Identification and Functional Analysis of Gene Expression Changes in Acute Myeloid Leukaemia

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Abbreviations

-7	monosomy 7
-7q	deletion of 7q
+8	trisomy 8
a.k.a	also known as
AKT	protein kinase B
AML	acute myeloid leukaemia
AML1	runt-related transcription factor 1
APL	acute promyelocytic leukaemia
ATM	ataxia telangiectasia mutated
ATRA	all-*trans* retinoic acid
BH	Benjamini-Hochberg
BMU	bone marrow unit
bp	base pairs
C/EBP	CCAAT enhancer binding protein
CBF AML	core binding factor AML (AML1-ETO and CBFβ-MYH11)
CBFB	core binding factor beta
CD90	cluster of differentiation 90
ChIP	chromatin immunoprecipitation
CHIP	microarray chip
CMAP	connectivity map
DC	dequalinium chloride
DMSO	dimethyl sulfoxide
ER	endoplasmic reticulum
ERK	extracelullar signal-regulated kinase
ETO	eight twenty one protein
FACS	flow cytometry

FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
FDR	false discovery rate
FL	human FLT3 ligand
FLT3-ITD	FLT3-Internal Tandem Duplication mutation
FLT3-TKD	FLT3-Tyrosine Kinase Domain mutation
FLT3-WT	FMS-like Tyrosine Kinase class III receptor
GEO	gene expression omnibus
GF	Growth factor
GM	granulocyte monocyte
GM-CSF	granulocyte macrophage colony-stimulating factor
GMR	IL-3/IL-5/GM-CSF hbc receptor
GMR-V449E	FDB1 cells expressing the hβc receptor V449E mutant
GSEA	gene-set enrichment analysis
h/m	human/mouse
HDAC	histone deacetylase
HOX	homeobox gene
HSC	haemopoietic stem cell
hβc	human GMR common beta subunit
IL-3	Interleukin 3
IMDM	Iscove's modified Dulbecco's medium
IMDM	Iscove's Modified Dulbecco's Medium
IPA	Ingenuity Pathway Analysis
JAK	Janus Kinase
kDa	kilo dalton
LIMMA	linear modelling for microarray analysis
Lod	log of odd ratio score which depicts the differential expression of a gene
LSC	leukaemic stem cell

M	Molar
M-CSFR	macrophage colony-stimulating factor receptor
MAPK	Mitogen activating protein Kinase
miR	micro-RNA
MLL	mixed-lineage leukaemia
MNC	mononuclear cells
MPD	myeloproliferative disorder
mRNA	messenger RNA
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MYH11	myosin, heavy chain 11, smooth muscle
NBM	normal bone marrow mononuclear cells
NFkB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NK	normal karyotype AML
PCR	polymerase chain reaction
PI	Propidium Iodide
PI3K	Phosphatidylinositol3 kinase
PML	promyelocytic leukaemia
PSG	Penicillin-Streptomycin-Glutamine
PTPN11	protein tyrosine phosphatase, non-receptor type 11; a.k.a SHP-2
Q-PCR	real-time quantitative PCR
r.p.m	revolutions per minute
RARA	retinoid acid receptor alpha
RMA	Robust Multichip Average
RNA	ribonucleic acid
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
RUNX1	runt-related transcription factor 1

RUNX1T1	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)
SCF	stem cell factor
SEM	standard error measurement
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMMHC	a.k.a MYH11
STAT	Signal Transducer and Activator of Transcription
TF	Transcription factor
vs	versus
Wnt	wingless-type MMTV integration site family
WT	wild-type

Abstract

Acute Myeloid Leukaemia (AML) is a malignant blood cancer characterised by uncontrolled growth of leukaemic blasts. This is associated with constitutive activation of key signalling molecules such as AKT, ERK1/2, STAT5 and NFκB and with aberrant transcription factor activity, which in many cases is associated with characteristic chromosomal translocations. Aberrant receptor signaling can constitutively activate the pathways associated with the above signaling molecules. For example, autocrine interleukin-3 (IL-3), and over-expression of IL-3 receptor alpha (*IL3RA/CD123*) have been found in AML, as has constitutive phosphorylation of the common beta subunit (hβc) for IL-3 and granulocyte-macrophage colony-stimulating factor receptor (GMR). Also mutation of the FMS-like tyrosine kinase 3 (FLT3) receptor is common in AML (~30% of patients) and the resultant aberrant FLT3 signaling contributes to enhanced survival, growth and a block in differentiation.

A focus in this thesis is the identification and dissection of the signaling pathways and downstream genes activated by a leukaemic mutant of GMR (GMR-V449E) and by the FLT3 activated mutants associated with AML. For these studies we make extensive use of the murine bi-potential myeloid cell line model FDB-1 in which these mutants induce factor-independent growth and survival and a block in differentiation. The use of this experimental approach together with bioinformatics has provided leads with regard to the role of the AKT/mTOR and ERK pathways downstream of these receptors, and important for cell proliferation, survival and differentiation. Additionally, we focused on the role of the *Growth Arrest and DNA Damage 45a* (*Gadd45a*) gene, repression of which is important for cell survival and the block in differentiation induced by the activated mutants.

A second focus has been extending the bioinformatic approaches to define the gene expression and pathways associated with the abnormal growth characteristics of AML. In

particular, we studied AML cases with numerical chromosomal abnormalities and translocation events. Extensive use is made of the Connectivity Map (CMAP) resource together with publicly available gene expression datasets to define agents with anti-leukaemic potential. We have tested drugs, selected using the inv(16) (CBFβ-MYH11) and MLL AML translocation signatures, for specificity and sensitivity on AML patient samples.

Declaration

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1. Powell *et al*, Blood, 2009 (**Appendix B**)
2. Perugini *et al*, Leukemia, 2009 (**Appendix D**)
3. Kok *et al*, Leukemia, 2010 (**Appendix E**)

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Chapter 1: Introduction

1.1 Acute Myeloid Leukaemia

Acute Myeloid Leukaemia (AML) is a cancer of the bone marrow, the organ which produces the majority of blood cells. AML is the most common subtype of leukaemia in adults and accounts for 15-20% of childhood leukaemia (Redaelli*, et al* 2004). Approximately 715 cases of AML are diagnosed each year in Australia which accounts for 0.8 percent of all cancers diagnosed (Leukaemia Foundation, http://www.leukaemia.org.au). AML is characterised by continued proliferation and suppressed differentiation of haemopoietic progenitors in the bone marrow with disease cells characterised by enhanced survival and self-renewal. Thus, accumulating numbers of immature haemopoietic progenitors replace the normal red blood cells, white blood cells and platelets. AML patients may experience symptoms, such as fatigue, anaemia and recurrent infections, all as a result of the decreased production of healthy blood and immune cells.

1.1.1 The classification and the prognostic outcome of AML

AML is divided into ten major FAB (French-American-British) subtypes (M0-M8) (Bennett*, et al* 1976, Tenen 2003) (see **Table 1.1**) or by World Health Organisation (WHO) classification (**Table 1.2**) which are the standard systems used to classify AML. The FAB subtypes are defined by the degree to which differentiation along one of the myeloid lineages is evident by morphology and immunophenotype (Scandura*, et al* 2002). Additionally, AML can also be categorised based on chromosomal abnormalities such as chromosomal translocations (**Table 1.2**). The most frequent chromosome translocations are those targeting retinoid acid receptor alpha (RARα), t(15;17)(q22;q12~21) *(PML-RARA)* which account for

Table 1.1. The use of the FAB category based on morphology and cytogenetics to classify AML (adapted from Bennett *et al*, 1976).

FAB	Name	Cytogenetics
M0	minimally differentiated acute myeloblastic leukemia	
M1	acute myeloblastic leukemia, without maturation	
M2	acute myeloblastic leukemia, with granulocytic maturation	t(8;21) (q22;q22), t(6;9)
M3	promyelocytic, or acute promyelocytic leukemia (APL)	t(15;17)
M4	acute myelomonocytic leukemia	inv(16) (p13q22), del(16q)
M4eo	myelomonocytic together with bone marrow eosinophilia	inv(16), t(16;16)
M5	acute monoblastic leukemia (M5a) or acute monocytic leukemia (M5b)	del (11q), t(9;11), t(11;19)
M6	acute erythroid leukemias, including erythroleukemia (M6a) and very rare pure erythroid leukemia (M6b)	
M7	acute megakaryoblastic leukemia	t(1;22)
M8	acute basophilic leukemia	

Table 1.2. The use of WHO category to classify AML (adapted from Gulley *et al*, 2010).

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* and 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

*Highlighted in red indicates chromosomal abnormalities.

6-7% of adult leukaemia (Rosenbauer and Tenen 2007). 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 (Brendel and Neubauer 2000, Chen*, et al* 2009). In addition, gain or loss of chromosomes also occurs in AML including most commonly trisomy 8 (+8), monosomy 7 (-7) or deletion 7q (-7q) (Chen*, et al* 2009). Overall across adult and paediatric AML patients, approximately 20-50% have a normal karyotype (NK) (Chen*, et al* 2009, Gulley*, et al* 2009, Hollink*, et al* 2009). This group is associated with a number of mutations (**Figure 1.1**) which can be used to stratify AML patient prognostic risk (**Table 1.3**) (Gulley*, et al* 2009, Hollink*, et al* 2009). For example, FLT3-ITD (FMS-like tyrosine kinase 3 receptor-internal tandem duplications, see **Section 1.6.2.1**) is associated with poor prognosis (Grimwade and Hills 2009). More recently nucleophosmin (*NPM1*) mutation has been included and combined with FLT3-ITD to define groups which respond differentially to treatment and disease outcome (Grimwade and Hills 2009, Haferlach 2008, Lowenberg 2008). Other mutations that influence the clinical outcome occur in *CEBPA* (CCAAT enhancer binding protein alpha), *GATA1* (GATA binding protein 1)*, SPI1* (*PU.1*) (spleen focus forming virus (SFFV) proviral integration)*, TP53* (tumour protein 53)*, RUNX1* (runt related transcription factor 1)*, KIT* (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene)*, RAS* (rat sarcoma viral oncogene)*, WT1* (Wilms tumour 1)*, TET2* (tet oncogene family member 2)*, CBL* (Cas-Br-M (murine) ecotropic retroviral transforming sequence), and *PTPN11* (protein tyrosine phosphatase, non-receptor type 11) (Grimwade and Hills 2009, Haferlach 2008, Lowenberg 2008, Rosenbauer and Tenen 2007). Recently, isocitrate dehydrogenase 1 (*IDH1*) and *IDH2* mutations have been found in 33% of NK AML (Marcucci*, et al* 2010). However, there is still approximately 20% of NK AML that is associated with unknown abnormalities (Hollink*, et al* 2009). There are also a number of

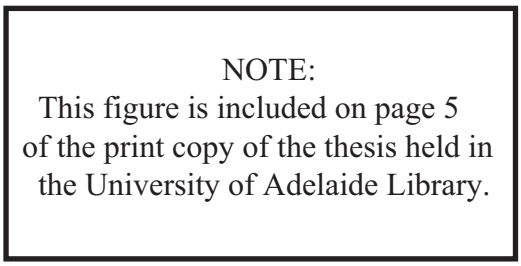


Figure 1.1. Relative frequencies of the recurrent cytogenetic abnormalities in AML. There is approximately 20% of cases of NK AML that have no identifiable genetic lesions (adapted from Hollink *et al*, 2009).

Table 1.3. The cytogenetic and mutation prognostic risk factors assignments of AML (adapted from Gulley *et al*, 2010).

Favorable risk factors
t(15;17)(q22;q12) *PML-RARA*
t(8;21)(q22;q22) *RUNX1-RUNX1T1*
inv(16)(p13;q22) or t(16;16)(p13;q22) *CBFB-MYH11*
NPM1 mutation when *FLT3* internal tandem duplication is absent and cytogenetics are normal
CEBPA mutation (correlates with erythroid differentiation and higher hemoglobin)
Intermediate risk group
Normal karyotype
FLT3 internal tandem duplication with *NPM1* mutation and normal cytogenetics
KIT mutation with t(8;21) or inv(16)
+8 only
t(9;11) *AF9-MLL* only
Abnormalities not otherwise listed
Unfavorable risk factors
Complex karyotype (3 abnormalities)
Monosomal karyotype (2 autosomal monosomies, or a single one plus 1 structural defect)
–5, –7 or other autosomal monosomy
del(5q) or del(7q)
11q23 *MLL* translocation, excluding t(9;11) *AF9-MLL*
MLL partial tandem duplication with normal cytogenetics
inv(3)(q21;q26) or t(3;3)(q21;q26*) RPN1-EVI1* or *MDS1-EVI1*
EVI1 overexpression
17p abnormality or *TP53* mutation
FLT3 internal tandem duplication when *NPM1* mutation is absent and cytogenetics are normal
t(9;22)(q34;q11) *BCR-ABL1*
t(6;9)(p23;q34) *DEK-CAN*
ERG overexpression without *FLT3* ITD when cytogenetics are normal
BAALC overexpression with normal cytogenetics
MN1 overexpression with normal cytogenetics
WT1 mutation with normal cytogenetics
TET2 mutation

over-expressed genes which contribute to poor outcome in AML such as *FLT3, EVI1* (ecotropic viral integration site 1)*, BAALC* (brain and acute leukemia cytoplasmic)*, ERG* (v-ets erythroblastosis virus E26 oncogene homolog)*,* and *MN1* (meningioma 1) (Grimwade and Hills 2009, Gulley*, et al* 2009, Haferlach 2008, Lowenberg 2008). The cytogenetics and mutations frequency for their lesions is summarized in **Figure 1.1** and **Table 1.4**.

1.1.2 Targeted therapies on AML

Despite extensive research, the prognosis for AML remains relatively poor for certain subtypes due to relapse (Doepfner*, et al* 2007). In addition, the current overall success rate for leukaemia treatment still remains relatively low with 5-year survival rates between 10-70% depending on the leukaemia subtype (**Figure 1.2**) (Giles*, et al* 2002, Gulley*, et al* 2009, Redaelli*, et al* 2004). The mainstay of treatment is still based on non-specific chemotherapy (Haferlach 2008). Chemotherapy treatment has significant side effects especially on elderly AML patients (Doepfner*, et al* 2007) and therefore this group is particularly difficult to treat and has very poor outcome. Increasing knowledge in understanding of the pathogenesis of AML, in particular the nature of genetic lesions and altered signalling pathways, has led to development of targeted therapy. Targeted therapy has resulted in improved outcome in the AML patients and is associated with fewer side effects than chemotherapy (Doepfner*, et al* 2007, Haferlach 2008).

Some examples of targeted therapies and the mechanism of action of the targeted therapies are summarised in **Table 1.5**. For example, all-*trans* retinoic acid (ATRA) has been successfully used to differentiate the blast cells from PML-RARα AML patients to granulocytes (Haferlach 2008, Nowak*, et al* 2009). Other approaches have focused on signal

Table 1.4. The frequency of the AML subgroups and the mutation of the transcription factors in AML (adapted from Rosenbauer and Tenen, 2007).

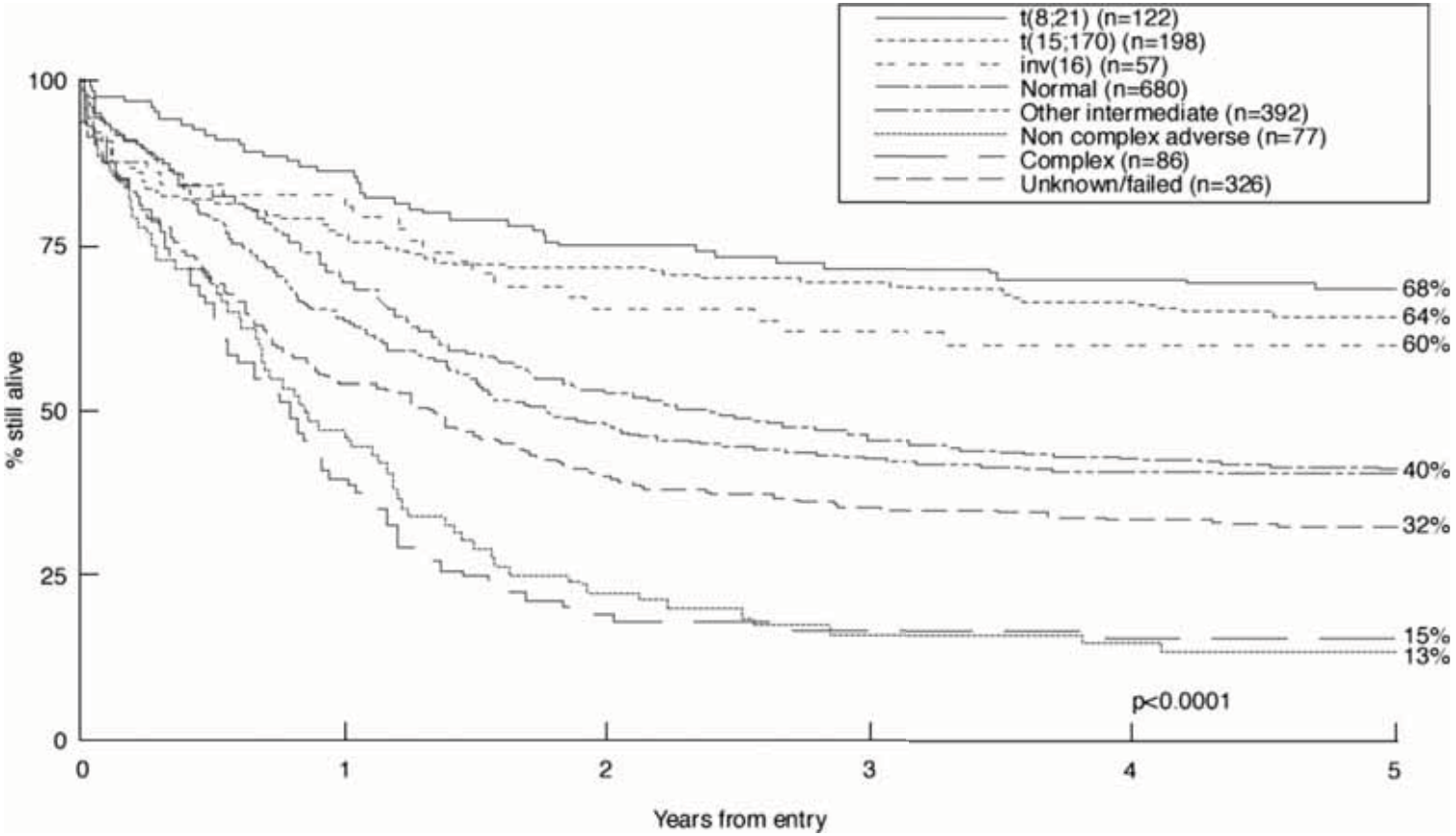


Figure 1.2. The prognosis of AML is strongly related with the cytogenetic findings. The prognosis for AML remains relatively poor for certain subtypes due to the relapse and complications associated with treatment (adapted from Gulley, 2010).

Table 1.5. The examples of the compound used and the mechanism of the current targeted therapies in AML (adapted from Haferlach, 2008).

transduction pathways (PI3K, mTOR, MAPK), farnesyltransferase inhibitors, surface molecules (CD123, CD33, CD44), and epigenetic modifications such as hypermethylation and histone acetylases (Chen*, et al* 2009, Doepfner*, et al* 2007, Haferlach 2008, Krause and Van Etten 2007, Nowak*, et al* 2009, Park*, et al* 2009). Additionally, there has been a focus on targeting activating FLT3 receptor mutation and generation of specific inhibitors. There are a number of FLT3 inhibitors in clinical and some in preclinical development (Weisberg*, et al* 2010). However, some targeted therapies have limitations when applied as monotherapy. For example, FLT3 and KIT inhibitor responders in clinical studies have been limited and transient due to the acquired resistance (Chu and Small 2009, Haferlach 2008).

Another approach is haemopoietic cell transplantation (HCT) which has been shown to improve clinical outcome compared to conventional chemotherapy in AML patients with FLT3-ITD (lower complete remission rate), or with certain high-risk patients if allogeneic HCT is received in first complete remission (Krause and Van Etten 2007, Meshinchi and Appelbaum 2009). Antisense drugs can be used to target particular lesions (e.g. Bcl-2 antisense (oblimersen Sodium), FLT3-targeted siRNA), however no conclusion can yet be drawn and the difficulties to deliver antisense *in vivo* is still a challenge (Moore*, et al* 2006, Walters*, et al* 2005). Due to the discrepancy between efficacy of some current and potential drugs in preclinical studies, and patients where a limited transient response is seen, there is a need to better understand the molecular mechanisms and genetic abnormalities involved in the regulation of proliferation, survival, self-renewal and differentiation of normal, and malignant haemopoietic cells.

1.2 Haemopoiesis: interplay between growth factor signalling and lineage-specific transcription factors

Haemopoiesis is the process of blood production such that all the haemopoietic lineages can be reconstituted from the haemopoietic stem cell (HSC) (**Figure 1.3A**) (Ceredig*, et al* 2009). Haemopoietic lineage specification is determined by extrinsic (haemopietic growth factors) and intrinsic factors (transcription factors). Many models have been proposed invoking that the process involves irreversible determination of fate between myeloid and lymphoid lineages (Ceredig*, et al* 2009). Each cell type in haemopoiesis expresses different levels of multiple surface markers which can be detected by flow cytometry techniques (Krause and Van Etten 2007, Rosenbauer and Tenen 2007). Generally, HSC have self-renewal and high proliferation potential while more committed progenitors have lost the self-renewal properties and gained lineage-specific expression programs (**Figure 1.3A**) (Rosenbauer and Tenen 2007). This model has been challenged recently with the finding that the intermediate progenitors have myeloid and lymphoid potential, and the lymphoid progenitors have myeloid potential (**Figure 1.3B**) (Ceredig*, et al* 2009). This provides new evidence that there is plasticity within the haemopoietic stem and progenitor cell compartment.

1.2.1 The importance of growth factors in haemopoiesis

The haemopoietic growth factors (GFs) constitute a large group of cytokines and growth factors shown to play crucial roles in regulating the survival and proliferation, differentiation and commitment, and end-stage cell functions of various haemopoietic lineages (Rieger*, et al* 2009, Socolovsky*, et al* 1998). Important groups of growth factors such as interleukin-3 (IL-

A.

B.

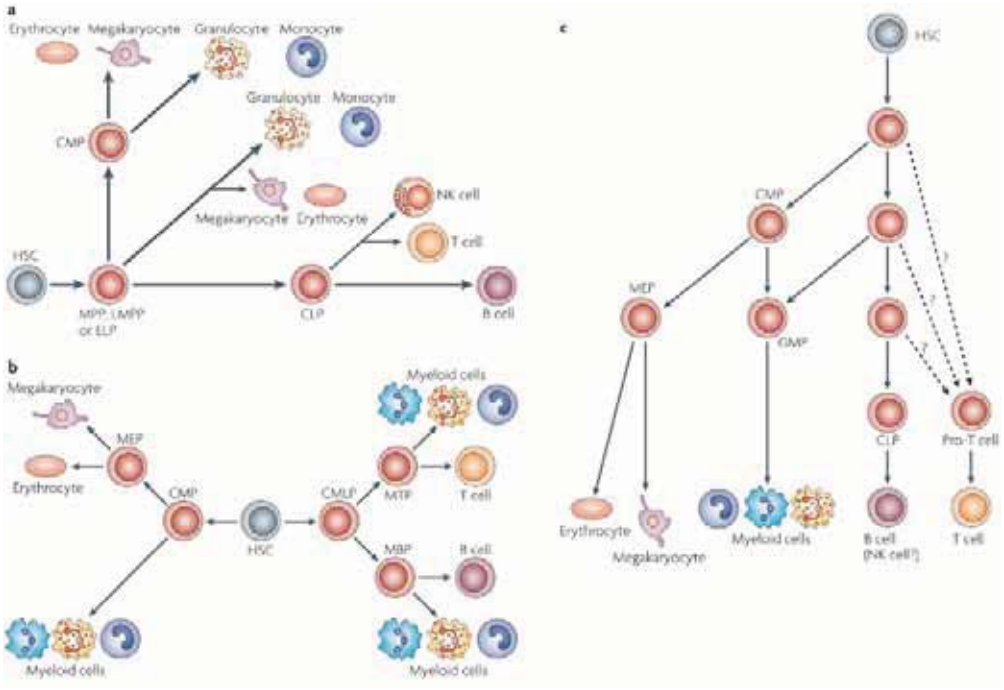


Figure 1.3. Alternative models of haemopoiesis. A. The classical model of haemopoiesis which determines either lymphoid or myeloid lineage choice during differentiation from haemopoietic stem cell (HSC) (adapted from Rosenbauer and Tenen, 2007). LT/ST HSC, Long-term/Short-term HSC; MPP, multipotential progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/monocyte progenitor; MEP, megakaryocyte/erythroid progenitor; MDP, macrophage/dendritic cell progenitor. Alternative models of haemopoiesis where intermediate progenitors have both myeloid and lymphoid potential, and the lymphoid progenitors have myeloid potential (adapted from Ceredig *et al*, 2009). There are three recent models of haemopoiesis shown in **B**. In model **a**., LMPP give rise to individual branches for myeloid and lymphoid cells. In model **b.**, CMLP have the potential to differentiate into myeloid and T cells, or into myeloid and B cells. In model **c.**, the lost of erythroid, neutrophil and myeloid potential is a requirement for commitment to lymphoid cell development. ELP, early lymphoid progenitor; LMPP, lymphoid-primed multipotent progenitor; MBP, myeloid–B-cell progenitor; MTP, myeloid–T-cell progenitor; CMLP, common myeloid-lymphoid progenitor.

3), IL-5, granulocyte macrophage colony-stimulating factor (GM-CSF), FLT3 ligand (FL), stem cell factor (SCF), erythropoietin (EPO), and granulocyte colony-stimulating factor (G-CSF) have key roles in regulation of haemopoiesis. For example, as shown in **Figure 1.4**, IL-3 modulates growth, differentiation and survival of progenitor cells whereas IL-5 is restricted to differentiation, activation and survival of eosinophils (Martinez-Moczygemba and Huston 2003, Socolovsky*, et al* 1998). GM-CSF regulates growth and differentiation of dendritic cells, myelomonocyte progenitors and granulocytes (Martinez-Moczygemba and Huston 2003). FL is a GF for dendritic cells (Naik*, et al* 2009, Waskow*, et al* 2008) and mediates differentiation of early progenitors. When combined with other growth factors such as SCF and IL-3, it supports proliferation of primitive haemopoietic progenitor, committed early myeloid and lymphoid precursors (Meshinchi and Appelbaum 2009). SCF is a multipotent haemopoietic GF involved in differentiation, proliferation and migration of mast cells (Masson and Ronnstrand 2009). SCF also has strong synergistic activities for early progenitor cells when combined with other GFs such as IL-3, G-CSF and EPO (Masson and Ronnstrand 2009). EPO is important for erythroid cells survival, proliferation and differentiation (Richmond*, et al* 2005). G-CSF is important for modulating proliferation and differentiation of granulocytes (Liu*, et al* 1996, Socolovsky*, et al* 1998). Growth factors induce activation of their cognate receptors which is associated with activation of a number of downstream signalling pathways including the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), mammalian target of rapamycin (mTOR), signal transducers and activators of transcription 5 (STAT5) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathways (de Groot*, et al* 1998, Masson and Ronnstrand 2009, Stirewalt and Radich 2003). As discussed in **Section 1.7** below, these pathways are important modulators of progenitor cell proliferation and survival, and aberrant activation is commonly seen in AML. Activation of these pathways induces activation and repression of downstream

Figure 1.4. The effects of growth factors in determining cell lineage specification during haemopoiesis. Some of the haemopoietic growth factors regulate the lineage specific differentiation are illustrated (adapted from Socolovsky *et al*, 1998). M-CSF, Macrophage colony stimulating factor; SCF, stem cell factor; Epo, erythropoietin; Tpo, thrombopoietin; CFU-GEMM, CFU granulocyte-erythroid-monocyte-megakaryocyte; CFU-GM, CFU granulocyte-monocyte; CFU-me, CFU megakaryocyte; CFU-E, CFU erythroid; CFU-Eo, CFU eosinophil; BFU-E, burst-forming unit erythroid.

genes that control the proliferation and differentiation of haemopoietic cell progenitors (Nowak*, et al* 2009). The capacity of GFs such as G-CSF and GM-CSF which induce myeloid differentiation, has been translated to the clinical settings to differentiate the AML blast cells. However, these haemopoietic GFs have limited capacity to differentiate AML cells and therefore their use in AML differentiation treatment has been negligible (Nowak*, et al* 2009).

1.2.2 Transcription factors that determine haemopoietic cell fates

Myeloid lineage specific transcription factors (TFs) such as *CEBPA, SPI1 (PU.1), GATA1, RUNX1, IRF8* (interferon regulatory factor 8)*, GFI1* (growth factor independent 1), and *GFI1B* (transcription repressor growth factor independent 1B transcription repressor) regulate each other and expression of differentiation programs associated with terminal differentiation (**Figure 1.5**). As shown in **Figure 1.5A-B**, each transcription factor has a specific role in regulating myelopoiesis. Regulation of growth factor receptors by these lineage-specific TFs specifies the response of progenitor cells to GFs, and signalling from GF receptors in turn modulates TF activity. For example, the C/EBPα is one of the critical myeloid transcription factors for granulocytic differentiation (Koschmieder*, et al* 2009). Forced expression of C/EBPα in the U937 (bipotential AML cell line) triggers granulocytic differentiation while suppressing the monocytic differentiation program (Radomska*, et al* 1998). A myeloid differentiation block was also observed in a C/EBPα-deficient mouse model (Friedman 2002, Zhang*, et al* 1997). Phosphorylation of C/EBPα on Ser^{21} is a key regulatory modification that can be regulated by GF receptor signalling, such as FLT3 (Radomska*, et al* 2006). In addition, other modifications on C/EBPα that affect or silence its functions and activities are discussed in **Section 1.3.1**. Another transcription factor *SPI1*

A.

B.

Figure 1.5. The role of transcription factors involved in determining cell fate during haemopoiesis. A. The stepwise requirement for transcription factors in determining lineage specificity during myeloid differentiation (adapted from Rosenbauer and Tenen, 2007). **B.** The mapping of transcription factors using the pairwise relationship model. Green colour indicates the transcription factor must be activated to direct the development of particular cell lineage. Red colour indicates the transcription factor must be inactivated to direct the the development of particular cell lineage (adapted from Ceredig *et al*, 2009). AP1, activator protein 1; C/EBP, CCAAT/ enhancer-binding protein-; EBF1, early B-cell factor 1; EDAG, erythroid differentiation-associated gene; EKLF, erythroid Kruppel-like factor; FOG1, friend of GATA1; GATA, GATA-binding protein; HSC, haematopoietic stem cell; PAX5, paired box protein 5.

(PU.1) is also important in the myeloid system especially for specification of the myelomonocytic lineage. Knockout of this gene significantly reduced mature myeloid cells (macrophages) and B cells and increased granulopoiesis (immature granulocytes), as well as being associated with defective HSC (Rosenbauer and Tenen 2007). There are multiple important transcription factors required for controlling the multistep process of haemopoiesis and phenotypes for these are summarised in **Table 1.6**.

1.3 AML biology and pathogenesis

Disruption or constitutive activation of GF signalling pathways and transcription factor function, as described above, contributes to leukaemic transformation. Aberrant GF signalling provides constitutive proliferation and survival pathways and can arise through mutations in GF receptors as seen with FLT3 (Masson and Ronnstrand 2009, Meshinchi and Appelbaum 2009). These mutations cooperate with aberrant TF activity induced by direct mutation or gene fusion, to induce or accelerate the progression of AML. However, single mutation in either a GF receptor pathway or transcription factors is not enough to give rise to AML. For example, introduction of FLT3-ITD mutation in the mouse model does not give rise to AML but generates a myeloproliferative disorder (MPD) (Grundler*, et al* 2005). Furthermore, FLT3-ITD mutation cooperates with PML-RARα or MLL-AF9 fusion protein to induce AML (Kelly*, et al* 2002a, Stubbs*, et al* 2008). This effect is consistent with the two 'hit' model of leukaemia pathogenesis where a minimum of two co-operating mutations, one each of a class I (transcription factors – interfering with transcription and differentiation) and class II (signal transduction molecules) mutation, are required for disease progression (**Figure 1.6A** and **Figure 1.6C**) (Dash and Gilliland 2001, Haferlach 2008). However, this is an over-simplification as activated GF receptor pathways can also contribute to the block in

Table 1.6. Summary of the transcription factors which determine specific cell lineage and their knockout effect in haemopoiesis (adapted from from Rosenbauer and Tenen, 2007).

NOTE:
This table is included on page 19
of the print copy of the thesis held in
the University of Adelaide Library.

A.

B.

C.

Figure 1.6. The effects and the frequency of two "hit" hypothesis for AML progression and development. A. Examples of cooperating Class I and Class II mutations in AML. Class I mutation normally interfere with transcription and hence contribute to the block in differentiation. Class II normally occur as receptor tyrosine kinase (RTK) mutations or result in constitutive activation of downstream signalling pathways. **B.** The frequency of the Class I and Class II mutation in AML (adapted from Haferlach, 2008). **C.** The normal haemopoiesis process is disrupted by the two "hit" hypothesis resulting in hyper-proliferation and blocked myeloid differentiation (adapted from Rosenbauer and Tenen, 2007).

differentiation; for example, FLT3-ITD phosphorylates Ser^{21} of C/EBPα which contributes to the block in granulopoiesis (Radomska*, et al* 2006). Other examples and the frequency of cooperating events are shown in **Figure 1.6A-B**.

1.3.1 Genetic alteration of transcription factors in AML

Deregulation or mutation of transcription factors, or the generation of fusion proteins (AML translocation) plays an important role in impairment of haemopoietic differentiation and hence progression to AML (**Figure 1.6C**). The most common balanced chromosomal translocations in AML (t(8;21), inv(16), t(15;17) or 11q23) target key lineage-specific transcription factors such as *CEBPA*, *SPI1 (PU.1)*, core binding factor (*CBF*), *RARA*, *MLL,* and members of the *HOX* gene family such as *HOXA9* (*NUP98-HOXA9*) (Doepfner*, et al* 2007). Additionally, direct mutations in *CEBPA* and *SPI1* (*PU.1)* occur relatively frequently in AML consistent with the critical role in myeloid differentiation described above. Down-regulation of C/EBPα expression has also been found in AML associated with AML1-ETO and CBFβ-MYH11 translocations, consistent with reduced activity of this TF being a critical event for leukaemogenesis (Koschmieder*, et al* 2009, Pabst and Mueller 2009). *CEBPA* is also frequently silenced by methylation in AML (Hackanson*, et al* 2008, Toyota*, et al* 2001). There are other mechanisms that inhibit C/EBPα activity such as sumolyation, the ratio of C/EBPα isoforms p42 and p44, and over-expression of calreticulin (*CALR*). Further details on these mechanisms have been reviewed by Koschmieder *et al* (Koschmieder*, et al* 2009). The frequency, effects and the FAB association of these transcription factor mutations in AML is summarised in **Table 1.4**.

1.4 Leukaemic stem cell

Leukemic stem cells (LSC) represent a small population 0.1-1% of total AML blasts (Bonnet and Dick 1997). Human LSC reside in $CD34^{+}CD38^{-}$ fraction of leukaemic cells. LSC generally lack the expression of CD90 (Thy-1) and CD117 (c-Kit) but highly express CD123 (IL3RA), CD47 and CD96 (Krause and Van Etten 2007, Lane and Gilliland 2009). The LSC population is quiescent with the properties of self-renewal and long-term survival (Lane and Gilliland 2009). There is increasing evidence that LSCs are able to resist anticancer treatment by escaping inhibitor or chemotherapeutic killing through the efflux pump, ATP-binding cassette sub-family B (MDR/TAP) member 1 *(ABCB1*) (Lane and Gilliland 2009), or by utilizing alternative survival pathways (reviewed in (Chu and Small 2009). LSC also evade macrophage killing and phagocytosis by expressing CD47 (Jaiswal*, et al* 2009). The level of transcription factor expression is also important in LSC. For example, the low expression of *CEBPA* or *SPI1 (PU.1)* beyond a critical threshold may be important for initiation of AML (**Figure 1.7**) (Rosenbauer*, et al* 2005). Characterisation of AML LSC populations using gene expression profiling and biochemical dissections is consistent with constitutive activation of PI3K/AKT, mTOR, homeobox (*HOX*) gene family, wingless-type MMTV integration site family (Wnt)-β-catenin, and nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB), all of which contribute to cell survival, self-renewal and proliferation (Krause and Van Etten 2007, Lane and Gilliland 2009). Furthermore, FLT3-ITD has been shown to be present in LSC (Levis*, et al* 2005) and is an independent predictor of poor prognosis. The size of LSC populations is correlated with increased residual disease detection and is associated with poorer survival after treatment (Lane and Gilliland 2009).

Figure 1.7. The important stem cell properties and low transcription factor activity in leukaemic stem cell. Leukaemic stem cells (LSC) have stem cell functions such as self-renewal and proliferation. However, HSC have the ability to differentiate to common myeloid progenitor (CMP), granulocyte monocyte progenitor (GMP) and subsequently undergo terminal differentiation. LSC give rise to blast cells with a block in differentiation. The development of LSC also requires two "hits" where lower transcription factor activity (such as *PU.1)* and constitutive activation of signalling pathways (such as FLT3-ITD) to give rise to LSC (adapted from Rosenbauer *et al*, 2005).

1.5 Receptor signalling in haemopoiesis

There are two classes of growth factor receptors which differ in regard to intrinsic tyrosine kinase activity. For example, the members of the type III receptor tyrosine kinases (RTKs) such as platelet-derived growth factors receptor (PDGFR), FMS-like tyrosine kinase 3 receptor (FLT3), Steel factor receptor (c-KIT) and macrophage colony-stimulating factor receptor (M-CSFR), have an intrinsic tyrosine kinase activity for auto-phosphorylation and thus downstream signalling activation (Gilliland and Griffin 2002, Masson and Ronnstrand 2009). The IL-3/IL-5/GM-CSF common beta chain receptor (GMR) which is a Type I transmembrane protein sharing signalling subunit for IL-3, IL-5 and GM-CSF, do not have intrinsic kinase activity and thus require intracellular tyrosine kinases such as Janus kinase 2 (JAK2) for phosphorylation and subsequently activation of downstream signalling pathways (Wang*, et al* 2009b).

1.5.1 IL-3/IL-5/GM-CSF receptor

Growth factors such as IL-3, IL-5 and GM-CSF are important regulators of myeloid development as described above. They exert their many functions by binding to surface receptor complexes which include a shared receptor subunit, the common beta chain subunit (βc), and specific alpha-subunits expressed on the surface of responding cells. They have overlapping biological activities and pleiotropic effects in stimulating survival, maintenance and stimulation of the proliferation and differentiation of multi-lineage haemopoietic progenitors and activation of neutrophils, eosinophils and monocytes (Geijsen*, et al* 2001). IL-5 exerts its effects on the eosinophil lineage specifically due to the lineage-restricted expression of the IL-5R α subunit while IL-3 influences the development of all five myeloid

lineages as well as the early maturation of erythroid cells. The actions of GM-CSF are restricted primarily to the neutrophil and monocyte lineages (Miyajima, *et al* 1993). This differential specificity of action is associated with the expression of the ligand-binding α subunits that are specific for each growth factor.

The unique growth factor-specific alpha (α) subunit (IL3Rα, IL5Rα or GMRα) binds to the specific growth factor with low affinity, but is unable to signal. The βc cannot detectably bind any of these factors alone, but complexes with the specific α subunits to form a high affinity, functional signalling complex (Miyajima, *et al* 1993). The structure of the ligand-bound GM-CSF dodecamer complex was recently reported (Hercus, *et al* 2009). As shown in **Figure 1.8A**, upon the growth factor (GM-CSF, IL-3 or IL-5) binding and subsequent receptor dimerisation, JAK2 activation occurs by trans-phosphorylation of two receptor-bound JAK2 molecules. Subsequently this leads to phosphorylation and activation of the downstream signalling pathways such as STAT5, PI3K/AKT, and MAPK/ERK pathways (de Groot, *et al* 1998, Hercus, *et al* 2009, Miyajima, *et al* 1993) which promotes cell survival, proliferation and differentiation effects depending on the target cells.

1.5.2 FMS-like tyrosine kinase 3 receptor (FLT3)

FLT3 is also known as Flk2 (fetal liver kinase 2), STK-1 (human stem cell kinase) and CD135. It encodes a 993 amino acid protein in human. It has important roles in haemopoiesis and the immune system, and is expressed on immature haemopoietic stem/progenitor cells in the bone marrow, thymus and lymph nodes as well as on dendritic cells (Szilvassy 2003). FLT3 comprises membrane bound receptors with five immunoglobulin-like extracellular domains, a transmembrane domain, a juxtamembrane domain, two intracellular tyrosine-

A. **B.**

NOTE:
These figures are included on page 26 of the print copy of the thesis held in the University of Adelaide Library.

C. **D.**

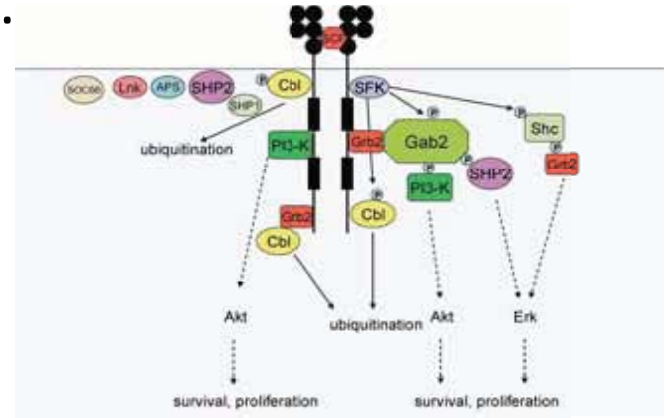


Figure 1.8. Receptor signalling in haemopoiesis and AML A. The signalling pathways activated by normal GMR for cell survival, differentiation and proliferation (adapted from Perugini *et al*, 2010). Please see text for more details. **B.** The signalling pathways activated by FLT3 receptor and constitutively activated receptor mutants. Constitutively activated pathways such as PI3K/ AKT, STAT5 and ERK1/2 are activated for cell survival and proliferation (adapted from Meshinchi *et al*, 2009). Please see text for more details. **C.** The signaling pathways activated by c-Kit receptor and its activated receptor mutation for cell survival and proliferation through PI3K/AKT and MAPK/ERK pathways (adapted from Masson *et al*, 2009). Please see text for more details **D.** The signalling pathways activated by GMR-V449E for cell survival and proliferation (adapted from Perugini *et al*, 2010). Please see text for more details.

kinase domains (TKDs) which are interrupted by a kinase insert domain and a C-terminal domain in the intracellular region as shown in **Figure 1.8B** (Kottaridis*, et al* 2003).

Unlike the GM-CSF family, which are always soluble growth factors, the human FL is a type I transmembrane protein (similar to c-KIT ligand and M-CSF) that can be released as a soluble homo-dimeric protein (Drexler and Quentmeier 2004). FL has several different isoforms that are biologically active (Stirewalt and Radich 2003) and has pleiotropic and potent effects on the proliferation, differentiation and survival of haemopoietic cells when synergised with other GFs (Drexler and Quentmeier 2004, Gilliland and Griffin 2002). Accumulated evidence indicates that FL stimulates proliferation and survival of FLT3-expressing primary AML cells and myeloid and monocytic leukaemia cell lines (Gilliland and Griffin 2002). FL-stimulated FLT3 leads to receptor dimerisation and auto-phosphorylation of tyrosine residues which further increases kinase activity (Stirewalt and Radich 2003, Weiss and Schlessinger 1998). The activated RTK then phosphorylates and binds multiple signalling molecules which leads to activation of different signalling pathways such as RAS/MAPK and PI3K/AKT/mTOR pathways (same as hβc signalling pathways) (**Figure 1.8B**). Consequently, this leads to cell proliferation, differentiation and survival of haemopoietic cells.

1.5.3 c-Kit receptor

The c-Kit ligand, SCF, has two alternative isoforms which are the membrane-bound and soluble form (Masson and Ronnstrand 2009). The structure of c-Kit is consisting of 976 amino acids with a molecular weight of 140 and 155 kDa depending on the extent of N-linked glycosylation (Masson and Ronnstrand 2009). SCF binding to the second and the third

domains in the extracellular region of c-Kit inducing dimerisation, auto-phosphorylation of tyrosine residues and activation of downstream signalling pathways, via a similar mechanism to FLT3. The activation of downstream pathways is important for cell survival, proliferation, self-renewal and differentiation of haemopoietic cells (**Figure 1.8C**) (Masson and Ronnstrand 2009).

1.6 Activated receptor mutants in AML

1.6.1 Constitutive activation of GMR induces AML

Our laboratory has identified a panel of constitutively active GMR mutants which can all confer factor-independence on the murine leukaemic cell line FDC-P1 (D'Andrea and Gonda 2000). These mutants have been utilised to identify transcriptional targets of signalling pathways and hence the effector molecules that influence proliferation, survival and differentiation of myeloid cells (Brown*, et al* 2006, Perugini*, et al* 2010). There are two classes of constitutively activated GMR mutant. They are the extracellular (EC) mutants, the best characterised of which is GMR-FIΔ, and transmembrane (TM) mutants, best represented by GMR-V449E (D'Andrea and Gonda 2000, Gonda and D'Andrea 1997). The TM mutant GMR-V449E is a major focus of this study as this mutant induces an AML-like disease in murine transgenic and bone marrow transplant model (McCormack and Gonda 1999).

1.6.1.1 GMR-V449E mutation in common beta chain

GMR-V449E contains an amino acid substitution in the transmembrane domain of the GMR common β-subunit, converting a valine residue at position 449 to glutamic acid (Jenkins*, et al* 1995). GMR-FIΔ is an EC mutant which contains a 37 amino acid duplication in the

membrane proximal region of the EC domain, encompassing the conserved WSXWS motif (D'Andrea*, et al* 1994). The extracellular GMR mutants activate limited aspects of signalling associate predominantly with phosphorylation of AKT (Perugini*, et al* 2010). Only the GMR-V449E mutant exhibits JAK2 activation associated with detectable constitutive βc tyrosine phosphorylation suggesting that activation of proliferation and survival pathways may not always require βc subunit tyrosine phosphorylation (D'Andrea and Gonda 2000, Perugini*, et al* 2010). This mutant most likely functions by constitutively inducing homodimerisation of hβc in the absence of GF (Jenkins*, et al* 1998). This is proposed to result in JAK2 activation by trans-phosphorylation and activation of downstream signalling pathways (e.g. JAK/STAT, PI3K/AKT and ERK1/2) (**Figure 1.8D**) (Perugini*, et al* 2010).

In mouse models generated by bone marrow transduction and reconstitution, or transgenesis, the TM and EC mutants have different biological outcomes. The most striking difference relates to leukaemogenic potential. GMR-V449E induces an acute, lethal leukaemia-like disorder characterised by blast cells in the blood, anaemia, splenomegaly and infiltration of blast cells and neutrophils into the spleen, liver and lung (McCormack and Gonda 1999). The disease occurred after a long and variable latency (mean 20 weeks) and was acute in onset (McCormack and Gonda 1999). Additionally, when expressed with PML-RARα, GMR-V449E cooperated to induce a lethal leukaemia-like syndrome in a mouse model with a latency of fewer than 21 days (Phan*, et al* 2003). In contrast, GMR-FIΔ induces a MPD in a transgenic model characterised by increased numbers of neutrophils, monocytes, erthrocytes and platelets (McCormack and Gonda 1999).

1.6.1.2 A critical motif in hβc regulates proliferation and survival

A secondary mutation of an intracellular tyrosine residue Tyr^{577} (Y577) to phenylalanine (Y577F) in GMR-V449E selectively abolishes factor independent proliferation but not survival in the factor-dependent myeloid cell line (FDB1, see **Section 1.9.1** for more details) (Brown, *et al* 2004). This secondary Y577F mutant of GMR-V449E also permits some differentiation to monocyte/macrophages in the absence of growth factor (Brown, *et al* 2004). This Y577F mutant of GMR-V449E was able to cooperate with PML-RARα to induce leukaemia *in vivo* but with longer latency compared to GMR-V449E alone (approximately 85 days vs 55 days respectively) (Phan, *et al* 2003). Tyr^{577} forms part of the binding site for Src homology 2 domain-containing transforming protein (SHC) and contributes to phosphorylation of PTPN11 (a.k.a SHP2) to activate RAS signalling (Mullally and Ebert 2010, Phan, *et al* 2003). Mutations in *PTPN11* have been found in 18% of childhood AML with the M5 FAB subtype (Tartaglia, *et al* 2005). Additionally, constitutively active PTPN11 cooperates with *HOXA10* and *ICSBP* to induce AML (Konieczna, *et al* 2008, Wang, *et al* 2009a). Thus, this key residue provides an important signal associated with cell proliferation and the block in differentiation via the *PTPN11* signalling pathway.

The Tyr^{577} residue has also been identified by Guthridge *et al* as part of the bidentate motif that regulates proliferation and survival signals for GM-CSF (Guthridge, *et al* 2006). In contrast to the proliferation effect associate with Tyr^{577}, the serine residue distal to Tyr^{577} (Ser^{585}) in GMR hβc has been reported to be involved in survival-only signalling (Guthridge, *et al* 2006). When CTL-EN cells were stimulated with low concentrations of GM-CSF (less than 10 pM), phosphorylation of Ser^{585} was observed and this was associated with cell

survival and activation of PI3K/AKT signalling. Guthridge *et al* reported that this Ser^{585}-dependent pathway of the hβc mediates the cell survival through the activation of PI3K-AKT-NFκB and the induction of BCL-2 (anti-apoptotic) (Guthridge*, et al* 2004). In contrast, when the cells were stimulated with higher concentrations of GM-CSF (10-10000 pM), phosphorylation of the Tyr^{577} residue was observed associated with proliferation, survival and activation of downstream signalling pathways such as JAK2-STAT5, with phosphorylation of Ser^{585} no longer observed. Importantly, the Ser^{585} residue was shown to be constitutively phosphorylated in many AML samples suggesting a possible contribution of this pathway to AML cell survival. Guthridge *et al* have proposed that there are two alternative pathways associated with the Tyr^{577} and Ser^{585} motif that mediate proliferation and survival, or survival-only signalling and these may be mutually exclusive (Guthridge*, et al* 2006).

1.6.2 FLT3

FLT3 is the most commonly mutated gene in AML of many FAB subtypes and approximately 30-35% of AML have acquired FLT3 mutations (Gilliland*, et al* 2004). The juxtamembrane and the activation loop domains have auto-inhibitory function that maintains the kinase in an inactive conformation (Gilliland and Griffin 2002, Griffith*, et al* 2004). The activation loop folds into the active site of the kinase domain, thus blocking access of ATP and substrates. Either phosphorylation or mutation of critical residues on the kinase domain allows the loop to fold out of the active site providing access to ATP and substrate, with subsequent activation of the kinase activity (Gilliland and Griffin 2002). The most common mutations are internal tandem duplications (ITD) in the juxtamembrane (JM) region and a point mutation within the activation loop of the second tyrosine kinase domain (TKD) as

shown in **Figure 1.8B**. Constitutive FLT3 activation occurs in 20-30% of adult AML due to internal tandem duplications (FLT3-ITD) in exons 14 and/or 15 (Gilliland and Griffin 2002) or mutation within the TKD in exon 20 (approx 5-10% of AML) (Abu-Duhier*, et al* 2001, Stirewalt and Radich 2003, Yamamoto*, et al* 2001). The most frequent FLT3-TKD mutation is a substitution mutation at aspartic acid (D) residue 835 in the second tyrosine kinase domain.

1.6.2.1 FLT3-ITD mutation

FLT3-ITD is expressed as an immature, under-glycosylated form with molecular weight of 130 kDa, whereas FLT3-WT exists as a mature glycosylated form with a molecular weight of 150 kDa. The immature form of FLT3-ITD is due to constitutive tyrosine phosphorylation in juxtamembrane which results in reduction of folding efficiency and retention in endoplasmic reticulum (Schmidt-Arras*, et al* 2005). Both FLT3 mutations (ITD and TKD) constitutively activate receptors resulting in auto-phosphorylation and downstream cytoplasmic signalling (**Figure 1.8B**) (Naoe and Kiyoi 2004). The most characteristic feature of FLT3-ITD is its modality of mutation. The length of an FLT3-ITD insertion varies significantly from 3 to 400 bp. Meshinchi *et al* observed that FLT3-ITD with length less than 48 bp has a lower relapse and more favourable outcome compared to longer length of FLT3-ITD (Meshinchi*, et al* 2008). FLT3-ITD duplication usually involves at least one of seven amino acid residues from codon 591 to 597 (YVDFREY) (Meshinchi*, et al* 2008). Interestingly, the codon 597 (YVDFREY) is duplicated with the highest frequency with 86% of AML (Meshinchi*, et al* 2008). However, Y597 is not critical for maintenance of the auto-inhibited conformation of FLT3 suggesting that other duplicated residues carry important function in FLT3-ITD (Meshinchi*, et al* 2008). For example, R595 (duplicated in 77% patients) is critical in FLT3-

ITD for conferring factor-independent growth and STAT5 activation (Vempati, *et al* 2007). Y589 and Y591 residues are important for the transforming potential and STAT5 activation in both FLT3-ITD and FLT3-TKD, and for ligand-dependent activation of FLT3-WT (Vempati, *et al* 2008). Y599 (YVDFREYEYDL) is also one of the residue duplicated frequently in AML and has been demonstrated to associate with and to phosphorylate SHP2 (Heiss, *et al* 2006, Meshinchi, *et al* 2008). In mouse models, FLT3-ITD mutations induce a MPD with a variable latency characterised by leukocytosis and comprised mainly of mature neutrophils (Grundler, *et al* 2005, Kelly, *et al* 2002b). These experiments indicate that the FLT3-ITD mutations are sufficient to promote a change associated with enhanced proliferation. However, they are not sufficient to induce overt acute leukaemia (see Section 1.3 for 2 'hit' hypothesis).

Signalling properties of FLT3 mutants have been well characterised. FLT3-ITD confers factor-independent proliferation on IL-3 dependent cell lines such as Ba/F3 and 32D cells (Choudhary, *et al* 2005, Fenski, *et al* 2000, Kim, *et al* 2005). Moreover, FLT3-ITD showed constitutive phosphorylation of AKT, ERK1/2 and STAT5 in murine bone marrow cells, Ba/F3 cells, 32D cells and primary cells (Choudhary, *et al* 2005, Grundler, *et al* 2005, Hayakawa, *et al* 2000, Mizuki, *et al* 2000). There are a number of genes that are dysregulated by FLT3-ITD and are important for transformation. These include *Pim1* (proviral integration site 1) (Kim, *et al* 2005), *Pim2* (Mizuki, *et al* 2003), *Rgs2* (regulator of G-protein signalling) (Schwable, *et al* 2005), *SPI1* (*PU.1)* and *CEBPA* (Mizuki, *et al* 2003, Zheng, *et al* 2004). Recent evidence in primary cells indicates that FLT3-ITD induces stronger STAT5 signalling than FLT3-TKD despite stronger auto-phosphorylation (Grundler, *et al* 2005, Irish, *et al* 2004). This data is also supported by a recent study that has shown FLT3-ITD induced *Pim-2* (*STAT3/5* target gene), although the comparison was not made with FLT3-TKD (Mizuki, *et*

al 2003). Furthermore, STAT5 activation has been shown to be more critical for FLT3-ITD transformation than for FLT3-TKD (Ono*, et al* 2009). As previously described in **Section 1.3**, FLT3-ITD constitutive activation of the MAPK/ERK pathway leads to phosphorylation of Ser^{21} on C/EBPα modifying its function, leading to a block in granulocytic differentiation (Radomska*, et al* 2006).

1.6.2.2 FLT3-TKD mutations

Of the FLT3-TKD mutations, substitution at aspartate residue D835 is the most frequent occurrence in AML (Bacher*, et al* 2008, Naoe and Kiyoi 2004). FLT3-TKD has some similar transforming properties to FLT3-ITD such as constitutive tyrosine activation and conferring factor-independent growth in 32D cells (Naoe and Kiyoi 2004). However, the downstream signalling of FLT3-TKD more resembles that of FLT3-WT than FLT3-ITD (Choudhary*, et al* 2005). Ono *et al* reported that FLT3-TKD is more sensitive to MEK inhibitors (inhibit MAPK/ERK pathway) than FLT3-ITD suggesting that the alternative pathways predominate in promoting leukaemogenesis (Ono*, et al* 2009). Interestingly, STAT3 has been reported to be selectively activated/phosphorylated by FLT3-TKD (Frohling*, et al* 2007). Choudhary *et al* also reported that FLT3-ITD and FLT3-TKD have different signalling pathways (Choudhary*, et al* 2005); in contrast to FLT3-ITD, FLT3-TKD failed to activate STAT5 and did not repress C/EBPα and PU.1 protein levels*,* or support clonogenic growth in semisolid media (Choudhary*, et al* 2005). FLT3-TKD is also more sensitive to FLT3 inhibitor (PKC412) compared to FLT3-ITD (Choudhary*, et al* 2005). The differences *in vitro* are also evident *in vivo*. For example, FLT3-TKD induces a lymphoid disease but not MPD in a murine bone marrow transplant model (Grundler*, et al* 2005). Both FLT3-ITD and FLT3-TKD require additional mutations to induce AML *in vivo,* or to accelerate the progression to

AML. However, unlike FLT3-ITD, there is no cooperativity between FLT3-TKD and AML1-ETO, as determined by rate of progression to AML (Schessl*, et al* 2005). The differences between FLT3-ITD and FLT3-TKD may be related to the observation that FLT3-ITD, but not FLT3-TKD or FLT3-WT is partially retained in the endoplasmic reticulum (ER) and does not reach the cell surface (Schmidt-Arras*, et al* 2009). Anchoring of FLT3-ITD in the ER activates STAT5 and its targets but not the PI3K and MAPK pathways, and also results in reduced global autophosphorylation. In contrast, membrane-bound FLT3-ITD activates PI3K and MAPK with reduced phosphorylation of STAT5 (Choudhary*, et al* 2009, Schmidt-Arras*, et al* 2009). Interestingly, the ER anchoring suppressed the FLT3-TKD transformation but not FLT3-ITD through STAT5 activation (Schmidt-Arras*, et al* 2009). In some AML patients, FLT3-ITD and FLT3-TKD display co-existence suggesting that these two mutants are not functionally redundant (Choudhary*, et al* 2005). This is supported by the differences in gene expression which are further discussed in the **Section 1.8.1** below.

1.6.3 The c-Kit-TKD mutation

The c-KIT receptor has also been shown to be constitutively activated in some cases of AML (Masson and Ronnstrand 2009). There is a high incidence of mutations in the receptor tyrosine kinase c-KIT in AML with reported frequencies of 9-48% for t(8;21) and 10-45% for inv(16) patients (Muller*, et al* 2008). Interestingly, the most common mutation of c-KIT in inv(16) AML is a substitution at aspartate 816 (D816) in the kinase domain which is equivalent to the aspartate 835 (TKD) mutation in FLT3 and is associated with an increased incidence of relapse in this AML subtype (Paschka*, et al* 2006). Moreover, AML1-ETO AML with KIT-D816 mutation is associated with worse overall survival and event-free survival (Schnittger*, et al* 2006). In addition to substitution at residue 816, insertions and

deletions have been previous reported (Doepfner, *et al* 2007). However, these mutations occur more frequently in gastrointestinal tumours (Masson and Ronnstrand 2009). As a result of gain-of-function mutations in c-KIT, there is constitutive activation of downstream signalling pathways similar to FLT3 mutations (**Figure 1.8C**). Some AML patients with c-KIT TKD mutations respond to the selective tyrosine kinase inhibitor imatinib or dasatinib providing a potential targeted therapy for core binding factor (CBF) AML (AML1-ETO and CBFβ-MYH11) (Cammenga, *et al* 2005, Schittenhelm, *et al* 2006).

1.7 Downstream signal transducers

Signalling events downstream of the RTK receptors have been studied extensively especially for FLT3 mutants. There is an increasing knowledge of the complexity of RTK signalling networks and this has provided leads for targeted therapies. As discussed above, constitutive activation of RTKs induces aberrant activation of major signalling pathways, such as PI3K/AKT, mTOR, RAS/MAPK/ERK, and JAK/STAT in AML. Many of these pathways are also important in other cancers (Doepfner, *et al* 2007) and there are now various inhibitors for these pathways which have been developed and used in clinical studies (Doepfner, *et al* 2007).

1.7.1 The PI3K/AKT/mTOR pathway

The PI3K pathway is one of the major pathways activated by RTK activation in AML. The PI3K family consists of eight enzymes which are subdivided into 3 classes (I–III) based on sequence homology and substrate specificity (Doepfner, *et al* 2007). Oncogenic Ras mutants, such as those in N-Ras and K-Ras, which are found commonly in AML, activate the PI3K

pathway inducing cell proliferation and survival. Upon activation of PI3K by either RTK or Ras mutation, several downstream targets of PI3K can be activated including AKT/PKB, phosphoinositide-dependent kinase-1 (PDK-1), forkhead transcription factors (FKHR), glycogen synthase kinase-3β (GSK-3β), tuberous sclerosis complexes 1 and 2 (TSC1, TSC2), BAD, Ras homologue enriched in the brain (Rheb), mammalian target of rapamycin (mTOR), S6 kinase (S6K), and 4E-binding protein (4E-BP1) (**Figure 1.9A**) (Doepfner*, et al* 2007). Constitutive activation of the PI3K/AKT pathway has been found in more than 50% of AML cases (Doepfner*, et al* 2007). Additionally, constitutive phosphorylation of AKT at Ser^{473} negatively correlates with the overall survival rate of AML patients (Doepfner*, et al* 2007). mTOR (a serine/thereonine kinase) also plays an important role in AML cell growth and survival and is activated by various RTK, GFs signaling, nutrient and energy status (Doepfner*, et al* 2007, Meric-Bernstam and Gonzalez-Angulo 2009). mTOR exists in two multiprotein complexes. These are mTOR complexes 1 and 2 (mTORC1 and mTORC2). mTORC1 activation results in phosphorylation of its downstream targets, the ribosomal S6K and 4E-BP1 whereas mTORC2 activation results in phosphorylation of AKT at Ser^{473} and protein kinase C alpha (PKCα) (**Figure 1.9A**) (Meric-Bernstam and Gonzalez-Angulo 2009). AKT Ser^{473} phosphorylation leads to full AKT activation and hence affects downstream targets as described above. The majority of AML samples have constitutive activation of S6K and 4E-BP1 which is consistent with aberrant activation of the mTORC1 as a feature of AML (Doepfner*, et al* 2007).

1.7.2 RAS/MAPK/ERK1/2 signalling

The MAPK signaling cascade is another important pathway for cell proliferation, differentiation and survival. Activation of the small GTP-binding protein Ras following RTK

A.

B.

Figure 1.9. The summary of PI3K/AKT/mTOR and MAPK pathways that are activated by RTK. A. The activated PI3K pathway induces the phosphorylation of AKT, mTOR and other downstream targets for cell survival and proliferation (adapted from Van der Heliden and Bernards, 2010). **B.** The MAPK pathway activated by RTK for cell survival and proliferation (adapted from Pratilas *et al*, 2010).

activation occurs via the adapter molecule Grb2 and the guanine nucleotide exchange factor Son of Sevenless (SOS). Subsequently, cytoplasmatic proteins such as Raf, MEK and ERK (MAPK) are activated resulting in the activation of the downstream targets such as ELK1 (ELK1, member of ETS oncogene family) as shown in **Figure 1.9B** (Doepfner*, et al* 2007, Pratilas and Solit 2010). Yamamoto *et al* reported that sustained activation of ERK1/2 down-regulates anti-proliferative genes such as *JunD* (Jun D proto-oncogene)*, Gadd45a* (growth arrest and DNA-damage-inducible, alpha)*, Ddit3 (*DNA-damage-inducible transcript 3), and *Tob1* (transducer of ERBB2 - known to regulate cell cycle progression negatively) in the G1 phase of cell cycle progression in NIH3T3 cells (Yamamoto*, et al* 2006). This may be an important mechanism for N-Ras and K-Ras mutants frequently mutated in AML. Constitutively activated MAPK/ERK1/2 signalling has been observed in approximately 50% of primary AML (Towatari*, et al* 1997). Furthermore, NF1 which encodes neurofibromin, a GTPase-activating protein and negative regulator of Ras, (**Figure 1.9B**) is inactivated in AML (Mullally and Ebert 2010, Parkin*, et al* 2010, Pratilas and Solit 2010). Activating RAS mutations are observed in approximately 20% of AML (Mullally and Ebert 2010). AML with inv(16) in particular is frequently associated with *NF1* deletions and hence alterations of RAS pathway (Haferlach*, et al* 2010). mTOR (downstream effectors of Ras and PI3K/AKT) has been shown to be negatively regulated by NF1 (Johannessen*, et al* 2005) and AML patients with normal *NF1* copy number and low *NF1* expression are more sensitive to mTOR inhibition than patients with an inactivating *NF1* mutation (Parkin*, et al* 2010).

1.7.3 JAK/STAT signalling

The Janus kinase (JAK) family is comprised of four members (JAK1, JAK2, JAK3, TYK2) which are characterised by JAK homology domains (JH1-JH7). JAK activation results in

phosphorylation of the receptor on Tyr residues, which subsequently allows binding of proteins such as the STAT, Src family kinases, phosphatases and adaptor proteins, which link to cell proliferation, survival and differentiation (Masson and Ronnstrand 2009). There is the crosstalk between MAPK and the STAT signalling (Masson and Ronnstrand 2009); in particular, STAT proteins located at the cytoplasm are directly phosphorylated by JAK which leads to dimerisation and translocation to nucleus. Activated STATs binds specifically to gamma-interferon-activated sequences (GAS) in DNA to initiate downstream transcription of target genes. For example, STAT5 induces expression of *Bcl2l1, Cish, Id1, c-Fos, Osm* and *Pim1* while STAT3 (activated by MEKK1, MEK kinase) activates the *c-Myc* gene (Masson and Ronnstrand 2009). Constitutive activation of STAT5 has been shown to promote cell proliferation and survival in Ba/F3 cells (Onishi*, et al* 1998). In AML, constitutive activation of STAT5 is detected in 22% of adult patients (Benekli*, et al* 2003). Constitutive DNA binding of STAT3 and STAT5 in AML blasts cells is one of the important signalling pathways downstream of FLT3 mutations for transformation (Masson and Ronnstrand 2009).

1.8 Application of gene expression profiling technology to AML

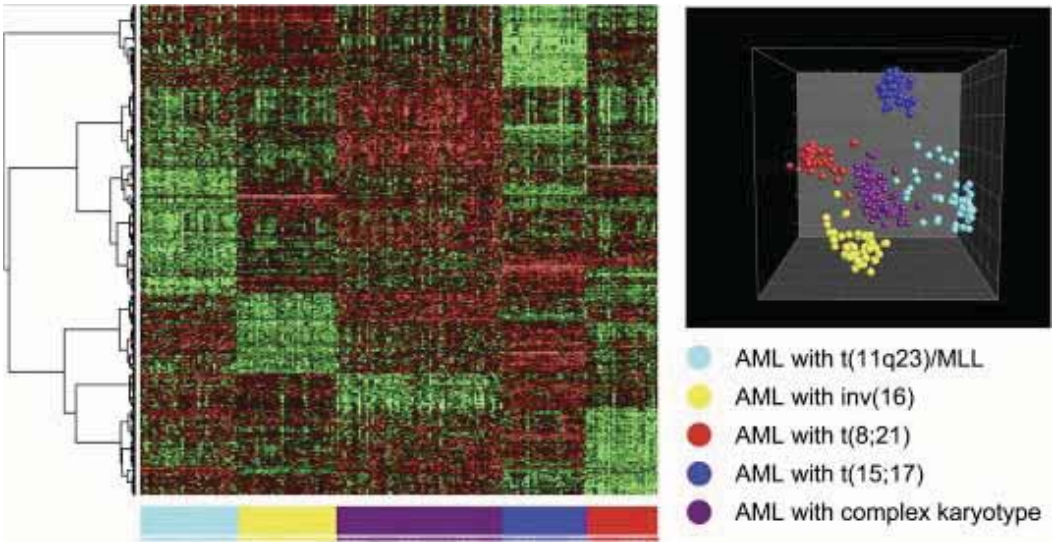
There has been widespread application of new technologies (microarray, SNP, LOH, methylation, next-generation sequencing and proteomics) to interrogate the pathogenesis and biology of AML (Goswami*, et al* 2009). Microarray gene expression profiling is a tool that enables measurement of the gene expression (mRNA level) of more than ten thousand genes at once and there has been numerous examples where microarray and transcriptomics data have been deposited at the Gene Expression Omnibus (GEO) and ArrayExpress databases for public usage (Bacher*, et al* 2009b). Microarray gene expression profiling has been applied extensively to the diagnostics, prognostics, mechanism, and therapeutic and drug discovery

in AML (Bacher, *et al* 2009b, Goswami, *et al* 2009). With freely available bioinformatics tools from the Bioconductor website (http://www.bioconductor.org/), it is now possible to identify the significant differentially expressed genes, explore network pathways, predict molecular mutations and cytogenetic abnormalities, and identify novel classes of AML using the publicly available microarray data.

1.8.1 Gene expression profiling for diagnostic and prognostic predictions

Efforts to identify the contribution of the translocation derived fusion proteins in leukemogenesis have included genome-wide transcriptional profiling of primary leukemic blasts. A number of these studies have described the unique features of each signature and reported gene expression changes associated specifically with the AML1-ETO, CBFβ-MYH11, MLL, or PML-RARα fusions (Bacher, *et al* 2009b, Ichikawa, *et al* 2006, Lee, *et al* 2006). AML with each of those key translocation events can be distinguished on the basis of gene expression demonstrating the unique properties associated with each fusion protein (Bacher, *et al* 2009a). Gene expression signatures have predictive power in classifying AML subtypes from patient samples (**Figure 1.10A**) (Bacher, *et al* 2009a) and several studies suggest that there are at least 15 AML subtypes based on gene expression (Bacher, *et al* 2009b, Bullinger, *et al* 2004, Schoch, *et al* 2002, Valk, *et al* 2004). Additionally, through supervised and unsupervised classification, the large normal karyotype AML group can be separated into at least two novel groups with significantly different prognostic value (Bullinger, *et al* 2004).

A.



B.

NOTE:
This figure is included on page 42
of the print copy of the thesis held in
the University of Adelaide Library.

Figure 1.10. Application of gene expression profiling for diagnosis and drug discovery. A. Classifying various subtypes of AML by hierarchical clustering heatmap and principal component analyses. Adult AML subtypes such as 11q23/*MLL*, t(8;21), t(15;17), inv(16), and AML with complex aberrant karyotypes are clearly distinct based on differential expression from 749 probe sets (adapted from Bacher *et al*, 2009)**. B.** The application of Connectivity map uses gene expression profiles derived from the treatment of cultured human cells with a large number of drugs to populate a reference database to facilitate identification of drugs that may be useful for treatment in a disease of interest (adapted from Lamb *et al*, 2006). Please see text for more details.

Gene expression profiling has also been useful in analysing different mutation groups in AML (Bacher, *et al* 2009b, Goswami, *et al* 2009). Several microarray studies have been performed to dissect the gene expression in FLT3-ITD (Bullinger, *et al* 2004, Bullinger, *et al* 2008, Gale, *et al* 2005, Goswami, *et al* 2009, Kim, *et al* 2007, Lacayo, *et al* 2004, Lu, *et al* 2007, Metzeler, *et al* 2008, Mizuki, *et al* 2003, Neben, *et al* 2005, Pekova, *et al* 2009, Radmacher, *et al* 2006, Stolzel, *et al* 2010, Valk, *et al* 2004, Verhaak, *et al* 2009), and these have identified several differentially expressed genes associated selectively with FLT3-ITD. There are also several microarray studies to investigate the gene expression of FLT3-TKD (Bullinger, *et al* 2004, Bullinger, *et al* 2008, Lu, *et al* 2007, Neben, *et al* 2005, Valk, *et al* 2004, Verhaak, *et al* 2009, Whitman, *et al* 2008, Yamaguchi, *et al* 2009) and these have also highlighted unique gene expression patterns between FLT3-ITD and FLT3-TKD. Several gene expression profiling studies have identified the FLT3 mutation groups with high accuracy (Bacher, *et al* 2009b, Goswami, *et al* 2009). These studies together have provided a better understanding of the FLT3 mutation pathogenesis and the association with adverse prognosis.

1.8.2 Gene expression profiling in target-based drug discovery

The observation that gene expression profiling can classify AML with distinct reciprocal translocations and mutations is consistent with each event activating unique downstream pathways and emphasizes that each of these groups of AML has unique characteristics that may provide for individualized therapy. The current bioinformatics technology, together with the rich resource of microarray datasets that have been deposited in the public domain make it possible to use these gene expression profiles to identify drugs that are associated with gene expression patterns of relevance to particular diseases. For instance, the Connectivity

Map (CMAP) that has been developed by Lamb *et al* (Lamb*, et al* 2006) has been used widely to identify potential drugs for target-based cancer treatment (Rosenbluth*, et al* 2008, Sanda*, et al* 2009, Stegmaier 2009, Vilar*, et al* 2009). The CMAP enables connection of human disease phenotype with drugs via comparison of gene expression profiles with a large bank of drug expression profiling experiments. This has enabled prediction of drug that may reverse disease characteristics (**Figure 1.10B**). CMAP uses a large reference database of genome-wide expression profiling from the cultured human cancer cell lines under the standard conditions (AML-HL60, prostate cancer-PC3, breast cancer-MCF7, and melanoma-SKMEL5) treated with small molecules and uses a pattern-matching algorithm to enable data mining (Lamb*, et al* 2006). More details on the use of the CMAP application can be found in Lamb *et al* (Lamb*, et al* 2006) and the review by Stegmaier (Stegmaier 2009). In the latest public CMAP release (Build 2), 1309 compounds have been arrayed including large number of FDA (US Food and drug administration)-approved drugs. CMAP is therefore a powerful tool for generating hypotheses that can be tested with experimental approaches (Stegmaier 2009). Successes using this application include identification of specific compounds for acute lymphoid leukaemia and solid tumours (Rosenbluth*, et al* 2008, Sanda*, et al* 2009, Vilar*, et al* 2009).

1.9 Cell line models to study AML

A widely accepted approach for studying the pathogenesis of AML is to use the immortalized myeloid leukaemia cell lines (HL-60, MV4-11, MOLM-13, K-562, Kasumi-1, KG-1, ME-1, and U937). Rucker *et al* reported that the leukaemic cell lines are faithful model systems that can provide insights into the pathogenesis of leukaemogenesis (Rucker*, et al* 2006). Indeed, gene expression profiling shows that they faithfully represent primary AML samples

(Rucker, *et al* 2006). For receptor signaling, it is common to use mouse myeloid cell lines (Ba/F3, 32D, FDCP1 and FDB1) expressing mutant receptors allowing ready dissection of signalling. In this study, we use the murine FDB1 cell line as a cell line model to dissect signaling and downstream events associated with RTK mutants such as FLT3 mutations and the constitutive activated GMR-V449E.

1.9.1 A cell line model of granulocyte-monocyte growth and differentiation, FDB1

A cell line model that has been used extensively in these studies is the FDB1 mouse bi-potential cell line. FDB1 is IL-3 dependent myeloid progenitor cell line (McCormack and Gonda 2000). It is a particularly useful model system to analyse the IL-3 and GM-CSF signalling pathways. This cell line proliferates continuously in murine IL-3 but undergoes complete growth arrest and granulocyte-macrophage terminal differentiation (neutrophils, monocytes and megakaryocytes) in response to murine GM-CSF (McCormack and Gonda 2000). This is unique among mouse myeloid cell lines (i.e. 32D cells differentiate to granulocytes upon treatment with G-CSF and Ba/F3 cells do not undergo differentiation). In FDB1 cells, IL-3 is dominant over GM-CSF and cells continue to proliferate without differentiation in a mixture of these two growth factors. Importantly, this cell line is strictly factor-dependent for survival as upon removal of IL-3, the cells die within 48 hours (Brown, *et al* 2004, McCormack and Gonda 2000). FDB1 thus constitutes a unique model of myeloid differentiation that permits dissection of signalling pathways associated with IL-3 and GM-CSF responses and their contribution to myeloid cell proliferation, survival, and differentiation. Importantly for these studies, activated receptor mutants have been introduced into this cell line resulting in factor-independent growth and survival, and

allowing extensive dissection of signalling and downstream events (Brown*, et al* 2004, Perugini*, et al* 2009).

1.10 Aims of the project

1.10.1 Overall Aims:

To further understand the mechanism of hβc and FLT3 mutations in normal haemopoiesis and AML, and to elucidate new approaches for treatment of AML.

1.10.1.1 Specific aims:

1. To identify differential expressed genes regulated by the critical Tyr^{577} and Ser^{585} in hβc using gene expression profiling, computational analysis and experimental validation.
2. To identify the mechanism of action of FLT3 mutations and the role of *Gadd45a* in AML through computational analysis and experimental validation.
3. To identify drugs that targets the most common and known translocations in AML through computational and experimental validation.

Chapter 2: Regulation of myeloid proliferation, differentiation and survival signals by the human GM-CSF/IL-3/IL-5 common beta chain

2.1 Introduction

Dysregulated cell survival and proliferation are hallmarks of AML. As discussed in **Chapter 1**, these effects can be associated with aberrant receptor signalling leading to constitutive activation of signalling pathways associated with proliferation and survival. For example, autocrine GM-CSF, over-expression of *IL-3RA*, also known as *CD123*, and constitutive serine phosphorylation of the hβc for GMR has been found in myeloid leukaemia (Guthridge*, et al* 2006, Krause and Van Etten 2007, Wang*, et al* 2007). It has also been shown that some subtypes of myeloid leukaemia, such as juvenile myelomonocytic leukaemia (JMML), and chronic myelomonocytic leukaemia (CMML) require GM-CSF to enhance proliferation and survival (reviewed in (Hercus*, et al* 2009)). Recently, Riccioni *et al* showed that the levels of human hβc were selectively high in AML with FLT3 mutations compared to wild-type FLT3 AML (Riccioni*, et al* 2009). The oncogenic capacity of a mutant form of the GMR β chain with the transmembrane V449E mutation (GMR-V449E) has previously been demonstrated. As discussed in **Section 1.6.1.1**, this mutant confers factor-independent proliferation, survival, and a block in differentiation *in vitro* and *in vivo* (Brown*, et al* 2004, McCormack and Gonda 1999, McCormack and Gonda 2000). Moreover, a secondary mutation of an intracellular tyrosine residue Tyr^{577} (Y577) to phenylalanine (Y577F) in GMR-V449E is able to induce AML *in vivo* when cooperated with PML-RARα, but with longer latency than GMR-V449E (see **Section 1.6.1.2**) (Phan*, et al* 2003). This is due to the Y577F mutant cells

is selectively abolished factor-independent proliferation, and with some monocyte differentiation, but not survival in FDB1 cells (Brown*, et al* 2004). Thus, in this system, this key residue provides an important signal associated with cell proliferation and the block in differentiation.

A serine residue proximal to Tyr^{577} (Ser^{585}) in the GMR has been reported to be involved in survival-only signalling (see **Section 1.6.1.2**) (Guthridge*, et al* 2006). Briefly, phosphorylation of the Ser^{585} was observed at low concentrations of GM-CSF, and this was associated with cell survival through PI3K/AKT signalling. In contrast, phosphorylation of the Ser^{585} switch to the Tyr^{577} residue was observed at higher concentrations of GM-CSF, and this associated with cell proliferation and survival through JAK-STAT5 pathway (Guthridge*, et al* 2006).

Given that the Tyr^{577} and Ser^{585} may have distinct roles in proliferation and/or survival, it is now important to characterise the nature of signalling associated with each of these residues and determine the relevance to AML. In this study, we have used the FDB1 cell line expressing the GMR-V449E mutant and determined the gene expression changes downstream of Tyr^{577}. We also analysed the gene expression associated with the Ser^{585} pathway. Connectivity map and pathway analysis was used extensively to identify pathways downstream of these two key residues. A comparison of both the Tyr^{577} and Ser^{585} signatures with AML gene expression profiles shows that AML with *CEBPA* mutations displays significant enrichment for these signalling pathways.

2.2 Materials and methods

2.2.1 Reagents

Recombinant murine IL-3 (mIL-3) and mGM-CSF were produced from Sf21-pIB-mIL-3 and Sf21-pIB-mGM-CSF conditioned media made in-house respectively. Rapamycin (a.k.a Sirolimus), Wortmannin and LY294002 were purchased from Calbiochem. Rapamycin (100 μM), Wortmannin (1 mM) and LY294002 (25 mM) were reconstituted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) as the stock solution and stored at -20^{o}C. 0.1% (v/v) DMSO was used as vehicle control. Propidium Iodide (PI) was purchased from Sigma-Aldrich. 100X Penicillin-Streptomycin-Glutamine (PSG) was purchased from Invitrogen. Iscove's Modified Dulbecco's Medium (IMDM) and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich.

2.2.2 Antibodies

Gr-1 (Ly-6G) and c-FMS (CD115, M-CSFR) mouse monoclonal antibodies and their isotype controls (IgG2b and IgG2a respectively) conjugated with PE were purchased from eBioscience. The anti-FLAG tag M2 primary antibody was purchased from Sigma-Aldrich. The Anti-mouse IgG-FITC secondary antibody was purchased from Beckman Coulter. Monoclonal phospho-Ser^{473} AKT and total AKT antibodies were purchased from Cell Signalling Technology.

2.2.3 Cell lines and culture conditions

FDB1 cells (myeloid suspension cells), FDB1-V449E and FDB1-V449E Y577F were maintained in IMDM supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 1X of PSG and 500 bone marrow unit (BMU)/mL mIL-3. FDB1-V449E and FDB1-V449E Y577F cells were maintained in 1 μg/ml of puromycin (Sigma-Aldrich) and cultured in a humidified incubator at 37°C with 5% CO_2.

2.2.4 Flow cytometry

Cells were harvested and washed with PBS and stained for 30 minutes on ice with the primary conjugated antibody (Gr-1 (1:1000)), c-FMS (1:100) and isotype controls) or unconjugated antibody (FLAG-M2 - 1:1000) in 100 μl of FACS wash (recipe in **Appendix A**). The cells were also incubated with 20 μl of Intragam (CSL) as a blocking Fc receptor antibody together with primary antibody. Subsequently, the cells were washed once with the FACS wash and then centrifuged at 1200 r.p.m for 5 minutes. For unconjugated antibody, supernatant was discarded prior the second layer staining (anti-mouse FITC in 1:100 dilution) for another 30 minutes on ice. Subsequently, the cells were washed with the FACS wash and centrifuged followed by removal of supernatant. The cells were resuspended in 300 μl FACS wash, analysed directly using a flow cytometer Beckman Coulter Epics XL-MCL, or stored in FACS FIX (recipe in **Appendix A**) to be analysed later. The flow data was analysed and generated using Flowjo software (http://www.flowjo.com/).

2.2.5 Differentiation, cell viability, apoptosis and proliferation assays

Cells were washed four times with IMDM without supplement, or PBS, and subsequently seeded at $1x10^5$ cells in 1 ml (24-wells format) and $5x10^3$ cells in 100 μl (96-wells format) of IMDM containing 10% FBS as described above. Cells were cultured in the absence of IL-3 or with 500 BMU/mL IL-3. The cells that expanded to over $1x10^6$ cells per well were split with a 1:10 dilution to a new well with fresh medium containing appropriate growth factor to prevent overgrowth and subsequently cell death. Cell proliferation was assessed in 96-wells format using MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)) assay (CellTiter 96 Aqeous One solution, Promega) as per manufacturer's protocols. To assess cell viability, the percentage of cells excluding trypan blue (Invitrogen) was determined microscopically using a haemacytometer. To assess cell apoptosis, aliquots of cells were stained with Annexin V (BD pharmingen) as per manufacturer's protocols. To assess cell differentiation, aliquots of cell populations were cytocentrifuged at 500 r.p.m onto glass slides and subsequently stained with May-Grünwald Giemsa for morphology assessment. Leukocyte differentiation antigens were also assessed by flow cytometry as described in **Section 2.2.4**. The flow histograms were generated using Flowjo software.

2.2.6 Cell cycle analysis

Cells were washed with PBS and fixed overnight with 70% ethanol at -20^{o}C. Subsequently, cells were spun down and incubated in PBS with 0.1% TritonX-100, 100 μg/ml RNase A and 40 μg/ml of Propidium Iodide (PI) for 30 minutes at room temperature in the dark prior to analysis by flow cytometry using Beckman Coulter Epics XL-MCL. The flow histograms were generated using Flowjo software.

2.2.7 Western blotting

The cells were first washed and starved in the absence of growth factor for 48 hours. Subsequently, the cells were either stimulated with IL-3 for 5 minutes or not stimulated. Then the cell lysates were prepared using Radio-Immunoprecipitation Assay (RIPA) buffer (recipe in **Appendix A**) and the protein quantitation was performed using the BioRad protein assay kit (BioRad) as per manufacturer's protocols. Quantitation analysis was calculated using Bradford assay using Microsoft Excel. Subsequently, 100 μg of proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Brown*, et al* 2004). Secondary antibody conjugated with Horseradish peroxidase (HRP) was used at 1:10000 and ECL chemiluminescent reagents were used for detection of the signal.

2.2.8 Bioinformatics analysis

2.2.8.1 Gene-set enrichment analysis using the Wilcoxon rank sum test

To test for association of published gene data sets with our data set or *vice versa*, we used the non-parametric Wilcoxon rank sum test as implemented in the R statistical program (http://www.r-project.org/). We constructed our reference set of genes based on ranking the log of odd ratio score (Lod) (Smyth 2004) for comparisons of reference-set and test-set. Gene-sets or reference-sets of interest were obtained from published studies. Only $p<0.01$ was considered as statistically significant. A significant result from the Wilcoxon rank sum test indicated that the gene-set of interest displays an association with the indicated reference-set.

2.2.8.2 Microarray dataset re-analysis

The raw CEL files from the previous study by Valk *et al* (Valk*, et al* 2004) were downloaded from NCBI GEO accession number GSE1159. Data was normalized with Robust Multichip Average (RMA) and the average gene expression change for each gene determined in each of the AML subtype groups, relative to the expression level in normal bone marrow mononuclear cells (NBM, n=5). Analysis was performed using linear modelling analysis (LIMMA) (Smyth 2004) to determined differentially expressed genes. The genes were adjusted for multiple testing using Benjamini-Hochberg (BH) methods. Genes that with BH adjusted p-value < 0.05 were considered as significant genes. All the statistical analysis was performed using the R statistical program. Heatmap was generated using MeV software (Saeed*, et al* 2006).

2.2.8.3 Connectivity map, pathway and gene ontology analysis

All the significant up- and down-regulated mouse V449E proliferation signature genes were first mapped to human orthologs that contained human Affymetrix HGU-133A probe-set IDs for each gene using in-house Perl scripts. Subsequently, the mapped Affymetrix probe-set IDs were uploaded to the Connectivity Map version 2 (http://www.broad.mit.edu/cmap). Small molecules or drugs were considered significant based on p-value < 0.05 and a mean connectivity score (>0.4). Protein and/or gene networks and ontology associated with the gene-set were derived using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, http://www.ingenuity.com).

2.2.8.4 Transcription factor prediction using microarray significant gene-set

To predict transcription factor regulation using the microarray gene signature, TFactS (Essaghir*, et al* 2010) was used. Briefly, all the significantly up-regulated and down-regulated genes (false discovery rate (FDR) $p<0.05$) were uploaded to TFactS and results were generated by TFactS and exported to Microsoft Excel. We used the author's recommended settings and only considered transcription factor regulation with Benjamini-Hochberg adjusted FDR p-value< 0.05 as statistically significant.

2.2.9 Statistical analysis

The statistical significance of various parameters was evaluated using the 2-tailed student's t-test otherwise indicated in the text. Only $p < 0.05$ was considered as statistically significant. The data is presented as mean ± standard error measurement (SEM) for at least 2 independent experiments. The figures including the dose-response curve were generated using GraphPad Prism version 5.0 (GraphPad Software, http://www.graphpad.com).

2.3 Results

2.3.1 V449E Y577F cells fail to proliferate but maintain viability

To dissect the nature of the proliferation pathway in the V449E mutant, we aimed to compare gene expression profiles between the parental V449E FDB1 cells and cells expressing the V449E Y577F second-site mutant which lacked the proliferation response. Expression of GMR-V449E or GMR-V449E Y577F (VY577F) in FDB1 cell populations was confirmed by detection of the anti-M2 FLAG epitope-tag using flow cytometry. As shown in **Figure 2.1A-B**, both cell populations expressed the receptors at similar levels. We first examined the

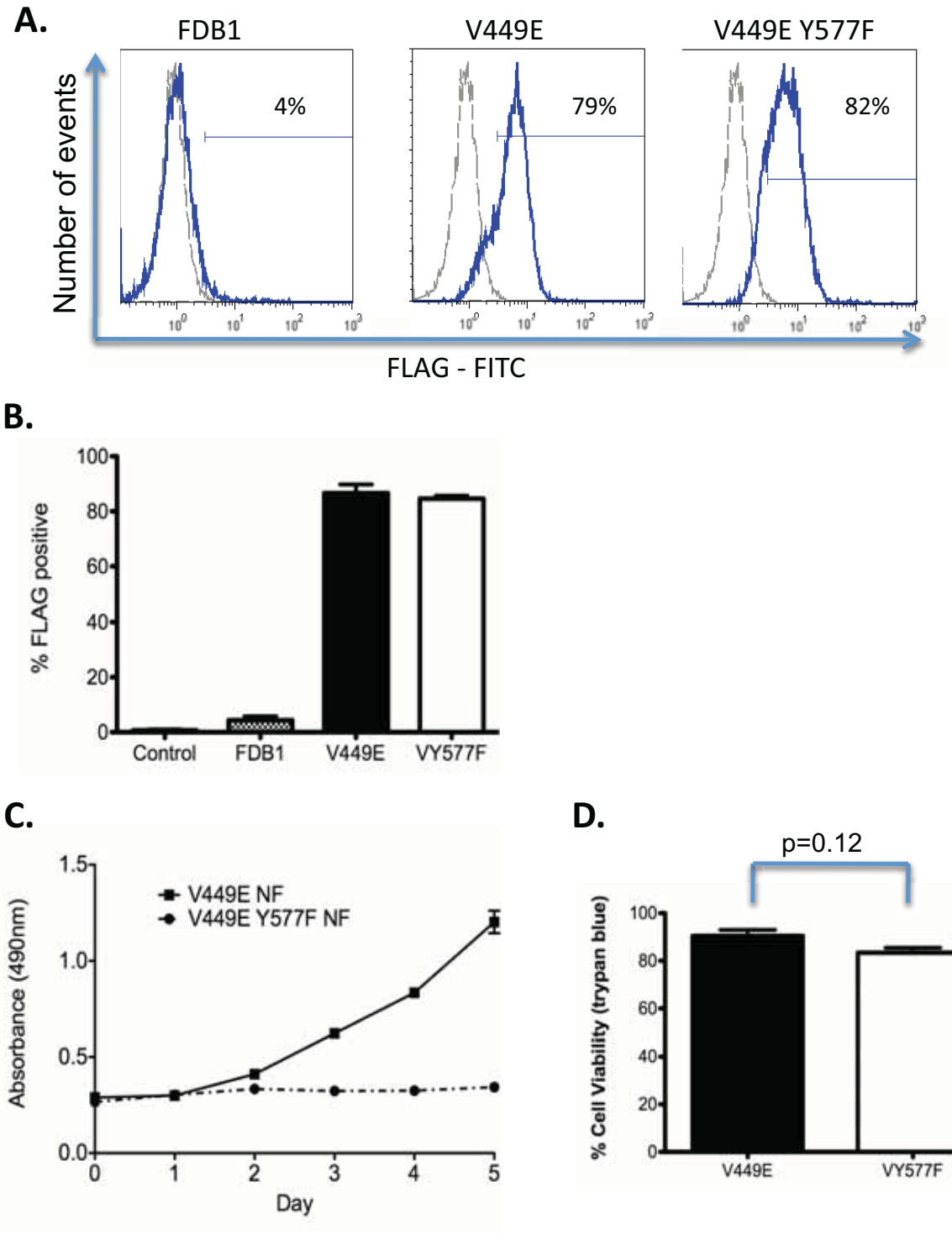


Figure 2.1. Effect of the GMR-V449E Y577F mutation on factor independent proliferation and viability in FDB1 cells. A. Cell surface expression of FLAG-tagged GMR-V449E and GMR-V449E Y577F receptors by flow cytometry after staining cells with an anti-FLAG M2 antibody followed by an anti-mouse FITC conjugated antibody. The control was the the cells stained with secondary antibody conjugated with FITC only. **B**. The summary of FLAG positivity in GMR-V449E and GMR-VY577F cells. **C.** FDB1 V449E and V449E Y577F cells were cultured without factor for 5 days and the growth was measured daily by MTS assay. **D.** The cell viability of V449E and V449E Y577F (VY577F) cultured without growth factor measured by trypan blue exclusion on day 5. Data represent mean ± SEM for at least 2 independent experiments. The statistical significance was determined by two-tailed student's t-test.

differences of these V449E and VY577F cell populations in cell proliferation assays. The MTS assay was used to examine the proliferation of each cell population over 5 days. As shown in **Figure 2.1C**, only V449E FDB1 cells grew exponentially in the absence of growth factor. As there was no cell growth in the VY577F cell populations, we then assessed cell viability. As shown in **Figure 2.1D**, there was no significant cell viability difference (p=0.12) at day 5 when VY577F cells were compared to V449E cell populations in the absence of growth factor. These results were consistent with those reported previously for these FDB1 cell populations (Brown*, et al* 2004).

Since there was no significant difference in cell viability, we next determined whether the lack of proliferation of the VY577F cell population was due to growth arrest. We assessed V449E and VY577F cell populations cultured without growth factor or with IL-3 for cell cycle distribution using PI staining. This analysis revealed that a significantly larger proportion of V449E Y577F cells accumulated in the G1 phase of the cell cycle at day 3 and day 5 compared to V449E cell populations (**Figure 2.2A-B**). There was no significant difference in cell cycle distribution between V449E and VY577F cell populations cultured in IL-3 as shown in **Figure 2.2B**.

We next used flow cytometry to analyse two myeloid markers on these cell populations (Gr-1 and c-FMS). Gr-1 is expressed by granulocytes and is also transiently expressed by monocytes during their development (Fleming*, et al* 1993). On the other hand, c-FMS (M-CSFR) is expressed by the monocytic lineage only (Sudo*, et al* 1995). As shown in **Figure 2.3A**, we found that in the absence of growth factor, the VY577F cell population displayed higher Gr-1 and c-FMS expression at day 3 compared to V449E cell populations. However,

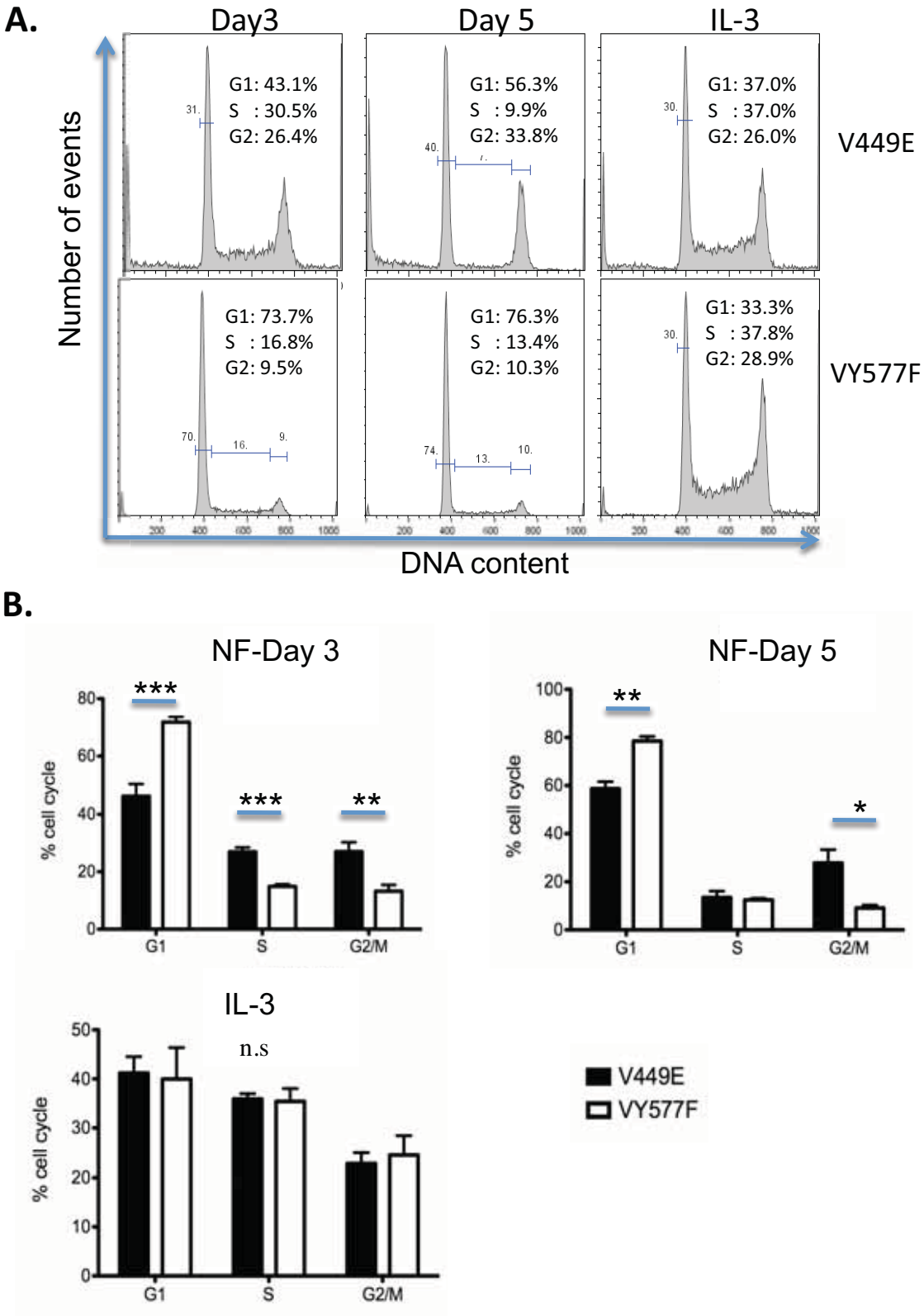


Figure 2.2. Cell cycle analysis of FDB1 cells expressing V449E or V449E Y577F. **A**. Cells were cultured in the absence of growth factor and the effect on cell cycle was assessed by PI staining and flow cytometric analysis at day 3 and day 5. Cells cultured with IL-3 were included as a comparison. **B.** The percentage of cells in the G1, S and G2/M phases under the condition described in part A were quantified for at least 2 independent experiments. Data represent mean ± SEM for at least 2 independent experiments. ***p<0.001, **p<0.01, *p<0.05 determined by two-tailed student's t-test compared to GMR-V449E. n.s indicates not significant.

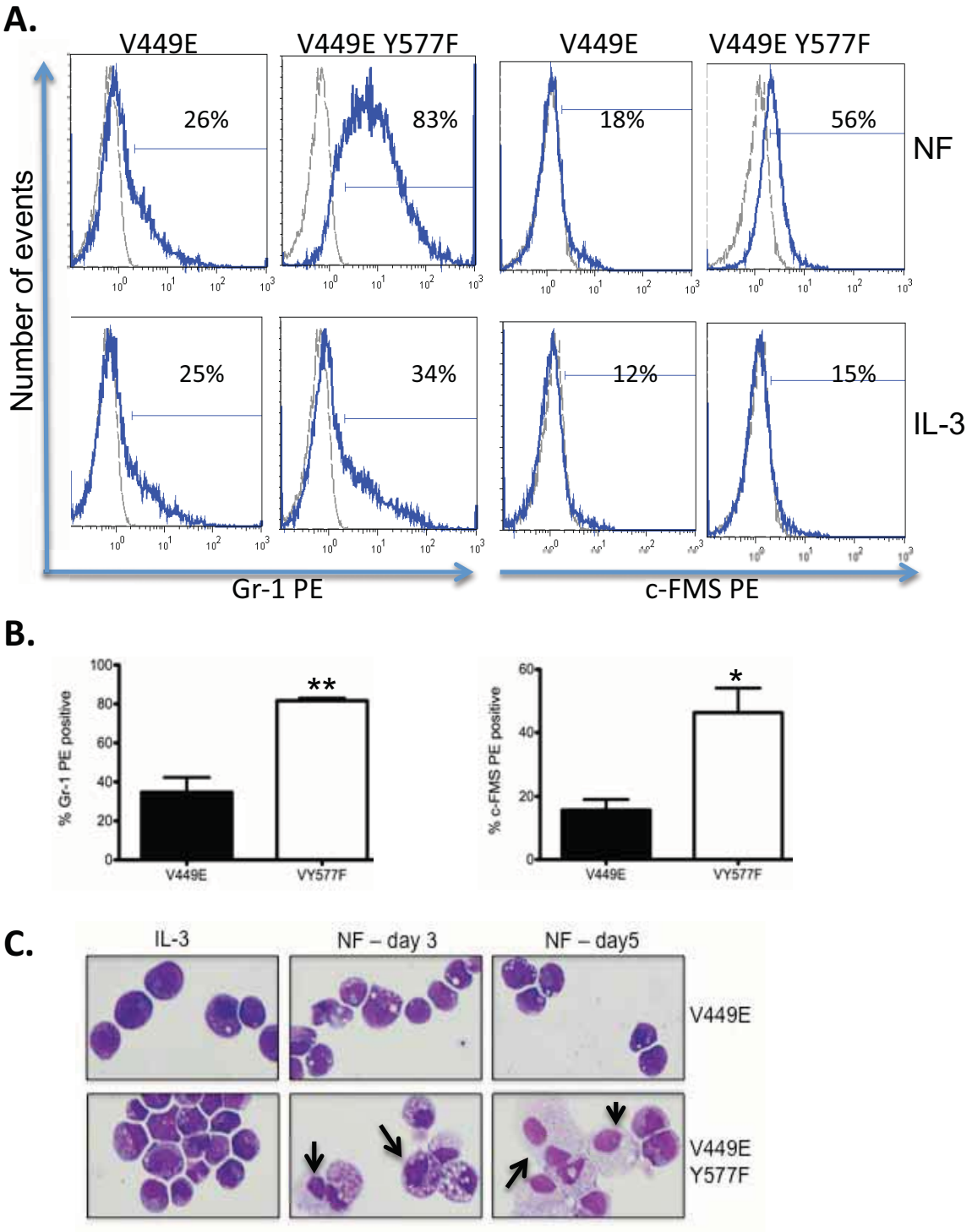


Figure 2.3. Expression of cell surface myeloid differentiation markers and morphology on FDB1 V449E and V449E Y577F cells. A. Cell surface expression of Gr-1 (granulocytes) and c-FMS (monocyte/macrophage) was assessed on GMR-V449E and GMR-V449E Y577F FDB1 cells at day 3 in the absence of growth factor by flow cytometry after staining cells with an anti-Gr-1 and anti-c-FMS antibody conjugated with PE antibodies respectively. The control was the the cells stained with isotype control conjugated with PE. Cells cultured with IL-3 were included as a comparison. **B**. The expression of Gr-1 and c-FMS expression under the condition described in part A were quantified for at least 2 independent experiments. **C.** The cell morphology was assessed under the conditions described in part A by cytocentrifugation and staining with May-Grünwald Giemsa. Data represent mean ± SEM for 2 independent experiments. **p<0.01, *p<0.05 determined by two-tailed student's t-test compared to GMR-V449E. Original magnification 200x. Arrows indicates monocytes and macrophages.

when VY577F cells were cultured with IL-3, Gr-1 and c-FMS expression was similar to the V449E cell population as shown in **Figure 2.3A**. The summary for Gr-1 and c-FMS expression of VY577F cells compared to V449E cells in the absence of growth factor is shown in **Figure 2.3B**. This surface marker expression is consistent with the microscopic analysis of the VY577F populations at day 3 and day 5 which showed cells morphologically identified as macrophages (**Figure 2.3C**). The high Gr-1 expression in the VY577F mutant is possibly due to the transient expression on the monocytes. These results are also consistent with the previous report by Brown et al (Brown*, et al* 2004) and indicate that the lack of proliferation signalling observed with VY577F mutant is associated with G1 cell cycle arrest and macrophage differentiation.

2.3.2 The V449E Y577F signature: A proliferation-associated signature

Given that the VY577F cells selectively lose proliferation while viability is maintained, gene expression profiling was performed to determine the gene signature associated with the Tyr^{577} proliferation signal and block in differentiation. We compared the gene expression in the VY577F and V449E FDB1 cells at 0 and 72 hr without growth factor using the approach previously described (Brown*, et al* 2006). From this analysis, we identified 419 genes (461 probes) for which there was significant up- or down-regulation in the VY577F mutant relative to V449E at 0 and 72 hr (FDR adjusted p-value < 0.05). This gene list was then referred to as the "V449E proliferation signature". **Figure 2.4** shows a heatmap representation of all the significant genes (n=419) and their differential expression between V449E and VY577F cells at 72 hr compared to 0 hr.

Figure 2.4. Differentially expressed GMR-V449E Y577F genes. Heatmap shows the mean $\log_2$ expression of 419 genes that are significantly differentially expressed (FDR p-value < 0.05) in GMR-VY577F FDB1 cells compared to GMR-V449E FDB1 cells. V449E indicates the comparison between 72 hr to 0 hr in V449E cells. VY577F indicates the comparison between 72hr to 0hr in VY577F FDB1 cells. Red indicates up-regulated expression and Green indicates down-regulated expression. NA indicates unannotated genes.

To determine the gene functions and categories that were affected by the VY577F mutant, the full gene list (n=419) was used to query the Ingenuity pathway analysis (IPA) software package (http://www.ingenuity.com/). As shown in **Table 2.1**, we found that the following categories: inflammatory response, cancer, haematological system development and function, and haemopoiesis were significantly enriched in this signature. Additionally, the V449E proliferation signature was also significantly enriched for genes associated with cell death, cell-to-cell signalling and interaction, growth and proliferation, morphology, and cell cycle as shown in **Table 2.1**. This analysis is consistent with the experimental data in the previous section showing an important role of the Tyr^{577} residue in regulating proliferation, morphology, and cell cycle arrest. We also used IPA to perform canonical pathway enrichment analysis using the V449E proliferation signature. As shown in **Table 2.1**, one of the top pathways is related to AML signalling. There are 6 genes (*CSF1R, CSF2RA, CSF3R, EIF4EBP1, RAC2, and STAT5A*) associated with AML signalling (**Figure 2.5**). Additionally, several other pathways such as 14-3-3-mediated signalling, apoptosis signalling, PI3K-AKT signalling, GM-CSF signalling and Wnt-β-catenin signalling were significantly enriched in this proliferation signature. Of note, this canonical pathway analysis indicated that the genes (*BCL2L1, EIF4EBP1, HLA-B, HSP90AB1, ITGA4 and RAC2*) were significantly (p=0.03) linked to an AKT network that is a key mediator downstream of the PI3K pathway and has been previously linked to proliferation and survival signalling in leukaemia (Martelli*, et al* 2009) (see **Section 1.7.1**). Of note, *EIF4EBP1* (regulator of cap-dependent translation) is down-regulated in the VY577F FDB1 mutant compared to the V449E mutant. *EIF4EBP1* is a key downstream target of oncogenic activation of the PI3K/AKT/mTOR and MAPK/ERK pathways as inhibition of both AKT and ERK suppress the phosphorylation of EIF4EBP1 and tumour growth *in vivo* (Dowling*, et al* 2010, She*, et al* 2010). These results suggest that the GMR-V449E mutant mediates proliferation and the block in differentiation via PI3K-

Table 2.1. Enrichment of gene ontology and canonical pathways associated with the GMR-V449E proliferation signature.

Name	p-value
Disease and Disorders	
Inflammatory response	$7.1x10^{-9}$ - $6.8x10^{-3}$
Cancer	$3.2x10^{-8}$ - $7.7x10^{-3}$
Molecular and cellular functions	
Cell death	$1.4x10^{-8}$ – $7.9x10^{-3}$
Cell-to-cell signalling and interaction	$4.9x10^{-8}$ – $7.9x10^{-3}$
Cellular growth and proliferation	$9.3x10^{-6}$ – $7.8x10^{-3}$
Cell morphology	$4.0x10^{-4}$ – $7.9x10^{-3}$
Cell cycle	$2.5x10^{-3}$ – $6.9x10^{-3}$
Physiological system and development and function	
Haematological system development and function	$2.1x10^{-8}$ – $7.8x10^{-3}$
Haemopoiesis	$7.6x10^{-6}$ – $5.9x10^{-3}$
Canonical pathways	
AML signalling	$3.5x10^{-3}$
14-3-3-mediated signalling	$2.4x10^{-2}$
Apoptosis signalling	$2.7x10^{-2}$
PI3K/AKT signalling	$3.3x10^{-2}$
GM-CSF signalling	$3.6x10^{-2}$
Wnt/β-catenin signalling	$4.8x10^{-2}$

419 genes were queried to IPA analysis and significant enrichment for gene ontology and canonical pathways were identified based on p-value < 0.05.

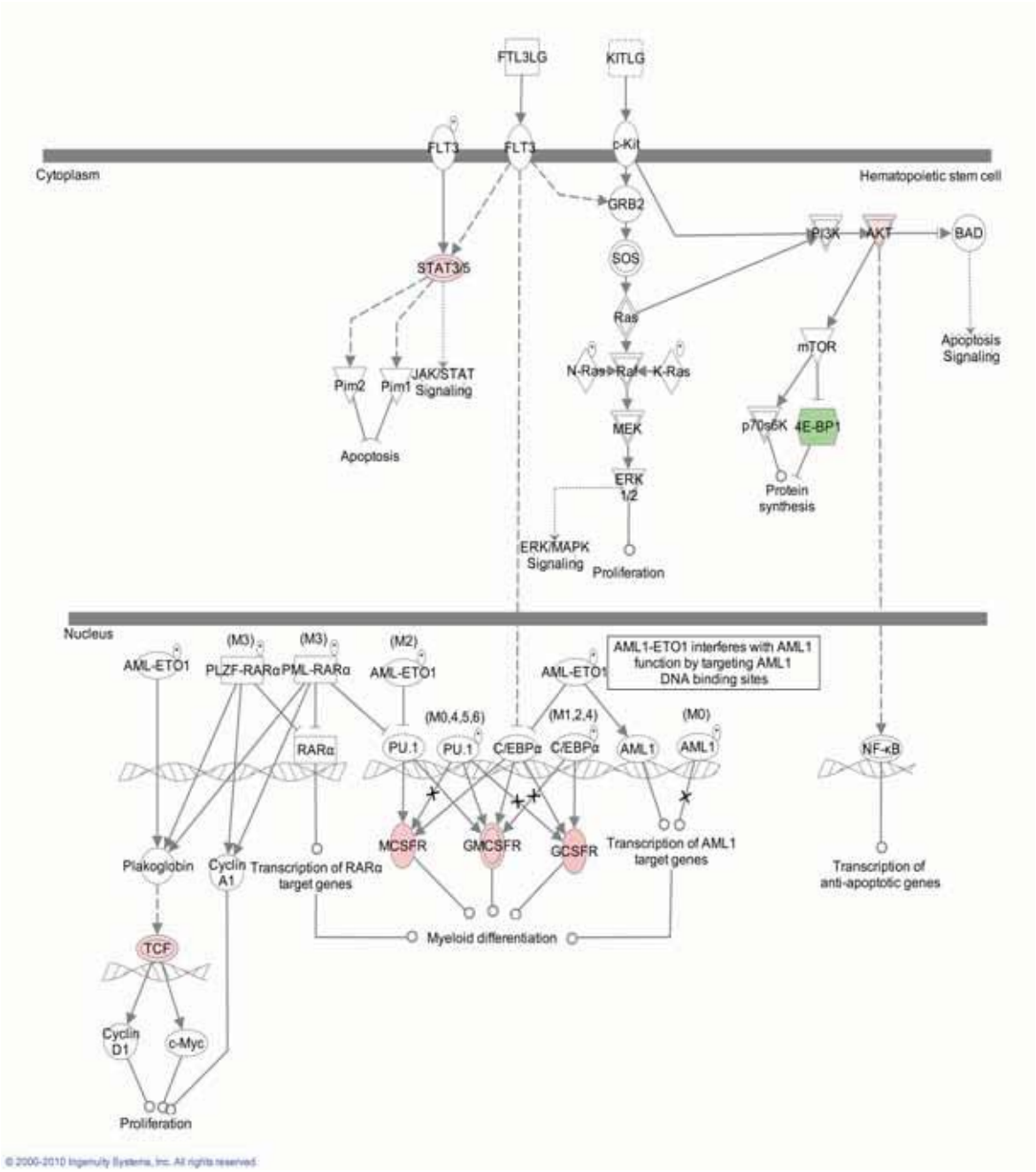


Figure 2.5. Significant AML signalling pathways associated with VY577F mutant cells. The proliferation signature was uploaded to IPA and identified the AML signalling pathway as one of the significant associations. Red indicates genes up-regulated in the proliferation signature and green indicates genes down-regulated in the proliferation signature.

AKT activation involving the Tyr^{577} residue. Additionally, the top networks that are associated with this proliferation signature are (1) Gene expression, cellular growth and proliferation, haematological system development and function, (2) Cancer, cell cycle, carbohydrate metabolism. These networks are shown in **Figure 2.6**.

In addition to pathway analysis, we also performed transcription factor prediction analysis using this proliferation gene signature. We used TFactS as described by Essaghir *et al* (Essaghir*, et al* 2010) as this bioinformatic tool has successfully predicted specific transcription factors regulated by PDGFB using a microarray gene signature, and these have subsequently been confirmed experimentally. We queried our proliferation gene signature (n=419) using TFactS and identified 91 transcription factors that were predicted to be significantly differentially regulated between VY577F and V449E. The top 10 transcription factors ranked based on FDR p-value are shown in **Table 2.2**. Consistent with this pathway mediating the block in differentiation, several key transcription factors of the GM lineage were detected. These include *SPI1 (PU.1), CEBPA*, and *EGR1* (early growth response protein 1). Detection of transcription factor (TCF)-β-catenin is consistent with the result above obtained from pathway analysis using IPA (see **Table 2.1**). To determine the direction of regulation of β-catenin in the VY577F mutant cells, we used two previously reported gene-lists of chromatin immunoprecipitation (ChIP) targets for *TCF7L2/TCF4* and *CTNNB1* (Hatzis*, et al* 2008, Yochum*, et al* 2007). As shown in **Table 2.3**, the expression of *Tcf7l2, Myb and Sox4* is up-regulated in the VY577F mutant while the negative regulator of Wnt-β-catenin pathway, *Sfrp2* is down-regulated. The promoter of *Sfrp2* is frequently methylated in 30% of AML patients, hence activating Wnt target genes and inreasing nuclear localisation of β-catenin (Valencia*, et al* 2009). This result suggests that β-catenin activation is

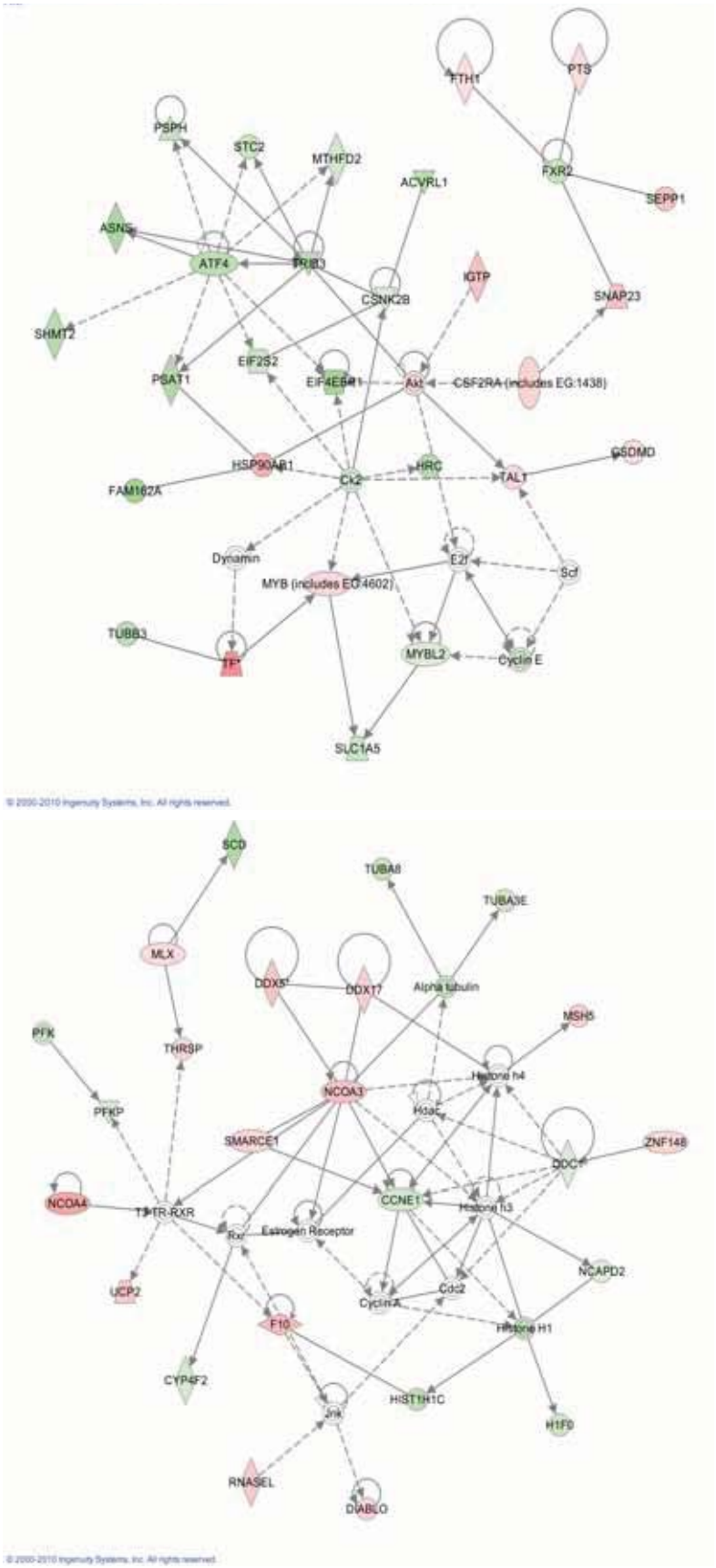


Figure 2.6. Proliferation signature network. Top networks generated by IPA that are significantly associated with the GMR-V449E proliferation signature. Red indicates genes up-regulated in the proliferation signature and green indicates genes down-regulated in the proliferation signature.

Table 2.2. Prediction of transcription factor regulation associated with the GMR-V449E proliferation signature.

Transcription factor prediction	FDR p-value (Benjamini-Hochberg)
ATF3	$5.5x10^{-4}$
SPI1 (PU.1)	$5.5x10^{-4}$
EGR1	$1.7x10^{-3}$
NFκB	$2.2x10^{-3}$
TCF/β-catenin	$2.8x10^{-3}$
WT1	$3.3x10^{-3}$
CREB1	$4.4x10^{-3}$
ETS1	$5.0x10^{-3}$
CEBPA	$7.7x10^{-3}$
MYC	$9.9x10^{-3}$

419 significant genes were queried to TFactS and transcription factors with Benjamini-Hochberg adjusted $p<0.05$ were considered statistically significant. The top 10 transcription factors associated with the GMR-V449E proliferation signature are shown.

Table 2.3. Identification of TCF7L2 and CTNNB1 target genes in VY577F gene list.

Gene Symbol	Direction	Fold Change (log2)	FDR p-value	β-catenin targets by SACO	TCF7L2/ TCF4 targets by SACO
Sox4	UP	0.808	0.008	-	+
Tgfbr2	UP	0.637	0.009	-	+
Smarce1	UP	0.542	0.035	-	+
Myb	UP	0.504	0.019	-	+
Tcf7l2	UP	0.489	0.029	-	+
Tal1	UP	0.465	0.021	-	+
Masp2	UP	0.361	0.039	+	-
H1f0	DOWN	-0.532	0.028	-	+
Bcl2l1	DOWN	-0.550	0.033	+	+
Sfrp2	DOWN	-1.015	0.006	-	+
Etv5	DOWN	-1.057	0.001	-	+
St6gal1	DOWN	-1.387	0.000	+	-

Up indicates the expression in VY577F is higher than in GMR-V449E and down indicates *vice-versa*. Both studies were performed using ChIP-CHIP microarray. SACO, serial analysis of chromatin occupancy.

suppressed by Tyr^{577} and the VY577F mutant cells are activating the Wnt-β-catenin pathway. Thus, in the context of VY577F mutant cells, the activation of Wnt-β-catenin pathway may be supporting survival and/or macrophage differentiation.

We also observed from the TFactS result that *WT1, MYC* and *NFκB* regulation are significantly enriched in this proliferation signature consistent with these transcription factors being required for growth or contributing to the differentiation block. For example, constitutive activation of PI3K-AKT-mTOR down-regulates PP2A (protein phosphatase 2) and GSK3β (glycogen synthase kinase 3 beta) which normally function to dephosphorylate Ser^{62}/Thr^{58} of Myc which are required for ubiquitination. Thus these pathways promote stabilisation of Myc which is a critical growth-promoting oncogene (Gustafson and Weiss 2010). WT1 and NFκB have also been reported to play an important role in promoting leukaemogenesis (Grandage*, et al* 2005, Grimwade and Hills 2009).

2.3.3 The Connectivity Map (CMAP) as a tool to explore the nature of the V449E proliferation signature

The Connectivity map (CMAP) and its use as a tool for drug discovery and pathway identification have been described in **Chapter 1 Section 1.8.2**. Recently, CMAP has been updated to version 2 with more compounds and FDA-approved drugs (1309 compounds). We used CMAP to identify compounds that are predicted to selectively inhibit proliferation and release the block in differentiation induced by the Tyr^{577} pathway. Briefly, all the significant up- and down-regulated genes in the proliferation signature (419 mouse genes) were first mapped using in-house Perl scripts to the human orthologs that also contained human

Affymetrix HGU-133A probeset IDs. There were 443 (237 up and 206 down) probesets mapped across to the human Affymetrix HGU-133A probe-set IDs and subsequently all of these probes were uploaded to the CMAP v2. CMAP ranks each of the drug-treated signatures based on fold change differential expression of each gene when compared to vehicle control arrays. Subsequently, CMAP determines the enrichment of the user-provided (V449E proliferation signature) gene-set in each drug gene expression profile to obtain a p-value using Kolmogorov-Smirnov test statistics (Lamb*, et al* 2006). By using this analysis, we identified 12 compounds that are significant associated with the Tyr^{577}-linked proliferation signature based on the criteria as described in the **Section 2.2.8.3** ($p<0.05$ and connectivity score mean > 0.4). **Table 2.4** shows all the 12 significant drugs or compounds ranked on p-value. Notably, several of the most significant matches were PI3K-AKT-mTOR pathway inhibitors; LY294002 (PI3K), Wortmannin (PI3K), Quinostatin (PI3K) (Yang*, et al* 2007) and Rapamycin (mTOR) as shown in **Figure 2.7A**.

The CMAP interface provides a bar-plot and summary table displaying enrichment of the user-provided gene-set to each of the drug profiles across 1309 compounds. The bar-plot consists of three colours (red - positive correlation to the input signature, grey - no correlation, and green - negative correlation). Bar plots for each of the PI3K-AKT-mTOR pathway inhibitors are shown in **Figure 2.7B**. LY294002, Rapamycin and Wortmannin are strong significant matches based on p-value. This enrichment associated with PI3K and mTOR strongly suggests that the signal maintaining proliferation and the block in differentiation in V449E involves the PI3K-AKT-mTOR pathway as shown in **Figure 2.7C**. This is also consistent with the result observed from interactive pathway analysis using IPA as described previously (see **Section 2.3.2**). Moreover, this analysis predicts that inhibition of PI3K-AKT-mTOR pathway with LY294002, Rapamycin, Wortmannin or Quinostatin in

Table 2.4. Top 10 Connectivity Map compounds identified using V449E proliferation signature.

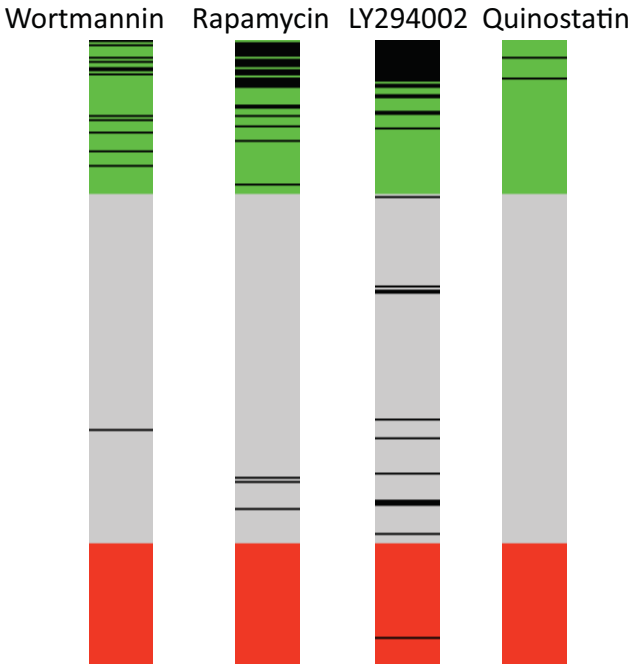
Compounds	Mean	n	p-value	Target
wortmannin	0.532	18	0	PI3K
rapamycin	0.525	44	0	mTOR
LY-294002	0.497	61	0	PI3K
gramine	0.565	4	0.00004	indole alkaloid
Prestwick-559	0.520	3	0.0029	unknown
ornidazole	0.431	5	0.0046	protozoan infections treatment
carcinine	0.434	4	0.0057	Histamine H3 receptor target
quinostatin	0.575	2	0.0074	PI3K
sulfadoxine	0.441	3	0.012	Malaria treatment
amoxicillin	0.405	4	0.029	Bacterial infection treatment
antazoline	0.412	4	0.030	Anti-histamine
menadione	0.503	2	0.046	Vitamin K3

Compounds or drugs were based on positive mean score of 0.4 cutoff and p-value < 0.05. The compounds were ranked by p-value. Mean indicates the connectivity score for correlation with the query gene-expression and n indicates the number of experiments performed using the indicated compounds.

A.

Rank	Compounds	Mean	n	p-value	Target
1	Wortmannin	0.532	18	0	PI3K
2	Rapamycin	0.525	44	0	mTOR
3	LY294002	0.497	61	0	PI3K
8	Quinostatin	0.575	2	0.00742	PI3K

B.



C.

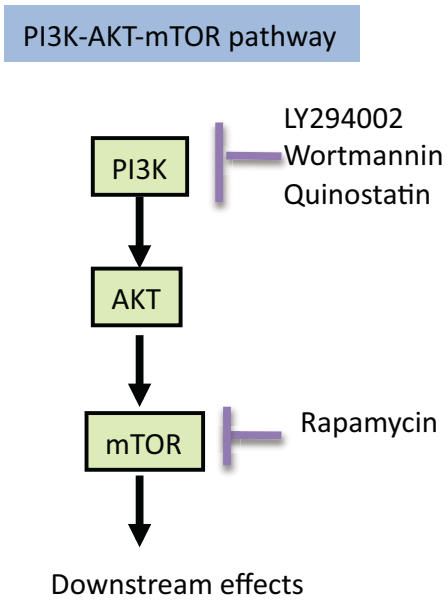


Figure 2.7. Differential activation of the PI3K pathway by V449E and VY577F. **A.** Significant PI3K and mTOR targeted drugs were identified by CMAP based on p-value < 0.05 and positive connectivity score of > 0.4. The list was ranked by p-value. Mean indicates the connectivity score for correlation with the query gene-expression and n indicates the number of experiments performed using the indicated compounds. **B.** Bar plot for each PI3K and mTOR targeted drugs. For more details of bar-plot, please see text. **C.** Summary of PI3K and mTOR pathway that is targeted by the CMAP identified drugs.

FDB1 cells expressing V449E would mimic the VY577F mutant. Thus, we next tested these findings from the CMAP and IPA interactive pathway analysis experimentally.

2.3.4 Experimental validation of CMAP results

Based on the CMAP results shown in **Section 2.3.3**, we predicted that there would be AKT activation in the V449E expressing FDB1 cells which would be lost in the VY577F FDB1 cell populations. We used western blot analysis to determine the level of AKT activation as determined by phosphorylation of Ser^{473}. The lysates were prepared from cell populations grown in the absence of growth factor for 48 hours, or in cells stimulated with IL-3 for 5 minutes. The V449E mutant displays low AKT Ser^{473} compared to the other cell lines such as FIΔ mutant which act predominantly via activation of AKT and IκB (Perugini*, et al* 2010). However, as shown in **Figure 2.8A**, 48 hours following growth factor withdrawal, we could detect phosphorylation of Ser^{473} of AKT in V449E cell populations and this was clearly reduced in the VY577F FDB1 cell population. When these cell lines were stimulated with IL-3 for 5 minutes, both showed robust activation of AKT indicating that neither of the cell lines had a deficiency in AKT activation. These findings were therefore consistent with the results obtained from CMAP and IPA analysis. We conclude that the V449E mutant activates AKT (albeit at a lower level than the other activated hβc mutant, FIΔ) with mutation of Tyr^{577} resulting in loss of AKT activation associated with loss of proliferation, and partial removal of the differentiation block.

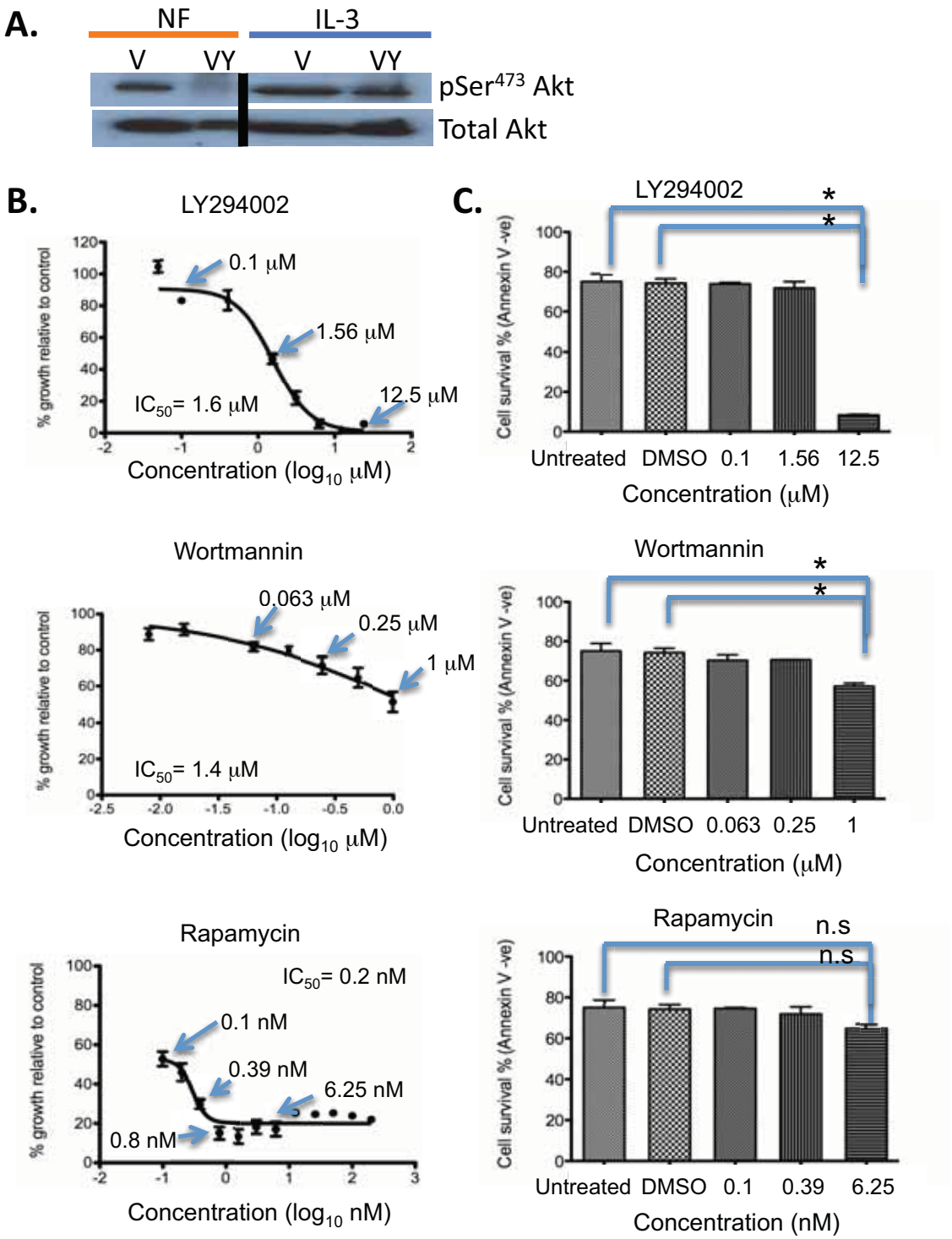


Figure 2.8. Inhibition of cell proliferation and survival in FDB1 cells expressing GMR-V449E related to PI3K-AKT-mTOR network. A. AKT activation (phospho Ser[473]) was assessed by western blot analysis in FDB1 expressing GMR-V449E (V) and VY577F (VY) at 48 hours in the absence of growth factors (NF) or stimulated with IL-3 for 5 minutes (IL-3). The Effect on PI3K and mTOR inhibitors on FDB1 GMR-V449E **B.** cell proliferation and **C.** cell survival cultured without growth factor were treated with the indicated concentrations of PI3K inhibitors: LY294002, Wortmannin, and mTOR inhibitor: Rapamycin. Cell growth relative to vehicle control (DMSO) was measured at day 5 using MTS assay. Cell growth is plotted. Data represent mean ± SEM for 2 independent experiments. Cell survival on GMR-V449E cells in the absence of growth factor was assessed by Annexin V staining using flow cytometry at day 3 with three different concentrations of PI3K and mTOR inhibitors. Data represent mean ± SEM of duplicate experiments. *p<0.05 determined by two-tailed student's t-test.

2.3.5 Treatment of GMR-V449E cells with compounds identified from the CMAP analysis

2.3.5.1 Treatment with the PI3K-AKT-mTOR pathway inhibitors

The CMAP analysis above predicts that inhibition of the PI3K-AKT-mTOR pathway in cells expressing the V449E mutant would result in a similar outcome to that seen with the Y577F second-site mutation i.e. a loss of proliferation associated with GM differentiation. We used the commercially available pathway inhibitors: LY294002 (PI3K), Wortmannin (PI3K) and Rapamycin (mTOR) to determine the effect on V449E FDB1 cell proliferation, apoptosis, cell cycle arrest and myeloid differentiation in the absence of growth factor.

We first examined the effects on cell growth in FDB1 cells expressing V449E. Cells were treated with inhibitors and growth was measured using the MTS assay for a 5-days period. As shown in **Figure 2.8B**, treatment with the PI3K inhibitor LY294002 in the range from 0.05 μM to 25 μM resulted in a dose-related inhibition of growth for the V449E FDB1 cell populations (in the absence of growth factor) at day 5. The IC_{50} for LY294002 on V449E FDB1 cell population was 1.6 μM (95% CI: 1.28-1.96 μM). The concentrations of LY294002 that were used in CMAP experiments were 0.1 μM and 10 μM which both are within the range that we used in this experiment.

In the case of the PI3K inhibitor, Wortmannin, while a dose-response was evident in the concentration range from 0.008 μM to 1 μM, none of these concentrations were able to

reduce the cell growth by greater than 50% compared to DMSO control (see **Figure 2.8B**). The highest concentration of Wortmannin (1 μM) in this study resulted in a 48% growth inhibition at day 5 with the predicted IC_{50} of Wortmannin calculated at 1.4 μM (95% CI: 0.8-2.5 μM). The concentration of Wortmannin used in CMAP was 0.01 μM which in our experiments resulted in an 11% reduction in cell growth. This reflects the sensitivity of gene expression in detecting cellular changes. However, at the 24 hour time point, we observed a larger effect on apoptosis with a significant 30% reduction in Annexin V negative cells at 1 μM, but there is no significant changes in other concentrations (data not shown).

Treatment with the mTOR inhibitor, Rapamycin, in the concentration range from 0.1 nM to 200 nM resulted in a pronounced growth inhibition effect as shown in **Figure 2.8B**. The IC_{50} of Rapamycin was 0.2 nM (95% CI: 0.1-0.3 nM) with the concentration between 0.1 nM and 0.2 nM sufficient to result in 50% or more growth reduction. The concentration of Rapamycin used in CMAP experiments was 100 nM which in our experiment resulted in 80% inhibition of cell growth. There was no further growth inhibition beyond the concentration of 0.8 nM as shown in **Figure 2.8B**.

2.3.5.2 Effects of the pathway inhibitors on survival of V449E FDB1

Since there was evidence of the pathway inhibitors reducing cell growth, we subsequently determined whether this growth inhibition effect is associated with apoptosis. Three different concentrations were used to treat FDB1 cells expressing V449E in the absence of growth factor as indicated in **Figure 2.8C** and the level of apoptosis determined by Annexin V staining at day 3. As shown in **Figure 2.8C**, a concentration of 12.5 μM LY294002, resulted

in a dramatic reduction in cell survival by more than 90% compared to DMSO or untreated control. This suggested that the reduced growth observed at this concentration of LY294002 was largely due to apoptosis. The other two lower concentrations (0.1 and 1.56 μM) did not alter the levels of apoptosis significantly compared to DMSO or untreated control at day 3 (**Figure 2.8C**). At a concentration of 1.56 μM, cell growth was inhibited by 50% without a significant induction of apoptosis. Thus, treatment with LY294002 at the concentration of 1.56 μM closely mimicked the effect of the VY577F mutation. Wortmannin resulted in only a small (18%) but significant reduction in cell survival at the highest concentration (1 μM) as shown in **Figure 2.8C**. The 48% reduction in cell growth at this concentration may therefore be associated with inhibition of both survival and proliferation.

Importantly, treatment of V449E FDB1 cells with Rapamycin had a dramatic effect on cell growth (see **Figure 2.8C**), but did not significantly increase apoptosis at all of the 3 concentrations (0.1, 0.39 and 6.25 nM) tested at day 3 (**Figure 2.8C**). The two highest concentrations inhibited cell proliferation by more than 70% without increasing Annexin V stained. Thus, V449E FDB1 cell populations treated with Rapamycin show strikingly similar features to the VY577F FDB1 cell populations.

2.3.5.3 Effect of pathway inhibitors on cell cycle status of V449E FDB1 cells

We next determined the contribution of cell cycle arrest to growth inhibition by the pathway inhibitors. We examined the cell cycle distribution using PI staining of V449E FDB1 cells treated with LY294002, Wortmannin or Rapamycin with 3 different concentrations at day 3. At a concentration of 1.56 μM LY294002, G1 cell cycle arrest was significantly induced as

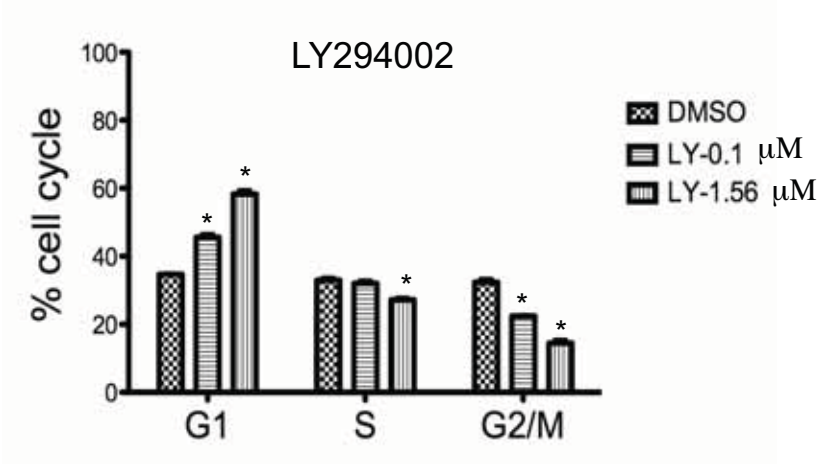
shown in **Figure 2.9A**. The assay was not performed for the LY294002 concentration of 12.5 μM due to the low viability. Approximately 60% of the cells treated with LY294002 1.56 μM accumulated in G1 phase (**Figure 2.9A**). Additionally, at this concentration, there was a significantly lower percentage of S and G2/M cells compared to the DMSO control. Thus, the PI3K inhibitor LY294002 induces G1 arrest consistent with a role of the PI3K pathway in proliferation. For V449E FDB1 cells treated with Wortmannin, at 1μM, there is significant accumulation of S and G2/M phase (**Figure 2.9B**). However, the effect is not as great as treatment with LY294002. The differential effects of LY294002 and Wortmannin are discussed further in the **Discussion Section 2.4** of this chapter.

Surprisingly for V449E FDB1 cells treated with Rapamycin, which display a large and significant reduction in cell proliferation without apoptosis, we observed only a slight but significant increase in cells in G1 phase and a slight decrease in cells in G2/M phase at 0.39 nM and 6.25 nM (**Figure 2.9C**). Taken together with the apoptosis data, this suggests that the reduction of cell growth with this inhibitor is only in part due to cell cycle arrest.

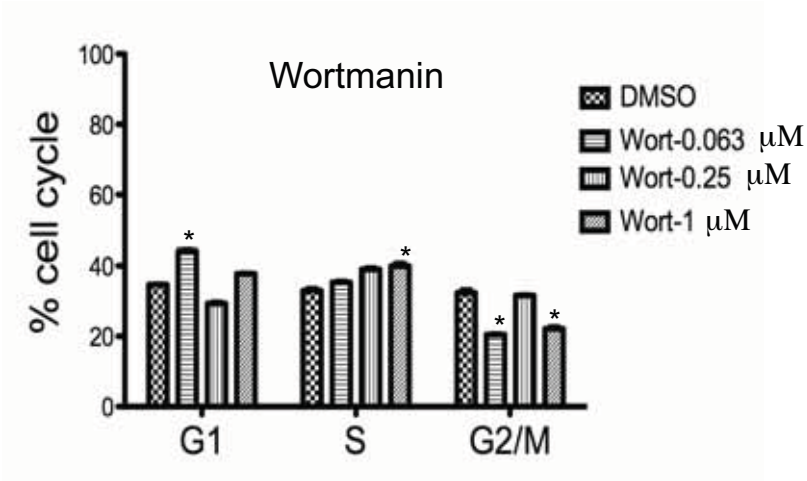
2.3.5.4 Effects of pathway inhibitors on myeloid differentiation of V449E FDB1 cells

Given that VY577F mutant cells display a level of myeloid differentiation, we measured the myeloid differentiation markers (Gr-1 and c-FMS) in V449E FDB1 cells after treatment with LY294002, Wortmannin and Rapamycin in the absence of growth factor at day 3. For cells treated with LY294002, Wortmannin or Rapamycin, there was no significant changes in Gr-1 and c-FMS expression in all three concentrations of LY294002, Wortmannin or Rapamycin tested as shown in **Figure 2.10A-C** respectively. We also examined the morphology of FDB1

A.



B.



C.

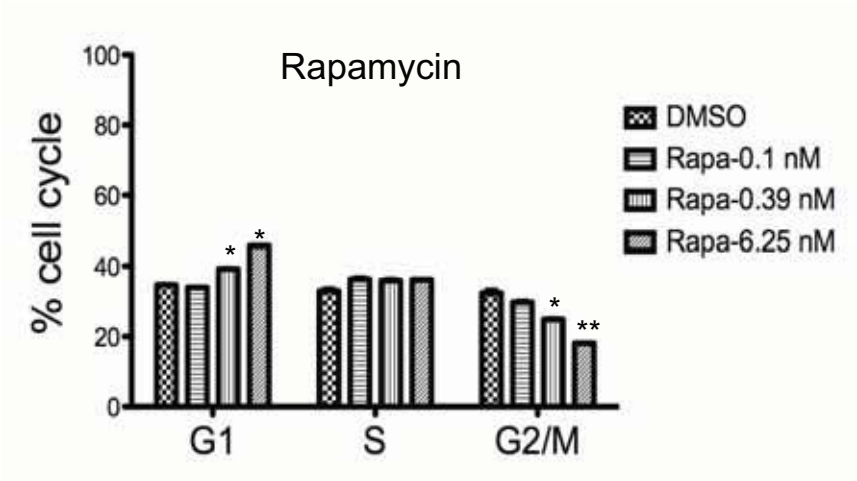


Figure 2.9. **Effect of LY294002 on the cell cycle status of FDB1 GMR-V449E cells**. FDB1 V449E cells were cultured without growth factor and treated with the indicated concentrations of **A.** LY294002 **B.** Wortmannin and **C.** Rapamycin for 3 days as determined by PI staining and flow cytometry. Data represent mean ± SEM of duplicate experiments. *p<0.05 determined by ANOVA with Bonferroni's multiple comparison test relative to DMSO control.

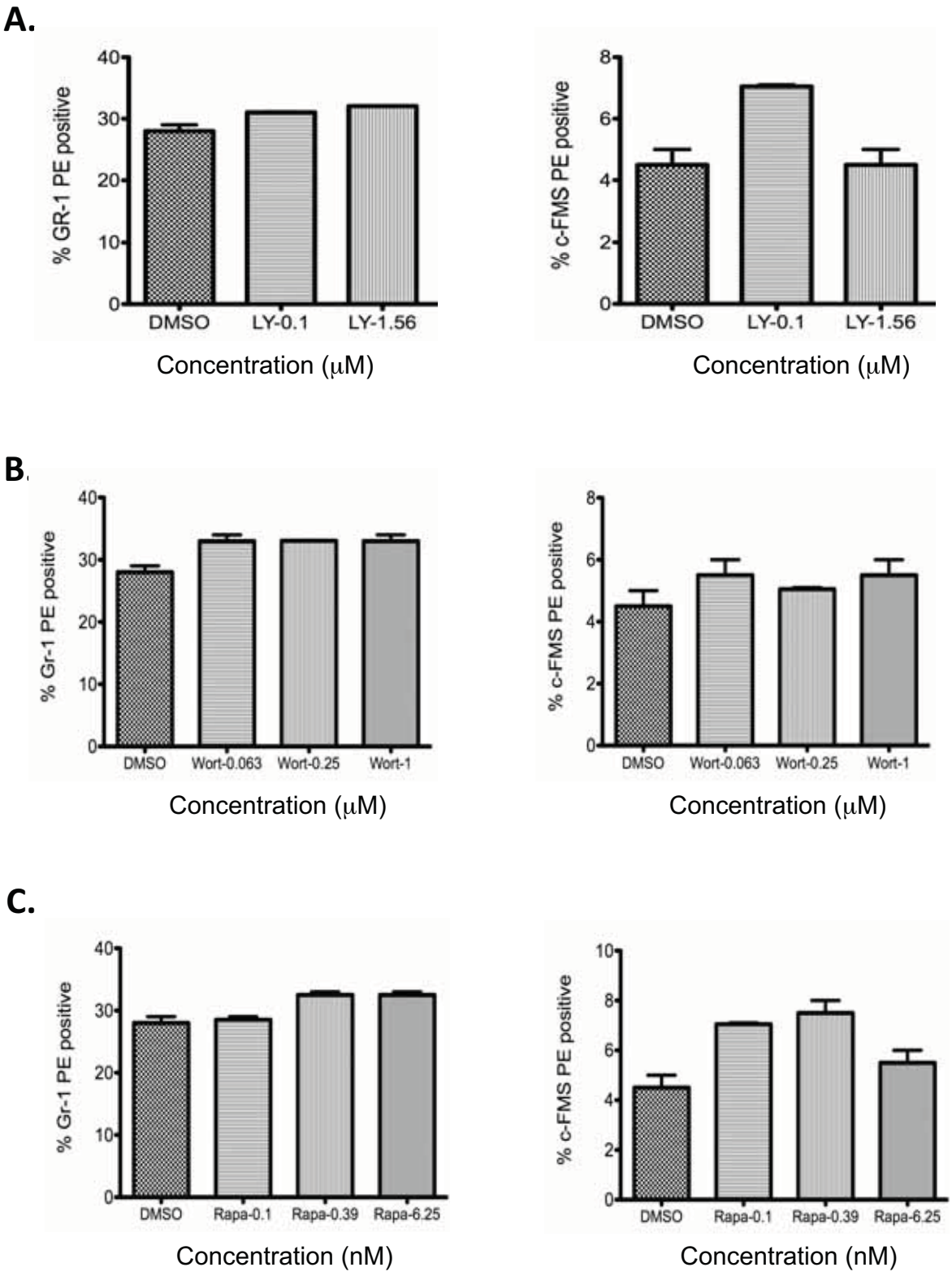


Figure 2.10. Expression of Gr-1 and c-FMS on FDB1 cells expressing GMR-V449E after treatment with PI3K and mTOR inhibitors. A. The mean of Gr-1 and c-FMS expression on V449E cell populations treated with 3 different doses of LY294002 (LY) at day 3. **B.** The mean of Gr-1 and c-FMS expression on V449E cell populations treated with 3 different doses of Wortmannin (Wort) at day 3. **C.** The mean of Gr-1 and c-FMS expression on V449E cell populations treated with 3 different doses of Rapamycin (Rapa) at day 3. Data represent mean ± SEM of duplicate experiments.

cells expressing GMR-V449E after treatment with PI3K or mTOR inhibitors in the absence of growth factor. We did not observe differences in morphology at day 3 or at day 5 for all 3 concentrations tested in these 3 inhibitors (data not shown) consistent with the results of Gr-1 and c-FMS expression.

2.3.6 Gene-set enrichment analysis (GSEA) of the V449E proliferation signature in AML

To examine the relevance of the V449E proliferation signature to AML, we performed a gene-set enrichment analysis using the expression signatures for AML described in the study by Valk *et al* (Valk*, et al* 2004). Briefly, average expression of each gene in the AML subtypes relative to the average of gene expression in normal bone marrow (NBM) was determined as described in **Section 2.2.8**. We then ranked the genes by their log of odds score (Lod) (Smyth 2004) in each AML subtype. The top 50 genes from the V449E Y577-associated proliferation signature were then used as a gene-set to determine enrichment in each AML subtype. We used $p < 0.01$ as threshold to determine the statistical significance associated with enrichment.

As shown in **Table 2.5**, the AML subtypes can be divided into 3 groups based on enrichment determined using the Wilcoxon rank sum test statistic. Several AML subtypes displayed a strong enrichment with the V449E proliferation signature ($p<0.001$). In particular, the group with *CEBPA* mutations displayed the top ranked level of enrichment ($p=0.000005$). This raises the possibility that there may be cooperation between *CEBPA* mutations that contribute to the block in differentiation, and activation of the hβc-associated pathway which may

Table 2.5. Gene-set enrichment analysis (GSEA) of top 50 V449E proliferation signature genes with indicated Valk *et al (*Valk *et al,* 2004*)* AML subtypes compared to NBM.

Wilcoxon rank sum test statistic was employed to calculate the enrichment p-value. Only $p<0.01$ was considered as statistically significant enrichment.

provide a proliferation signal. Similarly the MLL and PML-RARα translocations may cooperate with this proliferation pathway. The enrichment of the V449E proliferation signature with FLT3 AML suggests that constitutive activation of FLT3 may mimic the signal from GMR-V449E Tyr^{577}. Certainly, there is evidence that the PI3K-AKT-mTOR pathways are critical for both classes of the FLT3 mutants (Cai*, et al* 2006, Martelli*, et al* 2006, Weisberg*, et al* 2008). The effect of PI3K inhibitor LY294002 at 10 μM resulted in dephosphorylation of Bad but only a minimal apoptosis effect on 32D cells expressing FLT3-ITD (Minami*, et al* 2003). AML with FLT3-ITD is highly sensitive to Rapamycin with median IC_{50} 2.7 nM, and selective on the proliferative effect of clonogenic leukaemic cells (Recher*, et al* 2005).

It was also notable that amongst the chromosome aberrations, there is no significant enrichment ($p>0.01$) observed for the Tyr^{577}-associated genes expression with -7/7q. This raises the possibility that -7/7q AML use an alternative pathway for proliferation. A possibility is that an alternative proliferation pathway may be provided by K-RAS and N-RAS as these were not significantly enriched with the GMR-V449E proliferation signature. Haferlach *et al* identified that CBFβ-MYH11 AML has high frequency of NF1 deletions and thus alteration of RAS pathway. Haferlach *et al* also suggested that the RAS signalling pathway (RAS activated MEK/ERK) may provide a proliferation effect while PI3K/AKT activation may be associated with survival (Haferlach*, et al* 2010). As described in the **Chapter 1 Section 1.7.2**, mTOR inhibition is more sensitive to low expression of *NF1* but not *NF1* with inactivating mutation (Parkin*, et al* 2010). However, the response of the mTOR inhibition to *NF1* deletion is unknown, and our data for CBFβ-MYH11 is placed at the last of the significant list ($p=0.007$) (**Table 2.5**) suggesting that CBFβ-MYH11 may partially

response to mTOR inhibition due to the *NF1* deletion and thus aberration of RAS signalling pathway. Thus, we speculate that AML with RAS mutation and -7/7q may use MEK/ERK pathway for proliferation while other AML subtypes may use the PI3K-AKT-mTOR pathways. Additionally, there is evidence to support the cooperation between -7/7q and RAS mutations. For example, there is a higher frequency of RAS mutation in -7 myeloid diseases such as childhood -7 myeloproliferative states (Neubauer*, et al* 1991), myelodysplastic syndromes (Stephenson*, et al* 1995) and congenital neutropenia (Kalra*, et al* 1995).

2.3.7 Analysis of the hβc Ser^{585} Signature – a signature associated with survival-only signalling

In the attached publication Powell *et al* (**Appendix B**) (Powell*, et al* 2009), 138 genes were identified to be associated with Ser^{585} signalling. We generated a heatmap to summarize the changes in these 138 genes as shown in **Appendix B Figure 1G** (Powell*, et al* 2009). To identify the pathways associated with the Ser^{585} survival signature, IPA analysis and CMAP were used. As shown in **Appendix B Figure 3B**, CMAP v1 analysis revealed that LY294002 was significantly associated with the Ser^{585} signature (Powell*, et al* 2009). This suggested that the Ser^{585} survival-only pathway regulates the PI3K-AKT network. To further test this, we identified gene expression changes associated with LY294002 treatment and used GSEA with the Wilcoxon rank sum test to test for enrichment in the Ser^{585} signature. We first downloaded the publicly available microarray data from CMAP (10 μM LY294002 in MCF7 breast cancer cell line compared to vehicle control DMSO) and ranked the gene-list by the Lod score. The approach used is similar to that described above (**Section 2.2.8**). As shown in **Appendix B Figure 3C** (Powell*, et al* 2009), we identified a significant association of the hβc Ser^{585} gene-set with the gene expression induced by LY294002 ($p=1.8x10^{-8}$). The top

significant genes that were affected by both LY294002 treatment and Ser^{585} mutation were *MAT2A, TRIB3, PAK1IP1, SAT1, COX4NB, PPAN, HSPA8, MYC, VRK1* and *CDK8*.

We next queried the IPA database with the 138 genes of the Ser^{585} signature. This identified a significant association with the biological processes of cancer and cell death as shown in **Appendix B Figure 3D** (Powell*, et al* 2009). In addition, most of the Ser^{585} genes were connected to the PI3K-AKT pathway as shown in **Appendix B Figure 3E** (Powell*, et al* 2009).

Since submission of the publication, CMAP has been updated to version 2 (v2) and we have now queried this new CMAP interface with the Ser^{585} signature. From this analysis, we identified 71 compounds that associated with the Ser^{585} signature ($p<0.05$ and connectivity score mean > 0.4). The top 10 compounds are listed in **Table 2.6**. The histone deacetylase (HDAC) inhibitors (Trichostatin A and Vorinostat) were identified by CMAP analysis as having signatures related to the Ser^{585} gene-set. Interestingly, the HDAC inhibitors were not identified by CMAP v2 analysis using the V449E Tyr^{577} proliferation signature suggesting histone modification effects maybe unique to Ser^{585} signalling. Moreover, CMAP v2 analysis also identified LY294002, Wortmannin and Quinostatin as significant compounds associated with the Ser^{585} signature as shown in **Figure 2.11A**. These are all PI3K inhibitors and the bar plots for each inhibitors are shown in **Figure 2.11B**. Notably, Rapamycin, which inhibits mTOR, was not significantly associated suggesting that the PI3K-AKT signalling may act independently of mTOR for Ser^{585} survival-only signalling as shown in **Figure 2.11C**.

Table 2.6. Top 10 compounds predicted from CMAP analysis for Ser^{585} signatures.

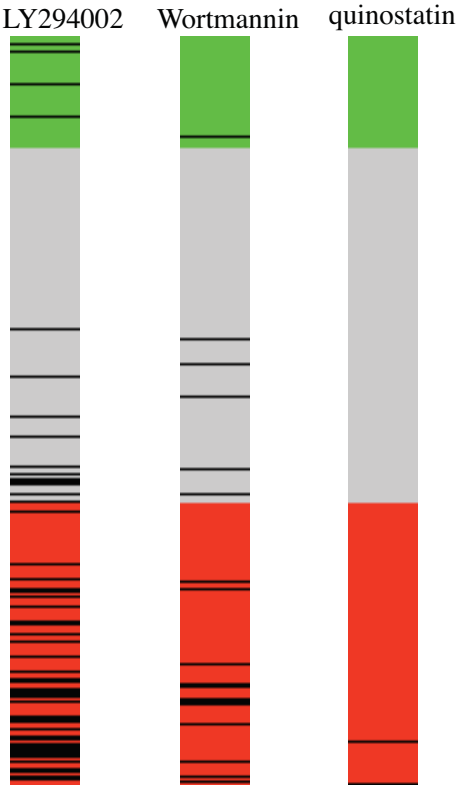
Compounds	Mean	n	p-value	Target
astemizole	0.782	5	0	histamine H1-receptor antagonist
pyrvinium	0.729	6	0	Anti-helminthic
resveratrol	0.616	9	0	phytoalexin
thioridazine	0.656	20	0	inhibitor of CYP1A2 and CYP3A2
vorinostat	0.583	12	0	HDAC inhibitor
prochlorperazine	0.572	16	0	Antipsychotic
trichostatin A	0.559	182	0	HDAC inhibitor
LY-294002	0.425	61	0	PI3K
trifluoperazine	0.539	16	0.00014	Antipsychotic
terfenadine	0.811	3	0.00026	Anti-histamine

The top 10 significant compounds or drugs were based on a p-value < 0.05 and negative connectivity score >0.4. Negative sign indicated the signature for phenotype of mutant Ser^{585} signature. The compounds were ranked by p-value. Mean indicates the connectivity score for correlation with the query gene-expression and n indicates the number of experiments performed using the indicated compounds.

A.

Rank	Compounds	Mean	n	p-value	Target
8	LY-294002	-0.425	61	0	PI3K
26	wortmannin	-0.412	18	0.00509	PI3K
30	quinostatin	-0.817	2	0.00706	PI3K

B.



C.

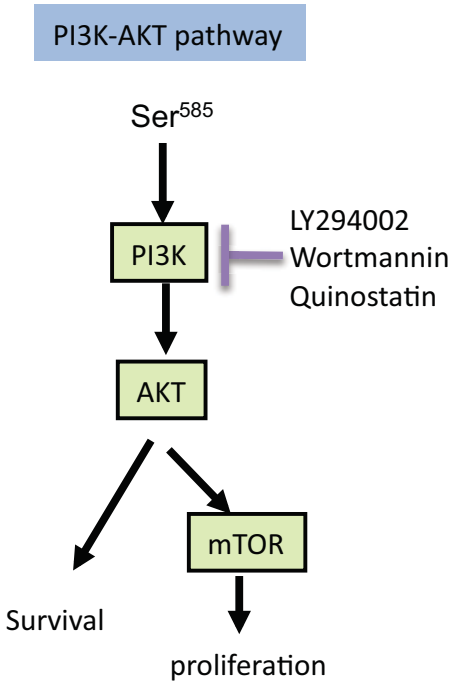


Figure 2.11. Identification of a PI3K-AKT network by CMAP analysis for the Ser^{585} survival-only signature. A. Significant PI3K and mTOR targeted compounds were identified by CMAP based on p-value < 0.05 and negative connectivity score >0.4. The list was ranked by p-value. Mean indicates the connectivity score for correlation with the query gene-expression and n indicates the number of experiments performed using the indicated compounds. **B.** Bar plot for each PI3K targeted drugs. For more details of bar-plot, please see text. **C**. Summary of PI3K pathway that is targeted by the CMAP identified drugs.

2.3.8 Relevance of the survival-only signature to AML

To examine the relevance of the Ser^{585} survival-only signature to AML, we performed a gene-set enrichment analysis using the Wilcoxon rank sum test as described in **section 2.3.6**. We used the Ser^{585} gene-set (138 genes) to determine the enrichment in each AML subtype. As shown in **Table 2.7**, the only significant enrichment ($p<0.01$) was AML with *CEBPA* mutations ($p=0.0035$). This result is again indicates that there may be cooperation between *CEBPA* mutations and activation of pathways associated with hβc.

2.4 Discussion

This work in this chapter follows on from the studies by Brown *et al* (Brown*, et al* 2004) and Guthridge *et al* (Guthridge*, et al* 2006). Brown *et al* previously demonstrated that the V449E Y577F mutation abolishes cell proliferation in FDB1 cells without affecting cell viability (Brown*, et al* 2004). Here, we further showed that the Y577F mutation resulted in G1 phase cell cycle arrest, increased expression of myeloid differentiation markers and morphological (macrophage) differentiation. These findings suggest that the Tyr^{577} residue is important for proliferation by permitting entry into G1 phase and for preventing macrophage differentiation. An important role of this residue may be to regulate the *TCF4/CTNNB1* (β-catenin) pathway which was significantly enriched in the Tyr^{577}-associated proliferation signature. We identified that the TCF4-β-catenin pathway is up-regulated in the VY577F mutant cells suggesting that this pathway is suppressed by Tyr^{577} signals. A role for this pathway in macrophage differentiation is consistent with other studies of GM-CSF induced macrophage differentiation using the FDB1 model (Brown AL and Salerno D, unpublished

Table 2.7. Gene-set enrichment analysis (GSEA) of Ser^{585} survival-only signature genes with indicated AML subtypes defined by Valk *et al* (Valk *et al,* 2004) compared to NBM.

138 Ser^{585} genes were used in the GSEA using Wilcoxon rank sum test statistic. Only $p<0.01$ was considered as statistically significant enrichment.

data). The Wnt/β-catenin pathway has also been shown to be involved in maintaining self-renewal and proliferation in stem and progenitor haemopoietic cells (Wang*, et al* 2010). As shown in **Figure 2.12**, Wnt/β-catenin can initiate differentiation through p300/beta-catenin whereas self-renewal and proliferation are maintained through the cAMP-response element-binding protein (CREB)-binding protein (CBP)/β-catenin arm (Takahashi-Yanaga and Kahn 2010). Thus in the VY577F mutant, p300/ β-catenin is likely involved in initiation of macrophage differentiation. The Tyr^{577} residue may also be important in stabilisation of Myc, and regulation of mTOR downstream targets such as *EIF4EBP1, S6K1, FOXO1, Cyclin D1, Cyclin D3, p27 and c-JUN* through the PI3K-AKT-mTOR pathway (Gustafson and Weiss 2010, Meric-Bernstam and Gonzalez-Angulo 2009).

Connectivity map (CMAP) analysis is a useful tool for identification of drugs or compounds with the potential to induce effects similar to an observed phenotype, based on similarities in gene expression profiles. This approach suggested that the Tyr^{577}-regulated proliferation pathway acts predominantly through PI3K-AKT-mTOR signalling. Our results are consistent with multiple pathways feeding into mTOR activation, as shown in **Figure 2.13**, as inhibition of PI3K by LY294002 resulted in growth arrest (**Figure 2.9**), while GMR-V449E is very sensitive to Rapamycin.

The finding that GMR-V449E promotes cell cycle progression, proliferation, and the differentiation block via mTOR downstream of PI3K is supported by the experimental findings with pathway inhibitors. GMR-V449E activated AKT and the effects of the mTOR inhibitor Rapamycin on GMR-V449E cells closely mimicked the Y577F mutation. When the GMR-V449E cells were treated with 6.3 nM Rapamycin, the proliferation was inhibited

Figure 2.12. The mechanism of Wnt/β-catenin pathway. The Wnt/β-catenin pathway initiates differentiation or self-renewal and proliferation through p300/β-catenin and CBP/β-catenin arms respectively (adapted from Takahashi-Yanaga *et al*, 2010).

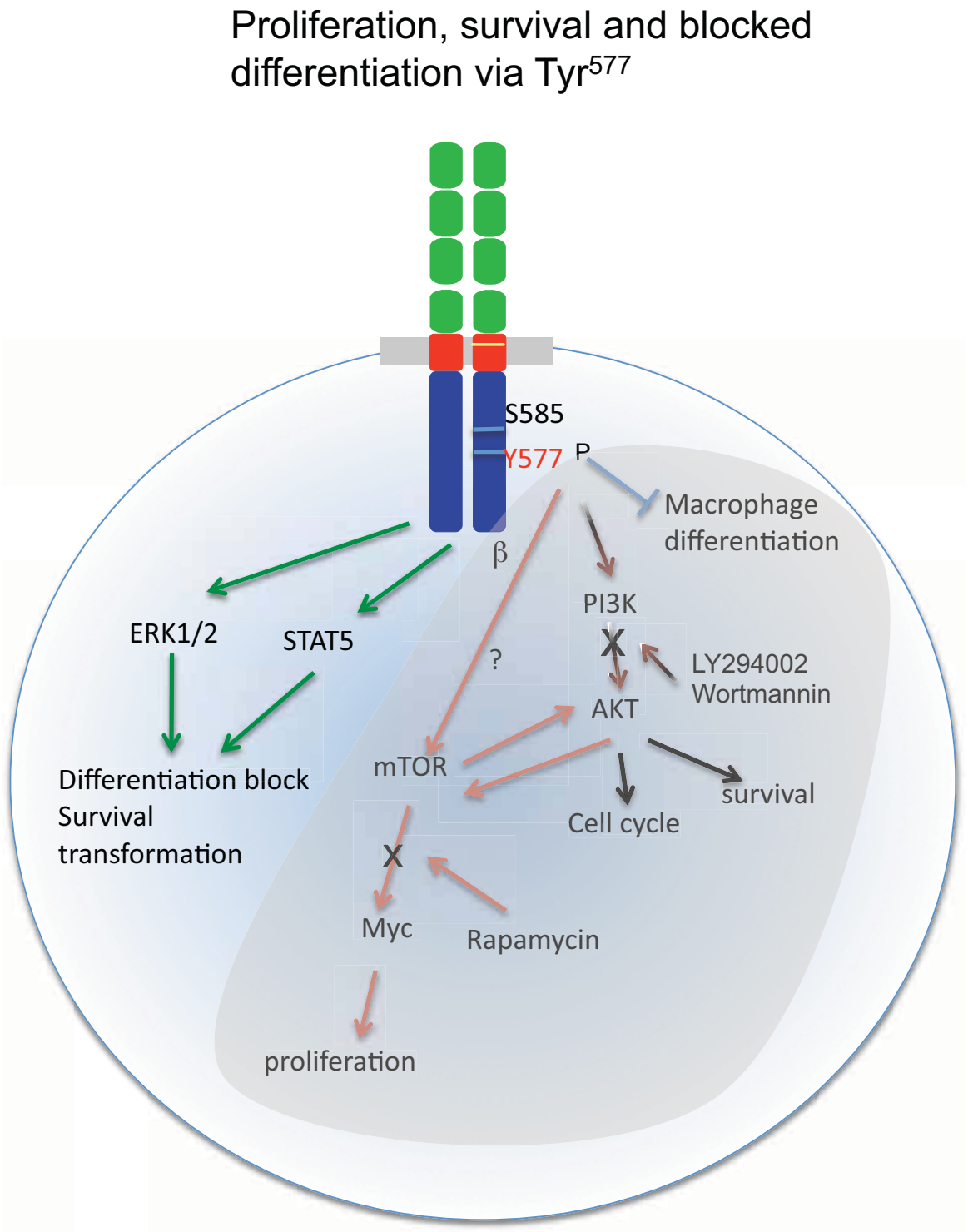


Figure 2.13. Summary of pathways regulated by GMR common beta chain residue Tyr^{577}. Proliferation is regulated through PI3K-AKT and mTOR pathways as indicated in the shaded area. Rapamycin, an mTOR inhibitor, inhibits cell growth without affecting the survival, possibly through downstream molecules such as Myc. Additionally, Tyr^{577} signalling also inhibits macrophage differentiation through down-regulation of TCF4/β-catenin pathway. Additionally, the inhibition of the granulocyte differentiation pathway may be driven by MEK-ERK1/2 pathway rather than PI3K-AKT-mTOR pathway in GMR-V449E FDB1 cells

without a significant increase in apoptosis and with evidence of cell cycle arrest in G1 phase. Zhou *et al* reported that mTOR supports long-term self-renewal, and is able to suppress mesoderm and endoderm activities of human embryonic cells (hESC) for maintenance of hESC pluripotency and controls fate decision in hESCs through inhibition of Wnt signalling (Zhou, *et al* 2009). These studies are consistent with the bioinformatics results suggesting activation of β-catenin in the VY577F mutant cells. This effect is consistent with reports using mTOR inhibitors on AML cells. It has been reported that RAD001 (Everolimus, mTOR inhibitor in phase I/II clinical trial (Tamburini, *et al* 2009)) did not induce apoptosis in AML cells while inhibiting cell growth (Park, *et al* 2008, Tamburini, *et al* 2009). Additionally, Kimball et al reported that AML cell lines treated with Rapamycin have an increase in doubling time (delayed in cell cycle progression), no cell cycle arrest and no apoptosis observed at nanomolar concentrations (Kimball, *et al* 2008). While mTOR appears to be specifically associated with proliferation, PI3K-AKT inhibition with LY294002 and Wortmannin clearly affects survival at the higher doses (see **Figure 2.8C**).

We found no evidence of myeloid differentiation when V449E FDB1 cells were treated with Rapamycin, suggesting that the block in differentiation may involve an mTOR-independent pathway (as shown in **Figure 2.13**) or alternatively full cell cycle arrest might be required for the induction of a differentiation effect. A recent study reported that treatment with Rapamycin in the murine MPRO cell line (mouse promyelocyte) induced terminal differentiation to neutrophils by inhibiting the level of *c-Myc* mRNA associated with polysomes (Wall, *et al* 2008). There have also been a number of studies reporting that treatment with Rapamycin induced myeloid differentiation in AML cells, however this effect occurred when combined with other agents such as HDAC inhibitors (Nishioka, *et al* 2008), ATRA (Nishioka, *et al* 2009), or Vitamin D3 (Yang, *et al* 2010). These studies may suggest

that inhibition of the proliferation pathway acting via mTOR could be a prerequisite for myeloid differentiation, and with additional differentiation agents, full differentiation can be induced. It would be interesting to test whether GMR-V449E FDB1 cells could undergo myeloid differentiation when treated with Rapamycin in combination with other drugs such as HDAC inhibitors. It is likely that PI3K activation also feeds into the mTOR activity as shown in **Figure 2.13**, however inhibitor experiments are most consistent with the presence of a PI3K independent pathway for mTOR as well. Also, in future experiments, we would like to test whether mTORC1 or mTORC2 is responsible for the cell proliferation effect. mTORC1 (activates protein synthesis by phosphorylating key regulators of mRNA translation) is sensitive to Rapamycin and can be activated by cytokines through the PI3K-AKT-mTOR pathway (Feldman*, et al* 2009, Meric-Bernstam and Gonzalez-Angulo 2009). On the other hand, mTORC2 is insensitive to Rapamycin and can phosphorylate Ser^{473} AKT (Feldman*, et al* 2009, Meric-Bernstam and Gonzalez-Angulo 2009). As VGMR-V449E cells are sensitive to Rapamycin and VY577F mutant cells have decreased Ser^{473} AKT phosphorylation, there is a possibility that the mutant cells have a reduction in the mTORC2 pathway. We will use the selective mTORC2 inhibitor PP242 (inhibition of cap-dependent translation) to determine whether the GMR-V449E cells will undergo growth arrest, differentiation and proliferation inhibition. As shown in **Figure 2.13**, the inhibition of the granulocyte differentiation pathway may be driven by MEK-ERK1/2 pathway rather than PI3K-AKT-mTOR pathway in GMR-V449E FDB1 cells; when GMR-V449E mutant cells are treated with MEK inhibitor U0126, granulocyte differentiation occurred as evidenced from increased Gr-1 expression and morphological changes (see **Chapter 3 Section 3.3.4.2**) (Perugini*, et al* 2009).

When the PI3K inhibitor LY294002 was used at higher dose (12.5 μM in this study), GMR-V449E FDB1 cell growth was inhibited, however this was associated with cell death suggesting that the PI3K-AKT pathway is also a primary signal for survival. A lower dose (1.6 μM) of LY294002, GMR-V449E FDB1 cell proliferation was inhibited by 50% without significant apoptosis and with cell cycle arrest. Thus at this lower concentration, LY294002 mimicked the phenotype of V449E Y577F FDB1 cells raising the possibility that the proliferation signal can be partially abolished while not dramatically reducing the survival signal. Further experiments to measure phosphorylation of AKT with these inhibitors are necessary to resolve these issues further.

With the PI3K inhibitor, Wortmannin, there was only slight effect on cell proliferation with the predominant effect being on cell survival. It has been reported that LY294002 and Wortmannin target PI3K signalling in different ways. While LY294002 competes with ATP for the ATP-binding site, Wortmannin uses covalent modification of Lys^{802} of the p110 catalytic subunit to inhibit PI3K (Luo*, et al* 2003). Moreover, LY294002 inhibits casein kinase 2 (CK2) activity and CK2-dependent AKT phosphorylation which is required for activation of PI3K. Wortmannin targets phospholipases C, D and A2 to inhibit the PI3K pathway (Martelli*, et al* 2006). Thus, our data is consistent with the PI3K activation being due to the CK2 activity, but not the phospholipase pathway. This could be tested experimentally by measuring the phospholipase activity as described in Burelout *et al* (Burelout*, et al* 2004).

A comparison of the genes regulated by Ser^{585} and Tyr^{577} showed that the Ser^{585}-regulated survival pathway is different from the signal associated with the Tyr^{577} residue. We identified

that the Ser^{585} residue is associated with a PI3K-AKT signature but as discussed our analysis above indicates this may act independently of mTOR signals. Guthridge *et al* reported that this Ser^{585}-dependent pathway mediates cell survival but not proliferation through the activation of PI3K-AKT-NFκB (**Figure 2.14**) and the induction of BCL-2 (anti-apoptotic) (Guthridge*, et al* 2004). Moreover, there are only 10 genes (*2310005L22Rik, Anxa2, Anxa5, Ero1l, Grhpr, Ptprs, Sat1, Trib3, Tuba4* and *Txk*) in common between the Ser^{585} (2.4%) and Tyr^{577} (7.2%) significant gene lists. This data is consistent with Ser^{585} and Tyr^{577} activate independent pathways as shown in **Figure 2.13 and Figure 2.14.**

The relationship of these Ser^{585} and Tyr^{577} residues in AML is still not clear. Guthridge *et al* reported that the Ser^{585} residue is constitutively phosphorylated in AML patient samples (Guthridge*, et al* 2006). We have shown that both the hβc GMR-V449E Tyr^{577} proliferation signature, and the survival-only signature associated with the hβc Ser^{585} are significantly enriched in some AML subtypes, particularly AML with *CEBPA* mutations. This raises the possibility that this subtype of AML is heavily dependent on the PI3K-AKT network for proliferation and/or survival. It is also possible that cooperation between hβc signalling and *CEBPA* mutations occurs in AML development. The PI3K-AKT signalling network has been previously reported to be constitutively activated in AML (Martelli*, et al* 2009). Kornblau *et al* reported that a high level of phospho-AKT activation but not total AKT was an adverse prognostic factor in AML (Kornblau*, et al* 2006). To date, there have been no studies investigating the sensitivity of AML with *CEBPA* mutations to PI3K-AKT-mTOR inhibition compared to other AML subtypes. This work suggests a number of future experiments. For example, further studies are warranted for screening the hβc gene (*CSF2RB*) for mutations in AMLs with *CEBPA* mutations. Alternatively, the hβc blocking antibody QP-1 (inhibitory

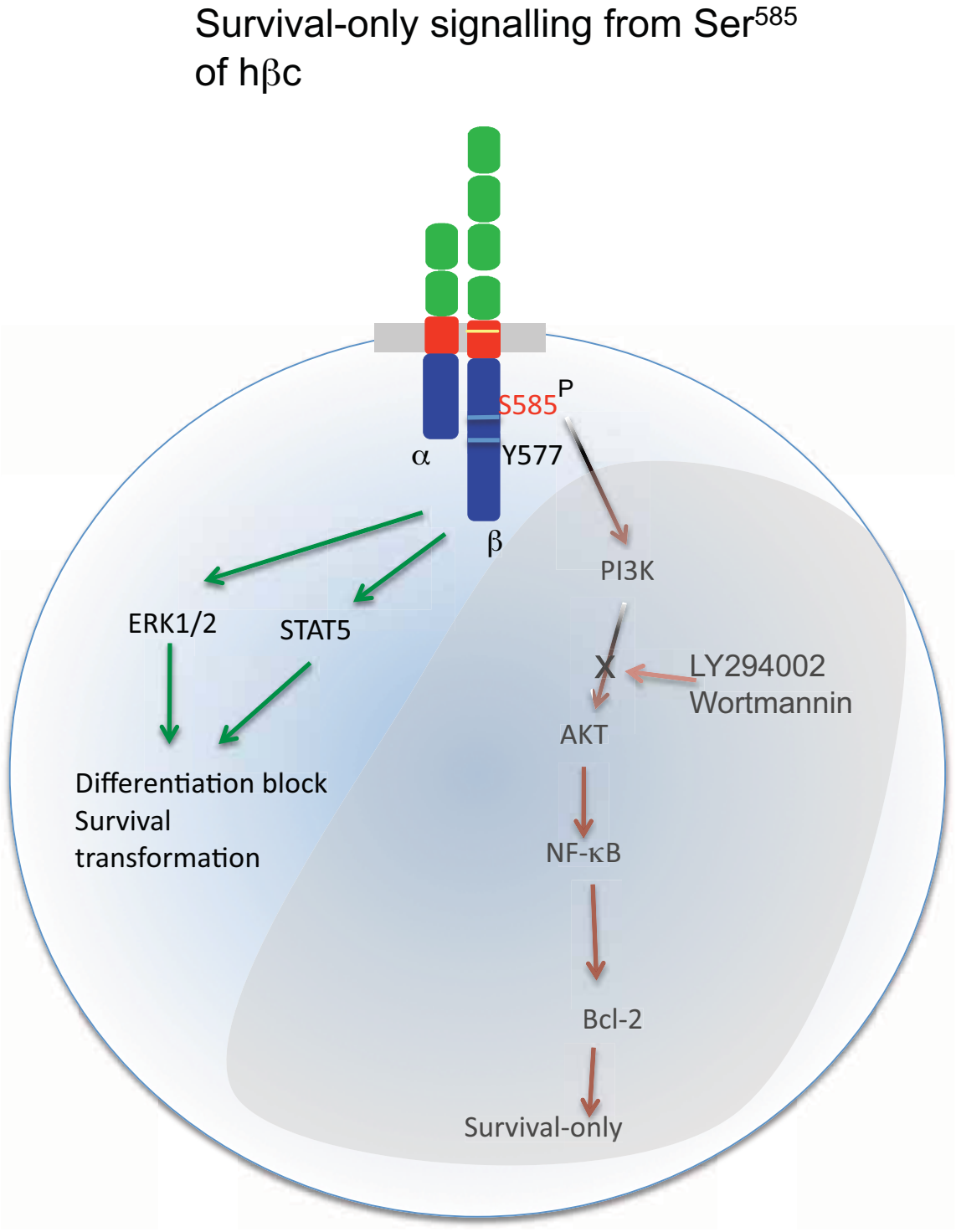


Figure 2.14. Summary of pathways regulated by GMR common beta chain residue Ser^{585}. When Ser^{585} mediated pathways are activated, there is no proliferation response but survival. This survival pathway is activated through PI3K-dependent AKT, NFκB and anti-apoptotic Bcl-2 pathways as shown in shaded area. In this system, mTOR mediated proliferation is not activated by Ser^{585}. LY294002 or Wortmannin (PI3K inhibitors) inhibit cell survival and cell growth through the PI3K dependent AKT pathway.

antibody) (Sun, *et al* 1996) or GM-CSF neutralisation antibodies (Nicola, *et al* 1993) could be used to block the activation of the hβc in AML with *CEBPA* mutations and AKT phosphorylation levels could be measured. Therapeutically, PI3K inhibitors and mTOR inhibitors (or the dual PI3K/mTOR inhibitor, PI-103) may be a good therapeutic approach for AML with *CEBPA* mutations. PI-103 has been reported to inhibit proliferation, induce G1 arrest, and apoptosis in primary AML cells and cell lines (Park, *et al* 2008) although whether it is more effective on specific subtypes of AML has not been investigated.

Taken together, we have shown that hβc Tyr^{577} mediates proliferation through PI3K-AKT-mTOR. This is likely associated with Myc (i.e. mTOR pathway inhibits PP2A leading to phosphorylation of Myc and stabilisation (Gustafson and Weiss 2010)) (see **Figure 2.13**). Ser^{585} regulates survival signals through PI3K-AKT-NFκB and BCL-2 pathways (see **Figure 2.14**). Gene-set enrichment analysis suggests that both of these pathways are active in AML and possibly particularly important in AML with *CEBPA* mutations. New agents that effectively target these pathways, for example, PI-103 may prove effective in this subtype of AML.

Chapter 3: Mechanisms associated with FLT3 mutations in AML

3.1 Introduction

It is well established that FLT3 mutations in AML constitutively activate MAPK/ERK, PI3K/AKT and STAT5 pathways (see **Chapter 1 Section 1.7**). Overall the occurrence of FLT3-TKD (~7%) is less frequent than FLT3-ITD (25-30%) (Scholl*, et al* 2008), although there is heterogeneity amongst subtypes. Core binding factor (CBF) AML is most commonly characterized by the presence of the translocation t(8;21)(q22;q22), or the inversion inv(16)(p13q22), which result in the AML1-ETO and CBFβ-MYH11 fusion proteins respectively (Muller*, et al* 2008). The frequency of CBF AML is approximately 8% of all AML cases (see **Chapter 1 Table 1.4**). As discussed in **Section 1.6.3**, CBF AML is characterised by a high frequency of mutations in the receptor tyrosine kinase c-KIT (Muller*, et al* 2008) and the most common mutation of c-KIT in inv(16) AML is a substitution at D816 residue which is equivalent to the FLT3-TKD D835Y, and is associated with an increased incidence of relapse in this AML subtype (Paschka*, et al* 2006). This suggests a role for specific signalling events in cooperating with the aberrant CBF complex. Such events may directly contribute to outcome and therefore may be important therapeutic targets. With regard to the FLT3-TKD mutation in inv(16) AML, findings from previous studies vary on the association of these two genetic lesions (Bacher*, et al* 2008, Mead*, et al* 2007) which could be due to the low occurrence of this subgroup of AML. Hence, the association of FLT3-TKD with inv(16) is still unclear.

Differences in signalling pathways induced by FLT3-ITD and FLT3-TKD mutants and c-KIT (Choudhary*, et al* 2005, Grundler*, et al* 2005) may be in part responsible for the preferential association with various translocation events. As discussed in **Chapter 1 Section 1.7,** activation of the PI3K/AKT, MAPK/ERK and STAT5 pathways have been shown to be important in FLT3-ITD and FLT3-TKD activity which may be important for transformation and survival. Constitutive activation of the MAPK/ERK pathway by FLT3-ITD leads to block in granulocytic differentiation through post-translational modification (Radomska*, et al* 2006). This suggests that the MAPK/ERK signalling is an important modulator of granulocytic differentiation and this signalling contributes to approximately 50% of primary AML (Towatari*, et al* 1997). Whether this is the only mechanism by which the FLT3 mutants contribute to the block in GM differentiation is unknown.

The FLT3 mutation is present in LSC and blast in $FLT3^{+}$ AML. As described in **Chapter 1 Section 1.4**, there is increasing evidence that LSCs are chemoresistant in part by using alternative survival pathways, and have an independent worse outcome (Chu and Small 2009). Majeti *et al* (Majeti*, et al* 2009) have previously analysed expression of LSC from a heterogeneous group of AML patients compared to normal haemopoietic stem cell (HSC). This defined a number of LSC-associate genes and revealed an acquisition of the Wnt signalling pathway associated with the LSC phenotype. However, to date, there have been no studies investigating the properties selectively associated with LSC from $FLT3\text{-}ITD^{+}$ in normal karyotype (NK) AML.

In this chapter, we will address the mechanisms associated with the FLT3 mutants and repression of the target gene, *Gadd45a,* using the combination of bioinformatics and

experimental analysis. We have re-analysed gene expression data derived from NK AML LSC to identify gene expression changes associated specifically with FLT3-ITD LSC in NK AML. This suggests pathways that may be contributing to the properties of FLT3-ITD$^+$ LSC and potentially to the poor outcome of this group.

3.2 Materials and Methods

3.2.1 Reagents

Recombinant murine IL-3 (mIL-3) and mGM-CSF were produced from Sf21-pIB-mIL-3 and Sf21-pIB-mGM-CSF conditioned media respectively made in-house. Gr-1 and Mac-1 (CD11b) mouse monoclonal antibodies together with the isotype controls (IgG2b) conjugated with FITC and PE respectively were purchased from eBioscience. IMDM and PBS were purchased from Sigma-aldrich.

3.2.2 Cell lines

FDB1 cells (myeloid suspension cells), GMR-V449E FDB1, FLT3-ITD FDB1 and FLT3-TKD FDB1 cells were maintained in IMDM supplemented with 10% (v/v) FBS, 500 BMU/ml mIL-3 and 1x PSG. GMR-V449E FDB1, FLT3-ITD FDB1 and FLT3-TKD FDB1 cells were maintained in 1 μg/ml of puromycin (Sigma-Aldrich) and cultured in a humidified incubator at 37°C with 5% CO_2.

3.2.3 Cell viability

Cell viability was determined using trypan blue (Invitrogen) exclusion by haemacytometer or using 7-Amino-Actinomycin D (7AAD) by flow cytometry. Briefly, aliquots of cells (100 µl) were added to 1 µl of 7AAD (1 mg/ml) (Sigma-Aldrich) directly into a tube. Subsequently, the cells were profiled by flow cytometry.

3.2.4 Primers

Mouse *Cebpe* primer sequences were obtained from a previously described report (Wang*, et al* 2006). Mouse *Cebpa, and Sfpi1* (*Pu.1*) primers sequences were obtained from Primer Bank (Spandidos*, et al* 2009). *Cebpa* sequence 5F: CAAGAACAGCAACGAGTACCG, 3R: GTCACTGGTCAACTCCAGCAC. *Cebpe* sequence 5F: CAGCCCTTGCGTGTCCT, 3R: CACGTCGCAGTCGGTACTCC. *Sfpi1* sequence 5F: GAACAGATGCACGTCCTCGAT, 3R: GGGGACAAGGTTTGATAAGGGAA.

3.2.5 Immunophenotyping by flow cytometry

Cells were harvested and washed with PBS and stained for 30 minutes on ice with the primary conjugated antibody Gr-1 (1:1000 dilution), Mac-1 (1:100) or their isotype controls in 100 µL of FACS wash (recipe in Appendix A). FDB1 cells were incubated with 20 µL of Intragam (CSL) as an Fc receptor blocking reagent together with the primary antibody. Subsequently, the cells were washed twice with the FACS wash and then centrifuged at 1200 r.p.m for 5 minutes followed by removal of supernatant. The cells were resuspended in 300 µL FACS wash, analysed directly using a flow cytometer Beckman Coulter Epics XL-MCL

or Beckman Dickinson Aria, or stored in FACS FIX to be analysed later. The histograms were generated using Flowjo software.

3.2.6 Bioinformatics analysis

Mouse myeloid differentiation program microarray data (Chambers, *et al* 2007) was downloaded from NCBI GEO (accession number GSE6506). Human myeloid differentiation data (Tonks, *et al* 2007b) was downloaded from EBI ArrayExpress database (http://www.ebi.ac.uk/microarray-as/aer/entry) accession number E-MEXP-583. The raw data was normalised using GC-RMA algorithm. For identifying the differential gene expression, the microarray data were analysed using the empirical Bayes moderated t-statistics, and LIMMA (Smyth 2004) obtained from the Bioconductor (http://www.bioconductor.org/). The FDR was controlled using Benjamini-Hochberg (BH) method. Only genes with BH adjusted p-value < 0.05 were flagged as statistically significant.

AML microarray data i.e. Valk *et al* (GSE1159) (Valk, *et al* 2004), Majeti *et al* (GSE17054) (Majeti, *et al* 2009), Ross *et al* (http://www.stjuderesearch.org/data/AML1) (Ross, *et al* 2004), and Verhaak *et al* (GSE6891) (Verhaak, *et al* 2009) were normalised using RMA. Expression intensity for each gene of interest in AML subtypes was extracted and was plotted using GraphPad Prism version 5 (GraphPad Software, http://www.graphpad.com). For identifying the differential gene expression, the microarray data were analysed as described above. Heatmap was generated using MeV software (Saeed, *et al* 2006).

3.2.6.1 Pathway analysis

All the significant genes (FDR p-value < 0.05 and with fold change > 2) were uploaded to Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, http://www.ingenuity.com). Protein and/or gene networks and ontology associated with gene-sets were identified using IPA with p-value < 0.05. To display the networks, we merged the available overlapping networks identified by IPA. If the networks were too big, then we merge only the top networks as suggested by IPA or otherwise indicated in the text. All the networks were filtered to remove indirect associations.

3.2.7 Statistical analysis

The statistical significance of comparison two sample groups was determined by using a 2-tailed student's t-test or otherwise indicated in the text. Only $p < 0.05$ was considered as significant. The data is presented as the mean ± standard error measurement (SEM) for at least 2 independent experiments. The data was analysed using GraphPad Prism version 5.0 (GraphPad Software, http://www.graphpad.com).

3.3 Results

3.3.1 Association of FLT3 mutations with inv(16) AML: a combined analysis of 734 reported cases of core-binding factor AML

As discussed in **Section 3.1**, for the FLT3-TKD mutation in CBF AML, findings from previous studies vary, particularly with regard to the association with the inv(16) lesion (Bacher*, et al* 2008, Mead*, et al* 2007). Here we examined more closely the association of the

FLT3-TKD mutations with CBF AML by performing a combined-analysis of previously reports in the literature between the year 2000 and May 2010. We limited this analysis to those CBF leukaemia studies that screened for FLT3 mutations in each sub-group. We also filtered to exclude overlapping studies and treatment protocols, using the latest year published reports. We also compared the frequency of the FLT3-TKD mutation to the frequency of FLT3-ITD in both sub-types of CBF AML.

There were 42 studies that have reported FLT3-ITD and/or FLT3-TKD status in AML with the inv(16) and t(8;21) translocations. However, after removing studies based on the criteria above, there are 19 adult AML studies with the total of 303 inv(16) patients and 431 t(8;21) patients. As shown in **Table 3.1**, there were 13 FLT3-ITD and 42 FLT3-TKD genotyped in the inv(16) group and 34 FLT3-ITD and 15 FLT3-TKD genotyped in the t(8;21) group. We examined the association of the mutations in each group using a chi squared test to determine if the frequency of FLT3-ITD and FLT3-TKD mutations varied significantly compared to the expected frequency in adult AML (25% and 7% respectively). In inv(16) AML, we found that the FLT3-TKD mutation was significantly over-represented (15.2%) while the FLT3-ITD mutation was under-represented (4.3%) ($p=8.8x10^{-8}$ and $p=8.4x10^{-17}$ respectively and see **Table 3.1**). In contrast we found that while FLT3-ITD was more frequent than FLT3-TKD in t(8;21) AML (7.9% vs 3.9% respectively) both mutations were significantly under-represented ($p=2.3x10^{-16}$ and $p=2.0x10^{-2}$ respectively) (**Table 3.1**). Thus there appears to be a unique association between the inv(16) subtype and the FLT3-TKD mutation. This analysis highlights a key difference between the two groups of CBF AML, and between inv(16) AML and other AML sub-types and suggests that the FLT3-TKD mutant may provide specific signals that cooperate with the CBFβ-MYH11 fusion to induce AML.

Table 3.1. The summary frequency of FLT3 mutations in CBF leukaemia in the 19 combined studies.

Adult Study (19 studies)	Country	period	Study and Treatment protocol	inv(16)			t(8;21)		
				Total	FLT3-ITD	FLT3-TKD	Total	FLT3-ITD	FLT3-TKD
Andersson *et al* (2004)	Sweden	1995-2002	Southern Sweden	1	0	0	2	0	0
Andreeff *et al* (2008)	US	---	MD Anderson Cancer Center IRB-approved protocol	7	0	1	5	1	0
Bao *et al* (2006)	China	07-2003 until 09-2004	JCML -28 major hospital, Shanghai	5	0	1	20	0	0
Boissel *et al* (2002)	France	---	ALFA-9000 prospective trial	13	0	---	10	0	---
Bullinger *et al* (2007)	Germany	11-1994 until 03-2004	German and Austrian AMLSG, AML-HD93, AML-HD98A, AML-HD98B	55	1	7	38	3	2
Carnicer *et al* (2003)	Spain	08-1998 until 03-2002	Hospital de la Santa Creu i Sant Pau in Barcelona	15	0	4	10	0	0
Figueroa *et al* (2010)	Netherlands	---	Dutch-Belgian HOVON, HO04/A, HO29, HO42, HO43	30	0	6	24	3	1
Gutiérrez *et al* (2005)	Spain	---	Spanish PETHEMA Cooperative group APL-96, AML-99	4	0	0	---	---	---
Haferlach *et al* (2010)	Germany	08-2005 until 10-2008	MLL Munich Leukemia Laboratory	37	3	3	---	---	---
Ishikawa *et al* (2009)	Japan	Jan-90	JALSG-92,97	3	1	0	19	0	2
Koh *et al* (2009)	korea	01-1997 till 12-2007	Seoul national University hospital, Seoul National University Bundang Hospital, Gachon Uni Gil Hospital	8	0	---	32	2	---
Krum *et al* (2009)	Brazil	09-2001 until 05-2005	Hospital Sao Paulo, Universidade Federal de Sao Paulo (Unifesp-EPM)	3	0	---	6	0	---
Mead *et al* (2007)	UK	---	UK MRC AML10, AML12	55	4	13	74	11	5
Palmisano *et al* (2007)	Italy	---	Institute Haematology and Medical oncology	6	2	0	2	1	0
Schessl *et al* (2005)	Germany	---	Ludwig Maximillians University	---	---	---	135	11	3
Shih *et al* (2004)	Taiwan	From 04-1991	Chang Gung Memorial Hospital, Taipei	1	0	1	---	---	---
Thiede *et al* (2002)	Germany	---	German SHG, AML-96, APL-93	43	1	5	41	2	1
Tiesmeier *et al* (2004)	Germany	---	Southern German AML Study group	3	0	---	3	0	---
Tomasson *et al* (2008)	US	---	CALGB cooperative group	14	1	1	10	0	1
			Grand total:	**303**	**13**	**42**	**431**	**34**	**15**
			Total FLT3-ITD genotyped:	**303**	**13**	**---**	**431**	**34**	**---**
			Total FLT3-TKD genotyped:	**276**	**---**	**42**	**380**	**---**	**15**

	Expected Freq:	Observed Freq:	95% CI:	X^2 p-value:
Total inv(16)-FLT3-ITD:	25.0	4.3	2.3-7.2	8.4E-17
Total inv(16)-FLT3-TKD:	7.0	15.2	11.2-20.0	8.8E-08
Total t(8;21)-FLT3-ITD:	25.0	7.9	5.5-10.9	2.3E-16
Total t(8;21)-FLT3-TKD:	7.0	3.9	2.2-6.4	2.0E-02

To examine the difference between the transcriptional changes induced by inv(16) and t(8;21) AML we analysed microarray expression data (GSE1159) from the study by Valk *et al* (Valk*, et al* 2004 2004). Data was normalized with RMA and the gene expression change for each gene determined in the inv(16) (n=19), t(8;21) (n=22), t(15;17) (n=18) or 11q23 (n=17) groups, relative to the expression level in normal bone marrow mononuclear cells (NBM, n=5). The analysis used gene expression from all cases irrespective of secondary mutations characterised. Differential expression was determined using the empirical Bayes moderated t-statistics and LIMMA package available from Bioconductor open-source software project (http://www.bioconductor.org/). The FDR was controlled using Benjamini-Hochberg method. To obtain genes specific to the CBF group of AML we filtered genes commonly differentially expressed (FDR p-value < 0.01) between all the translocation groups of AML, relative to NBM. There are 67 genes commonly differentially regulated (FDR p-value < 0.01) in both the t(8;21) and inv(16) sub-groups, while 96 genes displayed specific differential expression (FDR p-value < 0.01) in inv(16) AML relative to NBM, and 361 genes differentially expressed (FDR p-value < 0.01) and specific to t(8;21) AML (data not shown). Previous studies have reported selective activation of the β-catenin pathway in AML with t(8;21) (Muller-Tidow*, et al* 2004, Yeh*, et al* 2009). Consistent with this, 23 of the 361 genes specifically associated with the t(8;21) group are known direct targets of TCF4 or β-catenin, as defined by ChIP-CHIP microarray studies (see **Table 3.2**). While the number of genes in the inv(16) group is smaller, we also observed selective up-regulation of the Wnt signalling receptor *FZD2,* and down-regulation in this group of a number of negative regulators of β-catenin signalling (*AXIN1* and the genes encoding the *PP2A* beta subunit, *PPP2R5B*). Over-expression of the Frizzled related receptor (*FZD4)* has been shown to induce β-catenin stabilisation in 32D cells (Tickenbrock*, et al* 2008) suggesting that

Table 3.2. Direct target genes of TCF4/β-catenin in CBF leukaemia.

Type	Gene Symbol	Fold Change (log2) (t(8;21) // inv(16))	FDR p-value (BH adjusted)	TCF7L2/ TCF4 targets by SACO (Hatzis *et al*, 2008)	β-catenin targets by SACO (Yochum *et al*, 2007)
t(8;21) Specific	*PDGFC*	2.668	4.73E-04	+	-
t(8;21) Specific	*NCAM1*	2.011	4.06E-04	-	+
t(8;21) Specific	*SOX4*	2.000	0.006	+	-
t(8;21) Specific	*ITGB4*	1.012	3.45E-10	+	-
t(8;21) Specific	*PATZ1*	1.005	0.006	+	-
t(8;21) Specific	*KIFAP3*	0.982	0.003	+	-
t(8;21) Specific	*OBFC1*	0.971	0.008	-	+
t(8;21) Specific	*ZKSCAN1*	0.965	0.006	+	-
t(8;21) Specific	*ALS2CR8*	0.735	0.007	+	-
t(8;21) Specific	*EVPL*	0.671	4.65E-05	-	+
t(8;21) Specific	*TPO*	0.606	0.007	-	+
t(8;21) Specific	*DOCK6*	0.477	0.002	-	+
t(8;21) Specific	*TMEM161A*	0.362	0.002	-	+
t(8;21) Specific	*PPP2R1B*	-0.453	0.004	-	+
t(8;21) Specific	*CARHSP1*	-0.734	0.001	+	-
t(8;21) Specific	*KCNQ1*	-1.014	0.003	-	+
t(8;21) Specific	*GARNL4*	-1.027	0.003	-	+
t(8;21) Specific	*CDKN3*	-1.464	0.001	+	-
t(8;21) Specific	*LMO4*	-1.577	0.007	+	-
t(8;21) Specific	*S100A6*	-1.693	0.006	+	-
t(8;21) Specific	*ATXN1*	-2.064	1.10E-04	-	+
t(8;21) Specific	*NR4A2*	-2.960	0.003	+	-
inv(16) Specific	*MSLN*	4.249	6.15E-07	-	+
inv(16) Specific	*NRP1*	1.929	1.06E-11	+	-
inv(16) Specific	*RUNX1*	0.989	4.40E-04	-	+
inv(16) Specific	*H2AFJ*	0.570	0.005	+	-
CBF common	*CDC123*	0.710 // 1.067	0.008 // 4.76E-05	+	-
CBF common	*NARF*	-0.817 // -0.904	0.001 // 0.001	-	+
CBF common	*TBCD*	-0.682 // -0.725	0.003 // 0.003	-	+

Both studies were performed using ChIP-CHIP microarray. SACO, serial analysis of chromatin occupancy. FDR was adjusted using Benjamini-Hochberg (BH) method.

stabilisation of β-catenin and activation of downstream pathways may also contribute to inv(16) AML. Consistent with this, analysis of the 96 inv(16)-specific genes using DAVID database (http://david.abcc.ncifcrf.gov/) and IPA detected Wnt/β-catenin as a significant pathway with p=0.022 and p=0.0073 respectively. Wang *et al* (Wang*, et al* 2010) have recently reported that for some oncogenes β-catenin activation is essential for transformation and the gene expression analysis outlined here, using data from primary AML samples, suggests that activation of β-catenin maybe a particularly prominent feature CBF AML sub-group.

To investigate whether the FLT3-TKD has an impact on clinical outcome in inv(16) patients, we used the clinical outcome of overall survival and event-free survival provided in the study by Figueroa *et al* (Figueroa*, et al* 2010). The clinical characteristics for t(8;21) and inv(16) are summarised in **Table 3.3**. We observed there is no significant difference in overall survival and event-free survival of FLT3-TKD in inv(16) (**Appendix C**). However, the analysis is limited by the small sample size.

3.3.2 Gene expression and prognostic analysis of FLT3 mutations in normal karyotype AML blasts

3.3.2.1 Experimental design and patient selection

We wanted to examine the differences in gene expression associated with the two FLT3 mutants in AML blasts. Differences have been reported in signalling by FLT3-ITD and FLT3-TKD (Choudhary*, et al* 2005, Grundler*, et al* 2005), however gene expression profiling has not been compared directly in normal karyotype (NK) AML. We focused on NK AML,

Table 3.3. Patient clinical characteristic in CBF leukaemia.

	inv(16)	t(8;21)
Total	26	24
Mutation		
FLT3-ITD	0	3
FLT3-TKD	4	1
NPM1	0	0
RAS	9	4
CEBPA	0	0
EVI1	0	0
FAB		
M0	0	0
M1	0	1
M2	3	21
M3	0	0
M4	22	2
M5	1	0

as the gene expression differences would not be influenced by major karyotypic changes. This is also the most frequent subtype of adult AML occurring at a frequency of 40-49% (Mrozek*, et al* 2007) while FLT3 mutations occur in this subtype at a frequency of 28-33% (Bullinger*, et al* 2008). In addition to FLT3 mutants, AMLs within this group can have mutation in *CEBPA* (10%), *WT1*, and *NPM1* which are known to influence outcome (Grimwade and Hills 2009, Lowenberg 2008). Although a number of studies have described the gene expression associated with FLT3-ITD in AML patients with NK (Bullinger*, et al* 2008, Radmacher*, et al* 2006), and gene expression associated with FLT3-TKD in NK AML (Whitman*, et al* 2008), these studies have not directly compared gene expression between FLT3-ITD and FLT3-TKD. We used the microarray dataset (GSE6891) study by Verhaak *et al* (461 total adult AML) (Verhaak*, et al* 2009), and focused on AML patients with normal karyotype harbouring FLT3-ITD or FLT3-TKD mutations. The selection of these patients was independent of the *NPM1* mutation but patients with other mutations (*EVI1, K-RAS, N-RAS, CEBPA,* and double mutation *FLT3-ITD/FLT3-TKD*) were excluded to reduce the complexity associated with other molecular mutations. This approach has not been described previously. The study by Whitman *et al* identified a FLT3-TKD signature in NK AML (Whitman*, et al* 2008) but *NPM1* mutations were not excluded as the FLT3-TKD mutation is almost exclusively associated with the *NPM1* mutation, consistent with previous reported studies (Schnittger*, et al* 2005, Whitman*, et al* 2008). To exclude the differences that may due to *NPM1*, we also included FLT3-WT AML that either has *NPM1* mutation or is *NPM1* WT. As a result of this selection, there were a total of 68 normal karyotype AML patients with FLT3-ITD, 14 AML with FLT3-TKD and 60 FLT3-WT. The clinical characteristic of these three groups of AML is summarised in **Table 3.4**.

Table 3.4. The patient clinical characteristic of normal karyotype AML based on FLT3 mutations category.

	FLT3-WT	FLT3-ITD	FLT3-TKD
Total:	60	68	14
Median age (range):	50 (18-60)	47 (18-59)	45 (26-59)
Gender:			
Male	32	30	4
Female	28	38	10
NPM1:			
pos	28	53	13
neg	32	15	1
FAB:			
M0	1	0	1
M1	16	21	0
M2	14	14	2
M3	1	1	0
M4	10	11	2
M5	12	19	9
M6	2	1	0
RAEB	1	0	0
RAEB-t	2	0	0
unknown	1	1	0

3.3.2.2 Prognosis of FLT3 mutations in *NPM1*$^+$ NK AML

We determined the overall survival and event-free survival associate with *NPM1* mutation. We obtained the clinical information of a subset of these patients as provided in Figueroa *et al* study (Figueroa*, et al* 2010). For patients with NPM1 mutation, there is a significant decrease in overall survival in FLT3-TKD compared to FLT3-WT patients (**Figure 3.1**). When combined with the Valk *et al* study (to increase the sample size) (Valk*, et al* 2004), we observed the same trend with greater significance. We observed a similar effect on event-free survival for FLT3-TKD compared to FLT3-WT. We used FLT3-ITD as a comparison as it is known to be an independent poor prognostic outcome independent to the *NPM1* mutation (Grimwade and Hills 2009). However, Whitman *et al* also observed significant worse prognosis of disease-free survival but not overall survival when compared FLT3-TKD vs FLT3-WT (Whitman*, et al* 2008). Again their approach is different as this study used FLT3 mutations with *NPM1* mutation to compare the overall survival and the event-free survival with FLT3-WT NK AML.

3.3.2.3 Gene expression profiling of FLT3 mutation in NK AML

Gene expression differences for FLT3-ITD and FLT3-TKD were determined relative to the expression in NK AML with FLT3-WT regardless of *NPM1* mutation status as described in **Section 3.2.2.1**. There were 33 up-regulated probes and 14 down-regulated probes (greater than 2 fold-change and FDR $p<0.05$) in FLT3-ITD relative to FLT3-WT NK AML. In FLT3-TKD relative to FLT3-WT, there were more 272 up-regulated probes and 78 down-regulated probes. Of these, there were 5 commonly up-regulated probes and 19 commonly down-regulated probes in both types of FLT3 mutations relative to FLT3-WT.

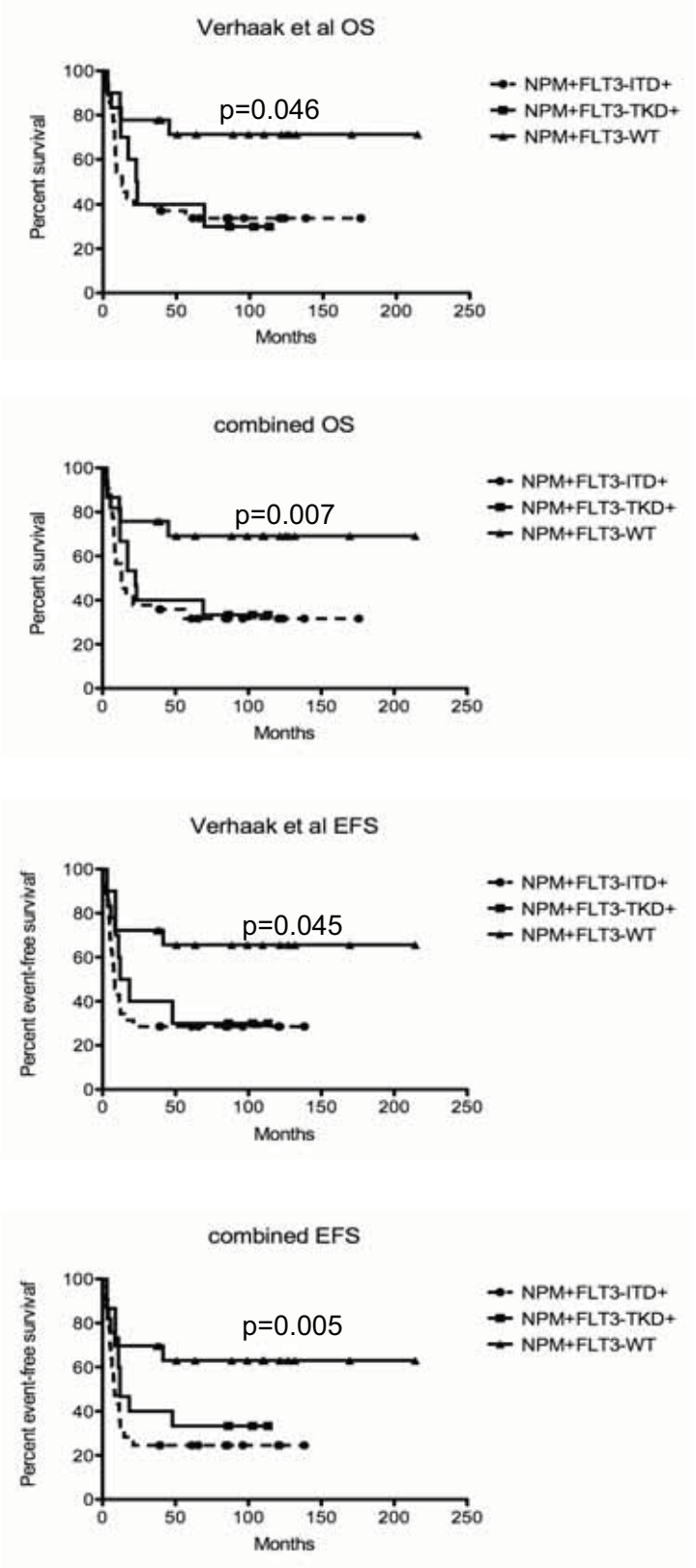


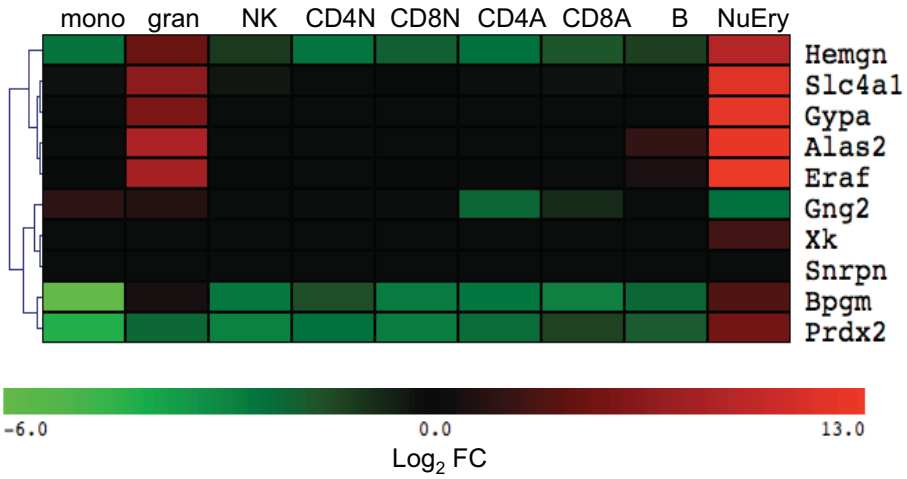
Figure 3.1. Effect of FLT3 mutations in NPM⁺ NK AML on overall survival and event-free survival. The overall survival and event-free survival for Verhaak *et al* study FLT3-ITD (n=35), FLT3-TKD (n=10) and FLT3-WT (n=18) and Combined data (Verhaak *et al* and Valk *et al*) FLT3-ITD (n=53), FLT3-TKD (n=15) and FLT3-WT (n=33). The statistical significance was assessed by Log-rank (Mantel-Cox) test.

As shown in **Figure 3.2**, in the common FLT3 mutations group, *HOXB3* was significantly up-regulated. *HOXB3* expression has previously been correlated with FLT3 activating mutations (Roche*, et al* 2004). In the commonly down-regulated group, we observed multiple genes that are associated with erythrocyte lineage as shown in **Figure 3.3A**. We also observed there is significant overlap with *GATA1* target genes based on gene expression profiling and transcription binding site consensus by gene-set enrichment analysis (GSEA) using hypergeometric statistics as shown in **Figure 3.3B**. This feature has not been previously reported. The transcription factor *GATA1* is important for the megakaryocyte-erythrocyte-basophil or mast-cell-eosinophil development continuum and regulates the Globin enhancer (Ceredig*, et al* 2009, Koschmieder*, et al* 2005). Consistent with the GSEA result, IPA also derived the *GATA1* network when the top 1 and 2 networks were merged together (**Figure 3.4**). This result suggests that both FLT3 mutations might inhibit *GATA1* pathways and drive myeloid lineage development. FLT3 up-regulation is important in sustained lymphoid-primed multipotent progenitor (LMPP – a progenitor common for the GM and lymphoid), granulocyte-monocyte progenitor (GMP) potential, and common lymphoid progenitor (CLP) potential, but not megakaryocyte-erythrocyte progenitor (MEP) potential (Adolfsson*, et al* 2005).

In the FLT3-ITD group, up-regulation of *SOCS2* was notable. *SOCS2* is a known target gene which is up-regulated by FLT3-ITD (Mizuki*, et al* 2003). In addition, we also identified *BIC (MIR155HG)*, a primary transcript (pri-miRNA) encoding miR-155, as over-expressed in the FLT3-ITD group. While this miR-155 has been reported to be over-expressed in FLT3-ITD group in NK AML, its regulation appears to be a least partly independent of FLT3-ITD

Figure 3.2. The gene expression differences of FLT3-ITD and FLT3-TKD compared to FLT3-WT in normal karyotype AML. Differential gene expression selectively regulated by FLT3-ITD and/or FLT3-TKD relative to FLT3-WT in normal karyotype AML is demonstrated in heatmap format. For the FLT3-TKD specific genes, only the top 100 genes based on FDR p-value are shown. Red and green colour indicates up-regulated and down-regulated genes respectively. The Venn diagram shows the number of differentially expressed probes by FLT3-ITD and/or FLT3-TKD compared to FLT3-WT. ** indicated gene discussed in the text. "---" indicates unannotated probe.

A.



B.

GSEA (Broad Institute)	p-value
TAKEDA_NUP8_HOXA9_8D_UP	6.25E-07
TAKEDA_NUP8_HOXA9_3D_UP	1.19E-06
VERHAAK_AML_NPM1_MUT_VS_WT_UP	1.23E-06
WELCH_GATA1	2.11E-04
V$GATA_C	2.70E-04
TAKEDA_NUP8_HOXA9_10D_UP	3.46E-04
TPA_SENS_MIDDLE_DN	8.82E-04
V$GATA_Q6	1.86E-03
V$GATA1_04	3.13E-03
V$GATA1_02	3.24E-03
V$GATA1_03	3.27E-03
GATAAGR_V$GATA_C	5.29E-03
VERHAAK_AML_NPM1_MUT_VS_WT_DN	2.10E-02
V$GATA3_01	3.40E-02
V$LMO2COM_02	3.40E-02

Figure 3.3. Both FLT3-ITD and FLT3-TKD repress *GATA1* target genes. A. The FLT3-ITD and FLT3-TKD commonly down-regulated genes were used to interrogate cell lineage specific gene expression data from mouse haemopoietic cells (Chambers et al, 2007). Each cell lineage was compared to the haemopoietic stem cell (HSC). Red colour indicates genes are up-regulated in the given cell lineage and green colour indicates genes are down-regulated in the given cell lineage. CD4N/CD8N = naïve T cells, CD4A/CD8A = Activated T cells, NuEry = nucleated erythrocyte, NK = natural killer, mono = monocyte, gran = granulocyte, B = B cells **B.** The commonly down-regulated genes show significant enrichment with *GATA1* transcription binding sites and other published datasets using Broad Institute GSEA (MSigDB) ranked by the p-value (hypergeometric statistics).

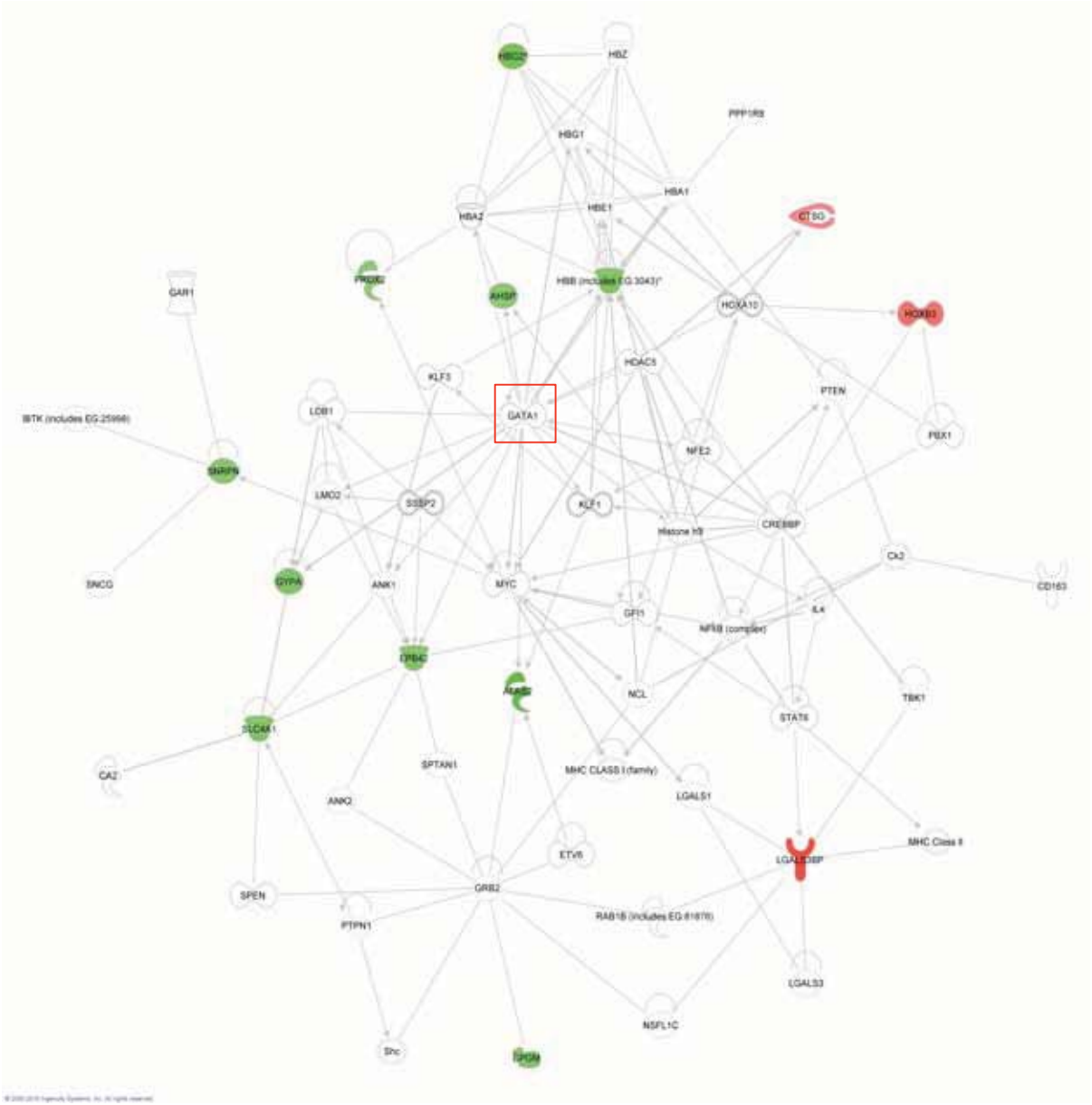


Figure 3.4. The network of the commonly up- and down-regulated genes by both FLT3-ITD and FLT3-TKD compared to FLT3-WT in NK AML. The top 2 networks derived from IPA were merged and filtered to remove indirect associations. Red indicates up-regulation in both FLT3 mutations and Green indicates down-regulation in both FLT3 mutations compared to FLT3-WT.

(Garzon, *et al* 2008). Recently, *HOXA9* has been reported to regulate miR-155 expression (Hu, *et al* 2010). To further understand the network of the differentially expressed genes, we used IPA to identify the network that is dysregulated by FLT3-ITD. As shown in **Figure 3.5**, IPA identified a network centred on Myc, NFκB and Fos suggesting that deregulation of these pathways may be important to FLT3-ITD. Kim *et al* reported that FLT3-ITD cell lines and patient cells showed over-expression of *MYC* (Kim, *et al* 2007). Knockdown of *MYC* expression by siRNA in a constitutively activated FLT3-ITD human cell line (MOLM-14) leads to decrease in cell proliferation suggesting the importance of Myc in providing cell proliferation signals from FLT3-ITD (Kim, *et al* 2007).

In the FLT3-TKD group, *TRIB1* was significantly up-regulated. *Trib1* and *Trib2* have been shown to induce AML in a mouse bone marrow transplant model (Dedhia, *et al* 2010), however up-regulation associated with FLT3 mutants has not been reported previously. A possible role for *TRIB1* downstream of FLT3-TKD is discussed further in the **Section 3.3.7.3**. Furthermore, we also identified *CSF2RA* (encoding the GM-CSF receptor alpha-subunit) and *CD68* (monocytic-macrophage marker) as significantly up-regulated in the FLT3-TKD NK AML group suggesting these may be markers for FLT3-TKD. Interestingly, we also identified *CD14* (macrophage marker) and *MAFB* (monocytic transcription factor) up-regulated in FLT3-TKD. This may suggest that FLT3-TKD and FLT3-ITD influence the lineage characteristics of the AML cells.

As shown in **Figure 3.6**, the network that was formed by FLT3-TKD is larger than FLT3-ITD as FLT3-TKD had more differentially expressed genes. Some of the FLT3-TKD central networks are the NFκB and C/EBPα networks. Both FLT3-ITD and FLT3-TKD have been

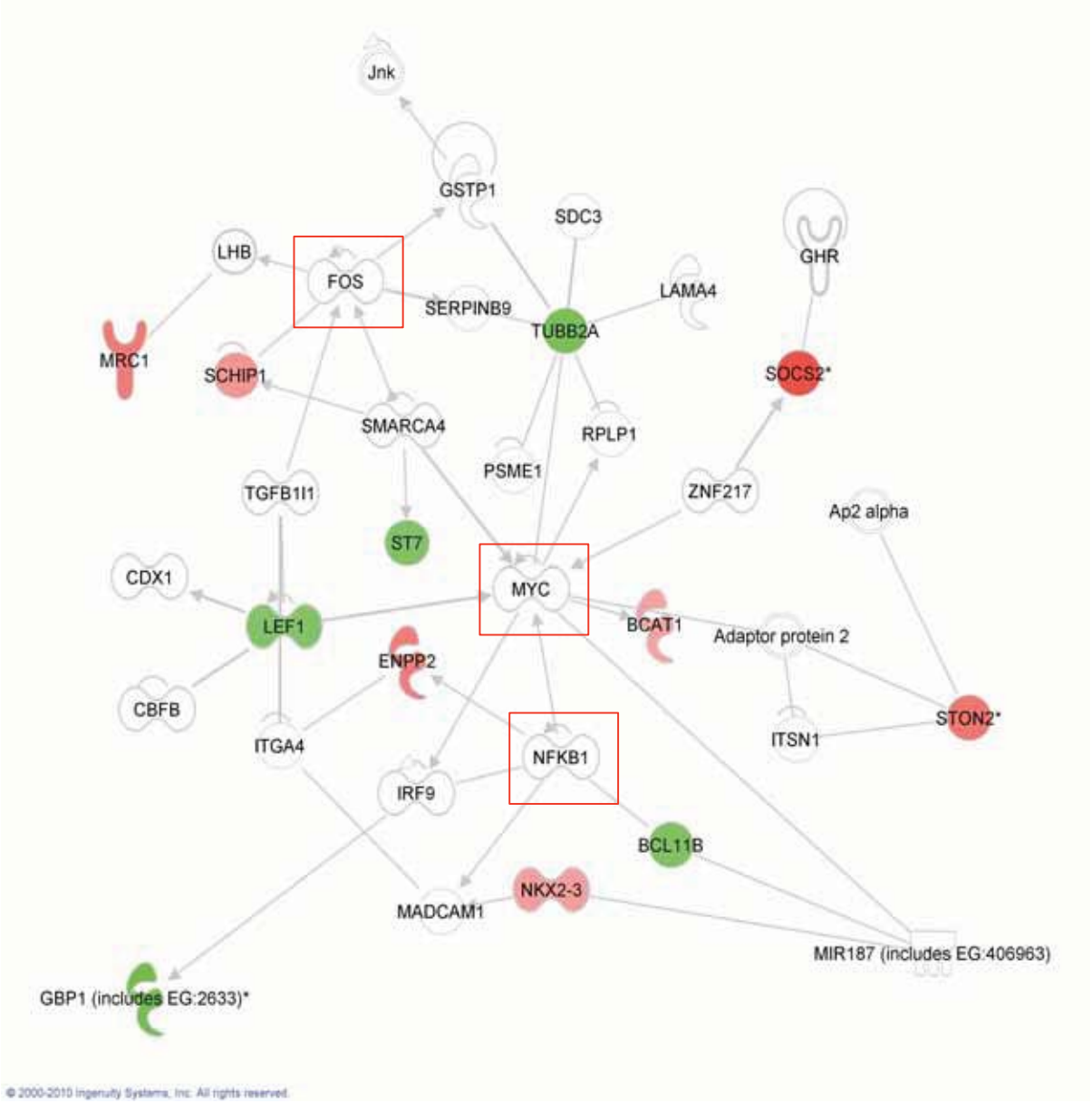


Figure 3.5. Differential gene expression of FLT3-ITD linked to a *MYC* network. The FLT3-ITD top 2 networks derived by IPA were merged together and filtered to remove indirect associations. Red indicates up-regulation in FLT3-ITD and Green indicates down-regulation in FLT3-ITD compared to FLT3-WT.

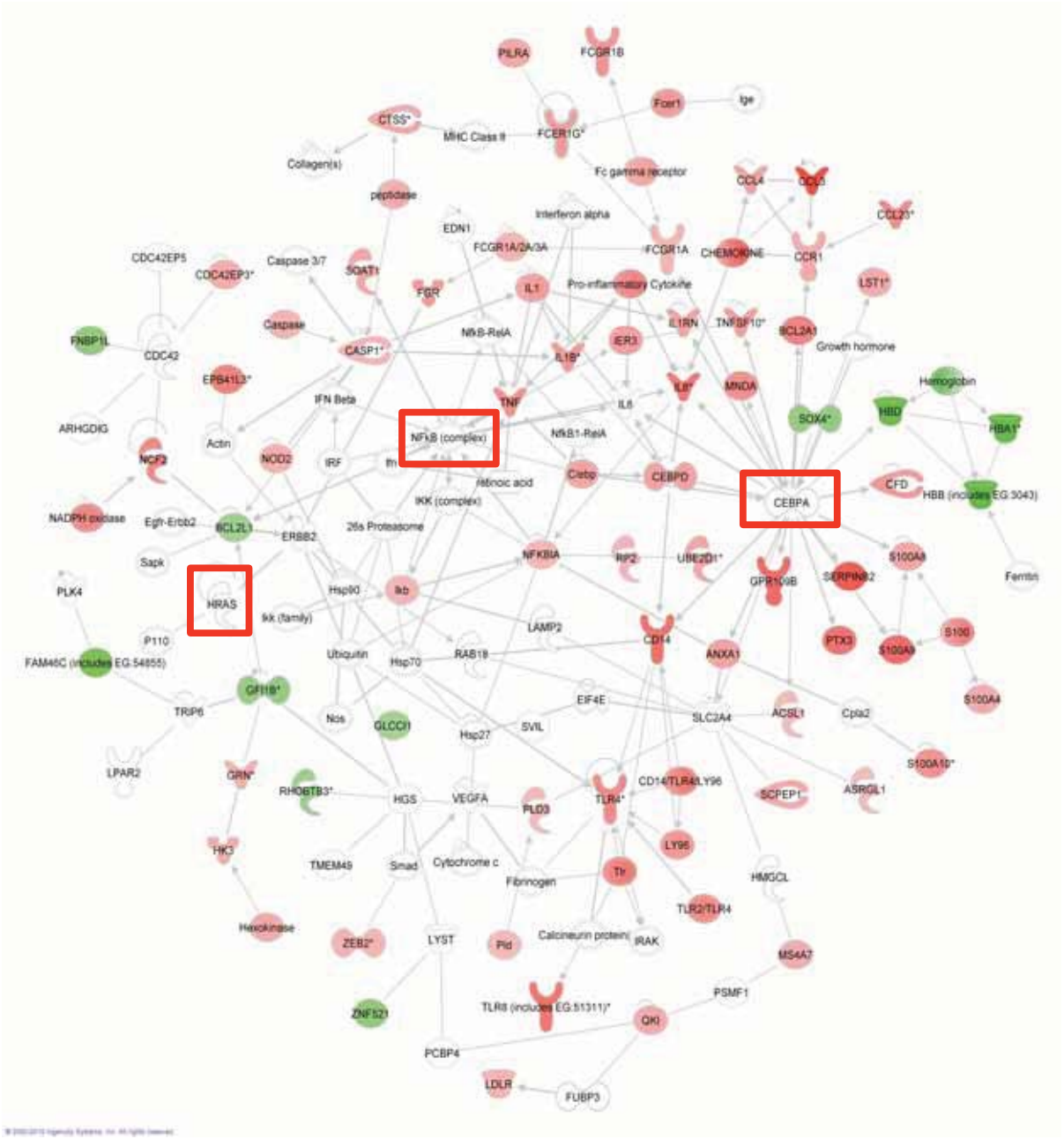


Figure 3.6. Differential gene expression of FLT3-TKD linked to a NFκB and *CEBPA* network. The FLT3-TKD top 5 networks and a network 10 derived by IPA were merged together and filtered to remove indirect associations. Red indicates up-regulation in FLT3-TKD and Green indicates down-regulation in FLT3-TKD compared to FLT3-WT.

previously reported to induce constitutive activation of NFκB in Ba/F3 cells expressing FLT3 mutants (Griessinger*, et al* 2007). Activation of NFκB is important for cell survival and therapeutic drug resistance (Karin 2006). As shown in **Figure 3.6**, H-Ras is part of FLT3-TKD network and Ono *et al* showed that FLT3-TKD is more sensitive to MEK inhibitor (U0126) than FLT3-ITD in the murine myelomonocytic cell line (HF6) expressing FLT3 mutants (Ono*, et al* 2009). This result suggests that FLT3-TKD might use RAS/MAPK/ERK pathway predominantly for transformation. With regard to C/EBPα, Choudhary *et al* showed that FLT3-ITD but not FLT3-TKD and FLT3-WT repress protein levels of C/EBPα (Choudhary*, et al* 2005). As shown in **Figure 3.6**, the expression for most of the genes that are linked to *CEBPA* is up-regulated suggesting the activation of C/EBPα in FLT3-TKD. Taken together, these gene expression results support that there are differences between FLT3-ITD and FLT3-TKD signalling.

3.3.3 Gene expression profiling of FLT3-ITD in NK acute myeloid leukaemia stem cell (LSC)

With a view to investigating the basis of disease in the NK group, we have analysed the gene expression profiles for the LSC from NK AML compared to normal HSC. The LSC AML dataset (Affymetrix HGU133plus2) available from NCBI GEO with accession number GSE17054 (Majeti*, et al* 2009) was reanalysed for this study. We focused on identification of genes in LSC that are differentially expressed in normal karyotype AML (n=6) compared to HSC (n=4). The patient details that were used in this analysis are shown in **Figure 3.7A**. However, in the Majeti *et al* study, the authors used meta-analysis to combine the LSC array data from University of Michigan (HGU133A or HGU133B) and Stanford University (Affymetrix HGU133plus2), and compared to HSC arrays from both side (University of

A.

Sample	Age/Gender	Karyotype	FLT3-ITD
SU001	59/F	normal	negative
SU004	47/F	normal	positive
SU008	64/M	normal	positive
SU014	59/M	normal	positive
SU018	21/M	normal	positive
SU032	47/M	normal	negative
SU006	51/F	unknown	negative
SU028	53/M	Complex	positive
SU031	31/F	inv(16)	negative

B.

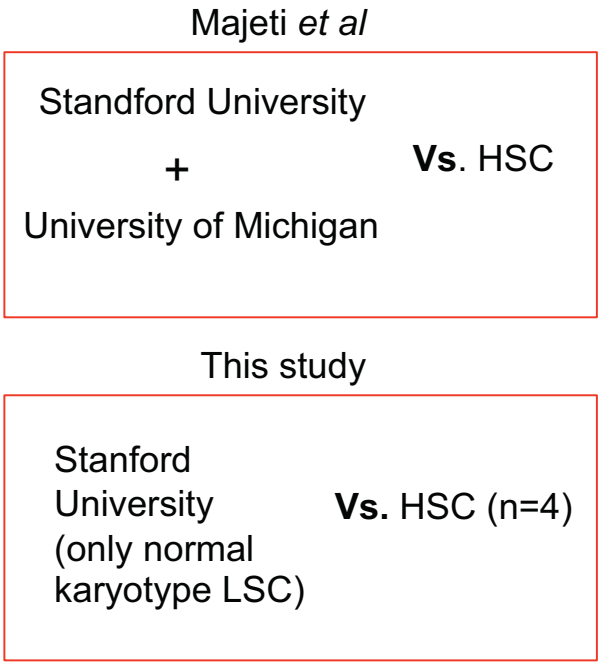


Figure 3.7. The microarray strategy to identify genes regulated by FLT3-ITD and/or FLT3-WT LSC in normal karyotype AML. A. Patient characteristics from our study (highlighted in red text) vs. Majeti *et al* study who used 9 LSC and 4 HSC from Stanford University, and 7 LSC and 3 HSC from University of Michigan. **B.** Microarray analysis strategy of our study and Majeti *et al*. The approach we use was different from Majeti *et al* as we focused on normal karyotype LSC relative to HSC. Please see text for more details.

Michigan, n=3; Stanford University, n=4) (**Figure 3.7B**) (Majeti*, et al* 2009). This is a complex analysis using different array platforms, and the isolation of LSC using different markers from University of Michigan are different from Stanford University. While, the University of Michigan isolates LSC with $Lin^-CD34^+CD38^-CD123^+$ and HSC ($Lin^-CD34^+CD38^-CD90^{lo}$), the Stanford University isolates LSC with $Lin^-CD34^+CD38^-CD90^-$ and HSC ($Lin^-CD34^+CD38^-CD90^+$) (Majeti*, et al* 2009). We normalized the raw data of NK LSC and HSC from Stanford University with RMA, and differentially expressed genes were identified using the empirical Bayes moderated t-statistics and LIMMA incorporating array quality weights (Smyth 2004). The FDR was controlled for multiple testing using Benjamini-Hochberg (BH) methods. We filtered the genes/probsets requiring at least a 2-fold change and a FDR p-value < 0.05.

We identified 3307 probes that are differentially expressed in NK LSC compared to HSC. There are 1319 probes that were identified in the previous analysis by Majeti *et al* (Majeti*, et al* 2009). We hypothesise that the additional 1988 probes identified by us may be specific for, or more pronounced in the NK AML group (data not shown). Of the genes that were identified in both analyses, known LSC marker genes or genes with known function that are critical for LSC (*IL3RA*, *CD96*, *CD47* and *ALOX5)* were identified as commonly up-regulated in NK LSC relative to HSC (**Figure 3.8**). The genes encoding the surface proteins (*CD99* and *CD109*), also displayed a significant association with NK LSC (**Figure 3.8**). These *CD99* and *CD109* genes have also been identified as differentially expressed genes in Majeti *et al* study suggesting that *CD99* and *CD109* are commonly dysregulated in LSC compared to HSC (Majeti*, et al* 2009). The expression of *CD99* is up-regulated while *CD109* is commonly down-regulated. Interestingly, *IL3RA* and *CD99* are closely linked at the pseudo-autosomal region Xp22.3 (Yp11.3). *CD99* encodes a cell surface glycoprotein, is

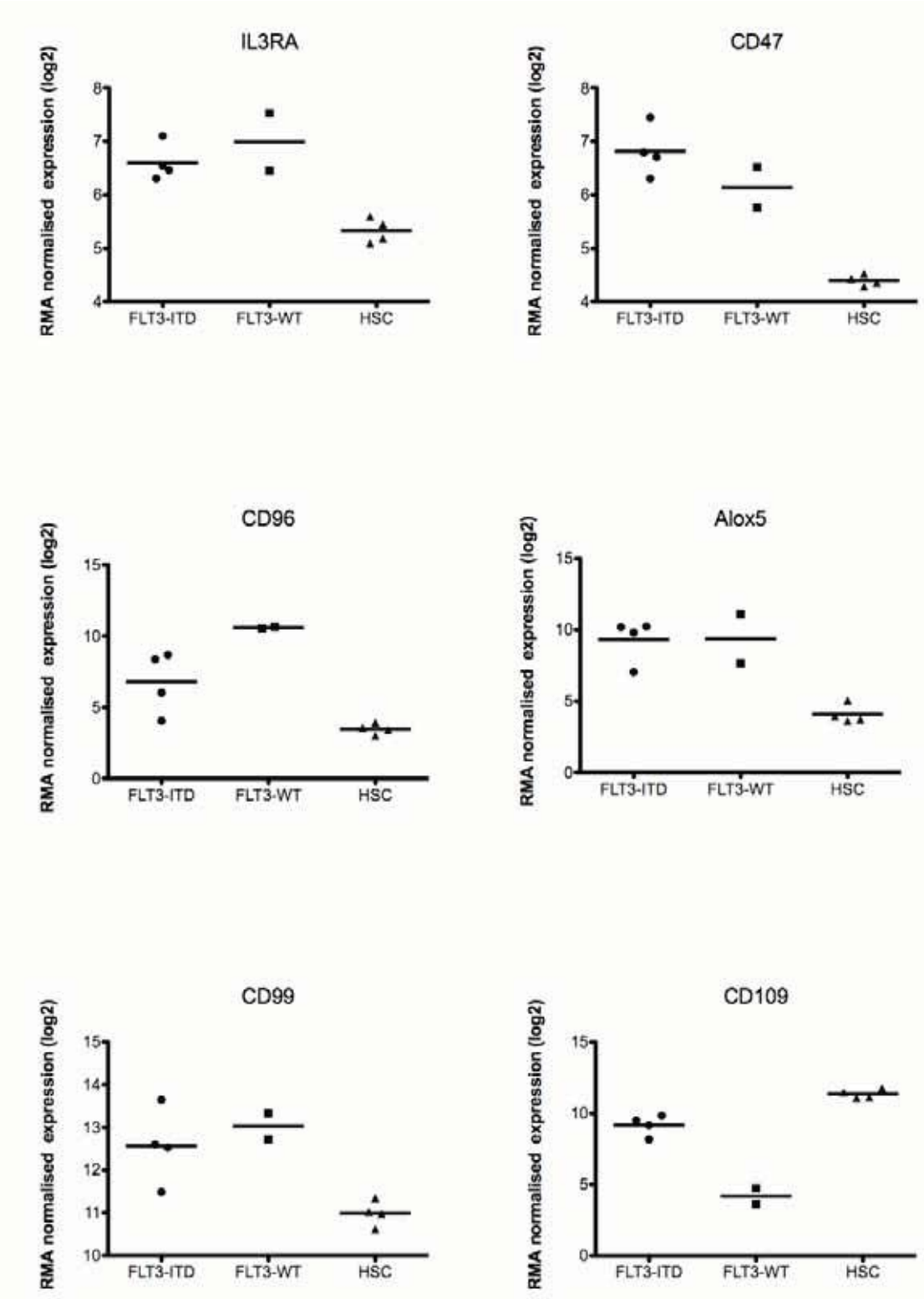


Figure 3.8. Commonly dysregulated genes in NK LSC AML. Known LSC genes such as *IL3RA, CD47, CD96* and *ALOX5* have been identified as differentially expressed genes (2 fold change and FDR p-value < 0.05) in LSC compared to HSC. Two genes *CD99* and *CD109* have not been previously identified associated with NK LSC.

involved in leukocyte migration and T-cell adhesion and has reported to be expressed in AML using immuno-histochemistry (Zhang, *et al* 2000). *CD109* is a glycophosphatidylinositol (GPI)-anchored membrane glycoprotein and is expressed at low levels on very primitive progenitor cells (Rappold, *et al* 1997). However, the expression or the functional role of *CD99* and *CD109* in AML LSC has not been investigated. Interestingly, we identified that expression of the Src family kinase *HCK* (out of 1988 probes as previously described above) is significantly (FDR p-value < 0.05) up-regulated (at least 16 fold change compared to HSC) in NK LSC. This most likely relates to the selected patient groups (NK AML vs all AML) and suggests that *HCK* expression is restricted to FLT3-ITD and/or NK LSC.

3.3.3.1 Focus of *HOX* genes that associated with FLT3-ITD in NK LSC

There are a number of *HOX* genes displaying differential expression specifically in NK LSC compared to HSC and interestingly identification of this differential *HOX* gene expression was not reported from the earlier comparison of LSC to HSC (Majeti, *et al* 2009). To investigate whether this differential *HOX* gene expression was related to the presence of FLT3 mutations in normal karyotype AML we further analysed this data to define differential expression associated with the FLT3-ITD (n=4) or FLT3 wild-type (FLT3-WT) (n=2) AML relative to HSC. The sample size in this analysis was small, nevertheless, using the approach above, we were still able to identify a number of LSC-associated genes displaying altered expression specifically in FLT3-ITD^{+} or FLT3-WT NK AML in comparison to HSC (**Figure 3.9A**). A limitation in this analysis relates to the representation of the NK FLT3-WT group with only 2 FLT3-WT samples, however this analysis suggests the possibility that there is differential expression of several of the *HOX* which may be selectively up-regulated in

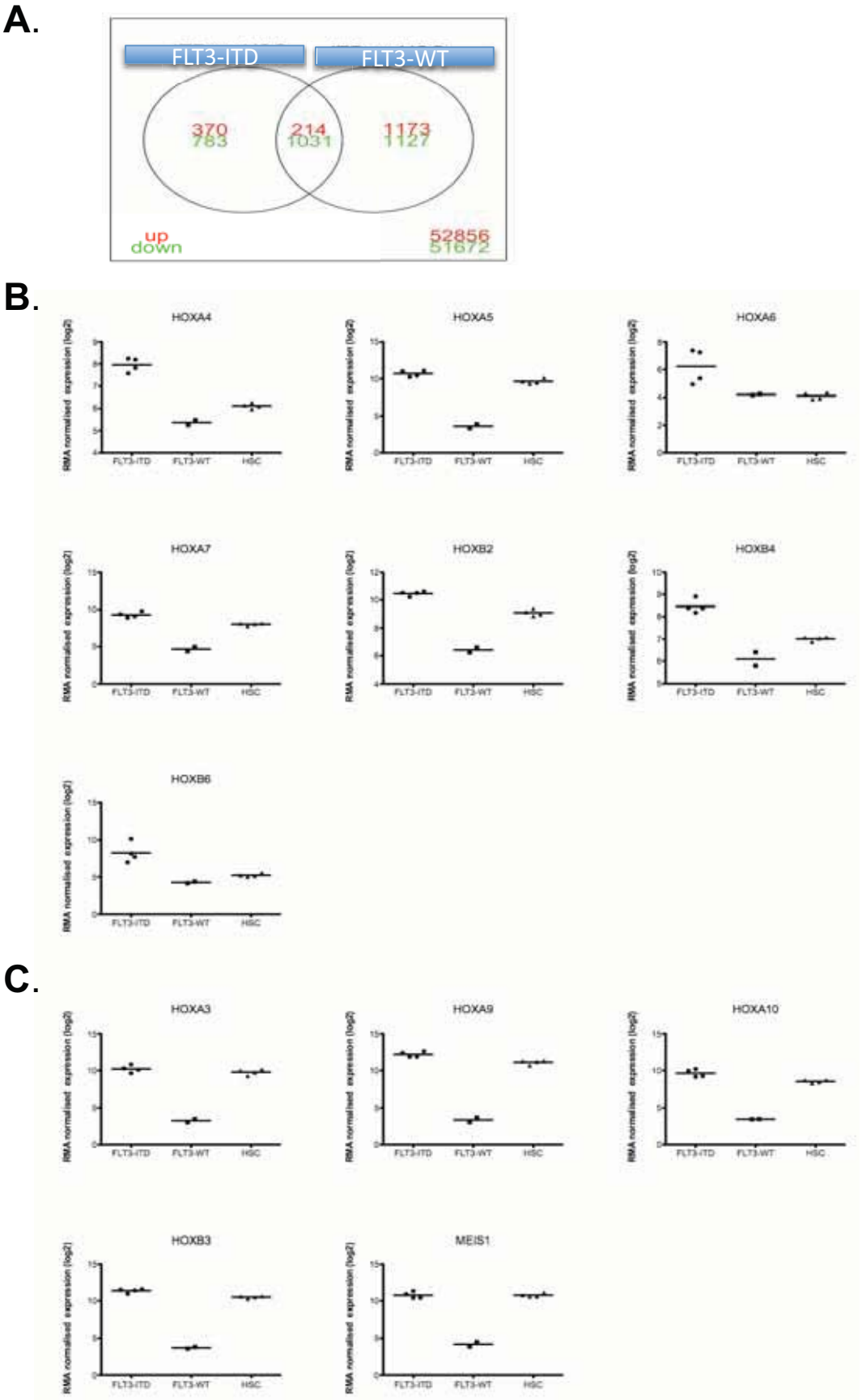


Figure 3.9. Differential expression of multiple HOX genes in FLT3-mutant LSC. A. Venn diagram shows total number of significant (FDR p-value < 0.05 and at least 2 fold change) probes in FLT3-ITD and/or FLT3-WT LSC compared to HSC. **B.** *HOX* genes (FDR p< 0.05 and fold change >2) that are dysregulated in FLT3-ITD NK LSC. **C.** *HOX* genes (FDR p< 0.05 and fold change >2) that are dysregulated in FLT3-WT NK LSC.

FLT3-ITD LSC. For example the *HOX* genes, *HOXA6, HOXA4, HOXA5, HOXB2, HOXA7, HOXB4* and *HOXB6* (**Figure 3.9B**) show selectively high expression in the FLT3-ITD group relative to LSC from FLT3-WT AML and HSC. The increased expression of selected *HOX* genes in NK AML may be associated with the presence of nucleophosmin (*NPM1*) mutations as *NPM1* is associated with normal karyotype AML and associated with high *HOX* gene expression (Mullighan*, et al* 2007). In this analysis we could not confirm the status of *NPM1,* as it is not defined in this dataset, however it is clear that the presence of FLT3-ITD in normal karyotype AML is independent of the presence of *NPM1* mutation (Grimwade and Hills 2009).

3.3.3.2 Genes selectively up-regulated in HSC and FLT3-ITD NK LSC

We also found that a number of *HOX* genes were commonly up-regulated in FLT3-ITD LSC and HSC compared to FLT3-WT LSC (**Figure 3.9C**). The selective high concordant expression of *MEIS1, HOXA9, HOXA10, HOXA3* and *HOXB3* in FLT3-ITD LSC and HSC suggests that FLT3-ITD^{+} AML may be derived from primitive progenitor or HSC, while FLT3-WT NK AML may initiate in a later progenitor or harboured *CEBPA* mutation (characterised by down-regulation of *HOXA* genes) (Kohlmann*, et al* 2010). This would be consistent with the high level expression of *HOXA9* and *MEIS1* in HSC and primitive cells of the myeloid lineage and CD34^{+}CD38^{-} LSC (compared to bulk AML cells) (Moore*, et al* 2007). Andreeff *et al* reported that low expression level of *HOXA* and *HOXB* genes is associated with favourable cytogenetic AML (Andreeff*, et al* 2008). Interestingly, by using the *HOX* gene family expression in LSC and Euclidean matrix hierarchical clustering, we can show that the FLT3-ITD group is readily separated from the FLT3-WT AML (**Figure 3.10**).

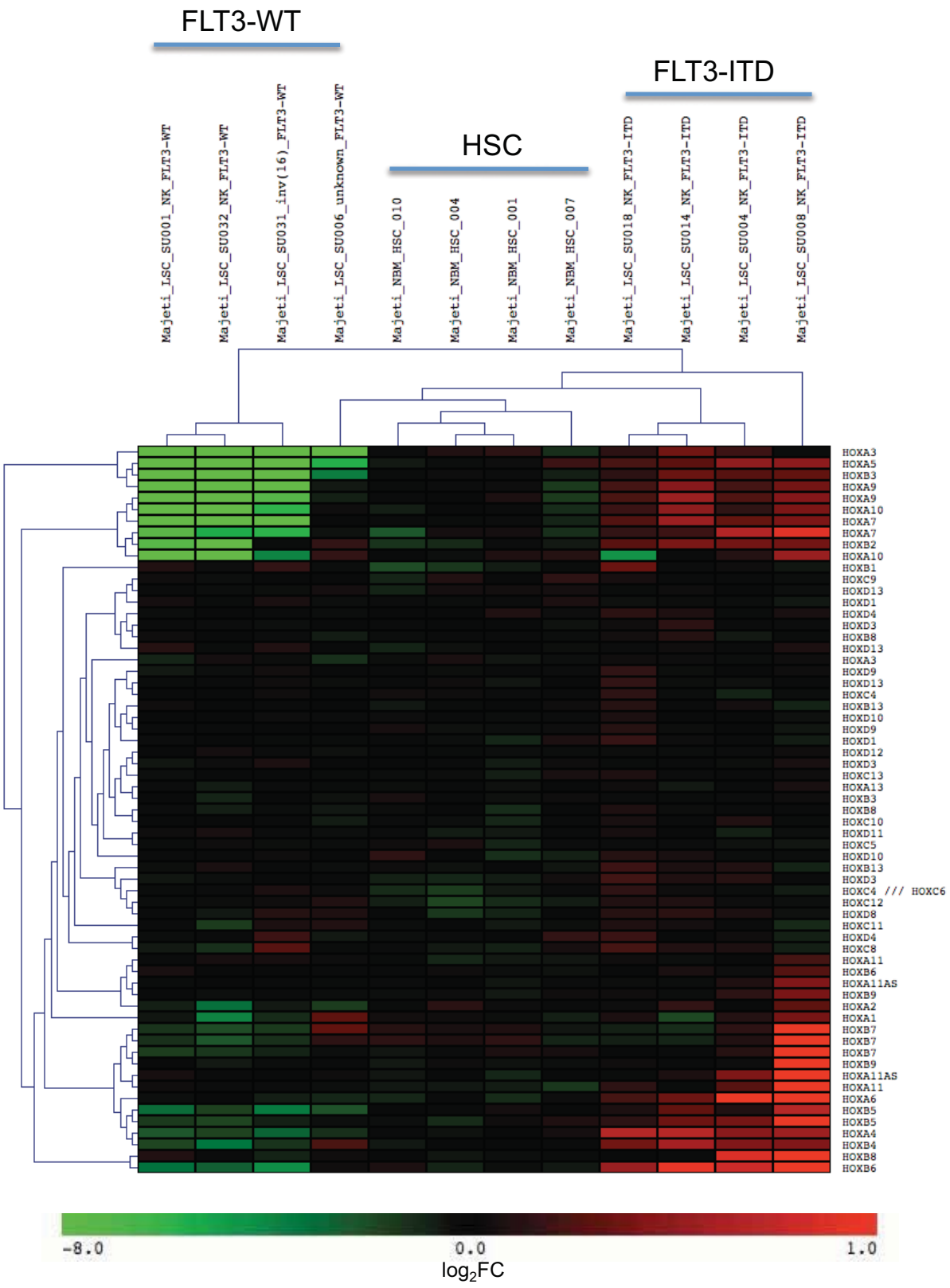


Figure 3.10. Hierarchical clustering of *HOX* gene family expression in LSC and HSC. All the LSC from Majeti *et al* (Stanford University) including AML other than normal karyotype were included in this analysis. The gene expression for each gene was median-centred. Hierarchical clustering with average linkage was used to cluster the genes and samples. Red indicates the expression is higher than the median-centred expression and green indicates the expression is lower than the median-centred expression.

This result confirms that with respect to *HOX* gene expression, FLT3-ITD LSCs are more closely related to HSC than FLT3-WT LSC AML.

3.3.3.3 *HOX* expression pattern in FLT3-ITD resembles normal HSC

We used Chambers *et al* gene expression data (Chambers*, et al* 2007) to identify the *HOX* gene expression patterns associated with normal haemopoietic cell differentiation. We identified that *HOXA9, MEIS1, HOXA2, HOXA5, HOXA10, HOXB5 and HOXB3* are down-regulated in all mature lineages assayed compared to HSC (**Figure 3.11**). *HOXA3, HOXB4, HOXA4 and HOXA1* were also down-regulated in mature lineages but not to he same degree. This is consistent with a role for this subset of *HOX* family genes maintaining the primitive phenotype, enhancing proliferation and expansion (Argiropoulos and Humphries 2007). The majority of *HOXA, HOXB* and *HOXC* genes are expressed in HSC and in immature progenitors (Argiropoulos and Humphries 2007). Thus, it is possible that FLT3-WT NK AML involves a distinct mechanism independent of *HOX* gene activation consistent with differential patterns of *HOX* gene expression being able to distinguish the FLT3-ITD from FLT3-WT NK LSC. Particular characteristics associated with these two groups, including differential response to therapy, may be related to this differential *HOX* gene expression.

3.3.3.4 Signalling pathways associated with NK LSC

To elucidate the pathway that is associated with NK LSC, we used IPA to analyse the differentially regulated genes. The top canonical pathways associated with the common LSC signature in NK AML were insulin-like growth factor 1 (IGF-1) signalling, phosphatase and tensin homolog (PTEN) signalling, and IL-3 signalling as shown in **Table 3.5**. Interestingly,

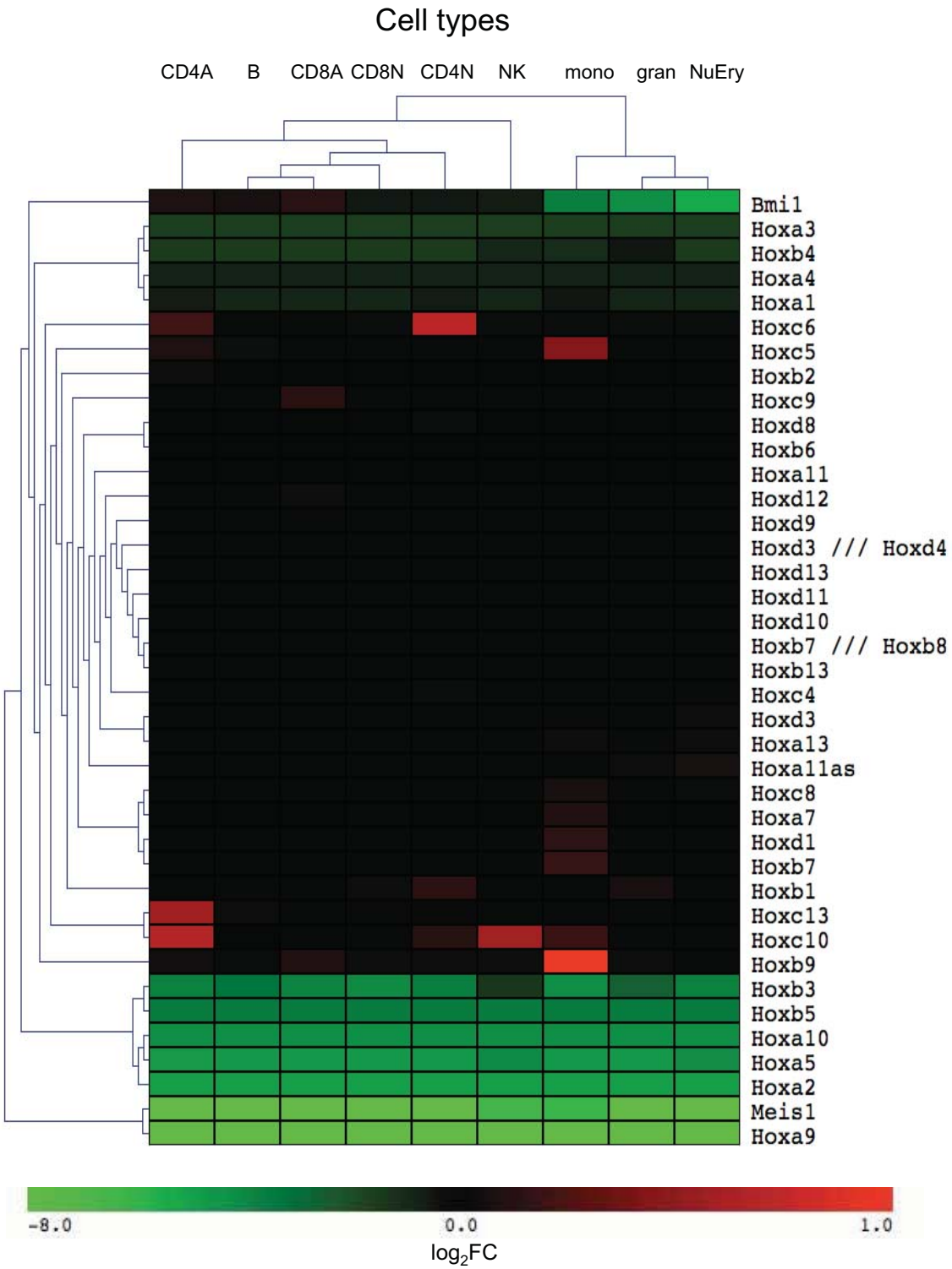


Figure 3.11. The gene expression pattern of the *HOX* gene family in mouse haemopoietic cell lineages. The gene for each cell lineage was compared to haemopoietic stem cell (HSC). The hierarchical clustering with average linkage was used to cluster the genes and samples. Red indicates the gene expression is higher in the lineage than in HSC and green is vice-versa. CD4N/CD8N = naïve T cells, CD4A/CD8A = Activated T cells, NuEry = nucleated erythrocyte, NK = natural killer, mono = monocyte, gran = granulocyte, B = B cells.

Table 3.5. Top significant canonical pathways regulated by LSC compared to HSC.

Top canonical pathways	LSC p-value
IGF-1 signalling	$9.5x10^{-5}$
PTEN signalling	$1.2x10^{-4}$
IL-3 Signalling	$1.7x10^{-4}$

Top canonical pathways	FLT3-ITD LSC p-value
Antigen presentation pathway	$1.7x10^{-4}$
Role of NFAT in regulation of the immune response	$2.0x10^{-3}$
ATM signalling	$4.6x10^{-3}$

Top canonical pathways regulated in NK LSC (top) and FLT3-ITD LSC (bottom) compared to HSC. The pathways were derived from IPA.

autocrine IGF-1/IGF-1R signalling has been shown to result in constitutive PI3K/AKT activation in 70% of AML at the blast cell level (Chapuis*, et al* 2009, Park*, et al* 2009) and our data suggests that this signal may initiate at the level of the LSC. Furthermore, the identification of PTEN signalling may indicate that LSC from NK AML have constitutive activation of the PI3K/AKT pathway which possibly contributes to survival and resistance to therapy (Grandage*, et al* 2005). The network that was derived from IPA pathway analysis indicates that the NK LSC differentially expressed genes are centrally linked to *MLL*, *GRB2, miR-124 and miR-1* (**Figure 3.12**). Whereas for FLT3-ITD LSC, the top canonical pathways derived from IPA are the antigen presentation pathway, role of nuclear factor of activated T-cells (NFAT) in regulation of the immune response, and ataxia telangiectasia mutated (ATM) signalling (**Table 3.5**). With ATM signalling, we found that there are 7 LSC genes that are dysregulated. Of these, 6 of those genes (*CCNB1, CCNB2, CDC2, CREB1, MDM4* and *NBN*) were down-regulated while only 1 gene (*ATF2*) is up-regulated suggesting that ATM signalling in LSC is down-regulated. This ATM signalling pathway is also identified by Majeti *et al* as one of the top 10 down-regulated pathways in LSC when compared to HSC (Majeti*, et al* 2009). The contribution of ATM signalling to AML will be discussed in the **Section 3.3.7.5**. Additionally, the networks analysis indicates that the FLT3-ITD NK LSC differentially expressed genes are centrally linked to AKT, MAPK*, miR-124, miR-1,* CREB*, histoneH3* and *histoneH4* (**Figure 3.13**). Interestingly, in this network, we identify the over-expression of the histone methyltransferase *DOT1L.* Krivtsov *et al* showed that inhibition of *DOT1L* by shRNA inhibits H3K79me2 resulting in reduced expression of *HOXA5* and *HOXA9* in human MLL-AF4 cell lines (Krivtsov*, et al* 2008).

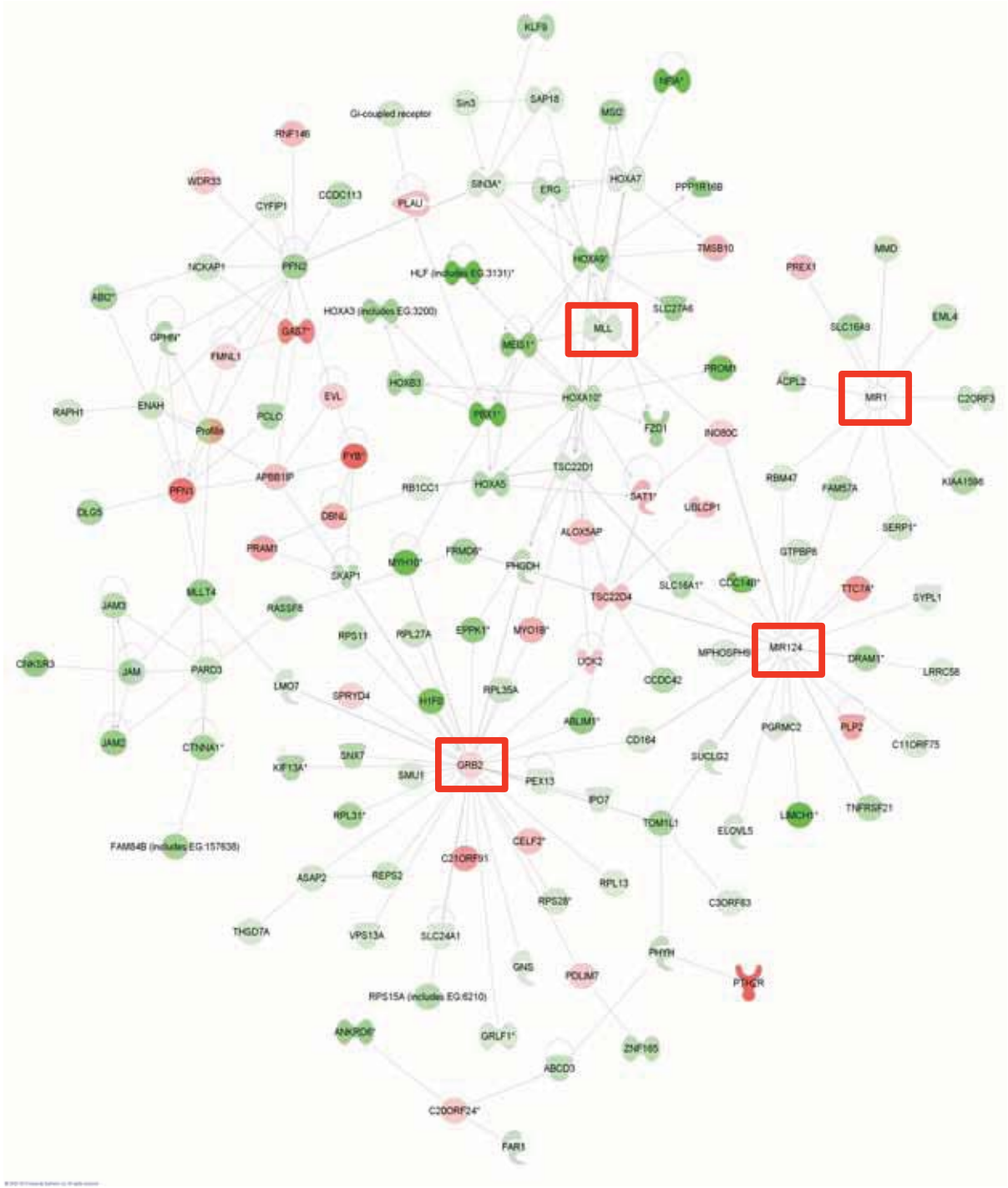


Figure 3.12. The network pathways derived from genes differentially expressed in NK LSC. The NK LSC top 5 networks (network1-5) that were derived by IPA were merged together and filtered to remove indirect associations. Red indicates genes up-regulation in NK-LSC and green indicates genes down-regulation in NK LSC compared to HSC.

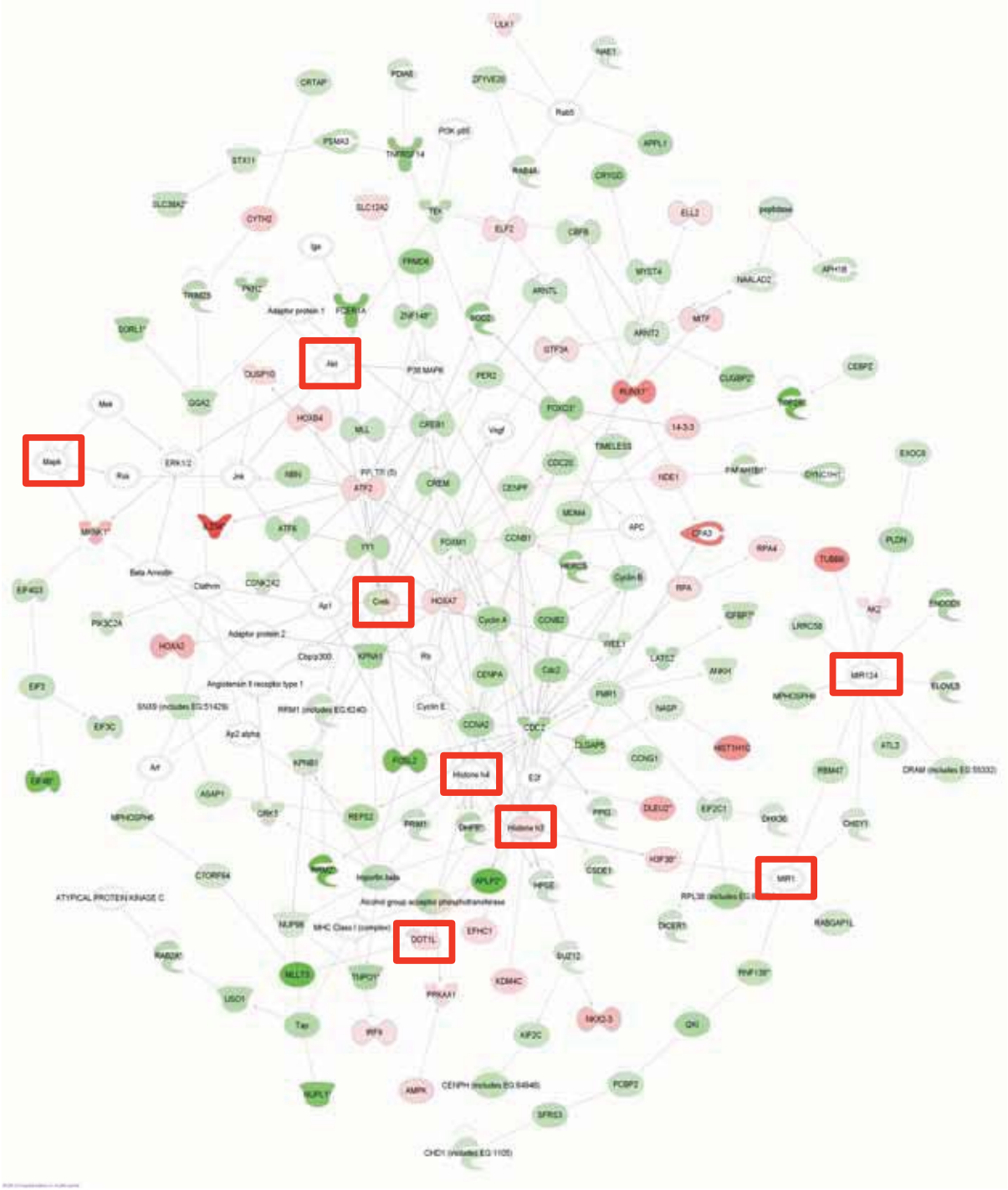


Figure 3.13. The network pathways derived from genes differentially expressed in FLT3-ITD NK LSC. The FLT3-ITD NK LSC top 5 networks and a network 7 that were derived by IPA were merged together and filtered to remove indirect associations. Red indicates genes up-regulation in FLT3-ITD NK-LSC and green indicates genes down-regulation in FLT3-ITD NK LSC compared to HSC.

3.3.4 Role of FLT3 mutants in the differentiation block in AML

3.3.4.1 FDB1 cells expressing FLT3 mutants or GMR-V449E are blocked in differentiation

To establish a model of FLT3 signalling, we generated FDB1 cells expressing either ITD (REYEYDL duplication) or TKD (D835Y) mutants of FLT3. To demonstrate that these FLT3 mutants are able to confer factor-independent growth in FDB1 cells, we performed cell proliferation assays with IL-3, GM-CSF, or in the absence of growth factor over a 5-day period. Parental FDB1 cells do not express endogenous FLT3 and do not respond to FLT3 ligand (FL) (range from 0 to 200 ng/ml) (data not shown). All the FLT3 and GMR-V449E FDB1 cell populations grew without growth factor indicating that all mutants were able to confer factor-independent growth. These results have been published in Perugini *et al* (see **Appendix D Figure 1C** and (Perugini*, et al* 2009).

The FDB1 system allows an assessment of the block in GM-CSF induced differentiation which is mediated by these activated receptor mutants. To examine differentiation, we cultured FDB1 cells expressing FLT3 mutants in GM-CSF for 5 days and determined the expression of myeloid differentiation markers (Gr-1 and Mac1) by flow cytometry. Gr-1 is a marker for granulocyte differentiation, however it is also detected transiently on intermediates in the monocyte lineage (Fleming*, et al* 1993). Mac-1 (CD11b) is a marker for monocytes and granulocytes (Ault and Springer 1981, Springer*, et al* 1979). All the mutants prevented an increase in expression of Gr-1 and Mac-1 in GM-CSF while parental FDB1 cells underwent differentiation into macrophages and neutrophils associated with increased Gr-1 and Mac-1 expression (**Figure 3.14A**) as reported previously

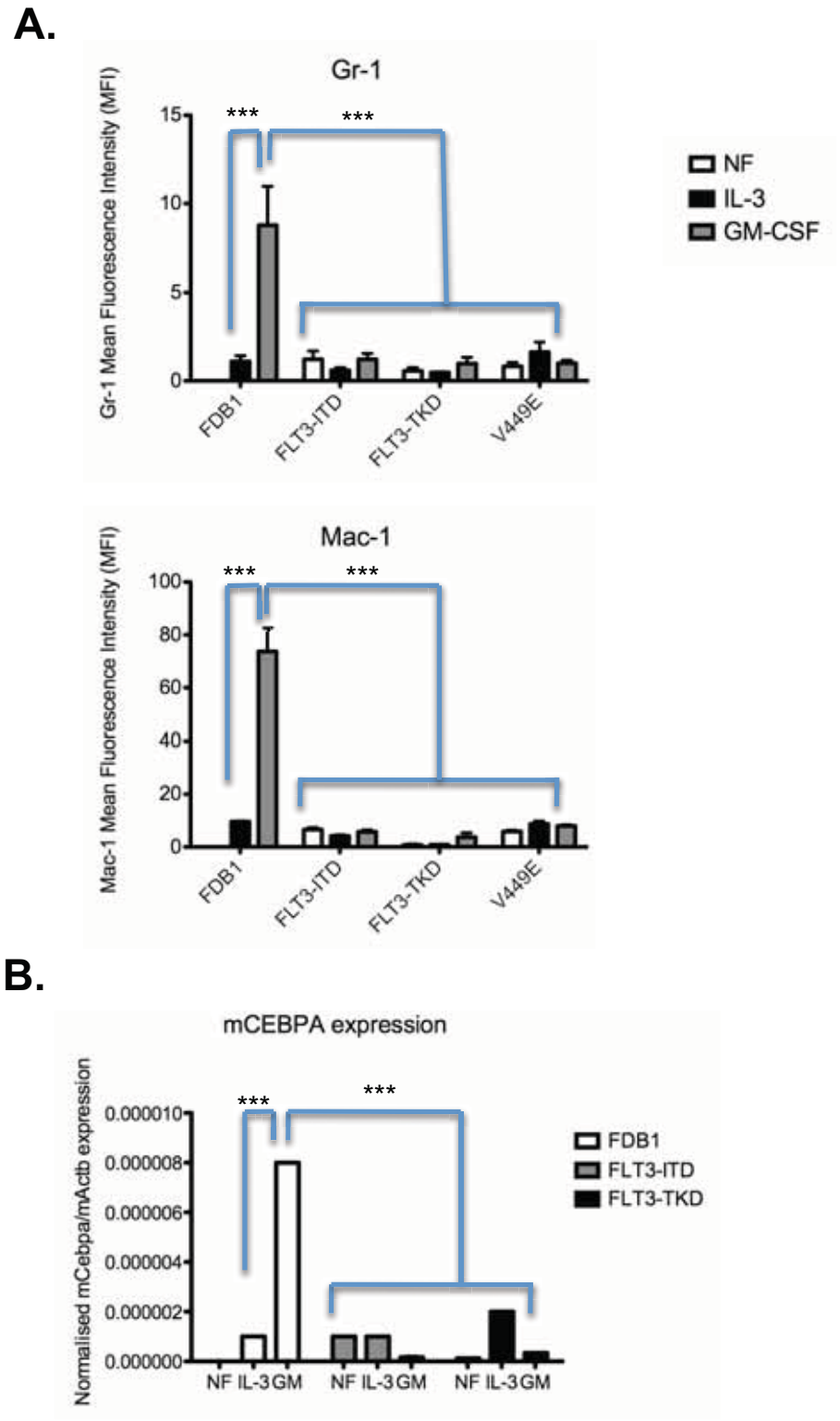


Figure 3.14. Assessment of myeloid differentiation in FDB1 cells expressing activated growth factor receptor mutants. A. Gr-1 (marker for granulocytes) and Mac-1 (marker for monocytes and granulocytes) mean fluorescence intensity level was determined by flow cytometry in FDB1 cells expressing FLT3-ITD, FLT3-TKD, GMR-V449E or parental cells in the absence of factor or with IL-3 or GM-CSF at day 5. **B.** The mRNA expression level of *Cebpa* quantitated by QPCR in FLT3 mutant FDB1 cells, cultured in the indicated conditions, at day 5. Data represent mean ± SEM for at least 2 independent experiments. *** $p<0.001$ determined by ANOVA compared to FDB1 in GM-CSF and corrected for multiple testing using Bonferroni.

(McCormack and Gonda 2000). In contrast, all the FLT3 mutants displayed low Gr-1 and Mac-1 mean fluorescence intensity (MFI) level at day 5. The Gr-1 and Mac-1 expression for all FLT3 mutants was comparable to parental FDB1 cells cultured in IL-3. The morphology of these FLT3 mutant cells was shown in Perugini *et al* **Appendix D Figure 1B** (Perugini*, et al* 2009). To further confirm the block in differentiation in FLT3 mutant FDB1 cells, we investigated the expression of the master regulator of granulocyte differentiation, *Cebpa*. In IL-3, all the mutants including parental FDB1 cells expressed low *Cebpa* expression (**Figure 3.14B**). As shown in **Figure 3.14B**, *Cebpa* expression is repressed by FLT3 mutants when cultured in GM-CSF whereas parental FDB1 cells induced *Cebpa* expression in the same condition. Expression of *Cebpa* in the FLT3 mutant cells in the absence of factors remained low with similar levels to cells in IL-3. Thus, the constitutive signalling induced by FLT3 mutants is associated with continuous growth and survival, and an effective block in GM-CSF-induced differentiation. Similar effects of the FLT3-ITD and FLT3-TKD mutant receptors have been reported in other murine cell lines (Hayakawa*, et al* 2000, Mizuki*, et al* 2003). As discussed in **Chapter 1 Section 1.9.1**, the FDB1 system provides an excellent tool for dissecting GM-CSF induced differentiation and for analysing leukaemic pathways that impact on this process. We next conducted experiments examining the pathways that may contribute to the block in differentiation.

3.3.4.2 The ERK1/2 pathway contributes to survival and blocks differentiation of FLT3 mutants in FDB1 cells

As previously described in **Section 1.7.2** and **Section 3.1**, constitutive activation of the MAPK/ERK pathway inhibits granulocytic differentiation via suppression of C/EBPα activity and we hypothesise that this may link in part to changes in *Gadd45a* expression. To

determine the effect of the ERK1/2 pathway in granulocytic differentiation in the FDB1 myeloid system, we first analysed the role of the ERK1/2 pathway in FDB1 cells expressing FLT3 mutants and GMR-V449E using the MEK inhibitor (targeting MAPK/ERK pathway), U0126 and its control U0124. We have previously shown that FDB1 cells expressing FLT3 mutants or GMR-V449E show constitutive activation of ERK1/2 signalling and this is associated with down-regulation of the key growth regulator *Gadd45a* (Perugini*, et al* 2009). Here, we have characterised the viability and myeloid differentiation of FDB1 cells expressing FLT3 mutants and GMR-V449E after treatment with U0126, or the control U0124, in the absence of growth factor for 48 hours.

As shown in **Figure 3.15**, in the absence of growth factor, FDB1 cells expressing FLT3-TKD and treated with U0126 were significantly reduced in cell viability at 24 hours. FDB1 GMR-V449E cells were less sensitive than FLT3-TKD cells, but their viability was also significantly reduced. At 48 hours, cell viability for all the mutants was significantly reduced with no viable FLT3-TKD cells remaining (**Figure 3.15**). Thus, the ERK1/2 signalling pathway is particularly important for survival in response to the FLT3-TKD mutant.

In addition to cell viability, we also examined differentiation of U0126-treated FDB1 populations expressing FLT3 mutants or GMR-V449E at 48 hours using Gr-1 expression. In the absence of growth factor, FDB1 cells expressing FLT3-ITD or GMR-V449E and treated with U0126 significantly increased the Gr-1 mean fluorescence intensity (MFI) by more than 2 fold as shown in **Figure 3.16A-B**. Additionally, for both FLT3-ITD and GMR-V449E in the presence of U0126, the morphology of the cells changed from immature (promyelocyte appearance) to have features such as lobed nucleus, lower nuclear:cytoplasmic ratio and

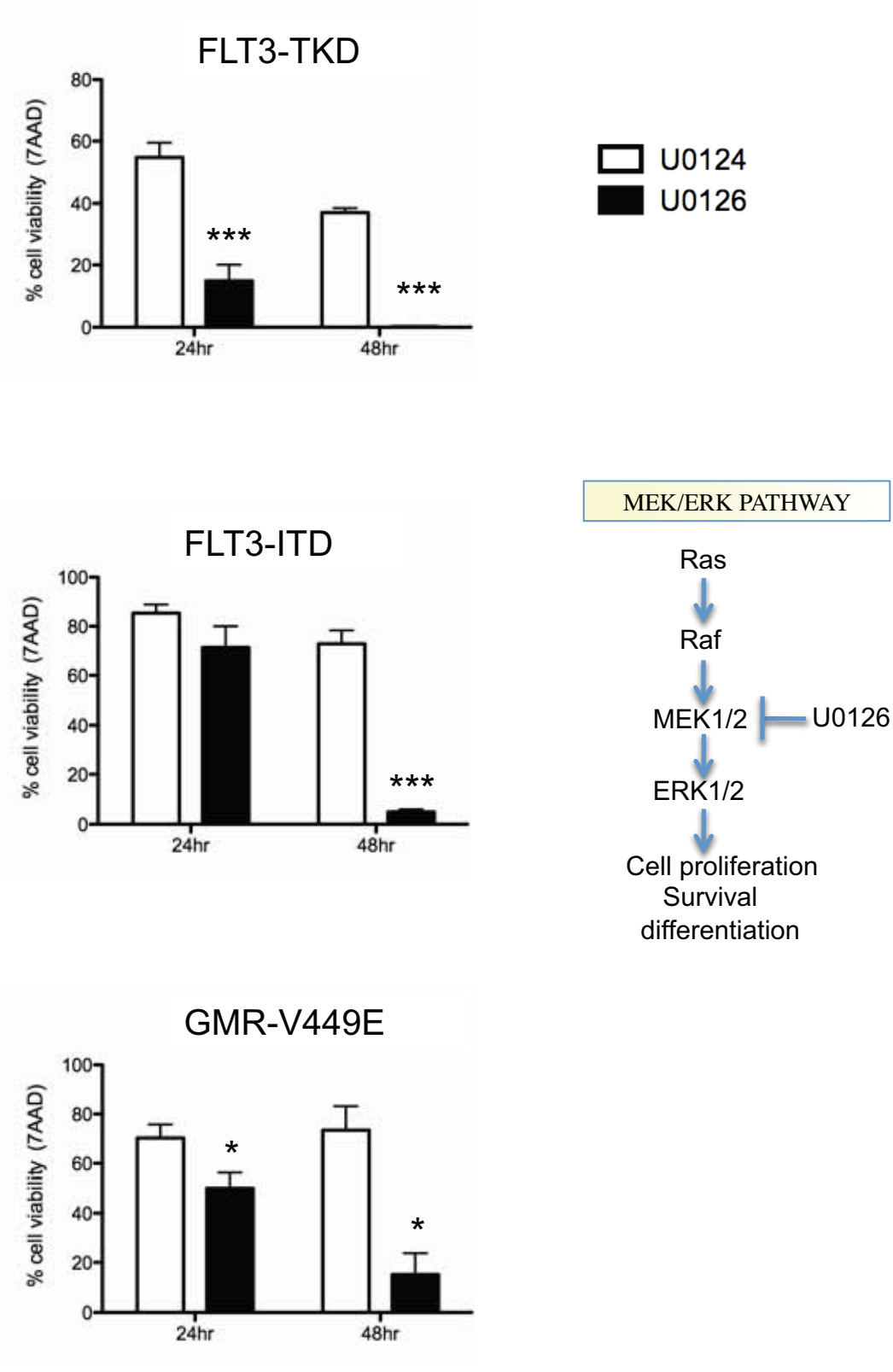


Figure 3.15. Effect of the MEK inhibitor, U0126, on cell viability. FDB1 cells, FLT3-ITD, FLT3-TKD and GMR-V449E cells were treated with U0126 and its control U0124 in the absence of growth factor for 24 hr and 48 hr. Cell viability was assessed using 7AAD staining and flow cytometry. Data represent mean ± SEM of at least 2 independent experiments**.** *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ determined by two-tailed student's t-test compared to the control U0124.

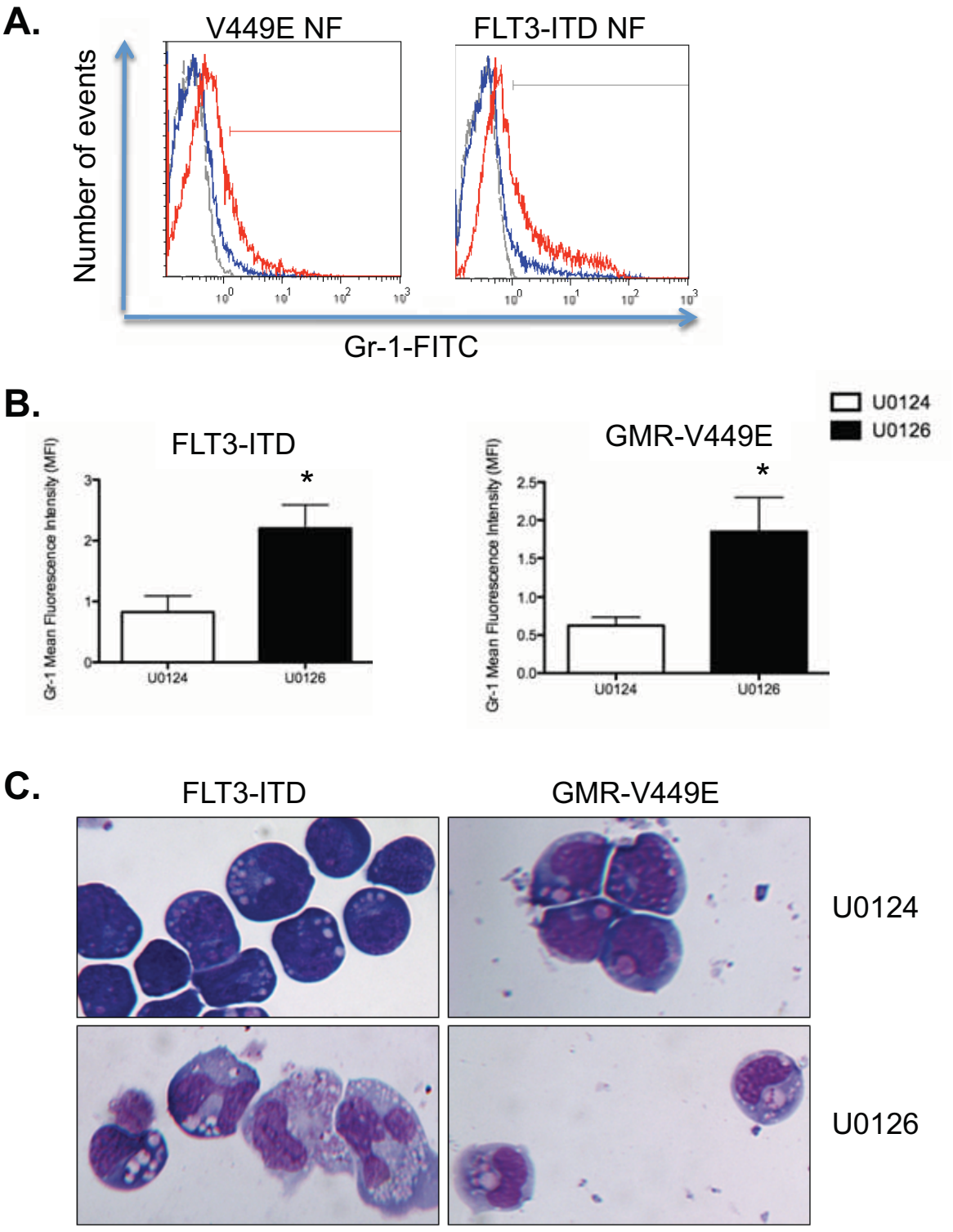


Figure 3.16. Treatment with U0126 induced Gr-1 myeloid differentiation on FDB1 cells expressing FLT3 mutants and GMR-V449E. A. Gr-1 expression was examined by flow cytometry on FLT3-ITD and GMR-V449E cells treated with the MEK inhibitor U0126, or its control, U0124 for 48 hours in the absence of growth factor. Grey indicates isotype control. Blue indicates cells treated with U0124. Red indicates cells treated with U0126. **B.** Mean fluorescence intensity of Gr-1 expression. Data represent mean ± SEM of at least 2 independent experiments. *p<0.05, **p<0.01 determined by two-tailed student's t-test compared to the control U0124. **C.** The morphology of FLT3-ITD and GMR-V449E cells treated with U0126 for 48 hours. The cells were cytocentrifuged and stained with May-Grunwald-Giemsa. Original magnification 400x.

lighter cytoplasm (**Figure 3.16C**). These results are consistent with constitutive activation of ERK1/2 inducing a block differentiation as suggested by Radomska *et al* (Radomska*, et al* 2006). As FLT3-TKD cells were not viable at 48 hours, it was not possible to assess differentiation status. There was no significance Mac-1 difference observed in the absence of IL-3 after treatment with U0126 in any of the cell lines (data not shown). Thus, ERK1/2 signalling is contributing to viability and the differentiation block in response to FLT3 activated mutants and GMR-V449E in this FDB1 system.

3.3.5 The role of *Gadd45a* downstream of FLT3 activated mutants

The expression of *Gadd45a* is induced by pro-apoptotic signals, and during myeloid differentiation. *GADD45A* is a tumour suppressor gene and is known to be mutated in some solid cancers (pancreatic adenocarcinoma) (Soliman*, et al* 2006). GADD45A is also silenced in cancers (e.g. breast cancer, osteosaroma and prostate cancer) through DNA methylation (Al-Romaih*, et al* 2008, Ramachandran*, et al* 2009, Wang*, et al* 2005). Interestingly, *GADD45A* expression has previously been shown to be repressed in response to sustained ERK1/2 signalling (Yamamoto*, et al* 2006) and is a known target gene of *CEBPA* and *MYC* (Constance*, et al* 1996, Tao and Umek 1999). Moreover, bone marrow from *Gadd45a* knockout mice has increased blast cells and reduced granulocytes (Gupta*, et al* 2006). These findings suggest that *Gadd45a* has important roles in myeloid differentiation and may be down-regulated by constitutively activated MAPK/ERK pathway either via suppression of C/EBPα activity, or via stablisation of c-Fos and enhanced AP-1 binding (Radomska*, et al* 2006, Sharrocks 2006). Given that this potential FLT3-MAPK/ERK-*GADD45A* pathway may be important in AML, we wished to explore the functional role of *Gadd45a* in the myeloid system and its potential as a tumour suppressor in AML. **These studies were**

performed in collaboration with Dr Michelle Perugini and were recently published in *Leukemia* with myself as co-first author (Perugini, *et al* 2009). Briefly, we identified the expression of *Gadd45a* as being commonly down-regulated in both FLT3-ITD and GMR-V449E leukaemic receptors through bioinformatic analysis and further confirmed by this Q-PCR. We then focused on the role of this commonly down-regulated gene. We showed that FLT3 mutants repress *Gadd45a* expression in the murine FDB1 cell line and in human AML patient samples (see **Appendix D Figure 2a and Figure 3a**) (Perugini, *et al* 2009). The expression of *Gadd45a* increased when treated with MEK inhibitor U0126 in FDB1 cells expressing FLT3 or GMR-V449E mutants (see **Appendix D Figure 2c-f**) consistent with *Gadd45a* expression being regulated by the MAPK/ERK pathway as reported by Yamamoto *et al* (Yamamoto, *et al* 2006). *Gadd45a* over-expression and knockdown in FDB1 cells and FLT3-ITD$^+$ AML cell lines also showed that *Gadd45a* expression is associated with granulocytic differentiation, as measured by increased Gr-1 expression, and induced cell survival respectively (see **Appendix D Figure 4-5**) (Perugini, *et al* 2009).

3.3.5.1 Role of *Gadd45a* in the differentiation block

Given the findings discussed above that *Gadd45a* suppresses growth and reduces survival of FDB1 expressing FLT3-ITD and GMR-V449E, and AML cell lines (see **Appendix D Figure 5**) (Perugini, *et al* 2009), we used our FDB1 model of FLT3 signalling to further examine the role and mechanism of *Gadd45a* down-regulation in the differentiation block induced by FLT3-ITD. We have reported previously that FLT3-ITD FDB1 cells appear to be blocked at a different stage of myeloid differentiation compared to cells expressing GMR-V449E (Perugini, *et al* 2009). In contrast to GMR-V449E cells, transduction of these FLT3-ITD FDB1 cells with *Gadd45a* did not induce Gr-1 marker expression (see **Appendix D Figure**

5d) (Perugini*, et al* 2009)) but there was a reduction in cell viability. To further investigate the changes induced by *Gadd45a* in the presence of FLT3-ITD or GMR-V449E differentiation block, we over-expressed the *Gadd45a* cDNA using the retroviral vector pRuf-IRES-eGFP and determined the mRNA expression level of the differentiation genes: *Cebpe* (granulocyte specific gene), *Cebpa* and *Sfpi1* (*Pu.1)* by QPCR.

We first determined the gene expression changes in GMR-V449E cell populations. As shown in **Figure 3.17A**, the expression level of *Cebpa, Cebpe* and *Sfpi1 (Pu.1)* increased following retroviral expression of *Gadd45a* in cells expressing GMR-V449E. GMR-V449E cells transduced with *Gadd45a* significantly increased *Cebpa* expression at day 0 but these normalised at day 3. Most notably, *Cebpe* expression level was significantly increased at both day 0 and day 3 after sorting for GFP (**Figure 3.17A**). In addition, *Sfpi1* (*Pu.1)* expression level increased 2 fold at day 0 and day 3 compared to empty vector control. These results are consistent with the increase in Gr-1 expression upon *Gadd45a* over-expression (see above) and provide strong evidence for induction of a granulocytic-monocyte differentiation program.

Interestingly, in FLT3-ITD cells, *Gadd45a* over-expression did not induce expression of *Cebpa* at day 0 but *Cebpa* expression level significantly increased at day 3 (**Figure 3.17A**). There was no change in the expression level of *Cebpe* or *Sfpi1* (*Pu.1*) at either day 0 or day 3 (**Figure 3.17A**). Thus, while over-expression of *Gadd45a* induced a significant increase in *Cebpa* expression in FLT3-ITD FDB1 cells, this was not sufficient to induce *Cebpe* or Gr-1 expression. In contrast, over-expression of *Gadd45a* in GMR-V449E FDB1 cells was associated with transient *Cebpa* expression and an increase in *Cebpe and Sfpi1 (Pu.1)*

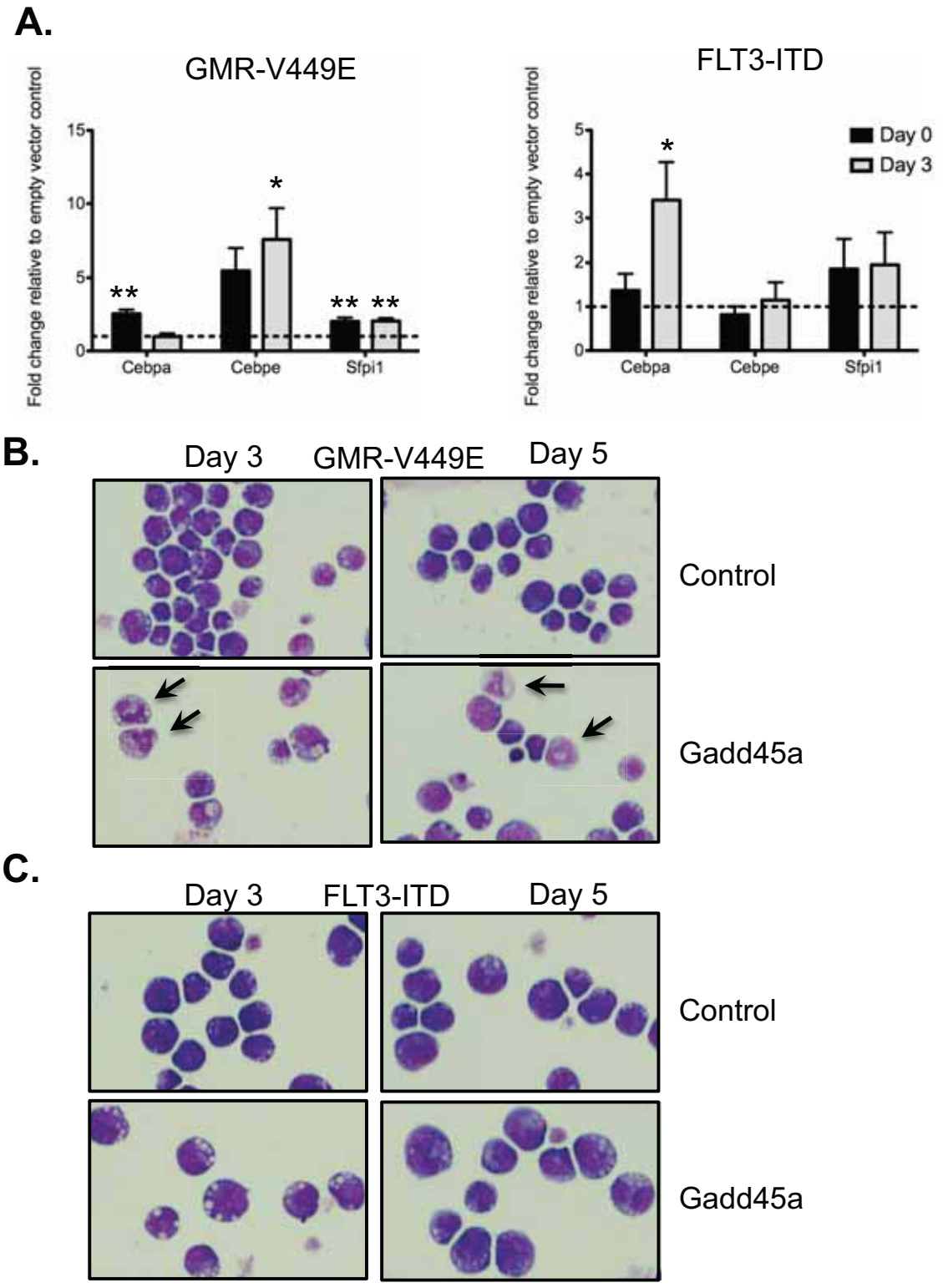


Figure 3.17. The effect of *Gadd45a* over-expression on the block in myeloid differentiation in GMR-V449E and FLT3-ITD FDB1 cells. A. GMR-V449E and FLT3-ITD cells were transduced with either pRuf-IRES-eGFP (control) or pRuf-*mGadd45a*-IRES-eGFP. Cells were enriched for GFP expression using flow cytometry and cells cultured without growth factors. Gene expression profiling of mouse *Cebpa, Cebpe, and Sfpi1(Pu.1)* at day 0 or day 3 after cell sorting, in the absence of growth factor. Data represent the mean ± SEM of 2 independent experiments**.** **p<0.01, *p<0.05 determined by two-tailed student's t-test relative to control. **B-C.** The morphology of GMR-V449E and FLT3-ITD cells 3 and 5 days after GFP sorting. The cells were May-Grunwald-Giemsa stained. Arrows indicate granulocytes. Original magnification 200x.

consistent with the previously observed increase in Gr-1 expression and the granulocytic morphology changes observed in these cells (**Figure 3.17B**). As shown in **Figure 3.17C**, there was no apparent morphology change in FLT3-ITD cells transduced with *Gadd45a*. The capacity of *Gadd45a* to induce differentiation (as measured by Gr-1 staining (Perugini*, et al* 2009) and gene expression) appears to be restricted to GMR-V449E FDB1 cells suggesting that the differentiation inducing activity of *Gadd45a* maybe stage-specific and/or require specific co-factors.

3.3.5.2 Regulation of *Gadd45a* expression level in haemopoiesis

We have previously observed that *Gadd45a* expression is increased during FDB1 differentiation in response to GM-CSF (see **Appendix D Figure 2a**) (Perugini*, et al* 2009) and *Gadd45a* deficient mice have reduced GM progenitors (Gupta*, et al* 2006). This data suggested that *Gadd45a* is involved in myeloid differentiation and has regulated gene expression. To investigate the regulation of *Gadd45a* in normal haemopoiesis, we performed a detailed analysis of *Gadd45a* expression using published gene expression profiling data. We used two published microarray datasets in which global gene expression changes were measured in different haemopoietic populations (Chambers*, et al* 2007, Tonks*, et al* 2007b). As shown in **Figure 3.18A**, in the human haemopoietic system (Tonks*, et al* 2007b), relative to immature cells ($CD34^+$), *GADD45A* expression level increases in granulocytes and erythrocytes. For comparison, *CEBPE* expression was also determined in this dataset and as expected, this increased selectively in granulocyte populations compared to $CD34^+$. We also included other known lineage-associated genes (*GFI1* - granulocytes*, IRF8* – monocytes, *GATA1* - erythrocytes) and show their appropriate regulation providing further validation for this microarray dataset. We also investigated *Gadd45a* expression in mouse haemopoietic

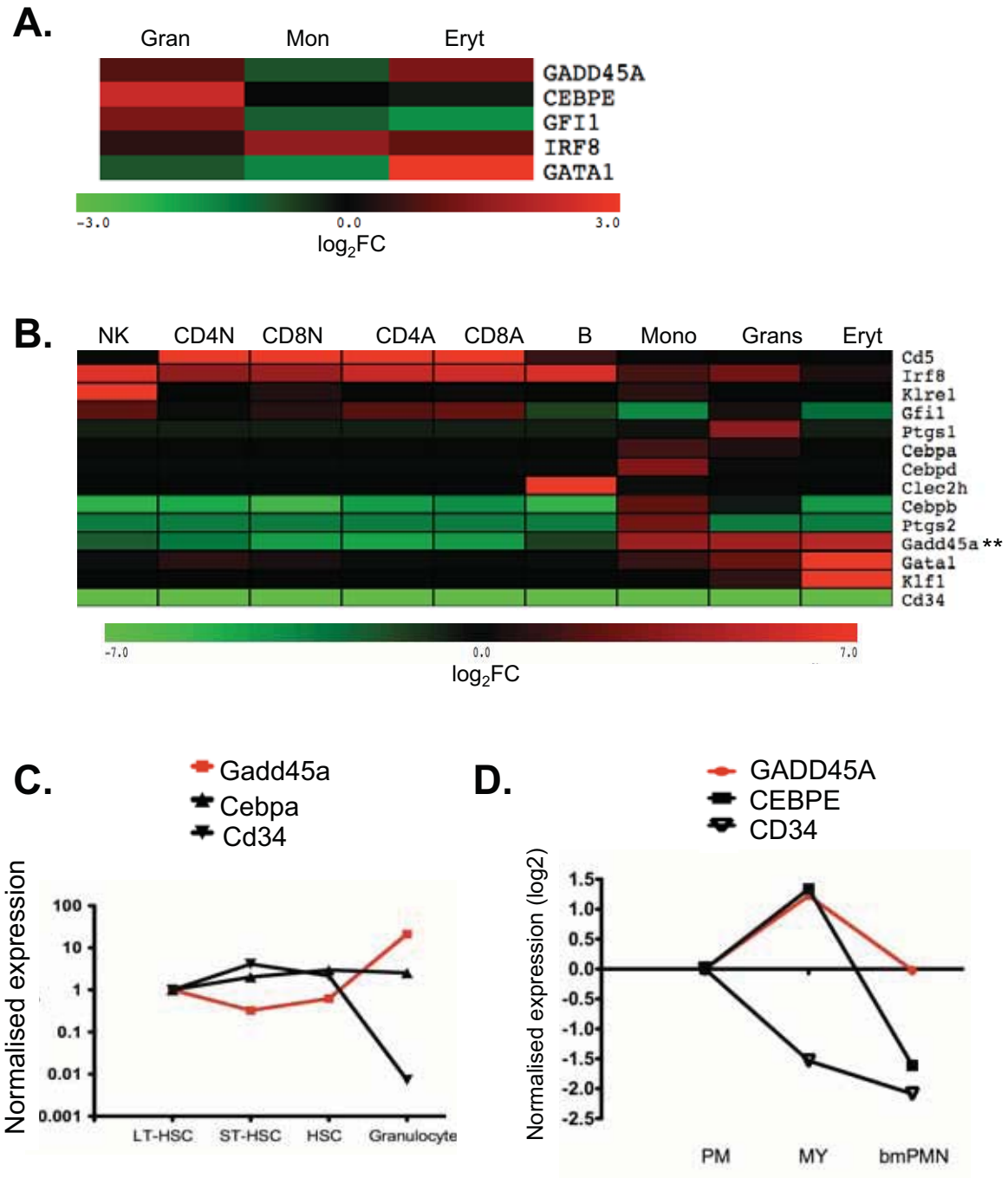


Figure 3.18. The *Gadd45a* expression pattern at various stages of haemopoiesis. A. Relative expression of *GADD45A* in indicated lineages compared to human CD34^{+} cells. *CEBPE* expression was used as specific granulocyte marker in this dataset. **B**. Relative expression of *Gadd45a* (asterisks) in indicated lineages compared to HSC. Genes other than *Gadd45a* have been identified by Chambers *et al* for specific lineage genes. CD4N/CD8N = naïve T cells, CD4A/CD8A = Activated T cells, NuEry = nucleated erythrocyte, NK = natural killer, mono = monocyte, gran = granulocyte, B = B cells. **C.** The expression pattern of mouse *Gadd45a* in granulocyte development from haemopoietic stem cell population (Sung *et al*). LT-HSC = long-term HSC, ST-HSC = short-term HSC. **D.** The expression pattern of human *GADD45A* in granulocyte development (Theilgaard-Monch *et al*). PM = promyelocyte, MY = Myelocyte, bmPMN = bone marrow polymorphonuclear.

lineages compared to HSC (Chambers, *et al* 2007). As shown in **Figure 3.18B**, increased *Gadd45a* expression is observed in the myeloid/erythrocyte lineages with increased expression relative to haemopoietic stem cell (HSC) observed in erythrocytes, granulocytes and monocytes. Interestingly, *Gadd45a* expression was down-regulated in lymphocyte populations relative to the HSC population. We again included the previously reported lineage-associated genes *Cd5, Irf8, Klre1, Gfi1, Ptgs1, Cebpa, Cebpd, Clec2h, Cebpb, Ptgs2, Gata1, Klf1 and Cd34* in this microarray dataset to provide validation for each lineage (Chambers, *et al* 2007). To further validate the increased expression of *Gadd45a* in the granulocyte lineage, we used two granulocyte gene expression datasets (Sung, *et al* 2006, Theilgaard-Monch, *et al* 2005). As shown in **Figure 3.18C**, *Gadd45a* expression increases more than 10 fold during mouse granulocyte differentiation (relative to HSC populations) in the microarray dataset (GSE5677) from Sung *et al* (Sung, *et al* 2006) (*Cebpa* and *Cd34* expression patterns are provided for comparison). Finally, the study by Theilgaard-Monch *et al* (Theilgaard-Monch, *et al* 2005) investigating gene expression in human promyelocyte, myelocyte and bone marrow polymorphonuclear cell populations shows a peak of *GADD45A* expression at the myelocyte stage, with *GADD45A* displaying a very similar profile to *CEBPE* (**Figure 3.18D**). This expression pattern of *GADD45A* is consistent with a role in promoting granulocyte differentiation of myeloid precursor cells, possibly with an additional role at the myelocyte stage of differentiation. Such a role is consistent with the phenotype of Gadd45a$^{-/-}$ mice where reduced mature granulocytes are observed together with increases in blast cells in bone marrow compared to WT control mice (Gupta, *et al* 2006).

3.3.6 The role of *GADD45A* as a tumour suppressor in AML

3.3.6.1 Repression of *GADD45A* expression is associated with MLL translocations in AML

We previously reported that lower *GADD45A* expression is associated with FLT3-ITD AML (Perugini*, et al* 2009). We next wished to examine the expression profile of *GADD45A* across different subtypes of AML patients. Thus, we investigated the expression of *GADD45A* in AML gene expression datasets. For this, we downloaded microarray datasets from Valk *et al*, Ross *et al* and Verhaak *et al* (Ross*, et al* 2004, Valk*, et al* 2004, Verhaak*, et al* 2009). We have previously reported the *GADD45A* expression level (Perugini*, et al* 2009) in the 16 distinct AML clusters defined from the gene expression profiling by Valk *et al* (Valk*, et al* 2004). Here, we also determined the *GADD45A* expression in the 285 AML patients from the Valk *et al* study (Valk*, et al* 2004) defined by karyotypic abnormality. This showed that *GADD45A* expression level was significantly lower in the MLL translocation group compared to NBM and normal $CD34^+$ controls (**Figure 3.19A**). Although the *GADD45A* expression was not significant in other AML groups, there is a trend of lower *GADD45A* expression in all groups compared to the normal controls. We also found that there is significantly lower expression of *GADD45A* in the MLL group compared to other karyotypes of AML in this dataset. Analysis of other AML microarray datasets was used to validate this finding (Ross*, et al* 2004, Verhaak*, et al* 2009). We found that the *GADD45A* expression level in paediatric AML was also significantly lower in MLL subtypes compared to other subtypes including adult AML (except for PML-RARα and T-ALL) (**Figure 3.19B**). In another study of 461 adult AML samples by Verhaak *et al* (Verhaak*, et al* 2009) *GADD45A* expression level was also significantly lower in the MLL group compared to other AML subtypes, apart from complex karyotype (data not shown). Thus, in AML associated with MLL translocations, there maybe selective silencing of *GADD45A.* This raises the

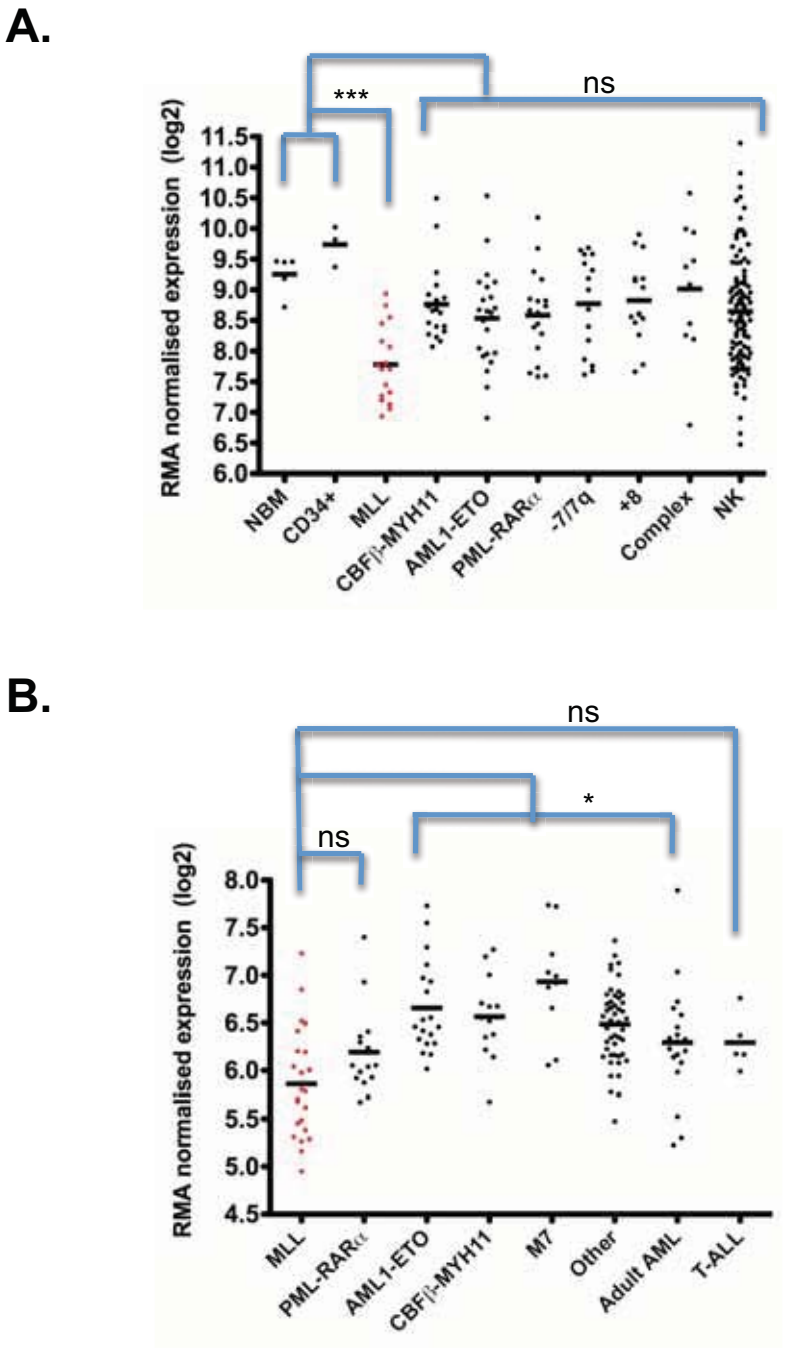


Figure 3.19. Expression of *GADD45A* in AML subtypes defined by karyotype. *GADD45A* expression was examined in AML with the indicated karyotypes using **A.** 285 adult AML data from Valk *et al*, **B.** 150 paediatric AML data from Ross *et al*. Horizontal line indicates the mean of expression. *** $p<0.001$, * $p<0.05$ determined by two-tailed student's t-test. ns indicates not significant.

possibility that in this subtype of AML, *GADD45A* is a target of CpG promoter methylation. Alternatively *GADD45A* maybe a target of the MLL fusion protein, or be under repression by *HOX* gene products which display elevated expression in this group of AML.

3.3.7 Discussion

3.3.7.1 FLT3-ITD and FLT3-TKD in CBF AML

The over-representation of FLT3-TKD in inv(16) AML but not t(8;21) AML in this study suggests that differences in the gene expression induced by the CBFβ-MYH11 fusion protein, compared to other Class I events, may be associated with cellular changes that preferentially cooperate with FLT3-TKD as a 'second hit' mutation. This is consistent with AML gene expression profiling studies showing that reciprocal translocations are associated with unique gene expression patterns (see **Chapter 1 Section 1.8.1**). Differences in cellular changes induced by the two different CBF AML fusions are also supported by *in vivo* studies which show that while CBFβ-MYH11 transgenic mice spontaneously develop leukaemia after 11-25 weeks (Kuo*, et al* 2006), transgenic mouse models of AML1-ETO develop leukaemia only in the presence of additional mutations (Muller*, et al* 2008).

Although FLT3-ITD is under-represented in AML1-ETO, the FLT3-ITD mutant is clearly still able to cooperate with this fusion oncoprotein in some contexts to block myeloid differentiation and contribute to survival and proliferation signals (Schessl*, et al* 2005). It is possible that the FLT3-TKD mutant provides a unique signal that cooperates with the cellular changes induced by the CBFβ-MYH11 fusion protein. A candidate for this maybe STAT3 which has been reported to be selectively activated by FLT3-TKD (Frohling*, et al* 2007) and

which is associated with enhanced cell proliferation and tumour growth in colorectal cancer (Corvinus*, et al* 2005). Constitutive activation of STAT3 induces nuclear stabilisation of β-catenin in colorectal cancer and is associated with activation of Notch signalling in cancer (Kawada*, et al* 2006, Ma*, et al* 2009). Thus, STAT3 may provide critical signals in the context of transformation by CBFβ-MYH11. In addition, the transcription co-activator *MN1,* which is over-expressed selectively in inv(16) compared to t(8;21), interacts with CBP/p300 (involved in the β-catenin pathway, see **Chapter 2 Discussion Section 2.4**) inducing growth or differentiation effects (Grosveld 2007). This suggests that inv(16) cells may have distinct mechanisms to t(8;21) cells which result in modulation of the β-catenin pathway, for example through STAT3 activation and/or *MN1* over-expression.

3.3.7.2 FLT3-ITD and FLT3-TKD mediated gene expression in NK AML

We have compared and contrasted FLT3-ITD and FLT3-TKD associated gene expression in normal karyotype AML blasts to further understand the pathways that are associated with the poor prognosis factor FLT3-ITD. Our approach to microarray analysis is different from others as we excluded known mutations (*RAS* mutations, *EVI1* over-expression, *CEBPA* mutation) that influence prognostic outcome to reduce complexity (see **Chapter 1 Section 1.1.1**). We compared gene expression in FLT3-mutant blasts to that in blasts from FLT3-WT AML. This revealed that there were many more genes differentially expressed associated with FLT3-TKD than FLT3-ITD in normal karyotype AML. This finding is consistent with the study by Verhaak *et al* who used a gene expression profiling approach with AML blasts (including normal karyotype AML) harbouring the FLT3-TKD mutation (Verhaak*, et al* 2009). The significant difference in gene expression may explain why signalling provided from these two alternatives activated FLT3 mutants preferentially cooperate with distinct

AML subtypes. Furthermore, we found that FLT3-ITD AML blasts over-expressed the *BIC* gene which encodes miR-155 that has been shown to down-regulate *SPI1 (PU.1)* expression, or modulate its target genes such as *TAB2, SHIP and CEBPB* (Chen*, et al* 2009, Hu*, et al* 2010). Interestingly, sustained expression of miR-155 induced a MPD *in vivo* (O'Connell*, et al* 2008). This suggests that FLT3-ITD up-regulation of miR-155 may act to repress the expression of *SPI1 (PU.1)* and hence contribute to the MPD phenotype observed previously in both FLT3-ITD knock-in and *SPI1 (PU.1)* knockout mice (Li*, et al* 2008, Rosenbauer and Tenen 2007).

Of note also the FLT3-TKD AML group displayed an up-regulation of *TRIB1* mRNA*,* which displayed significantly higher expression in FLT3-TKD than in either the FLT3-ITD or FLT3-WT groups. Over-expression of *TRIB1* has been shown to induce AML in a mouse model through degradation of C/EBPα and C/EBPβ working through the MEK1/ERK pathway (Dedhia*, et al* 2010, Yokoyama*, et al* 2010). Additionally, *TRIB1* inhibits granulocytic differentiation in G-CSF-treated 32D cells differentiation through degradation of C/EBPα (Dedhia*, et al* 2010).

3.3.7.3 Gene expression in NK LSC

Analysis of gene expression in NK LSC showed that *HCK* is significantly up-regulated in this group compared to HSC and this is independent of FLT3 mutation status. Mitina *et al* reported that Hck is recruited to tyrosine residues 589/591 of FLT3 and impairs FLT3 receptor maturation (see **Chapter 1 Section 1.6.2.1** for more details) through tyrosine phosphorylation on the juxtamembrane region of FLT3 (Mitina*, et al* 2007). The impairment of FLT3 maturation leads to a reduction in folding efficiency, and retention in endoplasmic

reticulum with altered downstream signalling (Choudhary, *et al* 2009, Schmidt-Arras, *et al* 2005). Indeed, the majority of AML blasts have constitutive activation of Src kinases such as Lyn and Hck (Roginskaya, *et al* 1999) raising the possibility that this cooperates with FLT3 mutation to amplify or modify the signalling events associated with the constitutively active receptors. Interestingly, suppression of Hck phosphorylation with anti-CD45RO that activates CD45 tyrosine phosphatase activity resulting in inhibition of Hck tyrosine kinase activity, or PP2 (Src inhibitor), down-modulates PI3K/AKT activation leading to inhibition of cell proliferation (Suh, *et al* 2005). Thus, Hck activation, associated with increased expression of *HCK* mRNA, may cooperate with FLT3 mutations and contribute to the altered growth and survival properties of LSC in the NK AML group.

3.3.7.4 FLT3 mediated *HOX* gene expression in NK LSC

The *HOX* family genes are important for HSC self-renewal and differentiation inhibition in AML (Pineault, *et al* 2002). In a small comparison of NK LSC and HSC we noted that several *HOXA* and *HOXB* genes were up-regulated selectively in FLT3-ITD LSC compared to normal HSC (**Figure 3.9** and **Figure 3.10**). In particular, the expression of *HOXB3* was up-regulated in NK AML blasts with both classes of FLT3 mutations (**Figure 3.2**). We also observed up-regulation of *HOXB3* in FLT3-ITD LSC (1.9 fold-change, FDR p=0.01) but it did not meet our criteria of 2-fold-change. Over-expression of *HOXB3* induces a lymphoid and myeloid disorder in a mouse model (Sauvageau, *et al* 1997) and this phenotype resembles the phenotypes of FLT3-ITD and FLT3-TKD mouse models suggesting that *HOXB3* may contribute to the LSC properties associated with FLT3 mutations. Moreover, the ability of *HOXB4* and *HOXB6* to expand HSC has been described (Fischbach, *et al* 2005) and *HOXB4* target genes have been identified (Lee, *et al* 2010). A recent study by Bach *et al*

closely examined the leukaemic potential of all the *HOXA* genes (*HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11, and HOXA13*) showing that, except for *HOXA2* and *HOXA5*, these blocked or delayed myeloid differentiation and cooperated with *MEIS1* to transform and immortalise the primary haemopoietic cells from 8-week-old BALB/C mice (Bach*, et al* 2010). The data in this study is consistent with important roles for *HOXA4, HOXA6, HOXA7, HOXB4* and *HOXB6.* Thus we suggest that in some NK AML, activation of *HOX* gene expression may provide a proliferation signal and differentiation block that cooperates with FLT3-ITD and other changes to induce leukaemia.

3.3.7.5 The association of ATM signalling pathway with FLT3-ITD LSC

The mechanism of genomic instability in AML is still unknown. Constitutive activation of FLT3 mutations is thought to endorse the incidence of additional mutations by suppressing the DNA damage response to mutagenic events (Weisberg*, et al* 2010). Our pathway analysis revealed down-regulation of ATM signalling pathways in FLT3-ITD LSC. ATM is involved in repair of double-strand DNA damage which prevents chromosomal aberrations or mutations. ATM has also been reported to mediate constitutive NFκB activation in AML (Grosjean-Raillard*, et al* 2009). Our findings are consistent with those of Boehrer *et al* who reported that in AML, the DNA damage response is suppressed (Boehrer*, et al* 2009). These studies suggest that down-regulation of ATM and the DNA damage response may contribute significantly to the LSC phenotype, possibly being important for the drug resistance and progression of AML. To date, there is no other study reporting that ATM down-regulation is selectively associated with FLT3-ITD and further studies of this pathway in NK LSC are now warranted.

3.3.7.6 *Gadd45a* and FLT3-ITD signalling

We showed that FLT3 mutants blocked granulocytic differentiation and enhance survival through constitutive ERK1/2 activation (**Figure 3.14** and **Figure 3.15**) associated with decreased *Gadd45a* expression (see **Appendix D Figure 2**). Interestingly, *Gadd45a* over-expression exerts growth inhibition effects selectively on FLT3-ITD but not FLT3-TKD. This differential sensitivity reflects the significant signalling and gene expression differences observed in FLT3-ITD and FLT3-TKD AML blasts (see **Chapter 1 Section 1.6.2** and discussion above **Section 3.3.7**), and again this may relate to the selective differential activation of STAT3 by the FLT3-TKD mutant (Frohling*, et al* 2007), or due to *TRIB1* up-regulation associated with this mutant.

FLT3-ITD signalling constitutively activates PI3K/AKT and hence causes repression of the transcription factor *FOXO3A* and pro-apoptotic BCL-2 family member *BIM* (Scheijen*, et al* 2004). This could subsequently deactivate *GADD45A,* as *GADD45A* is a known target gene of *FOXO3A* (Tran*, et al* 2002) and which has a number of functions that maybe important for AML growth and survival (discussed further in **Chapter 5 Section 5.1.2**). We have shown that *GADD45A* expression is reduced in FLT3-ITD^{+} AML (see **Appendix D Figure 3a**) and in another study examining gene expression downstream of FLT3-ITD in 32D cells, hierarchical clustering showed there were 2 distinct groups of FLT3-ITD which corresponded with alternative ITD variants (Pekova*, et al* 2009). These two groups of FLT3-ITD display significantly different *Gadd45a* gene expression levels. Interestingly, by analysing the difference in these two groups, we observed that the down-regulation of *Gadd45a* expression is associated with the most frequently occurring FLT3-ITD mutant (YVDFRE**Y**E**Y**DL) which duplicates the segment from codon 591 to codon 601 (data not shown). This variant of

FLT3-ITD is strongly associated with for STAT5 and MAPK/ERK activation, transformation and proliferation (see **Chapter 1 Section 1.6.2.1**) (Meshinchi*, et al* 2008). We have demonstrated that MAPK/ERK contributes to repression of *Gadd45a* expression (Perugini*, et al* 2009), however, whether STAT5 activation is involved in repression of *Gadd45a* is still unknown.

3.3.7.7 Mechanism of *Gadd45a* repression or silencing in AML

GADD45A expression is lower in $CD34^+$ cells and in HSC and increased expression occurs during granulocyte differentiation (**Figure 3.20A**) suggesting that *GADD45A* may be important in regulating myeloid differentiation. Consistent with the immature phenotype of AML blasts we observed *GADD45A* expression to be lower in AML compared to normal $CD34^+$ cells or NBM. Furthermore we showed that the expression of *GADD45A* was significantly lower in AML associated with MLL translocations (see **Figure 3.19**). The mechanism for this is unknown; however, the possible mechanisms (see **Figure 3.20B**) include regulation directly by MLL fusion proteins or *HOX* gene products. The reduced expression of *GADD45A* in MLL AML may also be due in part to increased expression of *Menin1* (*MEN1*); *MEN1* directly represses *JUND* expression (Gobl*, et al* 1999) and *JUND* binds to the third intron of *GADD45A* leading to activation of *GADD45A* (Daino*, et al* 2006). Further proposed mechanism of *Gadd45a* regulation is discussed in **Chapter 5 Section 5.1.2**.

A.

NOTE:
This figure is included on page 157 of the print copy of the thesis held in the University of Adelaide Library.

B.

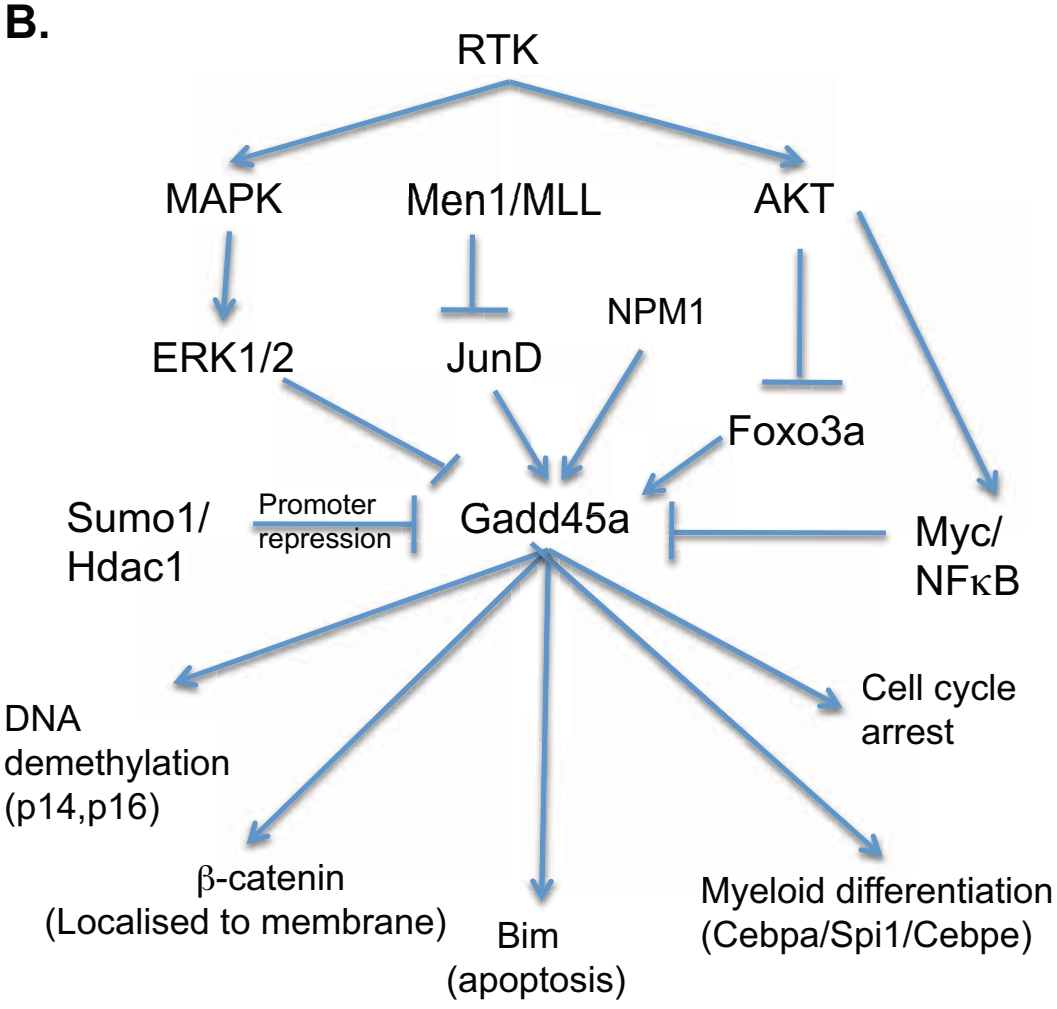


Figure 3.20. The proposed mechanism and expression pattern of *Gadd45a* in AML and normal haemopoiesis. A. The proposed *Gadd45a* expression pattern in normal haemopoiesis that is supported by the bioinformatics analysis of human (compared to $CD34^{+}$) and mouse haemopoiesis (compared to HSC) as described in Figure 3.18. The myelopoiesis figure is adapted from adapted from Rosenbauer and Tenen, 2007. **B.** The proposed mechanism and pathways that are regulated by *Gadd45a* based on the literature and this study.

Chapter 4: Use of bioinformatic approaches to determine key pathways and specific therapeutic approaches in AML subgroups

4.1 Introduction

Treatment for all subtypes of AML currently involves combination chemotherapy, with specific targeting of therapeutic agents limited to only one subtype, carrying the PML-RARα translocation, that can be successfully treated with ATRA (see **Chapter 1 Section 1.1.2**) (Nowak*, et al* 2009, Tallman 2008). Recent studies have used high throughput screening approaches such as chemical libraries to screen a wide range of drugs, including many approved by the US Food and Drug Administration (FDA), to identify new specific drugs for AML. Corsello *et al* identified corticosteroids and dihydrofolate reductase (DHFR) inhibitors through chemical genomics that specifically target AML cells and cell lines with the AML1-ETO translocation (Corsello*, et al* 2009). Wang *et al* also identified a GSK3β inhibitor as a specific drug active on AML cells with MLL translocations (Wang*, et al* 2008). While these approaches have provided promising leads, they are limited by access to large libraries and associated with high cost (Stegmaier 2009).

The current bioinformatics technology, in combination with the rich resource of microarray datasets that have been deposited in the public domain, makes it possible to use gene expression profiles to identify drugs that are associated with gene expression patterns of relevance to particular diseases. For instance, the CMAP that has been developed by Lamb *et al* (Lamb*, et al* 2006) has been used to identify potential drugs for cancer treatment (Rosenbluth*, et al* 2008, Sanda*, et al* 2009, Vilar*, et al* 2009). Successes using this application

include identification of specific compounds for acute lymphoid leukaemia and solid tumours (Rosenbluth*, et al* 2008, Sanda*, et al* 2009, Vilar*, et al* 2009). In this Chapter, we focus on applying this approach to identify drugs that may act on AML associated with the major translocations (PML-RARα t(15;17), AML1-ETO t(8;21), CBFβ-MYH11 inv(16), and MLL 11q23).

We also extend our bioinformatic approach to analysis of the major numerical chromosomal abnormality in AML. Trisomy 8 (+8) is the most frequent chromosomal abnormally in AML and is common in myeloproliferative neoplasm, myelodysplasic syndrome (MDS) and chronic myelomonocytic leukaemia (CMML) (Paulsson and Johansson 2007). Trisomy 8 has higher incidence in FAB subtype M5, is common in secondary AML and is associated with intermediate prognosis (Paulsson and Johansson 2007). It has been reported that gain of the extra copy of chromosome 8 does not necessarily confer higher gene expression for genes on that chromosome. In fact, +8 AML is associated with down-regulated *c-Myc* expression (Paulsson and Johansson 2007). Thus the contribution of +8 to AML pathogenesis is still unclear. Here we describe an analysis of a large AML gene expression dataset which revealed association between +8 and *HOX* gene expression.

4.2 Materials and Methods

4.2.1 Reagents

Pentoxyverine and Dequalinium choride were purchased from Sigma-Aldrich. The cytokines such as FL, IL-3, IL-6, SCF, G-CSF and GM-CSF (Peprotech, Rocky Hill, NJ) were kindly provided by Drs Jason Powell and Ian Lewis. Pentoxyverine was reconstituted in sterile

water at 10 mM stock concentration. Dequalinium chloride was reconstituted in sterile water at 10 mM stock concentration and pre-warmed at 37^{o}C to dissolve the compound prior to addition into cell medium. The stock solutions were stored at -20^{o}C.

4.2.2 AML patient samples thawing and culturing

Cryopreserved bone marrow biopsies from AML patients, taken at diagnosis after informed consent according to the Institutional Guidelines (Table below), were thawed in IMDM with 20% (v/v) FBS, 50 Unit/ml DNase, 1x PSG, washed and cultured at 37^{o}C in a waterbath. Viable mononuclear cells (MNC) were isolated by Ficoll-Hypaque density-gradient centrifugation as previously described (Powell*, et al* 2009) and the viable cells at cell density of 2x10^{5} cells/ml were cultured (IMDM with 0.5% FBS and 1x PSG) with or without growth factor cocktail (FL, IL-3, IL-6, SCF, G-CSF and GM-CSF, each 10 ng/ml) for downstream experiment validation.

Patient no.	**Gender**	**WBC Count (10^{9}/L)**	**% blast**	**Cytogenetic**	**Types**	**Relapse**	**FAB**
inv16-1	M	171.00	35	inv16	De novo	Yes	M4eo
MLL-1	F	6.21	ND	11q23	secondary	ND	M4

ND=not determined

4.2.3 Apoptosis, cell counts and differentiation

Apoptosis was analysed using Annexin V conjugated with FITC (BD Pharmingen) as per the manufacturers protocol. To examine differentiation, human CD11b and CD34 conjugated with PE (BD Biosciences) were used and examined by using flow cytometry. Total viable cell numbers were determined based on trypan blue exclusion or by APC-conjugated

fluorescent beads (Flow counts beads, BD Biosciences) examined using flow cytometry (Powell, *et al* 2009). To determine cell counts by APC-conjugated fluorescent beads, an equal amount of beads (100 μl) was added to each sample prior to analysis by flow cytometry. Viable cell number was assessed by using Flow-Count Fluorospheres (BD Biosciences). Relative cell number was calculated as the number of cells enumerated relative to a defined number of Flow-Count Fluorospheres (Powell, *et al* 2009).

4.2.4 Microarray re-analysis

The raw microarray data from Valk *et al* (Valk, *et al* 2004) was downloaded from NCBI GEO (GSE1159) and normalised with RMA that utilized probe level to fit a robust linear model. A total of 76 AML (18 PML-RARα, 17 MLL, 22 AML1-ETO and 19 CBFβ-MYH11) samples that carried either one of the four common translocation events were re-analysed. Differential expression was determined by using empirical Bayes moderated t-statistic and LIMMA that was available from Bioconductor open-source software project. Each AML translocation group was compared to the NBM group. The FDR was adjusted for multiple testing to control false discovery using Benjamini-Hochberg method. Each specific translocation group gene-list was defined by genes with a significant FDR $p<0.01$ for the specific translocation, but with FDR $p>0.01$ on other translocations. The analysis was done using the R statistical software package (http://www.R-project.org) and in-house Perl scripts. Heatmap was generated using MeV software (Saeed, *et al* 2006).

4.2.5 Connectivity Map analysis (CMAP)

The Affymetrix HGU133A ID extracted from the list for each specific translocation AML compared to NBM was used to upload to the CMAP v2 database (Lamb*, et al* 2006). Each of the top 200 up-regulated or down-regulated genes (FDR p-value < 0.01 and ranked based on fold-change) for each specific translocation AML subgroups were uploaded to CMAP database. Duplicated genes were removed based on FDR p-value ranking. For MLL translocation group, only 172 up- or down-regulated genes were uploaded to CMAP due to there being less than 200 up-regulated genes in MLL group (FDR p<0.01). Significant drugs were selected based on a negative connectivity score > 0.4 and p-value < 0.05.

4.2.6 Statistical analysis

The statistical significance of a comparison between two sample groups was determined using the 2-tailed student's t-test or otherwise indicated in the text. Only p<0.05 was considered as significant. The data is presented as mean ± standard error measurement (SEM) for technical duplicates. The data was generated using GraphPad Prism version 5.0 (GraphPad Software, http://www.graphpad.com).

4.3 Results

4.3.1 Rationale: Comparing AML gene expression to normal bone marrow mononuclear cells (NBM)

In this study, we determined gene expression changes, relative to NBM, for AML samples characterised by the key PML-RARα, AML1-ETO, CBFβ-MYH11 and MLL translocations,

regardless of other known molecular abnormalities (e.g. *FLT3*, *EVI1*, *RAS*, *CEBPA* or *NPM* mutations) or clinical features. This approach reduces the complexity of AML which is a heterogeneous disease and focuses on genes in each subgroup associated with the block in differentiation, increased self-renewal, increased survival and proliferative potential associated with AML. We hypothesised further that drugs identified through CMAP which show reversal of this expression pattern in a selective way may have specificity as therapy for AML associated with particular translocations.

4.3.2 Identification of 4 specific AML translocation gene lists

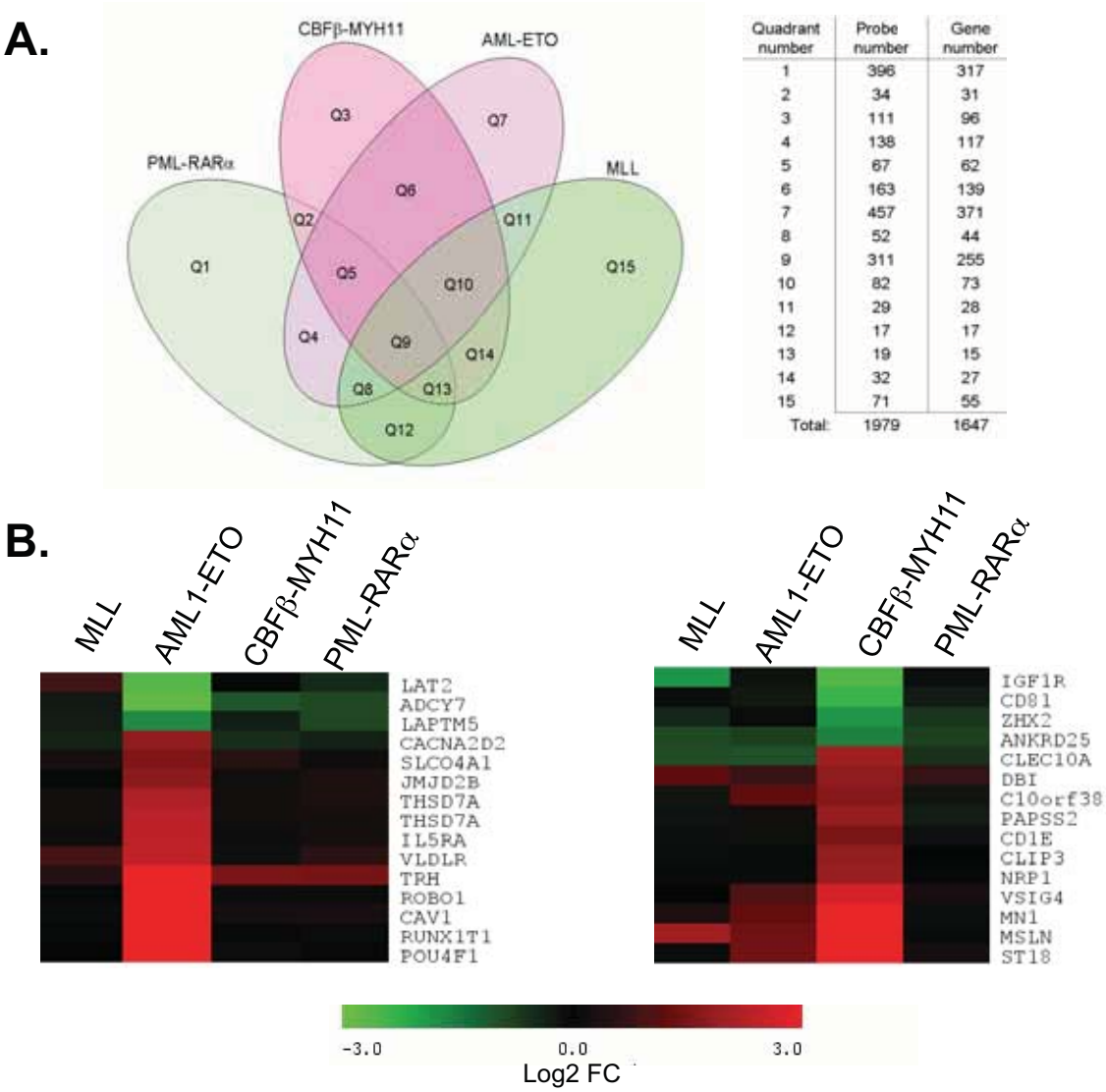
Expression patterns were determined using data from the landmark gene expression profiling study by Valk *et al* (Valk*, et al* 2004) which used the Affymetrix HGU133A array platform. In this study, 285 adult AML patient samples were defined into 16 distinct clusters of AML based on gene expression pattern similarity. This study was of great importance in that it was one of the largest tumour gene expression arrays reported (see **Chapter 1 Section 1.8.1**). The study included AML samples with substantial clinical characterisation and known karyotypic abnormalities, as well as normal $CD34^+$ and NBM control samples. AML samples were genotyped for *FLT3, RAS, CEBPA* mutations, and *EVI1* over-expression (Valk*, et al* 2004) and in a subsequent study samples were genotyped for *NPM1* mutations (Verhaak*, et al* 2009). This large and robust dataset has provided a unique opportunity to identify the translocation-associated gene expression changes for use in the CMAP approach described above.

Differentially expressed genes associated with each of the four major translocation events, and genes displaying differential expression in multiple subtypes are shown in the Venn

diagram (**Figure 4.1A**). **Figure 4.1B** shows heatmaps of the highest-ranked significant differentially expressed genes (FDR p-value < 0.01) displaying differential expression changes associated specifically with AML1-ETO and CBFβ-MYH11 relative to all other indicated translocation groups. Moreover, to examine the expression of individual genes across the whole cohort (285 patients) and to determine any association with the other major karyotypic abnormalities (e.g. trisomy 8, -7/7q), we generated plots of the normalised gene expression value in all AML samples, including samples with normal karyotype or defined karyotypic abnormalities. Representative plots are shown in **Figure 4.1C** for *ROBO1* (AML1-ETO specific gene) and *MN1* (CBFβ-MYH11 specific gene) respectively. The selective expression patterns of these genes were also identified by Valk *et al* (Valk*, et al* 2004). This analysis across the whole cohort allows a closer examination of individual gene expression profiles.

We performed the same analysis for the PML-RARα and MLL groups. In AML with PML-RARα, *FGF13* and *HGF* were among the highest ranked up-regulated genes that distinguished PML-RARα from the other translocations groups, as shown in **Figure 4.2A**. This is consistent with the reports by Valk *et al* and Gutierrez *et al* who found that higher expression of these genes is specific to PML-RARα (Gutierrez*, et al* 2005, Valk*, et al* 2004). The representative plot of *FGF13* is shown in **Figure 4.2A**.

The AML group with MLL translocations was characterised by high levels of expression of *HOXA9* and *HOXA10* as previously reported (Krivtsov and Armstrong 2007). In addition to *HOXA* genes, *MEIS1* was also shown to be significantly up-regulated ($p < 0.01$) compared to



Quadrant number	Probe number	Gene number
1	396	317
2	34	31
3	111	96
4	138	117
5	67	62
6	163	139
7	457	371
8	52	44
9	311	255
10	82	73
11	29	28
12	17	17
13	19	15
14	32	27
15	71	55
Total:	1979	1647

Figure 4.1. Genes that are specific to each of the 4 AML translocation events. A. Venn diagram showing genes differentially expressed in each specific translocation AML group with a FDR p-value < 0.01. Genes with a FDR p-value > 0.01 are not considered to be significant. **B.** Heatmap shows AML1-ETO and CBFβ-MYH11 AML specific gene list with p<0.01 and at least 2 fold change. Red indicates up-regulated gene expression and green indicates down-regulated gene expression compared to NBM. **C**. *ROBO1* and *MN1* expression level for individual patient across each indicated subtype of AML. ** indicates FDR p<0.01 compared to NBM.

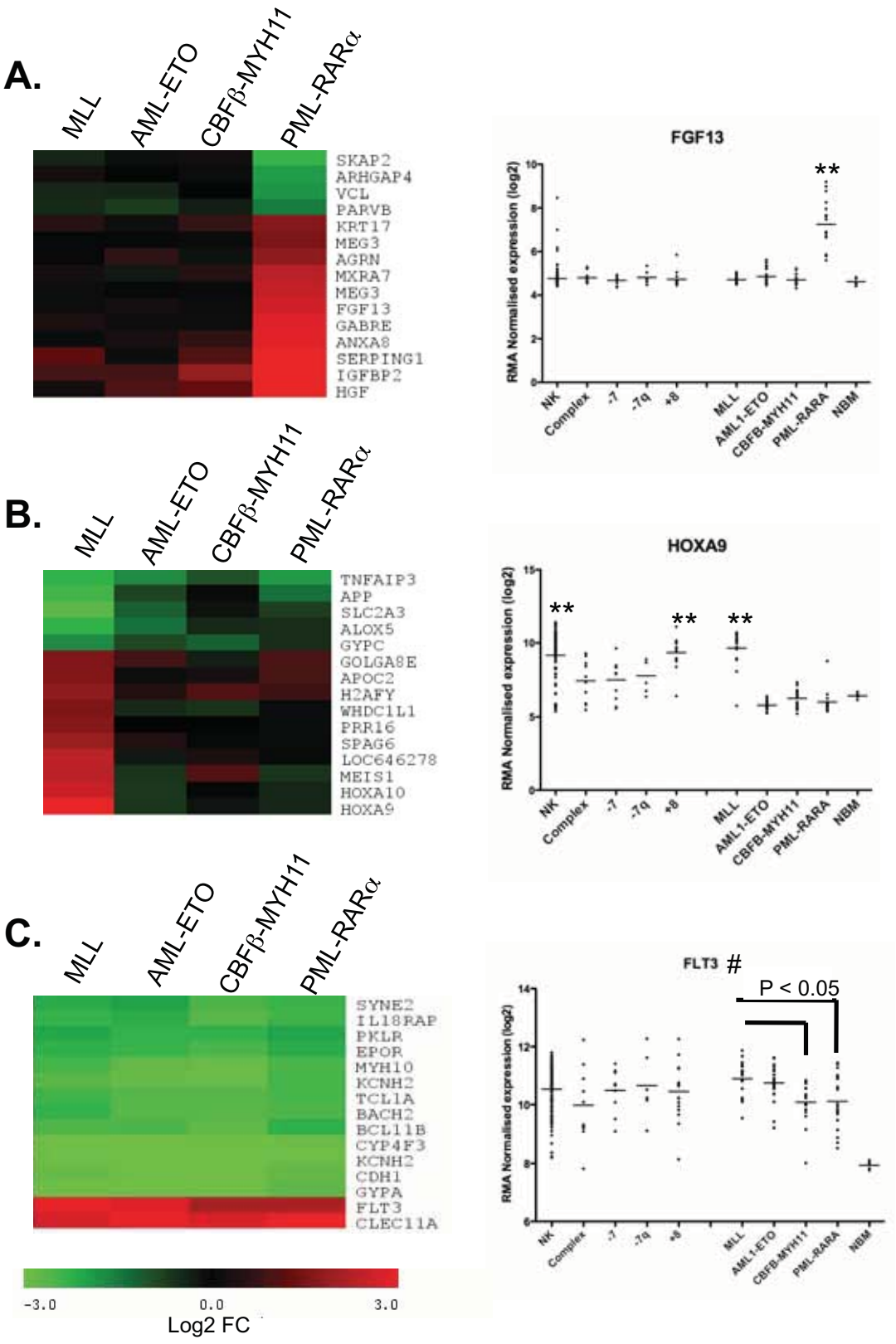


Figure 4.2. Identification of genes selectively regulated either in AML with PML-RARα, MLL or common to all four translocations groups. A. Heatmap shows PML-RARα AML specific gene list. *FGF13* expression level for each patients across each indicates subtypes of AML. **B.** Heatmap shows MLL AML specific gene list. *HOXA9* expression level for each patients across each indicates subtypes of AML. **C**. Heatmap shows AML commonly dysregulated gene list. *FLT3* expression level for patients across each indicated subtype of AML. ** indicates FDR p<0.01 compared to NBM. # indicates all the AML karyotypes are significantly (FDR p<0.01) differentially expressed compared to NBM. Red indicates up-regulated gene expression and green indicates down-regulated gene expression compared to NBM.

other subtypes as shown in **Figure 4.2B**. This also has been reported previously (Krivtsov and Armstrong 2007). This group of AML represents a poor prognosis group which may relate to the high expression level of *HOXA9* also as previously reported to be associated with poor outcome (Andreeff*, et al* 2008). The representative gene expression for *HOXA9* is shown in **Figure 4.2B**. In addition to high *HOXA9* expression in MLL group, we also found that the normal karyotype group has higher *HOXA9* expression as previously reported (Mullighan*, et al* 2007). There are two groups with divergent expression of *HOXA9* apparent in the normal karyotype group (Kok*, et al* 2010); the patient samples with lower expression of *HOXA9* in the normal karyotype group mainly harboured *CEBPA* mutations consistent with the study reported previously (Dufour*, et al* 2009, Kohlmann*, et al* 2010). Interestingly, we also identified high expression of *HOXA9* in AML with trisomy 8 (see **Section 4.4** for more details). These expression differences for known subtype-specific genes provide important validation for our analysis demonstrating that we have successfully identified gene-sets representing translocation-specific expression profiles.

4.3.3 Identification of gene expression changes associated with multiple translocations

4.3.3.1 Genes common to all 4 translocations

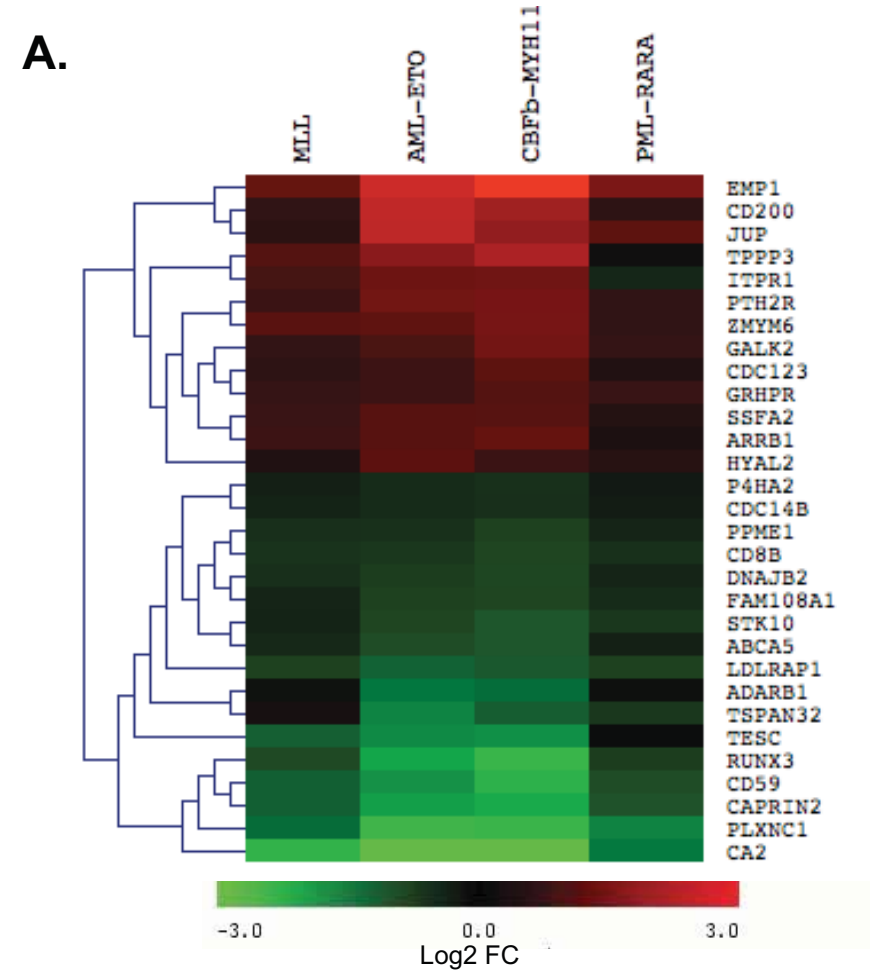
There are 255 genes commonly differentially regulated between AML and NBM (Quadrant 9) (**Figure 4.1**). This set of genes represent the general differences between immature AML blasts compared to NBM. One such gene commonly up-regulated in AML blasts relative to NBM was *FLT3* (**Figure 4.2C**). Differential *FLT3* up-regulation has previously been reported to be associated with MLL (Krivtsov and Armstrong 2007) and AML1-ETO (Dicker*, et al* 2007), and notably the MLL and AML1-ETO AML groups displayed

significantly (FDR p-value < 0.05) higher *FLT3* expression than CBFβ-MYH11 and PML-RARα (**Figure 4.2C**). Interestingly we observed that expression of several erythroid lineage genes were down-regulated (*PKLR, EPOR, GYPA, MYH10*) consistent with the disease-associated shift to myeloid cell characteristics.

4.3.3.2 Gene expression changes common to Core Binding Factor (CBF) AML

AML translocations with AML1-ETO and CBFβ-MYH11 are known as CBF-associated AML (see **Section 3.1**). There are several genes that we identified in this group of AML that have previously been reported to have an association with CBF leukaemia. For example, *RUNX3* (Cheng*, et al* 2008), *JUP* and *CD200* (Tonks*, et al* 2007a) (**Figure 4.3**). In addition to these genes, we also identified *CAPRIN2* as one of the highest ranked significantly (FDR p-value < 0.01) down-regulated genes in this CBF AML group (see **Figure 4.3**). **Figure 4.4A** clearly shows the low *CAPRIN2* expression in these two translocation groups using the data from Valk *et al* (Valk*, et al* 2004). To further validate these finding, we used another three previously published microarray datasets consisting of 461 adult AML (Verhaak*, et al* 2009), 151 paediatric AML (Ross*, et al* 2004) and 116 adult AML patients (Bullinger*, et al* 2004). These studies were also consistent with the results above (**Figure 4.4**) indicating that the expression of *CAPRIN2* is significantly down-regulated in CBF AML compared to other subtypes of AML.

CAPRIN2 is of interest because it is associated with apoptosis and enhanced canonical Wnt signalling via LRP5/6 phosphorylation (Aerbajinai*, et al* 2004, Ding*, et al* 2008). *CAPRIN2* expression has been studied in erythrocyte differentiation but not during myeloid



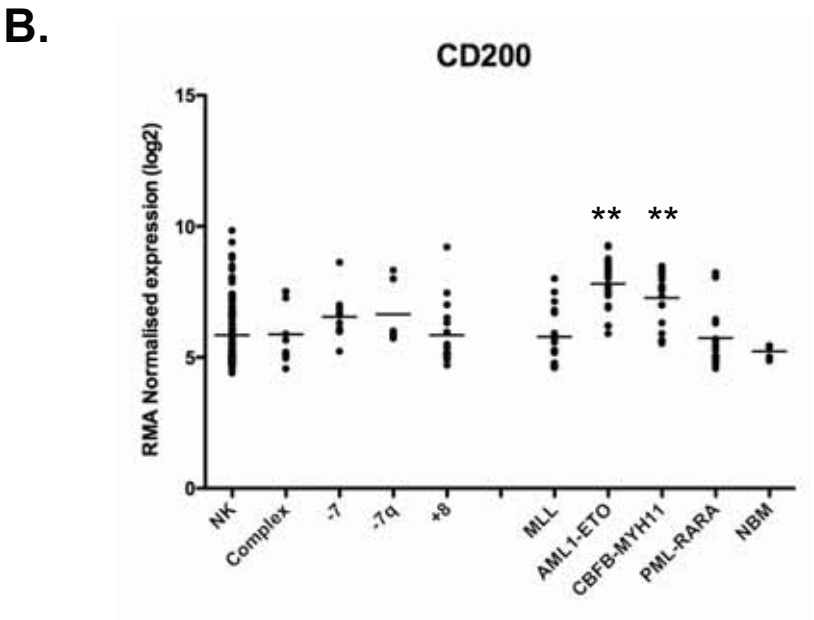


Figure 4.3. Identification of genes that are selectively regulated in CBF AML. **A**. Heatmap shows top 30 CBF leukaemia specific genes ranked based on FDR p-value. *CD200* expression level for patients across each indicated subtype of AML. ** indicates FDR p<0.01 compared to NBM. Red indicates up-regulated gene expression and green indicates down-regulated gene expression compared to NBM.

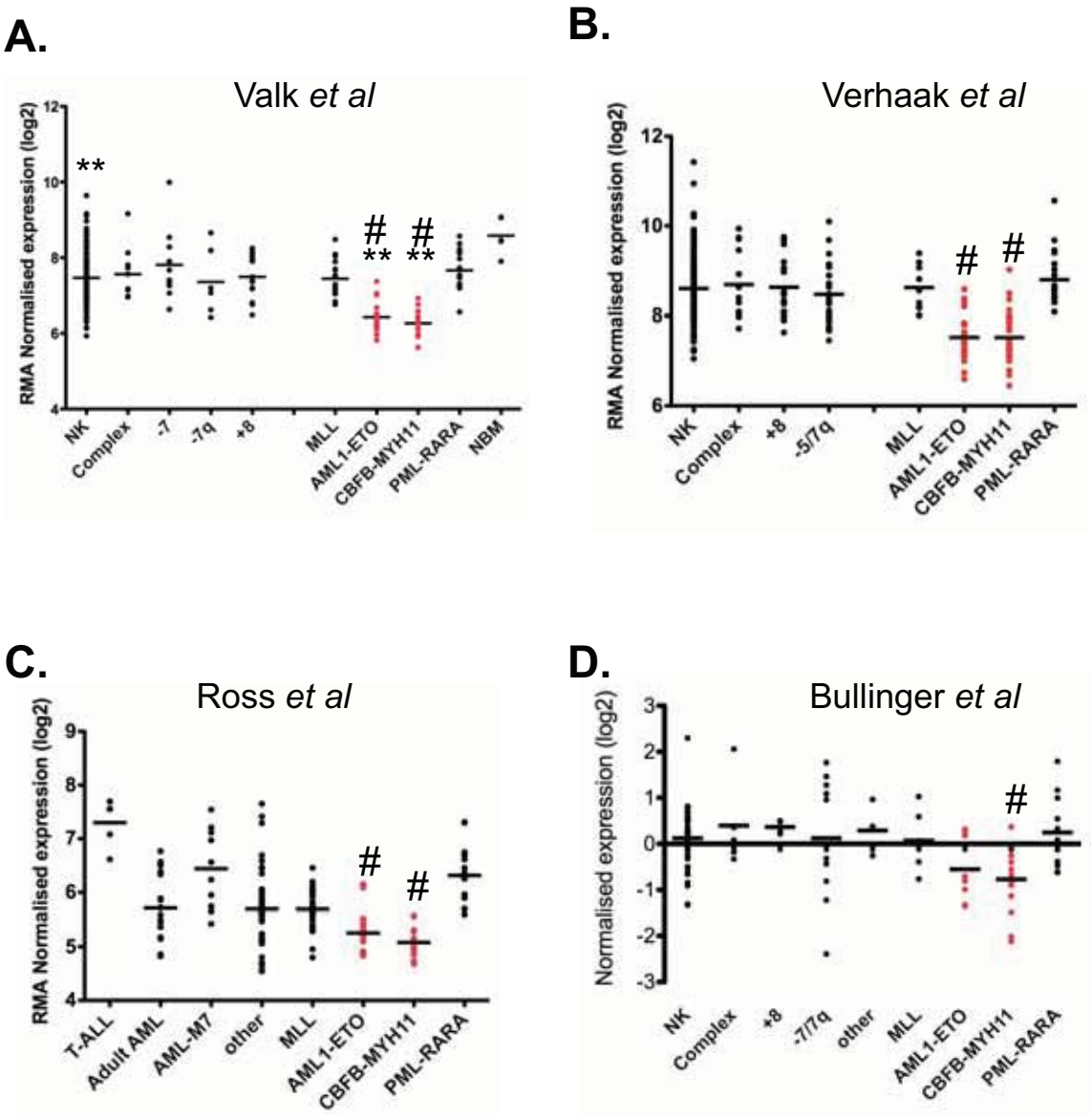
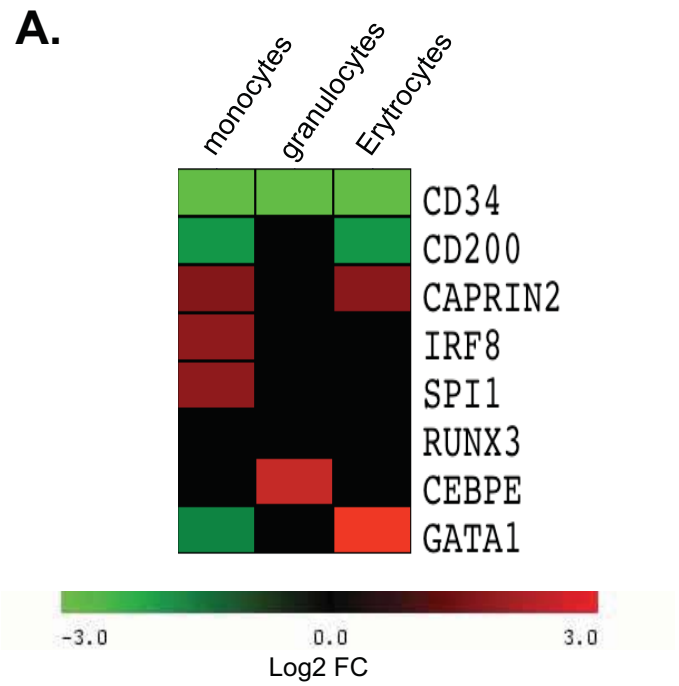


Figure 4.4. The expression of *Caprin2* in several AML microarray dataset. A. 285 adult AML patient microarray data from Valk *et al*, 2004. **B**. 461 adult AML patient microarray data from Verhaak *et al*, 2009. **C.** 151 paediatric AML patient microarray dataset from Ross *et al*, 2004. **D.** 116 adult AML patient microarray data from Bullinger *et al*, 2004. ** indicates FDR p<0.01 relative to NBM. # indicates Bonferroni corrected multiple comparison test p<0.05 relative to PML-RARA.

differentiation. Hence, we used publicly available microarray datasets to identify the gene expression pattern of *CAPRIN2* in human erythrocyte, monocyte and granulocyte populations relative to normal $CD34^+$ cells (Tonks*, et al* 2007b). Consistent with a previous report (Aerbajinai*, et al* 2004), *CAPRIN2* expression is significantly (FDR p-value = 0.004) increased (2.8 fold-change) in erythrocyte populations compared to $CD34^+$ cells as shown in **Figure 4.5A**. Interestingly, the *CAPRIN2* expression level was also significantly (FDR p-value = 0.002) increased in the monocyte population (2.6 fold-change) with no significant change in gene expression in granulocyte population. We used *CEBPE* (granulocyte lineage), *IRF8* and *SPI1 (PU.1)* (monocyte lineage), and *GATA1* (erythrocyte lineage) gene expression for comparison as shown in **Figure 4.5A**. We performed similar analysis using the raw data from the Chambers *et al* dataset (Chambers*, et al* 2007). Interestingly, in mouse haemopoiesis, *Caprin2* expression is generally significantly down-regulated relative to haemopoietic stem cells (HSC = $Lin^-Sca1^+ckit^+$, side population and Hoescht33342) (Chambers*, et al* 2007) suggesting that *Caprin2* is highly expressed in HSC (**Figure 4.5B**). This high level expression in HSC is not detected in the analysis using the $CD34^+$ cells, as HSC are a small percentage of this population. We also determined the lineage specific expression of the previously reported commonly regulated CBF genes, *Runx3 and Cd200* in haemopoiesis. We identified that *Runx3* expression is significantly down-regulated in monocyte, granulocyte and erythrocyte populations. Furthermore, this analysis revealed that *Cd200* expression is significantly down-regulated in monocyte and granulocyte populations but increased expression in T and B cell populations. Again to validate the microarray analysis, we used several genes known to have roles in specifying each lineage (see **Figure 4.5B**) (Chambers*, et al* 2007).



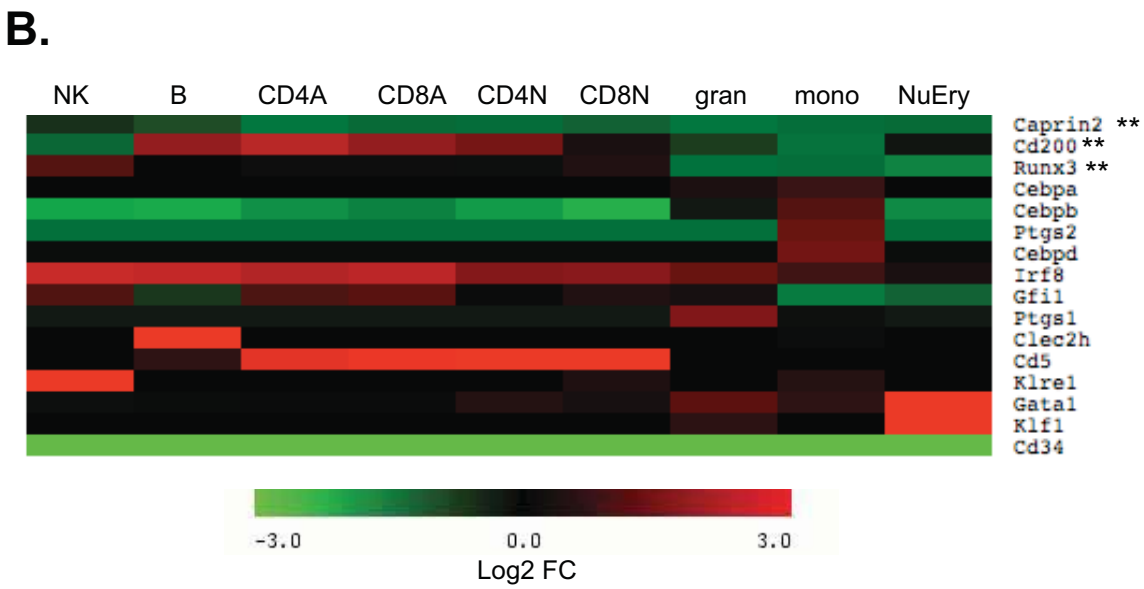


Figure 4.5. The expression pattern of *CAPRIN2* in human and mouse myeloid differentiation. A. The expression pattern of *CAPRIN2* in normal myeloid differentiation using microarray dataset derived from Tonks *et al*. Red indicates up-regulated expression and green indicates down-regulated expression compared to $CD34^+$ cells. **B**. The expression pattern of *Caprin2* in normal myeloid differentiation using the microarray dataset derived from Chambers *et al*. For more details of genes other than *Caprin2*, please see text. Red indicates up-regulated gene expression and green indicates down-regulated gene expression compared to HSC. CD4N/CD8N = naïve T cells, CD4A/CD8A = Activated T cells, NuEry = nucleated erythrocyte, NK = natural killer, mono = monocyte, gran = granulocyte, B = B cells. ** indicates CBF genes.

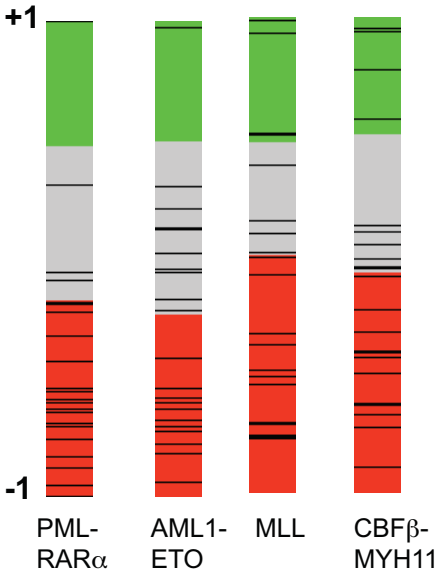
4.3.4 Identification of specific compounds and drugs using translocation signatures

We used the top 400 genes (FDR p-value < 0.01) associated with each specific AML translocation to query the CMAP database with the aim of identifying small molecules or drugs associated with reversal (i.e. a negative association) of the specific leukaemic signature in each case (Lamb*, et al* 2006). Validation of this approach was provided by our finding that the PML-RARα gene signature displays a significant and negative association with the ATRA signatures in CMAP as shown in **Figure 4.6A**. As discussed previously, ATRA is used successfully as a treatment for AML associated with the t(15;17) PML-RARα translocation (Tallman 2008). ATRA is a ligand that binds to retinoic acid receptors, releases corepressors from PML-RARα and restoration of retinoic acid receptors responsive genes such as *CEBPε* and *SPI1 (PU.1)*, and hence terminal granulocytic differentiation of PML-RARα AML cells (Nowak*, et al* 2009). Consistent with previous CMAP studies, this approach detects the overlap of ATRA gene expression and our PML-RARα signature regardless of cell lines, i.e. in CMAP array data is derived from the cell lines MCF7 (Breast cancer), HL-60 (acute promyelocytic leukaemia), PC-3 (prostate cancer) and SKMEL5 (melanoma) (Lamb*, et al* 2006). We observed the strongest overlap of the PML-RARα signature with expression derived from HL-60 treated with ATRA. None of the cell lines in the CMAP database express the PML-RARα fusion, however it is well known that ATRA can have effects on non PML-RARα cell lines as well. For example, ATRA treatment of MCF-7 induces growth arrest but not differentiation (Nobert*, et al* 2006, Yang*, et al* 1997). We further validated the overlap of the ATRA-induced gene expression with our PML-RARα signature by using a different GSEA approach. For this, we re-analysed the ATRA-

A.

All-*trans* retinoic acid (ATRA)

Name	n	Mean score	p-value
PML-RARα	22	-0.41	0.0002
AML1-ETO	22	-0.27	0.03
MLL	22	-0.19	0.3
CBFβ-MYH11	22	-0.16	0.09



B.

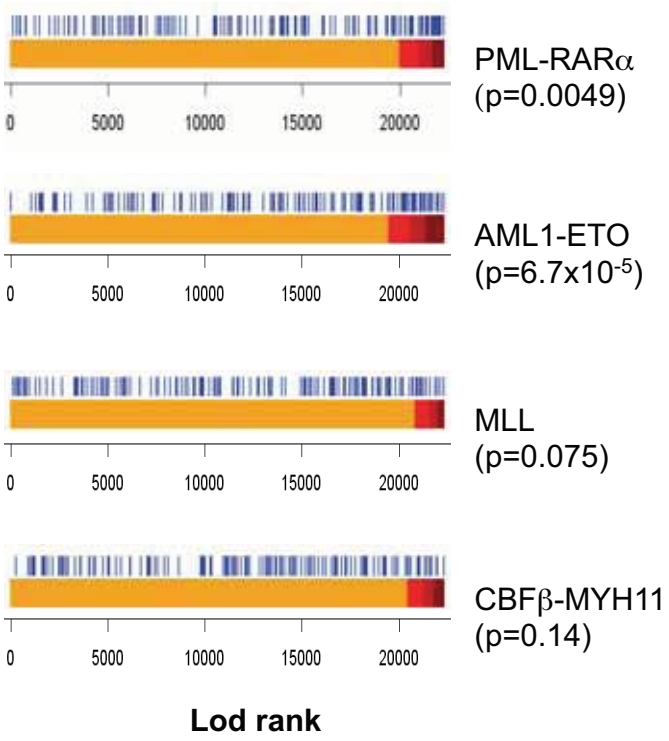


Figure 4.6. Validation of CMAP analysis by identification of ATRA in PML-RARα. A. Top 400 genes of PML-RARα (equal number of up- and down-regulated genes) were queried to CMAP. Bar plot shows the specificity of ATRA negatively associated (red) with PML-RARα and AML1-ETO. Each of the ATRA experiments comparisons (n = 22) is highlighted as a black bar on the bar plot, and colors represent a negative (red), no connection (gray), or positive connection (green) between the query signature and ATRA experiments for each array in the CMAP database. **B.** GSEA of differentially regulated genes from HL-60 cells treated with ATRA (top 150 genes based on FDR p-value) with AML translocation events using Wilcoxon rank sum test. Each blue line corresponds to an individual probe identified from the top 150 HL-60 treated HL-60 genes. The top 150 ATRA-treated HL-60 genes (blue lines) cluster with top-ranked PML-RARα and AML1-ETO significant genes (red region).

treated HL-60 microarray data derived from Stegmaier *et al* (Stegmaier*, et al* 2004) and examined overlap and enrichment of this data with the top 150 ATRA-responsive genes against each of the 4 AML translocation gene-sets using the Wicoxon rank sum test. As shown in **Figure 4.6B**, this ATRA-treated HL-60 gene-set was significantly enriched in the PML-RARα gene-list that we derived from the data of Valk *et al* (Valk*, et al* 2004) (p=0.0049). The ATRA-treated HL-60 gene-set was also enriched in the AML1-ETO gene-list ($p=6.7x10^{-5}$)(further discussion below), a finding also observed with our CMAP analysis (**Figure 4.6A-B**). The ATRA-treated HL-60 gene-set was not significantly enriched in the MLL or CBFβ-MYH11 (p>0.05) gene expression data and CMAP analysis did not detect ATRA association with these gene-lists (p>0.05). When acute promyelocytic leukaemic (APL) cells are treated with ATRA, the cells differentiate to granulocytes. A similar effect has also been reported with AML1-ETO AML cell lines and patient samples (Manfredini*, et al* 1999, Tagliafico*, et al* 2006).

With these results providing validation, we used a similar CMAP approach for the other three AML translocation signatures. **Table 4.1** shows the summary of the compounds detected with negative associations using this approach. In addition to ATRA, CMAP identified several other compounds that target specifically to the PML-RARα translocation and the other 3 specific translocation groups. Compounds were also detected that showed a significant negative association with all translocation signatures. This group included 8-azaguanine, 1,4-chrysenequinone, natamycin, and alpha-estradiol, as shown in **Table 4.1**. Of these compounds, alpha-estradiol at 10 μM has been shown to differentiate HL-60 promyelocytic cells to neutrophils (Stegmaier*, et al* 2004). 8-azaguanine is a purine analogue and has also been shown to induce granulocytic differentiation in the HL-60 cell line

Table 4.1. Significant connectivity scores for the AML translocation gene signatures.

Compounds	n	Connectivity score			
		PML-RARα	AML1-ETO	CBFβ-MYH11	MLL
Tretinoin (ATRA)	22	-0.4	-0.3	---	---
verteporfin	3	-0.7	---	---	---
lisinopril	3	-0.6	---	---	---
sitosterol	4	-0.6	---	---	---
chlorzoxazone	4	---	-0.6	---	---
ipratropium bromide	3	---	-0.6	---	---
mecamylamine	3	---	-0.6	---	---
pentoxyverine	4	---	---	-0.6	---
ellipticine	4	---	---	-0.6	---
gliclazide	4	---	---	-0.6	---
dequalinium chloride	4	---	---	---	-0.7
meptazinol	4	---	---	---	-0.7
etofenamate	4	---	---	---	-0.7
ginkgolide A	4	---	-0.6	-0.6	---
thioguanosine	4	---	-0.6	-0.6	---
norethisterone	4	---	-0.6	-0.6	---
8-azaguanine	4	-0.7	-0.7	-0.7	-0.6
1,4-chrysenequinone	2	-0.6	-0.7	-0.7	-0.7
natamycin	4	-0.6	-0.6	-0.6	-0.6
alpha-estradiol	16	-0.4	-0.4	-0.4	-0.4

All the compounds were significant ($p<0.05$) negatively associated with the relevant AML signature and ranked based on connectivity score. "---" indicates not significant $p>0.05$.

(Kretz*, et al* 1987). We also identified Ginkgolide A, Thioguanosine (anti-metabolites, cancer treatment in CLL) (Vora*, et al* 2006) and Norethisterone (progestogen) as CBF AML specific compounds (**Table 4.1**).

We next focused on drugs that are predicted to be selective for specific translocation subgroups. We focused in particular on pentoxyverine (a.k.a Carbetapentane citrate), as this is the highest ranked drug specific for the CBFβ-MYH11 translocation in AML (**Figure 4.7A** and **Table 4.1**) and Dequalinium chloride (DC), as the highest ranked specific drug for the MLL translocation group as shown in **Table 4.1** and **Figure 4.7B**. Pentoxyverine has not been previously tested on AML or as an anti-tumour compound. DC is an anti-microbial drug and induces loss of mitochondrial transmembrane potential and apoptosis (Sancho*, et al* 2007). The chemical structure for pentoxyverine and DC are shown in **Figure 4.7C**.

4.3.5 Experimental validation of CMAP results

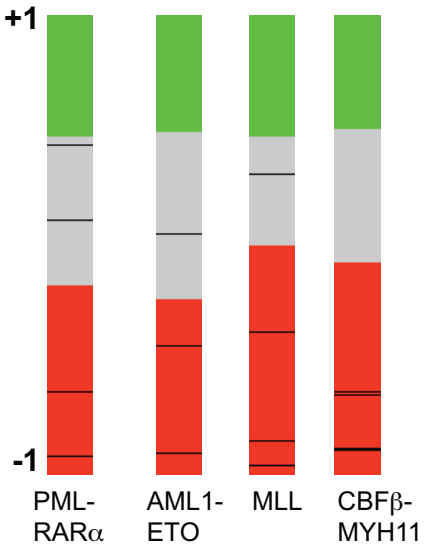
4.3.5.1 The effects of pentoxyverine on AML patient blasts

a) CBFβ-MYH11 AML. We first performed pentoxyverine treatment on MNC from a CBFβ-MYH11 patient (Patient inv16-1). Cells were either cultured with 10 ng/ml each of the GFs (FL, IL-6, IL-3, SCF, G-CSF and GM-CSF), herein referred to as the GF cocktail (see **Section 4.2.2**) or in the absence of growth factor. We analysed cell number, apoptosis, and differentiation markers (CD11b, CD14, CD15 and CD34) over a 4-day culture period. AML MNC cells were treated with either 10 μM (dose used in CMAP is 8 μM), or 50 μM of pentoxyverine, or vehicle control (water). As shown in **Figure 4.8A-B**, in the absence of the GF cocktail, there was reduced viable cell number and decreased cell survival when inv(16)

A.

Pentoxyverine (CBFβ-MYH11 specific)

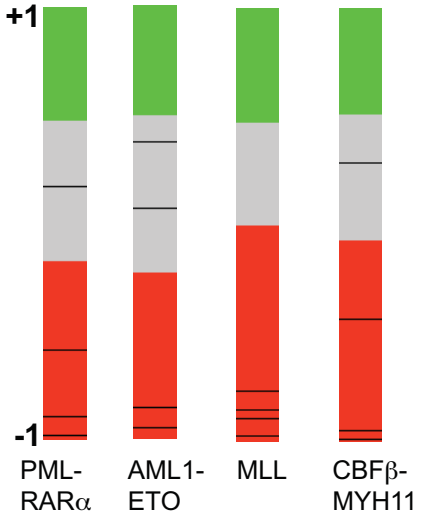
Name	n	Mean score	p-value
PML-RARα	4	-0.31	0.7
AML1-ETO	4	-0.50	0.2
MLL	4	-0.44	0.3
CBFβ-MYH11	4	-0.60	0.002



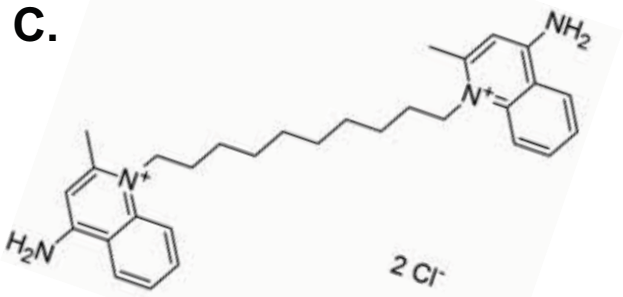
B.

Dequalinium chloride (MLL specific)

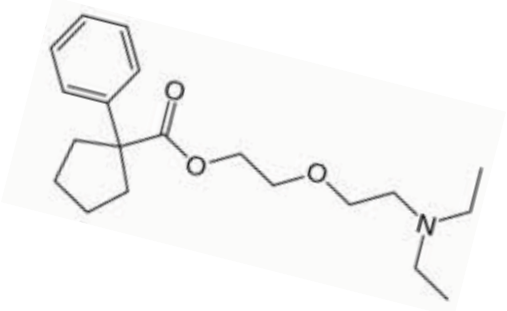
Name	n	Mean score	p-value
PML-RARα	4	-0.50	0.1
AML1-ETO	4	-0.37	0.4
MLL	4	-0.71	0.0004
CBFβ-MYH11	4	-0.53	0.2



C.



Dequalinium chloride



pentoxyverine

Figure 4.7. CMAP selectively identifies specific compounds for each AML translocation group. A. CMAP identifies pentoxyverine as negatively associated with the CBFβ-MYH11 gene signature. **B.** CMAP identifies dequalinium chloride as negatively associated with the MLL gene signature. Each of the pentoxyverine or dequalinium chloride experiments (n=4 for each compounds) is highlighted as a black bar on the bar plot, and colors represent a negative (red), no connection (gray), or positive connection (green) between the query signature and the compound experiments for each array in the Connectivity Map database. **C.** The chemical structure of pentoxyverine and dequalinium chloride.

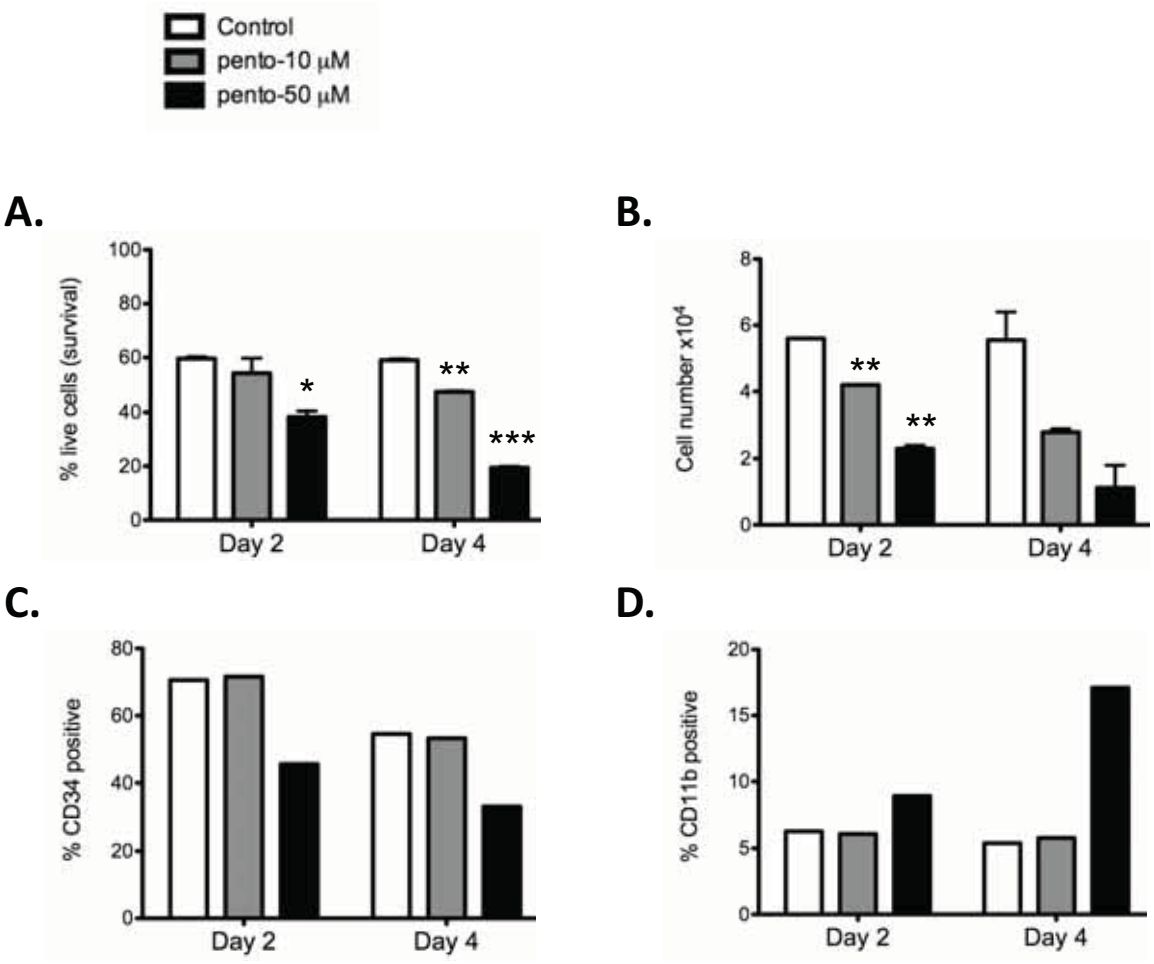


Figure 4.8. The effects of inv(16) patient MNC cells treated with pentoxyverine in the absence of growth factor cocktail. The blast cells (MNC) from the patient inv16-1 were treated with pentoxyverine to examine **A.** cell survival using Annexin V staining, **B.** total viable cell number, **C.** CD34 expression (n=1) and **D.** the expression of CD11b myeloid differentiation marker (n=1) in the absence of growth factor cocktail (IL-3, IL-6, SCF, FL, GM-CSF and G-CSF) at day 2 and day 4 after treatment with pentoxyverine. The control used was water. Data represent mean ± SEM for duplicates except where indicated otherwise. *** $p<0.001$, **$p<0.01$, *$p<0.05$ (unpaired student's t-test). Pento indicates pentoxyverine.

patient MNC cells were treated with pentoxyverine 50 μM. At 10 μM pentoxyverine, there was a small but significant decrease in cell survival at day 4 compared to water control.

Treatment of this sample with pentoxyverine also affected differentiation markers. At day 4, cells treated with 50 μM pentoxyverine in the absence of GF cocktail had decreased expression of CD34 and increased expression of CD11b compared to control or 10 μM pentoxyverine (**Figure 4.8C-D**). There were no obvious difference in staining for the monocytic marker CD14 or the granulocytic marker CD15 when cells treated at 10 μM or 50 μM pentoxyverine were compared to control (data not shown).

In the presence of the GF cocktail, 50 μM pentoxyverine decreased the cell survival of CBFβ-MYH11 MNC cells at day 4 (**Figure 4.9A**) with an associated decrease in cell number only at 50 μM pentoxyverine at day 4 (**Figure 4.9B**). As described above, we again observed decreased levels of CD34 (**Figure 4.9C**) and increased CD11b expression (**Figure 4.9D**) at 50 μM pentoxyverine compared to the control at day 4. There was no change in CD14 or CD15 expression when cells were cultured with pentoxyverine (data not shown). Thus, pentoxyverine had significant effects on survival and surface marker expression of this primary AML, particularly at 50 μM pentoxyverine, whether in the presence or in the absence of GFs.

b) MLL AML. To determine whether the effect of 50 μM pentoxyverine was selective to CBFβ-MYH11 AML cells, we thawed an MLL patient sample (Patient MLL-1) and treated

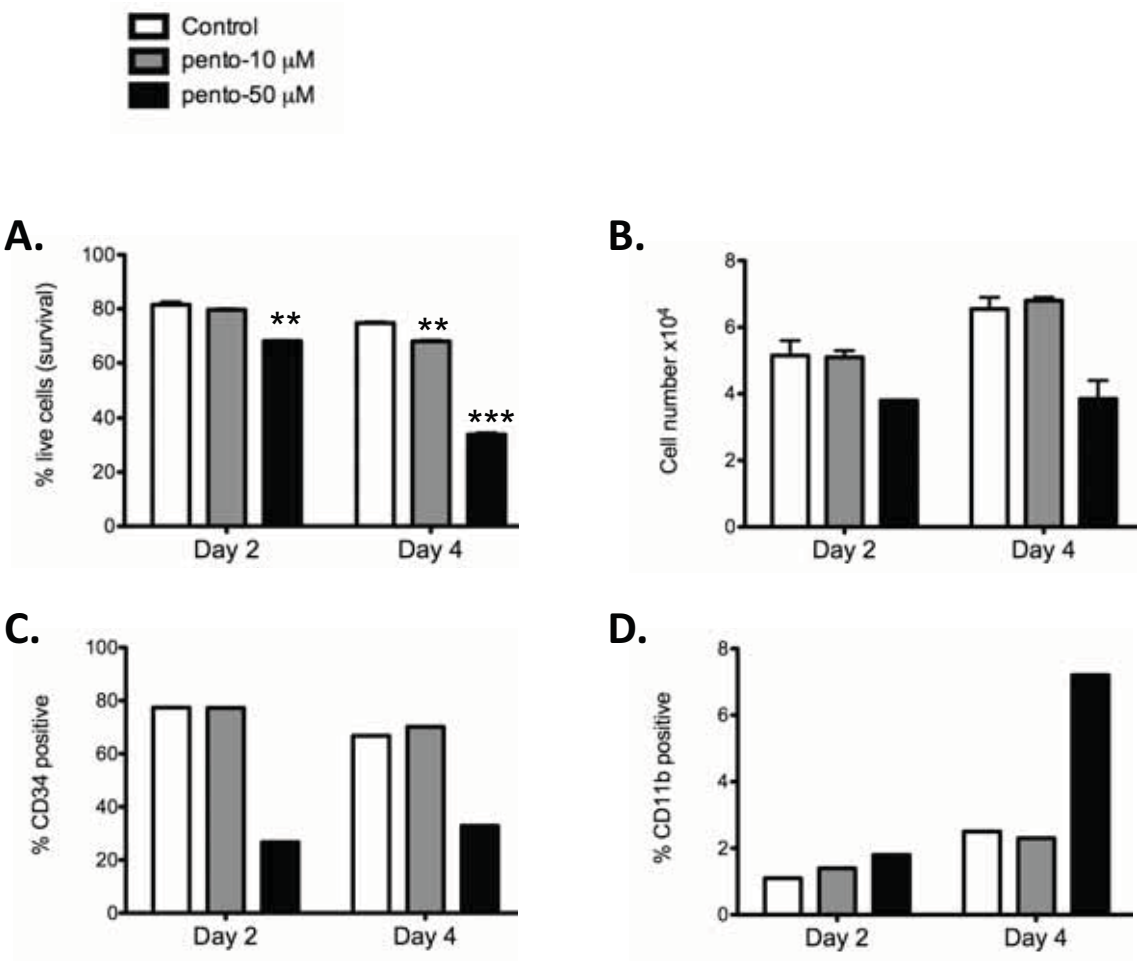


Figure 4.9. The effects of inv(16) patient MNC cells treated with pentoxyverine in the presence of growth factor cocktails. The blast cells (MNC) from the patient inv16-1 were treated with pentoxyverine to examine **A.** cell survival using Annexin V staining, **B.** cell number, **C.** CD34 expression (n=1) and **D.** CD11b myeloid differentiation marker expression (n=1) in the presence of growth factor cocktail (IL-3, IL-6, SCF, FL, GM-CSF and G-CSF) at day 2 and day 4 after treatment with pentoxyverine. The control used was water. Data represent mean ± SEM for duplicates except where indicated otherwise. *** $p<0.001$, **$p<0.01$ (unpaired student's t-test). Pento indicates pentoxyverine.

these cells with either 10 μM or 50 μM pentoxyverine or water control in the presence or absence of GF cocktail as described above. In the absence of GF cocktail, as shown in **Figure 4.10A**, there was a small decrease in cell survival at 50 μM pentoxyverine compared to control, although these cells displayed generally low viability in all conditions. There was also a small difference in cell number at 50 μM pentoxyverine compared to control at day 2 and day 4 (**Figure 4.10B**) suggesting that pentoxyverine may have a general affect on AML cell growth and survival. However, we observed no decrease in CD34 or increase in CD11b staining at either concentration of pentoxyverine when compared to control (**Figure 4.10C-D**).

In the presence of GF cocktail, both concentrations of pentoxyverine showed reduced in cell number compared to control. This reached significance at 50 μM day 4 but was not associated with changes in cell survival (**Figure 4.11A-B**). There was a small decrease in CD34 expression at both concentrations of pentoxyverine compared to control at day 4 (**Figure 4.11C**) and interestingly CD11b expression decreased at 50 μM pentoxyverine compared to control at day 4 (**Figure 4.11D**). Thus, we have detected significant differences in responses of these two patient samples to pentoxyverine, particularly with respect to induction of differentiation markers. Consistent with selectivity of pentoxyverine for the CBFβ-MYH11 AML cells as predicted by our bioinformatics analysis, we observed that 50 μM pentoxyverine displayed more pronounced effects on differentiation of primary inv(16) cells compared to cells with an MLL translocation. This analysis is limited by the difference in viability and growth of these primary samples and by the limited number of samples available at the time. Further studies are therefore required using more primary AML samples.

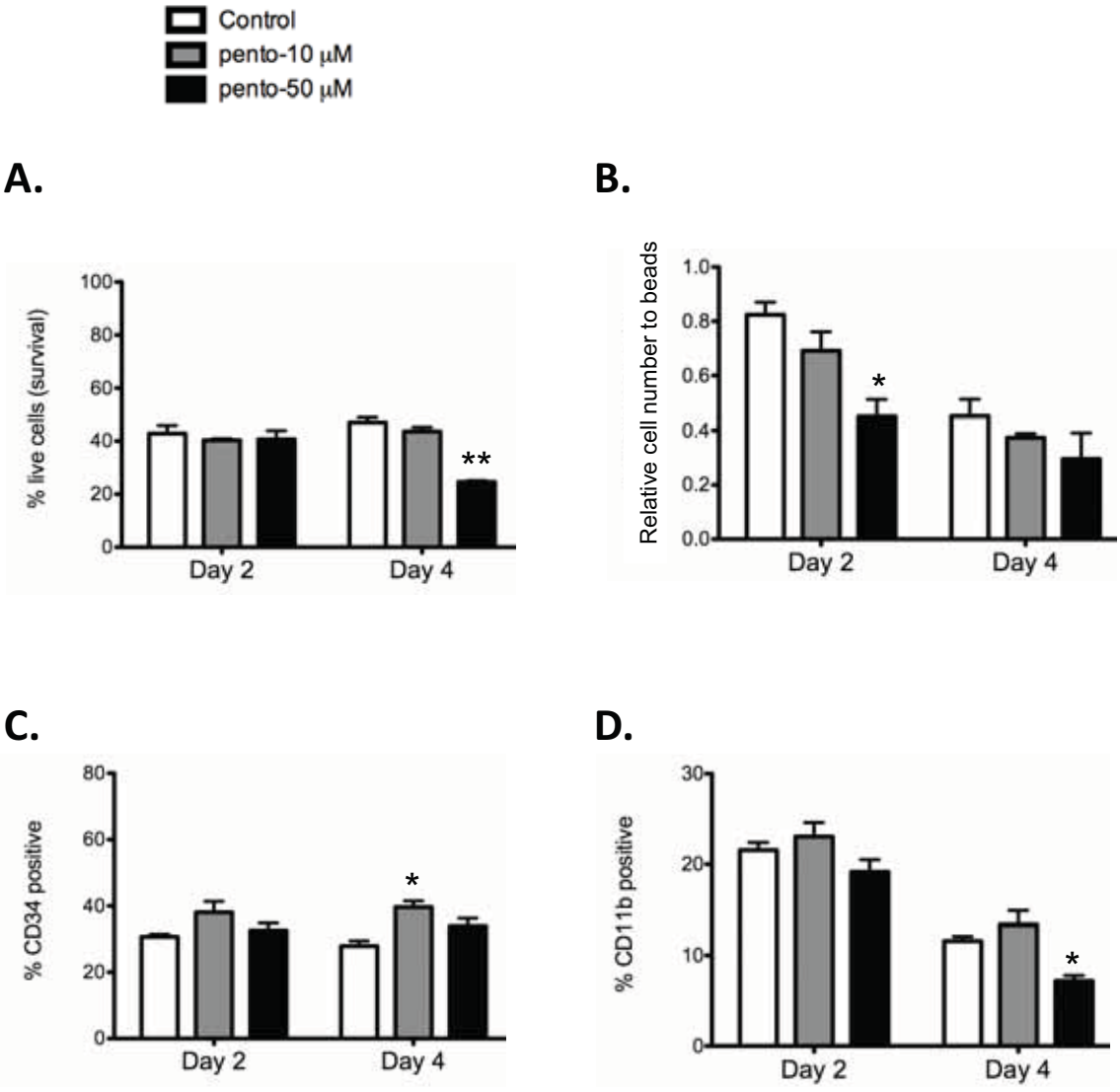


Figure 4.10. The effects of MLL patient MNC cells treated with pentoxyverine in the absence of growth factor cocktails. The blast cells (MNC) from the patient MLL-1 were treated with pentoxyverine to examine **A.** cell survival using Annexin V staining, **B.** total viable cell number using fluorescence-beads, **C.** CD34 expression and **D.** CD11b myeloid differentiation marker expression in the absence of growth factor cocktail (IL-3, IL-6, SCF, FL, GM-CSF and G-CSF) at day 2 and day 4 after treatment with pentoxyverine. The control used was water. Data represent mean ± SEM for duplicates experiments. ** $p<0.01$, *$p<0.05$ (unpaired student's t-test). Pento indicates pentoxyverine.

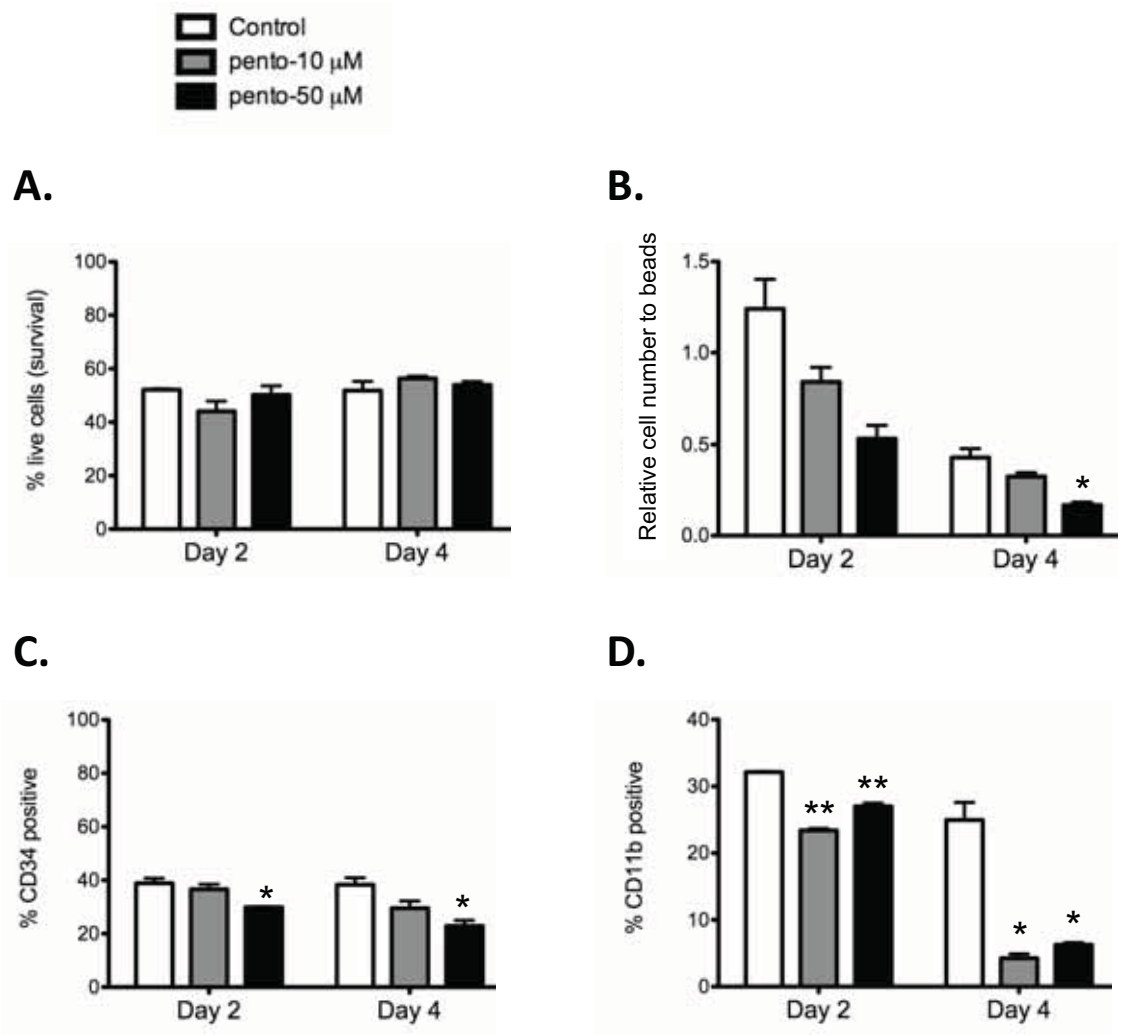


Figure 4.11. The effects of MLL patient MNC cells treated with pentoxyverine in the presence of growth factor cocktails. The blast cells (MNC) from the patient MLL-1 were treated with pentoxyverine to examine **A.** cell survival using Annexin V staining, **B.** total viable cell number using fluorescence-beads, **C.** CD34 expression and **D.** CD11b myeloid differentiation marker expression in the presence of growth factor cocktail (IL-3, IL-6, SCF, FL, GM-CSF and G-CSF) at day 2 and day 4 after treatment with pentoxyverine. The control used was water. Data represent mean ± SEM for duplicates experiments. ** $p<0.01$, *$p<0.05$ (unpaired student's t-test). Pento indicates pentoxyverine.

4.3.5.2 The effects of dequalinium chloride on MLL AML patient blasts

We next tested the effects of DC, which was identified by CMAP (7.6 μM) as specific for MLL AML on primary AML cells. We used the same MLL patient MNC cells from above (Patient MLL-1) and treated with 10 μM or 50 μM DC in the presence or absence of GF cocktail. In the absence of the GF cocktail, either 10 μM or 50 μM DC resulted in increased apoptosis measured by Annexin V staining, and a dramatic reduction in total viable cell number compared to the control (**Figure 4.12A-B**). Due to the extensive cell death, under these conditions, myeloid differentiation markers could not be measured.

In the presence of the GF cocktail, both concentrations of DC reduced survival at day 4 compared to the control with a dramatic reduction in cell number at day 2 and day 4 (**Figure 4.12C-D**). The mechanism of growth reduction is unclear given that only a small difference in survival at day 2 was detected using the APC-conjugated fluorescence beads and Annexin V assay as described in **Section 4.2.3**. Hence, it is important now to determine the effects on cell division, DNA replication and cell cycle. This could be achieved by using the carboxyfluorescein diacetate succinimidyl ester (CFSE), and PI or Bromodeoxyuridine (BrdU) staining assays respectively (Iglesias-Ara*, et al* 2010). Further insights to investigate the effects of this drug are discussed in the **Discussion Section 4.5**.

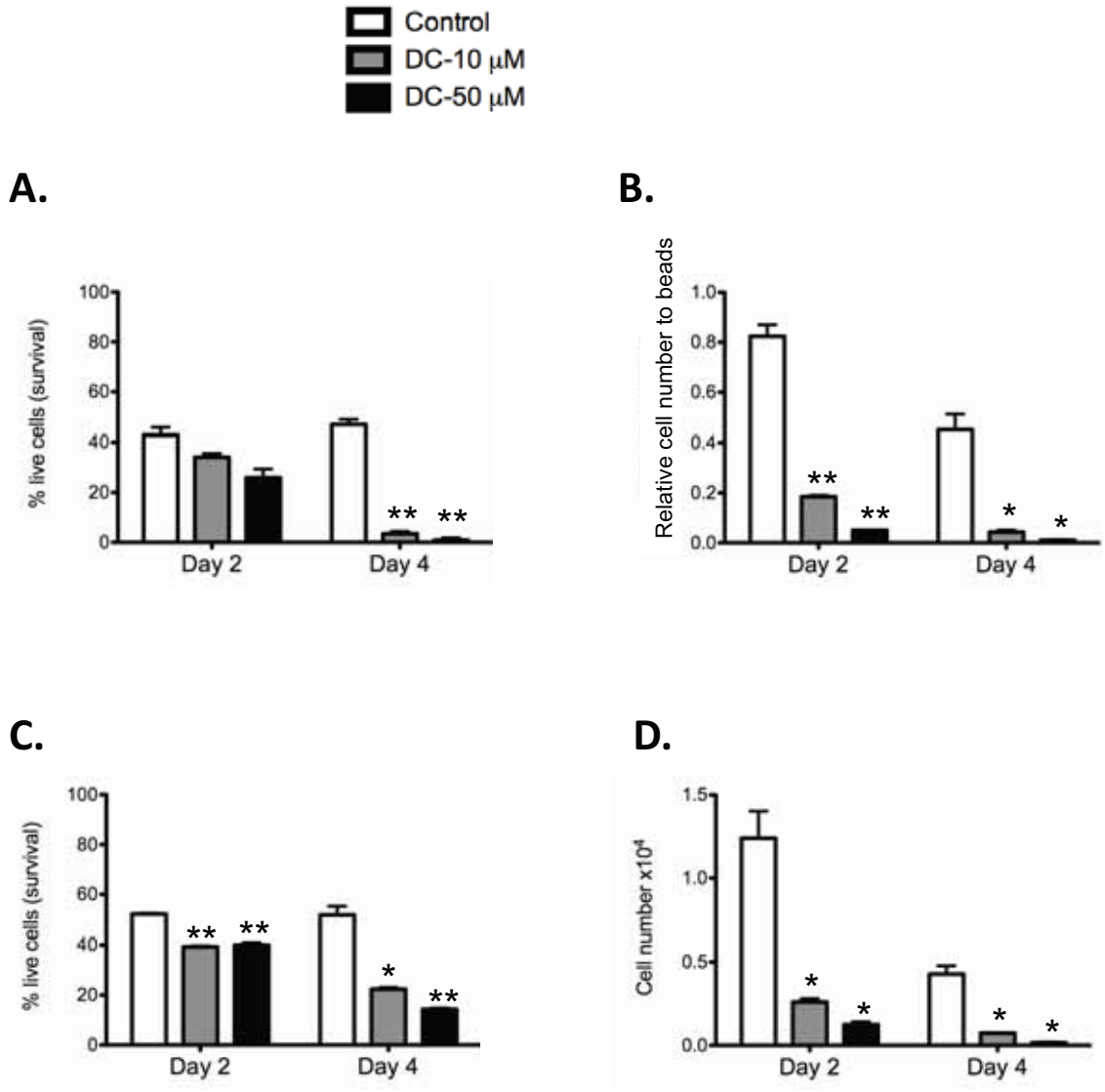


Figure 4.12. The effects of MLL patient MNC cells treated with dequalinium chloride in the absence or presence of growth factor cocktails. The blast cells (MNC) from the patient MLL-1 were treated with dequalinium chloride to examine cell survival using Annexin V staining and total viable cell number using fluorescence-beads in the absence **(A-B)** and in the presence **(C-D)** of growth factor cocktail (IL-3, IL-6, SCF, FL, GM-CSF and G-CSF) at day 2 and day 4 after treatment with dequalinium chloride. The control used was water. Data represent mean ± SEM for duplicates experiments. ** $p<0.01$, *$p<0.05$ (unpaired student's t-test). DC indicates dequalinium chloride.

4.4 HOXA9 is over-expressed in Trisomy 8 AML

4.4.1 Results presented as manuscript format

As described above (**section 4.3.2**), we identified high *HOXA9* expression in normal karyotype AML, AML associated with MLL as reported previously (Krivtsov and Armstrong 2007, Mullighan*, et al* 2007) and also in trisomy 8 AML. Here we described further analysis to investigate the contribution of trisomy 8 to leukaemogenesis. These results have been accepted for publication in the journal of Leukemia (Kok*, et al* 2010) (**Appendix E**). Briefly, we identified trisomy 8 AML patients have high level of *HOXA9, HOXA10* and *HOXA1* gene expression and IPA analysis revealed the connection between gene expression of *HOXA* and AKT/NFκB. Individual contributions of authors are summarised in the table below:

Name	Contribution %	Contribution types
Chung H Kok	40	Bioinformatics analysis, analysed the data, wrote the manuscript
Anna L Brown	15	Critical review of the manuscript, wrote the manuscript
Paul G Ekert	10	Contributed to the manuscript writing
Richard J D'Andrea	35	Supervised and designed the bioinformatics analysis, wrote the manuscript

4.4.1.1 Identification of compounds and drugs using a trisomy 8 signature

To identify compounds that are able to eliminate trisomy 8 AML cells, we utilised the CMAP analysis with the similar approach as outlined in **Section 4.2.5**. Gene set enrichment using version 2 of CMAP shows that the +8 gene signature displays significant negative connectivity with the signature for Sanguinarine (**Table 4.2**), a natural plant product that has been shown to be a potent inhibitor of NFκB activation in human myeloid ML-1a cells in response to a number of agents, for example TNF, PMA and IL-1 (Chaturvedi*, et al* 1997). Several other small molecules targeting the NFκB pathway such as Anabasine, Tyloxapol, Flunisolide and Benfotiamine are included in CMAP and while these also display significant negative connectivity with the +8 signature they failed to meet the specified mean cut-off (data not shown). Activation of the NFκB pathway is widespread in AML and the acquisition of +8 maybe one of several mechanisms resulting in increased NFκB activity that contributes to AML cell survival and resistance to chemotherapeutic agents (see **Discussion Section 4.5**) (Grandage*, et al* 2005, Karin 2006). The gene set enrichment analysis using CMAP suggests the possibility that Sanguinarine or other selected IκBKB or NFκB inhibitors may reverse some changes associated with acquisition of chromosome 8 and have therapeutic benefit.

4.5 Discussion

An analysis of a large 285 AML gene-expression dataset, using karyotypic abnormality as a primary criteria associated with gene expression, has identified unique signatures for each of the major translocations and revealed new insights regarding the role of some of the key players in AML such as *HOXA9*. There are other published studies which have reported the

Table 4.2. Drugs and/or small molecules that are negatively associated with the trisomy 8 AML signature.

Drug Name	Mean	p-value
MS-275	-0.674	0.00251
sanguinarine	-0.641	0.00875
dexpropranolol	-0.626	0.00863
rimexolone	-0.596	0.00533
apramycin	-0.586	0.00362
betahistine	-0.567	0.00497
imidurea	-0.549	0.03449
norethisterone	-0.544	0.01605
piribedil	-0.527	0.00633
carcinine	-0.511	0.00959
metamizole sodium	-0.509	0.00087
pseudopelletierine	-0.508	0.00479
roxarsone	-0.504	0.00195
zardaverine	-0.501	0.03272

All drugs from the Connectivity Map that met the criteria (mean < -0.5 and $p<0.05$) are listed. The list is ranked by the mean (connectivity score). A higher mean (regardless of the sign) indicates a better correlation between the drug gene expression and the query signature. A negative connectivity score indicates that the corresponding agent reverses the expression of the query signature.

gene expression differences among different karyotypic subgroups in AML especially the AML translocation subgroups (Bullinger*, et al* 2004, Valk*, et al* 2004). However, they have compared each AML subgroup to other AMLs or compared to normal $CD34^+$ or normal karyotype AML. In this study, we used a different approach by comparing AML subgroups to NBM. Within the gene signatures we have identified individual genes that may be of relevance to the pathogenesis of specific subtypes of AML. For example, we have shown that *CAPRIN2* expression is selectively lower in CBF AML compared to other subtypes. This finding has not been previously reported. The C-terminus of *CAPRIN2* has homology to the complement component C1q which is involved in clearance of damaged and/or harmful apoptotic cells (Aerbajinai*, et al* 2004, Tsiftsoglou*, et al* 2009). *CAPRIN2* mRNA is present normally in haemopoietic cells, including erythroid progenitor cells (Aerbajinai*, et al* 2004). Additionally, *CAPRIN2* expression is increased during mature erythroid differentiation from normal $CD34^+$ cells (Aerbajinai*, et al* 2004). A study has reported that over-expression of *CAPRIN2* during erythrocyte differentiation from $CD34^+$ cells induces apoptosis involving a mitochondrial mechanism that may be involve down-regulation of the anti-apoptotic gene *BCL-2* (Aerbajinai*, et al* 2004). Another recent study in zebrafish reported that *CAPRIN2* enhanced canonical Wnt signalling by regulating GSK3 mediated low-density lipoprotein receptor-related protein (LRP5/6) phosphorylation. Furthermore, *CAPRIN2* is able to stabilise cytosolic β-catenin, increases LEF1/TCF reporter gene activity, and expression of Wnt target genes such as *CDX4* and *TBX6* (Ding*, et al* 2008). It is not clear how these effects of *CAPRIN2* on apoptosis and Wnt signalling are related to its down-regulation in CBF AML. In t(8;21) AML, increased or stabilised β-catenin has been reported despite *CAPRIN2* down-regulation (Muller-Tidow*, et al* 2004), suggesting that stabilised β-catenin observed in t(8;21) is regulated through a mechanism other than increased expression of *CAPRIN2*. We speculate that *CAPRIN2* down-regulation may be as a consequence of the block in

macrophage and erythrocyte differentiation that is induced by these two fusion proteins. Further investigation of the functional role of *CAPRIN2* in CBF leukaemia is required to investigate.

The identification of specific AML translocation gene expression profiles, with reference to the normal population of BM cells undergoing growth and differentiation, allowed us to identify drugs or compounds by CMAP for each specific AML translocation and with potential for therapeutic application; i.e. we predicted that compounds with a significant negative association will induce growth suppression, differentiation and/or apoptosis. By using the CMAP database, we have successfully associated each specific AML translocation signature with drug responses. Identification of ATRA with the PML-RARα signature was an important validation of this approach with the strongest match against the HL-60 APL cell line treated with ATRA. We experimentally tested two of the compounds identified through CMAP analysis and generated cell-based data that was consistent with the bioinformatic analysis. The pentoxyverine exerted pronounced and selective effects particularly on differentiation of a primary inv(16) AML sample. There was also some growth reduction on MLL AML cells suggesting a possible general affect of pentoxyverine on growth. However, more detailed measurements of differentiation markers and an increase in number of samples are required to investigate the specificity and toxicity of pentoxyverine. The mechanism associated with the effect of pentoxyverine on inv(16) cells is unknown although it is a selective sigma receptor agonist (Wallace and Booze 1995). Sigma receptor ligand has been shown to exhibit anti-tumour activity both *in vitro* and *in vivo* through inhibition of cell proliferation (Berthois*, et al* 2003) and induces apoptosis through increases in intracellular calcium levels in colon and mammary adenocarcinoma cells (Brent*, et al* 1996). However, there have been no studies to investigate the effects of sigma receptor ligand or agonist on

AML cells. It must also be considered that these compounds may be acting via off-target effects. Thus, further investigation of inv(16) AML cells treated with pentoxyverine is needed to investigate the mechanism of action. Some further insights may be gained from gene expression profiling analysis and pathway analysis.

We also showed that DC effectively eliminated primary AML cells carrying the MLL translocation. Treatment with DC induced cell apoptosis as measured by the Annexin V marker and reduced viable cell number. It is now important to test the effect of DC on other AML samples and normal $CD34^+$ cells. It is also important to increase the sample size of MLL AML samples to test the effectiveness of this drug. To date, studies in our lab with AML cell lines are consistent with a selective effect (apoptosis and growth inhibition) in the MLL group (THP-1, MV4-11 and MOLM-13) compared to non-MLL group such as ME-1 and K562 cells (Salerno D, unpublished data). Studies with LSC from primary AML samples and normal HSC also have shown that MLL AML LSC are significantly more sensitive to DC than HSC (Powell J, unpublished data).

In contrast to the effect of pentoxyverine on AML cells, DC eliminated MLL cells by inducing cell death. There are multiple potential mechanisms for the activity of DC on AML MLL cells. There have been studies on myeloid leukaemic cell lines (NB4 and K562) reporting that the cell death effect of DC is mediated through loss of mitochondria transmembrane potential and apoptosis by the caspase3/9-dependent intrinsic pathway, enhanced reactive oxygen species (ROS) production and ATP depletion (Galeano*, et al* 2005, Sancho*, et al* 2007). While DC has also been reported to induce cell death on myeloid leukaemia cell lines (NB4 and K562) other than MLL fusion positive cell line (Galeano*, et al*

2005), there is no direct comparison of relative sensitivity of these cell lines or studies in primary AML samples. Elevated ROS levels may play a key role. Recently, elevation of ROS by iron-chelation was shown to induce AML differentiation by promoting monocytic differentiation of leukaemic cell lines, cultured AML blasts and LSC from patients, and normal HSC from cord blood (Abdel-Wahab and Levine 2010, Callens*, et al* 2010). Based on the studies above, it would be of interest to investigate ROS production by measuring the intracellular accumulation of superoxide anion using the fluorescent probe dihydroethidium (DHE) and flow cytometry analysis following treatment of AML cells (Sancho*, et al* 2007). It is also of interest to investigate the effect on mitochondrial transmembrane potential which could be done by the measurement of uptake and retention of rhodamine 123 (mitochondrial-specific membrane potential probe) in the mitochondria and the fluorescent can be detected by flow cytometry (Sancho*, et al* 2007).

Furthermore, DC has been reported to inhibit protein kinase C (PKC) in murine melanoma cells (Sullivan*, et al* 2000). Given recent evidence that MCL-1 levels are critical for cell survival of MLL-rearranged ALL cells (Stam*, et al* 2009), we propose that the mode of action for DC may involve the pathways illustrated in **Figure 4.13**. Briefly, DC inhibits the PKC pathway which is normally able to stabilise MCL-1 for cell survival. One mechanism for this may be through PKC mediated phosphorylation of AKT and GSK3 which has been shown to enhance cell survival through MCL-1 stabilisation (Baudot*, et al* 2009, Morel*, et al* 2009). Interestingly, high expression of MCL-1 is associated with prednisone resistance in MLL ALL further suggesting that alteration of MCL-1 levels may be important for drug effects on cells (Stam*, et al* 2009). Given the potential of DC as an anti-leukaemia therapy in the MLL group, we are now investigating the effects of this drug on LSC from MLL AML compared to normal HSC.

Figure 4.13. The proposed mechanisms of action of dequalinium chloride. Dequalinum chloride inhibits PKC which normally acts to phosphorylate AKT and leads to stabilisation of MCL-1 and cell survival. Dequalinium chloride is also able to enhance ROS production which is associated with LSC differentiation (Abdel-Wahab and Levine, 2010).

Using a +8 AML signature for IPA and CMAP analysis, we found there may be a link between the pathway AKT-NFκB and the *HOXA9* expression. Indeed, Costa *et al* demonstrated that *HOXA9* expression was inhibited by the PI3K inhibitor LY294002 but not Rapamycin (mTOR inhibitor) suggesting that the PI3K-AKT pathway regulates *HOXA9* expression independently of mTOR in glioblastoma cells (Costa*, et al* 2010). In addition, De Santa *et al* reported that NFκB directly regulates *JMJD3* expression and this resulted in higher expression of *HOXA9* in macrophages (De Santa*, et al* 2007). Phosphorylation of AKT in the +8 AML group was observed using a proteomic study (Kornblau*, et al* 2009). Together, these findings suggest that +8 AML might use a *AKT-IκBKB-HOXA9* pathway for cell survival and chemo-resistance. Consistent with the prominence of a PI3K-AKT-NFκB pathway in this subgroup, the gene set enrichment analysis using CMAP suggests the possibility that Sanguinarine or other selected IκBKB or NFκB inhibitors may reverse some changes associated with acquisition of chromosome 8 and have therapeutic benefit. To date only one IκBKB inhibitor has been tested in AML (Frelin*, et al* 2005). Further studies with such compounds focused on +8 AML are needed to confirm our findings and these may provide a targeted approach to improve the treatment outcome of this +8 AML group which is an intermediate risk group (see **Chapter 1 Table 1.3**).

In summary, we have identified several features uniquely associated with AML subgroups. Furthermore, the use of the CMAP tool has allowed identification of compounds that may be specific and effective for AML translocation events. Further testing on AML samples and a better understanding of the mechanism of action of pentoxyverine and DC in AML cells is now required.

Chapter 5: Final Discussion

AML is a heterogeneous disease in which there is a mix of leukaemic blast cells and stem cell populations. Constitutive activation of receptor signalling pathways and aberrant transcription factor activation leads to AML through aberrant self-renewal, increased cell survival, proliferation, and a block in differentiation (see **Chapter 1 Section 1.3**).

5.1 Receptor signalling in AML

5.1.1 Signaling from the GMR

Due to the complexity of leukaemic cell responses to oncogenic signalling pathways, an understanding of the mechanisms of activation of these pathways and the associated downstream events is required. This may provide new leads for targeted therapy. In **Chapter 2**, we showed the importance of Ser^{585} and Tyr^{577} residues of the GMR hβc in regulating the fates of myeloid cells as well as their survival and proliferation. Bioinformatic and functional approaches to analyse Tyr^{577} signalling led to a focus on the role of mTOR and a review of its association with AML. Guthridge *et al* suggested that the GMR could alternatively signal via Tyr^{577} or Ser^{585} which function respectively as a binary switch to control cell survival and proliferation; or cell survival-only (Guthridge*, et al* 2006). Our studies suggest that Tyr^{577} mediates cell growth via mTOR, while Ser^{585} survival-only signalling is associated with a PI3K, mTOR-independent pathway. This Tyr/Ser motif may play important roles in switching signalling pathways in normal progenitors in response to growth factors, with regulation of this switch being dysregulated by an undefined mechanism in AML, thus promoting aberrant survival (Guthridge*, et al* 2006).

From our GSEA analysis we predict that AML with FLT3-ITD mutations will display selective sensitivity to mTOR inhibitors while AML with *RAS* mutations are predicted to be resistant. This finding is supported by the recent screening for drug sensitivity in more than 1000 genetically-characterised cancer cell lines at the Sanger Institute (**http://www.sanger.ac.uk/genetics/CGP/translation/**); this showed that Rapamycin induced cell death in cell lines with FLT3-ITD at lower IC_{50} compared to other cell lines, in particular those cell lines with Ras mutations. There is wide range of responses to mTOR inhibitors in AML (Khwaja 2010) and the mTOR/Tyr^{577} signature that we describe in the **Chapter 2** may be useful in predicting the responsiveness of AML patients to these inhibitors. To further examine this we will use gene expression profiles from Rapamycin-treated HL-60 cells (available from CMAP) to further refine the signature and incorporate genes responsive to Rapamycin. This signature would then be tested using high-throughput QPCR on our diagnosis AML patient samples, independent of karyotypic abnormalities or mutations. To validate its accuracy and sensitivity, this expression data will be correlated with *in vitro* sensitivity of AML blast to mTOR inhibitors.

5.1.2 Constitutive activation of FLT3 receptor and the role of *Gadd45a*

FLT3 mutations are important in AML as they are present in 20-30% of AMLs, and contribute to a poor outcome (see **Chapter 1 Section 1.6.2**). In this study, we found differences between FLT3-ITD and FLT3-TKD AML with regard to gene expression and signalling pathways in AML blasts (see **Chapter 3 Section 3.3.2**). There may be cross-talk between FLT3-ITD and GMR signalling as FLT3-ITD up-regulates expression of the GMR (*CSF2RB*) compared to FLT3-WT in AML blasts (Riccioni*, et al* 2009), and FLT3-TKD up-regulated the gene encoding GMRα (*CSF2RA*) (see **Chapter 3 Section 3.3.2.3**).

A significant focus of the work in this thesis has related to *Gadd45a* and its role on AML. Down-regulation of *Gadd45a* by the constitutively activated receptors, and the link to the MLL-translocation sub-group is discussed in **Chapter 3**. Gadd45α has been reported to have several growth-inhibitory functions which may explain its down-regulation or silencing in AML (Cretu*, et al* 2009). In particular, Gadd45α promotes apoptosis by inducing BIM localisation to the mitochondria (Tong*, et al* 2005), and this maybe important for cell death in response to FLT3 inhibitors (see below). Gadd45α also associates directly with NPM1 which has been reported to localise Gadd45α from cytoplasm to nucleus where it functions with other co-factors to induce cell cycle arrest and apoptosis (Gao*, et al* 2005). *NPM1* mutations are most frequently found in normal karyotype AML (Rau and Brown 2009), and the Gadd45α-NPM1 interaction maybe important for characteristics of this group of AML. Gadd45α also regulates and/or reduces β-catenin levels (Hildesheim*, et al* 2004, Hildesheim*, et al* 2005, Ji*, et al* 2007), and hence may reduce the growth of LSC. Importantly we have shown that *Gadd45a* over-expression induces the expression of several differentiation genes such as *Cebpa, Cebpe and Sfpi1 (Pu.1)* (**Figure 3.17A**). Whether *Gadd45a* regulates *Cebpa* through a direct or indirect mechanism is unclear at this stage, however one possibility is that activation of these genes occurs through *Gadd45a*-induced DNA demethylation; Barreto *et al* showed that *Gadd45a* demethylates the tumour suppressor genes (e.g. *p16INK4a, p19ARF*) through a mechanism associated with DNA repair (Barreto*, et al* 2007). Sen *et al* also showed that *Gadd45a* promotes DNA de-methylation of skeletal muscle differentiation genes such as skeletal actin (*ACTA1*), tropomyosin (*TPM1*) and myosin heavy chain (*MYH8*) (Sen*, et al* 2010). Thus in some subtypes of AML down-regulation or silencing of *Gadd45a* could represent an important and novel mechanism of down-regulation for key tumour suppressor genes.

Although *Gadd45a* is commonly down-regulated by the leukaemic GMR-V449E mutant and both FLT3 mutations, the effect of *Gadd45a* overexpression in cells expressing the two FLT3 mutant receptors is different. There are several possible explanations for this observation. A recently published study shows that the protein Bim is more critical than Puma in FLT3 inhibitor-induced apoptosis of FLT3-ITD^{+} AML, consistent with reduced expression of *Gadd45a* in this group (Nordigarden*, et al* 2009). This could be further investigated with a more detailed study of the pro-apoptotic gene *Bim* in FLT3-TKD AML cells or cell lines.

Through bioinformatic analysis of gene expression profiling, FLT3-ITD was found to regulate MYC, NFκB, and ATM signalling in NK AML blasts and LSC. All of these pathways are involved in cell proliferation, survival and drug resistance. Most of these pathways are part of a network involving the AKT protein (**see Figure 3.13**), consistent with the FLT3-ITD mutant being selectively and highly dependent on AKT signalling pathways for cell survival (Brandts*, et al* 2005) (Sadras T, unpublished data). Sanchez *et al* suggested that genistein induced differentiation of HL-60 and NB4 AML cell lines through activation of the RAF/MEK/ERK pathway, and subsequently lead to ATM activation (Sanchez*, et al* 2009). It is not known whether ATM activity is influenced by the AKT network in AML with FLT3-ITD. However, a deficiency in ATM may generate genomic instability associated with FLT3-ITD mutations and this may contribute to the poor outcome.

As discussed in **Chapter 1 Section 1.6.2.2**, FLT3-ITD but not FLT3-TKD becomes trapped in ER, however, to date the mechanism associated with this has been unclear. We found that

STON2, a protein responsible for receptor endocytosis (Martina*, et al* 2001), is selectively overexpressed in FLT3-ITD NK AML blasts (see **Chapter 3 Figure 3.2**) and LSC (13 fold-change, FDR p-value = 0.048). In addition, *STON2* expression has been used as one of 86 probes that are able to accurately predict AML with FLT3-ITD expression (Bullinger*, et al* 2008). This suggests the possibility that the accumulation of FLT3-ITD in ER may be due to the up-regulation of *STON2* expression. Further functional studies on *STON2* are required in FLT3-ITD cells to validate this finding and determine the importance of *STON2* to FLT3-ITD responses.

As discussed in **Chapter 3 Section 3.3.7.2**, there are also unique features associated with FLT3-TKD signalling. For example, *TRIB1* which is selectively up-regulated in the FLT3-TKD^{+} group of AML blasts is a negative regulator of C/EBPα and C/EBPβ, and is a direct target of the MEK1/ERK pathway. Importantly, we have shown that FLT3-TKD is more sensitive to the MEK inhibitor (U0126) than FLT3-ITD (see **Chapter 3 Section 3.3.4.2**). It is possible that FLT3-TKD uses a different mechanism to down-regulate C/EBPα activity and it will be important to determine whether there is differential phosphorylation of C/EBPα Ser^{21} by the FLT3-ITD and FLT3-TKD mutants as this has been reported to be an important mechanism of C/EBPα inhibition for the FLT3-ITD mutant (Radomska*, et al* 2006).

Thus, despite the fact that FLT3 mutations arise in the same receptor, both FLT3 mutations have unique biological properties associated with differential signalling and transcription, and this should be considered when deciding which inhibitors should be used to treat FLT3 mutations AML.

5.2 Transcription factors in AML

5.2.1 *CEBPA* mutation

In addition to constitutive activation of signalling pathway from the receptors, differentiation-associated transcription factors are also involved in AML development by blocking the differentiation process. Our studies have highlighted an association between signalling from the GMR (Ser^{585} and Tyr^{577}) and AML with *CEBPA* mutations. The association could be due to strong activation of the PI3K/AKT/mTOR signalling pathways in *CEBPA*-mutant AML, or alternatively this association raises the possibility that mutations in the gene encoding the GMR hβc (*CSF2RB*) occur in association with *CEBPA* mutations. This cannot be excluded as this represents a small group of AML and to date there is only one group which has screened *CSF2RB* in a small number of AML patients (fewer than 40 patients). While they did not find oncogenic mutations (Freeburn*, et al* 1998, Freeburn*, et al* 1996, Freeburn*, et al* 1997) recent technology should be used in a more extensive study to exclude this possibility.

5.2.2 Over-expression of *HOX* genes

Increased expression of *HOX* genes is associated with HSC and has been observed in several AML subtypes, e.g. NK (Mullighan*, et al* 2007), +8 (this work and **Appendix E**) and the MLL AML subgroup (Krivtsov and Armstrong 2007). Interestingly, *HOXA9*$^{-/-}$ mice have impaired leukaemogenesis which is not observed in heterozygous *HOXA9*$^{+/-}$ cells (Lawrence*, et al* 1997). Moreover, our analysis of the *HOX* gene family in a small number of LSC samples was able to discriminate the FLT3-ITD and FLT3-WT groups at least in NK LSC.

The mechanisms for *HOX* gene up-regulation in the different subtypes possibly differ. For example, up-regulation of *HOX* genes is associated with *NPM1* mutation in NK AML group (Mullighan*, et al* 2007). In the +8 group we have speculated that increased expression may be associated with PI3K/AKT/NFκB signalling pathways (see **Appendix E**). In AML with MLL translocations, the fusion protein recruits the histone methyltransferase DOT1L resulting in up-regulation of some *HOXA* genes through DNA methylation and associated histone modifications (Shah and Sukumar 2010). In this study we observed up-regulation of DOT1L and some of the *HOX* genes in FLT3-ITD$^+$ LSC. This may suggest that FLT3-ITD up-regulates *HOX* gene expression via DOT1L and further investigation could determine if targeting the specific methyltransferase DOT1L is an approach for therapy in the FLT3-ITD$^+$ AML, possibly in conjunction with other targeted therapies.

5.3 CMAP

In AML, the most successful clinical treatment to date is ATRA which differentiates t(15;17) AML blasts to granulocytes (see **Chapter 1 Section 1.1.2**). For other subgroups, there are no FDA-approved drugs available. The development of new AML drugs that approved by FDA is slow and often failed in Phase II and III clinical trials (Woosley and Cossman 2007), requiring further testing in combination with standard chemotherapy regimens. We have shown that by using the CMAP database it is possible to identify drugs that are predicted to selectively act on tumour cell subtypes without harming normal cells. For example, our studies with dequalinium chloride are consistent with a selective sensitivity of MLL AML blasts and LSC to this compound with relative insensitivity of other translocation subtypes, and HSC. This approach provides an alternative method to the traditional drug-based assay, and is an inexpensive approach to finding drug leads that can be tested experimentally.

Moreover, this approach can be applied to other settings such as solid tumours as demonstrated by others (see **Chapter 1 Section 1.8.2**). In this thesis we have made extensive use of publicly available microarray data and this continues to provide an increasing resource. Together with the expansion of the CMAP drug database we believe this *in silico* approach will be important for identification of drug-leads that efficiently and more effectively treat AML.

Chapter 6: References

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Chapter 7: Appendix

7.1 Appendix A. Reagent Recipes

FACS WASH

PBS containing 5% FCS or Newborn Calf Serum, 0.2% Sodium Azide (w/v)**.

** made from 10% Sodium Azide stock, stored at 4^{o}C

FACS FIX

PBS containing 2% glucose (w/v), 1% formaldehyde (v/v), 0.02% Sodium Azide

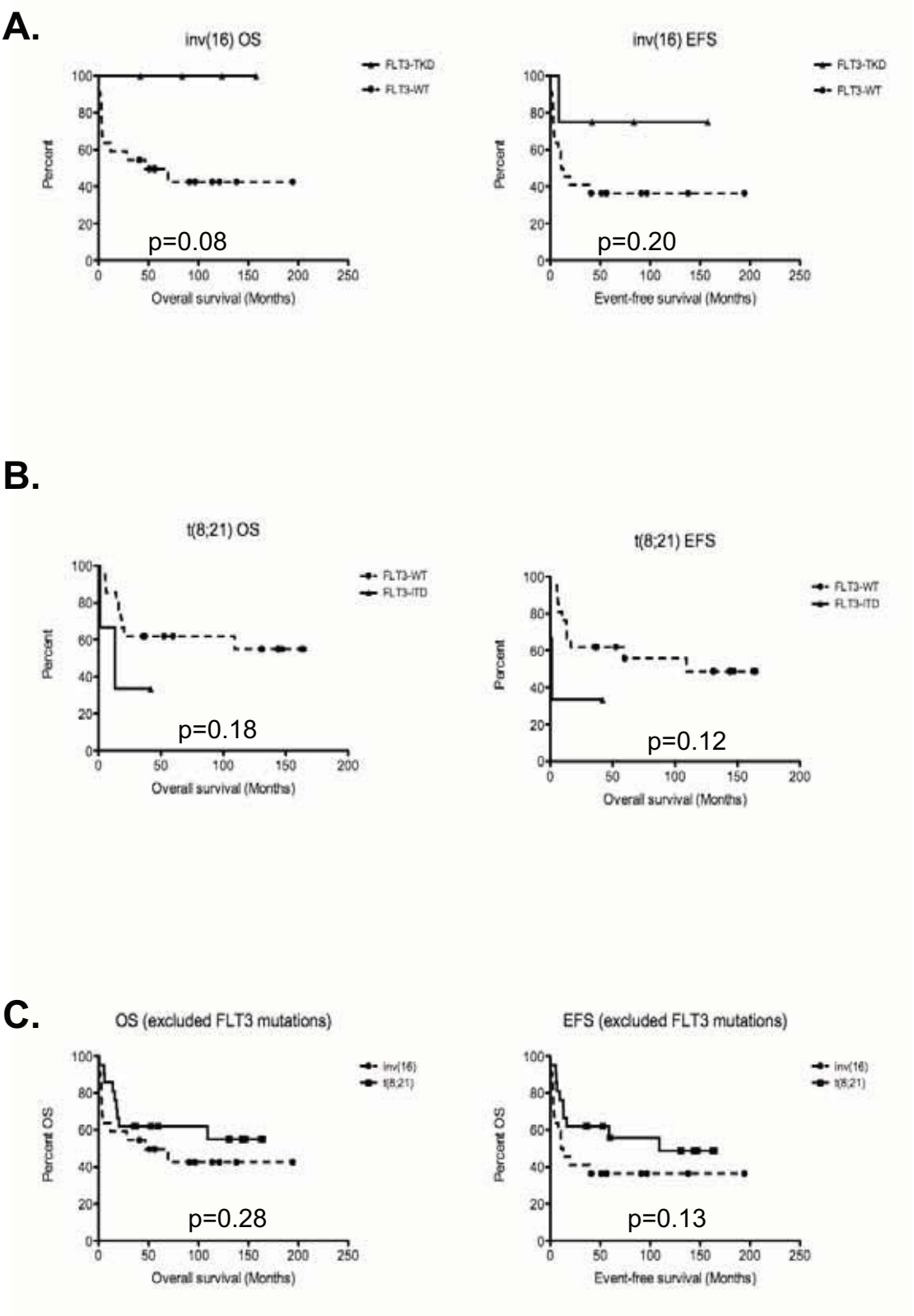
Modified RIPA Lysis Buffer

Tris-Hcl (pH. 7.4)	50mM
NP-40	1%
Na-deoxycholate	0.25%
NaCl	150mM
EDTA	1mM
Pefabloc	1:50 dil of 100mM stock
Complete Cocktail	1X
Na_3VO_4	1mM
NaF	2mM

7.2 Appendix B. Powell et al, 2009

Powell, J.A. et al. (2009) Expression profiling of a hemopoietic cell survival transcriptome implicates osteopontin as a functional prognostic factor in AML. *Blood, v. 114 (23), pp. 4859-4870, November 2009*

7.3 Appendix C. Effect of FLT3 mutations in CBF leukaemia on overall survival and event-free survival



Appendix C. Effect of FLT3 mutations in CBF leukaemia on overall survival and event-free survival. A. The overall survival and event-free survival of inv(16) FLT3-WT (n=22) and FLT3-TKD (n=4). **B.** The overall survival and event-free survival of t(8;21) FLT3-WT (n=20) and FLT3-ITD (n=3) excluding the FLT3-TKD (n=1). **C.** The overall survival and event-free survival of t(8;21) (n=20), inv(16) (n=22) excluding all the FLT3 mutations. The statistical significance was assessed using log-rank test. The clinical information extracts from the study Figueroa *et al (*Figueroa *et al*, 2010).

7.4 Appendix D. Perugini et al, 2009

Perugini, M. et al. (2009) Repression of Gadd45α by activated FLT3 and GM-CSF receptor mutants contributes to growth, survival and blocked differentiation. *Leukemia, v. 23, pp. 729-738, 2009*

7.5 Appendix E. Kok et al, 2010

Kok, C.H. et al. (2010) Gene expression analysis reveals HOX gene upregulation in trisomy 8 AML.
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