Role of the Notch signalling pathway in acute leukaemia

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DECLARATION

I, Marc R Mansour, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Date: 21st January 2013

ABSTRACT

The Notch signalling pathway is important in development and differentiation of a diverse range of both embryonic and adult tissues. There is now strong evidence implicating aberrant Notch signalling in the pathogenesis of T-cell acute lymphoblastic leukaemia (T-ALL), with over 50% of paediatric patients having activating mutations in *NOTCH-1*. This thesis aims to explore several aspects of the Notch pathway in both T-ALL and acute myeloid leukaemia (AML). Chapter one includes an overview of the clinical and biological characteristics of AML and T-ALL, and summarises the published data on the Notch signalling pathway, addressing the basic understanding of Notch activation through cell-to-cell interaction, as well as the mechanisms through which it is normally regulated. The role that Notch signalling plays in normal haematopoiesis is also discussed. Chapter two gives details of the reagents and methods used, and detailed methods are also given in the appropriate results chapters. Chapter three addresses the incidence and characteristics of *NOTCH-1* mutations in a cohort of adult patients with T-ALL in comparison to the published study of paediatric T-ALL, as well as in a cohort of patients with infantile leukaemia and AML. Secondly, the prognostic significance of *NOTCH-1* and *FBXW7* mutation status of adult T-ALL patients treated on the UKALLXII was investigated. Thirdly, a novel mutation affecting the LNR domain of *NOTCH-1* is reported. Chapter four includes data quantifying *NOTCH-1* mutation level in T-ALL patients, as well as the stability of *NOTCH-1* mutations at presentation and relapse, and attempts to interpret these findings in relation to the timing of acquisition of the mutation and clonal heterogeneity of T-ALL. Chapter five explores the functional and prognostic significance of a novel alternatively spliced isoform of the CSL transcription factor in AML, which was termed CSL-TREX (for TRuncates Exon X). The functional consequences of expressing CSL-TREX in CD34+ cells, in luciferase reporter assays and co-immunoprecipitation experiments with NOTCH-1 is reported. Chapter six summarises the overall implications of these findings to T-ALL and AML, and the future directions of research in this area.

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COMMONLY USED ABBREVIATIONS

APC	Allophycocyanin
bp	Base pairs
BM	Bone marrow
CD34+ cells	Multi-lineage haematopoietic progenitor cells expressing surface CD34
CFU	Colony forming unit
CR	Complete remission
DDW	Double-distilled water
DHPLC	Denaturing High Performance Liquid Chromatography
DMEM	Dulbecco's Modified Eagle Medium
D0	DMEM with 0% FCS
D10	DMEM with 10% FCS
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EFS	Event free survival
FAB	French American British
FACS	Fluorescent activated cell sorting
FCS	Foetal Calf Serum
FBXW7	F-box and WD repeat containing-7
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSI	γ-secretase inhibitor
HD	Heterodimerisation domain
HSC	Haematopoietic stem cell
ICN	Intracellular Notch
MRC	Medical Research Council, UK
MRD	Minimal residual disease
MUT	Mutant
OS	Overall survival
PB	Peripheral blood
PBS	Phosphate buffered saline
PBS-T	PBS plus 0.1% Tween
PEST	Proline Glutamic Acid Serine Threonine Rich domain
PCR	Polymerase chain reaction
R10	RPMI with 10% FCS
RT	Reverse transcriptase
SNP	Single nucleotide polymorphism
TCR	T-cell receptor
WCC	White blood cell count
WT	Wild type

CHAPTER 1. INTRODUCTION

1.1 Haematopoiesis

The blood system is comprised of a hierarchical organisation of different cellular lineages arising from pluripotent haematopoietic stem cells (HSCs) (Orkin and Zon, 2008). Because mature blood cells are short-lived, HSCs need to indefinitely self-renew throughout life, as well as dynamically respond to physiological stressors such as infection, hypoxia or haemorrhage. The mechanisms by which HSCs determine whether to self-renew or generate daughter cells that terminally differentiate toward a particular cell fate are highly complex, and involve multiple different extracellular cues such as cell-to-cell interaction with bone marrow (BM) stroma and stimulation by cytokines such as erythropoietin (EPO), thrombopoietin (TPO), granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Orkin and Zon, 2008).

HSCs are capable of committing daughter cells to all components of the blood system, including the development of red blood cells (erythropoiesis), myeloid cells (myelopoiesis), platelets (megakaryopoiesis), and lymphocytes (lymphopoeisis). Long-term HSCs reside in a BM niche in close proximity to osteoblasts, and give rise to short-term HSCs that have multi-lineage potential and can differentiate into either common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs) (Akashi et al., 2000). CMPs thence differentiate into either granulocyte/macrophage progenitors (GMPs) (which give rise to neutrophils, monocytes/macrophages, eosinophils and mast cells), or megakaryocyte/erythroid progenitor (MEPs) (which give rise to red blood cells and megakaryocytes). Although a wide range of transcription factors are involved in lineage determination, the level of signalling imparted by NOTCH-1 is one of the most important determinants of whether CLPs commit to either the B- or T-cell lineage, and also plays an important role in early definitive haematopoiesis, self-renewal of HSCs and interaction of HSCs with the stem cell niche (see section 1.6).

1.2 Acute leukaemia and its clinical presentation

Acute leukaemias are malignant clonal disorders resulting from chromosomal, genetic and epigenetic alterations arising in haematopoietic progenitors that lead to

unregulated cellular proliferation, a block in differentiation and enhanced self-renewal properties. The principal feature of leukaemia is the accumulation of malignant blast cells in the BM and often peripheral blood (PB), resulting in a failure of normal haematopoiesis and resultant PB cytopenias. Clinical symptoms can be broadly divided into those resulting from BM failure and those relating to the leukaemic cell burden. Typical clinical features occurring from BM failure include easy bruising or bleeding from thrombocytopenia, acute or chronic infection as a consequence of neutropenia, and pallor, fatigue and exertional dyspnoea from anaemia. The accumulation of leukaemic blasts often results in bone pain, a common presenting symptom. Extreme hyperleucocytosis can impair blood flow and oxygen transfer in the lung leading to respiratory failure. Other signs and symptoms include lymphadenopathy and hepatosplenomegaly, skin infiltration from malignant blasts (chloromas), widespread bleeding or thrombosis from disseminated intravascular coagulation (typically associated with acute promyelocytic leukaemia), features of superior vena cava obstruction from a mediastinal mass (typically associated with T-cell acute lymphoblastic leukaemia/lymphoma) and neurological symptoms from leukaemic infiltration of the central nervous system.

Accurate classification of acute leukaemia is essential in directing the correct treatment modality and in determining prognosis. Acute leukaemias are categorised according to the putative progenitor cell of origin and level of differentiation arrest, which is determined by a combination of morphological assessment of the BM or PB, blast cell immunophenotype, and the presence of particular cytogenetic abnormalities. At the broadest level of classification, acute leukaemias are divided into acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL), depending on whether the malignant blast cells are of myeloid or lymphoid origin respectively. Further subclassification is discussed below.

This thesis attempts to address the role of the Notch signalling pathway in both AML and ALL, with a particular focus on activating mutations in the *NOTCH-1* gene in T-cell ALL (T-ALL) and alternative splicing of the Notch pathway transcription factor *CSL* in AML. The introductory chapter offers a brief overview of both the clinical, molecular and prognostic features of AML and ALL, and discusses the role of Notch signalling in normal haematopoiesis. More detailed review of Notch signalling in

malignant haematopoiesis is discussed in the introduction of the relevant results chapters.

1.3 AML

1.3.1 Incidence of AML

AML has an annual incidence of 3.4 cases per 100,000 adults in the UK and occurs at higher frequency with increasing age, with two-thirds of cases occurring in people aged greater than 60 years of age (Milligan et al., 2006). Compared to ALL, childhood AML is relatively rare, with an annual incidence of 0.6 cases per 100,000 children between 1-14 years, although it is considerably more common in infants (<1 year of age), affecting 1.6 per 100,000 per year, where it is predominantly associated with translocations of the mixed lineage leukaemia gene (*MLL*) (data from the Cancer Research Website, www.cancerresearchuk.org, based on statistics from the National Registry of Childhood Tumours).

1.3.2 Causative/predisposing factors in AML

In the majority of cases, the cause of AML is idiopathic, although it has been associated with the following disorders (reviewed in Jabbour et al., 2006):

1. Transformation from acquired syndromes such as myelodysplasia (MDS), essential thrombocythaemia (ET), myelofibrosis (MF), chronic myeloid leukaemia (CML), polycythaemia vera (PRV), or paroxysmal nocturnal haemoglobinuria.
2. Genetic disorders:
    a. Chromosomal disorders: Kleinfelter's syndrome (XXY), Down's syndrome (trisomy 21), Patau syndrome (trisomy 13), familial monosomy 7 syndrome.
    b. Inherited/congenital genetic defects – ataxia telangiectasia, severe congenital neutropenia, Fanconi syndrome, Wiskott-Aldrich syndrome, Shwachman-Diamond syndrome, familial platelet disorder with predisposition to myeloid malignancy (associated with inherited mutations of the runt-related transcription factor 1 gene *RUNX1*), and inherited mutations of the CCAAT enhancer binding factor alpha (*CEBPA*) gene.

3. Radiation exposure – either from therapeutic radiotherapy, such as for a separate malignancy, or as seen with those exposed to ionising radiation, such as after the Chernobyl disaster or survivors of the atomic bombs in Japan.
4. Prior therapy with either alkylating agents, such as cyclophosphamide and melphalan, where the resultant AML is often associated with abnormalities of chromosomes 5 and 7, or topoisomerase II inhibitors such as etoposide and anthracyclines, where the AML is often associated with *MLL* gene rearrangements on chromosome 11q23 or with t(15;17), and has a short latency period of 1 to 3 years.
5. Exposure to other agents such as benzene, printing dyes and smoking.

Table 1.1. French-American-British (FAB) classification of AML

- **AML M0:** acute myeloblastic leukaemia without differentiation, with absent expression of myeloperoxidase (MPO)
- **AML M1:** acute myeloblastic leukaemia with minimal differentiation but with the expression of MPO that is detected by immunohistochemistry or flow cytometry
- **AML M2:** acute myeloblastic leukaemia with differentiation
- **AML M3:** acute promyelocytic leukaemia (APML)
    - **AML M3v:** APML, microgranular variant
- **AML M4:** acute myelomonocytic leukaemia
    - **AML M4Eo:** with eosinophilia.
- **AML M5:** acute monocytic leukaemia
    - **AML M5a:** without differentiation (monoblastic)
    - **AML M5b:** with differentiation
- **AML M6:** acute erythroid leukaemia
    - **AML M6a:** erythroleukaemia
    - **AML M6b:** pure erythroid leukaemia
- **AML M7:** acute megakaryocytic leukaemia

(According to Bennett et al., 1976; Bennett et al., 1985)

1.3.3 French-American-British (FAB) classification of AML

In 1976, the French-American-British (FAB) group was the first to comprehensively classify AML, based on morphological and histochemical staining patterns (Bennett et al., 1976). The classification system, including subsequent modifications (Bennett et al., 1985), is shown in Table 1.1.

1.3.4 World Health Organisation classification of AML

Although the FAB classification system is still used, its lack of prognostic power and the poor concordance between haemato-pathologists in assigning a FAB type has limited its clinical utility. Furthermore, it has now been superseded by improvements in immunophenotypic, cytogenetic and genetic characterisation of leukaemias, which can be standardised across laboratories and give a greater wealth of prognostic information. Such factors have been incorporated into the latest 2008 World Health Organisation (WHO) classification of AML (Arber DA, 2008; Falini et al., 2010; Vardiman et al., 2009) (Table 1.2). For instance, there is now the provisional inclusion of a subcategory for 'AML with mutated nucleophosmin' and 'AML with mutated CEBPA', because such patients have a superior prognosis. The presence of 20% or more blasts in PB or BM is required for the diagnosis of AML, except for cases harbouring t(8;21)(q22;q22), inv(16)(p13.1q22), or t(15;17)(q22;q12), which are now considered AML irrespective of the blast cell count. Additional changes include subcategories for 'AML with t(6;9)(p23;q34)' and 'AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2)', because patients with these translocations have a particularly poor outcome.

1.3.5 Molecular basis of AML

In a hypothesis explaining the malignant transformation to acute leukaemia it has been suggested that two molecular events or 'hits' are required – one leading to an increase in proliferation (so called class I mutation), and the other blocking differentiation (class II mutation) (Gilliland et al., 2004). For example, in a study by Kelly *et al* (2002), transgenic mice expressing the *PML-RARα* fusion transcript produced by the t(15;17) translocation only developed a low penetrance of leukaemia (approximately 20%) after 6 months or more, suggesting that a second mutational event was required for transformation. Similarly, when murine BM was transduced with a fms-like tyrosine kinase receptor gene (*FLT3*) internal tandem duplication

(ITD), a myeloproliferative disease developed, but not a transplantable leukaemia. However, when both *PML-RARα* and *FLT3-ITD* were expressed in murine marrow, AML developed with 100% penetrance, showing co-operativity between these two events. Furthermore, it has been argued that mutations in AML rarely affect more than one gene involved in differentiation or proliferation; for instance, *RAS* and *FLT3-ITD* mutations rarely occur together in the same leukaemic cell (Kelly and Gilliland, 2002).

Hanahan and Weinberg have described six hallmarks of cancer: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg, 2011). The two-hit model of leukaemogenesis does not take into account many of these features, and it is becoming apparent from whole genome sequencing data that there are a large number of somatically acquired genetic alterations in AML cases (Ding et al., 2012; Ley et al., 2010; Ley et al., 2008; Mardis et al., 2009). Many of these mutations are likely to be 'passenger' mutations that do not confer a selective advantage to cells, and may have been present by chance in the cell of origin or acquired as a result of genetic instability during tumour progression (Stratton et al., 2009; Welch et al., 2012). Determining which of the somatic mutations are so-called 'driver' mutations (those providing a survival or growth advantage to cells) is an area of on-going study, but is crucial in identifying dysregulated pathways that may serve as drug targets. Further details regarding the multi-hit model of tumourigenesis and clonal heterogeneity in leukaemia are discussed in the introduction of Chapter 4.

Table 1.2 World Health Organisation classification of AML

- **AML with recurrent genetic abnormalities**
  - AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
  - AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
  - Acute promyelocytic leukaemia (APML) with t(15;17)(q22;q12); *PML-RARA* (note that fusion partners of *RARA* other than PML are no longer considered together as the same entity)
  - AML with t(9;11)(p22;q23); *MLLT3-MLL*
  - AML with t(6;9)(p23;q34); *DEK-NUP214*
  - AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
  - AML with mutated *NPM1* (provisional entity)
  - AML with mutated *CEBPA* (provisional entity)
- **AML with myelodysplasia-related changes**
- **Therapy-related myeloid neoplasms**
- **Myeloid sarcoma**
- **Myeloid proliferations related to Down syndrome**
  - Transient abnormal myelopoiesis
  - Myeloid leukaemia associated with Down syndrome (usually megakaryocytic)
- **Blastic plasmacytoid dendritic cell neoplasm** (previously **'**agranular CD4+/CD56+ hematodermic neoplasm' and 'blastic NK-cell lymphoma')
- **AML Not Otherwise Specified (NOS)**
  - AML with minimal differentiation
  - AML without maturation
  - AML with maturation
  - Acute myelomonocytic leukaemia
  - Acute monoblastic and monocytic leukaemia
  - Acute erythroid leukaemia
  - Acute megakaryoblastic leukaemia
  - Acute basophilic leukaemia
  - Acute panmyelosis with myelofibrosis

(According to Arber DA, 2008; Vardiman et al., 2009)

1.3.6 Prognostication in AML

Although there are a multitude of international published studies that have reported on prognosis in AML, the following section focuses on data from the UK Medical Research Council (MRC) trials, which are broadly in line with other trials and generally consist of large patient cohorts. In the first AML MRC trial conducted between 1970-1979, there was a 5-year overall survival (OS) of approximately 5% for young adult patients (<60 years old). There has been an incremental improvement in OS with each consecutive MRC trial, relating partly from changes in therapy, but also to improvements in supportive care, and current 5-year OS approaches 50% (Burnett et al., 2011; Burnett et al., 2010; Grimwade et al., 1998). However, there is marked heterogeneity in outcome in particular patient subsets according to clinical, cytogenetic and molecular criteria. Thus, attempts have been made to develop prognostic models that can predict outcome, which would enable the development of a risk-adapted approach to patient therapy. In this way, low-risk patients could benefit by avoiding highly toxic therapies such as allogeneic stem cell transplantation, which could be reserved for high-risk patient subgroups.

1.3.7 Clinical prognostic factors

Clinical attributes have been shown to have a significant impact on patient outcome in multiple UK AML MRC trials (Burnett et al., 2011; Burnett et al., 2010; Wheatley et al., 1999). These include:

1. Age and performance status – advanced age has been associated with a poor prognosis in all adult UK MRC AML trials. Despite the advances in outcome with therapy for young adult patients (age 18 to 60 years), outcome for older patients (>60 years) remains dismal and has changed little. This partly relates to the higher proportion of patients in this category with poor performance status and co-morbidities who are unfit for intensive treatment with curative intent, experience higher treatment-related mortality and are not candidates for allogeneic transplantation. However, the biology of disease is somewhat different than younger patients, with a higher incidence of resistant disease in this age group, many with preceding MDS.

2. Blast cell count – a high PB blast cell count at presentation is associated with poor prognosis (Kottaridis et al., 2001; Wheatley et al., 1999).

3. Response to therapy - patients with resistant disease have a poorer OS. In the MRC AML10 trial, patients with greater than 15% blasts after their first course of induction had a 5 year OS of 22% compared to 53% in patients with less than 5% blasts (Wheatley et al., 1999). This does not hold true for acute promyelocytic leukaemia (APML), where the presence of greater than 5% blasts after induction is relatively common and does not convey an adverse prognosis. Minimal residual disease (MRD) analysis using flow cytometry or molecular abnormalities specific to the leukaemic blast population allows a more sensitive indication of response than is possible by morphological analysis (limit 1%) (Al-Mawali et al., 2009). Recent data using quantitative PCR (qPCR) directed at mutant nucleophosmin-1 *(NPM1)* as a means of assessing MRD, has shown that patients who are MRD-positive (>1 blast cell in 10^5 normal cells) at the end of double induction therapy have higher rates of relapse compared with MRD-negative patients (4-year cumulative incidence of relapse 6.5% for MRD-negative versus 53% for MRD-positive patients) (Kronke et al., 2011).

1.3.8 Cytogenetic abnormalities as prognostic factors

Data from over 1600 patients treated on the MRC AML10 trial and published 15 years ago showed that cytogenetic analysis has important prognostic implications for patient outcome (Grimwade et al., 1998). According to the particular cytogenetic abnormality, patients were divided into three risk groups, favourable, adverse, or intermediate:

- The favourable cytogenetic group (24% of patients) included patients with APML with the t(15;17) translocation, or core binding factor (CBF) leukaemias with inv(16) or t(8;21). The 5 year OS for this group was 72%, although patients with t(15;17) fared significantly better than those with CBF leukaemias.
- In the adverse cytogenetic group (16% of patients), abnormalities included a complex karyotype (five or more abnormalities), monosomy 5,

monosomy 7, del(5q), abn (3q), or t(9;22), and this group had a 17% 5 year OS. Of note, Lowenberg's group have more recently reported that patients with two or more distinct chromosome monosomies have a particularly poor prognosis with a 4-year OS of 3% (Breems et al., 2008).

- The intermediate group (60% of patients) included those without either favourable or adverse risk cytogenetics, or those with 11q23 abnormalities, trisomy 8, trisomy 21, trisomy 22, del(9q), or del(7q). This group had a 44% 5 year OS.

This data has now been updated with nearly 6000 patients from the AML10, AML12 and AML15 MRC trials (Grimwade et al., 2010). The expanded dataset confirms the original findings, and adds a further level of refinement to some of the rarer cytogenetic aberrations. For instance, prognosis related to chromosome 11q23 abnormalities (previously grouped in the intermediate risk group) involving the mixed lineage leukemia (*MLL*) gene depends on the fusion partner. Poor prognosis was associated with t(6;11)(q27;q23) and t(10;11)(p12;q23), involving the *MLLT4* (*AF6*) and *MLLT10* (*AF10*) genes respectively, while t(9;11)(p21−22;q23), resulting in the *MLLT3-MLL* fusion, or t(11;19)(q23;p13), leading to the *ELL-MLL* fusion remained in the intermediate risk group.

For non-APML patients, the current UK MRC AML17 trial includes a risk scoring system based on outcomes of the AML12 and AML15 trials, that takes into consideration these cytogenetic criteria (www.aml17.cardiff.ac.uk/aml17/), with the highest score indicating the worst prognosis (Table 1.3). This system divides patients into three risk groups with OS of 63%, 47% and 24% for scores of <1.85, 1.85-2.62 and high >2.63 respectively (Burnett, 2006).

Risk stratifying the large group of intermediate-risk patients, which includes over half of all patients, has been challenging and the focus of research for many groups, particularly with regards to molecular markers in this disease.

Table 1.3 Scoring system used on the UK MRC AML17 trial for risk stratification to determine prognosis

0.01325 x age (in years) + 0.16994 x sex (1=male, 0=female) + 0.22131 x diagnosis (0=de novo, 1 secondary) + 0.65082 x cytogenetics (1=favourable, 2=intermediate, 3 adverse) + 0.19529 x status post course 1 (1=complete remission, 2=partial remission, 3=no response) + 0.00169 x WBC ($x10^9$/l)

Patients are divided into three risk groups with OS of 63%, 47% and 24% for scores of <1.85, 1.85-2.62 and high >2.63 (Burnett, 2006).

1.3.9 Molecular markers as prognostic factors

Some of the somatic mutations identified in the presenting AML samples have proven to be important prognostic markers in multiple different AML trials (Lowenberg, 2008). Such markers have the potential to be particularly useful in patients with cytogenetically normal AML, who have a highly heterogenous outcome. One of the earliest prognostic molecular markers identified, associated with an increased relapse rate and poor outcome in AML was a *FLT3-ITD*, found in about one quarter of patients (Kottaridis et al., 2001). The discovery of *NPM1* mutations in approximately 30% of AML cases (Falini et al., 2005) added considerably to risk stratification and helped further stratify the *FLT3-ITD* positive group. For example, data from the MRC AML10 and 12 trials identified three prognostic groups – good risk (*FLT3-ITD* negative/*NPM1* positive), intermediate risk (*FLT3-ITD* negative/*NPM1* negative or *FLT3-ITD* positive/*NPM1* positive), and poor risk (*FLT3-ITD* positive/*NPM1* negative), with 5 year OS of 61%, 38% and 9% respectively (Gale et al., 2008).

Analysis of somatic mutations and aberrant expression of a number of other genes has also been useful in prognostication and some are summarised in Table 1.4. In recent years, whole genome sequencing (WGS) has been integral in identifying a number of recurrent mutations in AML that would have been hard to predict using a hypothesis-driven candidate gene approach. Interestingly, the first AML genome sequenced

identified a *FLT3-ITD* and an *NPM1* mutation, but no other recurrent mutations (Ley et al., 2008). The importance of depth of sequence coverage and appropriate bioinformatic analysis of WGS is best highlighted by the fact that repeat analysis from the same patient identified a DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*) mutation, which is highly recurrent in AML (approximately 20% of patients) and associated with a poor prognosis (Ley et al., 2010; Ribeiro et al., 2012; Thol et al., 2011). WGS was also used to identify mutations in isocitrate dehydrogenase *IDH1*, which occur in 7% of patients and are strongly associated with *NPM1* mutations and can be associated with a poor prognosis (Mardis et al., 2009; Schnittger et al., 2010). On the MRC trials, *IDH1* mutation status was an independent adverse factor for relapse in *FLT3-ITD* negative patients and a favourable factor in *FLT3-ITD* positive patients. Recurrent mutations have also been found in *IDH2* in approximately 10% of AML patients (Gross et al., 2010). The R140 mutation in *IDH2* was associated with an improved prognosis, while *IDH2* R172 mutations were associated with a poor prognosis and relapse rate of 72% (Green et al., 2011). Other recurrent mutations with potential prognostic significance include ten-eleven translocation-2 (*TET-2*) and BCL-6 co-repressor (*BCOR*), both of which have been associated with a poor prognosis (Chou et al., 2011; Grossmann et al., 2011; Weissmann et al., 2012).

With the ongoing sequencing effort in AML, it is likely that more genetic mutations will be identified, many of which are likely to be of low frequency and uncertain prognostic significance. Their clinical utility will have to be taken into account in the context of the other known prognostically significant molecular markers. For instance, one group recently reported that the favorable effect of *NPM1* mutations was restricted to patients with co-occurring *NPM1* and *IDH1* or *IDH2* mutations (Patel et al., 2012). How molecular stratification should influence treatment choice remains an area of controversy, and will need to be tested prospectively in large national trials.

Table 1.4 Selected gene mutations and aberrantly expressed genes shown to be associated with prognosis in AML

Gene mutations associated with poor prognosis	
	FLT3-ITD positive in *NPM1* WT patients (Gale et al., 2008; Rockova et al., 2011)
	WT1 mutation (Paschka et al., 2008; Virappane et al., 2008)
	MLL-PTD (Dohner et al., 2002; Patel et al., 2012)
	IDH1 mutation in *FLT3-ITD* negative patients (Green et al., 2010a)
	IDH2 (R172 mutation) (Green et al., 2011)
	DNMT3A mutation (Ribeiro et al., 2012; Thol et al., 2011)
	BCOR (Grossmann et al., 2011)
	TET2 (Chou et al., 2011; Weissmann et al., 2012)
Gene mutations associated with good prognosis	
	CEBPA biallelic mutations (Green et al., 2010b; Wouters et al., 2009)
	NPM1 mutation in *FLT3-ITD* WT patients (Gale *et al,* 2008)
	IDH1 mutation in *FLT3-ITD* positive patients (Green et al., 2010a)
	IDH2 (R140 mutation) (Green et al., 2011; Patel et al., 2012)
Gene overexpression associated with poor prognosis	
	ERG (Marcucci et al., 2007)
	MN1 (Langer et al., 2009)
	BAALC (Langer et al., 2008)

BAALC, Brain and leukaemia cytoplasmic gene. CEBPA, CCAAT enhancer binding factor gene. ERG, Ets-related gene. IDH, isocitrate dehydrogenase gene. MN1, Meningioma 1 gene. NPM1, Nucleophosmin 1 gene. PTD, Partial tandem duplication. WT, Wild type. WT1, Wilm's tumour-1 gene.

1.3.10 Current chemotherapeutic approaches in non-APML AML

Detailed guidelines for the treatment of AML in the UK have been published (Milligan et al., 2006). Treatment is broadly divided into induction and consolidation therapy. The standard approach in the UK has been to give two induction courses consisting of daunorubicin together with cytarabine (Ara-C), so called '3+7' due to the respective number of days given of each drug. If patients achieve remission after two courses of induction therapy, they require further consolidation therapy to prevent relapse from residual leukaemic blasts. Consolidation therapy typically consists of a course of MACE (amsacrine, cytarabine, etoposide) followed by MidAC (mitoxantrone, cytarabine).

Some of the areas of clinical uncertainty, which are currently under investigation, include:

1. Number of required consolidation cycles: this remains controversial. Both the MRC AML12 and AML15 trials found no benefit of a third consolidation course, with the reduction in relapse rate nullified by the toxicity of additional treatment. Patients on the AML12 and AML15 trial who were only able to receive three total courses of therapy had an outcome that was not statistically worse than those who received four. Thus, AML17 is prospectively randomising patients to a total of three versus four courses of therapy (www.aml17.cardiff.ac.uk/aml17/).
2. Optimal drug dosage: there is data to suggest that both elderly and young adult patients may benefit from higher dose daunorubicin (90mg/m^2 as opposed to standard dose 45mg/m^2) at induction (Lee et al., 2011; Lowenberg et al., 2009). Of note, high-dose daunorubicin was shown to have a survival benefit only among patients with *DNMT3A*, *NPM1* mutations or *MLL* translocations, but not among patients wild-type for these genes. High dose daunorubicin is currently being tested in the UK MRC AML17 trial.
3. Monoclonal antibody therapy: Gemtuzumab ozogamicin (Mylotarg), an anti-CD33 monoclonal antibody conjugated to the calicheamicin toxin, has recently received significant publicity. Although the drug has significant anti-leukaemic properties, it was associated with an increased risk of veno-occlusive disease and was withdrawn from the US market in 2010 following an increased risk of death in the Mylotarg arm at induction on the SWOG

S0106 trial (16 of 283 [5.7%] died at induction on the Mylotarg/cytarabine/daunorubicin arm vs. 4 of 281 [1.4%] on the cytarabine/daunorubicin alone arm, P=0.01) (Petersdorf 2009). However, on the UK MRC AML15 trial, there was a survival benefit for patients receiving Mylotarg in those with favourable cytogenetics and a trend towards improved survival without significant additional toxicity (Burnett et al., 2011). The French ALFA Group also showed an OS benefit for the addition of Mylotarg in patients aged 50-70 years (Castaigne et al., 2011). The differences in toxicity between the US and European trials likely relates to drug dosage and scheduling.

4. Alternative chemotherapeutic agents: cytarabine and daunorubicin have been the mainstay of induction therapy for over 20 years, and attempts to improve upon this combination have in general shown negative results. Large French and Japanese studies have shown no benefit in using idarubicin instead of daunorubicin at induction (Ohtake et al., 2011; Pautas et al., 2010). The UK MRC AML15 trial showed no difference in OS for FLAG-Ida (fludarabine/cytarabine/G-CSF/idarubicin) versus daunorubicin/cytarabine (Burnett et al., 2011).
5. Novel agents: a large number of novel agents are currently being tested in AML, and deciding on which should be included in national trials is challenging. A 'Pick-a-winner' approach has been adopted by the AML MRC trials (Hills and Burnett, 2011). This is a modification of the "Multi Arm Multi Stage" (MAMS) design of Royston *et al* (2003) (Royston et al., 2003) where, rather than sequentially testing agents against a control arm in a randomised fashion, multiple novel agents are tested in parallel against a single control arm or historical data. If an agent reaches a pre-specified outcome, it is selected for further study, while inferior drugs are dropped. This strategy has the potential to expedite early phase trial results and maximises the number of patients recruited into the investigational treatment arms. Such a strategy is underpowered to identify drugs that offer minor incremental benefits, and best reserved for patients whose outcome remains extremely poor and where the need for drugs with meaningful efficacy is greatest, such as for elderly AML patients or those at relapse (Hills and Burnett, 2011).

1.3.11 Use of transplantation in AML

The use of autologous transplantation (high dose chemotherapy with autologous stem cell rescue) instead of a course of consolidation chemotherapy was tested in the UK MRC AML10 trial. Although there was a modest improvement in OS in the first analysis, this was not substantiated in recruitment of 330 additional patients, and is no longer standard practice in the UK (Burnett et al., 1998). Furthermore, it had proven particularly difficult to obtain adequate stem cell harvest from patients following prior intensive chemotherapy.

It is universally accepted that patients with favourable cytogenetics, particularly APML, should not receive allogeneic transplantation in first remission. Deciding on which patients are most likely to benefit from allogeneic transplantation has been an area of interest and controversy worldwide (reviewed in Koreth et al., 2009). Myeloablative transplantation has consistently shown a reduction in relapse risk due to a graft-versus-leukaemia effect (GvL). However, in terms of OS, the reduction in relapse is offset by significant transplant-related mortality (TRM), which can be in excess of 40% in older patients groups treated with myeloablative non-sibling allografts (Alyea et al., 2005). Although it is less effective than myeloblative transplantation in reducing relapse risk, reduced intensity (non-myeloablative) transplantation significantly reduces TRM and is being increasingly used in patients more than 45 years of age (Alyea et al., 2005; Sorror et al., 2011; Storb, 2007). The use of prognostic markers that can identify patients at high-risk of relapse who may benefit most from such procedures is an area of ongoing study, and large prospective randomised studies taking into account the increasing number of markers are likely to be required to address this question comprehensively.

1.4 Acute lymphoblastic leukaemia (ALL)

ALL arises from lymphoid progenitor cells, and is subclassified as B-ALL or T-ALL depending on the expression of B-cell markers (cytoplasmic CD79a, CD19, HLA-DR) or T-cell markers (cytoplasmic CD3, with CD7 plus CD2 or CD5) (Swerdlow, 2008). Further subclassification of ALL is given below.

1.4.1 Incidence of ALL

ALL has a bimodal pattern of onset, with a major peak in early childhood (around 3 years of age), and an increase in incidence after middle age. Childhood ALL is the commonest childhood malignancy, accounting for 24% of all malignancies, with an annual incidence of 3-4 cases per 100,000 (Dores et al., 2012), whilst adult ALL has an incidence of 1.3 cases per 100,000 (Bassan et al., 2004).

1.4.2 Causative/predisposing factors in ALL

Similar to AML, the majority of cases of ALL are idiopathic. In both children and adults, there is a slight male preponderance, at a ratio of 1.4:1. For reasons that are not clear, the incidence is highest in higher socio-economic groups and in the most developed countries (Switzerland has the highest worldwide incidence), and there has been a moderate but steady increase in both childhood and adult ALL worldwide over the last 20 years (Dores et al., 2012). Hispanic groups are also at significantly higher risk of developing ALL than other ethnic groups. Other risk factors for ALL development (as reviewed in the National Institute of Health website: http://www.cancer.gov/cancertopics/pdq/treatment/childALL/) include:

1. Genetic disorders:
    a. Chromosomal disorders: Down's syndrome (trisomy 21), where there is a 20 fold increased risk for the development of ALL
    b. Inherited/congenital genetic defects – ataxia telangiectasia, Bloom syndrome, neurofibromatosis, and Shwachman-Diamond syndrome
2. X-ray exposure:
    a. Foetal exposure to X-rays
    b. Postnatal X-ray exposure. The classic example here being the so-called 'ringworm affair', in which over 200,000 children were treated with X-ray therapy in the 1940-1950s, predominantly to the scalp, for the treatment of tinea capitis (ringworm) (Crossland, 1956), resulting in brain tumours, skin cancers and ALL (Shore et al., 2003).[1]

[1] A selected abstract of the original Crossland paper (Crossland 1956) is worth noting here. 'Roentgen ray epilation [X-ray], which is at present the most important single weapon against M. audouini infection of the scalp, should not be withheld except in special circumstances, from a child who has the disease. It can quickly cure the disease and halt spread from one child to another. Harmful sequelae and medicolegal complications can be averted by competent use of the method…The Keinbock-Adamson technique of epilation can be recommended without hesitancy'

3. Single nucleotide polymorphisms (SNP). Genome-wide association studies have shown an association of specific SNPs at the *ARID5B* and *CEBPE* loci with an increased risk of B-ALL (Papaemmanuil et al., 2009).

1.4.3 WHO classification of ALL

Despite the FAB system categorising ALL according to morphological criteria (L1, L2 or L3 morphology) (Bennett et al., 1981), the majority of patients with L3 morphology (typically associated with the presence of numerous cytoplasmic vacuoles) have Burkitt's lymphoma and should be treated on appropriate protocols. Distinguishing between the L1 and L2 subgroups does not provide robust prognostic information or influence therapy, and for that reason is no longer used in routine practice. The 2008 WHO classification of ALL takes into account cellular immunophenotype and cytogenetic abnormalities, and is shown in Table 1.5 (Swerdlow, 2008).

Table 1.5 World Health Organisation classification of ALL

- B-lymphoblastic leukaemia/lymphoma not otherwise specified
- B-lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities:
    - t(9;22) *BCR-ABL*
    - t(v;11q23) 11q23 MLL rearrangement
    - t(12;21) *ETV6-RUNX1*
    - hyperdiploidy (>50 chromosomes)
    - hypodiploidy (<45 chromosomes)
    - t(5;14) IL3/IGH
    - t(1;19) E2A/PBX1
- T-lymphoblastic leukaemia/lymphoma

(According to Swerdlow, 2008)

1.4.4 European Group for the Immunological Characterization of Leukaemias (EGIL) classification of ALL

The EGIL classification relies purely on a flow cytometric assessment of blast immunophenotype to characterise ALL (Table 1.6), and the descriptive terminology used is still common in current practice. The advantages of this approach are that it assesses the extent of cellular differentiation, can be standardised between laboratories, has prognostic value in some categories, and predicts the presence or absence of particular underlying genetic/cytogenetic lesions (for instance, *MLL* gene rearrangements are common in CD10 negative B-ALL). The disadvantages are that, on occasion, some leukaemias do not display a characteristic immunophenotype despite being clearly of a particular lineage by cytochemical or morphological analysis, and it does not take into account cytogenetic abnormalities.

Table 1.6 Flow cytometric markers used to distinguish subtypes of ALL according to the EGIL classification

	Positive markers	Negative markers	Approximate percentage of ALL cases
B-ALL			
Pro B-ALL	HLA-DR+ TdT+ CD19+	CD10-, CyIg-	10%
Common ALL	CD10+ TdT+	CyIg-	50%
Pre-B-ALL	CyIg+ CD10+ TdT+		10%
Mature B-ALL*	SmIg+	TdT-, CD34-	4%
T-ALL			
Pro-T-ALL	TdT+ CD7+ CyCD3+	CD2-, CD4-, CD8-	7%
Pre-T-ALL	TdT+ CD7+ CyCD3+ CD2+	CD4-, CD8-	1%
Cortical/thymic T-ALL	TdT+ CD7+ CyCD3+ CD1a+ CD2+ CD5+ CD4+ CD8+		17%
Mature T-ALL	CD7+ SmCD3+ CD2+ CD4 or CD8+	TdT-, CD1a-	1%

B-ALLs can be CD79a±, and/or CD22±, and/or CD34±. T-ALLs can be CD33±, and/or CD34±, and/or CD13±. Cy, cytoplasmic. Ig, immunoglobulin; Sm surface. *Majority are Burkitt's lymphomas. Table according to (Bene et al., 1995).

1.4.5 EGIL classification of biphenotypic leukaemia

Biphenotypic leukaemias constitute a rare subgroup of leukaemias with a poor prognosis where the blast population co-expresses antigens of different cell lineages. This is distinct from the presence of two separate blast populations of different lineages, which should be termed 'bilineage leukaemia'. Using the EGIL classification points system, a score of greater than 2 for myeloid markers plus greater

than 1 for lymphoid markers is considered to meet the criteria for biphenotypic leukaemia (Table 1.7) (Bene et al., 1995).

The use of such scoring systems is important because it distinguishes these select subgroups from leukaemias that express a single marker of an alternate lineage, which is a common occurrence in both AML and ALL. For instance, approximately 20-25% of AML patients co-express T-cell markers such as CD7 and CD5 (Casasnovas et al., 2003). Since the 1980s it has been debated whether these aberrantly expressed markers relate to 'lineage promiscuity' or 'lineage infidelity'. Greaves proposed the lineage promiscuity argument as 'the existence of a transient phase of limited promiscuity of gene expression occurring in normal bipotential or multipotential progenitors and able to be preserved as a relic in leukaemic blast cell populations that are in maturation arrest' (Greaves et al., 1986), whilst McCulloch hypothesised that the aberrant expression of markers of different lineages related to lineage infidelity, explained as the 'misprogramming of differentiation in leukaemia' (McCulloch, 1983). It is very possible that both arguments hold true in particular settings. Such aberrant markers, whatever their origin, have proved useful in differentiating malignant clonal populations from normal cells when analysing samples for MRD by flow cytometry, as discussed below.

Table 1.7 EGIL classification for the diagnosis of biphenotypic leukaemia

<table>
<tr><th></th><th>B-lineage</th><th>T-lineage</th><th>Myeloid lineage</th></tr>
<tr><td>2 points</td><td>CD79+ IgM+ CD22+</td><td>CD3+
Anti TCR+</td><td>MPO+</td></tr>
<tr><td rowspan="3">1 point</td><td>CD19+</td><td rowspan="3">CD2+ CD5+ CD8+
CD10+</td><td>CD13+</td></tr>
<tr><td>CD10+</td><td>CD33+</td></tr>
<tr><td>CD20+</td><td>CD65+</td></tr>
<tr><td rowspan="2">0.5 point</td><td>TdT+</td><td rowspan="2">TdT+ CD17+ CD10+</td><td rowspan="2">CD14+
CD15+
CD64+
CD117+</td></tr>
<tr><td></td></tr>
</table>

MPO, myeloperoxidase. A score of >2 points for myeloid markers plus >1 point for lymphoid markers is considered to meet criteria for biphenotypic leukaemia. Table according to (Bene et al., 1995)

1.5 T-ALL

1.5.1 Clinical characteristics of T-ALL

T-ALL accounts for 10-15% of paediatric and approximately 25% of adult ALL cases (Pui and Evans, 1998). It has a male preponderance, at a ratio of 3:1, and is associated with very high circulating blast cell counts, the presence of a mediastinal mass, and increased risk of leukaemic infiltration of the central nervous system (CNS) at the time of diagnosis (Marks et al., 2009; Pui and Evans, 1998).

1.5.2 Cytogenetic aberrations in T-ALL

The most common cytogenetic abnormality (>70% of patients) in T-ALL is deletion of chromosome 9p. The deletions are usually large and occasionally homozygous, and involve the *CDKN2A* locus that encodes the cell cycle regulatory tumour suppressor genes *p16INK4A* and *p14ARF,* that are encoded from alternate reading frames of the same locus (Marks et al., 2009). Most of the important discoveries of the molecular anatomy of T-ALL have come from the study of rare, specific chromosomal translocations and intrachromosomal rearrangements. These typically juxtapose promoter elements or enhancers of the T-cell receptor (TCR) beta and alpha/delta genes (on chromosomes 7q34 and 14q11 respectively) to transcription factors that have roles in normal T-cell differentiation (Armstrong and Look, 2005; Ferrando et al., 2002; Look, 1997; Van Vlierberghe and Ferrando, 2012). Typical translocation partners of the TCR loci include basic helix-loop-helix (bHLH) transcription factors such as *TAL1* and *LYL1,* LIM-only domain (*LMO*) genes such as *LMO1* and *LMO2,* and the orphan homeobox genes *HOX11* and *HOX11L2* (Table 1.8) (Van Vlierberghe and Ferrando, 2012). Several transgenic mouse models have been developed where T-ALL is induced by overexpression of one of these transcription factors, including *Tal1, Lmo1/2,* and *Tlx*1 (De Keersmaecker et al., 2010; Larson et al., 1996; Tremblay et al., 2010). Translocations have also been identified juxtaposing TCR enhancer elements to oncogenes including intracellular *NOTCH-1 (ICN-1)* and *c-MYB* (Table 1.8), and although the translocations themselves are extremely rare, study of these genes has led to the identification of highly recurrent genetic abnormalities, including activating mutations of *NOTCH-1* in over 50% of patients and duplication of the *MYB* locus in 10% of patients (O'Neil et al., 2007b; Weng et al., 2004).

Table 1.8 Frequency of cytogenetic translocations identified in T-ALL

Gene family	Gene name	Cytogenetic aberration	Prognostic implication	Frequency in T-ALL
bHLH family members	*TAL1*	t(1;14)(p32;q11)	Good[1-3]	3%
		t(1;7)(p32;q34)	Good[1-3]	3%
		1p32 deletion	Good[1-3]	16%–30%
	TAL2	t(7;9)(q34;q32)		1%
	LYL1	t(7;19)(q34;p13)		1%
	BHLHB1	t(14;21)(q11.2;q22)		1%
LMO family members	*LMO1*	t(11;14)(p15;q11)		1%
		t(7;11)(q34;p15)		1%
	LMO2	t(11;14)(p13;q11)		6%
		t(7;11)(q34;p13)		6%
		11p13 deletion		3%
	LMO3	t(7;12)(q34;p12)		<1%
Homeobox family members	*TLX1(HOX11)*	t(10;14)(q24;q11)	Good[3]	5-10%
	TLX3(HOX11L2)	t(5;14)(q35;q32)		5-20%
	HOXA	inv(7)(p15q34)		3%
		t(7;7)(p15;q34)		3%
	HOXA (CALM-AF10)*	t(10;11)(p13;q21)		5-10%
	HOXA (MLL-ENL)*	t(11;19)(q23;p13)		1%
	HOXA (SET-NUP214)*	9q34 deletion		3%
		inv(14)(q11.2q13)		
	NKX2.1	inv(14)(q13q32.33)		5%
		t(7;14)(q34;q13)		
	NKX2.2	t(14;20)(q11;p11)		1%
Oncogene	*NOTCH-1*	t(7;9)(q34;q34)		<1%
	MYB	t(6;7)(q23;q34)		3%
	C-MYC	t(8;14)(q24;q11)		2%
Cell cycle	*CDKN2A/B*	Deletion 9p21		>70%
	CCND2	t(7;12)(q34;p13)		1%
		t(12;14)(p13;q11)		
	RB1	Deletion 13q14		4%
Signal transduction	*NUP214-ABL1*	Episomal amplification of 9q34	Poor[4]	4%

The 7q34 lesions involve the TCR beta enhancer/promoter and 14q11 lesions involve TCR alpha/delta enhancer/promoter. Other translocation partners are shown in brackets. *These translocations have been show to directly upregulate the *HOXA* cluster and so are included in the *HOXA* category here. Absent data in the prognosis column signifies no comprehensive outcome data available or have no prognostic significance. (Table modified from Van Vlierberghe and Ferrando, 2012), including additional data from http://atlasgeneticsoncology.org. [1](Kikuchi et al., 1993); [2](Bash et al., 1993); [3](Cave et al., 2004); [4](Graux et al., 2004). *ABL1* c-abl1 oncogene; *BHLHB1* Beta helix-loop-helix B1 gene; *C-MYC* myelocytomatosis viral oncogene homolog; *CCND2* Cyclin D2 gene; *CDKN2A* cyclin-dependent kinase inhibitor 2A; *HOXA* homeobox; *LMO* Lim domain only gene; *LYL1* lymphoblastic leukemia derived sequence-1 gene; *MYB* myeloblastosis viral oncogene homolog gene; *NKX* NK homeobox gene; *NUP214* nucleoporin 214kDa gene; *TAL* T-cell acute lymphoblastic leukemia gene; *RB1* retinoblastoma-1 gene; *TLX* T-cell leukemia homeobox gene.

1.5.3 Somatic mutations in T-ALL

Driven by technological advances such as copy number analysis by array comparative genomic hybridisation (aCGH) and SNP arrays, exome capture and whole genome sequencing, a wealth of somatic mutations have now been identified in T-ALL (Table 1.9). Many of these have been discovered since the work for this thesis was undertaken.

Table 1.9 Frequency of somatic mutations identified in T-ALL

Type of mutation	Mutated gene (approximate incidence in T-ALL)
Activating mutations (oncogenes)	• *NOTCH-1* (60%) (Weng et al., 2004) • *IL7R* (10%) (Zhang et al., 2012) • *JAK1* (1% children; 18% adults) (Flex et al., 2008) • *JAK3* (5%) (Zhang et al., 2012) • *NRAS* (5-10%) (Zhang et al., 2012) • *FLT3* (2%) (Zhang et al., 2012)
Inactivating mutations (tumour suppressor genes)	**Transcription factors:** • *RUNX1* (10-20%) (De Keersmaecker et al., 2010) • *LEF1* (10-15%) (Gutierrez et al., 2010b) • *ETV6* (13%) (Van Vlierberghe et al., 2011; Zhang et al., 2012) • *WT1* (10%) (Tosello et al., 2009) • *BCL11B* (10%) (Gutierrez et al., 2011; Zhang et al., 2012) • *GATA3* (5%) (Zhang et al., 2012) **Chromatin regulators:** • *PHF6* (20-40%) (Van Vlierberghe et al., 2010; Zhang et al., 2012) • *EZH2* (10-15%) (Ntziachristos et al., 2012; Simon et al., 2012; Zhang et al., 2012) • *SUZ12* (10%) (Ntziachristos et al., 2012; Zhang et al., 2012) • *EED* (10%) (Zhang et al., 2012) **Signal transduction:** • *PTEN* (10%) (Palomero et al., 2007; Zhang et al., 2012) • *NF1* (3%) (Zhang et al., 2012) **Ubiquitin ligase:** • *FBXW7* (20%) (Maser et al., 2007; O'Neil et al., 2007a; Thompson et al., 2007; Zhang et al., 2012)

1.5.4 Prognostication in T-ALL

Approximately 85-95% of paediatric patients and 35-50% of adult patients achieve long-term remission with ALL, depending on the treatment protocol (Goldstone et al., 2008; Hann et al., 2000; Larson, 2005). Historically, T-ALL had a particularly poor prognosis in comparison to B-ALL (Clavell et al., 1986; Steinherz et al., 1991) and, given the lack of effective agents, even led to an unsuccessful trial of thymectomy in these patients (Sallan, 1981). However, the introduction in recent years of intensified combination chemotherapy, particularly the inclusion of high dose methotrexate, has resulted in a remarkable improvement in the prognosis for this disease in children, with 5-year relapse-free survival rates now exceeding 80%, equivalent to B-ALL (Goldberg et al., 2003; Schrappe et al., 2000). In adult patients, the use of more aggressive combination chemotherapies has improved the OS rates to approximately 50%, which is a better prognosis than in adults diagnosed with B-lineage ALL (Goldstone et al., 2008; Hunault et al., 2004; Marks et al., 2009; Rowe et al., 2005).

Nonetheless, T-ALL patients with similar clinical features often respond quite differently to the same multi-drug regimen, creating a major obstacle to the development of risk-directed treatment strategies. Listed below are some of the variables that impact on patient outcome in ALL:

1) Age. Age remains one of the most important prognostic variables in terms of patient outcome for both B- and T-ALL. Excluding patients with infant ALL, who often harbour rearrangements of the *MLL* gene and fare particularly badly (Chessells et al., 1994), paediatric patients less than 10 years old tend to fare significantly better than those 10 years of age or more (Hann et al., 2000). Current paediatric treatment protocols in the UK (the UKALL 2003 protocol) offer reduced intensity therapy to those patients less than 10 years of age if they do not have other poor prognostic markers. In adults, age is a continuous variable inversely correlated with outcome, with lower complete remission (CR) rates, higher infective complications and less than 20% OS for patients greater than 60 years of age in the majority of studies (Larson, 2005; Sive et al., 2012). In B-ALL, a significant contribution can be attributed to a higher incidence in older patients of Philadelphia positive (Ph+) disease with the t(9;22) chromosomal translocation resulting in the *BCR-ABL* fusion transcript, which is associated with chemoresistance, and a lower incidence of the t(12;21) chromosomal translocation

resulting in the *ETV6-RUNX1* fusion transcript (known previously as *TEL-AML1*), which is associated with a better response to therapy.

Several studies have shown that adolescent patients and those in their early 20s have an inferior outcome when treated on adult ALL protocols compared to paediatric protocols (Boissel et al., 2003; de Bont et al., 2005; Hallbook et al., 2006; Ramanujachar et al., 2007; Usvasalo et al., 2008), and current amendments to protocols in the UK have attempted to address this. Although there are several other possible reasons for the disparate outcomes between paediatric and adult patients, such as higher cumulative doses of steroids and L-asparaginase, better tolerance to chemotherapy, and a lower incidence of protocol delays for paediatric patients, there appears to be an inherent difference in chemosensitivity between adult and paediatric ALL that as yet remains unexplained. For example, at the end of induction therapy, which consists of similar drug regimens in both children and adults, the incidence of MRD positivity is significantly higher in adults, suggesting that they have increased levels of inherent chemoresistance (Bruggemann et al., 2006; Patel et al., 2010). A comparison of the incidence of recently identified molecular abnormalities in these different age groups has the potential to elucidate mechanisms that may be associated with treatment response, which could add value both in terms of prognostication and, possibly, targeted therapy.

2) Presenting white cell count (WCC). WCC remains one of the most robust prognostic markers in both B- and T-ALL (Goldstone et al., 2008; Hoelzer et al., 1988; Rowe et al., 2005). For B-ALL a presenting WCC $<30x10^9$/l, and for T-ALL, a presenting WCC $<100x10^9$/l have been associated with improved OS.

3) Early treatment response. In the UK, it has become standard practice to perform BM aspirates on paediatric patients after one, two and four weeks of therapy to assess early treatment response. On the UK ALL2003 protocol, patients are classified by their marrow status at these time points as M1 (<5% blasts), M2 (5-25% blasts) and M3 (>25% blasts). This classification has significant prognostic value, with patients with M2 or M3 marrows faring significantly worse.

4) Minimal residual disease (MRD). In paediatric ALL, almost 60% of relapses occur from patients who have had a rapid treatment response by morphological criteria, which highlights the limited sensitivity of this technique for identifying residual leukaemia within the marrow. This has led to the development of MRD analysis, which aims to identify those patients with significant marrow disease that is below the limit of morphological sensitivity (1-5%), but which can be indicative of treatment failure. Ideally, MRD should be specific (able to distinguish malignant cells from normal cells), sensitive (able to detect at the very least one abnormal cell in 10^4 normal cells) and be amenable to standardisation so that results can be interpreted from different laboratories worldwide. There are two main approaches to MRD:
    a. Flow cytometry. Flow cytometric MRD requires the identification of an aberrant immunophenotype on the leukaemic clone that enables it to be distinguished from normal haematopoietic cells. For T-ALL, co-expression of T-cell markers such as CD2, CD7 and CD3 with markers of other lineages such the myeloid antigens CD13 or CD33, or the B-cell antigen CD19, can be used to identify an abnormal clone that is not present on normal cells (Campana and Coustan-Smith, 1999). The major limitations are the requirement for fresh samples, aberrant phenotypes are not always present on leukaemic cells, changes in immunophenotype can occur at relapse, and the sensitivity is inferior to qPCR techniques (limited to 1 leukaemic cell in 10^4-10^5 normal cells) (Coustan-Smith and Campana, 2010).
    b. Quantitative PCR (qPCR). Evaluation of rearrangements of the immunoglobulin genes to assess the levels of MRD at a variety of time points during therapy has proven to be highly predictive of relapse and outcome in B-ALL (Bruggemann et al., 2006). During normal T-cell development, lymphocytes first rearrange the Variable Diversity Joining (VDJ) segments of the T-cell receptor delta (*TCRδ)* and gamma *(TCRγ)* genes to form a *γδTCR*, and then the *TCRβ* and finally the alpha (*TCRα*) loci are rearranged to form a functional *αβ TCR* (Dadi et al., 2009; Krangel, 2009). VDJ recombination is mediated by the RAG recombinase-1 and-2 genes (*RAG-1* and *RAG-2*), and in order to produce a T-cell repertoire with extensive diversity, additional random DNA insertions and

deletions are introduced by the activity of terminal deoxynucleotidyl transferase (TdT). The cells then undergo a process of positive and negative selection that results in a polyclonal population of mature T-cells that are released from the thymus and are able to both recognise foreign antigens and tolerate self antigens. Consequently, PCR across the VDJ region of isolated T-cells in normal individuals produces a smear due to the extensive variation in DNA sequence length produced during VDJ recombination. However, in T-ALL, the clonal dominance of a T-cell clone can be identified by the presence of a specific intense band at PCR. The VDJ sequence is usually unique to the individual clone, therefore a primer designed to the specific sequence can be used for the sensitive detection of the clone by qPCR, which can then be expressed as a copy number or sensitivity, with a sensitivity as low as 1 abnormal cell in 10^6 normal cells (van Dongen et al., 2003). Monitoring of MRD by qPCR has proven highly predictive of outcome in large studies of T-ALL (Schrappe et al., 2011). Alternative methods, such as qPCR quantification of *SIL-TAL1* transcripts in patients harbouring the 1p32 microdeletion, have also been used successfully to monitor MRD in T-ALL (Breit et al., 1993).

5) Chromosomal aberrations. In B-ALL, there are well-defined cytogenetic groups that have robust associations with prognosis, such as improved outcome for patients with *ETV6-RUNX1* translocations and hyperdiploidy (>51 chromosomes), or poor outcome associated with hypodiploidy (<45 chromosomes), the *BCR-ABL* translocation, iAMP21 (*RUNX1* amplification) and rearrangements involving the *MLL* gene on 11q23 (Moorman et al., 2010). However, due to the rarity of recurrent translocations in T-ALL (Table 1.8), there are few cohort studies of adequate size to robustly address the impact of cytogenetics on outcome. The largest trial to date to assess this in adults is the UKALLXII/ECOG2993 trial, which identified complex karyotype (defined as 5 or more chromosomal abnormalities) as an adverse prognostic marker (Marks et al., 2009; Moorman et al., 2007). Deletions of the *CDKN2A* locus had no prognostic significance on this trial. Although it remains controversial, the majority of studies have shown improved outcome associated with translocations involving the *HOX11* or *TAL1* loci, and poorer prognosis associated with *NUP214-ABL1* episomal amplification

(Table 1.8) (Bash et al., 1993; Cave et al., 2004; Graux et al., 2004; Kikuchi et al., 1993). Apart from the latter, that may respond to treatment with tyrosine kinase inhibitors such as imatinib, these cytogenetic aberrations do not currently impact the choice of therapy in T-ALL.

6) Immunophenotype. Both adult and paediatric T-ALL with an immunophenotype similar to that of early cortical thymocytes (expressing CD1a) seem to respond particularly well to therapy (Marks et al., 2009). Whether chemosensitivity relates in some way to expression of *HOX11*, which is normally expressed during the early cortical stage and highly expressed in blasts with this immunophenotype, is unclear. It is possible that the association of *HOX11* translocation with improved prognosis reflects the differentiation arrest at the cortical stage, which is characteristic of such translocations (Asnafi et al., 2009; Cave et al., 2004).

7) Early T-cell progenitor (ETP) ALL. Patients with T-ALL and aberrant expression of CD13 and/or CD33 myeloid antigens have a poor prognosis (Marks et al., 2009), which raises the possibility that these cases originated from very early multipotent haematopoietic cells that are inherently more chemoresistant to ALL-directed therapy. Analysis of microarray data from 139 T-ALL patients identified a subgroup (12%) of T-ALL patients that had a gene expression signature highly similar to normal ETP cell subsets, and also overexpressed many myeloid-related genes including *CEBPA, GATA2, KIT* and *CD34* (Coustan-Smith et al., 2009). These patients had a characteristic flow cytometric immunophenotype with absent CD1a and CD8 expression, weak CD5 expression, and one or more of the following myeloid/stem cell antigens: CD117, CD34, CD13, CD33, CD11b or CD65. It should be noted that approximately half of the ETP-ALL patients would fulfil EGIL criteria for biphenotypic AML/T-ALL. In this study patients with ETP-ALL had a particularly dire outcome with a 10-year OS of 19% compared to 84% for their non-ETP counterparts. ETP leukaemias arise from early stem cells that have not undergone complete *TCRγ* rearrangement. An independent study used array comparative genomic hybridisation (aCGH) and qPCR to identify a subset of T-ALL patients with an absence of biallelic *TCRγ* deletion (so called 'ABD' patients) that were enriched in patients with the ETP phenotype and associated with a particularly poor prognosis (Gutierrez et al., 2010a).

8) Other factors. Historically, boys have tended to fare slightly worse than girls, partly, but not solely, due to the risk of testicular relapse, and this is addressed in current paediatric trials where maintenance therapy is extended by one year for boys. However, no difference in outcome according to sex was seen for adults with T-ALL entered onto the UKALLXII/ECOG2993 (Marks et al., 2009). Contrary to what one might expect, the presence of CNS disease at the presentation of T-ALL was not associated with a poorer prognosis on this trial (Marks et al., 2009).

1.5.5 Current chemotherapeutic approaches in ALL

A brief overview of the treatment approach for ALL in the UK is given below. Due to the differences in drug tolerability, adult and paediatric ALL treatment algorithms vary considerably between these two age groups. Paediatric regimens have also become increasingly risk-adapted (guided by age, cytogenetics, MRD, and marrow response), to enable treatment reduction in children at low risk of relapse and intensification for those at high risk. In adults, there is an increasing use of allogeneic transplantation, particularly non-myeloablative transplantation in older patients. Patients less than 25 years of age now receive a modified paediatric regimen in the UK.

On the paediatric UKALL2003 trial, the treatment regimen involves a sequential phase of induction, consolidation, intensification and maintenance therapy (www.ctsu.ox.ac.uk/research/mega-trials/leukaemia-trials/ukall-2003). This is given together with intrathecal CNS-directed therapy to treat and/or prevent CNS relapse. The treatment protocols are divided into regimens A, B and C depending on whether patients are standard-risk (children <10 years old with WCC<$50x10^9$/l), intermediate (children ≥10 years old or WCC >$50x10^9$/l) or high-risk (slow early response, high MRD at day 29, hypodiploid with less than 44 chromosomes), *BCR-ABL* positive, *RUNX1* amplification positive or *MLL* gene rearranged) respectively. Induction therapy using a three-drug regimen with steroids, vincristine, and asparaginase leads to remission in 95% of patients with standard-risk ALL (children <10 years old with WCC<$50x10^9$/l) (Pui and Evans, 2006). Intermediate and high-risk patients also receive daunorubicin at induction. Regimen A consists of two courses of interim maintenance (steroids, vincristine, 6-mercaptopurine and oral methotrexate), and two

courses of delayed intensification (steroids, vincristine, asparaginase, cyclophosphamide, cytarabine, 6-mercaptopurine), followed by maintenance therapy of steroids, vincristine, 6-mercaptopurine and oral methotrexate (2 years for girls, 3 years for boys). On the UKALLVIII trial, half of the patients were cured despite the lack of an intensification block (Eden et al., 1991), thus the UKALL2003 trial has randomised patients with low risk MRD between one and two intensification arms. Regimen B is similar but includes a consolidation phase after induction, consisting of cyclophosphamide, cytarabine, vincristine, asparaginase and 6-mercaptopurine (known as standard BFM consolidation). Regimen C is as for regimen B but the interim maintenance phases are replaced with the more intense escalating Capizzi regimen based on IV methotrexate.

Adult protocols tend to use the same drugs as for paediatric ALL but at differing doses and scheduling. On the adult ECOG2993/UKALLXII trial protocol (www.ctsu.ox.ac.uk/research/mega-trials/leukaemia-trials/adult-all/ukall-xii-ph-positive-patients/ukall-xii-archive/protocol/view), treatment consists of two courses of induction therapy (Phase I consists of steroids, vincristine, asparaginase, daunorubicin; phase II consists of cyclophosphamide, cytarabine and 6-mercaptopurine) and three courses of high dose methotrexate, followed by consolidation with further chemotherapy, autologous transplantation or allogeneic transplantation (as discussed below). Several centres worldwide have adopted the MD Anderson Hyper-CVAD regimen, which consists of courses of cyclophosphamide, vincristine, doxorubicin and dexamethasone, alternating with high dose methotrexate/cytarabine, with similar outcomes to the UKALLXII trial (Thomas et al., 2004). The addition of monoclonal antibodies against CD20 and CD22 for B-ALL and the use of the purine nucleoside analog nelarabine for T-ALL are currently being tested on the UKALL 14 trial.

Despite the similarity of the drugs used, the outcome between adult and paediatric ALL is markedly different. This, at least in part, relates to the high induction death rate in adults, which is 5-10%, as compared to less than 1% on most paediatric trials (Fielding, 2008). While a proportion of the mortality is related to higher risk of infective complications, there is a general poorer drug tolerance in adults (for instance, liver toxicity from asparaginase, cardiac toxicity from anthracyclines), and

significantly higher TRM. There was also a significant incidence of death in remission reported for patients on the UKALLXII protocol that may relate to drug and/or age-related complications (Goldstone et al., 2008).

1.5.6 Role of transplantation in adult and paediatric ALL

For adults entered on the UKALLXII/ECOG2993 trial, those less than 50 years old with an HLA-matched sibling donor received an allograft, whilst patients older than 50 years or lacking an HLA-matched donor were randomised to either consolidation therapy or an autologous transplant (Goldstone et al., 2008). This is the largest trial conducted to date in adult ALL and showed that patients who received autologous transplantation fared worse than those who received standard consolidation therapy (5 year OS 37% versus 46%, respectively). In a donor versus no-donor analysis, patients with a donor had a lower rate of relapse and the best outcome (5 year OS 53% versus 45%, respectively). As it is clear that patients who fail therapy have a very poor salvage rate, with only 7% of adult patients alive 5 years after relapse (Fielding et al., 2007), this would suggest that intensive therapy, including the use of allogeneic transplantation, should be given upfront to such patients, rather than being reserved for relapse. Unfortunately, the one group that did not benefit from allogeneic transplantation in terms of OS were those in the high-risk group (Goldstone et al., 2008). However, there was a higher TRM in these patients, relating, at least in part, from the older age of this subgroup. This has prompted the use of reduced intensity transplantation in older patients in the current UKALL14 trial.

In paediatric patients, allogeneic transplantation is generally reserved for patients at relapse. On the UKALL2003 trial, there are clear criteria for those at particularly high risk of relapse who should receive allogeneic transplant in first remission:

1. MLL rearrangement or hypodiploid (< 44 chromosomes) with day 28 M2 marrow (>5% blasts).
2. M3 marrow at day 28.
3. *BCR-ABL* positive ALL
4. t(17;19) (*E2A-HLF*) positive ALL
5. iAMP21 (amplification of *RUNX1*) with slow early response

1.6 The Notch signalling pathway

In 1917, the American geneticist and Nobel laureate Thomas Hunt Morgan first described a strain of the fruit fly, *Drosophila melanogaster*, with a notch in its wing margin that exhibited X-linked inheritance of the notch defect (Morgan, 1917). It was later discovered this was due to haploinsufficiency in a gene that earned its name from the defect - *notch*. In 1983, Spyros Artavanis-Tsakonas was the first to clone the *Drosophila notch* gene, which opened the way to many important discoveries relating to the mechanism of Notch signalling and its role in normal biology, embryology, and now cancer (Wharton et al., 1985). Because of the distinct phenotypes associated with either gain-of-function or loss-of-function of the Notch signalling pathway during development in *D.melanogaster* and *C.elegans*, many members of the Notch pathway were identified through forward genetic screens in these organisms. The gene terminology used for members of the Notch pathway in these species together with their human orthologues are given in Table 1.10 (modified from Bray, 2006). Whilst *D.melanogaster* have only one Notch gene, the number of Notch genes, ligands and members of the regulatory pathway increases with higher order species, reflecting the degree of tissue complexity.

1.6.1 Notch and embryonic development

Notch forms part of an ancient highly conserved evolutionary signalling pathway, with vital roles in the embryonic development of both invertebrates and vertebrates. The important developmental role of Notch was evident in Thomas Hunt Morgan's original 1917 publication (Morgan, 1917): *'*A female with notch wings carries the gene in one of her X-chromosomes and the normal allelomorph in the other X-chromosome. Half of her sons get the former and die, the other half get the latter X-chromosome and live.'

Consistent with this finding, homozygous *Notch-1* knockout mice die at E10.5 due to gross developmental abnormalities affecting multiple organs, many related to disorders in vascular development (Swiatek et al., 1994). Notch signalling has now been show to govern cell-fate specification of a wide number of different tissues, including the intestine, skin, retina, neuronal tissue, vasculature including cardiac tissue and valves, exocrine pancreatic tissue, mammary glands, and haematopoietic

tissue (Blaumueller and Artavanis-Tsakonas, 1997; Iso et al., 2003; Murtaugh et al., 2003; Sandy and Maillard, 2009; Uyttendaele et al., 1998; Wharton et al., 1985).

Table 1.10 Terminology used for members of the Notch pathway in different species

	Drosophila melanogaster	***Caenorhabditis elegans***	***Homo sapiens***
Notch receptors	*Notch*	*lin-12*	*NOTCH-1*
		glp-1	*NOTCH-2*
			NOTCH-3
			NOTCH-4
Notch ligands	*Delta*	*lag-2*	*Delta-like-1 (DLL-1)*
		apx-1	*Delta-like-3 (DLL-3)*
		arg-1	*Delta-like-4 (DLL-4)*
	Serrate	*dsl-1*	*Jagged-1 (JAG-1)*
			Jagged-2 (JAG-2)
CSL transcription factor	*Suppressor of hairless (Su(H))*	*lag-1*	*CBF1/RBPJ-K*
F-box E3 ubiquitin ligase	*Archipelago*	*sel-10*	*FBXW7/FBW7*
Co-activator	*Mastermind*	*lag-3*	*Mastermind-like-1 (MAML-1)*
			Mastermind-like-2 (MAML-2)
			Mastermind-like-3 (MAML-3)
Glycosyltransferase	*Fringe*		*Lunatic fringe (LFNG)*
			Manic fringe (MFNG)
			Radical fringe (RFNG)

(Modified from Bray, 2006)

1.6.2 The Notch pathway

The Notch signalling pathway is one of the most studied and best characterised in biology, and is outlined below (reviewed in Bray, 2006; Fortini, 2009; Kopan and Ilagan, 2009).

1.6.2.1 Notch receptors are activated by three cleavage steps

The Notch receptors are large single-pass type I transmembrane receptors of approximately 300kD. In humans there are four Notch receptors, *NOTCH-1, -2, -3* and *-4*, that share the same basic structure, exhibit approximately 70% sequence homology, and differ predominantly in the size of their extracellular domain. In the extracellular region they have a large epidermal growth factor (EGF)-like repeat domain responsible for binding to ligand (Figure 1.1). The extracellular LIN12-Notch-repeat (LNR) domain, comprised of 3 LNR regions (LNR-A, -B and -C) each coupled to a single calcium atom, was the first negative regulatory region (NRR) described in *C.elegans,* and associates with the heterodimerisation (HD) domain (Greenwald and Seydoux, 1990). Intracellular Notch (ICN) is comprised of an RBP-JK-associated module **(**RAM) domain, ankyrin repeat (ANK) domain, and C-terminal transactivation (TAD) and proline-glutamate-serine-threonine (PEST) domains (Figure 1.1), the functions of which are described below.

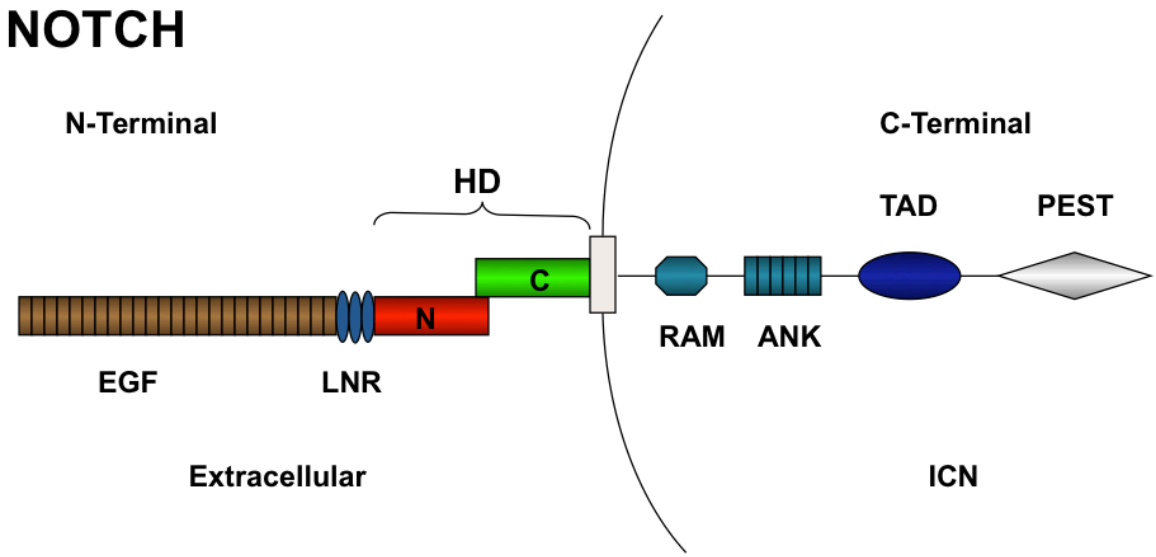

Figure 1.1 Structure of Notch receptors. ICN, intracellular Notch. EGF, Epidermal like growth factor repeats. LNR, Lin12-Notch repeats. HD, Heterodimerisation domain. RAM, RBP-Jkappa-associated module. ANK, Ankyrin repeat domain. PEST, proline (P) glutamic acid (E) serine (S) threonine (T) rich domain. Modified from Weng *et al* 2004.

When full-length Notch is *en route* to the cell membrane, it is first cleaved in the Golgi by a furin-like convertase at a site termed S1 in the HD domain at a position approximately 70 amino acids external to the transmembrane domain (Figure 1.2) (Logeat et al., 1998). The resulting two subunits of the Notch receptor, commonly referred to as the Notch extracellular [N(EC)] and Notch transmembrane [N(TM)] subunits, are held together by a non-covalent bond maintained by interaction between the LNR and HD domain. Crystallography data has shown that the LNR forms a tight protective packaging interface over the HD domain, preventing access of tumour necrosis factor-α converting enzyme (TACE/ADAM17) to the S2 cleavage site (Gordon et al., 2009; Gordon et al., 2007; Sanchez-Irizarry et al., 2004). Tight control of S2 cleavage is one of the key regulatory steps maintaining Notch in its quiescent state, and Notch alleles with a deletion of the LNR domain are constitutively active (Greenwald and Seydoux, 1990). The physical pulling force generated by endocytosis on the Notch extracellular region after ligand binding is thought to uncouple the LNR from its protective interaction with the HD, exposing the S2 site to cleavage by TACE, the so-called 'lift-and-cut' mechanism (Figure 1.3) (Gordon et al., 2008; Gordon et al., 2007).

Critical to stability of the LNR-HD interface are three disulphide bonds formed across cysteine residues in the LNR. In *D.melanogaster*, disruption of these bonds actually inactivates Notch signalling by activation of the unfolded protein response after protein misfolding in the endoplasmic reticulum (ER), such that membrane-bound Notch is not expressed (Tien et al., 2008). Interestingly, chelation of calcium by EDTA *in vitro*, which depletes calcium ions from the LNR, disrupts its stability leading to S2 cleavage and activation, a tool that has proved useful for *in vitro* Notch activation experiments without the requirement for ligand activation (Krejci et al., 2009; Rand et al., 2000).

Once Notch has undergone S2 cleavage, S3 cleavage is triggered at a site just intracellular to the transmembrane portion of N(TM) by γ-secretase (GS) (Figure 1.2). This consists of a four enzyme complex comprised of presenilin, nicastrin, PEN2 and APH1 (Okochi et al., 2002). ICN is then free to translocate to the nucleus to bind the transcription factor CSL.

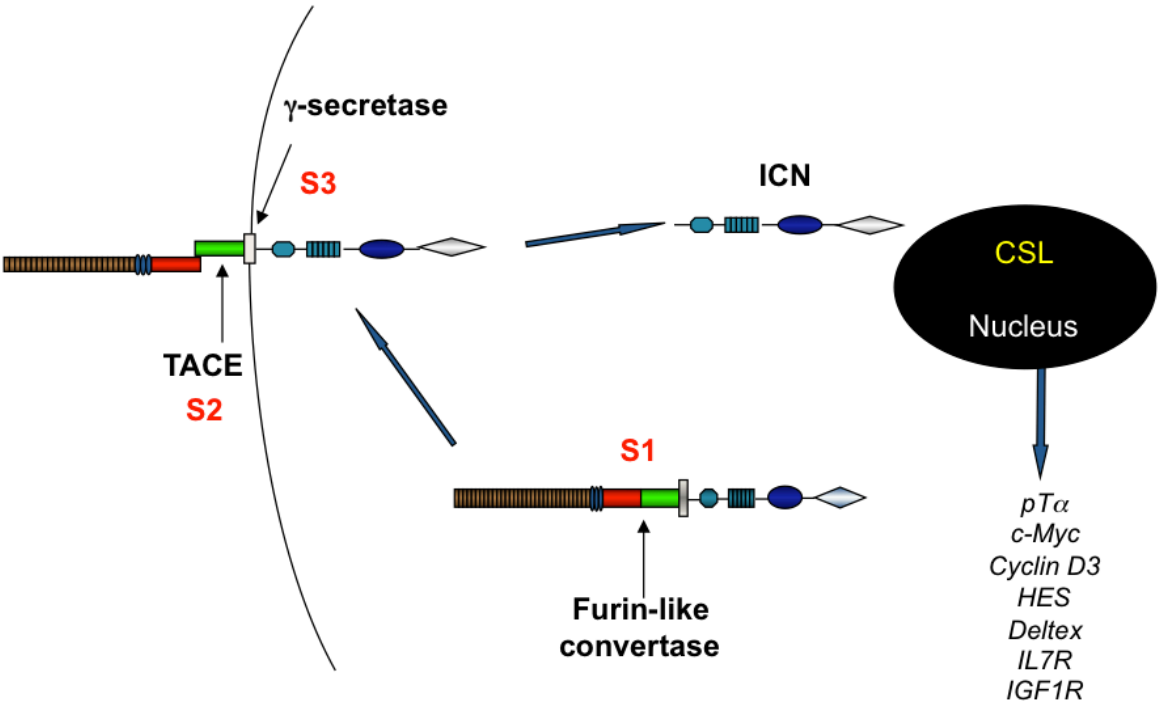

Figure 1.2 Notch is activated by three cleavage steps. In the Golgi, Notch is first cleaved in the HD domain by a furin-like convertase (S1 cleavage) and the HD domain held together by a non-covalent bond. On activation by ligand, the LNR domain is pulled from the HD, exposing the S2 cleavage site to proteolytic cleavage by TNFα-converting enzyme (TACE). This triggers S3 cleavage by the γ-secretase complex in the transmembrane domain, releasing ICN to translocate to the nucleus to bind CSL and activate a multitude of target genes, some of which are shown.

1.6.2.2 Notch ligand binding affinity is regulated by glycosylation of Notch receptors

Humans have five Notch ligands, *JAGGED-1* and *-2*, and *DLL-1, DLL-3* and *DLL-4*. These are single-pass type I proteins with a large extracellular domain containing up to 36 EGF-like tandem repeats. Receptor-ligand binding primarily involves the ligand EGF repeat interacting with EGF repeats 11-12 of NOTCH, with auxillary binding augmented by NOTCH EGF repeats 5-9 and 25-36 (Rebay et al., 1991; Xu et al., 2005). The receptor-ligand interaction has been shown, through atomic microscopy studies and cell-bead optical tweezer assay, to be of remarkably high affinity, presumably to withstand the high physical forces produced during endocytosis, a

mechanism that is dependent on dynamin, epsins, and actin (Ahimou et al., 2004; Meloty-Kapella et al., 2012; Musse et al., 2012; Shergill et al., 2012).

The affinity of the NOTCH-ligand interaction is regulated by modification of NOTCH by fucosylation (Stahl et al., 2008). O-fucosylation of EGF repeat 12 of NOTCH, the site of ligand binding, is first mediated by protein O-fucosyltransferase (*POFUT-1*) and then further fucose groups are added and extended by the Fringe family of fucosyltransferases, of which there are three family members in mammals: manic (*MNFG*), lunatic (*LNFG*) and radical (*RNFG*) fringe. *D.melanogaster* with mutant inactive *O-fut* (the orthologue of *POFUT-1*) have the classic phenotype seen with Notch haploinsufficiency and *Pofut1* -/- mouse ES cells have absent downstream Notch activity, highlighting the importance of fucosylation to physiological Notch signalling (Stahl et al., 2008).

As each of the different Notch ligands are individually capable of activating all four NOTCH receptors, O-fucosylation is thought to confer specificity through alterations in receptor-ligand affinity (Fitzgerald and Greenwald, 1995). This has been best established in the wing of *D.melanogaster*, where fringe-mediated O-fucosylation of Notch increases its affinity to Delta and decreases its affinity to Serrate (Haines and Irvine, 2003). Considering that humans have five ligands rather than the two seen in flies, and four Notch receptors rather than one, the role fucosylation plays in human Notch ligand-receptor affinity and specificity is likely to be more complex, especially as there are multiple other potential fucosylation sites in the EGF regions of both Notch and the ligands themselves that remain to be functionally tested.

On ligand binding to one of the four Notch receptors with cell-to-cell contact, endocytosis is activated from the so-called ‘sending cell’ (the cell expressing ligand), followed by internalisation of the Notch-ligand complex into a clathrin-coated pit and degradation in the lysosome (Figure 1.3) (Emery and Knoblich, 2006).

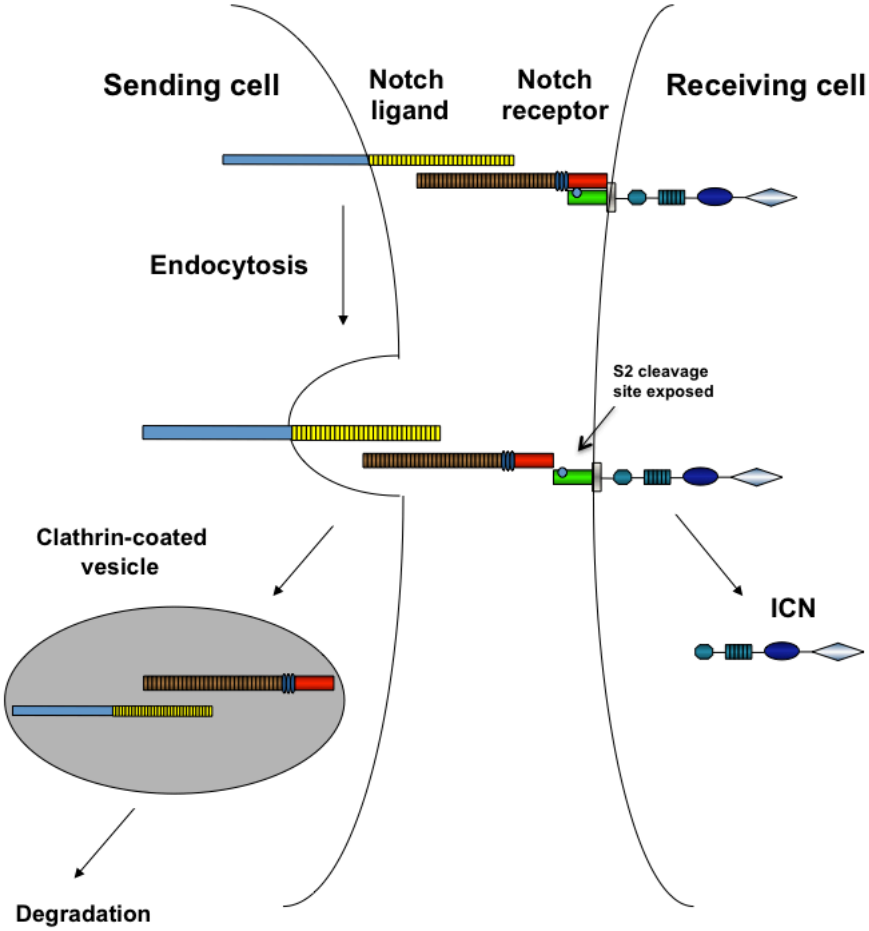

Figure 1.3 Notch is activated by ligand-binding and endocytosis. The 'sending cell' expresses a Notch ligand that binds to the EGF region of a Notch receptor of a neighbouring cell. Endocytosis mechanically pulls the LNR domain from the HD domain exposing the S2 site to cleavage, resulting in release of intracellular Notch (ICN), which translocates to the nucleus. The ligand-Notch extracellular domain is then degraded by the lysosome in the sending cell (Emery and Knoblich, 2006).

1.6.2.3 Structure/function of ICN

The RAM and ANK domains of ICN (Figure 1.1) are directly involved in binding both CSL and the co-activator Mastermind-like-1 (MAML-1). The PEST domain is the negative-regulatory domain and contains a threonine at position 2512, which is the target of the E3 ubiquitin ligase F-Box WD40 repeat-7 (FBXW7). Once T2512 is phosphorylated by cyclin dependent kinase-8 (CDK8), FBXW7 is capable of binding to and degrading ICN by ubiquitination (Fryer et al., 2004). ICN constructs containing a deletion of either the TAD or ANK domains lose transactivation potential *in vitro*

and their oncogenic potential *in vivo*, but the RAM domain is dispensable for these functions (Aster et al., 2000). ANK repeat numbers 2-7 govern the transactivation potential of ICN; the difference in the amino acids of this domain in ICN-4, which are conserved in the other three NOTCH family members, is thought to explain its inability to transform murine HSCs to T-ALL (Aster et al., 2011).

1.6.2.4 The ICN-CSL-MAML activation complex

The transcription factor CSL binds motifs with the sequence (C/T)GTGGGAA in both promoter and enhancer regions. In the absence of Notch activation, it acts as a repressor of its target genes (Tun et al., 1994) and is bound by co-repressors that include nuclear receptor co-repressor-2 (NCOR2, also known as SMRT), MSX2 interacting nuclear target (MINT) and corepressor interacting with RBPJ-1 (CIR-1). Rather than statically occupying target sites, as was originally thought, it is now believed to have a dynamic on-off interaction with DNA (Krejci and Bray, 2007). On entering the nucleus, ICN binds CSL, dislocating co-repressors and recruiting MAML, to form an activation complex that, with the assistance of acetyltransferases such as p300, leads to transcription of target genes. Although the basic-helix-loop-helix (bHLH) family of genes, such as hairy/enhancer of split (*HES*) and hairy/enhancer-of-split related with YRPW motif (HEY), are probably the best-characterised of the Notch targets, over 150 direct target genes have been identified, the regulation of which are cell context dependent (Palomero et al., 2006; Wang et al., 2011). In T-ALL, important direct targets of Notch signalling include *c-Myc*, the IL7 receptor (*IL7R*), insulin-like growth factor receptor-1 (*IGF1R*), and cyclin D3 (*CCND3*) (Figure 1.2) (Medyouf et al., 2011; Palomero et al., 2006; Wang et al., 2011; Weng et al., 2006). How Notch target genes are regulated in a cell context specific manner is not well understood, but may relate to promoter methylation, occupancy of promoter regions and enhancers by other transcription factor complexes, and chromatin state.

1.6.2.5 CSL structure

CSL is highly conserved amongst species; human CSL has over 80% protein homology with *D.melanogaster Su(H)* and is highly homologous to the *Rel* family of transcription factors. CSL consists of three integrated domains – the N-terminal domain (NTD), a central β-trephoil domain (BTD) and a C-terminal domain (CTD)

(Figure 1.4). The association of CSL with MAML, ICN and DNA has been characterised by crystallography of the CSL-ICN-MAML activation complex (Nam et al., 2006; Wilson and Kovall, 2006). The RAM domain of ICN binds to a loop of the BTD of CSL, whilst the C-terminal portion of CSL forms a groove with the ICN ankyrin repeats (ANK), in which MAML sits. The CSL-ANK interaction is thought to be a weaker bond than the CSL-RAM interaction. DNA binding occurs through an electropositive domain formed by the NTD and BTD of CSL, which bind the major and minor grooves of DNA respectively. Paired CSL-binding sites in the *Hes* promoter that are separated by 15–22 base pairs and oriented in a head-to-head fashion are required for Notch-mediated transcriptional activity (Bailey and Posakony, 1995; Cave et al., 2005). Such head-to-head dimerisation of the CSL complex on promoters is also required for its oncogenic potential, since an ICN-1 construct containing an R1985 mutation that inhibits CSL dimerisation cannot activate c-Myc or transform murine marrow (Liu et al., 2010).

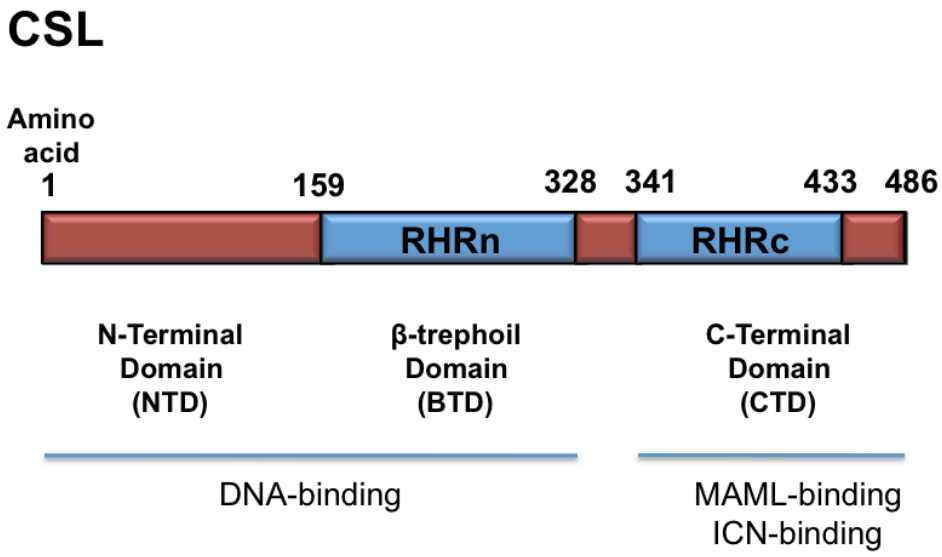

Figure 1.4 Structure of the CSL transcription factor. The N-terminal domain (NTD) of CSL and β-trephoil domain (BTD) bind the major and minor grooves of DNA respectively. The C-terminal domain (CTD) binds MAML and the RAM domain of ICN (Nam*, et al* 2006, Wilson and Kovall 2006). For details of the crystal structure, please see Figure 5.6 Page 175.

1.6.2.6 Lateral inhibition and binary cell fate decisions

In embryogenesis, binary cell fate decisions are regulated by Notch through lateral inhibition (Cabrera, 1990; Sternberg, 1988). This is the mechanism by which two equivalent neighbouring cells are prevented from adopting the same cell fate; one cell develops an active Notch signal (the so-called 'receiving cell') whilst the other down-regulates its Notch activity but increases expression of ligand (the so-called 'sending cell'). Initially, the two cells express equal amounts of Notch and ligand and, probably through a stochastic mechanism, one develops slightly higher Notch signal than the other. Notch activation increases the transcription of *Hes*, which in turn feeds back to increase Notch expression and decrease ligand expression, mediated by suppression of the acheate-scute complex (*ASCL1* in humans), creating an amplification loop within the cell. Meanwhile, the sending cell receives little ligand stimulation from its neighbour, which decreases Hes, and leads to increased ligand expression and decreased Notch expression. The increased ligand expression in turn activates Notch in the receiving cell, and thus a feedback amplification loop is established *in trans*. This sequence has been demonstrated, for example, in invertebrate neurogenesis, where the cell with the highest Notch activity is destined to become epidermal, whilst its neighbour with low Notch activity becomes a neuroblast. If the latter cell is laser ablated at an early time point, the neighbouring cell destined to an epidermal fate, no longer receiving ligand stimulation, instead becomes a neuroblast itself (Doe and Goodman, 1985).

Whilst binary cell fate decisions of this sort may suggest a cell is destined to only two potential outcomes, increasing and decreasing Notch expression iteratively as cells differentiate can lead to multiple outcomes (Figure 1.5) (Pourquie, 2003). Such iterative signalling has been well-described for the Notch pathway during somite formation in *D.rerio* and *D.melanogaster*, and a similar mechanism may be relevant to haematopoietic cell fate determinations (Jiang et al., 2000; Pourquie, 2003). For example, NOTCH-1 inhibits B-cell commitment at the common lymphoid progenitor stage, but is required for terminal differentiation of B-cells into antibody-secreting cells (Allman et al., 2002; Santos et al., 2007). The Notch pathway also displays oscillatory signalling during somitogenesis and neural stem cells development, with Hes-1 expression dynamically cycling between high and low levels over a period of hours before reaching levels critical for cell fate determination (Kageyama et al., 2009; Shimojo et al., 2008).

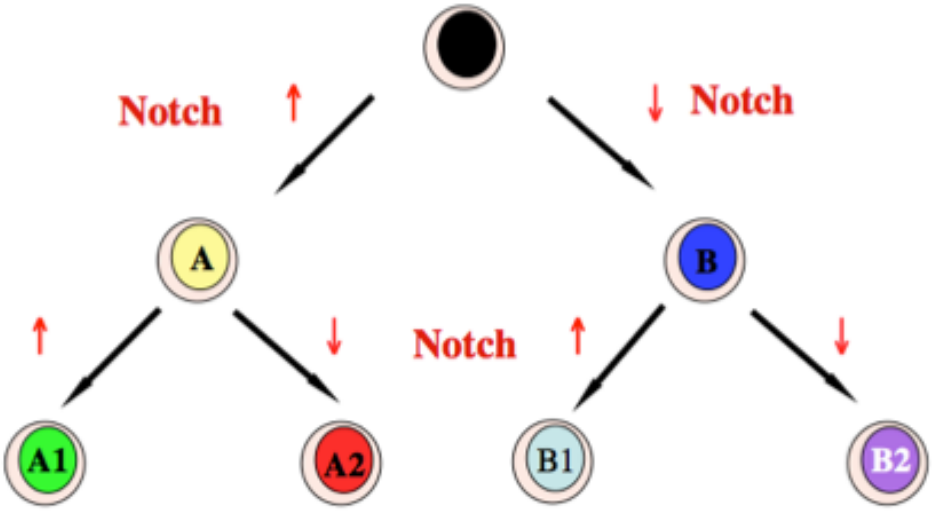

Figure 1.5 Mechanism through which a temporal expression of a single gene can result in multiple different outcomes. Notch is involved in binary cell fate decisions in embryogenesis and haematopoietic differentiation. The example here shows 4 different cellular outcomes (A1, A2, B1, B2) depending on the timing of Notch activation. Iterative signalling of this type is involved in somite formation and also likely to play a role in haematopoietic cell differentiation (Allman et al., 2002; Jiang et al., 2000; Pourquie, 2003).

1.6.3 Germ-line mutations in the Notch pathway in humans

In humans, germ-line mutations in the Notch pathway, either inherited or sporadically acquired, that lead to loss-of-function of Notch signalling cause a variety of syndromic diseases depending on the gene affected. These include:

i) Alagille syndrome

The autosomal dominant Alagille syndrome, caused by germ-line mutations (usually truncating) in the NOTCH ligand *JAGGED-1*, highlights the importance of Notch signalling in human embryonic development, with patients having a constellation of defects involving the heart, liver, vertebrae, eye and characteristic facial appearance (Krantz et al., 1997). Typical mutations described in *JAGGED-1* include P163L, R184H, and C714Y, which lead to abnormal folding of JAGGED-1 protein in the ER, and absent expression on the cell surface (Tada et al., 2012). Although the stem cell compartment has not been tested in detail in these patients,

haematopoiesis is not affected as determined by measurement of normal blood parameters (Krantz et al., 1997).

ii) CADASIL syndrome

CADASIL syndrome (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is a disease affecting the small vessels of the brain characterised by early onset dementia, migraine, and transient ischaemic attacks or stroke, usually in the $4^{th}/5^{th}$ decade of life, and arises from mutations in the *NOTCH-3* gene. The arteriopathy is in fact systemic and results from an accumulation of NOTCH-3 in the vascular smooth muscle, and thus can be diagnosed from NOTCH-3 immunostaining on a skin biopsy (Joutel et al., 2001). The majority of mutations described result in either the gain, or less frequently loss, of a cysteine residue in the *NOTCH-3* EGF region, leading to an odd number of cysteines (Joutel et al., 1996). Recent evidence suggests this results in unpaired disulphide bonding between mutant and wild type (WT) NOTCH-3, leading to protein multimerisation and a dominant-negative affect on Notch signalling (Monet-Lepretre et al., 2009; Opherk et al., 2009).

iii) Bicuspid aortic valve disease

Heterozygous inactivating mutations in the EGF region of *NOTCH-1* have been linked to bicuspid aortic valve disease in several kindreds, and the propensity to valve calcification in these families is thought to be due to loss of the osteogenic inhibitory signal normally conferred by NOTCH-1 in cardiac valves (Garg et al., 2005; Nigam and Srivastava, 2009).

iv) Spondylocostal dysostosis

Loss-of-function mutations of a variety of genes in the Notch signalling pathway, delta-like-3 (*DLL-3),* mesoderm posterior-2 *(MESP2),* lunatic fringe (*LFNG*) and hairy enhancer of split-7 *(HES-7),* lead to spondylocostal dysostosis types 1 to 4 respectively. These are inherited autosomal dominant disorders that are characterised by the presence of scoliosis, extensive hemivertebrae, truncal shortening and abnormally aligned ribs, with multiple bone fusions, and highlight the role of tightly regulated Notch signalling in normal bone development (Bulman et al., 2000; Sparrow et al., 2006; Sparrow et al., 2008).

1.7 The role of Notch in normal haematopoiesis

Whilst much of the embryonic lethality of homozygous *Notch-1* knockout mice can be explained by the widespread vascular defects, there is also an absence of definitive haematopoiesis, independent of the vascular abnormalities (Kumano et al., 2003; Kumano et al., 2001). Several groups have shown that the Notch pathway is required for the establishment of early embryonic haematopoietic stem cells and maintenance of haematopoietic stem cell self-renewal (Clements et al., 2011; Kumano et al., 2003; Kumano et al., 2001).

1.7.1 Expression of Notch and its ligands in haematopoietic tissue

All four *NOTCH* receptors are expressed in haematopoietic cells, although their expression pattern is dependent on the specific haematopoietic lineage and stage of differentiation (Kojika and Griffin, 2001). For instance, *NOTCH-1* and *NOTCH-2* are not expressed in erythroid precursors and have relatively low level expression in CD34+ cells, but are highly expressed in early myeloid cells and monocytes (Ohishi et al., 2003; Ohishi et al., 2000). Mast cells express *NOTCH-2* and *-3*, and monocytes also express *NOTCH-4* (Jonsson et al., 2001).

Activation of the Notch pathway in haematopoietic cells is thought to be mediated by direct contact with bone marrow or thymic stroma, or through cell-to-cell contact with neighbouring haematopoietic cells that express ligand. Bone marrow stromal cells predominantly express JAGGED-1, whilst thymic stromal cells also express JAGGED-2 and DLL-1, with the latter thought to be most important in determining T-lymphocyte lineage commitment (see below) (Anderson et al., 2001). Notch ligands are also expressed on haematopoietic cells, although, similar to the Notch receptors, expression patterns are dependent on cell type. JAGGED-1 is expressed on human CD34+ cells, megakaryocytes, and mast cells, JAGGED-2 on CD34+ cells. Thus there is a complex bidirectional cell-to-cell interplay between the various Notch receptors and their ligands in haematopoietic tissue, the subtleties of which are not yet fully understood. The density and ratio of Notch ligands and their receptors is likely to play an important role in cell fate specification and self-renewal signals imparted through Notch signalling (Delaney et al., 2005; Varnum-Finney et al., 2003).

1.7.2 Notch and haematopoietic lineage commitment

Just as it plays a vital role in cell fate determination of a wide variety of developing embryonic tissue, Notch is also a key regulator of haematopoietic cell fate commitment. In mice, Notch-1 has a non-redundant role in committing early lymphoid progenitors towards a T-cell fate, and conditional *Notch-1* knockout mice have thymic hypoplasia and a complete absence of T-cells (Radtke et al., 2004). ETP cells originate from CLPs, and in the thymus differentiate through the double-negative (DN) stage (in mice, DN stages 1-4 are characterised by the expression of CD44 and CD25) to become double positive (DP) cells expressing both CD4 and CD8, before their release into the circulation as single positive (SP) CD4+ or CD8+ T-cells (Dadi et al., 2009; Krangel, 2009). Progression of T-cell committed lymphoid progenitors through to the double-negative (DN3) stage of lymphocyte development is Notch-1 dependent (De et al., 2002; Pui et al., 1999). In early T-cells, the Notch-CSL activation complex directly activates pre-T-cell receptor-alpha (pTα) through binding to its promoter region, which leads to formation of the pre-T-cell receptor complex. However, once through the β selection checkpoint at the DN3b stage, there is downregulation of Notch-1 and terminal T-cell differentiation can occur in the absence of Notch-1 (Taghon et al., 2006; Witt et al., 2003). When Notch-1 was constitutively over-expressed by reconstituting lethally-irradiated mice with CD34+ cells transduced with a vector expressing ICN-1, there was a reduction in B-cells numbers, a marked increase in T-cells, thymic hyperplasia, and eventual development of an aggressive fatal T-cell leukaemia (Pear et al., 1996). Enforced expression of Notch-1 at and after the time of VDJ recombination, when signalling is normally on the decline, is thought to be when Notch confers its leukaemogenicity (Roy et al., 2007). The oncogenic role of Notch-1 in T-cells is discussed in more detail in Chapter 3.

NOTCH-2 enhances mast cell differentiation from CD34+ cells and also regulates marginal zone B-cell development, such that *Notch-2* haploinsufficient mice have a severe deficiency in marginal zone B-cell development in the spleen (Sakata-Yanagimoto et al., 2008; Witt et al., 2003). NOTCH-4 has recently been shown, both *in vitro* and *in vivo,* to be an important positive regulator of megakaryocyte lineage commitment, in part through an interaction with DLL-1 (Mercher et al., 2008). Umbilical cord blood-derived CD34+ cells undergo erythroid differentiation when co-

cultured with a stromal cell line expressing DLL-4 (Sugimoto et al., 2006). DLL-1, but not JAGGED-1, completely inhibits the differentiation of human CD34+ cells into B-cells, while promoting their development into T/NK precursor cells (Jaleco et al., 2001).

1.7.3 Notch and myeloid differentiation

There is still much controversy as to the role Notch signalling plays in myeloid differentiation. Conditional knock-out of *Notch-1* or *CSL* in bone marrow cells *in vivo* has been shown to have no effect on the myeloid lineage (Han et al., 2002). Additionally, overexpression of ICN-1 in bone marrow only affected lymphoid lineage commitment *in vivo*, with no apparent effect on myelopoiesis (Pui et al., 1999). *In vitro* experiments have shown conflicting results, with much of the published data using the murine 32D cell line. Two groups have shown that 32D cells, when either transduced with *ICN-1* or cultured on JAGGED-1 expressing stroma, have accelerated and increased granulocytic differentiation in response to G-CSF resulting from the up-regulation of Pu.1 by ICN-1 (Schroeder and Just, 2000; Tan-Pertel et al., 2000). Conversely, several independent groups have found that expression of ICN-1 in 32D cells markedly inhibited myeloid differentiation, and was associated with sustained levels of *GATA-2* (Bigas et al., 1998; Kumano et al., 2001; Milner et al., 1996; Tan-Pertel et al., 2000). This latter data is supported by the finding that umbilical cord blood-derived CD34+ cells exhibited a significant delay and inhibition in myeloid differentiation when cultured on JAGGED-2 expressing stroma, or when transduced with ICN-1 (Carlesso et al., 1999). Whilst one possibility for the conflicting data may relate to the propensity for 32D cell subclones to become aneuploid, altering their phenotype, it is likely that even small changes in the expression levels of ICN between experiments can have a significant impact on cellular phenotype. An elegant example of this was reported by Bernstein's group where they showed that human CD34+CD38- cord blood progenitors co-cultured with stroma expressing low densities of DLL-1 generated higher numbers of CD34+ cells and promoted early myeloid and lymphoid differentiation, but high density DLL-1 expression on stroma impaired myeloid differentiation and induced apoptosis of CD34+ cells (Delaney et al., 2005).

A further complexity is the possibility that NOTCH-1 may affect myeloid differentiation in a non-cell-autonomous manner. For example, Kawamata and colleagues noticed that when mice were transplanted with ICN-1-transduced haematopoietic cells, the neighbouring non-transduced population of marrow cells had increased numbers of terminally differentiated myeloid cells (Kawamata et al., 2002). This was not a result of direct cell-to-cell interaction, since conditioned medium from *ex vivo* cultures of ICN-1-transduced bone marrow significantly promoted myeloid maturation of normal marrow cultures.

Post-translational modifications of Notch may also be important to myelopoiesis. Fucosylation of the EGF repeats of Notch receptors markedly enhances their affinity for ligand binding. The $FX^{-/-}$ mouse, which is deficient in the FX protein that converts GDP-mannose to GDP-fucose in the de novo fucose synthesis pathway, develops a marked neutrophilia (Zhou et al., 2008). Whereas activation of Notch signalling of wild-type murine marrow by co-culture with OP9 stromal cells expressing either Dll-1, Dll-4, Jagged-1, or Jagged-2 receptors impaired terminal myeloid differentiation, ligand activation of $FX^{-/-}$ marrow exhibited no inhibition on myelopoiesis, suggesting that suppression of myelopoiesis by Notch is fucosylation-dependent (Zhou et al., 2008). The authors therefore hypothesised that the marked neutrophilia and marrow myeloid hypoplasia seen in $FX^{-/-}$ mice is mediated through loss of Notch-mediated repression on myeloid differentiation.

1.7.4 Notch and the stem cell niche

As long as ago as the 1970s, it was appreciated that higher numbers of HSCs reside nearest the bony surface of the marrow architecture and that as haematopoietic cells undergo differentiation, they move further from the endosteal surface of bone (Lord et al., 1975). In fact, the number of cells with colony forming potential obeys a negative linear relationship with the square of the distance from the endosteal surface (Lord et al., 1975). It has also been hypothesised for some time that HSC numbers are integrally related to signals received through direct cell-to-cell contact of HSCs and osteoblasts (Gong, 1978). More recently, using a mouse transgenic for a constitutively activated parathyroid hormone receptor (PTHr), David Scadden's group have elegantly shown that osteoblasts directly interact with and regulate HSCs (Calvi et al., 2003). These transgenic mice had an increased density of trabecular bone, higher

numbers of osteoblasts and more HSCs. The authors showed that this relationship was mediated by the interaction of Jagged-1 expressed on osteoblasts binding to and activating Notch-1 on HSCs; inhibiting this interaction abrogated the increased numbers of HSCs seen in the transgenic mice. As a consequence of this work, trials are underway using synthetic PTH in humans to enhance HSC numbers prior to collection of stem cells for autologous transplantation (Ballen et al., 2007).

1.7.5 Notch and self-renewal of HSCs

Stem cells are characterised by their ability to self-renew whilst maintaining multi-lineage potential, and it has been shown through a variety of approaches that up-regulation of Notch signalling can expand HSC numbers without leading to lineage commitment and differentiation. For instance, co-culture of HSCs with a stromal layer expressing Jagged-1 increased the number of primitive CD34+ CD38- cells and enhanced their repopulating capacity when transplanted into lethally irradiated NOD/SCID mice (Karanu et al., 2000; Varnum-Finney et al., 1998; Varnum-Finney et al., 2000). Similar data was obtained when HSCs were co-cultured with a stromal layer expressing Dll-1, although as discussed above, ligand density was a critical factor in determining self-renewal capacity, with low density expression promoting an increase in HSC numbers, and high density expression diminishing their numbers through the induction of apoptosis (Delaney et al., 2005; Varnum-Finney et al., 2003).

The utilisation of umbilical cord blood stem cells for bone marrow transplantation is often limited by inadequate numbers of HSCs, particularly for adult recipients. Thus, co-culture techniques capable of expanding HSC numbers without initiating a differentiation programme have genuine therapeutic potential. To this end, the Bernstein group, that have pioneered much of the initial work, established a Phase I trial whereby one unmanipulated cord blood unit and one cord blood unit expanded on stroma expressing DLL-1 were infused concurrently into a myeloablated patient (Dahlberg et al., 2011; Delaney et al., 2010). After co-culture with the stroma, there was an average 40-fold increase (range 4 to 140) in the number of CD34+ cells. For the thirty-two patients that have thus far been recruited, there has been rapid engraftment of the manipulated cord blood unit, with a median time to neutrophil and platelet engraftment of 15 days (range 9-42) and 40 (range 13-62) days respectively. Such an approach could thus potentially significantly shorten the dangerous

neutropenic period seen with cord blood transplantation and enable the inclusion of patients previously deemed unsuitable on the basis of inadequate numbers of donor cells for their body weight. Whether this affects long-term outcome and rates of graft-versus-host disease (a potential issue from increased T-cell numbers), awaits to be determined.

1.8 Aims of this thesis

At the time the work presented in this thesis was started, *NOTCH-1* mutations had not been described in adult T-ALL and could potentially account for the difference in prognosis between adult and paediatric patients. The incidence and characteristics of *NOTCH-1* mutations were therefore investigated in a cohort of adult T-ALL patients and these results are presented in Chapter 3. To address the prognostic implications of *NOTCH-1* and *FBXW7* mutations in adult T-ALL, the clinical outcome of patients treated on the E2993/UKALLXII trial was evaluated according to mutation status (Chapter 3). Alternative mechanisms of Notch pathway activation in T-ALL were explored through mutational screening of *NOTCH-3* as well as the LNR domain of *NOTCH-1*. Because AML patients often co-express T-cell antigens and the role of *NOTCH-1* signalling in myeloid cells is unclear, *NOTCH-1* mutational status was also investigated in this disease (Chapter 3). The timing at which *NOTCH-1* mutations occur in T-ALL was studied, specifically addressing whether they occur as disease initiators or as secondary events (Chapter 4). Finally, as *NOTCH-1* mutations were not detected in AML, expression of the downstream transcription factor *CSL* was investigated and the prognostic and functional significance of an alternatively spliced isoform, identified in a significant proportion of AML patients, were studied (Chapter 5).

CHAPTER 2. MATERIALS AND METHODS

Specific materials and methods are described in the relevant results chapters.

2.1 Cell culture

Unless otherwise specified, suspension cells were grown in RPMI-1640 medium with L-glutamine supplemented with 10% foetal calf serum (FCS) and penicillin-streptomycin (used at 100 units/ml and 100 µg/ml respectively) in a humidified incubator with 5% CO_2 at 37°C. Adherent cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS and penicillin-streptomycin under the same conditions. All cell lines used were growth factor independent. Specific techniques used for the isolation and culture of primary CD34+ cells is given in the methods of chapter 5.

2.1.1 Cell lines

- ALL-SIL - human T-cell acute lymphoblastic leukaemia, non-adherent
- HEK-293T - human embryonal kidney line, adherent
- HeLa – human cervical carcinoma, adherent
- HL-60 - human acute myelocytic leukaemia, non-adherent
- Jurkat - human T-cell acute lymphoblastic leukaemia, non-adherent
- KG-1 - human erythroleukaemia, non-adherent
- Monomacs-6 - human mature monocytic cell line, non-adherent
- NALM-6 - human B-cell acute lymphoblastic leukaemia, non-adherent
- NB-4 - human acute promyelocytic leukaemia, non-adherent
- RAJI - human Burkitt's lymphoma, non-adherent
- SEM - human B-cell acute lymphoblastic leukaemia, non-adherent
- THP-1 - human acute monocytic leukaemia, non-adherent
- U2OS - human osteosarcoma, adherent
- U937 - human histiocytic lymphoma, non-adherent

2.1.2 General cell culture reagents

- Dimethyl sulfoxide (DMSO) (Sigma Aldrich, Poole, UK)

- Dulbecco's Modified Eagle Medium (DMEM) – high glucose 4.5g/l with L-glutamine (Invitrogen, Paisley, UK)
- Ficoll-Paque™ gradient (Amersham Biosciences, Bucks, UK)
- Flow Check Fluorospheres (Beckman Coulter, High Wycombe, UK)
- Foetal calf serum (FCS) (Sigma Aldrich, Poole, UK), heat inactivated for 30 minutes at 56°C and filtered through 0.44micron filter (PAA Laboratories, Pasching, Austria)
- Intracellular flow kit (BD Bisocience Ltd.)
- MACS® Cell Separation Columns and Reagents (Miltenyi Biotec, Germany)
- Methocult™ (Stem Cell Technologies, Vancouver, Canada)
- Penicillin-Streptomycin containing 10,000 units/ml penicillin and 10,000 µg/ml streptomycin, used at 100 units/ml and 100 µg/ml respectively (Sigma Aldrich, Poole, UK)
- Phosphate buffered solution (PBS) (Invitrogen, Paisley, UK)
- Polyethylenimine (PEI) (Sigma Aldrich, Poole, UK)
- Polybrene (Sigma Aldrich, Poole, UK)
- RPMI-1640 medium with L-glutamine (Invitrogen, Paisley, UK)
- StemPro-34 with nutrients (Invitrogen, Paisley, UK)
- Trypsin-EDTA (0.25%) (Invitrogen, Paisley, UK)

2.1.3 Cell culture plastics

- T25, T75 and T175 flasks (VWR International, Merck, UK)
- 100mm and 145mm tissue culture dishes (Greiner Bio-One Ltd, Stonehouse, UK)
- 6, 12, 24 and 48 well tissue culture plates (Nalge Nunc, Rochester, NY, USA)
- 1.8ml cryovials (VWR International, Merck, UK)

2.1.4 Active compounds used in cell culture

- L-685,458 Potent selective gamma secretase inhibitor (GSI) (Sigma Aldrich, Poole, UK)

2.1.5 Antibodies

- Mouse anti-human CD11b antibody conjugated to allophycocyanin (APC) (Miltenyi Biotec, Germany)
- Mouse anti-human CD34 antibody conjugated to APC (BD Biosciences)

- CD34 microbead kit for isolation of human CD34+ haematopoietic cells (Miltenyi Biotec, Germany)
- Rat anti-human RBP-JK antibody (polyclonal clone SIM-2ZRBP3, T6719) (Cosmobio, Japan)
- Mouse anti-human Notch-1 antibody, specific to cleaved Notch-1 (monoclonal clone mN1A, N-6786) (Sigma Aldrich, Poole, UK)
- Mouse anti-FLAG M2 antibody, monoclonal (Sigma Aldrich, Poole, UK)
- Goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) (Cell Signaling Technology, Hitchin, UK)
- Goat anti-mouse IgG antibody conjugated to HRP (Cell Signaling Technology, Hitchin, UK)
- Goat anti-rat IgG antibody conjugated to HRP (Cell Signaling Technology, Hitchin, UK)

2.1.6 Recombinant growth factors

- Human FLT3-ligand (PeproTech, London, UK)
- Human granulocyte colony-stimulating factor (G-CSF) (PeproTech, London, UK)
- Human granulocyte macrophage colony-stimulating factor (GM-CSF) (PeproTech, London, UK)
- Human erythropoietin (PeproTech, London, UK)
- Human interleukin-3 (IL-3) (PeproTech, London, UK)
- Human stem cell factor (SCF) (PeproTech, London, UK)
- Human thrombopoietin (TPO) (PeproTech, London, UK)

2.1.7 Cell cryopreservation

A total of $2x10^6$ cells were frozen slowly in 1ml freeze mix consisting of 10% DMSO, 50% FCS and 40% RPMI-1640 medium, and stored in liquid nitrogen. When required, cells were thawed in a 37°C waterbath, then 10mls pre-warmed medium added dropwise to completely resuspend them. They were then centrifuged at 300g for 5 minutes at room temperature, the supernatant removed and replaced with fresh medium.

2.2 Lentivirus production

2.2.1 Production of lentivirus

A total of $1x10^7$ HEK-293T cells were suspended in 20mls DMEM/10% FCS in a 14.5cm plate, and transfected the following day when the cells were 70-80% confluent. For each plate, 40µg of the vector plasmid was added to 5mls serum-free DMEM, together with 10µg envelope plasmid VSVG-pMD.G2 and 30µg pCMV-deltaRp8.91, which codes for the helper and packaging plasmids, and filtered through a 0.2µm filter. One µl of 10 mM polyethylenimine (PEI, Sigma 40, 872-7) was added to 5mls serum-free DMEM per plate and filtered through a 0.2µm filter. The DNA and PEI solutions were mixed and left at room temperature for 20 minutes. After the cells were checked for confluence, the medium was removed and the cells were washed with PBS to remove FCS, and then 10mls of the DNA and PEI mixture was carefully added. The cells were incubated at 37^{o}C in 5% CO_2 for 4 hours, then the medium was replaced with DMEM/10% FCS. Incubation was continued for a further 48 hours, then the supernatant was harvested. The supernatant was filtered through a 0.4µm filter to remove cell debris, transferred to polyallomer centrifuge tubes and centrifuged at 50,000g for 90 minutes at 4^{o}C. The supernatant was decanted off and 50µl Stempro medium (Invitrogen) added to the viral pellet, which was resuspended by pipetting up and down, and left on ice for 1 hour. The mix was transferred to an Eppendorf tube and centrifuged for 10 minutes at 600g at 4^{o}C to remove cell debris. The viral supernatant was aliquoted into cryovials and stored at -80^{o}C until required.

2.2.2 Biological titre of lentiviral supernatants

HeLa cells were plated at a concentration of 5×10^4 cells per well in 1.5mls DMEM/10% FCS in a 12 well plate. The cells were incubated at 37^{o}C in 5% CO_2 for 4 hours, allowing time for them to adhere to the plate but not divide, thus keeping cell numbers constant. To create dilutions of the viral supernatant prepared above, 30ml supernatant was added to 1.47mls serum-free DMEM to make a starting dilution of 1:50. A total of 0.15mls was taken from this dilution and added to 1.35mls serum-free DMEM. This was repeated to prepare serial 1 in 10 dilutions of the supernatant to create six dilutions between 1:50 and 1:500,000. Cells were incubated for 48 hours at 37^{o}C in 5% CO_2 before the percentage of GFP positive cells was measured by flow cytometry.

The estimated titre of the virus was determined as below:

Viral titre = % GFP positive cells x 50,000 (cell number) x virus dilution factor

2.3 Molecular biology

2.3.1 Reagents for molecular biology, cloning and plasmid generation

- Acetonitrile (VWR International Ltd, Lutterworth, UK)
- Agar (Calbiochem, UK)
- Agarose (Bioline, London, UK)
- BIOTAQ DNA polymerase (Bioline, London, UK)
- Bioline buffer and magnesium chloride (Bioline, London, UK)
- Carbenicillin (Invitrogen, Paisley, UK)
- Chloroform (VWR International Ltd, Lutterworth, UK)
- Diethylpyrocarbonate (DEPC) treated water (Invitrogen, Paisley, UK)
- DH5α competent bacteria (Invitrogen, Paisley, UK)
- DNA size standard kit – 400bp or 600bp (Beckman Coulter UK Ltd., Buckinghamshire, UK)
- dNTPs (Bioline, London, UK)
- DTAB (dodecyl-trimethyl ammonium bromide) (Sigma Aldrich, Poole, UK)
- DTCS Quick Start kit (Beckman Coulter UK Ltd., Buckinghamshire, UK)
- EDTA (ethylenediaminetetraacetic acid, disodium salt) (Sigma Aldrich, Poole, UK)
- Ethanol 100% (VWR International Ltd, Lutterworth, UK)
- Ethidium Bromide (Invitrogen, Paisley, UK)
- Glycerol (VWR International Ltd, Lutterworth, UK)
- HEPES (Sigma Aldrich, Poole, UK)
- Isopropanol (VWR International Ltd, Lutterworth, UK)
- Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma Aldrich, Poole, UK)
- Luria-Bertani (LB) broth (Anachem, Luton, UK)
- Optimase proof-reading Taq polymerase (Transgenomic, Glasgow, UK)
- Optimase buffer and magnesium sulphate (Transgenomic, Glasgow, UK)
- Orthoboric acid (VWR International Ltd, Lutterworth, UK)

- Plasmid Mini and Mega prep kits (Qiagen, Crawley, UK)
- Oligonucleotide primers (Sigma Aldrich, Poole, UK)
- Promega Dual Luciferase Kit (Promega, Southampton, UK)
- QIAquick Gel Extraction Kit (Qiagen, Crawley, West Sussex, UK)
- QIAquick PCR Purification kit (Qiagen, Crawley, UK)
- Quick Ligase kit (New England Biolabs, Hitchin, UK)
- Restriction enzymes (New England Biolabs, Hitchin, UK)
- Reverse transcriptase Superscript III kit (Invitrogen, Paisley, UK)
- Sodium dodecyl sulfate (SDS) (Sigma Aldrich, Poole, UK)
- S.O.C. media (Super Optimal broth with Catabolite repression) (Invitrogen, Paisley, UK)
- Sodium acetate (VWR International Ltd, Lutterworth, UK)
- TOPO cloning kit (Invitrogen, Paisley, UK)
- TOP10F competent bacteria (Invitrogen, Paisley, UK)
- Triethylammonium acetate (TEAA) (Transgenomic, Glasgow, UK)
- Trizol reagent (Invitrogen, Paisley, UK)
- X-gal (Invitrogen, Paisley, UK)

2.3.2 DNA extraction

Cells were resuspended at $10x10^6$/ml in PBS and 2 volumes of DTAB solution (8% DTAB, 1.5M NaCl, 100mM Tris pH 8.0, 50mM EDTA pH 8.0) were added. The sample was thoroughly mixed and incubated at 68°C for 5 minutes. An equal volume of chloroform was added and the sample was mixed by end-over-end inversion for several minutes then centrifuged at 2000g for 10 minutes. To precipitate the DNA, the upper DNA-containing layer was removed and mixed with an equal volume of 100% ethanol. The precipitated DNA was then either removed with a pipette tip, if visible in solution, or centrifuged at 2000g for 10 minutes to form a pellet. The supernatant was removed and the precipitated DNA washed twice with 500µl 70% ethanol, resuspended in double distilled water (DDW) and stored at 4^oC.

2.3.3 RNA extraction

RNA extraction was carried out using the Trizol reagent. Between $1x10^6$ and $5x10^6$ cells were pelleted by centrifugation at 300g for 5 minutes, mixed with 1ml Trizol, vortexed to completely lyse the cells and then left at room temperature for 10 minutes. The sample

was then frozen at -20°C and further processing carried out in batches. After thawing, 200µl chloroform was added, the sample mixed thoroughly and centrifuged at 12,000g for 15 minutes at 4°C. The upper aqueous layer containing RNA was decanted off into a fresh tube and 500µl isopropanol added. The tube was incubated at room temperature for 10 minutes then centrifuged at 12,000g for 10 minutes at 4°C. The RNA pellet was then washed in 1ml 75% ethanol, centrifuged at 7,500g for 5 minutes, air dried and resuspended in DEPC-treated water.

2.3.4 Reverse transcription of RNA

Reverse transcription of RNA was performed using the Superscript III kit according to the manufacturer's recommendations (Invitrogen). A reaction mix containing 10µl reverse transcriptase reaction mix (which includes 2.5µM oligo(dT), 2.5ng/µl random hexamers, 10mM $MgCl_2$, and 2.5µM dNTPs), 2µl reverse transcription enzyme and 500ng RNA, 1µl RNAse inhibitor, made up to 20µl with DEPC-treated water. On a PCR thermocycler, the sample was incubated at 25ºC for 10 minutes, 50ºC for 30 minutes and 85ºC for 5 minutes, and then kept at 4ºC for 5 minutes. To remove residual RNA template, 1µl (2U) *E.coli* RNase H was added and the sample incubated at 37ºC for 20 minutes. The cDNA was stored at -20ºC until use.

2.3.5 Polymerase chain reaction (PCR)

For general PCR reactions, the non-proof reading enzyme BIOTAQ DNA polymerase (Bioline) was used. BIOTAQ DNA polymerase, Bioline buffer and magnesium chloride were used according to the manufacturer's recommendations. In order to amplify PCR products for DHPLC analysis, the proofreading Taq polymerase Optimase (Transgenomic) was used. This reagent has a low base substitution error rate due its 3' to 5' exonuclease activity and in general creates DHPLC chromatograms of superior quality to non-proof reading Taq polymerase. Optimase Taq polymerase, Optimase buffer and magnesium sulphate was used according to the manufacturer's recommendations. On the occasion when PCR products of adequate quality could not be obtained for DHPLC with Optimase, BIOTAQ DNA polymerase (Bioline) was used and is stated in the appropriate methods section. For both reagents, forward and reverse primers were used at 500nM, each dNTP at 200µM and a total of 100ng DNA template in a 20µl PCR reaction. For PCR reactions from cDNA, 2µl of cDNA (diluted 1 in 5 from the original 20µl RT

reaction) was used in the reaction. PCR conditions included an initial denaturation step at 95^{o}C (1 minute for BIOTAQ and 5 minutes for Optimase Taq) followed by successive cycles of denaturation (95^{o}C, 45 seconds), primer annealing (30 seconds) and extension (72^{o}C, time calculated according to PCR product size at 1kb per minute) for a total of 35 cycles, followed by a final 5 minute extension at 72^{o}C. Specific annealing temperatures are stated in the appropriate methods section.

2.3.6 Agarose gel electrophoresis

TBE (10x):108g Tris base, 55g Boric acid, 9.3g EDTA, made up to 1l with DDW.
Gel loading buffer: 3.9mls glycerol, 500µl 10% SDS, 25mg bromophenol blue, made up to 10mls with DDW.

Agarose gel electrophoresis was used to separate and visualise PCR products or plasmid DNA according to size. Agarose gel was made up to between 1%-2.5% weight/volume (depending on the anticipated size of the PCR products) in 35mls 1xTBE, melted in a microwave oven, then cooled before the addition of ethidium bromide (100ng/ml final concentration). PCR products were mixed with loading buffer and then run out in a horizontal submarine gel electrophoresis tank in 1xTBE at 70mA for 20-30 minutes. Products were visualised and photographed under UV light.

2.3.7 DNA Sequencing protocol

When screening for mutations, PCR products were sequenced from both the forward and reverse primers. PCR products were generated using BioTaq PCR, analysed by gel electrophoresis to ensure the correct size band was present, and then purified using the QIAquick PCR purification kit (Qiagen). The DNA concentration was determined using a Nanodrop spectrophotometer. The sequencing reaction was then performed using the CEQ™ DTCS Quick Start kit (Beckman Coulter) with 4µl mastermix, 20ng PCR product, and 3.2pmols oligonucleotide primer, made up to 10µl with DDW. The cycling parameters were 96°C for 20 seconds, 50°C for 20 seconds and 60°C for 4 minutes, repeated for 25 cycles. In order to adequately remove secondary structure of the template, an initial 2 minute denaturation step at 95^{o}C was performed when sequencing plasmids, and either 200ng plasmid DNA or 1µl of a bacterial culture was used in each 10µl reaction. The reaction products were precipitated by mixing with 0.4µl 500mM EDTA, 2µl 3M sodium acetate pH 5.2, 1µl 20ng/µl glycogen and 60µl 95% ethanol, and

then pelleted by centrifugation at 14,000g for 15 minutes. The pellet was washed twice in 200µl 75% ethanol, centrifuged at 12,000g, the supernatant removed, and allowed to air dry prior to resuspension in 38µl sample loading solution. The sample was then run on a CEQ™8000 Beckman Coulter DNA Genetic Analysis System.

2.3.8 TOPO cloning

PCR products prepared using BIOTAQ DNA polymerase were cloned using a TOPO kit (Invitrogen). The principle of this technique is that the A overhangs of the PCR product that are added by non-proof reading DNA polymerases are ligated into the T overhang of the TOPO vector. The ligation was performed according to the manufacturer's instructions and then used to transform TOP10F competent bacteria (Invitrogen). Here, 2µl TOPO reaction mix was incubated with 50µl TOP10F competent bacteria (Invitrogen) on ice for 30 minutes and then the bacteria were heat-shocked at 42°C for 30 seconds. SOC media (200µl) was then added, and the sample left on a shaker at 37°C for 1 hour. Agar plates containing 100µg/ml ampicillin and spread prior to use with 40µl 100mM IPTG and 40µl 40mg/ml X-gal were pre-warmed at 37°C for 30 minutes, spread with 100µl of the SOC/ bacterial culture, and left at 37°C overnight in a bacterial incubator. Blue colonies contain no insert, but white colonies, containing the PCR product, were then picked with a sterile pipette tip and inoculated into 200µl LB medium containing ampicillin and left at 37°C for 12-24 hours in a bacterial incubator. For sequencing, 1µl of the culture was added directly to the sequencing reaction (see section 2.3.7).

2.3.9 Plasmid preparation

Cloning reactions into retroviral and lentiviral vectors are described in the methods section of chapter 5. For bacterial transformations, 5-20ng plasmid DNA was incubated with 25µl DH5α competent bacteria (Invitrogen), left on ice for 30 minutes and then heat-shocked at 42°C for 30 seconds. SOC media (200µl) was then added, and the sample left on a shaker for 1 hour at 37°C. Agar plates containing 100µg/ml ampicillin were pre-warmed at 37°C for 30 minutes, spread with 100µl of the SOC/ bacterial culture, and left at 37°C overnight in a bacterial incubator. One colony was then picked with a sterile pipette tip, inoculated into 7ml LB medium containing 100µg/ml ampicillin and left at 37°C shaking at 200 rpm for 16 hours in a bacterial incubator. Glycerol stocks

were then made from the culture by mixing 500µl bacterial culture with 500µl 50% sterile glycerol, and frozen at -80°C. The remaining culture was used to make plasmid DNA using a Mini Prep kit (Qiagen) according to the manufacturer's instructions. The plasmid DNA was then sequenced using appropriate primers to cover the entire open reading frame of the cloned gene to exclude Taq errors. The glycerol stock (50µl) was used to inoculate a 500ml culture of LB containing 100µg/ml ampicillin, and left at 37°C shaking at 200 rpm for 16 hours in a bacterial incubator. The culture was then centrifuged at 6000g to pellet the bacteria, and plasmid DNA purified using a Maxi Prep kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA concentration was quantified using a Nanodrop spectrophotometer.

2.3.10 Denaturing High Performance Liquid Chromatography (DHPLC) using WAVE® analysis

For mutation detection, PCR products were analysed by DHPLC using the WAVE® DNA Fragment Analysis System (Transgenomic). The WAVE® system contains a DNASep column consisting of packed hydrophobic poly(styrene-divinylbenzene) beads to which the anionic DNA binds in the presence of the ion pairing reagent triethylammonium acetate (TEAA). Under increasing concentrations of acetonitrile, the ionic bond between the beads and PCR product gradually weakens until the DNA elutes off the column, as detected by UV absorbance.

PCR products containing a mutation or single nucleotide polymorphism (SNP) will form heteroduplexes after denaturation and cooling. A heteroduplex consists of one wild-type strand annealed to a complementary mutant strand, leading to the formation of a 'bulge' or mismatch at the position of the substitution (Figure 2.1). Such heteroduplexes are inherently less stable than their homoduplex counterparts, will denature more easily at a given temperature and acetonitrile concentration, and consequently will elute from the HPLC column first. Thus, whilst a wild-type PCR product (all homoduplexes) will elute as a single peak, a PCR product from a sample with a heterozygous substitution will

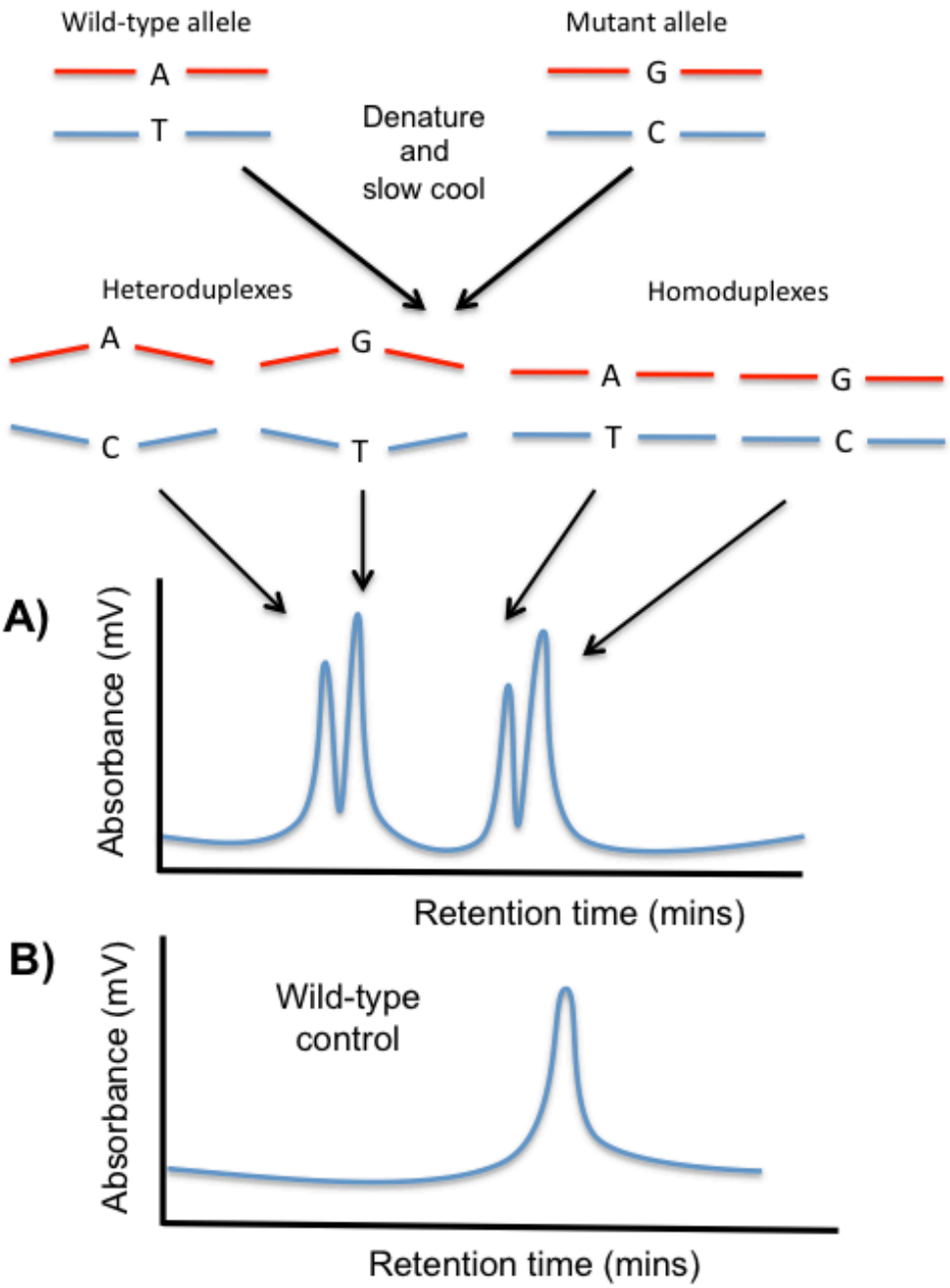

Figure 2.1 The principle of DHPLC analysis. A schematic showing the four possible combinations of a PCR product with a heterozygous A to G mutation after denaturation and slow cooling. **(A)** Heteroduplexes are more unstable than homoduplexes and elute earlier from the column, whilst homoduplexes are retained longer. **(B)** The wild-type control homoduplex elutes as a single peak.

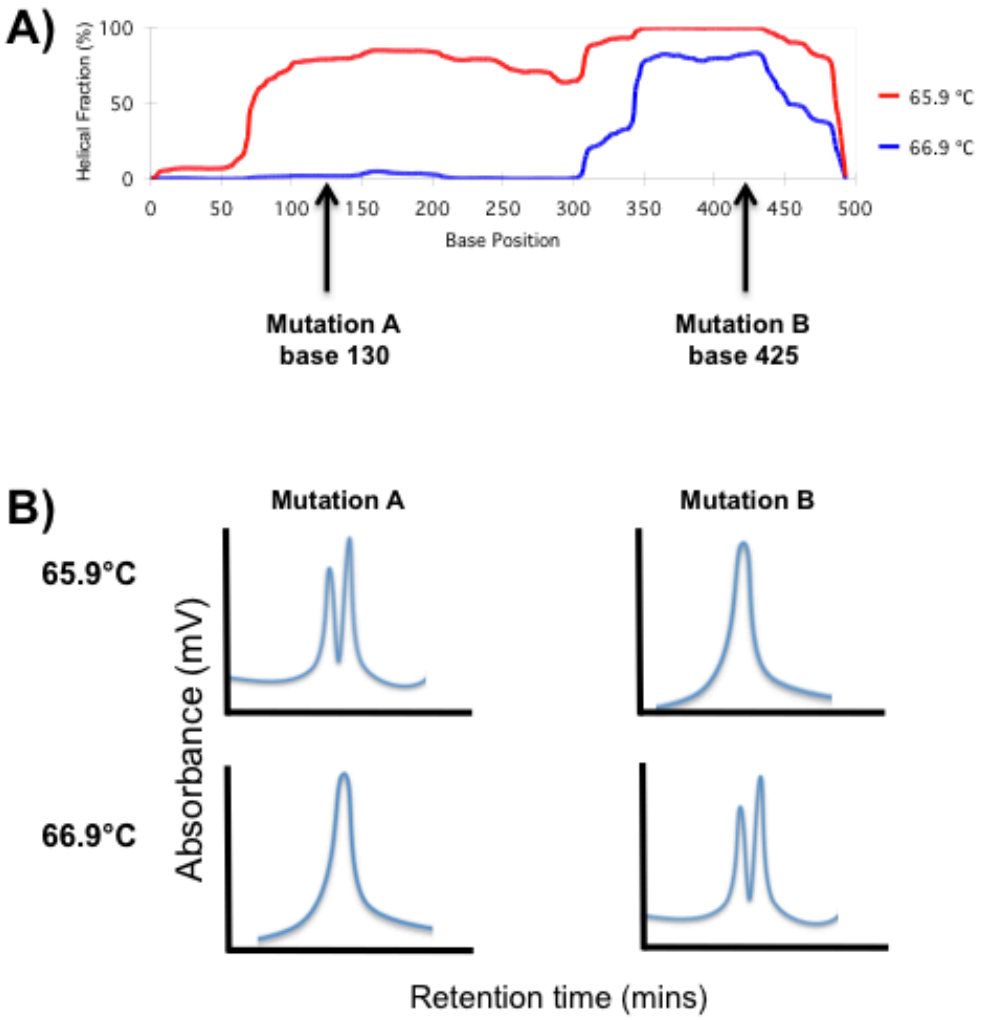

Figure 2.2 Example of a melting profile of a PCR product (A) A typical melting profile for a PCR fragment as analysed by WAVEMAKER® software which takes into account the size of the PCR product and GC content. On the Y-axis is the percentage of the PCR duplex predicted to be helical, such that the completely denatured portions of the PCR fragment are 0% helical, and non-denatured regions are 100% helical. On the X-axis is the base position of the PCR fragment. **(B)** Heteroduplexes are best detected when the PCR fragment is 50-70% helical. The schematic shows an example of why two or more temperatures are often required to identify mutations or SNPs residing in different regions of the PCR fragment. Mutation A would have been missed at 66.9°C as this portion of the PCR fragment is completely denatured at this temperature, whilst mutation B would have been missed at 65.9°C as this portion of the PCR fragment would still be 100% helical.

contain a combination of homo- and heteroduplexes that will elute at different time points (Figure 2.1). In theory, this should create four peaks consisting of all four possible combinations of annealed PCR product – the first two peaks will be the heteroduplexes, followed by the wild-type homoduplex and mutant homoduplex peaks. In practice, both homoduplexes often elute together as a single peak, and only two or three peaks are seen.

Optimal melting temperatures of the PCR products were calculated using WAVEMAKER® software, which takes into account the size of the PCR product and its GC content (Figure 2.2). Heteroduplexes are best detected when the PCR fragment is 50-70% helical; point mutations, insertions/deletions or SNP could potentially be missed (give a single peak rather than a double peak) if the helical fraction is outside of this range for the region harbouring the mutation (Figure 2.2). For this reason, PCR products often need to be eluted from the DNASep column (which is heated with an oven) at two or more temperatures. The optimal PCR product size for sensitive detection of heteroduplexes is 200-500 bp.

Temperature settings were optimised for each PCR product and a wild-type control was included with each run. In order to optimise heteroduplex formation, PCR samples were denatured at 95°C and slow-cooled to 25°C over 40 minutes before running on the column. One of the limitations of DHPLC is that it is not able to detect homozygous or hemizygous mutations (mutation of one allele, and loss of the other), although this can be overcome by mixing PCR products with a known wild-type PCR product prior to denaturation.

2.4 SDS-polyacrylamide gel electrophoresis (PAGE) and Western Blotting

2.4.1 Reagents

- Acrylamide/NN'methylene bis-acrylamide 30%:0.8% (National Diagnostics, Hull, UK)
- Ammonium persulfate (APS) (Sigma Aldrich, Poole, UK)
- Aprotinin (Sigma Aldrich, Poole, UK)
- Bovine Serum Albumin (BSA) (Sigma Aldrich, Poole, UK)
- BD-Perm/Wash™ (BD-Biosciences, Oxford, UK)
- Bradford protein assay kit (BIO-RAD, Hemel Hempstead, UK)

- DL-Dithiothreitol (DTT) (Sigma Aldrich, Poole, UK)
- Enhance chemiluminescence kits (ECL and ECL-plus) (Amersham Life Sciences, Bucks, UK)
- Hybond-C-Extra nitrocellulose membrane (Amersham Life Sciences, Bucks, UK)
- Hyperfilm™ high performance autoradiography film (Amersham Life Sciences, Bucks, UK)
- Leupeptin (Sigma Aldrich, Poole, UK)
- Microcystin LR (Sigma Aldrich, Poole, UK)
- Non-fat dried milk (Marvel)
- Nuclear extraction kit (Active Motif, Belgium)
- Pefabloc (Boehringer-Mannheim, Mannheim, Germany)
- Pepstatin A (Sigma Aldrich, Poole, UK)
- Prestained molecular weight markers (Sigma Aldrich, Poole, UK)
- Radio-Immunoprecipitation Assay (RIPA) Buffer (Sigma Aldrich, Poole, UK)
- Sodium dodecyl sulfate (SDS) (Sigma Aldrich, Poole, UK)
- *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) (Sigma Aldrich, Poole, UK)
- Tris base (Sigma Aldrich, Poole, UK)
- Tween-20 (Sigma Aldrich, Poole, UK)

2.4.2 Lysis buffers and cell lysis

For whole cell lysates, RIPA buffer (50mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS and 50mM Tris, pH 8.0) was supplemented with the following protease inhibitors (final concentrations given): 20mM NaF, 1mM Na orthovanadate, 10 µg/ml Aprotinin, 10µg/ml Pepstatin A, 10 µg/ml Leupeptin, 5µM Microcystin and 1mM Pefabloc. Cells were harvested by centrifugation at 300g for 5 minutes, washed once in ice-cold PBS, pelleted by centrifugation at 300g for 5 minutes, and snap frozen on dry ice. The cells were thawed on ice and resuspended in 3 volumes of RIPA buffer containing protease inhibitors (by approximating the cell pellet volume in µl), vortexed and left on ice for 10 minutes. They were then spun at 12,000g at 4°C for 15 minutes and the supernatant aspirated and stored at -80°C until required.

When immunoblotting for CSL, nuclear lysates gave superior quality bands by Western blot than when using whole cell lysates. Nuclear lysates were prepared from $5x10^6$

primary AML cells and cell lines using a nuclear extraction kit (Active Motif) according to the manufacturer's instructions. The cells were first resuspended in a hypotonic buffer to swell the cell membrane, before addition of a detergent to cause leakage of the cytoplasmic proteins into the supernatant, which was then removed by pipetting. The remaining proteins were enriched with nuclear proteins, which were then solubilised and extracted.

Protein quantification was performed using the Bradford Assay kit (BIO-RAD) according to the manufacturer's instructions. A standard curve was generated using dilutions of BSA at 2μg/ml, 1μg/ml, 0.5μg/ml, 0.25μg/ml, and 0.125μg/ml.

2.4.3 SDS-PAGE buffers

Gel running buffer: 30.3g Tris base, 144.2g Glycine, 10g SDS, made up to 1l with DDW.
Transfer buffer: 30.3g Tris base, 144.2g Glycine, 100mls Methanol, made up to 1l with DDW.
Gel loading buffer (4x): 17.5mls 1M Tris pH 6.8, 4g SDS, 2.315g DTT, 20mls Glycerol, 50mg Bromophenol blue, made up to 50mls with DDW.

2.4.4 Acrylamide gels

10% separating gel: 2.86mls deionised water, 3.74mls 1M Tris pH 8.8, 3.34mls acrylamide/N'N'-bis-methylene 30%:0.8%, 100μl 10% SDS, 75μl 10% APS, 9μl TEMED.
Stacking gel: 3.49mls deionised water, 0.625mls 1M Tris pH 6.8, 0.835mls Acrylamide/NN'methylene bis-acrylamide 30%:0.8%, 50μl 10% SDS, 38μl 10% APS, 7.5μl TEMED.
Preparation of gels: The Mighty-Small Hoeffer gel caster (Hoeffer Scientific Instruments, San Francisco, USA) was used to cast 10cm x 8cm gels. The separating gel was prepared using the reagents stated above and poured into the gel caster, layered with isobutanol to create a horizontal top interface, and left to polymerise at room temperature. After removal of the isobutanol layer, the gel was washed once with DDW, the stacking gel was poured on top of the separating gel and a 10 well comb inserted at the top of the gel. After the gel had polymerised, the comb was removed immediately prior to use and the wells washed with running buffer.

2.4.5 SDS-PAGE

15μg protein was mixed with 5μl 4x loading buffer, made up to 20μl with DDW, boiled for 5 minutes, and loaded onto the gel. A molecular size marker was loaded into one well. Proteins were resolved by electrophoresis in running buffer at 150V for 80 minutes and transferred electrophoretically to nitrocellulose membrane at 0.8mA/cm^2 for 1 hour. The membranes were pre-blocked for 1 hour in 5% skimmed milk in PBS/0.1% Tween (PBST). Immunoblotting was carried out with primary antibodies at dilution factors specified in the relevant chapters, all diluted in PBST and 5% skimmed milk, and left overnight at 4°C with rotation. The membrane was washed 5 times with PBST with rotation. Secondary HRP-linked IgG antibodies to mouse, rabbit or rat (Cell Signalling Technology) were diluted 1 in 10,000 in PBST and left on the membranes for 30 minutes. The membrane was washed 5 times with PBST with rotation, then ECL detection reagent was added and the membrane was exposed to autoradiography film for varying times in a dark room and developed.

CHAPTER 3. THE INCIDENCE AND SPECTRUM OF *NOTCH* AND *FBXW7* MUTATIONS IN T-ALL AND AML, AND THEIR PROGNOSTIC IMPACT

3.1 INTRODUCTION

3.1.1 Oncogenic potential of the Notch signalling pathway

The Notch pathway has been the focus of extensive research in the field of genetics and embryology since the 1920s, yet it was not apparent until the end of the 20th century that Notch pathway dysregulation also played a bona fide role in oncogenesis. Perhaps the first circumstantial evidence came in 1987 with the description of common retroviral integrations in the *Notch-4* locus (known then as *int-3*) in breast cancers initiated by mouse mammary tumour virus (MMTV) in feral mice (Gallahan & Callahan, 1987). This finding was corroborated by studies demonstrating that transgenic mice expressing *Notch-4* develop not only undifferentiated breast cancer at 2-7 months, but also salivary gland tumours (Jhappan *et al*, 1992).

In 1991 Ellisen and colleagues cloned the breakpoints of three paediatric cases of T-ALL harbouring a t(7;9)(q34;q34.3) translocation and revealed almost identical fusions in all three cases (Ellisen *et al*, 1991). The T-cell receptor-β (*TCR-β*) promoter on chromosome 7 was found to be juxtaposed to an intronic region in *NOTCH-1* (described at that time as the *TAN-1* gene) on chromosome 9, which led to constitutive expression of a truncated intracellular Notch (*ICN-1*) transcript lacking an N-terminal domain. Experiments in *Drosophila melanogaster* at that time showed that N-terminal truncations of this type led to a gain-of-function phenotype (Rebay *et al*, 1993), experiments in *Caenorhabditis elegans* having earlier demonstrated that the negative regulation of Notch cleavage was mediated by the HD and LNR domains (Greenwald & Seydoux, 1990). Although the t(7;9) translocation has turned out to be rare in larger studies of T-ALL, affecting only 1% of patients, it was the first description implicating aberrant Notch signalling in T-ALL. Furthermore, it was this finding that prompted Pear and colleagues to express *ICN-1* in murine marrow (Pear & Aster, 2004; Pear *et al*, 1996). All mice developed a highly penetrant and fatal T-ALL with a short latency. In this model, mice had a block in differentiation at the DN3 stage of T-cell development and expansion of polyclonal T-cells, before developing a monoclonal transplantable T-ALL. Interestingly, transgenic mice where *ICN-1* is driven by the *Lck*-promoter have a very

low penetrance of T-ALL, suggesting that overexpression of *ICN-1* in early haematopoietic progenitors, rather than just at the thymic stage, is important in *Notch-1*-induced leukaemogenesis (Robey *et al*, 1996). This is in contrast to the highly penetrant leukaemic phenotype in *Lck-ICN-3* transgenic mice, suggesting that Notch-1 and Notch-3 have oncogenic roles at different stages of T-cell maturation (Bellavia *et al*, 2000; Bellavia *et al*, 2002).

Insertional mutagenesis models have also implicated Notch-1 in the pathogenesis of T-ALL. For instance, when Moloney murine leukemia virus (MuLV) was used to accelerate tumour onset in *myc* transgenic mice, *Notch-1* was found to be a retroviral insertion site in 52% of the T-cell leukaemias that developed (Girard *et al*, 1996; Paquette *et al*, 1992). Remarkably many of the insertions occurred within 300bp of the intronic breakpoint described in the t(7;9) translocation (Girard *et al*, 1996) and commonly integrated in two distinct sites of *Notch-1*: in the extracellular HD and LNR domains leading to high expression of *ICN-1*, or in the PEST domain leading to expression of a truncated ICN-1 protein (Hoemann *et al*, 2000). Similar integrations were found in the *p27kip* null, *Eed* null and *AKXD* transgenic mouse models, suggesting that the insertions were not particular to *myc* transgenic mice and that Notch was capable of co-operating with different signalling pathways during T-cell leukaemogenesis (Akagi *et al*, 2004; Hansen *et al*, 2000; Suzuki *et al*, 2002). Given that a high proportion of the integration sites found in these models are generally 'false' discoveries, representing sites of preferred viral integration rather than relating to genuine oncogenes or tumour-suppressor genes, an important finding was that sustained Notch signalling was required for *in vitro* growth of some murine-derived T-ALL cell lines (Weng *et al*, 2003). Furthermore, deletions of either the 5' extracellular domain or the 3' PEST domain of *Notch-1* were found in over half of T-cell thymic lymphomas in mice when the tumours were initiated by radiation rather than viral mutagens (Tsuji *et al*, 2003).

3.1.2 Discovery of activating *NOTCH-1* mutations in paediatric T-ALL

Given the accumulating evidence implicating *NOTCH-1* in T-ALL, and with insertional integrations as a guide of where to focus their sequencing efforts, the groups of John Aster (Brigham and Women's Hospital, Boston) and A.Thomas Look (Dana-Farber Cancer Institute, Boston) chose to sequence *NOTCH-1* exons 26 (coding for the HD-N), 27 (the HD-C) and 34 (the PEST domain) in a cohort of 96 paediatric patients with T-

ALL. They found that a striking 56% of patients harboured mutations in one or more of these domains (Weng *et al*, 2004). Mutations in the HD domain tended to be missense, with a particular propensity for leucine to proline substitutions, or in-frame insertions or deletions in highly conserved amino acids. Mutations in the PEST domain tended to be out-of-frame insertions or deletions, often as large and complex *indels* leading to a premature stop codon and a truncated protein. Approximately 16% of patients had mutations in both domains *in cis*. Luciferase reporter gene assays showed that these mutations were activating and that the combination of HD and PEST mutations had a marked synergistic effect on activation. Furthermore, they demonstrated that five *NOTCH-1* mutated human T-ALL cell lines treated with a γ-secretase inhibitor (GSI) went into G0/G1 cell cycle arrest, although a further 25 cell lines remained resistant to Notch inhibition for reasons that were then uncertain.

3.1.3 Mechanisms of pathway activation by *NOTCH-1* mutations

Insights into the function of the Notch HD and LNR domains have come from crystal radiography and biochemical analyses and support a mechanism by which the LNR domain folds over the HD domain, protecting the metalloprotease site from cleavage by TACE (S2 cleavage) (Sanchez-Irizarry *et al*, 2004). Upon physiological ligand-binding to the Notch EGF region, a mechanical force is thought to uncouple the LNR domain from the HD domain, exposing the S2 site to cleavage by TACE, which is followed by constitutive S3 cleavage by γ-secretase in the transmembrane domain and subsequent release of ICN.

Mutations in the HD domain are thought to lead to constitutive S2 cleavage or hypersensitivity to ligand binding, leading to increases in ICN (Gordon *et al*, 2009). The HD domain mutations have been divided into three distinct classes (Malecki *et al*, 2006; Sulis *et al*, 2008). Class I mutations are missense amino acid changes or small in-frame insertions or deletions that disrupt the interaction between the HD and LNR domains at important residues leading to exposure of the S2 site. Class II mutations are larger in-frame insertions that duplicate the S2 cleavage site, exposing it to constitutive cleavage. An example of the latter is the 14 amino acid insertion found in the P12-Ichikawa T-ALL cell line, which was the most potently activating mutation of 14 mutants tested in an *in vitro* reporter assay (Malecki *et al*, 2006). A third class of mutation has been identified in a minority of patients involving duplications in exon 28 that result in

insertions in the juxtamembrane domain (so called juxtamembrane expansion mutations [JME]), although their mechanism of activation is unclear (Sulis *et al*, 2008).

The PEST domain of *NOTCH-1* contains a highly conserved serine at position 2512 followed by a threonine residue at position 2514, which have been identified as the site for FBXW7-mediated ubiquitination of ICN-1 (Oberg *et al*, 2001). The mutations described in the PEST domain are predicted to disrupt or truncate ICN-1 prior to these residues allowing ICN-1 to escape FBXW7-mediated degradation, with a subsequent increase in intracellular half-life.

3.1.4 NOTCH-1 activates oncogenes in T-ALL

Chromatin immunoprecipitation and microarray analysis of promoter occupancy (ChIP-on-chip), ChIP with DNA sequencing (ChIP-seq) and gene expression array analysis after Notch inhibition have all been instrumental in identifying the downstream targets of the ICN-1-CSL-MAML activation complex in T-ALL cells (Palomero *et al*, 2006a; Wang *et al*, 2011; Weng *et al*, 2006). The classic Notch target genes in embryonic development are basic helix-loop-helix transcriptional repressors such as hairy enhancer of split-1 and -5 (HES-1 and HES-5). Although their role in T-ALL pathogenesis is still unclear, HES-1 has been shown to bind and repress the *PTEN* promoter leading to down-regulation of PTEN protein expression, suggesting that ICN-1 can activate PI3K signalling in T-ALL (Palomero *et al*, 2007). In addition, a number of important oncogenes are direct transcriptional targets of ICN-1:

1) c-Myc. A number of published studies have identified MYC as a direct transcriptional target of Notch signalling (Palomero *et al*, 2006a) (Wang *et al*, 2011; Weng *et al*, 2006). The majority of T-ALL cell lines have high protein levels of MYC and inhibition of Notch signalling with GSI treatment leads to significant mRNA and protein depletion of MYC (Weng *et al*, 2006). Furthermore, a number of Notch-dependent T-ALL cell lines can be rendered resistant to GSI treatment by retroviral expression of MYC, suggesting that Notch-dependency is mediated through MYC in these cells (Weng *et al*, 2006). A compelling finding was demonstrated elegantly in the 8946 T-ALL cell line which had been generated from transduction of murine marrow with a doxycycline-repressible *c-Myc* transgene (Weng *et al*, 2006). This cell line proliferates rapidly *in vitro* and undergoes apoptosis on the addition of doxycycline

(Felsher & Bishop, 1999). The authors demonstrated that retroviral transduction with ICN-1 upregulates the expression of endogenous myc and inhibits cell death after doxycycline administration (Weng *et al*, 2006).

2) Cyclin D3. Overexpression of cyclin D3 has been identified in T-ALL cell lines as a consequence of direct transcriptional activation by NOTCH-1 and has been shown to regulate G1-S phase cell cycle progression in T-ALL cell lines and *in vivo* (Joshi *et al*, 2009). Furthermore, overexpression of cyclin D3 partially rescues Notch-dependent T-ALL cells from cell cycle arrest induced by GSI treatment.

3) IGF1R. More recently, signalling through the insulin growth factor receptor-1 (IGF1R), which activates the PI3K/AKT pathway, has been shown to be important for leukaemia-initiating cell activity in a serial transplant murine model of T-ALL (Medyouf *et al*, 2011). Although conditional deletion of the IGF1R had only a minimal impact on the latency of ICN-1 induced T-ALL, it dramatically reduced the ability to serially transplant tumours into secondary recipients. Furthermore, the authors identified that CSL-ICN-1 binds the IGF1R enhancer in intron 1, a finding that has since been corroborated in ChIP-seq studies (Wang *et al*, 2011).

4) IL7R. IL7R is highly expressed in the majority of T-ALL cases, and is directly transcriptionally regulated by Notch through a pathway that is important in normal T-cell development (Gonzalez-Garcia *et al*, 2009; Wang *et al*, 2011). An oncogenic role for IL7R has recently been described in T-ALL, with activating mutations occurring in approximately 10% of T-ALL patients (Shochat *et al*, 2011; Zenatti *et al*, 2011). The majority of mutations described thus far in T-ALL involve the insertion of a cysteine in the transmembrane domain leading to ligand-independent IL7R homodimerisation and activation of JAK1 and STAT5. Crystallography data has shown that when the wild-type receptor is expressed at high levels it too can homodimerise, suggesting that upregulation of wild-type IL7R by Notch may in itself activate JAK-STAT signalling in T-ALL, though this is yet to be proven (McElroy *et al*, 2012).

3.1.5 *FBXW7* – a frequently mutated tumour-suppressor in T-ALL

Ubiquitination describes the process by which proteins are first labelled by ubiquitin prior to their degradation by the proteosome. F-box and WD40 domain-containing

protein 7 (FBXW7) is an E3-ubiquitin ligase (also termed FBW7, CDC4, AGO or SEL-10) that is a member of the highly conserved SCF complex (named after its three members SKP1, CUL1 and F-box protein). This complex covalently attaches ubiquitin groups to proteins that harbour the consensus sequence of I/L-I/L/P-T-P-XXXX, where lysine and arginine are unfavourable in the X locations (Welcker & Clurman, 2008). This motif has been termed a phospho-degron, given that the threonine first requires phosphorylation prior to recognition by FBXW7, a process that often involves GSK3β (Fryer *et al*, 2004; McKenzie *et al*, 2006). FBXW7 can form homodimers mediated through its N-terminal dimerisation domain, although the role of dimerisation remains unclear given that monomeric forms can still target most of its known substrates. It may, however, have a role in determining particular affinity for cyclin E (Welcker & Clurman, 2007).

Crystallography data shows that FBXW7 consists of an F-box that interacts with SKP1 to form the SCF complex, followed by eight WD40 repeats that form a configuration similar to petals on a flower or blades of a propeller (Figure 3.1) (Orlicky *et al*, 2003b). Each of the propeller-like structures is capable of binding one or more substrates. Multiple important oncogenes have been shown to be substrates of FBXW7, some of which are listed below:

1) **c-Myc.** MYC is a direct transcriptional target of Notch signalling, and FBXW7 exerts another level of control on the Notch pathway by degrading MYC (Palomero *et al*, 2006a). Interestingly, in Burkitt's lymphoma, which in nearly all cases is driven by MYC over-expression, 60% of patients have mutations of MYC, the most common being an amino acid substitution at position T58 that leads to increased protein stability by the virtue of its ability to avoid FBXW7-mediated degradation (Chang *et al*, 2000). Furthermore, *fbxw7* conditional knockout (-/-) mice develop thymic lymphomas with high levels of myc (Onoyama *et al*, 2007).

2) **NOTCH-1.** The conserved threonine at position 2512 in the PEST domain of NOTCH-1 has been shown to be the target of phosphorylation by GSK3β, as well as by CDK8 and cyclin C, which is the priming event required for FBXW7 binding (Figure 3.1) (Fryer, White *et al.* 2004,(Wu *et al*, 2001). Conditional *in*

vivo deletion of FBXW7 leads to stabilisation of ICN-1, which in turn transcriptionally activates Myc (Onoyama *et al*, 2007). Presenilin-1, which forms part of the γ-secretase complex, is also targeted by FBXW7 for degradation, suggesting that FBXW7 controls Notch signalling both at the level of Notch cleavage and degradation of ICN (Li *et al*, 2002).

3) **Cyclin E.** Cdc4, the yeast homologue of FBXW7, was first discovered in a genetic screen of yeast mutants with an altered cell cycle (Yochem & Byers, 1987). Subsequently, multiple groups identified Cyclin E as the first bona fide Cdc4 target, and the regulation of cyclin E by mammalian FBXW7 was confirmed soon thereafter (Hao *et al*, 2007; Koepp *et al*, 2001; Orlicky *et al*, 2003a). Cyclin E controls G1 to S phase transition and is over-expressed in hepatocellular carcinomas (Geng *et al*, 2003). Cyclin E knockout mice exhibited almost total resistance to induction of hepatocellular carcinomas (Geng *et al*, 2003). However, the importance of cyclin E in T-ALL is still uncertain.

4) **mTOR.** In a search for proteins containing the conserved FBXW7 phospho-degron sequence, mTOR was also identified, and functionally validated, as a substrate of FBXW7 (Mao *et al*, 2008). mTOR can also be upregulated by Notch, supporting the hypothesis that Notch and FBXW7 collaborate to activate multiple pathways in T-ALL. Of importance to therapeutic approaches, increased sensitivity to mTOR inhibition was seen in cell lines harbouring mutations or deletions of FBXW7 (Mao *et al*, 2008).

5) Other important targets include the anti-apoptotic protein **MCL-1**, which is over-expressed at the protein level in T-ALL as a consequence of mutation of FBXW7, **MYB**, which is frequently duplicated in T-ALL, and **c-Jun** (Inuzuka *et al*, 2011; Kitagawa *et al*, 2009; Zhang *et al*, 2011)

Given its role in the degradation of such an array of oncogenes, it is not surprising that loss-of-function mutations, or more rarely, deletions, of the *FBXW7* gene have been identified in many types of human cancers, including cholangiocarcinomas (35%), and those affecting the endometrium (9%), colon (9%), stomach (6%) and head-and-neck (5%) (Agrawal *et al*, 2011; Akhoondi *et al*, 2007). The discovery of a high frequency of

mutations in *FBXW7* in T-ALL came about through analysis of thymic lymphomas from triple knockout mice ($Tp53^{-/-}$, $Terc^{-/-}$ and $Atm^{-/-}$), which harboured frequent deletions of this locus as evident by array CGH (Maser *et al*, 2007). Subsequent analysis of samples from T-ALL patients found a frequency of mutations of between 9-30% (Akhoondi *et al*, 2007; Mansour *et al*, 2009; O'Neil *et al*, 2007).

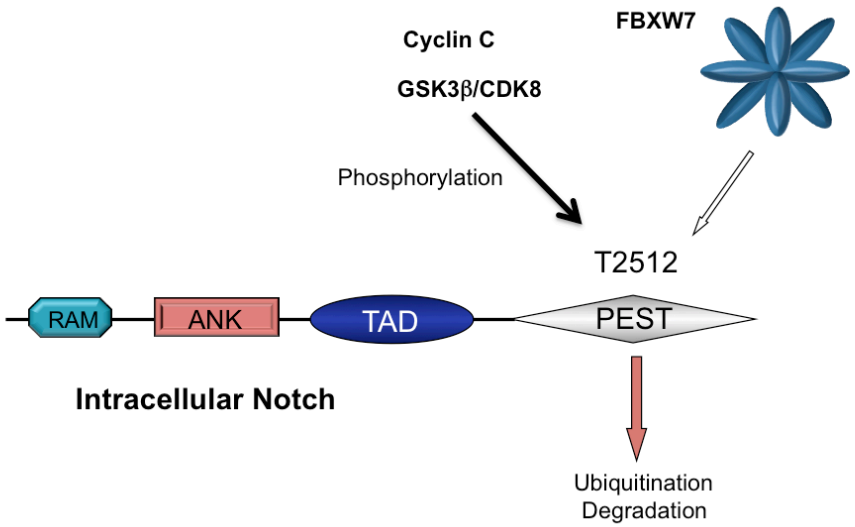

Figure 3.1 Schematic showing regulation of NOTCH through ubiquitination.
GSK3β, CDK8 and cyclin C are proposed to phosphorylate T2512 of the NOTCH PEST domain priming it for binding by the E3 ubiquitin ligase FBXW7 which leads to its degradation.

There are three particular amino acid mutation hotspots found in T-ALL - R465, R479 and R505. These arginine residues form the surface of the propeller blades in the WD40 domain required for substrate recognition. Somatic missense mutations of one of these arginines, often to either a histidine or cysteine, have been shown to abrogate FBXW7-mediated degradation of both Notch and Myc, leading to their increased stability (O'Neil *et al*, 2007). This mechanism is thought to play a role in resistance of some T-ALL cell lines to GSIs, whereby the increased stability of Myc through FBXW7 mutation uncouples it from its dependence on Notch activity (O'Neil *et al*, 2007). Given the ability of FBXW7 to homodimerise, mutations may also have dominant-negative activity on the wild-type protein (Akhoondi *et al*, 2007). Cytosine methylation has been identified at some of these hotspots in ovarian and endometrial cancer, which is interesting given that the observed mutations are often C to T transitions within a CG dinucleotide that can result from spontaneous deamination of 5'-methylcytosine to thymine (Akhoondi *et al*, 2007).

3.1.6 Prognostication in T-ALL

The various clinical and molecular features that are currently used in risk stratification in T-ALL are discussed in Chapter 1. A risk-adapted approach to the treatment of patients with ALL has the potential of improving survival in high-risk patients and reducing therapy-related long-term sequelae in those at low-risk. Whilst significant advances have been made in stratifying patient prognosis in AML through the use of cytogenetic and molecular characterisation of the presenting sample, there has been a paucity of informative prognostic markers in T-ALL. This can be attributed to at least three facts. Firstly, large cohort studies have been hampered by the relative rarity of T-ALL. Secondly, non-cryptic cytogenetic abnormalities are both infrequent and less prognostically informative in T-ALL as compared to AML. Thirdly, unlike the identification of a number of common molecular markers in AML, e.g. FLT3-ITDs, high frequency recurrent mutations in T-ALL had not been identified until relatively recently. Consequently, clinicians have had, and continue, to use criteria established as long ago as the 1980s for risk stratification, namely, presenting blast count and age (Hoelzer *et al*, 1988; Rowe *et al*, 2005). However, the marked heterogeneity in outcome among standard- and high-risk patients according to these criteria limits their clinical utility.

As discussed in chapter 1, minimal residual disease (MRD) kinetics using immunoglobulin or TCR rearrangements contributes significantly towards current stratification protocols (Bruggemann *et al*, 2006; Raff *et al*, 2007). However, there is some evidence suggesting that MRD in adult T-ALL is less robust at predicting prognosis and identifying early relapse than it is in adult B-ALL, or paediatric B- or T-ALL (Gameiro *et al*, 2002; Szczepanski *et al*, 2003; Willemse *et al*, 2002). Consequently, mutation screening strategies that predict outcome could prove invaluable in clinical decision making in adult T-ALL, particularly in the selection of patients for allogeneic haematopoietic stem cell transplantation, and the use of molecular markers that can complement or supersede current prognostication strategies are needed.

3.2 AIMS

This chapter describes studies to investigate a number of different questions regarding *NOTCH-1* and *FBXW7* mutations in acute leukaemia. Firstly, the incidence and spectrum of *NOTCH-1* and *FBXW7* mutations in a cohort of adult patients with T-ALL was

investigated. The first report of *NOTCH-1* mutations had been in a paediatric T-ALL cohort (Weng *et al*, 2004) and at the time the work in this thesis was started, the presence or incidence of *NOTCH-1* mutations in adult patients had not been described. Although the phenotype of adult T-ALL is similar to its paediatric counterpart, there are significant differences, in particular adult patients have a different frequency of cytogenetic abnormalities and oncogene expression and a poorer prognosis (Pui & Evans, 1998). Some of these differences may be attributable to the incidence and characteristics of *NOTCH-1* mutations in adult patients, which may impact on signalling events downstream of NOTCH-1 and, in turn, response to therapy.

The second aim of the studies presented in this chapter was to assess the association of *NOTCH-1* and *FBXW7* mutations with prognostic outcome in a cohort of adult patients with T-ALL treated on the MRC UKALLXII/ECOG2993 trial.

Thirdly, the potential for alternative mechanisms of Notch pathway activation in T-ALL patients were explored, specifically, the possibility that the LNR domain of *NOTCH-1* could also be a site for mutational disruption considering that it interfaces directly with the HD. Furthermore, given that ICN-3 can initiate leukaemogenesis in murine models, is highly over-expressed in T-ALL patients, and is structurally homologous to NOTCH-1 (Bellavia *et al*, 2002), the possibility that *NOTCH-3* is also activated by somatic mutations was also investigated.

The fourth aim was to investigate whether *NOTCH-1* mutations are also present in adult and infant AML patients. Mutational studies of *NOTCH-1* and *NOTCH-3* and the downstream pathway had not been carried out in AML, although gene array profile studies had shown increased expression of *NOTCH-1* and its ligand *JAGGED-1* in a subgroup of patients (Ross *et al*, 2004). Aberrant expression of T-cell markers such as CD2 and CD7 on AML blast cells has been reported in about 25% of adult AML cases (Casasnovas *et al*, 2003), and mixed-lineage surface antigen expression is particularly prevalent in infant AML (Biondi *et al*, 2000). The mechanism underlying this is unknown but may be due to either ‘lineage infidelity’, i.e. deregulation of lineage-affiliated genes during the leukaemic transformation process, or ‘lineage promiscuity’, i.e. reflecting the multipotency of the progenitor cell that is transformed (Greaves *et al*, 1986). Given that activated Notch signalling favours T-cell differentiation, mutational

activation of *NOTCH-1* may lead to aberrant expression of T-cell antigens, and also play a role in the increased self-renewal capacity and impaired differentiation found in AML blasts.

3.3 PATIENTS, MATERIALS AND METHODS

3.3.1 Patients

Genomic DNA samples were available from a total of 60 adult patients with T-ALL, of which 54 had been entered into the MRC UKALLXII trial, and 6 had samples collected locally after informed consent. DNA samples obtained from remission bone marrow were available from 15 patients. *NOTCH*-1 and *FBXW7* mutation data was made available to us from an additional 34 patients treated on ECOG2993 in collaboration with Adolfo Ferrando's group (who performed the mutational analysis) from Columbia University Medical Center, New York and Elisabeth Paietta's group (who collected the samples) from Our Lady of Mercy Cancer Center, New York. DNA samples from patients with infant leukaemia (patients<1 year of age) (n=29) and paediatric T-ALL (patients<13 years of age) (n=12) were obtained from Great Ormond Street Hospital in collaboration with Dr Phil Ancliff and Dr Nick Goulden. DNA samples were obtained from 156 young adult patients with AML from the tissue bank from patients entered onto the MRC/NCRI AML10, 12 and 15 trials. Ethical approval for the trials and tissue collection for research was obtained from the Multi-Centre Research Ethics Committees and local research ethics committees as appropriate and informed consent was provided according to the Declaration of Helsinki.

3.3.2 Denaturing High Performance Liquid Chromatography (DHPLC) Analysis

3.3.2.1 DHPLC screening of *NOTCH-1* mutations

NOTCH-1 mutation screening and identification were carried out using denaturing high-performance liquid chromatography (DHPLC) of PCR products followed by sequencing of samples with abnormal chromatograms. The principles and methods regarding PCR, DHPLC analysis and sequencing are discussed in Chapter 2. The LNR, HD-N, HD-C, TAD and PEST domains were amplified by 35 cycles of the PCR using the primer sequences and annealing temperatures shown in Table 3.1. Due to its size (646bp), which is not optimal for sensitive mutation detection by DHPLC analysis, the LNR was divided

into two overlapping fragments (298bp and 400bp) termed LNR-1 and LNR-2 for the 5' and 3' fragments respectively. The proofreading enzyme Optimase (Transgenomic) was used according to the manufacturer's specifications for the LNR, TAD and HD-C domains. Given the high GC content of these fragments, 1M betaine (final concentration) was added to enhance PCR efficiency. PCR products of adequate quality could not be obtained using Optimase for the HD-N and PEST domains, and these fragments were amplified using the non-proofreading enzyme BioTAQ DNA polymerase (Bioline). PCR products were run out on an agarose gel, denatured and slow-cooled to optimise heteroduplex formation (see Chapter 2.2.5 and Figure 2.1) and then analysed on the WAVE DNA Fragment Analysis System, Transgenomic.

Table 3.1 *NOTCH-1* primer sequences, PCR fragment sizes, PCR annealing temperatures and DHPLC analysis temperatures.

***NOTCH-1* Domain (Exon)**	**Primer name**	**Primer Sequence**	**PCR fragment size**	**PCR Annealing temp**	**DHPLC Analysis temp**
LNR-1 (exon 25)	25-A-F	5'-TGGCCCACCCCGACACCG-3'	298bp	63°C	64.5°C and 67.3°C
	25-A-R	5'-CAAGAGCCCGTTGAATTTGGCG-3'			
LNR-2 (exon 25)	25-B-F	5'-CGAGAGCCCCTTCTACCGTTG-3'	400bp	64°C	63.8°C and 66.7°C
	25-B-R	5'-CTCCCTCAGCCCCATGAGC-3'			
HD-N (exon 26)	26-F	5'-GGAAGGCGGCCTGAGCGTGTC-3'	494bp	67°C	65.9°C and 66.9°C
	26-R	5'-ATTGACCGTGGGCGCCGGGTC-3'			
HD-C (exon 27)	27-F	5'-GCCTCAGTGTCCTGCGGC-3'	320bp	Touch down 65-61°C	65.0°C
	27-R	5'-GCACAAACAGCCAGCGTGTC-3'			
JM (exon 28)	28-F	5'-TGATTAATCGCGTAGAAAATCACCT-3'	418bp	Touch down 60-56°C	N/A
	28-R	5'-CACCGGGGACCCAGAAGC-3'			
TAD (exon 34)	TAD-F	5'-GCTGGCCTTTGAGACTGGC-3'	483bp	62°C	63.5°C and 64.5°C
	TAD-R	5'-GCTGAGCTCACGCCAAGGT-3'			
PEST (exon 34)	PEST-F	5'-CAGATGCAGCAGCAGAACCTG-3'	520bp	64°C	64.4°C and 65.7°C
	PEST-R	5'-AAAGGAAGCCGGGGTCTCGT-3'			

N/A = Not applicable

Abnormal chromatograms were confirmed by repeat WAVE analysis. Fresh PCR products were then sequenced bidirectionally using the Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter) and analysed on the CEQ8000 Genetic Analysis System (Beckman Coulter). The nucleotide and amino acid numbering used corresponds to RefSeq accession number NM_017617.3 and Ensembl Transcript

ENST00000277541. For cases with low level heteroduplex formation by DHPLC analysis that were below the sensitivity of sequencing, the fragment collector facility of the WAVE was used to collect the heteroduplex peak for reamplification by PCR and sequencing. The technique and results of this method are discussed in more detail in chapter 4.

Juxtamembrane expansion (JME) mutations were analysed by PCR of exon 28 using BioTAQ DNA polymerase and buffer according to the manufacturers guidelines, and 35 cycles of PCR were performed with annealing temperature and primers shown in Table 3.1. PCR products were resolved by gel electrophoresis containing 2.5% agarose. DNA from the Jurkat T-ALL cell line, which contains a 51bp duplication in exon 28, was used as a positive control. As duplications were often too large and complex to resolve comprehensively by direct sequencing, PCR products were TOPO cloned prior to sequencing.

Table 3.2 *NOTCH-3* primer sequences, PCR fragment sizes, annealing temperatures and DHPLC melting temperatures.

***NOTCH-3* Domain (Exon)**	**Primer name**	**Primer Sequence**	**PCR fragment size**	**PCR Annealing temp**	**DHPLC Analysis temp**
LNR (exon 24)	24-F	5'-GTTCTGGGGTCCGCGTTGC-3'	605bp	63°C	66.5°C,68.3°C and 70.2°C
	24-R	5'-GGATGCATAGACAGACGGATCG-3'			
HD-N (exon 25)	25-F2	5'-CTCTGACCCCTGACTCCGC-3'	499bp	63°C	65.0°C and 66.8°C
	25-R	5'-TCTCCCCAGCCACCACGGC-3'			
HD-C (exon 26)	26-F	5'-ACCAGGGGGTGCATCGGGC-3'	298bp	62°C	63°C and 66.8°C
	26-R	5'-CGAGGGCGGGGCTTTGGC-3'			
TAD (exon 33)	TAD-F	5'-CGCTTGCTGGATCAACCCAGT-3'	451bp	61°C	65.3°C and 67.3°C
	TAD-R	5'-AATCGAGGGGCACAGCCACAG-3'			
PEST (exon 33)	PEST-F	5'-GGAGGATGTGTACTCAGCCTG-3'	535bp	60°C	64.0°C and 66.5°C
	PEST-R	5'-CCCCAAGATCTAAGAACTGACG-3'			

3.3.2.2 DHPLC for the screening of *NOTCH-3* mutations

NOTCH-3 mutation screening was carried out using DHPLC analysis and sequencing as before. The exons corresponding to the *NOTCH-1* LNR, HD-N, HD-C, TAD and PEST domains were identified by aligning the nucleotide sequences using BLASTN and showed between 73% and 86% homology for each domain. These regions were

amplified by 35 cycles of the PCR using the proofreading enzyme Optimase (Transgenomic), with the inclusion of 1M betaine for the HD-N and HD-C domain PCRs. The primer sequences were analysed by BLAST and in detail against the other Notch family members to ensure specificity to *NOTCH-3* and are shown in Table 3.2 together with the annealing temperatures. Optimal melting temperatures for DHPLC analysis were calculated using WAVEMAKER software and are given in Table 3.2. Nucleotide and amino acid numbering are according to RefSeq accession number NM_000435.2 (equivalent to ensembl ENSG74181).

3.3.2.3 DHPLC for the screening of *FBXW7* mutations

Screening for *FBXW7* mutations was only carried out for exons 8-12 since these exons encode the WD40 domain, which has been reported to harbour the vast majority of mutations in T-ALL. Samples were analysed by Optimase PCR and DHPLC heteroduplex analysis as before, followed by sequencing of abnormal chromatograms. Primer sequences, PCR fragment sizes, PCR annealing temperatures and DHPLC analysis temperatures are given in Table 3.3. Unlike mutation screening for *NOTCH-1*, where the variety of mutations precluded prediction of the mutation by analysis of the WAVE pattern, the characteristics of the abnormal heteroduplex patterns for *FBXW7* exons were highly predictive of the mutation, although all were confirmed by sequencing. The nucleotide and amino acid numbering used corresponds to Ensembl Transcript ENST00000281708.

Table 3.3 *FBXW7* primer sequences, PCR fragment sizes, annealing temperatures and DHPLC melting temperatures.

FBXW7 Domain (Exon)	Primer name	Primer Sequence	PCR fragment size	PCR Annealing temp	DHPLC Analysis temp
WD40 (Exon 8)	8F	5'-AGATAGACTACAAATTACTGTTCCTG-3'	255bp	55°C	56.0°C and 58.0°C
	8R	5'-CTTTGTGAAGTGTAGGAAGAGTAAAC-3'			
WD40 (Exon 9)	9F	5'-TCTACCCAAAAGTAATCATCTTAAGTG-3'	259bp	56.0°C	58.6°C
	9R	5'-ATAGAGGAAGAAGTCCCAACCAT-3'			
WD40 (Exon 10)	10F	5'-GTTTTTCTGTTTCTCCCTCTGCA-3'	300bp	58.0°C	56.0°C and 59.6°C
	10R	5'-ACCTTATGATTCATCAGGAGAGC-3'			
WD40 (Exon 11)	11F	5'-GTAATTGATAGGAAGAGTATCCATAC-3'	374bp	60.0°C	56.6°C and 59.0°C
	11R	5'-AACCATTCTGTATGAGGTTGACTC-3'			
WD40 (Exon 12)	12F	5'-CAAATTATAATGTAACTAACTCATAGCCA-3'	385bp	56.0°C	57.6°C and 61.5°C
	12R	5'-GAGTATATCGTCTACACAATTGGAC-3'			

3.3.3 Statistical analysis of clinical outcome data

Statistical analysis of the MRC UKALLXII/ECOG2993 cohort was performed by Georgina Buck and Sue Richards at the Clinical Trial Service Unit, Oxford, UK. The association between *NOTCH-1* and *FBXW7* mutation status with age (<35 years *vs.* ≥35 years) and WCC (<30x10^9/l *vs*. 30-99 *vs.* ≥100x10^9/l) at diagnosis were investigated using Fisher's Exact test in 2x2 tables and Mantel Haenszel test for trend. Outcome was analysed according to overall survival (OS) and event-free survival (EFS), the latter defined as time to relapse or death. Kaplan-Meier curves were used to assess survival, and differences between groups were compared by the log-rank test. Multi-variate analyses were performed using the Cox model. All p-values quoted are two-sided

3.4 RESULTS

At the commencement of these studies, *NOTCH-1* mutations had only been reported in paediatric patients with T-ALL, therefore the first question addressed was whether the incidence and characteristics of *NOTCH-1* mutations was similar in adult patients.

3.4.1 *NOTCH-1* mutations are highly prevalent in adult patients with T-ALL

DHPLC analysis was used to screen the HD-N, HD-C, TAD and PEST domains of *NOTCH-1* in samples from 60 adult patients. A wide range of different abnormal patterns were identified, and examples for the HD-N domain are shown in Figure 3.2. Given the wide variety of patterns, prediction of the specific mutation purely from the chromatogram was not possible, so all fragments with abnormal patterns were sequenced. Overall, heteroduplex patterns indicative of an alteration in at least one PCR fragment were seen in 40 of 60 (67%) patients (Table 3.4). However, in two patients (T17 and T26), the only alteration was a previously described single nucleotide polymorphism (SNP) (5094C>T, rs10521) which was synonymous at the amino acid level (D1699D) and thus not thought to be of functional or pathological significance. According to the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) it is found at a frequency of 0.43 in the normal population. Therefore, 38 patients (63%) had at least one *NOTCH-1* mutation, the details of which are listed in Table 3.4. Twenty-

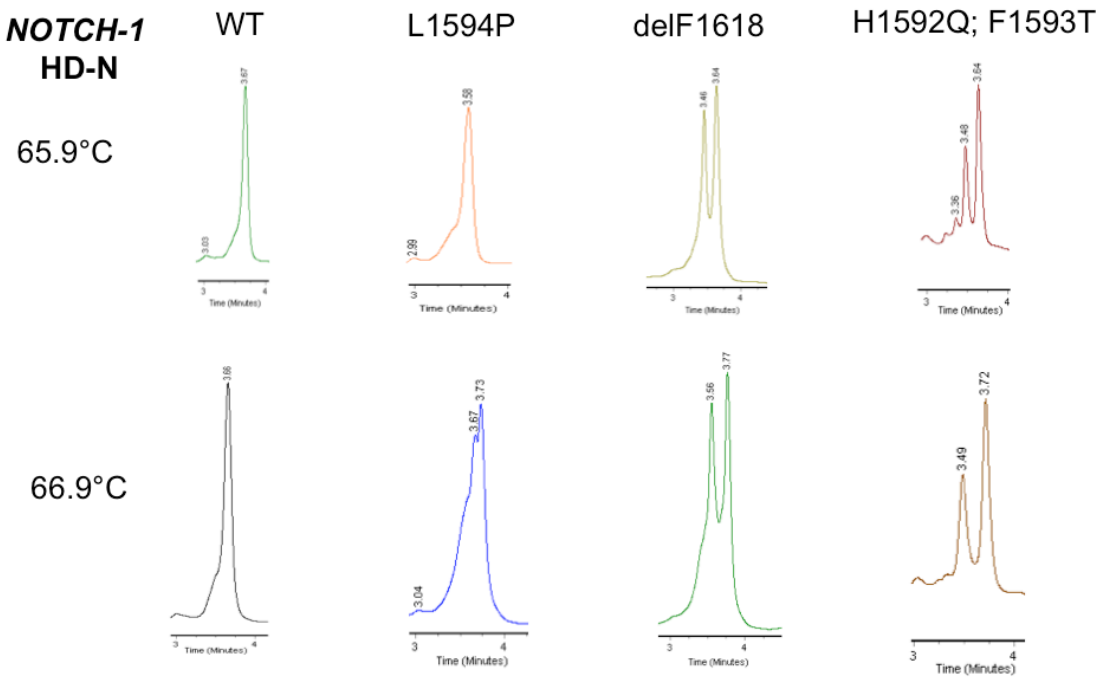

Figure 3.2 DHPLC patterns for the HD-N domain. The wild type HD-N PCR fragment has a single homoduplex peak, whereas heteroduplex peaks are detected in 3 representative examples of mutations identified in adult T-ALL patients.

three patients (38% of the total cohort) had mutations in the HD domain only, none in the TAD domain, five (8%) in the PEST domain only, two (3%) had JME mutations only, and eight (13%) had mutations in both the HD and PEST domains (Table 3.5).

Mutations in the HD-N domain were the most frequent, affecting 24 of the total cohort (40%), and tended to cluster in the conserved region between residues 1593 and 1597 (Table 3.4 and Figure 3.3). Thirteen patients had point mutations in the HD-N domain, of which eight were leucine to proline substitutions and eleven had in-frame insertions or deletions. Three of the latter had complex alterations with contiguous insertions and

Table 3.4 *NOTCH-1* and *FBXW7* mutational status of the 60 adult T-ALL patients studied

Case#	*NOTCH-1* mutation status	*FBXW7* mutation status	*NOTCH-1* Mutation (nucleotide)	NOTCH-1 Mutation (amino acid)	*FBXW7* Mutation (nucleotide)	FBXW7 Mutation (amino acid)
T1	PEST MUT	WT	7469-7470insAA	Y2491X		
T2	WT	WT	-	-		
T5*	HD-C + PEST MUT	WT	5011G>A (SNP); 5019-5020insGGG 7515T>G (SNP); 7327-7328insGAGGAAAA	V1672I (SNP); 1674-1675insG P2506P (SNP); fs@2444 STOP@2480	1944A>G (SNP)	L648L (SNP)
T9*	HD-N+ PEST MUT	WT	4721T>C+G4722G>T; 4754T>C+4755G>T 7387delG	L1575P and L1586P (different alleles) fs@2464, STOP@2477		
T11	HD-N MUT	MUT	4849-4851delTTC	1618delF	1393C>T	R465C
T15	HD-N MUT	WT	4787T>A	L1597H		
T16	WT	WT	-	-		
T17	WT	WT	5094C>T (SNP)	D1699D (SNP)		
T18	HD-N MUT	MUT	4778T>C	L1594P	1393C>T	R465C
T19	HD-C MUT	MUT	5026-5028delGTC†	1677delV†	1393C>T	R465C
T20	HD-N MUT	WT	4777-4778delCTinsGCTTT†	1594-1595insA†		
T21	HD-C MUT	WT	5094C>T (SNP); 5087C>A	D1699D (SNP); A1697D		
T22	HD-N MUT	MUT	4776-4777insGTCGCC†	F1593L+1593-1594insSP†	1393C>T†	R465C†
T23	WT	WT	-	-		
T24	JME MUT	WT	5205-5206ins20bp + 5206-5221duplication 16bp	1737-1738ins7aa+5 aa duplication 1737-1741		
T25	HD-N+ PEST MUT	WT	4847T>A 7301-7302insTGGGG	I1617N 2435-2436insGX		
T26	WT	WT	5094C>T (SNP)	D1699D (SNP)		
T27	HD-N MUT	WT	4778T>C	L1594P		
T28*	HD-N MUT	MUT	4849-4851delTTC	1618delF	1393C>T	R465C
T29	HD-N MUT	MUT	4721T>C	L1575P	1393C>T	R465C
T30	HD-N+ PEST MUT	WT	4774-4780delTTCCTGC+insCACTTCGATG 7535C>T	1593-1595delFLR+insHFDG P2513L		
T31	HD-N MUT	MUT	4841-4850delAGATGATCTT+insTGTGCCG	1615-1618delQMIF+insLCR	1436G>T	R479L
T32	HD-N+ PEST MUT	WT	4754T>C 7541-7542delCT	L1586P fs@2515, STOP @2518		
T33	JME MUT	WT	5229-5230insTT+5230-5269 duplication 40bp 5094C>T (SNP)	14aa duplication 1744-1757 D1699D (SNP)		

Case#	*NOTCH-1* mutation status	*FBXW7* mutation status	*NOTCH-1* Mutation (nucleotide)	NOTCH-1 Mutation (amino acid)	*FBXW7* Mutation (nucleotide)	FBXW7 Mutation (amino acid)
T36	HD-C MUT	WT	5160-5161insCCCCCGGGCAGTCTGCTG +5161-5163delGTG	1721-1722insPPGSL+1722delV		
T39	WT	WT				
T40	HD-N MUT	WT	4793G>C†	R1599P†		
T43	WT	WT				
T51	WT	WT				
T53	WT	WT				
T54	HD-N MUT	WT	4734-4735insGTG	1579-1580insV		
T55	HD-N MUT	WT	4817-4890del75bp	1607-1631delFKRDAHGQQMIFPYYGREEELRKHP		
T57	HD-N MUT	MUT	4842-4844delGAT	M1616I+1617delF	1393C>T	R465C
T58	HD-C MUT	WT	5160-5161ins66bp	1721-1722insASFLGALASLGSLNFPYRIEA+V1722D		
T59	WT	WT				
T60	PEST MUT	WT	7555C>T	Q2520X		
T61	PEST MUT	WT	7534C>T	P2513S		
T62	WT	WT				
T63	PEST MUT	WT	7429-7435delACCGCAG	fs@2478, STOP@2481		
T64	HD-N+ PEST MUT	WT	4778T>C 7487-7488insGCTACCCGTGTTT	L1594P fs@2497, STOP@2507		
T65	HD-C MUT	WT	5039T>A	I1681N		
T66*	HD-N+ PEST MUT	WT	4754T>C 7561-7562insGGCTAGCCCTCTACACT	L1586P 2495insG, STOP@2496		
T67*	PEST MUT	WT	7438-7439insACAGTACC	fs@2479, STOP@2481		
T70	HD-C MUT	WT	5033T>A	L1679Q		
T71	WT	WT				
T72	HD-N MUT	MUT	4747-4748insGTACCCACCCTAAGG	1583-1584insGTHPK	1268G>T	G423V
T73	WT	WT				
T74	WT	WT				
T75	HD-N MUT	MUT	4855delTA+4855-4856insCCAGGGTC	1619-1620insPGS	1393C>T	R465C
T78	WT	MUT			1436G>T	R479L
T83	WT	WT				

Case#	*NOTCH-1* mutation status	*FBXW7* mutation status	*NOTCH-1* Mutation (nucleotide)	NOTCH-1 Mutation (amino acid)	*FBXW7* Mutation (nucleotide)	FBXW7 Mutation (amino acid)
T86	WT	WT				
T87	WT	WT				
T88	WT	WT				
T89	WT	WT				
T101	WT	WT				
T102	WT	WT				
T103	HD-N+ PEST MUT	WT	4799T>C 7397-7398insGG	L1601P fs@2467 STOP@2478		
T107	HD-N MUT	WT	4793G>C	R1599P		
T109	HD-N MUT	WT	4775-4776insAAG	F1593L+1594insS		

T# local patient case number, * adult patient not entered onto UKALLXII study. MUT mutant, WT wild type, ins insertion, del deletion, SNP single nucleotide polymorphism, HD-N N-terminal heterodimerisation domain, HD-C C-terminal heterodimerisation domain, JME juxtamembrane expansion mutation, fs frameshift, X stop codon, †Low level mutation. Data on patients treated on ECOG2993 are shown in Appendix Table 1.

deletions (indels). Subsequent TOPO cloning of products from case T9 showed that the two leucine to proline mutations were on different alleles and were therefore either in *trans* in the same cell or present in different cells. Several mutations were low level as estimated visually from the WAVE pattern, which indicated either the presence of non-leukaemic cells in the sample studied or secondary mutations reflecting clonal evolution. The incidence of low level mutations, the methods used to identify them and their biological significance is presented in Chapter 4.

Of the seven patients with an HD-C domain mutation, four had an insertions or deletions, whereas all mutations previously described in this domain in the paediatric cohort were point mutations. Two of the three insertions (T36 and T58) contained the amino acid sequence GSL, which forms an integral part of the recognition motif for S2 cleavage (usually occurring at amino acid position 1711). Thirteen mutations were detected in the PEST domain, of which ten were insertions and/or deletions that led to premature stop codons, either through a frameshift or through direct insertion of a stop codon, truncating the receptor by 39 to 119 amino acids. In a further case there was an amino acid substitution (nucleotide 7535C>T resulting in P2513L), which had been initially reported as a SNP (Weng *et al*, 2004), but more recent studies have shown is recurrent in T-ALL (Medyouf *et al*, 2010). It is located at the FBXW7 binding site and is likely to be somatic, although remission DNA was not available to prove this definitively. All the PEST mutations would be predicted to abrogate binding of the negative regulator FBXW7. Notably, both insertions in the JME contained the amino acid sequence QLHF, as has been found in the Jurkat cell line and the majority of reported primary T-ALL cases (Sulis *et al*, 2008).

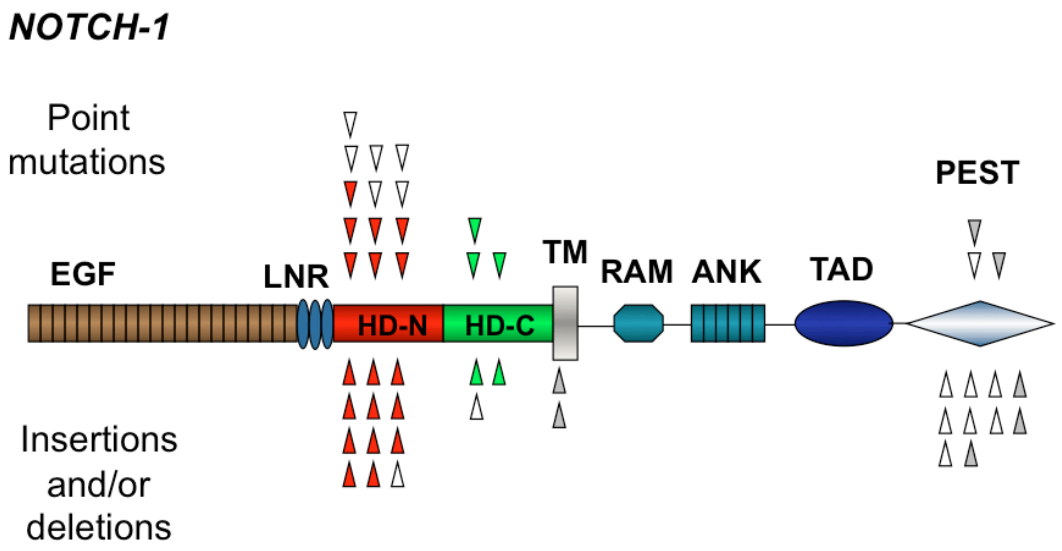

Figure 3.3. The location of *NOTCH-1* mutations identified in 60 adult T-ALL patients. Downward pointing arrowheads signify point mutation whilst upward arrowheads indicate insertions and or/deletions. Patients with more than one mutation are indicated with white arrowheads, whilst those with only one mutations are represented by a filled arrowhead. EGF Epidermal growth factor-like repeat, LNR Lin-Notch repeat, HD-N N-terminal heterodimerisation domain, HD-C C-terminal heterodimerisation domain, TM transmembrane domain, ANK Ankyrin domain,TAD transactivation domain, PEST proline (P) glutamic acid (E) serine (S) threonine (T) rich domain.

Remission DNA samples were available from 15 patients, nine of whom had a mutation at disease presentation. In each case the mutation was no longer detectable in remission, indicating they were somatic in origin.

NOTCH-1 mutations were therefore highly prevalent in an adult cohort of T-ALL patients. Although there was a higher number of insertions and/or deletions in the HD-C in adult T-ALL compared to those reported in paediatric patients (Weng *et al*, 2004), the overall incidence of *NOTCH-1* mutations were not significantly different between these two age groups (Table 3.5).

Table 3.5 A comparison of the frequency and characteristics of *NOTCH-1* mutations in 60 adult T-ALL patients versus those published for paediatric patients.

	Mutant in 96 paediatric patients†	Mutant in 60 adult patients	P value
Mutant in any domain (% of total cohort)	54 (56%)	36* (60%)	0.64
HD only	25 (26%)	23 (38%)	0.11
TAD/PEST only	12 (12%)	5 (8%)	0.42
HD + TAD/PEST	17 (18%)	8 (13%)	0.47
HD-N (% of all HD-N mutations)	(n = 34)	(n = 24)	0.56
Leucine to proline	14 (41%)	8 (35%)	0.82
Other point mutations	7 (21%)	5 (17%)	0.81
Insertions and/or deletions	13 (38%)	11 (48%)	0.42
HD-C (% of all HD-C mutations)	(n = 8)	(n = 7)	0.49
Leucine to proline	4 (50%)	0 (0%)	0.11
Other point mutations	4 (50%)	3 (42%)	0.81
Insertions and/or deletions	0 (0%)	4 (58%)	0.01
TAD/PEST (% of all TAD/PEST mutations)	(n = 29)	(n = 13)	0.24
Point mutations	10 (34%)	3 (23%)	0.23
Insertions and/or deletions	19 (66%)	10 (77%)	0.62

†Data from Weng et al. (Weng *et al*, 2004). *Data excludes JME mutations as they were not analysed by Weng et al. P values calculated using Chi-square test.

3.4.2 *FBXW7* mutations in adult patients with T-ALL

DHPLC analysis was used to screen the WD40 domain of *FBXW7* (exons 8-12) in samples from 60 adult patients. Twelve patients had an abnormal chromatogram pattern in one of these exons. In patient T5 the nucleotide change 1944A>G was found which is synonymous at the amino acid level (L648L), and although not reported on the dbSNP database, was present in the remission DNA sample and thus likely to be a private SNP. Thus overall, eleven patients (18%) had a mutation in *FBXW7* (Figure 3.4 and Table 3.4); eight with R465C, two R479L and one G423V, and, of note, all except one of these

mutations altered conserved arginine residues in the WD40 domain thought to be responsible for binding to the *NOTCH-1* PEST domain. Consistent with this finding, *FBXW7* mutations and *NOTCH-1* PEST domain mutations were mutually exclusive (Table 3.6). There was a positive association between having a mutation in the *NOTCH-1* HD domain only and an *FBXW7* mutation (10 of 22 patients with HD domain only mutations were *FBXW7* mutant vs. 1 of 37 other patients; Fisher's exact test P<.001) (Table 3.6). Such a combination of mutations been shown to be synergistically activating *in vitro* by a mechanism similar to that of dual HD and PEST domain mutations, ie. ligand-independent NOTCH cleavage and prolonged ICN half-life (Malyukova *et al*, 2007). A similar incidence of *FBXW7* mutations has been shown in paediatric T-ALL (Kox *et al*, 2010; Park *et al*, 2009; Zuurbier *et al*, 2010).

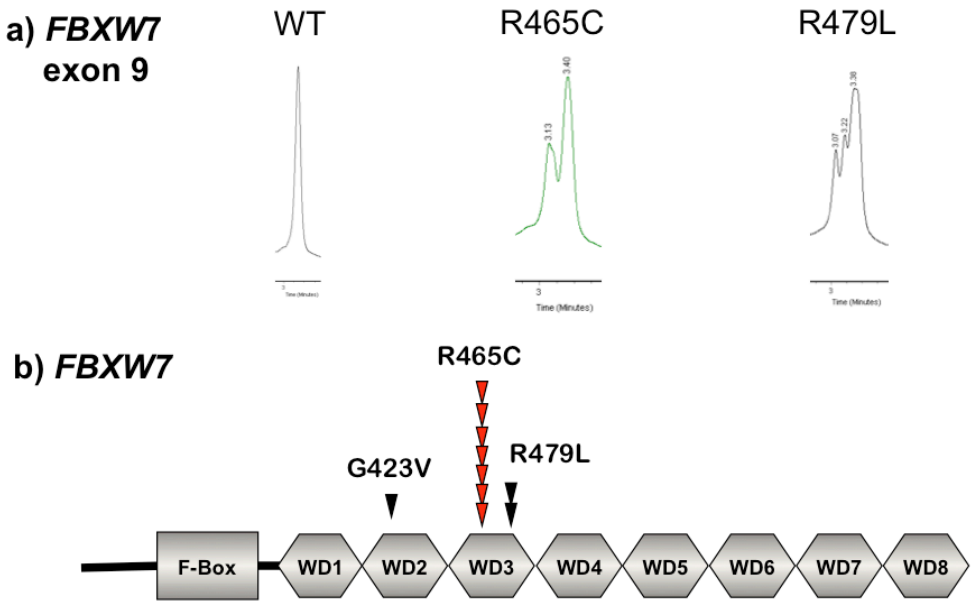

Figure 3.4 *FBXW7* mutations in adult T-ALL patients. (A) DHPLC patterns for *FBXW7*. Characteristic chromatograms for the R465C and R479Lmutations in exon 9 performed at 58.6°C. **(B) The location of *FBXW7* mutations identified in 60 adult T-ALL patients.** Schematic for FBXW7 showing position of mutations in the adult T-ALL patients. WD WD40 domain

Table 3.6 The association of *NOTCH-1* and *FBXW7* mutation status of 60 adult T-ALL patients.

(% of total cohort)	*FBXW7* WT (n=49)	*FBXW7* MUT (n=11)
***NOTCH-1* WT**	21 (35%)	1 (2%)
***NOTCH-1* HD only**	13 (21%)	10 (16%)
***NOTCH-1* JME**	2 (3%)	0
***NOTCH-1* PEST only**	5 (8%)	0
***NOTCH-1* HD+PEST**	8 (15%)	0

3.4.3 Prognostic implications of *NOTCH-1* and *FBXW7* mutations in adult T-ALL

Of the 60 patients studied, 54 were entered into the UKALLXII trial. Therefore, to address the prognostic implications of *NOTCH-1* and *FBXW7* mutations in adult patients with T-ALL, the data on these 54 patients were combined with data from 34 patients treated on the same protocol in the ECOG2993 trial (note ECOG2993 and UKALLXII were joint trials) and studied by Adolfo Ferrando's group. An outline of the trial protocol is shown in Figure 3.5. All the following data therefore refers to the combined group of 88 patients.

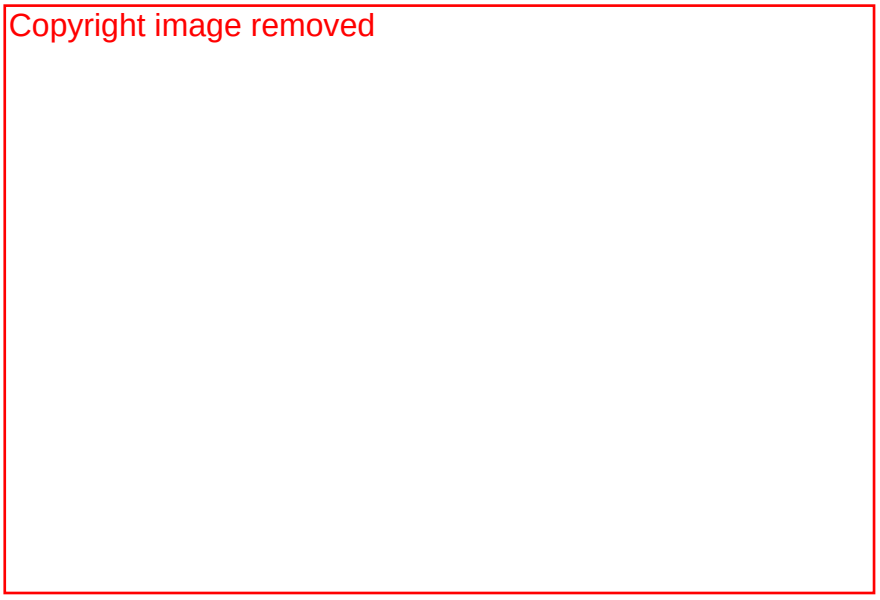

Figure 3.5 A simplified algorithm of the UKALLXII/E2993 treatment protocol. HD-MTX, high-dose methotrexate**.**

3.4.3.1 Clinical features of patient cohort

The cohort analysed was representative of all 356 T-ALL patients entered onto the clinical trial in terms of sex and median age, but had a higher presenting WCC, which probably reflects a bias towards the collection and banking of samples with abundant material (Table 3.7). Median follow-up for the 88 patients in the cohort was shorter than for all 356 patients, but complete remission rates, OS and EFS were similar.

Table 3.7 Comparison of the UKALLXII/ECOG 2993 trial T-ALL patients with and without molecular data

	Patients with *NOTCH-1/FBXW7* Mutation data	**Other UKALLXII T cell patients**	**P value**
Total	88	268	
Male	65 (74%)	195 (73%)	0.8
Median (range) age	30.5 (16-60)	28.5 (15-60)	0.8
Median (range) WCC x 10^9/l	50 (1-653)	31 (0.6-541)	0.003
Median follow-up (yrs)	3.6	7.9	<0.0001
Achieved remission	85 (97%)	251 (94%)	0.3
Survival *	49.4%	41.3%	0.98
Event free survival *	42.7%	44.9%	0.88

* Percents at 5 years; p-value = log rank over all follow-up

In the cohort analysed, there was no significant association with WCC or age according to either *NOTCH-1* or *FBXW7* mutational status (Mantel Haenszel test for trend $P>0.1$ for each case). Complete remission was achieved in 97% of patients; 33 of 35 (94%) *NOTCH-1*/*FBXW7* WT (ie. wild-type for both *NOTCH-1* and *FBXW7*) and 52 of 53 (98%) *NOTCH-1*/*FBXW7* MUT patients (ie. mutant for either *NOTCH-1* and/or *FBXW7*) (P=.56). The *NOTCH-1/FBXW7* WT and MUT groups received similar treatment (WT *vs.* MUT: 7 (20%) *vs.* 16 (30%) sibling allograft; 3 (9%) *vs.* 7 (13%) autograft; 3 (9%) *vs.* 3 (6%) matched unrelated donor allograft; 18 (51%) *vs.* 25 (47%) chemotherapy with maintenance alone; chi square P=.69).

3.4.3.2 *NOTCH-1* and *FBXW7* mutation incidence and features

The mutation rate for *NOTCH-1* was similar in the UKALL and ECOG cohorts (59% *vs.* 62% respectively, P=.77). Of the total 88 T-ALL patients analysed, 53 (60%) had at least

one *NOTCH-1* mutation; 36 patients (41%) had a mutation in the HD domain only, seven (8%) in the PEST domain only, six (7%) in both HD and PEST domains, one (1%) in the TAD and three (3%) had JME mutations (Table 3.4 and Appendix Table 1). For further analysis the TAD mutation was grouped with the PEST domain mutations, given that it truncated the PEST domain and would be functionally analogous to a PEST domain mutation.

The mutation rate for *FBXW7* was the same in the UKALL and ECOG cohorts (18% in both cohorts, *P*=1.0). Fifteen of the 16 patients had a mutation affecting one of the conserved arginine residues in the WD40 domain thought to be responsible for binding to the NOTCH-1 PEST domain (Table 3.4 and Appendix Table 1). Five of the *FBXW7* MUT patients (31%) were wild-type for *NOTCH-1*, whilst the other eleven *FBXW7* MUT patients (69%) had HD-N domain mutations. Details of the nucleotide and amino acid changes for *NOTCH-1* and *FBXW*7 for the 34 additional ECOG2993 patients are shown in Appendix Table 1.

3.4.3.3 Patient outcome according to mutation status

Importantly, patients with standard-risk disease (≤35years old with WCC <100x10^9/l) and high-risk disease (>35years and/or WCC ≥100 x10^9/l) were evenly distributed between the *NOTCH-1*/*FBXW7* MUT and WT groups. For WCC, 61% patients with WCC<100x10^9/l were *NOTCH-1*/*FBXW7* MUT *vs.* 62% patients with WCC ≥100 x10^9/l, Fisher's Exact test *P*=1.0. By age, 55% patients ≤35years were *NOTCH-1* MUT *vs.* 69% >35 years, *P*=.37). The overall outcome of all patients on the trial has recently been reported (Goldstone *et al*, 2008). Comparison of outcome in patients with a mutation in the Notch pathway (*NOTCH-1* and/or *FBXW7*) with those without a mutation showed a trend towards improved outcome in those with a mutation. At 5 years, EFS and 95% confidence interval (CI) were 51%±14% *vs.* 27%±19% (*P*=.10) in MUT and WT patients respectively (hazard ratio 0.6, 95% CI = 0.3-1.1) (Figure 3.6A). The OS at 5 years was 54%±14% and 41%±20% respectively (*P*=.30) (Figure 3.6B). Analysis of patients with or without a *NOTCH-1* mutation alone revealed a 5 year EFS of 49%±15% *vs.* 34%±18% (*P*=.2) (Figure 3.6C) and OS of 53%±15% *vs.* 45%±17 (*P*=.41) (Figure 3.6D) respectively. Comparison of patients with or without an *FBXW7* mutation alone showed a 5 year EFS of 53%±26 *vs.* 41%±13 (*P*=.72) (Figure 3.6E) and OS of 62%±24 *vs.* 47%±12% (*P*=.51) (Figure 3.6F). On the trial as a whole, treatment, age and WCC

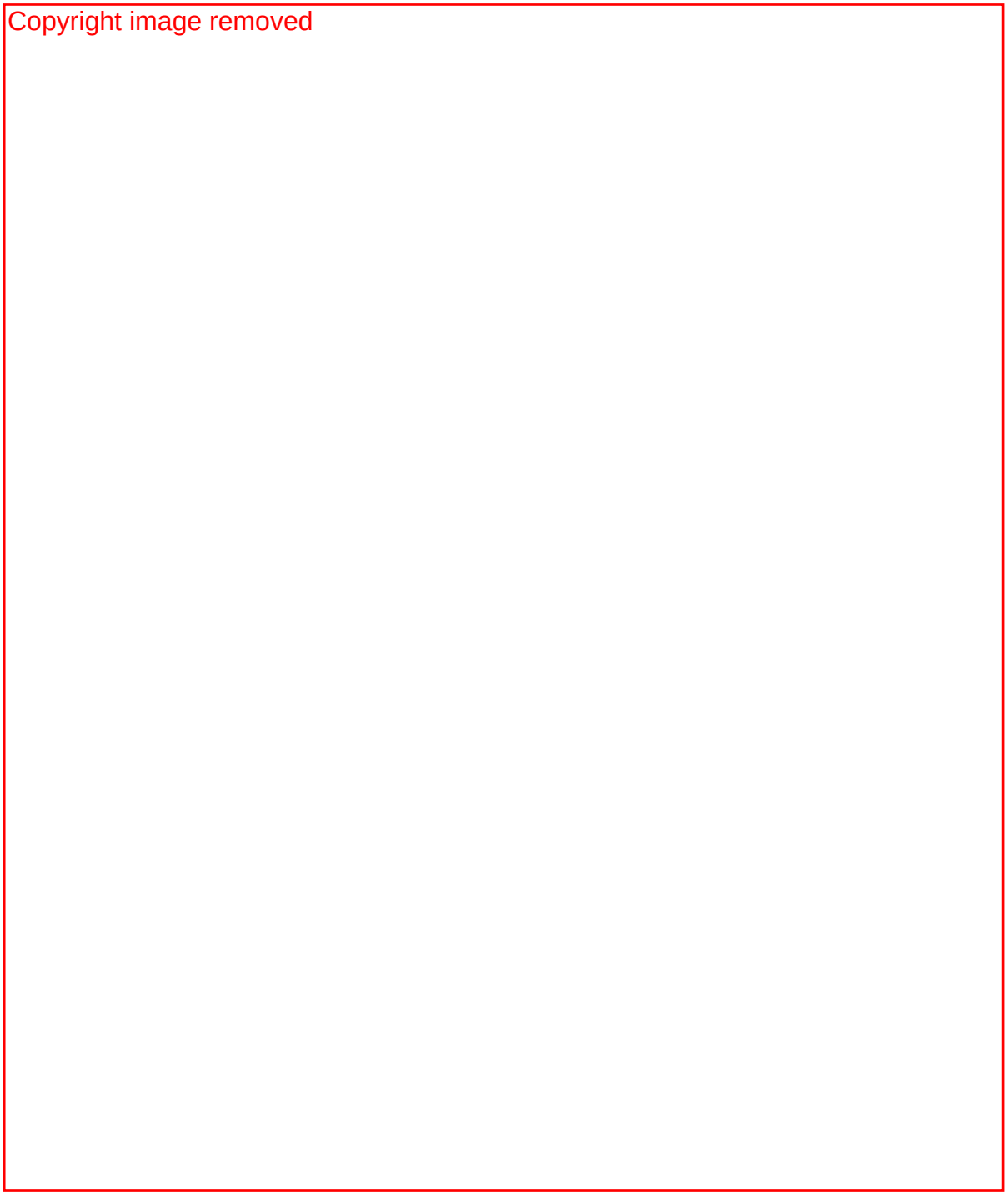

Figure 3.6. Outcome of adult patients with T-ALL treated on UKALLXII/E2993 protocol stratified by *NOTCH-1* and *FBXW7* mutational status. **(A)** Event-free survival (EFS) and **(B)** overall survival (OS) by *NOTCH1* and/or *FBXW7* mutation. **(C)** EFS and **(D)** OS by *NOTCH-1* mutation status alone. **(E)** EFS and **(F)** OS by FBXW7 mutation status alone. MUT mutated; WT wild type.

significantly influenced outcome (Goldstone *et al*, 2008; Rowe *et al*, 2005). Of these factors, only treatment received was significant in the Cox model in this smaller cohort of T-ALLs (*P*=.03), and inclusion of these variables did not materially affect EFS (Table 3.8). There was insufficient data to test the effect of transplant by means of a donor-versus-no donor comparison. Standard risk patients with a *NOTCH-1* and/or *FBXW7* mutation did not fair significantly differently from standard risk WT patients (EFS for patients with WCC<100 was 47.7% in patients with a *NOTCH-1* and/or *FBXW7* mutation *vs.* 30.3% for WT patients, *P*=.5; EFS for patients <35years was 45.5% in patients with a *NOTCH-1* and/or *FBXW7* mutation *vs.* 39.3% for WT patients, *P*=.6) and there was no interaction between effects of mutation and risk group.

Table 3.8 Multivariate analyses of event free survival for patients treated on UKALLXII/ECOG2993 according to age, WCC, treatment arm and *NOTCH-1/FBXW7* mutation status.

Model	Hazard ratio (95% confidence interval) for variable shown in italics
NOTCH-1/FBXW7	0.62 (0.34-1.11)
NOTCH-1/FBXW7, age, log(WCC+1)	0.66 (0.34-1.29)
NOTCH-1/FBXW7, age, log(WCC+1), treatment*	0.73 (0.37-1.44)
NOTCH-1	0.70 (0.39-1.24)
NOTCH-1, age, log(WCC+1)	0.77 (0.42-1.42)
NOTCH-1, age, log(WCC+1), treatment*	0.85 (0.45-1.61)
FBXW7	0.85 (0.38-1.91)
FBXW7, age, log(WCC+1)	0.91 (0.40-2.08)
FBXW7, age, log(WCC+1), treatment*	0.87 (0.36-2.13)

*First remission transplant or chemotherapy (n=84)

Considering the marked *in vitro* synergistic activation of the Notch pathway by dual *NOTCH-1* HD and PEST domain mutations or dual *NOTCH-1* HD domain and *FBXW7* mutations (Malyukova *et al*, 2007), the outcome of this 'synergistic' subgroup (n=17) was analysed versus the WT patients. The EFS was 62% versus 30% respectively, but this did not reach statistical significance (*P*=.17).

Overall, this data showed a trend towards improved EFS in patients with a Notch pathway mutation, but did not identify a subgroup of patients with a significantly favourable outcome to warrant treatment reduction on future trials.

3.4.4 Mutational screening of the LNR domain of *NOTCH-1*

The *NOTCH-1* NRR (negative regulatory region) consists of three LNR modules, termed LNR-A, -B and -C, that form a protective 'knuckle' over the hydrophobic core of the HD domain, preventing access of TACE to the S2 cleavage site in the absence of ligand. Given that the LNR-HD interface is an important negative regulator of NOTCH-1 activation, mutational disruption of the LNR could potentially activate Notch in those patients without HD mutations. This region was therefore screened in the 54 adult patients from the UKALLXII cohort. Twelve paediatric T-ALL patients were also analysed in case mutations of this domain happened to be a feature related to patient age. The age range of the latter patients was 5 months to 12 years (median age 4 years) and 7 (58%) were male.

Overall, seven of the patients had an abnormal WAVE pattern in the LNR domain. In five patients this was due to a SNP (rs61751542; 4132C>T; P1378S). Remission DNA from one patient showed an identical DHPLC pattern suggesting that the alteration was of germline origin. The reported frequency in the population is 0.02 on dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). The other two patients had somatic missense mutations that are detailed below.

- **Somatic H1545P LNR mutation**

  An H1545P mutation was identified in a sample from a 5 month old girl who was also found to have a PEST domain mutation (S2514F). Both mutations were absent in the remission sample. Although diagnostic RNA was available, it was not of adequate quality to amplify the relevant region of *NOTCH-1* mRNA in order to assess whether these particular mutations were in *cis, trans* or different clones. Given its likely functional significance, a collaboration was set up with Jon Aster and Stephen Blacklow's group at Brigham and Women's Hospital in Boston, USA, who were in the process of crystallising the NRR domain of NOTCH-1 and had developed assays to assess the activating potential of *NOTCH-1* mutations. The following data was produced in their laboratory, predominantly by Wendy Gordon, and is included here for interest and completeness (Gordon *et al*, 2008). Briefly, they identified by crystallography that H1545P was located in a loop of the LNR-C module, within the interface with the HD domain and the calcium-binding pocket. In luciferase reporter assays the mutation resulted in a 20-fold increase in ligand-independent reporter gene transcription, which was intermediate between the weak activating leucine-to-

proline mutations and the more potent type II mutations. Previous studies had shown that the LNR-C is directly involved in calcium binding, which is required to maintain this domain in its inhibitory conformation (Aster *et al*, 1999), and H1545 maps directly to the calcium-binding interface. It is therefore likely that the proline of H1545P activates signalling through disruption of calcium binding.

- **Somatic C1522G LNR mutation.**
  This mutation was identified in a sample from an adult patient who also had a PEST mutation (P2513S) but no HD mutation. Remission DNA was available from this patient and showed a wild-type DHPLC pattern, suggesting that the mutation was somatic in origin. The cysteine residue at position 1522 is thought to be one of three cysteine residues that provide disulphide bridging of the NOTCH-1 heterodimer after S1 cleavage. In order to further ascertain its function, Jon Aster's group expressed the C1522G mutation with a CSL luciferase reporter in U2OS cells. The mutation was not activating. Although speculative, one possible reason for the lack of activation is stimulation of the unfolded response by misfolding of the Notch heterodimer in U2OS cells that is tolerated in T-ALL cells. Another possibility is that the mutation increases sensitivity to stimulation by ligands that are expressed in T-ALL cells but not in U2OS cells.

3.4.5 Analysis of *NOTCH-3* in T-ALL and infant AML patients

The LNR, HD-N, HD-C, TAD and PEST domains of *NOTCH-3* were analysed by DHPLC using samples from 38 adult T-ALL patients and 30 infant leukaemia samples, followed by sequencing of those cases with abnormal chromatograms. No somatic mutations were identified. The following SNPs were identified:

- **rs1044006; 4639A>G; P1521P synonymous SNP.** This was identified in 6 of 38 patients (16%). The reported frequency in the population is 0.16 on dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).
- **rs56277836; intron 26, 4891+23 G>C heterozygous alteration 23 bp downstream of the exon 26 intron/exon boundary.** This was identified in 3 of 38 patients (8%). Remission DNA from one patient showed the same sequence alteration. The reported frequency in the population is 0.04 on dbSNP.

- **rs1044008; 6514G>A; A2416A synonymous SNP.** This was identified in 5 of 38 patients (13%). Remission DNA from one patient showed an identical sequence alteration. The reported frequency in the population is 0.03 on dbSNP.
- **rs1044009; 6744C>T; A2223V.** This was identified in 12 of 38 patients (31%). Remission DNA from three patients showed an identical sequence alteration. The reported frequency in the population is 0.4 on dbSNP.

3.4.6 *NOTCH-1* mutations are not a common feature of infant leukaemia

Of the 29 samples obtained from patients with infant leukaemia (occurring prior to the age of 1 year), immunophenotype data showed 13 cases had AML and 16 had B-ALL, of which six had pre-B-ALL and 10 pro-B ALL. Of the 26 patients where cytogenetic analysis was successful, 14 had a rearrangement involving the mixed lineage leukaemia gene (*MLL*) on chromosome 11q23. FISH data for MLL was not available. Sixteen of the 29 patients (55%) had expression of at least one T-cell marker in 10% or more of the cells, with 12 patients expressing CD7, eight with cytoplasmic CD3 expression, none had surface CD3 expression, and six had expression of CD4. DHPLC analysis was carried out for the *NOTCH-1* HD-N, HD-C, TAD and PEST domains and 12 patients (41%) were heterozygous for the 5094C>T SNP (rs10521) previously described in section 3.4.1. No other abnormalities were detected.

3.4.7 *NOTCH-1* mutations are not a common feature of adult AML

Samples from a cohort of 157 AML patients were screened for mutations in *NOTCH-1* as before. Of the cohort, 52 had good risk cytogenetics, of which three had t(8;21) and 48 had t(15;17) (acute promyelocytic leukaemia [APML]). Of the remainder, 77 had standard risk cytogenetics, three had poor risk and in 25 patients the cytogenetic data was not available. The cohort was intentionally enriched for patients with APML given reports of their particularly high frequency of aberrant T-cell marker expression. In one study of 196 APML patients, CD2 expression was detectable in 28% of cases (Guglielmi *et al*, 1998), whilst another study of 36 APML patients showed CD2 expression in 38% of cases and also the presence of TCR rearrangements in 15% (Chapiro *et al*, 2006). In this cohort, none of the T-ALL associated mutations were detected, although a number of SNPs were identified as outlined below.

Previously reported SNPs:

- **rs10521; 5094C>T; D1699D synonymous SNP.** The characteristic DHPLC pattern for the heterozygous 5094C>T SNP was identified in 46% patients in this cohort. The reported frequency in the population is 0.43 on dbSNP.
- **rs76371972; 4823G>A; R1609H SNP.** This was identified in two APML patients. Remission DNA from both patients showed persistence of the nucleotide substitution by sequencing, suggesting they were germline in origin. The reported frequency in the population is 0.006 on dbSNP.
- **rs61751489; 6853G>A; V2285I SNP.** This was identified in two AML patients. No remission/germline DNA was available for either patient, but this alteration is reported as a SNP on dbSNP with a frequency in the population is 0.02.

Nucleotide changes not previously reported and of uncertain significance:

- **4656G>A; A1553T.** This was identified in one AML-M2 patient. A1553 is just upstream of the HD-N domain. Remission or germline DNA samples were not available to assess whether or not this change was somatic, and it was not assessed in a functional assay.
- **4626C>T; D1543D synonymous.** No remission/germline DNA was available for this patient, but given the absence of alteration in the amino acid sequence, is unlikely to be of functional significance.
- **Intron 26 delTGAT 12bp upstream of the exon 27 intron/exon boundary.** This was identified in one APML patient. Unfortunately no remission/germline DNA was available to investigate whether this alteration was somatic. RNA was not available to investigate splicing, although it was thought unlikely to affect splicing given its distance from the acceptor site.
- **7510G>A; V2505M.** This was identified in one AML-M0 patient. No remission/germline DNA was available to investigate whether this alteration was somatic.

3.5 DISCUSSION

Overall, the frequency of *NOTCH-1* mutations detected in the UKALLXII cohort of 60 adult T-ALL patients studied was comparable with results reported in paediatric patients (61% versus 56% respectively), and remained the same when the cohort was increased to

88 patients by including the ECOG cohort (60%). The presence of mutations was not simply a feature of the young adult patients as 55% of patients less than 35 years of age were *NOTCH-1* mutant compared to 69% of patients over 35 years. This suggests that a similar pathogenic mechanism may underlie the development of most cases of T-ALL irrespective of age, and that the difference in curability in the two groups is not related to a differing frequency of *NOTCH-1* mutations.

Despite their marked heterogeneity, no particular type of mutation appeared to be associated with age. The majority of mutations identified were likely to be functionally significant since they either truncated the PEST domain or affected highly conserved residues in the HD domain. The importance of the latter is supported by crystallography data that has shown that it is the conserved amino acids in the HD domain that form the interface with the LNR domain, such that amino acid substitutions of these residues are likely to disrupt the HD-LNR interaction (Gordon *et al*, 2009). All the insertions and/or deletions in the HD domain were in-frame, consistent with the requirement of a functional ICN for leukaemic transformation. In the HD-N domain, there was a particular propensity for amino acid substitutions to prolines, which can be structurally detrimental because of their acute angle in the amino acid backbone. Eight of the 24 HD domain mutations detected in the UKALLXII cohort (L1594P, L1597H, R1599P, I1617N, I1681N, the S2 cleavage site duplication, L1601P and F1593L+1594insS) have been functionally tested in reporter assays in separate studies and shown to be activating (Chiang *et al*, 2008; Malecki *et al*, 2006; Palomero *et al*, 2006b).

Two patients had duplications of the S2 cleavage site in the HD-C domain. A similar duplication of the S2 cleavage site (also containing the GSL sequence) has been identified in the P12-Ichikawa cell line and was the most potently activating of 14 HD domain mutations tested (Malecki *et al*, 2006). Furthermore, this mutation is capable of initiating leukaemia in its own right in murine models, in comparison to the L1594P and L1601P mutations which are not transforming *in vivo,* but are able to accelerate KRas G12D-induced leukaemia (Chiang *et al*, 2008). It is likely that a dosage effect exists relating to the site and type of each individual mutation. *In vitro* reporter gene assays have demonstrated that HD domain mutations are more activating than PEST truncations

alone, whilst dual HD and PEST mutations *in cis* have a synergistic effect on Notch activation (Weng *et al*, 2004).

All the frame-shift mutations identified in the PEST domain truncated the protein prior to, or disrupted, the FBXW7 binding site at position T2512. However, in this study three missense mutations that would be predicted to affect the FBXW7 binding site were also detected: S2514F, P2513L and P2513S. Point mutations of this type had not previously been described. It would be of interest to functionally test these mutations to investigate whether they disrupt binding to FBXW7 in co-immunoprecipitation studies, and if they can activate signalling. The other mutation identified of particular interest was an H1545P substitution in the LNR-C domain. This does not appear to be a common mutational site and this was the first known description of an activating mutation affecting this domain in T-ALL. We were fortunate to collaborate with the Aster and Blacklow laboratories, who were able to model this mutation on their crystallography data and showed that it most likely inhibits calcium binding in the LNR-C which would result in loss of structural integrity and subsequent activation of Notch signalling. Such a mechanism is reminiscent of the ability of EDTA to activate Notch signalling through its ability to chelate calcium *in vitro* (Aster *et al*, 1999).

There was a significant positive association between having a *NOTCH-1* mutation in the HD domain only and an *FBXW7* mutation, and a negative association between having a *NOTCH-1* PEST domain mutation and an *FBXW7* mutation. This observation is consistent with the hypothesis that *NOTCH-1* HD domain and *FBXW7* mutations act in concert, similar to dual *NOTCH-1* HD and PEST domain mutations (Malyukova *et al*, 2007; Thompson *et al*, 2007). Although FBXW7 also targets c-Myc for degradation (O'Neil *et al*, 2007; Thompson *et al*, 2007), the association described here favours the concept that *FBXW7* mutations are acquired by T-ALL cells primarily as a means of increasing *NOTCH-1* signal strength. If *FBXW7* mutations were acquired by tumour cells predominantly to upregulate c-Myc, they would likely be found in conjunction with PEST mutations in some cases.

No *NOTCH-1* mutations were identified in the cohort of AML patients analysed, including those with APML or infant AML, where there is a high frequency of aberrant

expression of T-cell markers. However, it is still possible that Notch is activated by alternative mechanisms in AML. For instance, over-expression of *NOTCH-1* itself has been identified by microarray in a subset of AML patients with poor prognosis (Bullinger *et al*, 2004). Additionally, transduction of bone marrow cells with AML1-ETO, PML-RARA or PLZF-RARA fusion constructs was shown to increase expression of the Notch ligand JAGGED-1, a gene that is also up-regulated in some patients with AML (Alcalay *et al*, 2003; Ross *et al*, 2004). Other groups have similarly reported that *NOTCH-1* is not commonly mutated in AML (Fu *et al*, 2006), although one group identified an activating HD-N domain mutation in a single patient with AML-M0 from a cohort of 121 patients screened (Palomero *et al*, 2006b). There is, however, a rare select AML subset where *NOTCH-1* mutations have been described at higher frequency. A small group of six patients clustering together by gene set enrichment analysis (described as 'Cluster 4') in a large AML cohort of 285 patients have been identified as having frequent CEBPA promoter methylation and three had *NOTCH-1* mutations (Wouters *et al*, 2007), although it is unclear whether these patients should be truly classified as AML or would fulfil criteria described by the European Group for the Immunological Characterization of Leukemias (EGIL) for mixed lineage T-ALL/AML (Bene *et al*, 1995). A *NOTCH-1* mutation has also been described in a so-called 'lineage switch leukaemia', as identified in two sister cell lines (ML1 and ML2), where one cell line is derived from a patient with AML at presentation, and the other cell line from the same patient at relapse with T-ALL, with both cell lines having the same *NOTCH-1* mutation (Palomero *et al*, 2006b).

Despite the strong oncogenic potential of ICN-3 in inducing T-ALL in murine models, no mutations were found in *NOTCH-3*. Studies have shown that *NOTCH-3* is markedly over-expressed in the majority of T-ALL patients, in some cases this is secondary to direct transcriptional up-regulation by NOTCH-1 (Bellavia *et al*, 2002; Palomero *et al*, 2006a). As such, mutational activation of *NOTCH-3* may not add a selection advantage to cells in addition to a *NOTCH-1* mutation, and it is possible that a larger cohort of *NOTCH-1* mutation-negative patients needs to be screened to definitively exclude the possibility of mutational activation of *NOTCH-3* in T-ALL. However, it should be noted that it has not been reported whether T-ALL samples actually express ICN-3 protein, which would be indicative of NOTCH-3 activation rather than just over-expression of total NOTCH-3.

In terms of prognostic implications, the data presented here did not show a significantly improved outcome in those patients with mutations in the Notch pathway, although the trend towards a good prognosis is in accord with data previously presented in paediatric patients treated on the ALL-BFM protocol (Breit *et al*, 2006) and more recently in adults on the French LALA-94 and GRAALL-2003 trials (Asnafi *et al*, 2009), and in contrast to the association with poor prognosis reported for *NOTCH-1* mutations in adults by Zhu *et al*. When the three most robust prognostic factors on the overall UKALLXII/ECOG 2993 trial were taken into account (age, WCC and treatment arm), the differences in EFS and OS were unaffected. Additionally, the standard and high-risk patients had a similar incidence of *NOTCH-1/FBXW7* mutations, suggesting that the data was not confounded by a disparate representation of the mutation-positive patients in either risk group.

Whilst no significant difference in outcome in *NOTCH-1* or *FBXW7* MUT patients was observed when analysed individually, the combined *NOTCH-1/FBXW7* MUT group showed a trend towards improved outcome (*P*=.1) highlighting the importance of the addition of *FBXW7* status to that of *NOTCH-1.* More recent studies support the additional value of *FBXW7* mutation data to *NOTCH-1* mutation data in stratifying risk in both paediatric and adult patients (Asnafi *et al*, 2009; Kox *et al*, 2010), suggesting that it is the degree of Notch pathway activation itself that is important in determining treatment response. The marked variability in activation strength of individual mutations in *in vitro* assays suggests that validation of a robust marker of downstream Notch activation could be useful in prognosticating patients in future studies.

Other molecular markers have been reported to have some impact on prognostic outcome in adult T-ALL. For example, *TLX1* mRNA upregulation has been shown to be associated with an improved prognosis (Ferrando *et al*, 2004). It was not possible to evaluate this in the cohort studied here due to lack of RNA samples from all patients. However, patients with *TLX1* up-regulation constitute a minority of patients and are strongly associated with *NOTCH-1* mutations (20 of 21 patients with *TLX1* up-regulation were *NOTCH-1* MUT in the French study) (Asnafi *et al*, 2009). *NOTCH-1/FBXW7* mutations have also been shown to be prognostically important independently of *TLX1* (Asnafi *et al*, 2009).

Since the completion of these studies, a number of other groups have published data addressing outcome by *NOTCH-1/FBXW7* mutation status in T-ALL patients on different treatment cohorts and the results of these studies are summarised in Table 3.9. Consistent with the data from the UKALLXII/ECOG2993 cohort, most trials showed no association of *NOTCH-1/FBXW7* mutation status with WCC or age, though several groups showed a higher incidence of mutations in the cortical subgroup of patients (CD1a positive) and those with high expression of TLX3 (Baldus *et al*, 2009; Breit *et al*, 2006; Kox *et al*, 2010; Zuurbier *et al*, 2010). Data from the largest and best-characterised paediatric cohort comes from 301 patients treated on the ALL-BFM trial (Kox *et al*, 2010). This showed that paediatric patients with *NOTCH-1* and/or *FBXW7* mutations had an excellent early treatment response and favourable MRD at day 33. In terms of EFS, when patients were stratified by *NOTCH-1* mutation status alone or by *NOTCH-1* and/or *FBXW7* mutation status, the mutant-positive patients had a superior outcome, but this was not seen when stratifying by *FBXW7* mutation status alone. There was no association of EFS with *NOTCH-1* and/or *FBXW7* mutation status in their high-risk patient group (as defined by induction failure or poor early treatment response). This contrasts with the dismal prognosis seen in high-risk *NOTCH-1* mutant patients treated on the EORTC trial, which relates predominantly to a high-risk of CNS relapse (Clappier *et al*, 2010). The major difference in treatment approach between the BFM and EORTC trials is the use of prophylactic cranial radiation in the former, suggesting cranial radiotherapy may have a particular role in preventing CNS relapse in *NOTCH-1* mutant high risk T-ALL (Kox *et al*, 2010). In adult T-ALL, the largest study was reported by the French with 87 patients treated on the LALA-94 and 54 patients treated on the GRAAL-2003 protocols (Asnafi *et al*, 2009). The authors identified *NOTCH-1* and/or *FBXW7* mutations as an independent prognostic factor for improved outcome (P=.01), with an EFS for the WT group of 17 months compared to 36 months for those with either a *NOTCH-1* and/or an *FBXW7* mutation (P=.01). However, much larger studies of adult patients will be required to demonstrate this unequivocally, and differences in treatment protocols are likely to be an important variable. On the UKALLXII/ECOG2993 trial, there was an 80% power to detect a 30% increase in EFS. The data on this adult cohort does therefore indicate that, even if there is a better outcome in those with a Notch pathway mutation, the magnitude of the improvement in EFS is likely to be too low to consider de-intensification of therapy in this group of patients, as has been suggested by other groups (Asnafi *et al*, 2009).

Table 3.9 Summary of trials in paediatric and adult T-ALL that have investigated prognostic implications of *NOTCH-1* and *FBXW7* mutations

Reference	Age group	Protocol/trial	Number tested	% with *NOTCH-1* mutations	% with *FBXW7* mutations	Association of mutation status with outcome
Breit et al 2006	Paediatric	ALL-BFM 2000	157	52%	NT	*NOTCH-1* mutations associated with good early treatment response, improved EFS and low RR
(Zhu *et al*, 2006)	Paediatric and adult	VDCP induction/high dose MTX	53 paediatric 24 adult	38%	NT	Paediatric patients: No association with outcome Adult patients: *NOTCH-1* mutations associated with increased relapse rate and poor EFS
(van Grotel *et al*, 2008)	Paediatric	DCOG-ALL7/8/9	70	57%	NT	No association with outcome
(Larson Gedman *et al*, 2009)	Paediatric	POG 8704/9086/9295/ 9296/9297/9398	47	34%	11%	No association with outcome
(Park *et al*, 2009)	Paediatric	JACLS-ALL-97	55	40%	15%	*NOTCH-1* and/or *FBXW7* mutation positive patients associated with improved EFS
(Kox *et al*, 2010)	Paediatric	ALL-BFM 2000	350	50%	14%	*NOTCH-1* and/or *FBXW7* mutation associated with good early treatment response; improved EFS and OS with *NOTCH1* MUT alone but not *FBXW7* MUT alone
(Zuurbier *et al*, 2010)	Paediatric	DCOG-ALL7/8/9; COALL-97	141	56%	16%	Good early treatment response but no association with EFS. Trend towards worse outcome in *NOTCH1* MUT patients on DCOG cohort
(Clappier *et al*, 2010)	Paediatric	EORTC 58881/58951	134	60%	NT	Good early treatment response but no association with EFS or OS
(Mansur *et al*, 2012)	Paediatric	GBTLI-99	138	44%	19%	No association with outcome
(Jenkinson *et al*, 2012)	Paediatric	UKALL2003	162	62%	18%	Patients with double *NOTCH1* and/or *FBXW7* mutations had excellent OS (100%).
(Baldus *et al*, 2009)	Adult	GMALL 05/93; GMALL 06/99	126	57%	12%	No association with outcome
(Asnafi *et al*, 2009)	Adult	LALA-94; GRAALL-2003	141	62%	24%	*NOTCH-1* and/or *FBXW7* mutations independent prognostic markers for improved EFS and OS
(Mansour *et al*, 2009)	Adult	UKALLXII/ECOG2993	88	60%	16%	No association with outcome, though trend towards improved outcome in *NOTCH-1/FBXW7* MUT group

One question of particular interest, given the very high incidence of *NOTCH-1* and *FBXW7* mutations in T-ALL, is the mechanism by which these alterations occur. One possibility is that the high expression of Recombinase-activating gene 1 and 2 (*RAG-1* and *RAG-2*) leads to promiscuous double-strand DNA breaks with faulty mismatch repair, a mechanism that has been implicated in causing the abundance of translocations and genomic deletions found in lymphoid malignancies in general, a recent example being the PAR1 deletions leading to CRLF2 upregulation in B-ALL (Mullighan *et al*, 2009). During V(D)J recombination, RAG proteins recognise a recombination sequence consisting of a conserved heptamer, a 12 or 23bp non-conserved spacer, followed by a conserved nanomer – the so-called '12/23 rule' (Hesse *et al*, 1989). A search was performed to look for these conserved sequences in the vicinity of mutational hotspots of the HD and PEST domains in the UKALLXII cohort, but no such motifs were identified in these areas. Furthermore, acquisition of *Notch-1* mutations has been observed in the majority of *rag-2* deficient mice that develop T-ALL, indicating that, at least in this model, rag recombination is not involved in mediating these mutations (O'Neil *et al*, 2006). On the other hand, 11 of the 16 missense mutations identified in *FBXW7* were C to T transitions within CG dinucleotides and consistent with the mechanism of mutation acquisition due to deamination of methylated cytosines that was proposed originally by Arthur Bird (Bird, 1996). No studies on methylation within the *FBXW7* gene in T-ALL have been reported and this would be an interesting area for future study. A trend towards C to T transitions was not seen in the *NOTCH1* mutations identified in this cohort.

γ-secretase inhibitors (GSI) have been shown to induce cell cycle arrest *in vitro* in T-ALL cell lines harbouring *NOTCH-1* mutations (Weng *et al*, 2004), and the experience gained from clinical trials using GSIs for Alzheimer's disease, where they inhibit the production of beta amyloid peptide from amyloid precursor protein, has expedited trials of these agents in T-ALL. As discussed in further detail in chapter 6, they offer a rational molecularly targeted approach for these patients who, particularly in the older age group, have a poor outcome with current therapy.

CHAPTER 4. *NOTCH-1* MUTATIONS CAN BE SECONDARY EVENTS IN SOME PATIENTS WITH T-ALL

4.1. INTRODUCTION

4.1.1 Significance of low level mutations detected by DHPLC

Screening for *NOTCH-1* mutations, as outlined in Chapter 3, showed that a significant number of patients had abnormal DHPLC chromatograms where the heteroduplex peak was of a low level as compared to the wild-type peak. An example is shown in Figure 4.1. Such samples had no detectable mutation by direct sequencing of the PCR product, although the presence of a mutation could be detected by collection of the low level peak with the fraction collector facility of the WAVE®, followed by re-amplification by PCR and sequencing (as described below in section 4.3.2).

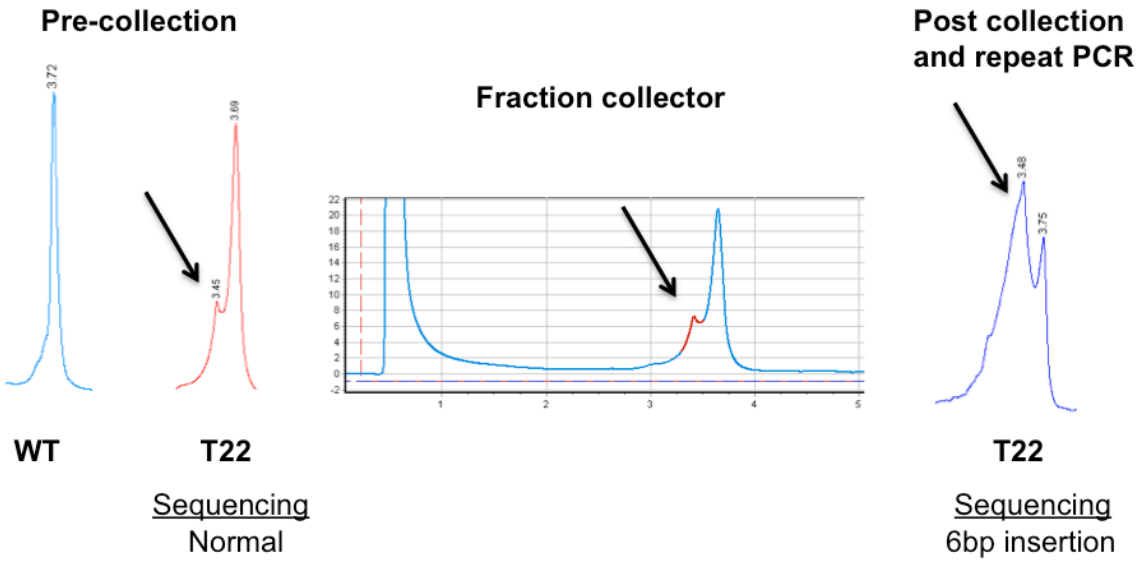

Figure 4.1 Enrichment of a low level mutation using the fraction collector facility of the WAVE®. The chromatogram for patient T22 showed a low level heteroduplex peak for the HD-N domain. The fraction collector of the WAVE® (DHPLC) was programmed to collect the heteroduplex peak (highlighted in red) which was then re-amplified by PCR. DHPLC analysis of the PCR product confirmed enrichment of the heteroduplex which was then detectable by sequencing..

Although contamination of tumour DNA with that of normal cells is a possible explanation for the presence of the low level mutations detected by DHPLC, if DNA is prepared from a pure population of tumour cells the most likely interpretation for this finding is that of subclonal evolution. In this scenario, the mutation is not present in the tumour-initiating cell but is acquired during disease pathogenesis/progression in a subclonal population of cells (Figure 4.2). An alternative explanation would be presence of the mutation in the initiating cell, but deletion of the mutant allele in the major population of cells during disease progression, although this would seem less likely if the mutation offered a significant survival advantage to cells.

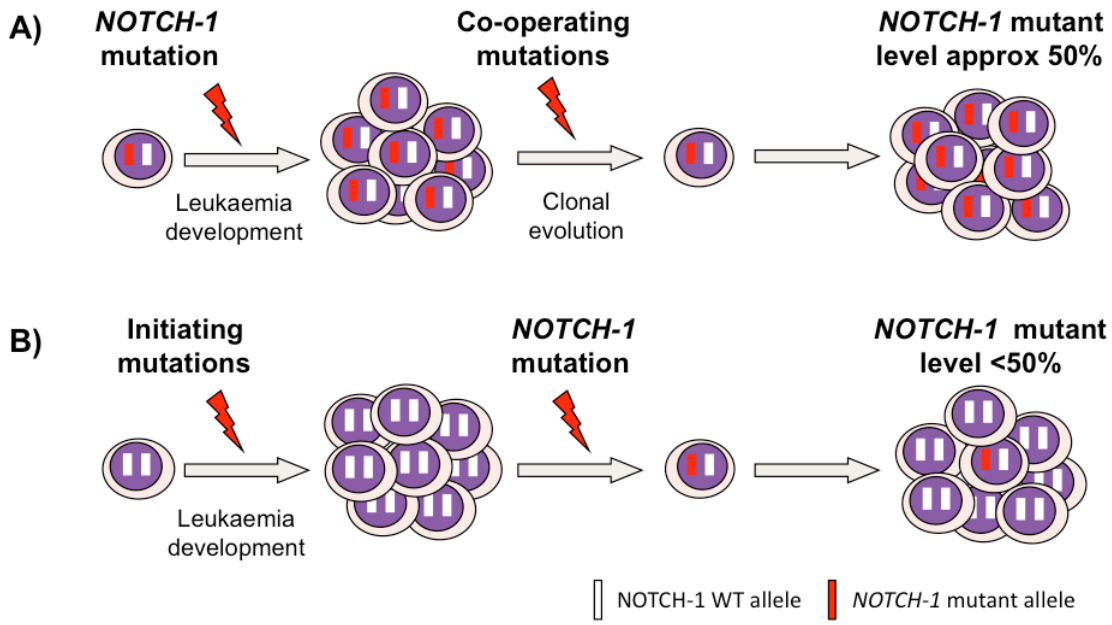

Figure 4.2 Timing of the acquisition of a *NOTCH-1* mutation can be reflected in the mutant level. (A) Occurrence of a *NOTCH-1* mutation in the leukaemia-initiating cell means that, even if there is clonal evolution with the acquisition of other mutations, the *NOTCH-1* mutant level will approximate 50%, assuming heterozygosity. **(B)** If the *NOTCH-1* mutation occurs as a late secondary event then the mutant level in the bulk population of cells is likely to be significantly less than 50%.

4.1.2 Multi-hit models and clonal heterogeneity in cancer

In 1953 Nordling hypothesised that an average of at least six mutations of a single cell are required to induce cancer, a figure he deduced by analysing statistics showing the association of cancer with increasing age (Nordling 1953). In 1971, Knudson published

his ‘two hit’ model of tumourigenesis, based on the age of onset of sporadic versus inherited forms of retinoblastoma (Knudson 1971), and this ‘multi-hit’ model was further refined by Vogelstein in a study of colorectal cancer (Fearon and Vogelstein 1990). Such studies and calculations were based on an assumption that cancers were of monoclonal origin, a model that was supported by the work of Nowell that proposed a carcinogen could induce multiple mutations within different cells, most of which were deleterious, but that only a single cell that had acquired a combination of mutations that conferred a selection advantage would go on to produce the tumour bulk (Nowell 1976). Fidler and Kripke went on to show that single cell clones derived from an individual tumour (in this case, melanoma) had marked differences in their ability to metastasise to the lungs in murine xenografts, one of the first convincing experimental examples of the clonal heterogeneity present in cancer (Fidler and Kripke 1977).

The exact number and type of mutations that are required to initiate tumourigenesis is highly variable. Work from the Weinberg laboratory has shown that anywhere between two and seven genetic pathways need to be perturbed for cellular transformation, dependent on cell type and species (Rangarajan*, et al* 2004). Other studies have identified up to 20 driver mutations in an individual tumour, consistent with a mathematical model in which each mutation provides a relatively small selective advantage to the cell (Beerenwinkel*, et al* 2007, Sjoblom*, et al* 2006).

With the recent development of next generation sequencing and high resolution copy number analysis, it should be noted that alternative models of tumourigenesis have been proposed. For instance, rather than the progressive accumulation of mutations occurring over time, the recent discovery of chromothripsis (the presence of tens to hundreds of genomic rearrangements and mutations on a single chromosome) has implicated the occurrence of a one-off catastrophic genetic event in some cancers such as osteosarcoma (Stephens*, et al* 2011). Furthermore, cells within a given tumour can be highly heterogeneous, as exemplified by the multiple different mutations, ploidy and gene expression signatures obtained from sampling different regions of an individual tumour (Gerlinger*, et al* 2012). The degree and complexity of clonal heterogeneity in ALL had not been established at the time the studies presented in this thesis were being performed, although several recent papers have addressed this in some detail (see Discussion).

4.1.3 'Passenger' and 'driver' mutations in cancer

Several authors have made analogies between Darwinian theories of evolution and cancer; tumour cells are under constant environmental pressure and genetic changes that offer a survival advantage will be selected for, whilst cells acquiring unfavourable genetic changes will be lost (Anderson*, et al* 2011, Greaves 2007, Merlo*, et al* 2006, Nowell 1976, Stratton*, et al* 2009). Despite the many thousands of somatic mutations identified by next generation sequencing in a variety of cancers, it is likely that most of them do not confer an advantageous phenotype to cells, and may have been present by chance in the cell of origin or acquired as a result of genetic instability during tumour progression. Such mutations have been described as 'passenger' mutations, as opposed to 'driver' mutations that provide a survival advantage to cells and have undergone positive selection (Stratton et al., 2009). This is of considerable importance when interpreting data from next generation sequencing and particularly in determining which mutations should be pursued as therapeutic targets.

4.1.4 *NOTCH-1* mutations - disease initiators or collaborative events in T-ALL?

After the seminal discovery of *NOTCH-1* mutations in the majority of paediatric patients with T-ALL, it was argued that the acquisition of a *NOTCH-1* mutation was the initiator of T-ALL in the majority of cases and that the other mutations or chromosomal aberrations found in T-ALL were acquired as secondary co-operating events after activation of the Notch pathway (Malecki*, et al* 2006). Given that NOTCH-1 is capable of T-cell lineage determination of uncommitted progenitors, the authors postulated that activating mutations of *NOTCH-1* were the 'first hit', causing expansion of the T-cell pool, prior to acquisition of other mutations. Support for this theory came from mouse models of T-ALL where animals transplanted with cells transduced with either ICN-1 or the Notch ligand Delta-like 4 developed a lethal T-cell leukaemia, suggesting that Notch activation in itself was sufficient to initiate leukaemia (Pear*, et al* 1996, van den Brandt*, et al* 2006, Yan*, et al* 2001). Furthermore, Mel Greaves' group at the Institute of Cancer Research, London, had analysed DNA extracted from a Guthrie card and shown that a *NOTCH-1* HD-N mutation was present prenatally in a child who developed T-ALL at 6 years of age (Eguchi-Ishimae*, et al* 2008). This patient had a *SIL-TAL1* deletion detectable at disease presentation that was not present in the Guthrie card sample suggesting that, at least in this particular case, the *NOTCH-1* mutation was acquired as the primary event.

However, many of the *NOTCH-1* mutations described in human T-ALL are insufficient in their own right to initiate disease in mouse models, and can only accelerate T-ALL onset in collaboration with activation of other oncogenic pathways such as Ras (Chiang*, et al* 2008, Kindler*, et al* 2008, Mansour 2009). *Notch-1* mutations have also been found in the majority of mice with *Tal-1*-induced T-ALL, as well as in mice that develop T-ALL as a result of deficiencies in various combinations of *Tp53, H2ax* and *Rag2* genes, suggesting that the mutations can occur as secondary events and play a collaborative role in T-ALL pathogenesis (Lin*, et al* 2006, O'Neil*, et al* 2006). Interestingly, the vast majority of the mutations acquired as secondary events in murine models are deletions of the PEST domain, with a conspicuous absence of HD mutations as compared to human disease. However, it has since transpired that in a high proportion of murine models of T-ALL, including the *Ikaros*-driven T-ALL model, ligand-independent activation of Notch-1 occurs by a different mechanism. Here, tumours acquire a deletion of the *Notch-1* promoter including exon 1 (type I deletion), or a deletion of sequences between exons 1-28 (type II deletion), which leads to transcription of a spliced transcript that is translated from an alternative start site at amino acid M1727 (murine sequence) within the transmembrane domain (Ashworth*, et al* 2010, Jeannet*, et al* 2010). Such a mechanism has recently been described in a single patient with human T-ALL who had a deletion of the 5' portion of *NOTCH-1* as detected by SNP array, which led to an alternative start site at M1668 in the HD-C domain that produced high levels of ICN-1 (Haydu*, et al* 2012).

4.1.5 Analysis of matched presentation-relapse samples

One possible way to dissect the chronology of mutation acquisition in T-ALL is to analyse paired presentation-relapse samples for mutations in particular genes. There are a number of possible outcomes to such analyses:

1. No detectable mutation at presentation or relapse
2. The same mutation is detectable at presentation and relapse
3. A mutation in the presentation sample is lost at relapse
4. A mutation in the relapse sample is not detectable at presentation
5. Mutations are detected in the presentation and relapse samples but are different

The most likely explanation for scenario 2 is that the mutation occurs early in the leukaemia-initiating cell, is present in all clones and is thus detectable at relapse, irrespective of which clone is responsible for relapse. This would be the expected scenario if the mutation was a primary disease initiator. An alternative possibility is that the mutation has been acquired as a secondary event that confers an additional survival advantage to the cell, for instance by increasing chemoresistance, and is present in the relapse sample, at which point the mutant level may be seen to increase. A third possibility is that the relapse clone by chance acquires the same mutation as a new event, which is plausible if the mutation was highly recurrent in that particular cancer, but much less likely if the mutation was unique. This would be technically difficult to prove, although a comprehensive analysis of other concurring mutations, including assessing mutant level, could assist in the interpretation of this finding.

Although there are technical explanations that could account for scenarios 3, 4 or 5, for instance, a sample mix-up with either incorrect patient identity, or incorrect time point (eg. remission sample), there are several biological possibilities that could lead to such occurrences. For instance, a mutation occurring as a late secondary event at presentation that is not present in the clone responsible for relapse is arguably the most likely cause for scenario 3. Another possibility is that the relapse is not derived from an ancestral clone but is in fact a true secondary leukaemia occurring after treatment for the primary T-ALL. Whilst most cases of relapsed T-ALL occur early in the first 18 months after the completion of therapy, secondary leukaemias typically occur after a longer interval and can be identified by the presence of a unique TCR-rearranged clonal sequence unrelated to the initial clone (Szczepanski*, et al* 2003).

Scenario 4 could be explained by the acquisition of a mutation during therapy that confers chemoresistance or a survival advantage to cells. Although the genetic damage induced by chemotherapy has the potential of introducing mutations that confer chemoresistance, several recent studies support a mechanism in which a resistant clone pre-exists at low level prior to the initiation of therapy, but is seemingly 'undetectable' by low sensitivity techniques such as Sanger sequencing. For instance, a study in mice transplanted with primary human T-ALL cells showed that engraftment often occurs from a population of cells that exist as a minor subclone in the diagnostic sample

(Clappier, *et al* 2011). In this study, analysis of paired presentation and relapse human samples showed that the murine xenograft leukaemias were genetically similar to the relapse clone, harbouring lesions such as *PTEN* deletions that could be identified at a low level in the diagnostic sample only when analysed by highly sensitive techniques such as quantitative PCR. A recent study of colorectal cancer by Vogelstein's group showed that resistance to the anti-EGFR antibody panitumumab occurs at 5-6 months after therapy and is mediated by cells harbouring *KRAS* mutations (Diaz, *et al* 2012). These tumours had been classified as *KRAS* wild-type at presentation when analysed by Sanger sequencing, but analysis by highly sensitive techniques identified the existence of rare minor clones harbouring *KRAS* mutations that became prominent by the selection pressure conferred by panitumumab therapy.

4.1.6 T-ALL secondary to gene therapy for X-linked Severe Combined Immunodeficiency (SCID)

Severe Combined Immunodeficiency (SCID) is a rare and often fatal congenital disorder that results in recurrent infections from impaired T-cell, B-cell and/or NK- cell function. A variety of loss-of-function mutations involving genes important in the T-cell differentiation or activation pathway have been described to date (Table 4.1). The first genetic defect described was in 1972, involving the adenosine deaminase gene (ADA) (Giblett, *et al* 1972), and since then at least 10 further genes have been identified as causing SCID including *IL2RG* (the *IL2* receptor γ-chain), *JAK3*, *IL7RA* (*IL-7* receptor α), *CD3δ, CD3ε, CD3ζ, CD45, RAG1, RAG2,* and *Artemis* (Buckley 2004). The most common cause of SCID, comprising nearly half of cases, is the X-linked type (known as SCID-X1) occurring as a result of mutations in *IL2RG. IL2RG* is also known as the common gamma chain because it heterodimerises with six other lymphocyte receptors including *IL2RB, IL4R, IL7R, IL9R, IL15R,* and *IL21R,* that are involved in a variety of functions including T-, B- and NK-cell proliferation and differentiation. Patients with SCID-X1 typically present in early childhood with apthous ulcers, intractable nappy rash, and recurrent infections (including oral candidiasis, Varicella and *Pneumocystis Jirovecci* pneumonia) and, without allogeneic bone marrow transplantation (BMT), usually succumb to their disease. Although allogeneic BMT can be curative, it carries a significant morbidity and mortality, particularly if the donor is not an HLA-matched matched sibling (Antoine, *et al* 2003, Buckley, *et al* 1999).

Table 4.1 The classification of SCID according to causative genes, mechanism of immunodeficiency, inheritance and cell type affected.

Mechanism	Genes involved	Inheritance	Cells affected
Induction of apoptosis	*ADA*	AR	T, B, NK
Defective cytokine signalling	*IL2Rγ*	X-linked	T, NK
	JAK3	AR	T, NK
	IL7RA	AR	T
Defective V(D)J rearrangement	*RAG1 or RAG2*	AR	T, B
	Artemis	AR	T, B
Defective TCR signalling	*CD45*	AR	T
	CD3 δ, ζ, ε	AR	T

AR, autosomal recessive; TCR T-cell receptor. Classification based on Cavazzana-Calvo and Fischer, 2007.

In 2000, a seminal French study was published showing that a lentiviral vector expressing a functional *IL2RG* gene could correct the T-cell defect in children with SCID-X1 (Cavazzana-Calvo*, et al* 2000), a finding that was reproduced in a British trial (Gaspar*, et al* 2004). However, a total of five of these patients have to date developed T-ALL, four on the French trial (of 10 treated) and one on the British trial (of 10 treated). In four of the five cases, the lentiviral insertion occurred in the vicinity of the LIM domain only 2 (*LMO2)* oncogene leading to its upregulation by the enhancer effect of the viral long-terminal repeats (LTR) (Hacein-Bey-Abina*, et al* 2008, Hacein-Bey-Abina*, et al* 2003a, Hacein-Bey-Abina*, et al* 2003b, Howe*, et al* 2008). Given that the viral insertions occur as the primary initiating event for the leukaemia, the presence of other somatic mutations are likely to have occurred as secondary events. Thus, the analysis of *NOTCH-1* mutation status in this setting may be informative as to whether *NOTCH-1* mutations occur exclusively as primary events.

4.2 AIMS

Although *NOTCH-1* mutations occur in all cytogenetic and molecular subgroups, it is unclear whether they are primary initiating or secondary cooperating events. The aim of the studies presented in this chapter was to investigate whether *NOTCH-1* mutations are always the primary initiating event in T-ALL and/or whether they can occur as secondary events. In order to address this issue, the relative level of *NOTCH-1* mutated alleles in a cohort of human patients at presentation with T-ALL was assessed, and the stability of these mutations examined at disease relapse. This has implications for the use of *NOTCH-1* mutations as MRD markers, as well as for the use of Notch inhibitors in clinical trials for T-ALL.

Whilst these studies were ongoing, a patient with SCID-X1 presented to Great Ormond Street Hospital with T-ALL secondary to gene therapy. In this case, the driver of leukaemogenesis was overexpression of the *LMO2* oncogene as a result of viral integration within its vicinity. The presence of pre-diagnostic serial samples gave a unique opportunity to investigate the chronology of acquisition of a *NOTCH-1* mutation during T-ALL pathogenesis.

4.3 MATERIALS AND METHODS

4.3.1 Patients and samples

Details on the samples from 60 patients with adult T-ALL were given in Chapter 3. DNA samples from 12 paediatric T-ALL patients at disease presentation were obtained from Great Ormond Street Hospital in collaboration with Dr Phil Ancliff, and from six adolescent T-ALL patients treated locally at University College Hospital. Samples were available from 16 patients at first relapse; three also had samples at second relapse. Ethical approval for tissue collection for research was obtained from the Multi-Centre Research Ethics Committee for patients entered into clinical trials or from local research ethics committees as appropriate, and informed consent was obtained according to the Declaration of Helsinki.

The patient with SCID-X1 was one of ten who received gene therapy for SCID-X1 at Great Ormond Street Hospital, and DNA samples were made available by Professor Adrian Thrasher. The patient/parents had received independent counselling prior to the

trial with regard to the potential risks of leukaemogenesis and fully informed parental consent was obtained. The study had ethical and regulatory approval from the Gene Therapy Advisory Committee (GTAC) and the Medicines and Healthcare Regulatory Authority (MHRA). The Gibbon ape leukaemia virus (GALV)–pseudotyped gammaretroviral vector encoding the *IL2RG* cDNA regulated by intact murine leukaemia virus (MLV) long terminal repeats (LTRs) and the transduction protocol have been described previously (Gaspar*, et al* 2004). A total of 7×10^6 $CD34^+\gamma c^+$ cells/kg were infused in the absence of conditioning.

4.3.2 *NOTCH-1* mutational analysis and enrichment of low level mutations

NOTCH-1 mutation screening and identification were carried out using heteroduplex analysis and sequencing as described in Chapter 3. For samples where the heteroduplex peaks were much smaller than the homoduplex peaks, the former were purified using the fraction collector facility of the WAVE® and sequenced. The procedure is based on the less stable heteroduplex peak eluting from the column prior to the homoduplex peak during DHPLC. At a given temperature the elution time is highly reproducible between successive runs, making it possible to utilise the fraction collector facility of the WAVE® to capture the desired fractions (Figure 4.1). The collected product can then be re-amplified by PCR, re-analysed by DHPLC to ensure the desired fraction had been collected, and sequenced. As outlined below, this strategy made it possible to identify mutations in a number of patients that would have been missed by direct sequencing strategies.

4.3.3 Quantification of *NOTCH-1* mutations by fluorescent PCR

In samples with insertions or deletions, relative mutant levels were quantified using the PCR methods described in Chapter 3 with the following modifications: one primer was fluorescently-labelled, only 30 cycles of amplification were performed, BioTAQ DNA polymerase was used for all reactions and primer concentrations were reduced to 60nM. Products were size-separated using fragment analysis on the CEQ8000 Genetic Analysis System and mutant size could be resolved to within two base pairs. Results for mutant level were expressed as a percentage of total alleles i.e. a heterozygous mutation in all cells would be expressed as 50% of total alleles.

4.3.3 T-cell receptor (TCR) rearrangements

TCR rearrangements were analysed by Drs Letizia Foroni and Veronique Duke at the Royal Free Hospital, London, using heteroduplex analysis and fluorescently-labelled primers at the *TCRγ* locus as previously reported (Gameiro*, et al* 2002, Szczepanski*, et al* 2003, van Dongen*, et al* 2003). For those patients where no suitable marker was detected at the *TCRγ* locus, the *TCRδ* locus was analysed.

4.3.4 Same patient identity

Same patient identity was confirmed in the paired presentation-relapse samples using size analysis at four highly informative polymorphic short tandem repeat (STR) loci (VWA, FES, D11S-554, F13I) (http://www.cstl.nist.gov/div831/strbase/) using primer sequences shown in Table 4.2 (Sellathamby*, et al* 2006). PCR reactions were carried out using BioTAQ DNA polymerase, a fluorescently-labelled forward primer (dye D4) at 60nM and 30 cycles of amplification, with annealing temperatures of 54°C for F131, FES and VWA, 61°C for D11S-554. Products were analysed for size by fragment analysis using the CEQ8000 Genetic Analysis System (Beckman Coulter).

Table 4.2 Primer sequences used in this study

Primer name	Primer sequence
FES-F	5'-GGGATTTCCCTATGGATTGG-3'
FES-R	5'-GCGAAAGAATGAGACTACAT-3'
VWA-F	5'-AGCTATATATATCTATTTATCAT-3'
VWA-R	5'-AGATACATACATAGATATAGG-3'
F13A1-F	5'-GAGGTTGCACTCCAGCCTTT-3'
F13A1-R	5'-ATGCCATGCAGATTAGAAA-3'
D11S-554-F	5'-GGTAGCAGAGCAAGACTGTC-3'
D11S-554-R	5'-CACCTTCATCCTAAGGCAGC-3'
NOTCH-1 26-F	5'-GGAAGGCGGCCTGAGCGTGTC-3
NOTCH-1 26-R	5'-ATTGACCGTGGGCGCCGGGTC-3
R1599P-R	5'-CACGTTGGTGTGCAGCACGG-3'

All forward primers were fluorescently-labelled with dye D4

4.3.5. Sensitive detection of R1599P mutation

Serial doubling dilutions of mutant (obtained at presentation from the SCID-X1 patient) and WT genomic DNA (obtained from NB4 cells) were performed to create mixtures of mutant and WT DNA of 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56%. These were then

amplified by PCR using *NOTCH-1* exon 26 primers and subject to DHPLC analysis of the HD-N as described in Chapter 3. This is based on the assumption that the presentation sample is 50% mutant, which was approximated by the DHPLC chromatogram and Sanger sequencing results (Figure 4.7)

To increase the sensitivity of detection of the *NOTCH-1* G4793C mutation (R1599P), an amplification-refractory mutation system (ARMS) was developed using a common forward primer (26-F), common reverse primer (26-R) and an internal mutation-specific reverse primer (R1599P-R), with primer sequences shown in Table 4.2. The principle of this assay is based upon the specificity of the internal primer being complementary to the mutant sequence (usually through the most 3' base of the primer), so that annealing and amplification of only the mutant allele should occur (Figure 4.3). Inclusion of all three primers in the same PCR reaction should generate just a single band for wild-type alleles, but a double band if mutant alleles are present due to the presence of either a heterozygous or homozygous mutation. Although other mismatches are often required to increase specificity of the mutant primer using this method, a single mismatch was sufficient in the mutation described here. To increase the sensitivity of the assay, a fluorescently-labelled forward primer was used and products were sized by fragment analysis. PCR reactions were carried out using BioTAQ DNA polymerase, $MgCl_2$ (1mM), dNTPs (200mM), with the primers NOTCH-1 26-F (dye D4) at 60nM, NOTCH-1 26-R at 60nm and R1599P-R at 40nM (Table 4.2). Cycling parameters were 95°C for 1 minute, 67°C for 1 minute and 72°C for 1 minute for 30 cycles. HD-N PCR product containing the R1599P mutation were TOPO-cloned and DNA was isolated from one mutant and one WT clone and quantified. Mutant DNA was then mixed with WT DNA in the following ratios: 1 in 10 (10% mutant), 1 in 100 (1%), 1 in 200 (0.5%), 1 in 1000 (0.1%) and 1 in 2000 (0.05%) and used to determine the sensitivity of the fluorescently-labelled ARMS assay.

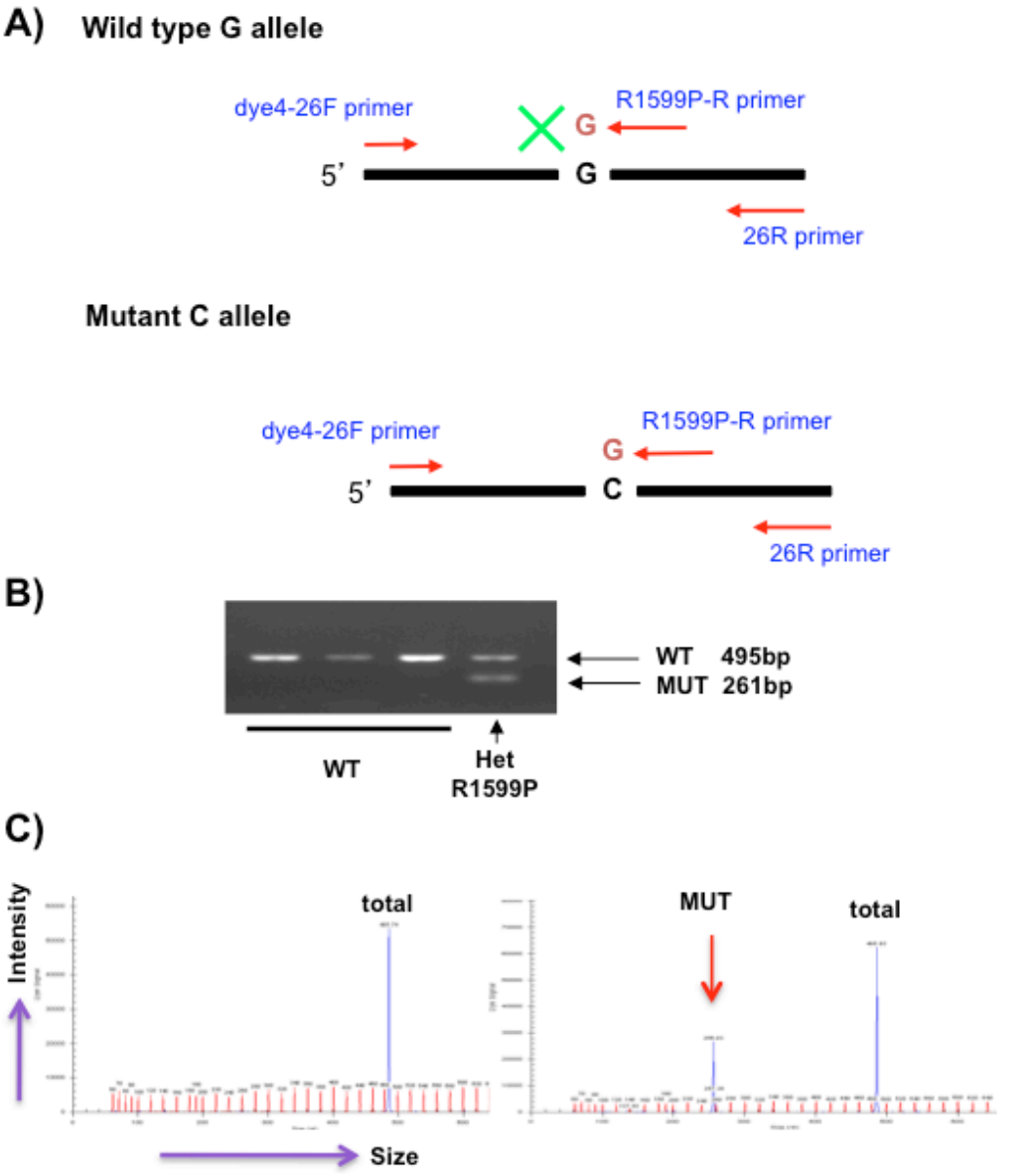

Figure 4.3 Principles of the amplification-refractory mutation system (ARMS) technique for the detection of the R1599P mutation. **(A)** The common outer primers 26-F and 26-R are used in the same reaction as a mutant-specific inner primer in which the most 3' base is mismatched to the wild-type sequence (G), but matched to the mutant sequence (C). A single band is generated for wild-type allele. The R1599P-R primer can anneal to the mutant base, creating a smaller additional band. **(B)** Agarose gel electrophoresis of the PCR products. **(C)** Fragment analysis of PCR products from a WT control (left) and the leukaemic presentation DNA sample from the SCID-X1 patient (right). Red peaks represent size standards.

4.4 RESULTS

4.4.1 *NOTCH-1* and *FBXW7* mutation status of paediatric and adolescent T-ALL patients

NOTCH-1 mutation screening was extended to include 12 paediatric and six adolescent patients for two reasons. Firstly, matched presentation and relapse samples were available from several of these patients. Secondly, it offered an opportunity to quantify *NOTCH-1* mutant level in a larger more diverse cohort of T-ALL patients. Overall, *NOTCH-1* mutations were identified in 16 of the 18 patients (89%) (Table 4.3). Seven patients had mutations in the HD-N domain only, three in the PEST domain only, three in both the HD-N and PEST domains, one in both the HD-C and PEST domain, one with mutations in both the HD-N and HD-C and one with mutations in the LNR and PEST domain (as reported in Chapter 3).

4.4.2 *NOTCH-1* mutation level

By DHPLC, in the total cohort of 78 patients analysed (60 adult patients and 18 paediatric/adolescent patients), there were a total of 66 mutations detected in the LNR, HD-N, HD-C, TAD and PEST domains of *NOTCH-1*. Mutations affecting the JME, which were screened by PCR, were not included in the study of *NOTCH-1* mutant level. Visual estimation of the WAVE pattern was consistent with a heterozygous mutation in the majority of cells for 56 of 66 mutations (85%). For 10 cases (15%), the relative height of the heteroduplex peak was considerably lower than the homoduplex peak, consistent with the presence of a low-level mutation or conversely a very high-level mutation (Figure 4.1 and 4.4). Sanger sequencing of these fragments failed to detect the presence of a mutation suggesting it was not the latter. However, after the early heteroduplex peaks were subjected to fraction collection and re-amplified by PCR, mutations were detectable with sequencing. These were unlikely to have occurred by sample-to-sample contamination because eight of the ten mutations were unique to this cohort of patients. In two further cases, Sanger sequencing of the collected fractions did not reveal any mutations and it is most likely that the abnormal chromatograms were due to the presence of DNA degradation that can, on occasions, resemble low-level mutations. These fragments were classed as wild-type when no mutation was identified after fraction collection on two attempts.

Table 4.3 *NOTCH-1* and *FBXW7* mutational status of the paediatric and adolescent T-ALL patients studied

Case#	*NOTCH-1* mutation status	*FBXW7* mutation status	*NOTCH-1* Mutation (nucleotide)	NOTCH-1 Mutation (amino acid)	*FBXW7* Mutation (nucleotide)	FBXW7 Mutation (amino acid)
PAEDIATRIC						
T3	WT	WT	-	-		
T6	HD-N	MUT	4799T>C	L1601P	1513C>T	R505C
T8	HD-N MUT	MUT	4733T>A	V1579G	1393C>T	R465C
T10	HD-N MUT	MUT	4721T>C	L1575P	1394G>A	R465H
T14	PEST MUT	WT	7475-7476insCCCCTTGACAGG	2492-2493insSPX		
T38	HD-N MUT	WT	4778T>C	L1594P		
T46	HD-N MUT	MUT	4721T>C	L1575P	1393C>T	R465C
T47	PEST MUT	WT	7326-7327insAGGAATCCGGT	fs@2444, STOP@2481		
T48	LNR MUT PEST MUT	WT	4631A>C 7538T>C	H1545P S2514F		
T49	PEST MUT	WT	7398-7390delGTinsTTAGAGAA	STOP@2468		
T50	HD-N MUT HD-C MUT	MUT	4817-4818insGGGACC† 5019-5027del9bp+ins18bp†	F1607L;1608-1609insGP† 1675-1676delSI+insTGSHA†	1436G>T	R479L
T108	HD-N	WT	4793G>C	R1599P		
ADOLESCENT						
T7	HD-N+ PEST MUT	MUT	4754T>C 7324-7331del8bp+ins16bp	L1586P 2443-2444insNGAX	1185-1186 insGGTCCGAATAGTT	396-397 insGPNS STOP@400
T34	HD-N+ PEST MUT	WT	4744-4746delCCG 7326-7327insGACCTGGTATG	1583delP fs@2444, STOP @2481		
T35	HD-N+ PEST MUT	WT	4799T>C 7534C>G	L1601P P2513A		
T44	HD-C+ PEST MUT	WT	5127-5128ins39bp† 7326-7327insCCTCATAGCTC†	1711-1712insVNSLNIPYKIEAV† fs@2444, STOP@2481†		
T45	HD-N MUT	WT	4847T>A	I1617H		
T69	WT	WT				

T# local patient case number. MUT mutant, WT wild type, ins insertion, del deletion, SNP single nucleotide polymorphism, HD-N N-terminal heterodimerisation domain, HD-C C-terminal heterodimerisation domain, fs frameshift, X stop codon, †Low level mutation

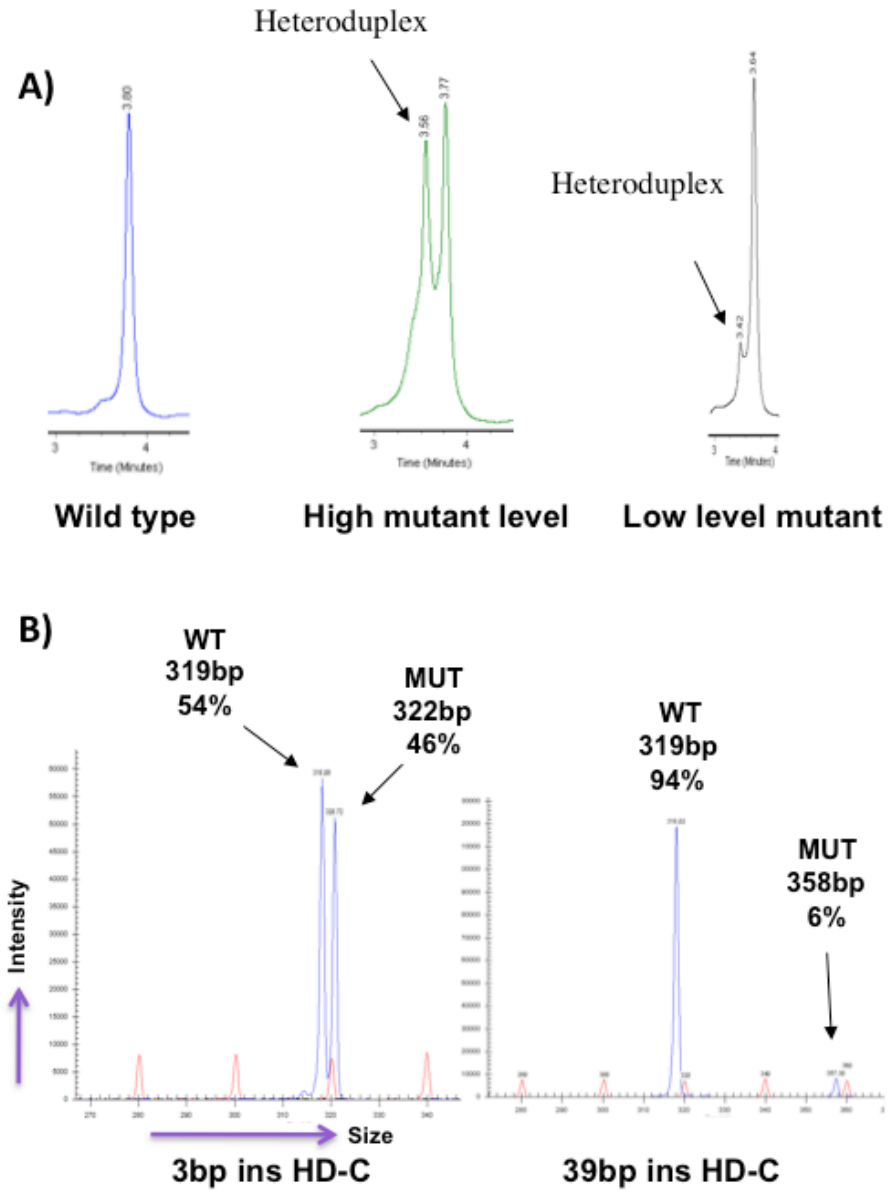

Figure 4.4 Examples of high and low level mutations of *NOTCH-1*. **(A)** DHPLC chromatograms showing a wild type peak (left), a high level 3bp insertion in the HD-C domain (middle) and a low level 3bp insertion in the HD-N domain (right). **(B)** Fragment analysis showing a high level 3bp insertion (left) and low level 39bp insertion in the HD-C domain (right). Red peaks represent size standards

Of the 66 mutations identified, 29 (44%) were nucleotide substitutions and not amenable to quantification using fragment analysis, which relies on size changes. However, only one of the 29 nucleotide substitutions (patient T40 – Table 3.4) had an apparent low level mutation by visual estimation of the WAVE chromatogram, all others were high level.

Of the 37 mutations with size changes, 32 were quantified by fragment analysis for the HD-N, HD-C and PEST domains. Examples of the appearance of the DHPLC chromatograms for high and low level mutants are shown in Figure 4.4, together with examples of fragment analysis traces. The median mutant level for the 32 investigated cases was 40% of total *NOTCH-1* alleles (range 5%-49%) (Table 4.4, Figure 4.5). The mutant level was greater than 30% of total alleles for 21 (66%) of the mutations, and therefore consistent with presence of a heterozygous mutation in the majority of cells (60% of cells or more). No mutant level was greater than 50%, suggesting that there was no loss of the wild-type allele or uniparental disomy.

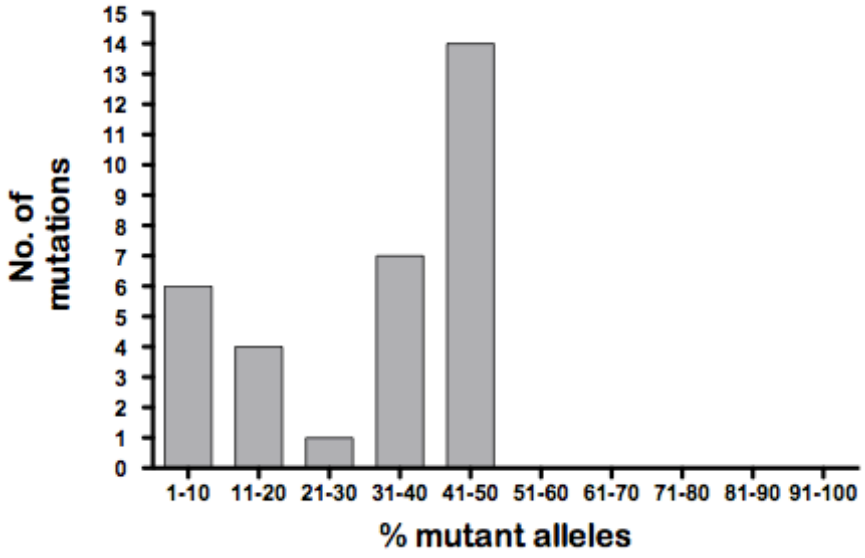

Figure 4.5 Distribution of the relative mutant level for 32 insertions or deletions in the *NOTCH-1* gene. Mutant level was quantified using fluorescently-labelled primers and fragment analysis for the HD-N, HD-C and PEST domains of *NOTCH-1*.

Table 4.4 Quantification of 32 insertions or deletions detected in the *NOTCH-1* gene in 28 patients

Patient number	*NOTCH-1* domain	Net ins/del‡	%mutant allele
T11	HD-N	3bp del	40%
T20	HD-N	3bp ins	10%
T22	HD-N	6bp ins	6%
T28	HD-N	3bp del	39%
T30	HD-N	3bp ins	49%
T31	HD-N	3bp del	42%
T34*	HD-N	3bp del	26%
T50*	HD-N	6bp ins	8%
T54	HD-N	3bp ins	48%
T55	HD-N	75bp del	50%
T57	HD-N	3bp del	13%
T5	HD-C	3bp ins	47%
T19	HD-C	3 bp del	9%
T36	HD-C	12bp ins	47%
T44*	HD-C	39bp ins	6%
T50*	HD-C	9bp ins	5%
T58	HD-C	66bp ins	19%
T1	PEST	2bp ins	46%
T5	PEST	8bp ins	44%
T7*	PEST	8bp ins	47%
T14*	PEST	12bp ins	43%
T25	PEST	5bp ins	45%
T32	PEST	2bp del	45%
T34*	PEST	11bp ins	38%
T43	PEST	5bp ins	19%
T44*	PEST	11bp ins	15%
T47*	PEST	11bp ins	46%
T49*	PEST	6bp del	39%
T63	PEST	7bp del	31%
T64	PEST	13bp ins	48%
T66	PEST	18bp ins	40%
T67	PEST	8bp ins	37%

Actual adult mutations are shown in Table 3.4 *****Patient <18yrs of age as per Table 4.3 ‡ Net ins/del refers to the net overall size difference of a particular insertion or deletion, or complex indel.

However, the mutant level was less than 30% for 11 of the mutations and in six of these it was 10% or less (Figure 4.5), indicating that in these patients 20% or less of cells carried the mutation (Table 4.4). Two of the low level mutations were in the same patient (T50 – Table 4.3). In each patient morphological examination revealed at least 75% leukaemic blasts (mean 87%). To exclude the possibility the mutations were present in contaminating normal cells as germline aberrations, mutational analysis was conducted from four patients where remission DNA was available from bone marrow samples. In no case was the mutation present at remission, consistent with the hypothesis that these were somatically acquired mutation. Furthermore, three of these patients had a high level mutation in another domain. None of the patients with low level mutations had evidence of ploidy or loss of genomic material of chromosome 9q by G-banding cytogenetics. These results suggest that the low level mutations were acquired in a subclone as a later event.

4.4.3 Paired presentation-relapse samples

The *NOTCH-1* mutation status of paired presentation-relapse samples from 16 patients with available material (patients designated as 'PR1-16' for Presentation-Relapse) was studied to examine the stability of these mutations at relapse. STR analysis of four loci confirmed that each paired sample was from the same individual. Seven cases were WT at both presentation and relapse (PR1-7, Table 4.5). Of nine mutant-positive patients at presentation, five relapsed with the same mutation(s) at approximately the same or increased mutant level (PR8-12). In one patient (PR10), the same mutation was also present at second relapse. In two patients (PR11, PR12) high level mutations were present in two different domains, both of which recurred at relapse, suggesting that they were in the same cell. In these five patients, it is likely that the *NOTCH-1* mutation occurred as an early event. However, in four mutant-positive patients there were changes at relapse.

At presentation, one patient (PR13, Table 4.5) had only a PEST domain mutation (insertion of stop codon at amino acid 2468), which was not detected at relapse. The same TCR Vγ1 clone was identified at both presentation and relapse. Another patient (PR14) had an 11bp insertion in the PEST domain at presentation which was not detected at first or second relapse (22 and 29 months respectively). However, a 39bp

Table 4.5 *NOTCH-1* mutational status at presentation and relapse in 16 patients

	Presentation /1st Relapse/ 2nd Relapse			
Patient no.	**HD-N**	**HD-C**	**PEST**	**Overall (% mutant) §**
PR1	-/-	-/-	-/-	WT/WT
PR2	-/-	-/-	-/-	WT/WT
PR3	-/-/-	-/-/-	-/-/-	WT/WT/WT‡
PR4	-/-	-/-	-/-	WT/WT
PR5	-/-	-/-	-/-	WT/WT
PR6	-/-	-/-	-/-	WT/WT‡
PR7	-/-	-/-	-/-	WT/WT
PR8	+/+ L1601P	-/-	-/-	MUT/MUT (H/H)
PR9	-/-	-/-	+/+* fs@2467	MUT/MUT (22/45)
PR10	-/-/-	-/-/-	+/+/+ fs@2444	MUT/MUT/MUT‡ (46/42/47)
PR11	-/-	+/+ insG1675	+/+ fs@2444	MUT/MUT (47/46; 44/37)
PR12	+/+ L1586P	-/-	+/+ 2442NGA-STOP	MUT/MUT (H/H;47/42)
PR13	-/-	-/-	+/- 2468-STOP	MUT /WT (39/-)
PR14	-/-/-	+/+/+* 13aa ins	+/-/- fs@2444	MUT/MUT/MUT‡ (6/21/33; 15/-)
PR15	-/+ H1592Q;F1593T	+/- delV1677	-/-	MUT/MUT† (-/H; 9/-)
PR16	+/- H1545P¶	-/-	+/+† S2514F/fs@2515	MUT/MUT† (H/-; -/34)

†different mutation at relapse, ‡post-allograft but donor cells undetectable; *increasing mutant level; §% mutant level as quantified by fragment analysis; H indicates high mutant level by visual estimation of WAVE chromatogram; fs frameshift; MUT indicates *NOTCH-1* mutation present; WT wild-type *NOTCH-1* status

insertion in the HD-C domain was present at a low level (6%) at diagnosis and progressively increased at each relapse (21% and 33% respectively) (Figure 4.6). Of note, at each time point the patient had the same TCR Vγ11 clone; no new clone was detectable. Case PR15 had a low level HD-C mutation at presentation (delV1677; 9% mutant) that was undetectable at relapse. However, the patient relapsed with a new high level HD-N mutation (H1592Q;F1593T). Furthermore, the same TCR Vγ1 clone was seen at both presentation and relapse. In these three cases the results suggest that the *NOTCH-1* mutations occurred after the TCR rearrangement.

Case PR16 presented at 5 months of age and relapsed 20 months later. Two high level mutations (H1545P and S2514F), which were detected at presentation, were lost at relapse, but a new high level (34% mutant) 2bp deletion in the PEST domain was acquired. TCR analysis at the gamma locus showed loss of the original clone and acquisition of a new unrelated clone (Vγ4 to Vγ2 clone respectively), with different CDR3 sequence. Therefore, in this patient the results most likely reflect development of a secondary leukaemia, similar to cases previously described (Szczepanski*, et al* 2003).

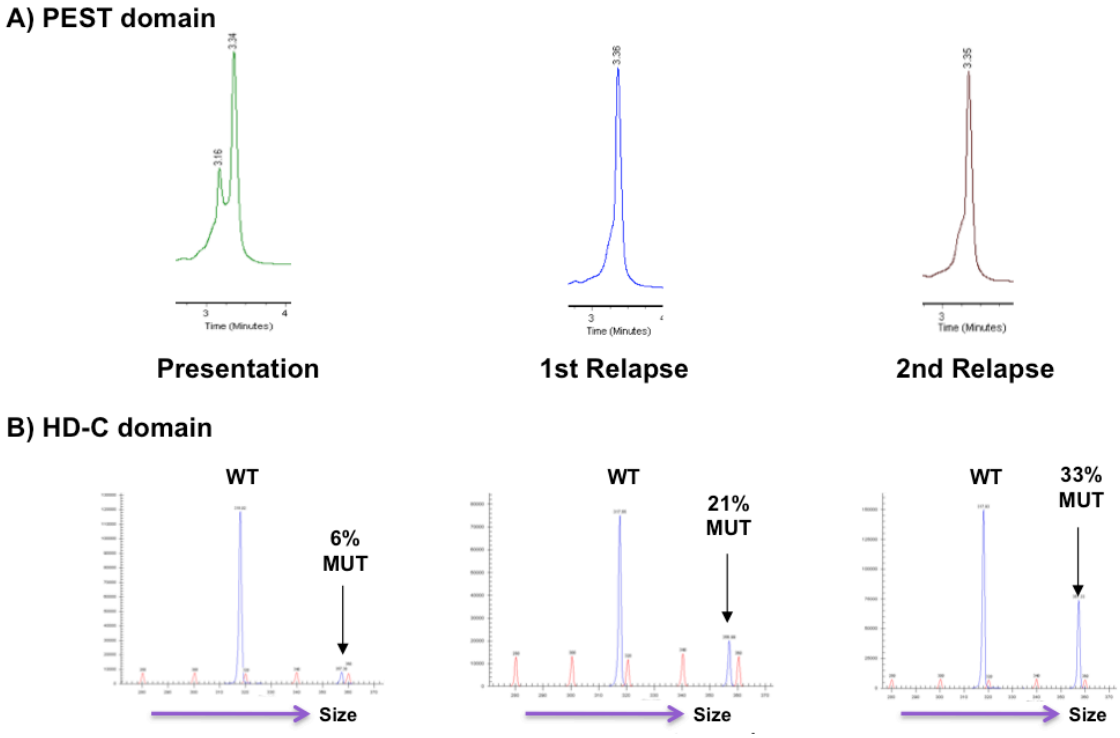

Figure 4.6 Changes in the *NOTCH-1* mutations detected at 1st and 2nd relapse in patient PR14. (A) An 11bp insertion in the PEST domain detected at presentation was not detectable at first and second relapse, as seen by DHPLC analysis. **(B)** Fragment analysis for the HD-C domain of the same patient showed progressive increase in mutant level of a 39bp insertion. Size standards are shown in red.

4.4.4 Clinical presentation and molecular characteristics of patient with SCID-X1

The patient presented at Great Ormond Street Hospital at the age of 10 months (Patient T108) and was shown to have an *IL2RG* C182Y mutation, which resulted in marked reduction in the expression of the IL2RG on the patient's lymphocytes (Howe*, et al* 2008). He received gene therapy with an *IL2RG*-expressing vector at the age of 13 months and regained normal T-cell numbers within 6 months of therapy. Despite normal haematological and immunological parameters when seen in clinic at 23 months post-treatment, he presented at 24 months (day 717) with T-ALL with a high WCC of 524×10^9/l, with circulating blasts. He was commenced on standard chemotherapy according to the UKALL2003 regimen B protocol and was in remission at 1 year post therapy, by which time he had reconstituted a normal T-cell count that retained expression of the IL2RG transgene.

Molecular characterisation of the diagnostic T-ALL sample at Great Ormond Street Hospital revealed marked over-expression of *LMO2*, *NOTCH1*, *HES1* and *c-MYC* by gene expression array and an antisense insertion of the viral vector 35 kb upstream of the transcription start site of the *LMO2* gene by LAM-PCR. A specific PCR that flanked the insertion site was positive at a sensitivity of 0.01-0.1% in a pre-leukaemic sample at day 539, but not before this time. Cytogenetics showed a t(1;7)(1p32;q35) translocation which juxtaposed the TCRβ promoter to TAL1, and SNP array analysis showed a deletion of 9p that included the tumour suppressor gene cyclin-dependent kinase 2A (*CDKN2A*) (Howe*, et al* 2008).

4.4.5 *NOTCH-1*/*FBXW7* mutation analysis of the gene therapy-induced T-ALL

DNA from the presentation T-ALL sample (day 717 after gene therapy) was screened for mutations in the LNR, JME, HD-N, HD-C, TAD and PEST domains of the *NOTCH-1* gene, and exons 8-12 of the *FBXW7* gene as described in Chapter 3. *FBXW7* was wild-type, but an R1599P missense mutation was identified in the HD-N domain of *NOTCH-1* (Figure 4.7). By DHPLC analysis, this mutation was not present in the DNA samples isolated from the patients' mononuclear cells prior to gene therapy, CD34+ cells after viral transduction, or CD3+ cells isolated at day 539 (178 days prior to presentation with T-ALL). Mixes of normal DNA with DNA from the T-ALL sample showed that DHPLC had a sensitivity of approximately 6% to detect the R1599P mutation (Figure 4.7).

Unfortunately, no cells or DNA were available for investigation from the clinic visit 1 month prior to presentation with T-ALL.

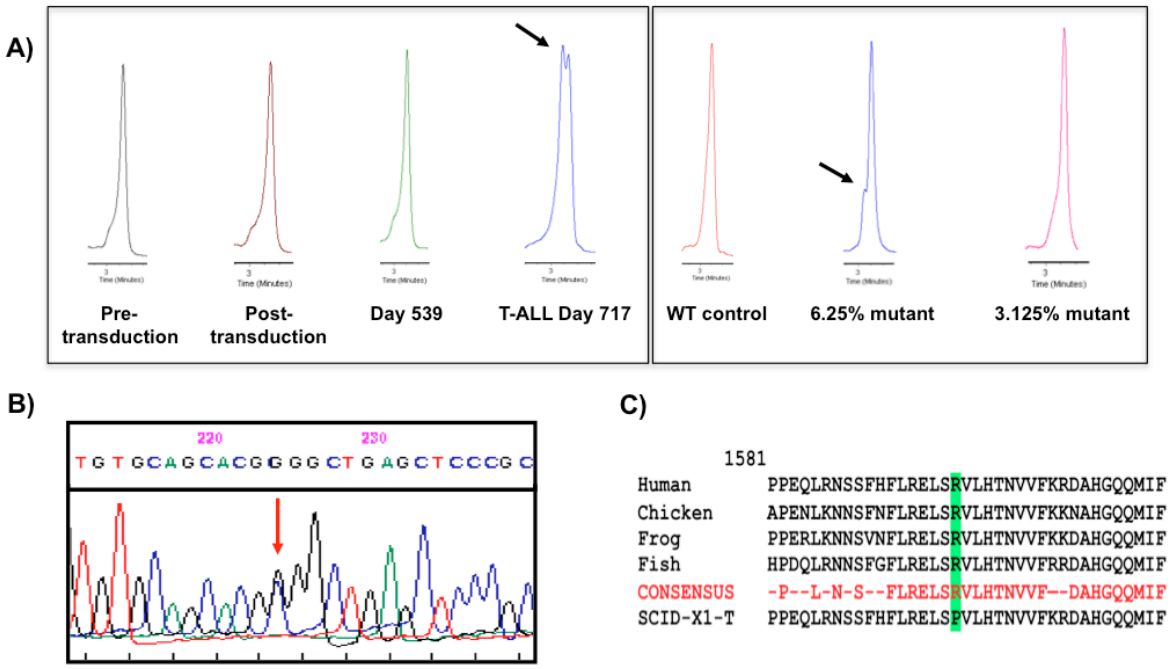

Figure 4.7 Identification of a *NOTCH-1* R1599P mutation in gene therapy-induced T-ALL. (**A)** DHPLC analysis of the HD-N of DNA isolated from mononuclear cells pre-transduction, CD34+ cells immediately post-transduction, CD3+ cells 539 days after transduction and blast cells at presentation with T-ALL. The right box shows wild-type control (WT) and dilutions of DNA at T-ALL presentation with normal control DNA. (**B)** Sequencing trace from the reverse (26-R) primer of the HD-N domain showing a C to G transition (G4796C). (**C)** The arginine at 1599 is highly conserved amongst diverse species, suggesting it has importance to the HD-N domain structure and/or function.

As the *LMO2* insertion would have occurred at the time of viral transduction, an important question was whether this occurred as the primary event with later acquisition of the *NOTCH-1* mutation, or whether by chance the CD34+ cell with the viral integration had a pre-existing *NOTCH-1* mutation. In an attempt to address this, a fluorescent amplification-refractory mutation system (ARMS) assay was developed (Figure 4.3). HD-N PCR products containing the R1599P mutation were TOPO-cloned, and DNA was isolated from one mutant and one WT clone and quantified. Mutant DNA was then mixed with WT DNA in the following ratios: 1 in 10 (10% mutant), 1 in 100 (1%), 1 in 200 (0.5%), 1 in 1000 (0.1%) and 1 in 2000 (0.05%). These dilutions were then used as input into the fluorescent ARMS assay and the products analysed by fragment analysis. The assay had a reliable and consistent sensitivity of 0.5% (one mutant clone in 199 normal clones) (Figure 4.8), equivalent to one heterozygous mutant cell in 99 normal cells. Although a small peak could be visualised on the majority of runs at 0.1% mutant (Figure 4.8), the peak height of the mutant was not always of adequate intensity above background to be definitive, and thus the limit of the assay was determined to be 0.5%.

Analysis of the available DNA samples from the SCID-X1 patient using the fluorescently-labelled ARMS assay was only positive in the presentation sample, but not in any of the preceding material, including the CD3-selected T-cell sample collected 178 days prior to T-ALL diagnosis (at which point the LAM-PCR was positive for the *LMO2* integration at a sensitivity of 0.01-0.1%). Thus, within the limits of the assay, there was no evidence of the *NOTCH-1* mutation prior to overt T-ALL.

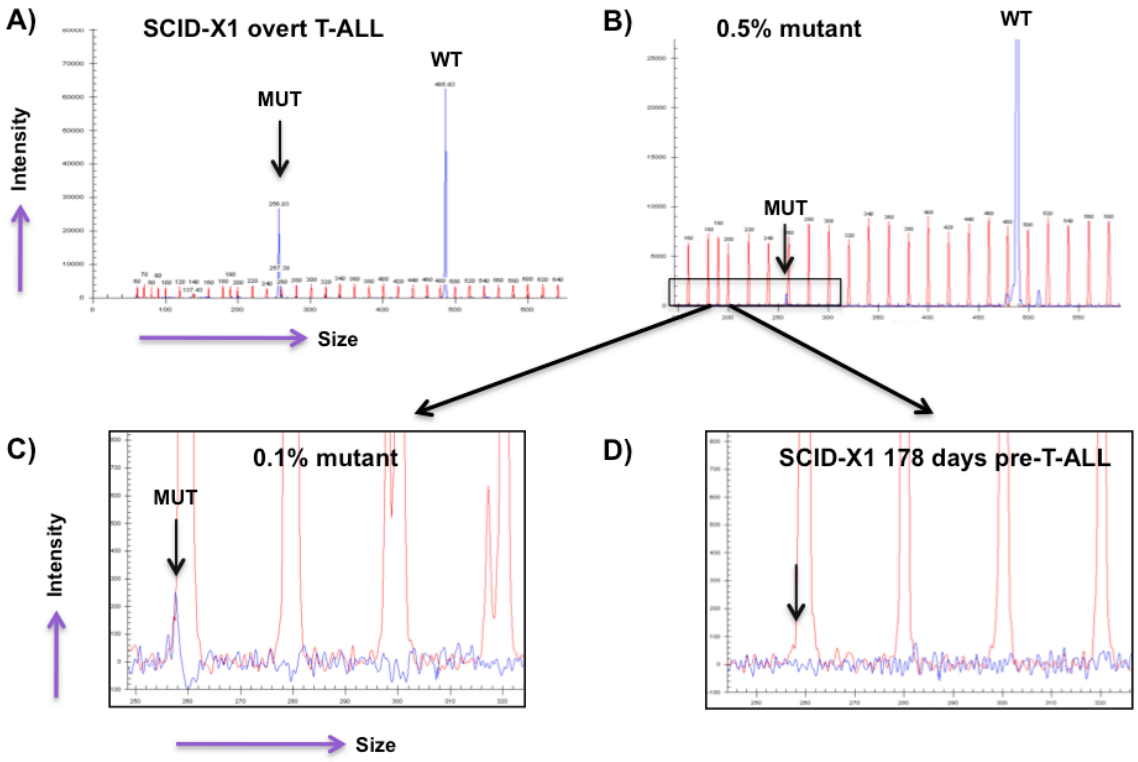

Figure 4.8 Fluorescent amplification-refractory mutation system technique for the detection of the R1599P mutation. **(A)** At presentation of overt T-ALL, the SCID-X1 patient had clear evidence of a peak at 256bp corresponding to the R1599P mutant allele. **(B)** A small peak was consistently seen when the assay is performed on mixes of 1 mutant clone TOPO PCR clone with 199 WT clones (0.5%). **(C)** Dilution of mutant TOPO clone 1 in 1000 (0.1%) with WT TOPO clones. D) DNA from CD3 selected T-cells from the patient 178 days prior to the diagnosis of T-ALL.

4.5 DISCUSSION

Cancer is thought to occur through the accumulation of multiple genetic events that perturb a number of cellular pathways that confer a growth and survival advantage to tumour cells over time (Nowell 1976). Hanahan and Weinberg recently described six hallmarks of cancer: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg 2011). Cells that acquire genetic events that contribute to these capabilities will ultimately prevail to form the tumour bulk, whilst those acquiring deleterious lesions will be lost. In a similar fashion,

cells that have acquired genetic lesions that confer resistance to chemotherapy (either prior to, or during treatment) will be responsible for disease relapse. Thus, maintaining a wide clonal heterogeneity of cells, together with the ability to adapt to selection pressure through the rapid accumulation of new genetic events, provides tumour cells with a powerful armoury for the evasion of targeted therapy.

As increasing numbers of genetic abnormalities are being described in T-ALL, it has become feasible to examine the chronology in which these molecular events are acquired. Such information could help to elucidate the mechanisms by which oncogenic pathways interact, further characterise the leukaemic cells responsible for relapse and possibly rationalise the molecular pathways that could be targeted for drug development, including combination therapies.

In murine models of T-ALL, Notch activation can both directly induce leukaemia and collaborate with other initiating genetic events to perpetuate leukaemic growth (Lin*, et al* 2006, O'Neil*, et al* 2006, Pear*, et al* 1996, van den Brandt*, et al* 2006). Mice transgenic for *Scl/Lmo1* have often acquired a *Notch-1* mutation by the time of overt transformation to T-ALL (Lin*, et al* 2006). Oligoclonality, as defined by the presence of clones with different TCR rearrangements, predates acquisition of the *NOTCH-1* mutation, but it is not until after the latter event that these thymocytes form transplantable tumours. It has also become apparent that activation of *Notch-1* as a secondary event occurs on a diverse genetic background in mouse models of T-ALL, suggesting that Notch can collaborate with multiple different genetic pathways. For instance, as well as the frequent *Notch-1* PEST domain mutations described in *lck-Scl/Lmo1, Tp53* null, *H2ax* null, and *rag-2* null backgrounds, activation of *Notch-1* through either PEST domain mutation or 5' deletion has also been shown in *Ikaros* deficient mice, transgenic *Kras* (G12D) mice and mice transplanted with marrow overexpressing *rasgrp1* (Jeannet*, et al* 2010, Kindler*, et al* 2008, O'Neil*, et al* 2006, Oki*, et al* 2012). Whether *NOTCH-1* mutations are an initiating or secondary collaborating event in human T-ALL is unclear. The results described in this chapter suggest that both events may occur.

Of the 32 mutations that were quantified in the patients studied here, the majority (66%) had high-level mutations (>30% mutant), which was consistent with the presence of a heterozygous mutation in the majority of cells. Furthermore, in five of nine (55%)

mutant-positive patients in the paired presentation-relapse cohort, the same mutation was present at relapse. In these patients it is likely that the mutation had occurred early in disease pathogenesis. Two of these patients had mutations in both the HD and PEST domains at presentation and relapse, suggesting that they were in the same cell. It is also noteworthy that two patients (PR9 and PR14) had evidence of increasing mutant level, and one (PR15) acquired a new high-level mutation at relapse, suggesting that Notch activation may offer a selection advantage to these cells.

Conversely, the results in some patients suggested that the *NOTCH-1* mutations occurred not as a primary event but later in the pathogenesis of the disease. Six (19%) were present at a particularly low level in the presentation sample. This was not due to contamination by large numbers of non-leukaemic haematopoietic cells, as review of the morphology showed that all cases had blast counts of 74% or greater. A low mutant level could also occur if the majority of leukaemic cells either lost genomic material including a mutated *NOTCH-1* allele or gained copies of the WT allele. However genomic loss of chromosome 9q34 has not been described by either genome-wide analysis or fluorescent in situ hybridisation in two substantial cohorts of T-ALL patients (Irving*, et al* 2005, Mullighan*, et al* 2007). Furthermore, the finding that murine T-ALL tumours frequently acquire a second synergistically activating *NOTCH-1* mutation suggests that leukaemic cells favour increased, rather than decreased, Notch signal strength, making genomic loss of mutated *NOTCH-1* alleles unlikely. Whilst homozygosity of the *NOTCH-1* mutated allele has been found in some human T-ALL lines as a result of loss-of-heterozygosity (Lin*, et al* 2006), the fact that no patient in our cohort had a mutant level of greater than 50% supports the finding of others that this is not a common phenomenon in human T-ALL (Irving*, et al* 2005, Mullighan*, et al* 2007, Wang and Armstrong 2007). *NOTCH-1* gene duplication has been described in T-ALL, occurring in a small population of cells as a subclonal phenomenon, which supports the hypothesis that Notch activation can be a late secondary event (van Vlierberghe*, et al* 2006). However, if this was the explanation for the low mutant levels observed in our cases, it would require duplication of the WT gene in the majority of cells, with the mutant-carrying clone in the minority. Our results therefore suggest that the low level mutants described here in 19% of the mutant-positive patients analysed are due to the gain of a mutation in a subclone, which, in the presence of high blast counts, must be a secondary event. These results were supported by the demonstration that three mutations were lost at relapse whilst the TCR rearrangements

remained unchanged. This suggests that the *NOTCH-1* mutation was acquired after the TCR rearrangement in these three patients, a situation analogous to the secondarily acquired *NOTCH-1* mutations in murine models of T-ALL.

The risks of T-ALL caused by gene therapy for SCID-X1 have proven to be substantial, with five of 20 patients developing the disease (Hacein-Bey-Abina, *et al* 2008, Hacein-Bey-Abina, *et al* 2003a, Hacein-Bey-Abina, *et al* 2003b, Howe, *et al* 2008). In four of the five cases described thus far, the viral integrations have occurred within the vicinity of the *LMO2* oncogene, with the other patient having an integration in the proximity of the *CCND2* gene. In the case described here, the viral LTRs led to upregulation of all genes within 200kb of the integration, which included *LMO2*. This mechanism of oncogenesis is similar to that induced by wild-type MuLV that sporadically effects feral mice (Cameron, *et al* 1996). Large-scale mapping of 572 retroviral integration sites (RISs) isolated from cells of 9 patients with SCID-X1 in the French trial showed non-random integration of the viral vector, with two-thirds of integrations occurring in or within the vicinity (<10kb) of genes that are expressed in normal CD34+ cells (Deichmann, *et al* 2007). This suggests that access of the gamma-retrovirus to sites of integration is influenced by the chromatin state of the cell, and the favouring of *LMO2* integrations may occur due to high expression of this gene in early haematopoiesis. Furthermore, activation of *LMO2* is capable of forming a transcriptional complex with *TAL1, RUNX1,* and *GATA3* to activate other oncogenic genes including *MYB*, *TRIB2* and *STAT5A* (Sharp 2005).

The SCID-X1 patient described here had many of the hallmarks of sporadic cases of T-ALL, suggesting that over-expression *LMO2* is not sufficient in its own right to induce T-ALL. They had deletion of the negative cell cycle regulator *CDKN2A* gene, which occurs in over 80% of T-ALL cases, and a translocation involving *TAL1*, which has been reported to lead to activation of TAL1. The latter occurs in 40-60% of patients with T-ALL (Ferrando and Look 2000, Ferrando, *et al* 2002). As TAL1 forms a transcriptional complex with LMO2, over-expression of both genes may act in concert to activate their downstream targets. Indeed, collaboration between *Tal1* and *Lmo2* in terms of accelerated T-ALL onset and increased disease penetrance has been shown in a mouse model (Larson, *et al* 1996).

The high incidence of *Notch-1* mutations (>70%) identified in *Lmo2* transgenic mice suggests strong collaboration between the Notch and Lmo2 pathways in murine T-ALL. The presence of the R1599P mutation identified here in *NOTCH-1* in the SCID-X1 patient suggests this collaboration also occurs in human T-ALL. The R1599P mutation has been described previously in sporadic T-ALL and has been shown to be strongly activating in reporter assays (Malecki*, et al* 2006). By visual estimation of the WAVE pattern it appeared to be of high level in the presentation sample, suggesting that it was present in the majority of cells. While specific PCR for the viral insertion site was able to detect the presence of the integration at day 539, 178 days prior to presentation with T-ALL, the R1599P mutation was not detectable at this time point. However, given the disparate sensitivities of detection in the two assays (0.5% for the ARMS for R1599P, 0.01-0.1% for the integration PCR), it is not possible to conclude definitively that the *NOTCH-1* mutation occurred as a late event after the viral integration. It seems unlikely that a CD34+ cell harbouring a pre-existing *NOTCH-1* mutation, *TAL*1 translocation and *CDKN2A* deletion would acquire a viral integration in LMO2.

Limiting dilution analysis of leukaemia initiating cells (LICs) has been performed in mice triple transgenic for *Tal1/Lmo1/ICN1,* and the time to overt T-ALL after transplantation of a single LIC is only 31 days (Tremblay*, et al* 2010). Although there are obvious caveats in comparing human and murine T-ALL, this rapid onset of leukaemia is far quicker than the onset of leukaemia seen in any of the five SCID-X1 patients, with the latter having a minimum latency of 18 months, more consistent with accumulative acquisition of secondary events. Such a latency is comparable with the *Lmo2* transgenic mice (12-18 months) (Larson*, et al* 1994, Larson*, et al* 1996). Furthermore, thymocytes from *Tal1/Lmo1* transgenic mice are blocked at the DN4 checkpoint and only able to proceed to the DP stage after acquisition of a *Notch-1* mutation, at which point they become fully transformed leukaemic cells capable of secondary transplantation (Tremblay*, et al* 2010). Although it is unclear exactly how Notch activation mediates this final step in transformation, the most compelling explanation is that it is, at least in part, mediated through the upregulation of MYC, activation of which has been shown to enable T-cells to evade negative selection and proceed through physiological T-cell checkpoints (Cameron*, et al* 1996, Palomero*, et al* 2006, Weng*, et al* 2006). It is conceivable that an analogous scenario occurs in gene therapy induced T-ALL;

activation of *LMO2* occurs through viral integration as the primary event at the time of *ex vivo* transduction, followed by progressive stepwise acquisition of mutations, each conferring an additional selective advantage to the clone, before overt leukaemic transformation.

There have now been several studies addressing the issue of clonal heterogeneity in ALL. Mel Greaves' group used FISH and copy number analysis (CNA) to study genetic alterations in *ETV6-RUNX1* positive childhood B-ALL at the single cell level (Anderson*, et al* 2011). They detected up to eight genetic abnormalities in a single cell and were able to assemble complex ancestral trees showing wide diversity in the clonal evolution of the leukaemia. *ETV6-RUNX1* translocations were presumed to be the early initiating event, but subclonal evolution of subsequent events was so heterogeneous that some subclones appeared to have independently acquired the same genetic alteration. They proposed a Darwinian model for relapse in cancer, whereby chemotherapy exerts a selection pressure that favours the expansion of resistant clones. James Downing's group used SNP arrays to analyse 61 cases of matched presentation-relapse B-ALL samples and showed that the CNAs in the relapse clones were often different from the primary leukaemia, with both loss of some alterations and gains of others (Mullighan*, et al* 2008). However, the new CNAs identified at relapse were detectable in the presentation sample when analysed by highly sensitive techniques. As discussed earlier, Jean Soulier's group have shown highly similar findings in human T-ALL samples transplanted into immuno-deficient mice (Clappier*, et al* 2011). John Dick's group used xenograft models and CNAs to model the clonal architecture of *BCR-ABL* positive B-ALL, and identified a branching multi-clonal rather than linear evolutionary path (Notta*, et al* 2011). Thus the results of the studies of *NOTCH-1* mutations in T-ALL presented in this chapter are consistent with the highly heterogeneous and complex clonal architecture of ALL, and possibly cancer in general.

The acquisition of *NOTCH-1* mutations at different stages of disease may influence the role of Notch in disease pathogenesis. Development of a mutation early in the disease process may lead to expansion of the T-cell population prior to acquisition of other pathogenic events, whereas with later development its impact on activation of other oncogenic pathways may be more important (Chan*, et al* 2007, Sharma*, et al* 2006). Dissecting this phenomenon, and the impact this has on disease pathogenesis and

response to therapy, will be challenging. The ability to now analyse insertion sites and the chronology of somatic mutations through next generation sequencing technology in gene therapy induced T-ALL offers a rare and unique opportunity to monitor individual clones through disease pathogenesis, and may shed light on the role of *NOTCH-1* mutations in T-ALL induction in humans.

Irrespective of the underlying mechanisms, however, results of the current study have implications for *NOTCH-1* as a target for both minimal residual disease (MRD) analysis and for therapy. *NOTCH-1* is an attractive target for MRD analysis due to specificity of many of the mutations observed in individual patients, although its application would also be challenging due to the high GC content of the gene. Nevertheless, the lack of mutation stability at relapse and the evident acquisition after the TCR rearrangement observed in a proportion of our patients means that caution should be exercised in using them as solitary markers for MRD analysis. In addition, there may be implications for Notch pathway inhibitors that are currently in clinical trials, which, even if effective against constitutively activated mutant-positive cells, may select out remaining WT cells which are unlikely to respond to such therapy.

CHAPTER 5. ALTERNATIVE SPLICING OF THE *CSL* TRANSCRIPTION FACTOR IN ACUTE MYELOID LEUKAEMIA

5.1 INTRODUCTION

5.1.1 Pre-mRNA splicing

The marked differences in length between nuclear and cytoplasmic RNA was first described in the 1970s, which led to the discovery that the entire gene locus containing both introns and exons is first transcribed into a pre-mRNA, before removal of the non-coding introns prior to translation (Berget *et al*, 1977; Sharp, 2005). This finding was of significant importance to basic cellular biology and genetics and was rewarded with the 1993 Nobel Prize in Physiology and Medicine to Richard Roberts and Philip Sharp. Regulation of splicing has turned out to be incredibly complex involving over 100 proteins and five small nuclear RNAs (snRNA), which together make up the spliceosome (Sharp, 2005). Recognition of exon-intron boundaries is mediated through identification of consensus sequences at the 5' intronic splice site (also referred to as the donor splice site) and 3' intronic acceptor site (Breathnach & Chambon, 1981). The 5' splicing typically occurs between an exonic AG and intronic GT sequence, although AG-GC donor sites are present in 0.6% of transcripts (Burset *et al*, 2000).

The first stage of splicing requires the recognition of a 5' splice site at the exon-intron boundary through binding of U1 snRNA by complementary base pairing, with assistance from serine-arginine rich (SR) proteins (Padgett *et al*, 1986). Together with members of the SF3 protein family, the branch point (an intronic adenine residue at a conserved distance from the 3' splice site) is then recognised by U2 small nuclear ribonucleoprotein (snRNP), prior to binding of U4, U5 and U6 snRNPs to the pre-mRNA. A two-step trans-esterification reaction then takes place that first involves the intronic branch point reacting with the first nucleotide of the 5' splice site to form what is known as an RNA lariat intermediate (so named because of its likeness to a lasso). This is followed by the released 5' exon reacting with the acceptor site such that the intronic lariat RNA is liberated and the exons are ligated.

The usage of particular donor or acceptor splice sites can vary considerably, such that strong splice sites are utilised in the majority of transcripts whilst weak splice sites contribute only to minor mRNA isoforms. This is in part governed by exonic flanking

sequences known as exonic sequence enhancers (ESEs) and exonic splicing silencers that promote or suppress splicing respectively. Sequences with similar roles have also been identified within nearby intronic regions (Fairbrother *et al*, 2002; Wang *et al*, 2004).

5.1.2 Alternative splicing

Alternative splicing (AS) involves the differential inclusion or exclusion of exonic and intronic sequences from pre-mRNA into the mature mRNA transcript (Luco *et al*, 2011). Genome wide analysis has shown that somewhere between 75-90% of genes are alternatively spliced, each gene often having multiple different transcripts (Pan *et al*, 2008). Considering the relatively small number of coding genes within the genome, AS is a mechanism of markedly increasing protein diversity as well as allowing tissue-specific protein regulation at the pre-translational stage (Diaz *et al*, 2012; Luco *et al*, 2011). The importance of AS in contributing to a cellular phenotypic is perhaps best exemplified by AS of the doublesex gene (*dsx*) in the fruitfly *D.melanogaster*. The sex-lethal gene (*sxl*) is only expressed in the female fly and leads to AS of *dsx* to produce a shorter isoform (termed dsx^f) that initiates the female gene expression signature, leading to female characteristics (Nagoshi *et al*, 1988). In the absence of *sxl*, *dsx* is not alternatively spliced and male characteristics are produced. Transcripts that occur through AS occur at approximately three times greater frequency in higher organisms than they do in lower organisms, which, at least in part, contributes to the increased tissue complexity that occurs in such organisms despite similar numbers of coding genes (Sharp, 2005).

There are a number of different AS events as exemplified in Figure 5.1:

1. Skipped exon
2. Retained intron
3. Alternative 5’ donor site (may change the frame of the downstream sequence)
4. Alternative 3’ acceptor site (may change the frame of the downstream sequence)
5. Mutually exclusive exons

5.1.3 Mechanisms of AS

Although splicing enhancer and silencer sequences contribute significantly to whether a particular splice site is utilised by the spliceosome, their presence cannot explain the tissue specificity seen with AS, nor the increased AS observed in various cancers

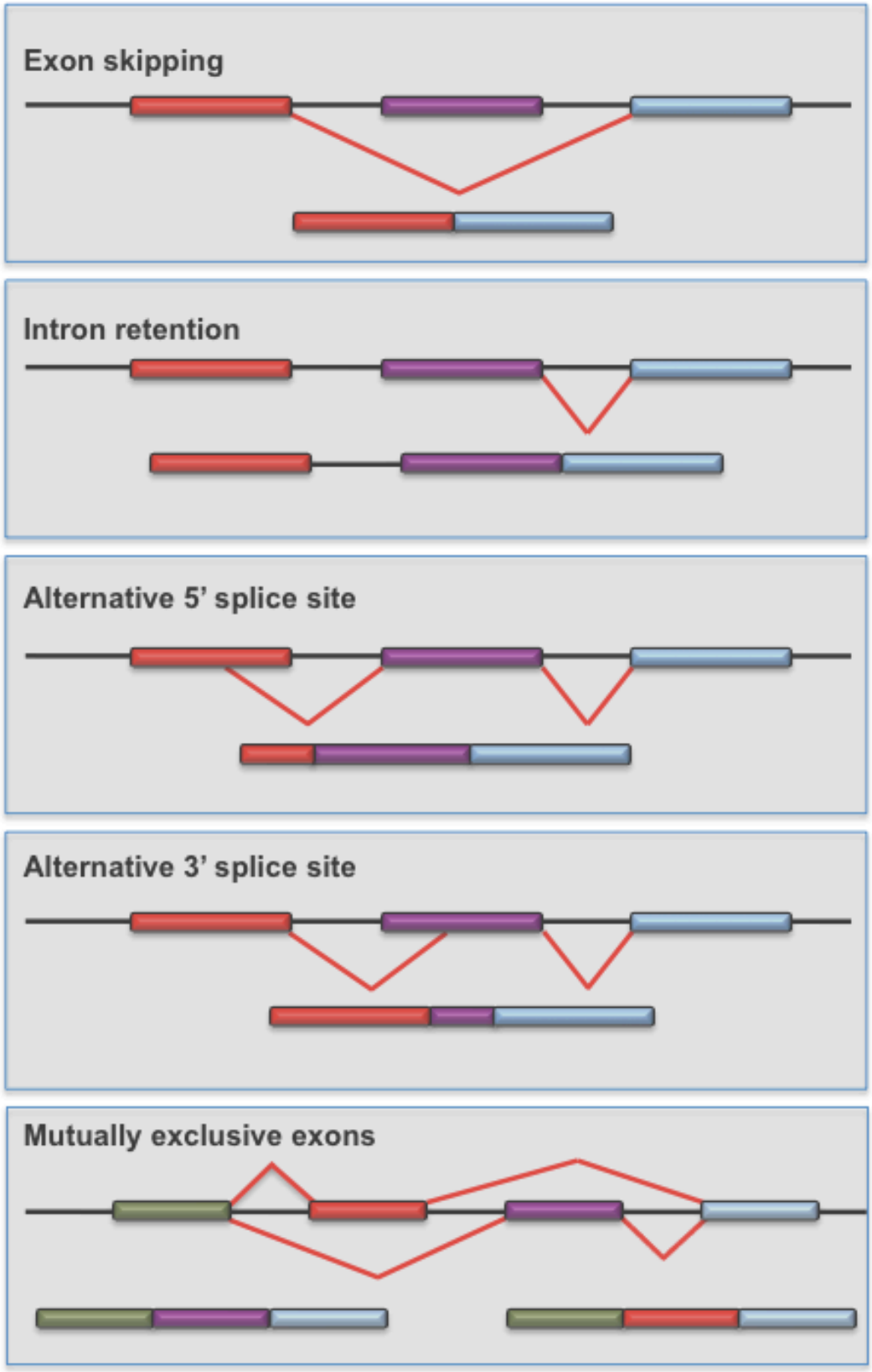

Figure 5.1 A schematic depicting some examples of alternative splicing patterns of RNA. Coloured bars represent exons whilst the black lines signify introns. The pre-mRNA is shown above the mature mRNA

(Faustino & Cooper, 2003). The mechanisms governing AS in cells remain poorly understood, although recent evidence has made conceptual advances in this regard.Firstly, the kinetics of RNA polymerase action during transcription has been shown to be important in determining the degree of AS, with slower transcription and pausing favouring utilisation of weak/alternative splice sites (de la Mata *et al*, 2003). Secondly, AS is influenced by chromatin and histone modifications, with of lysine 36 in histone H3 (H3K36me3) modification directly regulating splicing of a multitude of genes, for example, fibroblast growth factor receptor 2 (*FGFR2)* (Luco *et al*, 2010).

5.1.4 AS in disease

In their review of AS, Faustino and Cooper described four mechanisms of AS that can contribute to human disease (Faustino & Cooper, 2003).

1. *Cis*-acting mutations that disrupt the use of constitutive splice sites. These are usually single base pair substitutions affecting one of the bases of the exon-intron junction. The consequences of such mutations can vary, but most commonly they lead to loss-of-function transcripts through intron retention and nonsense mediated decay, although they can lead to exon skipping or use of alternative splice sites. Multiple examples of such mutations leading to loss-of-function in tumour suppressor genes such as neurofibromatosis-1 *(NF1),* phosphatase and tensin homolog (*PTEN), Tp53,* adenomatous polyposis coli (*APC)* and breast and ovarian cancer susceptibility protein 1/2 (*BRCA1/2)* have now been described in many cancer types (Charames *et al*, 2002; Jolly *et al*, 1994; Liu *et al*, 2012; Reiss *et al*, 1992; Welander *et al*, 2012).

2. *Cis*-acting mutations that alter the use of alternative splice sites. A well-characterised example of this is AS of the Wilm's tumour-1 gene *(WT1)* in Frasier syndrome, a disease causing urogenital developmental abnormalities and high risk of Wilm's tumour. *WT1* has two isoforms, a predominant +KTS isoform and a minor –KTS isoform, depending on the inclusion or exclusion respectively of 9 bases at the start of exon 9. In Frasier syndrome, an intronic splice site mutation at the intron/exon junction for exon 9 results in predominance of the –KTS isoform that is thought to cause the disease phenotype (Klamt *et al*, 1998).

3. *Trans*-acting mutations that disrupt the basal splicing machinery. Mutations in members of the U4/U6 snRNP complex, such as those affecting the pre-mRNA processing factor 3 homolog gene (*HPRP3)*, have been described in families with retinitis pigmentosa, although the mechanism by which this causes the disease is not known (Chakarova *et al*, 2002).

4. *Trans*-acting mutations that disrupt splicing regulation. Although many of the proteins involved in the regulation of AS are not fully characterised, Faustino and Cooper hypothesised that mutations affecting proteins that regulate the activity or specificity of the splicing machinery may be responsible for the widespread dysregulation of splicing observed in cancer (Faustino & Cooper, 2003). Indeed, mutations affecting the spliceosome have recently been discovered in myelodysplastic syndromes (MDS), and more rarely in AML, and these are addressed later in the discussion.

With the advent of whole genome sequencing and RNA-Seq technology, widespread dysregulation of splicing is now being recognised in human cancer, possibly occurring through dysregulation of the spliceosome machinery and subsequent selection of cells bearing transcripts favourable to maintenance of the neoplastic phenotype (Beerenwinkel *et al*, 2007; Xu & Lee, 2003). For example, AS can lead to loss of normal protein levels causing reduced tumour suppressor function, or generate neomorphic proteins that can function as oncogenes (Venables, 2004).

One of the best-characterised examples of the role AS plays in tumourigenesis is the extensive alternative splicing of CD44. CD44 encodes a transmembrane glycoprotein that can act as a tumour suppressor and is involved in cell proliferation, migration, adhesion and trafficking (Cheng & Sharp, 2006). It has 10 constitutive exons and 10 variable exons (encoded between constitutive exons 4 and 7), the inclusion or exclusion of which have significant consequences for downstream signalling. For instance, in human glioblastoma cells, the CD44v6 isoform (that contains variable exon 6) can bind to hepatocyte growth factor (HGF) and the tyrosine kinase receptor c-Met and activate downstream Ras signalling (Orian-Rousseau *et al*, 2002). This in turn alters CD44 splicing, favouring inclusion of variable exon 6, to form a feed-forward loop that generates the high levels of Ras signalling that are essential to cell growth (Cheng *et al*,

2006; Orian-Rousseau *et al*, 2002). It is not known whether this signalling axis also exists in AML cells, but expression of higher levels of the CD44v6 transcript in AML, correlates with poor survival, and a high proportion of AML patients also co-express HGF/MET (Kentsis *et al*, 2012; Legras *et al*, 1998).

As well as CD44, many alternatively spliced gene transcripts have been identified in AML such as CD13 (Dybkaer *et al*, 2001), RUNX1 (Miyoshi *et al*, 1995), PTPN6 (Beghini *et al*, 2000), PML (Pandolfi *et al*, 1992), and the α and β-intracytoplasmic chains of the granulocyte-macrophage colony stimulating factor (GM-CSF) receptor (Chopra *et al*, 1996; Gale *et al*, 1998). Although the relative contribution of many of these isoforms to the pathogenesis of AML remains to be determined, an alternatively spliced transcript detected in ALL has been shown to directly induce tumourigenesis *in vivo* in a mouse model. AS of the *Ikaros* gene produces non-DNA binding isoforms that have dominant-negative activity, are found at high level in childhood ALL, and induce T-ALL when expressed in murine marrow and transplanted into irradiated recipients (Hahm *et al*, 1994; Sun *et al*, 1999; Winandy *et al*, 1995).

5.1.5 Notch as a tumour suppressor

The consequences of Notch signalling are highly cell context-dependent. Whilst upregulation of Notch activity has been associated with a diverse range of different cancer types besides haematological malignancies, including breast, colorectal and pancreatic cancer, Notch has been shown to have a tumour suppressor role in several other cellular tissues (Lobry *et al*, 2011). Conditional *Notch-1* knockout mice develop cutaneous basal cell carcinomas, and mice chronically treated with gamma-secretase inhibitors (GSIs) are highly prone to carcinogen-induced skin cancers (Nicolas *et al*, 2003). Similarly, transgenic mice engineered to express dominant-negative *Maml-1* (that acts as a pan-Notch inhibitor) in the epidermis develop squamous cell carcinomas (SCC) of the skin (Proweller *et al*, 2006). One of the interesting features of the tumour-suppressor function for the Notch pathway in this context is that Notch may have a non-cell autonomous role in disease pathogenesis, as mice with conditional deletion of *Csl* in the mesenchymal compartment develop epithelial tumours (Hu *et al*, 2012). NOTCH-1 may also have a tumour suppressor function in the pathogenesis of hepatocellular carcinoma (HCC), as expression of *ICN-1* causes cell-cycle arrest in HCC cell lines, and

treatment with GSIs can accelerate tumour onset in a mouse model of HCC (Viatour *et al*, 2011).

More recently, loss-of-function mutations of *NOTCH-1* and *NOTCH-2* have been described in 75% of cutaneous SCCs, 12.5% of lung SCCs, and nearly 20% of head and neck SCCs (Agrawal *et al*, 2011; Wang *et al*, 2011). The majority (73%) of the mutations identified in SCC of the skin were G>A transitions typical of UV-induced DNA damage. There is also data to suggest that loss of *Tp53* activity in murine epithelial cells contributes to cellular transformation to SCC through the down-regulation of *Notch-1* (Lefort *et al*, 2007).

The presence of inactivating mutations of *NOTCH-1* and *NOTCH-2* in lung cancer is in contrast to the Notch pathway activation that had previously been described in 40% of patients with non-small cell lung cancer (NSCLC) (which includes the adenocarcinoma and SCC subtypes), either through activating mutations of *NOTCH-1* (10% of cases) or loss of expression of the negative regulator Numb (30% of cases) (Westhoff *et al*, 2009). Whilst the description of oncogenic and tumour suppressor functions for Notch in the same tumour subtype, namely SCC of the lung, is seemingly contradictory and requires further experimental validation, it suggests that the consequences of Notch pathway activation/inactivation may be influenced by the genetic landscape of the individual tumour.

The majority of studies of haematological malignancies have implicated an oncogenic role for mutations in the Notch pathway. For example, besides T-ALL, Notch activation through somatic mutation has also been described in chronic lymphocytic leukaemia (CLL) and mantle cell lymphoma (MCL) (Fabbri *et al*, 2011; Kridel *et al*, 2012; Puente *et al*, 2011; Robinson *et al*, 2011), and several of these studies have shown that *NOTCH-1* mutation status is associated with patient outcome. However, the role of Notch signalling in myeloid malignancies remains contentious. Over-expression of *NOTCH-1* mRNA has been identified by microarray analysis of samples from a subset of AML patients with poor prognosis (Bullinger *et al*, 2004). Tribbles homolog 2 (*Trib2*), a direct Notch target, enhances proliferation of myeloid progenitors and *Trib2* transgenic mice develop AML through degradation of Cebp/α, a transcription factor that is essential for myeloid differentiation (Keeshan *et al*, 2006). Notch signalling is also integrally

involved in the rare subset of patients with acute megakaryocytic leukaemia (AMKL) initiated by the *OTT-MAL* fusion transcript, whereby CSL is directly activated by OTT-MAL independently of NOTCH (Mercher *et al*, 2009). However, Notch signalling may also have a tumour suppressor role in myeloid cells. Loss of Notch signalling through compound deletion of *Notch-1* and *-2*, or through homozygous inactivation of the gamma-secretase complex by deletion of Nicastrin ($Ncstn^{-/-}$) in the haematopoietic compartment of mice leads to a myeloproliferative disease akin to chronic myelomonocytic leukaemia (CMML) (Klinakis *et al*, 2011).

5.1.6 CSL isoforms

CSL is a transcription factor that binds to NOTCH, MAML and DNA, and the structure of this complex is discussed in the general Introduction, sections 1.4.3.4 and 1.4.3.5. At the time of undertaking the practical work described in this thesis, four main isoforms were annotated on both the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org) websites, and are listed below. All are identical from exon 3 onwards, with the same amino acid sequence, but differ in their 5' regions and translational start sites. They are referred to here according to the NCBI annotation as isoforms 1-4. Figure 5.2 shows how the four isoforms differ in their 5' region and their putative transcriptional start sites.

- Isoform 1 NM005349 (ENST 342295; RBPSUH-201)
    - 12 exons
    - 2388 bp and 500 residues
- Isoform 2 NM015874 (ENST 348160; RBPSUH-202)
    - 11 exons
    - 2316 bp and 517 residues
- Isoform 3 NM203283(ENST 345843; RBPSUH-001)
    - 11 exons
    - 2272 bp and 485 residues
- Isoform 4 NM203284 (ENST 342320; RBPSH-003)
    - 12 exons
    - 2500 bp and 486 residues

5.2 AIMS

As described in chapter 3, no *NOTCH-1* mutations were detected in diagnostic samples in a substantial cohort of AML patients. To determine whether other components of the Notch signalling pathway were affected, all coding exons from CSL were screened by DHPLC analysis in samples from 100 AML patients from the same cohort. No mutations were detected (data not shown). Expression of CSL was therefore investigated by RT-PCR in primary AML patient samples, which led to the discovery of an additional unexpected smaller PCR product, often as the predominant band. Further analysis identified it as a novel alternatively spliced isoform. This chapter presents results of studies investigating the functional and prognostic significance of this isoform in AML.

5.3 MATERIALS AND METHODS

5.3.1 RNA samples from cell lines, patients and normal subjects

RNA samples were made from 13 different cell lines (ALL-SIL, HEK-293T, HL60, Jurkat, KG-1, Monomacs-6, NALM-6, NB4, RAJI, SEM, THP-1, U2OS and U937) and from CD34+ cells isolated as described in section 5.3.11 using the Trizol method as described in Materials and Methods, section 2.3.3. RNA samples from neutrophils (n=6) and T cells (n=6) purified from haematologically normal individuals and from diagnostic peripheral blood or bone marrow of 278 AML patients treated on the UK MRC/NCRI AML10 (n=15), 12 (n=27) or 15 (n=236) trials were available from the departmental tissue bank. All samples were anonymised. A panel of RNA from normal donor tissues was obtained from Ambion. cDNA was generated by RT-PCR as described in Chapter 2, section 2.3.4.

5.3.2 Identification of 5' CSL isoforms

In order to identify the predominant 5' *CSL* isoform expressed in primary AML cells, such that the relevant isoform could be cloned for functional studies, two separate RT-PCR reactions were performed. The first reaction used primers 2bF and 5R (Table 5.1), which amplify CSL isoforms 2 (expected product 208bp) and 4 (392bp) (Figure 5.2), and the second reaction used primers 1aF and 5R, which amplify CSL isoforms 1 (400bp) and 3 (284bp). Both reactions mixes were performed with Bioline PCR reagents as

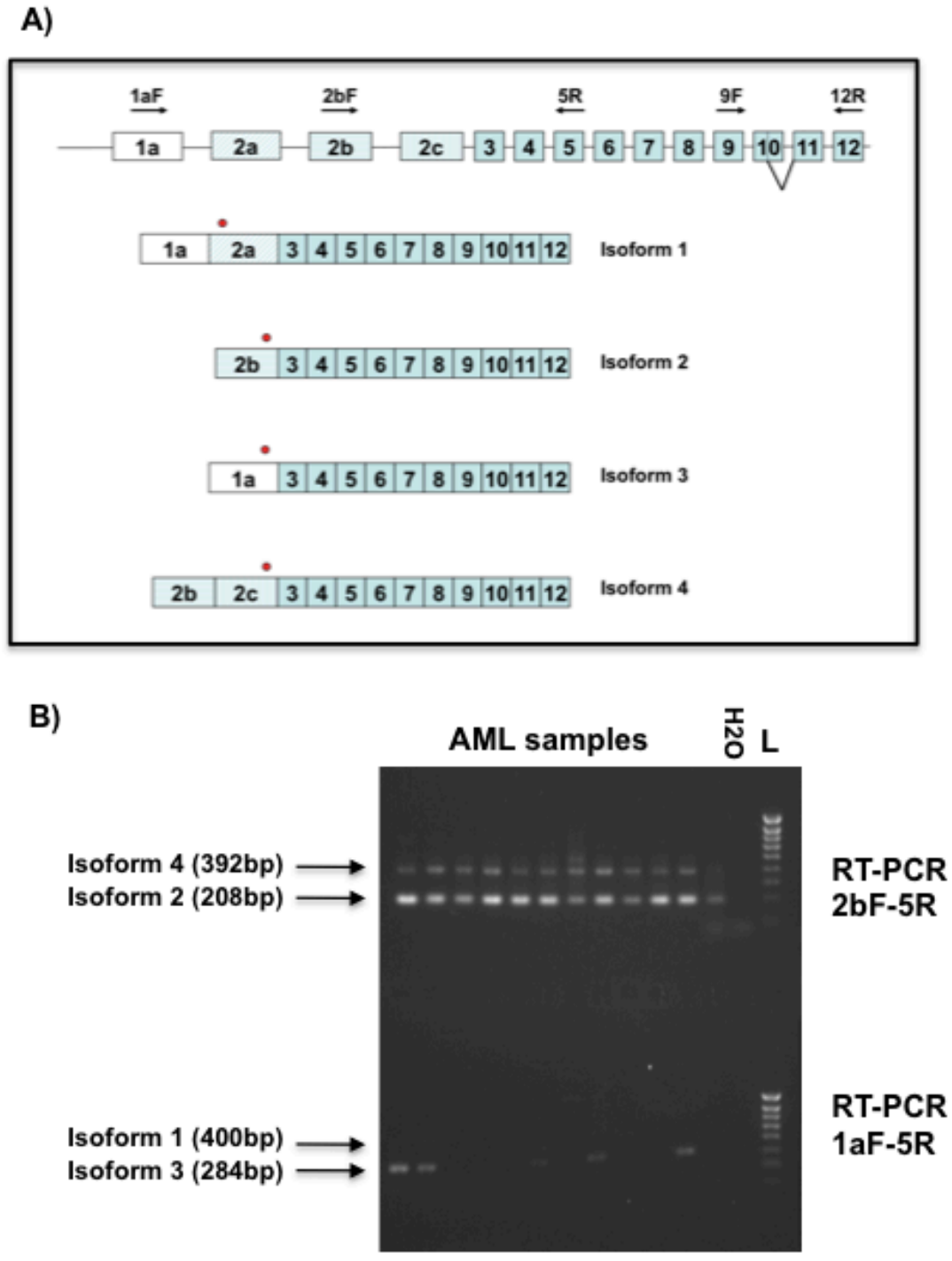

Figure 5.2 Alternative 5' CSL isoforms (A) A schematic showing annotated CSL mRNA isoforms. Coloured bars represent exons whilst the black lines signify introns. The red dots indicate predicted transcriptional start sites. The pre-mRNA is shown above the mature mRNA isoforms and the primers used in the study are shown with arrowheads. **(B)** RT-PCR results from primary AML patient samples using primers 2bF-5R (top) and 1aF-5R (bottom), suggesting that isoform 2 is the most predominant isoform in AML patients. L Hyperladder IV

described in Materials and Methods Chapter 2, section 2.3.5. PCR conditions were 95°C for 20 seconds, 60°C for 20 seconds, 72°C for 30 seconds, repeated for 32 cycles, followed by a final extension at 72°C for 5 minutes. PCR products were resolved by agarose gel electrophoresis as described in Materials and Methods Chapter 2, section 2.3.6, and are shown in Figure 5.2B.

5.3.3 Identification and quantification of *CSL-TREX*

In order to investigate the mRNA expression of *CSL* in haematopoietic cells, *CSL* exons 9 to 12 that are common to all four isoforms were amplified by RT-PCR from 2μl cDNA (diluted 1 in 5 from the original RT reaction) using BioTaq DNA polymerase (see Chapter 2, section 2.3.5) and primers CSL-9F and CSL-12R (Table 5.1) in a 20μl reaction. Cycling parameters were: denaturation at 95°C for 30 seconds, annealing at 62°C for 30 seconds, extension at 72°C for 30 seconds, repeated for 30 cycles, followed by a final 5 minute extension at 72°C. PCR products were examined by gel electrophoresis (see Chapter 2, section 2.3.6). PCR products were TOPO cloned (TOPO® TA, Invitrogen) and sequenced using M13 primers (see Chapter 2, section 2.3.8).

For accurate quantification of the two bands identified in the RT-PCR described above, size separation by fragment analysis was used with the same PCR reaction mix but with a fluorescently-labelled forward primer (CSL-9F-dye4). PCR amplification was limited to 28 cycles to avoid preferential amplification of the smaller PCR product. Optimised cycling parameters were: denaturation at 95°C for 1 minute, annealing at 62°C for 1 minute, extension at 72°C for 1 minute, with slow ramping of 0.5°C per second between cycles, followed by a final 10 minute extension at 72°C. PCR products were sized by fragment analysis (CEQ8000 Genetic Analysis System) and relative quantification was expressed as a proportion of total CSL alleles.

Table 5.1 Primer names and sequences used in the study

Primer name	Sequence 5' to 3'
CSL-1aF	5'-GTTTCGGGCGGCGAATTCCAG
CSL-2bF	5'-GTTTGTGGAAGATGGCGCCTG
CSL-5R	5'-AGATATACACAAGGAGGTGGGC
CSL-10F	5'-ATTTCAGGCCACTCCATGTCCA
CSL-10F-d4	5'-dye4-ATTTCAGGCCACTCCATGTCCA
CSL-12R	5'-ATGTCTGGGACGACACAGAGC
MP2932	5'-AACGTAGCCAATTGGGATCCGCCACCATGGCGCCTGTTGTGACAGGGA
MP2938	5'-GTTTCCGCACGGCACTCGAGTTAGGATACCACTGTGGCTGTAGATGATGTG
MP3739	5'-GGCCAGACTCCAGGATCCGCCACCATGGACTACAAAGACCATGACGGTGATTATAAAGATC
MP3740	5'-CCGCCGCTCTTGTCATCGTCATCCTTGTAATCGATGTCATG
MP3741	5'-ATGACGATGACAAGAGCGGCGGCGGCAGCGCGCCTG
MP3742	5'-TGAGAAAGGCACAAATACATTCTTTCTGTATC

5.3.4 Statistical analysis of patient outcome

The UK AML MRC trials are registered at http://www.controlled-trials.com under ISRCTN17833622 (AML12) and ISRCTN17161961 (AML15) (Burnett *et al*, 2011; Burnett *et al*, 2010). Consent was obtained from all patients at trial entry according to the Declaration of Helsinki. The trials were approved by the Wales Multicentre Research Ethics Committee and each participating institution's ethics review committee. Median follow-up was 40 months. The following definitions were used: overall survival (OS) is the time from randomisation to death; for remitters, disease-free survival (DFS) is the time from remission to first event (either relapse or death in CR), and the cumulative incidence of relapse (CIR) is the cumulative probability of relapse, with death as a competing risk. Mantel-Haenszel test for trend and chi-squared tests were used to test for differences in clinical and demographic data by *CSL-TREX* levels. Kaplan-Meier life tables were constructed for survival data and compared by means of the log-rank test. Multivariate Cox models were used to analyse CIR, DFS and OS. Mutational status of *FLT3* internal tandem duplications (*FLT3-ITD*), *FLT3*-tyrosine kinase domain (*FLT3-TKD*), and nucleophosmin 1 *NPM1* was available, as previously reported (Gale *et al*, 2008; Kottaridis *et al*, 2001; Mead *et al*, 2007).

5.3.5 Creation of constructs

The pcDNA3-ICN-1 plasmid was a kind gift from Jon Aster of Brigham and Women's Hospital Boston. For the creation of inserts for cloning, the proof-reading enzyme Phusion DNA polymerase (Finnzymes) was used for all PCR reactions. PCRs were set up in 50µl reactions with a mix consisting of 10µl Phusion buffer (which includes magnesium chloride at 1.5mM final concentration), 200µM each dNTP (final concentration), 500nM each primer (final concentration), 200ng template DNA, and 1U Phusion DNA polymerase. Primer sequences for the cloning reactions are given in Table 5.1. PCR cycling conditions were an initial denaturation step of 98°C for 2 minutes followed by 35 cycles of 98°C for 30 seconds, 64°C for 30 seconds, 72°C for 30 seconds per kb, followed by a final extension of 10 minutes at 72°C. Products were run out on a low-melt agarose gel, cut-out and extracted using a gel purification kit (Qiagen), and eluted in 100µl DDW. The relevant vector and PCR products were each digested with the appropriate restriction enzymes (New England Biolabs) in a 100µl reaction mix containing 5µl each enzyme, 10µl NEB buffer, and 1µl 100mg/ml BSA, left at 37°C for 2 hours. Vector and insert DNA were then ligated using the Quick Ligation Kit (New England Biolabs) in a 20µl reaction mix containing 1.5µl Quick ligase enzyme, 10µl Quick ligase buffer and a 1:1 molar ratio of vector:insert. A negative control reaction was set up using the same reaction mix but with digested vector alone and no insert. The reaction was left at room temperature for 10 minutes and then 2µl was used to transform DH5α cells as described in Chapter 2, section 2.3.9. Colonies were harvested and expanded (see Chapter 2, section 2.3.9) and plasmid DNA was prepared using a Maxi prep kit (Qiagen). The entire open reading frame of each construct was sequenced using the CEQ8000 sequencer.

5.3.5.1 Creation of MSCV-CSL-WT and MSCV-CSL-TREX constructs

MSCV-CSL-WT and MSCV-CSL-TREX plasmids were constructed as shown in Fig. 5.3. Full-length isoform 2 of CSL (which was considered the predominant isoform, according to its intensity by RT-PCR, Figure 5.2B) was amplified from normal T-cell cDNA for generation of CSL-WT, and from a primary AML cDNA sample for generation of CSL-TREX, using primers CSL-2bF and CSL-12R. Cycling parameters were: initial single denaturation step at 98°C for 2 minutes, denaturation at 98°C for 20

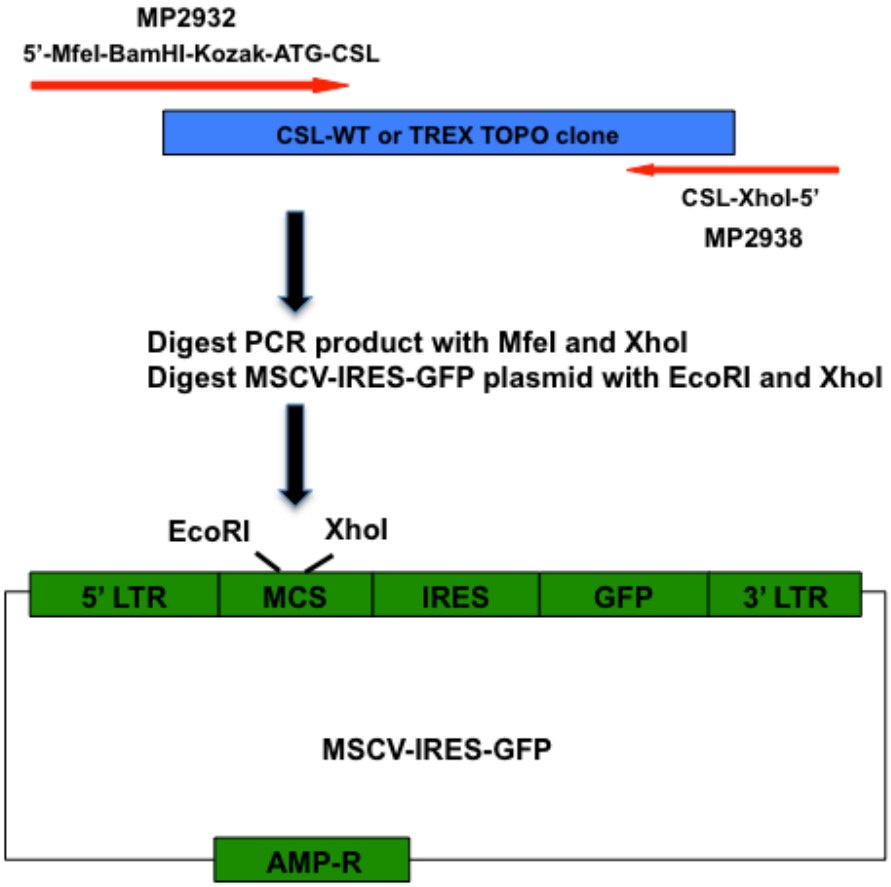

Figure 5.3 A schematic of the cloning strategy used to generate MSCV-CSL-WT and MSCV-CSL-TREX retroviral constructs. TOPO clones containing full-length isoform 2 CSL-WT or CSL-TREX were used as template for a PCR reaction using forward primer MP2932 which has an MfeI digest site at the 5'end, and reverse primer MP2938 which has an XhoI site at the 5'end. The PCR products were digested with MfeI and XhoI, the MSCV-IRES-GFP vector with EcoRI and XhoI, and the insert was ligated to create MSCV-CSL-WT and MSCV-CSL-TREX. Note MfeI and EcoRI overhangs are compatible. LTR long terminal repeats; MCS multi-cloning site; IRES internal ribosomal entry site; AMP-R ampicillin resistance gene.

seconds, annealing at 64°C for 20 seconds, extension at 72°C for 90 seconds, repeated for 35 cycles, followed by a final 10 minute extension at 72°C. PCR products were examined by gel electrophoresis. The PCR products were TOPO cloned and sequenced to identify the correct insert and to exclude Taq-errors, and were then used as template in subsequent reactions.

Insertion into the cloning site of the retroviral MSCV-IRES-GFP vector requires EcoRI/XhoI restriction digest sites. However, CSL has an internal EcoRI site and therefore an MfeI site, which has compatible overhangs with EcoRI but would not cut the CSL sequence, was substituted in the cloning strategy. A CSL coding sequence fragment was generated using a forward oligonucleotide (MP2932) containing 5' MfeI and BamHI digest sites, a Kozak sequence and ATG, followed by a region complementary to the 5' CSL sequence, and a reverse primer (MP2938) containing a 5' XhoI digest site followed by a region complementary to the 3' CSL sequence, using Phusion DNA polymerase and conditions as described above. The 1.5 Kb fragment was gel purified and digested with MfeI and XhoI, and then gel purified a second time. The MSCV-IRES-GFP plasmid was digested with EcoRI and XhoI and gel purified. The digested products were mixed and ligated at a vector: insert 1:1 molar ratio. The ligation was then used to transform DH5α cells and prepare purified plasmid DNA as described in Materials and Methods, section 2.3.9.

5.3.5.2 Creation of MSCV-FLAG-CSL-WT and MSCV-FLAG-CSL-TREX (5' triple FLAG sequence) constructs

A schematic representation of the cloning strategy to create 5' triple-FLAG tagged CSL constructs is shown in Figure 5.4. A triple FLAG construct was provided by Martin Pule in the department; this contained a Kozak-ATG sequence prior to three consecutive FLAG sequences. This was amplified by PCR (conditions given above, section 5.3.5) using forward oligonucleotide MP3739 (containing a BamHI restriction digest site in the 5' end) and reverse oligonucleotide MP3740 (containing a sequence complimentary to the start of the CSL transcript in the 5' end), to create a 100bp fragment coded BamHI-Kozak-ATG-FLAGx3; this was gel purified and termed fragment A.

Using MSCV-CSL-WT as template, a 5' CSL fragment was generated by PCR (conditions given above, section 5.3.5) using oligonucleotides MP3741 and MP3742, which codes for CSL up to amino acid 281 (prior to the alternative splice site) termed fragment B. Because 30% of primer MP3740 and MP3741 are complementary to each other, fragments A and B were ligated by mixing an equal ratio of DNA from each in a PCR reaction and amplifying with outer primers MP3739 and MP3742 (Figure 5.4), to create fragment C. This could then be cloned into MSCV-CSL-WT or MSCV-CSL-TREX using the BamHI site introduced in oligonucleotide MP2932 (Figure 5.3) and the internal CSL EcoRI site. MSCV-CSL-WT and MSCV-CSL-TREX and fragment C were digested by EcoRI and BamHI (5μl each enzyme, 10μl EcoRI buffer, 1μl 100mg/ml BSA, 30μl PCR product and 49μl DDW, incubated at 37°C for 2 hours) and ligated using the Quick ligase kit to produce MSCV-FLAG-CSL-WT and MSCV-FLAG-CSL-TREX. The ligation was then used to transform DH5α cells and make purified plasmid DNA as described in section 2.3.9.

5.3.5.3 Creation of pHR-SIN-CSL-WT-IRES-GFP and pHR-SIN-CSL-TREX-IRES-GFP constructs

The pHR-SIN-eGFP vector is a third generation self-inactivating vector based on the pHR-SIN-SE vector originally described by Demaison, designed for efficient transduction and expression in CD34+ cells (Demaison *et al*, 2002), and was a kind gift from Professor Adrian Thrasher at UCL Institute of Child Health. It has a central HIV polypurine tract that increases vector titres by about 10 fold, a woodchuck hepatitis virus, post-translational regulatory element (WPRE) that increases stability of mRNA, and a spleen focus forming virus (SFFV) promoter, which has strong promoter activity in haematopoietic cells. MSCV-CSL-WT and MSCV-CSL-TREX, or the triple FLAG-tagged versions, were digested with BamHI and NotI and cloned into the BamHI and NotI sites of pHR-SIN-eGFP using the Quick ligase kit (as described above in section 5.3.5). The ligation was then used to transform DH5α cells and make purified plasmid DNA as described in section 2.3.9.

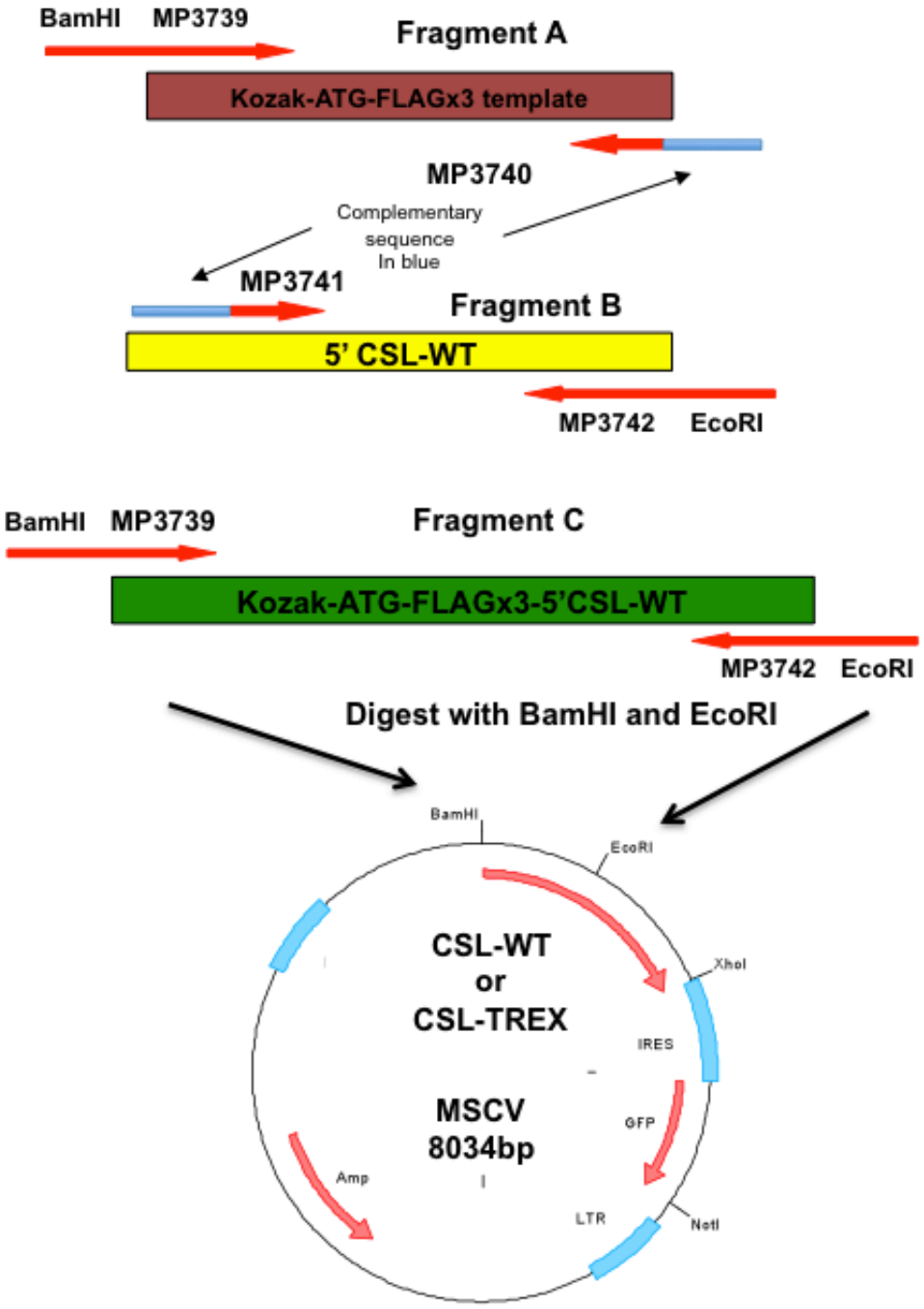

Figure 5.4 A schematic of the cloning strategy used to generate 5' triple FLAG tagged MSCV-CSL-WT and MSCV-CSL-TREX constructs. PCR products termed fragment A and fragment B were mixed and used as template in a PCR reaction using outer primers MP3742 and MP3739, leading to ligation of the fragments due to primer complementarity. Fragment C was cloned into the BamHI and EcoRI sites of MSCV-CSL-WT or MSCV-CSL-TREX.

5.3.6 Western blotting

Nuclear lysates from primary AML samples or cell lines were made from $5x10^6$ cells using a nuclear extraction kit (Active Motif) (see chapter 2.4.2). Proteins were resolved by electrophoresis on 10% SDS-polyacrylamide gels and transferred electrophoretically to a nitrocellulose membrane (see chapter 2.4.3). The membranes were pre-blocked for 1 hour in 5% skimmed milk dissolved in PBS containing 0.1% Tween (PBS-T). Immunoblotting was carried out as described in chapter 2, section 2.4.5 with the following primary antibodies, all diluted in PBS-T and 5% skimmed milk, and left overnight at 4°C: rat monoclonal anti-RBP-JK antibody diluted 1 in 500 (clone SIM-2ZRBP3, T6719, Cosmobio, Japan), mouse monoclonal anti-Notch-1 antibody specific to cleaved Notch-1 diluted 1 in 2000 (clone mN1A, N-6786, Sigma Aldrich, Poole, UK), mouse monoclonal anti-FLAG M2 antibody diluted 1 in 2000 (Sigma Aldrich, Poole, UK). Secondary horse-radish peroxidise (HRP)-linked IgG antibodies to mouse, rabbit or rat were diluted 1 in 10,000 in PBS-T (all from Cell Signalling Technology, Hitchin, UK) and left on the membranes for 30 minutes.

5.3.7 Co-immunoprecipitation experiments

HEK-293T cells were seeded at $1x10^5$/ml in a 10cm tissue culture plates in 10mls DMEM supplemented with 10% FCS and Penicillin-Streptomycin (100U/ml and 100μg/ml respectively) (D10). The following day, provided that the cells were at approximately 70% confluent, they were transfected by adding 18μl Genejuice reagent (Novagen) mixed with 800μl DMEM and a total of 8μg DNA and left at 37°C in 5% CO_2 for 48 hours. The negative control plate was transfected with 4μg pcDNA3-ICN-1 and 4μg MSCV-CSL-WT (no FLAG tag), the positive control plate with 4μg pcDNA3-ICN-1 and 4μg MSCV-FLAG-CSL-WT, and the test plate with 4μg pcDNA3-ICN-1 and 4μg MSCV-FLAG-CSL-TREX . The cells were then washed 2 times with PBS, scraped from the plate, transferred into a 15ml conical tube and pelleted at 400g at room temperature. After removal of the supernatant, the cells were resuspended in 200μl NP40 lysis buffer containing protease inhibitor cocktail, transferred into 1.5ml Eppendorf tubes, incubated on ice for 15 minutes and then pelleted at 16,000g at 4°C. An aliquot of 50μl of the supernatant was added to 50μl SDS loading buffer as the total extract control and stored at -80°C. The remainder of the supernatant was added to a tube containing 40μl FLAG-M2 agarose bead slurry (Sigma Aldrich) and rotated at 4°C for 2 hours. The

beads were then washed 6 times with ice-cold NP40 buffer containing protease inhibitor cocktail, and proteins were eluted with 40µl SDS loading buffer, and run on SDS-PAGE gels and Western blotted as described in Chapter 2, section 2.4.

5.3.8 Luciferase Reporter assays

Dual-luciferase reporter assays to assess Notch signalling were performed as described below using the Promega Dual Luciferase Kit with the adherent osteosarcoma cell line U2OS because of its low baseline Notch activity. The cell line was confirmed as wild type for *NOTCH-1* and *FBXW7* by DHPLC analysis as described in chapter 3. The Notch reporter plasmid CBF1x10-luc, which contains 10 TGGGAA motifs upstream of luciferase, was kindly given by Graeme McKenzie (McKenzie *et al*, 2006). In this assay, luciferase activity is proportional to the degree of Notch activity in the transfected cells, and the transfection efficiency and cell number are controlled for by co-transfection of a renilla luciferase construct. The kit contains a lysis buffer, a firefly luciferin substrate (LARII), and Stop-and-Glo, which quenches the firefly luciferin and provides a substrate for the renilla luciferase.

For the reporter assays, 4 x 10^4 U2OS cells in 500µl D10 per well were seeded the day before transfection in 24-well plates. For optimum transfection efficiency, cells were only used if they were 60-70% confluent at the time of transfection. Transfection and luminometry were performed in triplicate as follows:

a. Firstly, 0.75µl Genejuice (Novagen) was added to 20µl serum free medium, mixed by vortexing and left for 5 minutes at room temperature. The luciferase reporter and Renilla plasmids were added to the mastermix so that their concentrations were kept equal in each well. The DNA quantities used were 100ng CBFx10-luc reporter construct, 30ng renilla, 2.5ng pCDNA3.1-ICN1 and 50ng MSCV-CSL-WT or 50ng MSCV-CSL-TREX in a total volume of 9.25µl. Total DNA was made up to 250ng per well by adding 67.5ng MSCV-GFP. The mix was incubated for 15 minutes at room temperature.
b. The total volume of transfection mixture (30µl) was then added dropwise to each well, and the cells incubated for 48 hours at 37°C in 5% CO_2 before being harvested.

c. The culture supernatant was removed and the cells were washed once with PBS, then 100µl 2x Passive Lysis Buffer (Promega Dual Luciferase Kit) was added per well, and the plate left on a shaker for 15 minutes at room temperature.
d. To measure luminescence, 10µl lysate was then added to 50µl firefly reagent (LARII reagent) in a clear polystyrene tube, pipetted up and down 10 times, and the luciferase luminescence measured in a luminometer to assess expression of the reporter construct. Stop-and-Glo reagent (50µl) was then added, pipetted up and down 10 times, and the renilla luminescence measured.
e. The relative luminescence was then calculated to control for cell number and transfection efficiency. The ratio of luciferase to renilla luminescence was first calculated for each well, and then divided by the mean luciferase:renilla value of the three wells transfected with empty plasmid.

5.3.9 Lentiviral transduction of ALL-SIL cells and proliferation assays

ALL-SIL cells were obtained from DSMZ (Leibniz Institute, Germany) and grown in RPMI containing 10% FCS and penicillin-streptomycin (R10). Lentiviral supernatants were produced as described in Chapter 2, section 2.2. The cells were incubated overnight at 37°C in 5% CO_2 in a 12-well plate at a density of $1x10^5$/ml with 2mls of R10 per well. Cells were transduced by spinoculation at 400g for 60 minutes, a method that has been shown previously to enhance infectivity of lentiviral vectors by 40 fold (O'Doherty *et al*, 2000), with lentiviral particles expressing either GFP, FLAG-CSL-WT or FLAG-CSL-TREX at a multiplicity of infection (MOI) of 1 viral particle per cell. Such an MOI has relatively low efficiency but is unlikely to lead to cells with multiple viral integrations and exceptionally high transgene expression. The plates were then incubated at 37^{o}C in 5% CO_2 and after 24 hours the cells were washed, re-suspended in fresh R10 and returned to the incubator. At 48 hours from transduction, cells with equivalent GFP-positivity were selected by FACS and protein expression was determined by intracellular flow as described below in section 5.3.10. For proliferation assays, GFP-positive transduced cells were seeded at $1x10^5$/ml in triplicate in 96-well plate format. For ALL-SIL-GFP control cells, the GSI L685,458 (Sigma) was used at 1µM compared to DMSO control treated cells. Over a 7 day period, cell number was counted using an MTS assay:

20µl MTS tetrazolium compound (Promega, Southampton, UK) was added to each well, the plates were then incubated at 37°C in 5% CO_2 and after 4 hours the absorbance at 490nm was read.

5.3.10 Intracellular flow for anti-FLAG

To assess expression of the FLAG-tagged CSL-WT or CSL-TREX proteins, $2x10^5$ transduced and GFP-sorted ALL-SIL cells, as described in section 5.3.9, were spun down at 400g for 5 minutes, resuspended in 100µl fix-perm solution (BD Bioscience Intracellular flow kit), left for 15 minutes on ice, then pelleted and washed once in perm-wash (supplied in the kit). The cells were resuspended in 50µl perm-wash containing a 1:200 dilution of anti-FLAG M2 antibody (Sigma Aldrich), left for 30 minutes on ice, washed once in perm-wash and resuspended in 50µl perm-wash containing a 1:200 dilution of APC-anti-mouse secondary antibody. As negative controls, FLAG-CSL-WT and FLAG-CSL-TREX transduced cells were stained with primary antibody only, or secondary anti-mouse APC antibody without primary antibody. Samples were then analysed on a flow cytometer APC expression.

5.3.11 CD34+ cell selection and transduction

The use of surplus CD34+ cells from G-CSF-mobilised donors was approved by the Joint UCL/UCLH Ethics Committee on Human Research, REC study number 97/0282/amended. All donors had signed a consent form for the anonymous use of their cells in research. Magnetic cell selection of G-CSF-mobilised haematopoietic progenitor cells was performed using anti-CD34+ magnetic beads and MACS® cell separation columns and reagents (Miltenyi Biotec) according to the manufacturer's guidelines. In brief, cells were incubated with anti-CD34+ beads, loaded onto a MACS® cell separation column, washed and eluted. Following selection, a small aliquot of cells was tested for purity by flow cytometry using an anti-CD34-APC antibody (BD Pharmingen). If the cells were <95% CD34-positive, they were put through a second round of magnetic selection. The purified cells were washed in PBS and resuspended in Stem Pro-34 serum-free medium (SFM) containing 20ng/ml stem cell factor (SCF), 20ng/ml FLT3-ligand (FL), 45ng/ml thrombopoietin (TPO) at $1x10^6$/ml/well in a 24-well plate and incubated for 12 hours at 37°C in 5% CO_2 prior to lentiviral transduction.

Lentiviral supernatants (see section 2.2) were thawed on ice and added to the prepared cells at an MOI of 40. One non-transduced well was left as a control. The plates were

spinoculated at 400g for 60 minutes at room temperature then incubated at 37°C in 5% CO_2 in the above cytokine mix for 48 hours prior to assessment of transduction efficiency by flow cytometry for GFP.

5.3.12 Clonogenic assays of transduced CD34+ cells

To assess the consequences of CSL-WT or CSL-TREX overexpression on the clonogenic potential of CD34+ cells, purified CD34+ cells were transduced with a GFP, CSL-WT or CSL-TREX-expressing lentivirus as described in section 5.3.11. At 48 hours after transduction, the cells were sorted for GFP by FACS, counted and seeded into 12-well plates at 100 cells per well in 2mls methocellulose containing 10ng/ml SCF, 25ng/ml GM-CSF, 25ng/ml G-CSF, 30ng/ml interleukin-3 (IL-3), and 3U/ml erythropoietin (EPO). White cell colonies were counted after 2 weeks incubation at 37°C in 5% CO_2. The experiment was repeated 4 times using 4 separate donors and at least 12 wells were counted per vector.

5.4 RESULTS

5.4.1 Identification of a novel alternatively spliced isoform of CSL

By RT-PCR of the region spanning exons 9-12, *CSL* was expressed in haematologically normal T-cells (n=6), neutrophils (n=6), and CD34+ cells (n=9), as well as in all primary AML samples tested (n=278). While a single band was visualised in the neutrophils and T-cells, an unexpected smaller band was present in the majority of AML patients, often as the more abundant product (Figure 5.5A). By TOPO cloning and sequencing, the smaller band was identified as an alternatively spliced transcript containing a 78bp deletion of the C-terminus of exon 10, originating from a cryptic 5' donor splice site within the coding region of exon 10 that spliced in-frame to the start of exon 11 (Figure 5.5B). Unusually, the donor splice site originates at a GC, rather than a GT, donor site. This alternatively spliced transcript was termed *CSL-TREX* for Truncated Exon X. The deleted amino acids are part of a highly conserved region (Figure 5.5C). The observed deletion was modelled on the crystal structure of full-length wild-type CSL (CSL-WT) by Dr Steven Blacklow of Brigham and Women's Hospital, Boston, USA, and this indicated that it would delete the conserved bridge strand connecting the beta-trefoil

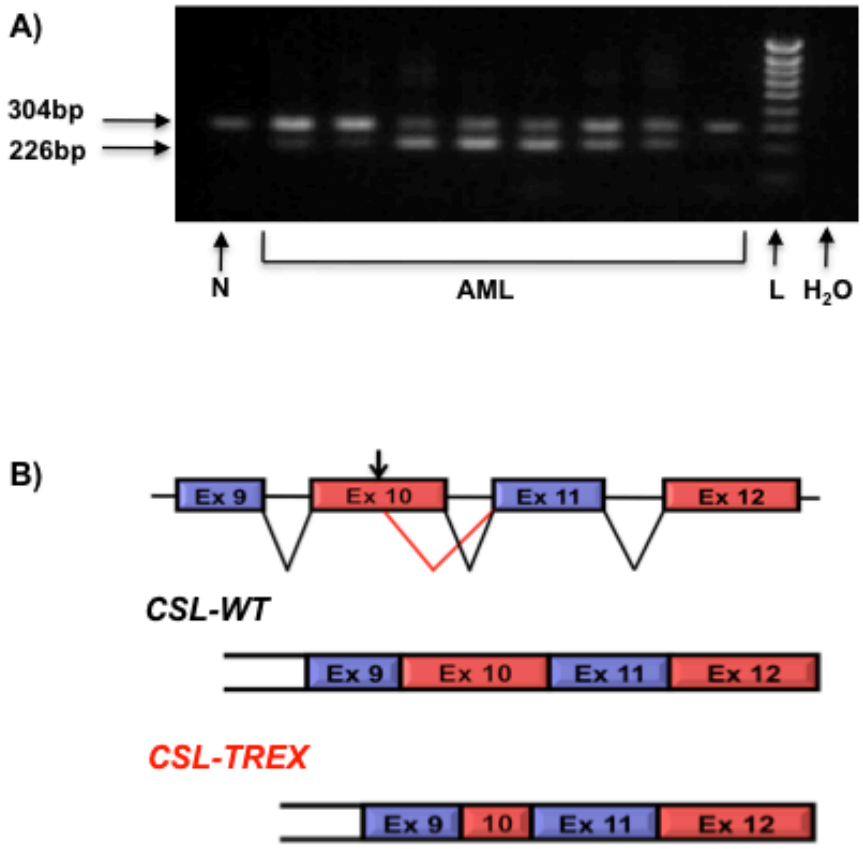

Figure 5.5 *CSL* is alternatively spliced at a cryptic donor site within exon 10 in samples from AML patients. (A) Agarose gel electrophoresis of RT-PCR products spanning *CSL* exons 9-12 from normal neutrophils (N) and 8 primary AML patient samples. *CSL-WT* product 304bp, *CSL-TREX* 226bp. L, Hyperladder IV. **(B)** Schematic showing splicing patterns at the 3' end of CSL. CSL-TREX splices in-frame from the cryptic donor site in exon 10 (downward arrow) to the start of exon 11. **(C)** The deleted amino acids of the *CSL-TREX* isoform are shown as the shaded region, as compared to the orthologous protein sequences from different species.

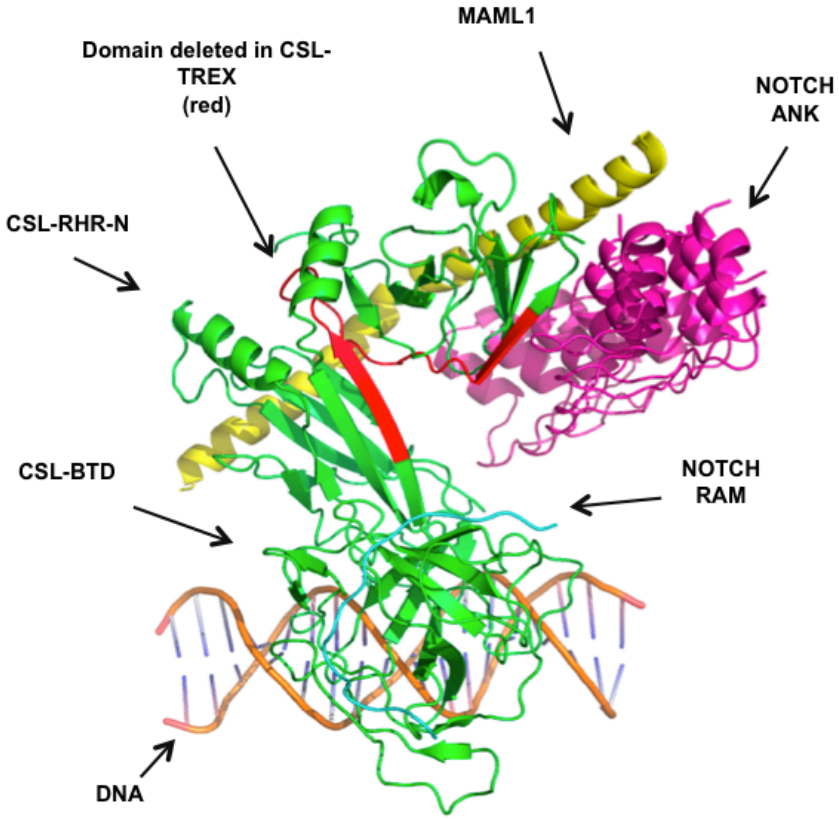

Figure 5.6 Modelling the CSL-TREX deletion on the crystal structure of the CSL-Notch-MAML1-DNA activation complex. Crystal structure modelling of the CSL-ICN-MAML-DNA activation complex showing MAML1 (yellow), CSL (green), DNA (orange) and ICN (purple). The spliced out portion of CSL is shown in red. Images were created by Dr Steven Blacklow from published crystallography data (Nam et al., 2006; Wilson and Kovall, 2006). RHR-N, Rel-homology region N-terminal; BTD, beta-trefoil domain; ANK, ankyrin repeat domain of Notch; RAM, RBPJ-associated molecule domain

domain to the C-terminal Rel homology region, and would be likely to interfere with the structural integrity of the protein (Figure 5.6) (Nam *et al*, 2006; Wilson & Kovall, 2006).

5.4.2 Relative quantification of *CSL-TREX*

The ratio of *CSL-TREX* to *CSL-WT* transcripts was quantified using the same exon 9-12 PCR as above but with a fluorescently labelled primer. The products were analysed by fragment analysis, with peaks for *CSL-WT* of 304bp and *CSL-TREX* of 226bp, as depicted in Figure 5.7A. Results were expressed as the percentage of *CSL-TREX* alleles as a proportion of total *CSL* alleles. The assay was highly reproducible. Repeated testing of two cDNA samples gave results of 64.1% ± 2.1% (n=12) and 73.1% ± 1.8% (n=7)

(mean ± 1 standard deviation) respectively (Figure 5.7B). As some of the AML trial samples can take up to 3 days to arrive in the laboratory from the time of bone marrow aspiration or phlebotomy, stability of the relative proportion of *CSL-TREX* transcripts was assessed in two fresh samples over time. RNA was made from half the sample within 6 hours of bone marrow aspiration, and the other half was left at room temperature for 72 hours before RNA extraction. There was no difference in the relative levels of *CSL-TREX* over the three day period, as shown in Figure 5.7C.

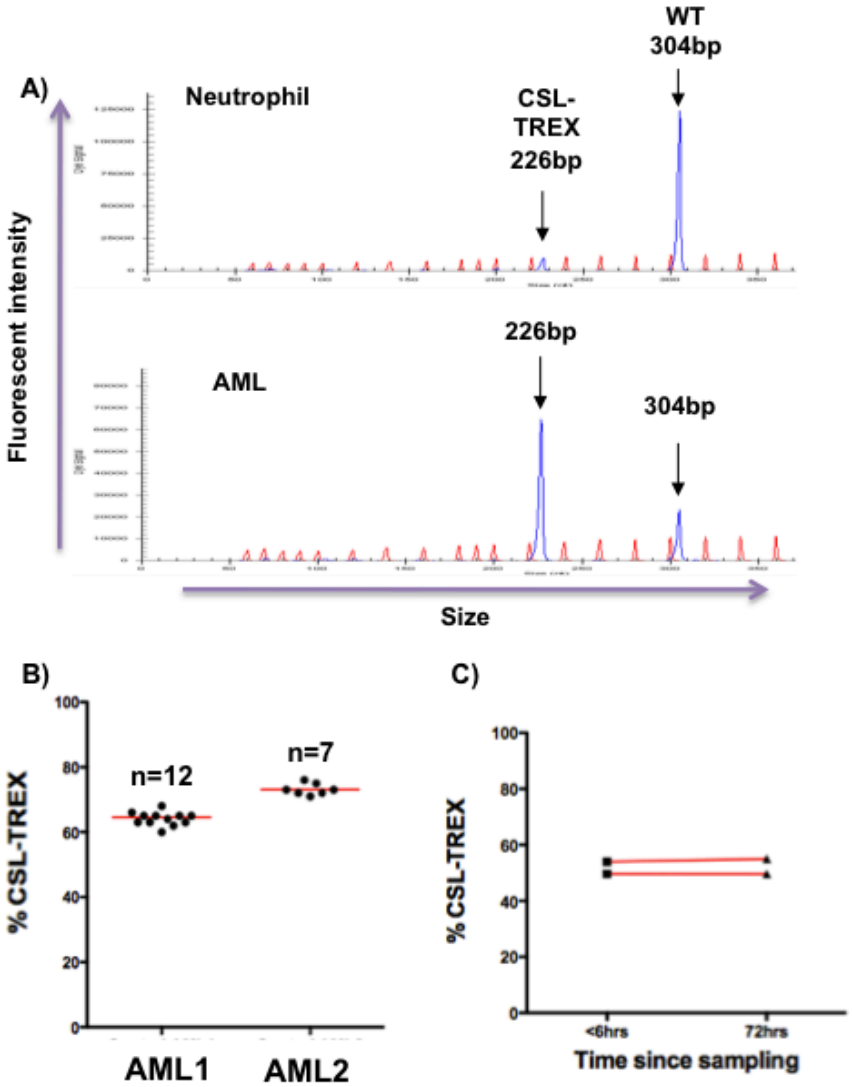

Figure 5.7 Quantification of CSL-TREX by fluorescently labelled PCR and fragment analysis. **(A)** Fragment analysis traces showing presence of the CSL-WT peak at 304bp and CSL-TREX peak at 226bp in a representative example from a normal neutrophil sample and an AML patient. **(B)** Reproducibility of the assay using two AML samples. **(C)** Stability of the relative proportion of the CSL-TREX isoform in two AML samples where RNA was prepared within 6 hrs or 72 hrs after sampling from the patient.

5.4.3 *CSL-TREX* is the predominant CSL isoform in many AML patients and is present in normal undifferentiated haematopoietic cells

Using fragment analysis, relative *CSL-TREX* mRNA levels were quantified in diagnostic samples from 278 young adult AML patients. Most patients expressed the *CSL-TREX* isoform, and in the majority it predominated over the *CSL-WT* isoform (median 57% *CSL-TREX* as a proportion of total *CSL* transcripts, range 0-100%) (Figure 5.8A). Normal terminally differentiated haematopoietic cells expressed low *CSL-TREX* levels (neutrophils (n=6), median 3%, range 0-8%; T-cells (n=6) median 0%, range 0-4%), whereas normal CD34+ selected haematopoietic cells expressed substantial levels (n=9, median 22%, range 4-41%) (Figure 5.8A), although they were significantly lower than in AML patient samples ($P < 0.001$). *CSL-TREX* levels were less than 10% in RNA analysed from 20 different normal tissues from the Ambion RNA panel, and from 13 cell lines (Figure 5.8B).

5.4.4 *CSL-TREX* levels decrease in AML blasts upon differentiation

To further explore the relationship of cell differentiation stage and levels of *CSL-TREX*, RNA available from primary AML blasts from six patients pre- and post- in vitro culture in the presence of IL-3, G-CSF and GM-CSF to induce myeloid differentiation was studied (Gale *et al*, 1998). At day 10 of culture there was a mean of 87% differentiated cells, range 50-98.5%. *CSL-TREX* levels markedly decreased in all six samples, with a mean difference before and after differentiation of 30.0% ($P<0.001$, paired t-test) (Figure 5.8C). These results indicate that high levels of *CSL-TREX* transcripts are a particular feature of myeloid leukaemic cells, moderate levels are present in undifferentiated haematopoietic cells, and low levels in differentiated T-cells and myeloid cells.

5.4.5 A band corresponding to CSL-TREX is expressed at the protein level in AML patients

To assess if *CSL-TREX* transcripts produced protein, endogenous *CSL* was immunoblotted from nuclear lysates obtained from six different leukaemia cell lines and 13 primary AML samples. Lysates from HEK-293T cells transfected with MSCV-CSL-WT were used as a size control. Only a single band corresponding to the expected size for CSL-WT was detected in the cell lines (Figure 5.9A), which had <10% *CSL-TREX* transcripts (Figure 5.8B). A smaller band corresponding to the predicted size of the CSL

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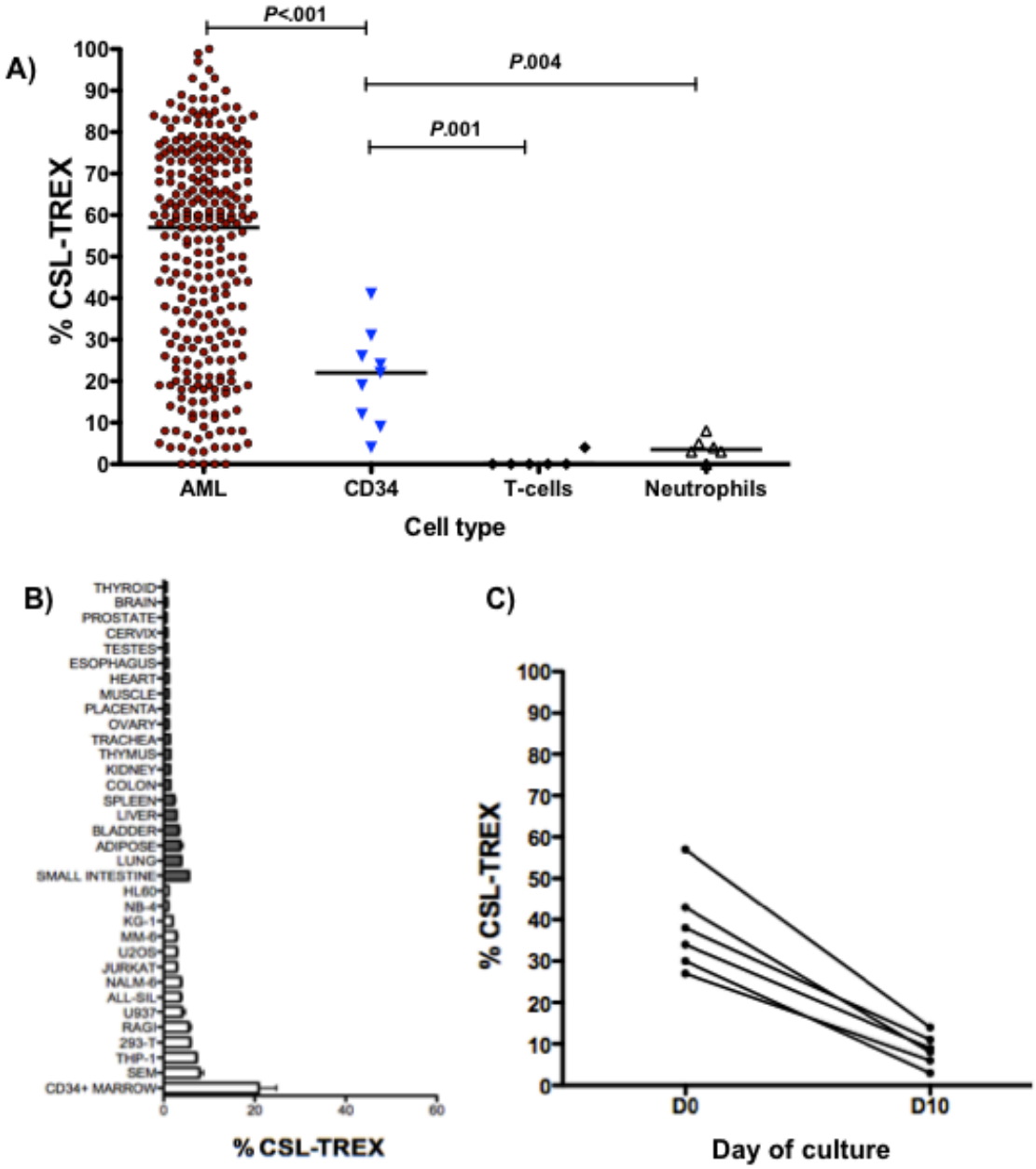

Figure 5.8 Quantification of *CSL-TREX* by fragment analysis in AML patients as compared to normal haematopoietic cells and tissues. **(A)** Scatter plot showing the relative percentage of *CSL-TREX* transcripts in samples from young adult AML patients, normal CD34+ cells, neutrophils and T-cells. Horizontal bars represent median levels. **(B)** Median percentage of *CSL*-TREX transcript levels from normal human tissues (each RNA sample pooled from 3 different individuals), normal CD34+ cells (n=9) and a variety of cell lines, as quantified by fragment analysis. **(C)** Percentage of *CSL-TREX* transcripts in primary AML blasts before and after *in vitro* differentiation with IL-3, GM-CSF and G-CSF. Data is shown for 6 independent samples.

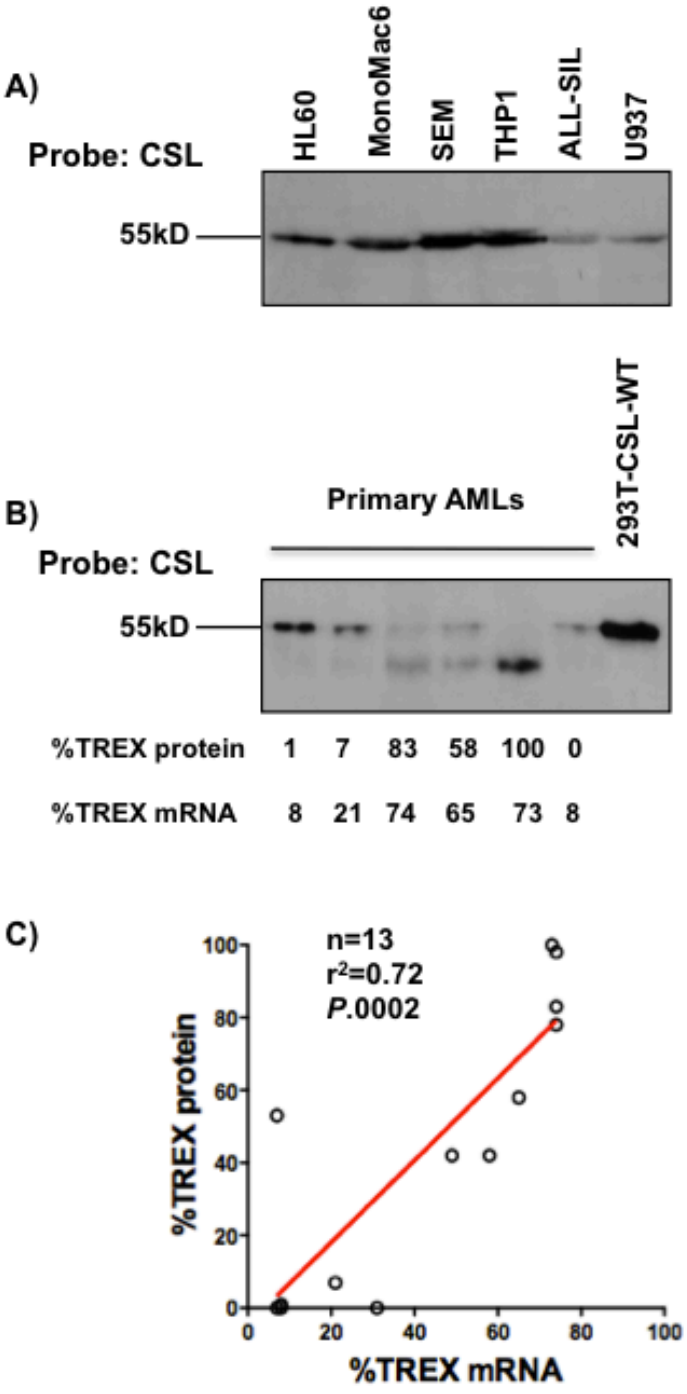

Figure 5.9 *CSL-TREX* protein expression in cell lines and primary AML samples. **(A)** Western blots of CSL in nuclear lysates from a variety of cell lines and **(B)** from primary AML cells (lanes 1-6). A whole cell lysate from MSCV-CSL-WT transfected 293T cells was included for size comparison (lane 7). The relative percentage of CSL-TREX protein (from densitometry) and cDNA (from fragment analysis) is shown below each lane. **(C)** Correlation of relative *CSL-TREX* transcripts with protein levels in 13 AML samples.

TREX isoform was also observed in 9 of the 13 primary AML samples studied, in one case as the only product (Figure 5.9B, lane 5). The relative amount of CSL-TREX isoform as a proportion of total CSL protein was quantified by densitometry and showed a significant correlation with transcript levels (n=13, r^2=0.72, P= 0.0002) (Figure 5.9C).

5.4.6 *CSL-TREX* levels have prognostic significance in AML

In order to assess biological relevance, the clinical features and outcome of the 278 young adult AML patients were analysed according to levels of *CSL-TREX.* There were no obvious cut off values (Figure 5.10A); the data was therefore analysed for groups divided into quartiles (Q1, *CSL-TREX* 0-28%; Q2, 29-57%; Q3 58-73%; Q4 74-100%). There was no association between *CSL-TREX* level and AML FAB subtype, age or sex, but higher levels were significantly correlated with a lower presenting white cell count (WCC, P=0.02, Pearson test for correlation) (Table 5.2). CSL-TREX was not associated with a particular cytogenetic subgroups, including APML. There was no association between CSL-TREX levels and rates of CR (odds ratio [OR] per quartile 0.79, 95% confidence intervals [CI] 0.57-1.09, P=0.14), resistant disease (OR per quartile 0.80, CI 0.55-1.16, P=0.20), or induction death (OR per quartile 0.79, CI 0.44-1.42, P=0.40), (Table 5.3). Patients with higher *CSL-TREX* levels had lower rates of relapse (CIR 31% vs 51% for highest [Q4] vs lowest [Q1] quartiles respectively at 5 years, hazard ratio [HR] 0.82, CI 0.68-0.99, P=0.04) (Figure 5.10B), improved DFS (63% vs 51% at 5 years, HR per quartile 0.77, CI 0.62-0.96, P=0.02), and improved OS (66% vs 45%, HR 0.82, CI 0.69-0.96, P=0.01) (Figure 5.10C, Table 5.3). Overall, 24% of patients (65 of 271 tested) had a *FLT3-ITD*, 10% (28 of 266 tested) a *FLT3-TKD* mutation and 34% (85 of 247 tested) an *NPM1* mutation. There was no association between *CSL-TREX* levels and genotype for *FLT3-ITD* (mean *CSL-TREX* levels in *FLT3-ITD*$^+$ vs *FLT3-ITD*$^-$ samples, 52% vs 50%, P=0.54), *FLT3-TKD* (42% vs 52%, P=0.07) or *NPM1* (55% vs 48%, P=0.09) that might account for the differences observed in survival according to *CSL-TREX* levels.

Table 5.2 Clinical and demographic characteristics of patients in the total AML cohort with follow up.

Demographic	Total (%)	Lowest quartile	Quartile 2	Quartile 3	Highest quartile	p-value for trend
% TREX		0-28%	29-57%	58-73%	74-100%	
Total per quartile	278	70	70	74	64	
Age						0.9
<15	3 (1%)	1 (1%)	0 (0%)	2 (3%)	0 (0%)	
15-29	38 (14%)	12 (17%)	7 (10%)	12 (16%)	7 (11%)	
30-39	38 (14%)	7 (10%)	11 (16%)	11 (15%)	9 (14%)	
40-49	86 (31%)	19 (27%)	26 (37%)	19 (26%)	22 (34%)	
50-59	86 (31%)	25 (36%)	17 (24%)	23 (31%)	21 (33%)	
60+	27 (9%)	6 (9%)	9 (13%)	7 (9%)	5 (8%)	
Sex						0.9
Female	138 (50%)	36 (51%)	32 (46%)	38 (51%)	32 (50%)	
Male	140 (50%)	34 (49%)	38 (54%)	36 (49%)	32 (50%)	
Secondary						0.6
No	259 (93%)	66 (94%)	63 (86%)	69 (93%)	61 (95%)	
Yes	19 (7%)	4 (6%)	7 (14%)	5 (7%)	3 (5%)	
Performance Status						0.08
WHO 0	165 (59%)	37 (53%)	40 (57%)	51 (69%)	37 (58%)	
WHO 1	89 (32%)	24 (34%)	25 (36%)	16 (22%)	24 (38%)	
WHO 2	14 (5%)	3 (4%)	5 (7%)	4 (5%)	2 (3%)	
WHO 3	7 (3%)	4 (6%)	0 (0%)	2 (3%)	1 (1%)	
WHO 4	3 (1%)	2 (3%)	0 (0%)	1 (1%)	0 (0%)	
WCC,						0.02
<10	119 (45%)	28 (40%)	30 (43%)	34(46%)	27 (43%)	
10-19.9	31 (13%)	10 (14%)	9 (13%)	7 (9%)	15 (24%)	
20-49.9	50 (18%)	9 (13%)	12 (17%)	16 (22%)	13 (21%)	
50-99.9	33 (12%)	6 (10%)	8 (11%)	12 (16%)	7 (11%)	
100+	33 (12%)	16 (23%)	11 (16%)	5 (7%)	1 (1%)	
Cytogenetics						0.8
Favourable	69 (28%)	21(33%)	16 (25%)	17 (27%)	15 (27%)	
Intermediate	153 (62%)	40 (62%)	36 (58%)	38 (61%)	39 (70%)	
Adverse	23 (10%)	3 (5%)	11 (17%)	7 (12%)	2 (3%)	
APL						0.7
No	246 (88%)	61 (87%)	63 (90%)	64 (86%)	58 (91%)	
Yes	32 (12%)	9 (13%)	7 (10%)	10 (14%)	6 (9%)	

WCC $x10^9/L$

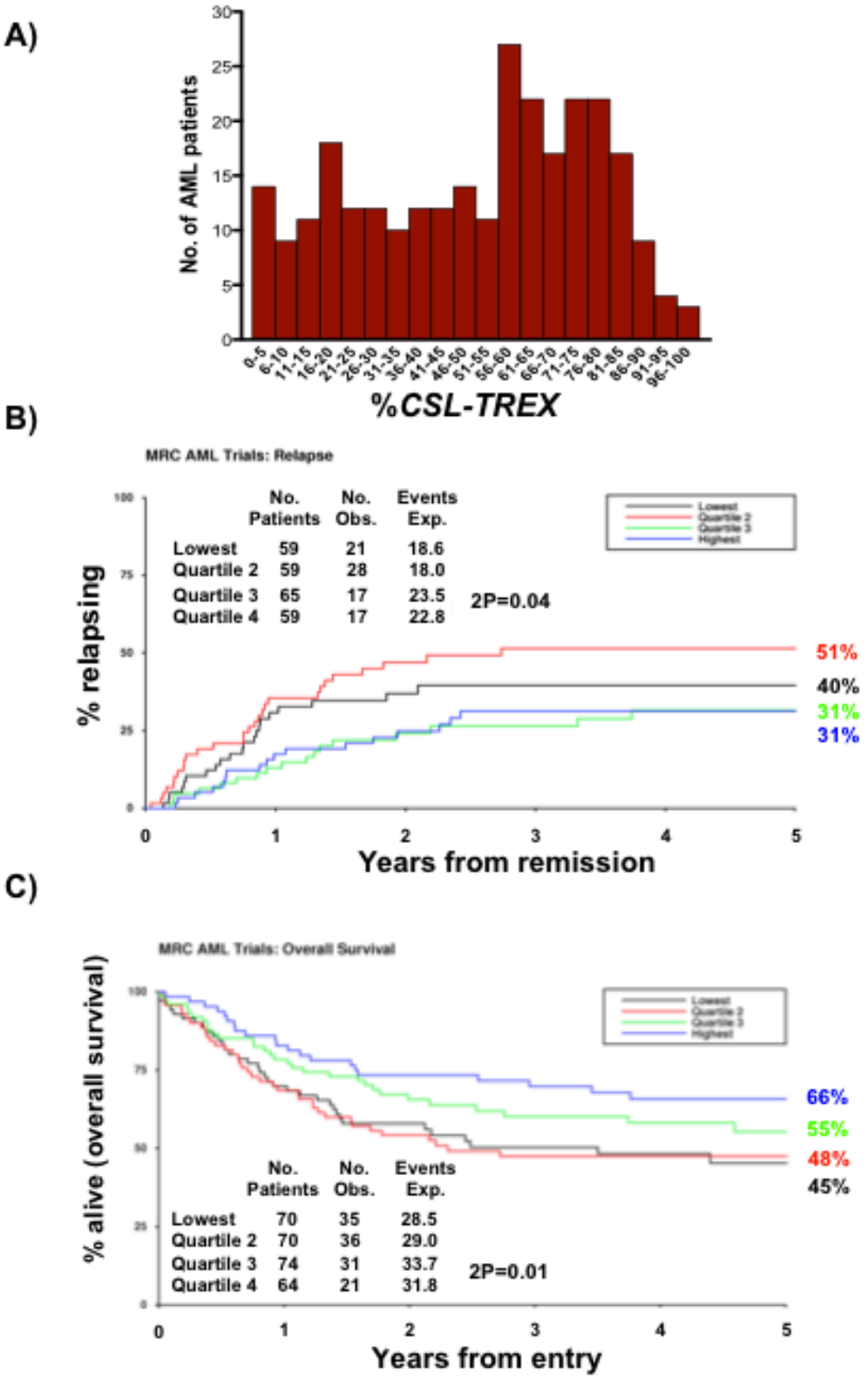

Figure 5.10 Prognostic outcome according to *CSL-TREX* levels in AML patients treated on the AML MRC trials. (A) Centile chart showing distribution of *CSL-TREX* levels as a proportion of total CSL transcripts in the 278 AML patient samples as quantified by fragment analysis. **(B)** Relapse rate and **(C)** Overall survival of young adult AML patients treated on the AML MRC trials stratified according to quartile levels of *CSL-TREX*

Table 5.3 Outcome of AML patients according to *CSL-TREX* levels

Outcome	Lowest quartile	Q2	Q3	Highest quartile	Unadjusted OR per quartile (CI)	Adjusted OR per quartile (CI)
CR/CRi	84%	84%	88%	92%	0.79 (0.57-1.09) p=0.14	0.76 (0.53-1.11) p=0.16
RD	11%	11%	8%	6%	0.80 (0.55-1.16) p=0.2	0.70 (0.44-1.12) p=0.14
ID	4%	4%	4%	2%	0.79 (0.44-1.42) p=0.4	0.98 (0.53-1.81) p=0.9
DFS (5yrs)	51%	43%	63%	63%	0.77, (0.62-0.96) p=0.02	0.79 (0.61-1.04) p=0.09
OS (5yrs)	45%	48%	55%	66%	0.82 (0.69-0.96) p=0.01	0.83 (0.69-1.00) p=0.05
CIR (5yrs)	51%	40%	31%	31%	0.82 (0.68-0.99) p=0.04	0.90 (0.72-1.13) p=0.4

Adjusted analyses use level as quartile and are adjusted for age, secondary disease, performance status, WCC and cytogenetics. CR Complete remission; Cri Complete remission with incomplete blood count recovery; RD Resistant disease; ID Induction Death; DFS Disease free survival; OS Overall survival; CIR Cumulative incidence of relapse. CI Confidence intervals

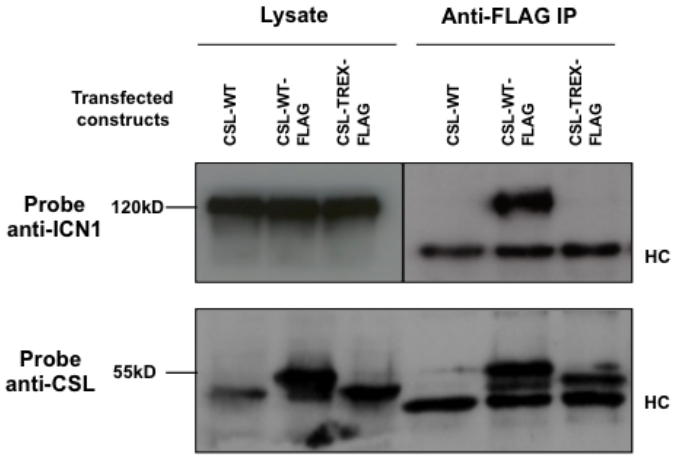

Figure 5.11 CSL-TREX cannot bind to ICN-1. Western blots of lysates (lanes 1-3) or anti-FLAG immunoprecipitates (lanes 4-6) of HEK-293T cells transfected with FLAG-tagged CSL constructs were probed with an anti-Notch-1 antibody (N6786, upper panel) or anti-CSL antibody (T6719, lower panel). HC, Heavy chain. The experiment was performed twice.

5.4.7 CSL-TREX is unable to bind NOTCH-1

To investigate the biological function of CSL, full-length isoform 2 (the predominant form expressed in haematopoietic cells) (Figure 5.1) was cloned into an MSCV retroviral vector. To assess whether CSL-TREX retained the capacity to bind to ICN-1, a co-immunoprecipitation experiment was performed in HEK-293T cells (Figure 5.11). The lower panel shows expression of the transfected CSL constructs using the anti-CSL antibody. The upper left panel shows a Western blot using a NOTCH-1 antibody and

indicates that the ICN-1 plasmid was equally transfected and expressed in all three cell lysates. The upper right panel (for FLAG-immunoprecipitated proteins) shows a strong ICN-1 band co-immunoprecipitating with FLAG-CSL-WT but not with FLAG-CSL-TREX. There was no apparent binding with non-FLAG-tagged CSL-WT. This is consistent with the findings from the crystal structure modelling that suggested CSL-TREX would lose structural integrity.

5.4.8 CSL-TREX does not have dominant-negative activity

In order to explore the possibility that the CSL-TREX isoform can activate Notch signalling in the absence of ICN, CSL constructs were expressed in U2OS cells (which have low endogenous Notch signalling) together with a luciferase reporter construct containing 10 CSL-binding sites. Neither CSL-WT nor CSL-TREX alone were able to significantly activate the reporter (Figure 5.12). To assess whether CSL-TREX could exert a dominant-negative effect on Notch signalling, CSL-WT or CSL-TREX were co-expressed with ICN-1 in the same assay. ICN-1 markedly activated luciferase activity on its own, and co-expression of CSL-TREX had no effect on the reporter activity induced by ICN-1, suggesting it does not exert a dominant-negative effect on Notch signalling (Figure 5.12). Of note, there was no interaction between CSL-WT and ICN-1 in these assays, suggesting that endogenous CSL abundance was not dose-limiting.

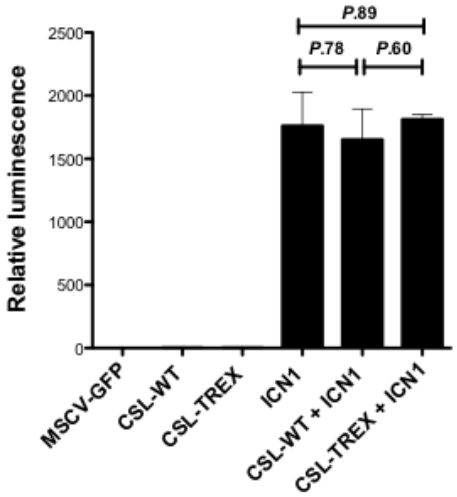

Figure 5.12 CSL-TREX does not show dominant-negative activity. Luciferase reporter assay of Notch activation in U2OS cells transfected with vector (MSCV-GFP), CSL and ICN-1 constructs. Data is shown for three independent experiments performed in triplicate and analysed by Student's t-test.

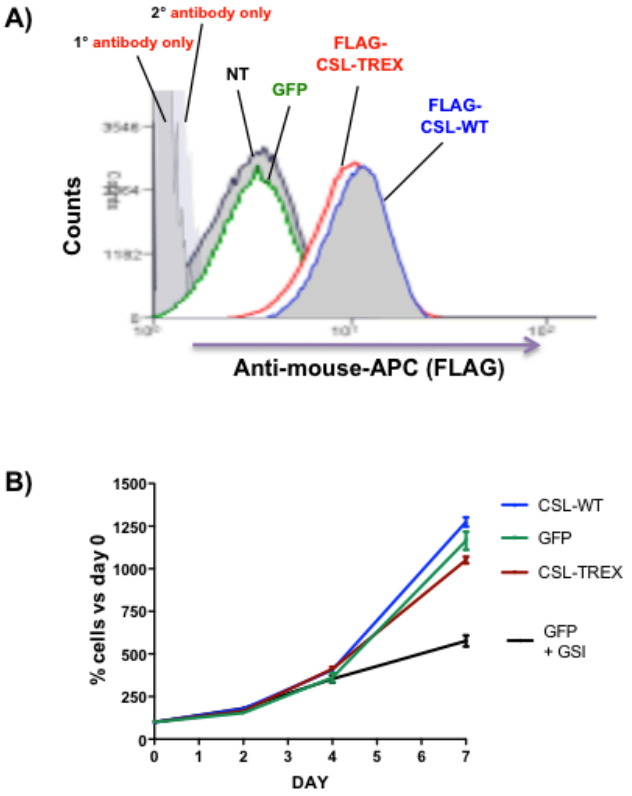

Figure 5.13 CSL-TREX does not show dominant-negative activity on growth of the Notch dependent T-ALL cell line ALL-SIL. (A) Intracellular flow cytometry of ALL-SIL cells transduced with GFP vector alone, FLAG-CSL-WT, FLAG-CSL-TREX, or non-transduced (NT) and stained with anti-FLAG primary (1^o) and APC-anti-mouse secondary (2^o) antibodies. Negative control cells were transduced with FLAG-CSL-WT and stained with primary but not secondary, or secondary but not primary antibody. Intensity of APC on X-axis versus cell count on the Y-axis**. (B)** Proliferation of transduced ALL-SIL cells after FACS sorting for GFP and then treated with vehicle (DMSO) or the γ-secretase inhibitor (GSI) L685,458 at 1μM. Cell numbers shown as percentage of cells at day 0.

This finding was corroborated by proliferation experiments in the T-ALL cell line ALL-SIL, which has activating mutations in *NOTCH-1* (L1575P in the HD-N domain, 2478X in the PEST domain) and requires sustained NOTCH-1 activity for optimal growth (Weng *et al*, 2004). ALL-SIL cells were stably transduced with a lentiviral vector expressing either GFP vector alone, FLAG-CSL-WT or FLAG-CSL-TREX, and cells of equal GFP intensity were selected by FACS 48 hours after transduction. Expression of FLAG, as assessed by intracellular flow cytometry, showed equivalent protein expression in FLAG-CSL-WT and FLAG-CSL-TREX transduced cells (Figure 5.13A). The rate of proliferation was not significantly affected by stable expression of either CSL-WT (mean fold increase in cell number at day 7 was 11.9±0.8 [mean ± 1SD]) or CSL-TREX (10.5±0.2) compared to GFP transduced cells (11.6±0.5, P=0.81 and 0.18

respectively), whereas treatment of GFP-transduced ALL-SIL cells with a GSI significantly retarded growth (5.8±0.3, P=0.002) (Figure 5.13B). This indicates that CSL-TREX does not exert a dominant-negative effect on the endogenous CSL-WT isoform /Notch signalling interaction.

5.4.9 CSL-WT, but not CSL-TREX, inhibits clonogenic potential of CD34+ cells

To assess whether CSL-TREX has an impact on the clonogenic potential of CD34+ cells that could be relevant to AML pathogenesis, normal CD34+ cells were transduced with lentivirus expressing GFP vector control, CSL-WT, or CSL-TREX, FACS sorted 48 hours later and seeded at 100 cells per well into methocellulose containing IL-3, G-CSF, GM-CSF, TPO, and EPO. The mean infection efficiency (± SD), calculated as the percentage of cells expressing GFP 48 hours after transduction was 50.4% (± 18.1%), 8.4% (± 5.2%) and 10.6% (± 6.4%) for GFP, CSL-WT and CSL-TREX infected CD34+ cells respectively (Figure 5.14A). In an attempt to obtain cells with equal transgene expression, cells of equivalent GFP fluorescent intensity were selected by FACS. The cells were left at 37°C for 2 weeks then colony-forming units (CFUs) were counted by light microscopy. The experiment was performed four times from four separate donors with a minimum of 12-wells per condition. The cell number required in these assays limited the ability to assess protein expression of the transgene, therefore 10,000 of the sorted cells were harvested for RNA and expression of the appropriate transduced gene confirmed by fragment analysis (Figure 5.14B). The presence of a CSL-WT peak in the GFP control was due to endogenous CSL, and the particular example shown had levels of CSL-TREX below the level of detection. Transduction with CSL-WT led to a marked and consistent reduction in CFUs compared to GFP control (mean 3.1 colonies/100 CD34+ cells for CSL-WT versus 10.6 for GFP control, P<0.0001) and CSL-TREX (8.5, P=0.001), while no significant difference was observed for cells transduced with CSL-TREX compared to GFP control (8.5, P=0.31) (Figure 5.14C). Furthermore, colonies from CSL-WT transduced CD34+ cells appeared smaller than control or CSL-TREX transduced cells, although this was not formally quantified (Figure 5.14D). This suggests that the CSL-WT isoform has a negative impact on the cell growth of haematopoietic progenitors, and raises the possibility that alternative splicing to a loss-of-function isoform may be a means of reducing CSL dosage in these cells.

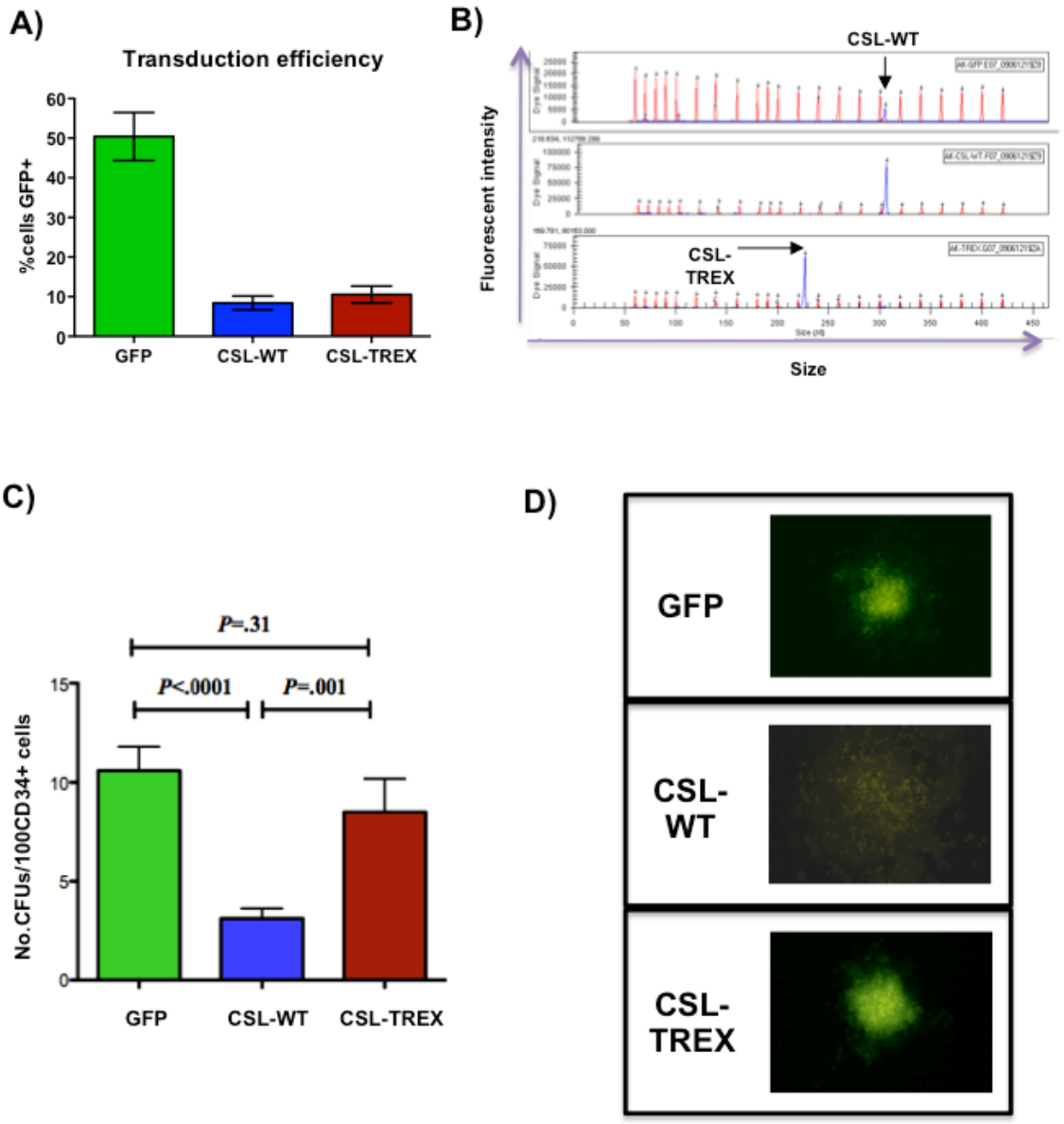

Figure 5.14 CSL-WT, but not CSL-TREX, markedly inhibits the clonogenic potential of CD34+ cells. (A) Transduction efficiency of CD34+ transduced cells as measured by flow cytometry. Data represents the mean ±SEM from 4 different donors **(B)** Expression of CSL isoforms in RNA from GFP-sorted CD34+ cells after lentiviral transduction with GFP vector only (upper panel), CSL-WT (middle panel), or CSL-TREX (lower panel) **(C)** Number of white cell colony forming units (CFUs) obtained from purified CD34+ cells, FACS sorted for GFP and cultured for 2 weeks in methocellulose and cytokines. Data is from 4 different CD34+ donor samples and was analysed by Student's t-test. **(D)** Representative images of individual colonies visualised by fluorescent microscopy at 2 weeks.

5.5 DISCUSSION

The analysis of CSL expression in AML by RT-PCR led to the serendipitous discovery of a smaller band in the majority of AML patient samples that was not present in differentiated haematopoietic cells (Figure 5.5). Further analysis identified the smaller band as an alternatively spliced transcript of CSL that deleted the last 78bp of exon 10, that was termed *CSL-TREX* (Figure 5.5). Fragment analysis and Western blotting showed a correlation between the *CSL-TREX* isoform at the RNA and protein level (Figure 5.9), suggesting that despite the predicted loss of structural integrity, CSL-TREX forms a stable protein. Although transcript artefacts can be created by strand skipping during reverse transcription as a result of RNA secondary structure, often occurring due to high GC content within the gene (Prasov *et al*, 2010), the presence of a protein band of the expected size and correlating in intensity to that seen at the cDNA level argues against this possibility for CSL.

The CSL-TREX isoform has not been previously reported and is not included as an alternatively spliced isoform on the Ensembl database, most likely because fragment analysis showed it is a rare transcript in a library of normal tissues (Figure 5.8), and databases such as Ensembl are created from such material (Flicek *et al*, 2012). Analysis of the CSL-TREX RNA and protein sequence using a basic local alignment search tool (BLAST) failed to identify matching sequences, so it is unlikely that this transcript results from a pseudogene (Altschul *et al*, 1997). The cryptic exon 10 donor splice site occurs at an AG-GC sequence; such sites are unusual in normal splicing (0.6% of donor sites), but are over-represented in transcripts occurring through alternative splicing and occur particularly at sites that create cryptic introns from exonic sequence, as found in this case (Burset *et al*, 2000; Thanaraj & Clark, 2001).

Co-immunoprecipitation studies showed that CSL-TREX does not form a complex with ICN-1 (Figure 5.11), but this does not exclude the possibility that it is able to bind other Notch family members, MAML1 or DNA, that were not tested in these studies. Predictions from crystallography data of the ICN-MAML1-CSL-DNA quaternary complex performed by Dr Steven Blacklow suggest that CSL would not maintain sufficient structural integrity to enable binding to either Notch or MAML1. To further test the possibility that the DNA-binding domain remained in tact, a collaboration was

established with Dr Jon Aster of Brigham and Women's Hospital, Boston, USA, who performed Electrophoretic Mobility Shift Assays using the constructs prepared in this thesis to investigate the interaction of CSL-WT and CSL-TREX with a radioactively labelled probe of the HES-1 promoter. Whilst there was significant gel shift with the CSL-WT isoform to both anti-CSL and anti-FLAG antibodies, CSL-TREX did not display evidence of HES-1 promoter binding (data not shown), which suggests that CSL-TREX is also unable to bind DNA, at least at canonical target sequences.

To test the possibility that CSL-TREX retained binding activity at other promoters and acted as a dominant-negative factor with regards to Notch signalling, CSL-TREX was expressed in ALL-SIL cells, a T-ALL cell line that is dependent on maintained Notch signalling for optimal growth. CSL-TREX did not alter the growth of these cells (Figure 5.13), suggesting that this isoform does not compete for DNA-binding with the CSL-WT isoform in a dominant-negative fashion. Nor did CSL-TREX appear to have Notch-independent constitutive activity at promoter sites because CSL-TREX alone did not significantly activate a CSL-luciferase reporter construct in U2OS cells (Figure 5.12). The most striking phenotype seen in a functional assay was the marked inhibition of the size and number of CFUs in *in vitro* culture after transduction of purified CD34+ cells with CSL-WT, which was not seen in cells transduced with CSL-TREX (Figure 5.14). One possible interpretation of these findings is that CSL-WT has an inhibitory effect on the proliferation of haematopoietic progenitors, and therefore the high levels of CSL-TREX observed in AML occur as a consequence of positive selection and outgrowth of cells bearing the highest levels of CSL-TREX. Thus, taken together, the data supports CSL-TREX as a loss-of-function isoform in regards to Notch signalling. Further studies are required to address whether AML patients with higher levels of CSL-TREX have lower levels of downstream Notch targets, such as *HES-1* and *DELTEX*.

There is an emerging role for a tumour suppressor function for the Notch pathway in myeloid malignancy. Not only does inactivation of Notch signalling in the haematopoietic compartment of mice lead to a CMML-like disease (Klinakis *et al*, 2011), but exon sequencing has also identified loss-of-function mutations in 12% of patients with CMML in a variety of components of the Notch pathway (*NCSTN*, *APH1*, *MAML1* and *NOTCH2*) in 12% of patients with CMML (Klinakis *et al*, 2011). Whether such mutations also exist in AML has not yet been reported. However, activation of

Notch signalling using a Jagged-1 peptide has been shown to induce apoptosis in AML cell lines, suggesting that Notch signalling may also have a tumour suppressor role in AML (Sutphin et al, 2006).

The association between high levels of CSL-TREX and improved prognosis was independent of *FLT3-ITD* and *NPM1* mutation status, and suggests that this isoform has clinico-biological relevance. Whether this association is direct (for instance, the chemosensitivity of AML blasts is influenced by presence of the CSL-TREX isoform itself) or indirect (for instance, presence of CSL-TREX reflects disordered AS on a wider scale, and it is this that influences chemosensitivity) remains to be determined. In support of the former hypothesis, AML blasts cultured on bone marrow stroma cells that express high levels of Jagged-1 and -2 exhibit marked chemoresistance *in vitro* (Nefedova *et al*, 2008), and a recent study demonstrated that AML patients with high mRNA expression of *NOTCH-1, JAGGED-1* and *DELTA-1* have a poorer prognosis (Xu *et al*, 2011). Thus it is possible that the activation of the Notch pathway that occurs through stromal interaction or cell-to-cell contact is abrogated in AML blasts that express a high proportion of CSL-TREX, which leads to lower levels of chemoresistance and is reflected in an improved prognosis.

Since completion of this work, mutations in the spliceosome machinery have been described as frequent somatic events in myelodysplastic syndromes (MDS). Thus far the genes affected are:

1. *U2AF1*. Mutations in exons 2 and 6 affect the two zinc finger domains of this protein, with the two most frequent mutations being S34F and Q157P (Graubert *et al*, 2012; Makishima *et al*, 2012). These have been found in 8-10% of patients with MDS, and are associated with a poor prognosis (Graubert *et al*, 2012; Makishima *et al*, 2012).
2. *SF3B1*. These mutations are most commonly found in exons 14 or 15, with the K700E mutation being the most recurrent (Makishima *et al*, 2012; Papaemmanuil *et al*, 2011). They have been detected in approximately 20% of MDS patients and at highest incidence (65%) in MDS-RARS (MDS refractory anaemia with ringed sideroblasts), a subtype of MDS with the best prognosis. In AML they occur in 5% or fewer of patients, and are typically found in patients who have transformed from MDS.

3. *SRSF2*. Almost all mutations are found at position P95 (in order of frequency: P95H, P95L, P95R). They are not associated with a particular MDS subgroup and are not associated with prognostic outcome. They are also found in CMML.
4. Rare mutations affecting <2% of MDS patients have also been found in other genes that are involved in splicing including *PRPF8, LUC7L2, ZRSR2, HCFC1, SAP130, SRSF6, SON* and *U2AF26.*

The functional consequences of mutations in the spliceosome machinery await to be determined. Although *SF3B1* mutations are associated with downregulation of genes associated with mitochondrial electron transport, specific splicing defects in this pathway have not been identified (Papaemmanuil *et al*, 2011). RNA-Seq data suggests that mutations in *SF3B1, U2AF* and *SRSF2* are not associated with widespread AS, but rather alter splicing at specific genes. For instance, *U2AF1* mutations result in retention of intron 5 of the *TET2* gene and intron 6 of the *RUNX1* gene (Makishima *et al*, 2012).

What is the mechanism of AS in AML, considering mutations affecting the spliceosome machinery are relatively rare in this disease? One possibility is that mutations affecting one or more of the splicing regulators exist but have yet to be discovered. Since chromatin and histone modifications have been shown to regulate AS (Luco *et al*, 2010), and a recent study has linked exonic methylation with increases in AS (Flores *et al*, 2012), a second hypothesis is that the presence of AS occurs secondary to widespread alterations of the methylation marks and histone/chromatin state that occurs in AML (Ameyar-Zazoua *et al*, 2012). Analysis of the methylation status of the CpG dinucleotide in exon 10 or nearby introns of CSL therefore warrants further study.

It is possible that transcripts generated through AS have neomorphic functions (i.e. a novel function outside of their canonical signalling pathway); determining whether CSL-TREX has functional consequences outside of the Notch pathway would therefore be of interest. Future studies could address whether CSL-TREX has novel protein binding partners, for instance, by pull-down mass spectrometry, or altered transcriptional activity, for instance, by chromatin immunoprecipitation coupled to massively parallel DNA sequencing, as compared to the CSL-WT form.

In conclusion, a novel alternatively spliced isoform of *CSL* occurring through a cryptic donor splice site within exon 10 was identified that is the predominant isoform in many patients with AML at both the mRNA and protein level. CSL-TREX has clinico-biological relevance given that it has prognostic significance in AML. Biochemical and *in vitro* studies all support CSL-TREX as a loss-of-function variant as regards Notch signalling, although a neomorphic function relevant to AML cannot be excluded. The presence of *CSL-TREX* in normal CD34+ cells, together with the negative impact on the clonogenic potential observed with *in vitro* overexpression of CSL-WT, support a model in which CSL dosage is controlled through alternative splicing, and the high CSL-TREX levels observed in AML occur as a result of selection against the CSL-WT isoform or proliferative outgrowth of high CSL-TREX expressing cells. While somatic mutations in the splicing machinery are frequent in MDS, these mutations are uncommon in *de novo* AML (Hahn & Scott, 2012) and so are unlikely to account for the high prevalence of CSL-TREX identified here. Predominance of the CSL-TREX variant isoform in AML patients together with its inability to support canonical Notch signalling support a tumour suppressor function for the Notch pathway in AML, although further experiments are clearly required to determine this definitively.

CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

This thesis set out to investigate the role of the Notch signalling pathway in acute leukaemia, focusing initially on the incidence, characteristics, and prognostic significance of activating mutations of *NOTCH-1* in adult T-ALL. Whether *NOTCH-1* mutations occur as primary initiating events or as late secondary events was studied by analysing mutation level, together with *NOTCH-1* mutation status in paired presentation-relapse samples. Finally, the functional and clinical significance of a novel isoform of *CSL* was investigated in AML.

6.1 *NOTCH-1* and *FBXW7* mutations, and their prognostic implications

There has been a steady improvement in the outcome of paediatric ALL over the past forty years, so what was once a terminal disease treated only with palliation is now curable in over 85% of cases (Pui & Evans, 1998, 2006). However, the outcome of adult ALL is still poor, with only 35-50% of adult patients achieving long-term remission (Goldstone *et al*, 2008; Hann *et al*, 2000; Larson, 2005). In B-ALL, this disparity in outcome can be partly explained by differences in the frequency of cytogenetic abnormalities that occur with age. For instance, Philadelphia-positive (Ph+) ALL, which is associated with poor outcome, occurs in only 2-3% of children, but 20-30% of adults (Jones & Saha, 2005; Moorman *et al*, 2007). The introduction of tyrosine kinase inhibitors for Ph+ ALL appears to be making significant improvements in outcome (Fielding, 2010), serving as a useful example that targeting high–risk molecular lesions has the potential to reduce the disparity in survival associated with age. In T-ALL, however, differences in the frequency of cytogenetic or molecular abnormalities between age groups that could explain the divergence in prognosis have not been clearly identified. Thus, after the description of activating mutations in *NOTCH-1* in paediatric T-ALL (Weng *et al*, 2004), it was important to assess the frequency and characteristics of *NOTCH-1* mutations in adult patients. In the cohort of 60 adult patients analysed in the studies presented in this thesis, 38 patients (63%) had at least one *NOTCH-1* mutation, (Chapter 3), which was very similar to the incidence of *NOTCH-1* mutations (56%) reported in paediatric T-ALL. Furthermore, the characteristics of the *NOTCH-1* mutations themselves were comparable between adult and paediatric patients, with similar frequencies of point mutations and insertions and/or deletions in the HD-N, HD-

C, TAD and PEST domains. Nor was there a difference in the incidence of *FBXW7* mutations in the adult cohort, with 11 of 60 patients (18%) affected, compared to 14-18% of paediatric T-ALL (Jenkinson *et al*, 2012; Kox *et al*, 2010; Park *et al*, 2009; Zuurbier *et al*, 2010). These results indicate that the difference in curability between the two groups is not related to a differing frequency or characteristics of *NOTCH-1/FBXW7* mutations.

This majority of paediatric studies have shown improved outcome associated with *NOTCH-1* and/or *FBXW7* mutations (Table 3.9). To directly assess whether this is the case in adult patients, the outcome of a combined cohort of 88 patients treated on the ECOG2993/UKALLXII trials was analysed. The 5 year EFS was 51% *vs.* 27% (*P*=.10) in *NOTCH-1* and/or *FBXW7* mutant-positive and WT patients respectively (Figure 3.6). The trend towards improved EFS in patients with a Notch pathway mutation on this trial was not of sufficient magnitude to warrant the recommendation of treatment reduction (for instance, avoidance of allogeneic transplant in first CR), as had been suggested by other groups (Asnafi *et al*, 2009).

An important issue, therefore, is whether there is a subgroup of T-ALL patients where a Notch pathway mutation could inform clinical decision-making. In adult patients, based on current data, the answer would appear to be no. Although *NOTCH-1/FBXW7* mutation status was an independent prognostic factor on the adult French LALA-94 and GRAALL-2003 trials (median EFS of 36 *vs.* 17 months, P = .01, in patients with *NOTCH1* and/or *FBXW7* mutations versus WT patients) (Asnafi *et al*, 2009), reducing treatment intensity for the mutant-positive group has to balanced against the extremely poor salvage rates of adult ALL at relapse (7% are long-term survivors after relapse). In paediatric patients, recent data from the UKALL2003 trial has shown a 100% OS for patients with double mutations (defined as either HD plus PEST mutations of *NOTCH-1* or both *NOTCH-1* and *FBXW7* mutations) (Jenkinson *et al*, 2012). Because 84% of the double mutation subgroup was considered high-risk (by virtue of slow early treatment response and/or MRD positivity), yet all survived, a possible question on future studies is whether this group truly requires treatment intensification. However, large numbers of patients will be required to test this definitively.

Due to its rarity, T-ALL does not have the luxury of large patient cohorts to sufficiently power studies to detect small effects on prognosis. A recent meta-analysis of published data from six paediatric (n=711) and three adult trials (n=253), which included the data presented in Chapter 3, failed to show a prognostic significance for *NOTCH-1* mutations in either age group (Ma & Wu, 2012). It should be noted that *FBXW7* mutation status and other possible confounders, such as rates of transplantation, were not included in this analysis. Whether the differences in prognostic impact of *NOTCH-1* mutation status observed between studies relates to differences in therapy or protocol design await to be determined.

There are a number of variables that need to be addressed in future studies. Firstly, no study has yet addressed the prognostic implication of *NOTCH-1/FBXW7* mutation status taking into account ETP disease. As discussed in Chapter 1, such patients fare particularly poorly and have a relatively low incidence of *NOTCH-1* (19%) and *FBXW7* mutations (6%) (Zhang *et al*, 2012). This means that in prognostic studies a higher proportion of patients with ETP will be represented in the WT group compared to the *NOTCH-1/FBXW7* mutated group, which could confound the outcome data, particularly in paediatric studies where the number of relapse events or induction failures is low. Secondly, activating mutations in *JAK1* have recently been identified in 21% of adult patients with T-ALL and are also associated with very poor outcome (Flex *et al*, 2008). All the *JAK1* mutated patients had *NOTCH-1* mutations, suggesting that there may be a group of *NOTCH-1* mutant *JAK1* WT patients that actually fare particularly well. Interestingly, *JAK1* mutations were found in only 1% of paediatric patients in this study, which raises the possibility that they may account for the difference in outcome seen between adult and paediatric disease generally, and perhaps explain the lack of association of *NOTCH-1* mutation status and outcome seen on some adult trials, including the data presented here, suggesting that *JAK1* mutation status may be an important prognostic variable that should be considered in future adult studies.

Whether the Notch pathway is activated by alternative mechanisms in those T-ALL patients without *NOTCH-1* or *FBXW7* mutations, would be an interesting area for future study. Only a weak correlation between *NOTCH-1*/*FBXW7* mutation status and activation of the downstream target gene *HES-1* was found by qPCR, with many WT patients having significantly higher *HES-1* transcript levels than mutant-positive patients

(Larson Gedman *et al*, 2009). Other possible mechanisms of activation include overexpression of one or more Notch ligands, inactivation of other negative regulators of the Notch pathway such as Numb, as has been reported in lung cancer (Westhoff *et al*, 2009), alternative mutational hotspots in *NOTCH-1*, or mutational activation of other *NOTCH* family members. With regards to the latter possibility, ICN-3 is able to initiate T-ALL in mice and *NOTCH-3* is overexpressed in human T-ALL (Bellavia *et al*, 2000; Bellavia *et al*, 2002), and therefore *NOTCH-3* was thought to be a good candidate for mutational screening. However, analysis of the HD, TAD and PEST domains of *NOTCH-3* in 38 adult T-ALL patients did not identify the presence of activating mutations (Chapter 3). A novel mutation of the *NOTCH-1* LNR domain (H1545P) was identified in a T-ALL patient who had a co-existing PEST domain mutation (Chapter 3), which was subsequently shown to be activating in functional studies performed by Jon Aster's group (Gordon *et al*, 2009). Mutations in this domain do not appear to be recurrent in other T-ALL cohorts that have since been analysed, although another LNR domain mutation (C1528R) was recently identified in a paediatric patient (Jenkinson *et al*, 2012).

Mutational analysis of samples from 157 AML and 30 infant leukaemia patients failed to identify somatic *NOTCH-1* or *FBXW7* mutations (Chapter 3). This indicates that Notch pathway activation through somatically acquired mutations is not responsible for the high incidence of aberrant expression of T-cell markers in these two groups of patients, but it does not exclude the possibility that Notch pathway activation by an alternative mechanism, such as through ligand activation, plays a role in these aberrant immunophenotypes.

6.2 *NOTCH-1* mutations as secondary events in T-ALL

In murine models of T-ALL, aberrant Notch activation can directly induce leukaemia or collaborate as a secondary event on other transgenic backgrounds (Lin *et al*, 2006; O'Neil *et al*, 2006; Pear *et al*, 1996; van den Brandt *et al*, 2006). Three lines of evidence, as presented in Chapter 4, support a model in which *NOTCH-1* mutations can occur as late secondary events in human T-ALL:

1. The *NOTCH-1* mutation level was <10% in nearly 20% of patients tested, suggesting that they often occurred in subclones.

2. Several patients with paired presentation-relapse samples showed a change in *NOTCH-1* mutation status at relapse, despite relapsing with a clone with the same TCR rearrangement.

3. A SCID-X1 patient who developed T-ALL after gene therapy due to viral integration and activation of *LMO2*, had acquired a *NOTCH-1* mutation by the time of overt disease.

The results of the studies of *NOTCH-1* mutations in T-ALL presented in Chapter 4 are consistent with the highly heterogeneous and complex clonal architecture of ALL that has since been described by others (Anderson *et al*, 2011; Mullighan *et al*, 2008). This data has two clinical implications. Firstly, given the lack of mutation stability at relapse, it limits the utility of using *NOTCH-1* mutations as markers for MRD analysis. Secondly, it has potential implications for the use of Notch pathway inhibitors that are currently in clinical trials as they may only target *NOTCH-1* mutant subclones and give a selection advantage to the remaining leukaemic *NOTCH-1* WT cells.

6.3 Notch pathway inhibitors for the treatment of T-ALL

Because HD and PEST-mutated NOTCH-1 receptors are still dependent on cleavage by the γ-secretase complex for activation, there is the potential to use GSIs to inhibit the Notch pathway in T-ALL. There has been long-standing interest from large pharmaceutical companies in the development of GSIs for the treatment of Alzheimer's disease, because amyloid precursor protein (APP) is cleaved to β-amyloid peptide (the major constituent of amyloid plaques responsible for disease) by a γ-secretase-dependent complex (Miyazaki *et al*, 1993). A Phase II study using an orally available GSI was already underway for Alzheimer's disease by the time *NOTCH-1* mutations were discovered in T-ALL (Barten *et al*, 2006). A phase II trial of the Merck compound MK-0752 was rapidly expedited at Dana-Farber Cancer Institute (DFCI 04-390 trial), and recruited eight patients that included seven patients with T-ALL and one patient with

AML (Deangelo, 2006). Although the first T-ALL patient to receive this drug had a transient response, there were no other responders. It should be noted that only four patients actually had a *NOTCH-1* mutation (one of which was the responder), and there were no studies performed to show efficacy of on-target effect such as a reduction in downstream Notch signalling or decreased ICN-1 protein in blast cells. There was also significant dose-limiting toxicity in the form of either grade III/IV diarrhoea and/or extreme fatigue, and the drug had to be discontinued in the majority of patients. Nonetheless, there are currently over ten ongoing trials assessing the safety and efficacy of GSIs in phase II trials, mostly of the drug RO4929097, recruiting patients with a wide range of tumours including osteosarcoma, melanoma, lymphoma, T-ALL, as well as breast, lung, and pancreatic cancer (www.clinicaltrials.gov).

There are a number of possible molecular reasons for the lack of efficacy of GSIs in T-ALL. The presence of *PTEN* deletions, present in 10-20% of T-ALL cases, has been associated with GSI-resistance, due to upregulation of the PI3K pathway (Palomero *et al*, 2007). *FBXW7* mutations have also been shown to cause resistance to GSI treatment through their impact on the oncogene c-MYC (O'Neil *et al*, 2007). T-ALL cells are dependent on c-MYC, which is transcriptionally activated by ICN and then targeted for degradation by FBXW7. Notch inhibition by GSIs results in a reduction in c-MYC, except in the presence of an inactivating *FBXW7* mutation, where c-MYC is stabilised at the protein level as a consequence of the loss of FBXW7 function.

Unfortunately, there is clear evidence that the gastro-intestinal toxicity of GSIs is an on-target rather than off-target effect of Notch inhibition, so developing increasingly potent drugs to achieve efficacy is unlikely to be a fruitful approach. Notch signalling regulates the cell fate of intestinal progenitor cells, such that active Notch signalling leads to differentiation towards the enterocyte lineage, whilst low Notch signals favours a goblet cell fate (Stanger *et al*, 2005). Prolonged treatment with GSIs increases goblet cell numbers (i.e. the mucous secreting cells) and disrupts villous architecture, leading to severe diarrhoea (van Es *et al*, 2005). Thus, although the DFCI 04-390 trial did not show evidence for Notch pathway inhibition in leukaemia cells, the development of diarrhoea in most patients suggests that adequate on-target effect was achieved, at least in the gut, which, together with the lack of clinical responses, has tempered excitement about the

future of such drugs. However, there has been recent development of three major strategies for overcoming gut toxicity *in vivo*:

1. Steroids - when steroids are used in combination with GSIs in mouse models, there is almost complete abrogation of GSI-induced gut toxicity (Real *et al*, 2009). Furthermore, GSIs have efficacy in overcoming steroid-resistance in T-ALL, and current trials of GSIs in T-ALL include the addition of dexamethasone (NCT01088763; www.clinicaltrials.gov).

2. Dosing schedules - use of short drug-holidays (for instance, a three-day-on, four-day-off schedule) avoids gut toxicity but retains efficacy in murine xenograft models of T-ALL (Tammam *et al*, 2009), and such dosing schedules have been used safely in phase I trials (Tolcher *et al*, 2012).

3. Specific targeting of *NOTCH-1* – GSIs inhibit activation of all four NOTCH family members, and gut toxicity occurs when both NOTCH-1 and -2 are inhibited concurrently, but not if they are inhibited individually (Riccio *et al*, 2008; Wu *et al*, 2010). Thus specific targeting of NOTCH-1 has the potential of targeting leukaemic cells without affecting gut homeostasis. A synthetic stabilised alpha-helical peptide (known as SAHM1) that acts to inhibit the interaction of MAML-1 with ICN-1 has shown efficacy *in vivo* in murine T-ALL xenograft models without causing gut toxicity (Moellering *et al*, 2009). Monoclonal antibodies that bind to and stabilise the LNR domain, and thereby inhibit activation of specific NOTCH receptors, have also shown promise in pre-clinical models of T-ALL without producing gastro-intestinal side effects (Wu *et al*, 2010).

NOTCH-1 has a tumour suppressor role in several cellular contexts. Mice chronically treated with GSIs are highly prone to skin malignancies (Nicolas *et al*, 2003) and, given a potential tumour suppressor role for the Notch pathway in the liver, lung and myeloid compartment, prolonged use of Notch pathway inhibitors must be approached with some caution. However, this is likely to be more relevant to long-term chronic use of such

drugs, for instance in Alzheimer's disease, rather than short-term intermittent use for T-ALL.

6.4 Alternative splicing of CSL in AML

As discussed in Chapter 5, the issue of whether Notch acts as an oncogene or tumour suppressor in AML remains contentious. Inactivation of *Notch-1* and *-2* in the haematopoietic compartment of mice leads to a myeloproliferative disease, and activation of Notch signalling using a Jagged-1 peptide has been shown to induce apoptosis in AML cell lines (Klinakis *et al*, 2011; Sutphin *et al*, 2007). Two very recent papers support a tumour suppressor function for Notch signalling in AML. The first identified low levels of *HES-1* in AML samples, despite high expression of *NOTCH-1* and *-2*, and showed that activation of the Notch pathway through expression of *ICN-1, ICN-2, HES-1* or with a DLL1 peptide, induced apoptosis in AML cells *in vitro* and *in vivo* (Kannan *et al*, 2013). The second paper recapitulated many of these findings, and also showed that overexpression of ICN-1 in an MLL-AF9 murine model of AML significantly delayed tumour onset (Lobry *et al*, 2013). Furthermore, mice with homozygous compound deletion of *Tet2* and *Ncstn*, leads to a fully penetrant transplantable AML-like disease.

The data presented in Chapter 5 is consistent with expression of a loss-of-function isoform of *CSL* in AML patients, in the majority of cases as the major isoform. CSL-TREX was unable to bind NOTCH-1 and did not have dominant-negative activity in reporter or biological assays. However, expression of CSL-WT in CD34+ cells profoundly inhibited *in vitro* cell growth, while expression of CSL-TREX did not, suggesting that splicing to CSL-TREX may be a means of regulating or fine-tuning Notch signalling. Further studies are required to demonstrate that this is a NOTCH-dependent mechanism. Whether expression of *CSL-TREX* functionally impacts on downstream signalling in AML, which could be assessed by analysis of *HES-1* and other downstream targets by qRT-PCR, is also worthy of further study.

Since moving to the Dana-Farber Cancer Institute, in the laboratory of Dr Tom Look, I have been able to continue some functional studies into *CSL-TREX*. With assistance from Dr Evisa Gjini, I have used a zebrafish model to examine whether *CSL-TREX* can rescue the phenotype of *CSL-WT* depletion. Notch signaling and *csl* expression are required for

normal vessel formation during early embryonic development (Siekmann & Lawson, 2007), therefore early embryogenesis offers an effective means to test CSL-TREX *in vivo*. Depletion of endogenous *csl* through morpholino injection at the single-cell stage of zebrafish embryogenesis causes a marked vessel defect, with characteristic alterations consisting of increased tortuosity and abnormal sprouting of the inter-segmental vessels (ISVs), and disrupted formation of the dorsal longitudinal anastomotic vessels (DLAV) (Siekmann and Lawson, 2007). Conversely, *notch* activation causes a global suppression of angiogenesis (Siekmann and Lawson, 2007). These features can be clearly visualised in the Tg(*fli*:EGFP) reporter zebrafish model and, given the high degree of conservation of the Notch pathway between humans and zebrafish, make it an ideal model in which to test *CSL* variants (Lawson & Weinstein, 2002; Siekmann & Lawson, 2007). No vessel defects were observed when *CSL-WT* or *CSL-TREX* cRNA were injected into Tg(*fli*-EGFP) embryos, showing that they do not have gain-of-function or dominant-negative activity (Figure 6.1 A, B and C). Use of the anti-*csl* morpholino, that specifically targets endogenous but not human *CSL*, resulted in characteristic vessel defects in an average of 76% of embryos injected, with increased tortuosity and abnormal sprouting of the ISVs and disrupted formation of the DLAV (Figure 6.1 D and H). Co-injection of human *CSL-WT* cRNA with the morpholino rescued the vessel phenotype in approximately half of the embryos (Figure 6.1 E and H; mean 38% of embryos with the phenotype, P=0.005 versus morpholino alone), whereas co-expression of *CSL-TREX* cRNA did not abrogate the effect of the morpholino (Figure 6.1 F and H; mean 69% of embryos with the phenotype, P=0.26 versus morpholino alone). These results indicate that *CSL-TREX* does not have dominant-negative activity and cannot compensate for the loss of endogenous *csl*, which strongly supports *CSL-TREX* as a loss-of-function variant.

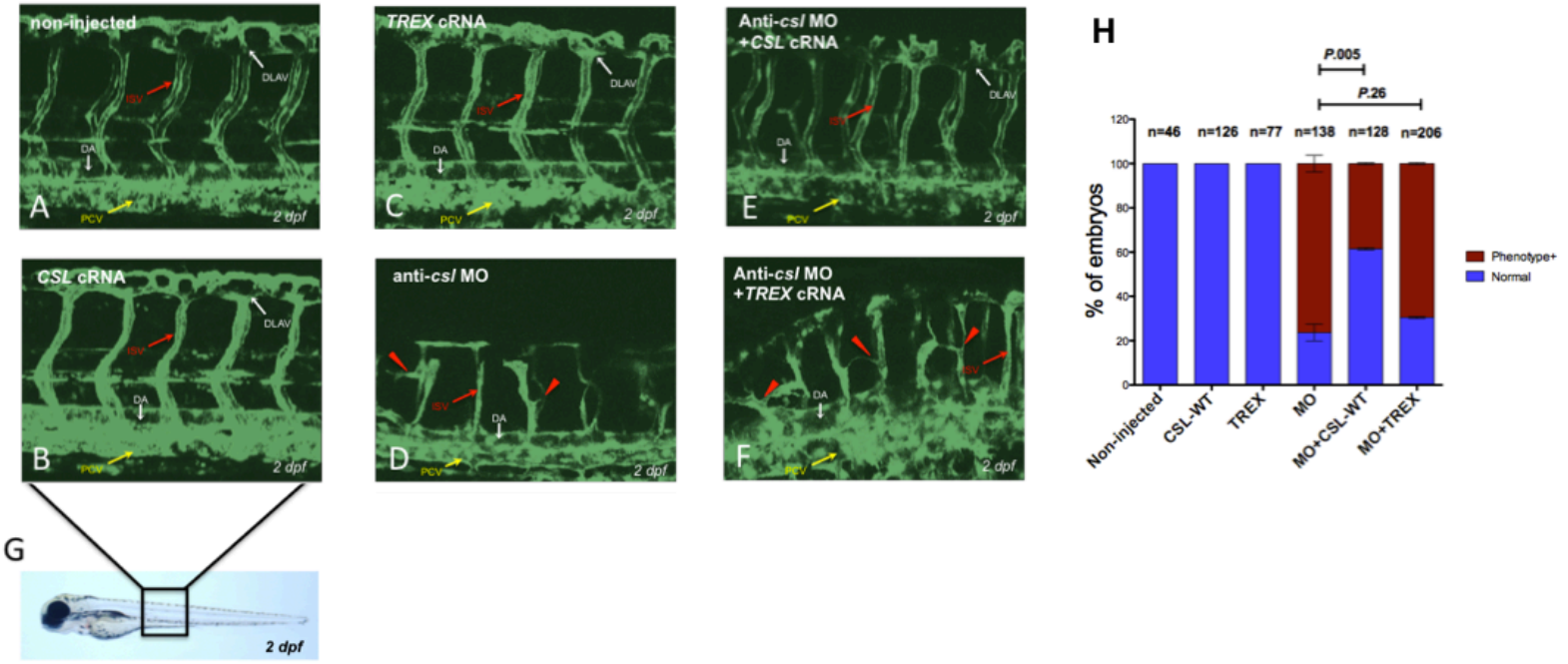

Figure 6.1 Knockdown of endogenous zebrafish *csl* induces a severe vessel deformity that can be rescued by human wild-type *CSL* cRNA but not *CSL-TREX*. **(A-F)** Transgenic *fli1*-EGFP embryos at 2 days post-fertilization (2dpf) visualised by fluorescent microscopy showing the dorsal aorta (DA), posterior cardinal vein (PCV), inter-segmental vessels (ISV) and dorsal longitudinal anastomotic vessels (DLAV). (A) Non-injected embryos. **(B)** Embryos injected with human *CSL*-WT cRNA. **(C)** Embryos injected with *CSL-TREX* cRNA. **(D)** Embryos injected with anti-*csl* morpholino (MO) that targets endogenous but not human *CSL*. Vessel deformities are indicated with red arrowheads. **(E)** Embryos injected with anti-*csl* morpholino and *CSL*-WT cRNA. (F) Embryos injected with anti-*csl* morpholino and *CSL-TREX* cRNA. **(G)** Light microscopy of a zebrafish embryo at 2dpf. **(H)** The percentage of injected embryos displaying the normal (blue) or *csl* loss-of-function vessel phenotype (red). Error bars represent standard error of the mean for 3 independent experiments, with the total embryo count given above the bars.

6.5 CONCLUSION

The Notch pathway plays a well-defined role in early definitive haematopoiesis and during lineage commitment of early haematopoietic cells. Activation of Notch signalling occurs in the majority of cases of T-ALL, and has more recently been implicated in other haematological malignancies including mantle cell lymphoma and CLL (Kridel *et al*, 2012; Puente *et al*, 2011). Notch also plays a tumour suppressor role in other malignancies, such as squamous cell carcinomas, and myeloid malignancies, including CMML and AML, highlighting the importance of cell context to the outcome of Notch pathway dysregulation.

A summary of the major findings in this thesis are listed below:

- *NOTCH-1* mutations are frequent in adult T-ALL, affecting over 50% of patients
- The characteristics and frequency of *NOTCH-1* mutations are similar in adult and paediatric T-ALL, and thus do not explain the difference in prognosis between these two groups of patients
- *NOTCH-1* and *FBXW7* mutation status are associated with a trend towards improved outcome in adult T-ALL, but not of significant magnitude to inform clinical decision-making
- *NOTCH-1* mutations are not a common feature of infant leukaemia or adult AML, and so do not account for the aberrant expression of T-cell markers frequently seen in these diseases
- A novel activating mutation of the LNR domain of *NOTCH-1* was identified in a single T-ALL patient
- No mutations were identified in *NOTCH-3* in T-ALL
- *NOTCH-1* mutations occur as late secondary events in a significant proportion of patients, often occurring in minor leukaemic subclones
- *NOTCH-1* mutations often show instability between presentation and relapse, suggesting they are not always the primary initiating event. This has potential implications for Notch targeted therapy, as well as the application of *NOTCH-1* mutations as markers for MRD analysis.
- In the majority of patients with AML, *CSL* is aberrantly spliced to an isoform lacking the last 78bp of exon 10 (termed *CSL-TREX*), which encodes a bridge-strand of the BTD, that is likely to result in loss of functional integrity

- The *CSL-TREX* isoform is also present at moderate levels in normal CD34+ cells, but not in differentiated T-cells, neutrophils or other normal tissues
- *CSL-TREX* levels decrease on differentiation of AML blasts *in vitro*
- *CSL-TREX* does not have dominant-negative activity
- Wild-type CSL markedly inhibits the clonogenic potential of CD34+ cells
- Together, this suggests splicing to *CSL-TREX* may be a means of fine-tuning CSL levels in haematopoietic cells, and supports a tumour suppressor function for the Notch pathway in AML.

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APPENDIX TABLE 1 *NOTCH-1*/*FBXW7* mutational status of adult T-ALL patients treated on ECOG2993 (data from of A. Ferrando)

Case#	UKALLXII/ ECOG TRIAL NO.	NOTCH-1 mutation status	FBXW7 mutation status	NOTCH-1 Mutation (nucleotide)	NOTCH-1 Mutation (amino acid)	FBXW7 Mutation (nucleotide)	FBXW7 Mutation (amino acid)
E1	ECOG13032	WT	WT				
E2	ECOG15451	HD-N MUT	WT	4799T>A	L1601Q		
E3	ECOG18654	TAD MUT	WT	7004-7005insGAGCACACAGGCCCCCT	fs@2336, STOP@2364		
E4	ECOG12535	HD-N MUT	WT	4778insACCATAACC	L1594H+insHNL		
E5	ECOG10726	HD-C MUT	WT	5045A>T	N1683I		
E6	ECOG12107	WT	WT				
E7	ECOG18774	WT	WT				
E8	ECOG13205	HD-N MUT	WT	4776insGGGCTC	F1573L+insGS		
E9	ECOG10614	WT	MUT			1436G>A	R479Q
E10	ECOG18296	WT	WT				
E11	ECOG16185	HD-N MUT	WT	4747-4748insCCG	1583-1584insP		
E12	ECOG12894	HD-N MUT	MUT	4793G>C	R1599P	1513C>T	R505C
E13	ECOG18849	HD-N MUT	WT	4721T>C	L1575P		
E14	ECOG18250	WT	WT				
E15	ECOG15445	HD-N MUT	WT	4823G>A	R1609Q		
E16	ECOG11496	HD-N MUT	WT	T4775T>C	F1593S		
E17	ECOG10496	WT	WT				
E18	ECOG11006	WT	WT				
E19	ECOG12004	WT	MUT			1436G>A	R479Q
E20	ECOG12874	HD-N MUT	WT	4747-4748insCCTTCT	1583-1584insPS		
E21	ECOG13684	HD-N MUT	MUT	4741-4742delC+insAGGGAAGAGAGGGCCA	1581-1582insREERA+P1582T	1513C>T	R505C
E22	ECOG13964	HD-N+ PEST MUT	WT	4757G>C 7021delTinsCC	R1587P fs@2342, stop@2354		
E23	ECOG14383	HD-N MUT	WT	4818delCinsACTTAATCCG	F1607L+1607-1608insLNP		
E24	ECOG17534	PEST MUT	WT	7367insGT	fs@2457, STOP@2478		
E25	ECOG8222	PEST MUT	WT	G7387T	A2465T		
E26	ECOG10766	HD-N MUT	WT	4813-4delGTinsCTCCCCCCTCCGGC	V1606L+insPPPA		
E27	ECOG11769	PEST MUT	WT	7541-7542delCT	fs@2515, STOP at 2518		
E28	ECOG12290	JME MUT	WT	5303-5371 duplication 69 bases	23 aa tandem duplication 1769-1792		
E29	ECOG10438	WT	WT				
E30	ECOG12487	WT	MUT			1394G>A	R465H
E31	ECOG15522	HD-N MUT	WT	4774-4775insGAG	1592-1593insE		
E32	ECOG13564	WT	MUT			1513C>T	R505C
E33	ECOG16911	HD-N MUT	WT	4754T>C	L1586P		
E34	ECOG11593	WT	WT				

E# local patient case number, WCC white cell count, MUT mutant, WT wild type, ins insertion, del deletion, SNP single nucleotide polymorphism, HD-N N-terminal heterodimerisation domain, HD-C C-terminal heterodimerisation domain, JME juxtamembrane expansion mutation, fs frameshift, X stop codon