A model to investigate the oncogenic activity of MLL-fusions in Acute Myeloid Leukaemia

Vanessa M. Walf-Vorderwülbecke

Department of Molecular Haematology and Cancer Biology

Institute of Child Health

University College London

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DECLARATION

I, Vanessa Walf-Vorderwülbecke, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

London, 27.8.2009 Signature:

Vanessa M. Walf-Vorderwülbecke

ABSTRACT

The *MLL* gene, located on 11q23, is involved in a large number of chromosomal translocations, including t(9;11)(p22;q23) and t(11;19)(p22;q23). These translocations encode the MLL-AF9 and MLL-ENL fusion transcription factors and are prevalent in infant acute leukaemia and therapy-related leukaemia. Leukaemias associated with these translocations have a particularly poor outcome. In order to conditionally express the MLL-AF9 fusion oncogene in primary haematopoietic progenitor cells, retroviral delivery of the Tet-off expression system was used. Progenitors were purified from murine bone marrow and co-infected with MSCV-TRE-fMLL-AF9 and MSCV-tTA retroviral supernatants. Using this approach, eight independent cell lines with conditional expression of MLL-AF9 and three independent cell lines with constitutive MLL-AF9 expression were generated. Treatment of the conditional cells with Doxycycline caused a decrease in MLL-AF9 mRNA and protein expression, and resulted in terminal differentiation of the cells. By analysing global changes in gene expression after treatment of cells with Doxycycline, using Mouse genome Affymetrix Gene Chips (430 2.0), we have identified a number of potential transcriptional target genes of the MLL-AF9 and MLL-ENL fusion oncogenes. In order to examine the importance of target genes for MLL-fusion mediated transformation, up-regulated target genes were knocked down *in vitro*. Knock-down of a small proportion of the target genes analysed caused MLL-ENL and MLL-AF9 immortalised cells to die. These data illustrate novel approaches to interfering with MLL-fusion activity in leukaemia. In order to establish the importance of MLL-fusion activity for leukaemia *in vivo*, and hence its dependence on the transcriptional target genes identified, MLL-ENL immortalised cell lines were chosen to be injected into primary recipients. Conditionally MLL-

ENL immortalised cell lines were found to induced leukaemia *in vivo*. Leukaemic cells isolated from primary recipient mice were shown to have acquired additional genetic abnormalities and, when transplanted into secondary recipients, induced leukaemia with shortened latencies. However, the leukaemic cells remained dependent on MLL-ENL expression *in vitro* and *in vivo*, and its ablation resulted in regression of established leukaemias.

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ABBREVIATIONS

2-ME	2-mercaptoethanol
7AAD	7-amino-actinomycin D
aa	amino acids
AF1p	ALL1 fused gene from chromosome 1p
AF4	ALL1 fused gene from chromosome 4
AF6	ALL1 fused gene from chromosome 6
AF5q31	ALL1-fused gene from chromosome 5q31
AF9	ALL1 fused gene from chromosome 9
AF10	ALL1 fused gene from chromosome 10
AF17	ALL1 fused gene from chromosome 17
AFX	fork head domain transcription factor AFX1
AGM	aorta-gonad-mesonephros
ALL	acute lymphoid leukaemia
AML	acute myeloid leukaemia
AML1	acute myeloid leukemia 1 protein
$AML1^{-/-}$	AML1 deficient mice
AMP^{r}	ampicilin resistence
APC (Wnt)	adenomatosis polyposis coli
APC	allophycocyanin
APS	Ammonium Persulfate
Ascl2	achaete-scute complex homolog-like 2
ASH2L	ash2 (absent, small, or homeotic)-like
axin	axis inhibition protein 1
βTRCP	beta-transducin repeat containing
Bcl2	B-cell lymphoma protein 2
BFU-E	burst-forming unit-erythroid
BM	bone marrow
BMI1	BMI1 polycomb ring finger oncogene
BSA	Bovine Serum Albumin
Cacybp/SIP	calcyclin binding protein/Siah-interacting protein
CB	cord blood

CBF	core binding factor
CBFβ	core binding factor β
CBP	cAMP binding protein
CDK9	cyclin dependent kinase 9
cDNA	complementary deoxyribonucleic acid
C/EBPα	CCAAT/enhancer binding protein alpha
CFU-GEMM	colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte
CFU-M	colony-forming unit-macrophage
CGH	comparative genomic hybridisation
Chr	chromosome
CK1	casein kinase 1
CLL	chronic lymphoid leukaemia
CLP	common lymphoid progenitors
cMA3	constitutive MLL-AF9 cell line 3
cME3	constitutive MLL-ENL cell line 3
CML	chronic myeloid leukaemia
CMP	common myeloid progenitors
c-Myb	avian myeloblastosis viral (v-myb) oncogene homolog
c-Myc	avian myelocytomatosis viral oncogene homolog
Cre	cyclization recombination
CREB	cAMP responsive element-binding protein
cRNA	complementary ribonucleic acid
C_T	cycle threshold
CTD	C-terminal domain
C-terminus	carboxyl-terminus
Ctnnb1	Catenin, beta-1
Cybb	cytochrome b-245 beta polypeptide
DMEM	Dulbecco's Modified Eagle's medium
DNA	deoxyribonucleic acid
DNMT1	DNA methyltransferase 1 homology domain
DOT1L	DOT1-like, histone H3 methyltransferase
DOX	Doxycycline
DTT	Dithiothreitol
DVL	dishevelled

E	embryonic day
EDTA	Ethylenediaminetetraacetic Acid Disodium Salt
EEN	extra eleven-nineteen leukemia fusion gene protein
EGFP	enhanced green fluorescent protein
ELL	elongation factor RNA polymerase II
ENL	eleven nineteen leukaemia
ER	oestrogen receptor
ETO	Eight-Twenty-One gene
EtOH	Ethanol
Evi2a	ecotropic viral integration site 2A
F1ori	origin of single stranded DNA production
FAB	French-American-British
FcγRII/III	Fcγ receptor-II/III
FCS	foetal calf serum
FKHRL1	forkhead in rhabdomyosarcoma-like 1
flagMLLAF9	pMSCV-neo-mod-fMLLAF9
Flt3	FMS-like tyrosine kinase 3
FOG-1	friend of GATA-1
Frat1	frequently rearranged in advanced T-cell lymphomas
Frat2	frequently rearranged in advanced T-cell lymphomas 2
Fzd7	frizzled homolog 7
GAS7	growth arrest-specific 7
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
GMP	granulocyte-monocyte restricted progenitors
GRG	groucho
GSK3β	glycogen synthase kinase 3 beta
GTG-banding	Giemsa banding
Gy	Gray
H3K4	histone H3 lysine 4
H3K79	histone H3 lysine 79
HAT	histone acetyltransferase
hCD2	human CD2
HDAC	histone deacetylases
Hexa	Hexachlorophene

HLF	hepatic leukemia factor
HMT	histone methyltransferase
HOM-C	homeotic
Hox	homeobox
HoxA	homeobox A cluster
HoxC9	homeobox C9
HPC	haematopoietic progenitor cells
HPC2	polycomb 2 homolog
Hpgd	hydroxyprostaglandin dehydrogenase 15-(NAD)
HSC	haematopoietic stem cells
HSP90	heat shock protein 90
HYG^r	hygromycin resistance
IL-3	interleukin-3
IL-6	interleukin-6
IL-7Rα	interleukin-7 receptor α-chain
INT	p-iodonitrotetrazolium
IPA	Ingenuity Pathway Analysis
IRES-EGFP	internal ribosomal entry site-EGFP
KAN^r	kanamycin resistance
KCl	Potassium Chloride
KEGG	Kyoto Encyclopaedia of Genes and Genome
LAF	lymphoid nuclear protein related to AF4
Laptm4b	lysosomal protein transmembrane 4 beta
LEF1	lymphoid enhancer factor 1
Lck	lymphocyte-specific protein tyrosine kinase
Lcn2	lipocalin 2
LEDGF	lens epithelium derived growth factor
L-GMP	leukaemic granulocyte-monocyte restricted progenitors
Lin	lineage-associated surface marker
Lmo2	LIM domain only 2
LMPP	lymphoid-primed multipotent progenitors
loxP	locus of X-over P1
LRP5	low density lipoprotein receptor-related protein 5
LSC	leukaemic stem cells
LSK	$Lin^-Sca1^+c\text{-}kit^{hi}$

Ltf	lactotransferrin
LT-HSC	long-term haematopoietic stem cells
LTR	long terminal repeat
M3434	methycellulose medium containing SCF, IL-6 and IL-3
MA	conditional MLL-AF9 cell line
mAb	monoclonal antibody
Mac-1	CD18 antigen
MACS	magnetic activated cell sorting
ME	conditional MLL-ENL cell line
Mef2c	myocyte enhancer factor 2C
Meis1	myeloid ecotropic viral integration site 1
MEN1	multiple endocrine neoplasia type 1
MeOH	Methanol
MEP	megakaryocyte-erythrocyte restricted progenitors
miRNA	micro ribonucleic acid
MLL	mixed-lineage leukaemia
MLL^C	carboxy-terminal proteolytic fragment of MLL
MLL^N	amino-terminal proteolytic fragment of MLL
$Mll^{-/-}$	Mll deficient mice
MM	mismatch
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
Mmp9	metalloproteinase 9
Mpo	myeloperoxidase
mRNA	messenger ribonucleic acid
Msi2	musashi homolog 2
mycMLLAF9	pMSCV-neo-mod-mMLLAF9
MYH11	myosin heavy chain 11
Mylk	myosin light chain kinase
NaCl	Sodium Chloride
NaN_3	Sodium Azide
NaOAc	Sodium Acetate
NaOH	Sodium Hydroxide
NEO	pMSCV-neo
NEO^r	neomycin resistance
NH_4Cl	Ammonium Chloride

NHEJ	non-homologous end joining
NK-Cells	natural killer cells
N-terminus	amino-terminus
Pax5	paired box 5
PBS	Phosphate-Buffered Saline
PBS-T	PBS-Tween
Pbx	pre-B-cell leukemia homeobox
PcG	polycomb group
PCR	polymerase chain reaction
PE	phycoerythrin
Peli1	pellino homolog 1
Peli2	pellino homolog 1
Per-CP	peridinin-chlorophyll protein complex
PGK	murine phosphoglycerate kinase promoter
PHD finger	plant homology domain
PlacZ	LacZ promoter
Plaur	plasminogen activator, urokinase receptor
PLZF	zinc finger protein 145
PM	perfect match
PML	promyelocytic leukaemia protein
Pol II	RNA polymerase II
Pontin	RuvB-like 1
PP2A	protein phosphatase 2A
PRO-B	B-cell progenitors
PRO-T	T-cell progenitors
pTEFb	positive transcription elongation factor b
PU.1	hematopoietic transcription factor PU.1
pUCori	origin of replication for propagation in E. coli
qPCR	real-time quantitative polymerase chain reaction
R	Discrimination score
RARα	retinoic acid receptor alpha
RbBP5	retinoblastoma binding protein 5
Reptin	RuvB-like 2
RIN	RNA Integrity Number
RNA	ribonucleic acid

RNA pol II	RNA polymerase II
rpm	revolutions per minute
Runx1	runt-related transcription factor 1
Runx2	runt-related transcription factor 2
S2	serine 2
SAPE	Streptavidin Phycoerythrin
Sca1	stem cell antigen 1
SCF	stem cell factor
SCL	stem cell leukaemia
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
SET	'Su(var)3-9, enhancer of zeste and trithorax' domain
Sh3bp5	SH3-domain binding protein 5
shRNA	short-hairpin ribonucleic acid
Siah1	seven in absentia homolog 1
siRNA	small interfering ribonucleic acid
Six1	sine oculis homeobox homolog 1
Six4	sine oculis homeobox homolog 4
SNL	sub-nuclear localisation domain
Sox4	SRY (sex determining region Y)-box 4
Stau	staufen, RNA binding protein, homolog 2
ST-HSC	short-term haematopoietic stem cells
τ	threshold
TA	transactivation domain
TALE	three amino acid loop extension
t-AML	therapy-related acute myeloid leukaemia
Tcf4	transcription factor 4
TEL	translocation-Ets-leukaemia
$TEL^{-/-}$	TEL deficient mice
TEMED	Tetramethylethylenediamine
Tnfaip2	tumor necrosis factor, alpha-induced protein 2
TRE	tetracycline-responsive promotor element
Tris-HCL	tris(hydroxymethyl)aminomethane
TRRAP	transformation/transcription domain-associated protein
tTA ($tTA2^{s}$)	transactivator protein

trx	trithorax
WDR5	WD-repeat protein 5
WHO	World Health Organisation
Wnt	wingless
Zfhx1b	zinc finger E-box binding homeobox 2
Ψ	viral packaging signal

PUBLICATION

The work described in Chapter VI has been published in Blood, 14 May 2009, Volume 113, Number 20 and has been attached to the Appendix.

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This thesis is dedicated to my family.

CHAPTER I - INTRODUCTION

1.1. Haematopoiesis

Haematopoiesis, the development of blood cells, has been well described in the mouse and serves as a model for human haematopoiesis. In vertebrates, establishment of a haematopoietic system starts early in the embryo and functions throughout embryonic and adult life. The extra-embryonic yolk sac produces the first haematopoietic cells, a process known as primitive haematopoiesis (reviewed in Orkin, 2000). Following this, the production of blood cells progresses to an intra-embryonic site defined as the aorta-gonad-mesonephros (AGM) and then to the foetal liver. After birth, haematopoietic precursors seed the bone marrow, which becomes the main site of haematopoietic activity (reviewed in Keller *et al.*, 1999). Haematopoiesis within the embryo is referred to as definitive haematopoiesis (reviewed in Orkin, 2000). Haematopoiesis takes place in a hierarchical order with haematopoietic stem cells (HSC) as the origin of multiple lineages. HSC have two characteristic capacities, they have the potential to self-renew and they can differentiate into various haematopoietic progenitor cells (HPC). HPC give rise to lineage specific precursors, which ultimately differentiate into mature blood cells (reviewed in Orkin, 2000). In murine haematopoiesis, HSC exist in a small fraction of bone marrow cells not expressing lineage-associated surface marker (Lin^-) but expressing high Sca1 and c-Kit ($Lin^-Sca1^+c\text{-}kit^{hi}$; LSK). Within the LSK fraction long-term self-renewal HSC (LT-HSC) reside in the $CD34^-CD38^+$ compartment and HSC with short-term self-renewal capacity (ST-HSC) reside in the $CD34^+CD38^-$ compartment (Randall *et al.*, 1996).

The classical model of the haematopoietic hierarchy (Figure 1) proposes that ST-HSC give rise to strictly lineage committed progenitor cells, either the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP). It has been shown that the interleukin-7 receptor α-chain (IL-7Rα) marks the CLP and that cells in the $Lin^{-}Sca1^{lo}c\text{-}Kit^{lo}IL\text{-}7R\alpha^{+}$ compartment give rise to T-Cells, B-Cells and natural killer (NK)-Cells (Kondo *et al.*, 1997). The CMP do not express IL-7Rα and reside in the $Lin^{-}Sca1^{-}c\text{-}Kit^{+}$ fraction. By including expression profiles of the Fcγ receptor-II/III (FcγRII/III), a marker for myeloid progenitors, and expression of CD34, this fraction can be further divided. CMP are $Fc\gamma RII/III^{lo}CD34^{+}$ and give rise to $Fc\gamma RII/III^{lo}CD34^{-}$ megakaryocyte-erythrocyte restricted progenitors (MEP) and $Fc\gamma RII/III^{hi}CD34^{+}$ granulocyte-macrophage restricted progenitors (GMP) (Akashi *et al.*, 2000). GMP and MEP produce only granulocyte-macrophage or megakaryocyte-erythrocyte lineage cells, respectively.

Contrary to this classical model of haematopoietic development, two alternative models have been proposed, which do not support an early division into lineage restriction of the progenitor cells (Adolfsson *et al.*, 2005). By including FMS-like tyrosine kinase 3 (Flt3) as a marker for a compartment of the LSK cells, it was shown that $LSKFlt3^{+}$ cells had the potential to produce granulocyte, macrophage, B-cell and T-cell lineage cells but no megakaryocyte-erythrocyte lineage cells (Adolfsson *et al.*, 2005). The $LSKFlt3^{+}$ cells were termed lymphoid-primed multipotent progenitors (LMPP) and asymmetric cell division of the ST-HSC into MEP and LMPP was proposed as the alternative model of haematopoietic development which does not include the existence of a CMP (Figure 2) (Adolfsson *et al.*, 2005).

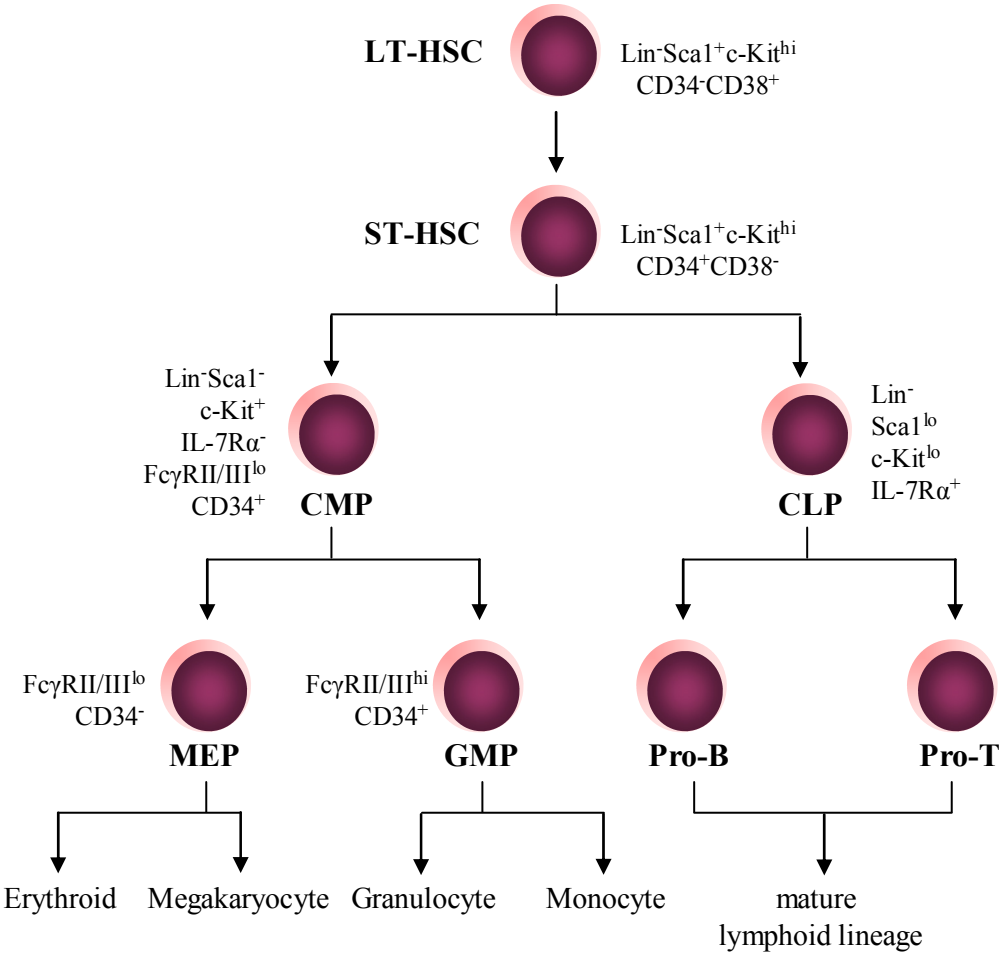


Figure 1: Classical model of adolescent haematopoiesis

Long-term haematopoietic stem cells (LT-HSC) have the potential to self renew and give rise to short-term HSC (ST-HSC) with limited self-renewal capacity. ST-HSC give rise to the common myeloid progenitors (CMP) and the common lymphoid progenitors (CLP). CMP can generate megakaryocyte-erythrocyte resricted progenitors (MEP) and granulocyte-macrophage restricted progenitors (GMP) which in turn give rise to the mature myeloid lineage cells. CLP can generate NK cells, B-cell progenitors (Pro-B) and T-cell progenitors (Pro-T) which in turn give rise to the mature lymphoid lineage cells (adapted from Traver *et al.*, 2001).

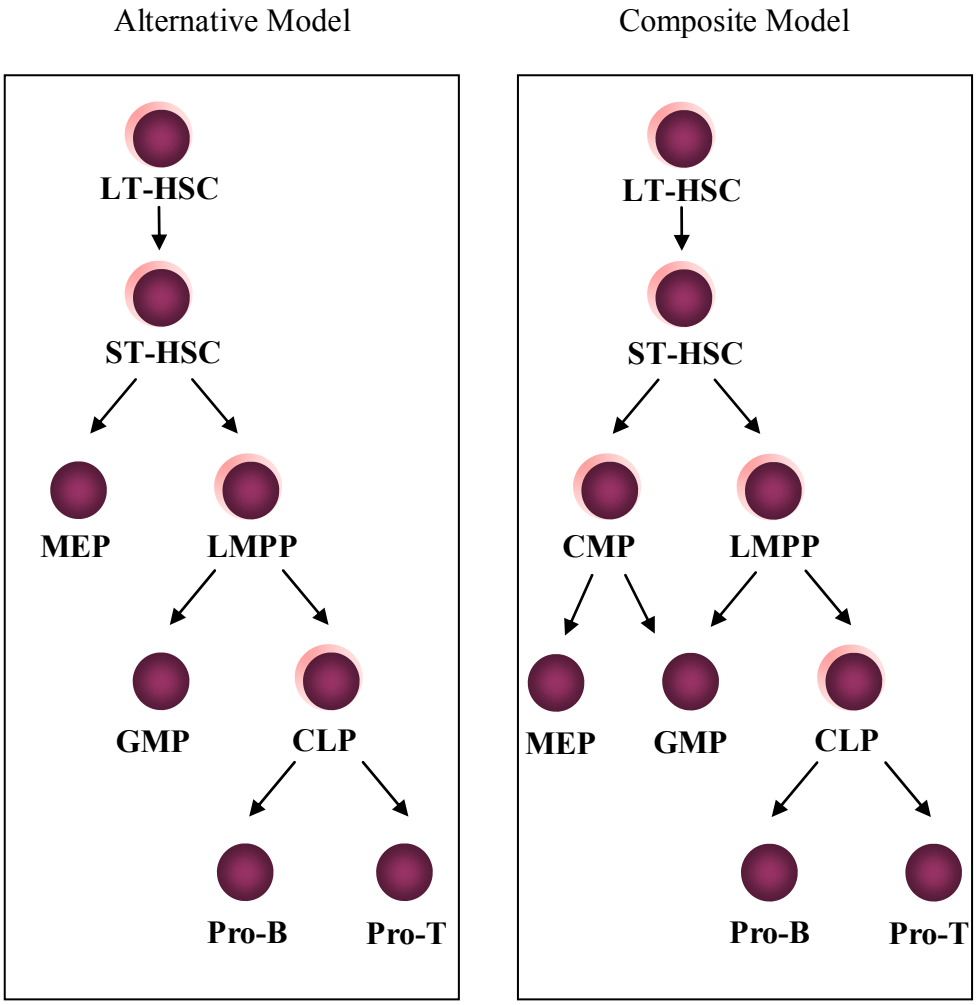


Figure 2: Alternative and composite model of haematopoiesis

The alternative model describes a less lineage restricted haematopoietic development by introducing a lymphoid-primed multipotent progenitors (LMPP). The alternative model was combined with the classical model into the composite model, in order to include the coexistence of the CMP and LMPP (adapted from Iwasaki and Akashi, 2007).

However, since it has been shown that CMP exist in the $Lin^{-}Sca1^{-}Kit^{+}IL\text{-}7R\alpha^{-}$ $Fc\gamma RII/III^{lo}CD34^{+}$ compartment (Akashi *et al.*, 2000), a composite model of haematopoiesis has been proposed (Figure 2). In the composite model, upon asymmetric cell division, the ST-HSC give rise to CMP and LMPP. CMP retain the potential to produce granulocyte-macrophage or megakaryocyte-erythrocyte lineage cells and LMPP have the potential to produce granulocyte-macrophage as well as B-cell and T-cell lineage cells (Adolfsson *et al.*, 2005; reviewed in Iwasaki and Akashi, 2007).

1.2. Transcriptional control of haematopoiesis

Self-renewal, lineage commitment and differentiation of HSC are complex processes that are orchestrated by expression of lineage specific transcription factors. The lineage specific function of any given transcription factor is analysed by loss-of-function or overexpression studies (reviewed in Iwasaki and Akashi, 2007). Experiments using these approaches have revealed transcription factors which are essential for haematopoiesis, such as the *Mixed lineage leukaemia* (*MLL*), *Acute myeloid leukaemia 1* (*AML1*) and *translocation-Ets-leukaemia* (*TEL*) genes. *Mll* deficient mice ($Mll^{-/-}$) were generated by homologous recombination in embryonic stem cells (Yu *et al.*, 1995; Yagi *et al.*, 1998). *Mll* deficiency was embryonic lethal, by embryonic day E10.5 (Yu *et al.*, 1995) or between E11.5-E14.5 (Yagi *et al.*, 1998). HPC isolated from both the yolk sac (Hess *et al.*, 1997) and foetal liver (Yagi *et al.*, 1998) of $Mll^{-/-}$ mice demonstrated impaired clonogenic capacity *in vitro*. It was shown that the number of colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), colony-forming unit-macrophage (CFU-M) and burst-forming unit-erythroid (BFU-E) was reduced in $Mll^{-/-}$ cultures (Hess *et al.*,

1997). However, *Mll*$^{-/-}$ cultures developed all cell types (Hess *et al.*, 1997). These studies indicate that *Mll* affects early haematopoiesis and functions in the control of HPC but is not required for later stages of differentiation. Two conditional *Mll* knock-out studies showed that *Mll* function is important for stem cell self-renewal capacity and promoting proliferation in progenitors (McMahon *et al.*, 2007; Jude *et al.*, 2007).

Another transcription factor, *AML1*, and its dimerising partner, *core binding factor β* (*CBFβ*), form a complex and are two of the most frequent targets of chromosomal rearrangements in human leukaemia. *AML1* deficient mice (*AML1*$^{-/-}$) showed a complete absence of foetal liver-derived haematopoiesis and were embryonic lethal around day E12.5 (Okuda *et al.*, 1996). These results indicate that *AML1* regulates target genes that are essential for definitive haematopoiesis of all lineages. Surprisingly, a conditional *AML1* knock-out study has recently shown that AML1 is not required for maintenance of HSC in adult haematopoiesis. However, it is necessary for maturation of megakaryocytes and differentiation of lymphocytes (Ichikawa *et al.*, 2004).

Tel encodes an Ets family transcription factor and is expressed in haematopoietic cells (Wang *et al.*, 1998). *Tel* deficient mice (*Tel*$^{-/-}$) are embryonic lethal at day E11.5 because of yolk sac angiogenic defect (Wang *et al.*, 1997). It was shown that in chimeras, *Tel* deficient embryonic stem cells contribute to foetal liver haematopoiesis but do not contribute to bone marrow myelopoiesis, erythropoiesis and lymphopoiesis (Wang *et al.*, 1998). Therefore, *Tel* function seems to be essential for the establishment of haematopoiesis of all lineages in the bone marrow. A conditional *Tel* knock-out model further showed that haematopoiesis in the bone

marrow depends on *Tel* expression. However *Tel* does not function as a master regulator but is specifically required for maturation of megakaryocytes and for survival of HSC in the bone marrow (Hock *et al.*, 2004).

Lineage specific gene expression depends on the presence of a combination of transcription factors. The main transcriptional regulators required for myeloid cell development include *PU.1*, *GATA-1* and *CCAAT/enhancer binding protein* (*C/EBP*). The transcription factor *PU.1*, an ETS family member, is only expressed in haematopoietic cells. High levels of *PU.1* are found in monocytic, granulocytic and B lymphoid lineages, whereas low levels are found in mature erythrocytes (reviewed in Gupta *et al.*, 2009). *PU.1* deficient mice lack mature macrophages, neutrophils, B- and T-cells and die shortly after birth (McKercher *et al.*, 1996). Different levels of *PU.1* gene expression are responsible for differentiation into macrophages, granulocytes or lymphocytes (reviewed in Gupta *et al.*, 2009). *PU.1* knock-down mice developed a block in macrophage and B-cell development (Rosenbauer *et al.*, 2004). These studies revealed that control of *PU.1* expression is important for normal haematopoietic development. A variety of other transcription factors have been shown to interact with *PU.1* and determine haematopoietic cell development. Among these are the megakaryocyte-erythroid transcription factor, *GATA-1*, and *C/EBPα*, which is expressed in immature myeloid cells (reviewed in Gupta *et al.*, 2009). PU.1 has been shown to block transcription of genes required for commitment to MEP lineage by blocking GATA-1 DNA binding (reviewed in Zhang *et al.*, 2000). The transcription factor *C/EBPα*, a basic leucine zipper transcription factor, is expressed in multiple haematopoietic lineages. *C/ebpα* deficient mice developed a myeloid differentiation block at the myeloblast stage. It was suggested that *PU.1* and *C/EBPα*

in combination regulate the differentiation of GMP into either granulocytes or monocytes (reviewed in Gupta *et al.*, 2009).

1.3. Wnt signalling in haematopoiesis

The Wnt signalling pathway is a highly conserved signalling system that plays a crucial role in embryonic development and regeneration of adult tissues, regulating processes such as proliferation, survival and differentiation (Figure 3). Transcriptional target genes of the Wnt signalling pathway act either as tumour suppressors or proto-oncogenes. It has been shown that aberrant activation of the Wnt signalling pathway, by inappropriate stabilisation and accumulation of cytoplasmic β-Catenin, is linked to a variety of cancers (reviewed in Klaus and Birchmeier, 2008). Following accumulation, β-Catenin translocates to the nucleus and constantly activates transcription of genes that are associated with cell proliferation and survival, such as c-Myc and CyclinD1 (reviewed in Klaus and Birchmeier, 2008). Studies have shown that aberrant accumulation of β-Catenin can be caused by mutations in factors controlling β-Catenin degradation, such as APC, axin or β-Catenin itself. Also, activating mutations of the Wnt-ligand co-receptor LRP5 can cause aberrant accumulation of β-Catenin (reviewed in Klaus and Birchmeier, 2008). Wnt signalling has also been shown to play a role in self-renewal of HSC as well as development of T- and B-cells. TCF-GFP reporter assays were used to demonstrate active Wnt signalling in HSC (Willert *et al.*, 2003). Deletion of *β-Catenin* in HSC showed that the distribution in cell cycle, multilineage differentiation and homing ability are unchanged in *β-Catenin*-deleted HSC but their self-renewal ability was impaired (Zhao *et al.*, 2007).

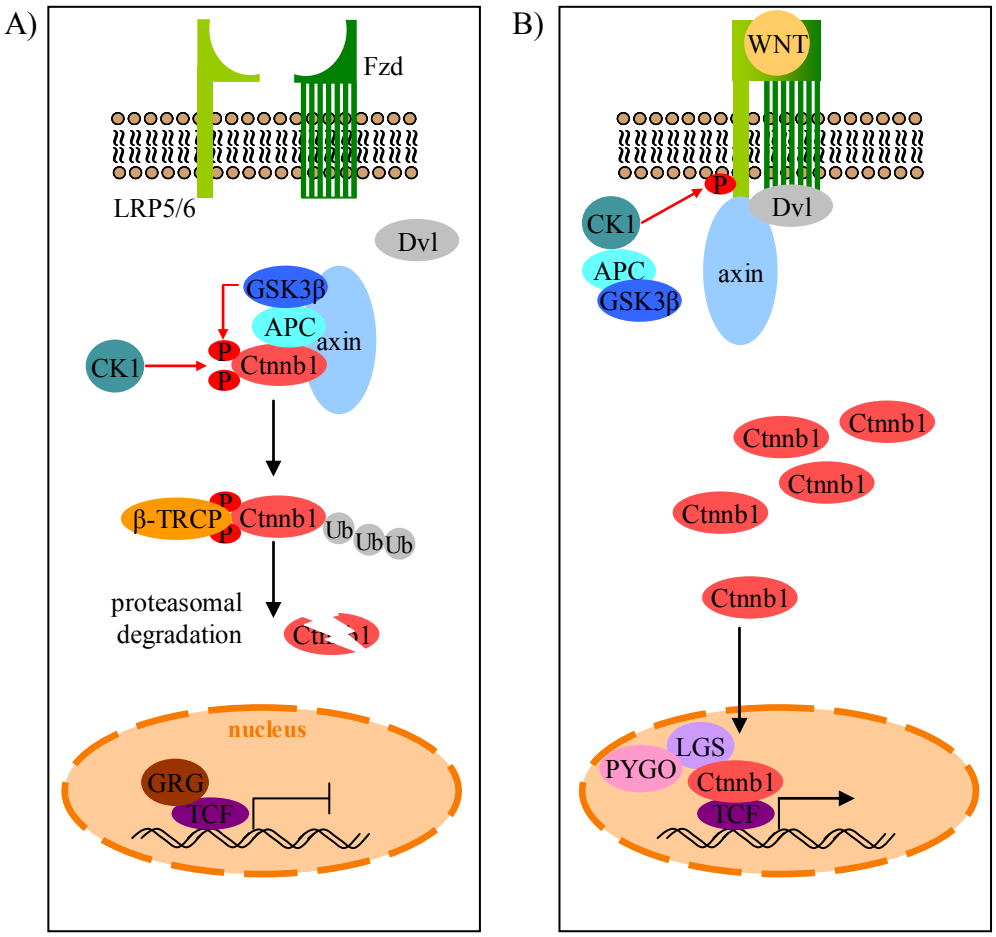


Figure 3: Wnt signalling pathway

A) In the absence of Wnt ligands β-Catenin (Ctnnb1) is linked with a destruction complex consisting of axis inhibitor (axin), adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen-synthase kinase 3 β (GSK3β), and N-terminally phosphorylated by CK1 and GSK3β. Upon N-terminal phosphorylation, Ctnnb1 is recognised by the E3 ubiquitin ligase, β-transducin-repeat-containing family (β-TRCP), and ubiquitylated, followed by proteasomal degradation. As a result levels of cytoplasmic Ctnnb1 are low and its transcriptional targets are repressed by intranuclear binding of the co-repressor Groucho (GRG) to the lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factor. B) Wnt ligand binding to the frizzled (Fzd) receptor and low-density-lipoprotein-receptor-related protein 5 and 6 (LRP5/LRP6) co-receptors, results in phosphorylation of LRP5/LRP6 by CK1 and GSK3β. Dishevelled (DVL) is then engaged on the plasma membrane with the Fzd receptor and axin, which also interacts with LRP5/LRP6. The degradation of cytoplasmic Ctnnb1 is inactivated and Ctnnb1 can accumulate in the cytoplasm and translocate into the nucleus. In the nucleus Ctnnb1 replaces GRG and activates transcription by forming a transcriptionally active complex with LEF/TCF (adapted from Staal and Cleevers, 2004; Klaus and Birchmeier, 2008).

Expression of a constitutively active form of β-Catenin in murine Bcl2-transgenic mice resulted in an increased number of HSC with enhanced ability to reconstitute lethally irradiated mice (Reya *et al.*, 2003). In contrast, a separate study showed that upon constitutive activation of β-Catenin, multilineage differentiation was negatively affected and numbers of HSC were depleted, following a transient increase in their number (Scheller *et al.*, 2006; Kirstetter *et al.*, 2006). These studies highlight the importance of maintaining the correct level of Wnt signalling in HSC. Furthermore, deregulated Wnt signalling has also been reported to be involved in the development of AML, CLL, CML and E2A-PBX1 induced pre-B-ALL. Müller-Tidow *et al.* showed that γ-Catenin was induced by the AML-associated translocation products, AML1-ETO, PML-RARα and PLZF- RARα (Müller-Tidow *et al.*, 2004). Another group reported that LEF1 and its down-stream target CyclinD1 were overexpressed in cells from patients with CLL (Lu *et al.*, 2004). Wnt signalling activation has been shown in blast crises of CML, using TCF-GFP reporter assays in BCR-ABL CML patients (Jamieson *et al.*, 2004). E2A-PBX1 has been shown to activate expression of *WNT16* (McWhirter *et al.*, 1999).

These studies show that Wnt signalling may contribute to the development of some haematological malignancies. β-Catenin has also been shown to interact with MLL. Sierra *et al.* reported that the C-terminal β-Catenin activation domain selectively associates with nuclear remodelling subunits of the TRRAP histone acetyltransferase (HAT) and the MLL histone methyltransferase (HMT) complexes and that β-Catenin promotes H3K4 histone trimethylation at the c-Myc gene (Sierra *et al.*, 2006).

1.4. Acute leukaemia in infants

Acute leukaemia is a cancer that arises in cells of the haematopoietic system and is the most common form of childhood cancer. Acute lymphoblastic leukaemia (ALL), a clonal expansion of lymphoblasts in the bone marrow or blood, is more frequent in children than acute myeloid leukaemia (AML), accounting for more than 50% of paediatric haematopoietic malignancies (reviewed in Downing and Shannon, 2002). Acute myeloid leukaemia is defined as a clonal expansion of myeloid blasts that comprise more than 20% of the nucleated cells in the bone marrow or blood, frequently resulting in haematopoietic insufficiency (reviewed in Downing and Shannon, 2002). AML is a heterogeneous disease, consisting of many recognized morphologic and biological subtypes. Acute myeloid leukaemias are divided by the French-American-British (FAB) classification into eight subtypes (Table 1), designated M0 through M7, based on the type of cell from which the leukaemia developed (Cancer Society, Cancer Reference Information, www.cancer.org).

Table 1: The French-American-British classification of human AML

FAB Subtype	**Name**	**Associated translocation, *gene***
M0	Undifferentiated AML	inv(3q26), *EVI1*
M1	Myeloblastic leukaemia with minimal maturation	n.a.
M2	Myeloblastic leukaemia with maturation	t(8;21), *AML1-ETO*
M3	Promyelocytic leukaemia	t(15;17), *PML-RARα*
M4	Myelomonocytic leukaemia	11q23, *MLL*
M4 eos	Myelomonocytic leukaemia with eosinophilia	inv(16), *CBFβ-MYH11*
M5	Monocytic leukaemia	11q23, *MLL*
M6	Erythroid leukaemia	unknown
M7	Megakaryoblastic leukaemia	unknown

The table lists the FAB subtypes of AML and the known associated translocations and genes.

A World Health Organisation (WHO) classification of AML has also been published (Table 2). The WHO classification of acute myeloid leukaemia attempts to be more clinically useful and to produce more meaningful prognostic information than the FAB criteria.

Table 2: WHO subtypes of AML

Name	Description
AML with characteristic genetic abnormalities	• AML with translocations between Chr 8 and 21 t(8;21); AML1-ETO • AML with inversions in Chr 16 inv(16); CBFβ-MYH11 • AML with translocations between Chr 15 and 17 t(15;17); RARα-PML
AML with multilineage dysplasia	• patients who have had a prior myelodysplastic syndrome (MDS) or myeloproliferative disease (MPD) that transforms into AML
AML and MDS, therapy-related	• patients who have had prior chemotherapy and/or radiation and subsequently develop AML or MDS • leukaemias may be characterized by specific chromosomal abnormalities
AML not otherwise categorized	• subtypes of AML that do not fall into the above categories

The table lists the WHO subtypes of AML and the associated subtypes.

Cytogenetic abnormalities are found in 80% of AML and ALL cases. Chromosomal rearrangements involving chromosome band 11q23 make up 60% of infant AML cases and 80% of infant ALL cases (Pui *et al.*, 1995) and they are prevalent in therapy-related secondary leukaemias (t-AML) (Felix, 1998). The rearrangements affecting the *MLL* gene are associated with poor outcome in ALL but

also in AML (reviewed in Felix and Lange, 1999). The young age at diagnosis (average six months for ALL) and studies of identical twins, concordant for infant ALL, suggest that *MLL* gene rearrangements arise *in utero* (reviewed in Eguchi *et al.*, 2003). Identification of a clonal but non-inherited breakpoint suggested that the clone containing the translocation is passed on from one twin to the other via the placenta. Furthermore, analysis of Guthrie neonatal bloodspots confirmed that translocations were already present at the time of birth (reviewed in Felix and Lange, 1999; Eguchi *et al.*, 2003). It has been suggested that a possible predisposition to develop leukaemia *in utero* could be the exposure of the mother to naturally occurring topoisomerase-II inhibitors, such as dietary bioflavonoids (Ross *et al.*, 1994).

1.5. MLL and its functional domains

The *MLL* gene is located on human chromosome 11, band q23. MLL is a large protein with several functional domains (Figure 4). The N-terminal part of MLL contains three AT hooks, two sub-nuclear localisation domains (SNL1 and SNL2), a DNA methyltransferase 1 homology domain (DNMT1), plant homology domains (PHD finger) and a bromodomain. The three **AT hooks**, located nearest to the N-terminus, recognize DNA structure and mediate binding to the minor groove of AT-rich regions (reviewed in Eguchi *et al.*, 2003; Ayton and Cleary 2001). These may stabilize protein-DNA complexes or mediate protein-protein interaction through binding to DNA (reviewed in Ayton and Cleary 2001). Two sub-nuclear localisation domains, **SNL1** and **SNL2**, direct the punctuate localisation pattern of the protein to the nucleus.

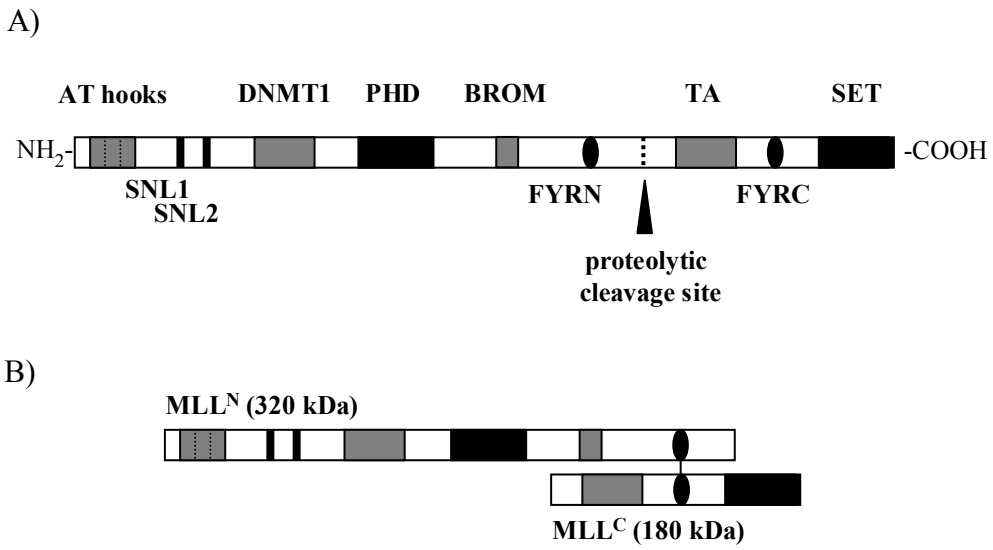


Figure 4: Domain organisation of the MLL protein

A) Three N-terminal AT hooks mediate binding to the minor groove of AT-rich DNA regions. Two small regions SNL1 and SNL2 specify the sub-nuclear localisation of the MLL protein. The DNA methyltransferase 1 homology domain (DNMT1) enables MLL to bind unmethylated CpG sequences with high specifity. The region around the DNMT1 domain has transcriptional repression activity through interaction with histone deacetylases (HDAC) or polycomb groub proteins HPC2 and Bmi-1. The PHD finger domain mediate protein-protein interactions. The bromodomain (BROM) binds to acetylated lysines on histones. The Transactivation domain (TA) is located between the PHD domain and the SET domain. TA enhances transcriptional activation through binding of MLL to cAMP binding protein (CBP). The SET (Su(var)3-9, enhancer of zeste and trithorax) domain possesses histone H3 lysine 4 specific methyltransferase activity. B) The mature MLL protein is cleaved in the cytoplasma by taspase-1 into an N-terminal p320 peptide (AT hooks, DNMT1 repression and PHD domain) and a C-terminal p180 peptide (TA and SET domain). The fragments translocate into the nucleus where they remain non-covalently associated through the FYRN and FYRC domain (adapted from Hess, 2004; Daser and Rabbitts, 2005).

These domains are two of the evolutionary conserved regions between *Drosophila trx* and MLL, suggesting an important functional role for the ability of MLL and *trx* to localise to specific regions within the nucleus (reviewed in Eguchi *et al.*, 2003). The **DNMT1** domain is a cysteine-rich CXXC domain that enables DNA binding of MLL to unmethylated CpG sequences with high specifity. This interaction with unmethylated CpG islands allows MLL to associate with active genes. The region around the DNMT1 domain has transcriptional repression activity through interaction with histone deacetylases (HDAC) or polycomb group (PcG) proteins; for example polycomb 2 homolog (HPC2) and BMI1 polycomb ring finger oncogene (BMI1) (reviewed in Eguchi *et al.*, 2003). The **PHD fingers** of MLL mediate protein-protein interactions, such as binding to methylated histones (Muntean *et al.*, 2008; reviewed in Eguchi *et al.*, 2003). The nuclear cyclophilin Cyp33 has been shown to bind the PHD fingers, mediating an enhanced binding of HPC2 and BMI1 to the transcriptional repression domain (Muntean *et al.*, 2008). This region also recruits histone deacetylases HDAC1 and HDAC2 to mediate transcriptional repression (reviewed in Daser and Rabbitts, 2005). Proximal to the PHD finger domains is the **bromodomain**, a domain that binds to acetylated lysines on histones (reviewed in Dou and Hess, 2007). The C-terminal part of MLL contains the transactivation domain (TA) and the 'Su(var)3-9, enhancer of zeste and trithorax' (SET) domain. The **transactivation** domain binds to cAMP binding protein (CBP), a histone acetyltransferase that activates gene expression. CBP in turn, mediates binding of cAMP responsive element-binding protein (CREB) to promote transcriptional activation (reviewed in Eguchi *et al.*, 2003). The **SET** domain at the C-terminus is a highly conserved domain with histone methyltransferase activity, that specifically methylates histone H3 at lysine 4 (H3K4) (reviewed in Dou and Hess, 2007). The mature MLL protein is proteolytically cleaved between the bromodomain

and the TA domain in the cytoplasm by the taspase-1 protease, into an N-terminal peptide (MLL^N 320kDa) and a C-terminal peptide (MLL^C 180kDa). Taspase-1 is the only protease in vertebrates that can cleave MLL (Takeda *et al.*, 2006). MLL^N and MLL^C fragments remain non-covalently associated through FYDN and FYDC domains after cleavage to avoid degradation and confer stability (reviewed in Eguchi *et al.*, 2003; Dou and Hess, 2007).

1.6. Transcriptional regulation by MLL

Post-translational histone modifications, including acetylation, methylation, phosphorylation, ubiquitination and sumoylation, alter chromatin accessibility and allow transcription factors to be recruited (reviewed in Linggi *at al.*, 2005). These histone modifications can have an activating, repressing or combined transcriptional role, creating a complex histone code. This histone code is created by histone modifiers such as kinases, histone methyltransferases and histone acetyltransferases and is then recognized by transcription factors. MLL is a histone-modifying factor that acts as a member of a macromolecular methyltransferase complex. It is recruited to target genes through interactions with menin and LEDGF and mediates trimethylation of histone H3K4 within the promoter region of genes occupied by RNA polymerase II (reviewed in Krivtsov and Armstrong, 2007). Trimethylation of histone H3K4 is generally associated with transcriptional activation and subsequent transcription of MLL target genes such as *homeobox* (*Hox*) genes (reviewed in Krivtsov and Armstrong, 2007).

The MLL^N and MLL^C fragments have different molecular functionalities to facilitate methylation, acetylation and DNA binding. The C-terminal part of MLL

interacts with a macromolecular complex with methyltransferase activity, including the WD-repeat protein 5 (WDR5), retinoblastoma binding protein 5 (RbBP5) and ash2 (absent, small, or homeotic)-like protein (ASH2L) (Figure 5 A) (reviewed in Dou and Hess, 2007). The protein WDR5, together with RbBP5, mediates association with the C-terminus of MLL, whereas ASH2L associates with RbBP5 only and mediates stabilisation of the complex (reviewed in Crawford and Hess, 2006). WDR5 binds preferentially to dimethylated histone 3 lysine 4 (H3K4) in order to facilitate MLL induced histone H3K4 trimethylation (reviewed in Berger, 2007). It has been shown that MLL requires the WDR5/RbBP5/ASH2L complex in order to trimethylate histone H3K4 and activate transcription (reviewed in Crawford and Hess, 2006). The N-terminal part of MLL directs targeting of the MLL complex. The tumour suppressor gene *MEN1* product, menin, is part of the MLL associated macromolecular complex. In contrast to most of the other MLL-interacting proteins, menin binds to the N-terminal domain of MLL (Hughes *et al.*, 2004; Yokoyama *et al.*, 2004). A high-affinity N-terminal conserved menin binding motif (RXRFP) has been identified in MLL, spanning amino acids six to ten (Yokoyama *et al.*, 2005). Recently, it was shown that menin and MLL interact with lens epithelium derived growth factor (LEDGF) to facilitate interaction with chromatin (Yokoyama and Cleary, 2008).

Following MLL induced trimethylation of histone H3K4 at the promoter, positive transcription elongation factor b (pTEFb) is associated with the active AF4-ENL/AF9 complex and recruited to the transcription machinery (Figure 5 B) (Bitoun *et al.*, 2006). pTEFb, a dimer of cyclin dependent kinase 9 (CDK9) and cyclin T, overcomes transcriptional pausing by phosphorylating the C-terminal domain (CTD) of RNA polymerase II at serine-2 (S2).

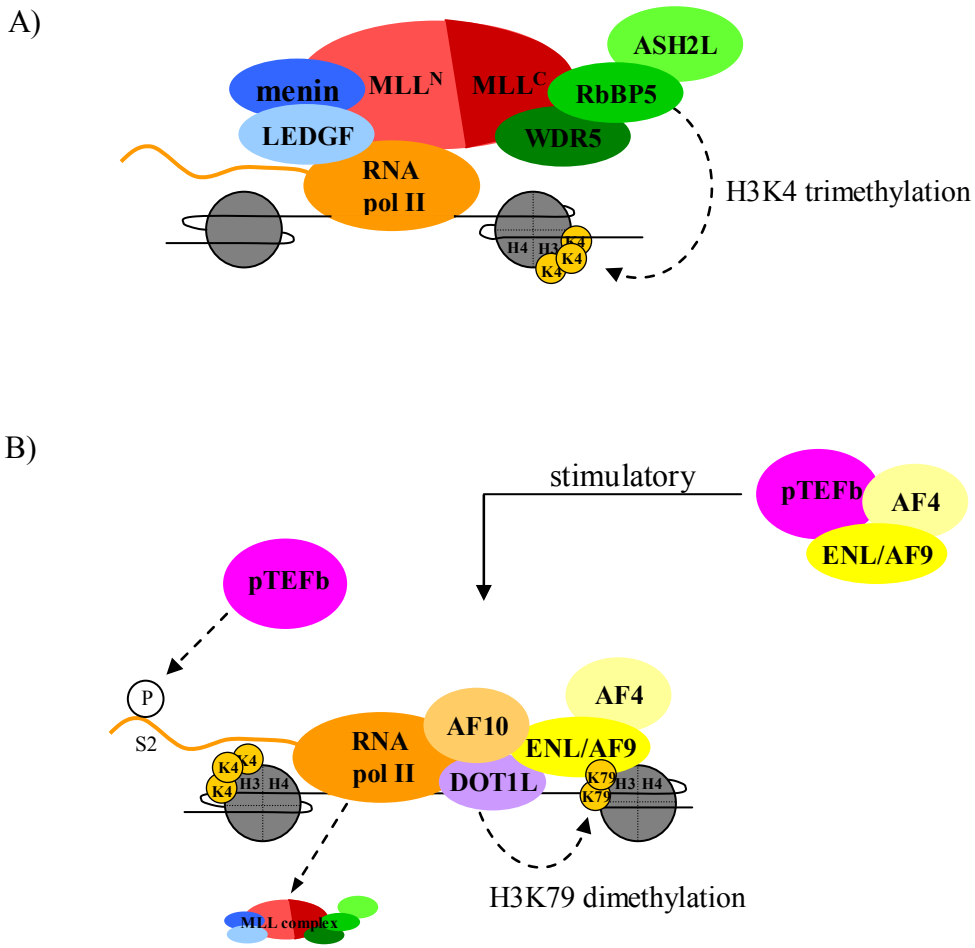


Figure 5: Transcriptional regulation by the MLL complex

A) MLL is a member of a macromolecular complex that mediates histone H3K4 trimethylation of the promoter regions of genes occupied by RNA pol II. The SET domain at the C-terminal part of MLL interacts with WDR5, RbBP5 and ASH2L forming a H3K4 specific methyltransferase complex. The N-terminal part of MLL interacts with menin which then interact with LEDGF to facilitate interaction with chromatin (adapted from Krivtsov and Armstrong, 2007; Dou and Hess, 2008). B) Following MLL mediated histone H3K4 trimethylation the pTEFb complex is recruited to phosphorylate the RNA pol II CTD at serine-2 (S2). AF4-ENL/AF9 complex associates with histone H3 and recruits AF10. This complex in turn can recruit the histone H3K79 specific methyltransferase DOT1L to the elongating RNA pol II and methylation of histone H3K79. The MLL complex separates from RNA polymerase II (adapted from Bitoun *et al.*, 2006).

Concurrently, ENL/AF9 associates with histone H3 and recruits AF10 to the AF4-ENL/AF9 complex. DOT1L is then recruited to elongating RNA polymerase II via interaction with ENL/AF9 and AF10 in order to methylate histone H3K79. As productive elongation continues the MLL complex dissociates from RNA polymerase II (Bitoun *et al.*, 2006).

1.7. Normal function of MLL

MLL is widely expressed during embryonic development, in most adult tissues, including myeloid and lymphoid cells (reviewed in Hess 2004). The *MLL* gene is a member of the trithorax group, a conserved family of proteins, and is homologous to the *Drosophila trithorax* (*Trx*) gene (reviewed in Hess 2004). *Trx* positively regulates *homeotic* (*HOM-C*) gene expression during development and is opposed by the polycomb group (PcG) proteins, which repress *HOM-C* gene expression (reviewed in Hess 2004). Mutations of the *Trx* gene cause loss or abnormal patterns of *HOM-C* gene expression resulting in segment identity defects (Breen and Harte, 1993). The *Mll* gene in mice has been disrupted and studied by homologous recombination (Yu *et al.*, 1995; Yu *et al.*, 1998; Yagi *et al.*, 1998; Ayton *et al.*, 2001). *Mll*$^{-/-}$ homozygous mice were embryonic lethal with completely absent expression of *HoxA7* and *HoxC9*. *Mll* heterozygous mice had retarded growth, axial skeletal abnormalities and disturbed pattern of *HoxA7* and *HoxC9* expression. Furthermore, heterozygous mice displayed haematopoietic abnormalities (Yu *et al.*, 1995). Later it was shown that *HoxA7* was initiated in Mll$^{-/-}$ embryos before E8.5 but not maintained (Yu *et al.*, 1998). Two conditional *Mll* knock-out models have shown that wild-type MLL is required for normal haematopoiesis and haematopoietic stem cell activity (Jude *et al.*, 2007; McMahon *et al.*, 2007).

MLL has a global role in transcription by associating along with RNA polymerase II to the 5' end of actively transcribed genes (as previously described). Studies with *Mll* knockout mice have shown that the transcriptional function of MLL includes *Hox* gene regulation (Yu *et al.*, 1995; Yu *et al.*, 1998). *Hox* genes encode transcription factors that are regulators of proliferation and differentiation of haematopoietic cells (Thorsteindottir *et al.*, 1997). 39 different *Hox* genes are clustered into four groups (HoxA, HoxB, HoxC and HoxD) on four different chromosomes (human/mouse: Chr7/Chr6, Chr17/Chr11, Chr12/Chr15 and Chr2/Chr2, respectively) and numbered from 1 to 13. No cluster contains a full set (Sauvageau *et al.*, 1994). *Hox* genes have expression patterns specific for the stage and lineage of progenitor cell development with a general down-regulation of *Hox* gene expression as the progenitors differentiate into mature blood cells (Sauvageau *et al.*, 1994). Generally, gene expression of 3' *Hox* genes within HoxA and HoxB clusters are found in the most immature haematopoietic cells. These genes are subsequently down-regulated and the Hox *loci* closer to the 5' end are expressed in the early stages of differentiation. *Hox* gene expression is lost upon terminally differentiation (Sauvageau *et al.*, 1994). It has been reported that the MLL protein interacts with the promoters of the *HoxA7*, *HoxA9* and *HoxA10* genes (Guenther *et al.*, 2005). However, transcriptional regulation extends beyond *Hox* gene regulation. MLL was found to be associated with many promoters in cell-line studies, suggesting a more global role in regulation of transcription (Guenther *et al.*, 2005).

1.8. MLL oncogenic rearrangements

Chromosomal rearrangements involving MLL are common in patients with AML and can take several forms such as balanced translocations, inversions and

duplications of 11q23 (reviewed in Dou and Hess, 2008). Balanced translocations are the most common rearrangement disrupting the *MLL* gene (Figure 6). The genomic breakpoints are located within an 8.3 kb breakpoint cluster region (bcr), spanning exons 8 to 12. It has been shown that breaks including the PHD fingers, located C-terminal of the bcr, are not compatible with transformation of HPC (Muntean *et al.*, 2008). The breakpoint cluster regions are always located within introns, invariably generating a novel in-frame chimaeric gene. Rearrangements result in the deletion of the PHD fingers and all other downstream domains of MLL (reviewed in Hess 2004). The bcr region contains topoisomerase-II-binding sites as well as Alu sequences and is thought to be prone to DNA double strand cleavage in response to topoisomerase-II inhibitors environmental exposure (reviewed in Eguchi *et al.*, 2003). Alternatively it was suggested that upon initiation of the apoptotic programme, double strand breaks in the *MLL* gene and the partner gene are generated and joined together by the error-prone non-homologous end joining (NHEJ) DNA repair pathway (reviewed in Greaves 2003). In the event that the apoptotic response is aborted, cells carrying the MLL rearrangement would survive (Betti *et al.*, 2001).

Balanced translocations fuse the genomic region encoding the N-terminal portion of MLL to sequences encoding the C-terminal portion of one of a number of translocation partners. This fusion generates an in-frame chimaeric gene that codes for a novel fusion protein with leukaemogenic potential (reviewed in Hess, 2004). Various studies have used retroviral gene transfer assays to assess the transforming capacities of MLL-fusions.

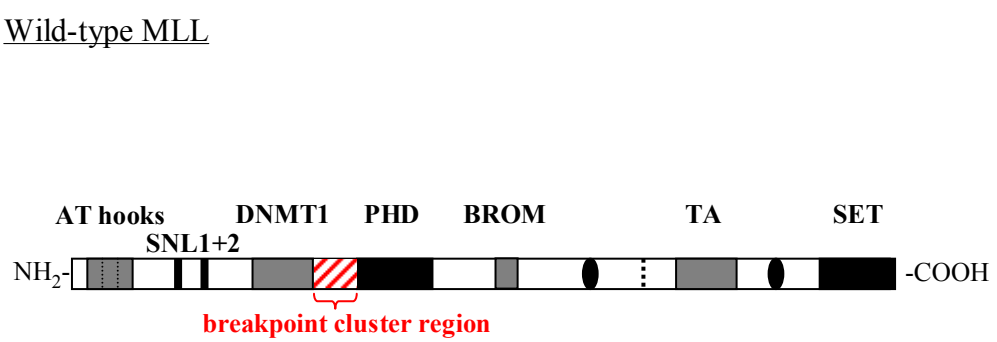


Figure 6: Balanced MLL translocation

The translocation breakpoints of MLL occur within a breakpoint cluster region (bcr) spanning exons 8 to 12. The translocation results in the deletion of the C-terminal portion of MLL including the PHD, TA and SET domains. The remaining N-terminal portion of MLL is fused to the C-terminal portion of one out of more than 40 fusion partners. As the result of this translocation, an in-frame chimeric fusion protein is generated (adapted from Hess, 2004).

We and others have shown that the fusion genes MLL-AF9 and MLL-ENL transform murine HPC *in vitro* and result in leukaemia development *in vivo* when transplanted into a mouse model (Lavau *et al.*, 1997; Ayton and Cleary, 2003; Cozzio *et al.*, 2003; Zeisig *et al.*, 2004; Horton *et al.*, 2005; Krivtsov *et al.*, 2006; Somervaille *et al.*, 2006; Horton, Walf-Vorderwülbecke *et al.*, 2009). Others have shown that the fusion genes MLL-GAS7 and MLL-AF4 also transform murine HPC *in vitro* and result in leukaemia *in vivo* (So *et al.*, 2003; Krivtsov *et al.*, 2008). Since heterozygous *Mll* knock-out mice do not have an increased incidence of leukaemia and neither the N-terminal region of MLL or wild-type ENL on their own had transforming capacities, a gain of function has been proposed as the mechanism by which MLL-fusion proteins transform HPC (Lavau *et al.*, 1997; reviewed in Ayton and Cleary 2001; Eguchi *et al.*, 2003; Hess 2004). Furthermore the generation of viable MLL-AF9 mutant mouse germlines confirmed that MLL function, vital for embryogenesis, is not blocked by MLL-AF9 *in vivo* (Dobson *et al.*, 1999).

It has been suggested that a subset of *HoxA* genes and *myeloid ecotropic viral integration site 1* (*Meis1*) are required for MLL-fusion proteins to transform HPC (Zeisig *et al.*, 2004; Horton *et al.*, 2005; reviewed in Dou and Hess, 2008). Meis1 is a member of the three amino acid loop extension (TALE) family of homeodomain-containing proteins. Meis1 is closely related to the pre B-cell leukaemia homeobox (Pbx) proteins. DNA binding by *Hox* transcription factors is enhanced by forming heterooligomeric complexes with the Pbx and Meis families (Mann and Chan, 1996). Despite persistent *MLL* expression during normal haematopoietic differentiation, *Hox* and *Meis1* gene expression are generally down-regulated as the progenitors differentiate (Sauvageau *et al.*, 1994). However, this regulation is disturbed by MLL-fusion proteins, leading to maintenance of high expression levels of *HoxA7*, *HoxA9*

and *Meis1*. Furthermore, murine models for MLL-AF9 and MLL-ENL fusion oncogenes showed that overexpression of a *HoxA* code (*HoxA4 – HoxA11*) appear to be important for MLL mediated leukaemogenesis (Kumar *et al.*, 2004; Horton *et al.*, 2005). This aberrant expression of *HOXA* genes and *MEIS1* is also observed in human leukaemias with MLL rearrangements (reviewed in Dou and Hess, 2008).

1.9. MLL translocation partner

Over 50 fusion partners of MLL have been identified. A few of the MLL partner proteins can be classified into either nuclear factors involved in transcriptional regulation (such as AF4, AF9, AF10, ENL, ELL) or signalling molecules that are localised in the cytoplasm (such as GAS7, EEN, AF1p, AF6) (reviewed in Ayton and Cleary, 2001). Some of the nuclear factors can be grouped into common gene families such as AF9/ENL, AF4/AF5q31/LAF4 and AF10/AF17 (Nakamura *et al.*, 1993; Chaplin *et al.*, 1995; Nilson *et al.*, 1997; Taki *et al.*, 1999). Table 3 lists examples for the identified MLL fusion partner genes with their known function and localisation.

The *AF9* (*ALL1 fused gene from chromosome 9*) gene at chromosome 9, band p22, and the *ENL* (*eleven nineteen leukaemia*) gene at chromosome 19, band p13, encode proteins with a 56% amino acid identity and are among the most common fusion partners (Nakamura *et al.*, 1993; Rubnitz *et al.*, 1994). The mouse *Af9* gene has been shown to be a controller of embryo patterning presumably *via* control of *Hox* gene regulation, which is similar to the role of *Mll* (Collins *et al.*, 2002).

Table 3: MLL fusion partner genes

Gene	Chr. Location	Function	Localisation
*AF4**	4q21	transcriptional activator	N
*AF6**	6q27	maintenance of cell-cell junctions	C
*AF9**	9p22	transcription factor	N
*AF10**	10p12	transcription factor	N
*ELL**	19p13.1	RNA pol II transcription elongation	N
*ENL**	19p13.3	transcriptional activator	N
AFX	Xq13	forkhead transcription factor	N
Septin6	Xq22	Septin family	C
AF1p	1p32	regulation of endocytosis	C, N
LAF4	2q11	transcriptional activator	N
GMPS	3q25	Guanosine monophosphatase synthetase	C
LPP	3q28	cell motility and focal adhesion	C, N
AF5q31	5q31	transcriptional activator	?
GRAF	5q31	negative regulator of RhoA	C
FKHRL1	6q21	forkhead transcription factor	N
AF9q34	9q34	Ras GTPase-activating protein	C
ABI1	10p11.2	cell motility	C
CALM	11q14-q21	regulation of endocytosis	C, N
LARG	11q23.3	activator of Rho GTPases	C
CBL	11q23.3	neg. regulator of receptor tyrosine kinase	C
GPHN	14q24	Gly and GABA receptors assembly	C
CBP	16p13	histone acetylase	N
GAS7	17p13	Actin assembly	C
AF17	17q21	transcription factor	N
AF17q25	17q25	Septin family	C
EEN	19p13.3	regulation of endocytosis	?
AF22	22q11	Septin family	C
p300	22q13	histone acetylase	N

The table lists MLL fusion partner genes with known function and localisation. N indicates nuclear localisation, C cytoplasmic localisation and ? not known localisation. The most common translocation partner are marked with * (adapted from Eguchi *et al.*, 2003).

AF9 is a 568 amino acid protein containing a serine-rich and a proline-rich domain as well as a nuclear localisation signal domain. ENL is a 559 amino acid protein containing multiple serine- and proline-rich domains. Both proteins contain an N-terminal YEATS domain with unknown function (Figure 7) (reviewed in Schulze *et al.*, 2009). However, the YEATS domain of ENL was found to bind histone H3 and histone H1 *in vitro*, suggesting a chromatin-modifying effect for YEATS domain-containing proteins (Zeisig *et al.*, 2005). The C-terminus of both AF9 and ENL proteins has been described to contain a domain possessing transcriptional activation properties. This domain is retained in the MLL fusion proteins (Rubnitz *et al.*, 1994).

As described in section 1.6., the MLL fusion partners have been shown to be involved in normal transcriptional elongation (Bitoun *et al.*, 2006). AF9 has been shown to interact with AF4, another MLL translocation partner, suggesting that the different MLL fusion partners may form a protein network in normal cells (Erfurth *et al.*, 2003). Interaction of MLL fusion partners has been further investigated by identifying ENL-associated proteins (Mueller *et al.*, 2007). The RNA polymerase II CTD kinase, pTEFb, associates with DOT1L, a non SET domain containing histone 3 lysine 79 (H3K79) specific methyltransferase. DOT1L also associates with the MLL translocation partner complex, ENL and AF4/AF5q31/LAF4, to promote transcriptional elongation (Bitoun *et al.*, 2006; Mueller *et al.*, 2007). These findings suggest a function of ENL and AF4 in histone modification and transcriptional elongation. In mice, it has also been shown that Af4 interacts with Af9 and/or Enl to recruit Af10 and mediate binding of Dot1l.

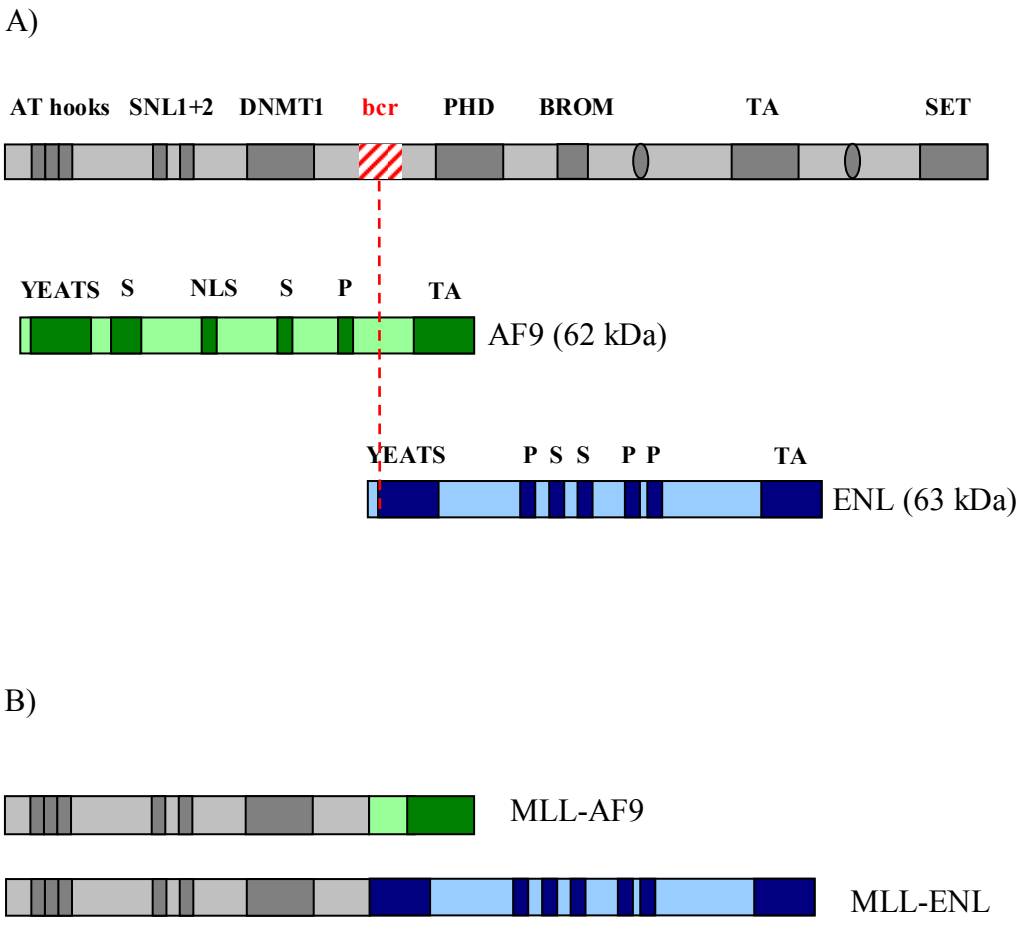


Figure 7: MLL-AF9 and MLL-ENL fusion protein

A) The domain organisations of the MLL protein is shown in grey, of the AF9 protein is shown in green and of the ENL protein is shown in blue. AF9 and ENL contain an N-terminal YEATS domain, multiple proline- (P) and serine-rich (S) domains and a C-terminal domain with transcriptional activation properties (TA). AF9 also contains a nuclear localisation signal (NLS). The breakpoint cluster region (bcr) of MLL and the corresponding fusion points within AF9 and ENL are highlighted in red. B) The figure illustrates the structure of the MLL-AF9 (grey-green) and MLL-ENL (grey-blue) fusion proteins.

Dot1l is able to methylate histone H3K79, which leads in conjunction with histone H3K4 methylation to active chromatin, and allows RNA polymerase II elongation (Meyer *et al.*, 2006; Bitoun *et al.*, 2007). Hence, the four most frequent MLL fusion partner AF4, AF9, AF10 and ENL have been found to interact in a protein network involved in histone H3K79 methylation and the transcriptional elongation processes.

1.10. Regulation of gene expression by MLL-fusion

The disruption of the wild-type MLL protein in infant and therapy-related leukaemia results in the loss of the C-terminal SET domain with histone H3K4 methyltransferase activity and its associated co-factors (reviewed in Krivtsov and Armstrong 2007). MLL fusion proteins always contain the AT hooks and the DNMT1 domain of MLL^N, which are required for MLL association with chromatin. Therefore, of the known associated co-factors only menin and LEDGF remain associated with the disrupted MLL fusion proteins (Yokoyama *et al.*, 2004; Yokoyama and Cleary, 2008). MLL fusion proteins interact with menin through multiple domains within the N-terminus including a high-affinity N-terminal conserved binding motif (RXRFP). It has been shown, that this high-affinity menin interaction domain is required for the fusion proteins MLL-ENL, MLL-GAS7 and MLL-AF10 to induce and maintain oncogenic transformation of myeloid progenitors (Yokoyama *et al.*, 2005).

Because of the particularly large number of possible translocation partner, a common mechanism of transformation for MLL-fusions has not been elucidated. However, a principle of transcriptional regulation for the most common nuclear

translocation partners, such as AF9, ENL, AF4 and AF10, has been proposed. As described earlier, the MLL fusion partners AF4, AF9/ENL and AF10 are part of a protein network that interacts with the DOT1L H3K79 histone methyltransferase (reviewed in Krivtsov and Armstrong, 2007). Methylation of histone H3K79 has also been linked with positive regulation of transcription (Lachner *et al*., 2003). It has been shown that the C-terminal region of ENL, essential for DOT1L binding, is also required for the transformation ability of MLL-ENL and therefore maintained in MLL-fusion proteins (Mueller *et al.*, 2007). Furthermore, high levels of histone H3K79 methylation at *HoxA9* locus has been observed in MLL-ENL and MLL-AF9 transformed cells (Milne *et al.*, 2005). Therefore, it has been suggested that the loss of histone H3K4 methyltransferase activity might be replaced by the acquisition of histone H3K79 methyltransferase activity in an MLL fusion protein (Figure 8). This is supported by results from a study, were the disruption of the AF9 interaction with AF4 with a synthetic peptide leads to a proliferation block of MLL-AF4 transformed cells (Srinivasan *et al.*, 2004).

1.11. Models of MLL-fusion mediated leukaemia

Several *in vivo* models have been established in order to investigate the leukaemogenic potential of MLL-fusion proteins utilizing different strategies. The first established *in vivo* model of MLL leukaemia was the *Mll-AF9* 'knock-in' mouse model (Corral *et al.*, 1996). Homologous recombination was used in order to fuse the 3'-terminal human *AF9* sequence with exon 8 of *Mll*. Therefore, expression of the *Mll-AF9* fusion gene was under the control of the endogenous murine *Mll* promoter and mice developed AML after 6 month of age.

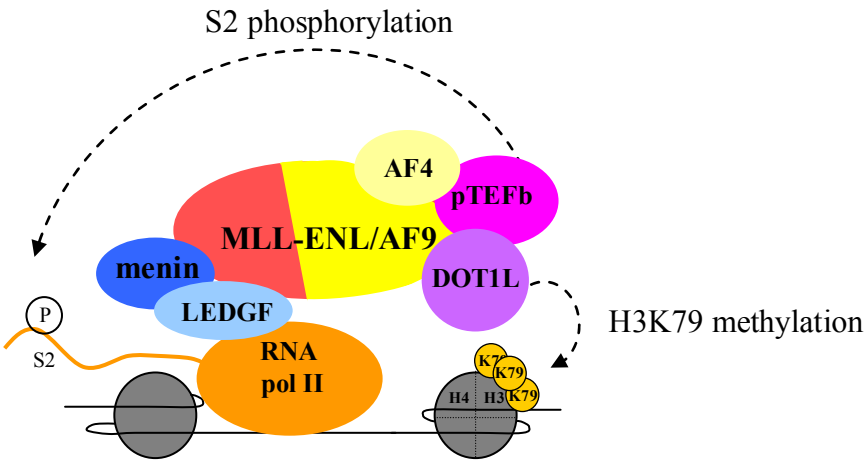


Figure 8: Proposed transcriptional regulation by the MLL-fusion

MLL-fusion oncogenes that lack the C-terminal SET H3K4 methyltransferase activity may recruit the H3K79 methyltransferase DOT1L. This mechanism allows recruitment of DOT1L to promoters that are normally occupied by MLL and promote H3K79 methylation which leads to abnormal activation of genes, such as genes in the HoxA cluster (adapted from Krivtsov and Armstrong, 2007; Dou and Hess, 2008).

The late onset of leukaemia suggested that secondary mutations were necessary for development of leukaemia (Corral *et al.*, 1996). However, the 'knock-in' approach did not allow for tissue or cell-specific fusion protein expression but expressed the fusion protein in all cells in which the endogenous *Mll* promoter was active.

To overcome this problem, the 'translocator' mouse model was developed in which chromosomal translocations were generated *de novo* during development (Collins *et al.*, 2000; Forster *et al.*, 2003; Drynan *et al.*, 2005). The 'translocator' mice were generated by introducing *locus* of X-over P1 (loxP) recombination sites into *Mll* and *Af9* (Collins *et al.*, 2000) or *Enl* (Forster *et al.*, 2003), and inter-bred with Cyclization Recombination (Cre)-expressing mice. The Cre-loxP system controls site specific recombination events in genomic DNA. Cre expression in cells with loxP sites in their genome causes a reciprocal recombination between the loxP sites. The double stranded DNA is cut at both loxP sites by the Cre recombinase and the strands are then rejoined with DNA ligase. In one model, the Cre recombinase was expressed from knock-in of the Cre cDNA into the haematopoietic regulator *Lmo2 locus* (Forster *et al.*, 2003; Drynan *et al.*, 2005). *Lmo2* is generally expressed in pluripotent stem cells and multipotent myeloid progenitors. This model gave rise to AML when either *Mll-Enl* or *Mll-Af9* translocations were formed in these progenitors.

A more frequently used method of generating murine models of MLL-fusion leukaemias is the retroviral transduction assay (Figure 9). Bone marrow derived HPC were transduced with retroviral MLL-fusion gene expression constructs and cultured in semi-solid methylcellulose medium containing myeloid growth factors.

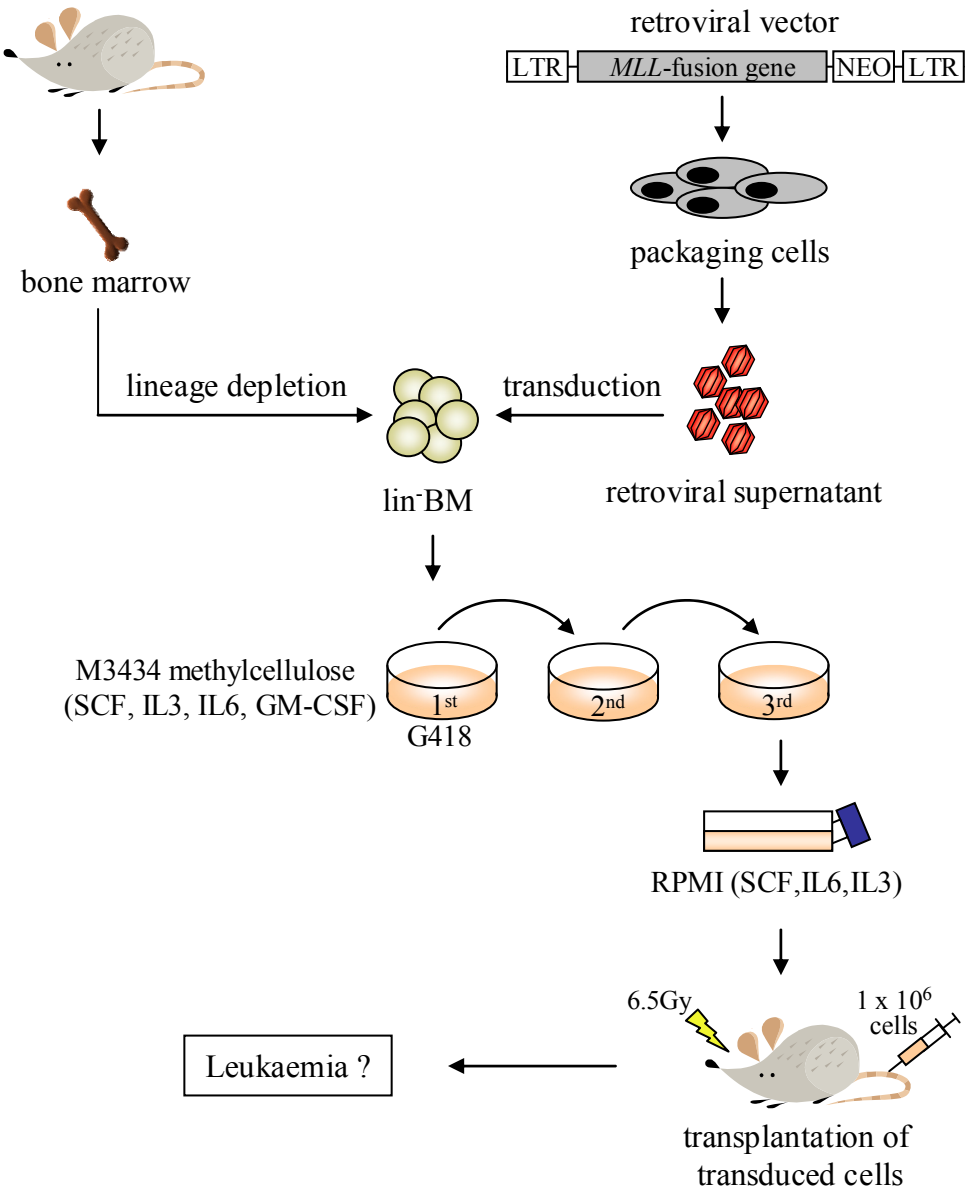


Figure 9: Retroviral transduction assay

Experimental strategy for transduction of myeloid progenitor cells. Bone marrow-derived HPC were transduced with retroviral MLL-fusion gene expression constructs and cultured in semi-solid methylcellulose medium containing myeloid growth factors (SCF, IL3, IL6, GM-CSF). Immortalised myeloid progenitors were serially replated and immortalised cell lines were established in liquid medium (RPMI containing SCF, IL3, IL6). Retrovirally transduced HPC were either analysed in vitro or injected into irradiated mice to assess leukaemia development (adapted from Eguchi *et al*., 2003).

HPC form myeloid colonies in methylcellulose. Immortalised myeloid progenitors retained their self-renewal and clonogenic potential and were able to serially replate in methylcellulose. In contrast, non-immortalised HPC terminally differentiated after two rounds of plating (reviewed in Ayton and Cleary, 2001; Eguchi *et al.*, 2003; Krivtsov and Armstrong, 2007). Retrovirally transduced HPC were either analysed *in vitro* or injected into irradiated mice to assess leukaemia development (Lavau *et al.*, 1997; Slany *et al.*, 1998; Zeisig *et al.*, 2004; Horton *et al.*, 2005; Horton, Walf-Vorderwülbecke *et al.*, 2009).

The first study using the retroviral transduction assay showed that MLL-ENL transduced cells could serially replate in methylcellulose and establish immortalised myeloid cell lines *in vitro* (Lavau *et al.*, 1997). Furthermore, the immortalised cell lines induced AML after 3 to 4 months when transferred into syngeneic recipients (Lavau *et al.*, 1997). The retroviral transduction assay was also used for studies of MLL fusion between ELL, AF10, AFX, FKHRL1, CBP, GAS7 and AF9 partner genes (DiMartino *et al.*, 2000; Lavau *et al.*, 2000; DiMartino *et al.*, 2002; So and Cleary 2002; So and Cleary 2003; So *et al.*, 2003, Krivtsov *et al.*, 2006). This approach was also used to express MLL-ENL and MLL-AF9 in lineage negative (lin^-) human cord blood progenitor cells (CB) and induce leukaemia in immunodeficient mice (Barabe *et al.*, 2007). Lin^-CB cells expressing MLL-ENL initiated B-lymphoid leukaemia in recipient mice, whereas lin^-CB cells expressing MLL-AF9 initiated both B-lymphoid and myeloid leukaemia upon transplantation. All recipients developed leukaemia with short latencies of less than 19 weeks and limiting dilution experiments estimated the frequency of leukaemia initiating cells to be less than 1:2000 (Barabe *et al.*, 2007). This model demonstrated that *MLL* fusion oncogenes possess a lympho-myeloid potential to induce leukaemia in primary

human HPC, which is influenced by the translocation partner and microenvironmental signals (Wei *et al.*, 2008).

1.12. The cell of origin of MLL-translocations

Although all MLL-fusion oncogenes may affect common pathways to induce malignant transformation, the different MLL translocations are often associated with haematopoietic malignancies of different lineages. The MLL-AF4 translocation t(4;11)(q21;q23) is the most common partner gene of MLL and is almost exclusively associated with infant ALL (reviewed in Felix and Lange, 1999). The MLL-AF9 translocation t(9;11)(p22;q23) is predominantly associated with infant AML and therapy related AML, while the MLL-ENL translocation t(11;19)(p13;q23) is found in both infant AML and ALL (Rubnitz *et al.*, 1996). Further insight into the distinct lineage association of these fusions in infant leukaemia, has been produced by experiments using the translocator mouse model (Forster *et al.*, 2003, Drynan *et al.*, 2005). The translocator mouse model allows the targeting of *de novo* chromosomal translocations to specific lineages, based on the pattern of expression of different Cre-cassettes. One translocator mouse model, in which chromosomal translocations of murine *Mll-Enl* occur in pluripotent stem cells (*Lmo2*-Cre), resulted in myeloid leukaemia in 100% of the mice (Forster *et al.*, 2003). A different translocator mouse model, with Cre under the control of the distal *Lck* promoter (*Lck*-Cre); demonstrated that translocation of *Mll-Enl* occurring in T-cells and their progenitors can be oncogenic in the lymphoid lineage (Drynan *et al.*, 2005). However, it was observed that murine *Mll-Af9* translocations induced in similar T-cell populations did not give rise to leukaemia, although *Mll-Af9* translocations in uncommitted progenitors did induce myeloid leukaemias in 100% of the mice (Drynan *et al.*, 2005).

These studies in mouse models suggest that although MLL-AF9 is highly homologous to MLL-ENL and may function via a common pathway to induce leukaemia, the two fusions are not functionally identical. As mentioned above, different MLL-rearrangements are often associated with leukaemias of different lineages. In order to identify the cell of origin of MLL-fusion induced leukaemia, murine models were used to analyse the effect of the *MLL*-fusion gene on haematopoietic cells. Two models, the instructive and the stochastic model, have been proposed in order to explain the lineage specificity of different *MLL*-fusion genes. The instructive model proposes that the translocation occurs in HSC, or uncommitted progenitors, providing lineage specific developmental signals which depend on the particular translocation (reviewed in Daser and Rabbitts, 2004). Therefore, MLL-AF9 or MLL-AF4 would provide signals for myeloid or lymphoid differentiation, respectively. The stochastic model proposes that MLL-translocations occur in committed myeloid or lymphoid progenitors, depending on the MLL-translocation (reviewed in Daser and Rabbitts, 2004). Therefore, fusion activities would only be compatible with leukaemogenesis in the appropriate lineage. Translocations occurring in an inappropriate lineage would have no consequence.

In order to analyse whether AML arises only from self-renewing stem cells or also from committed myeloid progenitors, HSC, CMP, GMP and MEP were purified and separately transduced with a retroviral expression vector encoding MLL-ENL and transplanted into recipient mice (Cozzio *et al.*, 2003). Transduction of HSC, CMP and GMP, but not MEP, generated myeloid immortalised cell lines and induced leukaemia in recipients with similar latencies and the same phenotype. All leukaemias exhibited a similar maturation arrest, at a late stage of myelomonocytic differentiation, independent of the starting population (Cozzio *et al.*, 2003). These

results suggest that the MLL-ENL oncogene does not expand the transduced progenitor population but initiates a specific arrest downstream of the GMP compartment (Cozzio *et al.*, 2003). Furthermore, it shows that myeloid leukaemias with the same immunophenotype can be initiated in HSC as well as committed progenitors.

1.13. Leukaemic stem cells

HSC are characterised by their ability to undergo asymmetric cell division and generate primitive HSC which indefinitely self-renew as well as generate more mature progenitors in order to sustain haematopoiesis (Ho *et al.*, 2005). It has been proposed that cancer cells contain rare cancer stem cells which also have the ability to indefinitely self-renew and therefore maintain the growth of the cancer (reviewed in Bonnet, 2005). In leukaemias these cells are termed leukaemic stem cells (LSC) which have either maintained or acquired the ability to self-renew through accumulation of genetic mutations (reviewed in Bonnet, 2005). Results from xenotransplantation experiments support the theory that AML LSC are transformed HSC. It was shown that only AML cells with a $CD34^{+}CD38^{-}$ phenotype, similar to the HSC phenotype, were able to transplant leukaemia in NOD/SCID mice (reviewed in Bonnet, 2005). On the contrary, Cozzio *et al.* showed that MLL-ENL induced the exact same leukaemia in HSC as well as progenitor populations (Cozzio *et al.*, 2003). Therefore, it seems possible that AML can arise in both immature HSC and more mature progenitor populations possibly depending on the nature of the associated oncogene.

1.14. Is the MLL-fusion oncogene sufficient to cause leukaemia?

The particularly short latencies of infant and therapy-related leukaemias with MLL rearrangements are very unusual for malignant cancer formation, which is usually a result of multiple genetic aberrations. Additionally, the high concordance of *MLL*-fusion gene positive leukaemia found in twins suggests that the *MLL*-fusion gene might be sufficient to initiate leukaemia (Greaves *et al.*, 2003). This fact has been supported by some of the data coming from animal models such as the *Mll-Enl* translocator mice which developed leukaemia with a rapid onset after 4 months of age (Forster *et al.*, 2003). Furthermore, mice that were engrafted with human transduced cells also showed short latencies (less than 5 months) to the onset of leukaemia (Barabe *et al.*, 2007). On the other hand, leukaemias with *MLL*-fusion gene rearrangements are often found to have acquired additional chromosomal abnormalities and gene mutations (reviewed in Eguchi *et al.*, 2003). In addition, some of the murine models of MLL leukaemias have demonstrated that leukaemia is induced with longer latencies, for example the *Mll-Af9* 'knock-in' mice (< 9 month) (Corral *et al.*, 1996). This longer latency possibly reflects the time necessary for secondary genetic events to occur. In general, different murine models of MLL leukaemias have shown to induce leukaemia with different latencies. Cytogenetic analysis of human leukaemias associated with MLL-ENL translocations identified additional genetic abnormalities (Moorman *et al.*, 1998). Whether additional abnormalities are required for the onset of leukaemia or are a consequence of MLL-ENL induced leukaemia is still unclear. However, in infant leukaemias it is possible that additional genetic mutations are also caused by the genotoxic agent that caused the MLL-rearrangement *in utero* and long latencies are therefore not required (reviewed in Eguchi *et al.*, 2003). Alternatively, MLL-fusion proteins could play a

role in promoting genomic instability by de-regulating the expression of MLL target genes (So and Cleary, 2002).

1.15. Identification of MLL-fusion protein target genes

Several previous studies have compared gene expression profiles of patients with 11q23 translocations (MLL leukaemias) to patients with leukaemias that lack this genetic lesion (other leukaemias) (Armstrong *et al.*, 2002; Ross *et al.*, 2004, Kohlmann *et al.*, 2005). In all studies, gene expression signatures were identified which were able to distinguish AML and ALL with 11q23 translocations from other ALL and AML subtypes ('MLL' vs 'non-MLL') (Figure 10). In some studies it was observed that although MLL leukaemias were clearly distinct from other leukaemia subtypes of the same cell lineage, they cluster primarily according to lineages independent of molecular abnormalities ('MLL AML with other AML' vs 'MLL ALL with other ALL') (Ross *et al.*, 2004, Kohlmann *et al.*, 2005). Kohlmann *et al.* then went on to demonstrate that MLL rearranged myeloid and lymphoid leukaemias can also be segregated with respect to their lineage but not with respect to their partner genes ('MLL AML' vs 'MLL ALL') (Kohlmann *et al.*, 2005). These results suggest common downstream pathways of MLL-fusion mediated transformation but do not explain why the presence of specific MLL rearrangements is associated with particularly lineages of leukaemia (Kohlmann *et al.*, 2005). However, another gene expression study shows that primary acute leukaemias and cell lines with the same primary genetic changes segregate based on their genetic rearrangement (Andersson *et al.*, 2005). Controversial results like these may be caused by inherent problems with comparing different kind of leukaemias with each other.

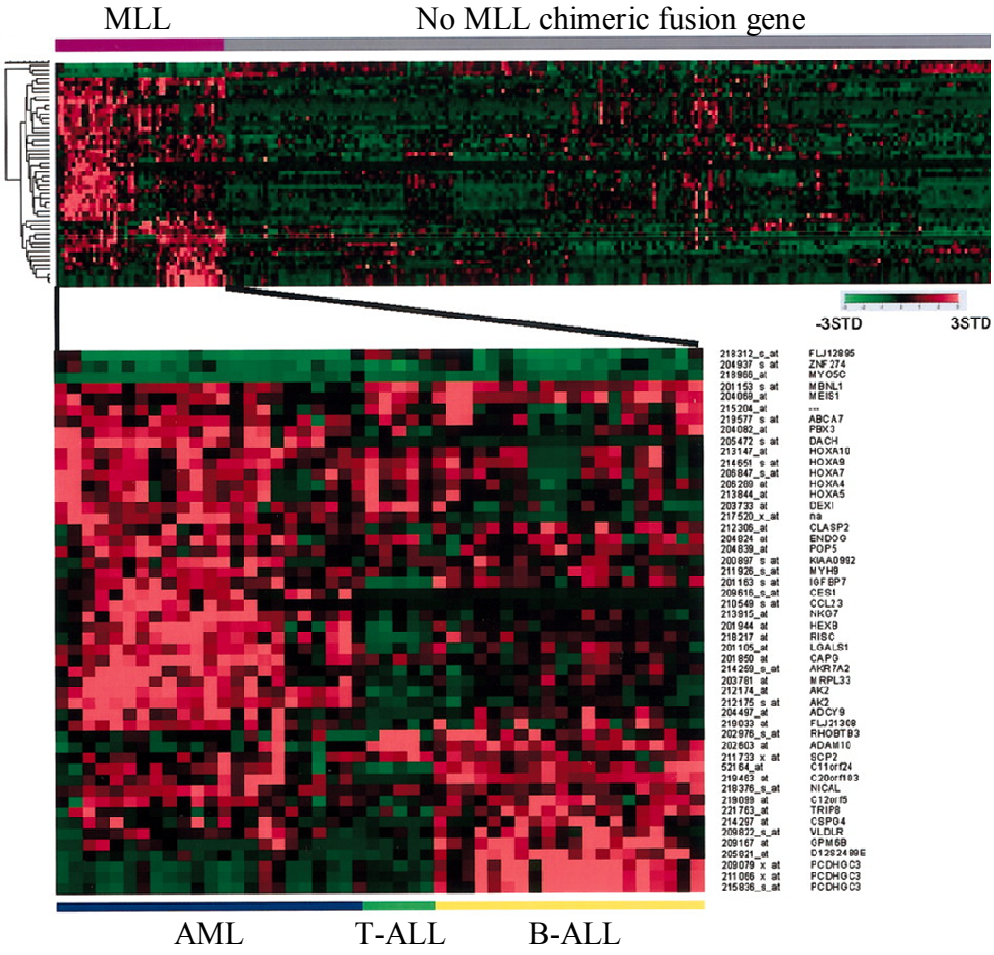


Figure 10: Top 50-ranked MLL discriminating genes

The figure illustrates the expression patterns of the top 50-ranked non-lineage restricted *MLL* class discriminating genes revealed by gene expression profiling of paediatric AML. All cases with *MLL* rearrangements were grouped on the left. The majority of the *MLL* class discriminating genes were overexpressed. Among these are genes that are expressed in the majority of *MLL* rearranged cases, such as *MEIS1*, *HOXA4*, *HOXA5*, *HOXA9*, *HOXA10* and *MYH9* (adapted from Ross *et al*., 2004).

In mice, previous gene expression studies with MLL-AF9 have analysed gene expression in leukaemic-GMP (L-GMP), an HPC sub-population (Krivtsov *et al.*, 2006). L-GMP is described to contain a high frequency of leukaemic stem cells (Krivtsov *et al.*, 2006). These studies have yielded important information, such as the characterisation of gene expression changes that occur during the transition from committed progenitor to LSC and the identification of a small set of direct MLL-AF9 activated target genes (Krivtsov *et al.*, 2006). However, this study has several potential drawbacks. Since LSC enrichment does not mean that LSC are only contained in the enriched population, it is unclear how many LSC were lost from the analysis because of the L-GMP purification protocol. One of the main problems associated with this approach of gene expression profiling is that the transformed cells permanently express MLL-AF9, which makes the definition of an appropriate control group difficult. In order to allow a comparison and identify differentially expressed genes, the gene expression profile of transformed cells was compared to the gene expression profile of non-transformed GMP (Krivtsov *et al.*, 2006). However, this resulted in an inadequate comparison. It is questionable if every GMP is capable of being transformed and therefore if the transformed GMP are different to the non-transformed GMP in terms of progenitor composition. Immortalised cells may represent an expansion of a small fraction of GMP, not equivalent to the total GMP pool. In order to exclude non-specific MLL-AF9 target genes, this study also performed an additional gene expression profiling shortly after transduction of HPC with MLL-AF9 (Krivtsov *et al.*, 2006). However, flow cytometry sorting and retroviral infection of the cells just before global gene expression profiling can cause high noise to signal ratio and therefore results in identification of only a small set of target genes.

Similar difficulties were encountered by Chen *et al.* using sorted HSC, CLP, CMP and GMP populations from pre-leuakemic *Mll-AF9* 'knock-in' mice and wild-type mice, to compare the early *in vivo* effects of MLL-AF9 on gene expression levels (Chen *et al.*, 2008). *Mll-AF9* 'knock-in' mice show myeloproliferation but are not leukaemic at this stage (Corral *et al.*, 1996, Chen *et al.*, 2008). Similar to Krivtsov *et al.*, the potential caveat of this study is that differentially expressed genes were identified based on the comparison between a population of transformed cells and a population of wild-type cells which may be completely different in their progenitor composition (Chen *et al.*, 2008). Furthermore, this study does not take into account that *Mll-AF9*-induced transformation varies within the different cell types, being most efficient in HSC and lowest in GMP. Differentially expressed genes were identified by analysing gene expression changes for all four *Mll-AF9* cell types compared to wild-type or by comparing *Mll-AF9* transformation sensitive HSC/CLP to *Mll-AF9* transformation-resistant CMP/GMP (Chen *et al.*, 2008). It is likely that an approach like this will only facilitate reliable identification of primary *Mll-AF9* target genes with high fold changes, such as *Evi1* and *HoxA5*, similar to the well-known MLL and MLL-fusion protein targets *HoxA9* and *Meis1*. Therefore, this system does not seem sensitive enough to identify downstream pathways required for *Mll-AF9* mediated transformation.

MLL-AF9 target genes were also identified in a study, using immortalised MLL-AF9 cell lines generated by retroviral transduction of human $CD34^+$ cord blood cells (Wei *et al.*, 2008). As comparison samples, Wei *et al.* used retrovirally immortalised cell lines, expressing one of the CBF fusion genes, *AML1-ETO* or *CBFβ-MYH11*. In order to further compare the gene expression profile of MLL-AF9 cells to primary AML samples, samples from two public data sets (Ross *et al.*, 2004,

Valk *et al.*, 2004) were combined and analysed for the most differentially expressed genes between CBF patient samples and MLL patient samples (Wei *et al.*, 2008). Differentially expressed genes in patient and culture samples were then compared and it was shown, that the gene expression profile of the MLL-AF9 expressing cell lines resembles human AML with 11q23 translocations (Wei *et al.*, 2008).

Zeisig *et al.* generated conditional MLL-ENL immortalised mouse cell lines by using a synthetic hormone-inducible system and performed a global gene expression analysis (Zeisig *et al.*, 2004). This system utilizes fusions between a mutated synthetic hormone-binding domain of the oestrogen receptor and the MLL-ENL fusion protein. In the absence of the synthetic hormone precursor tamoxifen, the fusion protein is held in an inactive state, presumably due to complex formation with HSP90. Addition of tamoxifen causes a conformational change that dissociates HSP90, resulting in activation of the fusion protein (Littlewood *et al.*, 1995). One caveat of this system was that the maintenance of MLL-ENL immortalised cell lines was dependent on addition of tamoxifen to the cell cultures. By excluding constitutive cell lines from the analysis, the effect of tamoxifen itself on gene expression was not taken into account (Zeisig *et al.*, 2004). Although biologically replicates are of critical importance for statistically and biologically valid conclusions, differentially expressed genes were identified by comparing RNA, from one cell line in triplicate experiments, cultured with and without tamoxifen (Zeisig *et al.*, 2004). Therefore, gene expression profiling using this approach resulted in only a small set of target genes.

Since leukaemia is a very heterogeneous disease, it is particularly difficult to define an appropriate control for global gene expression studies with patient

materials. One possibility is to compare different genetic subtypes with each other and identify genes that are differentially regulated between the compared subtypes of leukaemia. However, none of the leukaemic subgroups can be considered as a control group but rather as comparison samples. Furthermore, leukaemic cells from patient material often contain multiple genetic aberrations which can cause significant skewing of the gene expression profiles. The available data shows that there is a need for target gene analysis using a different, biologically and statistically more robust approach.

PROJECT AIM

The aim of my project was to investigate the transcriptional networks used by the MLL-AF9 and MLL-ENL fusion oncogenes to transform haematopoietic progenitor cells *in vitro* and induce leukaemia *in vivo*. It was envisaged that analysis of global gene expression changes in conditionally immortalised MLL-AF9 and MLL-ENL cell lines would identify genes which could play a role in the underlying transformation process induced by MLL-fusions. By changing MLL-fusion target gene expression, for example by knock down of target genes which are induced by the MLL-fusions, the importance of a particular target gene for MLL-fusion mediated transformation could be examined. Abrogated expression of target genes which affected MLL-AF9 and MLL-ENL immortalised cell lines *in vitro* could potentially play an important role in AML development *in vivo*. Therefore, conditional MLL-ENL cell lines were used to induce leukaemia *in vivo* and the dependence of the leukaemia on conditional expression of MLL-ENL was examined. This approach allowed the validation of targeting the transcriptional pathways controlled by MLL-fusions in order to abrogate leukaemia.

CHAPTER II - MATERIALS AND METHODS

2.1. Buffers and Solutions

Table 4: Buffers used in this study

Buffer	Components	Supplier
Red Cell Lysis	17 mM Tris(hydroxymethyl)methylamine (TrisHCl) [pH 7.2]	VWR
	0.144 M Ammonium Chloride (NH_4Cl)	VWR
TE Buffer	10 mM TrisHCl [pH 8.0]	
	1 mM Ethylenediaminetetraacetic Acid Disodium Salt (EDTA) [pH 8.0]	VWR
TNES	TE Buffer [pH 8.0]	
	0.1 M Sodium Chloride (NaCl)	VWR
	1% w/v Sodium Dodecyl Sulfate (SDS)	Sigma
Denaturation	1.5 M NaCl	
	0.5 M Sodium Hydroxide (NaOH)	VWR
Neutralisation	1 M TrisHCl [pH 7.7]	
	1.5 M NaCl	
Denhardt's solution (1x) [pH 7.0]	0.5% w/v Bovine Serum Albumin (BSA)	Sigma
	0.5% w/v Ficoll 400	Sigma
	0.5% w/v Polyvinylpyrrolidone	Sigma
Pre-hybridisation	0.1 g/ml Dextran Sulphate	Sigma
	4 x SSC (NaCl, Sodium Citrate) [pH 7.0]	NationalDiagnostics
	5x Denhardt's solution	
	0.5% w/v SDS	
2x Reducing Sample Buffer	200 mM Dithiothreitol (DTT)	Sigma
	2% w/v SDS	
	10% v/v Glycerol	Sigma
	0.1% w/v Bromophenol Blue	Sigma
	0.125 M TrisHCl [pH 6.8]	

(Table 4 continues)

Buffer	Components	Supplier
Triton X-100 Lysis Buffer	150 mM NaCl 20 mM TrisHCl [pH 7.4] 1 mM EDTA 1% v/v Triton X-100	Sigma
Upper Buffer	0.5 M TrisHCl [pH 6.8] 0.4% w/v SDS	
Lower Buffer	1.5 M TrisHCl [pH 8.8] 0.4% w/v SDS	
Running Buffer (1x)	0.192 M Glycine 25 mM TrisHCl [pH 8.3] 0.1% w/v SDS	VWR
Transfer Buffer (1x)	9.5 mM CAPS [pH 11.0]	Sigma
Western Wash Buffer	1x Phosphate-Buffered Saline (PBS) 0.1% v/v Tween-20	Oxoid Sigma
Stripping Buffer	50 mM 2-Mercaptoethanol (2-ME) 1% w/v SDS 62.5 mM TrisHCl [pH6.7]	Sigma
FACS Wash Buffer	PBS 0.1% w/v Sodium Azide (NaN_3)	Gibco Sigma
FACS Stain Buffer	PBS 0.1% w/v NaN_3 1% w/v BSA	

The table shows a list of all buffers used in this study.

2.2. Transformation of Bacteria

Electrocompetent cells:

To prepare electrocompetent cells, cells from the DH10βtrfA and DH10β bacterial strain were plated out from the glycerol stock onto LB Agar (1.5g Bacto Agar (BD)

per 100 ml LB broth) plates and incubated at 37ºC overnight. On the next day, one bacterial colony was picked and inoculated into 5 ml LB Broth (1% w/v Bacto Tryptone (BD), 0.5% w/v Bacto Yeast Extract (BD), 1% w/v Sodium Chloride (NaCl), [pH 7.0]) at 37ºC overnight with shaking at 240rpm. 5 ml of the overnight culture was inoculated in 500 ml of 2xYT (1.6% w/v Bacto Tryptone, 1% w/v Bacto Yeast Extract, 0.5% w/v NaCl) at 37ºC overnight with shaking at 240rpm. Cells were poured into 10 pre-chilled 50 ml falcon tubes and put on ice for five minutes to cool the cells quickly. Cells were centrifuged at 2260g for seven minutes at 4ºC and the supernatant was discarded. The pellets were gently resuspended in 4 ml chilled 10% glycerol and divided into 10 new 50 ml pre-chilled falcon tubes. 25 ml chilled 10% glycerol was added to each tube, centrifuged at 2260g for seven minutes at 4ºC and the supernatant was discarded. This step was repeated two times but the cells were pooled and divided into 5 new 50 ml chilled falcon tubes after centrifugation. Finally the pellets were resuspended in 8 ml chilled 10% glycerol and transferred into one pre-chilled 50 ml falcon tube. The cells were centrifuged at 2260g for five minutes at 4ºC and the supernatant was discarded. The pellet was resuspended in 250µl chilled 10% glycerol and the OD_{600nm} was measured. The cells were diluted to an OD_{600nm} reading of about 0.2.

1 µl DNA was incubated on ice and mixed with 25 µl electrocompetent cells, previously thawed on ice. The mixture was transferred to a 1 mm cuvette (Eppendorf) and pulsed once with 1.8 V using an electroporator (Micropulse, BioRad, bacteria program: EC1). The mixture was added to 1 ml LB broth and incubated for one hour at 37ºC with shaking. The transformed cells were plated onto LB Agar plates containing 100 µg/ml Ampicillin (Sigma) and cultured overnight at 37°C.

Chemocompetent cells:

Subcloning Efficiency DH5αCompetent Cells (Invitrogen) were transformed according to the manufacturer's instructions. After transformation the cells were treated as described earlier.

2.3. Isolation of plasmid DNA

For small amounts of DNA, used for cloning, a single bacterial colony was picked and inoculated into 3 ml LB broth containing 100 µg/ml Ampicillin and incubated overnight at 37ºC with shaking. Plasmid DNA was isolated from the bacterial cultures using High Pure Plasmid Isolation Kit (Roche) according to the manufacturer's instructions. To purify large quantities of plasmid DNA, used for transfections, a single bacterial colony was picked and inoculated into 3 ml LB broth containing 100 µg/ml Ampicillin for 6 hours at 37ºC with shaking. 250 µl of this starter culture was then transferred into 250 ml LB broth containing 100 µg/ml Ampicillin and incubated for at least 16 hours at 37ºC with shaking. Plasmid DNA was isolated from the bacterial cultures using Genopure Plasmid Maxi Kit (Roche) according to the manufacturer's instructions. The plasmid DNA concentration was determined by measuring the absorbance at 260 nm using a spectrophotometer (NanoDrop ND-1000, Labtech International). The ratio of absorbance at 260 nm to 280 nm was used to assess the purity of DNA. A ratio of ~1.8 is accepted as pure for DNA. If the ratio is lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm (NanoDrop user's manual).

2.4. Cloning

Polymerase chain reaction (PCR):

Template specific primers were designed and provided by Sigma. Table 5 shows the TRE PCR primers used in this study.

Table 5: TRE PCR primer

Name	Direction	Sequence (5'-3')	Vector
TRE 1F NEW	forward	GGATCCTCTCGAGTTTACCACTCC	pRevTRE
TRE 444R NEW	reverse	GTCGACGTACCGAGCTCGAATTCG	pRevTRE

The table shows the TRE PCR primers used in this study.

For the PCR reaction mix, 1 ng insert was mixed with 10x reaction buffer, 0.25 µl (1 unit) Taq polymerase, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.5 pmol forward primer, 0.5 pmol reverse primer and H_2O up to 50 µl total volume. The PCR was performed using the cycle conditions illustrated in Table 6.

Table 6: PCR cycle conditions

Stage 1	Stage 2 (35 cycles)	Stage 3
94ºC 5 minutes	94ºC 30 seconds	72ºC 10 minutes
	50ºC 1 minute	Hold: 10ºC 16 hours
	72ºC 1 minute	

The table shows the PCR cycle conditions.

Restriction enzyme digests:

Restriction enzymes (Promega) were used to cleave double stranded DNA. The digests were set up according to the manufacturer's instructions, with two- to ten-fold excess of enzyme over DNA and under 10% of the final volume. The digest was incubated for two hours at 37ºC, mixed with Orange G (Sigma) loading buffer and analysed on a 0.7% - 1.2% w/v (depending on the size of the fragments) Agarose gel (Agarose (Invitrogen), 1xTAE buffer (National diagnostics), 0.5% Ethidium Bromide (Sigma)).

Ligations:

A variety of insert to vector ratios (10:1, 3:1, 1:1 and 0:1) was set up for each ligation. The following equation was used to calculate the required amount of insert for a 3:1 ligation:

$$ng_{insert\ required} = [(50ng_{vector} \times kb_{insert})/kb_{vector}] \times 3$$

For the ligation reaction 0.3 µl (1 unit) T4 DNA ligase (Promega) was mixed with 1 µl 10x T4 ligase buffer (Promega), calculated amount of insert, 50 ng vector and made up to 10 µl total volume with H_2O. The reaction was left at room temperature for three hours for a sticky end ligation or overnight at 4ºC for a blunt end ligation.

Gel Extraction:

QIAquick Gel Extration Kit (Qiagen) was used to extract and purify DNA from Agarose gels according to the manufacturer's instructions. The concentration of purified DNA was determined using the NanoDrop ND-1000.

2.5. Retroviral constructs

Diagrams of all retroviral constructs used in this study are shown in Figure 11 and Figure 12.

pCR2.1-TREintermediate:

To generate the full length MLL-ENL conditional expression vector (pMSCV-neo-TRE-fMLLENL), the cDNA of the tetracycline-responsive promotor element (TRE) was amplified by PCR using pRevTRE vector (Clontech) as a template. A 0.5 kb fragment encoding the TRE was amplified using a forward primer incorporating a Sal1 and BamH1 site: 5'-CGACTTGTCGACGGATCCTCTCGAGTTTACCACTC CCTATC-3' and a reverse primer incorporating a Sal1 site: 5'-CGTTACGTCGACC CGGGTACCGAGCTCGAATTC-3' (added restriction sites are underlined). A poly A overhang was added to the 0.5 kb TRE fragment using Taq polymerase (Promega) to sub-clone into the pCR2.1 vector (Clontech). The resulting vector, pCR2.1TRE, was then digested with BamH1 and re-ligated to remove the first Sal1 restriction site for further cloning purposes (pCR2.1TREintermediate).

pCR2.1-TRE-fMLLENL:

pMSCV-neo-mod-fMLLENL was digested with Xho1 and the overhang was filled in with Klenow (Roche) to generate a blunt end. The vector was then digested with Sal1 to release the 6.1 kb fragment encoding a flag epitope upstream of the full-length MLLENL (fMLLENL) sequence. To be able to distinguish between the 6.5 kb vector fragment and the 6.1 kb fMLLENL fragment the vector was also digested with Cla1. The pCR2.1TREintermediate vector was digested with Not1 and the overhang was filled in with Klenow to generate a blunt end.

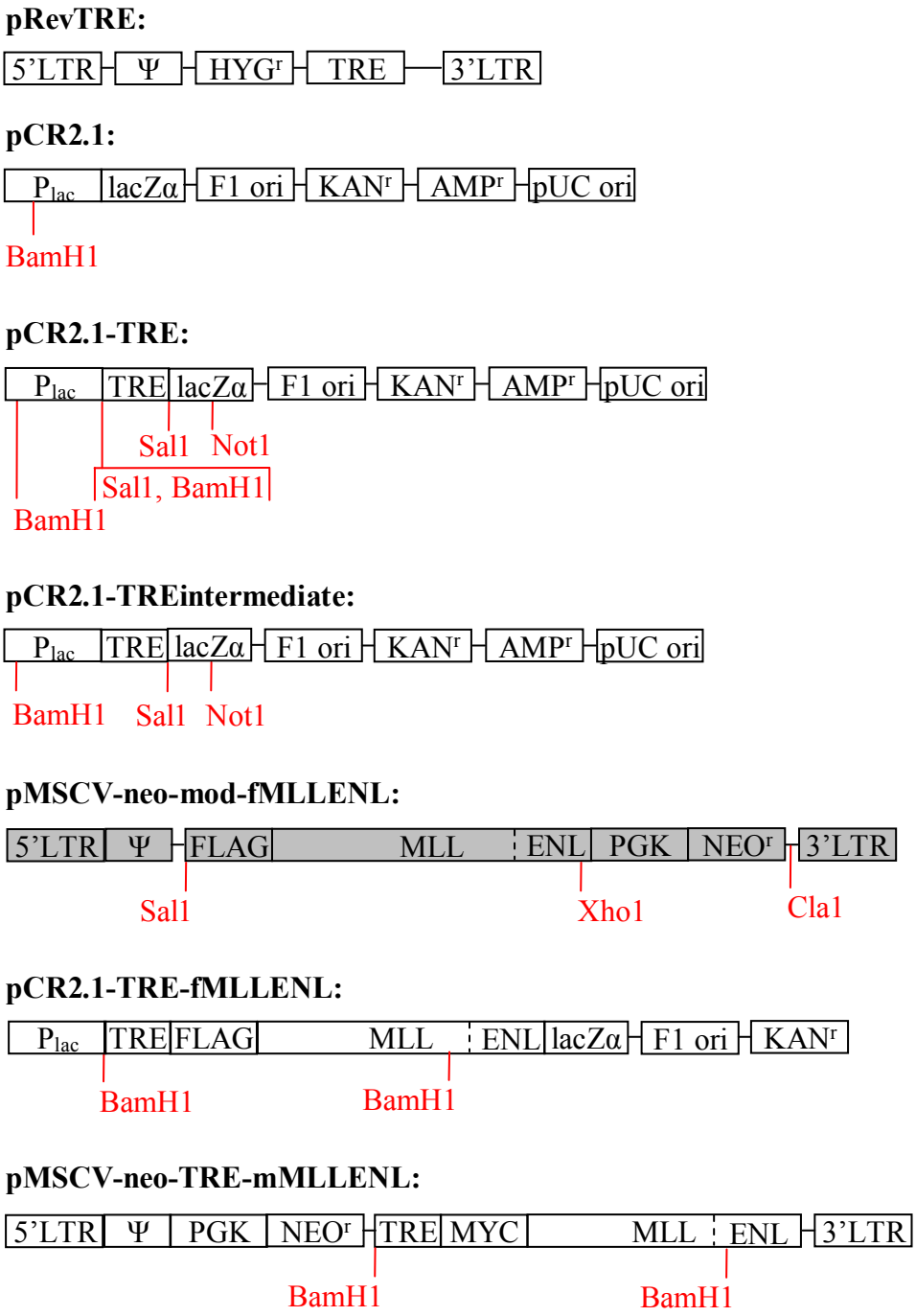
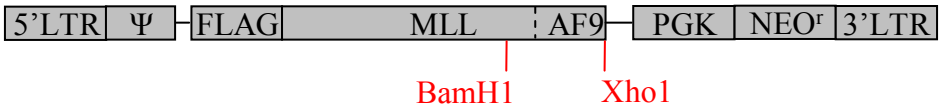


Figure 11: Retroviral constructs used in this study

LTR, long terminal repeat; Ψ, viral packaging signal; PKG, murine phosphoglycerate kinase promoter; NEOr, neomycin resistance gene; Plac, LacZ promotor; LacZα, LacZ gene fragment. Restriction enzyme sites shown in red were used for sub-cloning the full length MLL-ENL and MLL-AF9 conditional expression vector. Final vectors used in this study are highlighted in grey.

pMSCV-neo-TRE-fMLLENL:

pMSCV-neo-mod-fMLLAF9:



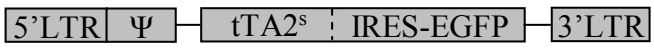
pMSCV-neo-mod-mMLLAF9:

pMSCV-neo:

pMSCV-neo-endMLLAF9:

pMSCV-neo-TRE-fMLLAF9:

pMSCV-tTA2^{s}-IRES-EGFP:



pMSCV-hCD2tailless-miR30:

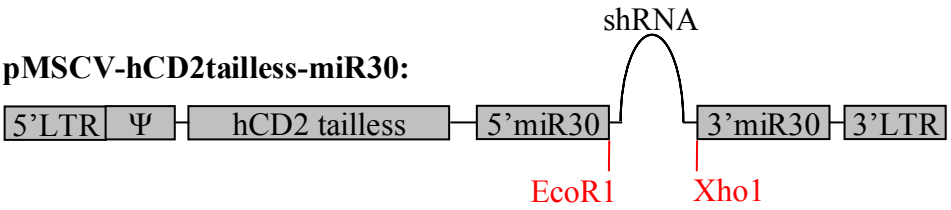


Figure 12: Retroviral constructs used in this study (continued)

LTR, long terminal repeat; Ψ, viral packaging signal; PGK, murine phosphoglycerate kinase promoter; NEOr, neomycin resistance gene. Restriction enzyme sites shown in red were used for cloning. Final vectors used in this study are highlighted in grey.

The resulting vector was then digested with Sal1 to generate a sticky end. The 6.1 kb fMLLENL fragment was then sub-cloned directionally into the pCR2.1TREintermediate vector.

pMSCV-neo-TRE-fMLLENL:

pCR2.1TRE-fMLLENL was digested with BamH1 to release the 4.3 kb TRE-fMLL fragment. This fragment was inserted into pMSCV-neo-TRE-mMLLENL which had previously been digested with BamH1, to generate pMSCV-neo-TRE-fMLLENL.

pMSCV-neo-endMLLAF9:

To generate the full length MLL-AF9 conditional expression vector (pMSCV-neo-TRE-fMLLAF9) pMSCV-neo-mod-fMLLAF9 vector was digested with BamH1 and Xho1 to release a 0.65 kb fragment encoding the 3' part of the MLL-AF9 fusion gene sequence. The pMSCV-neo vector was digested with BamH1 and Sal1 (Sal1 site can ligate with the Xho1 site) and the 0.65 kb fragment was then sub-cloned directionally into pMSCV-neo vector, downstream of the neomycin resistant gene to generate pMSCV-neo-endMLLAF9.

pMSCV-neo-TRE-fMLLAF9:

pMSCV-neo-TRE-fMLLENL vector was digested with BamH1 to release the 4.3 kb TRE-fMLL fragment. This fragment was sub-cloned into the pMSCV-neo-endMLLAF9 intermediate vector, which had been digested with BamH1, upstream of the 3' part of the MLL-AF9 fusion gene. The cloning sites were sequenced to check that no rearrangements had occurred while sub-cloning the conditional MLL-ENL and MLL-AF9 vector.

2.6. Culture of LinXE ecotropic retrovirus packaging cell line and NIH-3T3 fibroblast cells

The LinXE ecotropic retrovirus packaging cell line and NIH-3T3 fibroblast cells were cultured in a 10 cm culture dish (NUNC) in 10 ml Dulbecco's Modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated foetal calf serum (FCS, Sigma-Aldrich), 100 U/ml Penicillin (Gibco), 100 µg/ml Streptomycin (Gibco) and 2 mM L-glutamine (Gibco) (DMEM complete medium). LinXE cells were maintained in the presence of 7.5 µg/ml Hygromycin B (InvivoGen). To replate the cells, old medium was discarded, the plate was washed in 5 ml Phosphate-Buffered Saline (PBS, Gibco) and the cells were incubated with 2 ml 1xTrypsin/EDTA (Gibco) for 5 minutes. 2 ml of trypsinised cells were then added to 8 ml PBS mixed with 2 ml DMEM complete medium and centrifuged at 300g for 5 minutes at room temperature (Hettich Zentrifugen Rotina 46 R). The cell pellets were resuspended in 5 ml DMEM complete medium and counted. The cells were replated at 1:25 for 3 to 4 days.

2.7. Transfection of LinXE cells

Three days before transfection LinXE cells were seeded at a density of $5x10^5$ per 10 cm plate without Hygromycin B selection. For transfection 8 µg retroviral plasmid DNA mixed with 800 µl serum free medium Optimem (Gibco) was added drop-wise to 40 µl Lipofectamine (Invitrogen) mixed with 760 µl Optimem and incubated at room temperature for 15 to 45 minutes. LinXE cells were washed once in 8 ml Optimem. 6.4 ml Optimem was added to the transfection mix and incubated with the LinXE cells for five hours at 37°C. After five hours 8 ml DMEM complete

medium containing 20% FCS was added and incubated overnight at 37°C. One day after transfection, media on transfection plates was replaced with 8 to 10 ml fresh DMEM complete medium containing 10% FCS.

2.8. Determination of the viral titre

The viral titre of pMSCV-neo-TRE-fMLLAF9 was determined by titration on NIH-3T3 cells (Appendix Figure 74). Transfection of LinXE cells was carried out as described earlier. NIH-3T3 fibroblast cells were seeded at $5x10^4$ per well of a 6-well plate (NUNC) in 2 ml DMEM complete media. Two days after transfection virus supernatant was harvested and diluted with DMEM complete medium 1:10, 1:1000 and 1:100000 containing 5 μg/ml polybrene (Sigma-Aldrich). Medium of the cultured NIH-3T3 fibroblast cells was replaced with 2 ml of virus dilution and incubated overnight at 37°C. On the following day cells were split 1:20 into 10 cm plates and incubated over night at 37°C. The medium was replaced twice by 10 ml of fresh DMEM complete medium containing 1 mg/ml G418 (Invitrogen) and incubated for five days at 37°C. After this, the cells were washed with 5 ml PBS and stained with 5 ml methylene blue (0.1 g methylene blue (Sigma)/70 ml Methanol (MeOH)). The stain was incubated for 5 minutes on the plates, washed upside down in water 5 times and dried. The viral titre was calculated using the following formula:

viral titre (infectious particles/ml) = (number of colonies x dilution factor)/2

2.9. Determination of number of viable cells

Trypan Blue (Sigma) exclusion was used to determine the number of viable cells present in a cell suspension. The cell suspension was mixed with Trypan Blue (1:1) dye and then visually examined to determine whether cells had taken up or excluded dye and counted. Viable cells had a clear cytoplasm whereas nonviable cells had a blue cytoplasm.

2.10. Isolation of adult murine HPC from bone marrow

Mice were maintained in the animal facilities of the Western Laboratories (UCL Biological Services Unit) and experiments were performed according to institutional guidelines and Home Office regulations. Bone marrow was extracted from the femur and tibia and a single cell suspension was made. Red cells were lysed for 5 minutes with Red Cell Lysis buffer. Haematopoietic progenitor cells (HPC) were sorted by magnetic activated cell sorting (MACS) using the Lineage Cell Depletion Kit (Miltenyi Biotec). Lineage positive cells were labelled using 10 µl per $1x10^7$ cells of a biotin-conjugated monoclonal antibody cocktail (CD5, B220, CD11b, anti-GR-1, 7-4 and Ter-119). Then the cells were magnetically labelled using 20 µl per $1x10^7$ cells of an anti-biotin monoclonal antibody conjugated to MicroBeads. The magnetically labelled lineage positive cells were depleted by using a MACS LS or LD column in the magnetic field of the MACS separator. Lineage negative cells were seeded at a density of $1x10^6$/ml. HPC were cultured overnight in DMEM complete medium supplemented with 50 µM 2-mercaptoethanol (2-ME, Sigma), 100 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6 (all from Peprotech).

2.11. Transduction of HPC

Retroviral supernatants were produced as described earlier, using the ecotropic retrovirus packaging cell line LinXE. Isolated HPC were cultured for 24 hours in DMEM complete medium supplemented with 50 μM 2-ME, 100 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6. For unconcentrated virus, retroviral supernatants were filtered through a 0.45 μM filter (Sartorius) and used for infections. For concentration of virus, retroviral supernatants were centrifuged in a 15 ml falcon tube (NUNC) at 470g for 5 minutes to remove cellular debris. All supernatant but 1 ml was removed to a new 15 ml falcon tube and centrifuged again. 1 ml retroviral supernatant each was aliquoted into 1.5 ml tubes and centrifuged in a microcentrifuge (Eppendorf Centrifuge 5415R) at 15700g for 1 hour at 4°C. The virus pellet was resuspended in one tenth of the original volume in DMEM complete medium. Infections were performed in a 96 well flat bottomed plate (TPP) by spinoculation. $1x10^4$ cells were plated per well in 100 μl of virus supernatant supplemented with 50 μM 2-ME, 100 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6 and 5 μg/ml polybrene. In total $6x10^4$ cells were infected per virus. The plates were centrifuged at 680g for 45 minutes at 20°C. Infections were either performed on two consecutive days or alternatively 100 μl fresh DMEM complete medium, supplemented with growth factors was added to the infections on the second day.

2.12. Myeloid methylcellulose colony forming assays

On the third day of infection, the cells were pooled and $3.3x10^4$ cells were aliquoted in 3.3 ml methylcellulose medium containing SCF, IL-6 and IL-3 (MethoCult GF M3434, Stem Cell Technologies Inc), supplemented with 10 ng/ml

GM-CSF (Peprotech). The vials were shaken vigorously to mix growth factors and cells. Bubbles were allowed to settle for 5 minutes. 1.1 ml of M3434 and cell mix was dispensed into each of two 35 mm culture dishes (Stem Cell Technologies Inc.) using 2.5 ml syringes (BD) and blunt ended needles (Stem Cell Technologies Inc.). In some cases cells were selected with 1mg/ml G418 (Gibco) in the first round of methylcellulose plating. After 6 to 10 days colonies were counted. To harvest the cells, 1 ml MEMalpha (Gibco) was added to each plate, pipetted up and down to disperse the methylcellulose and transferred into 10 ml MEMalpha. The cells were centrifuged at 300g for 5 minutes at room temperature and resuspended in 1 ml MEMalpha to count. $3.3x10^4$ cells per 3.3 ml M3434 were replated into subsequent rounds without selection. In some cases 2 μg/ml Doxycycline (BDH) was added to subsequent rounds. Colonies were defined as clusters of more than 50 cells. To visualise colonies, cultures were stained with 1mg/ml p-iodonitrotetrazolium (INT, Sigma-Aldrich) in PBS.

2.13. Generation and maintenance of cell lines in liquid culture

After the third round of plating in M3434, cells were harvested, pooled and plated at a density of $1x10^5$/ml in RPMI-1640 (Gibco) with 10% FCS, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2 mM L-glutamine and 50 μM 2-ME (RPMI complete medium) supplemented with 100 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6. Cell lines were counted and replated every two to three days at a density between $5x10^3$ to $1x10^5$ per ml, depending on the proliferation rate of the cell line. The following calculation was used, to determine the rate of accumulation of the cell lines (Table 7).

Table 7: Determination of cell line proliferation rate

days in culture	fold increase	fold accumulation
n	-	1
n+x	[cell # $_{(n+x)}$] / [cell # plated$_{(n)}$]	[fold accumulation$_{(n)}$ x fold increase$_{(n+x)}$]

Determination of cell line proliferation rate, where x represents number of days after day n. Fold accumulation was plotted on a $\log_{10}$ scale.

2.14. Isolation of genomic DNA from eukaryotic cells

Typically 5 to 10x10^6 cells were centrifuged at 300g for 5 minutes, washed in PBS and resuspended in 400 µl TNES containing 0.5 mg/ml proteinase K (Roche). The mixture was incubated overnight a 37°C. The next day 0.5 ml phenol(25):chloroform(24):isoamylalcohol(1) solution (Sigma) was added and mixed by shaking vigorously for 1 minute. The mixture was centrifuged at 15700g for 5 minutes to separate the phases and the supernatant was mixed with 1 ml ethanol containing 3M NaOAc (Sigma) (25:1 mixture). After inverting the tubes several times the DNA was precipitated at room temperature and centrifuged for 10 minutes at 300g, then 1 ml 70% ethanol was added to the DNA pellet and centrifuged again for 2 minutes at 300g. The supernatant was taken off and the DNA pellet was air-dried for approximately 10 to 15 minutes. The DNA was redissolved in 100 µl TE buffer and left at 4°C overnight prior to freezing at -20°C.

2.15. Southern blot analysis

DNA isolated from MLL-ENL immortalised cell lines was digested overnight at 37°C with BamH1 to generate end fragments (30 µl DNA, 10 µl 10x buffer E, 4 µl

BamH1, 4 µl BSA, 5 µl RNAse in a total volume of 100 µl). DNA extracted from murine spleen or bone marrow was also digested and used as the negative control. For the positive control DNA from murine spleen or bone marrow was 'spiked' with 1 ng pMSCV-neo-TRE-fMLLENL plasmid DNA and digested. After that 10 µg of digested DNA was electrophoresed on a 0.8% w/v agarose gel overnight at 40V. Meanwhile pRev-TREneo was digested with BamH1 and HindIII and the fragments were gel purified to obtain the neo-fragment (0.8 kb), used as the hybridisation probe. Next the gel was washed three times for 20 minutes with shaking in denaturation buffer to denature the DNA. The gel was washed two times with H_2O prior to incubation with neutralisation buffer for 30 minutes with shaking. Thereafter, the gel was washed twice with 10x SSC buffer (National Diagnostics) and the nitrocellulose membrane Hybond-N+ (GE Healthcare) as well as Wattman paper (Fisher Scientific) were wet in 10x SSC buffer. The nitrocellulose membrane was placed on top of the gel, covered with 3 layers of Wattman paper and stacks of paper towels. Overnight, pressure was applied evenly to the gel, to transfer the DNA from the gel to the membrane by capillary forces. Next, the membrane was baked at 80°C for 2 hours to fix the DNA permanently to the membrane. The membrane was then briefly washed with 2x SSC and blocked with 100 ml pre-warmed pre-hybridisation buffer containing preheated (2 minutes, 100°C) 100 µl salmon testes DNA (Sigma) for 2 hours at 65°C shaking. To label the hybridisation probe, 100 ng probe in a total volume of 45 µl was denatured at 94°C for 3 minutes and placed on ice for 5 minutes. The probe was then used to resuspend the Ready-To-Go DNA Labeling Beads (-dCTP) (GE Healthcare), 5 µl of [α- ^{32}P]dCTP was added and incubated for one hour at 37°C. To purify the labelled probe, the ProbeQuant G-50 Micro Columns Purification Kit (GE Healthcare) was used according to the manufacturer's instructions. The labelled and purified probe was denatured at 94°C for 10 minutes,

added to the prehybridsation mix and incubated overnight at 65°C with rotation. After hybridisation, excess probe was washed off the membrane with 2x SSC buffer containing 0.1% SDS for one hour at 55°C. The pattern of hybridisation was visualized using a Typhoon phosphoimager (Amersham Biosciences) as well as on X-ray film by autoradiography.

2.16. Preparation of protein extracts for Western blot analysis

Cells were harvested and centrifuged at 300g for 5 minutes at 4°C. Cell pellets were lysed using 60 µl 2 x reducing sample buffer per $1x10^6$ cells. The lysate was incubated at 100°C for five minutes and vortexed for 10 seconds. Then the lysate was centrifuged at 15700g for 1 minute at 4°C in a microcentrifuge. The supernatant containing the protein extract was recovered and frozen at –20°C. To denature proteins before loading, samples were incubated at 100°C for five minutes and kept on ice until loading.

2.17. MLL-AF9 Western blot analysis

Table 8: Components of a 4% stacking gel

Solutions	Stacking Gel (4%)
30% Acrylamide/Bis Solution (29:1) (BioRad)	0.8 ml
Upper Buffer	1.6 ml
H_2O	3.8 ml
Temed (BioRad)	6.25 µl
10% w/v Amonium Persulfate (APS, BioRad)	31.3 µl

The table shows the components of a 4% stacking gel used for Western blot analysis.

Table 9: Components of resolving gels

Solutions	Resolving Gel		
	5%	**10%**	**12.5%**
30% Acrylamide/Bis Solution (29:1) (BioRad)	5 ml	10 ml	12.5 ml
Lower Buffer	7.5 ml	7.5 ml	7.5 ml
H_2O	17.5 ml	12.5 ml	10 ml
Temed (BioRad)	20 µl	20 µl	20 µl
10% w/v Amonium Persulfate (APS, BioRad)	100 µl	100 µl	100 µl

The table shows the components of 5%, 10% and 12.5% resolving gels used for Western blot analysis.

Hoeffer equipment (GE Healthcare) was used for all Western blot analysis. Protein samples were stacked in a 4% SDS-polyacrylamide gel (Table 8) and resolved on a 5% SDS- polyacrylamide gel (Table 9) at 65V overnight at room temperature. SDS- polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 1x running buffer. After that, samples were first transferred to a polyvinylidenefluoride (PVDF) membrane (Millipore) for 6 hours at 500 mA at 4ºC in 1 x CAPS and then membranes were blocked in PBS with 5% non-fat milk and 0.1% Tween-20 (Sigma) (PBS-T) overnight at 4°C. The membranes were then either incubated with anti-MLL^N/HRX [clone N4.4] monoclonal antibody (mAb) (Upstate) for 4 hours or with an anti-HSP90α/β antibody (Cell Signaling Technology) for 1 hour at room temperature. Excess antibody was removed by washing membranes six times with PBS-T for 10 minutes each. Proteins were detected using sheep anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP, GE Healthcare) or anti-rabbit antibody conjugated to HRP (GE Healthcare) and a chemiluminescent reagent (ECL, Amersham Biosciences) according to the manufacturer's instructions. To estimate the size of proteins, the High-Range

Rainbow Molecular Weight Marker (Amersham Biosciences) was used. In order to re-probe membranes with different antibodies, membranes were stripped of bound antibodies. Membranes were incubated at 50ºC for 30 minutes with agitation in stripping buffer. The membranes were then washed twice with PBS-T for 15 minutes and blocked again in PBS-T with 5% non-fat milk. Hypofilms ECL (Amersham) were exposed to membranes for various exposure times and then developed using a Xograph CompactX4 developer. Exposed films were scanned using a Calibrated Densitometer (GS-800, BioRad) and bands were quantitated using QuantityOne software (BioRad). The relative protein expression was calculated dividing the values of the protein of interest by the expression values of the protein loading control. Protein expression values from treated samples were normalised to untreated samples. Table 10 lists all antibodies used in this study and their corresponding HRP conjugated secondary antibodies, which were used for detection.

2.18. RNA isolation

Total RNA was isolated from the cells using either the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions or TRIzol reagent (Invitrogen). For the TRIzol reagent extraction method, a maximum of $1x10^7$ cells were centrifuged in a 15 ml falcon tube at 300g for 5 minutes. Tubes were flicked to dislodge pellets and the cells were lysed in 1 ml TRIzol reagent (Invitrogen) using repetitive pipetting. The lysate was transferred to a 1.5 ml tube and incubated at room temperature for 5 minutes. 200 µl chloroform (VWR) was added to the lysate, shaken vigorously for 15 seconds, incubated for 2–15 minutes at room temperature and centrifuged at 12000g for 15 minutes at 4ºC.

Table 10: Antibodies used in this study

Antibody (clone)	Supplier	Dilution	Secondary Ab
Actin (I-19)	Santa Cruz Biotechnology	1:500	α-goat (1:6000)
AF4 antiserum	provided by Prof KE Davies	1:1000	α-rabbit (1:10000)
Active-Ctnnb(8E7)	Upstate Millipore	1:2000	α-mouse (1:2000)
Total-Ctnnb (E-5)	Santa Cruz Biotechnology	1:500	α-mouse (1:2000)
c-Myb (H-141)	Santa Cruz Biotechnology	1:500	α-rabbit (1:10000)
HSP 90α/β (F-8)	Santa Cruz Biotechnology	1:10000	α-mouse (1:10000)
MLL^N/HRX(N4.4)	Upstate Millipore	1:400	α-mouse (1:2000)
Pontin52 (N-15)	Santa Cruz Biotechnology	1:500	α-goat (1:3000)
SIAH-1 (N-15)	Santa Cruz Biotechnology	1:500	α-goat (1:3000)
SIAH-2 (N-14)	Santa Cruz Biotechnology	1:500	α-goat (1:3000)
β-Tubulin (D-10)	Santa Cruz Biotechnology	1:500	α-mouse (1:2000)

The table lists all antibodies used for Western blot analysis in this study.

After centrifugation the upper aqueous phase containing the RNA was transferred to a fresh tube, mixed with 500 µl isopropanol, incubated at room temperature for 10 minutes and centrifuged at 12000g for 10 minutes at 4ºC. The pellet was washed in 1 ml 70% ethanol (EtOH) and centrifuged for 5 minutes at 5100g at 4ºC. The supernatant was discarded and the pellet was air dried. To redissolve the pellet carefully, 30 µl filtered H_2O was added and incubated at 55ºC for 5 minutes. The concentration of isolated RNA was determined using a spectrophotometer (NanoDrop ND-1000). The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA. A ratio of ~2.0 is accepted as pure for RNA. If the ratio is lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm (NanoDrop user's manual).

RNA quality of samples used for the Affymetrix Gene Chip Arrays was determined using the Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit) according to manufacturer's instructions. An indication for good RNA quality is a ratio of 18S to 28S ribosomal RNA between 1.6 and 2.0. Furthermore the entire electrophoretic trace of the RNA sample including the presence or absence of degradation products is calculated in a RNA Integrity Number (RIN). The RIN tool automatically assigns an integrity number to the RNA sample, with 1 representing poor quality and 10 representing good quality (Agilent 2100 Bioanalyzer 2100 Expert User's Guide).

2.19. cDNA preparation

RNA was converted into cDNA using a cDNA synthesis kit from Invitrogen according to the manufacturer's instructions. Samples were treated with DNase (Invitrogen) prior to reverse transcription. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen) was used to synthesize a complementary DNA strand.

2.20. Real-time quantitative PCR

A specific primer and probe set spanning the MLL-AF9 breakpoint region was designed and optimised to measure relative expression levels of MLL-AF9 in the cells by real-time quantitative PCR (qPCR). Primer and probe were designed using the PrimerExpress software v2.0 (Applied Biosystems). MLL-AF9 was amplified from cDNA using a forward primer 5'-CAAGTATCCCTGTAAAACAAA AACCA-3' (which binds to MLL) and a reverse primer 5'-CATTCACCATTCTTTA TTTGCTTATCTG-3' (which binds to AF9) and a probe 5'-TGCTTTGCTTTATTG

GACTTTTCACTTCAAGAATCTTT-3' (which spans the MLL-AF9 breakpoint) (Appendix, Figure 75). Various forward primer, reverse primer and probe concentrations were tested, to determine the optimal primer and probe concentration for measuring the MLL-AF9 transcript expression (Appendix, Figure 76). The optimal combination of 900 nM forward primer and 900 nM reverse primer concentration produced the lowest cycle threshold (C_T) value and the highest reaction (Rn) value (Appendix, Figure 76 A). The optimal probe concentration was 150 nM since this was the lowest concentration which did not affect the C_T value (Appendix, Figure 76 B). MLL-AF9 and 18S (endogenous control) qPCR reactions have to be equally efficient, in order to analyse the MLL-AF9 qPCR data using the $2^{-\Delta\Delta CT}$ relative quantitation method (Livak and Schmittgen, 2001). A standard curve was generated to determine the qPCR efficiency at low and high cDNA concentrations (0.39 ng to 50 ng), prepared from total RNA isolated from cMA3 (Appendix, Figure 77). MLL-AF9 reactions were set up with 900 nM forward primer, 900 nM reverse primer, 150 nM probe and 1 x TaqMan universal mastermix (Applied Biosystems). 18S reactions were set up with 1 x 18S primer and probe set (optimised by the manufacturer, Applied Biosystems) and 1x TaqMan mastermix. MLL-AF9 and 18S qPCR are reproducible at low and high cDNA concentrations with 0.9943 and 0.97 correlation coefficients of the MLL-AF9 and 18S standard curve, respectively. The efficiency of each reaction was calculated using the formula: PCR efficiency = $(10^{(1/-s)})$-1 (Ginzinger et al., 2002), where s is the gradient of the standard curve and a slope of 3.3 equals 100% PCR efficiancy. The efficiency of MLL-AF9 reaction was 99% and of 18S was 100%. Therefore the $2^{-\Delta\Delta CT}$ relative quantitation method can be used to determine the relative expression level of MLL-AF9. Expression of other genes was measured using inventoried qPCR primer-probe assays (Table 11).

Table 11: Inventoried Applied Biosystems primer-probe assays

Gene Name	Applied Biosystem Assay Number
18S	Hs99999901_s1
c-myb	Mm00501741_m1
c-myc	Mm00487804_m1
Ctnnb1	Mm00483039_m1
Frat1	Mm00484502_s1
Gapdh	Mm99999915_g1
HoxA9	Mm00439364_m1
Hprt	Mm00446968_m1
Mef2c	Mm01340842_m1
Meis1	Mm00487664_m1
Msi2h	Mm00475180_m1
Siah1a	Mm00845411_s1
Siah2	Mm00486114_m1
Mouse ACTB (20x)	4352933E

The table shows a list of all Applied Biosystem qPCR assays used in this study.

2.21. Cytospin analysis

$3x10^4$ cells were washed in PBS, centrifuged at 300g for 5 minutes and cell pellets resuspended in 100µl PBS. The cell suspension was added dropwise to a cytospin funnel and centrifuged onto a slide at 35g for 5 minutes at low deceleration using a cytospin 3 machine (Shandon). The slides were then fixed and stained with Wright-Giemsa stain using a Hematek (Bayer Health Care) automated staining machine in the Haematology Department at Great Ormond Street Hospital, London.

2.22. Flow cytometry

2 ml of wash buffer was added to the cells (maximal $1x10^6$ per stain). Cells were centrifuged at 300g for 5 minutes at 4°C and resuspended in 100 µl stain buffer containing unlabelled anti-Fcγ III/II receptor mAb supernatant (2.4G2) for 10 to 30 minutes on ice, in the dark. Cells were washed with 2 ml wash buffer and centrifuged at 300g for 5 min at 4ºC. After that, the cells were stained in a total volume of 100 µl stain buffer containing the required dilution of the appropriate antibody for 30 minutes on ice in the dark. In some cases, cells were washed and either a streptavidin-conjugated antibody or 7AAD was added and incubated for 15 minutes on ice, in the dark. The cells were washed again with 2 ml wash buffer, centrifuged at 300g for 5 minutes at 4ºC and resuspended in 300 µl wash buffer. Biotin conjugated antibodies and antibodies conjugated to either phycoerythrin (PE), allophycocyanin (APC) or peridinin-chlorophyll protein complex (Per-CP) fluorochromes were used in this study (Table 12) (eBioscience and BD). Flow cytometry was performed using the Cyan ADP analyser and Summit 4.3 software (Beckman Coulter).

2.23. Cell Proliferation assay

The viability of cells after culture with different cytokine combinations (SCF, IL-3, IL-6 or GM-CSF) was measured using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit according to manufacturer's instructions (Promega). Depending on the proliferation rate of the cell line, triplicates of $1x10^3$ or $1x10^4$ cells were seeded per well. Cells were seeded in a 96 well flat bottomed plate in a total volume of 100 µl per well containing different combinations of growth factors.

Table 12: Antibodies used for flow cytometry

Antibody	Clone	Isotype	Working dilution
c-kit^{PE} (eBiosience)	2B8	IgG_{2b}, κ	1:200
Gr-1^{PE} (eBiosience)	RB6-8C5	IgG_{2b}, κ	1:200
Gr-1^{APC} (eBiosience)	RB6-8C5	IgG_{2b}, κ	1:200
Mac-1^{PE} (eBiosience)	M1/70	IgG_{2b}, κ	1:200
F4/80^{PE} (eBiosience)	BM8	IgG_{2a}, κ	1:200
CD45.2^{PE} (eBiosience)	104	IgG_{2a}, κ	1:200
CD45.2^{BIO} (eBiosience)	104	IgG_{2a}, κ	1:100
hCD2^{PE} (eBiosience)	RPA-2.10	IgG1, κ	1:25
IgG_{2a}, κ ^{PE} (eBiosience)	MOPC-173	N/A	1:200
IgG_{2b}, κ ^{PE} (eBiosience)	A-1	N/A	1:200
IgG1, κ ^{PE} (eBiosience)	MOPC-21	N/A	1:200
Streptavidin^{Per-CP} (BD)	N/A	N/A	1:250

The table shows all eBioscience or BD antibodies used for flow cytometry in this study.

Cells were cultured for three days and than stained with the CellTiter 96 AQ_{ueous} One Solution Reagent for 4 hours in the incubator. Absorbance was recorded at 490 nm using a 96 well plate reader (Model 680, BioRad). To yield correct absorbance values the average was calculated for triplicates and the average 490 nm absorbance from wells containing medium alone was subtracted from all other absorbance values.

2.24. Leukaemogenesis assay

Leukaemogenesis assays were performed by Dr. Owen Williams as described in the Appendix: "Acute myeloid leukemia induced by MLL-ENL is cured by oncogene ablation despite acquisition of complex genetic abnormalities", Methods.

2.25. Giemsa banding

10 mg/ml Colecemid (KaryoMAX) was added to the cell cultures for at least 1 hour. The cells were then treated with 75 mM potassium chloride (KCl, VWR), centrifuged at 300g for 5 minutes at 4°C and fixed using a methanol and acetic acid mixture (3:1). Fixed cells were dropped onto a glass slide and visualised by Giemsa banding (analysis of slides was then performed by Steve Chatters, Paediatric Malignancies Cytogenetics Unit, Institute of Child Health and Great Ormond Street Hospital).

2.26. Array-based comparative genomic hybridisation (aCGH)

Array-based CGH analysis of genomic DNA isolated from immortalised and leukaemic cells, was performed by Miltenyi Biotec using 4 x 44K Agilent Mouse Genome CGH Microarrays (Agilent Technologies).

2.27. Affymetrix Microarrays

Total RNA was extracted, using TRIzol reagent, from three conditional (MA1, MA3, MA4) and one constitutive (cMA3) MLL-AF9 immortalised cell lines

and also from three conditional (ME4, ME5, ME7) and one constitutive (cME3) MLL-ENL immortalised cell lines, each of them cultured without or with 2μg/ml Doxycycline for 48 hours. The concentration and quality of isolated RNA was determined using the NanoDrop ND-1000. Since the quality of RNA is essential for the success of microarray analysis, the RNA quality was verified by ensuring the A_{260} to A_{280} ratio was between 1.8 and 2.0 as well as using the Agilent 2100 Bioanalyzer.

All subsequent steps were performed by Kerra Pearce and Nipurna Jina (ICH Microarray Center, UCL Genomics) using Affymetrix reagents according to the manufacturer's instructions. In brief, 5 μg RNA was reverse transcribed to cDNA using a poly-dT primer linked to a T7 promoter sequence. This was followed by second strand cDNA synthesis using DNA polymerase I. Double-stranded cDNA was then purified and used for synthesis of biotin-labelled complementary RNA (cRNA) using T7 RNA polymerase and biotinylated ribonucleotide analogue mix. The biotinylated cRNA was then fragmented to between 35 bp and 200 bp and hybridised to GeneChip Mouse Genome 430.2 arrays (Affymetrix). After hybridisation the arrays were washed and stained with Streptavidin Phycoerythrin (SAPE). The signal was then amplified by addition of biotynilated anti-Streptavidin antibody and subsequent staining with SAPE. The arrays were washed and scanned at 570 nm and levels of fluorescence converted to numeric data as a measure of gene expression (Expression Manual, Affymetrix).

GeneChip microarrays consist of small DNA fragments (probe), chemically synthesized at specific locations on a coated quartz surface. A transcript, represented as a probe set, is made up of a perfect match (PM) and mismatch (MM) probe pair.

Eleven pairs (probe set) are selected from the 3' end of each gene and are used to measure the level of transcription. The mismatch probe contains a single mismatch located directly in the middle of the 25-base probe sequence. While the perfect match probe provides measurable fluorescence when the sample binds to it, the paired mismatch probe is used to detect and eliminate any false or contaminating fluorescence within that measurement. The mismatch probe serves as an internal control for its perfect match partner because it hybridizes to non-specific sequences about as effectively as its counterpart (Affymetrix Technical Support, 2001). The hybridisation intensity data from the GeneChip expression probe arrays was analysed with Affymetrix Microarray Suite version 5.0 (MAS 5.0). The Detection algorithm implemented in MAS 5.0 used the probe pair intensities to generate a Detection *p*-value and assign a present, marginal or absent call. The Detection *p*-value for a probe set was calculated in two steps. The first step calculates the probe pair ability to detect its transcript (Discrimination score R) for all 11 probe pairs of each probe set. This is described by the transcript specific intensity difference between PM and MM relative to its overall hybridisation intensity (R = (PM-MM)/(PM+MM)). The second step compared each Discrimination score to a defined threshold τ (0.015 by default). Discrimination scores higher than τ indicate present transcripts and Discrimination scores lower than τ indicate absent transcripts. All Discrimination score to τ comparisons are summarised as a Detection *p*-value. The higher the Discrimination scores are above τ, the smaller the p-value and the more likely the transcript is present. Cut-offs were defined to define the Detection Calls present (Detection p-value < 0.04), marginal (0.04 < Detection *p*-value < 0.07) and absent (Detection *p*-value > 0.07) calls (Statistical Algorithms Reference Guide, Affymetrix).

2.28. Short hairpin RNA cloning and knockdown

Short hairpin RNA (shRNA) was expressed as sequences embedded in the miR30 microRNA sequence (Stegmeier *et al*., 2005). For each gene of interest three hairpin oligonucleotides were designed against the sense strand of the target gene, using either the katahdin prediction software (http://katahdin.cshl.org:9331/siRNA/RNAi.cgi?type=shRNA) or sequences published on the RNAi codex web site (http://codex.cshl.edu). All hairpin oligos used in this study are listed in the Appendix, Table 21. shRNA were cloned into pMSCV-hCD2tailless-miR30 (Figure 13) according to published protocol (Paddison *et al.*, Nature Methods, Vol.1 No 2, 163-167, Nov 2004). In brief, the target gene-specific oligonucleotides were designed to incorporate part of the miR30 microRNA (miRNA) sense and antisense sequences (sense: 5'-TGCTGTTGACAGTGAGCG-3', antisense: 5'-TCCGAGGCAGTAGGCA-3'). The template oligonucleotide was then amplified by PCR reaction, incorporating EcoRI and XhoI restriction enzyme sites (miRNA forward: 5'-GATGGCTGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG-3', miRNA reverse: 5'-GTCTAGAGGAATTCCGAGGCAGTAGGCA-3') and cloned into pMSCV-hCD2tailless-miR30 using EcoRI and XhoI. In this vector expression system human CD2 (hCD2) tailless (lacking part of the intracellular domains) cDNA is fused to the miR30 sequence. Therefore hCD2 is expressed from the same mRNA that gives rise to the cloned shRNA. The cloned shRNA construct was then transformed using Subcloning Efficiency DH5αCompetent Cells (Invitrogen) and sequenced using GATC Biotech sequencing services (www.gatc-biotech.com).

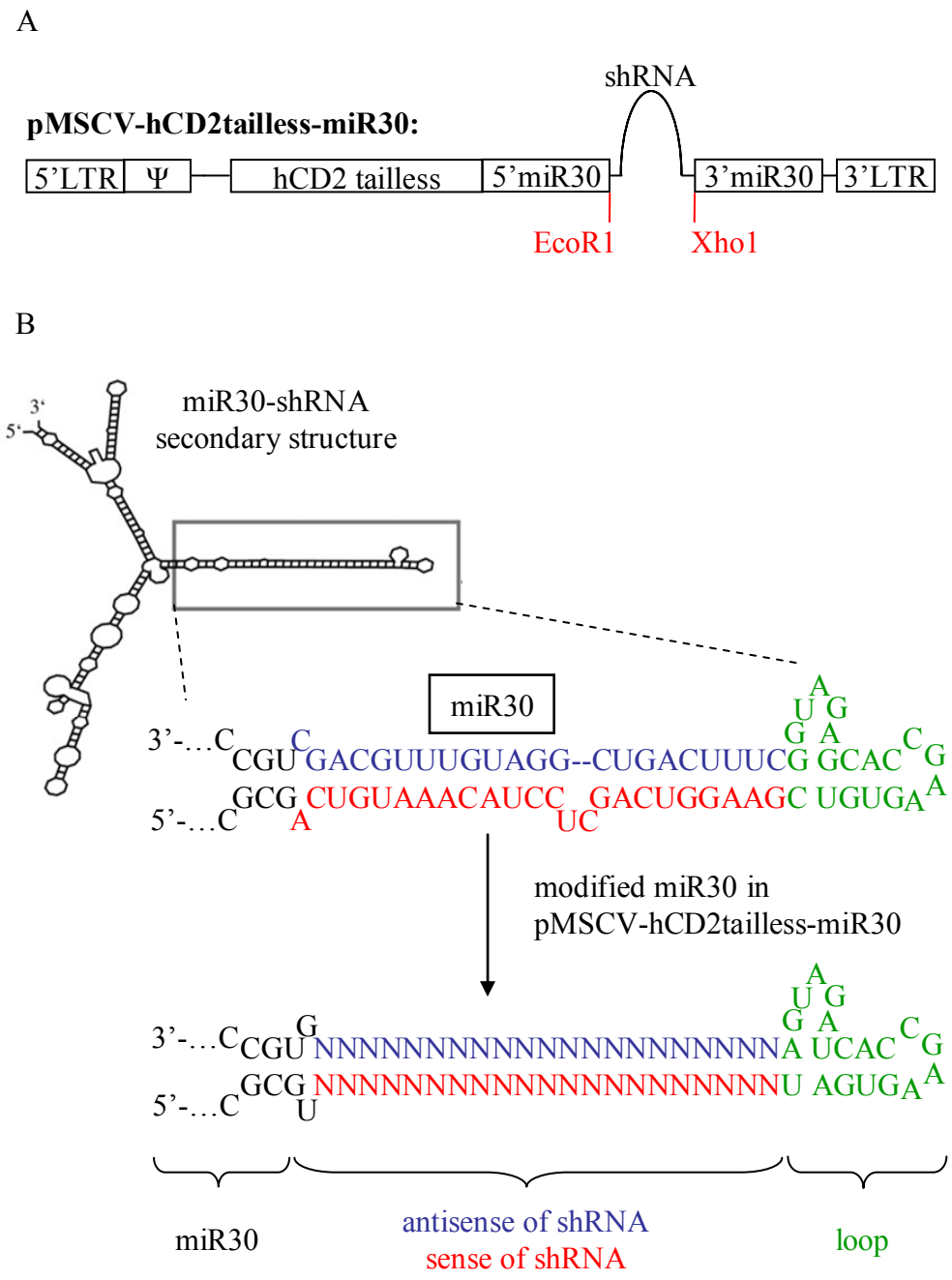


Figure 13: Retroviral construct used for shRNA gene knockdown

A) Restriction enzyme sides used for cloning the shRNA into pMSCV-hCD2tailless-miR30 are shown in red. B) Schematic representation of secondary structure fold of the miR30 based shRNA (adapted from Stegmeier *et al*., 2005). The mature miR30 miRNA region was replaced with the relevant gene of interest-specific shRNA (N = any nucleotide).

LinXE ecotropic retrovirus packaging cells were transfected with the pMSCV-hCD2tailless-miR30 shRNA vector as described earlier and MLL-AF9 and MLL-ENL immortalised cell lines were infected once with retroviral supernatants, also as described earlier. On the following day 100 µl of fresh RPMI complete medium supplemented with 100 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6 was added to the cells. The cells were replated and analysed on the flow cytometer every two to three days for expression of hCD2 as an indicator for the infection efficiency, after 48 hours, and after that as an indicator for the proportion of transduced cells in the culture. Cells were also analysed on the flow cytometer for expression of Gr-1 as an indicator for the state of differentiation. Cells were sorted by magnetic activated cell sorting for isolation of RNA. Cells were labelled using 100 µl MACS buffer per $1x10^7$ cells and hCD2-PE-conjugated antibody (1:25). The mixture was incubated for 10 minutes in the dark at 4°C. Then the cells were magnetically labelled using 20 µl per $1x10^7$ cells of an anti-PE monoclonal antibody conjugated to MicroBeads. A MACS MS column was used to retain the magnetically labelled hCD2 positive cells in the magnetic field of the MACS separator.

2.29. Tet-off system

Tetracycline controlled transcriptional activation is a stable method of expression, where transcription is turned on (Tet-on) or off (Tet-off) in the presence of the antibiotic Tetracycline or one of its derivatives, e.g. Doxycycline (Gossen and Bujard, 1992). In this study, MLL-AF9 or MLL-ENL expression is driven by the tetracycline-responsive promoter element (TRE). In the cotransduced cells, this expression depends on tetracycline-controlled transactivator protein ($tTA2^s$, Urlinger *et al.*, 2000) binding. In the absence of Doxycycline, $tTA2^s$ binds to the TRE and

allows transcription of the MLL-fusion cDNA (Figure 14). When Doxycycline is added to the system, it binds to $tTA2^s$ causing a conformational change. Therefore $tTA2^s$ is no longer able to bind to the TRE and can not activate transcription of MLL-AF9 cDNA.

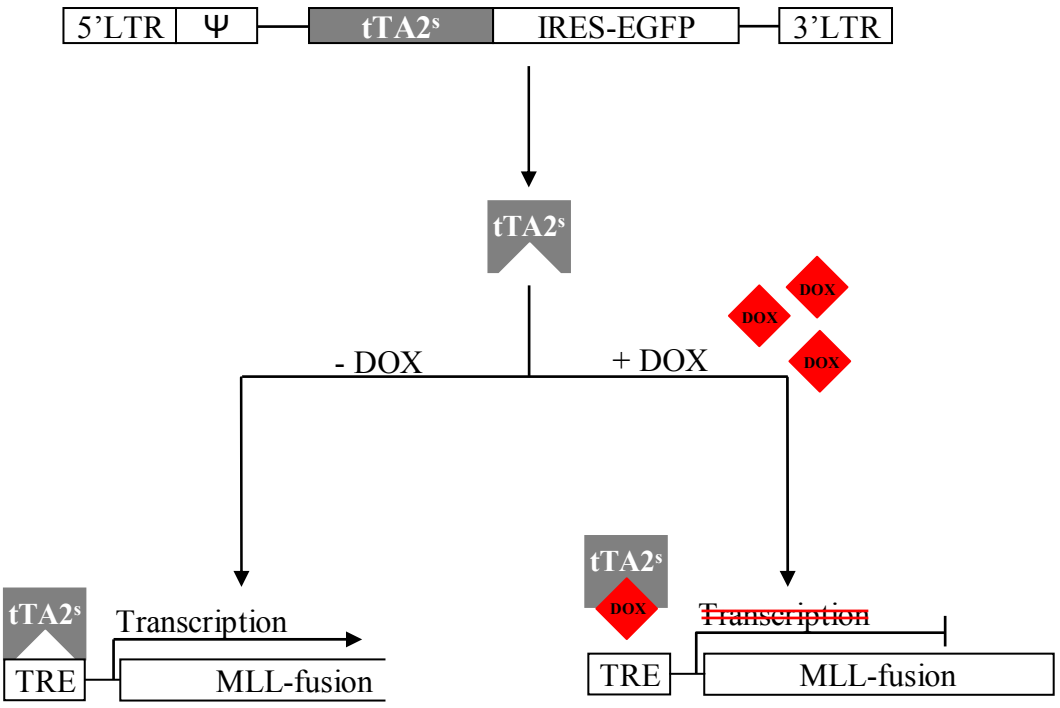


Figure 14: Tet-off expression system

In this system, MLL-AF9 expression is driven by the tetracycline-responsive promoter element (TRE). In the cotransduced cells this expression depends on tetracycline-controlled transactivator protein (tTA2^s) binding. In the absence of Doxycycline (DOX), tTA2^s protein binds to TRE and activates transcription of the MLL-AF9 cDNA. Upon addition of Doxycycline the antibiotic binds to the tTA2^s protein causing a conformational change. As a result the tTA2^s protein is blocked from binding to TRE and activation of transcription of MLL-AF9 cDNA is inhibited (adapted from Gossen and Bujard, 1992).

RESULTS

CHAPTER III - Generation and characterisation of immortalised cell lines

In order to generate conditional MLL-AF9 immortalised cells, retroviral delivery of the Tet-off expression system was used to conditionally express the MLL-AF9 fusion oncogene in primary haematopoietic progenitor cells. Serial replating of transduced HPC caused an outgrowth of immortalised cells with constitutive or conditional expression of the MLL-AF9 fusion oncogene. Immortalised cells were subsequently plated into liquid culture to generate cell lines. In total, eight independent cell lines with conditional MLL-AF9 expression and three independent cell lines with constitutive MLL-AF9 expression were generated. Five of the conditional and one of the constitutive immortalised cell lines were further characterised to determine the tTA dependent MLL-AF9 expression, morphology, immunophenotype and cytokine requirements of the cells. Furthermore, the response of immortalised cell lines with conditional MLL-AF9 expression to Doxycycline, was studied and compared to that of cells with constitutive MLL-AF9 expression.

3.1. Generating immortalised HPC with conditional full-length MLL-AF9 expression in methylcellulose

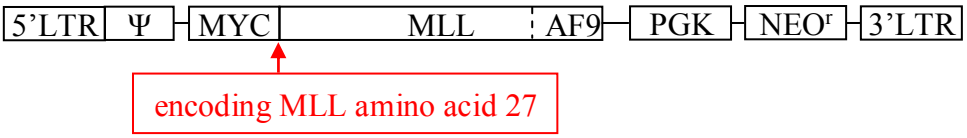
MLL fusion proteins interact with menin through multiple domains within the N-terminus, including a high-affinity N-terminal conserved binding motif (RXRFP) mapped to amino acids six to ten. It was shown that this high-affinity menin interaction domain is required for induction and maintenance of myeloid progenitor immortalisation by the MLL-fusion proteins, MLL-ENL, MLL-GAS7 and MLL-AF10 (Yokoyama *et al.,* 2005).

In previous studies in our laboratory, a myc-tagged conditional MLL-ENL expression vector, missing the high-affinity menin binding motif, has been successfully used to immortalise HPC (Horton *et al.*, 2005). The pMSCV-neo-mod-mMLLAF9 (mycMLL-AF9) vector with constitutive expression of MLL-AF9 also contains a sequence encoding a myc epitope instead of the first 27 amino acids of MLL (Figure 15 A). Therefore, this MLL-AF9 expression vector does not contain the region encoding the high-affinity menin binding motif (amino acids six to ten). Myeloid progenitors were transduced with the constitutive mycMLL-AF9 vector, to test if this construct could immortalise HPC. However, myeloid progenitors transduced with mycMLLAF9 exhausted their clonogenic activity after the second round of plating in methylcellulose culture, as did cells transduced with the empty pMSCV-neo vector (Figure 15 B). Therefore, the mycMLL-AF9 vector was not capable of immortalising HPC. In order to overcome this problem, an alternative vector, the pMSCV-neo-mod-fMLLAF9 (flagMLL-AF9) vector, was made. This vector drives constitutive expression of full length MLL-AF9 (Figure 16 A). HPC transduced with flagMLLAF9 formed large compact colonies in methylcellulose cultures, through at least three rounds of plating and gave rise to immortalised cell lines (Figure 16 B and C). This data suggested that the full-length MLL-AF9 protein, containing the high-affinity menin binding motif, is required for immortalisation of HPC.

For all further experiments, new conditional expression vectors with full length MLL-AF9 and MLL-ENL expression were generated (for cloning details see Materials and Methods, section 2.5).

A)

pMSCV-neo-mod-mMLLAF9:



B)

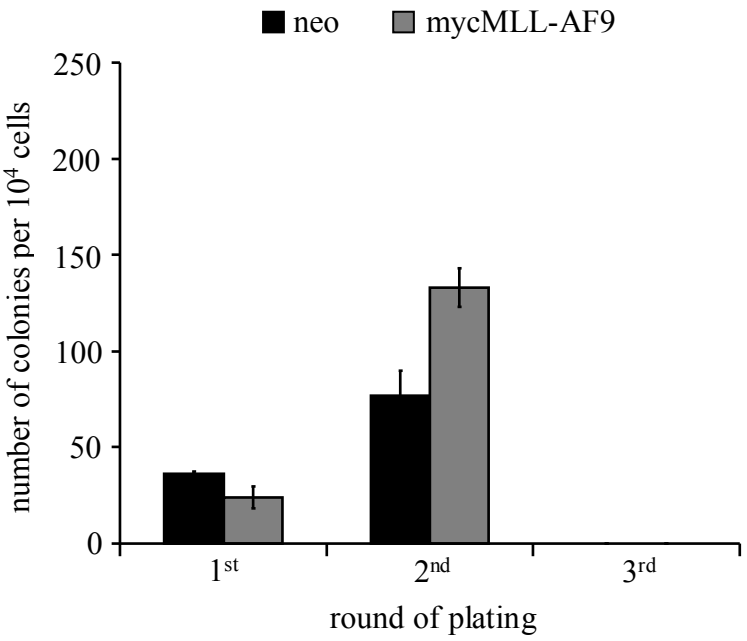


Figure 15: Constitutive expression of MLL-AF9 lacking the menin high-affinity binding motif does not immortalise HPC

HPC were transduced with the constitutive truncated MLL-AF9 expression construct or the empty neo vector as a control. 24 hours after transduction the cells were plated in methylcellulose culture with G418 selection. After 7 days the numbers of colonies were counted and 1x10^4 cells were plated without G418 selection into subsequent rounds. A) Schematic diagram of the constitutive truncated MLL expression construct: mycMLL-AF9. B) The graph shows the number of colonies after each round of plating. Black bars: empty vector, neo; grey bars: mycMLL-AF9. Error bars represent standard deviations of duplicate plates. Similar results were observed in four independent experiments.

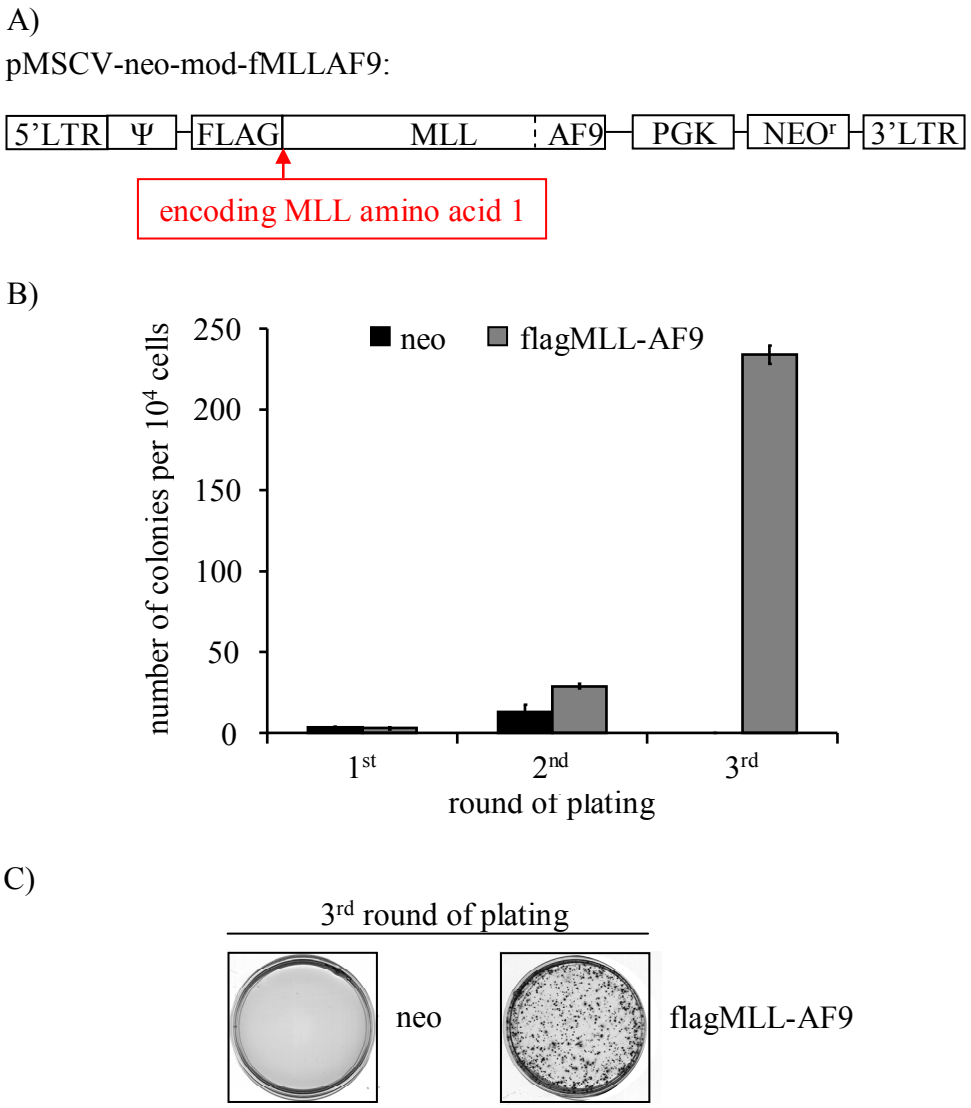


Figure 16: Constitutive expression of full length MLL-AF9 immortalises HPC

A) Schematic diagram of the constitutive full-length MLL expression construct: flagMLL-AF9. B) HPC were transduced with the constitutive full-length MLL expression construct (flagMLL-AF9, grey) or the empty neo vector as a control (neo, black). 24 hours after transduction the cells were plated in methylcellulose culture with G418 selection. After 7 days the number of colonies were counted and 1x10^4 cells were plated without G418 selection into subsequent rounds. The graph shows the number of colonies after each round of plating. Error bars represent standard deviations of duplicate plates. C) *p*-iodonitrotetrazolium stains of the third round methylcellulose cultures. Similar results were observed in seven independent experiments.

Retroviral delivery of the Tet-off expression system was used to conditionally express the MLL-AF9 or MLL-ENL oncogene, as done in previous experiments in our laboratory with the MLL-ENL oncogene (Horton *et al.*, 2005). In this system, MLL-AF9 or MLL-ENL expression is driven by the tetracycline-responsive promoter element (TRE). In the cotransduced cells, this expression depends on tetracycline-controlled transactivator protein (tTA) binding. Addition of Doxycycline to the system causes inhibition of the MLL-fusion cDNA transcription (Figure 14).

24 hours after purification, lineage negative HPC were cotransduced with unconcentrated pMSCV-neo-TRE-fMLLAF9 and ten times concentrated pMSCV-tTA2^{s}-IRES-EGFP retroviral supernatants. As a control, cells were transduced with unconcentrated pMSCV-neo-TRE-fMLLAF9 retroviral supernatant alone. In all experiments, cells cotransduced with pMSCV-neo-TRE-fMLLAF9 and pMSCV-tTA2^{s}-IRES-EGFP formed large compact colonies throughout five rounds of plating in methylcellulose cultures. Cells transduced with pMSCV-neo-TRE-fMLLAF9 alone exhausted their clonogenic activity after the second round of plating (Figure 17).

In order to examine the effect of Doxycycline on conditionally immortalised (pMSCV-neo-TRE-fMLLAF9 and pMSCV-tTA2^{s}-IRES-EGFP) cells, 2 μg/ml Doxycycline was added to the methylcellulose cultures in round four. In the absence of Doxycycline, the cells formed large compact colonies, which were composed of immature cells with a myeloblastic phenotype (Figure 18 A). The presence of Doxycycline caused a significant reduction in the number of colonies formed by cells conditionally immortalised with MLL-AF9 (Figure 18 B).

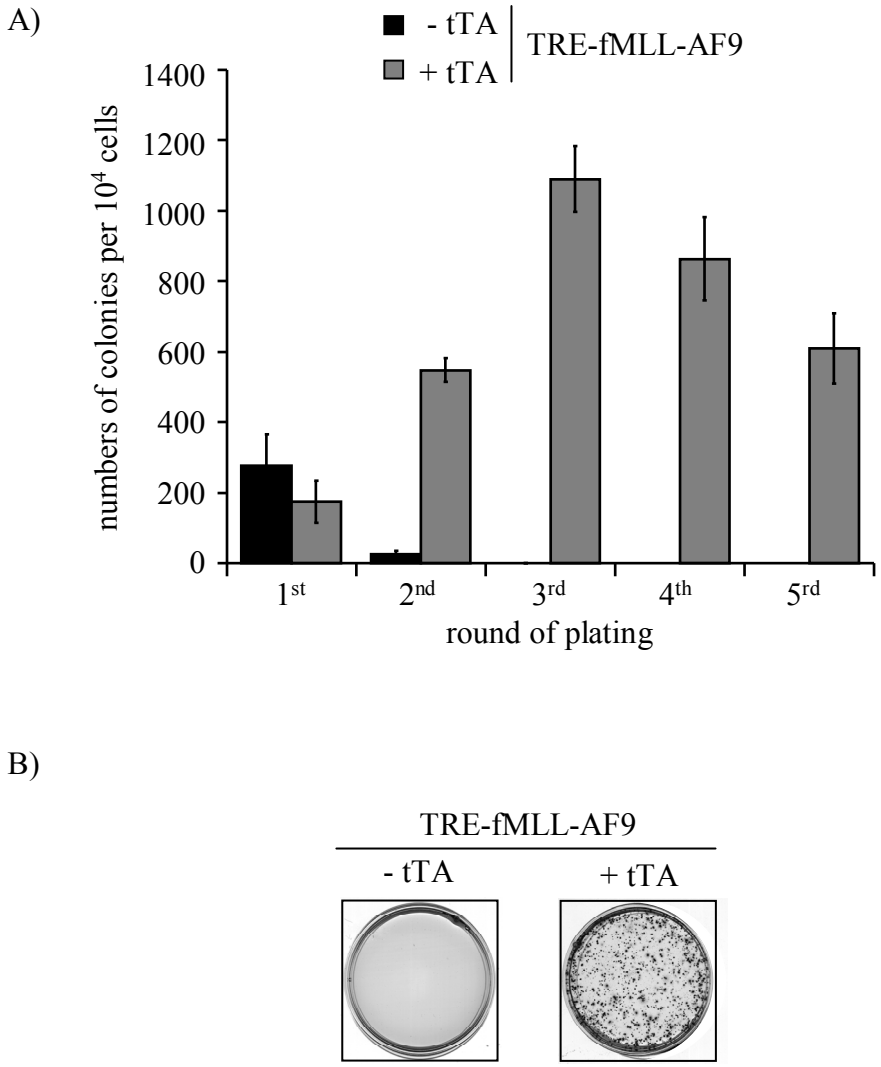


Figure 17: Conditional expression of MLL-AF9 immortalises HPC

HPC were co-transduced with the conditional MLL-AF9 expression construct (pMSCV-neo-TRE-fMLLAF9, unconcentrated virus) and the inducer tTA (pMSCV-tTA2s-IRES-EGFP, 10 x concentrated virus). As a control HPC were transduced with pMSCV-neo-TRE-fMLLAF9 alone. A) The graph shows the number of colonies after each round of plating. pMSCV-neo-TRE-fMLLAF9 + tTA (grey) and pMSCV-neo-TRE-fMLLAF9 alone (black). B) *p*-iodonitrotetrazolium stains of the third round methylcellulose cultures. Similar results were observed in eight independent experiments.

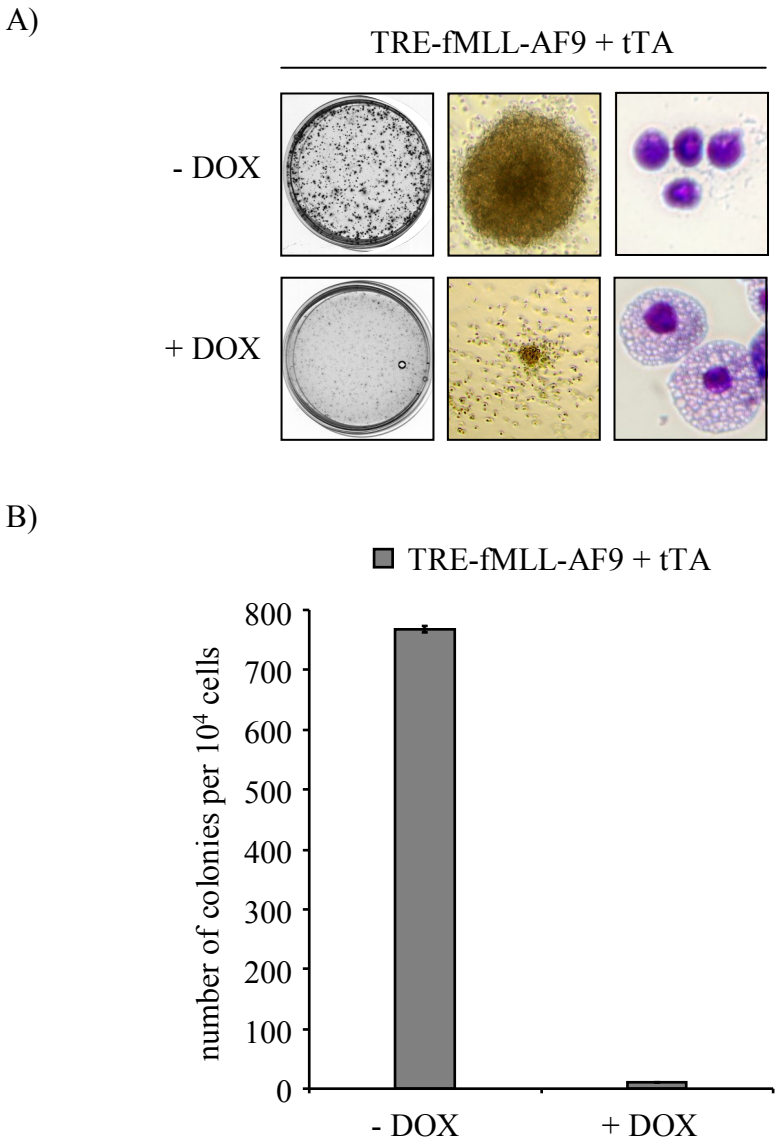


Figure 18: Effect of Doxycycline on MLL-AF9 immortalised cells

A) *p*-iodonitrotetrazolium stains of methylcellulose cultures, morphology of colonies (original magnification x 40) and morphology of cells (cytospin preparation followed by Wright's staining, original magnification x 400) in the fourth round methylcellulose cultures in the presence or absence of 2µg/ml Doxycycline for 5 days. B) The graph shows the number of colonies of immortalised HPC with conditional expression of MLL-AF9 cultured in the presence or absence of 2µg/ml Doxycycline for 5 days. Similar results were observed in five independent experiments.

The colonies that did form were composed of small clusters of cells displaying macrophage cytological features (Figure 18 A). This data suggested that HPC with conditional expression of MLL-AF9 were successfully immortalised in methylcellulose culture.

3.2. Generation and characterisation of immortalised myeloid cell lines with conditional or constitutive MLL-AF9 expression

Cell lines were established by plating the immortalised HPC in liquid culture after three to five rounds of replating in methylcellulose cultures. In total, eight conditional and three constitutive cell lines were maintained in RPMI medium supplemented with 100 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6. Due to their similar rate of proliferation the conditional cell lines MA1, MA2, MA3, MA4, MA6 and the constitutive cell line cMA3 were picked for further characterisation (Figure 19). All cell lines stably proliferated *in vitro* and could be maintained indefinitely in liquid culture. The conditional cell lines MA5, MA7 and MA8 could also be maintained indefinitely in liquid culture but showed a slower proliferation rate.

Having generated immortalised myeloid cell lines with constitutive and conditional MLL-AF9 expression, it was necessary to firstly confirm the MLL-AF9 protein expression in the cell lines and secondly test that this expression was conditional, by addition of 2μg/ml Doxycycline to the cultures. The conditional cell line MA1 and the constitutive cell line cMA3 were maintained in the presence and absence of Doxycycline for 48 hours and then harvested and cell pellets lysed for Western blot analysis (Figure 20).

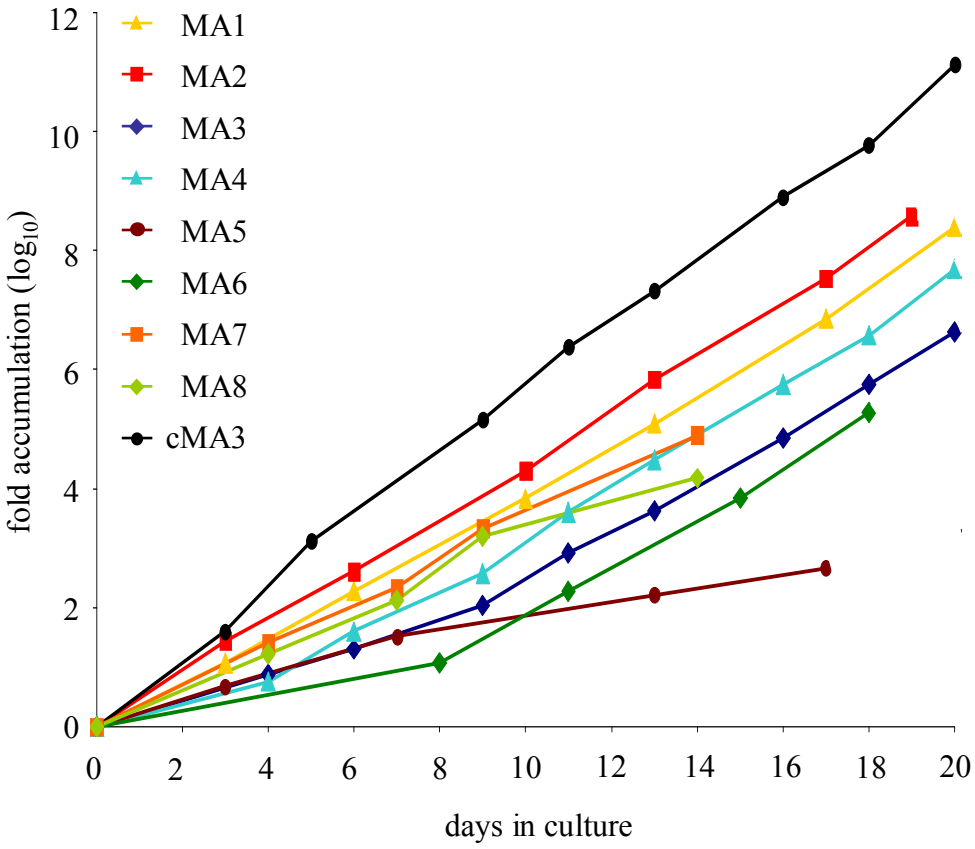


Figure 19: Generation of MLL-AF9 immortalized cell lines in liquid culture

The graph shows the fold accumulation in cell number, displayed as $\log_{10}$, of the conditional cell lines MA1 (yellow triangles), MA2 (red squares), MA3 (dark blue diamonds), MA4 (light blue triangles), MA5 (brown circles), MA6 (dark green diamonds), MA7 (orange squares), MA8 (light green diamonds) and of the constitutive cell line cMA3 (black circles). Similar results were observed in four independent experiments.

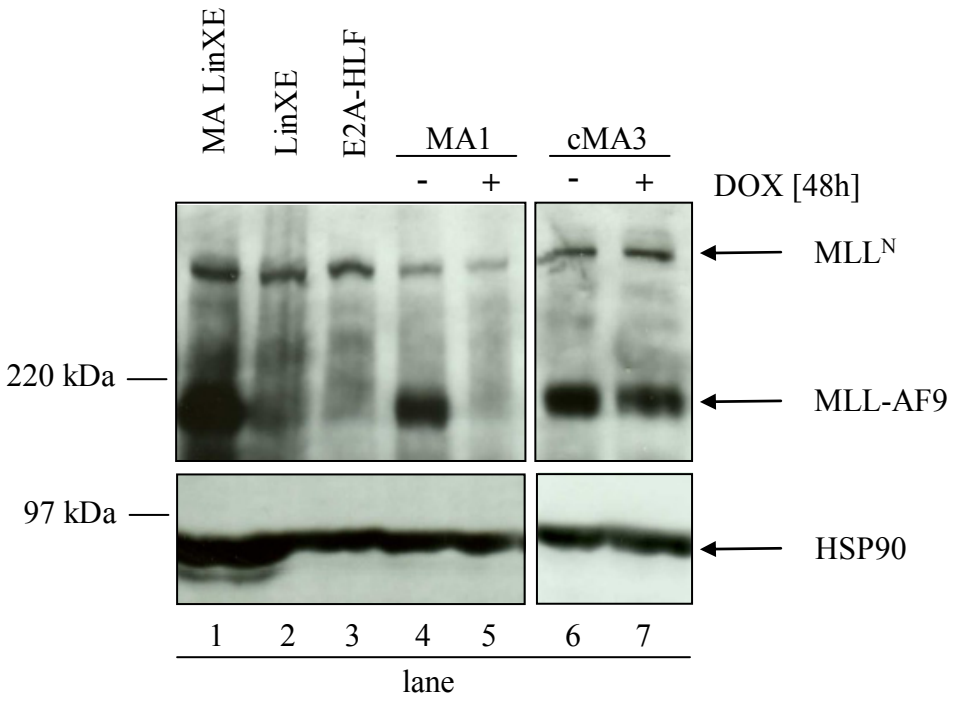


Figure 20: Detection of MLL-AF9 protein expression

The figure shows Western blot analysis of protein extracts from immortalized conditional (MA1) and constitutive (cMA3) cells. Cells were maintained for 48 hours with or without Doxycycline and then lysed using 2xDTT sample buffer. Protein lysates from LinXE cells transfected with the constitutive retroviral MLL-AF9 construct were used as a positive control (lane 1). As a negative control, protein lysates from untransfected LinXE cells (lane 2) and lysates from E2A-HLF immortalised myeloid cells (lane 3) were used. MA1 – DOX (lane 4), MA1 + DOX (lane 5), cMA3 –DOX (lane 6) and cMA3 + DOX (lane 7). Anti-MLL^N/HRX (clone N4.4) antibody was used to detect the endogenous N-terminal MLL fragment and the MLL-AF9 fusion protein. An anti-HSP90α/β antibody was used to control for protein loading. Proteins were resolved on a 5% resolving gel. Similar results were observed in four independent experiments.

Protein extract made from LinXE cells transfected with the constitutive retroviral MLL-AF9 vector, was used as a positive control. Untransfected LinXE cells and an E2A-HLF immortalised mouse myeloid cell line were used as negative controls (the E2A-HLF immortalised mouse myeloid cell line was kindly provided by Dr. Ramya Ramunjachar of the MHCB Unit). The anti-MLL^N/HRX (clone N4.4) antibody was used to detect MLL-AF9. The anti-MLL-N4.4 monoclonal antibody was raised against amino acid residues 161-356 of human MLL, and therefore recognises the N-terminal proteolytic fragment of MLL (~320 kDa), of both human and mouse.

MLL-AF9 was not detected in the untransfected LinXE cells (lane 2) and the E2A-HLF myeloid cell line (lane 3) (Figure 20). As expected, a 170 kDa band corresponding to full-length MLL-AF9 was detected in MA1 (lane 4) and cMA3 (lane 6) cells cultured without Doxycycline as well as in the MLL-AF9 transfected LinXE cells (lane 1). However, it was not detected in the conditional MA1 cells maintained in the presence of Doxycycline (lane 5). There was a small effect on MLL-AF9 protein expression by Doxycycline in the constitutive cells cMA3 (lane 7). An anti-HSP90α/β antibody was used to control for protein loading. The cell lines were then treated with Doxycycline for different periods of time, to further examine the effect of Doxycycline on MLL-AF9 protein expression. Conditionally immortalised MA1 cells and constitutively immortalised cMA3 cells were maintained in the absence and the presence of Doxycycline for two, four, eight and 16 hours, and cell pellets were then lysed for Western blot analysis. A significant down-regulation of MLL-AF9 protein expression was detected in conditional MA1 cells cultured in the presence of Doxycycline for 16 hours (Figure 21 A).

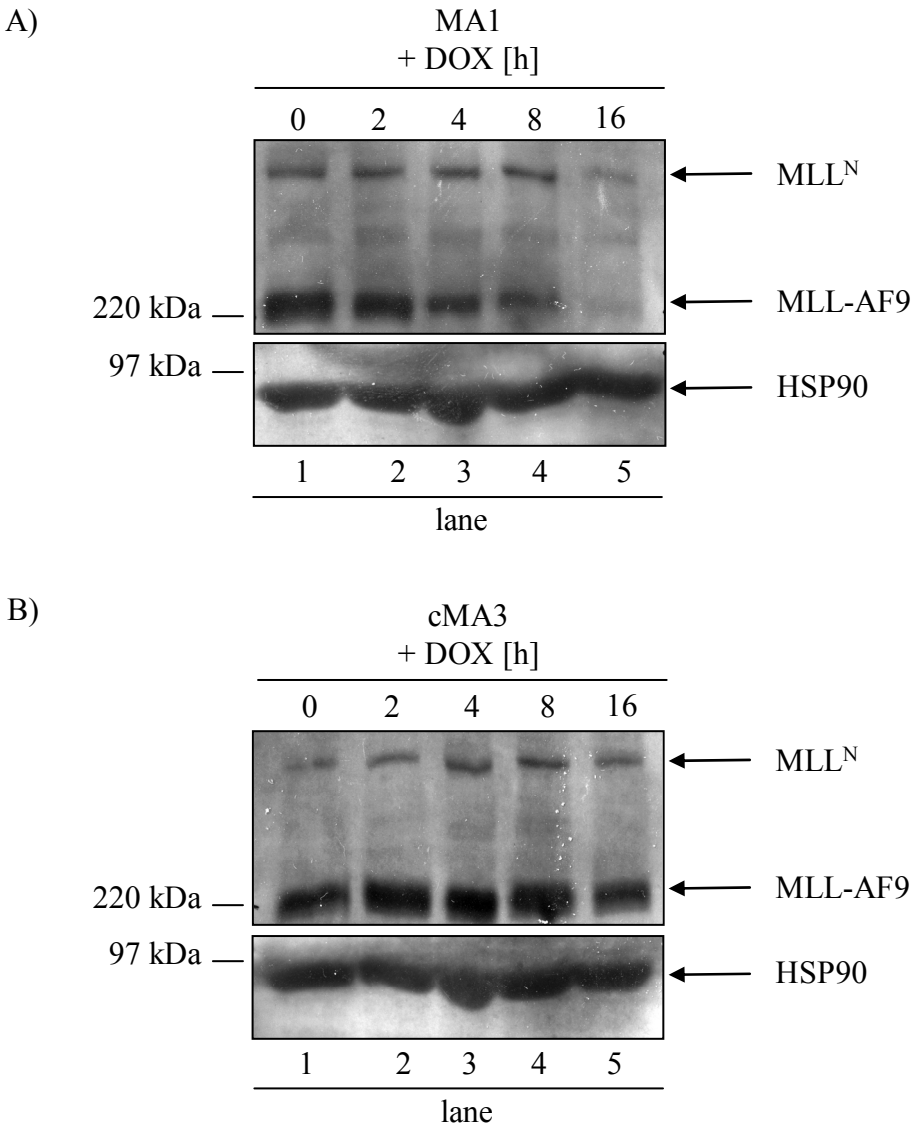


Figure 21: Detection of MLL-AF9 protein expression following a time course of Doxycycline treatment

The figure shows Western blot analysis of protein extracts from immortalized conditional MA1 (panel A) and constitutive cMA3 (panel B) cells. Cells were maintained for 0 hours (lane 1), 2 hours (lane 2), 4 hours (lane 3), 8 hours (lane 4) and 16 hours (lane 5) with Doxycycline and then lysed. Anti-MLL^N/HRX, clone N4.4 antibody was used to detect endogenous N-terminal MLL fragment and MLL-AF9 fusion protein. anti-HSP90α/β antibody was used as the protein loading control. Proteins were resolved on a 5% resolving gel. Similar results were observed in two independent experiments.

Addition of Doxycycline to the cultures of the constitutive cMA3 cells slightly reduced MLL-AF9 protein expression (Figure 21 B).

The effect of Doxycycline on *MLL-AF9* mRNA expression was examined by qPCR. A specific primer and probe set spanning the MLL-AF9 breakpoint region was designed and optimised to measure the relative expression levels of *MLL-AF9* (Figure 22). Total RNA was isolated from all cell lines maintained with or without Doxycycline for 48 hours and used for qPCR analysis. *MLL-AF9* expression was much lower in the conditional cell lines MA2 and MA6, compared to the other analysed cell lines. In the presence of Doxycycline, *MLL-AF9* expression was down-regulated in all five conditional cell lines. As expected, treatment with Doxycycline did not have an effect on *MLL-AF9* mRNA expression in the cMA3 cells. However, *MLL-AF9* appeared to be down-regulated to a greater degree at the protein than mRNA level. This is most likely due to the way the pMSCV-neo-TRE-fMLLAF9 vector is designed. In this vector, *MLL-AF9* expression is driven independently of the LTR by the tTA dependent TRE promoter. However, the qPCR assay will detect both the LTR driven mRNA, from which neomycin phosphotransferase but not MLL-AF9 is translated, and the independent, TRE driven MLL-AF9 mRNA, from which MLL-AF9 is translated.

In order to characterise the phenotype and morphology of the immortalised MLL-AF9 cells before and after turning off the expression of MLL-AF9, viable cell counts, flow cytometric analysis of cell surface markers and Wright-Giemsa stains of cytospin preparations, were performed. Cells were cultured in 2µg/ml Doxycycline for seven days and viable cells were counted using Trypan Blue exclusion after two, four and seven days (Figure 23).

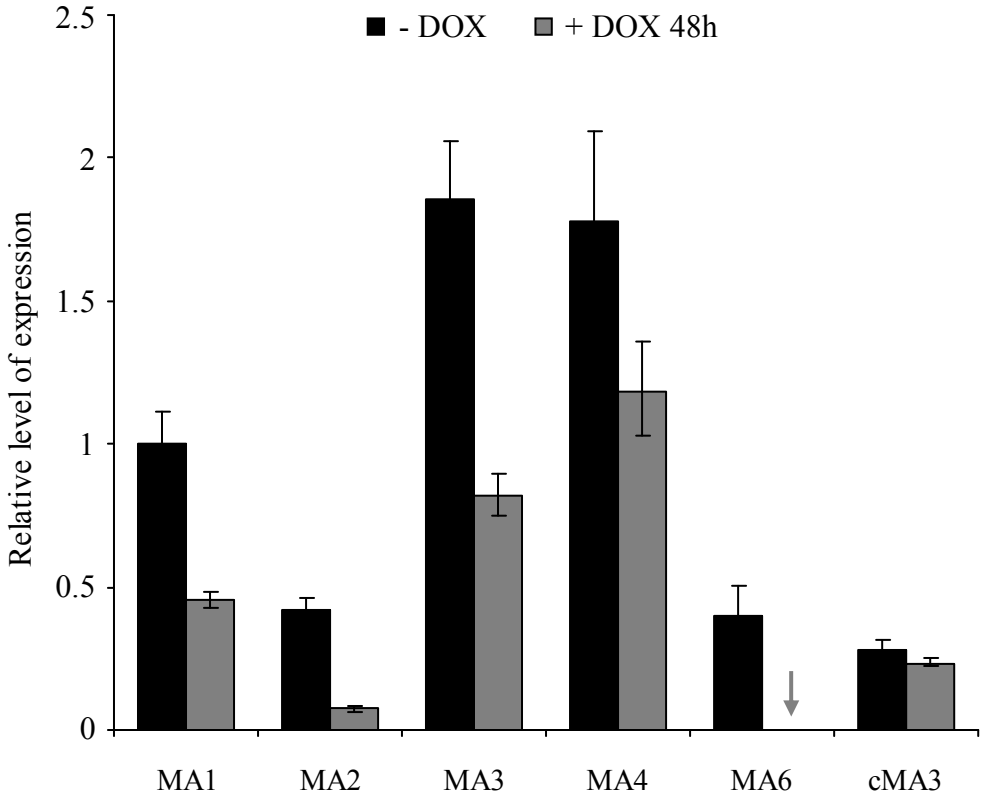


Figure 22: Relative MLL-AF9 mRNA expression in the presence or absence of Doxycycline

The graph shows the relative level of MLL-AF9 mRNA expression in immortalised MA1, MA2, MA3, MA4, MA6 and cMA3 cells after treatment (grey bars or indicated with arrow) or not (black bars) for 48 hours with 2µg/ml Doxycycline. Columns represent the mean of triplicate measurements, error bars represent the standard deviations. Similar results were observed in three independent experiments.

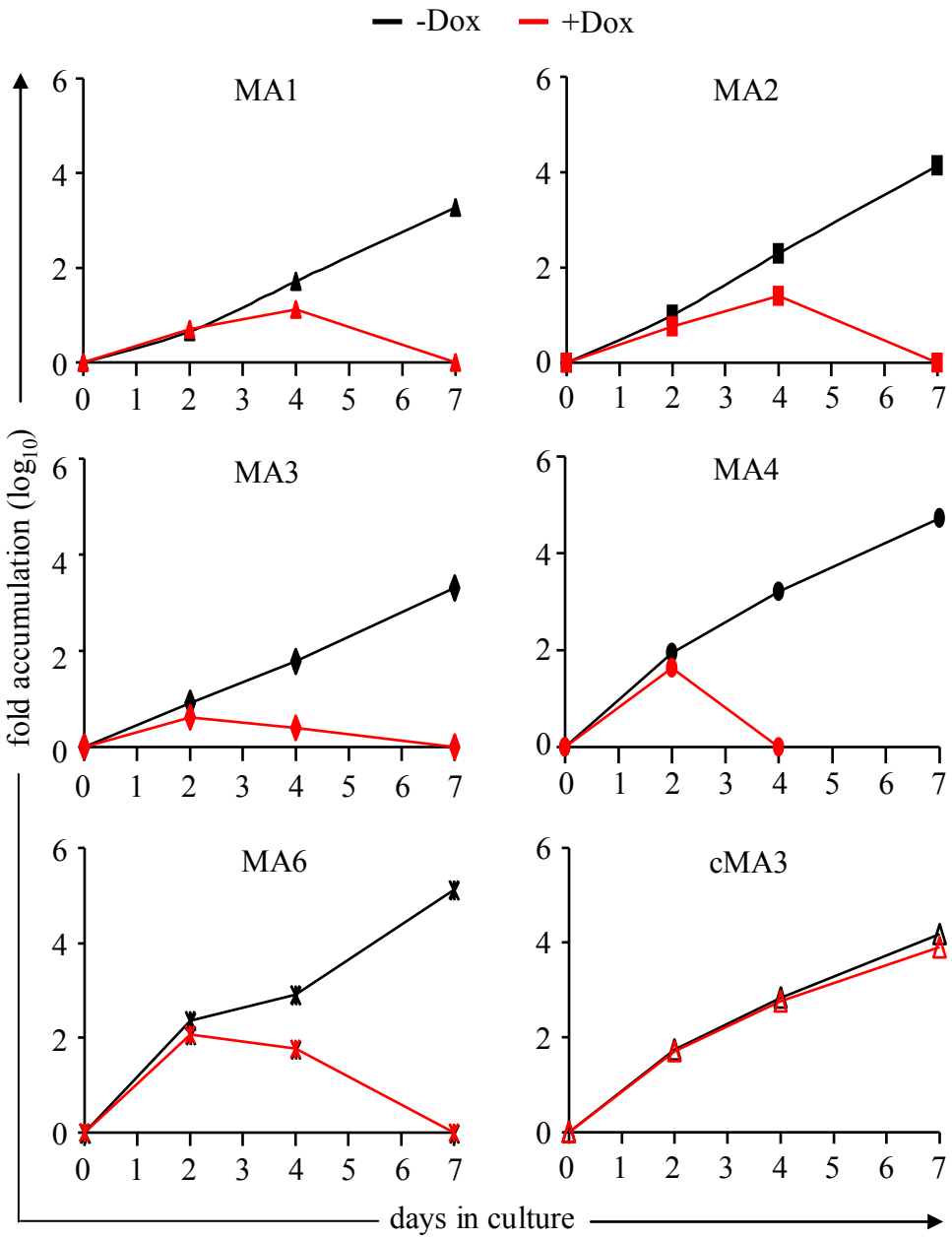


Figure 23: Effect of Doxycycline on cell accumulation

The graph shows the growth curves of immortalised cells MA1 (triangles), MA2 (squares), MA3 (diamonds), MA4 (circles), MA6 (stars) and cMA3 (open triangles) after treatment with 2 µg/ml Doxycycline. Viable cells were counted using Trypan Blue exclusion after 2, 4 and 7 days. The graph shows the $\log_{10}$ of the accumulation in cell numbers of cells treated with 2µg/ml Doxycycline (red) or untreated cells (black). Similar results were observed in three independent experiments.

After two days, the number of cells counted in cultures with or without Doxycycline was similar. By day four, the proliferation rate of cells in the presence of Doxycycline decreased and a decrease in cell numbers was observed. By day seven, all conditional cells cultured in the presence of Doxycycline died. MA4 cells had already terminally differentiated by day four. As expected, culture in the presence of Doxycycline did not have an effect on the proliferation capacity of cells with constitutive expression of MLL-AF9. Four days after addition of Doxycycline, the cells were analysed for the expression of the myeloid cell surface antigens Gr-1 and Mac-1, as well as the HPC surface marker c-kit, by flow cytometry (Figure 24). All cells maintained without Doxycycline had a characteristic immature myeloid phenotype, being c-kit^{+}Gr-1^{+}Mac-1^{hi}. Analysis by Wright-Giemsa stained cytospin preparations of these cells showed that the cells displayed immature myeloblastic morphology (Figure 25). Following four days in the presence of Doxycycline, most of the conditionally immortalised cells had terminally differentiated into neutrophils, as identified by their lobular shaped nuclei (Figure 25). Furthermore, a significant increase in Gr-1 expression and a decrease in c-kit expression were observed in all conditional cell lines (Figure 24). This c-kit^{-}Gr-1^{hi}Mac-1^{hi} phenotype is associated with a more differentiated myeloid phenotype. The conditional cell line MA4 differentiated more rapidly into neutrophils than the others and was therefore analysed after three days. As expected the constitutively immortalised cells did not differentiate in the presence of Doxycycline. They maintained the myeloblastic morphology and immature c-kit^{+}Gr-1^{+}Mac-1^{hi} phenotype (Figure 24 and Figure 25).

To determine the cytokine requirements of the cell lines, cell proliferation assays were carried out. Data for the representative conditional MA1 and constitutive cMA3 cell lines are shown in Figure 26.

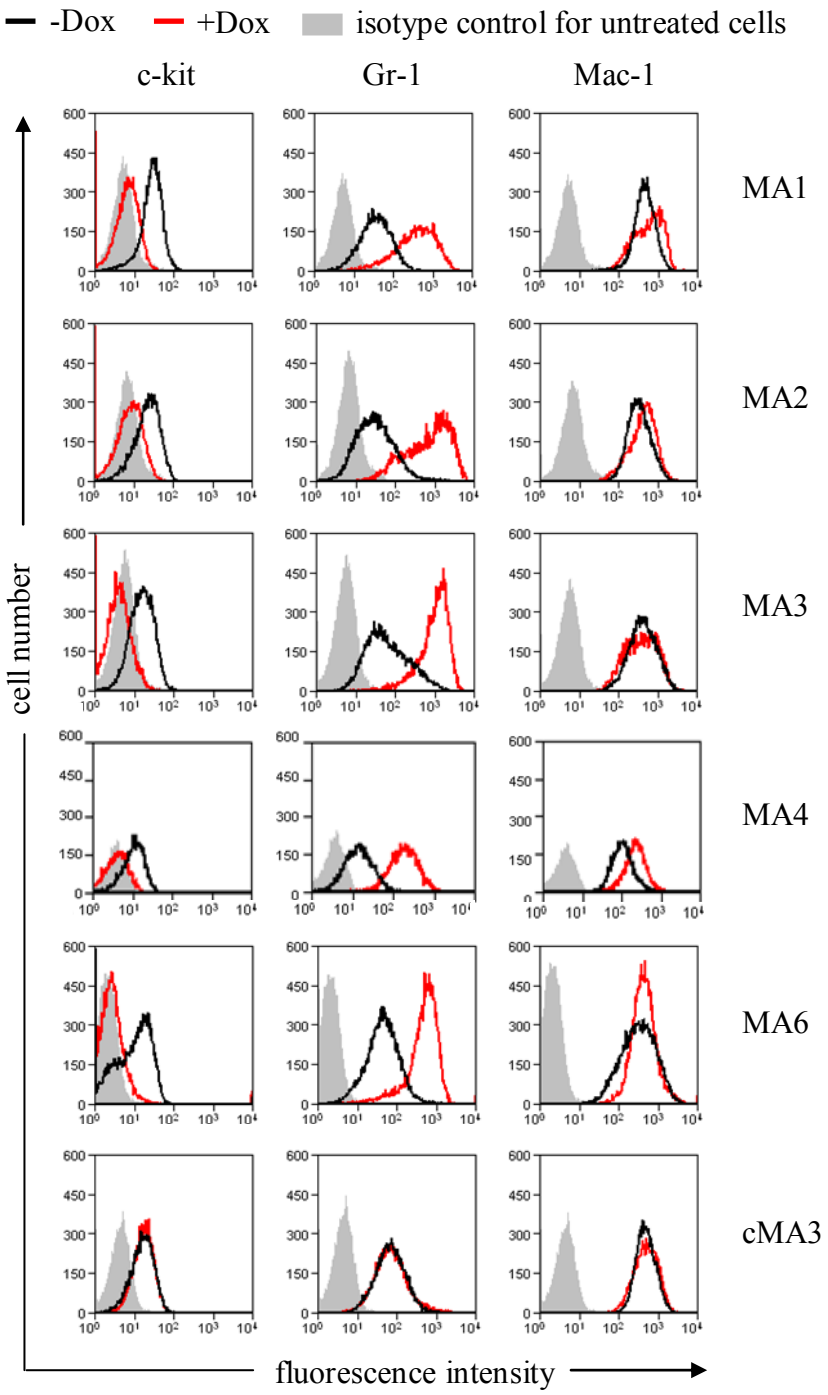


Figure 24: MLL-AF9 immortalized cell lines differentiate into mature cells after loss of MLL-AF9 expression

The graph shows flow cytometric analysis of cell surface antigen expression of immortalized cell lines after treatment with 2µg/ml Doxycycline for 96 hours (red line) compared to untreated cells (black line) and to the appropriate isotype control of untreated cells (filled grey area). Expression of the isotype control did not change in treated cells. MA4 was analysed after 72 hours with Doxycycline. Similar results were observed in three independent experiments.

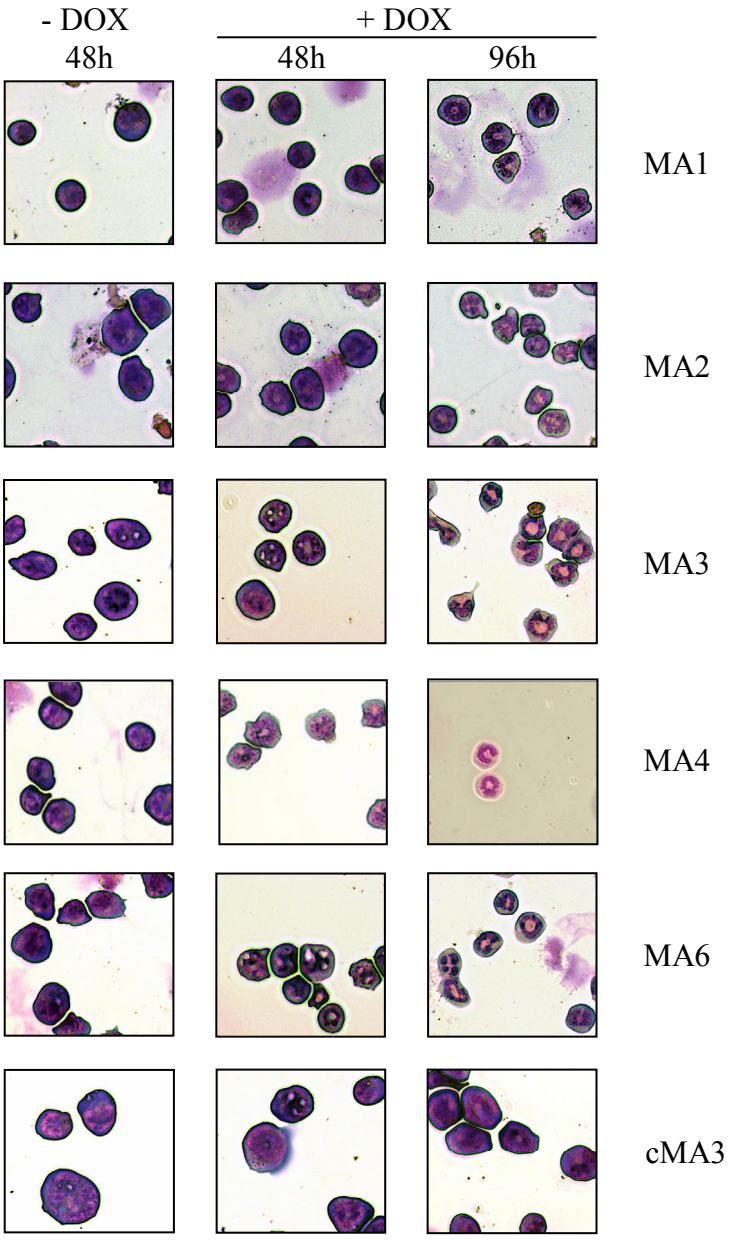


Figure 25: MLL-AF9 immortalized cell lines differentiate into mature cells after loss of MLL-AF9 expression in liquid culture

The figure shows the morphology of immortalised cells after treatment with 2µg/ml Doxycycline (48 hours and 96 hours) or not (48 hours), assessed by cytospin preparation and Wright-Giemsa staining (original magnification x 400). Only conditional cells differentiated into neutrophils after treatment with Doxycycline. Similar results were observed in three independent experiments.

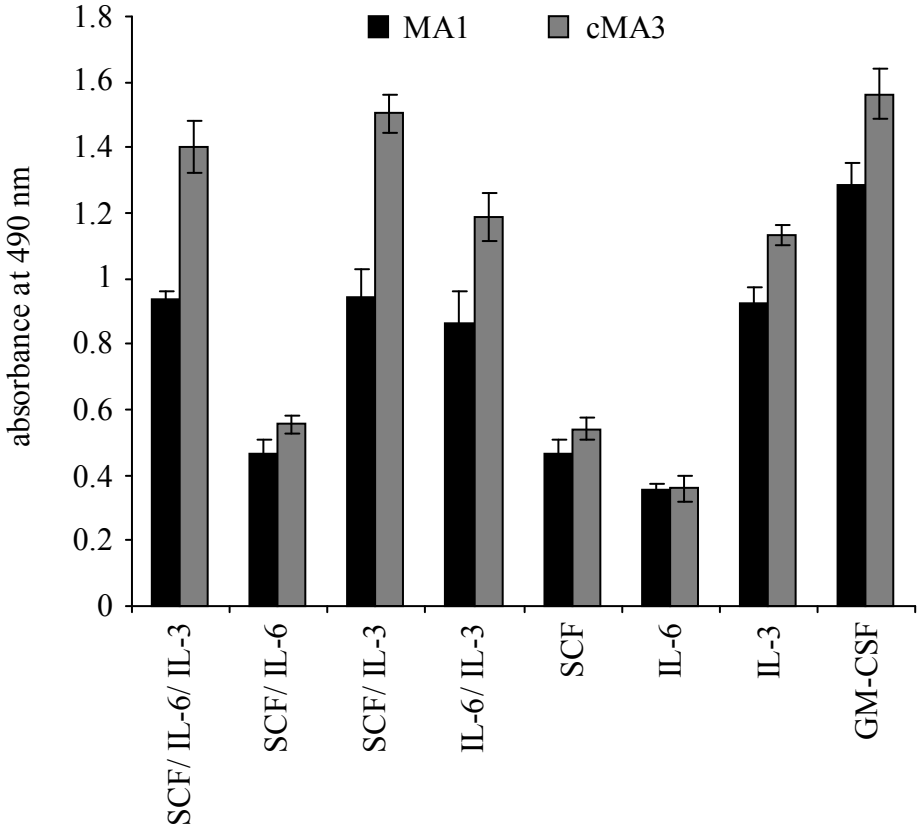


Figure 26: Cell proliferation assay to test the growth factor requirements for cell viability

Cells were seeded at $1x10^4$ cells per well and maintained for 3 days in a 96-well plate with various combinations of cytokines (SCF, IL-6, IL-3 or GM-CSF). The optical density, which is proportional to the number of viable cells, was measured at an absorbance of 490 nm. The graph shows the viability of MA1 (black) and cMA3 (grey) cells cultured in different cytokine combinations. Bars represent means of triplicate values and error bars their standard deviations. Similar results were observed in three independent experiments.

The cells were able to survive and proliferate at a similar rate in IL-3 alone, GM-CSF alone, IL-3 in combination with IL-6, IL-3 in combination with SCF or IL-3 in combination with IL-6 and SCF. A previous study in our laboratory (Dr. Sarah Horton of the MHCB Unit) had shown that the optimal cytokine combination for long term maintenance of MLL-ENL immortalised HPC is SCF, IL-3 and IL-6. Therefore, for long term maintenance, the MLL-AF9 immortalised cells were also cultured in the combination of SCF, IL-3 and IL-6 cytokines.

3.3. Discussion – Chapter III

It has been shown in several previous *in vitro* and *in vivo* studies that MLL-AF9 and MLL-ENL fusion proteins play an important role in leukaemogenesis (Corral et al., 1997; Dobson et al., 1999; Forster et al., 2003; Zeisig et al., 2004; Horton et al., 2005; Drynan et al., 2005; Krivtsov et al., 2006; Horton, Walf-Vorderwülbecke et al., 2009). In order to study the oncogenic activity of MLL-fusions, we have generated MLL-AF9 and MLL-ENL conditionally immortalised cell lines in an *in vitro* immortalisation assay.

In vitro myeloblastic immortalisation with conditional oncogene expression in primary HPC has been previously studied with the E2a-Pbx1 fusion gene (Sykes *et al.*, 2001). In this study, the oestrogen binding domain of the oestrogen receptor was fused to E2a-Pbx1 (E2a-ER-Pbx1) and fusion protein expression was dependent on addition of β-oestradiol to the transduced cells. This model was used to generate conditionally immortalised cell lines. Upon oestrogen withdrawal immortalised cells were found to differentiate into granulocytes and monocytes (Sykes *et al.*, 2001). This model was used to identify changes in gene transcription controlled by the E2a-Pbx1 oncogene (Sykes *et al.*, 2001). Two previous studies have also used a Tamoxifen-regulated, a synthetic oestrogen derivate, expression system (ER system) to generate conditionally immortalised MLL-ENL cell lines (Ayton and Cleary, 2003; Zeisig *et al.*, 2004). Due to size restraints for retroviral packaged DNA, one of these studies used a truncated ENL cDNA encoding only the last 129 amino acids of ENL to generate conditionally MLL-ENL immortalised cell lines (Zeisig *et al.*, 2004). The tamoxifen expression system is comparable to the Tet-on expression system where the introduction of Doxycycline to the system initiates the transcription

of the gene of interest. The Tamoxifen expression system is frequently used to study immediate downstream transcriptional and signalling activities of oncogenes, because of the rapid response kinetics. However, the Tet expression systems, which depend on transcription and subsequent translation of a target gene either in the presence (Tet-on) or absence (Tet-off) of Doxycycline, are not as fast acting as the Tamoxifen expression system, which stabilizes the already expressed target protein upon hormone administration. However, the On-systems (Tet-on and Tamoxifen expression system) suffer from the potential drawback that Doxycycline or Tamoxifen have to be continuously present for proliferation of the cells. This presents particular problems in studying the capacity of the conditional cell lines to generate leukaemia *in vivo*, where in order to express the fusion oncogene, the drug would have to be constantly administered to the animals. While these two Tamoxifen-regulated studies were published (Ayton and Cleary, 2003; Zeisig *et al.*, 2004), a different approach was used in a previous study in our laboratory in order to generate conditionally immortalised MLL-ENL cell lines using the Tet-off expression system (Horton *et al.*, 2005). The advantage of using the Tet-off expression system was that MLL-ENL immortalised cells can be maintained without addition of Doxycycline to the cultures. Although there was a small effect on MLL-AF9 protein expression by Doxycycline in the constitutive cells, this is presumably an effect of Doxycycline on the cell cycle but not a direct effect on the MLL-AF9 protein expression. Furthermore, it facilitates the identification of changes in gene expression required for maintenance of the immortalised phenotype.

The previous study in our laboratory used the conditional MLL-ENL expression vector to generate immortalised cell lines, which terminally differentiated upon addition of Doxycycline. However, as mentioned earlier, this vector contained

a sequence encoding a myc epitope instead of the first 27 amino acids of MLL (Horton *et al.*, 2005). Wild-type MLL associates with many proteins in a macromolecular complex through its C-terminal methylase domain (Yokoyama *et al.*, 2004). It was shown that menin, a member of this macromolecular complex, interacts with the N-terminus of MLL. Menin and LEDGF are the only known member of the complex that are not associated with the C-terminus and therefore remain associated with the disrupted MLL-fusion proteins (Hughes *et al.*, 2004; Yokoyama *et al.*, 2005; Yokoyama and Cleary, 2008). It has been shown in various studies that menin interaction with MLL is required for the fusion proteins MLL-ENL, MLL-AF9, MLL-GAS7 and MLL-AF10 to induce and maintain oncogenic transformation of myeloid progenitors (Yokoyama *et al.*, 2005; Caslini *et al.*, 2007). Furthermore, a high-affinity N-terminal conserved binding motif (RXRFP) has been identified, which spans amino acids six to ten (Yokoyama *et al.*, 2005).

The conditional MLL-ENL vector used in previous studies in our laboratory, does not contain the high-affinity binding motif (amino acids six to ten of MLL) but was successfully used to immortalise HPC (Horton *et al.*, 2005). Therefore, a similar myc-tagged vector containing the MLL-AF9 fusion cDNA was initially used in the present study. However, this truncated MLL-AF9 vector failed to immortalise HPC. In contrast, a full-length MLL-AF9 construct, encoding the intact MLL portion of the MLL-AF9 fusion gene, successfully immortalised HPC, resulting in replatable methylcellulose assays and long-term maintenance in liquid culture. Our data is consistent with a previous study using the MLL-AF9 fusion oncogene, suggesting that the high-affinity menin interaction domain is required for transformation (Caslini *et al.*, 2007). Therefore, MLL-AF9 full-length expression constructs were used to generate conditionally and constitutively immortalised MLL-AF9 cell lines.

Since known target genes of MLL-ENL, such as *myeloid ecotropic viral integration site 1* (*Meis1*) (Zeisig *et al.*, 2004), were not identified in the previous MLL-ENL Affymetrix study in our laboratory, we also decided to clone new full-length MLL-ENL expression constructs. The expression constructs were encoding the intact MLL portion of the MLL-ENL fusion gene, to generate new conditionally and constitutively immortalised MLL-ENL cell lines and screen again for MLL-ENL target genes (cell line ME4 was generated by myself, all other MLL-ENL cell lines were generated and characterised in our laboratory by Dr. Sarah Horton). Since MLL-AF9 and MLL-ENL are structurally very similar to each other but have different leukaemic activities in patients and animal models (Forster *et al.*, 2003; Drynan *et al.*, 2005), we were interested in comparing the transcriptional targets of these two MLL-fusion oncogenes. We hypothesised that both fusion oncogenes activate the same core pathways for transformation but also affect different transcriptional targets that are specific for MLL-ENL or MLL-AF9 transforming activity. This analysis would allow us to focus on the core pathways used by MLL-fusions to induce leukaemia.

By using retroviral delivery of the Tet-off expression system to conditionally express the MLL-AF9 or MLL-ENL oncogenes, immortalised cells were maintained in liquid culture without a requirement for continuous drug treatment. RNA isolated from conditionally MLL-AF9 and MLL-ENL immortalised cells expressing the fusion oncogene was compared to RNA isolated from cells upon loss of the oncogene. By characterising the immortalised cells before and after loss of the MLL-fusion expression we were able to choose a suitable time point for the gene expression study. A suitable time point for such analyses would be one at which the cells displayed the most marked change in oncogene expression and the expression

of known target genes but at the same time showed a minimal phenotypic change associated with differentiation. A significant down-regulation of MLL-AF9 protein expression was observed in conditional MA1 cells cultured with Doxycycline for 16 hours and no MLL-AF9 protein expression was detected in cells cultured with Doxycycline for 48 hours. However, MLL-ENL protein expression could not be detected by Western blot analysis. It is possible that levels of MLL-ENL protein expression are very low but nevertheless physiologically significant in order to immortalise HPC. Previous work in our laboratory has shown that down-regulation of known MLL-ENL target genes, such as *homeobox A7* (*HoxA7*) and *homeobox A9* (*HoxA9*), was more significant 48 hours after addition of Doxycycline to the cultures, compared to 24 hours after addition of Doxycycline (PhD Thesis Dr. Sarah Horton, London University). Furthermore, Wright-Giemsa staining of cytospin preparations of the cells showed that 48 hours after addition of Doxycycline most of the conditional cells still had a myeloblastic morphology. Hence, 48 hours incubation with Doxycycline was chosen as the time point for global gene expression analysis. This time point appears optimal in order to detect changes to transcript levels of target genes upon loss of the MLL-fusion expression and minimise differentiation related changes in gene expression. However, in order to completely exclude differentially expressed genes regulated by differentiation, MLL-fusion-independent differentiation can be induced by addition of the granulocyte colony-stimulating factor (G-CSF). Addition of G-CSF to the cultures of MLL-ENL immortalised cells was shown to induce terminal differentiation into neutrophils without abrogating MLL-ENL expression or resulting in decreased *HoxA7* and *HoxA9* expression (Horton *et al.*, 2005). Future comparisons of differentially expressed genes identified by G-CSF induced differentiation with differentially expressed genes identified upon

loss of MLL-fusion expression may facilitate the exclusion of genes that change as a result of differentiation.

CHAPTER IV - Gene Expression Profiling

Gene expression profiling facilitates simultaneous mRNA measurements on an entire genome, providing a global picture of the relative amount of mRNA expressed in cells under different conditions. Using the Tet-off conditional system, a stable assay with immortalised HPC was generated, expressing the MLL-AF9 fusion protein. Treatment of the conditional cells with Doxycycline caused a decrease in MLL-AF9 mRNA and protein expression, and resulted in terminal differentiation of the cells. In parallel to the generation of MLL-AF9 immortalised cell lines, MLL-ENL immortalised cell lines were also generated and characterised in the same way. MLL-AF9 and MLL-ENL fusion proteins are structurally very similar to each other but show differences in the presentation of leukaemia. Unlike MLL-AF9, which is predominantly found in infant AML and t-AML, MLL-ENL is commonly found in infant acute leukaemias of both the myeloid and lymphoid lineages (Ayton and Cleary, 2001; Daser and Rabbitts, 2004). However, because of their structural similarity, it is likely that both MLL-translocations would have the underlying oncogenic transformation mechanism in common. By analysing MLL-AF9 and MLL-ENL immortalised cells together, we aimed to define critical target genes involved in the immortalisation pathways of MLL-fusion oncogenes.

4.1. Identification of an appropriate time point for global gene expression analysis

In order to avoid measuring too many differentiation related gene expression changes, it was important to pick an early time point which would identify expression level changes of direct target genes of the MLL-fusion oncogenes, MLL-

AF9 and MLL-ENL. The results from the characterisation of the immortalised cell lines (Chapter III), and results from a previous study using MLL-ENL immortalised cells (Horton *et al.*, 2005), were considered for choosing a suitable time point for the global gene expression analysis. MLL-ENL immortalised cell lines generated for the previous study were missing the high-affinity N-terminal conserved binding motif for menin. However, it was shown that expression of the primary MLL-ENL transcriptional target genes *HoxA7* and *HoxA9* were still regulated by these N-terminal truncated constructs. Analysis showed that *HoxA7* and *HoxA9* gene expression levels were significantly decreased after 24 hours ($p < 0.07$, two-tailed students t-test) but not as significant as the decrease observed after 48 hours culture with Doxycycline ($p < 1.4 \times 10^{-5}$, two-tailed students t-test; data not shown). This was considered in order to pick a suitable time point for the present study. Changes in MLL-fusion protein expression were also considered but we were not able to detect MLL-ENL protein expression. However, detection of MLL-AF9 protein expression was possible and is shown in Chapter III. MLL-AF9 protein expression was significantly decreased after 16 hours incubation with Doxycycline (Figure 21) and completely lost by 48 hours (Figure 20). In order to allow time for changes to the transcript levels of primary MLL-fusion targets after loss of the MLL-fusion protein expression, 48 hours treatment with Doxycycline was considered an appropriate time point for the study of global gene expression.

4.2. Selection of cell lines for global gene expression analysis

It was important to validate that known MLL-AF9 and MLL-ENL transcriptional target genes were down-regulated upon loss of the MLL-fusion expression in conditionally immortalised cells, but not in the constitutive cells.

HoxA9 and *Meis1* have both been identified as primary target genes of MLL, MLL-AF9 and MLL-ENL (Yu *et al*., 1998; Kumar *et al*., 2004). Previous work from our laboratory has shown that *HoxA9* is highly expressed in MLL-ENL immortalised myeloid cell lines and that expression of *HoxA4-A11* genes is directly maintained by MLL-ENL (Horton *et al.,* 2005). The expression levels of *HoxA9* and *Meis1* in MLL-AF9 immortalised cells, and the expression levels of *HoxA9* and *MLL-ENL* in MLL-ENL immortalised cells, were measured by qPCR. Total RNA was extracted from all cell lines cultured with or without 2μg/ml Doxycycline for 48 hours and used for qPCR analysis.

As expected, a significant down-regulation of *HoxA9* expression was observed in the conditionally immortalised MLL-AF9 cell lines MA1 ($p < 0.0002$, two-tailed students t-test), MA2 ($p < 0.0042$, two-tailed students t-test), MA3 ($p < 0.0024$, two-tailed students t-test) and MA4 ($p < 8.33 \times 10^{-5}$, two-tailed students t-test) cultured with Doxycycline (Figure 27 A). *HoxA9* expression was significantly, but less markedly, down-regulated in MA6 ($p < 0.0047$, two-tailed students t-test) compared to the other conditional cell lines, after loss of MLL-AF9 expression. *Meis1* was expressed at similar levels in MA1, MA3, MA4 and cMA-3 cultured without Doxycycline (Figure 27 B). However, there was less expression of *Meis1* in MA6 and almost no detectable *Meis1* expression in MA2. A significant down-regulation of *Meis1* expression was observed in the conditionally immortalised cell lines MA1 ($p < 1.67 \times 10^{-5}$, two-tailed students t-test), MA3 ($p < 0.0028$, two-tailed students t-test), MA4 ($p < 0.0014$, two-tailed students t-test) and MA6 ($p < 0.0029$, two-tailed students t-test) cultured with Doxycycline.

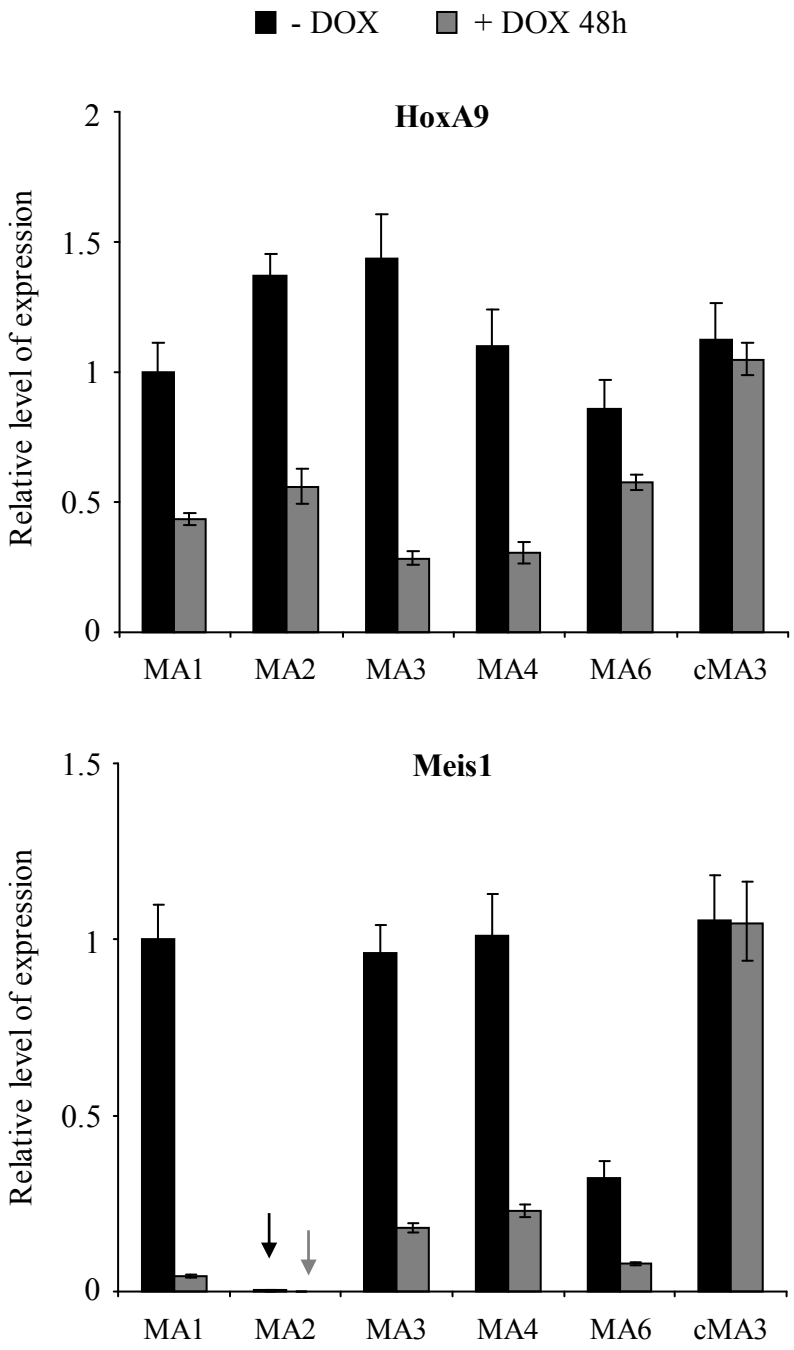


Figure 27: Expression of the MLL target genes *HoxA9* and *Meis1*

The graph shows the relative mRNA expression of *HoxA9* and *Meis1* in immortalised MA1, MA2, MA3, MA4, MA6 and cMA3 cells after treatment (grey bars or indicated with grey arrow) or not (black bars or indicated with black arrow) for 48 hours with 2µg/ml Doxycycline. Columns represent the mean of triplicate measurements, error bars represent the standard deviations.

As expected, there was no significant down-regulation of *HoxA9* ($p < 0.5$, two-tailed students t-test) and *Meis1* ($p < 0.9$, two-tailed students t-test) expression in the constitutively immortalised cells cMA3. For the gene expression profiling, at least three individual cell lines were required. This allows to group the samples as replicates, to remove differences and facilitate comparison analysis between Doxycycline treated and untreated samples. As a result of the *HoxA9* and *Meis1* expression analysis, the conditionally immortalised MLL-AF9 cell lines MA1, MA3 and MA4 were phenotypically most similar to each other and picked for gene expression profiling.

In all the conditionally immortalised MLL-ENL cell lines analysed, *MLL-ENL* expression was completely lost after 48 hours of Doxycycline treatment (Figure 28 A, experiment performed by Dr. Sarah Horton, MHCB Unit, ICH). The cell line ME6 had a higher level of *MLL-ENL* expression when compared to the other cell lines. A significant down-regulation of *HoxA9* expression was observed in all conditionally MLL-ENL cell lines (ME3 $p < 0.0019$, ME4 $p < 0.0002$, ME5 $p < 0.0011$, ME6 $p < 0.0276$, ME7 $p < 0.0048$ all two-tailed students t-test) cultured with Doxycycline (Figure 28 B experiment performed by Dr. Sarah Horton, MHCB Unit, ICH). The cell lines ME3 and ME6 had a slightly less marked down-regulation of *HoxA9* expression when compared to the other conditional cell lines. As expected, there was no significant down-regulation of *MLL-ENL* (cME3 $p < 0.14$, two-tailed students t-test) and *HoxA9* (cME3 $p < 0.046$, two-tailed students t-test) expression in the constitutively immortalised cells cME3. The conditionally immortalised cell lines ME4, ME5 and ME7 were picked for the gene expression profiling. These cell lines had similar expression levels of *MLL-ENL* and *HoxA9*, as well as similar levels of down-regulation of these genes in the presence of Doxycycline.

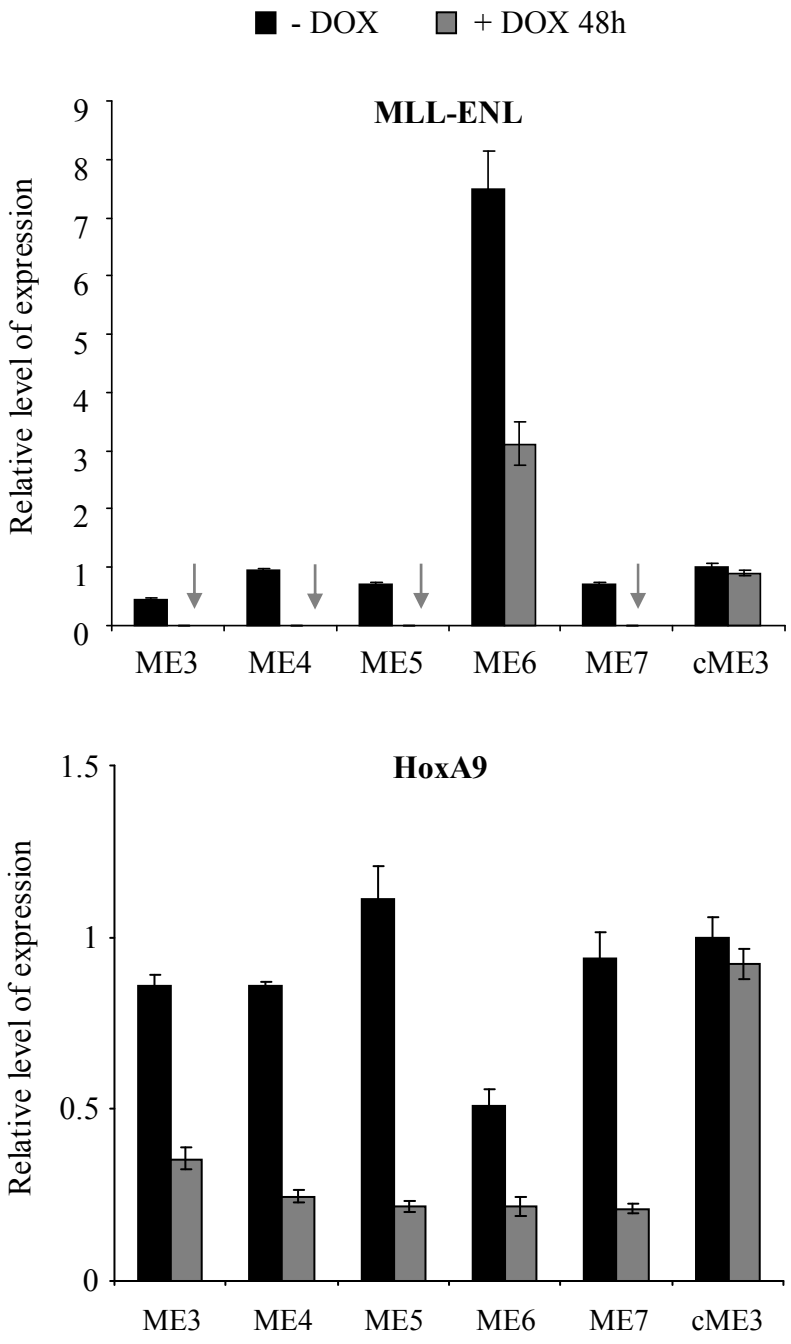


Figure 28: Expression of *MLL-ENL* and of the MLL target gene *HoxA9*

The graph shows the relative mRNA expression of *MLL-ENL* and *HoxA9* in immortalised ME3, ME4, ME5, ME6, ME7 and cME3 cells after treatment (grey bars or indicated with grey arrow) or not (black bars) for 48 hours with 2µg/ml Doxycycline. Columns represent the mean of triplicate measurements, error bars represent the standard deviations.

HoxA9 and *Meis1* expression levels were not changed in the constitutively immortalised cell lines cMA3 and cME3. This result indicated that these cell lines were suitable controls. By comparing gene expression changes in conditionally immortalised cell lines with changes in the constitutively immortalised cell lines it is possible to exclude genes which were affected by the Doxycycline treatment. Hence, a significant gene expression change in the constitutive as well as the conditional cells after treatment with Doxycycline would most likely have resulted from an effect of Doxycycline, unrelated to *MLL-AF9* or *MLL-ENL* expression. Consequently it was possible to exclude these false positive genes from the list of target genes.

4.3. Global gene expression analysis

Total RNA was extracted, using TRIzol reagent, from three conditional (MA1, MA3, MA4) and one constitutive (cMA3) MLL-AF9 immortalised cell lines and also from three conditional (ME4, ME5, ME7) and one constitutive (cME3) MLL-ENL immortalised cell lines, each of them cultured without or with 2µg/ml Doxycycline for 48 hours. The concentration and quality of isolated RNA was determined using the NanoDrop ND-1000 (Table 13), as well as using the Agilent 2100 Bioanalyzer (data not shown). Since the quality of RNA is essential for the success of microarray analysis, the RNA quality was verified by ensuring the A_{260} to A_{280} ratio was between 1.8 and 2.0.

In total 16 GeneChip Mouse Genome 430.2 arrays (Affymetrix) were hybridised and scanned (performed by K. Pearce and N. Jina, ICH Microarry Center, UCL Genomics). The data was analysed using two different methods and GeneSpring 7.3.1 software (Agilent).

Table 13: Concentration and quality of isolated RNA

Cell line	ng/µl (1:10 dilution)	A_{260}/A_{280}
cME3 -DOX	84.77	1.87
cME3 +DOX	161.07	1.83
ME4 –DOX	254.93	1.89
ME4 +DOX	246.78	1.85
ME5 –DOX	158.79	1.84
ME5 +DOX	154.94	1.83
ME7 -DOX	274.13	1.87
ME7 +DOX	224.38	1.85
cMA3 –DOX	159.2	1.88
cMA3 +DOX	204.11	1.91
MA1 –DOX	226.69	1.84
MA1 +DOX	149.06	1.82
MA3 –DOX	99.10	1.87
MA3 +DOX	34.42	1.99
MA4 –DOX	198.58	1.90
MA4 +DOX	38.06	1.89

The table shows the RNA concentration and quality of treated and untreated cell lines used in the microarray study.

For the first method of identifying differentially expressed target genes, MLL-AF9 immortalised cell lines and MLL-ENL immortalised cell lines were divided into two groups according to their MLL-fusion and analysed separately (Analysis I, Figure 29). The six arrays, hybridised with the conditional cell lines, were normalised to each other by firstly normalising each probe set intensity of an array to the median of all probe set intensities of that array (per chip normalisation). Secondly, each probe set intensity of an array was normalised by the median of its probe set intensities in all arrays (per gene normalisation). To allow pair-wise comparisons between cell lines treated with and without Doxycycline, the three conditional cell lines were grouped as replicates.

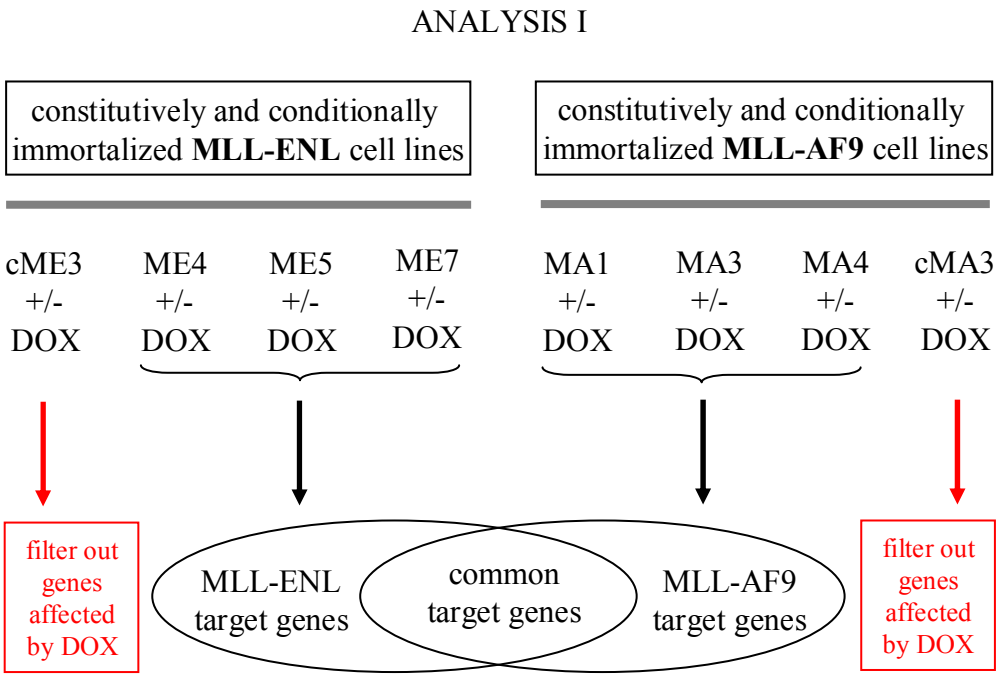


Figure 29: Analysis I

The figure shows a diagram of the Analysis I strategy. Genes which showed a significant change in gene expression in the constitutive cells after treatment with Doxycycline were filtered out.

The data were filtered by selecting for a probe set detection p-value smaller than 0.07, detected in at least two out of eight samples. This included the two arrays hybridised with the constitutively immortalised cells, with or without Doxycycline (Figure 30). Application of this filter reduced the number of detected genes to 25,618 in the MLL-AF9 group and to 25,307 genes in the MLL-ENL group. The data were filtered further by selecting for genes whose expression level changed more than 2-fold, with a defined p-value cut-off of less or equal than 0.05, upon treatment with Doxycycline. Application of this filter reduced the number of detected target genes to 2,486 in the MLL-AF9 group and 2,120 in the MLL-ENL group. In order to exclude genes which were affected by the Doxycycline treatment *per se*, all genes whose expression level changed more than 2-fold in the constitutively immortalised cells, upon treatment with Doxycycline, were excluded. In the MLL-AF9 group 131 genes could be excluded from the final target gene list. In the MLL-ENL group 127 genes could be excluded from the final target gene list.

The final MLL-AF9 target gene list included 2,335 differentially expressed genes of which 1,204 genes were up-regulated and 1,151 genes were down-regulated upon loss of MLL-AF9 expression. The final MLL-ENL target gene list included 1,993 differentially expressed genes of which 795 genes were up-regulated and 1,198 genes were down-regulated upon loss of MLL-ENL expression. In order to distinguish between common MLL-fusion target genes and unique MLL-ENL or unique MLL-AF9 target genes, the two lists of target genes were compared. The comparison showed that MLL-AF9 and MLL-ENL immortalised cells had 1,082 differentially expressed target genes in common (569 up-regulated, 513 down-regulated).

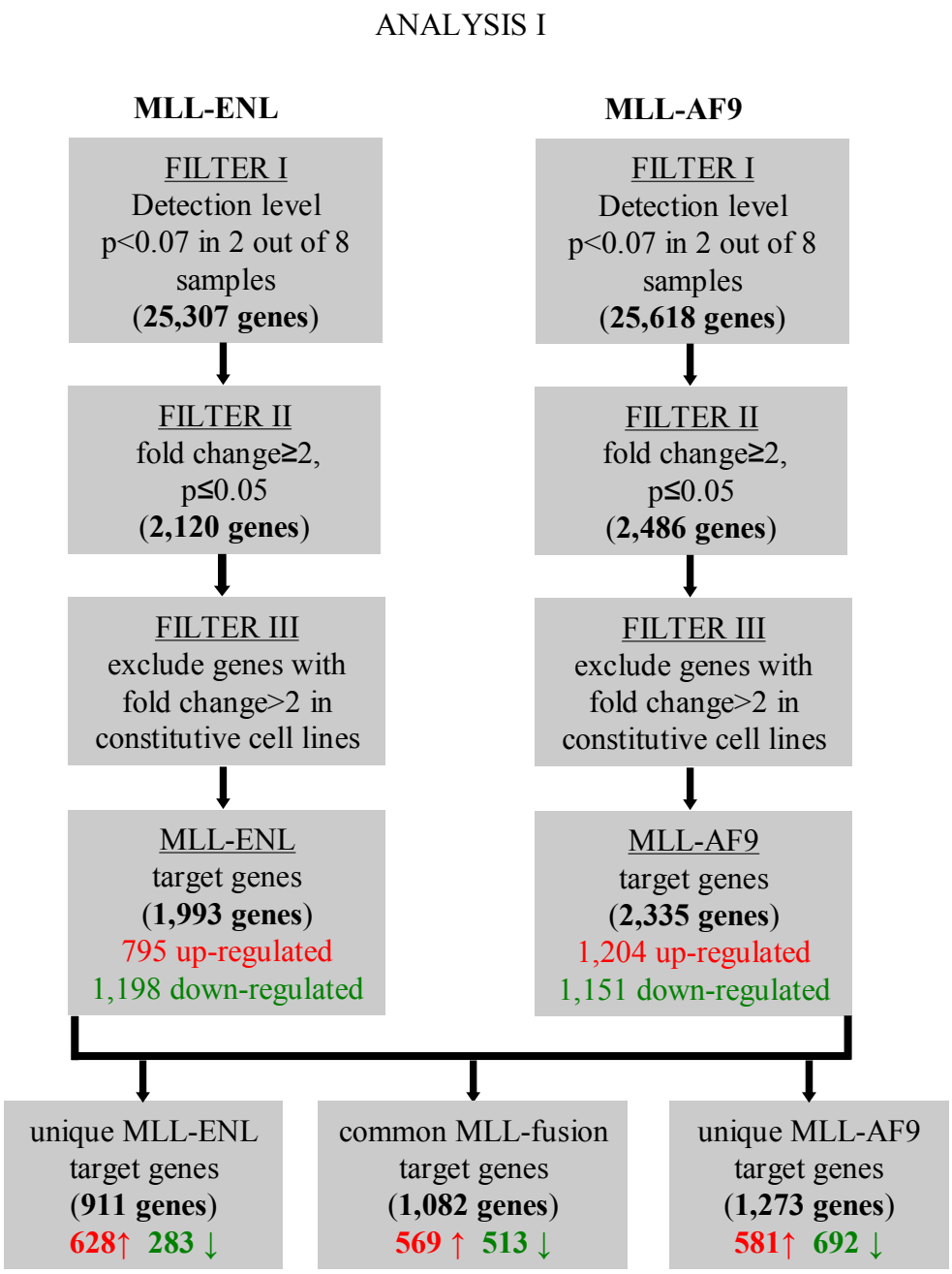


Figure 30: Generation of gene lists using the Analysis I strategy

MLL-AF9 immortalised cell lines and MLL-ENL immortalised cell lines were divided into two groups according to the MLL-fusion and analysed separately.

1,273 target genes were unique for MLL-AF9 (581 up-regulated, 692 down-regulated) and 911 target genes were unique for MLL-ENL (628 up-regulated, 283 down-regulated). Since MLL-AF9 and MLL-ENL immortalised cells had a high number of target genes in common and only three replicates per sample were used for the statistical analysis, a different method of analysing the data was applied.

For the second method, all MLL-AF9 and MLL-ENL immortalised cell lines were analysed together (Analysis II, Figure 31). Each probe set intensity of one array was normalised to the median of all probe set intensities of that array (per chip normalisation). Specified samples were than paired (Table 14) and normalised to one another. The probe set intensity for each gene in the experimental sample (with Doxycycline) was divided by the median of that genes probe set intensity in the corresponding control sample (without Doxycycline treatment) (per gene normalisation).

Table 14: Normalisation to control

Control samples (-DOX)	Experimental samples (+ DOX)
cMA3-	cMA3+
MA4-	MA4+
MA3-	MA3+
MA1-	MA1+
cME3-	cME3+
ME7-	ME7+
ME5-	ME5+
ME4-	ME4+

The table shows all pairs of control samples (-DOX) and experimental samples (+DOX) normalised to each other.

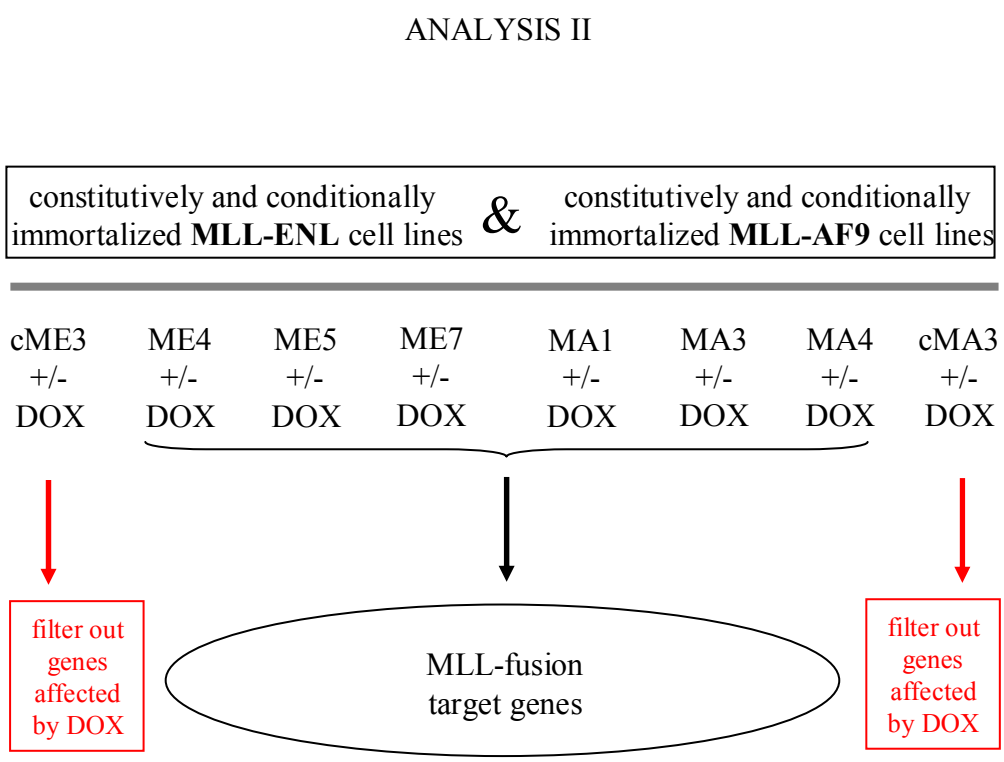


Figure 31: Analysis II strategy

The figure shows a diagram of the Analysis II strategy. Genes which showed a significant gene expression change in the constitutive cells after treatment with Doxycycline were filtered out.

The data were filtered by selecting for a probe set detection p-value smaller than 0.07, detected in at least two out of 16 samples. This included the two arrays hybridised with the constitutively immortalised cells, with or without Doxycycline (Figure 32). The number of detected genes was reduced to 29,100 after application of this filter. To allow pair-wise comparisons between treated and untreated cell lines, the six conditional cell lines with Doxycycline and the six conditional cell lines without Doxycycline were grouped as replicates. This step allowed filtering the data further by selecting for genes whose expression level changed more than 2-fold, with a defined p-value cut-off of less or equal to 0.05, upon treatment with Doxycycline. The p-value cut-off of 0.05 allows a 5% chance of error. That means that 5% of all tested genes could be genes that are found to be statistically different between conditions but are not in reality (false positives). The incidence of false positives is proportional to the number of tests performed and the p-value cut-off. In a microarray experiment, a t-test is performed on each gene separately. The very high number of genes on an array makes it likely that differential expression of some genes represent false positives. To control for the false positive discovery rate, the Benjamini-Hochberg method, a multiple testing correction procedure, was applied. Multiple testing correction adjusts the individual p-value for each gene to keep the overall false positive rate to less than or equal to the specified p-value cut-off (GeneSpring Analysis Guide, Multiple Testing Corrections).

Application of these filters reduced the number of 29,100 detected genes to 3,177 differentially changed target genes. In order to exclude genes which were affected by the Doxycycline treatment, all genes whose expression level changed more than 2-fold in the constitutively immortalised cells, upon treatment with Doxycycline, were excluded.

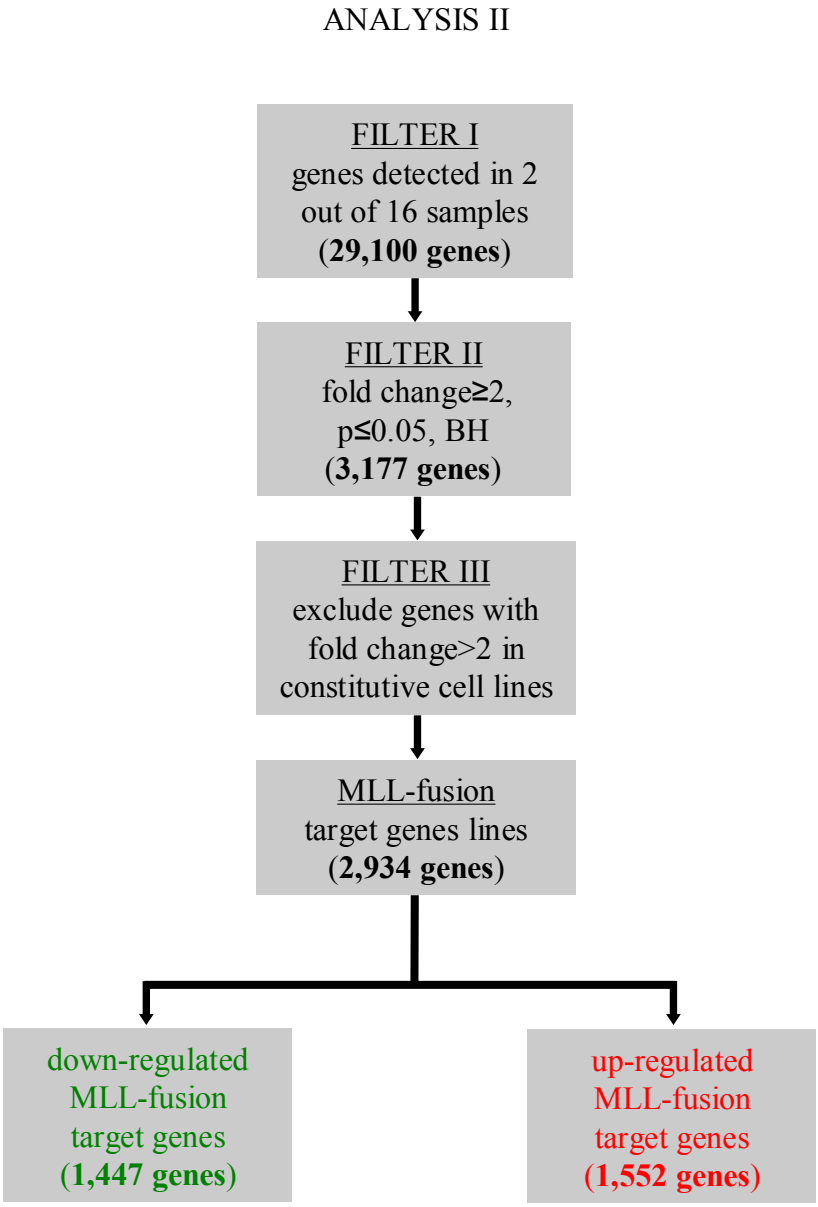


Figure 32: Generation of gene list using the Analysis II strategy

MLL-AF9 and MLL-ENL immortalised cell lines were analysed together. To control for the false positive discovery rate, the Benjamini-Hochberg (BH) method, a multiple testing correction procedure, was applied.

The final target gene list included 2,934 differentially expressed genes of which 1,552 genes were up-regulated and 1,447 genes were down-regulated upon loss of MLL-fusion oncogene expression.

Both gene expression analyses methods resulted in target gene lists with more than 1,000 entries. Analysis I showed that nearly half of the genes regulated by MLL-AF9, and more than half of the genes regulated by MLL-ENL, were the same. When the different analysis methods were compared to each other, only two genes of the common MLL-fusion target gene list generated by Analysis I were not contained in the target gene list generated by Analysis II (Table 15).

Table 15: Comparison of gene lists

Analysis I	Analysis II	number of concordant genes
common MLL-fusion: 1082 genes		1080 genes
unique MLL-ENL: 911 genes	2934 genes	608 genes
unique MLL-AF9: 1273 genes		607 genes

The table shows the comparisons of the three gene lists generated by Analysis I with the target gene list generated by Analysis II.

Replication of the biological samples is essential for drawing conclusions from the experiment. The six replicates of conditional untreated and treated samples in the Analysis II allowed application of more robust statistics than the minimum required three replicates of untreated and treated samples in the translocation specific analysis. Therefore Analysis II was generally considered more reliable. Furthermore, because of the significant overlap of genes regulated by MLL-ENL and MLL-AF9, the 2,934 genes generated by Analysis II were more closely inspected in order to identify common transcriptional target genes of MLL-fusion oncogenes.

4.4. Selection of target genes

Large numbers of genes were found to be changed, with a more or less equal number of up-regulated genes (1,552 genes) or down-regulated genes (1,447 genes). The genes *HoxA5*, *HoxA7*, *HoxA9*, *HoxA11* and *Meis1* were among the list of differentially expressed target genes and have been previously reported to be expressed in MLL-ENL and MLL-AF9 immortalised cell lines (Horton *et al.*, 2005; Zeisig *et al.*, 2004, Krivtsov *et al.*, 2006). Detection of the differential expression of these genes was considered as an internal positive control for the gene expression profiling. In a first attempt to group genes and find patterns among the regulated genes, gene ontology analysis was used to categorise the regulated genes (data not shown). However, this categorisation was not very helpful in terms of identification of MLL-fusion regulated pathways, given that genes were categorised into an unmanageable large number of possible pathways.

Genes were also sorted by rank and association with transcriptional regulation, cancer, leukaemia or MLL-fusions found in the literature. Table 22 in the Appendix, lists 41 genes which were picked by rank and publication from the list of genes that were up-regulated in the presence of MLL-fusions. Interestingly, inspection of the list revealed that 12 of the 41 picked genes were found to be associated with the wingless (Wnt) signalling pathway (Table 16).

Table 16: Genes involved in Wnt signalling pathway and found in the list of target genes

Gene Symbol	Accession Number	Fold Change
Ascl2	1422396_s_at	10.93
Sox4	1419156_at	8.23
Frat2	1455220_at	6.627
Six4	1425767_a_at	5.831
Six1	1427277_at	3.968
Pontin	1416585_at	3.916
c-Myc	1424942_a_at	3.724
Reptin	1422482_at	3.614
c-Myb	1423430_at	3.462
Frat1	1449814_at	3.047
Tcf4	1424089_a_at	3.04
Fzd7	1450044_at	2.46

The table lists genes, picked from the list of target genes, which are involved in the Wnt signalling pathway. The genes are sorted by fold change.

Following up this link, the Wnt signalling pathway derived from the Kyoto Encyclopaedia of Genes and Genome (KEGG) database was compared with the complete list of target genes, using GeneSpring 7.3.1 software, in order to detect further Wnt signalling associated target genes. The KEGG pathway database contains molecular interaction and reaction networks for metabolism and various cellular processes which are manually entered from published material. The genes, *Fzd7*, *Frat1*, *Frat2*, *Cacybp/SIP*, *Pontin* and *c-Myc* were found to be involved in the Wnt signalling network and up-regulated by MLL-fusions (Figure 33). The genes, *Siah-1* and *CyclinD*, were found to be down-regulated. Since the KEGG pathway entries are not validated relations, further inspection of the Wnt signalling pathway using Ingenuity Pathway Analysis (IPA) software, was performed.

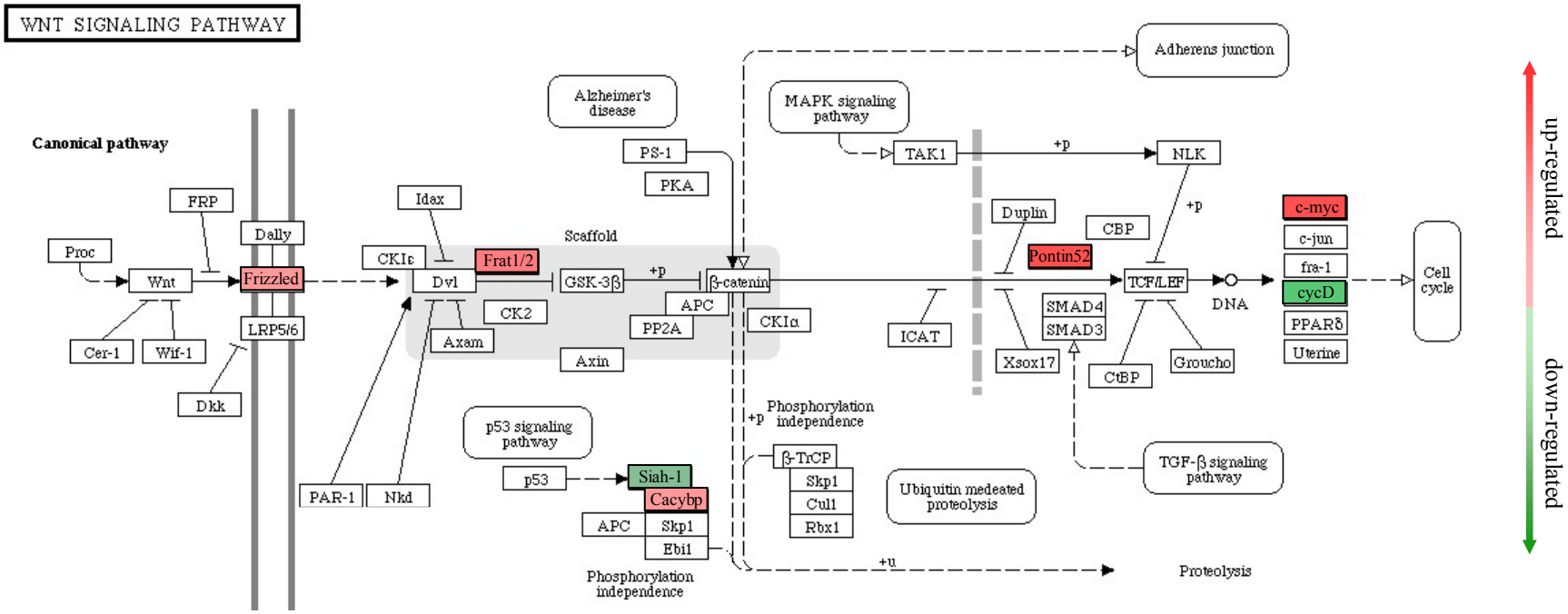


Figure 33: KEGG pathway analysis of the Wnt signalling pathway

The figure shows an overlay of the KEGG database derived Wnt signalling pathway with the MLL-fusion analysis target gene list. The level of gene up-regulation is indicated by increasing red intensity, the level of gene down-regulation is indicated by increasing green intensity. White boxes represent unchanged genes of the pathway.

The IPA software assembles genes with known relationships and can be used to compare pathways with experiments that generate gene lists. Using the IPA derived Wnt signalling pathway, the genes *Fzd7*, *Frat/GBP*, *SOX*, *c-Myc* and *TCF4* were found to be involved in the Wnt signalling network and up-regulated by MLL-fusions and the gene *PP2A* was found to be down-regulated (Figure 34). Discrepancies between the lists of genes associated with the Wnt signalling pathway result from the fact that the two different pathway analyses tools do not have the same entries of genes associated with Wnt signalling in their databases. Therefore it is helpful for the gene expression analysis to compare the results from different pathway analyses tools. Since a previous study demonstrated that *c-Myc* was required for MLL-ENL mediated transformation (Schreiner *et al.*, 2001), a Myc-network analysis was performed overlaying IPA derived Network 7 with the 2,934 differentially expressed target genes. Interestingly, almost all genes which have known relationships with *Myc* are entries of the MLL-fusion target gene list (Figure 35). Analysis with IPA software also revealed the most common identified canonical pathways when overlaid with the complete list of target genes (Table 17).

Table 17: Five most common canonical pathways identified by IPA software

Canonical Pathway	Number of genes up-regulated	Number of genes down-regulated
Neuregulin Signalling	5	5
Ceramide Signalling	2	7
Protein Ubiquitination Pathway	9	5
14-3-3-mediated Signalling	7	1
Dopamine Receptor Signalling	4	4

The table lists the five most common canonical pathways containing genes from the list of differentially expressed target genes. The number of up-or down-regulated genes is given.

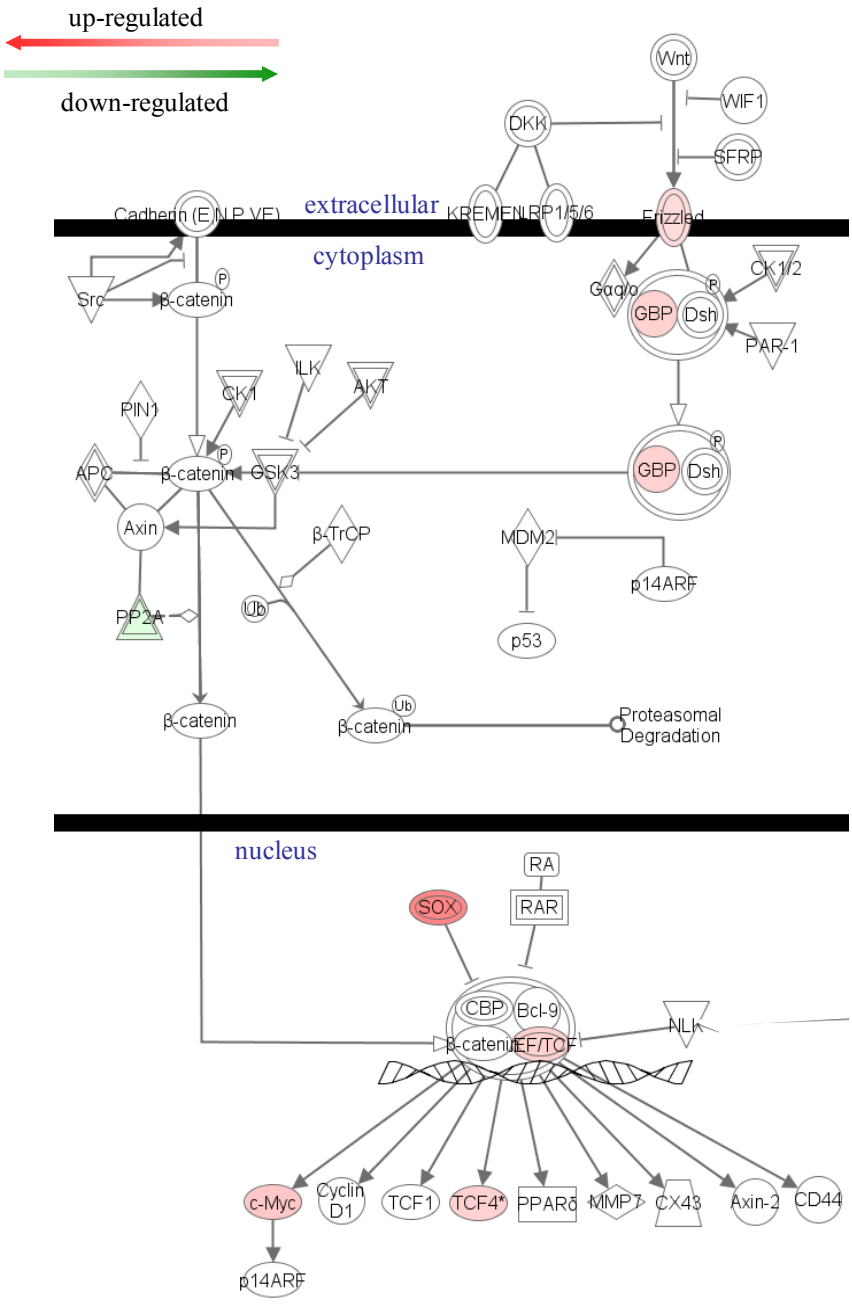


Figure 34: Ingenuity Pathway Analysis of the Wnt signaling pathway

The figure shows an overlay of the Wnt signaling pathway with the 2,934 differentially expressed genes derived from the Analysis II target gene list. Genes highlighted in red are up-regulated, genes highlighted in green are down-regulated. The level of fold change is indicated by increasing intensity of colouring. White boxes represent unchanged genes of the pathway.

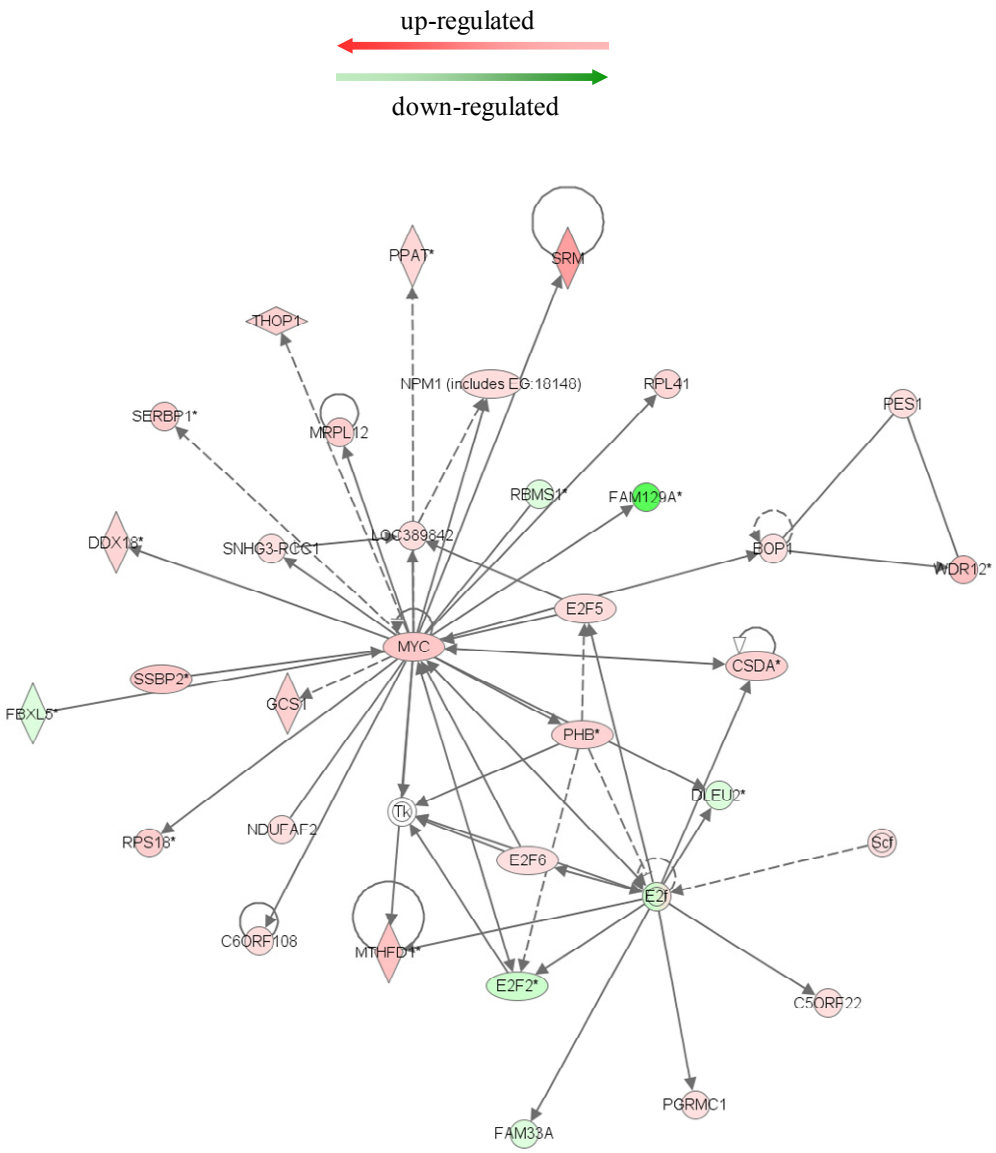


Figure 35: Ingenuity Pathway Analysis neighbourhood explorer of myc

The figure shows an overlay of the myc network (network 7) with the 2,934 differentially expressed genes derived from the Analysis II target gene list. Genes highlighted in red are up-regulated, genes highlighted in green are down-regulated. The level of fold change is indicated by increasing intensity of colouring. White boxes represent unchanged genes of the pathway.

To further identify genes that are affected by MLL-rearranged leukaemia, published data sets obtained from patient samples were compared to the genes identified in this study. Several of the genes identified in the present study are differentially expressed in patients with 11q23 rearrangements. Kohlmann *et al.* report the genes *FRAT1* and *ZFHX1B* to be common t(11q23)/MLL up-regulated target genes and Andersson *et al.* report *MSI2*, *MYB* and *SOX4* among the differentially expressed genes when comparing AML cell lines with ALL cell lines (Kohlmann *et al.*, 2005; Andersson *et al.*, 2005). The mouse homologues *Frat1*, *Zfhx1b*, *Msi2*, *c-Myb* and *Sox4* were also found to be up-regulated by the MLL-fusions in the present study. Furthermore, the genes *Evi2a*, *Ltf*, *Lcn2*, *Cybb*, *Sh3bp5*, *Plaur* and *Tnfaip2* were found to be down-regulated and their human orthologs have also been reported previously to be down-regulated in MLL-rearrangement leukaemia (Rozovskaia *et al.*, 2003; Yagi *et al.*, 2003; Ross *et al.*, 2004; Andersson *et al.* 2005).

4.5. Discussion – Chapter IV

In this study of global gene expression profiling of MLL-ENL and MLL-AF9 immortalised cell lines, we were able to confirm the presence of the most prevalent genes that have previously been identified in association with MLL-fusion mediated transformation. Two of the well characterised transcriptional target genes of MLL-fusion oncogenes are *HoxA9* and *Meis1* (Yu *et al*., 1998; Kumar *et al*., 2004; Zeisig *et al.*, 2004; Horton *et al.*, 2005). Analysis of the differential expression of these genes by qPCR, prior to global gene expression profiling, was used to select appropriate cell lines for the present gene expression study. In addition, the genes *HoxA5*, *HoxA7*, *HoxA9, HoxA10* as well as *HoxA11* were found to be differentially expressed. These genes have been previously reported to be expressed in MLL-ENL immortalised cell lines (Ayton and Cleary, 2003; So *et al.*, 2003) and have also been identified as part of a *HoxA* code expressed in MLL-ENL immortalised cell lines by our laboratory (Horton *et al.*, 2005).

The gene *Mef2c*, which has been identified as part of a subset of 11 genes immediately activated by MLL-AF9 expression (Krivtsov *et al.*, 2006), was also among the differentially expressed genes in the present study. These different previous gene expression analyses allowed the identification of MLL-fusion target genes with the most marked expression changes, such as genes of the *HoxA* cluster, *Meis1* and *Mef2c*, and underlined the importance of *Hox* gene regulation by MLL-fusion oncogenes (Ayton and Cleary, 2003; So *et al.*, 2003; Zeisig *et al.*, 2004; Horton *et al.*, 2005; Krivtsov *et al.*, 2006; Chen *et al.*, 2008). Many of these studies have restricted their further analyses to just a few hundred target genes, either because they only identified a small set of target genes (Zeisig *et al.*, 2004; Krivtsov

et al., 2006; Chen *et al.*, 2008) or only the top 100 most differentially expressed genes were considered (Ross *et al.*, 2004; Wei *et al.*, 2008). However, the experimental designs and the applied comparison methods are most likely not sensitive enough for identification of other not so markedly regulated underlying MLL-fusion mediated pathways and networks.

There are two different previous studies of conditional MLL-ENL expression in primary HPC. Our laboratory has previously optimised the Tet-off system and successfully used it in a study of conditional MLL-ENL expression in primary haematopoietic progenitor cells, for global gene expression profiling and to identify the Hox gene expression profile (Horton *et al.*, 2005; PhD Thesis, Sarah J. Horton, London University). Although the vector encoded an N-terminally truncated MLL-ENL construct, HPC were successfully immortalised and global *Hox* gene expression profiling revealed that these cells expressed a specific *HoxA* code, similar to the *HoxA* genes expressed in our study (Horton *et al.*, 2005). Later it was demonstrated that the MLL N-terminus contains a high-affinity conserved binding motif, which is required for interaction with the MLL co-factor menin (Yokoyama *et al.*, 2004). It was shown that *HoxA9* and *Meis1* are among the major effectors of menin in haematopoiesis (Chen *et al.*, 2006) This might be a potential explanation why for example the MLL-fusion target gene *Meis1* (Zeisig *et al.*, 2004; Krivtsov *et al.*, 2006) was not expressed (Horton *et al.*, 2005). Furthermore, it suggests that most likely other previously unidentified MLL-ENL target genes may not be identified by this truncated MLL-ENL vector. By generating a new full-length MLL-ENL cDNA conditional expression vector for the present study, we were able to confirm *Meis1* as a target gene.

The other conditional MLL-ENL model generated immortalised cell lines by using a synthetic hormone-inducible system (Zeisig *et al.*, 2004). Using this system, the maintenance of MLL-ENL immortalised cell lines was dependent on addition of tamoxifen to the cell cultures. This is a potential disadvantage, since the effect of tamoxifen itself on gene expression was not taken into account. It makes it unclear if differential expression of identified target genes was directly caused by MLL-ENL meditated transformation. Furthermore, biologically replicates are of critical importance for statistically and biologically valid conclusions. However, this study analysed one cell line in triplicates, therefore only the technical replicates were considered in the experimental design, whereas biological replicates were not included. Therefore, gene expression profiling using this approach resulted in a small set of target genes. In our study, we were able to overcome these problems by including immortalised cell lines with constitutive expression of the MLL-fusion as an internal control. This enabled us to eliminate differentially expressed genes, which change was caused by an effect of Doxycycline, unrelated to the loss of MLL-fusion expression. Furthermore, three different cell lines per fusion gene were used as biological replicates to identify differentially expressed genes.

Another gene expression study analysed MLL-AF9 target genes. Gene expression changes that occur during the transition from committed progenitor to LSC were characterised and a small set of 11 target genes directly activated by MLL-AF9 expression was identified (Krivtsov *et al.*, 2006). A potential caveat of this study is that only a very small HPC sub-population of cells, the leukaemic-GMP (L-GMP), was used to perform the global gene expression studies and it is unclear how many LSC are lost from the analysis because of the L-GMP purification protocol. Furthermore, in order to distinguish between gene expression changes

directly caused by MLL-AF9 and gene expression changes caused by the transformation process in general, an additional gene expression profiling shortly after infection of HPC with MLL-AF9 was performed. However, flow cytometry sorting and retroviral infection of the cells just before global gene expression profiling can cause a low signal to noise ratio and therefore results in a small set of 11 directly activated target genes. In contrast, the advantage of our study is that by using the Tet-off system the MLL-fusion genes are conditionally expressed and immortalised cells are maintained in the absence of Doxycycline. Thus, this system minimises manipulation of the cells which may affect gene expression itself. Furthermore, comparison of the 11 direct target genes with those identified in the present study revealed that eight of the 11 immediately activated genes, *Meis1*, *HoxA9*, *HoxA10*, *HoxA5*, *Mef2c*, *Mylk*, *Stau2* and *Itf2* (also known as *Tcf4*) were also identified in our study. The genes *Peli1*, *Runx2* and *Laptm4b* could not be identified as targets but instead the family members *Peli2* and *Runx1* were found to be differentially expressed.

One main problem of gene expression studies is the choice of a control population of cells for a gene expression analysis. The phenotype of the immortalised target population of cells can only be categorised into HSC and the various different progenitor populations. This is due to the lack of enough characterised cell surface marker, specific for the different sub-stages of haematopoietic cell maturation. Krivtsov *et al.* describe the target population of cells as a GMP-like population of leukaemic cells that is most likely a subset of GMP (Krivtsov *et al.*, 2006). Given that this is the fact, the defined progenitor population of GMP is still a heterogeneous population of progenitor cells with a heterogeneous gene expression pattern. Therefore, the population of GMP do not provide an appropriate group of cells for

this kind of gene expression studies. Another study uses a different approach and compares MLL-AF9 immortalised human $CD34^+$ cord blood cells with retrovirally immortalised cell lines, expressing one of the CBF fusion genes *AML1-ETO* or *CBF-MYH11* (Wei *et al.*, 2008). These comparisons will most likely only allow for identification of MLL-fusion target genes that have the most marked expression changes.

In our study, lineage depleted HPC, comprising a heterogeneous population of cells, including HSC and various progenitor populations, were transduced with MLL-fusion oncogenes under myeloid conditions. This resulted in an immortalised population of cells displaying phenotypic characteristics of differentiation arrest at a late state of myelomonocytic differentiation downstream of the GMP (c-kit^+Gr-1^+Mac-1^{hi} phenotype, Figure 24). A major advantage of using the Tet-off system is that upon loss of the MLL-fusion, the cells terminally differentiate and the phenotype of the population simultaneously shifts to c-kit^-Gr-1^{hi}Mac-1^{hi}, associated with a more differentiated myeloid phenotype. Since it is not possible to precisely define the phenotype of the target population of cells, it is better to analyse the loss of the oncogene in transformed cells. Comparing the gene expression profile of cells expressing the MLL-fusion with cells after loss of the MLL-fusion allows identifying pathways that are directly affected by MLL-fusion mediated transformation.

By not restricting the analysis to the top most differentially expressed genes but rather including all of the differentially expressed genes, we were able to identify several genes associated with the Wnt signalling pathway. Besides the crucial role of Wnt signalling in embryonic development and regeneration of tissue in adult organisms, several studies have implicated a role of Wnt signalling in

haematopoiesis. It was shown in studies with human $CD34^+$ cells and WNT-expressing co-cultures, that WNT signals can provide proliferative signals for immature HPC and HSC (Austin *et al.*, 1997; Van Den Berg *et al.*, 1998). Wnt signalling was also described in association with self-renewal of murine HSC (Reya *et al.*, 2003; Willert *et al.*, 2003). This is of interest, since self-renewal is an important feature of leukaemic stem cells, Wnt signalling might also play a role in leukaemia. Furthermore, among the direct transcriptional target genes of Wnt signalling are *c-Myc* and *c-Myb* (reviewed in Barker and Clevers 2006; Klaus and Birchmeier 2008). Both of these genes were among the differentially expressed genes of the present study and have been reported to be important in driving cancer formation. *c-Myb* is found to be overexpressed in many leukaemias and is required for normal HSC development (Mucenski *et al.*, 1991; Emambokus *et al.*, 2003). *c-Myc* is a central regulator of proliferation, differentiation, cell survival and neoplastic transformation and is found mutated or overexpressed in most human cancers, including myeloid leukaemia (reviewed in Levens, 2003). Therefore, it is not surprising that *c-Myc* and *c-Myb* were found to be up-regulated by MLL-fusion oncogenes in the present study. However, it has previously been reported that MLL-ENL requires *c-Myc* expression to establish a differentiation arrest of a myelomonocytic progenitor population (Schreiner *et al.*, 2001). Furthermore, the IPA pathway analysis tool was used to identify networks in which these genes might be implicated and which could potentially be regulated by the MLL-fusion genes. Strikingly, almost all genes which have known relationships with *c-Myc* were found to be differentially expressed. This high number of involved genes suggests that the *c-Myc* network may be targeted by MLL-fusion mediated transformation. Further analysis of immortalised cells after loss of *c-Myb* or *c-Myc* expression is required, in order to investigate the role of these genes as primary transcriptional targets of MLL-

fusion mediated transformation. In addition, MLL-fusion target genes, such as *HoxA9* and *Meis1*, are also found to be MLL target genes. In a study to identify human genomic binding sites of MLL1, Guenther *et al.* reported *c-Myc*, *Sox4*, *Pontin* and *Reptin* among the target genes of MLL1 (Guenther *et al.*, 2005). In total 236 of our identified target genes are also identified as target genes of human MLL1.

The human orthologs of the genes *Frat1*, *Msi2*, and *Sox4*, identified in this study, are also differentially expressed in patients with 11q23 translocations. The murine *Msi2* shares 75% amino acid identity with its paralogue *Msi1* and has been suggested to share its role in the maintenance and proliferation of neuronal stem cells (Sakakibara *et al.*, 2001). There is little evidence of a role in haematopoietic malignancies besides its association with 11q23 translocations. However, one study reported a novel in-frame *MSI2-HOXA9* fusion transcript which was found to be involved in the disease progression of CML (Barbouti *et al.*, 2003). Frat1 and Frat2 are positive regulators of Wnt signalling. By binding to GSK3, Frat prevents the phosphorylation of Ctnnb1 and allows the activation of downstream target genes by Ctnnb1/TCF complexes (reviewed in van Amerongen, 2005). There is evidence for involvement of *Frat1* in tumour progression of T-cell lymphomas and it was found to be overexpressed in t(11q23)/MLL leukaemias (Jonkers *et al.*, 1997, Kohlmann *et al.*, 2005). Since we also found *Frat1* to be up-regulated in transformed cells, maybe it plays a role in MLL-mediated transformation. Further analysis of these genes is required to elucidate their role in the underlying mechanism of oncogenic transformation induced by MLL-fusion proteins.

In general, gene expression profiling provides a global impression of the relative amount of mRNA expressed in cells under different conditions and allowed

us to identify genes regulated by MLL-AF9 and MLL-ENL. While global gene expression profiling lacks the quantitative accuracy of qPCR, it facilitates high-throughput identification of target genes that show statistically significant changes under different conditions, by analysing all genes of a cell simultaneously. A caveat of these experiments is that changes in RNA expression are not always reflected at the level of protein expression. Expression profiling cannot account for post-translational modifications of proteins or mechanisms regulating protein stability. Therefore, protein expression and functional analysis of target genes is necessary to confirm and validate gene expression analysis results.

CHAPTER V - Target Gene Validation

In order to validate the results from the Affymetrix global gene expression analysis, protein expression of target genes needs to be validated by Western blot analysis. In addition, target genes can also be functionally validated by knocking down up-regulated target genes or by over expressing down-regulated target genes. Expression of non-physiologically high amounts of a down-regulated target gene can produce non-specific effects, making the validation of down-regulated genes more complicated. Hence, we decided to initially choose the faster approach of knocking down up-regulated target genes in the MLL-AF9 and MLL-ENL immortalised cells, using shRNA. This method was used as an initial screen to select for critical target genes for further analysis. Following this initial screen, levels of knock-down and expression changes of the selected genes of interest, with and without Doxycycline treatment, were validated by qPCR and Western blot analysis. Both conditionally and constitutively immortalised cell lines were used for validation. Conditionally immortalised cells had to be used in order to analyse the effect of Doxycycline. Since we did not want to reduce the analysis to one type of immortalised cells, we also used constitutive cells for validation when possible. Additionally, using constitutive cell lines also validated the results of the Affymetrix screen, which was done with the conditionally immortalised cells.

5.1. Screening for critical target genes by knock-down

A selection of thirteen genes were picked for knock-down from the list of up-regulated target genes which were selected by rank and publication for further analysis, as described in Chapter IV (Appendix, Table 22). Although *β-Catenin*

(*Ctnnb1*) was not among the target genes identified by the Affymetrix gene expression analysis, it was also included for analysis because of its pivotal role in the Wnt signalling pathway. Furthermore, *Meis1* was included for analysis since it has been shown to be necessary for induction and maintenance of MLL-fusion induced transformation (Wong *et al.*, 2007). In order to knock down a target gene, hairpin oligonucleotides were designed against the sense strand of the target gene of interest (Appendix, Table 21). Constitutive MLL-AF9 and MLL-ENL immortalised cell lines were then retrovirally transduced with a vector that expresses hCD2 from the same mRNA giving rise to the cloned shRNA (Stegmeier *et al.*, 2005). As an indication of the effect of the knock-down on immortalised cells, enrichment or loss of hCD2 expression from the culture was monitored by flow cytometry (Table 18). Knock-down of the two genes, *c-Myb* and *Msi2*, had a marked effect on the transduced cells, whereas knock-down of *Frat1* and *c-Myc* had only a slight effect. In contrast, knock-down of the other selected target genes did not have any effect on the immortalised cells.

Constitutively immortalised cME3 cells were retrovirally transduced with one of three different shRNA knocking down *c-Myb* expression (22B, 23A and 24D) or with a non-silencing verified negative control shRNA (CO, Open Biosystems RHS1707). The effect of *c-Myb* knock-down on the percentage of hCD2 expressing cells in the cultures was measured by flow cytometry, two, seven and 14 days after transduction (Figure 36 A). A significant reduction of hCD2 expressing cells was observed seven days after transduction. Then, 14 days after transduction, hCD2 expressing cells had almost completely disappeared from the cultures. As expected, cells transduced with the control shRNA were not lost from the culture, shown by the relatively constant percentage of hCD2 expression in the control culture.

Table 18: Tested shRNA and their effect on hCD2 expressing cells

Gene Name	shRNA number	Loss of $hCD2^+$ expressing cells
Meis1	1	-
	2	-
	3	-
Zfhx1b	4	-
	5	-
	6	-
P2ry2	7	-
	8	-
	9	-
Hpgd	10	-
	11	-
	12	-
Frat1	13	-/+
	14	-/+
	15	-/+
Frat2	16	-
	17	-
	18	-
Fzd7	19	-
	20	-
	21	-
c-Myb	22	+
	23	+
	24	+
Msi2	25	+
	26	+
	27	+
Sox4	28	-
	29	-
	30	-
Gata2	31	-
	32	-
	33	-
Ctnnb1	34	-
	35	-
	36	-
c-Myc	37	-/+
	38	-/+

The table lists the 13 target genes and their corresponding shRNA identification number that were tested in this study. shRNA that had a significant effect on the percentage of hCD2 expressing cells are marked with +. Effects that were not clear are marked with -/+ and no effects are marked with -.

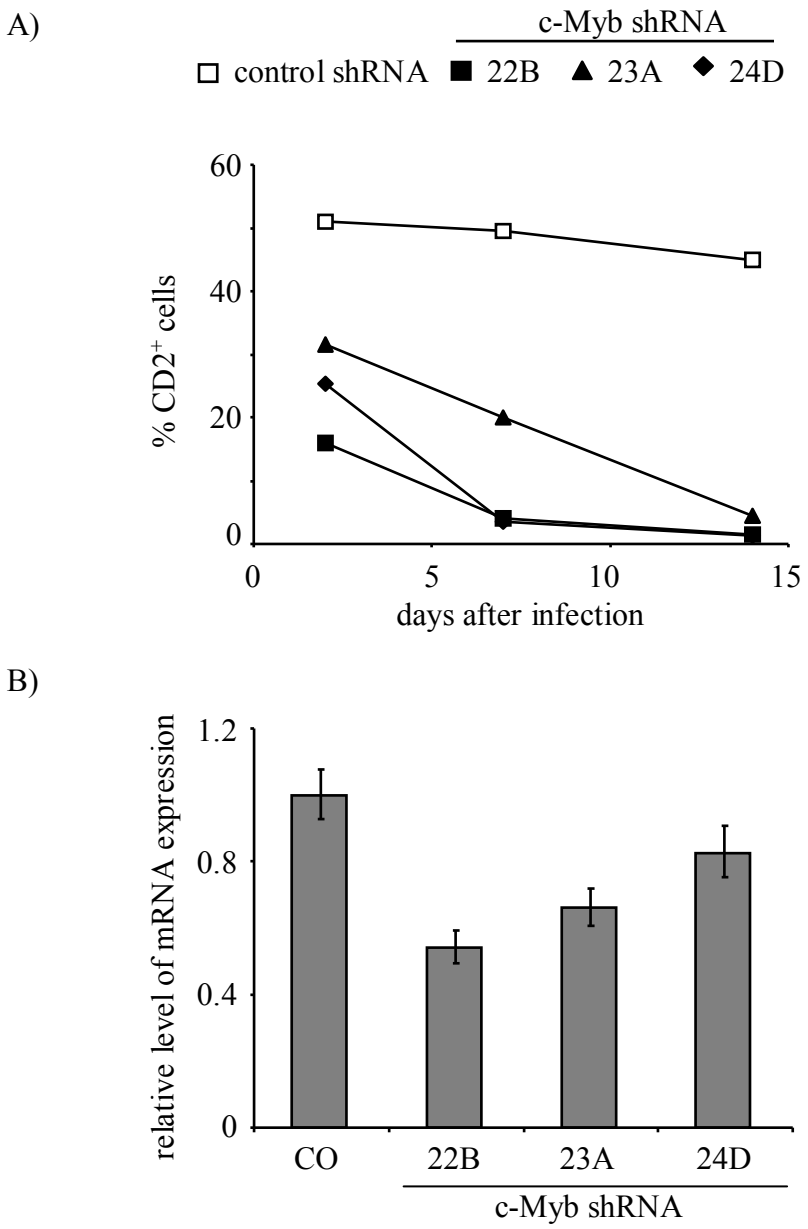


Figure 36: Analysis of c-Myb knock down using shRNA

A) The figure shows the percentage of hCD2$^+$ cME3 cells 2, 7 and 14 days after retroviral transduction with the c-Myb specific shRNA 22B (black squares), 23A (black triangles), 24D (black diamonds) and with control shRNA (open squares). Similar results were observed in three independent experiments. B) The graph shows the relative expression of c-Myb mRNA in hCD2$^+$ cells, 48 hours after infection with either one of the c-Myb specific shRNA (22B, 23A, 24D) or the non-silencing negative control shRNA (CO). Similar results were observed in two independent experiments. Columns represent the mean of triplicate measurements, error bars represent the standard deviations.

In order to measure the level of *c-Myb* knock-down, transduced cells were sorted for hCD2 expression by MACS, 48 hours after transduction, and total RNA was isolated from the purified $hCD2^+$ cells and used for qPCR analysis (Figure 36 B). Expression of *c-Myb* mRNA was reduced in all three analysed knock-downs (22B: $p < 0.0016$, 23A: $p < 0.0001$, 24D: $p < 0.29$; two-tailed students t-test). shRNA 22B showed the most marked reduction in *c-Myb* expression and shRNA 24D showed the weakest reduction. Interestingly, the level of mRNA knock-down did not correlate with the effect of the construct on the reduction of hCD2 expressing cells in culture.

The same experiment was performed using three different shRNA to knock down *Msi2* (25B, 26A and 27B) and hCD2 expression was measured two, seven, 14 and 21 days after retroviral transduction (Figure 37 A). Compared to the non-silencing control shRNA, all three *Msi2*-specific shRNA transduced cells showed a reduction in the percentage of hCD2 expression 14 days after retroviral transduction. *Msi2* mRNA expression in sorted hCD2 cells was significantly reduced in all three knock downs (25B: $p < 0.0003$, 26A: $p < 0.0005$, 27B: $p < 0.0002$, two-tailed students t-test) (Figure 37 B). Knock-down of the two genes, *Frat1* and *c-Myc*, did not have a consistent effect on the hCD2 expressing cells. A reduction of hCD2 expression in culture was observed only in cells transduced with the *Frat1* specific shRNA 14A and 15B, and the *c-Myc* specific shRNA 37B (Figure 38). The data for *Frat1* and *c-Myc* need to be further validated by analysing the level of knock-down of each shRNA by qPCR. If necessary, the level of gene knock-down could be improved by designing new shRNA or co-transducing the cells with multiple shRNA, targeting the same gene.

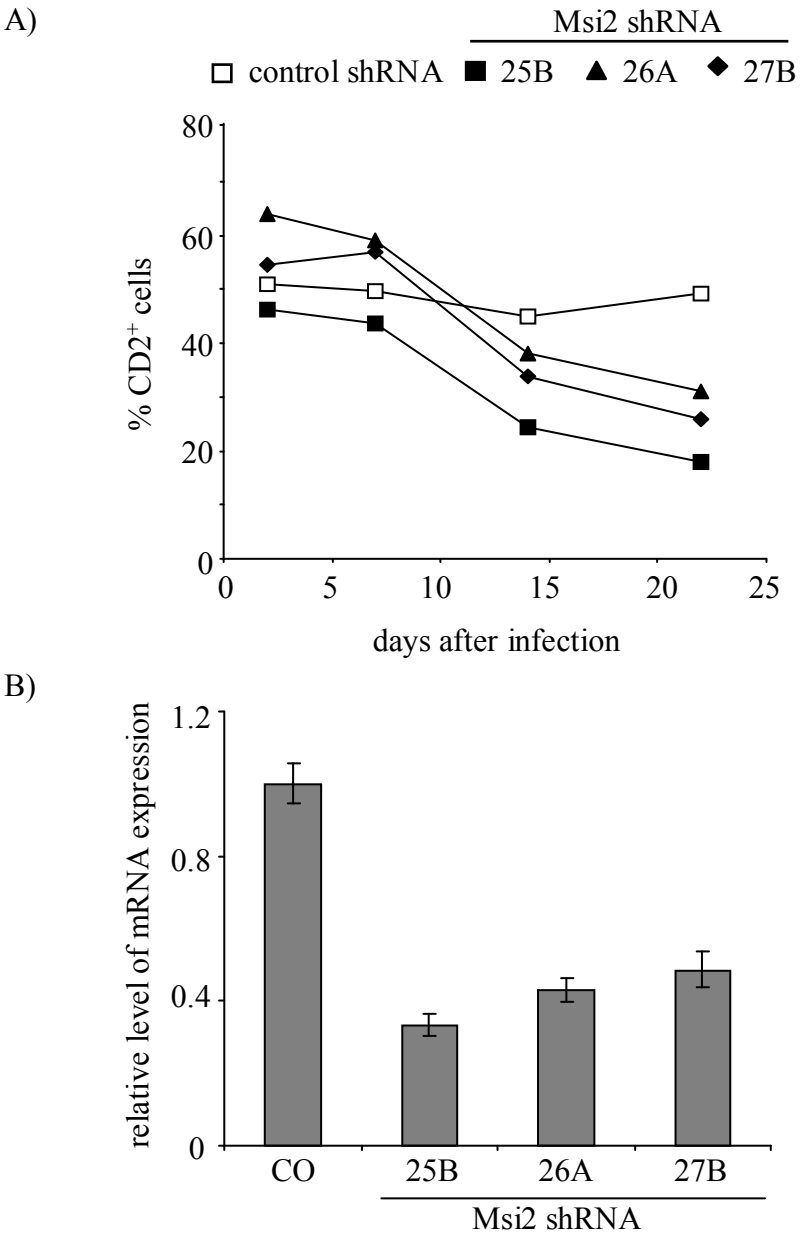


Figure 37: Analysis of Msi2 knock down using shRNA

A) The figure shows the percentage of hCD2$^+$ cME3 cells 2, 7, 14 and 21 days after transduction with the Msi2 specific shRNA 25B (black squares), 26A (black triangles), 27B (black diamonds) and with control shRNA (open squares). Similar results were observed in two independent experiments. B) The graph shows the relative expression of Msi2 mRNA of hCD2$^+$ cells 48 hours after infection with either one of the Msi2 specific shRNAs (25B, 26A, 27B) or the non-silencing negative control shRNA (CO). Columns represent the mean of triplicate measurements, error bars represent the standard deviations.

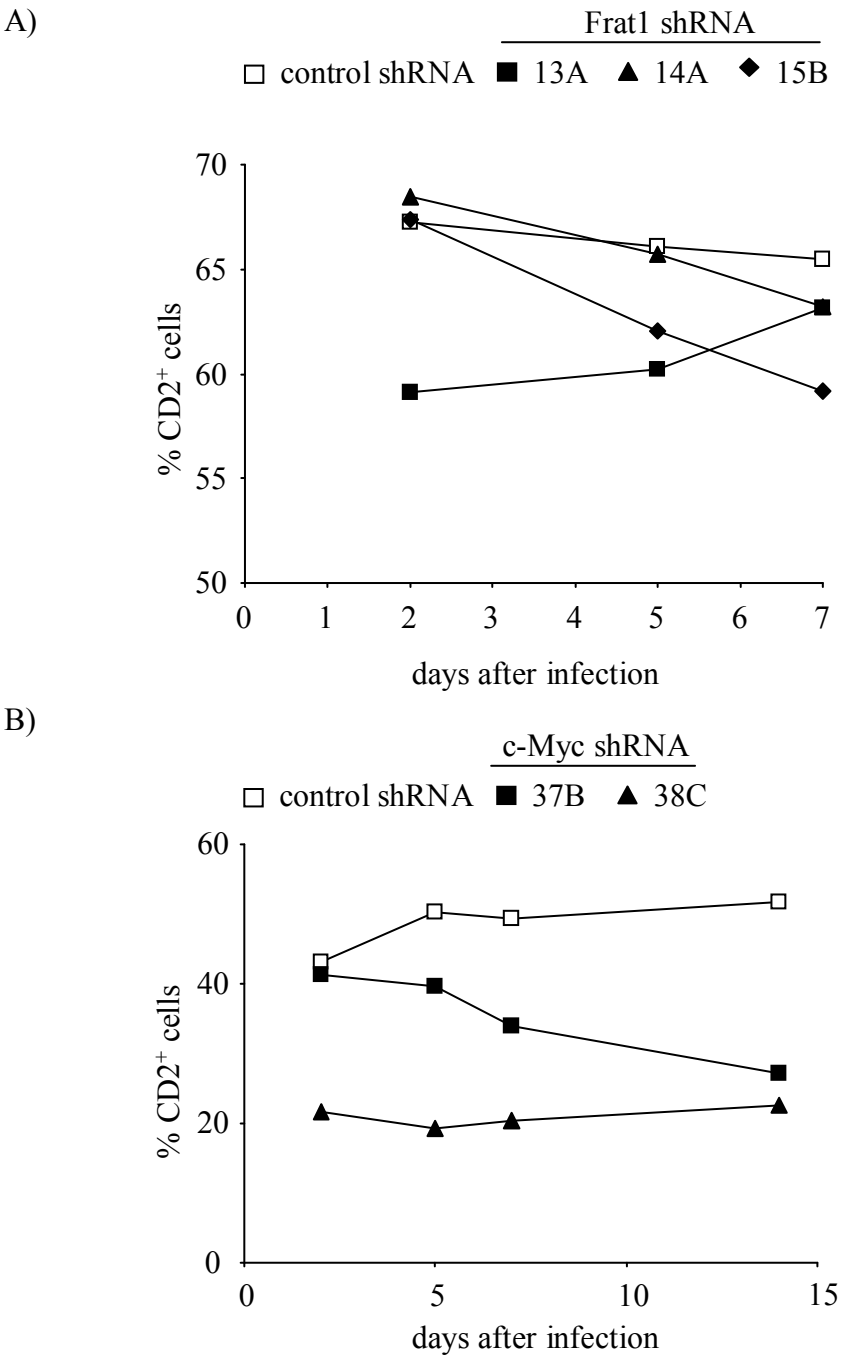


Figure 38: Analysis of Frat1 and c-myc knock down using shRNA

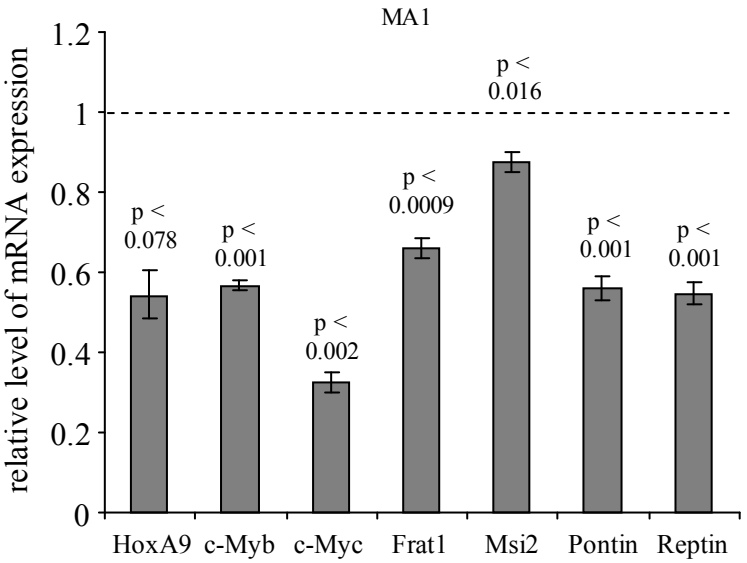
The figure shows the percentage of hCD2$^+$ cME3 cells 2, 5 and 7 days after transduction with the Frat1 specific shRNA 13A (black squares), 14A (black triangles), 15B (black diamonds) and with control shRNA (open squares) (A). Similar results were observed in three independent experiments. B) Percentage of hCD2$^+$ cME3 cells 2, 5, 7 and 14 days after transduction with the c-Myc specific shRNA 37B (black squares), 38C (black triangles) and with control shRNA (open squares).

If optimising *Frat1* and *c-Myc* knock-down reveals these genes to be important for MLL-fusion mediated immortalisation, they will be included in further analyses in future work.

5.2. Validation of mRNA and protein expression of selected target genes upon loss of MLL-fusion expression

The target genes *HoxA9*, *c-Myb*, *c-Myc*, *Frat1*, *Msi2*, *Pontin* and *Reptin* were up-regulated in the presence of the MLL-fusions and were selected for further validation. Although *Pontin* and *Reptin* were not part of the previous knock-down analysis, they were found in the list of up-regulated target genes and are involved in Wnt signalling (Chapter IV, Table 16) by interacting with *β-Catenin* as well as *c-Myc*. In order to validate the gene expression changes that were observed by the Affymetrix screen, expression levels of the target genes were validated by qPCR analysis. For validation, total RNA independent of the preparation used for the Affymetrix analysis was isolated from the conditional cell lines MA1 and ME4, maintained with or without Doxycycline for 48 hours. As expected, target gene expression was down-regulated upon loss of MLL-fusion expression in all cases (Figure 39). In addition, we were able to validate *c-Myb* and *Pontin* protein expression by Western blot analysis with antibodies we had available (Figure 40). Conditionally immortalised MA1 and ME4 cells were maintained in the absence and presence of Doxycycline for 48 hours and cell pellets were lysed for Western blot analysis. A significant down-regulation of *c-Myb* and *Pontin* protein expression was confirmed in MA1 and ME4 cells upon loss of MLL-fusion protein expression.

A)



B)

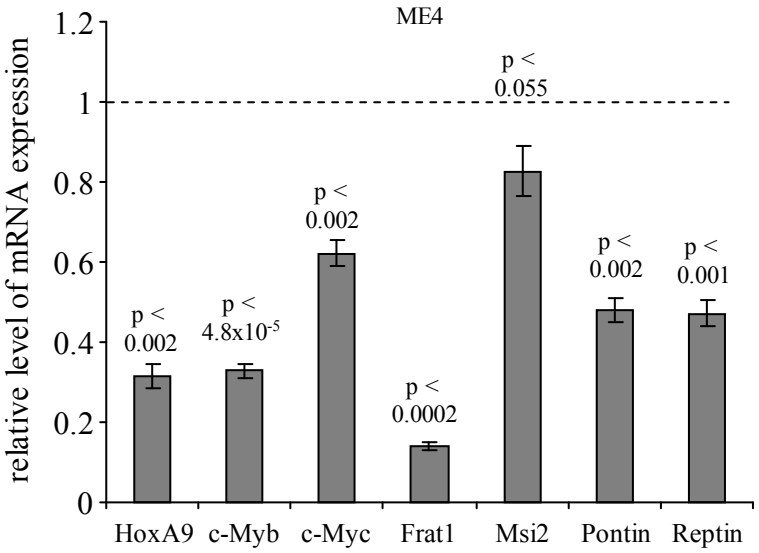


Figure 39: Validation of mRNA expression of target genes

The graphs show the relative expression of *HoxA9*, *c-Myb*, *c-Myc*, *Frat1*, *Msi2*, *Pontin* and *Reptin*, 48 hours after treatment with Doxycycline in MA1 (A) and ME4 (B). Each target gene is individually normalised to its expression level in untreated cells, shown as dotted line. Columns represent the mean of triplicate measurements, error bars represent the standard deviations.

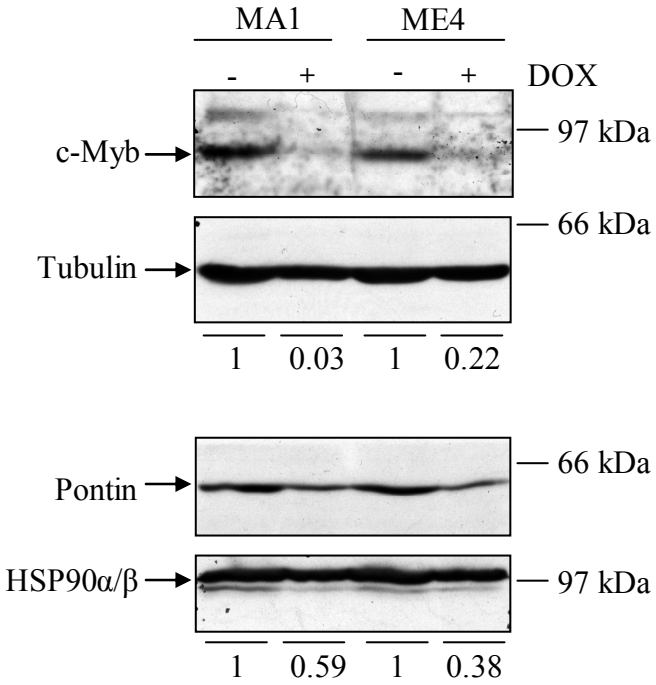


Figure 40: Validation of c-Myb and Pontin protein expression

The figure shows Western blot analysis of protein extracts from immortalized conditional MA1 and ME4 cells without Doxycycline (lane 1 and lane 3) or 48 hours with Doxycycline treatment (lane 2 and lane 4). Anti-c-Myb antibody was used to detect c-Myb (75 kDa) and anti-αTubulin (55 kDa) antibody was used as the protein loading control (10% resolving gel). Anti-Pontin52 antibody was used to detect Pontin (52 kDa) and anti-HSP90α/β (90 kDa) antibody was used as the protein loading control (12.5% resolving gel). Films were scanned and bands quantitated (see Materials and Methods). The relative protein expression, normalised to untreated cells, is given in numbers underneath.

In future experiments, *c-Myc*, *Frat1*, *Msi2* and *Reptin* protein expression protein expression will also be analysed and validated before further analysis of their role in MLL-fusion mediated transformation.

5.3. Validation of the role of Siah1a in MLL-mediated transformation

Although *β-Catenin* itself, the central regulator of Wnt signalling, was expressed in MLL-ENL and MLL-AF9 immortalised cells, its expression was not differentially regulated by the MLL-fusions. However, several of the Wnt signalling associated genes were detected as differentially expressed target genes. Therefore, we decided to reassess the expression levels of *β-Catenin* in Doxycycline treated conditional MA1 and ME4 cells by qPCR and Western blot analysis. In contrast to the results from the Affymetrix analysis, *β-Catenin* mRNA expression was down-regulated upon loss of MLL-fusion expression in both cell lines (Figure 41 A). Total β-Catenin protein expression was detected using an antibody that recognises both the inactive, phosphorylated and the active, dephosphorylated β-Catenin protein. Total cell lysates were prepared and examined by Western blot analysis. A significant down-regulation of total β-Catenin protein expression was detected in MA1 and ME4 cells cultured in the presence of Doxycycline for 48 hours (Figure 41 B).

Cytoplasmic levels of β-Catenin are usually regulated by phosphorylation through a destruction complex, consisting of APC, GSK3β and Axin, leading to ubiquitination and proteasomal degradation. β-Catenin can also be ubiquitinated and proteasomal degraded via an alternative Siah1a dependent pathway. The E3 ubiquitin ligase *Siah1a* was among the differentially expressed target genes and found to be down-regulated in the presence of MLL-fusion oncogenes.

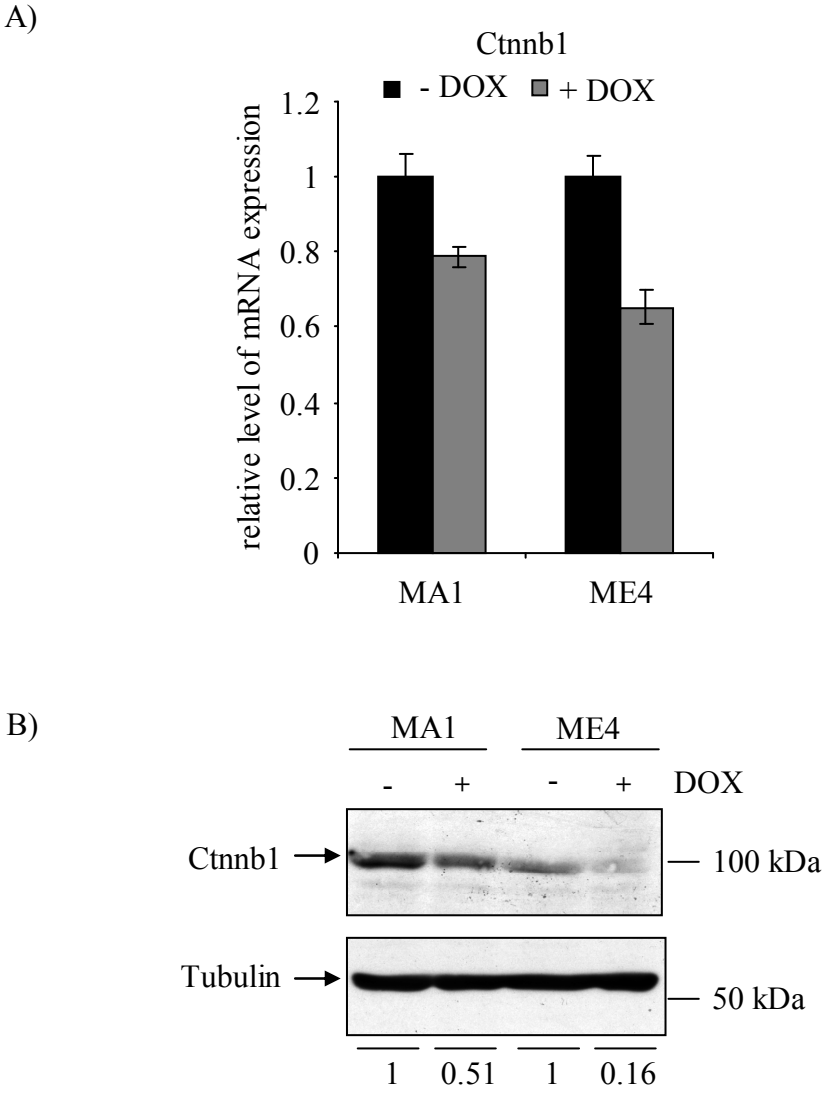
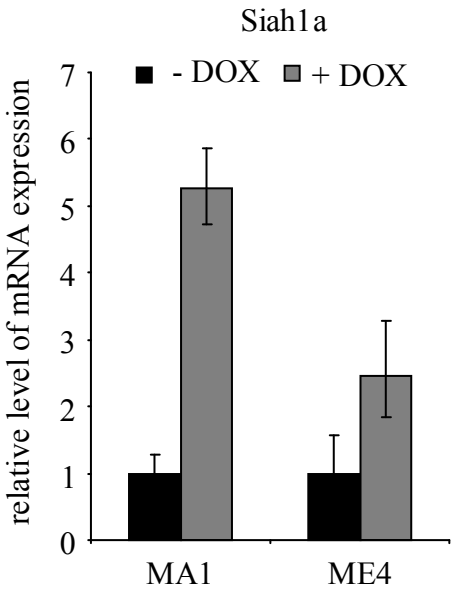


Figure 41: Validation of mRNA expression and protein expression of Ctnnb1

A) The graph shows the relative expression of *Ctnnb1* mRNA without (black) and 48 hours after treatment with Doxycycline (grey) in conditional immortalised MA1 ($p<0.012$) and ME4 ($p<0.003$) cells. Columns represent the mean of triplicate measurements, error bars represent the standard deviations. B) The figure shows Western blot analysis of protein extracts from immortalized conditional MA1 and ME4 cells without Doxycycline (lane 1 and lane 3) or 48 hours with Doxycycline treatment (lane 2 and lane 4). Anti-Ctnnb1 antibody was used to detect total Ctnnb1 (90 kDa) and anti-αTubulin (55 kDa) antibody was used as the protein loading control (10% resolving gel). Films were scanned and bands quantitated. The relative protein expression, normalised to untreated cells, is given in numbers underneath.

We decided to study Siah1a in greater detail because Siah1a targets not only β-Catenin for proteasomal degradation, but also AF4, a central component of the MLL-fusion complex, and c-Myb, an essential downstream mediator of MLL-fusion activity (Bursen *et al.*, 2004; Mueller *et al.*, 2007). qPCR analysis confirmed that in the absence of MLL-AF9 or MLL-ENL fusion gene expression, the relative level of *Siah1a* mRNA expression was up-regulated compared to cells expressing the MLL-fusion (Figure 42 A). In contrast to *Siah1a* mRNA expression, protein expression of Siah1a in MA1 and ME4 cells was down-regulated upon loss of MLL-fusion expression (Figure 42 B). However, this contrary observation could possibly be explained by the capacity of Siah1a to auto-ubiquitinate itself (Lorick *et al.*, 1999). In that case, increased expression of Siah1a may lead to increased auto-ubiquitination and consequently degradation, resulting in lower levels of the protein. Siah1a and Siah2 have previously been identified as AF4 binding proteins and Siah1a has been shown to promote proteasomal degradation of wild-type AF4 and the reciprocal MLL-fusion protein AF4-MLL (Bursen *et al.*, 2004). However, *AF4* was not detected as a differentially regulated target gene of MLL-ENL or MLL-AF9 immortalised cells, which was validated by qPCR analysis (Figure 43 A). *AF4* expression was found to be up-regulated upon loss of MLL-AF9 in MA1 cells but was not significantly regulated in ME4 cells. In order to measure AF4 protein levels, MA1 and ME4 cells were cultured for 48 hours with and without Doxycycline and total cell lysates were prepared and examined by Western blot analysis. Since AF4 has been described to be a Siah1a target, increased Siah1a activity in the absence of the MLL-fusion proteins would correlate with down-regulation of AF4 protein expression. Upon loss of MLL-fusion expression a significant down-regulation of AF4 protein expression was detected in both cell lines (Figure 43 B).

A)



B)

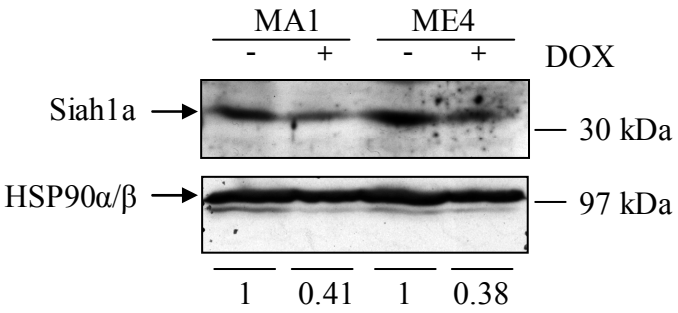


Figure 42: Validation of mRNA expression and protein expression of Siah1a

A) The graph shows the relative expression of *Siah1a* mRNA without (black) and 48 hours after treatment with Doxycycline (grey) in conditionally immortalised MA1 and ME4 cells. Columns represent the mean of triplicate measurements, error bars represent the standard deviations. B) The figure shows Western blot analysis of protein extracts from immortalized conditional MA1 and ME4 cells without Doxycycline (lane 1 and lane 3) or 48 hours with Doxycycline treatment (lane 2 and lane 4). Anti-Siah1 (N15) antibody was used to detect Siah1a (32 kDa) and anti-HSP90α/β (90 kDa) antibody was used as the protein loading control (12.5% resolving gel). Films were scanned and bands quantitated. The relative protein expression, normalised to untreated cells, is given in numbers underneath.

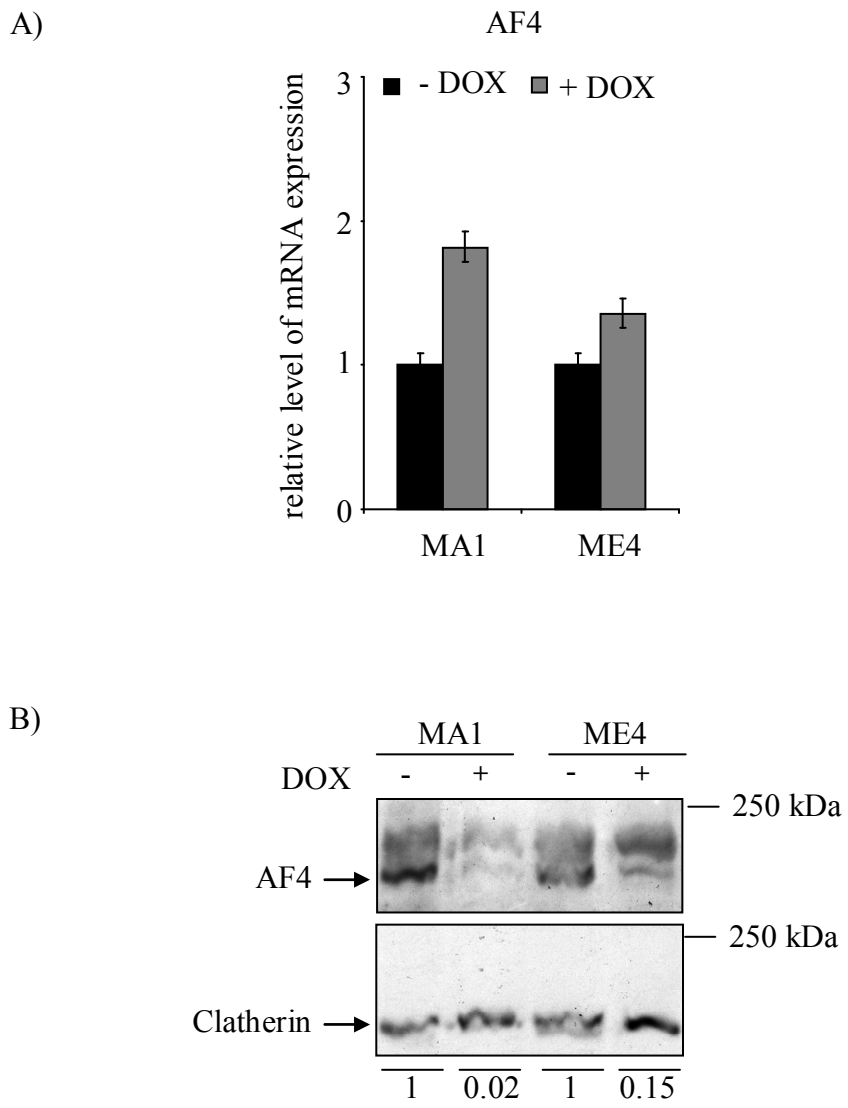


Figure 43: Validation of mRNA expression and protein expression of AF4

A) The graph shows the relative expression of *AF4* mRNA without (black) and 48 hours after treatment with Doxycycline (grey) in conditional immortalised MA1 and ME4 cells. Columns represent the mean of triplicate measurements, error bars represent the standard deviations. B) The figure shows Western blot analysis of protein extracts from immortalized conditional MA1 and ME4 cells without Doxycycline (lane 1 and lane 3) or 48 hours with Doxycycline treatment (lane 2 and lane 4). Rabbit anti-mouse AF4 antiserum (kind gift of E. Bitoun and K. Davies) was used to detect AF4 (193 kDa) and anti-Clatherin (190 kDa) antibody was used as the protein loading control (5% resolving gel). Films were scanned and bands quantitated. The relative protein expression, normalised to untreated cells, is given in numbers underneath.

Further experiments are required in order to assess MLL-fusion mediated regulation of Siah1a and link increased Siah1a activity in the absence of MLL-fusions to degradation of its described targets, such as AF4, β-Catenin and c-Myb.

5.4. Validation of the effect of Hexachlorophene on Siah1a in MLL-fusion transformed cells

In a cell based small-molecule screen, the antimicrobial compound Hexachlorophene (Hexa) was identified as an inhibitor of Wnt signalling (Park *et al.*, 2006). It was proposed that Hexachlorophene induces proteasomal degradation of β-Catenin in a Siah1a-dependent manner (Park *et al.*, 2006). In order to examine the role of Siah1a and Wnt signalling in MLL-fusion mediated transformation, Hexachlorophene was used to study induction of Siah1a function without abrogating the expression of MLL-AF9 or MLL-ENL. Previous studies demonstrated that Hexachlorophene inhibits the growth of human colon cancer cells in a concentration-dependent manner (Park *et al.*, 2006). The effect of different Hexachlorophene concentrations on the viability of conditionally immortalised MA1 and ME4 cells was therefore examined. Cells were cultured in the presence of 5μM, 10μM or 20μM Hexachlorophene or treated with the vehicle Methanol alone. The number of viable cells was determined on the day of plating (day 0) and 2 days and 3 days after treatment, by Trypan Blue exclusion (Figure 44). Similar to the results in human colon cancer cells, a strong concentration-dependent effect of Hexachlorophene on the viability of the MLL-fusion immortalised cell line was observed.

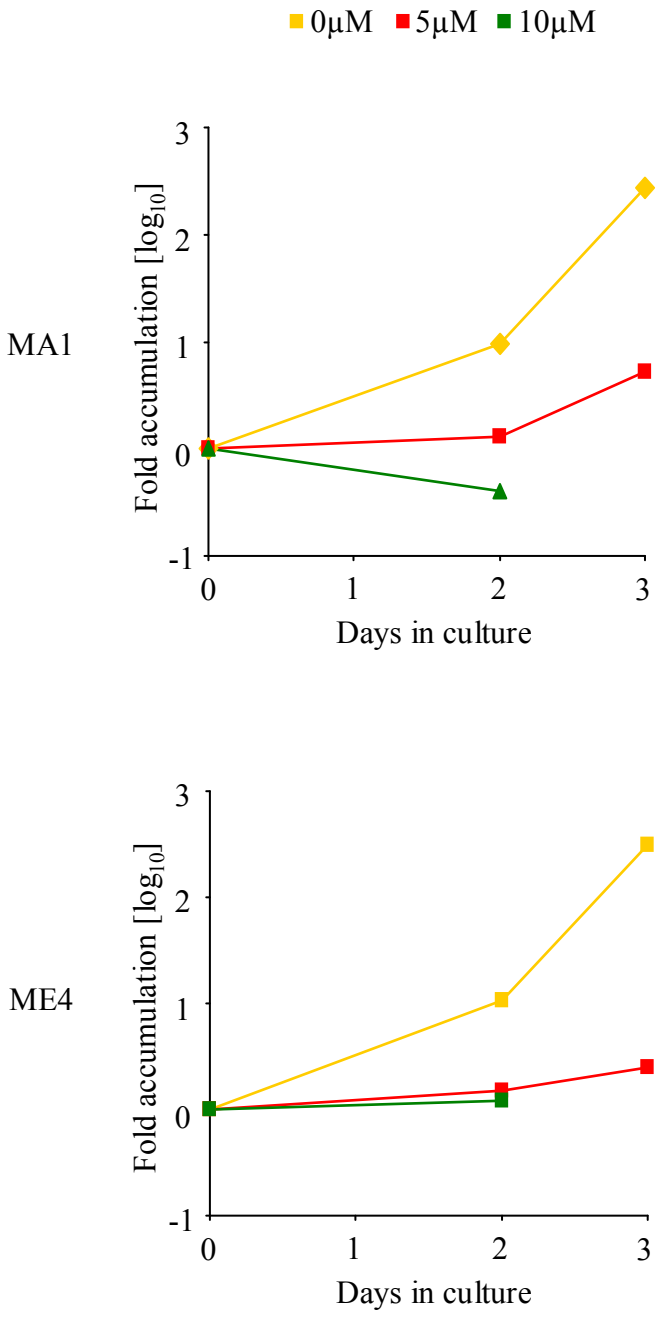


Figure 44: Concentration dependent effect of Hexachlorophene on viability of conditional MA1 and ME4 cells

The graph shows the number of viable cells 2 and 3 days after treatment with increasing concentrations of Hexachlorophene (0µM/yellow, 5µM/red, 10µM/green). There were no viable cells by day two with 20µM Hexachlorphene treatment.

In order to examine whether the effect of Hexachlorophene was a result of differentiation, the expression of the cell surface markers c-Kit and Gr-1 was measured by flow cytometry in the constitutively immortalised cME3 cells. Cells were cultured in the presence of 0.5μM, 1.25μM, 2.5μM, 5μM and 10μM or Methanol alone (0μM) for 24 hours and 5 days. Only treatment with 2.5μM, 5μM and 10μM Hexachlorophene caused up-regulation of the differentiation marker Gr-1 (Figure 45). The progenitor marker c-Kit was down-regulated in cells exposed to 1.25μM, 2.5μM, 5μM and 10μM Hexachlorophene for 5 days. Higher concentrations of Hexachlorophene were correlated with an earlier increase and decrease in expression of Gr-1 and c-Kit, respectively. These results suggest that MLL-fusion oncogene immortalised cells differentiate in response to Hexachlorophene treatment and that the rate of differentiation depends on the concentration of the drug. However, further experiments are necessary in order to verify whether or not MLL-fusion immortalised cells also directly undergo apoptosis in response to treatment with Hexachlorophene.

Since it was suggested that Hexachlorophene induces β-Catenin degradation through a Siah1a mediated mechanism (Park *et al.*, 2006), we decided to analyse the expression of Siah1a protein, following addition of Hexachlorophene to cME3 cells. Cells were cultured in the presence of 20μM Hexachlorophene, or Methanol alone, for 8 hours. Cell pellets were lysed and examined by Western blot analysis. As shown in Figure 46, Hexachlorophene did not induce Siah1a protein expression. However, the observed reduction of Siah1a protein expression upon treatment with Hexachlorophene is similar to the down-regulation of Siah1a protein expression observed upon treatment with Doxycycline (Figure 42).

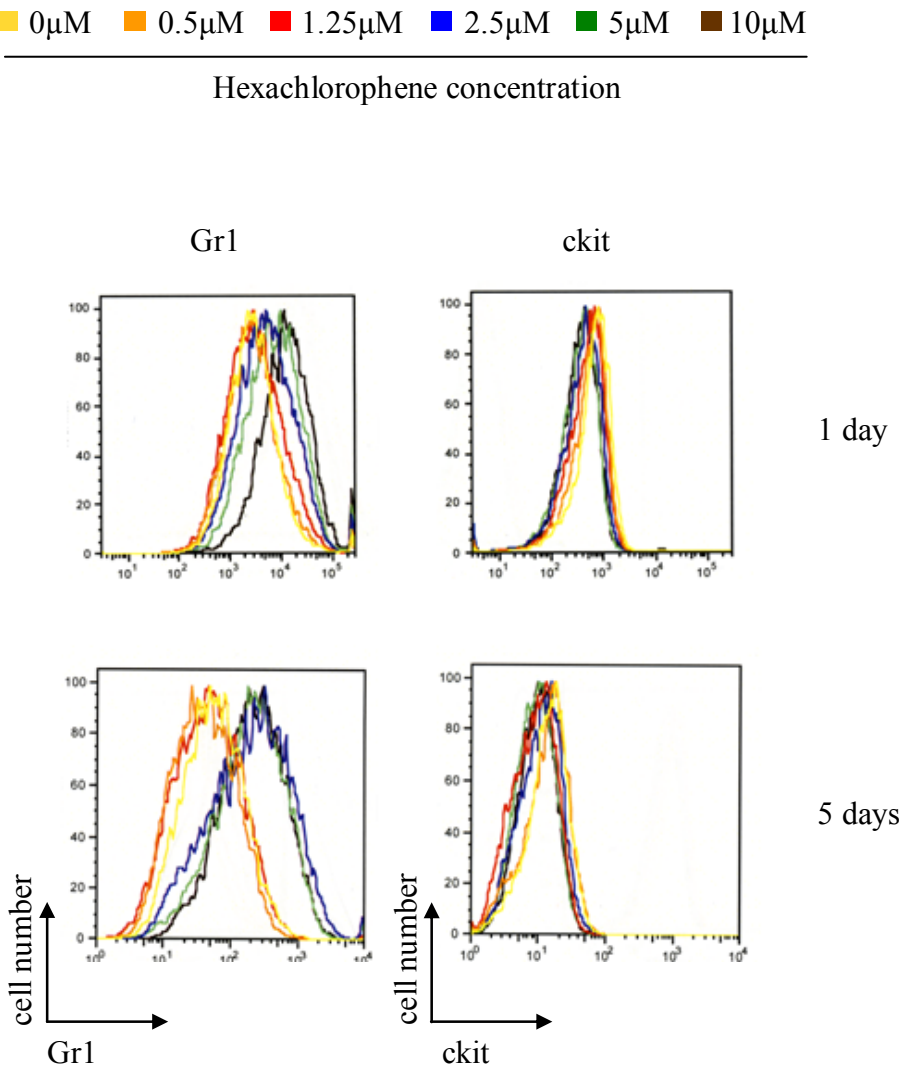


Figure 45: Differentiation analysis of cME3 cells treated with Hexachlorophene

The figure shows the expression levels of Gr-1 and c-Kit in cME3 cells treated with increasing concentrations of Hexachlorophene (0µM yellow, 0.5µM orange, 1.25µM red, 2.5µM blue, 5µM green and 10µM brown) for 1 day and 5 days.

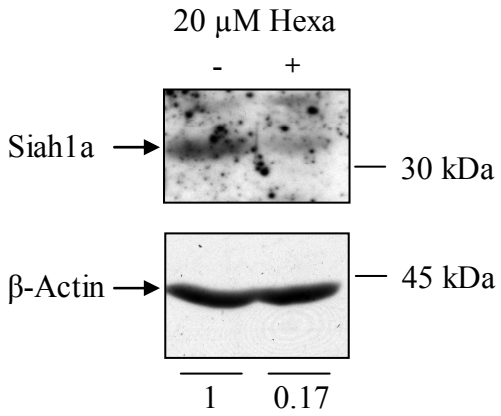


Figure 46: Effect of Hexachlorophene treatment on Siah1a protein expression

The figure shows Western blot analysis of protein extracts from immortalized constitutive cME3 cells cultured without (lane 1) or with 20µM Hexachlorophene (lane 2) for 8 hours. Cell pellets were lysed using 2xDTT sample buffer lysis method. (Anti-Siah1 (N15) antibody was used to detect Siah1a (32 kDa) and anti-β-Actin (45 kDa) antibody was used as the protein loading control (12.5% resolving gel). Films were scanned and bands quantitated. The relative protein expression, normalised to untreated cells, is given in numbers underneath.

Similarly to Doxycycline treatment, this result could possibly be explained by the regulation of elevated levels of Siah1a through auto-ubiquitination and proteasomal degradation. Although detectable Siah1a protein levels are decreased, its level of activity may be increased.

We next examined whether protein expression of Siah1a targets was affected by Hexachlorophene treatment. Protein expression of AF4 and c-Myb, degradation targets of Siah1a, was analysed. cME3 cells cultured in the presence of 20μM Hexachlorophene, or Methanol alone, for 8 hours were lysed and examined by Western blot analysis. In the presence of Hexachlorophene the protein expression of AF4 and c-Myb was significantly down-regulated (Figure 47). This result suggests that changes in AF4 and c-Myb protein expression correlate with Hexachlorophene induced increase of Siah1a activity, independent of the MLL-fusion proteins. However, the mechanism of Hexachlorophene induced Siah1a stimulation remains unclear at present. Park and colleagues have shown that Hexachlorophene induces Siah1a activity and degrades β-Catenin (Park *et al.*, 2006). By analysing the mRNA expression of *c-Myc*, a transcriptional target of c-Myb as well as the Wnt signalling pathway, the Hexachlorophene induced effect of c-Myb down-regulation was further examined. In order to compare *c-Myc* expression in cells treated with Hexachlorophene to that in cells treated with Doxycycline, conditional MA1 cells were maintained for 48 hours with 5μM Hexachlorophene or Methanol alone, or with Doxycycline. mRNA expression of *c-Myc* was significantly down-regulated with both treatments (Figure 48).

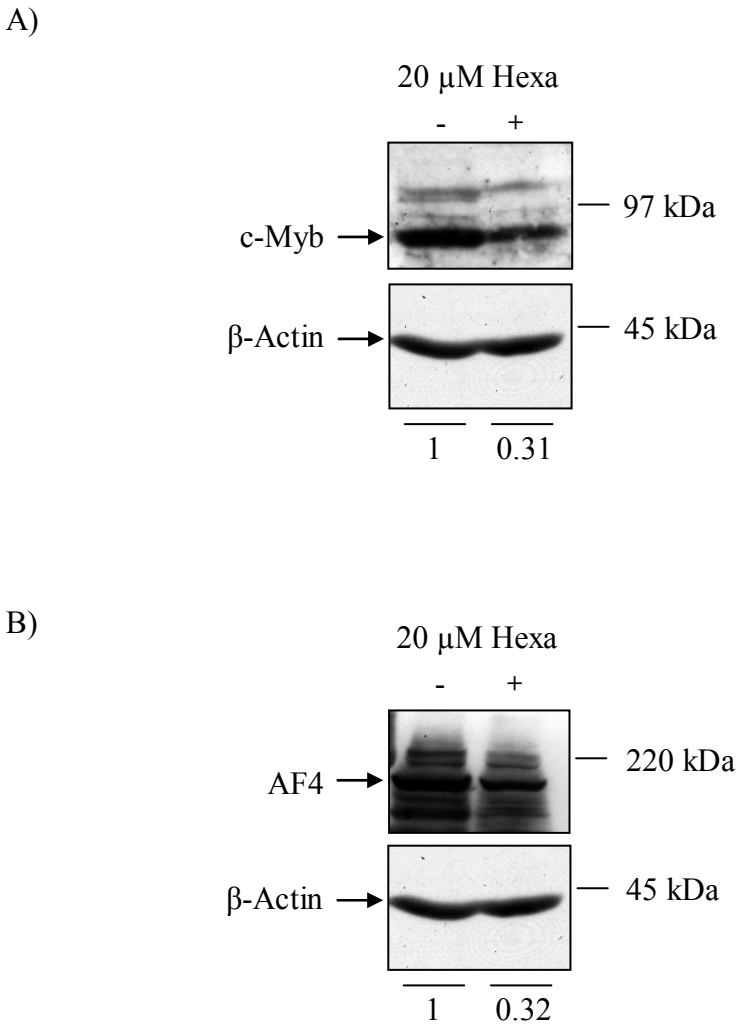


Figure 47: Effect of Hexachlorophene treatment on c-Myb and AF4 protein expression

The figure shows Western blot analysis of protein extracts from immortalized constitutive cME3 cells cultured without (lane 1) or with 20µM Hexachlorophene (lane 2) for 8 hours. Cell pellets were lysed using the 2xDTT sample buffer lysis method. Anti-cMyb antibody was used to detect c-Myb (75 kDa, 10% resolving gel) (A), AF4 antiserum to detect AF4 (193 kDa, 10% resolving gel) (B) and anti-β-Actin (45 kDa) antibody was used as the protein loading control. The relative protein expression, normalised to untreated cells, is given in numbers underneath.

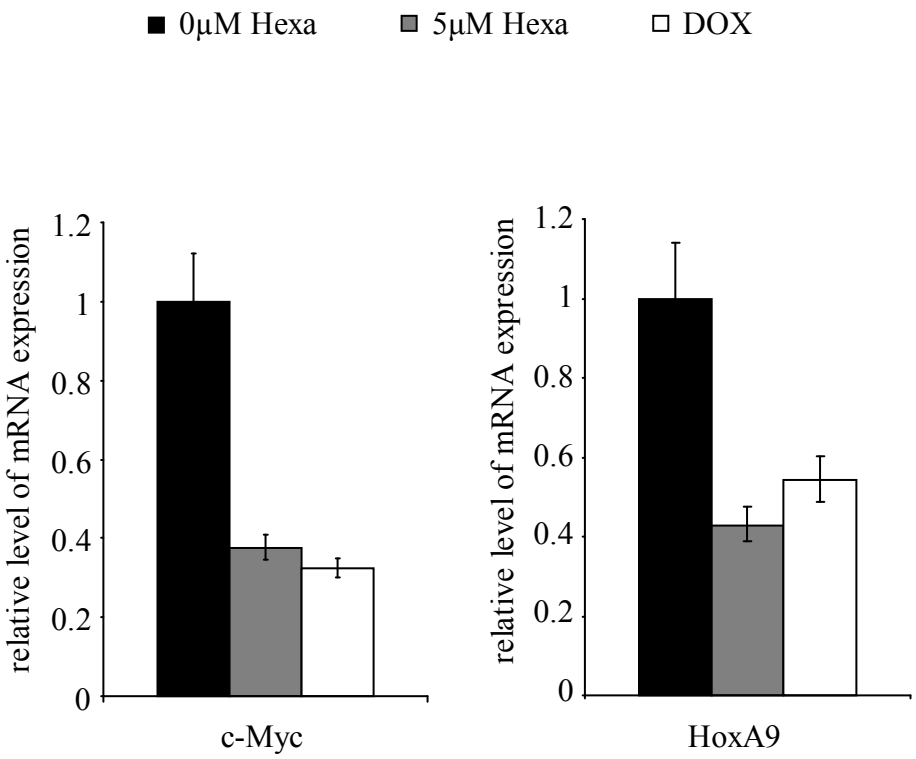


Figure 48: The effect of Hexachlorophene and Doxycycline on *c-Myc* and *HoxA9* mRNA expression

The figure shows the relative expression of *c-Myc* and *HoxA9* mRNA without (black), 48 hours after treatment with 5 μM Hexachlorophene (grey) and 48 hours after treatment with Doxycycline (white) in conditional immortalised MA1 cells. Columns represent the mean of triplicate measurements, error bars represent the standard deviations.

AF4 has been described as a critical member in a complex with MLL and several other MLL fusion partners, such as AF9/ENL and AF10, to recruit DOT1L and coordinate transcriptional elongation (Mueller *et al.*, 2007). Therefore, we decided to examine the mRNA expression of *HoxA9*, a known transcriptional target of this MLL-fusion complex (Figure 48). Since AF4 has been shown to be a Siah1a target for proteasomal degradation we reasoned that transcription of MLL-fusion target genes may be down-regulated upon induced activity of Siah1a. A significant down-regulation of *HoxA9* mRNA expression was observed with both treatments. These results suggest that target genes, identified by the global gene expression analysis upon loss of MLL-fusion expression, are also differentially regulated upon treatment with Hexachlorophene, presumably through induction of Siah1a activity. If it can be proven that Hexachlorophene induces Siah1a activity and has a similar effect to Doxycycline treatment, this would suggest that repression of Siah1a expression may be important for MLL-mediated transformation. Further experiments are necessary in order to prove the direct induction of Siah1a activity by Hexachlorophene and in order to show which of the Siah1a regulated targets are important for MLL-mediated transformation.

5.5. Discussion – Chapter V

Knock-down of the up-regulated target genes *c-Myb*, *Msi2*, *c-Myc* and *Frat1* had an effect on the proliferation of MLL-fusion immortalised cells. Interaction of these target genes with other genes or regulatory pathways was verified by using pathway analysis software. Using this approach, it was possible to identify *c-Myb*, *c-Myc*, *Ctnnb1*, *Siah1a*, *AF4*, *Pontin* and *Reptin* as MLL-fusion target genes which may be involved in a common biological process and represent more than chance associations.

In our study, several of the differentially regulated target genes have been found to be associated with the Wnt signalling pathway. The central mediator of the Wnt signalling pathway is β-Catenin. Wnt ligand binding-induced accumulation of unphosphorylated cytoplasmic β-Catenin results in translocalisation of β-Catenin into the nucleus, where it acts as a transcriptional co-activator. The translocation products AML1-ETO, PML-RARα and PLZF-RARα, all found in AML, have been shown to activate the Wnt signalling pathway in haematopoietic cells (Müller-Tidow *et al.*, 2004). Interestingly, it has been shown that the C-terminal activation domain of β-Catenin associates with the TRRAP and MLL1 SET-1-type chromatin-modifying complexes *in vitro* and that β-Catenin promotes histone H3K4 trimethylation at the *c-Myc* gene *in vivo* (Sierra *et al.*, 2006). Previous work has also shown that β-Catenin interacts with the TRRAP histone acetyltransferase complex subunits, Pontin and Reptin (Bauer *et al.*, 1998; Bauer *et al.*, 2000). Both genes *Pontin* and *Reptin* were found to be up-regulated in MLL-fusion expressing cells, in our study. Furthermore, Pontin protein expression was also shown to be up-regulated in MLL-fusion transformed cells. However, the roles of *Pontin* and *Reptin* in MLL-fusion mediated

transformation need to be further elucidated. This will be done by gene specific knock-down using shRNA or by introducing a mutant form of the *Pontin* protein that has been shown previously to block the expression of β-Catenin target genes (Feng *et al.*, 2003).

Levels of cytoplasmic β-Catenin are regulated by CK1 and GSK3β mediated phosphorylation, leading to βTRCP mediated ubiquitination and subsequently proteasomal degradation (reviewed in Staal and Clevers 2005; Klaus and Birchmeier 2008). A GSK3β and βTRCP independent pathway of β-Catenin degradation has been found to be mediated by APC and Siah1a (Liu *et al.* 2001). Siah1a is an E3-ubiquitin ligase that interacts with the C-terminus of APC and induces ubiquitination and proteasomal degradation of β-Catenin (Liu *et al.*, 2001). Human SIAH1 protein has been shown to contain an N-terminal RING domain, that is required for its proteasomal degradation function, and C-terminal sequences, required for oligomerisation and binding to target proteins (Hu *et al.*, 1997). It has been shown that Siah1 targets itself via RING dependent auto-ubiquitination for proteasomal degradation (Lorick *et al.*, 1999). In our study, *Siah1a* was one of the target genes down-regulated by the MLL-fusions in immortalised cells and was highlighted from the list of down-regulated target genes using pathway analysis tools. Furthermore, literature searches revealed that Siah1a is also a negative regulator of AF4, a critical member of the MLL-fusion complex, and c-Myb (Mueller *et al.*, 2007). It was possible to confirm a significant increase in *Siah1a* mRNA expression upon loss of MLL-fusion expression. However, Siah1a protein expression did not correlate with mRNA but decreased upon loss of MLL-fusion expression. This observed decrease may be a result of an increase in Siah1a activity and a consequent increased auto-ubiquitination and degradation. In order to examine increased Siah1a activity, we

examined protein expression levels of β-Catenin, c-Myb and AF4, all previously identified as targets of Siah1a mediated proteasomal degradation (Tanikawa *et al.*, 2000; Liu *et al.*, 2001; Bursen *et al.*, 2004). It is possible that genes such as *β-Catenin* and *AF4* were initially not identified as differentially expressed by the Affymetrix screen because significant changes in gene expression had to be detected in all six independent cell lines. However, we could show that β-Catenin and AF4 protein expression was significantly decreased in MA1 and ME4 cells, upon loss of the MLL-fusion expression. Furthermore, protein expression of c-Myb was also shown to be decreased. These data suggest that target genes are regulated at the level of transcription by the MLL-fusion oncogene and at the level of protein by MLL-fusion induced *Siah1a* repression.

Although deregulation of c-Myb is found in many cancers and leukaemias (reviewed in Ganter and Lipsick, 1999), this gene was recently described to be an essential transcriptional target of *HoxA9* and *Meis1* overexpression and by extension in MLL-ENL mediated transformation (Hess *et al.*, 2006). On the other hand, as mentioned earlier, c-Myb protein has also been described to be a p53-dependent degradation target of Siah1a (Tanikawa *et al.*, 2000). In our study, we demonstrated that c-Myb is up-regulated in MLL-ENL and MLL-AF9 immortalised cells and confirmed increased levels of mRNA and protein expression in the presence of fusion gene expression. However, the mechanism of MLL-fusion mediated c-Myb regulation remains unclear at present. Further analysis is necessary to elucidate whether regulation of *c-Myb* is solely at the level of transcription or also mediated by post-transcriptional regulation. Future experiments will include treating the cells with the peptide aldehyde MG132, a potent inhibitor of proteasome function, to analyse levels of ubiquitinated c-Myb. By comparing levels of ubiquitinated c-Myb in cells

expressing the MLL-fusion and cells upon loss of the MLL-fusion, we will be able to determine if c-Myb protein expression is changed due to ubiquitination and therefore proteasomal degradation. If we can identify an increase in ubiquitination and degradation of c-Myb upon loss of MLL-fusion expression, we can also treat the cells with Cycloheximide to block protein synthesis and analyse proteasomal degradation in the presence and absence of the MLL-fusion oncogenes.

Both MLL and its fusion partners, Af4, Af9, Af10 and Enl have been shown to play a direct role in transcriptional elongation (Bitoun *et al.*, 2007). Af4 has been described to recruit Af9 or Enl, Af10, Dot1 and pTEFb in a complex and positively regulate transcriptional elongation by RNA polymerase II. This leads to histone methylation (H3K79) resulting in increased and deregulated target gene expression (Bitoun *et al.*, 2007). The interaction domains of Af4, Af9/Enl and Af10 with Dot1 are retained in MLL-fusion proteins, suggesting that the fusion proteins mistarget Dot1 and pTEFb to transcriptional targets (Bitoun *et al.*, 2007). The observed decrease of Siah1a protein expression and increase of AF4 protein expression in MLL-fusion expressing cells possibly suggests that Siah1a degradation targets might be protected by the MLL-fusion oncogenes. Therefore, the transcriptional elongation complex, recruited by Af4, remains active in the presence of MLL-fusion oncogenes. Further experiments are necessary, in order to analyse if AF4 is critical in MLL-fusion mediated transformation. By performing an *AF4* specific knock-down we could possibly acquire more information about the role of AF4.

In order to elucidate the role of Siah1a down-regulation in MLL-ENL and MLL-AF9 immortalised cells, we decided to use the antimicrobial compound Hexachlorophene. Hexachlorophene has been suggested to inhibit Wnt signalling

through Siah1a-mediated proteasomal degradation of β-Catenin, although the mechanism of Siah1a stimulation is unknown at present (Park *et al.*, 2006; Min *et al.*, 2009). Our preliminary results suggested that upon addition of Hexachlorophene, MLL-fusion immortalised cells differentiated in a dose dependent manner. Furthermore, we observed that addition of Hexachlorophene caused a decrease in AF4 and c-Myb protein expression, both shown to be targets of Siah1a proteasomal degradation (Tanikawa *et al.*, 2000; Bursen *et al.*, 2004). This observation suggests a correlation between Hexachlorophene-mediated stimulation of Siah1a activity and decrease in protein expression of Siah1a degradation targets. Interestingly, the correlation between increased activity of Siah1a and down-regulation of Siah1a degradation targets is also observed following loss of MLL-fusion expression. Further experiments are necessary in order to confirm that Hexachlorophene induces Siah1a activity and to analyse if Siah1a is responsible for proteasomal degradation of AF4, c-Myb and β-Catenin.

The data presented in this chapter suggest that the target genes *c-Myb*, *c-Myc* and *Siah1a* together with *Ctnnb1* and *AF4* may be involved in a common biological process, induced by MLL-mediated transformation. These genes encode proteins that function together in different complexes. It is unclear at present if all the described protein complexes are required to interact or if they represent independent pathways that are regulated by MLL-fusion oncogenes. Since the use of Hexachlorophene in rats has been found to have toxic side effects such as cerebral swelling and brain damage, Hexachlorophene can not be used *in vivo*. However, inducing Siah1a activity by similar drugs may represent a novel therapeutic approach.

CHAPTER VI - Conditional MLL-ENL *in vivo* model

Global gene expression analysis was used to identify a number of potential transcriptional target genes of the MLL-AF9 and MLL-ENL fusion oncogenes. To examine the role of a particular target gene for MLL-fusion mediated transformation, up-regulated target genes were knocked down *in vitro*. In order to establish whether modulating the identified transcriptional networks used by MLL-fusions would block leukaemia *in vivo*, we induced leukaemia using the conditional MLL-ENL cell lines. We then examined the dependence of the leukaemia on MLL-ENL expression. The aim of these experiments was to validate the targeting of transcription pathways activated by MLL-fusions, in order to treat established leukaemias.

6.1. Immortalised MLL-ENL cells induce AML *in vivo*

Immortalised myeloid cells with either constitutive or conditional expression of MLL-ENL were generated and characterised in the same manner as the immortalised myeloid MLL-AF9 cell lines, described in Chapter III (generation of ME4 by myself, generation of all other immortalised MLL-ENL cell lines by Dr. Sarah Horton, Molecular Haematology and Cancer Biology Unit, UCL Institute of Child Health and Great Ormond Street Hospital). The relative expression levels of *MLL-ENL* in the cells and the tetracycline-transactivator dependent expression of *MLL-ENL* in conditional immortalised cells was measured by qPCR (Figure 49, experiment was performed by Dr. Sarah Horton). Total RNA was isolated from all selected cell lines cultured with or without 2μg/ml Doxycycline for 48 hours and used for qPCR analysis.

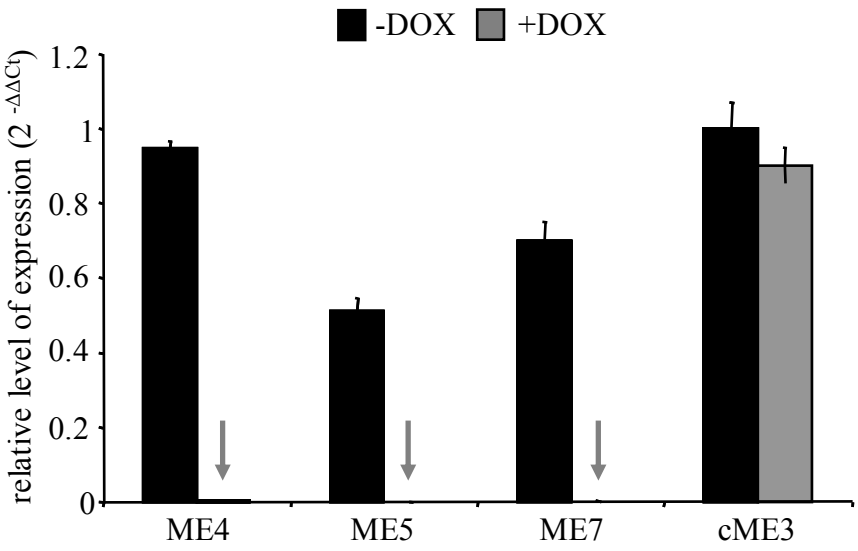


Figure 49: Relative *MLL-ENL* mRNA expression in the presence and absence of Doxycycline

The graph shows the relative level of *MLL-ENL* mRNA expression in ME4, ME5, ME7 and cME3 cells in the absence (black) or presence (grey bar or indicated by grey arrows) of 2µg/ml Doxycycline for 48 hours. Columns represent the mean of triplicate measurements, error bars the standard deviations.

Upon addition of Doxycycline to the cultures, *MLL-ENL* expression was completely abrogated. As expected, treatment of the cME3 constitutive immortalised cells with Doxycycline did not have an effect on MLL-ENL expression.

To determine if immortalised MLL-ENL cells could induce leukaemia *in vivo*, $1x10^6$ cells of the conditional immortalised MLL-ENL cell lines ME4, ME5, ME7 or of the constitutive immortalised cell line cME3 were transplanted intravenously into sublethally γ-irradiated (6.5 Gy) C57BL/6 or C57BL/6-CD45.1 mice (all transfers were performed by Dr. Owen Williams). After transplantation the primary recipients were closely monitored. The mice where immediately sacrificed as soon as they showed signs of distress such as hunched postures, abnormal breathing, weight loss, immobility and piloerection. When analysed, the recipients had pale bone marrow and had developed enlarged spleens, consistent with AML phenotype. To determine if the leukaemia could be transplanted into secondary recipients, $1x10^6$ leukaemic splenocytes from primary recipients were injected intravenously into sublethally irradiated C57BL/6-CD45.1 or C57BL/6 (CD45.2) mice. Peripheral blood was analysed at regular intervals for the presence of donor-derived leukaemic cells and recipients were sacrificed when they showed any signs of disease or at indicated time points (Figure 50).

The survival of all recipients was monitored and in order to further characterise the leukaemia, organs of leukaemic secondary recipients were analysed. MLL-ENL immortalised cells induced leukaemia in primary as well as secondary recipients with similar latencies of 81.25±4.4 days (n=8) and 26.89±6.8 days (n=44), respectively (Figure 51).

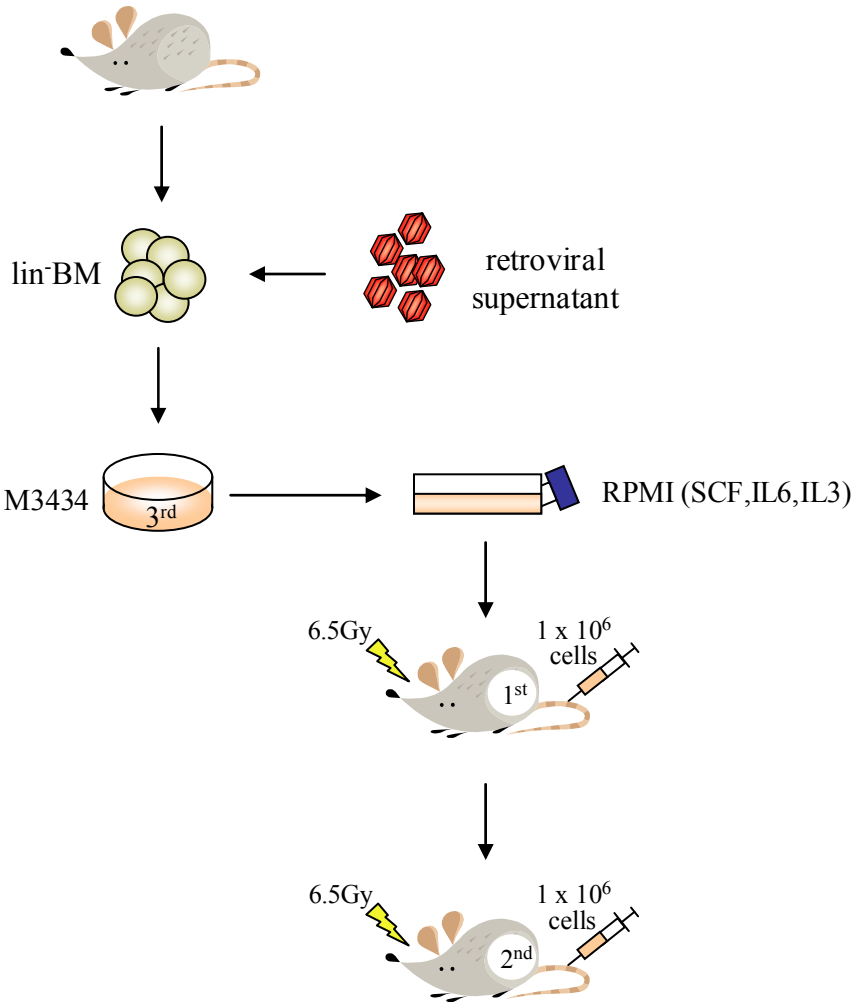


Figure 50: Experimental strategy for a conditional *in vivo* model of MLL-ENL leukaemia

Recipient mice were sublethally γ-irradiated with 6.5 Gray 24 hours prior to injections. Primary recipients were injected with 1x10⁶ immortalised cells. Secondary recipients were injected with 1x10⁶ leukaemic splenocytes derived from leukaemic primary recipients. Recipients were sacrificed when they showed any signs of disease.

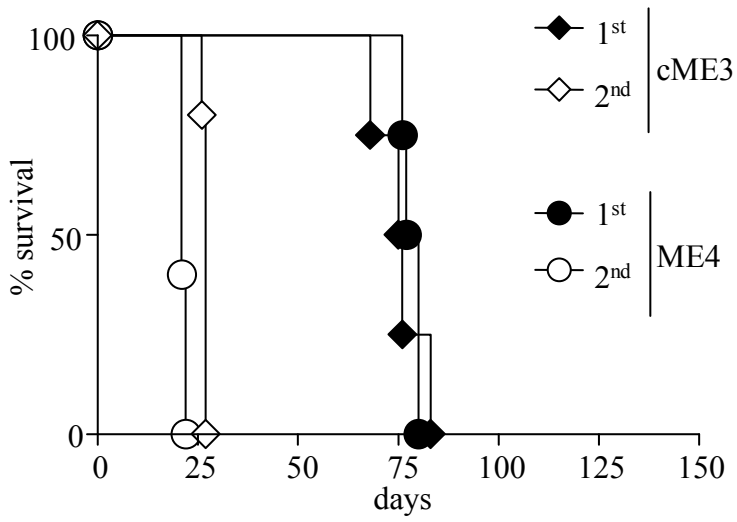


Figure 51: Secondary recipients develop disease with a shorter latency

The graph shows the survival curves of primary (filled symbols) and secondary (open symbols) recipients, transplanted with immortalised or leukaemic constitutive (cME3, diamonds, n=5) or conditional (ME4, circles, n=5) myeloid cells. In total MLL-ENL immortalised cells caused leukaemia in primary and secondary recipients with an average latency of 81.25±4.4 days (n=8) and 26.89±6.8 days (n=44), respectively.

Secondary recipients transplanted with leukaemic splenocytes from the primary recipients developed leukaemia with much shorter latencies than primary recipients transplanted with immortalised cells. In secondary recipients, leukaemic cells derived from conditionally immortalised cells could be identified by CD45.2 and EGFP expression. Leukaemic cells derived from constitutively immortalised cells did not express EGFP and could only be identified by CD45.2 expression (left panel Figure 52 A). Flow cytometric analysis of cell surface antigen expression revealed that leukaemic splenocytes derived from secondary recipients expressed high levels of the myeloid lineage marker Mac-1 and intermediate to high levels of the myeloid marker Gr-1 (right panel Figure 52 A). This phenotype is consistent with the phenotype of immature myeloid progenitors as well as with the phenotype of the immortalised MLL-ENL and MLL-AF9 cell lines (data for MLL-AF9 cell lines shown in Chapter III). Sections of liver harvested from secondary recipients and normal liver from C57BL/6 mice were fixed in formalin, set in paraffin and stained with hematoxylin & eosin (histopathology was performed by Dr. Neil Sebire, Department of Histopathology, UCL Institute of Child Health and Great Ormond Street Hospital). Liver sections from secondary recipients showed extensive leukaemic cell infiltration (Figure 52 B).

A possible explanation for the shortened latencies in secondary recipients could be the *in vivo* selection for an outgrowth of a minor aggressive clone in the primary recipients. In this scenario, a clone present as a minor population of the original immortalised cell line would compose the bulk of leukaemic cells isolated from primary leukaemic mice.

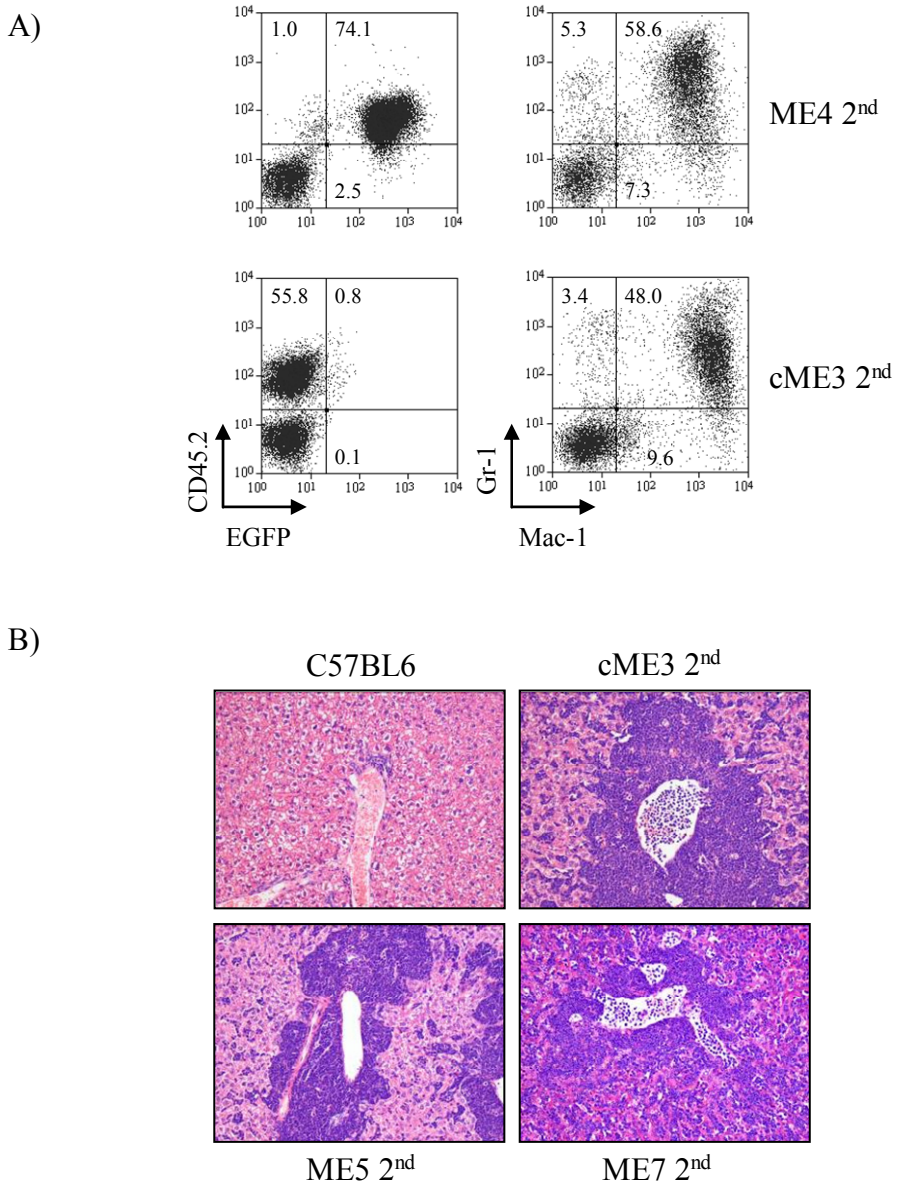


Figure 52: Spleen and liver analysis of diseased secondary recipients

A) The left panel of the dot plots shows the expression of CD45.2 and EGFP in leukaemic cME3 and ME4 splenocytes of secondary recipients. The right panel of the dot plots shows the expression of the myeloid markers Gr-1 and Mac-1 also in leukaemic cME3 and ME4 splenocytes of secondary recipients. Numbers in the plots refer to the percentages of cells within each quadrant. B) Photomicrographs of hematoxylin & eosin stained sections of leukaemic cell infiltrated liver from secondary recipients and normal liver from C57BL6 control mice (original magnification x100).

In order to compare the retroviral integration pattern of all immortalised cell lines and their corresponding leukaemic cells, southern blot analysis of end-fragments produced by BamHI digestion of integrated provirus and genomic DNA was performed (Figure 53 A). All leukaemic cell lines had the identical retroviral integration pattern as the immortalised cells from which they were derived (Figure 53 B). This result suggests that the outgrowth of a minor clone *in vivo* was unlikely.

Another possible cause of the shortened latencies could be that the immortalised cells became conditioned to the microenvironment *in vivo*. Hence, after transplanting those *in vivo* conditioned leukaemic cells into secondary recipients they were better able to interact with the bone marrow environment, engraft more efficiently and induced leukaemia at an accelerated rate. To test this hypothesis, splenocytes derived from primary leukaemic recipients were either directly transplanted into secondary recipients or cultured for one month *in vitro* before transplantation into secondary recipients (Figure 54 A). Prolonged culture of leukaemic cells *in vitro* would presumably lead to a loss of any acquired microenvironmental conditioning and the cells would have lost a possible engraftment advantage. However, the survival curves for mice transplanted with the leukaemic cells ME5a (Figure 54 B) show that leukaemic cells did not loose the ability to induce leukaemia with a shorter latency after one month *in vitro* culture prior to transplantation. Although there were some differences in latencies between *ex vivo* to post-culture transplanted leukaemic cells (Table 19), all leukaemic cells induced leukaemia with a shorter latency when compared to the immortalised parental cells.

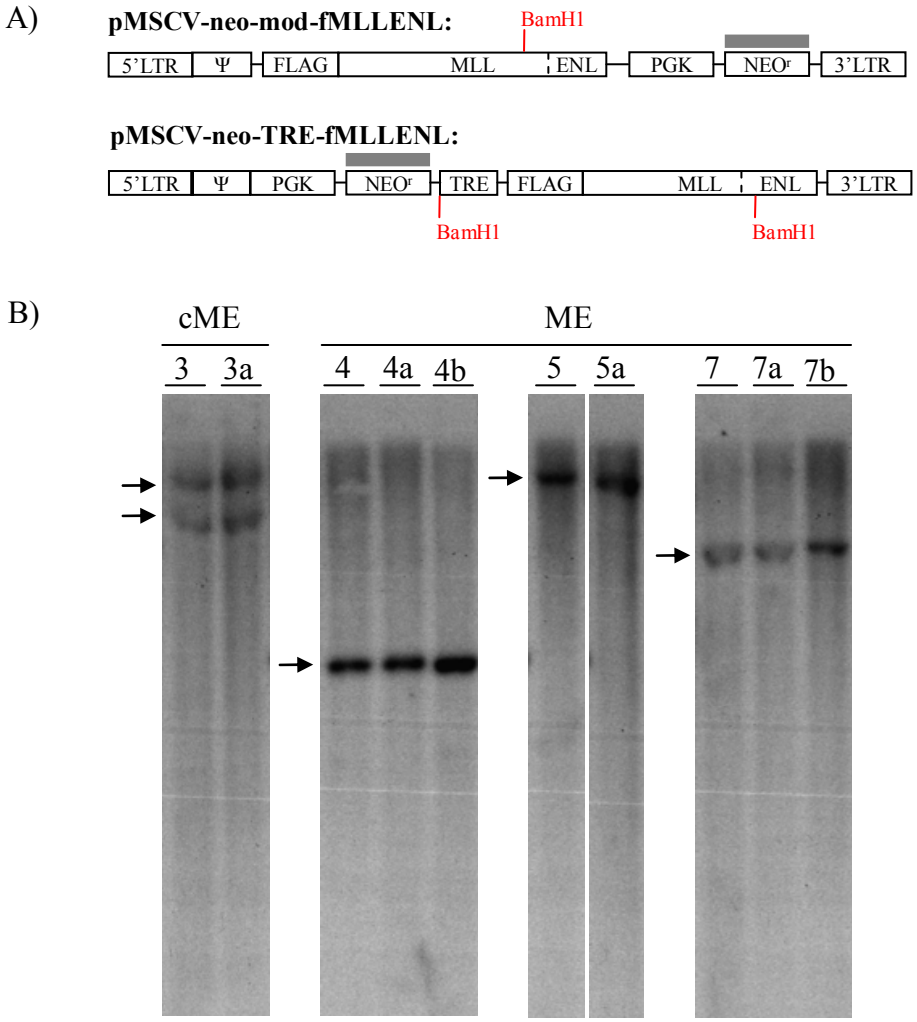
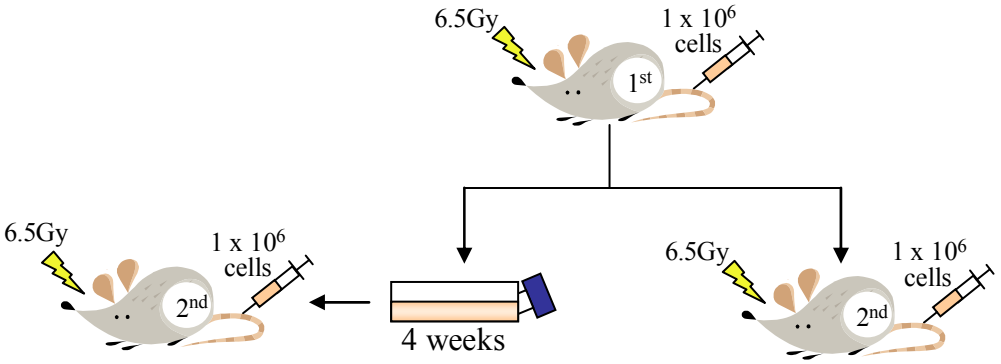


Figure 53: Leukaemic and immortalised cells have the same retroviral integration pattern

A) The figure shows diagrams of the constitutive (pMSCV-neo-mod-fMLLENL) and conditional (pMSCV-neo-TRE-fMLLENL) retroviral constructs. The BamH1 restriction endonuclease site and region corresponding to the probe (shown in grey) used in the southern blot analysis are indicated. B) Southern blot analysis of genomic DNA isolated from immortalised constitutive (cME3) and conditional (ME4, ME5 and ME7) cells and their leukaemic progeny (cME3a, ME4a, ME4b, ME5a, ME7a and ME7b). Blots show 5'(ME4, ME5, ME7, ME4a, ME4b, ME5a, ME7a and ME7b) and 3' (cME3 and cME3a) end-fragments produced by BamHI digestion of the integrated provirus and genomic DNA.

A)



B)

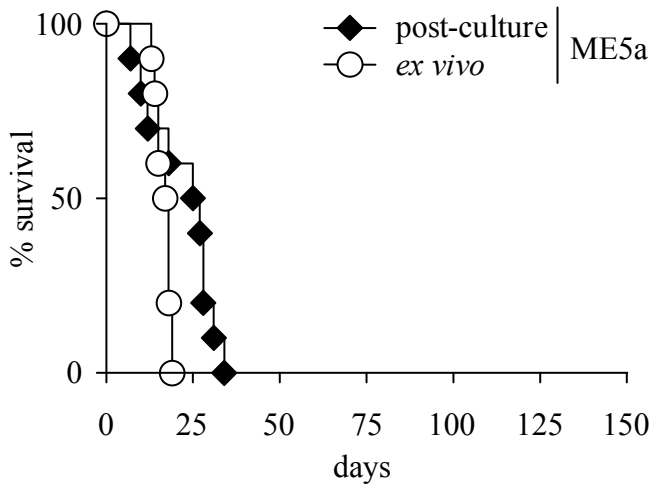


Figure 54: Cultured and freshly isolated leukaemic cells cause leukaemia in secondary recipients with similar latencies

A) Brief overview of the experimental strategy used to assess the effect of *in vitro* culture of primary leukaemic cells on latency of leukaemia in secondary recipients. B) Survival curves of mice transplanted with ME5a leukaemic cells, either freshly isolated (open circles) or following 4 weeks culture *in vitro* (filled diamonds). *In vitro* culture of the leukaemic cells caused a small but significant increase in ME5a latency (p=0.038, Mantel Haenszel logrank test).

Table 19: Latencies of secondary leukaemias ex vivo or post-culture

Leukaemic Cells	Latency of 2nd *ex vivo*	Latency of 2nd post-culture	p-value
ME4a	26.2 (±1.1, n=5)	17.8 (±9.2, n=5)	0.269
ME5a	16.6 (±2.2, n=5)	22.0 (±9.5, n=10)	0.038
ME7a	34.2 (±3.3, n=5)	14.2 (±3.3, n=5)	0.002

The table shows a summary of the comparisons between *ex vivo* and post-culture induced secondary latencies. ME4a leukaemic cells showed no significant difference in latencies, ME5a leukaemic cells showed a small but significant increase in latencies and ME7a showed a significant decrease in latencies.

An alternative explanation could be the acquisition of additional mutations by the leukaemic cells resulting in shortened latencies. In order to examine if additional mutations have occurred, immortalised cells and leukaemic cells were karyotyped (GTG banding and analysis of metaphase spreads was performed by Steve Chatters, Paediatric Malignancies Cytogenetics Unit, Institute of Child Health and Great Ormond Street Hospital). The microscopic analysis of metaphase spreads showed an increase in single cell abnormalities within leukaemic cultures when compared to the parental immortalised cell lines (Table 20). Most of the observed mutations were single cell abnormalities, such as polyploidy and chromosomal breaks (Figure 55). However, one set of leukaemic cells (ME7a) harboured a clonal abnormality, identified by GTG-banding as a trisomy 6 (Figure 56).

In the two cases of ME4b and ME7b, karyotyping could not reveal an increased number of mutations compared to the immortalised cell lines from which they were derived. A more sensitive approach was used to analyse changes in copy number of subchromosmal regions, by using array-based comparative genomic hybridisation (CGH) analysis (Agilent).

ME4a
Single cell abnormality

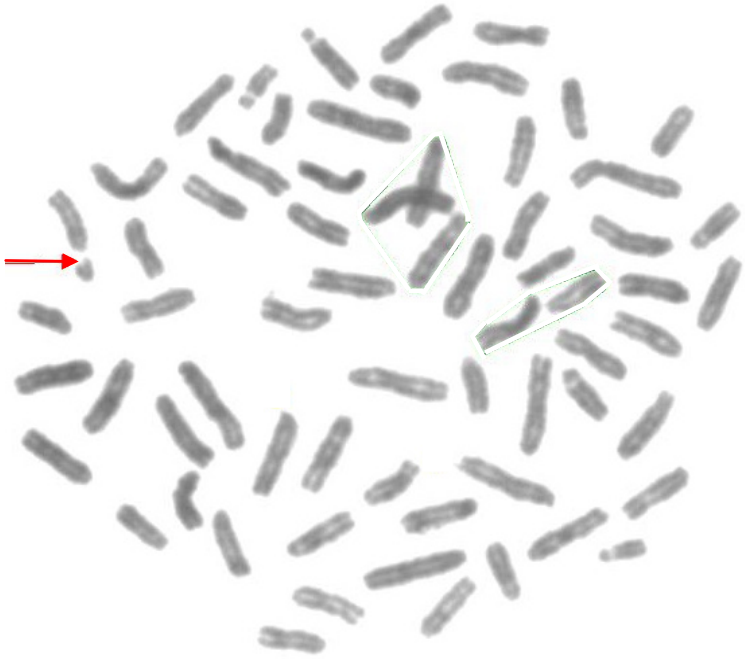


Figure 55: Leukaemic ME4a cells have acquired single cell abnormalities

Metaphase spread of ME4a cells (original magnification x1000) which shows a chromosome break (red arrow).

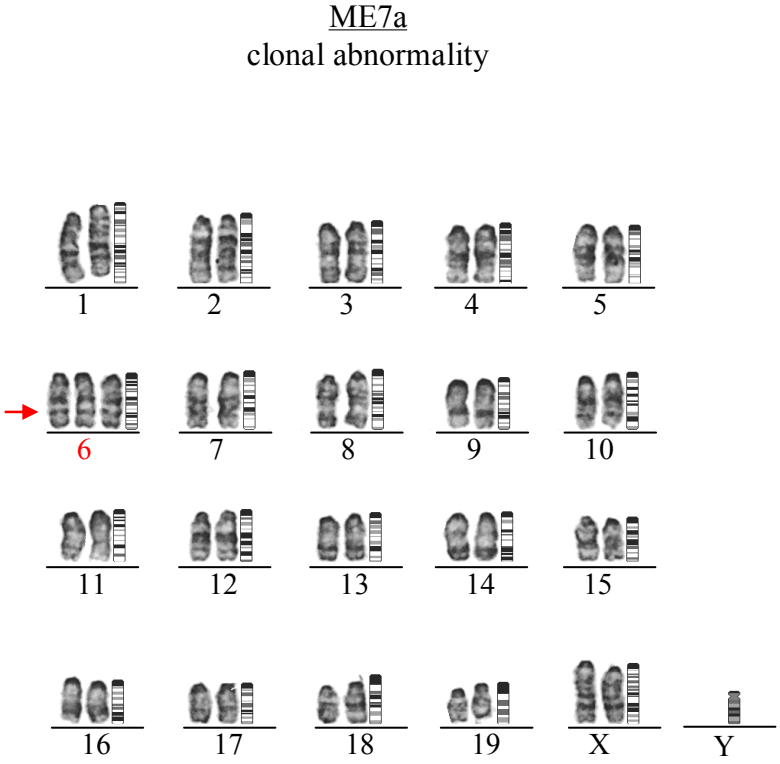


Figure 56: Leukaemic ME7a cells have acquired a clonal Trisomy 6 abnormality

GTG-banded karyotype of the leukaemic cell line ME7a. Ideograms are shown next to the corresponding chromosome pair (original magnification x1000).

Table 20: Leukaemic cells have cytogenetic abnormalities

Cell Line	Single Cell Abnormalities	Karyotype
ME4	0/50 abnormal	normal
	0/50 polyoloid	
ME4a	2/50 abnormal	normal
	14/50 polyoloid	
ME4b	2/50 abnormal	normal
	0/50 polyoloid	
ME5	0/30 abnormal	normal
	0/30 polyoloid	
ME5a	1/20 abnormal	normal
	14/20 polyoloid	
ME7	5/50 abnormal	normal
	0/50 polyoloid	
ME7a	1/50 abnormal	trisomy 6
	0/50 polyoloid	
ME7b	1/18 abnormal	normal
	0/18 polyoloid	
cME3	0/50 abnormal	normal
	0/50 polyoloid	
cME3a	1/50 abnormal	normal
	0/50 polyoloid	

The table shows a summary of the karyotype and the frequency of additional single cell mutations in all analysed immortalised cell lines and leukaemic cells.

Genomic DNA was isolated from leukaemic cells ME4b and ME7b and compared to genomic DNA from their parental immortalised cell lines, ME4 and ME7, respectively. Using this approach, changes in copy number could be detected. ME4b leukaemic cells showed a copy number increase of a region of chromosome 4 as well as a copy number decrease of a region of chromosome 15, when compared to the parental ME4 immortalised cells (Figure 57).

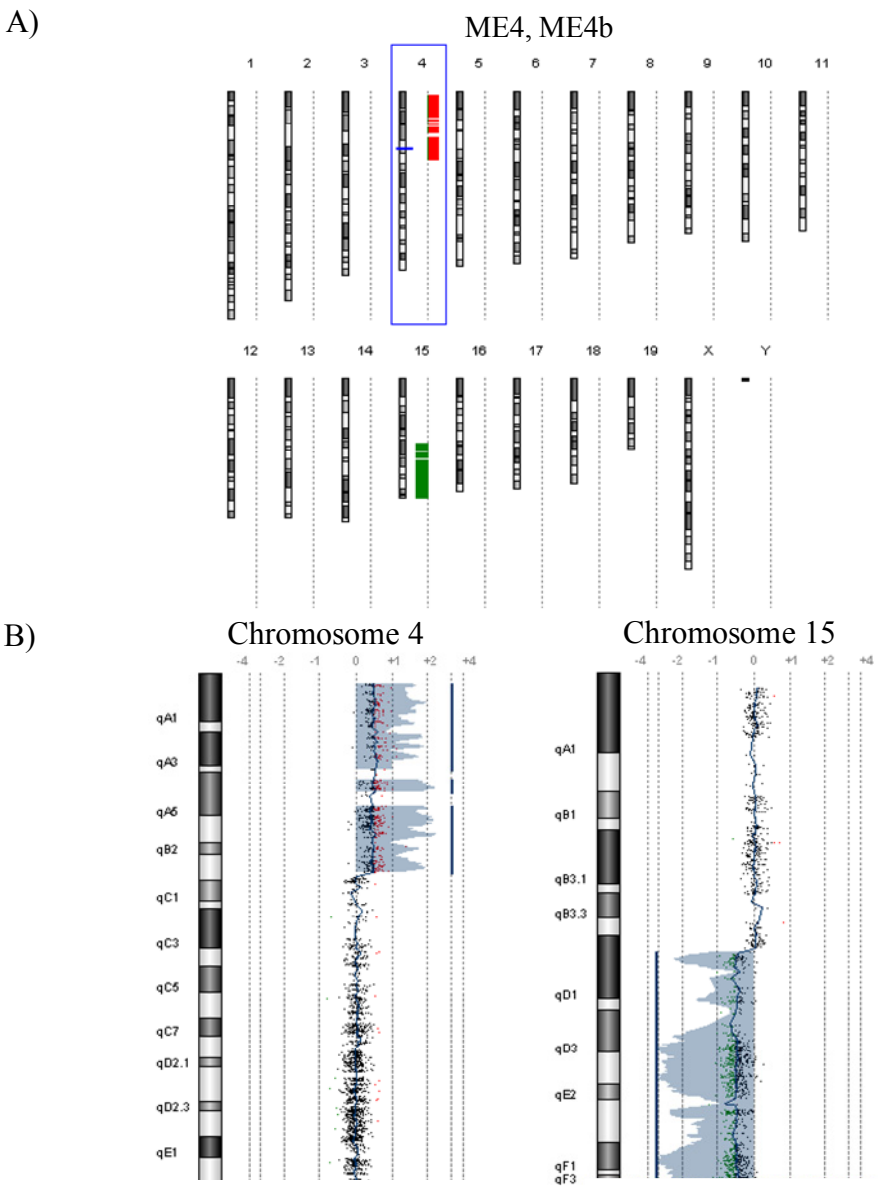


Figure 57: CGH analysis of ME4 and ME4b DNA samples

A) The figure illustrates the increase in copy number profile across all chromosomes. The copy number increase (red bars) of a region of chromosome 4 and decrease (green bars) of a region of chromosome 15 in ME4b leukaemic cells when compared to ME4 immortalised cells is shown. B) Detailed view of chromosome 4 and 15. Each point on the graph represents the log2 ratio value of a single oligonucleotide probe. The shaded area and vertical line indicate an aberrant region identified by the Z-score algorithm (CGH Analytics v3.4, Agilent Technologies). Array analysis performed by Miltenyi Biotec.

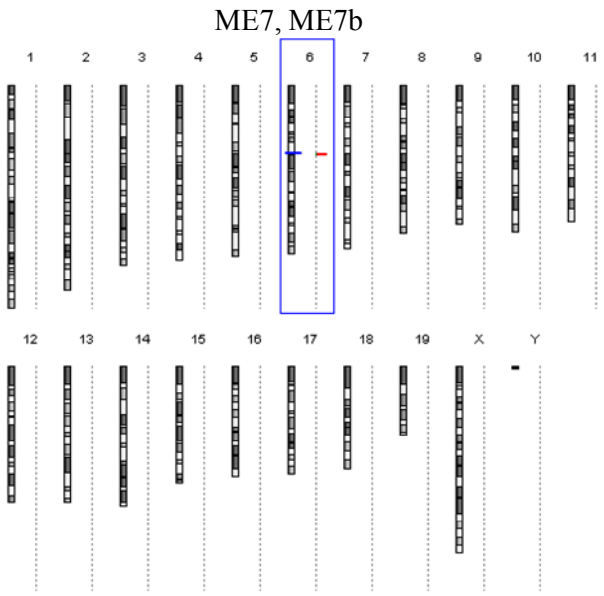
ME7b leukaemic cells showed a copy number increase of a small region of chromosome 6, when compared to the parental ME7 immortalised cells (Figure 58). It is tempting to speculate that the genes, which are mapped to the detected aberrant regions, could potentially play an important role for MLL-ENL induced leukaemogenesis. This possibility will be investigated further in future work.

If the provirus integration occurred at or proximal to the region of copy number alterations, this could result in either increased copy number of the MLL-ENL provirus or cause increased expression of the oncogene. Hence, those increases may have impacted on the latencies of secondary leukaemias. To examine this, genomic DNA and total RNA was isolated from all immortalised cell lines and leukaemic cells. The relative level of MLL-ENL DNA copy number (Figure 59 A) and relative level of MLL-ENL mRNA expression (Figure 59 B) was measured by qPCR. No significant change could be detected in MLL-ENL DNA copy number and no consistent increase could be detected in MLL-ENL mRNA expression of leukaemic cells compared to their parental immortalised cell lines.

6.2. *In vitro* characterisation of leukaemic cells

Leukaemic splenocytes were isolated from primary recipients and cultured *in vitro* in RPMI medium supplemented with 100 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6. Their rate of proliferation, growth factor requirements and response to Doxycycline was compared to the immortalised cells from which they were derived. Leukaemic cells proliferated steadily *in vitro* but in all cases analysed, showed a reduced rate of proliferation compared to the immortalised cells (Figure 60).

A)



B)

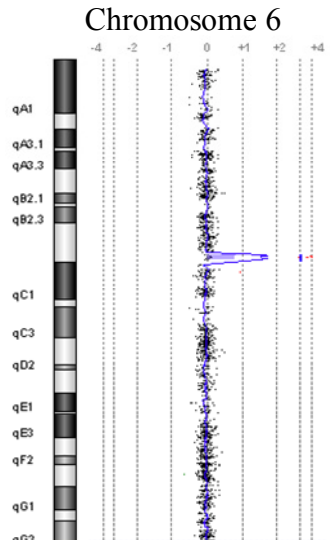


Figure 58: CGH analysis of ME7 and ME7b DNA samples

A) The figure illustrates the increase in copy number profile across all chromosomes. The copy number increase (red bars) of a region of chromosome 6 in ME7b leukaemic cells when compared to ME7 immortalised cells is shown. B) Detailed view of chromosome 6. Each point on the graph represents the log2 ratio value of a single oligonucleotide probe. The shaded area and vertical line indicate an aberrant region identified by the Z-score algorithm (CGH Analytics v3.4, Agilent Technologies). Array analysis performed by Miltenyi Biotec.

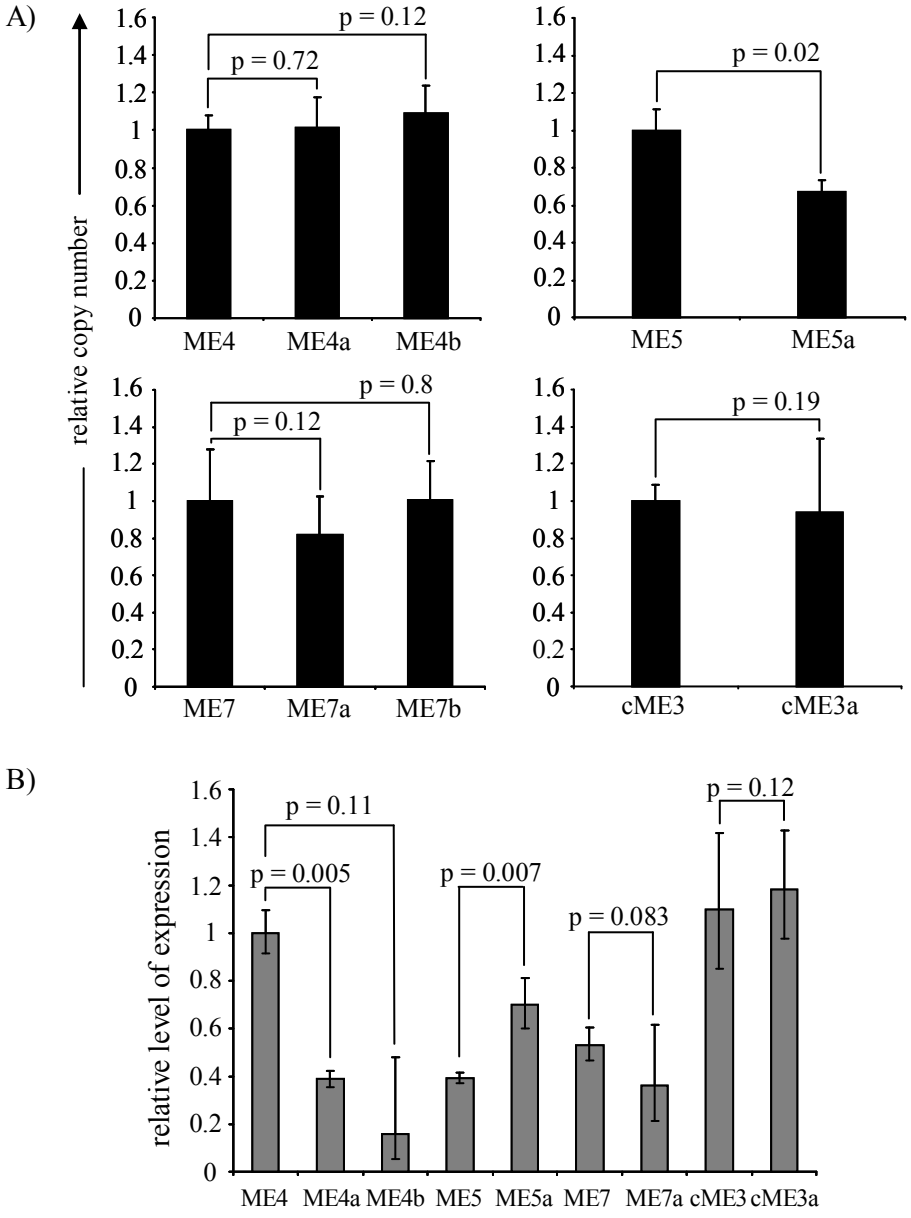


Figure 59: Relative MLL-ENL DNA copy number and mRNA expression

A) Relative MLL-ENL DNA copy numbers measured by qPCR. Copy numbers were calculated for ME4a and ME4b relative to ME4, for ME5a relative to ME5, for ME7a and ME7b relative to ME7 and for cME3a relative to cME3 and normalised to mouse β-Actin. Columns represent the mean of triplicate measurements, error bars represent the standard deviations. B) Relative level of *MLL-ENL* mRNA expression measured by qPCR shown for all cell lines normalised to ME4. Columns represent the mean of triplicate measurements, error bars represent the standard deviations.

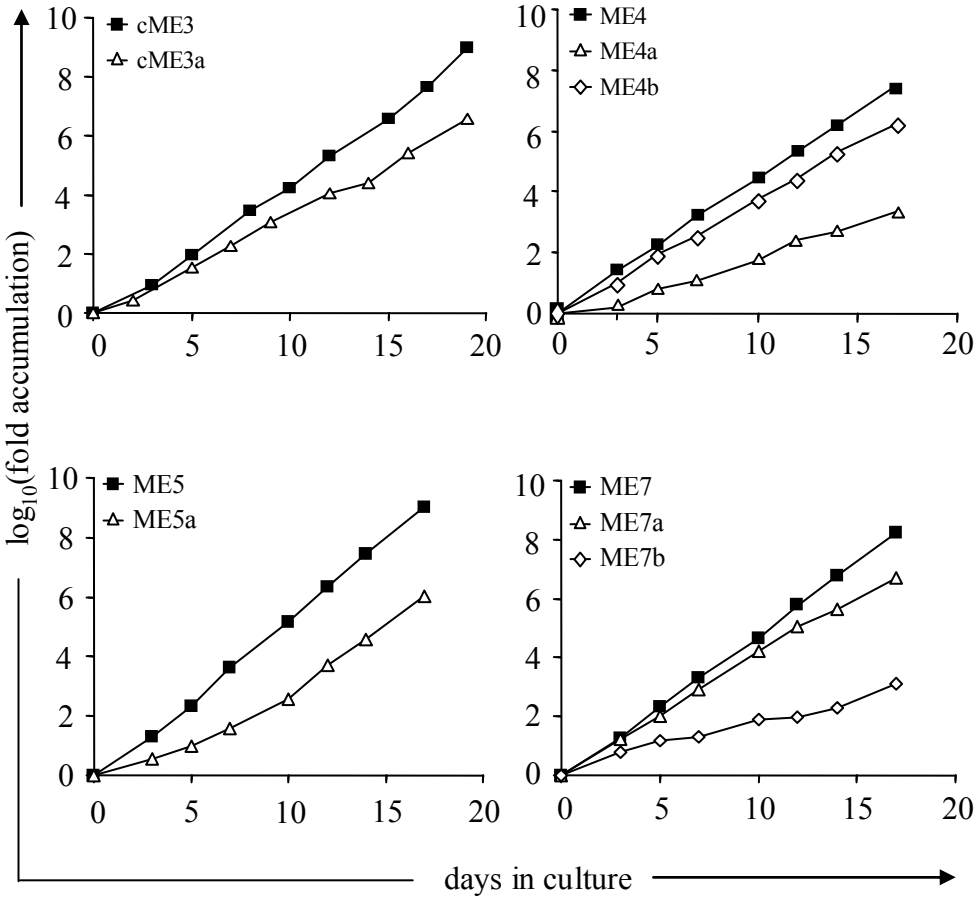


Figure 60: Leukaemic cells have a reduced proliferation rate *in vitro*

The graph shows the fold accumulation (log_{10}) in cell number of *in vitro* cell lines cME3, ME4, ME5 and ME7 (filled squares) and leukaemic cell lines cME3a, ME4a, ME5a, ME7a (open triangles), ME4b and ME7b (open diamonds).

To determine the cytokine requirements of the cell lines, cell proliferation assays were carried out. Data for all analysed cell lines are shown in Figure 61. The cells were cultured in various combinations of the cytokines SCF, IL-6, IL-3 and GM-CSF. The immortalised and the leukaemic cells remained growth factor dependent and showed a similar pattern of response to the cytokine combinations.

In order to examine the response to Doxycycline upon loss of MLL-ENL expression, leukaemic cells and immortalised cell lines were cultured in the presence of 2μg/ml Doxycycline for seven days (Figure 62). Viable cells were counted using Trypan Blue exclusion after two, four and six or seven days. After two days, both the leukaemic and immortalised cells had a similar cell number in cultures with and without Doxycycline. By day four, all conditionally immortalised cells and their corresponding leukaemic progeny, cultured in the presence of Doxycycline, showed a decrease in proliferation and by day six or seven no viable cells could be detected. This response was similar for all conditional cells apart from immortalised ME4 cells, which terminally differentiated by day four. As expected, addition of Doxycycline to the cultures of constitutively immortalised or leukaemic cells did not have an effect on their proliferation capacity. Analysis of Wright-Giemsa stained cytospin preparations showed that the leukaemic cells had the same myeloblastic morphology as the immortalised cells. Upon addition of Doxycycline to the cultures for four days most of the conditionally immortalised cells and their corresponding leukaemic progeny terminally differentiated into neutrophils, as shown by their lobular shaped nuclei (Figure 63).

To confirm the terminal differentiation of the cells into neutrophils, neutrophil-specific granule gene expression was examined.

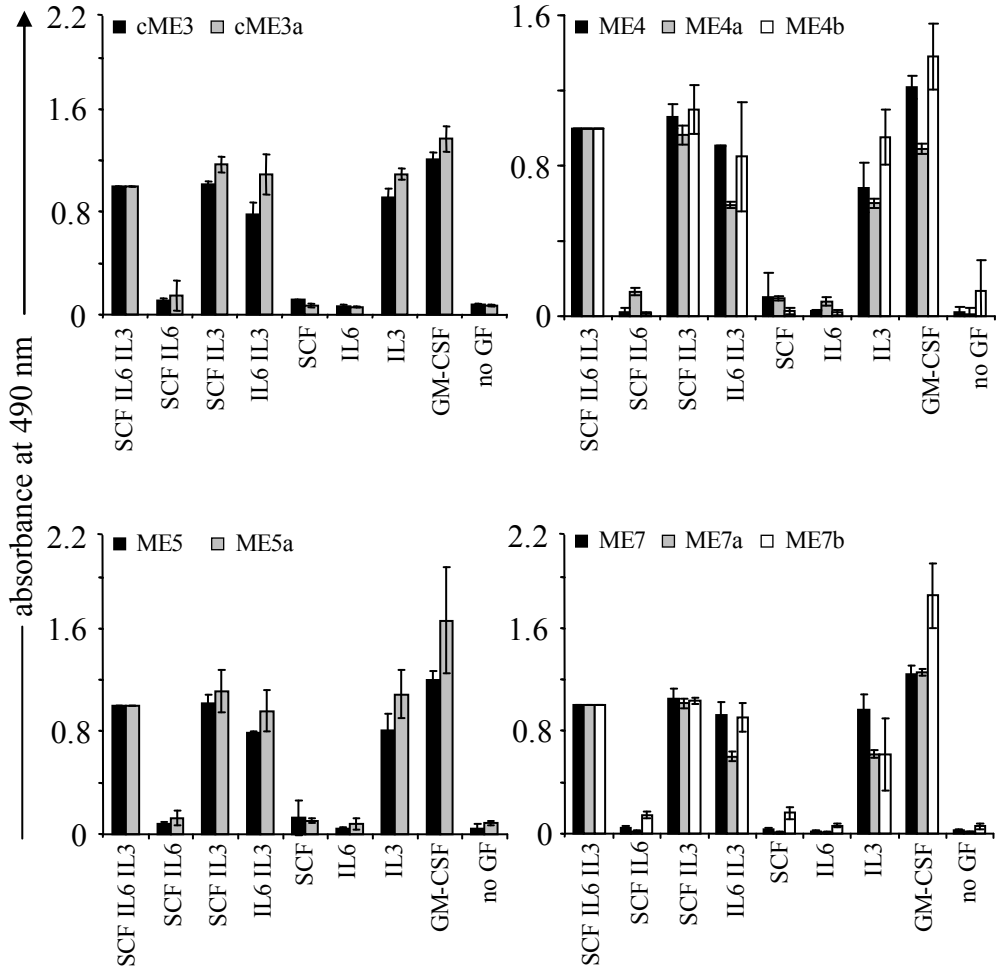


Figure 61: Cell proliferation assay to test growth factor requirements of immortalised and leukaemic cells

The graph shows the viability of cells cultured in various cytokine conditions (SCF, Il-6, IL-3 or GM-CSF) measured with an MTS assay at an absorbance of 490 nm. The cells were cultured for 72 hours in various cytokine combinations. The values are normalised to the growth of each cell line in the presence of SCF, IL-6 and IL-3. Bars represent means of triplicate values and error bars their standard deviations.

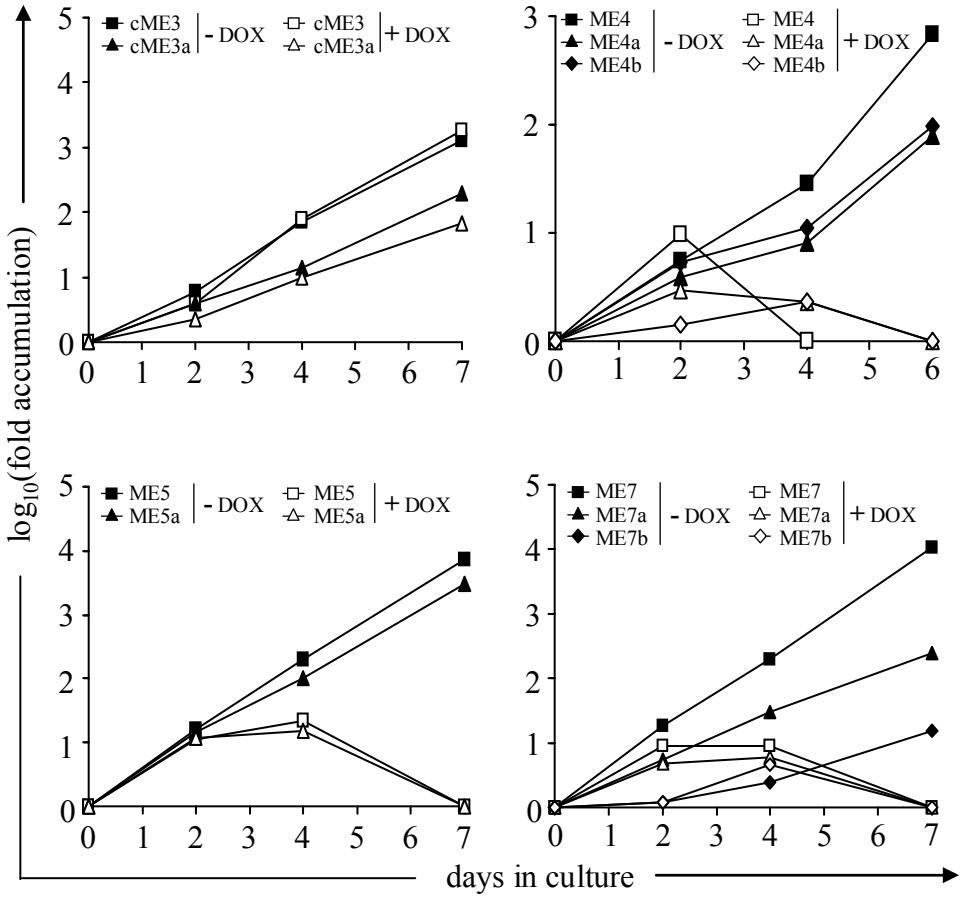


Figure 62: Effect of Doxycycline on viability of immortalised and leukaemic cells *in vitro*

The graph shows the fold accumulation (log_{10}) in cell number following maintenance of the cells without (filled symbols) and with 2 µg/ml Doxycycline (open symbols). Viable cells were counted using Trypan Blue exclusion after 2, 4 and 6 or 7 days.

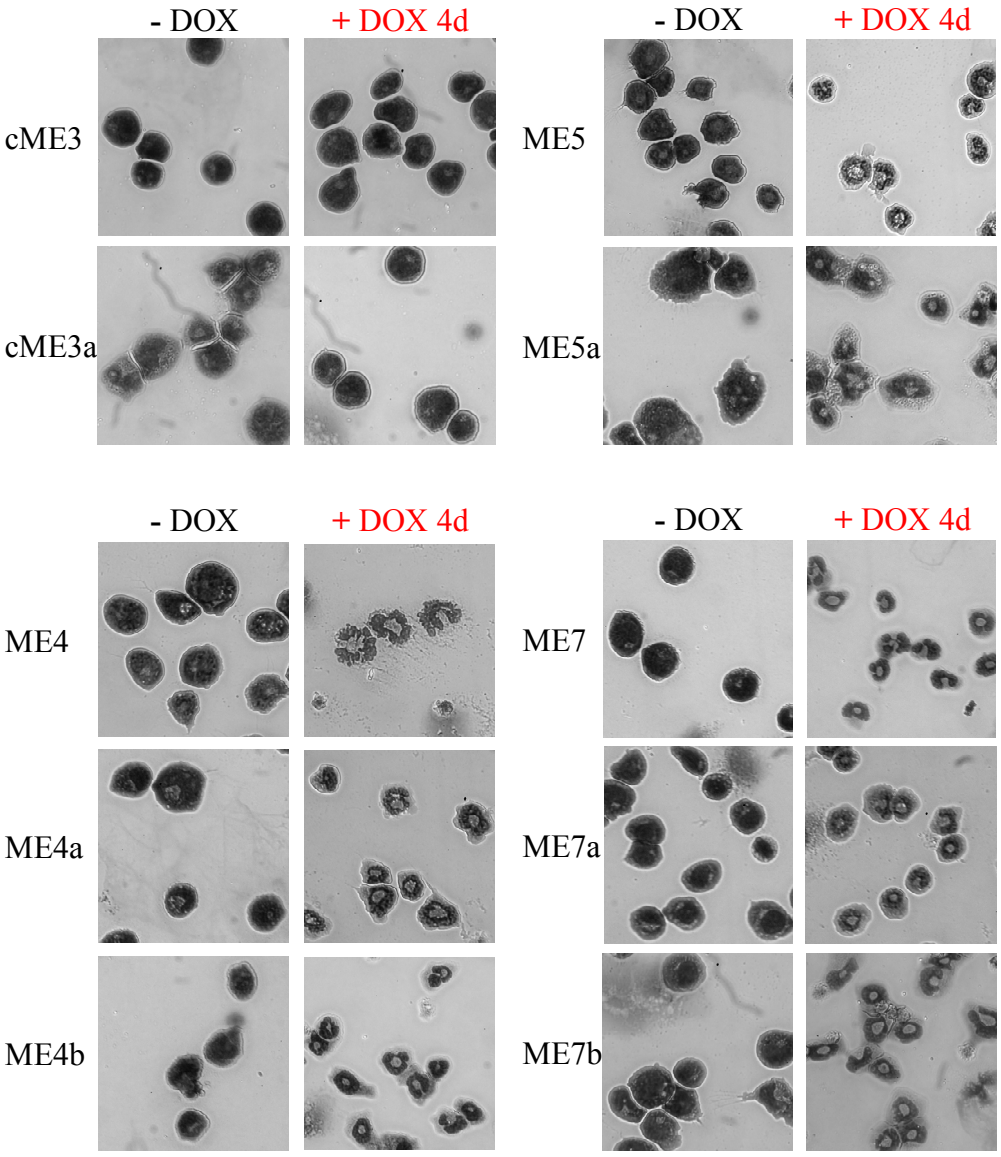


Figure 63: Differentiation of immortalised and leukaemic cells in response to Doxycycline

The figure shows the morphology of the cell lines following culture with and without 2µg/ml Doxycycline for 4 days, assessed by cytospin preparation and Wright-Giemsa staining (original magnification x 400).

Total RNA was isolated from the immortalised ME4 cells and the leukaemic ME4a cells cultured with or without 2μg/ml Doxycycline for 72 hours and used for qPCR analysis. In the presence of Doxycycline, the expression of the primary granule gene *Myeloperoxidase* (*Mpo*) was down-regulated while expression of the secondary granule gene *Lactoferrin* (*Ltf*) and the tertiary granule gene *Metalloproteinase 9* (*Mmp9*) were increased (Figure 64). These changes in neutrophil granule gene expression are consistent with terminal differentiation.

6.3. Leukaemic cells require MLL-ENL expression *in vivo*

These *in vitro* experiments suggested that leukaemic cells may also be sensitive to Doxycycline exposure *in vivo*. In order to examine this possibility, secondary recipient mice were transplanted with primary leukaemic splenocytes. Upon detection of donor derived leukaemic cells in the blood, one group of secondary recipients was treated with 200μg/ml Doxycycline and 5% sucrose in their drinking water for a specified period of time, and the other left untreated (Figure 65). Peripheral blood from secondary recipients was analysed at regular intervals for the presence of Mac-1^{+}EGFP^{+} leukaemic cells. Cells were stained using PE- or APC-conjugated antibodies to either c-Kit, Mac-1, Gr-1 or CD45.2 and used for flow cytometry analysis. (Experiments performed by myself, Dr. Sarah Horton and Dr. Owen Williams). Figure 66 illustrates an example of two mice transplanted with ME4a cells, one recipient treated with Doxycycline and one left untreated. An initial increase in the percentage of Mac-1^{+}EGFP^{+} leukaemic cells was detected in the peripheral blood of untreated as well as treated recipients after six days of Doxycycline treatment.

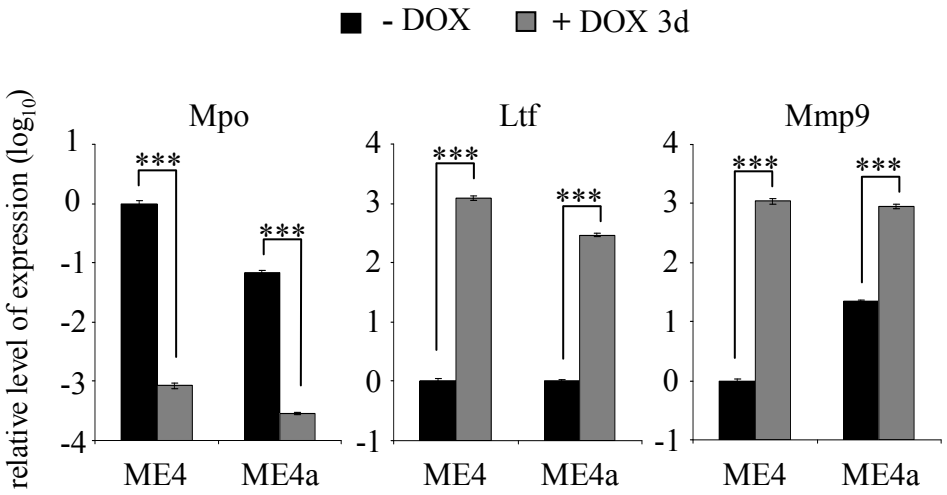


Figure 64: Neutrophil granule gene expression is changed in response to Doxycycline

The graph shows the relative level of neutrophil granule gene mRNA expression, measured by qPCR, in ME4 and ME4a cells after culture without (black bars) and with (grey bars) 2 μg/ml Doxycycline for 3 days. Plots show a significant ($p<0.001$) decrease after treatment with Doxycycline in primary granule *Mpo* and an significant ($p<0.001$) increase in secondary granule *Ltf* and tertiary granule *Mmp9* gene expression. Columns represent the mean of triplicate measurements, error bars represent the standard deviations.

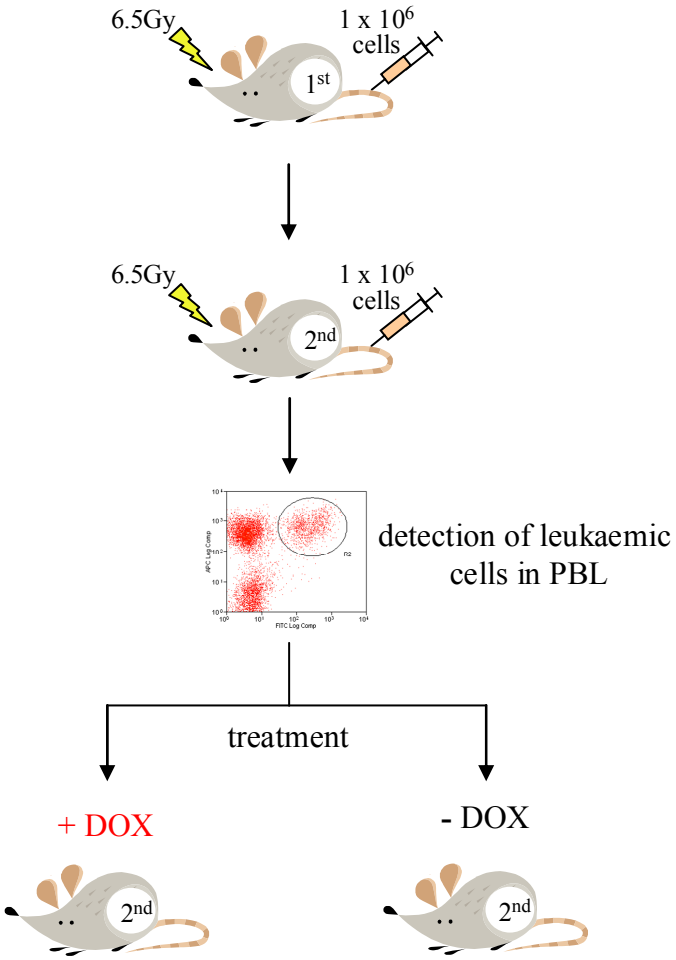


Figure 65: Experimental strategy for *in vivo* Doxycycline treatment

Secondary recipients were injected with 1x10⁶ leukaemic splenocytes derived from leukaemic primary recipients. Upon detection of leukaemic cells in the PBL Doxycycline was administered. Secondary recipients were divided into two groups and either administered 200 µg/ml Doxycycline and 5% sucrose or not in the drinking water. Mice were sacrificed when they showed any signs of disease.

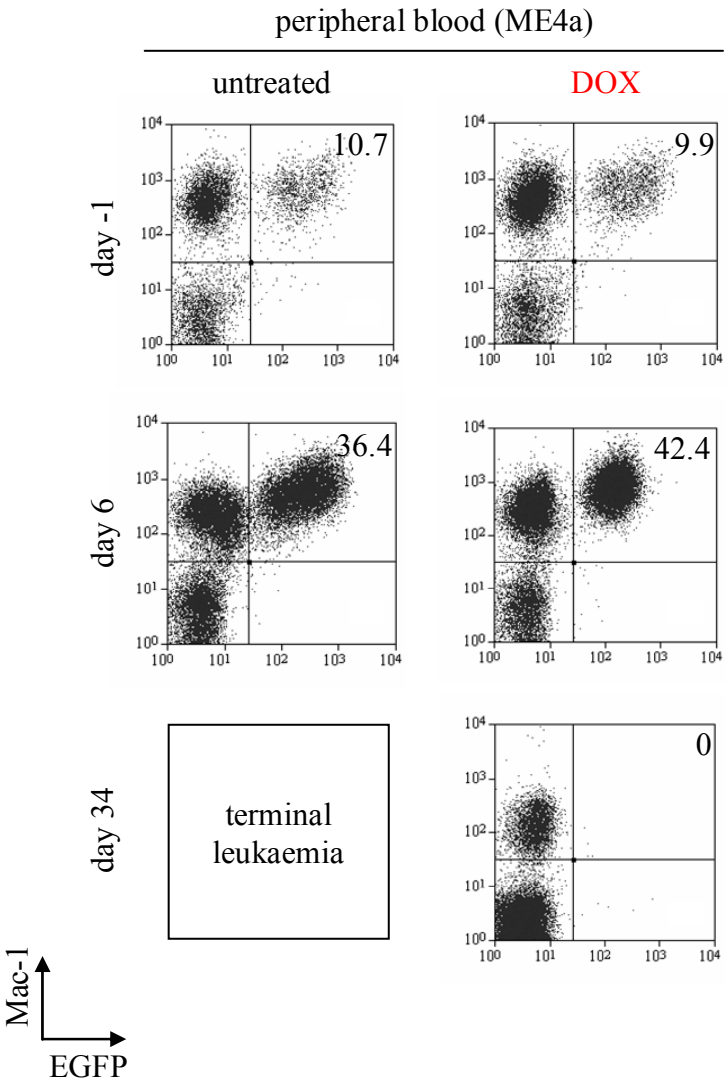


Figure 66: Elimination of conditional leukaemic cells from peripheral blood of Doxycycline treated mice

The dot plots show the presence of Mac-1^{+}EGFP^{+} leukaemic ME4a cells in the peripheral blood of recipient mice before (day -1) and after (day 6 and 34) their treatment (right panel), or not (left panel), with Doxycycline. Numbers in the top right quadrant represent Mac-1^{+}EGFP^{+} cells as a percentage of total Mac-1^{+} cells. None of the recipients in the untreated group survived until the last time-point shown.

However, 34 days later, recipients which received Doxycycline treatment were still alive and Mac-1^{+}EGFP^{+} leukaemic cells were completely eliminated from the peripheral blood. None of the untreated recipients survived until the last time point of analysis. All recipients which were transplanted with ME4a, ME5a and ME7c leukaemic cells, and which received Doxycycline treatment, survived for the entire period of the study (Figure 67). In all cases analysed, conditional leukaemic cells were completely eliminated from the peripheral blood (Figure 68). Interestingly, treatment duration for as little as seven days was sufficient to completely eliminate leukaemic cells from the peripheral blood (ME7c, Figure 67 and Figure 68). As expected, Doxycycline treatment did not have an effect on the survival of recipients which were transplanted with constitutive cME3a leukaemic cells. Moreover, Doxycycline treatment had no effect on the accumulation of leukaemic cells in the peripheral blood of mice transplanted with constitutive cells.

In order to examine the fate of Doxycycline treated leukaemic cells *in vivo*, bone marrow from secondary recipients was analysed. Bone marrow and spleen isolated from ME4b transfers with (n=3) and without (n=3) Doxycycline treatment showed loss of c-Kit expression (Figure 69 A). Leukaemic cells were further analysed for Gr-1 and c-Kit expression. Bone marrow from untreated transfers showed one population of cells with Gr-1^{hi}c-Kit^{lo} expression and one population of cells with Gr-1^{int}c-Kit^{hi} expression (Figure 69 B, upper panel). Upon treatment with Doxycycline the population of cells with Gr-1^{hi}c-Kit^{lo} expression increased and the population of cells with Gr-1^{int} expression showed reduced c-Kit expression indicating differentiation. An alternative possibility was that the cells underwent apoptosis *in vivo* as a direct result of MLL-ENL loss.

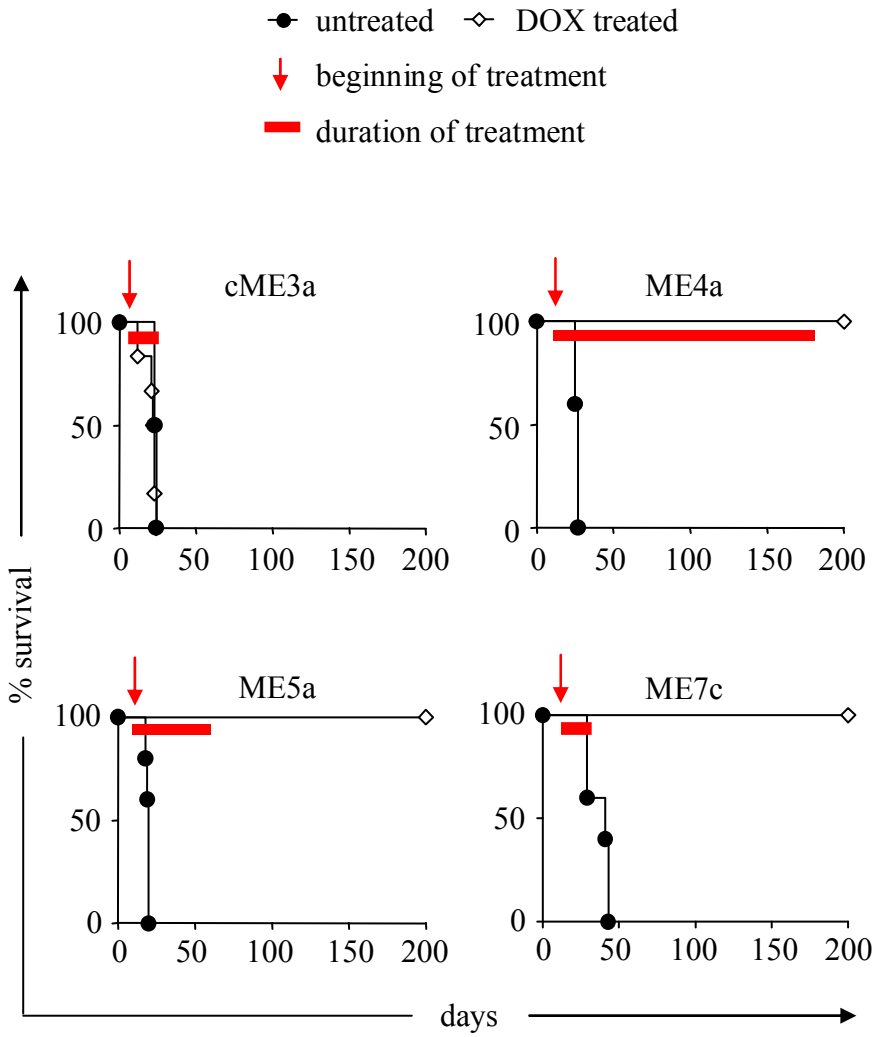


Figure 67: Effect of Doxycycline on survival of recipients

Recipients were transplanted with the indicated leukaemic cells and following their detection in the peripheral blood, one group was given Doxycycline in their water (open diamonds; cME3a n=6, ME4a n=5, ME5a n=5, ME7c n=4) and the other left untreated (filled circles; cME3a n=4, ME4a n=5, ME5a n=5, ME7c n=5). Red arrows indicate the point at which treatment started, red bars on the survival curves indicate the duration of treatment in each experiment. Doxycycline treated mice were still alive 350, 270 and 360 days after transplantation of ME4a, ME5a and ME7c cells, respectively.

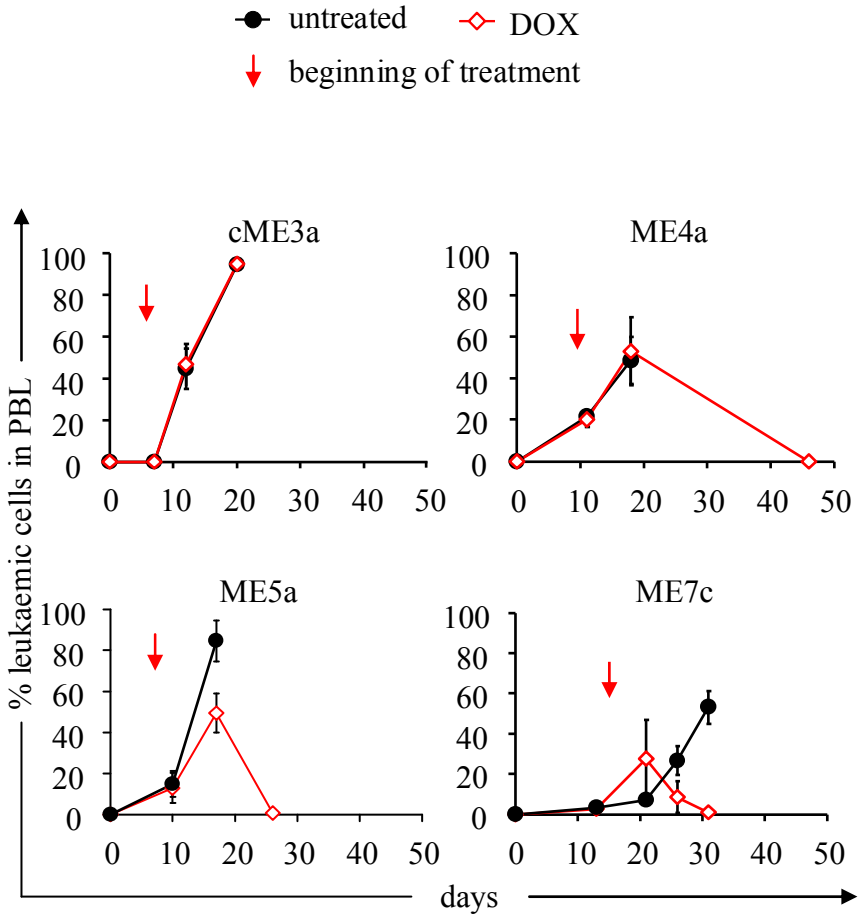


Figure 68: Effect of Doxycycline on the elimination of leukaemic cells from the peripheral blood

Recipient mice were transplanted with the indicated leukaemic cells and following their detection in the peripheral blood, one group was given Doxycycline in their water (open red diamonds; cME3a n=6, ME4a n=5, ME5a n=5, ME7c n=4) and the other left untreated (filled black circles; cME3a n=4, ME4a n=5, ME5a n=5, ME7c n=5). Red arrows indicate the point at which DOX treatment started. For conditional cells leukaemic cells were identified as (Mac-1$^+$EGFP$^+$)/Mac-1. For constitutive cells leukaemic cells were identified as (Mac-1$^+$CD45.2$^+$)/Mac-1.

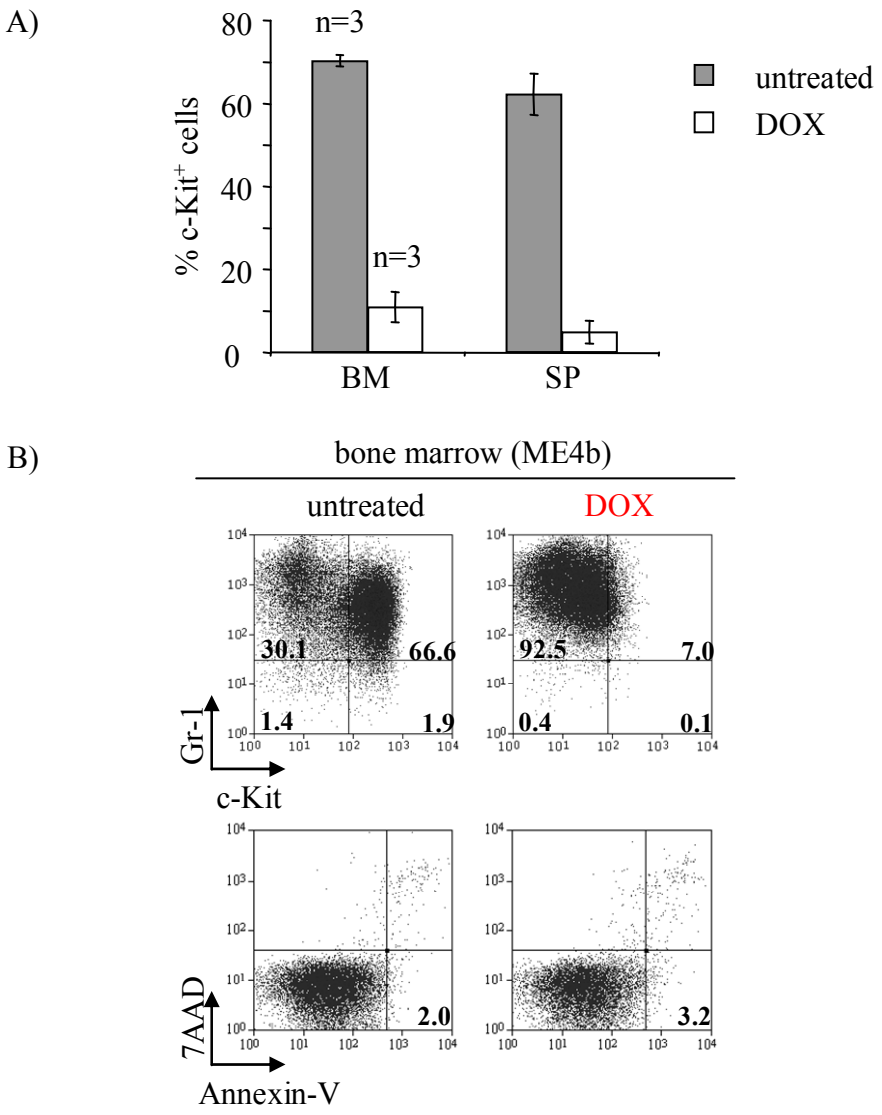


Figure 69: Leukaemic cells are not apoptotic in response to Doxycycline

A) Percentage of EGFP$^+$ ME4b cells expressing c-Kit in the bone marrow and spleen of the recipient mice, treated (grey bars) or untreated (white bars) with Doxycycline. Bars represent mean values (n=3), error bars represent the standard deviations. B) Dot plots show Gr-1 and c-Kit expression (upper panel), and staining with 7AAD and Annexin-V (lower panel), on gated EGFP$^+$ ME4b cells in the bone marrow of mice 18 days after transplantation with ME4b cells and 3 days after their treatment (right plot), or not (left plot), with Doxycycline. Numbers in the plots represent the percentage of cells in each quadrant (upper panel) and of apoptotic (7AAD$^-$, Annexin-V$^+$) EGFP$^+$ cells (lower panel).

Therefore, bone marrow from ME4b transfers was also analysed for 7-amino-actinomycinD (7AAD) and Annexin-V staining (Figure 69 B bottom panel). However, the cells showed no detectable apoptosis in response to Doxycycline *in vivo*. This was also shown for recipients transplanted with ME7c cells. Upon detection of leukaemic cells in the peripheral blood on day 18 after transfer, bone marrow from the first group of recipients (n=3) was harvested and analysed. On the same day Doxycycline treatment began for the remaining ME7c recipients. Bone marrow was then harvested and analysed three days as well as eight days after the beginning of Doxycycline treatment (Figure 70). At each time point, three individual ME7c recipients were sacrificed. Leukaemic $CD45.2^{+}EGFP^{+}$ cells with high c-Kit expression were detected in the bone marrow 18 days after transfer and before treatment with Doxycycline began. 21 days after the cells were transferred, and three days into Doxycycline treatment, the second group of ME7c recipients was sacrificed. Although leukaemic $CD45.2^{+}EGFP^{+}$ cells were still detected in the bone marrow, they expressed reduced levels of c-Kit. 26 days after the cells were transferred, and eight days into Doxycycline treatment, the third group of ME7c recipients was sacrificed. Leukaemic $CD45.2^{+}EGFP^{+}$ cells were almost completely eliminated from the bone marrow. Taken together with the *in vitro* data, this data suggested that loss of leukaemic cells results from terminal differentiation *in vivo*.

In some recipients, leukaemic cells reappeared in the peripheral blood and the mice relapsed with leukaemia (Figure 71 upper panel). All five recipients transplanted with ME4c leukaemic cells relapsed while they were still on Doxycycline treatment. One recipient transplanted with ME4b relapsed, also while still on Doxycycline treatment, and one recipient relapsed 101 days after the transfer, and 41 days after Doxycycline treatment was stopped.

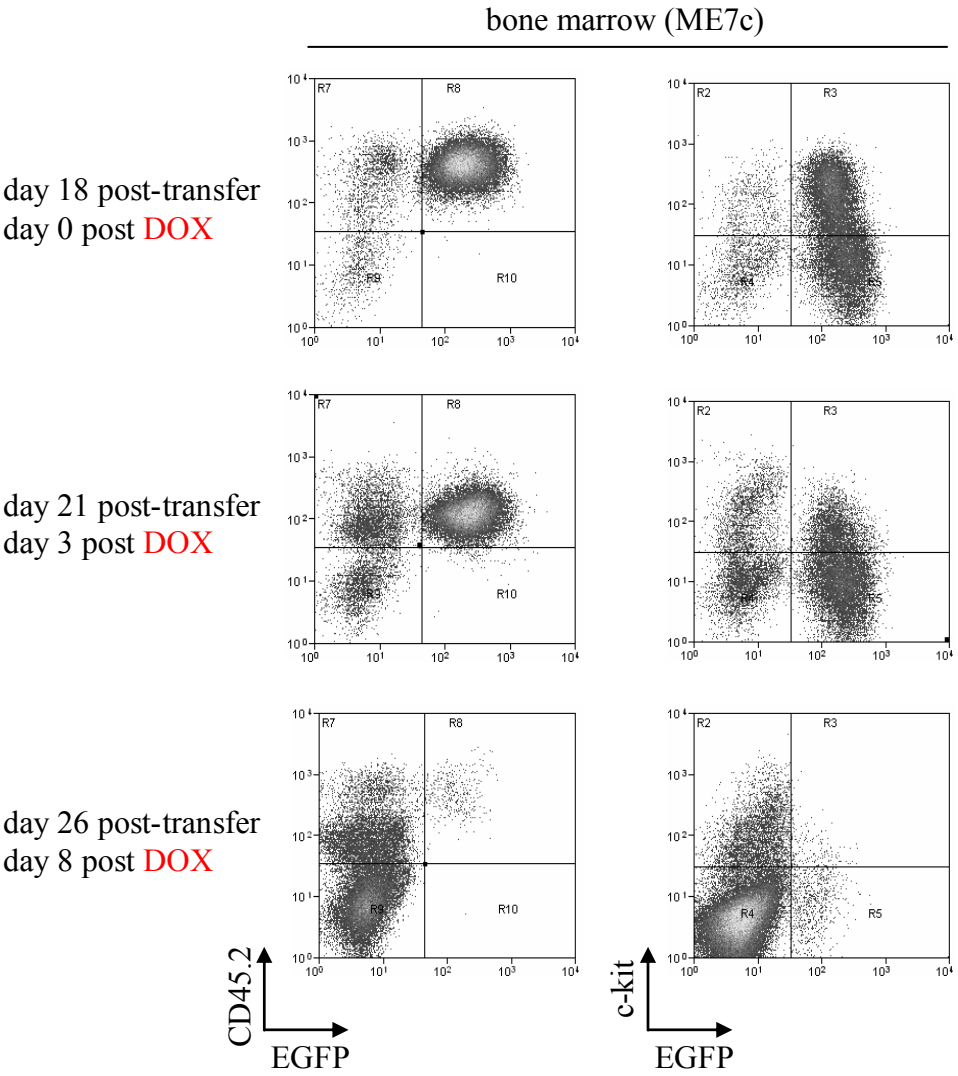


Figure 70: Doxycycline causes leukaemic cells to differentiate *in vivo*

The dot plots show the presence of c-Kit^{+}EGFP^{+} leukaemic ME7c cells in the bone marrow of three individual recipients after their treatment with Doxycycline (day 0, day 3 and day 8). This data is representative for three recipients per time point.

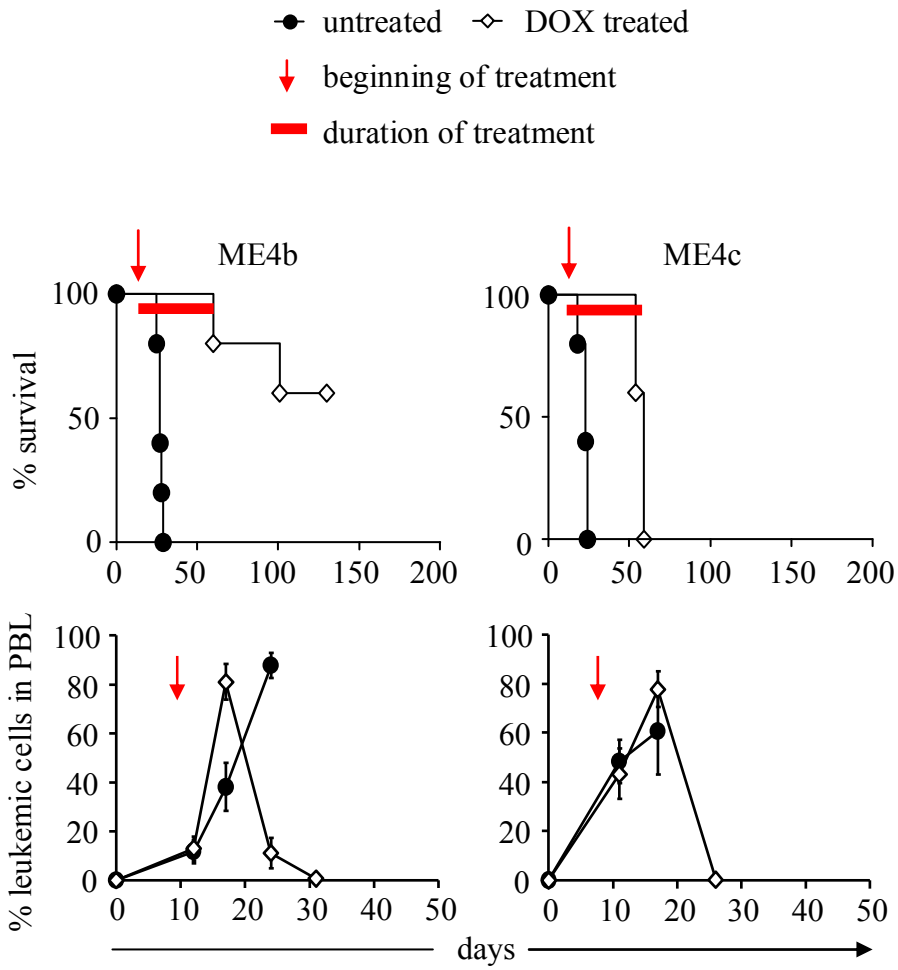


Figure 71: Some recipients relapsed during or after Doxycycline treatment

Recipient mice were transplanted with the indicated leukaemic cells and following their detection in the peripheral blood, one group was given Doxycycline in their water (open diamonds; ME4b n=5, ME4c n=5) and the other left untreated (filled circles; ME4b n=5, ME4c n=5). Red arrows indicate the point at which treatment started, red bars on the survival curves indicate the duration of treatment in each experiment. All of the mice transplanted with ME4c leukaemic cells relapsed while on Doxycycline treatment and two of the ME4b recipients relapsed: one while on Doxycycline treatment and one 41 days after Doxycycline withdrawal.

In all cases, the complete elimination of leukaemic cells from the peripheral blood was initially achieved, prior to the relapse (Figure 71 lower panel). In order to examine the expression of MLL-ENL in the relapses that occurred while recipients where being treated with Doxycycline, total RNA was isolated from splenocytes of the ME4cI, ME4cII and ME4bI relapses (Figure 72 A). All three analysed relapses had much higher *MLL-ENL* expression when compared to the parental immortalised cell line ME4, cultured with Doxycycline. Leukaemic cells were also isolated from the two ME4b recipients which relapsed, while on Doxycycline (ME4bI) and after (ME4bII) Doxycycline treatment. The isolated leukaemic cells were cultured in RPMI medium supplemented with 100 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6. MLL-ENL expression in response to Doxycycline treatment was analysed by qPCR (Figure 72 B). In both cases, the *MLL-ENL* expression was not abrogated by addition of Doxycycline. Although the ME4bII cell line, produced from the relapsed leukaemia, expressed relatively low levels of *MLL-ENL*, these were not reduced by Doxycycline treatment. In addition the low level of *MLL-ENL* expression in this cell line correlated with a particularly slow proliferation rate (data not shown). Furthermore, the relative level of *MLL-ENL* expression was much lower in ME4bII cells compared to ME4 and ME4bI, which could be a possible explanation for the much longer latency for the development of leukaemia in this case. These results suggested that MLL-ENL expression, in all of the relapsed leukaemias analysed had become Doxycycline-insensitive and treatment with Doxycycline could no longer abrogate the MLL-ENL expression.

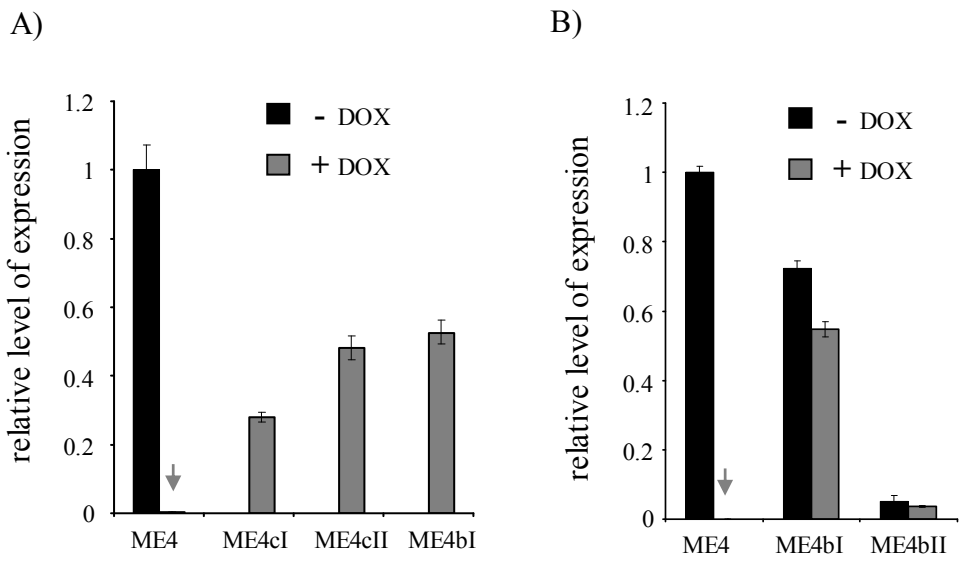


Figure 72: Leukaemic cells require MLL-ENL expression for survival *in vivo*

A-B) Relative level of *MLL-ENL* mRNA expression in immortalized ME4 cells after treatment (open bars or indicated with arrow) or not (filled bars) for 48 hours with 2 µg/ml Doxycycline. Also shown in panel A are levels of *MLL-ENL* mRNA expression in leukaemias isolated from the spleens of secondary recipient mice 54 days (ME4cI and ME4cII) after transplantation with ME4c leukaemic cells or 60 days (ME4bI) after transplantation with ME4b leukaemic cells (all on Doxycycline treatment). Also shown in panel B) are levels of *MLL-ENL* mRNA expression in ME4b leukaemias following culture in RPMI medium with (open bars) or without (filled bars) Doxycycline. The cells were isolated from the spleens of secondary recipient mice 60 days (ME4bI, on Doxycycline treatment) and 101 days (ME4bII, off Doxycycline treatment) after transplantation. Values determined by quantitative RT-PCR are normalised to untreated ME4 cells. Columns represent the mean of triplicate measurements, error bars represent the standard deviations.

6.4. Discussion – Chapter VI

Our study shows that constitutively and conditionally immortalised MLL-ENL cell lines induced leukaemia *in vivo* and that this leukaemia was transplantable into subsequent recipients. We observed that although leukaemic cells acquired additional genetic abnormalities, continuous MLL-ENL expression was required for maintaining leukaemia *in vivo*. Furthermore, we have shown that the leukaemia completely regressed upon loss of MLL-ENL activity. In the absence of MLL-ENL, leukaemic and immortalised cells underwent terminal myeloid differentiation *in vitro* and *in vivo*. Leukaemic cells remained growth factor dependent in culture, to the same combination of growth factors as the immortalised cells from which they were derived. However, the immunophenotype of leukaemic cells showed intermediate to high expression of the myeloid differentiation marker Gr-1. This heterogeneous leukaemic cell population is slightly different to the more homogeneous population of immortalised cells from which they were derived.

Some of the recipients relapsed with leukaemia after the complete elimination of leukaemic cells from the peripheral blood was initially achieved. Since all mice, apart from one case, that relapsed were still on Doxycycline treatment but nevertheless showed significant expression of *MLL-ENL* mRNA, it seems likely that MLL-ENL expression in these recipients had become tTA independent. Therefore, MLL-ENL expression could not be abrogated by addition of Doxycycline anymore. Two other *in vivo* studies have described a similar loss of conditional oncogene expression in response to Doxycycline or Tetracycline treatment. Felsher *et al.* used a Tet-regulatory system for *MYC* expression in mice and showed that 10% of the mice relapsed despite continuous Doxycycline treatment (Felsher *et al.*, 1999).

Furthermore, all the *MYC* expressing lymphoid tumours had acquired novel chromosomal rearrangements (Karlsson *et al.*, 2003). Koschmieder *et al.* developed a transgenic model in which tTA was placed under the control of the murine stem cell leukaemia (SCL) gene regulatory elements and showed that conditional expression of BCR-ABL resulted in reversible chronic myeloid leukaemia (CML) and B lymphoid blast crisis (Koschmieder *et al.*, 2005). However, one of these leukaemias did not regress upon Tetracycline treatment and tumour cells were shown to have retained BCR-ABL expression despite the presence of Tetracycline (Koschmieder *et al.*, 2005). Our similar findings suggest an *in vivo* selection for leukaemic cells with Doxycycline-independent MLL-ENL expression, which results in continuous MLL-ENL expression that cannot be abrogated. These data further support the critical importance of MLL-ENL expression for leukaemic progression *in vivo*.

We showed that secondary recipients transplanted with leukaemic splenocytes developed leukaemia with much shorter latencies than primary recipients transplanted with immortalised cells. Other *in vivo* MLL-fusion studies have also reported that secondary recipients develop leukaemia with shortened latencies (Lavau *et al.*, 1997; Krivtsov *et al.*, 2006; Somervaille *et al.*, 2006). However, the reason for this event has not been previously addressed. We analysed leukaemic cells from secondary recipients in order to examine whether the shortened latencies are caused by an outgrowth of a minor aggressive clone in the primary recipients, or are due to *in vivo* conditioning to microenvironmental signals, or due to additional acquired genetic abnormalities. Our data showed that immortalised and leukaemic cells have the same retroviral integration pattern, suggesting that the outgrowth of a minor clone *in vivo* was unlikely. We then showed that secondary recipients develop AML with similar latencies when comparing freshly isolated leukaemic splenocytes

to cells transplanted following culture for one month *in vitro*. This suggests that *in vivo* conditioning to microenvironmental signals does not cause shortened latencies. However, we have also shown that the leukaemic cells had acquired additional genetic abnormalities, which could not be detected in the immortalised cells from which they were derived. Additional secondary mutations have also been reported in human leukaemias associated with the MLL-ENL translocation but have not been reported in other murine studies (Moorman *et al.*, 1998). Since we demonstrated gross additional genetic abnormalities, such as the observed trisomy, or gains and losses of chromosomal regions, it is possible that these may be responsible for the reduced latency of AML upon secondary transplantation. Further analysis of the genes which are mapped to the regions of chromosomal gains or losses is necessary in order to examine whether some of these genes could be involved in the process of oncogenic transformation mediated by MLL-fusion oncogenes.

We and others have shown that cancer cells are still physiologically dependent on the continued activity of a specific oncogene for maintenance of their malignant phenotype (Felsher *et al.*, 1999; Koschmieder *et al.*, 2005; Horton, Walf-Vorderwülbecke *et al.*, 2009). Our data supports the theory of 'oncogene addiction' (Jonkers *et al.*, 2004; Weinstein *et al.*, 2006) suggesting that the immortalised and leukaemic cells are addicted to MLL-ENL expression. Since transcription factors, such as MLL-fusion oncogenes, are difficult to target with drugs, therapeutic approaches aiming to directly inhibit oncogene expression are unlikely to be effective. However, targeting downstream pathways which are regulated by MLL-fusions may be a possible way of interfering with the oncogenic transformation and reversing immortalisation (Krivtsov *et al.*, 2006; Wong *et al.*, 2007). Using this model we will be able to validate the importance of target genes, identified by the

Affymetrix microarray screen, *in vivo*. It will be necessary to examine if abrogating identified and validated target genes such as *c-Myb* and *Siah1a* will interfere with MLL-ENL mediated transformation *in vivo* and cause established AML to regress. Despite additional chromosomal mutations we have shown that MLL-ENL expression is still necessary to maintain leukaemia *in vivo*, offering a valid approach to target MLL-fusion activity. This specific targeting of oncogenic pathways maintained by MLL-fusions would offer new possibilities for therapy of MLL-fusion induced leukaemias.

CHAPTER VII - CONCLUSION

It was shown that the intact MLL portion of the MLL-AF9 fusion gene was required to immortalise murine haematopoietic progenitor cells. Myeloid constitutively and conditionally immortalised MLL-AF9 and MLL-ENL cells were generated using a retroviral transduction assay and methylcellulose culture. Stable constitutive and conditional cell lines were established in liquid culture and were maintained indefinitely in the presence of the myeloid growth factors SCF, IL-6 and IL-3. MLL-AF9 protein and mRNA expression was down-regulated in conditional cell lines in the presence of Doxycycline, but was not affected in constitutive cells. Similar results were observed for MLL-ENL mRNA expression. All of the characterised cell lines had a similar immature myeloid phenotype (c-Kit^{+}Gr-1^{+}Mac-1^{hi}) and myeloblastic morphology in the absence of Doxycycline. Upon loss of MLL-fusion expression, conditionally immortalised cells differentiated into more mature myeloid cells (c-kit^{-}Gr-1^{hi}Mac-1^{hi}). Constitutive cells were not affected by addition of Doxycycline to the cultures.

The known MLL-AF9 and MLL-ENL target genes, *HoxA9* and *Meis1*, were expressed in the characterised cell lines and their expression was down-regulated upon loss of MLL-fusion expression. Cell lines with similar expression levels of *HoxA9* and *Meis1*, as well as similar levels of down-regulation of these genes in the presence of Doxycycline, were selected for the gene expression analysis. Three conditional MLL-AF9 (MA1, MA3, MA4) and MLL-ENL (ME4, ME5, ME7) cell lines as well as one constitutive MLL-AF9 (cMA3) and MLL-ENL (cME3) cell lines were picked for the gene expression study and grouped as replicates. Global gene expression analysis confirmed the up-regulation of known MLL-fusion target genes,

such as genes of the *HoxA* cluster, *Meis1* and *Mef2c*, and identified a large number (2,934 genes) of differentially expressed MLL-fusion target genes. At least twelve up-regulated target genes were found to be associated with the Wnt signalling pathway.

Thirteen up-regulated target genes were selected for knock-down including some of the Wnt signalling associated genes and its key regulator *Ctnnb1*, which was not among the differentially expressed MLL-fusion target genes. Immortalised cell lines were transduced with shRNA knocking down specific target gene expression. Knock-down of two genes, *c-Myb* and *Msi2*, had a marked effect on the transduced cells, whereas knock-down of *Frat1* and *c-Myc* had a slight effect. Knock-down of all other selected target genes did not have any effect on the immortalised cells. In addition, down-regulation of *HoxA9*, *c-Myb*, *c-Myc*, *Frat1*, *Msi2*, *Pontin* and *Reptin* expression upon loss of MLL-fusion expression was confirmed by qPCR. Protein expression of c-Myb and Pontin was also validated by Western blot analysis. In contrast to the Affymetrix analysis, β-Catenin mRNA and protein expression were found to be down-regulated upon loss of MLL-fusion expression.

The E3 ubiquitin ligase Siah1a was found to be down-regulated in the presence of MLL-fusions. Since Siah1a targets not only β-Catenin for proteasomal degradation but also AF4, a member of the MLL-fusion complex, and c-Myb, a down-stream mediator of MLL-fusion activity, it was selected for further analysis. It was shown that *Siah1a* mRNA expression decreased in the presence of MLL-fusion proteins and that this regulation correlated with an increase in AF4 protein expression, a Siah1a degradation target. In order to analyse the role of Siah1a and Wnt signalling in MLL-fusion mediated transformation, Hexachlorophene was used

to study induction of Siah1a function without abrogating the expression of MLL-AF9 or MLL-ENL. It was shown that MLL-fusion immortalised cells differentiate in response to Hexachlorophene treatment at a drug concentration-dependent differentiation rate. The results suggested that MLL-fusion target genes, identified by the Affymetrix analysis, are also differentially regulated upon treatment with Hexachlorophene, presumably through induction of Siah1a activity. The consequence of induced Siah1a activity would be proteasomal degradation of its targets, such as AF4, c-Myb and β-Catenin. Therefore, the transcriptional elongation complex, recruited by AF4, could no longer remain active and MLL-fusion target gene expression would consequently be abrogated. However, further experiments are necessary in order to prove the direct induction of Siah1a activity by Hexachlorophene. Figure 73 illustrates a proposed model of oncogenic MLL-fusion activity.

Conditionally immortalised MLL-ENL cell lines were found to induce leukaemia *in vivo*. Secondary recipients transplanted with leukaemic cells developed leukaemia with much shorter latencies than primary recipients transplanted with immortalised cells. It was shown that the shortened latencies were not caused by an outgrowth of a minor aggressive clone in the primary recipients or by *in vivo* conditioning to microenvironmental signals. However, we have shown that the leukaemic cells isolated from primary recipients had acquired additional genetic abnormalities. The leukaemic cells remained dependent on MLL-ENL expression *in vitro* and *in vivo* and its ablation resulted in differentiation and regression of established leukaemias. We were able to show that MLL-ENL expression is still necessary to maintain leukaemia *in vivo*, validating the targeting of MLL-fusion transcriptional activity in leukaemic therapy.

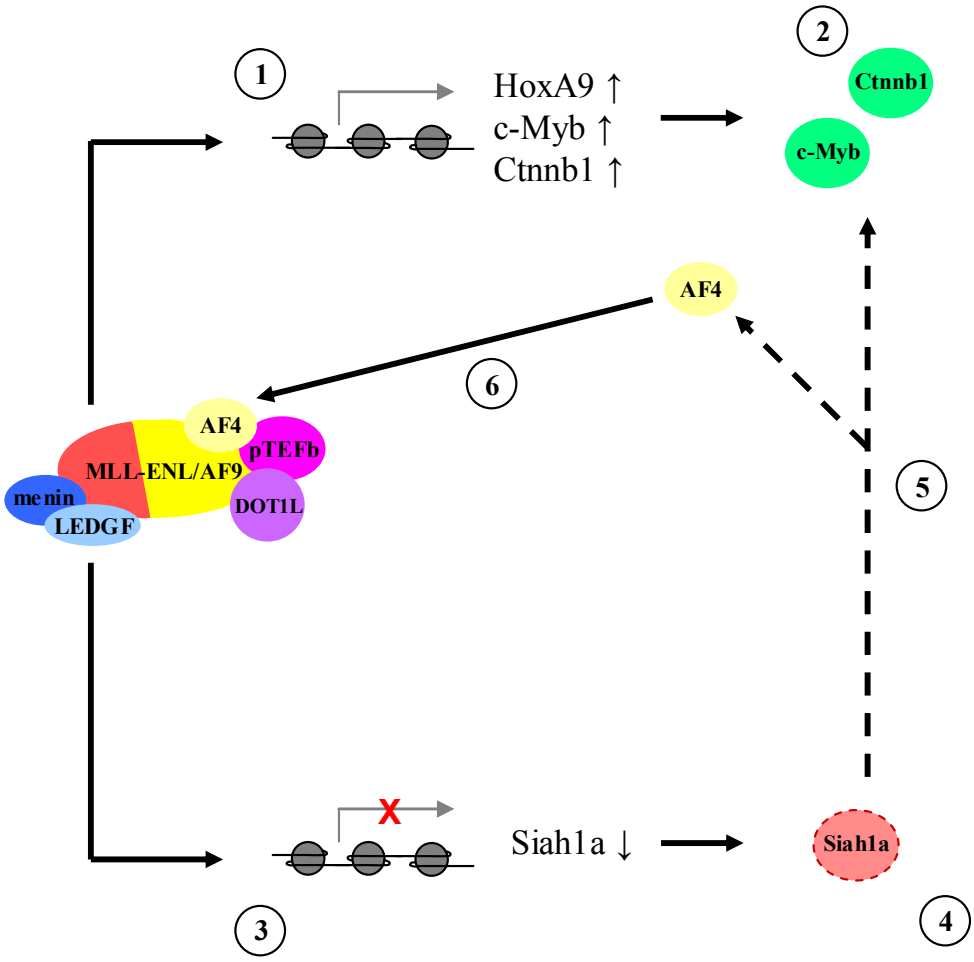


Figure 73: Proposed model of MLL-fusion activity

Target genes are regulated at the level of transcription by MLL-fusion oncogenes and at the level of protein by MLL-fusion induced Siah1a repression. 1) MLL-fusion up-regulate target genes, such as *HoxA9*, *c-Myb*, *Ctnnb1*; 2) level of c-Myb and Ctnnb1 protein expression are increased; 3) MLL-fusion down-regulate *Siah1a* mRNA expression; 4) Siah1a activity is decreased in the presence of MLL-fusions; 5) proteasomal degradation of Siah1a targets, such as c-Myb, Ctnnb1, AF4, is decreased; 6) transcription elongation complex recruits AF4 and remains active in the presence of MLL-fusions.

CHAPTER VIII - REFERENCES

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APPENDIX

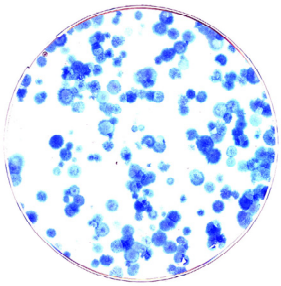
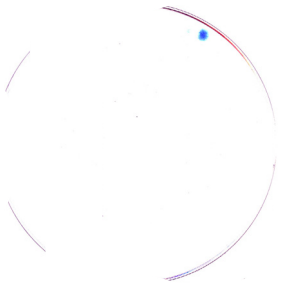
Virus dilution factor:	10^{-3}	10^{-5}
		
Infectious particle (ip) per ml:	1.47 x 10^6 ip/ml	1 x 10^6 ip/ml

Figure 74: Determination of the viral titre of pMSCV-neo-TRE-fMLLAF9

The viral titre was determined by titration on NIH-3T3 cells and was calculated using the following formula:

viral titre (infectious particles/ml) = (number of colonies x dilution factor)/2

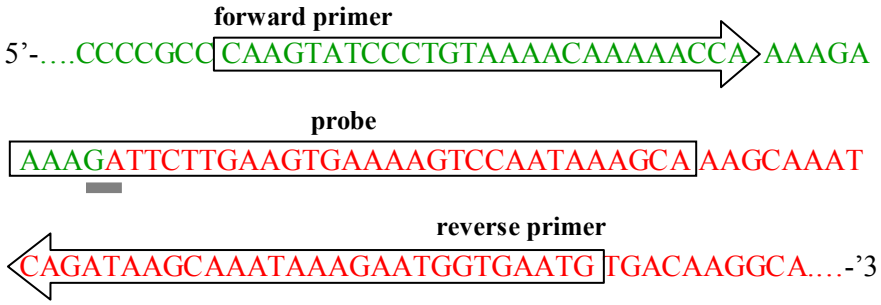


Figure 75: MLL-AF9 qPCR assay

The figure illustrates the location of the MLL binding forward primer, the AF9 binding reverse primer and the probe, which binds across the MLL-AF9 breakpoint. The MLL part of the construct shown in this figure is highlighted in green, the AF9 part of the construct shown in this figure is highlighted in red. The location of the breakpoint in this construct is underlined in grey.

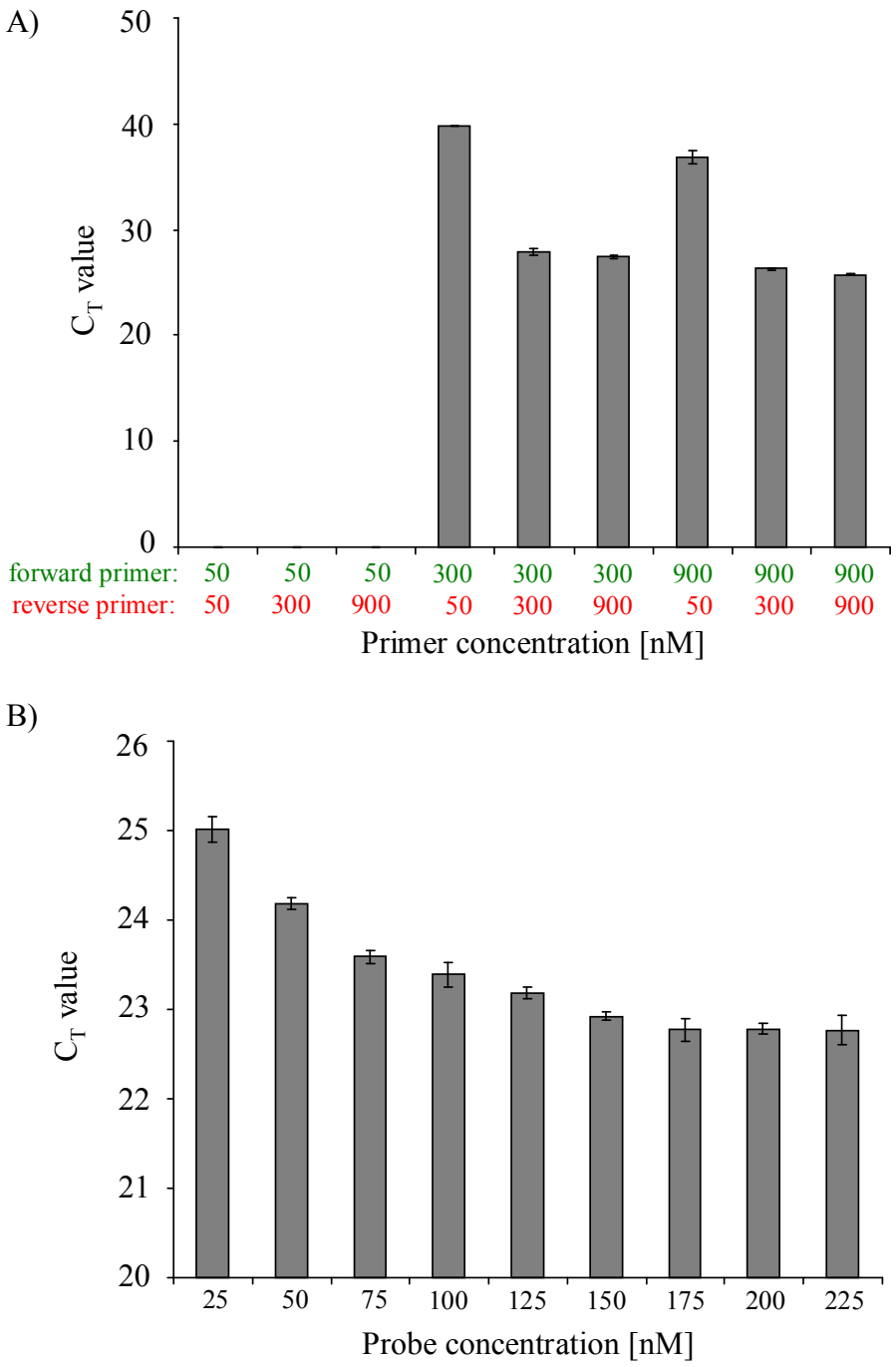


Figure 76: MLL-AF9 qPCR assay, primer and probe optimisation

A specific primer and probe set spanning the MLL-AF9 breakpoint region was designed and optimised to measure relative expression levels of MLL-AF9 by qPCR. A) The optimal primer concentration and B) the optimal probe concentration were determined by plotting the cycle threshold (C_T) value versus primer or probe concentration. Columns represent the mean of triplicates, error bars represent the standard deviations.

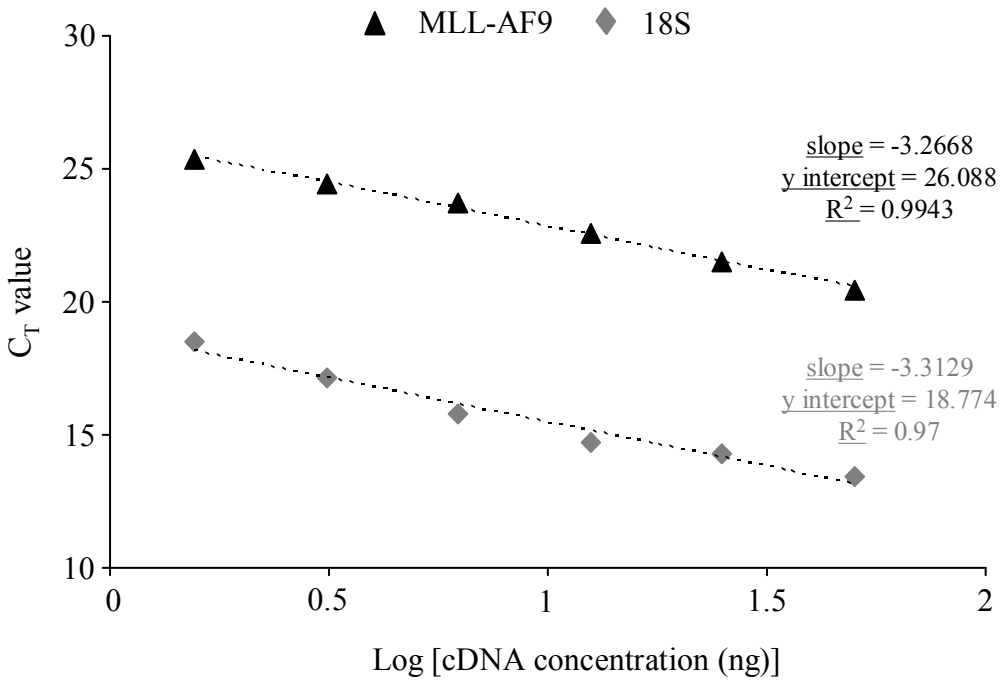


Figure 77: Standard curve for MLL-AF9 and 18S qPCR assays

The graph shows the standard curves for MLL-AF9 (black triangles) and for 18S (grey diamonds) generated by qPCR of serially diluted cDNA (0.39 ng to 50 ng). The slope reflects the efficiency of the assay, the y intercept reflects the sensitivity and R^2 indicated the reproducibility of the assay at low and high cDNA concentrations.

Table 21: List of all hairpin oligonucleotides designed and tested in this study

No.	Gene Name	Accession No.	Sequence (5'-miR30senseloopantisensemiR30-3')	Design tool
1	*Meis1*	NM_010789	TGCTGTTGACAGTGAGCGCGTGTCCTATCTTAGCATTTAATAGTGAAGC CACAGATGTATTAAATGCTAAGATAGGACACATGCCTACTGCCTCGGA	Kathadin
2	*Meis1*	NM_010789	TGCTGTTGACAGTGAGCGCCTGGATAACTTGATGATTCAATAGTGAAGC CACAGATGTATTGAATCATCAAGTTATCCAGTTGCCTACTGCCTCGGA	Kathadin
3	*Meis1*	NM_010789	TGCTGTTGACAGTGAGCGCCCACACTATGTGTGTTGTTTCTAGTGAAGC CACAGATGTAGAAACAACACACATAGTGTGGATGCCTACTGCCTCGGA	Kathadin
4	*Zfhx1b*	NM_015753	TGCTGTTGACAGTGAGCGCGCAAGAAATGTATTGGTTTAATAGTGAAGC CACAGATGTATTAAACCAATACATTTCTTGCTTGCCTACTGCCTCGGA	RNAi codex
5	*Zfhx1b*	NM_015753	TGCTGTTGACAGTGAGCGCCCACTAGACTTCAATGACTATTAGTGAAGC CACAGATGTAATAGTCATTGAAGTCTAGTGGTTGCCTACTGCCTCGGA	RNAi codex
6	*Zfhx1b*	NM_015753	TGCTGTTGACAGTGAGCGAAGACTGTAGCTTATCCTTTATTAGTGAAGC CACAGATGTAATAAAGGATAAGCTACAGTCTGTGCCTACTGCCTCGGA	Katahdin
7	*P2ry2*	NM_008773	TGCTGTTGACAGTGAGCGCGCTGGCAAATGTCAAACCATTTAGTGAAGC CACAGATGTAAATGGTTTGACATTTGCCAGCATGCCTACTGCCTCGGA	RNAi codex
8	*P2ry2*	NM_008773	TGCTGTTGACAGTGAGCGAGCCAATCCTATTTCAATTTAATAGTGAAGC CACAGATGTATTAAATTGAAATAGGATTGGCCTGCCTACTGCCTCGGA	RNAi codex
9	*P2ry2*	NM_008773	TGCTGTTGACAGTGAGCGCGGGAACCATAACTTATATGAATAGTGAAG CCACAGATGTATTCATATAAGTTATGGTTCCCATGCCTACTGCCTCGGA	RNAi codex

10	*Hpgd*	NM_008278	TGCTGTTGACAGTGAGCGAGGTGTAAGACTGAATGTCATTTAGTGAAGC CACAGATGTAAATGACATTCAGTCTTACACCGTGCCTACTGCCTCGGA	RNAi codex
11	*Hpgd*	NM_008278	TGCTGTTGACAGTGAGCGATTCCTGTATCTGATGTTAATATAGTGAAGC CACAGATGTATATTAACATCAGATACAGGAAGTGCCTACTGCCTCGGA	Katahdin
12	*Hpgd*	NM_008278	TGCTGTTGACAGTGAGCGATCACTCAGACTGCATCTTTACTAGTGAAGC CACAGATGTAGTAAAGATGCAGTCTGAGTGAGTGCCTACTGCCTCGGA	Katahdin
13	*Frat1*	NM_008043	TGCTGTTGACAGTGAGCGAGGAAACAAGAGTGGACTTAATTAGTGAAG CCACAGATGTAATTAAGTCCACTCTTGTTTCCCTGCCTACTGCCTCGGA	RNAi codex
14	*Frat1*	NM_008043	TGCTGTTGACAGTGAGCGACCAGGACTTAGGTTTGTTTAATAGTGAAGC CACAGATGTATTAAACAAACCTAAGTCCTGGGTGCCTACTGCCTCGGA	RNAi codex
15	*Frat1*	NM_008043	TGCTGTTGACAGTGAGCGCCGGCTCAGCCTGCTATGGAACTAGTGAAGC CACAGATGTAGTTCCATAGCAGGCTGAGCCGATGCCTACTGCCTCGGA	Katahdin
16	*Frat2*	NM_177603	TGCTGTTGACAGTGAGCGCGGGCTTCTAACAATACTTGAATAGTGAAGC CACAGATGTATTCAAGTATTGTTAGAAGCCCTTGCCTACTGCCTCGGA	RNAi codex
17	*Frat2*	NM_177603	TGCTGTTGACAGTGAGCGCCCTGATTTATAGGATTCATAATAGTGAAGC CACAGATGTATTATGAATCCTATAAATCAGGATGCCTACTGCCTCGGA	RNAi codex
18	*Frat2*	NM_177603	TGCTGTTGACAGTGAGCGCGGTGGTCCTGATTTATAGGATTAGTGAAGC CACAGATGTAATCCTATAAATCAGGACCACCATGCCTACTGCCTCGGA	RNAi codex
19	*Fzd7*	NM_008057	TGCTGTTGACAGTGAGCGCCCGGCTAGGGCCTAAGGGAAATAGTGAAG CCACAGATGTATTTCCCTTAGGCCCTAGCCGGTTGCCTACTGCCTCGGA	RNAi codex

20	*Fzd7*	NM_008057	TGCTGTTGACAGTGAGCGACCTTGTCAAGTGGTGGTCAAATAGTGAAGC CACAGATGTATTTGACCACCACTTGACAAGGGTGCCTACTGCCTCGGA	RNAi codex
21	*Fzd7*	NM_008057	TGCTGTTGACAGTGAGCGATGCGAGGCGCTCATGAACAAGTAGTGAAG CCACAGATGTACTTGTTCATGAGCGCCTCGCAGTGCCTACTGCCTCGGA	Katahdin
22	*c-myb*	NM_010848	TGCTGTTGACAGTGAGCGACCCTTGCAGCTCAAGAAATTATAGTGAAGC CACAGATGTATAATTTCTTGAGCTGCAAGGGCTGCCTACTGCCTCGGA	RNAi codex
23	*c-myb*	NM_010848	TGCTGTTGACAGTGAGCGAGGGAAACTTCTTCTGCTCAAATAGTGAAGC CACAGATGTATTTGAGCAGAAGAAGTTTCCCGTGCCTACTGCCTCGGA	RNAi codex
24	*c-myb*	NM_010848	TGCTGTTGACAGTGAGCGCAGAGACTCGGTGTTAGATAACTAGTGAAG CCACAGATGTAGTTATCTAACACCGAGTCTCTTTGCCTACTGCCTCGGA	Katahdin
25	*Msi2*	NM_054043	TGCTGTTGACAGTGAGCGACCAGCAAGTGTAGATAAAGTATAGTGAAG CCACAGATGTATACTTTATCTACACTTGCTGGGTGCCTACTGCCTCGGA	RNAi codex
26	*Msi2*	NM_054043	TGCTGTTGACAGTGAGCGCGCAAGTGTAGATAAAGTATTATAGTGAAG CCACAGATGTATAATACTTTATCTACACTTGCTTGCCTACTGCCTCGGA	RNAi codex
27	*Msi2*	NM_054043	TGCTGTTGACAGTGAGCGAACCCAGCAAGTGTAGATAAAGTAGTGAAG CCACAGATGTACTTTATCTACACTTGCTGGGTCTGCCTACTGCCTCGGA	Katahdin
28	*Sox4*	NM_009238	TGCTGTTGACAGTGAGCGACGCTCGATCGGGACCTGGATTTAGTGAAGC CACAGATGTAAATCCAGGTCCCGATCGAGCGCTGCCTACTGCCTCGGA	RNAi codex
29	*Sox4*	NM_009238	TGCTGTTGACAGTGAGCGCGGTTTATAGCTGTTGTGTTTATAGTGAAGC CACAGATGTATAAACACAACAGCTATAAACCTTGCCTACTGCCTCGGA	RNAi codex

30	*Sox4*	NM_009238	TGCTGTTGACAGTGAGCGACGAGATGATCTCGGGAGATTGTAGTGAAG CCACAGATGTACAATCTCCCGAGATCATCTCGCTGCCTACTGCCTCGGA	Katahdin
31	*Gata2*	NM_008090	TGCTGTTGACAGTGAGCGCGCCGCCATTACTGTGAATATTTAGTGAAGC CACAGATGTAAATATTCACAGTAATGGCGGCATGCCTACTGCCTCGGA	RNAi codex
32	*Gata2*	NM_008090	TGCTGTTGACAGTGAGCGAGGAGAGTGTTTGTTTGGAGAGTAGTGAAG CCACAGATGTACTCTCCAAACAAACACTCTCCGTGCCTACTGCCTCGGA	Katahdin
33	*Gata2*	NM_008090	TGCTGTTGACAGTGAGCGAGGCCTCTGTCTGTTTACCCAGTAGTGAAGC CACAGATGTACTGGGTAAACAGACAGAGGCCCTGCCTACTGCCTCGGA	Katahdin
34	*Ctnnb1*	NM_007614	TGCTGTTGACAGTGAGCGCGGACCAGGTGGTAGTTAATAATAGTGAAG CCACAGATGTATTATTAACTACCACCTGGTCCTTGCCTACTGCCTCGGA	RNAi codex
35	*Ctnnb1*	NM_007614	TGCTGTTGACAGTGAGCGACCAGTGTGGGTGAATACTTTATAGTGAAGC CACAGATGTATAAAGTATTCACCCACACTGGCTGCCTACTGCCTCGGA	RNAi codex
36	*Ctnnb1*	NM_007614	TGCTGTTGACAGTGAGCGCACTGTTGGATTGATTCGAAACTAGTGAAGC CACAGATGTAGTTTCGAATCAATCCAACAGTTTGCCTACTGCCTCGGA	Katahdin

All hairpin oligonucleotides were designed against the sense strand of the target gene, using either Katahdin prediction software (http://katahdin.cshl.org:9331/siRNA/RNAi.cgi?type=shRNA) or sequences published on RNAi codex web site (http://codex.cshl.edu).

Table 22: 41 up-regulated genes picked by rank and publication

Gene Symbol	Accession Number	Fold Change	Product
Hpgd	1419906_at	34.11	Hydroxyprostaglandin dehydrogenase 15
Cited4	1425400_a_at	12.2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain4
Ascl2	1422396_s_at	10.93	achaete-scute complex homolog-like 2 (Drosophila)
Sox4	1419156_at	8.23	SRY-box containing gene 4
P2ry2	1450318_a_at	7.474	purinergic receptor P2Y, G-protein coupled 2
Frat2	1455220_at	6.627	frequently rearranged in advanced T-cell lymphomas 2
Znrf3	1435608_at	5.839	zinc and ring finger 3
Six4	1425767_a_at	5.831	sine oculis-related homeobox 4 homolog (Drosophila)
Ppan	1423703_at	4.829	peter pan homolog (Drosophila)
Zfp248	1436473_at	4.775	zinc finger protein 248
Six1	1427277_at	3.968	sine oculis-related homeobox 1 homolog (Drosophila)
Hmgn1	1422495_a_at	3.924	high mobility group nucleosomal binding domain 1
Pontin	1416585_at	3.916	RuvB-like protein 1

Eif1a	1424343_a_at	3.822	eukaryotic translation initiation factor 1A
Pa2g4	1435372_a_at	3.779	proliferation-associated 2G4
Gas5	1455904_at	3.776	growth arrest specific 5
Zfhx1b	1442393_at	3.754	Zinc finger homeobox 1b
Tceal8	1418171_at	3.745	transcription elongation factor A (SII)-like 8
Nle1	1424731_at	3.726	notchless homolog 1 (Drosophila)
c-Myc	1424942_a_at	3.724	myelocytomatosis oncogene
Reptin	1422482_at	3.614	RuvB-like protein 2
Angptl4	1417130_s_at	3.587	angiopoietin-like 4
Pdcd4	1418840_at	3.571	programmed cell death 4
Mybbp1a	1423430_at	3.462	MYB binding protein (P160) 1a
c-Myb	1423430_at	3.462	MYB binding protein (P160) 1a
Nr4a2	1455034_at	3.151	Nuclear receptor subfamily 4, group A, member 2 (Nr4a2)
Taf4b	1435303_at	3.144	TAF4B RNA polymerase II, TATA box binding protein (TBP)-associated factor
Frat1	1449814_at	3.047	frequently rearranged in advanced T-cell lymphomas

Aatf	1432394_a_at	3.042	apoptosis antagonizing transcription factor
Tcf4	1424089_a_at	3.04	transcription factor 4
Eef1d	1428135_a_at	3.038	eukaryotic translation elongation factor 1 delta
Tada2l	1452310_at	2.994	transcriptional adaptor 2 (ADA2 homolog, yeast)-like
Pdrg1	1428309_s_at	2.855	p53 and DNA damage regulated 1
Eif2b3	1434524_at	2.855	eukaryotic translation initiation factor 2B, subunit 3
Msi2	1435521_at	2.786	Musashi homolog 2 (Drosophila)
Ptch1	1428853_at	2.767	patched homolog 1
Cnksr3	1433983_at	2.764	Cnksr family member 3
Gata2	1450333_a_at	2.744	GATA binding protein 2
Tnfrsf11a	1430259_at	2.602	tumor necrosis factor receptor superfamily, member 11a
Fzd7	1450044_at	2.46	frizzled homolog 7 (Drosophila)
Rab34	1416590_a_at	2.411	RAB34, member of RAS oncogene family

The table lists 41 genes which were selected by rank and publication from the list of genes that were up-regulated in the presence of MLL-fusions. The entries in the list are sorted by fold change.