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The Role of Mutant DNMT3a in Ageing and in the Regulation of Normal and Malignant Haematopoiesis

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Department of Medical Biology
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Abstract

DNA methyltransferase 3a (DNMT3a) is a *de novo* DNA methyltransferase that can establish DNA methylation signatures in cells. Recently, germline mutations in DNMT3a were found to cause an intellectual disability and overgrowth disorder named Tatton-Brown-Rahman syndrome and somatic mutations in *DNMT3a* constitute one of the most common mutations in haematological malignancies. The findings presented in this thesis inform on the role of the most common DNMT3a mutation, R882H, using a novel murine model with an emphasis on ageing, haematopoiesis and hematopoietic malignancies.

The mutant *Dnmt3a* mouse model was created using CRISPR/Cas9 genome editing technology to introduce the most common R882H mutation into the murine *Dnmt3a* locus at residue R878H (murine homologue of R882). Breeding of *Dnmt3a*^{R878H/+} mice revealed an inability of female *Dnmt3a*^{R878H/+} mice to deliver healthy offspring. This was a result of a maternal defect as surrogate mice could produce viable *Dnmt3a*^{R878H/+} pups through IVF. *Dnmt3a*^{R878H/+} mutant mice also had a shorter lifespan compared to their wt littermates when aged. The *Dnmt3a*^{R878H/+} aged mice were more susceptible to liver disease that was characterised by extensive hepatocyte steatosis and hepatocyte carcinoma and were also more likely to develop leukaemia with B cell morphology compared to their wt littermates.

To determine whether the *Dnmt3a*^{R878H/+} mutant mice had defects in haematopoiesis before overt haematological malignancy, the haematopoietic system was analysed under steady state conditions and in haematopoietic competition assays. There was evidence of a defect in early T cell development in the thymus characterised by significantly fewer immature T cell progenitors in *Dnmt3a*^{R878H/+} mutant mice compared to their wt littermates.

To resolve whether *Dnmt3a*^{R878H/+} mutant haematopoietic stem and progenitor cells (HSPCs) had a competitive advantage over wt HSPCs, HSPCs from the *Dnmt3a*^{R878H/+} mutant mice were competitively transplanted alongside wt HSPCs into lethally irradiated wt recipient mice. It was shown that *Dnmt3a*^{R878H/+} HSPCs and their descendants outcompeted their wt counterparts after 6 months, with some evidence that *Dnmt3a*^{R878H/+} HSPCs had already begun to accumulate after 3 months. These findings were extended to show that *Dnmt3a*^{R878H/+} HSPC-derived cells can also

outcompete wt HSPC-derived cells in other haematopoietic tissues, such as the thymus and spleen. Furthermore, it was also shown that *Dnmt3a*^{R878H/+} HSPCs also have an increased serial transplantation capacity compared to their wt counterparts.

To better understand how the *Dnmt3a*^{R878H} mutation promotes the development of haematological malignancies, a model of γ -irradiation induced thymic lymphoma was employed where the cancer cell of origin arises from a HSPC. It was shown that *Dnmt3a*^{R878H/+} mutant mice developed thymic lymphoma at a significantly faster rate than their wt littermates. Gene expression changes in *Dnmt3a*^{R878H/+} HSPCs that might account for their increased predisposition to leukaemogenesis revealed that *Dnmt3a*^{R878H/+} LSK cells have an underlying disturbance in Notch signalling and that upon γ -irradiation, they have a blunted induction of the p53 signalling network compared to wt HSPCs. Many other cellular pathways were also deregulated in *Dnmt3a*^{R878H/+} HSPCs, and they will be the subject of future experiments.

Overall, it was shown that heterozygous *Dnmt3a*^{R878H} mutations cause a vast array of abnormalities including problems in pregnancy, metabolic defects leading to obesity and liver pathologies as well as haematological disturbances leading to an accumulation of HSPCs in the bone marrow and a susceptibility to the development of haematological malignancies.

Declaration

This is to certify that:

- (i) This thesis comprises only of my original work towards the Doctor of Philosophy, except where indicated in the preface;
- (ii) Due acknowledgement has been made in the text to all other material used; and
- (iii) The thesis is fewer than 100,000 words in length, exclusive of table, figures, bibliographies and appendices.

Erin M. Lawrence

Preface

In accordance with the regulations of the University of Melbourne, I acknowledge that parts of the work presented in this PhD thesis involved collaboration with other people detailed below.

Marco Herold, Andrew Kueh, Martin Pal and Lin Tai created the *Dnmt3a*^{R878H} mouse model prior to my involvement with the project.

All sample collection and preparation for the RNA seq experiment was performed by me with the exception of the operation of the FACs sorting machines which was performed by the WEHI FACs facility staff. The RNA sequencing run on the Illumina was performed by Stephen Wilcox and the RNA sequencing data was analysed by Wei Shi and Yang Liao.

Organ dissection and fixation was performed by me, but the histology slides were prepared by the histology facility at WEHI and the pathology assessments were performed by the APN pathology service.

The remainder of this thesis comprises my original work. I assess my contribution to be 95%. The works presented in this thesis are unpublished.

This work was supported by a jointly funded PhD scholarship by Australian Rotary Health District 9650 and the Walter and Eliza Hall Institute. I also received a Melbourne University fee offset scholarship, a Walter and Eliza Hall top-up scholarship and a Melbourne abroad travel scholarship.

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I would also like to thank my PhD committee, Assoc. Prof. Marnie Blewitt, Prof. Jerry Adams and Prof. Doug Hilton, for their ongoing support and feedback during my PhD. In particular I would like to thank Doug, who has been my mentor since my undergraduate science degree, for opening up the doors of WEHI to me.

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List of Abbreviations

5-Fu	5-Flourouracil
5mC	5-methyl cytosine
ADD	ATRX-DNMT3-DNMT3L
A	adenine
Ac	acetylation
AML	acute myeloid leukaemia
APN	Australian phenomics network
B-ALL	B cell acute lymphoblastic leukaemia
bp	base pairs
C	cytosine
CHIP	clonal haematopoiesis of indeterminate potential
CLP	common lymphoid progenitor
CMML	chronic myelomonocytic leukaemia
CMP	common myeloid progenitor
CpG	cytosine preceding guanine
Cre	Cre recombinase
CSL	CBF1/Suppressor of Hairless/LAG-1
DF	degrees of freedom
DN	double negative
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMT1	DNA methyltransferase 1
DNMT3a	DNA methyltransferase 3a
DNMT3b	DNA methyltransferase 3b
DNMT3L	DNA methyltransferase-like
EFS1	EF1a promotor
ERK	extracellular signal-regulated kinase
EZH2	histone-lysine- <i>N</i> -methyltransferase enhancer of zeste homolog 2
FACS	fluorescence activated cell sorting
FDR	false discovery rate
G	guanine
γ -irradiation	gamma irradiation
GMP	granulocyte myeloid progenitor
GO	gene ontology
Gy	gray
H&E	haematoxylin & eosin
H3K4	histone 3 lysine 4
H3K9me3	histone 3 lysine 4 tri-methylation

H3K36me3	histone 3 lysine 36 tri-methylation
HAT	histone acetylase
HCT	haematocrit
HDAC	histone deacetylase
HDAC1	histone deacetylase 1
HGB	haemoglobin
HI-FCS	heat inactivated foetal calf serum
HKMT	histone lysine methyltransferase
HSC	haematopoietic stem cell
HSPCs	haematopoietic stem and progenitor cells
IV	intravenous
Ig	Immunoglobulin
IVF	<i>in vitro</i> fertilisation
KEGG	Kyoto encyclopedia of genes and genomes
KO	knockout
LSK	lineage negative SCA1+ cKIT- cells
LT-HSC	long term-haematopoietic stem cell
LUC	large unstained cell
MDS	myelodysplastic syndrome
Me	methylation
MEP	megakaryocyte erythroid progenitor
miRNAs	micro-RNAs
mL	millilitre
MPP	multipotent progenitor
mRNA	messenger RNA
Mtase	methyltransferase
Mx1	Mx dynamin-like GTPase 1
NGS	next gen
NK	natural killer
Notch-ICD	notch-intracellular domain
NSD1	nuclear receptor binding SET domain protein 1
OGID	overgrowth and intellectual disability disorder
ORF	open reading frame
PAM	protospacer adjacent motif
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
plpC	polyinosinic: polycytidylic acid
PLT	platelets
PWWP	proline-tryptophan-tryptophan-proline motif
RB	retinoblastoma tumour suppressor protein
RBC	red blood cell

RNA	ribonucleic acid
RNA seq	ribonucleic acid sequencing
SEM	standard error of the mean
sgRNA	single guide RNA
ST-HSC	short term-haematopoietic stem cell
T	thymine
T-ALL	T cell acute lymphoblastic leukaemia
TBR5	Tatton-Brown-Rahman Syndrome
TCR β	T cell receptor β
WBC	white blood cell
WEHI	Walter and Eliza Hall Institute
wt	wildtype
WT1	Wilms' tumour 1
μg	microgram
μL	microlitre
μM	micromolar

CHAPTER 1

INTRODUCTION

1.1 The human genome

Deoxyribonucleic acid (DNA) was first isolated from blood cells in 1869 by Friedrich Miescher ¹. Almost one hundred years later, the structure of DNA was solved. The discovery was credited to Francis Crick and James Watson in 1953 although their discovery was based on x-ray diffraction data collected by Raymond Gosling and Rosalind Franklin years earlier ². It was revealed that DNA existed in a helical structure made up of four different bases, or nucleotides. The DNA double helix has a phospho sugar backbone and is held together by hydrogen bond interactions between the four nucleotides adenine (A), cytosine (C), guanine (G) and thymine (T). The nucleotides are arranged such that adenine complementarily binds thymine and cytosine binds with guanine, referred to as base pairs (bp). When speaking of the discovery of the structure of DNA, Francis Crick was quoted as saying that they had “discovered the secret of life” ³. Indeed, DNA holds all of the information required for life packaged into a code consisting of just four units. How these four nucleotides, ATCG, could encode all of the information required for human development has captivated scientists for decades. There are 6.27-6.37 giga base pairs of nucleotides that encode for the human genome ⁴. To put that differently, if the DNA from a single human cell was laid out in front of you, it would be more than two metres long ⁴. J. Craig Venter was determined to decipher the DNA code and with private funding is credited with creating the first draft sequence of the entire human genome ⁵. When the human genome project was first conceived ⁶, one would not have predicted how far reaching the benefits would be. In medicine, deciphering the DNA code in humans has been essential for the progression of modern medicine. By sequencing the DNA and creating a reference genome, scientists have been able to determine the underlying genetic causes of many diseases. Deviations in the genetic code that result in irreversible modifications to the DNA are called mutations. These mutations have the potential to cause congenital defects ⁷ and diseases like cancer ⁸. Understanding the underlying genetic cause of disease is essential to producing targeted and effective therapeutics, however, not all diseases can be attributed to genetic mutations alone.

With an increasing understanding of how DNA encodes the building blocks of life, it became evident that there were additional layers of genetic regulation that were key to reading the genetic code. The same DNA sequence is present in all somatic cells of an organism, with few exceptions, and yet highly specialised cells with very different functions exist. One notable exception is in lymphocytes, specialised white blood cells (WBC), where segments of DNA are

removed in order to assemble specialised B and T cell antigen receptors ⁹. Another exception are red blood cells and platelets, which have no nucleus and no DNA, at least in mammals ¹⁰. Despite these exceptions, the DNA of all other somatic cells retains identical DNA. The diversity of cell types within the body raised the question of how DNA is regulated under different circumstances to instruct the development of different cell types.

1.1.1 The organisation and regulation of DNA

The organisation of the DNA is key to regulating cell diversity as it controls the regions of the DNA that are accessible and can be 'read' or are more inaccessible and therefore 'silent'. DNA can be subdivided into different regions which include open reading frames (ORF), that can produce gene products, and regulatory elements, that control the transcription of genes ¹¹. ORFs are regions in the DNA that begin with a start codon, contain a stop codon and end in transcriptional termination site ¹¹. ORFs are often associated with genes that encode protein products. The gene can be transcribed by RNA polymerase to 'read' and produce a complementary strand of RNA. Genes can have coding and non-coding regions called exons and introns, respectively. The RNA produced from the gene must undergo splicing to remove the introns to ensure that only the remaining coding regions are present in the mature messenger RNA (mRNA) ¹². Importantly, splice variants of genes can be produced by removing alternative exons which can lead to different gene products from the same gene ¹²⁻¹⁴.

The transcription of genes is tightly regulated through regulatory elements in the DNA. Gene transcription is initiated by the activation of the gene promotor. The promotor is found upstream (5') of the gene body and sits in close proximity to the transcriptional start site ¹⁵. Transcription factors, which are DNA binding proteins, can initiate gene transcription through complex formation and recruitment of RNA polymerase ¹⁶. Importantly, certain transcription factors can also repress gene expression, for example by blocking the access of RNA polymerase to the DNA thereby controlling gene expression ¹⁷. The expression of the gene can be further regulated by enhancer elements which are regions of DNA with regulatory activity that can sit up to 1 Mbp (1,000,000 bp) away from the gene itself ¹⁸. Coordination of which genes are transcriptionally active and which genes are repressed is determined by complex interactions that regulate genome accessibility to transcription factors.

1.2 The epigenome

The transcription of genes is not only regulated by the binding of transcription factors, promoters and enhancers but also by the physical accessibility of the gene to these regulatory elements. The physical accessibility of DNA can be regulated through modifications of the DNA itself that hinder the ability of transcription factors to bind the DNA and also through controlling the coiling of DNA around DNA-associated proteins, called histones. Together, the reversible modifications of the DNA and modifications to histones can further control gene transcription¹⁹. These reversible modifications and the 3-dimensional coiling of DNA are referred to as the epigenome and the proteins that coordinate these modifications are called epigenetic modifiers.

1.2.1 Histone modifications

As depicted in Figure 1.1, the 3-dimensional organisation of DNA is highly ordered. The DNA double helix is coiled around eight histones that facilitate the looping of DNA²⁰. Collectively, the histone and coiled DNA are termed nucleosomes. These nucleosomes can exist as loosely packed DNA around histones called euchromatin, or as tightly coiled DNA around histones called heterochromatin. The packaging of DNA into euchromatin or heterochromatin is determined by reversible histone tail modifications as depicted in Figure 1.1. Methylated histone tails are repressive marks and associated most strongly with heterochromatin²¹ whereas acetylation (Ac) marks active areas of the genome and is associated with euchromatin²². The physical coiling of DNA can partially control the accessibility of the DNA to transcription factors²². Genes that are associated with heterochromatin are generally inaccessible and therefore repressed. On the other hand, genes that are associated with euchromatin are more accessible and are therefore generally active.

The methylation and acetylation of histone tails is performed by specialised histone modifying enzymes. Briefly, histone methylation normally occurs on lysine (K) or arginine on the side chains of histones, called histone tails. Histone methylation can occur to different extents with lysine residues existing as mono-, di- or tri-methylated²³. Furthermore, arginine can also exist as mono- or di-methylated²³. The enzymes that facilitate the methylation of lysine residues are called histone lysine methyltransferases (HKMT)^{23,24}. The methylation of histone tails is associated with the repression of gene transcription. On the other hand, histone acetylation is

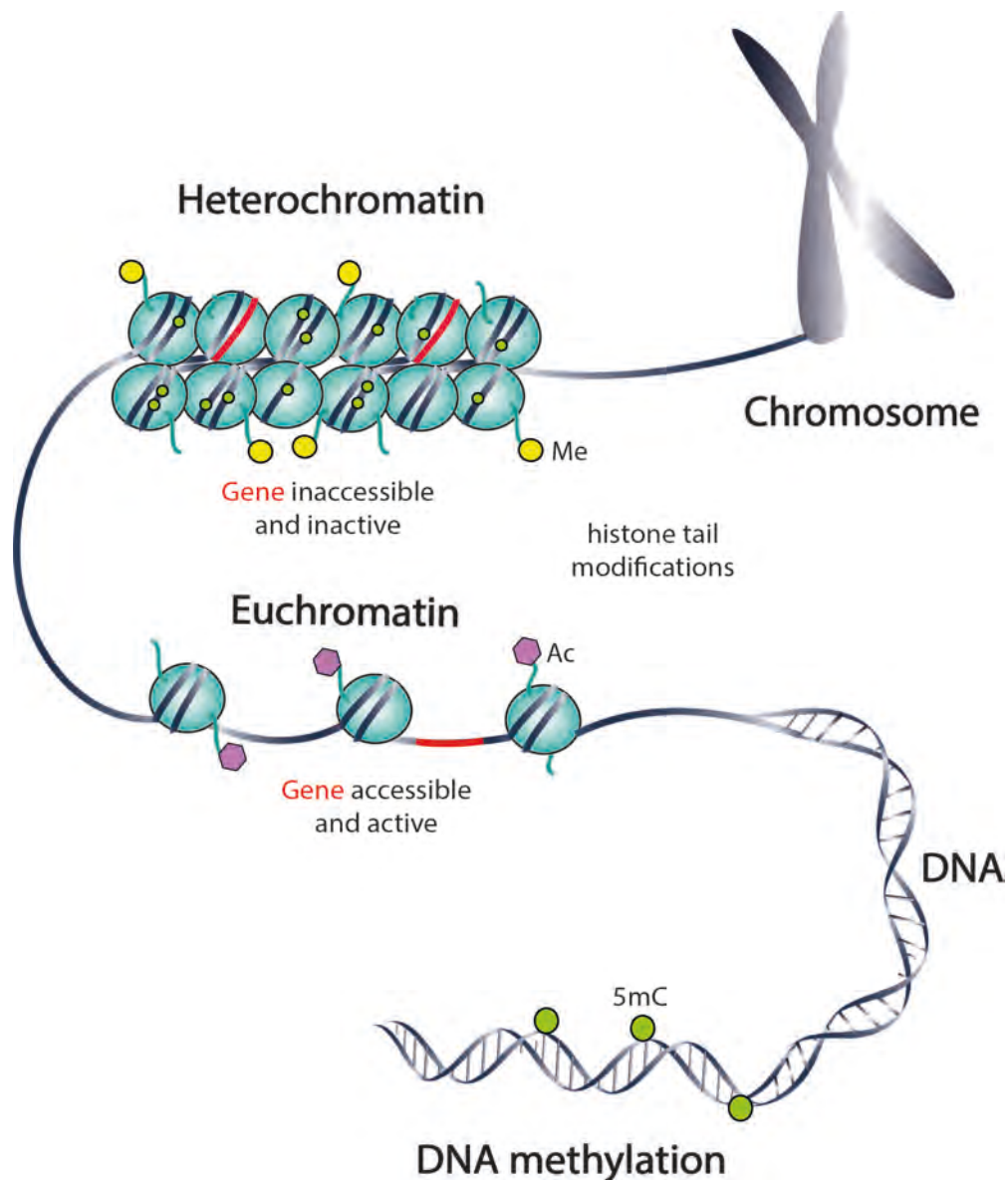


Figure 1.1 the 3-dimensional organisation of DNA

DNA is arranged into a 3-dimensional structure which dictates gene accessibility. DNA can be arranged into heterochromatin where DNA is tightly coiled around histones that are marked by repressive methylation (Me) marks. Genes in heterochromatin are inaccessible and therefore generally silenced. In euchromatin, DNA can be loosely coiled around nucleosomes that are characterised by acetylated (Ac) histones. Genes that are found in euchromatin are accessible and therefore more likely to be actively transcribed. Further regulation of gene transcription is controlled by DNA modifications like DNA methylation which can preclude the binding of transcription factors to the DNA and play a significant role in gene activation and silencing.

associated with active gene transcription. Histone acetylation is performed by histone acetyltransferases (HATs) and its effects are counteracted by the activity of the histone deacetyltransferases (HDACs) ²³. Together, these epigenetic modifiers regulate the accessibility of the gene, but are complemented by further regulatory modifications to the DNA itself.

1.2.2 DNA modifications

Reversible modifications to the DNA itself, such as DNA methylation further regulate the accessibility of a gene and therefore play an important role in the direct regulation of its transcription ^{25,26}. DNA methylation can be thought of as sitting at the apex of the DNA regulatory hierarchy as it makes up the highest order control over gene expression by directly altering the properties of DNA.

1.3 Introduction to DNA methylation

DNA methylation was first described in studies of pneumococcus around the same time that the structure of DNA was discovered ^{27,28}. The authors were trying to isolate the active compound that caused the transformation of one strain of pneumococcus into a more virulent strain with different morphology *in vitro*. The authors showed that the change in morphology was reproducible and inheritable and was caused by a nucleic acid with different properties to other nucleic acids. A few years later, modified cytosine was isolated from the thymus of a calf by showing that it would run separately from cytosine in chromatography. This led to the identification that DNA methylation marks primarily occurred on cytosine ²⁹. The methylated cytosine had a methyl group added to the C5 position of cytosine creating 5-methyl cytosine (5mC) ³⁰ as shown in Figure 1.2. While the proportion of methylated cytosines remains fairly stable throughout life, DNA methylation and demethylation are dynamic processes involved in the intricate regulation of gene expression at different stages of life ^{31,32}. Understanding when, where and why different regions in the genome are methylated or unmethylated has been the subject of much interest since DNA methylation was first described ^{27,28}.

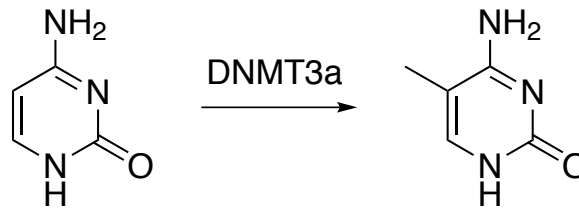


Figure 1.2 Cytosine is converted to 5-methyl cytosine by DNA methyltransferase enzymes

Chemical structure of cytosine (left) and 5-methyl cytosine (right). The enzyme depicted is DNA methyltransferase 3a (DNMT3a) which is one example of a protein capable of catalysing this conversion.

1.3.1 Temporal and spatial changes in DNA methylation.

DNA methylation is a dynamic process and the DNA methylation patterns of cells can vary drastically. DNA methylation has been shown to be critical during embryonic development where the methylation landscape of the zygote is wiped almost clean, before being re-established^{31,32}. This is to ensure that the methylation patterns of the terminally differentiated oocyte and sperm are abolished to restore totipotency to the zygote³³. However, some regions of the genome are protected from this demethylation, such as imprinted loci³⁴. The processes that determine when and how the DNA methylation pattern are restored are the subject of ongoing investigation. The new methylation pattern of the DNA makes up the template of somatic methylation signatures and is critical for genomic imprinting and X chromosome inactivation³⁵. DNA methylation has also been shown to be essential for silencing retroviral elements³⁶ as well as for coordinating tissue-specific gene expression during differentiation^{37,38}.

In addition to temporal changes in DNA methylation, spatial differences are also apparent. The genome can be separated into discrete regions based on the amount of DNA methylation as depicted in Figure 1.3. In vertebrates, at any given time, approximately 60-90% of CpG sites (cytosine preceding guanine) are methylated in the genome³⁹. However, not all CpG sites are methylated equally. Regions of the DNA that are rich in CpG dinucleotides are called CpG 'islands' and are associated with very low levels of 5mC⁴⁰. Unmethylated CpG islands are

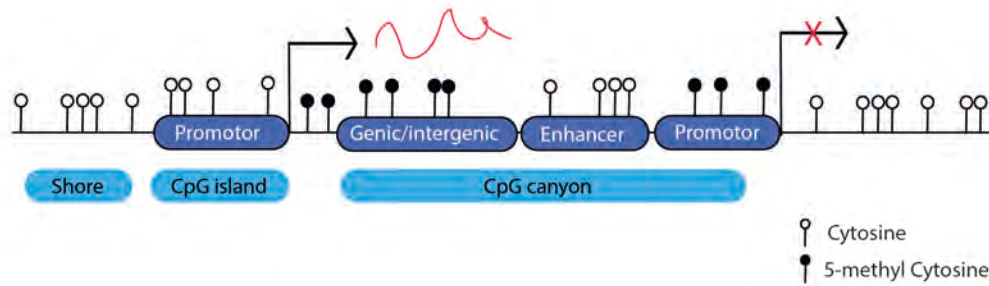


Figure 1.3 spatial composition of 5mC in the vertebrate DNA

DNA is depicted as a black horizontal line where unmethylated cytosine is shown by open circles and 5-methyl cytosine is shown by closed circles. Unmethylated CpG island promoters are associated with active transcription, whereas methylated CpG island promoters are associated with gene repression. CpG islands have higher frequency of CpG sites compared to shores and canyons.

conserved during development and between cell types ⁴¹. These islands are commonly located close to regulatory regions like enhancers and promoters of genes. While these islands are almost uniformly unmethylated, CpG islands are surrounded on each side by progressively few CpG dinucleotides which display variable methylation in a tissue-specific manner ^{38,42}. Regions flanking CpG islands are termed 'shores' and have been shown to be critical for transcriptional regulation in different cell types ⁴³. There is very little methylation found in gene bodies and these regions are aptly referred to as 'canyons'. While canyons can contain CpG islands when assessed overall, they show very sparse methylation. Genes expressed during embryonic development are often found within CpG canyons ⁴⁴. Despite low levels of methylation, associated with gene activation, many of the genes that regulate development residing in canyons are repressed. Although, the repression of these developmental genes is thought to occur through repressive histone 3 lysine 27 tri-methylation (H3K27me3) marks rather than being mediated by DNA methylation ⁴⁴. In general, promotor islands that are hypomethylated tend to correspond with genes that are actively transcribed, whereas islands that are hypermethylated are repressed ⁴⁵. The relevance of methylation at shores and canyons is less well understood but may be key in demarcating regulatory and non-regulatory regions of the genome ⁴⁴. Indeed, disrupting the demarcation by inhibiting DNA methylation through genetic

deletion of DNA methyltransferase enzymes can lead to the corrosion of CpG island shores and the aberrant expression of developmental genes, like *Ndn*, *Gtl2* and *Peg3*⁴⁴. The distribution of 5mC marks across shores, islands and canyons is coordinated by the DNA methyltransferase (DNMT) family of proteins.

1.4 DNA methyltransferase family

The enzymes that coordinate the pattern of methylation in vertebrates belong to the DNMT family. There are four members of the DNMT family; DNMT1, DNMT3a, DNMT3b and DNMT3L, all encoded by separate genes^{46,47}. Together these proteins catalyse the conversion of cytosine to 5mC, which helps to maintain tight regulation of gene transcription. DNMT1 is thought of as a maintenance methyltransferase as it primarily methylates CpG dinucleotides where the corresponding CpG dinucleotide on the other strand is already methylated (hemi-methylated)⁴⁸. Thereby, maintaining the pattern of DNA methylation that has been previously established prior to cell division. DNMT3a and DNMT3b are *de novo* DNA methyltransferases, meaning that they will establish new methylation patterns at sites that are unmethylated⁴⁹. DNA methyltransferase-like protein (DNMT3L), the last member of the DNMT family, lacks a catalytic domain and does not have methyltransferase activity but acts as an accessory protein to other family members. In particular, DNMT3L has been shown to form a heterotetramer with DNMT3a in certain conditions and it is thought to thereby increase the activity of DNMT3a⁵⁰.

1.4.1 DNA methyltransferase 3a

DNMT3a has many known binding partners and there are sure to be more that have not yet been described. As shown in figure 1.4, the DNMT3a protein has three major domains that are responsible for its methyltransferase activity and facilitate targeting to genomic locations through interactions with binding partners. The three domains are called the Pro-Trp-Trp-Pro (PWWP) domain, the ATRX-DNMT3-DNMT3L (ADD) domain and the catalytic methyltransferase domain (MTase)¹³. The N terminal, PWWP and ADD domains are regulatory regions that have been shown to possess a number of binding partners. The PWWP domain is conserved among 39 proteins with nuclear localisation and DNA binding affinity that can act as transcription factors to induce developmental genes⁵¹. The N-terminal region of DNMT3a1 has DNA binding affinity although it cannot distinguish between methylated and unmethylated CpG sites. It was

proposed that the N-terminal region acted as a DNA anchor and could thereby increase the methyltransferase activity of DNMT3a1 compared to DNMT3a2, two splice isoforms, by augmenting its association with the DNA ⁵². The PWWP domain of DNMT3a has also been shown to have weak DNA binding affinity ^{53 54}, although the function of this is unclear. In particular the PWWP domain has been shown to be essential for DNA methylation at heterochromatin as methylation in this region was absent when the PWWP domain was mutated ^{54 55}. The PWWP domain can also interact with histone modifications, like histone 3 Lysine 36 tri-methylation (H3K36me3) marks as well as histone 3 lysine 9 tri-methylation (H3K9me3) ⁵⁶ suggesting that this domain has a role in enhancing transcriptional repression associated with these histone modifications.

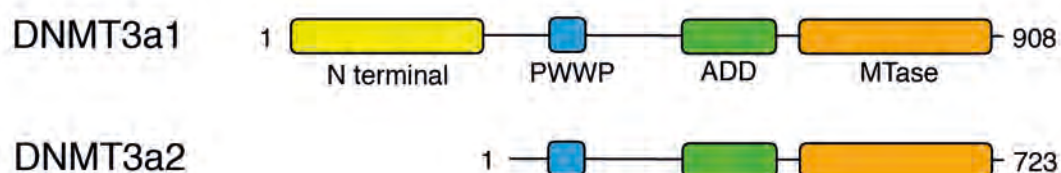


Figure 1.4 Major domains of the DNMT3a isoforms

Major domains of the two DNMT3a isoforms DNMT3a1 and DNMT3a2. DNMT3a1 (long) is the full-length protein and contains the N terminal region which is truncated in the DNMT3a2 (short) isoform. Both DNMT3a1 and DNMT3a2 possess three major functional domains; Pro-Trp-Trp-Pro (PWWP) domain (blue), the ATRX-DNMT3-DNMTL (ADD) domain (green) and the methyltransferase catalytic domain (MTase) (orange). Protein length indicated by number of amino acids.

The ADD domain creates an auto-inhibitory loop with the catalytic domain of DNMT3a. When the ADD domain recognises unmethylated histone 3 lysine 4 (H3K4), the auto-inhibitory interaction is disturbed and the methyltransferase activity is increased ⁵⁷. Furthermore, the ADD domain has been shown to bind to the histone deacetyltransferase 1 (HDAC1), a histone modifying enzyme, which can target DNMT3a to specific regulatory regions in the DNA ⁵⁸. The ADD domain can also interact with histone modifiers like the histone-lysine-*N*-methyltransferase enhancer of zeste homologue 2 (EZH2) to guide DNMT3a to different regions of the genome ⁵⁹ although this interaction with EZH2 did not always correspond with an induction of

methyltransferase activity of DNMT3a and therefore the function of this interaction is not clear⁶⁰.

The third domain of DNMT3a is the MTase domain where the methylation reaction occurs. The catalytic domain resides within the MTase domain of DNMT3a and contains five specialised motifs that are essential for DNA methyltransferase activity^{61,62}. Two of these motifs (I and X) bind essential cofactors and the remaining 3 motifs (IV, VI, VII) are involved in the catalytic conversion of cytosine to 5mC. Interestingly the DNMT3a protein is highly conserved among vertebrates with a 98% sequence homology between human and murine proteins⁶³.

To date there have been five different splice isoforms of DNMT3a reported^{13 64 65 66}. These are broadly categorised as the DNMT3a1 (long) and DNMT3a2 (short) isoforms (Figure 1.4). There are two transcripts, transcript 1 and 3, that encode for DNMT3a1 varying in their transcriptional start sites but both resulting in the same full-length protein. Transcript 2 encodes for the shorter DNMT3a2 protein which lacks the N-terminal region of the full-length protein. The fourth DNMT3a isoform lacks the catalytic domain as well as the PWWP and ADD domains necessary for interacting with binding partners. The fifth transcript is similar to DNMT3a2 but is missing the second exon⁶⁴. The significance of the DNMT3a1 and DNMT3a2 splice isoforms is still under investigation although there are differences in their tissue expression.

DNMT3a1 is expressed ubiquitously although at low levels in all tissues, whereas expression of DNMT3a2 is restricted to embryonic tissues, embryonic stem cells and in adults it is found in the testis, ovaries, thymus and spleen⁶⁷. In addition to different tissue expression, DNMT3a1 and DNMT3a2 also have different binding preferences. DNMT3a1 has been shown to navigate to methylated shores of CpG promoters which leads to an increase in hemi-methylated cytosines due to its *de novo* methyltransferase activity⁶⁸. It was proposed that DNMT3a1 was critical for the demarcation between methylated and unmethylated cytosines in CpG shores. Indeed, differences in nuclear localisation between DNMT3a1 and DNMT3a2 have been reported which might distinguish their activity in different contexts. While DNMT3a1 is associated with inactive heterochromatin, DNMT3a2 is associated with euchromatin⁶⁷, which is likely to correspond with actively transcribed genes. On this basis, DNMT3a2 is thought to be the more 'active' form due to its association with euchromatin.

1.4.2 Regulation of DNMT3a

1.4.2.1 Transcriptional regulation

DNMT3a has multiple transcriptional start sites that are controlled by at least three known promoters ⁶⁶. Sp-1 and Sp-3, which are zinc finger proteins, have binding sites at the transcriptional start site of DNMT3a, inducing transcription of DNMT3a. Indeed, overexpression of Sp-3 results in a 3- to 4-fold increase in *DNMT3a* mRNA which could be reduced by knockdown of Sp-3 ⁶⁹ indicating direct regulation of *DNMT3a* by Sp-3. *DNMT3a* expression can also be directly activated by Wilms' tumour 1 (WT1) which is a transcription factor required for embryonic development of the kidney, urogenital system and epicardium. WT1 can transactivate *DNMT3a* and alter the DNA methylation signature of kidney cells ⁷⁰. There is some evidence that extracellular signal-regulated kinase (ERK) can downregulate DNMT3a mRNA expression ⁷¹, but the molecular mechanism of this down regulation has yet to be identified. In addition to regulators that can activate *DNMT3a* transcription, there have also been factors identified that can downregulate *DNMT3a* expression. Indeed, RB/E2F which is involved in the regulation of cell cycling has been identified as a negative regulator of DNMT3a. Expression of retinoblastoma tumour suppressor protein (RB) results in the downregulation of DNMT3a mRNA and protein through interactions of E2F with the *DNMT3a* promotor ⁷². It is likely that there are many other factors that can transcriptionally regulate *DNMT3a* expression. Nevertheless, as the importance of DNMT3a expression in different tissues and at different times is identified, more regulators will likely emerge as coordinators of gene expression through altered DNA methylation status.

1.4.2.2 Translational regulation

MicroRNAs (miRs) are small non-coding RNAs that can regulate the expression of genes by interfering with the mRNA to directly inhibit their translation or even cause their degradation ⁷³. The *miR29* family, which includes *miR29a*, *miR29b* and *miR29c*, has a distinct sequence complementarity with the 3' UTR of *DNMT3a* and *DNMT3b* ⁷⁴. Indeed, *miR29* family member expression is inversely correlated with DNMT3a expression. Overexpression of *miR29* in lung cancer cell lines, which have high expression of DNMT3a, can reduce DNMT3a expression to normal levels, restore DNA methylation patterns and reactivate expression of silenced tumour suppresser genes as well as impairing the growth of tumours *in vivo* ⁷⁴. Similarly, *miR-143* has

also been shown to be inversely correlated with DNMT3a expression in colorectal cancer ⁷⁵. Enforced expression of *miR-143* into colorectal cancer cell lines reduced DNMT3a expression and inhibited tumour cell growth ⁷⁵. There is also some evidence that *miR-1741*, *miR-16c*, *miR-22* and *miR-1632* can also regulate DNMT3a expression ⁷⁶.

1.4.2.3 Post-translational modifications

There is so far very little known about post-translational regulation of DNMT3a. Sumoylation of the N-terminal region of DNMT3a ⁷⁷ can interfere with the ability of this region to interact with histone deacetylases, thus resulting in a failure to repress transcription of target genes ⁷⁸. Acetylation and phosphorylation of DNMT1 have been reported to alter protein stability and activity ⁷⁹, although these modifications have not been shown to occur on DNMT3a as of yet. DNMT3a can also be ubiquitinated leading to reduced DNA methylation. UHRF1 and UHRF2 are both E3 ubiquitin ligases that can ubiquitinate DNMT3a promoting its degradation ⁸⁰. This has important implications in cancer, where global DNA methylation is reduced, and this highlights the importance of retaining normal DNMT3a activity for tumour suppression.

1.4.3 Enzymatic activity of DNMT3a

DNMT3a is most active in a hetero-tetrameric form where DNMT3a dimerises and is then flanked by two DNMT3L units such that the protein is structured in a DNMT3L-DNMT3a-DNMT3a-DNMT3L arrangement ^{81,82}. This arrangement results in two catalytically active sites that are separated by a single helical turn in the DNA, meaning that upon binding of the DNMT3a hetero-tetramer two CpG sites are simultaneously methylated ⁸¹. When DNMT3L is not present, DNMT3a can form oligomers along the DNA resulting in extended DNA-associated DNMT3a fibres called nucleoprotein filaments ⁸³. These DNMT3a oligomers were reported to stabilise the protein and progressively increase the rate of DNA methylation activity ⁸⁴.

1.5 Mutations of DNMT3a in disease

Abnormal DNA methylation has been strongly associated with cancer, autoimmune diseases, metabolic disorders, neurological disorders and ageing ⁸⁵. Therefore, it does not come as a surprise that germline mutation of *DNMT3a* has been implicated in a developmental disorder called Tatton-Brown-Rahman syndrome (TBRS) and somatic mutations of *DNMT3a* have been

found in haematological cancers^{8,86}. Since DNA methylation sits at the apex of the gene regulatory hierarchy it follows logically that defects in methylation, due to mutations in DNMT3a, can have far reaching implications in many cell types and diverse cell signalling pathways. The most interesting feature of mutant DNMT3a in disease, is the frequency of mutations that occur at a particular residue. Although mutations in *DNMT3a* in both cancer and TBRS have been reported across all domains of the DNMT3a protein, in both diseases there is a strong enrichment of mutations at residue R882 (murine homologue R878). This suggests that the DNMT3a R882 mutant protein conveys some kind of selective advantage over cells with wt DNMT3a. Indeed, the DNMT3a R882H mutant has been shown to have reduced capacity for DNA methylation⁸². In patients with *DNMT3a* mutant acute myeloid leukaemia (AML) around 65% of mutations occur at R882⁸⁷⁻⁸⁹ and there is also an increased frequency of this mutation in patients with TBRS^{86,90}. Residue R882 sits within the MTase domain of DNMT3a⁸².

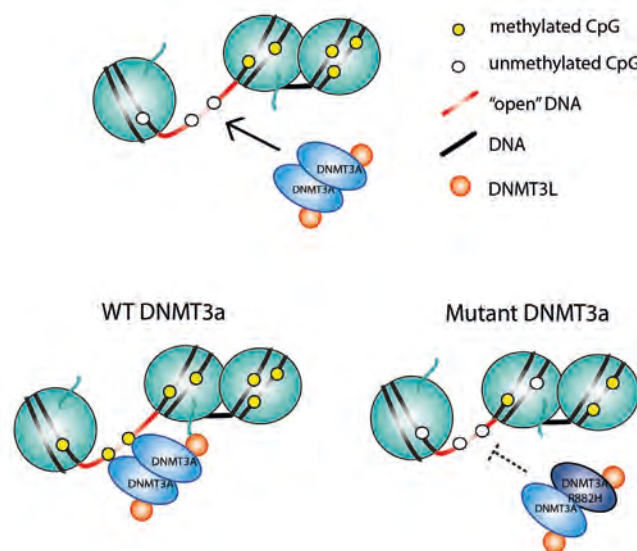


Figure 1.5 proposed consequences of DNMT3a R882H mutant on CpG methylation

The DNMT3L-DNMT3A-DNMT3L heterotetramer contains two catalytic domains that can methylate two CpG sites simultaneously. The DNMT3A R882H variant has been proposed to sequester wt DNMT3a and impair the ability of the heterotetramer to methylate DNA.

The most common mutation results in an arginine to histidine conversion that is predicted to interfere with the DNMT3a-DNMT3a binding interface⁸². It remains contested in the field why DNMT3a R882 mutations are so prevalent in disease. DNMT3a R882 mutations were initially proposed to act through a dominant-negative mode of action⁹¹⁻⁹⁴, although this was not supported by all groups⁹⁵. DNMT3a R882 has also been proposed to sequester wt DNMT3a through increased binding kinetics but lower methylation capacity⁹⁶. A model for the proposed reduced activity of the DNMT3L-DNMT3a^{WT}-DNMT3a^{R882}-DNMT3L tetramer is depicted in figure 1.5.

More recently, the DNMT3a R882 mutant was proposed to exert its oncogenic effects through an altered sequence flanking preference, meaning that it was methylating regions of DNA that it would not normally bind to^{97,98}. In doing so, mutant DNMT3a R882 was proposed to drive abnormal gene expression by methylating and hence inactivating genes it would normally not do. While there is yet to be a consensus on whether DNMT3a acts as a dominant-negative or displays an altered sequence preference there is no reason to believe that these two features must be mutually exclusive. What is clear, is that DNMT3a R882 disrupts the normal enzymatic activity of DNMT3a which has major consequences during the development and as a driver of cancer.

1.5.1 Tatton-Brown-Rahman Syndrome

In 2014 the first description of an intellectual disability and overgrowth syndrome caused by germline mutations in *DNMT3a* was described⁹⁹. The syndrome was named Tatton-Brown-Rahman Syndrome (TBRS) and was added to a class of overgrowth and intellectual disability (OGID) disorders. Initially, the authors described 13 patients identified with *de novo* germline *DNMT3a* mutations. Since the original publication, a further 55 patients were described by the authors and to this date less than 100 patients have been reported worldwide⁸⁶. As such, there is very little known about the disease and there is currently no treatment.

TBRS patients can present with a spectrum of *DNMT3a* mutations. These can include missense, stop, gain-of-function, frameshift variants, gene deletions, in frame deletions and splice site variants⁸⁶. Intriguingly, there is an enrichment of mutations at residue R882 in patients with TBRS, which overlaps with the R882 'hotspot' mutation observed in patients with

haematological malignancies ^{86,89,90}. However, since TBRS is caused by germline *DNMT3a* mutations the consequences of this mutation are far reaching. TBRS patients have a characteristic tall stature, overgrowth measured by both head circumference and body weight and abnormal facial characteristics ^{86,99}. Patients with TBRS often have thick, heavy set eyebrows, palpebral fissures and prominent front teeth ⁹⁹. All patients have some level of intellectual disability, although this can range from mild to severe ^{86,100}. Other common clinical characteristics include autism spectrum disorders and other mental health/neurological conditions; schizophrenia, personality disorders, aggression and seizures ^{86,100,101}. Other interesting clinical findings include a higher incidence of hypotonia (reduced muscle mass), kyphoscoliosis (spinal curvature) and congenital heart defects ⁸⁶. To date, there has been less than 100 patients with TBRS identified worldwide and as a result there is much to learn about the clinical course in these patients. The majority of the patients described are in childhood, adolescence or early adulthood and therefore we know very little about the disease progression beyond this time and what consequences this mutation will have as these patients age. Nevertheless, there is strong evidence that germline mutations in *DNMT3a* have widespread consequences for patients. Currently there are no preclinical models of TBRS and therefore it is important that a preclinical model is developed to inform clinicians on potential clinical outcomes that can be expected in TBRS patients as they age and to provide a tool to test potential therapies.

Some insight can be gained by comparing TBRS to other similar OGID disorders. TBRS has been compared to SOTOS syndrome, Weaver Syndrome, Malan syndrome ¹⁰² and the OGID disorder resulting from truncating *CHD8* gene mutations ¹⁰³. SOTOS and weaver syndromes are also caused by germline mutations in epigenetic regulators: Enhancer of zeste homolog 2 (*EZH2*) and nuclear receptor binding SET domain protein 1 (*NSD1*) respectively ¹⁰⁴⁻¹⁰⁶. SOTOS and Weaver syndromes have many overlapping features with TBRS, including intellectual disabilities and overgrowth. Interestingly, the defining characteristic of TBRS that separates it from the other OGID disorders is the higher prevalence of obesity ⁸⁶. TBRS patients do not generally require intensive clinical follow-up although most will require some form of support for their education ^{86,100}. It remains important to study TBRS in more detail as mutant *DNMT3a* is strongly associated with the development of acute myeloid leukaemia (AML) and it remains unclear whether TBRS patients may be at an increased risk for developing this or other haematological malignancies.

1.5.1.1 Cancer in Tatton-Brown-Rahman syndrome patients

It is unclear whether this patient cohort will have an increased propensity to develop cancer. To date, one patient has developed medulloblastoma ¹⁰⁷ and two have developed haematopoietic malignancies ^{108,109}. Somatic *DNMT3a* mutations are highly prevalent in haematological malignancies like AML and Adult T-cell acute lymphoblastic leukaemia (T-ALL) with an estimated prevalence of *DNMT3a* mutations of ~40% in AML and ~20% in T-ALL ^{89,110-112}. However, while *DNMT3a* mutant driven paediatric leukaemia is considered very rare ¹¹³, paediatric AML has already been reported in two TBRS patients ^{108,109}. With so few TBRS patients reported and only two reported cases of AML within this cohort it is too early to determine whether TBRS patients are at an increased risk of developing haematological malignancies. However, in the general population, somatic mutations in *DNMT3a* are strongly associated with the development of haematological malignancies and therefore it is essential that TBRS patients are monitored for signs of haematopoietic cancers.

1.5.2 Mutant *DNMT3a* in haematopoietic malignancies

1.5.2.1 Introduction to haematopoiesis

Haematopoiesis, the development of the components of the blood, is a tightly regulated process. All of the cell types of the blood are derived from haematopoietic stem and progenitor cells (HSPCs) which undergo self-renewal and differentiation to give rise to a number of highly specialised haematopoietic cells ¹¹⁴. As depicted in Figure 1.6, long term haematopoietic stem cells (LT-HSC) sit at the apex of the haematopoietic hierarchy as they have the potential to give rise to all haematopoietic lineages and cells. Haematopoietic stem cells (HSCs) possess two defining characteristics: self-renewal and the ability to give rise to all lineages of the blood (pluripotency) ¹¹⁴. HSC function has been examined experimentally through haematopoietic transplantation, where mice were rescued from lethal gamma irradiation (γ -irradiation), which ablates their haematopoietic system, by transplanting HSCs into their blood ¹¹⁵. These transplanted HSCs were able to rescue the mouse by repopulating the entire haematopoietic system with new functional cells ¹¹⁶. Even now, the ability of HSCs to repopulate the haematopoietic system of mice is considered the gold standard approach for measuring HSC function. By definition, an HSC must be able to repopulate the bone marrow of a lethally irradiated primary and at least one secondary recipient mouse ¹¹⁷⁻¹²¹.

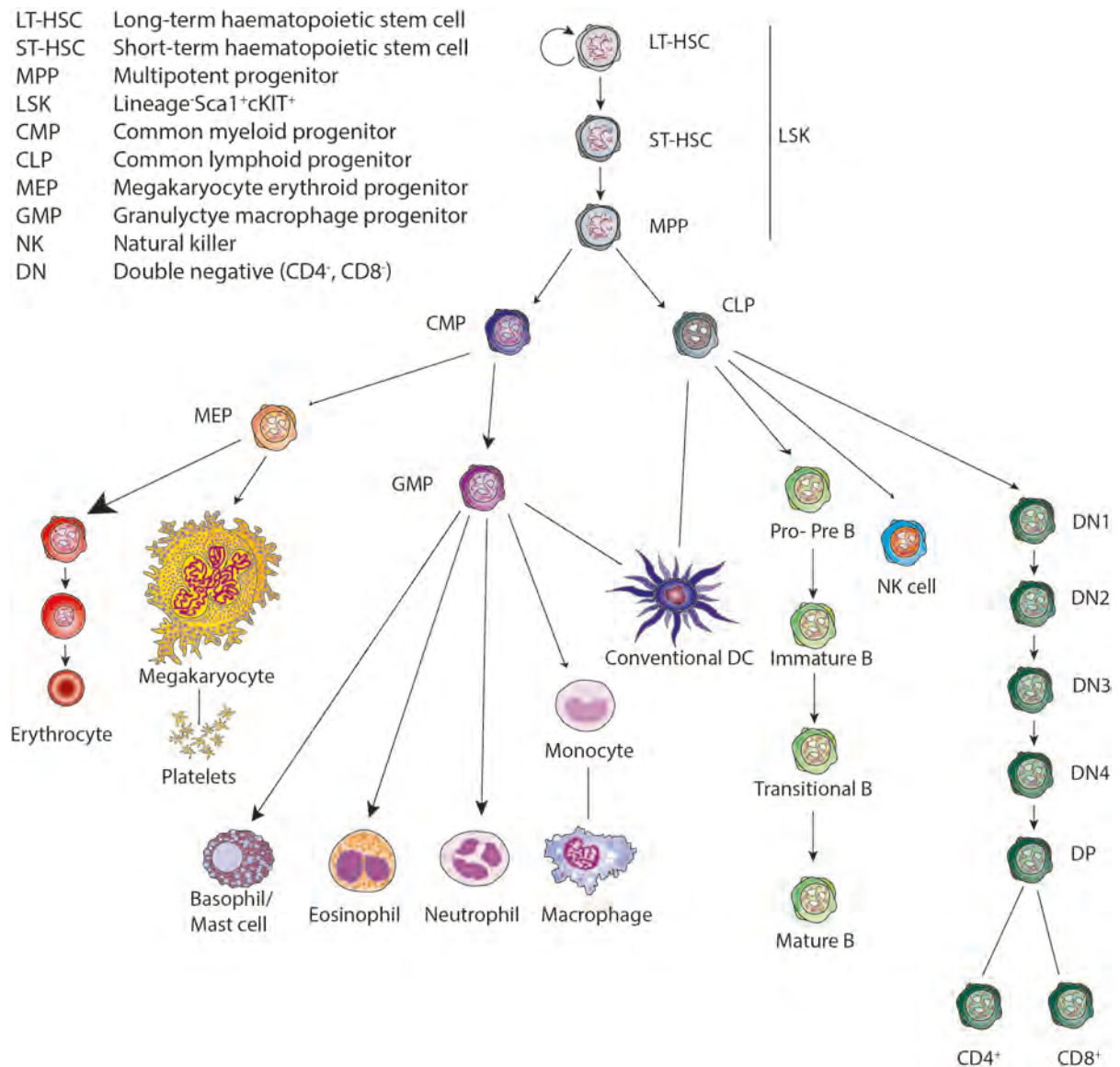


Figure 1.6 haematopoiesis represented as a hierarchy

Hierarchical depiction of haematopoiesis with long-term haematopoietic stem cells at the apex and progressively more differentiated progeny towards the bottom. Abbreviations are defined in the top left corner. For cells markers used to identify specific cell types refer to appendix I.

With each progressive step down the haematopoiesis hierarchy, or with each differentiation step, cells are thought to lose 'stemness' as they become more committed to a specific lineage. Short-term haematopoietic stem cells (ST-HSC) retain many of the properties of their long-term counterparts but are unable to provide lifelong haematopoietic reconstitution potential ^{122,123}. Haematopoietic multipotent progenitor cells (MPPs) also retain some stem cell features but do not have the longevity of true HSCs. MPPs can rescue mice from lethal γ -irradiation in the short-term (2-3 weeks) but are unable to sustain the production of all haematopoietic lineages as they do not possess true self-renewal capacity ¹²⁴. Collectively, LT-HSC, ST-HSC and MPPs are referred to as part of the haematopoietic stem/progenitor cell (HSPC) pool and can be defined by the absence of lineage specific markers but the presence of stem cell markers, such as SCA-1 and c-KIT ^{116,125,126}. This HSPC pool is commonly known as LSK cells (Lineage marker⁻SCA-1⁺c-KIT⁺).

The first major split in the haematopoietic tree occurs where the myeloid lineage is distinguished from the lymphoid lineage by the emergence of the common myeloid ¹²⁷ and common lymphoid progenitor cells ^{128,129}. In figure 1.6, as in most depictions of the haematopoietic system, the trajectory from HSPCs to fully differentiated mature haematopoietic cell appears quite linear with well-defined steps between each transition. This idea was formed on the basis of early studies into haematopoietic cell lineage fate but recent advances in the field have blurred the lines and suggested that haematopoietic cell development occurs through a much less rigid cascade and should be thought of more as a differentiation continuum ^{124,130,131}.

1.5.2.2 HSC self-renewal and cell fate decisions

The balance between HSC self-renewal and differentiation is critical to sustain haematopoiesis throughout life ¹³². The output of differentiated cells must be dynamic with continual turnover of haematopoietic cells during steady state as well as the ability to significantly ramp up blood cell production under conditions of haematopoietic stress. Haematopoietic stress can be induced as a result of infection, cancer, γ -irradiation or acute blood loss as a result of injury ¹³³. Under these conditions, HSC must exit their quiescent state and rapidly divide to ensure that haemostasis is restored. Activated and rapidly proliferating HSCs must then return to their dormant state following the stressor ^{134,135}.

The increase in cycling haematopoietic stem and progenitor cells (HSPCs) following

haematopoietic insults ultimately leads to an increase in differentiated haematopoietic cells. With each subsequent division different transcriptional networks must be activated and repressed¹³⁶. The control of these transcriptional networks occurs in part due to changes in DNA methylation, resulting in HSC transcriptional networks being repressed in more differentiated cells¹³⁷. The coordination of gene transcriptional networks is essential to ensure that haematopoietic cell numbers and their relative frequencies remain balanced. Defects in the regulation of transcriptional networks can quickly lead to uncontrolled proliferation and ultimately a blood cell cancer. *Dnmt3a* has been shown to coordinate the differentiation of LT-HSCs¹³⁸. Loss of DNMT3a in haematopoietic cells results in preferential self-renewal and a reduced capacity to fully differentiate into downstream progeny³⁷. The loss of DNMT3a in murine HSCs resulted in an inability to repress stem cell gene programs in more differentiated cells and hence accumulation of *Dnmt3a*-null HSCs³⁷. The self-renewal and stem cell advantage provided by loss of *Dnmt3a* is so strong that these *Dnmt3a*-KO HSCs have been shown to have almost indefinite serial repopulating capacity in murine transplantation models¹¹⁵. These studies have highlighted the importance of DNMT3a-mediated transcriptional control in HSCs and provide insight into why mutant DNMT3a is so common in haematological malignancies.

1.5.2.3 Mutant DNMT3a in haematological malignancies

DNMT3a mutations are prevalent in a range of haematological malignancies and have been identified less frequently in cancers of other origins as well⁸. Single cell sequencing in the blood of patients with AML can reveal the mutational timeline of malignant transformation. A mutational timeline can be constructed by determining which mutations certain cells harbour. Where all cells (leukaemic and non-leukaemic) in the sample carry a particular mutation, it can be established that this is the first mutation to occur in a sequence of mutations leading to cancer. Single cell sequencing of AML cells from patients have revealed that lesions in epigenetic modifiers are generally the first mutations to occur¹³⁹⁻¹⁴¹. Indeed, mutations in *DNMT3a* have been widely reported to be the first mutation to occur in the pathway to the development of AML^{139,141,142}, given that additional oncogenic mutations are only found in a subset of the leukaemic clones.

DNMT3A R882H is also strongly associated with the development of clonal haematopoiesis of indeterminate potential (CHIP)^{139,143}. CHIP is a phenomenon whereby all the blood cells of a

patient arise from a single haematopoietic cell. The blood becomes clonally derived and any mutations acquired in HSPCs are propagated through the haematopoietic lineages such that all blood cells carry particular mutations. The prevalence of CHIP increases with age and is a strong risk factor for the development haematological and cardiovascular disease ¹⁴³⁻¹⁴⁵. However, a patient can be perfectly healthy with CHIP, meaning that clonal haematopoiesis is a risk factor rather than a disease itself ¹⁴⁵. Mutant DNMT3a has been shown to be a potent driver of CHIP. Consistent with its role in driving CHIP, mutant DNMT3a is far more prevalent in age related haematological malignancies ¹¹¹. Indeed, mutant DNMT3a is extremely rare in paediatric leukaemia and present in only 1% of cases ¹¹³. The steps between the presence of CHIP and the development of leukaemia remain undefined, but there is little question that mutant DNMT3a is entwined in the development of both of these pathologies. How mutant DNMT3a can predispose haematopoietic cells to malignant transformation remains a subject of investigation. There is little insight into the role of mutant DNMT3a in preleukaemic cells due to a dearth of preclinical models.

Nevertheless, mutant DNMT3a is highly prevalent in haematological malignancies, suggesting that it plays a role in predisposing cells to neoplastic transformation. Indeed, mutant DNMT3a was found to be the third most common mutation present in patients with acute myeloid leukaemia (AML) ^{8,87}. Mutant DNMT3a is also highly prevalent in adult T-ALL ^{111 110}. Furthermore, even in AML cases where DNMT3a was not mutated, it was shown to be epigenetically silenced in 40% of patients with AML ¹⁴⁶, highlighting the importance of DNMT3a as a tumour suppressor in haematological malignancies. Furthermore, it is now fairly widely accepted in the field that mutant DNMT3a confers worse outcome for patients with haematological malignancies ^{147 87,148-151}. Of note, the DNMT3a R882 mutation is said to predict a worse outcome than non-R882 mutations in DNMT3a ¹⁵², further emphasising the need for accurate preclinical models.

1.6 Current models to study mutant DNMT3a and their limitations

Contributing to the lack of understanding of how mutant DNMT3a drives the initiation of cancer, is the dispute as to what the DNMT3a 882 mutation does at the protein level. The *DNMT3a* 882 mutation is agreed to alter the catalytic activity of the protein as well as DNA binding affinity ⁸², but it has proven difficult to ascertain whether the mutation exerts its effect as a dominant negative protein or if it has an altered flanking sequence preference with studies reporting

conflicting findings^{92,95,98}. These studies are complicated by the use of imperfect model systems. Indeed, much of what we know about mutant *Dnmt3a* has been extrapolated from studies that examined the impact of loss of one or both alleles of *Dnmt3a* *in vitro* and *in vivo*^{37,153}. These studies fail to shed light on why the R882 *DNMT3a* mutation is so strongly enriched in human blood cell cancers and preclude the possibility that the *DNMT3a* R882 mutation exerts its oncogenic actions through a mechanism other than loss of DNA methylation activity. The *DNMT3a* R882 mutation could cause a deviation in the normal behaviour of the protein. For example, it might result in an altered DNA flanking sequence preference causing aberrant promotor methylation⁹⁸ and thereby drive aberrant gene expression. There is some evidence that hypomethylation of gene promoters as a result of loss of DNMT3a expression results in an increased stem cell gene signature in HSCs¹¹⁵. It is also feasible that the DNMT3a R882 mutant protein has different binding partners or altered binding kinetics to sites in the DNA compared to the wt DNMT3a protein. There is some evidence that DNMT3a R882H increases the stability of large oligomers, which in turn reduces the enzymatic activity of the protein⁹². This reduction in enzymatic activity is on top of a reduced DNA binding affinity for the DNMT3a R882H protein as reported by Nguyen, et al.⁹². Importantly, these conclusions are based on *ex vivo* experiments and involve high overexpression of segments of the protein, like the catalytic domain or full-length protein, which do not have the same binding kinetics.

1.6.1 Viral expression of mutant *DNMT3a* R882H *in vitro*

Some groups have gone further by studying mutant DNMT3a *in vitro* and *in vivo*. As mentioned previously, the murine amino acid homologue of the human arginine (R) at position 882 is found in murine *Dnmt3a* at position 878. Much of what we known about mutant DNMT3a 882 has come from human cell lines with DNMT3a R882H mutations, but these are often compared to non-isogenic cell lines with wt DNMT3a making it very difficult to determine whether the observed result is due to the presence of the DNMT3a mutation or differences resulting from a different genetic background¹⁵⁴. Further studies have aimed to study the implications of mutant DNMT3a by using viral vectors to overexpress the DNMT3a R882H mutant in their cell line of choice^{155 154}. These viral vectors produce supra-physiological levels of mutant DNMT3A 882, which is suboptimal given that such large amounts of protein would be expected to cause abnormal methylation of the DNA making it difficult to determine whether the results are physiologically relevant.

Another group has approached this by using CRISPR/Cas9 to generate *DNMT3a* mutants with various truncating mutations in the K562 cell line ¹⁵⁵. The authors showed that *DNMT3a* mutant cells had decreased cellular proliferation and a higher tendency to undergo apoptosis both with and without application of the cellular stressor 5-Flourouracil ¹⁵⁵. The authors also showed that *DNMT3a* mutant cells were less able to differentiate into mature megakaryocytes, suggesting a differentiation defect in these cell lines. RNA sequencing revealed downregulation of the spliceosome and RNA degradation pathways, which was coupled with a trend towards reduced RNA stability and abnormal RNA splicing ¹⁵⁵. The authors found that the *DNMT3a* mutant cell lines also displayed signs of genome instability as there was evidence of abnormal cytogenetics. This finding is in contrast with human *DNMT3a* mutated cancers which are most commonly associated with normal cytogenetics ^{155 87}.

1.6.2 Viral expression of mutant *DNMT3a* R882H *in vivo*

Further knowledge about the roles of the *DNMT3a* R882 mutant have been gained from studies using viral transduction of expression vectors into HSPCs followed by their transplantation into mice and study of their progeny *in vivo*. Xu, et al. ¹⁵⁶ used a lentiviral transfection system to overexpress the human DNMT3a R882H variant in murine bone marrow cells. The infected bone marrow cells were then used for haematopoietic reconstitution of recipient mice. It was shown that DNMT3a R882H infected cells could accumulate in the bone marrow by four weeks post-reconstitution and that after a year there were significantly more granulocytes and monocytes in the blood, which was coupled by a loss of lymphocytes indicative of a myeloid differentiation bias, reminiscent of chronic myelomonocytic leukaemia (CMML) in humans ¹⁵⁶. The same approach was also used by Koya, et al. ¹⁵⁷. They did not observe an accumulation of DNMT3a R882H mutant cells in the bone marrow of transplanted mice and could not observe signs of leukaemia up to 1 year. However, DNMT3a R882H LT-HSCs and LSK cells did accumulate in the bone marrow of recipient mice. Isolation and re-transplantation of these LT-HSCs revealed normal haematopoietic repopulation capacity albeit with a myeloid bias. Most strikingly, DNMT3a R882H LT-HSCs were greatly overrepresented in secondary transplants and the mutation was proposed to drive aberrant accumulation of quiescent HSCs. Indeed, DNMT3a R882H LSK cells isolated from these mice were found to have downregulated genes associated with differentiation, like *Hox* family of genes ¹⁵⁷. While these studies reveal some plausible mechanistic insights into how DNMT3a R882H mutant contributes to the initiation of leukaemia,

the results may be hard to interpret as supraphysiological levels of DNMT3a R882H are likely to cause defects in DNA methylation that may or may not be present under physiological conditions.

1.6.3 *Dnmt3a R878H mutant models*

In an attempt to circumvent the problems with supraphysiological levels of mutant DNMT3a, a number of preclinical models have been created. The inducible Mx1-Cre; *Dnmt3a*^{R878H} murine model was created where the expression of mutant *Dnmt3a* R878H was induced by Mx dynamin-like GTPase 1 (Mx1) -Cre mediated recombination of a conditional *Dnmt3a* allele, replacing wildtype with the *Dnmt3a*^{R878H} allele^{34,154}. At 4-6 months following induction of the expression of mutant *Dnmt3a* R878H, mice succumbed to a fully penetrant AML characterised by extensive expansion of haematopoietic cells in the bone marrow³⁴. The mice survived a median of 230 days and showed significant accumulation of LSKs and myeloid progenitor cells. In contrast, Guryanova, et al.¹⁵⁴ did not see any evidence of leukaemia in their *Dnmt3a* R878H mice up to a year following recombination of the mutant allele in the absence of further cooperating cancer causing mutations. They did, however, also observe an increase in LSK cells in the bone marrow¹⁵⁴. A gene expression microarray assay was performed on heterozygous *Dnmt3a* R878H LSK cells isolated from mice 6 months after induction of the expression of the conditional mutant allele³⁴. A number of genes associated with HSC stemness, like *Mpl* and *Hlf*, were differentially expressed. Further, genes related to haematopoietic differentiation were drastically deregulated, corresponding with an up-regulation of myeloid differentiation genes and down-regulation of lymphoid associated genes³⁴. Collectively, these studies demonstrate that mutant *Dnmt3a* R878H induces HSC accumulation that is linked with an altered gene expression signature. However, there are some caveats to these murine models which are particularly relevant when studying HSCs.

1.6.4 *Caveats of current Dnmt3a mutant murine models*

The two murine models introduced above both rely on Cre mediated recombination to induce the expression of the mutant *Dnmt3a* R878H allele^{34,154}. The Mx1-Cre mouse model was first described by Kuhn, et al.¹⁵⁸ and relies on interferon dependent recombination which is induced by injection of polyinosinic: polycytidylic acid (pIpC). There are some major caveats to this system which are particularly relevant when studying the role of mutant DNMT3a on HSC

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function. The Mx1-Cre model has been shown to undergo spontaneous recombination in the absence of plpC injection and although this underlying recombination occurs at low levels (2-3% of cells)¹⁵⁸, if the transgene provides cells with an advantage this can have a significant effect on the interpretation of results¹⁵⁹. Spontaneous Mx1-Cre recombination was shown to be enhanced when HSPCs were transplanted into recipient mice, which is probably due to the interferon response caused by γ -irradiation that is required for haematopoietic ablation before bone marrow transplantation¹⁵⁹. Nevertheless, since Dnmt3a is often studied in the context of haematological malignancies, and where haematopoietic transplant experiments are at the core of haematopoietic research, murine models of *Mx1-Cre: Dnmt3a^{R878H}* have some obvious limitations. Furthermore, while there is great appeal to having an inducible mutation when studying the effects of DNMT3a there are also limitations surrounding the induction itself. Indeed, injection of plpC which is used to induce recombination, is known to cause proliferation of HSPCs^{160,161} as well as altering the immunophenotype of haematopoietic cells. In particular, plpC has been shown to transiently increase the expression of SCA-1 on haematopoietic cells which would not normally express it. SCA-1 is a marker commonly used to define HSC populations¹⁶¹ and therefore some data might suggest that the gene being studied has caused an increase in HSC numbers when actually the plpC has transiently induced SCA-1 expression on non-HSCs. Further, in some models of inducible heterozygous Dnmt3a R878H mutants, the cells are haplo-insufficient prior to recombination of the Dnmt3a mutant allele¹⁵⁴. Therefore, the phenotypes observed using these models are driven by the compounding effects of haplo-insufficiency during mouse development and up until recombination plus the effects of the Dnmt3a mutant allele after recombination. The compounding effects of haplo-insufficiency and mutant Dnmt3a on phenotype are critically important to consider when examining the phenotype of haematopoietic cells. Clearly, there is a need for a model of *Dnmt3a^{R878H}* that does not rely on Mx1-Cre plpC mediated induction.

1.7 Summary and project aims

The pathogenesis of DNMT3a R882 mutations is still poorly understood due to the dearth of preclinical models. Much of what is known about mutant DNMT3a is from observational studies of patients with TBRS caused by germline DNMT3a mutations or from sequencing studies of cancer patients with somatic DNMT3a mutations. In this thesis, I will present data from a novel *Dnmt3a^{R878H}* mutant mouse model that circumvents some of the caveats of using Mx1-mediated

recombination when studying the haematopoietic system. In the first results chapter, a general introduction and overview of the *Dnmt3a*^{R878H} mouse model will be presented with relevance to TBRS and also haematopoietic malignancies. The second results chapter will focus on the impact of mutant Dnmt3a^{R878H} on haematopoiesis. Heterozygous *Dnmt3a*^{R878H/+} cells will be examined in steady state conditions and characterised under sustained conditions of haematopoietic stress. In the final results chapter, deregulated gene expression pathways will be described in *Dnmt3a*^{R878H/+} HSCs with an emphasis on how *Dnmt3a*^{R878H/+} HSCs are primed for neoplastic transformation. Overall these results will lay the foundation for a better understanding of the impact of mutant DNMT3a in TBRS, haematological malignancies and may have further applications in other diseases.

CHAPTER 2

METHODS

2.1 Mice and Ethics

The use of animals in experiments was approved by the Walter & Eliza Hall Institute Animal Ethics Committee and was in accordance with the Australian Code of Practice for the care and use of animals for scientific purposes. All experiments herein were strictly in adherence with the conditions outlined by the aforementioned bodies. Animal welfare was monitored by experienced animal technicians, who made all decisions relating to ethical endpoints of experiments. All mice were maintained on a C57BL/6-CD45.2 background unless otherwise specified.

2.2 Generation of mouse strains for in vivo and in vitro assays

2.2.1 *DNMT3a*^{R878H/+} mice

The *Dnmt3a*^{R878H/+} mice were generated by the MAGEC laboratory at the Walter and Eliza Hall Institute (WEHI) as previously described by Kueh, et al. ¹⁶² on a C57BL/6 background. Briefly, Cas9 mRNA, single guide RNA (sgRNA) (CCTCGCCAAGCGGCTCATGT;) and the oligo donor (ccgcactcactcccttcctgccttctccacagGGTGTGGCTTCCCCGTCCACTACACCGACGTCTCTAACATGAGCCACTTGGCGAGGCAGAGACTGCTGGGCCGATCGTGGAGCGTGCCGGTCATCCGCCACCTCTT) were injected into fertilized single-cell stage embryos. Two-cell stage embryos were then transplanted into pseudo-pregnant female mice. Viable offspring were genotyped by next-generation sequencing. The *Dnmt3a*^{R878H/+} mice were continually backcrossed onto the C57BL/6 background.

2.2.2 *Lck-Notch1-IC9 transgenic mice*

The *Lck-Notch1-IC9 (Notch1)* mice, described previously ¹⁶³, were used to generate compound heterozygous *Dnmt3a*^{R878H/+}*Notch1*^{T/+} mice for the assessment of the role of *Dnmt3a*^{R878H/+} in the development of Notch1 driven T-ALL. *Dnmt3a*^{R878H/+} male mice were inter-crossed with Female *Notch1*^{T/+} mice. Pups were monitored for the development of leukaemia by experienced animal technicians. When signs of illness like laboured breathing or ruffled fur were detected, mice were euthanised and a bleed taken. Where possible, 200uL of blood was collected by retro-orbital bleed or heart puncture. Mice that had increased respiration were not bled by retro-orbital injection as thymoma precludes scruffing of the mice. Adequate amounts of blood for

ADVIA (200uL) was not always possible as the tumour mass can make it difficult to take bleeds by venous puncture. Bleeds were analysed by ADVIA and tumours were immunophenotyped by flow cytometry.

2.2.3 *p53 knock-out mice*

The KO50 (p53 deficient) (*Trp53*^{+/-}) mice have been described previously¹⁶⁴. Female *Trp53*^{+/-} mice were used to generate compound heterozygous *Dnmt3a*^{R878H/+}*Trp53*^{+/-} mice by inter-cross with male *Dnmt3a*^{R878H/+} mice due to breeding limitations of the *Dnmt3a*^{R878H} mutant mouse line. Mice were then aged to determine if the presence of the *Dnmt3a*^{R878H} mutation could accelerate *Trp53*^{+/-} tumour onset. The mice were monitored for signs of illness and were euthanised when they displayed signs of illness like lethargy, increased respiration, ruffled fur or enlarged organs/masses upon palpation. Mice were excluded from the survival curve for common illness known to affect mice on a C57BL/6 genetic background. These illnesses included malocclusion, fight wounds, ear infections or staph infections.

2.2.4 *Genotyping*

Genomic DNA was isolated from tail or ear clippings at weaning, by digesting tissue in 150 µL of DirectPCR Lysis Reagent (Viagen Biotech) with 0.4 µL Proteinase K (Sigma-Aldrich) at 56°C on a shaker overnight. Following digestion, Proteinase K was heat inactivated at 85°C for 30 minutes. Polymerase chain reactions (PCR) were performed with 1 µL Genomic DNA with 10 µL GoTaq Green Master Mix (Promega) with relevant primers at a final concentration of 0.5 µM (Table 1). PCR reactions were performed as outlined in table 2. PCR products were separated by Gel Electrophoresis (100 V for 30-40 min) on a 2 % agarose gel containing 0.5 µg/mL ethidium bromide (ThermoFisher). Gels were visualised on the ChemiDoc XRS+ machine with ImageLab software (Biorad).

‘KASP™ by Design’ (LGC Biosearch) was used for genotyping the *Dnmt3a*^{R878H} mouse strain. Since the PCR products of the wildtype (wt) and *Dnmt3a*^{R878H} allele could not be distinguished by size, the competitive allele-specific PCR had to be used to identify the mutant allele. Reactions were performed using 1 µL of Genomic DNA with 4 µL Nuclease-free water, 5 µL 2X KASP Master Mix (LGC Biosearch Technologies) and 0.14 µL KASP assay mix (including primers, table1) (LGC

Biosearch Technologies). Reactions were performed in 384 well plates and fluorescence was determined by biallelic discrimination on the ABI 7900 Fast real-time PCR system (Applied biosystems) or the Quantstudio 12K Flex real-time PCR system (Applied biosystems) as outlined in table 2. Results were visualised using the Quantstudio 12K Flex software.

Table 2.1 Genotyping primer information

Allele	Primers	Product
<i>Dnmt3a</i>^{R878H}	CAGTCTCTGCCTCGCCAAGC	Mut- FAM
	GCAGTCTCTGCCTCGCCAAGT	wt- HEX
	GTGTTTGGCTTCCCCGTCCACTA	Common
<i>Lck-Notch1-IC9</i>	CATGGACTACAAAGACGATGAC	T 250 bp
	GAGAACTACTGGCTCCTCAAAC	
<i>KO50 (Trp53)</i>	TTATGAGCCACCCGAGGT	KO 600 bp
	TATACTCAGAGCCGGCCT	wt 450 bp
	TCCTCGTGCTTTACGGTATC	
Mut; mutant allele, wt; wildtype allele, T; Transgene, KO; knockout		

Table 2.2 PCR and KASP programs

PCR reaction	PCR program		
Standard PCR	94°C	4 minutes	1 cycle
	94°C	40 seconds	
	55°C	30 seconds	30 cycles
	72°C	60 seconds	
	72°C	5 minutes	1 cycle
KASP	94°C	15 minutes	1 cycle
	94°C	20 seconds	
	61-55°C	60 seconds*	10 cycles
	94°C	20 seconds	
	55°C	60 seconds	26 cycles
*(drop 0.6°C/cycle)			

2.3 In vitro assays

2.3.1 Flow cytometry and Fluorescence Activated Cell Sorting

Haematopoietic organs were prepared for analysis by flow cytometry by firstly generating single cell suspensions. To do this, the tissue was gently passed through a 100 µM Nylon cell strainer (Falcon). For Blood, the cells were treated with 200 µL red blood cell lysis buffer for 5 minutes, followed by washing with phosphate buffered saline (PBS) supplemented with 5% heat-inactivated foetal calf serum (HI-FCS), herein referred to as wash buffer. Cells were then stained with a cocktail of antibodies against cell surface markers with Fc block in wash buffer, covered from light on ice for 30 minutes. Fc block was used to prevent immune cells binding to that antibodies via the Fc region rather than the antigen specific Fab region. Immediately prior to analysis by flow cytometry or fluorescence activated cell sorting (FACS), live cells were treated with a viability marker (PI, DAPI or Fluorogold). Flow cytometry was performed on either LSRIIW,

LSRIIC, BD Fortessa1, BD Fortessa X20 or Cytex Aurora. Cell sorts were performed by staff at the Walter and Eliza Hall FACS facility on the ARIA III or ARIA Fusion. All data was analysed using Flowjo v10.

Table 2.3 Antibodies for flow cytometry and FACS

Antigen	Clone	Antigen	Clone	Antigen	Clone
CD4	GK1.5-7	Ter119	TER119	CD34	RAM34
CD4	H129	IgD	11.26c.2a	CD135	A2F10
CD8	53-6.7	IgD	11.26C	CD48	HM48-1
CD8	YTS.169	NK1.1	PK136	CD150	TC15-12F12.2
TCRβ	H57.597	CD44	IM781	CD45.1	A20
B220	RA3-6B2	CD25	PC61	CD45.2	104
Mac1/CD11b	M1/70	Ly6G	1A8	5mC	5MC-CD
Gr1	RB6-8C5	CD2	RM2.1	γ-H2AX	20E3
cKIT/CD117	2B8	CD3	14-2C11	Anti-rabbit	
Sca1	E13-161.7	CD16/32	24G2	F4/80	
CD19	ID3	IgM	5.1	IgM	RMM-1

2.3.2 ADVIA

Blood was collected by retro-orbital or mandible venous puncture from live mice or by heart puncture of deceased mice into EDTA coated microvettes (Sarstedt). 200 μ L of blood was used for analysis by the high-volume hematology analyser ADVIA 2120 and 2120i (Siemens).

2.3.3 Histology

Mouse organs were dissected and immediately placed in 10% buffered Formalin (Formalin diluted in water) for at least 24hrs. The organs were then sent to the WEHI histology service for haematoxylin and eosin (H&E) staining. Some organs were sent to the Australian Phenomics Network (APN) at the University of Melbourne for H&E staining and assessment by a qualified pathologist.

2.4 Haematopoietic reconstitution experiments

2.4.1 General hematopoietic reconstitution

Recipient mice on a C57BL/6 Ly5.1 (CD45.1⁺CD45.2⁺) genetic background were used for all haematopoietic reconstitution experiments, unless otherwise stated. Recipient mice were lethally irradiated with 2 doses of 5.5 Gray (Gy), two hours apart. Recipient mice were then injected with haematopoietic stem cells derived from E13.5 foetal livers or whole bone marrow by intravenous tail injection. Recipient mice received a maximum of 2×10^6 cells in a maximum volume of 200 μ L of PBS intravenously (IV). Irradiated mice were given water supplemented with Neomycin (ThermoFisher) to prevent infection during recovery. Mice were monitored daily by experienced animal technicians for signs of reconstitution failure; i.e. pale feet, hunched, ruffled fur and heavy breathing. Mice that showed signs of reconstitution failure were euthanised immediately. Following successful reconstitution, mice were monitored weekly by animal technicians for signs of illness.

2.4.2 Competitive bone marrow reconstitutions

Bone marrow was harvested from 2 Femurs of donor mice. In a sterile fume hood, the bone marrow was gently flushed using a 23-gauge needle filled with PBS supplemented with 10% FCS. Bone marrow was counted using the Moxi Z mini automated (Orflo) cell counter. Competitor bone marrow derived from Ly5.1J (CD45.1⁺) mice was pooled together to ensure that both wt control and *Dnmt3a*^{R878H/+} test bone marrow was in competition with the same pool of CD45.1⁺ cells. The CD45.2⁺ bone marrow was harvested from either control wt mice or *Dnmt3a*^{R878H/+} mice, both of which were on a C57BL/6 genetic background. The CD45.2⁺ was not pooled together, to maintain separate biological replicates. Competitor (CD45.1⁺) and test (CD45.2⁺)

bone marrow was mixed together in a 1:1 (CD45.1: CD45.2) or 9:1 (CD45.1: CD45.2) ratio in PBS. Each mix of bone marrow was then prepared such that each recipient mouse received 2×10^6 cells in 150 μ L of PBS. There was a total of 3-4 biological replicates for each condition and 3-4 technical replicates of each biological repeat in each experiment. Mice were then aged for the specified period of time.

2.4.3 Serial competitive bone marrow reconstitutions

For serial competitive bone marrow reconstitutions, the primary reconstitutions were set up as outlined in section 2.4.2. Mice were left to age for 3- or 6-months following reconstitution. At the end of the primary endpoint, bone marrow was harvested from 2 femurs and gently flushed with PBS supplemented with 5% HI-FCS to generate a single cell suspension for cell counts. Bone marrow from each technical replicate was pooled together in equal ratios to minimise technical variation in bone marrow proportions. Prior to IV injection, the cells were washed and resuspended in PBS. A total of 2×10^6 bone marrow cells from each mix were IV injected into each lethally irradiated recipient mice. Blood, spleen, thymus and bone marrow were also collected for analysis by flow cytometry and histology.

2.5 In vivo treatments

2.5.1 5-Flourouracil treatment

Baseline bleeds were collected by mandible bleed one week prior to drug treatment to establish baseline blood composition. DNMT3a^{R878H/+} mice or wt control littermates received a single dose of 150mg/kg of 5-Flourouracil (5-Fu) by IV injection. Further mandible bleeds were taken on day 1, 4, 7, 10, 14 and 21 following treatment to monitor the recuperation of blood cells in response to the DNA damaging agent. Blood composition was analysed by the ADVIA blood analyser and flow cytometry using fluorescent conjugated antibodies against TCR β , CD4, CD8, B220, IgM, IgD, Gr1, Mac1, CD45.1 and CD45.2. Mice were monitored throughout the experiment for signs of illness by experienced animal technicians. All mice survived the treatment and were sacrificed at the final timepoint to collect organs.

2.5.2 Irradiation-induced thymic lymphoma model

Four-week-old DNMT3a^{R878H/+} or wt control litter mates received low dose (1.5Gy) γ -irradiation, once a week for 4 weeks (total 4 doses). Mice were then monitored for the development of thymic lymphoma. Mice were euthanised when they showed signs of illness such as laboured breathing, ruffled fur or lethargy. Where possible, blood was collected for analysis of blood cell composition by ADVIA. Thymic lymphoma was determined by thymus weight <50mg and/or abnormal CD4 and CD8 expression by flow cytometry. Overall survival was calculated in days from the last dose of irradiation.

2.6 RNA sequencing

2.6.1 Sample collection and RNA extraction

4-week-old Female DNMT3a^{R878H/+} or wt control littermates received a single low dose of γ -irradiation (2Gy) or no irradiation (Baseline). 2 hours after γ -irradiation, mice were sacrificed and both Femurs and Tibias were collected. The bone marrow was flushed with PBS supplemented with 5% FCS to generate a single cell suspension and then stained with biotinylated antibodies against TER119, Mac1, Gr1, B220, CD19 and TCR β . The bone marrow was then incubated for 5 minutes at room temperature with streptavidin conjugated magnetic selection beads (Magnisort™ MSNB-6002-71 Life technologies). Following incubation, each sample was placed on a magnet to remove cells expressing lineage markers. The lineage negative enriched bone marrow was then incubated with antibodies against cKit and Sca1 to identify HSPCs, as well as lineage markers to detect any remaining lineage committed cells. Prior to cell sorting the cells were incubated briefly with PI to determine live cells. Lineage⁻Sca1⁺cKit⁺ Live (PI⁻) cells were then sorted using an ARIA III or Fusion machines. LSK cells were kept on ice and immediately lysed in 700uL of TRIZOL (Invitrogen 15596026) and stored at -80°C until enough samples were collected. Once all samples were collected, RNA was extracted using the QIAGEN miRNeasy kit (Cat #/ ID 217084) with optional on-column DNase digestion (Cat#/ ID 79254).

2.6.2 cDNA synthesis and amplification

The SMART-seq HT Kit (Takara Bio USA, Inc) was used for cDNA synthesis and amplification due to the small number of LSK cells isolated from each mouse resulting in an RNA yield below 100

ng. RNA was amplified using the SMART (Switching Mechanism at 5' end of RNA template) technology as per the manufacturer's instructions. cDNA was quantified using the Agilent Tapestation high sensitivity D1000 Screentape assay.

2.6.3 Sequencing library preparation

Libraries were prepared using the Nextera XT DNA Library prep kit (Illumina) as per the manufacturer's instructions. Briefly, cDNA was tagged, indexed and then enriched. Following enrichment, the libraries were cleaned using NGS magnetic beads to remove contaminants. The indexed libraries were pooled and diluted to 1.5pM for paired end sequencing (2x 81 cycles) on a NextSeq 500 instrument using the v2 150 cycle High Output kit (Illumina) as per manufacturer's instructions. The base calling and quality scoring were determined using Real-Time Analysis on board software v2.4.6, while the FASTQ file generation and de-multiplexing utilised bcl2fastq conversion software v2.15.0.4.

2.6.4 Bioinformatic analysis

Each type of samples had two to five biological replicates, ending up with fourteen RNA-seq libraries in the analysis. The sequencing reads were aligned to the GRCm38/mm10 build of the *Mus musculus* genome using the Subread aligner [PMID: 23558742]. Genewise counts were obtained using featureCounts [PMID: 24227677]. Reads overlapping exons in annotation build 38.1 of NCBI RefSeq database were included. Genes were excluded from downstream analysis if they failed to achieve a CPM (counts per million mapped reads) value of greater than 1.0 in at least two libraries.

Counts were converted to log2-CPM, quantile normalised and precision weighted with the voom function of the limma package [PMIDs: 24485249 and 25605792]. A linear model was fitted to each gene, and empirical Bayes moderated t-statistics were used to assess differences in expression [PMID: 16646809]. The False Discover Rates (FDR) were adjusted across all the comparisons, and an FDR cut-off of 0.1 was applied for calling differentially expressed genes.

Known pathways enriched in the differentially expressed genes were detected by the Metascape on-line service [PMID: 30944313]. Heatmaps were generated to show the expression levels of all genes in each of the selected pathways that were called enriched in the differentially

expressed genes, and barcode plots measuring the enrichment levels of the pathways in our samples were generated using the barcodeplot function in limma. The significance levels of enrichment were calculated by the roast function in the same package [PMID:20610611]. Gene tracks were created in Integrated Genomic Viewer open access package from the Broad Institute.

2.7 Statistics

Prism software (Graphpad) was used to generate graphs and analyse all data unless otherwise indicated. Statistical methods used are referenced in the figure legends of relevant data. All data are presented as mean $\pm$ SEM unless otherwise specified. P value of <0.05 was deemed significant.

CHAPTER 3

CHARACTERISATION OF THE *Dnmt3a*^{R878H}

MUTANT MOUSE MODEL

3.1 Introduction

There is limited understanding of the consequences of DNMT3a R882 mutations on normal development and disease, which is due to the lack of preclinical models that faithfully mimic human disease. As discussed in Chapter 1, the majority of published work employs the use of murine *Dnmt3a* KO models, but this does not accurately reflect the human setting in which the majority of mutations in *DNMT3a* impair its function, but do not lead to a total lack of the protein. The only mutant *Dnmt3a*^{R878H} murine models that have been published previously are inducible models that require Cre recombination to induce the expression of the mutant protein^{34 154}. While there are some obvious benefits of having an inducible model, it precludes the examination of the impact of mutant *Dnmt3a*^{R878H} during embryonic development. Furthermore, the consequences of mutant *Dnmt3a*^{R878H} on the development of non-haematopoietic tissues has been vastly overlooked in the field.

The impact of the *Dnmt3a*^{R878H} mutations on embryonic development and on organs in the adult mouse is of particular interest given that Tatton-Brown-Rahman syndrome (TBRS), an intellectual disability and overgrowth syndrome, is caused by germline mutations in *DNMT3a*. Like patients with *DNMT3a* mutant AML, mutations in *DNMT3a* are also enriched at R882 in patients with TBRS⁸⁶. As highlighted in Chapter 1, germline mutations in *DNMT3a* have only recently been identified as the cause of this particular form of intellectual disability and overgrowth syndrome⁸⁶. Since there are so few cases of TBRS reported in the literature (only ~75 patients worldwide)^{86,101}, clinicians are offered only minimal resources to inform the strategy for clinical care of these patients. Further compounding this difficulty is the fact that many of the TBRS patients are children or adolescents, meaning there is little understanding of what complications patients with TBRS may face in adulthood and old age. Somatic mutations in *DNMT3a* are strongly associated with AML and T-ALL in adult populations but it remains unknown whether TBRS patients may also be at an increased risk of developing these malignancies. So far, only two patients with TBRS have developed AML^{108,109} but it is difficult to determine risk based on such small populations. Clearly there is a need for a preclinical model to study the impact of mutant *Dnmt3a* on development, ageing and cancer to provide some insight into expected clinical outcomes for patients with inherited *DNMT3a* R882H mutations.

To characterise the impact of mutant DNMT3a on embryonic development and in adult life, we

created a mouse model with a point mutation at residue R878 of *Dnmt3a*, which is the murine homologue of the human *DNMT3a* R882 mutation. This mouse model was used to study the impact of heterozygous *Dnmt3a*^{R878H} mutations in mice. As discussed in chapter 1, heterozygous DNMT3a R882H mutations are strongly enriched in patients with both AML and TBRS^{86,89}. In this chapter, data will be presented on the ability of *Dnmt3a*^{R878H/+} mice to undergo normal embryogenesis and an overview of organ morphology in young and aged *Dnmt3a*^{R878H/+} mice.

3.2 Generation and characterisation of the *Dnmt3a*^{R878H/+} mouse model

The *Dnmt3a*^{R878H/+} mouse model was created by the MAGEC laboratory using CRISPR/Cas9 gene editing. The guide RNA template was designed to introduce the murine homologue of the human ‘hostpot’ R882H mutation into exon 23 of the mouse *Dnmt3a* gene. The guide RNA template was designed with three-point mutations depicted in Figure 1A. Two silent mutations were introduced, the first one introduced a novel protospacer adjacent motif (PAM) site so that only the mutant allele could be retargeted by a sgRNA. The second mutation destroyed the original PAM site to prevent Cas9 from cutting the correctly targeted allele again. These mutations would not result in altered amino acid sequence in the protein and are therefore considered ‘silent’. The third mutation introduced at residue 878 resulted in a T to A mutation causing an arginine to histidine substitution in the protein. Importantly, in this model, the expression of *Dnmt3a*^{R878H} remains under the endogenous control of its endogenous promotor.

To introduce the *Dnmt3a*^{R878H} mutation into mice, the guide RNA and Cas9 mRNA were injected into the nucleus of single cell fertilised zygotes. Twenty-four hours later, two-cell stage embryos were transferred into the oviducts of pseudo-pregnant female mice. DNA collected from viable offspring was examined using next generation sequencing (NGS) to identify mice that had the intended mutations (data not shown). Correctly targeted mice were backcrossed to C57BL/6 mice for >4 generations to outbreed potential erroneous off-target mutations. To ensure that the *Dnmt3a*^{R878H} allele was expressed, RNA was isolated from LSK cells and subjected to next generation sequencing (further data from this RNA seq experiment is shown in Chapter 5). As shown in Figure 3.1 B, RNA from the mutant *Dnmt3a* allele was expressed and accounted for approximately 50% of the overall *Dnmt3a* transcripts, as expected. The three-point mutations are clearly visible as vertical lines in the RNA track, where blue corresponds to the mutation

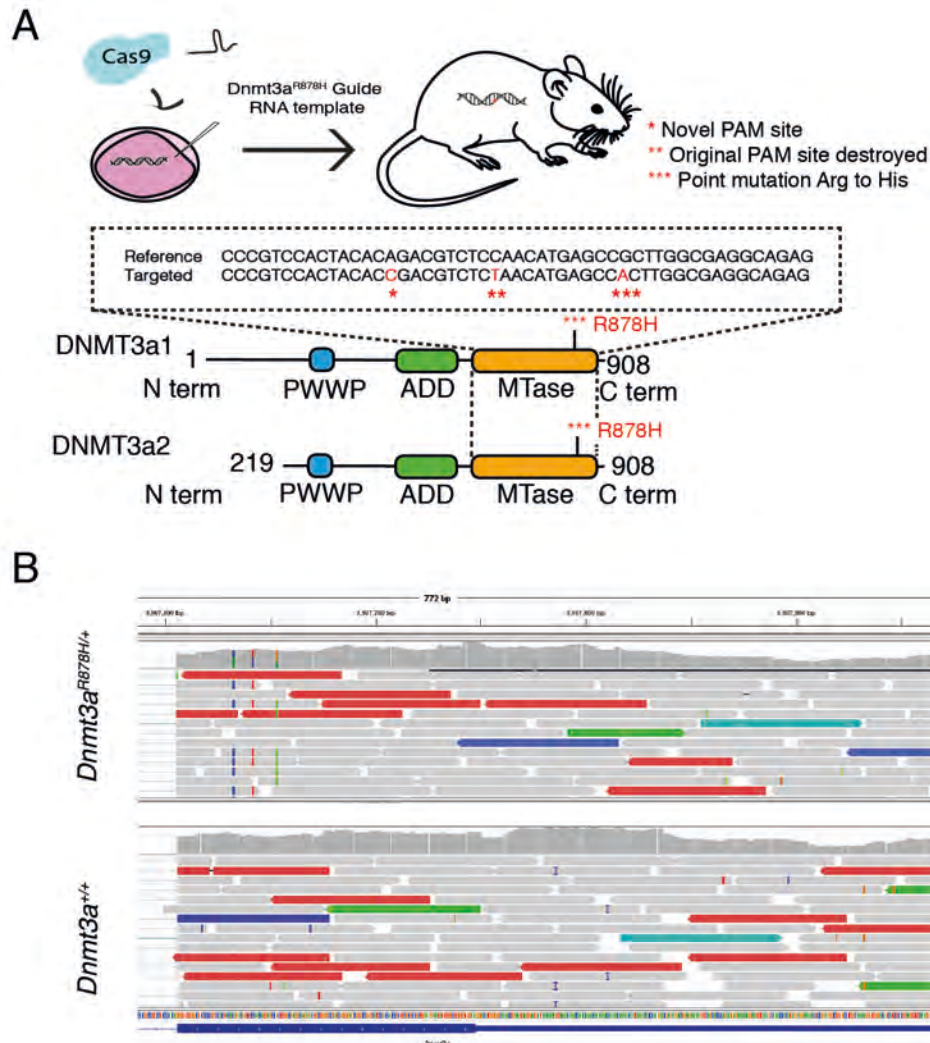


Figure 3.1 Generation of the *Dnmt3a*^{R878H/+} mutant mouse model.

A) Schematic showing the microinjection of Cas9 protein and *Dnmt3a*^{R878H} guide RNA template into the zygote. The guide RNA template included 3 mutations that are described in the text (* and ** were silent mutations). B) RNA track of exon 23 of *Dnmt3a*^{R878H/+} and wt LSK cells displaying single nucleotide changes as a vertical line where * = blue, ** = red and the R878H*** = green.

depicted by * in figure 3.1 A, the ** mutation is shown in red and the R878H mutation *** is shown in green.

3.2.1 *Dnmt3a*^{R878H/+} mice are born at a reduced Mendelian frequency but with the expected gender distribution

At weaning, *Dnmt3a*^{R878H/+} mice present at a slightly lower prevalence than expected by Mendelian ratios (Figure 3.2 A; Table 4) but there was no gender imbalance amongst the viable offspring (Figure 3.2 B). Interestingly, inter-crosses between female *Dnmt3a*^{R878H/+} and male C57BL/6 wt mice did not yield any viable offspring at weaning (Table 5). The female *Dnmt3a*^{R878H/+} mice did get pregnant but were unable to deliver the pups due to birthing difficulties. Upon inspection, it appeared that the pups had been partially reabsorbed suggesting that they had died sometime prior to the onset of contractions. The inability of the *Dnmt3a*^{R878H/+} female mice to give birth to live pups indicated that there was likely a problem with the ability of the female *Dnmt3a*^{R878H/+} mice to sustain a pregnancy rather than a developmental defect in the fetuses. Inter-crosses of *Dnmt3a*^{R878H/+} males with C57BL/6 females yielded normal, healthy offspring with only infrequent birthing difficulties. At this stage, it remains unclear what the underlying cause is for the inability of *Dnmt3a*^{R878H/+} females to produce viable offspring.

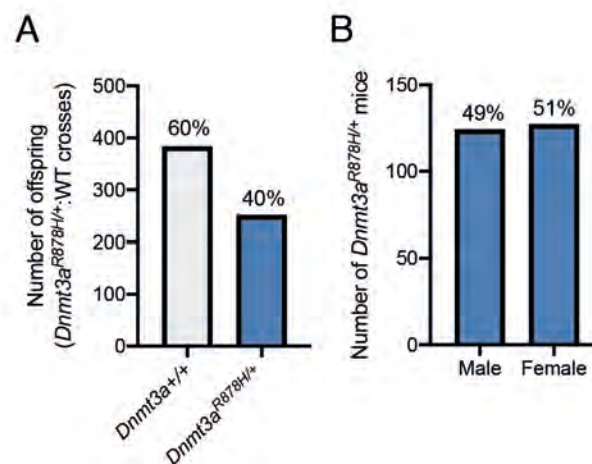


Figure 3.2 Frequency and gender distribution of *Dnmt3a*^{R878H/+} mice at weaning

A) Number of offspring and frequency of the indicated genotypes at weaning derived from inter-crosses of *Dnmt3a*^{R878H/+} and wt mice of the indicated genders. B) Gender distribution of *Dnmt3a*^{R878H/+} mice. Data presented as absolute numbers. Statistics on frequency of genotypes displayed in table 4. Gender differences compared using students t-test, not significant.

Table 3.1 Observed genotype distribution of offspring from inter-crosses of *Dnmt3a*^{R878H/+} mice with wt mice

^a <u>Male</u> <i>Dnmt3a</i> ^{R878H/+} x wt	+/+	R878H/+	R878H/R878H	n	χ^2 DF=1	p-value
Expected	50%	50%	0			
Actual	383 (60%)	251 (40%)	0	634	27.48	<0.0001

^b <u>Female</u> <i>Dnmt3a</i> ^{R878H/+} x wt	+/+	R878H/+	R878H/R878H	n	χ^2 DF=2	p-value
Expected	50%	50%	0			
Actual	0	0	0	0		

^c <i>Dnmt3a</i> ^{R878H/+} x <i>Dnmt3a</i> ^{R878H/+}	+/+	R878H/+	R878H/R878H	n	χ^2 DF=2	p-value
Expected	25%	50%	25%			
Actual (IVF)	14 (33%)	22 (57%)	4 (11%)	40	5.4	0.067

^a *Dnmt3a*^{R878H/+} male mice were inter-crossed with wt female mice and the genotype distribution at weaning is shown as numbers (percentages). Chi-square (χ^2) test was used to determine percentage. Degrees of freedom (DF).

^b *Dnmt3a*^{R878H/+} female mice were inter-crossed with wt male mice. No live pups were born and therefore no statistical analysis was performed.

^c IVF was performed to generate homozygous *Dnmt3a*^{R878H/+} mice and the genotype distribution at weaning is shown as numbers (percentages). Chi-square (χ^2) test was used to determine percentage. Degrees of freedom (DF).

In an attempt to investigate whether the *Dnmt3a*^{R878H} mutation caused defects in foetal development or impacted the ability of females to become pregnant, *in vitro* fertilisation (IVF) using *Dnmt3a*^{R878H/+} oocytes and *Dnmt3a*^{R878H/+} sperm was performed. The zygotes were then implanted into foster mice to examine the role of the heterozygous *Dnmt3a*^{R878H} mutation in the mum vs the developing embryo. Interestingly, such inter-crosses produced healthy *Dnmt3a*^{R878H/R878H} pups, albeit at a lower frequency than expected, and they survived to weaning. The *Dnmt3a*^{R878H/R878H} pups accounted for only 10.5% of the pups in the litters, which is well below the expected Mendelian ratio of 25% although this did not reach significance (chi square $p > 0.05$) (Table 3.1). This indicates that wt DNMT3a is critical for sustaining embryonic development in mice. Further experiments need to be done to determine the mechanism by which *Dnmt3a*^{R878H/+} mutations cause defects during embryonic development when the mum is heterozygous for *Dnmt3a*^{R878H}. It is tempting to speculate that the birthing difficulties and loss of the pups is intrinsic to *Dnmt3a*^{R878H/+} females and that this might result from placental abnormalities or abnormal hormonal signalling which is critical to the development of the foetus¹⁶⁵. However, further experiments will be required to determine at which stage of embryonic development Dnmt3a is critical either within the embryo or the mother.

Following the birth of *Dnmt3a*^{R878H/+} and *Dnmt3a*^{R878H/R878H} mice, pups were examined for morphological defects. *Dnmt3a*^{R878H/+} mice did not appear to have any gross morphological defects at birth. However, at weaning there were significantly fewer *Dnmt3a*^{R878H/+} mice compared with their wt littermates ($p < 0.0001$) (table 3.1). *Dnmt3a*^{R878H/R878H} pups were severely runted at weaning (Figure 3.3) and had to be euthanised by 4 weeks of age due to malocclusion, seizures upon scruffing or a general uncharacterised failure to thrive. Similar observations have been documented by Okano, et al.⁴⁹, who reported that complete genetic loss of Dnmt3a resulted in runted pups that were unable to survive beyond four weeks of age. The authors noted that the mice sometimes suffered from afebrile seizures which was also observed in one out of the four *Dnmt3a*^{R878H/R878H} pups generated in our colony. The difficulty in generating the *Dnmt3a*^{R878H/R878H} mice precluded further characterisation of these animals within the timeframe of this thesis. However, it would be interesting in the future to generate additional *Dnmt3a*^{R878H/R878H} mice and perform an extensive analysis on their cells and tissues. The following sections will focus on the impact of heterozygous *Dnmt3a*^{R878H} mutations on the general wellbeing of mice and the effect on ageing.



Figure 3.3 *Dnmt3a*^{R878H/R878H} mice are runted and fail to thrive.

Mice were littermates that were four weeks of age at the time of this photograph. The *Dnmt3a*^{R878H/R878H} mouse was euthanised as it had seizures upon scruffing.

3.3 *Dnmt3a*^{R878H/+} mice have normal organ morphology at 10 weeks of age

The *Dnmt3a*^{R878H} mutation was introduced into the germline of mice and therefore the mutant protein was expected to be expressed in all tissues of the mice. As demonstrated above we could detect expression of the *Dnmt3a* mRNA from the mutant and wt alleles at the same levels. To determine if there were any striking morphological consequences of expressing mutant DNMT3a in the adult, a histological examination was performed on the heart, kidney, lung, liver, spleen and bone marrow harvested from *Dnmt3a*^{R878H/+} mice and wt littermate controls. The organs were fixed in 10% buffered formalin and submitted to the WEHI histology service for Haematoxylin & Eosin (H&E) staining to identify different structures. As shown in Figure 3.3, there were no obvious defects in the heart, kidney, lung, liver, spleen or bone marrow of these mice at 10 weeks of age.

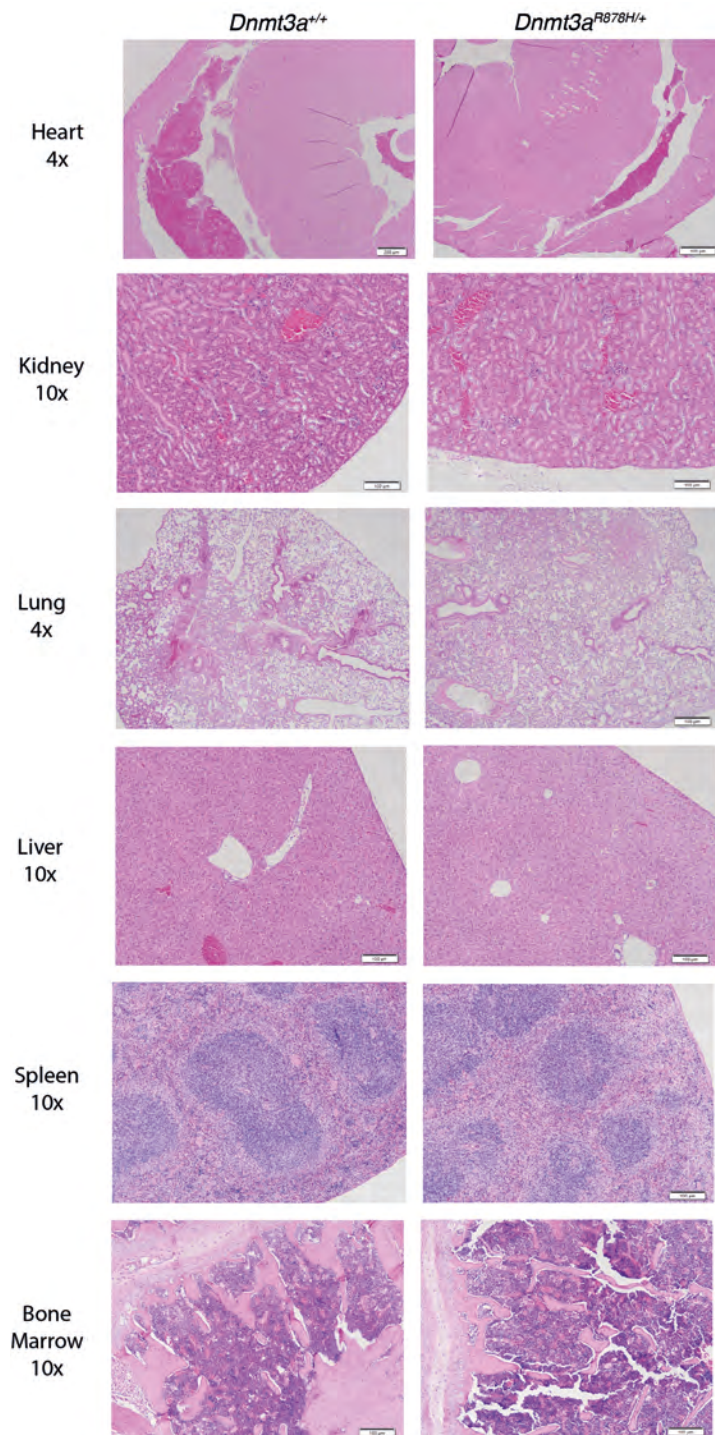


Figure 3.4 Histological examination of organs from $DNMT3a^{R878H/+}$ mice.

Organs were collected from $Dnmt3a^{R878H/+}$ mice and wt littermates at 10 weeks of age and fixed in 10% buffered formalin. Sections were stained with H&E and representative images of H&E stained sections from $Dnmt3a^{R878H/+}$ mouse and wt littermates are presented (n=2-3/group).

It is important to note that this was not an extensive analysis of organ morphology since only a few organs from *Dnmt3a* mutant mice were analysed by histological examination but not assessed by a pathologist. However, it was apparent that the collected organs from *Dnmt3a*^{R878H/+} and wt littermates animals did not significantly differ in their appearance. It must be noted that the histological analysis of the organs was conducted on young (10 week old) mutant *Dnmt3a*^{R878H/+} mice and it therefore remains unknown whether *Dnmt3a*^{R878H} mutations might exert adverse impact on the health of older mice.

3.4 *Dnmt3a*^{R878H/+} mice have a shorter overall lifespan compared to their wt littermates

To determine whether heterozygous *Dnmt3a*^{R878H} mutations resulted in adverse outcomes with increasing age, *Dnmt3a*^{R878H/+} mice were aged alongside their wt littermates. The overall survival of the aged mice is displayed in Figure 3.5. *Dnmt3a*^{R878H/+} mice had a significantly shorter overall survival compared to wt controls (455 vs 728 days, $p=0.001$). It was unclear why the *Dnmt3a*^{R878H/+} mice had an overall reduced survival compared to their wt littermates. Therefore, we wanted to determine, whether the reduced lifespan of *Dnmt3a*^{R878H/+} mice was due to a specific disease or whether they had a spectrum of defects. While somatic mutations in DNMT3a are strongly associated with haematopoietic malignancies in humans, the impact of these mutations in non-haematopoietic organs is less understood. Strikingly, when the aged *Dnmt3a*^{R878H/+} mice were visually inspected, it was clear that they were morbidly obese unlike their wt littermates.

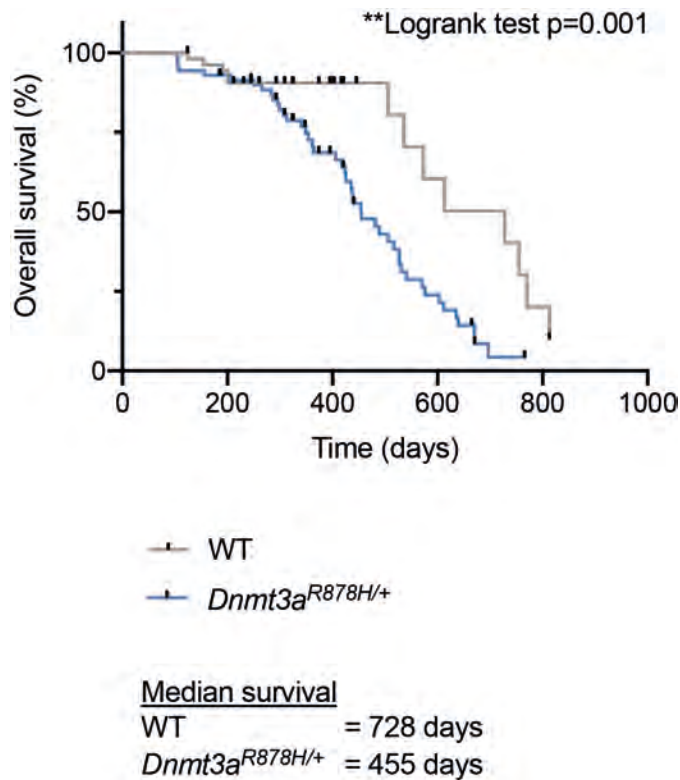


Figure 3.5 *Dnmt3a*^{R878H/+} have a poorer overall survival compared to their wt littermates

Kaplan-Meier survival curve comparing the overall survival of *Dnmt3a*^{R878H/+} mice to their wt littermates. Logrank test to compare curves, $p=0.001$. *Dnmt3a*^{R878H/+} $n=70$ (deaths 45), WT $n=54$ (deaths 13).

3.4.1 *Dnmt3a*^{R878H/+} mice develop obesity with increasing age

As shown in Figure 3.6 A, C, both male and female aged *Dnmt3a*^{R878H/+} mice were significantly heavier than their wt littermates (male $p<0.05$, female $P<0.0001$). This observation was intriguing as Tatton-Brown-Rahman syndrome (TBRS) patients carrying mutations in *DNMT3A* are characterised amongst other phenotypes by an abnormally increased weight, which is partly due to excess adipose tissue. Interestingly, in the *Dnmt3a*^{R878H/+} mouse model increased adiposity is also observed with increasing age (Figure 3.6 C). To determine whether these mice are born larger and sustain this increase in weight throughout life, *Dnmt3a*^{R878H/+} mice were weighed a few weeks after weaning (1.5 months old). At this age the *Dnmt3a*^{R878H/+} mice did not

weigh more than their wt littermates (Figure 3.6 B). The *Dnmt3a*^{R878H/+} male mice actually weighed significantly less than their wt littermates. This observation might be explained by another clinical characteristic of human TBRS where patients exhibit hypotonia, a decrease in muscle mass. While hypotonia was not experimentally verified in the *Dnmt3a*^{R878H/+} mice, visual inspection of the quadricep muscle of *Dnmt3a*^{R878H/+} male mice suggested that there was less muscle mass compared to male littermates. Clearly, the potential muscle defects of *Dnmt3a*^{R878H/+} mice should be further examined in more detail in the future although these initial observations already suggest that the *Dnmt3a*^{R878H/+} mouse model represents an accurate model for understanding the poorly characterised clinical course of TBRS patients.

TBRS patients are often overweight and obese, but the cause of this obesity is not known. However, there is some evidence in the literature from patients with Prader-Willi Syndrome, which is also caused by epigenetic defects, that the obesity in these patients is due to constant hunger¹⁶⁶. To determine whether the obesity observed in the *Dnmt3a*^{R878H/+} mice could be explained by an increase in food consumption, the volume of food ingested by *Dnmt3a*^{R878H/+} mice was measured over the course of 4 days. To this end, female *Dnmt3a*^{R878H/+} and wt mice aged 10-11 months that had been housed together since birth were separated into individual cages. Each morning the food was weighed before adding it into their cage. 24 hours later, the food was weighed again before adding a predetermined volume of food into the cage the next day. This was repeated over 4 days to reduce the initial confounding stress response of being separated. As shown in Figure 3.6 D there was no significant difference in food consumption between the two genotypes, suggesting that the abnormal increase in weight of the *Dnmt3a*^{R878H/+} mice could not be attributed simply to uncontrolled overeating.

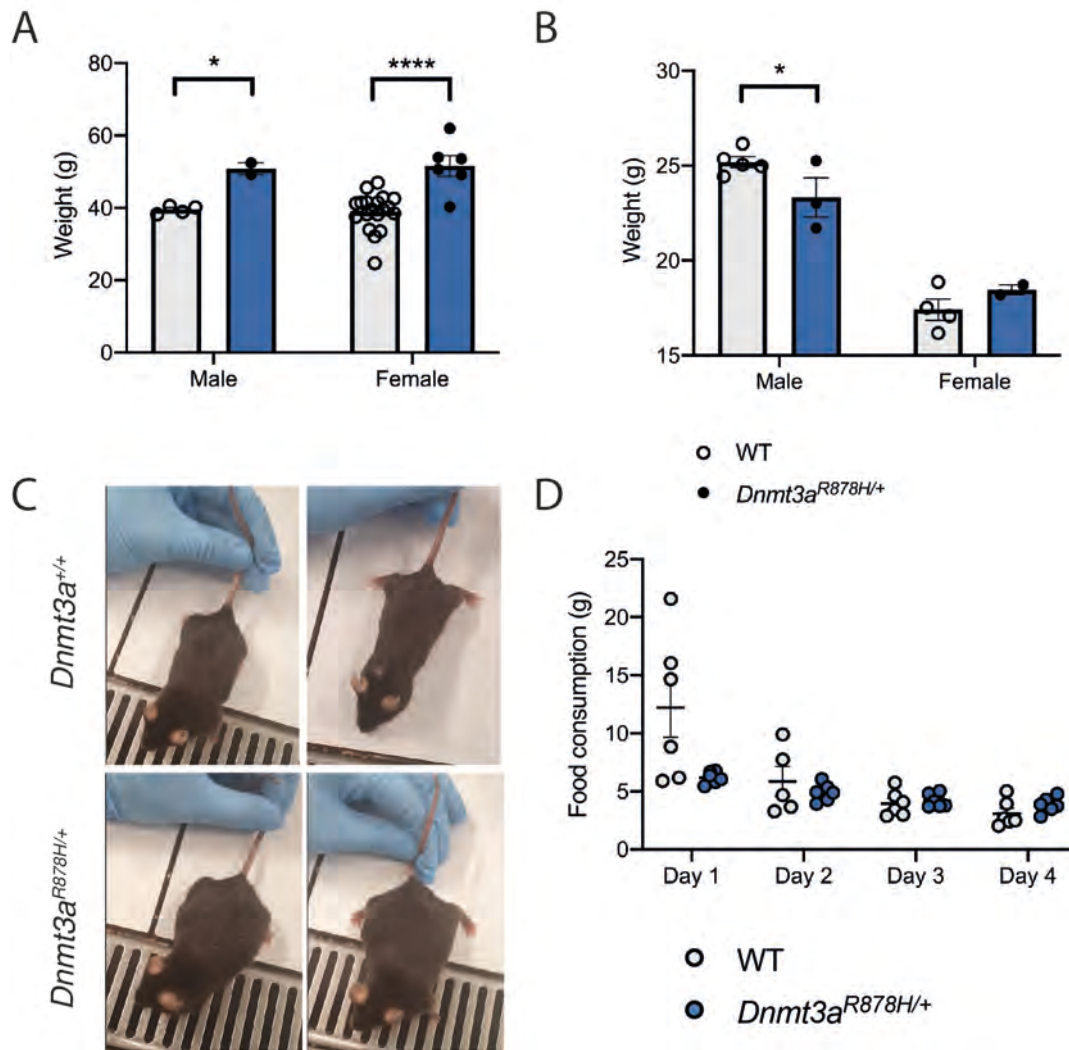


Figure 3.6 *DNMT3a*^{R878H/+} mice are obese and this is not due to increased food consumption

A) Weight of male and female mice (male mice 9.5 months, female mice 10-11 months) of the indicated genotypes. Males were housed together from birth *Dnmt3a*^{R878H/+} n=2, wt = 5. Female *Dnmt3a*^{R878H/+} n= 6, wt = 19. B) weight of mice of the indicated genotypes at 1.5 months of age, *Dnmt3a*^{R878H/+} n=3 males and n=2 females, wt n=5 males and n=4 females. C) images of *Dnmt3a*^{R878H/+} male mice and wt littermates (10.5 months). D) food consumption of female mice of the indicated genotypes aged 10-11 months. Mice were housed separately, and food was weighed each day to determine food consumption over 4 days. Data presented as mean±SEM. Students t-test, * p<0.05, *** p<0.001.

3.4.2 *Dnmt3a^{R878H/+} mice have a higher incidence of age associated liver disease*

Obesity is strongly associated with a significant reduction in life expectancy in humans. This is not surprising given that obesity is strongly associated with the development of cardiovascular disease, diabetes and cancer. It was shown in Figure 3.5 that *Dnmt3a^{R878H/+}* mice have a reduced overall survival compared to their wt littermates and that they also develop obesity with age (Figure 3.6). To determine the underlying cause of death in *Dnmt3a^{R878H/+}* mice, organs were collected from sick mice at ethical endpoint and assessed by histological examination for signs of abnormal organ morphology characteristic of disease.

The most striking abnormalities were observed in the liver of *Dnmt3a^{R878H/+}* mice. The livers were harvested from *Dnmt3a^{R878H/+}* and wt mice and fixed in 10% buffered formalin. After the samples were prepared, they were sent to the APN pathology service to be assessed by an experienced pathologist. These results are presented in Figure 3.7 and examples of liver histology are presented in Figure 3.8. There was evidence of lymphocytic infiltration into the livers of both the aged *Dnmt3a^{R878H/+}* mice and their littermates, and this was characterised as lymphatous infiltration and carcinoma infiltration of an unknown origin in the liver.

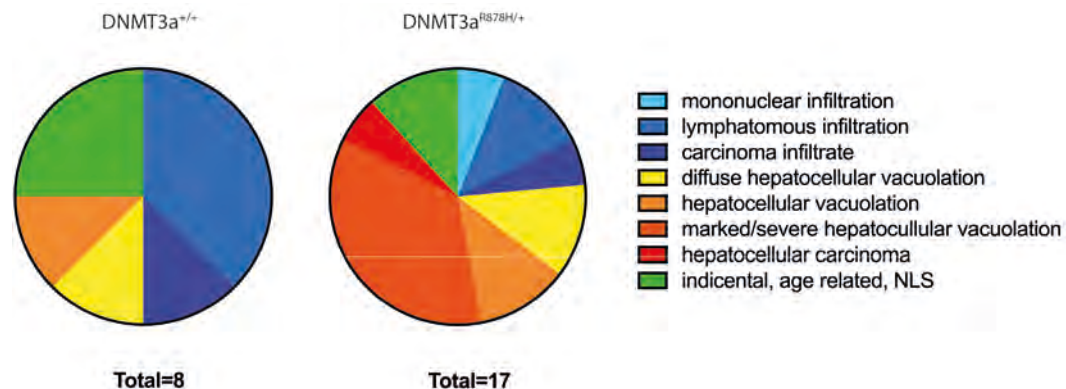


Figure 3.7 *Dnmt3a^{R878H/+} mice have a higher incidence and severity of liver disease associated with ageing compared to their wt littermates*

Liver histology slides were assessed by an experienced pathologist for signs of disease. NLS; no lesions of significance. Mean age of wt littermates was 561 days and for *Dnmt3a^{R878H/+}* mice was 491 days.

There was evidence of mononuclear cell infiltration into the liver of one *Dnmt3a*^{R878H/+} mouse which was not observed in the wt littermates (pale blue). Fewer wt mice were analysed overall and therefore the small sample numbers make it hard to draw a conclusion as to whether this mononuclear infiltration was more prevalent in *Dnmt3a*^{R878H/+} mice or if that was simply due to chance. However, there was undoubtedly a much higher incidence and greater severity of hepatocellular vacuolation in the *Dnmt3a*^{R878H/+} mice, as indicated by shades of yellow and orange in Figure 3.7. There was even evidence of progression to cancer in one *Dnmt3a*^{R878H/+} mouse, indicated in red.

Hepatocellular vacuolation/steatosis are terms used to describe fatty changes in the liver. As shown in Figure 3.7 there was some evidence of hepatocellular vacuolation in the wt littermates, although this was only evident in 2/8 mice assessed and was not graded as severe. In contrast, hepatocellular vacuolation was highly penetrant in the *Dnmt3a*^{R878H/+} mice and the severity was markedly higher, with evidence of hepatocellular carcinoma in one mouse. Vacuolation is caused by accumulation of lipids in the cytosol of hepatocytes. These lipid droplets can accumulate into large droplets (macro vesicular steatosis), which appear as large clear circles on histology slides as they displace the nucleus and other organelles to the periphery of the cell. Lipids can also accumulate in small droplets within the cytoplasm of the cell (micro vesicular steatosis) which give the cells a 'foamy' appearance. An example of micro vesicular steatosis is circled in the bottom left panel of *Dnmt3a*^{R878H/+} liver images in Figure 3.8. There was evidence of both macro and micro vesicular steatosis in the *Dnmt3a*^{R878H/+} mice.

The underlying cause of the macro and micro vesicular steatosis in the *Dnmt3a*^{R878H/+} mice is not known, although insight from previous studies suggests that the most common cause of micro vesicular steatosis is due to defects in mitochondrial beta oxidation of fatty acids ¹⁶⁷. The presence of micro vesicular steatosis is also commonly accompanied by evidence of visualisable mitochondria, termed megamitochondria ¹⁶⁸. However, megamitochondria were not reported by the pathologist in the liver sections from the *Dnmt3a*^{R878H/+} mice analysed. Of note, micro vesicular steatosis is associated with a poor prognosis in patients and commonly leads to liver failure ¹⁶⁸, which could account at least in part for the shortened lifespan observed of *Dnmt3a*^{R878H/+} mice.

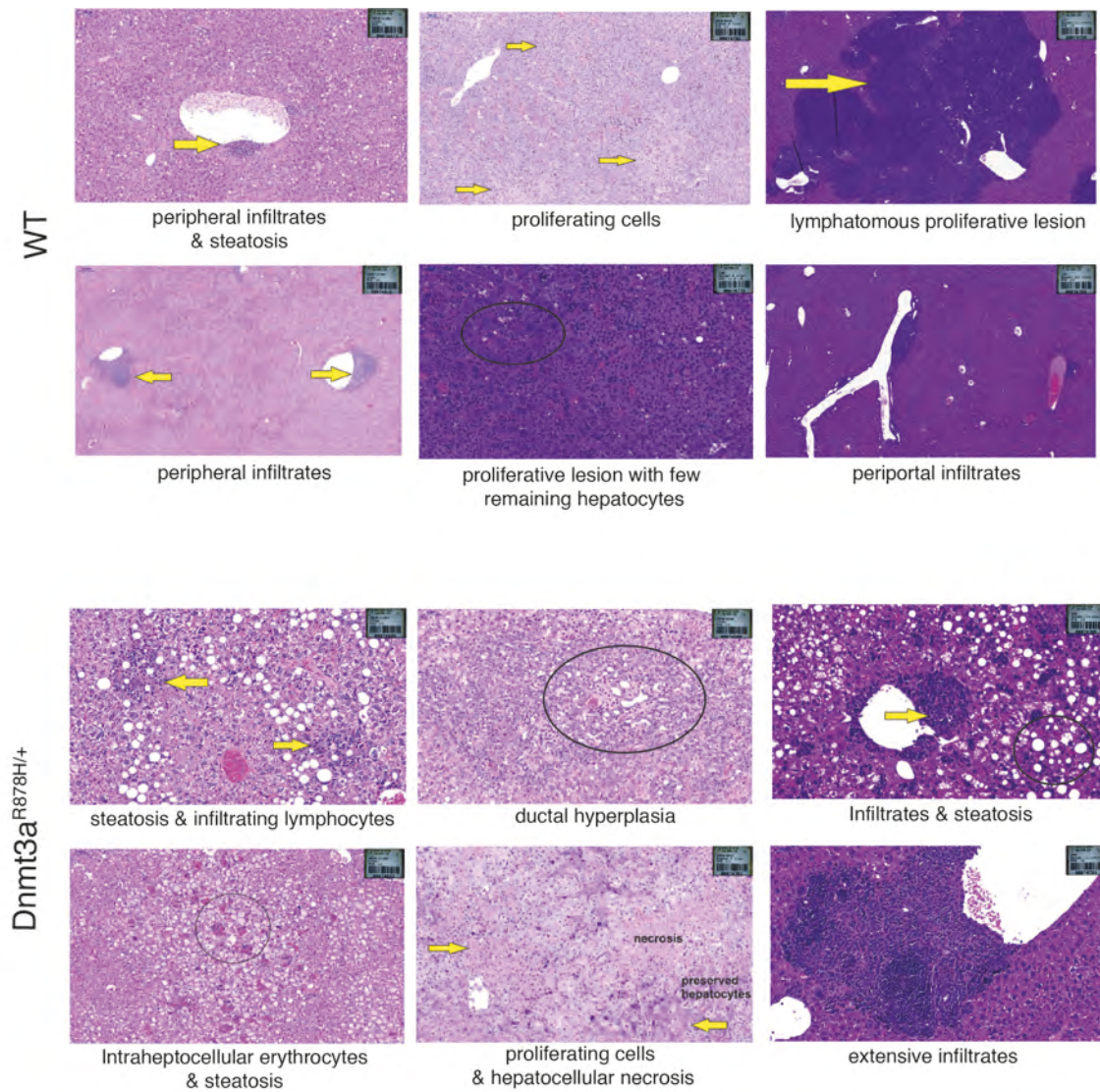


Figure 3.8 The livers of *Dnmt3a*^{R878H/+} mice exhibit more severe pathology compared to wt control mice.

Representative images of liver sections that had been stained with H&E and assessed by a pathologist. Livers were collected from sick aged mice at ethical endpoint. Yellow arrows point to foci of interest with descriptions below the image. Circles highlight regions of interest described below the image.

3.4.3 *Dnmt3a^{R878H/+} mice are more likely to develop lymphoma and leukaemia associated with age*

Further to an increase in the incidence and severity of liver pathology, abnormal spleen morphology was also prevalent in the aged mice. *Dnmt3a^{R878H/+}* mice but this was also seen in age-matched their wt littermates. When mice were deemed unwell by animal technicians, the mice were sacrificed, and their organs collected for assessment. Evidence of B cell lymphoma was present in a high proportion of mice, but this affected both *Dnmt3a^{R878H/+}* mice and their wt littermates. This suggests this could result from a susceptibility of the C57BL/6 genetic background of the mice rather than from the *Dnmt3a^{R878H/+}* mutation. It was noted that the *Dnmt3a^{R878H/+}* mice appeared to develop a broader range of both B cell lymphoma and histiocytic leukaemia at a younger age than their wt littermates. This suggests that the *Dnmt3a^{R878H}* mutation might accelerate age related disease. As shown in Figure 3.9, the *Dnmt3a^{R878H/+}* mice tended to have a broader range of spleen pathologies but there were similar frequencies of mice that showed incidental, age related or no lesions of significance in both genotypes.

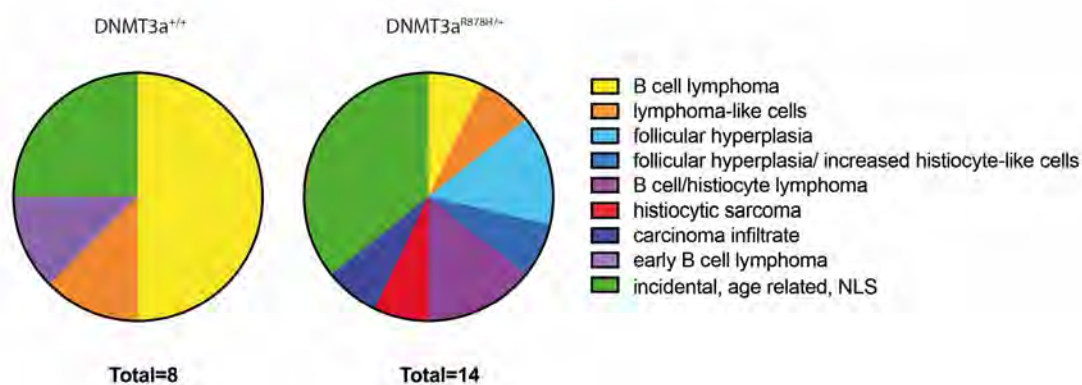


Figure 3.9 *Dnmt3a^{R878H/+} mice develop a range of B cell neoplasms with age but this is not different from their wt littermates.*

Splenic pathologies assessed by the APN pathologist from aged *Dnmt3a^{R878H/+}* mice and wt littermates. No significant lesions; NLS Mean age of wt littermates was 561 days, and for *Dnmt3a^{R878H/+}* mice it was 491 days.

Sections of spleens from aged *Dnmt3a*^{R878H/+} and wt littermate mice are shown in Figure 3.10, where examples of normal spleen architecture from an aged mouse with normal splenic morphology is displayed at the top and proliferative lesions in aged *Dnmt3a*^{R878H/+} mice and wt littermates are shown below. The scarcity of myeloproliferative diseases was surprising given that *DNMT3a*^{R882H} mutations are observed frequently in patients with MDS and AML. On this basis we hypothesised that the *Dnmt3a*^{R878H/+} mice would develop MDS and AML at a higher frequency than T-ALL or B-ALL. Unexpectedly, *Dnmt3a*^{R878H/+} mice, however, developed T-ALL only in rare cases with very few mice presenting with an enlarged thymus or lymph nodes with age. In patients, mutant *DNMT3a* is reported in around 20% of T-ALL cases¹¹¹. The incidence of T-ALL in our *Dnmt3a*^{R878H/+} mouse model is well below the expected frequency compared to the human populations. The high incidence of B cell neoplasms in the *Dnmt3a*^{R878H/+} mouse model was also perplexing. Mutant *DNMT3a* is rarely found in B-ALL in patients, although it does occur at very low frequencies^{169,170}. Understanding why the *Dnmt3a*^{R878H/+} mice have such a strong bias towards the development of B cell malignancies will require further characterisation of these animals under steady state conditions and in the context of oncogenic stimuli that drive B cell lymphoma development.

3.5 Discussion

In this chapter the novel *Dnmt3a*^{R878H/+} mouse model was introduced. The *Dnmt3a*^{R878H} point mutation was generated by microinjection of guide RNA and Cas9 into single cell stage zygotes. The *Dnmt3a*^{R878H/+} offspring were viable and correct gene targeting was confirmed by NGS. It was shown that at weaning *Dnmt3a*^{R878H/+} mice were present at lower than the expected Mendelian ratios, with only 40% of pups generated from *Dnmt3a*^{R878H/+} and wt inter-crosses carrying the heterozygous *Dnmt3a*^{R878H} mutation. The lower frequency of *Dnmt3a*^{R878H/+} pups at weaning suggested that the presence of the *Dnmt3a*^{R878H} allele was detrimental to normal development in a small subset of mice leading to the lower proportion of *Dnmt3a*^{R878H/+} pups. Interestingly, of the *Dnmt3a*^{R878H/+} pups that were born the ratio of male to female pups was equal. This indicates that both genders rely on wt Dnmt3a to the same extent for embryonic development.

3.5.1 *Dnmt3a*^{R878H/+} female mice cannot produce viable offspring

It was shown that Dnmt3a is essential for the maintenance of pregnancy/delivery of pups in female *Dnmt3a*^{R878H/+} mice. We were unable to obtain live pups from pregnant *Dnmt3a*^{R878H/+} mice. The *Dnmt3a*^{R878H/+} females appeared to carry the pregnancy to term and began labour but they were unable to deliver the pups. Upon visual inspection it appeared as though the pups were partially reabsorbed, suggesting that they had died some days prior to the proposed birth date (data not shown). To determine whether this was related to the foetus, placenta or hormonal signalling in the *Dnmt3a*^{R878H/+} pregnant females, IVF was performed. The purpose of performing IVF was for two reasons. Firstly, to determine whether *Dnmt3a*^{R878H/+} oocytes were capable of producing viable offspring in a wt mother and thereby informing us that the inability of *Dnmt3a*^{R878H/+} female mice to deliver pups was intrinsic to the mother and not related to dysfunctional oocytes and secondly, to determine whether mice that were homozygous for *Dnmt3a*^{R878H/R878H} were viable. This had not been shown previously by other groups and only mice that had homozygous loss of Dnmt3a had been reported by other groups ⁴⁹, Mice with a total deficiency of DNMT3a were runted and failed to survive beyond four weeks of age, which was similar to our *Dnmt3a*^{R878H/+} mice.

It was shown that IVF of *Dnmt3a*^{R878H/+} oocytes with *Dnmt3a*^{R878H/+} sperm and transplantation into wt foster mice, mice led to the birth of both *Dnmt3a*^{R878H/+} and *Dnmt3a*^{R878H/R878H} pups. These results suggest that the inability of *Dnmt3a*^{R878H/+} female mice to deliver pups is intrinsic to the mother and likely related to the development of the placenta or hormonal signalling that is essential for the maintenance of pregnancy and delivery of the pups. In the future, it would be interesting to determine at which embryonic day the *Dnmt3a*^{R878H/+} pups are dying and are absorbed. The initiation of labour is induced by an inflammatory response in the uterus ¹⁷¹ and therefore it would be interesting to determine whether there are defects in inflammatory signals in *Dnmt3a*^{R878H/+} mice that might cause problems during pregnancy and labour. The initiation of labour is thought to rely on an accumulation of proinflammatory cytokines, like IL1- β , in the amniotic fluid ¹⁷². The increase in proinflammatory cytokines corresponds with infiltration of neutrophils and macrophages into the uterine lining ^{173,174}, suggesting that haematopoietic cells are crucial during pregnancy and labour. It would be interesting to determine whether *Dnmt3a*^{R878H/+} neutrophils and macrophages are able to migrate into the uterine lining and effectively induce labour in *Dnmt3a*^{R878H/+} mice.

3.5.2 *Dnmt3a*^{R878H/+} mice have a reduced overall survival compared to wt littermates

Dnmt3a^{R878H/+} mice developed normally and were phenotypically almost indistinguishable from their wt littermates in the first few months of life, with the exception that the *Dnmt3a*^{R878H/+} mice appeared to have a reduced muscle mass, particularly around the thigh. This difference was subtle and not always visible, so it was not pursued any further. To determine whether wt *Dnmt3a* was essential for the development or function of particular organs, the morphology of major organs of *Dnmt3a*^{R878H/+} mice was assessed by histological examination. It was hypothesised that if wt *Dnmt3a* was essential for normal organ development then we would observe abnormal organ morphology in *Dnmt3a*^{R878H/+} mice. However, no overt phenotypic alterations were observed in the organs examined from young *Dnmt3a*^{R878H/+} mice. The normal organ morphology in young *Dnmt3a*^{R878H/+} mice suggested that any detrimental effects on development in the *Dnmt3a*^{R878H/+} mice could be overcome, possibly due to redundancy with other *Dnmt* family members, like *Dnmt3b*. *Dnmt3b* has been shown to be the predominant *Dnmt* functioning during embryonic development ⁴⁹.

To determine whether mutant *Dnmt3a*^{R878H} has impact on the ageing of mice, cohorts of wt and *Dnmt3a*^{R878H/+} mice were aged, and their overall survival recorded. It was shown that *Dnmt3a*^{R878H/+} mice had a shorter overall survival compared to their wt littermates. with a median survival of 728 days for wt compared to 455 days for the *Dnmt3a*^{R878H/+} mice. The *Dnmt3a*^{R878H/+} mice developed marked obesity with age which likely contributed directly and/or indirectly to their shorter lifespan. The two major causes of death were liver disease and haematological malignancies. There is accumulating evidence that steatosis and liver disease are associated with changes in DNA methylation that results in aberrant gene expression. For example, non-alcohol induced fatty liver disease is coupled with an increase in hepatic expression of the cell cycle checkpoint inhibitor *Cdkn1a* as a result of aberrant DNA methylation ¹⁷⁵. Further, the presence of differentially methylated regions in the hepatic genome of patients with steatosis has been proposed as a prognostic marker as it indicates a more severe form of disease ¹⁷⁶. There is little evidence directly linking mutant DNMT3a with the development of hepatic disease in patients. There is however, some evidence in murine models that DNMT3a may be critical for the prevention of liver disease. In a murine model, genetic loss of *Dnmt3a* resulted in hepatomegaly which was proposed to result from infiltration of monocytes into the

liver¹⁷⁷. The increase in infiltrating monocytes observed by Guryanova, et al.¹⁷⁷ was coupled with a higher frequency of HSCs in the liver of DNMT3a KO mice suggesting that Dnmt3a plays a role in preventing migration of haematopoietic cells. Interestingly, the liver resident HSCs in mice with conditional deletion of DNMT3a revealed a foetal-liver gene expression profile which may suggest that DNMT3a has a role in repressing developmental transcriptional programs in haematopoietic stem cells. An idea that is supported in other studies³⁷. Here, it was shown the heterozygous Dnmt3a^{R878H} mutation was sufficient to cause marked liver disease in mice further supporting the notion that DNMT3a is critical as a suppressor of liver disease. It remains unclear whether the liver disease is driven by aberrant methylation in the hepatocytes, the infiltrating haematopoietic cells or if it requires the combination of both. Further experiments would be required to unravel the role of Dnmt3a^{R878H/+} in the pathogenesis of liver disease. Future experiments that examine the gene transcription of Dnmt3a^{R878H/+} hepatocytes prior to and following the development of steatosis may reveal an aberrant gene signature which could drive this phenotype.

Dnmt3a^{R878H/+} mice were also more prone to the development of B cell malignancies. Dnmt3a KO mice have been shown to develop a range of haematopoietic malignancies with age¹⁵³, corroborating our findings. However, there was a scarcity of AML and T-ALL in the *Dnmt3a*^{R878H/+} mice which would have been expected given the high prevalence of somatic *DNMT3a*^{R882} mutations in human patients with these malignancies. AML has been particularly difficult to generate in our *Dnmt3a*^{R878H/+} model which may reflect a lymphoid/ leukaemic bias in murine models or the C57BL/6 genetic background. Indeed, the genetic background of the mice used in this thesis is known to impose susceptibility to B cell malignancies compared to other lymphoid and myeloid malignancies. Additionally, there is an important and often overlooked fundamental difference between the human and mouse haematopoietic systems: In mice, lymphocytes are the predominate circulating haematopoietic cell, whereas neutrophils are the predominant circulating cell in humans¹⁷⁸⁻¹⁸⁰. Therefore, the absence of AML and T-ALL in the *Dnmt3a*^{R878H/+} mouse model might reflect pre-established lineage biases in the mouse compared to humans. To determine whether such biases existed in premalignant haematopoietic cells of *Dnmt3a*^{R878H/+} mice, the haematopoietic system was characterised in healthy mice prior to signs of malignant transformation. In the following chapter, the consequences of heterozygous *Dnmt3a*^{R878H} mutations will be examined in the haematopoietic system under steady state and stress conditions prior to malignant transformation.

CHAPTER 4

ELUCIDATING THE ROLE OF HETEROZYGOUS

***Dnmt3a*^{R878H} MUTATIONS IN STEADY STATE**

AND EMERGENCY HAEMATOPOIESIS

4.1 Introduction

When mutations in *DNMT3a* were first reported in patients with AML, it was not clear how a mutation in an epigenetic modifier would contribute to the initiation, maintenance and chemotherapeutic response of these tumours. Insight from next generation sequencing approaches determined that mutations in DNMT3a were one of the first mutations to occur in the tumour lineage, as DNMT3a mutations were present in all leukaemic cells as well as in non-malignant haematopoietic cells^{181,182}. While mutations in *DNMT3a* are known to be one of the initial mutations in the development of leukaemia, mutant DNMT3a alone is not sufficient to cause neoplastic transformation of haematopoietic cells. Mutant DNMT3a has been identified in the blood cells of patients without myelodysplastic syndrome (MDS) or leukaemia¹⁴³⁻¹⁴⁵. However, mutant DNMT3a is strongly associated with clonal haematopoiesis of indeterminate potential (CHIP) which in turn is associated with an increased risk of developing MDS and leukaemia¹³⁹. Whether mutant DNMT3a is predictive of an adverse clinical outcome is still debated⁸⁹. However, in AML the presence of mutant DNMT3a is associated with an increased likelihood of malignant disease that is refractory to treatment and consequent early relapse¹⁸³⁻¹⁸⁶. Incomplete clearance of mutant DNMT3a expressing malignant cells has been proposed to drive relapse in patients with leukaemia, although it remains unclear how this occurs.

One theory for the frequency of relapse in patients with mutant DNMT3a expressing leukaemia is that non-malignant mutant DNMT3a haematopoietic cells persist after chemotherapy. Patients with mutant DNMT3a often have clonal haematopoiesis, meaning that all haematopoietic cells carry the DNMT3a mutation¹⁴³⁻¹⁴⁵. The persistence of mutant DNMT3a cells results in the recurrence of CHIP in these patients following treatment. CHIP in itself is a risk factor for the development of blood cell derived cancers but the steps that occur between the development of CHIP and haematopoietic malignancy remain poorly defined^{143,145}. Indeed, mutant DNMT3a driven CHIP can occur years or even decades before the development of leukaemia. With a scarcity of model systems to study the consequences of *DNMT3a* mutations in the haematopoietic system *in vivo*, much of what we know about these mutations has been inferred from observational studies in patients.

Some insight has been provided from mouse models with heterozygous or homozygous loss of DNMT3a in haematopoietic tissues^{37,115}. While there is insight to be gained about the

consequences of the loss of DNMT3a on the haematopoietic system, these models do not phenocopy the prevalent 'hotspot' DNMT3a mutation that is found frequently in leukaemia. Further, new studies are emerging that show that the hotspot DNMT3a R882H mutation results in an altered DNA binding sequence preference and it is proposed that it exerts its tumourigenic effects through this mechanism⁹⁷. Therefore, it is clear that the partial or complete loss of the DNMT3a protein does not effectively model the majority of cases of human leukaemia. Some insight can be gained from previously reported *Dnmt3a* R878H mouse models, however there are some limitations of these studies discussed in chapter 1^{34,154}. As a result, it remains unclear what effect the 'hotspot' DNMT3a mutant has on haematopoietic cells under steady state and in serial transplantation *in vivo* and whether expression of this mutant protein has distinct consequences from those seen with partial or complete loss of DNMT3a.

To determine the impact of the *Dnmt3a*^{R878H} mutation on the murine haematopoietic system, the *Dnmt3a*^{R878H/+} mouse strain, presented in the previous chapter was used. In the following section, data on the haematopoietic cell composition from blood, spleen, lymph nodes, thymus and bone marrow will be presented from *Dnmt3a*^{R878H/+} mice. Moreover, the chapter will present results from competitive and serial competitive bone marrow transplant experiments that had been conducted to determine the fitness and function of *Dnmt3a*^{R878H/+} haematopoietic stem and progenitor cells (HSPC).

4.2 Characterisation of the haematopoietic system of *Dnmt3a*^{R878H/+} mice under steady state conditions

4.2.1 *Dnmt3a*^{R878H/+} mice have slightly fewer lymphocytes in the blood compared to wt mice.

Blood was taken from *Dnmt3a*^{R878H/+} mice to determine whether this mutation resulted in abnormalities in blood cell composition. Blood was collected by retro-orbital bleeds of mice at 8-10 weeks of age and blood cell composition was determined by ADVIA, the results are shown in Figure 4.1. When comparing *Dnmt3a*^{R878H/+} mice to their wt littermates, there were no significant differences in the numbers of white blood cells (WBC) (Figure 4.1 A), red blood cells (RBCs) (Figure 4.1 B), platelets (Figure 4.1 C), neutrophils (Figure 4.1 E), monocytes (Figure 4.1 F), basophils (Figure 4.1 G) or eosinophils (Figure 4.1 H) ($P > 0.05$). However, there were significantly fewer lymphocytes (Figure 4.1 D) and large unstained cells (Figure 4.1 I) (large

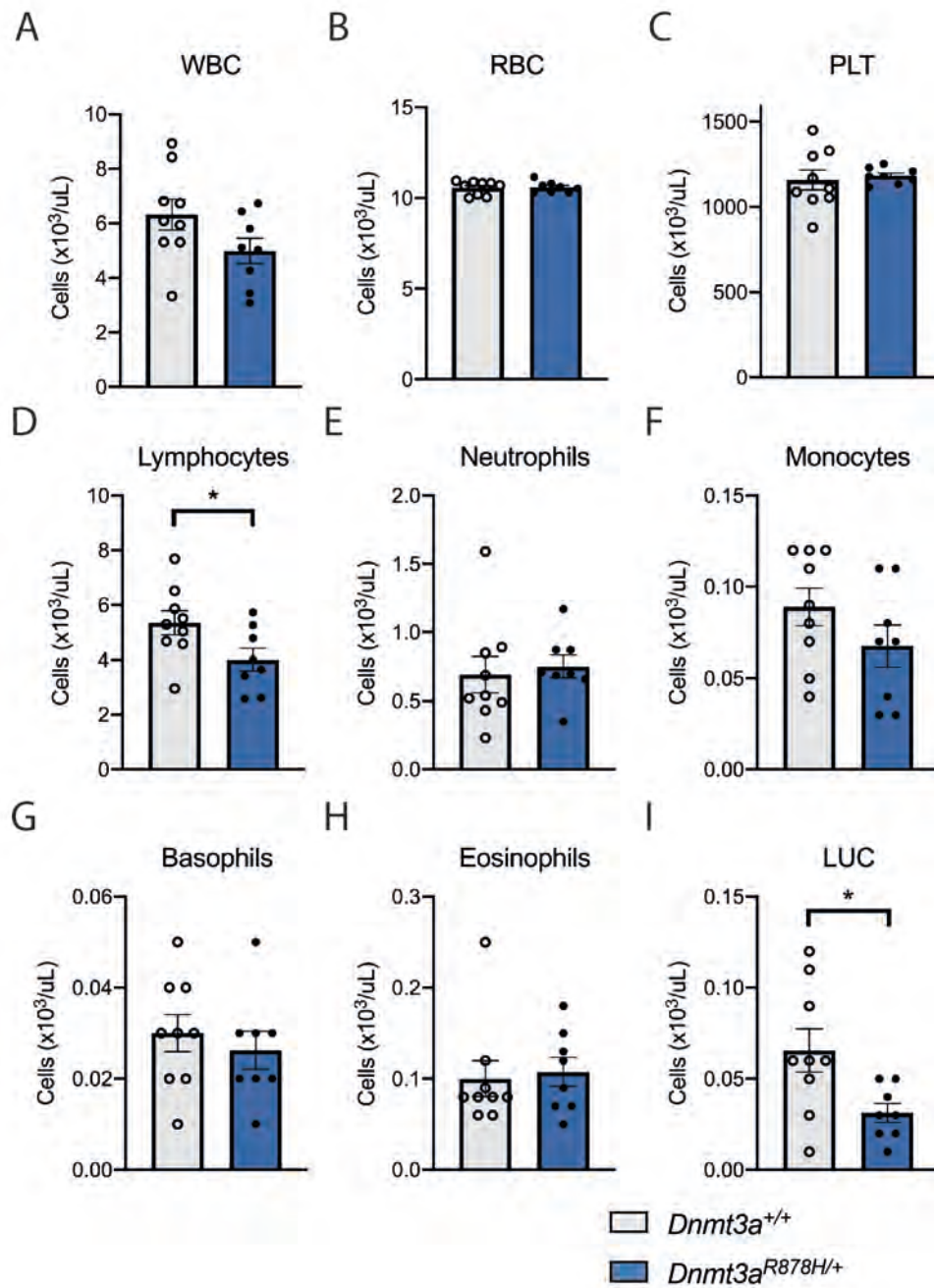


Figure 4.1 Subtle abnormalities in the numbers of mature blood cells in *Dnmt3a*^{R878H/+} mice, as determined by ADVIA.

Undiluted blood was collected by retro-orbital bleeds into EDTA coated tubes for analysis of cell numbers using the ADVIA haematology analyser. White blood cells; WBC, Red blood cells; RBC, Platelets; PLT, Large unstained cells; LUC. Graphs presented as mean ± SEM. Students t-test, * p < 0.05.

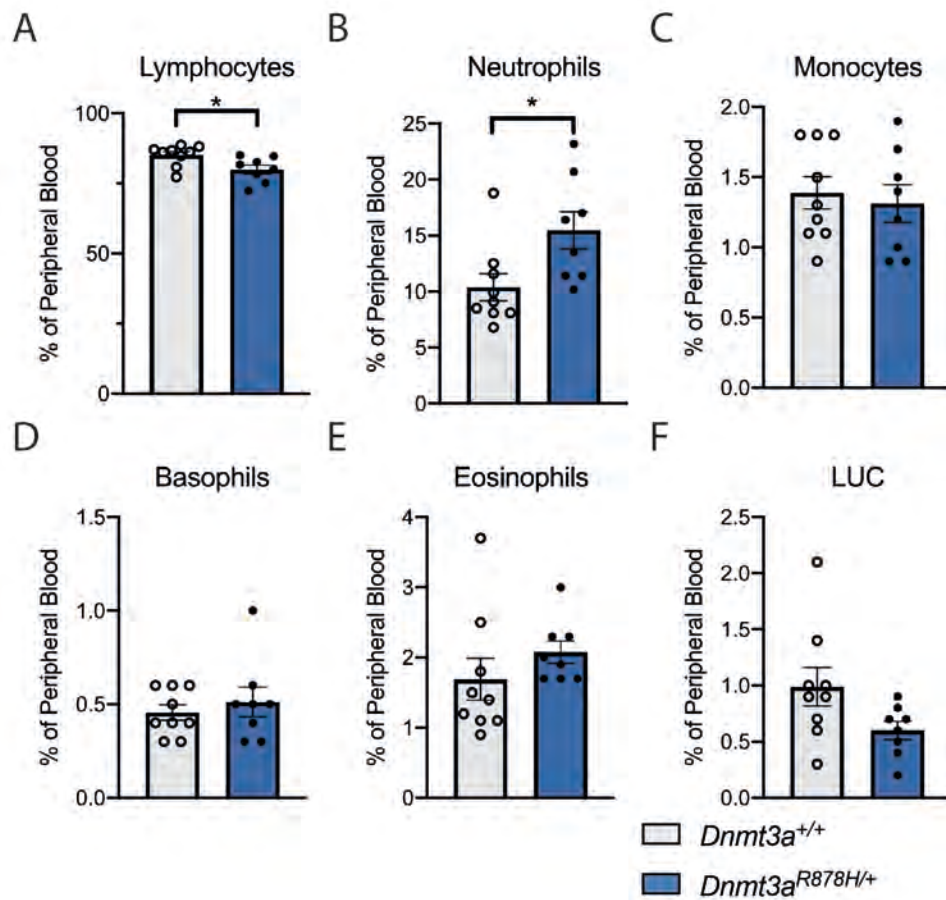


Figure 4.2 Increased frequency of neutrophils as a percentage of all white blood cells in *Dnmt3a*^{R878H/+} mice as determined by ADVIA.

Undiluted blood was collected by retro-orbital bleeds into EDTA coated tubes for analysis of blood cell composition (percentage) using the ADVIA haematology analyser Large unstained cells; LUC. Graphs presented as mean±SEM. Students t-test, * p<0.05.

peroxidase negative cells that cannot be categorised as lymphocytes, virocytes (virus infected lymphocytes) or haematopoietic stem cells) ($P < 0.05$). When blood cell subsets were measured as a percentage of peripheral blood, lymphocytes were significantly reduced in *Dnmt3a*^{R878H/+} mice (Figure 4.2 A). Neutrophils made up a significantly higher proportion of blood cells (Figure 4.2 B) despite their overall numbers being unchanged. Therefore, the higher percentage of neutrophils is probably a consequence of the decreased number of lymphocytes in the peripheral blood of *Dnmt3a*^{R878H/+} mice. Large unstained cells were not different between *Dnmt3a*^{R878H/+} and *Dnmt3a*^{+/+} wt littermates as a proportion of blood cells (Figure 34.2 F). Monocytes, basophils and eosinophils were normal compared to wt controls (Figure 4.2 C-E).

4.2.2 The blood, spleen and lymph nodes of *Dnmt3a*^{R878H/+} mice have normal haematopoietic cell populations

While blood composition determined by ADVIA provided a good overview of haematopoietic cells in the blood, cell types can be determined more stringently based on staining for cell type specific surface markers and subsequent analysis by flow cytometry. To determine blood cell composition in detail, blood as well as spleen, lymph nodes, thymus and bone marrow were analysed by flow cytometry. Different cell types were identified by staining the cells of each organ with fluorochrome conjugated antibodies against cell type specific antigens. The gating strategy to define the different cell types in the blood, spleen and lymph nodes is shown in Figure 4.3. There were no significant differences in the composition of the cell types analysed in the blood, spleen or lymph nodes with the exception of a reduction in the percentage of granulocytes in the blood (Figure 4.4 B) ($p < 0.05$). This reduction in granulocytes was unexpected as this was not supported by the ADVIA results presented above.

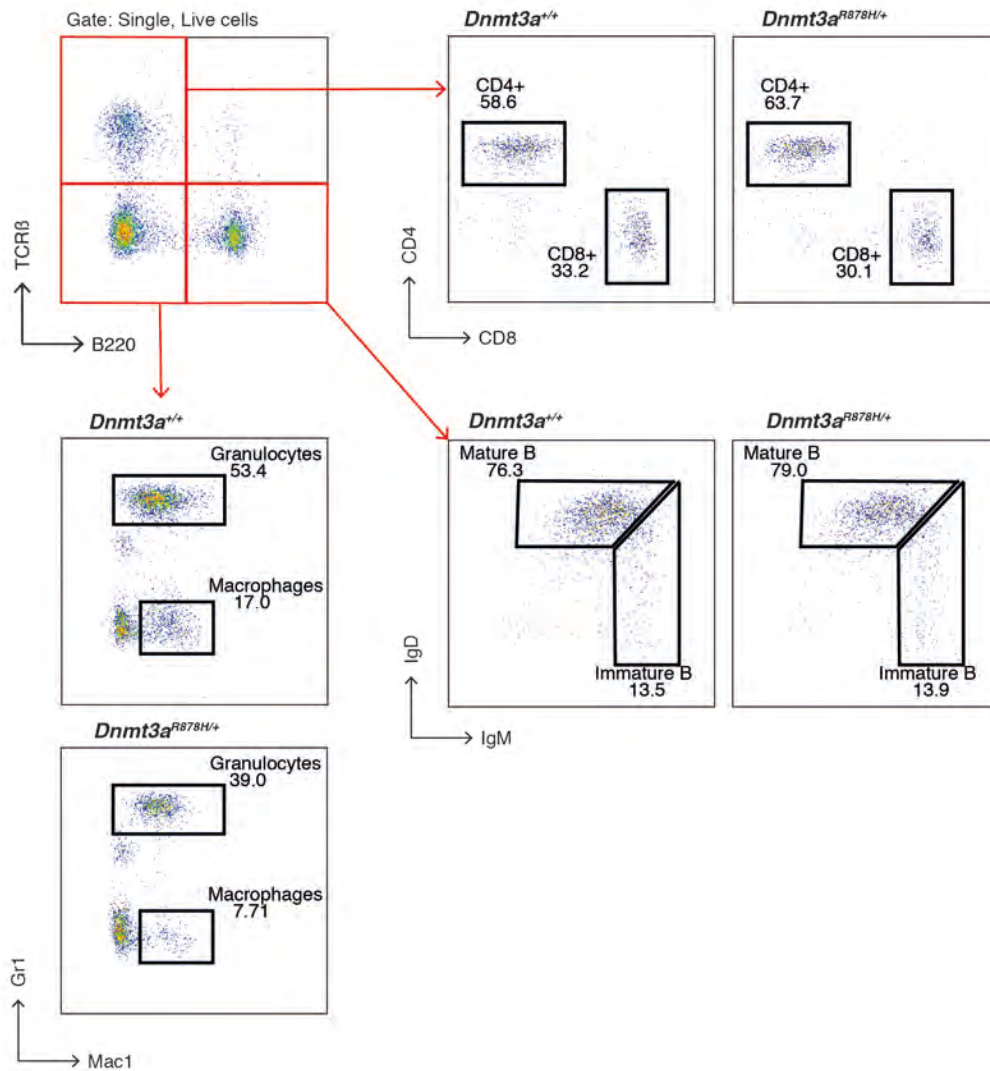


Figure 4.3 Gating strategy to define haematopoietic cell populations in the blood and Spleen by flow cytometry.

Leukocytes were gated by FSC-A and SSC-A, then cell doublets were gated out by FSC-H and FSC-A (not shown). Live cells were negative for dead cell markers PI, DAPI or Fluorogold. Cells were defined based on cell type specific marker expression; CD4⁺ T cells = TCRβ⁺CD4⁺CD8⁻, CD8⁺ T cells = TCRβ⁺CD4⁻CD8⁺, immature B cells = B220⁺IgD^{low}IgM^{high} and mature B cells = B220⁺IgD⁺IgM^{mid}, granulocytes = TCRβ⁻B220⁻Gr1⁺Mac1⁺ and macrophages = TCRβ⁻B220⁻Gr1⁻Mac1⁺.

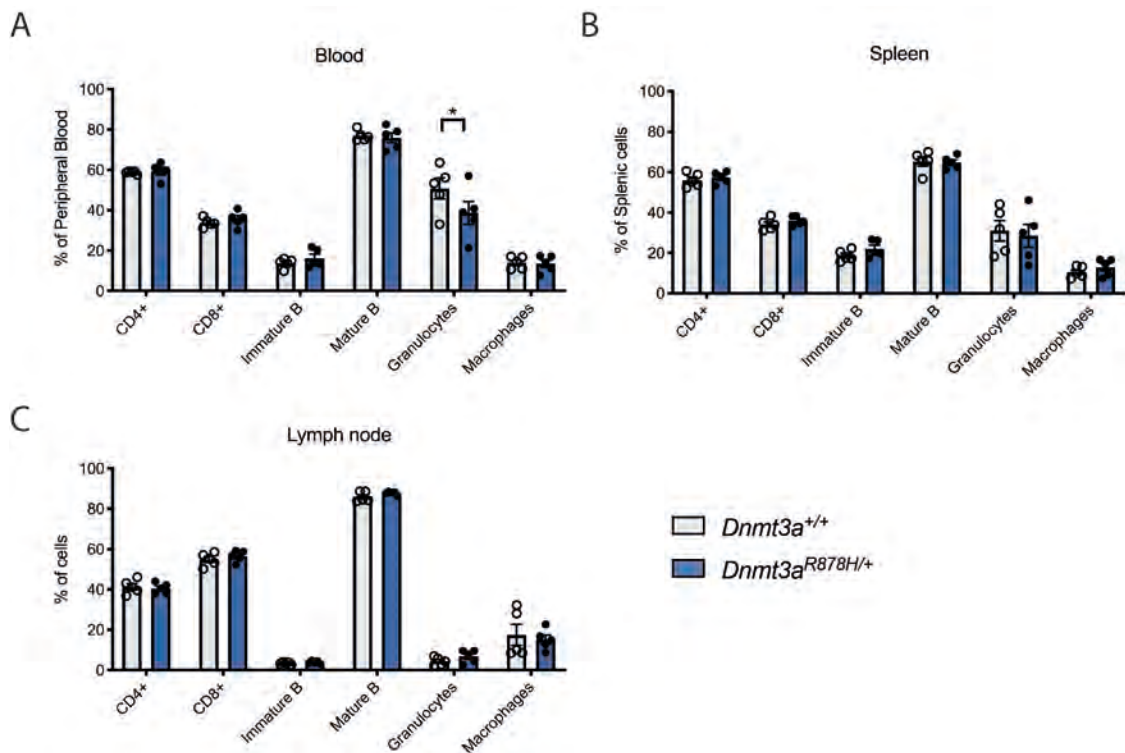


Figure 4.4 *Dnmt3a*^{R878H/+} mice have normal frequencies of haematopoietic cell subsets in the blood, spleen and lymph nodes.

Single cell suspensions were created by gently mashing the spleen and lymph nodes through a nylon filter. Blood was collected by retro-orbital bleeds into EDTA coated tubes. Prior to incubation with fluorescence conjugated antibodies, red blood cells were lysed in the blood and spleen with red blood cell lysis buffer. The cells were then stained with common haematopoietic markers and gated as depicted in figure 4.3 and analysed by flow cytometry. Percentages of haematopoietic cell subsets determined by flow cytometry in the A) blood, B) spleen and C) lymph nodes. Data are presented as mean±SEM. Students t-test, * p<0.05.

4.2.3 Early T cell development in the thymus is perturbed in *Dnmt3a*^{R878H/+} mice

In the thymus, there were no significant differences in the numbers of double negative (CD4⁻CD8⁻), double positive (CD4⁺CD8⁺), CD4⁺ or CD8⁺ single positive thymocytes in the *Dnmt3a*^{R878H/+} mice (Figure 3.8 A-E). However, the composition of early T cell progenitors was affected as there was a significant reduction in double negative CD4⁻CD8⁻CD44⁺CD25⁻ (DN1) cells ($p < 0.05$) and a trend towards a reduction in double negative CD4⁻CD8⁻CD44⁺CD25⁺ (DN2) cells ($p > 0.05$) (Figure 3.8 F-G). This did not constitute a differentiation block as there were normal numbers of double negative CD4⁻CD8⁻CD44⁻CD25⁺ (DN3) and double negative CD4⁻CD8⁻CD44⁻CD25⁻ (DN4) cells and normal numbers of single positive CD4⁺ and CD8⁺ T cells in *Dnmt3a*^{R878H/+} mice (Figure 3.8 H-I), demonstrating that this defect could be overcome. Since thymic T cell progenitors originate from the bone marrow, the cell populations in the bone marrow were examined next in *Dnmt3a*^{R878H/+} mice.

4.2.4 Bone marrow cell populations are not altered in *Dnmt3a*^{R878H/+} mice

B lymphocytes and myeloid cell populations in the bone marrow were defined using the gating strategy outlined in Figure 4.6 A. There were no significant differences in the cellularity of the bone marrow between 8-week-old *Dnmt3a*^{R878H/+} and wt mice. B lymphocyte development appeared normal with no significant differences in the proportions of pro-B/pre-B (IgM⁻B220^{low}), immature B (IgM⁺B220^{mid}), transitional B (IgM^{high}B220⁺) or mature B (IgM^{low}B220^{high}) cells in the bone marrow (Figure 4.6 C), although there was a trend towards increased numbers of mature B cells in *Dnmt3a*^{R878H/+} mice but this did not reach statistical significance. Similarly, there were no significant differences in the proportions of granulocytes (Gr1⁺Mac1⁺) or macrophages (Gr1⁻Mac1⁺) between *Dnmt3a*^{R878H/+} and wt mice (Figure 4.6 D-E). Despite little evidence of abnormalities in the mature cells of the bone marrow, it remained unknown whether the *Dnmt3a*^{R878H/+} stem and progenitor cells in the bone marrow may be abnormal.

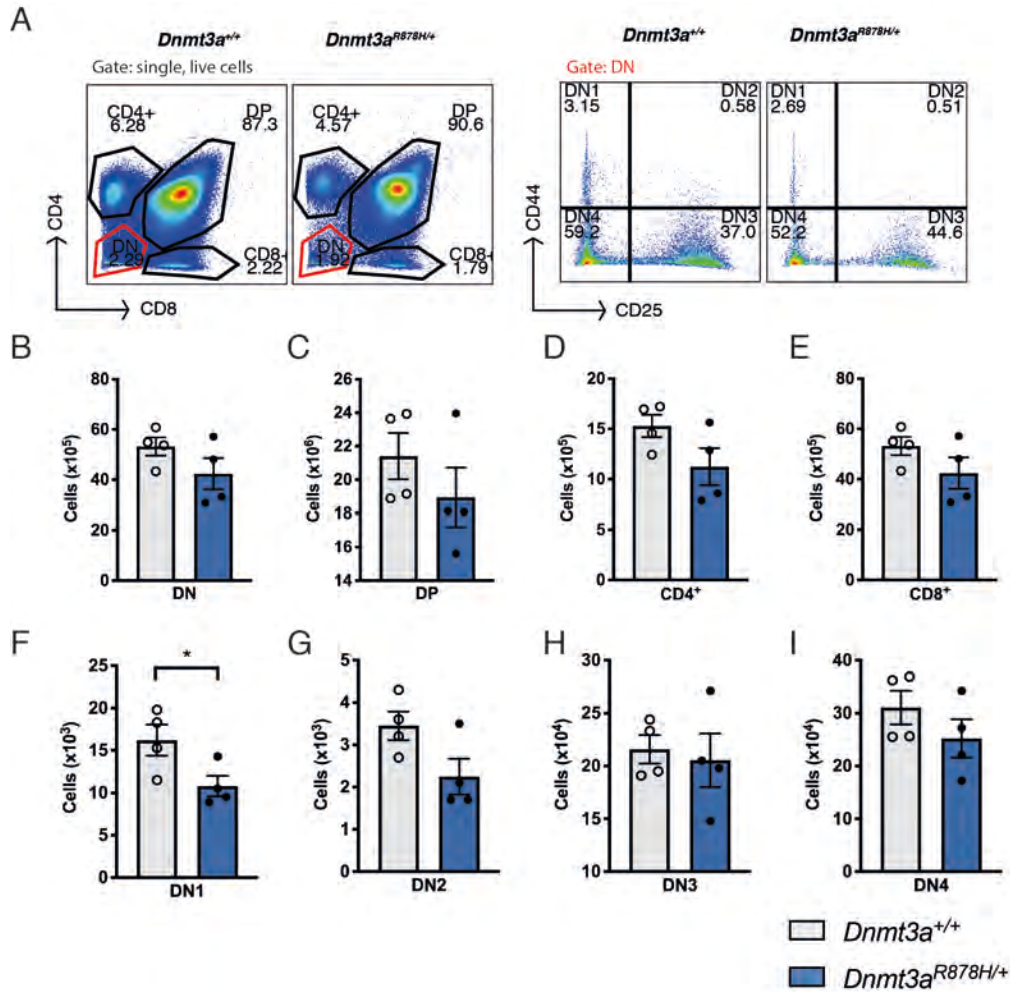


Figure 4.5 The thymi of *Dnmt3a*^{R878H/+} mice contain fewer DN1 cells compared to wt controls but this does not affect the production of the more mature thymic cell subsets.

Representative gating strategy to identify the different cell populations within the thymus by flow cytometry. Cell number of B) Double negative thymocytes (CD4-CD8-); DN, C) Double positive thymocytes (CD4+CD8+); DP, D) CD4+ single positive T cells, E) CD8+ single positive T cells, F) Double negative CD4-CD8-CD44+CD25- (DN1), G) Double negative CD4-CD8-CD44+CD25+ (DN2), H) Double negative CD4-CD8-CD44-CD25+ (DN3), I) Double negative CD4-CD8-CD44-CD25- (DN4). Graphs presented as mean±SEM. Students t-test, * p<0.05.

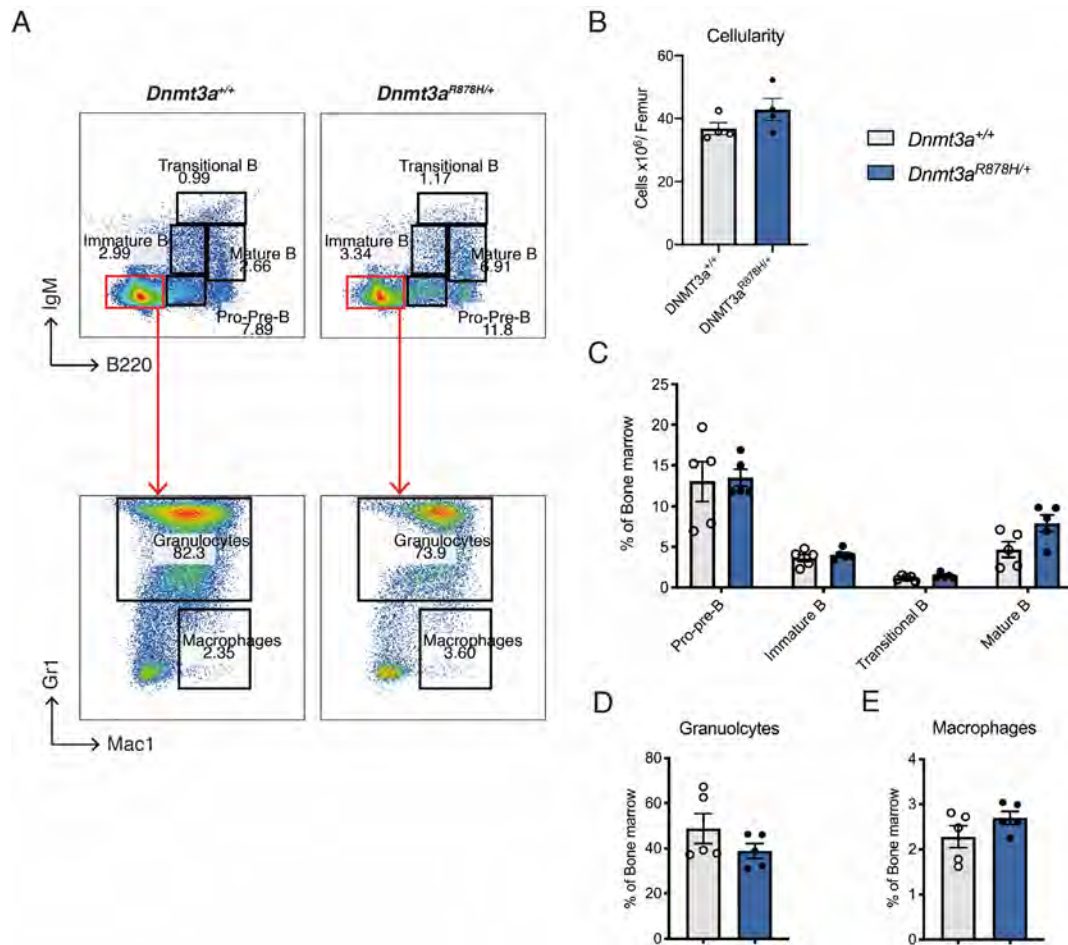


Figure 4.6 *Dnmt3a*^{R878H/+} mice have normal B lymphoid and myeloid cell subsets in the bone marrow

A single cell suspension of bone marrow was created by gently flushing the femur with PBS. Bone marrow cells were then stained with antibodies against common B cell and myeloid cell antigens. A) Gating strategy to identify different cell populations in the bone marrow by flow cytometry B) bone marrow cellularity, C) lymphoid cells in the bone marrow; pro-B/pre-B (B220^{mid}IgM⁻), immature B (B220^{mid}IgM^{mid}), transitional B (B220^{mid}IgM^{high}) and mature B (B220^{mid}IgM^{high}), D) granulocytes (B220⁻Gr1⁺Mac1⁺) and E) macrophages (B220⁻Gr1⁻Mac1⁺). Data are presented as mean ± SEM. Students t-test, p > 0.05.

The stem and progenitor cells in the bone marrow of *Dnmt3a*^{R878H/+} mice were next examined to determine if there were differences that could explain the subtle haematological abnormalities identified in the blood and thymus. Haematopoietic stem and progenitor cells were defined based on the staining for cell surface markers as shown in Figure 4.7A. As shown in Figure 4.7 B-J there were no significant differences in stem and progenitor cell populations in the bone marrow of *Dnmt3a*^{R878H/+} mice compared to wt control littermates. However, it is worth noting that there was a subtle, albeit non-significant, reduction in common lymphoid progenitors (CLP) in the *Dnmt3a*^{R878H/+} mice (Figure 4.7 C). This small reduction may account for the reduction of DN1 cells observed in the thymus (Figure 4.5 F). The reduced number of DN1 cells might reflect reduced influx of CLPs from the bone marrow into the thymus. However, more experiments would be necessary to determine if the small reduction of CLPs would be physiologically meaningful.

Additionally, there appeared to be a trend towards increased numbers of common myeloid (CMP), granulocyte myeloid (GMP) and megakaryocyte erythroid progenitors (MEP) in the bone marrow although this was not a robust increase and did not reach statistical significance (Figure 4.7 H-J). Interestingly, it appeared as though there was a trend towards increased long-term HSCs (LT-HSC) in the bone marrow of *Dnmt3a*^{R878H/+} mice which might account for the subtle non-significant increases in CMP, GMP and MEPs. The small non-significant increase in LT-HSCs may not reflect a physiologically meaningful difference but it was interesting given that *Dnmt3a* KO LT-HSCs have been reported to have increased self-renewal at the expense of differentiation³⁷. If *Dnmt3a*^{R878H/+} LT-HSCs also had increased self-renewal, then an increase in their cell number would make sense. Nevertheless, LT-HSC number appeared to be normal in the *Dnmt3a*^{R878H/+} mice at 8-weeks of age and this has not yet been examined in older mice.

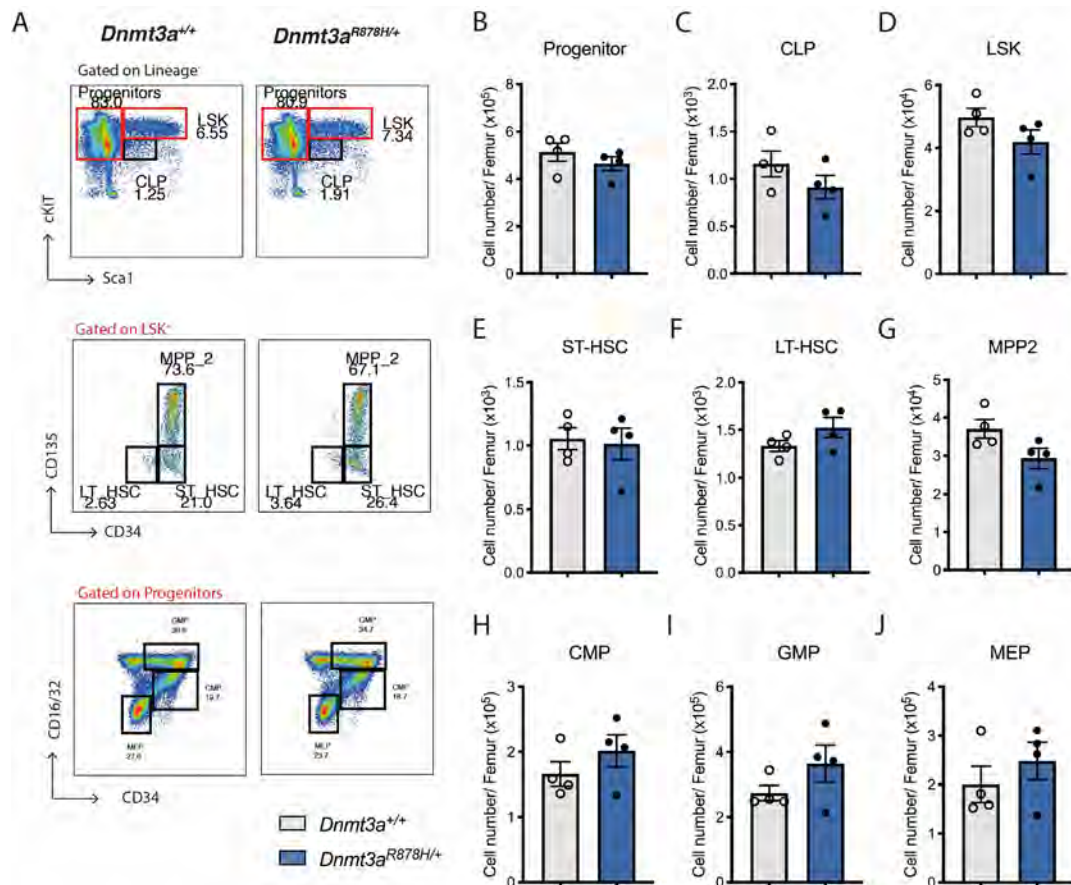


Figure 4.7 Haematopoietic stem and progenitor cell populations are normal in the bone marrow of 6-8-week-old *Dnmt3a*^{R878H/+} mice.

A) Gating strategy to identify haematopoietic stem and progenitor cells in the bone marrow, B-J) haematopoietic stem and progenitor cell numbers. Common lymphoid progenitor = Lin⁻cKIT^{mid}Sca1^{low} (CLP), LSK = Lineage⁻Sca1⁺cKIT⁺, Short-term haematopoietic stem cell = Lin⁻cKIT⁺Sca1⁺CD34⁺CD135⁻ (ST-HSC), Long-term haematopoietic stem cell = Lin⁻cKIT⁺Sca1⁺CD34⁺CD135⁻ (LT-HSC), Multipotent progenitor cell 2 = Lin⁻cKIT⁺Sca1⁺CD34⁺CD135⁺ (MPP2), Common myeloid progenitor = Lin⁻cKIT⁺Sca1⁺CD16/32^{mid}CD34⁺ (CMP), Granulocyte Myeloid progenitor = Lin⁻cKIT⁺Sca1⁺CD16/32^{mid}CD34⁺ (GMP), Megakaryocyte erythroid progenitor = Lin⁻cKIT⁺Sca1⁺CD16/32^{mid}CD34⁻ (MEP). Graphs presented as mean ± SEM. Students t-test, p > 0.05.

4.3 Examining the potency of *Dnmt3a*^{R878H/+} haematopoietic stem/ progenitor cells in short term competition assays in mice

Stem cell fitness is often assessed by the capacity of stem cells to completely reconstitute the haematopoietic system of a lethally irradiated host. For effective reconstitution to occur, whole bone marrow which includes HSPCs is injected into the tail vein of lethally irradiated mice. The HSPCs migrate to the bone marrow niche where they undergo rapid proliferation to give rise to functional mature haematopoietic cells of all lineages. In emergency haematopoiesis, it is essential that stem cells balance self-renewal with differentiation. Defects that result in an imbalance between self-renewal and differentiation will result in haematopoietic failure. To determine the fitness of *Dnmt3a*^{R878H/+} HSPCs relative to wt cells, competitive bone marrow reconstitutions were set up under different conditions. By introducing mixed wt: *Dnmt3a*^{R878H/+} bone marrow into mice, the fitness of *Dnmt3a*^{R878H/+} stem cells could be directly compared to wt cells *in vivo*. Typically, complete haematopoietic reconstitution occurs within three-four months following transplantation. At this time, residual radio-resistant cells from the recipient mice have been cleared and all haematopoietic cells are derived from LT-HSCs in the bone marrow.

To determine the fitness of *Dnmt3a*^{R878H/+} stem cells, lethally irradiated mice were transplanted with mixed whole bone marrow where 90% was wt (competitor) and 10% *Dnmt3a*^{R878H/+} (experimental) or 10% *Dnmt3a*^{+/+} (control). To distinguish the origin of the bone marrow, mice with different CD45.1 and CD45.2 genetic backgrounds were used. CD45 is expressed widely on haematopoietic cells and exists in two isoforms; CD45.1 and CD45.2. By carefully designing the experiment, haematopoietic cells from mice expressing different CD45 isoforms can be distinguished by flow cytometry. In the following experiments, competitor bone marrow was derived from wt mice expressing the CD45.1 variant (CD45.1⁺). The competitor cells were then mixed with the test cells (*Dnmt3a*^{R878H/+} or wt) expressing the CD45.2 isoform (CD45.2⁺). To ensure that only transplanted cells were included in the analysis, these cells were transplanted into host mice that expressed both the CD45.1 and CD45.2 isoforms (CD45.1⁺CD45.2⁺). Thereby making competitor cells CD45.1⁺, test cells CD45.2⁺ and host derived contaminating cells CD45.1⁺CD45.2⁺ as depicted in Figure 4.8.

As shown in the previous section, there were no significant differences between wt and *Dnmt3a*^{R878H/+} HSPC numbers and therefore it was not necessary to sort stem cells from whole

bone marrow to ensure equal numbers for injections. Bone marrow was derived from three independent wt and *Dnmt3a*^{R878H/+} mice. This bone marrow was kept separate and used to reconstitute two-three mice to function as technical replicates. Competitor bone marrow was derived from six mice and pooled together to ensure that the experimental and control conditions received the same mix of competitor bone marrow cells. Blood was sampled every 4 weeks by mandible bleed during recovery to monitor the proportions of *Dnmt3a*^{R878H/+} blood cells as shown in Figure 4.8.

Blood cell composition was monitored by flow cytometry every 4 weeks over the 16-week recovery period of the recipient mice. This was done to determine the overall cell composition

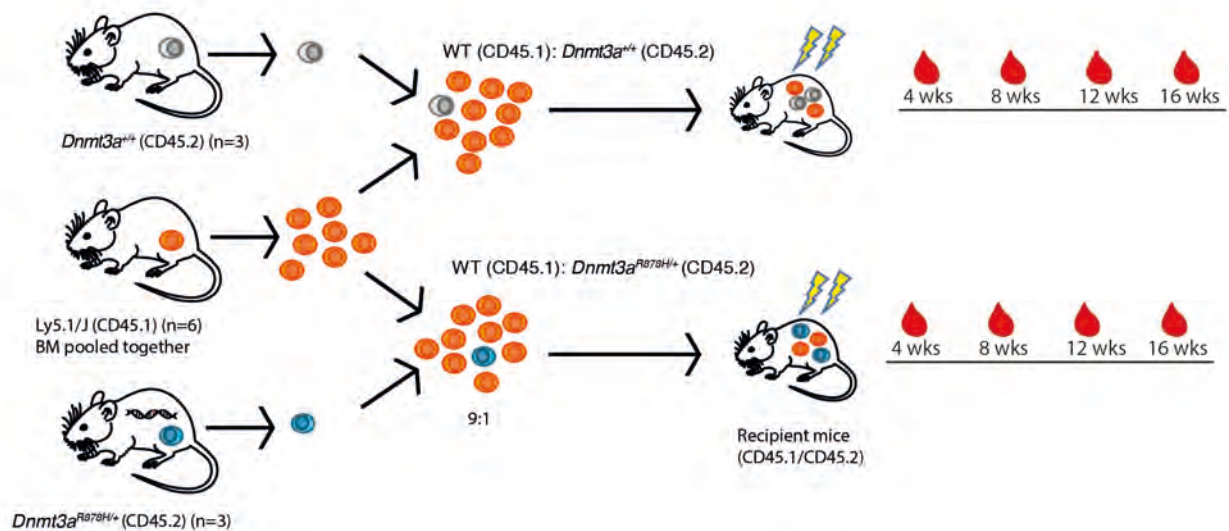


Figure 4.8 experimental set up for the 9:1 competitive bone marrow reconstitution experiment

Competitor bone marrow cells derived from CD45.1⁺ mice were pooled such that all mice received the same bolus of competitor cells. CD45.2⁺ test *Dnmt3a*^{R878H/+} or control *Dnmt3a*^{+/+} (wt) bone marrow was kept independent and used to reconstitute lethally irradiated recipient mice (CD45.1⁺/CD45.2⁺) in competition with the CD45.1 wt bone marrow cells. The ratio was 9 parts CD45.1⁺ competitor cells to 1 part test or control cells (CD45.2⁺). The relative contributions of CD45.1 vs CD45.2 cells in the in the different haematopoietic organs were assessed.

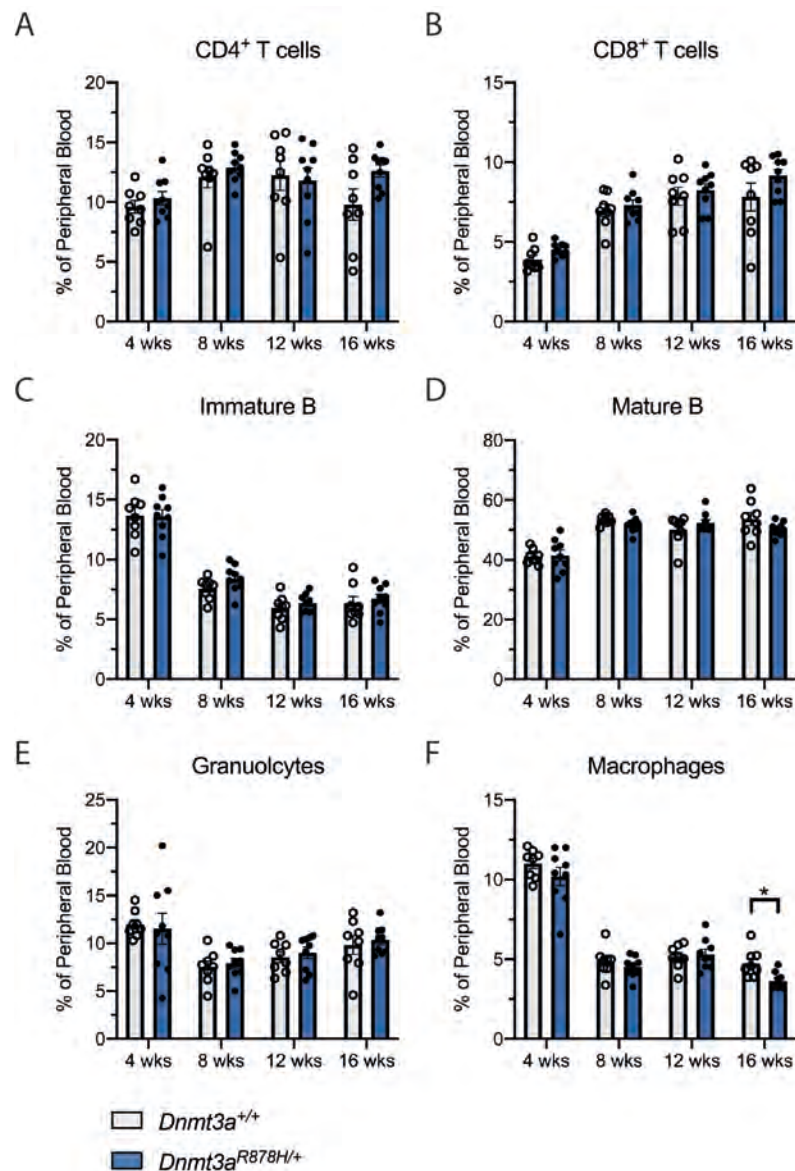


Figure 4.9 *Dnmt3a*^{R878H/+} bone marrow contributes to haematopoietic reconstitution in a manner comparable to wt bone marrow

Whole blood cell composition of lethally irradiated recipient mice that had been transplanted with wt: wt or wt: *Dnmt3a*^{R878H/+} mixtures of bone marrow, as determined by flow cytometry of A) CD4⁺ T cells = TCRβ⁺CD4⁺CD8⁻, B) CD8⁺ T cells = TCRβ⁺CD4⁻CD8⁺, C) immature B cells = B220⁺IgM⁺IgD^{mid}, D) mature B cells B220⁺IgM⁺IgD⁺, E) granulocytes TCRβ⁻B220⁻Gr1⁺Mac1⁺, F) macrophages TCRβ⁻B220⁻Gr1⁺Mac1⁺. Data are presented as mean±SEM. Students t-tests were performed, *p<0.05. Two-way ANOVA to compare the change in cell types over time, between WT: WT (Grey) and WT: *Dnmt3a*^{R878H/+} (blue) bone marrow mixture reconstituted mice (results reported in text).

of the blood of the reconstituted mice, as an indication of the health of these recipient mice. As shown in Figure 4.9 A-F, the composition of blood cells changed significantly over the 16 week recovery period for CD4⁺ (p<0.01), CD8⁺ (p<0.0001), immature B (p<0.0001), mature B (p<0.0001), granulocytes (p<0.0001) and macrophages (p<0.0001), although the overall response was not significantly different between the mice that had been reconstituted with wt: *Dnmt3a*^{+/+} or wt: *Dnmt3a*^{R878H/+} bone marrow mixtures. When comparing the two conditions there were no significant differences in any of the cell types at any time point with the exception of a significant reduction in the proportions of macrophages in the mice that had received the wt: *Dnmt3a*^{R878H/+} bone marrow mixture at the 16-week time point (p<0.05) (Figure 4.9 F).

Next, the proportions of CD45.2⁺ cells were calculated for each cell type to determine whether the *Dnmt3a*^{R878H/+} HSPCs had an advantage over wt cells. It was hypothesised that the *Dnmt3a*^{R878H/+} HSPCs would have an increased self-renewal capacity and that this would be reflected by an increase in the proportion of *Dnmt3a*^{R878H/+} HSPC derived cells in each haematopoietic cell subset tested. This hypothesis was largely based on the report that *Dnmt3a* KO HSPCs have abnormally increased self-renewal, and this has also been shown in mice transplanted with *Dnmt3a*^{Fl-R878H/+} HSPCs^{34,187}. However, both of these studies utilised plpC to activate expression of the *Dnmt3a*^{R878H} allele which is known to induce proliferation of HSPCs. It was therefore considered prudent to determine whether an advantage of *Dnmt3a*^{R878H/+} mutant HSPCs could be observed in our model, in the absence of a plpC mediated surge in interferons.

Contrary to the previous studies, there were no significant differences in the percentages of CD45.2⁺ derived CD4⁺ or CD8⁺ T lymphocytes between mice that had been transplanted with wt: *Dnmt3a*^{+/+} (CD45.2⁺) bone marrow mixtures (Figure 3.12 A-C) compared to mice transplanted with wt: *Dnmt3a*^{R878H/+} (CD45.2⁺) bone marrow mixtures (Figure 4.10 D-F) over 16 weeks. Interestingly, there was no significant differences in the percentages of CD45.2 derived granulocytes (yellow) or macrophages (orange) between the wt: *Dnmt3a*^{+/+} (CD45.2⁺) (Figure 4.10 A-C) vs the wt: *Dnmt3a*^{R878H/+} (CD45.2⁺) group (Figure 4.10 D-F). This result was surprising given that mutant DNMT3a^{R882H} was proposed to drive myeloid biased differentiation in humans, thereby leading to myelodysplastic syndrome and leukaemia^{143,145}. It is possible that the effects of the *Dnmt3a*^{R878H/+} mutation are muted due to the constitutive expression of this allele throughout development. It is feasible that these cells have developed a compensatory mechanism over time, leading to the subtle phenotype observed here. At 8-16 weeks post-

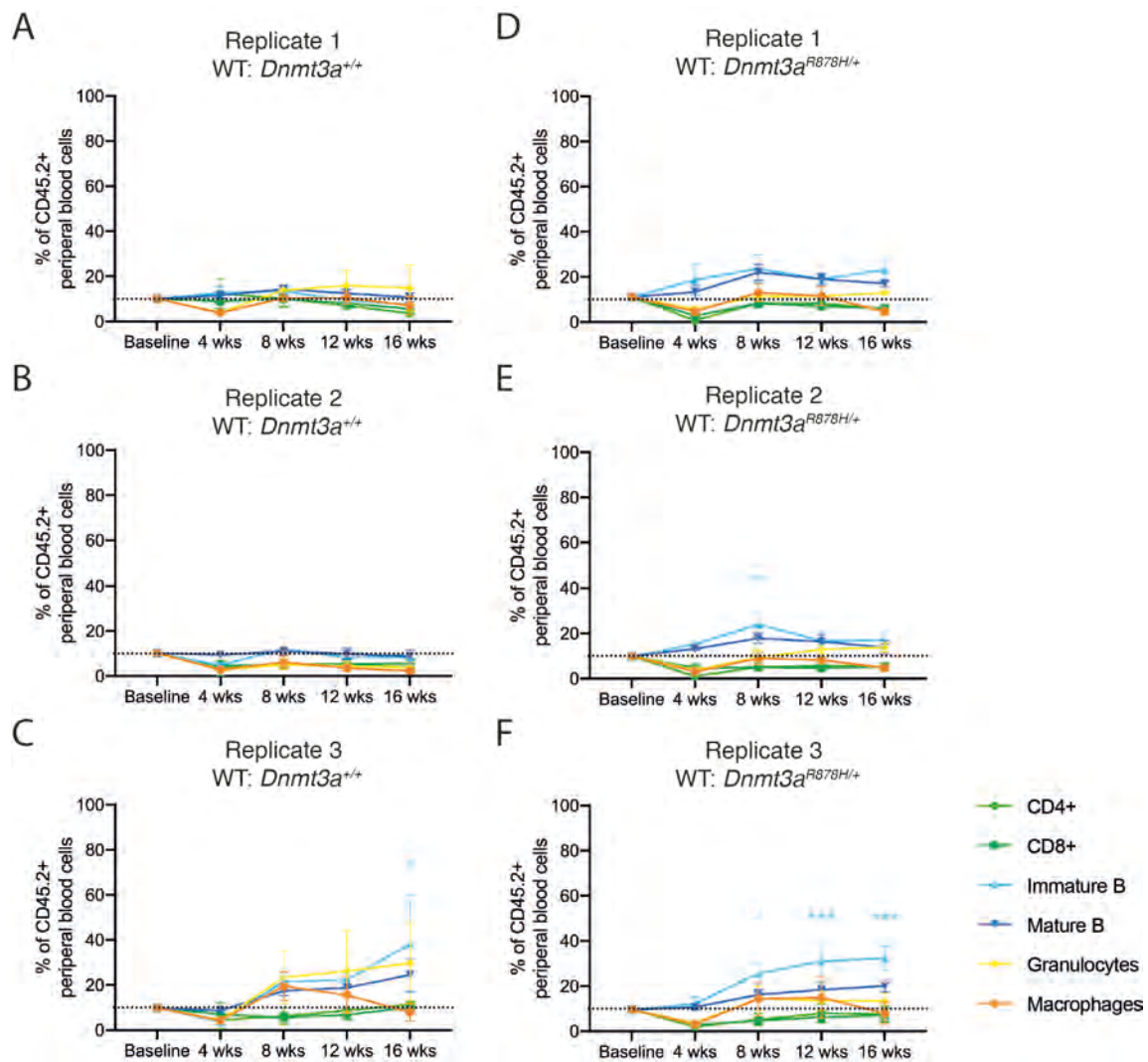


Figure 4.10 *Dnmt3a*^{R878H/+} Immature B cells have a competitive advantage over WT cells

Blood was collected by mandible bleed every 4 weeks after haematopoietic reconstituted. The proportion of *Dnmt3a*^{R878H/+} (CD45.2⁺) mutant cells in the wt: *Dnmt3a*^{R878H/+} reconstituted mice was monitored over time and compared with the control wt: *Dnmt3a*^{+/+} control reconstituted mice. A-C) % of CD45.2⁺ mature blood cells from wt: *Dnmt3a*^{+/+} control reconstitutions. D-F) % of CD45.2⁺ mature blood cells from wt: *Dnmt3a*^{R878H/+} reconstitutions. Each plot represents one biological replicate labelled 1-3. Data are presented as mean $\pm$ SEM of technical replicates, all groups had 3 technical replicates with the exception of C) which had 2. Mixed effects model with Tukey's post-hoc test, significant compared to baseline * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

transplantation, a higher proportion of immature B cells (light blue) were derived from *Dnmt3a*^{R878H/+} HSPCs (CD45.2⁺) in replicates two and three, with a similar trend in replicate one (Figure 4.10 D-F). In contrast, immature B cells (light blue) derived from wt CD45.2 mice were normal in replicates one and two but had increased significantly at 16 weeks in replicate three (Figure 4.10 A-C). There were no significant differences in the proportions of CD45.2 mature B cells in either group – i.e. comparing the mature B cells (blue) in the wt: *Dnmt3a*^{+/+} (CD45.2⁺) group with the wt: *Dnmt3a*^{R878H/+} (CD45.2⁺) group (Figure 4.10 A-F).

Preliminary results from this experiment suggest that the *Dnmt3a*^{R878H/+} HSPCs have a bias towards B cell differentiation which results in a subtle increase in the proportions of *Dnmt3a*^{R878H/+} immature B cells at the expense of their wt counterparts in the mice reconstituted with mixed bone marrow cells. Interestingly, this bias does not result in an increased proportion of these cells in the blood as a percentage of total blood cells (Figure 4.9 C). Additional experiments are required to extend the analysis of the bleeds over a longer period of time to determine whether *Dnmt3a*^{R878H/+} cells would accumulate in other lineages over a longer period of time. Furthermore, it would be interesting to determine if the proportions of certain *Dnmt3a*^{R878H/+} vs wt haematopoietic cell subsets are altered in other tissues.

As highlighted in the previous experiment, it remained unclear whether *Dnmt3a*^{R878H/+} cells would outcompete wt haematopoietic cells in other organs and therefore a more extensive analysis of haematopoietic organs was performed. In this experiment, wt (CD45.1⁺) bone marrow cells were mixed 1:1 with *Dnmt3a*^{R878H/+} (CD45.2⁺) or *Dnmt3a*^{+/+} (CD45.2⁺) bone marrow and used to reconstitute lethally irradiated (CD45.1⁺/CD45.2⁺) recipient mice as depicted in Figure 4.11. A 1:1 ratio was selected for this experiment as there were only subtle differences observed in the previous experiment with 10% test bone marrow cells. After 3 months, the recipient mice were sacrificed and the proportion of CD45.2⁺ cells was analysed by flow cytometry in the blood, spleen, thymus and bone marrow.

Firstly, the proportion of CD45.2⁺ cells in the bone marrow mixture used for transplantation was determined by flow cytometry to confirm the correct ratio prior to injection (Figure 4.12 A). Three months after transplantation, the percentage of CD45.2⁺ cells (Figure 4.12 B) was determined by flow cytometry for cells in the blood, spleen, thymus and bone marrow of the recipient mice (Figure 4.12 C-F). Similar to the previous experiment, there were no significant

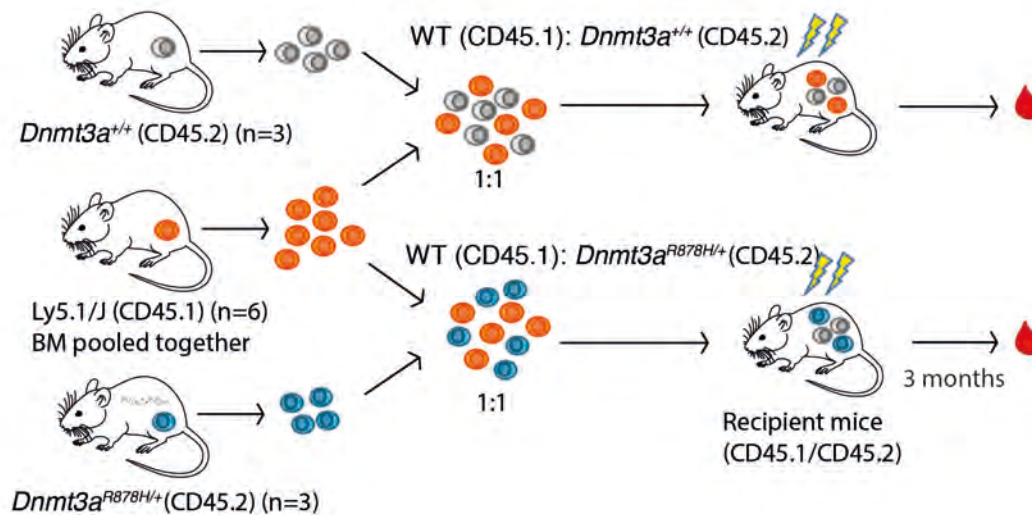


Figure 4.11 experimental design to set up short term 1:1 competition of wt and *Dnmt3a*^{R878H/+} haematopoietic cells

Competitor bone marrow cells derived from CD45.1⁺ mice were pooled such that all mice received the same bolus of competitor cells. CD45.2⁺ test *Dnmt3a*^{R878H/+} or control *Dnmt3a*^{+/+} (wt) bone marrow was kept independent and used to reconstitute lethally irradiated recipient mice (CD45.1⁺/CD45.2⁺) in competition with the CD45.1⁺ wt bone marrow cells. The ratio was 1 part CD45.1⁺ competitor cells to 1 part test or control cells (CD45.2⁺). The relative contributions of CD45.1 vs CD45.2 cells in the in the different haematopoietic organs were assessed.

differences in the contribution of *Dnmt3a*^{R878H/+} cells in the blood compared to the contribution from the wt (CD45.1⁺) competitor cells. This was mirrored in the spleen and thymus where there were no significant differences in the proportion of *Dnmt3a*^{R878H/+} CD45.2⁺ cells vs wt CD45.1⁺ competitor cells. Interestingly, there was a trend towards an increase in the proportion of *Dnmt3a*^{R878H/+} immature and mature B cells in the spleen, but this difference was not statistically significant (Figure 4.12 D) ($p > 0.05$).

In the bone marrow, there were significantly more *Dnmt3a*^{R878H/+} derived transitional B and mature B cells compared to those derived from the wt competitor bone marrow (Figure 4.12 F). Interestingly, there was also a trend towards an increased proportion of *Dnmt3a*^{R878H/+} pre-

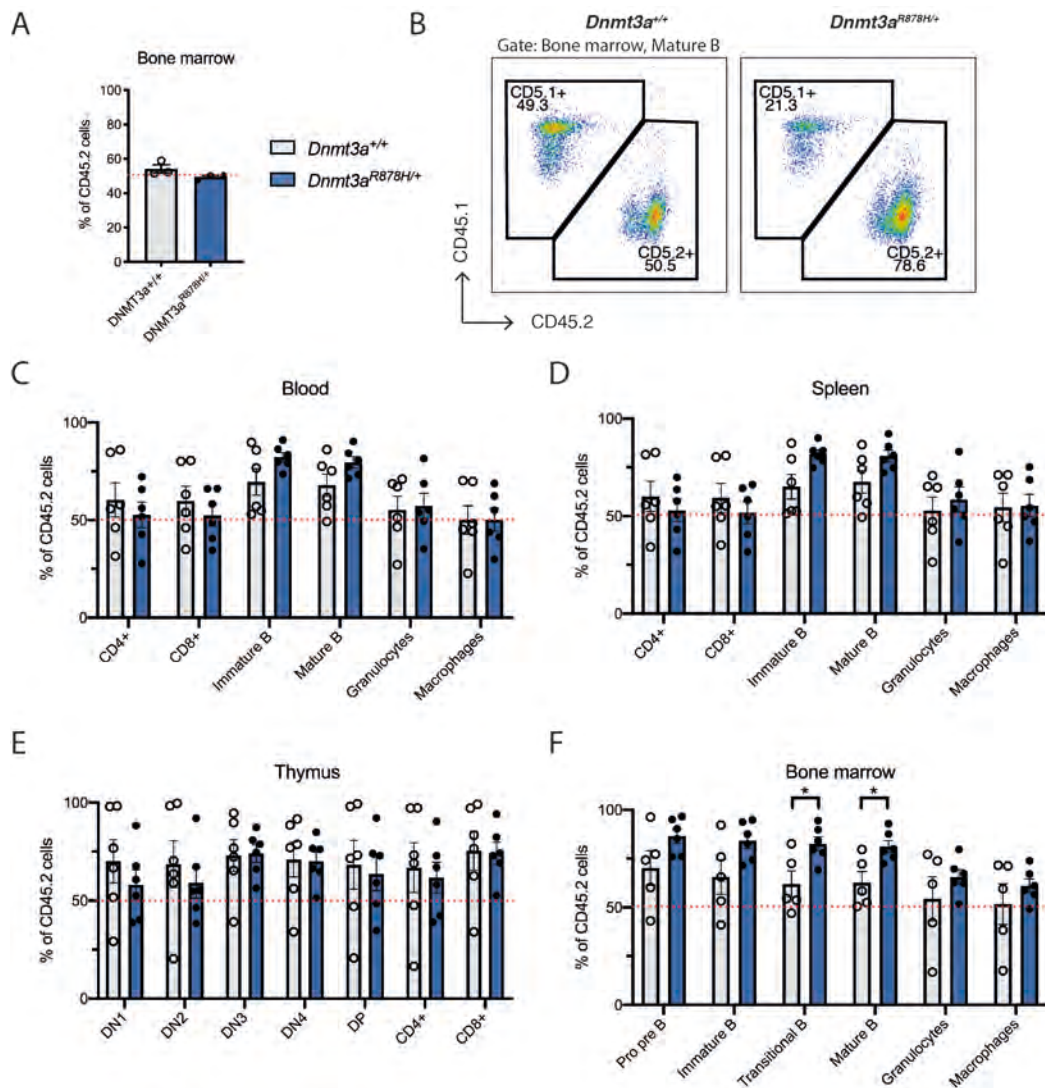


Figure 4.12 In short term competitive bone marrow transplantation assays immature and mature B cells $Dnmt3a^{R878H/+}$ accumulate in the bone marrow, but not in the blood, spleen or thymus.

A) Percentage of CD45.2⁺ cells in the bone marrow injected into lethally irradiated recipient mice. B) example gating of CD45.1⁺ and CD45.2⁺ gating in the bone marrow. Three months after reconstitution the proportions of CD45.2⁺ cells were determined by flow cytometry in C) blood, D) spleen, E) lymph nodes and F) bone marrow. Students t-test, *p<0.05.

B/pro-B cells and immature B cells in the bone marrow although this difference was not statistically significant ($p>0.05$). Since *Dnmt3a*^{R878H/+} B cells were accumulating in the bone marrow, this raised the question whether this was due to an increase in the proportions of *Dnmt3a*^{R878H/+} HSPCs. There were no significant differences between the proportions of *Dnmt3a*^{R878H/+} CMPs, GMPs or MEPs compared to their wt counterparts (Figure 4.13 A). This was not surprising given that there were no differences between *Dnmt3a*^{R878H/+} vs wt cells in the myeloid subsets of the haematopoietic organs. Interestingly, there appeared to be significantly more *Dnmt3a*^{R878H/+} short-term and long-term HSCs in the bone marrow compared to their wt counterparts after 3 months (Figure 4.13 B). This supports the notion that *Dnmt3a*^{R878H/+} stem cells have increased self-renewal capacity compared to their wt counterparts¹⁸⁷.

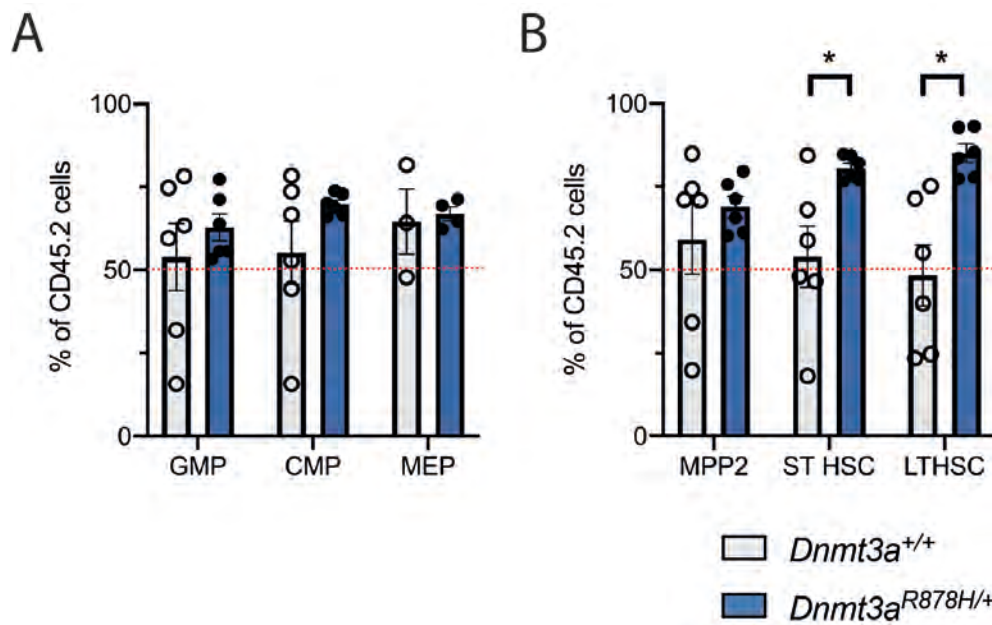


Figure 4.13 *Dnmt3a*^{R878H/+} short-term and long-term haematopoietic stem cells outcompete their WT counterparts after 3 months.

The proportions of CD45.2+ (WT or *Dnmt3a*^{R878H/+}) haematopoietic stem and progenitor cells in the bone marrow of competitively reconstituted mice were determined by flow cytometry. A) progenitor cells, B) stem and progenitor cells. Students t-test, * $p<0.05$.

These experiments showed that *Dnmt3a*^{R878H/+} HSPCs accumulated preferentially in the bone marrow during acute recovery from emergency haematopoiesis. Following an insult to the haematopoietic system, for example as a result of infection, trauma or treatment with cytotoxic therapies, HSPCs must undergo rapid proliferation and differentiation. It was proposed previously that *Dnmt3a* KO HSPCs preferentially underwent self-renewal rather than differentiation³⁷. This suggests that mice that have *Dnmt3a*^{R878H/+} HSPCs may not be able to overcome the stress induced by haematopoietic reconstitution. The results reported here demonstrate that mice with *Dnmt3a*^{R878H/+} HSPCs can effectively recover from haematopoietic stress as mature blood cells were present in normal frequencies in the blood of wt: *Dnmt3a*^{R878H/+} reconstituted mice. However, there was evidence of accumulation of *Dnmt3a*^{R878H/+} HSPCs in such animals at three months, which was only subtly reflected in the B cells of their haematopoietic organs. It remained unclear if *Dnmt3a*^{R878H/+} cells would accumulate in all organs and alter all haematopoietic lineages over a longer period of time or if the *Dnmt3a*^{R878H/+} HSPCs would remain biased towards the B cell lineage.

4.4 Examining the competition between wt and *Dnmt3a*^{R878H/+} haematopoietic stem/progenitor cells in long term competitive reconstitution assays

In the previous section, it was shown that *Dnmt3a*^{R878H/+} HSPCs could accumulate in the bone marrow of competitively reconstituted mice after three months. The increase in *Dnmt3a*^{R878H/+} HSPCs was only reflected in other haematopoietic organs by a similar increase in B cells. However, there was no evidence that the *Dnmt3a*^{R878H/+} HSPCs could also lead to an increased proportion of T cells or myeloid cells at this time point. To determine whether there was a competitive advantage of *Dnmt3a*^{R878H/+} cells over their WT counterparts over an extended period of time, lethally irradiated mice were reconstituted with a 1:1 mix of wt: *Dnmt3a*^{R878H/+} bone marrow cells and left to recover for nine months.

DNMT3a mutations in patients are primarily reported in elderly people. It was proposed that a mutation in *DNMT3a* could be present in the cells of patients decades prior to the emergence of MDS or haematological malignancies, suggesting that the accumulation of DNMT3a mutant cells occurred gradually over many years. Therefore, it was hypothesised that *Dnmt3a*^{R878H/+} cells would accumulate in all haematopoietic lineages in mice reconstituted with 50% wt plus 50%

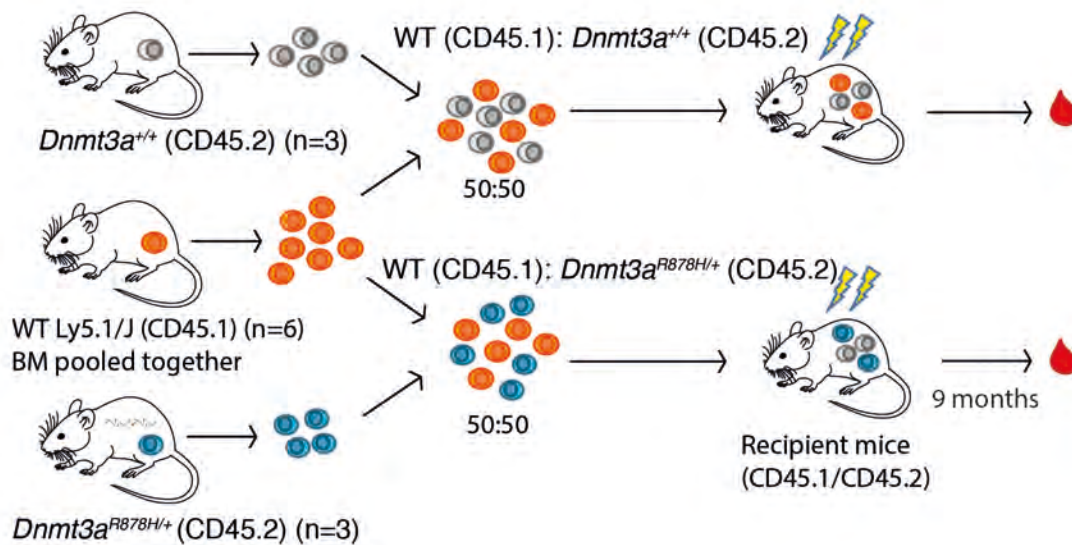


Figure 4.14 Schematic depicting the setup of the long-term competitive bone marrow transplantation assay

Competitor bone marrow cells derived from CD45.1⁺ mice were pooled such that all mice received the same bolus of competitor cells. CD45.2⁺ test *Dnmt3a*^{R878H/+} or control *Dnmt3a*^{+/+} (wt) bone marrow was kept independent and used to reconstitute lethally irradiated recipient mice (CD45.1⁺/CD45.2⁺) in competition with the CD45.1 wt bone marrow cells. The ratio was 1 part CD45.1⁺ competitor cells to 1 part test or control cells (CD45.2⁺). The relative contributions of CD45.1 vs CD45.2 cells in the in the different haematopoietic organs were assessed after 9 months.

Dnmt3a^{R878H/+} cells after nine months. To this end, chimeric mice were generated by combining wt (CD45.1⁺) competitor bone marrow with either *Dnmt3a*^{+/+} (CD45.2⁺) control or *Dnmt3a*^{R878H/+} (CD45.2⁺) experimental bone marrow in a 1:1 ratio. Mice were then left to age for nine months following transplantation (Figure 4.14). Bleeds were then taken and the proportions of *Dnmt3a*^{R878H/+} vs competitor wt cells were determined in each cell type.

In support of our hypothesis, *Dnmt3a*^{R878H/+} cells accumulated in all haematopoietic lineages. There were significantly more *Dnmt3a*^{R878H/+} (CD45.2⁺) CD4⁺ T cells compared to *Dnmt3a*^{+/+} (CD45.2⁺) controls (Figure 4.15 A). Similarly, there was a significantly higher proportion of *Dnmt3a*^{R878H/+} CD8⁺ T cells compared to wt controls, although this difference was not as

prominent as in other cell populations (Figure 4.15 B). Most strikingly, immature B and mature B cells were made up of almost 100% *Dnmt3a*^{R878H/+} cells and this was significantly higher than *Dnmt3a*^{+/+} cells in the control wt (CD45.1): *Dnmt3a*^{+/+} (CD45.2) transplantation setting (Figure 4.15 D-E). Furthermore, *Dnmt3a*^{R878H/+} granulocytes and macrophages accounted for approximately 80% of this cell type in the blood, which was significantly higher than the frequencies of the *Dnmt3a*^{+/+} cells in the control wt (CD45.1): *Dnmt3a*^{+/+} (CD45.2⁺) transplantation setting (Figure 4.15 F-G). If anything, in the control transplants the *Dnmt3a*^{+/+} (CD45.2⁺) cells tended to be less competitive than the wt (CD45.1⁺) cells across all cell types. The *Dnmt3a*^{+/+} (CD45.2⁺) cells often accounted for fewer than 50% of the cell types examined (Figure 4.15). This demonstrated the strong competitive advantage of *Dnmt3a*^{R878H/+} cells, given that all transplant recipient mice (i.e. both the wt (CD45.1⁺): *Dnmt3a*^{+/+} (CD45.2⁺) and the wt (CD45.1⁺): *Dnmt3a*^{R878H/+} (CD45.2⁺), received competitor cells from the same wt (CD45.1⁺) bone marrow pool.

The results from this experiment built upon and extended the results of the previous experiments which showed that *Dnmt3a*^{R878H/+} cells have a substantial advantage compared to their wt counterparts. After 9 months there were significantly more *Dnmt3a*^{R878H/+} CD4⁺ T cells, CD8⁺ T cells, immature B cells, mature B cells, granulocytes and macrophages in the blood of the competitively reconstituted mice. It remains unclear how long the *Dnmt3a*^{R878H/+} cells might be able to maintain their intrinsic competitive advantage and can continue to give rise to functional haematopoietic cells. Due to the slow kinetics of the accumulation of the *Dnmt3a*^{R878H/+} haematopoietic cells in this model, it was not feasible to age large cohorts of mice for a very long period to monitor for haematological abnormalities and the development of leukaemia. However, it would be interesting to examine the exhaustion kinetics of *Dnmt3a*^{R878H/+} stem cells compared to wt controls.

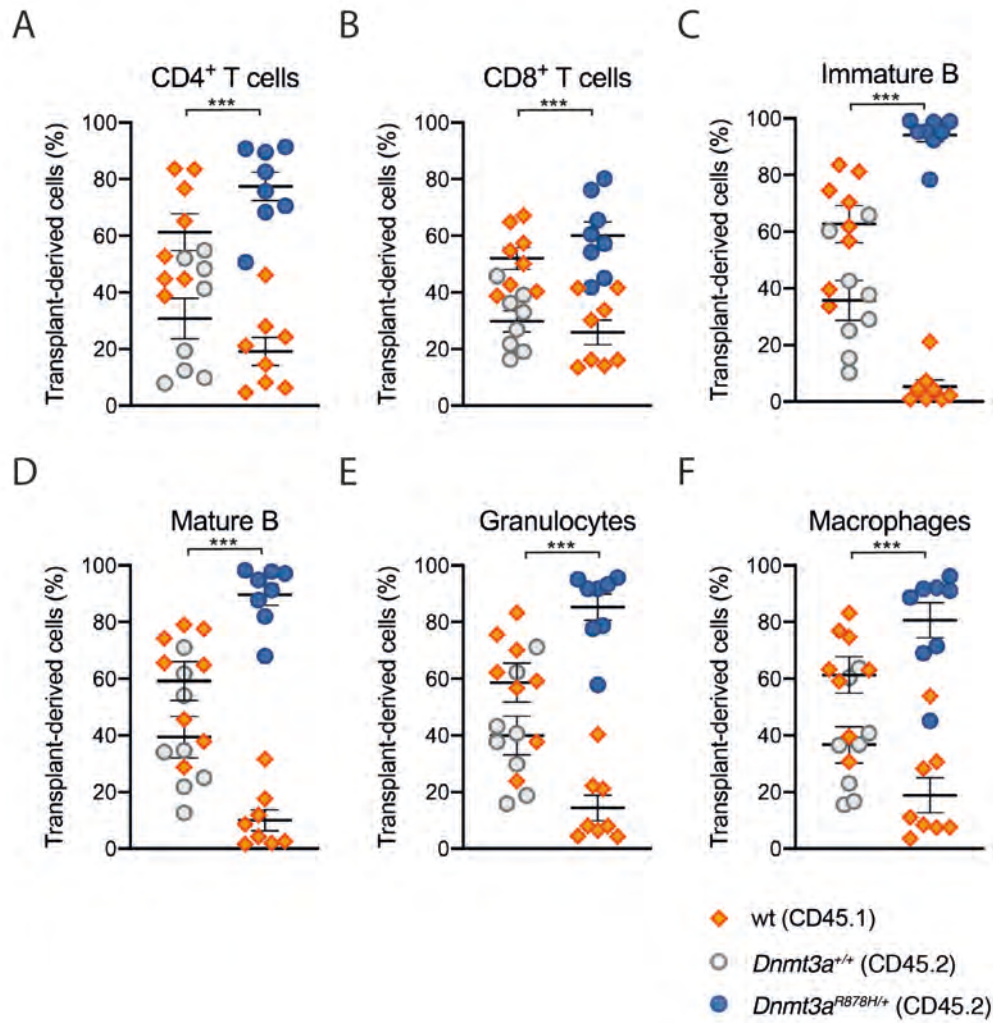


Figure 4.15 *Dnmt3a*^{R878H/+} cells accumulate in all haematopoietic lineages in the blood of competitively reconstituted mice over nine months

Contribution of transplanted CD45.1 and CD45.2 cells to mature blood cell types determined by flow cytometry after 9 months. A) CD4⁺ T cells = TCRβ⁺CD4⁺CD8⁻, B) CD8⁺ T cells = TCRβ⁺CD4⁻CD8⁺, C) immature B = B220⁺IgD^{low}IgM^{high}, D) mature B cells = B220⁺IgD⁺IgM^{mid}, E) granulocytes = TCRβ⁻B220⁻Gr1⁺Mac1⁺ and F) macrophages = TCRβ⁻B220⁻Gr1⁻Mac1⁺. Data presented mean±SEM, non-parametric t-test to compare *Dnmt3a*^{+/+} CD45.2 cells to *Dnmt3a*^{R878H/+} CD45.2 cells. ***p<0.001.

4.5 Examining the exhaustion of *Dnmt3a*^{R878H/+} haematopoietic stem/progenitor cells using serial competitive bone marrow transplantation assays

In the previous section it was shown that *Dnmt3a*^{R878H/+} cells could accumulate to disproportionate levels in all haematopoietic lineages in the blood of the competitively reconstituted mice. By nine months, *Dnmt3a*^{R878H/+} cells had almost completely outcompeted their wt counterparts. Thus, it was clear that the *Dnmt3a*^{R878H/+} cells had a competitive advantage over wt cells, but the question remained whether *Dnmt3a*^{R878H/+} cells had indefinite repopulating capacity. Studies of *Dnmt3a* KO stem cells have shown that these cells possess seemingly indefinite serial repopulating capacity *in vivo* (up to 11 transplantations) ¹¹⁵. It remains unanswered, whether the *Dnmt3a*^{R878H/+} cells have a similar phenotype. To assess stem cell exhaustion kinetics of *Dnmt3a*^{R878H/+} cells, serial competitive bone marrow reconstitutions were performed.

To determine the serial repopulating potential of *Dnmt3a*^{R878H/+} cells, competitive bone marrow transplants were performed as depicted previously in Figure 4.11. After three months, the bone marrow was harvested from primary recipient mice and equal amounts of bone marrow were pooled between technical replicates, maintaining biological replicates from the initial donor material separate. Pooled bone marrow was then used to reconstitute secondary recipients for three months. At each endpoint, bone marrow was treated as above and used to reconstitute the next round of recipients. Mice were monitored for signs of failure of haematopoietic reconstitution throughout the experiment. In total, the bone marrow was serially transplanted five times at the time of writing this thesis.

All mice survived primary, secondary and tertiary reconstitutions. However, wt: *Dnmt3a*^{+/+} bone marrow had weaker serial repopulating capacity compared to wt: *Dnmt3a*^{R878H/+} bone marrow, resulting in reconstitution failure of a number of the 4th generation recipient mice (Figure 4.16 A). Similarly, 5th generation recipient mice of wt: *Dnmt3a*^{+/+} bone marrow also suffered from reconstitution failure, whereas none of the recipients of wt: *Dnmt3a*^{R878H/+} bone marrow were adversely affected (Figure 4.16 B). To determine how wt: *Dnmt3a*^{R878H/+} bone marrow repopulated the recipients, blood from these mice was analysed by ADVIA.

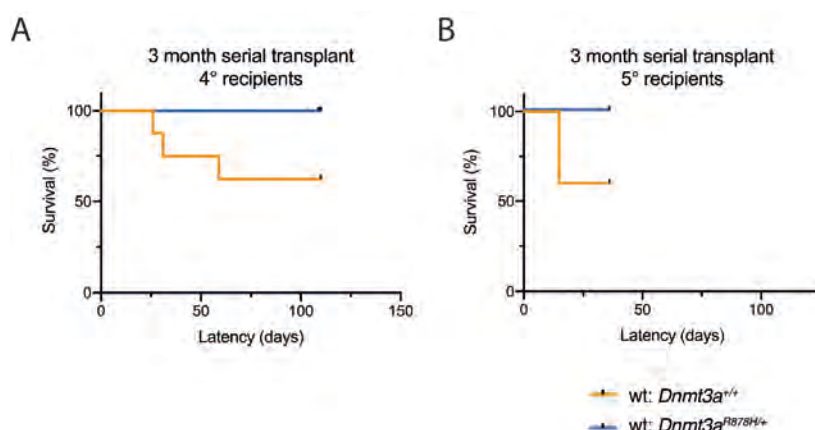


Figure 4.16 Mice reconstituted with wt: *Dnmt3a*^{R878H/+} mixtures of bone marrow cells are protected from stem cell exhaustion and reconstitution failure induced by serial transplantation

A) Survival of fourth generation (4°) recipients of serially transplanted bone marrow.
 B) Survival of fifth generation (5°) recipients of serially transplanted bone marrow.
 Log-rank (Mantel-Cox) test, ns.

There was a decrease in cellularity with each round of transplantation in both the wt: *Dnmt3a*^{+/+} and the wt: *Dnmt3a*^{R878H/+} groups as determined by ADVIA (Appendix II). The numbers of total WBCs, lymphocytes, monocytes, eosinophils and LUCs gradually reduced in cell number with each transplantation in all mice. Overall, there were very few differences between the blood cell composition of wt: *Dnmt3a*^{+/+} vs wt: *Dnmt3a*^{R878H/+} serially transplanted mice at any of the endpoints.

To examine the overall blood cell competition of reconstituted mice, the frequencies of mature blood cells was determined at each endpoint by flow cytometry to determine the general composition of the blood. As shown in Figure 4.17, there were no significant differences between wt: *Dnmt3a*^{+/+} and wt: *Dnmt3a*^{R878H/+} serially transplanted mice at the end of the primary reconstitution in CD4⁺ T cells (Figure 4.17 A), CD8⁺ T cells (Figure 4.17 B), immature B (Figure 4.17 C), mature B (Figure 4.17 D), granulocytes (Figure 4.17 E) or macrophages (Figure 4.17 F). However, at the end of the secondary transplantation study there were significantly fewer CD4⁺ T cells in the wt: *Dnmt3a*^{R878H/+} serially transplanted mice and this reduction was

maintained with each further round of transplantation (Figure 4.17 A). A similar trend was observed for CD8⁺ T cells in the wt: *Dnmt3a*^{R878H/+} serially transplanted mice with significantly fewer CD8⁺ T cells at the endpoints of the 3rd and 4th transplantation (Figure 4.17 B). Immature B cells made up a significantly higher proportion of peripheral blood in the wt: *Dnmt3a*^{R878H/+} reconstituted mice at the end of the secondary transplantation compared to the wt: *Dnmt3a*^{+/+} control group (Figure 4.17 C). However, they returned to similar proportions in the third and fourth round of serial transplantation. Similarly, mature B cells were also significantly higher in wt: *Dnmt3a*^{R878H/+} serially transplanted mice at the endpoint of the second and third transplantation but had returned to similar proportions as seen in the wt: *Dnmt3a*^{+/+} control group at the end of the fourth round of transplantation (Figure 4.17 D). Interestingly, there were no significant differences between the two groups in the proportions of granulocytes and macrophages at any of the time points measured (Figure 4.17 E-F).

While there were some differences in the blood cell composition between the two groups under sustained haematopoietic stress, the implications of these differences remain unclear. Granulocytes did increase as a percentage of total WBCs over time, but this increase seemed to be independent of the presence of *Dnmt3a*^{R878H/+} cells as granulocytes increased in the blood of both groups. This was somewhat unanticipated as *DNMT3a* 882 mutations in humans have been proposed to drive a myeloproliferative phenotype. It was interesting that both CD4⁺ and CD8⁺ T cells made up smaller proportions of total WBCs in the wt: *Dnmt3a*^{R878H/+} serially transplanted mice over time and that this was accompanied by an increase in the proportions of immature and mature B cells. However, these results do not discriminate whether these cell subsets are derived from wt (CD45.1⁺) or *Dnmt3a*^{R878H/+} (CD45.2⁺) cells. Therefore, it was important to discern the relative contribution of wt and *Dnmt3a*^{R878H/+} cells to each cell subset.

To determine whether serial competitive transplantation caused an increase in the proportions of *Dnmt3a*^{R878H/+} blood cells, haematopoietic cells were gated based on CD45 expression to examine the relative contributions of competitor (CD45.1⁺) and test (CD45.2⁺) cells. In serial transplantation, *Dnmt3a*^{R878H/+} (CD45.2⁺) cells made up a significantly higher proportion of CD4⁺ T cells at the endpoints of the second, third and fourth round of transplantation (Figure 4.18 A). The increase in *Dnmt3a*^{R878H/+} CD4⁺ T cells was most obvious at the endpoint of the secondary transplantation. While *Dnmt3a*^{R878H/+} T cells remained significantly more prevalent in serial transplantations, the proportion of *Dnmt3a*^{R878H/+} CD4⁺ T cells amongst total T cells gradually

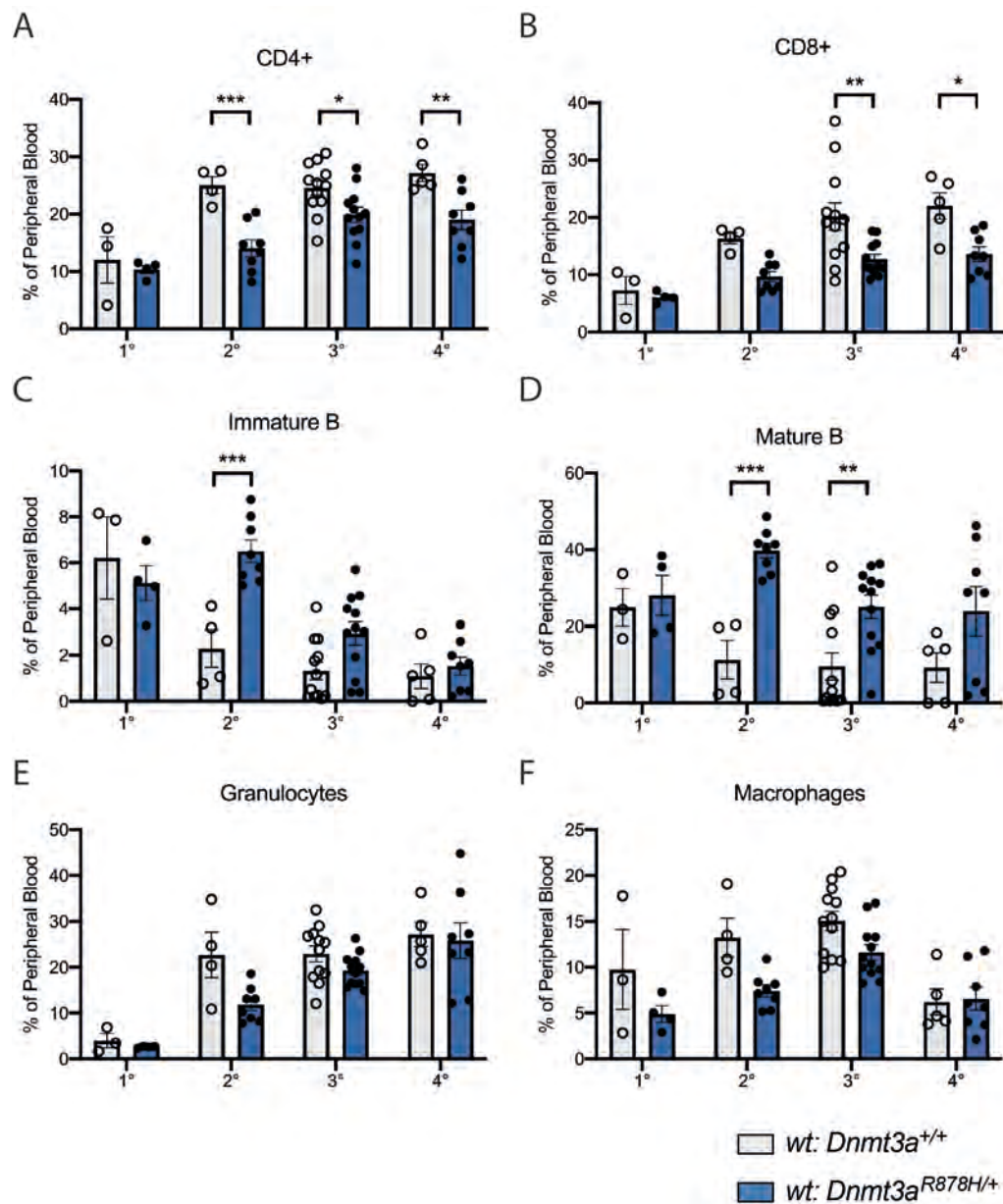


Figure 4.17 Blood cell composition of recipient mice during serial competitive bone marrow transplantation

Whole blood cell composition of mice serially transplanted with wt: wt or wt: *Dnmt3a*^{R878H/+} mixtures of bone marrow cells was determined by flow cytometry of A) CD4⁺ T cells = TCRβ⁺CD4⁺CD8⁻, B) CD8⁺ T cells = TCRβ⁺CD4⁻CD8⁺, C) immature B cells = B220⁺IgM⁺IgD^{mid}, D) mature B cells B220⁺IgM⁺IgD⁺, E) granulocytes TCRβ⁻B220⁻Gr1⁺Mac1⁺, F) macrophages TCRβ⁻B220⁻Gr1⁺Mac1⁺. Data are presented as mean±SEM. Students t-tests were performed, *p<0.05, **p<0.01, ***p<0.001.

declined. Interestingly, this was not due to the competition with the transplanted wt (CD45.1) cells but rather due to an increase in the radio-resistant recipient derived CD4⁺ T cells (CD45.1/CD45.2). While it was not anticipated that radio-resistant T cells would contribute so strongly to CD4⁺ T cells, this result is not altogether surprising given that T cells have previously been shown to be relatively resistant to γ -irradiation induced cell death and are able to expand in numbers owing to so-called homeostatic proliferation^{188,189}. Radio-resistant recipient derived cells may be fitter, as the transplanted wt: *Dnmt3a*^{R878H/+} cells had already undergone multiple rounds of transplantation. Nonetheless, *Dnmt3a*^{R878H/+} CD4⁺ T cells remained significantly higher than *Dnmt3a*^{+/+} CD4⁺ T cells at the endpoint of the 4th round of transplantation (Figure 4.18 B). The same results were true for CD8⁺ T cells. This supports the notion that *Dnmt3a*^{R878H/+} HSPCs have increased serial transplantation capacity compared to their wt counterparts.

Consistent with previous transplantation experiments, *Dnmt3a*^{R878H/+} immature and mature B cells were significantly enriched in the blood of recipients that had been serially transplanted with a wt: *Dnmt3a*^{R878H/+} mixture of bone marrow cells (Figure 4.19 A-B). *Dnmt3a*^{R878H/+} immature and mature B cells were significantly higher than *Dnmt3a*^{+/+} B cells during the second, third and fourth round of transplantation. In contrast to *Dnmt3a*^{R878H/+} T lymphocytes, recipient derived B cells did not appear to compete as much with the transplanted cells. However, by the fourth round of transplantation, the proportion of transplant derived cells was reduced. Nevertheless, the proportions of *Dnmt3a*^{R878H/+} immature and mature B cells remained significantly higher compared to the *Dnmt3a*^{+/+} B lymphoid cells in the control animals. This was not surprising as *Dnmt3a*^{R878H/+} B cells have been shown to outcompete their wt counterparts in previous transplantation experiments, as presented in this thesis.

Dnmt3a^{R878H/+} granulocytes and macrophages were also significantly enriched in the blood of recipient mice that had been serially transplanted with wt: *Dnmt3a*^{R878H/+} mixtures of bone marrow cells during the 2nd and 3rd round of transplantation (Figure 4.20 A-B). There was also a trend towards higher numbers of *Dnmt3a*^{R878H/+} granulocytes and macrophages in the blood of recipient mice that had been serially transplanted with wt: *Dnmt3a*^{R878H/+} mixtures of bone marrow cells compared to the control animals. Although this difference was not statistically significant, it was clear that in a subset of mice *Dnmt3a*^{R878H/+} granulocytes and macrophages remained elevated.

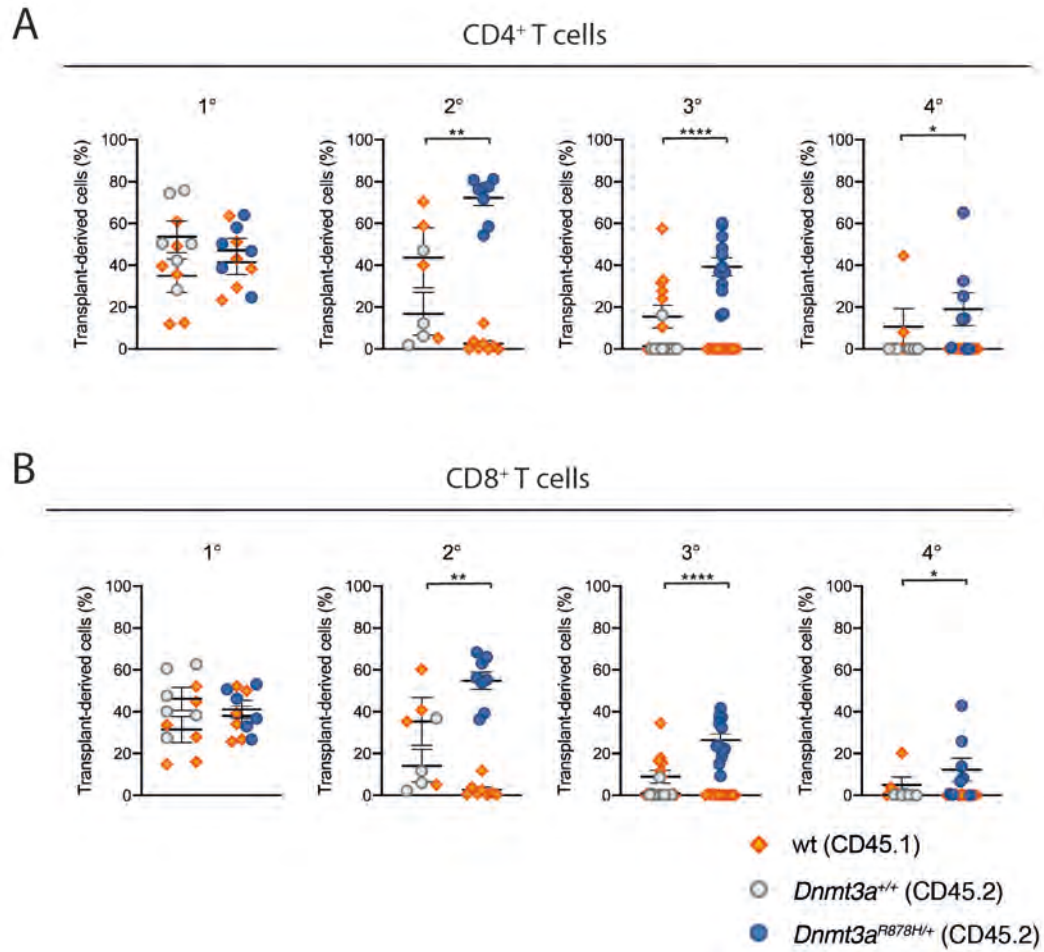


Figure 4.18 *Dnmt3a*^{R878H/+} CD4⁺ and CD8⁺ T cells outcompete their wt counterparts by the 2^o round of transplantation but are unable to maintain their advantage during the 3^o and 4^o rounds of transplantation.

The proportions of wt (CD45.1⁺) (Orange), *Dnmt3a*^{R878H/+} (CD45.2⁺) (Blue) and *Dnmt3a*^{+/+} (CD45.2⁺) (Grey) cells at the endpoints of the primary (1^o), secondary (2^o), tertiary (3^o) and quaternary (4^o) rounds of transplantation A) CD4⁺ T cells = TCRβ⁺CD4⁺CD8⁻, and B) CD8⁺ T cells = TCRβ⁺CD4⁺CD8⁺. Mann-Whitney test comparing CD45.2⁺ cells between groups, *p<0.05, **p<0.01, ****p<0.0001.

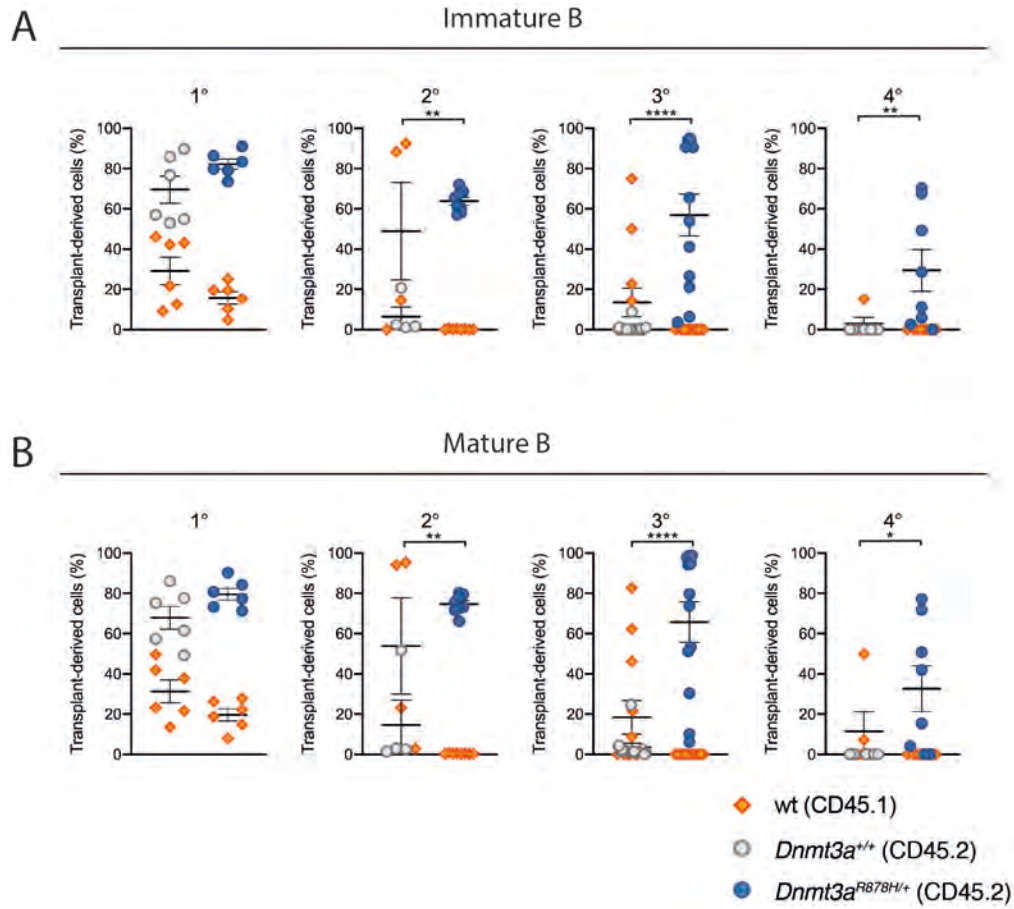


Figure 4.19 serial transplantation: analysis of immature and mature B cells

The proportions of wt (CD45.1⁺) (Orange), *Dnmt3a*^{R878H/+} (CD45.2⁺) (Blue) and *Dnmt3a*^{+/+} (CD45.2⁺) (Grey) cells were determined at the endpoints of the primary (1°), secondary (2°), tertiary (3°) and quaternary (4°) rounds of transplantation A) immature B cells = B220⁺IgM⁺IgD^{mid} and B) mature B cells B220⁺IgM⁺IgD⁺. Mann-Whitney test comparing CD45.2⁺ cells between groups, *p<0.05, **p<0.01, ****p<0.0001.

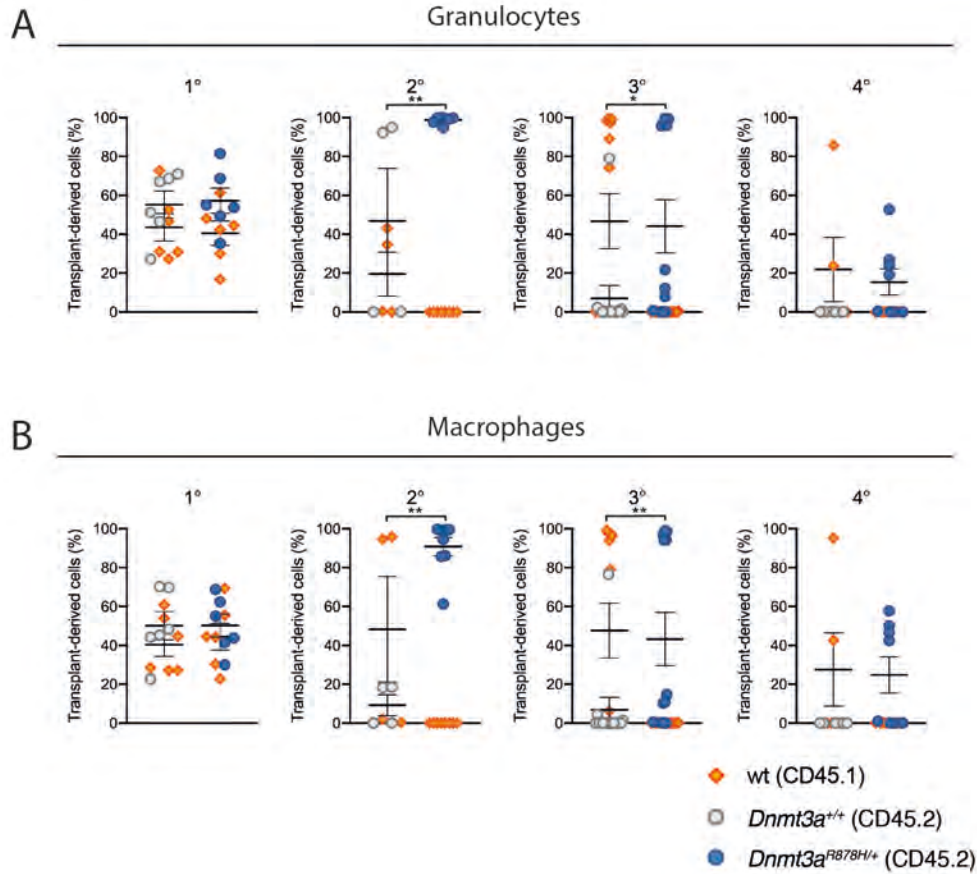


Figure 4.20 Proportions of *Dnmt3a*^{R878H/+} granulocytes and macrophages during serial transplantation

The proportions of wt (CD45.1⁺) (Orange), *Dnmt3a*^{R878H/+} (CD45.2⁺) (Blue) and *Dnmt3a*^{+/+} (CD45.2⁺) (Grey) cells was determined for the primary (1°), secondary (2°), tertiary (3°) and quaternary (4°) rounds of transplantation A) granulocytes TCRβ⁺B220⁺Gr1⁺Mac1⁺ and B) macrophages TCRβ⁺B220⁺Gr1⁺Mac1⁺. Mann-Whitney test comparing CD45.2⁺ cells between groups, *p<0.05, **p<0.01, ****p<0.0001.

The contribution of *Dnmt3a*^{R878H/+} cells to the different haematopoietic cell subsets was also examined in the spleens of mice at the endpoints of the 1st, 2nd and 3rd round of transplantation to determine whether *Dnmt3a*^{R878H/+} cells in the spleen behaved similarly to those found in the blood. To this end, single cell suspensions were generated by gently passing the spleen through a nylon filter as described in chapter 2. Then cells were incubated with fluorochrome conjugated antibodies against cell type specific surface markers as well as antibodies against CD45.1 and

CD45.2 to distinguish between competitor and test cells. Spleen cells from wt: *Dnmt3a*^{R878H/+} bone marrow mixture transplanted mice were analysed by flow cytometry to determine the proportions of *Dnmt3a*^{R878H/+} (CD45.2⁺) or *Dnmt3a*^{+/+} control (CD45.2⁺) derived cells in the various cell subsets. The proportions of CD4⁺ or CD8⁺ T cells, immature B cells, mature B cells, granulocytes and macrophages were determined by gating for these cell types on the basis of the expression of specific surface markers as shown previously in Figure 4.3. The results are presented in Figure 4.21, where each circle represents a single mouse. The three biological replicates of WT: *Dnmt3a*^{R878H/+} bone marrow mixture transplanted mice are shown in Figure 4.21 A, with each circle reflecting a single recipient mouse that had received that bone marrow mixture. The size of the circle represents the proportion of CD45.2⁺ test cells in the spleen. The smaller the circle, the smaller the proportion of CD45.2⁺ cells in the spleen. Equally, the larger the circle, the more CD45.2⁺ cells in the spleen of that mouse.

Figure 4.21 A-B shows the results at the end of the primary transplantation. Haematopoietic cells from all lineages are represented in the CD45.2⁺ *Dnmt3a*^{R878H/+} and *Dnmt3a*^{+/+} circles. In the primary transplanted mice, the sizes of the *Dnmt3a*^{+/+} (control) and *Dnmt3a*^{R878H/+} circles are similar, reflecting an equal contribution of wt and *Dnmt3a*^{R878H/+} cells at 3 months post transplantation. In contrast, after three months in the secondary transplant recipients the circles are much larger from the wt: *Dnmt3a*^{R878H/+} group (Figure 4.21 A) compared to the circles of the wt: *Dnmt3a*^{+/+} control group. This indicates that the *Dnmt3a*^{R878H/+} cells were outcompeting their WT counterparts. Furthermore, the proportions of CD4⁺ T cells, CD8⁺ T cells, immature B cells, mature B cells, granulocytes and macrophages were comparable between the biological replicates and similar to the primary endpoint. However, the opposite was true for the recipients that had been serially transplanted with wt: *Dnmt3a*^{+/+} mixed bone marrow cells. In this group the proportions of *Dnmt3a*^{+/+} (CD45.2⁺) test cells were significantly reduced compared to the endpoint of the primary transplantation, as depicted by a reduction in the circle size. Furthermore, the proportions of CD4⁺ T cells, CD8⁺ T cells, immature B cells, mature B cells, granulocytes and macrophages were altered compared to the endpoint of the primary transplantation. These observations were even more prominent in the recipient mice of the third round of transplantation. The results from the analysis of the spleen were interesting as it suggested that while *Dnmt3a*^{R878H/+} haematopoietic stem/progenitor cells retained the ability to give rise to cells of all haematopoietic lineages in serial transplantation studies, the *Dnmt3a*^{+/+} HSPCs became more lineage biased under these conditions.

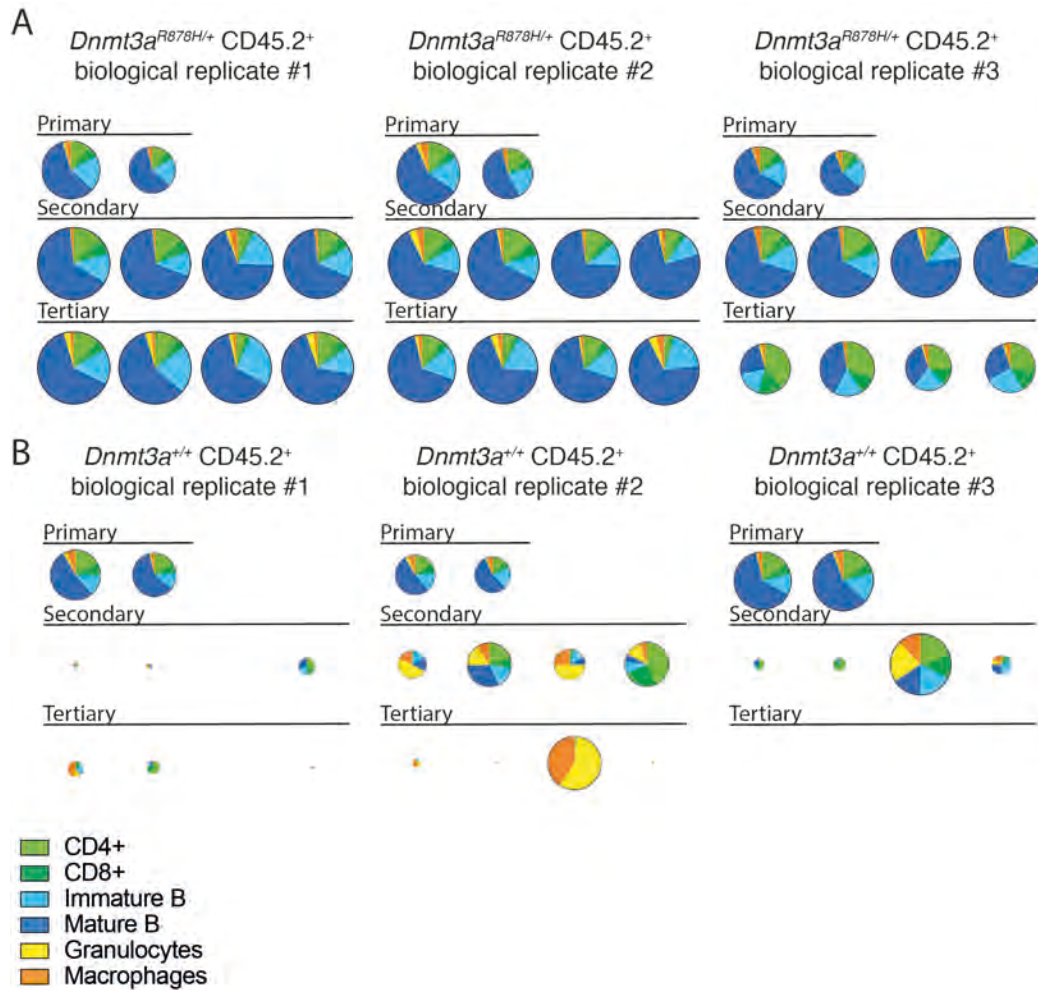


Figure 4.21 The ability of *Dnmt3a*^{R878H/+} cells to contribute to different mature blood cell types in serial competitive reconstitution studies

CD45.2⁺ cells derived from the spleens of serially transplanted mice. A) 1-3 biological replicates of recipient mice serially transplanted with wt: *Dnmt3a*^{R878H/+} bone marrow mixtures. B) 1-3 biological replicates of recipient mice serially transplanted with wt: *Dnmt3a*^{+/+} bone marrow mixtures. Each circle represents a single mouse. The size of the circle is reflective of the proportion of CD45.2⁺ cells in the spleen. The colours represent the proportions of the different cell subsets in the spleen. Cells types were defined by surface markers; CD4⁺ T cells = TCRβ⁺CD4⁺CD8⁻, CD8⁺ T cells = TCRβ⁺CD4⁻CD8⁺, immature B cells = B220⁺IgM⁺IgD^{mid}, mature B cells B220⁺IgM⁺IgD⁺, granulocytes TCRβ⁻B220⁻Gr1⁺Mac1⁺, macrophages TCRβ⁻B220⁻Gr1⁻Mac1⁺.

Next, the proportions of wt and *Dnmt3a*^{R878H/+} HSPCs in the bone marrow of the serially transplanted mice were determined. It was hypothesised that the increased proportions of *Dnmt3a*^{R878H/+} CD4⁺ T cells, CD8⁺ T cells, immature B cells, mature B cells, granulocytes and macrophages observed in the blood and spleen would be paralleled by an increase in *Dnmt3a*^{R878H/+} HSPCs in the bone marrow of the serially competitively reconstituted mice. To examine this, the proportions of CD45.1⁺ vs CD45.2⁺ LSK cells in the bone marrow were determined for each group. As shown in Figure 4.22, *Dnmt3a*^{R878H/+} LSK cells were increased compared to the wt LSK cells, although this difference was not statistically significant in the primary transplantation. In the secondary and tertiary transplanted mice, *Dnmt3a*^{R878H/+} LSK cells were markedly enriched in the bone marrow. In the fourth transplant the *Dnmt3a*^{R878H/+} LSK cells remained higher compared to the control LSKs, but this difference was no longer statistically significant.

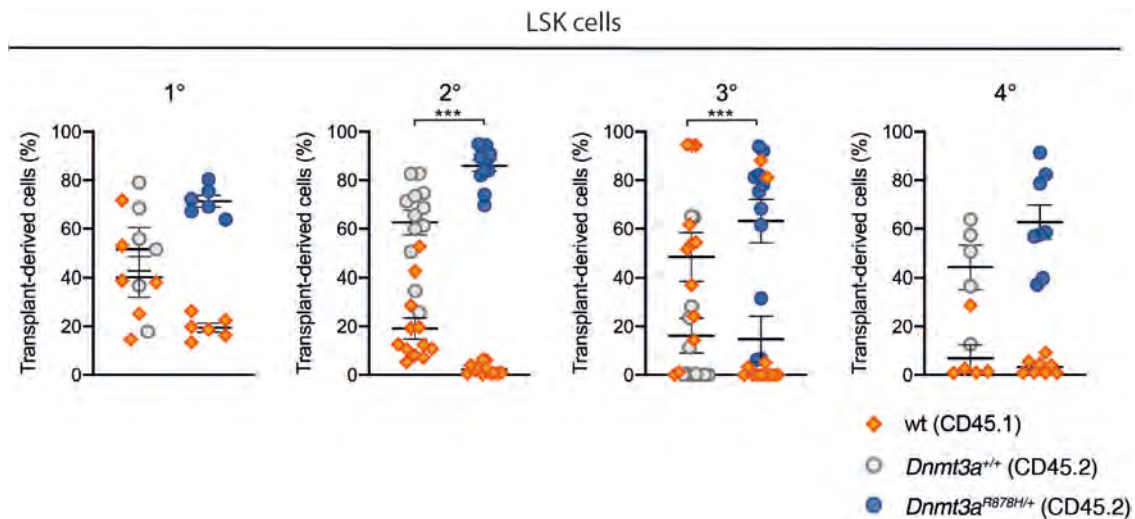


Figure 4.22 Representation of *Dnmt3a*^{R878H/+} LSK cells in the bone marrow during serial transplantation

The proportions of CD45.1⁺ and CD45.2⁺ LSK cells (Lin⁻Sca1⁺cKIT⁺) derived from the bone marrow of serially transplanted recipient mice at the end of the endpoint of the primary, secondary, tertiary and quaternary rounds of transplantation. Mann Whitney U test to compare *Dnmt3a*^{+/+} CD45.2⁺ cells with *Dnmt3a*^{R878H/+} cells, ***p<0.001. n=5-12 mice/ group.

Interestingly, the high proportion of *Dnmt3a*^{R878H/+} LSK cells in the bone marrow of the serially competitively transplanted recipient mice was not necessarily reflected in the haematopoietic cells of the blood and spleen. As shown previously, the proportions of *Dnmt3a*^{R878H/+} derived CD4⁺ T cells, CD8⁺ T cells, immature B cells, mature B cells, granulocytes and macrophages in the blood were all reduced in the quaternary transplantation group compared to the tertiary transplantation group (Figure 4.18-4.20). At the same time, *Dnmt3a*^{R878H/+} HSPCs remained high in the bone marrow (Figure 4.22), suggesting that the *Dnmt3a*^{R878H/+} HSPCs have increased self-renewal at the expense of differentiation. This explanation is consistent with similar studies of *Dnmt3a* KO cells where the authors reported that *Dnmt3a* KO HSPCs, supplemented with WT bone marrow, could be serially transplanted indefinitely but would not differentiate into mature haematopoietic cells beyond the fourth round of such transplantation ¹¹⁵. It is tempting to speculate that *Dnmt3a*^{R878H/+} HSPCs are also capable of indefinite serial transplantation and that this is accompanied by defects in differentiation into the different haematopoietic lineages. However, further experiments would be required to confirm this hypothesis.

The results of the serial competitive bone marrow transplant experiments presented above provided insight into how the *Dnmt3a*^{R878H} mutation altered the normal function of HSPCs in mice. There was clear evidence of progressively reduced fitness of wt HSPCs and blood cellularity over time as demonstrated by haematopoietic reconstitution failure in a subset of recipients that had been serially transplanted with wt: *Dnmt3a*^{+/+} bone marrow mixtures. In contrast, all mice that had been serially transplanted with wt: *Dnmt3a*^{R878H/+} bone marrow mixtures survived, reflecting the increased fitness of *Dnmt3a*^{R878H/+} stem cells under sustained stress. It was hypothesised that the sustained stress experienced by *Dnmt3a*^{R878H/+} HSPCs during serial transplantation might drive malignant transformation of these cells. However, there was no evidence of altered blood cell composition that would reflect haematological malignancy in the recipients that had been serially transplanted with wt: *Dnmt3a*^{R878H/+} bone marrow mixtures.

The results presented in this thesis demonstrated that four rounds of serial competitive transplantation every three months were not sufficient to drive malignant transformation of *Dnmt3a*^{R878H/+} haematopoietic cells. Since DNMT3a^{R878H} mutant human cancers are generally found in the ageing population, serial transplantation experiments were conducted in a way to include a longer time between rounds of transplantation. The rationale for this was that the *Dnmt3a*^{R878H/+} HSPCs would undergo rapid proliferation during each round of reconstitution of a

lethally irradiated recipient, followed by 6 months of recovery. At 6 months, the aged stem cells would be transplanted into secondary lethally irradiated recipients (Figure 4.23). It was hypothesised that the enforced proliferation of aged *Dnmt3a*^{R878H/+} HSPCs would increase the likelihood of acquiring additional cancer driving mutations, eventually leading to leukaemia.

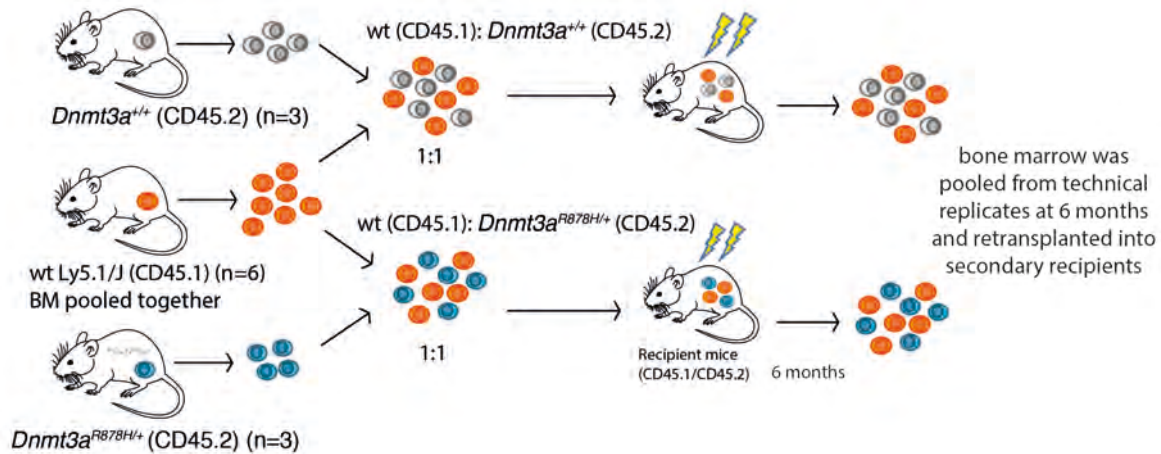


Figure 4.23 Schematic of the experimental setup for 6-month interval serial competitive bone marrow transplantation experiments.

Competitor bone marrow cells derived from CD45.1⁺ mice were pooled such that all mice received the same bolus of competitor cells. CD45.2⁺ test *Dnmt3a*^{R878H/+} or control *Dnmt3a*^{+/+} (wt) bone marrow was kept independent and used to reconstitute lethally irradiated recipient mice (CD45.1⁺/CD45.2⁺) in competition with the CD45.1 wt bone marrow cells. The recipient mice were aged for 6 months. At 6 months, the relative contributions of CD45.1 vs CD45.2 cells in the different haematopoietic organs were assessed. The bone marrow from 3 mice reconstituted with the same bone marrow cells was pooled and used to transplant secondary lethally irradiated recipient mice (CD45.1⁺/CD45.2⁺).

Blood cell composition was determined in primary and secondary bone marrow transplanted mice by ADVIA and flow cytometry. The relative contribution of wt and *Dnmt3a*^{R878H/+} blood cells in these mice was also determined. The increased recovery time of six months between transplantations did not have any effect on the contribution of *Dnmt3a*^{R878H/+} to the different cell types in the blood compared to the previously presented data from the short-term serial transplantation studies. Although, two mice that had been transplanted with the wt: *Dnmt3a*^{+/+}

bone marrow suffered from reconstitution failure. The inability of these wt cells to reconstitute lethally irradiated recipient mice could reflect impaired function with age, that does not occur in *Dnmt3a*^{R878H/+} HSPCs. Results from ADVIA and flow cytometric analysis of the blood of the six-month serial transplantation experiment are displayed in Appendix III. The ratio of wt: *Dnmt3a*^{R878H/+} cells was also determined in the thymus of primary recipient mice at 6 months but there was no significant difference compared to *Dnmt3a*^{+/+} cells (Appendix III).

Interestingly, a small proportion of reconstituted mice in each group developed thymic T cell lymphoma. This suggested initially that the aged stem cells were more prone to malignant transformation (Figure 4.24). As shown in Figure 4.24 B, the tumours from both the wt: *Dnmt3a*^{+/+} and the wt: *Dnmta*^{R878H/+} bone marrow mixture reconstituted mice consisted of DP and CD8⁺ T lymphoma cells. In contrast to the hypothesis that aged stem cells were more prone to malignant transformation, it was found that the tumours in all cases were derived from the recipient cells as indicated by tumour cells being positive for both CD45.1 and CD45.2 (Figure 4.24 C). This result was unexpected but suggested that aged serially transplanted stem cells were less fit, creating a need for the radio-resistant recipient derived HSPCs to proliferate and contribute to haematopoietic repopulation, which most likely led to the thymic lymphoma.

The incidence of lymphoma appeared to be somewhat lower in the wt: *Dnmt3a*^{R878H/+} reconstituted mice compared to the wt: *Dnmt3a*^{+/+} bone marrow mixture mice. This is consistent with the notion that *Dnmt3a*^{R878H/+} HSPCs are “fitter” than their WT counterparts; hence recipient HSPCs would have had to “work less hard” in mice that had been transplanted with the wt: *Dnmta*^{R878H/+} bone marrow mixture compared to those in mice that had been transplanted with wt: *Dnmt3a*^{+/+} bone marrow. Mutations in *DNMT3a* are thought to predispose patients to the development of leukaemia. So, it was surprising that despite conditions of sustained haematopoietic stress the *Dnmt3a*^{R878H/+} HSPCs did not give rise to leukaemia. It therefore remains unclear what events would drive malignant transformation of *Dnmt3a*^{R878H/+} haematopoietic cells.

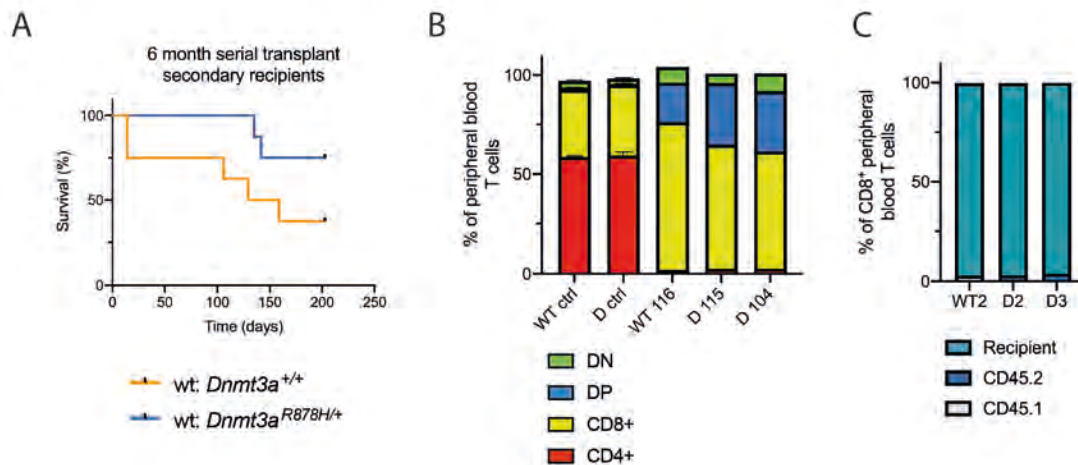


Figure 4.24 Some reconstituted mice develop recipient derived thymic T cell lymphoma upon serial transplantation of bone marrow cells.

A) Survival curve showing the percentage survival of mice during the secondary round of transplantation. Two wt: *Dnmt3a*^{+/+} (orange) mice failed reconstitution indicated by their early sickness with need for sacrifice. B) The blood from the wt: wt and wt: *Dnmt3a*^{R878H/+} bone marrow transplanted recipient mice was analysed by flow cytometry. B-D) The tumours were stained for TCRβ, B220, CD4 and CD8 markers. The blood from the mice was also analysed by ADVIA E-H). WBC; white blood cell, RBC; red blood cell. Data are presented as mean±SEM. Statistics were not performed as n numbers were too low.

4.6 Examining *Dnmt3a*^{R878H/+} stem and progenitor cells in emergency haematopoiesis induced by treatment with 5-Fluorouracil.

In this experiment, emergency haematopoiesis was induced by intravenous injection of 5-Fluorouracil (5-Fu) into mice that had previously been reconstituted with a 1:1 mixture of wt: *Dnmt3a*^{+/+} or wt: *Dnmt3a*^{R878H/+} bone marrow as depicted in Figure 4.25. To induce emergency haematopoiesis, animals were treated with 5-Fluorouracil (5-Fu), which induces cell death by inhibiting the synthesis of DNA (through inhibition of thymidylate synthase) in proliferating cells. 5-Fu treatment causes the depletion of most haematopoietic lineages, which leads to production of new blood cells involving mobilisation of LT-HSCs. It was hypothesised that *Dnmt3a*^{R878H/+} LT-HSCs would be more potent in repopulating the haematopoietic system and

hence cell populations would re-bound quickly in 5-Fu treated mice containing *Dnmt3a*^{R878H/+} HSPCs compared to those containing only wt HSPCs. This would be expected to lead to an increased representation of *Dnmt3a*^{R878H/+} cells in the haematopoietic lineages.

Blood cell composition changed over the time of treatment with 5-Fu and, as expected, returned to baseline levels by day 21. As shown in Figure 4.26, there were no significant differences in RBC (A), WBC (B), platelets (C), haematocrit (D), lymphocytes (E), neutrophils (F) or haemoglobin (G) between the wt: *Dnmt3a*^{+/+} vs the wt: *Dnmt3a*^{R878H/+} bone marrow mixture reconstituted mice.

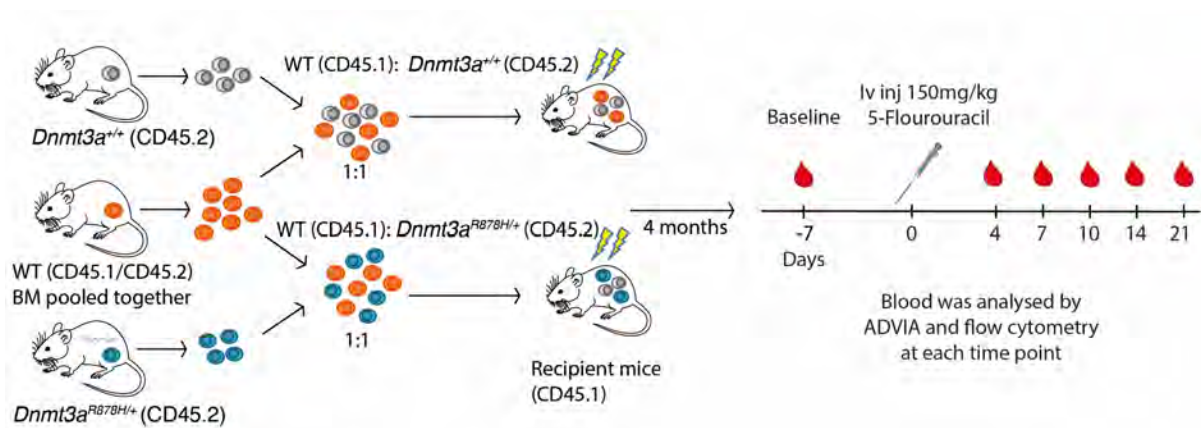


Figure 4.25 Schematic showing experimental design of competitive bone marrow reconstitution and treatment with 5-FU.

4 months after bone marrow transplantation but prior to treatment with 5-Fu, baseline blood cell composition was determined by ADVIA and flow cytometric analysis. Seven days after baseline bleeds, mice were weighed and then injected intravenously with 150 mg/kg of 5-FU. Mice were bled on days 4, 7, 10, 14 and 21 following 5-FU injection. All mice survived the treatment.

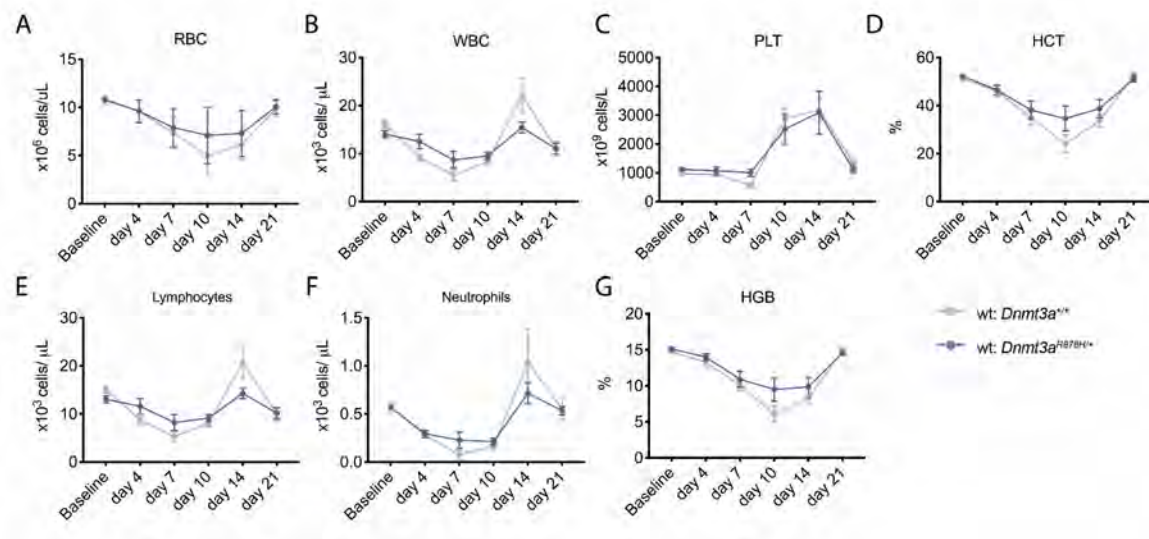


Figure 4.26 All bone marrow reconstituted mice responded normally to treatment with 5-FU

Blood cell composition was determined by ADVIA. A) red blood cells; RBC, B) white blood cells; WBC, C) platelets; PLT, D) haematocrit; HCT, E) lymphocytes, F) neutrophils and G) haemoglobin; HGB. Data are presented as mean $\pm$ SEM. Mixed effects model, ns. N=6-8/group

The change of the haematopoietic cell composition in the blood was also monitored throughout the experiment by flow cytometry. As shown in Figure 4.27 A, staining for cell type specific surface markers were used to identify the different blood cell types. Blood cells from mice that had been reconstituted with wt: *Dnmt3a*^{+/+} and wt: *Dnmt3a*^{R878H/+} bone marrow mixtures responded similarly to treatment with 5-FU. There were no significant differences between these groups of mice with respect to CD4⁺ and CD8⁺ T cells, mature B cells or macrophages at any of the time points after treatment with 5-FU (Figure 4.27 B-C, E, G). The numbers of immature B cells were significantly higher on days 7, 10 and 21 after treatment with 5-FU in the wt: *Dnmt3a*^{R878H/+} group compared to the control group (Figure 4.27 D). This result was not altogether unsurprising as immature B cells were also elevated in the secondary serial transplantation experiments as presented in Figure 4.18 C. These results raise suggest that either increased proliferation or enhanced survival of *Dnmt3a*^{R878H/+} immature B cells is responsible for their increases frequency in mice after treatment with cytotoxic agents, such as

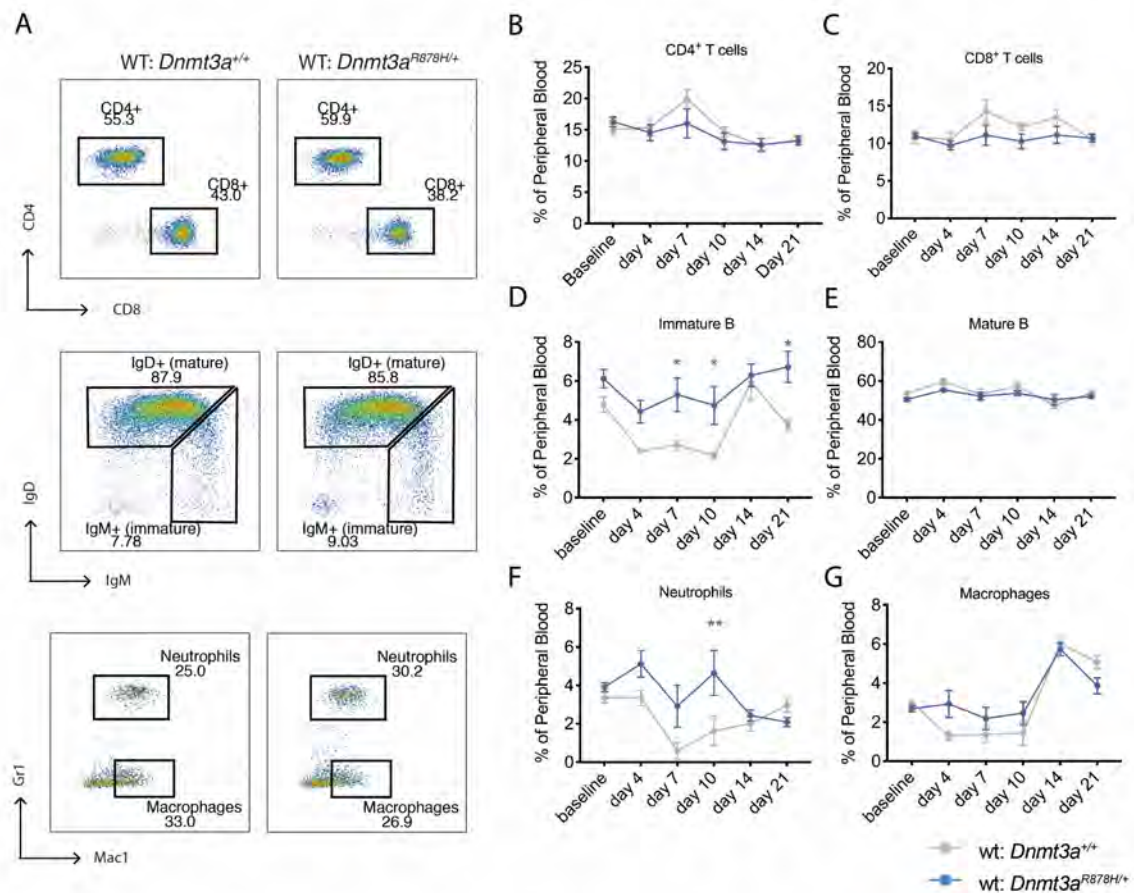


Figure 4.27 Flow cytometric analysis of cells derived from the blood of bone marrow reconstituted mice after treatment with 5-FU

Blood cell composition was determined by flow cytometric analysis. A) gating strategy to define cell populations, B) CD4⁺ T cells, C) CD8⁺ T cells, D) immature B, E) mature B, F) neutrophils, G) macrophages. Data are presented as mean $\pm$ SEM. Two-way ANOVA with Sidaks multiple comparisons test, N=8/group.

5-FU. However, further experiments would be required to determine whether this could be a mechanism of resistance in patients with B cell derived cancers harbouring *Dnmt3a*^{R878H/+} mutations. Similarly, neutrophils were also elevated in mice that had been reconstituted with wt: *Dnmt3a*^{R878H/+} bone marrow mixture at day 10 after treatment with 5-FU, although similar numbers of neutrophils were found in the two groups of mice at day 21 (Figure 4.28 G).

To determine whether *Dnmt3a*^{R878H/+} cells were more resistant or more sensitive to 5-FU treatment compared to wt cells, the proportions of different haematopoietic cell subsets in the competitively reconstituted mice was determined throughout the course of treatment with 5-FU. *Dnmt3a*^{R878H/+} cells made up a significantly larger proportion of immature B cells (Figure 4.29 D), mature B cells (Figure 4.29 E), granulocytes (Figure 4.29 F) and macrophages (Figure 4.29 G) at baseline compared to *Dnmt3a*^{+/+} cells in the control group. The *Dnmt3a*^{R878H} mutation did not appear to confer a competitive advantage in CD4⁺ and CD8⁺ T cells (Figure 4.29 B-C). There was a significant reduction in *Dnmt3a*^{+/+} cells in both CD4⁺ and CD8⁺ T cells subsets, but this was very small and unlikely to be physiologically relevant (Figure 4.29 B-C).

The treatment with 5-FU resulted in a significant reduction of *Dnmt3a*^{R878H/+} immature B cells at 21 days compared to baseline. This suggests that *Dnmt3a*^{R878H/+} immature B cells might be more sensitive to 5-FU treatment than WT cells. There was a small but significant increase in *Dnmt3a*^{R878H/+} mature B cells at 21 days compared to the pre-treatment levels. However, this increase was quite small and unlikely to result in a meaningful difference between *Dnmt3a*^{R878H/+} and *Dnmt3a*^{+/+} mature B cells. *Dnmt3a*^{R878H/+} granulocytes and macrophages returned to pre-treatment levels, although *Dnmt3a*^{+/+} granulocytes were significantly reduced at day 21 post treatment with 5-FU. This suggests that these cells are less fit compared to the *Dnmt3a*^{R878H/+} granulocytes.

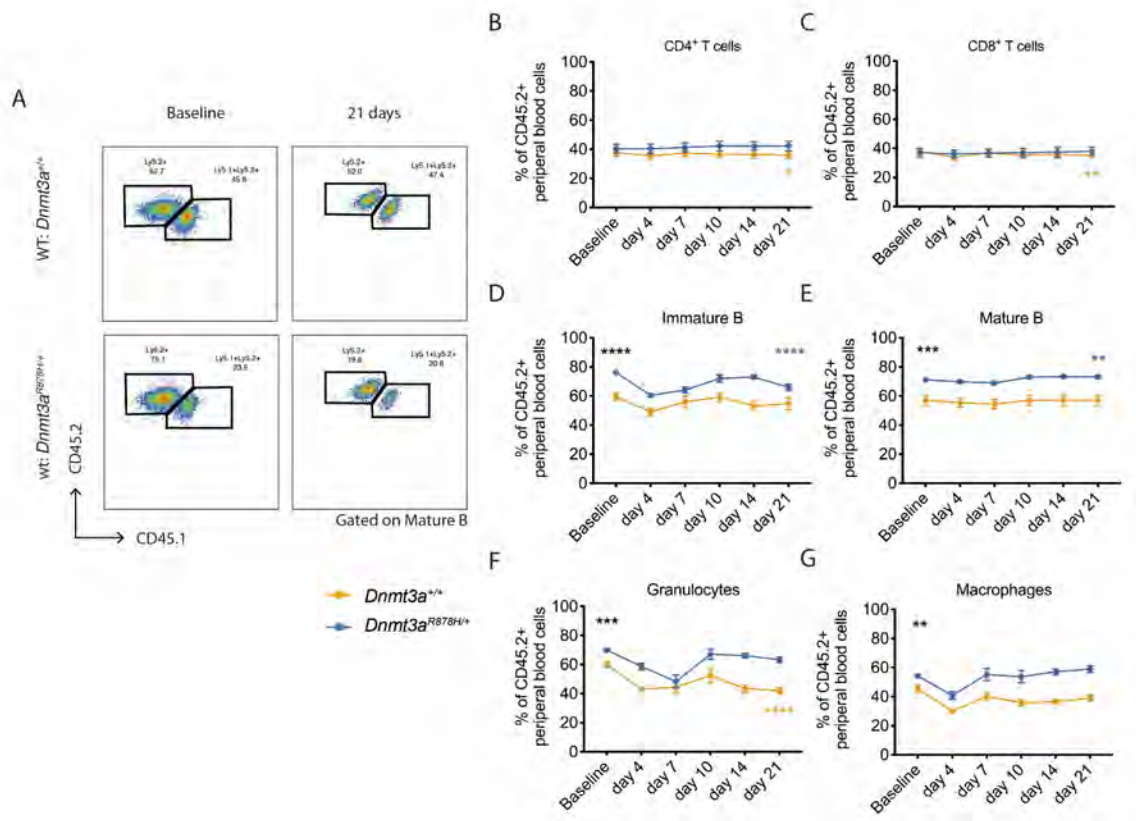


Figure 4.28 Treatment of competitive bone marrow reconstituted mice with 5-FU

Bleeds were analysed by flow cytometry. A) example gating strategy to define CD45.1 and CD45.2 populations. The percentage of CD45.2⁺ B) CD4⁺ T cells, C) CD8⁺ T cells, D) immature B, E) mature B, F) granulocytes and G) macrophages are displayed. mean±SEM, n=8 mice/group. Data were analysed by two-way ANOVA with Dunnetts post hoc test comparing CD45.2⁺ at 21 days to baseline (*Dnmt3a*^{R878H/+} (blue), *Dnmt3a*^{+/+} (orange)). Baseline CD45.2⁺ cell numbers were compared using a Mann-Whitney U test (black), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.7 Discussion

To investigate the impact of heterozygous *Dnmt3a*^{R878H} mutation on the murine haematopoietic system, mice were reconstituted with bone marrow cells derived from our newly developed mouse model. Firstly, in an attempt to reveal the functional implications of the *Dnmt3a*^{R878H} mutation on steady state haematopoiesis, the cell subset composition of blood and haematopoietic tissues was analysed in young mice. Secondly, to characterise the fitness of *Dnmt3a*^{R878H/+} stem and progenitor cells, emergency haematopoiesis was induced by either competitive and serial competitive bone marrow transplantation or by treatment with 5-FU.

4.7.1 *Dnmt3a*^{R878H/+} mice have subtle haematological abnormalities under steady state conditions.

A thorough analysis of the blood composition was performed in *Dnmt3a*^{R878H/+} bone marrow reconstituted mice under steady state conditions. As demonstrated in this chapter, these *Dnmt3a*^{R878H/+} bone marrow reconstituted mice displayed only mild haematological abnormalities compared to their controls. Cell numbers in the blood were comparable between *Dnmt3a*^{R878H/+} mice and wt littermates for the majority of cell types tested. An exception was that slightly fewer lymphocytes and a small reduction in LUC numbers were found in the blood of *Dnmt3a*^{R878H/+} mice. When data on these cell types were expressed as a percentage of total numbers of WBCs, the reduction in lymphocytes was compensated by a proportional increase in neutrophils in the *Dnmt3a*^{R878H/+} mice. The increase in neutrophils is exciting as it could reflect a myeloid differentiation bias in *Dnmt3a*^{R878H/+} mice. This is a tantalising conclusion given that *DNMT3A* R882H mutations are frequently found in patients with AML. However, flow cytometric analysis indicated that granulocytes appeared to be proportionately reduced in the blood of the *Dnmt3a*^{R878H/+} mice, casting doubt on such a conclusion. Granulocytes were defined as TCRβ⁻ B220⁻Gr1⁺Mac1⁺; these markers are often used to identify neutrophils, but other granulocytic cells, such as monocytes and eosinophils, would also share these cell surface markers. Therefore, the increase in neutrophils determined by ADVIA and the reduction in granulocytes found by flow cytometric analysis, may suggest that granulocytes other than neutrophils might differ in numbers between *Dnmt3a*^{R878H/+} vs control mice. Collectively, the results from the ADVIA and flow cytometric analyses indicate that there are subtle changes in blood cell composition between *Dnmt3a*^{R878H/+} vs wt mice but a more extensive analysis would need to be

performed to determine whether these are physiologically meaningful.

Interestingly, T Lymphocyte development was slightly perturbed in the *Dnmt3a*^{R878H/+} mice with significantly fewer DN1 stage cells detected in the thymus. This was accompanied by a proportional decrease in DN1 and DN2 stage cells in the thymus when expressed as a percentage (data not shown). It remains unclear what the physiological relevance of this observation is, as this abnormality in T lymphoid cell differentiation was overcome by the DN3 stage of T cell development in the thymus. There were no significant differences in the proportions of CD4⁺ or CD8⁺ cells in the blood, spleen, lymph nodes or thymus. This suggests that while the reduction in DN1 and DN2 stage cells in the thymus is intriguing it does not critically impair normal production of CD4⁺ or CD8⁺ T cells. However, other T cell populations, such as TCRγ/δ expressing cells in the intestines, were not examined in detail and therefore it is possible that *Dnmt3a*^{R878H/+} mice have abnormal T_{reg} populations that might account for the reduction in overall lymphocyte numbers in the blood. It also remains to be tested whether the *Dnmt3a*^{R878H/+} T cells function properly as this has not been explored in this thesis. Functional characterisation of the DNMT3a^{R878H/+} T cells and thymic epithelial cells is certainly warranted. In this experiment it was not possible to determine whether the reduction in DN1 stage cells in the thymus was due to a cell intrinsic or extrinsic effect of the *Dnmt3a*^{R878H/+} mutation, as all cells in this mouse, not just the T lymphoid cells, carried this mutation. Therefore, it is possible that early T lymphoid cell development in the thymus was impaired because the thymic stroma was compromised by the *Dnmt3a*^{R878H/+} mutation.

To determine whether the reduction in DN1 cells in the thymus could be traced back to defects in the bone marrow, the stem and progenitor cells were analysed in detail by flow cytometry. Interestingly there were no significant differences between stem and progenitor cell numbers between the *Dnmt3a*^{R878H/+} and WT mice, although there was a subtle trend towards reduced CLP numbers in the bone marrow. If this observation was confirmed in more mice, this might in part account for the reduction of DN1 cells in the thymus of the *Dnmt3a*^{R878H/+} mice.

While the characterisation of the haematopoietic system in this mouse model was by no means exhaustive, it was the first time that the consequences of the heterozygous *Dnmt3a*^{R878H} mutation has been assessed in steady state haematopoiesis in a mouse model without confounding treatments like poly-IC injection (causing a surge in interferons) and

haematopoietic transplantation. Therefore, these results are the first to show that in the absence of external stress stimuli, *Dnmt3a*^{R878H/+} haematopoiesis is grossly normal. The characterisation of the haematopoietic system revealed that mutant *Dnmt3a* cell composition did not markedly diverge from wt mice. The question remained as to why mutant DNMT3a is strongly enriched in human AML and T-ALL. The steps between the acquisition of a mutation in *DNMT3a* and malignant transformation remain unresolved. Therefore, the next step was to determine the response of *Dnmt3a*^{R878H/+} cells to conditions of haematopoietic stress.

4.7.2 *Dnmt3a*^{R878H/+} haematopoietic cells outcompete their wt counterparts in long-term but not in short-term competitive bone marrow reconstitution experiments

To test the fitness of *Dnmt3a*^{R878H/+} HSPCs, competitive bone marrow transplants were set up. Mice were transplanted with either a 1:1 or a 9:1 wt: DNMT3a^{R878H/+} mixture of bone marrow cells. It was found that *Dnmt3a*^{R878H/+} haematopoietic cells did not have a competitive advantage over their wt counterparts in the blood, spleen or lymph nodes after 3-4 months. However, there was some evidence that even during this short time, *Dnmt3a*^{R878H/+} stem and progenitor cells were accumulating in the bone marrow. The accumulation of these HSPCs was not yet reflected in the more mature haematopoietic cell types in peripheral haematopoietic organs but provided some insight that this might occur if the mice were left to age for a longer period of time. Therefore, long term competitive haematopoietic reconstitution experiments were conducted to expand upon the data from the short-term assays. As predicted, *Dnmt3a*^{R878H/+} cells were able to outcompete their wt counterparts as demonstrated by the predominance of *Dnmt3a*^{R878H/+} CD4⁺ T cells, CD8⁺ T cells, immature B cells, mature B cells, granulocytes and macrophages in the blood of the competitively reconstituted mice at nine months.

Unfortunately, bone marrow was not taken at the nine-month timepoint, so it remains unclear whether *Dnmt3a*^{R878H/+} HSPCs had entirely outcompeted their wt counterparts or if the wt stem cells were present but more likely to be quiescent. However, there were significantly more *Dnmt3a*^{R878H/+} HSPCs than wt HSPCs in the bone marrow three months after transplantation; so it appears reasonable to extrapolate that this could be observed to an even greater extent at nine months.

4.7.3 *Dnmt3a^{R878H/+}* stem cells have increased serial repopulating capacity compared to wt stem cells.

The serial competitive bone marrow transplantation experiment revealed an increase in serial repopulating capacity of *Dnmt3a^{R878H/+}* HSPCs. It was shown that upon secondary transplantation, *Dnmt3a^{R878H/+}* cells predominated in all haematopoietic cell subsets of the blood in mice that had been reconstituted with a wt: *Dnmt3a^{R878H/+}* bone marrow mixture. The competitive advantage of cells with the *Dnmt3a^{R878H}* mutation was maintained in both the lymphoid and myeloid lineages in the third round of transplantation, despite radio-resistant host derived cells also starting to contribute to certain cell subsets. By the fourth round of serial transplantation, there was significant contribution by radio-resistant recipient derived cells in several haematopoietic cell subsets. However, even under these conditions, *Dnmt3a^{R878H/+}* T cells and B cells were still significantly enriched in the blood compared to their wt counterparts. It was evident in these experiments that the presence of *Dnmt3a^{R878H/+}* cells provided a survival advantage to recipient mice. All mice reconstituted with wt: *Dnmt3a^{R878H/+}* bone marrow mixtures in the serial transplantation study survived, whereas several mice in the control arm of the experiment succumbed to haematopoietic reconstitution failure. This reveals that *Dnmt3a^{R878H/+}* HSPCs have increased serial repopulating capacity compared to their wt counterparts. Previous research has shown that *Dnmt3a* KO stem cells have indefinite serial repopulating capacity (up to 11 serial transplantations conducted) when transplanted alongside WT whole bone marrow cells ¹¹⁵. Intriguingly, despite the abundance of *Dnmt3a* KO stem cells in the bone marrow of the serially transplanted mice, *Dnmt3a* KO cells could not be observed in the blood of these mice after the fourth round of transplantation. The observations from this previous study support the findings of the results presented herein, where *Dnmt3a^{R878H/+}* cells were found to be less able to contribute to mature blood cell lineages in the 4th round of transplantation. It would be interesting to examine HSPC populations in the serially transplanted recipient mice at each time point to determine how these populations changed over time.

4.7.4 *Dnmt3a^{R878H/+}* haematopoietic stem and progenitor cells respond similar to their wt counterparts to emergency haematopoiesis induced by treatment with 5-FU.

In the previous section, serial competitive bone marrow transplantation experiments were performed to test the ability of *Dnmt3a^{R878H/+}* HSPCs to respond to repeated emergency haematopoiesis in the absence of DNA damage. To extend these findings using a model

incorporating treatment with a DNA damage inducing chemotherapeutic agent, mice that had been reconstituted with a wt: *Dnmt3a*^{R878H/+} bone marrow mixture were generated and then tested for their ability to overcome the deleterious effects on their haematopoietic system induced by treatment with 5-FU. There were no significant differences in the response to 5-FU between the wt: *Dnmt3a*^{R878H/+} vs the wt: *Dnmt3a*^{+/+} reconstituted groups. Mice from both groups were sacrificed at 21 days post 5-FU treatment; a time at which the haematopoietic cell populations had returned to baseline levels in the blood. In future experiments, it would be interesting to monitor the different haematopoietic cell populations over a longer period of time to determine whether the 5-FU treatment had any long-term effects on blood composition or overall survival in mice reconstituted with a mixture of wt: *Dnmt3a*^{R878H/+} bone marrow cells.

In conclusion, the results presented in this chapter are the first to show that *Dnmt3a*^{R878H/+} mice have a subtle abnormality in early stages of T cell development in the thymus. Moreover, *Dnmt3a*^{R878H/+} HSPCs are able to outcompete their wt counterparts in both lymphoid and myeloid lineages. The accumulation of *Dnmt3a*^{R878H/+} cells in the haematopoietic system is not characterised by aberrant proliferation of these cells, as the overall blood cell composition was not altered compared to wt mice. Furthermore, the *Dnmt3a*^{R878H/+} HSPCs are protected from exhaustion induced by serial transplantation. The increased fitness of the *Dnmt3a*^{R878H/+} HSPCs might provide some insight into the mechanism by which mutant *Dnmt3a* can predispose cells to leukaemic transformation.

CHAPTER 5

UNCOVERING GENE EXPRESSION CHANGES IN

HSPCs THAT ARE DRIVEN BY MUTANT

***Dnmt3a*^{R878H} AND ASSOCIATED WITH FASTER**

CANCER ONSET

5.1 Introduction

In the previous chapter it was shown that *Dnmt3a*^{R878H/+} HSPCs have a competitive advantage over wt cells in long-term and serial bone marrow reconstitution assays. Despite *Dnmt3a*^{R878H/+} mutant HSPC derived cells accumulating in the blood and haematopoietic organs over time, there was no evidence that this mutation was making these cells more prone to malignant transformation, at least under the conditions tested. Even under sustained conditions of haematopoietic stress, mice expressing mutant *Dnmt3a*^{R878H} in their haematopoietic cells did not succumb to cancer.

5.2 Irradiation induced thymic lymphoma model

Mutant DNMT3a alone is not sufficient to cause malignant transformation of haematopoietic cells. This is demonstrated by the observation that the presence of a mutation in *DNMT3a* on its own does not individually cause diseases like MDS or leukaemia^{143,145}. Clearly, additional mutations in other genes linked with the onset of cancer are required for full malignant transformation. Nevertheless, the high prevalence of mutant *DNMT3a* in patients presenting with leukaemia suggests that the mutation predisposes cells to neoplastic transformation⁸⁹.

The mechanism by which DNMT3a mutations predispose cells to leukaemia remains unknown but has been suggested to occur due to genomic instability as a consequence of aberrant DNA methylation patterns^{154,155}. Mutant *DNMT3a*^{R882} has been proposed to drive genome instability through defects in the detection of DNA torsional stress and chromosome remodelling in response to anthracyclines¹⁵⁴. Anthracyclines are a class of drug used for the treatment of cancer; they induce cell death primarily by reducing the ability of topoisomerase II to re-ligate double stranded breaks in the DNA. The authors stated that the presence of mutant *DNMT3a* in a range of human AML derived cell lines caused genomic instability, leading to increased mutagenesis and resistance to therapy. However, the evidence for this interpretation was weakened by the fact that the authors used a number of cell lines on different genetic backgrounds with different driver mutations, making it difficult to attribute genomic instability to mutant DNMT3a alone. Furthermore, the authors overexpressed the 'hotspot' *DNMT3a*^{R882} mutation in the Molm-13 cell line and concluded that the mutation caused an increase in DNA breaks. Whether endogenous levels of mutant DNMT3a could also increase DNA breaks remains

to be seen.

To test whether mutant DNMT3a results in an impaired DNA damage response and consequently affects the onset of cancer, as suggested by Guryanova, et al. ¹⁵⁴, we decided to use a model of γ -irradiation induced thymic lymphoma development. The murine γ -irradiation induced thymic lymphoma model was first described by Kaplan ¹⁹⁰. In this model, repeated low dose γ -irradiation was shown to start leukaemic transformation by acting on early HSPCs in the bone marrow. Experimentally, it was shown that the cancer cell of origin resided in the bone marrow because thymic lymphoma development could be prevented by shielding both femurs from γ -irradiation or by transplanting healthy haematopoietic cells into the γ -irradiated mice. It was concluded that the nascent leukemic cells must migrate from the bone marrow to the thymus where presumably acquisition of further genetic abnormalities cause a progression in lymphoma development. These results have been extended by Michalak, et al. ¹⁹¹, where it was shown that the development of thymic lymphoma is accelerated in mice with heterozygous loss of p53. p53 has been termed the 'guardian of the genome' as it can regulate key cellular processes like cell cycle, apoptosis and genome stability to prevent damaged cells to transform into malignant cells. The γ -irradiation induced DNA damage leads to the activation of the tumour suppressor p53 and this can induce expression of DNA repair proteins to fix the damage. Loss or mutation of the tumour suppressor p53 is found in around 50% of all human cancers ¹⁹². It was hypothesised that if mutant *Dnmt3a*^{R878H} caused genomic instability or impaired DNA repair then γ -irradiation would result in an increased acquisition of cancer-causing mutations in HSPCs and might thereby accelerate the development of thymic lymphoma compared to WT controls.

To test this hypothesis, *Dnmt3a*^{R878H/+} mice and WT littermates were given low dose (1.5 Gy) γ -irradiation, once per week from the age of four weeks for four weeks as outlined in Figure 5.1. Mice that displayed any signs of illness, such as increased respiration, pale feet (indicative of anaemia) or ruffled fur, were euthanised. Latency was determined from the date of the last dose of γ -irradiation until the ethical endpoint and hence sacrifice in days. Mice were said to have a thymic lymphoma if the thymus weight was >50 mg and/or a thymic T- cell population was present with abnormal CD4⁺ and CD8⁺ profile as determined by flow cytometry.

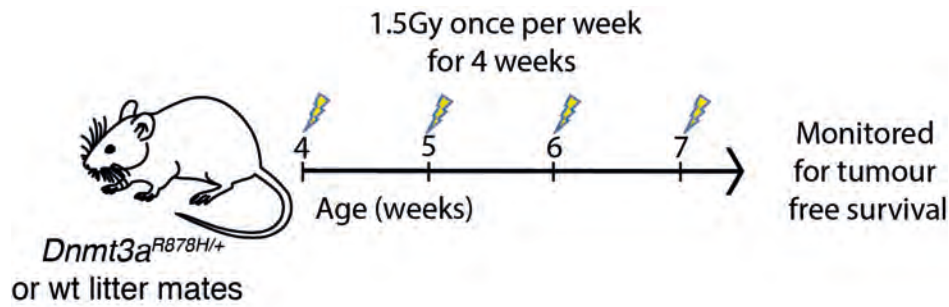


Figure 5.1 Schematic of Irradiation induced thymic lymphoma model

Dnmt3a^{R878H/+} or wt littermates were given low dose γ -irradiation (1.5 Gy), once per week for 4 weeks starting at 4 weeks of age. Mice were monitored for signs of illness, including increased respiration, pale feet and loss of condition. Mice were euthanised according to the animal ethics guidelines when this was deemed necessary by experienced animal technicians.

The development of thymic lymphoma was significantly accelerated in *Dnmt3a*^{R878H/+} mice compared to their wt littermates (Figure 5.2 A). *Dnmt3a*^{R878H/+} mice had a median survival of 92 days compared to 149 days for the wt mice (** $p < 0.0001$). At the time of sacrifice, the thymus and spleen were collected, and their weights are displayed in Figure 5.2 B-C. There were no significant differences in the thymus weights between the *Dnmt3a*^{R878H/+} and *Dnmt3a*^{+/+} control mice indicating that the tumour burden was similar between the different genotypes at sacrifice. There were no significant differences in the weights of the spleens but there was a trend towards higher spleen weights in the sick *Dnmt3a*^{R878H/+} mice.

Where possible, blood was collected at the time of sacrifice and the blood cell composition was determined by ADVIA and flow cytometric analysis. As shown in Figure 5.3, there were no significant differences in the blood cell composition of mice at the time of sacrifice between *Dnmt3a*^{R878H/+} and *Dnmt3a*^{+/+} mice with the exception of platelets that were significantly higher in the sick *Dnmt3a*^{R878H/+} mice (Figure 5.3 C). Unfortunately, it was not possible to get sufficient blood for ADVIA analysis from the majority of the mice in this experiment as the thymic lymphoma precluded scruffing of the mice to collect retro-orbital bleeds. Where possible, blood was collected via venous puncture of the heart. This was not an easy task as the heart was generally surrounded by thymic lymphoma mass in these mice and therefore the large amount

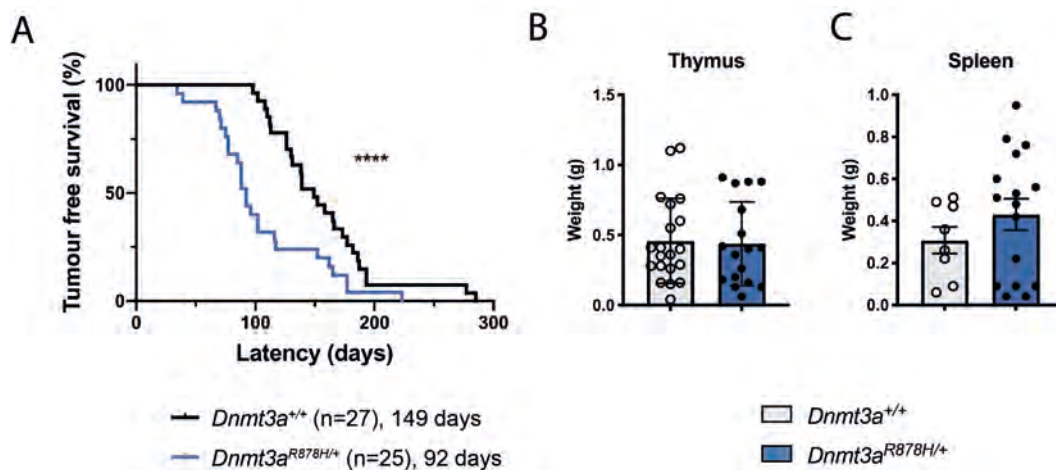


Figure 5.2 γ -radiation induced thymic lymphoma development is significantly accelerated in $Dnmt3a^{R878H/+}$ mice

A) Survival curves of $Dnmt3a^{R878H/+}$ mice and wt control littermates that had been treated with 4 weekly doses of 1.5 Gy γ -radiation starting at 4 weeks of age (latency shown from the last doses of γ -radiation). B) Thymus weight at ethical endpoint. C) Spleen weight at ethical endpoint. Data presented as mean $\pm$ SEM. Survival curves analysed using the Gehan-Breslow-Wilcoxon test and organ weights analysed by students t-test, ****p<0.0001.

of blood required for ADVIA analysis was very difficult to obtain. As a result, the higher platelet numbers in the $Dnmt3a^{R878H/+}$ mice might correspond with mice that had smaller thymic lymphoma burden and thus less advanced disease. A major limitation of this experiment is the difficulty in determining the correct time to sacrifice the mice. Thymic lymphoma can develop very quickly with the mass of the thymic lymphoma burden displacing the heart and lungs in the chest cavity. The thymic lymphoma can obstruct the lungs causing difficulty breathing, ultimately leading to suffocation if it is not picked up early enough. Mice will show signs of increased respiration due to the restriction on lung expansion as a result of the thymic lymphoma. Therefore, it is critical that animal technicians wait for signs of illness but do not wait until the mouse suffocates. Detecting increased respiration in mice is not easy as mice will often act as though they are well when being inspected. Given these difficulties it is possible that some of the mice were sacrificed too early, before thymic lymphoma had fully developed, but every attempt was made to exclude these mice on the basis of CD4 and CD8 expression profiles on

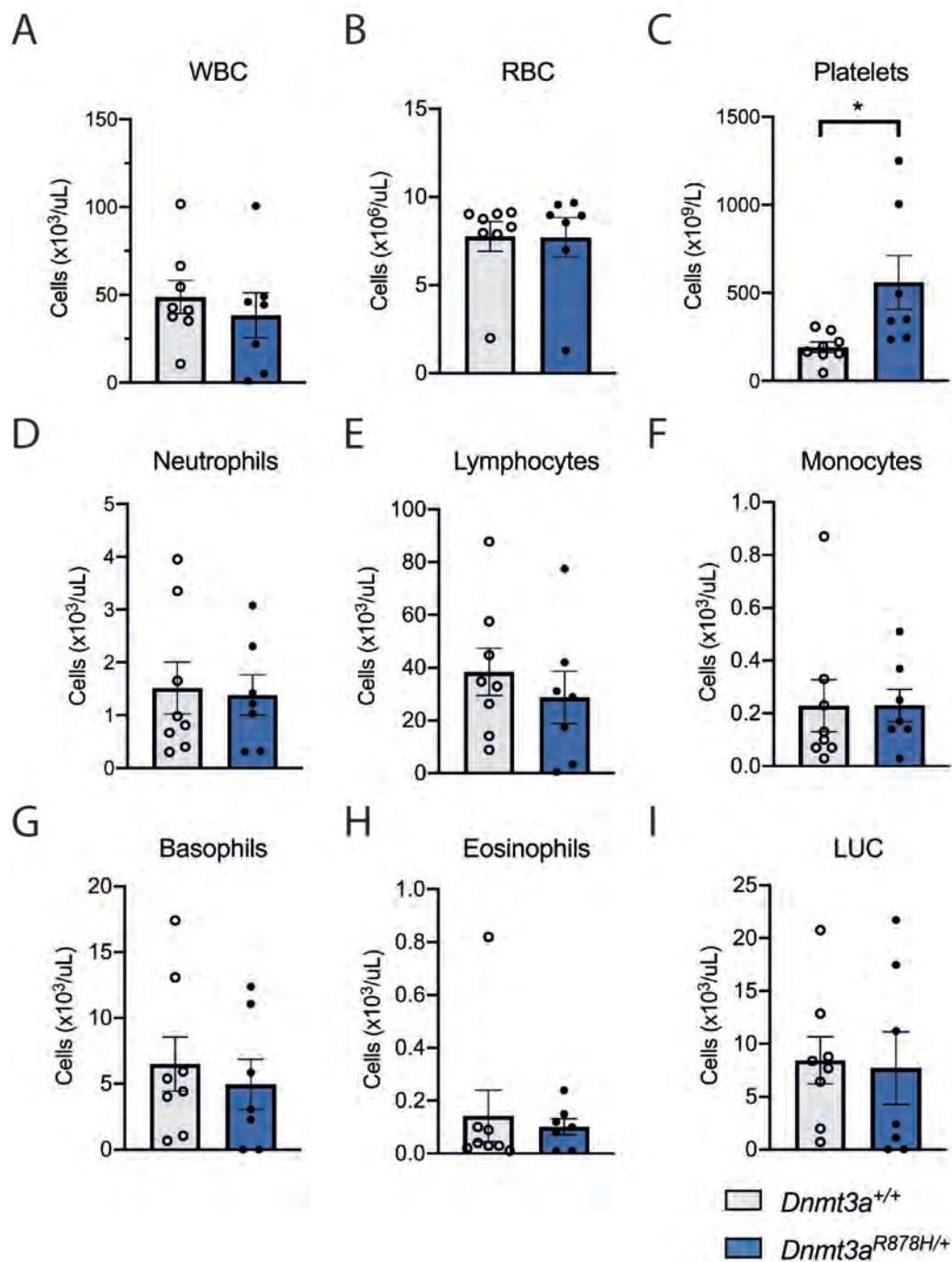


Figure 5.3 Increased white blood cell counts in mice with thymic lymphoma

Blood cell composition determined by ADVIA analysis. A) white blood cells; WBC, B) red blood cells; RBC, C) Platelets, D) neutrophils, E) lymphocytes, F) monocytes, G) basophils, H) eosinophils, I) large unstained cells, LUC. Graphs are presented as mean $\pm$ SEM, Students t test, *p<0.05.

cells from the thymus as determined by flow cytometry. Nevertheless, the ADVIA results overall showed that the blood cell composition of γ -irradiated *Dnmt3a*^{R878H/+} and wt mice was grossly abnormal compared to healthy mice (Chapter 4, Figure 4.1). WBC counts were ten-fold higher in the mice with thymic lymphoma compared to healthy control mice, although this increase occurred in both wt and *Dnmt3a*^{R878H/+} mice to the same extent. The blood cell composition results support the notion that both *Dnmt3a*^{R878H/+} mice and wt littermates had similar tumour burden at sacrifice.

To determine whether the presence of mutant *Dnmt3a* had any effect on the immunophenotype of the tumours, a subset of the thymic lymphomas was analysed by flow cytometry. Representative FACs plots are shown in Figure 5.4 A. There were no significant differences in Thy1 expression, a marker of T lymphoid cells found from the early progenitor cells all the way to the mature T cell stage), between *Dnmt3a*^{R878H/+} thymic lymphomas and WT controls (Figure 5.4 B). Intriguingly, *Dnmt3a*^{R878H/+} mice had a more consistent tumour phenotype characterised by double positive CD4/CD8 T cells with smearing into a CD8⁺ T cell phenotype. It was unclear why *Dnmt3a*^{R878H/+} thymic lymphoma cells were more likely to have a DP and CD8⁺ single positive T cells phenotype. One explanation is that Dnmt3a is involved in T cell fate decisions and that mutant Dnmt3a resulted in a bias towards CD8⁺ T cells. Indeed, Dnmt3a has previously been shown to be the major DNA methyltransferase that coordinated gene expression in T cells^{193,194}. Furthermore, knockdown of Dnmt3a in early thymic precursors favoured memory T cells differentiation over the production of effector T cells^{195,196}, highlighting that Dnmt3a function is important for normal T cell differentiation.

An alternative explanation arises from a recently identified subpopulation of single positive CD8 T cells. T cell maturation follows a well-defined pathway where immature DN cells mature to DP cells through acquisition of CD4 and CD8 expression. DP T cells then undergo selection to give rise to single positive CD4 and CD8 T cells. However, an immature single positive CD8 T cell that is molecularly distinct from mature CD8⁺ T cells has recently been described¹⁹⁷. These immature CD8⁺ T cells make up an intermediate population between DN and DP T cells. The differential gene signature between immature SP CD8⁺ T cells and more mature CD8⁺ T cells is driven by gene expression changes determined by bromodomain containing protein 4 (BRD4)¹⁹⁷. Therefore, it is possible that the increased frequency of CD8⁺ T cells in *Dnmt3a*^{R878H/+} tumours may in fact consist of immature CD8⁺ T cells arising from DN progenitors undergoing

development into DP thymocytes. This would be consistent with a defect in T lymphocyte development in the thymus of *Dnmt3a*^{R878H/+} mice which drives a more immature cancer phenotype. Indeed, as shown in Chapter 4, Figure 4.5, *Dnmt3a*^{R878H/+} mice have a developmental defect in DN thymocytes. While the impairment of T cell development at the DN stage can be overcome in healthy mice, it raises the possibility that this developmental defect drives an immature thymic lymphoma in the γ -irradiation induced thymic lymphoma model. In the future, it would be interesting to examine the CD8⁺ T cell molecular phenotype to determine whether these cells are indeed the molecularly distinct population of immature CD8⁺ T cells identified by Gegonne, et al.¹⁹⁷, or the more mature single positive CD8⁺ T cells.

Dnmt3a has been shown previously to be a haplo-insufficient tumour suppressor in CD8⁺ peripheral T cell lymphoma¹⁹⁸. In this study the authors generated mice that had heterozygous loss of *Dnmt3a* in HSPCs and this resulted in a CD8⁺ peripheral T cell lymphoma with a penetrance of around 12% when used to reconstitute lethally irradiated recipient mice. It was shown that homozygous loss of *Dnmt3a* resulted in genome wide hypomethylation in murine peripheral T cell lymphoma cells resulting in abnormal gene expression in these tumours. Furthermore, the authors were able to show that aberrant gene expression was conserved between murine and human peripheral T cell lymphoma samples, suggesting that the murine model system was reflective of the human malignant disease. Interestingly, murine peripheral T cell lymphomas with heterozygous or homozygous loss of *Dnmt3a* had downregulation of the p53 signalling pathway as determined by RNA expression of genes within this pathway. Reduced p53 signalling was also observed in pre-leukaemic T cells of mice at 9 months of age with heterozygous loss of *Dnmt3a*. Taken together, the work published by Haney, et al.¹⁹⁸ and the acceleration of γ -radiation induced thymic lymphoma development in the *Dnmt3a*^{R878H/+} mice reported here, suggest abnormal p53 signalling as a potential mechanism for accelerating lymphoma development in our model.

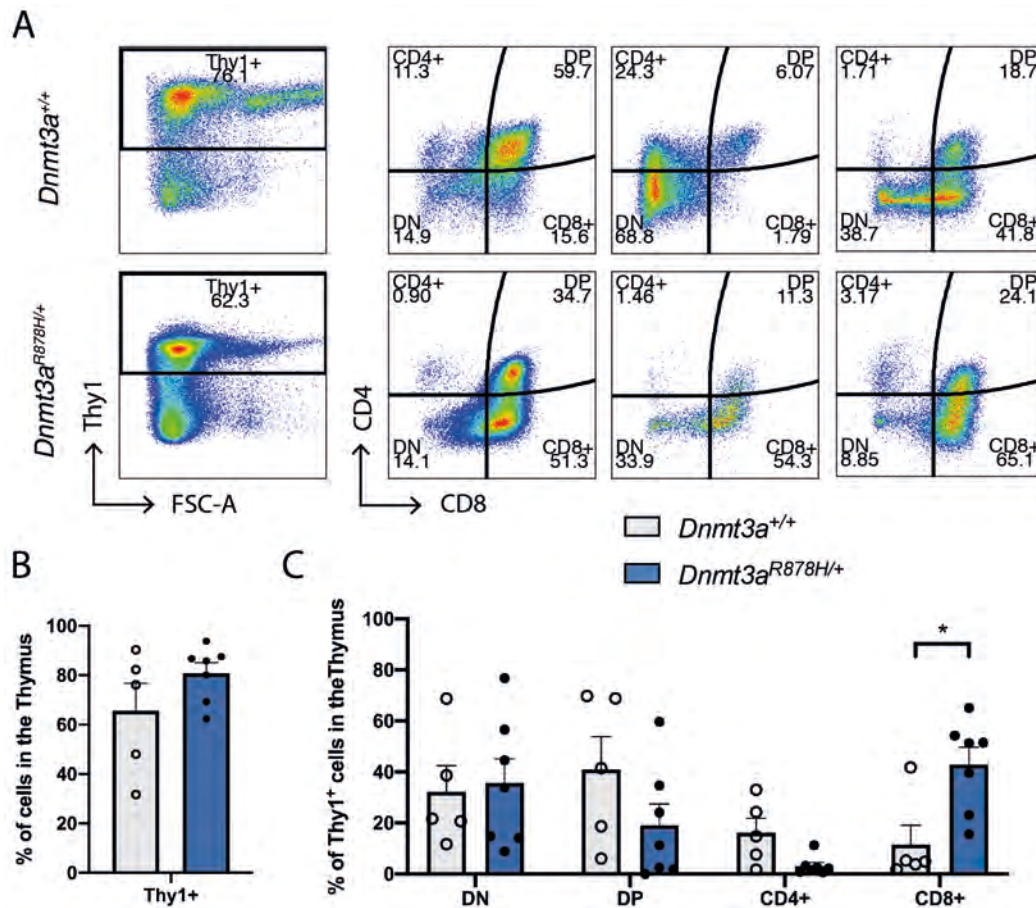


Figure 5.4 *Dnmt3a*^{R878H/+} thymic lymphomas are immunophenotypically more homogenous when compared to wt thymic lymphomas

A single cell suspension of cells from the thymus of tumour burdened mice was generated by gently passing the thymus through a nylon filter. The cells were then incubated with fluorochrome-conjugated antibodies against Thy-1, CD4 and CD8 and analysed by flow cytometry. A) Representative FACS plots of thymic lymphomas. B) percentages of Thy1⁺ cells in the thymus. C) CD4 and CD8 expression on thymic lymphomas. *Dnmt3a*^{R878H/+} lymphoma cells are shown in blue, *Dnmt3a*^{+/+} (wt) cells are shown in grey. Graphs are presented as mean±SEM, Students t test, *p<0.05.

5.3 *Dnmt3a*^{R878H/+} HSPCs have abnormal gene expression at baseline and in response to γ -irradiation

In the previous section it was established that *Dnmt3a*^{R878H/+} mice exhibited accelerated development of thymic lymphoma induced by repeated low dose γ -irradiation compared to wild-type controls. In this model, a haematopoietic stem/progenitor cell in the bone marrow is known to undergo the first steps towards malignant transformation before its descendants migrate into the thymus. The acceleration of thymic lymphoma development observed in *Dnmt3a*^{R878H/+} mice, the competitive advantage of *Dnmt3a*^{R878H/+} HSPCs in bone marrow reconstitution studies and the abnormal thymic T cell development in DNMT3a mutant mice revealed in chapter 4, led us to hypothesise that mutations in the epigenetic regulator DNMT3a caused dysregulated gene expression pattern in HSPCs.

To test this hypothesis, in an attempt to tease apart the underlying gene expression changes in HSPCs caused by mutations in DNMT3a, RNA sequencing (RNA-Seq) was performed on HSPCs from *Dnmt3a*^{R878H/+} mice and WT control littermates. Since we saw differences in the onset of thymic lymphoma development, gene expression was evaluated by RNA-Seq in *Dnmt3a*^{R878H/+} and WT HSPCs at baseline and in after treatment with γ -radiation. The experiment was set up as outlined in Figure 5.5. It was hypothesised that *Dnmt3a*^{R878H/+} HSPCs may have underlying changes in gene expression, but also may have an abnormal response to low dose γ -irradiation given that *Dnmt3a*^{R878H/+} mice were more susceptible to γ -irradiation induced thymic lymphoma development. In this model, mice receive low dose γ -irradiation once per week from the age of four weeks. Therefore, to match up the RNA sequencing with the γ -radiation induced thymic lymphoma development model, HSPCs were isolated from mice at four weeks of age (Figure 5.6 A). Litters were allocated into two groups; untreated (baseline) or γ -irradiated. Only female mice were used for this experiment to reduce confounding gender differences in the small sample size. The mice that were γ -irradiated received a single dose of 2 Gy and were then allowed to recover for 2 hours before being euthanised. Untreated mice were euthanised without any special treatment. To isolate LSK cells, the femurs and tibias of the mice were gently flushed to generate a single cell suspension of bone marrow cells. The bone marrow cells were then incubated with biotinylated antibodies against lineage specific surface markers, including TCR β , B220, CD19, Mac-1, Gr-1 and Ter119. Streptavidin conjugated magnetic beads were then added to the bone marrow samples prior to depletion of the unwanted cells using a magnet. Cells

expressing lineage markers bound by the biotin-conjugated antibodies will become attached to the streptavidin-coupled magnetic beads and physically pulled towards the magnet leaving only cells that do not express any of these lineage specific markers, including HSPCs, to flow through the column. The enriched cell populations were then stained with fluorochrome conjugated antibodies against c-KIT, SCA-1 and with streptavidin (to identify the non-depleted cells that had been stained with the biotin-conjugated lineage specific antibodies in the first step. Cell populations processed in this manner were then sorted to obtain Lin⁻SCA-1⁺c-KIT⁺ (LSK) cell populations, which contain MPPs, ST-HSCs and LT-HSCs. This cell sorting was repeated on as many bone marrow samples until sufficient numbers of LSKs from untreated and γ -irradiated *Dnmt3a*^{R878H/+} and wt control mice had been obtained.

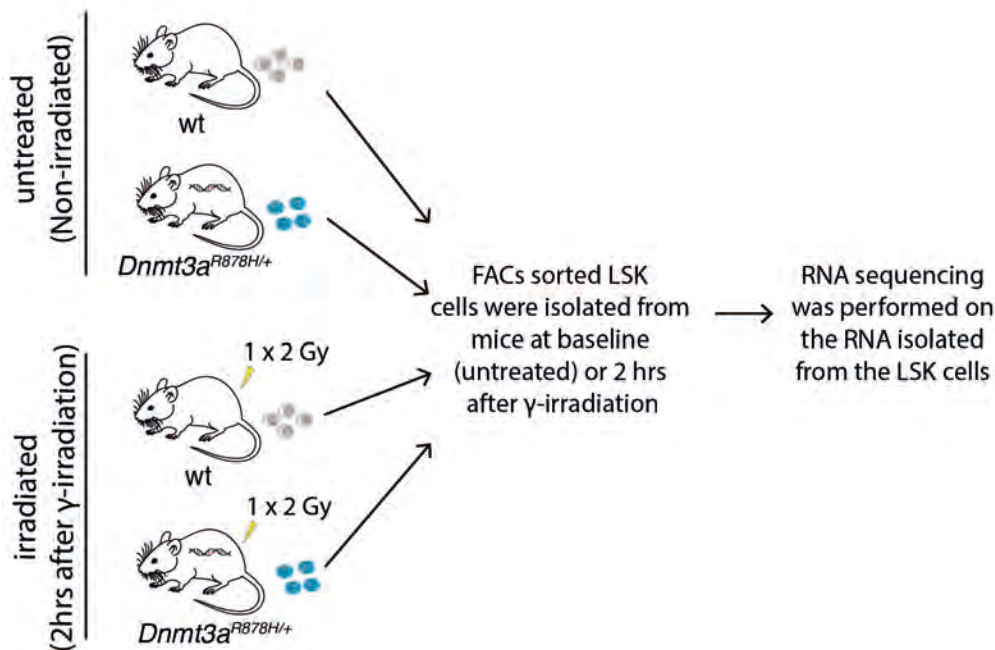


Figure 5.5 Outline of the experimental approach of the RNA-Seq experiment

Bone marrow was harvested from age-matched female *Dnmt3a*^{R878H/+} mice or female wt littermates. The mice were either left untreated or subjected to whole-body γ -irradiation at a dose of 2 Gy. The γ -irradiated mice were allowed to recover for 2 hours before their bone marrow was harvested. LSK (lineage marker- SCA-1⁺ c-KIT⁺) cells were sorted by FACS from bone marrow as described in the text. RNA was isolated from the sorted LSK cell populations and then sequenced using the Illumina platform.

Total RNA was isolated from sorted LSK samples from 4 groups of mice; untreated wt mice (n=4), untreated *Dnmt3a*^{R878H/+} mice (n=4), γ -irradiated wt mice (n=3) and γ -irradiated *Dnmt3a*^{R878H/+} mice (n=3). One of the samples from the γ -irradiated wt group had to be removed from the analysis as the resulting sequences could not be mapped sufficiently to the mouse genome.

As outlined in Figure 5.5, the experiment was designed to test two hypotheses. Firstly, that *Dnmt3a*^{R878H/+} LSK cells have underlying changes in gene expression (compared to wt LSK cells), most likely due to epigenetic dysregulation, that would reveal pathways that might explain the increased *Dnmt3a*^{R878H/+} stem cell fitness described in chapter 4. Secondly, it was hypothesised that *Dnmt3a*^{R878H/+} LSK cells would have an abnormal gene expression signature after treatment with γ -irradiation which would provide clues as to why γ -radiation induced thymic lymphoma development was significantly accelerated in *Dnmt3a*^{R878H/+} mice. To determine the time point after a single dose of γ -irradiation at which RNA should be isolated for RNAseq analysis, LSK cells were FACS sorted at different time points to identify the window where sufficient gene expression changes could be expected without too much cell death that would result in too little RNA being extracted for sequencing. As shown in Figure 5.6 B, very few LSK cells could be detected by flow cytometry 6 hours after γ -irradiation of mice due to substantial cell death. Therefore, a shorter 2-hour time point was investigated in an attempt to capture the LSK cells before they had undergone or even initiated cell death. The number of LSK cells obtained from cell sorting 2 hours after γ -irradiation demonstrated that they would be sufficient for RNA isolation (Figure 5.6 C). Low dose γ -irradiation has previously been shown to induce rapid changes in gene expression in human lymphocytes after just 1 hour¹⁹⁹. Hence, the 2 hour time point was deemed appropriate to determine the impact of the *Dnmt3a*^{R878H} mutation on the response of LSK cells to γ -irradiation.

5.3.1 *Dnmt3a*^{R878H/+} HSPCs demonstrate a blunted p53 response after treatment with γ -irradiation.

Ionising radiation causes double stranded DNA breaks in cells²⁰⁰. DNA damage is sensed and then repaired by a complex pathway involving ATM, CH1, CHK2 and p53 among other regulators depicted in Figure 5.7. The master regulator of this pathway is the tumour suppressor protein p53. P53 is negatively regulated by MDM2, an E3 ligase which ubiquitinates p53 and thereby marks it for degradation by the proteasome. In response to cellular stressors, like DNA damage,

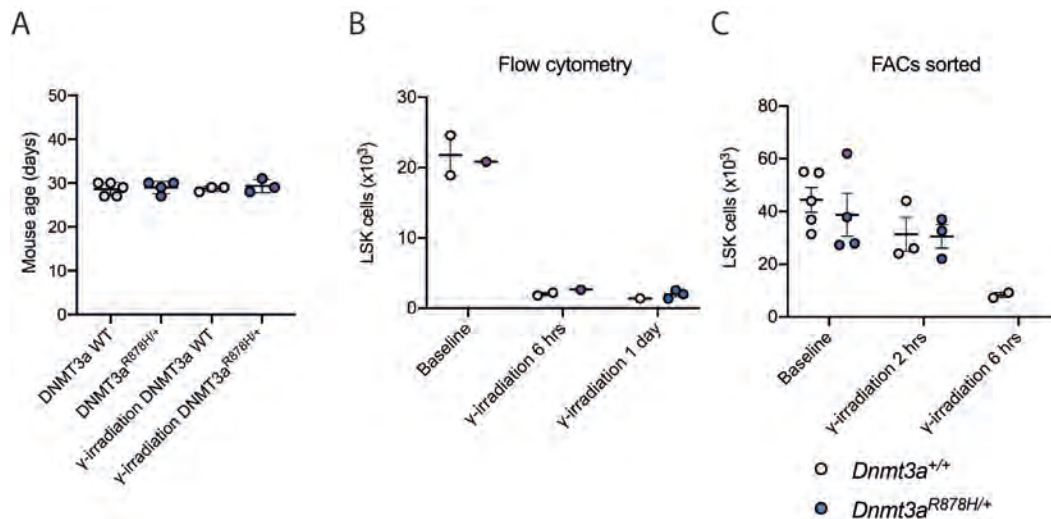


Figure 5.6 Optimisation of RNA-Seq timepoints

Determination of the numbers of LSK cells that could be isolated from 4 week-old mice that had been left untreated or had received a single 2 Gy dose of whole body γ -irradiation and were then allowed to recover for the specified time before being euthanised and the isolation of their LSK (Lin⁻SCA-1⁺c-KIT⁺) cells by FACS. A) Representation of the ages of the mice used for this experiment. B) Numbers of LSK cells obtained by FACS sorting from the bone marrow (2 femurs per mouse) from untreated mice (baseline) or mice 6 hours or 1 day after treatment with 2 Gy γ -irradiation. C) Numbers of LSK cells obtained by FACS sorting from the bone marrow (2 femurs plus 2 tibiae per mouse) from untreated mice (baseline) or mice 2 or 6 hours after treatment with 2 Gy γ -irradiation. Graphs presented as mean $\pm$ SEM.

reactive oxygen species (ROS) or oncogene activation, the p53 signalling pathway is induced by inactivation of MDM2 and post-translational modifications of p53 to enable it to bind to its specific recognition sites in the promoters of its target genes. Around 500 genes are thought to contain p53 binding sites and many more are thought to be indirectly regulated by p53 activation^{201,202}. These vast networks of genes can control a broad range of cellular responses, including apoptosis, cell senescence, cell cycle arrest, cell metabolism and the coordination of DNA repair damage repair, among others²⁰³.

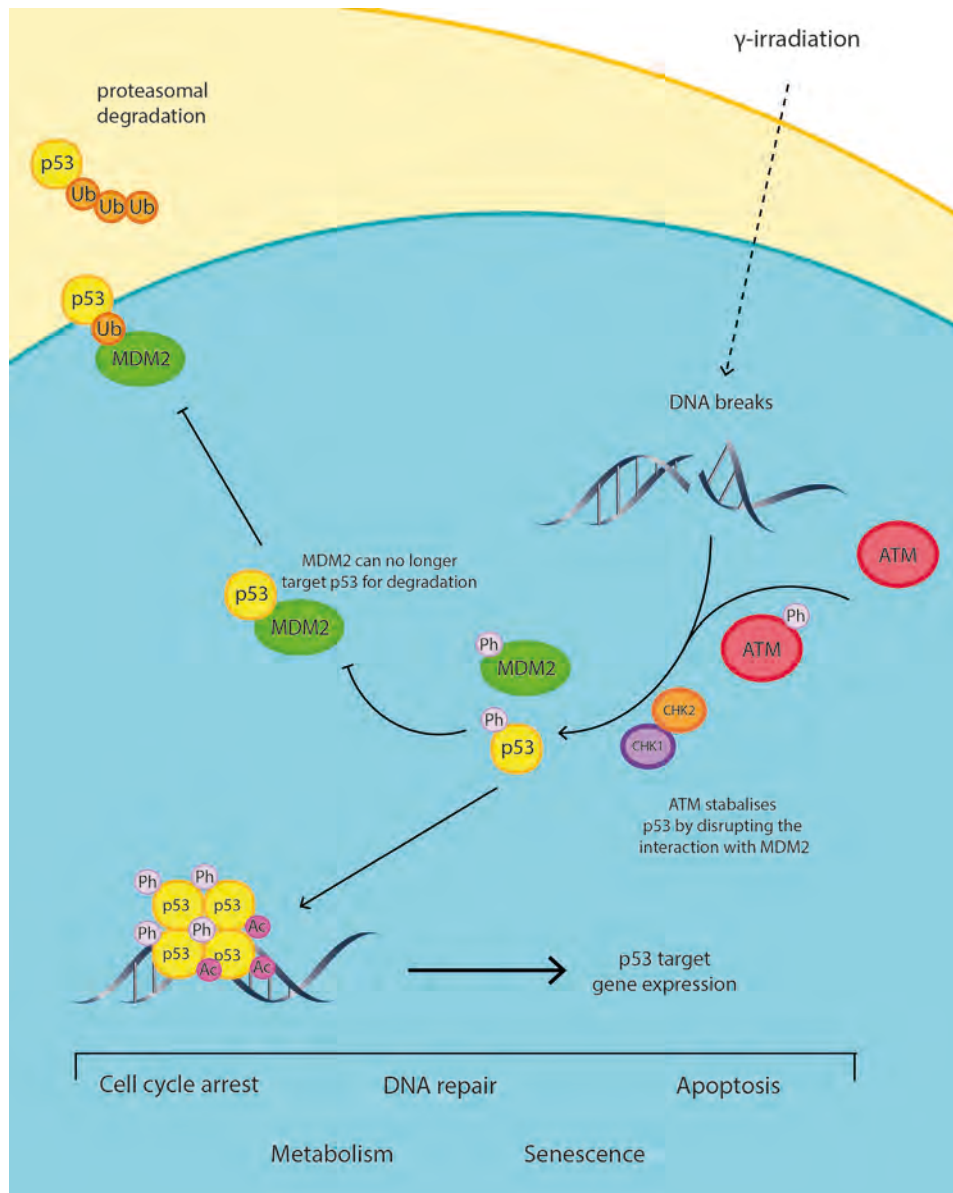


Figure 5.7 Schematic of p53 signalling in response to γ -irradiation

Schematic representing the effects of γ -irradiation on p53 signalling in cells. γ -irradiation induced double stranded DNA breaks are detected by ATM which is activated by phosphorylation. Through interactions with CHK1, MDM2 is phosphorylated causing destabilisation of the p53/MDM2 complex. The blockade of the p53-MDM2 interaction prevents the ubiquitination and proteasomal degradation of p53. The liberated p53 can accumulate in the nucleus. Further modifications of p53 through Acetylation facilitate binding of a p53 tetramer to specific sites in DNA to induce the activation of p53 target genes that regulate a number of cellular responses, such as apoptotic cell death or cell cycle arrest. Phosphorylation; Ph, Acetylation; Ac, Ubiquitination; Ub.

Gene set enrichment analysis was performed on the RNA sequencing data to determine pathways that were affected by the *Dnmt3a*^{R878H} mutation. Given that p53 signalling is crucial to the cellular response to DNA damage that results from γ -irradiation, we first examined this pathway as an internal validation that gene expression changes could occur within 2 hours following such treatment. It was reassuring to see that the p53 signalling pathway (KEGG mmu04115) was significantly enriched in the γ -irradiated LSK cells derived from both wt and *Dnmt3a*^{R878H/+} mice. The enrichment of the p53 signalling pathway in LSK cells upon γ -irradiation acted as an internal validation that within 2 hours of such treatment, changes in gene expression as measured by RNA do occur. As expected, there was no enrichment of the p53 signalling pathway in wt and *Dnmt3a*^{R878H/+} LSK cells at baseline (Figure 5.8 A), reflecting normal basal p53 signalling in *Dnmt3a*^{R878H/+} LSK cells. As described above, p53 was induced upon γ -irradiation in both wt and *Dnmt3a*^{R878H/+} LSK cells, which is presented in Figure 5.8 C-D barcode plots. Strikingly, when the enrichment of this pathway was compared between γ -irradiated wt and γ -irradiated *Dnmt3a*^{R878H/+} LSK cells it became apparent that activation of this pathway was significantly weaker in *Dnmt3a*^{R878H/+} LSK cells (Figure 5.8 B).

The dampened induction of the p53 signalling pathway in γ -irradiated *Dnmt3a*^{R878H/+} LSK cells was also visualised in a heatmap of individual genes functioning in this pathway (Figure 5.9). The first two columns of the heatmap, depicting baseline p53 signalling pathway genes in *Dnmt3a*^{R878H/+} and wt LSK cells, were quite similar (Figure 5.9). In contrast, it was clear that a number of genes were less highly induced in the γ -irradiated *Dnmt3a*^{R878H/+} LSK samples (fourth column) compared to the γ -irradiated LSK cells (third column). Despite the induction of the p53 pathway being significantly lower in *Dnmt3a*^{R878H/+} LSK cells in response to γ -irradiation, none of the genes were independently significantly differentially expressed compared to γ -irradiated wt LSK cells (data not shown). Significant changes in the expression of individual genes may have been observed at a later time point. It is possible that at 2 hours post γ -irradiated, changes in RNA expression were only just beginning to occur and that this time point had only captured the beginning of gene expression changes. Nevertheless, the diminished activation of the p53 pathway would be expected to have notable consequences for the cells, for example if DNA repair was not induced effectively. Interestingly, *Trp53* RNA did not appear to increase much in *Dnmt3a*^{R878H/+} LSK cells upon γ -irradiation when expressed as a z-score (Figure 5.9). This indicated that *Dnmt3a*^{R878H/+} LSK cells may be more prone to malignant transformation due to a

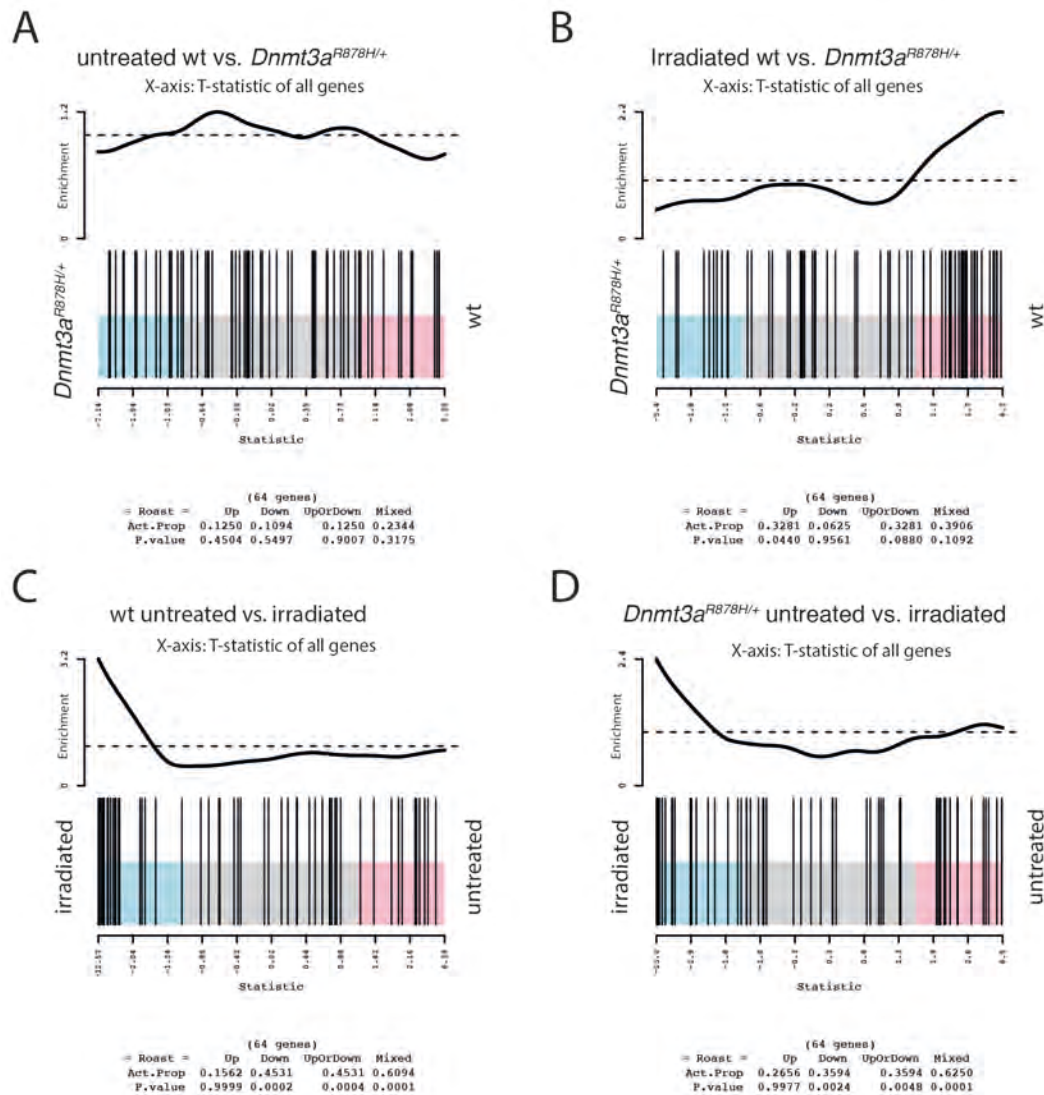


Figure 5.8 Compared to wt LSK cells *Dnmt3a*^{R878H/+} LSK cells display diminished induction of p53 signalling in response to γ -irradiation

Barcode plots showing enrichment of genes in the p53 signalling pathway (KEGG pathway mmu04115) in A) untreated *Dnmt3a*^{+/+} (wt) LSK cells vs untreated *Dnmt3a*^{R878H/+} LSK cells, B) γ -irradiated *Dnmt3a*^{+/+} LSK cells vs γ -irradiated *Dnmt3a*^{R878H/+} LSK cells, C) untreated *Dnmt3a*^{+/+} (wt) LSK cells vs γ -irradiated *Dnmt3a*^{+/+} (wt) LSK cells and D) untreated *Dnmt3a*^{R878H/+} LSK cells vs γ -irradiated *Dnmt3a*^{R878H/+} LSK cells. Red bars indicate up-regulated genes and blue bars depict down-regulated genes. The worms above the barcode plots show relative enrichment score for the genes in the gene set. $p < 0.05$ was considered significant.

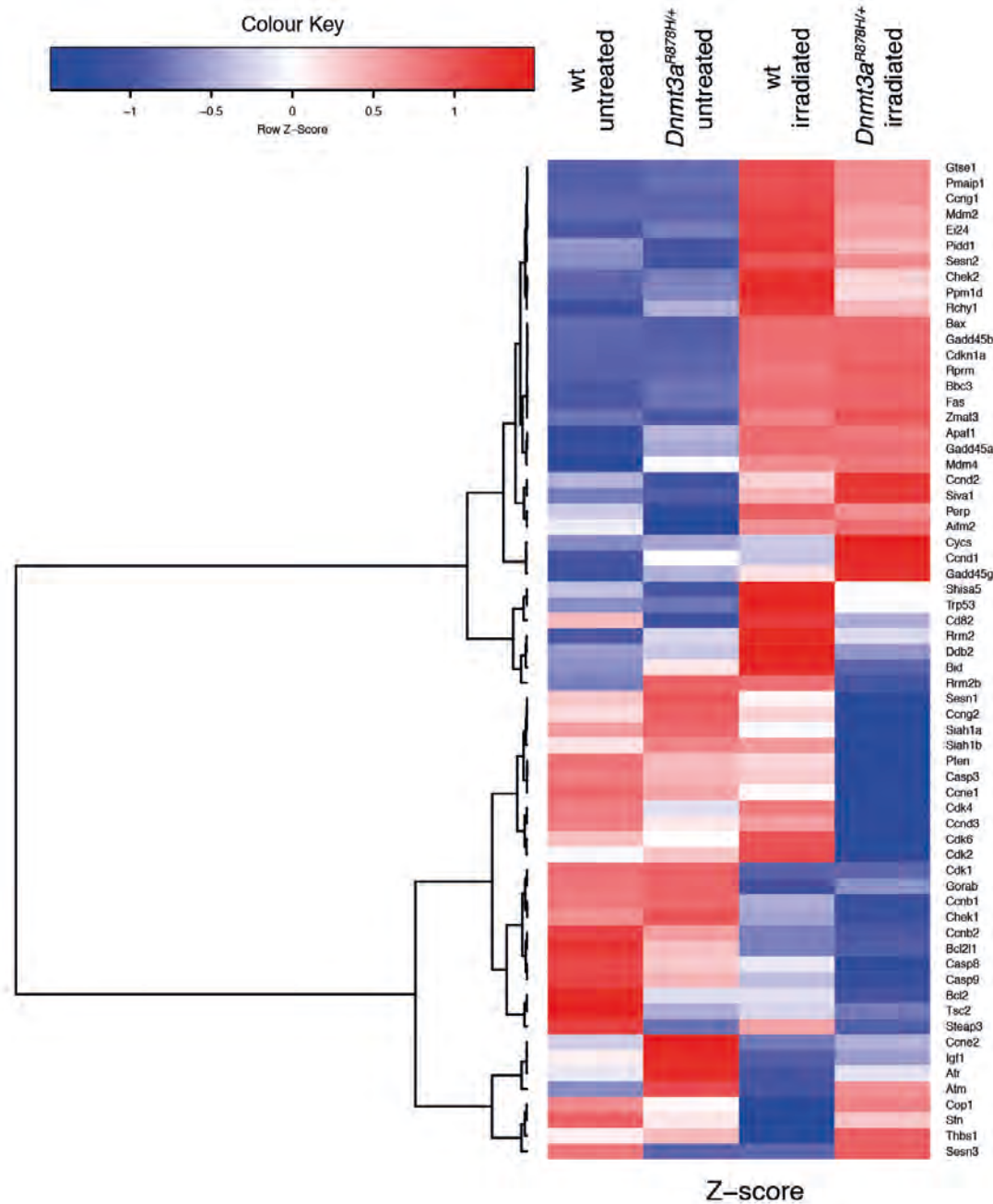


Figure 5.9 p53 signalling pathway RNA-Seq heatmap

Heatmap of RNA-Seq expression z scores of genes in the p53 signalling pathway (KEGG pathway mmu04115) between untreated WT LSK cells (column 1), untreated *Dnmt3a^{R878H/+}* LSK cells (column 2), γ -irradiated wt LSK cells (column 3) and γ -irradiated *Dnmt3a^{R878H/+}* LSK cells (column 4).

reduction in p53 itself, resulting in down-regulation of the entire pathway with possible implications for downstream genes transcriptionally regulated by p53. To visualise *Trp53* RNA in more detail, RNA tracks were generated for the *Trp53* gene (Figure 5.10). As shown in Figure 5.10 A, there was no difference in the *Trp53* RNA expression in untreated LSK cells between the wt and *Dnmt3a*^{R878H/+} samples. However, upon γ -irradiation there was a clear increase in *Trp53* expression in wt LSK cells (Figure 5.10 B) compared to baseline expression (figure 5.10 A). However, there was no induction of *Trp53* mRNA in the γ -irradiated *Dnmt3a*^{R878H/+} LSK cells compared to their untreated counterparts.

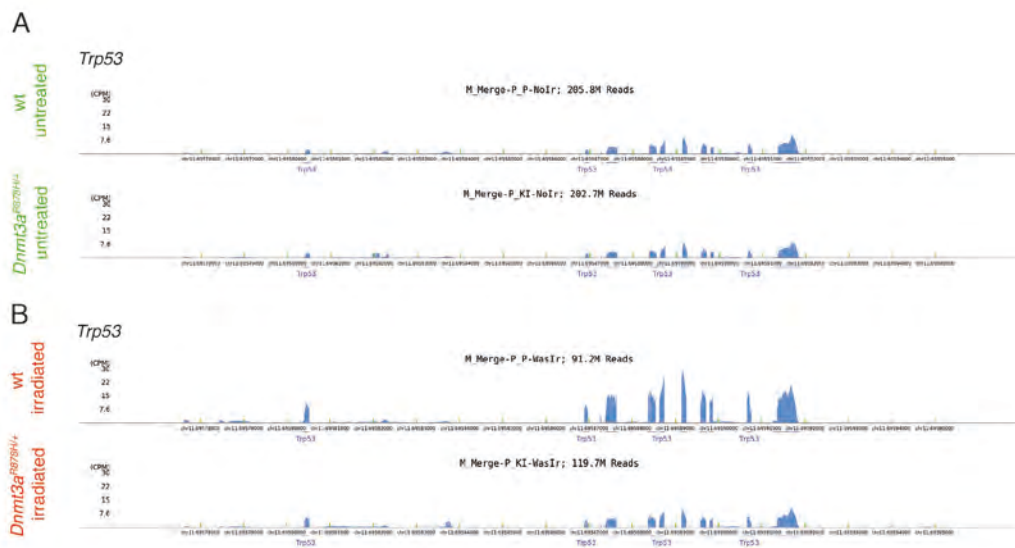


Figure 5.10 *Dnmt3a*^{R878H/+} LSK cells do not upregulate *Trp53* RNA in response to γ -irradiation

RNA was isolated from wt or *Dnmt3a*^{R878H/+} FACs sorted LSK cells at baseline (untreated) or 2 hours after low dose (2 Gy) whole body γ -irradiation. RNA tracks show sequencing coverage of the *Trp53* gene. Coding regions are depicted by horizontal blue lines below the tracks. *Trp53* RNA expression is displayed as counts per million (CPM) on the left axis. A) *Trp53* RNA tracks derived from untreated wt LSK cells and untreated *Dnmt3a*^{R878H/+} LSK cells are highlighted in green. B) *Trp53* RNA tracks derived from γ -irradiated wt LSK cells and γ -irradiated *Dnmt3a*^{R878H/+} LSK cells are highlighted in red.

The *Trp53* mRNA expression did not appear to change from baseline upon γ -irradiation in *Dnmt3a*^{R878H/+} LSK cells. Despite these visually convincing differences in *Trp53* expression between γ -irradiated wt LSK cells and γ -irradiated *Dnmt3a*^{R878H/+} LSK cells, *Trp53* did not come up as significantly differentially expressed in our analysis. One possible explanation is that there was high variability between individual samples within a genotype or treatment, which needs to be explored further as individual sample (mouse) differences were not examined in detail. Another possibility is that the difference in *Trp53* mRNA expression at 2 hours after γ -irradiation is too small and too variable. There is a clear trend towards reduced *Trp53* mRNA expression in *Dnmt3a*^{R878H/+} LSK cells at 2 hours post γ -irradiation compared to γ -irradiated wt LSK cells and this difference may become more robust at later time points, something that will be explored further in the future.

To determine whether *Trp53* activated target genes could be transcriptionally activated upon γ -irradiation in *Dnmt3a*^{R878H/+} LSK cells despite lower *Trp53* mRNA levels, two targets were examined in closer detail. The cyclin dependent kinase inhibitor 1a (*Cdkn1a*) and BCL-2 binding component 3 (*Bbc3* also called *Puma*) are both transcriptionally activated in response to p53. *Cdkn1a* encodes a protein called p21 that is a key regulator of cell cycle progression in the G1 phase²⁰⁴. The expression of p21 can be induced in both p53-dependent and p53-independent manners. In response to cellular stressors, like γ -irradiation, p21 is activated in a p53-dependent manner resulting in cell cycle arrest at the G1/S boundary. As shown in Figure 5.9, there was a strong induction of *Cdkn1a* expression upon γ -irradiation in both the *Dnmt3a*^{R878H/+} and WT LSK cells when expressed as z-scores. To visualise the RNA expression profiles, RNA tracks were generated. As shown in Figure 5.11 A, there was no detectable *Cdkn1a* expression in non-irradiated LSK cells from WT or *Dnmt3a*^{R878H/+} mice. The absence of baseline *Cdkn1a* expression was expected as these are unstressed cells and therefore basal p53 signalling would be very low. In contrast, there was a strong induction of *Cdkn1a* expression in both WT and *Dnmt3a*^{R878H/+} LSK cells in response to γ -irradiation (Figure 5.11 B). *Cdkn1a* mRNA expression was higher in *Dnmt3a*^{R878H/+} γ -irradiated LSK cells compared to wt cells. Since *Trp53* expression was blunted in *Dnmt3a*^{R878H/+} LSK cells in response to γ -irradiation but *Cdkn1a* expression was increased to a similar extent as that seen in γ -irradiated wt LSK cells, this raises the question as to how *Cdkn1a* expression was up-regulated. *Trp53* mRNA does not necessarily reflect p53 protein levels and therefore despite lower *Trp53* mRNA there may still be sufficient protein to activate *Cdkn1a* in

response to γ -irradiation. Measuring the relative abundance of these proteins in untreated and γ -irradiated LSK cells will need to be performed in the future. Interestingly, DNMT3a has been shown to form a co-repressive complex with c-MYC at the *Cdkn1a* promotor to reduce expression of *Cdkn1a* ²⁰⁵. Therefore, reduced or abnormal *Cdkn1a* promotor methylation as a result of mutant *Dnmt3a*^{R878H} could provide another explanation for the up-regulation of *Cdkn1a* expression observed in the γ -irradiated *Dnmt3a*^{R878H/+} LSK cells.

It is unclear whether the increased *Cdkn1a* RNA expression in *Dnmt3a*^{R878H/+} LSK cells could contribute to the accelerated γ -irradiation induced thymic lymphoma development that was observed in the *Dnmt3a*^{R878H/+} mice. Of note, the activity of p21 cannot be inferred from RNA expression alone since p21 can undergo post-translational modifications that alter its activity in regulating the cyclin dependent kinases. The ability of p21 to induce cell cycle arrest is dependent on a wide array of protein-protein interactions. The activity of p21 is therefore highly dependent on subcellular localisation of p21 protein to facilitate its binding to other proteins ²⁰⁶. Post-translational modifications of p21, including phosphorylation, have been reported to stabilise the protein in the cytosol and prevent its translocation into the nucleus. Therefore, further characterisation of p21 activity in *Dnmt3a*^{R878H/+} cells would be required to understand whether the increase in *Cdkn1a* RNA upon γ -irradiation was physiologically meaningful. Performing cell cycle analysis in untreated and γ -irradiated *Dnmt3a*^{R878H/+} and wt LSK cells would be interesting to do in the future.

Bbc3 also known as *Puma* is a transcriptional target of p53. When the p53 signalling pathway is initiated, *Puma* is transcriptionally activated resulting in an increase in PUMA protein ²⁰⁷. PUMA is a pro-apoptotic BH3-only member of the BCL-2 protein family that can bind to and inhibit the activity of pro-survival BCL-2 family members. In normal cells, the expression of pro-survival and pro-apoptotic BCL-2-family members is tightly regulated to ensure healthy cells survive and damaged cells undergo cell death. Increased expression of PUMA in response to p53 activation can swing the balance of a cell in favour of apoptotic cell death over cell survival thereby ensuring that damaged cells are killed rather than dividing with potentially oncogenic mutations. There were not any observable differences in *Puma* mRNA expression between *Dnmt3a*^{R878H/+} and wt LSK cells at baseline or in response to γ -irradiation (Figure 5.12).

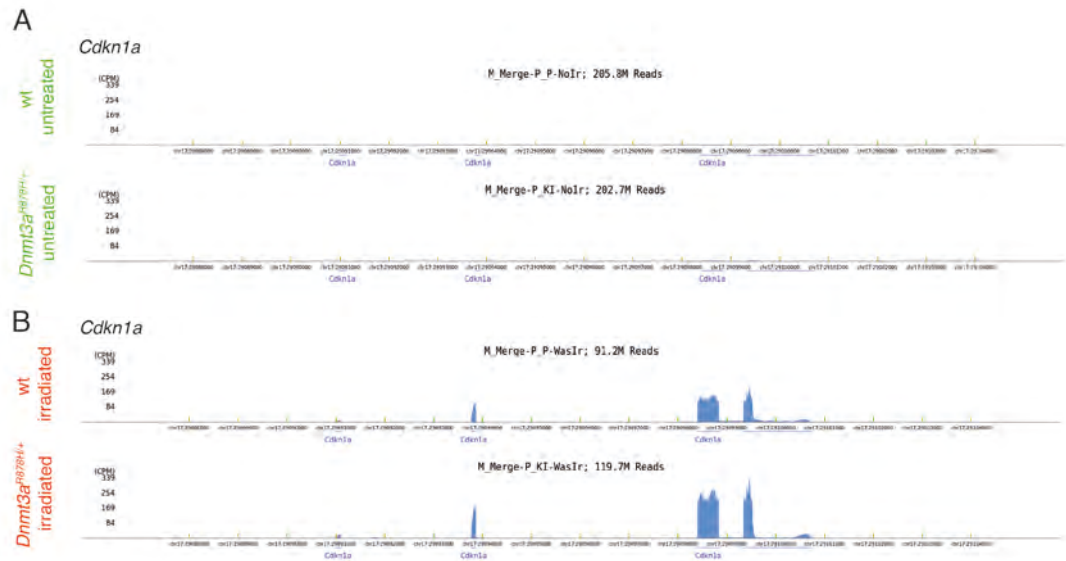


Figure 5.11 *Dnmt3a^{R878H/+}* LSK cells have a stronger *Cdkn1a* (p21) induction in response to γ -irradiation compared to wt LSK cells

RNA was isolated from wt or *Dnmt3a^{R878H/+}* LSK cells at baseline (untreated, i.e. non-irradiated) or 2 hours after low dose (2 Gy) whole body γ -irradiation. RNA tracks show sequencing coverage of the *Cdkn1a* gene. Coding regions are depicted by horizontal blue lines below the tracks. *Cdkn1a* mRNA expression is displayed as counts per million (CPM) on the left axis. A) *Cdkn1a* mRNA tracks derived from untreated wt and *Dnmt3a^{R878H/+}* LSK cells are highlighted in green. B) *Cdkn1a* mRNA tracks derived from γ -irradiated wt and *Dnmt3a^{R878H/+}* LSK cells are highlighted in red.

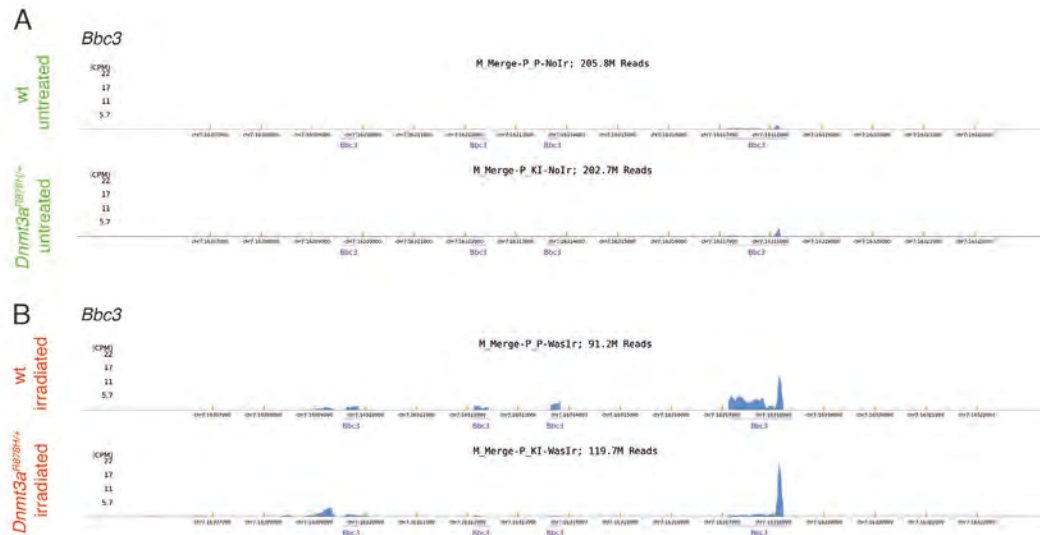


Figure 5.12 There are no differences in *Puma (Bbc3)* mRNA expression in *Dnmt3a^{R878H/+}* LSK cells in response to γ -irradiation

RNA was isolated from wt or *Dnmt3a^{R878H/+}* LSK cells at baseline (untreated, non-irradiated) or 2 hours after low dose (2 Gy) whole body γ -irradiation. RNA tracks show sequencing coverage of the *Puma (Bbc3)* gene. Coding regions are depicted by horizontal blue lines below the tracks. *Puma (Bbc3)* RNA expression is displayed as counts per million (CPM) on the left axis. A) *Puma (Bbc3)* mRNA tracks derived from untreated (non-irradiated) wt and *Dnmt3a^{R878H/+}* LSK cells are highlighted in green. B) *Puma (Bbc3)* mRNA tracks derived from γ -irradiated wt and *Dnmt3a^{R878H/+}* LSK cells are highlighted in red.

As mentioned previously, both *Puma (Bbc3)* and *Cdkn1a* are transcriptionally regulated by p53^{207,208}. Neither of these genes were independently significantly differentially expressed when comparing γ -irradiated *Dnmt3a^{R878H/+}* with γ -irradiated wt LSK cells. It was interesting that there tended to be higher *Cdkn1a* mRNA levels in γ -irradiated *Dnmt3a^{R878H/+}* LSK cells although *Dnmt3a^{R878H/+}* LSK cells had lower p53 expression. In the future it will be essential to explore which components of the p53 pathway are critical for the acceleration of γ -irradiation induced thymic lymphoma development. Given that none of the genes in the p53 signalling pathway were individually significantly differentially expressed between *Dnmt3a^{R878H/+}* and wt LSK cells upon γ -irradiation, it is likely that the shorter latency of γ -irradiation induced thymic lymphoma

development in *Dnmt3a*^{R878H/+} mice results from an overall reduction in the activity of the p53 signalling pathway.

5.3.2 Validation of mutant *Dnmt3a* as a driver of abnormal p53 signalling in vivo

Dnmt3a^{R878H/+} LSK cells were unable to induce p53 signalling in response to γ -irradiation to the same extent as wt LSK cells. I hypothesised that impaired p53 signalling in *Dnmt3a*^{R878H/+} LSK cells was a mechanism that was responsible (at least in part) for the accelerated development of γ -irradiation induced thymic lymphoma that was observed in the *Dnmt3a*^{R878H/+} mice. P53 mutations are found in approximately 50% of human cancers highlighting that its functioning is essential for tumour suppression.

Relevant to the acceleration of γ -irradiation induced thymic lymphoma development by mutant *Dnmt3a*, it was revealed that p53 signalling was blunted in *Dnmt3a*^{R878H/+} LSK cells in response to the stress induced by γ -irradiation. It was hypothesised that if mutant *Dnmt3a*^{R878H} induced aberrant methylation of p53 target genes, then concurrent genetic loss of p53 would not accelerate γ -irradiation induced thymic lymphoma development in *Dnmt3a*^{R878H/+} mice. In support of this hypothesis it should be noted that concomitant *Dnmt3a*^{R882} and p53 mutations are thought to be mutually exclusive in human cancers. In the TCGA database, p53 mutations were not reported in any patients with *DNMT3a* 'hotspot' mutated cancers, although there were a few examples of patients with p53 mutations and non-hotspot *Dnmt3a* mutations. Of the patients with p53 mutations, almost 3% also had a mutation in DNMT3a suggesting that the likelihood of having both mutations is extremely low ⁸.

In an attempt to determine whether heterozygous mutant *Dnmt3a*^{R878H} and loss of one allele of p53 could cooperate to accelerate tumour development, *Dnmt3a*^{R878H/+} male mice were inter-crossed with female p53^{+/-} mice to generate *Dnmt3a*^{R878H/+}/Trp53^{+/-} mice. The results of these crosses are presented in Table 5. As described in Chapter 3, *Dnmt3a*^{R878H/+} female mice could not be used for breeding. Therefore, to generate *Dnmt3a*^{R878H/+}/Trp53^{+/-} pups, male *Dnmt3a*^{R878H/+} mice were inter-crossed with female Trp53^{+/-} mice. These crosses were not particularly efficient in producing the desired *Dnmt3a*^{R878H/+}/Trp53^{+/-} offspring with only 25% of pups predicted to have this genotype based on Mendelian ratios. As shown in Table 5, the desired *Dnmt3a*^{R878H/+}/Trp53^{+/-} offspring were born significantly less frequently than predicted (born

17% vs expected 25% $p=0.0002$). $Dnmt3a^{R878H/+}/Trp53^{+/-}$ were also born less frequently than expected (born 12% vs expected 25%). The lower frequencies of mice carrying the $Dnmt3a^{R878H}$ allele was consistent with previous observations outlined in Chapter 3, where inter-crosses of $Dnmt3a^{R878H/+}$ with wt mice produced fewer $Dnmt3a^{R878H/+}$ pups than wt littermates (born 40% vs expected 50%). Therefore, it is likely that the low frequency of pups born that were $Dnmt3a^{R878H/+}/Trp53^{+/-}$ is likely due to the $Dnmt3a^{R878H}$ allele rather than an effect of the loss of one allele of *Trp53* or a compound effect.

Table 5.1 Offspring statistics from $Dnmt3a^{R878H/+}$ with $Trp53^{+/-}$ inter-crosses

<i>Trp53</i> <i>Dnmt3a</i>	+/+ +/+	+/- +/+	+/- R878H/+	+/ R878H/+	n	X2	p-value
Expected proportion	25%	25%	25%	25%			
Actual offspring # (%)	43 (36)	42 (34)	21 (17)	15 (12)	121	19.645	0.0002
Male: Female (%)	60:40	52:48	67:33	40:60			

To determine whether heterozygous mutant $Dnmt3a^{R878H}$ could cooperate with the loss of a single allele of *Trp53* to accelerate tumour development, the $Dnmt3a^{R878H/+}/Trp53^{+/-}$ mice were aged alongside $Dnmt3a^{+/+}/Trp53^{+/-}$ littermates and monitored for overall tumour-free survival. As shown in the survival curve in Figure 5.13, there were no significant differences in the overall and the tumour-free survival of the $Dnmt3a^{R878H/+}/p53^{+/-}$ and $Dnmt3a^{+/+}/p53^{+/-}$ controls. These findings indicate that mutant $Dnmt3a^{R878H/+}$ does not cooperate with the loss of *p53* to drive malignancy.

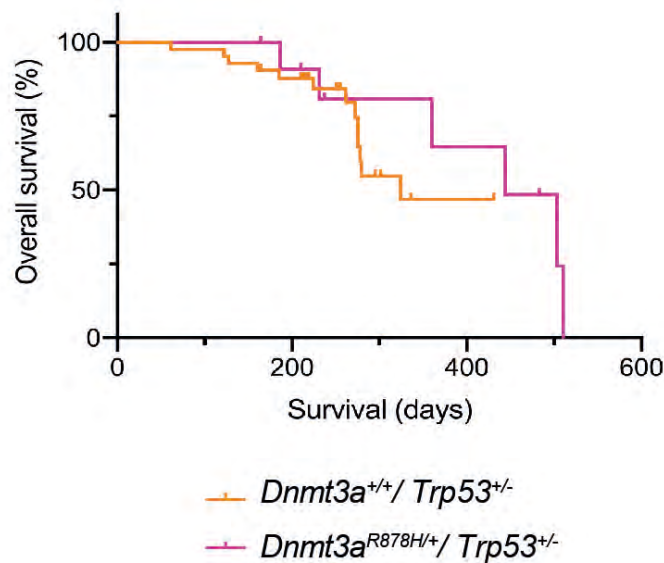


Figure 5.13 *Dnmt3a*^{R878H/+} does not cooperate with loss of one allele of *Trp53* to accelerate tumour development

Kaplan-Meier survival curve displaying tumour-free survival of *Dnmt3a*^{+/+}/*Trp53*^{+/-} (n=42) and *Dnmt3a*^{R878H/+}/*Trp53*^{+/-} (n=13). Mice were excluded if cause of death was not attributed to a tumour eg. malocclusion or fighting wounds. Significance of the curves were compared using log-rank (mantel-cox) test, ns.

To determine whether a relationship exists between mutant *Dnmt3a* and p53 more experiments will need to be performed. Firstly, the number of *Dnmt3a*^{R878H/+} p53^{+/-} mice need to be increased. However, this was quite challenging due to difficulties to obtain female p53^{-/-} mice, which would have been required to cross to *Dnmt3a*^{R878H/+} mice to obtain *Dnmt3a*^{R878H/+}/p53^{-/-} mice. However, p53^{-/-} female mice rarely survive to birth due to a failure to properly inactivate one of the X chromosomes²⁰⁹. Since *Dnmt3a*^{R878H/+} female mice cannot be used for breeding (see Chapter 3) the crossings are quite complex, and many more mice would need to be bred to generate enough *Dnmt3a*^{R878H/+} p53^{-/-} mice.

It remains to be seen how the cells derived from *Dnmt3a*^{R878H/+} p53^{+/-} mice respond to stress as examined in chapters 4 and 5. Since p53 is activated in response to certain cellular stresses, such as DNA damage, it would be interesting to determine whether γ -irradiation induced thymic

lymphoma development is further accelerated in *Dnmt3a*^{R878H/+}/Trp53^{+/-} mice beyond the acceleration observed in *Dnmt3a*^{R878H/+} mice presented in this thesis. It has been shown previously that p53^{+/-} mice develop γ -irradiation induced thymic lymphoma much more rapidly than wt mice ¹⁹¹. We have shown that the acceleration of thymic lymphoma development in the *Dnmt3a*^{R878H/+} mice is comparable to the acceleration that was previously reported for p53^{+/-} mice, notably with both strains on the same (C57BL/6) genetic background ¹⁹¹. This suggests that these genetic defects may work through the same mechanism.

It is extremely rare to find examples of human tumours that carry both *Dnmt3a* and *p53* mutations with only 1% of all tumours in the TCGA database having both mutations ^{8,210,211}. This might be due to the fact that Dnmt3a and p53 work through the same pathway. A tantalising explanation for the blunted p53 signalling pathway activation in *Dnmt3a*^{R878H/+} LSK cells upon γ -irradiation is that this may be due to aberrant DNA methylation of the p53 gene or its regulators. Indeed, there is some evidence that DNMT3a and p53 are intricately linked. Loss of p53 in murine embryonic stem cells was associated with increased Dnmt3a mRNA and protein ²¹². The authors concluded that Dnmt3a must be regulated by p53. ChIP-seq analysis revealed p53 binding sites near the promotor of Dnmt3a ^{212,213}, further suggesting a link with transcriptional regulation of Dnmt3a. On the other hand, Dnmt3a ChIP-seq has revealed that DNMT3a can bind super enhancers to modulate gene expression. Knockdown of DNMT3a using shRNA technology resulted in down-regulation of the p53 signalling pathway in human epidermal stem cells ²¹⁴. This supports the results presented in this thesis where *Dnmt3a*^{R878H/+} LSK cells also displayed reduced p53 pathway activation in response to γ -irradiation. The link between mutations or loss of DNMT3a activity and reduced p53 pathway signalling remain unknown. Indeed, little is known about the epigenetic regulation of p53 despite the wealth of knowledge surrounding its importance as a tumour suppressor. Perhaps mutant DNMT3a^{R882} regulates transcription of p53 through aberrant methylation of the p53 promotor. It is unlikely that wt DNMT3a normally binds to the p53 promotor as this would be predicted to cause high p53 mRNA levels when mutant DNMT3a^{R882H} is present. However, mutant DNMT3a^{R882H} is known to have an altered DNA sequence preference compared to wt DNMT3a ⁹⁷; therefore, mutant DNMT3a^{R882H} could perceivably cause aberrant methylation of the p53 promotor and subsequent impairment of p53 pathway activation.

Another explanation could be that DNMT3a and p53 associate at the protein level to regulate

p53 pathway activation. There is accumulating evidence that DNMT3a and p53 protein can directly bind to one another ²¹⁵. Importantly, p53 has been shown to repress the activity of DNMT3a by forming a Dnmt3a-p53 hetero-tetramer through the displacement of DNMT3L from DNMT3a. DNMT3L can facilitate binding of DNMT3a to target genes through interaction with histone tails. The displacement of DNMT3L from DNMT3a by p53 is proposed to inhibit the ability of DNMT3a to methylate target genes by interfering with guidance to certain loci. Intriguingly, the human *DNMT3a*^{R882} mutation which occurs distal to the proposed p53 binding site in the catalytic domain, was able to block p53 mediated displacement of DNMT3L from DNMT3a^{R882}. Furthermore, common cancer associated mutations in p53 (R248W and R273H) resulted in an inability of mutant p53 to displace DNMT3L from the DNMT3a-DNMT3L hetero-tetramer and thereby prevented p53 mediated repression of DNMT3a. Together, these results suggest that the *Dnmt3a*^{R882H} mutation might exert pro-tumorigenic effects through abnormal p53 protein interactions as well as defective DNA methylation.

It remains unclear why *Dnmt3a*^{R878H/+} LSK cells are less able to induce p53 pathway signalling in response to γ -irradiation. Further validation of the RNA-seq data is warranted and given that *Dnmt3a* regulates gene expression through DNA methylation, the obvious next step would be to determine whether there is evidence of aberrant methylation of p53 target genes that might explain the blunted p53 signalling response observed in *Dnmt3a*^{R878H/+} LSK cells. These experiments had not been completed by the time of writing this thesis.

5.4 Notch signalling is perturbed in *Dnmt3a*^{R878H/+} LSK cells

Our RNA-seq analysis revealed that the Notch signalling pathway was also altered in *Dnmt3a*^{R878H/+} LSK cells at baseline. Gene set enrichment analysis showed that the pathway was significantly enriched in the wt LSK cells at baseline compared to the *Dnmt3a*^{R878H/+} LSK cells (Figure 5.14 A). In response to γ -irradiation, the Notch signalling pathway was significantly down-regulated in wt LSK cells (Figure 5.14 C), whereas it was not significantly down-regulated in similarly treated *Dnmt3a*^{R878H/+} LSK cells (Figure 5.14 D). When comparing γ -irradiated wt with γ -irradiated *Dnmt3a*^{R878H/+} LSK cells there were no significant differences in Notch signalling (figure 5.14 B), which is likely due to the fact that the Notch signalling pathway was already downregulated in *Dnmt3a*^{R878H/+} LSK cells

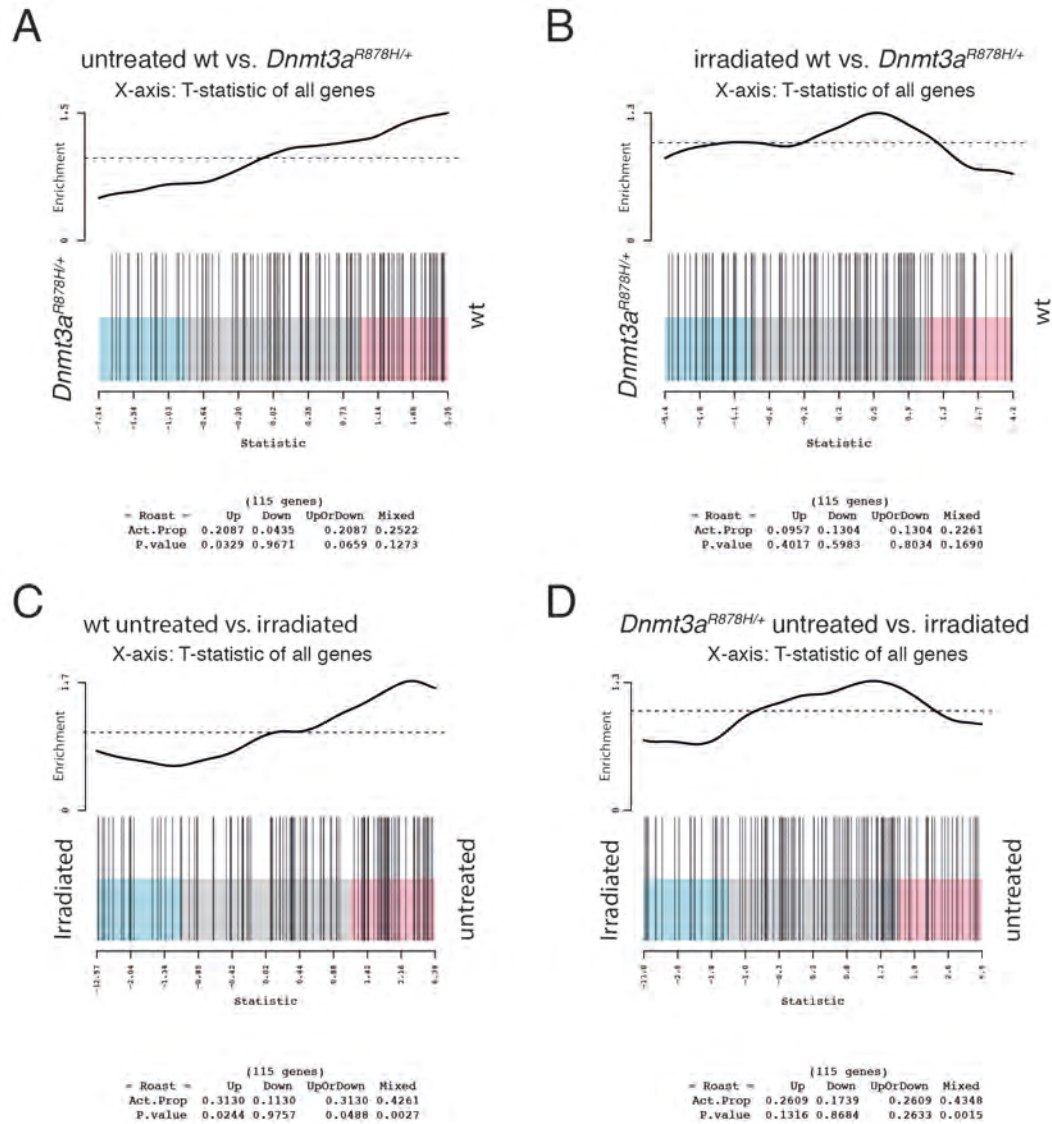


Figure 5.14 Notch signalling is down-regulated in *Dnmt3a*^{R878H/+} LSK cells under steady state conditions

Barcode plots showing enrichment of genes in the Notch signalling pathway (GO:0007219) in A) untreated (un-irradiated) *Dnmt3a*^{+/+} (wt) LSK cells vs untreated *Dnmt3a*^{R878H/+} LSK cells, B) γ -irradiated *Dnmt3a*^{+/+} (wt) LSK cells vs γ -irradiated *Dnmt3a*^{R878H/+} LSK cells, C) untreated *Dnmt3a*^{+/+} (wt) LSK cells vs γ -irradiated *Dnmt3a*^{+/+} (wt) LSK cells and D) untreated *Dnmt3a*^{R878H/+} LSK cells vs γ -irradiated *Dnmt3a*^{R878H/+} LSK cells. Red bars indicate up-regulated genes and blue bars depict down-regulated genes. The worms above the barcode plots show relative enrichment score for the genes in the gene set. $p < 0.05$ was considered significant

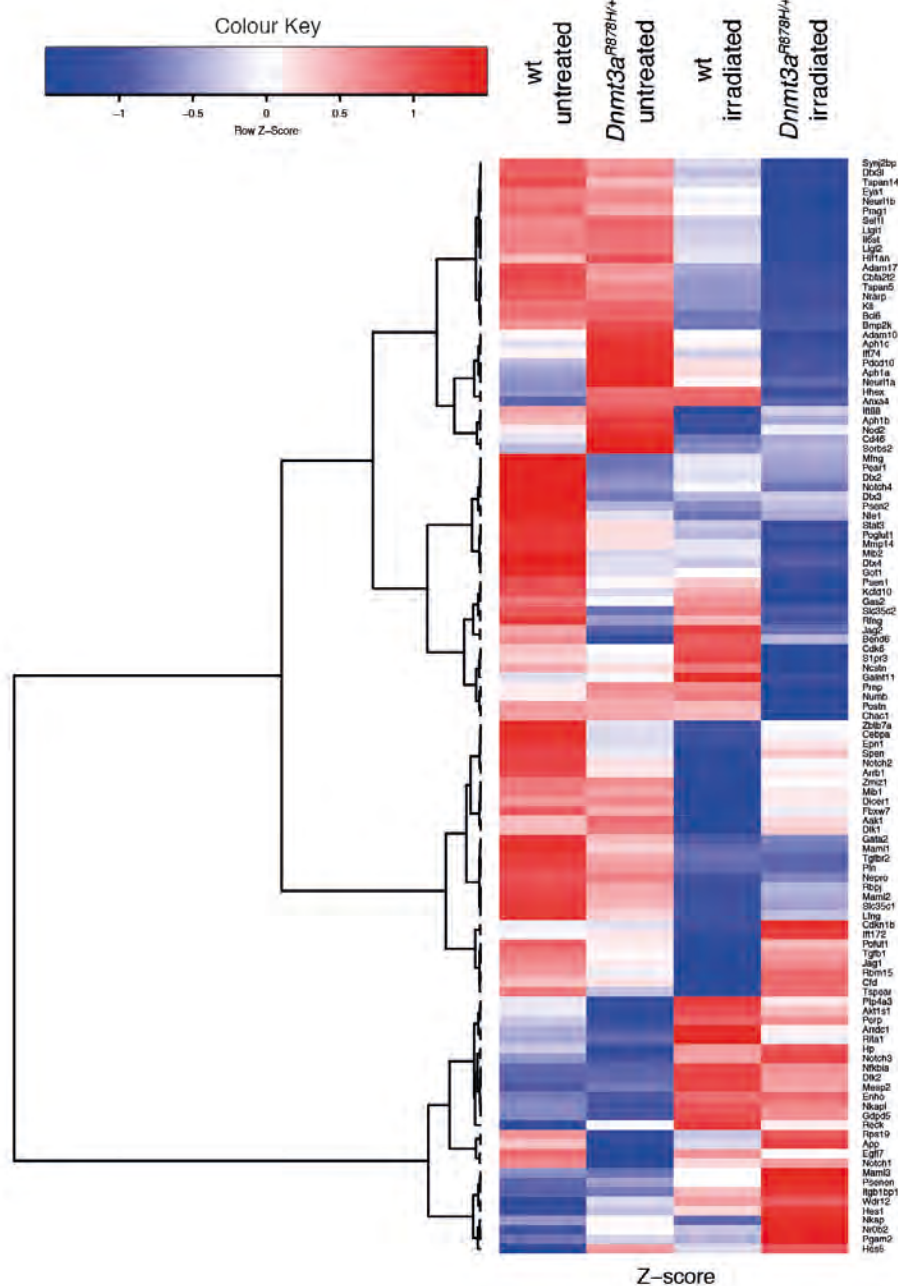


Figure 5.15 Notch signalling pathway heatmap showing the differences between wt and DNMT3a mutant LSK cells.

Heatmap of RNA-Seq expression z scores of genes in the Notch signalling pathway (GO: 0007219) comparing wt untreated (un-irradiated) LSK cells (column 1), untreated (un-irradiated) *Dnmt3a*^{R878H/+} LSK cells (column 2), γ -irradiated wt LSK cells (column 3) and γ -irradiated *Dnmt3a*^{R878H/+} LSK cells (column 4).

at baseline. Therefore, further down-regulation in response to γ -irradiation was not possible. The individual genes that were driving these differences are depicted in the heatmap in Figure 5.15. As shown in column 1 and 2, at baseline there was overall a higher Notch gene expression signature in wt LSK cells compared to *Dnmt3a*^{R878H/+} LSK cells, as indicated by the red squares. The Notch signalling pathway was not enriched in either group upon γ -irradiation, despite examples of different RNA expression in columns 3 and 4. None of the Notch signalling genes were independently significantly different between wt and *Dnmt3a*^{R878H/+} LSK cells at baseline or after γ -irradiation.

It was not immediately obvious how down-regulation of Notch signalling would affect LSK cells. Notch signalling fulfils different functions depending on the context and the cell type. Notch signalling has been reported to induce differentiation, proliferation and cell survival. It therefore appears logical that in response to γ -irradiation, which can cause cell cycle arrest and cell death, the Notch signalling pathway would be down-regulated. It is less clear why *Dnmt3a*^{R878H/+} LSK cells would have constitutively abnormally low Notch signalling at steady state and what selective advantage this might provide, although it could perceivably reduce the differentiation of HSPCs. The interpretation of the differences in Notch signalling between the *Dnmt3a*^{R878H/+} vs wt LSK cells is further complicated by the fact that LSKs are a highly heterogeneous pool of stem and progenitor cells, some of them quiescent and others rapidly cycling; i.e. Notch signalling is not necessarily uniform between these cell populations. Thus, differences in the relative representations of the sub-populations could explain the differences in Notch signalling between the *Dnmt3a*^{R878H/+} vs wt LSK cells.

The Notch pathway, depicted in Figure 5.16, is evolutionarily highly conserved and at first glance appears to be a relatively simple signalling pathway with only a few core pathway components. However, in mammals, there are four Notch receptors, aptly named Notch1-4, all presented on the cell membrane. The Notch receptors respond to five membrane-bound extracellular ligands, named Delta-like1, 3 and 4 and Jagged 1 and 2²¹⁶⁻²¹⁸. In canonical Notch signalling, the Notch receptors of one cell interact with the Notch ligands on a neighbouring cell to initiate proteolytic cleavage liberating the Notch intracellular domain (Notch-ICD). The Notch-ICD translocates to the nucleus where it binds to and consequently drives the expression of specific target genes by interacting with the DNA binding protein CBF1/Suppressor of Hairless/LAG-1 (CSL). In addition to cell-cell interaction mediated Notch signalling activation (*trans*-activation), certain

interactions of ligands with Notch receptors can inhibit signalling. Such inhibition of Notch pathway signalling occurs when ligands expressed on the cell interact with the Notch receptors on the same cell (so called *cis*-inhibition). The different ligands can induce diversity in cellular response by initiating different signalling dynamics. For example, delta like1 induces pulsatile signalling which can directly activate *Hes* genes whereas delta like4 elicits a sustained response which initiates *Hey1* and *HeyL* expression ²¹⁹.

Non-canonical Notch signalling can be initiated through non-canonical ligands or may not require the cleavage mediated release of the notch-ICD. Non-canonical Notch ligands may be membrane bound but can also include secreted proteins. While canonical Notch ligands bind a consensus sequence of the Notch receptor, non-canonical ligands do not have to bind to a discrete consensus sequence and can activate Notch signalling through gamma-secretase mediated cleavage or through direct protein interactions with CSL to induce Notch target gene expression ²²⁰. While non-canonical Notch signalling has been shown *in vitro*, the circumstances in which it is important *in vivo* remain poorly defined. Non-canonical Notch signalling is not critical for normal development unlike canonical notch signalling. However, non-canonical ligands likely act as modulators of Notch signalling in adult tissues. Therefore, Notch signalling activity can be diversely regulated in different cell types and at distinct developmental stages which can be achieved through canonical and non-canonical signalling.

An interesting feature of Notch signalling is the sensitivity of this pathway to ligand dosage. Upon Notch ligand binding to the Notch receptor, the Notch ICD is cleaved by gamma-secretase, initiating the signalling pathway thereby also rendering the Notch receptor unable to receive further signals. The Notch signalling pathway does not include any known amplification (feed-forward) events, meaning that the Notch signal remains in stoichiometric balance between the signal input and output. The strength of Notch signalling is likely key to maintaining the diversity in cell type and context specific Notch induced gene expression changes. Indeed, reduced Notch signalling as a result of haplo-insufficiency of genes encoding Notch pathway proteins results in congenital syndromes like Alagille syndrome, spondylocostal dysostosis and cadasil ²²¹. These congenital diseases are characterised by multi-organ developmental defects, revealing serious consequences of insufficient Notch signalling dosage in multiple organs.

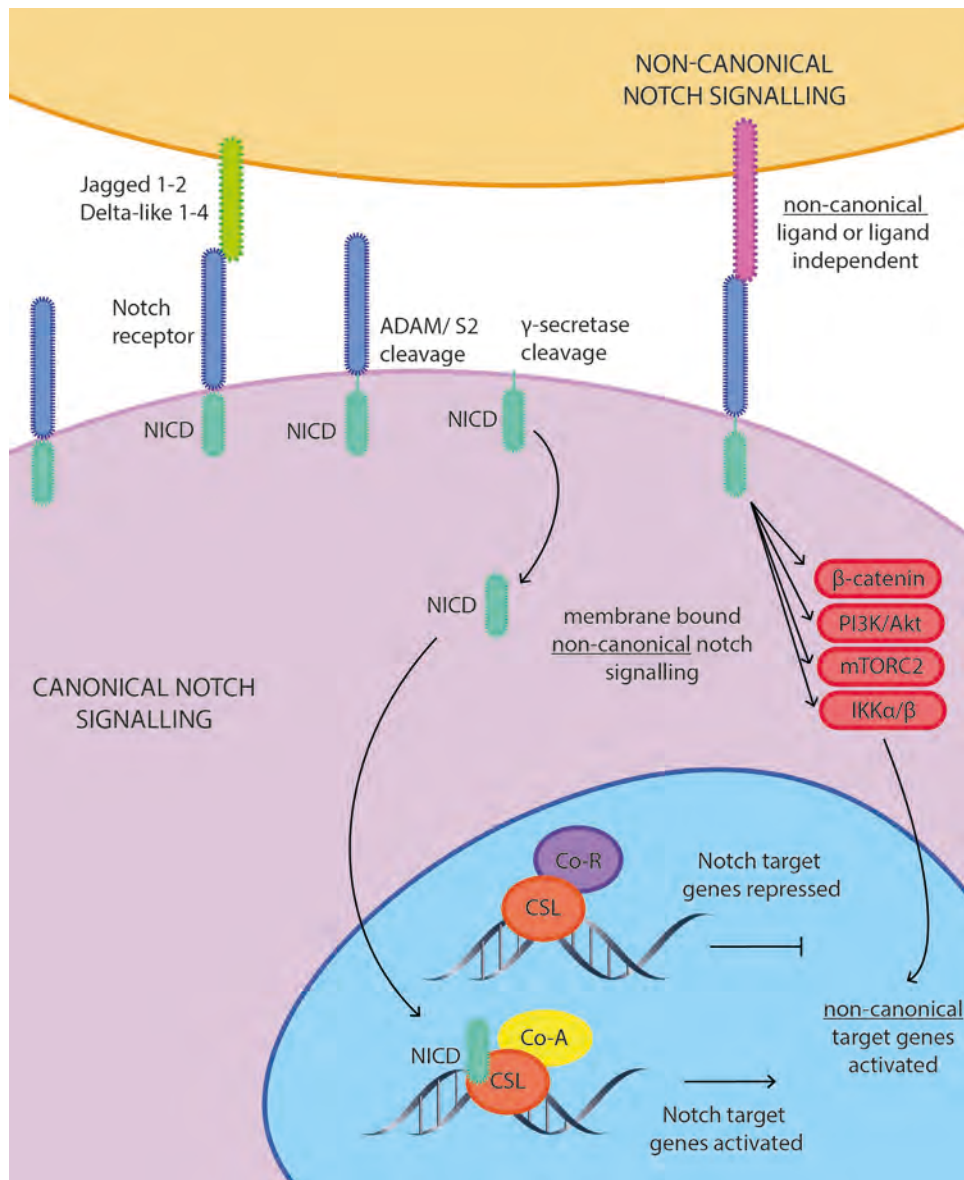


Figure 5.16 Schematic of canonical and non-canonical notch signalling

Notch receptors (Notch1-4) are activated upon binding by canonical ligands Jagged 1-2 or Delta-like 1-4. Upon ligand binding the Notch receptor is cleaved by ADAM/S2. The intracellular Notch receptor (NICD) is then cleaved from the transmembrane domain by gamma-secretase. The NICD translocates into the nucleus where it binds to CSL and co-activators (Co-A) to activate Notch target genes. Non-canonical ligand interactions with the Notch receptor may not require cleavage of the NICD for Notch target gene activation. Examples of non-canonical signalling are shown in red and these can activate non-canonical Notch target genes. Notch intracellular domain (NICD), A-Disintegrin And Metalloprotease (ADAM), Corepressor (Co-R), Coactivator (Co-A), DNA binding protein CBF1/Suppressor of Hairless/LAG-1 (CSL).

In the haematopoietic system, Notch signalling is essential to maintain homeostasis of blood cells by dictating haematopoietic cell proliferation, differentiation and death. While the importance of Notch signalling in HSCs is still debated, the role of Notch signalling in T cell development in the thymus is well established. T cell commitment is reliant on Notch signalling. In the absence of Notch signalling in MPPs, there is preferential differentiation into B lymphocytes rather than T lymphocytes ²²². In the previous chapter it was shown that in *Dnmt3a*^{R878H/+} mutant mice T cell development in the thymus was abnormal at early stages. There were significantly fewer DN1 progenitor cells in the thymus and there appeared to be a trend towards reduced CLPs in the bone marrow of *Dnmt3a*^{R878H/+} mice. This phenotype could be explained by impaired Notch signalling in the haematopoietic cells of *Dnmt3a*^{R878H/+} mice. If the reduction in Notch signalling identified in *Dnmt3a*^{R878H/+} LSK cells was carried through to the CLPs stage, it would provide a logical explanation for the impairment of T cell development in the *Dnmt3a*^{R878H/+} mice described in chapter 4. The transition of DN1 to DN2 cells in the thymus is highly dependent on Notch signalling. In this context, Notch signalling commits ETPs to the T cell lineage and antagonises B cell development. Thus, in the absence of adequate Notch signalling T cell development would be impaired and B cell development would be favoured. Accordingly, *Dnmt3a*^{R878H/+} mice demonstrated abnormally high frequencies of B cells in the blood, and this was even more obvious in competitive bone marrow transplanted mice described in chapter 4. This suggests that this abnormality could be due to impaired Notch signalling in other haematopoietic cells. More experiments are required to determine whether impaired Notch signalling would be observed in all *Dnmt3a*^{R878H/+} haematopoietic cells as it has only been shown in LSK cells at this point. Nevertheless, the underlying mechanism driving abnormal DN stage T cell development in the thymus as well as an increase in B lymphocyte bias in competitive bone marrow transplantation experiments could also be explained by impaired Notch signalling. Therefore, further experiments will be conducted to determine if impaired Notch signalling also occurs in *Dnmt3a*^{R878H/+} T and B lymphocytes.

It is unknown how mutant *Dnmt3a*^{R878H} causes reduced Notch signalling in LSK cells. There is only little published about epigenetic regulation of Notch signalling, although it is predicted to be important in creating diversity of Notch signalling intensity and outcomes in different cell types. The Notch signalling pathway is critical for normal development and *Dnmt3b* is the predominant DNA methyltransferase responsible for maintaining epigenetic regulation during embryonic development ⁴⁹. Therefore, it is possible that under normal conditions, *Dnmt3a* does not

regulate Notch signalling. However, there is some evidence that the *DNMT3a*^{R882} mutation results in a DNA binding affinity that is more similar to DNMT3b²²³ and this may explain the abnormal Notch signalling gene signature in *Dnmt3a*^{R878H/+} LSK cells. Further experiments to determine DNA methylation of genes that encode proteins that function in the Notch signalling pathway will reveal whether mutant Dnmt3a^{R878} can alter normal gene methylation signatures and this may reveal a mechanism for reduced Notch signalling in *Dnmt3a*^{R878H/+} mutant LSK cells.

5.1 Cooperation of mutant Dnmt3a and Notch1-ICD overexpression in a murine model of T-ALL

Impaired Notch signalling could reveal a mechanism for abnormal T cell development in the *Dnmt3a*^{R878H/+} mouse. However, it was unclear how reduced Notch signalling in the *Dnmt3a* mutant cells could contribute to γ -irradiation induced thymic lymphoma development. Interestingly, loss of function Notch1 mutations are rarely found in cancer. Instead activating Notch1 mutations are a potent driver of T-ALL and, interestingly, have been reported to co-occur with *DNMT3a* mutations in this malignancy. It seems counterintuitive to suggest that *Dnmt3a* mutant cells demonstrate impaired Notch signalling and yet co-occurrence of Notch1 gain-of-function mutations with *DNMT3a* mutations have been reported in T-ALL.

While *DNMT3a* mutations have been extensively characterised in patient cohorts with AML, the implications of mutant *DNMT3a* in T-ALL are less understood. *DNMT3a* mutations are associated with adverse survival outcomes in adult patients with T-ALL^{112,182,224}. Conversely, *DNMT3a* mutations are almost entirely absent in paediatric and adolescent T-ALL²²⁵, which is consistent with age-related acquisition of *DNMT3a* lesions reported in patients with AML^{143,145}. Indeed, *DNMT3a* mutations are strongly enriched in patients >40 years of age in T-ALL cohorts¹¹¹, making it difficult to tease apart the prognostic implications of a *DNMT3a* mutation from increasing age. Elderly patients are less likely to be eligible for intensive treatment regimens and therefore have worse overall survival outcomes compared to younger patients. An extensive analysis of T-ALL patients performed by Bond, et al.¹¹¹ provided evidence that *DNMT3a* mutations drive an aggressive, phenotypically immature disease with a higher risk of relapse, shorter event free survival and shorter overall survival independent of age.

Clinical data of T-ALL patients are accumulating but there remains a scarcity of preclinical models

to study the role of mutant DNMT3a in T-ALL. Mayle, et al. ¹⁵³ showed in mice that transplanted *Dnmt3a* KO HSCs underwent malignant transformation through acquisition of Notch1 activating mutations to cause T-ALL. While loss of one allele of *DNMT3a* is common in T-ALL, there was still some enrichment of the 'hotspot' R882 mutation in patient cohorts ^{111,226}. This suggests that this particular point mutation was important in malignant transformation. To build on the clinical data and extend mouse models of DNMT3a mutant T-ALL, we decided to cross the *Dnmt3a*^{R878H/+} mice with the *lck-Notch1-IC9* transgenic mouse line (Notch1) ¹⁶³. These mice express the Notch1-ICD under the control of the *lck* promotor, driving expression from the DN2 stage of T cell development in the thymus leading to T-ALL.

Thus far our data from the analysis of LSK cells would suggest that the enforced expression of constitutive active Notch in a *DNMT3a* mutant background might lead to a delay in the development of T-ALL or may even prevent this malignancy. Therefore, we crossed the *lck-Notch1-IC9* transgenic mouse line with *Dnmt3a* mutant mice.

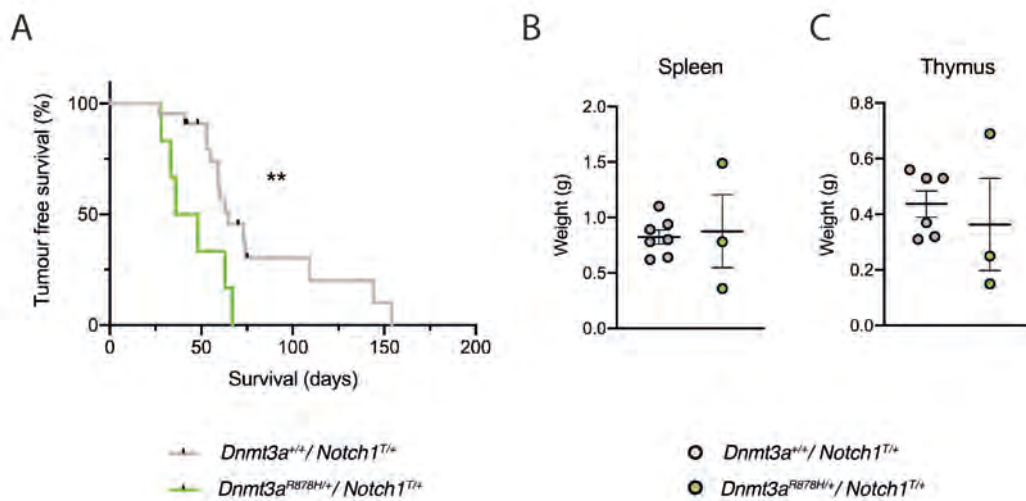


Figure 5.17 The *Dnmt3a*^{R878H} mutation accelerates the development of T-ALL in LCK-Notch1-IC9 mice.

A) Survival curves of *Dnmt3a*^{+/+}/*Notch1*^{T/+} n=7, *Dnmt3a*^{R878H/+}/*Notch1*^{T/+} n=7. B) weight of the spleen in mice of the indicated genotypes at ethical endpoint, *Dnmt3a*^{+/+}/*Notch1*^{T/+} n=7, *Dnmt3a*^{R878H/+}/*Notch1*^{T/+} n=3. Log-rank (Mantel-Cox) test, **P<0.01. C) weight of the thymus in mice of the indicated genotypes at ethical endpoint *Dnmt3a*^{+/+}/*Notch1*^{T/+} n=6, *Dnmt3a*^{R878H/+}/*Notch1*^{T/+} n=3. Students t-test, ns.

Strikingly, the *Dnmt3a*^{R878H/+} /*Notch1*^{T/+} mice showed accelerated development of T-ALL with a median tumour-free survival of just 42 days, as compared to the *Notch1*^{T/+} mice with a median survival of 65 days (Figure 5.17 A). There was no significant difference in the weight of the spleen (Figure 5.17 B) or the thymus (Figure 5.17 C) at the time of sacrifice, indicating similar tumour burden. Blood cell composition was also determined at ethical endpoint where possible and the results are displayed in Figure 5.13 A-H. There were no significant differences between the lymphomas from the *Dnmt3a*^{R878H/+} /*Notch1*^{T/+} mice vs those from the *Notch1*^{T/+}, These data suggest that the presence of the *Dnmt3a* mutation accelerate the development of mutant Notch driven T-ALL but does not alter severity of disease.

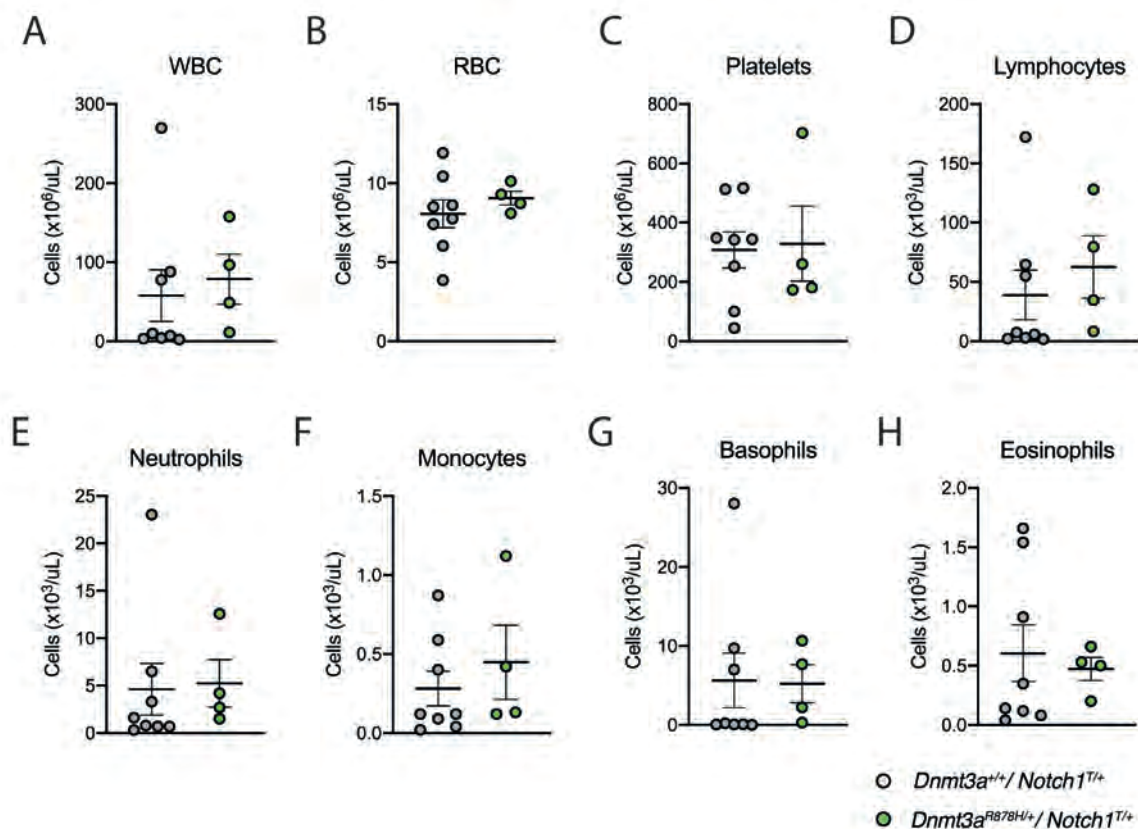


Figure 5.18 Notch tumour ADVIA

Blood cell composition of mice of the indicated genotypes at ethical endpoint as determined by ADVIA analysis. A) white blood cells, WBC, B) red blood cells; RBC, C) platelets, D) lymphocytes, E) Neutrophils, F) monocytes, G) basophils, H) eosinophils. Students t-test, ns.

To determine whether the presence of mutant *Dnmt3a* resulted in an altered immunophenotype of the lymphomas, the blood was analysed by flow cytometry. Briefly, red blood cells were lysed with red blood cell lysis buffer prior to incubation with antibodies. The blood was stained with antibodies against cell surface markers to distinguish cell populations in the blood. These included antibodies against TCR β , B220, CD4, CD8, IgM, IgD, MAC-1 and GR-1 which are common markers to identify T cells, B cells and macrophages and granulocytes. The gating strategy to define these populations is the same as that outlined in Chapter 4, Figure 4.3. Some example FACS plots of the blood cell analysis from sick *Dnmt3a*^{R878H/+}/*Notch1*^{T/+} mice and controls are presented in Figure 5.19. As shown in Figure 5.19 A, *Notch1*^{T/+} control lymphomas were positive for surface CD8 expression. The *Dnmt3a*^{R878H/+}/*Notch1*^{T/+} lymphomas did not vary drastically from the *Notch1*^{T/+} control lymphomas, although mouse #78 and mouse #84 appeared to have a large populations of immature B cells which was unusual.

The interplay between mutant Dnmt3a and Notch is interesting and warrants further exploration. It was previously shown by RNA seq analysis that mutant Dnmt3a^{R878H} reduced Notch signalling activity in LSK cells. On the other hand, mutant Dnmt3a^{R878H} and constitutive oncogenic Notch1 signalling cooperate to accelerate the development of T-ALL. Understanding the relationship between Dnmt3a and Notch signalling in tumorigenesis will help in the development of novel strategies for the treatment of patients with T-ALL or other malignancies that contain both mutant DNMT3a and oncogenic Notch-ICD. The *Dnmt3a*^{R878H/+}/*Notch1*^{T/+} doubly transgenic mice presented herein establish an attractive preclinical model to expand the understanding of the clinical course of Dnmt3a mutant T-ALL in patients. It remains unclear why the presence of the *DNMT3a*^{R882H} mutation in human T-ALL leads to poor survival outcomes for patients. Our model will allow us to better understand the chemotherapeutic response of *DNMT3a*^{R878H/+}/*Notch1*^{T/+} tumours, the likelihood of refractory disease and the implications for overall survival. In a continuation of this work, the previous points will be addressed in future experiments.

5.5 RNA sequencing of *Dnmt3a*^{R878H/+} LSK cells reveals a range of additional deregulated signalling pathways for future exploration

There were additional pathways identified as significantly differentially activated in LSK cells from *Dnmt3a*^{R878H/+} mice as shown in Figure 5.20. There were 13 pathways that were significantly enriched in the *Dnmt3a*^{R878H/+} LSK cells at baseline compared to wt LSK cells. These included pathways identified by gene ontology (GO) enrichment analysis and KEGG pathway analysis (prefix R-MMU). Given that DNA methylation is a critical process for the coordination of gene expression, it was no surprise that a wide variety of cellular pathways were affected. Some of these pathways were quite interesting given some of the abnormalities found in mutant *Dnmt3a*^{R878H/+} mice. For example, in Chapter 3 it was shown that *Dnmt3a*^{R878H/+} mutant mice became obese with increasing age. Here it was shown that the regulation of fat cell differentiation (GO:0045598) was enriched in *Dnmt3a*^{R878H/+} LSK cells. While it is not appropriate to extrapolate this finding to other cell types, it provides interesting insight which might suggest that *Dnmt3a*^{R878H/+} mutant mice have deregulated fat cell differentiation and abnormal glucose import (GO:0046324) which could in turn provide a mechanism causing the obesity.

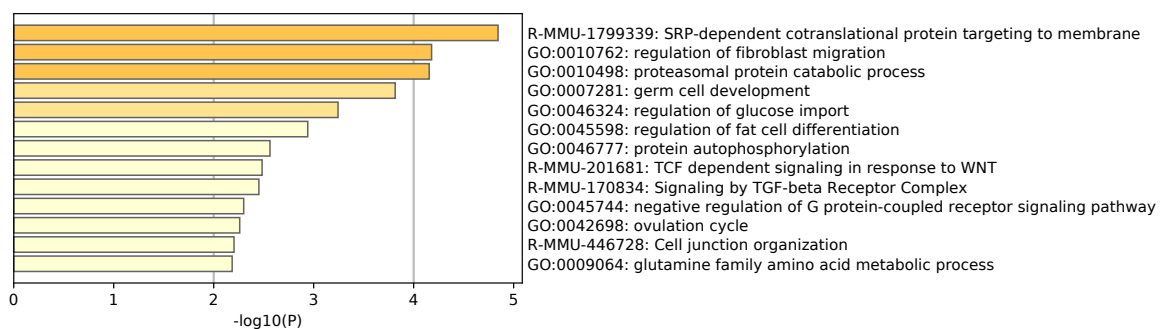


Figure 5.20 Pathways significantly enriched in *Dnmt3a*^{R878H/+} LSK cells under steady state conditions

To determine which pathways were enriched upon γ -irradiation in *Dnmt3a*^{R878H/+} mutant LSK cells but not in WT cells, a non-biased gene set enrichment analysis was performed. The top 20 pathways that were enriched in γ -irradiated *Dnmt3a*^{R878H/+} LSK cells but not in γ -irradiated WT LSK cells are shown in Figure 5.21. The full list of pathways that were enriched in γ -irradiated *Dnmt3a*^{R878H/+} LSK cells but not in wt LSK cells is shown in Appendix IV as well as common pathways in γ -irradiated LSK cells between *Dnmt3a*^{R878H/+} and wt groups and pathways that were

enriched in wt LSK cells upon γ -irradiation but not in *Dnmt3a*^{R878H/+} LSK cells. Transcriptional regulation by small RNAs (R-MMU-5578749) and oxidative phosphorylation (mmu00190) were both strongly enriched in γ -irradiated *Dnmt3a*^{R878H/+} LSK cells but not in γ -irradiated WT cells. There is very little reported about the role of DNMT3a as a regulator of small RNAs. While small RNAs have been reported to act as chaperones to direct DNMT3a to target genes²²⁷, there is little known about how DNMT3a might regulate the small RNAs themselves. Similarly, to my knowledge there is no known association between Dnmt3a and the regulation of oxidative phosphorylation. The enrichment of the oxidative phosphorylation gene signature in *Dnmt3a*^{R878H/+} LSK cells upon γ -irradiation will need to be validated and explored further in future experiments.

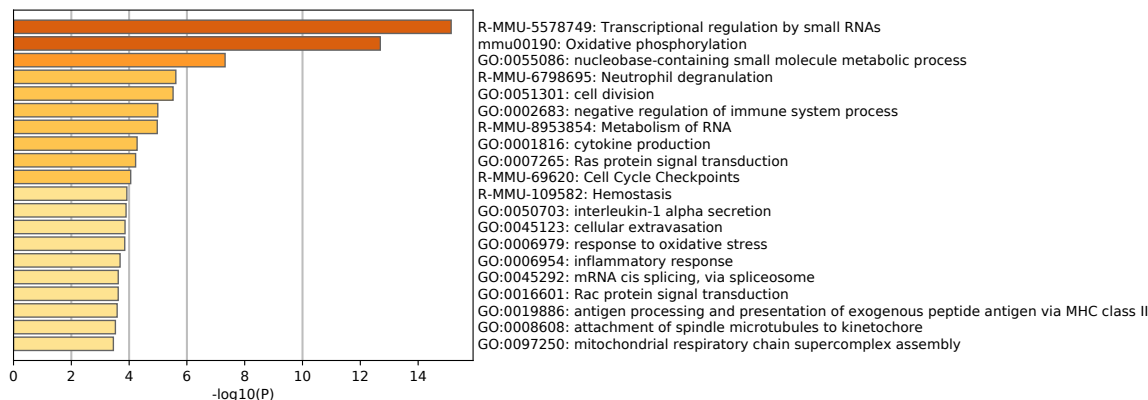


Figure 5.21 Pathways that were significantly enriched in *Dnmt3a*^{R878H/+} LSK cells upon γ -irradiation but not in γ -irradiated wt LSK cells

5.6 Discussion

In this chapter it was shown that *Dnmt3a*^{R878H/+} HSPCs are primed for leukaemic transformation induced by γ -irradiation induced DNA damage. Through gene expression analysis, it was shown that *Dnmt3a*^{R878H/+} HSPCs demonstrate blunted p53 signalling in response to γ -irradiation, revealing a potential mechanism that drives leukemic transformation. It is hypothesised that impaired p53 signalling occurs as a result of abnormal mutant *Dnmt3a* driven promotor hyper- or hypo-methylation. This can be addressed by methylation sequencing in future experiments. Nevertheless, the combination of the *Dnmt3a*^{R878H} mutation with loss of one allele p53 did not cooperate to induce faster onset of tumour development compared to each of the abnormalities

on their own. The lack of synergy and the low frequency co-occurrence of DNMT3a mutations and p53 mutations in human cancers suggest that the tumour suppressive effects of wt DNMT3a and wt p53 may occur through the same or closely related mechanism.

Despite abnormally low induction of p53 signalling in *Dnmt3a*^{R878H/+} HSPCs, no individual genes in this pathway were significantly differentially expressed on their own. Similarly, our RNA seq analysis demonstrated that *Dnmt3a*^{R878H/+} LSKs have an impaired Notch signalling pathway under steady state conditions. However, again no genes of the Notch signalling pathway were independently significantly differentially expressed. The dosage of Notch signalling appears to be critical to normal haematopoietic cell functioning as there was reduced Notch signalling in *Dnmt3a*^{R878H/+} LSK cells at baseline and yet mutant *Dnmt3a*^{R878H} and Notch activating mutations cooperate to drive an aggressive T-ALL in our mouse model. Using this model of T-ALL, future experiments will address gene expression changes in pre-leukaemic *Dnmt3a*^{R878H/+}/*Notch*^{T/+} T lymphoid cells as well as assessing the chemotherapeutic response of the malignant lymphomas.

While the development of cancer models and gene expression analysis reported in this chapter were critical in building the knowledge about mutant Dnmt3a and its role in cancer, there were some limitations to these experiments. Firstly, gene set enrichment analysis revealed abnormally reduced p53 and Notch signalling in *Dnmt3a*^{R878H/+} HSPCs but there was no individual significantly differentially expressed genes in either pathway. There are a number of possible explanations for this observation ranging from methodological to biological reasons. Firstly, the experimental design used only female mice to reduce confounding effects from gender differences in HSPC gene expression, but it is possible that we missed valuable gender specific differences and therefore the gene expression data cannot be generalised to males. Secondly, HSPCs were isolated from mice at 4 weeks of age which is a young age. *DNMT3a* mutations are primarily reported in the elderly and are thought to occur in cells years, even decades, before malignant transformation. Therefore, it is possible that HSPCs collected from older *Dnmt3a*^{R878H/+} mutant mice, may have much stronger differences in their gene expression compared to wt mice than we observed by looking at cells from young *Dnmt3a*^{R878H/+} mice. Nevertheless, there were interesting findings from the young mice and perhaps gene expression analysis could be performed on HSPCs isolated from older mice to extend the findings of the RNA seq analysis reported here. It is possible that HSPCs accumulate abnormal DNA methylation signatures with age, something that we are yet to show experimentally, and that this could be

reflected by further changes to gene expression. By performing RNA sequencing on HSPCs from old mice we can build upon and extend the findings of the gene expression changes reported here. Another consideration for the lack of individually differentially expressed genes in the p53 pathway could be due to the isolation of HSPCs after only 2 hours of recovery from γ -irradiation. While other groups have published that gene expression changes occur within 2 hours after stimulation ^{199,228}, it is also plausible that a longer time point after γ -irradiation would have revealed more robust changes in gene expression. Lastly, the RNA was isolated from a pool of HSPCs. It was reported that Dnmt3a is critical in LT-HSCs and that loss of Dnmt3a in these cells drove malignant transformation ³⁷. While LT-HSCs were present in the LSK pool, it would be interesting to assess gene expression changes specifically in LT-HSCs by single cell RNA-Seq rather than by population-based analysis. By using single cell technologies, we might find that a small subset of HSPCs have altered gene expression. Mutant Dnmt3a is known to be a potent driver of clonal haematopoiesis; so, it appears logical that only a small pool or even a single cell could give rise to clonally derived blood cells and ultimately blood cell derived cancers.

CHAPTER 6

CONCLUSIONS AND FUTURE OUTLOOK

6.1 Mutant *Dnmt3a*^{R878H/+} in mouse development and ageing

6.1.1 Mutant *Dnmt3a*^{R878H/+} in mouse development

In chapter 3, the *Dnmt3a*^{R878H/+} mouse was introduced as a novel preclinical model with potential applications for studying the role of mutant *Dnmt3a* in the context of TBRS and cancer. While *Dnmt3a*^{R878H/+} mice were viable, the female *Dnmt3a*^{R878H/+} mice exhibited recurrent birthing defects. We were unable to obtain live pups from these mothers and upon visual inspection, the pups appeared to be partially reabsorbed suggesting they had died some time prior to the onset of contractions. I did not perform a comprehensive analysis on the pups and these observations are only from around five pregnancies. There are many questions that remain unanswered surrounding the ability of *Dnmt3a*^{R878H/+} female mice to give birth to viable offspring that would require further experiments. Consistent with these developmental defects, mice with defects in *Dnmt1* due to genetic deletion of *Naa10*, are also born at lower frequencies and have evidence of impaired placental growth²²⁹. The resulting abnormalities in the mice arise from impaired imprinting due to reduced Dnmt1 activity²²⁹. Whether something similar occurs in the *Dnmt3a*^{R878H/+} mice remains to be seen. Interestingly, *Dnmt3a*^{+/-} mice develop normally and are fertile⁴⁹, highlighting a discrepancy between the loss of one allele of *Dnmt3a* and the *Dnmt3a* R878H mutation. A number of experiments should be performed to further understand the preliminary results reported in this thesis. Firstly, it is not clear at this stage if all female *Dnmt3a*^{R878H/+} mice are unable to deliver live pups as we stopped using them for breeding due to ethical reasons surrounding their use as breeders. It would be interesting to increase the sample size to determine the penetrance of this inability to deliver healthy pups by female *Dnmt3a*^{R878H/+} mice. Secondly, it remains unclear how long the pups survive and whether they die prematurely during development which causes the birthing defects in female *Dnmt3a*^{R878H/+} mice. To determine this experimentally, it would be interesting to harvest embryos at different developmental stages to assess viability and development in a controlled way. Collectively, these experiments could help us to understand why *Dnmt3a*^{R878H/+} female mice do not make effective breeders.

6.1.2 Mutant *Dnmt3a*^{R878H/+} mice are susceptible to liver disease and leukaemia

It was shown in chapter 3 that *Dnmt3a*^{R878H/+} mice had a reduced overall survival compared to their WT littermates. The two major causes of death in this mouse model were attributed to

liver disease and leukaemia or lymphoma. *Dnmt3a*^{R878H/+} mice developed extensive liver damage characterised by significant steatosis (Figure 3.8). In one case this had developed into a hepatocellular carcinoma. Aberrant DNA methylation has been shown in liver cancers, although this has been largely attributed to overexpression of DNMT3a rather than mutations²³⁰. It appears as though both overexpression and mutation of DNMT3a can contribute to carcinogenesis highlighting the importance of precise regulation of DNA methylation. However, it remains unclear if *Dnmt3a*^{R878H/+} mutant mice are more susceptible to liver cancer because of direct DNA methylation changes in hepatocytes favouring neoplastic transformation or if the obesity of these mice could predispose hepatocytes to malignant transformation. The *Dnmt3a*^{R878H/+} mice developed obesity with age which is consistent with reports from mice with inducible genetic loss of *Dnmt3a* in haematopoietic cells¹⁷⁷. Together, these results suggest that Dnmt3a is critical for normal fat metabolism, but it remains uncertain whether the increased adiposity occurs as a result of abnormal hepatocyte metabolism or defects in the regulation of fat storage in other cell types.

It was also established in chapter 3 that *Dnmt3a*^{R878H/+} mice were prone to haematopoietic malignancies. This observation is in line with previous studies where mice with an inducible *Mx1-Cre: Dnmt3a*^{R878H} allele developed AML and T-ALL following heterozygous and homozygous recombination. In these transplantation studies, the murine *Dnmt3a*^{R878H} allele was induced by injection of plpC resulting in mice succumbing to haematopoietic malignancies earlier than WT controls^{34,154,187}. In chapter 3, it was shown that *Dnmt3a*^{R878H/+} mutant mice were particularly susceptible to B cell neoplasms which was much more common in our mouse model than in the *Mx1-Cre* inducible *Dnmt3a*^{R878H} model^{34,153,154,187}. The major distinguishing feature between our novel mouse model and the previously reported *Mx1-Cre* based model is that we do not disrupt the HSC niche by plpC or haematopoietic transplantation and perhaps that is why we observe a different type of leukaemia. Another feature of our mouse model is that all cells in the mouse are *Dnmt3a*^{R878H/+} as this mutation is germline which also distinguishes it from the previous studies where mutant Dnmt3a was only expressed in select cell types.

6.1.3 Future directions

The *Dnmt3a*^{R878H/+} mutant mice introduced in chapter 3 share some similarities with features of TBRS. In particular, obesity, the defining characteristic of TBRS that differentiates it from other

overgrowth and intellectual disability disorders was also observed in the *Dnmt3a*^{R878H/+} mouse model. This suggests that this mouse model may be useful in understanding the clinical course of TBRS. One avenue that has yet to be explored is whether these mice recapitulate the intellectual disability observed in TBRS patients. Using the *Dnmt3a*^{R878H/+} mice as a model of TBRS associated intellectual disability is an attractive idea as there are currently no mouse models of this disease. Testing intellectual disability in murine models is difficult and often requires surrogate measurements of behaviours that can be extrapolated to diagnose intellectual disabilities²³¹. For example, grasping, anxiety and aggression can be used as surrogate measurements that may indicate a form of intellectual disability^{232,233}. In the future, it would be interesting to determine whether the intellectual disability reported in TBRS patients is recapitulated in the *Dnmt3a*^{R878H/+} mouse model. If it is, it will open up the possibility of examining how aberrant DNA methylation leads to intellectual disability and whether restoring the DNA methylation pattern during development or after birth, for example by drug mediated intervention, can reduce the severity or prevent intellectual disability all together.

6.2 The impact of mutant *Dnmt3a*^{R878H/+} on murine haematopoiesis

6.2.1 *Dnmt3a*^{R878H/+} haematopoietic cells have a competitive advantage over wt cells

In chapter 4, the impact of heterozygous *Dnmt3a*^{R878H} mutations on murine haematopoiesis were examined. This is the first time that preleukaemic haematopoietic phenotyping had been performed in mice with a germline *Dnmt3a*^{R878H/+} mutation. It was shown that while the haematopoietic system was grossly normal in *Dnmt3a*^{R878H/+} mutant mice under steady state conditions, there were some subtle but notable exceptions. For example, there was a defect in T cell development in the thymus as evidenced by significantly fewer DN1 thymocytes suggesting a defect in differentiation of immature T cell progenitors. This defect was not complete as the numbers of T lymphoid cells at later stages of development were normal. The HSC compartment in the bone marrow was also grossly normal although this was only examined in relatively young mice. Therefore, it would be interesting to repeat these experiments in mice as they age to determine whether there is an accumulation of HSCs in *Dnmt3a*^{R878H/+} mutant mice with age as has been shown previously in haematopoietic reconstitution studies using bone marrow from either *Dnmt3a*^{R878H/+}^{34,187} or *Dnmt3a* KO mice^{37,115}.

In chapter 4, it was established through competitive bone marrow transplantation assays that *Dnmt3a*^{R878H/+} haematopoietic cells have a strong advantage over their wt counterparts. *Dnmt3a*^{R878H/+} HSCs have been shown to have a competitive advantage over wt cells after 6 months which was accompanied by a higher contribution of *Dnmt3a*^{R878H/+} HSC-derived peripheral blood cells¹⁸⁷. However, the authors did not examine the relative contributions of *Dnmt3a*^{R878H/+} HSC-derived cells to each haematopoietic cell subtype and only examined peripheral blood. It has already been demonstrated several times that *Dnmt3a*^{R878H/+} HSC cells robustly outcompete wt cells and that *Dnmt3a*^{R878H/+} HSCs are biased towards B cell and myeloid cell differentiation as these cell types accumulate at the fastest rate in competitive bone marrow reconstitution studies.

It was also shown that *Dnmt3a*^{R878H/+} HSCs were capable of serial reconstitution and maintaining their growth advantage throughout multiple rounds of haematopoietic reconstitutions. This is the first time that the fitness of *Dnmt3a*^{R878H/+} HSCs has been examined in multiple rounds of haematopoietic reconstitution. These findings are consistent with the observed competitive advantage of *Dnmt3a* KO cells¹¹⁵. However, the results reported in this thesis provide a more extensive picture by examining the competitive fitness of *Dnmt3a*^{R878H/+} haematopoietic cells in the blood, bone marrow, thymus and spleen.

6.3 Impact of the *Dnmt3a*^{R878H/+} mutation on gene regulation in haematopoietic stem cells and cancer

6.3.1 *Dnmt3a*^{R878H/+} mutant HSC have abnormal gene expression which makes them susceptible to leukemia

In chapter 5, it was shown that γ -irradiation induced thymic lymphoma development was markedly accelerated in *Dnmt3a*^{R878H/+} mutant mice, although there was no increase in tumour burden compared to wt mice at the ethical endpoint. To understand why *Dnmt3a*^{R878H/+} mutant mice showed abnormally accelerated γ -irradiation induced thymic lymphoma development, a tumour that originates in HSPCs, a comprehensive gene expression analysis of HSPCs was performed by RNA seq. Since the leukaemia cell of origin in the γ -irradiation induced thymic lymphoma model is known to be a HSPC in the bone marrow, *Dnmt3a*^{R878H/+} mutant mice and wt littermates were exposed to low dose γ -irradiation and then their LSK cells were isolated from

the bone marrow. By sequencing the RNA and performing gene set enrichment analysis it was revealed that a number of signalling pathways were deregulated at baseline and in response to γ -irradiation in LSK cells from the *Dnmt3a*^{R878H/+} mutant mice compared to LSK cells from their wt littermates. *Dnmt3a*^{R878H/+} LSK cells had a significantly weaker induction of p53 signalling in response to γ -irradiation suggesting that impaired DNA damage repair and/or loss of other p53 activated processes may contribute to the faster onset of thymic lymphoma in the γ -irradiation model. The second pathway that was deregulated in *Dnmt3a*^{R878H/+} LSK cells was the Notch signalling pathway. Interestingly, it was shown that mutant *Dnmt3a*^{R878H} cooperated with oncogenic activation of Notch1 to drive an aggressive T-ALL in our mouse model.

6.3.2 Future directions

In the future, it will be necessary to validate the results of the RNA sequencing experiment. In this thesis, two pathways, Notch signalling and p53 signalling, were identified as being deregulated as a consequence of mutations in DNMT3a and discussed in detail. However, as shown in chapter 5 there were many additional pathways that were deregulated in *Dnmt3a*^{R878H/+} LSK cells at baseline and after exposure to γ -irradiation, meaning that there is still much more to understand about how mutant Dnmt3a predisposes cells to malignant transformation or increases the competitiveness of healthy HSCs. One of the major missing pieces of information is what effect mutant *Dnmt3a*^{R878H} has on the methylome of HSC cells and whether this directly correlates with the altered gene expression reported in this thesis. Moving forward, nanopore sequencing will be performed to determine methylation of the genome of cells from *Dnmt3a*^{R878H/+} mutant mice at baseline and after exposure to γ -irradiation to complement the RNA sequencing data set. The data presented within this thesis lay the groundwork for many exciting future research directions. This new mouse model has many applications in both embryonic development and in cancer.

6.4 Conclusion

In this thesis I have presented and characterised a novel *Dnmt3a*^{R878H} mutant mouse model which will be a useful tool to understand how aberrant DNA methylation contributes to diseases ranging from developmental abnormalities, like TBRS, to haematological and non-haematological cancers. I have shown that this new mouse model is relevant for studying

embryonic development and cancer. While I have investigated the important role of mutant Dnmt3a in haematopoiesis and haematopoietic malignancies, I have also shown that mutant Dnmt3a can cause birthing defects, obesity and liver disease. More work is required to understand how mutant Dnmt3a causes these abnormalities and whether there is an opportunity to reverse some of the pathologies that I have observed by restoring normal DNA methylation. Findings from other groups have recently suggested that DNA methylation can be partially reversed in *Dnmt3a* KO HSCs by overexpression of WT Dnmt3a²³⁴. This research opens up the possibility that Dnmt3a^{R878H} driven gene expression changes may be reversible which would be promising for the treatment of patients with TBRs and Dnmt3a^{R878H} mutation driven haematopoietic malignancies. This exciting research also opens up the possibility that aberrant DNA methylation could be reversed in preleukaemic cells and thereby halt the progression to cancer. This is particularly relevant to patients with mutant DNMT3a driven clonal haematopoiesis of indeterminate potential, where there is a great opportunity to reverse aberrant DNA methylation and potentially prevent the acquisition of secondary cancer driving mutations in these patients. Furthermore, this new mouse model will be of great value to validate potential drugs that may interfere with mutant Dnmt3a^{R878H} activity and facilitate the restoration of normal DNA methylation signatures in diseases, such as cancer or cardiovascular disease, which can both be sequelae of clonal haematopoiesis.

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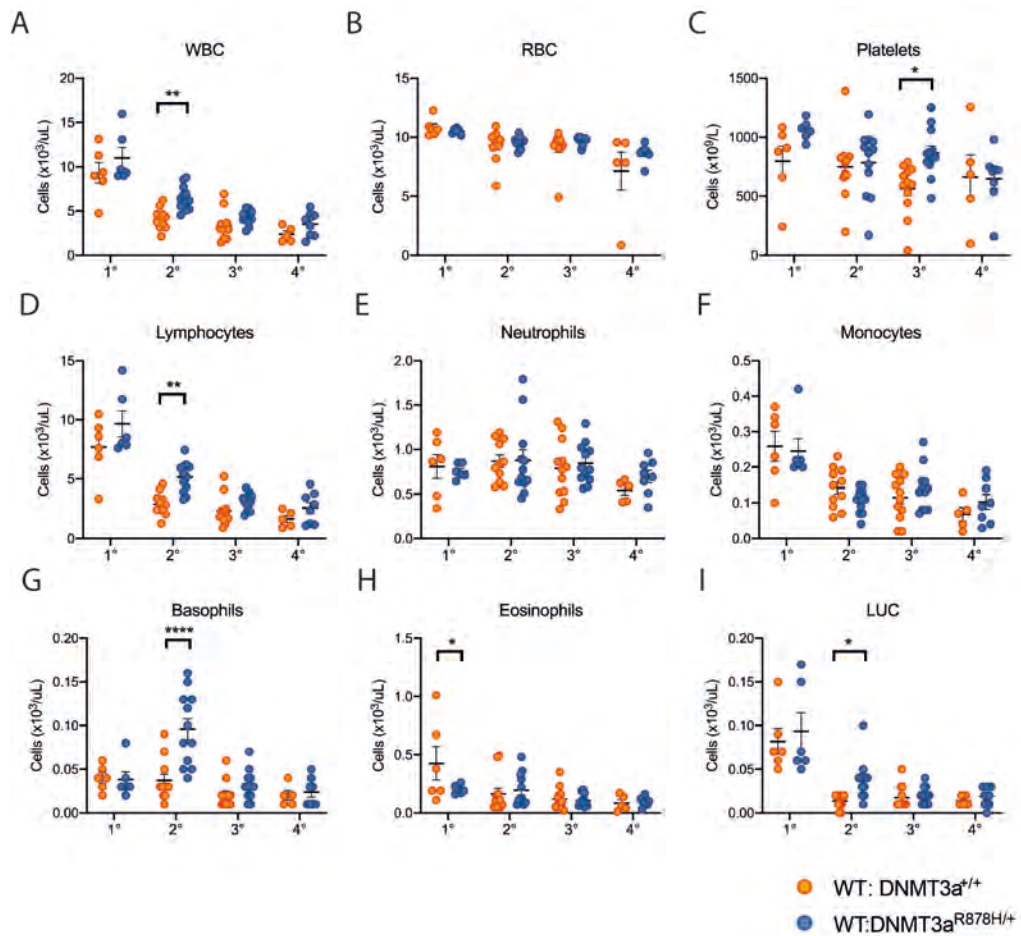
Appendix I

Cell markers used to define haematopoietic cells by flow cytometry

LT-HSC	Lin ⁻ Sca1 ⁺ cKIT ⁺ CD135/Flk2 ⁻ CD34 ⁻
ST-HSC	Lin-Sca1+cKIT+CD135/Flk2-CD34+
MPP	Lin-Sca1+cKIT+CD135/Flk2+CD34+
LSK	Lin-Sca1+cKIT+
CLP	Lin-Sca1midcKITmid
GMP	Lin-Sca1-cKIT+CD16/32+CD34+
CMP	Lin-Sca1-cKIT+CD16/32-CD34+
MEP	Lin-Sca1-cKIT+CD16/32-CD34-
Granulocytes	TCRβ-B220-Gr1+Mac1+
macrophages	TCRβ-B220-Gr1-Mac1+
Pro-pre B	TCRβ-B220midIgM-
Immature B	TCRβ-B220midIgMmid
Transitional B	TCRβ-B220+IgM+
Mature B	TCRβ-B220+IgM-
DN	CD4-CD8-
DN1	CD4-CD8-CD25-CD44+
DN2	CD4-CD8-CD25+CD44+
DN3	CD4-CD8-CD25+CD44-
DN4	CD4-CD8-CD25-CD44-
DP	CD4+CD8+
CD4+	CD4+CD8-
CD8+	CD4-CD8+
Lineage	CD4, CD8, CD11b/Mac1, Ter119, Ly6, B220, CD2, CD3, CD19, F4/80, NK1.1, Gr1

Appendix II

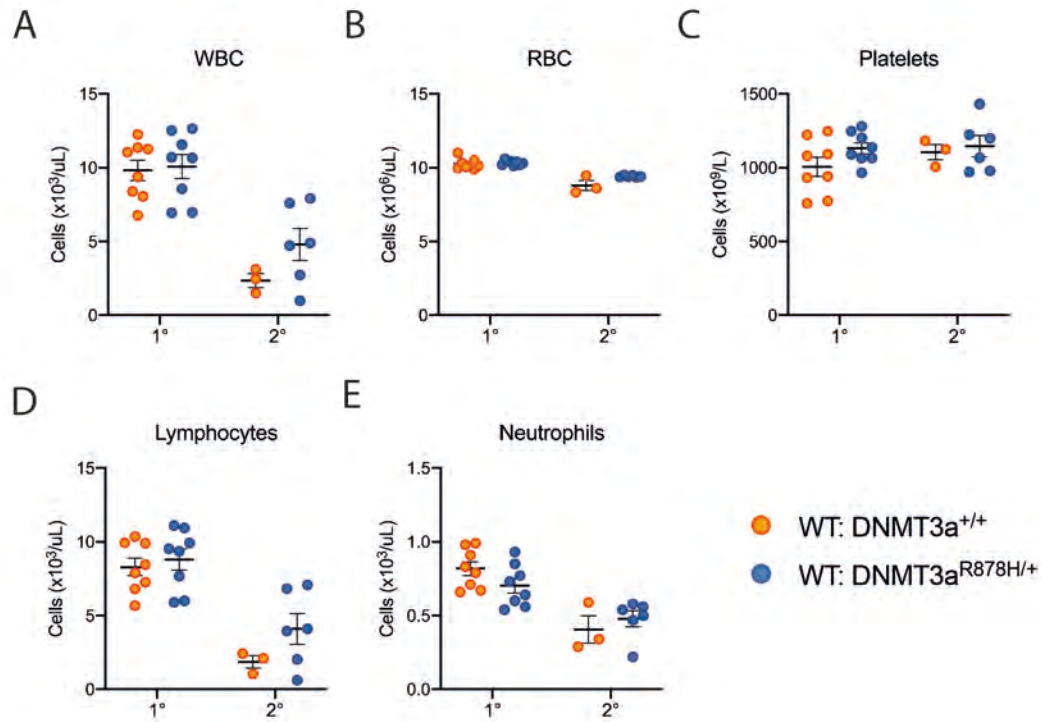
Blood cell composition determine by ADVIA at each endpoint during 3 month serial competitive bone marrow transplantation



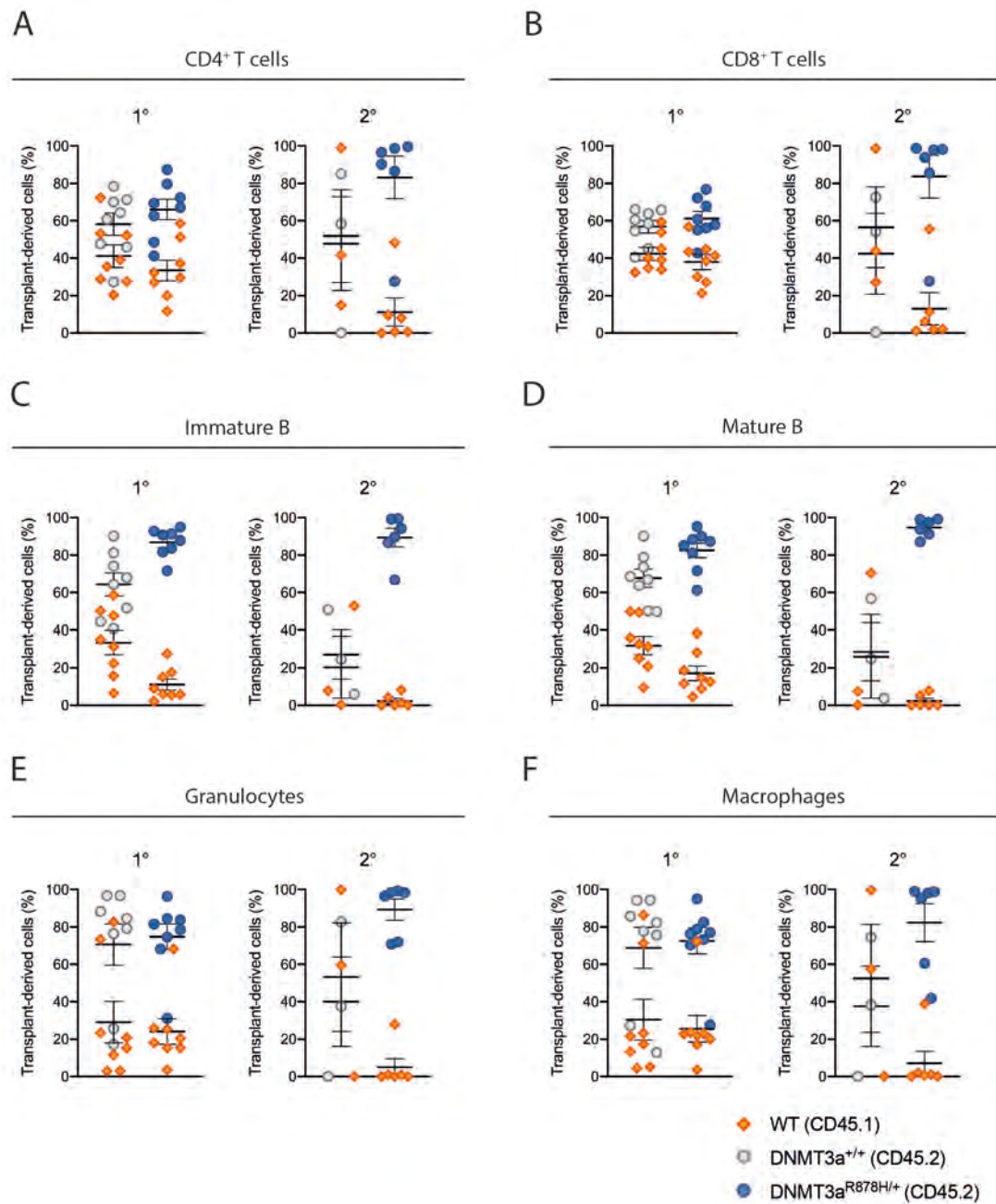
Blood cell composition was determined by ADVIA at the endpoint of each transplantation. White blood cells; WBC, Red blood cells; RBC, Large unstained cells; LUC. Students t-test *p<0.05, **p<0.01, ****p<0.0001.

Appendix III

Blood cell and thymocyte composition at each endpoint during 6 month primary and secondary serial competitive bone marrow transplantation

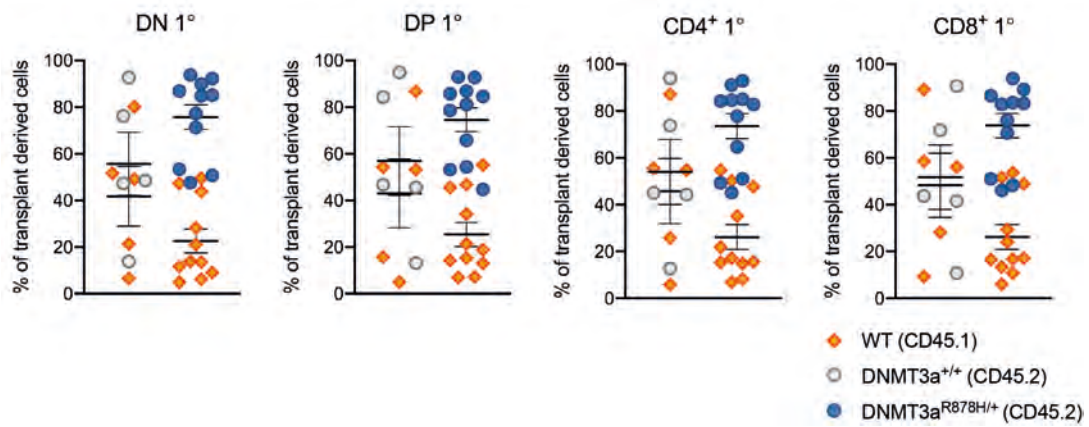


Mature Blood cells were analysed at the end of the primary and secondary endpoints of each transplant by ADVIA. A-E) The numbers of cells in the Blood of each cell type determined. WBC; white blood cell, RBC; red blood cell. Data are presented as mean $\pm$ SEM. Each data point reflects an individual mouse and four biological replicates.



Mature Blood cells were analysed at the end of the primary and secondary endpoints of each transplant by flow cytometry. The contribution of Dnmt3a^{R878H/+} (CD45.2⁺) (blue), Dnmt3a^{+/+} (CD45.2⁺) (grey) and wt (CD45.1⁺) cells are shown at the primary and secondary endpoints where cells were left for 6 months at a time. A) CD4⁺ T cells, B) CD8⁺ T cells, C) Immature B cells, D) Mature B cells, E) Granulocytes and F) Macrophages. Data are presented as mean $\pm$ SEM. Each data point reflects an individual mouse and four biological replicates.

Thymus

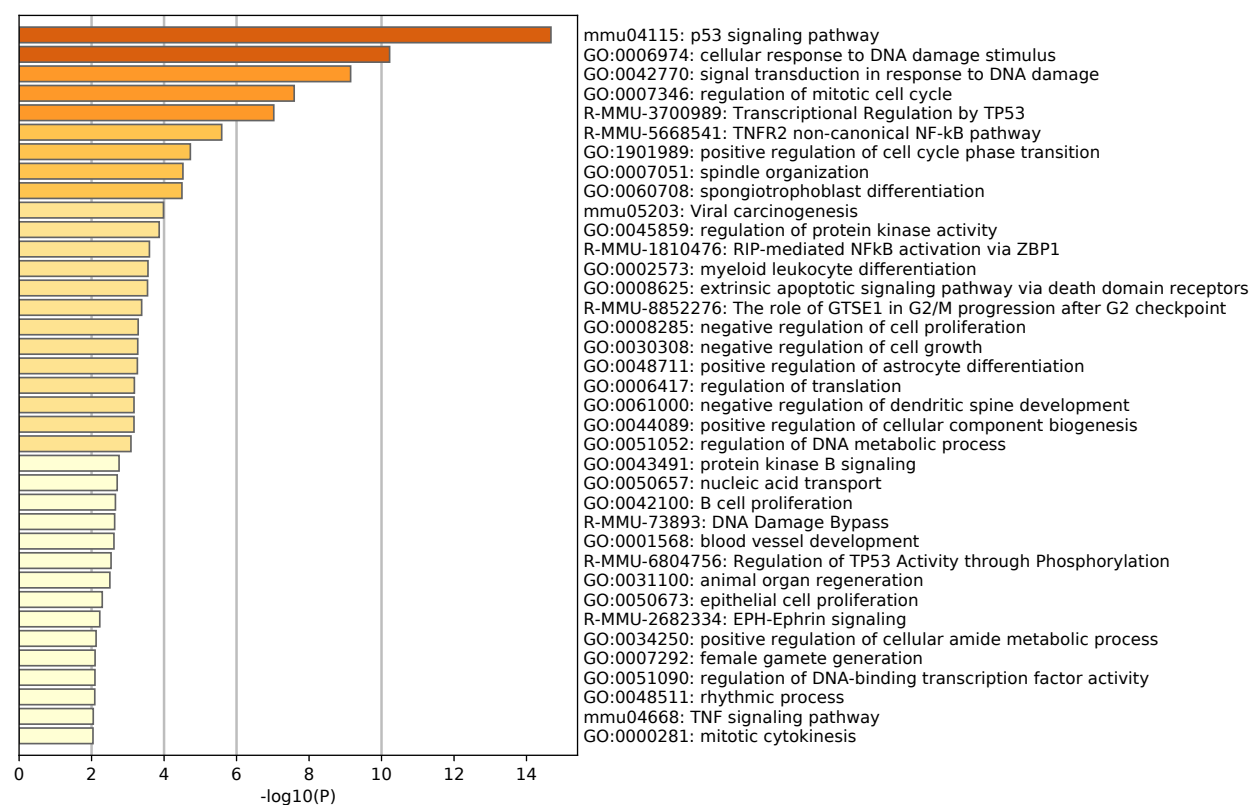


Thymocytes were analysed by flow cytometry. The contribution of *Dnmt3a*^{R878H/+} (CD45.2⁺) (blue), *Dnmt3a*^{+/+} (CD45.2⁺) (grey) and wt (CD45.1⁺) cells are shown at the primary endpoint of the 6-month competitive bone marrow reconstitution experiment. Mann-Whitney U test comparing CD45.2⁺ *Dnmt3a*^{+/+} (grey) with *Dnmt3a*^{R878H/+} (blue) cells, ns.

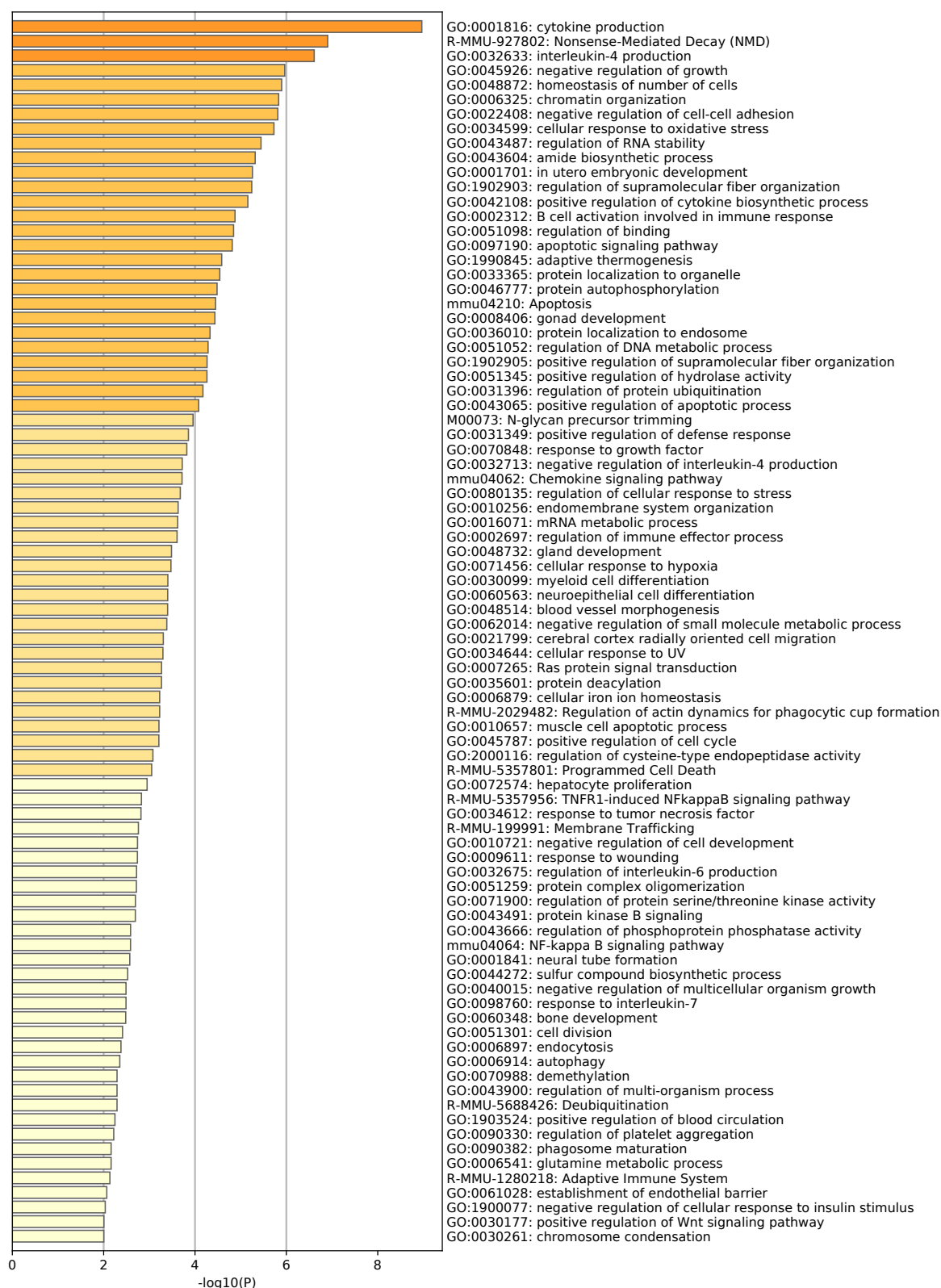
Appendix IV

Differentially regulated pathways between Dnmt3a^{R878H/+} LSK cells in response to γ -irradiation

Common pathways enriched in Dnmt3a^{R878H/+} and wt LSK cells upon γ -irradiation



Pathways enriched in wt LSK cells but not in Dnmt3a^{R878H/+} LSK cells upon γ -irradiation



Pathways enriched in Dnmt3a^{R878H/+} LSK cells but not in wt LSK cells upon γ -irradiation

