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An Investigation into the Role of MLL in Murine Haematopoiesis

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

Abstract

The *Mll* gene was originally identified through its role in infant acute myeloid and lymphoid leukaemias. *Mll* also has a role in normal haematopoiesis as identified using mouse knockout models. Homozygous mutation of *Mll* is embryonic lethal, which has limited research into its role in haematopoiesis.

To overcome this embryonic lethality, a conditional knockout mouse model of *Mll* was established. In this model, exons 9 and 10 of *Mll* were flanked by LoxP sites ('floxed'), which recombined to induce deletion of exons 9 and 10 in the presence of the Cre recombinase. Deletion of exons 9 and 10 lead to complete loss of the MLL protein as detected by immunoblotting. By breeding mice homozygous for the floxed *Mll* allele to mice that carried the Cre recombinase under the control of the Vav promoter, it was possible to delete *Mll* within the haematopoietic system.

Analysis of foetal and adult haematopoiesis in the absence of *Mll* was carried out using the new model. In embryos lacking *Mll*, the foetal liver showed a marked reduction in the number of colony forming cells as well as both long and short term haematopoietic stem cells. When transplanted into lethally irradiated recipients with wild type competitors, *Mll* deficient foetal liver cells were unable to contribute to reconstitution of the haematopoietic system.

In adult mice, removal of *Mll* had no apparent effect on the steady state haematopoietic system. Populations of myeloid, lymphoid and stem cells were unaffected. However, in competitive repopulation assays, *Mll* deficient bone marrow cells were unable to compete with wild type cells. This work suggests *Mll* is needed for the correct development of foetal liver haematopoiesis and also to maintain self-renewal potential in adult haematopoietic stem cells. However, it appears that *Mll* is not needed to maintain adult haematopoiesis under homeostatic conditions.

Abbreviations

5FU	5 Fluorouracil
AGM	Aorta Gonad Mesonephros
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloblastic Leukaemia
AML1	Acute Myeloid Leukaemia 1
ANOVA	Analysis of Variance
APS	Ammonium persulphate
BFU-E	Burst Forming Unit Erythroid
BM	Bone Marrow
BPTF	Bromodomain PHD finger Transcription Tactor
BSA	Bovine Serum Albumin
C/EBPα	CCAAT/Enhancer Binding Protein Alpha
CAFC	Cobblestone Area Forming Cell Assay
CAPS	N-Cyclohexyl-3-aminopropanesulfonic acid
CFU	Colony Forming Unit
CFU-GEMM	Colony Forming Unit Granulocyte, Erythrocyte
CMP	Common Myeloid Progenitor
CsCl	Caesium chloride
CTL	Cytotoxic T Cell
ddH_2O	Double Distilled water
DMEM	Dulbeccos Modified Eagle Media
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DP	Double Positive
DTT	Dithiothreitol
E	Embryonic Day
EBF	Early B cell Factor
EDTA	Ethylene-diamine-tetra-acetic Acid

ES cell	Embryonic Stem cell
FL	Foetal Liver
FOG-1	Friend of GATA-1
g	Grams
GMP	Granulocyte-Monocyte Progenitor
H3K4	Histone H3 Lysine 4
H3K4me3	Tri-methylated Histone H3 Lysine 4
H4	Histone H4
HBS	Hepes buffered saline
HCl	Hydrochloric acid
HDAC	Histone De-acetylase
HSC	Haematopoietic Stem Cell
Ing2	Inhibitor of Growth family 2
IRES	Internal Ribosomal Entry Site
kb	Kilobases
KO	Knockout
LIF	Leukaemia Inhibitory Factor
LMO2	LIM Domain Only 2
LMPP	Lympho-myleloid progenitor
LSK	$Lin^{-}Sca1^{+}c\text{-}kit^{hi}$
LTC-IC	Long Term Cell Initiating Culture
LT-HSC	Long Term Haematopoietic Stem Cell
Mcl-1	Myeloid Cell Leukaemia Sequence 1
mA	milliamps
MEF	Mouse embryonic fibroblast
MEP	Megakaryo-Erythroid Progenitor
MHC	Major Histocompatibility Complex
MLL	Mixed Lineage Leukaemia
MPP	Multipotent Progenitor
mg	Milligrams
MHC	Major Histocompatibility Complex

min	minutes
ml	Millilitres
MLL	Mixed Lineage Leukaemia
MPP	Multipotent Progenitor
MT Domain	MethylTransferase Domain
µl	microlitres
NaCl	Sodium chloride
NIMR	National Institute for Medical Research, Mill Hill
NK cell	Natural Killer cell
O/N	OverNight
P200	Gilson Pipetteman 200µl
PAGE	PolyAcrylamide Gel Electrophoresis
PAX5	Paired Box Gene 5
PBS	Phosphate Buffered Saline
PBX1	Pre-B-cell leukemia Homeobox 1
PcG	Polycomb Group
PCR	Polymerase Chain Reaction
PRC1	Polycomb Repressive Complex 1
P-sp	Paraortic splanchnopleura
Rb	Retinoblastoma gene
Rpm	Revolutions per minute
RT	Room Temperature
s	Seconds
Scl	Stem Cell Leukaemia
SD	Standard Deviation
SDS	Sodium Dodecyl-Sulphate
SEM	Standard Error of the Mean
SP	Single Positive
SSC	Sodium Chloride Sodium Citrate
ST-HSC	Short Term Haematopoietic Stem Cell
T_h cell	T Helper Cell

T_c cell	Cytotoxic T Cell
TE	Tris-EDTA solution
Tel	Translocation-Ets-Leukemia
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris(hydroxylmethyl)methylamine
V	Volts
WT	Wild Type

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Chapter 1

Introduction

1.1 Mll: The Mixed Lineage Leukaemia gene.

This study concentrates on the role of gene *Mll* (*Mixed Lineage Leukaemia)* in regulating haematopoiesis in foetal and adult mice. *Mll* was first discovered due to its involvement in chromosomal translocations in infant leukaemia (Daser and Rabbitts, 2004; Djabali et al., 1992; Ernst et al., 2002). Animal models and biochemical studies have shown that the *Mll* gene codes for a large protein involved in chromatin remodelling that the MLL protein has some role in regulating haematopoiesis in a normal context. This Chapter gives an overview of the processes that *Mll* may be involved in regulating and an introduction to the MLL protein itself.

1.2 Haematopoiesis

Haematopoiesis is defined as the formation and differentiation of red and white blood cells. In mammals, the continuous process of blood cell self renewal requires a population of haematopoietic stem cells (HSCs) that are able to give rise to all the different lineages of blood cells throughout the lifespan of an individual. These multi-potent stem cells are thought to undergo asymmetric cell division to produce progenitor cells which can then divide and differentiate to produce the lymphoid, myeloid and erythroid cells of the haematopoietic system (Cumano and Godin, 2007; Shizura et al., 2005; Weissman et al., 2001).

1.3 Haematopoietic development in mice

Embryonic haematopoiesis has been extensively studied in mice, and two distinct extra and intra-embryonic waves of haematopoiesis have been identified. In the first wave, primitive haematopoiesis begins in the blood islands of the yolk sac at

embryonic day 7.5 (E7.5) (Bonnet, 2003; Cumano and Godin, 2007; Fujikawa et al., 2003; Mikkola and Orkin, 2006; Wong et al., 1986). The first cells of haematopoietic origin to be produced in the embryo are found here and are thought to be derived from mesoderm cells of the primitive streak (Palis and Yoder, 2001). These are nucleated erythrocytes (so called primitive erythrocytes due to their resemblance to erythrocyte precursors in the adult bone marrow) that express foetal specific isoforms of haemoglobin (Cumano and Godin, 2007; Zhu and Emerson, 2002). At this time point, the yolk sac does not appear to contain haematopoietic stem cells capable of contributing to all adult haematopoietic lineages, as shown by repopulation assays (Cumano et al., 1996; Cumano and Godin, 2007; Godin and Cumano, 2002). However, it is possible that the yolk sac can support the expansion of definitive HSCs at later time points.

At E8.5, the first intra-embryonic haematopoietic precursors become apparent. These are situated in the paraortic splanchnopleura (P-sp) which then gives rise to the Aorta, Gonads, Mesonephros region (AGM) (Cumano et al., 1996; Godin and Cumano, 2002; Medvinsky and Dzierzak, 1996; Medvinsky et al., 1993). Cells which can give rise to long term reconstitution in *Rag-2*$^{-/-}$ *xyc*$^{-/-}$ mice have been detected in the AGM region by E10.5 (Cumano et al., 1996; Godin and Cumano, 2002; Medvinsky and Dzierzak, 1996; Muller et al., 1994). These cells, which reside in the ventral wall of the dorsal aorta, are thought to be the first definitive HSCs, which will eventually seed the foetal liver and then the bone marrow. There is also evidence to suggest that some definitive HSCs develop in the placenta (Gekas et al., 2005; Mikkola and Orkin, 2006).

Between E9.5 and E11.5, these HSCs transplant to the foetal liver, which becomes the primary site for haematopoiesis in the embryo (Cumano and Godin, 2007; Mikkola and Orkin, 2006). Until haematopoiesis transplants to the bone marrow at E15.5, the foetal liver is the main site of HSC expansion and differentiation. When cells from the foetal liver are transplanted into lethally irradiated mice, they are able to give rise to both long and short term haematopoiesis (Morrison et al., 1995; Rebel et al., 1996), showing the presence of HSCs that can function in the adult system. Following birth, the primary site of haematopoiesis is

the bone marrow, where HSCs reside in niches which are thought to protect their self renewal and multipotent capacities (Mikkola and Orkin, 2006).

1.4 Haematopoietic stem cells give rise to all the lineages of blood cells

In adult bone marrow, as in foetal liver, HSCs give rise to all the cells of the blood system. This small population of cells was originally identified through its ability to give rise to all mature blood cell lineages and to reconstitute the haematopoietic system when transplanted into lethally irradiated mice (Siminovitch et al., 1963; Smith et al., 1991; Spangrude et al., 1995).

The process by which HSCs divide and differentiate to give rise to mature cells is not known definitively, although several models have been proposed. (for review, see (Ema and Nakauchi, 2003).

One popular theory of HSC differentiation has been proposed by the group of Irving Weissman (Akashi et al., 2000; Kondo et al., 1997).In this model (see Figure 1.1,(Shizuru et al., 2005)) a subset of stem cells, the Long Term repopulating HSCs (LT-HSC) are capable of propagating haematopoiesis over the lifespan of an individual. These cells rest predominantly in the G_0 phase of cell cycle, and when transplanted into irradiated recipients, are able to maintain haematopoiesis for longer than 4 months (Forsberg et al., 2005; Forsberg et al., 2006). They can also reconstitute following serial transplantations into irradiated recipients, demonstrating their self-renewal capacity (Kiel et al., 2005). The LT-HSC gives rise to a population of more rapidly proliferating cells, the Short Term repopulating HSC (ST-HSC) which has a self-renewal lifespan of 6-8 weeks (Forsberg et al., 2006; Weissman, 2002) (Figure 1.1). The ST-HSC produces Multi-Potent Progenitors (MPPs) which have a self-renewal lifespan of only two weeks (Figure 1.1). All of these stem cell types, although they have different self-renewal capacity, are multipotent and can give rise to the different mature lineages of the haematopoietic system.

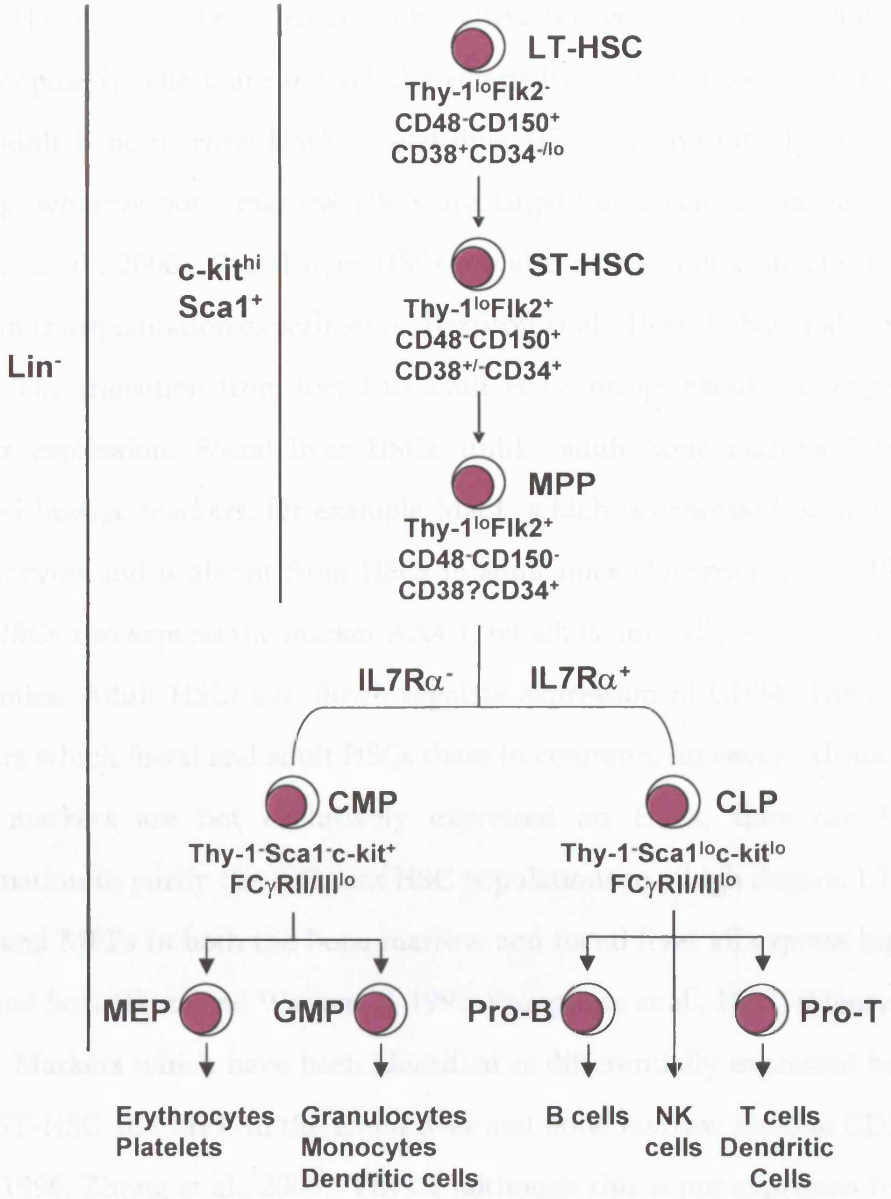

Figure 1.1. Haematopoiesis in Adult Mice.
LT-HSC – Long-Term HSC, ST-HSC – Short-Term HSC, MPP - Multi-Potent Progenitor CLP – Common Lymphoid Progenitor, CMP – Common Myeloid Progenitor, GMP – Granulocyte/Monocyte Progenitor, MEP – Megakaryocyte/Erythrocyte Progenitor. Figure adapted from Shizura et al., 2005 with additional information from Zhong *et al.* 2005 and Kiel *et al* 2005.

1.5 Characterisation of foetal and adults HSCs

HSCs have been extensively characterised in both foetal and adult haematopoiesis. There are several characteristics in which foetal liver HSCs differ from adult bone marrow HSCs. Foetal liver HSCs expand rapidly and are actively cycling, whereas bone marrow HSCs are largely quiescent (Cheshier et al., 1999; Nygren et al., 2006). Foetal liver HSCs are also able to out compete bone marrow HSCs in transplantation experiments (Harrison et al., 1997; Rebel et al., 1996).

The transition from foetal to adult HSCs brings about a change in surface marker expression. Foetal liver HSCs, unlike adult bone marrow HSCs, express selected lineage markers, for example Mac1, which is expressed on monocytes and granulocytes and is absent from HSCs in adult mice (Morrison et al., 1995). Foetal liver HSCs also express the marker AA4.1, which is normally expressed on B cells in adult mice. Adult HSCs also down regulate expression of CD34. There are several markers which foetal and adult HSCs share in common, however. Although some of these markers are not exclusively expressed on HSCs, they can be used in combination to purify the different HSC populations to a high degree. LT-HSCs, ST-HSCs and MPPs in both the bone marrow and foetal liver all express high levels of c-kit and Sca1 (Ikuta and Weissman, 1992; Spangrude et al., 1988) (Figure 1.1).

Markers which have been identified as differentially expressed between LT-HSC, ST-HSC and MPP in the foetal liver and bone marrow include CD38 (Randall et al., 1996; Zhong et al., 2005), Thy1.1 (although this is not expressed by all strains of mice (Spangrude et al., 1988)), and the SLAM family members CD48 and CD150 (Kiel et al., 2005; Kim et al., 2006) The expression of these markers on the different HSC subpopulations is detailed in Figures 1.1 and 1.2. The tyrosine kinase receptor Flk2/Flt3 can also be used to differentiate between LT and ST-HSC in adult bone marrow cells, but not foetal liver (Christensen and Weissman, 2001).

Phenotypic characteristics of the stem cells can also be used to isolate them from other haematopoietic populations. For example, treatment of mice with 5-fluoruracil (5FU) results in ablation of the majority of haematopoietic cells in the bone marrow. This is because 5FU targets actively cycling cells. HSCs, however, are

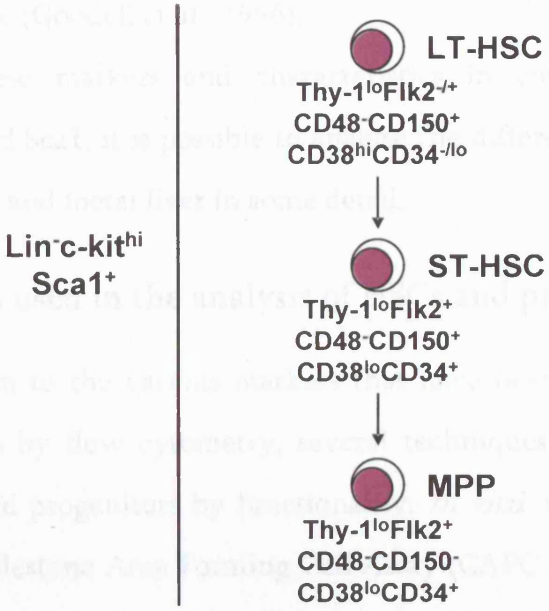

Figure 1.2. Identification of stem cells in foetal haematopoiesis.
The figure shows the model conceived by Irving Weissmans group. LT-HSC – Long-Term HSC, ST-HSC – Short-Term HSC, MPP - Multi-Potent Progenitor, CLP – Common. Figure adapted from Shizura et al., 2005 with additional information from Randall *et al,* 1996 and Kim *et al.* 1996.

predominantly quiescent and therefore immune to 5FU treatment. This means that HSCs are enriched in the bone marrow of mice following 5FU treatment, and can be purified in this manner (Ogata et al., 1995). HSCs are also more efficient at effluxing dyes using a multi-drug like transporter and this means that they can be purified by staining them with dyes such as Hoechst 33342 (Goodell et al., 1996). When the cells are analysed by flow cytometry, the HSCs form a so-called 'side population' of cells negative for Hoechst 33342, separating them from other haematopoietic cells, which are positive (Goodell et al., 1996).

Using these markers and characteristics in combination with Lineage markers, c-kit and Sca1, it is possible to analyse the different populations of HSC in the bone marrow and foetal liver in some detail.

1.6 Techniques used in the analysis of HSCs and progenitors

In addition to the various markers that have been characterised for use in identifying HSCs by flow cytometry, several techniques have been developed to identify HSCs and progenitors by functionality. *In vitro* methods to identify HSCs include the Cobblestone Area Forming Cell Assay (CAFC Assay) and the Long term Culture Initiating Cell Assay (LTC-IC Assay). Both of these techniques involve plating HSCs identified via flow cytometry onto irradiated bone marrow stroma cells. The HSCs then form so called 'cobblestone' areas which can be quantified by counting the cobblestone foci after limiting dilution (Ploemacher et al., 1989). The LTC-IC is defined as cells which can still produce clonogenic progenitors after 5 weeks in culture (de Wynter and Ploemacher, 2001) (Ploemacher et al., 1989).

In vitro assays for progenitor cell function predominantly take the form of methylcellulose colony forming assays. These assays measure the ability of individual progenitor cells to produce colonies when cultured in semi solid media containing cytokines to promote differentiation of the cells (Humphries et al., 1981; Johnson and Metcalf, 1977; Johnson et al., 1976). Different combinations of cytokines can be used to promote growth and assay progenitors of B cells, myeloid cells and erythroid or megakaryocyte cells. Following culture, each colony is counted as a Colony Forming Unit (CFU) thought to derive from one progenitor cell.

When assaying myeloid progenitors, it is possible to differentiate between the colony types by morphology and so assess whether the colony was formed by an early myeloid progenitor (CFU Granulocyte, Erythroid, Megakaryocyte, Macrophage) or later progenitors (Bust Forming Unit Erythroid, CFU Granulocyte, Macrophage).

The definitive *in vivo* assay for HSC function is still considered to be the repopulation assay. This involves transplanting cells from the bone marrow or foetal liver of one mouse to the bone marrow of a lethally irradiated recipient. On transplantation, the ability of the cells to reconstitute all of the mature haematopoietic lineages is an indication of their function as stem cells. Cells that can give rise to reconstitution past 3 months post transplantation, and can serially transplant have been termed LT-HSC. Cells that can reconstitute all lineages, but only transiently, for up to 4 weeks, have been termed ST-HSC (Ikuta and Weissman, 1992; Siminovitch et al., 1964; Spangrude et al., 1988). Transplantation assays have also been developed to compare the reconstitution capacity of mutant HSCs to wild type HSCs. These assays make use of the marker CD45 (Ly5), which is expressed on immature and mature haematopoietic cells (with the exception of mature erythrocytes and platelets). Different strains of laboratory mice express different alloantigens of CD45 – either CD45.1 or CD45.2 (Spangrude et al., 1988). Mutant cells of one strain and wild type competitors of another strain, can be transplanted together into lethally irradiated recipients. It is then possible to measure the contribution of each population of cells to reconstitution using flow cytometry to analyse CD45.1 and CD45.2 expression. This technique has been very useful in establishing the roles of different genes in the regulation of HSCs using knockout mouse models (Hock et al., 2004a; Katsumoto et al., 2006; Lawrence et al., 2005b).

1.7 Differentiation of HSCs to mature lineages

Following the progression from LT-HSC to MPP and concomitant loss of self-renewal capacity, stem cells must differentiate to the mature lineages of the haematopoietic system. There is some discussion as to how this occurs. Following the model of Irving Weissman, differentiation of the HSCs progresses with the

segregation of MPPs to committed progenitors of either the myeloid lineage or the lymphoid lineage. MPPs differentiate to become either common lymphoid progenitors (CLPs, IL-7R$^+$/c-kit$^+$/Sca1^{lo}, CD34$^+$), or common myeloid progenitors (CMPs, IL-7R$^-$/c-kit$^+$/Sca1^{lo}) (Weissman, 2002) (Figure 1.1).

The CMP differentiates to two further progenitor cells, the megakaryocyte/erythrocyte lineage restricted progenitor (MEP) and granulocyte/monocyte progenitor (GMP) (Akashi et al., 2000; Traver et al., 2001). These progenitors divide and differentiate to produce all the mature blood cell types of the myeloid, erythroid and megakaryocyte lineages. The CLP has the capacity to produce B and T-lymphoid cells and NK cells (Kondo et al., 1997). There is some disagreement in the literature as to whether this model is in fact correct, or whether there is more plasticity in some of the progenitor populations (Adolfsson et al., 2001; Adolfsson et al., 2005). However, this model is the most thoroughly researched at the present time (Adolfsson et al., 2001; Adolfsson et al., 2005; Forsberg et al., 2006).

1.8 Mature haematopoietic lineages: Lymphocytes

The lineages thought to be derived from the CLP include Natural Killer cells, and T and B lymphocytes. These cells are important in the response of the immune system to foreign antigens.

B lymphocytes function to produce antibody in response to external antigens and are an essential component of the adaptive immune system. Through progressive rearrangement of the light and heavy chain components of immunoglobulin genes, B cells can produce antibodies specific for an enormous number of antigens (Goldsby et al., 2003). B cells are thought to arise from CLP in the bone marrow (Kondo et al., 1997) and their subsequent development has been well characterised (Busslinger, 2004; Hardy and Hayakawa, 2001). A schematic detailing their development is shown in Figure 1.3. Following their production in the bone marrow and maturation in the spleen, they circulate the blood and lymph until they encounter their cognate antigen. Upon receiving additional signals from T Helper cells, they can begin to differentiate and begin secreting antibodies to mount an immune response to the encountered pathogen or irritant (Goldsby et al., 2003).

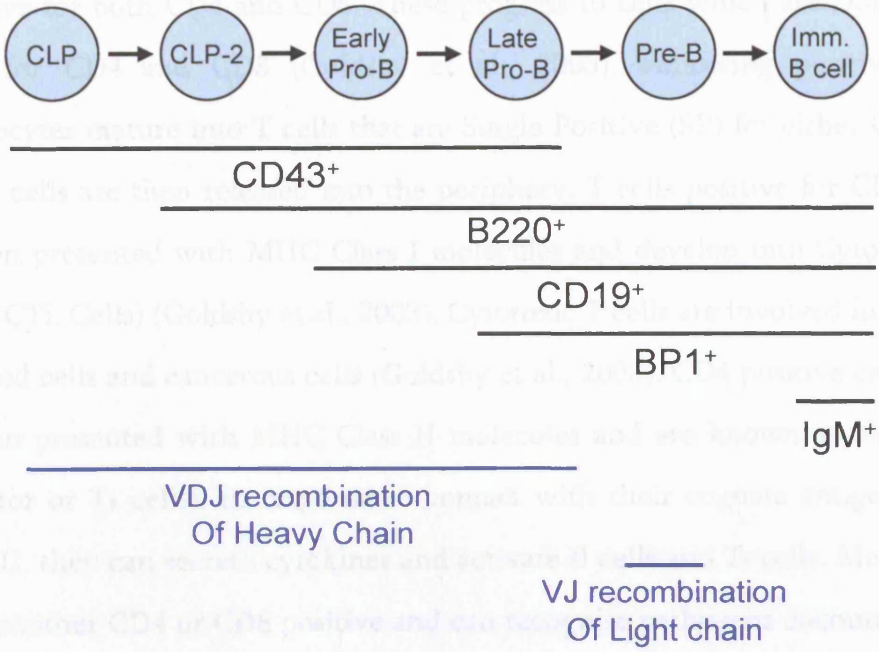

Figure 1.3. B cell development in the bone marrow.
The figure shows the progressive differentiation of B cells from common lymphoid progenitors (CLP) to immature B cells (Imm. B cell). The blue circles represent different B cell stages. Shown in black are the markers expressed by the cells at different stages of development. Shown in dark blue are times at which gene rearrangement of the heavy and light immunoglobulins occurs. Figure based on figures from Hardy and Hayakawa, 2001 and Busslinger, 2004.

T cells are also an essential component of the adaptive immune system. Unlike B cells, which can recognise free standing antigens, T cells can only recognise antigens when they are presented on the surface of an antigen presenting cell with the Major Histocompatibility Complex (MHC). T cells develop in the thymus, which is initially populated by progenitor cells derived from HSCs in the bone marrow (Schwarz and Bhandoola, 2004; Schwarz and Bhandoola, 2006). Once in the thymus, these cells are known as thymocytes. The earliest thymocytes are negative for both CD4 and CD8. These progress to cells which are Double Positive (DP) for CD4 and CD8 (Goldsby et al., 2003). Following positive selection, thymocytes mature into T cells that are Single Positive (SP) for either CD4 or CD8. These cells are then released into the periphery. T cells positive for CD8 recognise antigen presented with MHC Class I molecules and develop into Cytotoxic T cells (T_c or CTL Cells) (Goldsby et al., 2003). Cytotoxic T cells are involved in killing virus infected cells and cancerous cells (Goldsby et al., 2003). CD4 positive cells recognise antigen presented with MHC Class II molecules and are known as Helper T cells (effector or T_h cells). In response to contact with their cognate antigen and MHC Class II, they can secrete cytokines and activate B cells and T_c cells. Memory T cells can be either CD4 or CD8 positive and can recognise pathogens encountered during a previous infection to mount a swift immune response (Goldsby et al., 2003).

Natural Killer Cells are a form of lymphocyte which are part of the innate immune system. They are cytotoxic cells which are activated by cytokines or interferons. They can induce apoptosis in infected cells and are important in the response of the immune system to viral infections (Goldsby et al., 2003).

1.9 Mature haematopoietic lineages: Granulocytes and Macrophages

The cells of the myeloid lineage include granulocytes and macrophages. Both derive from the CMP, and then a further committed progenitor, the GMP (Granulocyte/Monocyte progenitor) (Akashi et al., 2000; Traver et al., 2001). Granulocytes (neutrophils, basophils and eosinophils) constitute up to 70% of circulating white blood cells and function in the defence against pathogens through phagocytosis and secretion of toxic substances (Goldsby et al., 2003). They are

recognisable through their expression of high levels of Gr1 (Ly6G) and Mac1 (CD11b) (Taylor et al., 2003). Macrophages begin life as monocytes in the bone marrow which then differentiate in tissues and have the ability to phagocytose and kill infected cells and pathogens and to present antigens to T-cells (Goldsby et al., 2003). Monocytes are recognisable by their expression of low levels of Gr1 and high levels of Mac1 (Taylor et al., 2003).

1.10 Mature haematopoietic lineages: Erythrocytes and Megakaryocytes

The other proposed derivative of the CMP is the MEP (Megakaryocyte/Erythrocyte Progenitor) (Akashi et al., 2000; Traver et al., 2001). The MEP is thought to give rise to erythroid cells and megakaryocytes cells. The cells of the erythroid lineage differentiate into enucleated erythrocytes. Erythrocytes consist mainly of haemoglobin, a complex molecule containing haeme groups whose iron atoms temporarily link to oxygen molecules in the lungs. The erythrocytes then distribute the oxygen around the body via the circulatory system (Goldsby et al., 2003).

Megakaryocytes are large cells responsible for producing platelets, which are involved in blood clotting. Megakaryocytes undergo endoreduplication that results in an increase in cell size (Zimmet and Ravid, 2000). Platelets are produced in and released from the cytoplasm of the megakaryocytes.

1.11 The genetic control of haematopoiesis: Initiation of primitive and definitive haematopoiesis

The renewal and differentiation of HSCs to progenitors and then mature cells is tightly regulated by a combination of factors. Some of these factors have been identified using mouse knockout and conditional knockout models. These include genes which are necessary for the establishment of primitive or definitive haematopoiesis, but whose expression is not necessary for adult stem cell self renewal.

In the earliest stages of primitive haematopoiesis, the transcription factors *scl* (*Stem Cell Leukaemia*) and *lmo2* (*LIM Domain Only 2*) are needed for the formation

of primitive erythroid cells. When mouse null models were analysed for of these genes, *scl*$^{-/-}$ and *Lmo2*$^{-/-}$ embryos died at E9.5-E10.5 (Robb et al., 1995; Warren et al., 1994). Both *scl*$^{-/-}$ and *lmo2*$^{-/-}$ embryos were anaemic and showed an absence of yolk sac haematopoiesis (Robb et al., 1995; Warren et al., 1994). Chimaeric mice made using *Scl* or *lmo2* deficient ES cells showed that these cells could not contribute to haematopoietic lineages in adult chimaeras, supporting a role for these genes in the establishment of haematopoiesis (Robb et al., 1996; Yamada et al., 1998). However, conditional deletion of *Lmo2* in early lymphocytes had no effect on the production of mature B and T cells (McCormack et al., 2003) in adult mice, suggesting that *Lmo2* is not essential for adult haematopoiesis. Likewise, conditional deletion of *scl* in the haematopoietic compartment of adult mice showed that *scl* is not needed for the proliferation and self- renewal of adult HSCs (Mikkola et al., 2003). These experiments showed that, whilst *Lmo2* and *scl* are needed for the establishment of primitive haematopoiesis in the yolk sac, they are not needed for the maintenance of adult haematopoietic cells.

Several genes are necessary for the establishment and maintenance of definitive haematopoiesis in the foetal liver. The most important of these is the transcription factor *Runx1/Aml1* (*Runt related transcription factor 1/ Acute Myeloid Leukaemia 1).* In the absence of *Runx1* expression, although yolk sac haematopoiesis is normal, no definitive haematopoietic cells are seen in the foetal liver. No haematopoietic cells were apparent in the AGM of *Runx1*$^{-/-}$ mice, and it has since been shown that *Runx1* expression is a defining marker of stem cells in the AGM region (Mukouyama et al., 2000; North et al., 2002). Conditional deletion of *Aml1* in the haematopoietic system of adult mice showed that, whilst it is necessary for the establishment of definitive haematopoiesis in the foetus, *Runx1* it is not needed in adult HSCs (Ichikawa et al., 2004).

Whilst *Runx1* is needed for the establishment of definitive haematopoiesis, there are other genes which, whilst not being needed for establishing definitive haematopoiesis, are necessary for the proper expansion and maintenance of HSCs in the foetal liver. These include the transcription factors *c-myb, GATA-2,* and *Pu.1.* In *c-myb* deficient foetal embryos, although sites of HSC development appear to be

functioning in the AGM, the only mature cells seen in the foetal liver are megakaryocytes and erythroid cells (Emambokus et al., 2003; Sumner et al., 2000). The foetal liver cells do not express high levels of c-kit and can form very few colonies in myeloid CFU assays, suggesting reductions in myeloid progenitors and stem cells. These results suggest that whilst *c-myb* is not necessary for establishing definitive haematopoiesis in the AGM, it is needed for the maintenance and self-renewal of HSCs and progenitors in the foetal liver. Embryos deficient in *GATA-2* had pan-haematopoietic defects and died at E10.5 (Tsai and Orkin, 1997). Embryos deficient in *Pu.1* die at E18.5 due to haematopoietic failure (Kim et al., 2004). Stem cells as defined by flow cytometry were absent or severely reduced in *Pu.1*$^{-/-}$ embryos, as were all myeloid progenitors (Kim et al., 2004). This suggests that *Pu.1* is needed for proper expansion of HSCs in the foetal liver.

1.12 The genetic control of haematopoiesis: Regulation of Adult HSCs

Following the transition of haematopoiesis to the bone marrow, HSC proliferation and differentiation is tightly regulated. In the foetal liver, HSCs must undergo large scale expansion in order to populate the foetal liver. This means that HSCs in foetal liver are predominantly actively cycling cells (Harrison et al., 1997; Nygren et al., 2006). In the bone marrow, the opposite is the case- HSC proliferation must be restrained in order to preserve the stem cell pool for the life-span of an individual. This requires, in some instances, regulation of the HSCs by a different set of genes to that which control the onset of primitive and definitive haematopoiesis. Genes which were necessary for the establishment of primitive and definitive haematopoiesis, such as *Aml1/Runx1* and *scl* are no longer needed for HSC self-renewal (Mikkola et al., 2003). However, as in foetal liver haematopoiesis, transcription factors play a large role in regulating HSC self renewal. A large part of this role is played through the regulation of cell cycle and apoptosis genes to maintain the self renewal capacity of HSCs. Another class of genes strongly linked to HSC regulation are chromatin remodelling proteins of the Polycomb and Trithorax groups.

One transcriptional repressor that is not needed for primitive haematopoiesis, but is crucial for the maintenance of adult HSCs is *Tel/Etv6* (*Translocation-Ets-Leukemia*). Although homozygous deletion of *Tel/Etv6* is embryonic lethal, yolk sac haematopoiesis is unperturbed in the absence of *Tel/Etv6*. However, induced deletion of *Tel/Etv6* in adult mice abrogates LT-HSC activity without affecting progenitor functions indicating that *Tel/Etv6* is essential for their survival HSCs (Hock et al., 2004b).

Other transcription factors have been identified as being important for HSC self-renewal and are thought to act via an effect on HSC proliferation. *GATA-2*, in addition to its role in regulating foetal liver and AGM haematopoiesis, also regulates HSC proliferation in adult mice (Ling et al., 2004). Mice heterozygous for *GATA-2* had reduced numbers of HSCs which had a competitive disadvantage against wild type cells in transplantation assays, and showed delayed recovery after 5FU treatment (Ling et al., 2004). This suggests that *GATA-2* is necessary for the normal self renewal of HSCs. The ETS transcription factor *Pu.1* is also needed to maintain the proliferation and differentiation of HSCs in adult mice, as shown using conditional knockout mice (Iwasaki et al., 2005).

In contrast, mice deficient for the transcription factor *MEF/ELF4* had a slightly higher number of BM HSCs as defined by flow cytometry. These cells were protected from cell cycle dependent toxicity due to a greater number of cells being in G_0. This suggests that *MEL1/ELF1* regulates the entrance of HSCs into the cell cycle at ready state, and prevents their over expansion (Lacorazza et al., 2006).

As expected, several genes involved in regulating cell cycle progression have also been identified as important regulators of stem cell self-renewal. Some cyclin dependent kinase inhibitors (CDKi) are thought to act to prevent HSC hyper or hypo proliferation. For example, $p21^{cip1/waf1}$ deficient mice have normal numbers of HSC but these HSC exhibit a serial repopulation defect and reduced resistance to 5FU treatment (Cheng et al., 2000b). This suggests that $p21^{cip1/waf1}$ is needed to maintain cells HSCs in G_0. In contrast, HSCs from mice deficient for $p18^{Ink4c}$ have improved long-term engraftment, suggesting that $p18^{Ink4c}$ functions to limit stem cell self renewal (Yuan et al., 2004). Mice deficient in $p27^{kip1}$, had normal numbers of

stem cells, and no transplantation defects in the HSC. However, they had increased numbers of progenitors that out competed wild type cells in transplantation assays (Cheng et al., 2000). Mice deficient for *p16^{INK4A}* had normal numbers of HSCs when young. However, in old mice, the numbers of both LT and ST HSC were increased, in comparison to wild type (Janzen et al., 2006). Bone marrow cells from old *p16$^{INK4A-/-}$* mice also performed better in competitive and serial transplantation experiments (Janzen et al., 2006). This suggests that expression of *p16^{INK4A}* is needed to regulate HSC pool size as mice age. The mosue models show that CDKis are closely involved in positively and negatively regulating HSC self renewal and proliferation. CDKis are thought to act through their inhibition of cyclin/CDK complexes, leading to prevention of the phosphorylation of Rb protein. Interestingly, conditional deletion in adult mice of *Rb*, led to no observed abnormalities in the numbers or repopulating capacity of HSCs (Walkley and Orkin, 2006). This suggests that the mechanism by which CDKis work to regulate proliferation in HSCs and progenitors is not dependent on *Rb*.

Three genes that encode proteins from the Polycomb (PcG) repressive complex 1 (PRC1) have been shown to be important in HSC function. Mice deficient in *mel18* had increased HSCs that had higher self-renewal activity and elevated expression of *Hoxb4* compared with wild type controls (Kajiume et al., 2004). Foetal liver HSCs and progenitors deficient in another PcG gene, *rae28* have impaired self-renewal capacity and were unable to reconstitute the bone marrow of lethally irradiated recipients (Ohta et al., 2002). Mice null for *Bmi1* had ten times as few phenotypic HSCs as wild type mice, with initially normal levels of progenitor and mature blood cells (Park et al., 2003). Gene expression profiles of BM mononuclear cells from *Bmi1* null mice showed increased expression of *p16^{Ink4A}* suggesting that *Bmi1* controls HSC renewal by releasing the cells from cell cycle arrest imposed by *p16^{Ink4A}*.

The transcription factor C/EBPα (CCAAT/Enhancer Binding Protein Alpha) is thought to play an antagonistic role to *Bmi1*. Disruption of *C/EBPα* in adult mice leads to an increase in expression of *Bmi1* and enhanced repopulation capacity in HSCs (Zhang et al., 2004).

Recently, an anti-apoptotic factor, *Mcl-1* (*Myeloid Cell Leukaemia Sequence 1*) was shown to regulate apoptosis in HSCs. Opferman *et al.* (2005) showed that the survival of bone marrow progenitors *in vitro* requires expression of *Mcl-1* induced by early-acting cytokines. *In vivo*, after conditional deletion of *Mcl-1*, progenitors and HSCs are gradually depleted.

The regulation of the self-renewal, proliferation and differentiation of the HSC pool is co-ordinately regulated by many factors, and involves measures to prevent inappropriate cell expansion and death.

1.13 The genetic control of haematopoiesis: Differentiation to mature lineages

Many different transcription factors work in concert and/or antagonistically to direct differentiation of HSCs to the different mature lineages of haematopoietic cells. Expression levels of the different transcription factors are particularly important as it is thought that, although some transcription factors are expressed in many lineages, only when their expression is above a threshold level will they drive progenitors down a particular developmental pathway.

Transcription factors important for erythropoiesis include *GATA-1, Lmo2, FOG-1* (*Friend of GATA-1*) *and scl/tal1* (Allen et al., 2006a; Cantor and Orkin, 2002; Mikkola et al., 2003; Tsang et al., 1998). As discussed above, *Lmo2* and *scl* are necessary for primitive erythropoiesis. Conditional deletion of *scl* in adult tissues also led to anaemia and thrombocytopenia in the mice, and an inability of the bone marrow cells to form BFU-E, suggestive of blocks in erythropoiesis and megakaryopoiesis (Hall et al., 2003). *Gata-1* and *Fog1* also regulate both erythropoiesis and megakaryopoiesis. Embryos deficient in *Fog1* die of anaemia and have blocked erythropoiesis and megakaryopoiesis (Tsang et al., 1998). Loss of *Gata1* leads to blocked maturation of erythrocytes and megakaryocytes and the failure of erythropoiesis (Orkin et al., 1998; Weiss and Orkin, 1995).

The two predominant transcription factors that are thought to regulate myelopoiesis are *Pu.1* and *C/EBP*α (Laiosa et al., 2006; Orkin, 2000). CMPs and

GMPs are absent in the foetal livers of E14.5 embryos lacking *Pu.1*, although MEPs were present (Kim et al., 2004). Adult mice in which *Pu.1* was conditionally deleted in HSCs after birth were unable to produce CMPs. However deletion of *Pu.1* after GMP formation resulted in excess granulocyte production (Dakic et al., 2005; Iwasaki et al., 2005). This suggests that *Pu.1* is needed for the formation of CMP but is also needed to restrict differentiation in the GMP. Adult mice in which *C/EBPα* was conditionally deleted produced very few GMPs, neutrophils, eosinophils and monocytes (Zhang et al., 2004). Conditional deletion of *C/EBPα* in the GMP population did not affect their capacity to produce colonies *in vitro*, suggesting that *C/EBPα* is needed for the formation of GMP, but not for the downstream differentiation of granulocytes and monocytes (Zhang et al., 2004).

Pu.1, in addition to regulating myelopoiesis, plays a further role in lymphoid development. Loss of *Pu.*1 results in an inability of HSCs to produce CLPs as well as CMPs (Dakic et al., 2005; Iwasaki et al., 2005). Another transcription factor needed for CLP formation is *Ikaros* (Allman et al., 2003) as evidenced by the fact that mice deficient in *Ikaros* have undetectable levels of CLPs.

Further differentiation of CLPs to mature B cells requires different levels of several transcription factors. Inactivation of transcription factors such as PAX5 (Paired Box Gene 5), E2A and EBF (Early B cell Factor) (Bain et al., 1994; Isnard et al., 2000; Lin and Grosschedl, 1995; Nutt et al., 1999) all result in disrupted B cell development. Loss of *EBF* or *Pax5* in adult mice leads to a block in B cell development at the ProB stage and loss of *E2A* leads to a loss of B cells early in development, before DJ immunoglobulin gene rearrangement (Bain et al., 1994; Lin and Grosschedl, 1995; Nutt et al., 1999) . Interestingly, *Pax5* deficient B cells can be induced to differentiate to cells of the myeloid lineage, and express genes of different lineage-affiliated gene programs (Nutt et al., 1999). Therefore, Pax5 plays an essential role in B-lineage commitment by suppressing alternative lineage choices.

Correct maturation of T-cells requires transcription factors such as *Runx1/Aml1*, *c-myb*, *E2A/HEB* and *GATA3* (Laiosa et al., 2006; Rothenberg and Taghon, 2005). *Aml1/Runx1* is needed for the proper expansion of DN thymocytes,

and also to suppress CD4 expression during the selection process for CD8 SP cells (Telfer et al., 2004; Woolf et al., 2003). *c-myb* is required during several stages of T cell development - transition through the DN stage in the thymus, survival of DP thymocytes, and differentiation of $CD4^+$ thymocytes (Bender et al., 2004). E2A and HEB are partially redundant bHLH transcription factors, but in the absence of either protein, T cell differentiation is partially blocked before the transition from DP to SP cells (Bain et al., 1997; Barndt et al., 1999). *GATA3* is the only transcription factor that appears to have a T cell specific role. It is needed for the formation of the earliest thymocytes, before the DN stage and has further roles in regulating the development of T_h cells (Ting et al., 1996; Zhu et al., 2004). *Notch1*, a component of the *Notch* signalling pathway is also important for T cell development. In mice in which *Notch1* has been conditionally deleted, T cell development is absent, and instead, B cell development occurs in the thymus (Radtke et al., 2004; Robey and Bluestone, 2004). This showed that *Notch1* signalling is needed to suppress B cell development in lymphoid progenitors in order for T cell development to occur. *Notch1* signalling is also required for later stages of T cell development (Radtke et al., 2004; Robey and Bluestone, 2004). Another group of signalling proteins important for T cell development are those of the Vav family (Tybulewicz, 2005). These are cytoplasmic guanine nucleotide exchange factors for Rho-family GTPases and are phosphorylated in response to T cell antigen receptor signalling (Bustelo et al., 1992). *Vav1* deficient mice showed a partial block at the transition from DN to DP cells (Turner et al., 1997). Mice deficient in all three Vav family proteins (Vav1, Vav2, Vav3) show a 100-fold reduction in number of DP and SP thymocytes, in comparison to a normal number of DN thymocytes. This demonstrates the importance of the Vav family in regulating the DN to DP transition (Fujikawa et al., 2003)

The genetic control of the differentiation of HSCs via committed progenitors to the mature lineages of the haematopoietic system requires careful coordination of a range of transcription factors, the levels of which can influence the cell fate of a specified progenitor.

1.14 The Hox cluster: A family of genes involved in haematopoiesis

Another group of genes shown to play an important part in regulating haematopoiesis are the genes of the *Hox* family. The HOX proteins are transcription factors that are important in determining cell fate and body patterning in the developing embryo. They are orthologous to the HOM-C proteins in *Drosophila* (Grier et al., 2005; McGinnis and Krumlauf, 1992) (Figure 1.4) that were first discovered through the analysis of *Drosophila* mutants (Gehring and Hiromi, 1986). Mutation of genes from the HOM-C cluster gave rise to homeotic transformations in the flies, involving the transformation of one body part e.g. an antennae, to another e.g. a leg (Gehring and Hiromi, 1986). This meant that HOM-C genes were needed for specifying the body plan during embryological development (Gehring and Hiromi, 1986). Following the discovery of the HOM-C cluster in *Drosophila, Hox* genes were found to reside in similar clusters in mammalian genomes (Hart et al., 1985; McGinnis et al., 1984).

The *Hox* genes code for transcription factors whose conserved motif is the homeodomain (Grier et al., 2005). This is a 61-amino-acid helix-turn-helix DNA-binding domain (Grier et al., 2005). Hox proteins may be involved in both transcriptional activation or transcriptional repression (Moens and Selleri, 2006; Owens and Hawley, 2002). In mammals, the *Hox* genes are organised in 4 clusters (see Figure 1.4 (Grier et al., 2005)), each containing 9-11 genes. They are expressed in precise temporal and spatial patterns during embryological development, and the importance of this expression is seen in the morphological abnormalities that can arise following targeted disruption of some of the *Hox* genes in mice. These include homeotic transformations of the skeleton, suggesting that their function is analogous to that of the *HOM-C* genes in *Drosophila* (Chen and Capecchi, 1997; Davis and Capecchi, 1994; Davis et al., 1995; Kostic and Capecchi, 1994).

Hox genes are also expressed postnatally in adult tissues, including blood cells (Pineault et al., 2002; Sauvageau et al., 1994). A survey of the CD34+ populations (which included HSCs and progenitors) showed expression of several genes from the *A, B* and *C Hox* gene clusters (Sauvageau et al., 1994). It is thought that 3' genes of

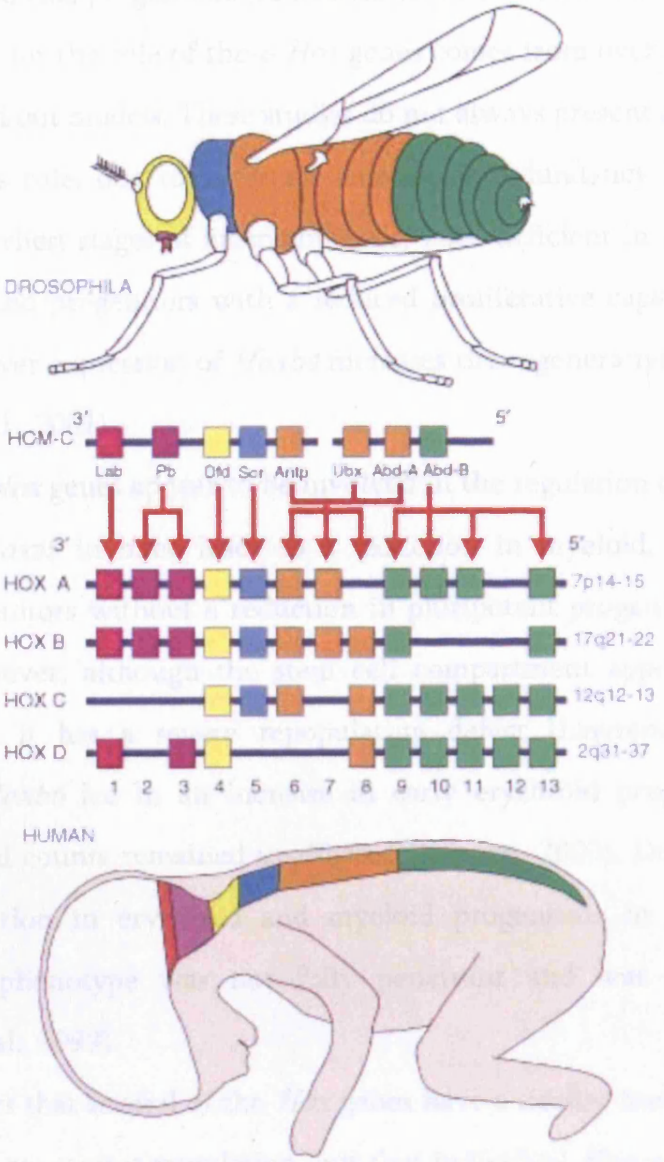

Figure 1.4. The HOM-C and HOX Cluster of Drosophila and Humans. Shown is the conservation between HOM-C genes of *Drosophila* and the *Hox* genes of mammals. Shown are the expression patterns of the genes during *Drosophila* and human development. Figure taken from Grier et al., 2005.

the *Hox* clusters are expressed in pluripotent progenitors, with 5' genes bring turned on in more committed progenitors (Lawrence et al., 1997; Sauvageau et al., 1994).

Evidence for the role of these *Hox* genes comes from over expression studies and mouse knockout models. These studies do not always present a clear picture of a particular gene's role, due to a certain amount of redundancy between the *Hox* genes. At the earliest stages of haematopoiesis, mice deficient in *Hoxb3* and *Hoxb4* produce HSCs and progenitors with a reduced proliferative capacity (Bjornsson et al., 2003), and over expression of *Hoxb4* increases the regenerative capacity of HSCs (Antonchuk et al., 2001).

Several *Hox* genes appear to be involved in the regulation of progenitor cells. Knockout of *Hoxa9* in mice leads to a reduction in myeloid, erythroid and B-lymphoid progenitors without a reduction in pluripotent progenitors (Lawrence et al., 1997). However, although the stem cell compartment appears normal, upon transplantation, it has a severe repopulating defect (Lawrence et al., 2005a). Knockout of *Hoxb6* led to an increase in early erythroid progenitors, although peripheral blood counts remained unaffected (Kappen, 2000). Disruption of *Hoxc8* led to a reduction in erythroid and myeloid progenitors in some individuals, although this phenotype was not fully penetrant and was not cell intrinsic (Shimamoto et al., 1999).

It appears that several of the *Hox* genes have a similar function in regulating the pluripotent progenitor population, but that individual *Hox* genes play different roles in the further differentiation of progenitors. Ernst et al. (2004) looked at the colony forming ability of cells from embryoid bodies differentiated from *Mll* deficient ES cells. These had reduced or absent expression of several *Hox* genes, and a much reduced ability to form myeloid colonies. When early progenitors from these embryoid bodies were transduced with individual *Hox* genes, *HoxA9, B4, A10* were able to rescue the phenotype and restore the ability of the cells to produce myeloid colonies. Subsequent differentiation, however, was influenced by the specific *Hox* gene expressed.

1.15 Hox gene cofactors Pbx1 and Meis1

Although HOX proteins have direct DNA binding capacity via the homeodomain, the sequence specificity of this binding is mediated by interaction with other homeodomain proteins such as PBX1 (Pre-B-cell leukemia Homeobox 1) and MEIS1 (Moens and Selleri, 2006). These cofactors can interact with a broad subset of HOX proteins and facilitate their binding to DNA (Moens and Selleri, 2006). It came as no surprise, considering the importance of their interaction to HOX protein function, when it was found that these HOX co factors were needed for haematopoiesis to develop normally. Homozygous deletion of either *Meis1* or *Pbx1* results in embryonic lethality in mice (DiMartino et al., 2001a; Hisa et al., 2004a). Studies using *Meis1*$^{-/-}$ and *Pbx1*$^{-/-}$ embryos show that they are both necessary for normal HSC function in the foetal liver. This evidenced by the fact that HSCs from both *Meis1*$^{-/-}$ and *Pbx1*$^{-/-}$ embryos have repopulating defects in transplantation experiments (DiMartino et al., 2001a; Hisa et al., 2004a).

1.16 The role of Hox genes and their cofactors in leukaemogenesis

In addition to their roles in normal haematopoiesis, its has been shown that several *Hox* genes and HOX cofactors play an active role in leukaemogenesis (Owens and Hawley, 2002). In human acute myeloblastic leukaemia, co-expression of *Hoxa7*, *Hoxa9* and *Meis1* are frequently observed (Afonja et al., 2000; Lawrence et al., 1999). In some leukaemias *Hox* genes and their cofactors may become fused to other genes as a result of chromosomal translocations, resulting in the formation of a chimaeric protein (Owens and Hawley, 2002). For example, in some human AMLs, *Hoxa9* can become fused to *Nup98* resulting in a chimaeric protein with oncogenic potential (Nakamura et al., 1996; Owens and Hawley, 2002). The over expression of several *Hox* genes also leads to the expansion of haematopoietic progenitor cells in mouse and human cells (Owens and Hawley, 2002). This suggests that the transformation potential of *Hox* genes is through the deviation of their normal role in HSC and progenitor proliferation (Owens and Hawley, 2002).

1.17 Mll – a gene rearranged in infant leukaemia

One of the upstream regulators of *Hox* genes in both normal and leukaemic haematopoiesis is *Mll*. The *Mll* gene was first identified through its role in infant and treatment-related secondary leukaemias (Djabali et al., 1992; Ernst et al., 2002; Zeleznik-Le et al., 1994). The most frequent cytogenetic abnormality found in these leukaemias is chromosomal rearrangement of the 11q23 region. When the locus spanning the breakpoint in 11q23 was cloned, the gene *MLL* (*ALL-1, HRX*) was identified (Cimino et al., 1991).

In leukaemic cells the N-terminal portion of *MLL* becomes fused, through chromosomal translocation, to one of up to 50 possible partner genes. These fusion genes can be present in cases of both Acute Myeloid Leukaemia (AML) and Acute Lymphoid Leukaemia (ALL) (Daser and Rabbitts, 2004; Ernst et al., 2002; Hess, 2004). The fusion partners of MLL tend to be either transcriptional activators (e.g. *Af9, Af4, ENL*) or dimerising proteins (e.g. *Gas7*) (Dobson et al., 1999; Horton et al., 2005; So et al., 2004; So et al., 2003). In primary murine haematopoietic cells, these fusion genes are often sufficient to induce immortalisation *in vitro* and transformation to leukaemia *in vivo* (Dobson et al., 1999; Horton et al., 2005; So et al., 2004; So et al., 2003).

It is thought that part of the action of MLL fusion proteins in leukaemia is through deregulated *Hox* gene expression (So et al., 2004). Murine cells immortalised with human MLL-ENL, a fusion gene which causes AML and ALL in infants, have upregulated expression of several *Hox* genes, notably genes at the posterior end of the *Hoxa* cluster, such as *Hoxa7, Hoxa9, Hoxa10* and *Hoxa11* (Horton et al., 2005; Zeisig et al., 2004). The *Hox* genes have also been shown to be direct targets of MLL fusion proteins which induce activating histone methylation at *Hox* loci (Milne et al., 2005a).

1.18 Mll is involved in Hox gene regulation in the context of normal development

Following the identification of the role of *Mll* in leukaemogenesis, research focused on whether the normal MLL protein was also involved in *Hox* gene regulation and the developmental processes which are normally regulated by the *Hox* genes. The *Mll* gene encodes a protein of 3639 amino acids (human), molecular weight 430kd that is homologous to the *Drosophila* protein Trithorax (TRX) (Mbangkollo et al., 1995). Trithorax is a member of the Trithorax group (trx-G) of proteins that positively regulate gene expression during *Drosophila* development. These proteins do not initiate, but instead, maintain correct temporal and spatial expression of their target genes in the HOM Complex (HOM-C). This is evidenced by the fact that flies mutant for trx-G genes have homeotic transformations (Hess, 2004).

To investigate whether *Mll* regulated *Hox* gene development during mammalian development, in a similar mechanism to *Trx*, mouse knockout models of *Mll* were established (Figure 1.5) (Ayton et al., 2001; Yagi et al., 1998; Yu et al., 1995) In the first model, *Mll* was truncated at exon 3 by insertion of a *lacZ/Neo* cassette (Yu et al., 1995). The resultant homozygous mutant mice died by E10.5. Both homozygotes and, to a lesser degree, heterozygotes show segmental abnormalities suggesting haploinsufficiency of *Mll* (Yu et al., 1998; Yu et al., 1995). The heterozygotes had abnormalities of the axial skeleton normally with *Hox* gene mis-expression (Yu et al., 1995). The homozygotes had gross abnormalities of the branchial arches (the structures which give rise to the bones of the face) again associated with Hox gene deregulation (Yu et al., 1998). Heterozygous embryos had shifted expression patterns of Hox genes in the embryo, and homozygous embryos were unable to maintain expression of *Hoxa7* in the somites following E9 (Yu et al., 1998). This confirmed that *Mll* was necessary for the maintenance of *Hox* gene expression during development, making it orthologous to *Trx*.

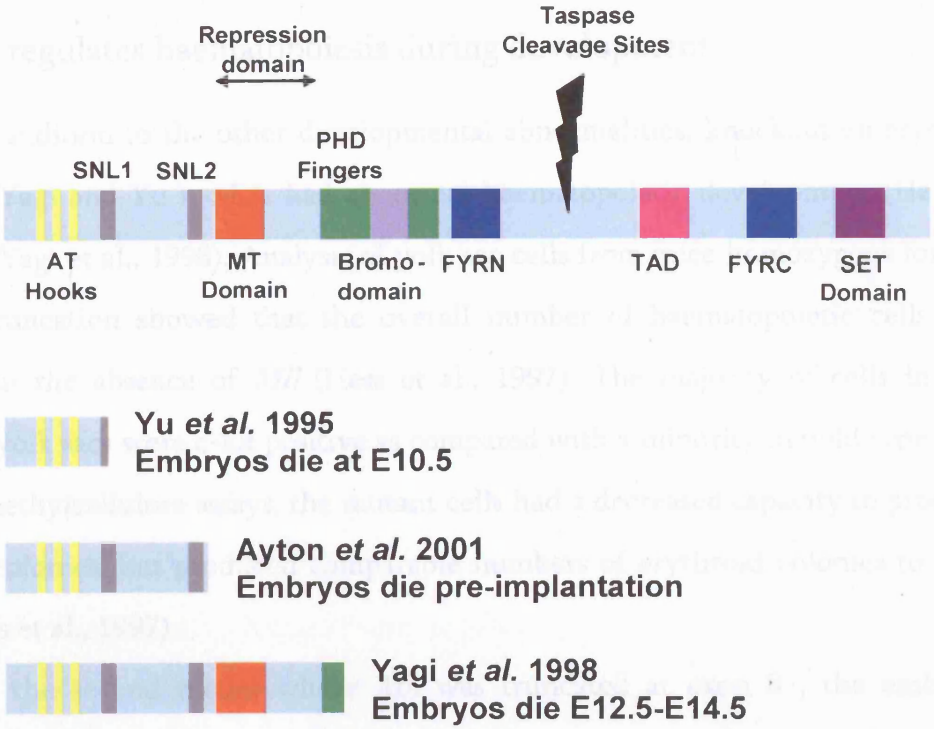

Figure 1.5. Knockout mouse models of MLL
The schematic shows the domains of MLL. Below are shown the point at which MLL is truncated in the different mouse knockout models, and the time at which mice homozygous for the truncation die. SNL – Subnuclear Localisation signal, MT domain - Methyltransferase domain, PHD fingers – Plant Homeodomain fingers, FYRN – N terminal dimerisation domain, FYRC – C-terminal dimerisation domain, TAD – Transactivation domain

In the second knockout model of *Mll*, the gene was truncated at exon 8 by the insertion of a neomycin resistance cassette (Figure 1.5). Although embryos homozygous for this truncation did not have the branchial arch abnormalities seen in the Yu knockout, they did have deregulated *Hox* gene expression (Yagi et al., 1998).

1.19 Mll regulates haematopoiesis during development

In addition to the other developmental abnormalities, knockout embryos of both the Yagi and Yu models had abnormal haematopoietic development (Hess et al., 1997; Yagi et al., 1998). Analysis of yolk sac cells from mice homozygous for the exon 3 truncation showed that the overall number of haematopoietic cells was reduced in the absence of *Mll* (Hess et al., 1997). The majority of cells in *Mll* deficient yolk sacs were c-kit positive as compared with a minority in wild type yolk sacs. In methylcellulose assays, the mutant cells had a decreased capacity to produce myeloid colonies, but produced comparable numbers of erythroid colonies to wild type (Hess et al., 1997).

In the second model where *Mll* was truncated at exon 8 , the embryos survived to E14.5, allowing analysis of foetal liver cells (Yagi et al., 1998). Homozygous mutant foetal livers had comparable numbers of mature cells, including myeloid cells, to wild type foetal livers, although the total cell number was reduced. In colony forming assays, the number and size of both erythroid and myeloid colonies was reduced in comparison to wild type (Yagi et al., 1998).

To further test the contribution of *Mll* mutant cells to haematopoiesis, mutant homozygous ES cells were used (Ernst et al., 2004a). These cells were derived from ES cells used in the Yu model. In stromal cultures, mutant ES cells were unable to give rise to B-lymphopoiesis. When transplanted into blastocysts, mutant ES did not contribute to mature NK cells, T-cells or B-cells in the adult *RAG-2* chimaeras. AGM cells from mutant embryos were unable to reconstitute haematopoiesis in sublethally irradiated recipients, as compared to wild type AGM cells, which gave 75% reconstitution (Ernst et al., 2004a).

When *Mll* deficient ES cells were differentiated down the haematopoietic lineage in embryoid body experiments, the cells were deficient in producing haematopoietic colonies (Ernst et al., 2004b). Q-PCR showed that in the absence of *Mll* induction of *Hoxa7, Hoxa9, Hoxa10, Hoxb4, Hoxb5, Hoxb6, Hoxb8,* and *Hoxc6* was reduced in the embryoid bodies (Ernst et al., 2004b). Ectopic expression of *Hoxa9, Hoxa10* or *Hoxb4* was able to rescue the reduction in colony frequency in $Mll^{-/-}$ embryoid bodies, suggesting that the haematopoietic abnormalities in *Mll* deficient embryos are due to *Hox* gene deregulation (Ernst et al., 2004b).

1.20 MLL regulates Hox gene expression via chromatin remodelling

More recent research has focused on elucidating the mechanism by which *Mll* controls the expression of *Hox* and other genes. The *Mll* gene codes for a large protein, with several different domains (Figure 1.5). Some of these domains are conserved with *Drosophila* TRX. These include the PHD fingers and the SET domain (Hess, 2004). It has been shown that the many of the domains of MLL act to regulate gene expression via chromatin remodelling. Chromatin remodelling is a means of controlling the transcriptional activation status of genes (Jenuwein and Allis, 2001; Kouzarides, 2007; Li et al., 2007).

Chromatin is the state in which DNA is packaged, and depending on its formation, it can influence gene transcription. For example, euchromatin is chromatin which is lightly packed and contains a higher proportion of genes which are being actively transcribed (Alberts et al., 1994). Heterochromatin is more tightly packed and contains fewer genes undergoing transcription (Alberts et al., 1994). Chromatin is composed of DNA strands wrapped around nucleosomes. Each nucleosome is composed of an octamer of four core histones (H3, H4, H2A, H2B) (Campbell and Reece, 2005). Each histone has two domains – a central region that interacts with the DNA and a tail region that projects outwards (Campbell and Reece, 2005).

Modification of amino acids in histone tails can weaken or strengthen their interaction with DNA, leading to a more open or a more closed chromatin formation (and the formation of heterochromatin) (Jenuwein and Allis, 2001). For example,

acetylation of amino acids in the histone tails can lead to a more open chromatin formation allowing access to the DNA of transcription factors (Jenuwein and Allis, 2001). Methylation of lysine and arginine residues in the histone tails can also lead to gene silencing or transcriptional activity (Jenuwein and Allis, 2001; Lachner and Jenuwein, 2002; Lachner et al., 2003). For example, methylation of Lysine 4 of Histone 3 (H3K4) has been shown to induce a transcriptionally active state whereas methylation of H3K9 has been shown to repress transcription (Milne et al., 2002; Nakamura et al., 2002).

The MLL protein is involved in both histone acetylation and histone methylation, either by the direct function of its domains, or by recruitment of other proteins via protein-protein interactions. The functions of the different domains of MLL are discussed below.

1.21 AT Hooks

There are three AT hooks located near the N-terminus of the MLL protein. AT hooks have been identified in several proteins and are thought to be able to bind to AT-rich regions in the minor groove of DNA (Broeker et al., 1996b). Using recombinant protein purified from bacteria, it has been shown in *in vitro* assays that the AT hook region of MLL can bind to cruciform DNA. This binding is not thought to be sequence specific but rather structure specific (Broeker et al., 1996a).

1.22 SNL1, SNL2

These short nuclear localisation domains confer a punctuate localisation pattern of the protein in the nucleus (Yano et al., 1997).

1.23 Methyl transferase Domain

The Methyltransferase (MT) domain is so called because this 100 amino acid region bares homology to the regulatory region of DNA methyltransferase 1 (DNMT1). The MT domain contains a CxxC motif shared with other proteins that are able to recognise and bind CpG dinucleotide sequences (Birke et al., 2002). Accordingly, recombinant MLL MT domain protein has been shown to bind

unmethylated CpG DNA *in* vitro and to bind to unmethylated DNA *in vivo* in VP16 reporter assays (Allen et al., 2006b; Ayton et al., 2004; Birke et al., 2002).

The MT domain lies in a larger region termed the 'repression domain', so-called because it can repress transcription when fused to the GAL4 DNA binding domain (Zeleznik-Le et al., 1994). This repression is thought to mediated through recruitment of histone de-acetylases, which remove acetyl groups from core histone tails in chromatin, thereby making it transcriptionally inert (Xia et al., 2003). It is also thought that HDACs may be able to recruit repressive complexes (Xia et al., 2003; Zeleznik-Le et al., 1994). The repression domain can also interact with the Polycomb Group proteins (PcG) HPC2 and BMI-1 (Xia et al., 2003). Exogenous expression of BMI-1 in reporter assays with the MT domain of MLL increase its repressive activity (Xia et al., 2003; Zeleznik-Le et al., 1994). This is significant because in *Drosophila* and mouse, PcG proteins act antagonistically to trx-G proteins to maintain silencing of *HOM-C* and *Hox* genes (Hanson et al., 1999).

1.24 PHD Fingers

The Plant Homeodomain (PHD) fingers, which are also present in TRX, are believed to play some part in the transcriptional regulation/ chromatin remodelling activity of MLL. This is because most proteins that contain PHD fingers are components of transcriptional regulation/ chromatin remodelling complexes, suggesting that these domains are intrinsic to their function (Eguchi et al., 2003). Further to this, the third PHD finger of MLL has been shown to interact with nuclear cyclophilin CYP33 (Fair et al., 2001). This is, in turn, thought to mediate the binding of HDAC1 to the MT domain (Xia et al., 2003; Zeleznik-Le et al., 1994)

Recent work has also shown that PHD fingers can act as highly specialised methyl-lysine binding domains (Li et al., 2006; Mellor, 2006; Pena et al., 2006). The PHD fingers of the chromatin remodelling proteins Ing2 (Inhibitor of Growth family 2) and BPTF (bromodomain PHD finger transcription factor) bind specifically to tri-methylated H3K4 (H3K4me3) (Li et al., 2006; Pena et al., 2006). H3K4me3 has been shown to be a mark for active chromatin and is found at the 5' end of genes together with acetylated lysines, another mark of active chromatin. It is thought

that the PHD finger of BPTF, a subunit of the NURF chromatin remodelling complex, recognizes H3K4me3 in coordination with the recognition of acetylated lysine by an adjacent bromodomain. This recognition helps target the chromatin remodelling complex to the active chromatin (Li et al., 2006; Wysocka et al., 2006). *Mll* contains 4 PHD fingers with a bromodomain between the 3rd and 4th fingers. It has not been shown whether the PHD fingers of MLL can bind to nucleosomes, as for ING2 and BPTF. However, H3K4me3 is positioned within a cage of 4 aromatic amino acids in the BPTF PHD finger and aromatic amino acids are present in similar positions in the 3rd MLL PHD finger, supporting the possibility that the 3rd PHD finger of MLL may have some nucleosome binding capacity. The proximity of the 3rd PHD finger to the bromodomain in MLL would support this.

1.25 Bromodomain

Little is known about the function of the bromodomain in MLL, however bromodomains are found in many chromatin associated proteins and nearly all histone acetyl-transferase (HAT)-associated transcriptional co-activators. They have been shown to interact with acetylated lysines, and to interact with PHD domains in tethering transcriptional HATs to the chromatin (Li et al., 2006; Wysocka et al., 2006). They may also be important for the assembly and activity of multiprotein complexes in transcriptional activation (Dhalluin et al., 1999).

1.26 Dimerisation Domains (FYRN and FYRC)

Within MLL, there are two sites for proteolytic cleavage (CS1 and CS2) and two dimerisation domains (FYRN and FYRC). MLL is cleaved before entry to the nucleus, at two protease recognition sites, CS1 and CS2 by Taspase1 to generate an N-terminal fragment, N320 and a C-terminal fragment C180 (Hsieh et al., 2003).(see Figure 1.6). The two fragments then interact through the FYRN domain of the N-terminal fragment and the FYRC domain of the C-terminal fragment. This dimerisation confers stability to the N-terminal fragment.

The N-terminal fragment of MLL contains mostly transcriptionally repressive domains, so fusion to the C-terminus, which contains the transactivating domain

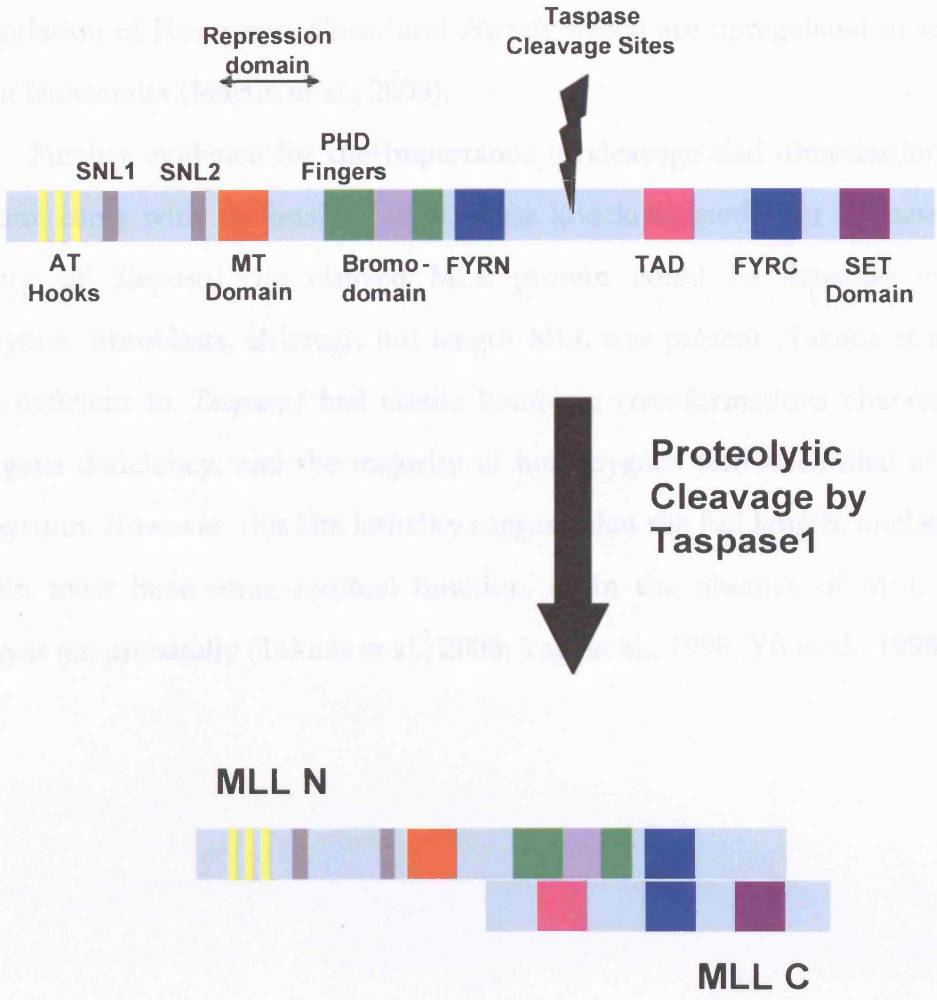

Figure 1.6. MLL is cleaved and forms a dimer.
Schematic showing the differnet domains of the MLL protein.
The protein is cleaved and then dimerises, resulting in net transcriptional activity. MLL N contains domains that repress transcription, MLL C contains domains that activate transcription.
Adapted from Eguchi et al., 2003 and Hess, 2004

and the SET domain, gives the protein an activating effect on transcription (Martin et al., 2003). Permanent dimerisation of MLL using pharmacologically dimerisable MLL fusion protein converts it to a transcriptional transactivator. This leads to upregulation of Hox genes *Hoxa7* and *Hoxa9*, which are upregulated in some MLL fusion leukaemias (Martin et al., 2003).

Further evidence for the importance of cleavage and dimerisation to MLL function came with the analysis of a mouse knockout model for *Taspase1*. In the absence of *Taspase1*, no cleaved MLL protein could be detected in murine embryonic fibroblasts, although full length MLL was present (Takeda et al., 2006). Mice deficient in *Taspase1* had classic homeotic transformations characteristic of *Hox* gene deficiency, and the majority of homozygous null mice died at 1-2 days postpartum. However, this late lethality suggests that the full length, uncleaved MLL protein must have some residual function, as in the absence of MLL function, embryos die prenatally (Takeda et al., 2006; Yagi et al., 1998; Yu et al., 1995).

1.27 Transactivation domain

The C-terminal fragment of MLL contains a transactivation domain (TAD). This activation domain can promote expression in Gal4 fusion experiments (Prasad et al., 1995). It is thought that the activity of this transactivation domain is based on its interaction with the CREB-CBP complex (Ernst et al., 2001). CREB (Cyclic AMP Response Element-Binding) is a signal dependent transactivator which becomes phosphorylated at serine 133 in response to stimuli that result in increased intracellular cyclic AMP. Upon phosphorylation, CBP (Creb Binding Protein) binds to CREB which results in a rapid induction of gene expression. This is through the direct recruitment of the basal transcriptional machinery and also through the acetyltransferase activity of CBP (Ernst et al., 2001). MLL has been shown to interact with the CREB-CBP complex *in vitro.* Point mutations which prevent this interaction also abrogate the transactivation potential of the MLL TAD in *in vivo* GAL4 fusion reporter assays (Ernst et al., 2001).

1.28 SET domain

The SET domain is perhaps the best characterised of all the MLL domains and is highly conserved with other MLL and SET family proteins. SET domains are found in many proteins demonstrated to mediate lysine-directed histone methylation (Lachner and Jenuwein, 2002).

The MLL SET domain has been shown to possess histone methyltransferase activity both *in vitro* and *in vivo* (Milne et al., 2002; Nakamura et al., 2002). The SET domain of MLL is responsible for maintaining H3K4 methylation on the promoter and enhancer regions of *Hoxc8* and *Hoxa9* (Milne et al., 2002; Nakamura et al., 2002). This, in turn maintains transcription of these genes. This is evidenced by the reduced expression and H3K4 methylation status in *Mll* null fibroblasts (Milne et al., 2002; Nakamura et al., 2002).

Recent work has shown that the SET domain is not as critical for MLL function as previously thought. A mouse knockout model has been established in which the MLL SET domain is absent. In the absence of the SET domain the stability

and expression of the MLL protein are unaffected (Terranova et al., 2006). Mice homozygous for the SET deletion mutant are healthy and there is no embryonic lethality. Although the mice show some homeotic transformations characteristic of *Hox* deregulation, and reduced expression levels of *Hoxc8* and *Hoxd4*, the phenotype is far milder than that of full MLL knockouts. This suggests that either MLL gene regulation is not solely based on the SET domain or that other SET domain proteins may be able to compensate for its function (Terranova et al., 2006).

1.29 The MLL protein family take part in large chromatin remodelling complexes

The presence of so many different domains in MLL would suggest that the protein can interact with several other proteins to perform different functions. This is apparently the case. MLL has been shown to participate in very large super complexes of 24 or more proteins (Nakamura et al., 2002). The proteins of the MLL super complex fall into eight different groups. These include core components of the nucleosome remodelling complex, members of the HDAC Sin3A complex, which is associated with transcriptional repression, components of the basal transcription machinery TFIID complex, and components of the yeast SET1 complex such as WDR5 (Nakamura et al., 2002) (Dou et al., 2006). Another protein recently found to complex with MLL is the tumour suppressor Menin (MEN1). It is thought that Menin functions to recruit MLL to the promoters of genes such as $p27^{kip1}$ (Milne et al., 2005c).

1.30 The MLL family of mammalian SET domain proteins

Several homologues of MLL that also contain SET domains have been found in mammals. These are termed MLL2, MLL3 and MLL4. Like MLL, these proteins have been shown to take part in chromatin remodelling complexes, although the components of these complexes vary (Goo et al., 2003; Hughes et al., 2004; Mo et al., 2006). MLL2 and MLL3 have both been shown to take part in complexes with H3K4 methylation activity that can positively regulate the expression of target genes (Goo et al., 2003; Hughes et al., 2004; Mo et al., 2006). *Mll2* has also been has been shown

to complex with Menin and WDR5, as for MLL (Dou et al., 2006; Hughes et al., 2004; Milne et al., 2005c; Mo et al., 2006). MLL2 null mouse die before E11.5 and have growth retardation, widespread apoptosis and neural tube defects (Glaser et al., 2006). Expression of *Hoxb1* collapsed in *Mll2*$^{-/-}$ embryos and expression of *Hoxb2* and *Hoxb5* was reduced in embryoid bodies differentiated from MLL2 null ES cells (Glaser et al., 2006). This suggests that MLL2 targets different a different set of *Hox* genes to MLL. However, there is some scope for redundancy between these proteins as both MLL and MLL2 complex with Menin, which in turn has been shown to bind to the *Hoxc8* locus (Hughes et al., 2004; Milne et al., 2005c).

1.31 Transcriptional targets of MLL

MLL functions as a chromatin remodelling factor with DNA binding capacity that can activate and repress transcription through histone modification, direct interactions with transcriptional activating complexes and DNA methylation. Some of the gene targets of this activity have been identified.

As discussed above, the *Hox* genes are well characterised targets of MLL. *Mll* homozygous null embryos have deregulated *Hox* expression leading to segmental abnormalities and *Mll* deficient foetal liver was shown to have reduced levels of *Hoxa10, Hoxa9, Hoxa7* and *Hoxc4* (Yagi et al., 1998; Yu et al., 1995). MLL also regulates the expression of several *Hox* genes, particularly of the *Hoxa* cluster, during embryoid body formation (Ernst et al., 2004b).

In non-haematopoietic cells MLL has been shown to bind to the promoters of the cell cycle inhibitors p27^{Kip1} and p18^{Ink4c} as part of a complex with Menin (Milne et al., 2005c). Expression of p27^{Kip1} and p18^{Ink4c} was also reduced in *Mll* deficient immortalised fibroblasts, which grow at a faster rate than wild type immortalised fibroblasts. This suggests that MLL, as well as regulating *Hox* gene expression, may play a role in the cell cycle in some cells by up-regulating cell cycle inhibitors.

Another recently identified target of MLL is *Gata3*. MLL has been shown to localise directly with and regulate H3K4 methylation at the *Gata3* locus in murine T_h cells (Yamashita et al., 2006). In *Mll* heterozygous mice the ability of memory Th2 cells to produce Th2 cytokines was reduced. This was because abnormal H3K4

methylation at the Th2 cytokine and *Gata3* gene loci resulted in reduced expression of these genes (Yamashita et al., 2006).

Further analysis of specific cell types, e.g. haematopoietic cells, may yet reveal more MLL targets.

1.32 The need for a conditional mouse model of Mll: Aims of this project

Due to the embryonic lethality of *Mll* deletion, there have not been any studies performed looking at the role of *Mll* in adult haematopoiesis. The limited material available from *Mll* deficient embryos has also limited the study of foetal haematopoiesis in the absence of *Mll* and the exact nature of the haematopoietic defect is not known. In order to study the role of *Mll* further, a new, conditional mouse model of *Mll* was needed. This study describes the establishment of a new mouse model that uses the Cre-lox system to induce haematopoietic specific deletion of MLL in adult mice. A full knockout model was also established to enable further study of foetal liver haematopoiesis. The aims of this project were as follows:

1. To establish new conditional and constitutive mouse knockout models of *Mll*.
2. To further analyse foetal liver haematopoiesis and characterise any effects on foetal HSCs in the absence of MLL.
3. To characterise the role of *Mll* in regulating adult haematopoiesis and HSCs.

Chapter 2

Materials and Methods

2.1 Materials

Reagents used in this project were from the following companies;

Perkin Elmer Life Sciences; Gene Screen Membrane.

Invitrogen; 1 kb ladder, Penicillin-streptomycin solution (10000 μ/10000 μg/ml), Dulbeccos Modified Eagle Media (DMEM), L-Glutamine solution (200 mM), 1x Phosphate Buffered Saline solution (1x PBS), 1x Trypsin-EDTA, 100x Minimal essential amino acids, Hepes Buffered Saline Solution (HBSS), MEM Alpha media, Karyomax Colcemid solution (10 μg/ml), G418 solution (50 mg/ml)

Promega; pGemT-Easy kit, restriction enzymes, klenow.

Sigma; General chemicals and reagents, Foetal Calf Serum (FCS) for MEF media, gelatine powder, Hepes Buffered Saline solution (HBS), Phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10 mM Tris pH 8.0, 1 mM EDTA, Genomic Red taq

Hyclone; ES cell FCS

VWR; General reagents and chemicals, Giemsa solution , Eukit, Superfrost© slides

Chemicon; ESGRO® (Leukaemia Inhibitory Factory)

NE Biosciences; Restriction enzymes, T4 DNA ligase

BD Biosciences; Advantage GC Taq polymerase kit

Upstate; Mll c-terminal antibody, Mll N-terminal antibody

Cell signalling technology; Hsp90 anitbody

Stem cell technologies; Methocult 3434, 3630

Peprotech; GMSCF

Pharmingen; Biotin-conjugated mouse lineage panel (for sorting FL cell)

Miltenyi; Lineage cell depletion kit, mouse (for sorting adult BM), MS and LS magnetic columns, anti-pe, anti-APC and anti-bio microbeads

Roche; Proteinase K (recombinant, PCR grade)

National Diagnostics; 20x SSC (Sodium Chloride Sodium Citrate)

Amersham Biosciences; ECL reagent, Sheep anti-mouse HRP

2.2 Methods

All buffers used in this work are detailed in Table 2.1

2.2.1 DNA sequencing and analysis

Vectors pMll197.A1 and pMLL197.2kb were sequenced by the Scientific Support services at the Wolfson Institute, UCL.

2.2.2 Culture of Mouse Embryonic Fibroblasts for ES cell feeder layers

The pluripotency of ES cells was maintained by growing the cells on a feeder layer of irradiated mouse embryonic fibroblasts (MEFs). Embryonic material from neomycin resistant $Rag2^{-/-}$ embryos at day 13-14 was provided by Ursula Menzel at NIMR. Primary embryonic fibroblasts were plated out in MEF medium (DMEM, 10% FCS, 5 ml penicillin/streptomycin solution, 2 mM L-Glutamine) and cultured at 37°C, 5% CO_2.

The cells were expanded over 3 passages from 1 to 25 175mm flasks. All flasks were then trypsinised, using 1% trypsin plus EDTA. The cells were pooled and counted and then γ-irradiated using a CIS Bio International ^{137}Cs irradiator, with a dose of 3500 rads. Cells were aliquoted at densities of $4x10^6$ and $2x10^7$ and frozen at -80°C in MEF freeze media (50% MEF media, 40% FCS, 10% DMSO).

2.2.3 Gelatinization of Plates for ES cell culture

Prior to laying down the feeder layer, plates were gelatinized. A solution of 0.1% gelatine in 1x PBS was left on the plates for up to an hour. The gelatine was removed and the plates left to dry before the feeder layer was seeded onto the plate or media added.

Buffer or Media	Components
1x TE	10 mM Tris-Cl,pH 7.5, 1 mM EDTA
caesium chloride/TE solution	1g/ml CsCl added to 1×TE
ES cell lysis buffer	0.1M NaCl, 1% SDS, 0.01M Tris-HCl, 0.001M EDTA, 0.5 mg/ml proteinase K Roche
Southern De-naturation buffer	1.5M NaCl, 0.5M NaOH
Southern Neutralisation buffer	1M Tris, 1.5M NaCl, pH 7.4
Southern Pre-hybridisation solution	5×SSC, 0.5% SDS, 10% dextran sulphate, 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA, 10 μg/ml denatured salmon sperm DNA
Hypotonic Solution	0.56% KCl in ddH_20
ES cell injection medium	90 ml DMEM plus HEPES, 10 ml ES cell FCS, 1.5 ml Penicillin/streptomycin solution, 1.5 ml L-Glutamine (2mM)
TNES lysis buffer	50mM Tris pH7.5, 50mM EDTA, 5% SDS plus 0.5mg/ml proteinase K
RBC lysis buffer	17 mM Tris (pH 7.2), 0.144 M NH_4Cl
2X SDS-DTT sample buffer	0.2 M DTT, 2% SDS, 10% glycerol, 0.125 M Tris-HCL, pH 6.8, 0.1% bromophenol blue
MACs buffer	1xPBS, 0.5% BSA, 2 mM EDTA
FACs running buffer	0.05% sodium azide, 1x PBS
FACS staining buffer	0.05% sodium azide, 0.1% BSA
Western Running Buffer	1.92M glycine, 250mM Tris base, 1% SDS
Western Transfer Buffer	1 x CAPS (N-Cyclohexyl-3-aminopropanesulfonic acid), pH11
TBS-T Buffer	0.14M NaCl, 0.025M Tris, 0.003M KCl, 0.4 μl Tween 20/ ml, pH7.4
1 x Stripping Buffer	200mM Tris-HCl, pH 7.0, 0.1% SDS, 0.1 x SSC

Table 2.1. Buffers used in materials and methods.

2.2.4 ES cell culture

PC3 ES cells (O'Gorman *et al.* 1997) at passage 14 were provided by Dimitris Kioussis, NIMR. Cells were routinely grown on a layer of irradiated mouse embryonic fibroblasts (MEFs) as a feeder layer. Feeder layers were laid down on gelatinized plates several hours before they were needed. Feeder layers were used in the densities shown in Table 2.2.

The ES cells were plated out onto MEF's and fed daily with ES cell medium (450 ml DMEM plus 75 ml ES cell FCS (Hyclone), 3.5 µl β-mercaptoethanol (98% solution), 5 ml Penicillin/streptomycin solution, 5ml L-Glutamine (2mM), 50 µl LIF (10^7 units/ml, ESGRO), 5 ml minimal essential amino acids (100X)). Cells were split at ratio of 1:3 to 1:8 when confluent, as assessed by colony size and density. To split, the cells were trypsinised for 5 minutes at 37°C using 1x trypsin-EDTA, washed once in ES cell media and plated onto a fresh feeder layer.

2.2.5 Preparation of the targeting vector for electroporation into ES cells

For electroporation into ES cells, it was necessary that the targeting vector DNA be cleaned of any bacterial genomic DNA and proteins. For this reason, targeting vector DNA prepared from *Escherichia coli* using a Quiagen Maxiprep kit (according to manufacturers instructions) was further cleaned by performing a Caesium Chloride preparation. Briefly, 10 ml of caesium chloride/TE solution was added to 1-2 mg targeting vector DNA dissolved in 500 µl TE. 4 ml of 10 mg/ml Ethidium bromide solution was added and the mixture transferred to an ultracentrifuge tube. The tubes were capped and sealed and spun overnight at 57,000 rpm, 20°C in an ultracentrifuge (Sorvall Discovery 100).

The following morning the tubes were removed and placed in a bench top clamp. A syringe needle was used to pierce the top of the tube to release any pressure, and then another needle and syringe were used to pierce the tube and extract the plasmid DNA. The DNA was transferred to a 15 ml tube, and 1 ml of NaCl-saturated isopropanol was added. After vortexing, the top layer was removed and the process repeated until the top layer was colourless. The bottom layer was

Size of Dish	Density of MEF's	Volume of Media
96-well dish	$2x10^4$ per well	50µl
24-well dish	$1.3x10^5$ per well	500µl
60mm plate	$2x10^6$	4ml
10cm plate	$4x10^6$	10ml

Table 2.2. Number of irradiated MEFs needed for feeder layers for ES cells.

removed and transferred to a 50 ml tube. Three volumes of ddH_2O were added to the DNA layer. Two volumes of 100% ethanol was added to this and the DNA left to precipitate for 10 minutes on ice. The tube was spun for 45 min at 4°C, 4000 rpm to pellet the DNA. The DNA was cleaned by spinning in 20 ml 70% ethanol for 15 min at 4000 rpm, the pellet dried and the DNA re-dissolved in sterile ddH_2O water.

The purified targeting vector was linearised using an *NsiI* site (shown in Figure 3.1), ethanol precipitated and re-suspended in sterile ddH_2O, ready for electroporation into ES cells.

2.2.6 Electroporation of ES cells

The linearised targeting vector was electroporated into PC3 ES cells at passage 16. The day before electroporation, 10 x 10 cm dishes were laid down with MEFs in MEF media at the appropriate density. The MEF media was replaced with ES cell media the morning of the electroporation. A 6 cm plate of ES cells was grown to 50-100% confluency. The cells were fed with ES cell media at least an hour before they were needed for electroporation.

The ES cells were trypsinised as before, and a live cell count performed. The cells were re-suspended in electroporation buffer (HBS/ $7x10^{-4}$% β-mercaptoethanol) at a concentration of 10^7 cells/ml. 0.8 ml cells was mixed with 25 μg targeting vector DNA (re-suspended in sterile ddH_2O) and the mixture transferred to a 4 mm electroporation cuvette. The cells were electroporated using an electroporator set at 400 v, 25 μF. Following electroporation, the cells were immediately transferred to a 15 ml tube containing 3.2 ml ES cell media.

400 μl of cells was added to each 10cm MEF plate, and the cells placed in a 37°C/5% CO_2 incubator. The following day, the media on all plates was changed to selection media (ES cell media plus 300 μg/ ml G418). The cells were left to grow under selection for the next 8-10 days, with a media change every 3 days.

2.2.7 Picking and Expansion of Selected ES cell clones

After 8-10 days of growth under selection, G418 resistant ES cell colonies were visible growing on the feeder layer. Each 10cm plate was examined for the

presence of G418 resistant colonies and the media replaced with 1 x PBS. 96-well MEF plates in ES cell media were prepared before picking any clones (see Table 2)

Using a phase-contrast microscope, colonies of a suitable size were picked from the MEF plates using a P200 pipette. Each colony was transferred to a 96-well U-bottom plate containing 30 µl trypsin/ well. The colonies were trypsinised for 5 minutes at room temperature and transferred to the pre-prepared 96-well MEF plate. Full 96-well plates were then transferred to 37°C/5% CO_2 incubator. Between 800-1000 colonies were picked in this way.

96-well plates were checked each day for confluent wells. Confluent wells were trypsinised and the cells were split to one DNA plate (gelatinised 96-well plate) and one freeze plate (96-well MEF plate). DNA plates were split to a further two DNA plates when confluent.

2.2.8 Freezing and of ES cell clones

Confluent 96-well plates of ES cells growing on MEFs were frozen at -80°C for future expansion. All wells of the plate were trypsinised with 50 µl trypsin per well, 50 µl ES cell media was then added to each well and the colonies made into single cell suspensions. 100 µl ES cell freeze medium (20% DMSO/80% ES cell FCS) was added to each well and the plates stored at -80°C.

2.2.9 DNA extraction from ES cell clones.

Confluent 96-well plates of ES cells growing on gelatine were lysed for DNA extraction. Media was removed from all wells and the wells washed once with 1 x PBS. 50 µl ES cell lysis buffer was added to each well and the plates incubated O/N at 37°C. Plates were then either frozen at -20°C for future use or DNA extracted directly.

To extract DNA, 100 µl ice cold isopropanol was added to each well. The DNA was left to precipitate at room temperature for an hour, after which the isopropanol was decanted off. 150 µl of 70% ethanol at room temperature was added to each well and then immediately decanted off. The wells were washed again with 70% ethanol, and the plates were left to dry. DNA was re-suspended either in 50 µl

1M Tris-HCl pH 7.5 or restriction digest buffer mix (according to manufacturer's instructions), and shaken O/N at 37°C.

2.2.10 Analysis of ES Cell DNA from 96-well plates

To prepare genomic DNA for Southern blot analysis, restriction digests were set up directly in the 96-well plates following DNA preparation. The DNA in each well was digested with 60 units of enzyme in a 65 µl volume for at least 16 hours before being used for Southern Blot analysis.

2.2.11 Southern Blot Analysis

DNA extracted from 96-well plates of ES cell clones was analyzed by Southern Blot analysis. Southern Blots were prepared using a standard protocol. DNA Samples were run on an agarose gel of the appropriate concentration (0.7%-0.8%) O/N at 24 v. The samples were run against 1Kb ladder for size approximation.

The following day, the gel was photographed, with a ruler to mark the position of the ladder, and then washed in dH_2O for 5 minutes. If large fragments were expected, the gel was de-purinated in 0.125M HCl for 10 minutes. Gels were then washed again for 5 minutes in dH_2O. This was followed by 2 x 30 minute washes in de-naturation buffer, 1 x 5 minute wash in dH_2O, 2 x 30 minute washes in neutralisation buffer. Gels were washed again for 5 minutes in dH_2O.

Transfer apparatus was set up as follows: A sponge soaked in 10 x SSC was placed in a tray. On top of this, in order, were placed 3 sheets of 3MM filter paper, the gel (face down), a sheet of Gene Screen membrane, 3 more layers of 3MM filter paper, paper towels and weights. All filter paper and membranes had previously been soaked in 10 x SSC. The gel was transferred overnight.

Following transfer, the membrane was washed in 2 x SSC. The membrane was baked at 80°C for two hours and re-hydrated in 2 x SSC. The membrane was blocked in pre-hybridisation solution for 2 hours at 65°C with agitation. 70 ng of purified ^{32}P d-CTP labelled probe was added to the pre-hybridisation solution and the probe left to bind O/N at 65°C. The probe (named the 3' probe) was obtained by digesting MLL genomic DNA, previously cloned into pBs, with *BstX*I/*Bgl*I which

released a 650 bp fragment from intron 13-14 of the gene. The blot was removed and washed the following day for 2 x 30 minutes in 3 x SSC/ 0.1% SDS, 2 x 30 minutes in 0.3 x SSC/ 0.1% SDS. The blot was then placed in a phosphoimager cassette and visualised using a Typhoon 8600 phosphoimager.

2.2.12 Expansion of Positive ES cell Clones

Clones found to be positive for the 3' and 5' LoxP sites were thawed and expanded. Whole 96-well plates of clones were thawed at 37°C and transferred directly to 24-well MEF plates with ES cell media. When confluent, clones were expanded first to 6 cm dishes and then 10 cm dishes. 6 cm dishes were used for karyotyping. 10 cm dishes, when confluent, were trypsinised and split in 4. Three-quarters of the plate were frozen in 3 aliquots in ES cell freeze media.

The remaining quarter of the plate was used for DNA extraction for Southern Blot analysis using a standard Phenol: Chloroform extraction protocol. Briefly, cells were lysed with ES cell lysis buffer as before, in a 1 ml volume. 1 ml of phenol:chloroform:isoamyl was added to the lysates and the tubes were vigourously shaken for 1 minute. The tubes were then spun at 13,000 rpm for 5 minutes to separate the hydrophobic and hydrophilic layer. The top aqueous layer was transferred to a fresh tube and an equal volume of chloroform was added to it. The tubes were again spun at 13,000 rpm for 5 minutes and the top layer transferred to a fresh tube. 1 ml of 100% ethanol: 3M sodium acetate 25:1 was added to each tube and then the tubes were placed on dry ice for up to an hour. The tubes were then spun at 13,000 rpm for 10 minutes to pellet the DNA and the supernatant was removed. 1 ml 70% ethanol was added to each tube and the tubes were spun at 13,000 rpm for 1-2 minutes. The ethanol was removed and the DNA pellets dried and resuspended in 1x TE.

2.2.13 Southern Blot Analysis of DNA from expanded clones

Southern Blot analysis was repeated using DNA from expanded clones. Southern Blots were performed as before using 5-10 μg DNA. Analysis was done

using both the *KpnI* and *NsiI* digests to ensure the presence of the LoxNeoLox cassette and the 5' LoxP site.

2.2.14 Karyotyping of ES Cells

Before using ES cells in blastocyst injections, it was necessary to karyotype them as culture of ES cells may result in aneuploidy in some cells. A 50-75% confluent 6cm plate of ES cells was used for karyotyping.

Cells were incubated in ES cell mediae plus 20 ng/ml Colcemid for 2 hours at 37°C. The cells were then washed with 1 x PBS and trypsinised. After a second wash in 1 x PBS the cells were re-suspended in 5 ml hypotonic solution and incubated for 6 minutes at room temperature. A few drops of fixative (3:1 methanol: acetic acid) were added to the hypotonic and the cells pelleted at 750 rpm for 5 minute. The hypotonic was removed and ice-cold fixative was added to the cells drop wise, to ensure an even suspension of cells. The cells were pelleted and washed in ice-cold fixative a further 3 times and finally re-suspended in an appropriate amount of fixative depending on the density of the suspension. Cells could be stored in fixative at -20°C for future use.

To make slides, Superfrost© slides were soaked in 5% acetic acid O/N and air dried. Fixed cells were dropped onto the slides using a Pasteur pipette and the metaphase spreads examined using a phase contrast microscope. Satisfactory spreads were left O/N at room temperature and stained the following day with 10% Giemsa stain for 20 minutes at room temperature. A coverslip was fixed to each slide with Eukit mountant. Metaphase spreads were examined using an oil immersion lenses and the chromosome counts made.

2.2.15 Blastocyst Injection

ES cell clones carrying the correct complement of chromosomes and positive for both LoxP sites and the Neo cassette were injected into C57Bl/6 blastocysts by Dimitris Kioussis at NIMR. Cells were prepared for injection as follows: a 50-75% confluent 6 cm plate of ES cells was fed at least two hours before they were needed for injection. When the blastocysts were ready for injection, the ES cells were

trypsinised and washed twice in ES cell media. The cells were resupended in injection media and taken on ice to be injected into blastocysyts. The blastocysts were implanted into pseudo-pregnant females and the resultant offspring analysed for levels of chimerism as shown by coat colour.

2.2.16 Mouse Genotyping

All chimeras and their descendents were housed at NIMR. Animal husbandry, re-derivations (necessary for transferral of the mice to clean animal facilities) and tail tipping were taken care of courtesy of staff at NIMR. DNA samples for genotyping were prepared from tail tips. Briefly, tips were digested O/N in 500 µl TNES lysis buffer plus 0.5 mg/ml proteinase K at 37°C or 55°C. The following day samples were spun at 13,000 rpm for 15 minutes and the supernatant transferred to a fresh microcentrifuge tube. 500 µl isopropanol was added to each tube and the DNA spooled out using an inoculating loop. DNA was washed once in 70% ethanol, re-suspended in 1 x TE and shaken O/N at 37°C.

The DNA was then analysed by Southern Blotting or PCR. Southern Blotting was carried out as before, using a *Kpn*I or *Nsi*I digest of 5-10 µg DNA and the 3' probe. PCR analysis was carried out using 3 combinations of primers. All primers and their binding sites in the different alleles are shown in Figure 4.2 and 4.8. Primers A and B were used to detected the presence of the Neo cassette. PCR reactions for genotyping the *Mll* allele were set up using the Advantage GC2 PCR kit according to manufacturers instructions (BD-Biosciences). Genotyping for Cre transgenes was done using Genomic RedTaq (Sigma) according to manufacturers instructions. All primer combinations and the programmes used are shown in Tables 2.3 and 2.4.

PC3 ES cells are homozygous for the protamine-Cre transgene (O'Gorman *et al.* 1997), so that all offspring of successful chimaeras were heterozygous for this transgene. To detect the presence of the protamine-Cre transgene, a PCR using primers PC3CreF and PC3CreR was used (Tables 2.3 and 2.4).

Primer Name	Primer Sequence
LoxPF	5′ TGCCCTAGGCCGACGTCGCATG 3′
LoxPR	5′ TCGCCCGGGTAGTCGACCTGCA 3′
Intron5-6MLLC	5′ GGTCTCTAGAAGACAGGAGG 3′
PCR2a	5′ CCCATGTACCTATCATCCAGC 3′
Exon5MLL	5′ CAGTTGCTGGCTCAGAAGATGCC 3′
Intron5-68410R	5′ CACACCCATCTTAGGACTACCTCC 3′
B	5′ GTCAGTGGTTGGTCACTGGG 3′
D	5′ TGTCTCCTCTGTGATGATGCC 3′
A	5′ GATCTCGTCGTGACCCATGG 3′
E2	5′ GCCAGTCAGTCCGAAAGTAC 3′
F2	5′ AGGATGTTCAAAGTGCCTGC 3′
G2	5′ GCTCTAGAACTAGTGGATCCC 3′
PC3CreF	5′ TTCCCGCAGAACCTGAAGATGTTCG 3′
PC3CreR	5′ GCCGATTACGTATATCCTGGCAGC 3′
VavF	5′ AGATGCCAGGACATCAGGAACCTG 3′
VavR	5′ ATCAGCCACACCAGACACAGAGATC 3′

Table 2.3. Primers used for cloning and genotyping.

PCR	Primers	Programme
Amplify LoxP	LoxPF, LoxPR	94°C 3 min, (94°C 1 min, 63°C 1 min, 72°C 1 min) ×35 cycles, 72°C 10 min
Amplify 5′ homology fragment	Exon5MLL, Intron5-68410R	97°C 3 min, (97°C 30s, 63°C 1 min, 72°C 2 min) ×3 cycles, (96°C 30s, 63°C 1 min, 72°C 2 min) ×7 cycles, (96°C 30s, 62°C 1 min, 72°C 2 min) ×10 cycles, (96°C 30s, 61°C 1 min, 72°C 2 min) ×10 cycles, 72°C 10 min
Amplify 3′ homology fragment	Intron5-6MLLC, PCR2A	97°C 3 min, (97°C 30s, 63°C 1 min, 72°C 2 min) ×3 cycles, (96°C 30s, 63°C 1 min, 72°C 2 min) ×7 cycles, (96°C 30s, 62°C 1 min, 72°C 2 min) ×10 cycles, (96°C 30s, 61°C 1 min, 72°C 2 min) ×10 cycles, 72°C 10 min
Genotyping: Detect 3′ LoxP	D, B	97°C 3 min, (97°C 30s, 63°C 1 min, 72°C 2 min) ×3 cycles, (96°C 30s, 63°C 1 min, 72°C 2 min) ×7 cycles, (96°C 30s, 62°C 1 min, 72°C 2 min) ×10 cycles, (96°C 30s, 61°C 1 min, 72°C 2 min) ×10 cycles, 72°C 10 min
Genotyping: Detect the Neo^r	A, B	97°C 3 min, (97°C 30s, 63°C 1 min, 72°C 2 min) ×3 cycles, (96°C 30s, 63°C 1 min, 72°C 2 min) ×7 cycles, (96°C 30s, 62°C 1 min, 72°C 2 min) ×10 cycles, (96°C 30s, 61°C 1 min, 72°C 2 min) ×10 cycles, 72°C 10 min
Genotyping: Detect the 5′ LoxP	E2, B E2, F E2, G2	97°C 3 min, (97°C 30s, 62°C 1 min, 72°C 2 min) ×3 cycles, (96°C 30s, 62°C 1 min, 72°C 2 min) ×7 cycles, (96°C 30s, 61°C 1 min, 72°C 2 min) ×10 cycles, (96°C 30s, 60°C 1 min, 72°C 2 min) ×10 cycles, 72°C 10 min
Genotyping: Detect the PC3 Cre	PC3CreF, PC3CreR	94°C 3 min, (94°C 40s, 62°C 40s, 72°C 30s) ×30 cycles, 72°C 5 min
Genotyping: Detect the *Vav-Cre*	VavF, VavR	94°C 3 min, (94°C 40s, 62°C 40s, 72°C 30s) ×30 cycles, 72°C 5 min

Table 2.4. PCR programmes used for cloning and genotyping

A genotyping PCR was also used to genotype mice carrying the *Vav-cre* transgene. Primers VavF and VavR were used to amplify a band of 250 bp (Tables 2.3 and 2.4).

2.2.17 Mouse Husbandry

All mouse husbandry was carried out by staff in the animal facilities in NIMR. All animal husbandry and experimental procedures were carried out according to UK Home Office regulations and Institute guidelines.

2.2.18 Timed matings

Where embryos were needed, timed matings were set up by members of the animal house staff at NIMR. Up to three females were left with one male from approximately 5pm in the evening to 7am the next morning. The females were checked for the presence of a vaginal plug. Those that had plugged were considered pregnant and were separated from the male. The day that the plug was found was counted as E0.5. Pregnant mothers were sacrificed for embryos on the appropriate day.

2.2.19 Dissection of embryos and preparation of foetal liver cells

Pregnant females at the appropriate timepoint were killed using Schedule 1 approved methods. The uteri were taken and placed in ice cold air buffered Iscoves Modified Dulbeccos Medium (AB IMDM). The embryos were dissected from the uterus and yolk sac and placed in ice cold AB IMDM in individual wells of 6-well plates. The fetal livers were removed from the embryos using dissecting forceps and the rest of the embryo removed to another dish. The heads of the embryos were used for genotyping. Each fetal liver was passed through a 70 μm cell strainer to produce a single cell suspension.

2.2.20 Removal of erythrocytes from single cell suspensions of hematopoietic cells

When analysing cells from haematopoietic organs and blood, it was sometimes necessary to remove any erythrocytes present. Single cell suspensions prepared from bone marrow, spleen, thymus, blood or fetal liver were pelleted at 1200 rpm for 5 minutes. The cells were resuspended in RBC lysis buffer and incubated at room temperature for 10 minutes. 10 ml of HBSS was then added to the tube and the cells were pelleted at 1200 rpm for 10 minutes. The cells were resuspended in the appropriate media.

2.2.21 Genotyping Embryos

Embryos were genotyped using the same PCR strategies that were used to genotype adult mice. Material from embryos e.g. the head was lysed in TNES lysis buffer plus 0.5 mg/ml proteinase K for 24 hours at 37°C or 55°C. DNA was then prepared as for tail lysates. PCRs were performed on the prepared DNA as for tail lysates.

2.2.22 Western Blotting to detect the MLL protein

All Western blotting was done by Samantha Hiew. The protocol was also optimized by Samantha Hiew. Whole-cell lysates for Western blotting were prepared using 2X SDS-DTT sample buffer. 1 x 10^6 cells were taken from single cell suspensions from foetal liver and spleen and resuspended in 60µl sample buffer. The lysates were then sonicated for 1 minute with a 50% pulse and then boiled for 5 minutes at 100ºC. The samples were centrifuged at 13,000 rpm for several seconds and then if not used immediately, stored at -80 ºC. The samples were loaded onto a SDS-PAGE gel with a 5% resolving gel and 4% stacking gel. The resolving and stacking gels were made according to standard protocols. Rainbow kaleidoscope ladder (Biorad) was used to assess protein fragment size. The gel was run at 50-65 v overnight in running buffer until the sample buffer had run off the end of the gel. The following day the protein was transferred onto PVDF membrane (Imobilon-P) in transfer buffer at 70 v/400 mA at 4ºC for 5-6 hours.

Following transfer, the membrane was blocked for 1 hour with 5 % skimmed milk in TBS-T buffer. Two antibodies were used to detect the N-terminal and C-

terminal fragments of the MLL protein. The N-terminal fragment runs at approximately 320 KD and the C-terminal fragment runs at approximately 180 kD (Hsieh et al., 2003). The anti-N-terminal antibody was raised against a maltose-binding protein (MBP) corresponding to human MLL amino acids 161-356 (clone N4.4) and the anti- C-terminal monoclonal antibody (Upstate) was raised against a maltose-binding protein (MBP) corresponding to human MLL amino acids 3084-3959 (clone 9-12). The binding positions of the antibodies are shown in Figure 2.1. The membrane was incubated overnight at 4ºC with either MLLN4.4 (1:400) or MLL Clone9-12 (1:250) diluted in 5% milk/TBS-T. Proteins were detected using a sheep anti-mouse horse radish peroxidase (HRP) and a chemiluminescent reagent (ECL). Membranes were stripped in 1x stripping buffer, the blocking step was repeated and the membranes were re-probed with anti-HSP90 (1:2000) for 2 hours at RT as a loading control.

2.2.23 Counting Cells using Trypan blue

To obtain viable cell counts, trypan blue exclusion was used. Single cell suspensions were diluted in trypan blue and counted using a haemocytometer.

2.2.24 Analysis of data

All flow cytometric data was analysed using Summit 4.1, 4.2 or 4.3 (Dakocytomation). All graphs were produced using either Microsoft Excel or Graphpad Prism 4. All statistical analyses were performed using Graphpad Prism 4 software, according to the guide provided with the software. When comparing two data sets, a two-tailed non-paired t-test was performed to assess whether the sets were significantly different. Paired two-tailed t-tests were also used to analyse flow cytometric data from *Mll* conditional mice. Where 3 data sets were analysed, One-way anova with Tukeys multiple comparison test was performed.

2.2.25 Methylcellulose Colony-forming assays with foetal liver cells

Single cells suspensions were prepared from E12.5 fetal liver. Following erythrocyte removal, 1.1 x 10^4 nucleated cells were added to 1.1 ml M3434

Figure 2.1. Binding positions of the antibodies used for western blots of MLL.
The schematic shows the MLL protein. See Figure 1.5 for details of all the domains shown. The N-terminal antibody – N4.4 - binds across the AT hooks. The C-terminal antibody – 9-12 – binds across the SET domain.

methylcellulose media (contains m-SCF, mIL-3, hIL-6, hEpo) plus GM-CSF at a concentration of 10 ng per ml. The cells were plated into 35 mm dishes and the cultures were placed in anther 10 cm dish containing a 35 mm dish of sterile 1 x PBS to prevent the plates from dessicating. The cultures were incubated at 37ºC in 5% CO_2 for up to 14 days. Colony Forming Units (CFUs) were scored after 7 and 14 days according to instructions from Stem Cell Technologies. Counts were made for CFU-Granulocyte, Macrophage (CFU-GM), CFU-Granulocyte, Erythrocyte, Macrophage, Megakaryocyte (CFU-GEMM) and Burst-Forming Unit Erythroid (BFU-E). The cells were then removed from the plate and then counted using trypan blue exclusion.

2.2.26 Cytospin analysis

To analyze the morphology of cells produced in CFU assays, cytopins were performed. Single cell suspensions were made and the cells washed twice in 1 x PBS. The cells were resuspended in MACs buffer at a concentration of 3 x 10^5 per ml. 100 µl of cell suspension was centrifuged onto slides at 35 g for 5 minutes at low deceleration using a cytospin 3 machine (Shandon). The slides were then fixed and stained with May-Grunwald-Giemsa using a Shandon varistain 24-4 automated staining machine in the Haematology department at Great Ormond Street Hospital.

2.2.27 General protocol for the preparation of samples for flow cytometric analyses

For flow cytometry, samples from fetal and adult mice were prepared as follows. Single cell suspensions were prepared from foetal liver, adult bone marrow, thymus, spleen, mesenteric lymph node or blood. Where necessary, erythrocytes were removed by incubation in RBC lysis buffer. The cells were counted and aliquoted at a concentration of up to 1x10^7 cells per ml into either 5 ml round bottom FACS tubes or v-bottomed 96 well microtitre plates. The cells were washed once in staining buffer. All incubations and washes were performed with staining buffer, unless otherwise indicated. The cells were incubated with anti-Fcγ III/II receptor $mAb^{2.4G2}$ (1:5 dilution, produced in house) for 15 minutes on ice to prevent non-specific antibody binding. The cells were then incubated with the primary

antibodies, at the specified dilution, on ice for 30 minutes. For details of all antibodies used, see Table 2.5. Isotype controls were routinely used for all experiments (Figure 2.2). Where a secondary antibody was used, the cells were washed once and incubated with the secondary antibody at the appropriate dilution for 15 minutes. The cells were then washed twice in running buffer. The cells were resuspended in 100-400 µl of running buffer and run on a Dakocytomation Cyan ADP flow cytometer. All samples were aquired using appropriate live gates (Figure 2.3). All flow cytometric data was analysed using Summit 4.1, 4.2 or 4.3 (Dakocytomation).

2.2.28 Magnetic Activated Cell Sorting

Where it was necessary to sort for a population of cells expressing a particular marker, the Miltenyi Magnetic Activated Cell Sorting System (MACS) was used. The system was used according to the manufacturers instructions. Briefly, cells prepared from foetal liver or bone marrow were incubated with an antibody to the marker of interest (e.g anti c-kit APC, anti-Ter119 PE, biotin-conjugated lineage markers). The cells were then washed and incubated with magnetic beads that bound to the conjugate present on the primary antibody (e.g Streptavidin beads, anti-APC, anti-PE beads). The cells were washed again and then passed down MACs MS or LS columns in the presence of a magnetic field. The column flow through was taken as cells negative for the marker in question, the cells attached to the column were taken as positive for the marker in question. The purity of all sorts was estimated following the sort by flow cytometry. Where necessary, cells were sorted twice to separate populations expressing different markers. For a detailed protocol, please see the manufacturers instructions (Miltenyi).

2.2.29 Analysis of foetal liver stem cells by flow cytometry

For analysis of fetal liver HSC populations, single cell suspensions were made from fetal livers from E13.5 embryos. Total live cell counts were obtained for each foetal liver using trypan blue. Cells were stained with a biotin-conjugated Lineage antibody cocktail (CD3e (145-2C), CD45R/B220 (RA3-6B2), Ly6G and Ly-6C (RB6-

Antibody	Clone	Isotype	Supplier	Working Dilution
Anti-Sca1-PE	D7	IgG2a κ	BD Pharmingen	1:200
Anti-Mac1-PE	M1/70	IgG2b κ	BD Pharmingen	1:200
Anti-Mac1-FITC	M1/70	IgG2b κ	eBioscience	1:100
Anti-Gr1-APC	RB6-8CS	IgG2b κ	eBioscience	1:300
Anti-Ter119-PE	Ter119	IgG2b κ	BD Pharmingen	1:100
Anti-B220-APC	RA3-6B2	IgG2a κ	eBioscience	1:100
Anti-CD19-PE	1D3	IgG2a κ	BD Pharmingen	1:200
Anti-Ly5.2-Bio	104	IgG2a κ	eBioscience	1:100
Anti-c-kit-APC	2B8	IgG2b κ	eBioscience	1:100
Anti-Sca1-FITC	D7	IgG2a κ	eBioscience	1:100
Anti-CD38-PE	90	IgG2a κ	eBioscience	1:600
Anti-Ly5.2-FITC	104	IgG2a κ	eBioscience	1:100
Anti-Ly5.1-PE	A20	IgG2a κ	eBioscience	1:200
Anti-CD4-PE	H129.19	IgG2a κ	BD Pharmingen	1:200
Anti-CD8-Tri	CT-CD8a	IgG2a κ	Caltag	1:100
Anti-IgG2A-PE	G155-178	IgG2a κ	BD Pharmingen	1:100
Anti-IgG2b-FITC	27-35	IgG2b κ	BD Pharmingen	1:100
Anti-IgG2b-APC	N/A	IgG2b κ	eBioscience	1:100
Streptavidin-PerCP	N/A	N/A	BD Pharmingen	1:200

Table 2.5. Antibodies used for flow cytometric analyses.

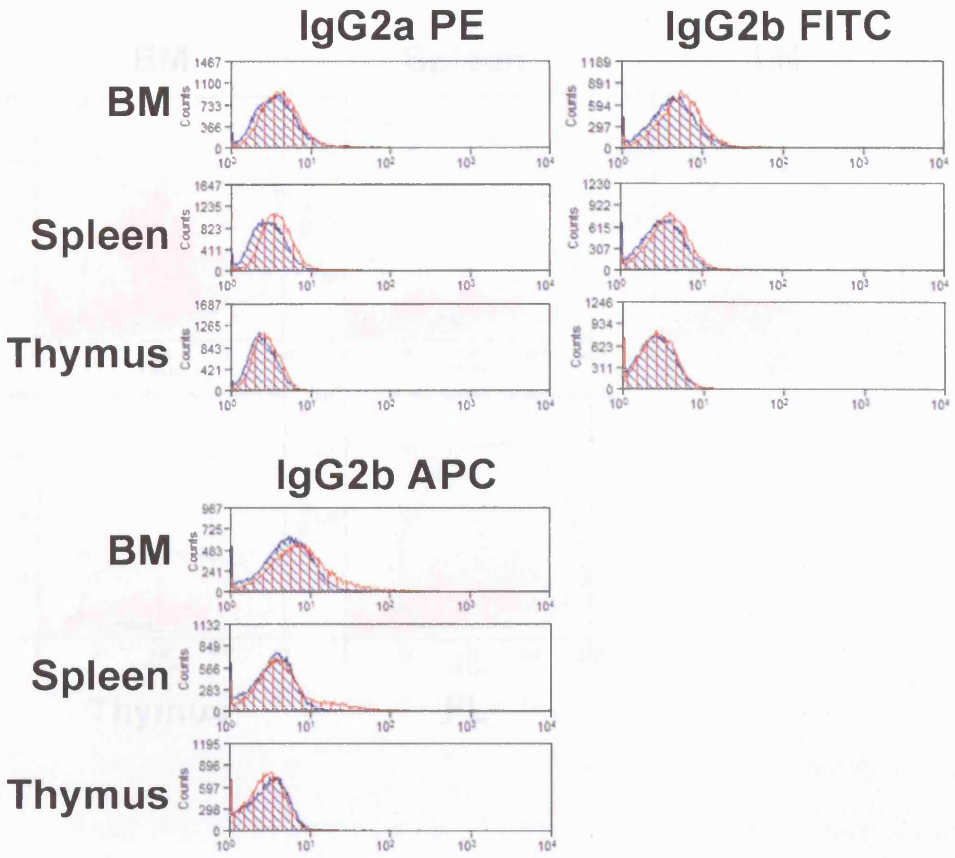

Figure 2.2. Isotype controls used for flow cytometry experiments. Representative histograms are shown for isotype controls to control for non-specific antibody binding. The blue plot is an unstained sample, the red plot is the isotype control stained sample.

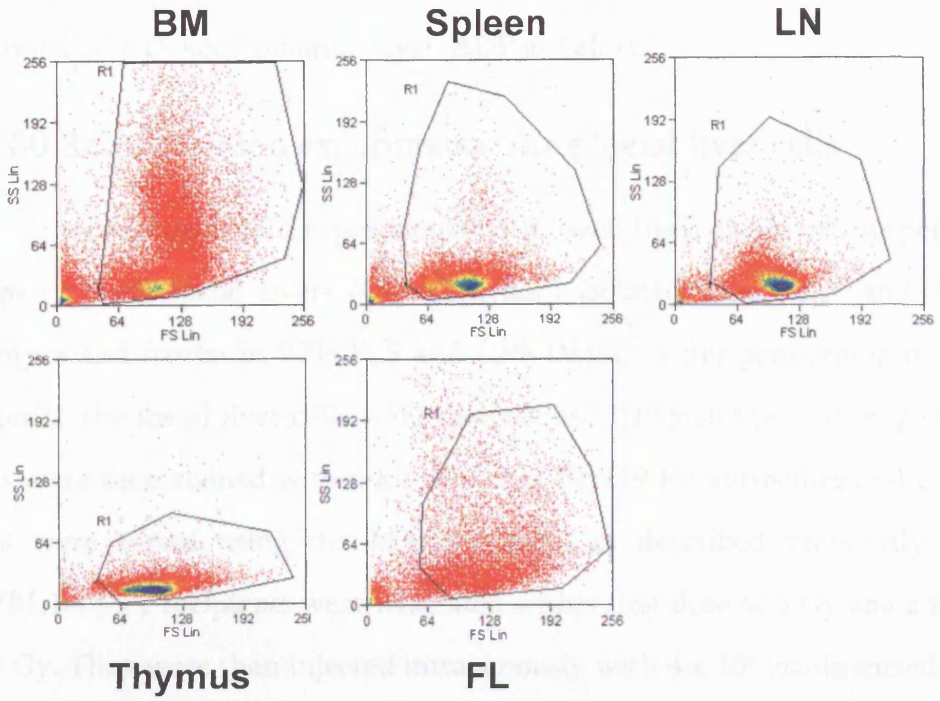

Figure 2.3. Live gates used for flow cytometric analyses.
Representative dot plots showing gating for live cell populations in bone marrow, spleen, lymph node, thymus and foetal liver.

8C5), and TER-119, Pharmingen). Lineage positive cells were depleted using magnetic sorting according to the manufacturer's instructions (MACS, Miltenyi). Lineage negative cells were incubated with anti-Fcγ III/II receptor $mAb^{2.4G2}$ to prevent non-specific antibody binding and then incubated with c-kit-APC, Sca1 FITC, CD38 PE, 7AAD (to gate out dead cells) and Streptavidin-PrCP. Cells were analysed on a Dakocytomation Cyan ADP as before.

2.2.30 Reconstitution experiments using foetal liver cells

For reconstitution experiments with foetal liver, single cell suspensions were prepared from foetal livers (CD45.2/Ly5.2$^+$) isolated from *Mll*$^{-/-}$ and *Mll*$^{+/+}$ E14.5 embryos and frozen in 90% FCS and 10% DMSO. After genotyping using embryo material, the foetal liver cells were thawed into HBSS and pooled by genotype. The cells were then stained with c-kit APC and Ter119 PE antibodies and c-kit$^+$Ter119$^-$ cells were sorted using the MACS system, as described previously (Miltenyi). C57BL/6 Ly5.1 recipients were irradiated with a first dose of 5 Gy and a second dose of 4 Gy. They were then injected intravenously with 4 x 10^4 viable sorted foetal liver cells plus a radioprotective dose of 2 x 10^5 C57BL/6 Ly5.1$^+$ bone marrow cells. All injections were performed by Owen Williams. Blood was taken from the recipients 1 month post-transplantation and the reconstitution levels were assessed by analysing the number of Ly5.1 and Ly5.2 positive cells present using flow cytometry. After 4 months, the recipients were sacrificed and the bone marrow, spleen and thymus analyzed for the presence of Ly5.1 and Ly5.2 positive cells by flow cytometry.

2.2.31 Analysis of adult haematopoietic populations by flow cytometry

Haematopoietic cells in adult *Mll* conditional mice were analysed by flow cytometry. 8 week old mice were killed using Schedule 1 approved methods. Spleen, thymus and mesenteric lymph nodes were taken from each individual. These were passed through 70 μM cell strainers to achieve single cell suspensions. The femurs and tibia were taken from each mouse and transferred to AB IMDM. Any muscle or skin was removed from the bones which were placed in fresh HBSS. The bone

marrow was then flushed from the bones using a 25 gauge needle. Erythrocytes were removed from all samples by incubation in RBC lysis buffer as before. Viable cell counts were made, and aliquots of 1 x 10^6 cells from each sample were transferred to 96 well v-bottom microtitre plates. The cells were stained with different combinations of the following antibodies: anti-CD4-PE, anti-CD8-Tri, anti-c-kit-APC, anti-Sca1-PE, Biotin-conjugated-Lineage panel, anti-B220-APC, anti-CD19-PE, anti-Gr1-APC, anti-Mac1-FITC, anti-IgG2a-PE, anti-IgG2b-FITC, anti-IgG2b-APC, anti-Ly5.2-FITC, anti-Ly5.1-PE, Steptavidin-PrCP. The cells were analysed on a Dakocytomation Cyan ADP flow cytometer as before.

2.2.32 Methylcellulose CFU assays using adult bone marrow cells

Single cells suspensions were prepared from bone marrow. For bone marrow Pre-B assays, 5.5 x 10^4 nucleated cells were plated into 1.1 ml M3630 methylcellulose media (containing hIL-7) and CFUs were counted after 7 days. For bone marrow myeloid assays, 2.7 x 10^4 nucleated cells were plated in 1.1 ml M3434 media and CFUs were scored after 10 days.

2.2.33 Reconstitution experiments using adult bone marrow cells

For competitive reconstitution assays with adult bone marrow cells, bone marrow was prepared from two mice for each genotype (*Mll*$^{Flox/+}$, *Mll*$^{Flox/-}$, *Mll* conditional). Erythrocytes were removed by lysis with RBC lysis buffer and the cells counted and pooled for each genotype. All injections were performed by Henrique Veiga-Fernandes. For the 10:1 ratio, 2 x 10^6 cells from each genotype were mixed with 2 x 10^5 WT C57Bl/6J Ly5.1$^+$ bone marrow cells and injected into 5 lethally irradiated C57Bl/6J Ly5.1$^+$ recipients. For the 1:1 ratio, 1 x 10^6 cells from each genotype were mixed with 1 x 10^6 WT competitor cells. After 4 weeks, blood was taken from the recipients for the analysis of reconstitution levels, as before. After 4 months, the recipients were sacrificed and bone marrow, spleen and thymus were taken for analysis as before.

2.2.34 Analysis of recombination in Mll cKO bone marrow and fetal liver stem cells

To sort bone marrow stem cells for DNA preparation, single cell suspensions were prepared from the bone marrow of 3 *Mll cKO* mice and pooled. The cells were stained with a biotinylated Lineage antibody cocktail (CD3e, CD11b, CD45R/B220, Ly6G and Ly-6C, and TER-119, Miltenyi), and the lineage positive cells were depleted by magnetic sorting. The remaining cells were stained with c-kit-APC and Sca1-PE. c-kit^{hi}Sca1^{+} cells were sorted from the population using a Beckman Coulter Epics Altra. DNA lysates were prepared from the cells and the DNA analyzed by PCR, using primers E2 and F to detect the floxed allele, and E2 and G2 to detect the deleted allele.

To sort c-kit^{+}Ter119^{-} fetal liver cells for DNA preparation, E13.5 fetal liver cells were stained with c-kit-APC and Ter119-PE. Ter119^{+} cells were depleted and then c-kit^{hi} cells were selected by magnetic sorting. DNA was prepared from the resulting cells for PCR analysis as above.

Chapter 3

Successful Targeting of Murine Embryonic Stem Cells with an *Mll* 'Floxed' allele

3.1 Introduction

As shown in previous studies, homozygous deletion of *Mll* results in embryonic lethality (Ayton et al., 2001; Yagi et al., 1998; Yu et al., 1995). This lethality had previously prevented the study of haematopoiesis in adult mice in the absence of *Mll*. It was therefore necessary to create a line of mice in which *Mll* could be conditionally removed only in the haematopoietic system. By removing *Mll* only in the haematopoietic system, it was hoped that the lethality of constitutively removing *Mll* would be overcome, enabling the study of adult haematopoiesis in the absence of *Mll*.

It was decided that the most efficient way to facilitate conditional removal of *Mll* was by utilizing the Cre-lox system. The bacterial recombinase Cre recombines DNA sequences flanked by so called loxP sites (Kuhn et al., 1995). loxP sites are unique, 34 bp long sequences of DNA, that are not normally present in mammalian DNA (Sauer, 1998). By flanking exons 9-10 of *Mll* with loxP sites, it was possible to create a 'conditional' or 'floxed' allele of gene. In the presence of the Cre recombinase, the loxP sites would be recombined to excise exons 9 and 10 of *Mll*. Cre is not normally expressed in mammalian cells, so by breeding mice carrying the 'floxed' allele to mice that express transgenic Cre under tissue specific promoters it was possible to produce mice where exons 9 and 10 of *Mll* were excised only in certain tissues, such as the haematopoietic system (Kuhn et al., 1995).

An Embryonic Stem (ES) cell targeting strategy was used to establish a mouse line bearing a 'floxed' allele of Mll. ES cells are pluripoitent cells derived from the inner cell mass of blastocysts. They can be cultured for many passages and still retain their pluripotency as shown by the fact that when they are reintroduced into blastocysts, they can contribute efficiently to the formation of chimaeras (Bradley et al., 1984). Using ES cells, mutations and foreign DNA can be introduced into the genome via gene targeting. This involves the homologous recombination of DNA sequences residing in the chromosome with newly introduced DNA sequences (Thomas and Capecchi, 1987). For example, as described here, a plasmid carrying introns 5-6 to 10-11 of MLL DNA, including the floxed exons 9 and 10 and a selection marker, was electroporated into PC3 ES cells (Figure 3.1). The floxed section of MLL was then integrated into the ES cell genome via homologous recombination (Figure 3.3). ES cells clones carrying the targeted allele were identified using the selection marker. These clones were karyotyped and injected into blastocysts to give rise to chimeric mice bearing the targeted *Mll* allele.

3.2 Construction of targeting vector pMll197.A1

The first targeting vector used for targeting was constructed by Dr Suzana Hadjur. A pBluescript vector containing introns 5-6 to 10-11 of mouse 129sv *Mll* genomic DNA was provided by Dr Terry Rabbitts (LMB, Cambridge) (pBS-Mll). pBS-Mll was linearised using a *Bgl*II site in intron 10-11 of the *Mll* genomic DNA and the sticky ends blunted. A LoxNeoLox cassette (a neomycin resistance cassette flanked by two loxP sites) was excised from the vector, pM197, using a *Sal*I/*Not*I double digest and the fragment ends blunted. The LoxNeoLox cassette was blunt-end ligated into the blunted *Bgl*II site of pBS-Mll. The resultant vector carrying was named pMll197.

pMll197 was digested with *Aat*II in intron 6-7 of the Mll genomic DNA and the ends blunted. A DNA fragment containing a loxP site was amplified from the vector, pGEM30, using the primers LoxPF and LoxPR (Table 2.3, Table 2.4). The 150bp fragment containing the loxP site was then digested with *Aat*II and *Sma*I and the sticky ends blunted. The loxP site fragment was then blunt-end ligated into the

blunted *Aat*II site of pMLL197. The final vector, containing a loxP site in intron 6-7 and a LoxNeoLox cassette in intron 10-11 of *Mll*, was named pMll197.A1 (Figure 3.1).

After sequencing, two tandem loxP sites were found to have been ligated into the 5' *Aat*II site Figure 3.1. It was thought that the presence of two loxP sites here would not interfere with the targeting strategy and the vector was used to target mouse ES cells.

3.3 Failure of targeting using pMll197.A1

Targeting of the *Mll* locus in PC3 ES cells by electroporation (O'Gorman et al., 1997) was attempted using pMll197.A1 (Figure 3.1). Over 800 clones were picked following selection in neomycin, and DNA prepared and screened by Southern Blot Analysis. Of 1000 neomycin resistant clones picked, 12 were found to be positive for the LoxNeoLox cassette using a *Kpn*I Southern blot strategy When these clones were screened for the 5' loxP site, none were found to be positive (data not shown). Homologous recombination had not been achieved at the 5' end of the targeting vector.

It was possible that the incomplete homologous recombination of the targeting vector in ES cells was due to a lack of *Mll* homology flanking the 5' loxP site or to there being too much space between the loxP sites. To increase the chances of correct recombination, a second targeting vector was constructed where the 5' loxP site was brought closer to the LoxNeoLox cassette and extra *Mll* homology was added to the 5' end of the targeting vector. In this second vector, exons 9 and 10 of *Mll*, which encode part of the first PHD finger of the protein, were flanked by loxP sites (Figure 3.2).

3.4 Construction of the second targeting vector pMLL197.2kb

To create the second targeting vector, pMll197 was used as the starting point. The 5' loxP site was amplified from pGEM30 as before and sub-cloned into pGEM-T Easy. An *Aat*II/*Sma*I digest was used to digest out the loxP site from pGEM-T Easy. The ends were blunted and the loxP site was this time ligated into a blunted *Nsi*I site

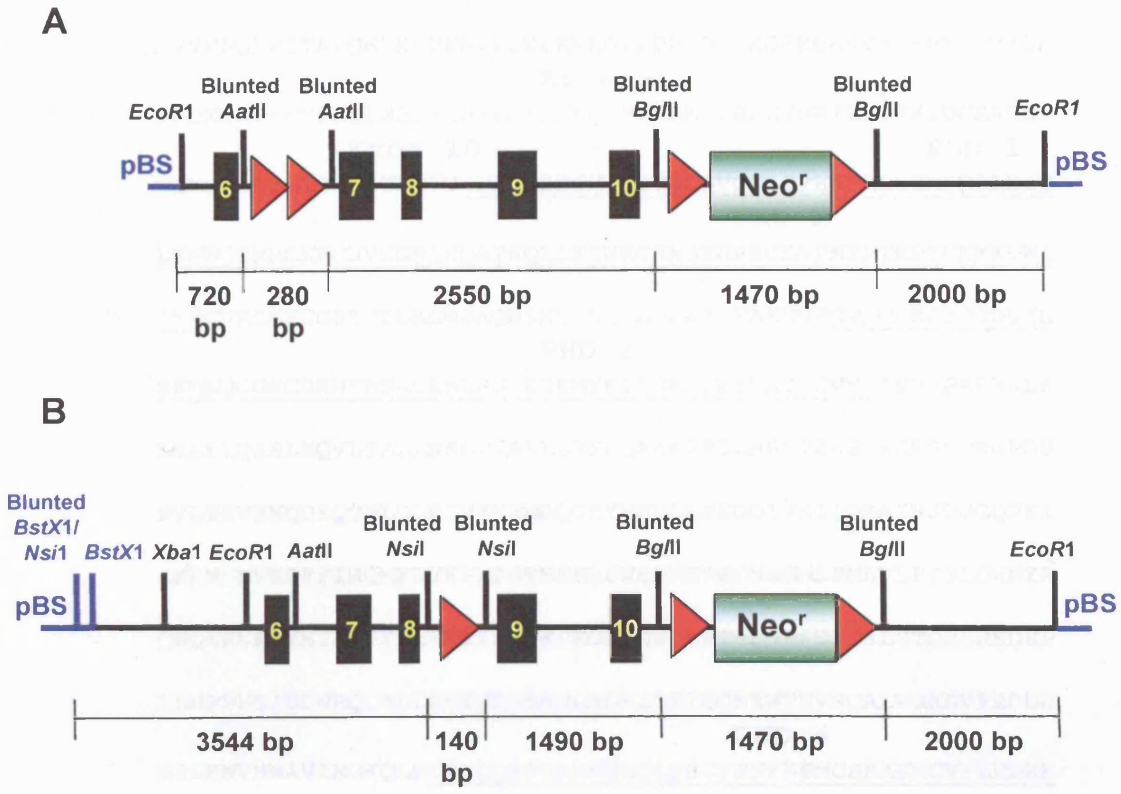

Figure 3.1. Schematics of the vectors used for targeting.

A) The first targeting vector, pMll197.A1.Filled black rectangles denote exons, red triangles denote loxP sites and Neo^r denotes the Neomycin resistance cassette. pBS indicates the pBluescript vector backbone. The blue denotes vector DNA, the black line denotes *Mll* genomic DNA

B) The second targeting vector Mll197.2kb

KCQNLQWMPSKASLQKQTKAVKKKEKKSKTTEKKESKESTAVKSPLEPAQKAAPPP

REEPAPKKSSSEPPPRKPVEEKSEEGGAPAPAPAPEPKQVSAPASRKSSKQVSQPA

AVVPPQPPSTAPQKKEAPKAVPSEPKKKQPPPPEPGPEQSKQKKVAPRPSIPVKQK

Exon 9

PKDKEKPPPVSKQENAGTLNILNPLSNGISSKQKIPADGVHRIRVDFKEDCEAENV

Exon 10 PHD 1

WEMGGLGILTSVPITPRVVCFLCASSGHVEFVYCQVCCEPFHKFCLEENERPLEDQ

PHD 2

LENWCCRRCKFCHVCGRQHQATKQLLECNKCRNSYHPECLGPNYPTKPTKKKVWI

CTKCVRCKSCGSTTPGKGWDAQWSHDFSLCHDCAKLFAKGNFCPLCDKCYDDDDYE

PHD 2

SKMMQCGKCDRWVHSKCESLSGTEDEMYEILSNLPESVAYTCVNCTERHPAEWRLA

LEKELQASLKQVLTALLNSRTTSHLLRYRQAAKPPDLNPETEESIPSRSSPEGPDP

PVLTEVSKQDEQQPLDLEGVKKRMDQGSYVSVLEFSDDIVKIIQAAINSDGGQPEI

KKANSMVKSFFIRQMERVFPWFSVKKSRFWEPNKVSNNSGMLPNAVLPPSLDHNYA

QWQEREESSHTEQPPLMKKIIPAPKPKGPGEPDSPTPLHPPTPPILSTDRSREDSP

ELNPPPGIDDNRQCALCLMYGDDSANDAGRLLYIGQNEWTHVNCALWSAEVFEDDD

PHD 4

GSLKNVHMAVIRGKQLRCEFCQKPGATVGCCLTSCTSNYHFMCSRAKNCVFLDDKK

VYCQRHRDLIKGEVVPENGFEVFRRVFVDFEGISLRRKFLNGLEPENIHMMIGSMT

Figure 3.2. Position of the PHD fingers in the MLL protein.
Exon 9 is highlighted in purple, exon 10 is highlighted in blue. The PHD fingers are underlined.
As shown above, exon 10 encodes a portion of the first PHD finger. The cysteine residues that form part of one of the zinc binding sites in the first PHD finger are highlighted in red. The sites of the PHD fingers were identified using information from Aasland *et al.* 1995 and Jose Saldanha (personal communication).

in intron 8-9 of pMLL197. Sequencing was used to check the orientation and sequence of the loxP site and to confirm the presence of only one loxP site.

The additional *Mll* 5' homology was PCR amplified in two fragments from genomic DNA extracted from PC3 ES cells (129sv background). A 5' fragment was amplified by PCR using primers Exon5MLL/Intron5-68410R and a 3' fragment was amplified using primers Intron5-6MLLC/PCR2A (Table 2.3, Table 2.4). Both fragments were sub-cloned into pGEM-T Easy and sequenced to check for mistakes that may have occurred during amplification. The 5' fragment was digested out of pGEM-T Easy using an *Xba*I/*Sal*I double digest and ligated into pGEM-T Easy containing the 3' fragment using an *Xba*I site in the 3' *Mll* sequence and a *Sal*I site in pGEM-T Easy. This vector was termed pGEM-T Easy1.4+1Kb.

The *MLL* homology in pGEM-T Easy1.4+1Kb1.1 was directionally cloned into the targeting vector using a blunt/sticky ended ligation. pGEM-T Easy1.4+1Kb1.1 was linearised using an *Nsi*I digest. The sticky ends were blunted and the vector digested again with *Aat*II, to release the fragment of homology. pBluescript MLL197 was linearised with *BstX*I and the ends blunted. The vector was then digested with *Aat*II. The homology from pGEM-T Easy1.4+1Kb1.1 was ligated into the 5' end of the vector using the *Aat*II in *Mll* and the blunted *BstX*1 to create the finished vector, pMLL197.2kb (Figure 3.1).

Restriction digest mapping was used to check that the resulting targeting vector was correct (all the components had been sequenced prior to assembly of the vector). In this targeting vector, the 5' loxP site and the 3' LoxNeoLox cassette flank exons 9 and 10 of *Mll* (Figure 3.1).

3.5 Targeting of ES Cells with the Second Targeting Vector

ES cells were electroporated with the second targeting vector, pMLL197.2kb. The targeting strategy is shown in Figure 3.3.

Eight-hundred neomycin resistant clones were picked following electroporation of PC3 ES cells with vector pMLL197.2kb. DNA was extracted from two samples of each clone for Southern Blot analysis. An aliquot of cells of each clone was frozen and kept at -70ºC.

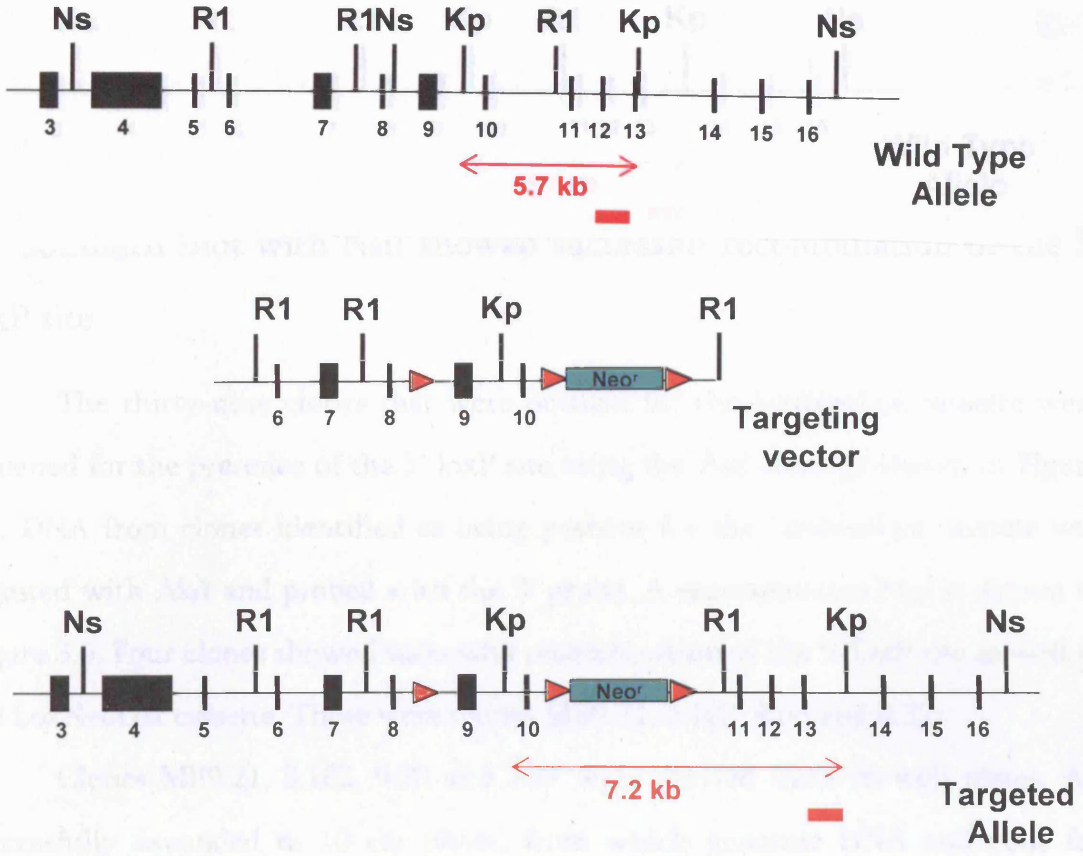

Figure 3.3. Targeting Strategy and Southern Strategy for identifying positive ES cell clones .

The figure shows the wild type *Mll* allele before targeting, and the targeted allele after homologous recombination with the targeting vector has taken place. Also shown are the position of the restriction sites and probe used in *Kpn*1 Southern Blot Analysis and the corresponding fragment sizes. Bs1- *BstX*1 Ns- *Nsi*1, R1- *EcoR*1, Kp- *Kpn*1.

The filled black boxes denote exons, the red triangles denote LoxP sites, Neo^r denotes the neomycin resistance gene and the filled red box denotes the 3′ probe.

3.6 Southern Blot with KpnI showed successful recombination of the LoxNeoLox cassette

DNA from over seven hundred clones was screened for the LoxNeoLox cassette using the *KpnI* Southern strategy shown in Figure 3.3. One gel per 96-well plate was run, as shown in Figure 3.4. The wild type allele gave a band of 5.7 kb and the targeted a band of 7.2 kb (due to the presence of the LoxNeoLox cassette) (Figure 3.4). Of approximately 500 clones screened, thirty-nine were found to be positive for the LoxNeoLox cassette.

3.7 Southern Blot with NsiI showed successful recombination of the 5' loxP site

The thirty-nine clones that were positive for the LoxNeoLox cassette were screened for the presence of the 5' loxP site using the *NsiI* strategy shown in Figure 3.5. DNA from clones identified as being positive for the LoxNeoLox cassette was digested with *NsiI* and probed with the 3' probe. A representative blot is shown in Figure 3.6. Four clones showed successful recombination of the 5' loxP site as well as the LoxNeoLox cassette. These were clones Mll9.21, 3.102, 9.33 and 3.37.

Clones Mll9.21, 3.102, 9.33 and 3.37 were thawed from 96-well plates. All successfully expanded to 10 cm plates, from which genomic DNA and cells for freezing were prepared.

3.8 Karyotyping of positive clones

When cultured, ES cells often develop chromosomal aneuploidy (the acquisition of extra chromosomes in addition to the normal complement of forty) (Koller, 1992). When injected into blastocysts, aneuploid ES cells can lead to developmental abnormalities in the resultant chimeras. For this reason it was important to karyotype the clones before injection. For Clone Mll3.102, 50% of the cells were aneuploid (Figure 3.7). Clones Mll9.21, 9.33 and 3.37 were found to contain over 90% normal cells (Figure 3.7). These clones, when grown in culture,

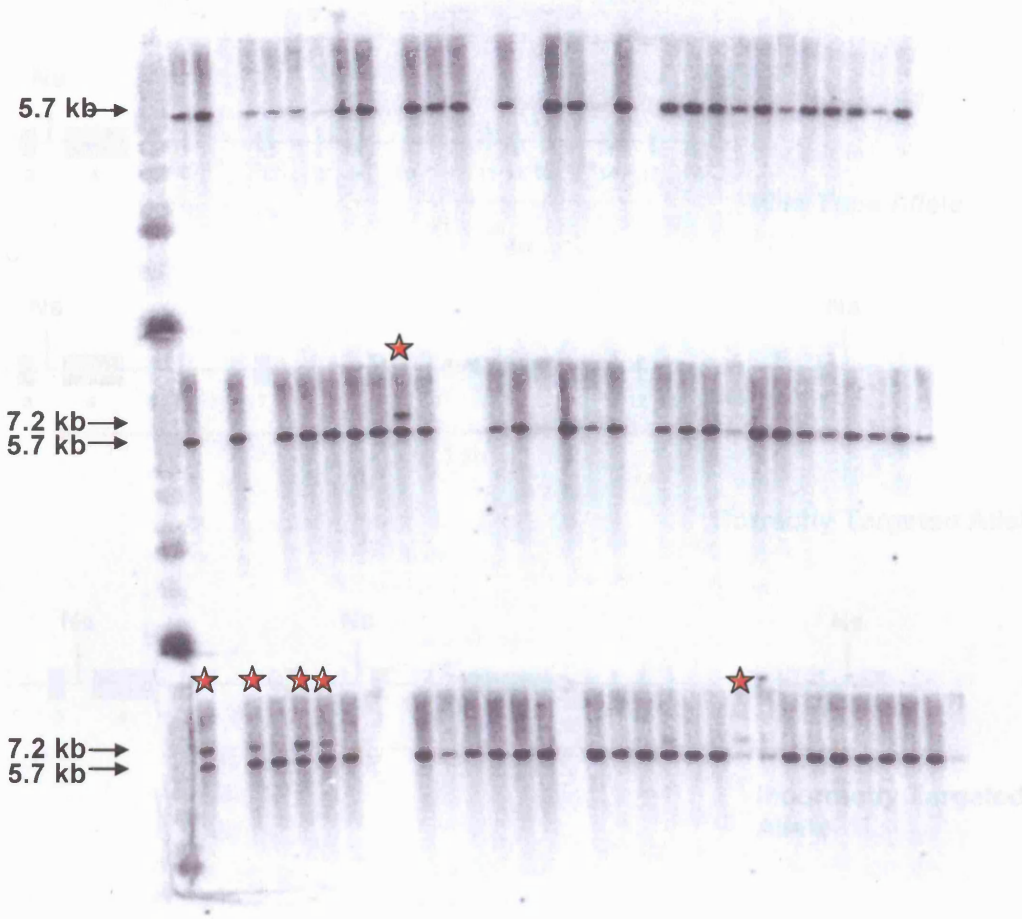

Figure 3.4. Southern Blot of DNA from G418 resistant clones. Genomic DNA from neomycin resistant ES cell clones grown in a 96 well plate was digested with *Kpn*I and then probed with the 3' probe.

Clones positive for the LoxNeoLox are highlighted by red stars. Wild type band = 5.7 kb. Targeted band = 7.2 kb

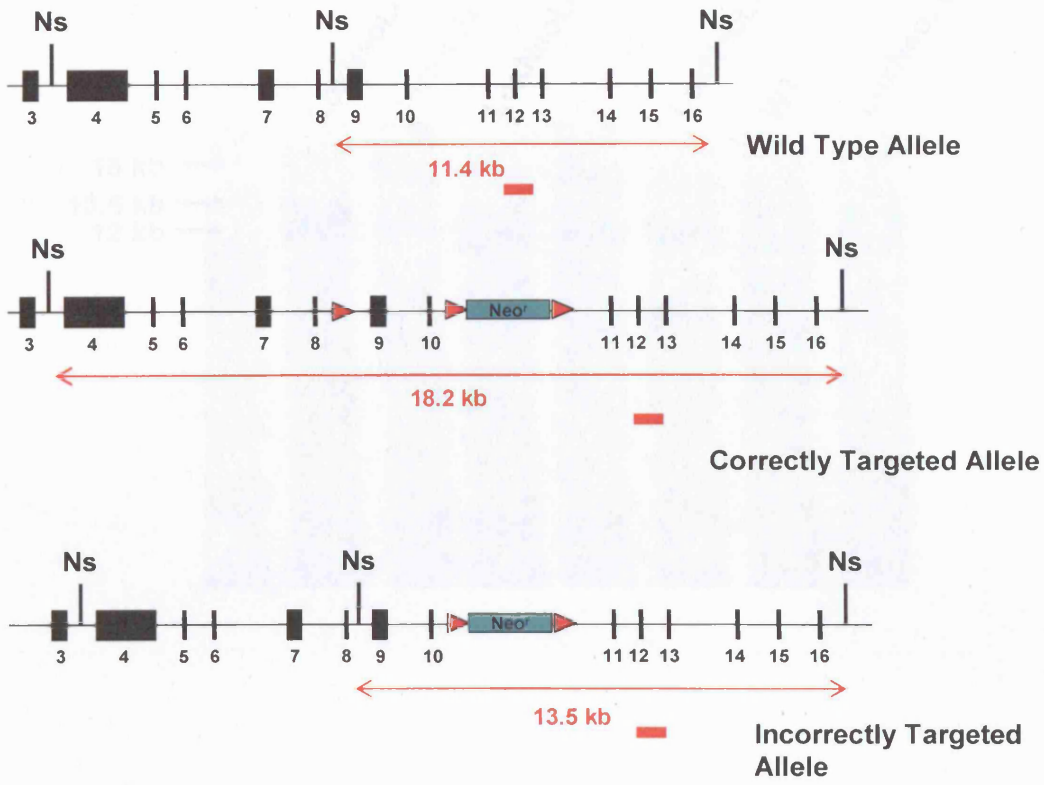

Figure 3.5. *Nsi*I Southern for the different Mll alleles.
The different fragment sizes seen in Southern Blot Analysis using *Nsi*I digest are shown. Ns- Nsi1. The filled black boxes denote exons, the red triangles denote loxP sites, Neo^r denotes the neomycin resistance cassette box and the red box indicates the 3′ probe.

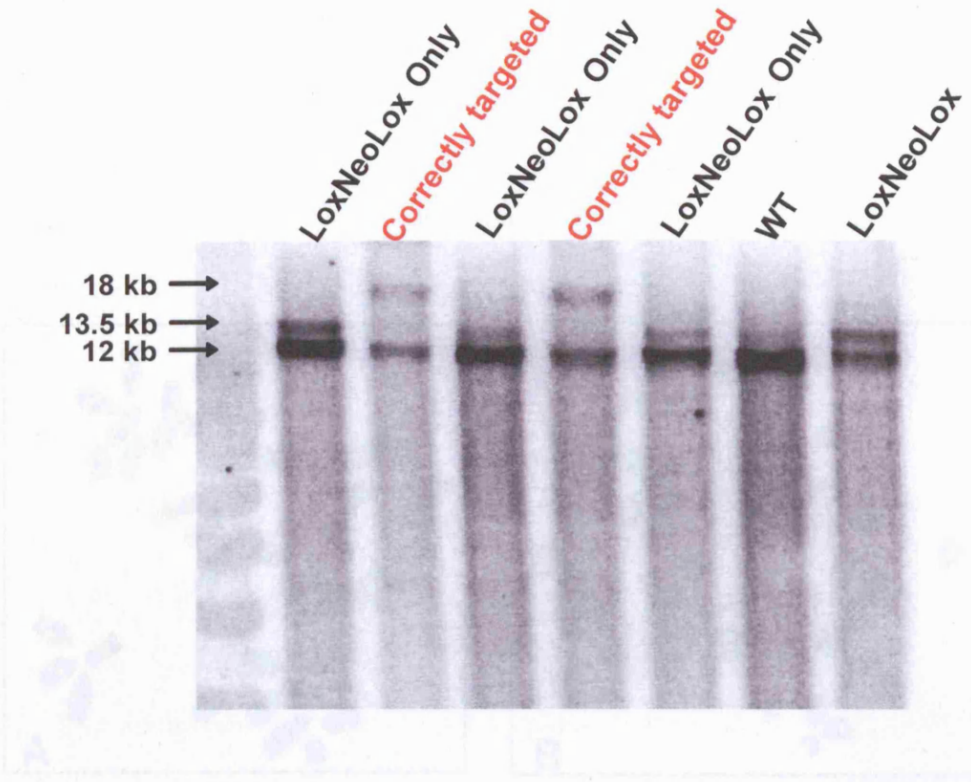

Figure 3.6. Southern blot of DNA from clones positive for the LoxNeoLox cassette.
DNA was digested with *Nsi*I and probed with the 3′ probe to look for the presence of the 5′ LoxP site. Wild type allele = 12 kb, incorrectly targeted allele (LoxNeoLox cassette only) = 13.55 kb, correctly targeted allele (plus the 5′ LoxP) = 18 kb.

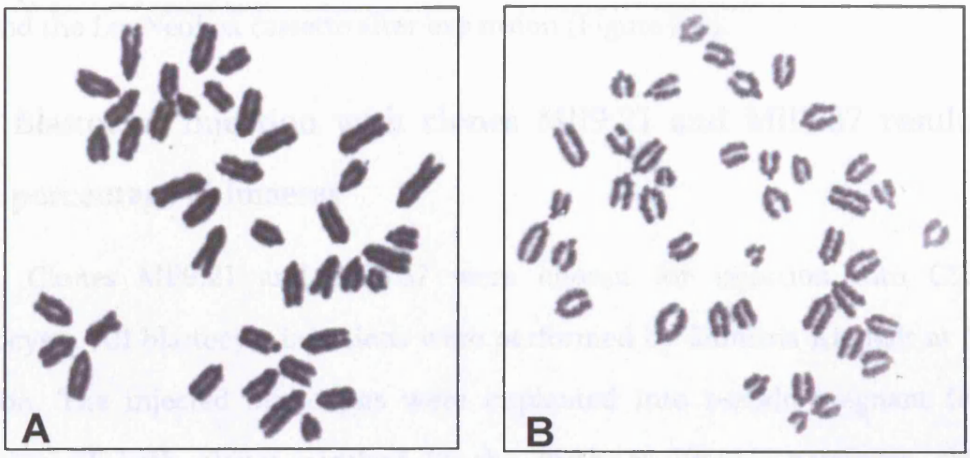

Figure 3.7. Examples of karyotypes of ES cell clones.

A) Karyotype of Clone Mll3.37, showing a normal complement of 40 chromosomes.

B) Karyotype of aneuploid clone Mll3.102 showing an abnormal complement of 41 chromosomes.

looked healthy and had 5-10% differentiation as expected for healthy, pluripotent ES cells.

3.9 Southern Blot analysis on DNA from expanded clones showed the correct clones were picked

Following expansion of Clones Mll9.21, 9.33 and 3.37, DNA from the clones was used for Southern Blot analysis using the *Nsi*I and *Kpn*I strategies mentioned previously (Figures 3.3 and 3.5). This was to ensure that the correct clones had been picked and that these clones were not mixed clones of correctly targeted and wild type ES cells. Clones Mll9.21, 9.33 and 3.37 were found to be positive for the 5' loxP site and the LoxNeoLox cassette after expansion (Figure 3.8).

3.10 Blastocyst injection with clones Mll9.21 and Mll3.37 resulted in high percentage chimaeras

Clones Mll9.21 and Mll3.37 were chosen for injection into C57BL/6J blastocysts. All blastocyst injections were performed by Dimitris Kioussis at NIMR, London. The injected blastocysts were implanted into pseudo-pregnant females. Injection of both clones resulted in the birth of litters containing chimeras exhibiting a high percentage of chimerism. The level of chimerism was indicated by amount of agouti coat colour that the mice had (PC3 ES cells are derived from 129Sv mice that carry agouti coat colour, C57BL/6 blastocysts carry black coat colour). A high level of chimerism suggested that the ES cells injected were contributing successfully to the tissues of the mice.

3.11 The injected ES cells successfully contributed to the germline of chimaeras

Male chimeras with a high percentage of chimerism were bred to C57Bl/6J females. All breeding pairs produced offspring with agouti coat colour, showing that the ES cells had successfully contributed to the germline of the chimeras. Chimeras

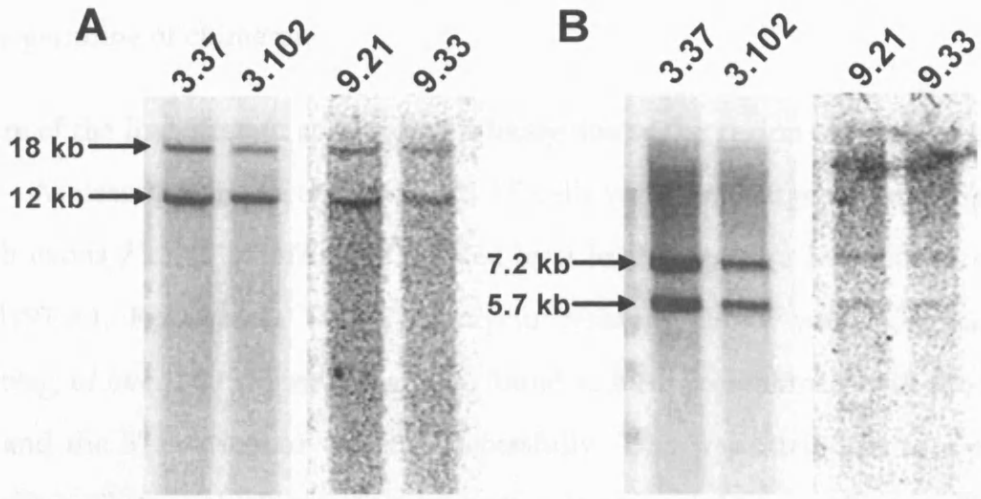

Figure 3.8. Southern blots to confirm the genotype of the expanded ES cell clones.

A) Southern blot analysis of clones Mll3.37,3.102, 9.21, 9.33 using a an *Nsi*I digest. Wild type band = 12 kb, targeted band = 18 kb.

B) Southern Blot analysis of the same clones using a *Kpn*I digest. Wild type band = 5.7 kb, targeted band = 7.2 kb.

derived from clone Mll9.21 gave rise to line Mll9.21, those derived from Mll3.37 gave rise to line Mll3.37.

3.12 Discussion

Establishing a conditional knockout model of the gene Mll

This chapter describes the successful targeting of mouse ES cells with a 'floxed' allele of *Mll* and the subsequent contribution of ES cells carrying this allele to the germline of chimeras.

Failure of the first attempt at targeting is likely due to the region of Mll targeted

As described in Section 3.3, PC3 ES cells were first targeted with a plasmid which exons 7 to 10 of *Mll* were flanked by a loxP site and a LoxNeoLox cassette (pMll197.A1, Figure 3.1). When neomycin resistant clones were screened after targeting, of over 800 clones, none were found to have recombined both the 5' loxP sites and the 3' LoxNeoLox cassette successfully. This was attributed to a possible lack of homology at the 5'end of the targeting vector and the large distance (2.55 kb) between the 5' loxP site and the 3' LoxNeoLox cassette. Previous work has shown that increased flanking homology can increase the likelihood of the recombination of non-homologous sequences (Koller, 1992; Thomas and Capecchi, 1987). It is also of note that this region of *Mll* is very close to the breakpoint cluster region of the gene, where chromosomal translocations occur (Scharf et al., 2006). When designing genotyping primers in this region, it was found that the majority of primers designed could not be used because of homology to sequences from several chromosomes. This would suggest that both the 5' loxP site and the 3' LoxNeoLox cassette were situated in introns with a large number of repetitive elements. This may have meant that when the ES cells were targeted, the LoxNeoLox cassette was incorporated into other sites in the genome other than the *Mll* locus, via homologous recombination with repetitive sequences. This may account for the large number of neomycin resistant clones that were picked that had not incorporated the LoxNeoLox cassette at the *Mll* locus.

The Second Targeting Vector - Targeting exons 9 and 10 of Mll

In the second, successful targeting vector, the loxP site and the LoxNeoLox cassette flanked exons 9 and 10 of *Mll* which encode two cysteine residues that form part of the first loop of PHD finger 1 of MLL (Figures 3.1 and 3.2). PHD fingers are zinc finger containing domains that are predominantly found in chromatin remodelling proteins (Bienz, 2006; Pena et al., 2006). As discussed in the introduction, they are important for the binding of nucleosomes and other ligands by chromatin remodelling proteins. So, by targeting the first PHD finger of the MLL, it was possible that important protein-protein interactions would be abolished, or that the protein's structure would be affected.

The second targeting vector was successfully used to target PC3 ES cells

Using the second targeting vector, PC3 ES cells were successfully targeted. Southern blot strategies where the probe bound externally to the targeted area of *Mll* were used to ensure that the *Mll* locus had been targeted correctly. Three clones were found that contained the correctly incorporated 5' loxP site and 3' LoxNeoLox cassette and that had a normal complement of chromosomes. Two of these clones were injected into blastocysts and both gave rise to chimeras showing high levels of chimerism. A high level of chimerism indicated that the ES cells used to create the chimera were healthy and able to contribute significantly to the embryo (Capecchi, 1989). When the male chimaeras were bred to C57BL/6 females, which, like the blastocysts, had a black coat colour, the resultant litters contained agouti offspring, the parental coat colour from the PC3 ES cells, which were derived from a 129sv background (O'Gorman et al., 1997). This indicated that the targeted ES cells had contributed to the germline of the chimeras.

Chapter 4

Deletion of Exons 9 and 10 of *Mll* Resulted in a Knockout Phenotype

4.1 Introduction

The previous chapter described the establishment of two mouse lines carrying an *Mll* allele where exons 9 and 10 were flanked by a LoxP site and a LoxNeoLox cassette. This chapter describes the production of mice carrying floxed and deleted alleles of *Mll*. In addition this chapter shows that the deletion of exons 9 and 10 lead to the loss of MLL protein expression.

The floxed and deleted alleles of *Mll* were produced using the PC3 Cre transgene which is present in PC3 ES cells (O'Gorman et al., 1997). The PC3 Cre transgene expresses the Cre recombinase under the control of the mouse protamine 1 (*Prm1*) promoter (O'Gorman *et al.* 1997). The *Prm1* promoter is active only during the terminal, haploid stages of spermatogenesis, so in those chimeras carrying the PC3 Cre transgene, Cre-mediated recombination occurred only in male spermatozoa. Therefore the offspring of male mice carrying the PC3 Cre transgene had different recombination events within the targeted *Mll* allele. As there are three LoxP sites in the targeted allele, three different recombination events can occur. These are termed Type I, Type II and Type III (Figure 4.1) (Cohen-Tannoudji and Babinet, 1998). These different recombination events successfully occurred in male chimeras carrying the targeted *Mll* allele, allowing the implementation of breeding strategies to propagate the different *Mll* alleles.

When the deleted *Mll* allele, lacking exons 9 and 10 was bred to homozygosity, it was embryonic lethal. Lysates prepared from embryos homozygous for the deleted allele showed an absence of MLL protein, indicating that a true knockout model of *Mll* had been established.

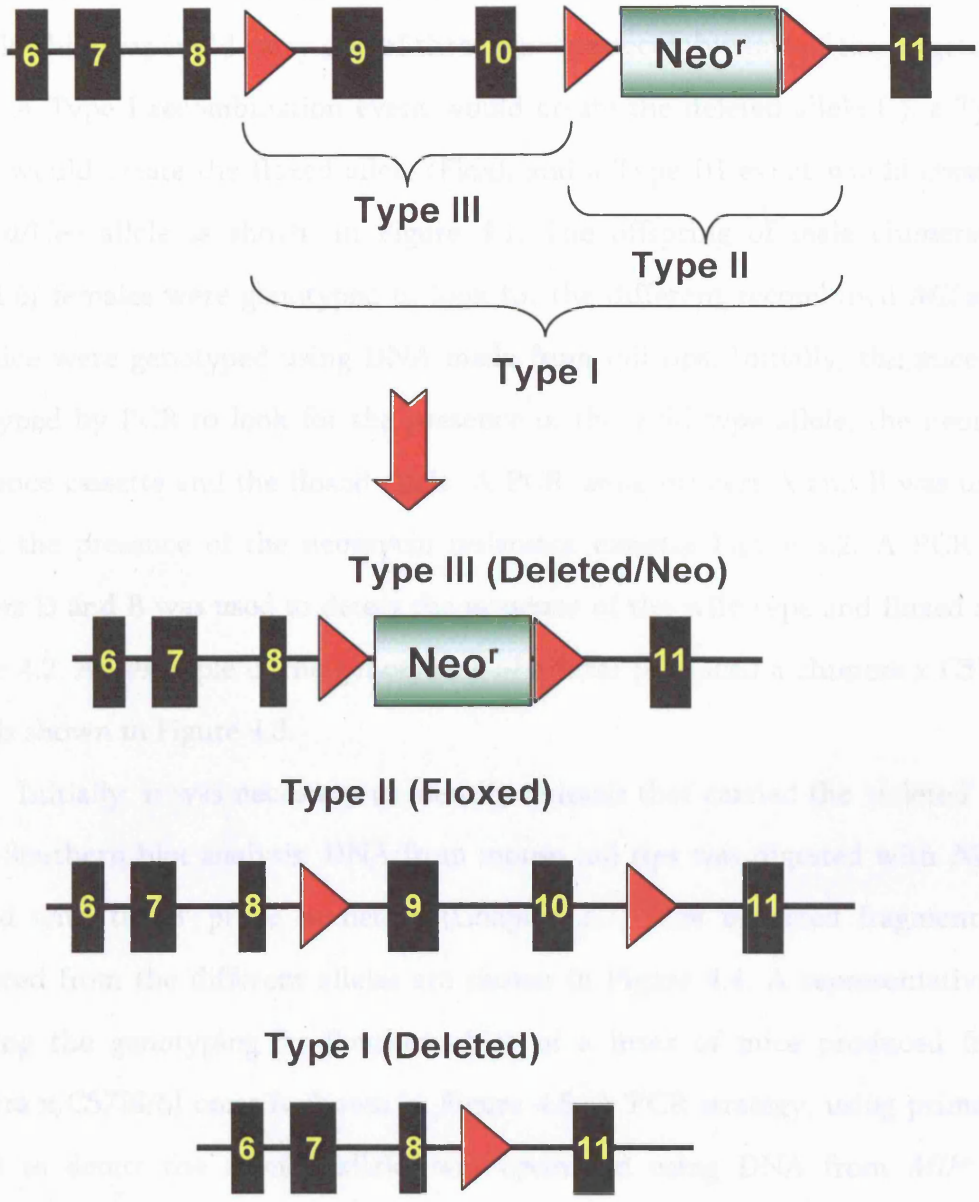

Figure 4.1. The different Mll alleles.
The different alleles that result from Cre-mediated recombination of the targeted allele.
The Cre recombinase recombines the LoxP sites (shown as red triangles). Black boxes denote exons, Neo^r denotes the neomycin resistance gene.

4.2 Recombination by the PC3-Cre recombinase occurred in the germline of male chimaeras

Due to PC3 Cre mediated recombination in the male germline, the offspring of male chimeras could carry one of three possible recombinants of the targeted *Mll* allele. A Type I recombination event would create the deleted allele (-), a Type II event would create the floxed allele (Flox), and a Type III event would create the deleted/Neo allele as shown in Figure 4.1. The offspring of male chimeras and C57Bl/6J females were genotyped to look for the different recombined *Mll* alleles. All mice were genotyped using DNA made from tail tips. Initially, the mice were genotyped by PCR to look for the presence of the wild type allele, the neomycin resistance cassette and the floxed allele. A PCR using primers A and B was used to detect the presence of the neomycin resistance cassette Figure 4.2. A PCR using primers D and B was used to detect the presence of the wild type and floxed alleles Figure 4.2. An example of the genotyping of a litter produced a chimera x C57Bl/6J cross is shown in Figure 4.3.

Initially, it was necessary to identify animals that carried the 'deleted' allele using Southern blot analysis. DNA from mouse tail tips was digested with *Nsi*1and probed with the 3' probe as before (Chapter 3.7). The expected fragment sizes produced from the different alleles are shown in Figure 4.4. A representative blot showing the genotyping by Southern blot of a litter of mice produced from a chimera x C57Bl/6J cross is shown in Figure 4.5. A PCR strategy, using primers E2 and B to detect the deleted allele, was optimised using DNA from $Mll^{+/-}$ mice identified in southern blot screening (Figure 4.2). As shown in Figure 4.6, the PCR using E2 and B was optimised to successfully detect the 'deleted' allele.

Recombination in the male germline of the chimeras had successfully produced $Mll^{+/-}$ and $Mll^{+/Flox}$ mice, which could be used to produce constitutive and conditional knockout *Mll* mice.

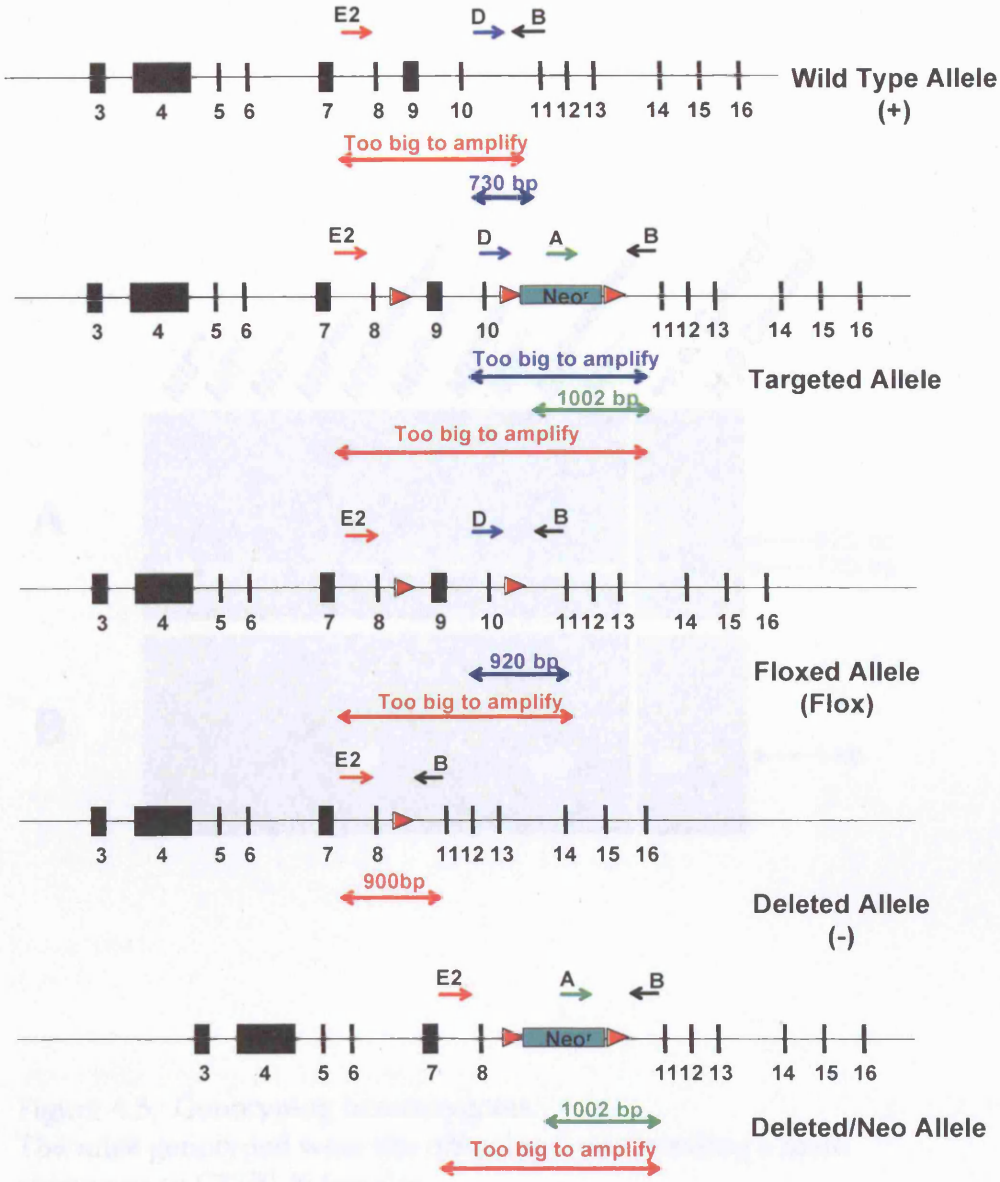

Figure 4.2. Original PCR strategy employed for genotyping the offspring from chimeras.
Primers A and B were used to detect the presence of the neomycin resistance cassette.
Primers D and B were used to detect the presence of the floxed and wild type alleles.
Primers E2 and B were used to detect the presence of the deleted allele.

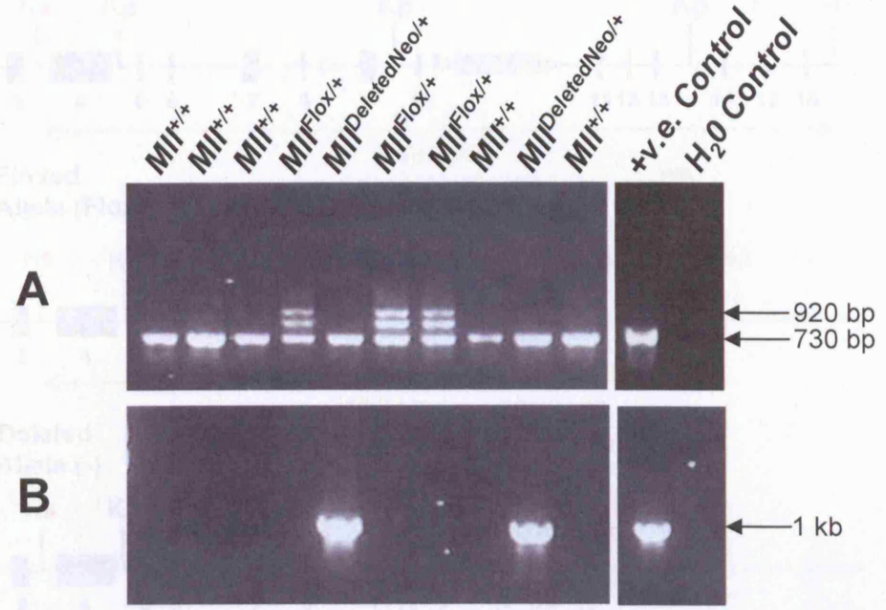

Figure 4.3. Genotyping heterozygotes.
The mice genotyped were the offspring from breeding a male chimaeras to C57BL/6 females.
A) PCR with primers Dnew and Bnew to identify mice carrying a wildtype or floxed allele. Wild type band – 730 bp, floxed band 920 bp. The third band present in PCRs for the floxed allele is a hybrid band where the floxed PCR product has bound to the WT PCR product. It disappeared upon denaturation of the DNA.
B) PCR with primers Anew and Bnew to identify mice carrying either the targeted allele or the deleted/neo allele. The band amplified from the Targeted and deleted/neo alleles was 1 kb.

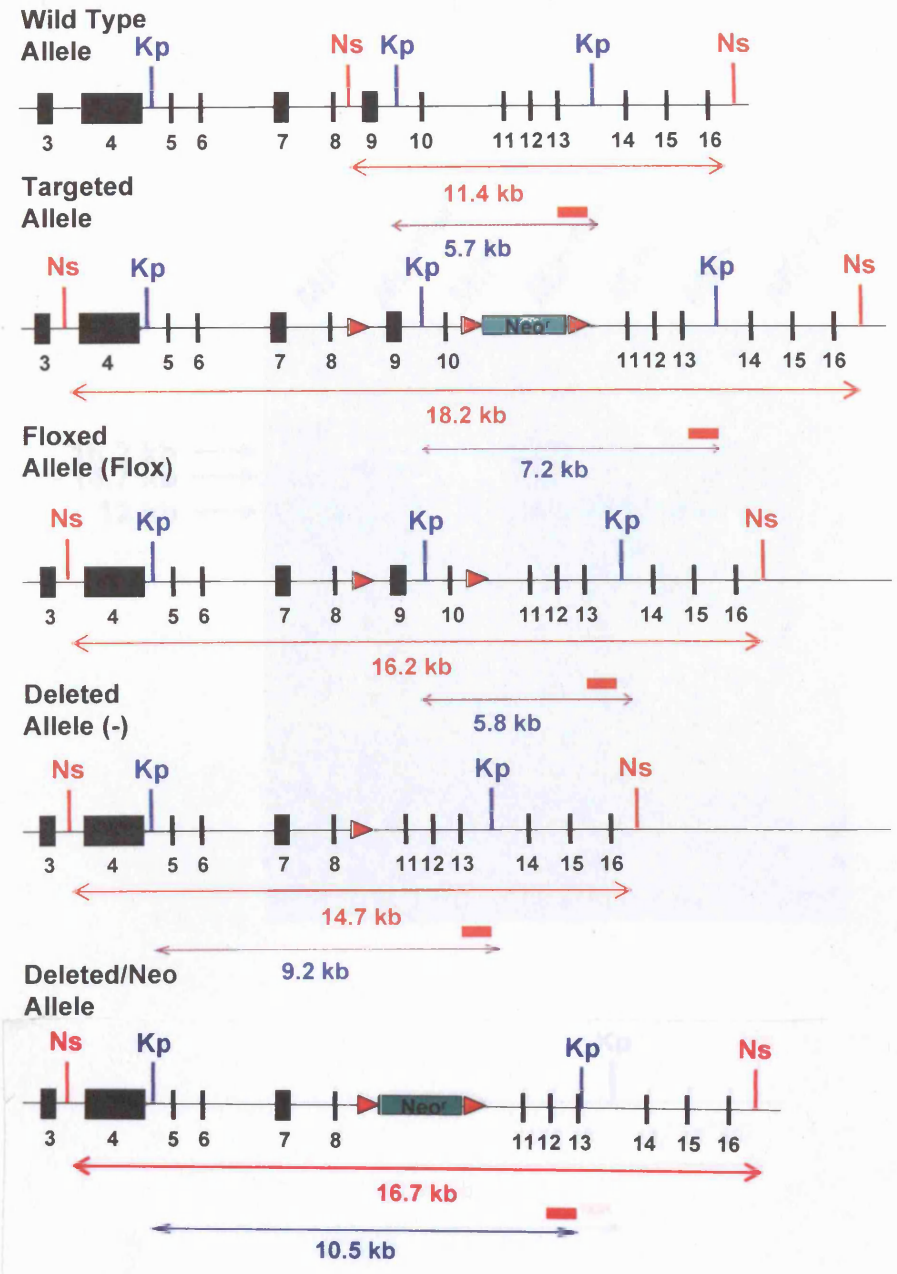

Figure 4.4. *Nsi*1 and *Kpn*1 Southern Blot Strategies for detecting the different *Mll* alleles.
Ns denotes *Nsi*1 sites and Kp denotes *Kpn*1 sites, the filled red box denoted the 3' probe.

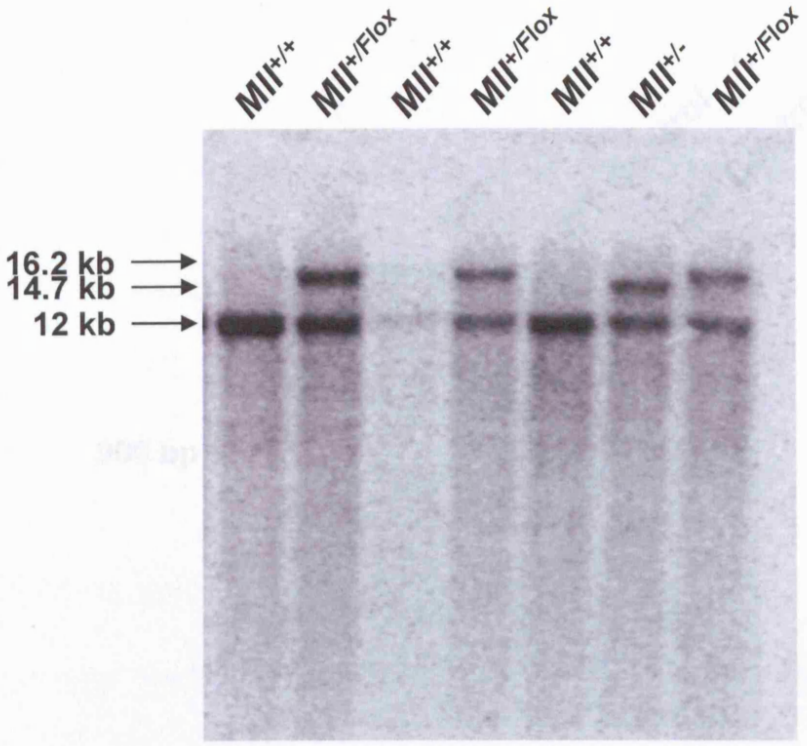

Figure 4.5. Genotyping by Southern blot of a litter of mice produced from a ♂chimera x ♀C57BL/6J cross.
DNA was digested with *Nsi*I, as in Figure 4.4. The floxed (Flox) band was 16.2 Kb, the deleted (-) band was 14.7 Kb and the wild type band was 12 Kb

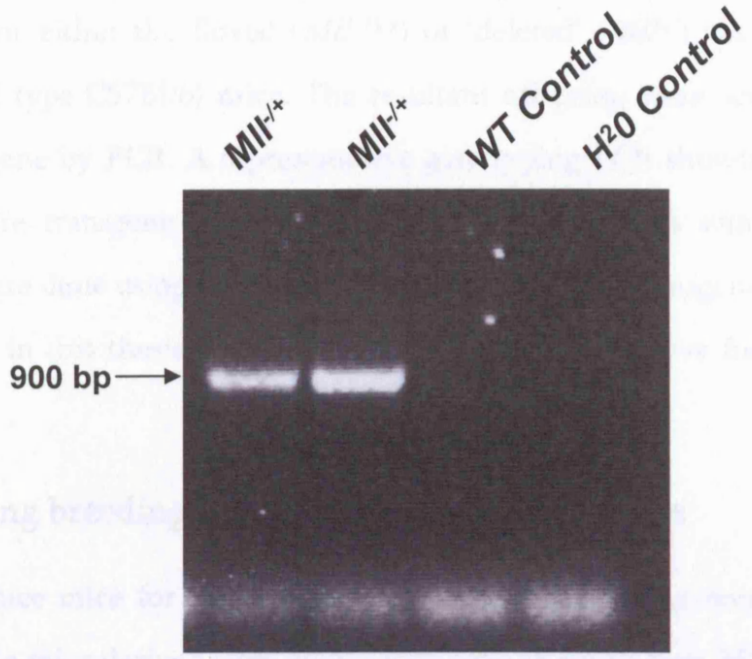

Figure 4.6 Optimisation of PCR using primers E and B to amplify the 'Deleted' allele.
The genomic DNA was had previously been genotyped by Southern blot. The expected band for the deleted allele was 900 bp.

4.3 Removal of PC3-Cre transgene

The PC3 ES cells used here were homozygous for the PC3 Cre transgene (O'Gorman et al., 1997). Hence all mice bred from chimeras where the PC3 ES cells had successfully contributed to the germline were heterozygous for the PC3 Cre transgene. Before establishing breeding strategies to produce experimental mice, it was necessary to remove the PC3 Cre transgene. Offspring of the chimeras heterozygous for either the floxed ($Mll^{+/Flox}$) or 'deleted' ($Mll^{+/-}$) alleles were back crossed to wild type C57Bl/6J mice. The resultant offspring were screened for the PC3 Cre transgene by PCR. A representative genotyping PCR showing genotyping for the PC3 Cre transgene is shown in Figure 4.7. Although some preliminary experiments were done using mice heterozygous for the PC3 transgene, the majority of experiments in this thesis were performed using mice negative for the PC3 Cre transgene.

4.4 Establishing breeding colonies for the two mice lines

To produce mice for experiments, two breeding colonies were established, one to propagate mice derived from Mll9.21 ES cells and one from Mll3.37 ES cells. $Mll^{-/+}$ and $Mll^{Flox/+}$ mice from both *Mll* strains were backcrossed to C57Bl/6J mice to begin the process of producing congenic strains. Breeding pairs were also established to produce mice conditional for *Mll* in the haematopoietic system – this breeding strategy is described in Chapter 6.

4.5 Failure of original genotyping PCRs

The initial PCR srategies used to detect the different *Mll* alleles were slightly unreliable in that they often failed due to undiagnosed reasons. For this reason, it was necessary to establish a new genotyping strategy. Primers E2 and F replaced primers D and B to detect the wild type and floxed alleles, and primers E2 and G2 replaced primers E2 and B to detect the deleted allele. The new PCR strategy and the band sizes expected are shown in Figure 4.8. This strategy proved to be more reliable.

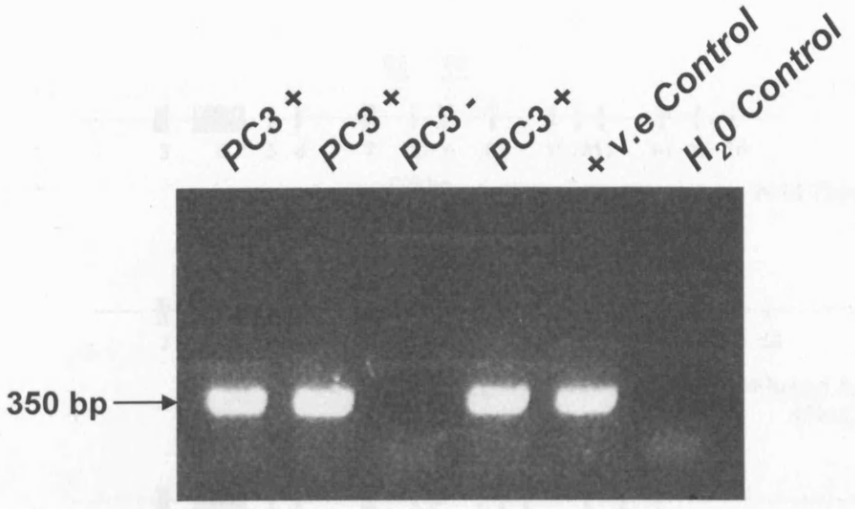

Figure 4.7. Removal of the PC3 Cre transgene from the mouse lines. The figure shows the genotyping for a litter of mice from a *Mll*$^{flox/+}$ X C57Bl/6J cross. The positive control was genomic DNA from PC3 ES cells, which were homozygous for the PC3 transgene. The expected band size was 350 bp

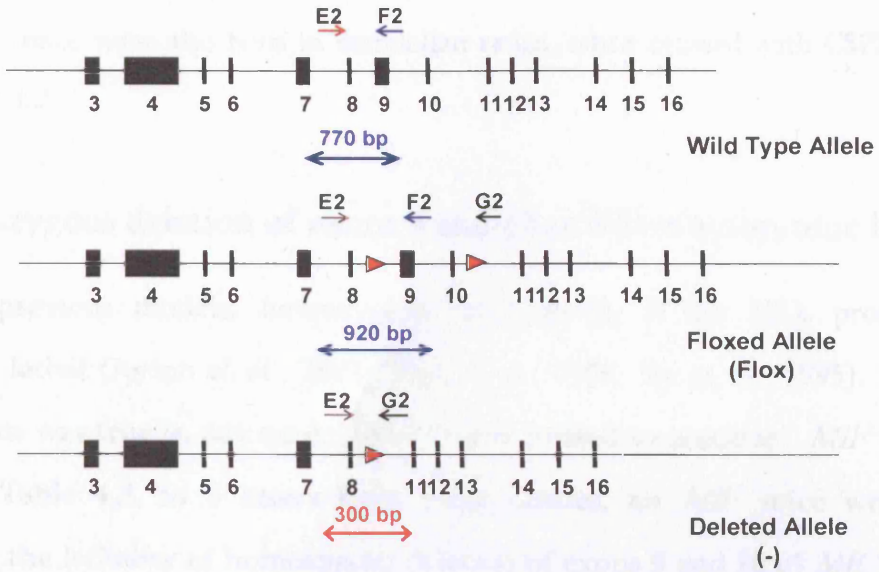

Figure 4.8. New PCR Strategy for genotyping the different *Mll* alleles.
Using Primers E2 and F, the band sizes are : Wild type – 730 bp, floxed – 920 bp.
Using primers E2 and G2, the deleted band is 300 bp. A band of 1.8 kb is also amplified from the floxed allele by E2 and G2, but is not reliably amplified.

4.6 Mll$^{Flox/+}$ and Mll$^{-/+}$ mice have no apparent phenotypic abnormalities

It was important to establish that mice carrying the floxed allele had a normal phenotype to ensure that targeting introns 8-9 and 10-11 of *Mll* had no effect on its function. *Mll$^{Flox/Flox}$* mice at 8-10 weeks of age were of normal weight (23.9 ± 2.3 for males, 19.4 ± 1.7 for females) compared to wild type mice (22.7 ± 2.2 for males, 20.9 ± 2.3 for females) and had no obvious anatomical abnormalities. *Mll$^{Flox/+}$* mice were of normal fertility, and were born in the expected mendelian ratios Table 4.1. The same was true of *Mllflox/flox* mice.

Mll$^{-/+}$ mice were also born in mendelian ratios, when crossed with C57Bl/6J mice (Table 4.2).

4.7 Homozygous deletion of exons 9 and 10 of Mll is embryonic lethal

In previous models, homozygous interruption of the MLL protein was embryonic lethal (Ayton et al., 2001; Yagi et al., 1998; Yu et al., 1995). To assess whether this was true in our model, *Mll$^{-/+}$* were crossed to produce *Mll$^{-/-}$* mice. As shown in Table 4.3, in 5 litters from these crosses, no *Mll$^{-/-}$* mice were born, confirming the lethality of homozygous deletion of exons 9 and 10 of *Mll*. To assess at which point of development *Mll$^{-/-}$* embryos died, timed matings were set up using *Mll$^{-/+}$* mice. As shown in Table 4.3, normal numbers of *Mll$^{-/-}$* embryos were present up to E12.5. Figure 4.9 shows a representive PCR for a litter of E12.5 embryos, showing the presence of an *Mll$^{-/-}$* embryo. After this point, the numbers of *Mll$^{-/-}$* embryos were gradually reduced, with the biggest drop occurring between E12.5 and E14.5. At these timepoints, dead *Mll$^{-/-}$* embryos were frequently seen (data not shown). Live *Mll$^{-/-}$* embryos frequently showed oedema and petechiae under the skin (data not shown). From E15.5 onwards, live *Mll$^{-/-}$* embryos were seen, but they were increasingly rare, only 2 live *Mll$^{-/-}$* embryos were seen from five litters.

4.8 Mll$^{-/-}$ embryos have no detectable MLL protein

The embryonic lethality seen in *Mll$^{-/-}$* embryos suggested that either the 1st PHD finger was integral to the function of MLL or that the levels of MLL were

Mll3.37			
Genotype	Males	Females	Total
Mll$^{+/flox}$	42%	48%	46% (n=22)
Mll$^{+/+}$	19%	25.9%	23% (n=13)
Mll$^{flox/flox}$	38%	25.9%	31% (n=15)

Mll9.21			
Genotype	Males	Females	Total
Mll$^{+/Flox}$	60%	42.9%	56% (n=15)
Mll$^{+/+}$	15%	25%	22% (n=6)
Mll$^{Flox/Flox}$	25%	14%	22% (n=6)

Table 4.1. *Mll*$^{flox/+}$ and *Mll*$^{+/+}$ mice are born in mendelian ratios.
Offspring from *Mll*$^{Flox/+}$ x *Mll*$^{Flox/+}$
Expected ratios are : *Mll*$^{Flox/+}$ 50%, *Mll*$^{+/+}$ 25%, *Mll*$^{Flox/Flox}$ 25%

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Mll9.21			
Genotype	Males	Females	Total
Mll$^{+/-}$	45.5%	43.9%	43% (n=54)
Mll$^{+/+}$	54.5%	56.1%	57% (n=73)

Mll3.37			
Genotype	Males	Females	Total
Mll$^{+/-}$	43.5%	41.4%	45% (n=50)
Mll$^{+/+}$	56.5%	58.6%	55% (n=62)

Table 4.2. *Mll*$^{+/-}$ and *Mll*$^{+/+}$ mice are born in mendelian ratios
Results of breeding pairs with *Mll*$^{-/+}$ x WT C57Bl/6J. Expected ratios are : *Mll*$^{+/-}$ 50%, *Mll*$^{+/+}$ 50%

	Percentage of live Embryos		
	Mll$^{+/+}$	*Mll*$^{+/-}$	*Mll*$^{-/-}$
E10.5 (n=19)	10.5%	47%	42%
E11.5 (n=16)	18%	62%	18%
E12.5 (n=28)	14.2%	50%	35.7%
E13.5 (n=35)	31%	48.9%	20%
E14.5 (n=51)	31%	54.9%	13.7%
E15.5 (n=11)	27%	63.9%	9%
E16.5 (n=9)	22%	66.7%	11%
Postnatal (n=38)	52.6%	47.4%	0%

Table 4.3. Embryonic lethality in *Mll*$^{-/-}$ embryos.
Expected Mendelian ratios were: *Mll*$^{+/-}$ 50%, *Mll*$^{+/+}$ 25%, *Mll*$^{-/-}$ 25%.
The table shows data pooled from Mll3.37 and Mll9.21 strains.
At earlier timepoints (E10.5, E11.5), live embryos were considered to be those that had a heartbeat. At later timepoints, live embryos were those that showed blood in the umbilical chord and veins.

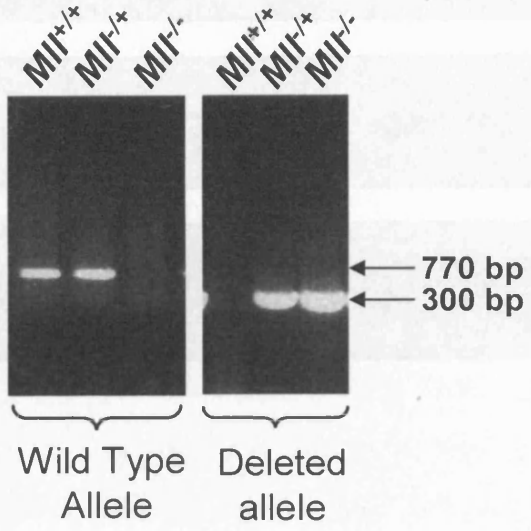


Figure 4.9. Genotyping of a litter of embryos from a *Mll*$^{+/-}$ x *Mll*$^{+/-}$ timed mating.
The wild type allele was detected using primers E2 and F and the band size was 770 bp.
The deleted allele was detected using primers E2 and G2 and the band size was 300 bp.

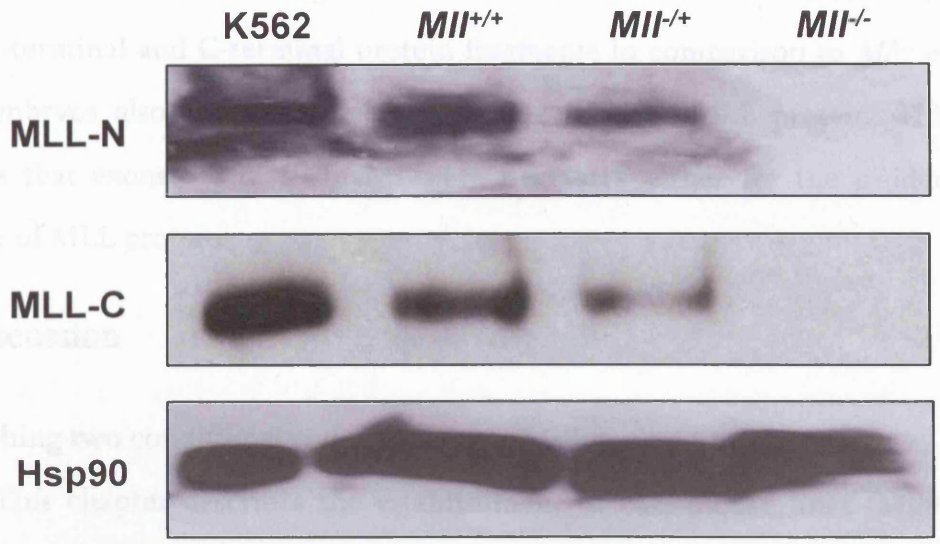


Figure 4.10. *Mll-/-* embryos show an absence of MLL protein.
Lysates made from foetal liver cells from *Mll*$^{+/+}$, *Mll*$^{-/+}$ and *Mll*$^{-/-}$ embryos were run alongside lysates made from K562 cells (positive control). The blots were probed with either and anti-MLL N-terminal antibody (MLL-N) or and anti-MLL C-terminal antibody (MLL-C). The HSP90 antibody was used as a loading control.

decreased. To ascertain the status of the MLL protein the absence of exons 9 and 10, protein lysates were made from the foetal liver cells of E13.5 *Mll*$^{+/+}$, *Mll*$^{+/-}$ and *Mll*$^{-/-}$ embryos. This analysis was done in collaboration with Samantha Y-L Hiew. Western blotting was performed with the lysates using antibodies to detect the N-terminal the C-terminal MLL fragments. The binding sites for the antibodies are shown in Figure 2.1. The HSP90 antibody was used to ensure equal loading of the protein lysates. As can be seen in Figure 4.10, *Mll*$^{-/-}$ embryos showed an absence of both MLL N-terminal and C-terminal protein fragments in comparison to *Mll*$^{+/+}$ embryos. *Mll*$^{+/-}$ embryos also showed a decrease in the level of MLL protein. This result suggests that exons 9 and 10 of MLL are necessary either for the production or stability of MLL protein.

4.9 Discussion

Establishing two conditional and constitutive *Mll* knockout mouse lines

This chapter describes the establishment of two mouse lines (Mll9.21 and Mll3.37) carrying a conditional and a constitutive knockout allele of *Mll*. When chimeras derived from *Mll* targeted ES cells were bred to C57BL/6J mice they produced offspring heterozygous for the targeted *Mll* allele, showing that the ES cells had successfully contributed to the germline of the chimeras. As expected, the offspring of the chimeras carried different recombinants of the targeted allele, showing that all the LoxP sites were functional and that the PC3 Cre was inducing recombination during spermatogenesis (O'Gorman et al., 1997). This also meant that mice were born from both lines heterozygous for the floxed and deleted alleles of *Mll*. These heterozygous mice were backcrossed to the C57Bl/6J to breed mice heterozygous for the *Mll* alleles but negative for the PC3 Cre transgene. Although the PC3 Cre transgene has not been shown to cause any phenotype, there is some very low level ectopic expression of the transgene in the brain (O'Gorman et al., 1997). In addition, male mice carrying the floxed allele would undergo recombination during spermatogenesis, which would affect the breeding strategies.

For these reasons, it was necessary to remove the PC3-Cre transgene by backcrossing the mouse lines to wild type C57BL/6J mice.

Establishment of breeding colonies to produce mice for experiments

Breeding colonies for each of the lines have been established to propagate the floxed and deleted alleles, and to begin the creation of congenic lines. The chimeras produced from the PC3 ES cells (which were derived from mice with a 129sv background) were bred to C57Bl/6J WT females. An important reason for doing this was that the C57Bl background has been used as a model in many studies of murine haematopoiesis (Gekas et al., 2005; Morrison et al., 1995; Spangrude et al., 1995; Worton et al., 1969). This is also true of previous *Mll* knockout studies (Ayton et al., 2001; Yagi et al., 1998). However, as the original ES cells used here were derived from a 129sv background, this meant that the mice used for experiments in this work were of a mixed C57Bl/6 and 129sv background. For this reason, in all experiments littermates were used as controls.

$Mll^{+/Flox}$ and $Mll^{-/+}$ mice have no apparent phenotypic abnormalities

As described, $Mll^{Flox/Flox}$ mice had no apparent abnormalities in terms of weight and fertility. More importantly, breeding the floxed allele to homozygosity was not embryonic lethal. This would suggest that targeting introns 8-9 and 10-11 of *Mll* has no obvious effect on the function of the gene. $Mll^{+/-}$ mice in our model had no apparent abnormalities in terms of fertility and weight. In the first *Mll* mouse knockout model, heterozygous females were hypofertile and heterozygotes of both sexes had retarded growth and reduced weight (Yu et al., 1995). However, as in the new model, in another *Mll* knockout model, heterozygotes were of normal weight and fertility (Ayton et al., 2001).

The new model represents a true knockout model of Mll

Although $Mll^{+/-}$ mice were apparently normal, $Mll^{-/-}$ mice were not detected after birth. The cause of death was not known. This showed that exons 9 and 10 were needed for MLL function. This theory was supported by Western blots using lysates made from foetal liver cells which showed a total absence of MLL protein in

Mll$^{-/-}$ embryos. Why would deletion of exons 9 and 10 of the gene lead to a complete loss of MLL protein? As shown in Figures 3.2 , two cysteine residues belonging to one of the putative zinc binding sites of PHD finger 1 of MLL are removed following deletion of exons 9 and 10 (Aasland et al., 1995). It is possible that the removal of these residues and probable loss of zinc binding in this location may have gross consequences for protein folding and maturation. This may lead then to degradation of the protein.

No previous knockout model of *Mll* has been directly shown to result in an absence of MLL protein in the resulting embryos (Ayton et al., 2001; Yagi et al., 1998; Yu et al., 1995). This means that our new model is the first proven true knockout of *Mll*. The phenotype of *Mll*$^{-/-}$ embryos in our model most closely resembled that of *Mll*$^{-/-}$ embryos in Yagi *et al.*, 1998, where *Mll* is truncated at exon 11 . When *Mll* is truncated at exon 11 , homozygous embryos die between E11.5 and E14.5 and show oedema and bleeding under the skin. This closely resembles the phenotype seen in *Mll*$^{-/-}$ embryos lacking exons 9 and 10. However, in the new model, lethality in *Mll*$^{-/-}$ embryos occurs at a slightly later timepoint. The reason for this is not clear, although it may be attributable to differences in strain background.

In three previous *Mll* knockout models, *Mll*$^{-/-}$ embryos all exhibited different phenotypes, depending at which point *Mll* was truncated at. The phenotype of the embryos also varied greatly. In the model by Ayton *et al.* from 2001, the embryos died before the implantation stage (Ayton et al., 2001; Yagi et al., 1998). In the study of Yu *et al.* the embryos died at E10.5 and showed severe branchial arch abnormalities (Yu et al., 1998). No such abnormalities were noted in the mice of Yagi *et al.* or in our mice (Yagi et al., 1998). The reasons for these differences in phenotype are not clear. It is possible that in the model of Yu *et al.*, a dominant negative was created. In this targeting, a *LacZ* cassette was inserted into exon 3 of *Mll* creating a fusion protein, which is detectable by immunohistochemistry (Scharf et al., 2007). This may account for the increased severity of phenotype in embryos homozygous for this fusion.

Mice carrying the floxed allele and the deleted allele can be used to study hematopoiesis in the absence of Mll

Mouse lines carrying deleted and floxed alleles of *Mll* were successfully established. The later lethality in *Mll*$^{-/-}$ embryos in this model compared to previous models meant that fetal liver hematopoiesis in the absence of *Mll* could be more extensively studied. The floxed allele was used to produce adult mice conditional for *Mll* as discussed in Chapter 6.

Chapter 5

Mll$^{-/-}$ Embryos Have Abnormal Foetal Liver Haematopoiesis

5.1 Introduction

It has been shown previously that *Mll* deficient embryos exhibit abnormalities in yolk sac and foetal liver haematopoiesis. In *Mll*$^{-/-}$ embryos these organs are reduced in cellularity and their cells have reduced colony-forming capacity (Hess et al., 1997; Yagi et al., 1998). Cells from the AGM (aorta-gonad-mesonephros) region of *Mll* deficient embryos are also unable to repopulate the bone marrow of irradiated mice, in comparison to WT controls (Ernst et al., 2004a). These studies all suggest that *Mll* is needed in a cell intrinsic manner for the proper progression of foetal haematopoiesis.

In previous mouse models of *Mll* deletion, the early lethality of homozygous truncation of the gene has precluded a more thorough study of the role of *Mll* in foetal haematopoiesis. In the model presented here, increased numbers of *Mll*$^{-/-}$ embryos at E12.5, E13.5 and E14.5 allowed further investigation into definitive haematopoiesis in the absence of *Mll*. These investigations have shown that whilst stem and progenitor cells are present in *Mll*$^{-/-}$ embryos, they are much reduced in number and do not have normal stem cell function in comparison to wild type cells.

5.2 Mll$^{-/-}$ embryos have reduced Foetal Liver Cellularity

To analyse foetal liver haematopoiesis in *Mll*$^{-/-}$ embryos in our mice, timed matings were set up between *Mll*$^{+/+}$ males and females. The females were taken at E13.5 and E14.5. At these time points, the foetal liver of *Mll*$^{-/-}$ embryos was visibly smaller before extraction (data not shown). To gain an accurate measurement of the reduction in foetal liver size, foetal livers were removed from *Mll*$^{-/-}$, *Mll*$^{+/-}$ and *Mll*$^{+/+}$

E13.5 (n=5-11) and E14.5 (n= 6-8) embryos. Single cell suspensions were made and viable cell numbers obtained for each foetal liver.

Total foetal liver cellularity was reduced 2-fold in *Mll*$^{-/-}$ embryos compared to *Mll*$^{+/+}$ embryos (Figure 5.1). However, a 5-fold expansion in foetal liver size was seen in both *Mll*$^{-/-}$ and *Mll*$^{+/+}$ embryos between E13.5 and E14.5. This suggests that the haematopoietic cells that are present in *Mll*$^{-/-}$ foetal livers are able to undergo some degree of expansion.

5.3 Myeloid Progenitors are reduced in Mll$^{-/-}$ embryos

The reduced cellularity of the foetal liver in *Mll*$^{-/-}$ embryos, suggested that *Mll*$^{-/-}$ embryos in our model might possess a foetal liver phenotype similar to that seen before in other *Mll* mouse models (Hess et al., 1997; Yagi et al., 1998).To assess this, the methylcellulose colony forming assays performed by Yagi *et al.* 1998 were replicated to assess the ability of *Mll*$^{-/-}$ foetal liver cells to form myeloid colonies. Cells from E12.5 foetal livers from *Mll*$^{-/-}$, *Mll*$^{+/-}$ and *Mll*$^{+/+}$ embryos were plated into methylcellulose with cytokines to induce myeloid colony growth (IL-3, IL-6, SCF, GM-CSF). After 7 days the colonies were counted and scored for the presence of Colony-Forming Unit Granulocyte, Macrophage (CFU-GM), Burst-Forming Unit Erythroid (BFU-E) and CFU- Granulocyte, Erythrocyte, Macrophage, Megakaryocyte (CFU-GEMM).

After 7 days in culture, the total number of myeloid CFUs was reduced 4-fold in cultures from *Mll*$^{-/-}$ foetal liver cells compared to *Mll*$^{+/+}$ cells (Figure 5.2). Cell numbers in *Mll*$^{-/-}$ cultures were reduced by 20 fold (Figure 5.2). Although there was a reduction in the total CFU number, there was no difference in the distribution of the different colony types between *Mll*$^{-/-}$ and *Mll*$^{+/+}$ cultures (Figure 5.3). Many of the colonies in *Mll*$^{-/-}$ cultures were smaller than in *Mll*$^{+/+}$ cultures, however, large colonies comparable to wild type colonies were also seen (Figure 5.4). The morphology of colonies in *Mll*$^{-/-}$ cultures also appeared normal (Figure 5.4). Cytospins of cells from *Mll*$^{-/-}$ cultures showed maturing macrophages similar to those seen in *Mll*$^{+/+}$ cultures (Figure 5.5). The normal distribution and appearance of the colonies would suggest that the defect seen in *Mll*$^{-/-}$ cells lies in an early progenitor

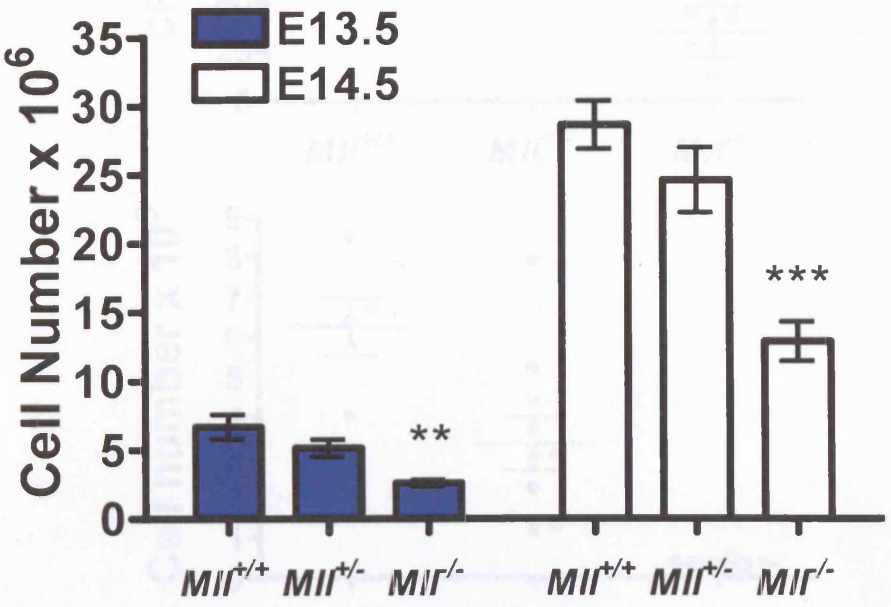


Figure 5.1. Foetal Liver cellularity is reduced in *Mll*$^{-/-}$ embryos.
Expansion between E13.5 and E14.5 in *Mll* $^{+/+}$, *Mll*$^{+/-}$ and *Mll*$^{-/-}$ fetal livers. Single cell suspensions were prepared from individual fetal livers and viable counts performed. *P* values were calculated using 1Way ANOVA, with Tukeys multiple comparison test. (***) $P \leq 0.001$, (**) $P \leq 0.01$, (*) $P \leq 0.05$. All samples were compared to *Mll*$^{+/+}$. The graph shows the mean (n=5-11) and standard error of the mean of cell number in the fetal liver.

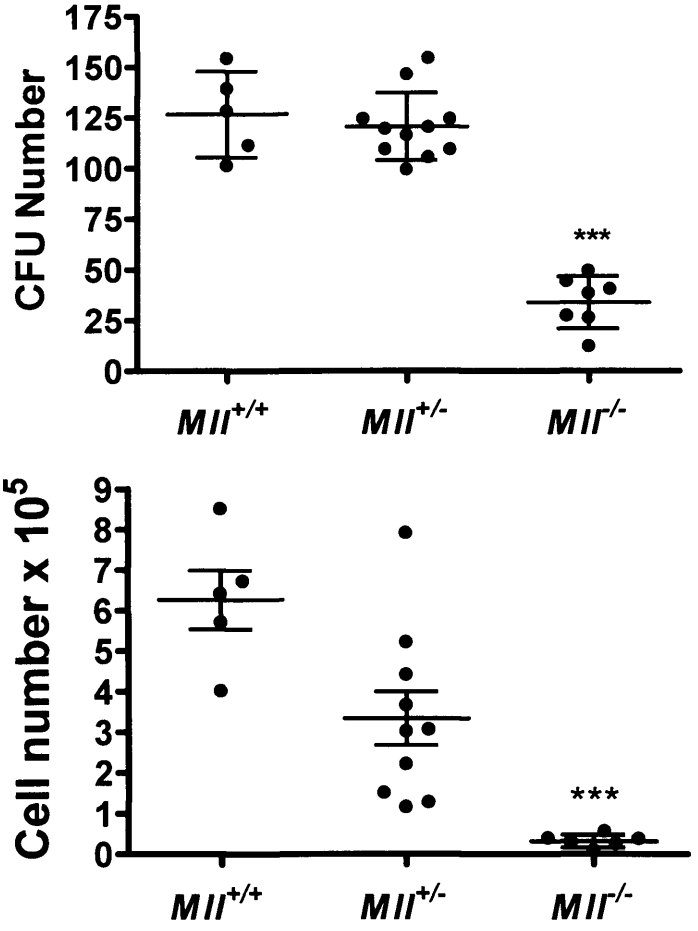


Figure 5.2 Reduction in Myeloid CFU number from *Mll*$^{-/-}$ foetal livers.
A. Myeloid CFU counts for E12.5 fetal liver cells. Following the removal of erythrocytes, 1.1 x 10^4 fetal liver cells were plated in methylcellulose and CFUs were counted after 7 days.
B. The number of cells present in CFU cultures from E12.5 fetal livers. 1.1 x 10^4 cells were cultured as above and after 7 days, single cell suspensions were made and viable counts performed.
P values were calculated using 1Way ANOVA, with Tukeys multiple comparison test. (***) $P \leq 0.001$, (**) $P \leq 0.01$, (*) $P \leq 0.05$. All samples were compared to *Mll WT.* The bars show the mean and standard error of the mean.

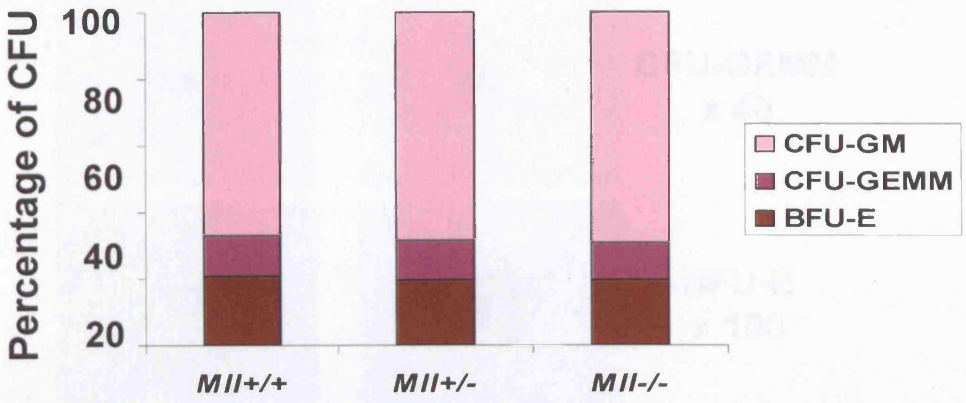


Figure 5.3 The proportions of different myeloid colony types are unchanged in *Mll*$^{-/-}$ embryos.
Proportion of different colony types produced by *Mll*$^{+/+}$, *Mll*$^{+/-}$ and *Mll*$^{-/-}$ E12.5 fetal liver cells after 7 days culture in myeloid methylcellulose. CFU-GM = Colony-Forming Unit Granulocyte, Macrophage. CFU-GEMM = Colony-Forming Unit Granulocyte, Erythroid, Macrophage, Megakaryocyte. BFU-E = Burst Forming Unit Erythroid.

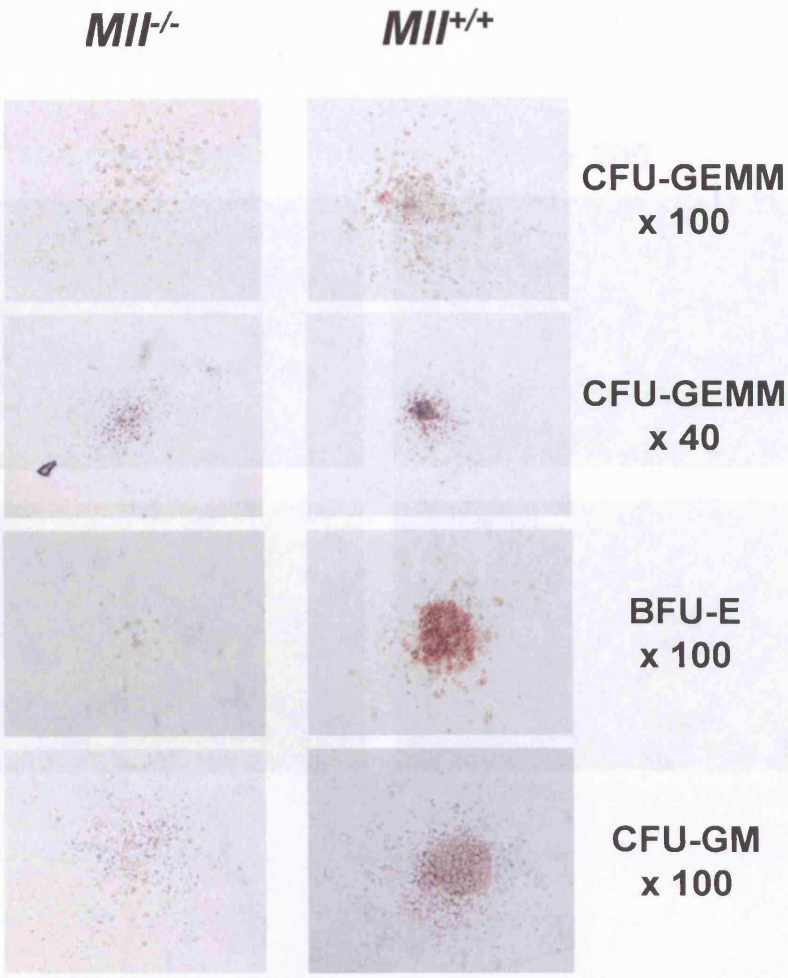


Figure 5.4 Colony morphology in *Mll*$^{-/-}$ cultures.
The photographs show individual colonies from *Mll*$^{-/-}$ and *Mll*$^{+/+}$ E12.5 foetal liver CFU assays at 7-9 days culture.
Photographs were taken at x 40 and x 100 magnification.

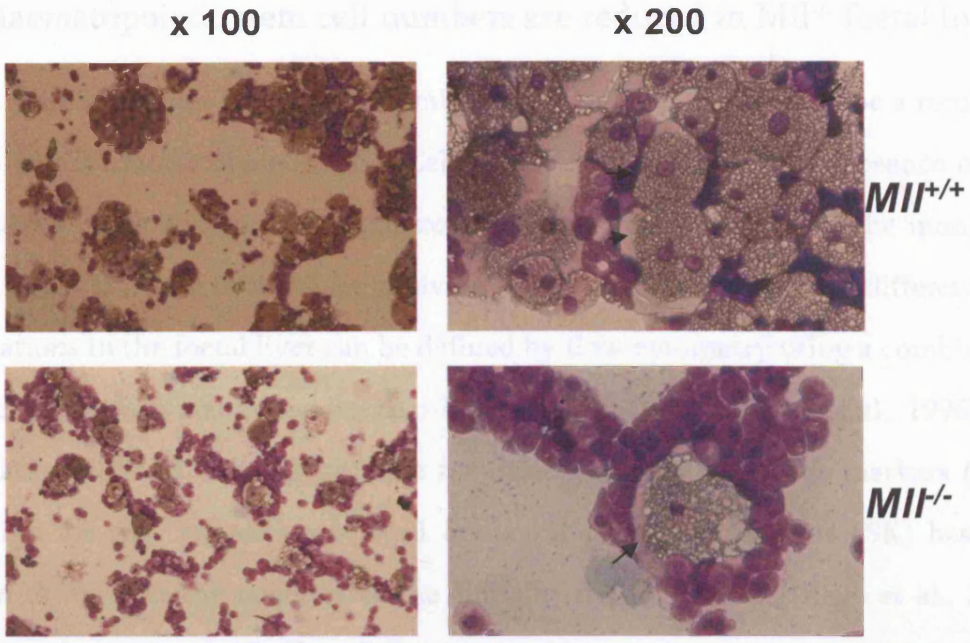


Figure 5.5. The morphology of cells produced in *Mll*$^{-/-}$ myeloid cultures.
The photographs show details from cytospins of fetal liver cells after 12 days in culture in methylcellulose M3434. The cells were stained with May-Grunwald (Giemsa). Photographs were taken at x 100 and x 200 magnification. The black arrows indicate maturing macrophages. The cytopins for both *Mll*$^{+/+}$ and *Mll*$^{-/-}$ cultures showed that the cultures were predominantly composed of maturing macrophages.

before the division between erythroid and other myeloid lineages. The presence of maturing macrophages in *Mll*$^{-/-}$ cultures would also suggest that those progenitors present are able to differentiate as normal. These results suggest a significant reduction in the number of early myeloid progenitors present in *Mll KO* foetal livers.

5.4 Haematopoietic stem cell numbers are reduced in Mll$^{-/-}$ foetal livers

The reduction in total cell number in *Mll*$^{-/-}$ foetal livers could be a result of a defect in the Haematopoietic Stem Cell (HSC) compartment in the absence of *Mll*. To assess this, flow cytometric analyzes were performed to examine the number of cells in the HSC pool in E13.5 foetal livers. It has been shown that the different HSC populations in the foetal liver can be defined by flow cytometry using a combination of lineage markers and antibodies to c-kit, Sca1 and CD38 (Randall et al., 1996). The population defined as being negative for the expression of lineage markers (Lin-), but high for the expression of Sca1 and c-kit (Lin$^-$Sca1$^+$c-kit^{hi} or LSK) has been shown to harbour the majority of the HSCs in the foetal liver (Reya et al., 2001). This population can be further defined by looking at the expression of the marker CD38. LSK foetal liver cells that express high levels of CD38 are highly enriched for Long Term HSCs (LT-HSCs), whilst those that express low levels of CD38 are enriched for Short Term HSCs (ST-HSCs) (Randall et al., 1996) Figure 1.2. Cells from the foetal livers of E13.5 *Mll*$^{-/-}$, *Mll*$^{+/-}$ and Mll$^{+/+}$ embryos were analysed by flow cytometry for the expression of these markers. Lineage depleted cells were stained with c-kit APC, Sca1 FITC, Streptavidin PrCP (to stain any remaining lineage negative cells), CD38 PE and 7AAD. The Lineage negative cells were gated for Sca1$^+$ cells, allowing the analysis of c-kit and CD38 expression in the Lin$^-$Sca1$^+$ population (Figure 5.6).

When the foetal liver stem cell compartment was analysed, a 2.5 fold reduction was seen in the number of LSK CD38^{lo} (ST-HSC) cells in *Mll*$^{-/-}$ foetal livers (mean = 1.5 ± 0.9 x 10^4 cells) compared to *Mll*$^{+/+}$ foetal livers (mean = 3.9 ± 1.3 x 10^4 cells) (Figure 5.7). A 5.7-fold reduction was seen in the number of LSK CD38^{hi} (LT-HSC) cells in *Mll*$^{-/-}$ foetal livers (mean = 0.78 ± 0.52 x 10^3 cells) compared to *Mll*$^{+/+}$

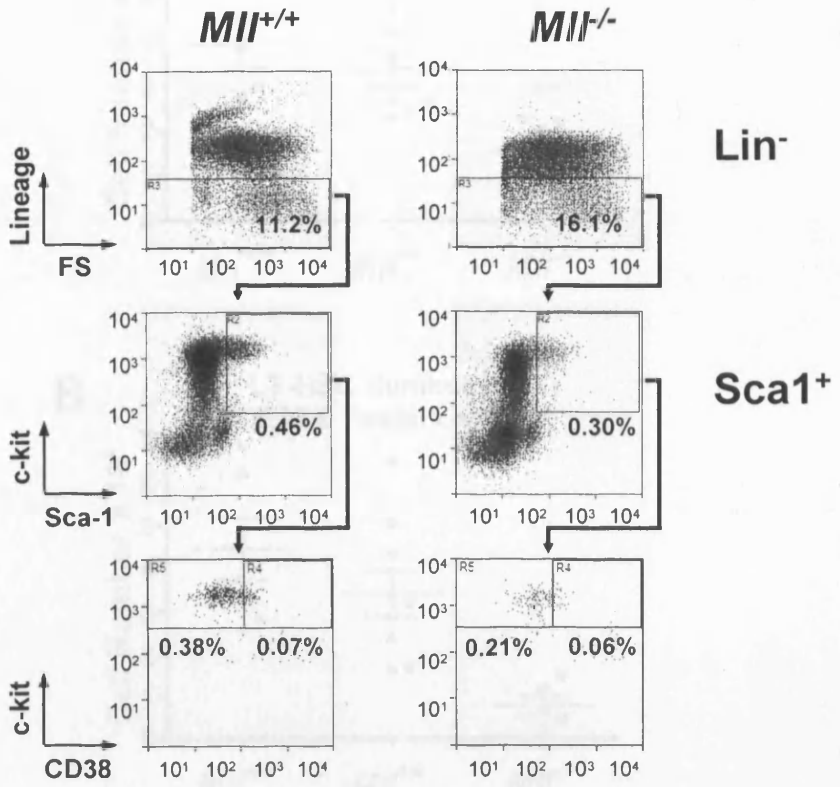


Figure 5.6. Identifying LT-HSCs and ST-HSCs. Cells from E13.5 fetal livers were depleted of lineage positive cells by magnetic sorting. A small sample was also stained with Lineage markers for flow cytometric analysis, to estimate the percentage of Lineage negative cells present in the fetal liver. The Lineage negative cells were stained with c-kit, Sca-1 and CD38, 7AAD and Streptavidin-PerCP. Any dead cells and Lineage$^+$ were gated out and Lin$^-$Sca1$^+$ cells were analyzed for the expression of c-kit and CD38.

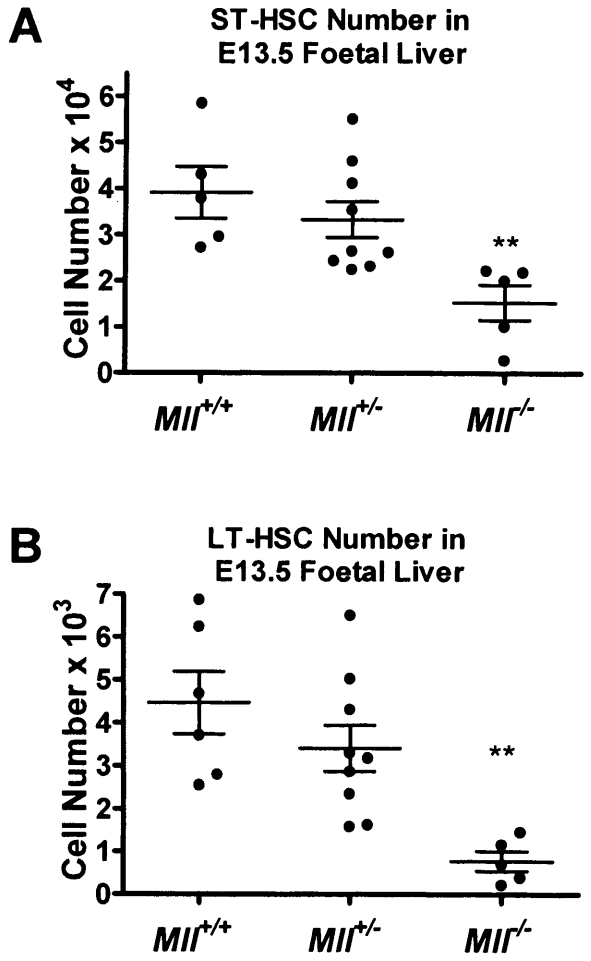


Figure 5.7. ST-HSC and LT-HSC Numbers in *Mll*$^{-/-}$ fetal livers.
Cells from E13.5 fetal livers were depleted of lineage positive cells by magnetic sorting. A small sample was also stained with Lineage markers for flow cytometric analysis, to estimate the percentage of Lineage negative cells present in the fetal liver. The Lineage negative cells were then stained with c-kit, Sca-1 and CD38, 7AAD and Streptavidin-PerCP. Any dead cells and Lineage+ were gated out and Lin-Sca1+ cells were analyzed for the expression of c-kit and CD38.
(*A*) Number of ST-HSCs present in E13.5 fetal livers. ST-HSCs were defined as Lin$^-$Sca1$^+$c-kit^{hi}CD38^{lo}.
(*B*) Number of LT-HSCs present in E13.5 fetal livers. LT-HSCs were defined as Lin$^-$Sca1$^+$c-kit^{hi}CD38^{hi}.
P values were calculated using 1Way ANOVA, with Tukeys multiple comparison test. (***) $P \leq 0.001$, (**) $P \leq 0.01$, (*) $P \leq 0.05$, ns = not significant. All samples were compared to *Mll*$^{+/+}$. The bars show the mean and standard error of the mean.

foetal livers (mean = 4.5 ± 1.8 x 10^3 cells (Figure 5.7). The percentage of LT-HSC as a proportion of the foetal liver was decreased 3-fold in *Mll*$^{-/-}$ foetal livers (mean = 0.019 ± 0.017%) compared to *Mll*$^{+/+}$ foetal livers (mean = 0.064 ± 0.031%) (Figure 5.8). However, the proportion of ST-HSC in *Mll*$^{-/-}$ foetal livers (mean = 0.54 ± 0.22%) was not significantly different to that seen in *Mll*$^{+/+}$ foetal livers (mean = 0.64 ± 0.26%) (Figure 5.8). The proportion of the foetal liver cells that were negative for lineage markers was also not significantly different in *Mll*$^{-/-}$ foetal livers (mean = 24.9 ± 7.5%) compared to *Mll*$^{+/+}$ foetal livers (mean = 23.8± 4.9%) (Figure 5.9). This data confirms a major loss in the number of HSCs present in foetal livers lacking *Mll*, suggesting that *Mll* is important for HSC development. The reduction in the proportion of LT-HSC present would suggest that these cells are the most severely affected by the absence of *Mll*.

5.5 Mll$^{-/-}$ foetal liver cells are unable to reconstitute the haematopoietic system

Although LSK CD38^{hi} cells were present in *Mll KO* foetal liver, it was not known whether these cells possessed normal stem cell function. To assess this, competitive repopulation assays were performed with c-kit$^+$Ter119$^-$ cells sorted from pooled, frozen foetal liver cells from E14.5 *Mll*$^{-/-}$ and *Mll*$^{+/+}$ embryos. 4 x 10^4 of the foetal liver donor cells (Ly5.2$^+$) were mixed with a radioprotective dose of 2 x 10^5 wild type congenic C57BL/6 Ly5.1$^+$ bone marrow cells and transplanted into lethally irradiated recipients. Analysis of blood taken from the recipients at 4 weeks post transplantation showed that *Mll*$^{+/+}$ cells reconstituted short term haematopoiesis efficiently, as measured by the percentage of Ly5.2$^+$ cells present in the periphery (Figure 5.10). By contrast, *Mll*$^{-/-}$cells were unable to give rise to any short term reconstitution (Figure 5.10). At 4 months post transplantation, the recipients were sacrificed and the bone marrow, spleen and thymus populations were analyzed for Ly5.2$^+$ cells (Figure 5.10B, 5.11). The organs of mice transplanted with *Mll*$^{+/+}$ cells contained high levels of Ly5.2$^+$ cells in all populations analyzed, demonstrating their ability to give rise to long term reconstitution (Figure 5.10B, 5.11). No Ly5.2$^+$ cells

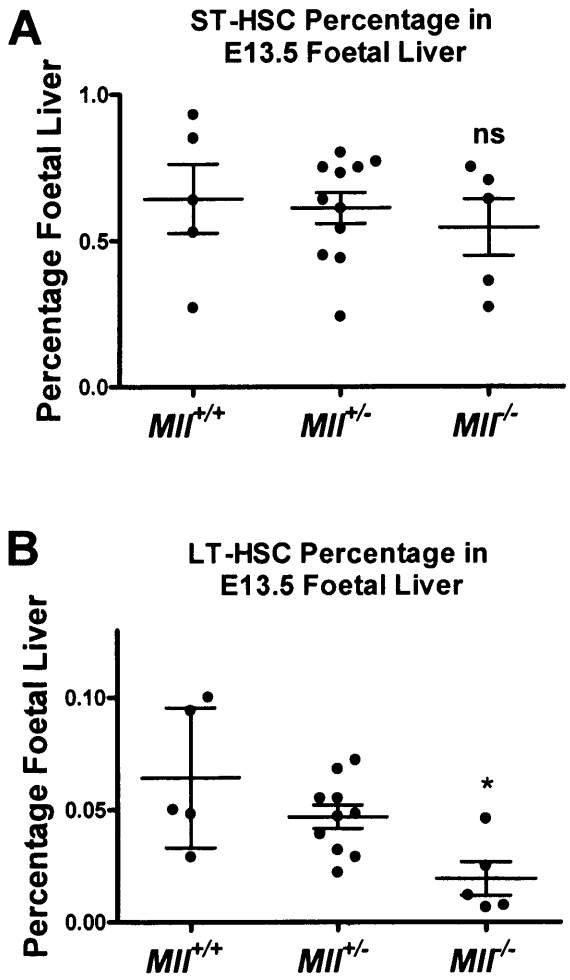


Figure 5.8. ST-HSC and LT-HSC Percentages in *Mll*$^{-/-}$ fetal livers.
A) Percentage of ST-HSCs present in E13.5 fetal livers. Lineage positive cells were depleted from E13.5 fetal liver cells and then the cells were analyzed by flow cytometry using markers c-kit, Sca1 and CD38. ST-HSCs were defined as Lin$^-$ Sca1$^+$c-kit^{hi}CD38^{lo}.
B) Number of LT-HSCs present in E13.5 fetal livers. LT-HSCs were defined as Lin$^-$Sca1$^+$c-kit^{hi}CD38^{hi}.
P values were calculated using 1Way ANOVA, with Tukeys multiple comparison test. (***) $P \leq 0.001$, (**) $P \leq 0.01$, (*) $P \leq 0.05$, ns = not significant. All samples were compared to *Mll*$^{+/+}$. The bars show the mean and standard error of the mean.

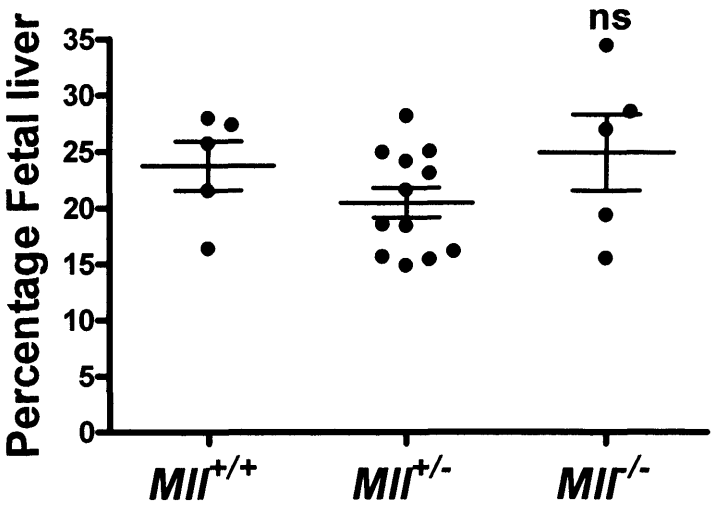
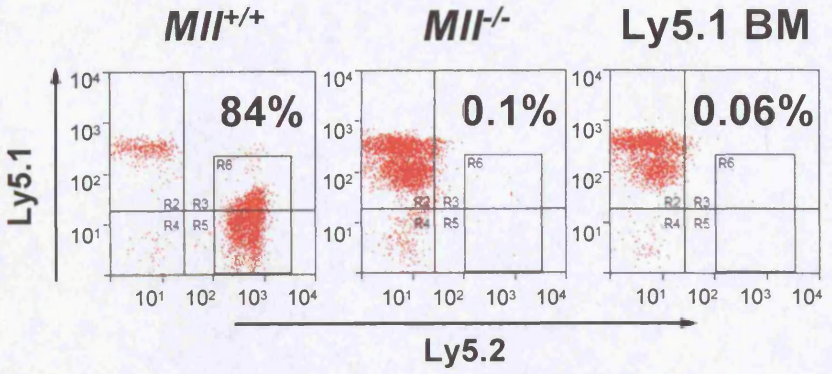


Figure 5.9. Percentage of *Mll-/-* foetal liver cells negative for Lineage markers.

Cells were stained with an antibody mix containing Gr1/Ly6G, B220, CD3, CD19 and Ter119 and analysed by flow cytometry.

P values were calculated using 1Way ANOVA, with Tukeys multiple comparison test. (***) $P \leq 0.001$, (**) $P \leq 0.01$, (*) $P \leq 0.05$, ns = not significant. All samples were compared to *Mll*$^{+/+}$. The bars show the mean and standard error of the mean.

A



B

Genotype	Short Term Reconstitution (1 Month)	Long Term Reconstitution (4 Months)
WT	5/5	5/5
KO	0/5	0/5

Figure 5.10. Failure of *Mll*$^{-/-}$ fetal liver cells to reconstitute lethally irradiated recipients.

c-kit^{+}Ter119^{-} cells were magnetically sorted from frozen E14.5 foetal liver cell suspensions. After sorting, 4 x 10^4 viable cells were transplanted into lethally irradiated recipient mice with a radioprotective dose of 2 x 10^5 wild type bone marrow cells. Ly5.2^{+} cells represent the cells derived from donor foetal liver, Ly5.1^{+} cells represent cells derived from wild type bone marrow.

A) Representative dot plots showing the percentage of Ly5.2+ (donor) cells present in the peripheral blood of irradiated recipients 1 month post-transplantation, as analysed by flow cytometry.

B) Table showing the number of irradiated recipients successfully reconstituted with either *Mll*$^{+/+}$ or *Mll*$^{-/-}$ fetal liver cells.

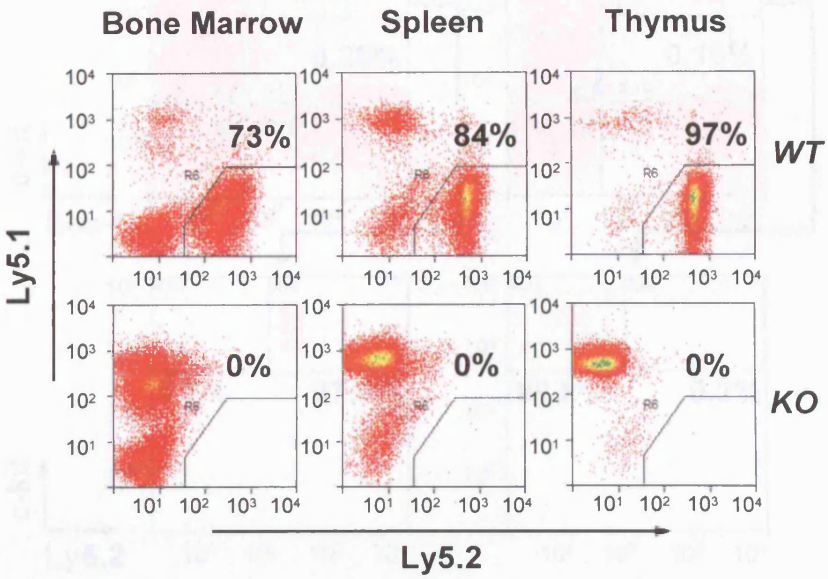


Figure 5.11. Failure of *Mll*$^{-/-}$ fetal liver cells to give rise to long term reconstitution in lethally irradiated recipients.

Representative dot plots showing the percentage of Ly5.2+ (donor) cells present in the hematopoietic organs of irradiated recipients 4 months post-transplantation. Single cell suspensions were prepared from the hematopoietic organs of recipient mice and the cells analyzed by flow cytometry for Ly5.2 and Ly5.1. Ly5.2+ cells represent the cells derived from donor foetal liver, Ly5.1+ cells represent cells derived from wild type bone marrow.

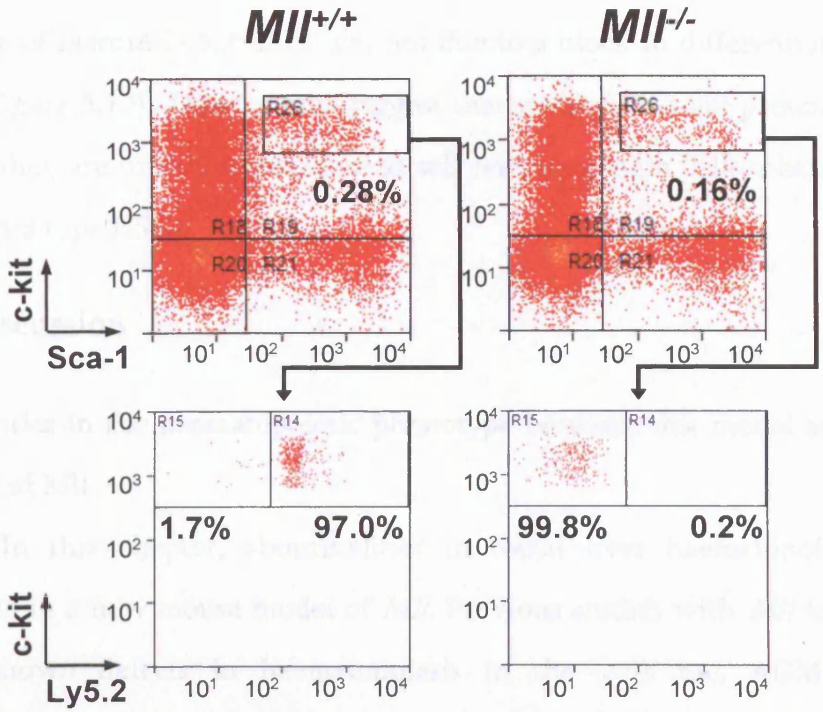


Figure 5.12. Failure of *Mll*$^{-/-}$ cells to contribute to the HSC compartment.

Representative dot plots showing the percentage of Ly5.2+ (donor) cells present in the bone marrow HSC compartment of irradiated recipients 4 months post-transplantation. Bone marrow cells were stained with Lineage markers, c-kit, Sca1 and Ly5.2 antibodies and the cells analysed by flow cytometry. The lineage positive cells were gated out and c-kit and Sca1 expression was analysed on the Lin$^-$ cells. Gates were set on the c-kit^{hi}Sca1$^+$ (LSK) cells. The percentage of Ly5.2$^+$ (donor foetal liver) cells in the LSK population was then analysed.

were detected in mice transplanted with *Mll*$^{-/-}$ donor foetal liver cells demonstrating that *Mll*$^{-/-}$ cells were unable to give rise to any long term reconstitution (Figure 5.10B, 5.11). Analysis of the bone marrow stem cell compartment also showed an absence of Ly5.2^{+} cells in mice that had received *Mll*$^{-/-}$ cells, showing that the absence of mature Ly5.2^{+} cells was not due to a block in differentiation of the stem cells (Figure 5.12). These results suggest that whilst HSCs are present in *Mll*$^{-/-}$ foetal livers, they are unable to function as self renewing stem cells when in competition with wild type HSCs.

5.6 Discussion

Similarities in the haematopoietic phenotype between this model and previous KO models of Mll

In this chapter, abnormalities in foetal liver haematopoiesis have been analysed in a new mouse model of *Mll*. Previous studies with *Mll* knockout models have shown defects in haematopoiesis in the yolk sac, AGM (Aorta-gonad-mesonephros) region and the foetal liver in the absence of *Mll* (Ernst et al., 2004a; Hess et al., 1997; Yagi et al., 1998). In two of these models, cells from *Mll*$^{-/-}$ yolk sacs (Hess, 2004) or foetal livers (Yagi et al., 1998) produced fewer colonies in myeloid CFU assays compared to *Mll*$^{+/+}$ cells. The data presented here is consistent with this, in that reduced numbers of colonies were seen in *Mll*$^{-/-}$ foetal liver cultures. The colony assays performed in this study, using cells from E12.5 foetal livers, were based on those performed in a previous study of an *Mll* mouse knockout (Yagi et al., 1998). When these cells were cultured, a 4- fold reduction in colony number was seen compared to wild type (Yagi et al., 1998). This bears striking similarity to the results in this study where a 5-fold reduction in colony number was seen. In the present study, no difference was seen in the proportions of the different colony types in *Mll*$^{-/-}$ cultures compared to *Mll*$^{+/+}$. This is in contrast to previous studies which saw reduced proportions of CFU-GEMM and CFU-GM produced (Hess et al., 1997; Yagi et al., 1998). The reason for these differences is not clear, although they

could be attributable to differences in the strain background of the mice, or to the levels of different cytokines used in the CFU assays.

The similarities between the foetal liver phenotype seen in *Mll*$^{-/-}$ embryos presented here and in previous studies support the protein evidence suggesting that deletion of exons 9-10 of *Mll* results in a null allele.

Mll is not needed for the establishment of definitive haematopoiesis in the embryo

The presence of myeloid progenitors in the foetal liver shows that, although *Mll* is needed for the proper progression of definitive haematopoiesis, it is not needed for its establishment. The presence of haematopoietic stem cells, as characterised by flow cytometry would support this. *Mll* is therefore not needed for the formation of HSCs in the AGM, the proposed site of origin for definitive HSCs (Godin and Cumano, 2002; Mikkola et al., 2003).

Mll$^{-/-}$ foetal liver stem cells are able to give rise to a limited amount of expansion

Although the stem cell compartment was severely reduced, those stem cells that were present in *Mll*$^{-/-}$ foetal livers were able to undergo some level of expansion, as shown by the size increase between E13.5 and E14.5 *Mll*$^{-/-}$ foetal livers. It is not clear what cell populations are responsible for this expansion in *Mll*$^{-/-}$ foetal livers but it is likely to be erythroid cells. By E14.5, approximately 80% of the wild type foetal liver is made up of late stage erythroblasts and mature erythrocytes (Katsumoto et al., 2006). Further analysis of foetal liver cells for the markers CD71 and Ter119, which are expressed on erythroblasts (CD71, Ter119) and erythrocytes (Ter119) would give more information as to whether the expansion seen in *Mll*$^{-/-}$ foetal livers is due to erythroid progenitors (Katsumoto et al., 2006; Socolovsky et al., 2001).

Foetal liver myeloid progenitors are severely affected by Mll disruption although this defect is not due to a block in differentiation

Although foetal liver expansion was seen in *Mll*$^{-/-}$ foetal livers, a profound defect was seen in myeloid CFU assays. Some myeloid differentiation could occur in the absence of *Mll* as shown by the normal colony morphology and the presence of

maturing macrophages in *Mll*$^{-/-}$ cultures. Further characterisation of the cells produced in *Mll*$^{-/-}$ cultures is necessary to assess whether an absence of Mll leads to any abnormal differentiation .The lack of any obvious block in differentiation would suggest that the reduction in colony number in *Mll*$^{-/-}$ cultures is due either to a reduced number of myeloid progenitors in the foetal liver or to a reduced capacity for those progenitors present to proliferate in response to cytokines.

As described above, there was no difference in the proportion of the different colony types produced in myeloid CFU cultures from *Mll*$^{-/-}$ cultures compared to *Mll*$^{+/+}$ cells. Myeloid cell differentiation from HSCs occurs through a series of populations of progressively more differentiated progenitors (Akashi et al., 2000; Kawamoto, 2006; Traver et al., 2001). Although there is some disagreement as to where the first myeloid progenitor arises, the work from Irving Weissman's laboratory has defined this as the Committed Myeloid Progenitor, or CMP. It has been shown, through *in vitro* colony forming assays, stromal culture assays and *in vivo* reconstitution assays that the CMP, is to give rise to lymphoid cells, but can give rise to erythroid, megakaryocyte and myeloid cells Figure 5.13 (Akashi et al., 2000; Traver et al., 2001). It is thought that two further progenitor cells are derived from the CMP - the Granulocyte/Monocyte Progenitor (GMP) which give rise to granulocytes and erythrocytes, and the Megakaryocyte/Erythrocyte progenitor (MEP), which gives rise to megakaryocytes and erythrocytes. You would therefore expect that a block in differentiation at either the GMP or the MEP stage would result in a reduction in the production of CFU-GM or BFU-E respectively in CFU assays. The equal reduction in all colony types in *Mll*$^{-/-}$ cultures would suggest that the defect is at the level of, or before, the CMP.

The reduction in myeloid CFU produced could be due to a reduction of myeloid progenitors in the foetal liver, or a block in proliferation. Flow cytometry could be used to assess whether myeloid progenitors are reduced in *Mll*$^{-/-}$ foetal livers. The CMP, MEP and GMP populations in the foetal liver can be identified by differential expression of the markers CD34, c-kit and FcγR (Traver et al., 2001). Further analysis by *in vitro* culture of foetal liver cells in media containing myeloid

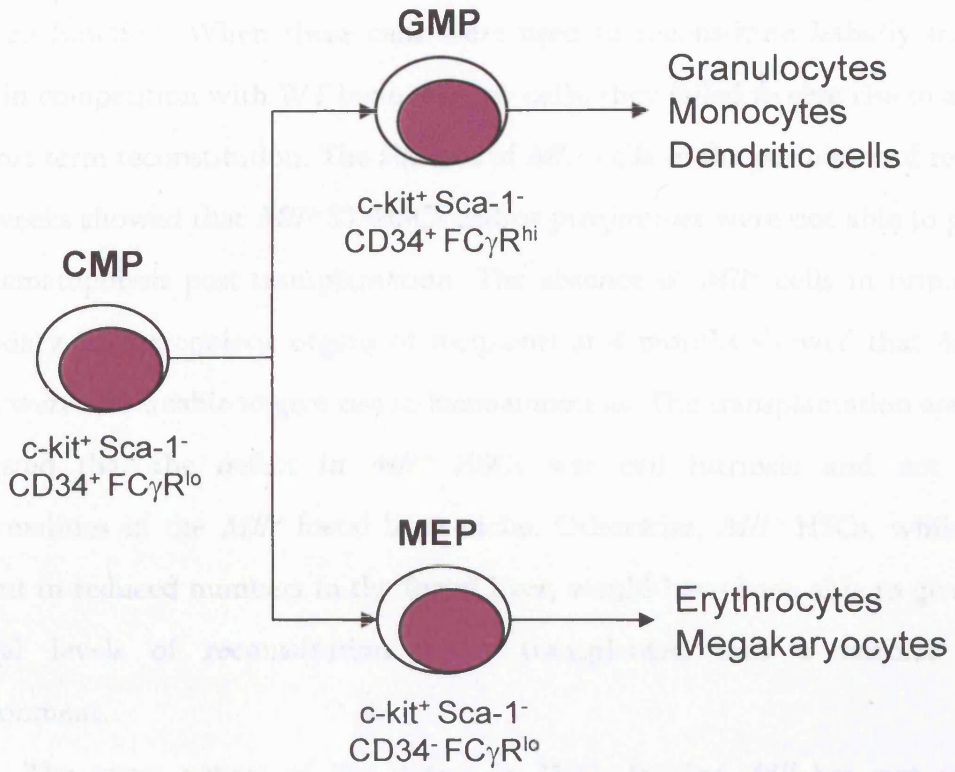


Figure 5.13. The development of myeloid cells.
The figure shows the model conceived by Irving Weissmans group (Akashi *et al.*, 2000, Traver *et al,*. 2001)
CMP – Common Myeloid Progenitor
GMP – Granulocyte/Monocyte Progenitor,
MEP – Megakaryocyte/Erythrocyte Progenitor
Shown below the cells are the markers used for isolation of the indicated populations.
Figure reproduced from Shizura et al., 2005.

cytokines, followed by cell proliferation assays could also show whether the myeloid progenitors have reduced proliferation capacity in response to cytokines.

Mll$^{-/-}$ foetal liver stem cells are not fully functional

The reduced numbers of cells and, in particular, the lower proportion of LT-HSC present in *Mll*$^{-/-}$ foetal liver suggested that *Mll*$^{-/-}$ HSCs, whilst present, had reduced function. When these cells were used to reconstitute lethally irradiated mice in competition with WT bone marrow cells, they failed to give rise to any long or short term reconstitution. The absence of *Mll*$^{-/-}$ cells in the periphery of recipients at 4 weeks showed that *Mll*$^{-/-}$ ST-HSCs and/or progenitors were not able to give rise to haematopoiesis post transplantation. The absence of *Mll*$^{-/-}$ cells in primary and secondary haematopoietic organs of recipients at 4 months showed that *Mll*$^{-/-}$ LT-HSCs were also unable to give rise to haematopoiesis. The transplantation assays also suggested that the defect in *Mll*$^{-/-}$ HSCs was cell intrinsic and not due to abnormalities in the *Mll*$^{-/-}$ foetal liver niche. Otherwise, *Mll*$^{-/-}$ HSCs, whilst being present in reduced numbers in the foetal liver, would have been able to give rise to normal levels of reconstitution when transplanted into a normal stromal environment.

The exact nature of the defect in HSCs lacking *Mll* has not yet been elucidated. However, there are several possible explanations. For example, it is possible that *Mll*$^{-/-}$ HSCs are incapacitated in their ability to home to the bone marrow and other haematopoietic niches when transplanted. HSCs normally reside in specific regulatory microenvironments in the foetal liver, bone marrow and other haematopoietic organs- so called 'niches' (Chute, 2006; Zhang et al., 2003). The HSCs are tethered to the niche cells via multiple cell adhesion molecule-ligand interactions. For example, in the bone marrow niche, haematopoietic stem cells reside in the endosteal lining of the bone marrow cavity, and are attached to specialised osteoblasts via a homotypic N-cadherin interaction (Calvi et al., 2003; Zhang et al., 2003). The niche is thought to provide a microenvironment that supports the HSCs and prevents their differentiation (Chute, 2006; Takubo et al., 2006; Wilson et al., 2004). When haematopoietic stem cells are transplanted into

irradiated recipients, it is necessary for the cells to home to these niches where they can then begin to propagate the haematopoietic system. This is evidenced by the fact studies showing that inhibition of the receptors and ligands necessary for the tethering of HSCs to the niches results in reduced homing and a reduction in HSCs in the bone marrow (Papayannopoulou et al., 2001; Potocnik et al., 2000; Sugiyama et al., 2006). It is possible that the reconstitution defect seen in *Mll*$^{-/-}$ HSCs could be due to a defect in their ability to home to the bone marrow niche. The ability of *Mll*$^{-/-}$ HSCs to transfer from the AGM to the foetal liver, and the results presented in Chapter 6 would suggest that this is not the case. However, to assess whether *Mll*$^{-/-}$ HSCs are compromised in their homing capacity, it would be necessary to perform homing assays, in which the foetal liver HSCs are labelled with a fluorescent dye (e.g. carbofloxyfluorescein diacetate, succinimidyl ester) and then transplanted into irradiated mice. The bone marrow of the long bones of the recipients can then be analysed 6 to 18 hours post-transplantation by flow cytometry or fluorescent microscopy to assess the homing efficiency of the donor cells (Wilson et al., 2004; Zhang et al., 2006).

It is interesting that the phenotype seen in *Mll*$^{-/-}$ foetal livers is similar to that seen in several other mouse gene knockout models. Of particular interest, is the similarities seen between the phenotype in the *Mll*$^{-/-}$ foetal livers and those seen when different *Hox* genes and their cofactors are deleted. For example, mice deficient in *Hoxa9* have normal numbers of LSK cells in the bone marrow, but in competitive reconstitution assays, these cells are severely compromised (Lawrence et al., 2005b). Mice deficient in the *Hox* cofactors *Pbx1* and *Meis1* establish foetal liver haematopoiesis, but the cells present have reduced myeloid CFU potential and are again compromised in competitive reconstitution assays (DiMartino et al., 2001b; Hisa et al., 2004b). In *Mll* deficient foetal liver, stem cells populations are present, but these stem cells are not able to function as normal in CFU assays and competitive reconstitution experiments.

Several studies have shown that MLL binds to the promoters and coding regions of *Hox* genes and promotes gene activation through the methylation of Histone3 Lysine4 (Milne et al., 2002; Milne et al., 2005b). The induction of several

HoxA, HoxB and *HoxC* cluster genes was severely reduced in embryoid bodies derived from *Mll* null ES cells (Ernst et al., 2004b), and *Mll* null embryos show disrupted *Hox* expression (Yagi et al., 1998; Yu et al., 1995). *Hox* gene expression was also severely reduced in murine embryonic fibroblasts derived from *Mll KO* embryos in our model (unpublished results, S. Y-L. Hiew and H. J. M. Brady).

It is possible that the stem and progenitor cell defects that we see in foetal haematopoiesis maybe due to a reduction in *Hox* gene expression in the absence of *Mll.* At this stage of development, it is thought that the expression of multiple *Hox* genes are needed to drive proliferation of stem and progenitor cells, and that *Mll* maintains this expression (Bjornsson et al., 2003; Ernst et al., 2004b; Lawrence et al., 2005b; Lawrence et al., 1997). In the absence of *Mll,* although stem cells are produced in the foetal liver, they are not able to expand as normal, either in the foetal liver or post transplantation, both situations where the expansion of stem cells is required.

It has been shown in other cell types that *Mll* activates *Hox* gene transcription through H3K4 methylation and also through its interaction with H4K16 histone deacetylases such as MOF (Dou et al., 2005) The phenotype of the mouse model of another histone acetyl-transferase, *Monocytic Leukaemia Zinc-finger* (*MOZ*) bears a striking similarity to the phenotype seen in $Mll^{-/-}$ embryos (Katsumoto et al., 2006). Embryos deficient in *MOZ* die by E15.5, and have a reduction in foetal liver size. When the foetal liver cells were analysed, they were found to have reduced numbers of myeloid progenitors, both by colony analysis and flow cytometry. Like $Mll^{-/-}$ foetal livers, the ratio of lineage-committed CFUs was not affected. As in $Mll^{-/-}$ foetal livers, the LSK population was severely reduced although present, but when *MOZ* deficient foetal liver cells were transplanted, they could not reconstitute lethally irradiated recipients. The expression of *Hoxa9,* a target of *Mll,* was also reduced. The common phenotype shared by *Mll* and *MOZ* deficient embryos suggests a possible interaction between the two proteins. As yet, MLL complexes have not been analysed in haematopoietic cells, so it is possible that MLL may interact with different proteins, including *MOZ,* in this context.

It is probable that MLL activates transcription of an unidentified set of target genes in early haematopoietic cells. *Mll* targets have been identified in other cell types, for example embryonic fibroblasts (Milne et al., 2005b; Scharf et al., 2006; Scharf et al., 2007; Schraets et al., 2003), but have not been analysed in haematopoietic cells, with the exception of embryoid bodies (Ernst et al., 2004b). The new exon 9-10 deletion mouse model presents a unique opportunity to analyse *Mll* targets in a haematopoietic specific context. Microarray analysis can be performed using $Mll^{-/-}$ foetal liver cells compared to $Mll^{+/+}$ cells to identify genes whose expression is altered in the absence of *Mll*. Identification of these targets may elucidate the mechanism by which *Mll* is involved in stem cell regulation.

Chapter 6

In the absence of *Mll*, homeostatic haematopoiesis is unaffected, but *Mll* deficient stem cells cannot self-renew when transplanted

6.1 Introduction

The defects in stem cell self renewal and progenitor growth seen in *Mll* deficient foetal livers would suggest that *Mll* plays an important role in foetal haematopoiesis. To assess whether this role was also needed in adult haematopoiesis, it was necessary to establish a line of mice in which MLL expression was abrogated only in haematopoietic tissues, leaving MLL expression intact in the rest of the mouse. This would overcome any embryonic lethal effects of *Mll* deletion. The creation of a mouse conditional for *Mll* could be achieved by breeding mice carrying the floxed allele of *Mll* to mice which carry the Cre recombinase under the control of different tissue specific promoters (Porret et al., 2006). Several strains have been developed for the study of genes important for haematopoiesis. The most widely used expresses the Cre recombinase under the control of the promoter for the *Mx1* gene (Hock et al., 2004b; Ichikawa et al., 2004; Opfer et al., 2005; Zhang et al., 2006). The *Mx1* promoter is normally silent, but can be transiently activated upon application of interferon α or β or a synthetic double stranded RNA (polyinosinic-polycytidylic acid or pI-pC). Activation of the promoter results in Cre mediated deletion in all cells. This model has been extensively used as it results in high levels of deletion in all haematopoietic cells including early stem cells. A more tissue

specific Cre shown to induce deletion in early haematopoietic cells is the *Tie2* Cre, where Cre is under the control of the *Tie2* promoter, a gene which is expressed in haematopoietic stem cells and endothelial cells (Kisanuki et al., 2001; Schlaeger et al., 2005).

Several models have also been developed that enable Cre mediated deletion in selected haematopoietic lineages. The Lck-Cre transgene can be used to induce LoxP mediated deletion in $CD4^+$ and $CD8^+$ T-cells (Hennet et al., 1995). The CD19 Cre and mb1 Cre transgenes can be used to induce deletion in pro-B and pre-B cells respectively (Hobeika et al., 2006; Rickert et al., 1997). The Lys2M Cre and CD11b Cre transgenes can be used to induce deletion in macrophages and granulocytes (Clausen et al., 1999; Ferron and Vacher, 2005). These mouse strains enable the study of the function of genes specifically in mature lineages of cells, whilst preventing any stem cell associated defects.

In this study, mice carrying the *Vav* Cre transgene have been used to create *Mll* conditional knockout mice. This transgene utilises the promoter elements of the *Vav* gene. The *Vav* promoter drives expression in haematopoietic cells of all lineages, including functional stem cells, from the foetal liver through to adult haematopoiesis (Almarza et al., 2003; de Boer et al., 2003; Ogilvy et al., 1999). Using this approach, Cre is continually expressed throughout haematopoietic development resulting in high levels of recombination in mature B lymphoid, T lymphoid and myeloid cells (de Boer et al., 2003). In this study, mice carrying a floxed allele of *Mll* were bred to mice carrying the *Vav* Cre transgene to produce mice conditional for *Mll* in the haematopoietic system. These mice were then analysed to assess the contribution of *Mll* to adult haematopoiesis.

6.2 Establishment of Mll conditional mouse lines

Vav Cre transgenic mice were kindly provided by Dimitris Kioussis and Alexandre Potocnik, NIMR, Mill Hill (de Boer et al., 2003). The mice had been backcrossed onto and were congenic for the C57Bl/6J background. The *Vav* Cre transgenic mice were bred to $Mll^{+/-}$ mice to produce $Mll^{+/-\ VavCre}$ mice. These mice were then bred to $Mll^{Flox/Flox}$ mice to give rise to $Mll^{Flox/-\ VavCre}$ mice. These mice, in

which *Mll* should be absent in the haematopoietic system, were termed '*Mll* conditional mice'. *Mll* conditional mice were born live with no apparent abnormalities.

6.3 High levels of recombination were seen in the haematopoietic cells of Mll conditional mice

To ensure that *Mll* was being efficiently deleted, the levels of recombination were assessed in the different haematopoietic organs of *Mll* conditional mice. Southern blot analysis confirmed that exons 9 and 10 of the gene were efficiently recombined in DNA lysates prepared from whole bone marrow, spleen, thymus and lymph node of *Mll* conditional mice (Figure 6.1). MLL protein was also undetectable in spleen lysates from *Mll* conditional mice, confirming abolition of MLL expression (Figure 6.2). To analyse the levels of deletion in haematopoietic stem cells, LSK cells were sorted from the bone marrow of *Mll* conditional mice by a combination of magnetic activated cell sorting and FACS. PCR analysis of DNA prepared from the cells showed efficient recombination of the floxed allele, although some residual unrecombined DNA remained (Figure 6.3). These results showed that the *Vav* Cre transgene was efficient in inducing recombination in adult haematopoietic cells.

6.4 Homeostatic haematopoiesis in adult mice is unaffected by the absence of Mll

To determine whether the haematopoietic system in *Mll* conditional mice was affected by the absence of *Mll*, cells from the bone marrow, spleen, thymus and lymph nodes were analysed by flow cytometry. $Mll^{Flox/+}$ mice were used as littermate controls, since these were comparable to wild type mice in similar assays (Figure 6.4). For all experiments, 8 week old males and females were used. There was no difference in the mean total cell number of the spleen and thymus between *Mll* conditional and $Mll^{Flox/+}$ control littermates (Figure 6.5). The populations of myeloid (Figure 6.6, 6.7), and lymphoid cells (Figures 6.8, 6.9, 6.10) were analyzed in the primary and secondary haematopoietic organs of *Mll* conditional and $Mll^{Flox/+}$ mice.

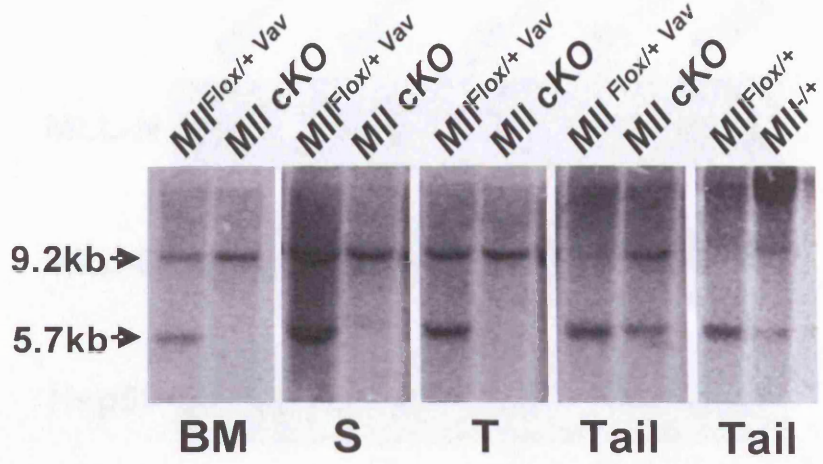


Figure 6.1. High levels of recombination were seen in the haematopoietic organs of *Mll* conditional mice.

Southern Blot analysis of DNA prepared from the bone marrow (BM), spleen (S) and thymus (T) of *Mll* conditional mice. DNA from $Mll^{Flox/+\ VavCre}$ mice was used as a control. Mll conditional mice are represented by *Mll cKO*. The DNA was digested with *Kpn*1 and incubated with the 3' probe shown in Figure 4.4. The floxed and the wild type bands ran at the same size (5.7 kb) and the deleted band was 9.2 kb. No floxed band was visible using DNA prepared from Mll conditional mice haematopoietic organs indicating very high levels of recombination. DNA prepared from the tail of the *Mll* conditional mouse shows the presence of the floxed band, as expected.

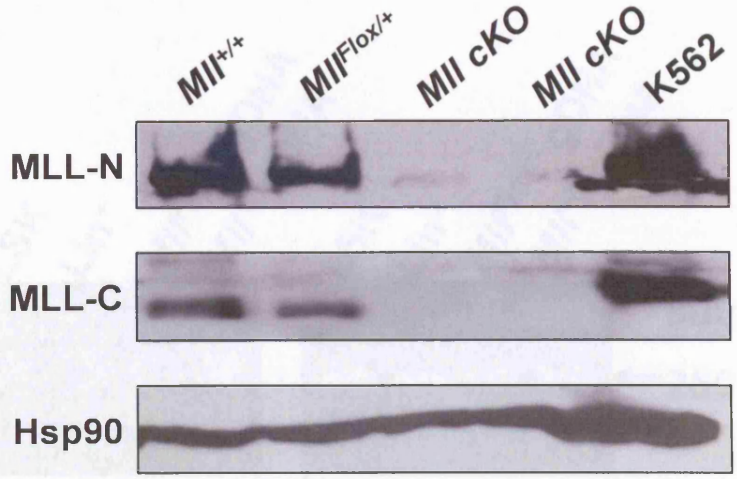


Figure 6.2. MLL protein is absent from the spleen cells of *Mll* conditional mice.
Western Blot of MLL protein in the spleens of *Mll* conditional mice. Whole cell lysates made from splenocytes were run with K562 cell lysates as a positive control. MLL N-terminal (MLL-N) and MLL C-terminal (MLL-C) antibodies were used to detect the presence of the MLL fragments as before. An antibody against Hsp-90 was used as a loading control.
Mll cKO denotes *Mll* conditional.

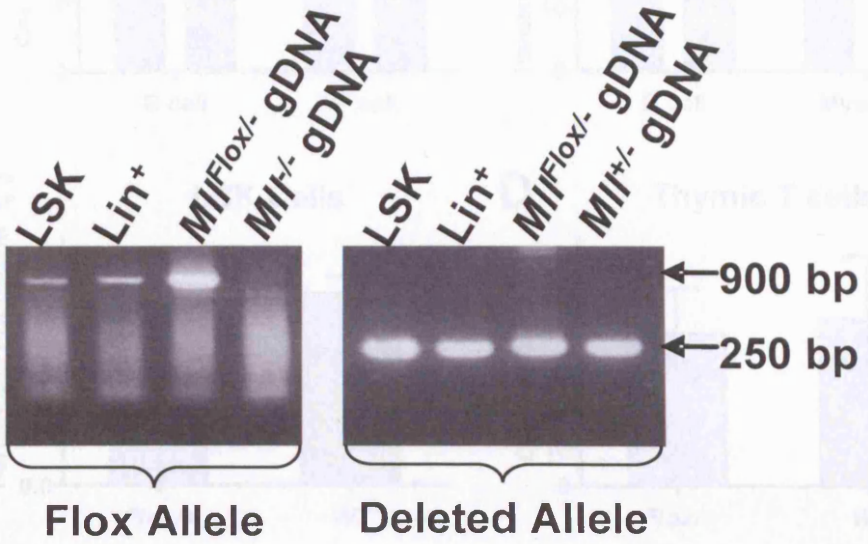


Figure 6.3. Recombination in *Mll* conditional HSCs.
Recombination of the floxed allele in *Mll* conditional LSK and lineage positive cells. Lineage positive cells were depleted from bone marrow cells by magnetic sorting. c-kit^{hi}Sca1^{+} (LSK) cells were sorted from the lineage negative cells by FACS and DNA was prepared from them. PCR analysis was then performed on the DNA using primers E and F and E and B. Genomic DNA from an *Mll*$^{Flox/-}$ mouse and an *Mll*$^{-/-}$ embryo were used as positive and negative controls. Flox allele - 900 bp, deleted allele - 250 bp.

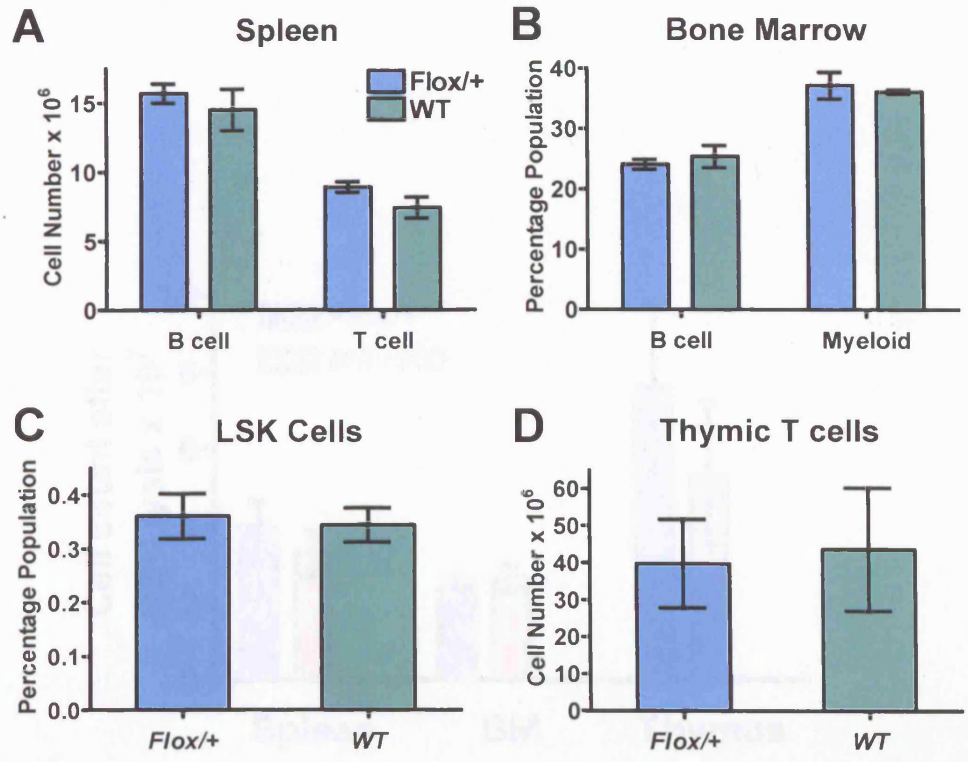


Figure 6.4. Haematopoiesis is comparable to wild type in *Mll*$^{Flox/+}$ mice. Flow cytometric analysis of the haematopoietic system of *Mll*$^{Flox/+}$ control mice showed that they were comparable to wild type animals. For all charts, n = 2-3. All mice were 8 week old males. The graphs show the mean and the Standard Error of the Mean (SEM).
A) B cell and T cell numbers in the spleen. B cells were defined as B220^{+} and T cells were defined as CD4^{+} or CD8^{+}.
B) B cell and myeloid cell percentages in the bone marrow. B cells were defined as above, myeloid cells were defined as Gr1^{+} Mac1^{+}.
C) LSK (HSC) cell percentages in the bone marrow.
D) T cell numbers in the thymus. T cells were defined as above.

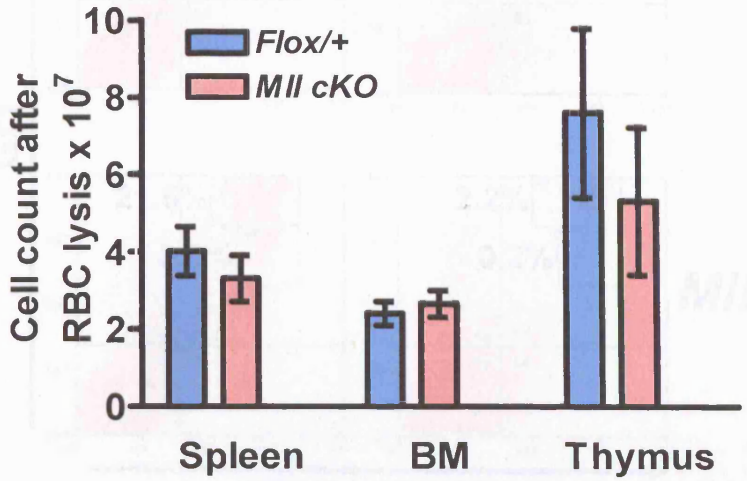


Figure 6.5. Cell numbers in the haematopoietic organs of *Mll* conditional mice are equivalent to controls.
Single cell suspension were prepared from the spleen, bone marrow (BM) and thymus of $Mll^{Flox/+}$ (*Flox/+*)and *Mll* conditional (*Mll* cKO) mice. Erythrocytes were removed and live cell counts taken. For the thymus, n=3-4, for the spleen and bone marrow, n=3-6. All mice were 8 week old females. The graph shows the mean and SEM.

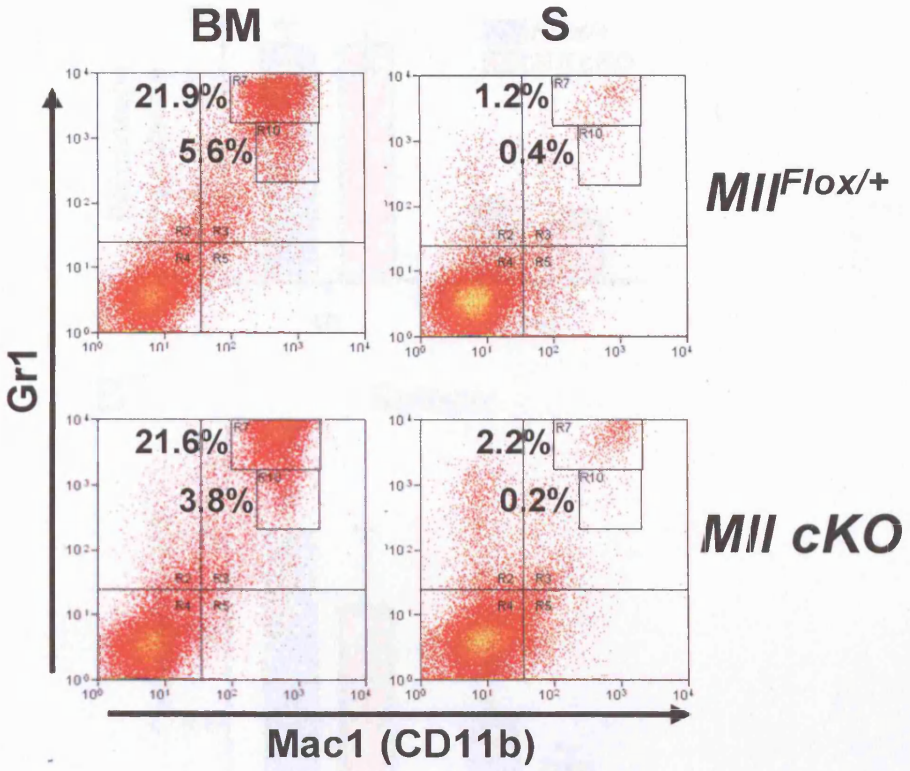


Figure 6.6. Myeloid cell populations are unaffected in the absence of *Mll*.

Representative dot plots showing flow cytometric analysis of myeloid cells in the bone marrow (BM) and spleen (S). Granulocytes were defined as $Gr1^{hi}$ $Mac1^{+}$ and monocytes were defined as $Gr1^{lo}$ $Mac1^{+}$.

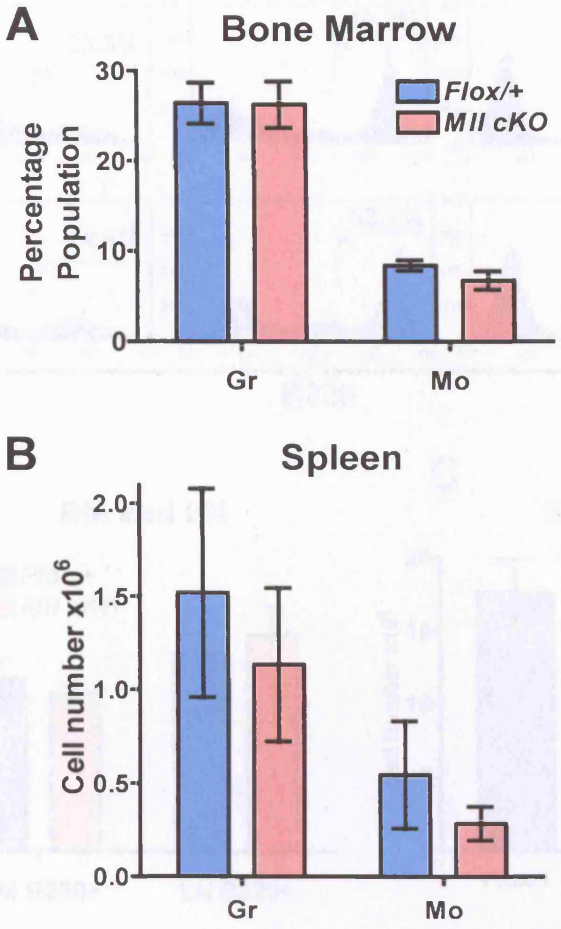


Figure 6.7. Myeloid cell populations are unaffected in the absence of *Mll*.

Flow cytometric analysis of the myeloid cell populations in the bone marrow and spleen of *Mll* conditional mice. For all charts, n = 3-4. *Flox/+* denotes $Mll^{Flox/+}$ control mice, *Mll cKO* denotes *Mll* conditional mice. The graphs show the mean and the SEM.

Granulocytes were defined as $Gr1^{hi}$ $Mac1^{+}$ and monocytes were defined as $Gr1^{lo}$ $Mac1^{+}$.

A) Granulocyte and monocyte populations in the bone marrow of *Mll* conditional mice. Percentages are shown as the cell numbers obtained were not accurate.

B) Granulocyte and monocyte numbers in the spleens of *Mll* conditional mice.

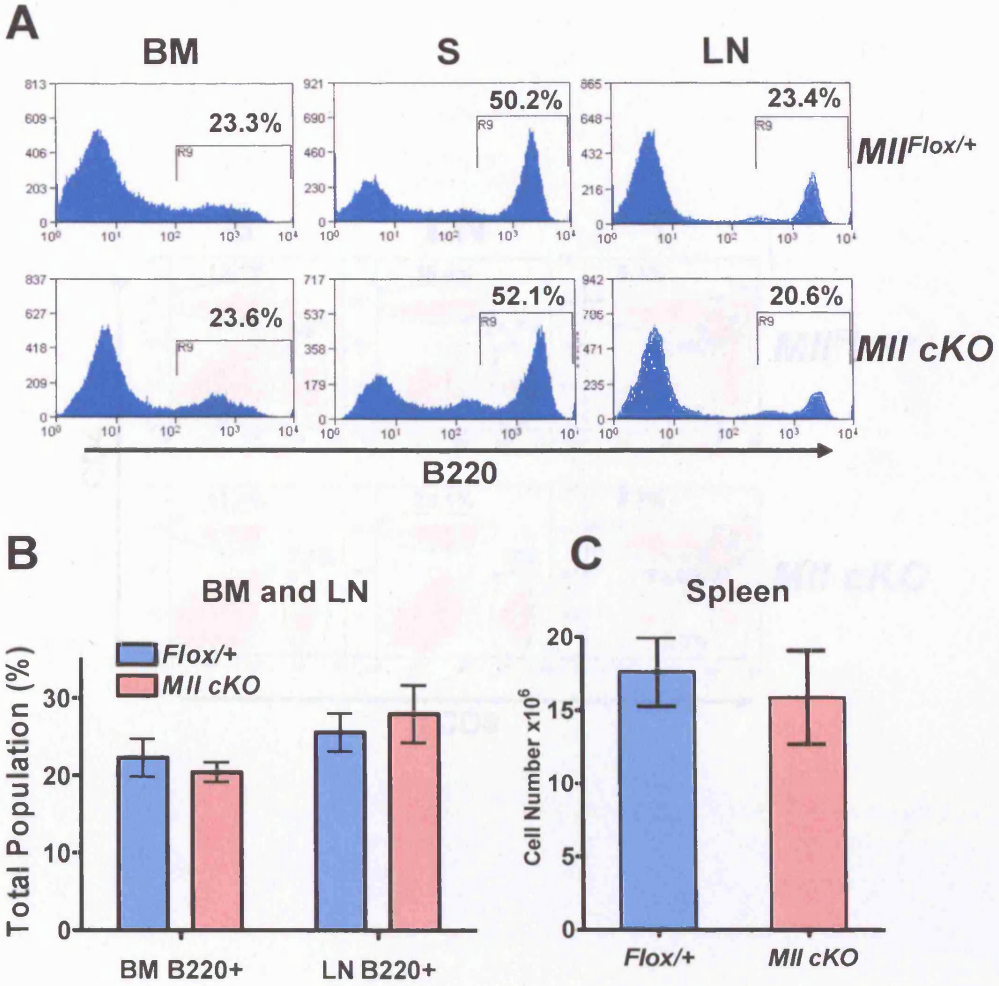


Figure 6.8. B lymphoid populations are unaffected in the absence of *Mll*.

Flow cytometric analysis of the B cell populations in the bone marrow and spleen of Mll conditional mice. For all charts, n = 4. The graphs show the mean and the SEM

A) Representative histograms showing flow cytometric analysis of B cell populations in bone marrow (BM), Spleen (S) and Lymph Node (LN).

B) B cell populations in the bone marrow and lymph node of *Mll* conditional mice. Percentages are shown as the cell numbers obtained were not accurate.

C) B cell numbers in the spleen of *Mll* conditional mice. B cells were defined as $B220^{+}CD19^{+}$.

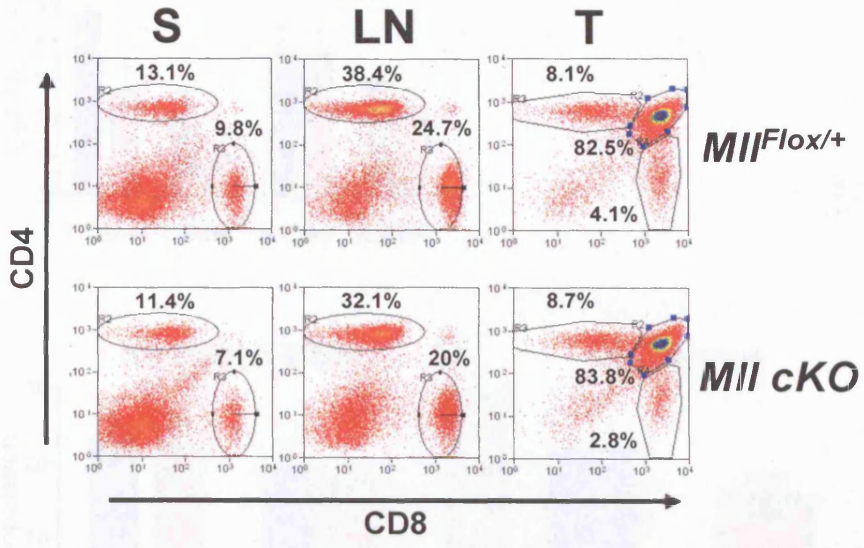


Figure 6.9. T lymphoid populations are unaffected in the absence of *Mll*.

Representative dot plots showing flow cytometric analysis of T cells in the and spleen (S), lymph node (LN) and thymus (T) of *Mll* conditional mice. In the spleen and lymph node the numbers of $CD4^+$ and $CD8^+$ cells were analysed. In the thymus, $CD4^+CD8^+$ cells were also analysed.

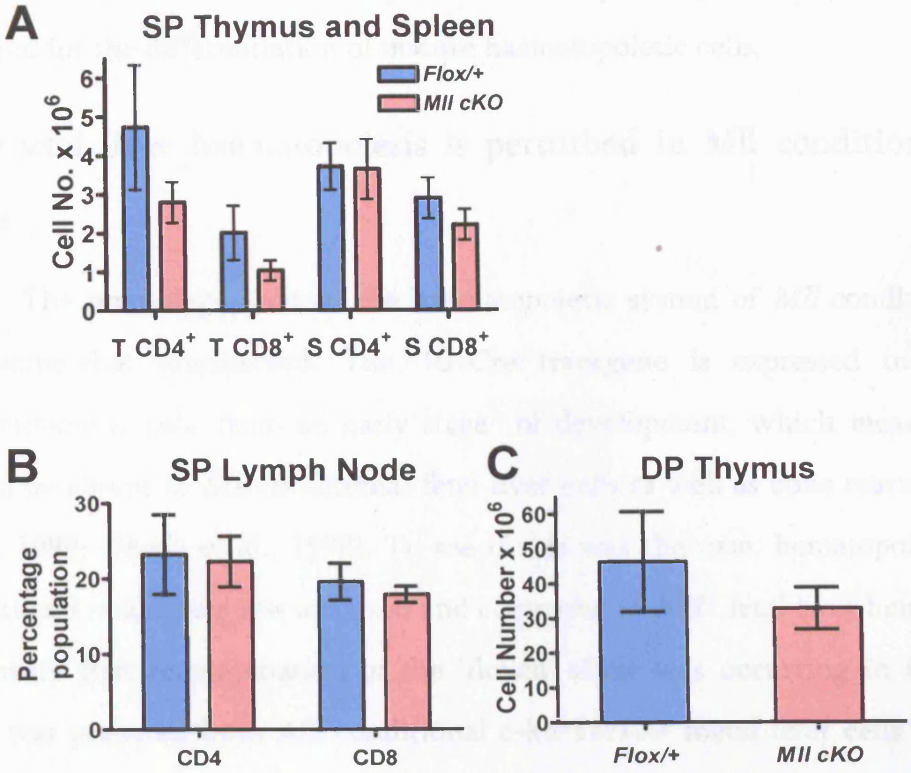


Figure 6.10. T lymphoid populations are unaffected in the absence of *Mll*.

Flow cytometric analysis of the T cell populations in the spleen, lymph node and thymus of Mll conditional mice. For all charts, n = 1 male and 3 females. The graphs show the mean and the SEM. *Flox/+* denotes $Mll^{Flox/+}$ control mice, *Mll cKO* denotes *Mll* conditional mice.

A) Numbers of CD4 and CD8 single positive cells in the thymus and spleen of *Mll* conditional mice.

B) Percentage of CD4 and CD8 single positive cells in the mesenteric lymph node of *Mll* conditional mice. Percentages are shown as the cell numbers obtained were not accurate.

C) Numbers of CD4 and CD8 double positive cells in the thymus.

No significant differences were found between *Mll* conditional and $Mll^{Flox/+}$ mice in any of the major blood cell populations, when the data sets were compared, both with paired and un-paired t-tests. This surprising result suggests that *Mll* is not required to establish normal steady-state haematopoiesis postnatally. *Mll* is also not required for the differentiation of mature haematopoietic cells.

6.5 Foetal liver haematopoiesis is perturbed in Mll conditional foetal livers

The phenotype seen in the haematopoietic system of *Mll* conditional mice was somewhat unexpected. The *Vav*Cre transgene is expressed in definitive haematopoietic cells from an early stage of development, which means that *Mll* should be absent in *Mll* conditional fetal liver cells as well as bone marrow (Ogilvy et al., 1999; Okada et al., 1998). To see if this was the case, hematopoiesis in *Mll* conditional fetal livers was analysed and compared to $Mll^{-/-}$ fetal liver hematopoiesis. To ensure that recombination of the 'floxed' allele was occurring in foetal liver, DNA was prepared from *Mll* conditional c-kit$^+$Ter119$^-$ foetal liver cells from E13.5 embryos. PCR analysis of this DNA showed high levels of recombination (Figure 6.11). Stem cells from *Mll* conditional, $Mll^{Flox/+}$, $Mll^{Flox/Flox}$, and $Mll^{Flox/-}$ E13.5 foetal liver were analyzed by flow cytometry, using LSK markers and CD38 as described in Chapter 5.4. Surprisingly, there was no significant difference in the number of ST-HSCs or LT-HSCs in *Mll* conditional foetal livers compared to control $Mll^{Flox/Flox}$ or $Mll^{Flox/+}$ (Figure 6.12). To further analyze haematopoiesis in *Mll* conditional embryos, myeloid CFU assays were performed with cells from E12.5 foetal livers as before. A 2-fold reduction in colony number was seen in *Mll* conditional cultures compared with $Mll^{Flox/-}$ (Figure 6.13A). A 2-fold reduction in cell number was also seen (Figure 6.13B). Southern blot analysis of DNA extracted from methylcellulose cultures showed high levels of recombination of the floxed allele in *Mll* conditional cultures (Figure 6.14). The reductions in colony number and cell number were not as great as that seen between $Mll^{-/-}$and $Mll^{-/+}$cultures, where there were 3.5-fold and 10-fold reductions, respectively (Figure 5.2).

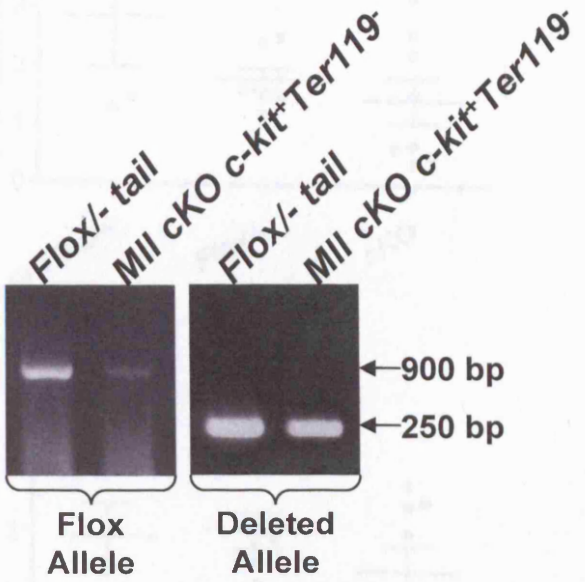


Figure 6.11. The *Mll* floxed allele is efficiently recombined in the foetal liver of *Mll* conditional embryos.
Recombination of the floxed allele in *Mll* conditional fetal liver cells. c-kit^{+}Ter119^{-} cells were sorted from E13.5 fetal livers by magnetic sorting and DNA prepared from them. Primers E and F2 were used to amplify the floxed allele (900 bp) and primers E and G2 were used to amplify the deleted allele (250 bp).Tail DNA from a $Mll^{Flox/-}$ mouse was used as positive control.

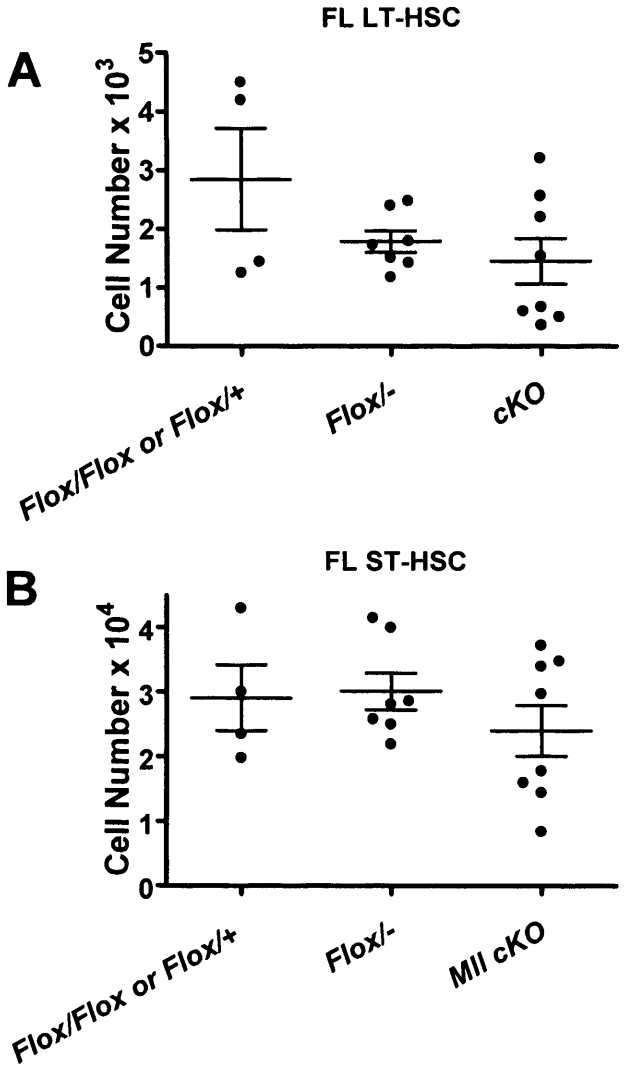


Figure 6.12. *Mll* conditional foetal livers have normal numbers of haematopoietic stem cells.

A) Number of LT-HSCs present in *Mll* control (Flox/Flox or Flox/+) $Mll^{Flox/-}$ (*Flox/-*) and *Mll* conditional (*Mll cKO*) E13.5 fetal livers. Lineage positive cells were depleted from E13.5 fetal liver cells and then the cells were analyzed by flow cytometry for the markers c-kit, Sca1 and CD38. LT-HSCs were defined as $Lin^{-}Sca1^{+}c\text{-}kit^{hi}CD38^{hi}$.

B) Number of ST-HSCs present in *Mll* conditional E13.5 fetal livers. ST

HSCs were defined as $Lin^{-}Sca1^{+}c\text{-}kit^{hi}CD38^{lo}$.

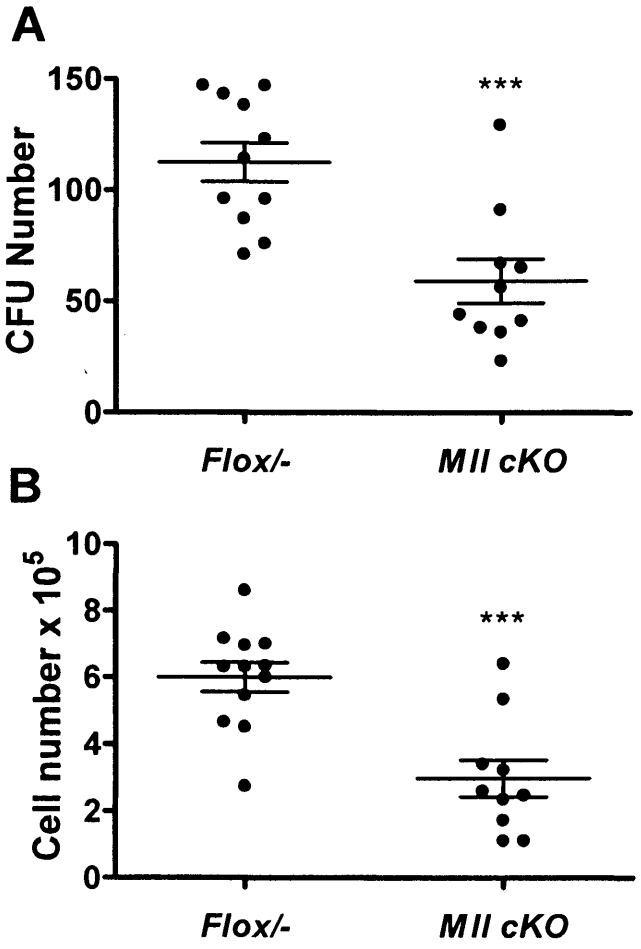


Figure 6.13. Myeloid CFU are reduced in *Mll* conditional foetal liver.
The graphs show the mean and the SEM.
A) Myeloid CFU counts for *Mll cKO* E12.5 fetal liver cells. Following the removal of erythrocytes, 1.1 x 10^4 fetal liver cells were plated in methylcellulose and CFUs were counted after 7 days.
B) The number of cells present in CFU cultures from E12.5 fetal livers. 1.1 x 10^4 cells were cultured in methylcellulose and after 7 days, single cell suspensions were made and viable counts performed.

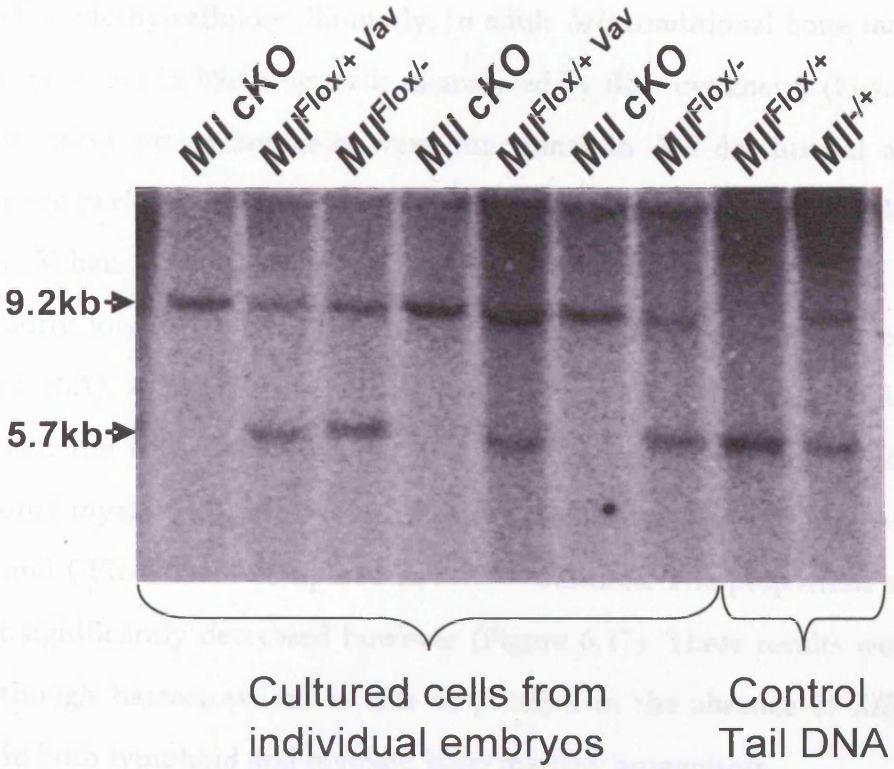


Figure 6.14. High levels of recombination were seen methylcellulose cultures of *Mll* condtional foetal liver cells.

Southern Blot analysis of DNA prepared from cells in methylcellulose cultures of the foetal livers of from a litter of embryos. All embryos were genotyped by PCR using material from the head. Mll conditional embryos are represented by *Mll cKO*. The DNA was digested with *Kpn*1 and incubated with the 5′ probe shown in Figure 4.4. The floxed and the wild type bands ran at the same size (5.7 kb) and the deleted band was 9.2 kb.

6.6 Reduced colony-forming capacity in Mll conditional bone marrow cells

Although there was no phenotype in *Mll* conditional foetal liver stem cells as analyzed by flow cytometry, a functional defect was seen when the cells were cultured in methylcellulose. Similarly, in adult *Mll* conditional bone marrow there was no reduction in LSK stem cells as analysed by flow cytometry (Figure 6.15). To assess whether progenitor cells were functional in *Mll* conditional adults, CFU assays were performed with cells from *Mll* conditional, $Mll^{Flox/Flox}$ *and* $Mll^{Flox/+}$ bone marrow. When cultured in lymphoid conditions, *Mll* conditional cells produced a significantly lower number (3-fold) of Pre-B cell CFUs compared to control cells (Figure 6.16A). A significant reduction in the number of CFUs (1.6-fold) was also seen when the cells were cultured in myeloid conditions (Figure 6.16B). In *Mll* conditional myeloid cultures, a significant decrease was seen in the proportion of BFU-E and CFU-GEMM compared to $Mll^{Flox/+}$ cultures. The proportion of CFU-GM was not significantly decreased however (Figure 6.17). These results would suggest that, although haematopoiesis is able to proceed in the absence of *Mll*, there are defects in both lymphoid and myeloid bone marrow progenitors.

6.7 Bone marrow cells from Mll conditional mice have a profound reconstitution defect

The reduction in CFUs produced from *Mll* conditional progenitor cells suggested that *Mll* conditional stem cells may also have had some hidden functional defects. To assess this, competitive repopulation assays were performed to test whether bone marrow stem cells lacking *Mll* would be able to compete with *Mll* wild type stem cells. $Ly5.2^+$ bone marrow cells from *Mll* conditional, $Mll^{Flox/-}$ and $Mll^{Flox/+}$ mice were transplanted into lethally irradiated recipients with C57BL/6J $Ly5.1^+$ wild type competitor bone marrow cells in a 1:1 ratio or 10:1 ratio. 4 weeks post-transplantation, blood from the recipients was analyzed for the presence of $Ly5.2^+$ and $Ly5.1^+$ cells in the periphery. When transplanted at a 1:1 ratio, *Mll* conditional bone marrow cells gave very low levels of reconstitution (0.9 ± 0.5%

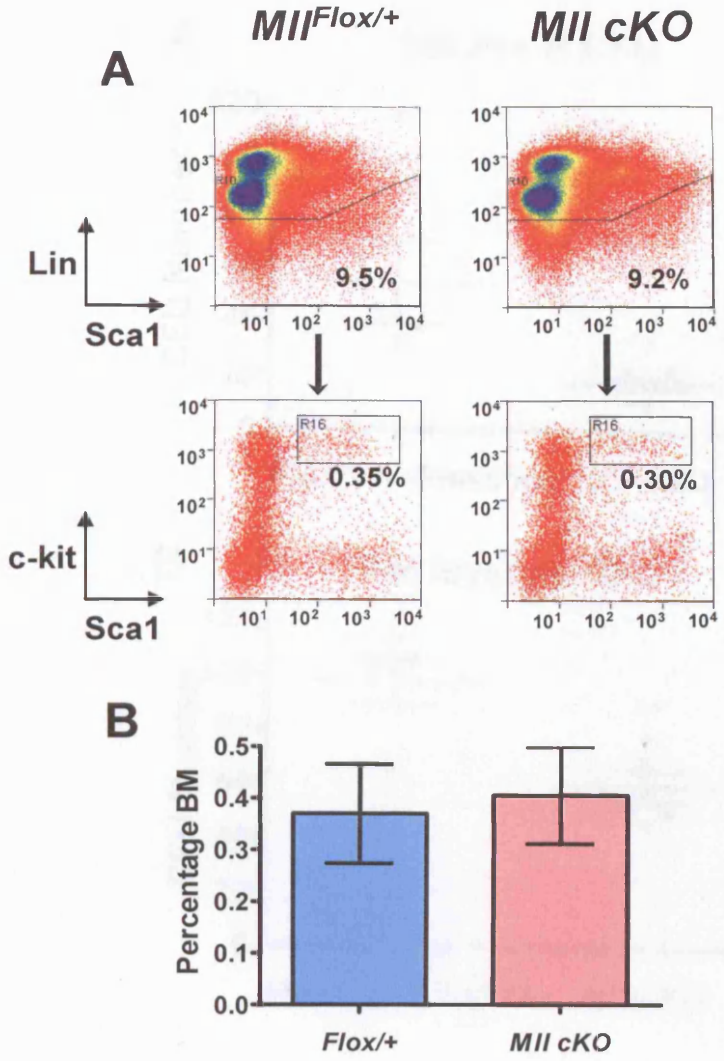


Figure 6.15. Bone marrow HSC number is unaffected in the absence of *Mll*.

Flow cytometric analysis of the HSC population in the bone marrow of *Mll* conditional mice. For all charts, n = 3-4 females. The graphs show the mean and the SEM.

A) Representative dotplots showing flow cytometric analysis of the LSK population in bone marrow. LSK cells were defined as Lin^{-} $Sca1^{+}c\text{-}kit^{hi}$.

B) LSK population in the bone marrow of *Mll* conditional mice. Percentages are shown as the cell numbers obtained were not accurate.

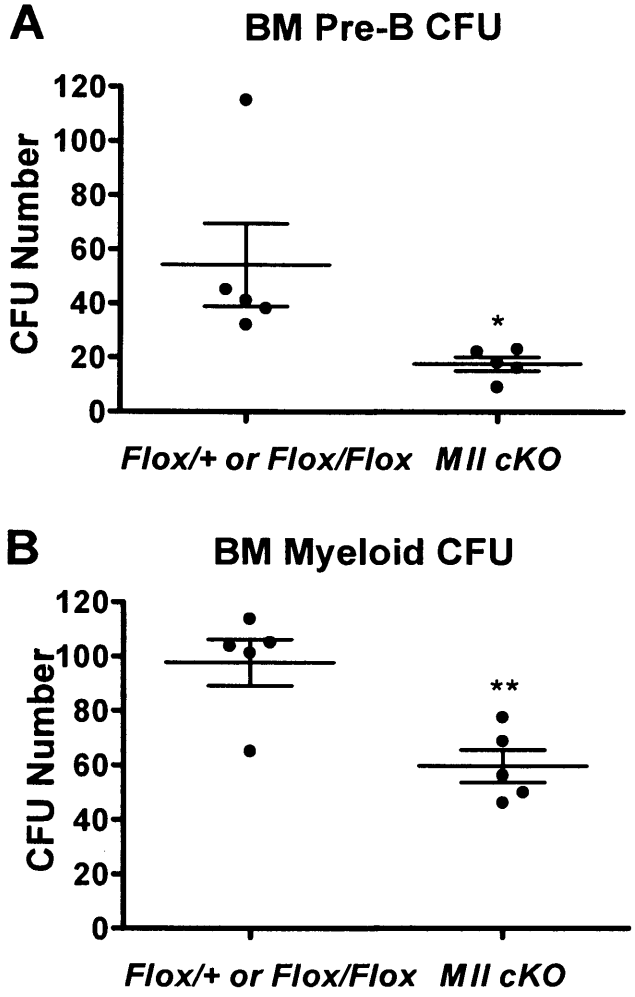


Figure 6.16. Reduced CFU numbers in *Mll* conditional bone marrow.
The graphs show the mean and the SEM.
A) Pre B CFU counts for *Mll Flox/+* and *Mll* conditional (*Mll cKO*) bone marrow cells. 5.5 x 10^4 cells were plated in methylcellulose and CFUs were counted after 7 days.
B) Myeloid CFU counts for *Mll Flox/+* and Mll conditional (Mll *cKO)* bone marrow cells after 10 days in culture in methylcellulose. 2.7 x 10^4 cells were plated in methylcellulose and CFUs were counted after 10 days.

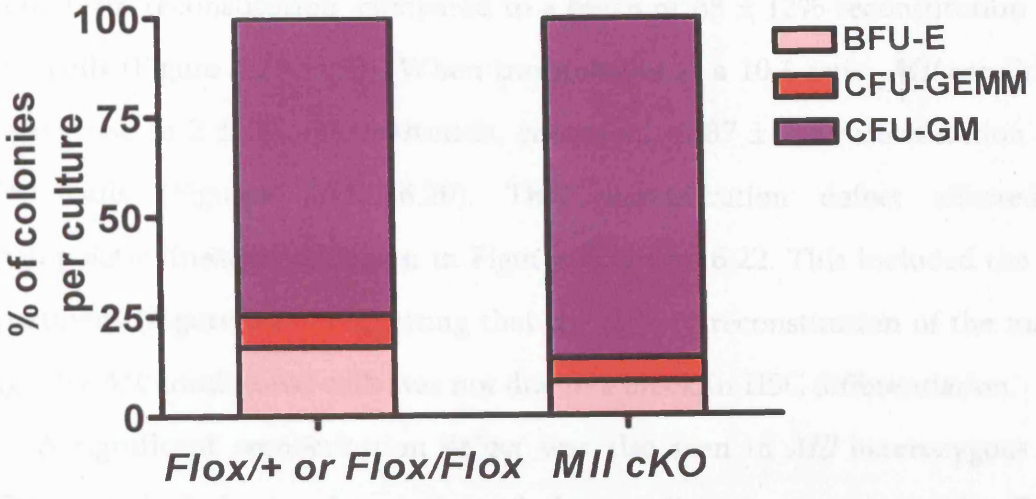


Figure 6.17. *Mll* conditional bone marrow cells produced reduced frequencies of BFU-E and increased frequencies of CFU-GM.
Myeloid CFU counts for *Mll* $^{Flox/+}$ and *Mll* conditional (Mll *cKO)* bone marrow cells after 10 days in culture in methylcellulose. 2.7 x 10^4 cells were plated in methylcellulose and CFUs were counted after 10 days. The graph shows the proportions, in percentages, of BFU-E, CFU-GEMM and CFU-GM in methylcellulose cultures of bone marrow cells after 10 days of culture. The frequency of BFU-E was significantly reduced (p = 0.0125) and the frequency of CFU-GM significantly increased (p = 0.014) in *Mll* conditional (*Mll cKO*) cultures compared to *Mll*$^{Flox/+}$ and *Mll*$^{Flox/Flox}$. For both *Mll* conditional and *Mll Flox* controls, n = 5.

Ly5.2$^+$ cells in the peripheral blood) compared to *Mll*$^{Flox/+}$ cells (37.6 ± 2.7%) (Figure 6.18). *Mll* conditional cells also gave very low levels of reconstitution when transplanted at a 10: ratio (10.35 ± 2.6%) compared to *Mll*$^{Flox/+}$ cells (81.7 ± 1.0%) (Figure 6.18).

At 4 months post-transplantation, the recipients were sacrificed and the bone marrow, spleen and thymus analysed for the presence of Ly5.2$^+$ cells. When transplanted at a 1:1 ratio with wild type cells, *Mll* conditional cells gave less than 1% long term reconstitution, compared to a mean of 68 ± 12% reconstitution with *Mll*$^{Flox/+}$ cells (Figure 6.19, 6.20). When transplanted at a 10:1 ratio, *Mll* conditional cells gave rise to 2 ± 2% reconstitution, compared to 87 ± 7% reconstitution with *Mll*$^{Flox/+}$ cells (Figures 6.19, 6.20). This reconstitution defect affected all haematopoietic lineages, as shown in Figures 6.21 and 6.22. This included the HSC compartment (Figure 6.22) suggesting that the lack of reconstitution of the mature lineages by *Mll* conditional cells was not due to a block in HSC differentiation.

A significant reconstitution defect was also seen in *Mll* heterozygous cells (*Mll*$^{Flox/-}$), particularly in the ability of these cells to reconstitute the T cell compartment (Figures 6.18- 6.22). These results show that, while *Mll* deficient haematopoietic stem cells are able to generate steady state haematopoiesis, their ability to proliferate and self-renew is greatly compromised when they are placed in competition with wild type cells.

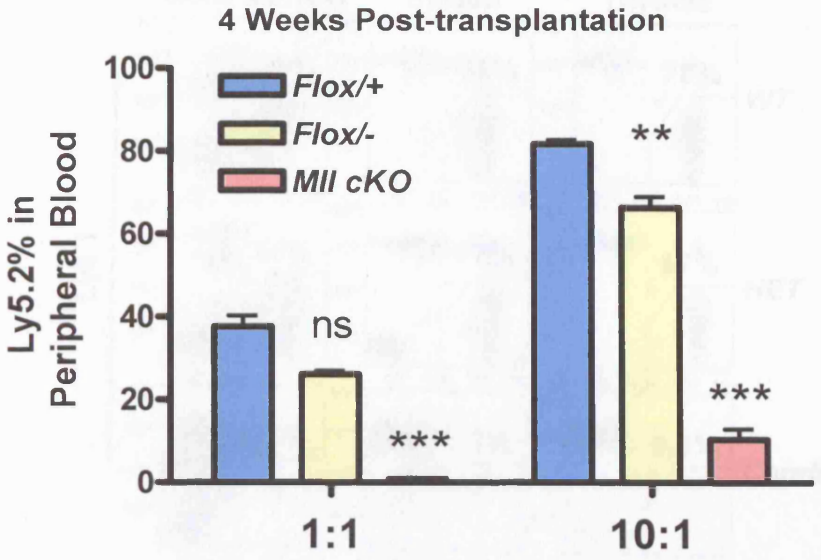


Figure 6.18. *Mll cKO* bone marrow cells have a severe short term repopulating defect.
Reconstitution at 4 weeks post transplantation by *Mll* conditional (*Mll cKO*) bone marrow when mixed with wild type competitors in 1:1 ratio and 10:1 ratios. The graph shows the percentage of Ly5.2+ cells present in the peripheral blood of recipients. For all samples, n=5, except *Flox/+* 1:1, where n=4. The graphs show the mean and the SEM.
(***) $P \leq 0.001$, (**) $P \leq 0.01$, (*) $P \leq 0.05$. All samples were compared to $Mll^{Flox/+}$.

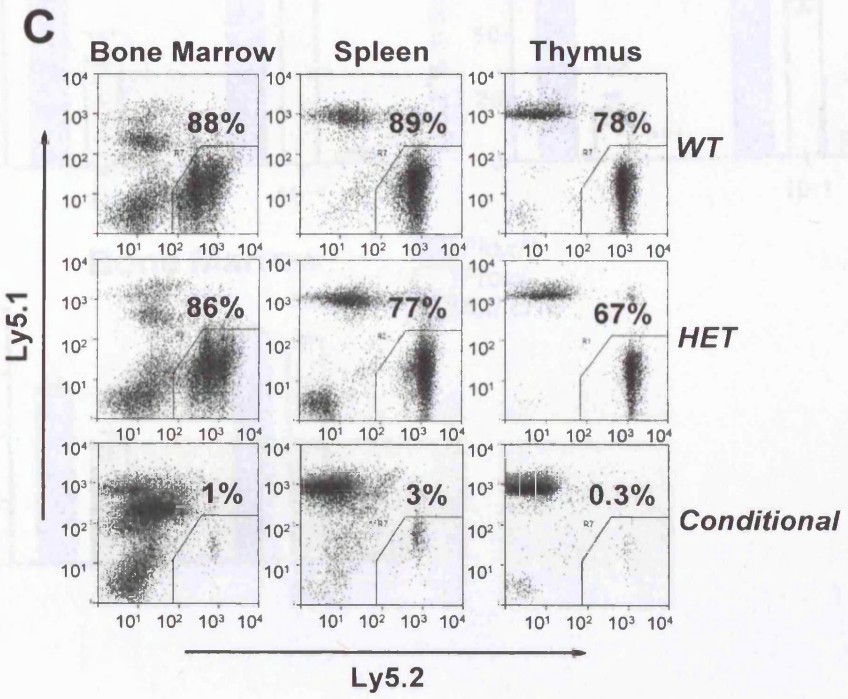


Figure 6.19. *Mll cKO* bone marrow cells have a severe long term repopulating defect.
Reconstitution at 4 weeks post transplantation by *Mll* conditional (*Mll cKO*) bone marrow when mixed with wild type competitors in a 10:1 ratio. Representative flow cytometric plots showing reconstitution levels in the organs of recipients. Single cell suspensions were prepared from spleen, bone marrow and thymus and $Ly5.1^+$ and $Ly5.2^+$ cells were analysed by flow cytometry.

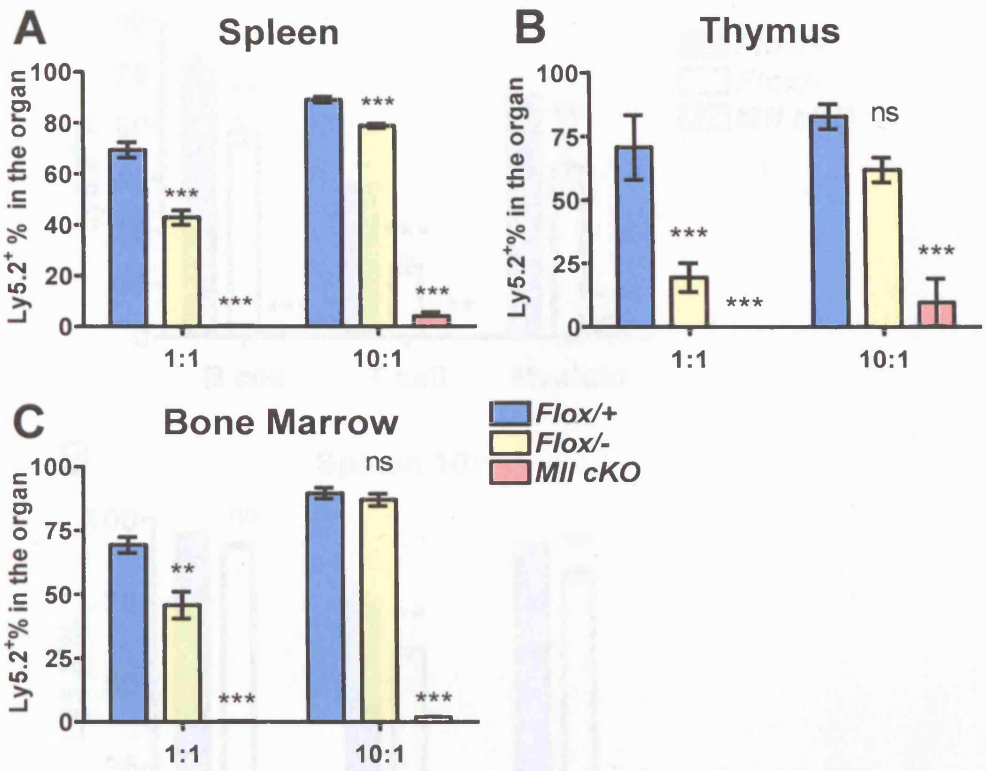


Figure 6.20. *Mll cKO* bone marrow cells have a severe long term repopulating defect.

Reconstitution at 4 months post-transplantation by *Mll* conditional (*Mll cKO*) bone marrow when mixed with wild type competitors in 1:1 ratio and 10:1 ratios. For all samples, n= 4-5, except *Flox/+* 1:1, where n= 3. The graphs show the mean and the SEM. (***) $P \leq 0.001$, (**) $P \leq 0.01$, (*) $P \leq 0.05$. All samples were compared to $Mll^{Flox/+}$.

A) Reconstitution levels in the spleens of recipients as shown by the percentage of $Ly5.2^+$ cells present.

B) Reconstitution levels in the thymuses of recipients.

C) Reconstitution levels in the bone marrow of recipients.

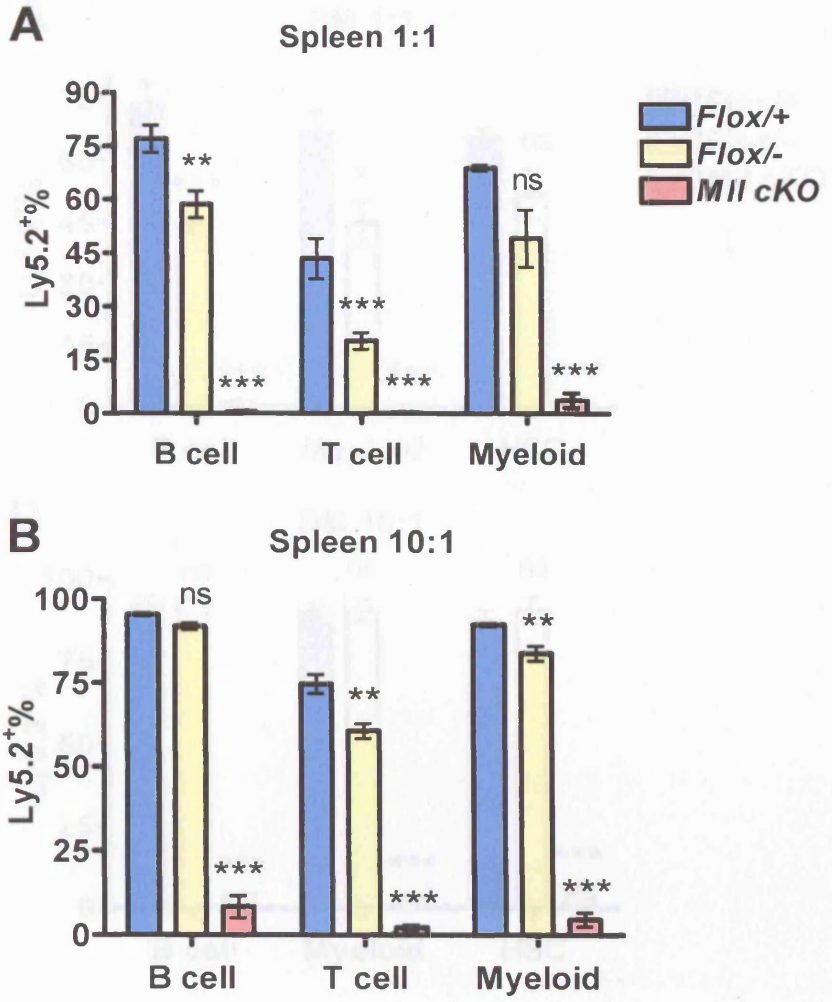


Figure 6.21. *Mll cKO* bone marrow cells have a severe long term repopulating defect affecting all haematopoietic lineages.
Long term contribution, as shown by the percentage of Ly5.2+ cells, to the lineages of the spleen. B cells were defined as $B220^{+}CD19^{+}$, T cells as $CD4^{+}$ or $CD8^{+}$ and myeloid cells as $Gr1^{+}Mac1^{+}$. The graphs show the mean and the SEM
For all samples, n= 3-5. (***) $P \leq 0.001$, (**) $P \leq 0.01$, (*) $P \leq 0.05$. All samples were compared to $Mll^{Flox/+}$.
A) Reconstitution levels when cells were transplanted at a 1:1 ratio with WT competitors.
B) Reconstitution levels when cells were transplanted at a 10:1 ratio with WT competitors.

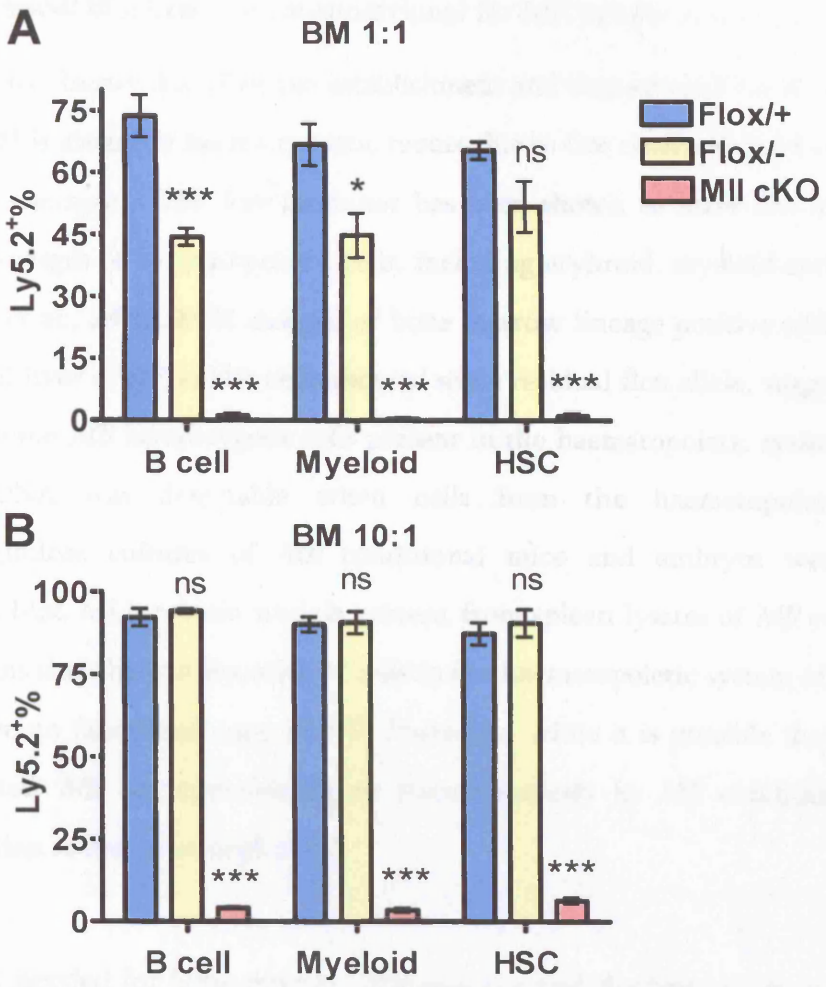


Figure 6.22. *Mll cKO* bone marrow cells have a severe long term repopulating defect affecting all haematopoietic lineages.
Long term contribution, as shown by the percentage of Ly5.2+ cells, to the lineages of the bone marrow. B cells were defined as $B220^{+}CD19^{+}$, HSCs as $Lin^{-}Sca1^{+}c\text{-}kit^{hi}$ and myeloid cells as $Gr1^{+}Mac1^{+}$. The graphs show the mean and the SEM
For all samples, n= 3-5. (***) $P \leq 0.001$, (**) $P \leq 0.01$, (*) $P \leq 0.05$. All samples were compared to $Mll^{Flox/+}$.
A) Reconstitution levels when cells were transplanted at a 1:1 ratio with WT competitors.
B) Reconstitution levels when cells were transplanted at a 1:1 ratio with WT competitors.

6.8 Discussion

Establishment of a line of mice conditional for Mll in haematopoietic tissues

This chapter describes the establishment and characterisation of a line of mice in which *Mll* is absent in haematopoietic tissues due to Cre mediated deletion driven by the *Vav* Cre transgene. The *Vav* promoter has been shown to drive Cre expression in all mature lineages of haematopoietic cells, including eryhroid, myeloid and lymphoid cells (de Boer et al., 2003). PCR analysis of bone marrow lineage positive cells and LSK cells, and foetal liver c-kit$^+$Ter119$^-$ cells showed some residual flox allele, suggesting that there are still some *Mll* heterozygous cells present in the haematopoietic system. However, no floxed DNA was detectable when cells from the haematopoietic organs and methylcellulose cultures of *Mll* conditional mice and embryos were analyzed by Southern blot. MLL protein was also absent from spleen lysates of *Mll* conditional mice. This means that the vast majority of cells in the haematopoietic system of *Mll* conditional mice have no functional copy of *Mll*. Therefore, while it is possible that cells that have not deleted *Mll* are contributing to haematopoiesis in *Mll* conditional mice, their contribution seems to be negligible.

Mll is not needed for bone marrow homeostasis and the formation and maintenance of HSCs

It was surprising to see no effect on the development of mature lineages in the bone marrow of adult mice in the absence of *Mll*. A similar effect has been reported for other genes known to be important for foetal liver haematopoiesis when deleted in adults. For example, the *Aml1* and *Scl* genes are essential for the initiation of definitive haematopoiesis in the embryo. However, when these genes are knocked out conditionally using the Cre-Lox system, no effect is seen on adult HSCs (Ichikawa et al., 2004; Mikkola et al., 2003). It has been suggested that these genes are necessary to form the first definitive HSCs but are not needed for their maintenance. This is not the case with *Mll*, however, as definitive haematopoiesis does occur in *Mll*$^{-/-}$ embryos in the absence of *Mll* but it is severely reduced.

Differences in phenotype between Mll conditional and Mll$^{-/-}$ embryos

Although recombination levels appear to be high in the foetal livers of *Mll* conditional embryos, the haematopoietic cells exhibit a far less severe phenotype than that seen in *Mll*$^{-/-}$ embryos. In particular, the reduction of the HSC pool that is seen in *Mll*$^{-/-}$ embryos, is not seen in *Mll* conditional embryos. There are two possible reasons for this difference. The first is that the *Vav* Cre transgene is active after the point where *Mll* expression is critical. A previous study of *Mll* knockout embryos has shown that *Mll* is needed for the proper formation of the first definitive haematopoietic stem cells in the AGM (Ernst et al., 2004a). It is possible that there are also defects in the AGM in the *Mll*$^{-/-}$ embryos presented here. This would result in fewer HSCs seeding the foetal liver and have a knock on effect on foetal liver haematopoiesis. Although *Vav* is expressed in the AGM (Okada et al., 1998), it is not known if the *Vav* Cre transgene is active in the AGM. It is possible that in *Mll* conditional mice, *Mll* is deleted after the point at which it is necessary for producing normal numbers of stem cells to seed the foetal liver.

Another possibility is that the few cells that escape *Mll* deletion in conditional embryos, either in the AGM or the early foetal liver, are able to give rise to sufficient stem cells to establish foetal liver and then adult haematopoiesis successfully. It would be impossible to detect the presence of these cells as continued expression of the Cre throughout development would mean that their progeny would be homozygous for the *Mll* deletion.

Mll is needed for in vitro expansion of lymphoid and myeloid progenitors

Although no reduction was seen in the number of HSCs in *Mll* conditional embryos, a defect was seen in myeloid progenitors. A similar situation was also observed in the bone marrow of *Mll* conditional mice, where the number of HSCs was equivalent to controls, but the number of colonies formed in both myeloid and Pre-B CFU assays were reduced. So, *Mll* does have a critical function in progenitor cell expansion of both the myeloid and lymphoid lineages. This function is apparent in *in vitro* culture, but is not reflected in the production of mature myeloid and B-lymphoid cells *in vivo*. This supports the data from *Mll*$^{-/-}$ embryos, suggesting that

Mll is necessary for the expansion of progenitor cells in response to cytokines, as is seen in methylcellulose colony forming assays. Preliminary analysis by flow cytometry in *Mll* conditional mice suggested that the numbers of Pre B cells in the bone marrow were normal in comparison to controls (data not shown). This would suggest that the defect seen in Pre B CFU assays is not due to a reduced input, but to an intrinsic defect in the Pre B cells to produce colonies. Further analysis by flow cytometry of the CMP population in the bone marrow will confirm whether this population is also normal in *Mll* conditional mice. If this is the case, the reduction in CFU numbers may be attributable to a defective response of the cells to cytokines or a proliferative defect.

Mll deficient stem cells suffer are unable to self-renew in competitive repopulation assays

The hidden defect in *Mll* deficient progenitor cells led to further examination of the HSC compartment in these mice. Whilst the HSC population showed no apparent abnormalities under homeostatic conditions in *Mll* conditional mice, these cells crashed spectacularly when transplanted with wild type competitors into lethally irradiated recipients. Even when transplanted at a 10:1 ratio, they contributed to less than 10% of the major haematopoietic lineages 4 months post transplantation. The reconstitution defect extended to all mature lineages analysed, suggesting that the lack of reconstitution was due to a defect in the HSCs. This defect appeared to affect both long and short term stem cells, as out competition of the *Mll* conditional cells was seen at both 4 weeks and 4 months post transplantation (Christensen and Weissman, 2001; Spangrude et al., 1995).

It has been noted that the transplantation of HSCs into irradiated recipients leads to increased cell cycle activity of the cells, and proliferative stress (Allsopp et al., 2001; Cheng et al., 2000b; Spangrude et al., 1995). Under these conditions, it is thought that sufficient stem cells need to remain quiescent to enable stem cell self renewal capacity to be maintained post transplantation. It is possible that *Mll* deficient mice have a higher proportion of cycling BM HSCs, which would lead to an inability to self renew when placed under proliferative stress.

There are several other genes which, when deleted, appear to have no or even a positive effect on the numbers of HSCs present in the bone marrow. However, when these stem cells are transplanted, particularly when in competition with wild type cells, they are unable to give rise to effective reconstitution or to serially transplant. These genes include the cyclin dependent kinase (CDK) inhibitor, *p21$^{cip1/waf1}$*. Mice deficient in *p21$^{cip1/waf}$* have apparently normal haematopoiesis and normal numbers of stem cells. However, the stem cell pool contains fewer quiescent cells, and in serial transplantations, these cells show a self renewal defect. This suggests that *p21$^{cip1/waf}$* is needed to maintain stem cells in a quiescent state upon proliferative stress (Cheng et al., 2000b). Mice deficient in *Gfi-1*, a transcriptional repressor which is thought to positively regulate expression of *p21$^{cip1/waf}$*, have slightly elevated numbers of LT-HSC, with a higher proportion or the cells in cycle. However, when these cells are transplanted in competition with wild type cells, very poor levels of reconstitution were seen from the *Gfi-1$^{-/-}$* cells (Hock et al., 2004a). These two genes are of particular interest as *Gfi-1$^{-/-}$* has been shown by luciferase reporter assays to be a target of *Hoxa10*, and by micro array analysis, a target of *Hoxa9* (Ferrell et al., 2005; Magnusson et al., 2007). *Hoxa9* has been identified as a direct target of *Mll* and *Hoxa10* is down regulated in embryoid bodies derived from *Mll$^{-/-}$* ES cells (Ernst et al., 2004b; Milne et al., 2005a; Milne et al., 2002; Milne et al., 2005b). The *Hoxa9* knockout mice share some phenotypes with the *Mll* conditional mice – they have normal numbers of HSCs but these have greatly reduced repopulation capacity when transplanted with wild type competitors (Lawrence et al., 2005b). This similarity in phenotype between *Hoxa9*, *Gfi-1* and *p21$^{cip1/waf}$* deficient HSCs, suggests a common causality by down regulation of *p21$^{cip1/waf1}$* as shown in the model presented in Figure 6.23. This would also suggest that the phenotype seen in *Mll* deficient stem cells is due to a failure to self renew under proliferative stress, caused by the down regulation of *p21$^{cip1/waf}$* and an inability of the stem cells to remain quiescent. Analysis by Q-PCR of the levels of *Hoxa9*, *Hoxa10*, *Gfi-1* and *p21$^{cip1/waf}$* in *Mll* deficient stem cells may confirm if this is indeed the case. Cell cycle analysis would also reveal whether the cell cycle profile of *Mll* deficient HSCs was abnormal, resulting in fewer cells residing in G_0.

It is interesting to note that the phenotypes seen in mature myeloid and lymphoid cells in the above knockout models are quite divergent. The *p21$^{cip1/waf1}$* deficient mice have no apparent abnormalities in the lymphoid or myeloid compartments, whereas *Gfi1* deficient mice have no granulocytes and reduced numbers of lymphoid cells and progenitors (Cheng et al., 2000b; Hock et al., 2004a). The *Hoxa9* mice also have reduced numbers of myeloid and lymphoid cells (Lawrence et al., 2005c; Lawrence et al., 1997). These differences in phenotype would suggest that after HSC differentiation, the functions of these proteins diverges, and they may no longer be part of the same pathway.

It is also possible that *Mll* may be directly responsible for the regulation of CDK inhibitors. For example, in *Mll* deficient immortalised fibroblasts, MLL has been shown to cooperate with the tumour suppressor Menin to regulate the transcription of p27 (Milne et al., 2005c). It is possible that *Mll* may be active in regulating other CDKis such as *p21$^{cip1/waf1}$* in a cell context dependent manner (Figure 6.23). Therefore, the identification of *Mll* targets in HSCs would prove whether this is the case and also identify other genes contributing to the knockout phenotype.

The effect of Mll deletion is dose dependent

The reduced repopulating capacity of heterozygote (*Mll$^{Flox/-}$*) cells shows that the dosage of *Mll* is also important. Although the heterozygous cells had an intermediate phenotype in the reconstitution of all haematopoietic cell types, the most profound effect was seen on the T cell compartment. The reconstitution of all T cells was more severely reduced in comparison to *Mll$^{Flox/+}$* control cells, and reconstitution in the thymus was only 20% when the cells were transplanted in a 1:1 ratio with wild type cells (Figure 6.20B). This suggests a further role for *Mll* specifically in the expansion of T cells.

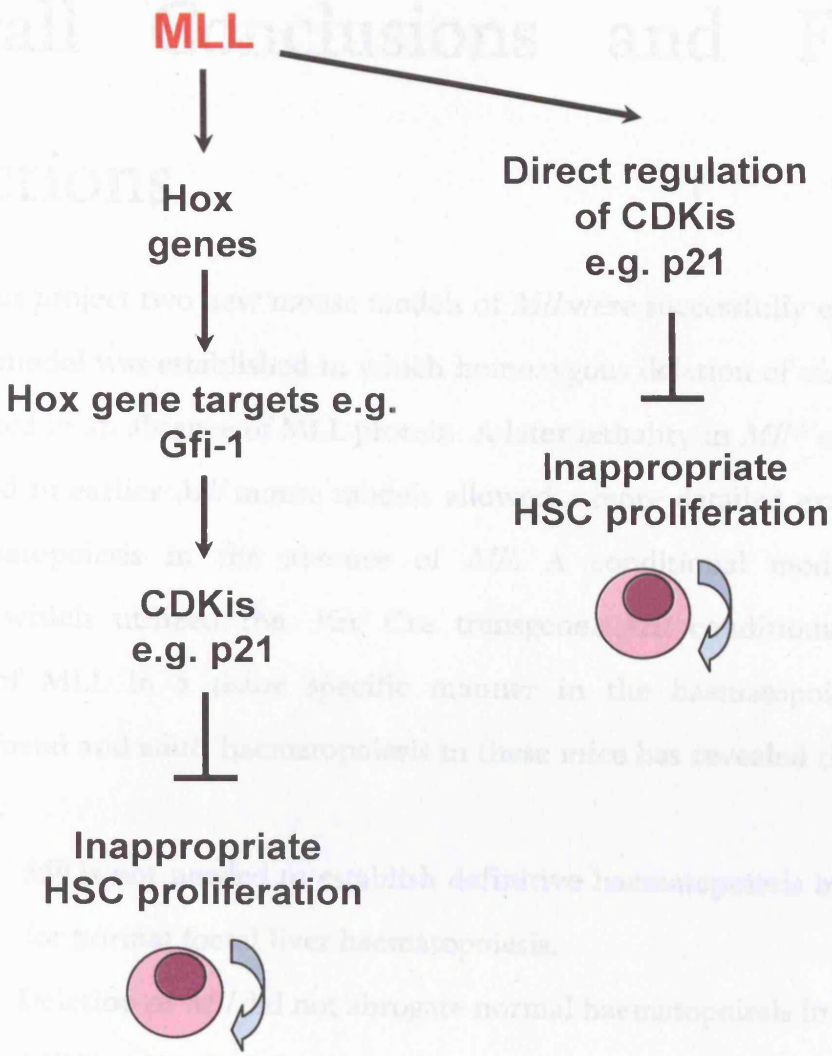


Figure 6.23. A Possible mechanism for the function of *Mll* in the control of HSC self renewal.
MLL may positively regulate control of the expression of multiple *Hox* genes. The targets of these *Hox* genes include *Gfi-1*, which in turn regulates the expression of $p21^{cip1/waf1}$. $p21^{cip1/waf1}$ acts to maintain the quiescent state of long term HSCs. *Mll* may also directly control the expression of cyclin dependent kinase inhibitors.

Chapter 7

Overall Conclusions and Future Directions

In this project two new mouse models of *Mll* were successfully established. A null mouse model was established in which homozygous deletion of exons 9 and 10 of *Mll* resulted in an absence of MLL protein. A later lethality in $Mll^{-/-}$ embryos than was observed in earlier *Mll* mouse models allowed a more detailed examination of foetal haematopoiesis in the absence of *Mll.* A conditional model was also established which utilized the *Vav* Cre transgene. *Mll* conditional mice lost expression of MLL in a tissue specific manner in the haematopoietic system. Analysis of foetal and adult haematopoiesis in these mice has revealed the following findings:

- *Mll* is not needed to establish definitive haematopoiesis but is needed for normal foetal liver haematopoiesis.
- Deletion of *Mll* did not abrogate normal haematopoiesis in adult mice.
- *Mll* foetal and adult stem cells cannot compete with wild type cells in competitive reconstitution assays.
- *Mll* is needed for the proper expansion of progenitor cells in vitro.
- The *Mll* phenotype is dose dependent.

Mll conditional mice: a useful model for future investigations into Mll function

The new conditional mouse model for *Mll* provides a useful tool for studying the role of *Mll* in a variety of processes. Further work is still needed to clarify the exact role of *Mll* in the regulation of adult haematopoietic stem cells, including

analysis of the downstream targets in *Mll* conditional mice and cell cycle analysis of *Mll* deficient HSCs. Further work is also needed to assess whether *Mll* is needed outside of normal homeostatic haematopoiesis, during the response to immunological challenge. The apparent role of *Mll* in regulating GATA-3 and cytokine production in memory Th2 cells (Yamashita et al., 2006) would suggest further roles in the T and B lymphoid response to infection. This is supported by the effect on repopulation of the T cell compartment by *Mll* heterozygous cells seen here.

The new conditional model can be crossed to other tissue specific Cre expressing mice, to assess whether *Mll* functions outside of the haematopoietic system. For example, there is growing evidence for a role for *Mll* in neurodevelopment. *Mll* has been linked to H4 acetylation in the adult brain and neuronal differentiation (Kim et al., 2007; Wynder et al., 2005). Using transgenic mice expressing Cre under the control of central nervous system specific promoters, e.g. the Glial Fibrillary Acidic Protein (GFAP), a mouse model where *Mll* is conditional in the central nervous system could be established (Zhuo et al., 2001). This could be used to further investigate the role of *Mll* in neural development. Other tissue specific Cre transgenes could be used to elucidate the role of *Mll* in other cell types and processes. The new conditional model described here represents an exiting opportunity to explore the function of *Mll* in murine development.

Chapter 8

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