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CORINNE KOX -MASTER BIOLOGY
BORN IN LUXEMBOURG, LUXEMBOURG

ORAL EXAMINATION:

MOLECULAR PATHWAYS IMPORTANT FOR THE PATHOGENESIS AND RISK STRATIFICATION OF CHILDHOOD T-ALL

REFEREES:

DR. Andreas Ladurner
PROF. DR. Christof Niehrs

Summary

Paediatric T-cell acute lymphoblastic leukaemia (T-ALL) is a rare type of cancer that represents 10-15% of ALL cases. Intensified chemotherapy led to improvements of the overall survival rates of childhood T-ALL, reaching approximately 80%. However, the prognosis of relapses remains poor, emphasising the need for new molecular markers that identify high risk patients in the earliest stages of the disease. In addition, understanding of the precise mechanisms of T-ALL leukaemogenesis allows for the development of molecular targeted therapies that could potentially improve the quality of life of children with T-ALL, in general, and the response in patients that develop recurrent disease, in particular. Frequent genetic abnormalities in T-ALL are mutations in the NOTCH pathway which are thought to drive T-ALL development.

In the first part of this study, the prognostic and predictive value of mutations in the NOTCH pathway was investigated in T-ALL patients, at initial presentation and at relapse. Activating mutations in the *NOTCH1* gene and inactivating mutations in the *FBXW7* gene, encoding for a negative regulator of the pathway, were correlated with early treatment response and long-term outcome. At initial presentation, *NOTCH1* mutated patients treated according to the ALL-BFM 2000 protocol show improved early treatment response and better long-term outcome. Interestingly, the NOTCH1 effect is restricted to the ALL-BFM 2000 good responders only. In the high risk group, patients with and without *NOTCH1* mutations do not show a significant difference in long-term outcome. In contrast, inactivation of the FBXW7 protein correlates with excellent early response, but does not associate with better long-term outcome. At relapse, event-free survival rates were low in the ALL-REZ 2002 study, not exceeding 40%. In particular, *FBXW7* mutated patients with recurrent disease showed a poor response to treatment. This study laid foundations for the possible application of molecular markers in T-ALL risk stratification.

In the second part of this PhD work, the effect of up-regulated NOTCH signalling on microRNA expression was analysed to gain insight into the pathogenic mechanisms of NOTCH1 in T-ALL. In a cell-based assay, NOTCH1 was knocked down in a T cell line and mature microRNA expression was monitored by microarray profiling. Two normalisation methods were employed for the microarray analysis. The first method identified 5 microRNAs down-regulated upon NOTCH1 knock-down, including members of the miR-17~92 and miR-106~363 clusters. However, no major changes in miRNA expression were observed when using the second normalisation method. The validation of the microarray experiments by microRNA-specific quantitative PCR confirmed the results from the second microarray normalisation method. In comparison, a qPCR approach detected a down-regulation of primary microRNA transcripts, such as the transcript of the miR-17~92 cluster. In a

second setting, the effect of *NOTCH1* mutations on microRNA expression was determined in primary T-ALL patient samples. Marginal differences in expression were detected in members of the miR-17~92 cluster and the miR-181 family. Further work will be required to elucidate the mechanism of action of miRNA regulation by NOTCH1 in T-ALL.

Zusammenfassung

Die T Zell lymphoblastische Leukämie (T-ALL) ist ein seltener Krebstyp, der meistens bei Kindern auftritt, und nur 10-15% der ALL ausmacht. Die Krankheit zeichnet sich in den letzten Jahren durch immer höhere Überlebenschancen aus. Eine Ausnahme stellen rezidivierte Patienten dar, weswegen die Früherkennung von Hochrisikopatienten und deren schnelle intensive Therapie besonders wichtig ist. Eine individuelle Behandlung ab der Diagnose könnte die Überlebensrate der Hochrisikopatienten verbessern. Um die Diagnose des Rezidivrisikos sicher vorhersagen zu können, fehlen allerdings zuverlässige Marker. Des Weiteren ist es wichtig die Mechanismen der Pathogenese der T-ALL besser zu verstehen und hierauf aufbauend zielorientierte Therapien zu entwickeln. Individuelle Behandlungen könnten die Lebensqualität der Kinder mit T-ALL, und auch speziell das Therapieansprechen von rezidivierten Patienten verbessern. Häufige genomische Veränderungen in der T-ALL sind Mutationen im NOTCH Signalweg, die auch mitverantwortlich sind für die Entwicklung der Leukämie.

Im ersten Teil der vorliegenden Studie wurden die Mutationen in Genen des NOTCH-Signalweges mit der Prognose der T-ALL Patienten korreliert. *NOTCH1* enthält aktivierende Mutationen in 50% der Patienten, während FBXW7, ein Repressor von NOTCH1, inaktiviert wird durch Mutationen in weniger als 20% der T-ALL Patienten. Diese Mutationen wurden verglichen mit dem Ansprechen auf die Therapie, sowie mit dem Langzeitergebnis in der Erstdiagnose und im Rezidiv. In der Erstdiagnose der ALL-BFM 2000 Studie konnte ein positiver Effekt von *NOTCH1* Mutationen auf das Therapie- und das Lanzeitergebnis nachgewiesen werden. Der NOTCH1-Effekt konnte lediglich in den Patienten festgestellt werden, die gut auf die Therapie angesprachen. In der Gruppe der Hochrisikiopatienten übte die *NOTCH1* Mutation keinen Unterschied auf die Überlebensrate aus. Inaktivierende *FBXW7* Mutationen korrelierten auch mit exzellentem Ansprechen der Patienten, es konnte allerdings keine Assoziation zwischen diesem Typ Mutation und eines verbesserten ereignisfreiem Überleben festgestellt werden. In Übereinstimmung mit den Erwartungen, überschritt die Überlebensrate bei rezidierten Patienten der ALL-REZ 2002 nicht mehr als 40%. Insbesondere Patienten mit *FBXW7* Mutationen schlugen allgemein schlecht auf die Rezidivtherapie an. Diese Studie hat den Grundstein für den möglichen klinischen Gebrauch von Mutationen des NOTCH Signalweges gelegt.

Im zweiten Teil dieser Studie wurde der Effekt von *NOTCH1* Mutationen auf die Expression von microRNAs untersucht, um die Konsequenzen der NOTCH1 Aktivierung zu verstehen. In einem Zell-basierten Assay, wurde NOTCH1 in einer T-Zelllinie herabreguliert und die Expressionsprofile von reifen microRNA-Molekülen mit einer Microarray-Analyse untersucht. Zwei Normalisierungsmethoden wurden für die Analyse angewendet. Die erste Methode identifizierte

5 microRNA-Moleküle, die herabreguliert waren, darunter Mitglieder der miR-17~92 und miR~106-363 Cluster. Eine zweite Normalisierung konnte dagegen keinen Unterschied in den Expressionsprofilen von Kontroll- und NOTCH1-herabregulierten Experimenten aufzeigen. Die spezifische Amplifizierung der microRNA-Kandidaten durch quantitative PCR (qPCR) bestätigte die Resultate der zweiten Normalisierungsmethode, da keine Unterschiede in der Expression der microRNA-Moleküle entdeckt werden konnten. Die qPCR-Analyse konnte allerdings zwei primäre microRNA-Transkripte identifizieren, die nach einer Herabregulation von NOTCH1 ebenfalls herabreguliert waren: die Transkripte des miR-17~92-und des miR-491-Clusters. Zusätzlich wurden microRNA-Profile von T-ALL-Erstdiagnoseproben auf den Effekt von *NOTCH1* Mutationen überprüft. Marginale Unterschiede in der Expression von microRNA-Molekülen aus dem miR-17~92-Cluster und der miR-181-Familie wurden ermittelt. Zukünftige Studien werden notwendig sein, um die genauen Mechanismen der miRNA-Regulation von NOTCH1 in T-ALL zu untersuchen.

Dedicated to my family

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List of Abbreviations

Ago	Argonaute
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
BFM	Berlin-Frankfurt-Münster
BM	bone marrow
BMT	bone marrow transplantation
bp	base pair
Brd	Bearded
BrEt	ethidium bromide
°C	degree Celsius
C. *elegans*	*Caenorhabditis elegans*
C_t	Threshold cycles
C-terminus	carboxy-terminus
CCR	continuous complete remission
cDNA	complementary DNA
CLL	chronic lymphoblastic leukaemia
CLP	common lymphoid precursor
CML	chronic myeloid leukaemia
CMP	common myeloid precursor
CPD	Cdc4 phospho-degron
CR	complete remission
CSL	CBF1, Suppressor of Hairless, Lag-1
D. *melanogaster*	*Drosophila melanogaster*
DEAE	Diethylaminoethyl cellulose
Dll	Delta-like
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DP	double positive
dsRNA	double-stranded RNA
E. *coli*	*Escherichia coli*

E(spl)	Enhancer of split
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
env	envelope
ETP	early T lineage precursors
FBS	fetal bovine serum
FBXW7	F-box and WD-40 domain protein 7
g	gram
gag	group antigen
GC	glucocorticoid
GFP	green fluorescent protein
GSI	γ-secretase inhibitor
GSK3	Glycogen synthase kinase-3
h	hour
H2O	water
HCL	hierarchical clustering
HD-C	C-terminal of heterodimerisation domain
HD-N	N-terminal of heterodimerisation domain
hsa	*Homo sapiens*
HSC	haematopoietic stem cell
ICN1	intracellular NOTCH1
Ig	Immunoglobulin
J	Jagged
l	liter
LB	Luria-Bertani
LEF1	Lymphoid enhancer-binding factor 1
LN	Lin12-NOTCH1
LSC	leukaemic stem cell
µ	micro
M	mol/l
MAML	mastermind-like
MB	medulloblastoma
min	minute
miRISC	miRNA-containing ribonucleoprotein induced silencing complex
miRNA	microRNA

miRNP	microRNA-containing ribonucleo-protein
mol	moles
MRD	minimal residual disease
mRNA	messenger RNA
NaCl	Sodium chloride
NEAA	Non-essential Amino Acids
ng	nanogram
nM	nanoMolar
N-terminus	amino-terminus
O-Fut	*O*-Fucosyl transferase
PBS	phosphate-buffered saline
pCIR	probability of cumulative incidence of relapse
PCR	polymerase chain reaction
pEFS	probability of event-free survival
Pen/Strep	Penicillin/Streptomycin
PEST	Proline-Glutamate-Serine-Threonine
PFA	Paraformaldehyde
PGR	prednisone good response
PolyA	poly-adenylate
PPR	prednisone poor response
Pre-miRNA	precursor miRNA
Pri-miRNA	primary miRNA
RAM	RBPJk-associated molecule
RNase	ribonuclease
RNA	ribonucleic acid
RNA Pol II	RNA polymerase II
RPMI-1640	Roswell Park Memorial Institute-1640
RT	reverse transcription
sec	seconds
SCF	SKP1, CUL1 and F-box protein
SDS	sodium dodecyl sulphate
shRNA	short hairpin RNA
SP	single positive
T-ALL	T-cell acute lymphoblastic leukaemia
TAD	transactivation domain

Taq	Thermophilus aquaticus
TBE	tris/borate/EDTA
TBS	tris-buffered saline
TCR	T-cell receptor
Tm	melting temperature
Tris	tris(hydroxymethyl)aminoethane
TU	transducing units
U	units
V	Volts
v/v	volume/volume
VSVG	vesicular stomatitis virus glycoprotein
WBC	white blood cell
w/v	weight/volume
%	per cent

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Zum Schluss, wëll ech ménger Famill villmools Merci soën, Mama, Papa, Julie a Jean. Ouni äere Réckhalt wär ech nët sou wäit komm, an hätt mat Sëcherheet d'Bengele bei d'Tromm geschmass. Just ze wëssen dat dir do sidd, an dass ech ëmmer rëm Heem "an d'Nascht" ka kommen, gët mer Sëcherheet an e Grond heiduerch ze marchéieren. Ech soën dat ni, mee ech hun iech gär.

1. Introduction

1.1 Paediatric T-cell acute lymphoblastic leukaemia

In the human bone marrow (BM) reside the haematopoietic stem cells (HSCs) that give rise to all cellular blood components (Wilson and Trumpp, 2006). These stem cells comprise only 0.01-0.05% of the total bone marrow population, but the number of HSCs remains constant due to their ability of self-renewal (He et al., 2009). The division of HSCs is asymmetrical giving rise to a new pool of HSCs and to the haematopoietic progenitor cells (HSPs). The formation of mature and functional blood cells is owed by the property of pluripotency of the HSCs. HSPs divide several times in a symmetrical fashion, and with every division become increasingly committed to one of two blood cell lineages, the myeloid or the lymphoid lineage. The common myeloid progenitors (CMP) will differentiate towards erythrocytes, megakaryocytes or towards monocytes, macrophages and granulocytes (neutrophils, eosinophils and basophils), whereas from the common lymphoid progenitors (CLP) T cells, B cells and Natural Killer (NK) cells are formed (Figure 1.1). The final maturation of all blood cells takes place in the BM, except T cells that mature in the thymus.

Many regulatory processes in haematopoiesis are involved in the generation of sufficient and functional mature blood cells of all types. Deregulation of differentiation and development of these cells results in haematological disorders and cancer. Leukaemia, a type of haematological malignancy, is characterised by uncontrolled proliferation of immature white blood cells and impairment of their differentiation. The accumulation of leukaemic cells in the BM impairs the production of normal blood cells causing a deficiency in red blood cells (anaemia, reduced oxygenation), a deficiency in platelets (thrombocytopenia, internal bleeding) and a deficiency in normal white blood cells (increased risk for infections). In addition, leukaemic blasts can spread into the peripheral circulation which eventually leads to organ infiltration. Without treatment, leukaemia is a fatal disease.

Depending on the lineage origin of the leukaemic blasts, one differentiates between lymphoid or myeloid leukaemia. Lymphoid leukaemias are further subdivided into T- or B-lineage leukaemias. Another subdivision differentiates between the acute and chronic forms of leukaemia. Acute leukaemia is characterised by a rapid accumulation of immature leukaemic blasts and occurs mainly in children and young adults. In chronic leukaemia, the proliferation of blasts is slower, and the cells are characterised by a more mature differentiation state. The chronic form occurs mostly in elderly people. The leukaemia classification consists of 4 main categories: acute lymphoid

leukaemia (ALL), chronic lymphoid leukaemia (CLL), acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML).

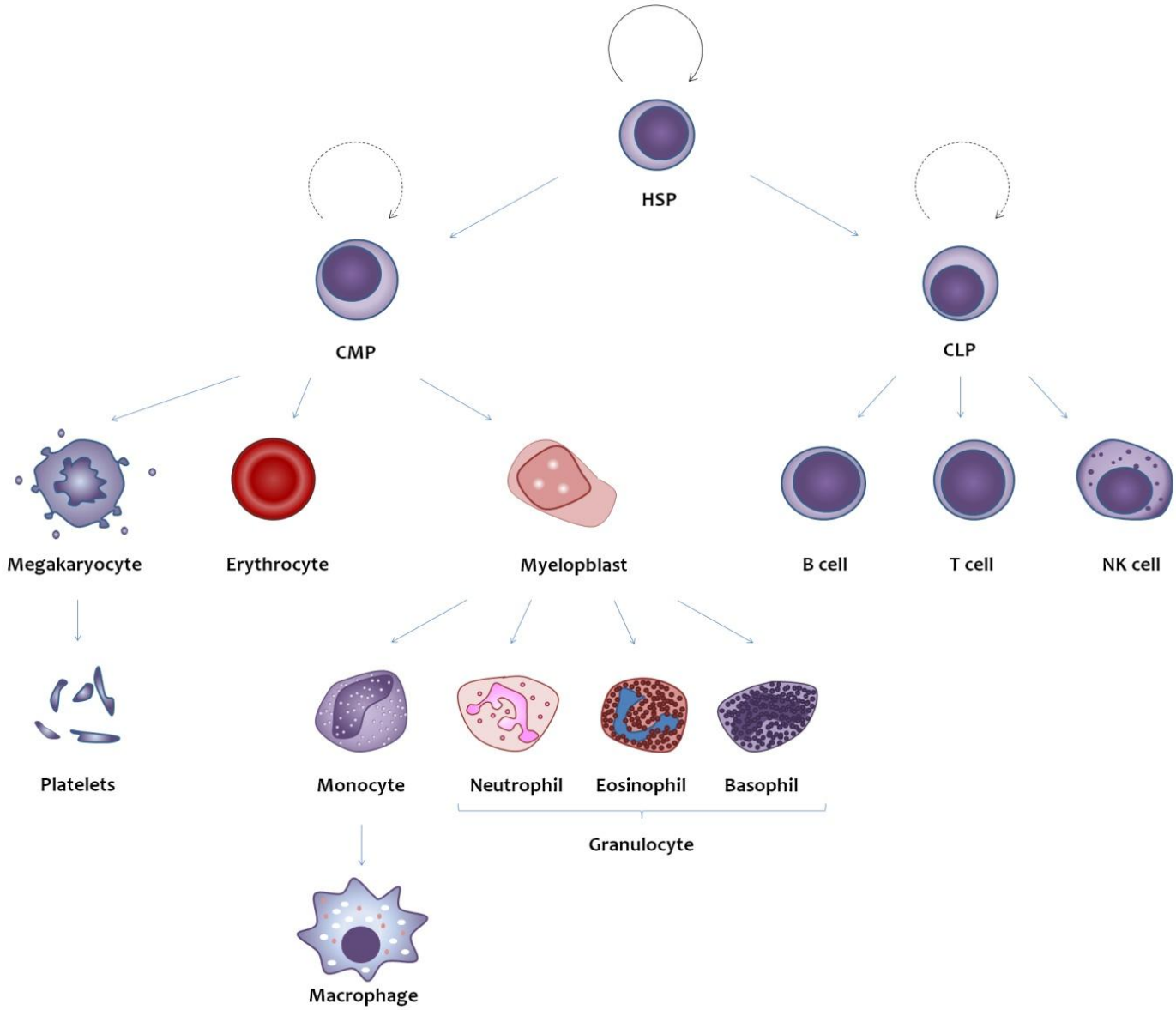


Figure 1.1 **Simplified schematic representation of the human haematopoiesis**
(HSP, haematopoietic stem cell; CMP, common myeloid precursor; CLP, common lymphoid precursor)

In children, leukaemias represent the most common type of cancers. The ALL form represents the most common leukaemia with around 85% of the cases (Pui et al., 2004). The age peak of paediatric ALL occurs between 2 and 5 years. Interestingly, more boys than girls are affected by ALL (Van Vlierberghe et al., 2010). 10-15% of ALLs represent T-ALL, a particularly aggressive form of leukaemia with a high white blood cell (WBC) count at diagnosis. T-ALL is triggered by the

proliferation of immature thymoblasts. In T-ALL, leukaemic thymoblasts can be arrested at different intrathymic maturation stages. Immunophenotyping by using common cell surface markers helps to distinguish between the maturation stages. There are 4 main phenotypes: pro-T, pre-T, cortical and mature phenotype (Bene et al., 1995). The main treatment strategies include intensive chemo- and radiotherapy (Conter et al., 2010). The introduction of these new therapeutic strategies has substantially improved the treatment outcome of paediatric T-ALL patients. However, relapses are usually fatal due to therapy-resistance. Compared to childhood T-ALL, adult T-ALL remains a difficult cancer to cure with survival rates between 40-60% (Heesch et al., 2010; Marks et al., 2009). Although, the outcome in childhood T-ALL has improved drastically, individualised treatment approaches would be desirable for the cure of patients with high relapse risk. In addition, reduction of therapy intensity for good responders could diminish treatment-related toxicity and severe long-term sequelae.

A study group that was involved in major advances in paediatric ALL treatment is the multi-centre study group, ALL-BFM (Berlin/Frankfurt/Münster), founded in Germany. The ALL-BFM treatment protocol is applied in more than 70 hospitals in Germany, Austria and Switzerland. The latest protocol with a long follow-up (ALL-BFM 2000) is divided into 4 blocks, the induction phase, the consolidation phase, the re-induction phase and the maintenance. Each patient undergoes a pre-phase which includes the administration of prednisone (or dexamethasone) and intrathecal methotrexate. After the induction phase, patients are stratified into one of three risk groups dependent on their early treatment response, assessed by prednisone response on day 8 and by minimal residual disease (MRD) kinetics on days 33 and 78. Prednisone response corresponds to the direct count of blasts in the blood. Patients with less than 1000 blood blasts/µL were defined as prednisone good responders (PGR), whereas those with 1000 or more blood blasts/µL were considered as prednisone good responders (PPR) (Dordelmann et al., 1999). MRD quantification is carried out by allele-specific detection and amplification of leukaemic clonal immunoglobulin (*Ig*) and T-cell receptor (*TCR*) gene rearrangements using the quantitative polymerase chain reaction (PCR) technique (Szczepanski et al., 2002a).

The three risk groups correspond to

- the standard risk (SR) group defined by a prednisone good response (PGR) and negative MRD results on days 33 and 78;
- the high risk (HR) group defined by a prednisone poor response (PPR), a MRD response of $\geq 10^{-3}$ on day 78, or both;
- and the intermediate risk (MR) group which regroups all remaining patients.

Importantly, all T-ALL patients are given prophylactic cranial irradiation to prevent CNS relapse.

T-ALL patients, that suffer a relapse, undergo intensified treatment with chemotherapy, radiotherapy and, in most cases, allogeneic stem-cell transplantation (Tallen et al., 2010).

The aetiology of leukaemia is still under debate, and only rare reports describe a familial occurrence. Hypotheses of what causes leukaemia include environmental factors or infections (Greaves, 2006). Molecular insight into the genetic causes of T-ALL was gained by the discovery of abnormal karyotypes in about 50% of the patients (Graux et al., 2006). Chromosomal translocations frequently involve fusions of the *TCR* genes with genes involved in transcription regulation, such as *TAL1*, *HOX11*, *LYL1*, *LMO1*, *LMO2* or *NOTCH1* resulting in their ectopic expression (Graux et al., 2006). Non-TCR translocations result in the generation of fusion genes with oncogenic properties encoding chimeric proteins like SIL-TAL1. These chromosomal translocations result from illegitimate TCR rearrangements during T cell development. Other genetic loci could also be prone to recombination when present in an open chromatin configuration. Interestingly, some diagnosed ALL patients have already harboured chromosomal abnormalities prenatally which, in combination with further genetic events, induce leukemogenesis at a later age (Gale et al., 1997; Greaves et al., 2003). Even in the absence of chromosomal abnormalities, these T cell oncogenes are often aberrantly expressed (Ferrando et al., 2002). Other genomic abnormalities have been identified by genomic profiling including amplifications or deletions affecting transcription factor genes (*MYB*, *LEF1*) and genes involved in signalling pathways (*PTEN* (Phosphatase and Tensin homolog)), cell cycle progression (*CDKN1B*, *CDKN2A*, *CDKN2B*) and apoptosis (*CASP8AP2*) (Burkhardt et al., 2008; Lahortiga et al., 2007; Mullighan et al., 2007; Remke et al., 2009). Furthermore, epigenetic silencing is affected in T-ALL, involving hypermethylation of promoter regions (Dunwell et al., 2010; Dunwell et al., 2009; Roman-Gomez et al., 2005). Moreover point mutations have been found in *NOTCH1* (>50% of the patients), in its negative regulator, *FBXW7* (15-30%), in genes involved in the PI3K-Akt (phosphatidyl-inositol-3-kinase) pathway (Gutierrez et al., 2009; Jotta et al., 2010; Remke et al., 2009), in a transcriptional regulator, *PHF6* (Van Vlierberghe et al., 2010), and less frequently in a receptor tyrosine kinase, *FLT3* (Van Vlierberghe et al., 2005).

1.2 The NOTCH pathway in paediatric T-ALL

1.2.1 NOTCH1, a transmembrane receptor with a transcription activating function

1.2.1.1. The structure and the function of NOTCH1

NOTCH mutants were first described in *Drosophila melanogaster* that carried notches in the wing blades (Morgan, 1917). Later this phenotype was described to be the result of a haploinsufficiency of the gene NOTCH (Kidd et al., 1986; Wharton et al., 1985). NOTCH is a family of evolutionary conserved type I transmembrane receptors. In mammals four different NOTCH receptors (NOTCH1-NOTCH4) and five corresponding ligands (Delta-like1 (Dll1), Dll3, Dll4, Jagged1 (J1), and J2) are found.

NOTCH is a transmembrane receptor which contains 2 subunits, an extracellular and a transmembrane subunit (Figure 1.2). These two subunits are associated non-covalently through the heterodimerisation domains in a Ca^{2+}-dependent manner. The extracellular part contains epidermal growth factor (EGF)-like repeats including ligand binding sites, followed by three cysteine-rich Lin12-Notch (LIN) repeats that are involved in preventing NOTCH signalling in the absence of a ligand. The transmembrane part contains in the intracellular region:

- RBPJk-associated molecule (RAM) region in the juxtamembrane region harbouring a binding site for CSL (**C**BF1, **S**uppressor of Hairless, **L**ag-1), a DNA-binding transcription factor;
- six ankyrin repeats involved in docking of CSL and members of the MAML (Mastermind-like) family, transcriptional coactivators;
- transactivation domain (TAD);
- PEST (Proline-Glutamate-Serine-Threonine rich) domain involved in the degradation of the intracellular part. This domain contains a WSSSSP motif and a Cdc4 phospho-degron (CPD) which is recognised by the E3 ubiquitin ligase FBXW7 (see 1.2.2). Both motifs are involved in NOTCH1 ubiquitylation (Chiang et al., 2006; Thompson et al., 2007).

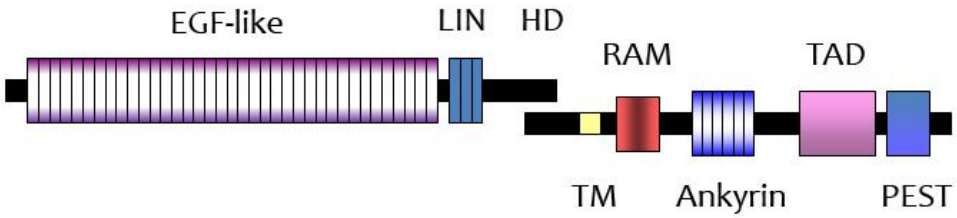


Figure 1.2 **The transmembrane receptor NOTCH1 and its domains.**
LIN (Lin12-Notch), HD (Heterodimerisation domain), TM (Trans-membrane domain), RAM (RBPJk-associated molecule), TAD (Transactivation domain), PEST Proline-Glutamate-Serine-Threonine rich domain)

For proper function, NOTCH1 has to undergo post-translational modifications. The *O*-fucosyl transferase (*O*-Fut) adds a first fucose on the glycosylation sites located in the EGF-like domains (Figure 1.3). *O*-Fut is also essential for the proper folding and transport of NOTCH to the plasma membrane. Other glycosyl-transferases (members of the Fringe family) elongate the carbohydrate chains in the EGF-like domains. Interestingly, differential glycosyl-modifications generate NOTCH molecules with different ligand binding affinities. Another important modification is the S1 cleavage by the furin-like convertase, which generates the extracellular and the transmembrane parts of NOTCH1 (Blaumueller et al., 1997; Logeat et al., 1998). The activation of the transmembrane receptor is triggered by specific Delta-like or Jagged ligands to the extracellular part of NOTCH1. Binding of the ligand to the EGF-like repeats triggers two successive cleavages which in turn cause the release of the intracellular part of NOTCH1 (ICN1) into the cytoplasm. The first activating cleavage (S2) involves the ectodomain of NOTCH and is dependent on a metalloprotease of the ADAM/TACE/Kuzbanian family (Brou et al., 2000). The second activating cleavage (S3) within the TM is catalysed by the γ-secretase complex (Mumm et al., 2000). This complex is necessary to release the intracellular portion of NOTCH1 into the cytoplasm from where it is translocated into the nucleus. When in the nucleus, ICN1 interacts with a transcription factor, CSL, and transcriptional coactivators from the MAML family. In the absence of ICN1, CSL is associated to transcriptional co-repressors and inhibits the expression of NOTCH1 target genes. The association of ICN1 and CSL, however, results in the release of CSL from the repressors, probably due to a conformational change. CSL, when bound to ICN1, will then act as a positive transcriptional regulator. The ICN1/CSL/MAML complex is able to activate the transcription of NOTCH1 target genes. Although, a CSL-independent signalling of NOTCH has been proposed, the underlying mechanism is so far unknown (Martinez Arias et al., 2002). The termination of NOTCH signalling is achieved by ICN1 degradation. CDK8 phosphorylates NOTCH1

rendering it susceptible for ubiquitylation by FBXW7, which is an E3 ubiquitin ligase. FBXW7 specifically binds and ubiquitinates a CPD within the PEST domain. Furthermore, the WSSSSP motif is also required for efficient degradation, but the potential kinases phosphorylating the serine residues have not been identified yet.

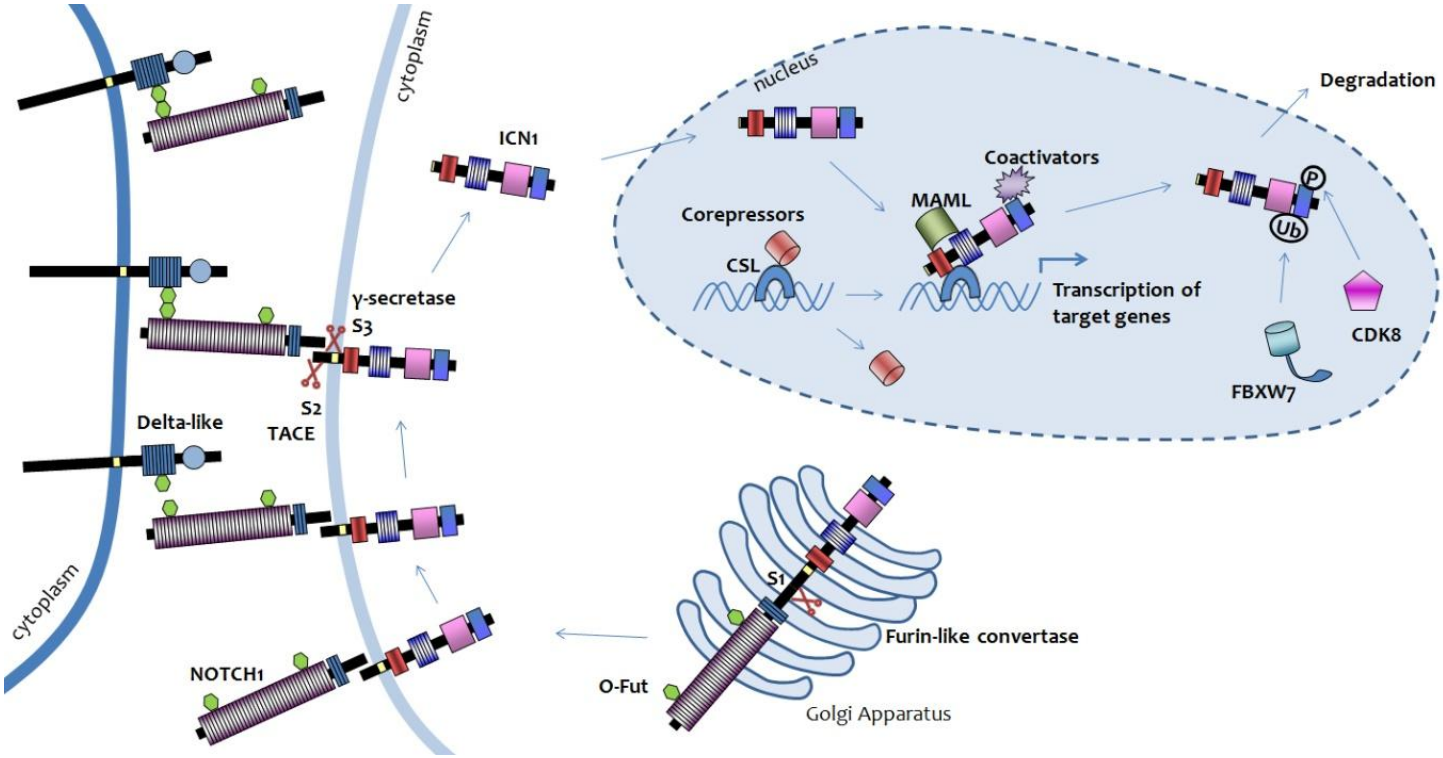


Figure 1.3 **The NOTCH1 pathway**

1.2.1.2 The role of NOTCH1 in haematopoiesis

The NOTCH pathway functions broadly; it is involved in lateral inhibition (Greenwald, 1998), lineage decisions (Hartenstein and Posakony, 1990; Louvi and Artavanis-Tsakonas, 2006), and forming boundaries or niches (Bray, 1998). The developmental and physiological processes in which the NOTCH pathway is involved are very diverse. In mammals, essential functions of NOTCH occur at various stages in haematopoiesis (Radtke et al., 2010). NOTCH receptors and their ligands are widely expressed in the haematopoietic system. During murine embryonic development, NOTCH1 signalling is required for the generation of HSCs (Kumano et al., 2003). The best characterised NOTCH function in haematopoiesis is its role during lymphoid development, and specifically during thymic T cell lineage commitment and maturation (Radtke et al., 2004; Tanigaki

and Honjo, 2007). Various NOTCH receptors and ligands are expressed in lymphoid progenitors, suggesting redundant and non-redundant functions (Kitamoto et al., 2005). Specifically, NOTCH1 was reported to be necessary and sufficient to specify T cell fate. Bone marrow progenitors depleted of NOTCH1 launch a B lymphoid program when entering the thymus, whereas constitutively active NOTCH1 in these cells causes an ectopic production of immature T cells at the expense of B cells (Pui et al., 1999; Radtke et al., 1999; Wilson et al., 2001). In early T lineage precursors (ETPs) NOTCH signalling is still required as these cells maintain their non-T cell potential (Bell and Bhandoola, 2008; Feyerabend et al., 2009). Interestingly, although weak NOTCH activity is able to suppress B lineage development, it is not sufficient to induce T cell development (Hoebeke et al., 2006; Schmitt et al., 2004; Visan et al., 2006). Altogether, these data suggest that the NOTCH pathway is used to target different genes depending on the amount of activated NOTCH1 molecules.

1.2.1.3 The role of NOTCH1 in paediatric T-ALL

Tight regulation of the duration of NOTCH signalling and the identity of its targets is important for the proper development and function of cells and tissues. Deregulation of its activity is, not surprisingly, implicated in the pathology of several malignant diseases, including paediatric T-cell acute lymphoblastic leukaemia (T-ALL). The first indication of NOTCH1 function in T-ALL came with the identification of a chromosomal translocation, t(7;9) (q34:q34.3), involving the juxtaposition of the *NOTCH1* gene on chromosome 9 to the *TCRB* locus on chromosome 7 (Ellisen et al., 1991; Reynolds et al., 1987). Interestingly, only the intracellular part of NOTCH1 is juxtaposed to the promoter, and enhancer sequences of the TCRβ receptor gene induce an overexpression of a constitutively active NOTCH1. In murine models, constitutive activation of NOTCH1 causes aggressive T-ALL, providing evidence of its oncogenic function (Pear et al., 1996). However, the t(7;9) chromosomal translocation occurred in a minor fraction of children with T-ALL (<1%). Later Weng *et al.* described activating mutations in *NOTCH1* in over 50% of all T-ALLs (Weng et al., 2004). Most of these mutations lie in the heterodimerisation domains which are hypothesised to reduce the affinity between the two subunits. This would lead to ligand-independent cleavage of the transmembrane subunit. Some other mutations cause a premature stop codon in the TAD or the PEST domain, resulting in an increase of the stability of ICN1 due to the absence of the degradation signal. Both, the translocations and the mutations are thought to result in an aberrant activation of NOTCH1 and an increase of its activity.

An important research focus became the identification of possible target genes of NOTCH1. Several studies reported an up-regulation of c-Myc upon ectopic NOTCH1 expression, and

identified the *MYC* gene as a direct transcriptional target of NOTCH1 (Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006). Another finding represents the transcriptional activation of the IκB kinase complex, thereby inducing the activation of the NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) pathway (Vilimas et al., 2007). Phosphorylation specific protein microarrays detected the mTOR (mammalian Target of Rapamycin) pathway as a positively regulated target of NOTCH1 (Chan et al., 2007). How this effect is mediated, is only partially understood. In addition, aberrant NOTCH1 signalling has a direct effect on cell cycle progression by inducing the proteasome-mediated degradation of CDKN1B, a cyclin-dependent kinase inhibitor, and the expression of SKP2, the S phase kinase-associated protein 2 (Dohda et al., 2007). It was shown that both CDKN1B and CDKN2D are induced in T-ALL cell lines treated with γ-secretase inhibitors (GSI) (Rao et al., 2009). Furthermore, in mice with aberrantly expressed NOTCH1, p53 is suppressed through an ARF-mdm2-p53 mechanism, which led to inhibition of apoptosis (Beverly et al., 2005). Moreover, upon ectopic NOTCH1 expression, a signaling protein with anti-apoptotic functions, GIMAP5 (GTPase of immunity-associated nucleotide binding protein 5), was shown to be up-regulated (Chadwick et al., 2009). Additionally, NOTCH1 induces the expression of a chemokine receptor, CCR7 (CC-chemokine receptor 7), in T-ALL, which is essential for CNS infiltration (Buonamici et al., 2009). In summary, aberrant NOTCH1 signalling affects cell growth, survival and tissue infiltration of T cells by interfering with various molecules and pathways.

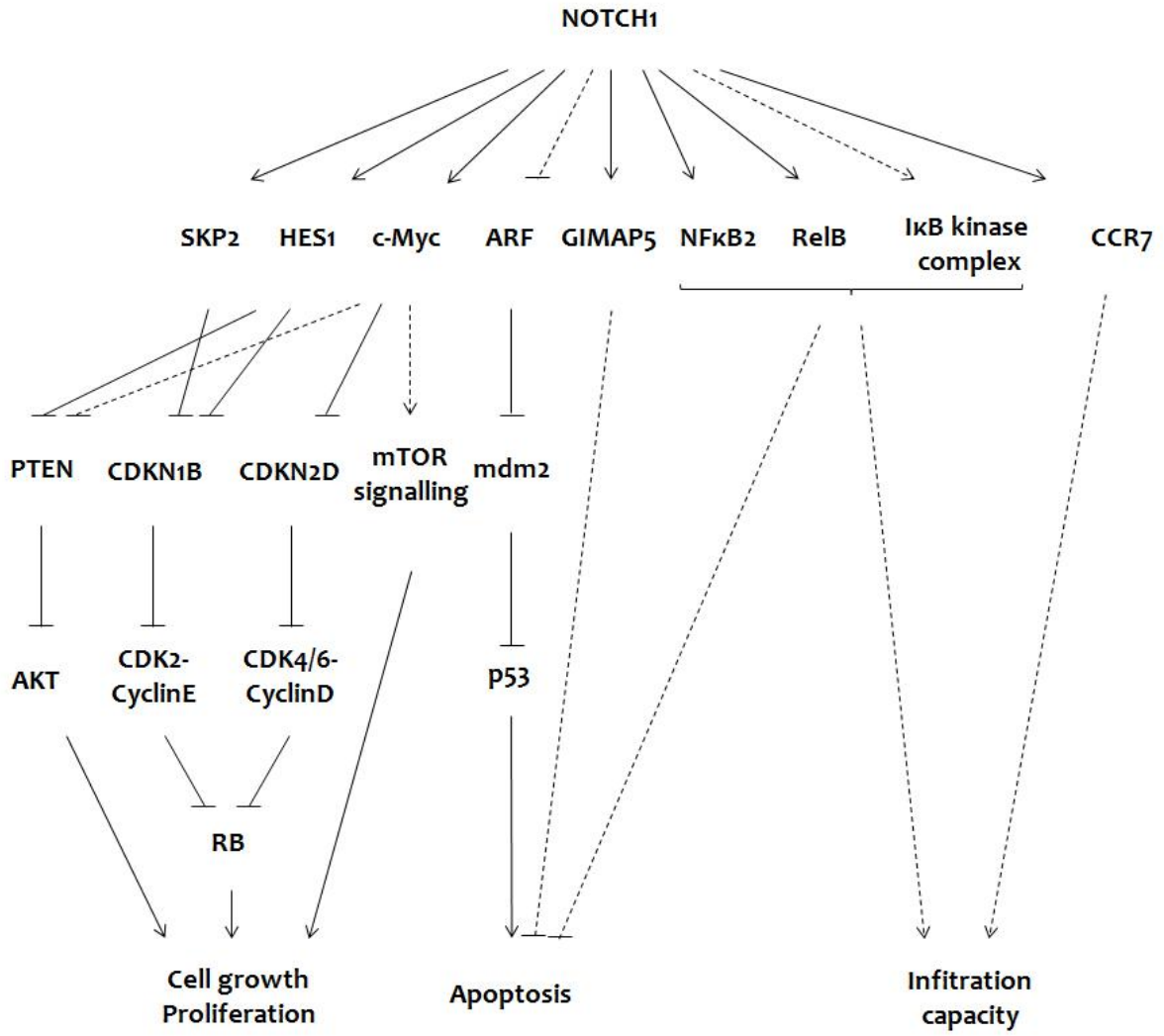


Figure 1.4 **Targets of the aberrant NOTCH1 activation in T-ALL**

1.2.2 FBXW7, a negative regulator of the NOTCH pathway

FBXW7 (F-box and WD repeat domain-containing 7), an E3 ubiquitin ligase, is a component of the SCF complex (SKP1, CUL1 and F-box protein) that is responsible for the ubiquitylation of proteins destined for proteasomal degradation. FBXW7 was first described in genetic screens in budding yeast as Cdc4 (Hartwell et al., 1973). Cdc4 controls the cell cycle checkpoint by causing the disposal of cyclin-dependent kinase (CDK) inhibitor, Sic1 (Feldman et al., 1997; Verma et al., 1997). The human *FBXW7* gene is located on chromosome 4, and is able to produce three different transcripts by alternative splicing, FBXW7α, β and γ. These isoforms vary only in their N-terminal sequence and are in principle functionally identical. However, they occupy different cellular locations (Kimura et al., 2003; Welcker et al., 2004). FBXW7α and γ contain both a NLS (nuclear

localisation signal) and are localised in the nuclear and in the nucleolar region, respectively. FBXW7β contains a TMD (transmembrane domain) and is found in a cytoplasmic distribution. The FBXW7 protein contains three major domains, a dimerization domain (DD), an ubiquitin ligase (F-box) and 8 repeats of WD domains (WD40). The F-box domain interacts with Skp1 from the SCF complex (Bai et al., 1996), whereas the WD40 domains mediate substrate binding by specific recognition of a phospho-epitope, called Cdc4 phospho-degron (CPD) (Hao et al., 2007). Homodimerisation of FBXW7 seems to be dispensable for ubiquitylation of some substrate proteins, and may depend on the strength of binding to the CPD in the substrate. FBXW7 targets some of the most broadly acting proteins for degradation, these include MYC, PSEN1, c-JUN, cyclin E1, SREBP (sterol regulatory element-binding protein), mTOR or Aurora-A (Koepp et al., 2001; Li et al., 2002; Mao et al., 2004; Nateri et al., 2004; Sundqvist et al., 2005; Yada et al., 2004). Noticeably, FBXW7 regulates several proteins within the NOTCH pathway, including NOTCH1, the γ-secretase complex, PSEN1 (presenilin1), and several NOTCH1 downstream effectors, like c-MYC or mTOR.

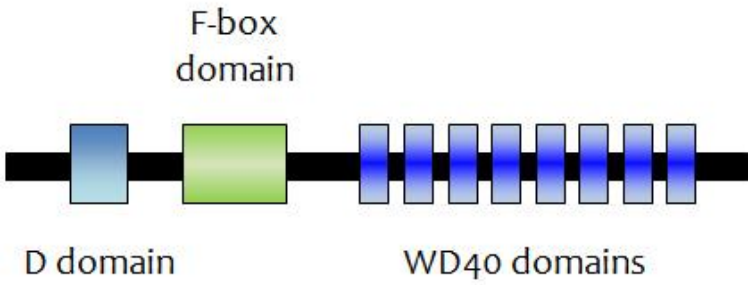


Figure 1.5 **The E3 ubiquitin ligase FBXW7 and its domains.**
FBXW7 contains a dimerisation domain (D domain), an ubiquitin ligase function (F-box domain) and 8 repeats of the WD40 domains involved in substrate binding.

FBXW7 null mice die *in utero* at embryonic day 10.5-11.5 of gestation because of defective cardiovascular and hematopoietic development. In these mice, an accumulation of NOTCH led to increased expression of NOTCH pathway downstream effectors (Tetzlaff et al., 2004; Tsunematsu et al., 2004). It was reported that a specific loss of FBXW7 in the HSCs resulted in their premature depletion due to active proliferation and p53-induced apoptosis. When p53 was suppressed, most animals developed T-ALL (Matsuoka et al., 2008). In mice with a conditional FBXW7 deficiency in the T cell lineage, immature T cells hyper-proliferate whereas mature T cells show a cell-cycle arrest followed by p53-dependent apoptosis (Onoyama et al., 2007).

Not surprisingly, it was discovered that FBXW7 is deregulated in several human malignant diseases by chromosomal aberrations or point mutations, e.g. endometrial cancer (Spruck et al., 2002), colorectal cancer (Rajagopalan et al., 2004) or T-ALL (Maser et al., 2007). The highest mutational incidence was found in cholangiocarcinoma (35%) (Akhoondi et al., 2007). In T-ALL, *FBXW7* mutations were described in about 11-30% (Akhoondi et al., 2007; Larson Gedman et al., 2009; Maser et al., 2007). The heterozygous mutations found in *FBXW7* are inactivating missense or nonsense mutations. Of interest, T-ALL contains mostly missense mutations. The mode of action of the mutated FBXW7 proteins varies depending on the location of the mutation. Most missense mutations target arginine residues in the WD40 region that mediate specific substrate binding. The D domain however, stays intact, and by dimerisation with a functional FBXW7 molecule, the truncated FBXW7 is hypothesised to function as dominant-negative. Nonsense mutations downstream of the D domain could also produce potential dominant-negative proteins, whereas stop codons upstream of the D domain likely produce non-functional alleles.

Binding to NOTCH1 is mediated by a CPD in the PEST domain, a threonine residue located on position 2512. Most *NOTCH1-PEST* mutations in T-ALL interfere with this residue by directly mutating it or causing premature stop codons upstream of this region. Interestingly, only rarely a *NOTCH1-PEST* mutation is found in combination with a *FBXW7* mutation. This almost complete mutual exclusivity suggests that the main target of FBXW7 in T-ALL is NOTCH1, and that no additional selection advantage is provided by combining a *NOTCH1-PEST* and *FBXW7* double mutation (Maser et al., 2007; O'Neil et al., 2007). If also other target proteins are affected by *FBXW7* mutations in T-ALL, is not known.

1.3 MicroRNAs in paediatric T-ALL

1.3.1 MicroRNA biogenesis

Mature microRNAs (miRNAs) are single-stranded non-coding RNAs of 19-25 nucleotides in length that negatively regulate gene expression. Mature miRNAs are incorporated in a ribonucleoprotein complex (RISC complex). MiRNAs are predicted to be one of the largest gene families, counting for 1% of the total genome. The miRNA genes are mostly localised in intergenic regions, some in introns, protein-coding or non-coding (in the sense or antisense orientation) regions. Often, miRNA genes encode for multiple miRNAs, the so-called miRNA clusters that form polycistronic transcription units. MiRNAs exist in animals, plants and viruses, but have not been identified in yeast.

MiRNAs are transcribed as long primary transcripts (pri-miRNAs) by the RNA Polymerase II (PolII) (Figure 1.4). The pri-miRNAs contain a 5' cap structure, one or more hairpin-loop structures as well as 3' polyA tails (Cai et al., 2004; Lee et al., 2004). It is believed that the pri-miRNA tertiary structure determines the recognition by a microprocessor complex consisting of the RNase type III endonuclease, Drosha, and DGCR8 (DiGeorge syndrome critical region gene 8) (Denli et al., 2004; Lee et al., 2003; Zeng and Cullen, 2003; Zeng et al., 2005). A 70-nucleotide long stem-loop RNA is cleaved, called the precursor-miRNA (pre-miRNA). MiRNAs are evolutionarily highly conserved in their stem sequence, but diverge consistently in the regions flanking the stem-loop (Kim, 2005). The Ran-GTP dependent exportin-5 is responsible for the active transport of the pre-miRNA into the cytoplasm (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). The cytoplasmic RNase III Dicer in association with TRBP (HIV-1 transactivating response (TAR) RNA binding protein) processes the pre-miRNA into a mature duplex miRNA (Hutvagner et al., 2001; Knight and Bass, 2001). Mostly only one strand is incorporated into the miRNA-containing RNA induced silencing complex (miRISC), whereas the second one is degraded. The single-stranded mature miRNA tends to be selected on the basis of the thermodynamic instability of the base pairs at the end of the duplex; the strand with the weaker 5'end base-pairing relative to the complement will preferentially be incorporated into miRISC (Khvorova et al., 2003; Schwarz et al., 2003). This complex is composed of members of the highly conserved Argonaute (Ago) family and a number of other protein components with RNA binding or helicase activity (Sontheimer, 2005). The mature miRNA finally mediates the binding of the miRISC with the target mRNA. In plants, most miRNAs condemn the mRNAs to destruction by cleavage and degradation by an almost perfectly complementary binding in a single site (Hake, 2003). In contrast, in animals multiple binding sites exist mainly in the 3'UTR region of the mRNA, but base-pairing is only partially complementary. Here the binding is mediated by an almost perfect annealing of the "seed" sequence embodied in the nucleotides 2 to 8 (Tomari and Zamore, 2005). In general, the fate of the target mRNA depends on the stability of the mRNA-miRNA base pairing; a perfect match causes the degradation of the mRNA (Hutvagner and Zamore, 2002), whereas partial complementarity represses its translation (Bartel, 2004). Up to date, how the translational repression is achieved remains elusive. Biochemical data show an association of miRNPs with polyribosomes suggesting an interference with protein synthesis (Nottrott et al., 2006). Alternative mechanisms include interference with the recognition of the 5' cap to prevent translational initiation (Pillai et al., 2005; Thermann and Hentze, 2007), deadenylation of the polyA tail (Eulalio et al., 2009; Wu et al., 2006) and recruitment of eIF6, a ribosome inhibitory factor (Chendrimada et al., 2007). Moreover, colocalisation of Ago proteins with processing bodies (P bodies) lead to the hypothesis of miRNA-targeted mRNAs sequestration into P bodies which

show an accumulation of endonucleases and decapping proteins, but lack ribosomes and initiation factors (Liu et al., 2005; Sen and Blau, 2005). An interesting observation was described in cells entering quiescence in which miRNAs trigger a translational activation rather than a repression (Vasudevan et al., 2007).

Regulation of miRNA biogenesis probably occurs mostly on the transcriptional level, as expression of miRNAs is controlled by methylation of the promoter region (Saito et al., 2006) and by transcription factors (He et al., 2007; O'Donnell et al., 2005). However, alternative mechanisms were proposed including pri-miRNA editing which can influence positively or negatively the cleavage by Drosha (Scadden, 2005; Yang et al., 2006), regulation of the Drosha-DGCR8 complex (Davis et al., 2008; Han et al., 2009) and regulation of the cleavage by Dicer (Forman et al., 2008; Rybak et al., 2008). Although miRNA degradation pathways have been identified in plants (Ramachandran and Chen, 2008), the mechanisms of miRNA half-life regulation in animals await further investigation.

The latest release of the miRBase database lists more than 900 human miRNAs that have been identified by cloning and through computational analysis. Computational analyses estimate that 20-30% of the genome might be regulated by miRNAs, and that on average any given miRNA might target up to 200 mRNAs (Lim et al., 2005). One of the biggest limitations in the functional analysis of miRNA is the lack of reliable tools for target prediction. Development of algorithms for miRNA-target prediction (i.e. target mRNAs) is rather difficult, because in mammals the miRNA-mRNA recognition occurs through partial complementarity. Hence, target prediction always requires validation. A number of studies based on different biochemical approaches not only validated miRNA targets, but also show that miRNA interference/overexpression has a widespread effect on post-translational gene regulation (Baek et al., 2008; Beitzinger et al., 2007; Easow et al., 2007; Karginov et al., 2007; Selbach et al., 2008).

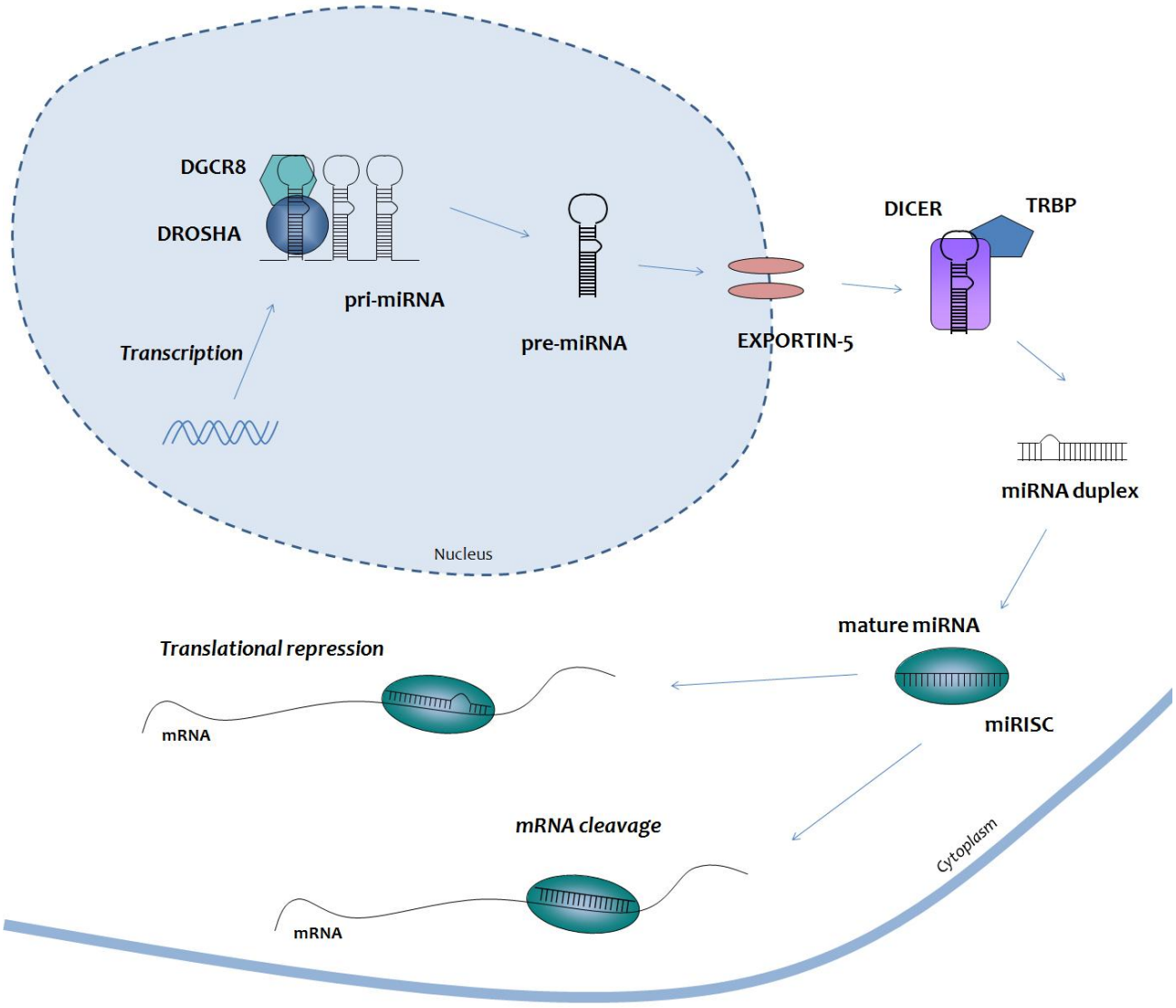


Figure 1.6 **MicroRNA biogenesis and function**

1.3.2 MicroRNA in haematopoiesis

MiRNAs are expressed during different developmental stages and in different tissues. They are involved in various processes, including cell proliferation and apoptosis, stem cell maintenance and cell fate specificity. Not only is their expression in time and space of importance, but also their expression levels need to be tightly regulated.

Specifically in haematopoiesis, controlled miRNA expression is crucial for proper development of all blood cells. A functional role for miRNAs in haematopoiesis has previously been predicted, as miRNA genes are frequently found close to chromosomal breakpoints (Calin et al., 2004b). A general role for miRNAs in haematopoiesis was suggested as the conditional deletion of Dicer in murine lymphoid progenitors resulted in impaired T cell development (reduction of helper T cell proliferation and survival, reduction of regulatory T cells) (Cobb et al., 2006; Muljo et al., 2005) and in a block at the pro- to pre-B cell transition (Koralov et al., 2008). First indications of miRNAs involved in haematopoiesis came from studies describing the expression of individual miRNAs in the different haematopoietic compartments, including BM, thymus and peripheral blood (Chen et al., 2004; Monticelli et al., 2005). Interestingly, miRNA expression profiles differ not only between haematopoietic cell types, but are also temporarily regulated in the various developmental stages in haematopoiesis (Monticelli et al., 2005; Neilson et al., 2007).

In lymphopoiesis, important miRNAs are miR-181, miR-142, miR-223, miR-150 and miR-17~92. miR-181, highly expressed in thymocytes and mature B cells, has prominent functions in B cell development. Over-expression studies of miR-181 in murine HPCs showed an increase of B cells, but a reduction of the T cell lineage. Enforced expression of miR-223 and miR-142 resulted in an increased T cell number, and a modest effect on the myeloid lineage (Chen et al., 2004). An additional function of miR-181 is to positively regulate T cell sensitivity and selection (Li et al., 2007). Murine B cell development is also substantially impaired when miR-150 is depleted or ectopically expressed (Xiao et al., 2007; Zhou et al., 2007). Similarly, depletion of the miR-17~92 cluster inhibits B cell development by stabilisation of the pro-apoptotic protein, BIM (BCL-2-interacting mediator of cell death; Ventura et al., 2008). Furthermore regulation of the T- and B-cell responses is under the control of miR-155 expression. miR-155 knock-out mice were shown to be immunodeficient (Rodriguez et al., 2007; Thai et al., 2007). miR-155 also functions in dendritic cells by regulating the pathogen binding activity through the down-regulation of DC-SIGN (DC-specific Intercellular Adhesion Molecule-3 Grabbing Non-integrin) (Martinez-Nunez et al., 2009) (Table 1.1).

Multiple links between myeloid development and miRNA expression have been proposed. miR-223 has several roles during different stages of myeloid cell development by regulating granulocyte generation and function, in addition to modulating neutrophil sensitivity (Fazi et al., 2005; Fukao et al., 2007; Johnnidis et al., 2008). Moreover, members of the miR-17~92 cluster and its homologue cluster miR-106~363 regulate monocyte proliferation and maturation (Fontana et al., 2007). Although not described in detail here, many other miRNAs seem to play an extensive role in myelopoiesis and in the innate immune response, such as miR-155, miR-146 or miR-221/222 (Felli et al., 2005; Jurkin et al., 2010; O'Connell et al., 2008; O'Connell et al., 2007; Taganov et al., 2006) (Table 1.1).

miRNA	Haematopoietic lineage	Function	References
miR-181	lymphopoiesis	regulates B cell development positively, but T cell development negatively; modulates T cell sensitivity and selection	Chen et al., 2005 Li etal., 2007
miR-142	lymphopoiesis	negatively regulates the T-lymphoid development	Chen et al., 2005
miR-150	lymphopoiesis	inhibits B cell differentiation at the proB- to pre-B cell transition	Xiao et al., 2007 Zhou et al., 2007
miR-106~363	myelopoiesis	miR-106a inhibits monocytic differentiation and maturation	Fontana et al., 2007
miR-146	myelopoiesis	role for miR-146 in control of Toll-like receptor and cytokine signalling; ectopic miR-146a expression influences DC sensitivity to PGN-induced activation	Taganov et al., 2006 Jurkin et al., 2010
miR-221/222	myelopoiesis	expression of miR-221/222 causes impaired proliferation and accelerated differentiation of erythropoietic cells	Felli et al., 2005
miR-223	lymphopoiesis myelopoiesis	ectopic expression of miR-223 causes a 30 to 40% increase in the T-lymphoid lineage ; levels of miR-223 control the differentiation fate of promyelocytic precursors; delimits neutrophil production and activation	Chen et al., 2003 Fazi et al., 2005 Fukao et al., 2007 Johnnidis et al., 2008
miR-155	lymphopoiesis myelopoiesis	involved in the in vivo control of specific differentiation processes in the immune response; regulates the function and response of B and T lymphocytes, and of dendritic cells; modulates pathogen binding ability in dendritic cells; important in the primary macrophage response to different types of inflammatory mediators	Rodriguez et al., 2007 Thai et al., 2007 Martinez-Nunez et al., 2009 O'Connell et al., 2007 O'Connell et al., 2008
miR-17~92	lymphopoiesis myelopoiesis	in miR-17~92 deficient embryos a reduction in pre-B cells was observed, due to increased apoptosis; regulates B cell survival; miRNAs 17-5p and 20a inhibit monocytic differentiation and maturation	Ventura et al., 2008 Fontana et al., 2007

Table 1.1 **MicroRNAs involved in haematopoiesis**

1.3.3 MicroRNA in cancer

Given their important role in development, it is not surprising that deregulation of miRNA expression is involved in cancerogenesis. More than half of the known human miRNA genes are located near chromosomal breakpoints or cancer-associated genomic regions indicating an implication of miRNAs in leukaemogenesis (Calin et al., 2004b). Other than chromosomal aberrations, potential alternative causes for abnormal miRNA expression in cancer are the deregulation of miRNA processing mechanisms (Kumar et al., 2007; Kumar et al., 2009) and epigenetic deregulation of miRNA genes (Saito et al., 2006). Still under investigation is the role of relatively infrequent SNPs, mutations in miRNAs or mutations in miRNA seed sequences in target genes (Ryan et al., 2010). MiRNAs were shown to have tumour suppressor and/or oncogenic activity, which is dependent on the cell type and the pattern of expression (He et al., 2005; O'Donnell et al., 2005). The first evidence of tumour suppressor activity came from homozygous deletions in B-cell CLL involving two clustered miRNAs, miR-15 and miR-16 (Calin et al., 2002). Later, it was shown that the effect of the deletions is partly due to the stabilisation of the Bcl-2 mRNA, encoding an anti-apoptotic protein (Cimmino et al., 2005). Given its extensive role in lymphoid and myeloid development, it is not surprising that the miR-17~92 and miR-106~363 clusters are involved as oncogenes in B-cell lymphomas, in CML and in T-cell leukaemia (He et al., 2005; Landais et al., 2007; Mavrakis et al., 2010; Ota et al., 2004; Venturini et al., 2007).

High throughput methods such as miRNA expression profiling have been useful to determine candidate miRNAs directly involved in tumourigenesis. These techniques might also serve to identify miRNA signatures with diagnostic and prognostic implications. Diagnostic utility of circulating miRNAs in serum was described in patients with diffuse large B-cell lymphoma (miR-155, miR-210, miR-21) and ALL (miR-16) compared to a healthy cohort (Kaddar et al., 2009; Lawrie et al., 2008). MiRNA patterns differ between tumour entities, and between different subclasses of the same tumour type (Calin et al., 2004a; Lu et al., 2005). As miRNA expression can also predict therapy outcome in haematological malignancies, miRNA profiling could advance to a novel prognostic tool (Calin et al., 2005; Garzon et al., 2008; Zhang et al., 2009).

1.3.4 MicroRNAs and the NOTCH pathway

An interaction between miRNA expression and the NOTCH pathway was expected, given the widespread regulation of multiple biological processes by both. The first indication for a possible interplay between miRNAs and the NOTCH pathway was described by Stark et al. who identified NOTCH target genes (Enhancer of split (E(spl), Bearded (Brd) complex genes) regulated

by miR-7, miR-4, miR-79, miR-2 and miR-11 in *Drosophila* (Lai et al., 2005; Stark et al., 2003). Another miRNA in *Drosophila*, miR-1, regulates the NOTCH pathway by targeting Delta, a NOTCH ligand, and thereby affecting cardiac differentiation (Kwon et al., 2005). *Vice versa*, NOTCH was shown to activate transcription of miR-61, a miRNA specific in *C.elegans*. miR-61 in turn inhibits the expression of VAV-1, a negative regulator of NOTCH. This positive feedback loop promotes vulval precursor cells in *C.elegans* to adopt a secondary fate (Yoo and Greenwald, 2005). An additional miRNA targeted by NOTCH in *C.elegans* is let-7a, which was also shown to be positively regulated in human 293T cells (Solomon et al., 2008). In a mouse myoblast cell line, it was reported that miR-146a regulates muscle cell differentiation by inhibiting expression of Numb, a NOTCH inhibitor (Kuang et al., 2009). Cross-talks have also been identified in cancer. miR-34 targets NOTCH1 in many cancer cell types (e.g. glioma cells, pancreatic cancer cells), thereby inhibiting cell growth and inducing apoptosis (Ji et al., 2008; Ji et al., 2009; Li et al., 2009; Pang et al., 2010). Another miRNA involved in regulating NOTCH signalling in tumour development is miR-199b-5p, which targets HES-1 in medulloblastoma (MB) tumours causing a decrease in cell growth (Garzia et al., 2009) (Table 1.2).

miRNA	**Member of NOTCH pathway**	**Interaction**	**Model**	**Model**
miR-7 miR-4 miR-79 miR-2 miR-11	E(spl) complex genes Brd complex genes*	miRNAs negatively regulating E(spl) and Brd complex gene expression	*D.melanogaster*	Stark et al., 2003 Lai et al., 2005
miR-1	Delta	miR-1 negatively regulating Delta expression	*D.melanogaster*	Kwon et al., 2005
miR-199-5p	HES1	miR-199-5p negatively regulating HES-1 expression	medulloblastoma	Garzia et al., 2009
miR-61	NOTCH	NOTCH activating miR-61 expression	*C.elegans*	Yoo and Greenwald, 2005
let-7a	NOTCH	NOTCH positively regulating miR-61 expression	*C.elegans* 293T cells	Solomon et al., 2008
miR-34	NOTCH	miR-34 negatively regulating NOTCH expression	glioma cells pancreatic cancer cells	Ji et al., 2008 Ji et al., 2009 Li et al., 2009 Pang et al., 2010
miR-146a	NUMB	miR-146a negatively regulating NUMB expression	mouse myoblast cells	Kuang et al., 2009

Table 1.2 **Interaction between miRNAs and members of the NOTCH pathway**
*Enhancer of split (E(spl)); Bearded (Brd)

1.4 Molecular markers in paediatric T-ALL

Although overall treatment outcome of children with T-ALL has improved dramatically over the years due to intensified treatment, a small number of patients develop recurrent disease and then respond poorly to the administered treatment. Molecular markers would therefore be of substantial value to identify high risk patients. In addition, good responders could benefit from therapy reduction, in order to avoid treatment-related toxicity and late complications.

A risk stratification using commonly used clinical and laboratory prognostic markers like age, WBC, hyperdiploidy, presence of translocations, has not been shown to be useful because of their low predictive value (Goldberg et al., 2003; Pullen et al., 1999). In many study groups, risk assessment is based on early drug response and minimal residual disease (MRD) measurements (Cave et al., 1998; Conter et al., 2010). With the discovery of chromosomal translocations and aberrant expression of oncogenes, the search for new risk markers continues. Ferrando *et al.* firstly demonstrated the possible use of gene expression profiles in stratifying T-ALL patients according to risk. As epigenetic profiles in cancer differ from those in healthy tissues, epigenetic profiling showed to be potentially useful for risk prediction in other tumour types (Miyamoto and Ushijima, 2005). In T-ALL, specific DNA methylation profiles associate with good prognosis (Roman-Gomez et al., 2005). Other genomic imbalances in T-ALL, identified by genomic profiling, promise to be useful for early prognosis (Burkhardt et al., 2008; Remke et al., 2009). Recently, the frequency of *NOTCH1* and *FBXW7* mutations has become interesting for their application in risk prediction. In the ALL-BFM 2000 study group the presence of *NOTCH1* mutations correlated with excellent long-term outcome (Breit et al., 2006). This result was confirmed by some studies (Asnafi et al., 2009; Malyukova et al., 2007; Park et al., 2009), but contradicted by others (Baldus et al., 2009; Larson Gedman et al., 2009; Mansour et al., 2009; van Grotel et al., 2008; Zhu et al., 2006).

1.5 Aim of the present study

The aim of this study was to analyse important pathways in the pathogenesis of T-ALL. A fundamental mechanism in leukaemogenesis is the aberrant up-regulation of the NOTCH1 pathway. Understanding the underlying mechanisms and the accompanying effects of NOTCH1 signalling in T-ALL could have fundamental implications for T-ALL therapies.

In the first part of this study, the clinical potential of the activated NOTCH pathway was determined to develop diagnostic and treatment strategies. Specifically, the predictive value of *NOTCH1* and *FBXW7* mutations on the prognosis of paediatric T-ALL patients was characterised. The analysis was performed in a T-ALL patient cohort at first presentation and in patients with a relapse.

Further, the regulation of miRNAs by the NOTCH1 pathway was investigated in paediatric T-ALL. MiRNAs acting as oncogenes or tumour suppressors in several types of cancer could be potential players in T-ALL malignancy. To address the question of a possible role of miRNAs in T-ALL, a cell-based assay was set up in which miRNA expression was monitored upon NOTCH1 knockdown. Moreover, miRNA expression profiles from paediatric T-ALL patients were examined depending on the NOTCH1 mutational status. In addition, the miRNA signatures were tested for their prognostic usefulness.

2. Experimental procedures

2.1 Materials

2.1.1 Frequently used reagents and chemicals

Glycine, Tris-(hydroxymethyl)-methylamine (Tris), sodium chloride (NaCl), methanol, ethanol, isopropanol and sodium dodecyl sulphate (SDS) were from Carl Roth GMBH (Karlsruhe, Germany). Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute-1640 (RPMI-1640), Opti-MEM®, fetal bovine serum (FBS), non-essential amino acids (NEAA), sodium pyruvate and penicillin/streptomycin (Pen/Strep) were all from Gibco BRL div. of Invitrogen (Karlsruhe, Germany). Hexadimethrine bromide (Polybrene), diethylaminoethyl (DEAE)-dextran, paraformaldehyde (PFA) and chlorophorm were obtained from Sigma-Aldrich GmbH (Taufkirchen, Germany). Bacto™ Agar, Bacto™ Yeast Extract, Bacto™ Tryptone, cell strainers and FACS tubes were from BD Biosciences (Franklin Lakes, NJ, US). Cell culture multi-well plates, dishes, flasks and falcons were obtained from Sarstedt (Nümbrecht, Germany). PVDF membranes were from GE Healthcare (Little Chalfont, UK), PBS from PAA laboratories (Pasching, Austria), ABsolute™ QPCR SYBR® Green Capillary mix from Thermo Scientific Inc. (Waltham, MA, US), *Trans*IT®-LT1 Transfection Reagent from Mirus Bio (Madison, WI, US), Cryovials from Nunc (div. of Thermo Fisher Scientific Inc., Waltham, MA, US), Steriflip-HV Filter from Millipore (Billerica, MA, US), ultracentrifuge tubes from Beckman Coulter (Brea, CA, US), QIAzol Lysis Reagent from Qiagen (Hilden, Germany), Biocoll separating solution from Biochrom AG (Berlin, Germany) and TRIzol® from Invitrogen (Karlsruhe, Germany).

2.1.2 Enzymes

The restriction enzymes were obtained from New England Biolabs (NEB) (Ipswich, MA, USA) and were used according to the NEB guidelines. The reverse transcriptase, RevertAid™ H Minus M-MuLV, was from MBI Fermentas (Burlington, Ontario, Canada). PCR reactions were performed with Taq Polymerase or with HiFi Taq Polymerase (Roche).

2.1.3 Buffers, Solutions and Media

All buffers and solutions used in this study are listed in table 2.1.

Buffers and Solutions	**Preparation**
Antibiotic solutions (1000x)	100 mg/ml ampicillin in H_2O 50 mg/ml kanamycin in H_2O
40 mM 6-amino-4-hexanoic acid	0.01% (w/v) SDS pH 9.4
DNA loading buffer (6x)	0.9% (w/v) bromophenol blue 60% (v/v) glycerol 60 mM EDTA pH 8.0
Luria-Bertani (LB) agar (autoclaved)	15 g/l Bacto™ Agar in LB broth
LB broth (autoclaved)	10 g/l Bacto™ Tryptone 5 g/l Bacto™ Yeast extract 5 g/l NaCl pH 7.6 (adjusted with 5 N NaOH)
TBE (10x)	0.89 M Tris base, pH 8.3 0.89 M boric acid
Paraformaldehyde (PFA) 1%	1 g PFA up to 100ml PBS filter-sterilise
Growth medium (T cell line)	10% FBS 1% Penicillin/Streptomycin in RPMI
Growth medium (293FT)	10% FBS 1% Penicillin/Streptomycin 1x Non essential amino acids 1% Sodium Pyruvate in DMEM
Diethylaminoethyl-Dextran (DEAE-Dextran) 5 mg/ml	5 mg DEAE-Dextran up to 1 ml PBS filter-sterilise
Hexadimethrine bromide (Polybrene) 6 mg/ml	6 mg Polybrene up to 1 ml H_2O filter-sterilise

Table 2.1 **List of buffers and solutions**

2.1.4 Kits

Listed below in table 2.2 are the kits that were used in this work. Unless stated otherwise, the manufacturer's recommendations were followed.

Application	Name	Supplier
Plasmid DNA extraction from agarose gels	NucleoBond Xtra Midi® , NucleoSpin Plasmid ®	Macherey-Nagel, Düren, Germany
PCR clean-up	NucleoSpin Extract II ®	Macherey-Nagel, Düren, Germany
DNA preparation from E.coli	NucleoSpin Extract II ®	Macherey-Nagel, Düren, Germany
RNA extraction	miRNeasy ®	Qiagen, Hilden, Germany
genomic DNA extraction	QIAamp ®	Qiagen, Hilden, Germany

Table 2.2 **List of kits**

2.1.5 Plasmids

A description of the plasmids used and their respective sources are documented in table 2.3. Plasmids that were kindly provided by other laboratories are indicated as such. The integrity of all DNA constructs employed in the experimental studies was verified by forward and reverse DNA sequencing (GATC biotech AG, Konstanz, Germany).

Plasmid Name	Template	Insert	Fluorophore	Source
MISSION® Control Vector pLKO.1-puro CMV-TurboGFP™	pLKO.1-puro	-	Turbo-GFP™	Sigma-Aldrich
MISSION® Non-Target shRNA Control Vector	pLKO.1-puro	Non-Target shRNA	-	Sigma-Aldrich
MISSION® pLKO.1-puro-NOTCH1 shRNA1	pLKO.1-puro	NOTCH1 shRNA	-	Sigma-Aldrich
MISSION® pLKO.1-puro-NOTCH1 shRNA2	pLKO.1-puro	NOTCH1 shRNA	-	Sigma-Aldrich

MISSION® pLKO.1-puro-NOTCH1 shRNA3	pLKO.1-puro	NOTCH1 shRNA	-	Sigma-Aldrich
MISSION® pLKO.1-puro-NOTCH1 shRNA4	pLKO.1-puro	NOTCH1 shRNA	-	Sigma-Aldrich
MISSION® pLKO.1-puro-NOTCH1 shRNA5	pLKO.1-puro	NOTCH1 shRNA	-	Sigma-Aldrich
pMD2.G		VSVG	-	Dr Violaine Rosenstiel-Goidts (DKFZ)
psPAX2		gag, rev, pol	-	Dr Violaine Rosenstiel-Goidts (DKFZ)

Table 2.3 **List of plasmids**

2.1.6 Oligonucleotides

All primers were purchased from Sigma-Aldrich (Taufkirchen, Germany) or biomers.net (Ulm, Germany). PCR primers were obtained in desalted, lyophilized form, and were diluted in H2O. All primers used in this work are listed in table 2.4.

Sequencing of genes (see 2.2.2.1)

Genes	FW 5'-3'	RV 5'-3'
NOTCH1 HD-N	agccccctgtacgaccagta	gagggcccaggagagttg
NOTCH1 HD-C	gtggcgtcatgggcctca	tagcaactggcacaaacagc
NOTCH1 TAD	agactggcccacctcgtctct	gctctccactcaggaagctc
NOTCH1 PEST	aaacatccagcagcagcaaa	aaaggaagccggggtctc
FBXW7 exon9	ccaggccagagctatcataac	agacaaaacgctatggctttcc
FBXW7 exon10	aaccttgactaaatctaccatgtt	ctggatcagcaatttgacagtg

Sequencing of plasmids (see 2.2.2.1)

Plasmid	FW 5'-3'	RV 5'-3'
pLKO.1-puro	ggactatcatatgcttaccgt	tctttcccctgcactgtacc

quantitative PCR (see 2.2.1.10.1)

mRNA/miRNA	FW 5'-3'	RV 5'-3'
ACTG1	tacagcttcaccaccacggc	tacccaggaaggaaggctgg
NOTCH1	tgcactgcgaggtcaacac	caggcacttggcaccattc
HES1	aacacgacaccggataaacc	cgagtgcgcacctcggta
primary ACTG1	tttccccagtcattttctgc	gccagtgtgatgtgtggaga
pri-miR-17~92	gtggggattgtgaccagaag	gcaaccccaaaagtgaaatg
pri-miR-491	gtggggaatggggaattaag	gtgctaacatcgagcagtgg

miQPCR (see 2.2.1.10.2)

mature miRNA	FW 5'-3'	RV 5'-3'
hsa-miR-106a	aaaagtgcttacagtgcaggtagg	linker primer (patented)
hsa-miR-106b	taaagtgctgacagtgcagatgg	linker primer (patented)
hsa-miR-15a	gcagcacataatggtttgtgg	linker primer (patented)
hsa-miR-16	tagcacgtaaatattggcgg	linker primer (patented)
hsa-miR-17	caaagtgcttacagtgcaggtagg	linker primer (patented)
hsa-miR-181a	caacgctgtcggtgagtg	linker primer (patented)
hsa-miR-181b	cattgctgtcggtgggtg	linker primer (patented)
hsa-miR-181c	cattcaacctgtcggtgagtg	linker primer (patented)
hsa-miR-181d	acattgttgtcggtgggtg	linker primer (patented)
hsa-miR-18a	taaggtgcatctagtgcagataggg	linker primer (patented)
hsa-miR-18b	taaggtgcatctagtgcagttagg	linker primer (patented)
hsa-miR-19a	tgtgcaaatctatgcaaaactgag	linker primer (patented)
hsa-miR-20a	taaagtgcttatagtgcaggtaggg	linker primer (patented)

hsa-miR-20b	caaagtgctcatagtgcaggtagg	linker primer (patented)
hsa-miR-223	tgtcagttgtcaaataccccagg	linker primer (patented)
hsa-miR-25	tgcacttgtctcggtctgag	linker primer (patented)
hsa-miR-363	aattgcacggtatccatctgtagg	linker primer (patented)
hsa-miR-491-3p	cttatgcaagattcccttctacgg	linker primer (patented)
hsa-miR-92a	cttgtcccggcctgtg	linker primer (patented)
hsa-miR-92b	cccggcctccgg	linker primer (patented)
hsa-miR-93	gtgctgttcgtgcaggtagg	linker primer (patented)
RNU6	gcttcggcagcacatatactaa	linker primer (patented)

Table 2.4 **List of oligonucleotides**

2.1.7 Bacterial strains

For propagation of vectors, the heat shock competent *E.coli* strain, XL1 blue, (La Jolla, CA, USA) was used. The genotype of this strain is the following: hsdR17, supE44, recA1, endA1, gyrA96, thi, relA1, lac/F´[proAB+ lacIa, lacZΔM15:Tn10(TetR)].

2.1.8 Cell lines

The virus producer cell line 293FT was obtained from Invitrogen. Cells were cultured in flasks at 37°C, 5% CO_2 in Dulbecco's minimal essential medium (DMEM), supplemented with 10% foetal bovine serum (FBS), 1% antibiotics (penicillin/streptomycin), 1x non-essential amino acids (NEAA) and 1% Sodium Pyruvate.

The T cell line (CCRF-CEM) was purchased from DMSZ (Braunschweig, Germany). Cells were cultured in flasks at 37°C, 5% CO_2 in Roswell Park Memorial Institute-1640 (RPMI-1640), supplemented with 10% foetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin).

2.2 Methodologies

2.2.1 Molecular Biology

2.2.1.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used to selectively amplify particular DNA segments for the mutation sequencing screen. The reaction mixture was typically for a final volume of 50 µl consisting of: DNA polymerase buffer, 1.5 mM MgCl2, 5% DMSO, 0.2 µM of each primer (sense and anti-sense), 0.2 mM dNTP, 2 units of DNA polymerase and 50 ng of template DNA in dH_2O. This PCR mixture was used to amplify plasmids, exons 27 (HD-N) and 34 (PEST domain) of *NOTCH1* and exons 9 and 10 of *FBXW7*. For PCR amplification exons 28 (HD-C) and 34 (TAD domain) of *NOTCH1*, betaine 5 M was added in addition to the previous mixture.

A typical temperature program was as follows:

10’	95°C	denaturation	1 cycle
30”	95°C	denaturation	40 cycles
30”	57°C	annealing	
1’	72°C	elongation	
10’	72°C	final extension	1 cycle

The PCR products were separated using gel electrophoresis to estimate the quality and the quantity. For DNA sequencing, PCR products were sent to GATC biotech AG, Konstanz, Germany.

2.2.1.2 Transformation of bacteria

Fifty nanograms of plasmid DNA were mixed with 100 µl of the transformation-competent *E.coli* strain, XL1 blue, incubated on ice for 30 min. The cells are heat-shocked for 90 s at 42°C on ice and subsequently incubated on ice for 3 min. 600 µl of LB medium was added and the mixture was incubated for 1h at 37°C. The transformed bacteria were then spread on LB agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

2.2.1.3 Bacterial culture and isolation of plasmid DNA

Single bacterial clones were incubated in 200 ml LB medium containing the appropriate antibiotic in the shaker, overnight at 37°C. Cells were pelleted by centrifugation at 4500 g for 15 min, 4°C in a 5415R centrifuge (Eppendorf). To prepare large amounts of plasmid DNA, the NucleoBond Xtra Midi® kit was used according to the manufacturer's instructions. Lentiviral vectors were treated as low copy-number plasmids.

2.2.1.4 Ficoll-Hypaque density gradient centrifugation

Fresh blood samples were collected at the "blood bank", INF 305, Heidelberg, Germany. The samples were diluted with an equal volume of 1x PBS and mixed by inversion. This mix was overlaid slowly with an equal volume of Ficoll reagent in a 50 ml Falcon tube and subsequently centrifuged without brake at 800 g for 20 min at room temperature in a 5810R centrifuge from Eppendorf. The interphase (white cell layer containing the lymphocytes) wass carefully transferred into a fresh 50ml Falcon tube. 30 ml of 1x PBS were added, mixed by inversion and centrifuged at 500 g for 10 min. The supernatant was discarded and the cell pellet was washed in 20 ml 1x PBS. The cells were centrifuged at 500 g for 5 min. The remaining cell pellet was resuspended in 2 ml 1x PBS to determine the cell number. The cells were collected once more by centrifugation at 500 g for 5 min, and resuspended in TRIzol® reagent. Resuspended cells in TRIzol® were stored at -20°C or total RNA was extracted directly from these samples.

2.2.1.5 Genomic DNA extraction

To extract genomic DNA, the QIAamp® kit (Qiagen) was used according to the manufacturer's instructions.

2.2.1.6 Determination of DNA concentration and quality

DNA concentration was determined using the Biophotometer®, Eppendorf (Hamburg, Germany). Sequencing of all DNA constructs and all PCR products was performed to confirm the sequences by GATC Biotech AG (Konstanz, Germany). Sequence analysis was performed by Chromas Lite (Technelysium Pty Ltd) or Mutation Surveyor™ (Softgenetics, US).

2.2.1.7 DNA agarose gels

DNA samples mixed with DNA loading buffer were loaded on 1% agarose (w/v) gels containing ethidium bromide (EtBr). 250 ng of a 100 bp or a 1 kb DNA ladder used as a size marker allowed the estimation of the size of DNA fragments. Agarose gels were usually run at 120V, and visualised using a UV transilluminator (Herolab UVT-28 ME).

2.2.1.8 Total RNA extraction

Two RNA extraction methods were used in this study. For both methods, cells were lysed in QIAzol Lysis Reagent and stored at -20°C until RNA extraction. In the first protocol (named miRNeasy protocol), the miRNeasy Mini® kit was used according to the manufacturer's instructions. To prepare total RNA using the second method (named TRIzol® method), chloroform is added (1/5 of TRIzol® volume) to the tube, and shaken for 15 sec. After 3 min of incubation at room temperature, the mix is centrifuged at 12000 g for 15 min at 4°C. Three different phases were formed. The upper aqueous phase was carefully transferred into a fresh tube. For RNA precipitation, isopropanol was then added (1/2 volumes of TRIzol® volume) and the mix centrifuged at 12000 g for 15 min at 4°C. The pellet formed was washed using 1 ml of ethanol 75% and spun again for 10 min (12000 g; 4°C). The supernatant was discarded completely, and the pellet was resuspended in $H2O_{bidest}$ by incubating the tubes for 5 min at 50°C.

2.2.1.9 Determination of RNA concentration and quality

RNA concentration was measured using the Biophotometer®, Eppendorf (Hamburg, Germany). To check the RNA quality, 1 µl of sample was run on the 2100 Bioanalyzer using the RNA Nano 6000 kit (Agilent, Santa Clara, US) according to the manufacturer's instructions.

2.2.1.10 cDNA synthesis and quantitative PCR

2.2.1.10.1 Quantitative PCR for mRNA

For quantitative PCR (qPCR), 1 µg of total RNA was used for reverse transcription (RT). The RNA and 1 µl of random primers (0.2 µg/µl) were denatured at 70°C for 10min, and cooled down on ice for 2min. The reverse transcription reaction mixture contained a total volume of 25 µl consisting of RT buffer, 0.4 mM dNTPs, 200 units of RevertAid H Minus M-MuLV Reverse

Transcriptase, 1 µl random primers (0.2 µg/µl) and 1 µg of denatured total RNA. The mix was incubated at 42°C for 90 min, then at 70°C for 10min for stopping the reaction. After cDNA clean-up using the NucleoSpin Extract II ®, the cDNA was eluted in 50 µl H2O. For quantitative PCR (qPCR), the reaction mix (20 µl) contained ABsolute™ QPCR SYBR® Green Capillary mix, 0.4 µM of the forward and reverse primers and 2 µl of cDNA. The qPCR mixture was run on a StepOnePlus™ from Applied Biosystems (ABI) according to the following thermocycle protocol:

10'10''	95°C	denaturation	1 cycle
3''	95°C	denaturation	40 cycles
10''	60°C	annealing	
20''	72°C	extension	
15'	95°C	final extension	1 cycle
1'	60°C		1 cycle
60°C to 95°C		dissociation step	

Threshold cycles (C_t) were defined as the fractional cycle number at which the fluorescence passed the fixed threshold. C_t values were extracted by using the 7500 software v2.0.1 (ABI) and calculations for normalisation and analysis were done in Excel (Microsoft Office).

2.2.1.10.2 miQPCR

For miQPCR (patent application EP 09 002 587.5), total RNA was first dephosphorylated for 30 min at 37°C, followed by 5 min at 90°C. The reaction mixture for a final volume of 3.5 µl contained 500ng of total RNA, 10 U calf intestinal phosphatase (CIP; Ambion) and CIP buffer. Total RNA was then tailed to a linker adaptor by mixing the dephosphorylated RNA with NEB buffer, 17% PEG, 2.4mM ATP, 20 U RNase Inhibitor (NEB), 40 U T4 RNA ligase (NEB) and 5 µM RNA linker in a total volume of 15 µl. The mix was incubated for 120 min at 37°C, followed by 5 min incubation at 85°C. To reverse transcribe (RT) the RNA, the mix was incubated with 10 mM dNTP and 100 µM of Upm3 primer (total volume 22 µl) for 3 min at 80°C, then cooled down on ice for 5 min. A mix of 7 µl, containing RT buffer and 0.1M DTT, was added, and incubated at 48°C for 3 min. The cDNA synthesis was completed by incubating the reaction mix with 200 U superscript II

(Invitrogen), for 60 min at 48°C. The reaction was stopped by incubating the mix for 10min at 70°C. 1 µl of RNase H (NEB) was added, and incubated for 30 min at 37°C. The cDNA was then diluted with 215 µl of H_2O. For each qPCR reaction, 5 ng of cDNA were used. cDNAs were amplified by using 0.5 µM of miRNA- and linker- specific primers and SYBR Green (Applied Biosystems (ABI)). The mixture was run on a ABI 7500 instrument. The amplification protocol was as follows:

2'	50°C		1 cycle
10'	95°C	denaturation	1 cycle
15''	95°C	denaturation	50 cycles
1'	60°C	annealing and extension	
60°C to 95°C		dissociation step	1 cycle

Threshold cycles (C_t) were defined as the fractional cycle number at which the fluorescence passed the fixed threshold. C_t values were extracted by using the 7500 software v2.0.1 (ABI) and calculations for normalization and analysis were done in Excel (Microsoft Office).

2.2.2 Cell culture

2.2.2.1 Freezing and thawing cell lines

Confluent 293FT and CCRF-CEM cells were washed in PBS and detached briefly with 1ml trypsin/EDTA. After adding 10 ml of medium, cells were centrifuged at 120 g for 4 min. The cell pellet was washed in 10 ml PBS, and centrifuged at 120 g for 4 min. The pellet was carefully resuspended in 1 ml DMEM (293FT), or RPMI-1640 (CCRF-CEM), containing 10% DMSO. The cell suspension was transferred into a cryovial and frozen at -80°C overnight. The vial was then transferred to a liquid nitrogen tank for storage. For thawing cells, a frozen vial was immediately transferred from liquid nitrogen into a water bath at 37°C until completely thawed. The cells were subsequently transferred into a 50ml falcon containing 10ml fresh medium, and centrifuged at 120 g for 4min. The cell pellet was taken up in medium and transferred into a 75cm^2 flask. 24h after thawing, the medium was replaced to remove all traces of DMSO.

2.2.2.2 Lentiviral production

The 293FT cell line was cultured in growth medium in 175cm^2 flasks to a 50-70% confluence. Transfection of DNA constructs was carried out using *Trans*IT®-LT1 Transfection Reagent. 100 µl of *Trans*IT®-LT1 Reagent was diluted in 6 ml of Opti-MEM® and incubated for 10 min at room temperature. A mixture of 20 µg psPAX2, 10 µg pMD2.G and 20 µg of a lentiviral construct (pLKO.1-puro) was added to the diluted *Trans*IT-LT1 Reagent, mixed gently by inversion and incubated for 20 min at room temperature while the growth medium was changed to 20 ml fresh growth medium. The *Trans*IT-LT1 Reagent-DNA complex was added dropwise to the cells and the flask gently rocked back and forth. The transfected 293FT cells were incubated 48h at 37°C, 5% CO_2. Growth medium was harvested in 50 ml falcon tubes after 48h and centrifuged at 800 g for 5 min. The supernatant was filtered using a 0.45 µm Steriflip-HV Filter (SE1M003M00, Millipore) and stored in a sealed bag at 4°C overnight. 25ml fresh growth medium was added to the cells which were incubated for another 24 hours at 37°C, 5%CO_2. After 72h post-transfection, growth medium was harvested, centrifuged and the supernatant filtered. The filtered medium was transferred into Beckman ultracentrifuge tubes, and centrifuged for 2h, at 24000 rpm at 4°C in a Beckman Optima L-70 ultra-speed centrifuge (rotor SW28). The supernatant was decanted and the viral particles taken up in approximately 200 µl PBS. The concentrated viral particles were distributed equally into 3 cryovials and stored at -80°C until further usage.

2.2.2.3 Lentiviral titration

10^5 of 293FT cells were plated overnight in 2 ml growth medium in each well of a 6-well plate. Growth medium was then replaced by 1 ml of fresh growth medium containing 6 µg/ml polybrene. 1 µl of concentrated viral particles was added to 1 well (1 ml). To achieve 10-fold serial dilutions of the viral particles, 100 µl of the diluted particles were added to the next well. The dilutions ranged from 10^{-3} to 10^{-7}. 24h post-transduction the growth medium containing the viral particles was removed and replaced by fresh medium. 72h post-transduction cells were fixed in PFA 1% (see 2.2.2.5). Cells were taken up in 1 ml of PBS, passed through a 70 µm cell strainer and collected in FACS tubes. The titration was measured by BD FACScan™ Flowcytometer measuring GFP (BD Biosciences, Franklin Lakes, US) (see 2.2.2.6).

2.2.2.4 Lentiviral transduction

For each lentiviral transduction, $2x10^5$ CCRF-CEM cells were added to 1ml growth medium (RPMI, 10% foetal bovine serum (FBS), 1% antibiotic (penicillin/streptomycin)) containing 8µg/ml DEAE. Between $4x10^7$ and $7x10^7$ TU/µl (transducing units/µl; see 2.2.2.7) were added to the each well. A 30 min centrifugation at 200 g improved the binding of the virus to the surface of the cells. Cells were resuspended in the same medium, plated in 6-well plates and incubated for 24h at 37°C, 5% CO_2. At 24h post-transduction, the growth medium was replaced by 2ml fresh growth medium. At 72h, 120h and 168h post-transduction, the number of cells was counted for each transduction by using the Neubauer counting chamber. The GFP expressing (GFP^+) cells were fixed (see 2.2.2.5) and scanned using the BD FACScan™ Flowcytometer (BD Biosciences, Franklin Lakes, US) to estimate the transduction efficiency (see 2.2.2.6). All other cells were suspended in QIAzol Lysis Reagent and stored at -20°C.

2.2.2.5 Cell fixation

Cells were washed in 1x PBS and trypsinized for 5 min. Cells taken up in 1x PBS were then collected by centrifugation at 200 g, 4 min and fixed in 100 µl PFA 1%. For fixation, cells were incubated 10min at room temperature. 1.5 ml of PBS was added and cells were centrifuged again at 200 g, 4min.

2.2.2.6 Determination of transduction efficiency

Cells expressing GFP were detected by the BD FACScan™ Flowcytometer (BD Biosciences, Franklin Lakes, US). To determine the number of GFP^+ cells, the CellQuest™ software version 3.3 from Becton Dickinson was used according to the manufacturer's instructions.

2.2.2.7 Calculating the lentiviral titre

The transducing titre is expressed in transducing units (TU). To calculate TU, the following formula was used:

$$\mathrm{TU}/\mu\mathrm{l} = \frac{\frac{P}{100} \times C}{V}$$

where P = average (% GFP^+ cells in dilution 10^n; 10 x % GFP^+ cells in dilution 10^{n-1}); C = number of cells at time of transduction; V = µl of viral supernatant added (dilution 10^n).

To measure the percentage of GFP^+ cells, the BD FACScan™ Flowcytometer was used. The CellQuest™ version 3.3 software from Becton Dickinson determined the percentage. From the dilutions ranging from 10^{-3} to 10^{-7}, the 2 samples were used that contained a measurable number of GFP^+ cells, but that were non-saturated (<95% of GFP^+ cells). Lentiviral titres varied between $2x10^5$ and $5x10^5$ TU/µl.

2.2.3 MiRNA microarray analysis

Sample preparation, hybridisation and chip scanning were done by Sabine Schmidt and Mirco Castoldi at EMBL. MiChip image analysis was done by loading the chip images into Genepix Pro version6.0 software (Axon Instruments) generating raw "gpr files".
The raw miRNA microarray files concerning the knock-down experiments were analysed using the MiChip package (http://www.bioconductor.org/packages/2.6/bioc/html/MiChip.html; by Jonathon Blake, EMBL) on the Bioconductor software. This package is based on a whole chip median normalisation and includes a set of functions for loading data from several MiChip hybridisations, flag correction, filtering and summarising the data. The normalised output files were loaded into Excel. The ratio was calculated of each experiment by dividing the NOTCH1 shRNA sample to the corresponding non-target shRNA (scramble) sample. For the 72h experiments, these ratios were then analysed for differential gene expression analysis by T-test ($p<0.02$ for both NOTCH1 shRNA experiments). The fold change was calculated by dividing the mean of the NOTCH1 shRNA samples by the mean of the scramble samples. From the generated list of candidate miRNAs were filtered out those that were not or lowly expressed. This list was further filtered by comparing if the same trend was observed also in the pool of experiments at 120h.

The miRNA microarray data from the T-ALL patient samples were analysed using two normalisation methods. In Figures 4.6 and 4.7, the MiChip package was used. In Figure 4.8 a normalisation based on a linear model was employed by Wolfgang Huber (EMBL) and Julia Engelmann (Regensburg).
For hierarchical clustering (HCL), the MiChip-normalised data was uploaded into TIGR MeV 4.0 (MultiExperimentViewer; http://www.tm4.org; Wang et al., 2008).

2.2.4 Statistical analyses

Differences in the distribution of prednisone response and MRD kinetics measurements among patient subsets were analysed using the Fisher's exact test. Estimated probability of 5-years EFS (pEFS) and estimated cumulative incidence of relapse (pCIR) analyses (at first presentation) were done by Martin Zimmermann, Hanover. Estimated probability of 5-years EFS (pEFS) analyses (at relapse) were done by Jana Hof, Berlin. The pEFS curves include all events (relapse, second malignancy, death in continuous complete remission and death before complete remission).

3. Results 1: The role of *NOTCH1* and *FBXW7* mutations as molecular biomarkers in paediatric T-ALL

In precursor B-cell lymphoblastic leukaemia some prognostic markers have been identified that are of clinical relevance (hyperdiploidy, hypodiploidy and specific translocations e.g. t(9;22)) (Fletcher et al., 1992; Martinez-Climent, 1997; Trueworthy et al., 1992). However, in paediatric T-cell leukaemia such valuable markers are still missing. One of the purposes of this work is to study the possible use of *NOTCH1* and *FBXW7* mutations as prognostic biomarkers in paediatric T-ALL, both at initial presentation and at relapse.

3.1 *NOTCH1* and *FBXW7* mutations in paediatric T-ALL at initial presentation

It has been reported that 50% of children with T-ALL carry *NOTCH1* activating mutations (Weng et al., 2004). The first report, in which the potential of *NOTCH1* mutations was investigated, was undertaken in the context of the ALL-BFM 2000 study group (Breit et al., 2006). This study showed that children with *NOTCH1* mutations showed an improved long-term outcome compared to those without *NOTCH1* mutations. We now extended the study to 301 patients enrolled in the ALL-BFM 2000 study, the largest cohort of paediatric T-ALL samples analysed to date.

3.1.1 Frequency of *NOTCH1* and *FBXW7* mutations in paediatric T-ALL at initial presentation

Of 301 T-ALL patients, 30 (10%) carried mutations in both genes, *FBXW7* and *NOTCH1*, 120 (40%) harboured *NOTCH1* mutations only, and 12 (4%) contained *FBXW7* mutations only (Figure 3.1). All mutations were heterozygous.

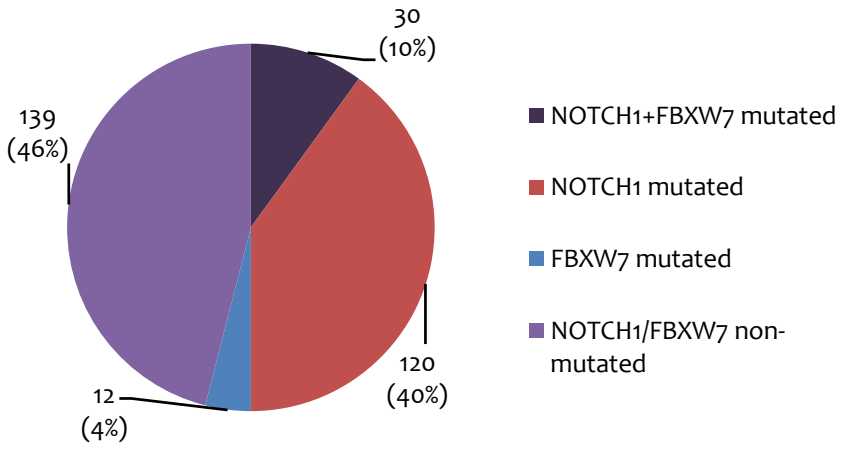


Figure 3.1 **Frequency of *NOTCH1* and *FBXW7* mutations in childhood T-ALL at initial presentation**

In the 150 patients (50%) with *NOTCH1* mutations, 94 children (31.2%) had a mutation in the heterodimerisation domain (HD), 29 (9.6%) in the TAD-PEST domain, and 27 (9 %) in both. All *NOTCH1* mutations affecting these domains are hypothesised to result in an aberrant up-regulation of NOTCH1 signalling (Weng et al., 2004).

FBXW7 mutations, found in 42/301 patients (14%), were all located in exons 9 and 10, coding for the WD repeats 2 to 4 (Figure 1.5). The D domain in the N-terminal part of the protein is unaffected by the mutations and functions properly. By sequestering non-mutated FBXW7 proteins of the normal allele, the truncated FBXW7 is hypothesised to function as dominant-negative. The frequency of mutations is similar to results published by other study groups (Baldus et al., 2009; Malyukova et al., 2007; Weng et al., 2004).

3.1.2 *NOTCH1* mutations correlate with improved early treatment response and a better long-term outcome

Patients were grouped according to their *NOTCH1* mutational status, and were compared in relation to their early treatment response (assessed by prednisone response on day 8 and MRD kinetics on days 33 and 78). Prednisone good response (PGR) was measured in 102/149 patients (68%) with *NOTCH1* mutations, but only in 73/145 (50%) patients without *NOTCH1* mutations (p=0.002) (table 3.1).

MRD measurements on day 33 were available for 267 patients (138 *NOTCH1* mutated, 129 *NOTCH1* non-mutated) and for 274 patients on day 78 (140 *NOTCH1* mutated and 134 *NOTCH1* non-mutated). 47/138 (34%) *NOTCH1* mutated patients and 23/129 (18%) *NOTCH1* non-mutated patients showed a favourable response on day 33 (p=0.003). On day 78, 103/140 (74%) of the *NOTCH1* mutated patients showed a favourable response compared to only 58/134 (43%) patients without *NOTCH1* mutations (p<0.0001).

	Prednisone Response (Day 8) *			
	PPR (%)	**PGR (%)**	**Data not available**	**P‡**
NOTCH1 mutated	47 (32)	102 (68)	1	p=0.002
NOTCH1 non-mutated	72 (50)	73 (50)	6	
	MRD (Day 33) †			
	Unfavourable (%)	**Favourable (%)**	**Data not available**	**P‡**
NOTCH1 mutated	91 (66)	47 (34)	12	p= 0.003
NOTCH1 non-mutated	106 (82)	23 (18)	22	
	MRD (Day 78) †			
	Unfavourable (%)	**Favourable (%)**	**Data not available**	**P‡**
NOTCH1 mutated	37 (26)	103 (74)	10	p<0.0001
NOTCH1 non-mutated	76 (57)	58 (43)	17	

Table 3.1 **_NOTCH1_ mutations associate with favourable early treatment response in paediatric T-ALL.**
***** PPR: ≥1000 leukaemic blood blasts/µL on treatment day 8; PGR: <1000/µL.
† Unfavourable MRD: $\geq 10^{-4}$; Favourable MRD: $<10^{-4}$.
‡ P-value; Fisher's Test.

Similar results were obtained for long-term outcome with a pEFS of 87% in the *NOTCH1* mutated groups, but only 74% in the non-mutated group (p=0.005; Figure 3.2A). Moreover, a difference in the relapse rate was observed, being of 7% in the mutated group, and of 17% in the non-mutated group (p=0.007; Figure 3.2B). In summary, the present analysis of a large group of patients confirms that activating *NOTCH1* mutations correlate strongly with improved early treatment response and with excellent long-term outcome.

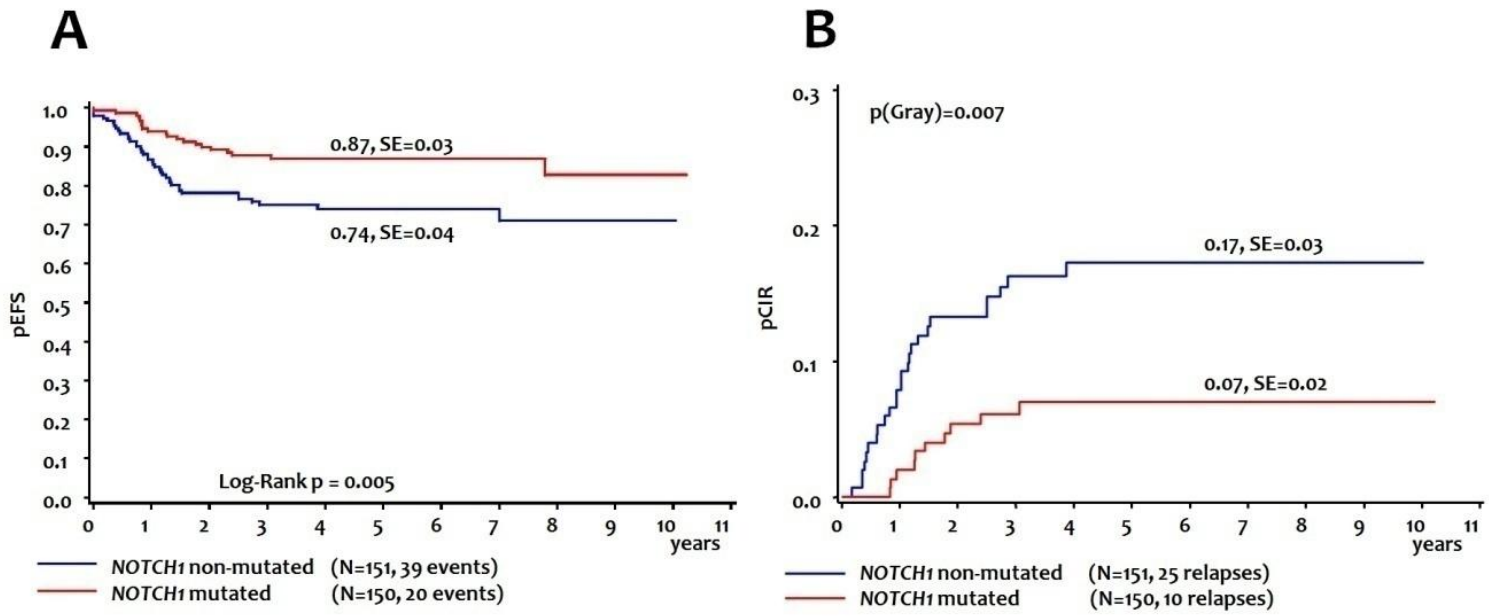


Figure 3.2 **_NOTCH1_ mutations correlate with excellent long-term outcome in T-ALL.**
(A) Kaplan-Meier estimate of the probability of event-free survival (pEFS) in *NOTCH1* mutated and *NOTCH1* non-mutated patients. (B) Cumulative incidence of relapse (pCIR) in *NOTCH1* mutated and *NOTCH1* non-mutated patients

In addition, initial CNS involvement was analysed in the total cohort of T-ALL patients. Clinical data on the presence of initial CNS involvement were available in 254 patients. Twelve out of 127 (9%) patients with *NOTCH1* mutations showed initial CNS involvement compared to 18/127 (12%) in the *NOTCH1* non-mutated group. The incidence of relapses with CNS involvement was also similar between *NOTCH1* mutated and non-mutated patients, both in the entire group (pCIR 2%±1% vs 4%±2%), and in the high risk subgroup of patients (pCIR 3%±3% vs 5%±3%). Overall, the rate of CNS-involved relapses was low.

3.1.3 The favourable effect of *NOTCH1* mutations is restricted to the standard and intermediate risk groups

The next aim was to analyse the effect of *NOTCH1* mutations on pEFS and relapse in the different risk groups. These groups are defined in the ALL-BFM 2000 protocol by the *in vivo* early treatment response of the patients. The standard risk patients (SR) show a PGR and negative MRD measurements on days 33 and 78. The high risk group (HR) is defined by a PPR, a MRD

response ≥10^{-3} on day 78, or both. All remaining patients are stratified into the intermediate risk group (MR).

The SR/MR groups encompassed 168 patients. The *NOTCH1* mutated patients showed an excellent pEFS of 95% compared to only 83% in the *NOTCH1* non-mutated patients (p=0.02; Figure 3.3A). A trend towards a lower relapse rate in the *NOTCH1* mutated group (pCIR 0.04), compared to the *NOTCH1* non-mutated patients (pCIR 0.11), was observed (p=0.11; Figure 3.3B). Interestingly, no significant difference in pEFS could be observed in the HR group between *NOTCH1* mutated (72%) and non-mutated patients (66%; p=0.49; Figure 3.3C). Although non-significant, a trend towards a lower relapse rate in the *NOTCH1* mutated group (pCIR 0.12) was observed, compared to the *NOTCH1* non-mutated group (pCIR 0.23; p=0.13; Figure 3.3D). In contrast, the rate of non-relapse events is slightly higher in the *NOTCH1* mutated group (8/51) than in the non-mutated group (9/82; Table 3.2).

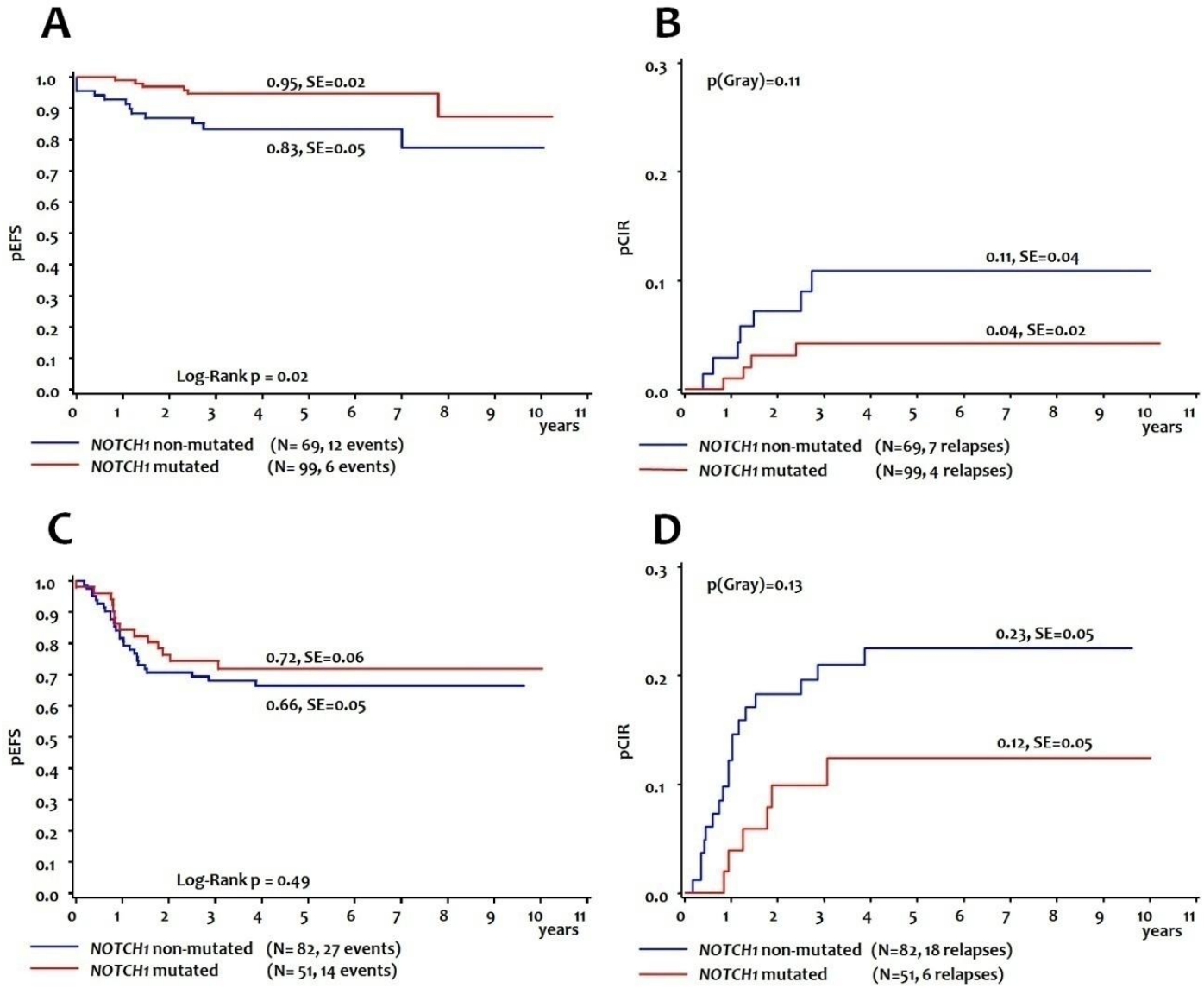


Figure 3.3 **The favourable effect of *NOTCH1* mutations on long-term outcome is restricted to the standard and intermediate risk groups.**
(A) Kaplan-Meier estimate of the probability of event-free survival (pEFS) in *NOTCH1* mutated and *NOTCH1* non-mutated SR/MR patients. (B) Cumulative incidence of relapse (pCIR) in *NOTCH1* mutated and *NOTCH1* non-mutated SR/MR patients. (C) Kaplan-Meier estimate of the probability of event-free survival (pEFS) in *NOTCH1* mutated and *NOTCH1* non-mutated HR patients. (D) Cumulative incidence of relapse (pCIR) in *NOTCH1* mutated and *NOTCH1* non-mutated HR patients.

In conclusion, the favourable effect of *NOTCH1* mutations on long-term survival is restricted to patients characterised already by a good response to treatment protocol of the ALL-BFM 2000.

	SR/MR		HR	
	***NOTCH1* mutated**	***NOTCH1* non-mutated**	***NOTCH1* mutated**	***NOTCH1* non-mutated**
Non-relapse Event	**2**	**5**	**8**	**9**
Death in CCR*	0	1	7	8
Death before CR†	0	3	1	0
Second malignancy	2	1	0	1
Relapse	**4**	**7**	**6**	**18**
All Events	**6**	**12**	**14**	**27**

Table 3.2 **List of relapse and non-relapse events in the SR/MR and HR groups**
* Death in CCR (continuous complete remission)
† Death before CR (complete remission)

3.1.4 *FBXW7* inactivating mutations correlate with early treatment response but do not predict favourable late treatment response or better long-term outcome

Previously, inactivating *FBXW7* mutations have been identified in paediatric T-ALL, although in a lower frequency than *NOTCH1* mutations. The *FBXW7* mutations are mainly found in the WD40 repeats which mediate substrate recognition. In patients with *FBXW7* mutations, it is hypothesised that NOTCH1, as a FBXW7 target, is not degraded, so that NOTCH1 signalling remains active. Therefore, it is plausible that these patients have similar clinical characteristics and that both types of mutations synergise to sensitise leukaemia cells for treatment.

Prednisone response was available for all the patients with *FBXW7* mutations and in 252/259 patients without *FBXW7* mutations. PGR was observed in 37/42 patients (88%) in the *FBXW7* mutated group compared to 138/252 (55%) in the non-mutated group (p=0.0003; Table 3.3). Sixteen out of 39 *FBXW7* mutated patients showed a favourable MRD response on day 33 compared to 174/228 in the non-mutated group (p=0.03; Table 3.3). On day 78, however, only a non-significant trend could be observed; favourable MRD was observed in 28/39 patients with *FBXW7 mut*ations and 133/235 patients without *FBXW7 mut*ations (p=0.08; Table 3.3).

The predictive value of *FBXW7* mutations was lost at long-term outcome; both groups have a similar pEFS (76% in the *FBXW7* mutated group versus 81% in the *FBXW7* non-mutated group) (p=0.48; Figure 3.4A) and a similar pCIR (10% compared to 13%, p=0.6, Figure 3.4B).

	Prednisone Response (Day 8) *			
	PPR (%)	**PGR (%)**	**Data not available**	**P‡**
FBXW7 mutated	5 (12)	37 (88)	0	p=0.00003
FBXW7 non-mutated	114 (45)	138 (55)	7	
	MRD (Day 33) †			
	Unfavourable (%)	**Favourable (%)**	**Data not available**	**P‡**
FBXW7 mutated	23 (59)	16 (41)	3	p= 0.03
FBXW7 non-mutated	174 (76)	54 (24)	31	
	MRD (Day 78) †			
	Unfavourable (%)	**Favourable (%)**	**Data not available**	**P‡**
FBXW7 mutated	11 (28)	28 (72)	3	p=0.08
FBXW7 non-mutated	102 (43)	133 (57)	24	

Table 3.3 **_FBXW7_ mutations associate with excellent prednisone response, but have only marginal effects on MRD kinetics.**
***** PPR: ≥1000 leukaemic blood blasts/µL on treatment day 8; PGR: <1000/µL.
† Unfavourable MRD: $\geq 10^{-4}$; Favourable MRD: $<10^{-4}$.
‡ P-value; Fisher's Test.

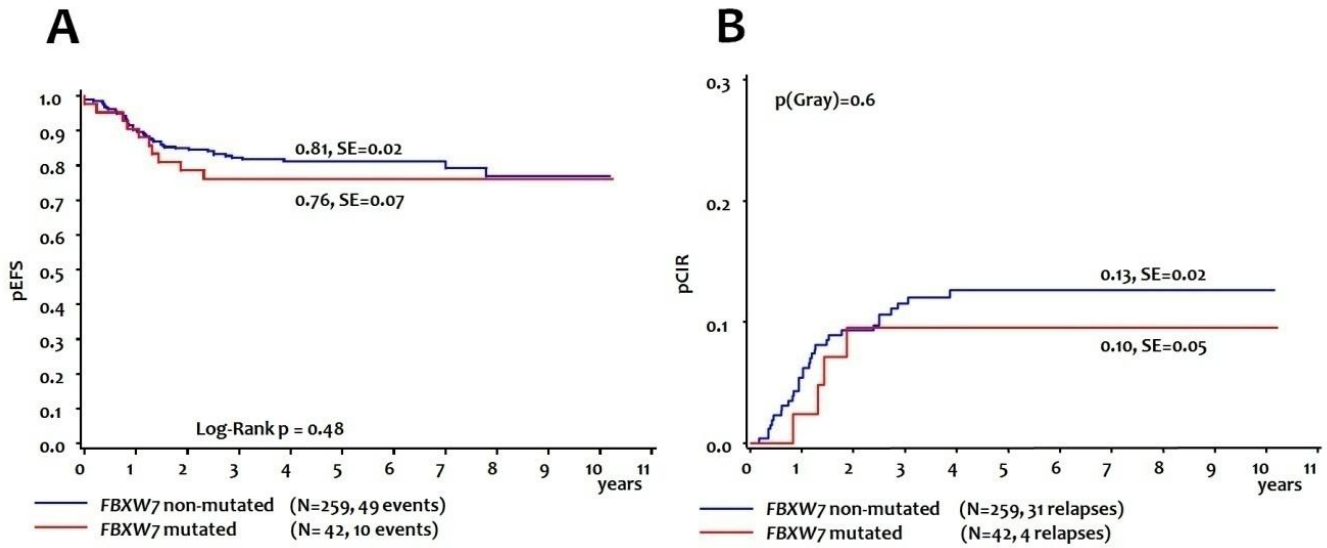


Figure 3.4 **_FBXW7_ mutations do not associate with better long-term outcome in T-ALL.**
(A) Kaplan-Meier estimate of the probability of event-free survival (pEFS) in *FBXW7* mutated and *FBXW7* non-mutated patients. (B) Cumulative incidence of relapse (pCIR) in *FBXW7* mutated and *FBXW7* non-mutated patients.

In summary, the data indicate that *FBXW7* mutations confer a high sensitivity to very early treatment in the ALL-BFM 2000 protocol (administration of prednisone (or dexamethasone) and intrathecal methotrexate). In contrast, they do not affect the response to drugs used later on during induction, intensification and re-induction.

3.1.5 The combination of *NOTCH1* and *FBXW7* mutations results in excellent early treatment response but does not signify a more favourable long-term outcome

It was previously observed that *FBXW7* mutations and *NOTCH1-PEST* mutations are mutually exclusive. In our cohort only one out of 30 patients showed this combination. This almost complete mutual exclusivity suggests that no additional selective advantage is provided by a *NOTCH1-PEST* and *FBXW7* double mutation and that the biological function in T-ALL of both types of mutation is similar.

In adult T-ALL, it has already been reported that *FBXW7* mutations add significant prognostic value to *NOTCH1* mutational status. It is therefore possible that both types of mutations may synergise clinically, also in the context of the paediatric ALL-BFM study.

Prednisone response was available for 294 patients from the total cohort. Twenty-eight out of 30 (93%) patients with *NOTCH1* and *FBXW7* mutations showed a PGR compared to 74/139 (62%) in the *NOTCH1* mutated only group and 9/12 (75%) in the *FBXW7* mutated only group. Only 64/133 (48%) of cases with no mutations in *NOTCH1* or *FBXW7* presented a PGR.

On day 33, the group with double mutations had the highest number of cases with a favourable MRD (16/29; 55%) compared to the *NOTCH1* mutated group with 31/109 (28%) patients and the group without mutations with 23/119 (19%). Conversely all 10 cases with only *FBXW7* mutations showed unfavourable MRD. On day 78, the lowest MRD loads were present in the groups with mutations in both genes (24/30; 80%) and with *NOTCH1* mutations alone (79/110; 72%). Four out of 9 patients with only *FBXW7* mutations (44%) and 54/125 (43%) patients with neither mutation showed a favourable MRD (Table 3.4).

	Prednisone Response (Day 8) *			
	PPR (%)	**PGR (%)**	**Data not available**	**P‡**
NOTCH1+FBXW7 mutated	2 (7)	28 (93)	0	p<0.00001
NOTCH1 mutated	45 (38)	74 (62)	1	p=0.03
FBXW7 mutated	3 (25)	9 (75)	0	p=0.13
NOTCH1/FBXW7 non-mutated	69 (52)	64 (48)	6	

	MRD (Day 33) †			
	Unfavourable (%)	**Favourable (%)**	**Data not available**	**P‡**
NOTCH1+FBXW7 mutated	13 (45)	16 (55)	1	p=0.0003
NOTCH1 mutated	78 (72)	31 (28)	11	p=0.12
FBXW7 mutated	10 (100)	0 (0)	2	p=0.21
NOTCH1/FBXW7 non-mutated	96 (81)	23 (19)	20	

	MRD (Day 78) †			
	Unfavourable (%)	**Favourable (%)**	**Data not available**	**P‡**
NOTCH1+FBXW7 mutated	6 (20)	24 (80)	0	p=0.0004
NOTCH1 mutated	31 (28)	79 (72)	10	p=0.00001
FBXW7 mutated	5 (56)	4 (44)	3	p=1.00
NOTCH1/FBXW7 non-mutated	71 (57)	54 (43)	14	

Table 3.4 **The combination of *NOTCH1* and *FBXW7* mutations result in an excellent prednisone response and early but not late MRD.**
***** PPR: ≥1000 leukaemic blood blasts/µL on treatment day 8; PGR: <1000/µL.
† Unfavourable MRD: $\geq 10^{-4}$; Favourable MRD: $<10^{-4}$.
‡ P-value; Fisher's Test.

The groups with mutations in both genes and *NOTCH1* mutations alone show similar event-free survival (83% versus 88%) and relapse rates (8% versus 6%). The group of patients with *FBXW7* mutations includes only 12 patients, and therefore does not allow meaningful statistics. This group shows a poor long-term outcome (pEFS 58%) with 5 non-relapse events, and only 1 relapse. In terms of relapse rates, the groups with *NOTCH1* or *FBXW7* mutations do not show statistically significant differences. Patients with no mutations show a pEFS of 75%, and a high pCIR of 18% (21/139; Figure 3.5).

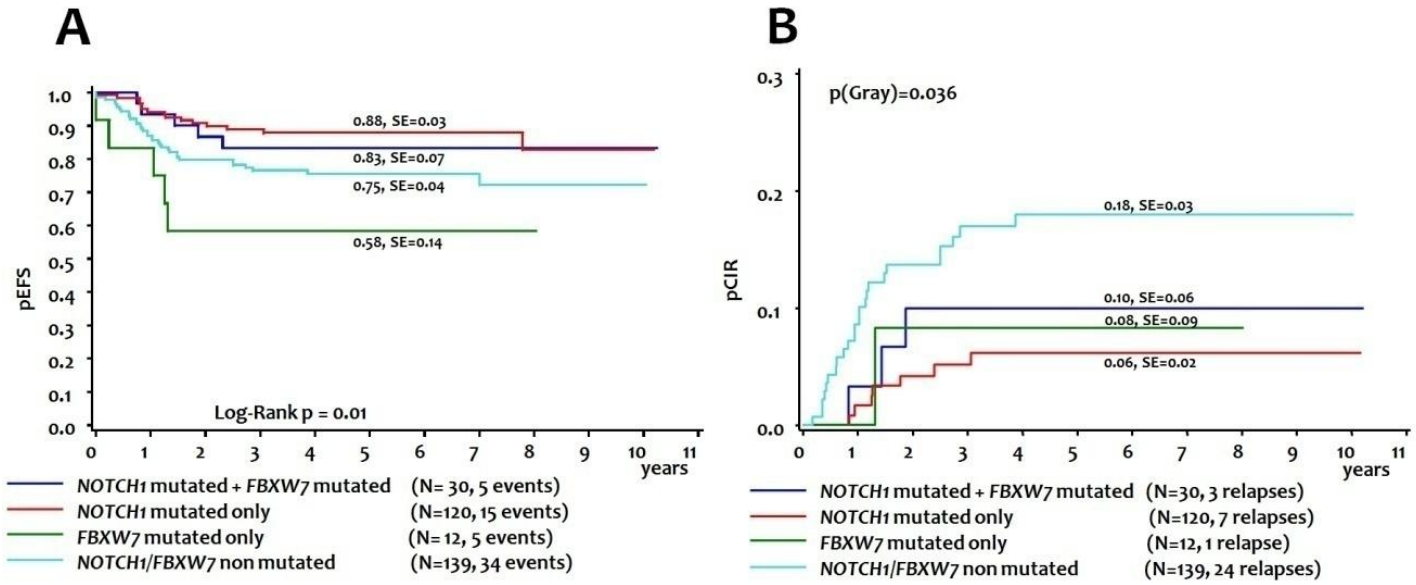


Figure 3.5 **_NOTCH1_ and _FBXW7_ mutations do not act together to predict a favourable long-term outcome.**
(A) Kaplan-Meier estimate of the probability of event-free survival (pEFS) in *NOTCH1+FBXW7* mutated (dark blue), *NOTCH1* mutated (red), *FBXW7* mutated (green) and *NOTCH1/FBXW7* non-mutated (light-blue) patients. (B) Cumulative incidence of relapse (pCIR) in *NOTCH1+FBXW7* mutated (dark blue), *NOTCH1* mutated (red), *FBXW7* mutated (green) and *NOTCH1/FBXW7* non-mutated (light blue) patients.

In summary, *NOTCH1* and *FBXW7* mutations synergise clinically during early treatment response in the ALL-BFM 2000 treatment protocol. In contrast, *FBXW7* mutations do not add predictive value to the *NOTCH1* mutations at long-term outcome of paediatric T-ALL patients.

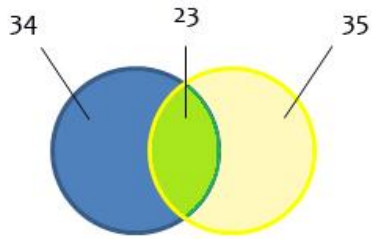
3.2 *NOTCH1* and *FBXW7* mutations in paediatric T-ALL at relapse

Having shown in the previous part that, at initial presentation, *NOTCH1* mutations, but not *FBXW7* mutations, correlate with good prognosis in T-ALL, it was now analysed if these mutations associate with long-term outcome at relapse.

3.2.1 Clinical characteristics of the cohort of relapsed T-ALL patients

In the cohort of 301 T-ALL patients analysed at initial diagnosis (Figure 3.1), 35 had a relapse (Figure 3.2B). From these 35 children, relapse material was available in 23 cases. In addition, 21 patients with a relapse were analysed (Figure 3.6A). The total relapse cohort (n=34) was treated at initial presentation according to the ALL-BFM 2000 protocol and at relapse according to the ALL-REZ 2002 protocol (including the corresponding pilot protocol). To determine if this cohort is a representative relapse group, the clinical characteristics of the 34 ALL-REZ 2002 treated patients were compared to the relapsed patients from the first presentation study (n=35; Figure 3.2). Striking is the inclusion of only 18/34 (53%) very early relapses in the analysed cohort, whereas the very early group from the ALL-BFM 2000 represented 74% (26/35; Figure 3.6B). This recruitment bias was probably based on the very early T-ALL relapses not being treated according to the ALL-REZ protocol, but only in a palliative fashion. Another discrepancy was the presence of 8 (23%) patients that developed recurrent disease after allogeneic BMT (bone marrow transplantation) in the ALL-BFM group, compared to none of the patients in the analysed cohort. Most relapsed patients with allogeneic BMT receive palliative care, which explains the absence of this group of patients in our relapse analysis.

A



B

	ALL-REZ 2002 n=34 (%)	ALL-BFM 2000 n=35 (%)
Time-point of relapse *		
very early	18 (53)	26 (74)
early	7 (21)	5 (14)
late	9 (26)	4 (12)
On/Off treatment †		
on	23 (68)	22 (63)
off	11 (32)	5 (14)
after allogeneic BMT	0	8 (23)
Site of relapse		
Isolated BM	22 (65)	18 (52)
Isolated extramedullary	1 (3)	7 (20)
Combined BM	11 (32)	10 (28)
First presentation study		
ALL-BFM 2000	34 (100)	35 (100)
Relapse study		
ALL-REZ 2002 (including pilot study)	34 (100)	17 (49)
Other	0	18 (51)

Figure 3.6 **Clinical characterisation of the cohort of T-ALL patients with a relapse.**
(A) Venn diagram of the overlap of relapses occurring in the initial presentation study group (n=35, yellow, Figure 3.2) and relapses analysed in this study (n=34, blue). Twenty-three patients with a relapse were represented in both studies (green).
(B) Comparison of the clinical characteristics of the cohort of relapsed patients analysed in this study (n=34) with those of the patients with a relapse (n=35) enrolled in the ALL-BFM 2000 study group at initial presentation (Figure 3.2B).
***** Time of relapse: very early (≤18 months after initial diagnosis), early (≥18 months after initial diagnosis and <6 months after completion of initial therapy), late (≥6 months after completion of initial therapy);
† On/off treatment: on (relapse during treatment of initial T-ALL); off (relapse after treatment of initial T-ALL); after allogeneic BMT (after allogeneic bone marrow transplantation).

3.2.2 Frequency of *NOTCH1* and *FBXW7* mutations in paediatric T-ALL at relapse

First, the *NOTCH1* and *FBXW7* mutational status was determined in the T-ALL patient samples at relapse. Five out of 34 (15%) patients harboured mutations in both genes, *NOTCH1* and *FBXW7*, 13 (38%) were mutated in only *NOTCH1* and 2 (6%) had *FBXW7* mutations only. All other 14 (41%) did not carry any mutations in either *NOTCH1*, or *FBXW7* (Figure 3.7A).

When compared to the frequency of mutations at initial presentation (n=301; Figure 3.1), no major changes could be observed (Figure 3.7B). Intriguingly, it was previously stated (Figure 3.4B) that ALL-BFM 2000 treated patients, that develop a recurrent disease, are mostly *NOTCH1*/*FBXW7* non-mutated (24 *NOTCH1*/*FBXW7* non-mutated compared to 11 mutated). In the relapse cohort, however, 59% of the patients carried mutations in either *NOTCH1* or *FBXW7*. A plausible explanation for this finding could be that the cohort studied is not representative of the general relapse population, and a recruitment bias towards patients with mutations was introduced. Indeed, 19/24 (79%) relapses without *NOTCH1* or *FBXW7* mutations (in Figure 3.6B) were very early relapses. An additional explanation could be that, in non-mutated patients, new mutations were acquired at relapse.

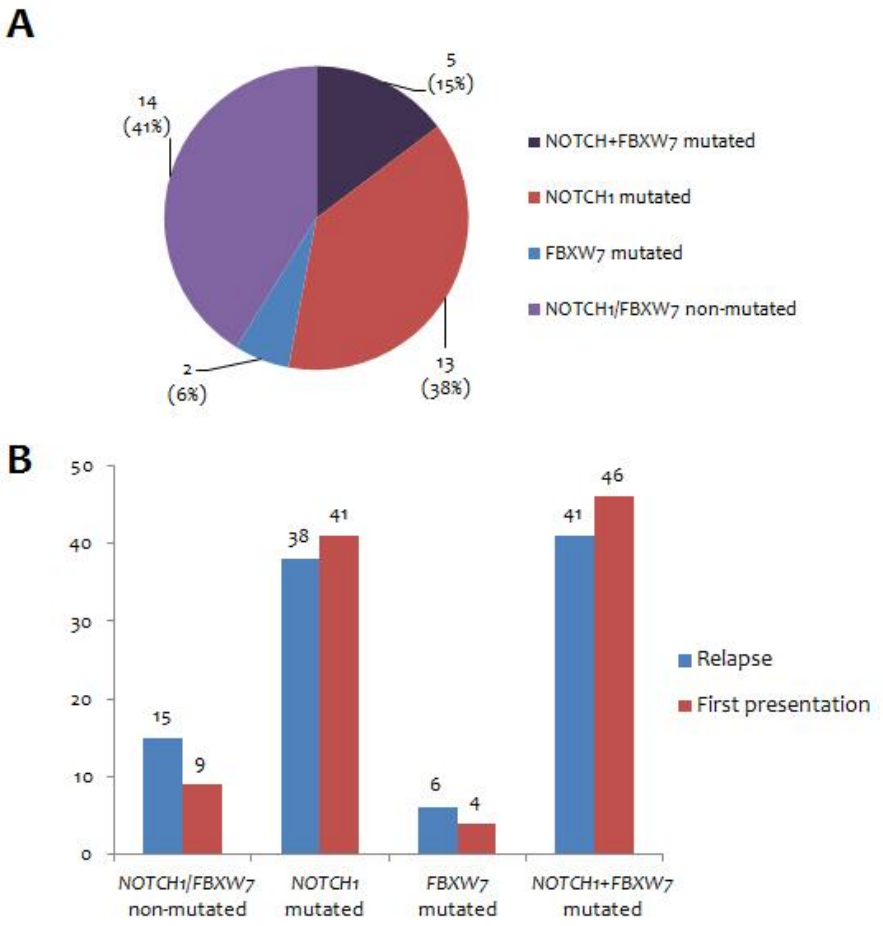


Figure 3.7 **Frequency of *NOTCH1* and *FBXW7* mutations in childhood T-ALL at relapse**
(A) Distribution of *NOTCH1* and *FBXW7* mutations in paediatric T-ALL patients at relapse. (B) Comparison of the frequency of *NOTCH1* and *FBXW7* mutations in initial presentation samples (n=301; Figure 3.1) and in relapse samples (n=35).

3.2.3 At relapse, *NOTCH1* or *FBXW7* mutations can be lost or newly acquired

In 23 cases, the *NOTCH1* and *FBXW7* mutational status could be determined in matched initial presentation-relapse samples. Depending on possible gains or losses of mutations, patients were categorised into 4 classes: no mutations at first presentation/relapse (class 1), the same mutation(s) at first presentation/relapse (class 2), loss of *NOTCH1* or *FBXW7* mutation at relapse (class 3) and gain of mutation at relapse, but absent at initial presentation (class 4).

Nine T-ALL patients did not present any mutations in *NOTCH1* or *FBXW7* at initial presentation or at relapse (class 1). The same mutational status was found in 5 patients (class 2). One patient had a *NOTCH1* and a *FBXW7* mutation in the initial T-ALL, and lost the *NOTCH1* mutation at relapse (class 3). Five patients lost mutations detected at first presentation, and simultaneously acquired novel

mutation(s) at relapse (classes 3 and 4). Three (12.5%) cases acquired novel mutations at relapse without losing those from the first T-ALL (class 4) (Table 3.5).

Patient no.	Initial Presentation *								Relapse *								Class †
	HD-N	HD-C	TAD	PEST	**NOTCH1**	exon9	exon10	**FBXW7**	HD-N	HD-C	TAD	PEST	**NOTCH1**	exon9	exon10	**FBXW7**	
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
10	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	+	2
11	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	2
12	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	2
13	-	+	-	-	+	-	-	-	-	+	-	-	+	-	-	-	2
14	+	-	-	-	+	+	-	+	+	-	-	-	+	+	-	+	2
15	+	-	-	-	+	+	-	+	-	-	-	-	-	+	-	+	3
16	-	-	-	-	-	+	-	+	+	-	-	-	+	-	-	-	3 4
17	-	+	-	-	+	-	-	-	+	-	-	-	+	-	-	-	3 4
18	+‡	-	+	-	+	-	-	-	+‡	-	+	-	+	-	-	-	3 4
19	-	+	-	-	+	-	-	-	+	-	-	+	+	-	-	-	3 4
20	-	-	-	+‡	+	-	-	-	-	-	-	+‡	+	-	-	-	3 4
21	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	4
22	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	+	4
23	+	-	-	-	+	-	-	-	+	-	-	-	+	+	-	+	4

Table 3.5 **List of 23 pairs of initial presentation/relapse samples with the corresponding *NOTCH1* and *FBXW7* mutational status.**
***** Initial Presentation/Relapse: "-" = non-mutated; "+" = mutated;
† Class: 1 = no mutation at initial presentation and at relapse; 2 = same mutation at initial presentation and at relapse; 3 = loss of *NOTCH1* or *FBXW7* mutation at relapse; 4 = gain of mutation at relapse, but absent at initial presentation;
‡ Patients with a different mutation in the same protein domain at initial presentation and at relapse.

Classes 1 and 2 were further grouped into "*NOTCH1*/*FBXW7* mutational status unchanged" and classes 3 and 4 into "*NOTCH1*/*FBXW7* mutational status changed". Fourteen out of 24 (61%) cases did not present any changes in their *NOTCH1*/*FBXW7* mutational status; however 9 (39%) patients lost or acquired novel mutations at relapse. The instability of *NOTCH1* and *FBXW7* mutations in more than a third of the relapsed patients could suggest that a minor resistant sub-clone, already present, but undetectable at diagnosis, expands during the relapse. Another possibility is the

existence of a leukaemic stem cell that gives rise to a new clonogenic pool at relapse. However, further analyses are needed to differentiate between these hypotheses.

3.2.4 *FBXW7* mutations tend to predict poor long-term outcome in relapse T-ALL

To test whether *NOTCH1* and *FBXW7* mutations have a predictive value in relapsed T-ALL, they were correlated with long-term survival. From the cohort of 34 patients, 29 patients were taken into account for long-term analysis, as they were treated according to protocol. In the group of 29 patients, 15 were *NOTCH1* mutated and 14 *NOTCH1* non-mutated. As expected, the survival rate was very poor in the total group. However, no difference in pEFS was observed between the *NOTCH1* mutated and the *NOTCH1* non-mutated groups (33% vs 25%; p=0.16; Figure 3.8).

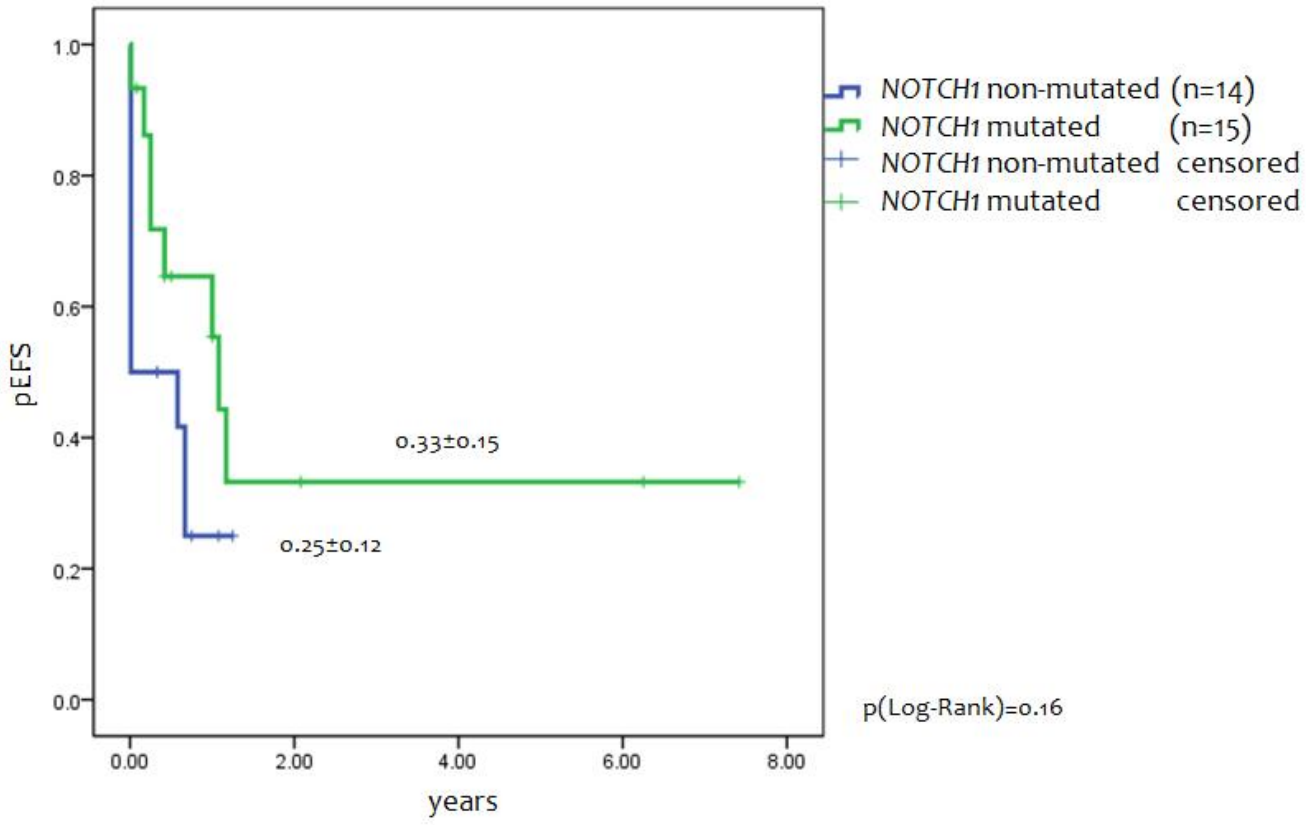


Figure 3.8 **No significant difference in long-term outcome at relapse was observed between patients with or without *NOTCH1* mutations.**
Kaplan-Meier estimate of the probability of event-free survival (pEFS) in *NOTCH1* mutated and *NOTCH1* non-mutated relapsed T-ALL patients.
Patients are censored when no event occurred (relapse, no response to treatment, etc.) and their observation time was shorter than 7.5 years.

In this group, seven children carried *FBXW7* mutations. Event-free survival was not significantly different, but a trend towards better long-term outcome of relapsed patients without *FBXW7* mutations was observed (35% and 14% in the *FBXW7* non-mutated and *FBXW7* mutated group, respectively; p=0.32; Figure 3.9).

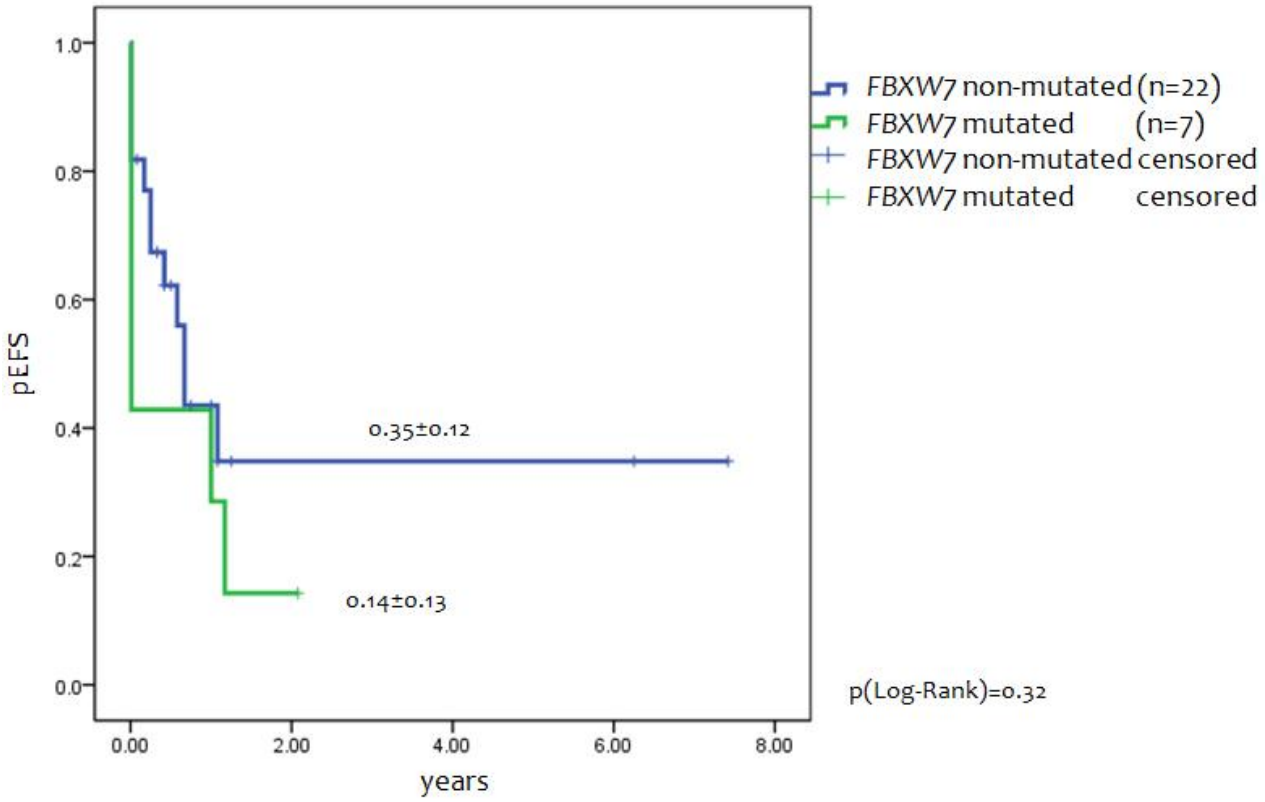


Figure 3.9 **_FBXW7_ mutations show a trend towards lower event-free survival.**

Kaplan-Meier estimate of the probability of event-free survival (pEFS) in *FBXW7* mutated and *FBXW7* non-mutated relapsed T-ALL patients.
Patients are censored when no event occurred (relapse, no response to treatment, etc.) and their observation time was shorter than 7.5 years.

When combining the mutational status of *NOTCH1* and *FBXW7*, four patients harboured mutations in both genes, eleven contained mutations only in *NOTCH1* and three had mutations only in *FBXW7*. The other eleven patients were not mutated in either *NOTCH1* or *FBXW7*. The groups with *NOTCH1* and *FBXW7* mutations, and with *NOTCH1* mutations alone showed a pEFS of 25% and 40%, respectively, whereas the group with no mutations in either gene had a pEFS of 32%. Strikingly, all patients with *FBXW7* mutations alone did not respond to the intensified treatment (p=0.028;

Figure 3.10). Although the statistical analysis is significant, a larger group of patients would be of value to strengthen the relevance of these data.

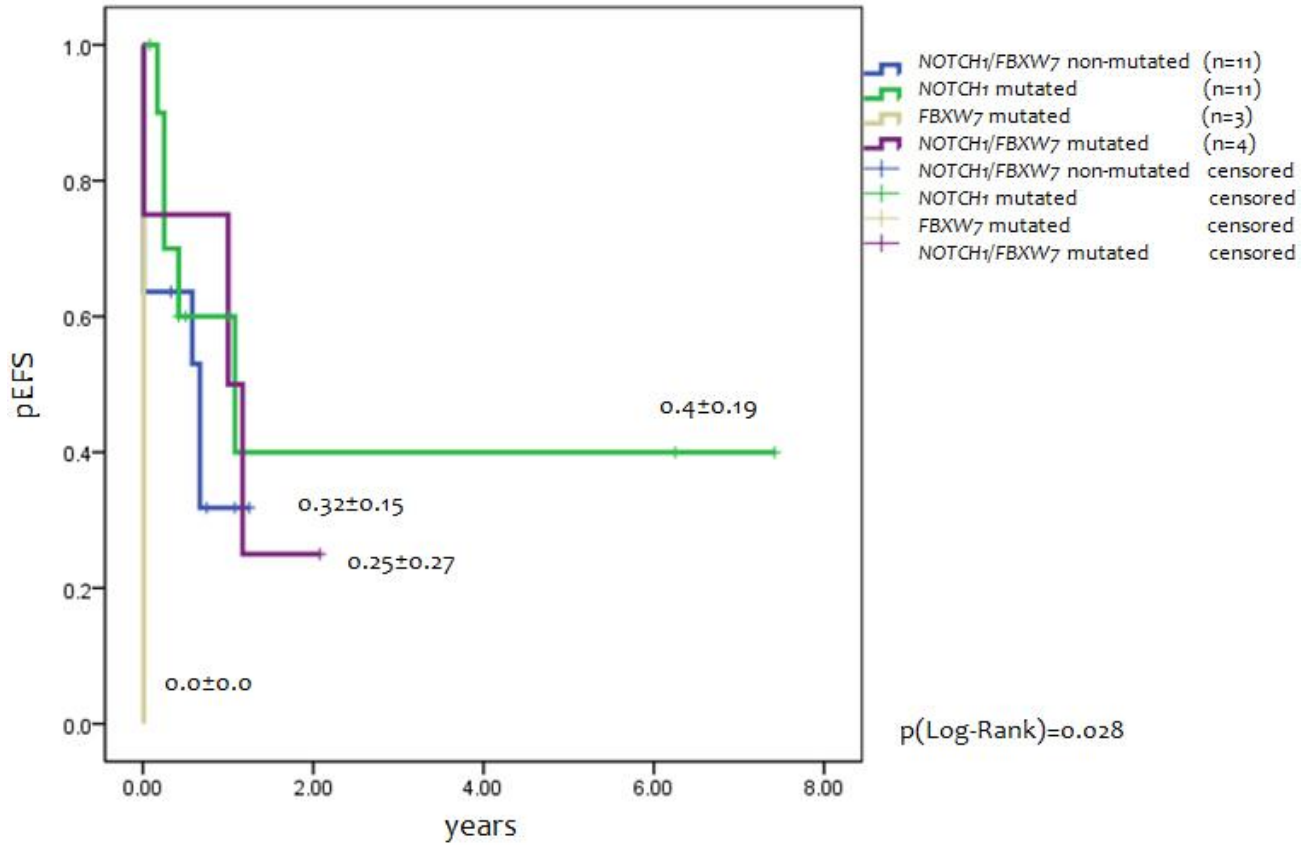


Figure 3.10 **Relapsed patients with *FBXW7* mutations alone do not respond to the intensified treatment.**
Kaplan-Meier estimate of the probability of event-free survival (pEFS) in *NOTCH1+FBXW7* mutated (violet), *NOTCH1* mutated (green), *FBXW7* mutated (grey) and *NOTCH1/FBXW7* non-mutated (blue) relapsed T-ALL patients.
Patients are censored when no event occurred (relapse, no response to treatment, etc.) and their observation time was shorter than 7.5 years.

In conclusion, the results obtained in this study give preliminary insights into the usefulness of *NOTCH1* and *FBXW7* mutations in predicting outcome of relapsed T-ALL patients. It would now be of importance to confirm these results in a larger patient group.

4. Results 2: Characterisation of miRNA expression in paediatric T-ALL

NOTCH1 is mutated in over 50% of all paediatric T-ALL patients. During the last years, research has been focussed on the identification of downstream effects of these activating mutations. A yet unexplored question is whether *NOTCH1* mutations affect miRNA expression. MiRNAs act as tumour suppressors or oncogenes and could thus be powerful downstream effectors. In this part of the thesis, it was explored if miRNA expression is under the control of NOTCH1 in paediatric T-ALL. In the first experimental setting, cell-based assays were employed to monitor miRNA expression upon NOTCH1 knock-down. A miRNA-specific microarray platform, miChip, allowed for direct analysis of miRNA expression (Castoldi et al., 2006). In a second step, miRNA expression in primary T-ALL patient samples was analysed depending on the *NOTCH1* mutational status of the patients.

4.1 miRNA expression is dependent on NOTCH1

Here it was analysed if the deregulation of NOTCH1 in T cell lines interfered with primary and mature miRNA expression. For our experiments, the CCRF-CEM T cell line was chosen because of its high endogenous NOTCH1 expression. Knock-down of NOTCH1 was established by using 5 different shRNAs targeting the mRNA of NOTCH1. Two shRNAs (shRNA2, shRNA3) were selected for further experiments on the basis of the highest knock-down efficiency. A non-target shRNA (scramble) was used as a negative control. To monitor the transduction efficiency, TurboGFP™ expression was measured. The shRNAs and TurboGFP™ were cloned in the lentiviral vector pLKO.1.

4.1.1 Knock-down of NOTCH1 decreases proliferation of a T cell line

After transduction, cells were harvested at 2 different time-points (72h and 120h). Firstly, NOTCH1 mRNA expression was analysed to determine the knock-down efficiency. NOTCH1 mRNA levels were decreased upon NOTCH1 knock-down, by approximately 45% after 72h and by 65% after 120h for both shRNAs (Figure 4.1A). To determine if the decrease of NOTCH1 mRNA has functional effects on downstream targets, HES1 mRNA levels were analysed. The expression levels of HES1 mirrored the NOTCH1 mRNA levels with a 40% decrease at 72h, and 50% at 120h (Figure 4.1B).

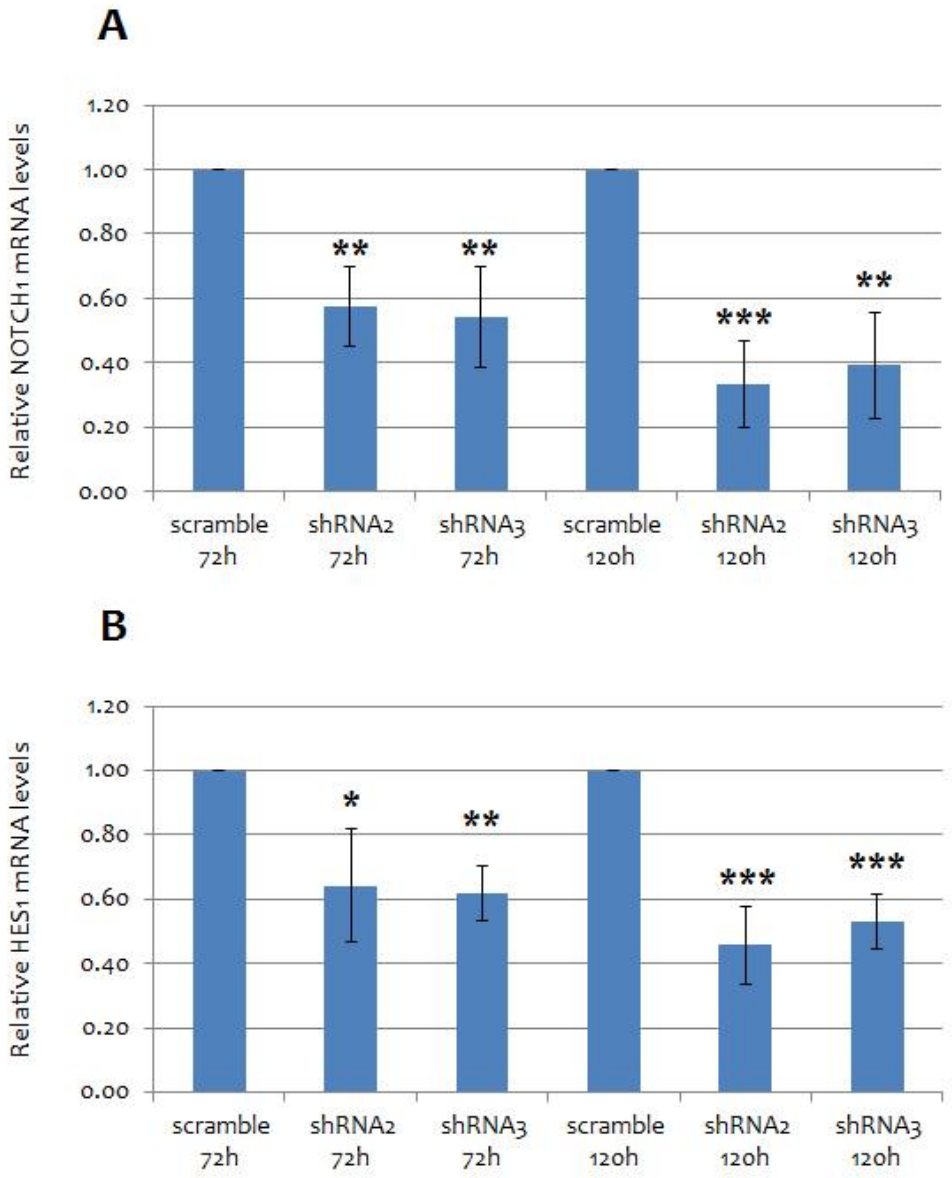


Figure 4.1 **NOTCH1 knock-down decreases NOTCH1 and HES1 mRNA levels.**
(A) NOTCH1 mRNA expression and (B) HES1 mRNA expression were detected by qPCR in CCRF-CEM cells that were transduced with either a non-target shRNA virus (scramble) or a NOTCH1 shRNA (shRNA 2, shRNA3) virus targeting the mRNA of NOTCH1, at 72h and 120h post-transduction.
The scramble sample was set to 1 (n=3; $p<0.05$ *; $p<0.01$ **; $p<0.001$ ***).

Protein levels could not be analysed due to insufficient protein material available for western blot. In parallel to mRNA expression analysis, the cell proliferation was measured at 3 different time points by counting the cells with the Neubauer counting chamber (72h, 120h, 168h) (Figure 4.2). The expression of shRNA2 and shRNA3 had drastic effects on the proliferation rates of CCRF-CEM. At 168h, the cell number diminished by 40% and by 60% upon NOTCH1 knock-down using shRNA2 and shRNA3, respectively. Altogether, these data show that the NOTCH1 knock-down is functional, and that NOTCH1 is essential for growth of CCRF-CEM.

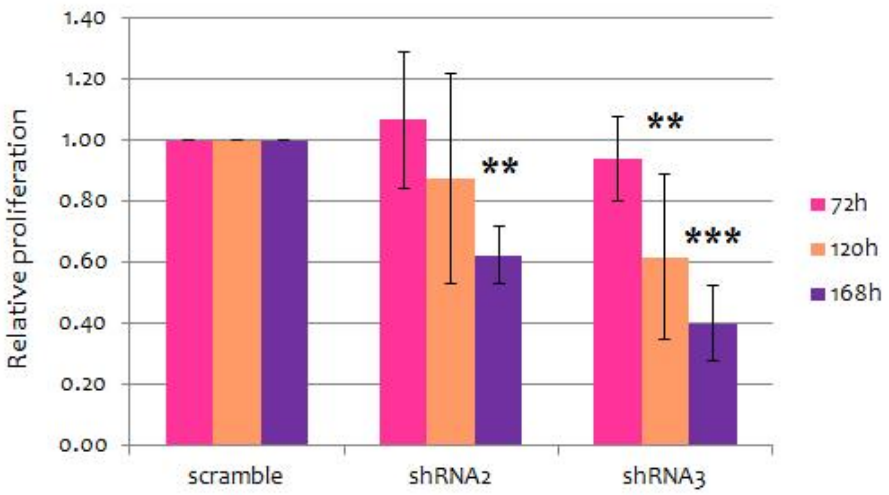


Figure 4.2 **NOTCH1 knock-down decreases proliferation in the CCRF-CEM cell line.**
The scramble sample (non-target shRNA) was set to 1. (n=6; p<0.01 **; p<0.001 ***)

4.1.2 MiRNAs are differentially expressed upon NOTCH1 knockdown

Next, it was examined if the NOTCH1 knock-down caused changes in the miRNA expression. Mature miRNA expression was monitored by miChip, a miRNA-specific microarray platform, and miQPCR, a miRNA-specific qPCR (Castoldi et al., 2006; Ibberson et al., 2009).

4.1.2.1 Mature miRNAs differentially expressed upon NOTCH1 knock-down

For monitoring mature miRNA expression, total RNA, harvested at 72h and at 120h from three independent NOTCH1 knock-down experiments, was hybridised on miChip. Total RNA extracted from the 72h time point was hybridised on miChip (9 chips), whereas total RNA from the 120h time point was pooled before hybridisation on miChip (3 chips). Raw data files were analysed using the miChip package in the Bioconductor software (Gentleman et al., 2004). Normalised data files were further analysed in Excel applying T-test and fold change calculations (Figure 4.3). Candidate miRNAs were chosen on the basis of different criteria:

(1) the miRNA expression was significantly up- or down-regulated at 72h ($p<0.02$) upon NOTCH1 knock-down using both shRNAs (shRNA2 and shRNA3);
(2) the same trend of regulation (up- or down-regulation at 72h) should be observed at 120h.

Based on the above described criteria, a list of 5 candidate miRNAs was generated: miR-18a, miR-18b, miR-181b, miR-15a and miR-491-3p. The 5 mature miRNAs were all down-regulated upon NOTCH1 knock-down (Figure 4.4).

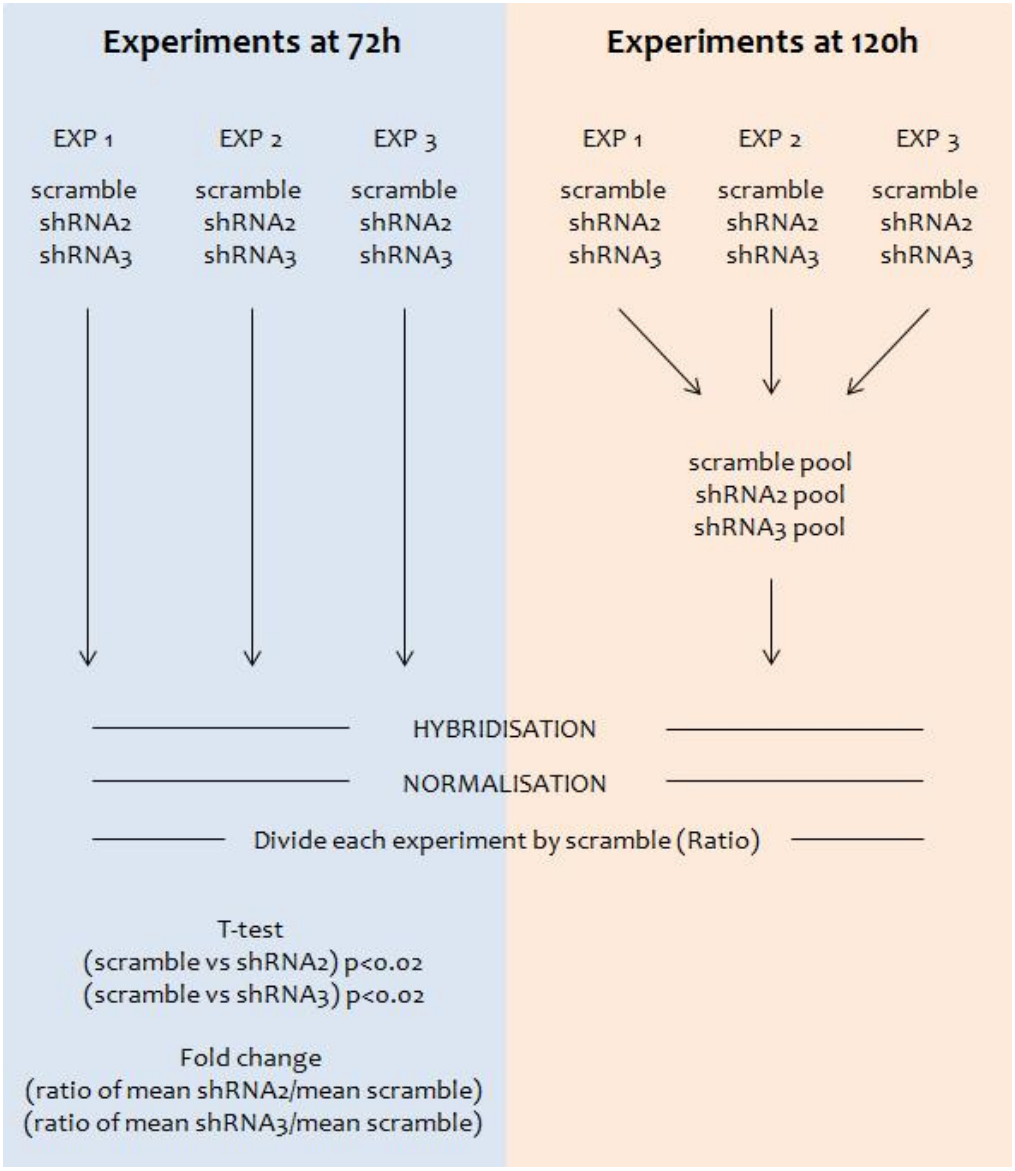


Figure 4.3 **Microarray workflow process**

A

	shRNA2 72h		shRNA3 72h	
mature miRNA	p-value (T-test)	Fold change	p-value (T-test)	Fold change
miR-15a	0.01	1.63 fold down	0.007	2.11 fold down
miR-181b	0.01	1.60 fold down	0.01	1.74 fold down
miR-18a	0.003	2.06 fold down	0.003	2.33 fold down
miR-18b	0.0006	1.88 fold down	0.01	1.96 fold down
miR-491-3p	0.0006	1.47 fold down	0.01	1.60 fold down

B

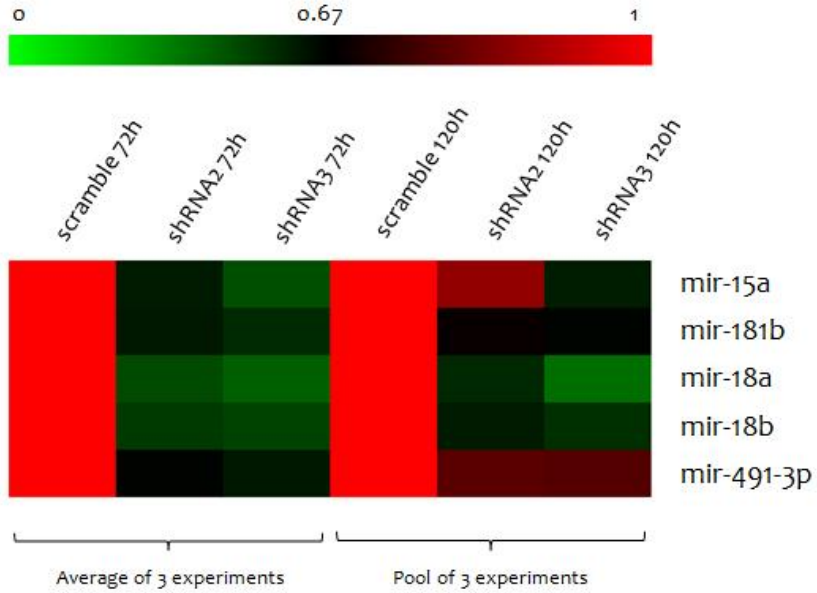


Figure 4.4 **Candidate miRNAs differentially expressed upon knock-down of NOTCH1.**
(A) p-values (T-test) were calculated comparing the miRNA expression at 72h in cells expressing the non-target shRNA and one of the NOTCH1 shRNAs (shRNA2 or shRNA3). The fold change was calculated by dividing the mean of the NOTCH1 shRNA samples (either shRNA2 or shRNA3) by the mean of the non-target shRNA samples (see Figure 4.3).
(B) Heat map of expression levels of the candidate miRNAs. The colour scale represents the ratios (scramble set to 1) ranging from 0 (green) to 1 (red).

In parallel, a second normalisation method, vsn (variance stabilisation normalisation) (Huber et al., 2002), was applied (in cooperation with Wolfgang Huber, EMBL). The application of this alternative normalisation method showed that the miRNA expression profiles of the cells expressing either a NOTCH1 shRNA or a non-target shRNA were very similar (Figure 4.5), and no miRNA could be identified that was significantly de-regulated upon NOTCH1 knock-down.

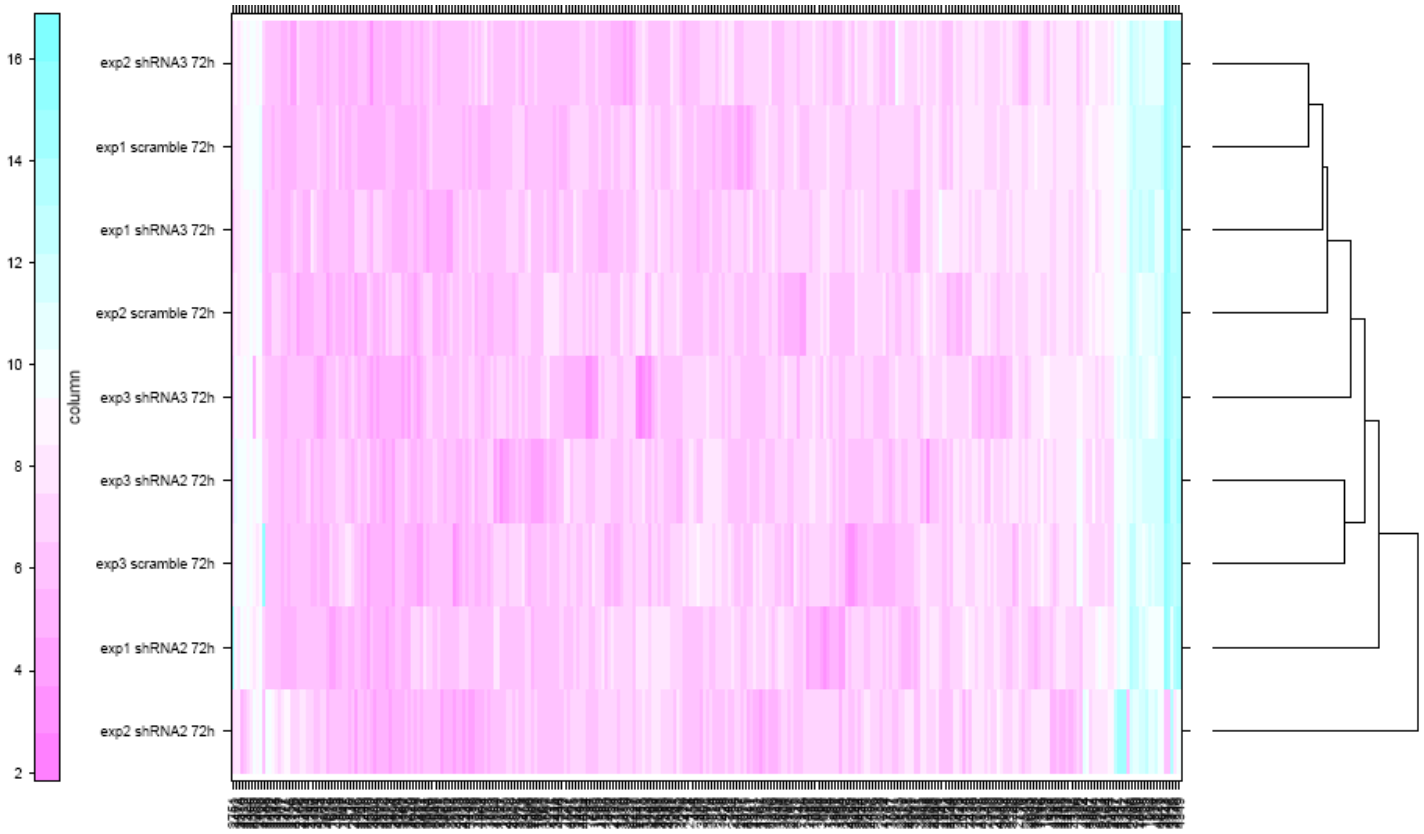


Figure 4.5 **Heat map of vsn-normalised miRNA expression**
The colour scale represents the log normalised intensities ranging from 2 to 17. The left shows the different experiments at 72h. The right panel represents the hierarchical clustering of the experiments. The x-axis shows all human miRNAs of the miChip (in collaboration with Wolfgang Huber).

To validate the miRNA expression using a second technique, a miRNA-specific qPCR approach (miQPCR) was used. The five candidate miRNAs identified by the miChip-normalised microarray analysis (in Figure 4.4) were amplified in three independent experiments (72h). However, none of these miRNA could be validated by the miQPCR approach (Table 4.1).

	shRNA2 72h		shRNA3 72h	
mature miRNA	**p-value (T-test)**	**Fold change**	**p-value (T-test)**	**Fold change**
miR-15a	0.33	1.09 fold up	0.22	1.17 fold down
miR-181b	0.10	1.44 fold up	0.90	1.02 fold down
miR-18a	0.34	1.16 fold down	0.13	1.63 fold down
miR-18b	0.15	2.08 fold up	0.68	1.11 fold down
miR-491-3p	0.81	1.09 fold up	0.09	1.34 fold down

Table 4.1 **miQPCR analysis of the candidate mature miRNAs from NOTCH1 knock-down experiments.**
The t-test was used to compare the miRNA expression in the NOTCH1 shRNA2 and shRNA3 samples to the non-target shRNA samples.

In summary, miRNA microarrays, when normalised using the miChip package in the Bioconductor software, could detect differentially expressed miRNAs upon NOTCH1 knock-down. However, a second normalisation method (vsn) showed that the miRNA signatures from cells expressing non-target shRNAs or NOTCH1 shRNAs do not differ. A miRNA-specific qPCR confirmed the second normalisation method, as the qPCR results showed that the expression of five miRNAs (shown to be down-regulated by the miChip package) was not altered after NOTCH1 knock-down.

4.1.2.2 Primary miRNA transcripts differentially expressed upon NOTCH1 knock-down

To further analyse the effects of the NOTCH1 knock-down on miRNA expression, the primary transcripts of the five candidate miRNAs were examined (Table 4.2). The expression of the primary transcripts at 72h and at 120h was measured in three independent experiments by qPCR.

mature miRNA	primary transcript
miR-15a	pri-miR-15a~16-1
miR-181b*	pri-miR-181a-1~181b-1 pri-miR-181a-2~181b-2
miR-18a	pri-miR-17~92
miR-18b	pri-miR-106a~363
miR-491-3p	pri-miR-491

Table 4.2 **List of candidate mature miRNAs and the corresponding primary transcripts**
***** The mature miRNA miR-181b could potentially be produced by the primary transcripts, pri-miR-181a-1~181b-1 or pri-miR-181a-2~181b-2.

Interestingly, the pri-miR-17~92 and pri-miR-491 were down-regulated two-fold upon NOTCH1 knock-down (Figures 4.6A and 4.6B), whereas the primary transcript levels of miR-181a-1~181b-1 were not significantly changed (Figure 4.6C). The levels of pri-miR-181a-2~181b-2 were also down-regulated for shRNA 2 and shRNA3 at 72h compared to the scramble shRNA, but were unchanged at 120h (Figure 4.6D). Pri-miR-15a~16-1 levels did not show any significant changes at 72h; only in shRNA3 expressing cells at 120h, the primary transcript of miR-15a~16-1 was down-regulated at 120h (Figure 4.6E). Pri-miR-106a~363 could not be detected by qPCR.

Altogether, significant changes in miRNA expression upon NOTCH1 knock-down could be found, when analysing the levels of primary transcripts of miR-17~92, miR-491 and miR-181a-2~181b-2. However, if these expression changes have an effect on the corresponding mature miRNA levels is unclear, as miRNA microarray experiments produced opposite results depending on the normalisation method used. The maximal decrease in pri-miRNA expression levels was two-fold, possibly causing only subtle changes in mature miRNA expression, which could not be detected by qPCR.

In conclusion, deregulation of NOTCH1 by knock-down decreased proliferation in the T-ALL cell line, CCRF-CEM. Primary transcripts of pri-miR-17~92 and pri-miR-491 were down-regulated two-fold upon NOTCH1 knock-down. Microarray experiments could detect a decrease of the corresponding mature miRNAs, miR-18a and miR-491-3p (2-fold and 1.5-fold, respectively). However, mature miRNA expression could not be validated by qPCR.

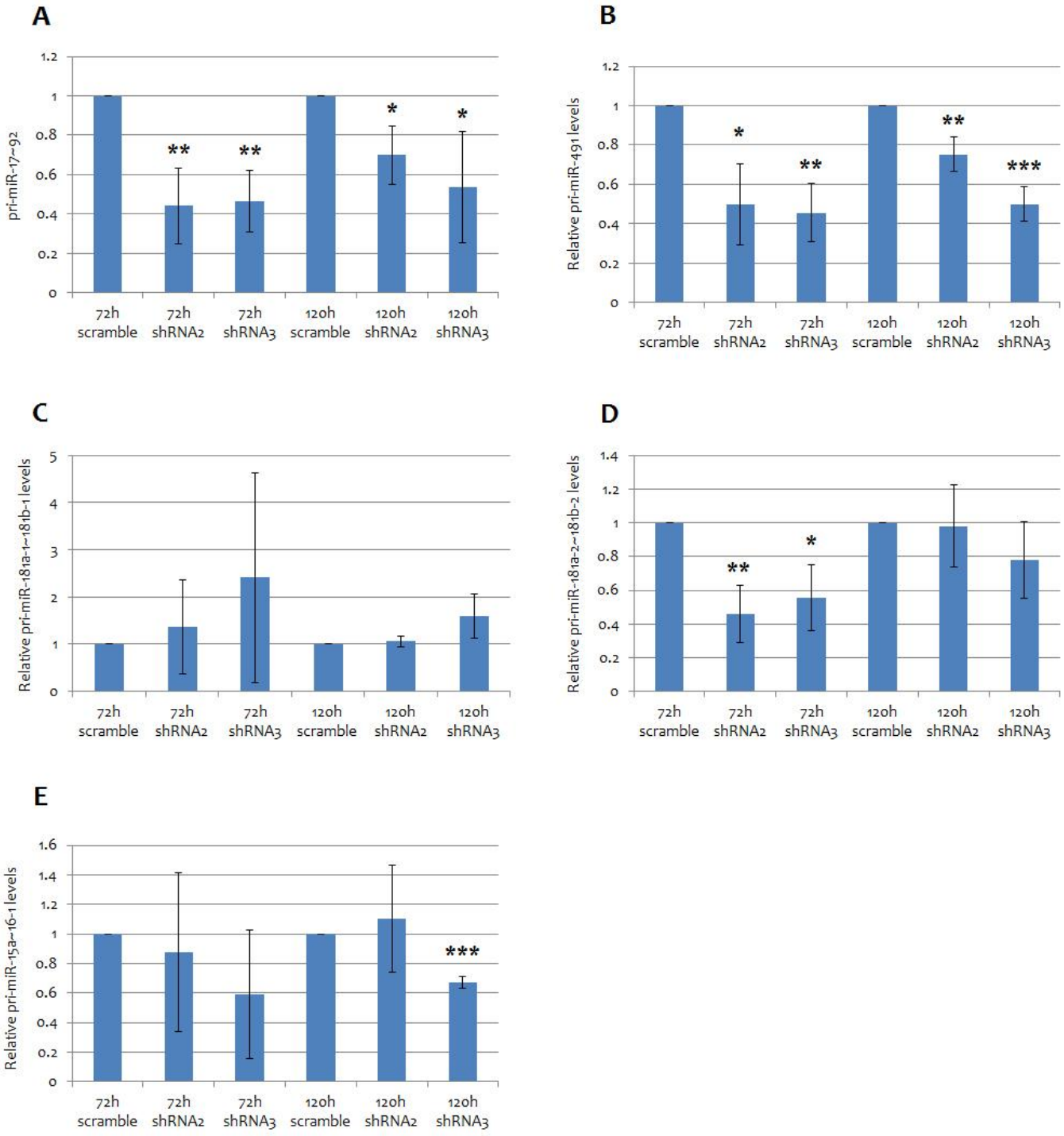


Figure 4.6 **NOTCH1 knock-down decreases primary miRNA expression levels.**
(A) pri-miR-17~92 expression, (B) pri-miR-491 expression, (C) pri-miR-181a-1~181b-1, (D) pri-miR-181a-2~181b-2 and (E) pri-miR-15a~16-1 detected by qPCR in CCRF-CEM cells transduced with either a non-target shRNA virus (scramble) or a NOTCH1 shRNA virus (shRNA 2, shRNA3) targeting the mRNA of NOTCH1 , at 72h and 120h post-transduction.
The scramble sample was set to 1 (n=3; $p<0.05$ *; $p<0.01$ **; $p<0.001$ ***).

4.2 miRNA expression in primary paediatric T-ALL samples

To explore miRNA expression in T-ALL patients, primary T-ALL samples were collected either from the ALL-BFM study group or directly from different hospitals.

4.2.1 MiRNA expression profiles show significant variations depending on the processing of diagnostic peripheral blood and bone marrow samples

Forty-one different paediatric T-ALL samples, as well as 3 adult T-ALL and 4 preB-ALL samples were collected. Thirty-one T-ALL specimens originated from the sample bank of the ALL-BFM study group. After collection of bone marrow from the patient at the hospital, the samples were sent by regular mail to the study centre, where they were frozen in 10% DMSO and stored in liquid nitrogen until further usage. These RNA samples were referred to as "frozen" samples. All other ALL samples corresponded to fresh peripheral blood or bone marrow samples. Within thirty minutes after sample collection, leukaemic blasts were prepared by Ficoll gradient analysis, resuspended in TRIzol® and immediately frozen. These samples are named "fresh" samples. After shipment to our institute on dry ice, total RNA was extracted. In addition, total RNA of lymphocytes extracted from a buffy coat was added as controls to the hybridisations. Importantly, two different RNA extraction methods were employed in this study, the TRIzol® extraction method and a column-based method (miRNeasy protocol, Qiagen). Those RNA samples extracted by the TRIzol® method are marked by an asterisk (*) in Figure 4.8.

Because one hybridisation includes a maximum of 24 samples, the ALL specimens were distributed into three sets as follows:

- Set1: 22 "frozen" paediatric T-ALL samples
- Set2: 9 "frozen" paediatric T-ALL samples and 1 "fresh" paediatric T-ALL sample
- Set3: 10 "fresh" paediatric T-ALL samples (1 patient represented by 2 RNA samples extracted by 2 different extraction methods), 3 "fresh" adult T-ALL samples and 4 "fresh" paediatric preB-ALL samples.

Each hybridisation included two (set1) or three (set2 and 3) lymphocyte control samples. After hybridisation and data acquisition of the chips, "miChip" normalisation was applied. The median of the signal intensities of the lymphocyte samples were calculated for each hybridisation (hyb) was calculated and each ALL patient sample was divided by this median. When, in most samples, no data was available for a probe set, the corresponding miRNA set was removed. This left a total of 426 miRNAs for final analysis (Figure 4.7). The data were uploaded into TIGR MeV 4.0 for hierarchical clustering (HCL).

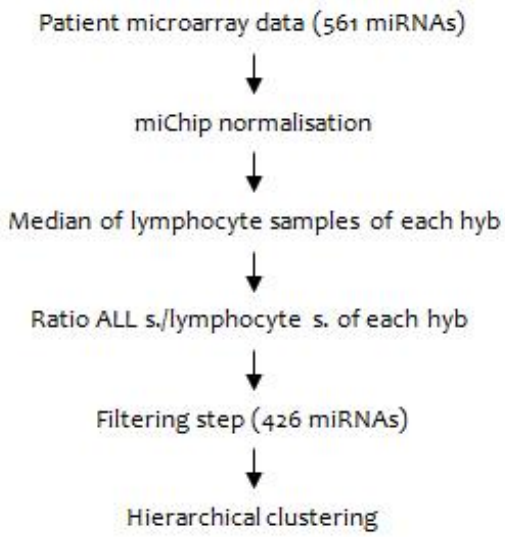


Figure 4.7 **Analysis of the diagnostic ALL microarray data before hierarchical clustering**

Upon hierarchical clustering, two major clusters formed representing mostly the "fresh" and the "frozen" samples (Figure 4.8). The adult T-ALL specimens were comprised in the cohort of "fresh" samples in between the paediatric T-ALLs, indicating that profiles of adult and paediatric T-ALL samples are highly similar. Unexpectedly, one preB-ALL sample (preB-ALL4) was found in between the "fresh" T-ALL samples, whereas the other three preB-ALL were sufficiently distinct to form a separate cluster in the "fresh" cluster. This observation is consistent with the notion that these leukaemias correspond to different clinical entities, but indicates also that miRNA signatures of preB-ALL and T-ALL samples contain many similarities. Two "fresh" RNA samples were extracted by the column-based method (T-ALL4 and T-ALL9), whereas all other "fresh" ones were extracted using TRIzol® method. Their profiles are found mixed in the "fresh" cluster. In addition, sample T-ALL9 from the second hyb set clusters closely with the samples from the third set. This indicates that miRNA expression profiles were mostly affected by the pre-analytical handling ("fresh/frozen") that overrides the changes introduced by the RNA extraction method and the different hybridisations.

In conclusion, depending on the processing of blood or bone marrow samples, huge variations in the miRNA expression profiles occur. In this study, miRNA signatures alter mostly upon differential sample processing. However, possible differences caused in the profiles by the RNA extraction methods and the hybridisations would also be expected, but are overridden in these samples by the "fresh/frozen" signature.

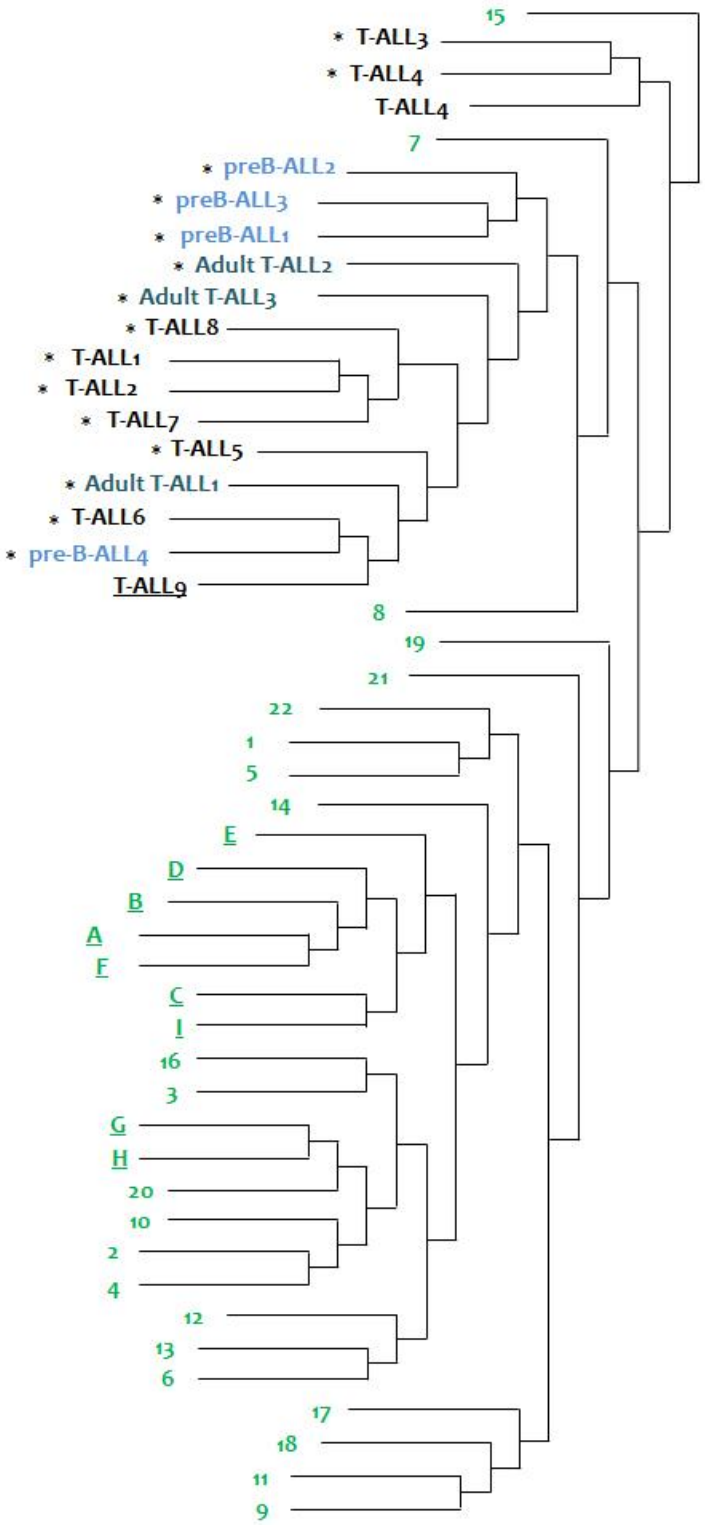


Figure 4.8 **Hierarchical clustering of 3 sets of ALL patient samples.**
The processing of patient samples introduces significant differences in the miRNA profiles.
set1 = 22 "frozen" paediatric T-ALL samples (numbered 1-22, green)
set2 = 9 "frozen" paediatric T-ALL samples (numbered A-I, green, underlined) and 1 "fresh" paediatric T-ALL sample, **T-ALL9** (black, underlined)
set3 = 9 "fresh" paediatric T-ALL samples (black), 3 "fresh" adult T-ALL samples (dark blue) and 4 "fresh" paediatric preB-ALL samples (light blue).
***** Classical TRIzol® RNA extraction.

4.2.2 Differentially expressed miRNAs in patients with or without *NOTCH1* mutations

The microarray analysis of the T-ALL patient samples, using the miChip package, showed that the differential sample processing severely affects miRNA signatures. Therefore, it was not possible to search for miRNAs that are differentially expressed depending on clinical or biological characteristics. To allow the identification of miRNAs that are differentially expressed in patients with (n=16) or without *NOTCH1* mutations (n=24), a different normalisation method was applied (in collaboration with Julia Engelmann, Regensburg and Wolfgang Huber, EMBL). For 1 patient, the *NOTCH1* mutational status was not available. In this normalisation procedure, a linear model was introduced that eliminates effects of the batches of hybridisation and of the differential pre-analytical handling. The analysis identified 10 candidate miRNAs that were differentially expressed in patients with or without *NOTCH1* mutations: miR-223, miR-181a, miR-181b, miR-221, miR-191, miR-768-5p, miR-181c, miR-19a, miR-181d and miR-518e*/519a*/519b-5p/519c-5p/522*/523* (Figure 4.9). It has to be noted that the differences of the median intensities in the *NOTCH1* mutated and non-mutated groups were modest. With the exception of miR-518e*/519a*/519b-5p/519c-5p/522*/523*, these miRNAs showed a higher expression in the patients without NOTCH1 activating mutations. Interestingly, a member of the miR-17~92 cluster, miR-19a, as well as, members of the miR-181 family were amongst the candidates, that were also described in the NOTCH1 knock-down experiments (Figure 4.4). In contrast to the cell-based assay experiments, the miRNAs showed a higher expression in the *NOTCH1* non-mutated group, whereas upon NOTCH1 knock-down in the cell line, these miRNAs were down-regulated. These opposite results await further analysis, and will be discussed in the following chapter.

The validation of the expression of individual miRNA in the T-ALL patient samples was not possible, because of the microarray normalisation procedure. To validate these miChip data by another approach, another high-throughput format would be required that allows a similar normalisation method than the linear model for the miChip data.

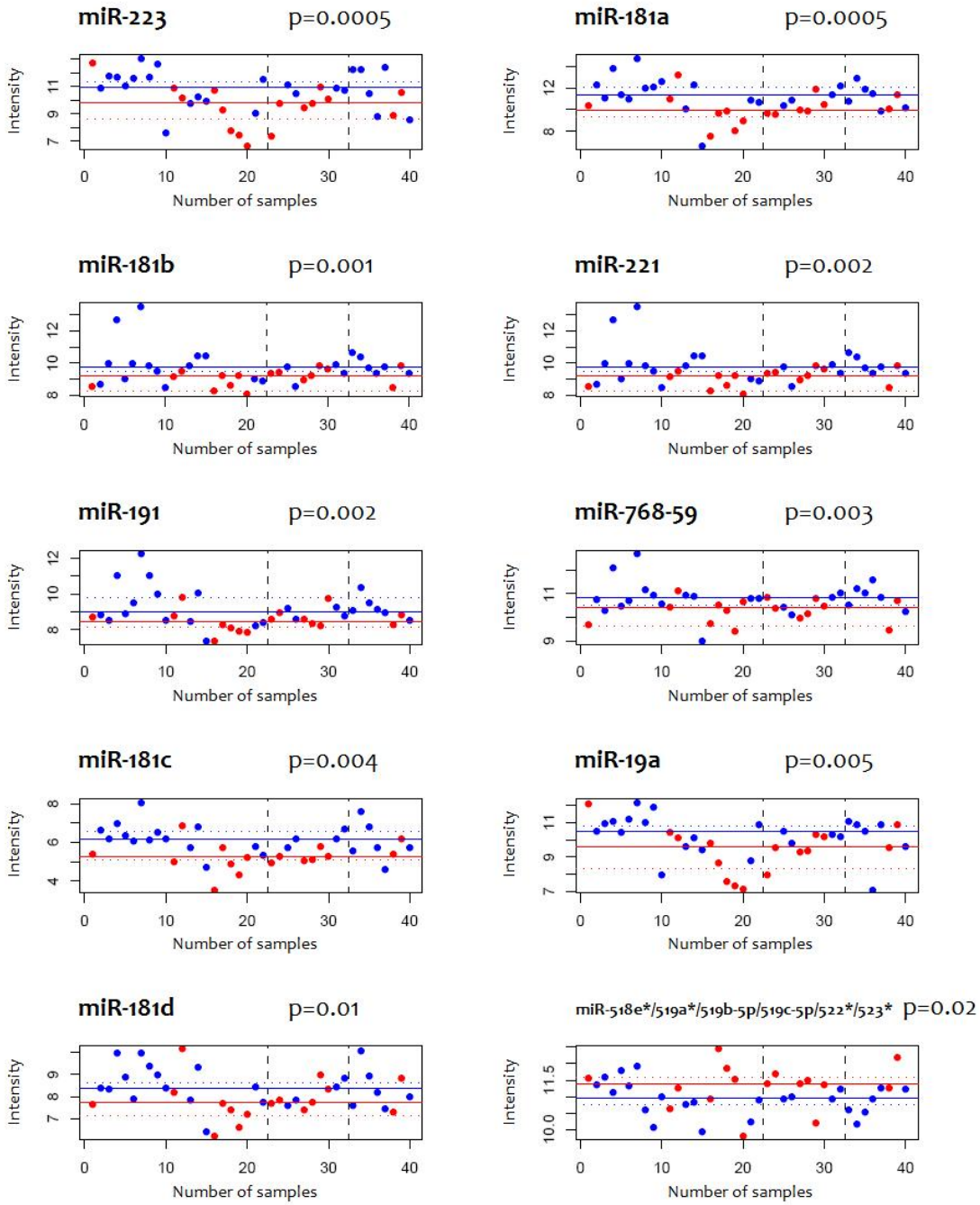


Figure 4.9 **Candidate miRNAs differentially expressed in T-ALL patients with or without *NOTCH1* mutations.**

- *NOTCH1* mutated
- *NOTCH1* non-mutated
- Median of the *NOTCH1* mutated group after including the hybridisation and the "fresh/frozen" effects
- Median of the *NOTCH1* mutated group after including the hybridisation and the "fresh/frozen" effects
- Median of the *NOTCH1* mutated group before including the hybridisation and the "fresh/frozen" effects
- Median of the *NOTCH1* mutated group before including the hybridisation and the "fresh/frozen" effects

In conclusion, miRNA members of the mir-17~92 cluster and of the miR-181 family were found to be differentially expressed depending on the *NOTCH1* mutational status in paediatric T-ALL patients. In addition, the analysis of these patient samples showed that differential sample processing can significantly interfere with the miRNA expression profiles. However, the effects of variable pre-analytical handling and data generation can be levelled out using a linear model during the normalisation procedure.

5. General Discussion and Conclusions

5.1 Molecular markers in risk stratification in paediatric T-ALL

A way of improving treatment outcome in patients with leukaemia is to successfully stratify the patients according to risk. In paediatric T-ALL, the aim of personalised treatment strategies are an improvement of the survival rate of high risk patients, but also a decrease of treatment-dependent toxicity in low risk patients. The current risk stratification protocol in the ALL-BFM 2000 is based on the response to induction therapy, assessed by the measurement of prednisone response and MRD kinetics (Conter et al., 2010). This method of risk assessment has been the most powerful tool to predict long-term outcome in childhood T-ALL. However, it would be favourable to treat the patients according to their risk at the earliest stage of the disease. Therefore, there is great interest in identifying clinical or molecular prognostic markers that allow individualised treatment already at diagnosis.

In this study the clinical application of two potential molecular risk factors was characterised in paediatric T-ALL: point mutations in *NOTCH1* and *FBXW7*, and miRNA expression profiles.

5.1.1 Mutations in the NOTCH pathway to predict long-term outcome in T-ALL?

5.1.1.1 Outcome prediction using mutations in the NOTCH pathway is treatment-dependent

In this study, the *NOTCH1* and *FBXW7* mutational statuses were analysed for their possible application in risk stratification in T-ALL. The advantage of these mutations is their high frequency in childhood T-ALL, with over 50% of the patients containing *NOTCH1* mutations and up to 20% harbouring *FBXW7* mutations, and the simple and fast detection by sequencing.

At initial presentation, *NOTCH1* mutations strongly predict better early treatment response and improved long-term outcome (Table 3.1; Figure 3.2). This effect was restricted to the standard and intermediate risk groups, whereas in the high risk group similar survival rates were observed for patients with and without *NOTCH1* mutations (Figure 3.3). Study groups with similar treatment strategies also observed the positive effect of *NOTCH1* mutations on treatment outcome (Asnafi et al., 2009; Malyukova et al., 2007; Park et al., 2009). However, in other groups no association was described between *NOTCH1* mutations and long-term outcome (Baldus et al., 2009; Larson Gedman et al., 2009; Mansour et al., 2009; van Grotel et al., 2008; Zhu et al., 2006; Zuurbier et al., 2010). These results suggest that the effect of *NOTCH1* mutations depends on the treatment

protocol applied. It is known from other cancer types that markers can be treatment-dependent ($p16^{INK4A}$ and Bcl-2 expression in prostate cancer (Lopergolo and Zaffaroni, 2009) or microvessel count in breast cancer (Esteva and Hortobagyi, 2004)). Therefore, the clinical utility of prognostic factors has to be rigorously validated for each treatment protocol.

The reason why patients with *NOTCH1* mutations respond particularly well to the ALL-BFM protocol is currently unknown. Because treatment protocols use multi-agent therapies and vary in the nature of the drugs and the corresponding doses administered, a simple comparison between protocols will not be possible to identify the agent(s) that induce a positive response in the *NOTCH1* mutated group. However, an indication towards a favourable treatment strategy for the *NOTCH1* group came from the comparison between our ALL-BFM 2000 study and the EORTC study group (Clappier et al., 2010). The chemotherapy in the EORTC protocol is in large parts similar to the ALL-BFM protocol, but does not include cranial irradiation. Interestingly, in the EORTC study group, *NOTCH1* mutated patients were characterised by a better response to the prephase and higher incidence of low MRD level than the patients without *NOTCH1* mutations. In contrast to the ALL-BFM, however, the long-term outcome of T-ALL patients with or without *NOTCH1* mutations was similar (Clappier et al., 2010). In particular, the *NOTCH1* mutated group treated according to the high risk protocol showed a poor prognosis in long-term outcome. (pEFS of 0% and 42%, respectively; Figure 5.1; Clappier et al., 2010). They also identified a non-significant trend towards a higher number CNS relapses in the *NOTCH1* mutated group, which are generally rare in the ALL-BFM 2000 study. It could be hypothesised that in the ALL-BFM 2000, cranial radiotherapy prevents CNS relapses in the high risk patients with *NOTCH1* mutations and thereby improve overall outcome of this group. Because of the radiation-associated late complications, such as second malignancies and decline in growth rate (Sklar et al., 1993; Walter et al., 1998), concerns have been raised about the use of prophylactic cranial irradiation in childhood ALL. The occurrence of these late sequelae led to omission of cranial radiotherapy or dose reduction in some protocols (Conter et al., 1997; Kamps et al., 1999; Pui et al., 2009). Therefore, the comparison of the ALL-BFM 2000 and EORTC study groups is of great importance, because it led to the possible identification of a patient group that could truly benefit from cranial irradiation.

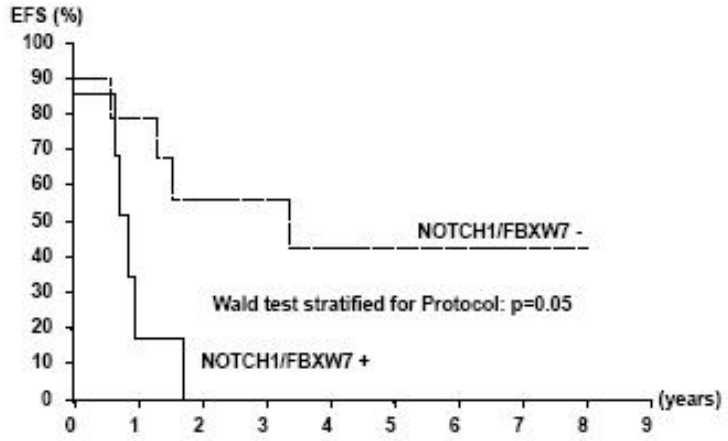


Figure 5.1 **Event-free survival of T-ALL patients in EORTC-CLG trials according to *NOTCH1*/*FBXW7* mutations in patients with high MRD level (>10-2) (with the permission of Clappier et al.)**
Patients without *NOTCH1* and *FBXW7* mutations (with high MRD level) had a better EFS than the patients with *NOTCH1* or *FBXW7* mutations (0% vs 42%; p=0.05; Clappier et al., 2010).

In accordance with the higher rate of CNS relapses in the EORTC-treated *NOTCH1* mutated group, is the finding in a T-ALL mouse model that NOTCH1 causes CNS involvement via controlling the expression of CCR7 (Buonamici et al., 2009). The chemokine receptor, CCR7, and its ligand, CCL19 expressed in brain epithelial cells, are essential for CNS infiltration by the leukaemic T cells. CCR7 is a key regulator of the immune system, demonstrated by migrating defects of the immune cells, including T cells, into the lymph nodes and the ineffective tolerance to self-antigens leading to multi-organ autoimmunity in CCR7-deficient mice (Davalos-Misslitz et al., 2007; Schneider et al., 2007). The role of CCR7 in CNS involvement in human T-ALL is currently unknown. A more detailed understanding of the mechanism of CCR7-induced CNS infiltration could pave the way for new treatment strategies of CNS-directed therapy. In addition, the detection of CCR7 expression at diagnosis could provide a potential predictive value for CNS involvement.

5.1.1.2 Patients with *NOTCH1* and *FBXW7* mutations respond differently to treatment

Because FBXW7 is part of the NOTCH pathway, it was hypothesised that patients with *FBXW7* mutations respond similarly to the ALL-BFM treatment than those with *NOTCH1* mutations. In addition, *NOTCH1-PEST* and *FBXW7* mutations are found in a mutual exclusive manner (with the exception of 1 out of 301 patients), suggesting that no additional selective advantage is offered by mutating both proteins that target the same degradation pathway. Indeed, early treatment response (assessed by prednisone (or dexamethasone) response) was excellent in patients with

FBXW7 mutations. Unexpectedly however, late MRD kinetics and pEFS were not significantly different between the *FBXW7* mutated and non-mutated groups. Therefore, it seems that the effect of both types of mutations is separable. A possible explanation could be that FBXW7 interferes with other oncogenic pathways and proteins like c-MYC, PSEN1, c-JUN, cyclin E1, SREBP, mTOR or Aurora-A (Koepp et al., 2001; Li et al., 2002; Mao et al., 2008; Nateri et al., 2004; Sundqvist et al., 2005; Yada et al., 2004). In line with this, is the fact that *NOTCH1* mutations are specifically found in T-ALL, whereas *FBXW7* mutations have been identified in many different tumour types raising the hypothesis of NOTCH-independent effects of *FBXW7* mutations (Rajagopalan et al., 2004; Spruck et al., 2002). The mutual exclusiveness of *NOTCH1-PEST* and *FBXW7* mutations, however, suggests that FBXW7 primarily targets NOTCH1 as a means of activating NOTCH1 signalling. As a "side effect" of inactivating mutations of the E3 ubiquitin ligase, the half-life of other FBXW7 targets could be affected, and explain the different response to treatment of patients with *FBXW7* mutations.

Of particular interest for individualised treatment, are patients with isolated *FBXW7* mutations that show a particularly low pEFS at initial presentation (58%; n=12; Figure 3.5) and at relapse (0%; n=3; Figure 3.10). Although the low number of patients does not allow meaningful statistics, the analysis of a larger cohort could potentially identify a group of high risk patients. Nevertheless, due to the diversity of events in this group (1 second malignancy, 3 deaths before or in complete remission, 1 relapse at initial presentation; 2 "non-response to treatment", 1 death before CR at relapse), it would be difficult to define a uniform therapy for this heterogeneous group.

In contrast to *FBXW7* mutations, *NOTCH1* mutations correlate with a good prognosis of outcome. This effect is however restricted to the SR/MR groups, and no difference in EFS was observed in the HR group (Figure 3.3). The events in the *NOTCH1* non-mutated group, treated according to the high risk protocol, were mostly relapses (18/27), whereas in the *NOTCH1* mutated group, the number of non-relapse events was higher (including 7 deaths in CR). It can be speculated that the treatment was too intensive for these *NOTCH1* mutated patients, and that this group might have benefitted from a SR or MR therapy. The validation of these data by a prospective study could favour the inclusion of *NOTCH1* mutations as a prognostic marker in the ALL-BFM treatment protocol.

5.1.1.3 The NOTCH pathway and the response to glucocorticoids

As discussed previously, the mechanism of response of the *NOTCH1* mutated patients to the ALL-BFM treatment protocol will be difficult to entangle. However, already during the early

prephase (administration of prednisone/dexamethasone and intrathecal methotrexate), the group of *NOTCH1* and *FBXW7* mutated T-ALLs show an excellent response to the early treatment. This stands in striking contrast to studies in cell-based assays and in mouse models, in which NOTCH1 actively induces glucocorticoid (GC) resistance (De Keersmaecker et al., 2008; Deftos et al., 1998). Treatment with GSI reverses this effect, and dexamethasone (a glucocorticoid) is able to induce apoptosis (De Keersmaecker et al., 2008; Real et al., 2009). NOTCH1-induced GC resistance is mediated by up-regulation of the pro-survival factor, Bcl-2 (B-cell lymphoma 2; Deftos et al., 1998), and of HES-1, which inhibits the transcription of the glucocorticoid receptor gene, *NR3C1* (Real et al., 2009). A Bcl-2-independent mechanism of resistance, is the NOTCH1-mediated down-regulation of SRG3 (SWI3-related gene product), a component of the chromatin-remodelling complex, SWI/SNF (SWItch/Sucrose NonFermentable) (Choi et al., 2001; Pottier et al., 2008). How the level of SRG3 expression determines GC sensitivity remains elusive. Moreover, dexamethasone-induced apoptosis in lymphoma cells is prevented by GSK3 inhibition (Spokoini et al., 2010). GSK3 (Glycogen synthase kinase-3) causes apoptosis by interacting with pro-apoptotic BIM, possibly activating the mitochondrial apoptotic pathway. Inhibition of NOTCH1 signalling results in GSK3 inhibition via activation of Akt signalling, thus causing apoptotic sensitivity (Spokoini et al., 2010).

Why glucocorticoid correlates positively with the presence of *NOTCH1* or *FBXW7* mutations in T-ALLs, treated according to the ALL-BFM protocol response, remains to be investigated. The *in vivo* response in human T-ALL might be more complex as proposed in the experimental settings. Alternatively, NOTCH1-induced GC resistance might be present in only a subset of T-ALL patients (e.g. *NOTCH1* mutated high risk group). This group could represent a subgroup of patients in whom the concomitant use of a NOTCH1 inhibitor (GSI) with glucocorticoids may be beneficial, sparing them from the side and late effects of intensified chemotherapy. It could also be speculated that the better early prephase response in the mutated group is not only related to the administration of glucocorticoids, but also to the administration of intrathecal methotrexate.

5.1.1.4 The mechanism of T-ALL relapses

The comparison of matched diagnosis and relapse samples unravelled the instability of *NOTCH1* and *FBXW7* mutations in approximately one third of the patients. In these patients, a gain or a loss of *NOTCH1*/*FBXW7* mutations was described at relapse. This result confirms a previous study in which 4/16 patients showed a variable *NOTCH1* mutational status in the primary and the recurrent leukaemia (Mansour et al., 2007). These findings raise the question about the

mechanism of relapse. One hypothesis for the acquisition of novel mutations is the existence of a leukaemic stem cell that gives rise to a new clonogenic pool at relapse (Bonnet and Dick, 1997; George et al., 2001). Another possibility is the selection and further expansion of a minor resistant sub-clone that was already present, but undetectable, at diagnosis (Choi et al., 2007; Li et al., 2003; Mullighan et al., 2008). To differentiate between these hypotheses (Figure 5.2), additional leukaemic-specific characteristics need to be analysed, such as *TCR* rearrangements or chromosomal aberrations. From a clinical perspective, the detection of minor resistant clones at first presentation would allow an additional way to improve T-ALL therapy, by intensification of the treatment at initial diagnosis.

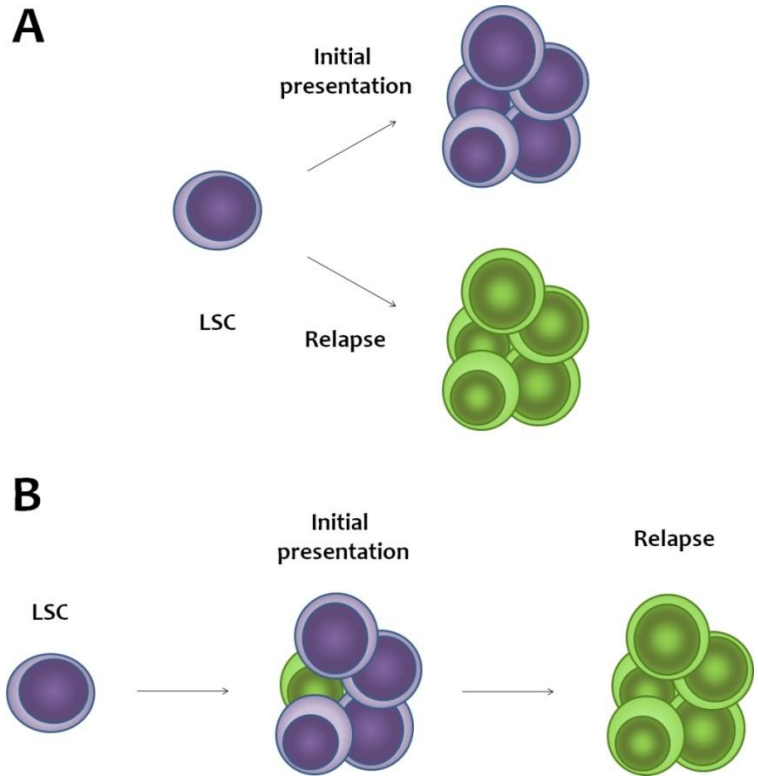


Figure 5.2 **Two alternative models for T-ALL relapse**
(A) The relapse clone develops from a leukaemic stem cell (LSC).
(B) During treatment of the first T-ALL, resistant leukemic cells are selected.

5.1.1.5 NOTCH pathway mutations as MRD markers?

In the ALL-BFM 2000 study group, MRD measurements are DNA-based. This is achieved through leukaemic clonal specific detection and amplification of *Ig* and *TCR* gene rearrangements

by quantitative PCR. Due to the clonal evolution of *Ig* and *TCR* gene rearrangements, at least two sensitive MRD markers are analysed to prevent false-negative MRD (Szczepanski et al., 2002b). The relatively high frequency of *NOTCH1* and *FBXW7* mutations in paediatric T-ALL makes them potential MRD marker candidates. However, a downside of the use of these mutations is the lack of their stability at relapse (Table 3.5). Although NOTCH1 is a key player in the pathogenesis of T-ALL and is a potent inducer of leukaemia in mice (Pear et al., 1996), the activating mutations can also occur as secondary events (Chiang et al., 2008; Lin et al., 2006; Mansour et al., 2007; O'Neil et al., 2006). Consequently, the mutational status of *NOTCH1* would not be sufficiently reliable to serve as a sole MRD marker, with the uncertainty of missing NOTCH1-independent leukaemic clones.

5.1.2 mRNA and miRNA expression profiling to predict long-term outcome in T-ALL?

In recent years, many expression profiles have been analysed with success to identify different cancer types or clinical subtypes of the same entity (Calin et al., 2004a; Golub et al., 1999; Lu et al., 2005). Gene expression profiling provided a new approach for defining new tumour subtypes, and for assigning samples to already defined subclasses (Golub et al., 1999; Ross et al., 2004; Yeoh et al., 2002). Moreover, matched first diagnosis-relapse samples could identify differences in gene expression at relapse (Staal et al., 2003; Yeoh et al., 2002). The comparison of first diagnosis samples from patients that were in CR or relapsed, allowed the prediction of recurrent disease development. In addition, patients with a different treatment-response or characterised by drug resistance showed different patterns in gene expression (Cario et al., 2005; Cheok et al., 2003; Holleman et al., 2004; Lugthart et al., 2005; Moos et al., 2002).

In paediatric T-ALL, Ferrando et al. described different mRNA expression profiles that were indicative of the arrest in the developmental stage (Ferrando and Look, 2003). Each group with similar expression patterns was characterised by aberrant expression of a known oncogene in T-ALL. Importantly, the gene expression signatures were of clinical relevance as, for example, the group with high HOX11 expression was associated with favourable prognosis. T-ALL samples could be distinguished with high accuracy from preB-ALL samples by comparing expression signatures of a subset of genes (Fulci et al., 2009; Haferlach et al., 2005; Moos et al., 2002; Yeoh et al., 2002).

As miRNA expression profiles have also shown to be useful for subtype classification, risk stratification or prognosis (Calin et al., 2005; Fulci et al., 2009; Lu et al., 2005), miRNA profiling was undertaken in this study using 40 paediatric T-ALL samples obtained from different hospitals and institutes in Germany. Peripheral blood or bone marrow samples were either sent to the ALL-

BFM study centre for storage in the sample bank or directly stored in trizol after collection from the patient. Differential pre-analytical sample processing caused major changes in the miRNA expression signatures, overriding possible biological differences resulting from the arrest in a specific developmental stage, expression of oncogenes or point mutations. Similar variation due to pre-analytical handling is expected in mRNA expression signatures (Breit et al., 2004; Micke et al., 2006; Schoenberg Fejzo and Slamon, 2001). Therefore, careful pre-analytical handling needs to be guaranteed to obtain comparable and meaningful expression profiles. To date however, it would be practically impossible to apply the techniques in the setting of a multi-centre study, due to difficulties in homogenous sample processing and due to the long-lasting experimental set-up. Major criteria for microarray studies in a multi-centre study include short and cold storage of patient material, purification of leukaemic cells, identical RNA extraction method and microarray protocol/platform/analysis (Staal et al., 2006; Staal et al., 2003).

In summary, although mRNA and miRNA expression profiles could be powerful predictors of risk in paediatric T-ALL, their application harbours major difficulties ranging from pre-analytical handling to tedious data generation. Therefore, the application of genomic markers such as point mutations would be favoured for fast and simple risk assessment in a clinical setting.

5.1.3 Towards a molecular risk profile in paediatric T-ALL?

Although *NOTCH1* mutations predict improved long-term outcome in paediatric T-ALL patients, there is substantial overlap between poor and good responders in the *NOTCH1* non-mutated group. To more stringently identify high risk patients with increased risk for relapse, additional markers are needed. Multiple risk factors could be combined into a molecular risk profile determined at the time of diagnosis. Attractive factors could be chromosomal aberrations, (such as deletions or gains of specific chromosomal regions), point mutations or expression levels.

Interesting candidate markers are *CASP8AP2* gene deletions predicting unfavourable treatment response in ALL-BFM 2000 (12% in paediatric T-ALL; Remke et al., 2009) and *PTEN* inactivating mutations/deletions (between 5-25% of T-ALL patients; Maser et al., 2007), that were shown to have a negative prognostic impact in the GBTLI ALL-99 study (Jotta et al., 2010) and in a combined study of the Children's Oncology Group 9404 and Dana-Farber Cancer Institute 00-001 clinical trials (Gutierrez et al., 2009). In contrast, the Pediatric Oncology Group (POG) study group failed to associate the PTEN inactivation with treatment outcome (Larson Gedman et al., 2009). In the

Children's Oncology Group 9404 and Dana-Farber Cancer Institute 00-001 study groups, also *LEF1* micro-deletions were analysed that showed a trend towards better overall survival (Gutierrez et al., 2010). Other point mutations include *WT1* inactivating mutations (found in approximately 15% of the paediatric T-ALLs). However, the EORTC study group (Renneville et al., 2010), as well as the DCOG and the COALL-97 groups (Tosello et al., 2009) did not observe a prognostic significance of these mutations in their cohorts. A recent study on *PHF6* (PHD finger protein 6) mutations (identified 16% in paediatric T-ALLs) did not detect an association with patient outcome treated according to the DCOG protocols (Van Vlierberghe et al., 2010).
Although the assessment of mRNA expression levels is more difficult compared to the assessment of genomic markers (see 5.1.2), the expression of T-ALL oncogenes shows prognostic relevance in a number of studies. Indeed, high HOX11 expression levels correlates with good prognosis in T-ALL, whereas HOX11L2 is mostly associated with a poor outcome (Ballerini et al., 2002; Bergeron et al., 2007; Cave et al., 2004; Ferrando et al., 2002).

In sum, the collection of several prognostic markers could potentially result in accurate risk stratification and in an appropriate treatment strategy for each newly diagnosed T-ALL patient.

5.2 The role of miRNAs in paediatric T-ALL

5.2.1 miRNA expression in T-ALL is dependent on NOTCH1

In recent years, major advances in understanding the pathophysiology of T-ALL have been made. An important contribution came by Weng et al. with the discovery of activating *NOTCH1* mutations in more than 50% of the paediatric T-ALL patients (Weng et al., 2004). Some of the downstream effects of increased NOTCH1 signalling include the up-regulation of c-MYC transcription or the activation of the mTOR pathway (Chan et al., 2007; Palomero et al., 2006; Sharma et al., 2006). However, each downstream effect only partially explains the phenotype observed by aberrant NOTCH1 activation. Because an interplay between NOTCH1 and miRNAs has been described in development and in cancerogenesis (Wang et al., 2010), we hypothesised that NOTCH1 activation in T-ALL also affects miRNA expression.

To investigate this question, a cell-based assay was set-up in which NOTCH1 expression was knocked down and miRNA expression was monitored by miRNA-specific microarray-based profiling. Interestingly, NOTCH1 down-regulation drastically decreases cell proliferation confirming the role of NOTCH1 in maintaining T-ALL cell growth, even in cultured cells (Palomero

et al., 2006; Weng et al., 2003). MiRNA signatures were analysed using two different normalisation methods. Using the miChip method (Castoldi et al., 2006), a number of miRNAs showed differential expression upon NOTCH1 knock-down. The candidate miRNAs with the largest changes were miR-18a, miR-18b, miR-181b, miR-15a and miR-491-3p that were all down-regulated when NOTCH1 was knocked down. By contrast, the vsn method did not detect any major differences between non-target and NOTCH1 shRNA overexpression (Huber et al., 2002). To validate the miChip-normalised microarray data, a miRNA-specific qPCR, miQPCR, was used (patent application EP 09 002 587.5). However, the expression of the 5 miRNAs could not be recapitulated. Possible reasons include that the vsn normalisation is the more appropriate method for normalisation. Contrasting this view, expression analysis of the primary transcripts indicated that both, the transcripts coding for the miR-17~92 cluster and miR-491, were down-regulated two-fold upon 72h of NOTCH1 knock-down.

MiRNA expression analysis in paediatric T-ALL samples (n=41) was performed with the application of a linear model in the normalisation procedure. This method allowed the identification of a small number of miRNAs differentially expressed in patients with *NOTCH1* mutations. Prominent miRNA candidates were miR-223, members of the miR-181 family and mir-19a, a member of the miR-17~92 cluster. Unexpectedly, miR-181 members and miR-19a showed higher expression levels in the *NOTCH1* non-mutated group, contrasting the data of the cell-based assays.

In the cell-based assays, highest expression differences in mature and primary miRNA expression were two-fold changes. These expression changes are maybe too subtle to be detected by all methods. The discrepancy of miRNA expression between patient samples and cell lines could be explained by the cell lines having compensated the effect on miRNA expression. Alternatively, due to the problems of pre-analytical handling, the opposite findings could be based on the poor quality of the patient data. An important difference between the two methods is that the cell-based assay permitted the assessment of direct effects of NOTCH1 deregulation. In primary T-ALL patient samples, the NOTCH1 effect might be weakened or overridden by other clinical or biological differences.

In conclusion, additional approaches could be useful to confirm the effect of NOTCH1 on miRNA expression, such as T-ALL mouse models.

5.2.2 Role of miRNAs in human T cell development

In the past years, research groups have studied mRNA/miRNA expression in leukaemia by comparing profiles of patients with a different leukaemia subtype (Fulci et al., 2009; Moos et al., 2002), with different molecular (Ferrando and Look, 2003) or clinical (Cario et al., 2005; Lugthart et al., 2005) characteristics. Another way to learn about aberrant miRNA expression is to compare profiles of primary T-ALL samples to profiles of T cell development.

T cell development is characterised by differentiation and maturation of haematopoietic progenitor cells (HPCs) into mature T cells in the thymus. When HPCs enter the murine thymus, they undergo positive and negative selection, and progress from a double negative (DN; CD4-CD8-) to a double positive (DP; CD4+CD8+), and finally mature to a single positive stage (SP; CD4+ or CD8+) (Staal et al., 2001). In T-ALL, the leukaemic blasts are characterised by a block in one of these stages, and the stage differs between the T-ALL patients.

It was previously shown that expression profiling of murine and human T cell development is possible (Dik et al., 2005; Neilson et al., 2007). In a murine model, miRNA profiles were generated from different developmental stages, demonstrating a dynamic regulation of miRNAs during T cell development (Neilson et al., 2007). Due to difficult accessibility of human thymus, most studies on maturation of thymocytes were done in the mouse. However in an exploratory study, several distinct stages of T lymphocyte development were separated and mRNA expression profiling carried on each stage (Dik et al., 2005). By a qPCR and a microarray approach, TCR gene rearrangement could be followed through several maturation steps, and possible regulators of TCR recombination could be identified.

To gain insight in deregulated miRNA expression in T-ALL, the direct comparison of T-ALL samples to each stage of T cell maturation would be of great interest. Furthermore, T-ALL patients could be accurately assigned to a particular developmental stage. This information, in turn, could be used to identify novel prognostic subgroups.

5.2.3 The functional role of the miR-17~92 cluster and of its homologues in paediatric T-ALL

The miR-17~92 cluster includes miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a and miR-92a-1, whereas the miR-106~363 cluster encodes for miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92a-2, and miR-363 and the miR-106b~25 cluster is composed of miR-106b, miR-93 and miR-25. The oncogenic function of these clusters has been widely described in leukaemias and in solid tumours (He et al., 2005; Volinia et al., 2006).

Also in T-ALL, miR-17~92 was shown to play a role (Mavrakis et al., 2010). Specifically, miR-19 delivers the oncogenic activity, and cooperates with NOTCH1 to induce T-ALL leukaemogenesis. The oncogenicity of miR-19 is partly driven by targeting BIM, PTEN and PPP2R5E. Interference with the expression of BIM, an anti-survival factor, could result in apoptosis repression, whereas translational repression of PTEN and PPP2R5E (subunit of PP2A), that inhibit PI3K-Akt signalling, could affect proliferation. Interestingly, the PI3K-Akt pathway was reported to also be up-regulated by the activation of NOTCH1 (Palomero et al., 2007). Furthermore, frequent chromosomal aberrations and mutations were found in the PI3K-Akt pathway, demonstrating its essential role in the pathogenesis of T-ALL (Maser et al., 2007; Remke et al., 2009). Other targets of this cluster include E2F1, CDKN1A/p21 and Cyclin D1 identified in other human malignancies (Inomata et al., 2009; O'Donnell et al., 2005; Petrocca et al., 2008; Yu et al., 2008). Interesting is also the fact that c-MYC, a NOTCH1 transcriptional target in T-ALL, is able to activate the expression of miR-17~92 (O'Donnell et al., 2005). The decrease in expression of pri-miR-17~92 observed upon NOTCH1 knock-down could be mediated by c-MYC in T-ALL. The mechanism behind this regulation however remains unclear and awaits further investigation.

Moreover, a homologue cluster of miR-17~92, miR-106a~363, is highly expressed in T-cell leukaemia and induces anchorage independence in NIH-3T3 cells (Landais et al., 2007). Only recently, a molecular mechanism was discovered why boys develop T-ALL more frequently than girls. An X-chromosome-targeted analysis identified inactivating mutations in *PHF6*, a transcriptional regulator, that were found almost exclusively in males (Van Vlierberghe et al., 2010). Intriguingly, the gene encoding miR-106a~363 is also located on the X chromosome. It could be hypothesised that the high expression of miR-106a~363 in T-cell leukaemia is caused by chromosomal aberrations or epigenetic changes in this chromosomal region. It remains to be investigated if a gender-specific role of miR-106a~363 in leukaemogenesis exists, and which mature miRNA in this cluster harbours T-ALL oncogenicity.

5.2.4 The functional role of the miR-181 family in paediatric T-ALL

Despite important functions in haematopoiesis and in solid tumours (Chen et al., 2004; Li et al., 2007; Shi et al., 2008), tumourigenic activity of the miR-181 has only scarcely been described (patent application WO/2009/148631; Pekarsky et al., 2006). Of particular interest is the finding that miR-181a is able to influence the expression of negative regulators of the NOTCH and the pre-TCR pathways, and thereby to modulate these signalling pathways in T-ALL (patent application WO/2009/148631). Overexpression of miR-181a in murine double positive (DP: CD4+CD8+)

thymocytes induces an accumulation of DP cells, but inhibits maturation into single positive (SP) CD8+ thymocytes. Among miR-181a targets, are NRARP, a negative regulator of NOTCH signalling (Lamar et al., 2001), and multiple phosphatases, such as SHP2, involved in pre-TCR signalling suppression (Nguyen et al., 2006). Although in this experimental setting, miR-181c was not able to influence DP development, the *in vivo* role of the other miR-181 family members in T-ALL leukaemogenesis, namely miR-181b and miR-181d, needs to be determined.

6. Future Perspectives

How to improve treatment strategies and survival of children and adolescents with T-cell acute lymphoblastic leukaemia?

The use of intensive chemotherapy in the treatment protocols has contributed to the improved survival rates of ALL patients. Treatment failure in relapsed patients and late sequelae, however, mandate a search for new therapy strategies.

A way to increase overall outcome, is a stringent risk stratification protocol that accurately assigns patients to different risk groups in the earliest stages of the disease. Nowadays, molecular markers, that could allow early risk assessment, are still missing. The search for novel markers in T-ALL could be very fruitful in guiding the development of very early risk-adapted therapies, as highlighted by this PhD study.

In addition, the remarkable progress in understanding the pathogenesis of the disease harbours the possibility of developing targeted therapeutic strategies. In particular, inhibitors of the NOTCH pathway, such as the γ-secretase inhibitor (GSI), have been suggested. Unfortunately, the clinical trials of GSIs in T-ALL were hampered through severe gastrointestinal toxicity and low induction of apoptosis in leukaemic cells (Deangelo et al., 2006; Roy et al., 2007). In a recent study using a mouse model, these effects were reversed by a combination therapy of GSIs and glucocorticoids (Real et al., 2009). Further identification of cellular signalling pathways in T-cell malignancy could open new avenues for novel treatment approaches.

Tailoring the intensity of treatment with the help of molecular prognostic factors, and using target-specific therapeutics could help in answering the above question…

7. Appendix

Publication of part of the work presented in this thesis:

Kox, C., Zimmermann, M., Stanulla, M., Leible, S., Schrappe, M., Ludwig, W.-L., Koehler, R., Tolle, G., Bandapalli, O. R., Breit, S., Muckenthaler, M. U., Kulozik, A. E., "The favorable effect of activating *NOTCH1* receptor mutations on long-term outcome in T-ALL patients treated on the ALL-BFM 2000 protocol can be separated from *FBXW7* loss of function", *Leukemia,* in press.

Publication on paediatric T-cell acute lymphoblastic leukaemia:

Remke, M., Pfister, S., Kox, C., Toedt, G., Becker, N., Benner, A., Werft, W., Breit, S., Liu, S., Engel, F., Wittmann, A., Zimmermann, M., Stanulla, M., Schrappe, M., Ludwig, W.-D., Bartram, C.R., Radlwimmer, B., Muckenthaler, M.U., Lichter, P., and Kulozik, A.E., "High-resolution genomic profiling of childhood T-ALL reveals frequent copy-number alterations affecting the TGF-β and PI3K-AKT pathways and deletions at 6q15-16.1 as a genomic marker for unfavorable early treatment response", *Blood*, 2009, *114* (1053-1062).

Manuscripts in preparation:

Kox, C., Rausch, T., Stuetz, A., Schnitzler, P., Happich, M., Muckenthaler, M.U., Korbel, J., Kulozik, A.E., "Next generation sequencing screening for viral sequences in childhood acute leukaemia".

Kox, C., Hof, J., Happich, M., Tolle, G., Kulozik, A.E., Kirschner-Schwabe, R., Muckenthaler, M.U., "*NOTCH1* and *FBXW7* mutations in relapsed childhood T-ALL".

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