciferase assay *Renilla* luciferase reporter vector pSV40-Renilla (5 ng) was also incorporated into each transfection (Promega, Madison, WI, USA) for normalization. The total amount of transfected DNA was kept constant by adding empty vector. At 36 h post-transfection, the cells were lysed in a reporter lysis buffer (Passive Lysis Buffer; Promega, Madison, WI, USA) at 100 µl/well. Firefly and pSV40-Renilla derived luciferase activities were measured in each sample with the Dual luciferase Assay System (Promega, Madison, WI, USA) using a Model TD-20/30 luminometer (Turner Designs). Light emission was measured for 10 s after injection. The specific luciferase activity of different transfections was determined in triplicate samples and was normalized on the *Renilla* luciferase activity. Data were expressed as means±s.d. of at least three independent experiments. Luciferase and renilla activity were assayed with a dual-luciferase assay system (Promega). Transient transfection of Jurkat cell lines with siNotch3 (Code – sc-37135) and its negative control (Code nos – AM4611), was carried out with Amaxa Nucleofection (Lonza)

3.11) miRNA transfection

Jurkat T-ALL cell lines were transiently transfected with 10 µM Control (*CN-001000-01-05*) or Mimic (*C-300580-07-0005*) of hsa-miR-223 or 10 µM Control (*IN-001005-01-05*) or AntimiR (*IH-300580-0005*) of hsa-miR-223 (Thermo Scientific), using Amaxa nucleofection . Protein were extracted 48 h post transfection as described in Western blot section.

3.12) Western Blot

Pelleted cells were resuspended in lysis buffer (*Tris 20 mM pH 7.5, NaCl 150 mM, NaF 1mM, Triton X-100 1%, Sodium-Orthovanadate 1 mM, PMSF 1mM, Sodium Butyrate 10mM*) plus protease inhibitors for 20 mins on ice. After a centrifugation at 13000 rpm for 20 min, supernatants were boiled for 5 min in Laemmli sample buffer (Biorad, Hercules, CA, USA). Concentration of Protein was determined by Bradford (BioRad). 40 µg of protein was separated on 8% or 10% gel. After electrophoretic separation, proteins were electrotransferred on a Nitrocellulose membrane (Perkin Elmer). The membrane was then blocked for 1 h at room temperature with blocking agent 5% in TBS/Tween 0.05%, probed overnight with a primary antibodies against the proteins of interest, and finally revealed with a secondary antibodies HRP conjugated (Santa Cruz) and ECL Advance system (Cyanagen). The β actin or β tubulin were used as reference.

3.13) miR-223 Promoter Construct

The fragment containing the putative miR-223 promoter region was amplified by PCR from human genomic DNA using the primers :-

Forward –5' *CAAAGTCAACTACTTTCTTCTCCCTT* 3'

Reverse – 5' *CCAGATGGAATTGGGCTTT* 3'

The PCR products were separated by agarose gel electrophoresis, and DNA fragments were then isolated and subcloned in topo TA-cloning vector (Invitrogen, Carlsbad, CA, USA). Minimal functional promoter were generated by cleaving with NdeI. The miR-223 promoter-luciferase fusion plasmids were constructed by cleaving with NdeI and EcoRV and inserted into the NdeI and EcoRV sites upstream of the luciferase cDNA of pGL4-basic vector (Promega, Madison, WI, USA).

3.14) Mutagenesis

Plasmid pGL4 containing miR-223 promoter fragment was used to generate mutant having the deletion of few base pair from the nested CSL/NFκB binding sites. The primers designed to introduce the site-directed mutation were :

Forward - 5'-ccaggttcctcaattattctaacctcttgaccac-3'

Reverse - 5'-gtgggtcaaggaggttagaataattgagggaacctgg-3'

The melting temperature (T_m) was calculated with the formula given by Stratagene (<http://www.stratagene.com/manuals/200519.pdf>). In brief, Two complimentary oligonucleotides containing the desired mutation flanked by unmodified nucleotide sequence were synthesized by PCR using 1µl of *PfuUltra* HF DNA polymerase. The extension reaction was initiated by pre-heating the reaction mixture to 95°C for 1 mins; 18 Cycles of 95°C for 50 sec, 60°C for 50 sec, 68°C for 1 min followed by incubation at 68°C for 7 mins. 1 µl of *Dpn* I restriction enzyme was added and incubated at 37°C for 1 hours. 2 µl of *Dpn* I treated DNA was transferred to ultracompetant cells. Transformation was done with heat pulse for 30 sec at 42°C. 0.5 ml of preheated NZY⁺ broth was added and incubated at 37°C for 1 hr. Transformant was plated and selected by Amphotericin. A total of 10 colonies were selected and their plasmids were isolated by mini-prep. The positive mutants were selected by respective restriction enzymatic digestion. The mutation was detected by sequencing.

3.15) Cell cycle , Cell growth and Apoptosis Assay

Cell Cycle analysis was done by using 7AAD (A9400) (Sigma Aldrich).. In Brief, the cells were washed with PBS and Fixation/Permeabilization solution (eBioscience, San Diego, CA, USA), was added. It was vortexed and stored at 4°C for 2 hours. Then washed 2 times with Permeabilization buffer (eBioscience, San Diego, CA, USA). After eliminating supernatant 7AAD solution was added and incubated at Room Temperature for 30mins (or Overnight). To access cell growth, trypan blue was used to count the viable cell number. Apoptosis was

detected by using Annexin V Apoptosis Detection Kit (00-6990; eBioscience, San Diego, CA, USA).

3.16) Cell sorting

Thymocyte suspensions from wt and Notch3^{IC} tg mice were prepared and stained with anti-CD4-FITC and anti-CD8-PE antibodies as described before. CD4⁺CD8⁺ DP subsets were then isolated (purity level $\geq 95\%$) with the FACS Aria cell sorter (BD Biosciences).

3.17) Chromatin Immunoprecipitation (ChIP)

Protein complexes were cross-linked to DNA in living nuclei by adding formaldehyde (Sigma, Inc.) directly to fresh Jurkat cell lines to a final concentration of 1%. Crosslinking was allowed to proceed for 10 min at 37°C and was then stopped by the addition of glycine to a final concentration of 0.125 M. Cross-linked cells were washed twice with phosphate-buffered saline and pelleted. Nuclei were extracted with a buffer containing 10 mM Tris pH 8, 0,25% Triton-X 100, 10 mM Na-EDTA, 0.5 mM Na-EGTA and protease inhibitors, pelleted by microcentrifugation and lysed by incubation in SDS lysis buffer (0.5% sodium dodecyl sulfate, 5 mM Na-EDTA, 50 mM Trischloride pH 8), containing protease inhibitors. The resulting chromatin solution was sonicated for 18 pulses of 15 s at 80% power to generate 300–1000 bp DNA fragments. After microcentrifugation, the supernatant was diluted 1:5 with a dilution buffer (0.01% sodium dodecyl sulfate, 1% Triton X-100, 1 mM EDTA, 20 mM Tris-chloride pH 8, 150 mM NaCl, containing protease inhibitors), and aliquoted. After precleaning with Salmon Sperm DNA/Protein agarose (Upstate Biotechnology) 5 μ g of anti-RBPJ κ or normal rabbit IgG, anti-p65 or normal mouse (Santa Cruz Biotechnology Inc.) were added to each aliquot of chromatin and incubated on a rotating platform for 12–16 h at 4°C. Antibody–protein–DNA complexes were isolated by immunoprecipitation with Salmon

Sperm DNA/Protein agarose (Upstate Biotechnology). Following extensive washing, bound DNA fragments were eluted and analyzed by subsequent PCR with the following primers:

Forward 5' *GTGATTTGGAGTCCATGGGG* 3'

Reverse 5' *ACCAACTCCCAGCTGACCTTC* 3'

3.18) Transduction of cells with lentiviral vectors.

The silencing of Notch3 in Human leukemia Molt3 cell lines was done with pLKO-puro-IPTG-1xLacO lentivirus particles expressing shRNA for human Notch3 (TRCN0000020234, Sigma Aldrich). In brief, lentivirus particles was added to the Molt3 cell lines. The cells were centrifuged at 800 x g for 30 minutes at 32 °C. Each cell pellet was resuspended in 2 mL of media by gently pipetting the pellet up and down, and each resuspended pellet was transferred to its own well in a 6-well plate tissue culture plate. The negative selection of the transduction was done by puromycin. Cells were maintained in puromycin until the non-transduced negative control cells have died as determined by inspection or Trypan Blue staining. After the transduction, the transfectants were selected for 7 days with puromycin (2µg/ml) followed by the treatment with IPTG for induction of shRNA against Notch3.

4. Results

4.1) Microarray profiling of Notch-regulated miRNAs in T-ALL

Aimed to characterize miRNAs that are regulated by Notch signaling in T-ALL leukemia, we carried out miRNA expression profiling of freshly isolated double positive CD4⁺CD8⁺ (DP) thymocytes from 6-weeks-old wild type and N3^{IC} tg mouse T-ALL developing models [61]. As shown in Figure 10A, in all the mice analyzed, the thymocytes subset distribution is similar with respect to CD4 and/or CD8 expression. The overexpression of the N3^{IC} in the DP thymocytes from the transgenic mice was verified by the western blot when compared to wild type mice. The Notch1 expression was same in DP thymocytes from both N3^{IC} transgenic mice and wild type mice (Figure 10B). The overexpression of N3^{IC} protein in DP thymocytes from transgenic mice is correlated with the increased mRNA expression of known Notch target genes such as Hes1 and pT α when compared with the wt counterparts (Figure 10C). Total RNA was then isolated and the expression of genomic miRNAs was analyzed by qRT-PCR using rodent TaqMan Low Density miRNA Arrays (TLDA). Data analyzed by RealTime StatMiner™ v3 software (Integromics™ SL, Spain) revealed a significant up-regulation, of up to two folds, of 40 miRNAs and a considerable downregulation, of up of two fold, of 4 miRNAs (adjusted *P* value < 0.7) (Table 4). The unsupervised hierarchical clustering analysis shows that the strongest significantly up-regulated miRNAs, in N3^{IC} tg when compared with the wt DP thymocytes, were miR-337-5p, miR-31, miR-223 and miR-146a and the more significative down-regulated were miR-150, miR-700, miR-126-3p and miR-378 (Figure 11). Subsequently, immature murine M31 T cells [191] were transiently transfected with the N3^{IC} or N1^{IC} plasmid (Figure 12A) to simulate the Notch pathway activation. As shown in figure 12B the forced expression of the activated Notch domains resulted in the increased expression of the Notch target genes Hes1 and c-Myc when compared with the control vector transfected cells. Total RNA from these samples were

isolated and analyzed for the expression of differentially regulated miRNAs by qRT-PCR using rodent TLDA. Amplification plots which did not amplify in all the samples, or/and had very high variation, or/and which expression was too low was excluded from the analysis by manual inspection. Relative miRNAs expression was normalized against the endogenous control U6 snRNA using the comparative $\Delta\Delta\text{CT}$ method and calculated by using SDS RQ manager ver. 1.2 software. As shown in Table 5, 92 miRNAs were upregulated and 60 miRNAs were downregulated when compared between N3^{IC} vs pCDNA transfected M31 T cells as their expression levels varied up to two fold. The unsupervised hierarchical clustering analysis showed that, in N3^{IC} transfected M31 T cells when compared with the control vector transfected cells, the strongest up-regulated miRNAs were miR-223, miR-875 and miR-452 and the more significant down-regulated miRNAs were miR-665, miR-let7-c-1 and miR-694 (Figure 12C). In order to evaluate the ability of Notch3 to regulate specific miRNAs, Notch3 expression was, also, ectopically silenced in either Molt3 and Jurkat human leukemia T cell lines, known to express Notch3, as shown Figure 13A and 14A. The strength of the silencing was evaluated by the downregulation of Notch target genes expression (Figure 13B and 14B). The total RNA from the above samples was subjected to miRNA profiling by qRT-PCR using human TLDA and then analyzed manually as previously described for the M31 T cells. The result revealed 236 miRNAs upregulated and 199 miRNAs downregulated in Molt3 T cell lines (Table 6) and 34 miRNAs upregulated and 130 miRNAs downregulated in Jurkat T cell lines (Table 7), as their expression levels varied up to two fold.

The subsequent unsupervised Hierarchical clustering of miRNAs differentially expressed between the Notch3 silenced cell lines versus the controls showed that the strongest up-regulated miRNAs were miR-497*, miR-144 and miR-34c-5p in Molt3 and miR-137, miR-628-5p, and miR-299-3p in Jurkat and the more significant down-regulated miRNAs were miR-892b, miR-1267 and miR-1276 in Molt3 (Figure 13C) and miR-1267, miR-198 and miR-127-3p in Jurkat cell lines (Figure 14C). Interestingly, according to Venn diagram

analysis, there are 11 miRNAs, which were commonly upregulated in N3^{IC} overexpressed models (Figure 15A). Moreover, the intersection of the miRNAs profile of Molt3 and Jurkat silenced for Notch3 revealed 95 miRNAs, which were commonly downregulated (Figure 15B). Finally, as shown in Figure 15C and in Table 1, only 7 miRNAs were significantly modulated in response to either N3^{IC} overexpression or Notch3 silencing. The putative promoter analysis on these miRNAs showed only miR-223 and miR-139 to contain the RBPJκ/CSL binding site in both human and mouse, and miR-182 which is a member of cluster miR-183/93/182 contain CSL/RBPJκ binding site only in Mouse, thus showing non conservation of CSL/RBPJκ binding site, in the putative promoter of clustered miR-182 (Table 3). The literature reviewed did not gave any much information regarding the oncogenic role miR-139-5p in T-ALL. Interesting it was previously shown that ectopic expression of miR-223 on hematopoietic lineage differentiation in vitro increased T-lymphoid lineage with little or no reduction in the B-lymphoid [192]. In another study cross-comparison of miRNAs expression profiles in human T-ALL identified miR-223 as one of the highest expressed miRNA in T-ALL. Moreover, the same authors indicated its oncogenic role in cancer promotion in a mouse model of T-ALL leukemia by regulating the expression of the E3 ligase FBW7 [188]. The above results showing miR-223 modulations by TLDA microfluid cards were reconfirmed through qRT-PCR by specific stem-loop primers for miR-223 (Figure 16) on all the above mentioned models. These evidences made us to focalize the study on the relation between Notch signaling and miR-223 regulation and its role in T-ALL context.

4.2) miR-223 expression is regulated by Notch and NFκB signalling pathway.

The locus of miR-223 maps on the X chromosome and is transcribed independently of any recognized genes [193] [194]. Independent studies were focalized on the regulation of miR-223 revealing the transcriptional activation by CAAT enhancer-binding protein alpha

(C/EBP α). Fazi and his colleagues proposed that C/EBP α and the Nuclear Factor I-A (NFIA) regulate miR-223 expression competing for the same binding site 709 bp upstream of the pre-miR-223 sequence [193]. Controversy, Fukao et al. demonstrated that the miR-223 transcription is regulated by C/EBP α and PU.1 located 3420 bp in front the pre-miR in a conserved promoter region upstream the transcription start site of the pri-miR [194]. A very recent study confirmed that the miR-223 expression is mostly regulated by the promoter region upstream the transcription start of the pri-miR suggesting that the previously described promoter region could represent a regulatory region [195]. Subsequently in-silico analysis, by Genomatix Maltinspector software, of the conserved human pri-miR-223 5' proximal genomic region, confirmed the C/EBP and PU.1 consensus binding sites at 39bp and 51/ 63bp respectively, as previously demonstrated. Interesting, the analysis revealed a novel putative RBPJ κ /CSL and NF κ B-p65 overlapping evolutionary conserved binding sites between 280-299 bp upstream the transcription start site of the pri-miR (Figure 17A). In order to investigate the role of the Notch signaling in the transcriptional regulation of the miR-223, we generated a luciferase reporter vector containing 690bp of the human pri-miR-223 5' proximal genomic region (Figure 17B) or mutant construct which lack the consensus binding site for CSL/RBPJK nested with p65 (Figure 17C). The bonafide promoter region was confirmed by transfecting HEK293T cell lines with or without human-C/EBP- α expression vector (Figure 17D). As expected, the ectopic overexpression of Notch3 transcriptional complex or p65-NF κ B resulted in an increased activity of the luciferase reporter, moreover co-expression of Notch3 transcriptional complex with p65-NF κ B displayed a synergic effect on the promoter activation (Figure 17D) . Notably, the p65-induced promoter activity was dramatically decreased, when HEK-293T cells were transiently transfected with a deletion mutant reporter that lack the nested RBPJ κ /CSL/NF κ B consensus binding site (Mut miR-223 promoter), but unexpectedly the same effect was not seen by Notch3 transcriptional complex, suggesting that this nested RBPJ κ /CSL/NF κ B consensus binding site is more important for

p65-NFκB mediated transcriptional regulation (Figure 17D). In order to understand the relationship between Notch and NFκB in the regulation of miR-223, we performed an *in vivo*, chromatin immunoprecipitation assay using sonicated chromatin from Jurkat Human Leukemia T cell lines. Chromatin extracts were administered to immunoprecipitation with both anti-RBPJκ/CSL or anti-p65-NFκB antibodies and subjected to PCR amplification with the use of specific primers that amplified the region containing the RBPJκ/CSL and NFκB binding region of the miR-223 promoter. As shown in Figure 18A, only anti-p65 antibody recovered a 300-bp fragment of the proximal promoter containing the previously described binding site, while no signal was detected in PCR of RBPJκ/CSL immunoprecipitated DNA fragment suggesting that p65 play a direct role in transcriptional regulation of miR-223, in contrast to Notch transcriptional complex which could either regulate miR-223 through other mechanism independently of this RBPJκ/CSL and NFκB binding region or could play an indirect role in the transcriptional regulation of miRNA-223 likely through the activation of NFκB signaling which for its part is able to do this directly by binding to the promoter. In order to deepen our understanding on the direct role of p65 in transcriptional regulation of miR-223, we tried to use phosphorylation-defective IκBα S32A/S36A (IκBα PD) that is known to sequester NFκB in cytoplasm [196]. As shown in (Figure18B) co-transfecting p65 with IκBα PD completely abrogated miR-223 promoter activation. Moreover, the NFκB pharmacological inhibition using BAY11-7082 or activation using 12-O-tetradecanoyl phorbol-13-acetate (TPA) resulted respectively in the inhibition or in the activation of the endogenous miR-223 expression in Jurkat cells lines (Figure 18C).

Our previous works demonstrated the ability of Notch3 to trigger the canonical p65-NFκB pathway [189] [197], confirming, on one hand, that NFκB signaling plays a key role in miR-223 transcriptional regulation in T-ALL leukemia other than C/EBP and PU.1 known regulatory pathways in acute myeloid leukemia (AML) and, on the other hand, in this regulatory mechanism Notch signaling plays an important role indirectly by activating the

NFκB signaling. In detail, these results demonstrate that the transcriptional regulation of miR-223 is mainly linked to the activity of NFκB that directly activates the promoter. Therefore, in contexts in which the activity of NFκB is closely dependent on the Notch pathway activation, as demonstrated in several human cell lines and murine models of T-ALL leukemia where NFκB, it was identified as one of the major mediators of Notch-induced tumorigenesis [198], [199] [61] [189], Notch could have a key role in the miR-223 gene expression.

4.3) miR-223 plays a role in regulating cell growth of T-ALL.

In the last years, emerging evidence suggested miR-223 as novel oncogene by directly targeting the onco-suppressor FBXW7/hCdc4 (FBXW7) in different tumor contexts such as human gastric cancer [200], in oesophageal squamous cell carcinoma [201] in primary mouse embryo fibroblasts [202]. Moreover, loss of functional mutations in Fbxw7 were linked with many human diseases and tumors including T-ALL [203] [204]. Interestingly, the enforced over-expression of the mimic-miR-223 in Jurkat T cell resulted in remarkable decrease of the FBXW7 protein expression and conversely, the inhibition of the miR-223 by antago-miR-223 increased the FBXW7 expression (Figure 19A).

Notably, several studies have revealed multiple targets of the FBXW7 ubiquitin ligase activity [205] [206] [207] [208] [202] which are known plays pivotal roles in many key cellular processes including cell proliferation, differentiation and apoptosis, and were founded often deregulate in a wide range of human cancers [209]. Consistently, the FBXW7 modulation in miR-223-induced Jurkat T cells caused the substantial regulation of all the previously mentioned FBXW-7 target proteins. As shown in Figure 19A, Jurkat T cells transfected with the mimic-miR-223 resulted in the increased protein levels of the Notch1 intracellular domain (N1^{IC}), c-Myc and Cyclin E while the inhibition of the miR by transfecting antago-miR-223 caused the opposite effects. Additionally, the cell number was increased in Jurkat T cells

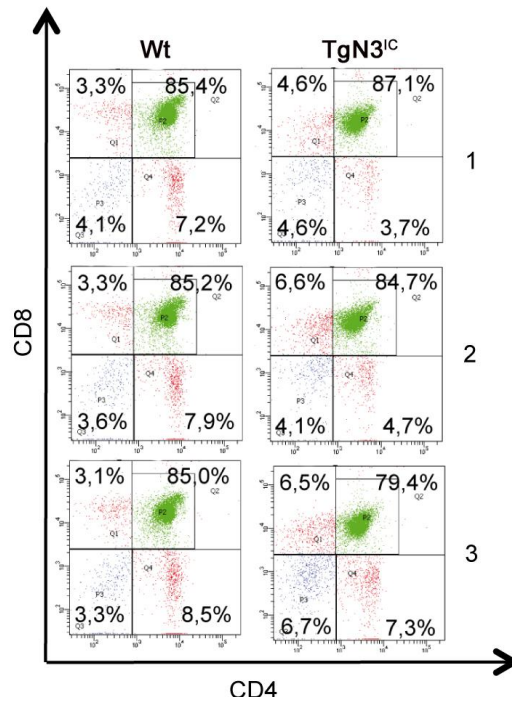
transfected with the mimic-miR-223 when compared to its relative control, thus showing increased cell growth (Figure 19B). While the reverse effect was seen with antagomiR as the cell growth decreased (Figure 19C). Moreover, cell cycle analysis by flow cytometry showed decreased number of cells accumulated in G0/G1 phase (from 51% to 43%) and increased in S phase (from 27% to 37%) comparing transfected cells with mimic-miR-223 to the control cells (Figure 19D) without affecting the apoptosis rate (Figure 19E). Overall, these results demonstrated that miR-223 inhibiting the tumor suppressor FBWXW-7 expression stimulates Jurkat T cell proliferation, revealing for the first time its oncogenic role in T-cell Leukemia.

4.4 miR-223 confers resistance to γ -secretase inhibition in T-ALL cell lines.

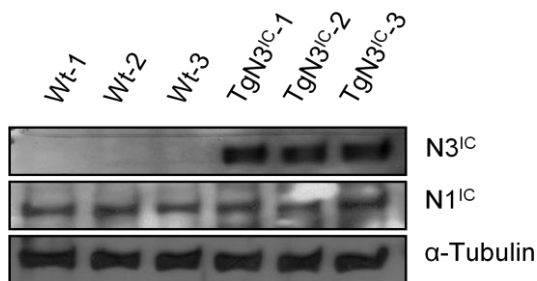
In quest to see the effect of inhibition of Notch signalling on miR-223 expression, we utilise γ -Secretase inhibitor IX (DAPT), a chemical compound, which is known as strong inhibitors of the Notch signalling, unexpectedly we saw an increase in endogenous expression of miR-223 in both Jurkat and Molt3 cell lines (Figure 20A & 20B). Consistently a recent publication also revealed increase in miR-223 expression when the T-ALL cell lines were treated with Compound E (another γ -Secretase inhibitor) [210]. This result was completely opposite to what we observed when we specifically inhibited Notch3 using specific siRNA. The solution of this dilemma was unveiled by the subsequent comparison of the Notch1 activated form and the phosphorylated p65-NF κ B protein expression between Jurkat and Molt3 T cells DAPT-treated versus the control cells. As shown in Figure 20C, although DAPT inhibited Notch signalling by blocking the Notch S3 cleavage, simultaneously, it induced the activation of the NF κ B canonical signaling.

Furthermore, analysis of miR-223 expression showed a reduced transcription of this miR in stable cell line of Jurkat in which the NF κ B signaling was inhibited by the silencing of IKK γ (Jurkat IKK $\gamma^{-/-}$) when compared with wt counterpart Jurkat T cell lines (Figure 21A). The

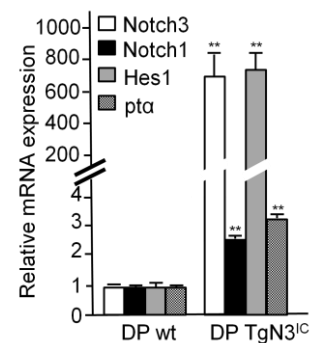
inhibition of NFkB signalling was confirmed by the decreased phosphorylation of p65-NFkB and by the increased accumulation of IKB α in the total extracts from the Jurkat IKK γ ^{-/-} when compared with the Jurkat wt. (Figure 21A). Cell count of viable cell using trypan blue staining showed decreased cell number in Jurkat IKK γ ^{-/-} (Figure 21B), which had a low expression of miR-223 when compared to Jurkat wt, thus indicating that miR-223 could play a role cell growth and proliferation. To access the role of miR223 in rendering sensitivity to GSI resistant cell, we treated Jurkat wild type and Jurkat IKK γ ^{-/-} cell lines with DMSO and GSI-IX (DAPT), and accessed their growth rate every 2 days until day 14. As shown in Figure 21C, Jurkat wild type cell lines treated with DAPT or DMSO, both continued to grow normally. Instead Jurkat IKK γ ^{-/-} cell line showed decreased growth rate when they were treated with DAPT in comparison to its counterpart DMSO treated (Figure 21D).



A



B



C

Figure 10 : N3^{IC} and its target gene expression in DP Thymocytes from N3^{IC} transgenic Mice :
 A) CD4⁺ and/or CD8⁺ subset distribution of thymocytes derived from N3^{IC} Transgenic and Wild Type (wt) mice. B) Western blot analysis of whole-cell extracts from the sorted DP thymocytes from Transgenic and wild type mice, probed with Notch3 & Notch1 antibodies. The α -Tubulin expression was used as loading control. C) Relative mRNA expression of Notch3 downstream target genes as analyzed by RT-PCR. Data are represented as mean \pm s.d. from triplicate tests. *P<0.05; **P<0.01.

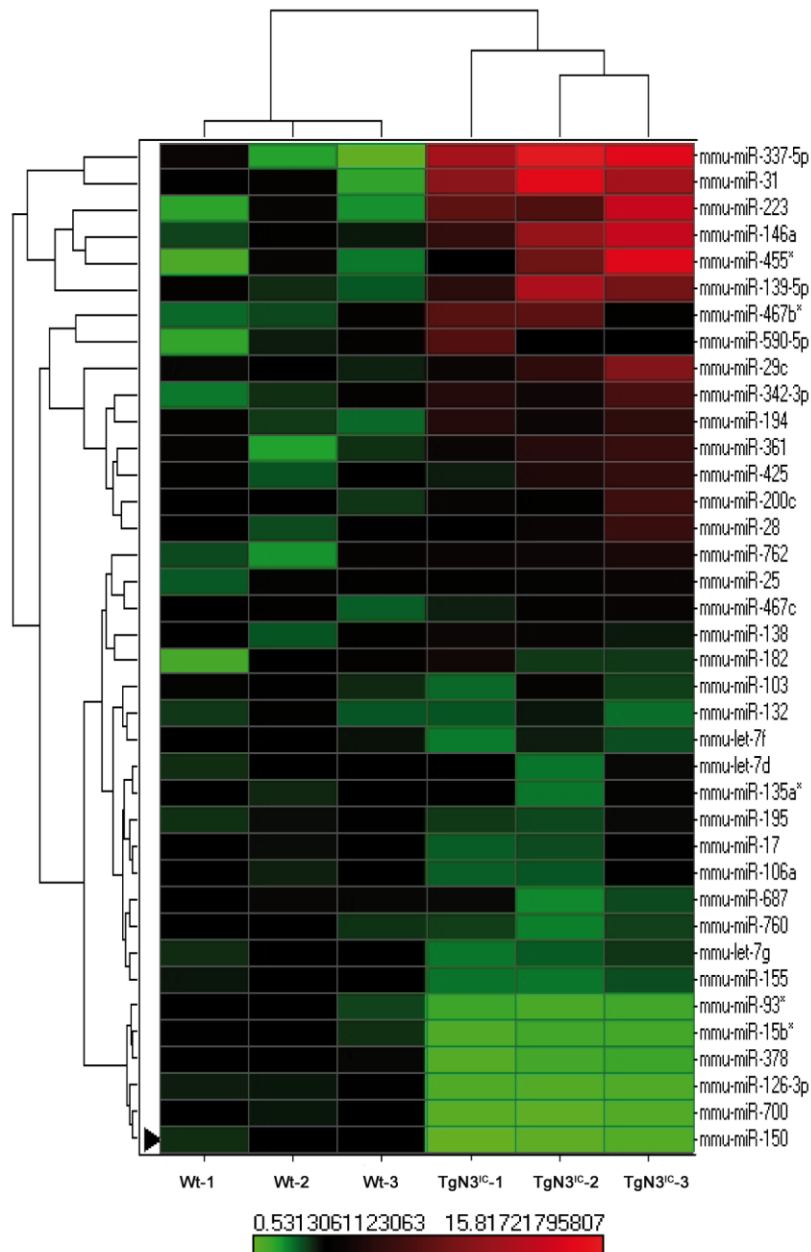


Figure 11 : Heat map shows distinct miRNAs expression profiles in N3^{IC} Transgenic mice relative to wild-type (WT) counterpart : Each column represents one of three RNA samples and each row represents one miRNA gene. Relatedness in miRNA expression across samples is shown by a hierarchical tree on the Y axis through standard linkage. Expression levels are depicted according to the color scale at the bottom with increased expression ranging from green to red.

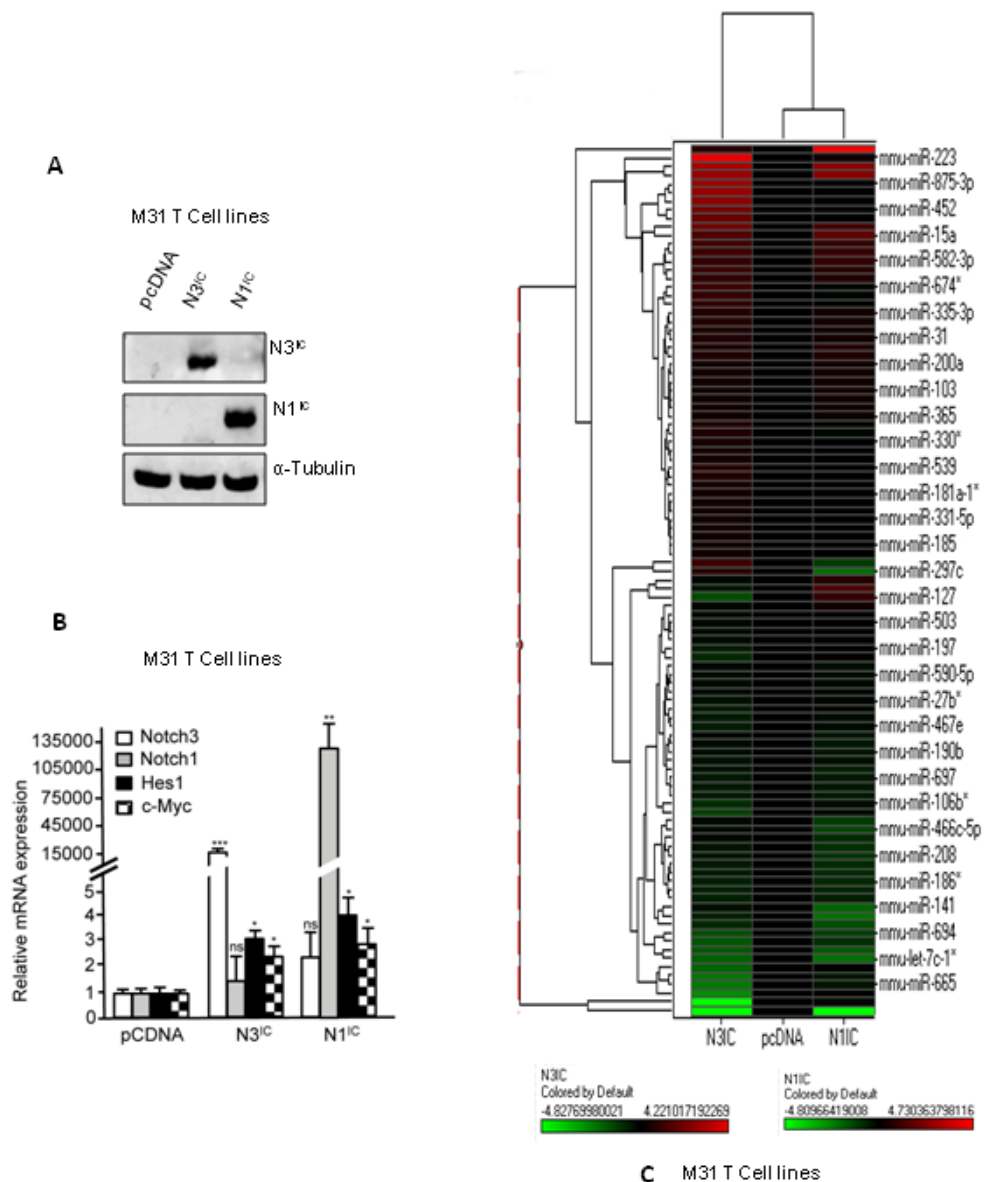


Figure 12 : Differential miRNAs expression profiling of M31 T Cell lines overexpressed with N3^{IC} , N1^{IC} or empty vector : **A)** Western blot against N3^{IC} or N1^{IC} confirmed transfection and overexpression of N3^{IC} or N1^{IC} protein. **B)** RT-PCR shows increased mRNA expression of Notch target gene in N3^{IC} or N1^{IC} transfected M31 Cell lines compared to M31 transfected with only empty vector. **C)** Heat map shows genomic miRNAs expression profiles of M31 transfected with N3^{IC} or N1^{IC} relative to pcDNA empty vector. Each column represents one RNA samples and each row represents one miRNA gene. Relatedness in miRNA expression across samples is shown by a hierarchical tree on the Y axis through standard linkage. Expression levels are depicted according to the color scale at the bottom with increased expression ranging from green to red.

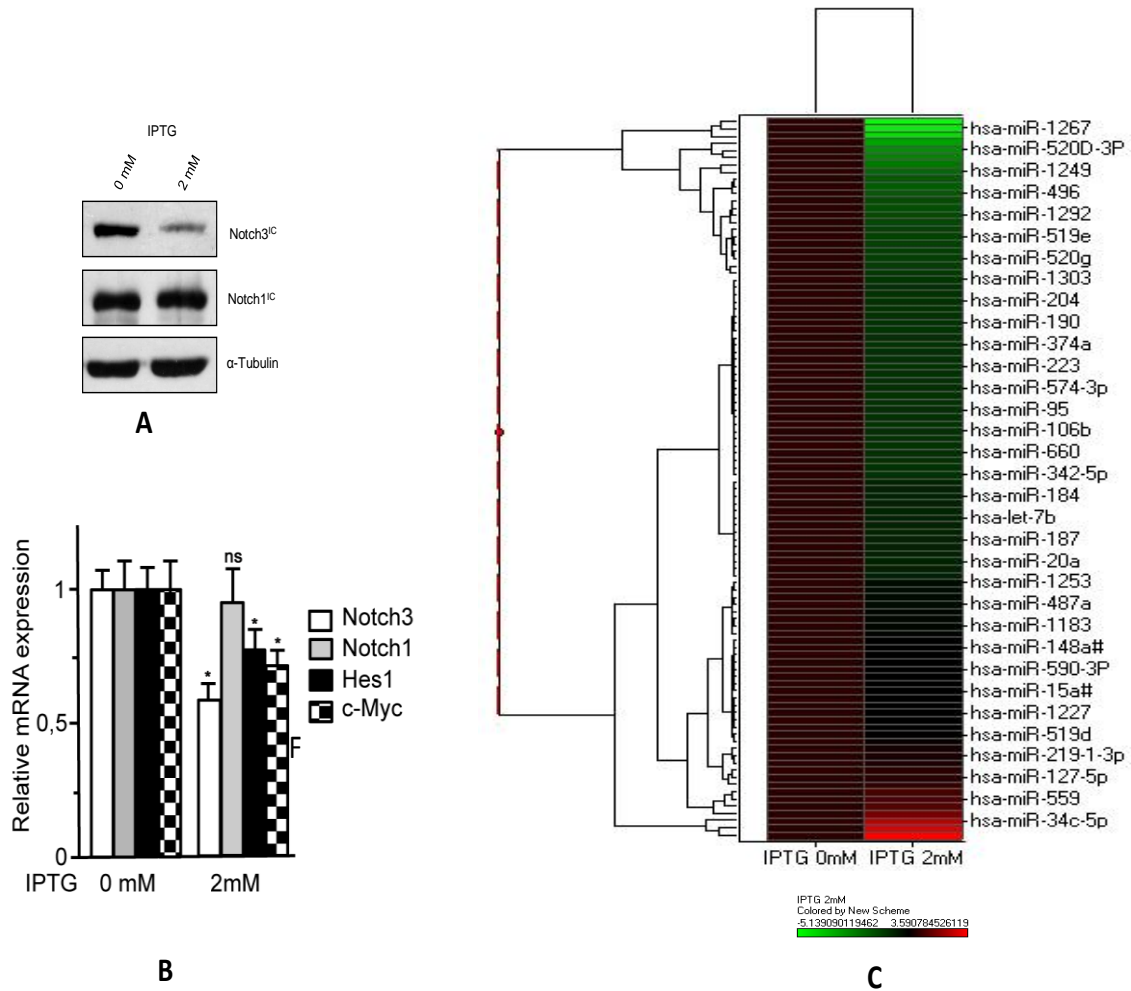


Figure 13 : Silencing of N3^{IC} in Molt3 showed distinct set of miRNAs which were modulated: **A)** Western blot against N3^{IC} showing efficiency of Notch3 silencing by lentivirus. The silencing was induced by treatment with IPTG. N1^{IC} protein expression level showed no modulation . **B)** RT-PCR shows down modulation of Notch target gene in N3^{IC} silenced Molt3 IPTG treated cell lines compared to untreated counterpart. **C)** Heat map shows differential regulated miRNAs expression profiles of Molt3 IPTG treated and untreated samples. Each column represents one RNA samples and each row represents one miRNA gene. Relatedness in miRNA expression across samples is shown by a hierarchical tree on the Y axis through standard linkage. Expression levels are depicted according to the colour scale at the bottom with increased expression ranging from green to red.

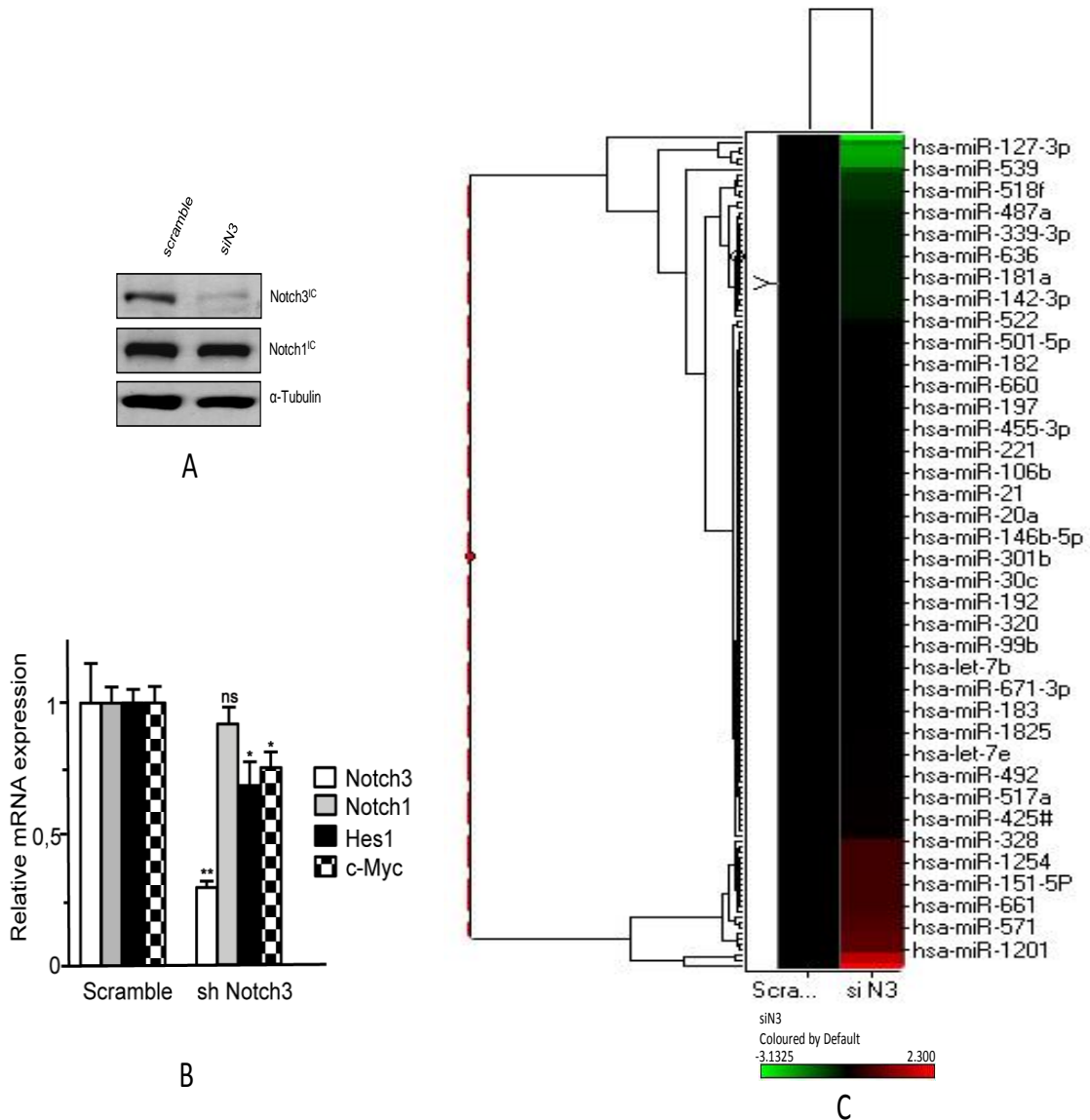


Figure 14 : Silencing of Notch3 in Jurkat shows significant effect on differential expression of miRNAs: Western blot against N3^{IC} and N1^{IC} showing efficiency of Notch3 transient silencing by siRNA without affecting the N1^{IC} protein expression level. **B)** RT-PCR shows down modulation of Notch target gene in N3^{IC} silenced Jurkat to Control counterpart. **C)** Heat map shows genomic miRNAs expression profiles of Notch3 silenced Jurkat relative to control sample . Each column represents one RNA samples and each row represents one miRNA gene. Relatedness in miRNA expression across samples is shown by a hierarchical tree on the Y axis through standard linkage. Expression levels are depicted according to the colour scale at the bottom with increased expression ranging from green to red.

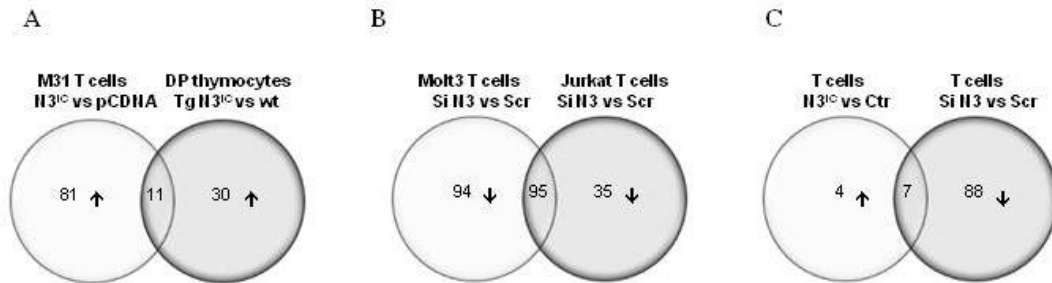


Figure 15 : Notch3 affects expression of specific miRNAs in both Human and Mouse T Cell Leukemia Models. (a) The Venn diagram shows the number of the significantly up-regulated miRNAs caused by Notch3 overexpression. The intersection gives a view of miRNAs that are induced in response to Notch3. (b)The Venn diagram shows the number of significantly down-regulated miRNAs resulted from Notch3 interference. The intersection gives a view of miRNAs that are decreased in response to Notch3 silencing. (c) The Venn diagram shows the number of significantly modulated miRNAs in response to either Notch3 overexpression and its silencing. The intersection gives a direct view of the miRNA that are positively regulated by N3 signaling.

Table 2 : Relative expression level of 7 miRNAs positively regulated by Notch3 Signalling

<u>miRNA</u>	<u>Tg Mice N3</u>	<u>M31 N3IC</u>	<u>Jurkat siN3</u>	<u>Molt3 siN3</u>
miR-223	10,5385	16634,7850	0,5113	0,2642
miR-182	3,2713	2,1239	0,5008	0,5346
let-7d	2,4870	2,0882	0,5222	0,2639
miR-425	4,0177	2,0806	0,5044	0,5394
miR-25	3,3795	2,0681	0,5074	0,5433
miR-139-5p	8,1822	2,0525	0,5150	0,5431
miR-103	2,5001	2,1346	0,5077	0,2752

Table 3 : Chromosomal location of 7 regulated miRNAs and CSL/RBPJK binding site in their putative promoter region

<u>miRNA</u>	<u>Chrom Loc Hum</u>	<u>Chrom Loc Mouse</u>	<u>Host gene name</u>	<u>CSL/RBPJK</u>	
				<u>Human</u>	<u>Mouse</u>
miR-223	chrX: 65238712-65238821	chrX: 96242817-96242926	AL034397.1	yes	yes
miR-182	chr7: 129410223-129410332 [-]	chr6: 30165918-30165992 [-]	-	no	yes
let-7d	chr9: 96941116-96941202 [+]	chr13: 48536012-48536114 [-]	RP11-2B6.1	no	no
miR-425	chr3: 49057581-49057667 [-]	chr9: 108568777-108568861 [+]	DALRD3	-	-
miR-25	chr7: 99691183-99691266 [-]	chr5: 138165321-138165404 [-]	MCM7	no	no
miR-139-5p	chr11: 72326107-72326174 [-]	chr7: 101475376-101475443 [+]	PDE2A	yes	yes
miR-103	-	-	PANK2	no	no

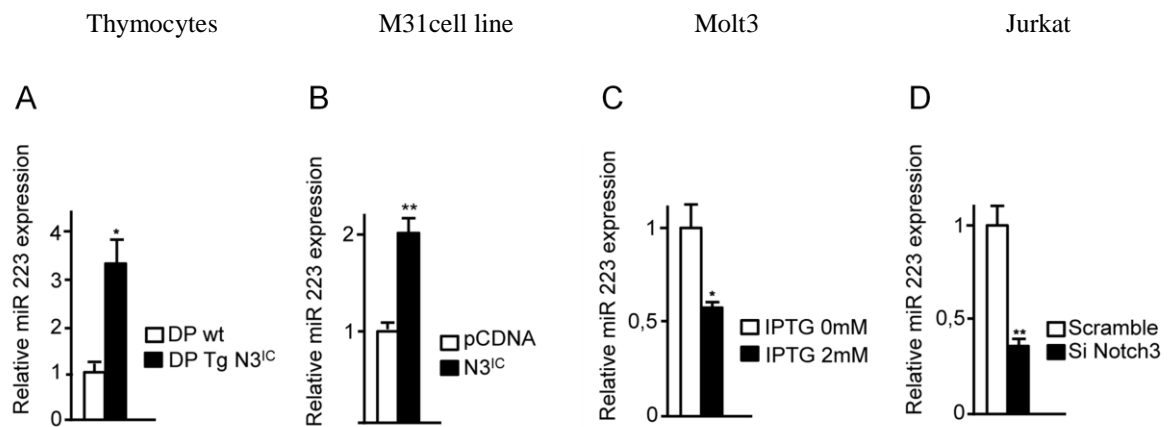


Figure 16 : miR-223 expression is dependent on Notch3 signaling in both human and mouse models of leukemia. The level of miR-223 was compared by quantitative RT-PCR in: (A) DP Thymocytes from tgN3^{IC} mice versus wt mice, (B) M31 T cell lines N3^{IC} transfected versus control vector transfected, (C) Molt3 and (D) Jurkat T cell lines Notch silenced respectively versus the control cells. Data are represented as mean \pm s.d. from triplicate tests. *P<0.05; **P<0.01.

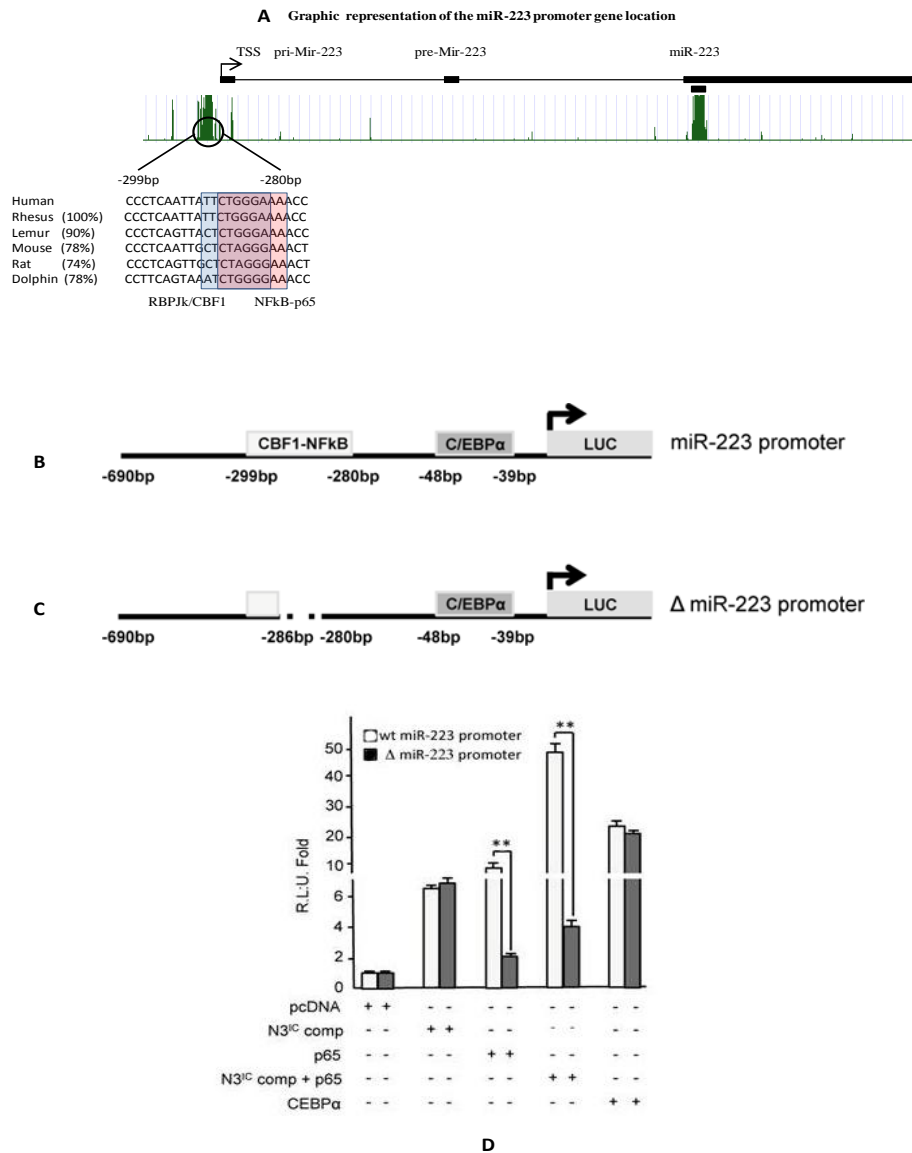


Figure 17 : Regulation of human miR-223 by Notch3 and NFκB (p65)

A) Graphical representation of human miR-223 promoter region. Conserved regions are shown as green peak as retrieved from USCS genome browser. RBPJκ and NFκB binding site in conserved region are shown in Blue and Pink and their intersection shows nested region. **B)** Schematic representation of the miR-223 promoter reporter constructs. The putative consensus site for C/EBPα from -39bp to -48bp and the nested binding site RBPJκ/NFκB from -280bp to -299bp from the start of transcription, fixed as +1bp, are indicated by boxes (wt miR-223 promoter). **C)** The dotted line refers to the internal deletion generated inside the RBPJκ /NFκB from -280bp to -286bp of the mutated Δ miR-223 promoter. **D)** Luciferase assay performed on HEK 293T cells after the co-transfection with a luciferase reporter construct containing the wt or mutated hsa-miR-223 promoter and C/EBPα, N3^{IC}, or NFκB-p65 vectors. (C/EBPα is used as Positive control). The results showed in the figure are expressed as the means average deviations of three separate experiments and bars indicate s.d. *P<0.05; **P<0.01, ***P<0.001.

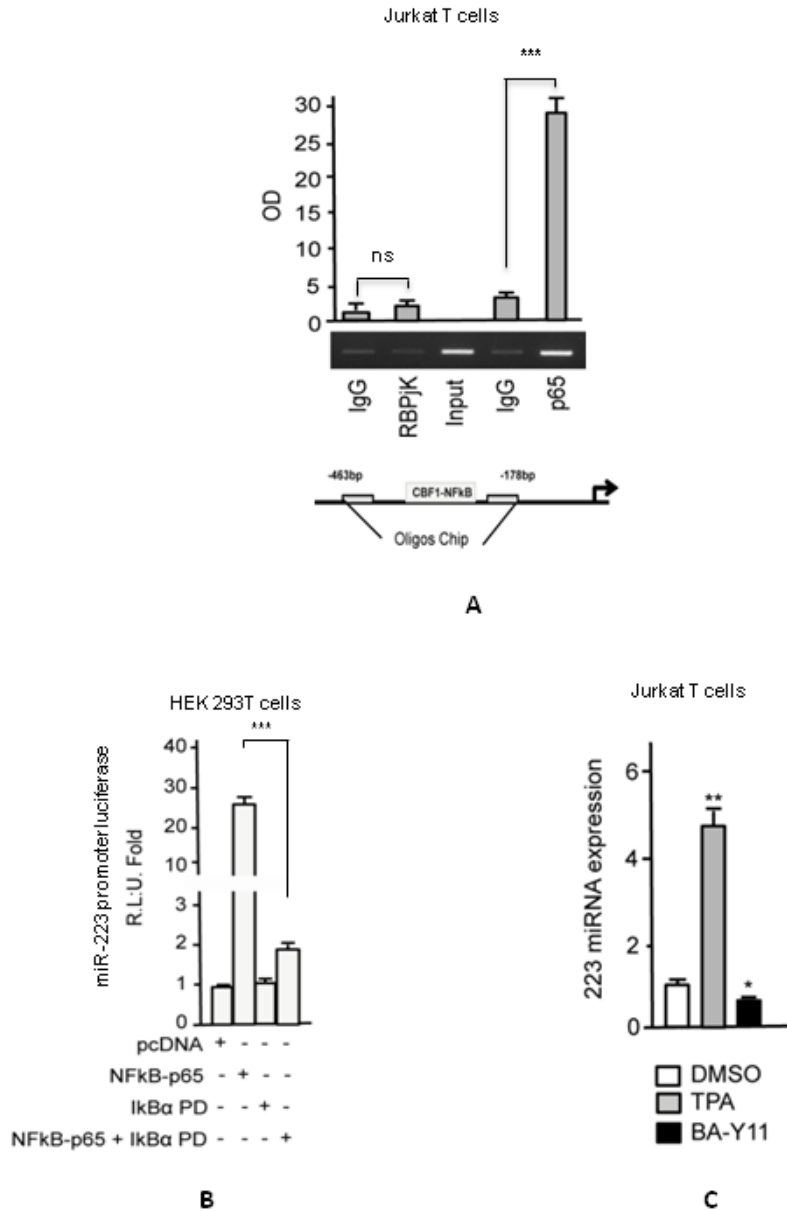


Figure 18 : NFκB (p65) directly regulate human miR-223 expression : A) Jurkat T cells were processed for chromatin preparation and then subjected to immunoprecipitation with antibodies against either NFκB-p65 , RBPJk or IgG as control . The immunoprecipitates were analyzed by PCR with human miR-223 promoter-specific primers. The upper panels show the relative quantification as determined by optical densitometry (OD). Schematic representation of miR-223 promoter regions assessed by chromatin immunoprecipitation is indicated in the lower panel. B) Luciferase assay performed on HEK 293T cells after the co-transfection with a luciferase reporter construct containing the wt miR-223 promoter, NFκB-p65 and IκBα PD alone or in combination. C) The expression levels of endogenous miR-223 in Jurkat T cells treated with either TPA, BA-Y11-7082 or with the vehicle alone were examined by quantitative real-time RT-PCR as detailed in the Material and Methods. All the results showed in the figures are expressed as the means average deviations of three separate experiments and bars indicate s.d. *P<0.05; **P<0.01, ***P<0.001.

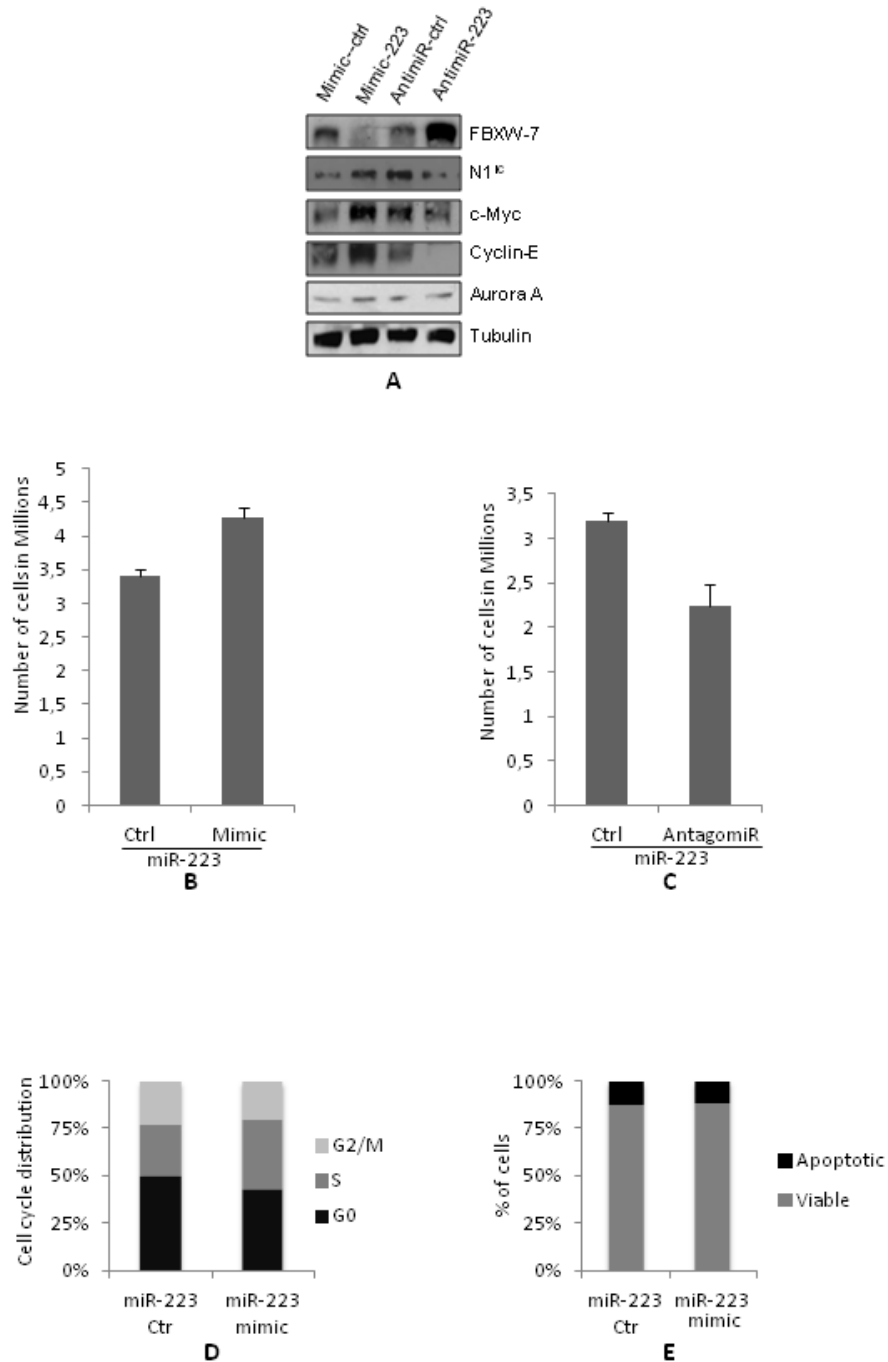


Figure 19 : miR-223 affects cell cycle by repressing FBXW7

A) Expression level of FBXW7 and its downstream target proteins such as Notch1, c-Myc, and CyclinE as analysed by western blot against anti-Notch1, anti-c-Myc, and anti-CyclinE, in Jurkat transiently transfected with either miR-223 Mimic or Antago-miR and relative controls. B) Increased cell growth of Jurkat transfected with Mimic of miR-223 compared to Jurkat transfected with Control, as shown by the cell count using trypan blue staining. C) Decreased cell growth of Jurkat transfected with antago-miR of miR-223 compared to Jurkat transfected with Control, as shown by the cell count using trypan blue staining. D & E) Jurkat cells transiently transfected with mimic of miR-223 or control subjected to fluorescence-activated cell sorting (FACS) analysis for cell cycle or Annexin assay for Apoptosis.

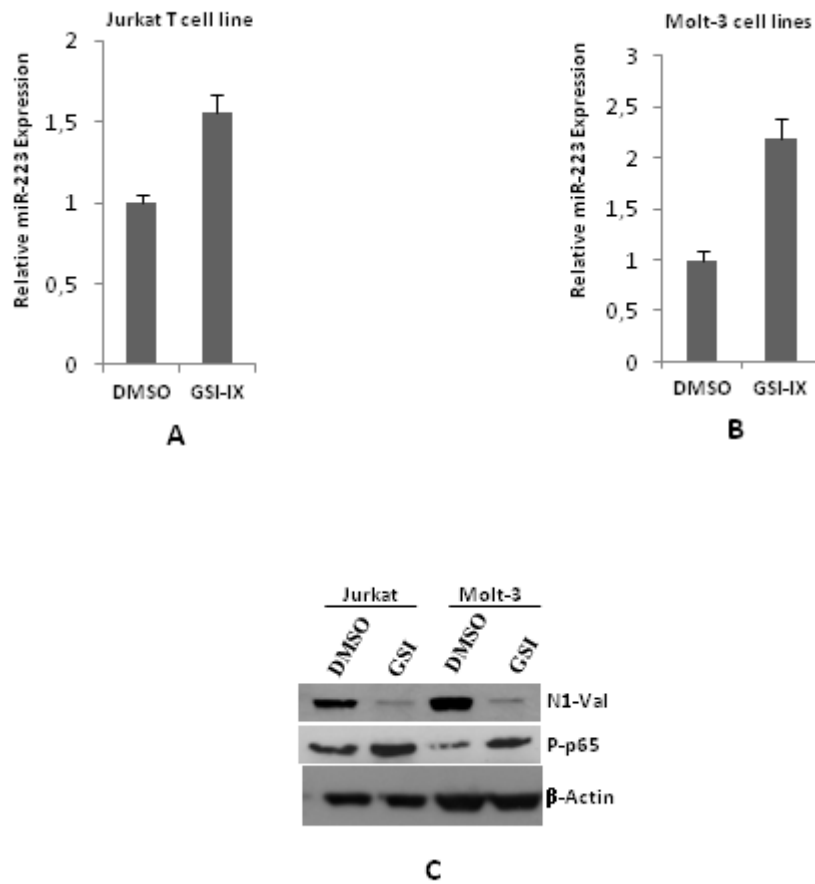


Figure 20 : GSI treatment induce miR-223 expression through NFκB pathway: A) & B) Relative expression of endogenous miR-223 in GSI IX or DMSO treated Jurkat and Molt3 T-ALL cell lines. **C)** Whole cell extract from DMSO and GSI IX treated Jurkat cells were subjected to western blot against antibody for Val-Notch1 and Phospho-p65. β-actin is used as loading control.

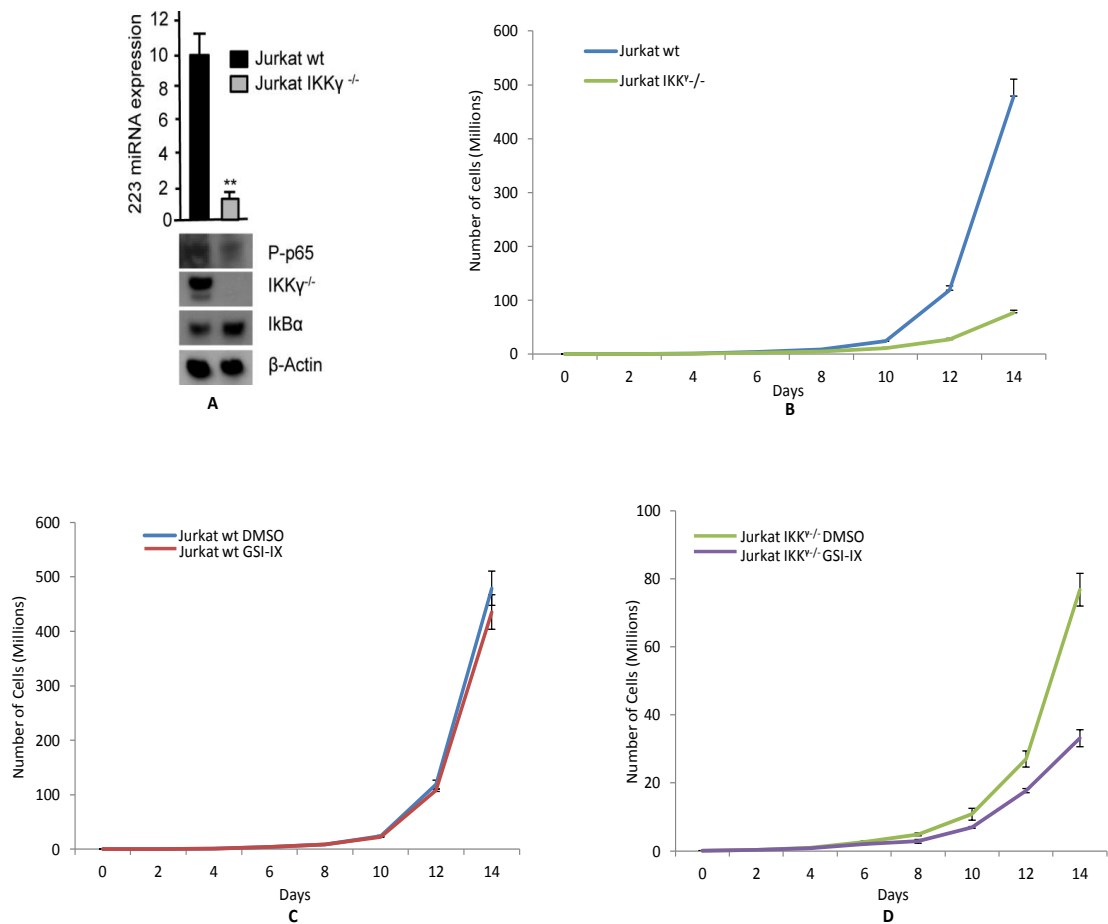


Figure 21 : Stable Deletion of NF κ B regulator IKK γ in Jurkat showed decreased miR-223 expression and cell-growth: A) Upper panel : RT-PCR shows decreased expression of endogenous miR-223 in Jurkat IKK γ ^{-/-} compared to Jurkat wild type (wt). Down panel : Western blot analysis of whole-cell extracts from Jurkat wt and Jurkat IKK γ ^{-/-} T cells probed with pospho-NF κ B p65, IKK γ and I κ B α antibodies. The α -actin expression was used as loading control. B) The graph shows decrease in cell growth of Jurkat IKK γ ^{-/-} compared to Jurkat wt until 14 days of culture as analysed by the cell count using trypan blue staining. C) Treatment with DMSO or GSI-IX (DAPT) in Jurkat wild type cell lines and its effect on cell growth as analysed by cell count until 14 days. D) Treatment with DMSO or GSI-IX (DAPT) in Jurkat IKK γ ^{-/-} cell lines and its effect on cell growth as analysed by cell count until 14 days.

Table 4 : Differential regulated miRNAs in N3^{IC} Transgenic Mice relative to its wild type counterpart.

<u>Up-Regulated miRNAs</u>				<u>Down-Regulated miRNAs</u>			
miRNA	Detector code	RQ (>2fold)	P value	miRNA	Detector code	RQ	P value
miR-337-5p	4395645	17,2411	1,5E-03	miR-150	4373127	0,5366	6,31E-02
miR-31	4373331	12,7473	5,1E-04	miR-700	4381057	0,5917	5,16E-02
miR-223	4395406	10,5385	2,0E-03	miR-126-3p	4395339	0,7590	2,41E-01
miR-146a	4373132	9,2801	1,0E-03	miR-378	4395354	0,8920	6,81E-01
miR-139-5p	4395400	8,1822	1,1E-03				
miR-455*	4378098	7,8619	2,1E-02				
miR-467b*	4381092	6,0277	3,7E-03				
miR-501-3p	4381069	5,9578	4,2E-02				
miR-29c	4395171	5,8174	3,4E-03				
miR-342-3p	4395371	5,5059	1,9E-03				
miR-361	4373035	5,1301	3,8E-03				
miR-194	4373106	5,0249	1,1E-03				
miR-590-5p	4395176	4,6243	8,9E-03				
miR-21	4373090	4,4293	3,8E-02				
miR-762	4395565	4,4134	4,7E-03				
miR-200c	4395411	4,2655	3,8E-03				
miR-425	4380926	4,0177	9,8E-03				
miR-28	4373067	3,9939	6,6E-03				
miR-138	4395395	3,4855	2,4E-03				
miR-25	4373071	3,3795	4,7E-03				
miR-182	4395729	3,2713	1,8E-02				
miR-467c	4395647	3,2052	1,0E-02				
miR-449a	4373207	3,0697	2,9E-02				
miR-20b	4373263	2,8252	9,3E-03				
miR-328	4373049	2,6988	1,2E-02				
miR-92a	4373013	2,6931	5,3E-03				
miR-181c	4373115	2,6809	2,0E-02				
miR-98	4373009	2,6504	3,3E-02				
miR-467b	4381084	2,6111	1,1E-02				
miR-195	4373105	2,5059	1,2E-02				
miR-103	4373158	2,5001	3,7E-02				

let-7d	4395394	2,4870	9,6E-03
miR-135a*	4395343	2,4549	7,6E-03
miR-17	4395419	2,4072	2,0E-02
miR-106a	4395589	2,3132	2,0E-02
miR-132	4373143	2,3082	4,6E-02
let-7f	4373164	2,1927	4,3E-02
miR-687	4386750	2,1460	1,0E-02
miR-760	4395439	2,0921	1,6E-02
let-7g	4395393	2,0827	4,0E-02

Table 5 : Differential regulated miRNAs in M31 Cell lines transiently transfected with N3^{IC} relative to M31 transfected with empty vector pcDNA.

<u>Up-Regulated miRNAs</u>				<u>Down-Regulated miRNAs</u>			
miRNA	Detector Code	RQ (>2fold)	Log ₁₀ (RQ)	miRNA	Detector Code	RQ (< 2fold)	Log ₁₀ (RQ)
miR-223	4395406	16634,7850	4,2210	miR-679	4381077	1,487E-05	-4,8277
miR-546	4381044	1332,2702	3,1246	miR-675-3p	4386762	1,601E-05	-4,7957
miR-875-3p	4395677	691,1303	2,8396	miR-363	4378090	1,821E-03	-2,7396
let-7g*	4395622	650,5439	2,8133	miR-665	4395737	4,566E-03	-2,3405
miR-742	4395573	641,8850	2,8075	miR-743a	4395599	4,995E-03	-2,3014
miR-99a	4373008	346,1544	2,5393	miR-9	4373285	1,122E-02	-1,9500
miR-452	4373281	133,5740	2,1257	let-7c-1*	4395609	1,373E-02	-1,8625
miR-497	4381046	128,3268	2,1083	miR-411	4381013	1,869E-02	-1,7284
miR-451	4373360	89,6460	1,9525	miR-101a*	4395637	2,067E-02	-1,6846
miR-15a	4373123	28,4902	1,4547	miR-694	4381090	2,924E-02	-1,5340
miR-701	4381058	15,9166	1,2019	miR-127	4373147	3,021E-02	-1,5199
miR-681	4381080	14,7949	1,1701	miR-196a*	4395607	4,981E-02	-1,3027
miR-674*	4386773	14,5647	1,1633	miR-106b*	4395491	1,240E-01	-0,9066
miR-698	4381055	12,3537	1,0918	miR-702	4381059	1,395E-01	-0,8553
miR-672	4395438	12,3158	1,0905	miR-875-5p	4395314	1,411E-01	-0,8506
miR-92a*	4395626	9,4531	0,9756	miR-708*	4395453	1,956E-01	-0,7087
miR-151-3p	4373304	8,6860	0,9388	miR-878-3p	4395671	2,123E-01	-0,6730
miR-582-3p	4395697	8,4844	0,9286	miR-186*	4395704	2,148E-01	-0,6679
miR-409-3p	4395443	7,7586	0,8898	miR-721	4381073	2,228E-01	-0,6521

miR-297c	4395610	5,8229	0,7651	miR-467e	4395698	2,421E-01	-0,6161
miR-206	4373092	5,7802	0,7619	miR-501-3p	4381069	2,513E-01	-0,5998
miR-335-3p	4395296	4,9526	0,6948	miR-197	4373102	2,522E-01	-0,5983
miR-450b-5p	4386779	4,6840	0,6706	miR-671-3p	4395433	2,552E-01	-0,5932
miR-539	4378103	4,6084	0,6636	miR-509-3p	4395651	2,584E-01	-0,5877
miR-380-5p	4395731	4,5820	0,6611	miR-138	4395395	2,588E-01	-0,5871
miR-125a-5p	4395309	4,3750	0,6410	miR-34b-3p	4395748	2,602E-01	-0,5846
miR-322*	4395636	4,1529	0,6183	miR-193b	4395597	2,645E-01	-0,5776
miR-99b*	4395307	3,9656	0,5983	miR-697	4381054	2,690E-01	-0,5703
miR-676	4386776	3,8213	0,5822	miR-294*	4395725	3,059E-01	-0,5144
miR-218	4373081	3,7559	0,5747	miR-208	4373091	3,121E-01	-0,5057
miR-31	4373331	3,7463	0,5736	miR-801	4395562	3,360E-01	-0,4736
miR-191*	4395706	3,1237	0,4947	miR-449c	4386747	3,565E-01	-0,4480
miR-330*	4373337	2,8549	0,4556	miR-17*	4395673	3,667E-01	-0,4357
miR-449b	4395669	2,6087	0,4164	miR-27b*	4395285	3,855E-01	-0,4140
miR-673-5p	4386772	2,5662	0,4093	miR-141	4373137	3,860E-01	-0,4134
miR-200a	4378069	2,3016	0,3620	miR-190b	4395374	4,496E-01	-0,3472
miR-491	4381053	2,2319	0,3487	miR-383	4381093	4,841E-01	-0,3151
miR-331-5p	4395344	2,2096	0,3443	miR-361	4373035	5,028E-01	-0,2986
miR-375	4373027	2,1795	0,3384	miR-501-5p	4381048	5,034E-01	-0,2981
miR-181a	4373117	2,1741	0,3373	miR-7a*	4381118	5,085E-01	-0,2937
miR-297a*	4395584	2,1503	0,3325	miR-7a	4378130	5,089E-01	-0,2933
miR-181a-1*	4373086	2,1404	0,3305	miR-23b	4373073	5,110E-01	-0,2916
miR-103	4373158	2,1346	0,3293	miR-466c-5p	4395631	5,120E-01	-0,2907
miR-365	4373194	2,1269	0,3277	miR-503	4395586	5,139E-01	-0,2891
miR-182	4395729	2,1239	0,3271	let-7d*	4378108	5,165E-01	-0,2869
miR-450a-5p	4395414	2,1068	0,3236	miR-547	4395694	5,167E-01	-0,2867
miR-467b*	4381092	2,1031	0,3229	miR-33*	4395247	5,176E-01	-0,2860
miR-106b	4373155	2,1021	0,3227	miR-26b*	4395555	5,210E-01	-0,2831
miR-185	4395382	2,0976	0,3217	miR-590-5p	4395176	5,238E-01	-0,2808
miR-19a	4373099	2,0882	0,3198	miR-696	4381051	5,244E-01	-0,2803
let-7d	4395394	2,0882	0,3198	miR-22*	4395412	5,271E-01	-0,2781
miR-532-3p	4395466	2,0867	0,3195	let-7f*	4395528	5,316E-01	-0,2744
miR-324-5p	4373052	2,0863	0,3194	miR-34c*	4395714	5,358E-01	-0,2710
miR-183	4395380	2,0807	0,3182	miR-20a*	4395621	5,364E-01	-0,2705

miR-425	4380926	2,0806	0,3182	miR-467e*	4395699	5,453E-01	-0,2634
miR-339-5p	4395368	2,0786	0,3178	miR-741	4395587	5,531E-01	-0,2572
miR-328	4373049	2,0765	0,3173	miR-805	4395577	5,584E-01	-0,2530
miR-21*	4395623	2,0742	0,3169	miR-666-5p	4386770	5,682E-01	-0,2455
miR-574-3p	4395460	2,0735	0,3167	ath-miR159a	4373390	5,741E-01	-0,2410
miR-676*	4386775	2,0715	0,3163	miR-699	4381056	5,895E-01	-0,2295
miR-29a	4395223	2,0710	0,3162				
miR-652	4395463	2,0701	0,3160				
miR-25	4373071	2,0681	0,3156				
miR-140	4373374	2,0647	0,3149				
miR-27b	4373068	2,0624	0,3144				
let-7b	4373168	2,0619	0,3143				
miR-322	4378107	2,0618	0,3142				
miR-26a	4395166	2,0612	0,3141				
miR-467a	4395717	2,0612	0,3141				
miR-142-5p	4395359	2,0563	0,3131				
miR-24	4373072	2,0558	0,3130				
let-7i	4395332	2,0548	0,3128				
miR-708	4395452	2,0547	0,3127				
miR-872*	4395672	2,0529	0,3124				
miR-139-5p	4395400	2,0525	0,3123				
miR-30c	4373060	2,0520	0,3122				
let-7a	4373169	2,0516	0,3121				
miR-872	4395375	2,0515	0,3121				
miR-532-5p	4380928	2,0498	0,3117				
miR-362-3p	4395746	2,0489	0,3115				
miR-301a	4373064	2,0484	0,3114				
miR-500	4395736	2,0482	0,3114				
miR-188-5p	4395431	2,0481	0,3113				
miR-32	4395220	2,0472	0,3112				
miR-582-5p	4395696	2,0451	0,3107				
let-7e	4395517	2,0426	0,3102				
miR-30e*	4373057	2,0381	0,3092				
miR-378	4395354	2,0295	0,3074				
miR-27a	4373287	2,0293	0,3074				

miR-467d	4395648	2,0271	0,3069
miR-467c	4395647	2,0061	0,3023
miR-881	4395739	2,0041	0,3019

Table 6: Differential regulated miRNAs in Molt3 T-ALL cell lines stably transduced with shRNA for Notch3 relative to its control counterpart.

<u>Down-Regulated miRNAs</u>				<u>Up-Regulated miRNAs</u>			
miRNA	Detector Code	RQ (< 2fold)	Log ₁₀ (RQ)	miRNA	Detector Code	RQ (>2fold)	Log ₁₀ (RQ)
miR-892b	2214	7,260E-06	-5,1391	miR-497#	2368	3897,4857	3,5908
miR-1267	2885	1,416E-05	-4,8490	miR-144	2676	1868,1169	3,2714
miR-1276	2843	3,090E-05	-4,5100	miR-34c-5p	4373036	633,2197	2,8016
miR-1247	2893	8,262E-04	-3,0829	ath-miR159a	4373390	336,0763	2,5264
miR-520D-3P	2743	1,920E-03	-2,7168	miR-1282	2803	112,9981	2,0531
miR-525-5p	4378088	3,330E-03	-2,4776	miR-665	2681	49,8670	1,6978
miR-644	1596	7,042E-03	-2,1523	miR-559	1527	32,0573	1,5059
miR-1249	2868	1,355E-02	-1,8680	miR-765	2643	30,3561	1,4822
miR-522	4395524	2,942E-02	-1,5314	let-7a#	2307	17,6805	1,2475
miR-567	1534	2,967E-02	-1,5276	miR-127-5p	4395340	13,7401	1,1380
miR-496	4386771	3,210E-02	-1,4936	miR-1301	2827	12,6245	1,1012
miR-551b	4380945	5,282E-02	-1,2772	miR-521	4373259	8,9110	0,9499
miR-215	4373084	5,593E-02	-1,2523	miR-219-1-3p	4395206	8,5010	0,9295
miR-1292	2824	6,172E-02	-1,2096	miR-151-3p	2254	7,7339	0,8884
miR-18b	4395328	7,333E-02	-1,1347	miR-520f	4373256	4,4012	0,6436
miR-33a	2135	1,000E-01	-0,9998	miR-519d	4395514	4,2449	0,6279
miR-519e	4395481	1,106E-01	-0,9564	miR-29b-2#	2166	4,1180	0,6147
miR-539	4378103	1,127E-01	-0,9482	miR-424#	2309	4,0478	0,6072
let-7f-2#	2418	1,180E-01	-0,9280	miR-1227	2769	3,9661	0,5984
miR-520g	4373257	1,201E-01	-0,9206	miR-589	1543	3,9111	0,5923
miR-96	4373372	1,368E-01	-0,8638	miR-1255B	2801	3,8809	0,5889
miR-299-3p	4373189	1,665E-01	-0,7785	miR-15a#	2419	3,8486	0,5853
miR-1303	2792	2,334E-01	-0,6319	miR-17#	2421	3,8481	0,5852
miR-935	2178	2,343E-01	-0,6302	miR-151-5P	2642	3,8442	0,5848
miR-34a#	2316	2,346E-01	-0,6297	miR-590-3P	2677	3,8373	0,5840

miR-204	4373094	2,374E-01	-0,6245	miR-27a#	2445	3,8080	0,5807
miR-523	4395497	2,411E-01	-0,6178	miR-363#	1283	3,8035	0,5802
miR-148b#	2160	2,424E-01	-0,6155	miR-148a#	2134	3,7209	0,5706
miR-190	4373110	2,428E-01	-0,6147	miR-639	1583	3,7191	0,5704
miR-181a	4373117	2,502E-01	-0,6017	miR-662	1607	2,5298	0,4031
miR-202	4395474	2,633E-01	-0,5795	miR-1183	2841	2,4364	0,3868
miR-374a	4373028	2,636E-01	-0,5791	miR-1825	2907	2,3719	0,3751
miR-19a	4373099	2,638E-01	-0,5787	miR-628-5p	4395544	2,2537	0,3529
let-7d	4395394	2,639E-01	-0,5786	miR-487a	4378097	2,1139	0,3251
miR-223	4395406	2,642E-01	-0,5781	miR-99b	4373007	2,1088	0,3240
miR-30c	4373060	2,652E-01	-0,5765	miR-505	4395200	2,0022	0,3015
miR-374b	4381045	2,658E-01	-0,5755	miR-1253	2894	2,0015	0,3013
miR-574-3p	4395460	2,659E-01	-0,5752				
miR-501-5p	4373226	2,667E-01	-0,5740				
miR-31	4395390	2,673E-01	-0,5730				
miR-95	4373011	2,676E-01	-0,5726				
miR-744	4395435	2,681E-01	-0,5718				
miR-346	4373038	2,681E-01	-0,5717				
miR-106b	4373155	2,682E-01	-0,5716				
miR-579	4395509	2,688E-01	-0,5705				
miR-27a	4373287	2,689E-01	-0,5704				
miR-660	4380925	2,695E-01	-0,5694				
miR-340	4395369	2,696E-01	-0,5693				
miR-185	4395382	2,697E-01	-0,5691				
miR-342-5p	4395258	2,702E-01	-0,5684				
miR-494	4395476	2,712E-01	-0,5667				
miR-107	4373154	2,716E-01	-0,5660				
miR-183	4395380	2,719E-01	-0,5656				
miR-520e	4373255	2,720E-01	-0,5655				
let-7c	4373167	2,721E-01	-0,5653				
miR-210	4373089	2,723E-01	-0,5650				
miR-34a	4395168	2,735E-01	-0,5630				
miR-542-3p	4378101	2,739E-01	-0,5625				
miR-597	4380960	2,739E-01	-0,5623				
miR-103	4373158	2,752E-01	-0,5603				

miR-301a	4373064	2,759E-01	-0,5593
miR-520a-5p	4378085	2,903E-01	-0,5371
miR-129-3p	4373297	2,959E-01	-0,5289
miR-23a	4373074	3,022E-01	-0,5198
miR-379	4373349	3,098E-01	-0,5089
miR-872	4395375	3,111E-01	-0,5070
miR-190b	2263	3,580E-01	-0,4461
miR-191#	2678	4,408E-01	-0,3557
miR-181c#	2333	4,683E-01	-0,3295
miR-505#	2087	4,723E-01	-0,3257
miR-720	2895	4,731E-01	-0,3251
miR-664	2897	4,748E-01	-0,3235
miR-20a#	2437	4,776E-01	-0,3210
miR-1248	2870	4,793E-01	-0,3194
miR-31#	2113	4,794E-01	-0,3193
miR-335#	2185	4,798E-01	-0,3189
miR-519a	4395526	4,805E-01	-0,3183
miR-425#	2302	4,806E-01	-0,3182
miR-15b#	2173	4,843E-01	-0,3149
miR-106b#	2380	4,843E-01	-0,3149
miR-192#	2272	4,852E-01	-0,3141
miR-16-1#	2420	4,853E-01	-0,3140
miR-564	1531	4,886E-01	-0,3111
miR-126#	451	4,890E-01	-0,3107
miR-941	2183	4,946E-01	-0,3058
miR-744#	2325	5,033E-01	-0,2981
miR-1201	2781	5,054E-01	-0,2964
miR-548d-3p	4381008	5,065E-01	-0,2954
miR-200b	4395362	5,128E-01	-0,2900
let-7e	4395517	5,136E-01	-0,2893
miR-19b	4373098	5,149E-01	-0,2883
miR-23b	4373073	5,169E-01	-0,2866
miR-296-5p	4373066	5,192E-01	-0,2846
miR-449b	4381011	5,208E-01	-0,2833
miR-323-3p	4395338	5,222E-01	-0,2821

miR-212	4373087	5,252E-01	-0,2796
miR-483-5p	4395449	5,291E-01	-0,2765
miR-21	4373090	5,302E-01	-0,2755
miR-518e	4395506	5,306E-01	-0,2752
miR-299-5p	4373188	5,308E-01	-0,2751
miR-331-3p	4373046	5,322E-01	-0,2740
miR-193a-5p	4395392	5,325E-01	-0,2737
miR-15a	4373123	5,340E-01	-0,2725
miR-29c	4395171	5,341E-01	-0,2723
miR-92a	4395169	5,344E-01	-0,2722
miR-182	4395445	5,346E-01	-0,2720
miR-532-5p	4380928	5,348E-01	-0,2718
miR-222	4395387	5,349E-01	-0,2717
miR-625	4395542	5,356E-01	-0,2711
miR-106a	4395280	5,359E-01	-0,2709
has-miR-155	4395459	5,360E-01	-0,2709
miR-330-3p	4373047	5,361E-01	-0,2708
let-7g	4395393	5,361E-01	-0,2708
miR-491-5p	4381053	5,363E-01	-0,2706
miR-30b	4373290	5,367E-01	-0,2703
miR-142-5p	4395359	5,369E-01	-0,2701
miR-128	4395327	5,370E-01	-0,2700
miR-502-3p	4395194	5,370E-01	-0,2700
miR-18a	4395533	5,371E-01	-0,2700
miR-342-3p	4395371	5,371E-01	-0,2699
miR-150	4373127	5,371E-01	-0,2699
miR-301b	4395503	5,371E-01	-0,2699
miR-181c	4373115	5,382E-01	-0,2691
miR-132	4373143	5,385E-01	-0,2688
miR-362-5p	4378092	5,386E-01	-0,2688
miR-454	4395434	5,388E-01	-0,2686
miR-28-3p	4395557	5,389E-01	-0,2685
miR-361-5p	4373035	5,389E-01	-0,2685
miR-324-5p	4373052	5,391E-01	-0,2684
miR-195	4373105	5,393E-01	-0,2682

miR-200c	4395411	5,393E-01	-0,2681
miR-425	4380926	5,394E-01	-0,2681
miR-146b-5p	4373178	5,400E-01	-0,2676
miR-203	4373095	5,401E-01	-0,2675
miR-636	4395199	5,403E-01	-0,2674
miR-193b	4395478	5,403E-01	-0,2673
miR-484	4381032	5,405E-01	-0,2672
miR-509-3-5p	4395266	5,406E-01	-0,2671
miR-130b	4373144	5,406E-01	-0,2671
miR-328	4373049	5,406E-01	-0,2671
miR-326	4373050	5,407E-01	-0,2670
miR-320	4395388	5,409E-01	-0,2669
miR-17	4395419	5,416E-01	-0,2664
miR-28-5p	4373067	5,418E-01	-0,2662
miR-616	4395525	5,419E-01	-0,2661
miR-93	4373302	5,420E-01	-0,2660
miR-455-3p	4395355	5,427E-01	-0,2654
miR-27b	4373068	5,427E-01	-0,2654
miR-576-3p	4395462	5,429E-01	-0,2653
miR-424	4373201	5,429E-01	-0,2653
miR-139-5p	4395400	5,431E-01	-0,2652
miR-192	4373108	5,431E-01	-0,2651
miR-26a	4395166	5,432E-01	-0,2651
miR-26b	4395167	5,433E-01	-0,2650
miR-25	4373071	5,433E-01	-0,2649
miR-221	4373077	5,438E-01	-0,2646
miR-671-3p	4395433	5,438E-01	-0,2645
miR-363	4378090	5,442E-01	-0,2643
miR-339-3p	4395295	5,442E-01	-0,2642
miR-324-3p	4395272	5,443E-01	-0,2641
miR-500	4395539	5,445E-01	-0,2640
miR-98	4373009	5,451E-01	-0,2635
miR-518d-3p	4373248	5,452E-01	-0,2635
miR-186	4395396	5,453E-01	-0,2634
miR-489	4395469	5,454E-01	-0,2633

let-7f	4373164	5,455E-01	-0,2632
miR-197	4373102	5,455E-01	-0,2632
miR-130a	4373145	5,458E-01	-0,2630
miR-140-5p	4373374	5,460E-01	-0,2628
miR-148a	4373130	5,466E-01	-0,2624
miR-101	4395364	5,474E-01	-0,2617
miR-142-3p	4373136	5,481E-01	-0,2611
miR-551b#	2346	5,491E-01	-0,2604
miR-590-5p	4395176	5,492E-01	-0,2603
let-7a	4373169	5,497E-01	-0,2599
miR-140-3p	4395345	5,500E-01	-0,2597
miR-652	4395463	5,500E-01	-0,2597
miR-20b	4373263	5,519E-01	-0,2582
miR-422a	4395408	5,526E-01	-0,2576
miR-502-5p	4373227	5,531E-01	-0,2572
miR-532-3p	4395466	5,537E-01	-0,2568
miR-618	4380996	5,538E-01	-0,2567
miR-335	4373045	5,548E-01	-0,2559
miR-629	4395547	5,552E-01	-0,2556
miR-126	4395339	5,558E-01	-0,2551
miR-24	4373072	5,566E-01	-0,2544
miR-661	1606	5,568E-01	-0,2543
miR-184	4373113	5,577E-01	-0,2536
miR-29a	4395223	5,586E-01	-0,2529
miR-1	4395333	5,601E-01	-0,2517
let-7b	4395446	5,612E-01	-0,2509
miR-16	4373121	5,619E-01	-0,2504
miR-518b	4373246	5,633E-01	-0,2492
miR-187	4373307	5,691E-01	-0,2448
miR-365	4373194	5,729E-01	-0,2420
miR-9	4373285	5,738E-01	-0,2412
miR-20a	4373286	5,759E-01	-0,2397
miR-486-5p	4378096	5,805E-01	-0,2362
miR-1298	2861	5,827E-01	-0,2345

Table 7: Differential regulated miRNAs in Jurkat cell lines silenced for Notch3 relative to its scramble counterpart.

Down-Regulated miRNA			Up-Regulated miRNA		
miRNA	Detector Code	RQ (>2fold)	miRNA	Detector Code	RQ (>2fold)
miR-1267	2885	8,58232E-06	miR-137	4373301	15352,415053
miR-198	4395384	0,000737	miR-628-5p	4395544	7946,627478
miR-127-3p	4373147	0,007004	miR-299-3p	4373189	202,319140
miR-337-5p	4395267	0,007091	miR-133b	4395358	42,648704
miR-1298	2861	0,008838	miR-99a	4373008	36,339508
miR-124	4373295	0,009365	miR-1201	2781	4,582948
miR-539	4378103	0,058724	miR-1300	2902	4,490683
miR-449b	4381011	0,116513	miR-215	4373084	4,201883
miR-491-5p	4381053	0,124069	miR-193b#	2366	4,044530
miR-331-5p	4395344	0,128924	miR-571	1613	3,174796
miR-518f	4395499	0,137446	miR-424	4373201	3,120773
miR-122	4395356	0,139273	miR-1262	2852	2,823383
miR-129-3p	4373297	0,194106	miR-500	1046	2,411622
miR-105	4395278	0,204880	miR-661	1606	2,305753
miR-487a	4378097	0,239804	miR-30a-5p	417	2,273452
miR-200c	4395411	0,244434	miR-190b	2263	2,266146
miR-130b	4373144	0,247882	miR-543	2376	2,265451
let-7a	4373169	0,248395	miR-151-5P	2642	2,251496
miR-339-3p	4395295	0,249094	miR-1260	2896	2,251487
miR-486-5p	4378096	0,250567	miR-223#	2098	2,248335
miR-195	4373105	0,250718	miR-1290	2863	2,243967
miR-500	4395539	0,251805	miR-26b#	2444	2,240106
miR-636	4395199	0,251836	miR-26a-1#	2443	2,238476
miR-494	4395476	0,253856	miR-126#	451	2,228261
miR-744	4395435	0,254502	miR-1254	2818	2,225146
miR-27a	4373287	0,255139	miR-663B	2857	2,218681
miR-181a	4373117	0,255194	miR-938	2181	2,216853
miR-132	4373143	0,255857	miR-378	567	2,210304
miR-29b	4373288	0,255864	miR-106b#	2380	2,177423
miR-20b	4373263	0,256223	miR-1296	2908	2,167619
miR-142-3p	4373136	0,260388	miR-518d-3p-	4373248	2,099646
let-7f	4373164	0,263536	miR-328	4373049	2,042388
miR-15b	4373122	0,271889	miR-194	4373106	2,041546
miR-302a#	2381	0,295837	miR-1227	2769	2,035154
miR-522	4395524	0,426182			
miR-874	4395379	0,464569			
miR-1294	2785	0,486192			
miR-217	4395448	0,487889			
miR-501-5p	4373226	0,492347			
miR-17	4395419	0,496901			
miR-331-3p	4373046	0,497826			
miR-652	4395463	0,500312			

miR-182	4395445	0,500773
miR-18b	4395328	0,500893
miR-519d	4395514	0,501203
miR-484	4381032	0,501513
miR-660	4380925	0,502092
miR-362-5p	4378092	0,504181
miR-425	4380926	0,504361
miR-196b	4395326	0,504464
miR-197	4373102	0,504837
miR-149	4395366	0,504907
miR-126	4395339	0,505109
miR-29a	4395223	0,505239
miR-455-3p	4395355	0,505315
miR-29c	4395171	0,505946
miR-483-5p	4395449	0,506506
miR-345	4395297	0,506624
miR-221	4373077	0,506681
miR-363	4378090	0,506844
miR-128	4395327	0,507212
miR-25	4373071	0,507407
miR-106b	4373155	0,507718
miR-103	4373158	0,507736
miR-150	4373127	0,507777
miR-422a	4395408	0,507880
miR-95	4373011	0,508274
miR-21	4373090	0,508377
miR-146a	4373132	0,508996
miR-138	4395395	0,509051
miR-106a	4395280	0,509280
miR-20a	4373286	0,509351
miR-526b	4395493	0,509899
miR-324-3p	4395272	0,509902
miR-30b	4373290	0,510112
miR-146b-5p	4373178	0,510461
miR-296-5p	4373066	0,510466
miR-19a	4373099	0,510560
miR-590-5p	4395176	0,510625
miR-301b	4395503	0,510940
miR-532-3p	4395466	0,510973
miR-301a	4373064	0,511083
miR-342-3p	4395371	0,511111
miR-30c	4373060	0,511135
miR-223	4395406	0,511350
miR-28-3p	4395557	0,511471
miR-26b	4395167	0,511559
miR-192	4373108	0,511707
miR-505	4395200	0,511900

miR-361-5p	4373035	0,511955
miR-222	4395387	0,512022
miR-320	4395388	0,512317
miR-455-5p	4378098	0,512784
miR-19b	4373098	0,512996
miR-140-5p	4373374	0,513135
miR-99b	4373007	0,513361
miR-93	4373302	0,513432
miR-24	4373072	0,513722
miR-374a	4373028	0,514210
let-7b	4395446	0,514218
miR-210	4373089	0,514761
miR-532-5p	4380928	0,514777
miR-139-5p	4395400	0,515020
miR-671-3p	4395433	0,515055
miR-140-3p	4395345	0,515375
miR-454	4395434	0,515641
miR-26a	4395166	0,516401
miR-183	4395380	0,516477
miR-324-5p	4373052	0,516772
miR-148a	4373130	0,516957
let-7g	4395393	0,517136
miR-1825	2907	0,517330
miR-18a	4395533	0,517983
miR-186	4395396	0,518778
miR-374b	4381045	0,519032
let-7e	4395517	0,519188
miR-9	4373285	0,519737
miR-92a	4395169	0,520252
miR-28-5p	4373067	0,520277
miR-492	4373217	0,521188
let-7d	4395394	0,522238
miR-213	516	0,536599
miR-942	2187	0,545913
miR-517a	4395513	0,546139
miR-629	1562	0,553075
miR-340#	2259	0,553100
rno-miR-7#	1338	0,553745
miR-425#	2302	0,554031
miR-130b#	2114	0,556442
miR-941	2183	0,558926
miR-520a-3p	4373268	0,589622

5. Discussion

Canonically, Notch acts as a membrane bound co-transcription factor that is released in response to ligand binding by two proteases acting sequentially. The freed intracellular domain enters the nucleus, where it interacts with a DNA-bound protein complex to activate transcription of selected target genes. Despite, dysregulated Notch signaling constitutes one of the major signaling pathway in inducing T-ALL, it has not been fully elucidated all the target genes of Notch including miRNAs, how these target are regulated and consequence of this in T-ALL development. Thus in this work we aimed to understand if Notch3 was able to regulate some miRNAs, both in-vivo and in cell models which were modulated with different expression of Notch3. Here we utilized different cell models from mouse to human and modulated the expression of Notch3 in order to get more universal miRNAs which could be regulated by Notch3 in different cell contexts. The miRNAs profiling revealed 7 significant miRNAs which were positively regulated by modulation of Notch3. Out of these miRNAs, miR-223 exhibited the most dynamic up and down-regulation after Notch3 overexpression or knockdown. Single miR-223 validation by RT-PCR in our models confirmed the regulation of miR-223 in response to modulation of Notch3, thus the result obtained by the array analysis was not false. Although the identification of differentially regulated genes by microarray analysis is important to understand transcriptional networks, it provides only limited information on the regulation of genes as microarray analysis cannot distinguish between direct and indirect targets. The presence of a CSL binding site in the promoter of a gene could be an indicative of direct regulation by Notch signaling. Promoter analysis of miR-223 revealed the conserved CSL/RBPJ κ binding site which was nested with NF κ B. Consistent with our finding of miR-223 overexpression in Notch3 models, a recent study identified miR-223 as the most abundant miRNA in T-ALL patient samples and revealed its oncogenic role by virtue of its ability to accelerate Notch-induced T-ALL in a murine model mice [188]. Another

studies identified miR-223 as overexpressed in CD4⁺ naive T-lymphocytes from individuals affected with rheumatoid arthritis [211]. These evidences made us to focus on miR-223 in relation on how it was regulated by Notch3. As expected, the luciferase assay on mir-223 promoter revealed a direct regulation by Notch3 transcriptional complex, but interestingly also p65 induced the promoter activation as the CSL/RBPjK binding site on miR-223 was nested with NFκB. Thus revealing that miR-223 promoter could be regulated in part directly by Notch3 and indirectly by activating NFκB signaling pathway during T Lymphocyte development. This is also true as our lab has already published that in Notch3 transgenic mice which develop symptoms of T-ALL there is constitutive activation of NFκB [61] [189]. As miR-223 has been shown to exert diverse role depending on the cell context [212] [213] [214] [215] [200] so it would be unlikely that its transcriptional regulation is controlled by single transcriptional factor. Previous reports have been already described about the transcriptional regulation of miR-223 by CEBPα , NFI-A [193] P.U1 [194] and E2F1 [216], but all these studies were done on Acute Myeloid Leukemia (AML). Although mir-223 has been reported to be more up regulated in AML compared to ALL, [217], it cannot be assumed that its expression is fully repressed in ALL patients, thus there must be some mechanism which are responsible for regulation of miR-223 in ALL patients apart from myeloid specific transcription factors such as CEBPa & P.U1. Here we report for the first time that miR-223 regulation in T-ALL context was dependent on Notch3 and NFκB signaling. The luciferase assay on the mutant of miR-223 promoter deleted for NFκB/CSL/ RBPjK consensus site and the Chip analysis gave us the strong evidences that p65 really played a direct role in regulation of miR-223 at transcriptional level. Moreover the luciferase assay on miR-223 promoter with p65 in presence of IκBα-sr and pharmacological approach such as TPA (which is known to induce NFκB signaling pathway) and BAY-11-7082 (which is known to repress NFκB pathway) on endogenous miR-223 in Jurkat cell lines justified more appropriately that p65 was involved in miR-223 regulation.

As we were able to show that miR-223 could directly regulate FBXW7 by transient transfecting mimic and antimiR-223 in Jurkat cell lines and this regulation affected the stabilization of FBXW7 targets such as c-myc, cyclin-E , Notch1 etc which were already reported to be involved in cell-cycle, proliferation and growth, suggesting the oncogenic role of miR-223 in T-ALL through repression of tumor suppressor FBXW7. Previous reports have already suggested that down modulation of Notch signaling in T-ALL could result in growth arrest by modulating Notch direct target gene such as c-Myc, but till now no report has highlighted the relationship on Notch3 and miRNAs in cell proliferation and growth. Our study could suggest another aspect by which Notch could regulate cell growth and proliferation in T-ALL by regulating miR-223 through p65. This functional aspect of miR-223 modulation in cell cycle in T-ALL was confirmed when mimic of miR-223 showed an increase in cell growth and increase in percentage of cells in S phase compared to negative control by FACS analysis on Jurkat cell lines. To deepen our knowledge on functional aspect of miR-223 in growth regulation, we utilized Jurkat cell lines which were stably deleted for IKK γ . These cell lines showed decrease phospho-p65 and less induction of miR-223 when compared with Jurkat wild type. The stable clone of IKK γ $-/-$, showed decrease cell growth as expected, compared to Jurkat wild type when they were kept in culture for 14 days. Although it cannot be excluded that the effect of IKK γ deletion on other signaling pathways can also be responsible for decrease in cell growth.

We had already validated that down modulation of Notch3 specifically by Sh or siRNA in Molt3 and Jurkat leads to down modulation of miR-223. Keeping this aspect we tried to use DAPT (GSI- IX) (γ -Secretase Inhibitor-IX) to decrease miR-223 expression by blocking Notch signaling, but unexpectedly we got an opposite result as DAPT instead of suppressing had an inductive effect on miR-223 expression both in Jurkat and Molt-3 T-ALL cell lines. Keeping this aspect in consistent, a very recent publication revealed that GSI-XXI Compound

E treatments increased miR-223 levels in several T ALL cell lines such as Jurkat and RPMI 8402 cells and by this they suggested that active Notch signaling could repress miR-223 expression by regulating an intermediate repressor protein. In this study the authors hypothesized Notch signaling positively regulate the expression of some transcriptional repressor of miR-223 and therefore GSI-XXI treatments inhibiting Notch signaling, indirectly, leads the increased miR-223 expression. Unfortunately, the authors didn't identify the specific molecules responsible of the miR-223 gene regulation. Moreover, the authors demonstrated that neither Hes1 and Myc are involved in this mechanism as enforced expression of both, did not prevent GSI-XXI-dependent miR-223 overexpression [210]. The regulation miR-223 by DAPT treatment was completely opposite to what we observed when we down modulated Notch3 by Sh or siRNA, without modulating other Notch receptors. As the specific silencing of Notch3 is more reliable to see the effect of Notch3 compared to DAPT treatment. This results suggest that GSI could not be used as a sole compound to better understand the Notch signaling mechanism exerted by different Notches as GSI treatment block all Notch receptors making it difficult to distinguish the effect of different Notch receptors on signaling, and also being a chemical compound it cannot be excluded that it can have diverse effect on different proteins and signaling pathway. To solve this dilemma we tried to focus on status of NFκB in DAPT treated and untreated Jurkat and Molt3 cell lines. To our surprise we saw an activated p65 (Phosho-p65) in DAPT treated cell lines thus resolving our problem of why DAPT could up regulated miR-223. But we were not able to find out how DAPT treatment leads to phosphorylation of p65, and thus its need further investigation. Utilising this finding we used Jurkat IKK γ ^{-/-} cell lines for DAPT treatment, as in this cell lines, we were blocking both, Notch mediated proliferation by blocking Notch and miR-223 mediated proliferation by blocking NFκB through IKK γ deletion, we saw decrease cell growth in Jurkat IKK γ ^{-/-} treated. Thus making resistance T-ALL sensitive, by combination of Notch and NFκB inhibition. This could give us the reason why some T-ALL patient or Cell

lines are resistant to GSI treatment. In this scenario our results strongly suggest a combinatorial therapeutic approach aimed to block, both Notch and NF κ B pathway for the treatment of childhood T-ALL.

Moreover, it has already been known that Acute lymphoblastic leukemia (ALL) of T-cell type is a heterogeneous group of ALL at least by immunophenotyping [218] and recently the overexpression of miR-223 was shown to be used to identify a subset of adult T-cell acute lymphoblastic leukemia with myeloid-like gene features [214]. Thus it could be possible that other subset of T-ALL overexpress miR-223. In this regard, our finding could give a new point of view to better understand the mechanistic event undergoing inside the subset of T-ALL, making it possible to design the more effective combinatorial drugs.

Considering another aspect of miRNAs in regulation, a recent report has shown that miRNAs can be secreted inside microvesicle and can be transported to other distant target cells by peripheral blood to induce their effect on that targets. Among them miR-223 was identified as one of the miRNA which was also secreted by macrophage-derived microvesicles transported to target cells and was functionally active [219]. Thus it would be interesting to understand if the same effect is seen in T-ALL, in which the miR-223 secreted by the Lymphocytes of T-ALL is transported by microvesicles to the target cell in the microenvironment, thus making the cells in microenvironment more potent for growth which in turn can promote the T-ALL tumorigenesis .

6. Conclusion and Future direction:-

In this study we identified 7 miRNAs which were regulated by Notch3 modulation in different Models. Among them the induction of miR-223 was most pronounced. Previous study had already identified the transcriptional regulation of miR-223 by CEBP α and PU.1 in Myeloid lineage . Recently miR-223 was shown to be up-regulated in T-ALL patients and also in Notch1 over-expressing transgenic mice. Thus suggesting some other transcriptional regulators of this miR in T-ALL context. Our study found a novel conserved CSL/RBPJ κ binding site which was nested to NF κ B consensus, in its putative promoter region. The luciferase assay on the wild type and mutated promoter construct for RBPJ κ /NF κ B binding site showed the regulation by both NF κ B & Notch3 signalings. Consistently, endogenous ChIP assay confirmed direct involvement of NF κ B in the regulation of miR-223. Moreover, ectopic expression of miR-223 strongly down-regulated the tumor suppressor FBXW7, thus up-regulating its targets such as c-Myc, Cyclin-E, known to be involved in proliferation, while the opposite effects was seen when antagomiR was used. Transient transfection of miR-223 affect Jurkat cell growth as revealed by increased cell number and percentage of cells in S phase of cell cycle. Interestingly, GSI resistant T-ALL cell lines treated with GSI showed increase in miR-223 expression and NF κ B activation without affecting their proliferation rate. The same cell lines deleted for NF κ B pathway, showed less expression of miR-223 when compared with wild type and lost their ability to resist GSI treatment, suggesting the involvement of miR-223 in the mechanism of GSI resistivity. In this regard we are investing the miR-223 expressing in human TALL cell lines, and its effect in cell proliferation, where we are using the combinatorial drugs to block both Notch and NF κ B pathways.

More over we found miR-223 to be highly expressed in all the human TALL patient analysed, thus accessing this miRNA as a novel therapeutic target for T-ALL treatment.

As, our results suggest the involvement of miR-223 in development of T-ALL leukemia and its role in GSI resistivity, thus compelling us to go further in details of its functional role. In

this regard we are generating human T-ALL cell lines stably overexpressing / silencing miR-223 by Lentivirus vectors to magnify the functional effect as obtained by transient transfection.

Overall our study provide an insight into the regulatory mechanism of miR-223 in Notch induced T-ALL, and its role in GSI restivity. The combinatorial therapeutics or therapeutic targeting of oncogenic miRNAs in leukemia can give an edge to the pharmacological therapy, where the conventional protocols show their limits of action and can obviate the toxicity associated to the classical chemotherapy approaches.

References:

1. Poulson, D.F., *Chromosomal Deficiencies and the Embryonic Development of Drosophila Melanogaster*. Proc Natl Acad Sci U S A, 1937. 23(3): p. 133-7.
2. Welshons, W.J. and E.S. Von Halle, *Pseudoallelism at the notch locus in drosophila*. Genetics, 1962. 47: p. 743-59.
3. Wharton, K.A., et al., *Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats*. Cell, 1985. 43(3 Pt 2): p. 567-81.
4. Kidd, S., M.R. Kelley, and M.W. Young, *Sequence of the notch locus of Drosophila melanogaster: relationship of the encoded protein to mammalian clotting and growth factors*. Mol Cell Biol, 1986. 6(9): p. 3094-108.
5. Brou, C., et al., *A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE*. Mol Cell, 2000. 5(2): p. 207-16.
6. Mumm, J.S., et al., *A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1*. Mol Cell, 2000. 5(2): p. 197-206.
7. Lieber, T., S. Kidd, and M.W. Young, *kuzbanian-mediated cleavage of Drosophila Notch*. Genes Dev, 2002. 16(2): p. 209-21.
8. Bray, S.J., *Notch signalling: a simple pathway becomes complex*. Nat Rev Mol Cell Biol, 2006. 7(9): p. 678-89.
9. Xu, T. and S. Artavanis-Tsakonas, *deltex, a locus interacting with the neurogenic genes, Notch, Delta and mastermind in Drosophila melanogaster*. Genetics, 1990. 126(3): p. 665-77.
10. Coyle-Thompson, C.A. and U. Banerjee, *The strawberry notch gene functions with Notch in common developmental pathways*. Development, 1993. 119(2): p. 377-95.
11. Lai, E.C. and G.M. Rubin, *Neuralized is essential for a subset of Notch pathway-dependent cell fate decisions during Drosophila eye development*. Proc Natl Acad Sci U S A, 2001. 98(10): p. 5637-42.
12. Itoh, M., et al., *Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta*. Dev Cell, 2003. 4(1): p. 67-82.
13. Tamura, K., et al., *Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H)*. Curr Biol, 1995. 5(12): p. 1416-23.
14. Blank, V., P. Kourilsky, and A. Israel, *NF-kappa B and related proteins: Rel/dorsal homologies meet ankyrin-like repeats*. Trends Biochem Sci, 1992. 17(4): p. 135-40.
15. Rechsteiner, M., *Regulation of enzyme levels by proteolysis: the role of pest regions*. Adv Enzyme Regul, 1988. 27: p. 135-51.
16. Dou, S., et al., *The recombination signal sequence-binding protein RBP-2N functions as a transcriptional repressor*. Mol Cell Biol, 1994. 14(5): p. 3310-9.
17. Doyle, T.G., C. Wen, and I. Greenwald, *SEL-8, a nuclear protein required for LIN-12 and GLP-1 signaling in Caenorhabditis elegans*. Proc Natl Acad Sci U S A, 2000. 97(14): p. 7877-81.
18. Petcherski, A.G. and J. Kimble, *LAG-3 is a putative transcriptional activator in the C. elegans Notch pathway*. Nature, 2000. 405(6784): p. 364-8.
19. Kumano, K., et al., *Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells*. Immunity, 2003. 18(5): p. 699-711.
20. Robert-Moreno, A., et al., *RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells*. Development, 2005. 132(5): p. 1117-26.
21. Varnum-Finney, B., et al., *Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling*. Nat Med, 2000. 6(11): p. 1278-81.
22. Stier, S., et al., *Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome*. Blood, 2002. 99(7): p. 2369-78.

23. Varnum-Finney, B., C. Brashem-Stein, and I.D. Bernstein, *Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability*. *Blood*, 2003. 101(5): p. 1784-9.
24. Kunisato, A., et al., *HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo*. *Blood*, 2003. 101(5): p. 1777-83.
25. Shojaei, F., et al., *Hierarchical and ontogenic positions serve to define the molecular basis of human hematopoietic stem cell behavior*. *Dev Cell*, 2005. 8(5): p. 651-63.
26. Calvi, L.M., et al., *Osteoblastic cells regulate the haematopoietic stem cell niche*. *Nature*, 2003. 425(6960): p. 841-6.
27. Mancini, S.J., et al., *Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation*. *Blood*, 2005. 105(6): p. 2340-2.
28. Han, H., et al., *Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision*. *Int Immunol*, 2002. 14(6): p. 637-45.
29. Duncan, A.W., et al., *Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance*. *Nat Immunol*, 2005. 6(3): p. 314-22.
30. Mercher, T., et al., *Notch signaling specifies megakaryocyte development from hematopoietic stem cells*. *Cell Stem Cell*, 2008. 3(3): p. 314-26.
31. Bernard, O.A., D.G. Gilliland, and T. Mercher, *[The OTT-MAL fusion oncogene: another Notch in megakaryoblastic leukemia]*. *Med Sci (Paris)*, 2009. 25(8-9): p. 676-8.
32. Yoshida, T., et al., *Early hematopoietic lineage restrictions directed by Ikaros*. *Nat Immunol*, 2006. 7(4): p. 382-91.
33. Arinobu, Y., et al., *Reciprocal activation of GATA-1 and PU.1 marks initial specification of hematopoietic stem cells into myeloerythroid and myelolymphoid lineages*. *Cell Stem Cell*, 2007. 1(4): p. 416-27.
34. Dias, S., et al., *E2A proteins promote development of lymphoid-primed multipotent progenitors*. *Immunity*, 2008. 29(2): p. 217-27.
35. Stehling-Sun, S., et al., *Regulation of lymphoid versus myeloid fate 'choice' by the transcription factor Mef2c*. *Nat Immunol*, 2009. 10(3): p. 289-96.
36. Ikawa, T., et al., *E proteins and Notch signaling cooperate to promote T cell lineage specification and commitment*. *J Exp Med*, 2006. 203(5): p. 1329-42.
37. Ng, S.Y., et al., *Genome-wide lineage-specific transcriptional networks underscore Ikaros-dependent lymphoid priming in hematopoietic stem cells*. *Immunity*, 2009. 30(4): p. 493-507.
38. Benz, C. and C.C. Bleul, *A multipotent precursor in the thymus maps to the branching point of the T versus B lineage decision*. *J Exp Med*, 2005. 202(1): p. 21-31.
39. Schwarz, B.A., et al., *Selective thymus settling regulated by cytokine and chemokine receptors*. *J Immunol*, 2007. 178(4): p. 2008-17.
40. Singh, N., et al., *Expression of notch receptors, notch ligands, and fringe genes in hematopoiesis*. *Exp Hematol*, 2000. 28(5): p. 527-34.
41. Maeda, T., et al., *Regulation of B versus T lymphoid lineage fate decision by the proto-oncogene LRF*. *Science*, 2007. 316(5826): p. 860-6.
42. Yan, X.Q., et al., *A novel Notch ligand, Dll4, induces T-cell leukemia/lymphoma when overexpressed in mice by retroviral-mediated gene transfer*. *Blood*, 2001. 98(13): p. 3793-9.
43. Dorsch, M., et al., *Ectopic expression of Delta4 impairs hematopoietic development and leads to lymphoproliferative disease*. *Blood*, 2002. 100(6): p. 2046-55.
44. Koch, U., et al., *Subversion of the T/B lineage decision in the thymus by lunatic fringe-mediated inhibition of Notch-1*. *Immunity*, 2001. 15(2): p. 225-36.
45. Wilson, A., H.R. MacDonald, and F. Radtke, *Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus*. *J Exp Med*, 2001. 194(7): p. 1003-12.
46. Visan, I., et al., *Regulation of intrathymic T-cell development by Lunatic Fringe- Notch1 interactions*. *Immunol Rev*, 2006. 209: p. 76-94.

47. Maillard, I., et al., *Notch-dependent T-lineage commitment occurs at extrathymic sites following bone marrow transplantation*. *Blood*, 2006. 107(9): p. 3511-9.
48. Starr, T.K., S.C. Jameson, and K.A. Hogquist, *Positive and negative selection of T cells*. *Annu Rev Immunol*, 2003. 21: p. 139-76.
49. Izon, D.J., et al., *Deltex1 redirects lymphoid progenitors to the B cell lineage by antagonizing Notch1*. *Immunity*, 2002. 16(2): p. 231-43.
50. Felli, M.P., et al., *Expression pattern of notch1, 2 and 3 and Jagged1 and 2 in lymphoid and stromal thymus components: distinct ligand-receptor interactions in intrathymic T cell development*. *Int Immunol*, 1999. 11(7): p. 1017-25.
51. Bellavia, D., et al., *Combined expression of pTalpha and Notch3 in T cell leukemia identifies the requirement of preTCR for leukemogenesis*. *Proc Natl Acad Sci U S A*, 2002. 99(6): p. 3788-93.
52. Pui, J.C., et al., *Notch1 expression in early lymphopoiesis influences B versus T lineage determination*. *Immunity*, 1999. 11(3): p. 299-308.
53. Radtke, F., et al., *Deficient T cell fate specification in mice with an induced inactivation of Notch1*. *Immunity*, 1999. 10(5): p. 547-58.
54. Robey, E., et al., *An activated form of Notch influences the choice between CD4 and CD8 T cell lineages*. *Cell*, 1996. 87(3): p. 483-92.
55. Washburn, T., et al., *Notch activity influences the alphabeta versus gammadelta T cell lineage decision*. *Cell*, 1997. 88(6): p. 833-43.
56. Fowlkes, B.J. and E.A. Robey, *A reassessment of the effect of activated Notch1 on CD4 and CD8 T cell development*. *J Immunol*, 2002. 169(4): p. 1817-21.
57. Wolfer, A., et al., *Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes*. *Immunity*, 2002. 16(6): p. 869-79.
58. Bellavia, D., et al., *Notch3, another Notch in T cell development*. *Semin Immunol*, 2003. 15(2): p. 107-12.
59. Ellisen, L.W., et al., *TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms*. *Cell*, 1991. 66(4): p. 649-61.
60. Deftos, M.L., et al., *Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes*. *Immunity*, 2000. 13(1): p. 73-84.
61. Bellavia, D., et al., *Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice*. *EMBO J*, 2000. 19(13): p. 3337-48.
62. Pear, W.S., et al., *Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles*. *J Exp Med*, 1996. 183(5): p. 2283-91.
63. Aster, J.C., et al., *Essential roles for ankyrin repeat and transactivation domains in induction of T-cell leukemia by notch1*. *Mol Cell Biol*, 2000. 20(20): p. 7505-15.
64. Allman, D., et al., *Separation of Notch1 promoted lineage commitment and expansion/transformation in developing T cells*. *J Exp Med*, 2001. 194(1): p. 99-106.
65. Artavanis-Tsakonas, S., M.D. Rand, and R.J. Lake, *Notch signaling: cell fate control and signal integration in development*. *Science*, 1999. 284(5415): p. 770-6.
66. Lee, J.S., et al., *Murine leukemia provirus-mediated activation of the Notch1 gene leads to induction of HES-1 in a mouse T lymphoma cell line, DL-3*. *FEBS Lett*, 1999. 455(3): p. 276-80.
67. Feldman, B.J., T. Hampton, and M.L. Cleary, *A carboxy-terminal deletion mutant of Notch1 accelerates lymphoid oncogenesis in E2A-PBX1 transgenic mice*. *Blood*, 2000. 96(5): p. 1906-13.
68. Eddy, S.R., *Non-coding RNA genes and the modern RNA world*. *Nat Rev Genet*, 2001. 2(12): p. 919-29.
69. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14*. *Cell*, 1993. 75(5): p. 843-54.

70. Reinhart, B.J., et al., *The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans*. Nature, 2000. 403(6772): p. 901-6.
71. Kozomara, A. and S. Griffiths-Jones, *miRBase: integrating microRNA annotation and deep-sequencing data*. Nucleic Acids Res, 2011. 39(Database issue): p. D152-7.
72. Lau, N.C., et al., *An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans*. Science, 2001. 294(5543): p. 858-62.
73. Lagos-Quintana, M., et al., *Identification of novel genes coding for small expressed RNAs*. Science, 2001. 294(5543): p. 853-8.
74. Lee, Y., et al., *MicroRNA maturation: stepwise processing and subcellular localization*. EMBO J, 2002. 21(17): p. 4663-70.
75. Lee, Y., et al., *MicroRNA genes are transcribed by RNA polymerase II*. EMBO J, 2004. 23(20): p. 4051-60.
76. Borchert, G.M., W. Lanier, and B.L. Davidson, *RNA polymerase III transcribes human microRNAs*. Nat Struct Mol Biol, 2006. 13(12): p. 1097-101.
77. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function*. Cell, 2004. 116(2): p. 281-97.
78. Lee, Y., et al., *The nuclear RNase III Drosha initiates microRNA processing*. Nature, 2003. 425(6956): p. 415-9.
79. Denli, A.M., et al., *Processing of primary microRNAs by the Microprocessor complex*. Nature, 2004. 432(7014): p. 231-5.
80. Gregory, R.I., et al., *The Microprocessor complex mediates the genesis of microRNAs*. Nature, 2004. 432(7014): p. 235-40.
81. Han, J., et al., *The Drosha-DGCR8 complex in primary microRNA processing*. Genes Dev, 2004. 18(24): p. 3016-27.
82. Okamura, K., et al., *The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila*. Cell, 2007. 130(1): p. 89-100.
83. Ruby, J.G., C.H. Jan, and D.P. Bartel, *Intronic microRNA precursors that bypass Drosha processing*. Nature, 2007. 448(7149): p. 83-6.
84. Bohnsack, M.T., K. Czapinski, and D. Gorlich, *Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs*. RNA, 2004. 10(2): p. 185-91.
85. Lund, E., et al., *Nuclear export of microRNA precursors*. Science, 2004. 303(5654): p. 95-8.
86. Yi, R., et al., *Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs*. Genes Dev, 2003. 17(24): p. 3011-6.
87. Hutvagner, G., et al., *A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA*. Science, 2001. 293(5531): p. 834-8.
88. Haase, A.D., et al., *TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing*. EMBO Rep, 2005. 6(10): p. 961-7.
89. Kok, K.H., et al., *Human TRBP and PACT directly interact with each other and associate with dicer to facilitate the production of small interfering RNA*. J Biol Chem, 2007. 282(24): p. 17649-57.
90. Lee, Y., et al., *The role of PACT in the RNA silencing pathway*. EMBO J, 2006. 25(3): p. 522-32.
91. Kawamata, T. and Y. Tomari, *Making RISC*. Trends Biochem Sci, 2010. 35(7): p. 368-76.
92. Yoda, M., et al., *ATP-dependent human RISC assembly pathways*. Nat Struct Mol Biol, 2010. 17(1): p. 17-23.
93. Wang, Y., et al., *Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex*. Nature, 2008. 456(7224): p. 921-6.
94. Iki, T., et al., *In vitro assembly of plant RNA-induced silencing complexes facilitated by molecular chaperone HSP90*. Mol Cell, 2010. 39(2): p. 282-91.
95. Liu, J., et al., *Argonaute2 is the catalytic engine of mammalian RNAi*. Science, 2004. 305(5689): p. 1437-41.

96. Leuschner, P.J., et al., *Cleavage of the siRNA passenger strand during RISC assembly in human cells*. EMBO Rep, 2006. 7(3): p. 314-20.
97. Lewis, B.P., et al., *Prediction of mammalian microRNA targets*. Cell, 2003. 115(7): p. 787-98.
98. Gu, S., et al., *Biological basis for restriction of microRNA targets to the 3' untranslated region in mammalian mRNAs*. Nat Struct Mol Biol, 2009. 16(2): p. 144-50.
99. Lee, Y.S. and A. Dutta, *The tumor suppressor microRNA let-7 represses the HMGA2 oncogene*. Genes Dev, 2007. 21(9): p. 1025-30.
100. Krek, A., et al., *Combinatorial microRNA target predictions*. Nat Genet, 2005. 37(5): p. 495-500.
101. Brennecke, J., et al., *Principles of microRNA-target recognition*. PLoS Biol, 2005. 3(3): p. e85.
102. Grimson, A., et al., *MicroRNA targeting specificity in mammals: determinants beyond seed pairing*. Mol Cell, 2007. 27(1): p. 91-105.
103. Yekta, S., I.H. Shih, and D.P. Bartel, *MicroRNA-directed cleavage of HOXB8 mRNA*. Science, 2004. 304(5670): p. 594-6.
104. Behm-Ansmant, I., et al., *mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes*. Genes Dev, 2006. 20(14): p. 1885-98.
105. Pillai, R.S., C.G. Artus, and W. Filipowicz, *Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis*. RNA, 2004. 10(10): p. 1518-25.
106. Beilharz, T.H., et al., *microRNA-mediated messenger RNA deadenylation contributes to translational repression in mammalian cells*. PLoS One, 2009. 4(8): p. e6783.
107. Iwasaki, S., T. Kawamata, and Y. Tomari, *Drosophila argonaute1 and argonaute2 employ distinct mechanisms for translational repression*. Mol Cell, 2009. 34(1): p. 58-67.
108. Wakiyama, M., et al., *Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system*. Genes Dev, 2007. 21(15): p. 1857-62.
109. Humphreys, D.T., et al., *MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function*. Proc Natl Acad Sci U S A, 2005. 102(47): p. 16961-6.
110. Mathonnet, G., et al., *MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F*. Science, 2007. 317(5845): p. 1764-7.
111. Pillai, R.S., et al., *Inhibition of translational initiation by Let-7 MicroRNA in human cells*. Science, 2005. 309(5740): p. 1573-6.
112. Thermann, R. and M.W. Hentze, *Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation*. Nature, 2007. 447(7146): p. 875-8.
113. Wang, B., et al., *Recapitulation of short RNA-directed translational gene silencing in vitro*. Mol Cell, 2006. 22(4): p. 553-60.
114. Wang, B., A. Yanez, and C.D. Novina, *MicroRNA-repressed mRNAs contain 40S but not 60S components*. Proc Natl Acad Sci U S A, 2008. 105(14): p. 5343-8.
115. Maroney, P.A., et al., *Evidence that microRNAs are associated with translating messenger RNAs in human cells*. Nat Struct Mol Biol, 2006. 13(12): p. 1102-7.
116. Nottrott, S., M.J. Simard, and J.D. Richter, *Human let-7a miRNA blocks protein production on actively translating polyribosomes*. Nat Struct Mol Biol, 2006. 13(12): p. 1108-14.
117. Bhattacharyya, S.N., et al., *Relief of microRNA-mediated translational repression in human cells subjected to stress*. Cell, 2006. 125(6): p. 1111-24.
118. Liu, J., et al., *MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies*. Nat Cell Biol, 2005. 7(7): p. 719-23.
119. Orkin, S.H. and L.I. Zon, *Hematopoiesis: an evolving paradigm for stem cell biology*. Cell, 2008. 132(4): p. 631-44.
120. Gangaraju, V.K. and H. Lin, *MicroRNAs: key regulators of stem cells*. Nat Rev Mol Cell Biol, 2009. 10(2): p. 116-25.

121. Georgantas, R.W., 3rd, et al., *CD34+ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control*. Proc Natl Acad Sci U S A, 2007. 104(8): p. 2750-5.
122. Merkerova, M., et al., *MicroRNA expression profiles in umbilical cord blood cell lineages*. Stem Cells Dev, 2010. 19(1): p. 17-26.
123. Gruber, J.J., et al., *Ars2 links the nuclear cap-binding complex to RNA interference and cell proliferation*. Cell, 2009. 138(2): p. 328-39.
124. Argiropoulos, B. and R.K. Humphries, *Hox genes in hematopoiesis and leukemogenesis*. Oncogene, 2007. 26(47): p. 6766-76.
125. Garzon, R., et al., *Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin*. Proc Natl Acad Sci U S A, 2008. 105(10): p. 3945-50.
126. Mansfield, J.H., et al., *MicroRNA-responsive 'sensor' transgenes uncover Hox-like and other developmentally regulated patterns of vertebrate microRNA expression*. Nat Genet, 2004. 36(10): p. 1079-83.
127. Popovic, R., et al., *Regulation of mir-196b by MLL and its overexpression by MLL fusions contributes to immortalization*. Blood, 2009. 113(14): p. 3314-22.
128. Shen, W.F., et al., *MicroRNA-126 regulates HOXA9 by binding to the homeobox*. Mol Cell Biol, 2008. 28(14): p. 4609-19.
129. Li, Z., et al., *Distinct microRNA expression profiles in acute myeloid leukemia with common translocations*. Proc Natl Acad Sci U S A, 2008. 105(40): p. 15535-40.
130. Felli, N., et al., *MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation*. Proc Natl Acad Sci U S A, 2005. 102(50): p. 18081-6.
131. Merkerova, M., M. Belickova, and H. Bruchova, *Differential expression of microRNAs in hematopoietic cell lineages*. Eur J Haematol, 2008. 81(4): p. 304-10.
132. Monticelli, S., et al., *MicroRNA profiling of the murine hematopoietic system*. Genome Biol, 2005. 6(8): p. R71.
133. Wu, H., et al., *miRNA profiling of naive, effector and memory CD8 T cells*. PLoS One, 2007. 2(10): p. e1020.
134. Sandberg, R., et al., *Proliferating cells express mRNAs with shortened 3' untranslated regions and fewer microRNA target sites*. Science, 2008. 320(5883): p. 1643-7.
135. Cobb, B.S., et al., *T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer*. J Exp Med, 2005. 201(9): p. 1367-73.
136. Muljo, S.A., et al., *Aberrant T cell differentiation in the absence of Dicer*. J Exp Med, 2005. 202(2): p. 261-9.
137. Xiao, C., et al., *Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes*. Nat Immunol, 2008. 9(4): p. 405-14.
138. Li, Q.J., et al., *miR-181a is an intrinsic modulator of T cell sensitivity and selection*. Cell, 2007. 129(1): p. 147-61.
139. Rodriguez, A., et al., *Requirement of bic/microRNA-155 for normal immune function*. Science, 2007. 316(5824): p. 608-11.
140. Johnston, R.J., et al., *Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation*. Science, 2009. 325(5943): p. 1006-10.
141. Yu, D., et al., *The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment*. Immunity, 2009. 31(3): p. 457-68.
142. Du, C., et al., *MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis*. Nat Immunol, 2009. 10(12): p. 1252-9.
143. Chong, M.M., et al., *The RNaseIII enzyme Drosha is critical in T cells for preventing lethal inflammatory disease*. J Exp Med, 2008. 205(9): p. 2005-17.
144. Zhou, X., et al., *Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity*. J Exp Med, 2008. 205(9): p. 1983-91.

145. Kohlhaas, S., et al., *Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells*. J Immunol, 2009. 182(5): p. 2578-82.
146. Huang, B., et al., *miR-142-3p restricts cAMP production in CD4+CD25- T cells and CD4+CD25+ TREG cells by targeting AC9 mRNA*. EMBO Rep, 2009. 10(2): p. 180-5.
147. Cobb, B.S., et al., *A role for Dicer in immune regulation*. J Exp Med, 2006. 203(11): p. 2519-27.
148. Ambs, S., et al., *Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer*. Cancer Res, 2008. 68(15): p. 6162-70.
149. Childs, G., et al., *Low-level expression of microRNAs let-7d and miR-205 are prognostic markers of head and neck squamous cell carcinoma*. Am J Pathol, 2009. 174(3): p. 736-45.
150. Datta, J., et al., *Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis*. Cancer Res, 2008. 68(13): p. 5049-58.
151. Nasser, M.W., et al., *Down-regulation of micro-RNA-1 (miR-1) in lung cancer. Suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by miR-1*. J Biol Chem, 2008. 283(48): p. 33394-405.
152. Melkamu, T., et al., *Alteration of microRNA expression in vinyl carbamate-induced mouse lung tumors and modulation by the chemopreventive agent indole-3-carbinol*. Carcinogenesis, 2010. 31(2): p. 252-8.
153. Kwon, C., et al., *MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling*. Proc Natl Acad Sci U S A, 2005. 102(52): p. 18986-91.
154. Ivey, K.N., et al., *MicroRNA regulation of cell lineages in mouse and human embryonic stem cells*. Cell Stem Cell, 2008. 2(3): p. 219-29.
155. Ji, Q., et al., *MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells*. PLoS One, 2009. 4(8): p. e6816.
156. Bommer, G.T., et al., *p53-mediated activation of miRNA34 candidate tumor-suppressor genes*. Curr Biol, 2007. 17(15): p. 1298-307.
157. Chang, T.C., et al., *Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis*. Mol Cell, 2007. 26(5): p. 745-52.
158. He, C., et al., *Functional elucidation of MiR-34 in osteosarcoma cells and primary tumor samples*. Biochem Biophys Res Commun, 2009. 388(1): p. 35-40.
159. Lodygin, D., et al., *Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer*. Cell Cycle, 2008. 7(16): p. 2591-600.
160. Li, Y., et al., *MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes*. Cancer Res, 2009. 69(19): p. 7569-76.
161. Ji, Q., et al., *Restoration of tumor suppressor miR-34 inhibits human p53-mutant gastric cancer tumorspheres*. BMC Cancer, 2008. 8: p. 266.
162. Bellon, M., et al., *Deregulation of microRNA involved in hematopoiesis and the immune response in HTLV-I adult T-cell leukemia*. Blood, 2009. 113(20): p. 4914-7.
163. Hurst, D.R., et al., *Breast cancer metastasis suppressor 1 up-regulates miR-146, which suppresses breast cancer metastasis*. Cancer Res, 2009. 69(4): p. 1279-83.
164. Bhaumik, D., et al., *Expression of microRNA-146 suppresses NF-kappaB activity with reduction of metastatic potential in breast cancer cells*. Oncogene, 2008. 27(42): p. 5643-7.
165. Kuang, W., et al., *Cyclic stretch induced miR-146a upregulation delays C2C12 myogenic differentiation through inhibition of Numb*. Biochem Biophys Res Commun, 2009. 378(2): p. 259-63.
166. Stylianou, S., R.B. Clarke, and K. Brennan, *Aberrant activation of notch signaling in human breast cancer*. Cancer Res, 2006. 66(3): p. 1517-25.
167. Pece, S., et al., *Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis*. J Cell Biol, 2004. 167(2): p. 215-21.
168. Osipo, C., et al., *Off the beaten pathway: the complex cross talk between Notch and NF-kappaB*. Lab Invest, 2008. 88(1): p. 11-7.

169. Iorio, M.V., et al., *MicroRNA signatures in human ovarian cancer*. *Cancer Res*, 2007. 67(18): p. 8699-707.
170. Murakami, Y., et al., *Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues*. *Oncogene*, 2006. 25(17): p. 2537-45.
171. Garzia, L., et al., *MicroRNA-199b-5p impairs cancer stem cells through negative regulation of HES1 in medulloblastoma*. *PLoS One*, 2009. 4(3): p. e4998.
172. Ladeiro, Y., et al., *MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations*. *Hepatology*, 2008. 47(6): p. 1955-63.
173. Burk, U., et al., *A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells*. *EMBO Rep*, 2008. 9(6): p. 582-9.
174. Gregory, P.A., et al., *The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1*. *Nat Cell Biol*, 2008. 10(5): p. 593-601.
175. Korpai, M., et al., *The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2*. *J Biol Chem*, 2008. 283(22): p. 14910-4.
176. Park, S.M., et al., *The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2*. *Genes Dev*, 2008. 22(7): p. 894-907.
177. Li, Y., et al., *Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells*. *Cancer Res*, 2009. 69(16): p. 6704-12.
178. Li, X., et al., *Repression of tumor suppressor miR-451 is essential for NOTCH1-induced oncogenesis in T-ALL*. *J Exp Med*, 2011. 208(4): p. 663-75.
179. Ghisi, M., et al., *Modulation of microRNA expression in human T-cell development: targeting of NOTCH3 by miR-150*. *Blood*, 2011. 117(26): p. 7053-62.
180. Song, G., Y. Zhang, and L. Wang, *MicroRNA-206 targets notch3, activates apoptosis, and inhibits tumor cell migration and focus formation*. *J Biol Chem*, 2009. 284(46): p. 31921-7.
181. Wang, Z., et al., *Cross-talk between miRNA and Notch signaling pathways in tumor development and progression*. *Cancer Lett*, 2010. 292(2): p. 141-8.
182. Sayed, D. and M. Abdellatif, *MicroRNAs in development and disease*. *Physiol Rev*, 2011. 91(3): p. 827-87.
183. Chen, B., et al., *Roles of microRNA on cancer cell metabolism*. *J Transl Med*, 2012. 10: p. 228.
184. Mavrakis, K.J., et al., *Genome-wide RNA-mediated interference screen identifies miR-19 targets in Notch-induced T-cell acute lymphoblastic leukaemia*. *Nat Cell Biol*, 2010. 12(4): p. 372-9.
185. Mei, J., R. Bachoo, and C.L. Zhang, *MicroRNA-146a inhibits glioma development by targeting Notch1*. *Mol Cell Biol*, 2011. 31(17): p. 3584-92.
186. Manca, S., et al., *Oxidative stress activation of miR-125b is part of the molecular switch for Hailey-Hailey disease manifestation*. *Exp Dermatol*, 2011. 20(11): p. 932-7.
187. Chen, L., et al., *P53-induced microRNA-107 inhibits proliferation of glioma cells and down-regulates the expression of CDK6 and Notch-2*. *Neurosci Lett*, 2013. 534: p. 327-32.
188. Mavrakis, K.J., et al., *A cooperative microRNA-tumor suppressor gene network in acute T-cell lymphoblastic leukemia (T-ALL)*. *Nat Genet*, 2011. 43(7): p. 673-8.
189. Vacca, A., et al., *Notch3 and pre-TCR interaction unveils distinct NF-kappaB pathways in T-cell development and leukemia*. *EMBO J*, 2006. 25(5): p. 1000-8.
190. Talora, C., et al., *Pre-TCR-triggered ERK signalling-dependent downregulation of E2A activity in Notch3-induced T-cell lymphoma*. *EMBO Rep*, 2003. 4(11): p. 1067-72.
191. Primi, D., et al., *Rearrangement and expression of T cell receptor and immunoglobulin loci in immortalized CD4-CD8- T cell lines*. *Eur J Immunol*, 1988. 18(7): p. 1101-9.
192. Chen, C.Z., et al., *MicroRNAs modulate hematopoietic lineage differentiation*. *Science*, 2004. 303(5654): p. 83-6.

193. Fazi, F., et al., *A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis*. Cell, 2005. 123(5): p. 819-31.
194. Fukao, T., et al., *An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling*. Cell, 2007. 129(3): p. 617-31.
195. Eyholzer, M., et al., *Complexity of miR-223 regulation by CEBPA in human AML*. Leuk Res, 2010. 34(5): p. 672-6.
196. Natoli, G., et al., *Nuclear factor kB-independent cytoprotective pathways originating at tumor necrosis factor receptor-associated factor 2*. J Biol Chem, 1998. 273(47): p. 31262-72.
197. Felli, M.P., et al., *PKC theta mediates pre-TCR signaling and contributes to Notch3-induced T-cell leukemia*. Oncogene, 2005. 24(6): p. 992-1000.
198. Aifantis, I., T. Vilimas, and S. Buonamici, *Notches, NFkappaBs and the making of T cell leukemia*. Cell Cycle, 2007. 6(4): p. 403-6.
199. Vilimas, T., et al., *Targeting the NF-kappaB signaling pathway in Notch1-induced T-cell leukemia*. Nat Med, 2007. 13(1): p. 70-7.
200. Li, J., et al., *MicroRNA-223 functions as an oncogene in human gastric cancer by targeting FBXW7/hCdc4*. J Cancer Res Clin Oncol, 2012. 138(5): p. 763-74.
201. Kurashige, J., et al., *Overexpression of microRNA-223 regulates the ubiquitin ligase FBXW7 in oesophageal squamous cell carcinoma*. Br J Cancer, 2012. 106(1): p. 182-8.
202. Xu, Y., et al., *MicroRNA-223 regulates cyclin E activity by modulating expression of F-box and WD-40 domain protein 7*. J Biol Chem, 2010. 285(45): p. 34439-46.
203. Park, M.J., et al., *FBXW7 and NOTCH1 mutations in childhood T cell acute lymphoblastic leukaemia and T cell non-Hodgkin lymphoma*. Br J Haematol, 2009. 145(2): p. 198-206.
204. Thompson, B.J., et al., *The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia*. J Exp Med, 2007. 204(8): p. 1825-35.
205. Wang, Z., et al., *Tumor suppressor functions of FBW7 in cancer development and progression*. FEBS Lett, 2012. 586(10): p. 1409-18.
206. Oberg, C., et al., *The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog*. J Biol Chem, 2001. 276(38): p. 35847-53.
207. Yada, M., et al., *Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7*. EMBO J, 2004. 23(10): p. 2116-25.
208. Strohmaier, H., et al., *Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line*. Nature, 2001. 413(6853): p. 316-22.
209. Cheng, Y. and G. Li, *Role of the ubiquitin ligase Fbw7 in cancer progression*. Cancer Metastasis Rev, 2012. 31(1-2): p. 75-87.
210. Gusscott, S., et al., *Notch-mediated repression of miR-223 contributes to IGF1R regulation in T-ALL*. Leuk Res, 2012. 36(7): p. 905-11.
211. Fulci, V., et al., *miR-223 is overexpressed in T-lymphocytes of patients affected by rheumatoid arthritis*. Hum Immunol, 2010. 71(2): p. 206-11.
212. Wong, Q.W., et al., *MicroRNA-223 is commonly repressed in hepatocellular carcinoma and potentiates expression of Stathmin1*. Gastroenterology, 2008. 135(1): p. 257-69.
213. Stamatopoulos, B., et al., *microRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukemia and improves disease risk stratification*. Blood, 2009. 113(21): p. 5237-45.
214. Chiaretti, S., et al., *Gene expression profiling identifies a subset of adult T-cell acute lymphoblastic leukemia with myeloid-like gene features and over-expression of miR-223*. Haematologica, 2010. 95(7): p. 1114-21.
215. Laios, A., et al., *Potential role of miR-9 and miR-223 in recurrent ovarian cancer*. Mol Cancer, 2008. 7: p. 35.
216. Pulikkan, J.A., et al., *Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia*. Blood, 2010. 115(9): p. 1768-78.

217. Mi, S., et al., *MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia*. Proc Natl Acad Sci U S A, 2007. 104(50): p. 19971-6.
218. Burger, R., et al., *Heterogeneity of T-acute lymphoblastic leukemia (T-ALL) cell lines: suggestion for classification by immunophenotype and T-cell receptor studies*. Leuk Res, 1999. 23(1): p. 19-27.
219. Ismail, N., et al., *Macrophage microvesicles induce macrophage differentiation and miR-223 transfer*. Blood, 2013. 121(6): p. 984-95.