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Investigation of the Role and Mechanism of Beta-Catenin Activation in Acute Myeloid Leukaemia

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Abbreviations

AML	Acute myeloid leukaemia
AML-ETO	AML1-Eight-Twenty One oncoprotein fusion
APC	Adenomatous polyposis coli
βc	Common beta-subunit
BCL-2	B-cell lymphoma 2
BM	Bone marrow
ChIP	Chromatin immunoprecipitation
ChEA	ChIP enrichment analysis
GSEA	Gene set enrichment analysis
CISH	Cytokine-inducible SH2-containing protein
CK1α	Casein kinase 1 alpha
CML	Chronic myeloid leukaemia
CSC	Cancer stem cell
CSF2RB	Colony stimulating factor 2 receptor beta
EGR-1	Early growth response protein 1
ERK	Extracellular regulated kinase
FAB	Frech-American-British
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FDM	Factor-dependent myeloid
FDR	False discovery rate
FRA-1	Fos-related antigen 1
GFP	Green fluorescent protein
GSK3β	Glycogen synthase kinase 3 beta
GM	Granulocyte-macrophage
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Granulocyte-macrophage progenitor

HSC	Haematopoietic stem cell
HOX	Homeobox
IL-3	Interleukin-3
IL-3R	Interleukin-3 receptor
IL-3Rα	Interleukin-3 receptor alpha-subunit
IL-5	Interleukin-5
ITD	Internal tandem duplication
JAK	Janus kinase
LEF	Lymphoid enhancer factor
LIMMA	Linear models for microarray data analysis
LSC	Leukaemic stem cell
mAb	Monoclonal antibody
MIG	MSCV-IRES-GFP
MLL	Mixed-lineage leukaemia
miR	Micro RNA
MSigDB	Molecular signatures database
PI3K	Phosphoinositol 3 kinase
PIM1	Proviral integration site for Moloney-murine leukaemia virus 1
PML-RARα	Promyelocytic leukaemia-retinoid acid receptor alpha
PTGS2	Prostaglandin-endoperoxide synthase 2
QRT-PCR	Quantitative real-time polymerase chain reaction
RUNX1	Runt-related transcription factor 1
STAT	Signal transducer and activator of transcription
TCF	T-cell factor
WHO	World Health Organization
Wnt	Wingless-type
WT	Wild-type

Abstract

Aberrant activation of β-catenin is a common event in Acute Myeloid Leukaemia (AML), and accumulating evidence indicates this pathway plays a critical role in the establishment and maintenance of myeloid neoplasms. In AML, increased β-catenin signalling has been associated with activating mutations in the FLT3 receptor, and the oncogenic AML1-ETO and PML-RARα translocation products. In the absence of these lesions, however, it remains unclear which mechanisms may activate β-catenin in AML more broadly. Here we have explored a potential role for the multipotent haematopoietic cytokine, interleukin-3 (IL-3) in the regulation of β-catenin signalling in myeloid and leukaemic cells.

We show that IL-3 can induce the dose dependent stabilisation of β-catenin in a myeloid model of Hox oncogenesis, and that β-catenin is required for IL-3 driven colony formation and growth. Enforced expression of β-catenin in this system allows cell survival at sub-optimal concentrations of IL-3 which may contribute to leukaemic transformation by providing a survival advantage to blast cells in the haematopoietic niche.

We also demonstrate that IL-3 can promote β-catenin activation in the IL-3 dependent human erythroleukaemia cell line, TF-1.8, and in primary AML cells. Furthermore, Affymetrix gene expression analysis of bone marrow cells from four AML patients treated ± IL-3 revealed a strong correlation between the IL-3 induced signature and Wnt/β-catenin gene networks. Interestingly, the IL-3 receptor alpha subunit (IL-3Rα) has been previously shown to be overexpressed in AML leukaemic stem cells and progenitors compared to normal counterparts, and elevated levels of IL-3Rα are associated with poor prognosis and overall survival.

Consistent with the regulation of β-catenin by IL-3, we show that a neutralising monoclonal antibody (7G3) which targets IL-3Rα, inhibits IL-3 mediated activation of β-catenin in TF-1.8 and primary AML cells. Modified versions of 7G3 are currently undergoing clinical trials for patients with AML, and our data indicates that this therapy may be more effective for patients with elevated levels of oncogenic β-catenin.

As previous studies have demonstrated that cytokines can induce the inhibitory phosphorylation of GSK3β via activation of the PI3K/AKT pathway, we have also made use of pharmacological PI3K and AKT small molecule inhibitors to determine the importance of this axis in the IL-3 mediated regulation of β-catenin.

On the whole, the work in this thesis reveals a novel mechanism which may contribute to β-catenin activation in AML, and provides further insight into the amplitude of IL-3 signalling in normal and malignant haematopoiesis**.**

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CHAPTER 1

Introduction

1.1 Haematopoiesis

Haematopoiesis is a life-long process sustained by pluripotent haematopoietic stem cells (HSC) and progenitor cells in the bone marrow, which in steady state give rise to more than one million cells per second [1, 2]. In a tightly regulated process, HSC and their progeny give rise to a range of precursors which ultimately differentiate into all mature cells of the lymphoid and myeloid lineages **(Figure 1.1)**. This differentiation is accompanied by a progressive reduction in self-renewal capacity as cells become progressively more committed to a particular fate, resulting in a proposed hierarchical organisation of blood cells. Haematopoiesis is driven by pool of cytokines and their cognate receptors which finely tune haematopoietic cell survival, growth and differentiation through the activation of intracellular signalling cascades and cell-specific transcription profiles [3-5]. Thus, it is not surprising that aberrations in cytokine-mediated signalling pathways which disrupt the balance between differentiation and proliferation/self-renewal underpin the pathology of many haematological malignancies which are characterised by dysregulation of proliferation and lack of differentiation into mature cell lineages.

HSC constitute less than 0.01% of cells in the bone marrow and are defined by their unique self-renewal capacity and the ability to give rise to any of the haematopoietic cell lineages throughout the lifetime of an individual [1, 6]. The critical capacity of HSC for self-renewal is illustrated by their ability to colonise the bone marrow of lethally irradiated mice and reconstitute the entire haematopoietic system in a transplantation setting [1, 6].Over the last few decades

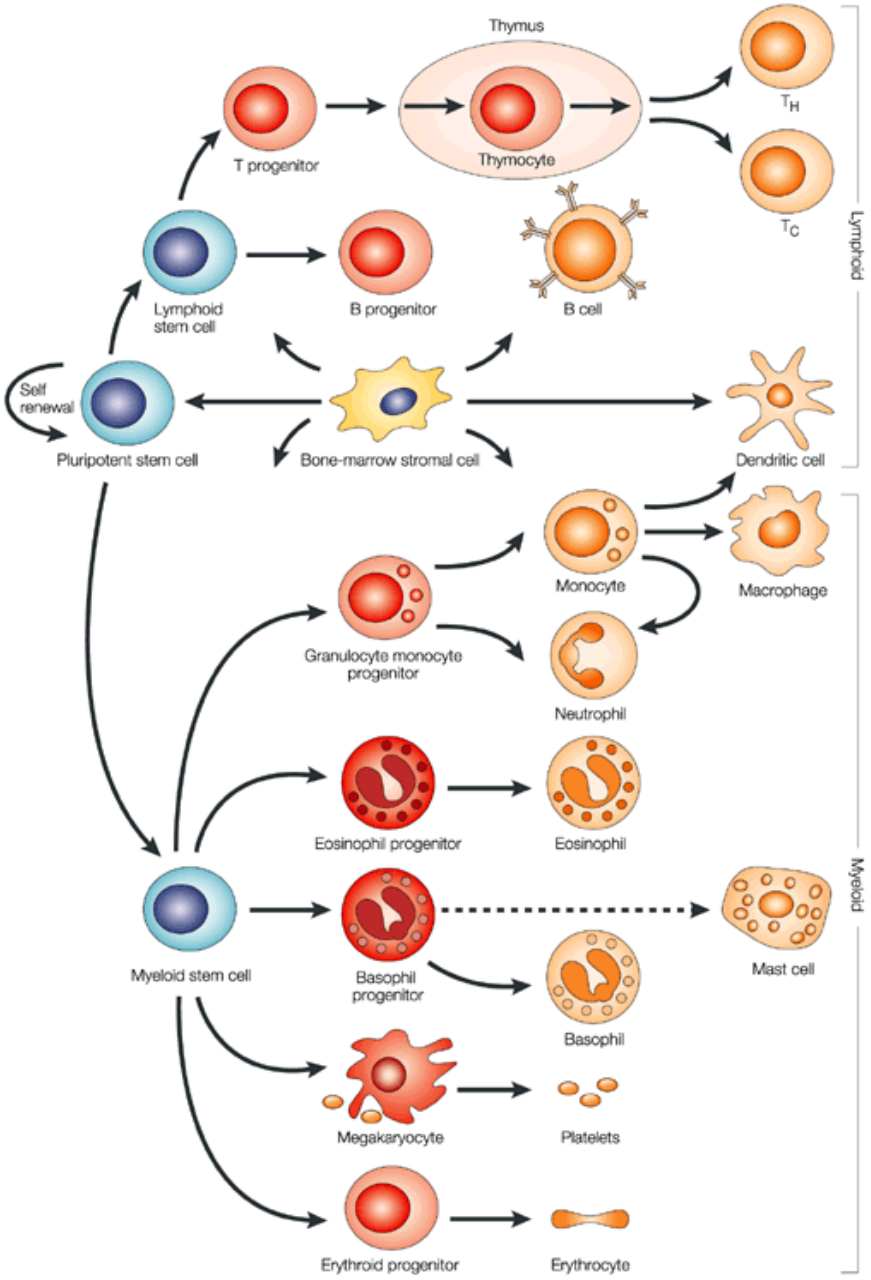


Figure 1.1 Haematopoiesis. In a hierarchical and tightly regulated process, haematopoietic stem cells (HSC) in the bone marrow give rise to all the progenitors and terminally differentiated mature blood cells of the lymphoid and myeloid lineages. *CMP*= common myeloid progenitor; *CLP*= common lymphoid progenitor.

Taken from Nabel (2002) [7]

a great deal of research has focused on understanding the signalling pathways involved in the maintenance of HSC, and it is becoming increasingly apparent that the bone marrow microenvironment also plays a critical role in the function of these cells [8-10]. Currently, HSC remain the best characterised of adult stem cells, although further understanding of the regulatory signalling networks governing the maintenance of these stem cells will improve efficiency of bone marrow transplants in the treatment of blood and bone marrow disorders as well as provide an insight into the pathogenesis of stem-cell based haematopoietic malignancies such as leukaemias and lymphomas.

1.2 Acute myeloid leukaemia

Acute myeloid leukaemia (AML) is the most common type of acute leukaemia in adults with an incidence of ~ 3.7 cases/100,000 people causing up to 180,000 deaths each year world-wide [11, 12]. AML is an aggressive heterogeneous disorder of myeloid precursor cells characterised by an accumulation of immature myeloid cells in the bone marrow and peripheral blood, as a result of sustained and deregulated proliferation and survival together with a block in differentiation. The inability for the haematopoietic progenitors to differentiate normally and to respond to normal regulators of proliferation leads to infection, bleeding and organ infiltration resulting in death within one year of diagnosis in the absence of treatment [11-13]. Chemotherapy, radiation therapy, and allogeneic bone marrow transplantation are the most effective treatments currently available but these therapies frequently fail to maintain long term eradication of the disease, and 75% of AML patients experience a relapse within 2 years of remission [12, 14, 15].

In this section the classification and associated prognosis of the molecular lesions underlying the development and progression of AML are reviewed, and the concept of the leukaemic stem cell is introduced. In addition, we emphasise how a more complete understanding of these areas will facilitate the generation of more efficient therapies for AML.

1.2.1 The molecular pathology of AML

Despite substantial advances in the diagnosis of the different subtypes of AML and the development of therapeutics, AML remains particularly difficult to treat, largely due to the immense phenotypic and genetic heterogeneity associated with the disease. The current overall success rate for AML treatment still remains considerably low with 5-year survival rates between 10-70% depending on the leukaemia subtype [16-18]. To date, over 200 chromosomal aberrations, as well as a wide range of karyotypic abnormalities and gene mutations affecting proteins such cell surface receptors, intracellular signalling proteins and transcription factors, have been linked with the development and progression of AML [19-21]. The prognosis and genetics of AML are tightly linked, and pre-treatment karyotype has long been recognised as the most important independent predictor of clinical outcome in this disease [22-25]. Furthermore, differential treatment of certain cytogenetic subgroups appears to markedly improve prognosis [26-28], emphasising the importance of understanding the genetics underlying AML for the development of strategic and more proficient therapeutics.

Over the last few decades there has been increasing progress in the classification of AML into reproducible, clinically relevant groups. The French American

British (FAB) scheme classified AML subtypes based on comprehensive morphological and cytochemical criteria, and each subtype (M0-M7) corresponded to a differentiation block at a particular stage [29]. While molecular heterogeneity was observed within subtypes, recurrent chromosomal translocations were specifically associated with some of the subtypes, (e.g. FAB M4 with inv(16)). More recently, initially in 2001, and updated in 2008 [30, 31], the World Health Organization (WHO) has incorporated genetic alterations into the classification of subtypes of AML **(Table 1.1)**.

The most frequent chromosome translocations in AML are those targeting retinoic acid receptor alpha (RARα), t(15;17)(q22;q12~21) (PML-RARα) which occur in 6-7% of patients [18, 32]. The other major translocations are t(8;21)(q22;q22) (RUNX1-RUNX1T1; AML1-ETO), inv(16)(p13q22)/t(16;16)(p13;q22) (CBFβ-SMMHC; CBFβ-MYH11), and translocations disrupting the mixed-lineage leukaemia (MLL) gene at 11q23 . In addition, gain or loss of chromosomes also occurs in AML including most commonly trisomy 8 (+8), monosomy 7 (-7) or deletion 7q (-7q) [17, 18]. These cytogenetic abnormalities are associated with a distinct prognostic risk factor, and are subdivided into favourable, intermediate or adverse risk classes as summarised in **Table 1.2**.

Although detectable clonal chromosomal aberrations occur in 50-60% of AML patients, and are central to the diagnosis and classification of AML, the remaining 40-50% of individuals present with no cytogenetic abnormalities [14, 24, 25]. This category of patients is broadly associated with intermediate prognostic risk (Table 1.2). However, it has become increasingly clear that AML patients with a

Table 1.1 The World Health Organisation (WHO) classification of AML.

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22) RUNX1-RUNX1T1 (CBFA-ETO)
AML with inv(16)(p13q22) or t(16;16)(p13;q22) CBFB-MYH11
APL with t(15;17)(q22;q11–12) PML-RARA
AML with t(9;11)(p22;q23) MLLT3-MLL & other balanced translocations of 11q23 (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 (megakaryoblastic) with t(1;22)(p13;q13) RBM15-MKL1
AML with mutated NPM1
AML with mutated CEBPA
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemias
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome (+21)
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasms

Adapted from Gulley *et al* (2010). [33]

normal karyotype encompass a range of submicroscopic molecular aberrations which are also germane to the pathogenesis and prognosis of AML (summarised in **Table 1.3**). Importantly, the prognostic significance of some of these lesions does not only provide critical clinically applicable information in patients with normal cytogenetics, but can modify the prognosis when associated with recurrent cytogenic abnormalities or other molecular lesions **(Table 1.3)**.

Table 1. 2 AML prognostic risk stratification by cytogenetic abnormalities at diagnosis.

Associated outcome	Cytogenetic Abnormality	Incidence
Favourable	t(15;17)(q24;q21) t(8;21)(q22;q22) inv(16)(p13.1q22)/ t(16;16)(p13.1;q22)	5%–13% 5%–7% 5%–8%
Intermediate	t(9;11)(p21;q23) normal karyotype −Y +8	2% 40%–45% 3% 10%
Adverse	inv(3)(q21q26)/t(3;3)(q21;q26) −5/ del(5q)/ add(5q) −7/ del(7q)/ add(7q) t(8;16)(p11;p13) t(11q23) [excluding t(9;11)] t(6;9)(p23;q34) t(9;22)(q34;q11.2) −17/ abn(17p) Complex (≥3 unrelated abnormalities)	1%–2% 4%–12% 8% <1% 3%–4% 1%–2% 1% 5% 6%–14%

* Favourable risk classification is irrespective of additional cytogenetic abnormalities. Adverse risk classification is applicable only in the absence of favourable cytogenetic abnormalities.

Adapted from Morrissette & Bagg (2011). [18]

For instance, internal tandem duplication (ITD) mutations in the FLT3 receptor (FLT3-ITD) which occur in 20-25% of AML patients are associated with a poor prognosis independently of the effects of cytogenetic abnormalities [34, 35]. Further marking the importance of molecular lesions in the prognosis of AML, the 2008 WHO edition has expanded the "AML with recurrent genetic abnormalities"

Table 1.3 Gene mutations in AML: Contextual frequency and prognostic significance.

	Incidence/Typical (Cyto)genetic context			
Gene	**Normal Karyotype**	**Associated Cytogenetic Abnormality**	**Mutations**	**Prognostic Significance**
NPM1	50–60%	14–15%	~50 variants. Mutation A (TCTG tetra-nucleotide duplication) commonest (70%–80%)	Favorable (with NK, in the absence of*FLT3*-ITD)
CEBPA	10–15%	9q deletions (40%)	Out-of-frame insertions and deletions in the N-terminal region; In-frame insertions and deletions in the C-terminal region	Favorable (when biallelic, with NK, in the absence of *FLT3*-ITD)
FLT3	25–30%	t(6;9) (70%) t(15;17) (40%)	ITD (75–80%) TKD (20%–25%)	Poor (when there is aUPD)
KIT	2–8%	t(8;21) (16–45%) inv(16)/t(16;16) (29%) trisomy 4	TKD D816 TKD N822	Poor
RUNX1	5–33%, *MLL*-PTD (20%) *FLT3* (16%) *NRAS* (9%)	trisomy13 (90%)	Missense, nonsense, frameshift mutations	Poor
MLL	5–11%, *FLT3*	trisomy 11 (25–45%)	PTD	Unclear (# poor)
WT1	1-2% *FLT3*-ITD (8%)	#	Insertions and deletions within exons 7 and 9	Unclear (# poor with *FLT3*-ITD)
NRAS	10–13%	inv(16)/t(16;16) (37%) t(8;21) (7%) inv(3)/t(3;3) (23%)	Missense, codons 12, 13, and 61	Unclear
KRAS	2.5%	inv(16)/t(16;16) inv(3)/t(3;3) (24%)	Missense, codons 12, 13	Unclear
TP53	1–11%	Complex karyotype with or without 17p deletion (66–88%)	Missense (most common), nonsense, splice site, deletions, insertions	Poor
IDH1	7–8%	Monosomy 8	Missense (R132)	Unclear (#poor)
IDH2	8–9%	Unclear	Missense (R140, R172)	Unclear (# poor)
DNMT3A	22%	Unclear	Missense	Poor
ASXL1	8–25%	9%, including favorable [eg, t(8;21)) and poor (eg, −7, i(17q)]	Exon 12	Unknown
TET2	12–17%	Unknown	Deletion, frameshift nonsense	#poor
CBL	2–4%	CBP-AML (3–5%)	Missense	Unknown

* ITD= internal tandem duplication; TKD= tyrosine kinase domain; PTD= partial tandem duplication; aUDP= acquired uniparental disomy; NK= normal karyotype; #= currently unknown or questionable

Adapted from Morrissette & Bagg (2011). [18]

class to include the provisional incorporation of AML with mutated nucleophosmin (NPM1) and CEBPA, both of which are associated with a favourable prognosis in the absence of FLT-3-ITD mutations [36-38].

The oncogenic events underlying AML pathogenesis are often separated into at least two complementation classes (Class I and Class II) based on the classical two-hit model of leukaemogenesis. Class I mutations confer a proliferation and/or survival advantage through increased signal transduction and Class II mutations induce a block in myeloid differentiation and contribute to self-renewal capacity [12, 23, 39]. The cooperation between Class I and Class II mutations disrupts the intricate balance between proliferation and differentiation, resulting in the AML phenotype. Examples of Class I mutations include those affecting FLT-3 and NRAS, whereas examples of Class II lesions include the chromosomal PML-RARα and mutations in CEBPA.

This theory suggests that a common functionality among different mutation in AML may underlie the pathogenesis of this disease, and presents an integrated molecular principle that may be useful for understanding how unique mutations can generate similar biological outcomes amongst the AML subtypes. Support for this model comes from a range of studies which show that mutations from the two different classes often occur together in the same AML patient, whereas mutations within complementation groups are rarely observed in the same AML [23, 39-41]. Furthermore, studies in mice directly demonstrate cooperativity between mutations of the two different classes and show that this collaboration is sufficient to generate AML *in vivo* [42-45].

More recently, it has become apparent that there are at least two additional mutation classes (Class III and Class IV). Class III genes encompass epigenetic modifiers such as DNMT3A, IDH1, IDH2, TET2, ASXL1 and EZH2, while the fourth class covers mutations in tumour suppressors including WT1 and TP53 [46-48]. These new mutation classes highlight the complexity of AML pathogenesis, and it is likely that combination therapies which target at least two classes of mutations may provide a more efficient or even synergistic approach for the treatment of AML. In addition, advances in genomic technologies are now allowing large-scale DNA sequencing and methylation analyses of primary AML genomes which will be valuable for the identification of novel DNA mutations and epigenetic modifications that are responsible for the pathogenesis of AML [49-52].

1.2.3 The leukaemic stem cell

At the pinnacle of current research in cancer initiation and progression, lies the paradigm of the *cancer stem cell* (CSC). The CSC hypothesis postulates that tumours are sustained by a subset of cells, which have the idiosyncratic ability to self-renew, and give rise to the more differentiated neoplastic cell types that make up the bulk of the tumour [53-55].

The origins of CSC remain debatable; however, they share many common functional properties with normal adult stem cells such as self-renewal capacity and quiescence. Critically, despite the long-term proliferative capacity of CSC which drives continual expansion of the population of malignant cells, these cells remain largely quiescent allowing them to evade traditional chemotherapy which

targets rapidly dividing tumour cells [56-58]. The resistance of these potent CSC to chemotherapy and customary cancer therapeutics is likely to account for the high rates of disease recurrence associated with many cancers, and targeted eradication of these stem cells will be crucial for permanent elimination of the disease and improved overall outcome.

The existence of cancer stem cells in leukaemia was first proposed in the 1960s following the observation that only a small fraction of leukaemic cells isolated from patients can form colonies *in vitro* or can proliferate *in vivo* [59]. However it was not until the 1990s that a series of studies in which fluorescence-activated-sorted human AML cells were engrafted into non-obese diabetic-severe combined immune-deficient (NOD-SCID) mice, led to the definitive identification of a sub-population of primitive cells which were able to initiate and maintain the disease burden in irradiated recipients [60, 61]. This population of cells with *leukaemic initiating* ability were labelled leukaemic stem cells (LSC), and were shown to share the $CD34^{+}CD38^{-}$ immune-phenotype of normal haematopoietic stem cells. Additionally, it was shown that the $CD34^{+}CD38^{-}$ cell fraction was capable of reconstituting the leukaemia in irradiated mice regardless of the AML classification, suggesting that while the various AML subtypes display considerable developmental and phenotypic heterogeneity, there is a degree of functional conservation with regard to the more primitive leukaemic cells [61]. Subsequent studies have found that while the LSC represent a small portion of the total leukaemic cells, the size of the LSC compartment can vary widely between patients [62, 63], and importantly the LSC frequency at diagnosis was recently shown to be a relevant prognostic factor, as AML patients with increased

frequencies of $CD34^{+}CD38^{-}$ displayed significantly higher levels of minimal residual disease and decreased long term survival [64, 65].

As for other cancer stem cells, LSC can notionally arise from normal stem cells which have acquired mutations affecting normal growth regulation, or from more committed progenitor cells which gain self-renewal properties **(Figure 1.2)** [66, 67]. While the origin of LSC remains somewhat elusive, normal HSC are a prospective candidate as these cells intrinsically possess an array of pathways necessary for self-renewal, and in addition, their increased longevity allows sufficient opportunity for these cells to accumulate the genetic changes required for the leukaemic transformation. However, some recent studies indicate that certain mutations may be able to transform more committed progenitor cells that lack inherent self-renewal capacity suggesting that the early oncogenic events of stem cell pathogenesis may be noticeably different depending on the cell of origin [68-71].

1.2.3.1 Characterisation of the LSC phenotype

Nonetheless, LSC trigger a cascade of cell divisions which results in an ordered heterogeneous pool of cells with differing self-renewal potential, including mature AML blasts [61, 72-74]. This mirrors the hierarchical organisation of cells normally observed in the haematopoietic system which is driven by HSC and their progenitors, and there has been increased interest in the identification of cell-surface markers that are selectively or primarily expressed on LSC compared to normal HSC. An in-depth characterisation of the LSC phenotype will allow critical insight into the elemental mechanisms which may govern the leukaemic

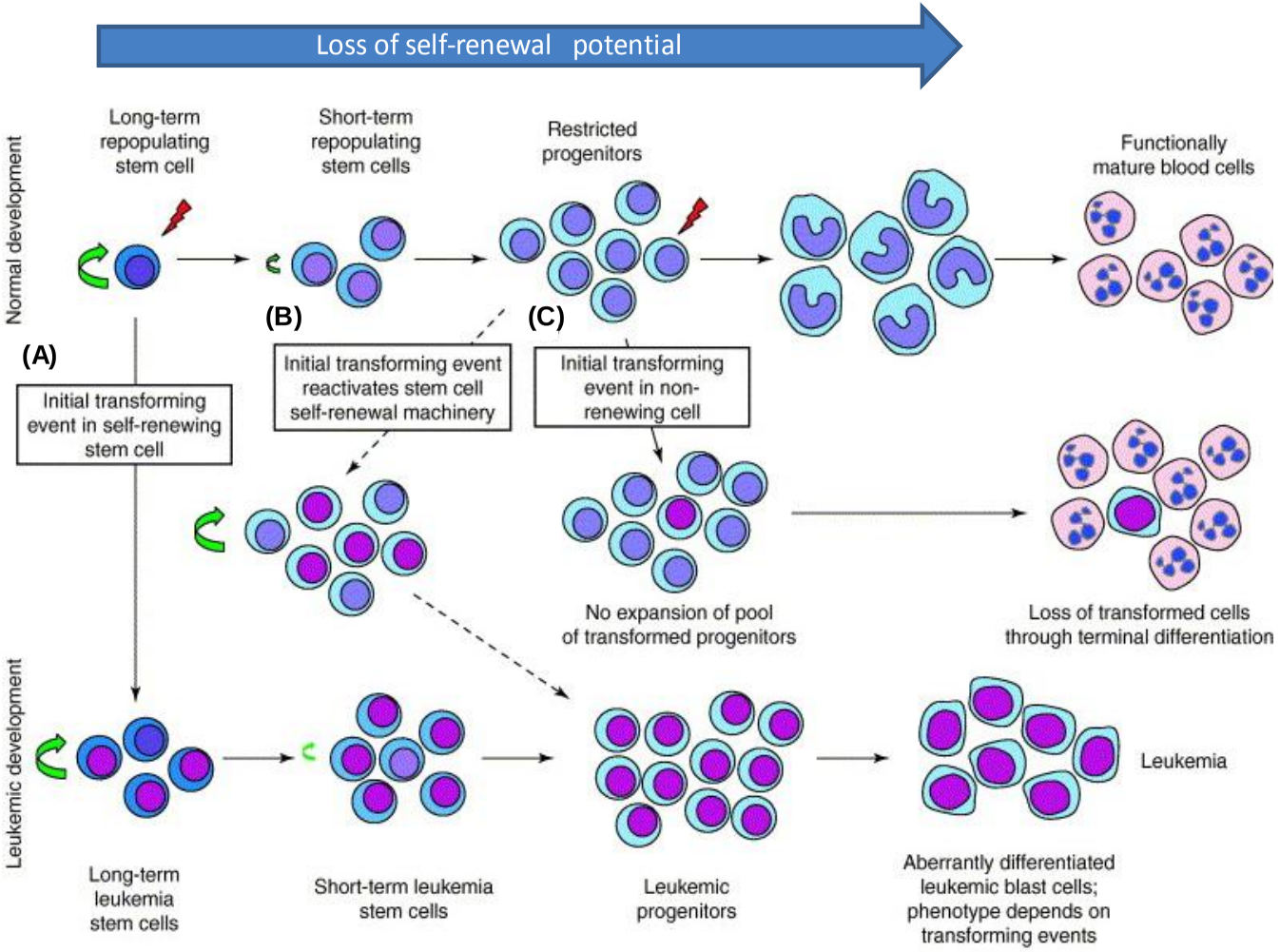


Figure 1.2 Differing initiating lesions and target cells in the formation of leukaemic stem cells. Self-renewal is a key property of both normal and leukaemic stem cells. Hence, less mutations are required to transform stem cells in which self-renewal properties are already active **(A)**, as compared with committed progenitors in which self-renewal must be obtained through additional lesions **(B)**. In the case where a committed progenitor with a limited life-span acquires a genetic mutation that does not confer increased self-renewal **(C)**, that cell will likely undergo apoptosis or terminal differentiation before sufficient mutations occur to initiate a complete leukaemic transformation.

Taken from Wang & Dick (2005) [74]

transformation, and will also facilitate the development of therapies targeted for specific elimination of LSC. Previous studies have identified a selection of cell surface markers which are differentially expressed on LSC compared to normal haematopoietic progenitor and stem cells. These include the absence of CD90 surface expression, the upregulation of IL-3Rα/CD123, CLL-1, CD96, CD47, and Tim3 [75-77].

IL-3Rα, which is the alpha-subunit of the interleukin-3 receptor (IL-3R), was one of the first surface antigens to be described as preferentially up-regulated on LSC [78], and the biological function of IL-3 signalling in normal and malignant myeloid cells is further described in section **4.1**. Importantly, it has been demonstrated that administration of 7G3, a monoclonal neutralising antibody to IL-3Rα, reduced the homing, engraftment, and serial transplantation of AML cells in immunodeficient mice [79]. Targeting of IL-3Rα is currently being investigated in the clinic, and trials are undergoing using a humanised version of 7G3. Furthermore, an IL-3-diphteria toxin fusion protein and IL-3Rα-directed single-chain Fv antibody immunotoxin fragments have also been developed as potential approaches to target IL-3Rα in AML [80-82].

Other studies have reported comparable results for a range of other surface molecules selectively expressed on AML LSC (Reviewed in [75, 76]), and given the recent advances in monoclonal antibody-based therapies, the targeting of LSC-specific surface antigens is becoming a more promising approach for AML therapy as it is clear that the eradication of this cell population may be critical to achieve durable remission [83-85].

In addition to cell surface proteins, identifying *intracellular signalling proteins* which are aberrantly expressed in LSC compared to HSC, will also provide novel venues for therapeutic targets. Recently, Majeti *et al* [86], performed a genome-wide microarray expression analysis to directly compare the expression profiles of highly enriched HSC and AML LSC. The analysis identified over 3000 differentially expressed genes and a range of pathways, many of which have been previously implicated in leukaemia including the JAK/STAT and NFκB pathways. Of particular interest, the Wnt/β-catenin pathway, which has established roles in cancer development, was one of the Top 10 signalling networks shown to be deregulated in the AML LSC populations. The remainder of this review will focus on the normal and aberrant regulation of this pathway, and how this protein may contribute to the development of AML.

1.3 The Wnt/β-catenin pathway in Cancer and Haematological malignancies

β-catenin is a multi-functional protein; its role largely determined by its subcellular localisation. At the plasma membrane β-catenin is an important component of the adherens junctions and links cadherin adhesion proteins to the actin cytoskeleton. A second, cytoplasmic pool of β-catenin, acts as the primary effector of the canonical Wingless-type (Wnt) signalling pathway [87-91].

The highly conserved canonical Wnt/β-catenin signalling pathway plays a role in the proliferation, survival, differentiation and migration of cells during development, and plays a critical role in maintaining homeostasis in a range of

adult tissues [87, 89, 92]. Importantly, disruptions in this pathway have been implicated in the development of many diseases, most notably cancer [93-95]. This section reviews the molecular regulation of the Wnt/β-catenin pathway, and outlines the current evidence supporting a role for activated β-catenin signalling in cancer with an emphasis on AML and other haematological malignancies.

1.3.1 Molecular regulation of the canonical Wnt/β-catenin pathway

In the absence of Wnt signalling, free cytoplasmic β-catenin is rapidly targeted for degradation by a multiprotein complex containing *(i)* the scaffold proteins axin and adenomatous polyposis coli (APC), and *(ii)* the serine/threonine kinases casein kinase 1 α (CK1α) and glycogen synthase kinase 3β (GSK3β) [96, 97]. After binding axin and APC, β-catenin is initially phosphorylated by CK1α at Ser45, which primes β-catenin for subsequent GSK3β-dependent phosphorylation of Ser33, Ser37 and Thr41 residues [96, 97]. Ser/Thr-phosphorylated β-catenin is then recognised by an E3 ubiquitin ligase complex containing β-transducin repeat containing protein (β-TrCP), which marks β-catenin for proteosomal mediated degradation **(Figure 1.3A)**.

Upon binding to their cell-surface receptor, consisting of Frizzled (FZ) and the associated low-density lipoprotein receptor-related protein 5/6 (LRP 5/6), the cysteine-rich Wnt proteins induce the stabilisation of β-catenin via the recruitment of axin and the inhibition of GSK3β **(Figure 1.3B)** [96, 98]. Stabilised β-catenin then accumulates in the cytoplasm, and translocates to the nucleus where it interacts with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) subfamily of transcription factors to induce expression of target genes including

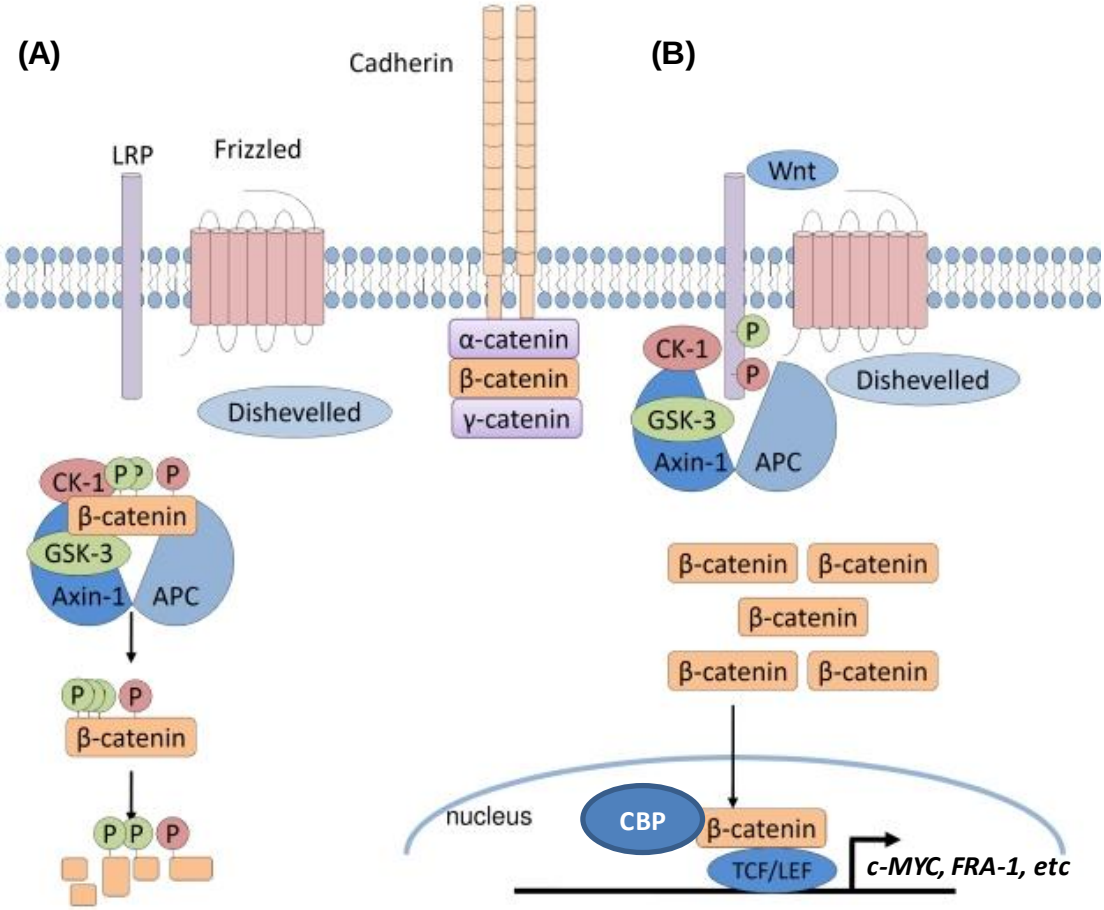


Figure 1.3 The canonical Wnt/β-catenin signalling pathway. (A) In the absence of Wnt signalling, β-catenin is phosphorylated in N-terminal Ser/Thr residues and marked for degradation by a regulatory complex containing axin, adenomatous polyposis coli (APC), and the serine/threonine kinases casein kinase 1 α (CK1α) and glycogen synthase kinase 3β (GSK-3β). Binding of Wnt proteins to transmembrane Frizzled/LRP 5/6 receptors **(B)** induces the stabilisation of β-catenin by inhibiting the β-catenin regulatory complex. Stabilised, β-catenin then translocates to the nucleus where it interacts with HMG-box containing TCF/LEF transcription factors, and activates transcription of target genes via the displacement of co-repressor and the recruitment of transcriptional co-activators such as CBP. Adapted from Voskas *et al* (2010). [99]

c*yclin D1* and *c-myc* and *fra-1* [87, 92]. In the absence of β-catenin, the high-mobility group (HMG)-box containing TCF/LEF transcription factors associate with co-repressors such as Groucho and CtBP, and repress the expression of Wnt/β-catenin target genes. Binding of β-catenin to the N-terminal domain of TCF/LEFs, is thought to displace any bound co-repressors and recruit a series of transcriptional co-activators such as CREB-binding protein (CBP)/p300, pygopus (Pygo) and legless (LGS)/BCL9, hence resulting in an active transcription-promoting complex **(Figure 1.3B)** [96, 97].

It is now evident, that in addition to the core factors mentioned above, there are many other cellular proteins that can directly or indirectly interact with β-catenin and other components of the Wnt pathway and act to modulate their localisation and activity [96, 100-102]. Based on the versatile role of β-catenin as a transcription factor and the critical role it plays in distinct cell types and developmental stages, it is likely that the expression and activity of the biomolecular components regulating Wnt/β-catenin signalling display a marked diversity amongst tissues. Furthermore, normal or abnormal fluctuations in regulatory proteins and pathways may alter the intensity of β-catenin signalling and have a profound effect on the fate of a cell.

1.3.2 Activation of β-catenin in cancer

Wnt/β-catenin signalling plays a critical role in regulating the self-renewal, proliferation and differentiation of cells in several stem-cell niches, including the skin and hair follicle, the mammary gland, the intestinal crypt and the bone marrow [87, 89, 92]. Consistent with this role, constitutive activation of β-catenin

signalling has been associated with tumorigenesis in these tissues [94, 101, 103, 104].

The prototypical example of aberrant activation of Wnt/β-catenin signalling in neoplastic disease is colorectal cancer. Inactivating mutations in APC, which lead to the stabilisation and accumulation of β-catenin, are observed in up to 85% of patients diagnosed with sporadic colorectal cancer [105, 106]. Importantly, constitutive activation of β-catenin has now been recognised as the rate-limiting and initiating event of this disease [107, 108]. The aberrant activation of β-catenin mediated transcriptional signalling in the intestinal crypt, disturbs the tightly regulated balance between epithelial cell proliferation and self-renewal/differentiation paving the way for tumorigenesis. Remarkably, the small fraction of colorectal cancers which lack APC mutations, have identifiable mutations in β-catenin which render the protein resistant to GSK3β-mediated phosphorylation and subsequent proteolytic degradation [108].

In addition, mutations in β-catenin and other components of the Wnt signalling pathway including axin and TCF4, all of which result in constitutive β-catenin signalling, have been detected in a range of other cancers including hepatocellular carcinoma, melanoma, and gastric, ovarian and colon cancers [93, 97]. Importantly, numerous studies have recently focused on the role of Wnt/β-catenin signalling in leukaemia, and accumulating evidence suggests that aberrant activation of this pathway may be a common event in haematological diseases.

1.3.2.1 β-catenin in normal and malignant haematopoiesis

Akin to the established role of Wnt/β-catenin in the homeostatic maintenance of numerous adult tissues, β-catenin has been shown to be an important regulator of the growth and survival of blood cells. Initial evidence for a role of Wnt signalling in haematopoiesis came from a series of studies which demonstrated that exposure of primitive haematopoietic cells to Wnt ligands significantly increased their proliferation *in vitro* and maintained the immature phenotype of these cells [109-111]. Accordingly, Wnt ligands and receptors were shown to be expressed in the HSC and primitive haematopoietic progenitors of both mice and humans, and these proteins are also expressed in the cells that make up the bone marrow microenvironment [110, 112].

These early findings were extended by the work of Reya *et al* [113], who directly examined the role of Wnt/β-catenin signalling in purified mouse HSC. In this study, HSC were isolated from mice overexpressing the anti-apoptotic *Bcl-2* gene and retrovirally transduced with a constitutively active form of β-catenin [113]. Overexpression of β-catenin extensively increased the proliferation of HSC *in vitro* and maintained the immature phenotype of HSC in long term cultures. In addition, cultured HSC expressing constitutively active β-catenin were capable of engrafting irradiated mice in contrast to the untransduced counterparts. Similarly, increased proliferation and also the maintenance of an immature phenotype and function was observed in human and mouse HSC induced to express β-catenin by the inhibition of GSK3β [114]. Furthermore, using a TCF/LEF-1 reporter, it was shown that HSC normally activate Wnt/β-catenin signalling *in vivo* and that

signalling is reduced as these stem cells give rise to more committed myeloid progenitor cells [115]. In accordance with this, enforced expression of constitutively active β-catenin in normal lymphoid or myeloid progenitors led to an increase in self-renewal and a block in differentiation [116], further supporting a role for β-catenin signalling in the maintenance of 'stem-like' properties**.**

While the studies described above support a role for Wnt/β-catenin signalling in HSC function, these investigations have mainly focused on the effects of abnormally elevated levels of β-catenin in the haematopoietic system. The reciprocal knockout studies however have generated controversial results. The deletion of β-catenin, the β-catenin homologue γ-catenin (plakoglobin) or both β-catenin and γ-catenin in adult mice using Mx1-Cre showed no defects in haematopoiesis of lymphopoiesis [117, 118], and similar observations were observed by an independent group [119]. These initial results led the authors to exclude a role for the canonical Wnt pathway in haematopoiesis. However, it was subsequently shown that TCF/LEF-dependent transcription remained intact in the combined absence of β-catenin and γ-catenin, and it has been suggested that partially functional β-catenin protein may persist in these animals [119, 120].

In contrast, a study by Zhao *et al* [121] which used Vav-Cre to induce β-catenin deletion in HSC, found that while the formation of HSC was normal in the absence of β-catenin, the self-renewal of these cells was significantly impaired when transplanted into recipient mice. Furthermore, Wnt3a deficiency, which correlates with reduced canonical Wnt signalling *in vivo,* led to a five-fold reduction in the numbers HSC and progenitor cells in the foetal liver (FL) and a

large reduction in self-renewal potential [122, 123]. One explanation for the discrepancies between the studies described above may lie in the developmental stage and cell-types in which β-catenin and/or γ-catenin genes were deleted. While the IFN-induced deletion of β-catenin or γ-catenin in the Mx1-Cre mice occurs in adulthood, the Vav-Cre system used by Zhao *et al* drives the deletion of β-catenin from embryonic life. This suggests that a requirement for β-catenin may be pronounced in foetal stem cells in line with the strong demand for proliferation and expansion of this cell population, an effect which may be otherwise obscured in adults and during homeostasis. The idea that β-catenin may be critical in conditions were extensive self-renewal and proliferation are required, is also supported by the observation that stem cell renewal defects in models with deficiencies in Wnt/β-catenin signalling were only apparent in secondary transplantation assays. Another difference between the two induction systems is the targeted cell-type; Mx1-Cre affects gene deletion primarily in HSC and mesenchymal stem cells [124], while Vav-Cre induces deletion in HSC and endothelial cells [125], which may also contribute to the discrepancies observed in these studies.

While more work will be required to delineate the precise role of Wnt/β-catenin in normal HSC, data from numerous laboratories support an important function for this pathway in haematopoiesis. Additional, studies in normal HSC development have prompted the exploration of Wnt/β-catenin pathway in the pathogenesis of leukaemia. The aberrant activation of β-catenin has now been reported in a range of haematological malignancies, and the evidence supporting a role for this pathway in the pathogenesis of leukaemia is described in the following sections.

1.3.2.2 β-catenin activation in AML

Analysis of primary AML samples revealed that these cells express significantly higher levels of β-catenin mRNA and protein than normal haematopoietic progenitors [126] and studies on AML cohorts, have similarly shown that constitutively active Wnt/β-catenin can be detected the majority of patients (over 60%) [127, 128]. Moreover, aberrant expression of additional Wnt-signalling components including Wnt-1, Wnt-2b and LEF-1 are observed in a large proportion of these cases indicative of a positive feedback loop [127].

To determine whether β-catenin expression in AML correlates with clonogenic capacity, Ysebaert *et al* [128] performed methylcellulose based colony formation and replating assays with primary AML samples which were classified for the presence or absence of β-catenin. It was found that AML cells expressing β-catenin had enhanced clonogenic capacity and self-renewal potential suggesting that activation of this pathway in AML may play an important role in the leukaemic transformation. Expression of β-catenin was subsequently documented as an important prognostic factor in AML, and overexpression of this signalling molecule has been associated with both shortened relapse-free survival and poor overall survival [128-130]. Critically, the Wnt/β-catenin pathway was found to be one of the key signalling networks deregulated in LSC compared to normal haematopoietic stem cells HSC [86]. Imaging flow cytometry has confirmed that there is increased β-catenin nuclear localisation in AML LSC compared to normal progenitors, consistent with the idea that this pathway may play an active role in the LSC compartment [131].

While a requirement for the activation of β-catenin in the maintenance of LSC across all AML subtypes is not clearly established [131], recent *in vivo* studies suggest that β-catenin activity may be particularly critical for the maintenance and drug-resistant properties of AML LSC in patients with mixed-lineage leukaemia (MLL) translocations [132, 133]. To determine whether β-catenin also plays a role in the establishment of MLL LSC, inducible deletion of β-catenin was performed in pre-LSC, defined in this model as early transduced primary cells carrying the initiating event with the capacity to induce leukaemia with a long latency. MLL pre-LSC with ablated β-catenin failed to induce leukaemia in syngeneic mice, indicating that β-catenin is not only essential for the long-term maintenance of LSC, but also for the establishment of these cells *in vivo* [133]. In addition, *in vitro* analysis of primary AML samples supports a critical role for β-catenin in the self-renewal potential of MLL LSC, and introduces a novel window for therapeutic targeting of LSC in patients with MLL leukaemia [133]. There has been increasing interest in the pathogenesis of MLL-AML since the demonstration that certain MLL fusion proteins possess the ability to confer stem cell renewal properties to normal myeloid progenitors, together with the fact that MLL leukaemia represents one of the most aggressive and worst prognostic groups among all haematological malignancies [69, 134]. Of importance, the activation of β-catenin has been shown to be required for the leukaemic transformation of granulocyte macrophage progenitors by Meis1/HoxA9 or MLL-AF9 *in vivo*, further supporting a pivotal function for this oncogene in AML associated with MLL fusions or HOX genes [132].

While the mechanisms underlying β-catenin stabilisation in leukaemia are still not fully understood, the translocation products AML1-ETO and PML-RARα, as well as commonly occurring activating mutations in the receptor tyrosine kinase FLT3, have been shown to activate β-catenin signalling in leukaemic cells [135, 136]. In addition, Wnt antagonist genes are commonly transcriptionally repressed by promoter methylation in AML, and this is directly associated with activation of Wnt/β-catenin signalling and a poor prognosis [137]. Interestingly, the close structural and functional homologue of β-catenin, γ-catenin, was recently demonstrated to be overexpressed in AML, inducing β-catenin stabilisation and nuclear translocation in leukaemic cells, but not in normal $CD34^+$ haematopoietic progenitor cells suggesting that normal Wnt regulatory elements may be bypassed or altered in AML [138]. It is likely that several pathways and molecular mechanisms may result in the accumulation of β-catenin in AML, however, the molecular mechanisms that govern β-catenin regulation, in the absence of these other lesions, remains poorly understood.

1.3.2.3 β-catenin activation in CML and other leukaemias

The aberrant activation of Wnt/β-catenin has also been implicated in the development and progression of CML. In contrast to the complex molecular genetics of AML, CML displays and overt and well-defined pathology and is consistently associated with the 9;22 chromosomal translocation and the presence of the Bcr-Abl oncoprotein which drives the malignant expansion of leukaemic cells [139].

CML originates as an indolent disease, and its initial *chronic phase* is characterised by the Bcr-Abl-mediated hyperproliferation of transformed myeloid cells without a distinctive block in differentiation. In the majority of cases, CML progresses from chronic phase to *blast crisis*, the terminal phase of the disease [140]. Blast crisis has been strongly associated with the upregulation of Bcr-Abl mRNA and protein, and the prominent expansion of committed granulocyte-macrophage (GM) precursors. Unlike normal GM progenitors, however, the GM precursors from CML blast crisis display self-renewal capacity *in vitro* [141]. Importantly, β-catenin levels are significantly increased during the transition to blast crisis, and this event has been specifically linked to the enhanced self-renewal and leukaemic potential of these GM precursors [141].

Interestingly, Bcr-Abl was found to directly interact with β-catenin and promote its stabilisation and nuclear accumulation [142]. Furthermore, silencing of β-catenin by targeted siRNAs reduced the proliferation and clonogenicity of Bcr-Abl$^+$ CML cells *in vitro* [142]. It is not clear, however, whether the activation of β-catenin observed in blast crisis is a direct result of increased Bcr-Abl levels; it is possible that other mechanisms may contribute to the stabilisation of β-catenin in CML. Critically, Zhao *et al.* [121] recently demonstrated that β-catenin signalling is required for the progression of Bcr-Abl mediated CML *in vivo*. In this study conditional β-catenin-/- mice were generated using the Cre/LoxP system under the control of vav regulatory elements, to induce the deletion of β-catenin predominantly in the haematopoietic system. While control Bcr-Abl$^+$ CML leukaemic cells propagated leukaemia in 65% of the recipient mice following transplantation, leukaemic cells from β-catenin-/- mice did not propagate CML

formation in any of the recipient mice. Further analysis revealed that loss of β-catenin prevented CML formation by reducing the long-term propagation, i.e. self-renewal, of these cancer cells [121].

In addition to a role for aberrantly activated Wnt/β-catenin in the pathogenesis of myeloid leukaemias described above, emerging studies suggest that β-catenin may also be implicated in lymphoid neoplasia. Activation of Wnt/β-catenin in both B- and T- cell acute lymphoblastic leukaemia, has been associated with the expansion of these malignancies, and the upregulation of canonical pathway genes including Wnt16, Frizzled 3 and TCF-3 is commonly observed in these leukaemias [143-145]. Overexpression of β-catenin has also been documented in multiple myeloma (MM), a haematological malignancy characterised by the overproduction of terminally differentiated B-cells, and importantly inhibition of β-catenin transcriptional activity in MM cells decreases growth and survival [146]. A potential role for Wnt/β-catenin signalling has also been suggested in mast cell leukaemia and chronic lymphocytic leukaemia [147, 148]. More work is required, however, to determine the pathways which contribute to the activation of β-catenin in these neoplasms.

The widespread action of oncogenic Wnt/β-catenin signalling in diverse haematological malignancies outlined in this section, suggests that targeting of this pathway may be an efficient therapeutic strategy for several of these diseases. While Wnt/β-catenin inhibitors for the treatment of haematological disorders have not reached clinical trials, pre-clinical studies in AML, CML and MM provide a

promising outlook for this approach [149, 150], and the identification and development of novel and specific small molecule Wnt inhibitors will be critical.

1.3.3 How does β-catenin signalling contribute to leukaemogenesis?

While accumulating data supports a role for activated Wnt/β-catenin in the pathogenesis of AML and other leukaemias, relatively little is known about the specific transcriptional and molecular contributions of this pathway to leukaemogenesis. In this section, we propose two non-exclusive mechanisms by which aberrantly activated β-catenin may promote oncogenesis in AML:

(i) activation of pro-proliferative target genes,

(ii) maintenance of the leukaemic stem cell population: signalling within the haematopoietic niche.

1.3.3.1 Activation of pro-proliferative target genes

As described in section 2.1, stabilised β-catenin translocates to the nucleus where it interacts with members of the TCF/LEF family of transcription factors to drive expression of target genes. Many recognized Wnt/β-catenin targets such as *c-myc, fra-1* and *cyclin D1* are proto-oncogenes, and the constitutive activation of these genes as a result of β-catenin overexpression in cancer, likely contributes to the oncogeneic potential of this pathway [103, 151].

However, it is clear that Wnt/β-catenin can induce distinct genetic programmes in a tissue specific manner such that a given cell will contain a specific combination of Wnt-responsive and -non responsive genes [92, 152, 153]. Numerous

endogenous Wnt/β-catenin target genes have been identified and confirmed in a range of tissues although the relevance of these genes to the transcriptional activity of β-catenin in leukaemia remains largely unknown. To date no large-scale analysis has been performed to identify the genes targeted by Wnt/β-catenin signalling in the haematopoietic system, and studies have focused on select candidate genes.

The expression of *c-myc* correlates with that of β-catenin as this proto-oncogene is expressed in immature myeloid cells and is normally downregulated during myeloid differentiation [154, 155]. Accordingly, activation of β-catenin in AML has been associated with increased TCF/LEF-dependent transcription of *c-myc* [136, 156]. The c-Myc protein has a critical role in cell-cycle regulation and proliferation, and the ability for c-Myc to promote cell transformation and tumour progression in a range of cancers makes this gene an important downstream effector of β-catenin in AML [151, 157].

The Wnt/β-catenin target gene, survivin, has also been shown to be highly expressed in the majority of AML patients [158]. Survivin belongs to the family of inhibitor of apoptosis proteins (IAPs), and is a key regulator of cell division and programmed cell death [159]. Interestingly, overexpression of survivin is detected in most cancers, and is associated with a poor prognosis and increased risk of relapse [159-161]. The strong anti-apoptotic effect of survivin likely contributes to the deregulated balance between proliferation and cell death in AML, and possibly plays an important role in chemoresistance. Furthermore, activation of Wnt/β-catenin signalling in normal HSC was shown to upregulate

the expression of transcription factors and cell-cycle regulators implicated in HSC self-renewal including *Notch1* and *HoxB4* [114, 162]. The aberrant activation of these genes in primitive leukaemic cells may hence contribute to the clonogenicity of AML cells.

It is likely that stabilised β-catenin activates an additional range of genes important for the proliferation, cell-cycle progression, and survival of leukaemic cells. Nonetheless, a large-scale analysis of Wnt/β-catenin target genes in normal and malignant haematopoietic cells using the combination of microarray technology and chromatin immunoprecipitation based assays, will be crucial to gain a better understanding of the downstream molecular responses associated with activated Wnt/β-catenin transcriptional signalling in AML.

1.3.3.2 Maintenance of the leukaemic stem cell population: signalling within the haematopoietic niche

Although the ability to self-renew is believed to be essential for the leukaemic potential of LSC, the molecular pathways which regulate this process remain poorly defined. AML is organised in a hierarchy of functionally distinct cells which arises from the LSC pool, and hence LSC appear to retain some of the processes that normally regulate stem cell developmental programs [61, 72]. Interestingly the polycomb group gene *Bmi-1,* a key regulator of HSC self-renewal, has also been implicated in maintaining the self-renewal capacity of LSC [163, 164]. Nonetheless, the sustained expansion of the leukaemic clone suggests that self-renewal may be deregulated in LSC, and transplantation studies have

indicated that LSC posses significantly higher self-renewal potential than normal HSC [61].

Critically, a recent genome-wide microarray study comparing the expression profiles of highly enriched HCS and AML LSC identified Wnt/β-catenin as a central pathway deregulated in AML LSC [86]. Furthermore, there is increasing evidence supporting a role for activated β-catenin signalling in the pathogenesis of AML, and a requirement for β-catenin in the maintenance of LSC from patients harbouring MLL fusion proteins (see section 3.2.1).

In addition to intrinsic mechanisms, extrinsic signals emanating from the microenvironment niche are crucial in the regulation of HSC self-renewal and quiescence [8-10]. The HSC niche is located within the bone marrow cavity and comprises osteoblasts, oesteclasts, stromal fibroblasts, and elements of the extracellular matrix, which synthesise or secrete a range of signalling molecules, growth factors and cytokines [10, 165]. Importantly, Wnt/β-catenin signalling between cancer cells and the stromal microenvironment has been found to contribute to the maintenance of tumours, and may play a similar role in the maintenance of LSC [95]. LSC, like HSC, require direct contact with the bone marrow niche to maintain their self-renewal capacity and quiescent nature suggesting that LSC may exploit the normal mechanisms that are involved in the long-term preservation of HSC [166]. Furthermore, treatment of AML cells with compounds which prevent LSC homing and engraftment to the bone marrow, impede the development of AML in transplantation experiments [83, 167]. Recent studies have shown that Wnt/β-catenin signalling between HSC and the

bone marrow microenvironment plays an important role in the functional maintenance of these stem cells and activation of Wnt/β-catenin in the bone marrow niche appears to be crucial for the preservation of HSC self-renewal and quiescence [168-170]. It is hence plausible that aberrant β-catenin signalling in LSC may instigate a positive feed-back loop resulting in increased secretion of Wnt ligands, which in turn may enhance Wnt/β-catenin activity in the bone marrow stroma and reinforce the self-renewal potential of these leukaemic cells.

1.4 Molecular regulation of β-catenin stabilisation in AML: a potential link with IL-3 signalling

While mutations in components of the Wnt-signalling pathway are commonly identified as the source of constitutive β-catenin signalling in an array of solid cancers [89, 94, 97], mutations in β-catenin or its central regulatory proteins are rarely identified in leukaemias [127, 142, 146, 171] indicating that additional non-canonical mechanisms underlie β-catenin stabilisation in these haematological malignancies.

As previously described (refer to section 3.2.1), numerous chromosomal translocations associated with AML pathogenesis including AML1-ETO and PML-RARα, as well as the β-catenin homolog γ-catenin activate Wnt/β-catenin signalling in leukaemic cells [136]. Stabilisation of β-catenin in AML has also been associated with activating mutants of the cytokine receptor FLT3 [135, 172]. Interestingly, β-catenin stabilisation in mast cell leukaemia has been attributed to activating mutations in the c-Kit receptor, and signalling of the T-cell receptor in

primary human T-cells has also been demonstrated to activate the β-catenin pathway [147, 173].

Together, these studies suggest that the stabilisation of β-catenin by activated haematopoietic receptors may be a common mechanism underlying β-catenin accumulation in normal and malignant haematopoiesis, and in this work we have investigated a potential role for the IL-3 receptor in the modulation of β-catenin signalling in myeloid and leukaemic cells.

1.4.1 IL-3 receptor biology, signalling and role in AML pathogenesis

IL-3 is a multi-potent growth factor which belongs to a family of secreted glycoprotein hormones that bind with high affinity to their cognate receptors and regulate the production and function of haematopoietic cells [174, 175]. IL-3 acts on a range of haematopoietic cell lineages promoting self-renewal of early multi-potential cells, proliferation, survival and differentiation of myeloid progenitors and survival and activation of mature haematopoietic cells such as mast cells, eosinophils and macrophages [174-176]. The IL-3R is comprised of a ligand-specific 60-70kDa α-chain (IL-3Rα/CD123), and a 130-160kDa common β-chain (βc) which is shared with the receptors for GM-CSF and IL-5 [174, 175, 177]. Although there are some redundancies in the pathways activated by these receptors due to the sharing of a common βc subunit, specificity is achieved through differential expression of the α-subunits, together with conferred specificity of ligand binding to the cytokine receptor complex. In mice, cytokine specificity is facilitated by the existence of an additional IL-3-specific β-subunit ($\beta^{IL\text{-}3}$) which is used in preference to the βc for IL-3R formation [178].

IL-3 binding to IL-3Rα induces the recruitment and interaction with βc resulting in the formation of a high affinity receptor and subsequent activation of intracellular signalling. While the majority of signal transduction appears to be mediated by tyrosine phosphorylation of βc, the cytoplasmic domain of IL-3Rα is also critical for signalling as deletion of the membrane proximal cytoplasmic region abrogates receptor signalling, whilst binding of the receptor to ligand remains unaffected [179]. To date, three principal signalling cascades have been associated with the IL-3 receptor: the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), the mitogen-activated protein kinase (MAPK), and the phosphatidylinositol 3-kinase (PI3K)-Akt pathways **(Figure 1.4)** (Reviewed in [174]). The fine-tuning of these signalling pathways by IL-3 and other haematopoietic cytokines is critical for the regulation of cell proliferation, survival and differentiation, and deregulations in these networks resulting from activating mutations in pathway components or elevated cytokine receptor signalling is commonly observed in AML.

Supporting a role for deregulated cytokine signalling in the development of leukaemia, autocrine production of IL-3 and other cytokines and growth factors such as GM-CSF, C-CSF, IL-6, IL-8, FLT3 ligand and VEGF by AML blast cells has been well documented [180-183]. Furthermore, *in vitro* studies have shown that primary AML cells exhibit a degree of proliferation in response to stimulation with these factors, and IL-3 in particular, promotes the clonogenicity and proliferation of more than 85% of AMLs [184-186]. At a functional level, a critical role for IL-3 was demonstrated in CML *in vivo*, as IL-$3^{-/-}$ BM cells transformed with Bcr-Abl display impaired leukaemogenic potential compared to

WT BM cells [187]. In AML, IL-3 has been shown to cooperate with *Hox* genes to induce aggressive and transplantable myeloid leukaemias in mice [188-190]. This is of particular interest, as β-catenin was recently shown to be critical for the leukaemic transformation of granulocyte macrophage progenitors by Meis1/HoxA9 *in vivo* (see section 3.2.1), supporting the idea that there may be a functional link between the IL-3 and β-catenin pathways.

More recently, overexpression of IL-3Rα has been reported on AML blasts, $CD34^+$ progenitors and LSC relative to normal haematopoietic cells [78, 191-193]. The upregulation of IL-3Rα in AML is not simply a passive marker, as overexpression of this protein on AML blasts confers increased proliferation and survival [193]. In addition, the overexpression of IL-3Rα in FDCP-1 cells was shown to allow cell proliferation at sub-optimal concentrations of IL-3, supporting a functional role for elevated levels of IL-3Rα in AML.

At a clinical level, elevated IL-3Rα expression is associated with increased blast counts at diagnosis, lower relapse-free survival and lower overall survival [193]. The significantly increased expression of IL-3Rα on LSC compared to normal HSC, has not only provided a good marker for the isolation of LSC, but also provides a promising target for the therapeutic eradication of the AML LSC population (see section 2.2.1). More work is now required to dissect the functionally relevant pathways activated by elevated IL-3 signalling in leukaemic cells.

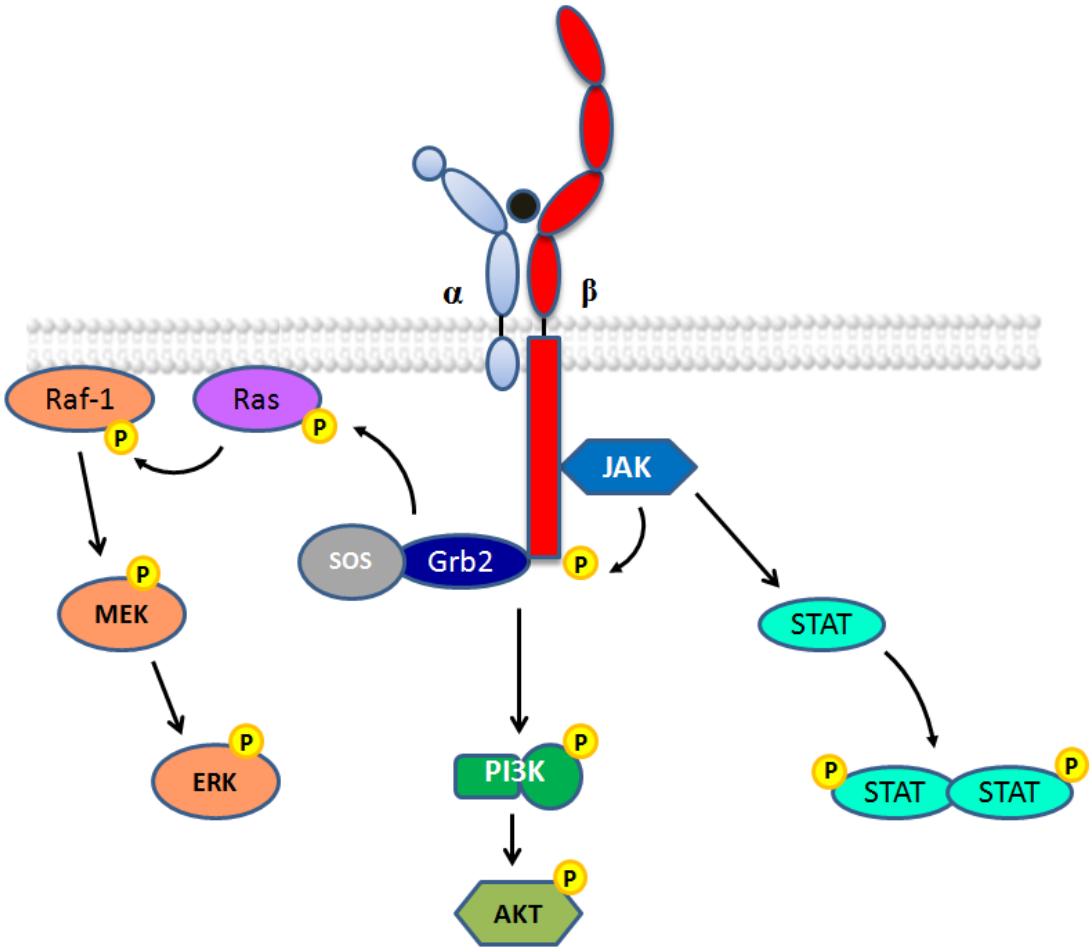


Figure 1.4 Intracellular signal transduction pathways activated by IL-3. Binding of IL-3 by IL-3Rα leads to recruitment of βc, tyrosine phosphorylation of βc cytoplasmic residues, and activation of the JAK/STAT, MAPK and PI3K/AKT pathways. The ultimate phosphorylation of STAT, ERK and AKT proteins subsequently result in activation of transcriptional programmes which regulate proliferation, survival and differentiation. Although signalling pathways are represented separately, there may be significant overlap between these pathways.

1.5. Project overview and aims

Accumulating evidence supports a pivotal role for β-catenin signalling in the development of AML. Critically, β-catenin levels are elevated in LSC compared to normal HSC, and a functional requirement for this pathway in the establishment and maintenance of LSC has been demonstrated in AML with MLL rearrangements.

While several recognised molecular lesions have been associated with increased β-catenin levels in AML, the mechanism by which β-catenin occurs in the absence of these lesions remains unknown. Another protein which is highly expressed on leukaemic blast and LSC compared to normal haematopoietic progenitors, is IL-3Rα. While the exact consequences of this increased expression are not well understood, studies suggest it may sensitise AML cells to IL-3 signalling. As several haematopoietic receptors have been linked to β-catenin in both normal and malignant haematopoietic settings, we propose that IL-3 may contribute to the regulation of β-catenin in AML.

The specific aims of this project were:

1. Investigate the potential link and mechanism between IL-3 signalling and the stabilisation of β-catenin in myeloid and leukaemic cell lines and primary AML patient samples.
2. Determine the functional significance of β-catenin downstream of IL-3 mediated growth and survival.

3. Utilise microarray gene expression technology to investigate the genome-wide gene expression changes induced by IL-3 in primary AML cells, and identify novel pathways which may contribute to a cytokine-mediated growth and survival signals in leukaemia.

CHAPTER 2

Materials and Methods

2.1 Cell culture protocols

2.1.1 Culture medium and supplements

All cells were cultured in medium supplemented with 2mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cultures were maintained in a Sanyo humidified incubator with 5% CO_2 at 37°C. Recombinant murine IL-3 (mIL-3) was produced from baculoviral vectors supplied by Dr Andrew Hapel (John Curtin School of Medical Research, Canberra). Recombinant human IL-3 (hIL-3) was purchased from Peprotech.

2.1.2 Cell lines and AML patient samples

The FDM cell lines utilised in this investigation were generated by Gabriela Brumatti (Walter and Eliza Hall Institute of Medical Research, Melbourne). FDM cells were maintained in DMEM (1000 mg/L glucose) with 10% Fetal Calf Serum (FCS) and 10ng/mL mIL-3. TF-1.8 cells were maintained in RPMI-1640 medium with 10% FCS and 2ng/mL hIL-3. Primary AML samples were obtained from the AML apheresis tissue bank at the IMVS, Adelaide. All patient samples were collected at diagnosis with informed patient consent, in accordance with the Declaration of Helsinki. For *in vitro* assays, primary AML patient samples were thawed and recovered overnight in IMDM with 20% FCS and DNase (50U/mL) and mononuclear cells were subsequently enriched using Lymphoprep (Axis-Shield PLC). AML cells were then cultured in IMDM with 10% FCS ± 15ng/mL hIL-3. All cell manipulation was carried out in an Oliphant Laminar Flow cabinet in a PC2, OGTR approved facility.

2.1.3 Production of retrovirus and transduction of haematopoietic cells

HEK293T cells were seeded at $1x10^6$ cells in 5 mL of medium per $25cm^2$ tissue culture flask and transfected with Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. The DNA constructs utilised, MSCV-IRES-GFP (MIG), MIG-Cre and MIG-β-catenin-S33A (MIG- βcatS33A), were transfected into HEK293T cells together with the ecotrophic pEQ packaging plasmid (kind gift of Dr. Elio Vanin, St. Jude Childrens Research Hospital, Memphis). 24 hours post-transfection HEK293T medium was replaced with complete medium relevant to the desired target cell line for collection of viral supernatant. Supernatant was harvested at 48 and 72 hours post transfection. FDM cells were transduced at $1x10^6$ cells per 2.5 mL viral supernatant supplemented with 4μg/mL polybrene. Cells were spinoculated two times with an intermittent incubation of 6 hours at 1800 rpm (Eppendorf Centrifuge 5810R) for 60 minutes. Cells were then further incubated for 24 hours in viral supernatant. Cells expressing GFP were subsequently sorted using a BD FACS Aria Flow Cytometer/Cell Sorter.

2.2 Bacterial techniques

2.2.1 Media and Solutions

All bacterial cell culture media and glassware was autoclaved prior to use and aseptic techniques were used to prevent contamination. Bacterial cell culture was carried out in Luria broth (LB- 10g Bacto-tryptone, 5g Bacto-yeast extract and 5g NaCl made up to 1L with Milli-Q water, pH 7.6) supplemented with 50mg/ml

ampicillin to select for transformed populations. Bacterial cultivation was carried out in an orbital shaker (Paton Industries, Australia) at 37°C overnight.

2.2.2 Midi-prep Plasmid Purification

Plasmid purifications were carried out using the QIAfilter Plasmid Midi Kit from QIAGEN. This method was used for the purification of DNA used in transfections and retroviral transductions. These were performed following manufacturer's instructions and DNA concentrations were determined using a DNA spectrophotometer (OD 260).

2.3 Pharmacological inhibitor and 7G3 Assays

2.3.1 Pharmacological inhibition of PI3K/AKT

FDM cells were treated with a dose response of the PI3K inhibitors LY294002 or Wortamannin or with the Akt inhibitor, Akt Inhibitor X (all from Calbiochem) for 16 hours in the presence of continuous mIL-3 (10ng/mL). DMSO was used as a vehicle control.

2.3.2 Treatment of leukaemic cells with 7G3

Leukaemic cells grown in the absence of IL-3 were treated ± 100nM of the IL-3 neutralising antibody 7G3 or an IgG2a isotype control (clone BM4) on ice for 20 minutes. Cells were then cultured in the presence or absence of 15ng/mL of hIL-3 for 3 hours. Antibodies were supplied by Dr. Hayley Ramshaw (Division of Immunology, Centre for Cancer Biology, SA Pathology, Adelaide).

2.4 Western immunoblotting

2.4.1 Isolation of protein from cell lines and primary AML samples

Cells were lysed for 30 minutes on ice in modified RIPA lysis buffer (1% Np-40, 0.1% SDS, 0.1% NaDeoxycholate, 100mM 19 NaCl, 2.5mM EDTA, 2.5mM EGTA, 50mM Hepes pH 7.4) containing protease and phosphatase inhibitors (Pefabloc, PhosSTOP and Complete Protease Inhibitors, all from Roche). Protein concentrations were determined using the Biorad DC Protein Assay, according to the manufacturer's instructions and 5X SDS load buffer (0.5M Tris-HCl (pH7.4); 0.8ml glycerol; 1.6ml 10% sodium dodecyl sulfate (SDS); 0.4ml β-mercaptoethanol; 0.2ml 0.5% (w/v) bromophenol blue; 4ml Milli-Q water) was added 1:4 (v/v) to each sample and boiled at 95°C for 5 minutes.

2.4.2 SDS-PAGE gel and western blotting

40-100 µg of each sample was separated using 8% SDS-polyacrylamide gel electrophoresis at 100V (Bio-Rad power supply, model 200/2.0, Bio-Rad Laboratories, Hercules, CA). Samples were subsequently transferred to PROTRAN nitrocellulose transfer membrane (Schleicher & Schuelle, USA) using BioRad wet transfer apparatus at 100 volts for 1 hour. The membranes were blocked for 1 hour at room temperature in PBST (1X PBS, 0.1% Tween-20) with 5% non-fat dry milk. The membranes were incubated overnight at 4°C in PBST with 5% non-fat dry milk containing anti-β-catenin (BD Biosciences), anti-dephospho(aa)35-50-β-catenin (Enzo LifeSciences), anti-Actin (Sigma), anti-phosphoS473-Akt (Cell Signaling), anti-Akt (Cell Signaling), anti-phosphoS9-

GSK3β (Cell Signaling), anti-GSK3β (Cell Signaling), anti-phosphoS552-β-catenin (Cell Signaling), anti-Bcl-2 mouse preferred (Cell Signalling), anti-Bcl-xL (Cell Signaling) or anti-phosphoY694/699-Stat5a/b (Millipore). All antibodies were used at the manufacturer's recommended concentration. Antibody binding was detected with a mouse or rabbit horseradish peroxidase conjugated secondary antibody (Pierce) followed by chemiluminescence detection (SuperSignal WestPico or WestDura Chemiluminescent Substrate, Pierce) and developed using the ImageQuant LAS 4000 digital imaging system (GE Healthcare).

2.5 Cellular assays

2.5.1 Cell Growth Assay

Cells were seeded in triplicate at 5 x10^4 cells/mL in a 96-well plate for each time-point (100 µL per well). Readings were taken every 24 hours for a total of 4 days. To assess cell growth at each time-point, 25 µL of CellTiter 96 AQueous One Solution (Promega) was added to each well and the plates were incubated at 37°C for 4 hours. Plates were read using the Opsys MR (Dynex Technologies) plate reader with a wavelength of 490nm.

2.5.2 Colony formation

Equal numbers of FDM cells transduced with MIG-Cre or the MIG control were seeded in semi-solid methylcellulose (StemCell Technologies) with 10ng/mL of mIL-3 three days after sorting for GFP. Colonies were counted in triplicate plates after seven days in methylcellulose culture at 37°C.

2.5.3 3H Thymidine incorporation assay

5 x10^4 AML mononuclear cells were seeded in triplicate in 96-well plates with a titration of IL-3 for 48 hours at 37ºC. 3H-thymidine (MP Biomedicals Australasia, Sydney, Australia) was added for the final 24 hours at a concentration of 0.5µCi per well. Cells were deposited onto glass fiber paper using a Packard Filtermate cell harvester (PerkinElmer Life and Analytical Sciences, Melbourne, Australia) and counted using a Top Count (PerkinElmer).

2.5.4 Cell Viability analysis

Cells were diluted 1:1 with Trypan Blue (Invitrogen) and viable and non-viable cells were manually counted based on Trypan Blue exlusion using a Neubauer hemacytometer slide on an inverted microscope.

2.5.5 Cell-cycle analysis

1x10^6 cells were washed twice with cold PBS and fixed overnight with 70% ethanol at 4°C. Cells were spun and incubated in PBS, 0.1% Triton X-100, 100µg/mL RNase A and 40 µg/mL propidium iodide for 30 min at 37 °C in the dark. Cells were subjected to flow cytometric analysis of DNA content.

2.5.6 Morphology

Up to 30, 000 cells were centrifuged onto glass slides using a Thermo Shandon Cytospin 4 cytocentrifuge at 500 rpm for 5 min. Slides were air-dried then Wright-Giemsa stained, courtesy of the IMVS Core Laboratory (SA Pathology, Adelaide). Slides were mounted using DEPEX mounting medium (Sigma) and

cover-slipped for analysis. Photographs were taken using an Olympus BX51 microscope and DP70 camera system.

2.5.7 Surface marker expression

2.5.7.1 Differentiation marker analysis of FDM cell lines

1x 10^4 to 10^6 cells were resuspended in 50μL of FACS wash solution (5% FCS and 1% sodium azide in 1x PBS) containing Phycoerythrin (PE) conjugated anti Gr-1 (eBioscience), c-FMS (eBioscience) or F4/80 (BD Biosciences) antibody, then incubated in the dark for 30 min on ice. Antibodies were used at the manufacturer's recommended concentration. Cells were then washed twice in FACS wash and fixed in 200-400 μL of FACS fix solution (2% glucose, 1% formaldehyde and 0.02% sodium azide in 1x PBS) and stored at 4°C prior to flow cytometric analysis.

2.5.7.2 IL-3R surface expression analysis of primary AML cells

Following ficoll centrifugation, 1x 10^6 primary AML cells from each patient sample were resuspended in 50μL of FACS wash solution (5% FCS and 1% sodium azide in 1x PBS) containing anti IL-3Rα (eBioscience, clone 9F5), βc (provided by Hayley Ramshaw, clone IC1) or IgG1 isotype control (BD Biosciences) antibody, then incubated for 30 min on ice. Cells were washed twice in FACS wash then incubated for a further 30 min on ice with Biotinlyated horse anti-mouse IgG (Vector Laboratories). Cells were then washed again, and incubated on ice in the dark for 30 min with Streptavidin-PE (Invitrogen). Following staining, cells were washed twice in FACS wash and fixed in 200 μL of

FACS fix solution (2% glucose, 1% formaldehyde and 0.02% sodium azide in 1x PBS) and stored at 4°C prior to flow cytometric analysis.

2.6 Immunofluorescence staining

$5x10^4$ cells were cytospun onto glass microscope slides using a Thermo Shandon Cytospin 4 cytocentrifuge at 500 rpm for 5 min, and fixed with 1C Fixation Buffer (eBioscience) for 20 minutes at room temperature. Cells were washed three times and permeabilised with 1 x Permeabilization Buffer (eBioscience). Anti-β-catenin (BD Biosciences) was used at 1:500 in 1 x Permeabilization Buffer overnight at 4°C. Slides were washed three times with 1 x Permeabilization Buffer and subsequently incubated with 1:100 goat anti-mouse Alexa-Fluor-488 (Invitrogen) for 1 hour at room temperature in the dark. Slides were washed again three times with 1 x Permeabilization Buffer, and mounted with ProLong Gold reagent with DAPI (Invitrogen). Slide were viewed and photographed using a BioRad Radiance 2100 confocal microscope with Laser Sharp Software version 5.

2.7 Quantitative Real-time PCR (QRT-PCR)

Total RNA was isolated using either the Qiagen miRNeasy Mini kit or the Invitrogen Trizol protocol according to the manufacturer's instructions. Quantitation of RNA was performed using a NanoDrop Spectrophotometer. Reverse transcription was performed using QuantiTect Reverse Transcription kit (Qiagen) with the gDNA Wipeout Buffer to remove genomic DNA contamination, according to manufacturer's instructions. QRT-PCR reactions were set up using the Roche FastStart Taq system (Roche Applied Systems)

according to manufacturer's instructions, and the reactions were performed on a Corbett RotorGene 6000 instrument with the following cycling conditions: 95°C for 10 min (1 cycle); 95°C for 25 sec - 60°C for 25 sec - 72°C for 30 sec (40 cycles, acquiring to green); 72° for 1 min (1 cycle). For each sample duplicate reactions were performed for detection of the gene of interest and a control gene (*ACTB* or *GAPDH*). QGene Software was used for analysis of relative mRNA expression levels. Oligonucelotide primer sequences are shown in **Table 2.1.**

2.8 Microarray Analysis of Primary AML patient samples

Total RNA was extracted using Qiagen miRNeasy kit. Microarrays were performed using Affymetrix Human Gene 1.0 ST arrays. The raw data were normalised using RMA as implemented in aroma.affymetrix. Differential gene expression for IL-3 treatment was analysed using moderated t-statistic linear model for microarray analysis (LIMMA), after adjusting for pairing and the Empirical Bayes. Differential gene expression was adjusted for multiple testing to control the false discovery rate (FDR) using Benjamini-Hochberg method. Genes with FDR $p<0.05$ were considered statistically significant. All statistical analyses were performed using R statistical software. Gene-set enrichment analysis (GSEA) was performed against Broad Institute Molecular Signature Database (MSigDB, v3.1) to determine significant enrichment between IL-3 differential gene expression and MSigDB signatures. To determine statistically significant enrichment of the differential IL-3 gene expression and transcription factors based on ChIP, ChIP enrichment analysis (ChEA GUI version) was performed against 197 transcription factors based on a 212 publications database.

Table 2.1 Sequence of oligonucleotide primers used for QRT-PCR analysis of gene expression.

Detects	Species	Forward Primer (5'-3')	Reverse Primer (5'-3')
Actb (control)	*Mus musculus*	AGTGTGACGTTGACATCCGTA	GCCAGAGCAGTAATCTCCTTCT
ACTB (control)	*Homo sapiens*	AAGAGCTACGAGCTGCCTGAC	GTAGTTTCGTGGATGCCACAG
Bcl-2	*Mus musculus*	ATGCCTTTGTGGAACTATATGGC	GGTATGCACCCAGAGTGATG
Bcl-xL	*Mus musculus*	GACAAGGAGATGCAGGTATTGG	TCCCGTAGAGATCCACAAAAG
FANCC	*Homo sapiens*	CTGCCATATTCCGGGTTGTTG	AGCACTGCGTAAACACCTG
CISH	*Homo sapiens*	GAACTGCCCAAGCCAGTC	CTATGCACAGCAGATCCTCC
FRA-1	*Homo sapiens*	CAGGCGGAGACTGACAAACTG	TCCTTCCGGGATTTTGCAGAT
GAPDH (control)	*Homo sapiens*	GAGCGAGATCCCTCCAAAATC	GGCTGTTGTCATACTTCTCATGG
PTGS2	*Homo sapiens*	TTCAAATGAGATTGTGGAAAAATTGCT	AGATCATCTCTGCCTGAGTATCTT
MYC	*Homo sapiens*	GTCAAGAGGCGAACACACAAC	TTGGACGGACAGGATGTATGC
UPP1	*Homo sapiens*	CTGTCAGTCATGGTATGGGC	GAGCACCGGGCATAGTAC

2.9 Statistical Analysis and Presentation of Data

Error bars represent the standard error of the mean. Probability and statistical significance were analysed using the two-tailed student *t* test with a confidence interval of either 95% ($p<0.05$) or 99% ($p<0.01$). Experiments were carried out a minimum of three times ($n=3$) unless otherwise stated.

CHAPTER 3

IL-3 Mediated Regulation of β-catenin in Myeloid and Leukaemic Cell Lines

3.1 Introduction

β-catenin is a key signalling molecule that is critical to normal development and adult tissue homeostasis, and is involved in pathways that govern self-renewal, cell growth and survival, and differentiation [92, 104, 194]. Aberrant activation of this signalling pathway has been implicated in the pathogenesis of malignant neoplasms, including a range of haematological malignancies such as acute myeloid leukaemia (AML), multiple myeloma, acute lymphoblastic leukaemia and chronic myeloid leukaemia [121, 127, 145-147].

In AML, increased expression of β-catenin is associated with enhanced blast clonogenicity and is clinically associated with reduced relapse free and overall survival [128, 130]. The stabilisation and activation of β-catenin in AML has been previously associated with activating internal tandem duplication (ITD) mutations in the FLT3 receptor which occur in around 20-25% of patients. FLT3-ITD has been demonstrated to directly interact with β-catenin resulting in its tyrosine phosphorylation, nuclear translocation and ultimately activation of β-catenin target genes [135, 147, 177]. The oncogenic fusion proteins AML1-ETO and PML-RARα, and the close structural and functional homolog of β-catenin, γ-catenin, have also been associated with increased β-catenin activity in leukaemic cells [136, 138]. In addition, abnormal promoter methylation of specific Wnt/β-catenin inhibitors has been reported in myeloid malignancies [137, 195, 196]. Aberrant β-catenin activation, however, has been observed in patients in the absence of these other lesions indicating that other molecular mechanisms are involved in the deregulation of β-catenin signalling in AML.

Consistent with the role of β-catenin signalling in the regulation of cell proliferation, survival and self-renewal, accumulating evidence indicate that aberrant activation of β-catenin also occurs in the highly specialised leukaemic stem cell (LSC) population [86, 131-133]. The Wnt/β-catenin pathway was identified as one of the key signalling networks deregulated in the LSC population in a recent study which compared the expression profiles of highly enriched haematopoietic stem cells (HSC) and AML LSC, and increased β-catenin nuclear localisation has been directly observed in AML LSC compared to normal $CD34^+$ progenitors [86, 131]. Functionally, β-catenin activity has been shown to be critical for the maintenance and drug-resistant properties of AML LSC in patients with mixed-lineage leukaemia (MLL) translocations, a subtype of AML which is characterised by increased expression of *HOX* genes, and a particularly poor prognosis [132, 133]. In addition, β-catenin is required for the leukaemic transformation of granulocyte macrophage progenitors (GMP) by Meis1/HoxA9 or MLL-AF9 *in vivo*, further supporting a pivotal function for this oncogene in AML associated with MLL fusions or Hox genes [132].

Another defining feature of AML LSC is the increased expression of the alpha subunit of the interleukin-3 receptor (IL-3Rα) relative to normal HSC [78, 191-193]. The overexpression of IL-3Rα in AML has been associated with higher blast counts at diagnosis, lower complete remission rate, and reduced overall survival [193]. IL-3 is a multipotent haematopoietic growth factor that acts on a variety haematopoietic lineages; promoting self-renewal of early multipotent cells, proliferation, survival and differentiation of myeloid progenitors and survival and activation of mature haemapoietic cells such as mast cells and basophils [4, 197,

198]. The receptor for IL-3 (IL-3R) comprises the ligand-specific α-chain and a common β-chain (βc) which is shared with the receptors for GM-CSF and IL-5. IL-3Rα binds IL-3 with high specificity but with low affinity, and the interaction of IL-3Rα with βc results in a high affinity receptor complex with subsequent activation of intracellular signalling cascades including activation of the JAK/STAT, MAPK and PI3K/AKT signalling pathways (reviewed in [174]). While activities of IL-3 overlap considerably with those of GM-CSF, IL-3 has the unique property of acting as a proliferation and/or survival factor in HSC and has been reported to promote self-renewal [199-201]. Importantly, retained responsiveness to IL-3 and other haematopoietic cytokines is observed in leukaemic cells, and recombinant IL-3 can promote the growth and clonogenicity of more than 80% of AMLs *in vitro* [184, 186, 202]. *In vivo,* enforced expression of IL-3 in haematopoietic cells induces a myeloproliferative disorder in mice, and results in an aggressive transplantable myeloid leukaemia when expressed in conjunction with HoxB8 [188]. Critically, given the recent link between *Hox* genes and β-catenin, this raises the possibility that IL-3 may act, in part, through the induction of the β-catenin pathway.

In this chapter we have investigated a link between IL-3 signalling and the regulation of β-catenin stabilisation and activity in myeloid and leukaemic cell line systems. In addition, as previous studies have demonstrated that cytokines can induce the inhibitory phosphorylation of GSK3β via activation of the PI3K/AKT pathway, we have made use of pharmacological PI3K and AKT small molecule inhibitors to determine the importance of this axis in the IL-3 mediated regulation of β-catenin. Lastly, we have used a model of Hox oncogenesis based

on HoxB8 transformation of IL-3 dependent myeloid cells to investigate the functional significance of β-catenin downstream of IL-3 signalling.

3.2 Results

3.2.1 β-catenin is stabilised and activated by IL-3 signalling in FDM myeloid cells

We utilised an IL-3 dependent myeloid cell line model to investigate the link between IL-3 signalling and the activation of β-catenin in haematopoietic cells. These factor-dependent myeloid (FDM) cell lines were generated by the transduction of E14.5 murine foetal liver progenitors with HoxB8 retrovirus in the presence of saturating levels of IL-3 (as previously described in [203]) and provided a unique system to analyse IL-3 signalling in a model of Hox-oncogenesis.

To determine if IL-3 can modulate β-catenin protein expression, we firstly analysed β-catenin protein levels in FDM cells cultured in the presence or absence of 10ng/mL mIL-3 or in a time-course of IL-3 stimulation by western blotting. As shown in **Figure 3.1 A**, total β-catenin protein levels were reduced in FDM cells after a 16h withdrawal of IL-3, and accumulated in response to IL-3 stimulation over 120 min. Next, to assess whether IL-3 regulates the functional activation of β-catenin in these cells, we utilised an antibody which detects only the N-terminally non-phosphorylated form of β-catenin which is considered to be the transcriptionally active form of the protein [204]. Consistent with functional activation by IL-3, non-phosphorylated β-catenin could only be detected above

the basal level in the presence of continuous IL-3 **(Figure 3.1 A)**. However, in contrast to total levels of β-catenin protein which displayed notable increase from starved levels after a 120min IL-3 stimulation **(Figure 3.1 A)**, increased non-phosphorylated β-catenin levels in response to IL-3 stimulation were not observed before 4-8 hours **(Appendix A)**. The composite dynamics and kinetics of β-catenin accumulation have been addressed in recent studies, and it is apparent that the correlation of total, phosphorylated and non-phosphorylated β-catenin levels is more complex than would be predicted from the model of canonical β-catenin post-translational regulation [205, 206]. Our results suggest that although β-catenin levels start to re-accumulate in stimulated FDM cells as early as 1h after the addition of IL-3, steady-state expression levels are not restored until at least 3h later.

Variations in the dynamics and associated functional outcome of β-catenin stabilisation is typical for signalling cascades such as the Wnt/β-catenin pathway which play critical yet distinct functions in a range of developmental processes and adult tissues. In addition, the timing and *levels* of β-catenin accumulation in response to a stimulus can induce diverse cellular responses [207-211]. We hence wanted to determine whether β-catenin levels in FDM cells are dependent on the concentration of IL-3 present or if there is a threshold response. To address this question, parental FDM cells were cultured in a titration of IL-3 for 72 hours and β-catenin protein levels were analysed by western blotting. Interestingly, this revealed that the stabilisation of β-catenin proteins in FDM cells is dependent on the dose of IL-3 **(Figure 3.1 B)**, suggesting that different concentration of IL-3

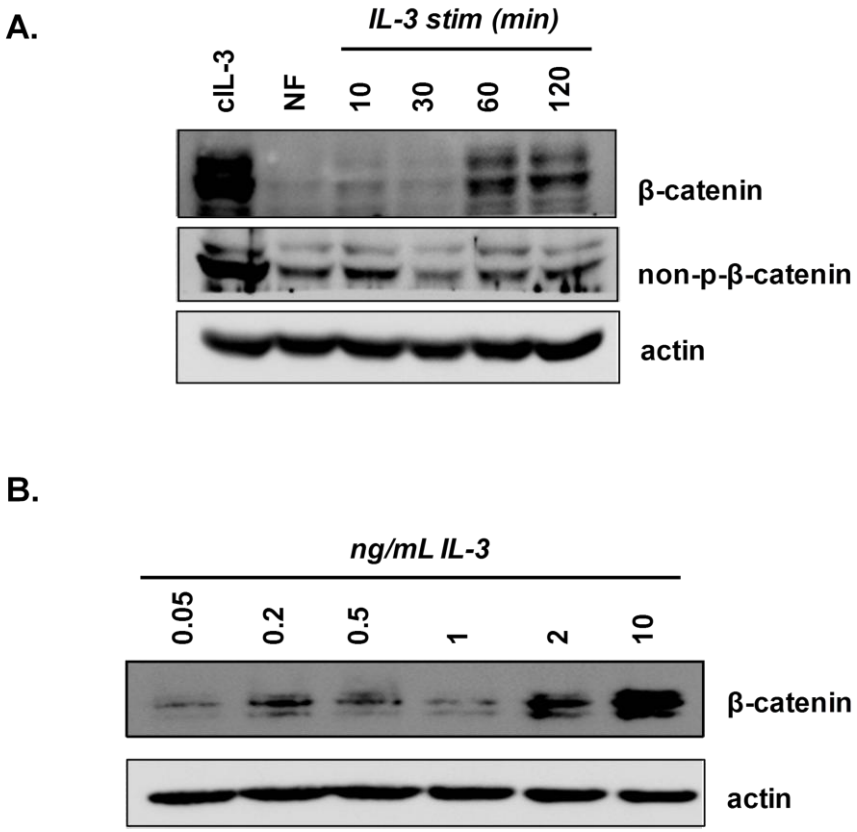


Figure 3.1 Modulation of β-catenin levels by IL-3. (A) FDM cells were grown continuously in IL-3 (cIL-3), starved of IL-3 for 16 hours (NF, no factor), or starved and then stimulated with 10ng/mL of mIL-3 for 10, 30, 60, or 120 min. Lysates were analysed by western blotting for total β-catenin and N-terminally non-phosphorylated β-catenin. **(B)** FDM cells with growth factor removed, were cultured in a range of mIL-3 concentrations for 72 hours. Lysates were analysed by western blotting for expression of β-catenin.

may be able to elicit distinct cellular responses in part by modulating levels of β-catenin in the cell.

We next investigated the mechanism by which IL-3 regulates β-catenin levels in FDM cells. Cytokine receptor signalling has been previously associated with the inhibitory phosphorylation of GSK3β at Serine 9 through the activation of the PI3K/AKT pathway [212, 213], and as GSK3β is a key player in the regulatory network associated with β-catenin protein stability [214], we looked at phospho-GSK3β levels in parental FDM cells following a timecourse of IL-3 stimulation. As shown in **Figure 3.2**, the phosphorylation of GSK3β (Ser9) decreased upon removal of IL-3 and was rapidly restored after the addition of IL-3. The activating phosphorylation of AKT (Ser473) was also reduced following withdrawal of IL-3, and phosphorylation was restored upon IL-3 stimulation for 10 min up until 120 min **(Figure 3.2)**.

In addition to the canonical regulation of β-catenin by GSK3β, β-catenin can also be directly phosphorylated on Serine 552 by AKT resulting in its stabilisation, nuclear accumulation and increased transcriptional activity [215-217]. In order to determine whether the PI3K/AKT pathway is required for IL-3 mediated stabilisation of β-catenin, and whether this is through the inactivation of GSK3β or directly via phosphorylation of β-catenin (Ser552), we treated parental FDM cells with a range of doses of PI3K inhibitors LY294002 and Wortmannin, and also the AKT specific inhibitor AKT Inhibitor X. PI3K or AKT inhibition resulted in reduced AKT phosphorylation (Ser473) as expected, and also reduced total β-catenin levels, suggesting that AKT is important for IL-3 mediated stabilisation of

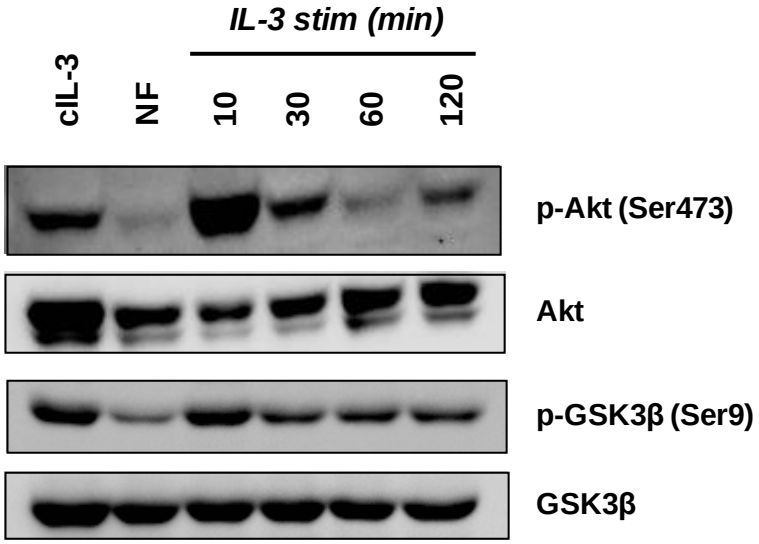


Figure 3.2 IL-3 mediated regulation of AKT and GSK3β phosphorylation in FDM cells. FDM cells were grown continuously in IL-3 (cIL-3), starved of IL-3 for 16 hours (NF), or starved and then stimulated with 10ng/mL of mIL-3 for 10, 30, 60, or 120 min. Lysates were analysed by western blotting for phospho-AKT (Ser 473), AKT, phospho-GSK3β (Ser 9) and GSK3β.

β-catenin **(Figure 3.3)**. Interestingly, phospho-GSK3β (Ser9) levels did not change with AKT inhibition **(Figure 3.3)**, suggesting that GSK3β is not a key mediator of IL-3 induced stabilisation of β-catenin. Conversely, in cells treated with LY294002 or the AKT inhibitor X where there was complete inhibition of AKT, we observed almost complete loss of phospho-β-catenin (Ser552). This was not observed with Wortmannin, likely due to the less efficient inhibition of AKT observed at the doses tested for this inhibitor. Overall, these results suggest that IL-3 mediates β-catenin stabilisation through AKT activation associated with specific phosphorylation of Ser552 and not through a GSK3β mediated pathway.

3.2.2 Modulation of β-catenin levels in FDM cells influences IL-3 mediated growth and survival signals

As the β-catenin signalling pathway has an established role in the regulation of cell survival, proliferation and differentiation, and an increasingly apparent role in the development of myeloid leukaemias, we wished to determine whether the activation of β-catenin by IL-3 has a functional significance in the context of Hox transformation in myeloid cells. In this section we explored the role for β-catenin downstream of IL-3 signalling, by induced deletion or overexpression of β-catenin in IL-3 dependent FDM cells.

3.2.2.1 β-catenin is required for IL-3 driven colony formation and growth

To investigate the requirement for β-catenin in IL-3-mediated cell growth and survival we utilised IL-3–dependent FDM cell lines generated from C57BL/6 mice containing a floxed β-catenin gene. These cell lines were derived as

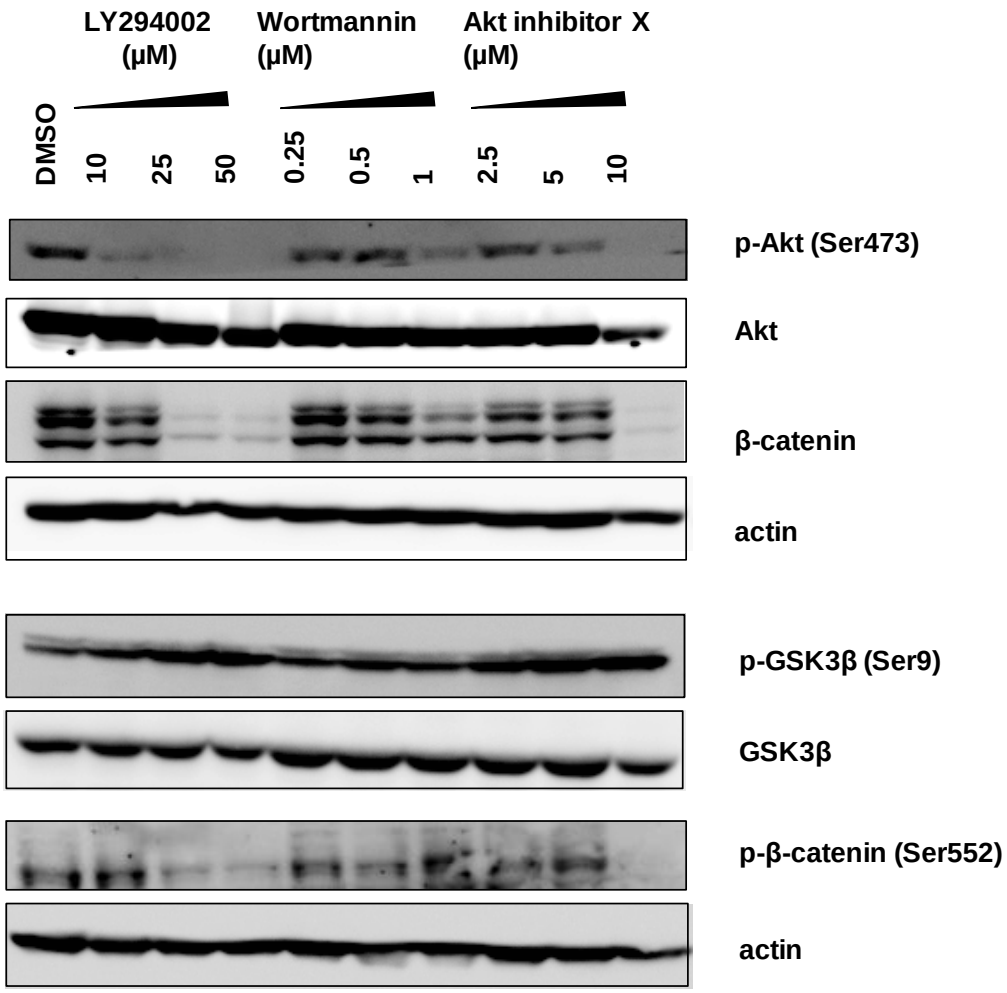


Figure 3.3 IL-3 regulates β-catenin through AKT-specific phosphorylation of β-catenin at Ser552. FDM cells cultured in continuous IL-3 (10ng/mL) were treated for 16hr with increasing concentrations of the PI3K inhibitors LY294002, Wortmannin, or AKT Inhibitor X, prior to analysis of phospho-AKT (Ser 473), AKT, total β-catenin, phospho-GSK3 β (Ser 9), GSK3β, and phospho-β-catenin (Ser552).

previously described [203], and represent a system which is amenable to modulation of both IL-3 levels and also β-catenin expression. For induced deletion of β-catenin, FDM β-catenin$^{flox/flox}$ cells were transduced with retrovirus encoding Cre-recombinase in the MSCV-IRES-GFP vector system (MIG-Cre) or the MSCV-IRES-GFP vector control (MIG) and cells were sorted for GFP expression by fluorescence-activated cell sorting (FACS) to obtain pure transduced cell populations **(Figure 3.4 A)**. Deletion of β-catenin was confirmed by western blotting in cell lysates prepared seven days following viral transductions **(Figure 3.4 B)**.

To measure the colony-forming potential of FDM cells with deleted β-catenin, equal numbers of FDM cells transduced with MIG-Cre or the MIG control were seeded in semi-solid methylcellulose with 10ng/mL of mIL-3 three days after sorting for GFP. Colonies were counted in triplicate plates after seven days in methylcellulose culture. As shown in **Figure 3.4 C**, Cre-mediated deletion of β-catenin in FDM cell lines resulted in approximately 70% loss of colony forming potential in response to IL-3. Consistent with colony forming assays, we observed a significant reduction in the growth of FDM cells in liquid culture with mIL-3 (10ng/mL) over four days following deletion of β-catenin **(Figure 3.4 D)**, suggesting that this pathway plays an important role in the growth response mediated by IL-3.

To determine if the marked reduction in IL-3 dependent growth resulting from β-catenin deletion was due to cell-cycle arrest, FDM cell populations were fixed in 70% ethanol at 4°C and subsequently stained with propidium iodide. Stained cells

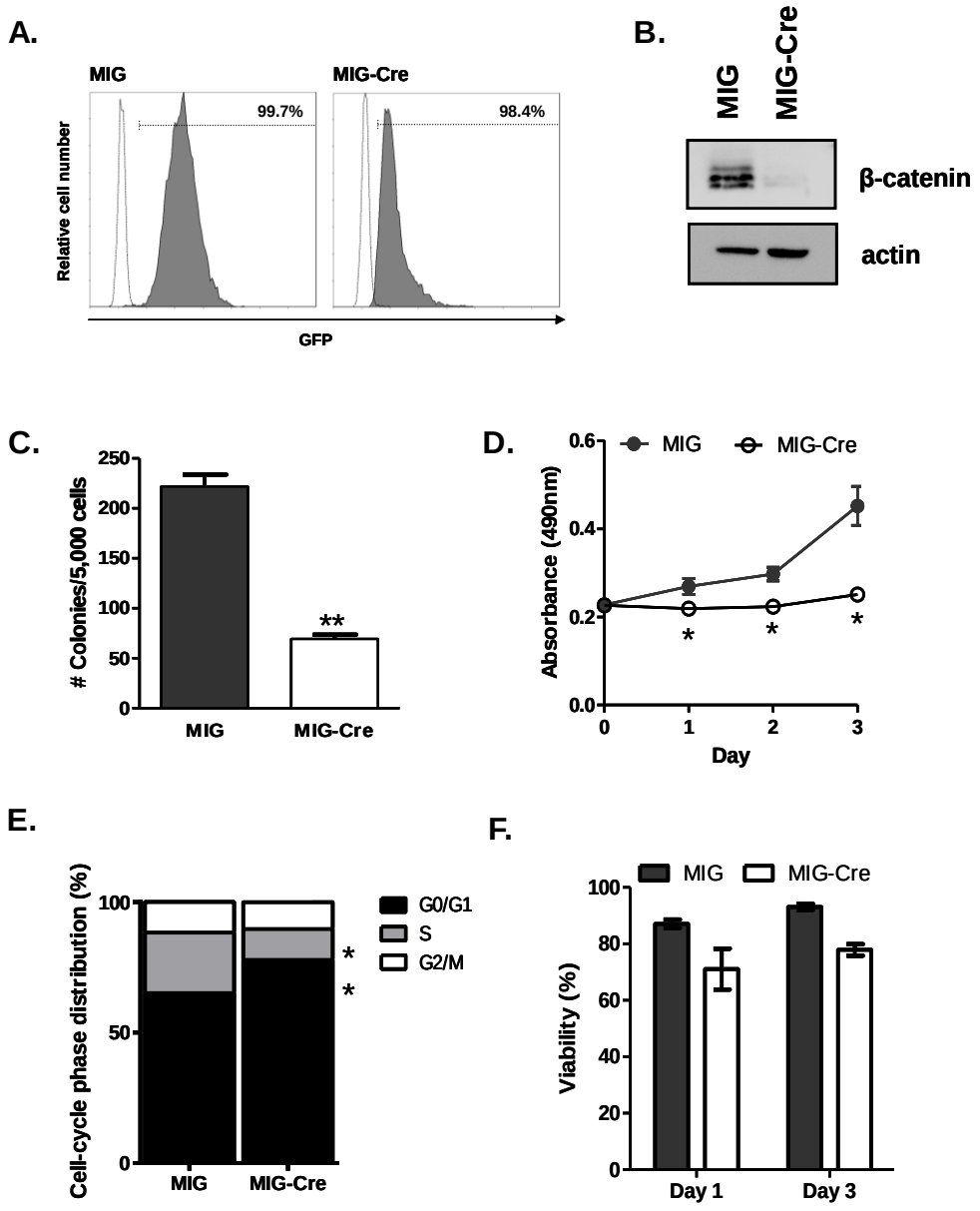


Figure 3.4 Ablation of β-catenin in IL-3 dependent FDM cells derived from β-catenin flox/flox mice. (A) GFP expression of FDM cells derived from C57BL/6 mice containing a floxed β-catenin gene retrovirally transduced with MIG-Cre or an MSCV-IRES-GFP vector control (MIG) was measured by flow cytometry following FACS purification of GFP positive cells. **(B)** β-catenin protein levels were analysed by western blot analysis to confirm deletion of β-catenin. FDM cells transduced with MIG-Cre or MIG were analysed for **(C)** colony formation in semi-solid methylcellulose in the presence of 10ng/mL mIL-3, 7 days after seeding **(D)** liquid culture proliferation in the presence of mIL-3 in an MTS assay (Promega) over three consecutive days **(E)** cell cycle distribution by propidium iodide staining and flow cytometry analysis **(F)** cell viability by trypan blue exclusion. Error bars represent SEM (n= 3). *p≤0.05; **p≤0.01.

were analysed by flow cytometry, and we observed a significant increase in cells in the G0/G1 cell-cycle stage, with a concomitant decrease in cells in the DNA-replication associated S-phase in the FDM β-catenin$^{flox/flox}$ cell populations transduced with MIG-Cre **(Figure 3.4 E)**. This observation suggests that cell cycle arrest in G0/G1 contributes in part to the reduction in cell growth observed in the absence of β-catenin. Viability was measured by trypan blue exclusion over four days following deletion of β-catenin, and while FDM cells transduced with MIG-Cre displayed a trend for decreased viability this did not reach significance **(Figure 3.4 F)**.

In order to assess any affects on differentiation resulting from the deletion of β-catenin in the FDM cell lines, we next measured surface expression of the myeloid antigen markers *Gr-1* and *F4/80,* which are expressed on FDM cells consistent with the myeloid nature of this cell line, and the erythroid marker *Ter-119*. We found no difference in expression between the cells transduced with MIG-Cre or MIG **(Figure 3.5 A)**. Consistent with this finding, the morphology of cells with induced deletion of β-catenin was similar to control cells as visualised by Giemsa staining of cytocentrifuged cells **(Figure 3.5 B)**. Together, these results suggest that the significant growth reduction seen with β-catenin deletion in response to IL-3 is largely due to cell-cycle arrest at G0/G1, with little observed effect on cell viability or differentiation. Our observations are in line with previous studies which show that shRNA mediated knockdown of β-catenin in AML and multiple myeloma (MM) cell lines results in a significant reduction in proliferation associated with cell-cycle arrest, with negligible effects on cell viability [218, 219]. However it is possible that in addition to cell-cycle arrest, the

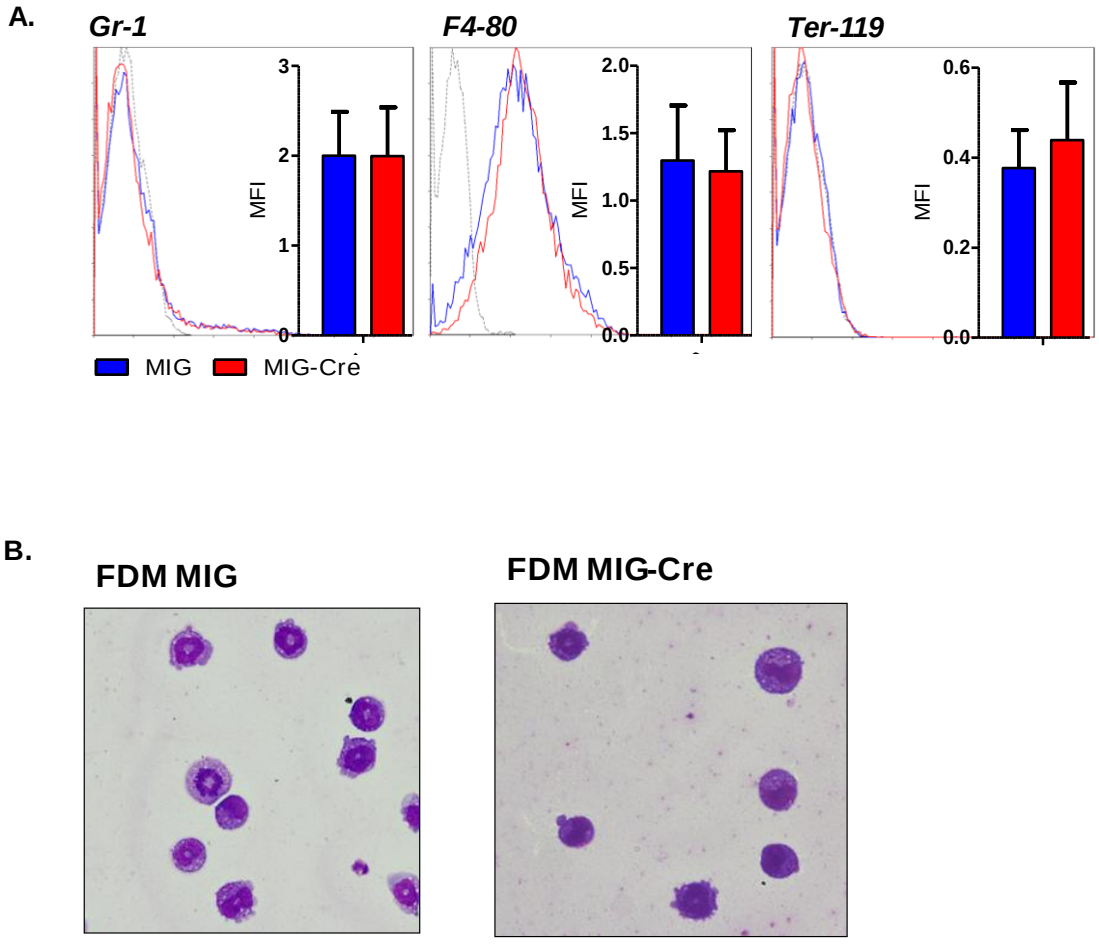


Figure 3.5 Surface antigen expression and morphology of FDM cells with deleted β-catenin. (A) FDM β-cateninflox/flox cells transduced with MIG-Cre or MIG were analysed by flow cytometry for surface expression of haematopoietic markers *Gr-1*, *F4/80* and *Ter-119* using monoclonal PE-conjugated antibodies. Shown are representative flow cytometry profiles, and inset graphs depict the mean of three independent experiments (error bars represent SEM). **(B)** Cells were centrifuged onto glass slides and stained with May-Grunwald-Giemsa to determine morphology.

deletion of β-catenin may have influenced other cellular processes such as cell senescence, which were not included in our analysis.

3.2.2.2 Ectopic expression of β-catenin promotes survival at low concentrations of IL-3

As deletion of β-catenin induced a dramatic reduction in IL-3 mediated colony formation and growth, we wished to determine whether ectopic expression of β-catenin in parental FDM cells could promote growth or survival at sub-optimal concentrations of IL-3. To this end, FDM populations expressing a constitutively active form of β-catenin or GFP-vector control were generated by retroviral transduction and expanded in 10ng/mL of mIL-3. The constitutively active β-catenin mutant (β-catS33A) contains 4 amino acid substitution mutations (S33A, S37A, T41A and S45A) that prevent its phosphorylation by casein kinase-1 and GSK3β and its subsequent proteosomal-mediated degradation. Pure populations were obtained by FACS **(Figure 3.6 A)**, and the expression of β-catenin in FDM MIG-β-catS33A transduced cells was verified by western blotting **(Figure 3.6 B)**.

FDM cells expressing MIG-β-catS33A or MIG were cultured in a titration of IL-3 for 72h, and cell proliferation and viability were assessed by thymidine incorporation and trypan blue exclusion resepctively. Overexpression of β-catenin did not significantly alter the proliferation of FDM cells in response to IL-3 when compared to the vector control **(Figure 3.6 C)**. This suggests that while it is possible that β-catenin is required for IL-3 driven cell growth, the endogenous level of β-catenin in this system is sufficient for cell growth in the conditions studied here. Interestingly, the overexpression of β-catenin had a striking effect of

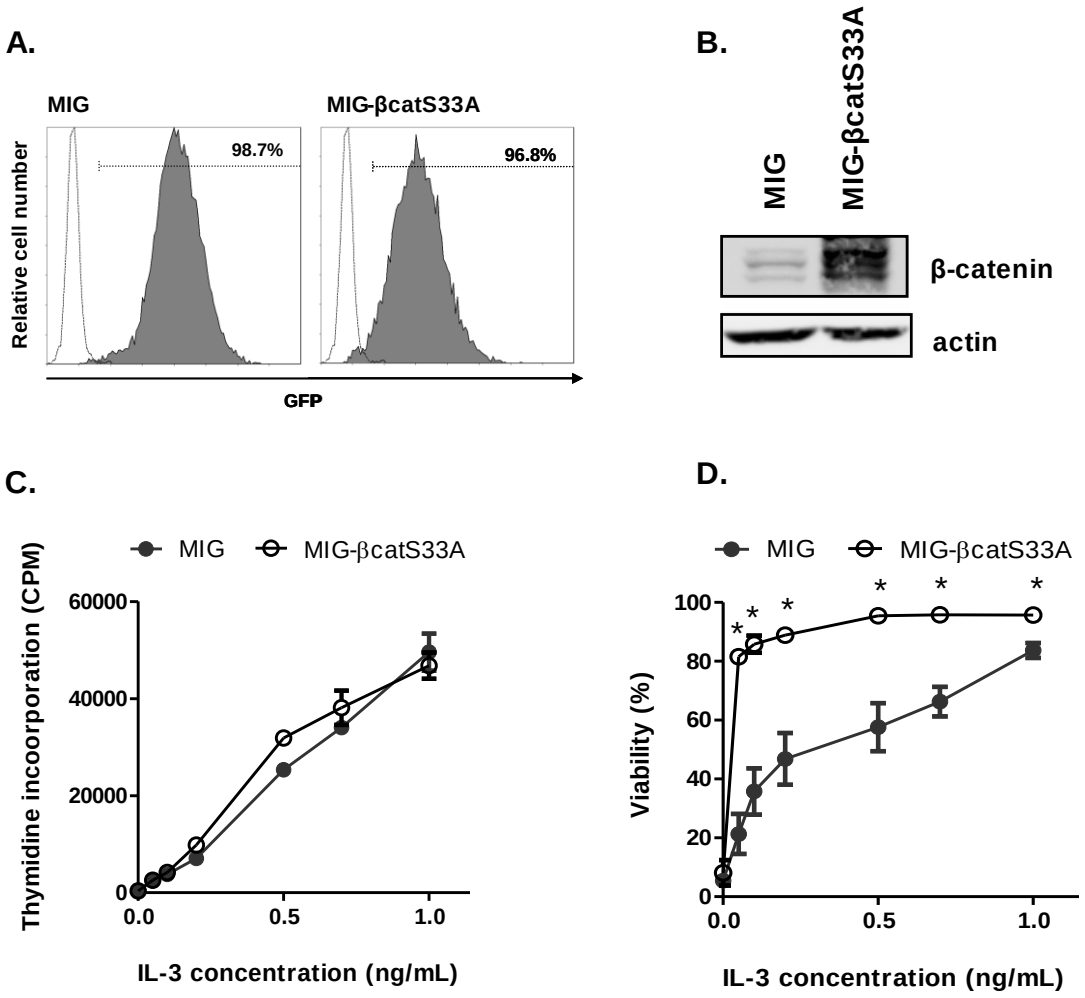


Figure 3.6 Constitutive activation of β-catenin lowers the dependency on IL-3 for cell survival. (A) GFP expression of FDM cells transduced with the constitutively active β-catenin mutant (MIG-βcatS33A) or MIG was measured by flow cytometry following FACS purification of GFP positive cells. **(B)** Overexpression of β-catenin was confirmed by western blotting in FDM MIG-βcatS33A cells. FDM MIG-βcatS33A and MIG cells were washed in PBS 3 times to remove traces of factor, and cultured in a range of mIL-3 concentrations for 3 days. Cells were then analysed for **(C)** proliferation by 3H-thymidine incorporation at a concentration of 0.5μCi per well; radioactive uptake was measured on a scintillation counter 16 hours after the addition of thymidine, and percentage viability was determined using trypan blue exclusion **(D)**. Error bars represent SEM (n= 3). *$p \leq 0.05$.

cell viability at suboptimal concentrations of IL-3 **(Figure 3.6 D)**. FDM MIG-β-catS33A cells maintained high viability, >80%, at IL-3 doses that markedly reduced the viability of control cells **(Figure 3.6 D)**. Although able to maintain a high level of viability at low IL-3 concentrations, FDM cells overexpressing β-catenin were not factor-independent, as removal of IL-3 resulted in complete loss of viability. This suggests that β-catenin alone is not sufficient for factor-independent survival, but may act by amplifying the IL-3 survival signal in situations where there is sub-optimal cytokine concentrations to maintain cell viability.

Cell death resulting from cytokine withdrawal is primarily regulated by the Bcl-2 family of proteins and overexpression of anti-apoptotic Bcl-2 members Bcl-2 and Bcl-xL has been shown to prevent apoptosis of factor-dependent myeloid cell lines in the absence of IL-3 [220-222]. In contrast, deletion of pro-apoptotic Bcl-2 members, Bax and Bak, renders cells highly resistant to apoptosis following growth factor withdrawal [223, 224] highlighting the importance of this protein family in cytokine mediated survival. In this study, we focused on the role of Bcl-2 as there is evidence that the anti-apoptotic Bcl-2 gene may be a direct β-catenin target in some cell types [225-227]. Studies in colorectal cancer cell lines have shown that β-catenin binds the *BCL-2* promoter and induces *BCL-2* mRNA and protein expression [225, 226]. In AML, siRNA knockdown of β-catenin in purified LSC results in downregulation of *BCL-2* mRNA suggesting that it may be an important downstream modulator of β-catenin driven survival [227]. Hence, we aimed to investigate the link between β-catenin activation and Bcl-2 expression in an IL-3 dependent system.

To determine if Bcl2 is a target of β-catenin in FDM cells we firstly analysed *Bcl-2* mRNA levels by QRT-PCR in parental FDM cells transduced with MIG-β-catS33A. As shown in **Figure 3.7 A**, overexpression of β-catenin induced a significant up-regulation in *Bcl-2* mRNA levels compared with parental FDM cells expressing the MIG control. Consistent with this, FDM β-catenin$^{flox/flox}$ cells transduced with MIG-Cre to induce deletion of β-catenin, displayed a significant two-fold reduction in basal *Bcl-2* mRNA levels **(Figure 3.7 B)**.

We next investigated whether modulation of *Bcl2* levels is a potential mechanism by which increased β-catenin expression promotes cell survival at suboptimal IL-3 concentrations. To address this, we performed QRT-PCR to measure *Bcl-2* mRNA expression in FDM MIG-β-catS33A and parental FDM cells expressing the MIG control, cultured in a titration of IL-3 for 16h **(Figure 3.8 A)**.We also measured mRNA expression of the related pro-survival Bcl-2 family member *Bcl-xL*, to determine if any differences in expression were specific to *Bcl-2* or extended to other members of the same family **(Figure 3.8 B)**. Interestingly, compared to control cells, we observed a significant increase in *Bcl-2* mRNA levels in the FDM cells overexpressing β-catenin at the higher doses of mIL-3 (1 and 0.7 ng/mL), with no difference observed in the lower concentrations of IL-3. We did not observe any significant differences in *Bcl-xL* mRNA expression between the FDM MIG-β-catS33A and parental FDM MIG cells at any dose of IL-3 **(Figure 3.8 B)** suggesting that β-catenin may selectively regulate *Bcl-2*.

To determine if increased β-catenin signalling modulates Bcl-2 at the protein level, we also analysed Bcl-2 and Bcl-xL protein expression by western blotting

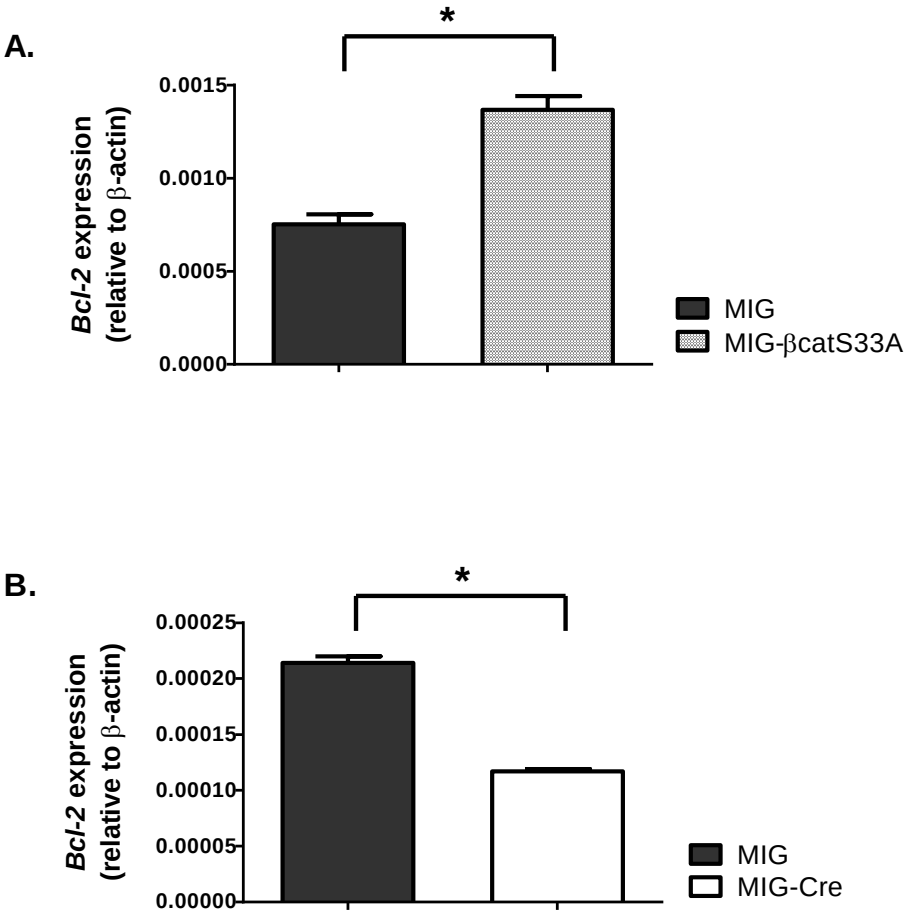


Figure 3.7 β-catenin modulates Bcl-2 mRNA expression in FDM cells. Bcl-2 mRNA expression was measured by QRT-PCR in RNA extracted from FDM cells expressing MIG-βcatS33A **(A)** and in FDM β-catenin flox/flox cells transduced with MIG-Cre **(B)** and compared to FDM cells transduced with a vector control. Results were normalised to β-actin expression. Error bars represent SEM (n=3, A. n=2, B). *p≤0.05.

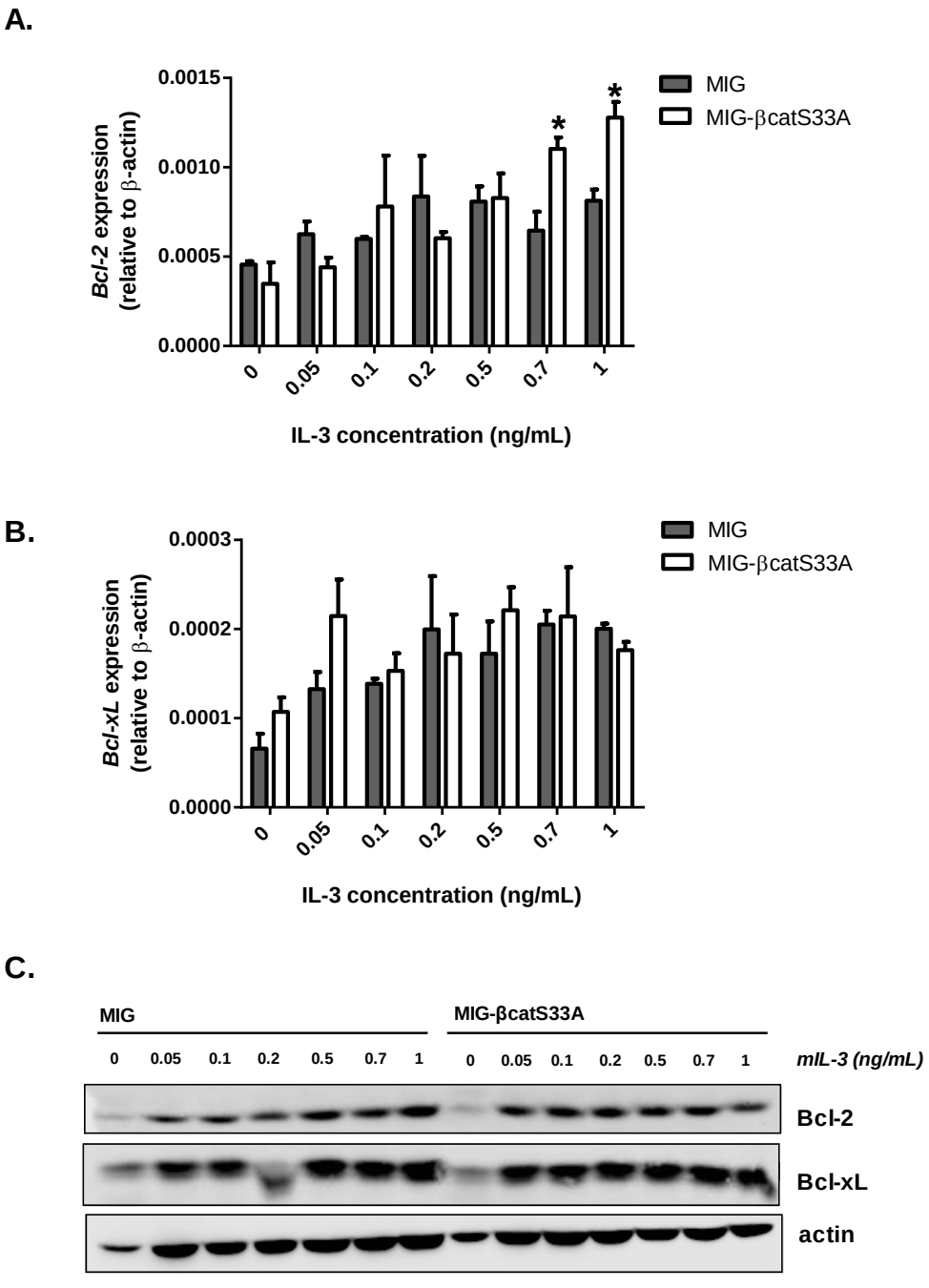


Figure 3.8 Regulation of Bcl-2 expression by β-catenin is dependent on IL-3. FDM MIG-βcatS33A and MIG cells were washed in PBS 3 times to remove traces of factor, and cultured in a range of mIL-3 concentrations for 16h for mRNA and protein analyses. Extracted RNA was analysed for Bcl-2 **(A)** and Bcl-xL **(B)** mRNA by QRT-PCR and results were normalised to β-actin expression. **(C)** Cell lysates were analysed for Bcl-2 and Bcl-xL protein expression by western blotting. Error bars represent SEM (n= 3). *p≤0.05.

in lysates prepared from FDM MIG-β-catS33A and parental FDM MIG cells treated with various doses of IL-3 as above. There was no observable difference in Bcl-xL protein expression between the FDM MIG-β-catS33A and parental FDM MIG cells and Bcl-xL expression was only reduced in both cell lines following complete IL-3 withdrawal **(Figure 3.8 C)**. This suggests that Bcl-xL expression is less sensitive to changes in IL-3 concentration than Bcl-2, as Bcl-2 protein levels increased in a dose dependent manner in response to increasing IL-3 concentrations in parental FDM MIG cells **(Figure 3.8 C)**. Interestingly, in FDM-MIG-β-catS33A cells the dose-responsive effect was not apparent, with high levels of Bcl-2 protein maintained at the low doses of IL-3 **(Figure 3.8 C)**. Bcl-2 levels were noticeably reduced in both cell lines when IL-3 was withdrawn completely for 16h. Bcl-2 protein expression however, did not correlate with our analysis of mRNA expression in FDM β-catS33A cells, as Bcl-2 mRNA was only significantly higher than control cells at the highest concentrations of IL-3 suggesting that β-catenin may modulate Bcl-2 expression at the mRNA and post-translational level in context of IL-3 signalling through different mechanisms. The notion that the regulation of Bcl-2 mRNA and protein expression by β-catenin is dependent on the dose of IL-3 is consistent with our previous results showing that deletion of β-catenin in FDM cells does not affect the viability of cells cultured in high doses of IL-3 **(Figure 3.3 F)**. We observed, however, that FDM cells with deleted β-catenin were more sensitive to IL-3 withdrawal than control cells **(Appendix B)**. More extensive timecourse experiments may be important to further interpret the discrepancies between the differences observed in protein and mRNA levels of Bcl-2 downstream of β-catenin.

Additional studies are also required to determine the significance of this pathway downstream of IL-3 and could involve selective knockdown of Bcl-2. Further, it is possible that other mechanisms also contribute to the survival advantage observed in FDM cells overexpressing β-catenin.

3.2.3 IL-3 regulates β-catenin protein levels, nuclear localisation and transcriptional activation in leukaemic TF-1.8 cells

In the previous section we showed that β-catenin is regulated by IL-3 signalling in IL-3 dependent myeloid cells. As IL-3Rα is overexpressed on AML blast and progenitor cells and associated with a poor prognosis and outcome [78, 193], we next wished to determine whether IL-3 can modulate β-catenin levels in leukaemic cells. To this end, we used the factor-dependent TF-1.8 erythroleukaemia cell line which responds to IL-3 signalling for growth and survival.

To determine if IL-3 signalling regulates β-catenin in TF-1.8 cells we analysed β-catenin protein levels in cells cultured in the presence or absence of 2ng/mL hIL-3 by western blot analysis. As shown in **Figure 3.9**, β-catenin levels were reduced in TF-1.8 cells following IL-3 withdrawal for 16h in low serum (2% FCS), and increased again upon a 6h IL-3 stimulation (2ng/mL). This was similar to the well characterised STAT5 response [228, 229] which was measured by phosphorylation of STAT5A/B (Tyr694/699) (**Figure 3.9)**. In the same experiment we treated IL-3-starved TF-1.8 cells with 100nM of the IL-3 receptor neutralising monoclonal antibody (mAb) 7G3 [230] or an IgG2a isotype control

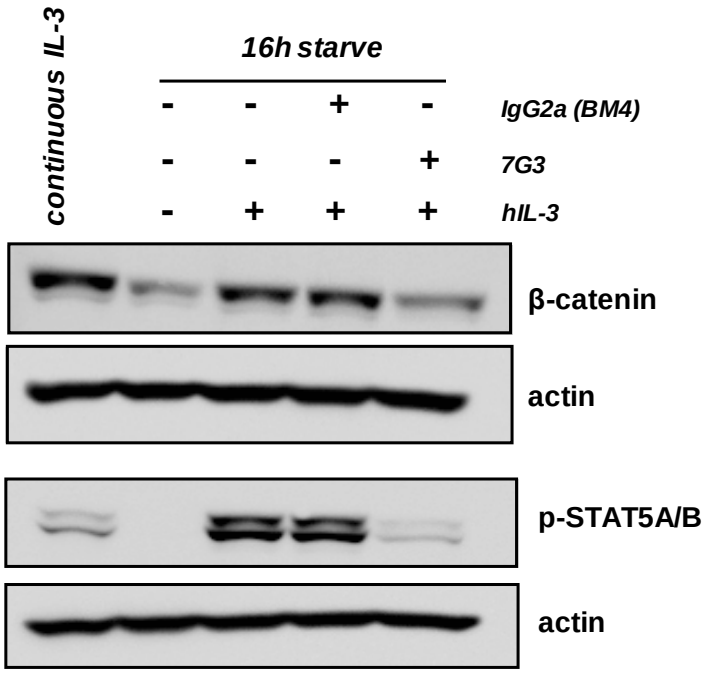


Figure 3.9 IL-3 promotes β-catenin stabilisation in TF-1.8 leukaemic cells. Lysates were prepared from TF-1.8 cells grown for 16h ± 2ng/mL hIL-3 (continuous culture conditions) or re-stimulated with 10ng/mL hIL-3 for 6h ± a 20 minute pre-treatment with 100nM of the IL-3 neutralizing antibody 7G3 (or IgG2a isotype control). β-catenin and p-STAT5A/B (Tyr694/699) and protein levels were analysed by western blotting.

for 20 minutes on ice, prior to the re-addition of IL-3. The mAb 7G3 recognizes and binds the N-terminal domain of IL-3Rα resulting in inhibition of ligand binding, and has been shown to effectively antagonise IL-3 mediated activation of STAT5A/B and cell proliferation in primary AML cells [79]. Our results show that as well as blocking STAT5A/B phosphorylation, pre-treatment of TF-1.8 cells with 7G3 inhibited IL-3 induced accumulation of β-catenin **(Figure 3.9)**.

A common measure of β-catenin activity is its subcellular localisation to the nucleus where it can transcriptionally activate its target genes. We hence assessed β-catenin subcellular localisation in TF-1.8 cells in response to IL-3, using immunofluorescence staining and confocal microscopy **(Figure 3.10)**. TF-1.8 cells cultured in the presence or absence of 2ng/mL hIL-3 were stained with a mouse monoclonal anti-β-catenin antibody followed by an Alexa-488 conjugated secondary antibody on glass slides. To allow visualisation of nuclei, the slides were mounted with antifade fluorescence medium containing DAPI. Consistent with functional activation, the β-catenin in TF-1.8 cells growing continuously in hIL-3 was primarily localised to the nucleus **(Figure 3.10 A-B** - single cell and field views). Removal of IL-3 for 16 hours in low serum (2% FCS) induced cytoplasmic localisation of β-catenin, which translocated back into the nucleus upon the re-addition IL-3 for one hour. These results show that in addition to regulating total β-catenin protein levels, IL-3 signalling regulates the subcellular localisation of β-catenin in TF-1.8 cells.

To further support the notion that IL-3 regulates transcriptional activation of β-catenin in TF-1.8 cells, we also analysed the mRNA expression of recognised β-

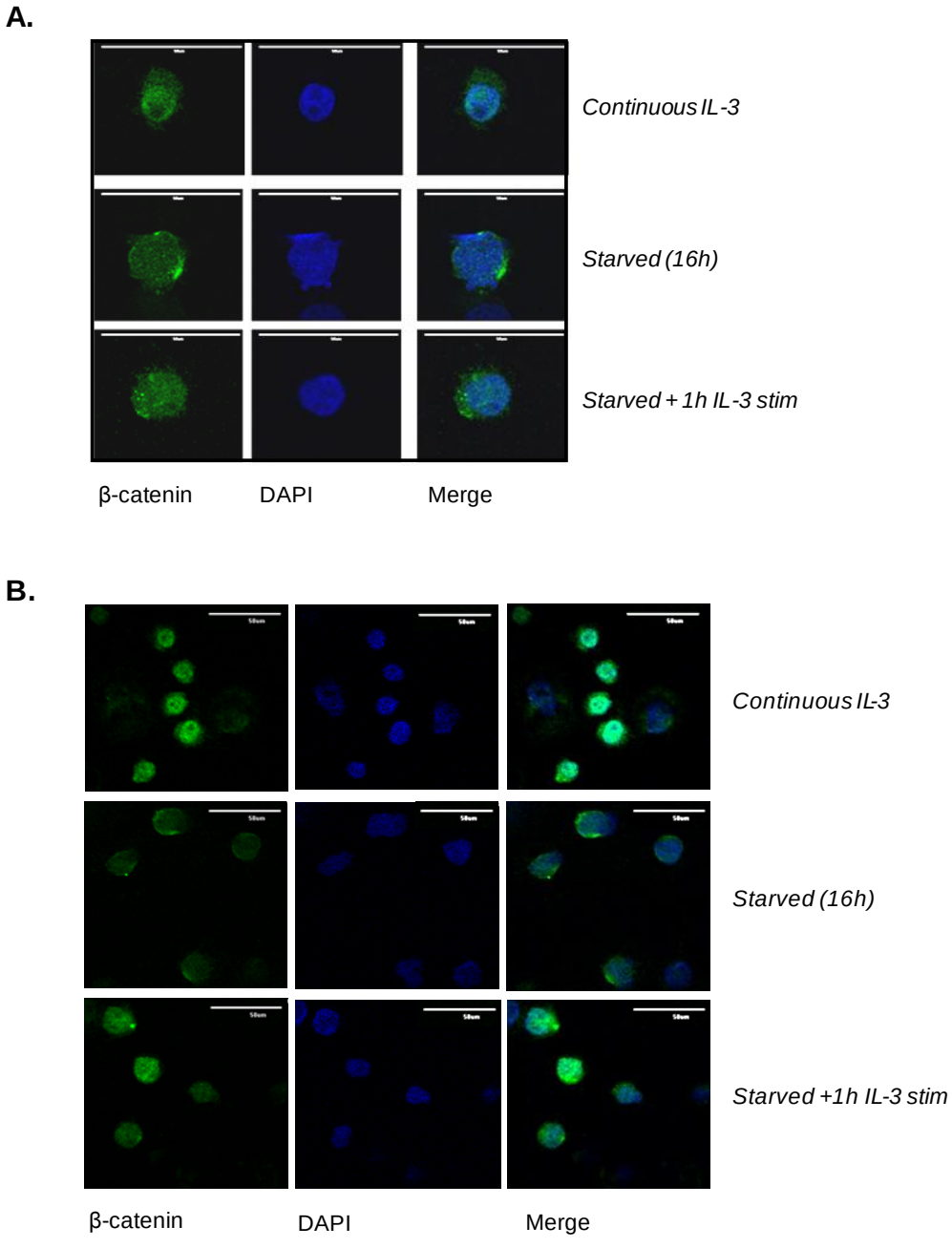


Figure 3.10 IL-3 promotes β-catenin nuclear localisation in TF-1.8 leukaemic cells. TF-1.8 cells ± hIL-3 (2ng/mL), or restimulated with 2ng/mL hIL-3 were spun onto glass slides, fixed and permeabilised with commercial solutions (eBiosciences) and stained overnight with an anti-β-catenin monocolonal antibody. Slides were then washed with permeabilisation solution, and stained with an Alexa488 secondary antibody (green), and mounted with antifade fluorescence medium containing DAPI. Slides were viewed using the BioRad Radiance 2100 confocal microscope, and photos were captured at 60x **(A)** or 40x **(B)** magnification with Laser Sharp Software version 5. Scale bar = 50μm.

catenin target genes in response to IL-3. The transcriptome regulated by the canonical Wnt/β-catenin pathway has been well studied in a range of normal and malignant tissues, and while there is a large overlap it is clear that there are system and context specific β-catenin target genes [152, 194, 208]. To date however, there has not been a genome wide analysis of β-catenin target genes in haematopoietic cells, so we selected genes from a publicly available compilation of verified β-catenin targets (http://www.stanford.edu/ group /nusselab/cgi-bin/wnt/targetgenes) which we confirmed to be expressed in TF-1.8 cells by analysis of a microarray previously performed in our laboratory (unpublished data). We performed QRT-PCR to measured mRNA expression of three of these genes, *c-MYC, FRA-1* and *PTGS2* in RNA extracted from TF-1.8 cells growing in the presence or absence of 2ng/mL hIL-3 for 16h, or re-stimulated with hIL-3 for 6h following 16h IL-3 withdrawal. Interestingly, we did not observe any significant changes in *c-MYC* expression in response to modulating IL-3 levels, although there was a trend for reduced expression upon withdrawal of IL-3 **(Figure 3.11 A)**. There was however, a significant regulation of the β-catenin targets *FRA-1* and *PTGS2* by IL-3 **(Figure 3.11 B-C)**, and the direct STAT5 target gene *CISH* which was included as a control **(Figure 3.11 D)**.

3.3 Discussion

In this Chapter, we have identified β-catenin as a novel downstream target of IL-3 cytokine signalling in myeloid and leukaemic cell line systems. Given the normal role of β-catenin in the regulation of cell proliferation and self-renewal, and the commonly observed aberrant activation of β-catenin in cancer, we suggest that

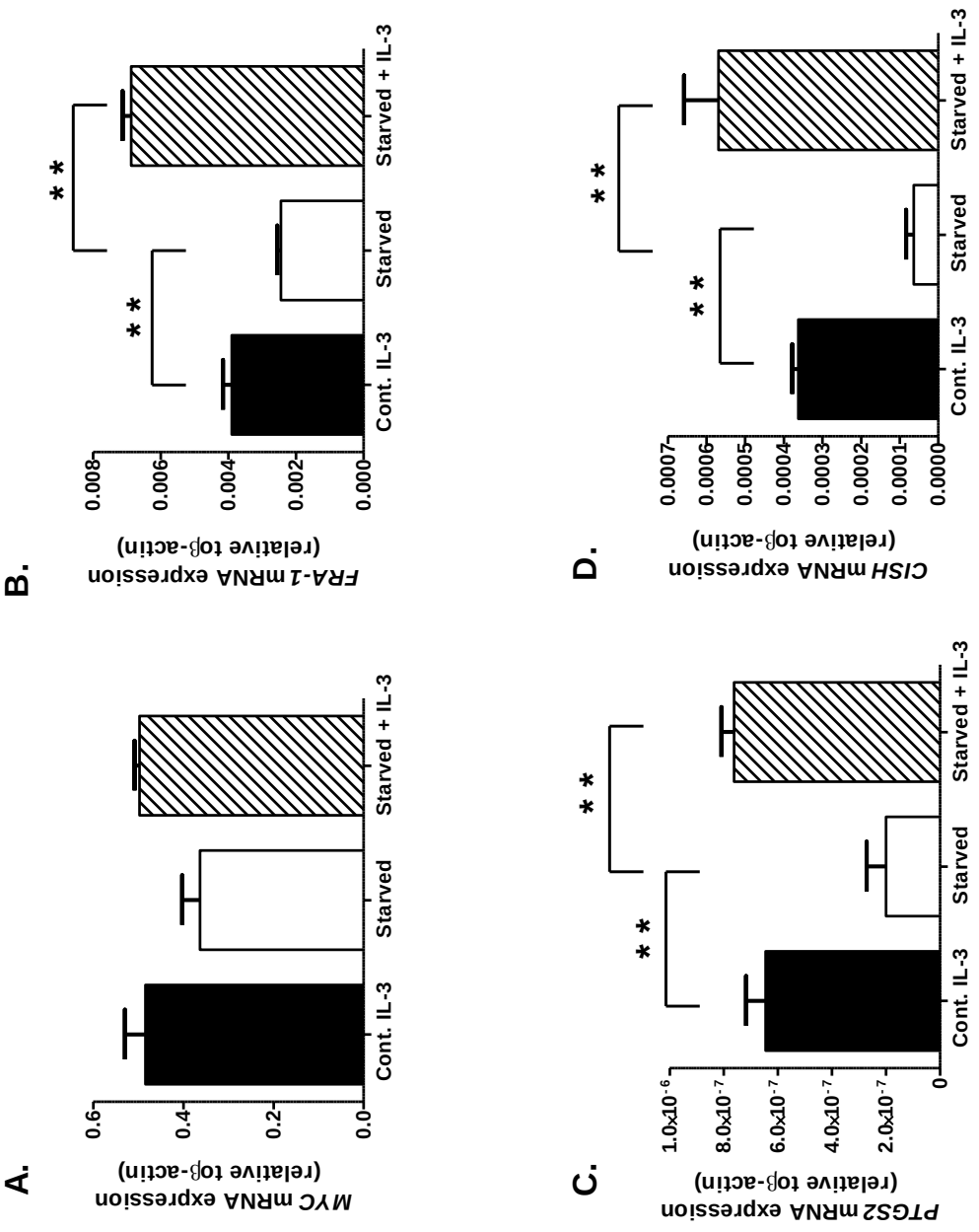


Figure 3.11 IL-3 regulates mRNA expression of β-catenin target genes in TF-1.8 cells. Expression of recognised β-catenin targets genes *MYC* **(A)** *FRA-1* **(B)** and *PTGS2* **(C)** was measured in RNA prepared from TF-1.8 cells grown for 16h ± 2ng/mL hIL-3 or re-stimulated with 10ng/mL hIL-3 for 6h by QRT-PCR, and normalised to β-actin expression. Expression of the direct IL-3 target, *CISH*, was also measured as a control **(D)**. Error bars represent SEM (n=2). **p≤0.01. Cont IL-3 = continuous IL-3 (2ng/mL hIL-3).

this is a mechanism by which IL-3 may contribute to normal and malignant haematopoiesis.

The FDM cell lines used in this study were generated by the transformation of murine foetal liver progenitors with HoxB8 in the presence of saturating levels of IL-3, and provided a unique system to explore the link between IL-3 signalling and β-catenin in a model of Hox oncogenesis. Utilising this model, we have shown that IL-3 promotes β-catenin protein stabilisation in a dose dependent manner. Consistent with functional activation of β-catenin, we also observed an accumulation of transcriptionally active, N-terminal non-phosphorylated β-catenin in response to IL-3 stimulation. In addition we show that the IL-3 mediated stabilisation of β-catenin in FDM cells occurs through AKT specific phosphorylation of β-catenin which can be modulated by PI3K and AKT inhibitors *in vitro.* Specifically, our results suggest that the IL-3 induced phosphorylation of AKT results in stabilisation of β-catenin independently of GSK3, through the AKT mediated phosphorylation of β-catenin on Ser552. Given the limitations associated with the use of pharmacological inhibitors, however, it will be of importance to further test the requirement for AKT signalling in the IL-3 induced regulation of β-catenin. A number of approaches to this are possible, including siRNA knockout of AKT in FDM cell lines, or the use of a myristoylated version of AKT which has been previously demonstrated to result in constitutive activation of AKT [231, 232]. In addition, it will be of interest to determine whether this mechanism of regulation is active in AML cells.

In this study we have also investigated whether the activation of β-catenin influences cytokine-mediated growth and survival signals, utilising IL-3 dependent FDM cells with ablated or enforced expression of β-catenin. Our results showed that the induced deletion of β-catenin in FDM cells abolishes IL-3 dependent growth and colony formation of the HoxB8 transformed cells, in part due to cell-cycle arrest at G0/G1. Our findings are of particular significance, as a critical function for β-catenin has been demonstrated in the development and maintenance of AML stem cells associated with MLL fusion proteins, and activation of β-catenin in combination with Meis/HoxA9 expression is sufficient to induce leukaemic transformation of GMP *in vivo* [132, 133]. *Hox* genes have also been shown to cooperate with IL-3 signalling to generate a transplantable myeloid leukaemia in mice [188], and our work suggests that IL-3 may cooperate with *Hox* genes through the activation of β-catenin.

Interestingly, enforced expression of β-catenin in FDM cells did not affect cell proliferation, but instead we found that this event reduced the dependency on IL-3 for cell survival. The significant effect of constitutive β-catenin expression on IL-3 mediated survival at sub-optimal concentrations of cytokine, with no apparent effect on proliferation is consistent with previous studies with human IL-3 and GM-CSF which have characterised the uncoupling of survival and proliferation signals in response to low, picomolar, concentrations of cytokine [233-235]. We have shown that ectopic expression of β-catenin increases cell survival at picomolar concentrations of *murine* IL-3 (<1ng/mL) and our data suggests that β-catenin may act by amplifying survival-specific signals downstream of mIL-3, in part through an upregulation of Bcl-2. However, more experiments will be

required to definitively define the role of Bcl-2 downstream of β-catenin in myeloid cells.

Lastly, as both β-catenin and the alpha subunit of the IL-3 receptor (IL-3Rα) are over-expressed in a large subset of AMLs, we also wished to investigate whether IL-3 signalling can modulate β-catenin in human leukaemic cells. We demonstrate that IL-3 stimulation of the erythroleukaemic TF-1.8 cell line can induce β-catenin protein accumulation, which is inhibited by treatment of cells with the IL-3Rα neutralising monoclonal antibody 7G3. Consistent with a functional activation of β-catenin by IL-3, we also show that IL-3 promotes the nuclear localisation of β-catenin in TF-1.8 cells, and induces expression of the recognised β-catenin/Tcf4 target genes *FRA-1* and *PTGS2*.

In addition to a critical role for IL-3 signalling in the growth and survival of AML cells, accumulating evidence suggests that elevated expression of the IL-3Rα subunit can also contribute to the development of an aggressive leukaemic phenotype [193, 236]. Overexpression of IL-3Rα on leukaemic blasts confers increased cell proliferation and survival, and clinically, elevated IL-3Rα expression is associated with increased blast counts at diagnosis, and a poor prognosis [78, 193]. Interestingly, overexpression of IL-3Rα in FDC-P1 cells was shown to increase sensitivity to IL-3 and allowed cell proliferation in the presence of suboptimal concentrations of cytokine, suggesting that elevated IL-3Rα levels on AML blasts might provide leukaemic cells with a proliferative advantage in the presence of low IL-3 levels [237]. It is possible that increases in IL-3Rα and β-catenin expression are early events associated with leukaemic transformation and

contribute to leukaemia development by sensitising the cells to growth and survival signals from IL-3 respectively. In turn, enhanced or altered signalling resulting from IL-3Rα overexpression, may result in increased growth, promoting downstream signalling including activation of the β-catenin pathway.

In the following Chapter we have made use of primary AML patient samples to further investigate the networks and signalling pathways activated by IL-3 in leukaemia.

CHAPTER 4

Dissecting the Signalling and Global Gene Expression Changes Induced by IL-3 in Primary AML Cells

4.1 Introduction

IL-3 and other haematopoietic cytokines play a critical role in maintaining the growth and survival of leukaemic cells and increasing evidence suggests that (a) cytokine levels, (b) cytokine production and (c) blast-cell responsiveness to cytokine stimulation are abnormal in leukaemia patients. Levels of IL-3, IL-6, IL-8, GM-CSF, and SCF have been found to be elevated in patients with myeloid leukaemias compared with healthy controls [238-240], and importantly, studies have identified an association between levels of circulating cytokines/chemokines and prognosis [241-243]. In addition, autocrine production of IL-3 and other cytokines and growth factors such as GM-CSF, G-CSF, IL-6, IL-8, FLT3 ligand and VEGF by AML blast cells has been well documented [180-183], and the majority of AMLs exhibit a degree of proliferation in response to stimulation with these factors *in vitro* [244, 245]. Furthermore, recent studies suggest that in combination with the basal levels of signalling proteins, the cytokine-induced signalling profiles of AML cells may provide critical insight for patient diagnosis and prognosis, including predicted response to therapeutic agents [39-41].

Particular interest has been placed on the role of IL-3 signalling in AML since the discovery that IL-3Rα surface levels are elevated on $CD34^+$ leukaemic progenitors and LSC relative to their normal counterparts [78, 193]. Increased expression of IL-3Rα in AML appears to be more than a passive marker as accumulating evidence suggests that elevated expression of this receptor subunit may contribute to the development of a particularly malignant leukaemic phenotype. Specifically, overexpression of IL-3Rα on leukaemic blasts is associated with increased cell

proliferation and survival [78, 193], and another study demonstrated that enforced expression of IL-3Rα in FDC-P1 cells allowed cell proliferation at suboptimal concentrations of IL-3 [237]. Clinically, elevated IL-3Rα expression is associated with increased blast counts at diagnosis, lower relapse-free survival and lower overall survival [78, 193].

In addition to abnormalities in IL-3Rα expression in AML [184, 186, 202], a functional role for IL-3 in leukaemia is supported by evidence that IL-3 can cooperate with oncogenic lesions in the development of chronic and acute myeloid leukaemia. In the context of CML, it has been demonstrated that Bcr-Abl transformed haematopoietic cell lines, as well as primitive $CD34^{+}$/Bcr-Abl^{+} cells from CML patients, establish an autocrine IL-3 loop which results in a stimulation of phosphorylated STAT5 and factor-independent growth *in vitro* [187, 246, 247]. Further, it has been shown that the treatment of Bcr-Abl transformed BM cells with a neutralising anti IL-3Rα antibody induces a significant reduction of proliferation *in vitro*, and IL-$3^{-/-}$ BM cells transformed with Bcr-Abl display impaired leukaemogenic potential *in vitro* and *in vivo* compared to WT BM cells [187]. Interestingly, autocrine IL-3 production by Bcr-Abl transformed haematopoietic cell lines can protect cells from Bcr-Abl tyrosine kinase inhibitor induced apoptosis [248], and additionally, the IL-3 produced by imatinib resistant CML blasts can render other leukaemic blast cells resistant through paracrine stimulation [249].

In contrast to the well-defined pathology of CML which is associated with the presence of the Bcr-Abl oncoprotein, AML is a highly heterogeneous disease

associated with a plethora of cytogenetic and molecular aberrations. The complexity of the numerous molecular interactions underlying the initiation and development of AML has been intensively studied, and oncogenic events are often separated multiple complementation classes (for more details see Chapter 1, section 1.2.1). Briefly, the classical Class I lesions (e.g. activating mutations in FLT3 or c-Kit) provide a proliferation and/or survival advantage and Class II mutations (e.g. PML-RARα or MLL-AF9) induce a block in myeloid differentiation and contribute to self-renewal [12, 39, 42, 45]. The cooperation between Class I and Class II aberrations disrupts the intricate balance between haematopoietic cell proliferation and differentiation, resulting in the AML phenotype.

Importantly, and consistent with a significant role for IL-3 in the leukaemic transformation, a range of AML-associated genetic lesions have been demonstrated to cooperate with IL-3 in the induction of a malignant leukaemic phenotype. Early studies discovered that the proto-oncogenes HoxB8, Myb and Evi-1 could immortalise IL-3 dependent haematopoietic cells by inducing a block in the normal differentiation process while allowing intact proliferation and survival signalling downstream of IL-3[250-253]. *In vivo* studies have further shown that IL-3 can cooperate with well characterised oncogenes such as HoxB8 and PML-RARα to induce aggressive and transplantable myeloid leukaemias in mice [188-190]. Similarly, a constitutively active mutant of the IL-3R β-subunit (V449E) can cooperate with the PML-RARα fusion to induce leukaemia *in vivo* [189, 190]. Together, these results suggest that in conjunction with molecular or cytogenetic lesions which result in a block in differentiation, IL-3 signalling may

reproduce the proliferation, survival and self-renewal programmes typically seen with Class I mutations. In line with this hypothesis, it has been shown that the growth and proliferation gene expression programme activated downstream of FLT3-ITD in myeloid 32Dcl3 cells more closely resembles that induced by IL-3 than by ligand activated FLT3-WT [254]. As mutations in the components of the IL-3 receptor are not frequent in leukaemia, this raises the possibility that the signal transduction downstream of IL-3 is potentiated in AML cells as a result of increased expression of IL-3Rα and autocrine IL-3 production, although more work is required to understand the signalling events induced by IL-3 in leukaemic cells.

In Chapter 3 we have demonstrated that IL-3 induces the stabilisation and activation of β-catenin in myeloid and leukaemic cell line systems, and in this Chapter we wished to further explore the relationship between IL-3 signalling and the regulation of β-catenin using primary AML patient samples. Importantly, aberrant activation of the Wnt/β-catenin axis has a well documented role in AML, and our findings which link IL-3 signalling and β-catenin accumulation, provide critical insight into a novel mechanism for the modulation of this pathway in leukaemia. In addition, we have performed microarray analysis coupled with a range of bioinformatic approaches to investigate the gene signatures activated by IL-3 in primary leukaemic cells. Using this approach, we identify early, late and sustained gene sets regulated by IL-3 in AML cells, and demonstrate significant enrichment of IL-3 induced gene expression with proliferation, self-renewal and oncogenic gene networks, further supporting a critical role for IL-3 in leukaemia.

To our knowledge, this is the first study which has looked directly at global gene regulation changes occurring downstream of IL-3 signalling in AML.

4.2 Results

4.2.1 β-catenin is stabilised by IL-3 in primary AML cells

To determine whether IL-3 can induce the stabilisation of β-catenin in primary AML cells, we analysed the response of four AML patient apheresis samples cultured in the presence or absence of IL-3. Patient information is summarised in **Table 4.1**; critically the peripheral blood leukaemic blast percentage was above 50% in all four patients studied. Primary AML cells were thawed and recovered overnight in high serum media (20% FCS), purified by ficoll-centrifugation and incubated in presence or absence of hIL-3 for 3, 6 or 16 hours. Cell lysates were analysed for STAT5A/B phosphorylation and total β-catenin protein expression by western immunoblotting.

Table 4.1 Clinical information of AML patients utilised in study.

Patient ID	Karyotype	FAB subtype	FLT3 mutation status	NPM1 mutation status	PB blasts (%)
AML 1	t(9;11), 11q23	M5	negative	negative	94
AML 2	q11	M1	negative	negative	51
AML 3	normal	M4	FLT3-TKD	positive	55
AML 4	normal	M1	FLT3-ITD\	positive	96

* PB=peripheral blood

Following a 3 hour IL-3 stimulation, all four AML patients showed increased phosphorylation of STAT5A/B consistent with an IL-3 signalling response. In patients AML 1, AML 2, and AML 3, we also observed an increase in β-catenin levels in cells stimulated with IL-3 **(Figure 4.1 A)**. Similar results were observed following a 6 and 16 hour stimulation with IL-3 **(Figure 4.1 B-C)**. These results are supportive of our cell line data (Chapter 3), and demonstrate that β-catenin levels can be induced by IL-3 stimulation in primary leukaemic cells. We did not however, observe any changes in β-catenin levels in response to IL-3 in AML 4 despite detectable stimulation of phospho-STAT5A/B. Interestingly, AML 4 was the only patient harbouring a FLT3-ITD mutation which has been previously associated with increased basal expression of β-catenin in AML [156].

In the previous chapter we have shown that treatment of starved TF-1.8 leukaemic cells with the IL-3Rα neutralising mAb 7G3 can inhibit the stimulation of β-catenin by IL-3. Hence, as a further measure of IL-3 signalling specificity, we next wished to determine whether 7G3 can block the IL-3 induced accumulation of β-catenin observed in the primary AML samples. To address this, AML 1-4 apheresis samples were processed as described above, and treated for 20 minutes on ice with 100nM of 7G3 or an IgG2a isotype control, and cultured in the presence or absence of 15ng/mL of hIL-3 for 3 hours. Consistent with the neutralising capacity of 7G3 which has been previously characterised in primary AML cells [79], IL-3 induced STAT5 phosphorylation was inhibited in all patient samples by the addition of the blocking antibody **(Figure 4.2 A-D)**. Importantly, treatment of AML 1, AML 2 and AML 3 cells with 7G3 prior to IL-3 stimulation blocked the accumulation of β-catenin, particularly in AML 1 and AML 2 which

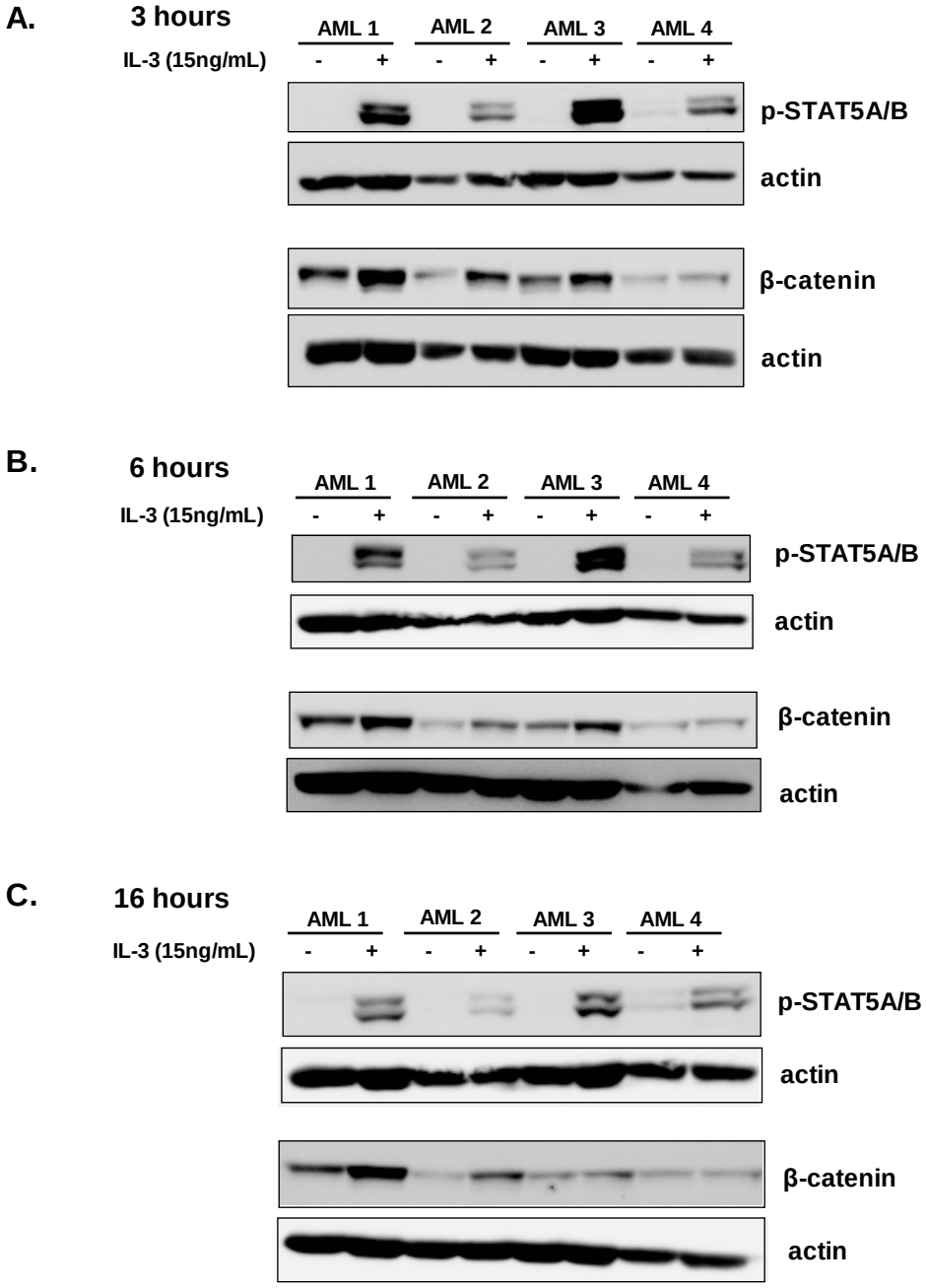


Figure 4.1 IL-3 regulates β-catenin levels in primary AML cells. Mononuclear cells from AML patient samples AML1-4 were thawed overnight in high serum media (IMDM + 20% FCS), purified by ficoll-centrifugation and re-cultured in IMDM + 10% FCS. Samples were then treated ± 15ng/mL hIL-3 at 37ºC for 3h **(A)** 6h **(B)** or 16h **(C)**. Cell lysates were analysed for p-STAT5A/B (Tyr694/699) and β-catenin protein levels by western blotting.

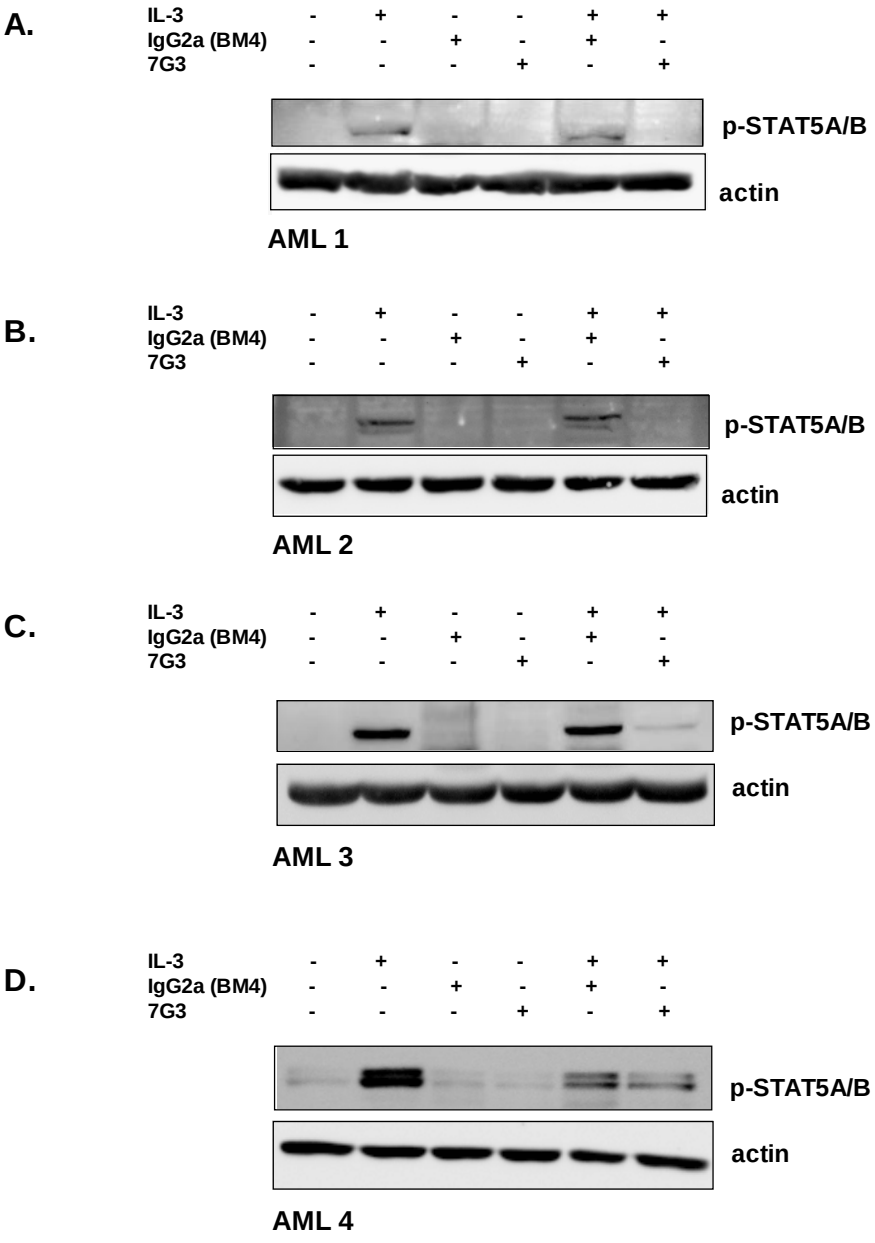


Figure 4.2 7G3 inhibits IL-3 mediated phosphorylation of STAT5A/B in primary AML cells. Mononuclear cells from AML patient samples AML1-4 were thawed overnight in high serum media (IMDM + 20% FCS), purified by ficoll-centrifugation and re-cultured in IMDM + 10% FCS. Samples were then treated ± 100nM of the IL-3 neutralising antibody 7G3 or an IgG2a isotype control (clone BM4) for 20 minutes on ice. Samples were subsequently cultured ± 15ng/mL hIL-3 for 3h at 37ºC. Cell lysates from AML 1-4 **(A-D)** were analysed for p-STAT5A/B (Tyr694/699) expression by western blotting.

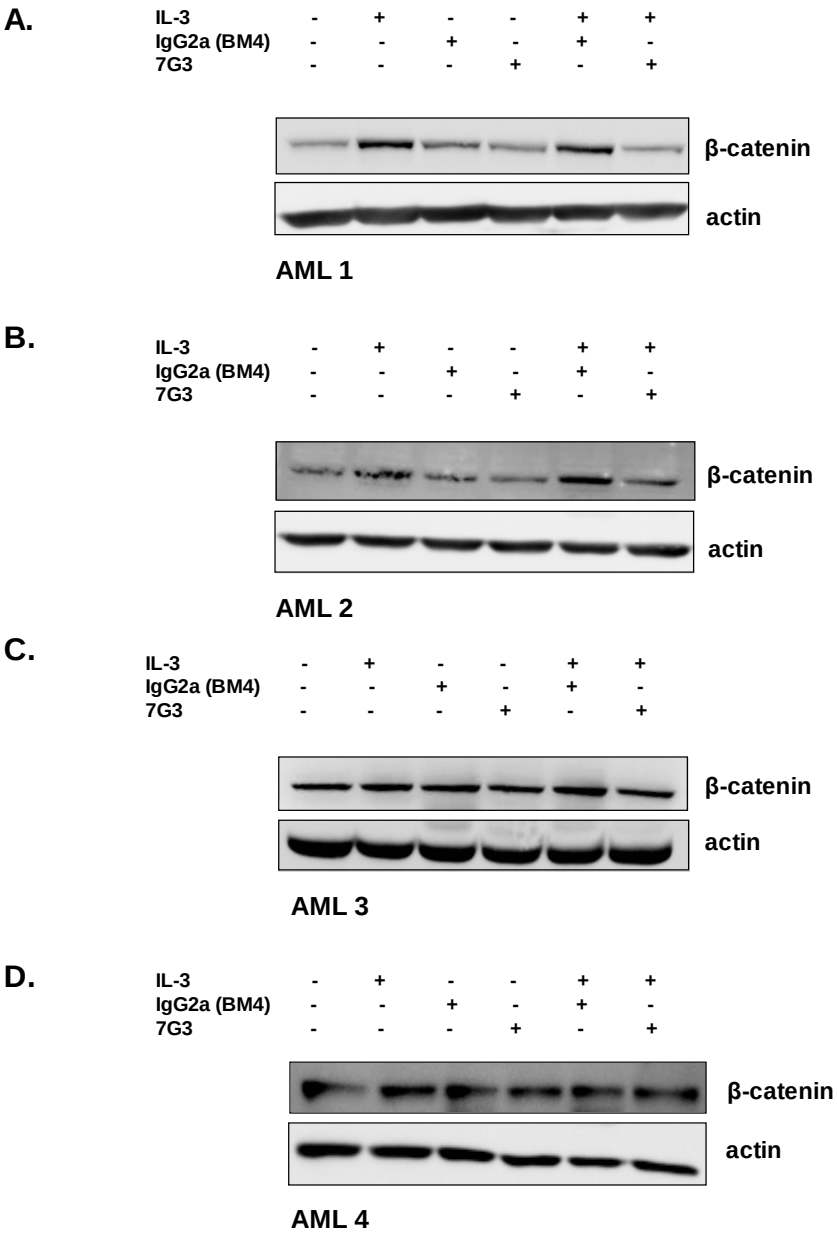


Figure 4.3 7G3 inhibits IL-3 mediated stabilisation of β-catenin in primary AML cells. Mononuclear cells from AML patient samples AML1-4 were thawed overnight in high serum media (IMDM + 20% FCS), purified by ficoll-centrifugation and re-cultured in IMDM + 10% FCS. Samples were then treated ± 100nM of the IL-3 neutralising antibody 7G3 or an IgG2a isotype control (clone BM4) for 20 minutes on ice. Samples were subsequently cultured ± 15ng/mL hIL-3 for 3h at 37ºC. Cell lysates from AML 1-4 **(A-D)** were analysed for β-catenin expression by western blotting.

showed the largest increase in β-catenin protein expression in response to IL-3 **(Figure 4.3 A-C)**. As may be predicted, however, treatment of AML 4 with 7G3 did not affect β-catenin protein levels which were unresponsive to IL-3 stimulation in this patient **(Figure 4.3 D)**.

To determine if the differences observed in the stimulation of β-catenin by IL-3 between the four patients could be due to differences in IL-3R expression, we performed flow cytometry analysis on the AML samples using anti-IL-3Rα and anti-βc primary antibodies, followed by Biotin and Streptavidin-PE staining. As shown in **Figure 4.4**, while all four patient samples displayed high levels of surface IL-3Rα expression, this was lowest for AML 4. Similarly, AML 4 had considerably lower expression of the βc subunit compared to AML 1-3 and it would be of interest to explore the correlation between IL-3R subunit expression and the IL-3 mediated regulation of β-catenin in a larger cohort of patients. It is also possible that the absence of β-catenin stimulation downstream of IL-3 in AML 4 may be a result of patient-specific alterations in signalling molecules downstream of the cytokine receptor.

Given reported differences in the proliferative response of primary AML cells to cytokines *in vitro,* we also measured proliferation of cells in response to IL-3 by thymidine incorporation of the AML samples cultured in a titration of IL-3 over 72 hours. Interestingly, while we observed dose-dependent proliferation in response to IL-3 for all samples **(Figure 4.5)**, the proliferation of AML 4 was notably reduced in comparison to the other patients supporting the idea that

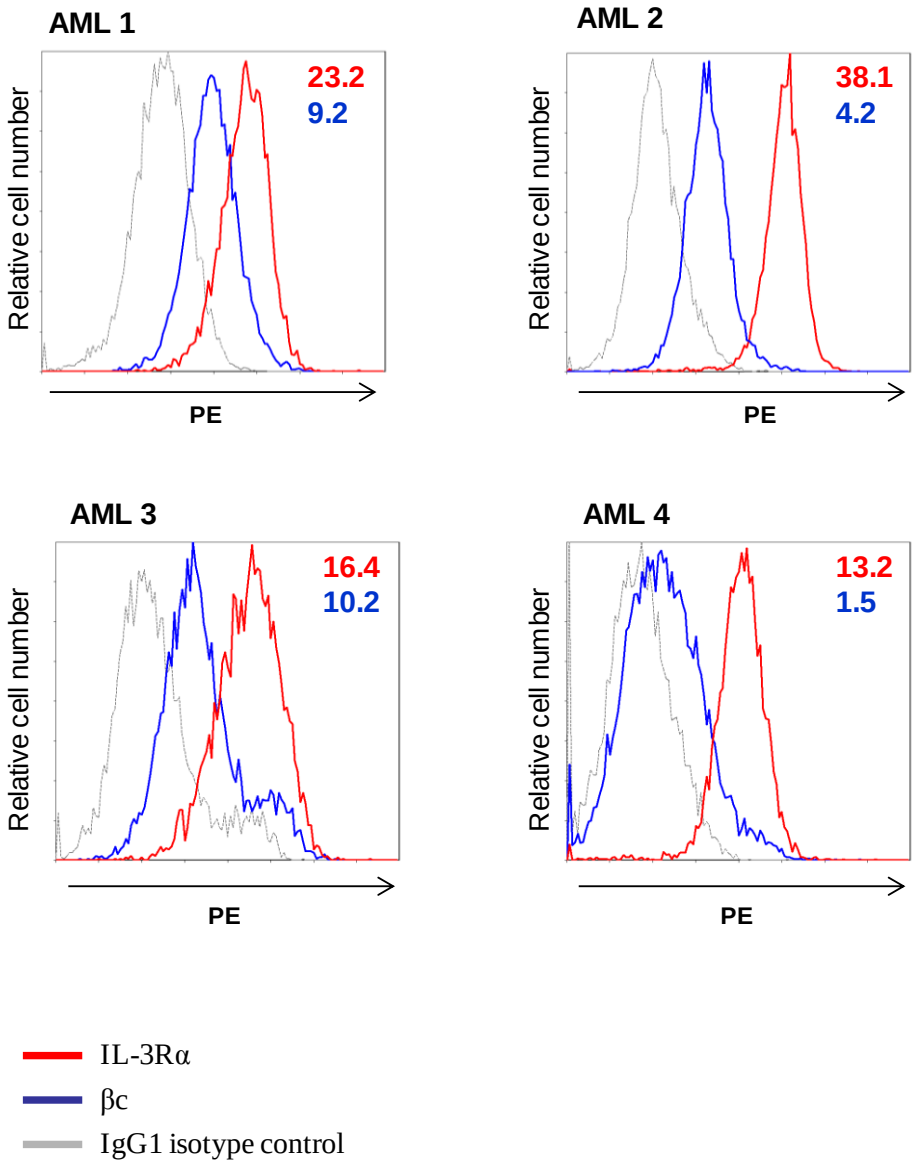


Figure 4.4 IL-3R surface expression of AML patient samples 1-4. AML mononuclear cells from AML1-4 were stained using an IL-3Rα, βc or isotope control antibody, followed by a Biotin and Streptavidin-PE tri-layer stain. Cells were fixed, and analysed by flow cytometry. A minimum of 50,000 live cells were analysed for each sample. Inset numbers indicate the MFI for IL-3Rα (red) or βc (blue) relative to isotype.

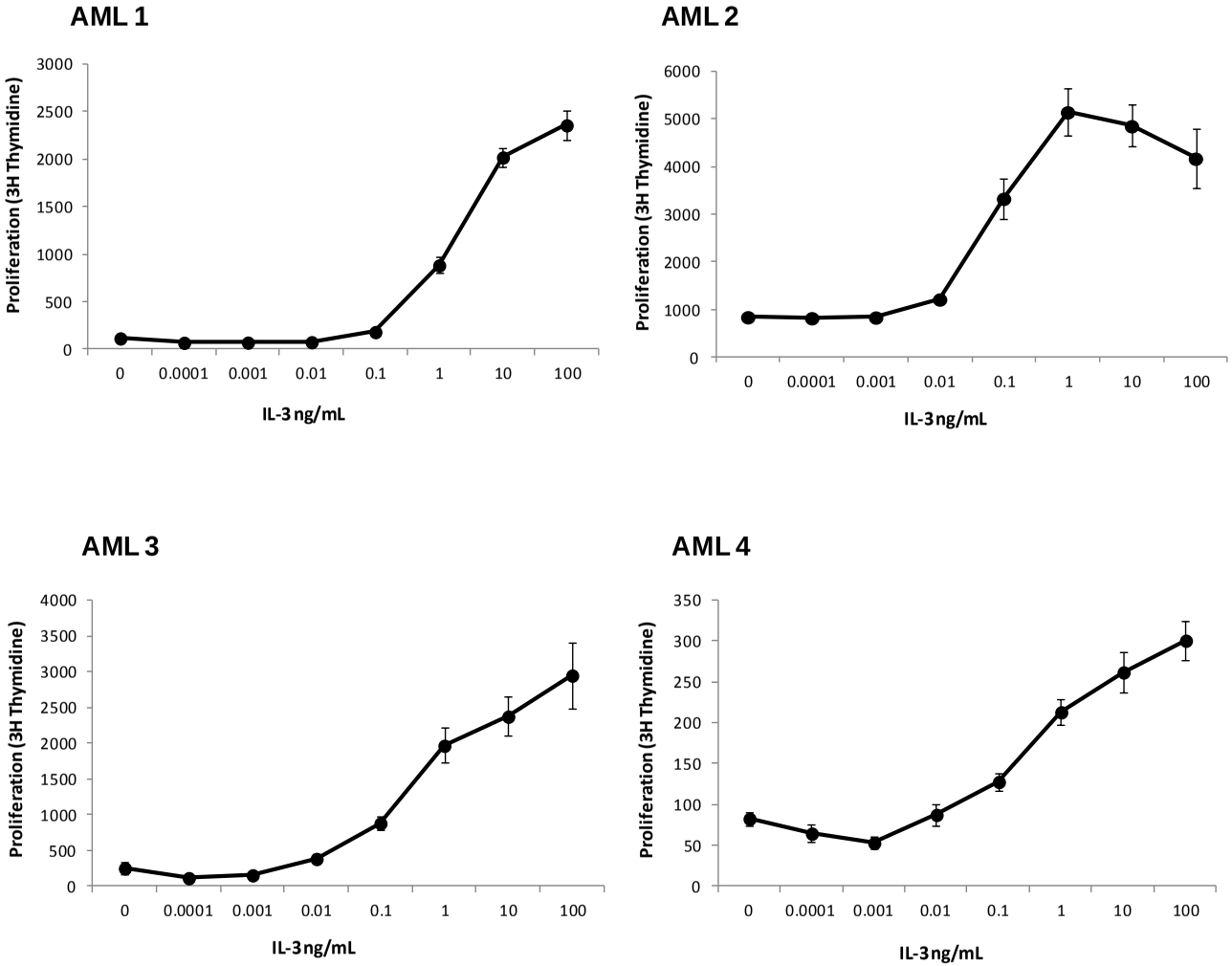


Figure 4.5 *In vitro* proliferation of AML patient samples 1-4 in response to IL-3. Mononuclear cells from AML patients were incubated in IMDM + 10% HI-FCS with a titration of IL-3 for 48 hours at 37ºC. Proliferation was measured by the addition of 3H thymidine for the final 24 hours at a concentration of 0.5µCi per well. Data shown represents the mean ± SEM of 6 replicates per patient.

This experiment was performed by Hayley Ramshaw and Sue Heatley from the division of Immunology, Centre for Cancer Biology, SA Pathology, Adelaide (2012).

activation of β-catenin, in addition to STAT5 may be critical for growth signals induced by IL-3.

4.2.2 Microarray analysis of global changes in gene expression induced by IL-3 in primary AML cells

In addition to large-scale studies analysing expression of IL-3Rα in AML and other leukemias [78, 192, 193, 255], IL-3 and other cytokines have been studied in the context of AML using several approaches, many of which have been recently facilitated by the advent of multiplex protein and substrate technologies, next-generation sequencing and genome wide gene expression and epigenetic assays. Several groups have shown that levels of IL-3 and other cytokines such as GM-CSF, IL-8 and TNF-α are elevated in patients with AML and other haematological malignancies compared to normal controls [238, 239, 241, 243]. This may be in part a result of autocrine cytokine production by leukaemic blasts [180, 246, 256]. Other recent studies have focused on comparing basal and IL-3 induced phospho-signalling networks in primary AML cells [257-259], however these have been limited to the analysis of the canonical IL-3 pathways such as the PI3K/AKT and JAK/STAT axes. To our knowledge, a genome wide analysis of the gene networks induced by IL-3 in AML has not been previously reported. Thus, in this study we wished to explore the global gene expression changes induced by IL-3 in primary AML cells with the aim to gain insight into the genes, gene signatures, and miRNA profiles regulated by IL-3 in leukaemia. In addition, as we have shown a novel link between IL-3 and β-catenin regulation in AML, we

aimed to determine whether IL-3 stimulation resulted in the activation of β-catenin/Tcf4 gene signatures in these leukaemic cells.

We performed microarray gene-expression analysis of the same four primary AML patient samples described in section 4.2.1 treated in the presence and absence of hIL-3, for which a functional IL-3 response has been demonstrated by the IL-3 induced phosphorylation of STAT5A/B. The AML patient samples were thawed and recovered overnight in high serum media, purified by ficoll-centrifugation, and cultured in the presence or absence 15ng/mL of hIL-3 for 6 or 16 hours. RNA was extracted using the miRNeasy mini kit (Qiagen) which allows the effective purification of miRNAs in addition to total RNA. RNA quality was confirmed using an Agilent Bioanalyzer (**Appendix C**). Microarrays were performed at the Adelaide Microarray Centre using Affymetrix gene platforms and differential gene expression was determined using the Linear Models for Microarray data Analysis (LIMMA) method [260]. Microarray data analysis was assisted by Chung Kok (Centre for Cancer Biology, SA Pathology).

4.2.2.1 IL-3 induces a range of transcriptional changes in AML cells at 6 and 16h

We observed large transcriptional changes in the AML patient cells in response to IL-3: 664 gene probes showed significant regulation at 6h, and 3150 at 16h, the majority of which increased in expression on IL-3 treatment. The top 50 IL-3 induced genes at each time point (selected by their FDR adjusted p-value <0.05 and listed by fold-change) and their differential expression between IL-3 treated or untreated cells are represented as heatmaps in **Figure 4.6** and **Figure 4.7**

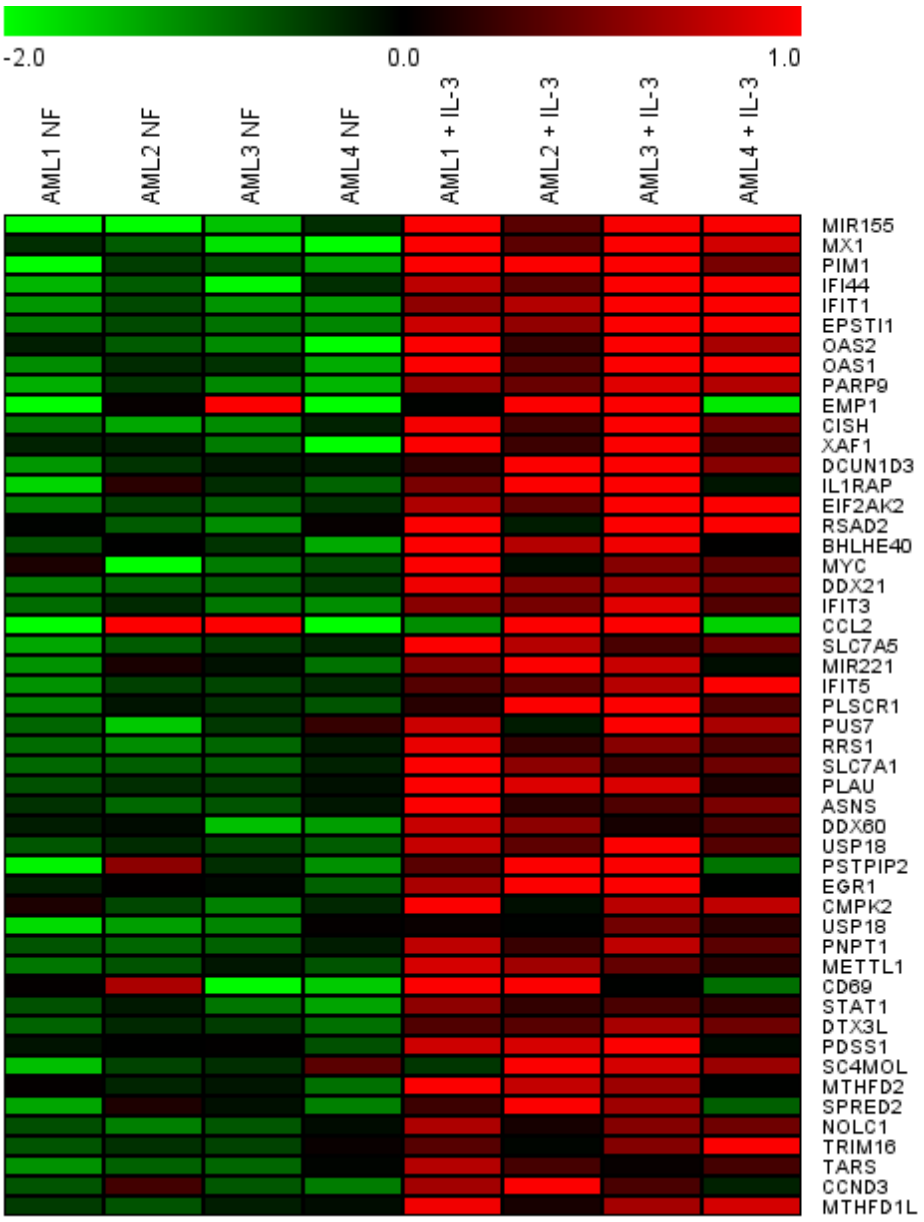


Figure 4.6 Highest ranking genes induced by IL-3 in primary AML cells after 6h stimulation. RNA extracted from mononuclear cells from four AML patients (AML 1-4) incubated in IMDM + 10% HI-FCS and treated ± 15ng/mL hIL-3 for 6h was analysed for global gene expression using an Affymetrix gene array. Differential gene expression was determined using paired analysis of all 4 samples (LIMMA method). Represented in a heatmap, are the top 50 genes displaying significant expression differences induced by IL-3 treatment at 6h (selected by their FDR p-value and Fold Change). The heatmap was generated using the MultiExperimental Viewer (MeV) software v4.8. NF = no factor.

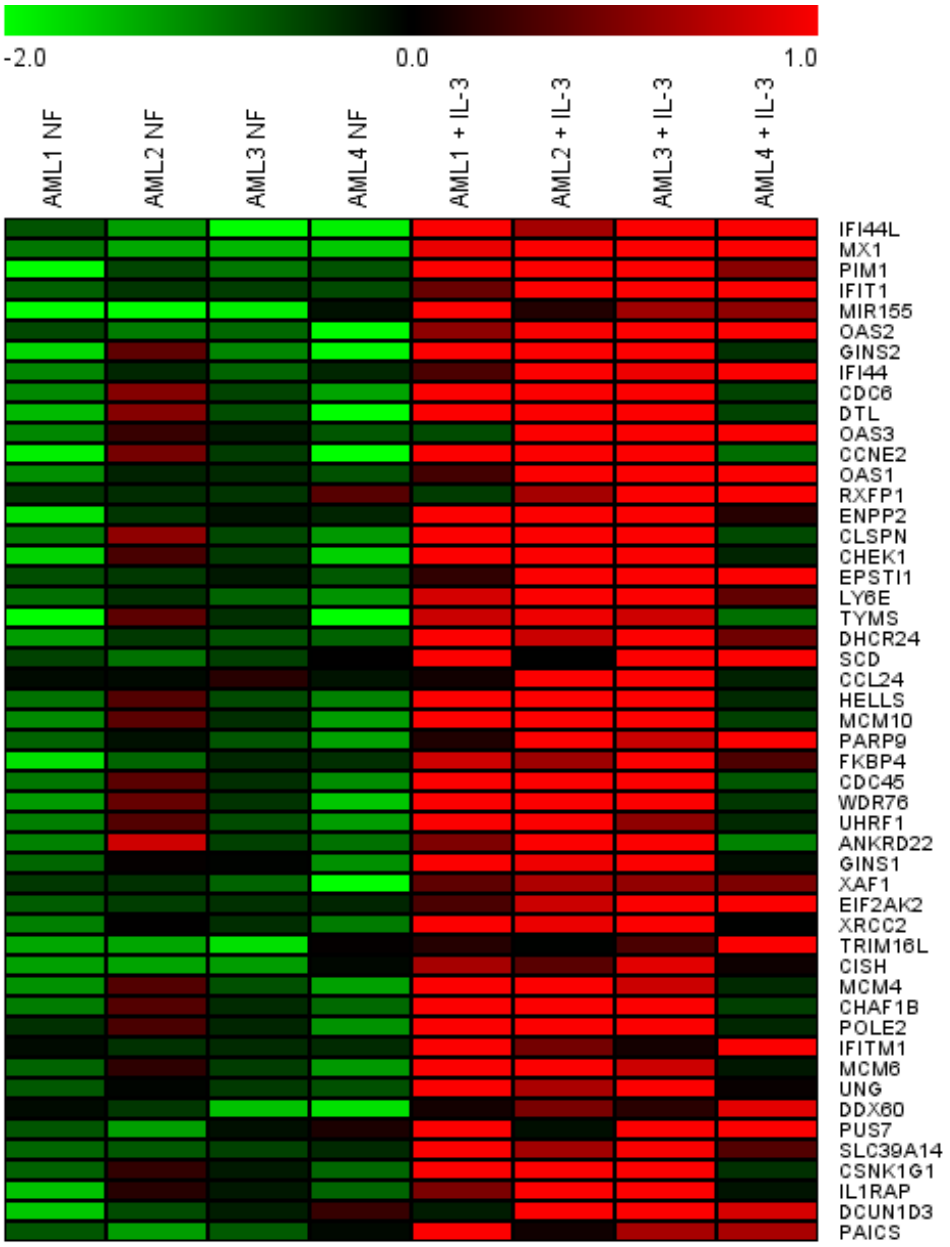


Figure 4.7 Highest ranking genes induced by IL-3 in primary AML cells after 16h stimulation. RNA extracted from mononuclear cells from four AML patients (AML 1-4) incubated in IMDM + 10% HI-FCS and treated ± 15ng/mL hIL-3 for 16h was analyzed for global gene expression using an Affymetrix gene array. Differential gene expression was determined using a paired analysis of all 4 samples (LIMMA method). Represented in a heatmap, are the top 50 genes displaying significant expression differences induced by IL-3 treatment at 16h (selected by their FDR p-value and Fold Change). The heatmap was generated using the MultiExperimental Viewer (MeV) software v4.8. NF = no factor.

respectively (additional information in **Appendix D**). Of interest, several miRNAs with reported roles in proliferation and oncogeneis, including miR-155 and mi-221, were amongst the genes with the highest fold change, and these will be further discussed in section 4.2.2.3.

We also identified a significant number of genes that were induced by IL-3 at either 6h or 16h, and another group that were regulated at both timepoints. We have analysed these groups of genes separately and refer to the different gene sets as early (only changed at 6h), late (only changed at 16h) or sustained (changed at both 6 and 16h). The top 30 IL-3 induced genes from each group (selected by their FDR adjusted p-value < 0.05 and listed by fold change) are represented as a heatmap in **Figure 4.8** and **Appendix E**. Consistent with other studies which have shown a rapid and transient increase in *EGR-1* mRNA expression in response to IL-3 in myeloid and leukaemic cells [261-263], *EGR-1* was one of the early IL-3 responsive genes with the highest fold change (p=0.03; FC=2.30). The haematopoietic transcription factor *GATA-2* (p=0.02; FC=1.84) and the inflammation associated cytokine *TNF* (p=0.02; FC=1.62) were also significantly upregulated by IL-3 after a 6h stimulation.

Interestingly, another of the highest ranking early response genes was *IL-3Rα* (p=0.03; FC=1.60). Autoregulation of receptor components is commonly observed for growth factors and cytokines and represents an integral part of transduction regulation and control. It has been shown that not only IL-3, but also the related cytokines GM-CSF and IL-5 increase *IL-3Rα* mRNA levels in eosinophils [264]. Stimulation of eosinophils with IL-3, GM-CSF or IL-5 also

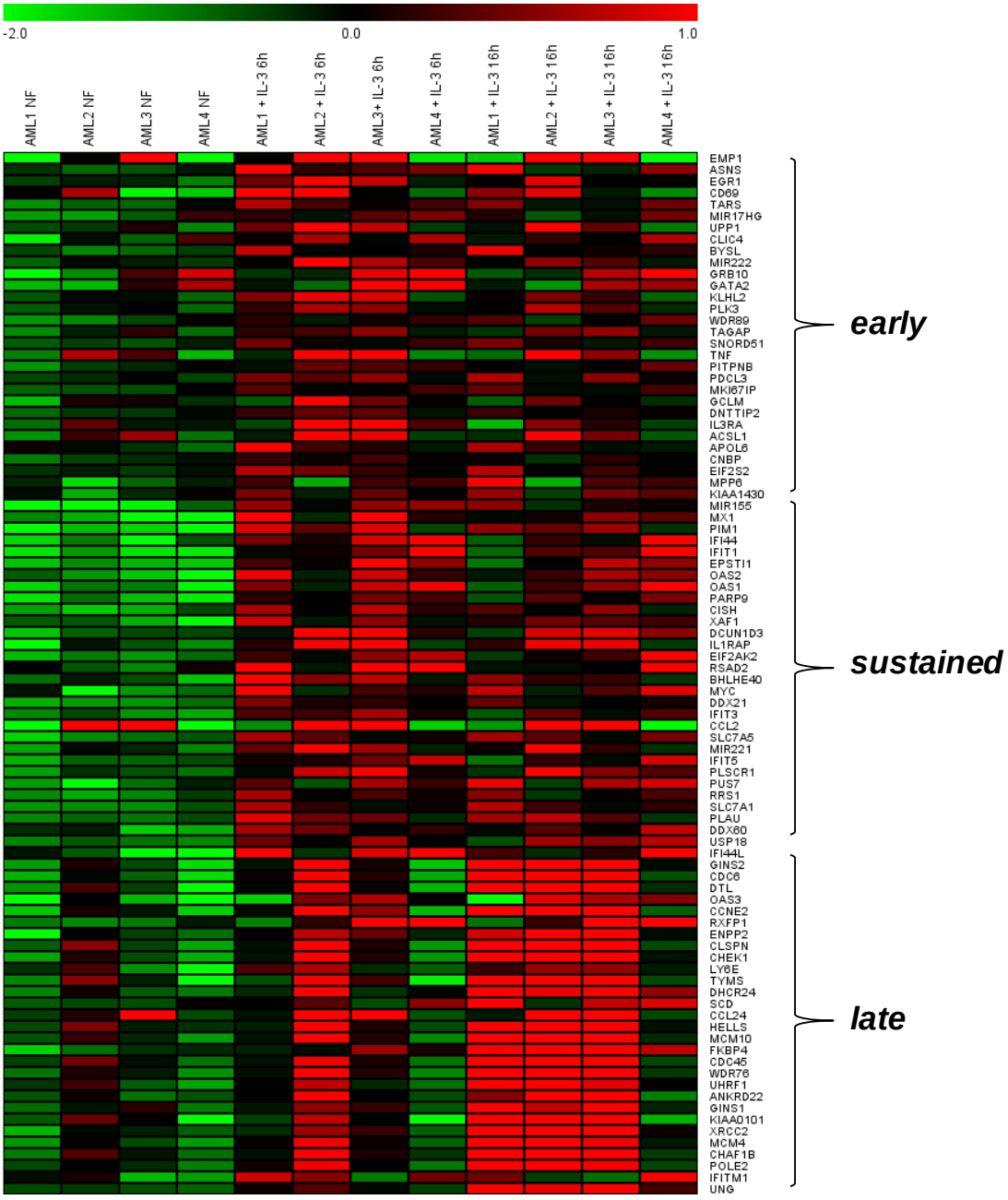


Figure 4.8 Classification of highest ranking early, sustained and late IL-3 induced genes in primary AML cells. Differential gene expression induced by IL-3 in AML1-4 was determined using a paired analysis of all 4 samples, and significant genes were classified as early (only modulated at 6h), sustained (modulated at both 6 and 16h) and late (only modulated at 16h) genes. Represented in a heatmap, are the top 30 genes from each category displaying significant expression differences induced by IL-3 treatment (selected by their FDR p-value and Fold Change). The heatmap was generated using the MultiExperimental Viewer (MeV) software v4.8. NF = no factor.

resulted in increased mRNA expression of *GM-Rα* and the common *β-chain* (*βc*) and a decrease in *IL-5Rα* levels [264]. It has also been demonstrated that the cytokine-induced accumulation of *IL-3Rα, GM-Rα* and *βc* mRNA is not a result of increased transcription, but through a reduction in mRNA degradation [264]. The coordinated regulation of α- receptor subunit expression induced by IL-3, GM-CSF and IL-5 may be specific to eosinophil differentiation as we did not observe any significant changes in *GM-Rα* or *IL-5Rα* mRNA levels in response to IL-3 in the four AML patient samples. *βc* levels, however, were significantly increased by IL-3 at both 6 (p=0.04; FC=1.70) and 16h (p=0.005; FC=1.90), suggesting that upregulation of IL-3 receptor mRNA may constitute an important feedback loop downstream of IL-3 in AML cells.

In contrast to the early IL-3 induced genes, which by our definition were only significantly modulated by hIL-3 at the 6h timepoint, the majority of the genes significantly regulated after a 6h IL-3 stimulation (504/664) were in the sustained category and remained differentially regulated at 16h. Several of the genes that were commonly upregulated at both timepoints are well characterised cytokine-responsive genes including *PIM1*, *PIM2* and *CISH* consistent with the known functions of IL-3 signalling**.** Notably *PIM1* and *CISH* were amongst the top 50 ranking genes with the highest fold change at each timepoint **(Figure 4.6 & 4.7)**. To validate expression of these genes in the individual AML patient samples, we performed QRT-PCR analysis**. Figure 4.9 A-B** shows mRNA expression of *PIM1* and *CISH* in AML1-4 cultured in the presence or absence of hIL-3 for 6 or 16 hours. Consistent with the microarray data, we observed a significant increase in both *PIM1* and *CISH* mRNA expression at both timepoints ($p<0.05$).

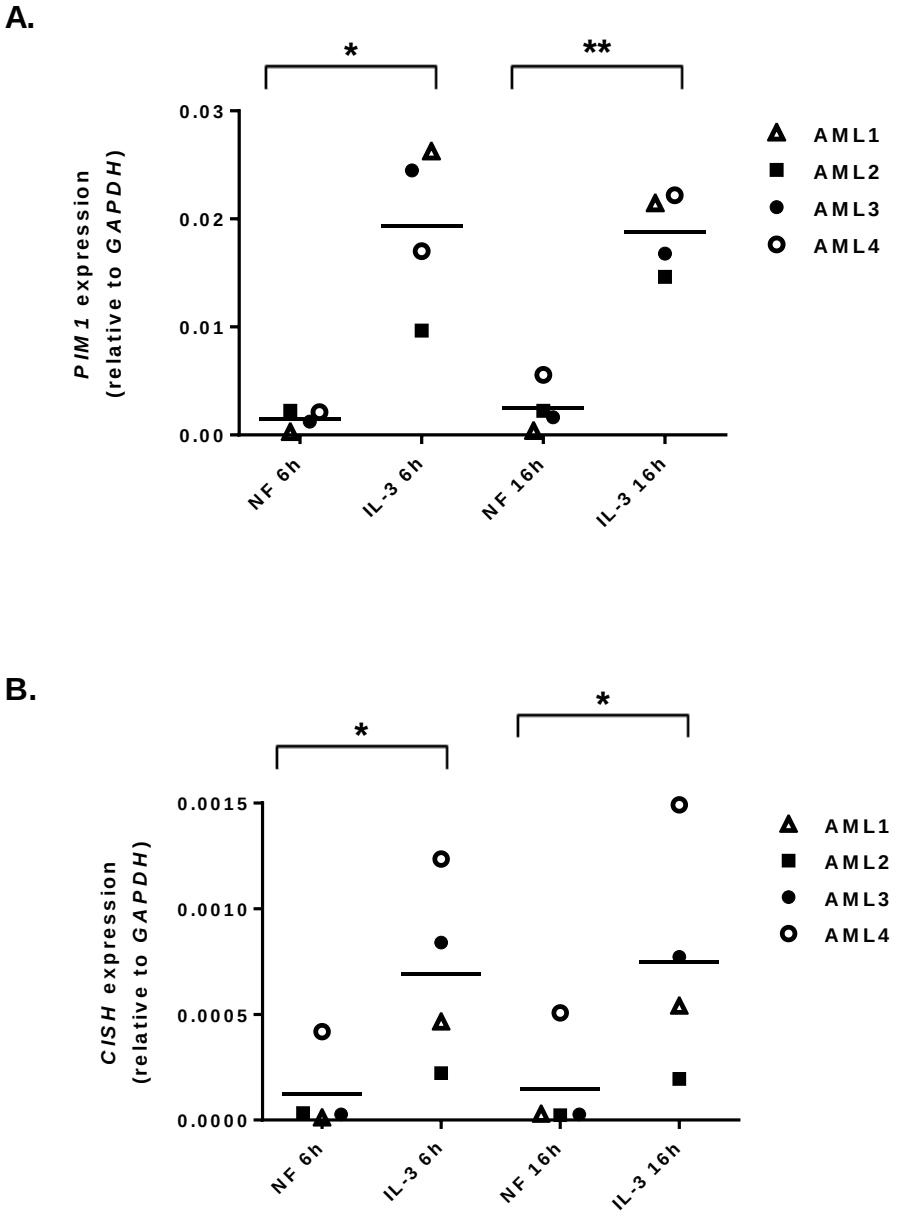


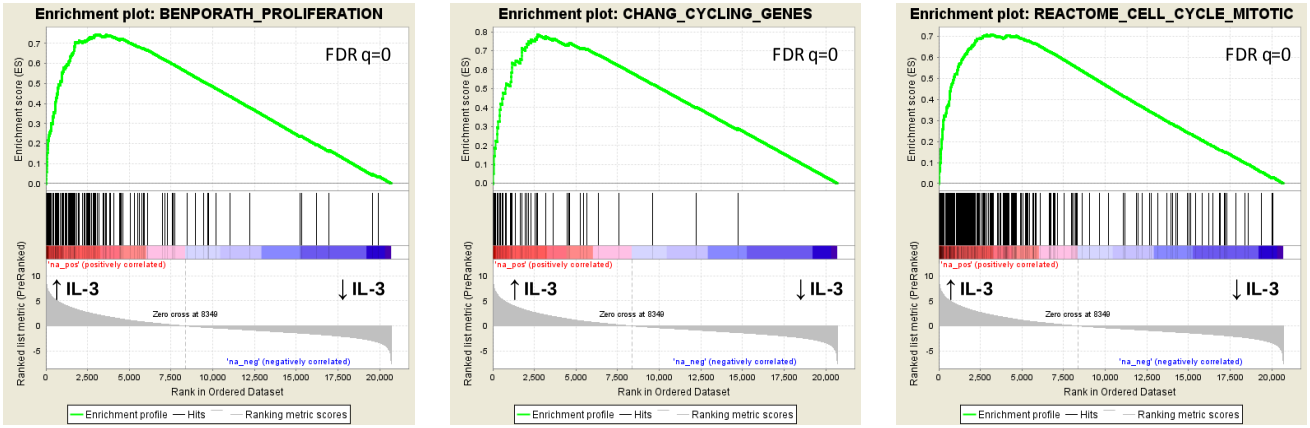
Figure 4.9 Validation of PIM1 and CISH mRNA expression in primary AML samples treated with hIL-3. (A) *PIM1* and **(B)** *CISH* mRNA expression was measured by QRT-PCR in RNA extracted from patient samples AML1-4 treated ± 15ng/mL hIL-3 for 6 or 16h. The RNA was the same as used for microarray analysis of gene expression. Results were normalised to *GAPDH* expression. Horizontal lines depict mean expression of the four samples. Results were analysed using a paired t-test, were *$p \leq 0.05$; **$p \leq 0.01$. NF = no factor.

Lastly, we identified >2500 late genes induced by IL-3 only at 16h**.** Not surprisingly, given the known proliferative and cycling function of IL-3 on myeloid and leukaemic cells, several of the highest ranking late IL-3 induced genes such as *CDC6* (p=0.003; FC=3.94), *CCNE2* (p=0.002; FC=3.80), *CHECK1* (p=0.002; FC=3.34), and *CDC45* (p=0.005; FC=2.90), are key players in the mitotic cycle and cell division pathways. Further supporting the known functions of IL-3, Gene Set Enrichment Analysis (GSEA) performed using the Broad Institute Molecular Signature database (MSigDB) curated genesets (c2, version 3.1) identified a strong correlation between the gene expression changes induced by IL-3 at 16h and gene signatures related to proliferation and cell cycle control as with haematopoiesis and stem cell associated signatures **(Figure 4.10)**.

It should be noted here, that while the statistical analysis performed on the microarray data to determine the genes which were significantly regulated by IL-3 comprised paired analysis of the four AML patient samples, this information did not provide insight into the patient-specific differences in gene expression. In contrast, the heatmaps, adjusted to the median centre for each represented gene, allow a visual comparison of variations in gene expression induced by IL-3 in the four AML patients. Given the known heterogeneity of AML, it is not surprising that although the four patients displayed similar trends in gene alterations, there is a distinct expression profile observed for each patient. For instance, compared to the other AML patient samples, AML 2 showed high expression of the majority of the genes we classified as late genes at 6h **(Figure 4.8)**. This suggests that several of the late genes, were upregulated considerably earlier in AML 2, and remained elevated up to 16h following IL-3 stimulation despite statistically being classified

A.

Proliferation and cell division



B.

Haematopoiesis and stem-cell function

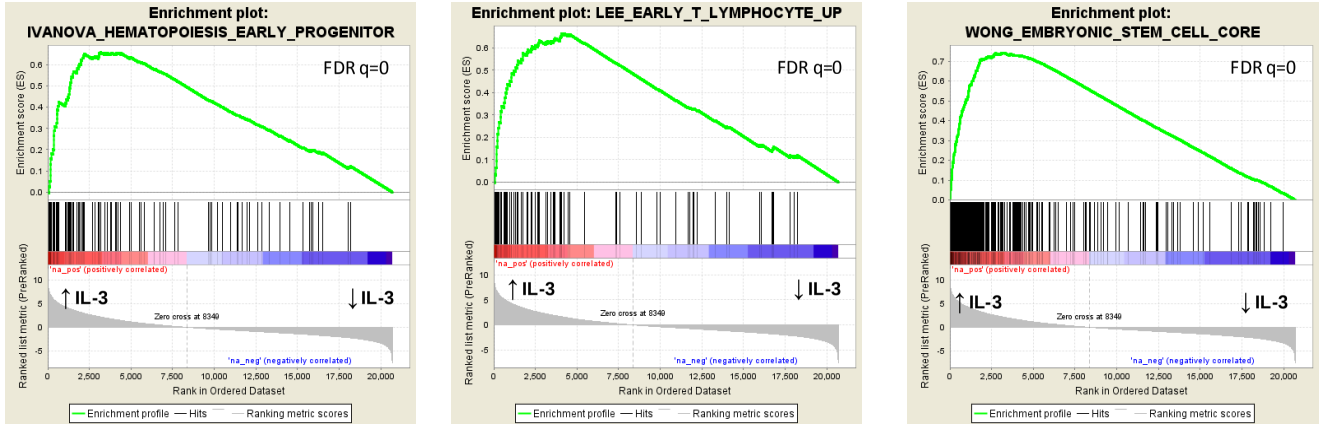


Figure 4.10 Enrichment plots of proliferation and haematopoiesis related signatures identified by GSEA analysis of gene expression changes induced by IL-3 in primary AML cells. The dataset of gene expression differences resulting from the 16h IL-3 stimulation of primary AML cells was used for GSEA using the Broad Institute Molecular Signature database (MSigDB) curated genesets (c2, version 3.1). Summarised are enrichment plots of **(A)** proliferation and cell division associated signatures and **(B)** haematopoiesis and stem cell associated signatures which displayed a significant enrichment for genes modulated by IL-3 in the primary AML cells ($p<0.05$).

as late (only changed at 16h) when considering the four AML patient samples together. This result cannot be attributed to differences in IL-3R expression (**Figure 4.4**), however it suggests that while IL-3 may induce similar gene expression changes across different AML patient samples, the dynamics of transcriptional activation may vary considerably across AMLs.

Also of interest, the gene expression profile induced by IL-3 in AML 4 appeared the most reduced compared to AML 1-3 **(Figures 4.6-8)**. In addition, principle component analysis and unsupervised hierarchical clustering analysis revealed AML 4 as an outlier of the gene expression profiling when compared with AML 1-3 **(Figure 4.11)**. Importantly, the distinct and reduced gene signatures activated by IL-3 in AML 4 compared to the other AML patients may be related to the absence of β-catenin protein stabilisation, which may in turn explain the diminished proliferation response we observed in this patient sample following treatment with IL-3.

4.2.2.2 Stimulation of primary AML cells with IL-3 induces the activation of β-catenin and Tcf4 transcriptional signatures

In this study we have identified β-catenin as a novel downstream target of IL-3 signalling, and have demonstrated that IL-3 induces the stabilisation and activation of β-catenin in myeloid and leukaemic cell line systems (Chapter 3). In addition we have extended these studies to primary AML cells, and showed that β-catenin protein was stabilised in response to IL-3 stimulation in 3 out of the 4 patient samples we tested (Section 4.2.1). To establish if increased β-catenin levels in the primary AML cells corresponded with activation of β-catenin

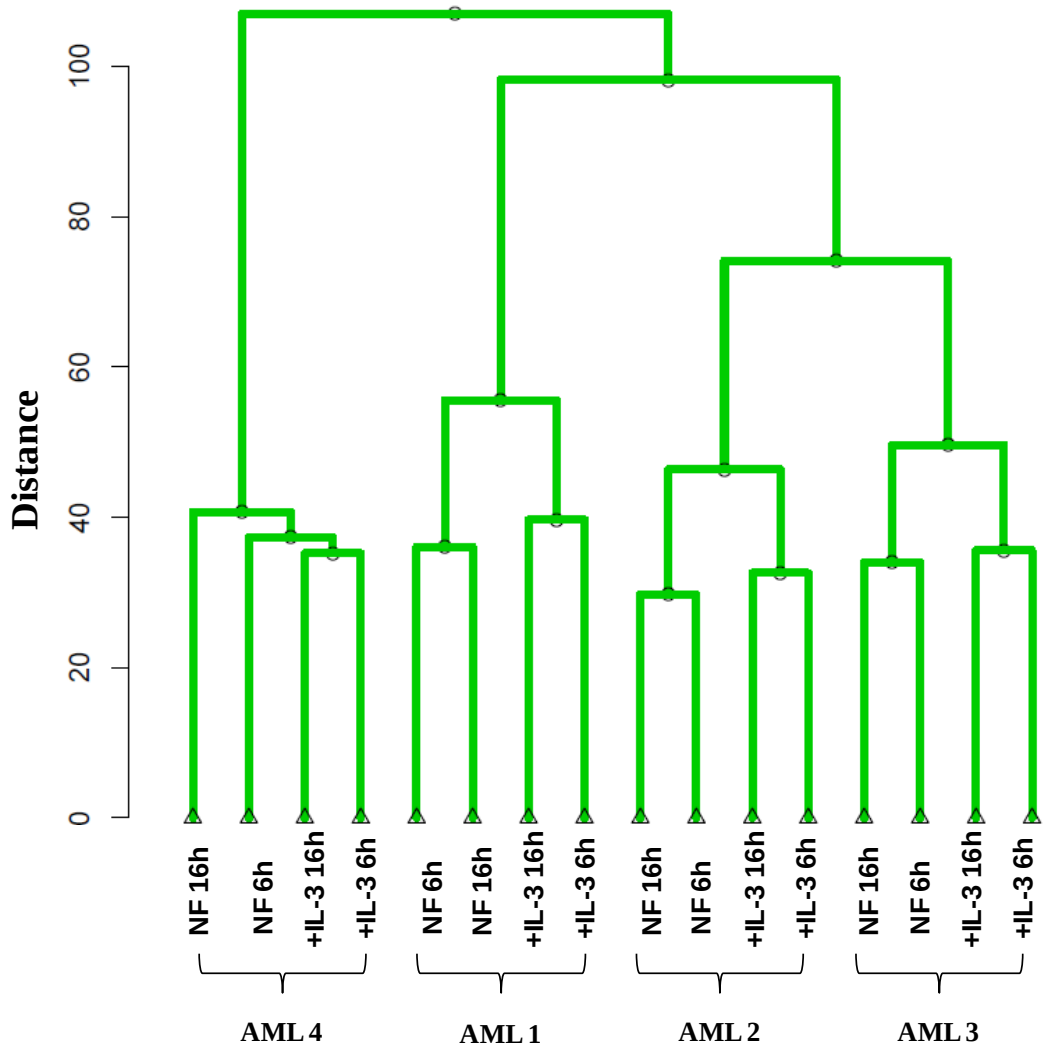


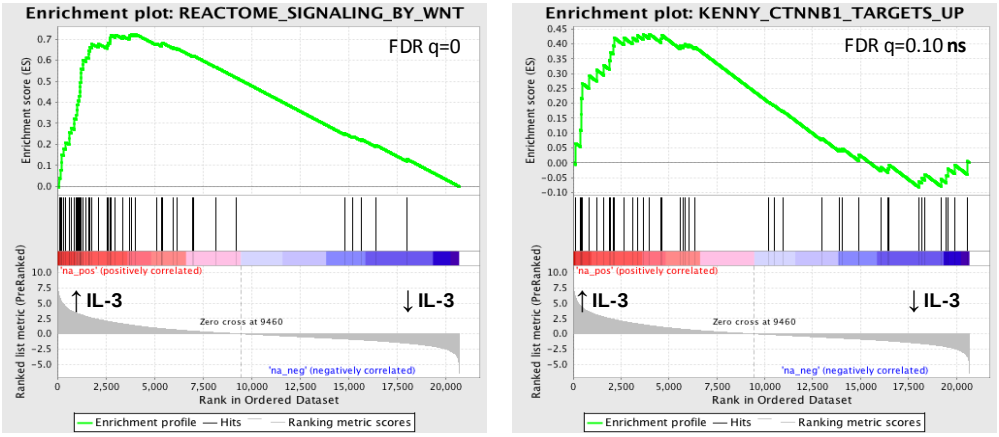
Figure 4.11 Hierarchical clustering of AML patient samples analysed in microarray. RNA extracted from mononuclear cells from four AML patients (AML 1-4) treated ± 15ng/mL hIL-3 for 6h or 16h was analysed for global gene expression using an Affymetrix gene array. Unsupervised clustering was performed using the Euclidian method based on the gene expression of each sample. NF= no factor.

transcriptional activity, we performed GSEA and ChIP Enrichement Analysis (ChEA) of the microarray data to determine whether the IL-3 modulated gene networks were enriched for Wnt/β-catenin/Tcf4 gene signatures.

GSEA analysis was performed on the datasets of gene expression differences resulting from IL-3 treatment of the primary AML cells at 6 and 16h. In support for a role for IL-3 in the functional activation of β-catenin in primary AML cells, we identified a significant enrichment for gene signatures associated with Wnt and β-catenin signalling in the IL-3 regulated genes ($p>0.05$, **Figure 4.12**). Enrichment of the *Wnt* signature **(Figure 4.12 A)** was significant for both the 6 and 16h timepoints, while the *Ctnnb1/β-catenin* signature **(Figure 4.12 B)** was only significant with the genes modulated by IL-3 at 16h. Although we detected increased β-catenin protein levels as early as 3h following IL-3 stimulation **(Figure 4.1 A)**, these results suggest that detection of β-catenin transcriptional output is more evident after 16h. This is consistent with previously published information about the dynamics of β-catenin transcriptional activation which requires β-catenin protein stabilisation, nuclear localisation, interaction with DNA-binding co-transcription factors such as Tcf4 and Lef-1, and subsequent regulation of downstream target genes (see Chapter 1, section 1.3.1**).**

To further study the association between IL-3 signalling and β-catenin transcriptional activity in AML cells, we performed ChEA on the genes significantly regulated by IL-3 at 6 and 16h with a fold-change > 1.5. ChEA revealed significant overlap of genes modulated by IL-3 with published β-catenin/Tcf4 ChIP studies at both the 6h **(Table 4.2)** and 16h **(Table 4.3)**

A.



B.

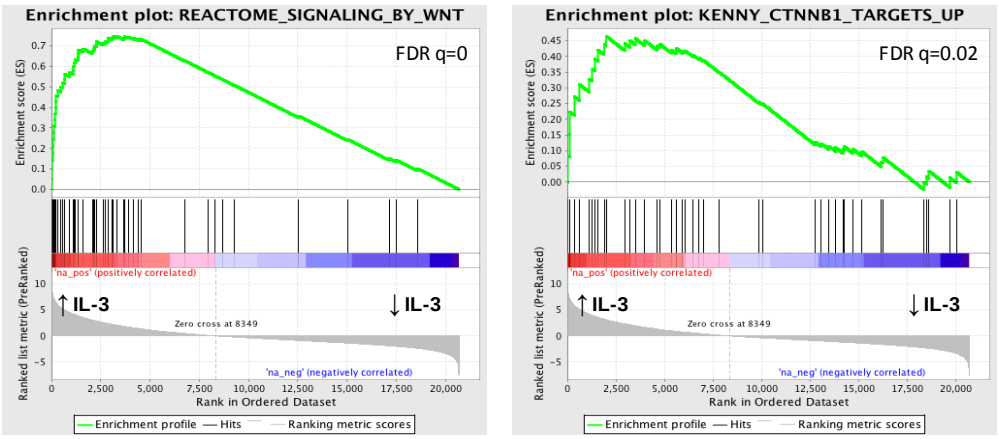


Figure 4.12 Genes modulated by IL-3 in primary AML cells show significant enrichment for Wnt and β-catenin signatures. The dataset of gene expression differences resulting from the 6h **(A)** and 16h **(B)** IL-3 stimulation of primary AML cells was used for GSEA using the Broad Institute Molecular Signature database (MSigDB) curated genesets (c2, version 3.1). Analysis identified a significant enrichment with a *Wnt Reactome* signature, $p<0.05$ for the 6 and 16h gene sets, and a *β-catenin (Ctnnb1) signature*, $p<0.05$ for the 16h gene set.

timepoints. Three of these target genes, *UPP1*, *MYC* and *FANCC*, were validated by QRT-PCR on RNA extracted from the individual AML patient samples cultured in the presence or absence IL-3 for 6 or 16h **(Figure 4.13)**. Consistent with the microarray results, we observed a significant upregulation of *UPP1* and *MYC* at 6 and 16h, and a significant upregulation of *FANCC* at 16h following IL-3 stimulation.

Table 4.2 ChEA analysis showing β-catenin and TCF4/TCF7L2 ChIP target genes significantly enriched in primary AML cells treated with IL-3 for 6h. Genes significantly regulated by IL-3 (FC > 1.5) after a 6h stimulation of primary AML cells were used against the ChEA database. Significant enrichment was identified for the IL-3 regulated genes and published β-catenin and TCF4/TCF7L2 ChIP studies ($p<0.05$). Indicated are the overlapping genes. Genes marked in bold were selected for QRT-PCR validation of expression in the primary AML samples.

From PMID 20460455 (Bottomly *et al*, 2010)	FC	From PMID 21901280 (Norton *et al*, 2011)	FC
BHLHE40	2.643	LYRM1	1.658
CSF2RB	1.692	MKI67IP	1.590
EML4	1.672	MTHFD2	1.805
FBXO32	-1.686	PIM1	5.824
LYRM1	1.658	PITPNB	1.619
MTHFD1L	2.002	PPIL1	1.533
MYC	2.621	RUVBL1	1.566
NOLC1	2.052	SLC39A14	1.701
PPIL1	1.533		
PPRC1	1.798		
TIMM17A	1.559		
UPP1	1.963		
UTP15	1.758		
WDR74	1.542		
YARS	1.583		

* ChIP studies:
Bottomly *et al*, 2010 - β-catenin ChIP (Colon cancer)
Norton *et al*, 2011 - TCF4/TCF7L2 ChIP (Hepatic tissue)
PMID= PubMed Identification

Table 4.3 ChEA analysis showing β-catenin and TCF4/TCF7L2 ChIP target genes significantly enriched in primary AML cells treated with IL-3 for 16h. Genes significantly regulated by IL-3 (FC > 1.5) after a 16h stimulation of primary AML cells were used against the ChEA database. Significant enrichment was identified for the IL-3 regulated genes and published β-catenin and TCF4/TCF7L2 ChIP studies ($p<0.05$). Indicated are the overlapping genes. Genes marked in bold were selected for QRT-PCR validation of expression in the primary AML samples.

From PMID 20460455 (Bottomly *et al*, 2010)	FC	From PMID 21901280 (Norton *et al*, 2011)	FC	From PMID 20615089 (Pedrosa *et al*, 2010)	FC
AIMP2	1.537	AGMAT	1.594	CCL24	3.072
AKAP1	1.520	BCAS3	-1.567	DUX4	-1.637
ARHGEF3	-1.547	FADS2	2.275	FAM86B1	1.300
BCAS3	-1.567	**FANCC**	1.712	GNG7	-1.516
BHLHE40	1.804	GMNN	1.929	LOC349196	-2.054
BRCA1	2.047	GNG7	-1.516	MCM7	1.934
BRIP1	2.291	LYRM1	1.696	MRPL21	1.672
BTBD11	-1.623	MAD2L1	1.849	POLE	1.570
C4ORF21	1.623	MTHFD2	1.525	PRMT5	1.593
C4ORF46	2.073	NHEDC2	1.747	PRPS1	1.627
C6ORF108	1.554	NUP205	1.544	PXMP2	1.654
CENPN	1.853	PIM1	5.109	REXO1L1	-1.829
CKS1B	1.737	PPIL1	1.588	REXO1L2P	-1.609
CSF2RB	1.895	PRMT1	1.643		
DTL	3.878	PSPH	1.619		
EML4	1.747	RAD51AP1	2.032		
EZH2	1.707	RUVBL1	2.089		
FBXO32	-1.800	SLC39A14	2.513		
GNG7	-1.516	TRMT1	1.603		
HBB	-1.885	TTLL12	1.568		
HELLS	3.059	ZBTB20	-1.563		
LARP4	1.625				
LDLR	1.915				
LYRM1	1.696				
MTHFD1L	1.907				
MYC	1.764				
NOLC1	2.063				
PPIL1	1.588				
PPRC1	1.716				
PRKDC	1.579				
RANBP1	2.242				
SLC12A2	1.545				
SPATS2L	1.990				
STEAP3	1.564				
UTP15	1.949				
UTP20	1.917				
WDR74	1.760				
YARS	1.562				
ZBTB20	-1.563				

* ChIP studies: Bottomly *et al*, 2010 - βcatenin ChIP (Colon cancer); Norton *et al*, 2011 - TCF4/TCF7L2 ChIP (Hepatocytes); Pedrosa *et al*, 2010 - β-catenin ChIP (Brain tissue). PMID= PubMed Identification

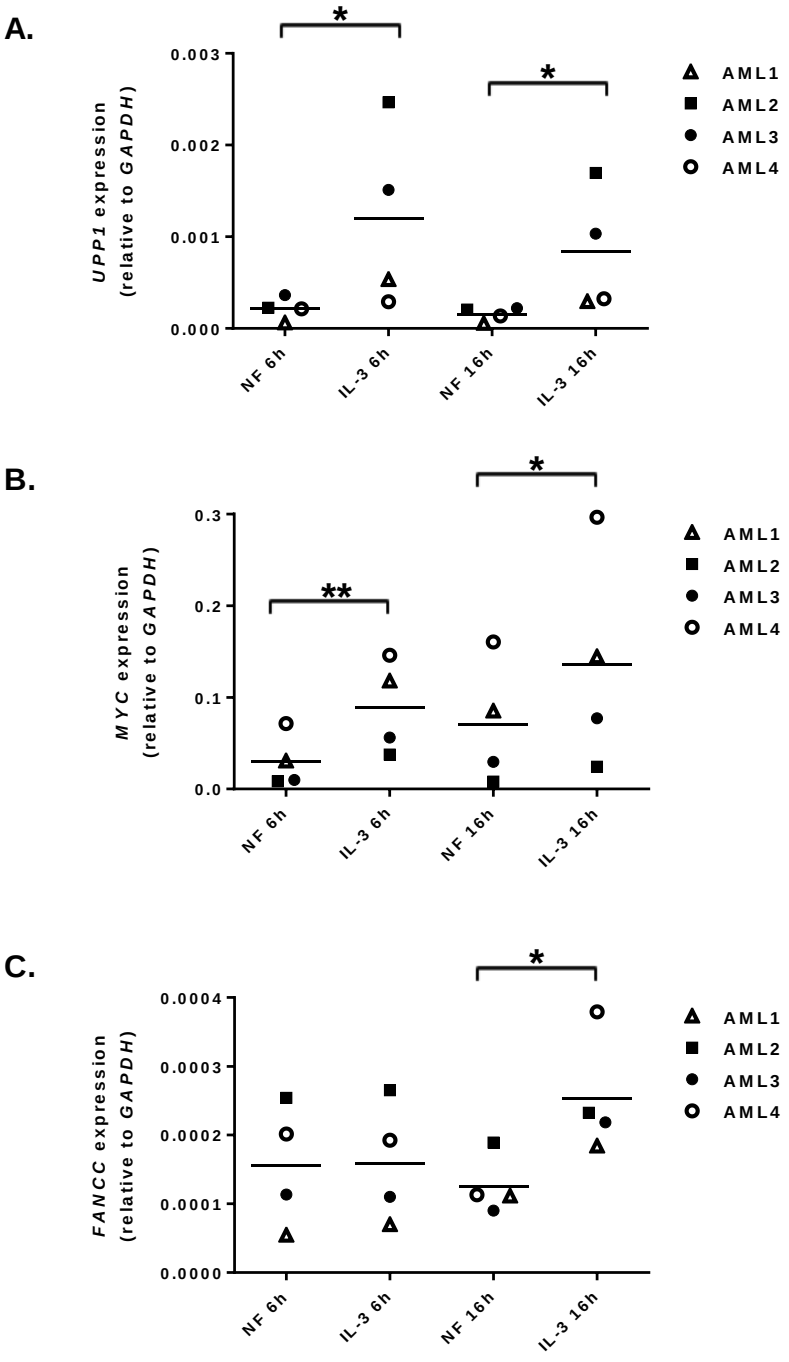


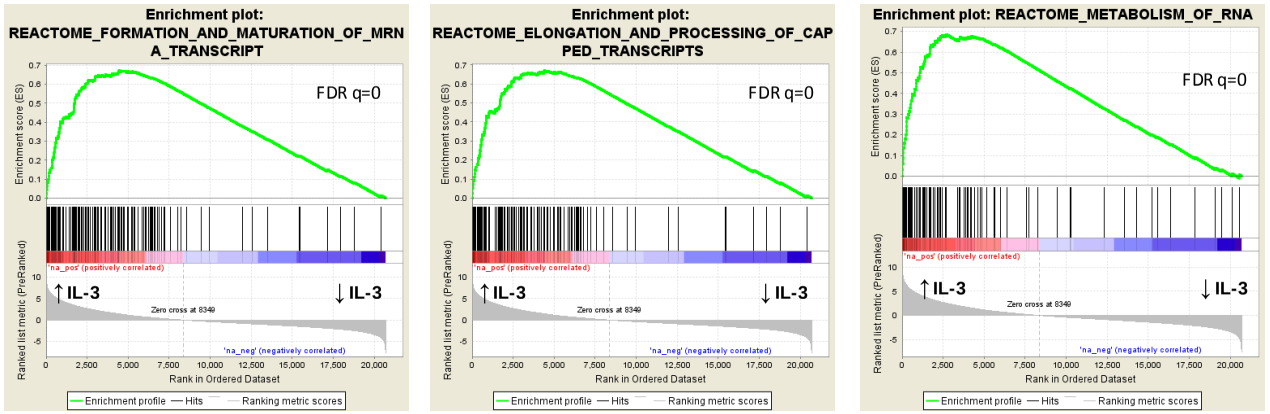
Figure 4.13 Validation of β-catenin target genes identified by ChEA analysis in primary AML samples treated with hIL-3. Three β-catenin target genes indentified by ChEA analysis of genes significantly regulated by IL-3 at 6 and 16h (with fold change >1.5)- see Table 4.2 and Table 4.3- were validated by QRT-PCR in RNA extracted from patient samples AML1-4 treated ± 15ng/mL hIL-3 for 6 or 16h. The RNA was the same as used for microarray analysis of gene expression. Shown, is the mRNA expression of **(A)** *UPP1* **(B)** *MYC* and **(C)** *FANCC* normalised to GAPDH expression. Horizontal lines depict mean expression of the four samples. Results were analysed using a paired t-test, were *p≤0.05; **p≤0.01. NF = no factor.

4.2.2.3 Regulation of RNA modification pathways and miRNAs by IL-3 in AML cells

In this study we have investigated the global gene expression changes induced by IL-3 in primary AML cells. We utilised GSEA, to compare IL-3 induced gene signatures to curated gene-sets (using the MSigDB curated genesets c2, version 3.1). This analysis revealed a significant enrichment between IL-3 modulated genes and a range of RNA processing and modification gene-sets, including genes associated with RNA processing, metabolism, splicing and transport ($p<0.05$, **Figure 4.14**). As further illustrated in **Figure 4.15**, genes significantly regulated by IL-3 encode factors involved in the formation of the 5'Cap Binding complex, the spliceosome and the nuclear pore complex, as well as a range a heterogeneous nuclear ribonucleoproteins (hnRNPs) and gene products involved in the polyadenylation of the 3'UTR of mRNAs. Given the importance of these regulatory pathways in the formation, stability and translation of mature transcripts, our results infer that IL-3 signalling may influence global or gene-specific post-transcriptional regulation of mRNA in AML cells.

In line with our findings, a recent study by Ernst *et al* [265] showed that IL-3 stimulation of 32Dcl3 cells results in the stabilisation of a large range of mRNA transcripts which map to key functional pathways characteristic of the IL-3 response. Many of the transcripts significantly stabilised by IL-3 were involved in cell-cycling and proliferation, and interestingly, the authors identified a strong enrichment for 3' UTR AU-rich elements (AREs). These represent sites which are commonly bound by RNA stabilising or destabilising proteins, and are highly

A. *Processing and metabolism of RNA*



B. *Splicing and transport of RNA*

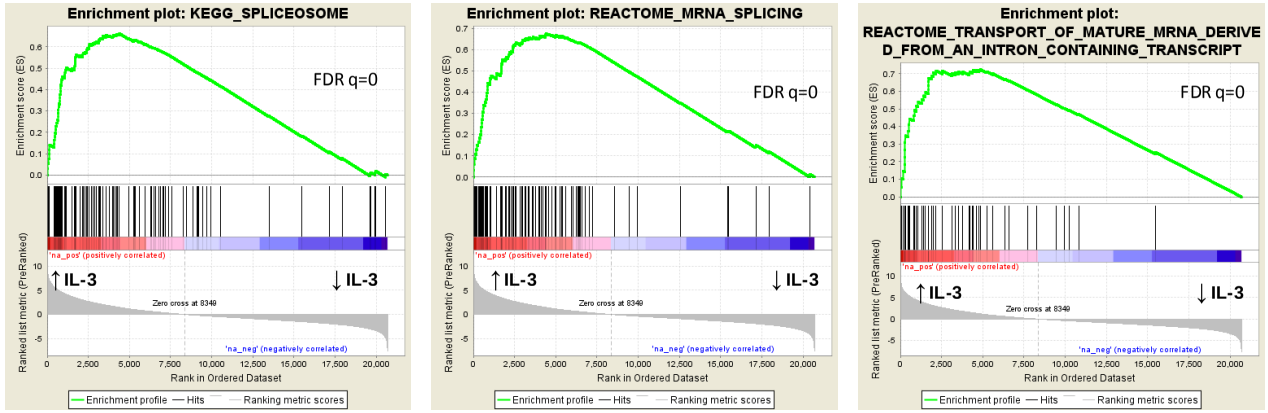


Figure 4.14 Enrichment plots of RNA processing signatures identified by GSEA analysis of gene expression changes induced by IL-3 in primary AML cells. The dataset of gene expression differences resulting from the 16h IL-3 stimulation of primary AML cells was used for GSEA using the Broad Institute Molecular Signature database (MSigDB) curated genesets (c2, version 3.1) . Summarised are enrichment plots of **(A)** processing and metabolism of RNA associated signatures and **(B)** splicing and transport of RNA associated signatures which displayed a significant enrichment for genes modulated by IL-3 in the primary AML cells ($p<0.05$).

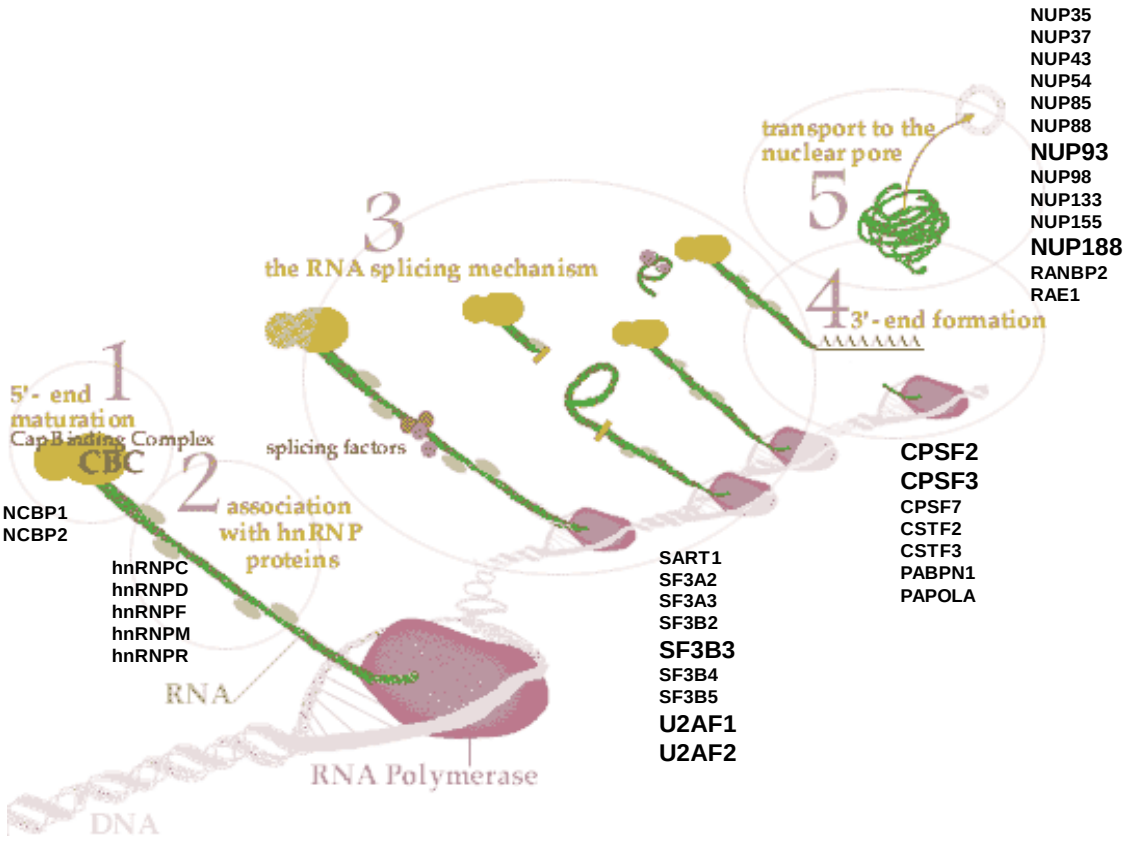


Figure 4.15 Critical genes modulated by IL-3 in primary AML cells involved in consecutive steps of the mRNA processing pathway. Represented in this figure are genes significantly regulated by IL-3 in primary AML cells ($p<0.05$) which play an important role in the processing an dmaturation of mRNA transcripts, from (1) 5' maturation and cap formation to (5) transport of mRNAs through the nuclear pore. Indicated in larger font, are genes which were also identified to have increased mRNA stability following IL-3 stimulation of a murine myeloid cell line (32Dcl) [Ernst *et al*. (2009)].

Schematic modified from:
http://www.nobelprize.org/educational/medicine/dna/a/splicing/index.html

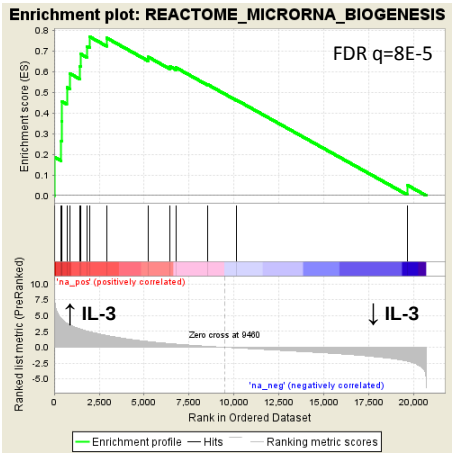
represented in the transcripts stabilised by IL-3 [265]. In addition, it was shown that IL-3 significantly induced the stabilisation of transcripts involved in RNA processing [265], and some of these such as the cleavage stimulating factors *CSTF2* and *CSTF3* involved in 3' end cleavage and polyadenylation of pre-mRNAs, the splicing factors *U2AF1*, *U2AF2* and *SF3B3*, and numerous nucleoporins (central components of the nuclear pore complex) including *NUP93* and *NUP188* were also identified as significantly regulated genes downstream of IL-3 in our microarray analysis of primary AML cells **(Figure 4.15)**. Common to both studies was also the stabilisation or upregulation of components of the exosome including *EXOSC8* and *EXOSC10*, involved in the degradation of mRNA, ribosomal RNA and other small RNA species. The parallel increase in expression of negative regulators of mRNA stability in response to IL-3 may serve to limit cytokine-induced post-transcriptional effects or to facilitate degradation of apoptotic or antiproliferative transcripts. Together, these findings suggest that IL-3 may regulate the expression of a subset of target genes partly through post-transcriptional regulation. As many of these genes are involved in RNA processing themselves, this suggests an ability of IL-3 to re-programme mRNA processing and confer dynamic wide-spread gene expression changes. However, more work will be required to understand the mechanism and functional relevance of this IL-3 induced regulatory network in AML.

In addition to the post-transcriptional gene regulatory pathways described above, microRNA (miRNA) networks play a critical role in the regulation of gene expression by inducing the degradation or translational suppression of their target mRNAs. The biology and known roles of miRNAs in normal and malignant

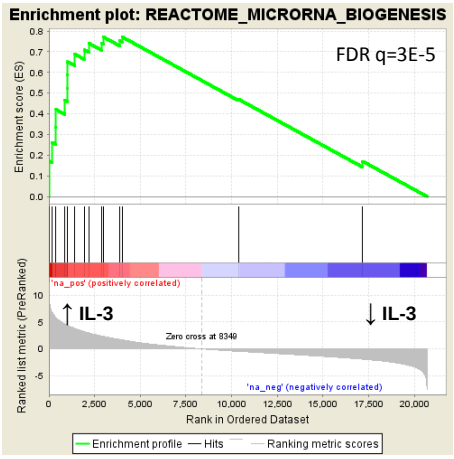
hematopoiesis have been elegantly described in recent reviews [266-268], and although several studies have looked at the expression profiles of miRNAs in primary AML cells and other haematopoietic malignancies [269-272], cytokine-induced miRNA networks have not been well studied in leukaemia. Interestingly, the GSEA analysis of the list of IL-3 modulated genes performed in this study also identified a significant enrichment with a signature associated with *miRNA Biogenesis* ($p<0.05$, **Figure 4.16 A-B**) leading us to look for any differential expression of miRNAs resulting from the IL-3 stimulation of the AML samples. We predicted that IL-3 may upregulate miRNAs which act on tumour suppressors, such as negative regulators of the cell-cycle, and downregulate miRNAs which target genes involved in pro- proliferation and -survival pathways.

Figure 4.16 C-D shows the miRNAs which were significantly regulated by IL-3 at 6 or 16 hours. Notably, the two miRNAs with the highest fold change at the 6 hour timepoint, miR-155 ($p=0.004$; FC=6.27) and miR-221 ($p=0.006$; FC=2.42) remained significantly upregulated, although expression of these was reduced at 16 hours ($p=0.003$; FC=4.64 and $p=0.04$; FC=1.61 respectively). The expression and functional significance of miR-155 and miR-221 has been previously studied in the context of leukaemia, and oncogenic potential has been attributed to elevated levels of these miRNAs [273-275]. miR-155 in particular, has been shown to induce a myeloproliferative disorder in mice when overexpressed in HSC, and increased expression of this miRNA has been reported in cytogenetically-normal AML compared to normal controls [273, 274]. In line with a pro-proliferative function for miR-155 it has been demonstrated to target and

A.



B.



C.

miRNA	Category	Direction	P. value	Fold Change
miR-155	sustained	↑	0.0040	6.27
miR-221	sustained	↑	0.0064	2.42
miR-17HG	early	↑	0.0110	1.96
miR-222	early	↑	0.0135	1.90

D.

miRNA	Category	Direction	P. value	Fold Change
miR-155	sustained	↑	0.0027	4.64
miR-221	sustained	↑	0.0387	1.61
miR-320A	late	↓	0.0438	-0.67
miR-30E	late	↓	0.0330	-0.73
miR-223	late	↓	0.0229	-0.74
miR-34A	late	↓	0.0442	-0.79
miR-99B	late	↓	0.0191	-0.80
miR-197	late	↓	0.0439	-0.81

Figure 4.16 IL-3 regulates a subset of miRNAs in primary AML cells . The dataset of gene expression differences resulting from the 6 and 16h IL-3 stimulation of primary AML cells was used for GSEA using the Broad Institute Molecular Signature database (MSigDB) curated genesets (c2, version 3.1). A significant enrichment was identified with *miRNA biogenesis* signature ($p<0.05$) at both 6h **(A)** and 16h **(B)**. miRNAs significantly regulated by IL-3 at 6h **(C)** and 16h **(D)** are indicated.

reduce the expression of the haematopoietic differentiation transcription factor PU.1, as well as Pten and Ship1, and correlating with increased levels of phosphorylated AKT (Ser473) [273, 274]. The miRNAs which were downregulated by IL-3 (Figure 14D) have been less studied, but there is some evidence that these may act as tumour suppressors [276-278].

4.3 Discussion

The role that the multipotent haematopoietic cytokine IL-3 may play in the pathogenesis of AML has received increasing interest since the discovery that IL-3Rα surface levels are elevated on $CD34^+$ leukaemic progenitors and LSC relative to their normal counterparts [78, 193]. IL-3Rα has been proposed as a prospective therapeutic target in AML as it provides the possibility to selectively eliminate AML blasts and LSC while sparing normal haematopoietic progenitor and stem cells. Currently, several approaches to target IL-3Rα including neutralising monocolonal antibodies and diphteheria-toxin fusions have been developed and are being trialled for the treatment of AML [79, 81, 191, 279]. At a molecular level, some evidence suggests that increased expression of IL-3Rα may potentiate the signal transduction downstream of IL-3 in AML cells [78, 193, 237], although more work is required to understand the signalling networks induced by this cytokine in leukaemia.

In the previous Chapter we have identified β-catenin as a novel downstream target of IL-3 signalling, and have demonstrated that IL-3 induces the accumulation and activation of β-catenin protein in myeloid and leukaemic cell line systems. In this Chapter we extended these studies to primary AML cells, and showed that β-

catenin protein accumulated in response to IL-3 stimulation in 3 out of the 4 patient samples we tested. Interestingly, the patient sample which did not display an upregulation of β-catenin protein after treatment with IL-3 showed reduced proliferation and distinct and reduced overall transcriptional changes in response to IL-3 stimulation suggesting that activation of β-catenin by IL-3 may be a critical event for the function of this cytokine.

Importantly, the results from this chapter are also consistent with a functional activation of β-catenin by IL-3, as microarray analysis on the primary AML cells treated in the presence or absence of IL-3 revealed that IL-3 induced Wnt/β-catenin and Tcf4 transcriptional gene signatures in these cells. In addition, we demonstrated that pre-treatment of the primary AML cells with the IL-3Rα neutralising antibody 7G3 blocked the IL-3 stimulation of β-catenin, particularly in the two patient samples which displayed the largest increase in β-catenin protein expression in response to IL-3. This is the first study to demonstrate a link between IL-3 signalling and the activation of β-catenin in AML, and given the important role of β-catenin in cell proliferation and self-renewal, activation of this pathway provides a novel mechanism whereby IL-3 may contribute to malignant haematopoiesis. Our results are of particular interest as both IL-3Rα and β-catenin are over-expressed in a subset of leukaemic patients, and increased expression of these proteins has independently been associated with a poor prognosis in AML [78, 128, 130, 193]. Furthermore, as expression of both IL-3Rα and β-catenin has been shown to be elevated in the LSC fraction compared to normal HSC, our results suggest that IL-3 signalling may contribute to the

maintenance of β-catenin levels in these specialised leukaemic cells, although the importance of this pathway in LSC remains to be explored in detail.

The significant insight gained from studying the signalling transduction induced in cancerous cells in response to environmental cues, is highlighted in reports that show that potentiated activation of the JAK/STAT and PI3K/AKT signalling pathways in response to the cytokine stimulation of primary AML samples correlates with a poor response to chemotherapy [257, 259]. There has been mounting interest in the study of cytokine-induced signalling profiles in leukaemic cell populations, and it has been proposed that the signalling 'potential' of leukaemic cells, that is the manner in which phospho and transduction proteins perform in signalling networks in response to cytokine stimulation, may be more relevant to understanding the pathology of AML than the absolute level of signalling proteins themselves [257, 259]. Importantly, given the results presented in this study and the established role of β-catenin in growth and survival, it would be of interest to determine the relevance of IL-3 mediated activation of β-catenin in a larger cohort of patients, and we predict that analysis of β-catenin levels in response to IL-3 stimulation may provide further insight into the classification of deregulated networks in patients with AML.

The advent of microarray technology, which allows the large scale analysis of gene expression, has proven to be a powerful tool in the investigation and characterisation of AML. A growing amount of studies, many of which included large cohorts of AML samples, have utilised gene expression profiling with the aim of (a) refining the classification of AML subgroups or identifying new

clusters of AML patients with distinct gene signatures, (b) more accurately defining prognosis of patients within specific cytogenetic and molecular subgroups (c) comparing and identifying differentially regulated genes between leukaemic progenitor/stem cells and normal bone marrow (d) gaining insight into the biological pathways and mechanisms involved in the pathology of the disease [86, 280-283]. Previous studies, however, have focused on basal levels of gene expression and to our knowledge our investigation is the first to analyse global gene expression changes in primary AML cells following cytokine stimulation. Consistent with the IL-3 induced phosphorylation of STAT5A/B that we observed in all four AML patient samples analysed in this study, our microarray analysis revealed significant increases in mRNA expression of the STAT5 target genes *PIM1* and *CISH* in response to IL-3. Also in line with the known functions of IL-3, we identified a strong correlation between the gene expression changes induced by IL-3 in the primary AML cells, and gene signatures related to proliferation, cell cycle control and stem cell function.

In addition, we identified a significant enrichment of IL-3 modulated genes with a range of RNA modification pathways including gene-sets associated with RNA processing, metabolism, splicing and transport. This is in line with other studies which have shown that physiological stimuli such as cytokines, growth factors and hypoxia can induce changes in mRNA stability [284, 285]. Importantly, the coordinated stabilisation or destabilisation of multiple mRNAs in response to external stimuli allows coherent and rapid changes in gene expression and cellular phenotype, and it has been suggested that functionally related transcripts may share similar regulatory sequences and overlapping stability control pathways.

Furthermore, concordant or discordant changes in mRNA stability interplaying with changes in gene transcription could result in amplified or diminished expression of particular genes, adding and additional level of gene regulation to the cell. The importance of post-transcriptional control in maintaining cellular homeostasis is highlighted by the aberrant regulation of mRNA turnover resulting from mutations in 3'-UTR regulatory regions of proto-oncogenes or alterations in levels or activity of RNA binding proteins (RNABPs) has been implicated in the development of disease [286-291].

Of interest, IL-3 has been reported to increase the stability of its own receptor in eosinophils [264], and although microarray analysis does not distinguish between changes in gene transcription or mRNA stability we observed a significant increase in both *IL-3Rα* and *βc* expression in the primary AMLs after stimulation with IL-3. Taking into consideration that we identified robust changes in a range of genes involved in the modification of mRNA processing and stability, we expect that in addition to a putative stabilisation of IL-3R transcripts, IL-3 may affect the post-transcriptional regulation of a series of mRNAs. This is supported by a recent study which demonstrated that the IL-3 stimulation of factor-deprived 32Dcl3 cells resulted in the stabilisation of hundreds of mRNA transcripts which map to key functional networks characteristic of the IL-3 response [265]. However, the mechanisms by which IL-3 may influence the stability of specific transcripts have not been previously defined and our microarray analysis provides a valuable insight into a range of IL-3 modulated genes and pathways which may contribute to the post-transcriptional regulation of mRNAs in leukaemic cells.

CHAPTER 5

Final Discussion

5.1 Final discussion

AML is a heterogeneous disease with large variability in the genetic and molecular events underlying its development and progression, as well as variations in prognosis and response to therapy. Ultimately, the molecular, cytogenetic and/or epigenetic lesions which arise during the leukaemic transformation, cooperate to drive the increased proliferation and survival, and blocked differentiation which is characteristic of AML blasts. While significant progress has been made into the classification and pathology of AML, the overall 5-year survival rates remain considerably low (40-50%), and as low as 10-20% for some subtypes [16]. A more thorough understanding of the molecular mechanisms underlying myeloid leukaemogenesis, is hence critical for the development of more efficient therapeutic approaches for the treatment of AML.

Aberrant activation of the β-catenin signalling pathway occurs in a large proportion of AML patients, is associated with increased blast clonogenicity and has been documented as an independent predictor of poor prognosis [127, 128]. However, in contrast to solid tumours in which mutations in components of the Wnt-signalling pathway are commonly identified as the source of constitutive β-catenin signalling [89, 94, 97], mutations in β-catenin or its canonical regulatory proteins are rarely identified in AML or other leukaemias [127, 142, 146, 171]. Consistent with the notion that additional non-canonical mechanisms likely underlie β-catenin stabilisation in AML and other haematological malignancies, the stabilisation of β-catenin has been associated with activating mutations of the FLT3 receptor [135, 147], and oncogenic chromosomal translocations including

PML-RARα and AML-ETO [136]. To date however, the mechanisms which lead to the accumulation of β-catenin in AML in the absence of these lesions remain largely unknown.

The findings in this thesis describe a novel mechanism for the regulation of β-catenin in myeloid and leukaemic cells. We have demonstrated that the multi-potent haematopoietic cytokine, IL-3, modulates β-catenin protein expression in both Hox-transformed myeloid cells, and also in leukaemic cell lines and primary AML patient samples. Our results are also consistent with functional activation of β-catenin by IL-3: in Chapter 3 we have shown that IL-3 induces β-catenin nuclear localisation and activation of target genes in TF-1.8 leukaemic cells; and in Chapter 4 our microarray analysis on primary AML patient cells treated in the presence or absence of IL-3 revealed that IL-3 induces Wnt/β-catenin and Tcf4 transcriptional gene signatures in these cells. Furthermore, we have explored the mechanism by which IL-3 induces the accumulation of β-catenin in Hox-transformed IL-3 dependent FDM cells, and demonstrated that this is mediated by AKT specific phosphorylation of β-catenin at Serine 552, which can be modulated by PI3K and AKT inhibitors *in vitro*. Our results are of significance for leukaemia, as the IL-3 receptor is expressed on the majority of AML blasts, and more than 80% of AML patient samples display a proliferative response to this cytokine *in vitro* [78, 185, 191]. In addition, an enhanced response to IL-3 signalling in AML progenitor cells is likely to occur through autocrine IL-3 production and increased expression of the IL-3 receptor α-subunit (IL-3Rα) [180, 193, 237], suggesting that this pathway may be a more general mechanism by which β-catenin is regulated in AML.

Of interest internal tandem duplication (ITD) mutations in the FLT3 receptor, and point mutations in c-Kit which result in the constitutive activation of these cytokine receptors in AML and mast-cell leukaemia respectively, have been previously been shown to activate β-catenin via direct tyrosine phosphorylation and stabilisation [135, 147, 177]. Signalling downstream of the T-cell receptor in primary human T-cells has also been linked to β-catenin stabilisation through activation of the PI3K/AKT axis, although in contrast to our findings in the context of IL-3, this appeared to be dependent on GSK3β [173]. While our data indicates a strong link between AKT mediated β-catenin activation downstream of IL-3, we cannot exclude a possible role for IL-3-induced tyrosine phosphorylation of β-catenin as this was not covered in our investigation.

Taken together, these findings suggest that the stabilisation of β-catenin may be a common event downstream of haematopoietic receptors which are activated by mutation or through their congnate ligands (summarised in **Figure 5.1**). Importantly, as differing mechanisms are employed by haematopoietic receptors in the regulation of β-catenin by, it is likely that the activation of β-catenin may play an important role in cytokine driven growth and survival signals.

In line with this, in Chapter 3, we made use of FDM cells, a murine model of Hox/IL-3 myeloid progenitor transformation to explore the functional significance of β-catenin downstream of IL-3. Our results showed that induced deletion of β-catenin in this system significantly reduced the colony formation and growth of FDM cells in response to IL-3, consistent with an essential role for β-catenin in growth signals downstream of this cytokine. Interestingly, *Hox* genes have been shown to co-operate with IL-3 signalling to generate a transplantable myeloid

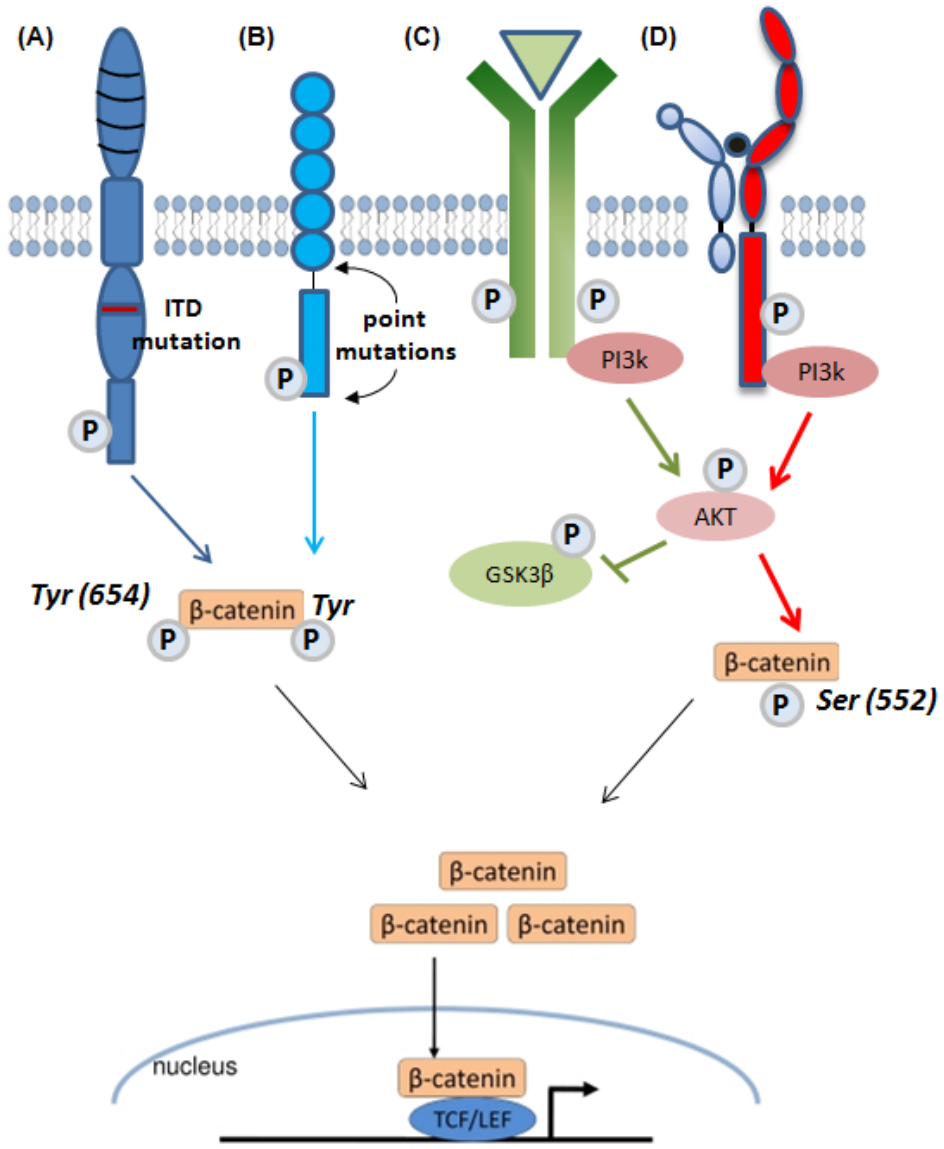


Figure 5.1 Wnt-independent regulation of β-catenin by haematopoietic receptors activated by mutation or their cognate ligands. Internal tandem duplication (ITD) mutations in the FLT-3 receptor (A) or point mutations in the c-Kit receptor (B) which result in ligand-independent constitutive activation of these receptors in AML and mast-cell leukaemia, respectively, have been shown to directly phosphorylate tyrosine residues on β-catenin resulting in its stabilisation and nuclear translocation. β-catenin tyrosine residue 654 has been shown to be critical for FLT-3-ITD regulation of β-catenin, while the specific tyrosine residues phosphorylated by c-Kit are not yet defined. (C) In primary human T-cells, signalling through ligand activated T-cell recpetor induces the accumulation of β-catenin through PI3k/AKT mediated inhibitory phosphorylation of GSK3β (Ser9). (D) Our studies have shown that IL-3 induces the stabilisation of β-catenin in myeloid and in AML cells via PI3K/AKT mediated phosphorylation of β-catenin at Ser552, resulting in subsequent protein accumulation and activation.

leukaemia in mice [188], and recent studies that show Hox genes and β-catenin cooperate in myeloid transformation [132, 133]. Thus the results from this thesis provide a plausible explanation for the co-operative role of IL-3 in Hox-mediated myeloid transformation through activation of the β-catenin pathway.

This significant functional role for β-catenin downstream of IL-3 may explain the contribution of IL-3 to the leukaemic transformation. The overexpression of IL-3 in haematopoietic cells and activated mutants of the IL-3R β-subunit have been demonstrated to co-operate with the oncogenic fusion protein PML-RARα to induce myeloid leukaemia in mice [189, 190]. In addition, enforced expression of IL-3 alone is sufficient to induce a myeloproliferative disease in mouse models [292], and our results suggest that the oncogenic activity of IL-3 may be reduced in the context of β-catenin$^{-/-}$ haematopoietic cells. Furthermore, several studies, as well as unpublished data from our laboratory suggest that the overexpression of IL-3Rα increases cell sensitivity to signal transduction and proliferation downstream of IL-3 [193, 237], and it would be of interest to determine whether elevated expression of IL-3Rα in haematopoietic cells mirrors to oncogenic effects of IL-3 overexpression *in vivo*, and whether this effect is reduced in the absence of β-catenin.

More studies are required to understand the functional contribution of elevated IL-3Rα in AML, the discovery that IL-3Rα levels are significantly higher in leukaemic stem cells (LSC) compared to normal haematopoietic stem cells has made IL-3Rα a prospective therapeutic target in the treatment of AML [78, 191]. Several approaches which have shown promising potential *in vitro* and *in vivo*

have now been developed to target IL-3Rα, including a neutralising antibody targeting IL-3Rα (7G3), IL-3-diphteria toxin fusion proteins and IL-3Rα-directed single-chain Fv antibody immunotoxin fragments [79, 81, 82, 293]. In this thesis we have demonstrated that 7G3 blocks the IL-3 mediated accumulation of β-catenin in TF-1.8 leukaemic cells and primary AML patient samples. A humanised version of 7G3 has reached clinical trials for the treatment of AML, and our findings suggest that AMLs with increased expression of HOX genes, and particularly the unfavourable AML subtype associated with MLL fusion proteins, may be suitable for such treatments that target IL-3 signalling and β-catenin activation.

A limitation of these studies was that we were only able to analyse a small number of AML patient samples due to the scarcity of primary material. We identified a noticeable increase in β-catenin levels following IL-3 stimulation in three out of four AML patients studied. Interestingly, the reduced gene expression response observed in the AML patient which did not show an increase in β-catenin in response to IL-3 raises the possibility that β-catenin may be a key driver of transcriptional changes in response to IL-3. It would be now be of interest to determine the frequency, and prognostic relevance of β-catenin stimulation by IL-3 using a larger cohort of patients.

In addition to the identification and characterisation of a link between IL-3 signalling and the regulation of β-catenin, we also wished to gain insight into the gene networks regulated by IL-3 signalling in AML. To achieve this aim, in Chapter 4 we performed microarray analysis coupled with a range of

bioinformatic approaches to investigate the gene signatures activated by IL-3 in primary AML cells. Using this system, we identified early, late and sustained gene sets regulated by IL-3, and demonstrated a significant enrichment with proliferation, self-renewal and oncogenic gene networks which further support a critical role for IL-3 in leukaemia. We identified a subset of IL-3 regulated miRNAs which have known roles in the regulation of cell survival, proliferation and differentiation including miR-155 and miR-221. We also identified a significant enrichment of the IL-3 modulated genes with a range of gene expression patterns associated with RNA modification including gene-sets associated with RNA processing, metabolism, splicing and transport. These results are consistent with other studies which have shown that physiological stimuli such as cytokines, growth factors and hypoxia can induce changes in mRNA stability [284, 285], and suggest that a subset of IL-3 target genes may be regulated at a post-transcriptional level in myeloid and leukaemic cells. Our results are also in line with recent reports of recurrent somatic mutations in splicing machinery components identified in myelodysplastic syndrome and AML patients [197, 294, 295], further highlighting the importance of these networks in normal cellular function and leukaemogenesis.

It would now be of interest to investigate the link between IL-3 signalling and the regulation of RNA modification components in a larger cohort of AML patients. It will also be of importance to identify the RNA modification pathways and miRNAs which are differentially regulated by IL-3 in AML cells compared to normal bone marrow.

5.2 Summary

In summary, the work presented in this thesis has provided two central findings which will provide further understanding of the molecular mechanisms underlying the pathogenesis of AML:

(1) We have characterised a novel link between IL-3 signalling and the activation of the β-catenin in myeloid and leukaemic cells, and we have proposed that the Wnt-independent activation of β-catenin may be a common event downstream of haematopoietic receptors that are activated by mutation or their cognate ligands, in normal and malignant haematopoiesis. Further, given the elevated expression of IL-3Rα in AML LSC, and recent studies which show a requirement for β-catenin in the establishment and maintenance of LSC in patients with MLL translocations, our results suggest that the targeting of β-catenin in this subset of patients may be a therapeutic strategy that could be achieved through clinically approved monoclonal antibodies directed to IL-3Rα.

(2) To our knowledge, this is the first study which has looked at global gene regulation changes occurring downstream of IL-3 in leukaemia. Our results have provided important insight into the gene networks induced by IL-3 in primary AML cells, and we have identified a subset of regulated genes involved in post-transcriptional gene regulation, including several oncogenic miRNAs. Further studies will be required to delineate the importance of these pathways in IL-3 mediated growth and survival.

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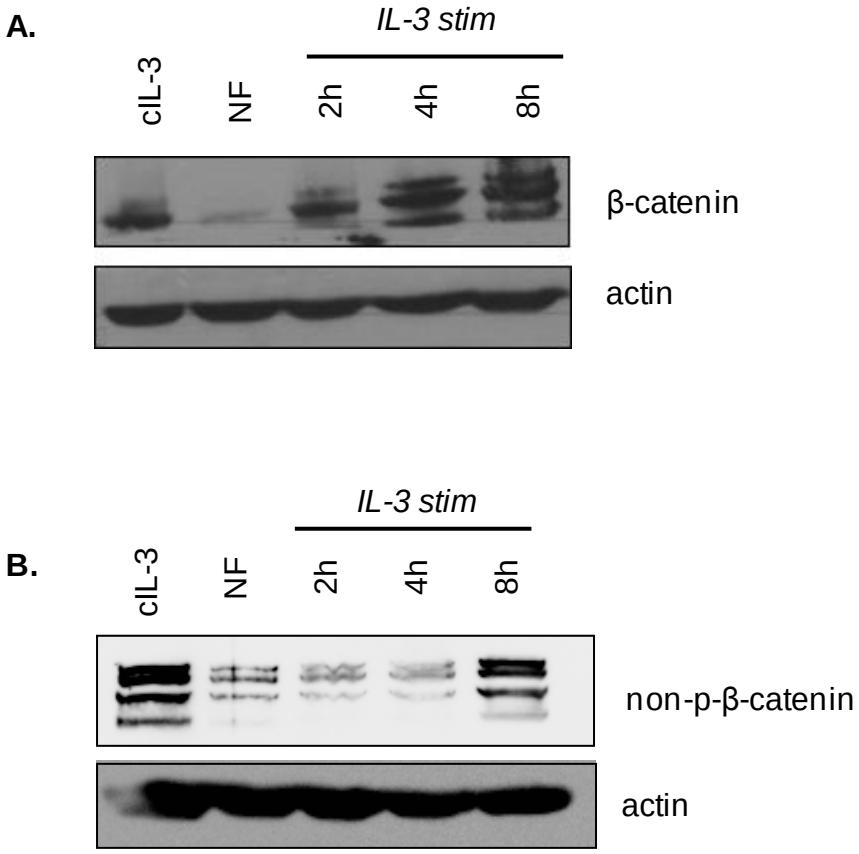
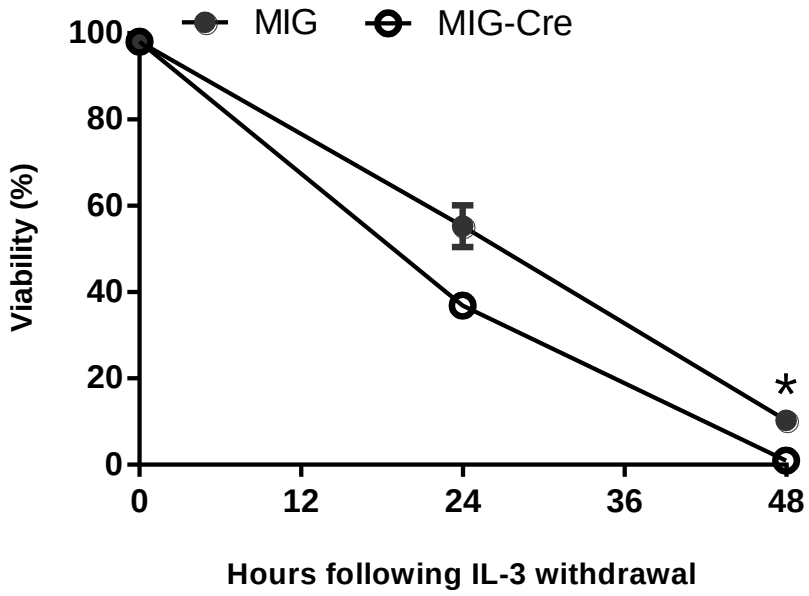
APPENDIX A



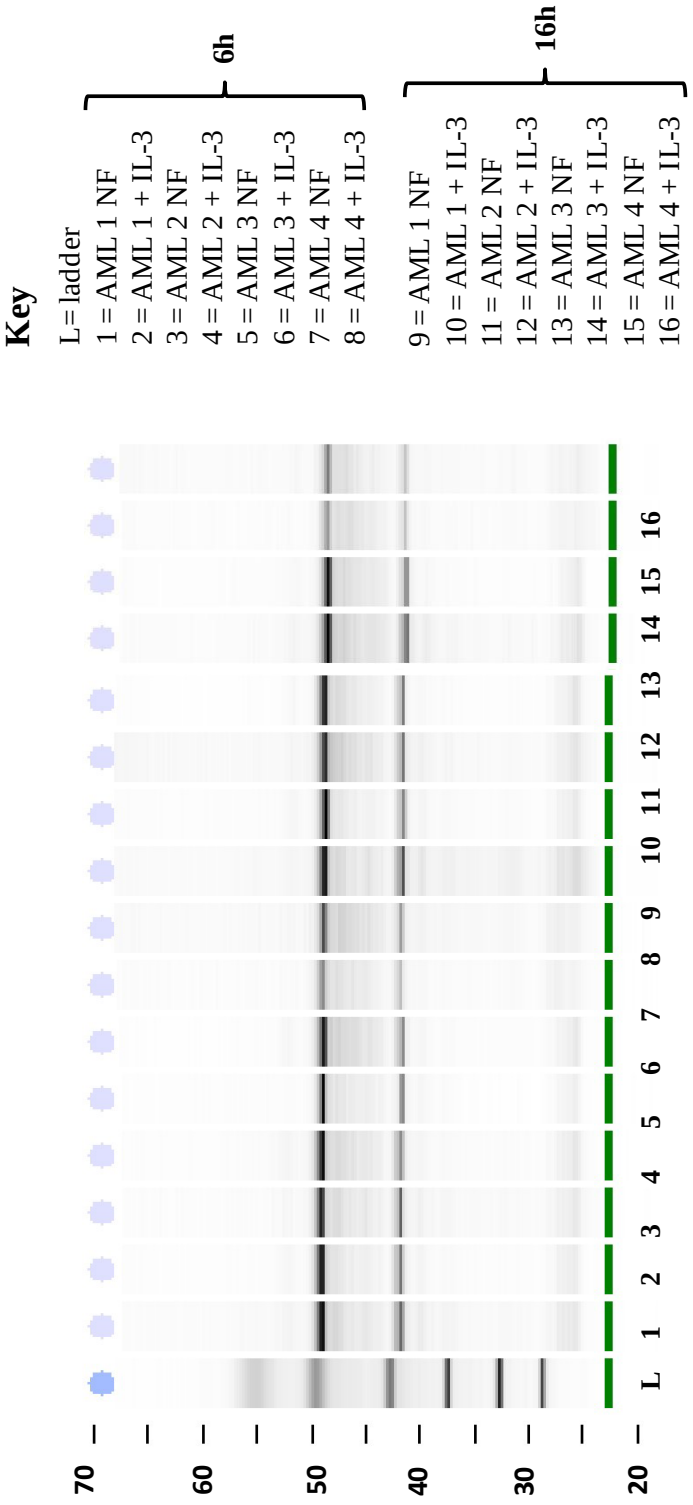
Modulation of β-catenin levels by IL-3. (A) FDM cells were grown continuously in IL-3 (cIL-3), starved of IL-3 for 16 hours (NF), or starved and then stimulated with 10ng/mL of mIL-3 for 2, 4, 8 hours. Lysates were analysed by western blotting for **(A)** total β-catenin and **(B)** N-terminally non-phosphorylated β-catenin.

APPENDIX B



Deletion of β-catenin sensitises FDM cells to IL-3 withdrawal. FDM β-cateninflox/flox cells transduced with MIG-Cre or vector control and sorted for GFP expression, were washed 3 times in PBS and cultured in the absence of IL-3. Viability was measured by trypan blue exclusion analysis. Error bars represent SEM (n=2). *p≤0.05.

APPENDIX C



Assessment of RNA quality with Agilent Bioanalyzer prior to microarray analysis. The quality of RNA extracted from mononuclear cells from four AML patients (AML 1-4) treated ± 15ng/mL hIL-3 for 6h or 16h was analysed with an Agilent Bioanalyzer to confirm quality of material. This was performed by the Adelaide Microarray Facility staff at the IMVS, Adelaide.

NF= no factor.

APPENDIX D

Highest ranking genes induced by IL-3 in primary AML cells after 6h stimulation.

Gene symbol	Gene name	FDR adjusted p-value	Fold change
MIR155	microRNA 155	0.004	6.27
MX1	myxovirus resistance 1, interferon-inducible protein p78	0.005	5.99
PIM1	pim-1 oncogene	0.006	5.82
IFI44	interferon-induced protein 44	0.006	4.45
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	0.008	4.15
EPSTI1	epithelial stromal interaction 1	0.004	4.07
OAS2	2'-5'-oligoadenylate synthetase 2	0.009	4.03
OAS1	2',5'-oligoadenylate synthetase 1	0.009	3.68
PARP9	poly (ADP-ribose) polymerase family, member 9	0.004	3.32
EMP1	epithelial membrane protein 1	0.045	3.25
CISH	cytokine inducible SH2-containing protein	0.009	3.04
XAF1	XIAP associated factor 1	0.015	2.99
DCUN1D3	DCN1, defective in cullin neddylation 1, domain containing 3	0.006	2.98
IL1RAP	interleukin 1 receptor accessory protein	0.005	2.85
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	0.003	2.80
RSAD2	radical S-adenosyl methionine domain containing 2	0.021	2.66
BHLHE40	basic helix-loop-helix family, member e40	0.007	2.64
MYC	v-myc myelocytomatosis viral oncogene homolog	0.008	2.62
DDX21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	0.002	2.61
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	0.005	2.58
CCL2	chemokine (C-C motif) ligand 2	0.026	2.56
SLC7A5	solute carrier family 7, member 5	0.014	2.51
MIR221	microRNA 221	0.006	2.42
IFIT5	interferon-induced protein with tetratricopeptide repeats 5	0.003	2.39
PLSCR1	phospholipid scramblase 1	0.002	2.39
PUS7	pseudouridylate synthase 7 homolog	0.004	2.37
RRS1	RRS1 ribosome biogenesis regulator	0.003	2.37
SLC7A1	solute carrier family 7, member 1	0.005	2.33
PLAU	plasminogen activator, urokinase	0.026	2.31
ASNS	asparagine synthetase (glutamine-hydrolyzing)	0.015	2.31
DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	0.045	2.30
USP18	ubiquitin specific peptidase 18	0.018	2.30
PSTPIP2	proline-serine-threonine phosphatase interacting protein 2	0.032	2.28
EGR1	early growth response 1	0.030	2.28
CMPK2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	0.007	2.25
USP18	ubiquitin specific peptidase 18	0.030	2.24
PNPT1	polyribonucleotide nucleotidyltransferase 1	0.003	2.20
METTL1	methyltransferase like 1	0.009	2.18
CD69	CD69 molecule	0.015	2.16
STAT1	signal transducer and activator of transcription 1	0.005	2.10
DTX3L	deltex 3-like	0.009	2.08
PDSS1	prenyl (decaprenyl) diphosphate synthase, subunit 1	0.010	2.07
SC4MOL	sterol-C4-methyl oxidase-like	0.048	2.06
MTHFD2	methylenetetrahydrofolate dehydrogenase 2	0.005	2.05
SPRED2	sprouty-related, EVH1 domain containing 2	0.045	2.05
NOLC1	nucleolar and coiled-body phosphoprotein 1	0.003	2.05
TRIM16	tripartite motif-containing 16	0.016	2.04
TARS	threonyl-tRNA synthetase	0.017	2.02
CCND3	cyclin D3	0.005	2.01
MTHFD1L	methylenetetrahydrofolate dehydrogenase 1-like	0.009	2.00

RNA extracted from mononuclear cells from four AML patients (AML 1-4) treated ± 15ng/mL hIL-3 for 6h was analysed for global gene expression using an Affymetrix gene array. Differential gene expression was determined using paired analysis of all 4 samples (LIMMA method). Represented in table are the top 50 genes displaying significant expression differences induced by IL-3 treatment at 6h (selected by their FDR p-value and Fold Change).

Highest ranking genes induced by IL-3 in primary AML cells after 16h stimulation.

Gene symbol	Gene name	FDR adjusted p-value	Fold change
IFI44L	interferon-induced protein 44-like	0.008	8.97
MX1	myxovirus resistance 1, interferon-inducible protein p78	0.001	5.87
PIM1	pim-1 oncogene	0.002	5.11
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	0.002	4.71
MIR155	microRNA 155	0.003	4.64
OAS2	2'-5'-oligoadenylate synthetase 2	0.002	4.47
GINS2	GINS complex subunit 2 (Psf2 homolog)	0.001	4.25
IFI44	interferon-induced protein 44	0.002	4.13
CDC6	cell division cycle 6 homolog	0.003	3.93
DTL	denticleless homolog	0.001	3.88
OAS3	2'-5'-oligoadenylate synthetase 3	0.005	3.86
CCNE2	cyclin E2	0.002	3.78
OAS1	2',5'-oligoadenylate synthetase 1	0.003	3.74
RXFP1	relaxin/insulin-like family peptide receptor 1	0.026	3.67
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	0.003	3.59
CLSPN	claspin	0.003	3.42
CHEK1	CHK1 checkpoint homolog	0.002	3.33
EPSTI1	epithelial stromal interaction 1	0.002	3.31
LY6E	lymphocyte antigen 6 complex	0.002	3.28
TYMS	thymidylate synthetase	0.002	3.25
DHCR24	24-dehydrocholesterol reductase	0.002	3.16
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	0.006	3.12
CCL24	chemokine (C-C motif) ligand 24	0.044	3.07
HELLS	helicase, lymphoid-specific	0.002	3.06
MCM10	minichromosome maintenance complex component 10	0.001	3.02
PARP9	poly (ADP-ribose) polymerase family, member 9	0.002	2.91
FKBP4	FK506 binding protein 4	0.002	2.88
CDC45	cell division cycle 45 homolog	0.005	2.86
WDR76	WD repeat domain 76	0.003	2.86
UHRF1	ubiquitin-like with PHD and ring finger domains 1	0.002	2.82
ANKRD22	ankyrin repeat domain 22	0.035	2.82
GINS1	GINS complex subunit 1	0.004	2.81
XAF1	XIAP associated factor 1	0.007	2.79
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	0.001	2.77
XRCC2	X-ray repair complementing defective, Chinese hamster cells 2	0.003	2.77
TRIM16L	tripartite motif-containing 16-like	0.000	2.76
CISH	cytokine inducible SH2-containing protein	0.005	2.74
MCM4	minichromosome maintenance complex component 4	0.002	2.69
CHAF1B	chromatin assembly factor 1, subunit B	0.008	2.64
POLE2	polymerase (DNA directed), epsilon 2	0.002	2.63
IFITM1	interferon induced transmembrane protein 1	0.036	2.62
MCM6	minichromosome maintenance complex component 6	0.002	2.60
UNG	uracil-DNA glycosylase	0.003	2.60
DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	0.009	2.57
PUS7	pseudouridylate synthase 7 homolog	0.001	2.54
SLC39A14	solute carrier family 39 (zinc transporter), member 14	0.001	2.51
CSNK1G1	casein kinase 1, gamma 1	0.004	2.48
IL1RAP	interleukin 1 receptor accessory protein	0.003	2.46
DCUN1D3	DCN1, defective in cullin neddylation 1, domain containing 3	0.004	2.45
PAICS	phosphoribosylaminoimidazole carboxylase	0.000	2.44

RNA extracted from mononuclear cells from four AML patients (AML 1-4) treated ± 15ng/mL hIL-3 for 6h was analysed for global gene expression using an Affymetrix gene array. Differential gene expression was determined using paired analysis of all 4 samples (LIMMA method). Represented in table are the top 50 genes displaying significant expression differences induced by IL-3 treatment at 16h (selected by their FDR p-value and Fold Change).

APPENDIX E

Differential gene expression induced by IL-3 in AML1-4 was determined using a paired analysis of all 4 samples, and significant genes were classified as early (only modulated at 6h), sustained (modulated at both 6 and 16h) and late (only modulated at 16h) genes. Represented in tables A-C, are the top 30 genes from each category displaying significant expression differences induced by IL-3 treatment (selected by their FDR p-value and Fold Change).

(A) Early genes

Gene symbol	Gene name	FDR adjusted p-value	Fold change
EMP1	epithelial membrane protein 1	0.045	3.25
ASNS	asparagine synthetase (glutamine-hydrolyzing)	0.015	2.31
EGR1	early growth response 1	0.030	2.28
CD69	CD69 molecule	0.015	2.16
TARS	threonyl-tRNA synthetase	0.017	2.02
MIR17HG	microRNA 17HG	0.011	1.96
UPP1	uridine phosphorylase 1	0.035	1.96
CLIC4	chloride intracellular channel 4	0.038	1.94
BYSL	bystin-like	0.004	1.93
MIR222	microRNA 222	0.013	1.90
GRB10	growth factor receptor-bound protein 10	0.023	1.83
GATA2	GATA binding protein 2	0.020	1.79
KLHL2	kelch-like 2, Mayven (Drosophila)	0.008	1.77
PLK3	polo-like kinase 3	0.025	1.69
WDR89	WD repeat domain 89	0.026	1.69
TAGAP	T-cell activation RhoGTPase activating protein	0.024	1.64
SNORD51	small nucleolar RNA, C/D box 51	0.007	1.63
TNF	tumor necrosis factor	0.025	1.62
PITPNB	phosphatidylinositol transfer protein, beta	0.023	1.62
PDCL3	phosducin-like 3	0.027	1.61
MKI67IP	MKI67 (FHA domain) interacting nucleolar phosphoprotein	0.012	1.59
GCLM	glutamate-cysteine ligase, modifier subunit /	0.024	1.58
DNTTIP2	deoxynucleotidyltransferase, terminal, interacting protein 2	0.045	1.58
IL3RA	interleukin 3 receptor, alpha (low affinity)	0.034	1.57
ACSL1	acyl-CoA synthetase long-chain family member 1	0.020	1.56
APOL6	apolipoprotein L, 6	0.011	1.56
CNBP	CCHC-type zinc finger, nucleic acid binding protein	0.030	1.56
EIF2S2	eukaryotic translation initiation factor 2, subunit 2 beta	0.034	1.55
MPP6	membrane protein, palmitoylated 6	0.039	1.54
KIAA1430	KIAA1430	0.043	1.51

(B) Sustained genes

Gene symbol	Gene name	FDR adjusted p-value	Fold change
MIR155	microRNA 155	0.004	6.27
MX1	Myxovirus resistance 1, interferon-inducible protein p78	0.005	5.99
PIM1	pim-1 oncogene	0.006	5.82
IFI44	interferon-induced protein 44	0.006	4.45
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	0.008	4.15
EPSTI1	epithelial stromal interaction 1	0.004	4.07
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	0.009	4.03
OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	0.009	3.68
PARP9	poly (ADP-ribose) polymerase family, member 9	0.004	3.32
CISH	cytokine inducible SH2-containing protein	0.009	3.04
XAF1	XIAP associated factor 1	0.015	2.99
DCUN1D3	DCN1, defective in cullin neddylation 1, domain containing 3	0.006	2.98
IL1RAP	interleukin 1 receptor accessory protein	0.005	2.85
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	0.003	2.80
RSAD2	Nradical S-adenosyl methionine domain containing 2	0.021	2.66
BHLHE40	basic helix-loop-helix family, member e40	0.007	2.64
MYC	v-myc myelocytomatosis viral oncogene homolog	0.008	2.62
DDX21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	0.002	2.61
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	0.005	2.58
CCL2	chemokine (C-C motif) ligand 2	0.026	2.56
SLC7A5	solute carrier family 7, member 5	0.014	2.51
MIR221	microRNA 221	0.006	2.42
IFIT5	interferon-induced protein with tetratricopeptide repeats 5	0.003	2.39
PLSCR1	phospholipid scramblase 1	0.002	2.39
PUS7	pseudouridylate synthase 7 homolog	0.004	2.37
RRS1	RRS1 ribosome biogenesis regulator homolog	0.003	2.37
SLC7A1	solute carrier family 7, member 1	0.005	2.33
PLAU	plasminogen activator, urokinase	0.026	2.31
DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	0.045	2.30
USP18	ubiquitin specific peptidase 18	0.018	2.30

(C) Late genes

Gene symbol	Gene name	FDR adjusted p-value	Fold change
IFI44L	interferon-induced protein 44-like	0.008	8.97
GINS2	GINS complex subunit 2 (Psf2 homolog)	0.001	4.25
CDC6	cell division cycle 6 homolog	0.003	3.93
DTL	denticleless homolog	0.001	3.88
OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	0.005	3.86
CCNE2	cyclin E2	0.002	3.78
RXFP1	relaxin/insulin-like family peptide receptor 1	0.026	3.67
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	0.003	3.59
CLSPN	claspin	0.003	3.42
CHEK1	CHK1 checkpoint homolog	0.002	3.33
LY6E	lymphocyte antigen 6 complex, locus E	0.002	3.28
TYMS	thymidylate synthetase	0.002	3.25
DHCR24	24-dehydrocholesterol reductase	0.002	3.16
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	0.006	3.12
CCL24	chemokine (C-C motif) ligand 24	0.044	3.07
HELLS	helicase, lymphoid-specific	0.002	3.06
MCM10	minichromosome maintenance complex component 10	0.001	3.02
FKBP4	FK506 binding protein 4, 59kDa	0.002	2.88
CDC45	cell division cycle 45 homolog	0.005	2.86
WDR76	WD repeat domain 76	0.003	2.86
UHRF1	ubiquitin-like with PHD and ring finger domains 1	0.002	2.82
ANKRD22	ankyrin repeat domain 22	0.035	2.82
GINS1	GINS complex subunit 1 (Psf1 homolog)	0.004	2.81
KIAA0101	KIAA0101	0.001	2.80
XRCC2	X-ray repair complementing defective, Chinese hamster cells 2	0.003	2.77
MCM4	minichromosome maintenance complex component 4	0.002	2.69
CHAF1B	chromatin assembly factor 1, subunit B (p60)	0.008	2.64
POLE2	polymerase (DNA directed), epsilon 2 (p59 subunit)	0.002	2.63
IFITM1	interferon induced transmembrane protein 1 (9-27)	0.036	2.62
UNG	uracil-DNA glycosylase	0.003	2.60