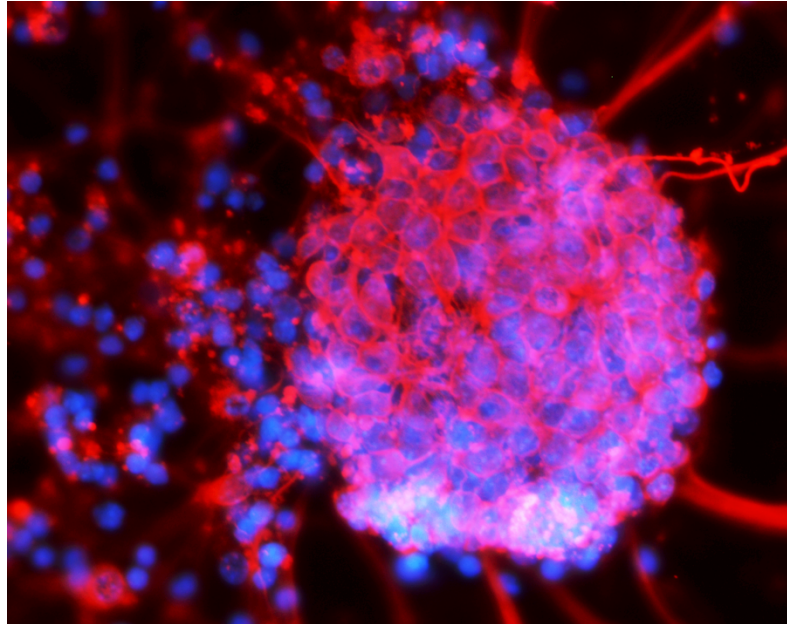


Assessing the role of histone acetylation during development

Maria I. Bergamasco



Student number 587419

Supervisors

A/Prof Anne Voss

A/Prof Tim Thomas

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Immunofluorescence of mouse neurons following differentiation of a neural stem cell neurosphere culture, stained for DAPI (blue) and β III tubulin (red). 100x magnification. Performed by Maria Bergamasco.

Abstract

The modulation of chromatin architecture is a key mechanism in the regulation of gene expression. The establishment of gene expression profiles permissive to normal development is underpinned by chromatin modifications, which render distinct gene loci accessible or inaccessible to the transcriptional machinery. Accordingly, interference with these modifications or the enzymes that mediate them can have negative consequences during development of the mammalian embryo.

Histone acetylation is a major form of chromatin modification and is associated with reduced nucleosomal density and enhanced gene activity at affected gene loci. The MYST family histone acetyltransferase, KAT6B (MYST4, MORF, QKF) is required within neural stem cells (NSCs) of the developing brain, as well as the adult subventricular zone (SVZ), where it drives stem cell proliferation and self-renewal. In the mouse model, KAT6B deficiency results in a failure to thrive and heterozygous mutation of the *KAT6B* gene causes intellectual disability disorders in humans. In addition, KAT6B fusion to CREB-binding protein (CBP) or KANSL1 has been shown to mediate acute myeloid leukaemia (AML) and leiomyomata, respectively. Currently, there is a lack of understanding regarding the role of KAT6B within the developing brain and during haematopoiesis and its effect on gene expression within these systems. As such, the underlying mechanism by which KAT6B disruption underlies disease is poorly understood.

Here I demonstrate that KAT6B coordinates gene expression within the developing brain and in neural stem and progenitor cells (NSPCs), such as to drive NSPC self-renewal, proliferation and neuronal development. I also identify key genes altered by differential *Kat6b* expression, amongst which are a number of SRY-related HMG-box (*Sox*) family transcription factor genes, including the gene encoding the pioneer transcription factor required for commitment to the neural cell lineage, *Sox2*.

In addition, I demonstrate a novel role for KAT6B within the developing haematopoietic system and in haematopoietic stem cells (HSCs). I demonstrate that KAT6B is required for normal HSC pool sizes in the embryonic and foetal liver and that *Kat6b* homozygous loss impairs HSC reconstitution in irradiated recipients. Lastly, I present a platform for assessing

potential therapeutics in *Kat6b* heterozygous animals, through the assessment of peripheral blood histone acetylation. I show that treatment with a histone deacetylase (HDAC) inhibitor can increase histone acetylation levels in heterozygous *Kat6b* deficient animals to wild type levels.

Together, the results presented in this thesis show that KAT6B promotes proliferation within both neural and haematopoietic stem cell populations, sheds light on the unique and conserved roles of KAT6B within these developmental systems and provides a platform upon which the defects of *Kat6b* mutation can be functionally assessed and therapeutically ameliorated.

Declaration

This is to certify that:

- i) This thesis comprises only my original work towards the degree of Doctor of Philosophy, except where indicated in the Preface.
- ii) Due acknowledgment has been made in the text to all other materials used.
- iii) This thesis is fewer than 100,000 words in length, exclusive of figures, tables, bibliographies and appendices

Maria I. Bergamasco

Preface

All data presented within this thesis were generated during the course of my PhD.

For RNA-seq experiments on E12.5 dorsal telencephalon and cultured NSPCs, I performed the tissue dissection and primary cell culture, as well as the RNA extraction and library preparation required for sequencing. Sequencing was performed by Dr. Stephen Wilcox. The bioinformatics analysis of the resultant data was performed by Dr. Alexandra Garnham, Dr. Yifang Hu and Professor Gordon Smyth. All graphs and tables related to the analysis of differential gene expression were generated by myself, with the exception of the correlation analysis between the RNA-seq dataset presented in this thesis and the RNA-seq data set from Bertolini et al., 2019, which was generated by Dr. Alexandra Garnham.

For foetal liver transplants I dissected foetal livers and prepared the cells for transplantation. Cells were injected into irradiated recipients by a trained animal technician. Peripheral blood, bone marrow, spleen and thymus samples were collected by a trained animal technician. Flow cytometry was performed on blood and bone marrow samples by myself. Flow cytometry on thymus samples was performed by Dr. Helen McRae and Dr. Yuqing Yang. Flow cytometry on spleen samples was performed by Ms. Angela D'Amico and Dr. Simon Willis. Analysis of flow cytometry data was performed by myself. For foetal liver transplants using sorted HSC^{SLAM} cells, I dissected foetal livers and prepared the cells prior to sorting. Cells were sorted by a trained FACS technician and injected into irradiated recipients by a trained animal technician. Peripheral blood and bone marrow samples were collected by a trained animal technician. Flow cytometry was performed on blood and bone marrow samples by myself. For colony formation using foetal liver cells, foetal livers were dissected and prepared by myself. Colony formation was performed by Ms. Ladina de Rago. Colony quantification and identification was performed by myself.

Lastly, BrdU injections into adult mice were performed in the morning by a trained animal technician and in the evening by myself. Perfusion fixation and tissue dissection was performed by myself. Tissue embedding and sectioning of adult cortices for BrdU staining was performed by the WEHI Histology Department. BrdU staining on cortical sections and analysis of BrdU positive cells was performed by myself.

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List of Abbreviations

ac	Acetylated
Acetyl-CoA	Acetyl coenzyme A
AGM	Aorto-Gonad-Mesonephros
AML	Acute myeloid leukemia
BAC	Bacterial Artificial Chromosome
bFGF	Basic Fibroblast Growth Factor
BM	Bone marrow
BMI1	BMI1 Proto-Oncogene, Polycomb Ring Finger
bp	Base pair
BPES	Blepharophimosis-Ptois-Epicanthus Inversus Syndrome
BRPF	Bromodomain and PHD finger containing
BrdU	Bromodeoxyuridine
BSA	Bovine serum Albumin
CBP	CREB binding protein
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CNS	Central Nervous system
Cr	Crotonylation
CTCL	Cutaneous T cell Lymphoma
DG	Dentate Gyrus
DLX	Distalless
DN2	Double negative type 2
DN3	Double negative type 3
DN4	Double negative type 4
DNA	Deoxyribonucleic acid
DNMT	DNA Methyltransferase
DP	Double positive
E (followed by a number)	Embryonic day
<i>E.coli</i>	<i>Escherichia coli</i>
EAF6	Esa1-associated factor 6
EGF	Epidermal growth factor
ENU	N-ethyl-N-nitrosourea
EP300	E1A binding protein p300
ESC	Embryonic stem cell
ETP	Early T cell progenitor
FDA	Food and Drug Administration
Fo	Follicular B cell
FRAP	Fluorescence recovery after photobleaching
g	Gravitational Force

GCAT	Gcn5-related acetyltransferase
GMP	Granulocyte-monocyte progenitor
GNAT	Gcn5-related N-acetyltransferases
GO	Gene Ontology
GPS	Genitopatellar syndrome
H&E	Haematoxylin and eosin
HBS	Hank's Balanced Salt Solution
H2A	Histone protein 2A
H2B	Histone protein 2B
H3	Histone protein 3
H3K4	Histone 3 Lysine 4
H3K9	Histone 3 Lysine 9
H3K14	Histone 3 Lysine 14
H3K23	Histone 3 Lysine 23
H3K27	Histone 3 Lysine 27
H3K36	Histone 3 Lysine 36
H3R2	Histone 3 Arginine 2
H4	Histone protein 4
H4K16	Histone 4 Lysine 16
HAT	Histone acetyltransferase
HBO1	HAT binding to the origin of replication (ORC) 1
HDAC	Histone deacetylase
HOX	Homeobox
HSC	Haematopoietic stem cell
HPC-1	Haematopoietic progenitor cell type 1
HPC-2	Haematopoietic progenitor cell type
ICM	Inner cell mass
indel	Insertion/deletion
ING	Inhibitor of growth
JADE	Gene for apoptosis and differentiation-family PHD finger
K	Lysine
KANSL1	KAT8 Regulatory NSL Complex Subunit 1
KAT	Lysine acetyltransferase
KAT5	Lysine acetyltransferase 5
KAT6A	Lysine acetyltransferase 6A
KAT6B	Lysine acetyltransferase 6B
KAT7	Lysine acetyltransferase 7
KAT8	Lysine acetyltransferase 8
kDa	Kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LMPP	Lymphoid-primed multipotent progenitor
lox	loxP flanked allele (floxed allele)

MAPK	Mitogen activated protein kinase
MBT	Malignant Brain Tumour
me	Methylated
me2	Dimethylated
me3	Trimethylated
MEF	Mouse Embryonic Fibroblast
MEP	Megakaryocyte-erythrocyte progenitor
MFI	Median Fluorescence Intensity
MGI	Mouse Genome Informatics database
MGZ	Marginal zone B cell
MLL	Mixed lineage leukemia
MOF	Males-absent On the First protein
MORF	Moz-related factor
MOZ	Monocytic leukemia zinc protein
MQ-H ₂ O	Milli-Q® ultrapure Type 1 water
MPP	Multipotent progenitor
mRNA	Messenger RNA
MYST	Moz, Tbf2/Sas3, Sas2 and Tip60-containing family
N	Sample size
NAD	Nicotinamide Adenine Dinucleotide
NBF	Neutral Buffered Formalin
NCOA	Nuclear Receptor Co-activator
NEMM	N-terminal park of Enok, MOZ or MORF
NMR	Nuclear Magnetic Resonance
NSC	Neural stem cell
NSPC	Neural stem and progenitor cell
OB	Olfactory bulb
OCT4	Octamer-binding transcription factor 4
O/N	Overnight
ORC1	Origin Recognition Complex subunit 1
ORF	Open reading frame
p	P value
PBS	Phosphate buffered solution
PBST	0.1% Tween20 in PBS
PCAF	P300/CBP-associated factor
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PHD	Plant homeodomain
pM	Pico Molar
PRC1	Protein Regulator of cytokinesis 1
psi	Pounds per square inch
PTCL	Peripheral T-cell lymphoma

PWWP	Proline-Tryptophan-Tryptophan-Proline
PZP	PHD-Zinc knuckle-PHD
QKF	Querkopf
qPCR	Quantitative PCR
R	Arginine
RGC	Radial Glial Cell
RMS	Rostral migratory stream
RNA	Ribonucleic acid
RNA Pol II	RNA Polymerase II
RNA-seq	RNA-sequencing
RT	Room temperature
RT-qPCR	Reverse transcriptase qPCR / Real time qPCR
RUNX1	Runt-related transcription factor 1
RUNX2	Runt-related transcription factor 2
S	Serine
SBBYSS	Say-Barber-Biesecker-Young-Simpson syndrome
SEC	Super Elongation Complex
s.e.m.	Standard error of the mean
SGZ	Sub-granular zone
shRNA	Short hairpin RNA
SOX	SRY-related HMG-box
SVZ	Subventricular zone
T	Threonine
TBX1	T-box transcription factor 1
Tg	Transgene
TGF α	Transforming growth factor alpha
TIF2	Transcriptional Intermediary Factor 2
TSS	Transcription start site
TIP60	HIV Tat interactive protein-60
WEHI	Walter and Eliza Hall Institute
VPA	Valproic Acid

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Chapter 1: Literature Review

1.1 Chromatin-mediated regulation of gene expression

Eukaryotic chromatin comprises DNA in close association with histone proteins (Kornberg, 1974). Two copies of four histone proteins: H2A, H2B, H3 and H4, are surrounded by 147 base pairs (bp) of DNA, to form the nucleosomal subunits of chromatin (Luger et al., 1997). Nucleosomal organisation represents a major regulator of gene expression, rendering different gene loci accessible or inaccessible to transcriptional machinery. As such, the cell can mediate dynamic changes in gene activity, by reorganising nucleosomal subunits.

One mechanism, by which a cell can modulate chromatin structure, is through the post-translational modification of histone residues (Allfrey et al., 1964). Histones are subject to a range of post-translational modifications by specialised chromatin-modifying enzymes (reviewed in (Allis & Jenuwein, 2016)). The dynamic addition and removal of small chemical modifications to histone residues promotes a more active or inactive chromatin state, with different modifications and combinations of modifications associated with predictable changes in gene activity (Bannister et al., 2001; Carmen et al., 2002; Lachner et al., 2001; Nishioka et al., 2002; Zegerman et al., 2002; Zeng & Zhou, 2002) (Table 1.1). Contemporary analyses of histone modifications and chromatin binding proteins, suggest the existence of 5 to 51 distinct chromatin organisational states, depending on the parameters and organism being tested (de Graaf & van Steensel, 2013; Kundaje et al., 2012). While this is consistent with the complexity observed in biological systems, it remains to be determined how cellular signals are translated into specific chromatin modifications and subsequently, changes in chromatin architecture. At present, three models aim to explain this process.

The histone charge hypothesis proposes that histone modifications alter the chemistry of histone proteins (Hansen et al., 1998; Wolffe & Hayes, 1999). Different modifications are thought to strengthen or weaken the electrostatic interactions between histone proteins and the surrounding negatively charged DNA, as well as between adjacent nucleosomal subunits. In this way, histone modifications can facilitate or sterically hinder the binding of transcriptional machinery to gene loci. For example, histone acetylation neutralises the positive charge of lysine, thereby weakening electrostatic interactions between acetylated histone proteins and DNA and promoting a more open chromatin structure (Vidali et al., 1968). Consistently, acetylated histones are easier to displace from DNA *in vivo* (Reinke & Horz, 2003; Zhao et al., 2005) and *in vitro* (Chandy et al., 2006; Hassan et al., 2006; Ito et al., 2000). Furthermore,

histone acetylation is associated with reduced chromatin compaction and increased transcriptional activity at affected loci (Robinson et al., 2008; Tse et al., 1998; Vidal et al., 1968; Wang et al., 2001), with high resolution tiling microarray analysis showing that the level of histone acetylation is proportional to the rate of transcription in yeast (Pokholok et al., 2005). Inconsistent with this model, histone lysine methylation, which does not affect lysine's charge, is associated with both increased gene expression (Litt et al., 2001; Strahl et al., 1999) and heterochromatin formation (Loyola et al., 2009; Pinheiro et al., 2012), depending on the specific histone lysine residue that is methylated.

By comparison, the histone code hypothesis suggests that histone modifications act in a combinatorial manner to generate or remove binding sites for chromatin-modifying machinery (Callebaut et al., 1999; Qiu et al., 2002; Qiu & Li, 2002; Strahl & Allis, 2000). Different modifications are thought to recruit 'reader' proteins bearing appropriate recognition domains that subsequently mediate changes in chromatin structure or recruit transcriptional machinery. In accordance with this theory, a number of proteins with the capacity for chromatin modification contain domains capable of recognising modified histone residues. For example, proteins containing Tudor, chromo, PWWP (Proline-Tryptophan-Tryptophan-Proline), MBT (Malignant Brain Tumour) and PHD (Plant Homeodomain) domains are all capable of recognising methylated histone residues {Strahl, 2000 #479; Wismar et al., 1995}. This model does not, however, resolve how the cell regulates opposing complexes bearing the same recognition domain, such as PHD domains that are observed in both HAT and HDAC complexes (Zhang, 2006).

Lastly, the signalling network hypothesis proposes that histone modifications act in a manner analogous to phosphorylation within the Mitogen activated protein kinase (MAPK) pathway, recruiting other modifying enzymes that modify both chromatin and each other (Kouzarides, 2000; Schreiber & Bernstein, 2002). In this way, the relative rates of activity of different protein recruits, regulates chromatin structure and gene expression. This final model provides a mechanism by which subtle changes in the expression or activity of a single factor, can result in alterations within an entire network, however does not resolve how the cell initiates such signalling cascades in accordance with cellular requirements.

While all three theories hold credence, none alone is able to account for all observed effects of histone modifications on chromatin structure. In reality, the complexity of gene regulation may

be explained by all three theories, in a context-dependant manner. For example, consistent with the histone charge hypothesis, acetylation of Histone H3 Lysine 14 (H3K14) occurs across large regions of a gene and has been shown to prevent gene silencing and enable *de novo* gene expression (Flury et al., 2017; Reddy et al., 2011; Wang et al., 2012), while acetylation of Histone H4 Lysine 16 (H4K16) decreases Sir3-mediated heterochromatin formation (Oppikofer et al., 2011) by disrupting the electrostatic contacts between Sir3 and chromatin (Armache et al., 2011). By comparison, Histone H3 Lysine 9 (H3K9) acetylation is more commonly limited to gene promotor regions (Liang et al., 2004; Roh et al., 2005), and has been shown to recruit chromatin reader proteins such as SEC (super elongation complex) to enable the release of paused RNA Polymerase II from gene promoters (Gates et al., 2017), following a histone code mechanism. As such, histone modifications may mediate different effects on gene activity, depending on the type of modification as well as its magnitude and location within or across the gene. This is supported by the observation that the same modification can have divergent consequences at different gene elements. When SET2-mediated methylation of Histone H3 Lysine 36 (H3K36) is artificially shifted from within the open reading frame (ORF) to the promoter region, it results in transcriptional repression rather than activation (Landry et al., 2003; Strahl et al., 2002). Unfortunately, this does not simplify the current issue surrounding chromatin-mediated gene regulation. The difficulty now becomes determining which theory applies to which modification, and, more interestingly, how the cell controls how even very similar modifications are translated into distinct physiological responses.

1.1.1 Histone acetylation and deacetylation

Histone acetylation is the first described histone post-translational modification (Phillips, 1963) and one of the most extensively studied. Catalysed by histone acetyltransferase (HAT) enzymes, histone acetylation involves the addition of an acetyl group from acetyl-Co enzyme A to lysine residues on the unstructured tails of histone proteins (Hodawadekar & Marmorstein, 2007; Roth et al., 2001). This is correlated with reduced nucleosomal density at affected gene loci and an increase in gene expression (Allfrey et al., 1964; Pogo et al., 1966). Conversely, the removal of these acetyl groups is catalysed by histone deacetylases (HDACs) (Inoue & Fujimoto, 1969; Taunton et al., 1996) and associated with increased nucleosomal density at corresponding gene loci and decreased gene activity (Winkler et al., 2010; Wiren et al., 2005) (Fig 1.1).

In mammals there are 17 canonical HATs (Allis et al., 2007) that can be structurally defined within three families; the GNAT family (GCN5-related N-acetyltransferases) (Clements et al., 1999; Lin et al., 1999), CBP and P300 family (Delvecchio et al., 2013) and the MYST family, named for its founding members Moz, Ybf2/Sas3, Sas2 and Tip60 (Yan et al., 2000). By comparison, there are 18 HDACs that are broadly categorised as either zinc-dependant or nicotinamide-adenine-dinucleotide (NAD)-dependent, on the basis of their mechanism of action (Gregoret et al., 2004). Based upon sequence similarity, zinc-dependant HDACs are further categorised into one of Class I (HDAC 1, 2, 3 and 8), Classes IIA (HDAC 4, 5, 7 and 9) or IIB (HDAC 6 and 10), Class III Sirtuins (SIRT 1-7) or Class IV (HDAC11) (Gao et al., 2002; Grozinger et al., 1999; Hu et al., 2003; Kao et al., 2000; W. M. Yang et al., 1997). In the following, I will concentrate on the MYST family of histone acetyltransferases.

1.1.2 MYST family of histone acetyltransferases

Within the mammalian genome, the largest family of HATs is the MYST family. This family represents about one third of mammalian HATs and subsequently about 33% of the mammalian genomes capacity for regulation of gene expression at the level of histone acetylation. MYST proteins are characterised by the presence of a MYST histone acetyltransferase domain and can be sub-grouped based on domain conservation outside this catalytic domain (Utley & Cote, 2003; Yang, 2004) (Fig 1.2). KAT6A (monocytic leukaemia zinc finger protein, MOZ, MYST3) and KAT6B (QKF, MORF, MYST4) form one subgroup, sharing a NEMM domain, two plant homeodomains (PHD), an acidic domain and serine- and methionine-rich domains (Champagne et al., 1999; Thomas et al., 2000). MOF (males absent on first, MYST1, KAT8) and TIP60 (HIV Tat interacting protein of 60 kDa, KAT5) form a second group with shared chromodomain (C) (Hilfiker et al., 1997; Kamine et al., 1996; Neal et al., 2000) and HBO1 (histone acetyltransferase binding to ORC1, MYST2, KAT7) stands alone, with unique serine-rich and zinc finger domains (Iizuka & Stillman, 1999). In recognition of the capacity of many HATs to also acetylate non-histone targets within cell-free acetylation assays, the MYST family proteins TIP60, MYST3, MYST4, HBO1 and MOF have recently been renamed KAT5, KAT6A, KAT6B, KAT7 and KAT8, respectively (Allis et al., 2007). Unfortunately, this new nomenclature no longer allows stratification of HATs based on the similarity of their HAT domain or identification of the MYST family by its unique acetylation domain. In addition, it

likely creates a KAT designation for proteins that are yet to be verified as genuine lysine acetyltransferases.

The importance of MYST family HATs during early life is highlighted in the consequences of their mutation (Fig 1.3). Embryos lacking *Kat5* (*Kat5*^{-/-}) die prior to implantation (Hu et al., 2009), while those lacking *Kat8* (*Kat8*^{-/-}) do not progress past the peri-implantation stage (Thomas et al., 2008). *Kat7* deficient animals (*Kat7*^{-/-}) arrest in development at embryonic day 8.5 (E8.5) and die two days later (Kueh et al., 2011). On a *C57BL/6* inbred background, animals lacking *Kat6a* (*Kat6a*^{-/-}) do not progress past E15.5 (Voss et al., 2009; Voss et al., 2012); however, *Kat6a*^{-/-} animals on a mixed genetic background (Thomas & Voss, unpublished) and animals lacking the 3' portion of exon 16 (*Kat6a*^{Δ/Δ}) on an outbred background (Thomas et al., 2006), survive to birth, but die shortly afterwards. In addition, animals that are homozygous for a mutation in the catalytic domain of KAT6A (*Kat6a*^{HAT-/-}) survive gestation, however, 40% die within 6 months of age (Perez-Campo et al., 2009). Lastly, animals deficient in 90% of *Kat6b* (*Kat6b*^{gt/gt}) survive to birth, however 80% die in the first two days of life and the remaining animals fail to thrive (Thomas et al., 2000).

1.1.3 KAT6A

KAT6A was originally identified as monocytic leukaemia zinc finger protein (MOZ) in a chromosomal translocation fusing it to CREB-binding protein (CBP) in cases of acute myeloid leukaemia (AML) (Borrow et al., 1996). Consistent with this observation, endogenous KAT6A has since been shown to be essential for normal haematopoiesis, such that loss of the KAT6A protein, either by deletion of the 3' portion of exon 16 (*Moza*^{Δ/Δ}) (Thomas et al., 2006) or targeted disruption of exons 3 to 17 (*Moza*^{-/-}) (Katsumoto et al., 2006), results in haematopoietic defects in the mouse model, including reduced numbers of lineage progenitors (Thomas et al., 2006), arrest of erythroid maturation (Katsumoto et al., 2006; Thomas et al., 2006) and a complete loss of definitive haematopoietic stem cells (HSCs) capable of reconstituting an irradiated recipient (Katsumoto et al., 2006; Thomas et al., 2006).

In addition to fusion with CBP, KAT6A has been reported in a number of chromosomal translocations involving E1A binding protein p300 (EP300, KAT3B) (Chaffanet et al., 2000)

and transcriptional intermediary factor 2 (TIF2) (Carapeti et al., 1998), resulting in myeloid malignancies with poor patient responses to chemotherapy and short median survival. Of these fusion proteins, KAT6A-TIF2 has also been shown to induce AML in mice (Deguchi et al., 2003; Huntly et al., 2004) and zebrafish (Zhuravleva et al., 2008) and to display transforming activity in cultured murine haematopoietic progenitor cells (Huntly et al., 2004). Various *KAT6A-TIF2* deletion mutants have demonstrated that loss of the PHD fingers of KAT6A, capable of histone recognition, and the CBP-binding domain of TIF2, eliminate the capacity of these fusion proteins to induce AML in mice (Deguchi et al., 2003; Huntly et al., 2004). Interestingly, mutation of the catalytic MYST domain of KAT6A was also shown to reduce AML penetrance from 88% to 50% (Deguchi et al., 2003), suggesting that the leukemic properties of the KAT6A-TIF2 fusion protein can be partially attributed to the HAT activity of KAT6A and partially to the recruitment of CBP to KAT6A chromatin binding sites. It should be noted that in these experiments, the requirement of the KAT6A HAT domain in the absence of CBP HAT function, was not tested and the contribution of other regions of the fusion protein to the remaining 50% penetrance of AML was not experimentally resolved (Deguchi et al., 2003).

Studies using a mouse mutant carrying a catalytically inactive KAT6A variant, due to a Q654E/G657E double mutated acetyl-CoA binding site within the HAT domain, demonstrated that reduction of KAT6A HAT activity to less than 10% of wild type levels, prevented AML induction in mice (Shima et al., 2014). This was supported by a second study that demonstrated that *KAT6A* heterozygosity was sufficient to reduce B cell numbers in normal mice and to increase the median survival in an *Eμ-Myc* aggressive pre B/B cell lymphoma mouse model by ~4 fold (Sheikh et al., 2015b). As such, KAT6A represents a potential target for the treatment of B-cell-associated malignancies. In recognition of this, a targeted inhibitor of the acetyl-Co A binding site of both KAT6A and KAT6B, was recently found to induce cell cycle exit and cellular senescence in a zebrafish model of hepatocellular carcinoma and to arrest the progression of lymphoma in mice (Baell et al., 2018).

KAT6A has also been identified as an inhibitor of cellular senescence. Both mouse embryonic fibroblasts (MEFs) carrying a catalytically inactive KAT6A (*KAT6A^{HAT-/-}*) (Perez-Campo et al., 2009; Perez-Campo et al., 2014) or disrupted *Kat6a* locus (*Kat6a^{-/-}*) (Sheikh et al., 2015c), displayed transcriptional upregulation of *p16^{INK4a}* and premature entry into replicative

senescence. This was associated with decreased transcriptional activity of cell cycle and senescence genes *Cdc6*, *Ezh2* and *Melk* and correlated with a reduction in H3K9 and H3K27 acetylation at their gene promoters. Interestingly, KAT6A has also been implicated in senescence avoidance in neural stem cells (NSCs) and shown to mediate NSC self-renewal by silencing *Cdkn2a* (Perez-Campo et al., 2014). While a cellular and molecular role for KAT6A in brain development is yet to be extensively explored, heterozygous mutations of the human *KAT6A* gene underlie an intellectual disability and facial dysmorphogenetic syndrome, now recognised as KAT6A syndrome (Arboleda et al., 2015; Millan et al., 2016; Tham et al., 2015) (Fig 1.4 A). The majority of these mutations have been mapped to *KAT6A*'s acidic domain and predicted to result in protein truncation, however mutations within the NEMM, PHD and Ser- and Met rich domains have also been reported (Table 1.2). Given that some of these mutations may leave the catalytic MYST domain intact, they would not be expected to eliminate KAT6A HAT activity, however could impair or alter it.

Molecular analyses of mouse embryos lacking KAT6A has shown a decrease in acetylation levels of H3K9 at specific gene loci (Voss et al., 2009). Consistently, patient-derived dermal fibroblasts from individuals with heterozygous mutations in *KAT6A* displayed a reduction in acetylation at H3K9 and an increase in acetylation at H3K18 (Arboleda et al., 2015). The reduction in H3K9 acetylation suggests that the truncating mutations assessed constitute a loss of function, whereas the increase in H3K18 acetylation may indicate a gain of abnormal function effect. Alternatively, the observed increase in H3K18 acetylation may be due to upregulation of an H3K18-targeting complex, such as CBP/p300 (Ferrari et al., 2008; Horwitz et al., 2008; Jin et al., 2011), or increased chromatin access by this complex, due to loss of the KAT6A complex. It is also of note that individuals have been identified with mutations upstream of the acetyl-coenzyme A binding portion of the catalytic MYST histone acetyltransferase domain (Millan et al., 2016). Interestingly these individuals have not been reported to display a more severe phenotype than other KAT6A-disorder patients (Millan et al., 2016), suggesting that their mutations have a minimal effect on the acetyltransferase capacity of KAT6A.

KAT6A is also required for proper *Homeobox (Hox)* gene regulation and body segmentation (Voss et al., 2009). *Hox* genes confer body segment identity, such that their mutation results in the absence, duplication or misspecification of corresponding body segments (Graham et al., 1989; Lewis, 1978; Schneuwly et al., 1987). *Kat6a* loss in the mouse model results in an anterior

homeotic transformation and reduced expression of *Hoxa1*, *a2*, *a3*, *a4*, *a5* and *a6* and *Hoxb2*, *b3*, *b4*, *b5*, *b6* and *b8*, coupled to hypoacetylation of H3K9 at the promoters of these genes (Voss et al., 2009). Consistently, ENU-mutagenesis and morpholino inactivation of the zebrafish *Kat6a* orthologue reduces *Hox* gene expression and affects segment identity in the pharyngeal arches (Miller et al., 2004). Studies in embryonic stem cells (ESCs) have demonstrated that KAT6A activity in regulating *Hox* gene expression opposes the role of polycomb repressive complex 1 (PRC1) member, BMI1 (Sheikh et al., 2015a), the mutation of which results in a posterior homeotic transformation (van der Lugt et al., 1996; van der Lugt et al., 1994). *Kat6a*;*Bmi1* double homozygous mutation was shown to rescue the anterior and posterior homeotic transformations observed in *Kat6a*^{Δ/Δ} and *Bmi*^{-/-} single homozygous mutants, respectively, and rectify aberrant *Hox* gene expression patterns, but interestingly did not rescue the levels of *Hox* gene expression in these mutants (Sheikh et al., 2015a).

Finally, *Kat6a*^{Δ/Δ} (Thomas et al., 2006) and *Kat6a*^{-/-} (Voss et al., 2009) mice display craniofacial abnormalities, including cleft palate and thymic hypoplasia, as well as cardiac septum and aortic arch abnormalities, with the latter attributed to abnormal development of the fourth pharyngeal arch (Voss et al., 2012). The cardiac septum and aortic arch defects observed in *Kat6a*^{Δ/Δ} and *Kat6a*^{-/-} mice have also been replicated by mesoderm-specific deletion of *Kat6a* using *Mespl*-driven *Cre*-recombinase, indicating cell-autonomous effects of KAT6A within this tissue (Vanyai et al., 2015). The craniofacial, cardiac and aortic arch defects in these animals resemble human DiGeorge syndrome, which results from a heterozygous microdeletion on chromosome 22q11, including the *TBX1* (T-Box protein 1) gene locus (Chieffo et al., 1997) or, more rarely, by heterozygous mutation of *TBX1* alone (Yagi et al., 2003). Indeed, *Kat6a*^{-/-} mutant mice displayed reduced *Tbx1* mRNA levels and H3K9 hypoacetylation at the *Tbx1* locus (Voss et al., 2012). Furthermore, DiGeorge syndrome-like features were observed when *Kat6a*^{+/-} heterozygosity was combined with *Tbx1* haploinsufficiency and in *Kat6a*^{+/-} heterozygous animals treated with retinoic acid (Voss et al., 2012), highlighting a susceptibility of KAT6A-mediated gene regulation to environmental impact. Notably, only the cardiac septum defects were rescued by transgenic overexpression of *Tbx1* in *Kat6a* homozygous mutants, suggesting that normal levels of KAT6A gene targets other than *Tbx1* are a prerequisite for normal craniofacial and aortic arch development (Voss et al., 2012). Later studies demonstrated that KAT6A also regulates distal-less homeobox (DLX) transcription factors *Dlx1*, 2, 3 and 5 (Vanyai et al., 2019), required for normal craniofacial development (Acampora et al., 1999; Depew et al., 1999; Jeong et al., 2012; Qiu et al., 1997; Qiu et al., 1995). A 25 –

55% reduction in these *Dlx* members, as well as the *Dlx* target genes, *Hand2*, *Gsc*, *Gbx2*, *Osr1*, *Osr2* and *Sim2* was observed in *Kat6a* deficient animals (Vanyai et al., 2019). Of these genes, KAT6A was shown via ChIP-seq and ChIP-qPCR to directly occupy the *Dlx5* locus and to be required for normal levels of H3K9 acetylation at its promoter (Vanyai et al., 2019).

Given the domain modularity of KAT6A, it is likely that individual domains mediate or contribute more significantly than others, to the multiple functions of KAT6A. Studies in mice carrying a catalytically inactive KAT6A (*Kat6a*^{HAT^{-/-}}) variant have shed light on the role of KAT6A's acetyltransferase activity more specifically (Perez-Campo et al., 2009). Unlike *Kat6a*^{-/-} animals (Katsumoto et al., 2006), *Kat6a*^{HAT^{-/-}} mice survived gestation, however 40% died within 6 months of age (Perez-Campo et al., 2009). These animals exhibited a significant reduction in HSCs and committed progenitors, as well as developmental defects in B-cell development, compared to the complete absence of HSCs observed in embryos lacking the KAT6A protein (Katsumoto et al., 2006; Thomas et al., 2006). This suggests that, while the HAT activity of KAT6A is not essential for cell specification during prenatal haematopoiesis, it is indispensable for regulating the balance between proliferation and differentiation in haematopoietic precursors. Further studies utilising single domain loss of KAT6A will be essential in exploring the roles of these different domains within tissue systems, during embryogenesis, foetal development and postnatal life.

1.1.4 KAT6B

Originally identified by its sequence similarity to the MYST family member, KAT6A (Champagne et al., 1999), KAT6B was initially characterised in a gene trap screen aiming to identify genes essential for normal brain development (Thomas et al., 2000). *Kat6b* gene trap mice (*Kat6b*^{gt/gt}), deficient in 90% of *Kat6b* mRNA, displayed low body weights, low set ears, small eyes and occluded nasolacrimal ducts, as compared to wild type, littermate controls (Thomas et al., 2000). In addition, *Kat6b*^{gt/gt} mutants displayed thin cortical plates during development, with the *Kat6b*^{gt/gt} mutant mice that survived to adulthood consistently displaying small cerebral cortices, with reduced numbers of large pyramidal neurons and cortical interneurons (Thomas et al., 2000). *Kat6b*^{gt/gt} mutant mice also displayed reduced adult forebrain NSCs, migrating neuroblasts within the rostral migratory stream (RMS) and olfactory bulb interneurons (Merson et al., 2006). Congruent with a role for KAT6B in adult NSCs, high

Kat6b gene expression levels correlated with adult NSC functions (Sheikh et al., 2012) and microarray analyses demonstrated that *Kat6b* deficiency in both the embryonic dorsal telencephalon and adult cortex (Kraft et al., 2011) affects expression of genes encoding insulin signalling pathway proteins, which modulate NSC proliferation, differentiation and survival (Aberg et al., 2000; Aberg et al., 2003; Arsenijevic & Weiss, 1998; Arsenijevic et al., 2001; Yu et al., 2008); MAPK signalling pathway proteins, required for region-specific apoptosis in the developing brain (Kuan et al., 1999; G. Y. Yang et al., 1997) and for cell fate specification of neuronal cell types (Menard et al., 2002; Paquin et al., 2005) and wnt signalling pathway proteins, essential for neural tube closure (Carter et al., 2005; Perry et al., 1995; Wu et al., 2012), proliferation and specification of neural progenitors (McMahon & Bradley, 1990; Thomas & Capecchi, 1990) and development of the hippocampus (Lee et al., 2000).

In accordance with these findings, *de novo*, heterozygous mutations in human *KAT6B* underlie mental disability syndromes (Fig 1.4 B). Point mutations and indels within KAT6B's carboxy-terminal serine (Ser) and methionine (Met)-rich domains result in the Say-Barber-Biesecker-Young-Simpson (SBBYS) variant of Ohdo syndrome (Clayton-Smith et al., 2011; Szakszon et al., 2013), while the majority of those within the acidic domain cause the more severe Genitopatellar syndrome (GPS) (Campeau et al., 2012a; Simpson et al., 2012; Szakszon et al., 2013). In addition, a single patient diagnosed with a Noonan syndrome-like disorder, carried a balanced chromosomal translocation that disrupted the *KAT6B* locus (Kraft et al., 2011), while another individual, displaying features of Blepharophimosis-Ptosis-Epicanthus-Inversus (BPES) syndrome carried a nonsense 2 bp insertion in the carboxy-terminal region of *KAT6B* (Yu et al., 2014) and two additional patients with point mutations in the acidic domain were clinically diagnosed with Lin-Gettig syndrome (Bashir et al., 2017) (Table 1.3).

The clinical ambiguity of KAT6B disorders can in part be attributed to the pleiotropic effects of *KAT6B* mutation. In addition to intellectual disability, KAT6B disorder patients present with genital abnormalities, visual impairment, hearing loss and skeletal defects, including scoliosis, syndactyly, underdeveloped or absent patellae and club feet (Table 1.4), with both the presence and severity of these features dissimilar even between patients with similar mutations. Reflective of this, one of two individuals clinically diagnosed with Lin-Gettig syndrome was found to carry a p.Ser1402Cysfs*8 mutation previously described in an individual clinically diagnosed with SBBYSS (Clayton-Smith et al., 1994), suggesting that this particular Lin-Gettig syndrome patient could have otherwise been diagnosed as having SBBYSS.

Current thinking supports KAT6B underlying two distinct developmental disorders; SBBYSS and GPS, with otherwise diagnosed individuals representing atypical cases of either of these syndromes. While it is yet to be experimentally addressed, it is thought that features common to GPS and SBBYSS are loss-of-function effects, derived from truncation of the carboxy-terminal domains of *KAT6B* (Campeau et al., 2012b). By comparison, more clinically distinct features may arise when variants are sufficiently distal to allow the transcript to escape nonsense-mediated decay and produce a truncated protein product with decreased histone acetyltransferase activity. Such variants, may also display gain-of-abnormal-function effects or dominant negative effects, due to alterations in critical binding sites, which may result in more severe phenotypes, relative to loss of KAT6B function alone (Campeau et al., 2012b).

This discussion has been further complicated by recent reports of individuals carrying *KAT6B* mutations that are clinically indistinguishable as SBBYSS or GPS patients, showing features described in either and both disorders. Interestingly, mutations in these individuals mapped to *KAT6B*'s acidic domain, between the mutational clusters for SBBYSS and GPS (Gannon et al., 2015; Vlckova et al., 2015). These reports have put into question whether GPS and SBBYSS are in fact distinct syndromes or should be reclassified within a spectrum of KAT6B-related disorders. To date, only one study has formally assessed this, clustering patients with *KAT6B* mutations based on their phenotypic features but blinded towards their diagnoses. This study was able to identify two clusters that correlated with the classification of patients as GPS or SBBYSS (Vlckova et al., 2015). Nonetheless, the authors suggested that KAT6B disorder patients should be categorised within four groups rather than two; Group 1, comprising individuals carrying proximal variants, currently associated with SBBYSS or atypical SBBYSS; Group 2 comprising individuals carrying variants associated with GPS, which lack a portion of the acidic region and the Ser- and Met-rich domains and may escape nonsense mediated decay; Group 3, comprising individuals carrying variants also expected to produce an intact or truncated acidic protein, but lacking the Ser and Met-rich transcriptional activation domains and Group 4, comprising individuals with variants located within the Ser- and Met-rich domains and exclusively associated with SBBYSS. Under this classification, Group 3 individuals are considered as a transition between Groups 2 and 4, showing features of both SBBYSS and GPS (Vlckova et al., 2015). More recently, (Radvanszky et al., 2017) suggested expanding this classification to six groups by splitting the former first group into two new groups and an additional, hypothetical Group 6, of variants distal to p.1912, within the Met-

rich region. To date, no variants have been reported for Group 6, such that any associated phenotypes remain unknown.

In addition to intellectual disability disorders, somatic *KAT6B* translocations have been shown to result in AML and myelodysplastic syndromes, with weak responses to chemotherapy, poor patient prognoses and leiomyomata (Fig 1.5 A). *KAT6B* fusion with *CBP* drives AML (Panagopoulos et al., 2001; Vizmanos et al., 2003) and therapy-related myelodysplastic syndrome (Kojima et al., 2003) (Fig 1.5 B – C). The *KAT6B*-*CBP* protein product contains the MYST HAT domain of *KAT6B* as well as the HAT domain of *CBP*, such that hyperacetylation of *CBP* and/or *KAT6B* target genes may drive disease (Panagopoulos et al., 2001). *KAT6B* fusion to *KANSL1* drives leiomyomata, which are benign uterine tumours (Panagopoulos et al., 2015) (Fig 1.5 D). Compared to *KAT6B*-*CBP*, the *KAT6B*-*KANSL1* fusion protein product contains only the NEMM domain of *KAT6B*, including a histone H1/H5 domain, capable of chromatin binding (Kitabayashi et al., 2001b) and the PEHE domain of *KANSL1*, involved in interactions with the MYST family protein, *KAT8* (Cai et al., 2010; Marin, 2003). Notably, *KAT8* is essential for cell cycle progression and survival across a number of cell types (Dou et al., 2005; Gupta et al., 2005; Smith et al., 2005; Sykes et al., 2006; Taipale et al., 2005), hence, *KAT6B*-*KANSL1* fusion may indirectly drive abnormal cell proliferation in a *KAT8*-dependant manner. Consistently, *KAT8* conditional deletion in mice has been shown to reduce tumour burden and improve survival in an *MLL*-*AF9* leukemogenesis model (Valerio et al., 2017).

In addition to driving malignancy within fusion proteins, *KAT6B* is significantly overexpressed in highly aggressive ovarian carcinomas, with its knockdown shown to reduce cell proliferation, migration and cell cycle progression within three ovarian cancer cell lines (Liu et al., 2019). Furthermore, the Ser- and Met-rich domains of *KAT6B* have been shown to bind and positively regulate the transcriptional activity of the gene encoding the runt-related transcription factor, *RUNX1*, *in vitro* (Pelletier et al., 2002). *RUNX1* is essential for foetal liver haematopoiesis (Chen et al., 2009; Yokomizo et al., 2001) and a frequent target of leukaemia-associated chromosomal translocations (Sood et al., 2017). The Ser- and Met-rich domains of *KAT6B* display transcriptional activation activity (Champagne et al., 1999; Champagne et al., 2001), such that fusion protein formation may separate these domains, or fuse them to another protein, facilitating dysregulation of *RUNX1*-mediated transcription that, in turn, may drive haematological malignancy.

Recently, *Kat6b* has also been identified as a regulator of haematopoietic differentiation, in a short hairpin RNA (shRNA) screen to identify epigenetic regulators of myeloid versus B-lymphoid differentiation (Khokhar et al., 2020). This study went on to demonstrate that *Kat6b* is highly expressed in HSCs, but its expression decreases with age, consistent with previous studies that have demonstrated a reduction in *Kat6b* in aging murine HSCs (Sun et al., 2014) and a reduction in active enhancer-associated chromatin modifications H3K4me1 and H3K27ac, at a *KAT6B*-proximal enhancer in aged human HSCs (Adelman et al., 2019). Furthermore, shRNA knockdown of *Kat6b* in young HSCs, following lentiviral transduction and 48 h *ex-vivo* culture, skewed myeloid production at the expense of erythroid cell differentiation in an *in vitro* myeloid CFU assay and *in vivo*, based on HSC transplantation into irradiated recipients (Khokhar et al., 2020). Notably, the assessment of donor-derived cells in irradiated recipients was only assessed at 1 month following transplantation in the peripheral blood and not at later time points or in the bone marrow, thymus or spleen, such that the effect of *Kat6b* knockdown on HSC and progenitor population reconstitution is unknown. In addition, the lentiviral transduction and 48 h *ex-vivo* culture of transplanted HSCs would have resulted in a number of HSCs differentiating into progenitors, which may have impacted later reconstitution results. As such, an improved understanding of the role of KAT6B in haematopoiesis and within HSCs is required in order to determine how KAT6B fusion proteins drive disease and may also reveal additional treatment options for KAT6B-mediated malignancy.

1.1.5 KAT6A and KAT6B multi-protein co-regulatory complexes

In cell-free assays using just the enzymatic domain, chromatin modifiers, including HATs, exhibit extensive target promiscuity, capable of modifying virtually any target with which they are provided (Baell & Miao, 2016; Dahlin et al., 2015; Voss & Thomas, 2018; Wapenaar & Dekker, 2016). This absence of *in vitro* specificity is thought to be resolved *in vivo* through the formation of multi-protein complexes, with auxiliary complex members acting to refine the target specificity of their associated chromatin modifier. Mass spectrometry analysis of HeLa cell extracts has shown that KAT6A and KAT6B form mutually exclusive catalytic subunits of a complex comprising inhibitor of growth family member 5 (ING5), bromodomain and PHD finger containing 1/2/3 (BRPF1/2/3) and Esa1-associated factor 6 homologue (hEAF) (Doyon et al., 2006; Ullah et al., 2008) (Fig 1.6). *In vitro* studies in Sf9 insect cells have demonstrated

that BRPF1/2/3 acts as a scaffold protein, with a short N-terminal EPcA domain containing two homology subdomains, serving as a docking site for KAT6A/KAT6B, hEAF6 and ING5 (Avvakumov et al., 2012). Adjacent to this, two PHD domains, linked by a zinc knuckle, comprise a PZP domain that can promote the catalytic activity of KAT6A or KAT6B *in vitro* (Lalonde et al., 2013). At its carboxy-terminus BRPF1 contains a proline-tryptophan-tryptophan-proline (PWWP) domain that can bind H3K36me3-rich regions at the coding regions of active genes (Vezzoli et al., 2010) and may serve to direct the complex to gene regulatory elements. BRPF1 also contains a bromodomain capable of binding H2AK5ac, H4K12ac and H3K14ac residues (Poplawski et al., 2014) and BRPF1 can be crystallised with H2AK5ac or H4K12ac peptides (Lubula et al., 2014a; Lubula et al., 2014b). BRPF1 also links KAT6A/B to hEAF6 (Ullah et al., 2008), the role of which is uncharacterised, and ING5, which contains a single PHD finger specific for H3K4me3 (Pena et al., 2006; Ullah et al., 2008). As such, ING5 may act as an adaptor subunit, conferring preferential acetylation of H3K4 peptides and stabilising the KAT6A/KAT6B complex at H3K4me3-rich regions (Ullah et al., 2008).

While the mechanism by which the cell mediates complex target specificity remains unresolved, in the context of the MYST family member KAT7 (HBO1), scaffold subunits have been suggested to confer target selection for Histone H3 or H4. Tandem-affinity purification from HeLa cell extracts has shown that KAT7 can co-purify with both gene for apoptosis and differentiation-family PHD finger (JADE), as well as BRPF scaffold proteins ((Avvakumov et al., 2012; Doyon et al., 2006; Miotto & Struhl, 2010), with the exchange of alternate scaffold subunits BRPF1/2/3 and JADE1/2/3 shown to target the KAT7 complex to H3 or H4 residues, respectively. A short EPcA-related amino-terminal domain within BRPF and JADE subunits was suggested to mediate this effect (Lalonde et al., 2013). While deletion of this region in JADE subunits was shown to result in KAT7 acetylation of both H3 and H4, its deletion in BRPF1 only reduced acetylation at H3, but did not redirect this to H4 (Lalonde et al., 2013). Furthermore, it was not demonstrated whether this amino-terminal portion, if transferred between scaffold subunits, is sufficient to redefine target specificity of the chromatin modifier complex. Nonetheless, this finding suggests that scaffold subunits are required for not only complex formation, but also for complex target selection.

In regards KAT6A and KAT6B, these proteins share an identical domain structure (Champagne et al., 1999; Thomas et al., 2000). A conserved N-terminal part of Enok, MOZ or MORF (NEMM) domain is thought to mediate nuclear localisation (Kitabayashi et al., 2001b). Tandem

plant homeodomain (PHD) fingers have been shown to recognise unmodified histone H3 tails within *Escherichia. coli* and Rosetta pLysS cell expression systems (Koh et al., 2008), with fluorescence spectrometry, ITC and analysis of NMR chemical shift perturbations demonstrating that this binding affinity is increased ~3 fold upon acetylation of H3K9 or H3K14 (Ali et al., 2012). Lastly, C-terminal Ser- and Met-rich domains, unique to KAT6A and KAT6B, are thought to interact with transcription factors RUNX1 and RUNX2, as well as the ETS transcription factor, PU.1, based on *in vitro* studies in 293 cell and Sf9 insect cell expression systems (Katsumoto et al., 2006; Kitabayashi et al., 2001b).

In addition to H3K14, the PHD domains of KAT6A have been shown to bind unmodified H3R2, an interaction that is inhibited upon methylation of this residue or trimethylation of H3K4 (Qiu et al., 2012). KAT6A has also been shown to acetylate H3K23, based on RNAi experiments in U87 and LN229 cells, with KAT6A interference also reducing acetylation of H3K9 and H3K14, but to lesser degrees (Lv et al., 2017). Mechanistically, the crystallised structure of KAT6A, bound to an H3K14ac peptide, has suggested that peptide interaction with the PHD domains forces adoption of a helical structure. This exposes the H3K9 residue of the peptide for potential acetylation by the KAT6A MYST domain. As such, the crystal structure of KAT6A favours its acetylation of H3K9, supported by *in vivo* data that shows H3K9ac is reduced at specific gene promoters when KAT6A is lost (Voss et al., 2009; Voss et al., 2012).

By comparison, the *Drosophila* homologue of KAT6B, Enok, has been shown to acetylate H3K23 (Huang et al., 2014). This is supported by a recent study using purified complexes containing BRPF1, ING4/5, MEAF6 and KAT6B fused to a FLAG tag. Complexes purified from K562 cells were shown to acetylate unmodified H3 peptides, with complex catalytic activity shown to decrease ~12 fold when peptides were acetylated at Lys23 and ~1.2 fold when acetylated at Lys14. Similar results were observed when the complex was expressed in 293T cells, with peptide acetylation reduced ~7 fold upon acetylation of Lys23 and ~1.2 fold when acetylated at Lys14. Mechanistically, the PHD domain of KAT6B, co-crystallised with an H3K14cr peptide, demonstrated a high degree of superimposition with structures for KAT6A-PHD-H3K14cr (Xiong et al., 2016) and KAT6B-PHD-H3K14bu (Brea-Fernandez et al., 2019), indicating that the acyllysine-binding mechanism is conserved between these proteins and domains. The binding affinity to H3K14cr, H3K14ac and H3 peptides was reduced ~10³ fold upon introduction of a F287A substitution mutation to the PHD domain and the catalytic activity of the mutated complex was reduced 2 fold, indicating that a functional PHD domain

is essential for the catalytic activity of KAT6B. Furthermore, ChIP on 293T cells expressing FLAG-tagged KAT6B or F287A – mutated KAT6B showed decreased association of the mutant protein, relative to wild type, while ChIP for H3K23ac and H3K14ac showed that expression of wild type KAT6B-FLAG, but not the F287A mutant, increased H3K23ac levels at gene promoters, while H3K14ac was unaffected by this KAT6B mutation (Klein et al., 2019)

As mentioned, KAT6A and KAT6B multi-protein complexes have also been shown to interact with the runt-related transcription factors RUNX1 and RUNX2 as well as the ETS transcription factor, PU.1. RUNX1 is implicated in the regulation of HSC proliferation and differentiation (North et al., 2004) and is commonly altered in leukaemia-associated translocations (Speck & Gilliland, 2002). This is consistent with the implication of both KAT6A and KAT6B in haematological malignancies and suggests that KAT6A/B translocation – driven malignancies may result from aberrant co-activation of RUNX1 gene targets. KAT6A has also been shown to interact with the ETS transcription factor PU.1 (Katsumoto et al., 2006; Kitabayashi et al., 2001a), required for normal development of myeloid and lymphoid lineages (Anderson et al., 1998; Scott et al., 1994), which may also contribute to the haematological abnormalities observed in animal models lacking KAT6A, as well as human patients carrying KAT6A fusion proteins.

By comparison, RUNX2 is essential for osteoblast development during skeletogenesis (Komori et al., 1997; Otto et al., 1997), with *Runx2*^{-/-} mouse mutants exhibiting a complete absence of bone ossification (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). By comparison, *Kat6a* mutant mice on an *FvBxBalb/C* hybrid background exhibit cleft palate at 100% penetrance (Voss et al., 2012) and *Kat6b* genetrap mutant mice display a squared, flattened skull and long first digits (Thomas et al., 2000). Notably, no ossification defect has been described in either mutant. This discrepancy may result from KAT6A and KAT6B having overlapping functions in skeletal development, such that the presence of any 2 functional alleles of the 4 combined *Kat6a* and *Kat6b* alleles, may be sufficient to prevent the more severe *Runx2* mutant phenotype. While the observation of distinct phenotypes in both *Kat6a* and *Kat6b* animal models, suggests that their roles are not entirely overlapping, studies in *Kat6a;Kat6b* double mutants will be required to expose the overlapping roles of these proteins in skeletal development. Alternatively, given that the putative interaction between KAT6A/KAT6B and RUNX2 was described in the context of an *in vitro* overexpression system, this interaction may

not occur *in vivo*. As such, assessment of the effect of *Kat6a/Kat6b* expression on *Runx2* mRNA and RUNX2 protein levels also needs to be assessed *in vivo*.

1.1.6 Chromatin modifications as therapeutic targets

During the acquisition of cellular identity, chromatin modifications underlie the progressive restriction in gene expression observed as cells commit to a specific cell lineage (Levine & Davidson, 2005). This ultimately ensures that only genes required for the cell's specialised functions are expressed, while non-essential genes become progressively silenced. As such, disruptions to the process of identity acquisition or to the regulation of gene activity during development, is ultimately reflected in the developed individual, with disruptions to gene regulation seen to underlie disease.

The requirement for chromatin-mediated regulation of gene expression during development, its disruption in disease and the reversibility of chromatin modifications, makes chromatin modifying enzymes attractive therapeutic targets. In recognition of this, a number of drugs are currently in use that disrupt or alter the activities of chromatin modifying enzymes, with a drive to develop more selective therapies and better understand the mechanistic effects of these compounds. For example, decitabine (5-Aza-2'-dC) is an FDA-approved agent that targets and prevents the action of DNA methyltransferases (DNMTs). Decitabine is currently used for the treatment of myelodysplastic syndromes and AML (Kantarjian et al., 2006), however its anti-proliferative effects have also been tested on testicular (Biswal et al., 2012), lung (Momparker, 2013), breast (Vijayaraghavalu & Labhasetwar, 2013), colorectal (Mossman et al., 2010), glioblastoma (Cui et al., 2015) and prostate cancer (Fialova et al., 2013; Hagelgans et al., 2013; Tian et al., 2012) cell types. Vorinostat and Romidepsin are FDA-approved HDAC inhibitors used against refractory CTCL (Cutaneous T-cell lymphoma) and PTCL (Peripheral T-cell lymphoma) (Coiffier et al., 2012; Duvic et al., 2009; Kavanaugh et al., 2010), shown to reactivate silenced tumour suppressor genes and act in an anti-tumorigenic capacity to promote growth arrest, apoptosis and cell differentiation (Cameron et al., 1999). Lastly, valproic acid (VPA) is a broad spectrum HDAC inhibitor currently used for the clinical treatment of epilepsy (Loscher, 2002; Rossi, 2013), as a first-line treatment for tonic-clonic seizures, absence seizures and myoclonic seizures, as well as a second-line treatment for partial seizures and infantile spasms (Mattson et al., 1978). VPA is also used for the treatment of migraine headaches (Linde et al., 2013; Reiter et al., 2005), bipolar disorder (Friedman et al., 2004;

Macritchie et al., 2001) and schizophrenia (Facciola et al., 1999; Sharma et al., 2006), however evidence for the efficacy of VPA in these disorders is based on randomized, controlled trials. Despite its pharmacological worth, VPA is also a potent teratogen (Johannessen, 2000; Phiel et al., 2001; Tunnicliff, 1999), with exposure in early pregnancy linked to neural tube defects and congenital malformations, including atrial septal defects, cleft palate, hypospadias, polydactyly and craniosynostosis (Jentink et al., 2010; Nau et al., 1991; Werler et al., 2011). VPA exposure has also been linked to the dysregulation of chromatin-mediated gene regulation within the brain, with two independent studies demonstrating that transient, VPA-induced hyperacetylation of H3 and H4 in the embryonic brain, is associated with postnatal autistic-like behaviour (Kataoka et al., 2013; Moldrich et al., 2013). Additionally, VPA has also been shown to act by non-chromatin-mediated mechanisms, shown to inhibit GSK3 β and promote Wnt signalling during the neuronal differentiation of cortical intermediate progenitors and axonal remodelling during neuronal network development (Chen et al., 1999; Hall et al., 2002). VPA exposure can also disrupt developing neuronal circuitry via GABA (Gamma-Aminobutyric acid), inhibiting GABA-degrading transaminases whilst enhancing the activity of the GABA synthesising enzyme, glutamic acid decarboxylase, to elevate GABA levels and disrupt the balance of neuronal subtypes within the developed cortex (Loscher, 1979, 1989). The breadth of action of VPA illustrates the wide range of long-lasting effects that changes to the chromatin landscape can elicit, both directly and indirectly and highlights the need for highly specific inhibitors of chromatin modifying enzymes.

1.2 Stem cells and embryonic development

Stem cells are defined by their capacity for limitless self-renewal and potential to give rise to multiple, specialised cell types (Hall & Watt, 1989; Lajtha, 1979; Lovell-Badge, 2001). The range of this differentiation demarcates a stem cell hierarchy. Atop this hierarchy is the totipotent zygote, capable of giving rise to all differentiated cell types of the mature organism, in addition to extra-embryonic structures of the placenta (Evans & Kaufman, 1981; Leblond, 1964; Martin, 1981). By comparison, embryonic stem cells (ESCs), derived from the inner cell mass (ICM) of a developing blastocyst, are able to give rise to all three germ layers of the developing embryo and described as pluripotent (Chambers, 2004). Following development, tissue-specific, multipotent stem cells are limited to give rise to specific cell types of the tissue

to which they belong. These act as cellular reservoirs, replacing cells lost or damaged throughout adult life and enabling cellular plasticity in developed tissues (Clarke et al., 2000; Ferrari et al., 1998; Krause et al., 2001; Lagasse et al., 2000; Mezey & Chandross, 2000). The capacity of adult stem cells to promote tissue integrity has made their therapeutic manipulation an attractive option for the treatment of disease, generating an ever-increasing drive to understand the mechanisms that regulate them *in vivo*.

1.2.1 Chromatin regulation in stem cell potency

As a stem cell differentiates into a specialised cell type, it undergoes a regulated restriction in gene expression, progressively losing expression of pluripotency or multipotency-associated genes, whilst simultaneously activating expression of genes specific to the cell lineage to which it will commit (Levine & Davidson, 2005). Immunofluorescence and chromatin immunoprecipitation (ChIP) studies have shown that differentiation is coordinated both genome-wide, through the establishment of global chromatin architecture permissive to cell fate allocation, as well as locally, through activation of lineage-specific genes by DNA-binding transcription factors.

Distinct differences can be observed between the chromatin of ESCs and their differentiated progeny. ESCs display fewer and more diffuse, transcriptionally inactive heterochromatin foci than differentiated cell types (Meshorer & Misteli, 2006; Meshorer et al., 2006). Upon differentiation, ESC chromatin is observed to condense into a more repressed state, associated with a global increase in the incorporation of the histone variant macroH2A and concentration of heterochromatin proteins, such as HP1, at discrete foci (Dai & Rasmussen, 2007; Meshorer et al., 2006). Fluorescence Recovery After Photobleaching (FRAP) studies in ESCs have revealed that loosely bound or soluble chromatin proteins become more stably associated with chromatin during differentiation, such that exchanging linker histone H1 with a more tightly, chromatin-bound version, inhibits cell fate commitment, whereas replacement of core histone H3 with its variant H3.3, a marker of active transcription, accelerates this process (Meshorer et al., 2006). Lineage specification and differentiation is also accompanied by a global decrease in activating trimethylated Histone H3 lysine 4 (H3K4me3) and increase in repressive trimethylated Histone H3 lysine 9 (H3K9me3) (Azuara et al., 2006; Lee et al., 2004; Meshorer et al., 2006).

Another hallmark of undifferentiated cell types is the presence of trimethylated Histone H3 lysine 27 (H3K27me3) and trimethylated Histone H3 lysine 4 (H3K4me3) bivalent domains at gene promoters (Bernstein et al., 2006; Noordermeer et al., 2014). The juxtaposition of an active H3K4me3 and repressive H3K27me3 mark defines a poised gene, as the removal of either modification will shift the locus to an inactive or active state, respectively (Bernstein et al., 2006; Noordermeer et al., 2014). In ESCs, bivalent domains are thought to silence developmental genes that would otherwise activate differentiation (Azuara et al., 2006; Bernstein et al., 2006). When differentiation initiates, only genes required for the specific lineage will be activated, whilst others will persist in a repressed state. Interestingly, poised domains seem to be enriched for evolutionarily conserved genes, including transcription factors critical for development and body patterning, while lineage-specific poising is more evolutionarily divergent (Burglin & Affolter, 2016; Lesch et al., 2016).

At the gene specific level, genes required for cell fate allocation are selectively activated by DNA binding transcription factors. The transcription factors OCT4, SOX2 and NANOG form a core transcriptional network that interact biochemically to promote and maintain embryonic stem cell potency and regulate lineage commitment to distinct germ layers (Boiani & Scholer, 2005; Boyer et al., 2005; Chew et al., 2005; Ivanova et al., 2006; Kuroda et al., 2005; Loh et al., 2006; Masui et al., 2007; Rao & Orkin, 2006; Remenyi et al., 2003; Rodda et al., 2005; Yeom et al., 1996). Expression of zygotic *Oct4* begins at the 4-cell stage, and is subsequently restricted to cells of the inner cell mass (ICM), germ cells and ESCs. *Oct4* deficiency induces differentiation of the ICM and ESCs into trophectoderm and later, cell death (Nichols et al., 1998), whereas its overexpression in ESCs promotes differentiation into the primitive endoderm and mesoderm lineages (Ivanova et al., 2006; Niwa et al., 2000). By comparison, *Nanog* expression is restricted to pluripotent cells and required for maintaining the pluripotency of ICM cells and ESCs (Mitsui et al., 2003). While its deficiency does not prevent ESC formation, it does result in their differentiation into primitive endoderm (Chambers et al., 2003; Mitsui et al., 2003). Lastly, SOX2 is essential for the maintenance of ESCs (Fong et al., 2008; Masui et al., 2007) and is the earliest marker of ICM formation (Guo et al., 2010), with loss of zygotic *Sox2* resulting in mouse embryo lethality at implantation (Avilion et al., 2003) and depletion of both zygotic and maternal *Sox2* mRNA preventing early cleavage events and development of the trophectoderm (Keramari et al., 2010). SOX2 is also required to inhibit mesodermal differentiation and promote differentiation into neural ectoderm (Taniguchi et al., 2017) with neural-specific loss resulting in neural malformations and foetal death (Ferri et al.,

2004). Beyond the initial commitment of a cell to one of the three germ layers, it undergoes further differentiation into a specialised cell type of the developed organism, with specific transcription factors acting at each differential stage to drive lineage-specific gene expression. Together, the global and specific regulation of gene expression enables cell differentiation during development and promotes cellular homeostasis following cell fate allocation. As such, understanding the factors involved in cell differentiation and chromatin regulation is essential to understand how different cell types normally develop and how transcription is coordinated in a cell type-specific manner.

1.2.2 Neural Stem Cells

Neural stem cells (NSCs) are stem cells of the nervous system (Cattaneo & McKay, 1990; Gage, 2000; Kilpatrick & Bartlett, 1993; Reynolds & Weiss, 1992; Temple & Qian, 1996). During cortical development, NSCs reside within the polarised epithelium of the ventricular zone (VZ) and undergo proliferative and differentiating cell divisions to give rise to the cellular diversity of the developing neocortex (Haubensak et al., 2004; Noctor et al., 2004). Following embryogenesis, NSCs persist within the subgranular zone (SGZ) of the hippocampus (Altman & Das, 1965; Kaplan & Bell, 1984; Seri et al., 2004) and the subventricular zone (SVZ) that lines the lateral ventricles (Altman, 1969; Lois & Alvarez-Buylla, 1994). Within the dentate gyrus (DG), NSCs of the SGZ migrate to the granule cell layer and are thought to contribute to memory formation and learning (Eriksson et al., 1998), while NSCs within the SVZ migrate as neuroblasts through the rostral migratory stream (RMS) towards the olfactory bulbs, where they give rise to olfactory bulb interneurons (Altman, 1969; Kornack & Rakic, 2001; Lois & Alvarez-Buylla, 1994; Pencea et al., 2001).

1.2.3 Neurogenesis and cortical patterning

The neocortex is the seat of higher order cognitive functions, including sensory perception, spatial awareness, problem solving, conscious thought and language. Tangentially, this structure is organised into functional areas that differ in cytoarchitecture, afferent and efferent connectivity and gene expression (O'Leary & Nakagawa, 2002; Rash & Grove, 2006; Sur & Rubenstein, 2005) (Fig 1.7). During development the cortex forms from the rostral portion of

the forebrain, via a monolayered neuroepithelium known as the dorsal telencephalon. Neuroepithelial cells (NECs) within the VZ (ventricular zone) show apico-basal polarity and undergo symmetric divisions to expand the stem cell pool and give rise to neural progenitor cells (NPCs) (Huttner & Brand, 1997; Wodarz & Huttner, 2003). While retaining their polarity, NECs elongate and begin to express glial markers, including glial fibrillary acidic protein (GFAP), astrocyte-specific glutamate transporter (GLAST), brain lipid binding protein (BLBP) and tenascin C (TNC) (Arai & Taverna, 2017; Gotz & Barde, 2005; Taverna et al., 2014) and are termed radial glial cells (RGCs). RGCs retain their cell body within the VZ but extend their long basal process to the basal lamina, allowing it to act as a scaffold for neuronal progenitors during their migration to the cortical plate (Miyata et al., 2004; Noctor et al., 2004). RGCs divide both symmetrically to expand the RGC pool, as well as asymmetrical to give rise to intermediate progenitors (Haubensak et al., 2004; Malatesta et al., 2000; Miyata et al., 2001; Miyata et al., 2004; Noctor et al., 2001; Noctor et al., 2004). These cells lack apico-basal polarity, allowing them to delaminate from the VZ and migrate upwards to the SVZ, where they divide symmetrically before undergoing a terminal division and giving rise to two neuronal daughters that migrate upwards through the developing cortical layers (Noctor et al., 2004; Sessa et al., 2008). This neurogenic capacity of radial glial cells has also led to the proposal that they are in fact neural stem cells of the developing cortex (Gotz & Barde, 2005; Heins et al., 2002).

The migration of neuronal progenitors occurs in an inside out manner, such that newly born cells migrate to form the lower cortical layers 5 and 6, while later born neurons migrate through older layers to form upper layer 2 – 4 (Nadarajah et al., 2001; Rakic, 1972; Tabata et al., 2009; Tabata & Nakajima, 2003). Following neuronal migration, the specificity of neural circuitry relies on the guidance of growing axons to their synaptic targets. To mediate axonal guidance, developing axons are tipped with a motile sensory structure known as a growth cone (Sperry, 1963; Yamada et al., 1970). This structure binds guidance molecules in the extracellular environment that in turn dictate growth cone morphology and directionality through reorganisation of the cytoskeleton. In this way, axon guidance molecules can act as chemo-attractants or chemo-repellents, such as to fine tune neuronal connections and inhibit synapse formation where it is not required (Carri & Ebendal, 1983; Colamarino & Tessier-Lavigne, 1995; Gundersen & Barrett, 1979; Heffner et al., 1990; Pini, 1993; Tessier-Lavigne, 1995).

Through each stage of neurogenesis, transcriptional and chromatin regulators play vital roles in dictating proliferation and cell fate decisions. As such, perturbation to a number of these factors has been shown to result in cell type imbalances and alterations in neuronal output and cortex size. For example, as RGCs undergo elongation, there is a redistribution of the repressive histone mark H3K27me3 to tight junction-associated genes (Albert et al., 2017) and gain of the activating mark H3K4me3 at glial-specific genes, including *Slc1a3/Glast* and *Fabp7/Blbp* (Albert et al., 2017; Schuettengruber et al., 2017), allowing these cells to gain glial cell markers and extend their basal process upwards through the developing cortex. H3K27me3 also regulates the balance between RGC self-renewing and differentiating cell divisions via the PRC2 histone methyltransferase, *Ezh2* (Pereira et al., 2010), such that *Ezh2* depletion in the developing neocortex shifts RGC divisions from self-renewing to differentiating, resulting in overproduction of intermediate progenitors at the expense of RGCs and reducing neuronal output and cortex size (Pereira et al., 2010). *Sox2* is also highly expressed in RGCs (Hutton & Pevny, 2011) and essential for their self-renewal, with single cell RNA-sequencing (scRNA-seq) of *in utero* electroporation (IUE) cortices identifying SOX2 as a negative regulator of genes that promote NPC proliferation, including *cyclin D1* (Hagey & Muhr, 2014). Upon differentiation into intermediate progenitors, *Sox2* is repressed by NEUROG2, allowing de-repression of *cyclin D1* and intermediate progenitor proliferation (Hagey & Muhr, 2014). Genes encoding other factors important in intermediate progenitor generation and neuronal differentiation, such as the transcription factor genes *Insm1*, *Eomes*, *Neurog1/2* and *Neurod1/2*, are H3K27me3-positive during the expansion phase of NSCs (Albert et al., 2017), such that loss of this repressive mark may contribute to their precocious activation, altering the balance of neocortical cell types. This has already been demonstrated for *Eomes*, the CRISPR/Cas9-mediated disruption of which results in an acute reduction in intermediate progenitors and increased neuronal differentiation (Kalebic et al., 2016).

Following progenitor pool expansion, NPCs undergo cell fate specification in order to give rise to specialised neuronal subtypes. The transcriptional repressor FOXG1 is required and sufficient to induce deep-layer neurogenesis, switching the transcriptional program of progenitor cells to acquire upper layer neuron identity through the repression of *Tbr1*, a transcription factor and marker of early born post-mitotic neurons (Hevner et al., 2001; Toma et al., 2014). The transcription factors FEZF2 and CUX2 are markers of deep and superficial layer neurons, respectively, and are required for cell fate specification (Guo et al., 2013; Nieto et al., 2004), while the hedgehog pathway transcription factor GLI3 promotes intermediate

progenitor production from RGCs and their fate-specification into superficial neurons (Wang et al., 2011).

Following neuronal development, remaining NSCs and progenitors switch to gliogenesis, generating astrocyte and oligodendrocyte supporting cell types of the nervous system (Levison et al., 1993; Qian et al., 2000). The timing of this shift is critical for brain development, as it dictates the final number of cortical neurons produced and ensures sufficient supporting cell types to maintain the newly formed neural circuitry. This timing is regulated by a number of factors that coordinate to promote the expression and repression of neurogenic and astrogenic genes, depending on the stage of cortical development.

During the neurogenic phase, PcG proteins promote expression of the pro-neural transcription factor gene, *Neurog1*, as well as by directly repressing the key astrogenic marker gene, *Gfap*, in order to prevent the onset of gliogenesis (Sparmann et al., 2013). NEUROG1 in turn induces neuronal development by binding CBP-SMAD1 transcriptional complexes, which recruit it to the promoters of neuronal genes, including NEUROD transcription factors. Simultaneously, the binding of NEUROG1 to CBP-SMAD1 complexes, prevents their binding to STAT1/3, which otherwise induces expression of GFAP and glial cell differentiation (He et al., 2005; Sun et al., 2001). H3K27me3 by PcG complexes progressively increases at the *Neurog1* promoter, such as to gradually suppress *Neurog1* expression. This results in PcG proteins inhibiting *Neurog1* expression at the neurogenic-to-astrogenic switch, restricting neurogenic competence in NSPCs in order to allow astrogenesis to proceed (Hirabayashi et al., 2009; Sparmann et al., 2013).

The initial development of neuronal networks during embryogenesis is followed by their refinement in early postnatal life by synaptic pruning. Synaptic pruning involves the selective elimination of axons that are inappropriately synapsed or seldom activated (Hua & Smith, 2004; Katz & Shatz, 1996; Sanes & Lichtman, 1999). This degeneration adjusts the size of neuronal populations in accordance with the functional needs of their projection fields and in so doing, represents both a fine-tuning mechanism for neuronal circuitry, as well as means of enabling maturation and plasticity within the developing nervous system. As such, disruption to this process may impede cortical maturation and the development of higher order brain processes, an idea supported by the observation that synaptic pruning is inefficient in a number of autism spectrum disorders (Casanova et al., 2002; Casanova et al., 2006; Tang et al., 2014;

Wegiel et al., 2010), such that autism spectrum disorders may arise in part, due to hyperconnected or aberrantly connected cortical networks.

1.2.4 Adult neural stem cells

Following development, the adult cortex retains two NSC populations, mentioned briefly above; one in the SGZ that contributes to memory formation and learning (Altman, 1962; Altman & Chorover, 1963; Altman & Das, 1965, 1966; Kaplan & Hinds, 1977; Seri et al., 2001) and one within the SVZ, involved in olfaction and olfactory memory (Lois & Alvarez-Buylla, 1993) (Fig 1.8). The SGZ is a narrow layer of cells located between the granule cell layer and hilus of the dentate gyrus, comprised of NSCs in various stages of development, as well as astrocytes, endothelial cells and blood vessels, which together regulate NSC proliferation, migration and differentiation (Seri et al., 2004). Lineage tracing experiments have demonstrated that NSCs undergo consecutive developmental stages in order to become functional neurons and integrate into hippocampal circuitry (Kempermann et al., 2008; Seri et al., 2001). NSCs firstly give rise to type 1 radial glial cells (RGCs) that express GFAP, Nestin and SOX2. These serve as non-neuronal support cells, with their cell bodies in the SGZ and radial processes extending into the molecular layer to act as a scaffold upon which newly formed neurons can migrate (Bonaguidi et al., 2011; Lugert et al., 2010). RGCs then divide asymmetrically to form one new RGC and one intermediate progenitor cell. Intermediate progenitor cells are highly proliferative type 2 neuronal progenitors that rapidly expand the precursor pool and represent a transitory state in NSC development, during which NSCs lose glial cell characteristics and gain neuronal traits including expression of NEUROD (Steiner et al., 2006) and PROX1 (Pleasure et al., 2000). Transit amplifying cells subsequently give rise to type 3 neuroblasts that express DCX and PSA-NCAM, which proliferate and in turn form post-mitotic neurons that develop the morphology of hippocampal granule cells, marked by extension of dendrites into the DG molecular layer, axonal growth into the CA3 region and formation of synaptic connections (Brown et al., 2003; Rao & Shetty, 2004).

The rate of adult hippocampal neurogenesis is strongly associated with learning and memory, with newborn neurons from the SGZ believed to provide functional plasticity within hippocampal circuitry. These networks are also heavily influenced by environmental inputs. Animals reared in an enriched environment possess adult neurons with a greater number of

inputs from local and distal neurons, as well as mammillary bodies and interneurons in the CA1 and CA3 of the hippocampus, which are absent in animals reared in a non-enriched environment (Bergami et al., 2015). This suggests that reorganisation of postsynaptic connectivity occurs in an experience-dependant manner and that reorganised neurons may modulate synaptic integration and information processing within the DG (Kempermann et al., 1997).

The second site of adult neurogenesis is the SVZ. SVZ – derived NSCs ultimately generate interneurons of the olfactory bulbs (Lois & Alvarez-Buylla, 1994), which contribute to fine odour discrimination and odour-reward association (Grelat et al., 2018; Li et al., 2018; Lledo & Saghatelian, 2005). Within the SVZ, B1 cells represent NSCs of the adult forebrain (Doetsch et al., 1999a). These cells apically contact CSF and blood vessels via a long basal process and are surrounded at their apical surface by multi-ciliated ependymal cells. B1 cells undergo symmetrical self-renewal and can also generate B2 cells (Obernier et al., 2018), a population of proliferative astrocytes, with a currently unknown function (Doetsch et al., 1997). Like B1 cells, B2 cells contact the vasculature, however do not contact the lateral ventricle. Both B1 and B2 cells give rise to transit amplifying intermediate progenitors (C cells), which divide three to four times before generating rapidly amplifying neuroblasts (A cells) (Doetsch et al., 1999b; Ponti et al., 2013). Neuroblasts are migratory cells that enter the RMS to reach olfactory tissue, where they differentiate into olfactory bulb interneurons and integrate into pre-existing circuitry (Doetsch et al., 1999b; Lois & Alvarez-Buylla, 1994; Lois et al., 1996). To mediate their tangential migration, the RMS comprises a layer of ciliated ependymal cells that interface the RMS and cerebrospinal fluid (CSF) of the adjacent lateral ventricles (Doetsch et al., 1997). These cells act as both a structural barrier and transporter of CSF components, including growth factors TGF α (Jordan et al., 2007) and bFGF (Hayamizu et al., 2001), using the coordinated beating of their cilia to generate chemical guidance cues that direct migrating neuroblasts towards the olfactory bulbs (Sawamoto et al., 2006). Three-dimensional imaging shows that the SVZ niche lies in close proximity to blood vessels (Q. Shen et al., 2008; Tavazoie et al., 2008). Unlike vasculature at other neurogenic regions, SVZ blood vessels are devoid of the astrocytic foot processes and pericyte coverage that habitually maintain the impermeability of the blood brain barrier. This allows exchange of small molecular weight factors between the blood vessels and niche and is particularly pertinent to injury, where changes in blood composition can mediate neuroblast divergence to sites of damage, including to regions where

post-natal neurogenesis does not usually occur (Arvidsson et al., 2001; Magavi et al., 2000; Parent, 2002).

Despite the observation of high proliferative activity in the SVZ and RMS of humans, the low reliance of humans on olfaction and small size of the olfactory bulbs, relative to other species, has put the extent of olfactory bulb postnatal neurogenesis into question (Bergmann et al., 2012; Curtis et al., 2007; Sanai et al., 2011). Rather, it has been postulated that SVZ NSCs in humans also support circuitry of the frontal cortex. In support of this, substantial numbers of striatal neurons are generated within the SVZ of rodents and monkeys following stroke (Arvidsson et al., 2001; Tonchev et al., 2005; Tonchev et al., 2003), suggesting that SVZ NSCs can be diverted to sites of injury, potentiating their use as therapeutic tools in traumatic brain injury. In addition, neuroblasts and newborn neurons derived from the SVZ have been shown to migrate to the frontal cortex and cingulate cortex in the infant brain, with the numbers of these cells shown to dramatically drop between 4-6 months and 8-18 months (Sanai et al., 2011), while in the adult human cortex, DCX and PSA-NCAM-expressing neuroblasts are present in both the SVZ and adjacent striatum, indicative of their migration to these regions and confirmed by IdU in striatal interneurons and retrospective birth dating of striatal neurons (Ernst et al., 2014). Given that birth represents the largest environmental change a human will experience, neurogenesis and neuroblast migration to the human frontal cortex during perinatal periods may provide additional plasticity within the brain to support higher order neuronal processes. Beyond this early period, the steady-state turnover of SVZ-derived NSCs may enable additional plasticity within cortical networks, as well as a response system to cortical trauma such as stroke.

1.2.5 Differences between embryonic and adult neural stem cells

While adult neurogenesis has been viewed as a simple continuation of embryonic neurogenesis, there are notable functional differences between NSPCs derived from the embryonic and adult brain that demonstrate that this is not the case. Despite being morphologically similar, their proliferation, fate, molecular mechanisms and the niches to which these cells are exposed, are notably distinct. In addition, although many transcriptional regulators are shared in neurogenesis at embryonic and adult stages, the mechanisms by which neuronal fate

determinants control fate acquisition and maintenance differs between development and adulthood.

The first difference between adult and embryonic NSPCs is the environment to which these cells are exposed. In the embryonic brain, RGCs are surrounded by other radial glial cells as well as migrating neuroblasts, with the majority of NSPCs driven to form neuronal cell types at the expense of glial cell development (Miller & Gauthier, 2007). By comparison, adult NSPCs are directly contacted by ependymal cells and a unique vascular network that largely drives gliogenesis and limits neurogenesis to the SGZ of the DG and SVZ of the lateral ventricles. This difference in cellular output between the environments of the embryonic and adult brain has been demonstrated in transplantation studies using embryonic and adult NSPCs. While both cell types show a strong neurogenic capacity *in vitro*, neither can efficiently generate neurons when grafted in the adult brain, and rather, show a strong gliogenic tendency (Cao et al., 2002; Enzmann et al., 2005; Herrera et al., 1999; Hofstetter et al., 2005). The same is true following injury within the CNS, with most cells shown to be incapable of regenerating neurons, rather because glial cell types or remaining undifferentiated *in vivo* (Barnabe-Heider et al., 2010; Ohori et al., 2006; Yamamoto et al., 2001; Yang et al., 2006).

Niche differences also influence the modes of action of transcriptional regulators of neurogenesis. Different intrinsic levels of neurogenic fate determinants such as PAX6, GSX2, DLX, ASCL1, NEUROG2 and NEUROD1, influence the capacity of embryonic and adult NSPCs to give rise to neurons in culture. In a low-density primary culture system, adult NSPCs, with low mRNA levels of a number of neurogenic factors (Beckervordersandforth et al., 2010), did not directly differentiate into neurons, but rather generated a series of transit amplifying cells and neuroblasts through sequential cell divisions, which result in the progressive upregulation of neurogenic determinants (Costa et al., 2011; Ninkovic et al., 2013). By comparison, embryonic NSPCs have intrinsically high levels of neuronal determinants and are able to undergo direct neuronal differentiation (Haubst et al., 2004; Heins et al., 2002).

Another significant difference between embryonic and adult NSPCs is their rate of division. Where most RGCs of the developing brain have a short cell cycle, ranging from 10-18 h (Gotz and Huttner, 2005; Dehay and Kennedy, 2007; Lange and Calegari, 2010; Arai et al., 2011), adult RGCs have a cell cycle of several days to 2 weeks (Morshead et al., 1994; Bonaguidi et al., 2011; Lugert et al., 2012). This reflects the need in embryogenesis for tissues and structures

to be formed relatively quickly, requiring a short cell cycle and active proliferation, while in the adult, cell division is rare to minimise the accumulation of genomic mutations and shortening of telomeres in cells that must reside within the adult brain for the majority of adult life. Further to the point of proliferation, cell division requirements differ between the embryonic and adult brain. During development, NSPCs are required to generate a fixed number of neuronal and glial cell types, limited by the final size of the brain. Conversely, adult NSPCs are not limited in this manner, with neuronal and glial cell output based on the requirement for plasticity within developed neural networks or during times of injury to the CNS.

1.2.6 Neural stem cell isolation

The existence of NSCs was first definitively demonstrated through their long-term maintenance in culture and differentiation into major neuronal cell types, *in vitro* (Lois & Alvarez-Buylla, 1993; Reynolds & Weiss, 1992). This work led to the establishment of the neurosphere culture assay, in which NSCs are isolated from embryonic and adult brain tissue, dissociated into a single cell suspension and cultured in defined, serum-free conditions in the presence of specific mitogenic factors EGF and bFGF (Gritti et al., 1999; Morshead et al., 1994; Reynolds & Weiss, 1992, 1996; Vescovi et al., 1999). Under these conditions NSCs can give rise to proliferating, floating colonies known as neurospheres, which demonstrate self-renewal through long-term passaging and differentiation into neuronal and glial cell types. The defined conditions for isolation and expansion of neural precursors subsequently led to rapid advancements in the understanding of NSC biology, permitting the assessment of neural precursor proliferation, survival, self-renewal and differentiation and has since been adapted for the expansion of brain cancer cells (Buffo et al., 2008) and non-neural precursors such as carotid body stem cells (Pardal et al., 2007).

Unfortunately, there are notable caveats to the *in vitro* study of NSCs. Sustained exposure to FGF alters both cellular and temporal identity, inducing upregulation of ventral markers *Olig2* and *Mash1*, while restricting neuronal differentiation into GABAergic interneurons (Gabay et al., 2003; Hack et al., 2004) and inducing expression of EGFR, a marker typically found in late stages of embryogenesis (Ciccolini et al., 2005; Gabay et al., 2003; Pollard et al., 2008). In addition, experimental analysis may be compromised by cellular heterogeneity, as only a small

proportion of cells within a neurosphere represent NSCs, with the potential to form secondary neurospheres (Reynolds & Rietze, 2005; Reynolds & Weiss, 1996). Furthermore, apoptosis within the core of the sphere, where cells are poorly exposed to growth factors or nutrients of their culture medium, may complicate experimental manipulation of these cells and increase variability across samples or culture passages. Alternatively, NSCs can be cultured as adherent, clonal lines (Sun et al., 2008), which are amenable to chemical or genetic screens, biochemical studies and population-based analysis of gene expression and transcriptional regulation. Despite these advantages, it is possible that in the absence of non-NSC neurosphere cell types, adherent NSCs behave discordantly *in vitro* than *in vivo*. As such, all *in vitro* results should be supported and validated by *in vivo* studies.

In vivo, NSCs can be identified by their long cell cycle and hence, long-term retention of thymidine analogues (Morshead et al., 1998; Morshead et al., 1994). The synthetic thymidine analogue BrdU (5-bromo-2-deoxyuridine), incorporated into replicating DNA and subsequently detected using anti-BrdU antibodies, can be used to identify proliferating cells. While rapidly proliferating cells dilute the label through continued cell divisions or migrate away from the region of interest, slowly proliferating, stationary cells such as resident NSCs, retain the label over time. NSCs can also be identified *in vivo* using intracellular and cell surface markers. The co-expression of nestin (Lendahl et al., 1990) and SOX2 (D'Amour & Gage, 2003) identifies NSCs, however, the intracellular distribution of these proteins limits their use as selective antigens for live cells. Alternatively, NSCs can be identified using the cell surface markers CD133 and SSEA1/CD15 (Capela & Temple, 2002; Corti et al., 2007), with SSEA1 expressed in subpopulations of CNS cells during development and adulthood (Allendoerfer et al., 1995; Capela & Temple, 2002; Yamamoto et al., 1985) and CD133 demarcating a cell population capable of generating neurospheres and differentiating into the three neuroectodermal lineages (Corti et al., 2007).

1.2.7 Haematopoietic Stem Cells

Haematopoiesis generates all mature blood cell types during embryogenesis and adulthood, balancing blood cell production requirements with the precise regulation of each cell type (Dieterlen-Lievre, 1975; Godin et al., 1993; Medvinsky et al., 1993; Moore & Owen, 1965). Haematopoiesis is sustained by self-renewing, multipotent haematopoietic stem cells (HSCs)

that differentiate into progressively more lineage restricted progenitors, capable of rapid proliferation, but lacking long-term self-renewal (Krause et al., 2001; Notta et al., 2011). Multipotent progenitors (MPPs) give rise to oligopotent common myeloid progenitors (CMPs) and multi-lymphoid progenitors (MLPs). CMPs in turn differentiate into granulocyte/macrophage progenitor (GMPs) and megakaryocyte/erythroid progenitors (MEPs) while MLPs represent the progenitor cell type for common lymphoid progenitors (CLPs), from which B, T and natural killer (NK) cells are derived.

Recently, single cell RNA-seq analysis of human bone marrow has put the traditional hierarchical paradigm of haematopoiesis into question. Optimised single cell functional assays and genetic analyses have demonstrated that classically defined CMPs and MEPs exist as a heterogeneous population, largely composed of uni-lineage committed cells (Notta et al., 2016). A second study has gone on to show that both HSCs and their immediate progenitors exist within a spectrum of low-primed, undifferentiated haematopoietic stem and progenitor cells (HSPCs), which undergo progressive transcriptomic lineage priming into more discrete populations (Velten et al., 2017). These findings suggest that HSCs and MPPs can give rise to unipotent progenitors without an intermediate oligopotent progenitor state and that previously defined cell populations, based on shared cell surface markers, represent substantially broader sub-categories than previously thought (Fig 1.9).

1.2.8 Haematopoiesis

During embryogenesis, haematopoiesis occurs in waves, with the site of haematopoiesis and the major cell type produced observed to shift in accordance with embryonic requirements (Fig 1.10). The first wave occurs between E7.0 and E7.5 in mice, with the ingression of extra-embryonic mesoderm through the posterior primitive streak and its haematopoietic differentiation in the yolk sac (Lawson et al., 1991; Silver & Palis, 1997). From E7.5 bipotent mesodermal cells of the epiblast, termed hemangioblasts (Choi et al., 1998; Eichmann et al., 1993; Shalaby et al., 1995) migrate posteriorly to enter the yolk sac (Sabin, 2002), committing to an endothelial or haematopoietic lineage (Ueno & Weissman, 2006) and contacting yolk sac endodermal cells to initiate blood island formation within this tissue (M Belaussoff, 1998). The first major haematopoietic output of the yolk sac are large, nucleated erythrocytes, as well as primitive macrophages and megakaryocytes (Fukuda, 1973; Luckett, 1978). These serve as the

primary source of embryonic red blood cells (Gekas et al., 2005; Godin et al., 1993; Medvinsky et al., 1993; Moore & Metcalf, 1970; Palis et al., 1999; Palis & Yoder, 2001). Notably, lineage-tracing studies using multicolour chimeric mice have shown that at E7 – E8.5, each yolk sac blood island contains multiple clones of either endothelial or blood cells, but not both lineages (Ueno & Weissman, 2006), suggesting that a single hemangioblast blood island does not give rise to both blood and endothelial cells.

The second wave of foetal haematopoiesis is characterised by the development of erythroid-myeloid progenitors (EMPs) (Boiers et al., 2013; McGrath et al., 2015). These progenitors emerge in the yolk sac at around E8.5 from hemogenic endothelial cells (Bertrand et al., 2010; Boisset et al., 2010; de Bruijn et al., 2002; Kissa & Herbomel, 2010) and, compared to the first wave that produces primitive, nucleated cells, the second wave gives rise to definitive erythrocytes and myeloid cells (Lux et al., 2008; Palis et al., 1999). The third wave initiates at E9 and is marked by a surge of multipotent progenitors (MPPs) from the yolk sac and aorta-gonad-mesonephros (AGM) (Godin et al., 1995; Inlay et al., 2014), some of which demonstrate a lymphoid lineage bias (LMPPs) (Boiers et al., 2013; Cumano et al., 1996; Lu & Auerbach, 1998; Lu et al., 1996; Nishikawa et al., 1998; Yokota et al., 2006; Yoshimoto et al., 2011; Yoshimoto et al., 2012).

From E9-E11, the precursors of long-term repopulating HSCs develop from the hemogenic endothelium of the AGM. These cells transition from pro-HSCs to pre-HSC type I and pre-HSC type II, which give rise to definitive HSC (Chen et al., 2011; Gekas et al., 2005; Kumaravelu et al., 2002; Medvinsky & Dzierzak, 1996; Rybtsov et al., 2014; Rybtsov et al., 2011; Taoudi et al., 2008). All HSC precursor stages express cKit (Rybtsov et al., 2014), with the cKit ligand, SCF, identified as a key regulator of precursor HSC in the AGM (Rybtsov et al., 2014). Correspondingly, there is a decline in HSC activity in SCF mutant mice (Ding et al., 2012). At all stages these cells express endothelial markers such as vascular-endothelial cadherin and CD31 and subsequently upregulate haematopoietic surface markers, CD41 (pro-HSCs), CD43 (pre-HSC type I) and CD45 (pre-HSC type II). Unlike definitive HSCs, pre-HSCs cannot reconstitute the adult haematopoietic system by direct transplantation, as they still require maturation in an embryonic or neonatal environment (Rybtsov et al., 2014; Rybtsov et al., 2011; Taoudi et al., 2008).

At E11.5 definitive HSCs migrate from the AGM to the foetal liver, with their proliferation in this organ peaking at E14.5. Migration is mediated via chemotaxis, with CXCL12, expressed on stromal cells and its receptor, CXCR4, on HSCs (Ma et al., 1998; McGrath et al., 1999) and further amplified by cytokine receptor binding of SCF and KIT (Broxmeyer et al., 1991; Christensen et al., 2004) as well as the transcription factor PITX2, which must be expressed on stromal cells to support HSC function (Kieusseian et al., 2006). Finally, from E16.5, foetal liver HSCs migrate to the bone marrow, where they will reside into adulthood (Christensen et al., 2004; Gekas et al., 2005).

The establishment of haematopoiesis, its shift from primitive to definitive stages and the lineage commitment of haematopoietic stem and progenitor cells, is subject to regulation by transcription factors. At the gene specific level, transcription factors TAL1 (Porcher et al., 1996; Shivdasani et al., 1995), LMO2 (Boehm et al., 1991; Larson et al., 1995; Royer-Pokora et al., 1991) and GATA2 (Tsai et al., 1994; Tsai & Orkin, 1997) drive the initiation of haematopoiesis, such that embryos lacking in any one of these factors fails to establish primitive or definitive haematopoiesis. RUNX1 is crucial for the early stages of definitive haematopoiesis, with *Runx1* null embryos showing normal yolk sac haematopoiesis but absent foetal liver haematopoiesis (Okuda et al., 1996; Wang et al., 1996). The requirement of RUNX1 in haematopoiesis relies on its binding partner CBF β , to increase the DNA binding affinity of their associated complex and protect RUNX1 from ubiquitination-dependant degradation, thereby extended the enzymes half-life (Huang et al., 2001). Notably, it has been demonstrated that RUNX1 can drive nascent HSC formation in the zebrafish embryo in the absence of CBF β (Bresciani et al., 2014), suggesting that RUNX1 can work independently of CBF β in some contexts. In addition, RUNX1 regulates multiple target genes, many of which acts as transcriptional regulators in the formation of haematopoietic lineages, including the ETS family transcription factor PU.1 (Hoogenkamp et al., 2007; Huang et al., 2008). PU.1 regulates lineage commitment in an expression-dependant manner, with high PU.1 expression driving myeloid lineage commitment and lower PU.1 levels directing differentiation down the lymphoid pathway (Nerlov & Graf, 1998; Scott et al., 1994). PU.1 mutant embryos display a variable onset of anaemia from E14.5 – 17.5, resulting in perinatal lethality (McKercher et al., 1996; Scott et al., 1994), accompanied by an absence of macrophages, impaired neutrophil development and an early block in B cell maturation (McKercher et al., 1996). RUNX1 has also been shown *in vitro* to interact with the C-terminal serine and methionine rich domains of

KAT6A and KAT6B (Pelletier et al., 2002), with KAT6A required to regulate HSC function and *Kat6a* loss resulting in foetal liver cells unable to reconstitute haematopoiesis in irradiated recipients (Katsumoto et al., 2006; Thomas et al., 2006).

1.2.9 Haematopoietic stem cell niche

As described above, the site of haematopoiesis shifts during development in accordance with embryonic requirements. In adulthood, however, haematopoiesis occurs predominately in the bone marrow. To support HSC maintenance and cellular output, a number of factors, including perivascular, stromal, osteo-lineage and neuronal cells, contribute to HSC pool viability and lineage commitment. In addition, regulatory signals emanating from these cells and from physical cues such as oxygen concentration, shear stress and contractile forces also regulate niche output and response to environmental stresses (Adamo et al., 2009; Takubo et al., 2010) (Fig 1.11).

HSC migration to the bone marrow coincides with the production of cartilage and bone from mesenchymal progenitors. In addition to serving as the structural components of the niche, mesenchymal progenitors, osteoblasts and adipocytes also contribute to HSC maintenance and regulation. Osteoblasts drive expansion of haematopoietic progenitors *in vitro* and *in vivo* (Marusic et al., 1993; Taichman & Emerson, 1994; Taichman et al., 1996), such that their loss results in reduced HSC numbers (Visnjic et al., 2004), while their constitutive activation elevates numbers of long-term repopulating HSCs (Calvi et al., 2001; Zhang et al., 2003). By comparison, adipocytes in the bone marrow negatively regulate HSC activity, providing osteospondin, which limits HSC expansion, as well as angiopoietin-1 and thrombopoietin, which bind the TIE2 and MPL receptors, respectively, and promote HSC quiescence (Arai et al., 2004; Naveiras et al., 2009; Qian et al., 2007; Stier et al., 2005; Yoshihara et al., 2007).

Perivascular cells of the niche localise to HSCs and sympathetic nerve fibres. These cells highly express CXCL12, SCF and IL-7 to promote HSC maintenance and niche retention (Cordeiro Gomes et al., 2016; Ding & Morrison, 2013; Ding et al., 2012; Greenbaum et al., 2013). Perivascular cells also sheathe endothelial cells lining the surface of bone marrow blood vessels, which provide angiocrine factors such as FGF2, DLL1, IGFBP2, ANGPT1, DHH and EGF that drive HSC proliferation (Ding et al., 2012; Doan et al., 2013; Hooper et al., 2009;

Kobayashi et al., 2010). Sympathetic nerves release adrenergic signals into the bone marrow to regulate HSC trafficking into the bloodstream (Mendez-Ferrer et al., 2008), causing downregulation of HSC maintenance genes including CXCL12, ANGPT1, KITL and VCAM-1 to drive HSC egress from the bone marrow (Lucas et al., 2008; Mendez-Ferrer et al., 2008; Mendez-Ferrer et al., 2010; Sugiyama et al., 2006).

HSC location within the niche is another important factor regulating the maintenance and functionality of these cells. Three-dimensional imaging has shown that ~80% of HSCs lie within 50% of the distance to the bone surface, while only ~20% are found within 50% of the distance to the central vein (Kiel et al., 2007; Lo Celso et al., 2009; Nombela-Arrieta et al., 2013). Intravital microscopy imaging of the mouse calvarium has revealed that, following injection into non-irradiated mice, HSCs home and localise within 16 μ m of the vasculature (Lo Celso et al., 2009), while transplantation into irradiated mice results in HSCs homing within 15 μ m of the endosteum, where they were able to generate all peripheral blood lineage cells (Lo Celso et al., 2009). The relationship between HSCs and the niche has been further refined using whole-mount confocal immunofluorescence to image mouse bone marrow, showing that quiescent HSCs typically localise with small arterioles of the endosteal region, however, following activation, migrate away from this region to the perisinusoidal niche (Kiel et al., 2005a; Kunisaki et al., 2013; Nilsson et al., 2001).

1.2.10 Isolating HSCs and haematopoietic progenitors

Both HSCs and their progenitors can be isolated from the peripheral blood and haematopoietic organs, with *in vivo* and *in vitro* assays developed to test HSC and progenitor self-renewal, multipotency, proliferation and reconstitution. Based on cell surface markers, HSCs are enriched within the LSK population (LIN⁻ Sca-1⁺ cKit⁺), defined by the absence of differentiated or lineage-specific markers and co-expression of Sca1 and c-Kit (Ikuta & Weissman, 1992; Spangrude et al., 1988). This population can be further divided using the SLAM (signalling lymphocytes activation molecule) markers CD48 and CD150, to identify HPC-1 (CD48⁺ CD150⁻), HPC-2 (CD48⁺ CD150⁺) and MPP (CD48⁻ CD150⁻) progenitor populations as well as HSCs (CD48⁻ CD150⁺) (Kiel et al., 2005b; Oguro et al., 2013). An alternative method of stem cell identification uses the markers FLT3 (CD135) and CD34

(Adolfsson et al., 2001; Osawa et al., 1996), identifying CD34^{hi} FLT3^{lo} and CD34^{lo} FLT3^{lo} stem cell populations. This system also defines MPPs as CD34^{hi} FLT3^{hi} and LMPPs as CD34^{hi} FLT3^{hi} (Adolfsson et al., 2001; Christensen & Weissman, 2001; Yang et al., 2005).

More recently, single cell transcriptomics and lineage tracking experiments have re-categorised HSC and MPP cell types, demonstrating that the haematopoietic compartment is composed of distinct subsets of quiescent, activated and lineage-primed HSCs and MPPs, which support haematopoiesis through continuous, parallel differentiation (Benz et al., 2012; Dykstra et al., 2007; Lu et al., 2011; Muller-Sieburg et al., 2004; Muller-Sieburg et al., 2002; Pietras et al., 2015) as compared to the distinct differentiation hierarchy previously assumed. In the adult bone marrow, LSKs can be subdivided into HSCs (CD34[−]CD135[−]CD150⁺CD48[−]), which are dormant or activated, based on long-term BrdU-retention assessment; metabolically active MPP1 (CD34⁺CD135[−]CD150⁺CD48[−]) cells, capable of short-term self-renewal and similar to ST-HSCs; MPP2 (CD34⁺CD135[−]CD150⁺CD48⁺) cells, which are largely megakaryocyte/erythroid biased; MPP3 (CD34⁺CD135[−]CD150[−]CD48⁺) cells that are myeloid biased and MPP4 (CD135⁺CD150[−]CD48⁺CD34⁺) cells that represent a distinct, lymphoid biased population (Cabezas-Wallscheid et al., 2014; Pietras et al., 2015; Wilson et al., 2008).

Functionally, the transplantation assay is the most stringent test of HSC self-renewal, during which the haematopoietic compartment of a mouse is ablated using radiation, and reconstituting donor cells then injected (Jacobson et al., 1951; Lorenz et al., 1951). The reconstitution of irradiated recipients has two overlapping phases; the short-term phase, during which progenitor cells with limited self-renewal proliferate rapidly to generate mature blood cells (Jones et al., 1989) and the long-term phase, during which reconstitution is taken over by self-renewing, multipotent HSCs (Jones et al., 1989). The contribution of progenitors to peripheral blood in recipient mice is generally assessed 30 days post-transplantation, while the contribution of stem cells is analysed at 16 – 20 weeks. At 16-20 weeks the multipotency of HSCs is assessed by their multi-lineage contribution to the re-forming system, while the proliferative and reconstitutive capacity of injected cells is demonstrated in the survival of recipient animals. Despite its stringency, the transplantation assay does not quantitatively assess HSC activity. For this, a competitive reconstitution experiment is required (Harrison, 1980). In the competitive reconstitution assay, experimental donor HSCs are combined with competitor cells and the relative contributions of these starting populations assessed in the HSC

compartments of irradiated recipients. To distinguish the progeny of these starting populations the allelic variants of the leukocyte cell surface marker CD45, known as CD45.1 (Ly5.1) and CD45.2 (Ly5.2) are most commonly used. A known ratio of CD45.1 and CD45.2 cells can be injected into lethally irradiated recipients and the readout of chimerism in different blood lineages can provide a quantitative indication of functional differences in HSCs from the starting populations (Sykes et al., 1989).

Another method of investigating HSC stem and progenitor proliferation and potency, uses *in vitro* colony formation. In the colony forming assay (CFU), bone marrow or foetal liver cells are cultured in the presence of growth factors SCF, EPO and IL3 (Bradley et al., 1967a; Bradley et al., 1967b). The number of colonies formed serves as a functional readout of early haematopoietic progenitor number, while the size of individual colonies reflects their proliferative capacity. Cell potency and lineage potential can also be determined by identifying the relative proportions of colony forming unit erythrocytic (CFU-E), blast forming unit erythrocytic (BFU-E), colony forming unit granulocyte and monocytic (CFU-GM) and colony forming unit granulocytic, monocytic, erythrocytic and megakaryocytic (CFU-GEMM) colony types.

1.2.11 HSCs as potential clinical tools

The capacity of HSCs to stably reconstitute the haematopoietic system of lethally irradiated recipients has led to their clinical use following chemotherapy and radiotherapy and recognition as a potential treatment for lysosomal storage disorder (Biffi et al., 2013); immunodeficiencies (Aiuti et al., 2013; Hutter et al., 2009) and amyotrophic lateral sclerosis (Appel et al., 2008). Despite the many advances in our understanding of haematopoietic development, maintenance and disruption, there remains much to be understood in regards to HSC function and manipulation. This is highlighted by the multitude of chromatin regulators with identified roles in haematopoiesis, as well as their combinatorial modes of action. An improved understanding of steady state haematopoiesis and reconstitution following transplantation may facilitate the development of novel therapies in haematopoietic disease as well as previously unrecognised gene targets with vulnerability to oncogenic transformation.

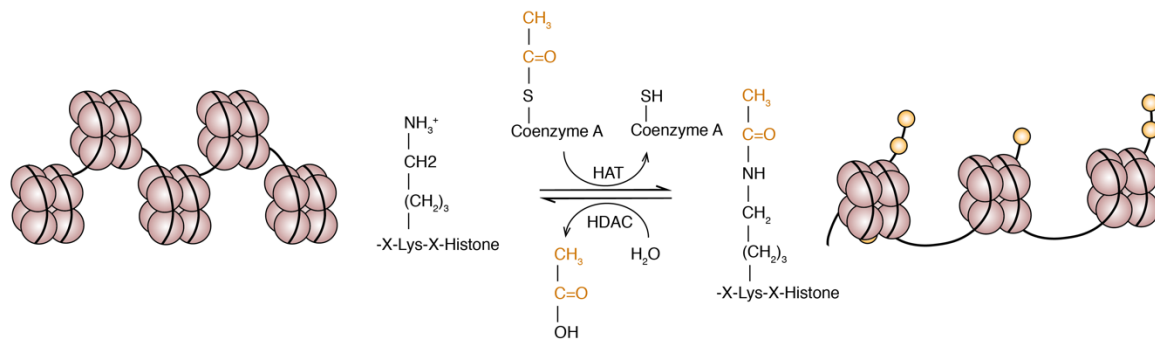


Figure 1.1 Schematic drawing of histone acetylation with partial chemical structures shown

Histone acetylation involves the addition of an acetyl group from acetyl coenzyme A to histone lysine residues. This reaction is catalysed by histone (lysine) acetyltransferases (HATs). Histone acetylation is associated with reduced nucleosomal density at affected loci. The opposing reaction is catalysed by histone deacetylases (HDACs) and associated with increased nucleosomal density.

Redrawn based on (Rodd et al., 2012)

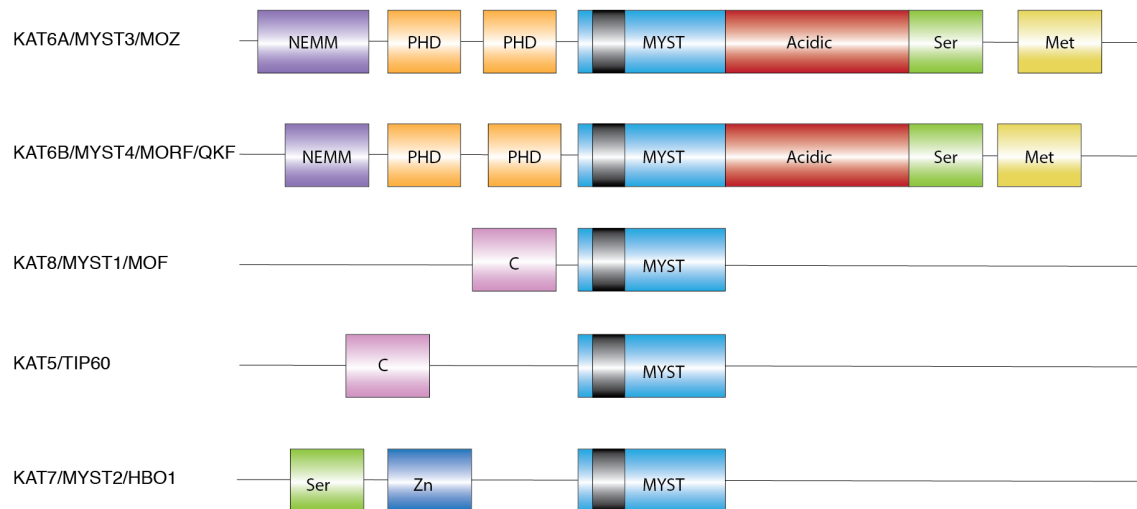


Figure 1.2 Schematic drawing of the MYST family of histone acetyltransferases with domain structure shown

The MYST family of histone acetyltransferases, named after its founding members Moz, Ybf2/Sas3, Sas2 and Tip60, comprises five members in mammals, KAT6A (MYST3, MOZ), KAT6B (MYST4, QKF), KAT8 (MYST1, MOF), KAT5 (TIP60) and KAT7 (MYST2, HBO1). Enzymes are defined by their MYST histone acetyltransferase domain and can be further divided into 3 subgroups based on a shared NEMM (N-terminal park of Enok, MOZ or MORF), PHD (Plant homeodomain) or C (chromodomain), as shown above

Redrawn based on (Avvakumov & Cote, 2007; Voss & Thomas, 2009)

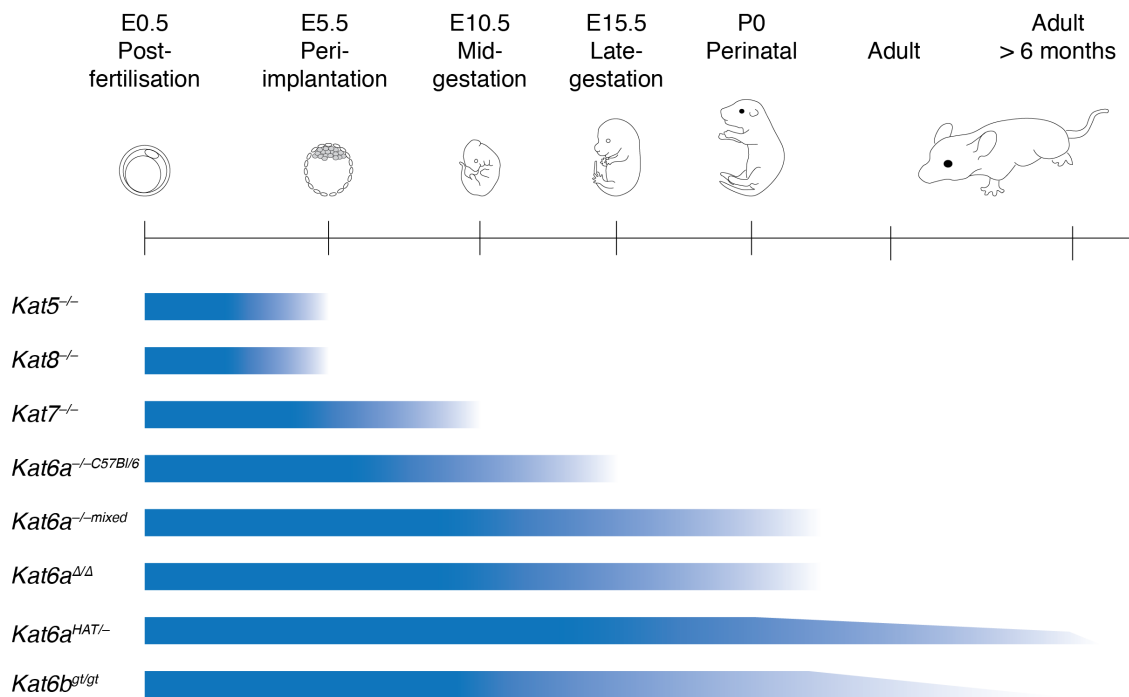


Figure 1.3 Timeline of lethality from MYST family deficiency and loss

Schematic representation of embryonic, peri- and post-natal lethality in mouse models deficient in or lacking MYST family histone acetyltransferases. Embryos lacking *Kat5* (*Kat5*^{-/-}) die prior to implantation, while those lacking *Kat8* (*Kat8*^{-/-}) do not progress past the peri-implantation stage. *Kat7* deficient animals (*Kat7*^{-/-}) arrest in development at embryonic day 8.5 (E8.5) and die two days later. On a *C57BL/6* inbred background animals lacking *Kat6a* (*Kat6a*^{-/-}) do not progress past E15.5; however, *Kat6a*^{-/-} animals on a mixed genetic background and animals lacking the 3' portion of exon 16 (*Kat6a*^{ΔΔ}) on an outbred background survive to birth but die shortly afterwards. Animals that are homozygous for a mutation in the catalytic domain of *Kat6a* (*Kat6a*^{HAT/-}) survive gestation; however, 40% die within 6 months of age. Animals deficient in 90% of *Kat6b* (*Kat6b*^{gt/gt}) survive to birth; however, 80% die in the first two days of life and the remaining animals fail to thrive.

Figure generated using data from (Hu et al., 2009; Thomas et al., 2008; Kueh et al., 2011; Voss et al., 2009; Voss et al., 2012) and Thomas & Voss unpublished data.

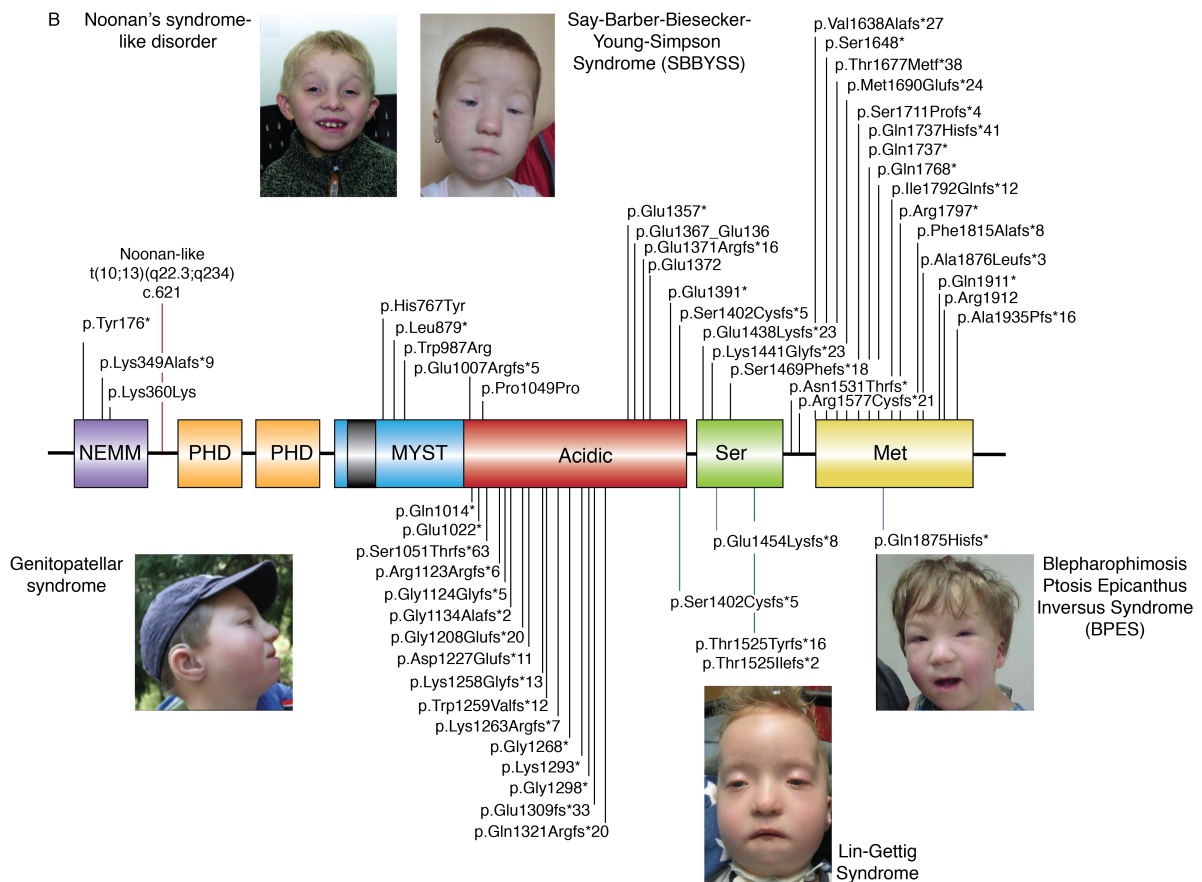
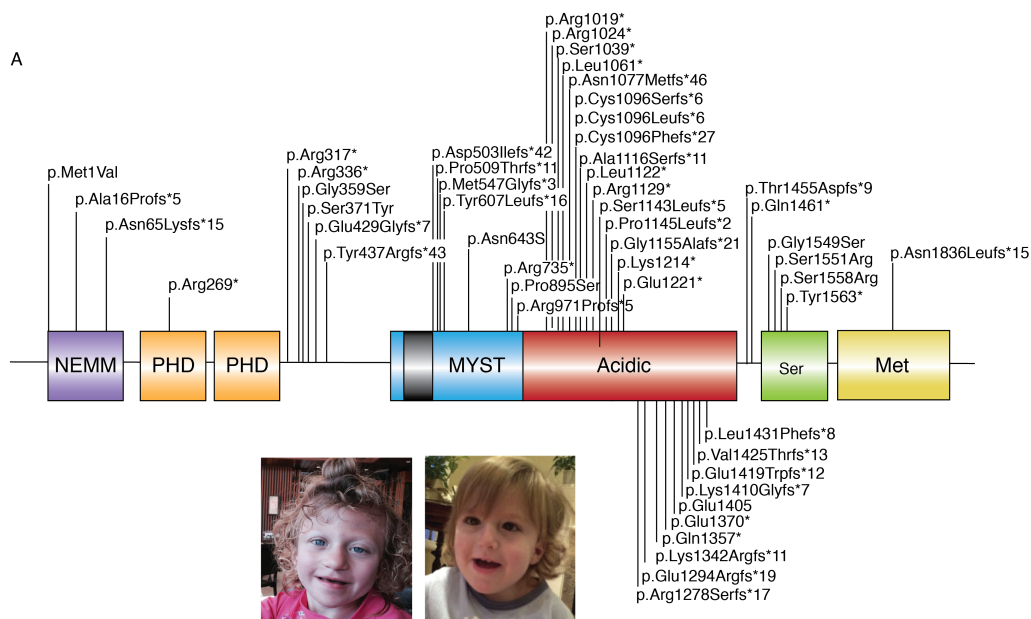


Figure 1.4 Schematic of identified mutations in human KAT6A and KAT6B disorder patients with protein domain structure shown

Schematic drawing of the KAT6A (A) and KAT6B (B) proteins, with domain structure shown. Identified mutations in human patients are marked and representative photos of human patients from published reports are shown. Mutations in KAT6A are broadly categorised as KAT6A congenital disorders. KAT6B mutations underlie five clinical disorders; most commonly the Say-Barber-Biesecker-Young-Simpson variant of Ohdo syndrome (SBBYSS) and Genitopatellar syndrome (GPS), as well as individuals who display features of both syndromes. In addition, *KAT6B* mutation has been identified in individuals diagnosed with a Noonan's syndrome-like disorder (red line), Blepharophimosis-Ptoisis-Epicanthus Inversus-Syndrome-like phenotype (BPES) (purple line), Lin-Gettig syndrome (green line).

Figure generated using data and images from (Arboleda et al., 2015; Bashir et al., 2017; Brea-Fernandez et al., 2019; Campeau et al., 2012a; Champagne et al., 1999; Clayton-Smith et al., 2011; Gannon et al., 2015; Hamaguchi et al., 2019; Kennedy et al., 2019; Kraft et al., 2011; Lundsgaard et al., 2017; Lyu et al., 2019; Marangi et al., 2018; Millan et al., 2016; Radvanszky et al., 2017; Simpson et al., 2012; Szakszon et al., 2013; Tham et al., 2015; Urreizti et al., 2020; Yu et al., 2014)

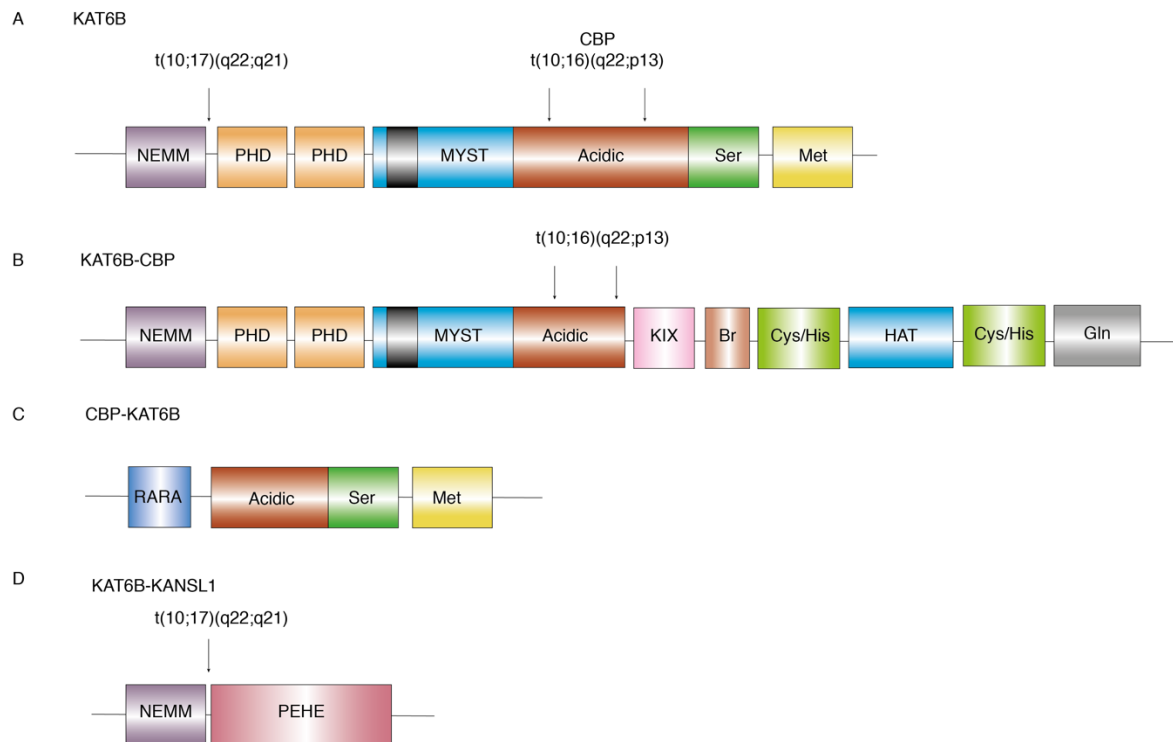


Figure 1.5 Schematic of KAT6B fusion proteins underlying malignancy with protein domain structure shown

KAT6B (A) has been identified in chromosomal translocations encoding KAT6B protein domains fused with CBP and KANSL1. KAT6B fusion to CBP results in two fusion protein products; one product contains the catalytic histone acetyltransferase domain of both KAT6B and CBP (B), while the reciprocal product (C) contains the RARA domain of CBP and a portion of the KAT6B C-terminus, including the serine and methionine rich domains. The KAT6B-KANSL1 fusion protein product comprises the NEMM domain of KAT6B, which contains a histone H1/H5 domain capable of binding chromatin and the PEHE domain, involved in interactions with another MYST family protein, KAT8.

Figure generated using data from (Carapeti et al., 1998; Kitabayashi et al., 2001b; Kojima et al., 2003; Panagopoulos et al., 2001; Panagopoulos et al., 2015; Pelletier et al., 2002).

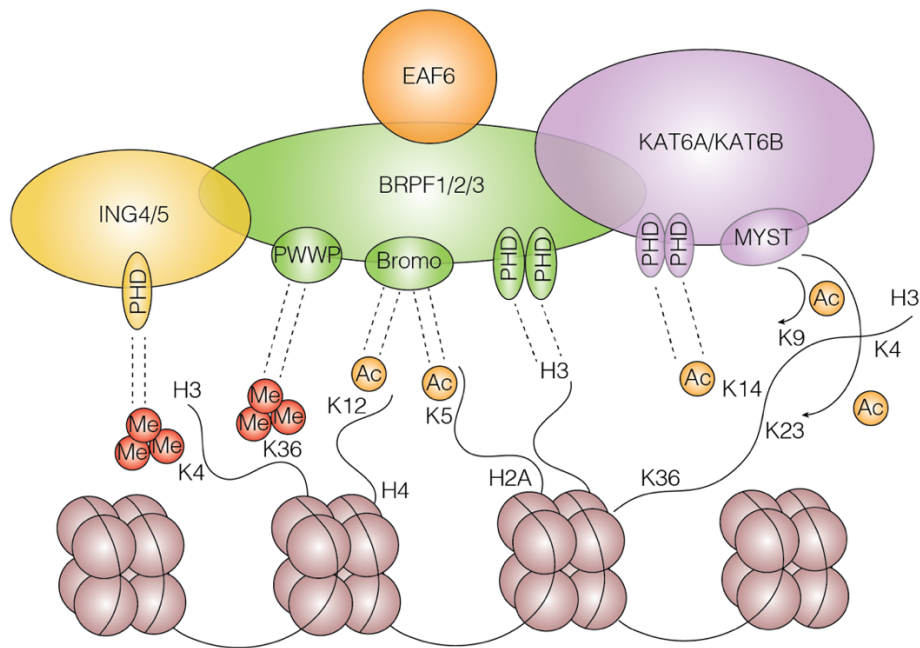


Figure 1.6 Schematic of the KAT6A/KAT6B multi-protein complex with chromatin recognition domains indicated

Schematic representation of the KAT6A/B chromatin-binding protein complex. BRPF1/2/3 act as scaffold subunits for ING4/5, EAF6 and the catalytic subunit, KAT6A or KAT6B. Together, these proteins are capable of chromatin binding, through the recognition of varied modified histone residues and subsequent acetylation of specific lysine residues (K) in the relatively unstructured amino-terminal portion of histones (histone tails).

Figure based on (Klein et al., 2019; Klein et al., 2014).

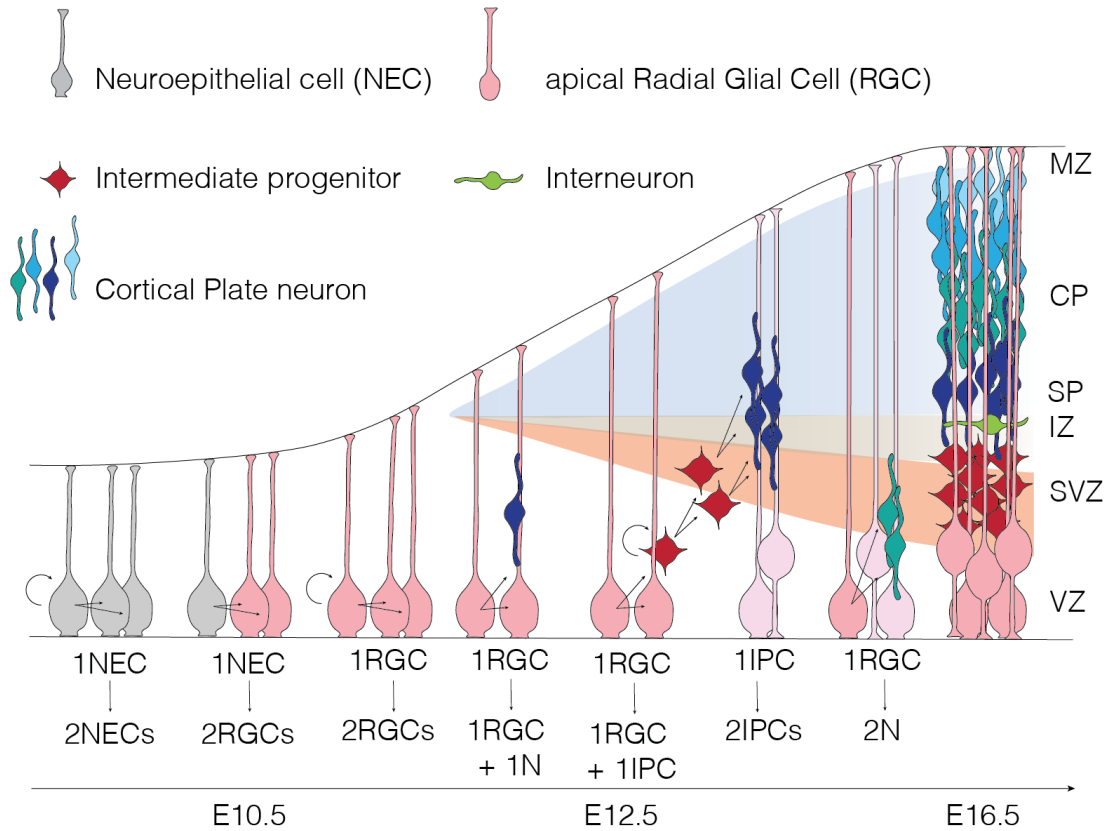


Figure 1.7 Cortical layer development in mice

The neocortex is organised into six distinct layers, the organisation of which is precisely regulated in a temporal and spatial manner during embryonic development. Neuroepithelial cells (NECs) within the ventricular zone (VZ) and subventricular zone (SVZ), give rise to radial glial cells (RGCs) that in turn give rise to intermediate progenitors (IPs) and neurons (N). Cortical layers develop in an inside-out manner, such that early born progenitors (dark blue) form lower cortical layers, while later-born progenitors (mint green and light blue) migrate through early layers to form upper cortical layers. Embryonic (E) time points associated with cortical development in mice are shown at the bottom of the diagram.

MZ: marginal zone; CP : cortical plate; IZ: intermediate zone; SP: subplate; E; embryonic day

Figure based on (Jiang & Nardelli, 2016; Mukhtar & Taylor, 2018)

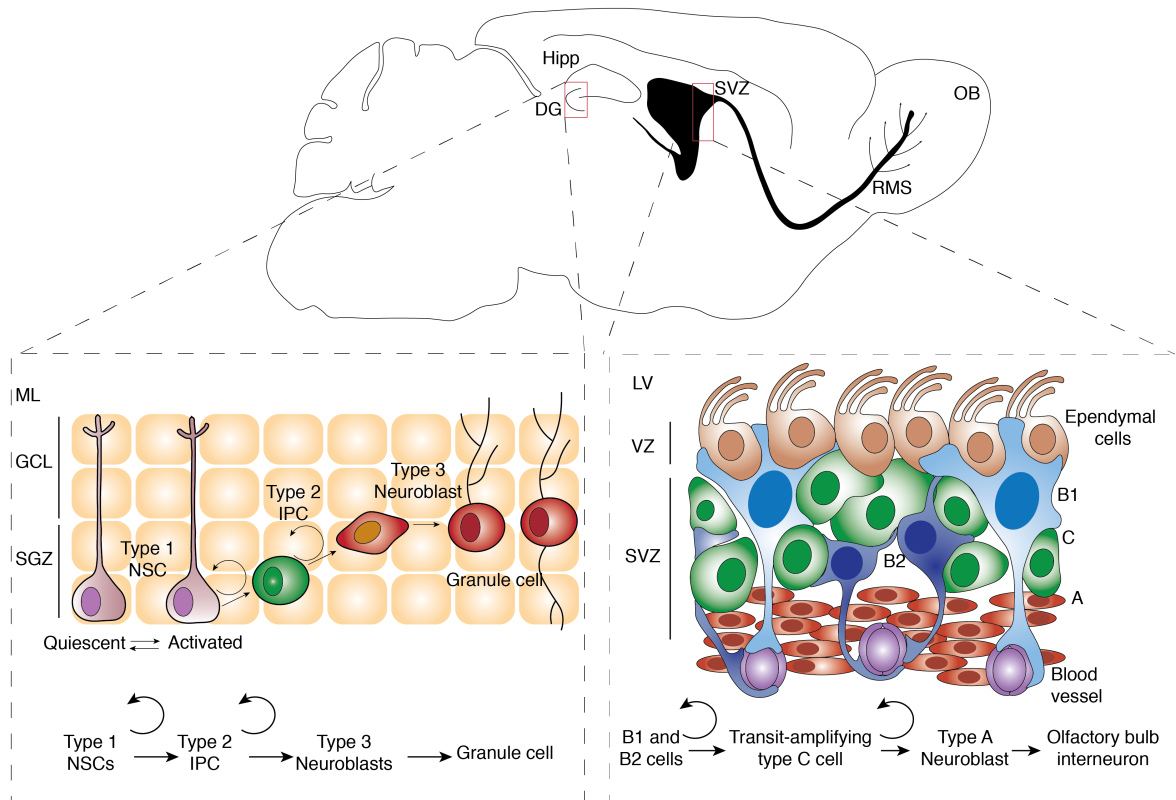


Figure 1.8 Schematic of mammalian adult neural stem cell niches

Schematic of the organisation of the adult subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus (Hipp) and subventricular zone (SVZ) in the walls of the lateral ventricles (LV). Within the DG, NSCs give rise to Radial glial cells (RGCs). These in turn give rise to Type 2 intermediate progenitors (IPCs), that rapidly expand the precursor pool, prior to the development of neuroblasts. Neuroblasts in turn form post-mitotic neurons that integrate into hippocampal circuitry and enable synaptic plasticity that underlies memory and learning. In the SVZ, B1 cells function as the NSCs. These cells exhibit astroglial characteristics and are surrounded by multi-ciliated ependymal cells at their apical surface. B1 cells can also give rise to B2 cells, which similarly exhibit astroglial characteristics and contact blood vessels, but lack an apical contact. B1 cells generate transit amplifying cells (C cells), which in turn give rise to rapidly dividing Type A neuroblasts. Neuroblasts migrate through the rostral migratory stream (RMS) to reach the olfactory bulbs (OB) and differentiate into olfactory bulb interneurons.

Figure based on (Alvarez-Buylla & Garcia-Verdugo, 2002; Fuentealba et al., 2012; Obernier & Alvarez-Buylla, 2019)

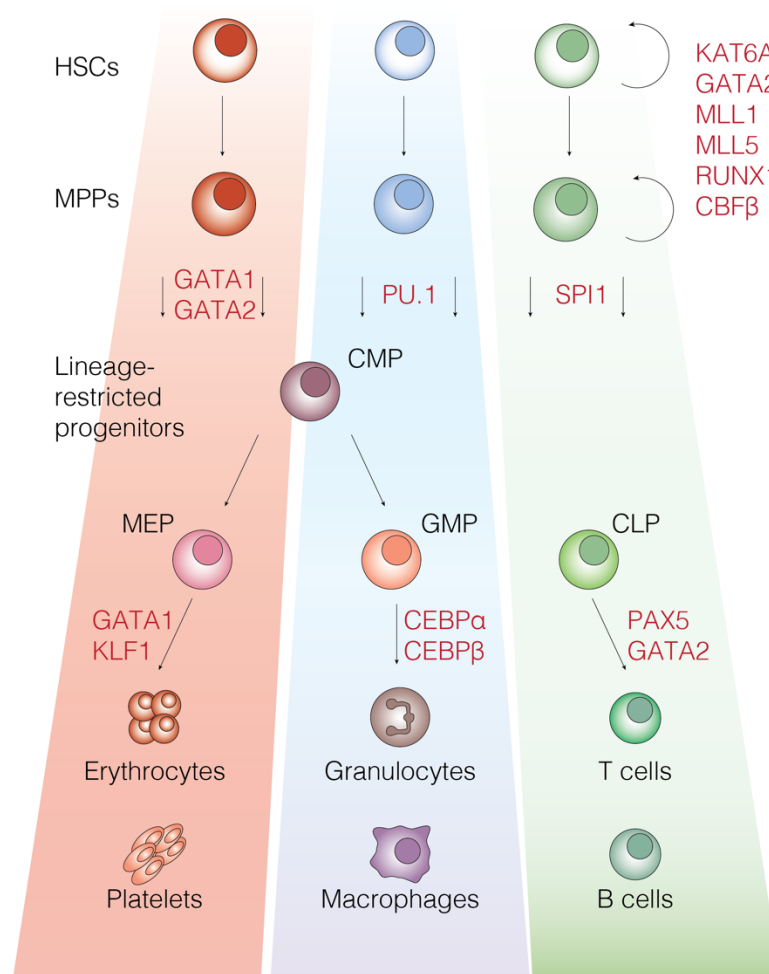


Figure 1.9 Continuous model of haematopoiesis and key transcriptional regulators

Contemporary evidence suggests that haematopoietic differentiation exists as a spectrum, with haematopoietic stem cell (HSC) and multipotent progenitors (MPPs) demonstrating heterogeneity and lineage biases as they progress through differentiation, to give rise to megakaryocyte/erythroid, granulocyte or lymphoid lineages. At each stage of maturation key transcriptional regulators (shown in red) contribute to cell maintenance and differentiation programs. Figure based on (McRae et al., 2019).

HSC: Haematopoietic stem cell; MPP: Multipotent progenitor; CLP: Common Lymphoid progenitor; GMP: granulocyte, macrophage progenitor; MEP: megakaryocyte, erythrocyte progenitor

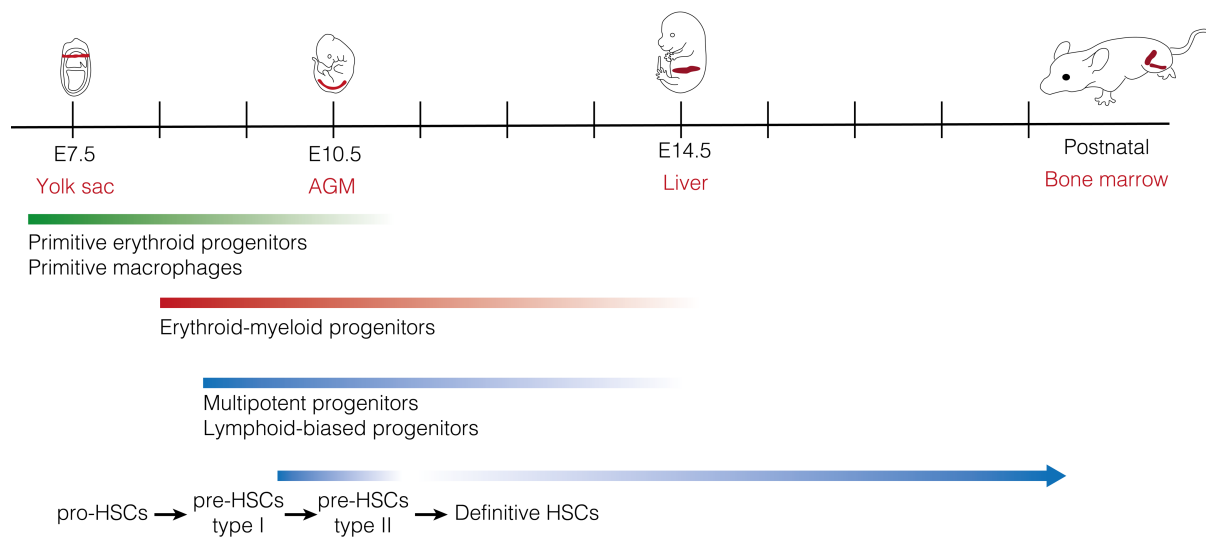


Figure 1.10 Schematic representation of primitive and definitive haematopoiesis during mouse development

During mouse embryonic development, the first detectable haematopoietic cells arise in the blood islands of the yolk sac at E7.5. These cells derive from bipotential hemangioblast cells of the epiblast, which can form short lived, primitive macrophages and primitive erythroid progenitors. The second wave is marked by the appearance of definitive erythroid-myeloid progenitors (EMPs) and the third by the development of multipotent progenitors (MPPs) and lymphoid-biased multipotent progenitors (LMPPs). EMPs, MPPs and LMPPs are derived from hemogenic endothelial cells of the yolk sac and aorto-gonad-mesonephros (AGM). The final wave of embryonic haematopoiesis gives rise to definitive HSCs. HSC precursors are detectable in the AGM from E9 – E11. These undergo maturation from pro-HSC (E9.5) to pre-HSC type I (E10.5) and pre-HSC type II cells (E11.5), before generating definitive HSCs capable of reconstituting haematopoiesis following transplantation into an irradiated recipient. Definitive HSCs within the AGM migrate to the embryonic liver at E11.5, with their proliferation peaking at this location at E14.5. At E16.5 these cells then migrate to the bone marrow (BM), where haematopoiesis is sustained into postnatal life.

Figure based on (Hoeffel & Ginhoux, 2015; Ivanovs et al., 2017)

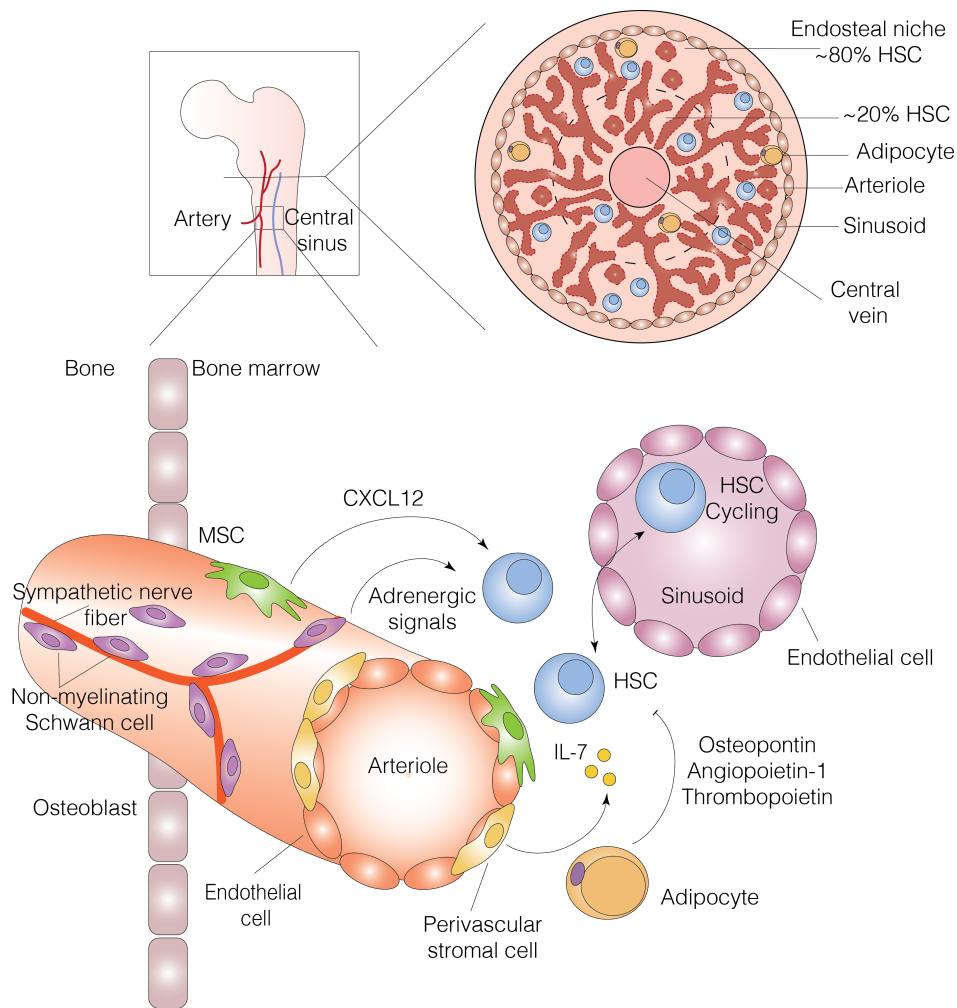


Figure 1.11 Schematic of the haematopoietic stem cell niche in the bone marrow

The bone marrow represents the major haematopoietic niche in adult animals, providing key signalling components that regulate the proliferation and survival of haematopoietic stem cells (HSCs). The outer endosteal niche contains largely quiescent HSCs, while the central, perivascular niche contains actively dividing HSCs. Perivascular stromal cells provide factors including IL-7, to promote HSC proliferation, while adipocytes negatively regulate HSC turnover by providing osteopontin, angiopoietin-1 and thrombopoietin, which repress HSC proliferation and promote quiescence. Mesenchymal stem cells (MSCs) provide CXCL12 and non-myelinating Schwann cells lining synthetic nerve fibres provide adrenergic signals to promote HSC proliferation.

Figure based on (Giles et al., 2016; Kunisaki et al., 2013)

Table 1.1 Histone modifications and their general effect on transcription

Modification	Residue		Writer	Effect on transcription
Methylation	H3	K4	SET7/9	Activation
			MLL	
			Smyd3	
		K9	SUV39h1	Heterochromatin
			SUV39h2,	
			G9a, Eu-	
			HMTase1	
			SETDB1	
			EZZH2	
			EZH1/2	
Methylation	H4	K27	EZH1/2	Repression and Heterochromatin
		K36	HSD1	Recruitment of Rpd3S to repress internal initiation
		K79	DOT1	Activation
		K20	SET7	Silencing
			SUV4-20	Heterochromatin
			NSD1	
			EZH2	
		R2	PRMT4	Activation
Arginine Methylation	H3	R17	PRMT4	Activation
		R26	PRMT4	Activation
		R3	PRMT1	Activation
Phosphorylation	H3	S10	MSK1/2	Activation
			Akt	
			Aurora B	
		S28	MSK1/2	Activation

			JNK1	
			Aurora B	Chromosome condensation
Ubiquitination	H2B	K120	UBE2A/B	Activation
		K123	Bre1	Activation
	H2A	K119	PRC1	Repression
Acetylation		K9	KAT6A, Gnc5, Pcaf1	Activation
		K14	KAT7	Activation
		K23	KAT6A, KAT6B	Activation
		K27	CBP/p300	Activation
		K56	CBP/p300	Activation
	H4	K16	KAT8	Activation

Table compiled from data in (Baek, 2011; Bannister & Kouzarides, 2005; Das et al., 2009; Ferrari et al., 2014; Kim et al., 2009; Li et al., 2007; Margueron et al., 2008; X. Shen et al., 2008; Wang et al., 2004)

K, lysine; R, arginine; S, serine

Table 1.2 Mutations in KAT6A-related developmental disorders

Exon	Mutation	Type of mutation	Predicted protein change	Publication
1	c.1A>G	Loss of start codon	p.Met1Val	Kennedy et al., 2018
2	c.46del	indel	p.Ala16Profs*5	Kennedy et al., 2018
2	c.195_198del	indel	p.Asn65Lysfs*15	Kennedy et al., 2018
4	c.805C>T	nonsense	p.Arg269*	Kennedy et al., 2018
Intron 5	c.907+1delG	Altered splice site	Unknown.	Kennedy et al., 2018
6	c.949C>T	nonsense	p.Arg317*	Kennedy et al., 2018
7	c.1075G>A	missense	p.Gly359Ser	Urriezti et al., 2020
7	c.1096C>T	nonsense	p.Arg366*	Kennedy et al., 2018
7	c.1112C>A	missense	p.Ser371Tyr	Kennedy et al., 2018
7	c.1283_1284insT	indel	p.Glu429Glyfs*7	Kennedy et al., 2018
7	c.1308_1309insCGCAA	indel	p.Tyr437Argfs*43	Kennedy et al., 2018
8	c.1364-2A>T	Altered splice site	Unknown.	Kennedy et al., 2018
9	c.1507delG	indel	p.Asp503Ilefs*42	Kennedy et al., 2018
10	c.1638_1640del	indel	p.Met547Glu fs*3	Kennedy et al., 2018
11	c.1819dupT	indel	p.Tyr607Leufs*16	Kennedy et al., 2018
Intron 11	c.1903-1G>A	Altered splice site	Unknown.	Kennedy et al., 2018

12	c.1928A>G	missense	p.Asn643Ser	Millan et al., 2016
13	c.2203C>T	nonsense	p.Arg735*	Kennedy et al., 2018
15	c.2683C>T	missense	p.Pro895Ser	Kennedy et al., 2018
15	c.2911dupC	indel	p.Arg971Profs*5	Kennedy et al., 2018
16	c.3040-1_3040delGA	Altered splice site	Unknown	Millan et al., 2016
16	c.3055C>T	nonsense	p.Arg1019*	Kennedy et al., 2018
16	c.3070C>T	nonsense	p.Arg1024*	Arboleda et al., 2015
16	c.3116_3117delCT	indel	p.Ser1039*	Tham et al., 2015
16	c.3182T>G	nonsense	p.Leu1061*	Kennedy et al., 2018
16	c.3230delA	indel	p.Asn1077Metfs*56	Millan et al., 2016
16	c.3286_3287insC	indel	p.Cys1096Serfs*6	Kennedy et al., 2018
16	c.3286dupT	indel	p.Cys1096Leufs*6	Kennedy et al., 2018
16	c.3287delG	indel	p.Cys1096Phefs*27	Millan et al., 2016
16	c.3345_3346insAGTCAGATGA	indel	p.Ala1116Serfs*11	Kennedy et al., 2018
17	c.3365T>G	nonsense	p.Leu1122*	Kennedy et al., 2018
17	c.3385C>T	nonsense	p.Arg1129*	Arboleda et al., 2015
17	c.3427_3428insTA	indel	p.Ser1143Leufs*5	Urreizti et al., 2020
17	c.3434del	indel	p.Pro1145Leufs*2	Kennedy et al., 2018
17	c.3462delA	indel	p.Gly1155Alafs*21	Millan et al., 2016
17	c.3515delG	indel	p.Gly1172Aspfs*4	Kennedy et al., 2018
17	c.3640A>T	nonsense	p.Lys1214*	Urreizti et al., 2020

17	c.3661G>T	nonsense	p.Glu1221*	Kennedy et al., 2018
17	c.3830_3832insTT	indel	p.Arg1278Serfs*17	Tham et al., 2015
17	c.3879dupA	indel	p.Glu1294Argfs*19	Tham et al., 2015
17	c.4025del	indel	p.Lys1342Argfs*11	Kennedy et al., 2018
17	c.4042C>T	nonsense	p.Gln1348*	Kennedy et al., 2018
17	c.4069C>T	nonsense	p.Gln1357*	Kennedy et al., 2018
17	c.4108G>T	nonsense	p.Glu1370*	Tham et al., 2015
17	c.4254_4257del	indel	p.Glu1419Trpfs*12	Kennedy et al., 2018
17	c.4273_4274del	indel	p.Val1425Thrfs*13	Kennedy et al., 2018
17	c.4228_4232del	indel	p.Lys1410Glyfs*7	Kennedy et al., 2018
17	c.4292dupT	indel	p.Leu1431Phefs*8	Tham et al., 2015
17	c.4362dupG	indel	p.Thr1455Aspfs*9	Kennedy et al., 2018
17	c.4381C>T	nonsense	p.Gln1461*	Kennedy et al., 2018
17	c.4645G>A	missense	p.Gly1549Ser	Kennedy et al., 2018
17	c.4653T>G	missense	p.Ser1551Arg	Kennedy et al., 2018
17	c.4672A>C	missense	p.Ser1558Arg	Kennedy et al., 2018
17	c.4688_4689del	indel	p.Tyr1563*	Gauthier-Vasserot et al., 2016

Table 1.3 Mutations in KAT6B-related developmental disorders

Phenotype	Exon	Mutation	Type of mutation	Predicted protein change	Publication
Atypical SBBYSS	3	c.527dup	indel	p.(Tyr176*)	Clayton-Smith et al. 2011
SBBYSS	8	c.1078G>A	missense	p.(Glu360Lys)	Clayton-Smith et al. 2011
SBBYSS	15	c.2959T>C	missense	p.(Trp987Arg)	Gannon et al., 2015
SBBYSS	15	c.3018del	indel	p.(Glu1007Argfs*5)	Clayton-Smith et al. 2011
SBBYSS	16	c.3040C>T	nonsense	p.(Gln1014*)	Gannon et al., 2015
SBBYSS	16	c.3064 G>T	nonsense	p.(Glu1022*)	Gannon et al., 2015
SBBYSS	16	c.3147 G>A	missense	p. Pro1049Pro	Gannon et al., 2015
SBBYSS	16	c.3366_3369del	indel	p.(Lys1124Glyfs*5)	Gannon et al., 2015
SBBYSS	16	c.3367_c.3370delAGAA	indel	p.Arg1123Argfs*6	Lyu et al., 2019
SBBYSS	17	c.3401_3402del	indel	p. (Gly1134Ala fs*2)	Gannon et al., 2015
GPS	17	c.3598_3622dup25	indel	p.(Gly1208Glu fs*19)	Gannon et al., 2015
GPS	18	c.3962_3963del	indel	p.(Gln1321Argfs*20)	Gannon et al., 2015
SBBYSS	18	c.4069G>T	nonsense	p.(Glu1357*)	Clayton-Smith et al. 2011
SBBYSS	18	c.4074_4079del	indel	p.(Glu1367_Glu1368)	Gannon et al., 2015
SBBYSS	18	c.4114G>T	nonsense	p.(Glu1372*)	Gannon et al., 2015
SBBYSS.	18	c.4205_4206del	indel	p.(Ser1402Cysfs*5)	Clayton-Smith et al. 2011

SBBYSS	18	c.4312del	indel	p.(Glu1438Lysfs*23)	Gannon et al., 2015
SBBYSS	18	c.4320_4321del	indel	p.(Lys1441Glyfs*23)	Gannon et al., 2015
SBBYSS	18	c.4405dup	indel	p.(Ser1469Phefs*18)	Clayton-Smith et al. 2011
SBBYSS	18	c.4572_4573dup	indel	p.Thr1525Ilefs*25	Rodrigo et al., 2019
SBBYSS	18	c.4728_4729del	indel	p.(Arg1577Cysfs*21)	Gannon et al., 2015
SBBYSS	18	c.4911_4921del	indel	p.(Val1638Alafs*27)	Clayton-Smith et al. 2011
SBBYSS	18	c.4943C>G	nonsense	p.S1648*	Lundsgaard et al., 2017
SBBYSS	18	c.5030del	indel	p.(Thr1677Metfs*38)	Clayton-Smith et al. 2011
SBBYSS	18	c.5064_5071del	indel	p.(Met1690Glufs*24)	Szakson et al. 2013
SBBYSS	18	c.5131del	indel	p.(Ser1711Profs*4)	Gannon et al., 2015
SBBYSS	18	c.5201_5210dup	indel	p.(Gln1737Hisfs*41)	Clayton-Smith et al. 2011
SBBYSS	18	c.5209 C>T	nonsense	p.(Gln1737*)	Gannon et al., 2015
SBBYSS	18	c.5302 C>T	nonsense	p. (Gln1768*)	Gannon et al., 2015
SBBYSS	18	c.5370_5373dup	indel	p.(Ile1792Glnfs*12)	Clayton-Smith et al. 2011
SBBYSS	18	c.5734A>T	nonsense	p.(Arg1912*)	Clayton-Smith et al. 2011
SBBYSS	18	c.5389C>T	nonsense	p.(Arg1797*)	Clayton-Smith et al. 2011
SBBYSS	18	c.5424C>A	nonsense	p. (Tyr1808*)	Gannon et al., 2015
SBBYSS	18	c.5442_5445del	indel	p.(Phe1815Alafs*8)	Gannon et al., 2015
SBBYSS	18	c.5623_5624dup	indel	p.(Gln1875Hisfs*5)	Yu et al. 2014
SBBYSS	18	c.5624_5625del	indel	p.(Ala1876Leufs*3)	Gannon et al., 2015
SBBYSS	18	c.5731C>T	nonsense	p.(Gln1911*)	Gannon et al., 2015

SBBYSS	18	c.5802delA		p.A1935Pfs*16	Brea-Fernandez et al., 2018
GPS	18	c.3680_3695del	indel	p.(Asp1227Glu fs*11)	Simpson et al. 2012
GPS	18	c.3773dup	indel	p.(Trp1259Val fs*12)	Simpson et al. 2012
GPS	18	c.3768_3771del	indel	p.(Lys1258Gly fs*13)	Simpson et al. 2012
GPS	18	c.3769_3772del	indel	p.(Lys1258Gly fs*13)	Campeau et al. 2012
GPS	18	c.3788_3789del	indel	p.(Lys1263Arg fs*7)	Campeau et al. 2012
GPS	18	c.3802G>T	nonsense	p. (Gly1268*)	Campeau et al. 2012
GPS	18	c.3877A>T	nonsense	p.(Lys1293*)	Simpson et al. 2012
GPS	18	c.3892G>T	nonsense	p.(Gly1298*)	Campeau et al. 2012
GPS	18	c.3925dup	indel	p.Glu1309fs*33	Hamaguchi et al., 2019
GPS	18	c.4110dupA	nonsense	p.(Glu1371Arg fs*16)	Gannon et al., 2015
GPS	18	c.4360_4368delins AAAAACCAAAA	indel	p.(Glu1454Lys fs*8)	Campeau et al. 2012
GPS/SBBYSS	3	c.1045_1049delTTAAA	indel	p.L349Afs*9	Marangi et al., 2017
GPS/SBBYSS	16	c.3152delG	indel	p.S1051Tfs*63	Brea-Fernandez et al., 2018
GPS/SBBYSS	18	c.4592delA	indel	p.Asn1531Thr fs*18	Radvansky et al., 2017
GPS/SBBYSS	18	c.5302C>T	nonsense	p. (Gln1768*)	Gannon et al., 2015
BPES-like phenotype	18	c.5623_5624dupCA	indel	p.Gln1875His fs*	Yu et al., 2014
Lin-Gettig	18	c.4572dupT	indel	p.Thr1525Tyr fs*16	Bashir et al., 2017

Lin-Gettig	18	c.4205_4206delCT	indel	p.Ser1402Cysfs*5	Bashir et al., 2017
Noonan syndrome-like phenotype	3	c.621+26310_c.621+26311	translocation	t(10;13)(q22.3;q34)	Kraft et al., 2011

Table 1.4 General features associated with KAT6B-related disorders

Symptoms	Noonan-like phenotype	SBYSS	GPS	BPES-like phenotype	Lin-Gettig syndrome
Neurological					
Intellectual disability	+	+	+	+	+
Visual impairment		+		+	
Microcephaly		+	+		
Absent/thin corpus callosum			+		
Dental/oral					
Micrognathia		+	+		
Dental abnormalities		+	+		
Articular difficulty	+				
Facial					
Nose	Short and broad	Broad	Broad	Short and broad	Broad
Craniosynostosis		NR	NR		+sagittal
Cleft palate		+	+		U-shaped
High arched palate	+			+	

Blepharophimosis	+		+	+	
Ptosis	+		+	+	+
Small chin	+			+	
Aural					
Ear abnormalities	Low set	Low set	Low set		Low set, posteriorly rotated
Hearing difficulty		+	+	+	+
Skeletal					
Chest abnormalities		Kyphoscoliosis, costal anomalies and narrow chest	Pectus		Wide spaced nipples
Contractures		Hips, knees and talipes	Lower joint stiffness		Hips, fingers and knees
Hypotonia	+	+	+		+
Underdeveloped/absent patellae		+	+		+
Long first digits		+	+		+
Camptodactyly		+	+		+
Short stature	+				

Palpebral fissures		+	
Scoliosis		+	
Club feet		+	
Genitourinary	Cryptorchidism	Male: Cryptorchidism, micropenis, scrotal hypoplasia and hypospadias Female: Clitoromegaly, labial hypoplasia	Male: Cryptorchidism, micropenis and hypospadias
Cardiac	+	+ ASD, PFO and VSD	+ PFO and ASD
Gastrointestinal	Feeding difficulties	Feeding difficulties	Feeding difficulties
Thyroid abnormalities	+	+	

+ indicated presence of a symptom

This is a generalised schematic. Features are variable in their penetrance and severity

Table generated using data from (Campeau et al., 2012a; Campeau et al., 2012b; Clayton-Smith et al., 2011; Kraft et al., 2011; Yu et al., 2014)

NR; not reported, NA; not applicable, VSD; ventricular septal defect, PFO; patent foramen ovale, ASD; atrial septal defect, NEC; necrotizing enterocolitis

Chapter 2: Aims

De novo, heterozygous mutations in the histone acetyltransferase, KAT6B, underlie congenital disorders associated with intellectual disability (Bashir et al., 2017; Campeau et al., 2012a; Clayton-Smith et al., 2011; Kraft et al., 2011; Simpson et al., 2012; Yu et al., 2014). Studies in mice have demonstrated that KAT6B is an important regulator of embryonic and adult neurogenesis (Merson et al., 2006; Thomas et al., 2000). It is highly expressed within the embryonic dorsal telencephalon and foetal cortex (Thomas et al., 2000), as well as within a small subset of cells of the adult SVZ (Merson et al., 2006). While *Kat6b* deficiency has been shown to impair NSC proliferation *in vitro* and *in vivo*, as well as neuronal differentiation *in vitro* (Merson et al., 2006), the effect of its overexpression has not been assessed, nor has there been an assessment of gene expression differences resultant from complete *Kat6b* loss or overexpression.

This study aims to increase our understanding of the role of KAT6B in regulating neurogenesis and NSCs, to better understand how KAT6B acts within these cells and how its mutation underlies human intellectual disability disorders. More specifically, this study aims to interrogate the molecular requirement for KAT6B and identify key genes affected by *Kat6b* loss and overexpression within the developing brain and NSCs.

While no haematopoietic defects have been described for *Kat6b* mutant animals, the MYST histone acetyltransferase, KAT6A, with which KAT6B shares identical domain structure, is essential for HSC development (Katsumoto et al., 2006; Thomas et al., 2006). As KAT6B and KAT6A represent mutually exclusive catalytic subunits of the same multi-protein complex (Ullah et al., 2008) and fusion of either factor to CBP results in AML (Borrow et al., 1996; Panagopoulos et al., 2001), it is possible that they also share roles in haematopoiesis. As such, this study aims to assess the effect of *Kat6b* loss and gain on haematopoiesis and HSC transplantation, as well as the effect of *Kat6a* and *Kat6b* combined heterozygosity on these parameters.

Aims

- (i) Investigate the function of KAT6B during embryonic cortex development and within NSCs, by examining the effects of *Kat6b* overexpression and the effects of a complete absence of KAT6B.
- (ii) Elucidate the molecular mechanism of KAT6B during neurogenesis and identify key genes and pathways affected by its loss or overexpression.
- (iii) Functionally characterise the role of KAT6B within the haematopoietic system.

Chapter 3: Materials and Methods

3.1 Mice

All mouse husbandry and experiments were conducted in accordance with the guidelines set by the Australia Code of Practice for the Care and Use of Animals for Scientific Purposes and with the approval of the Walter and Eliza Hall Animal Ethics Committee. Mice were housed 4 – 6 to a box in micro-ventilated cages (AirLaw) and provided with γ -irradiated feed (Barastoc, Ridley AgriProducts) and sterilised water, with a 14 – hour light/10 – hour dark cycle.

3.1.1 *Kat6b* null allele

Two constructs carrying a *Kat6b* floxed allele were generated by Dr Mathew Dixon and Natalie Downer in my host laboratory and Jeanette Rientjes (Monash). Construct 1 carried *loxP* sites flanking Exon 1 and construct 2, *loxP* sites flanking exons 10 – 11 of the wild type *Kat6b* allele. Construct 1 was introduced into mouse embryonic stem (ES) cells by the WEHI microinjection facility, to generate germ line chimeras, which were used to produce *Kat6b* exon 1 – deleted heterozygous mice. Blastocysts from these mice were used by Natalie Downer to isolate ES cells that were subsequently retargeted with construct 2 by Natalie Downer, resulting in a *Kat6b* allele carrying four *loxP* sites, flanking exon 1 and exons 10 – 11 (Fig 3.1 B). ESC chimeras carrying this 4-*lox* allele were generated by Anne Voss, Tim Thomas and Andrea Griffo and these were crossed to a *Cre*-deleter mouse strain (Schwenk et al., 1995), resulting in Cre-mediated deletion of exons 1-11 of the wild type *Kat6b* gene and yielding a *Kat6b* null allele, with single remaining *loxP* site (Fig 3.1 C). Animals were maintained on a C57BL/6 genetic background.

3.1.2 *Kat6b* overexpression construct

A pBACe3.6 (U80929) construct carrying the wild type *Kat6b* allele was obtained from the AGRF and microinjected into mouse pronuclei by Julie Scott. Seven copies of the transcript inserted in tandem at a random autosomal site in the mouse genome (Fig 3.1 D). Animals were maintained on a *FvbxBALB/c* genetic background.

3.1.3 *Kat6a* null allele

Mice carrying the *Kat6a*⁻ allele as described in (Voss et al., 2009) were used. In these mice exons 3 – 7 of the wild-type *Kat6a* locus has been excised by *Cre*-mediated deletion, with a single *loxP* and *frt* site remaining. Animals were maintained on a *C57Bl/6* genetic background (Fig 3.1 G).

3.1.4 *Sox2-GFP allele*

Sox2-GFP mice as described in (Arnold et al., 2011) were used. These mice carried EGFP (Ellis et al., 2004) that had been knocked into the endogenous *Sox2* locus in V6.5 ESCs, leaving *Sox2* regulatory elements intact.

3.1.5 *Staging embryos and fetuses*

To obtain embryos of known gestational time points, noon of the morning following a vaginal plug was considered embryonic day 0.5 (E0.5). Pregnant mothers were sacrificed on the specific gestational day by cervical dislocation.

3.1.6 *PCR genotyping embryonic, foetal and postnatal tissue*

Genomic DNA was extracted from tail tissue of embryonic or adult mice using DirectPCR (Tail) lysis buffer (Viagen, 102-T) and Proteinase K (10 mg/ml, Roche) in a 50:1 ratio. Samples were digested at 55°C and 600 rpm in a Thermomixer (Eppendorf) for 1 h for embryonic tissue or O/N for adult tissues, followed by 95°C for 10 – 30 min to inactivate the Proteinase K. 2 µl extracted DNA was used per sample, using 1x MyTaq buffer (Bioline, BIO-37112) and 10 µM allele-specific primers (Table 3.10). All PCRs were run on an S1000 ThermoCycler (Bio Rad). Samples were run on a 2% agarose gel (Bioline, BIO-41025) and imaged under UV light.

3.1.7 *BrdU administration*

BrdU (Sigma-Aldrich, B5002) was prepared as a stock solution of 10 mg/ml in 0.007 N NaOH and 0.9% (w/vol) NaCl in MQ-H₂O. To assess NSC numbers in the SVZ of adult animals, mice were intraperitoneally injected every 12 h with BrdU at a dose of 100 µg/g body weight, for 12 days, followed by 14 days without treatment. Mice were perfusion fixed as per section 3.4.2

and their brains dissected and fixed for a further 48 h in 4% paraformaldehyde (PFA) (Sigma-Aldrich, P6148) in MT-PBS (Gibco, 141901144) followed by 2x 70% EtOH washes, each for 24 h. Brains were cut using a scalpel to remove the olfactory bulbs and hindbrain and given to the WEHI histology department for embedding in paraffin and cutting. 10 µm coronal serial sections were cut and dewaxed for each animal. Comparable sections from all samples were taken, 6 slides per sample, from the start of the cortex to the section with the most rostral aspect of the fimbria hippocampi.

3.1.8 Valproic acid administration and blood collection

Valproic acid (VPA) sodium salt (Sigma, P4543) was delivered by a trained animal technician, twice daily for 2 weeks by oral gavage, at a dose of 100 mg/kg body weight. For the following 2 weeks, mice were fed VPA-supplemented chow (20 g VPA per kg chow). For each treated animal there was a sex, age and genotype matched, untreated control.

200 µl retro-orbital eye bleeds were collected by a trained animal technician at 2 and 4 weeks after the start of treatment. At 4 weeks animals were euthanised by CO₂ asphyxiation and the SVZ removed as per section 3.2.5.

3.1.9 Foetal liver transplants

E14.5 foetal livers were dissected as per section 3.2.6 and passed through a 40 µm cell sieve (Corning, 352340) into 5 ml 2% FACS buffer (Table 3.1). Adult bone marrow was collected as per section 3.2.5 into 5 ml 2% FACS buffer and 300 µl used for ADVIA 2120i haematology analyser (Siemens Healthineers) WBCB counts. Foetal liver cells were centrifuged (220 x g, 5 min) and resuspended in 5 ml RCRB (Table 3.1) to lyse red blood cells. This was performed twice. Following the second lysis cells were centrifuged (220 x g, 5 min) and resuspended in 2% FACS buffer. Foetal liver cells were either resuspended in 5 ml 2% FACS buffer and WBCB counts determined using an ADVIA 2120i haematology analyser, or resuspended in 100 µl 2% FACS buffer containing cell surface markers to identify HSC^{SLAM} cells prior to cell sorting.

Based on ADVIA 2120i haematology analyser WBCB counts, 500,000 foetal liver white blood cells were combined with 1×10^6 adult bone marrow WBCB cells and injected into 3 x irradiated recipients (2 x doses of 5.5 Gy, 3 h apart) or 50 sorted HSC^{SLAM} cells were combined with 200,000 adult bone marrow cells and injected into one irradiated recipient (2 x doses of 5.5 Gy, 3 h apart).

3.2 Dissections

3.2.1 Embryo and foetus removal from pregnant females

On the desired embryonic day (E), pregnant females were sacrificed by cervical dislocation and embryos collected as described in section 3.1.5. Under a dissecting microscope (Stemi 2000-C, Carl Zeiss), embryos or foetuses were removed from their individual yolk sacs using fine scissors. A portion of the tail was removed for genotyping and placed in a chilled 1.5 ml Eppendorf tube (Eppendorf).

3.2.2 Embryonic dorsal telencephalon

On embryonic date 12.5, pregnant females were sacrificed by cervical dislocation. Embryos were isolated, as per section 3.2.1. Using fine forceps, the skin was removed from the head and forebrain hemispheres pinched away from the developing skull. Forebrain hemispheres were separated and turned so that medial surfaces were facing upwards. Fine forceps were used to pinch along the lateral ganglionic eminences and detach the dorsal telencephalon.

3.2.3 Foetal cortex

On embryonic date E16.5 or E18.5, pregnant females were sacrificed by cervical dislocation. Embryos were isolated, as per section 3.2.1. For dissection of E16.5 cortices for cortical neuron culture, fine forceps were used to peel back the skin and skull and reveal the brain. The skull surrounding the brain was carefully pulled away and the brainstem snipped to release the brain from the rest of the skull. Fine forceps were used to separate the forebrain from the mid and hindbrain sections and the meninges gently peeled away. Forebrain hemispheres were

separated and turned so that medial surfaces were facing upwards. Fine forceps were used to pinch along the lateral ganglionic eminences and detach the foetal cortex.

For cortical layering analysis of E18.5 cortices, small scissors were used to decapitate E18.5 fetuses at a region just below the brainstem. The brain was left within the skull for subsequent processing.

3.2.4 Adult cortex

For adult cortices, animals were euthanised by CO₂ asphyxiation. Animals were perfusion fixed as per section 3.4.2. Using large scissors, the skull was removed from the body and fur cut away. Forceps were used to hold the skull in place, with the dorsal surface facing upwards, while a small pick was used to scrape along the sutures of the skull. Curved forceps were then used to pull the skull away from the cortex. Large scissors were used to cut the brainstem and curved forceps used to gently ease the brain away from the skull, cutting the optic and trigeminal nerves to release the brain. Brains were placed into 4% (w/vol) PFA for a further 48 h.

3.2.5 Adult subventricular zone (SVZ)

Animals were sacrificed by CO₂ asphyxiation. Large scissors were used to remove the head and all fur. A straight pick was used to scrape along the sutures of the skull, allowing the skull to be peeled back from the brain using fine forceps. The brainstem, optical nerves and trigeminal nerves were severed with fine scissors to separate the brain from the skull. A scalpel blade was used to slice coronally at the optic chiasm, revealing the SVZ in the rostral section. Under a dissecting microscope (Zeiss) curved forceps were used to isolate the SVZ from the lateral ventricles.

3.2.6 Embryonic and foetal liver

On embryonic day E12.5 or E14.5, embryos and fetuses were collected and their livers dissected and passed through a 40 µm cell sieve (Corning, 352340). Cells were isolated by centrifugation (220 x g, 5 min, 4°C) and stained for appropriate cell surface markers for 1 h on

ice. Unbound antibody was removed by washing cells using 2x 5 ml 2% FACS buffer and cells collected by centrifugation (220 x g, 5 min, 4°C). Cells were analysed using the BD™ LSR II W (FACS LSR™ II SORP, BD Biosciences) at a rate of less than 7,500 events per second.

3.2.7 Adult bone marrow

Adult bone marrow from femurs or hips was flushed using an 18 G needle (Clifford Hallam, BD 302017) into 5 ml 2% FACS buffer. 300 µl undiluted bone marrow was used for ADVIA haematology analysis.

Remaining cells were isolated by centrifugation (220 x g, 5 min, 4°C) and stained for appropriate cell surface markers for 1 h on ice. Unbound antibodies were removed by washing cells in 2x 4 ml 2% FACS buffer and cells collected by centrifugation (220 x g, 5 min, 4°C). Cells were analysed using the BD™ LSR II W (FACS LSR™ II SORP, BD Biosciences) at a rate of less than 7,500 events per second.

3.2.8 Adult thymus

Thymi were dissected, partially dissociated using a scalpel and passed through a 40 µm cell strainer (Corning, 352340). Cells were isolated by centrifugation (220 x g, 5 min, 4°C) and stained for appropriate cell surface markers for 1 h on ice. Unbound antibodies were removed by washing cells in 2x 4 ml 2% FACS buffer and cells collected by centrifugation (220 x g, 5 min, 4°C). Cells were analysed using the BD LSRFortessa™ X-20 (FACS LSRFortessa™ X20 SORP, BD Biosciences) at a rate of less than 7,500 events per second.

3.2.9 Adult spleen

Spleens from adult mice were dissected, partially dissociated using a scalpel and passed through a 40 µm cell strainer (Corning, 352340). Cells were isolated by centrifugation (220 x g, 5 min, 4°C) and red blood cells depleted using 4.5 ml RCBC for 30 sec on ice. Cells were washed twice in 2% FACS buffer and stained for appropriate cell surface markers for 1 h on ice. Unbound antibodies were removed by washing cells in 2x 4 ml 2% FACS buffer and cells

collected by centrifugation (220 x g, 5 min, 4°C). Cells were analysed using the BD LSRFortessa™ X-20 (FACS LSRFortessa™ X20 SORP, BD Biosciences) at a rate of less than 7,500 events per second.

3.2.10 Peripheral blood

200 µl eye bleeds were collected in EDTA blood collection tubes (Sarstedt Inc, 20.1341.102). Peripheral blood was depleted of red blood cells using RCRB (2x 10 ml incubated for 10 min at RT) and white blood cells isolated by centrifugation (220 x g, 5 min, 4°C) and stained for appropriate cell surface markers for 1 h on ice. Unbound antibodies were washed using 2x 4 ml 2% FACS buffer and cells resuspended in 200 µl 2% FACS buffer. Blood cells were analysed using the BD™ LSR II W (FACS LSR™ II SORP, BD Biosciences) at a rate of less than 7,500 events per second.

3.3 Cell culture

All cell culture experiments were carried out using standard sterile techniques.

3.3.1 Neurosphere proliferation culture

Primary neural stem and progenitor cells (NSPCs) were dissected from the SVZ of adult mice or dorsal telencephalon of E12.5 embryos. Tissue was triturated into a single cell suspension in 1 ml complete neurosphere medium (Table 3.2) and passed through a 70 µm cell sieve (Corning, 431751). Cells were plated onto uncoated 6 – well cell culture plates (Corning, 353046) containing 2 ml complete neurosphere medium (Table 3.2) and grown at 37°C and 5% CO₂. Cells were passaged every 4 – 5 days before neurospheres became too large and before apoptosis became apparent within the neurosphere core. Floating neurospheres were collected and centrifuged at 200 x g for 5 min. Supernatant was removed and cells were resuspended in 1 ml Accutase™ (StemCell Technologies, 07922) and incubated for 5 min in a 37°C water bath. Cells were resuspended in 5 ml complete neurosphere medium and centrifuged at 200 g for 5 min. Supernatant was removed and cells resuspended in 1 ml fresh complete neurosphere medium and counted using a Countess™ automated cell counter (Invitrogen), excluding dead

cells using trypan blue (Sigma, T8154). Cells were seeded at 10,000 cells/cm² in 3 ml fresh complete neurosphere medium. The proliferative capacity of neurosphere cultures was assessed using cell counts at each passage to determine cumulative cell numbers.

3.3.2 Neurosphere differentiation assay

To determine the differentiation capacity of neurospheres, 10,000 cells/cm² were plated onto 4 – well chamber slides (Thermo Scientific™, 154526) pre-coated with 15 µg/ml poly-L-ornithine (Sigma-Aldrich, P4957) for 2 h at 37°C and 5% CO₂, followed by 4.5 µg/ml laminin (Sigma-Aldrich, L2020) O/N at 37°C and 5% CO₂, with a MT-PBS wash in between coating solutions. Cells were grown in neurosphere differentiation medium (Table 3.2) for 5 – 7 days at 37°C and 5% CO₂.

3.3.3 Secondary neurosphere formation assay

Secondary neurosphere formation was assessed as described in (Merson et al., 2006). Briefly, primary neurosphere cultures were dissociated using 1 ml Accutase™, as described in section 3.3.1 and cell counts determined using a Countess™ cell counter, excluding dead cells with trypan blue. Cells were re-plated into 96 well plates in 100 µl complete neurosphere medium (Table 3.2), at concentrations of 160, 80, 40, 20, 10, 5, 2.5 and 1.25 cells per well, in triplicate. Cells were grown in 37°C and 5% CO₂. Secondary neurosphere formation was quantified after 7 days of culture. Average secondary neurospheres formed per sample across technical triplicates, were compared between mutant and control samples.

3.3.4 E16.5 cortical neuron culture

E16.5 foetal cortices were dissected, washed in MT-PBS and mechanically triturated into a single cell suspension in 1 ml cortical neuron culture medium (Table 3.2). Cells were passed through a 70 µm cell sieve (Corning, 431751) and cell number determined using a Countess™ automated cell counter (Invitrogen). 10,000 cells/cm² were plated onto chamber slides (Sigma, C6932) pre-coated with 15 µg/ml poly-L-ornithine (Sigma-Aldrich, P4957) for 2 hr at 37°C and 5% CO₂, followed by 4.5 µg/ml laminin (Sigma-Aldrich, L2020) O/N at 37°C and 5%

CO₂, with a MT-PBS wash in between coating solutions. Cells were grown at 37°C and 5% CO₂ for 5 days to allow neurite outgrowth.

After 5 days, cells were fixed in 4% paraformaldehyde (PFA) in MT-PBS for 20 min at RT. Cells were permeabilised using 0.25 % Triton-X 100 for 1 h at RT and incubated with Mouse anti- β III tubulin IgG₁ (1:1000, Promega, G7121) in 10% FCS in MT-PBS for 1 h at 37°C. Cells were washed in MT-PBS (2 x 5 min at RT) and incubated with Goat anti-mouse IgG₁ γ AlexaFluor 568 conjugated secondary antibody (1:1000, Molec. Probes, A21124) for 1 h at 37°C. Cells were washed in MT-PBS (2 x 5 min at RT) and incubated with DAPI (1:1000, Invitrogen, D1306) for 5 min on ice. Cells were washed in MT-PBS (2 x 5 min at RT) and mounted in Dako fluorescent mounting medium (Dako, S3023). Cells were photographed using a fluorescent microscope (Zeiss) and digital camera (Axioplan 2, Zeiss).

For neurite outgrowth analysis, neurites were traced in Adobe Illustrator (Adobe, 2019 version). Only neurons that were sufficiently isolated such that neurites could be individually defined, were traced and analysed. Neurite tracings were defined as objects in Adobe Illustrator and object length converted from mm into μ m, based on the image field of view.

3.3.5 *Viral transduction of neurospheres*

The *Sox2* coding sequence (NM011443.4) was cloned into a pMSCV-IRES-GFP II (pMIG II) vector, using Xho1/EcoR1 restriction enzyme sites, by GeneArt Subcloning and Plasmid Services (ThermoFisher Scientific), to generate a *Sox2* expression construct. The *Sox2* construct was virally packaged using the Phoenix-AMPHO (ATCC ® CRL-3213TM) human cell line. Phoenix cells were plated at a density of 3×10^6 cells/10 cm plate in DMEM + 10% FCS for 24 h prior to transfection. The following day, 5.0 μ g *Sox2* construct was combined with 250 μ l 0.5 M CaCl₂, 250 μ l MQ-H₂O and 500 μ l 2x Hank's Balanced Salt Solution (HBS) (Sigma, H4385), vortexed for 10 sec and allowed to incubate for 10 min at RT. The transfection mixture was added dropwise over Phoenix cells, swirling the plate to ensure even distribution. The following morning, transfection medium was replaced with 6 ml complete neurosphere medium. Viral supernatant was collected 24 and 48 h later and passed through a 0.45 μ m filter syringe (Sartorius Australia, 16533-K). Filter supernatant was added to freshly passaged neurospheres.

3.4 Histology and immunohistochemistry

3.4.1 Gelatine coating of slides

Superfrost glass slides (Menzel-Glaser, AG00008032E) were submerged in 0.5% (w/vol) gelatine (Sigma, G-1890) with 0.05% (w/vol) chromium potassium sulphate (Ajax Chemicals, 7788990). Slides were dried O/N at RT and baked at 85°C for 2 h.

3.4.2 Perfusion Fixation

Mice were euthanised by CO₂ asphyxiation. Small scissors were used to expose the thoracic cavity, taking care not to damage any blood vessels. Scissors were used to cut the diaphragm adjacent to the rib cage and the ribs along both sides of the sternum. The sternum was lifted upwards and fixed in place with a needle to expose the lungs and heart. Curved tweezers were used to separate the heart from the pericardium and small scissors used to make a small incision in the right atrium. A 21G needle (Clifford Hallam, BD 302017), attached to a fluid line with RT, filtered MT-PBS, was inserted into the left ventricle. Mice were perfused with MT-PBS for 5 min until fluid emerging from the right atrium was clear and colourless, followed by RT, filtered, 4% PFA in MT-PBS for 5 min.

3.4.3 Cryostat sections

Dissected tissues were frozen at -80°C in O.C.T. embedding tissue freezing/mounting medium (Tissue Tek®, 4583) in cryomolds (Tissue Tek® Tek, SA62532-01). 10 µm tissue sections were cut using the Cryostat Microm 550 (Thermo Scientific) and placed on gelatine-coated slides. Sections were stored at -80°C until staining.

3.4.4 BrdU immunohistochemistry

Coronal sections from cortices of animals injected with BrdU, as described in section 3.1.7, were cut and mounted by the WEHI histology department. The day of staining, selected slides were dewaxed by the WEHI histology department and stored in MQ-H₂O. Antigen unmasking was performed by boiling slides in a microwave for 20 min in 1 L antigen unmasking solution

(Vector Laboratories, H-3300), diluted 9.4 ml/L in ultrapure H₂O (MilliporeSigma™ Milli-Q™ Advantage A10 Water Purification System, MilliporeSigma™ Z00Q0V0US). Endogenous peroxidase activity was blocked using 0.3% H₂O₂ in 50% (v/v) MeOH (Millipore, 67561) in H₂O for 20 min at RT, followed by 2x 5 min washes in MT-PBS, using Coplin jars. Non-specific sites were blocked using PBSG (0.25% (w/v) gelatine (Sigma, G-1890) in MT-PBS, for 20 min at RT in a Coplin jar. Slides were incubated with anti-BrdU antibody (Dako, M0744, clone Bu20a; 1:10 dilution in PBSG) for 1 h at RT in a wet chamber, followed by 2x 5 min MT-PBS washes at RT in a Coplin jar. Sections were incubated with biotinylated universal anti-mouse/rabbit IgG (H + L) (Vector Laboratories, Vectastain Universal Elite ABC Kit, PK-6200) in 1% normal horse serum (10 µl/ml) in MT-PBS for 1 h at RT in a wet chamber, followed by 2x 5 min washes in a Coplin jar. Slides were incubated with the coupling solution of Vectastain ABC kit (Vector Laboratories, PK-6200), according to the manufacturer's instructions. In the dark, slides were incubated with 0.5 mg/ml 3,3'-diaminobenzidine (Fluka 32750) in 0.01 % H₂O₂ in 0.05 M Tris-HCl, pH 7.6 for 2 – 10 min, until a colour reaction was observed. Washed slides were counterstained with 0.3% methyl green and mounted by the WEHI Histology Department.

BrdU positive SVZ cells were counted at six evenly spaced levels between the most rostral histological section of the frontal cortex and the most rostral section of the fimbria hippocampi. NSCs were identified as BrdU positive cells within the SVZ of the lateral wall of the lateral ventricle with a round morphology. Elongated cells, apoptotic cells or cells with very weak staining were not included. Volumetric analysis of the cortex was performed by the Cavalieri method, as described in (Coggeshall, 1992).

3.4.5 Triple antibody stain of differentiated neurosphere cultures

Cells were cultured in 4 – well chamber slides (Thermo Scientific™, 154526) pre-coated with 15 µg/ml poly-L-ornithine (Sigma-Aldrich, P4957) for 2 h at 37°C and 5% CO₂, followed by 4.5 µg/ml laminin (Sigma-Aldrich, L2020) O/N at 37°C and 5% CO₂, with a MT-PBS wash in between coating solutions. Cells were cultured under differentiation conditions, as described in section 3.3.2. Differentiated cells were fixed in 4% (w/vol) PFA for 20 min at RT, washed in MT-PBS (2x 5 min) and blocked in 10% (w/vol) FCS at 37°C for 1 h. Cells were incubated with mouse anti-O4 IgM antibody (1:500, Millipore, MAB345) in 10% (w/vol) NGS for 1 h at

37°C, followed by a 2x 5 min MT-PBS washes. Washed slides were incubated with goat anti-mouse IgM FITC-conjugated secondary antibody (1:500, Vector Laboratories, FI2020) for 1 h at 37°C. Following further washes cells were incubated with mouse anti- β III tubulin IgG₁ (1:1000, Promega, G7121) and rabbit anti-GFAP (1:500, Dako, Z0334) in 10% (w/v) NGS and 0.25% (w/vol) Triton-X 100 for 1 h at 37°C. Slides were washed in MT-PBS (2x 5 min) and incubated with goat anti-mouse-IgG₁ AlexaFluor 568 (1:1000, Molec. Probes, A21124) and goat-anti rabbit-IgG (H + L) AMCA-conjugated (1:500, Jackson, 111156003) secondary antibodies in 10% (w/vol) NGS for 1 h at 37°C. Slides were washed in MT-PBS (2x 5 min) and the walls of chamber slides removed. Slides were mounted in Dako fluorescent mounting medium (Dako, S3023) and allowed to dry O/N at 4°C. Slides were imaged using a fluorescent microscope (Axioplan2, Zeiss) and a digital camera (AxioCam HRC, Zeiss).

3.4.6 Cortical layer staining of E18.5 cortices

Frozen 10 μ m coronal cryostat sections from E18.5 cortices were thawed to RT for 30 min. In Coplin jars, cryosections were fixed in 4% (w/vol) PFA for 20 min at RT, washed in MT-PBS (2x 5 min), permeabilised in 0.25 % (w/vol) Triton X-100 (Sigma, T8787) for 20 min, washed in MT-PBS (2x 5min), blocked in 10% (w/vol) NGS in MT-PBS for 30 min and incubated with 1:250 anti-TBR1 (Abcam, ab31940), 1:500 anti-CTIP2 (Abcam, ab18465) and 1:50 anti-SATB2 (Abcam, ab31940) in 10% NGS in MT-PBS for 2 h in a wet chamber at RT. Slides were washed in MT-PBS (2x 5min). Slides were incubated for 1 h at RT in a wet chamber with 1:200 anti-rabbit IgG (H+L), AMCA (Jackson, 111-156-003), 1:1000 anti-rat IgG Alexa568 (A-11077) and 1:1000 anti-mouse IgG Alexa488 (Invitrogen, A11029) secondary antibodies and washed in MT-PBS (2x 5 min). Slides were mounted in fluorescence mounting medium (Dako, S3023) and left to dry at 4°C O/N. Slides were imaged using a fluorescent microscope (Axioplan2, Zeiss) and a digital camera (AxioCam HRC, Zeiss).

3.4.7 Colony forming assay

E14.5 foetal livers were dissected and total white blood cell counts (WBCB) determined using an ADVIA 2120i haematology analyser. 10,000 WBCB were cultured in 0.3% agar (Bioline, BIO-41025) and stimulated with purified murine growth factors, interleukin 3 (IL3; 10^{-3} U/ml; made in house), stem cell factor (SCF; 100 ng/ml; made in house) and erythropoietin (EPO; 2

U/ml; Janssen-Cilag, 113427240), with saline as a negative control. Cultures were incubated for 7 days prior to fixation and staining. Colonies were counted and qualified as previously described in (Metcalf, 1984).

3.5 Molecular biology and biochemistry

3.5.1 RNA extraction

RNA was extracted from dissected E12.5 dorsal telencephalon tissue or cultured neurospheres using a RNeasy Mini Kit (Qiagen, 74104) as per the manufacturer's instructions with the optional DNase digest step included. RNA quality and concentration were determined by automated electrophoresis using the Agilent 4200 Tapestation (Agilent).

3.5.2 RNA library construction and Next Generation sequencing

1 µg RNA, as determined by the Agilent 4200 Tapestation, was used per sample for library construction. Stranded mRNA libraries were generated using the Illumina TruSeq mRNA stranded library kit (Illumina), according to the manufacturer's instructions. Constructed libraries were validated and concentrations determined, using the Agilent 4200 Tapestation. Indexed libraries were pooled and diluted to 1.5 pM. Paired end sequencing was performed by Dr. Stephen Wilcox on a NextSeq 500 instrument (Illumina) using the v2 150 cycle High Output kit (Illumina), to yield 2.5×10^7 reads per sample. Subsequent bioinformatics analyses were performed by Prof. Gordon Smyth, Dr. Yifang Hu and Dr. Alexandra Garnham. Base calling and quality scoring were determined using Real-Time Analysis on board software (v2.4.6). FASTQ files were generated and de-multiplexed using bcl2fastq conversion software (v2.15.04). FASTQ files were aligned to the mouse genome, mm10, using the Rsubread aligner (1.21.12) (Liao et al., 2013). Reads overlapping each Entrez gene were summarised into counts using featureCounts (Liao et al., 2014) with inbuilt RefSeq annotation. Differential expression analyses were performed independently for *Kat6b*^{-/-} and *Tg(Kat6b)* samples and littermate controls using edgeR (Robinson et al., 2010) and limma (Ritchie et al., 2015) software packages. Unknown genes, pseudo genes, predicted genes, rRNA genes and genes without official gene symbols were removed. P-values were adjusted to control for a false discovery rate below 5% using the Benjamini and Hochberg method. Gene ontology analysis were carried

out using limma's goana function. Multi-dimensional scaling (MDS) plots, mean-difference (MD) plots and barcode plots were generated using limma's plotMDS, plotMD and barcodeplot function, respectively. RNA-seq data has not been made publicly available at this time, however will be made so upon publication of the results presented within this thesis

3.6 Flow cytometry and cell sorting

Cells were kept on ice until the time of analysis to maintain cell viability. Peripheral blood, embryonic liver, foetal liver and bone marrow cells were analysed on the BDTM LSR II W (FACS LSRTM II SORP, BD Biosciences); thymus and spleen cells were analysed on the BD LSRFortessaTM X-20 (FACS LSRFortessaTM X20 SORP, BD Biosciences). Cell sorting of foetal liver and bone marrow cells for transplant experiments was carried out on the Aria W (FACS AriaTM II SORP, BD Biosciences) cell sorter. All collected data was subsequently analysed using FlowJo 10.1r5 (Tree Star Inc.). RNA-seq data has not been made publicly available at this time, however will be made so upon publication of the results presented within this thesis.

3.6.1 Histone acetylation in peripheral blood

Peripheral blood samples were collected into EDTA-coated tubes by an animal technician. Red blood cells were lysed using 2x 10 min washes in 10 ml red cell removal buffer (RCRB) (Table 3.1) at RT. White blood cells were pelleted by centrifugation (220 x g, 5min, 4°C). Cells were resuspended in 100 µl MT-PBS containing 1:1000 dilution of LIVE/DEAD Fixable Green Dead Cell stain (Thermo Scientific, L23101) and incubated on ice for 30 min. Cells were washed once in 5 ml 2% FACS buffer and stained for 1 h on ice for cell surface markers to differentiate peripheral white blood cell populations (Table 3.3). Cells were washed in 3–4 ml 2% FACS buffer and pelleted by centrifugation (220 x g, 5 min, 4°C). Cells were fixed and permeabilised using the FOXP3 Transcription Factor Staining Buffer Set (Thermo Scientific; 00-5523-00). Fix/perm buffer was washed using 4 ml 2% FACS buffer and cells pelleted by centrifugation (220 x g, 5 min, 4°C). Cells were resuspended in 500 µl 2% FACS buffer containing a 1:500 dilution of anti-Acetyl Histone H3 Lysine 9 antibody (Cell Signalling, 9649S) O/N at 4°C on a roller.

The following morning cells were washed in 3– 4 ml 2% FACS buffer and resuspended in 100 µl 2% FACS buffer containing anti-rabbit Pacific Blue secondary (1:500, Jackson ImmunoResearch Laboratories, Inc, 711476152) and incubated for 1 h on ice. Cells were washed in 3– 4 ml 2% FACS buffer and resuspended in 200 µl 2% FACS buffer. Cells were processed with the BD™ LSR II W (FACS LSR™ II SORP, BD Biosciences) and analysed using FlowJo 10.1r5 (Tree Star Inc.).

3.6.2 Sox2-GFP in dorsal telencephalon tissue

E12.5 embryos were collected and the dorsal telencephalon dissected as per section 3.2.2. Dissected tissue was triturated into a single cell suspension in 1 ml 2% FACS buffer. Cells were centrifuged at 220 x g for 5 min. Cells were resuspended in 50 µl 2% FACS buffer, containing 1:200 dilution of CD133-APC (eBiosciences, 17-1331-81) and SSEA1/CD15 (BD Biosciences, 347420) antibodies and incubated on ice for 1 h. Cells were washed in 5 ml 2% FACS buffer and centrifuged at 220 x g for 5 min. Cells were resuspended in 50 µl 2% FACS buffer containing 1:200 dilution of human anti-mouse PE-conjugated secondary antibody (made in house) and incubated for 1 h on ice. Cells were washed in 5 ml 2% FACS buffer and centrifuged at 220 x g for 5 min. Cells were resuspended in 100 µl 2% FACS buffer and kept on ice prior to analysis.

3.6.3 Cell sorting

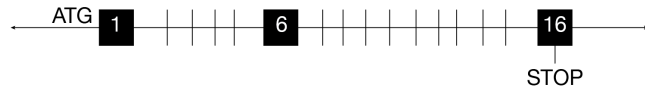
Foetal livers or adult bone marrow were isolated and stained as per sections 3.24 and 3.25. For primary transplants foetal liver HSC^{SLAM} (CD48 – CD150 +) cells were sorted by a trained operator on the BD FACS Aria W (FACS Aria™ II SORP, BD Biosciences) cell sorter, using a 70 µm nozzle at 70 psi and less than 20,000 events per second. Cells were sorted into a 1.5 ml Eppendorf containing 100 µl 2% FACS buffer.

For secondary transplants, adult bone marrow-derived CD45.2 + HSC^{SLAM} cells were sorted by a trained operator on the BD Influx™ cell sorter (BD Influx™, BD Biosciences) using a 70 µm nozzle at 70 psi and less than 20,000 events per second. Cells were sorted into a 1.5 ml Eppendorf containing 100 µl 2% FACS buffer.

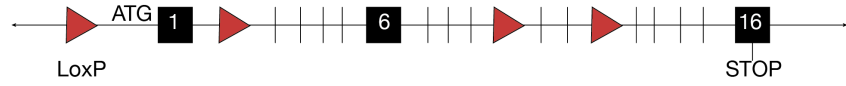
3.7 Statistical Analysis

All data are presented as mean $\pm$ s.e.m. Statistical analysis was performed using Prism GraphPad Version 8.3.1 for Mac (GraphPad Software). Statistical tests used and the number of biological or technical replicates are specified in the figure legends of the results section.

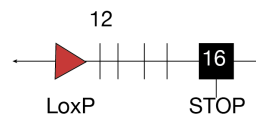
A *Kat6b* wild type allele (*Kat6b*⁺)



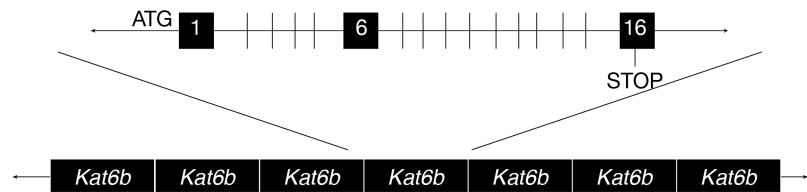
B *Kat6b* floxed allele



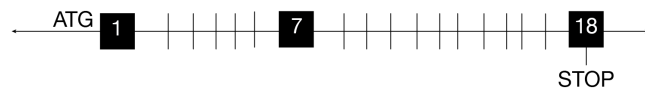
C *Kat6b* null allele (*Kat6b*⁻)



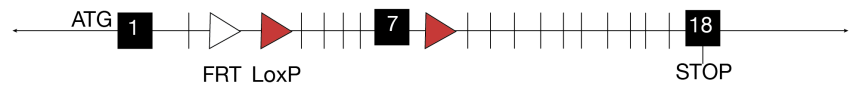
D *Kat6b* overexpression construct (*Tg(Kat6b)*)



E *Kat6a* wild type allele (*Kat6a*⁺)



F *Kat6a* floxed allele



G *Kat6a* null allele (*Kat6a*⁻)

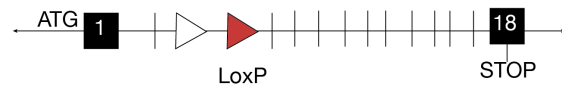


Figure 3.1 *Kat6b* and *Kat6a* allele schematic with exon structure shown

Mice used for this work were previously generated in the host laboratory. Full details of allele generation can be found in section 3.1.

(A – C) The *Kat6b* wild type allele (*Kat6b*⁺) (A) was double targeted by homologous recombination to introduce *loxP* sites flanking exon 1 and exons 10 – 11 (B). *Cre*-mediated recombination removed exons 1 – 11, comprising 130 kb of DNA and yielded the *Kat6b* null allele (*Kat6b*⁻) (C), lacking an internal initiating methionine, the NEMM domain, PHD domains, catalytic MYST domain and a portion of the acidic domain. Any product produced would lack any acetyltransferase activity and would be out of frame, thereby lacking any resemblance to the KAT6B protein.

(D) *Kat6b* overexpressing transgenic mice were generated by introducing seven additional copies of the wild type *Kat6b* sequence into a pBACe3.6 (U80929) BAC vector.

(E – G) The wild type *Kat6a* allele (E) was targeted by homologous recombination to introduce an *frt*-flanked neomycin phosphotransferase selection cassette and *loxP* sites flanking exons 3 – 7 of the endogenous *Kat6a* locus. Following removal of the *frt*-flanked neomycin phosphotransferase selection cassette, the floxed allele contained *loxP* sites flanking exons 3 – 7 and single remaining *frt* site (F). *Cre*-mediated recombination yielded the *Kat6a* null allele, lacking exons 3 – 7 with single remaining *loxP* site (G).

Table 3.1 Buffers and solutions

Buffer	Recipe
2% FACS buffer pH 7.1-7.2	2% (vol/vol) FCS, 150 mM NaCl, 3.7 mM KCl, 2.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.8 mM K_2HPO_4 , 1.2 mM KH_2PO_4 , 11.5 mM HEPES, made up in MQ- H_2O
MT-PBS pH 7.4	16 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.4 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 150 mM NaCl, in MQ- H_2O , pH 7.4
RCRB pH 7.2	150mM ammonium chloride (NH_4Cl), 0.1 mM EDTA, 12 mM NaHCO_3 made up in MQ- H_2O
Tail Lysis Buffer	10mM Tris.Cl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS made up in MQ- H_2O

Table 3.2 Cell culture media

Medium	Recipe
Neurosphere basal medium	5mM HEPES (Sigma H4034), 13.4 mM Sodium Carbonate (NaHCO_3), 0.6% D-glucose, 100 Units/ml Penicillin, 100 $\mu\text{g/ml}$ Streptomycin (Gibco, 15140-122), 1x DMEM/F12 sachet/L (Gibco 12500-062), made up in MQ- H_2O
Complete neurosphere medium	25 $\mu\text{g/ml}$ Insulin (Sigma 16634), 9.6 $\mu\text{g/ml}$ (60 μM) Putrescine dihydrochloride (Sigma P6149), 100 $\mu\text{g/ml}$ (30 nM) Selenium (sodium salt) (Sigma S9133), 6.3 ng/ml (20 nM) Progesterone (Sigma P6149), 10 ng/ml Fibroblast Growth Factor (bFGF) (Roche 1104616), 4 $\mu\text{g/ml}$ Heparin (sodium salt) (Sigma H3149), 20 ng/ml Epidermal Growth Factor (EGF) (BD 354010) and 0.2 % BSA (2 mg/ml) (Sigma A3311) made up in neurosphere basal medium
Neurosphere differentiation medium	25 $\mu\text{g/ml}$ Insulin (Sigma 16634), 9.6 $\mu\text{g/ml}$ (60 μM) Putrescine dihydrochloride (Sigma P6149), 100 $\mu\text{g/ml}$ (30 nM) Selenium (sodium salt) (Sigma S9133), 6.3 ng/ml (20 nM) Progesterone (Sigma P6149), 4 $\mu\text{g/ml}$ Heparin (sodium salt) (Sigma H3149), 0.2 % BSA (2 mg/ml) (Sigma A3311) and 1% (vol/vol) FCS made up in neurosphere basal medium
Cortical neuron medium pH 7.2	25 $\mu\text{g/ml}$ Insulin (Sigma 16634), 9.6 $\mu\text{g/ml}$ (60 μM) Putrescine dihydrochloride (Sigma P6149), 100 $\mu\text{g/ml}$ (30 nM) Selenium (sodium salt) (Sigma S9133), 6.3 ng/ml (20 nM) Progesterone (Sigma P6149), 4 $\mu\text{g/ml}$ Heparin (sodium salt) (Sigma H3149), 0.2 % BSA (2 mg/ml) (Sigma A3311) and 1% (vol/vol) FCS made up in neurosphere basal medium

Table 3.3 Peripheral blood FACS antibodies

Marker	<i>Fluorophore</i>	<i>Clone number</i>	<i>Order ref</i>	<i>Dilution</i>
B220	A700	B220 clone 16A	Made in house	1/200
CD19	PE/Cy7	BD Pharmingen clone 1D3	#552854	1/200
CD4	APC	GK1.5	BD 553730	1/200
CD8a	PE	Clone 56.3.7	Made in house	1/200
GR1	A594	Clone 1A8	Made in house	1/200
MAC1	PerCp/Cy5.5	BioLegend clone ICRF44	#301327	1/200
CD45.1	BV650	BD Horizon clone A20	#563754	1/200
CD45.2	FITC	BD clone A20	BD 553775	1/200
Viability	Fluorogold	Polyclonal	AB153-I	1/120

Table 3.4 Embryonic and foetal liver FACS antibodies

Marker	Fluorophore	Clone number	Order ref	Dilution
LIN (B220, CD19, CD4, CD8, Gr1, Ter119, Lyg6	A700	B220 clone 16A, CD19 clone 1D3, CD4 clone 17A2, CD8a clone 53-6.7, Gr1 clone RB6-8C5, Ter119 clone TER-119 (not for erythroid development), LyG6	Made in house	1/400
cKIT	PerCp/Cy5.5	BD clone 2B8	#563160	1/200
SCA1	A594	Clone E13	Made in house	1/200
CD48	PE/Cy7	Ebioscience clone HM48-1	#25-0481-80	1/200
CD150	A647	Bioleged clone TC15-12F12.2	#115918	1/200
CD34	FITC	eBioscience clone RAM34	#11-0341-82	1/200
CD135/FLT3	PE	BD clone A2F10.1	BD 553842	1/200
CD71	FITC	2B8	BD 553266	1/200
TER119	PE	TER-119	BD 553673	1/200
Viability	Fluorogold	Polyclonal	AB153-I	1/120
H3K9ac	Unconjugated	C5B11	Cell signalling #9649	1/500
H3K14ac	Unconjugated	D4B9	Cell signalling #7627	1/500

H3K23ac	Unconjugated	EPR17712	ab177275	1/500
Anti-rabbit secondary	Pac Blue	Polyclonal	ThermoFisher P-10994	1/500

Table 3.5 Bone marrow FACS and cell sort antibodies

Marker	Fluorophore	Clone number	Order ref	Dilution
LIN (B220, CD19, CD4, CD8, Gr1, Mac1 (BM only), Ter119, LYG6	A700	B220 clone 16A, CD19 clone 1D3, CD4 clone 17A2, CD8a clone 53-6.7, Gr1 clone RB6-8C5, Mac1 , Ter119, LyG6	Made in house	1/400
cKIT	PerCp/Cy5.5	BD clone 2B8	#563160	1/200
SCA1	A594	Clone E13	Made in house	1/200
CD48	PE/Cy7	eBioscience clone HM48-1	#25-0481-80	1/200
CD150	A647	Biolegend clone TC15-12F12.2	#115918	1/200
CD34	A647	BD clone RAM34	#560230	1/100
CD135/FLT3	PE	Clone A2F10	Made in house	1/100
CD127/IL7R α	PE	Clone A7R34	Made in house	1/200
CD16/32	PE/Cy7	Biolegend clone 93	#101317	1/200
CD45.1	BV650	BD Horizon clone A20	#563754	1/200
CD45.2	FITC	BD clone A20	BD 553775	1/200
Viability	Fluorogold	Polyclonal	AB153-I	1/120

Table 3.6 Spleen FACS antibodies

Marker	Fluorophore	Clone number	Order ref	Dilution
CD45.1	FITC	S450-15-2	Made in house	1/400
CD19	Pacific Blue	1D3	Made in house	1/300
CD23	PE/Cy7	B3B4	Biolegend 101614	1/200
CD21/35	APC-eFluor 780	eBio8D9	eBioscience 47-0211-80	1/200
CD93	PE	AA4.1	BD Pharmingen 558039	1/200
IgM	PerCP-eFluor 710	II/41	eBioscience 46-5790-82	1/400
CD138	Brilliant Violet 650	281-2	Biolegend 142517	1/500
CD98	Alexa Flour 647	RL388	Biolegend 128210	1/500
CD45.1	Biotin	A20.1	Made in house	1/50
CD45.2	FITC	BD clone A20	BD 553775	1/200
Streptavidin	BV 786		BD Biosciences 563858	1/300
Viability Dye	eFluor 506		eBioscience 65-0866-14	1/500

Table 3.7 Thymus FACS antibodies

Marker	Fluorophore	Clone number	Order ref	Dilution
B220	Biotin	clone RA3-6B2	Made in house	1/200
CD25	PerCp/Cy5.5	clone PC61	#561112	1/200
CD4	PE	clone GK1.5	Made in house	1/400
CD44	FITC	clone 1M7	Made in house	1/400
CD45.1	BV650	clone A20	#563754	1/200
CD45.2	A700	clone S450-15-2	Made in house	1/200
CD8	PE/Cy7	clone 53-6.7	#552877	1/200
cKIT	A647	clone ACK4	Made in house	1/200
MAC1	Biotin	clone M1/70	Made in house	1/200
TER119	Biotin	clone TER-119	Made in house	1/200
Streptavidin	E450	Polyclonal	Cat#48-4317-82	1/400

Table 3.8 Dorsal telencephalon FACS antibodies

Marker	Fluorophore	Clone number	Order ref	Dilution
CD133	APC	clone 2F8	eBiosciences 17-1331-81	1/200
SSEA1/CD15	Unconjugated	Clone MMA	BD Biosciences 347420	1/200
Anti-mouse secondary	PE		Made in house	1/400
Viability	Fluorogold	Polyclonal	AB153-I	1/120

Table 3.9 Immunofluorescence antibodies

Marker	Order reference	Dilution
β III tubulin mAb IgG ₁	Promega #G7121	1/2000
GFAP	Dako Corp, #Z0334	1/500
O4	Millipore, MAB345	1/500
DAPI	Invitrogen, D1306	5 mg/ml
TBR1	Abcam, ab31940	1/250
SATB2	Abcam, ab51502	1/500
CTIP2	Abcam, ab18465	1/50
Goat α -mouse IgM (FITC)	Vector #F1-2020	1/400
Goat α -mouse IgG _γ AlexaFluor568	Invitrogen, A21124	1/800
Goat α -rabbit IgG (H + L), Jackson Laboratories, AMCA-conjugated	Jackson ImmunoResearch, 111-156-003	1/200

Table 3.10 Genotyping primers

<i>Targeted sequence</i>	<i>Primer sequence 5' → 3'</i>	<i>PCR amplicons</i>
<i>Kat6b</i> +	F1 TGCCTCAGAAAGCCATTACC	WT = 230 bp
<i>Kat6b</i> –	F2 CAGACAAATCAGCCCCAGAT	Del = 600 bp
	R GAGGCTCAGGGCTACAAGTG	
<i>Kat6a</i> +	F1 GATCCCTGTCCGCACCCTCTATG	WT = 300 bp
<i>Kat6a</i> –	F2 GCCAGTCCCTTCCCGCTTCAGTG	Del = 400 bp
	R ACACAACGTCTGCGTGAGGC	
<i>Cre</i>	F GCGCGGTCTGGCAGTAAAAAC	Cre = 500 bp
	R GCAGATGGCGCGGCAACAC	
<i>Sox2-GFP</i>	F AAGTTCATCTGCACCACCG	GFP = 173
	R TCCTTGAAGAAGATGGTGCG	
<i>SacB in BAC</i>	F CACCTCAATCGACAGCTGGA	SacB = 190 bp
	R GGCTTTGTTTGCCGTAATGT	
<i>Y repeat</i>	F TTGGAAGGCCATCATTCTAGG	Y repeat = 250 bp
	R CATCCCACTCCAGTTGTCCT	

Table 3.11 Peripheral blood cell surface markers

Cell type	Cell surface markers
B cells	B220 + CD19 +
CD4 T cells	CD4 + CD8 –
CD8 T cells	CD4 – CD8 +
Monocytes	GR1 lo MAC1 +
Neutrophils	GR1 hi MAC1 +

3.12 Erythroid maturation cell surface markers

Cell type	Cell surface markers
S0	LIN (B220, CD19, CD4, CD8, Gr1, Lyg6) –, Ter119 lo, CD71 lo
S1	LIN –, Ter119 lo, CD71 med-hi
S2	LIN –, Ter119 med, CD71 med-hi
S3	LIN –, Ter119 hi, CD71 med-hi
S4	LIN –, Ter119 hi, CD71 med
S5	LIN –, Ter119 hi, CD71 lo

3.13 Bone marrow HSC and progenitor cell surface markers

Cell type	Cell surface markers
LSK	LIN −, cKIT +, SCA1 +
LK progenitors	LIN −, cKIT +, SCA1 −
HPC-1	LIN −, cKIT +, SCA1 +, CD48 +, CD150 −
HPC-2	LIN −, cKIT +, SCA1 +, CD48 +, CD150 +
HSC ^{SLAM}	LIN −, cKIT +, SCA1 +, CD48 −, CD150 +
MPP ^{SLAM}	LIN −, cKIT +, SCA1 +, CD48 −, CD150 −
HSC ^{CD34loFlt3lo}	LIN −, cKIT +, SCA1 +, CD34 lo, FLT3 lo
HSC ^{CD34hiFlt3lo}	LIN −, cKIT +, SCA1 +, CD34 hi, FLT3 lo
LMPP	LIN −, cKIT +, SCA1 −, CD34 hi, FLT3 med
MPP	LIN −, cKIT +, SCA1 −, CD34 hi, FLT3 hi
GMP	LIN −, cKIT +, SCA1 −, CD16/32 +, CD34 hi
CMP	LIN −, cKIT +, SCA1 −, CD16/32 +, CD34 hi
MEP	LIN −, cKIT +, SCA1 −, CD16/32 −, CD34 lo

3.14 Spleen B and progenitor cell surface markers

Cell type	Cell surface markers
Immature B cells	CD19 +, CD93 + B220 +
T1 B cells	CD19 +, CD23 –, CD93 +
T2 B cells	CD19 +, CD23 +, CD93 +, IgM +
T3 B cells	CD19 +, CD23 +, CD93 +, IgM –
Follicular B cells	CD19 +, CD23 +, CD93 –
Marginal zone (MGZ) B cells	CD19 +, CD23 –, CD93 +, CD21/35 +, IgM +
B1 B cells	CD19 +, CD23 –, CD93 +, CD21/35 –, IgM +
Mature B cells	CD19 +, CD93 –, B220 +
Plasma cells	CD138 + CD98 +

3.15 Thymus T and progenitor cell surface markers

Cell type	Cell surface markers
ETP	cKIT +, CD44 +, CD25 –, CD4 –, CD8 –
DN2	cKIT +, CD44 +, CD25 +, CD4 –, CD8 –
DN3	cKIT +, CD44 –, CD25 +, CD4 –, CD8 –
DN4	cKIT +, CD44 –, CD25 –, CD4 –, CD8 –
DP	CD44 +, CD4 +, CD8 +
CD4	CD4 +, CD8 –
CD8	CD4 –, CD8 +

Chapter 4

The role of KAT6B in neural stem cells and cortical development

Introduction

The histone acetyltransferase, KAT6B, is required for normal cortex development. In mice, a *Kat6b* gene-trap allele that produces only 10% of the normal mRNA levels, results in small cerebral cortices, with reduced or absent layer V pyramidal neurons (Thomas et al., 2000). Furthermore, NSPCs derived from *Kat6b* – deficient animals show reduced potency and proliferation *in vitro* and *in vivo* (Merson et al., 2006; Sheikh et al., 2012), as well as a reduced capacity for neuronal differentiation in culture (Merson et al., 2006). In this chapter I aimed to further investigate the requirement for KAT6B within the developing brain and in NSPCs.

4.1 Results

4.1.1 Characterising the *Kat6b* null phenotype in mice

To investigate the requirement of KAT6B during development, exons 1 – 11 of endogenous *Kat6b*, encompassing the NEMM, double PHD finger domains and most of the catalytic MYST domain, were excised by *Cre*-mediated deletion, removing 130 kb of DNA (Fig 3.1 B – C). Given the size of the region to be removed and unavailability of CRISPR technology at the time of allele generation, it was not possible to target the 130 kb region with a single construct, hence, the method of double targeting was employed. This large region was also removed, as opposed to only deleting the catalytic MYST domain, as there is an abundant 1 kb mRNA transcribed from exon 1, of currently unknown function that is 10x more abundant in the brain than the full length *Kat6b* (Thomas et al., 2000), which would not have been deleted if only the catalytic domain had been targeted. Furthermore, only deleting exon 1 could still have allowed for alternate slicing that could have produced an in-frame mRNA that still expressed the MYST domain. By RT-qPCR, *Kat6b*^{-/-} animals showed no detectable mRNA from the excised exons in either dorsal telencephalon tissue or neurospheres (Fig 4.1 A – B). In the absence of reliable antibodies against KAT6B, it is possible that a protein product is still produced from exons 12 to 16 of the *Kat6b* null allele, however, the entire product would be out of frame and lack an internal initiating methionine, such that any abnormal product would lack all resemblance to the KAT6B protein. Nevertheless, it is possible that such an abnormal protein would have functional effects.

Phenotypically, homozygous *Kat6b* mutants (*Kat6b*^{-/-}) did not show any overt abnormalities at E12.5, compared to heterozygous or wild type controls (Fig 4.1 C), however at E18.5, displayed an underdeveloped lower jaw (Fig 4.1 D – E), as has previously been reported in *Kat6b* gene trap mutants, lacking 90% of *Kat6b* mRNA (Thomas et al., 2000). Despite being observed at a normal Mendelian ratio *in utero* up to E18.5 (Fig 4.2 A), *Kat6b*^{-/-} mutants showed perinatal lethality at 100% penetrance on a *C57BL/6* background. To address this perinatal lethality, *Kat6b* wildtype and mutant pups were observed following caesarean delivery at E19.5 (Table 4.1). Relative to heterozygous or wild type siblings, *Kat6b* null mutants were substantially delayed in inflating their lungs ($p < 0.01$) and oxygenating their blood ($p < 0.01$), as indicated by the acquisition of a pink skin colour. There were also significantly delayed in establishing regular, rhythmic, high-frequency and shallow breathing ($p < 0.01$), as compared to the deep, gasping breaths taken by all pups early after delivery. Nonetheless, new-born *Kat6b*^{-/-} pups vocalised upon contact and displayed spontaneous movement, indicating that they could respond to maternal cues. Furthermore, collection of *Kat6b*^{-/-} pups within 1 – 2 hours after birth showed stomachs full of milk, indicating that they were capable of suckling and do not suffer from significant heart or lung defects, given the high energy expenditure required for this task. Furthermore, despite reaching basic milestones after birth, animals heterozygous for the *Kat6b* mutant allele (*Kat6b*^{+/-}) were under-represented at weaning relative to wild type controls (Fig 4.2 C; $\chi^2 p < 0.01$). This indicates that both the complete loss of *Kat6b* and reduction in KAT6B, impair survival in early life.

4.1.2 Characterising the *Kat6b* overexpression phenotype in mice

The effect of *Kat6b* gain was assessed using BAC transgenic animals (*Tg(Kat6b)*), carrying seven additional copies of the wild type *Kat6b* allele (Fig 3.1 D). By RTqPCR this resulted in a 3.5 – 4-fold increase in *Kat6b* mRNA in the dorsal telencephalon and cultured NSPCs (Fig 4.1 A – B). *Tg(Kat6b)* animals were phenotypically normal at E12.5 and E18.5 compared to wild type, littermate controls (Fig 4.1 C – E). Analogous to *Kat6b*^{+/-} animals, despite being observed at a normal Mendelian ratio *in utero* up to E18.5 (Fig 4.2 B), *Tg(Kat6b)* animals were underrepresented at weaning relative to wild type controls (Fig 4.2 C; $\chi^2 p < 0.0001$). In addition, adult male and female *Tg(Kat6b)* animals displayed heightened aggression, resulting in the necessity for euthanasia due to fight wounds, as well as spontaneous epilepsy from 1 year of age, both perhaps suggesting abnormal brain function.

4.1.3 KAT6B in cortical layering

Kat6b deficiency results in a thin cortical plate during development, and subsequently small cerebral cortices in adulthood (Merson et al., 2006; Thomas et al., 2000). To assess the effect of KAT6B loss and gain on cortical layer development, coronal sections from the frontal, parietal and occipital cortex of *Kat6b* mutant and control animals were stained to detect the transcription factors SATB2, CTIP2 and TBR1, which mark superficial, intermediate and deep layers of the developing cortex, respectively. For the purposes of quantification, cortices on stained sections were divided into 10 pial to ventricular bins and the proportion of each cell type quantified therein.

In both *Kat6b*^{-/-} and *Tg(Kat6b)* mice, compared to wild type controls, all cortical layers were present in the correct order and showed distinct layer boundaries (Fig 4.3 A and Fig 4.4 A). In addition, no significant differences were found in the proportion of cell types within each bin (Fig 4.3 B and Fig 4.4 B), indicating that *Kat6b* status does not affect cortical layer development. The *Kat6b*^{-/-} mutant cortical plate histopathology was not assessed in this thesis and so waits further investigation.

4.1.4 KAT6B promotes NSPC proliferation *in vitro* and *in vivo*

Neural stem and progenitor cells (NSPCs) can be grown in culture as free-floating clusters known as neurospheres. It has already been shown that *Kat6b* loss results in impaired proliferation in NSPCs both *in vivo* and *in vitro* (Merson et al., 2006), with *Kat6b* expression levels positively correlated with NSC self-renewal (Sheikh et al., 2012). In addition, *Kat6b* loss is observed to result in an impaired capacity for NSCs to differentiate into neurons in culture (Merson et al., 2006). To interrogate whether KAT6B is a prerequisite for NSC function or a driver of NSC proliferation and neurogenesis, I assessed the effect of *Kat6b* overexpression on NSPC proliferation and neuronal differentiation.

NSPCs derived from the adult subventricular zone (SVZ) of *Tg(Kat6b)* animals and wild type controls were propagated *in vitro* as neurospheres. Over consecutive passages *Tg(Kat6b)*-derived NSPCs showed significantly enhanced proliferation relative to wild type controls (Fig 4.5 A, $p < 0.0001$). In addition, the neurospheres formed from *Tg(Kat6b)* animals had a larger average diameter at each passage, compared to wild type cells (Fig 4.5 B – C, $p < 0.001$) and

were capable of giving rise to significantly more secondary neurospheres when plated in a dilution series (Fig 4.5 D, $p = 0.007$), indicating that KAT6B gain drives NSPC proliferation and self-renewal.

To assess NSPC proliferation *in vivo*, *Tg(Kat6b)* animals and controls were treated with the thymidine analogue BrdU for a 2 week period, followed by 2 weeks without treatment (Fig 4.6 A). Treated animals were sacrificed and their cortices sectioned and stained for BrdU positive cells within the SVZ. Given the relatively low division rate of NSCs to other cell types, BrdU was expected to be detected within stem cells (long-term label – retaining cells) and not in more actively dividing or migrating cell types. The number and depth of coronal sections was used to determine the rostro-caudal extent of the cortex, while BrdU positive cells were quantified at six forebrain levels along the rostro-caudal axis. *Tg(Kat6b)* animals showed no difference in the rostro-caudal length of the cortex, relative to wild type controls (Fig 4.6 B, $p = 0.30$), however, *Tg(Kat6b)* mice showed significantly more BrdU positive cells within the SVZ across all cortex levels (Fig 4.6 C – D; $p < 0.0001$), indicating that KAT6B overexpression can drive NSC proliferation and self-renewal *in vitro* and proliferation of SVZ NSCs *in vivo*.

4.1.5 KAT6B drives neuronal differentiation and neurite outgrowth *in vitro*

Kat6b deficiency has been shown to impair the ability of cultured NSPCs to give rise to neurons *in vitro* (Merson et al., 2006). To test the differentiation potential of NSPCs overexpressing *Kat6b*, neurospheres were subjected to differentiation culture conditions for 7 days and their capacity to form neurons, astrocytes and oligodendrocytes examined using immunofluorescence, using the cell type-specific markers β III tubulin, GFAP and O4, respectively. *Tg(Kat6b)* NSPCs gave rise to proportionally more neurons ($p < 0.01$) and fewer astrocytes ($p < 0.01$), compared to controls, with no difference observed in the proportion of oligodendrocytes formed between mutant and control samples ($p = 0.2$) (Fig 4.7 B – C). This indicates that *Kat6b* overexpression can drive neuronal differentiation at the expense of astrocyte differentiation, the opposite effect to what has been described when KAT6B is lost (Merson et al., 2006).

Given that *Kat6b* status altered neuronal differentiation, the effects of *Kat6b* expression on neuronal arborisation and neurite outgrowth were also assessed *in vitro*. E16.5 cortical neurons

from *Kat6b*^{-/-} (Fig 4.8), *Tg(Kat6b)* (Fig 4.9) and control animals were cultured for 5 days, prior to fixation and staining for the neuronal marker β III tubulin and nuclear marker DAPI. Neurites from individual neurons were then traced to determine the number and average length of 1°, 2°, 3° and 4° neurites per cell. *Kat6b* loss resulted in significantly shorter primary ($p = 0.003$) and secondary neurites ($p = 0.02$) (Fig 4.8 C), as well as fewer secondary neurites ($p = 0.04$) (Fig 4.8 D) on average per cell. Conversely, *Kat6b* overexpression resulted in significantly longer primary ($p = 0.003$) and secondary neurites ($p = 0.03$) (Fig 4.9 C), as well more secondary neurites ($p = 0.02$) (Fig 4.9 D) on average per cell, relative to wild type controls. This indicates that in addition to driving neuronal differentiation in NSPCs, KAT6B can also drive 1° and 2° neurite number and 2° neurite length *in vitro*.

4.2 Discussion

In this chapter, I investigated the effect of *Kat6b* loss and overexpression on cortical layering and NSPC proliferation and differentiation, demonstrating that KAT6B drives proliferation in NSPCs both *in vivo* and *in vitro*, as well neuronal differentiation and neurite outgrowth in culture. Previous studies of *Kat6b*^{gt/gt} mutants, deficient in 90% of *Kat6b* mRNA, have shown that *Kat6b* deficiency results in a failure to thrive phenotype and animals with craniofacial abnormalities and small cerebral cortices, deficient in or lacking layer V pyramidal neurons (Thomas et al., 2000). The work described in this chapter builds upon these findings by assessing the effects of *Kat6b* loss as well as its overexpression. Here, I showed that *Kat6b* homozygous loss results in perinatal lethality, while both *Kat6b* heterozygosity and overexpression impair survival in early postnatal life. This indicates that disruption of *Kat6b* expression in either direction, disrupts early survival and highlights the requirement for specific expression of chromatin modifiers during development and in early life. At E18, *Kat6b* homozygous mutants had underdeveloped jaws, consistent with shortened frontal bones previously described in *Kat6b*^{gt/gt} mutants (Thomas et al., 2000). By comparison, *Tg(Kat6b)* mutants were phenotypically normal at E18, relative to littermate controls, however displayed high levels of aggression and spontaneous epilepsy in adulthood, indicative of neurological abnormalities. While *Kat6b*^{gt/gt} E15.5 and adult brain histopathology revealed substantial anomalies in the E15.5 cortical plate and the adult cortex (Thomas et al., 2000), the brain histopathology of the *Kat6b*^{-/-} fetuses or foetal or adult *Tg(Kat6b)* brains was not assessed in this thesis. Therefore, these aspects of the effects of the complete loss and gain of KAT6B

await further study. In addition, the *Kat6b*^{-/-} mutants do not survive to adulthood and therefore a direct comparison between the data on GAD67+ interneurons in adult *Kat6b*^{gt/gt} brains as published previously (Thomas et al., 2000) is not possible. For the purpose of publication, it may be of interest to repeat this experiment on *Kat6b*^{+/-} animals and *Tg(Kat6b)* animals.

4.2.1 *Kat6b* germ line deletion is perinatal lethal

While *Kat6b*^{-/-} mutants are born at the expected Mendelian ratio, KAT6B loss of function is perinatal lethal with 100% penetrance on a *C57BL/6* background. Nevertheless, *Kat6b*^{-/-} mutants were able to reach early perinatal milestones and were able to suckle. It is possible that, despite being able to suckle, *Kat6b*^{-/-} mutants have gastrointestinal abnormalities, resulting in their inability to efficiently digest milk. Alternatively, the observed delay in lung oxygenation may impair oxygen delivery to organ systems, including the brain, which may affect later behaviours, resulting in mutants that respond abnormally to maternal cues and are subsequently neglected. In addition, the craniofacial abnormalities in *Kat6b* null mutants may contribute to the perinatal death observed in these mutants. *Kat6b* null mutants have small, flattened skulls, relative to wild type controls, which may prevent adequate growth of the brain, possibly due to craniosynostosis, a condition known to result in intellectual disability and impaired survival (Becker et al., 1997; Gault et al., 1992; Shimoji et al., 2002). It is also possible that the perinatal lethality is a cumulative result of these defects. In this regard, the use of tissue-specific *Cre*-deletion may shed light on the tissue-specific requirements of KAT6B and their contributions to the perinatal death observed in germline null mutants. In addition, *Kat6b* heterozygous animals, despite surviving perinatally, were underrepresented at weaning. Given that these mutants did not display any overt phenotype at birth that could account for their underrepresentation, it is likely that they display milder phenotypes or behavioural abnormalities than homozygous null mutants, sufficient for the first few days of life, but progressively detrimental to survival in a subset of the heterozygous animals over time.

By comparison, *Tg(Kat6b)* overexpressing animals did not show perinatal lethality, however were also under-represented at weaning. In this case, the aggressive behaviours observed in this strain may manifest early and account for the underrepresentation of these animals at weaning, either due to fighting with their siblings or neglect from their mother. What these findings demonstrate is that both *Kat6b* deficiency and overexpression result in reduced

survival, reflecting the requirement for a precise balance in chromatin modifying enzymes and the detrimental consequences of their hypo and hyperactivity during early life.

4.2.2 *KAT6B drives NSPC proliferation and neuronal differentiation*

Tg(Kat6b) NSPCs showed enhanced proliferation in culture and *Tg(Kat6b)* adults had more long-term BrdU-retaining NSCs within the SVZ. In addition, *Kat6b* overexpression resulted in increased neuronal differentiation *in vitro* and increased neuronal arborisation in cultured E16.5 cortical neurons. Comparatively, *Kat6b*^{-/-} cortical neurons formed fewer and shorter neurites relative to wild type controls, complementing the finding that *Kat6b* loss impairs neurogenesis *in vitro* (Merson et al., 2006).

It is possible that reduced neuronal development, coupled to reduced neuronal arborisation, may result in neural networks insufficiently complex to support higher order brain processes, possibly accounting for the failure to thrive in *Kat6b* deficient animals and intellectual disability in human patients with heterozygous mutation of *KAT6B*. Conversely, the aggression and epilepsy observed in *Tg(Kat6b)* animals, which may reflect hyperexcitability, may be the result of excess neuronal development and arborisation. To further investigate this, neurite density could be assessed in *Kat6b* deficient and overexpressing brains, to determine whether *KAT6B* status also affects neuronal development *in vivo*. Furthermore, it would be of interest to determine whether *Kat6b* not only drives neurogenesis, but also affects the development of different neuronal subtypes, by assessing the proportions of excitatory and inhibitory neurons within the brains of *Kat6b* mutant and control mice. Lastly, the manifestation of these differences in adulthood would also be of interest. Behavioural assessment and electroencephalograms of *Kat6b* deleted and overexpressing mice may shed further light on particular brain regions most susceptible to differential *Kat6b* expression.

	Time to inflate lungs (min)	Time to pink skin (min)	Time to establish regular breathing (min)	Vocalise	Spontaneous movements	Milk in stomach
<i>Kat6b</i> ^{-/-}	8.44 ± 1.48	11.25 ± 1.40	34.19 ± 5.75	Yes	Yes	Yes
<i>Kat6b</i> ^{+/-}	3.36 ± 0.55	5.64 ± 0.51	7.82 ± 1.3	Yes	Yes	Yes
<i>Kat6b</i> ^{+/+}	2.35 ± 0.38	4.34 ± 0.39	5.55 ± 0.53	Yes	Yes	Yes

Table 4.1 Developmental milestones in E19.5 *Kat6b*^{-/-} and control pups

Summary of the time taken (min) to reach basic developmental milestones by E19.5 *Kat6b* mutant and control pups collected by caesarean section or within 1 – 2 h after birth. *Kat6b*^{-/-} pups take significantly longer than wild type controls to inflate their lungs ($p < 0.01$), oxygenate their blood ($p < 0.01$) and establish regular breathing ($p < 0.01$). By comparison, *Kat6b*^{+/-} mutants do not take significantly longer than wild type controls to inflate their lungs ($p = 0.47$), oxygenate their blood ($p = 0.25$) or establish normal breathing ($p = 0.89$). Despite the delay in these vital functions, *Kat6b*^{-/-} mutants vocalise, display spontaneous movements and have a stomach full of milk if collected 1 – 2 h after birth.

N = 4 – 7 E19.5 pups per genotype.

Milestones compared between genotypes by a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor.

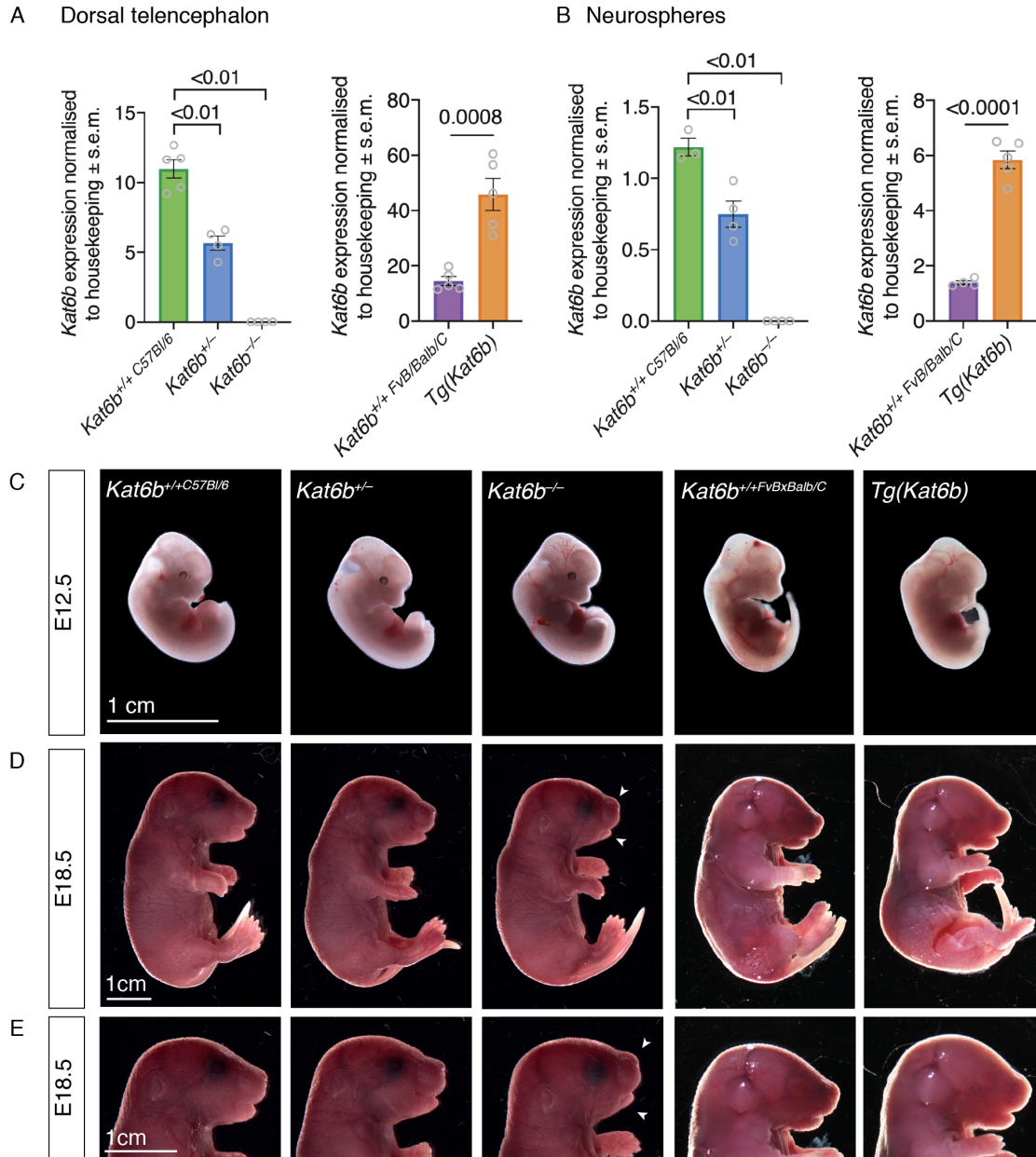


Figure 4.1 *Kat6b* loss and overexpression during embryonic development

(A) Bar graph depicting *Kat6b* mRNA levels, normalised to housekeeping, in E12.5 dorsal telencephalon RNA from *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+ C57Bl/6}, *Kat6b*^{+/+ FvBxBalb/C} and *Tg(Kat6b)* embryos. *Kat6b*^{+/-} ($p < 0.01$) and *Kat6b*^{-/-} ($p < 0.01$) displayed significantly lower *Kat6b* expression, relative to wild type. *Tg(Kat6b)* dorsal telencephalon tissue displayed 3.5 – 4 fold greater *Kat6b* expression than *Kat6b*^{+/+ FvBxBalb/C} ($p = 0.0008$).

(B) Bar graph depicting *Kat6b* mRNA levels, normalised to housekeeping, in cultured neurosphere RNA from *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* E12.5 dorsal telencephalon – derived neurosphere cultures. *Kat6b*^{+/-} (p < 0.01) and *Kat6b*^{-/-} (p < 0.01) cells displayed significantly lower *Kat6b* expression, relative to wild type. *Tg(Kat6b)* neurospheres displayed 3.5 – 4 fold greater *Kat6b* expression than *Kat6b*^{+/+FvBxBalb/C} (p = 0.0001).

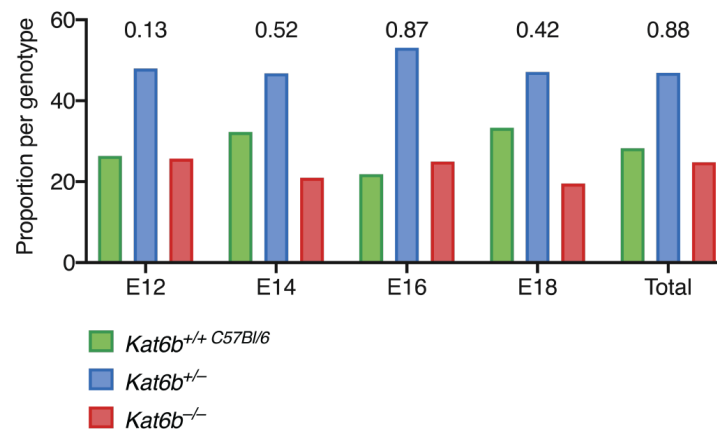
(C) Representative images of *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* E12.5 embryos. *Kat6b* status did not affect gross embryonic morphology at E12.5 Scale bar = 1 cm.

(D) Representative images of *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* E18.5 fetuses. *Kat6b*^{-/-} fetuses has an underdeveloped lower jaw (white arrows), relative to *Kat6b*^{+/-}, *Kat6b*^{+/+C57Bl/6} fetuses. *Tg(Kat6b)* fetuses were not overtly different from wild type controls. Scale bar = 1 cm.

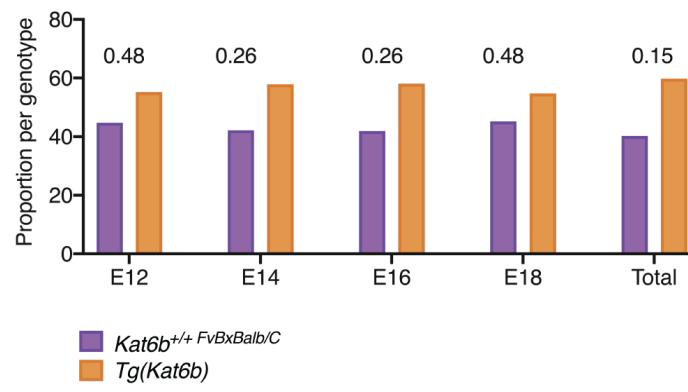
(E) Representative images of *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* E18.5 fetuses, focusing on the skull. *Kat6b*^{-/-} animals had an underdeveloped lower jaw (white arrows) relative to *Kat6b*^{+/-}, *Kat6b*^{+/+C57Bl/6} fetuses. *Tg(Kat6b)* fetuses were not overtly different from wild type controls. Scale bar = 1 cm.

N = mRNA extracted from 4 – 5 dorsal telencephalon tissue or cultured neurosphere samples per genotype. All bar graphs are displayed as individual data points with mean ± s.e.m., where data points represent individual E12.5 dorsal telencephalon or neurosphere mRNA samples from a single embryo. *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+C57Bl/6} mRNA expression levels were compared between samples using a one-way ANOVA, followed by a Bonferroni correction for multiple comparisons, using genotype as the independent factor. *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* mRNA expression levels were compared using a two tailed Student's t-test.

A



B



C

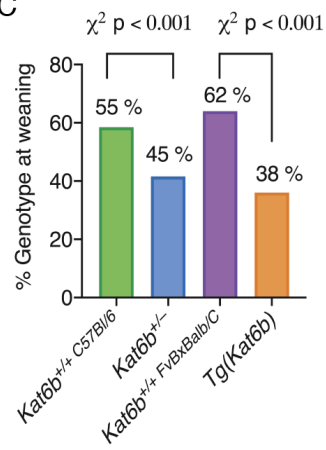


Figure 4.2 Effect of *Kat6b* deletion and overexpression on Mendelian ratios during development and at weaning

(A) Bar graph depicting the proportion of *Kat6b*^{-/-}, *Kat6b*^{+/-} and *Kat6b*^{+/+C57Bl/6} embryos and fetuses at E12, E14, E16, E18 and cumulatively across these embryonic time points. χ^2 p value indicated above each embryonic time point. *Kat6b* mutant and control animals are present at the expected Mendelian ratios *in utero*.

(B) Bar graph depicting the proportion of *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* embryos and fetuses at E12, E14, E16, E18 and cumulatively across these embryonic time points. χ^2 p value indicated above each embryonic time point. *Kat6b* transgenic and control animals are present at the expected Mendelian ratios *in utero*.

(C) Bar graph depicting the percentage of *Kat6b*^{+/-}, *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* animals present at weaning. *Kat6b*^{+/-} animals are present at a significantly lower percentage than expected, relative to *Kat6b*^{+/+C57Bl/6} (χ^2 p < 0.0001). *Tg(Kat6b)* animals are also present at a significantly lower percentage than expected, relative to *Kat6b*^{+/+FvBxBalb/C} (χ^2 p < 0.0001). Note that *Kat6b*^{-/-} animals are not shown due to the perinatally lethality of this genotype on a C57Bl/6 genetic background. Only *Kat6b*^{+/-} and *Kat6b*^{+/+C57Bl/6} animals from *Kat6b*^{+/+} x *Kat6b*^{+/-} matings are shown. Animals from *Kat6b*^{+/-} x *Kat6b*^{+/-} matings were excluded from this analysis.

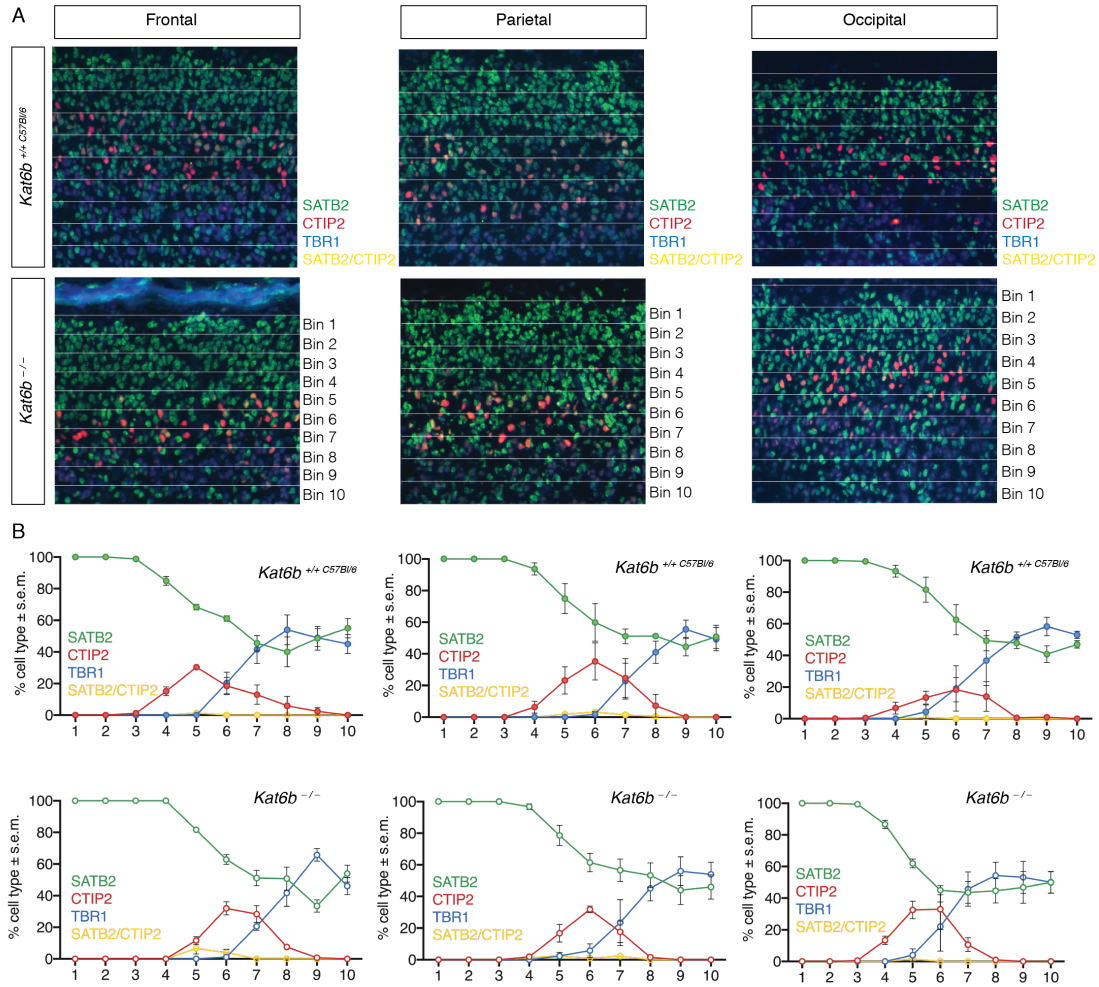


Figure 4.3 *Kat6b* loss in cortical layering

(A) Representative images of frontal, parietal and occipital coronal sections from E18.5 *Kat6b*^{-/-} and wild type pups, stained for SATB2, CTIP2 and TBR1, as markers of superficial, intermediate and deep cortical layers, respectively. Immunofluorescence images show no gross morphological differences comparing *Kat6b*^{-/-} mutant to control animals. For the purposes of quantification, images were sectioned into 10 bins from the pial (1) to the ventricular surface (10) and the proportions of SATB2+, CTIP2+, TBR1+ and SATB2+ CTIP2+ cells compared between mutants and controls. Fluorophore colour code indicated in the bottom right hand corner of images in the top row.

(B) Graphs depicting cell proportions within each bin for *Kat6b*^{+/+ C57Bl/6} and *Kat6b*^{-/-} frontal, parietal and occipital cortex sections. Data displayed as average percentage of each cell type ± s.e.m. across each bin. No significant difference was observed between genotypes across bins

or cortex sections. Lines colour coded in accordance with immunofluorescence images. Cell type colour code indicated in the bottom left hand corner of each graph.

Cell type percentages across 10 bins were compared between mutant and control samples in the frontal, parietal and occipital cortex using a two-way ANOVA with genotype and bin number as the independent factors. There was no statistically significant difference between mutant and control samples for any bin.

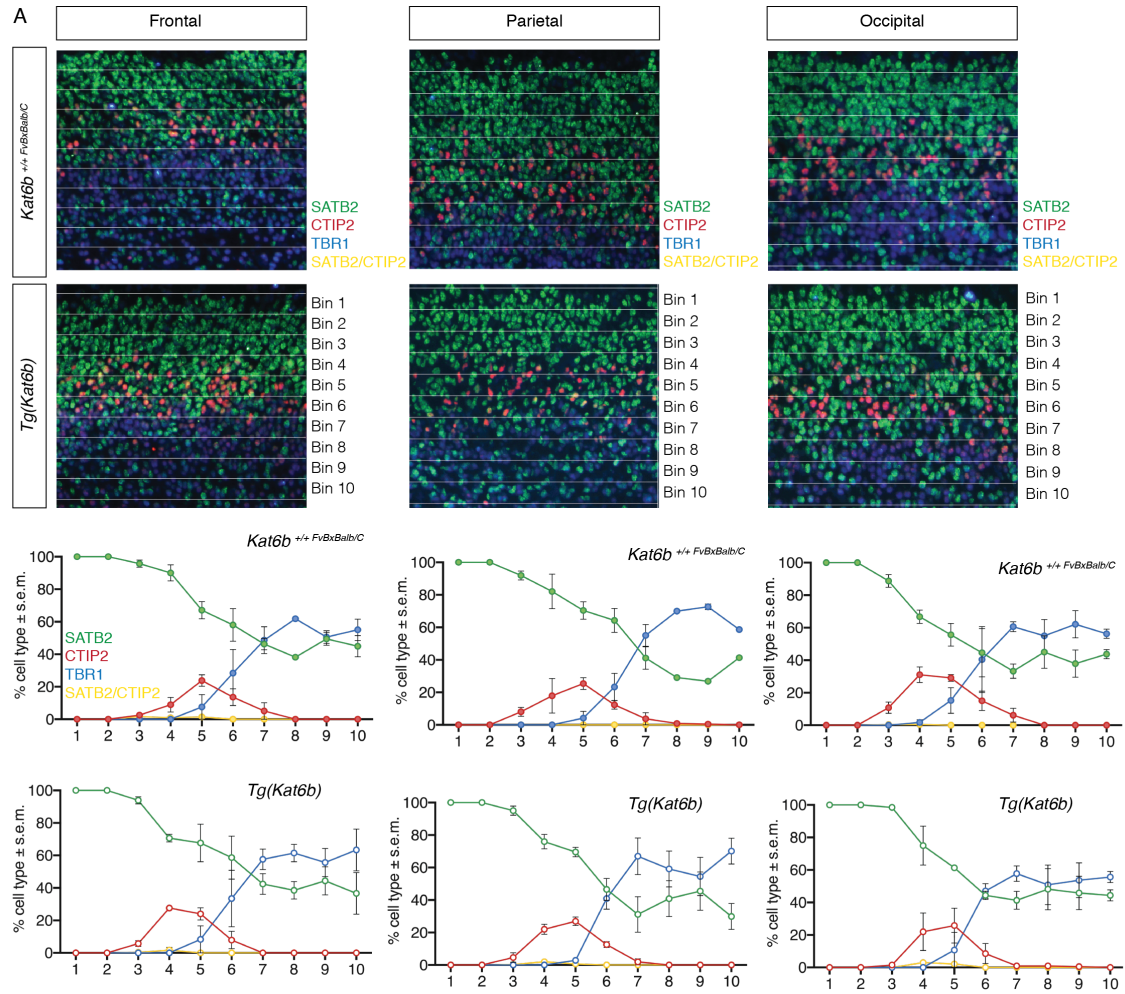


Figure 4.4 *Kat6b* overexpression in cortical layering

(A) Representative images of frontal, parietal and occipital coronal sections from E18.5 *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* pups, stained for SATB2, CTIP2 and TBR1, as markers of superficial, intermediate and deep cortical layers, respectively. Immunofluorescence images show no gross morphological differences comparing *Tg(Kat6b)* mutant to control animals. For the purposes of quantification, images were sectioned into 10 bins from the pial (1) to the ventricular surface (10) and the proportions of SATB2+, CTIP2+, TBR1+ and SATB2+ CTIP2+ cells compared between mutants and controls. Fluorophore colour code indicated in the bottom right hand corner of images in the top row.

(B) Graphs depicting cell proportions within each bin for *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* frontal, parietal and occipital cortex sections. Data displayed as average percentage of each cell type ± s.e.m. across each bin. No significant difference was observed between genotypes across

bins or cortex sections. Lines colour coded in accordance with immunofluorescence images. Cell type colour code indicated in the bottom left hand corner of each graph.

Cell type percentages across 10 bins were compared between mutant and control samples in the frontal, parietal and occipital cortex using a two-way ANOVA with genotype and bin number as the independent factors. There was no statistically significant difference between mutant and control samples for any bin.

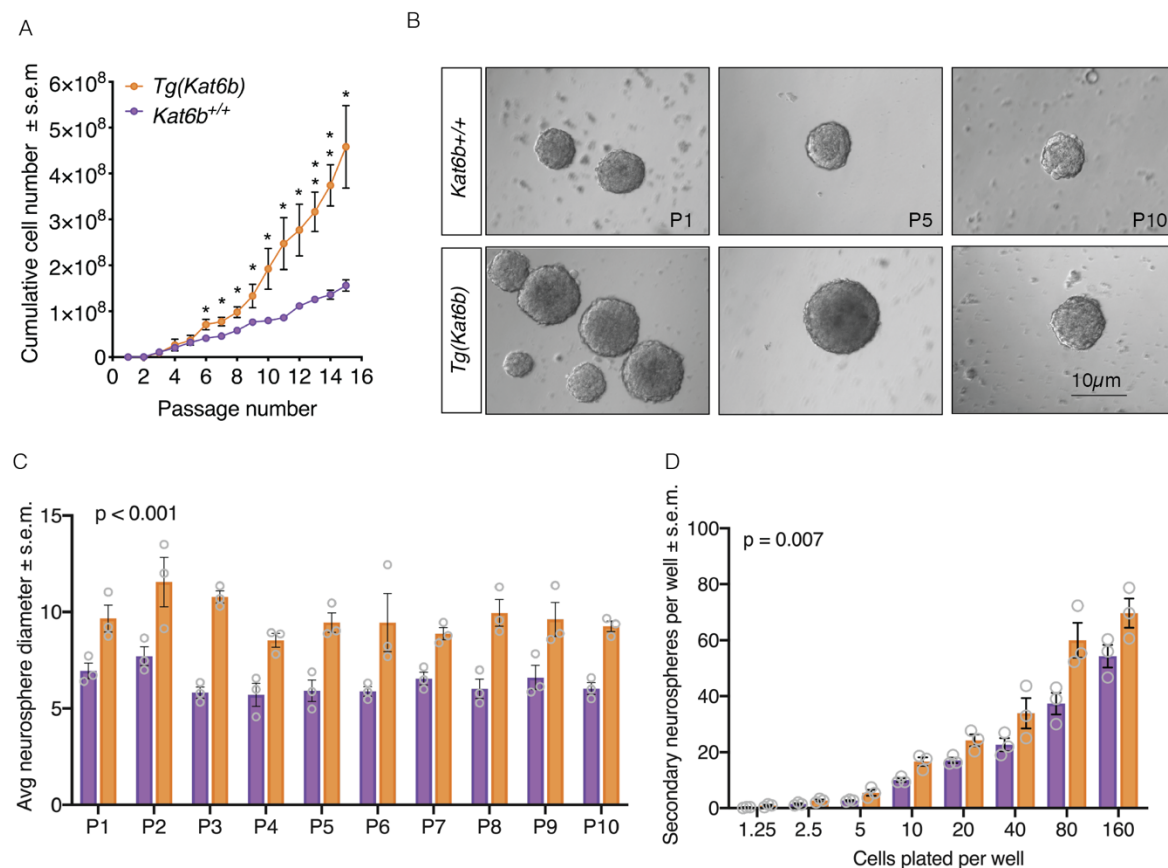


Figure 4.5 *Kat6b* promotes NSPC proliferation and neurosphere size *in vitro*

(A) Cumulative growth curve of SVZ-derived NSPCs from *Tg(Kat6b)* and wild type control 8 week old animals. Over consecutive passages, *Tg(Kat6b)* neurospheres showed significantly enhanced proliferation, relative to wild type control cells ($p < 0.001$). $N = 3$ SVZ – derived neurosphere cultures from 3 adult animals, per genotype. *Kat6b*^{+/+} and *Tg(Kat6b)* growth curves were compared using a two-way ANOVA, using genotype and passage number as the independent variables. Cell counts were taken at the time of passage.

(B) Representative neurosphere images from *Tg(Kat6b)* and *Kat6b*^{+/+}*FvBxBalb/C* controls taken just prior to passage (P) 1, 5 and 10. Scale bar = 10 μm .

(C) Bar graph depicting average neurosphere diameter from *Tg(Kat6b)* and *Kat6b*^{+/+}*FvBxBalb/C* neurosphere cultures just prior to each passage. *Tg(Kat6b)* neurospheres are, on average, larger than *Kat6b*^{+/+} neurospheres over consecutive passages ($p < 0.001$). Bar graph displayed as individual data points with mean $\pm$ s.e.m., where data points represent the average neurosphere

diameter of 100 – 150 neurospheres per sample at each passage. N = 3 SVZ-derived neurosphere cultures from 3 adult animals, per genotype. *Kat6b*^{+/+} and *Tg(Kat6b)* neurosphere diameters were compared using a two-way ANOVA, using genotype and passage as the independent variables.

(D) Bar graph depicting secondary neurosphere formation versus cell number plated, from *Tg(Kat6b)* and *Kat6b*^{+/+} neural stem and progenitor cells. Cells were plated at limiting dilution and secondary neurosphere formation assessed. *Tg(Kat6b)* neural stem and progenitor cells form more secondary neurospheres across all dilutions assessed as compared to wild type controls ($p < 0.007$). Bar graph displayed as individual data points with mean $\pm$ s.e.m., where data points represent the average secondary neurosphere formation, per sample, across technical triplicates. N = 3 SVZ – derived neurosphere cultures from 3 adult animals, per genotype at each dilution. *Kat6b*^{+/+} and *Tg(Kat6b)* secondary neurosphere formation were compared using a two-way ANOVA, using genotype and dilution as the independent variables.

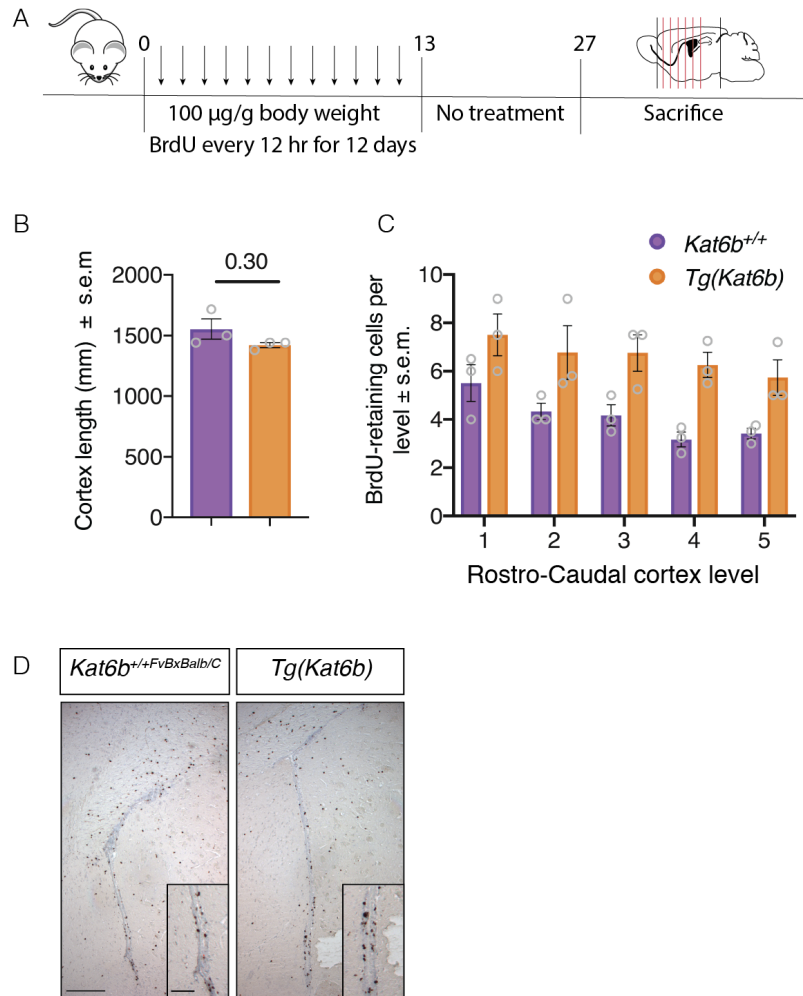


Figure 4.6 *Kat6b* overexpression results in more SVZ NSCs *in vivo*

(A) Schematic of BrdU treatment schedule. Adult mice were treated with BrdU at a dose of 100 $\mu\text{g/g}$ body weight every 12 h for 12 days. Animals were untreated for the following 2 weeks prior to sacrifice and cortex dissection.

(B) Bar graph depicting cortex rostro-caudal extent of *Kat6b*^{+/+} and *Tg(Kat6b)* adult animals. There is no difference in cortex length between genotypes ($p = 0.30$). Bar graph displayed as individual data points with mean $\pm$ s.e.m., where data points represent the cortex length of one animal. $N = 3$ adult animals per genotype. *Kat6b*^{+/+} and *Tg(Kat6b)* cortex length compared using an two-tailed Student's t-test.

(C) Bar graph depicting the number of BrdU-retaining cells counted at 5 levels along the rostro-caudal axis of the cortex where the SVZ was present. *Tg(Kat6b)* animals had increased

numbers of BrdU + cells within the SVZ across all levels ($p < 0.0001$). Bar graph displayed as individual data points with mean $\pm$ s.e.m., where data points represent the number of BrdU + cells for one animal at each level. The number of BrdU + cells was compared between control and transgenic samples using a two-way ANOVA with genotype and cortex level as the independent factors. N = 3 adult brains per genotype.

(D) Representative images of *Kat6b*^{+/+} and *Tg(Kat6b)* coronal cortex sections stained for BrdU + NSCs Scale in D = 500 μ m and 100 μ m in the insert.

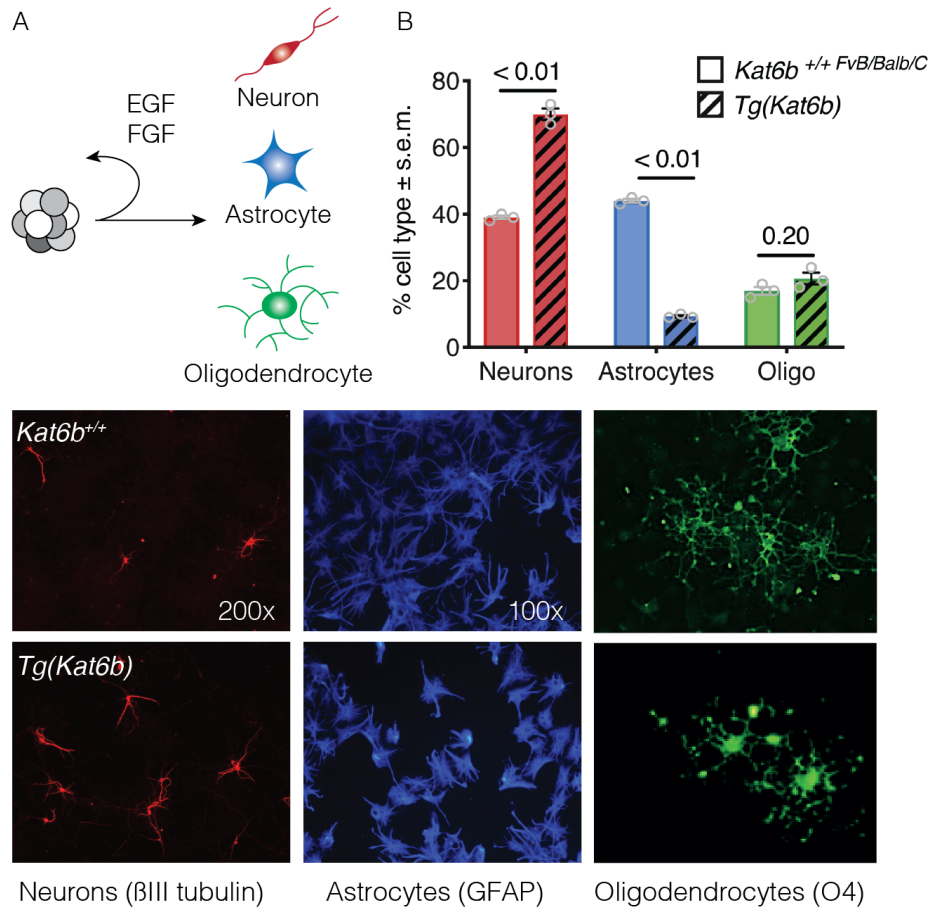


Figure 4.7 *Kat6b* promotes neuronal differentiation *in vitro*

(A) Schematic of neurosphere proliferation and differentiation. In the presence of growth factors of EGF and FGF, neurospheres will retain potency and undergo self-renewal. When EGF and FGF are removed, neurosphere cells will differentiate into one of neurons, astrocytes or oligodendrocytes.

(B) Bar graph depicting the percentage of neurospheres, astrocytes and oligodendrocytes formed from $Kat6b^{+/+}$ and $Tg(Kat6b)$ neurospheres, following 1 week of differentiation in the absence of EGF and FGF. $Tg(Kat6b)$ neurospheres form a significantly greater percentage of neurons ($p < 0.01$) and significantly lower percentage of astrocytes ($p < 0.01$), than $Kat6b^{+/+}$ cultures. There is no difference in the percentage of oligodendrocytes formed between genotypes ($p = 0.20$). Bar graph displayed as individual data points with mean $\pm$ s.e.m., where data points represent a neurosphere culture derived from one adult SVZ.

N = 3 SVZ – derived neurosphere cultures from 3 adult animals, per genotype. The percentage of each cell type was compared between *Kat6b*^{+/+} and *Tg(Kat6b)* samples using a two tailed Student's t-test.

(C) Representative images of neurons, astrocytes and oligodendrocytes derived from *Kat6b*^{+/+} and *Tg(Kat6b)* neurosphere cultures, following 7 days of differentiation. Cell types were identified by immunofluorescence, staining neurons with β III tubulin, astrocytes with GFAP and oligodendrocytes with O4. Neuron images at 100 X magnification. Astrocyte and oligodendrocyte images at 200 X magnification.

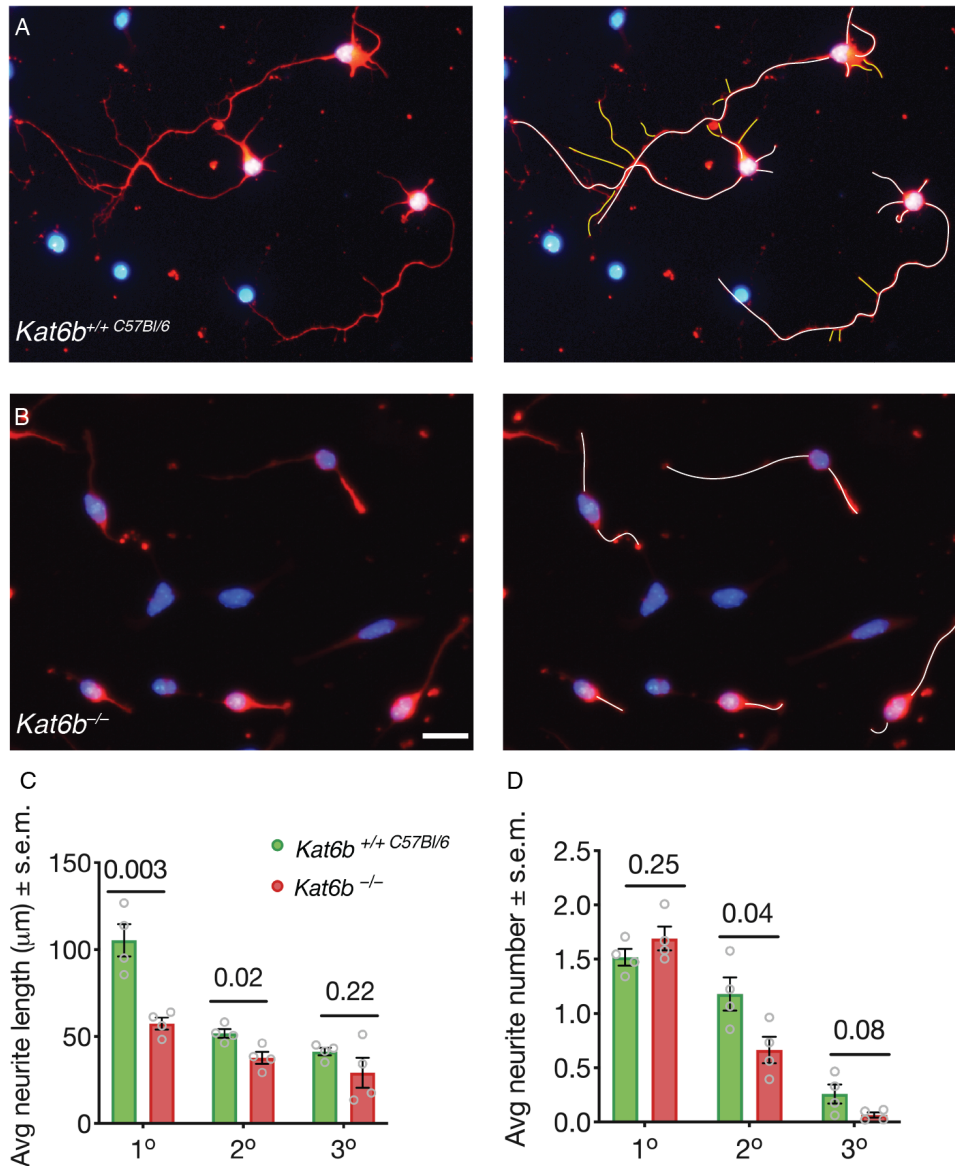


Figure 4.8 Effect of *Kat6b* loss on neurite outgrowth

(A) Representative images of traced and untraced E16.5 cortical neurons from *Kat6b*^{+/+}C57Bl/6 cortices, stained for βIII tubulin (red) and DAPI (blue). Scale bar = 50 μm.

(B) Representative images of traced and untraced E16.5 cortical neurons from *Kat6b*^{-/-} cortices, stained for βIII tubulin (red) and DAPI (blue). Primary neurites are coloured in white, secondary neurites in yellow and tertiary neurites in green. Quaternary neurites are not shown in these images. Scale bar = 50 μm.

(C) Bar graph depicting the average neurite length (μm) of 100 –150 cortical neurons derived from *Kat6b*^{-/-} and *Kat6b*^{+/+*C57Bl/6*} cortices. *Kat6b* loss results in significantly shorter 1° neurites ($p = 0.003$) and 2° neurites ($p = 0.02$) on average per cell, relative to wild type cells. Genotype colour code indicated in the top left hand corner.

(D) Bar graph depicting the average neurite number of 100 –150 cortical neurons derived from *Kat6b*^{-/-} and *Kat6b*^{+/+*C57Bl/6*} cortices. *Kat6b* loss results in significantly fewer 2° neurites ($p = 0.04$) on average per cell, relative to wild type cells.

Bar graphs displayed as individual data points with mean $\pm$ s.e.m., where data points represent the average of 100 – 150 cortical neurons from one E16.5 cortex. N = cortical neuron cultures from 3 E16.5 cortices per genotype. *Kat6b*^{-/-} and *Kat6b*^{+/+*C57Bl/6*} cortical neuron neurite number and length were compared using a two tailed Student's t-test.

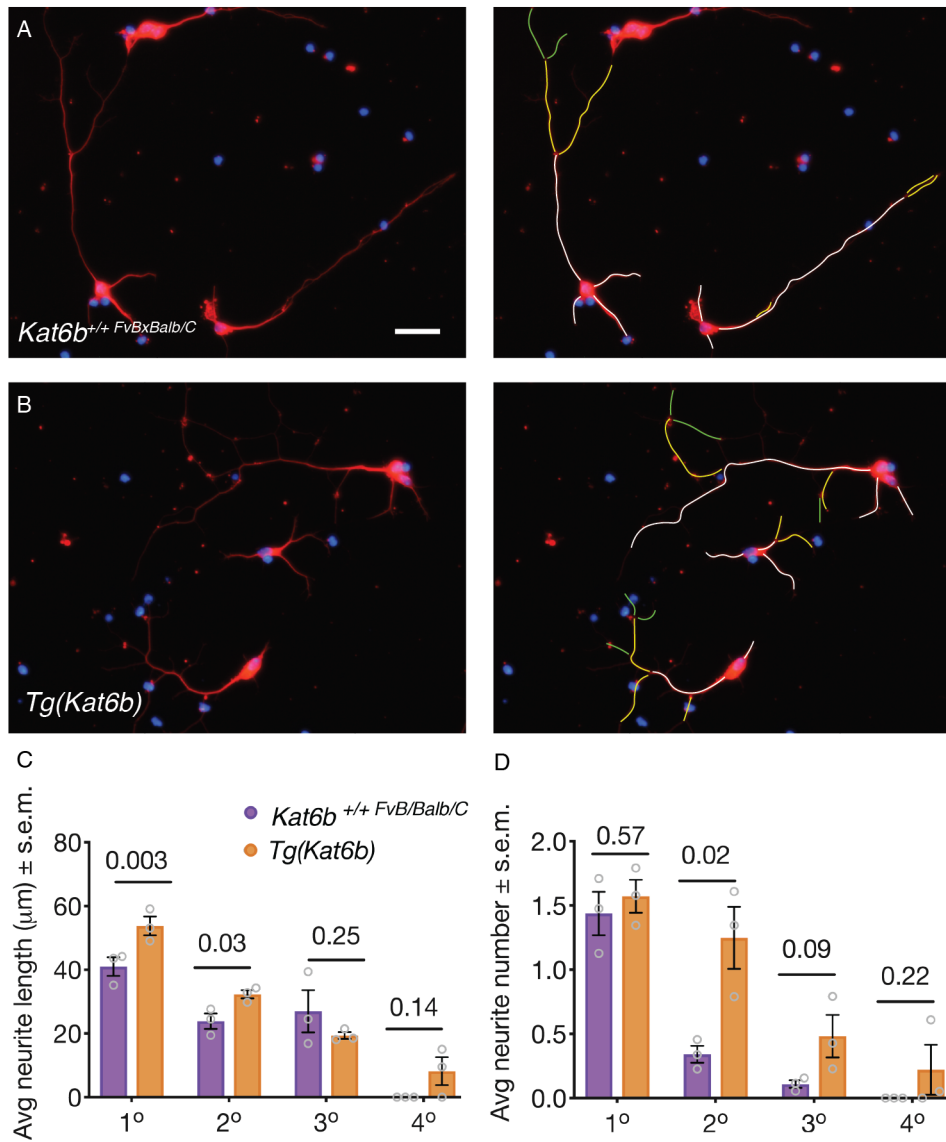


Figure 4.9 Effect of *Kat6b* overexpression on neurite outgrowth

(A) Representative images of traced and untraced E16.5 cortical neurons from *Kat6b*^{+/+}FvBxBalb/C cortices, stained for βIII tubulin (red) and DAPI (blue). Scale bar = 50 μm.

(B) Representative images of traced and untraced E16.5 cortical neurons from *Tg(Kat6b)* cortices, stained for βIII tubulin (red) and DAPI (blue). Primary neurites are coloured in white, secondary neurites in yellow and tertiary neurites in green. Quaternary neurites are not shown in these images. Scale bar = 50 μm.

(C) Bar graph depicting the average neurite length (μm) of 100 –150 cortical neurons derived from *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* cortices. *Kat6b* overexpression results in significantly longer 1° neurites ($p = 0.003$) and 2° neurites ($p = 0.03$) on average per cell, relative to wild type cells. Genotype colour code indicated in the top left hand corner.

(D) Bar graph depicting the average neurite number of 100 –150 cortical neurons derived from *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* cortices. *Kat6b* overexpression results in significantly more 2° neurites ($p = 0.02$) on average per cell, relative to wild type cells.

Bar graphs displayed as individual data points with mean $\pm$ s.e.m., where data points represent the average of 100 – 150 cortical neurons from one E16.5 cortex. N = cortical neuron cultures from 3 E16.5 cortices per genotype. *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* cortical neuron neurite number and length were compared using a two tailed Student's t-test cells.

Chapter 5

KAT6B's molecular mechanism in neural stem cells and the developing cortex

Introduction

In the mouse model and human patients, deficiency or mutation in KAT6B underlies a failure to thrive (Thomas et al., 2000) and intellectual disability disorders (Bashir et al., 2017; Clayton-Smith et al., 2011; Kraft et al., 2011; Simpson et al., 2012; Yu et al., 2014), respectively. In addition, KAT6B has been identified as a driver of NSC proliferation and multipotency, both *in vivo* and *in vitro* (Merson et al., 2006), with a progressive loss in *Kat6b* activity observed as NSCs undergo differentiation (Sheikh et al., 2012). Furthermore, microarray analysis of *Kat6b^{gt/gt}* and control E12.5 dorsal telencephalon and adult cortex tissue, as well as HEK293 and HeLa cells following *KAT6B* siRNA knockdown, identified a number of pathways affected by *Kat6b/KAT6B* loss (Kraft et al., 2011). In the adult cortex significantly altered pathways included networks of neuronal development, such as the neuroactive ligand-receptor interaction, axon guidance, calcium signalling and MAPK signalling pathways. By comparison, in the E12.5 dorsal telencephalon, affected pathways included MAPK, insulin, Wnt and GnRH signalling pathways, which were similarly affected in both HEK293 and HeLa cells, following *Kat6b* knockdown. While these results demonstrate that *Kat6b* expression affects pathways associated with neuronal development and axon guidance, more contemporary technologies such as RNA-seq would enable detection of more lowly abundant transcripts, enabling a broader dynamic range for detection of differentially expressed genes and an improved understanding of the role of KAT6B within the developing and adult brain.

In this chapter I aimed to further elucidate KAT6B's molecular mechanism during neurogenesis. Given that KAT6B is a histone acetyltransferase (HAT), phenotypic differences observed due to its loss and gain are anticipated to be the result of differential gene expression. I assessed differential gene expression in the developing cortices and cultured neural stem and progenitor cells (NSPCs) of embryos lacking or overexpressing *Kat6b*, relative to wild type controls. While previous studies on KAT6B have focused on adult SVZ-derived NSPCs, this chapter will focus on embryonic brain tissue and embryonic cortex-derived NPSCs. Despite there being notable differences between these cells types, as discussed in section 1.2.5, in human patients, heterozygous, *de novo* mutations in *KAT6B* underlie intellectual disability disorders, indicating that altered KAT6B activity within the developing brain is incompatible with the development of normal intellect. As such, embryonic brain tissue was considered the more relevant cell type to assess KAT6B's molecular mechanism in the brain and shed light on genes and signalling pathways susceptible to altered *Kat6b* expression.

5.1 Results

5.1.1 *KAT6B* regulates pathways associated with NSPC proliferation and neuronal differentiation

To better understand the contribution of KAT6B to gene expression profiles within the developing brain, RNA-seq was performed using dorsal telencephalon tissue and cultured neural stem and progenitor cells (NSPCs) from E12.5 *Kat6b*^{-/-} and *Tg(Kat6b)* embryos, with littermate controls. The E12.5 dorsal telencephalon was chosen for this analysis as this structure displays high *Kat6b* expression, based on *in situ* hybridisation (Thomas et al., 2000), suggesting that this is a time during which KAT6B is active during neurogenesis. Secondly, the E12.5 dorsal telencephalon is the precursor structure to the cerebral cortex, allowing assessment of how KAT6B contributes to early gene expression profiles permissive to normal brain development and how these profiles are disrupted upon its loss or gain. Lastly, as I observed differences in NSPC proliferation and self-renewal, but not in cortical layer formation (Chapter 4), the later stages of cortical development were considered to be less promising subjects for this analysis. To complement this dataset, RNA-seq was also performed on neurospheres, to more specifically assess the role of KAT6B within the NSPC population and within an *in vitro* system. Neurospheres were derived from the E12.5 dorsal telencephalon from *Kat6b*^{-/-} and *Tg(Kat6b)* embryos, with littermate controls and collected for RNA isolation 3 days following passage 5, when still relatively small and before any apoptosis was observed within the neurosphere cores. Comparisons between *Kat6b* mutant and control samples within the dorsal telencephalon and NSPC datasets were based on genes with FDR < 0.05, unless stated otherwise.

As histone acetylation is associated with increased gene expression, KAT6B gene targets were expected to be downregulated in *Kat6b* null mutants and upregulated in overexpressing transgenic mice. Differentially expressed genes were assessed for enriched Gene Ontology (GO) and KEGG pathway terms and lists of potential gene targets generated based on the current literature and animal models of individual gene mutations, based on the Mouse Genome Informatics (MGI) database. Comparisons between *Kat6b* mutant and control samples within the dorsal telencephalon and NSPCs datasets were based on genes with a p value ≤ 0.05.

In the dorsal telencephalon, relative to wild type controls, *Kat6b* loss resulted in downregulation of 1029 genes, and *Kat6b* overexpression in upregulation of 779 genes, with 298 of these overlapping between these mutants (Fig 5.1 A – B). Genes downregulated in *Kat6b*^{-/-} mutants were significantly enriched for GO terms associated with nervous system development ($p = 2.12 \times 10^{-22}$) and forebrain development ($p = 3.33 \times 10^{-8}$), as well as neuronal development ($p = 2.73 \times 10^{-14}$), synaptic signalling ($p = 4.22 \times 10^{-11}$) and neuron projection development ($p = 1.27 \times 10^{-13}$) (Fig 5.1 C). Similarly, enriched KEGG pathways in downregulated genes included axonal guidance pathways ($p = 0.0021$) as well as Wnt signalling ($p = 0.00069$), involved in neuronal progenitor proliferation and differentiation (Dickinson et al., 1994; Megason & McMahon, 2002; Zechner et al., 2007), axon development and guidance (Ciani et al., 2011; Ciani & Salinas, 2005; Hall et al., 2000; Lucas & Salinas, 1997; Rosso et al., 2005; Wayman et al., 2006) and Rap1 signalling ($p = 0.025$), implicated in neural cell polarity, axonal formation (Shah et al., 2017), synaptic growth and growth cone activation (Heo et al., 2017; Lilja et al., 2017) (Fig 5.1 D).

By comparison, *Tg(Kat6b)* dorsal telencephalon samples, relative to wild type controls, showed upregulation of genes associated with nervous system development ($p = 6.88 \times 10^{-25}$), neuron differentiation ($p = 1.16 \times 10^{-22}$) and synaptic signalling ($p = 3.45 \times 10^{-29}$) GO terms (Fig 5.1 E). Upregulated KEGG pathways included cAMP signalling ($p = 6.01 \times 10^{-7}$), signalling pathways regulating pluripotency ($p = 0.017$) and the FoxO signalling pathway ($p = 0.0021$), required for maintenance of the NSPC pool during development and in adulthood (Paik et al., 2009; Renault et al., 2009; Yeo et al., 2013), consistent with *Tg(Kat6b)* animals having more long-term BrdU retaining NSCs within the SVZ. Other affected KEGG pathways included ErbB signalling ($p = 0.032$), required in neuronal migration, axon and dendrite development and synapse formation (Fazzari et al., 2010; Flames et al., 2004; Krivosheya et al., 2008), consistent with *Tg(Kat6b)* E16.5 cortical neurons developing more secondary neurites and longer primary and secondary neurites in culture, relative to wild type controls (Fig 5.1 F).

In cultured NSPCs, *Kat6b* loss resulted in downregulation of 5691 genes and *Kat6b* overexpression in upregulation of 4152 genes, relative to wild type controls, with 1830 of these overlapping (Fig 5.2 A – B). *Kat6b* loss resulted in downregulation of genes associated with fatty acid beta-oxidation ($p = 2.62 \times 10^{-6}$), neuronal differentiation ($p = 3.07 \times 10^{-7}$) and axonal development ($p = 0.0004$) GO terms, consistent with *Kat6b* deficient NSPCs showing reduced proliferation and neuronal differentiation *in vitro* (Merson et al., 2006) (Fig 5.2 C). Enriched

KEGG pathways included fatty acid metabolism ($p = 0.004$), metabolic pathways ($p = 0.011$) and signalling pathways regulating pluripotency ($p = 0.011$), congruent with the role of KAT6B as a driver of NSPC proliferation and observation that *Kat6b* expression is progressively lost as NSPCs undergo differentiation (Sheikh et al., 2012). Enriched KEGG pathways also included the mTor signalling pathway ($p = 0.01$), required for neuronal development and the disruption of which is associated with autism-spectrum disorders and epilepsy in human patients (Crino, 2007, 2015; Kassai et al., 2014; Sarnat et al., 2012; Takei & Nawa, 2014); the hippo signalling pathway ($p = 0.02$), involved in regulating stem cell proliferation and maintenance (Yu et al., 2013) and the notch signalling pathway ($p = 0.02$), essential for neuronal development and regulating the balance between NSC maintenance and differentiation during cortical development (Ables et al., 2010; Breunig et al., 2007; Imayoshi et al., 2010; Lugert et al., 2010) (Fig 5.2 D). By comparison, genes upregulated in *Tg(Kat6b)* samples NSPC samples were similarly enriched for GO terms associated with cellular respiration ($p = 9.60 \times 10^{-16}$) and neural precursor proliferation ($p = 0.004$), as well as neurogenesis ($p = 0.0001$) and axon development ($p = 0.007$) (Fig 5.2 E). Similar to terms enriched in genes downregulated in *Kat6b*^{-/-} samples, enriched KEGG pathways in *Tg(Kat6b)* samples included oxidative phosphorylation ($p = 1.86 \times 10^{-24}$), fatty acid metabolism ($p = 2.32 \times 10^{-5}$) and signalling pathways including mTor ($p = 0.00063$), neurotrophin ($p = 0.0023$), wnt ($p = 0.011$) and hippo signalling ($p = 0.024$) (Fig 5.2 F)

Genes altered in the opposing direction were not enriched for GO terms or KEGG pathways relevant to nervous system development or NSPCs, in either mutant within the dorsal telencephalon or NSPC data sets. Genes upregulated in *Kat6b*^{-/-} dorsal telencephalon tissue included GO terms associated with the extracellular matrix and cell adhesion, while enriched KEGG pathways included ECM-receptor interactions, protein processing and glycan degradation. By comparison, genes downregulated in *Tg(Kat6b)* dorsal telencephalon tissue were enriched for blood vessel development and morphogenesis, as well as cell motility and epithelium development, while enriched KEGG pathways included protein digestion and absorption and cellular senescence. In NSPCs, genes upregulated in *Kat6b*^{-/-} samples included GO terms associated with RNA processing and RNA binding, while enriched KEGG pathways included RNA biogenesis, spliceosome and DNA replication. Genes downregulated in *Tg(Kat6b)* NSPCs were enriched for GO terms associated with the extracellular region, cell surface and cell adhesion, while enriched KEGG pathways included extracellular matrix receptor interaction, purine metabolism and mismatch repair. As these terms did not correlate

with the phenotypes observed within the developing brains or NSPCs of *Kat6b* mutant samples, genes upregulated in *Kat6b*^{-/-} samples or downregulated in *Tg(Kat6b)* samples, were not further investigated.

To identify genes commonly affected by differential *Kat6b* expression within the developing brain and neurosphere cultures, genes downregulated in *Kat6b*^{-/-} dorsal telencephalon and NSPCs data sets were compared against those upregulated in *Tg(Kat6b)* dorsal telencephalon and NSPC samples. For the purpose of this analysis, the FDR value cut-off for differentially expressed genes was increased to 0.1, to account for the cell and tissue type differences between the dorsal telencephalon and NSPC datasets and to generate gene lists with a sufficient number of genes to carry out subsequent analyses. 482 genes were commonly affected in all mutant datasets (Fig 5.3 A). Analysis of these genes using DAVID (Huang et al., 2009a, 2009b) revealed that these genes were enriched for brain development ($p = 0.03$), nervous system development ($p = 1.50 \times 10^{-11}$), neuronal migration ($p = 0.03$) and synapse assembly ($p = 0.03$) GO terms (Fig 5.3 B), while enriched KEGG signalling pathways included the notch ($p = 0.003$), hippo ($p = 0.007$) and ErbB signalling pathways ($p = 0.04$) (Fig 5.3 C), previously identified in the separate dorsal telencephalon and NSPC analyses, suggesting that KAT6B targets components of these pathways in both NSPCs and the developing cortex.

5.1.2 *Kat6b* affects expression of transcriptional regulators in the dorsal telencephalon

In addition to GO and KEGG pathways affected by differential *Kat6b* expression, individual gene lists were generated, based on the current literature and animal models of individual gene mutations as described in the Mouse Genome Informatics database (Bult et al., 2019). Based on this assessment it was observed that *Kat6b* loss resulted in significant downregulation in a number of genes known to drive cortical development and neurogenesis, including the transcription factors *Neurod6* (Baxter et al., 2012), *Neurod2* (Olson et al., 2001), *Emx1* (Gulisano et al., 1996; Hong et al., 2007), *Lhx2* (Chou & O'Leary, 2013; Hsu et al., 2015; Subramanian et al., 2011) and *Otx1* (Acampora et al., 1999; Suda et al., 1996; Weimann et al., 1999) (Fig 5.4 A), consistent with *Kat6b* loss resulting in reduced neuronal differentiation *in vitro* (Merson et al., 2006) and reduced neurite outgrowth in cultured E16.5 cortical neurons, as shown here. By comparison, transgenic overexpression of *Kat6b* resulted in upregulation of the transcription factor *Cux2*, known to regulate proliferation and neuronal differentiation in neuronal progenitors (Cubelos et al., 2008; Iulianella et al., 2008) (Fig 5.4 B), consistent with

the enhanced neuronal differentiation observed in cultured *Tg(Kat6b)* NSPCs described in Chapter 4.

5.1.3 *Kat6b* loss transcriptionally resembles *Sox2* loss in cultured NSPCs

RNA-seq data sets from both the E12.5 dorsal telencephalon and cultured neurospheres demonstrated differential expression of a number of SOX family transcription factors (Fig 5.4 C – D), including *Sox2*. SOX2 is a pioneer transcription factor required in the establishment and maintenance of neural progenitor cells, such that its loss hinders self-renewal and proliferation in NSPCs (Bylund et al., 2003; Graham et al., 2003). To further investigate the relationship between KAT6B and SOX2, RNA-seq results from *Kat6b*^{-/-} dorsal telencephalon tissue and cultured neurospheres, were compared to publicly available, cell type-matched datasets from *Sox2* null samples (Bertolini et al., 2019). A correlation analysis revealed that *Kat6b*^{-/-} dorsal telencephalon tissue showed a trend for a positive correlation with *Sox2* null dorsal telencephalon tissue, which did not reach statistical significance ($p = 0.06$; Fig. 5.5 A), while *Kat6b*^{-/-} NSPCs showed a strong positive correlation with *Sox2* null NSPCs ($p = 0.003$; Fig. 5.5 B), demonstrating that *Kat6b* loss transcriptionally resembles *Sox2* loss in NSPCs. Similarly, in *Tg(Kat6b)* mutants, there was a trend for a negative correlation between *Tg(Kat6b)* dorsal telencephalon samples and *Sox2* null dorsal telencephalon samples ($p = 0.05$; Fig. 5.5 C). Interestingly, in NSPCs, differentially expressed genes tended to be similar between *Tg(Kat6b)* and *Sox2* null samples and their controls, but this was not statistically significant in regards the directional changes of these genes (Fig 5.5 D).

5.1.4 *KAT6B* loss reduces *SOX2* promoter activity in the E12.5 dorsal telencephalon

Given the differential expression of *Sox2* in the dorsal telencephalon and NSPCs of *Kat6b* null and transgenic samples and positive correlation in gene expression differences between *Kat6b*^{-/-} and *Sox2* null NSPCs, I investigated whether KAT6B affects the promoter activity of the *Sox2* locus. To assess this, *Kat6b*^{+/-} animals were crossed to animals carrying GFP inserted into the *Sox2* locus (*Sox2-GFP^{T/+}*) (Arnold et al., 2011) and their offspring inter-crossed to assess the effect of *Kat6b* status on *Sox2* promoter activity (Fig 5.6 A). GFP levels in the dorsal telencephalon of mutant and control embryos were assessed by FACS, gating on the SSEA1+ CD133+ NSC population and using median fluorescence intensity (MFI) of GFP as a readout of *Sox2* promoter activity (Fig 5.6 B).

GFP levels were significantly reduced in *Kat6b*^{+/-} (p = 0.02) and *Kat6b*^{-/-} (p = 0.001) embryos relative to *Kat6b*^{+/+} controls and in *Kat6b*^{-/-} samples relative to *Kat6b*^{+/-} samples (p = 0.01), demonstrating a gene dose effect of KAT6B on *Sox2* promoter activity. Interestingly, no difference was observed in SOX2-GFP MFI levels in *Tg(Kat6b)* animals relative to littermate control samples (p = 0.81) (Fig 5.6 C). Wild-type animals (lacking the *Sox2* knock-in allele) showed only background levels of GFP expression.

5.1.5 *Sox2* overexpression can partially rescue the proliferative defect in *Kat6b*^{-/-} NSPCs

Having identified KAT6B as a driver of *Sox2* gene expression and promoter activity, I postulated that *Sox2* overexpression may rescue the proliferative defect described in *Kat6b*^{-/-} NSPCs *in vitro*. To assess this, *Kat6b*^{-/-} NSPCs were virally transfected with a *Sox2* overexpression construct (Fig 5.7 A) and propagated *in vitro*, with cell numbers counted over consecutive passages. Indeed, *Kat6b*^{-/-} cells overexpressing *Sox2* (*Kat6b*^{-/-}; *Tg(Sox2)*) showed a partial rescue of the proliferative defect seen in *Kat6b*^{-/-} cells, exhibiting intermediate proliferation between *Kat6b*^{-/-} and *Kat6b*^{+/+} cells (Fig 5.7 B). In addition, *Kat6b*^{+/+} NSPCs overexpressing *Sox2* (*Kat6b*^{+/+}; *Tg(Sox2)*) showed a further, statistically insignificant, increase in proliferation, above *Kat6b*^{+/+} control cultures. This is consistent with *Sox2* being a driver of NSPC proliferation, however, demonstrates that the full effect of *Sox* overexpression on NSPC proliferation cannot be observed in the absence of *Kat6b*.

5.2 Discussion

In this chapter, I assessed the effect of *Kat6b* deletion and overexpression on gene expression within the early developing cortex and in cultured NSPCs. This analysis demonstrated that KAT6B drives expression of genes involved in forebrain development, neuronal development and neuronal projection, including a number of transcriptional regulators associated with NSPC proliferation and potency. Amongst these transcriptional regulators were a number of SOX family genes, including the pioneer transcription factor, *Sox2*.

5.2.1 *Kat6b* regulates expression of transcription factors that drive cortical development and neurogenesis

Loss of *Kat6b* resulted in downregulation of a number of transcription factors and chromatin modifiers required in early brain development, including *Foxg1*, *Otx1*, *Emx1*, *Lhx2*, *Neurod2*, *Neurod6* and *Neurog2*, suggesting that KAT6B may be important in establishing gene expression profiles required for normal brain development. In support of this, mouse mutants of these genes show an overlap in phenotypes with *Kat6b* mutant mice. For example, FOXG1 promotes NSC potency (Hanashima et al., 2004; Martynoga et al., 2005), with *Foxg1* mutant mice dying perinatally, with thin cortical plates and small cerebral cortices (Hebert & McConnell, 2000), as well reduced proliferation of neuronal progenitors (Xuan et al., 1995). Conversely, lentiviral overexpression of *Foxg1* in NSPCs results in enhanced proliferation, improved viability and increased neuronal differentiation at the expense of glial differentiation (Brancaccio et al., 2010), similar to the effects of loss and gain of KAT6B shown here. By comparison, OTX1 is required for proper brain development and is expressed in layer V neurons in order to promote their maturation and axonal refinement (Weimann et al., 1999). *Otx1*-expressing cells have also previously been shown to be reduced in the hypomorphic *Kat6b* gene trap mutants (Thomas et al., 2000) and, similarly to *Kat6b*^{-/-} mutants, homozygous *Otx1* germ line mutants die within 24 h of birth, presenting with small cerebral cortices (Acampora et al., 1999; Suda et al., 1996). *Otx1* deficiency also results in aberrant neuronal excitability, with reduced burst firing but increased excitability, while its overexpression increases neuronal firing but impedes the strength of electrical signals (Acampora et al., 1999; Acampora et al., 1996; Zhang et al., 2015). Lastly, NEUROD2 promotes neuronal differentiation, inducing cell cycle arrest in neuronal progenitors to drive their differentiation and maintain their survival (Olson et al., 2001), while NEUROD6 regulates mitochondrial organisation prior to axonal growth (Baxter et al., 2012), and promotes neuronal integrity by interacting with the cytoskeleton of neuronal cell types (Baxter et al., 2012; Uittenbogaard et al., 2010). While the parallels in phenotypic anomalies resulting from mutations in these factors, with those seen upon *Kat6b* disruption, suggest a functional link, in order to distinguish direct from indirect gene targets of KAT6B, ChIP-sequencing is needed to assess KAT6B occupancy at the gene loci encoding these factors and their known target genes.

Interestingly, both *Dcx* and *Dscam* were downregulated in the E12.5 dorsal telencephalon of *Kat6b*^{-/-} mutants and upregulated in the E12.5 dorsal telencephalon of *Tg(Kat6b)* mutant samples. DSCAM is an important factor involved in synapse strengthening and the timing of synaptic transmission (Li et al., 2009), as well as the regulation of neural circuit formation (Alves-Sampaio et al., 2010), dendrite formation and synaptogenesis (Jia et al., 2011; Perez-

Nunez et al., 2016; Schmucker & Chen, 2009; Zhu et al., 2006). DSCAM is also important for regulating morphogenesis in cortical pyramidal neurons, with *Dscam* mutant mice showing reduced cortical thickness and reduced branching in layer V pyramid neurons in early postnatal life (Maynard & Stein, 2012). This is consistent with *Kat6b* genetrapped mutants showing reduced layer V pyramidal neurons, relative to wild type controls (Thomas et al., 2000). By comparison, DCX is involved in neuronal migration and promoting stability in microtubules (Deuel et al., 2006; Gleeson et al., 1999). While I observed no differences in cortical layering between *Kat6b* mutants and littermate controls, the differential expression of *Dcx* in mutant dorsal telencephalon samples may result in too subtle an effect on cell migration to detect in E18.5 cortical layers.

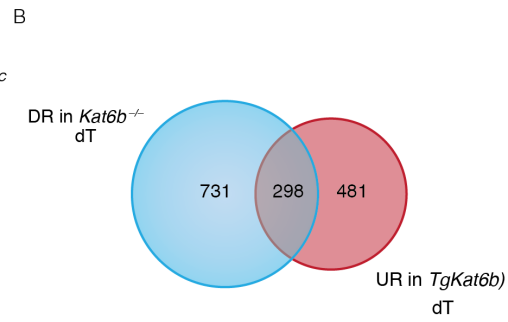
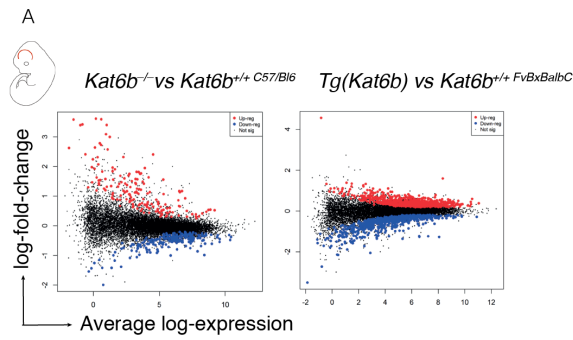
5.2.2 *KAT6B* drives expression of SOX family transcription factors

Amongst the transcriptional regulators differentially expressed upon *Kat6b* deletion or overexpression were a number of SOX family genes, including the pioneer transcription factor required for commitment to the neural lineage, *Sox2*. Supporting *Sox2* as a KAT6B gene target, *Sox2* compound heterozygous mice carrying one *Sox2* null allele and another lacking a neural-specific enhancer, exhibit small cerebral cortices (Ferri et al., 2004), while *Nestin-Cre* conditional *Sox2* deletion results in minor brain abnormalities, yet is lethal at birth (Miyagi et al., 2008). Consistent with *Sox2* as a KAT6B gene target, *Sox2* and *Kat6b* deficiency show substantially overlapping effects in NSPCs. Similar to loss of KAT6B, loss of SOX2 impairs self-renewal and proliferation in NSPCs (Bylund et al., 2003; Graham et al., 2003), with *Sox2* expression shown to be high in undifferentiated cells but decline as cells differentiate (Cavallaro et al., 2008). This has also been shown for *Kat6b*, the expression of which correlates with NSPC potency and self-renewal (Sheikh et al., 2012). In addition, *Sox2* deficiency impairs neuronal differentiation, as has previously been shown for *Kat6b* deficient NSPCs (Merson et al., 2006), while *Sox2* overexpression by lentiviral transfection promotes neurogenesis, while repressing gliogenesis (Cavallaro et al., 2008), just as observed here for *Kat6b* overexpression in *Tg(Kat6b)* NSPCs (Chapter 4).

Kat6b expression also affected *Sox2* promoter activity, based on FACS analysis of the dorsal telencephalon from embryos deficient in or lacking *Kat6b* and carrying GFP at the *Sox2* locus. Notably, as the *Sox2-GFP* allele is knocked into the *Sox2* locus, these results are in the context of *Sox2* heterozygosity and may underestimate the effect of *Kat6b* on *Sox2* promoter activity.

Nonetheless, *Sox2* overexpression by viral transfection was able to partially rescue the proliferative defect in *Kat6b*^{-/-} NSPCs, demonstrating that the role of KAT6B on NSPC growth is mediated at least in part, via SOX2. As demonstrated in the RNA-seq datasets, differential *Kat6b* expression affects a large number of genes, including a number of transcription factors. As such, it would not be expected that any deficits resulting from *Kat6b* loss could be restored to completely normal levels through the reintroduction of a single gene. Therefore, it is quite remarkable that overexpression of *Sox2* in *Kat6b*^{-/-} NSPCs results in a partial rescue of the proliferative defect of these cells. Furthermore, *Kat6b*^{+/+} neurospheres overexpressing *Sox2* showed a further increase in proliferation above wild type samples lacking the *Sox2* construct. This demonstrates that SOX2 must act downstream of KAT6B to control NSPC proliferation and that the full effect of *Sox2* overexpression on NSPC proliferation is not observed in the absence of *Kat6b*.

Taken together, this investigation has demonstrated that KAT6B regulates expression of a number of transcriptional regulators in the early developing brain, to coordinate early neurogenesis. It is thus unsurprising that its disruption results in intellectual impairment in human patients, or that its overexpression results in heightened aggression and spontaneous epilepsy in the mouse model. What remains to be assessed are the functional consequences of individual *KAT6B* mutations in human patients, how these affect the expression of downstream KAT6B gene targets and how individual mutations underlie pleiotropic disease traits of dissimilar severity. The effects of loss and gain of KAT6B on histone acetylation in NSPCs will also be the subject of future investigation.



C GO term analysis

Kat6b^{-/-} vs Kat6b^{+/+} C57Bl/6

Gene Ontology term	p.value
Nervous system development	2.12E-22
Neurogenesis	8.00E-17
Neuron differentiation	8.02E-17
Neuron development	2.73E-14
Neuron projection development	1.27E-13
Neuron projection morphogenesis	6.08E-12
Synaptic signaling	4.22E-11
Synaptic vesicle cycle	5.72E-10
Neurotransmitter transport	4.04E-09
Forebrain development	3.33E-08

D KEGG pathway analysis

Kat6b^{-/-} vs Kat6b^{+/+} C57Bl/6

KEGG pathway	p.value
Wnt signaling pathway	0.00069
Sphingolipid signaling pathway	0.00079
Dopaminergic synapse	0.0015
Neuroactive ligand-receptor interaction	0.0016
Axon guidance	0.0021
Cholinergic synapse	0.0023
Serotonergic synapse	0.0027
Calcium signaling pathway	0.018
Phosphatidylinositol signaling system	0.020
Rap1 signaling pathway	0.025

E *Tg(Kat6b) vs Kat6b^{+/+} FvBxBalb/C*

Gene Ontology term	p.value
Synaptic signaling	3.45E-29
Nervous system development	4.20E-25
Neuron development	6.88E-25
Neuron projection development	1.06E-23
Neurogenesis	6.86E-23
Neuron differentiation	1.16E-22
Generation of neurons	3.24E-21
Regulation of neuron projection	7.77E-21
Modulation of chemical synapses	3.61E-20
Regulation of cell projection organisation	3.43E-14

F *Tg(Kat6b) vs Kat6b^{+/+} FvBxBalb/C*

KEGG pathway	p.value
Axon guidance	2.53E-07
cAMP signaling pathway	6.01E-07
Dopaminergic synapse	1.31E-06
Glutamatergic synapse	3.78E-06
MAPK signaling pathway	7.62E-06
Synaptic vesicle cycle	0.00012
GABAergic synapse	0.00012
FoxO signaling pathway	0.0021
Signaling pathways regulating pluripotency	0.017
ErbB signaling pathway	0.032

Figure 5.1 Gene Ontology terms and KEGG pathways altered in dorsal telencephalon RNA-seq

(A) Mean-difference plot of differentially expressed genes between *Kat6b*^{-/-} and *Kat6b*^{+/+C57Bl/6} and between *Tg(Kat6b)* and *Kat6b*^{+/+FvBxBalb/C} E12.5 dorsal telencephalon RNA samples. Each dot represents an individual gene. The x-axis shows the average log-expression for each gene and the y-axis shows the log-fold change of each gene. Differentially expressed genes are highlighted in red and blue for up and down regulation, respectively. Only genes with a FDR value < 0.05 are shown.

(B) Venn diagram depicting the number of genes downregulated in the *Kat6b*^{-/-} E12.5 dorsal telencephalon, relative to *Kat6b*^{+/+C57Bl/6} (blue) and upregulated in the *Tg(Kat6b)* E12.5 dorsal telencephalon, relative to *Kat6b*^{+/+FvBxBalb/C} (red). Circle area is proportional to the number of genes within the circle. Only genes with a FDR < 0.05 are shown.

(C) Table depicting the top gene ontology (GO) terms enriched in genes downregulated in the *Kat6b*^{-/-} E12.5 dorsal telencephalon, relative to *Kat6b*^{+/+C57Bl/6} controls, relevant to neurogenesis. P values are adjacent to each GO term.

(D) Table depicting the top KEGG pathway terms enriched in genes downregulated in the *Kat6b*^{-/-} E12.5 dorsal telencephalon, relative to *Kat6b*^{+/+C57Bl/6} controls, relevant to neurogenesis. P values are adjacent to each KEGG term.

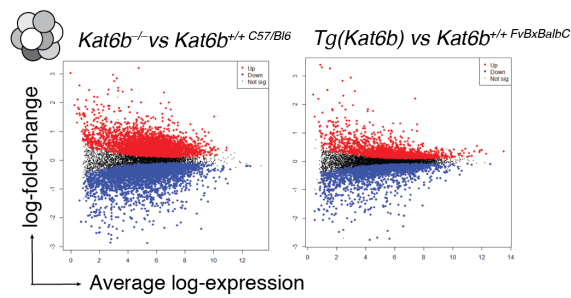
(E) Table depicting the top gene ontology (GO) terms enriched in genes upregulated in the *Tg(Kat6b)* E12.5 dorsal telencephalon, relative to *Kat6b*^{+/+FvBxBalb/C} controls, relevant to neurogenesis. P values are adjacent to each GO term.

(F) Table depicting the top KEGG pathway terms enriched in genes upregulated in the *Tg(Kat6b)* E12.5 dorsal telencephalon, relative to *Kat6b*^{+/+FvBxBalb/C} controls, relevant to neurogenesis. P values are adjacent to each KEGG term.

DR = Downregulated; UR = Upregulated

N = 3 – 4 E12.5 dorsal telencephalon tissue samples per genotype.

A



B



C GO term analysis

Kat6b^{-/-} vs *Kat6b*^{+/+} C57Bl/6

Gene Ontology term	p.value
Nervous system development	7.63E-12
Synapse assembly	3.78E-09
Regulation of neurogenesis	8.14E-09
Generation of neurons	6.69E-08
Neuron differentiation	3.07E-07
Regulation of cell projection organisation	1.68E-06
Regulation of cell communication	1.84E-06
Fatty acid beta-oxidation	2.62E-06
Axon guidance	0.0001
Axon development	0.0004

D KEGG pathway analysis

Kat6b^{-/-} vs *Kat6b*^{+/+} C57Bl/6

KEGG pathway	p.value
Axon guidance	5.44E-05
Fatty acid metabolism	0.0040
PPAR signaling pathway	0.0083
Tryptophan metabolism	0.0089
mTOR signaling pathway	0.010
Metabolic pathways	0.011
Signaling pathways regulating pluripotency	0.011
SNARE interactions in vesicular transport	0.012
Hippo signaling pathway	0.020
Notch signaling pathway	0.020

E *Tg(Kat6b)* vs *Kat6b*^{+/+} FvBxBalb/C

Gene Ontology term	p.value
Cellular respiration	9.60E-16
ATP synthesis coupled electron transport	4.84E-12
Cell communication	2.99E-10
Mitochondrial ATP synthesis	7.67E-10
Aerobic respiration	1.38E-09
Regulation of nervous system development	7.48E-05
Negative regulation of cell communication	8.51E-05
Neurogenesis	0.0001
Neural precursor proliferation	0.004
Axon development	0.007

F *Tg(Kat6b)* vs *Kat6b*^{+/+} FvBxBalb/C

KEGG pathway	p.value
Oxidative phosphorylation	1.86E-24
Metabolic pathways	6.51E-10
Fatty acid metabolism	2.32E-05
mTOR signaling pathway	0.00063
PPAR signaling pathway	0.0019
Neurotrophin signaling pathway	0.0023
Wnt signaling pathway	0.011
AMPK signaling pathway	0.017
PI3K-Akt signaling pathway	0.020
Hippo signaling pathway	0.024

Figure 5.2 Gene Ontology terms and KEGG pathways altered in NSPC RNA-seq

(A) Mean-difference plot of differentially expressed genes between *Kat6b*^{-/-} and *Kat6b*^{+/+*C57Bl/6*} and between *Tg(Kat6b)* and *Kat6b*^{+/+*FvBxBalb/C*} NSPC RNA samples. Each dot represents an individual gene. The x-axis shows the average log-expression for each gene and the y-axis shows the log-fold change of each gene. Differentially expressed genes are highlighted in red and blue for up and down regulation, respectively. Only genes with a FDR < 0.05 are shown.

(B) Venn diagram depicting the number of genes downregulated in the *Kat6b*^{-/-} NSPCs, relative to *Kat6b*^{+/+*C57Bl/6*} (blue) and upregulated in the *Tg(Kat6b)* NSPCs, relative to *Kat6b*^{+/+*FvBxBalb/C*} (red). Circle area is proportional to the number of genes within the circle. Only genes with a FDR < 0.05 are counted.

(C) Table depicting the top gene ontology (GO) terms enriched in genes downregulated in *Kat6b*^{-/-} NSPCs, relative to *Kat6b*^{+/+*C57Bl/6*} controls, relevant to neurogenesis. P values are adjacent to each GO term.

(D) Table depicting the top KEGG pathway terms enriched in genes downregulated in the *Kat6b*^{-/-} NSPCs, relative to *Kat6b*^{+/+*C57Bl/6*} controls, relevant to neurogenesis. P values are adjacent to each KEGG term.

(E) Table depicting the top gene ontology (GO) terms enriched in genes upregulated in the *Tg(Kat6b)* NSPCs, relative *Kat6b*^{+/+*FvBxBalb/C*} controls, relevant to neurogenesis. P values are adjacent to each GO term.

(F) Table depicting the top KEGG pathway terms enriched in genes upregulated in the *Tg(Kat6b)* NSPCs, relative *Kat6b*^{+/+*FvBxBalb/C*} controls, relevant to neurogenesis. P values are adjacent to each KEGG term.

N = 3 – 4 E12.5 NSPC cultures derived from 3 – 4 E12.5 dorsal telencephalon tissue samples, per genotype.

DR = Downregulated; UR = Upregulated

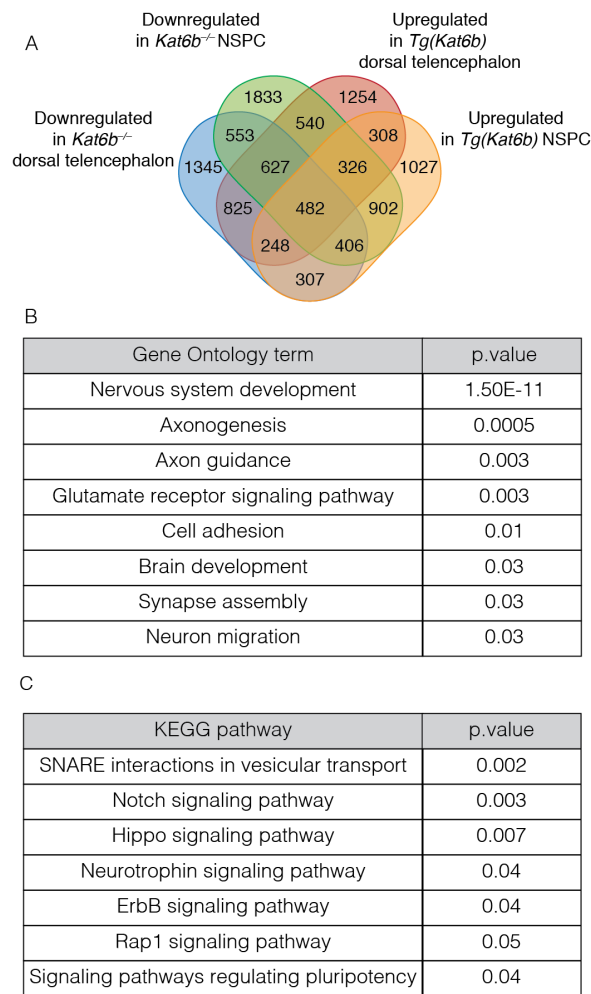


Figure 5.3 Genes altered by *Kat6b* expression in common in the dorsal telencephalon and NSPCs

(A) Venn diagram depicting the overlap in differentially expressed genes in *Kat6b*^{-/-} and *Tg(Kat6b)* E12.5 dorsal telencephalon and NSPC RNA samples, compared to littermate controls. Graph depicts the number of shared, differentially expressed genes between samples and data sets. Genes with a FDR < 0.1 were used in this analysis. 482 genes are downregulated in *Kat6b*^{-/-} E12.5 dorsal telencephalon and NSPC RNA samples, relative to wild type controls and also upregulated in *Tg(Kat6b)* E12.5 dorsal telencephalon and NSPC RNA samples relative to wild type controls.

(B) Table depicting the top GO terms enriched within the 482 genes commonly differentially expressed between *Kat6b* mutant and control RNA samples in both the dorsal telencephalon and NSPCs. Associated P values are shown adjacent to each term.

(C) Table depicting the top KEGG pathway terms enriched within the 482 genes commonly differentially expressed between *Kat6b* mutant and control RNA samples in both the dorsal telencephalon and NSPCs. Associated P values are shown adjacent to each term.

N = 3 – 4 E12.5 dorsal telencephalon tissue samples or NSPC cultures derived from 3 – 4 E12.5 dorsal telencephalon tissue samples, per genotype.

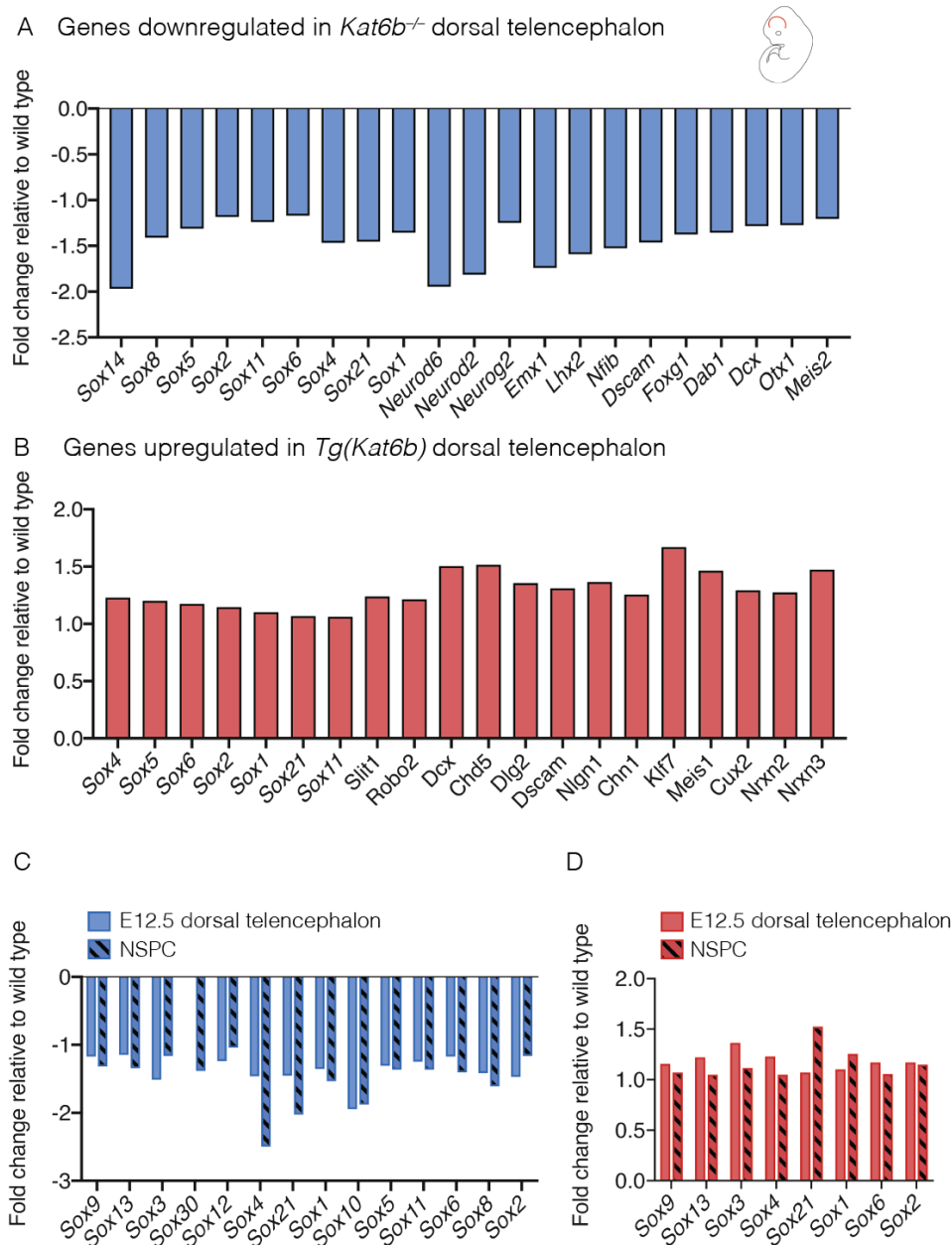


Figure 5.4 KAT6B regulates expression of transcriptional regulators required in early neurogenesis

(A) Bar graph depicting the fold change relative to wild type of genes downregulated in *Kat6b*^{-/-} E12.5 dorsal telencephalon RNA samples, with identified roles in cortex development. Only genes with a FDR value < 0.05 were considered for this analysis.

(B) Bar graph depicting the fold change relative to wild type of genes upregulated in *Tg(Kat6b)* E12.5 dorsal telencephalon RNA samples, with identified roles in cortex development. Only genes with a FDR value < 0.05 were considered for this analysis.

(C) Bar graph depicting the fold change relative to wild type of *Sox* family genes downregulated in *Kat6b*^{-/-} dorsal telencephalon and NSPC RNA samples. Only genes with a FDR value < 0.05 were considered for this analysis.

(D) Bar graph depicting the fold change relative to wild type of *Sox* family genes upregulated in *Tg(Kat6b)* dorsal telencephalon and NSPC RNA samples. Only genes with a FDR value < 0.05 were considered for this analysis.

N = 3 – 4 E12.5 dorsal telencephalon tissue samples or NSPC cultures derived from 3 – 4 E12.5 dorsal telencephalon tissue samples, per genotype.

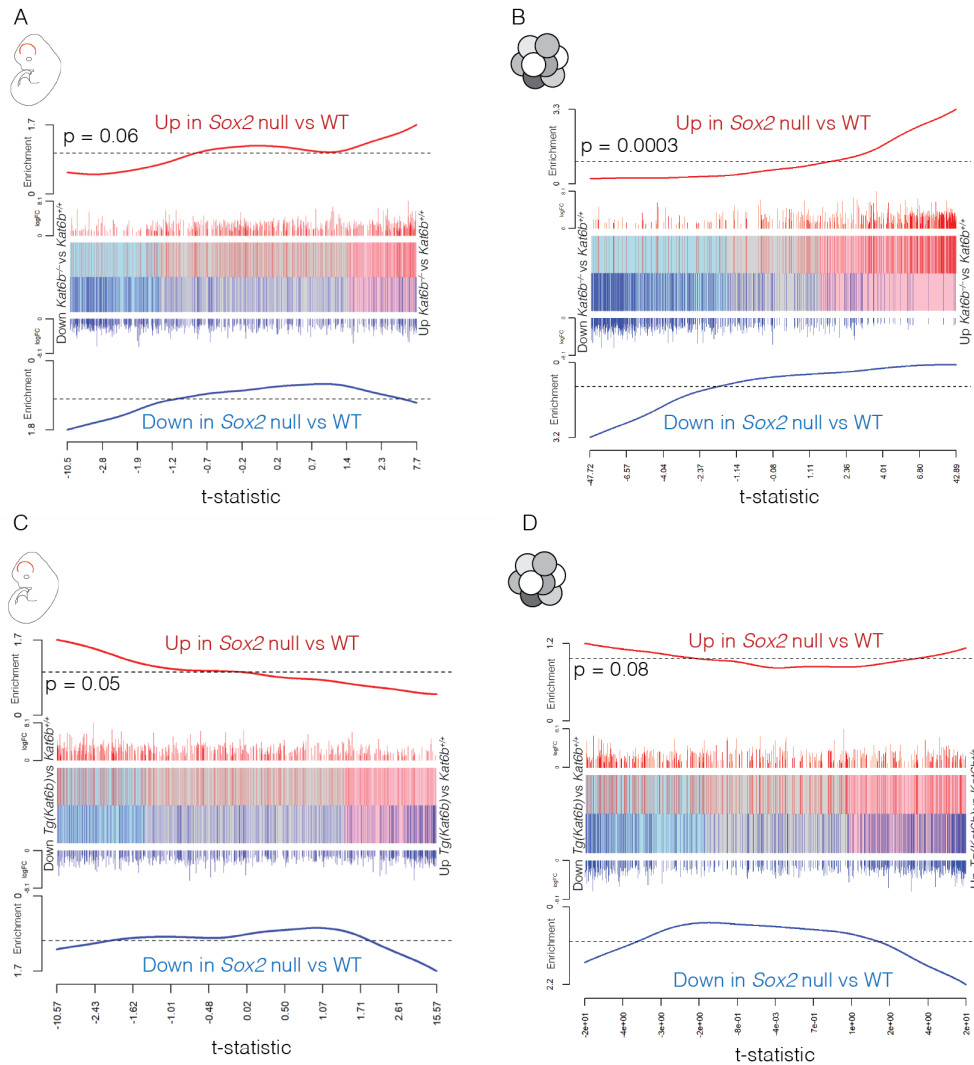


Figure 5.5 The effects of *Kat6b* deletion on gene transcription in NSPCs resemble the effects of *Sox2* deletion

(A) Barcode enrichment plot comparing gene expression changes in *Kat6b*^{-/-} dorsal telencephalon relative to *Kat6b*^{+/+}*C57Bl/6* RNA samples, to gene expression changes between *Sox2* null dorsal telencephalon tissue relative to controls from Bertolini et al., 2019. A correlation analysis shows a statistically insignificant positive correlation ($p = 0.06$) between the data sets.

(B) Barcode enrichment plot comparing gene expression changes in *Kat6b*^{-/-} NSPCs relative to *Kat6b*^{+/+}*C57Bl/6* RNA samples, to gene expression changes between *Sox2* null NSPCs relative to controls from Bertolini et al., 2019. A correlation analysis shows strong positive correlation ($p = 0.0003$) between the data sets.

(C) Barcode enrichment plot comparing gene expression changes in *Tg(Kat6b)* dorsal telencephalon relative to *Kat6b*^{+/+FvBxBalb/C} RNA samples, to gene expression changes between *Sox2* null dorsal telencephalon tissue relative to controls from Bertolini et al., 2019. A correlation analysis shows a slight negative correlation ($p = 0.05$) between the data sets.

(D) Barcode enrichment plot comparing gene expression changes in *Tg(Kat6b)* NSPCs relative to *Kat6b*^{+/+FvBxBalb/C} RNA samples, to gene expression changes between *Sox2* null NSPCs relative to controls from Bertolini et al., 2019. A correlation analysis shows no statistically significant negative correlation ($p = 0.08$) between the data sets.

In all barcode enrichment plots the horizontal axis shows t-statistics for all differentially expressed genes in *Kat6b*^{-/-} vs *Kat6b*^{+/+C57Bl/6} and *Tg(Kat6b)* vs *Kat6b*^{+/+ FvBxBalb/C} dorsal telencephalon and NSPC datasets. Vertical lines represent differentially expressed genes in the *Sox2* null vs wild type dorsal telencephalon and NSPC datasets from Bertolini et al., 2019. Red and blue shaded areas indicate genes that are upregulated and downregulated, respectively, in the *Kat6b*^{-/-} and *Tg(Kat6b)* samples, relative to littermate controls. Red and blue worms indicate the relative enrichment of genes differentially expressed between *Sox2* null and wild samples, with the *Kat6b* null and transgenic samples.

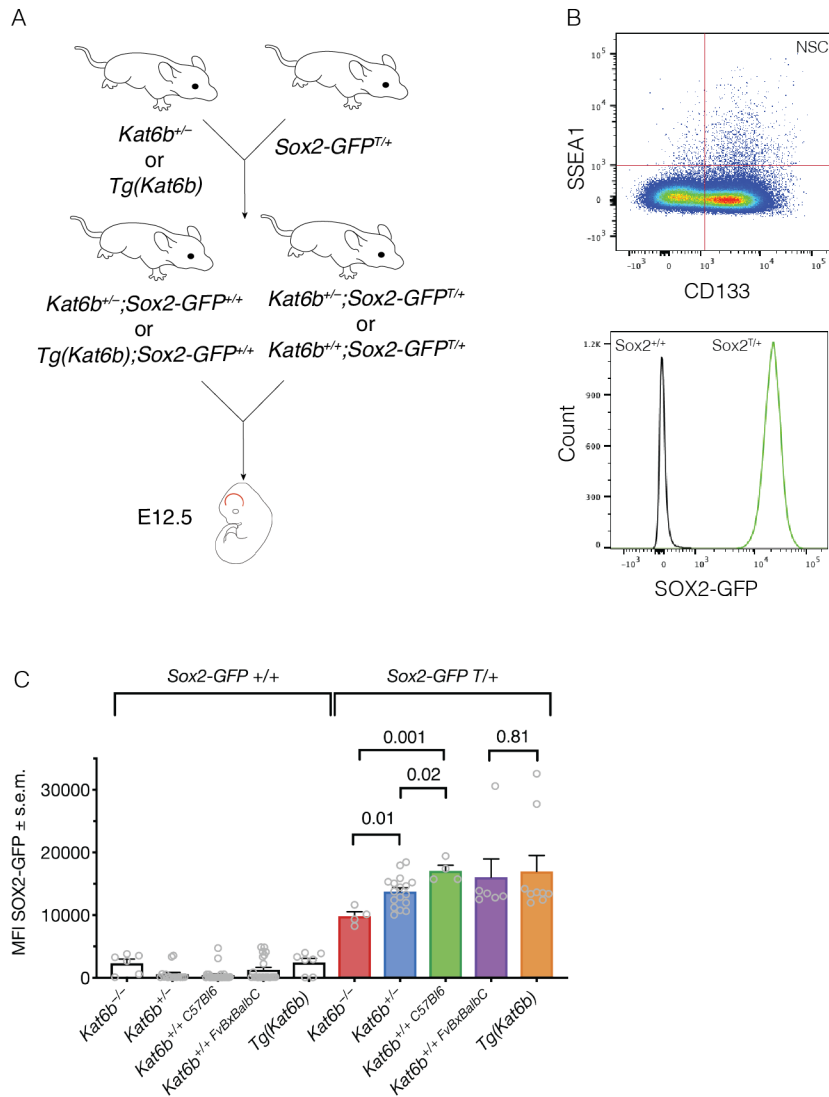


Figure 5.6 KAT6B affects *Sox2* promoter activity in the dorsal telencephalon

(A) Diagram showing the breeding scheme to assess the effect of *Kat6b* status on *Sox2* promoter activity. Animals carrying GFP knocked into the *Sox2* locus were crossed to *Kat6b*^{+/-} and *Tg(Kat6b)* animals and offspring interbred.

(B) Representative FACS plot showing the gating for neural stem cells within the CD133 and SSEA1 double positive population. NSCs were gated on total live cells. A histogram of *Sox2*-GFP and wild type *Sox2* is shown below. The MFI of GFP was used as a readout of *Sox2* promoter activity.

(C) Bar graph depicting the MFI of GFP in NSCs from E12.5 dorsal telencephalon samples of *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* embryos, with or without GFP knocked into the *Sox2* locus. Samples lacking the *Sox2* knock-in allele (*Sox2-GFP* +/+) express only background levels of GFP. Of the samples carrying the GFP knock-in allele (*Sox2-GFP* T/+), *Kat6b*^{-/-} samples show significantly lower levels of GFP than *Kat6b*^{+/-} (p = 0.01) or *Kat6b*^{+/+C57Bl/6} (p = 0.001). In addition, *Kat6b*^{+/-} samples show significantly lower levels of GFP relative to *Kat6b*^{+/+C57Bl/6} (p = 0.02). There is no statistically significant difference between *Tg(Kat6b)* and control samples (p = 0.81).

Bar graph displayed as individual data points with mean ± s.e.m., where data points represent individual E12.5 dorsal telencephalon samples.

N = 4 – 18 E12.5 dorsal telencephalon samples per genotype. *Kat6b*^{-/-}, *Kat6b*^{+/-} and *Kat6b*^{+/+C57Bl/6} samples were compared using a one-way ANOVA followed by a Bonferroni correlation for multiple comparisons, with genotype as the independent factor. *Tg(Kat6b)* and *Kat6b*^{+/+FvBxBalb/C} samples were compared using a two-tails Students t-test.

MFI: Median Fluorescence Intensity.

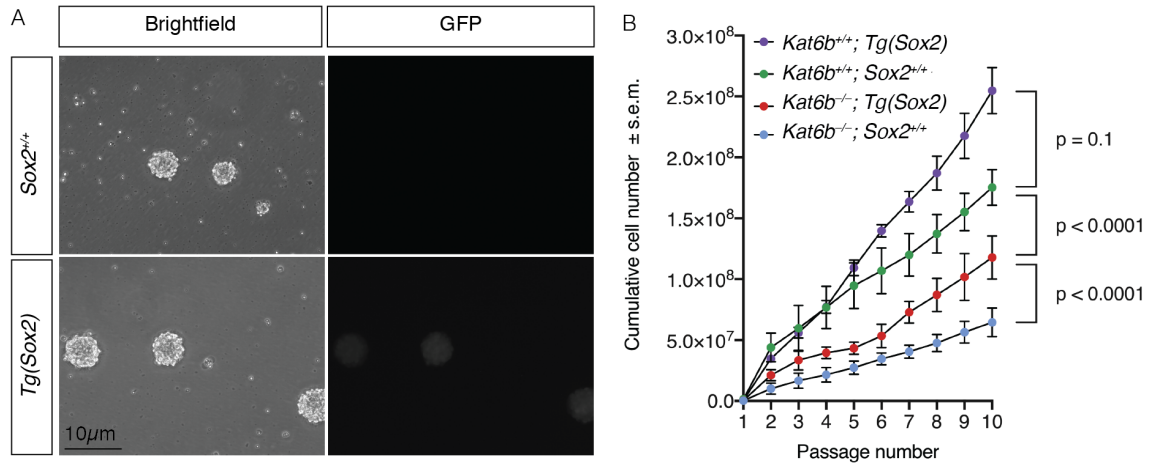


Figure 5.7 *Sox2* overexpression partially rescues the proliferative defect in *Kat6b*^{-/-} NSPCs and pioneer transcription factor SOX2 cannot exert its full effect without the chromatin-modifier KAT6B

(A) Representative brightfield and fluorescent images of NSPCs that are *Sox2*^{+/+} or *Tg(Sox2)*. GFP is within the *Sox2* expression vector. Scale bar = 10 μm.

(B) Cumulative growth curve of NSPCs from *Kat6b*^{-/-}; *Sox2*^{+/+}, *Kat6b*^{+/+}; *Sox2*^{+/+}, *Kat6b*^{-/-}; *Tg(Sox2)* and *Kat6b*^{+/+}; *Tg(Sox2)* samples, over 10 consecutive passages. Over consecutive passages, *Kat6b*^{-/-}; *Sox2*^{+/+} NSPCs grow significantly slower, relative to *Kat6b*^{+/+}; *Sox2*^{+/+} controls. When *Sox2* is overexpressed [*Tg(Sox2)*], *Kat6b*^{-/-}; *Tg(Sox2)* neurospheres show an intermediate growth rate between to *Kat6b*^{-/-}; *Sox2*^{+/+} and *Kat6b*^{+/+}; *Sox2*^{+/+} samples, which is still significantly lower than *Kat6b*^{+/+}; *Sox2*^{+/+} samples (*p* < 0.0001), but also significantly higher than *Kat6b*^{-/-}; *Sox2*^{+/+} samples (*p* < 0.0001). *Sox2* overexpression in *Kat6b*^{+/+} cells (*Kat6b*^{+/+}; *Tg(Sox2)*) did not result a significant increase in cumulative growth over all passages (*p* = 0.1).

N = 2 NSPC cultures derived from *Kat6b*^{+/+} or *Kat6b*^{-/-} E12.5 dorsal telencephalon tissue samples per genotype. Samples compared using a two-way ANOVA, followed by a post hoc correction for multiple comparisons, using genotype and passage number as the independent variables.

Chapter 6

KAT6B in haematopoietic stem cells

Introduction

KAT6B and its MYST family member, KAT6A, share identical domain structure. The shared MYST histone acetyltransferase domain shows 96.7% similarity or identity (Voss & Thomas, 2009), the double PHD domain 93%, the serine-rich domain 96.5% similarity and the methionine-rich domain 68% similarity (Thomas et al., 2000). Given their structural similarity, these proteins may also share functional roles within biological systems. Indeed, both proteins have identified roles in NSCs, with loss of KAT6B observed to impair NSC proliferation, self-renewal and neurogenesis (Merson et al., 2006). Loss of KAT6A similarly impairs NSC self-renewal, while enabling cells to avoid senescence (Perez-Campo et al., 2014). In human patients, heterozygous mutation of either *KAT6B* (Bashir et al., 2017; Clayton-Smith et al., 2011; Kraft et al., 2011; Simpson et al., 2012; Yu et al., 2014) or *KAT6A* (Arboleda et al., 2015; Millan et al., 2016; Tham et al., 2015) underlies congenital disorders associated with intellectual disability. Similarly, both KAT6A and KAT6B have been described in fusion proteins that drive malignancy with weak responses to chemotherapy and poor patient prognoses (Carapeti et al., 1998; Kitabayashi et al., 2001b; Moore et al., 2004; Panagopoulos et al., 2001; Panagopoulos et al., 2015). While KAT6A has been identified as an essential factor in the development of haematopoietic stem cells (HSCs) (Katsumoto et al., 2006; Thomas et al., 2006) and their maintenance in adulthood (Sheikh et al., 2016), a role for KAT6B in haematopoiesis has not been experimentally assessed.

In this chapter I investigate a role for KAT6B in haematopoiesis, both during development and in competitive transplantations in adult mice. I demonstrate that foetal liver-derived *Kat6b* null HSCs have an impaired ability to reconstitute the haematopoietic system of lethally irradiated mice and *Kat6b* null animals have smaller foetal liver HSC pools during development. Furthermore, I show that heterozygosity at both *Kat6b* and *Kat6a* loci reduces HSC pools in the foetal liver and severely impairs reconstitution of irradiated recipients, highlighting the requirement for adequate levels of both factors in HSC development and function.

6.1 Results

6.1.1 *KAT6B* regulates HSC numbers within the embryonic and foetal liver

At E18.5, *Kat6b*^{-/-} mutants showed small ($p = 0.0002$), pale spleens relative to wild type controls (Fig 6.1 A – B). *Tg(Kat6b)* E18.5 spleens are of a normal size ($p = 0.55$) and appearance, relative to wild type (Fig 6.1 A – B). To determine the effect of loss and gain of *Kat6b* on definitive haematopoiesis, HSC populations were assessed in the E12 and E14 liver by FACS. Stem cells were quantified within the HSC-enriched lineage negative (LIN-), cKIT + and SCA1 + (LSK) population, assessing CD48-, CD150+ (HSC^{SLAM}) (Kiel et al., 2005a) and CD34hiFlt3lo (HSC^{CD34hiFlt3lo}) or CD34loFlt3hi (HSC^{CD34loFlt3hi}) populations (Adolfsson et al., 2001; Christensen & Weissman, 2001; Yang et al., 2005).

Loss of one and both alleles of *Kat6b* resulted in a dose-dependent reduction in HSC populations at E12 and E14. Within the LSK population, the percentage of HSC^{SLAM} cells was significantly lower in *Kat6b*^{-/-} samples at E12 ($p < 0.01$) and E14 ($p < 0.01$) and in *Kat6b*^{+/-} samples at E14 ($p = 0.01$), relative to *Kat6b*^{+/+C57Bl/6} (Fig 6.2 B). The percentage of ST-HSC^{CD34hiFlt3lo} was significantly lower in *Kat6b*^{-/-} samples at E12 ($p < 0.01$) and E14 ($p = 0.03$) and in *Kat6b*^{+/-} samples at E12 ($p = 0.05$) and E14 ($p = 0.02$), relative to *Kat6b*^{+/+C57Bl/6} (Fig 6.2 D). Lastly, the percentage of HSC^{CD34loFlt3lo} was significantly lower in *Kat6b*^{-/-} samples at E12 ($p < 0.01$) and E14 ($p = 0.04$) and in *Kat6b*^{+/-} samples at E12 ($p < 0.01$) and E14 ($p = 0.02$), relative to *Kat6b*^{+/+C57Bl/6} (Fig 6.2 F).

By comparison, *Kat6b* gain resulted in a larger percentage of HSC^{SLAM} cells at E12 ($p = 0.04$) and E14 ($p = 0.01$) (Fig 6.2 C), ST-HSC^{CD34hiFlt3lo} cells at E12 ($p = 0.04$) and E14 ($p = 0.01$) (Fig 6.2 E) and HSC^{CD34loFlt3lo} at E14 ($p = 0.01$) (Fig 6.2 G), relative to *Kat6b*^{+/+FvBxBalb/C} controls, demonstrating that KAT6B can drive HSC proliferating in the liver.

6.1.2 Loss of *Kat6b* does not affect HSC homing to the bone marrow

To investigate HSC homing in *Kat6b* mutant and control embryos, 5×10^6 E14 foetal liver cells (CD45.2+) were combined with 5×10^6 wild type adult bone marrow competitor cells (CD45.1+) and injected into CD45.1 and CD45.2 double positive, lethally irradiated recipients (Fig 6.3 A). At 18 h post-transplant, the proportions of CD45.1 and CD45.2 single positive cells were assessed in the total live cells and HSC enriched LIN-SCA1+ (LS) populations, excluding host-derived CD45.1/2 double positive cells (Fig 6.3 B). No difference in the ratio of CD45.1 to CD45.2 single positive cells was observed between *Kat6b*^{-/-} ($p > 0.99$) or *Kat6b*^{+/-} ($p > 0.99$) samples relative to wild type controls in the total live cell population (Fig

6.3 C). No difference in the ratio of CD45.1 to CD45.2 single positive cells was observed between *Kat6b*^{-/-} (p = 0.07) or *Kat6b*^{+/-} (p = 0.44) samples relative to wild type controls in the LIN – SCA1+ (LS) populations (Fig 6.3 D). In addition, no difference was observed in the ratio of CD45.1 to CD45.2 between *Tg(Kat6b)* and control samples in the total live cell (p = 0.84) (Fig 6.3 C) or LIN – SCA1+ (p = 0.49) populations (Fig 6.3 D).

6.1.3 Loss of Kat6b functionally impairs HSC reconstitution in foetal liver haematopoietic cell transplantation experiments in vivo

The requirement for KAT6B in HSC function was assessed by determining its effect on the reconstitution of lethally irradiated recipient mice in competitive foetal liver transplantation experiments. 5 x 10⁵ foetal liver test cells (CD45.2 +) from *Kat6b*^{-/-}, *Kat6b*^{+/-} or wild type E14 fetuses were combined with 1 x 10⁶ adult bone marrow competitor cells (CD45.1+) and injected into CD45.1+ lethally irradiated recipients. In addition, 5 x 10⁵ foetal liver cells (CD45.2) from *Tg(Kat6b)* or control E14 fetuses were combined with 1 x 10⁶ adult bone marrow competitor cells (CD45.1/2+) and injected into CD45.1/2 lethally irradiated recipients. For both transplants, the proportion of CD45.2+ cells was assessed in the peripheral blood at 1, 3 and 5 months after transplantation and at 5 months also in the bone marrow, thymus and spleen. Each starting population of cells was injected into three irradiated recipients. Foetal liver donors and recipients were matched for genetic background and accordingly, no graft-versus-host disease was observed in any recipient during the course of the experiment.

At 1 month in the peripheral blood, *Kat6b*^{-/-} recipients showed a 50 – 60% reduction in the percentage of CD45.2+ cells across all cell types, relative to *Kat6b*^{+/+} recipients (Fig 6.4 B). This same effect was also observed at 3 months (Fig 6.4 C) and 5 months (Fig 6.4 D), demonstrating that loss of *Kat6b* impairs reconstitution of irradiated recipients across all peripheral blood cell lineages. Reconstitution in *Kat6b*^{+/-} recipients did not significantly differ from that of wild type recipients in the peripheral blood at 1 month (Fig 6.4 B), 3 months (Fig 6.4 C) or 5 months (Fig 6.4 D). Conversely, *Tg(Kat6b)* recipients showed a ~10% increase in CD45.2 in peripheral blood monocytes at 1 month (p = 0.04; Fig 6.5 B) and a 10 – 20% increase in CD45.2 in monocytes (p = 0.04), neutrophils (p = 0.04) and B cells (p = 0.01) at 3 months (Fig 6.5 C). This increase persisted at 5 months in monocytes (p = 0.04), neutrophils (p = 0.04) and B cells (p = 0.03) (Fig 6.4 D). These results demonstrate that, while *Tg(Kat6b)* foetal liver

cells show an increased capacity to reconstitute peripheral blood cells relative to wild type, this effect is specific to the myeloid and B-cell lineages and excludes CD4 and CD8 T cells.

In the bone marrow at 5 months post-transplantation, *Kat6b*^{-/-} recipients demonstrated a strikingly consistent 50 – 60% reduction in CD45.2, relative to *Kat6b*^{+/+} recipients, across all stem and progenitor cell types analysed (Fig 6.6 C). Reconstitution in *Kat6b*^{+/-} recipients did not differ significantly from *Kat6b*^{+/+} recipients in any cell type analysed (Fig 6.6 C). By comparison, a 20 – 30% increase in CD45.2+ cells was observed in *Tg(Kat6b)* recipients, relative to wild type controls, across all bone marrow cell types, excepting the ST-HSC^{CD34hiFlt3lo} (p = 0.07), HSC^{SLAM} (p = 0.4) and MPP^{SLAM} (p = 0.7) populations, which were not significantly different (Fig 6.7 C).

In the spleen, *Kat6b*^{-/-} recipients displayed a 50 – 60% reduction in CD45.2+ cells, relative to *Kat6b*^{+/+} recipients, consistently across all B lineage cell types analysed (Fig 6.8 D). Reconstitution in *Kat6b*^{+/-} recipients was not statistically different from *Kat6b*^{+/+} recipients for any splenic population (Fig 6.8 D). Comparatively, a ~10% increase in CD45.2+ cells was observed in *Tg(Kat6b)* recipients for all B lineage cell types, except T2 progenitors (p = 0.09), marginal zone B cells (p = 0.69) and plasma cells (p = 0.34), which were not significantly changed (Fig 6.9 C).

Lastly, in thymic T cell and progenitor cell types, *Kat6b*^{-/-} recipients displayed a 50 – 60% reduction in CD45.2+ cells across all cell types (Fig 6.10 D). Reconstitution in *Kat6b*^{+/-} recipients was not significantly different from *Kat6b*^{+/+} recipients for any cell population (Fig 6.10 D). By comparison, no difference in CD45.2+ cells was observed between *Tg(Kat6b)* and control cell recipients (Fig 6.11 C). This is consistent with *Kat6b* overexpression having no effect on CD4 or CD8 reconstitution in the periphery at 1, 3 and 5 months and demonstrates that the enhanced reconstitution observed in *Tg(Kat6b)* recipients across some cell types, excludes the T cell lineage.

6.1.4 Functional assessment of defined numbers of HSCs

Given that *Kat6b* status affects HSC numbers within the E14 foetal liver, the differential reconstitution observed in recipients of *Kat6b*^{-/-} and *Tg(Kat6b)* foetal liver cells may reflect numerical differences in HSCs of the foetal livers of mutant and control samples, rather than

competitive differences within the HSCs themselves. To determine if there were functional differences between *Kat6b* mutants and controls in addition to numerical differences, 50 HSC^{SLAM} cells were sorted prior to transplantation and combined with 2 x 10⁵ whole adult bone marrow cells from CD45.1 + or CD45.1/2 + adult mice. Contribution to reconstitution was assessed in recipients at 1 month in the peripheral blood and at 3 months in the peripheral blood and bone marrow (Fig 6.12 A). Owing to limiting numbers of HSC^{SLAM} cells within the E14 foetal liver, HSC^{SLAM} cells from each donor were injected into one irradiated recipient.

Consistent with the initial transplantation experiments, at 1 month in the peripheral blood, *Kat6b*^{-/-} recipients showed an ~60% reduction in CD45.2+ representation across all cell types (Fig 6.12 B). *Kat6b*^{+/-} recipients were not significantly different from wild type recipients for any peripheral blood cell population (Fig 6.12 B). *Kat6b*^{-/-} recipients still showed a ~60% reduction in CD45.2 at 3 months in the peripheral blood (Fig 6.12 C). Interestingly, at 3 months, *Kat6b*^{+/-} recipients showed a 30 – 40% reduction in CD45.2+ cells, relative to wild type recipients, for all peripheral blood cell populations except neutrophils (Fig 6.12 C). At 3 months in the bone marrow, *Kat6b*^{-/-} recipients showed a 70 – 80% reduction in CD45.2, relative to wild type recipients, across all bone marrow populations, while *Kat6b*^{+/-} recipients showed a 30 – 40% reduction in CD45.2 across all bone marrow populations (Fig 6.12 D), compared to wild-type controls.

By comparison, *Tg(Kat6b)* HSC recipients showed increased CD45.2+ representation at 1 month post-transplant in all peripheral blood cell types, including CD4 (p = 0.02) and CD8 T cells (p = 0.02) (Fig 6.13 B). This effect was still apparent at 3 months, in peripheral B cells (p = 0.01) and neutrophils (p = 0.04), but no longer in CD4 (p = 0.20) or CD8 (p = 0.14) T cell populations (Fig 6.13 C). In the bone marrow at 3 months, *Tg(Kat6b)* recipients showed increased CD45.2+ representation in ST-HSC^{CD34hiFlt3lo} (p = 0.02) and HSC^{SLAM} (p = 0.04) populations, but not in any other cell type (Fig 6.13 D).

6.1.5 *KAT6B* does not affect haematopoietic progenitor lineage potential *in vitro*

The effect of *Kat6b* status on the ability of stem and progenitor cells to undergo haematopoietic colony formation was assessed *in vitro*. E14 foetal liver cells were subjected to a colony-forming assay, exposed to SCF, IL3 and EPO, with saline-only used as a negative control (Fig 6.14 A). The total number of colonies formed was used as an indicator of the number of

primitive myeloid progenitors within foetal liver samples, while the colony type, identified based on (Metcalf, 1984), was used as an indicator of differentiation potential. No colonies were observed in any of the saline-only negative controls.

Kat6b^{-/-} foetal liver cells formed fewer colonies compared to wild type controls ($p = 0.01$) (Fig 6.14 B), while significantly more colonies were observed from *Tg(Kat6b)* foetal liver cells ($p = 0.0016$) (Fig 6.14 B). In addition, *Kat6b*^{-/-} HSCs developed proportionally more erythroid colonies, relative to *Kat6b*^{+/-} samples ($p = 0.04$), however this was not statistically significant between *Kat6b*^{-/-} and wild type samples ($p = 0.42$) or between *Kat6b*^{+/-} samples and wild type controls ($p = 0.48$) (Fig 6.14 C). This indicated a potential effect of *Kat6b* status on erythroid development. No difference in the proportion of any colony type was observed in *Tg(Kat6b)* samples relative to their controls (Fig 6.14 D). Furthermore, no differences were observed in erythropoiesis in the foetal livers of *Kat6b* null, heterozygous or transgenic mutants, relative to wild type (Fig 6.15), indicating that KAT6B does not affect erythroid development in the foetal liver *in vivo*, but may affect the differentiation response to *in vitro* culture conditions.

6.1.6 The overlapping and unique roles of KAT6A and KAT6B in haematopoietic stem cells

The MYST family histone acetyltransferase, KAT6A, is essential for the embryonic development of HSCs (Katsumoto et al., 2006; Thomas et al., 2006) and their continued maintenance in adulthood (Sheikh et al., 2016). The results presented in this chapter demonstrate that KAT6B is also required in HSC proliferation and functionality during development, raising the possibility of overlapping roles for these closely related MSYT family members within HSCs.

To functionally assess the combined effects of *Kat6a* and *Kat6b* on HSC development and function, *Kat6a*^{+/-} and *Kat6b*^{+/-} animals were inter-crossed to quantitate foetal liver HSCs in wild type, single and double heterozygous embryos (Fig 6.16 A). At E14, HSC^{SLAM} proportions in *Kat6b* single heterozygous mutants were statistically lower from wild type controls ($p < 0.01$), while *Kat6a* single heterozygous animals also had fewer HSC^{SLAM} cells compared to wild type controls ($p < 0.01$). Double heterozygous mutants showed an even greater reduction in HSC^{SLAM} proportions, relative to wild type ($p < 0.01$) (Fig 6.16 B).

To functionally assess these cells, 50 HSC^{SLAM} cells from CD45.2+ wild type, *Kat6a* heterozygous, *Kat6b* heterozygous and double heterozygous mutants were sorted and combined with 2 x 10⁵ CD45.1+ adult bone marrow cells and injected into one irradiated CD45.1+ recipient (Fig 6.17 A). The contributions of these starting populations to the reconstitution of irradiated recipients was assessed at 1 and 3 months in the peripheral blood and at 3 months in the bone marrow.

At 1 and 3 months in the peripheral blood and at 3 months in the bone marrow, double heterozygous recipients showed a significant reduction in CD45.2 representation across all cell types (Fig 6.17 B – C). Single heterozygous recipients also showed reduced reconstitution, with the effect size larger in *Kat6a*^{+/-} recipients compared to *Kat6b*^{+/-} recipients (Fig 6.17 B – C), consistent with the published data (Katsumoto et al., 2006; Thomas et al., 2006) and *Kat6b* transplants reported in this study. The same effect was seen at 3 months across all bone marrow HSC and progenitor populations (Fig 6.17 D).

6.1.7 KAT6B acetylates H3K9 in the foetal liver

Given that KAT6B is a histone acetyltransferase, acetylation of the current putative histone acetylation targets of KAT6B, H3K14 (Doyon et al., 2006) and H3K23 (Simo-Riudalbas et al., 2015) as well as the histone target of the closely related MYST family member KAT6A, H3K9 (Voss et al., 2009), was analysed in the E14 foetal liver in HSC^{SLAM}, HSC^{CD34loFlt3lo} and ST-HSC^{CD34hiFlt3lo} cells by FACS, using median fluorescence intensity (MFI) as a readout of histone acetylation levels.

H3K9ac was significantly reduced in *Kat6b*^{-/-} E14 foetal livers in HSC^{SLAM} ($p < 0.01$), HSC^{CD34loFlt3lo} ($p = 0.02$) and ST-HSC^{CD34hiFlt3lo} ($p = 0.02$) populations (Fig 6.18 A). Conversely, H3K9ac was increased in *Tg(Kat6b)* foetal livers in HSC^{SLAM} ($p = 0.006$), HSC^{CD34loFlt3lo} ($p = 0.005$) and ST-HSC^{CD34hiFlt3lo} ($p = 0.01$) populations (Fig 6.18 A). In contrast, H3K14ac was unchanged in any HSC population in *Kat6b*^{-/-} foetal livers, relative to wild type (Fig 6.18 B). However, H3K14ac was reduced in *Tg(Kat6b)* liver HSC^{SLAM} ($p = 0.05$), HSC^{CD34loFlt3lo} ($p = 0.02$) and ST-HSC^{CD34hiFlt3lo} ($p = 0.0007$) populations (Fig 6.18 B). No difference was observed in H3K23ac in any HSC population in either *Kat6b*^{-/-} or *Tg(Kat6b)* foetal livers, relative to controls (Fig 6.18 C).

6.1.8 HDAC inhibition can restore H3K9ac levels in *Kat6b*^{+/-} adult mice

Given that loss of one or two alleles of *Kat6b* affects histone H3 lysine 9 acetylation levels in foetal liver HSC populations in a gene dose dependant manner (Fig 6.18), I aimed to determine if the deficiency of H3K9ac in *Kat6b*^{+/-} mice might be ameliorated by treatment with an HDAC inhibitor. In these initial exploratory experiments, I used a broad-spectrum HDAC inhibitor, valproic acid (VPA). Rather than activating KAT6B or other HATs, the predominant action of VPA is to inhibit the enzymes that remove modifications laid down by HATs. As such, VPA will not functionally alter KAT6B, but may act to modulate the effect of *Kat6b* deficiency and any subsequent deficiency in histone acetylation resulting from KAT6B loss.

In peripheral blood cells types, *Kat6b*^{+/-} adults show a 20 – 40% reduction in H3K9ac across all cell types (Fig 6.19 A). To determine whether treatment with the HDAC inhibitor, valproic acid (VPA) can increase H3K9ac levels in *Kat6b*^{+/-} animals, *Kat6b*^{+/-} and wild type controls were treated with VPA (Sigma, P4543) from 2 weeks of age, twice daily for 2 weeks by oral gavage (100 mg/kg body weight), followed by 2 weeks with VPA-supplemented chow (20 g/kg chow) (6.19 B).

Assessment of H3K9ac levels in the peripheral blood after 2 weeks of treatment with VPA showed that H3K9ac levels in *Kat6b*^{+/-} treated animals were increased, relative to untreated *Kat6b*^{+/-} animals. H3K9ac levels in *Kat6b*^{+/-} treated animals were not significantly different from *Kat6b*^{+/+} untreated controls across all cell types, while untreated *Kat6b*^{+/-} animals showed significantly lower H3K9ac in B cells ($p = 0.04$), but not other peripheral blood populations (Fig 6.19 C).

After 4 weeks of treatment with VPA, *Kat6b*^{+/-} treated animals remained statistically indistinguishable from *Kat6b*^{+/+} untreated controls (Fig 6.19 D) demonstrating that the histone acetylation defect in *Kat6b* deficient animals is amenable to intervention and can be increased to wild type levels. Interestingly, untreated *Kat6b*^{+/-} animals showed significantly less H3K9ac across all peripheral blood cell types at 4 weeks, relative to untreated *Kat6b*^{+/+} animals (Fig 6.15 D), suggesting that the reduction in H3K9ac observed in heterozygous mutants may be progressive in early life.

6.2 Discussion

Here, I identify KAT6B as a novel regulator of embryonic and foetal haematopoietic stem and progenitor cells, both in the embryonic and foetal liver and during reconstitution in irradiated recipients. Within the embryonic liver, definitive haematopoiesis initiates at E11.5, peaking at E14.5, before dropping as haematopoiesis begins to shift to the bone marrow. *Kat6b* loss resulted in reduced HSC pools at E12 and E14, while *Tg(Kat6b)* overexpressing embryos and fetuses showed increased HSC numbers at both time points analysed. *Tg(Kat6b)* transgenic haematopoiesis still followed the trend of wild type samples, with HSC numbers increasing from E12 – 14. This suggests that *Tg(Kat6b)* overexpression results in enhanced HSC proliferation, but does not affect the timing of haematopoiesis. In addition, *Kat6b* status did not affect HSC homing to the bone marrow, as demonstrated by the presence of HSCs within recipient bone marrow, 18 h following transplantation.

Prior to this thesis, there had not been an investigation into KAT6Bs acetylation target in the haematopoietic system. H3K9, H3K14 and H3K23 were selected as putative acetylation targets given that H3K14 is a KAT6B acetylation target based on studies in *in vitro* overexpression systems, while H3K23 is the target of KAT6Bs orthologue in *Drosophila*, Enok and has been identified as a KAT6B gene target in a study using purified KAT6B - containing complexes expressed in 293T cells. H3K9 was also chosen as this is one of the proposed histone acetylation targets of KAT6A. This thesis demonstrated that *Kat6b* loss reduced acetylation at H3K9 in foetal liver HSCs, while *Kat6b* overexpression increases acetylation of this residue. Neither H3K14ac or H3K23ac was affected in the cell types analysed.

Kat6b null HSCs showed substantially impaired reconstitution both in the short and long term, while *Tg(Kat6b)* HSCs showed enhanced reconstitution capacities in a cell type specific manner. Given that all cell lineages are affected by *Kat6b* loss, this indicates a stem cell defect. In contrast, the enhanced reconstitution seen in recipients of *Tg(Kat6b)* cells largely excluded T cell progenitor and mature cell types. Transgenic overexpression of *Kat6b* did not affect the numbers of common lymphoid progenitors (CLPs) or other early T cell progenitors in sorted HSC^{SLAM} transplants, reflected in the normal numbers of T cells in the mature tissue and peripheral blood. The effect of *Kat6b* overexpression on T cell populations may also result from the selective environment within the thymus, which may remove cell abundance differences during rounds of negative and positive selection.

Further to this point, one might expect that gain of KAT6B in the *Tg(Kat6b)* would have opposing effects on cell biology and gene expression compared to loss of KAT6B in the *Kat6b*^{-/-} mutant mice. While this is the case for some parameters examined in this thesis, the nature of the function of KAT6B as the enzymatic domain of a large histone acetylation complex, with selection of chromatin adapter proteins (BRPF1/2/3, ING5) and EAF6 (Doyon et al., 2006), would suggest that while a loss of function is easy to achieve, a gain of function of this complex is not expected to be 100% successful when only overexpressing one complex component. Therefore, it was interesting that in the case of some parameters, such as peripheral blood B cells and granulocytes and in bone marrow stem cells, that the opposing effect was achieved, suggesting that in these cell types KAT6B, but not the other complex members, was limiting. It is also possible that the additional KAT6B "recruited" complex members that would have ordinarily associated with KAT6A. With respect to other parameters, overexpression did not have prominent effects, such as peripheral blood and thymic T cells, suggesting that more of KAT6B did not result in more histone acetylation at key loci affecting these parameters.

Another possible explanation for the mixed results of KAT6B overexpression could be that in some cell types histone acetylation was already at a maximally possible level and increasing KAT6B had no effect, while in other cell types additional KAT6B resulted in an increase in acetylation. However, this latter explanation appears to be less likely, because in the instance where histone acetylation was checked in HSC^{SLAM}, HSC^{CD34loFLT3lo} and HSC^{CD34hiFLT3lo} cell types, overexpression of KAT6B was seen to result in an increase in acetylation levels and Histone H3K9.

6.2.1 Discrepancy between transplantation results in the bulk and sorted HSC^{SLAM} transplants

In the bulk foetal liver haematopoietic cell transplant, *Tg(Kat6b)* recipients showed enhanced reconstitution in monocytes in the peripheral blood at 1 month and in monocytes, neutrophils and B cells in the peripheral blood at 3 and 5 months. *Tg(Kat6b)* recipients also showed enhanced reconstitution in most HSC and progenitor populations in the bone marrow, in most splenic B cell populations, however not in a thymic T cell population. By comparison, when HSC^{SLAM} cells were sorted and injected into irradiated recipients, *Tg(Kat6b)* recipients showed enhanced reconstitution in B cells, neutrophils and monocytes in the peripheral blood at 1 month and in B cells and neutrophils at 1 and 3 months, comparable to what was seen in the

bulk transplant. In the bone marrow at 3 months, HSC but not progenitor populations showed enhanced reconstitution in *Tg(Kat6b)* recipients. The thymus and spleen were not assessed.

While the results between the two transplants are similar in that *Tg(Kat6b)* foetal liver cells and HSC^{SLAM} recipients showed enhanced reconstitution across a number of cell populations, there are more affected populations in the bulk foetal liver cell transplant than when cells were sorted. There are a number of reasons that this could be the case. Firstly, as described in section 6.1.1, *Tg(Kat6b)* E14 foetal livers had a greater proportion of HSC^{SLAM} cells than littermate controls. As such, when 5×10^5 bulk foetal liver cells were injected into irradiated recipients, the total number of HSCs would have differed between *Tg(Kat6b)* and *Kat6b*^{+/+FvBxBalb/C} recipients. By comparison, when a defined number of sorted HSC^{SLAM} cells were injected into irradiated recipients, this numerical difference in the starting population was removed. The sorting process also removed multipotent progenitors which may have contributed to reconstitution in the bulk transplant. In addition, the process of sorting the cells using a fluorescence-activated cell sorter introduced an additional mechanical stress to the cells, which was not the case when bulk foetal liver cells were transplanted. Coupled to this, only a small number of sorted HSC^{SLAM} cells were transplanted into recipients, such that variability across recipients in regards the number of cells successfully sorted into tubes and subsequently injected into recipients, was greater than when bulk cells were transplanted, increasing the variability in reconstitution across recipients and decreasing the ability to detect significant changes.

6.2.2 Functional overlap between *Kat6a* and *Kat6b* in HSCs

KAT6A has already been identified as a critical factor in the development of HSCs, such that homozygous *Kat6a* loss is incompatible with HSC generation during development (Katsumoto et al., 2006; Thomas et al., 2006). I have now demonstrated that the closely related protein, KAT6B, is required for the generation of normal numbers of HSCs during development and for HSC reconstitution capacity following transplantation. Notably, even the loss of one allele of *Kat6b* compromised HSC reconstitution capacity. Furthermore, despite HSCs being detected in the E14 foetal liver of *Kat6a/Kat6b* double heterozygous animals, these cells displayed a severely reduced ability to reconstitute an irradiated recipient, demonstrating that these cells are significantly functionally impaired. These results highlight that both KAT6A and KAT6B

are essential for haematopoietic stem cell function and that the full complement of the combined four alleles of *Kat6a* and *Kat6b* are required for normal haematopoiesis.

6.2.3 Discrepancy in the role of KAT6B in developing and aging HSCs

It has previously been demonstrated that KAT6B expression is reduced during aging, in both murine HSCs (Sun et al., 2014) and in aging human HSCs (Adelman et al., 2019). Coupled to this, shRNA knockdown of *Kat6b* in sorted murine HSCs has been shown to result in a myeloid bias at the expense of erythroid development *in vitro* and in a myeloid bias in the periphery, 1 month after transplantation into irradiated recipients (Khokhar et al., 2020). By comparison, the data described in this chapter demonstrates that *Kat6b* loss in foetal HSCs impairs reconstitution in irradiated recipients across all cell types in the periphery, bone marrow, thymus and spleen.

The likely reason for this discrepancy is two-fold. Firstly, divergent results may be due to the use of *Kat6b* shRNA knockdown, as compared to germ line deletion used in this study. The use of shRNA knockdown meant that the HSCs analysed in (Khokhar et al., 2020) had developed with normal levels of KAT6B and within HSC niches that had normal levels of KAT6B, which was not the case for this study and may account for functional differences between these KAT6B mutant cells. Secondly, the viral transduction and 48 h culture of adult sorted HSCs for shRNA knockdown would have allowed for a number of cells to differentiate towards a progenitor state, which may have affected the behaviours of these cells in later experiments, as is acknowledged by the authors. A similar difference in *in vitro* and *in vivo* results was observed in this study, as *Kat6b* null foetal liver cells showed an erythroid bias *in vitro* in a colony forming assay, however showed no difference in erythropoiesis within the foetal liver, demonstrating that the environment to which these cells are exposed is important in directing their lineage commitment. Lastly, (Khokhar et al., 2020) assess adult HSCs, while this study focuses on embryonic and foetal HSCs. The differing functional requirements of HSCs at these different time points may also result in their functional differences within *in vitro* and *in vivo* assessments of HSC function.

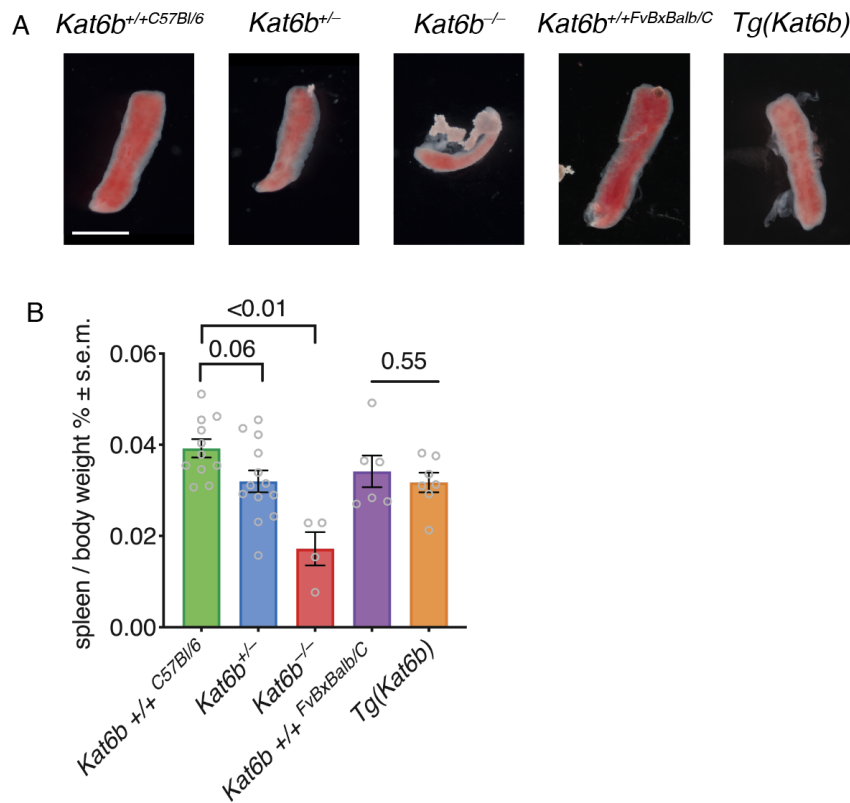


Figure 6.1 Loss of *Kat6b* results in small, pale spleens in E18.5 embryos

(A) Representative photographs of *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* spleens at E18.5. Scale bar = 1 mm

(B) Bar graph of spleen weight as a percentage of total body weight for *Kat6b* wild type and mutant embryos at E18.5. *Kat6b*^{-/-} mutants have small spleens, relative to wild type controls (p < 0.01), while *Kat6b*^{+/-} spleen weights were not statistically different from wild type controls (p = 0.06). *Tg(Kat6b)* mutant spleen weights were not statistically different from littermate controls (p = 0.55).

Kat6b^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} samples were analysed using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples were analysed using a two-tailed Student t-test. Data in bar graphs are displayed as individual data points with mean ± s.e.m, where data points represent individual foetuses. N = 4 – 13 E18.5 samples per genotype.

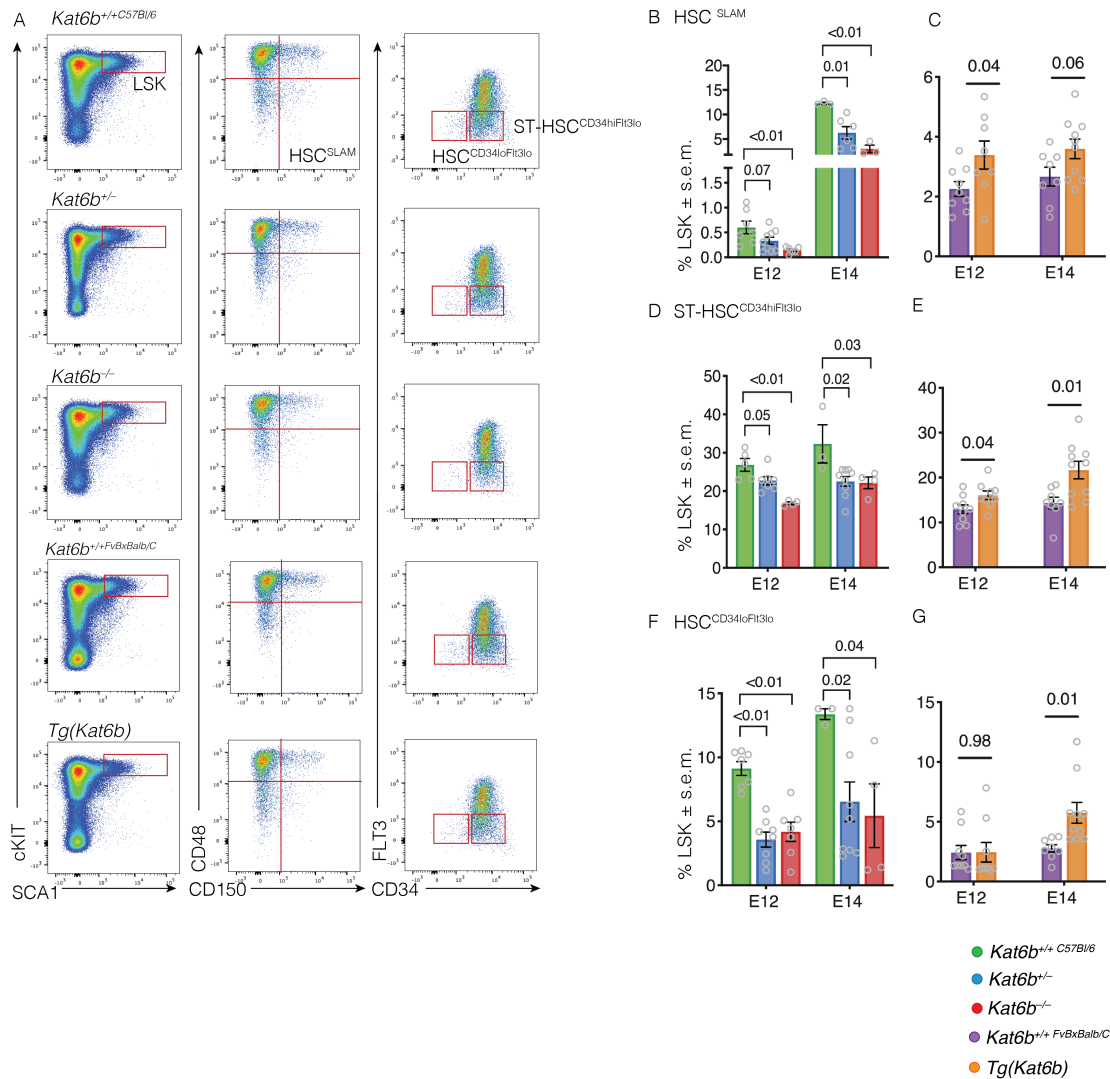


Figure 6.2 Loss and gain of *Kat6b* affects haematopoietic stem and progenitor cell populations in the E12 and E14 embryonic and foetal liver

(A) Representative FACS plots of HSC^{SLAM}, HSC^{CD34^{lo}Flt3^{lo}} and ST-HSC^{CD34^{hi}Flt3^{lo}} populations in E14 foetal livers of *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/-}C57Bl/6, *Kat6b*^{+/-}FvBxBalb/C and *Tg(Kat6b)* samples. HSC populations are gated on LSK cells.

(B) Bar graph of the percentage of HSC^{SLAM} cells within the LSK population in the E12 and E14 livers of *Kat6b*^{-/-}, *Kat6b*^{+/-} and *Kat6b*^{+/-}C57Bl/6 samples. *Kat6b*^{-/-} samples have significantly lower proportions of HSC^{SLAM} cells relative to controls at E12 ($p < 0.01$) and E14 ($p < 0.01$). *Kat6b*^{+/-} samples have significantly lower proportions of HSC^{SLAM} cells relative to controls at E14 ($p = 0.01$) but not at E12 ($p = 0.07$).

(C) Bar graph of the percentage of HSC^{SLAM} cells within the LSK population in the E12 and E14 livers of *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples. *Tg(Kat6b)* samples have significantly more HSC^{SLAM} cells relative to wild type controls at E12 (p = 0.04) but not at E14 (p = 0.06).

(D) Bar graph of the percentage of ST-HSC^{CD34hiFlt3lo} cells within the LSK population in the E12 and E14 livers of *Kat6b*^{-/-}, *Kat6b*^{+/-} and *Kat6b*^{+/+C57Bl/6} samples. *Kat6b*^{-/-} samples have significantly lower proportions of ST-HSC^{CD34hiFlt3lo} cells relative to controls at E12 (p < 0.01) and E14 (p = 0.03). *Kat6b*^{+/-} samples have significantly lower proportions of ST-HSC^{CD34hiFlt3lo} cells relative to controls at E14 (p = 0.02) but not at E12 (p = 0.05).

(E) Bar graph of the percentage of ST-HSC^{CD34hiFlt3lo} cells within the LSK population in the E12 and E14 livers of *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples. *Tg(Kat6b)* samples have significantly more ST-HSC^{CD34hiFlt3lo} cells relative to wild type controls at E12 (p = 0.04) and E14 (p = 0.01).

(F) Bar graph of the percentage of HSC^{CD34loFlt3lo} cells within the LSK population in the E12 and E14 livers of *Kat6b*^{-/-}, *Kat6b*^{+/-} and *Kat6b*^{+/+C57Bl/6} samples. *Kat6b*^{-/-} samples have significantly lower proportions of HSC^{CD34loFlt3lo} cells relative to controls at E12 (p < 0.01) and E14 (p = 0.04). *Kat6b*^{+/-} samples have significantly lower proportions of HSC^{CD34loFlt3lo} cells relative to controls at E12 (p < 0.01) and E14 (p = 0.02).

(G) Bar graph of the percentage of HSC^{CD34loFlt3lo} cells within the LSK population in the E12 and E14 livers of *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples. *Tg(Kat6b)* samples do not have significantly different proportions of HSC^{CD34loFlt3lo} cells relative to wild type controls at E12 (p = 0.98), however have significantly more at E14 (p = 0.01).

For all graphs, *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} samples were analysed using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples were analysed with a two-tailed Student t-test. All bar graphs are displayed as individual data points with mean ± s.e.m., where data points represent individual liver samples. N = 3 – 10 liver samples per genotype and developmental timepoint. Colour code of genotypes indicated below G.

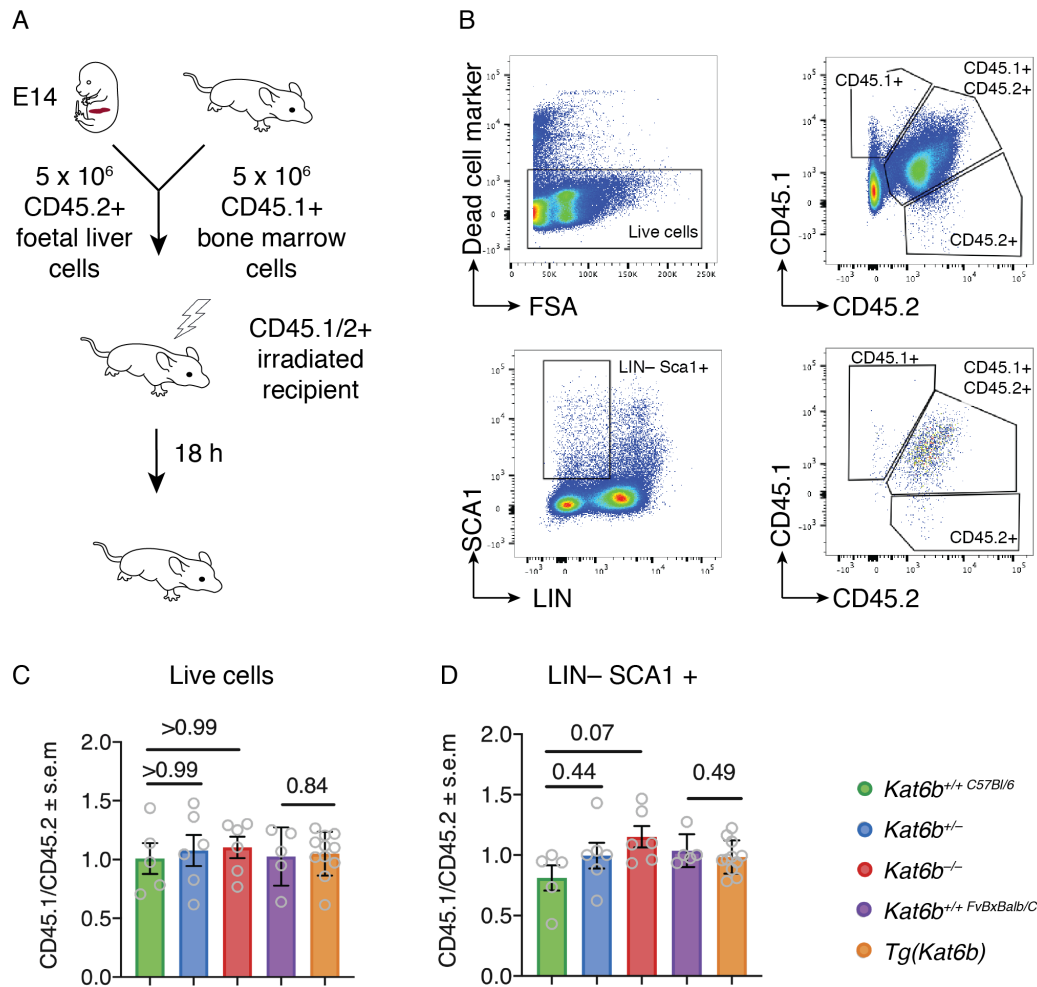


Figure 6.3 Loss and gain of *Kat6b* does not affect haematopoietic stem and progenitor cell homing to the bone marrow

(A) Diagram of transplantation experimental design. 5 x 10⁶ CD45.2+ foetal liver cells were combined with 5 x 10⁶ CD45.1+ adult bone marrow cells and injected into a CD45.1/2+ irradiated recipient. 18 h following injection, recipient bone marrow was collected.

(B) Representative images of the FACS gating strategy on recipient bone marrow. The proportions of CD45.1+ (adult bone marrow), CD45.2+ (foetal liver) and CD45.1/2+ (host-derived) cells were assessed in the total live cells and HSC-enriched SCA1+ LIN- populations.

(C – D) Bar graphs depicting the ratio of CD45.1/CD45.2 in the total live cells and LIN – SCA1+ populations. There is no statistically significant difference in the ratio of CD45.1/CD45.2 between genotypes in either the total live cells or LIN– SCA1+ populations.

Kat6b^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} samples were analysed using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples were analysed using a two-tailed Student's t-test.

Bar graphs are displayed as individual data points with mean $\pm$ s.e.m, where data points represent individual irradiated recipients. N = 5 – 11 foetal liver cells per genotype, injected into 1x irradiated recipient.

LIN – throughout this figure refers to a lack of B220, CD19, GR1, TER119, CD4, CD8 and LYG6. Colour code of genotypes indicated beside (D).

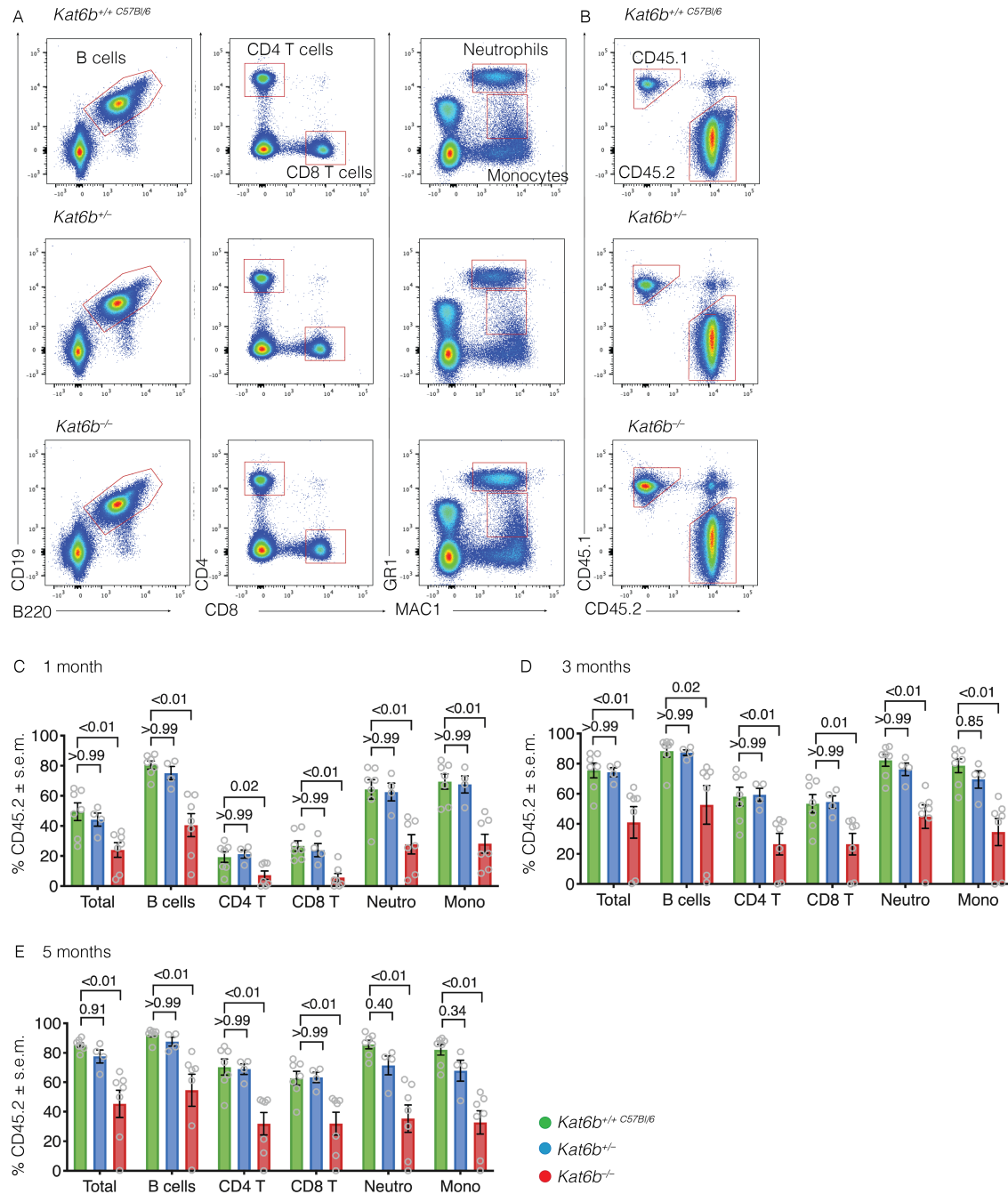


Figure 6.4 Loss of *Kat6b* reduces contribution of foetal liver cells to the production of peripheral blood cells at 1, 3 and 5 months in competitive transplantation assays

(A) Representative FACS plots from *Kat6b*^{+/+}C57Bl/6, *Kat6b*^{+/-} and *Kat6b*^{-/-} recipient peripheral blood at 5 months, showing gating for B cells, CD4 T cells, CD8 T cells, neutrophils and monocytes. All populations are gated on total live cells.

(B) Representative FACS plots from *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} recipient peripheral blood at 5 months, showing gating for CD45.1 vs CD45.2. Plots gated on B cells.

(C) Bar graph representing the percentage of CD45.2+ cells within peripheral blood populations of transplant recipients 1 month after transplantation. *Kat6b*^{-/-} recipients show significantly reduced contribution to all peripheral blood populations, relative to *Kat6b*^{+/+C57Bl/6} controls. *Kat6b*^{+/-} recipients are not statistically different from *Kat6b*^{+/+C57Bl/6} controls for any cell population.

(D) Bar graph representing the percentage of CD45.2+ cells within peripheral blood populations of transplant recipients 3 months after transplantation. *Kat6b*^{-/-} recipients show significantly reduced contribution to all peripheral blood populations, relative to *Kat6b*^{+/+C57Bl/6} controls. *Kat6b*^{+/-} recipients are not statistically different from *Kat6b*^{+/+C57Bl/6} controls for any cell population.

(E) Bar graph representing the percentage of CD45.2+ cells within peripheral blood populations of transplant recipients 5 months after transplantation. *Kat6b*^{-/-} recipients show significantly reduced contribution to all peripheral blood populations, relative to *Kat6b*^{+/+C57Bl/6} controls. *Kat6b*^{+/-} recipients are not statistically different from *Kat6b*^{+/+C57Bl/6} controls for any cell population.

In all bar graphs *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} samples were analysed using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. All bar graphs are displayed as individual data points with mean $\pm$ s.e.m., where data points represent the average of three transplant recipients from a single donor. N = 4 – 7 foetal liver donors per genotype. Colour code of genotypes indicated beside (E).

Neutro: Neutrophil, Mono: Monocyte

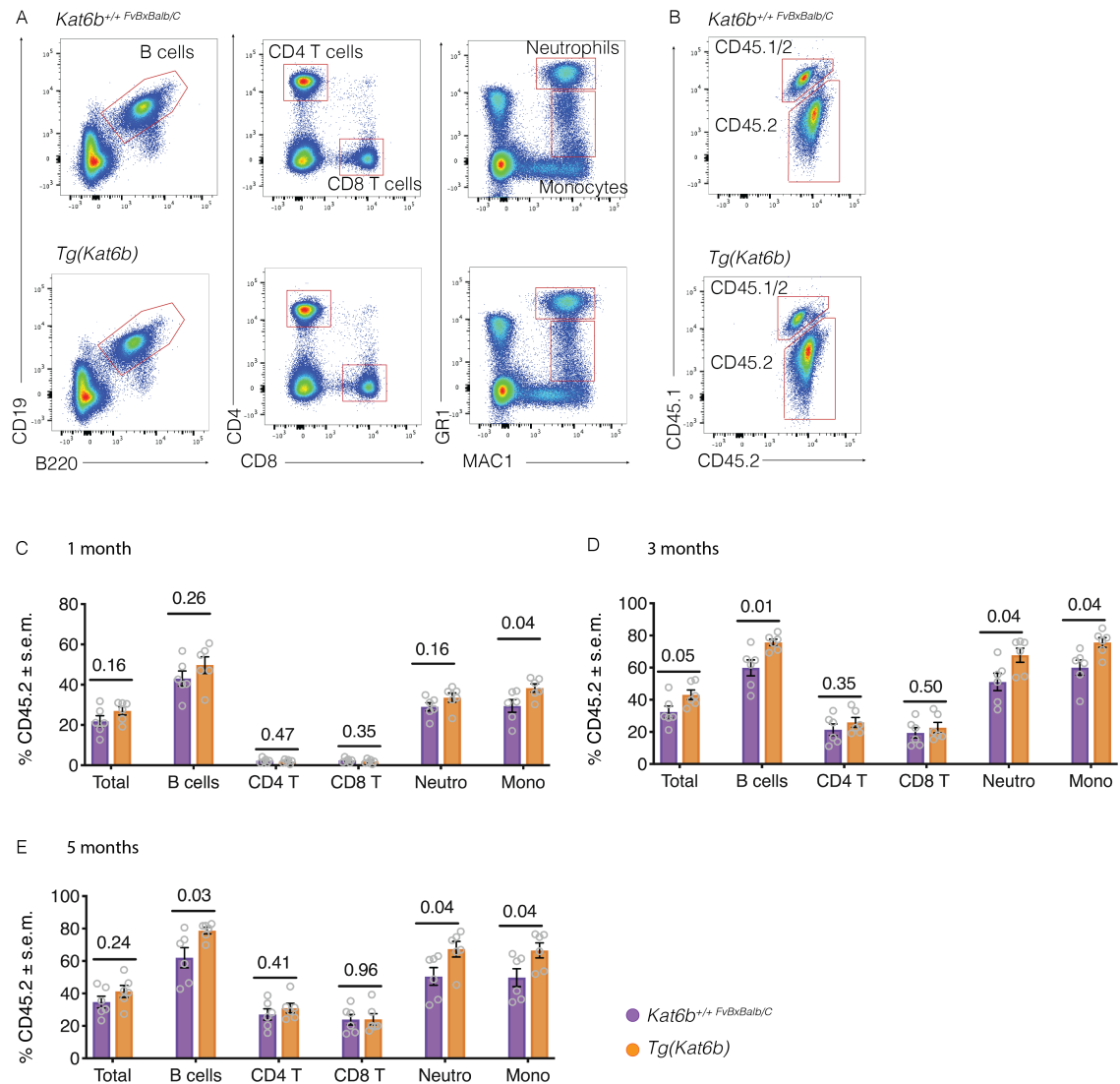


Figure 6.5 *Kat6b* overexpression increases the contribution of foetal liver cells to the production of some peripheral blood cells at 1, 3 and 5 months in competitive transplantation assays

(A) Representative FACS plots from *Kat6b*^{+/+}FvBxBalb/C and *Tg(Kat6b)* recipient peripheral blood at 5 months, showing gating for B cells, CD4 T cells, CD8 T cells, neutrophils and monocytes. All populations are gated on total live cells.

(B) Representative FACS plots from *Kat6b*^{+/+}FvBxBalb/C and *Tg(Kat6b)* recipient peripheral blood at 5 months, showing gating for CD45.1 vs CD45.2. Plots gated on B cells.

(C) Bar graph representing the percentage of CD45.2+ cells within peripheral blood populations of transplant recipients 1 month after transplantation. *Tg(Kat6b)* recipients show significantly increased contribution to peripheral blood monocytes, relative to *Kat6b^{+/+}FvBxBalb/C* controls (p = 0.04), however are not statistically different from controls in any other peripheral blood population.

(D) Bar graph representing the percentage of CD45.2+ cells within peripheral blood populations of transplant recipients 3 months after transplantation. *Tg(Kat6b)* recipients show significantly increased contribution to peripheral blood B cells (p = 0.05), monocytes (p = 0.04) and neutrophils (p = 0.04), relative to *Kat6b^{+/+}FvBxBalb/C* controls, however are not statistically different from controls in CD4 (p = 0.35) or CD8 T cells (p = 0.50).

(E) Bar graph representing the percentage of CD45.2+ cells within peripheral blood populations of transplant recipients 5 months after transplantation. *Tg(Kat6b)* recipients show significantly increased contribution to peripheral blood B cells (p = 0.03), monocytes (p = 0.04) and neutrophils (p = 0.04), relative to *Kat6b^{+/+}FvBxBalb/C* controls, however are not statistically different from controls in CD4 (p = 0.41) or CD8 T cells (p = 0.96).

In all bar graphs *Kat6b^{+/+}FvBxBalb/C* and *Tg(Kat6b)* samples were analysed using a two-tailed Student's t-test. All bar graphs are displayed as individual data points with mean $\pm$ s.e.m., where data points represent the average of three transplant recipients from a single donor. N = 4 – 7 foetal liver donors per genotype. Colour code of genotypes indicated beside (E).

Neutro: Neutrophil, Mono: Monocyte

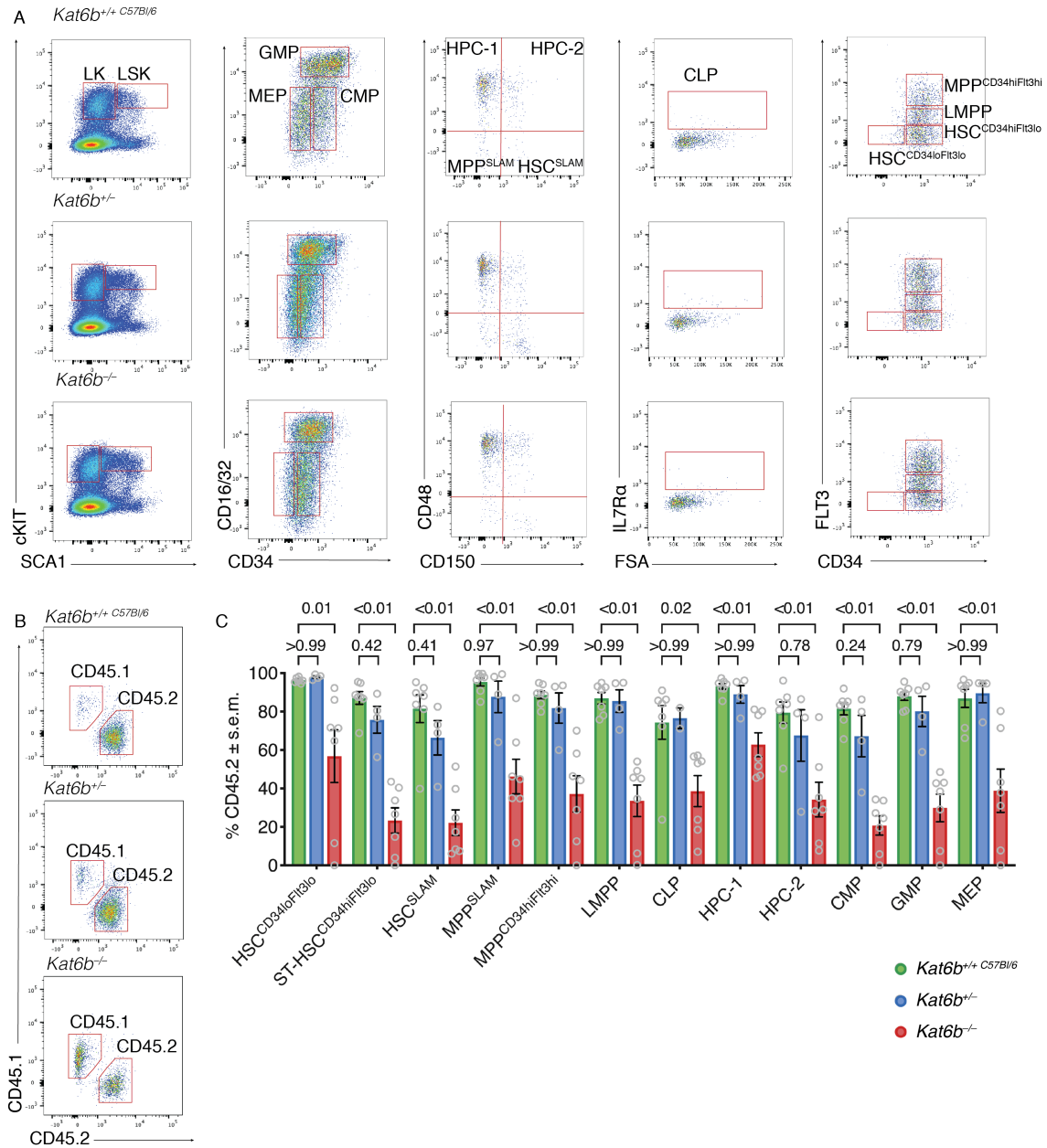


Figure 6.6 Loss of *Kat6b* reduces contribution of foetal liver cells to the production of bone marrow stem and progenitor cells in competitive transplantation assays

(A) Representative FACS plots from *Kat6b*^{+/-}C57Bl/6, *Kat6b*^{+/-} and *Kat6b*^{-/-} recipient bone marrow at 5 months, showing haematopoietic stem and progenitor cell populations. CMP, GMP and MEP populations are gated on LK cells. HPC-1, HPC-2, HSC^{SLAM}, MPP^{SLAM}, MPP^{CD34hiFlt3hi}, LMPP, ST-HSC^{CD34hiFlt3lo} and HSC^{CD34loFlt3lo} are gated on LSK cells.

(B) Representative FACS plots from *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} recipient bone marrow at 5 months showing CD45.1 vs CD45.2. Cells gated on the LSK population.

(C) Bar graph representing the percentage of CD45.2+ cells within bone marrow haematopoietic stem and progenitor cell populations of transplant recipients 5 months after transplantation. *Kat6b*^{-/-} recipients show significantly reduced contribution to all bone marrow stem and progenitor cells, relative to wild type controls. *Kat6b*^{+/-} recipients are not statistically different from wild type controls in any cell population analysed. Colour code of genotypes indicated below (C).

Kat6b^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} samples were analysed using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. Graph is displayed as individual data points with mean $\pm$ s.e.m., where data points represent the average of three transplant recipients from a single donor. N = 4 – 7 foetal liver donors per genotype.

LK: LIN⁻ cKIT⁺; LSK: LIN⁻ cKIT⁺ SCA1⁺; MPP: Multipotent progenitor, LMPP: Lymphoid-primed multipotent progenitor, CLP: Common lymphoid progenitor, HPC-1: Restricted haematopoietic progenitor type 1, HPC-2: Restricted haematopoietic progenitor type 2, CMP: Common myeloid progenitor GMP: Granulocyte, macrophage progenitor, MEP: Megakaryocyte, erythrocyte progenitor.

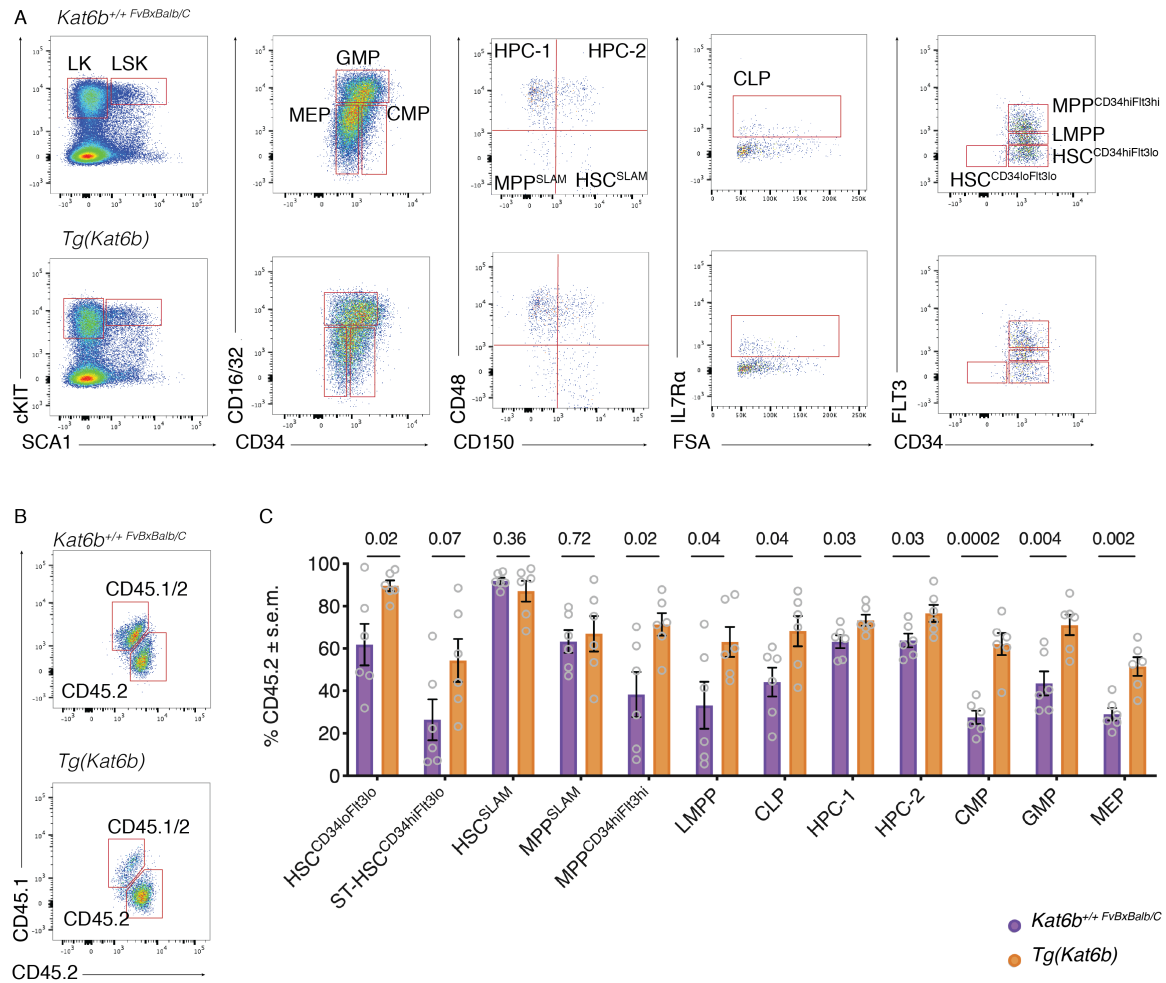


Figure 6.7 *Kat6b* overexpression increases contribution of foetal liver cells to the production of bone marrow stem and progenitor cells in competitive transplantation assays

(A) Representative FACS plots from *Kat6b*^{+/+} *FvBxBalb/C* and *Tg(Kat6b)* recipient bone marrow at 5 months, showing haematopoietic stem and progenitor cell populations. CMP, GMP and MEP populations are gated on LK cells. HPC-1, HPC-2, HSC^{SLAM}, MPP^{SLAM}, MPP^{CD34hiFlt3hi}, LMPP, ST-HSC^{CD34hiFlt3lo} and HSC^{CD34loFlt3lo} are gated on LSK cells.

(B) Representative FACS plots from *Kat6b*^{+/+} *FvBxBalb/C* and *Tg(Kat6b)* recipient bone marrow at 5 months showing CD45.1/2 vs CD45.2. Cells gated on the LSK population.

(C) Bar graph representing the percentage of CD45.2+ cells within bone marrow haematopoietic stem and progenitor cell populations of transplant recipients, 5 months after transplantation. *Tg(Kat6b)* recipients show significantly increased contribution to HSC^{CD34loFlt3lo}.

($p = 0.02$), MPP^{CD34^{hi}Flt3^{hi}} ($p = 0.02$), LMPP ($p = 0.04$), CLP ($p = 0.04$), HPC-1 ($p = 0.03$), HPC-2 ($p = 0.03$), CMP ($p = 0.0002$), GMP ($p = 0.004$) and MEP ($p = 0.002$) populations, relative to wild type controls. The percentage of CD45.2 in ST-HSC^{CD34^{hi}Flt3^{lo}}, HSC^{SLAM} and MPP^{SLAM} populations was not statistically different between genotypes. Colour code of genotypes indicated below (C).

Kat6b^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples were analysed with a two-tailed Student t-test. All bar graphs are displayed as individual data points with mean $\pm$ s.e.m., where data points represent the average of three transplant recipients from a single donor. N = 4 – 7 foetal liver donors per genotype.

LK: LIN[–] cKIT⁺; LSK: LIN[–] cKIT⁺ SCA1⁺; MPP: Multipotent progenitor, LMPP: Lymphoid-primed multipotent progenitor, CLP: Common lymphoid progenitor, HPC-1: Restricted haematopoietic progenitor type 1, HPC-2: Restricted haematopoietic progenitor type 2, CMP: Common myeloid progenitor GMP: Granulocyte, macrophage progenitor, MEP: Megakaryocyte, erythrocyte progenitor.

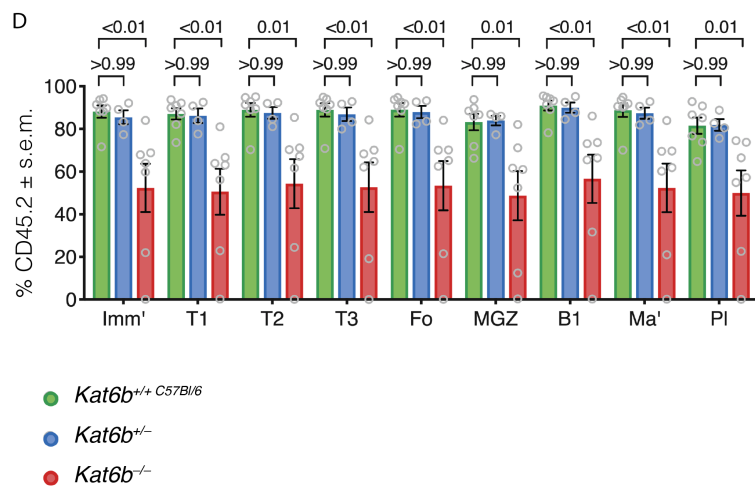
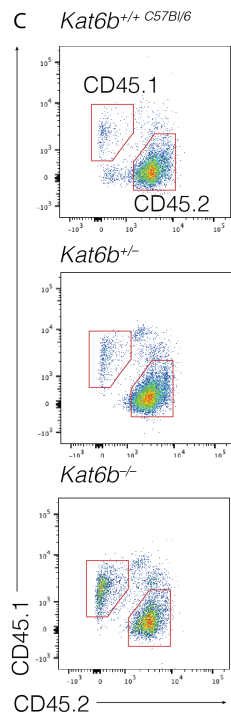
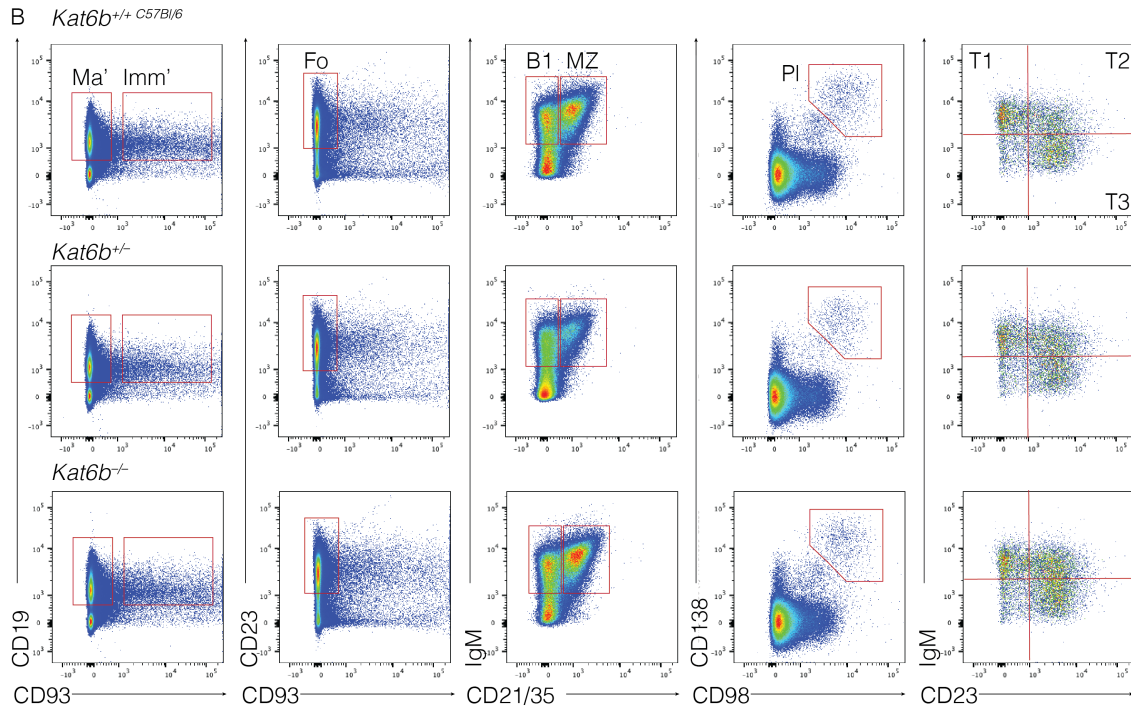
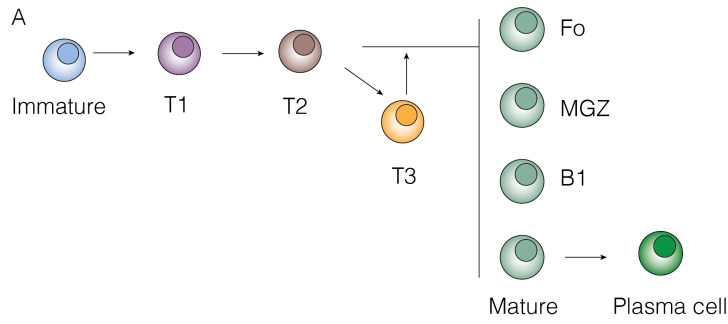


Figure 6.8 Loss of *Kat6b* reduces contribution of foetal liver cells to the production of splenic B cell and progenitor cells in competitive transplantation assays

(A) Simplified diagram displaying B cell maturation in the spleen. Immature B cells enter the spleen and mature through transitional stages T1 – 3, prior to differentiating into Follicular B cells (Fo), Marginal zone B cells (MGZ), B1 or mature B cells, which can subsequently form plasma cells.

(B) Representative FACS plots from *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} recipient spleens at 5 months, showing splenic B cell and progenitor cell populations. Ma' and Imm' B cells gated on total live cells. MZ, B1, Fo, Pl, T1, T2 and T3 cells gated on CD19+ cells.

(C) Representative FACS plots from *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} recipient spleens at 5 months, showing CD45.1 vs CD45.2. Cells gated on the CD19+ cell population.

(D) Bar graph representing the percentage of CD45.2+ cells within splenic B cell and progenitor cell populations of transplant recipients, 5 months after transplantation. *Kat6b*^{-/-} recipients show significantly reduced contribution to all splenic B cell and progenitor cells, relative to wild type controls. *Kat6b*^{+/-} recipients are not statistically different from wild type controls in any cell population analysed.

Kat6b^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} samples were analysed using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. Graph is displayed as individual data points with mean $\pm$ s.e.m., where data points represent the average of three transplant recipients from a single donor. N = 4 – 7 foetal liver donors per genotype. Colour code of genotypes indicated below (D).

Ma': Mature B cells; Imm': Immature B cells; Fo; Follicular B cells; MGZ: Marginal Zone B cells; Pl: Plasma cells; T1: Transitional B cell Type 1; T2: Transitional B cell Type 2; T3: Transitional B cell Type 3.

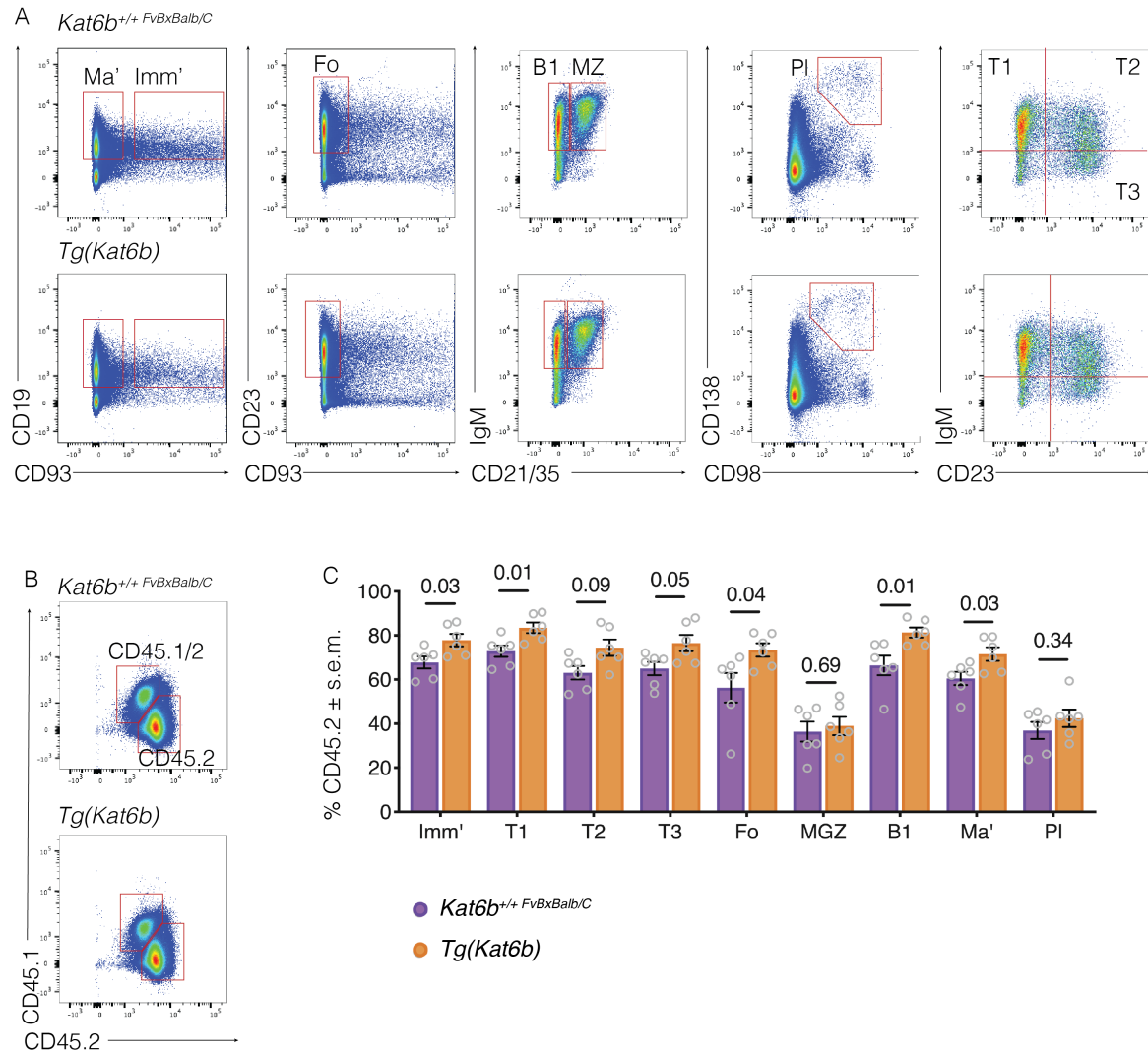


Figure 6.9 *Kat6b* overexpression increases the contribution of foetal liver cells to the production of splenic B cell and progenitor cells in competitive transplantation assays

(A) Representative FACS plots from *Kat6b*^{+/+}FvBxBalb/C and *Tg(Kat6b)* recipient spleens at 5 months, showing splenic B cell and progenitor cell populations. Ma' and Imm' B cells gated on total live cells. MZ, B1, Fo, PI, T1, T2 and T3 cells gated on CD19+ cells.

(B) Representative FACS plots from *Kat6b*^{+/+}FvBxBalb/C and *Tg(Kat6b)* recipient spleens at 5 months showing CD45.1/2 vs CD45.2. Cells gated on the CD19+ cell population.

(C) Bar graph representing the percentage of CD45.2+ cells within splenic B cell and progenitor cell populations of transplant recipients 5 months after transplantation. *Tg(Kat6b)* recipients show significantly increased contribution to Imm' (p = 0.03), T1 (p = 0.01), Fo (p = 0.04), B1 (p = 0.01) and Ma' (p = 0.03) populations, relative to controls, however are statistically insignificant from controls for T2, T3, MGZ and Pl cell populations.

Kat6b^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples were analysed using a two-tailed Student's t-test. All bar graphs are displayed as individual data points with mean $\pm$ s.e.m., where data points represent the average of three transplant recipients from a single donor. N = 4 – 7 foetal liver donors per genotype. Colour code of genotypes indicated below (C).

Ma': Mature B cells; Imm': Immature B cells; Fo; Follicular B cells; MGZ: Marginal Zone B cells; Pl: Plasma cells; T1: Transitional B cell Type 1; T2: Transitional B cell Type 2; T3: Transitional B cell Type 3.

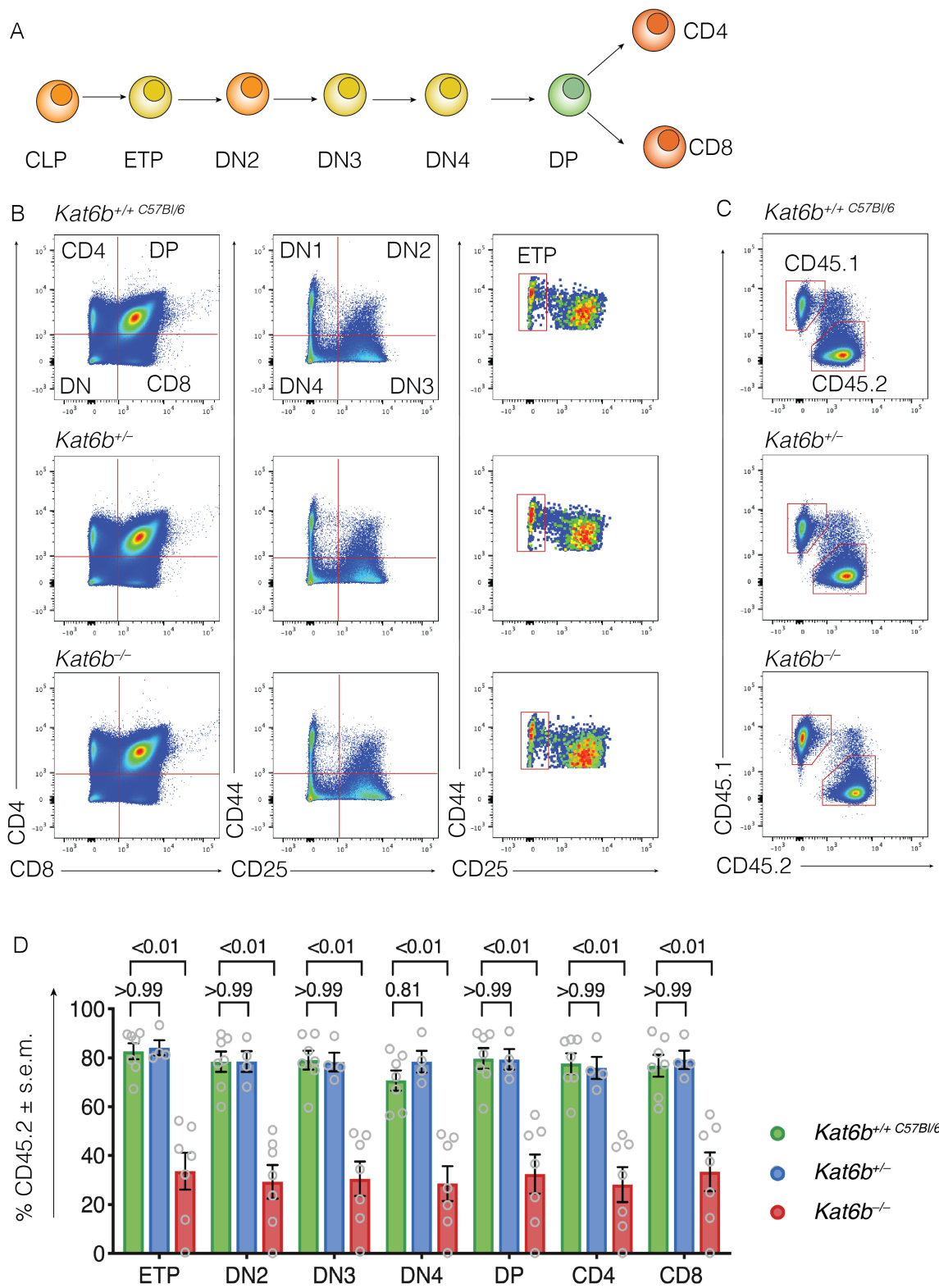


Figure 6.10 Loss of *Kat6b* reduces contribution of foetal liver cells to the production of thymic T cell and progenitor cells in competitive transplantation assays

(A) Diagram of T cell maturation in the thymus. CLPs enter the thymus and mature through transitional stages ETP, DN1, DN2, DN3, DN4 and DP, prior to differentiating into CD4 or CD8 mature T cells.

(B) Representative FACS plots from *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} recipient thymi at 5 months, showing thymic T cell and progenitor cell populations. DN, CD4, DP and CD8 populations are gated on live cells. DN1, DN2, DN3 and DN4 are gated on DN. ETP are gated on cKIT⁺ CD44⁺ cells.

(C) Representative FACS plots from *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} recipient thymi at 5 months, showing CD45.1 vs CD45.2. Cells gated on the CD4 population.

(D) Bar graph representing the percentage of CD45.2⁺ cells within thymic T cell and progenitor cell populations of transplant recipients 5 months after transplantation. *Kat6b*^{-/-} recipients show significantly reduced contribution to all thymic T cell and progenitor cells, relative to wild type controls. *Kat6b*^{+/-} recipients are not statistically different from wild type controls in any cell population analysed.

Kat6b^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} samples were analysed using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. Graph is displayed as individual data points with mean $\pm$ s.e.m., where data points represent the average of three transplant recipients from a single donor. N = 4 – 7 foetal liver donors per genotype. Colour code of genotypes indicated beside (D).

CLP: Common Lymphoid Progenitor, DN: Double negative; DP: Double positive

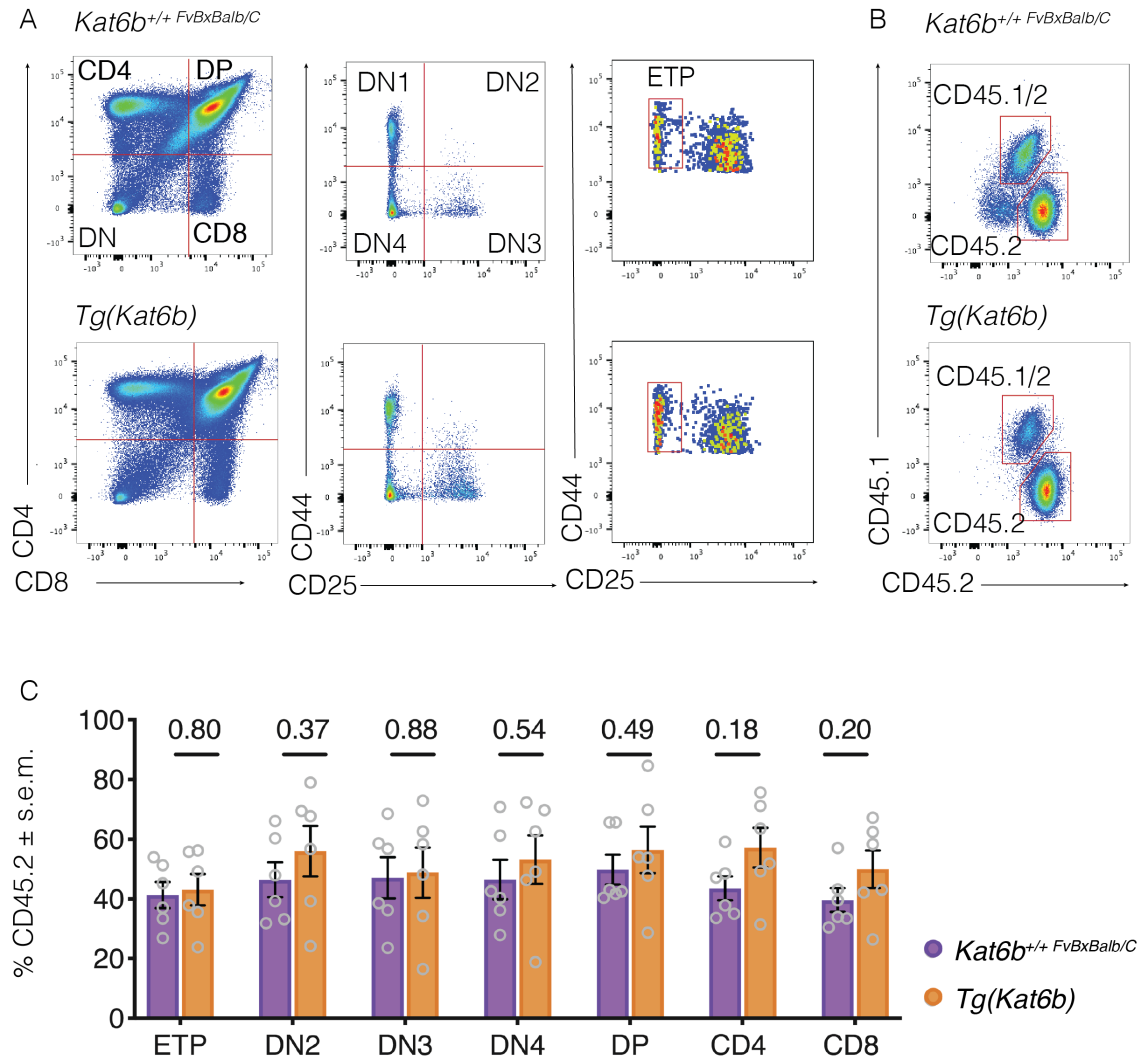


Figure 6.11 *Kat6b* overexpression does not affect the contribution of foetal liver cells to the production of thymic T cell and progenitor cells in competitive transplantation assays

(A) Representative FACS plots from *Kat6b*^{+/+}*FvBxBalb/C* and *Tg(Kat6b)* recipient thymi at 5 months, showing thymic T cell and progenitor cell populations. DN, CD4, DP and CD8 populations are gated on live cells. DN1, DN2, DN3 and DN4 are gated on DN. ETP are gated on cKIT⁺ CD44⁺ cells.

(B) Representative FACS plots from *Kat6b*^{+/+}*FvBxBalb/C* and *Tg(Kat6b)* recipient thymi at 5 months, showing CD45.1/2 vs CD45.2. Cells gated on the CD4 population.

(C) Bar graph representing the percentage of CD45.2+ cells within thymic T cell and progenitor cell populations of transplant recipients 5 months after transplantation. *Tg(Kat6b)* recipients do not show significantly different contribution to any thymic T cell and progenitor cells, relative to wild type controls.

Kat6b^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples were analysed with a two-tailed Student's t-test. All bar graphs are displayed as individual data points with mean $\pm$ s.e.m., where data points represent the average of three transplant recipients from a single donor. N = 4 – 7 foetal liver donors per genotype. Colour code of genotypes indicated beside (C).

CLP: Common Lymphoid Progenitor, DN: Double negative; DP: Double positive

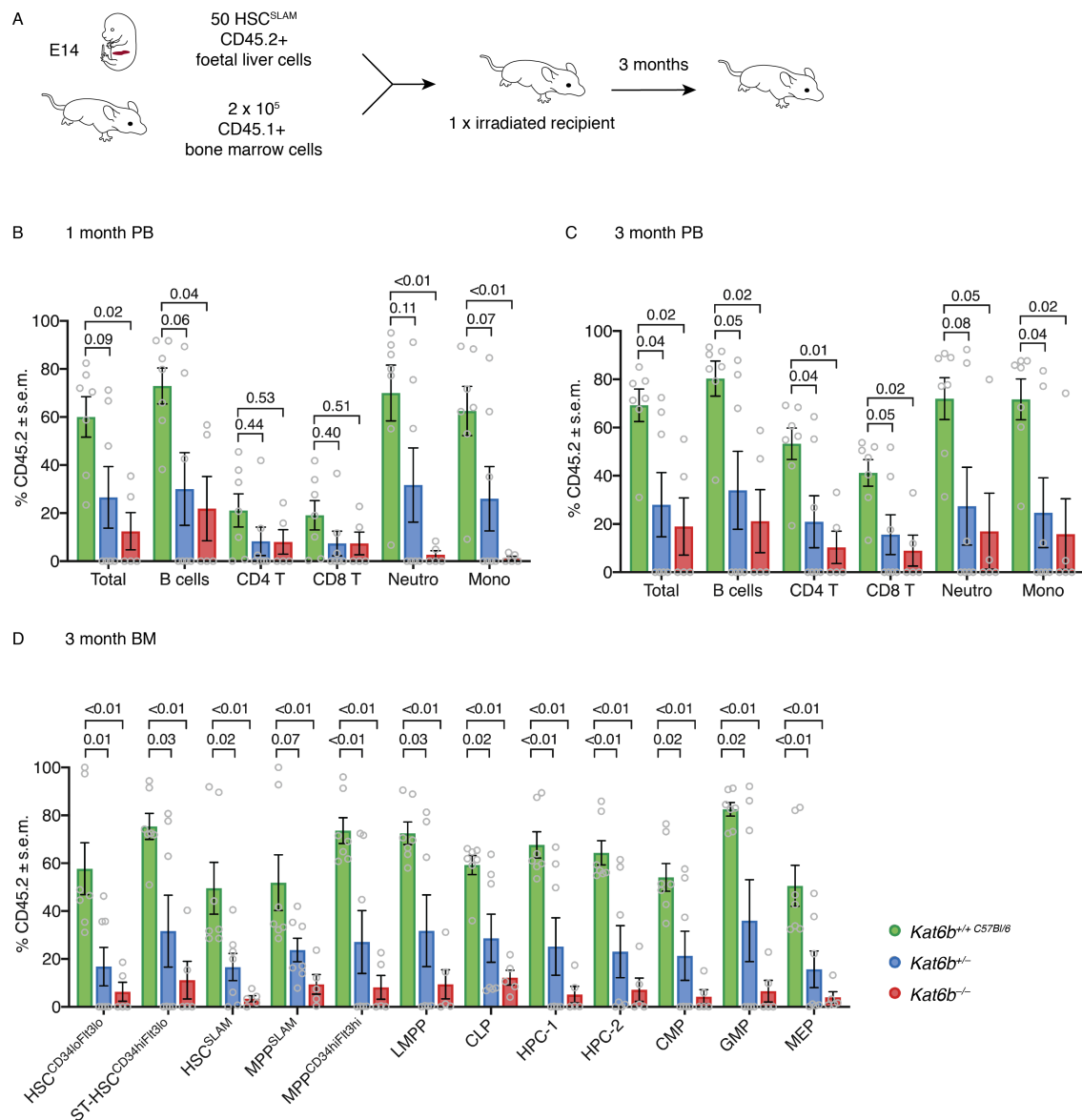


Figure 6.12 Sorted *Kat6b*^{+/+} and *Kat6b*^{-/-} HSC^{SLAM} cells show impaired multi-lineage reconstitution in the peripheral blood and bone marrow at 1 and 3 months after transplantation

(A) Diagram displaying the experimental design for the transplantation. 50 HSC^{SLAM} CD45.2+ cells from *Kat6b*^{+/+}C57Bl/6, *Kat6b*^{+/+} or *Kat6b*^{-/-} E14 foetal livers were combined with 2 x 10⁵ CD45.1+ adult bone marrow cells and injected into 1x irradiated recipient. The proportion of CD45.2 was assessed in the peripheral blood at 1 and 3 months after transplantation and in the bone marrow at 3 months.

(B) Bar graph depicting the percentage of CD45.2+ cells in peripheral blood cell populations at 1 month after transplantation. Relative to wild type recipients, *Kat6b*^{-/-} foetal liver cells show a significantly reduced contribution to total live cells (p = 0.02), B cells (p = 0.04), neutrophils (p < 0.01) and monocytes (p < 0.01). There is no significant difference between *Kat6b*^{-/-} and wild type cell contributions to peripheral blood CD4 T cells (p = 0.53) or CD8 T cells (p = 0.51). *Kat6b*^{+/-} recipients are not statistically significant from wild type recipients in any cell population.

(C) Bar graph depicting the proportion of CD45.2+ cells in peripheral blood cell populations at 3 months after transplantation. Relative to wild type recipients, *Kat6b*^{-/-} foetal liver cells show a significantly reduced contribution to total live cells (p = 0.02), B cells (p = 0.02), CD4 T cells (p = 0.01), CD8 T cells (p = 0.02), neutrophils (p = 0.05) and monocytes (p = 0.02). *Kat6b*^{+/-} cells show a significantly reduced contribution to total live cells (p = 0.04), B cells (p = 0.05), CD4 T cells (p = 0.04), CD8 T cells (p = 0.05) and monocytes (p = 0.04), but not neutrophils (p = 0.08).

(D) Bar graph depicting the proportion of CD45.2+ cells in bone marrow stem and progenitor cell populations at 3 months after transplantation. Both *Kat6b*^{+/-} and *Kat6b*^{-/-} recipients show a significant reduction in the contribution to all bone marrow stem and progenitor cells, relative to wild type recipients.

In all bar graphs, *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} samples were analysed using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. Graphs are displayed as individual data points with mean ± s.e.m., where data points represent one transplant recipients from a single donor. N = 4 – 7 foetal liver donors per genotype. Colour code of genotypes indicated beside (D).

PB: Peripheral blood, BM: Bone marrow, Neutro: Neutrophil, Mono: Monocyte, HSC: Haematopoietic stem cell, MPP: Multipotent progenitor cell, LMPP: Lymphoid-primed multipotent progenitor, CLP: Common lymphoid progenitor, HPC-1: Restricted haematopoietic progenitor type 1, HPC-2: Restricted haematopoietic progenitor type 2, CMP: Common myeloid progenitor, GMP: Granulocyte, macrophage progenitor, MEP: Megakaryocyte, erythrocyte progenitor.

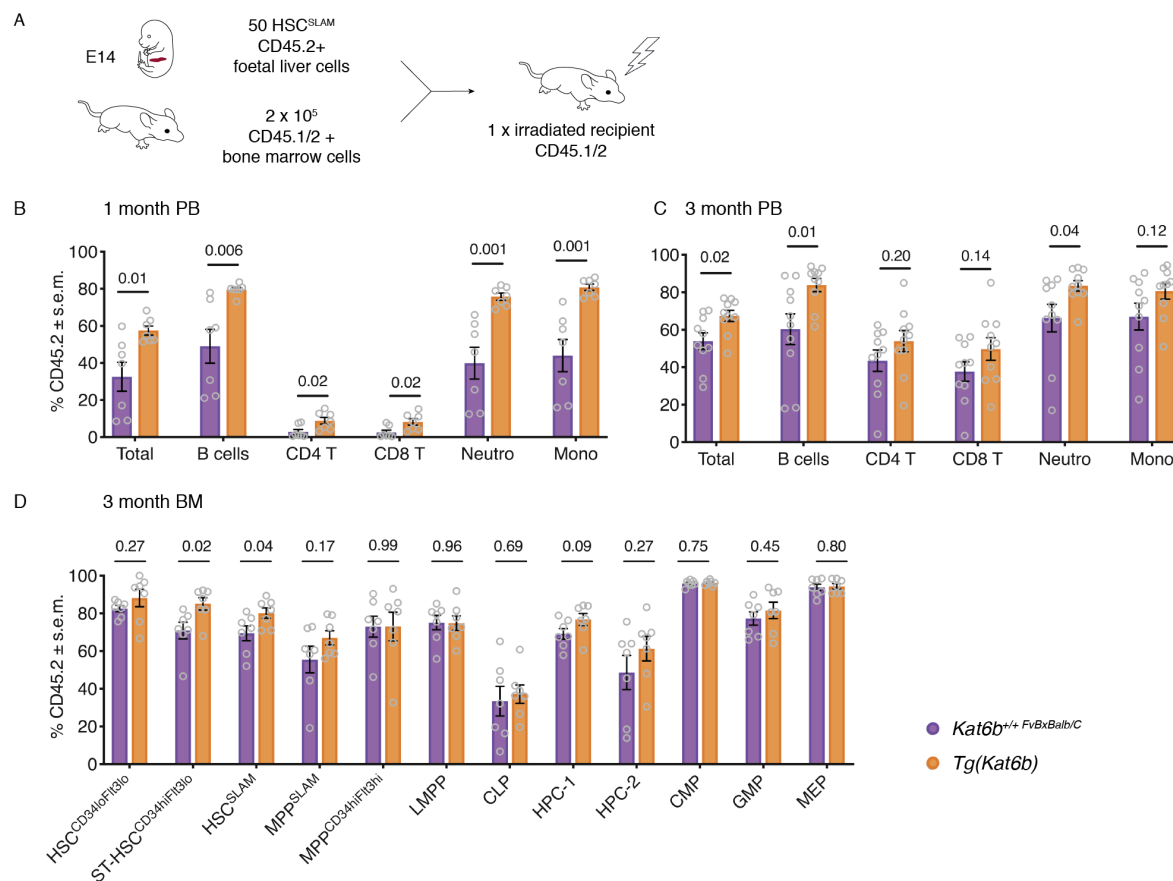


Figure 6.13 Sorted *Tg(Kat6b)* HSC^{SLAM} cells show enhanced multi-lineage reconstitution in peripheral blood B cells and myeloid cells and bone marrow HSC^{CD34hiFlt3lo} and HSC^{SLAM} populations at 1 and 3 months after transplantation

(A) Diagram displaying the experimental design for the transplantation. 50 HSC^{SLAM} CD45.2+ cells from *Kat6b*^{+/+}*FvBxBalb/C* or *Tg(Kat6b)* E14 foetal livers were combined with 2 x 10⁵ CD45.1/2 adult bone marrow cells and injected into 1x irradiated recipient. The proportion of CD45.2 was assessed in the peripheral blood at 1 and 3 months after transplantation and in the bone marrow at 3 months.

(B) Bar graph depicting the percentage of CD45.2+ cells in peripheral blood cell populations at 1 month after transplantation. *Tg(Kat6b)* recipients show a significantly larger percentage of CD45.2+ cells in peripheral blood total live cells ($p = 0.01$), B cells ($p = 0.006$), CD4 T cells ($p = 0.02$), CD8 T cells ($p = 0.02$), neutrophils ($p = 0.001$) and monocytes ($p = 0.001$), relative to *Kat6b*^{+/+}*FvBxBalb/C* recipients.

(C) Bar graph depicting the percentage of CD45.2+ cells in peripheral blood cell populations at 3 months after transplantation. *Tg(Kat6b)* recipients show a significantly larger proportion of CD45.2+ cells in peripheral blood total live cells ($p = 0.02$), B cells ($p = 0.01$) and neutrophils ($p = 0.04$), but are no longer significantly different from wild type recipients for CD4 T cells ($p = 0.20$), CD8 T cells ($p = 0.14$) or monocytes ($p = 0.12$), relative to *Kat6b*^{+/+FvBxBalb/C} recipients.

(D) Bar graph depicting the percentage of CD45.2+ cells in bone marrow stem and progenitor cell populations at 3 months after transplantation. *Tg(Kat6b)* recipients show a significantly larger percentage of CD45.2+ cells in ST-HSC^{CD34^{hi}Flt3^{lo}} ($p = 0.02$) and HSC^{SLAM} ($p = 0.04$) populations, relative to *Kat6b*^{+/+FvBxBalb/C} recipients. The percentage of CD45.2 is not statistically significant between genotypes in any other population. Colour code of genotypes indicated beside (D).

Data in all bar graphs were analysed using a two-tailed Student's t-test. All bar graphs are displayed as individual data points with mean $\pm$ s.e.m., where data points represent a single transplant recipient from a single donor. N = sorted HSC^{SLAM} cells from 10 E14 foetal livers per genotype, each injected into 1x irradiated recipient. Peripheral blood cells populations gated as shown in Fig 6.5. Bone marrow stem and progenitor cell populations gated as shown in Fig 6.7.

PB: Peripheral blood, BM: Bone marrow, Neutro: Neutrophil, Mono: Monocyte, HSC: Haematopoietic stem cell, MPP: Multipotent progenitor cell, LMPP: Lymphoid-primed multipotent progenitor, CLP: Common lymphoid progenitor, HPC-1: Restricted haematopoietic progenitor type 1, HPC-2: Restricted haematopoietic progenitor type 2, CMP: Common myeloid progenitor GMP: Granulocyte, macrophage progenitor, MEP: Megakaryocyte, erythrocyte progenitor.

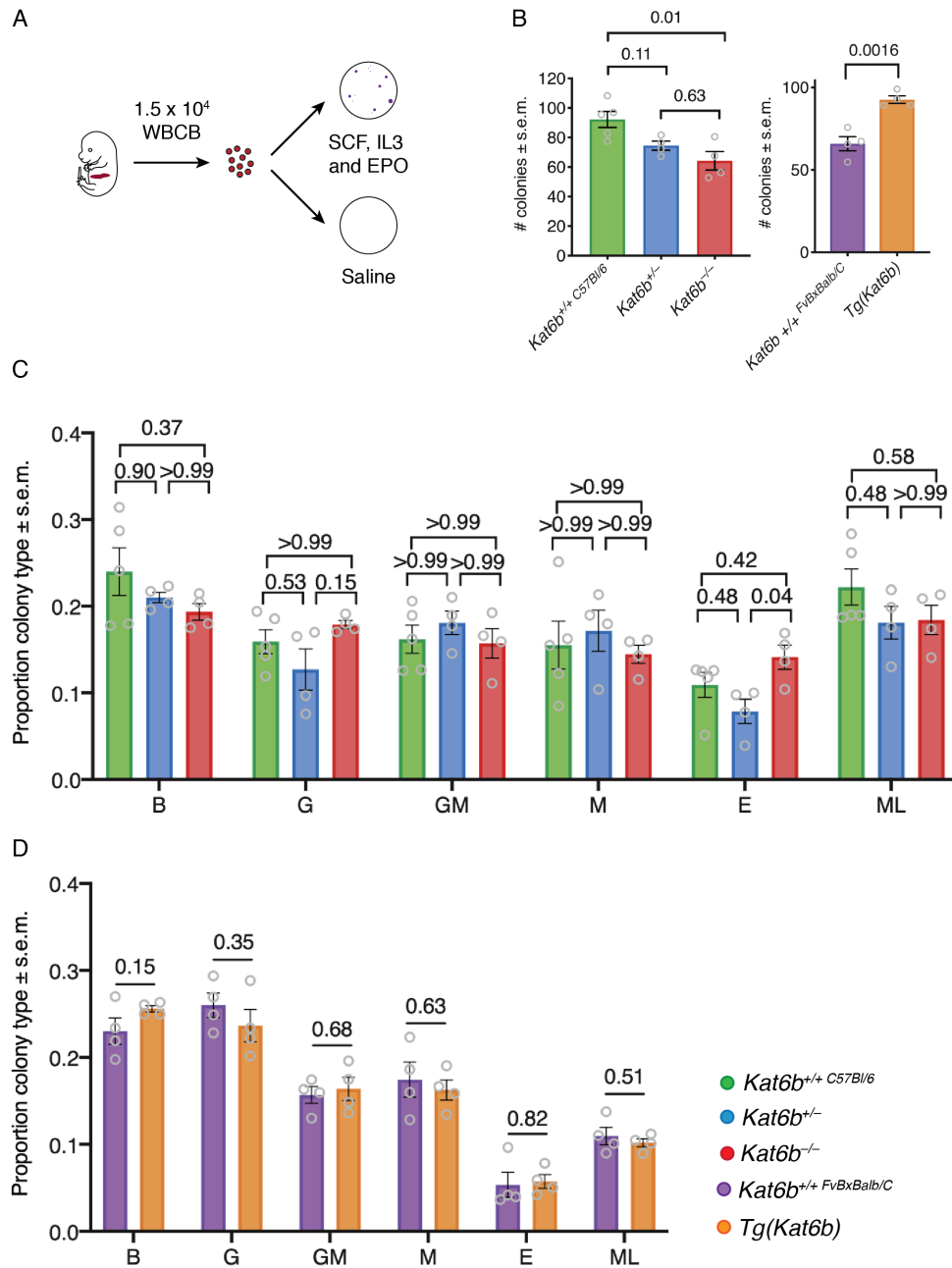


Figure 6.14 Loss of *Kat6b* reduces progenitor cell proliferation *in vitro* and increases the proportion of erythroid colonies

(A) Diagram displaying experimental design for colony forming assay. 1.5 x 10⁴ WBCB cells from E14 *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+}C57Bl/6, *Kat6b*^{FvbxBalb/C} and *Tg(Kat6b)* foetal livers were grown in the presence of SCF, IL3 and EPO to induce proliferation and differentiation, or in saline-only as a negative control.

(B) Bar graph depicting the number of colonies formed per sample. *Kat6b*^{-/-} foetal liver cells formed fewer colonies than wild type controls ($p = 0.01$) but were not significantly different from *Kat6b*^{+/-} cells ($p = 0.63$). The number of colonies formed from *Kat6b*^{+/-} foetal liver cells was not significantly different from wild type cells ($p = 0.11$). More colonies were formed from *Tg(Kat6b)* cells, relative to *Kat6b*^{+/+FvBxBalb/C} cells ($p = 0.0016$). No colonies were observed in saline-only negative controls (data not shown).

(C) Bar graph depicting the proportion of each colony type in *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+C57Bl/6} samples. *Kat6b*^{-/-} samples formed proportionally more erythroid colonies than *Kat6b*^{+/-} samples ($p = 0.04$). The proportion of no other colony type was significantly different between genotypes. *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} samples were analysed using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. Graph is displayed as individual data points with mean $\pm$ s.e.m., where data points represent colonies derived from a single foetal liver sample. $N = 4 - 5$ samples per genotype

(D) Bar graph depicting the proportion of each colony type in *Kat6b*^{FvbxBalb/C} and *Tg(Kat6b)* samples. The proportion of no colony type was significantly different between genotypes. *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples were analysed using a two-tailed Student's t-test. Graph is displayed as individual data points with mean $\pm$ s.e.m., where data points represent colonies derived from a single foetal liver sample. $N = 4$ samples per genotype. Colour code of genotypes indicated below (D).

WBCB: White blood cell count; B: Blast colony; G : Granulocyte colony; GM : granulocyte/macrophage colony; M : megakaryocyte colony; E : erythroid colony; ML : mixed lineage colony

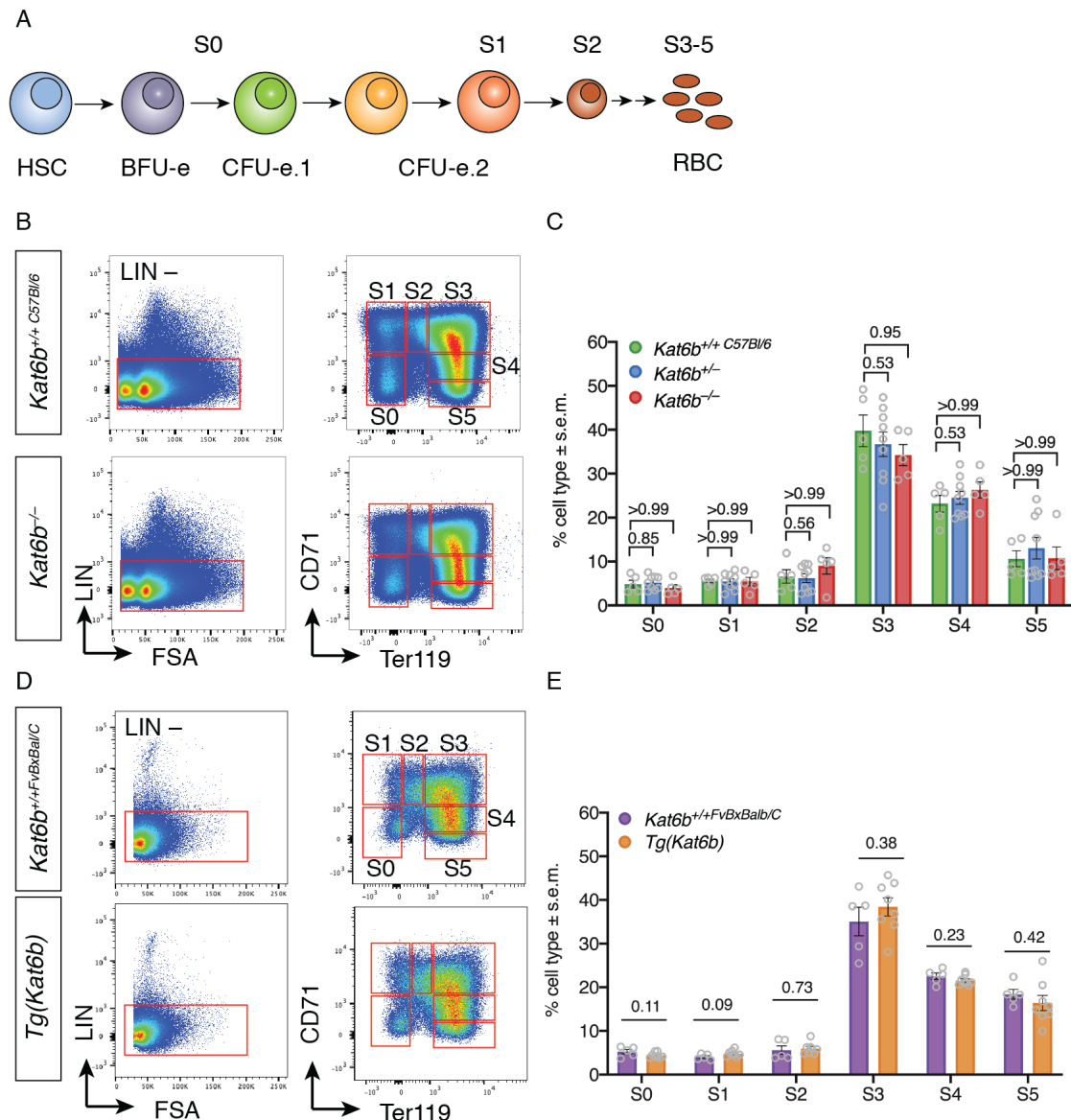


Figure 6.15 Loss or gain of *Kat6b* does not affect erythroid development in the foetal liver

(A) Diagram displaying erythroid development. HSCs goes through progressive maturation stages (S0 – 5), prior to generating mature red blood cells (RBCs).

(B) Representative FACS gating for *Kat6b*^{-/-} and *Kat6b*^{+/+}*C57Bl/6* E14 foetal liver samples. LIN- cells were gated on total live cells. S0 – S5 stages were gated on the LIN- population.

(C) Bar graph depicting the percentage of LIN- cells within S0 – S5 stages for *Kat6b*^{-/-}, *Kat6b*^{+/-} and *Kat6b*^{+/+}*C57Bl/6* samples. There is no statistically significant difference between

genotypes for any cell population. *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} samples were analysed using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. Graph is displayed as individual data points with mean $\pm$ s.e.m., where data points represent a single foetal liver sample. N = 5 – 9 samples per genotype. Genotype colour code is displayed in the top left hand corner.

(D) Representative FACS gating for *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples. LIN⁻ cells were gated on total live cells. S0 – S5 stages were gated on the LIN⁻ population.

(E) Bar graph depicting the percentage of LIN⁻ cells within S0 – S5 stages for *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples. There is no statistically significant difference between genotypes for any cell population. *Kat6b*^{+/+FvBxBalb/C} and *Tg(Kat6b)* samples were analysed using a two-tailed Student's t-test. Graph is displayed as individual data points with mean $\pm$ s.e.m., where data points represent a single foetal liver sample. N = 5 – 8 samples per genotype. Genotype colour code is displayed in the top left hand corner.

HSC = Haematopoietic stem cells; BFU-e = Burst-forming unit-erythroid; CFU-e.1 Colony forming unit-erythroid 1; CFU-e.2 = Colony forming unit-erythroid 2; LIN : lineage (B220, CD19, CD4, CD8, LyG6 and Gr1); FSA : Forward Scatter Area; RBC: Red Blood Cell

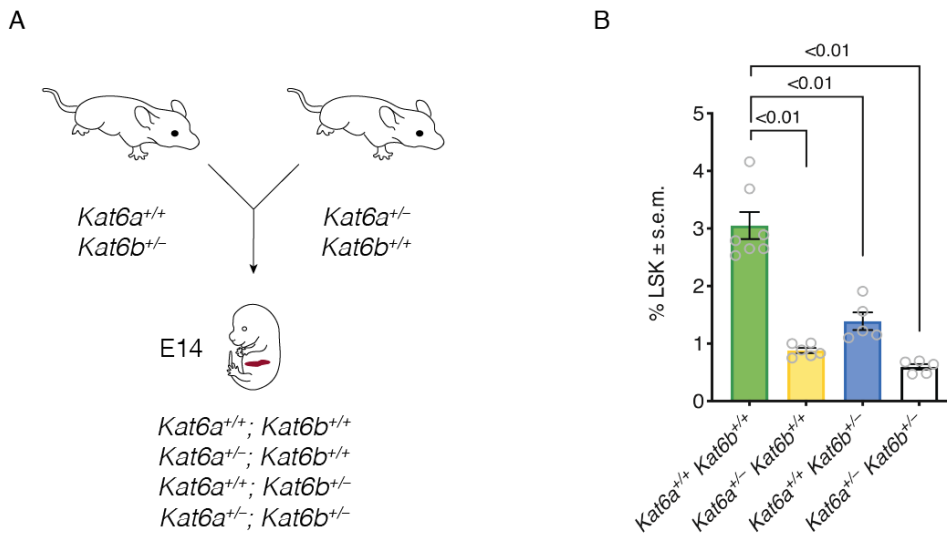


Figure 6.16 *Kat6a*^{+/-}; *Kat6b*^{+/-} double heterozygosity reduces the percentage of HSCs in the foetal liver compared to wild type or *Kat6b*^{+/-} single heterozygous samples

(A) Diagram displaying breeding strategy to obtain mutant and control E14 embryos. *Kat6a*^{+/-}; *Kat6b*^{+/-} double heterozygous animals were intercrossed to yield *Kat6a*^{+/+}; *Kat6b*^{+/+}, *Kat6a*^{+/-}; *Kat6b*^{+/+}, *Kat6a*^{+/+}; *Kat6b*^{+/-} and *Kat6a*^{+/-}; *Kat6b*^{+/-} fetuses.

(B) Bar graph depicting the percentage of HSC^{SLAM} cells within the LSK population of E14 foetal livers from *Kat6a*^{+/+}; *Kat6b*^{+/+}, *Kat6a*^{+/-}; *Kat6b*^{+/+}, *Kat6a*^{+/+}; *Kat6b*^{+/-} and *Kat6a*^{+/-}; *Kat6b*^{+/-} foetal livers. *Kat6a*^{+/-}; *Kat6b*^{+/+} foetal livers have a significantly lower proportion of HSC^{SLAM} cells relative to wild type controls ($p < 0.01$). *Kat6a*^{+/+}; *Kat6b*^{+/-} have a significantly lower proportion of HSC^{SLAM} cells relative to wild type controls ($p < 0.01$). *Kat6a*^{+/-}; *Kat6b*^{+/-} have a significantly lower percentage of HSC^{SLAM} cells relative to wild type controls ($p < 0.01$).

Samples compared to wild type controls using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. Graph is displayed as individual data points with mean ± s.e.m., where data points represent a single foetal liver sample. $N = 3 - 5$ foetal liver samples per genotype.

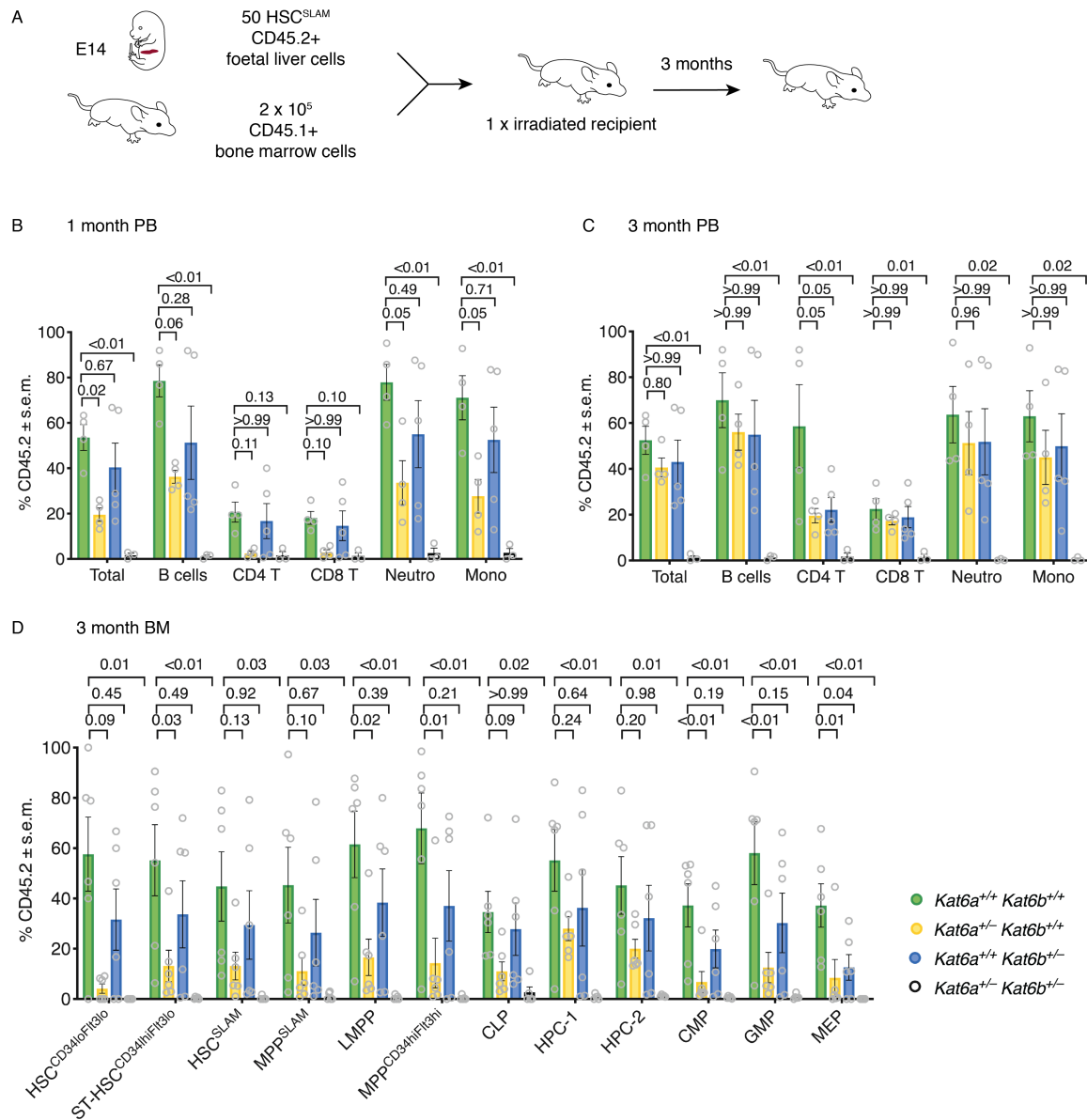


Figure 6.17 *Kat6a*^{+/-};*Kat6b*^{+/-} double heterozygosity severely impairs HSC reconstitution in a competitive transplant

(A) Diagram of transplantation experimental design. 50 sorted CD45.2⁺ HSC^{SLAM} cells from E14 foetal livers of *Kat6a*^{+/+}; *Kat6b*^{+/+}, *Kat6a*^{+/-}; *Kat6b*^{+/+}, *Kat6a*^{+/+}; *Kat6b*^{+/-} and *Kat6a*^{+/-}; *Kat6b*^{+/-} fetuses were combined with 2 x 10⁵ CD45.1⁺ adult bone marrow cells and injected into an irradiated recipient. The contribution of each starting population to the recipient reconstituting system was assessed in the peripheral blood at 1 month and in the peripheral blood and bone marrow at 3 months.

(B) Bar graph depicting the contribution of foetal liver HSC and adult bone marrow cells to irradiated recipient peripheral blood cell populations 1 month post-transplant. *Kat6a*^{+/-}; *Kat6b*^{+/-} recipients show significantly reduced contribution to total live cells ($p < 0.01$), B cells ($p < 0.01$), neutrophils ($p < 0.01$) and monocytes ($p < 0.01$), but not for CD4 T cells ($p = 0.13$) or CD8 T cells ($p = 0.10$). *Kat6a*^{+/-}; *Kat6b*^{+/+} recipients show a significantly reduced contribution to total live cells (0.02), neutrophils ($p = 0.05$) and monocytes ($p = 0.05$) but no other cell population. *Kat6a*^{+/+}; *Kat6b*^{+/-} recipients are not significantly different from *Kat6a*^{+/+}; *Kat6b*^{+/+} recipients at 1 month post-transplant.

(C) Bar graph depicting the contribution of foetal liver HSC and adult bone marrow cells to irradiated recipient peripheral blood cell populations 3 months post-transplant. *Kat6a*^{+/-}; *Kat6b*^{+/-} recipients show significantly reduced contribution to total live cells ($p < 0.01$), B cells ($p < 0.01$), CD4 T cells ($p < 0.01$), CD8 T cells ($p = 0.01$), neutrophils ($p = 0.02$) and monocytes ($p = 0.02$). *Kat6a*^{+/-}; *Kat6b*^{+/+} recipients show significantly reduced contribution to CD4 T cells ($p = 0.05$) but no other population. *Kat6a*^{+/+}; *Kat6b*^{+/-} recipients show significantly reduced contribution to CD4 T cells ($p = 0.05$) but no other population.

(D) Bar graph depicting the contribution of foetal liver HSC and adult bone marrow cells to irradiated recipient bone marrow stem and progenitor cell populations 3 months post-transplant. *Kat6a*^{+/-}; *Kat6b*^{+/-} recipients show significantly reduced contribution to all bone marrow cell populations analysed. *Kat6a*^{+/-}; *Kat6b* recipients show significantly reduced contribution to ST-HSC^{CD34hiFlt3lo} ($p = 0.03$), LMPP ($p = 0.02$), MPP^{CD34hiFlt3hi} ($p = 0.01$), CMP ($p < 0.01$), GMP ($p < 0.01$) and MEP ($p = 0.01$) populations. *Kat6a*^{+/+}; *Kat6b*^{+/-} recipients show significantly reduced contribution to MEP cells ($p = 0.04$), but no other population analysed.

Data in all bar graphs were analysed using a one-way ANOVA, followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. All bar graphs are displayed as individual data points with mean $\pm$ s.e.m. where data points represent one transplant recipient from a single donor. N = 4 – 7 foetal liver donors per genotype. Peripheral blood cell populations gated as shown in Fig 6.4. Bone marrow populations gated as shown in Fig 6.6.

PB: Peripheral blood, BM: Bone marrow, Neutro: Neutrophil, Mono: Monocyte, HSC: Haematopoietic stem cell, MPP: Multipotent progenitor cell, LMPP: Lymphoid-primed multipotent progenitor, CLP: Common lymphoid progenitor, HPC-1: Restricted haematopoietic progenitor type 1, HPC-2: Restricted haematopoietic progenitor type 2, CMP: Common myeloid progenitor, GMP: Granulocyte, macrophage progenitor, MEP: Megakaryocyte, erythrocyte progenitor.

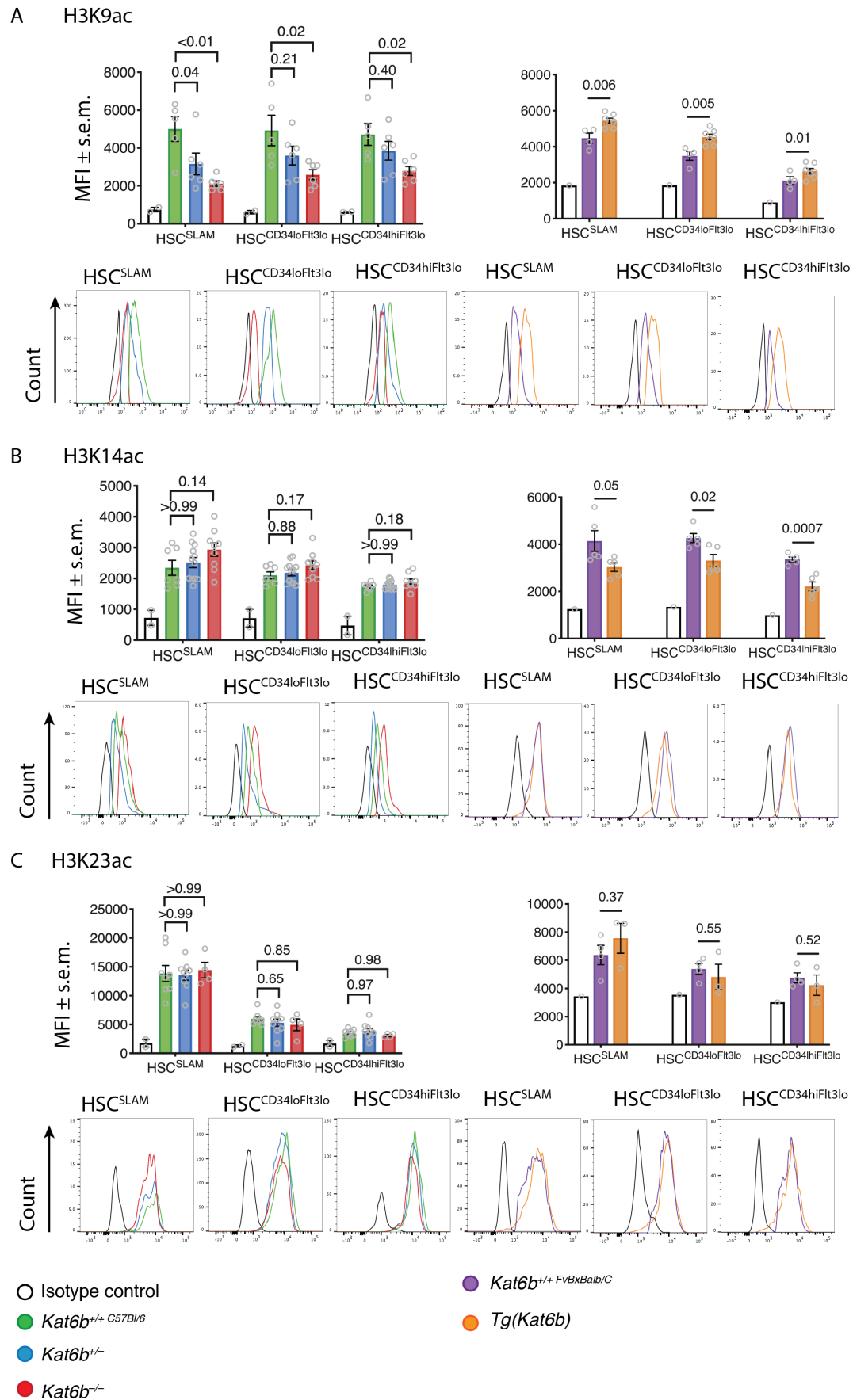


Figure 6.18 KAT6B acetylates histone H3 lysine 9 in foetal liver stem cell populations

(A) Bar graph depicting MFI of H3K9ac levels in E14 foetal liver HSC^{SLAM}, ST-HSC^{CD34^{lo}Flt3^{lo}} and HSC^{CD34^{lo}Flt3^{lo}} populations, with an isotype negative control. Representative histograms of H3K9ac in *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+ C57Bl/6}, *Kat6b*^{+/+ FvBxBalb/C} and *Tg(Kat6b)* samples shown below each bar graph. *Kat6b*^{+/-} samples shown significantly reduced H3K9ac levels in HSC^{SLAM} ($p = 0.04$) cells, relative to wild type controls. *Kat6b*^{-/-} shown significantly reduced H3K9ac levels in HSC^{SLAM} ($p = 0.01$), HSC^{CD34^{lo}Flt3^{lo}} ($p = 0.02$) and ST-HSC^{CD34^{hi}Flt3^{lo}} ($p = 0.02$) populations, relative to wild type controls. *Tg(Kat6b)* samples show significantly increased H3K9ac levels in HSC^{SLAM} ($p = 0.006$), HSC^{CD34^{lo}Flt3^{lo}} ($p = 0.005$) and ST-HSC^{CD34^{hi}Flt3^{lo}} ($p = 0.01$) populations, relative to littermate controls.

(B) Bar graph depicting MFI of H3K14ac levels in E14 foetal liver HSC^{SLAM}, ST-HSC^{CD34^{hi}Flt3^{lo}} and HSC^{CD34^{lo}Flt3^{lo}} populations, with an isotype negative control. Representative histograms of H3K14ac in *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+ C57Bl/6}, *Kat6b*^{+/+ FvBxBalb/C} and *Tg(Kat6b)* samples shown below each bar graph. *Kat6b*^{-/-} and *Kat6b*^{+/-} samples do not show significantly different levels of H3K14ac in any HSC population. *Tg(Kat6b)* samples show significantly lower levels of H3K14ac in HSC^{SLAM} ($p = 0.05$), HSC^{CD34^{lo}Flt3^{lo}} ($p = 0.02$) and ST-HSC^{CD34^{hi}Flt3^{lo}} ($p = 0.0007$) populations, relative to littermate controls.

(C) Bar graph depicting MFI of H3K23ac levels in E14 foetal liver HSC^{SLAM}, ST-HSC^{CD34^{hi}Flt3^{lo}} and HSC^{CD34^{lo}Flt3^{lo}} populations, with an isotype negative control. Representative histograms of H3K23ac in *Kat6b*^{-/-}, *Kat6b*^{+/-}, *Kat6b*^{+/+ C57Bl/6}, *Kat6b*^{+/+ FvBxBalb/C} and *Tg(Kat6b)* samples shown below each bar graph. There is no significant difference in H3K23ac in any HSC population between mutant and control samples.

Graphs are displayed as individual data points with mean $\pm$ s.e.m., where data points represent an individual foetal liver sample. *Kat6b*^{+/+C57Bl/6}, *Kat6b*^{+/-} and *Kat6b*^{-/-} samples were analysed using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. *Kat6b*^{+/+ FvBxBalb/C} and *Tg(Kat6b)* samples compared using a two tail Students t-test. $N = 5 - 6$ foetal liver samples per genotype. HSC^{SLAM}, ST-HSC^{CD34^{hi}Flt3^{lo}} and HSC^{CD34^{lo}Flt3^{lo}} gated as shown in Fig 6.6. MFI: Median Fluorescence Intensity. Colour code for genotypes shown below (C).

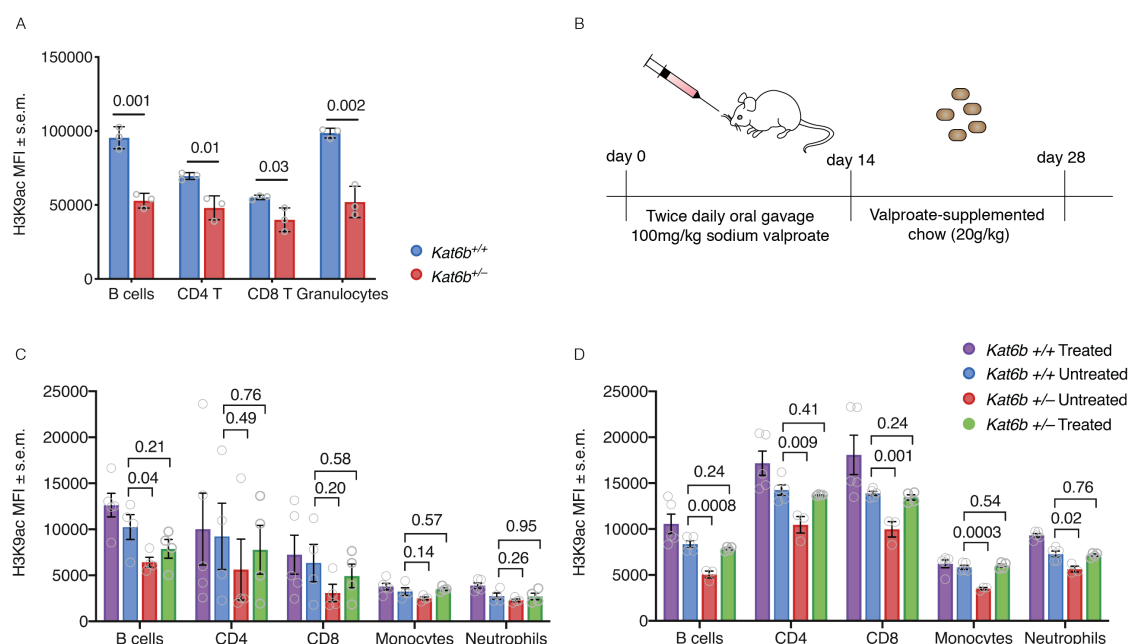


Figure 6.19 Valproic acid treatment of *Kat6b*^{+/-} animals increases H3K9ac in the peripheral blood

(A) Bar graph depicting H3K9ac levels, based of MFI in peripheral blood cell populations of *Kat6b*^{+/+}*C57Bl/6* and *Kat6b*^{+/-} 8 week old animals. *Kat6b*^{+/-} animals show significantly lower H3K9ac levels in peripheral blood B cells ($p = 0.001$), CD4 T cells ($p = 0.01$), CD8 T cells ($p = 0.03$) and granulocytes ($p = 0.002$), relative to wild type controls. Graphs are displayed as individual data points with mean $\pm$ s.e.m., where data points represent one peripheral blood cell sample. *Kat6b*^{+/+}*C57Bl/6* and *Kat6b*^{+/-} samples were analysed using a two tailed Student's t-test. Genotype colour code is shown to the right of the graph.

(B) Diagram displaying the treatment regime for *Kat6b*^{+/+}*C57Bl/6* and *Kat6b*^{+/-} animals. Animals were given VPA by oral gavage from 2 – 4 weeks of age at a dose of 100 mg/kg body weight. From 4 – 6 weeks animals were given chow supplemented with VPA at 20 g/kg chow. Peripheral blood samples were collected on day 14 and 28 from the first treatment.

(C) Bar graph depicting H3K9ac levels, based of MFI, in peripheral blood cell populations of *Kat6b*^{+/+}*C57Bl/6* and *Kat6b*^{+/-} treated and untreated animals after 2 weeks. Untreated *Kat6b*^{+/-} animals show significantly lower levels of H3K9ac, relative to untreated wild type controls, in B cells ($p = 0.04$), but no other populations. Treated *Kat6b*^{+/-} animals do not show significantly

different levels of H3K9ac, relative to untreated wild type controls, in any cell population analysed.

(D) Bar graph depicting H3K9ac levels, based of MFI in peripheral blood cell populations of *Kat6b*^{+/+C57Bl/6} and *Kat6b*^{+/-} treated and untreated animals after 4 weeks. Untreated *Kat6b*^{+/-} animals show significantly lower levels of H3K9ac, relative to untreated wild type controls, in B cells ($p = 0.0008$), CD4 T cells ($p = 0.009$), CD8 T cells ($p = 0.001$), monocytes ($p = 0.0003$) and neutrophils ($p = 0.02$). Treated *Kat6b*^{+/-} animals do not show significantly different levels of H3K9ac, relative to untreated wild type controls, in any cell population analysed.

Graphs in C and D are displayed as individual data points with mean $\pm$ s.e.m., where data points represent an individual animal. Samples were analysed using a one-way ANOVA followed by a Bonferroni correction for multiple comparisons, with genotype as the independent factor. Peripheral blood cell populations gated as shown in Fig 6.4.

MFI: Median Fluorescence Intensity; VPA: Valproic acid

Chapter 7

General discussion and future directions

7.1 Summary of major findings

Within this study I have investigated the effects of *Kat6b* deletion and overexpression within the developing brain and cultured NSPCs. I have demonstrated that KAT6B can drive proliferation in neural stem and progenitor cells *in vivo* and *in vitro*, as well as neuronal differentiation and neurite outgrowth *in vitro*. At the level of gene expression, I have shown that KAT6B promotes expression of a number of transcriptional regulators required in early brain development and the maintenance of NSPC populations, including a number of members of the SOX family of pioneer transcription factors. Amongst the affected transcription factors was *Sox2*, essential for stem cell commitment to the neural lineage. I have demonstrated that *Kat6b* status affects *Sox2* promoter activity in dorsal telencephalon NSCs and that the proliferative defect in cultured *Kat6b*^{-/-} NSPCs can be partially rescued by overexpressing *Sox2*.

In addition to further elucidating the requirement for KAT6B during neurogenesis, I have identified a novel role for KAT6B as a driver of proliferation in HSCs, showing that *Kat6b* status affects HSC pools within the developing liver and functionally alters the capacity of transplanted HSCs to reconstitute an irradiated recipient. I have demonstrated that *Kat6b* status does not affect HSC lineage differentiation *in vitro* or the capacity of transplanted HSCs to home to the bone marrow. I have also shown that KAT6B loss reduces acetylation at H3K9 in foetal liver HSC populations, while *Kat6b* heterozygous adult animals show a 20 – 30% reduction in acetylation at H3K9 in peripheral blood cells. Furthermore, I have shown that the levels of H3K9ac in *Kat6b*^{+/-} animals can be increased to wild type levels following treatment with an HDAC inhibitor, demonstrating that a molecular deficit resulting from *Kat6b* deficiency, can be brought to normal levels by therapeutic intervention.

The major findings of this thesis are as follows:

Within the developing cortex and cultured neural stem and progenitor cells (NSPCs)

- KAT6B drives NSPC proliferation *in vitro* and *in vivo*, resulting in more SVZ NSCs in *Tg(Kat6b)* cortices and enhanced proliferation in cultured neurospheres, over consecutive passages, as well as enhanced self-renewal (secondary neurosphere formation).
- KAT6B drives neuronal development and neurite outgrowth *in vitro*.
- KAT6B promotes expression of a number of transcriptional regulators in E12 dorsal telencephalon tissue, known to be required for normal cortical development.
- KAT6B drives expression of SOX family members in both the developing cortex and in cultured NSPCs, notably *Sox2*.
- *Sox2* overexpression in cultured NSPCs partially rescues the proliferative defect in *Kat6b*^{-/-} NSPCs.

Within the haematopoietic system and in haematopoietic stem cells (HSCs)

- KAT6B drives HSC proliferation in the embryonic and foetal liver.
- KAT6B loss impairs HSC reconstitution in irradiated recipients across all cell lineages.
- Heterozygosity at both the *Kat6b* and *Kat6a* loci severely impairs HSC reconstitution in irradiated recipients.
- KAT6B acetylates H3K9 in foetal liver HSC populations.
- H3K9ac levels in the peripheral blood are reduced in *Kat6b*^{+/-} animals. This deficiency can be increased to wild type levels upon treatment with the HDAC inhibitor, valproic acid (VPA).

7.2 Considerations when investigating *Kat6b* overexpression

Within this study, all assessments of the consequences of *Kat6b* loss are complemented by an assessment of its overexpression, using *Tg(Kat6b)* animals. While the effects of *Kat6b* deficiency or loss are generally mirrored by its overexpression, it is possible that there are gain-of-abnormal function effects in *Tg(Kat6b)* animals, especially in situations where the *Tg(Kat6b)* phenotype is more severe than the heterozygous or null phenotype. For example, in regards to gene expression differences, the RNA-seq data sets for *Tg(Kat6b)* animals relative to their genetic background matched controls, are dominated by upregulation of neurogenesis-associated genes, rather than upregulation in the transcriptional regulators that are downregulated in *Kat6b*^{-/-} samples. Even though *Tg(Kat6b)* animals may overexpress a number of genes downregulated in null mutants, the dominant effect of excess *Kat6b* in the developing nervous system is to drive neuronal development-associated genes, masking any other effect on transcriptional regulators of cortical development.

On the other hand, there may also be an upper limit to this effect. While *Kat6b* is in excess, the auxiliary complex members with which it associates are not similarly overexpressed. This may not only limit KAT6B activity, but may contribute to the acquisition of abnormal functions if excess KAT6B interacts with other protein partners that are not affected when KAT6B is present at normal levels. In addition, given that KAT6B and KAT6A represent mutually exclusive catalytic subunits of a shared multi-protein complex, it is also possible that excess KAT6B may limit the proportion of KAT6A-containing complexes, hence skewing the balance between these two HATs during development. However, this would only be true if auxiliary complex members were limiting within cells and, as no KAT6A-associated phenotypes have been observed in *Tg(Kat6b)* animals, this is unlikely to be the case.

7.3 Functional co-operation vs. overlap between KAT6B and KAT6A

I have demonstrated that heterozygosity at both the *Kat6a* and *Kat6b* loci severely impairs HSC reconstitution in irradiated recipients. Interestingly, although double heterozygous mutants have fewer HSCs within the foetal liver, relative to wild type controls, there are still HSCs present. This suggests that at least one functional allele of both *Kat6a* and *Kat6b* is sufficient to support HSC development, albeit at a markedly reduced rate relative to single heterozygous animals or wild type controls. Nonetheless, the impact of loss of one allele each at the *Kat6a*

and *Kat6b* loci became obvious when specific numbers of HSCs were transplanted to assess their function. One remaining allele each at the *Kat6a* and *Kat6b* loci is not sufficient to generate functionally competent HSCs, able to give rise to haematopoietic cell types within irradiated recipients. In future studies it would be of interest to assess how the combined homozygous loss of *Kat6a* and *Kat6b* affects other tissue systems and development. Reciprocally, it would be interesting to determine whether overexpression of *Kat6a* or *Kat6b* could ameliorate or rescue the defects resulting from loss of the other.

Furthermore, given that *de novo* mutations in both *KAT6A* and *KAT6B* are associated with congenital intellectual disability disorders in human patients, it is possible that *KAT6A* and *KAT6B* play similarly overlapping roles in brain development, as has been shown here for haematopoiesis. An investigation into the role of *KAT6A* during neurogenesis and the effect of heterozygosity at both the *Kat6a* and *Kat6b* loci, may shed light on unique and conserved roles of these factors during neurogenesis.

7.4 Chromatin modifiers within different cell populations

Within this thesis I have demonstrated that *KAT6B* acts both with NSC and HSC populations. In NSCs, I have shown that *KAT6B* drives proliferation *in vivo* and *in vitro*, promoting expression of a number of transcriptional regulators known to drive NSC self-renewal and proliferation, including *Sox2*. I have also shown that *KAT6B* promotes neuronal differentiation at the expense of astrocyte development in cultured NSPCs, as well as neurite outgrowth in cultured cortical neurons. By comparison, in HSCs I have demonstrated that *KAT6B* is required for normal HSC pools in the embryonic and foetal liver, acetylating H3K9 in foetal liver stem cell populations, and that its loss functionally impairs HSCs in competitive transplants, however does not affect lineage commitment in this cell type. It is of note that such requirements for a chromatin regulator within more than one cell type is not an observation unique to *KAT6B*.

For example, the histone acetyltransferase *KAT6A* regulates heart development by activating *Tbx1* and *Tbx5* gene expression and is required for normal H3K9ac levels at the promoters of these genes (Voss et al., 2012). The *KAT6A* complex also acts during B cell development, at *Meis1* and *Hoxa9* gene loci to maintain their expression and promote B cell progenitor proliferation (Sheikh et al., 2015b) and is recruited to *Hox* genes during gastrulation, to

correctly assign body segment identity (Miller et al., 2004; Sheikh et al., 2015a; Voss et al., 2009).

By comparison, the PcG protein BMI1 is required for the development and expansion of cerebellar granule neuron progenitors, acting as a downstream target of Shh signalling (Leung et al., 2004). BMI1 is also required for the maintenance of adult NSCs by repressing the *Ink4a-Arf* locus (Jacobs et al., 1999a; Jacobs et al., 1999b) to prevent cell senescence and depletion of neural stem and progenitor cell pools (Molofsky et al., 2003). BMI1 similarly modulates expression of *Ink4a-Arf* in adult HSCs, to prevent cellular senescence (Park et al., 2003) and regulates HSC potency by preventing premature expression of developmental regulators of the B cell lineage, *Ebf1* and *Pax5* (Oguro et al., 2010).

Common roles for chromatin regulators across cell types alludes to conserved mechanisms that may govern the maintenance, proliferation and differentiation of stem and progenitor cell populations. The results presented in this thesis demonstrate that KAT6B now adds a further example of a chromatin regulator orchestrating roles across more than one cell type. Importantly, in order to comprehensively understand how KAT6B affects HSC proliferation and function, it will be necessary to assess differential expression in HSCs and haematopoietic progenitors, such as to identify KAT6B gene targets within the haematopoietic system. Furthermore, to distinguish direct from indirect targets of KAT6B, ChIP-seq will be required in NSC and HSC populations, to assess KAT6B occupancy genome wide.

7.5 The potential of HDAC inhibition as a treatment for Kat6b deficiency

While the major structure of the brain and its cortical layers develop during embryogenesis, the maturation of these structures, including the refinement of neuronal synapses, myelination of axons and development of glial cell types, mainly occurs during postnatal life. Furthermore, these stages of post-natal cortex maturation can be correlated with the acquisition of behavioural milestones. For example, in humans, the disappearance of primary neonatal reflexes such as the palmer grasp reflex (Herschkowitz et al., 1997) and the ability to retrieve recently acquired memories, coincide with an increase in synaptic density and dendritic arborisation at 7 – 10 months of age (Herschkowitz et al., 1997). As such, therapeutic intervention, if possible, in infancy and childhood would be expected to produce a notable effect on overall cognition. Given that KAT6B is an enzyme with the opposing enzymatic

activity of HDACs, a disturbed balance between histone acetylation and deacetylation may be amenable to modulation using HDAC inhibitors, some of which are already in clinical use, e.g. valproic acid (VPA) for the treatment of epilepsy, bipolar disorder, migraine headaches and schizophrenia.

In addition to shedding light on the requirements of KAT6B during development, this thesis has exhibited the amenability of *Kat6b* deficiency to therapeutic intervention, showing that the loss of H3K9ac in the peripheral blood of *Kat6b*^{+/-} animals can be increased to wild type levels using treatment with the HDAC inhibitor, VPA. What this demonstrates is that a molecular defect resulting from *Kat6b* deficiency can be restored to normal levels through therapeutic intervention in post-natal animals, at least in peripheral blood cells. What remains to be determined, is whether this restoration also occurs in the brain and corresponds with a cognitive improvement in treated animals, to establish whether VPA represents a potential therapy for KAT6B disorder human patients. Notably, VPA is a common treatment for epilepsy, migraine headaches, bipolar disorder and depression. As such, it can be prescribed postnatally and in adults without severe side effects and it would be feasible to suggest treating KAT6B disorder patients with an agent such as VPA, following a genetic diagnosis, in order to ameliorate cognitive defects resulting from KAT6B deficiency.

One important caveat to this proposition, is that there has not been sufficient functional assessment of individual *KAT6B* mutations to enable personalised treatment of KAT6B-disorder patients. A detailed functional understanding of each individual mutation, whether it represents a loss-of-function or gain-of-abnormal function mutation and whether a truncated or altered protein product is formed needs to be clearly established prior to therapeutic intervention. For example, a gain-of-function mutation would be worsened by treatment with an HDAC inhibitor such as VPA. To that end, the gene expression analyses performed within this study have identified key genes and signalling pathways affected by altered *Kat6b* activity, which may be investigated in KAT6B disorder patients. These can then be used as a platform to assess gene expression within KAT6B patients, which would help to better understand the functional consequences of their mutation, prior to therapeutic intervention.

Chapter 8

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