The regulation of erythropoiesis by Hedgehog (Hh) signalling

by

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Abstract

Dhh (Desert Hedgehog) is one of three Hedgehog (Hh) proteins essential for testis development and Schwann cell function. Gli1, Gli2, and Gli3 are dedicated transcription factors that transduce the Hh pathway. A study published by our group showed significant changes in erythrocyte differentiation in Dhh-deficient mice, under steady state and stress-induced conditions, implicating Dhh as a negative regulator of murine erythropoiesis (Lau et al., 2012). To investigate these phenotypic differences, we created an mCherry reporter mouse line for Dhh using Bacterial Artificial Chromosome ‘recombineering’ and hypothesised that Dhh is produced by the splenic stroma, especially red pulp fibroblasts (RPFs), under steady state and anaemic stress. Erythroblast kinetics were also analysed in Hedgehog-related mouse lines during haematopoietic recovery.

After validating the mCherry line as a faithful reporter of Dhh using testes, brain and thymic tissue, we found that Dhh (mCherry) is expressed by a fraction of all splenic ICAM-1+ stromal cells in particular RPFs and also fibroblast reticular cells (FRCs), disproving our steady state hypothesis. During anaemia, Dhh was not upregulated but showed most expression in the RPF subset in support of our hypothesis, while some follicular dendritic cells (FDCs) and marginal reticular cells (MRCs) significantly upregulated Dhh. Interestingly, Dhh was more highly detected in antigen-presenting cells (APCs) and some non-APCs under normal and anaemic conditions, potentially implicating Dhh in white pulp splenic functions.

Irradiating Dhh-deficient mice corroborated Dhh as a negative erythropoietic regulator by analysing reticulocytes and splenic erythroid progenitors, in line with Dhh-deficient mice exhibiting less Gli-binding activity in their BM and spleen. In Gli3-heterozygote mice elevated Shh but reduced Dhh expression was found in the spleen, supporting our hypothesis that Gli3 represses Shh. Irradiating Gli3-heterozygote mice showed significant expansion of erythroblast differentiation in the BM but not the spleen, highlighting the intricacies of the Hh signalling pathway in erythropoiesis.
“Let us pick up our books and pencils. They are our most powerful weapon.”

- Malala Yousafzai

_Education activist and Nobel Prize laureate_
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All primers were purchased from Sigma, except for Dhh-mCherry and mCherry Internal that come from IDT……………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………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### Abbreviations

-/-  Knockout  
+/−  Heterozygote  
+/+  Wild-type  
Aire  Autoimmune regulator  
APC  Antigen presenting cell  
Au  Arbitrary unit  
BAC  Bacterial artificial chromosome  
BMP  Bone morphogenetic protein  
Bp  Base pair  
BSA  Bovine serum albumin  
CCR  Chemokine receptor  
CD  Cluster differentiation  
cDNA  Complementary DNA  
CMP  Common myeloid progenitor  
cTEC  Cortical thymic epithelial cells  
DC  Dendritic cell  
ddH₂O  Double-distilled water  
Dhh  Desert Hedgehog  
DNA  Deoxyribonucleic acid  
dNTP  Deoxynucleotide triphosphate  
gDNA  genomic DNA  
FACS  Fluorescence activated cell sorter  
FCS  Fetal calf serum  
FDC  Follicular dendritic cell  
FITC  Fluorescein isothiocyanate  
FMO  Fluorescence minus one  
FRC  Fibroblastic reticular cell  
FSC  Forward Scatter  
GATA  Gata binding protein 3  
GBS  Gli binding site  
GFP  Green fluorescent protein  
Gli  Glioma associated oncogene  
GMP  Granulocyte macrophage progenitor  
Gy  Gray unit  
Het  Heterozygote  
Hh  Hedgehog  
Hhip  Hedgehog-interacting protein  
HPRT  Hypoxanthine guanine phosphoribosyl transferase  
HSC  Haematopoietic stem cell  
Ihh  Indian Hedgehog  
IL  Interleukin  
Kb  Kilobase  
Kif7  Kinesin family member 7  
KO  Knockout  
LSK  Lineage-negative Sca1-positive C-kit-negative  
Mb  Megabase  
mCh  monomer Cherry  
MEP  Myeloid erythroid progenitor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>Mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>MRC</td>
<td>Marginal zone reticular cell</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mTEC</td>
<td>Medullary thymic epithelial cell</td>
</tr>
<tr>
<td>nfw</td>
<td>Nuclease-free water</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
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<td>P</td>
<td>p-value</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
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<td>Ptch</td>
<td>Patched</td>
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<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPF</td>
<td>Red pulp fibroblast</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>Smo</td>
<td>Smoothened</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>TEC</td>
<td>Thymic epithelial cell</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>VEC</td>
<td>Vascular endothelial cells</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>XT</td>
<td>Extra toe (Gli3+/-)</td>
</tr>
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</table>
Chapter One: Introduction

1.1. Murine Haematopoiesis

Haematopoiesis is a delicately orchestrated process by which haematopoietic stem cells (HSCs) develop into different lineages of mature functional blood cells. HSCs are pluripotent progenitors, capable of self-renewal and responsible for the production of all the blood cells. Haematopoiesis takes place in different tissues during embryogenesis and in adult life. The process responsible for the differentiation of red blood cells is known as erythropoiesis. During embryonic development, two sequential phases of haematopoiesis called primitive and definitive haematopoiesis take place. Primitive erythrocytes differentiate in the yolk sac from a bipotent precursor called a haemangioblast, which also gives rise to endothelial cells, by embryonic day (E) 7.5 in mice. Differentiation then produces progenitor precursors for macrophages, megakaryocytes and erythrocytes (Palis, Robertson, Kennedy, Wall, & Keller, 1999; Speck & Gilliland, 2002; Tober et al., 2007).

Subsequently, fetal-liver or ‘definitive’ haematopoiesis is initiated by the aorta-gonad-mesonephros (AGM) region at E10-11, and starts to produce HSCs during mid-gestation, around E10.5 in mice (Medvinsky & Dzierzak, 1996; Speck & Gilliland, 2002). Shortly after this, haematopoiesis migrates to the foetal liver and remains there up until birth. HSC-derived blood lineage progenitors such as myeloid and lymphoid progenitors start to appear from the foetal liver (Orkin & Zon, 1997). After birth and throughout adult life, HSCs are continuously produced in the bone marrow to generate all the haematopoietic progenitors that proliferate and differentiate into committed lineages as shown in Figure 1.1.
Figure 1.1. Schematic showing both primitive erythropoiesis (foetus only) and definitive haematopoiesis (foetus and adult), and their defined cells lineages. Lineage-negative Sca1-positive C-kit-positive (LSK) cells give rise to the common lymphocyte progenitor (CLP) or the common myeloid progenitor (CMP). The latter in turn gives rise to either the megakaryocyte-erythroid progenitor (MEP) or the granulocyte-macrophage progenitor (GMP). CLP, MEP and GMP progenitors give rise to a distinct set of committed populations as shown in this Figure.

Although various models for haematopoietic lineage commitment have been proposed, the classical model suggests that HSCs have the potential to give rise to either one of the two distinct haematopoietic lineage progenitors: the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). The CLP differentiates into T and B lymphocytes and natural killer (NK) cells. The CMP gives rise to all myeloid cells by first differentiating into the megakaryocyte-erythroid progenitor (MEP) or the granulocyte-macrophage progenitor (GMP). MEP, as its name implies, give rise to erythrocytes and megakaryocytes (that eventually develop into platelets) while GMP give rise to granulocytes and monocyte lineages (Lieu & Reddy, 2012). It is thought however that a degree of plasticity can occur between CMP and CLP lineage commitment, and this is seen for example in the thymus.
where early thymic progenitors (ETP) are capable of giving rise to myeloid cells and T cells (Bell & Bhandoola, 2008).

1.1.1. Erythroid development
Erythrocytes (red blood cells) are small, highly specialized cells that originate from the megakaryocyte-erythroid progenitor (MEP), which in turn differentiates from a common myeloid progenitor (CMP). MEP differentiates to become the earliest committed erythroid progenitor called the burst-forming unit erythroid (BFU-E). The BFU-E then differentiates into the colony-forming unit erythroid (CFU-E) after limited self-renewal divisions. The process of terminal differentiation then follows and involves the induction of erythroid genes, chromatin condensation, synthesis of increasing amounts of haemoglobin and enucleation. This process is characterised by the sequential differentiation of four morphologically distinct erythroblast populations (I to IV) called proerythroblasts (I), basophilic (II), polychromatophilic (III) and orthochromatophilic (IV) erythroblasts (Lau et al., 2012; Pishesha et al., 2014).
Figure 1.2. The erythroid pathway in mice. Myeloid progenitors (CMP) give rise to erythrocytes and platelets from the MEP committed pathway, and neutrophils, basophils, eosinophils and macrophages from the GMP committed pathway. Erythroid development goes through distinctive steps of development, including erythroblast populations I-IV characterised by increasing levels of Ter119 and decreasing levels of CD71 expression, before maturing to reticulocytes and mature red blood cells.
In mice, these four subpopulations are distinguishable by their varying surface expression of CD71 (transferrin receptor) and Ter119 (Ly76) as shown in Figure 1.2. Erythroblast populations III and IV contain less nucleic acids, and are smaller compared to populations I and II. GlyA (serine hydroxymethyltransferase) expression also distinguishes populations I to IV, with a marked increase in GlyA visualised on population II compared to I, that is retained up till population IV (Pishesha et al., 2014). Orthochromatic erythroblasts then enucleate to become reticulocytes, defined as erythroid cells that still retain residual RNA despite having lost their nucleus. After enucleation, reticulocytes are released into circulation after a brief period, and lose their residual RNA prior to maturing to erythrocyte. Under steady-state homeostasis, rodent reticulocyte maturation lasts between 2 to 3 days, where around half of this time is spent in the bone marrow, and half in circulation (Ney, 2011). This represents a short yet dynamic period compared to the average 40-day life span of an erythrocyte in mice, during which reticulocytes undergo significant changes such as the loss of membrane-bound organelles and ribosomes, cell size and membrane remodelling, prior to differentiating into smaller discoid erythrocytes (An et al., 2014; Putten, 1958).

1.1.2. The regulation of erythropoiesis

A range of transcription factors control the regulation of erythrocyte differentiation, such that during steady-state conditions, erythropoiesis predominates in the bone marrow but is shifted to the spleen under erythropoietic stress, such as hypoxia. The best studied transcription factors that are crucial in erythroblast differentiation include GATA-1, a zinc finger transcription factor, and Kruppel-like factor (KLF) EKLF/KLF1 (Gordon et al., 2008). GATA-1 was identified through a GATA-1 knock-out study that caused failure of erythropoiesis in mice early in gestation, and embryo death at E10.5 due to severe anaemia (Fujiwara, Browne, Cunniff, Goff, & Orkin, 1996). GATA-1 has also been reported to interact with other transcription factors relevant to erythroid development, including Lmo2, FOG-1, E2A, Scl and Ldb-1, which form protein-complexes that regulate the transcription of GATA target genes (Wadman et al., 1997). KLF1 was identified as a CACC box-binding factor that binds to many erythroid gene promoters including β-globin in transient reporter assays (Hodge et al., 2006), and in which a total deficiency causes mouse embryo death at E16 due to severe anaemia (Perkins, Sharpe, & Orkin, 1995). Within the KLF family of...
transcription factors, KLF2-5, 8 and 11-13 are also expressed in erythroid cell lines, of which most bind to globin promoters. KLF13 has been more recently identified as a critical factor that activates the promoters of erythroid genes including γ-globin, GATA-1 and porphobilinogen deaminase (involved in heme biosynthesis) in vitro. In fact KLF13-knockout mice were shown to undergo a high degree of erythropoietic stress and splenomegaly (Gordon et al., 2008).

Terminal erythroid differentiation (the process by which immature precursors become erythrocytes) is also regulated by histone deacetylases and a key growth factor called erythropoietin (EPO), a glycoprotein hormone. Epo binds to its receptor EpoR to control cell survival, proliferation and differentiation of the erythroid lineage (Lin, Lim, D’Agati, & Costantini, 1996). The proliferation of the primitive erythroid lineage is dependent on the Epo signal. However in the definitive erythroid lineage, Epo acts as a survival factor, as well as a growth factor for the survival and proliferation of proerythroblasts (Koury & Bondurant, 1990). Histone deacetylase 2 has been identified as indispensable to chromatin condensation, which occurs prior to enucleation of murine erythroblasts in vitro (Migliaccio, 2010).

1.2. The Hedgehog signalling pathway

The Hedgehog (Hh)-signalling pathway and its family of secreted proteins is a multifunctional system involved in mammalian embryonic patterning and development (Jiang & Hui, 2008). The pathway was first identified in a large-scale screen for Drosophila mutations in 1980, which identified the importance of Hh proteins in the development of the larval body plan of Drosophila (Nüsslein-Volhard & Wieschaus, 1980). The Hh signalling pathway has a wide range of roles in cell differentiation, survival and cell fate. Indeed, it is involved in the embryogenesis of several tissues including limb and lung development and neural patterning, as well as the homeostasis of adult tissues (Crompton, Outram, & Hager-Theodorides, 2007). Although the signalling pathway is not yet completely understood, it is known that several of its components are highly conserved in vertebrates (Varjosalo & Taipale, 2008).
Figure 1.3. Schematic of the mammalian Hedgehog (Hh) pathway. Right: The binding of Hh protein to its receptor complex Patched 1 (Ptch1) releases the inhibition of Smoothened (Smo). Smo signals allows the Gli multi-protein complex, which also consists of Kif7 and Sufu, to dissociate from the microtubule. Activator forms of Gli proteins (GliA) are released and translocate to the nucleus, leading to transcription of Hedgehog-specific target genes. Left: In the absence of Hh binding, Patched 1 inhibits the activity of Smo. This allows protein kinase A (PKA) to phosphorylate Gli proteins, generating repressor forms of Gli2 and Gli3 (GliR). This inhibits downstream transcriptional activation.

The three highly related Hh proteins are called Desert (Dhh), Sonic (Shh), and Indian (Ihh). The three proteins act through the 12-span transmembrane Patched 1 receptor (Ptch1), which relieves the inhibition of Smoothened (Smo). Smo is a 7-span transmembrane protein that transduces the Hh signal by activating a signalling cascade, which activates and alters gene expression through the Gli-family of transcription factors (Briscoe & Thérond, 2013;
When Ptch1 is not bound by Hh protein, it represses the action of Smo. This prompts the phosphorylation of kinase recognition motifs near the C-termini of Gli proteins by Protein Kinase A (PKA), Glycogen Synthase Kinase-3 (GSK3) and Casein Kinase-I (CKI) (Pan, Wang, & Wang, 2009; Price & Kalderon, 2002), marking them for ubiquitination (Tempé, Casas, Karaz, Blanchet-Tournier, & Concordet, 2006; W. Zhang et al., 2005). For ubiquitination to occur, the Gli proteins must associate with the scaffolding protein Kif7. Together with a serine/threonine kinase called Fused (Fu), they form the hedgehog signalling complex (Endoh-Yamagami et al., 2009; Sisson, Ho, Suyama, & Scott, 1997; W. Zhang et al., 2005). Another important cell surface receptor called the Hedgehog-interacting protein (Hhip) inhibits the Hh pathway by sequestering the Hh ligand, but does not appear to be actively involved in signal transduction itself (Beachy, Hymowitz, Lazarus, Leahy, & Siebold, 2010) (see Figure 1.3).

1.2.1. The Gli family of transcription factors

In mammalian cells, Gli1, Gli2, and Gli3 (Glioma-associated oncogene family members 1, 2, and 3) are dedicated transcription factors for signal transduction of the Hedgehog (Hh) pathway. They act as bifunctional transcription factors; Gli1 contains only a C-terminal transcriptional activation domain, whereas both Gli2 and Gli3 possess C-terminal activation and N terminal repression domains (Hui & Angers, 2011). Mutant studies in mice have demonstrated that Gli2 and Gli3 are the primary mediators of Shh signalling and are essential for embryogenesis (Ding et al., 1998).

Although Gli2 and Gli3 act as the principal transcriptional activator (GliA) and repressor (GliR) of the pathway, respectively, all three Gli proteins can share an overlapping activator function (Bai, Auerbach, Lee, Stephen, & Joyner, 2002; Hashimoto-Torii et al., 2003). Gli3 and, to a lesser extent, Gli2 can function as repressors of transcription (GliR), whereas all three Gli proteins can act as transcriptional activators (GliA). Gli3 is the principal repressor of the mammalian Hh pathway and is responsible for suppressing Hh target genes in the absence of Hh protein. As such, Gli3-deficient adult mice are polydactylyous, and lack AP symmetry compared to their wild-type counterparts (Hui & Angers, 2011). Due to their polydactylyous phenotype, Gli3 heterozygote mice are commonly referred to as ‘extra toe’ or
In response to Hh stimulation, Gli2 acts as the principal activator to trigger the expression of Gli1, a robust activator of Hh target gene transcription, and additional Gli targets. As Gli1 is a target gene of the Hh signalling pathway, the detection of its transcription level can indicate the degree of Hh signalling occurring in a given population of cells (Hui & Angers, 2011).

### 1.2.2. The regulation of Hh activity

Hh proteins act as morphogens to regulate different developmental processes, whereby they diffuse to form varying concentration gradients, with varying effects on the cells of a developing embryo. Shh is the most extensively studied vertebrate homolog and morphogen that controls the pattern of cellular differentiation by acting both as a short-range, contact-dependent and long-range morphogen. It is critical in regulating vertebrate organogenesis such as digit growth on limbs, and the organisation of the brain (Choy & Cheng, 2012).

The duration of the received signal is another important factor that influences differentiation outcome (J Briscoe & Ericson, 1999). Hh proteins therefore control cellular development dependent on the responding cell type, the concentration and the duration of exposure to Hh by target cells (Harfe et al., 2004; Varjosalo & Taipale, 2008). It has also been shown that the amount of Hh signal received by a cell correlates with the concentration of Gli protein. Additionally, the ratio of the Gli activator to Gli repressor forms of the protein is affected by the Hh signal gradient received by the target cell. This means that cells that are closer to the Hh secreting source will increase the amount of activator forms of Gli proteins, whereas the repressor forms will be inhibited and vice versa (Stamatakii, Ulloa, Tsoni, Mynett, & Briscoe, 2005).

### 1.2.3. Hedgehog protein-associated developmental defects

Although the three Hh proteins share a common signalling pathway with similar physiological effects, they are implicated in a wide range of different biological functions in development. These differences are attributed to their distinctive expression patterns. In line with this, independent Hh protein deficiencies cause mild to severe discrete developmental
defects (Varjosalo & Taipale, 2008).

Shh expression is found in a wide range of tissues, and is in fact the most broadly expressed Hh protein in mammals. Shh plays essential roles in many developmental processes that control cellular differentiation and proliferation (Varjosalo & Taipale, 2008). During embryogenesis, Shh is expressed in midline tissues and regulates the patterning of embryonic tissues such as the spinal cord, axial skeleton and limb development (Chiang et al., 1996). Shh is also involved in organogenesis, including the development of eyes, ears and the kidney (Varjosalo & Taipale, 2008). Thus aberrations in Shh signalling cause serious developmental defects in many tissues, leading to embryonic lethality (Heussler & Suri, 2003).

Ihh expression appears more restricted compared to that of Shh. Ihh knockout embryos die mid-gestation or just before birth due to a poorly developed yolk sac (Cridland, Keys, Papathanasiou, & Perkins, 2009). Later in development Ihh is crucial in bone growth, as shown by studies that observe reduced proliferation of chondrocytes and osteoblasts in the context of Ihh deficiency (Razzaque, Soegiarto, Chang, Long, & Lanske, 2005; St-Jacques, Hammerschmidt, & McMahon, 1999). The role of Dhh however has been defined in the proliferation of the male germ-line, Schwann cell function (as described in chapter 4, section 4.2.2), erythropoiesis and T cell function in the spleen (Bitgood, Shen, & McMahon, 1996; Furmanski et al., 2013; Lau et al., 2012; Parmantier et al., 1999).

1.2.4. The role of Hedgehog signalling in haematopoiesis and erythropoiesis

There has been a growing body of evidence implicating Hedgehog signalling in various stages of haematopoiesis and erythropoiesis. The first studies that demonstrated a role for hedgehog signalling in haematopoietic differentiation were conducted by Detmer et al. (2000) and our lab (Outram et al. (2000). The former study observed a significant reduction in erythroid maturation when culturing haematopoietic progenitors in formulated semi-solid medium in the presence of cyclopamine, a Hh signal transduction inhibitor. However treatment with recombinant (r)-Shh increased the number of granulocyte and monocyte colonies, while the erythroid colonies were also noticeably larger (K Detmer, Walker, Jenkins, Steele, & Dannawi, 2000). In support of the Detmer study (2000), another study found that human primitive haematopoietic progenitors were significantly increased, along
with raised cell proliferation, when treated with soluble forms of Shh (Bhardwaj et al., 2001).

Outram et al. (2000) however demonstrated a role for Hh signalling as a negative regulator of T cell development, based on the observations that foetal thymocyte development is arrested under r-Shh protein treatment, but double positive cells increase in response to Hh inhibition (Outram, Varas, Pepicelli, & Crompton, 2000; Shah et al., 2004). Other Hedgehog-related studies continued to support the importance of Hedgehog signalling in haematopoiesis. A study on Ptch-knockout mice for example showed that the HSC pool is expanded when the Hh signal is enhanced (Trowbridge, Scott, & Bhatia, 2006), while a later study on Gli-knockout mice observed a reduction in granulocyte production, as well as defects in myeloid differentiation and stress haematopoiesis (Drakopoulou et al., 2010; Merchant, Joseph, Wang, Brennan, & Matsui, 2010). A more recent study using human pluripotent stem cells (hPSCs) also revealed that a reduction in Hh signalling (by truncation of Gli3 to its repressor form) is necessary and sufficient for developmental progression of haematopoiesis throughout human ontogeny, from embryonic to adult haematopoiesis (McIntyre et al., 2013).

In conflict with these studies, other studies have proposed that Hh signalling is not required for haematopoietic homeostasis, including two studies that observed no differences in the regulation of adult HSC and progenitor homeostasis differentiation and maintenance in the steady state or under stress conditions (Gao et al., 2009; Hofmann et al., 2009). Although another study showed that loss of Smo impairs stem cell renewal and decreases induction of CML (chronic myelogenous leukaemia) (Zhao et al., 2009).

Other studies have also investigated the role of Hh signalling with a focus on the erythroid lineage. Apart from the published study in 2000 described above, Detmer et al. later showed that Hh signalling inhibition specifically delays the morphological change of erythroid maturation, and alters the formation of erythroid progenitor colonies (Kristina Detmer et al., 2005). More recently, a murine study found that embryos died from mid-gestational anaemia when lacking two copies of Ihh, proposing Ihh as a crucial Hh protein in definitive erythropoiesis (Cridland et al., 2009). Following this study, our lab investigated the role of Dhh in erythropoiesis and found significant findings, as discussed in the next section (Lau et al., 2012).
1.2.4.1. Dhh as a negative regulator of erythropoiesis

A recent study conducted by our group showed that Dhh acts as a negative regulator of erythropoiesis at distinct developmental stages in the bone marrow and spleen. During haematopoiesis, all progenitors, as well as the common myeloid progenitors (CMP) were found to be increased in the bone marrow of Dhh knockout (Dhh-/-) mice compared to wild-type (WT). Additional findings concluded that the differentiation from CMP to MEP (megakaryocyte erythroid progenitor) was increased in Dhh-/- mice, as well as the MEP progenitor population itself. Consistent with these findings, higher percentages of immature erythroblast populations III and IV were detected in the bone marrow, and II to IV in the spleens of Dhh-/- mice (see Figure 1.4).
Figure 1.4. **Schematic of erythropoiesis**, showing the progenitor and erythroblast populations that were increased in the bone marrow (blue arrows) and spleens (red arrows) of Dhh deficient mice, compared to WT controls (Lau et al., 2012).

Functional experiments in this study revealed that erythroblast populations I-IV were Dhh-responsive *in vitro* and *ex vivo*, and the recovery of erythropoiesis under irradiation or drug-induced anaemic conditions was faster in Dhh-/- mice compared to WT. Together, this data suggested that accelerated erythroid differentiation occurs in the context of Dhh deficiency suggesting that Dhh negatively regulates erythroblast differentiation (Lau et al., 2012).
1.3. Objectives

In continuation of the study conducted by our lab (Lau et al., 2012), chapter 3 describes the making of the mCherry reporter line for Dhh, developed to investigate the specific source of Dhh in the bone marrow and spleen, as well as the molecular/cellular mechanisms behind this newly attributed regulatory role for Dhh. After successful completion of the new mouse line, it was first validated to confirm it is a faithful reporter for Dhh (chapter 4). BM and spleen experiments under steady state and stress-related anaemic conditions (spleen only) were then undertaken to investigate the pattern of Dhh expression in these tissues, as shown in chapter 5. Based on evidence that Dhh acts as a negative regulator of erythropoiesis, we hypothesised here that Dhh is more highly expressed in red pulp fibroblasts compared to white pulp stromal cells. In chapter 6, stress-induced erythropoiesis by sublethal irradiation was investigated in the context of complete Dhh deficiency and partial Gli3 deficiency, to investigate the interactions of Dhh with other components of the Hh pathway in erythropoiesis. Based on the finding (described in this chapter) that Dhh is reduced in Gli3-heterozygote splenocytes, we hypothesised that erythropoiesis is accelerated in the spleen and BM in this context, compared to WT controls. Total Gli-binding activity using a WT and Dhh-/- GFP reporter line was also investigated here under steady state and stress-induced erythropoiesis. Finally, Chapter 7 summarises all the findings described in results chapters 3 to 6, and suggests future avenues of experimentation.
Chapter Two: Materials & Methods

2.1. Mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
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</thead>
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<tr>
<td>C57BL/6</td>
<td>B&amp;K Universal (UK)</td>
</tr>
<tr>
<td>Dhh&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Gift from Andrew McMahon (Harvard University, Cambridge, MA)</td>
</tr>
<tr>
<td>Shh&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Gift from Philip Beachy (The John Hopkins University School of Medicine, Baltimore, MD)</td>
</tr>
<tr>
<td>Ihh&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Gift from Andrew McMahon (Harvard University, Cambridge, MA)</td>
</tr>
<tr>
<td>Gli3&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Purchased from Jackson Laboratories (USA)</td>
</tr>
<tr>
<td>GBS-GFP</td>
<td>Gift from James Briscoe (Balaskas, Ribeiro et al. 2012)</td>
</tr>
<tr>
<td>mCherry-Dhh</td>
<td>Made as part of my PhD project</td>
</tr>
</tbody>
</table>

Table 2.1. Mice strains were maintained and bred at the University College London (UCL) under the United Kingdom Home Office regulations.

Adult mice were defined as 4 weeks of age or older. All mice were backcrossed onto C57BL/6 for at least 16 generations.

2.2. Genotyping

2.2.1 DNA Extraction

Mouse DNA was extracted from 2mm tail tips or ear biopsies by resuspending these samples in 100 µl of lysis buffer (50mM KCL, 10mM Tris HCL (pH 8.5), 1.5 mM MgCL<sub>2</sub>, 0.01% gelatin, 0.45% Noident P-40 and 0.45% Tween20) and 0.5 µg/ml of Proteinase K (Sigma-Aldrich) in ultra pure water (Life Technologies) and incubated overnight on a shaker at 56°C and 500rpm. Samples were subsequently spun at 13000rpm in a micro-centrifuge for 5 minutes prior to use.
2.2.2 Polymerase chain reaction (PCR)

A PCR reaction was made-up of a 20 µl mix containing 1 µl (~ 1 µg) of template genomic DNA (prepared as described in 2.2.1), 50% 2x GreenTaq DNA Polymerase (Sigma-Aldrich) and 10µM of each relevant primer (see table 2) made up with ultra pure water (Life Technologies). All PCR reactions were run on a Robocycler (Stratagene) according to manufacturer’s instructions with the following conditions (except for Dhh-mCherry and mCherry internal); 5 minutes at 94°C followed by 30-40 cycles for 90 seconds at 94°C (see Table 2.2 for specific cycle number), a primer-specific annealing step (Table 2.2), 60 seconds at 72°C and 10 minutes at 72°C. For Dhh-mCherry (93/88) and mCherry internal primers, PCR reactions were run with the following conditions; 30 seconds at 94°C, followed by 38 cycles for 30 seconds at 94°C, 30 seconds at 60°C, 45 seconds at 68°C and 5 minutes at 68°C. As Shh+/−, Gli3+/− and Ihh+/− were used and not knockouts, only mutated primer pairs were required to screen for heterozygote mice, not WT primer pairs.
<table>
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<tr>
<th>Transcript</th>
<th>Primer Direction</th>
<th>Oligonucleotide Sequence</th>
<th>Annealing T°C</th>
<th>Duration (Sec)</th>
<th>Cycle No</th>
<th>Product Size (bp)</th>
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<td>60</td>
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<td><strong>Dhh mutated gene</strong></td>
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<td></td>
<td>Reverse</td>
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<tr>
<td><strong>Dhh-mCherry (93/88)</strong></td>
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<td>602</td>
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<td><strong>mCherry Internal</strong></td>
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<td>Reverse</td>
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</tr>
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<td><strong>Shh mutated gene</strong></td>
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<td>60</td>
<td>34</td>
<td>~1300</td>
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<td></td>
<td>Reverse</td>
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<td><strong>Gli3 mutated gene</strong></td>
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<td>CTGTGCTGTAGACCAC</td>
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</tbody>
</table>

**Table 2.2.** List of genotyping WT, mutant and mCherry-Dhh primers used for PCR product amplification, together with their respective primer-specific PCR conditions (annealing temperature, annealing duration and total cycle number) and final amplicon sizes. All primers were purchased from Sigma, except for Dhh-mCherry and mCherry Internal that come from IDT.

PCR products were resolved on a gel made up of 1.5 - 2% agarose (Sigma-Aldrich), 1x TAE (Life Technologies) and stained with Gel Red (Biotium). A 100bp molecular weight marker (Life Technologies) was used to estimate band size. After electrophoresis, the gel was analysed under ultraviolet light (Herolab, Germany) and photographed (Sony).
2.2.3. Flow cytometry
Adult GFP reporter mice (GBS-GFP-transgenic line) were genotyped by acquiring 10 000 events from whole blood (2 µl diluted in 500 µl phosphate-buffered saline (PBS)) on the BD FACS Accuri flow cytometer, and measuring FL-1 GFP fluorescence against a known GFP-negative control.

2.3. Mouse cell isolation

2.3.1. Isolation of bone marrow cells
Bone marrow cells were flushed out through the femur via a 25G syringe in cold AIM-V medium (Life Technologies, Gaitersburg, MD) and washed in FACS buffer, made up of 1mM EDTA (Invitrogen), 1.5% Fetal Calf Serum (FCS, Gibco) and 0.01% Sodium Azide (Severn Biotech) in 1x PBS (Sigma Aldrich).

2.3.2. Isolation of splenocytes, testicular and brain cells
Cells from spleen, testicular and brain tissue were obtained by crushing tissue using the sterile flat end of a syringe through a 70 mm filter in AIM-V medium, with a final wash in FACS buffer prior to antibody staining.

2.3.3. Isolation of splenic stromal cells
Mouse spleens were excised and digested in RPMI 1640 medium (Gibco) containing 10% FCS, 1 mg/ml collagenase D (Roche) and 20 mg/ml DNase I (Roche), at 37°C with regular agitation for 30 minutes. Cells were passed through a 70 mm cell strainer and washed in cold RPMI 1640 medium with 10% FCS at 1400 rpm for 5 minutes. Cell pellets were resuspended in 2 ml of RBC lysis buffer (eBioscience) at room temperature (RT) for 5 minutes, followed by two further washes in cold medium and finally in FACS buffer prior to antibody staining (Hou et al., 2010).

2.3.4. Isolation of adult thymic epithelial cells (TEC)
Mouse thymi were excised into ~ 2 mm tissue sections and rolled for 20 minutes in RPMI 1640 with 10% FCS to release thymocytes. After aspirating the medium, the remaining tissue sections were digested in 25 mg/ml Liberase (Roche) and 100 mg/ml DNase I (Roche) for 30 minutes at 37°C with regular agitation. 0.5M EDTA was added to the suspensions, gently mixed and washed in cold medium at 1400rpm for 5 minutes, and once more in FACS buffer prior to antibody staining (Saldaña et al., 2016).
2.3.5. Cell counting
All cells were counted using the Accuri C6 flow cytometer (Becton Dickinson, New Jersey, USA) using a sample of cells diluted 1:20 in FACS buffer.

2.4. In vivo treatment of mice

2.4.1 Sub-lethal irradiation
Adult mice aged between 5 – 10.5 weeks were given a sub-lethal total body dose of 6 Grays (Gy) from a $^{60}$Co gamma-ray source. Mice were placed in ventilated canisters during irradiation.

2.4.2 Phenylhydrazine (PHZ) treatment
Anemia was induced in mice by intraperitoneal injection of 0.6% PHZ (Sigma-Aldrich) in 1x PBS at a dose of 60 mg/kg body weight.

2.5. Antibodies and Flow Cytometry

2.5.1. Surface staining
BM and spleen erythroblasts were analysed using CD71 against Ter119, and granulocytes/macrophages using Gr-1 versus Mac-1. See Table 2.3 for antibody details. All antibodies were diluted in FACS buffer made up of 1mM EDTA (Invitrogen), 1.5% Fetal Calf Serum (FCS, Gibco) and 0.01% Sodium Azide (Severn Biotech) in 1x PBS (Sigma Aldrich). These cell subsets were acquired on the Accuri C6 flow cytometer (Beckton Dickinson) or the LSRII (BD Pharmingen). Live cells were gated using forward scatter (FSC) against side scatter (SSC). Approximately 100 000 events within the live gate were acquired.

BM and spleen LSK and myeloid progenitor cells were analysed by staining with PE conjugated lineage-specific (LIN) antibodies and progenitor markers. Progenitors (defined as cKit+ Sca1.1-) and LSK cells (defined as cKit+ Sca1.1+) were analysed within the LIN negative gate. The progenitor antibody anti-CD34 Ax700 replaced anti-CD34 FITC when staining for GFP+ cells. GMP, CMP and MEP progenitor populations were defined within cKit+ Sca1.1+progenitor cells, as CD16/32+CD34+, CD16/32-CD34+ and CD16/32-CD34- respectively. To ensure accurate gating of GMP, CMP and MEP populations, fluorescence
minus one (FMO) controls were stained in parallel. Approximately 1-1.5 million events were acquired within the live gate to analyse progenitors on the LSRII (BD Pharmingen).

Splenic stromal cells were analysed by staining for CD45, epithelial cell adhesion molecule (EpCAM), CD54 (ICAM-1), CD49e (integrin alpha 5), major histocompatibility complex (MHC) I and MHC II, and analysing all surface markers within the CD45 negative gate. Medullary and cortical thymic epithelial cells (mTEC and cTEC respectively) were analysed by staining for ulex europaeus agglutinin I (UEA-1), Ly51, EpCAM, CD45 and MHC II. mTEC were defined as CD45+EpCAM+ UEA-1+ Ly51- while cTEC were defined as CD45+EpCAM+ UEA-1- Ly51+. mCherry-positive testicular cells and brain cells were acquired unstained for antibodies.

For mCherry positive splenic stromal cells, TEC cells, testicular, brain cells, whole splenocytes and bone marrow cells, approximately 1-1.5 million events were acquired within the live gate on the Aria III (BD Pharmingen) in order to determine mCherry expression using the PE-Texas Red channel. As mCherry spills over heavily into the PE channel, all PE-conjugated antibodies were avoided in these experiments.

All flow cytometry experiments were compensated using whole splenocytes stained for lineage markers with an unstained control. All cells were stained for 30 minutes with the relevant antibody cocktail in 100 – 200 µl FACS buffer in the dark at 4°C, then washed in FACS buffer at 1400 rpm for 5 minutes and filtered through a 70 mm mesh (for cell yields above 1 million) prior to acquisition. For TEC cells, all surface antibodies and UEA1-Biotin were stained in a first 30-minute incubation, washed in FACS buffer and stained for Streptavidin-ef450 alone in a second 20-minute incubation, followed by a final washing and filtering step in FACS buffer.

See table 2.3 for all antibody details.
<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>Fluorochrome</th>
<th>Staining Purpose</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>PE</td>
<td>LIN-</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD3</td>
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<td>LIN-/mCh whole spleen and stroma panel</td>
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<td>PE/PeCy7</td>
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<tr>
<td>CD49e</td>
<td>Anti-armenian hamster IgG Biotin plus Streptavidin APC or APCCy7</td>
<td>mCH splenic stroma panel</td>
<td>eBioscience (unlabelled CD49e &amp; Biotinylated ab), Biolegend &amp; BD (Streptavidin ab)</td>
</tr>
</tbody>
</table>

Table 2.3. List of all surface fluorochrome-conjugated antibodies (ab) used for flow cytometry analysis of progenitors, erythroblasts, granulocytes/macrophages, splenic stromal cells, TEC cells and reticulocytes. CD34 Axf700, CD34 Pacific Blue and CD71 PE were used as alternative antibodies to FITC-conjugated antibodies when staining GFP+ cells.
Reticulocytes were measured by staining murine blood with FITC RETIC-COUNT reagent (BD) in 1x PBS for half an hour at RT according to the manufacturer’s protocol, and acquiring directly on the C6 (BD), LSRII (BD) or Aria III (BD). A FITC-negative PBS control was always stained in parallel. Approximately 100,000 events were acquired uncompensated (FITC only).

All flow cytometry experiments were analysed using Flowjo software v10.0.8r1 (Tree Star, US) or FACSDiva v8.0.1. (BD).

2.6. Histology & Immunohistofluorescence

2.6.1. Paraffin-embedding sections
Mouse spleens were formalin-fixed and cut using a microtome at a thickness of 3 microns by Alex Virasami at the Histopathology Department of Great Ormond Street Hospital (GOSH), London, UK.

2.6.2. Deparaffinisation & antigen retrieval of paraffin sections
Paraffin slides were immersed twice in fresh xylene (Genta Medical), then twice in 100% EtOH, followed by two immersions in 70% EtOH (Haymankimia), with gentle agitation for 5 minutes (all incubations). Slides were then immersed twice in fresh distilled water with gentle agitation for 5 minutes, and once in 0.01 M sodium citrate pH6, made by mixing 9 ml of 0.1 M citric acid (BDH) with 41 ml of 0.1 M trisodium citrate (BDH) and 450 ml distilled water. While immersed in the 0.01 M sodium citrate, the slides were heated in the microwave for 2 minutes at full power, followed by 5 minutes at low power, and finally left to cool at RT prior to antibody staining.

2.6.3. Haematoxilin & Eosin histological staining of spleen
Formalin-fixed spleens were processed for standard H&E staining for white pulp (WP) and red pulp (RP) histology, and analysed under the Nikon Eclipse NI upright microscope with a 2x or 4x objective at the Histopathology Department of GOSH. ImageJ software (v. 2.0.0) was used to measure WP and RP surface area for ratio calculation.

2.6.4. Preparation of cryosections
Mouse organs including spleens, thymi, testes and brains were immersed in optimal cutting compound (OCT, Thermo Scientific), snap-frozen on dry ice and stored at -80°C for future
use. Prior to immunostaining, frozen samples were cut using the BDRC Bright Cryostat machine at a thickness of 7.5 microns and air-dried for 30 minutes in preparation for antibody staining.

2.6.5. Immunofluorescent staining of cryosections and paraffin sections

Cryosections were fixed with 100 µl of 4% paraformaldehyde (PFA) in 1x PBS for 5 minutes and washed three times for 5 minutes in wash buffer, made up of 0.1% Triton X (Sigma) in 1x PBS. Paraffin sections were unfixed at this stage and treated as cryosections from the next blocking step onwards. All sections were blocked with 100 µl of blocking solution, made up of 0.1% Tween (Biorad) and 0.2% Fish Gelatin (Sigma) in 1x PBS at RT for 1 hour. 100 µl of primary antibody diluted in blocking solution was subsequently added for 1 hour at RT in the dark (see Table 2.4 for appropriate dilutions used). After three more washes, 100 µl of secondary antibody diluted in blocking solution was added for 1 hour at RT in the dark. Sections were washed again three times. In the case of spleen sections (which are highly autofluorescent), 100 µl of filtered Sudan Black (Sigma) fluorescence quenching solution (0.1% in 70% EtOH) was applied for 5 minutes at RT and washed twice for 5 minutes with an alternative low-Triton wash buffer (0.02% Triton X in 1x PBS). All sections (spleen or other) were then immersed in DAPI solution (Invitrogen) for 10 minutes and washed once in 1x PBS. Sections were mounted in DPX mountant (Fisher Scientific) and analysed under the BDRC LEICA fluorescent microscope with a 10x, 20x, or 40x objective.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-Shh N-19 (1:40)</td>
<td>Donkey anti-goat Axfluor488 (1:500) Jackson Immune Research</td>
</tr>
<tr>
<td>SantaCruz</td>
<td></td>
</tr>
<tr>
<td>Goat anti-mCherry (1:200)</td>
<td>Donkey anti-goat Axfluor594 (1:500) Jackson Immune Research</td>
</tr>
<tr>
<td>Sicgen</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.4.** Primary and secondary antibodies used in immunofluorescence experiments, with their respective applied dilutions in blocking solution, selected after antibody titration testing.

Spleen cryosections obtained from GFP+ mice were left unstained and simply fixed in 4% PFA, and immersed in DAPI as described above, prior to LEICA microscope analysis.
All fluorescent images analysed from paraffin or cryosections under the LEICA microscope were later analysed using ImageJ software (v. 2.0.0).

2.7. RNA extraction
RNA was extracted using the Absolutely RNA Miniprep kit (Agilent Technologies) for samples containing $1 \times 10^6$ cells or more, according to manufacturer’s instructions. For samples with lower cell yields, the Arcturus Picocure RNA isolation kit (Applied Biosystems) was used using manufacturer’s instructions which includes all samples sent for RNA sequencing (section 2.9). The Qiagen DNA digestion kit was used in both protocols. RNA concentration (ng/µl) and quality (260/280) was evaluated using the Nanodrop (ND-1000).

2.8. cDNA synthesis
cDNA was then synthesized using up to 2 µg of extracted RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s protocol.

2.9. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)
RT-PCR reactions were carried out on the CFX Connect (Biorad) using cDNA, iTaq Universal SYBR Green Supermix (Bio-Rad) and Quantitect primers (Qiagen), according to manufacturers’ instructions, in sample triplicates. Hypoxanthine Guanine Phosphoribosyl Transferase (HPRT) Quantitect primers (Qiagen) were used as a housekeeping gene for normalization. HPRT and target gene amplification was quantitated using a 1:10 dilution series of cDNA synthesized from murine embryo head RNA. A standard PCR reaction was made up of 0.5 µl cDNA of varying concentration, 1µl of combined 0.3M forward and reverse primers, 5ul SYBR Green Supermix made up to 10 µl with HPLC grade water.

The $Dhh$ target gene was run by RT-PCR using Quantitrect primers purchased from Qiagen (Quantitect primer assay). Relative target gene expression against HPRT was calculated using HPRT and target gene mean standard quantities extrapolated from their respective standard curves.
2.10. Molecular Cloning; constructing a conditional targeting vector by using Bacterial Artificial Chromosome (BAC) recombineering to generate an mouse mCherry reporter line for Dhh

Dhh-containing BAC clone RP23-349E22 (BAC PAC Resources) purchased, BAC DNA miniprep and BAC transfer into recombineering-able SW106 bacterial cells. Sections 2.11.5 – 2.11.6.

Construction of Loxp-Neo targeting vector (pl452 in this project). Section 2.11.7.

BAC DNA modification with pl452, completed targeting vector. Section 2.11.8.

Arabinose-Cre mediated neo deletion of modified BAC. Section 2.11.9.

Sequencing of final modified BAC clone (2.2.4), BAC midiprep purification and extra checks before sending for pronuclear injection in mouse embryos Section 2.11.10.

In parallel to BAC project; Efficient fluorescent protein expression (mCherry) confirmed in mammalian HEK293T cells by flow cytometry Section 2.11.11.

Figure 2.1. Overview of the cloning project that generated an mCherry reporter for Dhh using BAC recombineering technology.
Section 2.10 describes the various techniques used in making a new mCherry reporter mouse line for *Dhh*, described in detail in Chapter 3.

### 2.10.1. Recipes for LB broth and agar plates

**Basic LB broth:**
12.5 g LB Broth (Miller Merck Millipore)  
500 ml nuclease-free water (nfw) → autoclaved

**Basic LB-Agar plates:**
12.5 g LB Broth  
7.5 g Agar (Acros Organics)  
500 ml nfw → autoclaved and plated out ~ 20 ml per agar plate

For **CAM+ plates/LB**
1:1000 chloramphenicol (12.5 mg/ml) added in EtOH

For **AMP+ plates/LB**
1:500 ampicillin (50 mg/ml) added

For **KAN+ plates/LB**
1:200 kanamycin (i.e. neomycin, 10mg/ml) added

For **ZEO+ plates/LB**
1:4000 zeomycin (100 mg/ml) added

All antibiotics were purchased at Sigma-Aldrich.

### 2.10.2. Agarose gel electrophoresis

1 – 2% agarose gels were made up by dissolving agarose powder (Sigma-Aldrich) into 1X TAE (Tris-acetate-EDTA) buffer (Life Technologies) and gel red solution (Biotium). The gel percentage was chosen based on size of expected product. A 100 bp or 1 kb ladder solution
with loading buffer was applied to each gel (NEB) depending on expected size of the PCR product, and run in a gel electrophoresis tank (135 V, ADVANCE). The gels were visualised for DNA bands under UV light (Herolab, Germany) and photographed (Sony) to confirm successful PCR amplification.

2.10.3. DNA purification
All QIAGEN DNA purification kits were carried out using the manufacturer’s protocol (Qiagen). Final DNA elution was always carried out in nuclease-free water. DNA was purified in preparation for digestion, ligation or PCR reactions. DNA purification of PCR reactions was carried out using the PCR-purification kit. DNA was purified from excised gel fragments by the gel-purification kit while DNA purification from minicultures was carried out using the miniprep isolation kit.

2.10.4. Sanger sequencing and analysis
5 µl of DNA/sequencing reaction was sent at a concentration of 100ng/µl and primers (IDT) were sent at a concentration of 3µM to Source Bioscience for Sanger sequencing. Sequencing results were analysed using the Staden package. Alignment of sequencing results was determined using an alignment algorithm, Kalign, using a GUI software, EbioX.

2.10.5 RP23-349E22 BAC DNA transfer into SW106 cells
Four 5 ml LB cultures of SW106 cells (see section 3.2.3. for strain details) were made-up and incubated overnight at 32°C. The following day, 2.5 ml was aliquoted from one of the overnight cultures and added into a 500 ml flask containing 50 ml of LB. The culture was incubated for 1 hour at 32°C and the OD value measured using a colorimeter (Cell Density Meter WPA CO 8000). Values fell within the expected range of 0.2 - 0.3. The flask was then chilled in an icebox for 10 minutes with gentle agitation. 10 ml was aliquoted into a chilled 50 ml falcon, and spun at 5000 rpm for 5 minutes at 0°C. The supernatant was discarded and the pellet resuspended in 1 ml of chilled 1mM HEPES (Gibco 15360-080) by pipetting, then transferred to a 1.5 ml eppendorf. The eppendorf was spun at 10 000 rpm for 1 minute at 0°C and resuspended in 1 ml of chilled 1mM HEPES. This wash was repeated twice. After the final wash, the pellet was resuspended in a total volume of 50 µl 1mM HEPES containing 10 µl of BAC DNA solution. Exactly 50 µl of this solution was transferred to a chilled electroporation cuvette (Gene pulser cuvette, Biorad). Electroporation was carried out with the following settings; 1.75 kV, 200 Ω, 25 µF, while the time constant was controlled between 3.7 - 4.5. 1 ml of SOC media (NEB Biolabs) was immediately added at
the end of electroporation and the GF values (ms of voltage) recorded. This 1 ml volume was then transferred to a 15 ml tube and incubated for 1 hour at 32°C followed by centrifugation at 10 000 rpm for 1 minute. The pellet was resuspended in 100 µl of SOC media, plated onto an LB+Cam (12.5 µg/ml) plate and incubated overnight at 32 °C. The following day, four 5ml LB cultures were made up and inoculated each with a single colony. These were incubated overnight on a shaker (200 rpm) at 32°C.

2.10.6. RP23-349E22 BAC DNA miniprep DNA isolation

Two glycerol stocks were made up of transfected SW106 cells from two of the four 5 ml minicultures set up overnight. 500 µl were pipetted from two minicultures (Dhh-1 & Dhh-2), resuspended each in 500 µl of 50% glycerol solution and frozen immediately at -80 °C for future use (streaking of plates for new colonies).

The remaining samples in minicultures Dhh-1 & Dhh-2 were spun at 10 000 rpm for 4 minutes. Each pellet was transferred and resuspended in an eppendorf containing 1 ml PBS. A second 10 000 rpm spin followed for 4 minutes. The pellets were resuspended in 150 µl Buffer P1 (Qiagen) and topped up with 150 µl of Buffer P2 (Qiagen). The tubes were gently mixed by inversion for maximum 5 minutes, topped up with 150 µl of Buffer P3/S3 solution (Qiagen) and again gently inverted. Another 13 200 spin followed for 10 minutes and the supernatants were transferred into two new eppendorfs. 450 µl isopropanol was added to each tube for 5 minutes, the tubes were gently inverted and spun at 13 200 rpm for 5 minutes. The supernatants were discarded and the pellets resuspended in 500 µl of 80% EtOH. A final spin at 13 200 rpm for 5 minutes was carried out, the two supernatants were completely removed, air dried for 5 minutes, resuspended in 30 µl dd-H2O (double-distilled water) and chilled on ice for 1 hour before storing at -20°C.

2.10.7. Constructing a conditional targeting vector in pl452

This step involved inserting mCherry-polyA (monomer Cherry-polyA) (Addgene) as well two homology arm sequences into the original pl452-neo plasmid (National Cancer Institute, USA). The two homology sequences were carefully designed to equate to a specific region of the Dhh gene in the BAC clone to be exchanged with mCherry-polyA during homologous recombination in BAC targeting. mCherry-polyA was made by excising the mCherry sequence from the pFPV-mCherry plasmid (Addgene), ligating it into the excised N1-
FtSlow/pFN1 plasmid (Addgene) containing the polyA sequence, and excising combined mCherry-polyA. See Figures 2.2 - 2.3, and section 3.3.2.

**Figure 2.2.** Schematic of the original pl452 vector containing the neomycin and ampicillin selectable markers, used for generating the targeting cassette containing mCherry (from UCL, USA).
Figure 2.3. Schematic showing the Dhh exon1, coding region in the BAC replaced by mCherry-polyA in pl452, in addition to the flanking homology sequences (H1/H2) of the region to be replaced.

This step involved many PCR reactions, digestion reactions and ligation reactions. See results section 3.3.2 for details. High-fidelity PCR was used to generate DNA fragments used for inserts in various ligation reactions. Standard PCR was used to check for ligation success using ‘colony’ DNA (picked colony diluted in 20µl LB solution) or purified DNA (miniprep or gel purified) as template DNA by running them using gel electrophoresis.

2.10.7.1. PCR reactions
All PCR reactions (Standard/Turbo/OneTaq) used in this project were run on the Peltier Thermal Cycler (MJ Research). All primers were purchased from IDT.

2.10.7.1.1. Standard TAQ PCR reactions
All standard TAQ PCR reactions (NEB reagents) used in this BAC project were run with the following cycle settings; initial denaturation 95°C, annealing temp 60°C and extension temp 68°C for 37 cycles.
### STANDARD TAQ PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>25 μl reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector DNA</td>
<td>0.5 μl/template (~10 – 100 ng)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Taq DNA polymerase 0.125 μl</td>
</tr>
<tr>
<td>10μM Forward/Reverse Primers</td>
<td>0.5 μl/primer</td>
</tr>
<tr>
<td>dNTP 10mM</td>
<td>dNTP 0.5 μl</td>
</tr>
<tr>
<td>Buffer</td>
<td>10X Standard Taq Buffer 2.5 μl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>20.375 μl</td>
</tr>
</tbody>
</table>

Table 2.5. Standard TAQ PCR reaction set up.

#### 2.10.7.1.2. High-fidelity PCR reactions

All high-fidelity Turbo PCR (Agilent Technologies) reactions used in this BAC project were set up with the following cycle settings; initial denaturation 95°C, annealing temp 52°C and extension temp 72°C for 37 cycles.

### PFU TURBO PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>25 μl reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector DNA</td>
<td>0.5 μl (~10 ng)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Pfu turbo DNA polymerase 0.5 μl</td>
</tr>
<tr>
<td>10μM Forward/Reverse Primers</td>
<td>Primers F &amp; R 0.5 μl/0.5 μl</td>
</tr>
<tr>
<td>dNTP (10 mM)</td>
<td>dNTP 0.5 μl</td>
</tr>
<tr>
<td>Buffer</td>
<td>10X Turbo buffer 2.5 μl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

Table 2.6. Standard PFU Turbo PCR set up.

This PCR was used to generate various DNA inserts for ligation reactions. High-fidelity, compared to standard, bears a lower mutation rate during PCR amplification.
2.10.7.1.3. OneTaq PCR reactions for genomic DNA

OneTaq PCR reactions (NEB reagents) were carried out specifically for crude genomic DNA with the following cycle settings; initial denaturation 94°C, annealing temp 60°C, extension temp 68°C for 37 cycles.

<table>
<thead>
<tr>
<th>Component</th>
<th>25 µl reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector DNA</td>
<td>0.5 µl (1 – 10 ng)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.125 µl OneTaq DNA polymerase</td>
</tr>
<tr>
<td>10µM Forward/Reverse Primers</td>
<td>0.5 µl / 0.5 µl</td>
</tr>
<tr>
<td>dNTP (10 mM)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Buffer</td>
<td>5X OneTaq Standard Reaction Buffer 5 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>17.875 µl</td>
</tr>
</tbody>
</table>

Table 2.7. Standard One Taq PCR set up.

2.10.7.2. Digestion reactions

Table 2.8 shows two typical double digestions reactions, using the digestion of FpV as an example. All digestion reactions (NEB reagents) used a relevant digestion buffer specifically tailored to the digestion enzyme(s) used (10% of total reaction volume).
### DIGESTION

<table>
<thead>
<tr>
<th>Component</th>
<th>50 µl reaction</th>
<th>50 µl reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector DNA</td>
<td>FpV (mCherry)</td>
<td>pFN1 (polyA)</td>
</tr>
<tr>
<td></td>
<td>19.5 µl (~760 ng)</td>
<td>3 µl (~260 ng)</td>
</tr>
<tr>
<td>Restriction Enzyme</td>
<td>XbaI 1 µl</td>
<td>Nhel 1 1 µl</td>
</tr>
<tr>
<td>Restriction Enzyme</td>
<td>BsrG1 1 µl</td>
<td>BsrGI 1 1 µl</td>
</tr>
<tr>
<td>Buffer</td>
<td>NeBuffer 2.1 5 µl</td>
<td>NeBuffer 2.1 5 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>23.5 µl</td>
<td>40 ul</td>
</tr>
</tbody>
</table>

→ 1 hour at 37 °C

**Table 2.8.** Two typical double digestion reaction set-ups.

### 2.10.7.3. Ligation reactions

Table 2.9 shows a standard reaction using the Shrimp alkaline phosphatase (SAP) enzyme (NEB). SAP treatment was carried out on linearized vectors prior to carrying out ligation (Table 2.10), to inhibit vector self-ligation by phosphate group removal.

### Phosphatase treatment

<table>
<thead>
<tr>
<th>Component</th>
<th>20 µl reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector DNA</td>
<td>N1 10 µl</td>
</tr>
<tr>
<td>Alkaline phosphatase Enzyme</td>
<td>rSAP 1 µl</td>
</tr>
<tr>
<td>Buffer</td>
<td>rSAPB 2 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>7 µl</td>
</tr>
</tbody>
</table>

→ 37°C for 30 minutes & 65°C for 5 minutes

**Table 2.9.** Standard SAP reaction set-up.
Table 2.10 shows the ligation of N1 plasmid and mCherry as an example (NEB reagents).

<table>
<thead>
<tr>
<th>Component</th>
<th>20 µl reaction (complete)</th>
<th>20 µl reaction (NEG CTRL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector DNA</td>
<td>Modified N1 1 µl</td>
<td>Modified N1 1 µl</td>
</tr>
<tr>
<td>Insert DNA</td>
<td>mCherry 3 µl</td>
<td>N/A</td>
</tr>
<tr>
<td>Ligation enzyme</td>
<td>T4 DNA ligase 1 µl</td>
<td>T4 DNA ligase 1 µl</td>
</tr>
<tr>
<td>Buffer</td>
<td>10X T4 DNA ligase buffer 2 µl</td>
<td>10X T4 DNA ligase buffer 2 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>13 µl</td>
<td>16 µl</td>
</tr>
</tbody>
</table>

→ 16°C for 2 hours

Table 2.10. Standard ligation reaction.

2.10.7.4. **USER strategy for constructing insert (mCherry_polyA-H1) and ligating into conditional vector (pk2 vector)**

USER cloning generates long, complementary custom-made overhangs in both PCR products (H1/mCherry_polyA) and a destination vector (psK_USER). These overhangs can anneal to each other to form a stable hybridization product that can be used to transform DH5α cells without prior ligation. See Figure 2.4.
For the generation of overhangs in the PCR products (at either end of H1 and mCherry_polyA, and in the overlapping region of H1(end) and mCherry_polyA (start)), a short 8-nucleotide sequence that ends in a single deoxyuridine residue was included as an upstream extension in each primer used to amplify the target DNA sequences.

In parallel, overhangs were generated in the destination vector (psK_USER), where digestion with a restriction and a nicking enzyme (PmeI and Nt.BbvCl) create the desired overhangs. The resulting PCR products were treated briefly with USERTM enzyme mix (NEB), which removed the four single deoxyuridine residues at either end and in the overlapping region of the PCR products, enabling the dissociation of the short, single-
stranded fragments lying upstream from the cleavage sites and spontaneous ligation into the pSK_USER vector by complementary binding. See table 2.11.

<table>
<thead>
<tr>
<th>USER LIGATION</th>
<th>16.75 µl reaction (complete)</th>
<th>16.75 µl reaction (NEG CTRL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector DNA</td>
<td>PsK_USER 1 µl</td>
<td>PsK_USER 1 µl</td>
</tr>
<tr>
<td>Insert DNA</td>
<td>H1 arm 7.5 µl</td>
<td>N/A</td>
</tr>
<tr>
<td>Insert DNA</td>
<td>mCherry-PolyA 7.5 µl</td>
<td>N/A</td>
</tr>
<tr>
<td>Ligation enzyme</td>
<td>USER 0.75 µl</td>
<td>USER 0.75 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>N/A</td>
<td>15 µl</td>
</tr>
</tbody>
</table>

$\rightarrow 37^\circ C$ for 15 minutes & 25°C for 15 minutes

Table 2.11. Standard USER ligation reaction set up.

2.10.7.5. Transformation of DH5α cells

A highly competent strain of *E.Coli* bacteria called DH5α (NEB) were used for several transformation experiments in order to express target DNA under various forms;

DH5α cells were thawed on ice for 5 minutes, of which 25 µl was inoculated with 3 µl complete ligation DNA, and the other 25 µl with 3 µl negative control ligation DNA (in certain case the volume of bacterial cells and DNA was increased however the ratio remained the same). After gentle mixing, the two mixtures were put on ice for 10 minutes and then heat shocked in a 42°C water bath for 30 seconds. After heat shock, the tubes were quickly put back on ice for 3 minutes, topped up with 450 µl SOC media and incubated for 10-30 minutes at 37°C on a 250 rpm shaker. Once the incubation time compete, agar plates (with or without antibiotics depending on nature of experiment) were classically streaked with 200 µl, 75 µl and 20 µl respectively of the ‘complete’ transformed bacterial mixture. An agar plate was also streaked with 200 µl of the negative control transformed bacterial mixture. All plates were incubated overnight at 37°C.
2.10.8. RP23-349E22 BAC DNA modification/targeting

The targeting of the original BAC clone within SW106 cells by H1-mCherry-polyA_Neo_H2 (cut from completed pl452 cassette with KpnI/SaclII enzymes) was carried out as follows.

Two chloramphenicol agar plates were streaked with BAC-containing SW106 cells (SW106-Dhh-1 and SW106-Dhh-2 respectively) from glycerol stocks and incubated at 32°C overnight. A good number of colonies were retrieved the following day, from which four 5 ml LB-chloramphenicol minicultures were set up, two from each plate, and left at 32°C overnight.

2.5 ml from two of the four minicultures (one each from SW106 Dhh-1 and Dhh-2) was inoculated into two warm flasks each containing 50 ml of warm LB-chloramphenicol (labelled SW106-Dhh-1/Dhh-2 respectively), and left at 32°C for 1 hour (with agitation). Both solutions were measured for their OD, giving values of 0.19-0.2. 10 ml of each bacterial solution was pipetted into two empty pre-warmed flasks and incubated at 42°C for 13 minutes with gentle agitation. The change in temperature should allow homologous recombination to occur after electroporation. After the incubation, the flasks were chilled in ice water for 10 minutes with gentle agitation, and the contents then transferred into two chilled falcon tubes.

After a centrifugation at 5000 rpm for 5 minutes at 0°C, the pellets were resuspended in 1 ml of chilled 1mM HEPES solution and transferred to 1.5 ml tubes. Three subsequent washes followed in 1mM HEPES at 10 000 rpm for 1 minute at 0°C. After the final wash the pellets were resuspended in 40 µl 1mM HEPES and transferred to a chilled electroporation cuvette. 10 µl target DNA (eluted in nuclease-free water ~1µg) was added to both cuvettes and electroporation was carried out with the following conditions; 1.75kV 200Ω, 25µF. SOC media was immediately added to each cuvette and the total bacterial solutions were transferred to two individual 15 ml tubes for a 1-hour incubation at 32°C.

Three chloramphenicol/kanamycin-containing agar plates were streaked with 10 µl, 100 µl and 700 µl bacterial cells per clone (SW106-Dhh-1 and SW106-Dhh-2 respectively) and incubated at 32°C overnight.
Six minicultures were set up using picked colonies from these plates into 5ml LB+Cam+Kan and left at 32°C overnight. The following day, six glycerol stocks were made by aliquoting 500 µl from each miniculture into 500 µl glycerol and storing them at -80°C. The minicultures were also used for confirming successful targeting by PCR, described in the following section.

### 2.10.9. Arabinose-Cre-mediated neo deletion

Two minicultures of SW106 cells containing zeo-targeted BAC (see section 3.3.5) were used for Ar-Cre-mediated neo deletion.

1 ml of the each culture was inoculated into 20 ml LB+Cam in a 100 ml flask, and incubated at 32°C for 1 hour. The culture was then topped up with 0.2 ml of 10% arabinose, gently mixed and incubated again at 32°C for 1 hour. A dilution series was then set up where 10 µl of the bacterial culture was added to 10 ml of LB-Cam (10⁻³ dilution), with a further serial dilution to provide 10⁻⁴ and 10⁻⁵ dilutions. Four LB-Cam plates were then independently streaked with 50 µl from each dilution series per clone, and incubated overnight at 32°C.

The following day, colonies from all four plates were used to set up six individual minicultures in 5 ml LB-Cam, and left overnight at 32°C. The following day, glycerol stocks were made and frozen while the remaining minicultures cells were DNA-purified using the QIAGEN miniprep isolation kit and diluted 1:10 in nuclease-free water (nfw) for standard PCR experiments.

### 2.10.10. Amplification and purification of BAC DNA for pronuclear injection

The nucleobond midiprep plasmid DNA purification kit (Clontech) was used according to the manufacturer’s protocol to purify BAC DNA in preparation for pronuclear injection of mouse C57BL/6 embryos. The low-copy DNA option for cell lysis, and the NucleoBond Xtra Midi option for DNA precipitation were followed in this protocol. In preparation for this purification, two minicultures of clone 3 and 19 SW106 cells were made in LB-CAM and left overnight at 32°C. The following day, the cells were harvested and treated with the above protocol, once an O.D. value of 2 was reached for each culture. At the end of the protocol, DNA was reconstituted in 200 µl of Tris-EDTA pH8 buffer for each clone, measured on the nanodrop
and sent to the Institute for Viral Research, Kyoto, Japan for pronuclear injection. In a total of two pronuclear injections, approximately 300 pure C57BL/6 eggs were injected and implanted into around 40 C57BL/6 females, giving rise to 30 pups by caesarean section, of which six were screened positive for mCherry. One of the six mCherry positive founders later died around 10 weeks of age of unspecified causes.

2.10.11. Maintenance, breeding and genotyping of mCherry+ mice
The five mCherry+ mice (founders) were transported from Kyoto, Japan to Charles River Laboratories, UK around 8-9 weeks of age where they were maintained, pathogen-tested, ear punched and later bred with respective WT partners. Around 9 months of age all five founders were sent to the Institute of Child Health, UCL where they were maintained and bred under UK Home Office regulations with WT or Dhh+/- partners. Two primer pairs were designed to screen mCherry+ mice; 93/88, which spans a Dhh – mCherry boundary within the inserted BAC, and ‘mCherry internal’ which amplifies a sequence in the middle of the mCherry region. For primer sequences please refer to section 2.2.

2.10.12. Measuring mCherry BAC copy number in mCherry+ mice
Genomic RT-PCR reactions were carried out on the CFX Connect (Biorad) using genomic DNA (gDNA), iTaq Universal SYBR Green Supermix (Bio-Rad) and mCherry primers according to manufacturers’ instructions, in sample triplicates. mCherry primers (109/108) amplifying an internal sequence of mCherry, designed and used in constructing the BAC clone were used here to measure the mCherry copy number in mCherry+ gDNA from all founders and F1. CD25 (130/131) was used as a housekeeping gene for normalization. Prior to RT-PCR, all 11 genomic samples to be analysed (5 mCh+ founders, 5 mCh+ F1 and a WT control) were DNA purified using the GeneElute mammalian genomic DNA miniprep kit (Sigma Aldrich), eluted in 150 µl and measured for quantity (ng/µl) and quality (260/280) on the nanodrop (ND-1000). gDNA concentration ranged from 15 – 125.5 ng/µl.

A standard PCR reaction was made up of 0.5 µl gDNA, 0.5 µl of 10µM forward and reverse primers respectively, 5µl iTaq Universal SYBR Green Supermix (Biorad) made up to 10µl with HPLC grade water. The Quantititect PCR protocol (section 2.8) was run with a modified
annealing temperature of 58°C. Fold expression values in all samples using Ct obtained from 109/108 and CD25 were calculated using the equation $2^{-\Delta Ct}$.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primer Direction</th>
<th>Oligonucleotide Sequence</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mCherry</strong> (109/108)</td>
<td>Forward</td>
<td>GCCGTACATGAACTGAGGGG</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGGCGAGGGAGGATAACATGG</td>
<td></td>
</tr>
<tr>
<td><strong>CD25</strong> (gDNA control)</td>
<td>Forward</td>
<td>CAGGAGTTTCCTAAGCAACG</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTGTGTCTGTATGACCCACC</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.12. mCherry and CD25 baseline control primers used in the Qiagen Quantitect primer assay program to determine mCherry-Dhh BAC copy number in mCherry+ founders.

2.10.13. 293T HEK cell transfection with mCherry/GFP plasmids by lipofection & freezing

The following plasmids courtesy of Dr. Masahiro Ono (Imperial College London), were used for HEK 293T cell transfection in duplicates;

1. pMCs-IRES-mCherry x2
2. pMCs-USER-IG-GFP x2

Prior to transfection, HEK 293 T cells were visualized under the microscope to ensure they were in exponential phase before proceeding. Plasmid DNA was also measured by the Nanodrop and the required volume was determined to obtain ~2 µg.
The lipofection mixture (Fugene, Promega kit) was made up as follows and left at RT for 15 minutes as shown in Table 2.13.

<table>
<thead>
<tr>
<th>TRANSFECTION</th>
<th>91 µl reaction</th>
<th>91 µl reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA (added last)</td>
<td>pMCs-IRES-mCherry (2 µg)</td>
<td>pMCs-USER-IG (GFP) (2 µg)</td>
</tr>
<tr>
<td>Fugene (Promega)*</td>
<td>6 µl</td>
<td>6 µl</td>
</tr>
<tr>
<td>RPMI (no FCS)*</td>
<td>80 µl</td>
<td>80 µl</td>
</tr>
</tbody>
</table>

Fugene and RPMI mixed first and left 5 minutes at RT before mixing with plasmid DNA → 15 minutes at RT

**Table 2.13.** Transfection reaction set up.

After 15 minutes, each 91 µl plasmid mixture was added to a 24-well culture plate containing HEK 293 T cells in a drop-wise manner, and incubated at 37°C for 48 hours. After 48 hours the transfected cells were washed in sterile 1x PBS and treated with trypsin-EDTA. The detached cells were centrifuged in RPMI with 10% FCS for 3 minutes at 1600rpm. Each respective supernatant were discarded and the pellets resuspended in 3 ml PBS with 2 % FCS. The cells were filtered through a 70 mm cell strainer before being transferred into sorting tubes ready for FACS acquisition on the FACS Aria.

Once mCherry and GFP fluorescence by the transfected cells was confirmed on the BD FACSAriaIII, the cells were frozen for future use for compensation purposes. Approximately 10 million transfected cells were spun down at 1400 rpm for 3 minutes and resuspended in 600 µl FCS. 600 µl of 20% DMSO in RPMI was added drop-wise with gentle vortexing, evenly aliquoted amongst three cryotubes and immediately stored at -80°C.

### 2.11. Statistical analyses

A paired t test using the two-tailed p value was used to test the significance of differences between wild-type and heterozygote/knock-out littermates. Values of p <0.05 were considered to be significant.
Chapter Three: Making an mCherry reporter for Desert hedgehog – a Bacterial Artificial Chromosome recombineering approach

3.1 Abstract
This chapter describes the BAC recombineering approach to make a new murine mCherry reporter for Desert Hedgehog. The introduction describes the desirable features of the different cloning elements used in the project, supporting the combination of BAC technology and recombineering as our cloning system of choice. Indeed, in the case of a morphogen such as the Dhh protein, it is important to be able to accurately identify Dhh-producing cells within a field of cells, in order to understand the dynamics of its gradient and location. It was therefore instrumental to carefully consider all cloning options available, in order to determine the best approach in achieving an efficient and reliable mouse reporter for Dhh. The successful acquisition of mCherry-positive founder and F1 mice, and the success in efficiently visualising Dhh-producing cells by mCherry expression via FACS and immunofluorescence, shown in chapters 4 and 5, has validated our approach.

3.2 Introduction

3.2.1. Bacterial Artificial Chromosome (BAC) transgenics
BAC transgenic mice are becoming an increasingly applied tool in basic and biomedical research for studying gene regulation and function, by virtue of their large insert libraries, clone stability and ease of purification. Importantly, BACs are also less likely to cause chimerism (Maximiliano L Suster, 2009).

The BAC cloning system, motivated by the Human Genome Project, came about because of a requirement in the cloning field to generate physical maps of chromosomes, allowing the isolation of short DNA fragments for direct sequencing and other applications. Although the YAC (Yeast Artificial Chromosome) system had already been developed to fit this purpose, and was capable of carrying DNA as large as one megabase (mb), important limitations were soon revealed. The main inconvenience was the impractical requirement to
transfer DNA from yeast cells to bacteria, added to the difficulties of purifying cloned DNA, due to the long and shearing-susceptible linear structure of YACS. The arrival of BAC technology overcame these limitations and thus became a more attractive option to molecular biologists, who are unfamiliar with yeast cells. BAC transgenesis involves *E. Coli* cells, and requires only one direct transfer of DNA for manipulation. This BAC system is based on the *E.Coli* F-factor, a well-known supercoiled, circular low-copy plasmid. This quickly proved to be a stable cloning system, allowing unusually large insert sizes, of up to 300 kb, to be manipulated and efficiently purified during recovery from bacterial cells (Shizuya & Kouros-Mehr, 2001; Warming, Costantino, Court, Jenkins, & Copeland, 2005).

In addition to physical gene mapping and complete genome sequencing, the large availability of BAC libraries stretches to many other functional applications, such as molecular cytogenetics, analysis of single nucleotide polymorphisms (SNP) in regulatory and coding sequences, the rescuing of mutant phenotypes, and the regulated expression of human disease-related genes in mice. Several reporter BACs encoding fluorescent proteins have been designed by ‘recombineering’ in bacteria, allowing the visualization of specific cell types or tissues *in vivo* in mice.

To name a couple of examples of many, BAC technology has been instrumental in the field of neurobiology where the large-scale generation of BAC transgenic GFP reporter mice represented the first real attempt to visualise specific cell populations in the brain by gene expression patterning (Huang & Zeng, 2013; Shizuya & Kouros-Mehr, 2001; Warming et al., 2005). BAC technology was also instrumental in discovering the breast cancer susceptibility gene BRCA1. In the latter example, positional cloning was first used to identify specific loci responsible for this disorder (and others), followed by the analysis of these loci for individual genes using BAC ‘contig assembly’ to create a physical map (Shizuya & Kouros-Mehr, 2001).

For BAC applications in mice and zebrafish, transgenesis of modified and purified BAC DNA is carried out by microinjection into the pronucleus of murine oocytes or the cytoplasm of fertilised eggs in zebrafish. In the case of mice, it is reported that ~ 5-20% of injected embryos that survive and develop successfully integrate the DNA, while this Figure is drastically lower in zebrafish at only ~ 1-3% (Maximiliano L Suster, 2009).
3.2.2. What is recombineering?

The term recombineering, a short hand for recombination-mediated genetic engineering, relates to a new form of chromosome engineering. It allows the modification of DNA via lambda Red-mediated homologous recombination within *E. Coli* cells. One of its numerous applications includes the introduction of *loxP* sites (spanning a selectable marker such as neomycin resistance) and/or a fluorescent marker, by homologous recombination anywhere in a gene of interest. The selectable markers/fluorescent proteins are encoded within a targeting cassette designed with appropriate homology arms spanning the gene of interest contained within a plasmid, BAC or PAC (P1-artificial chromosome). This process allows the gene of interest to be 'targeted'. Using this method, the construction of a targeting cassette is less laborious as it omits the need to find appropriate restriction sites in or near the gene of interest, where these may not always be available (Copeland, Jenkins, & Court, 2001; P. Liu, Jenkins, & Copeland, 2003; Warming et al., 2005).

Because of this system’s efficiency and requirement for short homology sequences, the potential applications for recombineering are vast. As mentioned above, it can be used to insert selectable or non-selectable markers in plasmids, BAC or bacterial chromosome DNA, and to generate transgenic reporter constructs using BAC transgenics, including lacZ and fusion tags such as GFP and mCherry. It can also be used to generate knock-in or knockout alleles in embryonic stem cells, as well as minor modifications in bacterial chromosome DNA or BACs without a requirement for a selectable marker or site-specific recombination. But like any technology, despite its efficiency, this system is not limitation-free. Paradoxically, the practical requirement for short homology sequences means that regions containing many repetitive sequences can be tricky to manipulate if these are genomic areas of interest. Additionally, the target region sequences must be known, but this is not usually an issue as the genomes of the most commonly used organisms have been completely sequenced (Sharan, Thomason, Kuznetsov, & Court, 2009).

3.2.3. Homologous recombination in recombineering-specific SW106 cells

This process of targeting the gene of interest (within a BAC in the case of this project) is possible in variant forms of *Escherichia coli* strains, and is much less laborious and more time-effective compared to working with yeast. The process of homologous recombination itself within these bacterial cells is carried out by specific phage-encoded proteins encoded
by the Red genes of bacteriophage λ for example, allowing double-stranded DNA to be inserted into DNA cloned on a plasmid, BAC or PAC (Copeland et al., 2001; P. Liu et al., 2003). See Figure 3.1.

Figure 3.1. Schematic showing the process of homologous recombination by phage-encoded proteins within recombineering-able E.Coli cells. In the case of the defective λ prophage, three Red genes are present in the prophage called exo, beta and gam. Only exo and beta are required for homologous recombination. Gam however inhibits the RecBCD exonuclease activity of E.Coli. Gene expression is controlled by the strong phage promoter pL, under stringent control of the temp-sensitive repressor, cI857.

In this defective prophage system as shown above, the recombination genes are expressed by a promoter, which is itself controlled by a temperature-sensitive repressor (cI857). At 32°C, the three recombination genes are not expressed, but are induced to high levels as soon as the temperature is increased by 10°C for as little as 10-15 minutes. This allows the point at which homologous recombination takes place, and therefore targeting of your gene of interest, to be carefully controlled - a desirable characteristic in any cloning experiment.
This project made use of the recombineering strain SW106 that, like all recombineering *E.Coli* strains, possesses a temperature-sensitive repressor controlling homologous recombination. This strain specifically also possesses an arabinose-inducible *Cre* promoter and lacks the *galK* gene involved in galactose metabolism. This means that when SW106 electrocompetent cells are exposed to an arabinose source and transformed with a plasmid, for example pl452 containing neomycin and two flanking *loxP* sites, *Cre* recombinase expression is induced. This causes the deletion of the neomycin gene and a single *loxP* site (Sharan et al., 2009; Warming et al., 2005).

As mentioned above, another unique feature of SW106 cells is their lack of the *galK* gene necessary for galactose metabolism, allowing *galK*-based positive and negative selection for the deletions of sections in a BAC clone. *Galk* is one of four genes present in the *E.Coli* galactose operon, the other three being *galE*, *galT* and *galM*, and are required for the growth and utilisation of galactose as the only carbon source. *Galk* encodes galactokinase which catalyses the phosphorylation of galactose in the first step of the degradation pathway. Galactokinase also catalyses the phosphorylation of a galactose analog called 2-deoxy galactose (DOG); this product cannot be further metabolised and leads to a toxic build up. Because of these two functions, *galK* can be used for positive and negative selection of recombinant clones in SW106 cells using the right media (galactose or DOG-rich) (Warming et al., 2005).

### 3.2.4. mCherry fluorescent protein use in reporter lines

mCherry is a bright red monomer fluorescent protein and increasingly chosen in transgenic reporter lines due to its photostability and rapid maturation. The latter allows fluorescent emission to be detected as soon as transcription of the gene of interest is initiated. The monomeric fluorescent protein was derived from the Anthozoa DsRed protein through extensive mutagenesis. Part of the red mFruit protein series, mCherry has a 610 nanometer emission peak, with an intrinsic brightness of approximately 75% of enhanced green fluorescent protein (EGFP). Several studies using mCherry reporter lines have shown bright and stable mCherry expression via a variety of techniques, including flow cytometry and immunofluorescence (Abe & Fujimori, 2013).
3.2.5. Objectives

The objective of this project was to make use of BAC cloning and recombineering in order to produce a faithful mouse reporter for Desert Hedgehog (Dhh) using the mCherry fluorescent tag. In doing so, the goal was to investigate the precise cellular source of Dhh production in the sites of erythropoiesis (spleen and bone marrow), as well as the dynamics between Dhh and other components of the hedgehog signalling pathway relevant to haematopoiesis and erythropoiesis.
3.3. Results; A BAC transgenic approach for generation of a reporter mouse line for Desert Hedgehog

3.3.1. Confirming the authenticity of the Dhh-containing BAC clone RP23-349E22

The BAC clone RP23-349E22 containing Dhh was purchased for this project. To confirm that the purchased BAC clone was in fact RP23-349E22, three primer pairs (IDT) were designed to span three clone regions within the BAC clone (Figure 3.2). These represented the region between Dhh and Lmbr1I (primer pair 1) and the intragenic regions of Tuba1b (primer pair 2) and Wnt1 (primer pair 3).

Figure 3.2. Overview of the originally purchased BAC clone RP23-349E22 before modification, showing Dhh and several other adjacent genes (ncbi).
The following three primer pairs were designed to amplify these three regions:

**Primer pair 1**

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Expected Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP23_349E22_133985F (DHH-LMBR1L)</td>
<td>TCC CAT CCT GGA AAC CAT GC</td>
<td>718 bp</td>
</tr>
<tr>
<td>RP23_349E22_134703R (DHH-LMBR1L)</td>
<td>CAG CCT GCT CTG TGA AGT GA</td>
<td></td>
</tr>
</tbody>
</table>

**Primer pair 2**

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Expected Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP23_349E22_24587F (Wnt1)</td>
<td>ACA CAC CGA CGC TTA CAC TC</td>
<td>288 bp</td>
</tr>
<tr>
<td>RP23_349E22_24875R (Wnt1)</td>
<td>TGT CCT CTC GAA GTC CGT CT</td>
<td></td>
</tr>
</tbody>
</table>

**Primer pair 3**

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Expected Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP23_349E22_169686F (Tuba1b)</td>
<td>GGT TAC CGT TCC CGA CAA CT</td>
<td>359 bp</td>
</tr>
<tr>
<td>RP23_349E22_170045R (Tuba1b)</td>
<td>AAA ACA GCG GAC ATG CCA AG</td>
<td></td>
</tr>
</tbody>
</table>

Two BAC clones of RP23-349E22, labelled Dhh-1 and Dhh-2, were tested using these primers. 6 PCR reactions were therefore set up and run on a 2% agarose gel;

**Figure 3.3.A and 3.3.B. Confirmation of the authenticity of BAC RP23-349E22.** Reactions 1-2, 3-4 and 5-6 correspond to primer pairs 1, 2 and 3 respectively carried out in duplicates on both BAC templates. **A.** An 100 bp ladder (lane 1) was run alongside reactions 1-2-3-4. **B.** Reactions 5-6 (lanes 1 and 2) were run alongside a 1 kb ladder in lane 3.
The electrophoresis gels shown in Figures 3.3.A and 3.3.B confirmed that the purchased BAC clone was in fact RP23-349E22. Now that this authenticity was confirmed, two BAC clones (Dhh-1 and 2) were transferred into two SW106 lines prior to the construction of an mCherry-containing targeting cassette (methods 2.10.8).

3.3.2. Construction of targeting cassette in pl452

The next step in this project was to construct a targeting cassette within the pl452 plasmid, containing mCherry-polyA, and two homology arms (H1 and H2) complementary to short sequences spanning a specific region in the Dhh gene of the original BAC clone. In this manner, the mCherry-polyA protein within the targeting cassette would be later exchanged with the BAC clone. The original BAC clone would therefore be 'targeted' with the completed cassette, allowing mCherry-polyA to be inserted in a specific point of interest in the BAC clone. See Figure 3.4.

![Figure 3.4](image-url)

**Figure 3.4.** Schematic showing the targeting of the RP23-E922 BAC clone by the mCherry-polyA targeting fragment (cut out of the pl452 plasmid once the cassette was completed). Homologous recombination (shown by the crosses) was carried out here on a small coding region of the Dhh gene at the beginning of the 5' end. The vertical black line near the origin of replication represents a LoxP site.
3.3.2.1. Acquisition of Dhh homology arm 2

A high-fidelity PCR reaction was carried out to generate H2 using the following designed primer pair, in order to generate the desired ~500 bp homology 2 DNA fragment;

<table>
<thead>
<tr>
<th>Dhh homology arm 2</th>
<th>GCG GCC GCT CTG GTC TGG TAA CCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP23_349E22_NotI_132755F</td>
<td>GCG GCC GCT CTG GTC TGG TAA CCT</td>
</tr>
<tr>
<td>RP23_349E22_BamHI_133265R</td>
<td>GGA TCC GTA AGG AGA GTT TCT CCC CAC</td>
</tr>
</tbody>
</table>

Desired Homology arm 2 sequence; 3'
TCTGGTCTGGTAACCTGACTCTTGTGAGCTCTTCTTTTTCCAGCCCCCTTCTTTTGGCTCACAGAGCCTGGACAGCACAGCCAGGCAGGCTAAGTGAAGCTTATGCAGTCTACCTATCATAGCCAGCTCCTGGCAGCCAAGCTCCAGTATGGAAGCTGGTGGTGGTAGAGCAGGTGGGGAGAAACTCTTAC

The completed high-fidelity PCR reaction was run on a 1% agarose gel, and this confirmed the amplification of the homology arm 2 (511 bp) containing a BamHI (3') and NotI sequence (5') at either end (data not shown).
3.3.2.2. *Introducing the H2 arm into linearised pl452 vector*

Now that the H2 fragment was generated and confirmed, the next step taken towards constructing the targeting cassette within pl452, was to insert the H2 fragment inside the pl452 vector by ligation, as described in the following steps.

3.3.2.2.1. *Digestion of pl452 vector and H2 arm*

The pl452 vector and H2 arm were both double-digested (linearised) with BamH1 and NotI to generate sticky ends for ligation (Figure 3.5). Both digestion reactions were run on a 1% agarose gel as shown in Figure 3.5.

![Image](image_url)

**Figure 3.5. Linearisation of pl452 and H2.** The gel shows double-digested linearised pl452 (~4.5 kb) and H2 (~0.5 kb), in comparison to a 1 kb ladder (lane 1).

Both bands representing linearised pl452 and H2 were independently excised from the gel and DNA purified. Shrimp alkaline phosphatase (SAP) treatment was then carried out on linearised pl452 prior to ligation.
3.3.2.2.2. Ligation of linearised pL452 with H2 arm and confirmation of insert

A ligation reaction was set up between the gel-extracted insert (H2) and a SAP-treated linearised pL452 vector, and DH5α bacterial cells were transformed with the ligated DNA. The transformed cells were streaked on kanamycin+ plates, and colonies were obtained after an overnight incubation, with low background on the negative control.

Twelve miniculture s were set up in LB-kanamycin and used to obtain concentrated DNA plasmid samples after DNA purification. All twelve DNA samples were double-digested with BamHI and NotI and run on a 1% agarose gel alongside a 1 kb ladder to assess H2 ligation as shown in Figure 3.6.

![Figure 3.6. Clone 5 is positive for H2 (circled).](image)

Figure 3.6. Clone 5 is positive for H2 (circled). A 1 kb ladder (lane 1) was run alongside twelve digestion reactions on a 1% agarose gel.

Figure 3.6 shows that only one clone (clone 5) was positive for the H2 arm shown at the 0.5 kb mark. This clone was sent for Sanger sequencing to confirm the desired H2 sequence, alongside the following primer;

\[
pL452_4511F \quad \text{AGAGCTTGCGGAACCCTTA} \\
\]

Sequencing confirmed that the H2 arm insert had the correct sequence (see Figure 3.7). Kalign alignment showed that our desired H2 sequence (3armR) is fully aligned with the
sequencing results of primer 46, which read the entire homology arm 2 sequence of the sent DNA sample.

Figure 3.7. Sequencing confirms that the H2 arm insert had the correct sequence. Figure shows Kalign alignment of the desired H2 sequence (3armR) with the sequencing result of the H2-containing clone (Primer46 read). EbioX software was used to analyse this sequencing data.

3.3.2.3. Acquisition of mCherry-polyA
The next step in constructing this pl452 targeting cassette was to generate an mCherry-polyA fragment to later ligate inside the pl452-H2 vector. As we were not in possession of a vector containing mCherry followed by a polyA sequence, the following experiments were undertaken to obtain an mCherry-polyA fragment.

3.3.2.3.1. Generating the N1-mCherry vector
Two plasmids called N1-FtSlow/pFN1 (containing a polyA sequence) and FpV (containing mCherry) were digested using NheI/BsrGI and XbaI/BsrGI enzymes respectively, and run on a 1% agarose gel. The FpV (mCherry) plasmid digestion allowed the mCherry sequence alone to be excised. The digestion of the N1 plasmid (containing a polyA sequence
originally derived from the SV40 virus) allowed a sequence named FtSlow to be excised which is equivalent in size to mCherry (~0.9 kb). Both digestion reactions were run on a 1% agarose gel. The excised mCherry fragment and ‘empty’ N1 vector (no longer containing the FtSlow fragment,) were gel-excised and DNA purified. Both DNA samples were run on a 1% agarose gel alongside a 1 kb ladder for band confirmation (Figure 3.8).

![Figure 3.8. Excised and purified N1 plasmid and mCherry fragment confirmed prior to carrying out ligation. Both reactions were run alongside a 1 kb ladder (lane 1).](image)

Shrimp alkaline phosphatase (SAP) (NEB Biolabs) treatment was then carried out on the N1 vector DNA prior to ligation with mCherry. The ligation reaction was set up involving the 'empty' N1 vector and the mCherry fragment with a 1:3 ratio of N1-mCherry vector for improved efficiency, alongside a negative control (no mCherry).

A highly competent strain of *E.Coli* bacteria called DH5α was used for transforming the completed ligation reactions using heat shock. Once transformed, the bacterial cells were streaked onto kanamycin+ agar plates and left to incubate at 37°C overnight. 10-100 colonies were obtained from the positive ligation plates the following day, while 1-2 only
were detected on the negative control plates, indicating low negative background and high transformation efficiency.

### 3.3.2.3.2. Confirmation of mCherry insert into the N1 vector

Four minicultures were set up by picking four colonies from the positive kanamycin+ plates in LB solution. The minicultures were then incubated overnight at 37°C and then DNA-purified. Two of the four DNA samples were sent for Sanger sequencing to confirm the mCherry insert into the N1 plasmid, along with the following primer pair;

FT/-sl-/f/GFP_start_F
ATGGTGAGCAAGGGCGAGGA
EcoRI_polyA_N1_R (70520R)
GAATTCCGCCTTAAGATACATTGATGAGTTTGG

The primer pair allowed both mCherry and the polyA sequence within the N1 vector to be read. eBiox analysis was used to confirm successful ligation of mCherry into N1 by alignment analysis of the sequence result with a known desired sequence file of mCherry (see Figure 3.9).
Figure 3.9. Successful ligation of mCherry into the N1 vector. Figure shows Kalign alignment of desired mCherry sequence with sequencing result of mCherry-containing N1 vector.
3.3.2.3.3. USER ligation of H1-mCherry-polyA into pI452 vector

3.3.2.3.3.1. Obtaining H1_USER and mCherry-polyA_USER fragments & ligation into the pk2 (psK_USER) vector

A high-fidelity PCR was carried out using the following designed primer pairs in order to make two new DNA sequences, namely H1 arm and mCherry with unique sticky ends;

H1 arm
USER_Sall_Dhh_H1_F   GGCCGTT/ideoxyU/GTCGACGGAGGTAGAAGGATGCTTATC
USERj1_mCherry_2_H1_R ACCATGGG/ideoxyU/GCCGTGGACTTGGGTCGGAA

mCherry-polyA
USERj1_Dhh_H1_2_mCherry_F   ACCCATGG/ideoxyU/GAGCAAGGGCGAGGAGGATA
USER_EcoRI_PolyA_R   GGTTGTT/ideoxyU/GAATTCCGCTTAAGATACATTGATGAG

Once completed the reactions were run on a 1% agarose gel to confirm the desired PCR amplification of each product, as shown in Figure 3.10.

![Figure 3.10. Confirmation of the amplification of H1 and mCherry.](image)

H1 and mCherry products at ~500 bp and ~900 bp are clearly shown alongside a 1kb ladder (lane 1) on a 1% agarose gel.

The plasmid/destination vector Pk2 (psK_USER) was then digested by the PmeI enzyme for linearisation, and also treated with the nicking enzyme Nt.BbvCI after an overnight
incubation of Pmel to generate unique sticky ends for later H1, mCherry-polyA and pk2 ligation. The USER ligation was then set up between the vector and two inserts, and the reaction was used to transform DH5α bacterial cells with the ligated pk2 vector on ampicillin+ agar plates. A number of colonies were obtained the following day on all amp+ plates, and there was low background on the negative plate. See section 2.10.7.4 for further details on USER strategy.

3.3.2.3.3.2. **Confirmation of H1 arm and mCherry-polyA insertion into the pk2 vector**

To determine whether any positive colonies were obtained with the mCherry-polyA-H1 insert inside pk2, 6 minicultures were set up to obtain purified DNA for digestions. 6 digestion reactions were then set up using ECORI and SALI and run on a 1% agarose gel;

![Figure 3.1](image)

**Figure 3.11. All clones positive for mCherry-polyA_H1.** ~ 1.4 kb mCherry-H1 products and 2960 kb linearized pk2 vector products are shown for all colonies confirming the insert in each, with a 1kb ladder for reference in lane 1.

Figure 3.11 shows that all clones were positive for mCherry-polyA_H1.

For additional confirmation, two of the six clones were also sent for Sanger sequencing, confirming full alignment and therefore successful ligation of mCherry-polyA-H1 insertion into the pk2 vector.
3.3.2.3.3. Ligation of mCherry_polyA-H1 into the pl452-H2 vector

In order to ligate the mCherry_polyA_H1 fragment into the pl452-H2 vector, the two pk2_mCherry-polyA_H1 clones were digested with EcoRI and Sall in order to obtain the mCh_H1 fragment. The two digestion reactions were run on a 1% agarose gel for mCh-H1 excision;

![Image](image-url)

**Figure 3.12. Digestion of pk2_mCherry-polyA_H1 clones.** Expected ~ 1.4 kb mCh_polyA-H1 fragments are shown for both clones alongside a 1 kb ladder (lane 1).

Both mCherry-H1 fragments shown around the 1.5 kb mark were carefully excised from the gel and DNA-purified (Figure 3.12). Both samples were merged into one. A ligation reaction was then set up between mCh_polyA_H1 and linearised pl452-H2 (cut by EcoRI/Sall) and DH5α cells were transformed with the ligated DNA, and streaked on kanamycin+ plates. Several colonies were obtained the following day on the positive plates.

3.3.2.3.3.4. Confirmation of mCherry_polyA-H1 insertion into pl452-H2 vector

To check for successful ligation, 20 colony PCR reactions were carried out on 20 picked colonies from the kanamycin+ plates (each inoculated in 20ul LB-Amp as a template for the PCR) using the following primer pair;

- **RP23_349E22_Sall_134073R**: GTCGACGGAGGTAGAAGGATGCTTATC
- **EcoRI_polyA_N1_R(70520R)**: GAATTCCGCCTTAAGATACATTGATGAGTTTG
The reactions were run on a 1% agarose gel to check for ~1.5 kb bands corresponding to an mCh-H1 fragment insertion. The gel (data not shown) showed that several colonies were positive for the mCh-H1 sequence, of which two colonies were chosen to further confirm the insertion by setting up minicultures in LB-Kana and purifying DNA from them in the usual way. Two digestion reactions were carried out on the purified DNA representing both clones using EcoRI and SalI and run on a 1% agarose gel to confirm successful mCh_polyA_H1 ligation;

![Image](image.jpg)

**Figure 3.13. Successful insertion of mCh-H1 into pl452-H2.** Both digested clones were run and show an expected band at the ~1.5kb mark of the the 1 kb ladder (lane 1).

Both clones show an expected band at ~1.5kb mark with reference to the 1 kb ladder, confirming successful insertion of mCh-H1 into pl452-H2 (Figure 3.13). For additional confirmation, both clones were sent for Sanger sequencing, confirming successful ligation into the pl452 vector and the correct desired sequence of mCherry-polyA_H1.
3.3.3. Targeting of BAC clone RP23-349E22 with pl452_H1_mCh-polyA_Neo_H2_Dhh

As the targeting cassette pl452_H1_mCh-polyA_Neo_H2_Dhh was now complete and confirmed, the H1_mCherry_PolyA_Neo_H2 fragment within the cassette was excised in preparation for targeting of the BAC clone.

3.3.3.1. Isolation of H1_mCherry_polyA_Neo_H2 fragment

The H1_Mcherry_PolyA_Neo_H2 fragment was isolated from the now completed pl452 targeting cassette (pl452_H1_mCh_polyA_Neo_H2) using a KpnI/SacII double digestion.

The total digestion reaction was run in two lanes in the following 1.2% agarose gel;

![KpnI/SacII digestion of pl452 targeting cassette for excision](image)

*Figure 3.17. KpnI/SacII digestion of pl452 targeting cassette for excision.* Both total digestion reactions were run in two lanes (lanes 2 and 3). The top bands were excised, merged and DNA-purified for BAC DNA modification.

The top bands in both replicates corresponding to the 3.9 kb H1_Mcherry_PolyA_Neo_H2 targeting fragments were excised (lower band DNA corresponding to ~ 2.8 kb 'empty' was discarded). The duplicate excised gel fragments were merged together and DNA-purified (Figure 3.17).
3.3.3.2. RP23-349E22 BAC DNA modification

Now that the H1_mCherry_PolyA_Neo_H2 targeting fragment was acquired, the original SW106 cells containing the original BAC was now targeted with the targeting fragment for temperature-induced homologous recombination using electroporation (methods section 2.10.8). The transformed SW106 cells were streaked on chloramphenicol/kanamycin+ plates, from which 6 colonies were picked and set up as minicultures (LB-KAN/CAM) to purify BAC DNA.

3.3.3.3. Confirming successful BAC RP23-349E22 targeting

Two primer pairs were designed to confirm successful targeting of the BAC clone with the designed mCherry targeting cassette. The primer pairs were designed to confirm the region spanning the outside of homology 2 (5’ side) up to neomycin, and the region spanning mCherry until the outside of homology 1 (3’ side). In order to distinguish random integration and recombination, one of the primers was set outside the cassette. See Figure 3.15.

![Figure 3.15. Schematic showing both designed primer pairs (1 and 2) together spanning the H2 junction, H2, neo, polyA, mCherry, H1 and the H1 junction.](image)

The following primer pairs were designed:

**Primer pair 1 (outside H2 and within Neo)**

Dhh_H2targeting_132524F   GTTCAGTGCCCCTGTAGC  
PL452_4159_H2targeting_R   TCGTGCTTTACGGTATCGCC  
**Expected product size:** 0.918 Kb

**Primer Pair 2 (within mCherry and outside H1)**

mCherry_304_H1targetingF  CCG CCG TCC TCG AAG TTC AT
Six purified DNA samples (targeted BAC) were run with both primer pairs, and the 12 reactions were run on a 1% agarose gel to confirm which BAC clones were successfully targeted as shown in Figure 3.16.

![Image of gel with bands for primer pairs 1 and 2, showing bands at 918 bp and 899 bp, respectively.](image)

**Figure 3.16. Four out of six BAC clones were successfully targeted.** 12 PCR reactions were run; clones 1-6 run on both primer pairs respectively, alongside a 1 kb ladder (lane 1).

Clones 1, 4, 5 and 6 came up positive for the targeting as they produced PCR bands for both primer pairs (Figure 3.16). This was confirmed on a subsequent gel (data not shown), showing that successful targeting of the BAC clone had taken place.

### 3.3.4. Zeo targeting of modified BAC clone RP23-349E22

Now that the Dhh BAC clone was successfully targeted with the neo cassette, the next step to undertake was LoxP site modification to delete a WT LoxP site in the BAC backbone to avoid any unwanted BAC deletions by Cre induction.

#### 3.3.4.1. Acquisition of LoxZeo fragment for Dhh BAC modification

3 µg of the p23LoxZeo plasmid (total length of 4570bp) was digested by EcoRI and the reaction volume was run on a 1% agarose gel by electrophoresis alongside a 1 kb ladder. The lower 1.9 kb bands on the gel representing the targeting fragment (designated as LoxZeo), now cut from the original p23LoxZeo vector (now only ~2.9 kb), were excised from the gel, merged and DNA-purified (Figure 3.17).
3.3.4.2. Dhh BAC modification with LoxZeo

In parallel, four minicultures were made up in LB-CAM-KAN using Dhh BAC-containing SW106 plate colonies obtained in section 2.10.5, in preparation for BAC modification. BAC DNA modification was carried out in the same way as described in methods section 2.10.8.

Three LB-CAM+KAN agar plates were streaked with 20µl, 90µl (first culture) and 200 µl (second culture) bacterial cells and incubated at 32°C overnight. The following several colonies were observed from the plates from which 6 minicultures were set up in 5 ml LB-CAM+KAN+ZEO, and left again overnight at 32°C.

3.3.4.3. Confirming LoxZeo recombination of the targeted BAC locus

Colony PCR reactions on miniculture DNA obtained in 3.3.4.2 were carried out to confirm successful LoxZeo recombination with the targeted LoxP site on the Dhh BAC clone. The following primer pair was used;

**Zeo-23U**

CGGCAAATGGTACTTGTTCACTGA

TCACG

**Zeo-AS**

ACCGCGCTGATGAACAGGGTCAC

GTCGTCC

Expected product size; 1059 bp
After PCR completion, the 6 reactions were run on a 1% agarose gel.

![Image of agarose gel](image)

**Figure 3.18. Successful zeo targeting confirmed.** 1 kb ladder run alongside 6 PCR reactions, as well as negative control and positive controls for loxzeo recombination.

Figure 3.18 confirmed that zeo targeting had been successfully carried out on all clones. The modified Dhh BAC was now ready for neomycin gene deletion from two of these six clones, as described in the next section.

### 3.3.5. Ara-Cre-mediated neo deletion

Two of the six minicultures containing bacteria with confirmed zeo-targeted BAC were used for Ara-Cre-mediated neo deletion (see methods 2.10.9). Once the arabinose neo deletion was completed and the plates streaked, several colonies were obtained the following day from which six individual minicultures were set up in 5 ml LB-Cam, and left overnight at 32°C. Six standard PCR reactions were then set up to check which of the six clones had been successfully neo-deleted, using the following designed primer pair;

- GAGCTTGCGGAACCCTTAAT (forward primer within two loxp sites of neo gene)
- GTT CAG TGC CCC TCT GTA GC (reverse BAC targeting primer outside H2 junction)

**Expected product size:** ~ 900bp

The 6 reactions were run on a 1% agarose gel;
According to the gel run shown in Figure 3.19, clone number 3 (circled) looks neo cassette-deleted and was therefore used for further experiments.

As a second neo-del clone was required, the entire procedure (arabinose-induced neo deletion) was repeated as above to obtain a second neo-deleted ‘clone 19’ (data not shown).

3.3.6. Sequencing of modified Dhh BAC clones 3 and 19

Now that the BAC clone in SW106 bacterial cells had been successfully modified with the targeting cassette, and that the neomycin gene was successfully removed, sequencing of the final modified region of the BAC clone was carried out. Five primer pairs were designed to generate 5 overlapping genetic regions of the cassette by PCR, which could then be sent for Sanger sequencing (Figure 3.20)
Region 1 (R1): Primer Pair 1; spans a region of 500bp outside H2 and within H2

R1F_seqfinal_DhhmCherry
CTGAACCTGCTCTCAGCAATG
R1R_seqfinal_DhhmCherry
TCCTTGGCACGGGAAAGGTA
Expected product size: 413 bp

Region 2 (R2): Primer Pair 2; span H2 arm and loxp site

Dhh_H2targeting_132524F
GTT CAG TGC CCC TCT GTA GC
RP23_349E22_BamHI_133265R
GGATCCGTAAGGAGAGTTTCTCCCCAC
Expected product size: 747 bp

Region 3 (R3): Primer Pair 3; spans mid-H2 arm until the beginning of mCherry

RP23_349E22_Notl_132755F
GCGGCGCTCTGGTCTGGTAACCTGAC
bet_pA_loxp_Foxp39982F_Target1
TGCAGCCCAATTCGGATCA
Expected product size: 602 bp
Region 4 (R4): Primer Pair 4; spans region from LoxP (BamH1 site) to the end of mCherry

```
R4F_seqfinal_DhhmCherry: CCTCGAGGGACCTAATAACTTCG
FT_common_12F: GGGCGAGGAGGATAACATGG

Expected product size: 1051 bp
```

Region 5 (R5): Primer Pair 5; spans mid-mCherry to the outside of H1

```
FT_common_219R: GCCGTACATGAACTGAGGGG
RP23_349E22_134703R: CAGCCTGCTCTGTGAAGTGA

Expected product size: 1604 bp
```

**Figure 3.20.** Figure shows gene region of modified BAC clone (schematic) along with designed primer pairs corresponding to each primer ‘R’ region shown by a horizontal line.

Using these 5 designed primer pairs, 10 PCR reactions were ran to generate the 5 corresponding regions (R1 to R5) for both BAC clones 3 and 19. After PCR completion, the 10 reactions were run on a 1% agarose gel with a 1kb ladder;

![PCR amplification results](image)

**Figure 3.21.** PCR amplification of regions 1 to 5 for clones 3 and 19. 10 PCR products were run alongside a 1kb ladder (lane 1). Products 1-5 (R1-R5) correspond to clone 3, and 6-10 (R1-R5) to clone 19.

Once successful PCR was confirmed on the gel, where all bands corresponded to the expected product size (Figure 3.21), all PCR samples were DNA-purified and sent for Sanger sequencing along with the same 5 primer pairs used to make the PCR products.
3.3.7. Final checks of BAC clones 3 & 19 before sending to Kyoto for pronuclear injection

The midiprep column purification kit was used to purify large SW106 bacterial cultures containing BAC clones 3 and 19 (see methods 2.10.10). Once these two large-scale purifications were completed on each clone, both DNA samples were measured on the Nanodrop for their concentration (ng/µl) and quality (260/280 absorbance ratio);

<table>
<thead>
<tr>
<th></th>
<th>ng/µl</th>
<th>260/280</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 3</td>
<td>80.5</td>
<td>1.98</td>
</tr>
<tr>
<td>Clone 19</td>
<td>800.85</td>
<td>1.915</td>
</tr>
</tbody>
</table>

Table 3.1. Nanodrop values of midiprep purification samples; clones 3 and 19.

To further confirm the high purity of both DNA clones, they were run on a 0.6% agarose gel.

Figure 3.22. Gel shows purity of clones 3 and 19. A supercoiled (SC) ladder (lane 1) was run alongside clone 3 and 19 midiprep-purified DNA.

Figure 3.22 shows the characteristic band ‘smudge’ of supercoiled BAC DNA run without smear, indicating that the DNA was not degenerated.
To ensure the purified DNA samples still had intact mCherry-polyA expression in the desired regions of the BAC clones, standard PCR was carried out. Two primer pairs spanning the mCherry-polyA region, as well as the two outside junction regions spanning mCherry polyA (Figure 3.24), were run by PCR using the following two primer pairs:

**Primer pair 1:**
Dhh_H2targeting_132524F  
GTT CAG TGC CCC TCT GTA GC
bet_pA_loxp_Foxp39982F_Target1  
TGCAGCCCAATTCCGATCA

**Primer pair 2:**
FT common_219R  
GCCGTACATGAACTGAGGGG
bet_pA_loxp_Foxp39982F_Target1  
TGCAGCCCAATTCCGATCA

![Diagram](image.png)

**Figure 3.23.** Regions of interest to be amplified by both primer pairs (represented by upper and lower arrow pair) in BAC DNA for confirmation of sequence integrity.
The four PCR reactions were run on a 1% agarose gel;

![Image of agarose gel showing bands at 700 bp and 1.5 kb for lanes 3 and 19, with a 1 kb ladder.]

**Figure 3.24. mCherry-polyA is intact.** A 1 kb ladder was run alongside standard PCR reactions 1 and 2 (one for each primer pair) for BAC clones 3 and 19 respectively.

Figure 3.24 confirmed that the mCherry-polyA region in addition to the two flanking sequences remained intact after midiprep purification, as both primer pair DNA amplifications took place with the expected product size.

The next step was to design two new primer pairs for genomic DNA to screen pups for pronuclear injection and screening in Kyoto, Japan. The following primer pairs were designed to amplify a ~450 bp region within and outside mCherry (Figure 3.25);

**Primer pair 1**
- mCherry_304_H1targetingF: CCG CCG TCC TCG AAG TTC AT
- Dhh_mC_gPCR2R: GGT ACT CTG GGA CCT TCT CTA TCT

**Primer pair 2**
- RP23_349E22_NotI_132755F: GCGGCCGCTCTGGTCTGGTAACCTGAC
- bet_pA_loxp_Foxp39982F_Target1: TGCAGCCCCATTCCGATCA

Our Kyoto collaborators usually perform PCR reactions using a Taq DNA Polymerase from Thermus aquaticus YT-1 on crude-extracted DNA (i.e. lysed tissue) as template DNA. We therefore chose to use the same polymerase, OneTaq, to optimise the genomic PCR that
would later be used in Kyoto to screen potential mCherry positive pups after pronucleus injection and implantation.

To ensure the designed primer pairs would amplify the region of interest, OneTaq PCR (a PCR reaction mixture adapted for genomic DNA) was run using the primer pairs on crude C57BL/6 WT genomic DNA mixed with BAC DNA from clone 3, as well as on clone 3 BAC DNA alone (positive control), genomic DNA alone and nuclease-free water alone (two negative controls). The crude genomic DNA and BAC DNA were mixed at a ratio of 1:100) to mimic the physiological ratio of BAC DNA to genomic DNA in a potential positive founder pup. Two, rather than one primer pair, were designed to maximize the success rate of the amplification of the region of interest.

![Figure 3.25](image)

**Figure 3.25.** One region of interest that was amplified by both primers (represented by the single arrow pair) in genomic DNA of pups. Both primers amplify a near-identical sequence with the exception of 10-20 bases.
The eight OneTaq PCR reactions (4 reaction conditions run by both primer pairs) were run on a 1% agarose gel;

![Image of gel with bands labeled L, CTRL, BAC+g, g(-), nfw(-), ~450 bp, ~490 bp]

**Figure 3.26. Efficiency of designed primer pairs confirmed.** An 100 bp ladder was run alongside OneTaq PCR reactions 1-8; 4 PCR conditions (BAC clone 3 alone positive CTRL, BAC clone 3 mixed with crude WT genomic DNA, genomic WT DNA alone negative CTRL and nuclease-free water (nfw) negative CTRL) run using primer pairs 1 and 2 respectively.

Figure 3.26 confirmed that both primer pairs were successful in amplifying the region of interest. Both primer pairs were sent to Kyoto along with BAC DNA extracts for pronuclear injection.

3.3.8. Transfection of HEK293T cells with mCherry fluorescent protein

In parallel to generating a Dhh BAC clone with the desired mCherry-polyA sequence in the steps described above, it was important to confirm that mCherry fluorescence is effectively detected by flow cytometry. To confirm this, a human cell line HEK293T (Human Embryonic Kidney 293 T-antigen cells) were transfected by lipofection with two retroviral plasmids; pMCs-IRES-mCherry retroviral plasmid and pMCs-USER-IG-GFP (both courtesy of Dr. Masahiro Ono, Imperial College London). GFP transfection was also carried out for comparison with mCherry (methods section 2.10.13).

Once the cells were transfected, they were filtered and acquired using the FACS AriaIII. The
FACS Aria was compensated with ~1 million untransfected cells (negative control) and single colour GFP and mCherry controls (previously transfected and known to express mCherry and GFP respectively) before acquisition. 10 000 events from the live FSC/SSC gate were then acquired on the four mCherry and GFP transfection samples (transfected in duplicates) and analysed using BD flowjo software. See Figure 3.27 for excitation and emission values of both proteins.

<table>
<thead>
<tr>
<th></th>
<th>Ex (nm)</th>
<th>Em (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type GFP</td>
<td>396,475</td>
<td>508</td>
</tr>
<tr>
<td>mCherry</td>
<td>587</td>
<td>610</td>
</tr>
</tbody>
</table>

**Figure 3.27. GFP and mCherry have distinct emission spectra.** A. The table shows the excitation and emission values of GFP and mCherry (nm). 488nm and 561nm lasers in the BD Aria III were used to activate GFP and mCherry, respectively. B. Figure showing the absorbance and emission spectra of GFP (blue/green) and mCherry (orange/purple).

The transfection experiment was successful and showed that mCherry expression in mammalian cells is well detected with high resolution by flow cytometry (FACSAria III, BD), confirming our choice of fluorescent protein for generating a reporter mouse line for Dhh (Figure 3.27).
Figure 3.28. mCherry is efficiently detected by flow cytometry. Dot plots show untransfected HEP293T cells (negative control), GFP-transfected HEK293T cells and mCherry-transfected HEK293T cells respectively (from left to right). Data was acquired on the BD FACSARia III.

The degree of mCherry transfection was measured at around 60% based on flow cytometry data.

3.3.9. mCherry genomic screening of five founders positive for mCherry-BAC
After successful mCherry pronuclear injection and implantation in female C57BL/6 mice in Kyoto, six mCherry-positive pups were screened and transported to Charles River, UK, of which one died (section 2.10.10). An ear biopsy was taken from each for genomic (g) DNA extraction and purification. The gDNA was screened for the presence of mCherry-BAC using the two validated mCherry primer pairs prior to breeding and an mCherry-negative WT was run as negative control.
Figure 3.29. All five founders; 300, 301, 302, 304 and 306 (lanes 2-6 and 8-12) screen positive for both mCherry primer pairs. Additionally, the WT mCh negative controls (lanes 7 and 13) were both negative as expected. A 1 kb ladder was used as a reference (lane 1). 1.5% agarose gel.

Figure 3.29 confirmed that all five founders 300, 301, 302, 304 and 306 were positive for mCherry, using mCherry primer pairs ‘Dhh-mCherry’ (93/88) and ‘mCherry internal’ (methods section 2.2.2), prior to breeding with WT partners to establish an mCherry line.

3.3.10. Measuring the mCherry copy number in mCh+ founders and F1 mice

Prior to carrying out qRT-PCR experiments to assess the mCherry copy number in the genomes of all mCh-positive founders and F1, a quality control step was carried out. Primers 109/108 (mCherry) and 130/131 (CD25) were checked using a standard genomic PCR on founder mCh-positive 301 and mCh-positive 104, as well as an mCh-negative WT control. Both CD25 and mCherry primer pairs showed a single positive band for both 301 and 104, while the WT control only showed a single band for CD25 and was negative for the mCherry primers. This confirmed that both primer pairs were specific in amplifying the correct region of interest (namely the CD25 gene and a section of mCherry).
Figure 3.30. Genomic qRT-PCR shows mCherry fold expression values using $2^{\Delta Ct}$ in all mCherry+ founders (A) and mCherry+ F1 mice and their parent founder 301 (B). Both graphs show a negative WT control as a baseline reference. $\Delta Ct$ equates to the difference in Ct values between mCherry (109/108) and CD25 (130/131). This data is representative of one of two RT-PCR reactions carried out in triplicates. Errors bars are based on normalized SD of CD25 and mCherry Ct values.
Figure 3.30 A shows that mouse 301 has the highest mCherry copy number of all the founders, while the other founders show expression values within a similar range. Only founder 301 gave rise to transgene+ F1 progeny. Based on this graph and the respective fold expression ratios between 301 and the other four founders, it is reasonable to hypothesise that 301 has a copy number of 5 whilst all other founders have 1. Additionally, the WT mCherry-negative control is undetected, which confirms that PCR product amplification was specific. This further supports the later hypothesis for a copy number of 1 in 4 out of 5 founders, which represents the most common statistic in BAC reporters. Graph B shows more variability even though all the F1 mice (104, 196, 230, 231 and 247) should have the same copy number as their parent 301. This is likely due to variations in DNA concentration and processing reagents used for genomic DNA digestion.
Figure 3.31. Graph shows mCherry (X axis) versus CD25 (Y axis) Ct values (averages of sample triplicates) obtained from genomic qPCR on gDNA of the 5 mCherry-positive founders, 5 mCH+ F1 and a WT (mCh- control). Two lines of best fit were plotted around both groups; founders 300, 302, 304 and 306 (blue line) and founder 301 and his first generation offspring (red line). Plotted Ct values represent the averages of sample triplicates. Duplicate values represent two independent qPCR experiments carried out in triplicates.

Figure 3.31 further supports the hypothesis that 301 and its F1 offspring have the same copy number, whereas the four other founders contain the same copy number as one another, despite variations in RT-PCR values, based on the independent clustering of the two sets of mice on different axes.

The blue line of best fit represents an $R^2$ value of 0.43, whereas the red line represents an $R^2$ of 0.91, both calculated from the gradients of each line. The higher R value representing the red line is far more accurate, being very close to 1.0, and reflects the tight clustering of
each biological replicate along the same axis, as well as the number of biological replicates available. This provides added confidence that 301 and its F1 progeny all have the same copy number (a loss of copy number between generations is extremely unlikely). Although the other founders do not have biological replicates, having not transmitted mCherry to their offspring, it is nevertheless most likely that they have a copy number of 1. This is also reflected by their clustering along the blue line, while the WT negative control being distinctively unclustered. The implications for the differences in mCherry copy number are discussed in section 4.5.1. To exclude technical error, this RT-PCR experiment was carried out twice in sample triplicates on separate days showing very similar fold expression values (shown in Figure 3.31). See methods section 2.10.12.

3.4. Discussion

3.4.1. Rationale for creating a new transgenic reporter for Dhh

Although the already established Dhh-/- mouse line has so far provided much data and clues as to the regulatory function of Dhh in erythropoiesis, the precise location of Dhh expression in the bone marrow and spleen remained unclear. The extent to which the Dhh gene is transcribed in these two sites of erythropoiesis, and how gene activity is affected by different proteins of the Hedgehog family under steady state and stress-induced erythropoiesis was also unclear. No effective antibody exists against the Dhh protein to date due to the high homology of the three mammalian Hh proteins, and previous attempts to identify the localisation of Dhh transcripts through in-situ hybridisation had failed (Outram et al., 2000).

With several successful fluorescent BAC reporter transgenics coming to light (Fiorini et al., 2013; Imayoshi et al., 2013), generating a new reporter mouse line for Dhh seemed like the next logical and promising step in elucidating both Dhh expression in murine organs as a whole, and within specific cells in various tissues. The added advantage to generating a transgenic mouse line is the availability of both flow cytometry and immunofluorescence to look at the expression of your gene of interest. Both techniques give answers at varying degrees of sensitivity and magnitude, therefore providing a bigger picture as to the physiology of the gene of interest when combined together. In this project, endogenous expression of mCherry was efficiently detected by flow cytometry but not by
immunohistofluorescence, entailing the need for an mCherry-specific antibody, which then allowed mCherry expression to be detected in spleen, brain and testicular cryosections. The flow cytometry and immunofluorescence data obtained from this project will be shown in detail in chapters 4 and 5.

### 3.4.2. Choosing BAC recombineering over conventional transgenics

One of the many advantages of BAC cloning is its flexibility to work with large inserts. Based on the size of the mCherry targeting cassette (~ 4 kb) to be constructed and inserted into the large Dhh gene (> 5.5 kb), including adjacent intergenic regions to Dhh, conventional transgenics was not a rational approach (Preston, 2003). Furthermore, such a conventional approach would have been more time consuming without the possibility of being able to target a BAC clone with a large cassette using homologous recombination.

Other cloning approaches include knocking in or out the endogenous gene of interest, however this was not the aim of our reporter model. For our purpose, it was important to preserve the endogenous function of Dhh in order to produce a reporter in which mCherry fluorescence reflects the real physiological expression of Dhh as accurately as possible. For this reason, mCherry was engineered using unique homology arms to replace a specific portion of exon 1 of Dhh in the BAC clone, while the other two exons were left unmodified (Figure 2.3 & 3.4). Replacing this portion of exon 1 with mCherry caused the deletion of the exon 1 signal peptide, allowing mCherry to be expressed (all promoter/adjacent regulatory sequences of Dhh remaining intact) but not secreted in the functional reporter line. This allows effective fluorescent detection in Dhh-producing cells. Maintaining the endogenous and physiological activity of Dhh also avoided the risk of creating dominant negatives, where non-functional Dhh can bind to its receptor and internalise it.

BAC recombineering was therefore the reporter of choice, allowing us to work with large fragment sizes and maintaining the regulatory regions of the Dhh gene important for its physiological expression, by the use of high-efficiency homologous recombination (Farrar & Donnison, 2007; Sharan et al., 2009).
3.4.3. Selecting mCherry as the fluorescent protein of choice

mCherry was the fluorescent protein of choice for making this reporter mouse line due to its stable expression, increasing use in transgenic mouse models (section 3.2.1), and efficient detection by flow cytometry (Figure 3.28) and immunofluorescence (F. Zhang et al., 2011). Prior to starting the BAC project, stable and reliable mCherry expression was confirmed by flow cytometry on transfected mammalian HEK293T cells with a retroviral plasmid containing mCherry (section 3.8).

Additionally, mCherry (red) and GFP (green) have distinctive emission spectra and can therefore be combined. GFP is a common reporter protein of choice, and this allows for the possibility of crossing the mCherry reporter line with other established reporters, namely the Gli binding site (GBS) GFP reporter mouse line for Gli-binding activity (Hh-mediated transcription). Such crosses would allow the detection of two distinct expressions within the same tissues or cells, providing more clues as to the dynamic expression pattern of Desert hedgehog, its interaction with other Hedgehog proteins, and the implications of this in erythropoiesis and other relevant biological processes. Indeed, both fluorochromes are commonly used together in co-localisation and co-expression experiments due to their distinctive spectral properties (Doherty, Bailey, & Lewis, 2010). Due to time limitations, this step has not yet been reached, but will no doubt be carried out once sufficient data has been obtained on the newly established mCherry mouse line.

3.4.4. Determining the mCherry copy number in founders and F1

Based on the mCherry qRT-PCR data obtained on the genomic DNA of all 5 mCh+ founders, 5 mCh+ F1 mice and WT negative control, we hypothesise that founder 301 had 5 tandem repeats of the BAC-mCh clone in his genome, and that he therefore transmitted this copy number of 5 to his offspring (Figure 3.30). Although mCherry fold expression values between 301 and his offspring show variable levels, we are confident that a copy number of 5 was maintained within the second filial generation (as a loss of copy number during transmission is highly unlikely). This is supported by Figure 3.31 which shows that all F1 mice and 301 cluster along the same axis (blue), with a very reliable $R^2$ value close to 1. Despite the same copy number, variability in RT-PCR values (Figure 3.30) is most likely due to differences in how the gDNA of 301 and the F1 mice were digested and purified (different
kits used), with a marked difference in age at which biopsies were taken between founders and F1 mice.

It is also reasonable to hypothesise that all other founders 300, 302, 304 and 306 had a copy number of 1 (common in BAC transgenics), as shown by their tight clustering along the red line (Figure 3.31) and similar range of fold expression values (Figure 3.30), while being unclustered to the WT control. High variability due to technical error was ruled out as all duplicate qRT-PCR reactions were independently repeated and tightly clustered (Figure 3.31). Based on this data, it is reasonable to assume that the other 4 founders had a single BAC-mCh copy in their genome, despite a lack of biological replicates (F1 samples) for each of these founders. This represents the most common copy number statistic in BAC reporter lines. As the other 4 founders did not transmit mCherry to their offspring, it was not possible to relate copy number differences to mCherry expression intensity in younger mice.

It is noteworthy that while all 5 founders were mCherry-positive, they were distinct due to the unique BAC-mCherry integration sites within their genome. However assuming that the BAC was successfully integrated into the genome and not an episome in the nucleus, this would not have affected the founder’s ability to be a true and faithful Dhh reporter. This is because BAC fluorescent expression is driven by the gene of interest’s promoter and enhancers and synchronises with endogenous gene expression, regardless of the random site at which the BAC was inserted into the genome. Indeed, as BAC transgenes are large (between 150 - 200kb of mouse genome) and usually have low copy numbers, they are unlikely to be affected by positional effects after integration, and consequently recapitulate endogenous gene regulation more efficiently than shorter transgenes (M. Liu, Guo, Battle, & Stiles, 2011).

3.4.5. Project limitations
Throughout the BAC project, the main limitation experienced involved inserting homology 1 (H1) and mCherry-polyA within the H2-containing pl452 vector. Although H2 was easily inserted into pl452 via restriction digestion and ligation, followed by a high transformation efficiency of DH5α cells, this approach was not successful to double ligate H1/mCherry-polyA. Despite several attempts using variations of experimental conditions such as incubation time and bacterial cell number used during transformation, the low-probability
double (simultaneous) 'blunt end' ligation of mCherry-polyA with H1 and pl452 did not occur. To circumvent this problem, the ligation of H1 and mCherry-polyA was carried out using USER technology (Bitinaite et al., 2007), which was then successfully singly introduced inside the targeting vector (see 2.11.7.4).

Several months later when breeding the five mCherry-positive founders, issues were faced with low transmission rates of mCherry in founders to their offspring. Within the year and a half that all 5 founders were being bred with WT partners, only one male founder (301) successfully transmitted mCherry to his offspring, and therefore became the focus for breeding. Although 301 did transmit mCherry, this founder also exhibited low transmission rates. It is not uncommon to see low non-mendelian transmission rates between founders and F1, as pronuclear injection may have induced undesirable mutations or chromosomal aberrations. These chromosomal aberrations are progressively ‘cleaned out’ as the transgene is passed through generations however, which is why BAC reporter founders are always crossed with WT partners to avoid the creation of undesirable homologous loci. Indeed, although mCherry transmission from 301 to his offspring was low, Mendelian rates of mCherry transmission were quickly achieved between F1 and F2 mice after 2-3 litters.

Additionally F1 mCherry+ mice always appeared runty at birth and for the first few weeks of life. This may be linked to other genes present on the BAC clone, such as Wnt1, interfering with normal development. Due to these low transmission rates, the target number of mCherry experiments was not achieved for this PhD. However thanks to two healthy F1 female mCherry mice from 301 born in December 2015 and January 2016 respectively, who continue to breed well and transmit mCherry at a Mendelian rate, the line has finally been established. This should allow a range of future experiments to be carried out beyond the PhD.

3.5. Conclusions
In conclusion, an mCherry reporter for Desert Hedgehog has been successfully created and shown to be a faithful reporter for Desert Hedgehog, as shown in chapters 3 and 4. This validates our approach in selecting both BAC technology and recombineering as a time-effective and efficient cloning system of choice. Choosing mCherry as our fluorescent protein tag allowed effective detection by flow cytometry and immunofluorescence due to its
photostability and brightness. Having distinctive emission spectra to GFP, mCherry was an obvious choice, enabling the possibility of crossing the newly established reporter line with other established and relevant GFP reporter lines in the future. This step was not achieved during the PhD due to the late establishment of the new mCherry line, owing to time limitations and low mCherry transmission rates. This will however be part of a long agenda of experiments that should be realised after the PhD, which also includes stress-related erythropoiesis experiments.
Chapter Four: The validation of a new mCherry reporter mouse line for Desert Hedgehog

4.1. Abstract
The successful establishment of our new mCherry reporter mouse line for Dhh produced mCherry-positive F1 mice, of which some were kept to maintain the mouse line, and others used for validation. This chapter describes the validation experiments that were carried out on both founder and F1 mCherry-positive mice described in chapter three, in order to determine the efficiency of the new line as a faithful reporter for Dhh expression. Fortunately, our validation experiments confirmed our line as an accurate reporter of Dhh physiological expression. Indeed, our analysis of Dhh expression by mCherry readout in the testes, brain and thymus mirrored expected patterns of expression in Sertoli cells, the brain and thymic epithelial cells (TEC) of the thymus respectively. Additionally, we found significantly higher mCherry expression in Dhh+- tissue compared to WT (Dhh+/+)
tissue that were both positive for mCherry. We also found that Dhh is expressed in medullary TECs (mTECs) but not in cortical TECs (cTECs), a trend that is supported by recent microarray and RNA sequencing (RNA-Seq) data. The successful validation of our line set the stage for the subsequent set of experiments described in the next chapter (chapter 5), which shows our in-depth analysis of Dhh in the spleen.

4.2. Introduction
Dhh is known to be expressed in the thymus, testes and brain, and its expected expression pattern and function in these three tissues is discussed below.

4.2.1. The thymus

4.2.1.1. Adult murine thymic epithelial cells
Thymic epithelial cells (TECs) are an important constituent of the thymic stroma of the thymus, alongside dendritic cells, endothelial cells, macrophages, neural crest-derived pericytes, fibroblasts and other mesenchymal cells (Xing & Hogquist, 2014). They are essential for normal T cell development in the thymus where they specify T cell fate, and
present peptide antigens (self antigen presentation) that interact with T cell receptors (TCRs) on developing thymocytes. In doing so, they provide a unique microenvironment for T cell development. TECs are subdivided into cortical thymic epithelial cells (situated in the thymus cortex) and medullary thymic epithelial cells (situated in the thymus medulla) (Danzl, Jeong, Choi, & Alexandropoulos, 2014) (see Figure 4.1).

**Figure 4.1. Schematic of the murine thymus and stromal cell interactions involved in T cell development.** After T cell receptor β-selection, double-positive (DP) cells move throughout the cortex and interact with cTECs (blue) for positive selection. After CD4 or CD8 lineage commitment, single-positive (SP) thymocytes rapidly move to the medulla and interact with medullary antigen-presenting cells including dendritic cells (DCs, purple) and mTECs (pink), for their entire residency.

Specifically, cortical thymic epithelial cells (cTECs) provide Delta-like ligands to activate Notch1 in early progenitors, and specify T cell fate. They are also required for positive selection of thymocytes, and express genes for antigen presentation, including Cathepsin-L and Prss16. Medullary thymic epithelial cells (mTECs) mediate negative selection of auto-
reactive T cells by expressing the Aire gene and Cathepsin-S. The upregulation of RANKL (the receptor for the NF-kB ligand) and CD40 ligand (CD40) on positively selected thymocytes has been found to regulate and maintain mature mTEC development, highlighting the importance of cross talk between thymocytes and TEC (Danzl et al., 2014; Saldaña et al., 2016). Both types of TECs are believed to originate from a common embryonic progenitor although this is not yet fully understood.

In the adult thymus both types of TECs are negative for CD45, and positive for Epcam1, CD40 and MHC Class II (MHCII). However cTECs are positive for Ly51 and CD205, while mTECs are negative for these two but stain positive with plant lectin ulex europeus agglutinin-1 (UEA-1). mTECs are further classified into three distinct subpopulations by their varying expression of CD80/86, MHCII and Aire (St-Pierre, Trofimov, Brochu, Lemieux, & Perreault, 2015). Immature and mature mTECs are defined by low or high expression of CD80 and MHCII molecules respectively, in which mature Aire+CD80$^{\text{Hi}}$ or Aire+ MHCII$^{\text{Hi}}$ develop from immature Aire-CD80$^{\text{Low}}$ or Aire-MHCII$^{\text{Low}}$ mTECs. The notion that Aire is expressed by the mature subset has been supported by the finding that Aire + mTECs are post-mitotic (Nishikawa et al., 2010).

4.2.1.2. The role of Hh proteins in thymic epithelial cells
Shh is secreted by TECs in both foetal and adult mouse thymus. In humans and mice, immunohistochemistry has shown Shh to be expressed by epithelial cells in the subcapsular region, as well as in the medulla and cortico-medullary junction (Crompton et al., 2007). To corroborate this data, Shh transcripts were also detected in both cTECs and mTECs in microarray datasets carried out on purified TEC populations (Barbarulo et al., 2016; Saldaña et al., 2016).

Immunohistochemistry and RT-PCR analysis also identified Indian Hedgehog (Ihh) expression in stromal cells (Outram et al., 2000; Sacedon et al., 2003). Furthermore, Ihh expression was also found in double positive (DP) thymocytes as well as the stroma, in E16.5 murine fetal thymi (Outram et al., 2009; Sacedon et al., 2003). Dhh has been shown to be expressed primarily by stromal cells throughout human thymic parenchyma but not in thymocytes, by both RT-PCR analysis and immunofluorescence (Sacedon et al., 2003). More recently, the role of Hedgehog signalling in TEC differentiation has been investigated.
TECs were found to actively transduce Hh signals in both foetal and adult thymi through a Gli Binding Site GFP reporter mouse. Interestingly, active Hh signalling was reportedly higher in mature mTECs compared to cTECs. The specific role of Shh was then investigated in normal TEC differentiation. Shh-deficient foetal thymus organ cultures (FTOC) exhibited fewer TECs compared to WT controls, while Gli3-deficient thymi, which are generally believed to have higher overall Hedgehog signalling, exhibited increased numbers of TECs. Taken together, these observations attributed Shh a new and important role in TEC development (Saldaña et al., 2016).

It is noteworthy that Hedgehog proteins are required for T cell development in the thymus. Indeed, mRNA studies have shown that thymocytes can transduce the Hh signal, as Ptc1 and Smo are present in foetal and adult thymocytes (Outram et al., 2000). Sonic Hedgehog (Shh) has been found to be a key player in the regulation of early T cell proliferation and differentiation, while Indian hedgehog has also been found to regulate T cell development and homeostasis in adult and foetal thymi by controlling thymocyte numbers (Outram et al., 2009, 2000; Saldaña et al., 2016; Shah et al., 2004).

So far, Shh has been the only morphogen that has been directly implicated in TEC differentiation. A role for Ihh has only been identified in T cell development, while Dhh has not yet been attributed any functional role in the thymus as a whole.

4.2.2. Desert Hedgehog in murine testes, Schwann cells and the brain
The mouse Desert hedgehog (Dhh) gene is mapped to the distal region of chromosome 15 (Marigo et al., 1995). Dhh is expressed by the Schwann cells of peripheral nerves and controls the development of peripheral nerve sheaths. Dhh upregulates Patched 1 (Ptc1) and Hip (Hedgehog-interacting protein) in nerve fibroblasts (Parmantier et al., 1999). Indeed, Dhh deficiency both in mice and humans causes peripheral neuropathy (Renault et al., 2013; Umehara et al., 2000). Dhh is also known to be expressed in the mouse brain (Hegde et al., 2008), but not as highly as Shh (Traiffort, Angot, & Ruat, 2010).

Dhh is also expressed in the Sertoli cells of the male testes, but not the ovary in females. By generating a Dhh knockout mouse line, Dhh was found to be produced in Sertoli cell precursors soon after Sry (sex determining gene) activation, highlighting Dhh as a crucial
protein in mammalian spermatogenesis. Sertoli cells are the multifunction ‘nurse’ cells of the testes situated within the seminiferous tubule. They offer structural support to surrounding germ cells, guide sperm cells towards the lumen and control the availability of nutrients for germ cells. They also express androgen receptors for testosterone and follicle-stimulating hormone (FSH), two essential hormones in spermatogenesis (Griswold, 1998) (Figure 4.2).

Consistent with these findings, Dhh knockout male mice are viable but infertile due to a lack of mature sperm production. Additionally, they show a 90% size reduction of their testes by 6 weeks compared to WT littermates. Females on the other hand do not show any obvious phenotype (Bitgood et al., 1996).

![Figure 4.2. Schematic of the seminiferous tubules of the testes, and the location of sertoli cells, leydig cells (involved in testosterone production), spermatogonia (undifferentiated male germ cells) and resultant spermatozoa within the seminiferous tubule lumen. Sertoli cells are the epithelial cells of the seminiferous epithelium that extend from the basal lamina to the lumen, where they release mature spermatozoa in the latter (spermiation). Spermatogonia (diploid) undergo mitosis and two meiotic divisions before becoming mature spermatozoa (haploid).](image)

Interestingly, *Ptch* was also shown to have male-specific transcription restricted to Leydig
cells (involved in testosterone production) of the gonad, and this is completely abrogated when *Dhh* is absent. This indicates that while Sertoli cells are the source of Dhh production, Leydig cells may be the direct targets of Dhh signalling (Bitgood et al., 1996). Such findings are consistent with human studies. One study described a patient with a homozygous missense mutation in the start codon of exon 1 of the *Dhh* gene 46, which was associated with both XY partial pure gonadal dysgenesis (PGD) and minifascicular neuropathy (Umehara et al., 2000). Individuals with this disorder have a 46, XY karyotype, normal Mullerian ducts, bilateral streak gonads and female external genitalia (Berkovitz et al., 1991). Another study reported three cases of 46, XY pure PGD were attributed to two *Dhh* mutations; a homozygous L162P mutation of exon 2 and a homozygous 1086delG mutation in exon 3 (Canto, Söderlund, Reyes, & Méndez, 2004). Dhh expression in the spleen is also documented, where it is believed to be restricted to the splenic stroma (Lau et al., 2012; Perry et al., 2009). This will be discussed in detail in the next chapter, section 5.2.1.3.

### 4.2.3. Objectives

This chapter describes the first experiments carried out on the newly established mCherry reporter for Desert Hedgehog to test the hypothesis that our line is a faithful reporter for Dhh. To this end, immunofluorescence and flow cytometry were carried out on tissues and cells where Dhh expression is documented, such as the brain, testes and thymic epithelial cells of the thymus.
4.3. Results

The following results section describes the mCherry validation experiments that were carried out in the testes, brain and thymus. For reference, all mCherry-positive mice contain two copies of endogenous Dhh (Dhh+/+) and are therefore described as ‘WT’.

4.3.1. A fraction of F1 mice are positive for mCherry by genomic PCR

As with the five positive mCherry founders (300, 301, 302, 304 and 306) that were obtained from our mCherry BAC project (Chapter 3, Figure 3.32), genomic PCR also confirmed the acquisition of new F1 mCherry-positive pups from our 301 mCherry founder only (Figure 4.1). Indeed, for all genomic PCR experiments on F1 litters, the parent 301 or another mCherry-positive founder was used as a positive control, while an mCherry-negative WT always represented the negative control, as shown in Figure 4.3.

Figure 4.3. Typical genomic PCR showing that one F1 pup out of a 6-pup litter is mCherry-positive (sixth ‘F1’ band). The F1 positive mCherry band (amplified by the ‘mCherry internal’ primer pair) shows the same degree of amplification as the positive control (founder 301), while the negative control (mCherry-negative WT) did not show any amplification. The litter was derived from founder 301 crossed with a WT mCh-negative female and the mCherry amplicon product is 208 bp.

Figure 4.3 shows a typical genomic PCR carried out with the same primer pairs used to test the founders, showing that F1 mCherry pups show the same band intensity as the positive founder control. Once mCherry-positive pups were acquired, a few aged between 2.5 and 6
weeks were used for the analysis of their thymus, testes (in males) and brain to validate the line, as described in the next section. Our 301 founder aged 1 year and 7 months was also culled after acquiring an untreatable eye infection, and used in validation experiments. Testes were obtained from this founder for validation, but not the thymus, due to the impact of age on thymic involution and significantly low numbers of TECs as a result. This validation was a necessary step prior to carrying out larger scale experiments on tissues, in which the pattern of Dhh expression is less known and under investigation.

4.3.2. Dhh (mCherry) is expressed in murine testicular cells and brain tissue

As described in the introduction, the testes, as well as peripheral nerves and the brain, are well known sources of Dhh for its role in spermatogenesis and Schwann cell function, respectively (Bitgood et al., 1996; Parmantier et al., 1999; Renault et al., 2013). The testes were therefore obvious organs of choice in a first instance to measure mCherry expression by flow cytometry and immunofluorescence in newly established F1 WT (Dhh+/+) and Dhh+/- mCherry-positive mice. The brain was also analysed in parallel despite its lower expression of Dhh (Hegde et al., 2008).
Figure 4.4. Dhh (mCherry) is detected by flow cytometry in the testes, especially in Dhh+/- tissue compared to WT, but not in the brain. A. Dot plots representative of mCherry Dhh expression in the testes of a 4-week old F1 Dhh+/- mCh+ reporter mouse (top left) and F1 WT (Dhh+/+) reporter mouse (bottom left), with their respective mCh- WT littermates as controls (top and bottom right). Dhh+/+ data is representative of four experiments, and Dhh+/- of one experiment. B. Dot plots representative of mCherry Dhh expression in undigested whole brain tissue of WT founder mCh+ 301 (left) and its WT mCh- littermate control (right).

Figure 4.4 A shows that Dhh expression by mCherry fluorescence emission is detectable in the testes in both WT (Dhh+/+) and Dhh+/- mCherry-positive mice by flow cytometry. The Dhh+/+ data is representative of four mCherry-positive WT mice that were measured for testicular mCherry expression, all falling within the range of 8 to 13.9% mCherry expression. mCherry gating was based on a WT mCherry-negative control. Interestingly, this Figure shows that mCherry expression was more highly detected in the testes of the Dhh+/- mCherry mouse, with around 2-fold higher than the WT. Figure 4.4 B shows that Dhh expression by mCherry is not detectable in the WT brain of the mCherry founder 301 despite its known expression in the brain.

Taken together, our analysis showed that Dhh expression by mCherry emission is efficiently detectable in tissues of high Dhh expression such as the testes by flow cytometry, but not in the brain, where Dhh is less expressed. This preliminary data also showed us that our mCherry reporter mirrors true Dhh expression in these tissues, and that losing a copy of Dhh promotes further Dhh gene promoter binding, and therefore higher mCherry emission. It is noteworthy that BAC reporters reflect endogenous activity of the gene of interest, as the
fluorescent-containing BAC construct integrated in the genome synchronises with endogenous transcription of the gene of interest, in our case, *Dhh*. 
Figure 4.5. Dhh (mCherry) is more highly expressed in reporter Dhh+/- testes compared to WT, and also well detected in the reporter WT brain by immunofluorescence. A. Immunofluorescence images (20x resolution) of testes cryosections obtained from a 6-week-old WT mCh- mouse stained with anti-mCherry antibody (left), and its WT mCh+ (104) littermate without antibody staining (right). B. Top panel; Immunofluorescence images of the WT 104 mCh+ testes cryosections stained with anti-mCherry antibody at a 20x resolution (left) and 10x resolution (right). Bottom panel; Immunofluorescence images of testes cryosections obtained from a 4-week old Dhh+/- mCh+ (230) stained with anti-mCherry antibody at a 20x resolution (left) and 10x resolution (right). C. Immunofluorescence images (20x resolution) of brain cryosections obtained from a 6-week-old WT mCh- mouse (left) and its WT 104 mCh+ littermate (right), both stained with anti-mCherry antibody. Goat anti-mCh antibody (Sicgen) and a donkey anti-goat secondary Ax594-conjugated antibody (Jackson Immune Research) were used to stain cryosections, together with DAPI, a nuclear counter-stain. All immunofluorescence images were acquired on the LEICA fluorescent microscope.

Figure 4.5 A shows that the negative WT mCh- control (left) is in fact negative, as no Alexafluor594 was detected in the red channel, confirming that the mCherry fluorescence that was detected in the mCherry sections (panels B and C) represented true expression. The 104 mCh+ testes section was that not stained with anti-mCherry antibody did not reveal any fluorescence expression, which suggests that endogenous expression by our reporter line is detectable by flow cytometry but not by immunofluorescence. Figure 4.5 B (top panel) shows clear mCherry expression in the seminiferous tubules of the testes in the WT mCh+ mouse. The 10x resolution provides a clear image of the delineation of the testes tubules compared to the 20x resolution. The images show high Dhh expression as mCherry fluorescence deep within the tubules in the Sertoli cells, whilst the basement membrane remains negative for mCherry. This mirrors published reports describing Dhh’s known expression within Sertoli cells, where it plays an important role in spermatogenesis (Bitgood et al., 1996).
The bottom panel of Figure 4.5 B shows the same pattern of mCherry expression within the Sertoli cells of the seminiferous tubules of a Dhh+/− reporter mouse, and also shows higher expression compared to the 104 WT, which is consistent with our flow cytometry testes data (Figure 4.4). Interestingly, Figure 4.5 C shows that Dhh by mCherry expression is efficiently detected in the brain of the WT mCh+ mouse by immunofluorescence, and not in the WT mCh- littermate, showing true Dhh expression. This suggests that Dhh in the brain is only detectable using an antibody (immunofluorescence), but not high enough to be detected endogenously (flow cytometry).

Taken together, the flow cytometry and immunofluorescence data show that endogenous mCherry is efficiently detected by flow cytometry when Dhh is highly expressed in tissues, and detectable using an anti-mCherry antibody in tissues with low expression. The data also shows that our new mCherry line is a faithful reporter for Dhh expression, as our analysis mirrored expected patterns of high Dhh expression in Sertoli cells of the testes, and significantly lower expression in the brain.

4.3.3. Dhh (mCherry) is more highly expressed in mature medullary thymic epithelial cells (mTECs) than cortical TECs (cTECs)

Although the testes and brain data shown above provided satisfactory validation of our new mCherry reporter line, we chose to extend our analysis to the thymus. As part of our extended validation, this organ was of interest to us, as Dhh expression has been documented in thymic epithelial cells (TEC) yet its precise expression within particular TEC subsets remains debateable (Barbarulo et al., 2016; Sacedon et al., 2003; Saldaña et al., 2016). This section describes our in-depth mCherry analysis of TEC subsets within the thymi of mCherry mice as part of an extended validation of the reporter line, but also to improve our understanding of the dynamics of Dhh expression in thymus.
**Figure 4.6.** Dhh (mCherry) is expressed in medullary thymic epithelial cells and not cortical thymic epithelial cells of the thymus. **A.** Panel A shows the gating strategy for thymic epithelial cells. cTEC and mTEC subsets are gated as Ly51+ UEA1- and Ly51- UEA1+ respectively, within CD45- EpCAM+ epithelial cells that are positive for CD40 and MHC Class II. **B.** Histograms representative of Dhh mCherry expression (red) in CD45- EpCAM+, CD45- EpCAM-, CD45+EpCAM-, mTEC and cTEC cells from a 6-week old F1 mCh+ WT (Dhh+/+) reporter overlaid with its WT mCh-control littermate (blue). Data is representative of three experiments.

Figure 4.6 A depicts the gating strategy employed to analyse TEC subsets, as mentioned in the introduction. Cells that were CD40 and MHCII positive were gated on thymic epithelial cells that were negative for CD45 and positive for the epithelial cell adhesion molecule (EpCAM). Gating on CD45-positive EpCAM-negative leucocytes that are largely negative for CD45 helped to define the positive gate for CD40+CD45-EpCAM+ TEC cells. Figure 4.6 B shows that Dhh by mCherry fluorescence is expressed in CD45-negative epithelial cells, which are positive for EpCAM, but barely detected in CD45-negative EpCAM-negative non-TEC stromal cells (Rode et al., 2015), and CD45-positive leucocytes of a 6-week old WT (Dhh+/+) mCh+ mouse. The observed trends confirm published reports that describe stromal cells of the thymic parenchyma, and not thymocytes, as the source of Dhh in the
thymus (Sacedon et al., 2003), which further validates our new mCherry mouse line as a reporter for Dhh. Interestingly, when looking at mTEC and cTEC subsets in the same Figure, mCherry expression was detected in the former but not in the latter. This supports recent microarray and RNA-Seq data on adult and foetal thymi respectively, as described in the discussion.

4.4. Discussion

Once our new mCherry reporter mouse line for Dhh was established, and the acquisition of mCherry-positive pups from our original mCherry-positive founder 301 was confirmed by genomic PCR as shown in section 4.3.1, validation experiments were carried out. Following the establishment of a reporter line, the validation stage is instrumental, as it determines whether the new reporter in question accurately reflects true physiological expression of the gene of interest in cells and tissues. In terms of validation, genomic PCR is limited as it only confirms whether the BAC construct containing the fluorescent protein is present in the nucleus, but not whether genomic integration actually took place or whether the reporter is expressed. Indeed, in rare cases, the BAC construct may sit in an episome within the nucleus outside the genome, in which case it cannot act as a reporter, but will still be amplified by genomic PCR. Another rare possibility is that the BAC construct is efficiently integrated in the genome, but not expressed (known as a positional effect), as the random integration may have inserted it into a silent part of the genome (see chapter 3, section 3.4.4) (Liu et al., 2011).

Although these events are not common, it is important to rule them out. It was therefore crucial to measure actual protein expression of our reporter gene by fluorescence, in cells or tissues where Dhh is known to be expressed. For the case of Dhh, the obvious tissues to analyse in the first instance were the testes, knowing the importance of Dhh in spermatogenesis (Bitgood et al., 1996). The brain and thymus were also analysed, where Dhh expression is well documented, as discussed in the introduction (Hegde et al., 2008; Saldaña et al., 2016). Both immunofluorescence and flow cytometry were carried out in parallel in all experiments.

Prior to carrying out a complete validation analysis, optimisation experiments were carried out for both flow cytometry and immunofluorescence. Various combinations of antibodies
were tested in order to determine the best panel for the measurement of mCherry within our cells of interest by flow cytometry. The immunofluorescence optimisation experiments took longer to finalise, as we soon realised that mCherry expression was only detected by fluorescent microscopy when the tissue cryosections were stained with anti-mCherry antibody, and not endogenously. This was shown on testicular (shown in Figure 4.5 A) as well as spleen cryosections (shown in chapter 5). Endogenous mCherry expression was therefore undetectable by immunofluorescence, as opposed to flow cytometry. This was expected prior to the validation stage, as fluorescence microscopy is generally less sensitive than flow cytometry (Marjanovic et al., 2014). After testing two different primary antibodies, the goat anti-mCherry antibody from Sicgen, coupled with a donkey anti-goatAx594 secondary antibody (Jackson Immune Research), were selected for use in all immunofluorescence mCherry experiments.

Section 4.3.2 describes our Dhh analysis on the testes and brain. In terms of testes analysis, only one F1 Dhh+/- reporter mouse was available for analysis as the 301 founder WT mCherry-positive cross with a Dhh+/- mCherry-negative female was carried out much later in the project. Four mCherry-positive WT (Dhh+/+) testes were analysed however, which included the only mCherry-positive founder who transmitted mCherry to his offspring (301) and three of his WT F1 mCherry-positive pups (104, 378, 374). Interestingly, when we compared the analysis of both WT and Dhh+/- mCherry-positive genotypes, we found that mCherry was more highly expressed in the Dhh+/- mouse, as shown in Figures 4.4 A (flow cytometry) and 4.5 B (immunofluorescence). One possible explanation for this significant rise in mCherry expression in the Dhh+/- genotype, is that as one copy of Dhh is lost, more transcription factors are available to bind the promoter of the Dhh reporter. This would in turn translate into higher mCherry emission. The fact that both flow cytometry and mCherry immunofluorescence techniques were consistent in showing this result suggests that the difference observed is a real biological result.

Interestingly, when analysing brain tissue, we found that mCherry expression was not detected endogenously in undigested whole brain cells by flow cytometry, yet efficiently detected by immunofluorescence when stained with anti-mCherry antibody on brain cryosections. Dhh is not highly expressed in brain tissue, and our analysis was carried out on undigested tissue (Hegde et al., 2008). This suggests that the low Dhh expression
present was most likely diluted out and not sufficient to be detected endogenously, but can be detected by antibody staining.

As an extension to our validation, we analysed thymic tissue in our mCherry mice to confirm documented Dhh expression profiles in thymic epithelial cells (TEC), as described in section 4.3.3. Thymic epithelial analysis was not carried out on our ageing (> 1.5 years old and above) founder mice, but only on young mice aged 4 to 6 weeks old. This is due to age-related thymic involution, a process by which thymi gradually decrease in size, resulting in low epithelial cell yields and changing TEC profiles with increasing age (Gui, Mustachio, Su, & Craig, 2012). Our finding that Dhh (mCherry) is expressed by CD45-negative EpCAM-positive stromal cells, but not detected, or barely, in CD45-positive leucocytes confirms published reports that describe thymic parenchyma stromal cells as the source of Dhh in the thymus, and not thymocytes (Sacedon et al., 2003). Interestingly, we also found that CD45-negative EpCAM-negative stromal cells do not express Dhh, which suggests that the source of Dhh is restricted to EpCAM-positive epithelial cells of the thymic stroma, like Shh (Saldaña et al., 2016). These observations may point to a possible functional role of Dhh in TEC differentiation.

The finding that Dhh is expressed in mTECs but not cTECs is in line with recent publicly available microarray data carried out by George Hollander’s Lab, that reveals a > 2-fold increase in Dhh in mTEC compared to cTEC subsets of the adult murine thymus (Zuklys et al., 2012). Dhh was detected in the cTEC subset in this microarray project however, contrary to our flow cytometry analysis that did not show any mCherry expression. These discrepancies may be attributed to the fact that microarray analysis measures gene expression, while our flow cytometry reflects protein expression. Additionally, recently published RNA-Seq data on 14 thymi of 7-days old C57BL/6 mice describes RPKM (Reads Per Kilobase of transcript per Million mapped reads) values for Dhh of 0.044 and 18.86 for cTECs and mTECs respectively. This also shows significant Dhh expression in mTECs, which is barely detectable in the cTEC subpopulation (GSE44945). Taken together, these published studies combined with our findings, further support the validation of our new mCherry line for Dhh. This propelled the project into its next stage, which involved in-depth analysis of Dhh in the spleen stroma.

A recent study carried out by our lab concluded that Shh is an important morphogen for
normal TEC development, with particular influence on the mTEC lineage (Saldaña et al., 2016). This conclusion was drawn by several observations. The first was that Shh-deficient foetal thymi contain fewer TEC, in particular mTECs compared to cTECs, in comparison with WT controls. Additionally, Shh deletion in TEC of adult thymi using a TEC conditional knockout mouse line, caused changes in TEC differentiation, in particular a reduction in mature Aire-positive mTECs, and consequently changes in T cell development. Finally, they also observed that the neutralisation of endogenous Hh proteins by soluble recombinant (r)H-hip in WT foetal thymic organ cultures, also caused a reduction in TEC numbers and Aire-positive mTECs. Shh is known to be expressed by EpCAM+ TEC like Dhh, especially at the cortical medullary junction of the thymus, while Ihh is expressed by both stromal cells and thymocytes, and has been implicated in T cell development, as previously mentioned (Outram et al., 2009; Sacedon et al., 2003).

The finding that Dhh is expressed in mTECs, but barely detected or absent in cTECs, together with the knowledge that Shh is tightly implicated with mTECs, opens up an interesting avenue of research. Indeed, these observations may suggest that both morphogens cooperate together in the differentiation and development of thymic epithelial cells.

4.5. Conclusion

In conclusion, our validation experiments have confirmed our hypothesis that our new mCherry mouse line is an accurate reporter of murine Dhh physiological expression. Indeed, our analysis of Dhh expression by mCherry readout in the testes, brain and thymus mirrored expected patterns of expression in Sertoli cells, brain cells and EpCAM-positive thymic epithelial cells (TEC) respectively by both immunofluorescence and flow cytometry. The use of an anti-mCherry antibody allowed us to overcome a limitation to our line, in which endogenous expression could not be visualised by immunofluorescence, but by flow cytometry only. We also discovered that Dhh+/- tissue bears significantly higher mCherry expression compared to WT tissue by both techniques. This technical consistency further supported our line as a true reporter of Dhh. As part of an extended validation, we carried out a complete analysis of TEC subsets, in which we found that Dhh is more highly expressed in mTECs compared to cTECs. This trend is consistent with recent microarray and RNA-Seq published data, which further corroborates the successful validation of our
line. In light of these observations, and the known influence of Shh on the mTEC lineage and Ihh in T cell development, further experiments to confirm this finding would represent a salient step towards understanding the role of Dhh in TEC differentiation and development.

With this successful validation stage completed, we were confident in using this mouse line towards achieving our main goal; to identify the precise source of Dhh in the spleen, which harbours a proportion of steady-state erythropoiesis, and represents the main site of stress-induced erythropoiesis.
Chapter Five: Characterisation of Dhh expression in the spleen using the mCherry reporter line for Dhh

5.1. Abstract

Published reports concluded that Dhh is a negative erythropoietic regulator in the BM and spleen under steady state and stress-induced conditions, and have also suggested that its expression originates from the spleen stroma (Lau et al., 2012). In continuation to this, we further investigated the precise source of Dhh within the spleen in the steady state, and after PHZ treatment, using our newly validated mCherry reporter line for Dhh. In doing so, we first confirmed that Dhh is not expressed by haematopoietic progenitors and erythroblasts (Lau et al., 2012), which validated our line as a true reporter for splenic tissue. When studying splenic cells in more depth, our findings supported our hypothesis that Dhh is produced by the spleen stroma, but we also detected Dhh at an even higher frequency within haematopoietic cells, including antigen-presenting cells (APCs) and non-APCs in the steady state. When analysing specific splenic subsets, we found that a fraction of red pulp fibroblasts (RPFs) and fibroblastic reticular cells (FRCs) express Dhh at the same frequency in the steady state, as well as all other ICAM-1-positive stromal cells to a lesser degree. This finding was supported by immunofluorescence data indicating that Dhh is scattered throughout the spleen. After PHZ treatment however, RPFs did not upregulate Dhh but maintained the highest expression of Dhh compared to other ICAM-1-positive stromal cells as FRCs downregulated Dhh after treatment. Interestingly, a fraction of follicular dendritic cells (FDCs), marginal zone reticular cells (MRCs) and vascular endothelial cells (VECs) significantly upregulated Dhh after treatment. This supported our hypothesis that RPFs express the most Dhh under anaemic stress, but not in the steady state. Combining these findings, together with the fact that both APCs and non-APCs also upregulated Dhh after treatment, suggests that Dhh has a potential function in white pulp splenic processes, such as B cell differentiation, in addition to erythropoiesis.
5.2. Introduction

5.2.1. The spleen

5.2.1.1. Spleen structure and function

The spleen is the largest secondary lymphoid organ and the body’s largest blood filter. Located in the abdomen underneath the diaphragm, the spleen is surrounded by a fibrous capsule of connective tissue (Mebius & Kraal, 2005). The spleen mediates the removal of old erythrocytes from circulation and the removal of blood-borne microorganisms and cellular debris. In fact the spleen contains about a fourth of the body’s total lymphocytes, which are crucial in initiating immune responses to blood-borne antigens. Due to its multitude of functions, the spleen combines both innate and adaptive immune responses in a uniquely organized way. The spleen is also a site of haematopoiesis, especially in the embryo. Extra medullary haematopoiesis occurs in adults but at a decreased rate, except under stress-induced conditions such as anaemia, inflammation, decreased bone marrow production or neoplasia (Losco P, 1992).

The spleen is largely made up of two parts; the red pulp and the white pulp (see Figure 5.1). The red pulp is a blood filter that removes foreign material and damaged and effete erythrocytes, and acts as a storage site for iron, erythrocytes, and platelets. The cords of the red pulp are lined with fibroblasts and reticular fibres that help provide immune defence via the production of cytokines, including IL-6. In fact the red pulp is an important site of antibody production, where plasma cells and plasmablasts (immature precursors of plasma cells) migrate here after antigen-specific differentiation inside the follicles of the white pulp. The anti-microbial functions of the red pulp are mediated by the cord-filled macrophages that selectively phagocytose ageing erythrocytes that have developed stiffening membranes and can therefore no longer continue their passage through the cords. These macrophages also have an important role in iron recycling in cooperation with liver macrophages (Cesta, 2006; Mebius & Kraal, 2005; Mueller & Germain, 2009).
Figure 5.1. Cross section of a wild-type mouse spleen stained with Haematoxylin & Eosin (H&E). Dark purple stained areas represent the white pulp and the light purple stained areas represent the red pulp of the spleen.

The white pulp of the spleen is made up of three compartments; the follicles, the marginal zone and the periarteriolar lymphoid sheath (PALS). The follicles contain dividing B-lymphocytes and are important in the opsonisation of extracellular organisms, such as encapsulated bacteria. Germinal centres may be present here, which arise upon antigenic stimulation. The marginal zone is situated between the red pulp, PALS and follicles, and contains antigen-presenting cells (APCs), therefore playing an important role in antigen processing. The PALS predominantly contain T lymphocytes, as well as dendritic cells and migrating B-cells, where plasma cell formation occurs. The organization and maintenance of the white pulp is under control of specific chemokines (Cesta, 2006; Mebius & Kraal, 2005).

5.2.1.2. Splenic stromal cells and their characterisation

Stromal cells have an important role in both white and red blood cell homeostasis, and adaptive immune responses. Amongst their many roles, they control splenic blood flow, prompt the removal of dyeing red blood cells and provide appropriate chemokines and cytokines at the right time and place. They also act as scaffolds for cell trafficking, and contribute to tolerance induction and active immune responses based on the antigens and adhesion/inhibitory molecules they express. Their numerous functions are reflected by their
heterogeneity and localisation within different compartments of the spleen (Mueller & Germain, 2009). Indeed, distinct subsets of stromal cells are present in both the red pulp, and discrete areas within the white pulp. Broadly speaking, the cellular composition of secondary lymphoid organs, such as the spleen, can be subdivided into haematopoietic and non-haematopoietic cells based on the expression of CD45. Known CD45-negative cells contain all bona fide mesenchymal-derived stromal cells, blood endothelial cells and lymphatic endothelial cells (Park et al., 2014; Roozendaal & Mebius, 2011).

Red pulp stromal cells play an important role in supporting myeloid progenitor cells, helping to localise effector cells such as plasma cells, and assisting in the removal of dyeing erythrocytes (den Haan, Mebius, & Kraal, 2012). One subset of red pulp stromal cells are known as red pulp fibroblasts, and are thought to contribute to the function of localisation through the expression of various integrin chains and IL-6. One such role that has been largely studied involves the localisation of plasma cells in the red pulp. This is mediated by the expression of ICAM-1 (CD54) and the chemokine CXCL12 on red pulp fibroblasts, which bind to the receptors LFA-1 and CXCR4 respectively, present on short-lived plasma cells (Ellyard, Avery, Mackay, & Tangye, 2005). Red pulp fibroblasts are also known to have a role in acute infections such as malaria, where they activate and fuse together in response to IL-1 and/or endotoxins, thereby restricting blood flow and parasite access to splenic cords during acute stress. Red pulp fibroblasts are characterized by the expression of ICAM-1 (CD54), ER-TR7, desmin, laminin, the four integrins α3, α4, α5 and β1, PDGFR and IL-6 and CXCL12 production (Mueller & Germain, 2009).

Endothelial cells within the red pulp are also important stromal cells. These cells contain stress fibres that are attached to annular fibres that run around venous sinuses. Contraction of these stress fibres allows erythrocytes to leave the sinuses for the venous system of the spleen. Endothelial cells assist in the removal of dyeing erythrocytes by trapping ageing erythrocytes that bear stiffened membranes. Endothelial cells have also been associated with haematopoietic niches in red pulp sinuses. However the precise role of stromal cells in the maintenance of myeloid progenitor cells (which eventually give rise to red pulp macrophages) and extramedullary haematopoiesis remains unclear (den Haan et al., 2012). (Please refer to Figure 5.2 for the localisation of stromal cell subsets in the spleen, and Table 5.1 for a detailed characterisation of the surface markers used to define them).
The stromal cell subsets attributed to the white pulp are numerous. In the T-cell zones of the white pulp, CD4+, CD8+ and DCs are supported by a subset of stromal cells called fibroblastic reticular cells (FRCs). Their production of CCL19 and CCL21 allows the delineation of T-cell zones, T cell homeostasis, and promotes the migration of DCs and lymphocytes that are positive for the CCL19 and CCL21 receptor, CCR7. In the B cell follicles of the white pulp, B cells are supported by follicular dendritic cells (FDCs) and other stromal cells present at the boundary of these follicles. In terms of chemokines, FDCs produce CXCL13, which attracts B cells that express the chemokine receptor CXCR5 into follicles (den Haan et al., 2012; Mueller & Germain, 2009).
Table 5.1. Stromal cell subsets in the spleen. This table shows a summary of the markers used to define all splenic stromal cells, as well as their respective functions.

Another reported stromal subset known as marginal reticular cells (MRCs) are found in the marginal zone. Their precise functions are less understood but they are thought to interact with marginal zone metallophilic macrophages in helping to deliver antigens to the B cell follicles. They may also be involved in splenic structure during organogenesis. Vascular endothelial cells within the white pulp are also important stromal cells that recruit leukocytes entering the spleen through the marginal sinuses in the marginal zone (den Haan et al., 2012; Mueller & Germain, 2009). (Please refer to Figure 5.2 for the localisation of stromal cell subsets in the spleen, and Table 5.1 for a detailed characterisation of the surface markers used to define them).

5.2.1.3. Splenic stromal cells and Desert Hedgehog expression

Various groups have investigated the expression of Dhh in the spleen, and have all concluded that Dhh expression is restricted to the stroma of the spleen (Lau et al., 2012; Perry et al., 2009). One such study suggested Dhh to be expressed by non-haematopoietic stromal cells of the spleen during recovery from phenylhydrazine (PHZ)-induced anaemia. This hypothesis was based on the observation that both BMP-4 and Dhh were co-
expressed in the spleen by immunohistofluorescence and western blot analysis during anaemic recovery (24 hours after PHZ treatment), having previously shown BMP-4 to also be induced in non-haematopoietic stromal cells of the red pulp during recovery (Perry et al., 2009).

A later murine study from our lab also showed that Dhh expression is restricted to the non-haematopoietic cells of the spleen stroma using RT-PCR. As a negative control Dhh knockout mice spleens were isolated and did not show any Dhh expression in their stroma. Additionally, Dhh was not detected in purified erythroblast populations (I to III), suggesting that Dhh expression is restricted to non-haematopoietic stromal cells (Lau et al., 2012). Interestingly, Dhh expression was also detected by RT-PCR in the bone marrow stroma of mice (Lau et al., 2012) and in the mouse-derived OMA-AD bone marrow mesenchymal cell line (Hegde et al., 2008). A recent study also showed that Dhh expressed by the splenic stroma has a role in T cell function in the spleen. They observed that splenic CD4+ T cells from Dhh-KO mice cultured in Th2 skewing conditions upregulated Gata3, a regulator of T cell differentiation, less efficiently than WT CD4+ T cells (Furmanski et al., 2013).

However despite these murine studies suggesting it is the stromal cells in the spleen that express Dhh, this has not been documented conclusively. In addition, the precise subset of stromal cells and their localisation in the spleen, responsible for Dhh production, have not yet been investigated.

5.2.2. Objectives

Once our new mouse line was validated as an efficient reporter of Dhh, immunofluorescence and flow cytometry experiments on the spleen were carried out to test the hypothesis that Dhh is expressed by the stroma of the spleen. Furthermore, the objective of these experiments was to identify the precise stromal cell subsets responsible for Dhh production, and to check whether Dhh is also expressed in leucocyte subsets. We also wanted to test the hypothesis that Dhh is more highly expressed in red pulp fibroblasts (RPFs), particularly under anaemic stress but also in the steady state, based on Dhh’s known function in erythropoietic regulation in the red pulp under normal and stress-induced conditions. The spleen was focused on as the main site of stress-induced erythropoiesis that also harbours a portion of steady state erythropoiesis. Splenic stromal cells are also
easily extracted from the spleen, compared to the BM, and the spleen additionally offers the possibility to visualise Dhh expression using immunofluorescence.

5.3. Results

The stroma of the spleen is a complex tissue, comprised of distinct types of stromal subsets, all involved in distinct functions of the spleen as described in section 5.2.1.3. Our knowledge of the dynamics of Dhh expression is limited to its known expression in non-haematopoietic cells of the spleen stroma (Lau et al., 2012). After successful validation of our new mCherry reporter line for Dhh (described in chapter 4), experiments were carried out to investigate unknown Dhh cellular expression patterns within the various compartments of the spleen stroma under steady state and stress-induced conditions. With this objective in mind, we first wanted to test whether Dhh was not also expressed by haematopoietic cells of the spleen, as this has not been previously investigated. We focused this in-depth analysis of Dhh on the spleen rather than the BM, because Dhh is functionally important in the spleen, and due to the ease of manipulation and the added possibility of analysing spleen tissue by immunofluorescence, in addition to flow cytometry (Furmanski et al., 2013; Perry et al., 2009). We nevertheless carried out a basic flow cytometry analysis of Dhh expression in haematopoietic versus non-haematopoietic cells of the BM as a preliminary basis for future BM experiments, as well as in blood reticulocytes. For reference, all mCherry-positive mice contain two copies of endogenous Dhh (Dhh+/+) and are therefore described as ‘WT’.

5.3.1. Dhh (mCherry) is detected in some splenic CD11b+, CD3+ and B220+ B cells as well as BM B220+ B cells, but not in blood reticulocytes

Prior to analysing Dhh in leucocyte subsets, we measured its expression in bone marrow and spleen progenitors and erythroblasts, known not to express Dhh, as an additional validation measure and negative control for our new mCherry line. Blood reticulocytes were also measured for Dhh expression by mCherry as this has not been previously documented. In our analysis of Dhh expression in leucocytes of the BM and spleen, we measured the following populations; progenitors, B220+ (B220-positive) B cells, CD3+ cells, CD11b+ cells and granulocytes in the BM and spleen. Monocytes, neutrophils, natural killer cells, CD8+ dendritic cells, granulocytes, macrophages and a subset of CD8+ T cells all express the CD11b integrin receptor, making it a useful marker to analyse (Christensen, Andreasen, Christensen, & Thomsen, 2001).
Figure 5.3. Dhh (mCherry) is not expressed in bone marrow haematopoietic progenitors.

A. Histogram and FACS plots show the gating strategy employed to measure the different types of progenitors in the spleen or BM. After excluding lineage-positive cells (including T cells, B cells, NK cells and granulocytes/monocytes) from live spleen or BM cells, the progenitor population is gated as C-Kit positive and Sca1.1 negative, while LSK cells (parent progenitor population) are gated as double-positives. The C-Kit+ Sca1.1- progenitor population is then gated against FcγRIII and CD34 to subdivide the three distinct progenitor populations (CMP, GMP and MEP) as shown. MEP progenitors that differentiate into the erythroblast lineage are negative for both these markers.

B. Histograms showing Dhh expression by mCherry fluorescence (red) in all live BM cells gated using FSC/SSC, LSK cells, C-Kit+ Sca1.1- progenitors and the three distinct progenitor subpopulations CMP, GMP and MEP from a 4-week old Dhh+/- mCh+ mouse (230) overlaid against a WT mCh- littermate control (blue). This data is representative of three experiments.
Figure 5.3 A shows the gating strategy that was employed to measure distinct progenitor populations in the BM and spleen throughout this project. Figure 5.3 B shows that Dhh, as shown by mCherry readout, is not expressed in LSK cells and C-kit+ Sca1.1- progenitors of the BM. As CMP, GMP and MEP BM progenitor populations arise from C-kit+ Sca1.1- progenitors, no mCherry was detected in these former populations. mCherry expression was also measured in spleen progenitor cells but not detected, as seen in the BM progenitors (data not shown), confirming overall that Dhh is not expressed by haematopoietic progenitors of the spleen or BM as previously published (Lau et al., 2012). Having confirmed that Dhh is not expressed by haematopoietic progenitors, we went on to confirm that Dhh is not expressed by erythroblasts as published by Lau et al. (Lau et al., 2012), as an added validation measure for our mouse line. Blood reticulocytes were also measured.
Figure 5.4. Dhh (mCherry) is not expressed in BM and spleen erythroblasts and blood reticulocytes. A. Dot plots (left) show the gating strategy for erythroblast populations I to IV, and the Ter119+ subset (collective populations II to IV). Histograms (right) show Dhh expression by mCherry (red) in erythroblast population I and the Ter119+ subset from a 4-week old Dhh+/- mCh+ mouse (230) overlaid with a WT mCh- littermate control (blue). B. The left histogram shows the gating strategy for reticulocytes as FITC-positive events (staining for DNA/RNA), while the right histogram shows Dhh expression by mCherry (red) in reticulocytes of a 6-week old WT mCh+ mouse (104) overlaid with a WT mCh- littermate control (blue). Erythroblast data is representative of eight experiments while the reticulocyte data is representative of 6 experiments.
Figure 5.4 shows that Dhh is not expressed by erythroblasts as expected (Lau et al., 2012), nor blood reticulocytes. The latter finding is an unsurprising result, as Dhh has never been detected as being expressed in the blood. Having ruled out Dhh expression in haematopoietic progenitors, erythroblasts and reticulocytes using our reporter line, we went onto measure Dhh in BM and spleen leucocytes.
Endogenous Dhh (mCherry) is detected in leukocytes of the BM and spleen except for BM B220+ B cells. Histograms show the gating strategy for BM and spleen CD3+ T cells, CD11b+ cells and B220+ B cells on the left-hand side of panels A, B and C respectively, and Dhh expression by mCherry (red) in each respective cell subset on the right-hand side from a 6-week old Dhh+/- mCherry-positive mouse (230), all overlaid with a WT mCh- littermate control (blue). Data is representative of 3 experiments.

Figure 5.5 shows our analysis of Dhh expression in T cells, CD11b+ cells and B cells of the BM and spleen. As shown, mCherry was detected in these spleen leucocyte subsets, but not in the BM except for B220+ B cells. As the CD11b receptor is expressed on many cell subsets as previously mentioned, further experiments are required to identify the precise leucocyte subset responsible for Dhh expression. Nevertheless this data was interesting, as it suggests that Dhh may not only be expressed in the spleen stroma. It is noteworthy however that earlier published studies that looked at Dhh in the spleen did not exclude CD45+ cells for characterisation of stroma (Lau et al., 2012; Perry et al., 2009). It is also interesting to note that Dhh expression in these leucocyte subsets was only detected in the spleen, but not in their BM counterparts, except for B cells. This raises interesting questions
as to the possible role of Dhh in relation to the function of these leucocyte subsets in the unique microenvironment of the spleen.

5.3.2. Dhh (mCherry) is detected in the spleen by immunofluorescence

Having measured mCherry fluorescence reflecting Dhh expression in BM and spleen progenitors, erythroblasts and leucocytes, we carried out an in-depth analysis of Dhh in splenic stromal subsets. Both immunofluorescence and flow cytometry were used, to paint an image of Dhh expression dynamics in splenic stromal cells. To start with, we employed fluorescence microscopy to determine whether mCherry could be detected in the spleen by immunofluorescence, and if we could observe differences in expression across the morphological landscape of the spleen.
Figure 5.6. Dhh (mCherry) expression is detected in the spleen stroma of young and old mCherry-positive mice by immunofluorescence, and its detection requires an anti-mCherry antibody. A. Immunofluorescence images (20x resolution) of spleen cryosections obtained from a 6-week-old WT mCh- mouse stained with anti-mCherry antibody (top left) and its WT mCh+ (104) littermate without antibody staining (top right), and of 104 and 15.5 month-old 304 WT mCh+ founder both stained with anti-mCherry antibody (bottom left and right). B. Same immunofluorescence images (20x resolution) of 104 and 304 spleen cryosections stained with anti-mCherry antibody as in panel A, but separately showing both the red (mCherry) and blue (DAPI) channels (left and right respectively). Goat anti-mCh antibody (Sicgen) and a donkey anti-goat secondary Ax594-conjugated antibody (Jackson Immune Research) were used to stain cryosections, together with DAPI, a nuclear counter-stain. All immunofluorescence images were acquired on the LEICA fluorescent microscope.

Figure 5.6 shows the immunofluorescence images obtained from our mCherry experiment on splenic cryosections. Figure 5.6 A shows that mCherry is not detected in the WT mCh+ spleen cryosection (104) endogenously, but detected when treated with anti-mCherry antibody, as seen for the testes in Chapter 4. For comparison, we also stained a splenic cryosection obtained from the 18 month-old WT mCh+ founder mouse (304) with anti-mCherry antibody, and detected mCherry expression within a similar range, albeit at a lower intensity compared to 6-week old 104. Composite images (showing both mCherry and DAPI together) for this experiment are shown at the bottom of panel A.
Figure 5.6 B shows immunofluorescence images separately for each channel (red and blue) from the same antibody-stained composite images in panel B. By looking at the separate channels, we see clearly that mouse 104 shows higher mCherry expression in the spleen compared to the old founder. This data is consistent with known differences in mCherry copy number between mouse 304 and 104, of 1 and 5 respectively (see chapter 3, section 3.3.10). Indeed, a higher BAC-mCherry copy number contains more mCherry proteins, resulting in higher mCherry fluorescence emission. Inherent differences in Dhh endogenous expression in the spleens of young and older mice may also be contributing to mCherry fluorescence intensity differences. Further experiments would be required to investigate whether such age differences do occur.

Additionally, DAPI (a dye that stains nuclei) was used to distinguish white pulp (WP) areas from red pulp (RP) areas. This is based on the fact that WP regions have a higher cellular concentration compared to RP regions (Mcilroy et al., 2009). As shown in Figure 5.6 B, Dhh appears scattered throughout the spleen, with no precise pattern visualised across the WP and RP in the spleen sections of both mice.

Overall, these results show that Dhh by mCherry fluorescence can be detected in the spleen by immunofluorescence using our BAC reporter line with the use of an anti-mCherry antibody. Although the precise cell subsets responsible for Dhh production cannot be visualised using this technique, it shows the overall pattern of morphogen expression throughout a typical cross-section of a WT spleen. No obvious differences in mCherry fluorescence in terms of intensity and surface area were observed across the morphological landscape of the spleen, suggesting that Dhh is scattered throughout the spleen. This preliminary data already provides some evidence that our hypothesis that Dhh is more highly expressed by red pulp fibroblasts compared to other spleen stromal subsets may not be true. However, to fully test our hypothesis, flow cytometry was carried out in the next stage of experimentation, as a complementary technique to investigate endogenous mCherry expression within specific splenic cellular subsets, as depicted in the following section.
5.3.3. Dhh (mCherry) expression is more highly detected in some haematopoietic cells than ICAM-1-positive stromal cells, while ICAM-1-negative cells do not express Dhh in the steady state.

This section describes the flow cytometry experiments carried out in various stromal subsets of the spleen under steady-state conditions. Based on our finding that Dhh is expressed in CD11b+, Gr-1+ and CD3+ splenic leucocytes, we also analysed CD45+ subsets using the leucocyte-related markers available in our pre-designed antibody panel for spleen stroma analysis.

A

Gating strategy for CD45- cells
mCherry (Dhh) expression in WT CD45- stromal subsets
Figure 5.7. Endogenous Dhh (mCherry) is expressed in CD54-positive CD45-negative stromal subsets, but not CD54-negative stromal cells of the spleen (vascular endothelial cells).

A. Gating strategy employed to analyse stromal subsets of the spleen that are all CD45-negative (CD45-). To determine accurate gating of CD54 (ICAM-1)+ cells within CD45- cells, EpCAM positive and negative cells were gated against the CD54 axis, as EpCAM+ are all CD54+. Once CD54+/− gating was established on CD45- cells, CD45-CD54+ cells were further subdivided and gated as MHC Class I Low or High. B. Top dot plots are representative of mCherry fluorescence detected in CD45- cells of a 4-week old WT mCh+ (374) and a WT mCh- littermate control respectively. Middle and bottom panel histograms are representative of mCherry expression (red) in all CD45- stromal cells, and specifically in CD45- CD54- VECs (vascular endothelial cells), all CD45- CD54+ stromal cells (all stromal cells except VEC), CD45- CD54+ MHC I High FRCs (fibroblast reticular cells) and CD45- CD54+ MHC I Low cells, in the spleen of mCh+ mouse 374 overlaid with its WT mCh- littermate (blue). CD54+ MHC I Low cells correspond to three stromal cell subsets, namely FDCs (follicular dendritic cells), MRCs (marginal reticular cells) and RPFs (red pulp fibroblasts). Data are representative of three experiments.

Figure 5.7 A shows the gating strategy employed to identify various splenic stromal subsets, as described in the Figure legend. All known splenic stromal subsets express ICAM-1 (CD54) except VECs (vascular endothelial cells). Additionally, MHC Class I (MHCI) is a common selectable marker to identify CD54-positive FRCs but not other CD54-positive stromal cells. Therefore by default, CD45-negative cells that expressed ICAM-1 but low MHCI were defined collectively as FDCs, MRCs and RPFs, and MHCI-High cells as FRCs. EpCAM, an epithelial marker also expressed on splenic dendritic cells (DCs) was used to define CD54-positive and negative cells, as most EpCAM-positive cells were found to express ICAM-1 (Borkowski, Nelson, Farr, & Udey, 1996).

Figure 5.7 B shows that mCherry (Dhh) is expressed by some ICAM-1 expressing stromal cells, but not by ICAM-1-negative VECs. Within ICAM-1-positive stromal subsets, similar degrees of expression were detected between FRCs, and FDCs, MRCs and RPFs collectively. However as FDCs, MRCs and RPFs were grouped together in these
experiments, further experiments described in the next section were carried out to analyse RPFs exclusively, to more accurately identify the source of Dhh within these distinct stromal cell subsets.

Nevertheless, based on the known function of these stromal subsets described in the introduction (section 5.2.1.2), this data suggests that Dhh may not only act in the red pulp as part of erythropoiesis, and be produced by red pulp cells via RPF and red pulp macrophage (Immgen) secretion, but may also be produced and play a part in the formation of secondary immune responses in the white pulp.
Figure 5.8. Endogenous Dhh (mCherry) is expressed in CD45+ haematopoietic cells, especially in antigen-presenting cells (APCs) compared to non-APCs. A. Gating strategy employed to analyse CD45+ subsets of the spleen. APCs were defined as CD45+ MHC II+, in which DCs were gated as EpCAM+ and B cells and macrophages as EpCAM-. Non-APCs were defined as MHC II−, which comprise all other immune subsets including T cells, NK cells and haematopoietic stem cells. B. Dot plots are representative of mCherry fluorescence in CD45+ spleen cells of the WT mCh+ (374) mouse (left) and its mCh− littermate control (right). Histograms represent mCherry expression (red) in the various CD45+ subsets described in panel A in WT mCh+ mouse 374, overlaid with its WT mCh− littermate control (blue). N.B. Data is representative of three experiments.

Figure 5.8 A describes the gating strategy employed to analyse CD45+ subsets using the available markers present in our stroma antibody panel. As MHC Class II and EpCAM were available, we were able to analyse Dhh expression by mCherry readout in APCs, and specifically DCs due to their expression of EpCAM, as well as non-APCs (Borkowski et al., 1996). CD45+ non-APCs however comprise a large array of immune subsets, including T cells, NK cells, NKT cells, granulocytes and haematopoietic stem cells. Figure 5.8 B shows that Dhh is expressed by CD45+ cells, in line with our findings shown in Figure 5.5. The
WT mCh-negative control confirmed that the mCherry fluorescence visualised in the transgenic cells was true. mCherry expression was detected more highly in APCs compared to non-APCs under steady-state conditions. In relation to the APCs, similar patterns of Dhh expression between DCs, and B cells/macrophages were observed. These findings validate the mCherry expression that was visualised in CD11b+ splenic cells depicted in Figure 5.5, which consist of macrophages and CD8+ dendritic cells (APCs) (Schliehe et al., 2011), and non-APCs including monocytes, neutrophils, NK cells, granulocytes and a subset of CD8+ T cells (Christensen et al., 2001). Detecting mCherry in APCs here is also consistent with our previous finding that B220+ B cells express mCherry (Figure 5.5).

Taken together, this data suggests that Dhh is mainly expressed by APCs within CD45+ cells, as well as non-APCs such as T cells, albeit at a smaller level. Further experiments will be required to determine whether Dhh is also specifically expressed by NK cells, granulocytes, monocytes and neutrophils, which are also positive for CD11b. This data, combined with our findings that Dhh is also expressed by white pulp stromal subsets, suggests that Dhh is not only expressed by non-haematopoietic cells of the spleen stroma, and corroborates a potential role for Dhh in white pulp-related functions, additionally to erythropoiesis. Indeed, our data shows that more CD45+ haematopoietic cells express Dhh than CD45- stromal cells.

5.3.4. Dhh (mCherry) expression is raised in FDC, MRC and VEC stromal cells and haematopoietic cells, but not in FRC and RPF stromal cells after PHZ treatment

Having analysed Dhh expression via mCherry readout in stromal cells and CD45+ subsets of the spleen under steady-state conditions, we investigated its expression in the same subsets under stress-induced conditions. PHZ treatment was used to induce stress-induced erythropoiesis (anaemia) in the spleen as a model to amplify Dhh production in splenic cells, as previously published (Perry et al., 2009). Please refer to the chapter 6 introduction for a detailed background on PHZ treatment and its applications.
mCherry (Dhh) expression in PHZ versus no-PHZ stromal cells

A

B

CD45

Integrin α5

MHC I
Figure 5.9. Dhh (mCherry) is upregulated in CD45- stromal cells, particularly in FDCs and MRCs upon 24-hour PHZ treatment. RPFs express Dhh at the same degree under steady state and stress-induced conditions. A. Typical image of two WT spleens, one left untreated (left) and one treated with PHZ for 24 hours (right). Ruler scale is in cm. B. Histograms represent the gating strategy employed to measure splenic stromal subsets. CD54 was gated on CD45- cells in the usual way as described in Figure 5.7. CD45-CD54+ stromal cells that are positive for integrin α5 are defined as RPFs while those that are negative for this receptor are classified as MHC Class I Low or High, defining FDCs and MRCs, and FRCs respectively. C. The three dot plots represent mCherry (Dhh) expression in splenic CD45- cells of the same WT mCh+ mouse (357) treated with PHZ, the untreated 3-month old WT mCh+ (376) and a WT mCh- littermate control, respectively (top). The three middle histograms show this mCherry expression in splenic CD45- cells (left), CD45-CD54+ cells (middle) and CD45-CD54- VECs (right) of the PHZ-treated WT mCh+ mouse (pink) overlaid with its non-treated mCh+ control (beige). Bottom histograms represent mCherry expression in splenic RPFs (left), FRCs (middle) and FDCs and MRCs (right) of the PHZ-treated
mCh+ mouse (pink) overlaid with its non-treated mCh+ control (beige). The data in this Figure is representative of three experiments.

The efficacy of the 24-hour PHZ treatment in this experiment was confirmed by the significant difference in spleen size observed between untreated and treated spleens, of which one representative no PHZ/PHZ pair is shown in Figure 5.9 A. This Figure shows that after just 24 hours of PHZ treatment, the treated spleen is markedly increased and darker compared to the untreated control, reflecting the rapid expansion of erythropoiesis as a result of anaemic stress (Lau et al., 2012).

Figure 5.9 B shows the gating strategy employed to measure stromal subsets in this experiment. The same stroma panel was used as in the steady-state experiment, however an additional antibody targeted towards the surface marker α5 integrin (CD49e) was used to further identify red pulp fibroblasts (RPFs), which exclusively express this marker. CD49e was an important inclusion as we wanted to test the hypothesis that red pulp fibroblasts, involved in red blood cell clearance in the spleen, increase their Dhh expression upon anaemia-inducing PHZ treatment, where a rapid expansion of erythropoiesis occurs during recovery.

The dot plots in Figure 5.9 C show that mCherry differences are clearly visualised in splenic CD45- cells between the mCh+ PHZ-treated and mCh+ no-treatment mice. The WT mCh-control, in which few events are seen, shows that the mCherry expression observed in the mCherry-positive mice is true. The significant increase in Dhh observed in splenic CD45- and CD45+ cells of the mCherry-positive mouse after only 24 hours of PHZ treatment is consistent with Perry et al.’s study, which also showed that Dhh is expressed at low levels in the spleens of untreated mice, but upregulated during the first 48 hours of anaemic recovery in PHZ-treated mice, with a peak expression after 24 hours.

Interestingly, we found that Dhh is upregulated in CD45-CD54+ stromal cells. We also found significant Dhh expression in CD45-CD54- VECs despite it being undetected in the no-PHZ control (also shown in Figure 5.7). With regards to CD54-positive stromal cells, Dhh was not upregulated in FRCs and RPFs after treatment, but contained the highest overall Dhh expression in the steady state (as well as after treatment for RPFs only). Finally, FDCs and MRCs were found to have the lowest Dhh expression in the steady state, but showed
the most upregulation of the morphogen after PHZ treatment. No significant differences were noted in the percentages of the different stromal cells before and after treatment (data not shown).
Figure 5.10. Dhh (mCherry) is upregulated in haematopoietic cells, including APCs after 24-hour PHZ treatment. A. The three dot plots represent mCherry (Dhh) expression in splenic CD45+ cells of a 3.5-month old WT mCh+ mouse (357) treated with PHZ, an untreated 3-month old WT mCh+ (376) and a WT mCh- littermate control, respectively. The histogram shows the same mCherry expression in splenic CD45+ cells of the PHZ-treated WT mCh+ mouse (pink) overlaid with its non-treated WT mCh+ control (beige). B. Histograms represent mCherry expression in DCs (left), B cells and macrophages (middle) and non-APCs (right) of PHZ-treated WT mCh+ mouse 376 (pink) overlaid with its non-treated WT mCh+ control (376). N.B. This data is representative of one experiment. For the gating strategy employed here please refer to Figure 5.6.

Figure 5.10 shows that Dhh is significantly upregulated in professional APCs, particularly in EpCAM- APCs (B cells macrophages and possibly macrophages), as well as non-APCs to a lesser extent, after PHZ treatment. In this Figure, the gating strategy was not shown as it corresponds to the same one shown in Figure 5.8 A. The dot plots depicted in panel A show that mCherry is clearly upregulated after PHZ compared to the untreated control, and is absent in the untreated WT mCh- control, confirming true mCherry expression. No significant differences were noted in the percentages of the different haematopoietic cells before and after treatment (data not shown). Further experiments would be required to determine whether macrophages also express mCherry, in addition to B cells within EpCAM- APCs.
5.4. Discussion

This section discusses the results obtained from various mCherry experiments described above, which investigated Dhh expression profiles in different cell subsets of the spleen, under steady state and stress-induced conditions. These cell subsets include both haematopoietic cells and stromal cells. Flow cytometry and immunofluorescence were used to meet this objective.

5.4.1. Dhh (mCherry) is detected in some splenic CD11b+, CD3+ and B220+ B cells as well as BM B220+ B cells, but not in blood reticulocytes

Our finding that mCherry (Dhh) is not expressed by BM and splenic haematopoietic progenitors and erythroblasts is consistent with published reports (Lau et al., 2012). This provided further validation for our mCherry line as a faithful reporter of Dhh in the spleen, as depicted in section 5.3.1. In this section, we also showed that Dhh is not expressed by blood reticulocytes, which answers a previously undocumented question.

Interestingly, when studying leucocyte subsets, Dhh by mCherry expression was detected in CD11b-positive, B220-positive, and to a smaller degree, CD3-positive splenic cells. mCherry was not detected in BM leucocyte subsets however, except for B220+ B cells. Although Dhh expression in CD45-negative haematopoietic cells has not been previously documented as part of functional or phenotypic studies, gene expression databases published in ‘The Immunological Genome Project’ (Immgen) on ~ 6-week old mice, also document Dhh expression in BM and splenic B cells, splenic CD8+ and CD8- plasmacytoid DCs and classical splenic CD8-CD4-CD11b+/- DCs. The fact that mCherry was not detected in BM leucocytes except for B220+ B cells also highlights the unique microenvironments of the BM and spleen, and also suggests additionally to erythropoiesis, Dhh may have distinct functions in both tissues.

5.4.2. Dhh (mCherry) is detected in the spleen by immunofluorescence

Section 5.3.2 depicts the immunofluorescence experiments that were carried out on mCherry spleen cryosections. This technique was applied in order to determine whether Dhh could be detected by immunofluorescence, and whether differences in red pulp and white pulp expression could be visualised. Our results show that mCherry cannot be detected endogenously in the spleen using this reporter line. Having obtained the same
result on testes cryosections, which express more Dhh than the spleen, this finding was expected. Anti-mCherry antibody treatment revealed a clear mCherry signal in both mCherry-positive cryosections from a 6-week old (104) and 18-month old founder mouse (304). The higher mCherry signal observed in cryosections from mouse 104 compared to 304, is consistent with our copy number analysis described in chapter 3, section 3.3.10, which attributed a BAC-mCherry copy number of 5 and 1 to founder 301 (which includes F1 mouse 104) and 304, respectively.

Although this technique only shows broad expression profiles within the spleen, it once again confirms that Dhh can efficiently be detected using our reporter line, even in tissues with low Dhh expression such as the spleen (Lau et al., 2012). The immunofluorescence data also suggests that Dhh is scattered throughout the spleen, and not restricted to red or white pulp areas.

5.4.3. Dhh (mCherry) expression is more highly detected in some haematopoietic cells than ICAM-1-positive stromal cells, while ICAM-1-negative cells do not express Dhh in the steady state

Experiments conducted on splenic stromal cells under steady state conditions described in section 5.3.3, showed that Dhh is expressed by a fraction of all known ICAM-1-positive stromal subsets, whereby both groups of stromal cells (FRCs and FDCs, MRCs and RPFs respectively) showed similar expression levels. Dhh was not detected in ICAM-1-negative VECs however. These results suggest that in the steady state, Dhh may also play a role in the white pulp, where FDCs, MRCs and FRCs reside, in addition to its role as a negative regulator of erythropoiesis in the red pulp.

mCherry expression by Dhh was also more highly detected in CD45+ haematopoietic cells. Combining this data on haematopoietic cells in section 5.3.3, with that of section 5.3.1, suggests that within APCs, Dhh is expressed by a subset of DCs and B cells, and possibly macrophages. The latter possibility would need to be independently analysed however to be conclusive. In terms of non-APCs, the only subset that was independently analysed was CD3+ cells - other subsets such as granulocytes, neutrophils and NK cells could be evaluated for potential Dhh expression in future experiments. So far, these findings supported our hypothesis that Dhh is expressed by a fraction of the spleen stroma, but also
suggests that Dhh is more highly expressed by a heterogeneous group of haematopoietic cells of the spleen in the steady state.

5.4.4. Dhh (mCherry) expression is raised in FDC, MRC and VEC stromal cells and haematopoietic cells, but not in FRC and RPF stromal cells after PHZ treatment

As mentioned above, our findings that mCherry (Dhh) is upregulated after 24 hours PHZ treatment in CD45- and CD45+ cells tie in published reports that used the same treatment modality (Perry et al., 2009). This confirmed that this model is effective in amplifying the Dhh response, as a means to improve our understanding of its expression profile in the spleen. This initial finding further supported our hypothesis that Dhh is expressed by the spleen stroma, but also provided additional evidence that haematopoietic cells also express it.

Interestingly, we found that ICAM-1-negative stromal cells (VECs), which are involved in blood transport and cell recruitment into the spleen, express Dhh after treatment but not in the steady state. In terms of ICAM-1-positive stromal cell analysis, it was also interesting to observe that a fraction of RPFs, that reside in the red pulp, consistently express Dhh at the same levels with or without anaemia. Additionally, a public gene expression database in Immgen identified significant Dhh expression by red pulp macrophages in the steady state, which play a role in recycling iron from effete erythrocytes.

The fact that Dhh production by RPFs remains unchanged during anaemia compared to the steady state, suggests that the additional production of Dhh observed during anaemia is coming from other sources. This ties in with the finding that VECs, FDCs and MRCs all upregulated the morphogen after treatment. Red pulp macrophages may also upregulate their production of Dhh after treatment, but this remains to be evaluated. This is also consistent with our immunofluorescence data shown in section 5.3.2, which indicated that Dhh is not only restricted to the red pulp, but rather scattered throughout the spleen.

As PHZ has a direct effect on erythroblasts, it was surprising to observe increased Dhh production in the FDCs and MRCs of the white pulp. However the red pulp does not only play a role as a blood filter, but also receives plasma cells and plasmablasts from the white
pulp (after antigen-specific differentiation), and therefore represents an important site of antibody production (Cesta, 2006; Mebius & Kraal, 2005; Mueller & Germain, 2009). Therefore it is unsurprising that significant changes in erythroblast/red blood cell dynamics and frequencies in the red pulp as a result of anaemia induction invariably have an effect on incoming plasma cells in the red pulp, and therefore white pulp processes. This is consistent with the fact that MRCs represent a layer through which the migration of cells into the white pulp occurs, and interact with marginal zone macrophages to help deliver antigens to B cell follicles, which are in turn supported by FDCs (den Haan et al., 2012; Katakai, 2012). Additionally, Dhh was found to be upregulated in haematopoietic cells, particularly in B cells/macrophages compared to DCs, as well as non-APCs. Importantly, Dhh was expressed in a higher number of haematopoietic cells than stromal cells in the steady state.

Taken together, these observations suggest that Dhh function may not only be restricted to erythropoiesis in the red pulp, but may also take part in white pulp processes, such as B cell differentiation. Our hypothesis that Dhh is produced by the stroma of the spleen has therefore been validated, although our findings have also shown it to be significantly expressed by haematopoietic cells as well under stress and steady state conditions, with potential functional implications. Additionally, our hypothesis that Dhh is more highly expressed in RPFs is supported in the context of anaemic stress only, as RPCs expressed Dhh at the same level as FRCs in the steady state. Dhh will not have been the first Hh protein to be potentially attributed a white pulp function, as Shh expression by FDCs in germinal centres (GCs) of the follicles has also been documented, where it signals to and acts as a survival signal for GC B cells in the spleen (Sacedón et al., 2005).

Additionally, Ihh expression has also been identified in the spleen, at the same frequency before and after PHZ treatment, although its precise source in the spleen is not yet known (Perry et al., 2009). As such, once a clearer picture of the potential function of Dhh in other splenic processes other than erythropoiesis have been identified, it would be worthwhile to study it in conjunction with other Hh proteins in the spleen.
5.5. Conclusion

Taken together, the data shown here supports our hypothesis that Dhh is expressed by stromal cells of the spleen, and also supports our hypothesis that it is more highly expressed by red pulp fibroblasts under anaemic stress only. Under steady state conditions however, a fraction of FRCs of the white pulp were also found to express Dhh at the same rate as RPFs, while all other ICAM-1-positive stromal subsets also expressed Dhh at a lower frequency. This observation is in line with the immunofluorescence data shown in Figure 5.6, whereby Dhh was found to be scattered throughout the spleen. Furthermore, VECs, FDCs and MRCs significantly upregulated Dhh upon PHZ treatment, implying possible changes in white pulp reactions as a result of erythroblast expansion in the red pulp to compensate for PHZ-inducing anaemia induction. These observations, combined with the finding that Dhh is expressed by a higher frequency of haematopoietic cells compared to stromal cells in the steady state, and is upregulated in APCs and non-APCs after treatment, point to a potential role for Dhh in the white pulp. Further experiments would be required to confirm these preliminary findings, however the evidence so far suggests that Dhh may not be limited to erythropoietic regulation in the spleen. Investigating this further may be a worthwhile step towards improving our understanding of the role of Hedgehog signalling in secondary immune response formation in the spleen.
Chapter Six: The dynamics of Hedgehog signalling in steady-state erythropoiesis and during erythropoietic recovery following irradiation and phenylhydrazine treatment

6.1. Abstract

Stress erythropoiesis is a process by which the rapid expansion of erythrocytes in the spleen occurs in response to anaemia or hypoxia stimuli. In continuation of the study by our lab that has implicated Dhh as a negative regulator of erythropoiesis, this chapter tests the impact of mutation of various Hedgehog (Hh)-related genes on stress erythropoiesis after sublethal irradiation or PHZ treatment, to further investigate the role of Hh signalling pathway components in erythropoiesis (Lau et al., 2012). In the Dhh/-/- context, an irradiation experiment supported the hypothesis that splenic MEP progenitors and reticulocytes are increased in Dhh/-/- mice compared to WT, suggesting accelerated erythropoiesis kinetics during haematopoietic recovery, corroborating Dhh as a negative regulator of erythropoiesis. In line with this, a study on one of our Hedgehog reporter mouse models, (a GBS-GFP transgenic) that measures Gli activity, showed that Dhh is responsible for a significant portion of Hedgehog pathway activation in erythroblasts. In the context of Gli3 mutation, our hypothesis that Shh is elevated in the Gli3+/- spleen was supported by our immunofluorescence data. However RT-PCR analysis found Dhh to be reduced in the Gli3+/- spleen, suggesting that Gli3 may not act directly on Dhh, but rather acts to repress Shh. Following this, we tested the hypothesis that erythroblast differentiation in the BM and spleen is increased during haematopoietic recovery and in steady state conditions in the Gli3+/- context. This was based on the rationale that erythroblasts may be receiving a lower negative regulatory signal, if steady state spleen Dhh expression profiles also correspond to the BM and the stress-induced state. Overall erythroblast differentiation was significantly increased in Gli3 mutants during haematopoietic recovery in the BM, but not in the spleen. However a key reduction in spleen size was observed in the spleen during steady state and stress-induced conditions, as well as reduced RP/WP ratios in XT mice compared to WT. This underscores the differences in spleen and bone marrow (BM) microenvironments that signal to erythroblasts during haematopoietic recovery, as well as the complexity of the role of Hedgehog signalling in erythropoiesis, which requires further investigation.
6.2. Introduction

In the basal state, the bone marrow (BM) plays the main role in producing erythrocytes, but this is shifted to the spleen under erythropoietic stress. The spleen thus serves as a reserve site for erythroid progenitors, which are propelled to rapidly differentiate into erythrocytes when needed in response to anaemia and hypoxia stimuli. This process is referred to as stress erythropoiesis. Rapid and efficient stress erythropoiesis is essential to compensate for critical blood loss in clinical settings, which may be caused by radiotherapy, chemotherapy or anaemia. Animal models have widely been used to study stress erythropoiesis in the spleen, by using sublethal irradiation (4–6 Grays) or cytotoxic drug treatment. This causes suppression of haematopoiesis in the BM and apoptosis of haematopoietic cells and erythroid progenitors, in which recovery of haematopoiesis can be studied in the spleen and BM (Peslak et al., 2011, 2012). Interestingly, studies have also shown that extramedullary stress erythropoiesis can also occur in the murine liver when the spleen is absent, in a BMP-4 dependent manner (Lenox, Shi, Hegde, & Paulson, 2009).

Key factors that have been identified as regulators of stress erythropoiesis include erythropoietin (EPO), Stem Cell Factor (SCF), Bone Morphogenetic Protein 4 (BMP4), hypoxia (Perry et al., 2009; Perry, Harandi, & Paulson, 2007) and Dhh (Lau et al., 2012). Anaemia leads to tissue hypoxia (low oxygen tension), which stimulates EPO production by kidney peritubular cells (Socolovsky et al., 2007). EPO then binds its receptor EPO-R, expressed on erythroid progenitors and proerythroblasts, resulting in the proliferation and differentiation of erythroid cells via the induction of Bcl-xL (an anti-apoptotic protein crucial for erythroid survival) (Todaro et al., 2013). In line with this, Fas/FasL are significantly down-regulated on the surface of splenic proerythroblasts and basophilic erythroblasts during stress (Socolovsky et al., 2007).

It has also been suggested that BMP-4 is induced by EPO production in stress erythropoiesis. Additionally, culturing spleen cells with stem cell factor (SCF) in hypoxic conditions has been found to significantly increase stress erythroid progenitor numbers (Koury & Bondurant, 1990; Perry et al., 2007). A more recent study has also suggested a model in which a distinct subset of BFU-E stress progenitors, referred to as ‘stress BFU-E’ exists in the spleen that have migrated from the BM. This subset is though to respond quicker to erythropoietic stress compared to steady-state BM BFU-E progenitors, and is
regulated by BMP-4 (Lenox, Perry, & Paulson, 2005). According to this model, hypoxia is also required for a maximal response of stress progenitors to BMP-4 and SCF (Paulson, Shi, & Wu, 2011; Perry et al., 2007).

Additionally, it has been suggested that Hh signalling and its interaction with the BMP-4 pathway are essential for the maintenance of the stress progenitor pool in the spleen. In that study, treatment of BM with Shh resulted in an increased number of stress erythroid progenitors and induced BMP4 expression. This indicated that Shh signalling maintains BMP4 expression in response to the induction of stress erythropoiesis (Perry et al., 2009). Interestingly, phenotypic analysis went on to show that Dhh is expressed at low levels in untreated mice (steady-state), but upregulated and maintained throughout anaemic recovery in PHZ-treated mice, which suggests a role for Dhh during recovery. Ihh however was also expressed in the spleens of untreated mice, but not raised after PHZ treatment (Perry et al., 2009).

To investigate the role of Dhh in stress-related erythropoietic recovery, a study published by members of our lab used sublethal irradiation to study the first three weeks of recovery of erythrocyte differentiation in the BM and spleen of Dhh-/- mice. They found that erythropoiesis recovered more quickly in both the spleen and BM of Dhh-/- mice compared to WT littermates, and both returned to pre-irradiation values by day 21 post-irradiation. In the same study, Dhh-/- and WT mice were treated with PHZ to investigate the role of Dhh in stress erythropoiesis in the spleen. Dhh-/- mice also recovered more quickly than WT mice, with red blood cell (RBC) counts returning to pre-PHZ values by day 14. Interestingly, MEP progenitors were significantly increased in Dhh-/- spleens compared to WT, in line with the increased mobilisation of 'stress progenitors' to the spleen in Dhh-/- mice compared to WT. Taken together, this accelerated erythroid differentiation in the context of Dhh deficiency observed under irradiation and PHZ-induced stress conditions, supported their hypothesis that Dhh acts as a negative regulator of erythropoiesis (Lau et al., 2012). This study formed the basis for the irradiation and PHZ experiments described in this chapter.

**6.2.1. Objectives**

Based on these recent studies on Dhh in stress erythropoiesis (Lau et al., 2012; Perry et al., 2009), the following sublethal and PHZ experiments were carried out with two objectives in
mind. The first was to confirm Dhh as a negative regulator of erythropoiesis using irradiated Dhh-/- mice. In doing so, we wanted to test the hypothesis that spleen progenitors and reticulocytes are raised in Dhh-/- mice during post-irradiation recovery. The second was to study other available Hedgehog-related mouse lines in the context of stress erythropoiesis, to further delineate the potential roles of different Hedgehog signalling factors and their interactions during erythropoietic recovery. WT GFP-GBS transgenic reporter mice (measuring Gli-binding activity and hence Hh pathway activation) were treated with PHZ, and Dhh-/- GFP-GBS reporter transgenic mice were studied under steady state conditions to further investigate Dhh activity indirectly in erythroblasts. Additionally, we investigated Shh and Dhh expression levels in Gli3+/- mice, which generally exhibit heightened Hedgehog signalling. Based on the finding that Dhh is decreased in the spleen in this context, we sublethally irradiated Gli3+/- and WT mice to test the hypothesis that erythroblast differentiation is increased in the spleen, and consequently the BM during haematopoietic recovery.

6.3. Results

6.3.1. Reticulocytes and splenic progenitors are raised in Dhh-/- mice during irradiation-induced haematopoietic recovery

As previously mentioned, Lau et al. showed that erythropoiesis recovers more quickly in both the spleen and BM of Dhh-/- mice compared to WT littermates, but they did not measure reticulocyte numbers during recovery (Lau et al., 2012). At 7 days post-irradiation, they observed that erythroblast population II is already detectable in the BM and spleens of Dhh-/- mice, but not in WT. 14 days post-irradiation, Dhh-/- spleens were noticeably larger compared to their WT counterparts, and contained more cells in erythroblast subsets II and III. Overall, differentiation along the erythroid lineage is accelerated in Dhh-/- BM and spleen compared to WT, during irradiation-induced haematopoietic recovery, indicating that Dhh acts as a negative regulator of erythropoiesis.

In order to test the hypothesis that blood reticulocytes and splenic erythroid progenitors mirror the dynamics of erythropoiesis in Dhh-/- mice during irradiation-induced haematopoietic recovery described above, another irradiation experiment was carried out. Dhh-/- and WT mice were sublethally irradiated (dose; 6 Grays) and culled (for progenitor
analysis) or tail bled (for reticulocyte analysis) at various time points during the first four weeks post-irradiation. As a control, non-irradiated mice were maintained throughout this period and stained for their reticulocytes and progenitors in parallel. Reticulocytes were measured by staining for the FITC-labelled BD Retic-Count reagent. The reagent works by binding DNA and RNA present in reticulocytes, but absent from mature erythrocytes.
Figure 6.1. Dhh-/- reticulocytes (red) surpass WT reticulocytes during the reticulocyte surge at day 13 post-irradiation. A. FACS plots show reticulocyte percentages (red) of non-irradiated WT control mice (left), irradiated WT (middle) and irradiated Dhh-/- (right) at each time point, in a representative experiment. Reticulocytes were defined as FITC-positive events. Plots were overlaid against a FITC-negative PBS control (blue) to set the gate for the reticulocytes. B. Average percentages of blood reticulocytes measured in irradiated WT and Dhh-/- mice during the first 4 weeks post-irradiation at days 6, 11, 13, 21 and 28, in parallel to non-irradiated WT mice as controls. The graph represents one non-irradiated WT mouse and 2 irradiated WT/Dhh-/- pairs per time point, except for day 28 where only one irradiated pair was stained.

Figure 6.1 shows the reticulocyte results obtained in this experiment. Interestingly, between day 0 and 13 post-irradiation, reticulocyte percentages were similar between WT and Dhh-/- mice, remaining well below no-irradiation values (<5%). After 13 days post-irradiation, a dramatic surge in reticulocytes was observed for both genotypes with a peak around day 21. During this surge, WT reticulocyte percentages reached 33.6% while Dhh-/- reticulocytes were increased to more than 40%. After day 21, reticulocyte numbers drastically decreased. WT reticulocytes reached WT no-irradiation values by day 28, while Dhh-/- reticulocytes also fell but remained around 5% higher than WT reticulocytes, above
no-irradiation values. As a steady state control, WT no-irradiation reticulocyte values stayed below 10% as an expected range throughout the 4 weeks.

Interestingly, the rapid surge in reticulocytes observed at day 13 reflects the rapid known recovery of erythropoiesis that begins to occur already after day 7 in the spleen and BM, prior to reticulocyte formation (Lau et al., 2012). Once the surge occurs and sufficient reticulocytes have been produced during recovery, a dramatic decrease in reticulocyte percentages follows after day 21, before homeostasis is reached at a steady state. This data supported our hypothesis that reticulocytes are increased in Dhh-/- mice during post-irradiation recovery.

To determine whether erythrocyte progenitor (MEP) dynamics in WT and Dhh-/- mice reflect post-irradiation erythropoiesis and reticulocyte trends respectively, all splenic progenitors were measured at day 13, 21 and 28 post-irradiation, in addition to no-irradiation controls, as shown in Figure 6.2. As mentioned in the introduction, the spleen was focused on, being the site of infiltrated stress progenitors for stress erythropoiesis. As a comparison, BM progenitors were also measured at day 13 (data not shown).
Figure 6.2. Splenic MEP progenitors are increased in Dhh-/− compared to WT under steady-state conditions, and during recovery at 13 and 21 days post irradiation. BM MEP progenitors are raised in WT mice at 13 days post irradiation. A. FACS plots show splenic progenitor percentages of WT and Dhh-/− mice at 13, 21 and 28 days post irradiation, with a non-irradiated control pair, as labelled. Two pairs per time point were measured in this experiment. B. Photographs represent typical spleens from a WT/Dhh-/− pair at days 13 (top) and 28 (bottom) post-irradiation, with two non-irradiated pairs as a control. Ruler scale is in cm.

Figure 6.2 A shows that under steady-state conditions and during post-irradiation haematopoietic recovery, splenic MEP progenitors are increased in Dhh-/− mice compared to WT both at day 13 and 21. By contrast, MEP percentages in Dhh-/− mice fall just under WT levels at day 28 post-irradiation. Interestingly, the range of progenitor percentages observed during recovery fall within the no-irradiation range, except for day 21 post-irradiation, where a reduction in progenitor percentages is seen in both the WT and Dhh-/−. Figure 6.2 B shows that spleen sizes remain consistently larger in Dhh-/− mice compared to WT during both steady state and stress-induced erythropoiesis.

These results show that by day 13 post-irradiation, a high proportion of splenic progenitors in the range of no irradiation levels are already present, and most likely start to rise several days before. This is in line with the idea that progenitors emerge early to replenish the erythroblast and reticulocyte niche, consistent with the rapid and early expansion of erythroblasts seen during recovery (Lau et al., 2012), and the significant reticulocyte surge seen by day 13 (Figure 6.1). The elevated percentage of splenic MEP progenitors in Dhh-/− mice during haematopoietic recovery (day 13 and 21) also supports published data suggesting that increased infiltration of erythroid progenitors to the spleen occurs in Dhh-/− mice compared to WT, where stress-induced erythropoiesis occurs (Lau et al., 2012). This
is consistent with splenic progenitor trends observed during PHZ-induced anaemic recovery (Lau et al., 2012).

Furthermore, the higher proportion of MEP progenitors in the steady-state Dhh-/ mouse spleen compared to WT is also consistent with Lau et al.’s observation that erythroblast differentiation is increased in the spleen under steady-state conditions in Dhh-/ mice, as it is in the BM. Although the BM is the main site of steady-state erythropoiesis, this shows that erythropoiesis continues to occur in the spleen under steady-state conditions, while this is increased in the context of Dhh deficiency, due to the absence of Dhh as a negative regulator of erythropoiesis. This view is also supported by the observation that spleens of Dhh-/ mice are larger compared to WT during post-irradiation recovery (Figure 6.2 B), and in the steady state, as a result of increased erythropoiesis.

It is unclear why a reduction in MEP progenitors in the Dhh-/ mouse is observed at day 28 after full irradiation recovery. This may be part of a temporary negative feedback system in response to MEP progenitors having remained consistently high throughout recovery, before reverting back to the no-irradiation steady state. In fact, the spleen sizes at day 28 post-irradiation shows that the Dhh-/ spleen remains considerably larger compared to the WT, indicating that despite a decrease in MEP progenitors, erythropoietic differentiation remains high. It is noteworthy that when comparing our data to published irradiation data (Lau et al., 2012), the difference in irradiation dose of 2 Grays used between both studies should be considered.

6.3.2. Gli-binding activity is reduced in Dhh-/ mice under steady state conditions, and raised under PHZ-induced stress conditions in WT mice

To assess total Gli binding activity in erythroblasts under steady-state conditions in the context of Dhh deficiency, WT and Dhh-/ GBS-GFP mice were generated and analysed, as shown in Figure 6.3. In this transgenic mouse line, GFP readout on a flow cytometer corresponds to a measure of total Gli activity, as multiple Gli-binding sites in this reporter model drive transgene expression. Gli-binding activity was measured in erythroblast population II and all Ter119+ erythroblasts in the BM and spleen. In addition to the Ter119+ subpopulation (erythroblast populations II to IV), population II was shown as an independent
population, as Ptch (the surface receptor for Hh proteins) transcription, is known to be highly expressed in this population, compared to populations III and IV (Lau et al., 2012).
Figure 6.3. Total GFP-binding activity is decreased in splenic and BM Dhh-/- erythroblasts compared to WT under steady-state conditions, and barely detectable in MEP BM progenitors. A. Histograms represent total Gli binding activity (red) measured as a percentage of GFP within WT and Dhh-/- BM and splenic basophilic erythroblasts (population II) and Ter119+ cells (erythroblast populations II – IV), overlaid with a WT GFP-negative control (blue). B. Histograms represent total Gli binding activity in MEP BM progenitors in the same WT/Dhh-/- pair, overlaid with a WT GFP-negative control (blue). This data is representative of three experiments.

Figure 6.3 shows that a high proportion of WT basophilic erythroblasts (population II) and Ter119+ cells exhibit Gli-binding activity, with similar ranges of GFP expression between both the BM and spleen. In the context of full Dhh deficiency, this proportion is decreased compared to WT, particularly in the BM, where a reduction of up to 17% is observed. It is not surprising that the differences observed in the spleen are less striking, as these data were collected under steady-state conditions, where the majority of steady-state erythropoiesis occurs in the BM. WT and Dhh-/- BM MEP progenitors were also measured but did not reveal a significant level of Gli-binding activity, suggesting that progenitors act as the precursors of immature erythroblasts only, but do not directly interact with Hh proteins in the erythropoiesis process. These results suggest that Dhh is responsible for a significant
portion of Hh signalling activity within erythroblasts, which further supports its role as a (negative) regulator in erythropoiesis.

To assess Gli-binding activity under stress-induced conditions, two WT GFP+ mice out of four were treated with PHZ to induce anaemia, to study the spleen as the site of stress erythropoiesis. Due to the ease of detecting GFP in spleen cryosections in this mouse model, immunofluorescence was selected to assess GFP expression. A GFP negative mouse treated with PHZ was used as a negative control. Figure 6.4 shows representative data of one analysed GFP+ pair (with and without PHZ treatment.

A
Figure 6.4. Gli binding activity is raised in the WT spleen upon PHZ treatment. **A.** Endogenous immunofluorescence showing total Gli binding activity by GFP expression (green) with (left) or without (right) DAPI in the same spleen cryosections of WT mice with (top panel) and without (bottom panel) anaemia-inducing PHZ treatment. A WT GFP-negative spleen cryosections treated with PHZ is shown as the negative control (bottom left). Mice were culled 10 days after PHZ injection. One representative PHZ/No PHZ WT pair is shown out of two stained pairs. **B.** Spleen image of typical WT spleens with and without PHZ treatment. Ruler scale is in cm.

This immunofluorescence experiment shows that Gli binding activity is increased in the spleen upon PHZ treatment, reflected by the increase in endogenous GFP observed (Figure 6.4 A). Less concentrated areas of DAPI (which stains for cell nuclei), which most likely reflect red pulp areas where erythropoiesis occurs, appear to correspond with higher GFP expression in both the PHZ and non-PHZ sections. The increase in GFP observed 10 days after PHZ treatment compared to the no PHZ control, is in line with the rapid expansion of erythroblasts (known to exhibit high Gli-binding activity as shown in Figure 6.4), that takes place during anaemic recovery in the spleen by day 7 onwards (Lau et al., 2012). The GFP-negative PHZ-treated control showed that the GFP that was visualised in GFP-positive spleen sections was true expression. Figure 6.4 B shows that PHZ treatment was successful based on the large spleen size of the treated WT compared to the no treatment control.
6.3.3. Shh is elevated and \textit{Dhh} is reduced in XT mice under steady state conditions

As Gli3 partial deficiency causes increased Hedgehog signalling in some tissues, we wanted to test the hypothesis that Shh is also elevated in the spleen in this context (Meyer & Roelink, 2003). To this end, Shh was measured in Gli3+/- (Gli3 heterozygote) mice under steady-state conditions. For reference, Gli3+/- mice are also commonly referred to as ‘extra-toe’ or ‘XT’ due to their polydactylous nature. Anti-Shh antibody was used to stain spleen paraffin sections processed from two pairs of non-irradiated WT and XT mice, as part of an immunofluorescence experiment. To test the hypothesis that Dhh expression is also raised in the XT context like Shh, RT-PCR experiments were run due to the lack of a reliable and specific immunofluorescence antibody targeted against Dhh. Two independent RT-PCR experiments using \textit{Dhh} primers were carried out on cDNA converted from the RNA isolated from two pairs of WT and XT spleens, under steady-state conditions.

A

\begin{figure}
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\includegraphics[width=\textwidth]{shh_xtWT_DAPI_shh_xtWT.png}
\caption{Immunofluorescence images showing Shh expression in WT and XT spleens.}
\end{figure}
Figure 6.5. Shh expression is increased and Dhh is decreased in XT compared to WT spleens under steady state conditions. A. Immunofluorescence images of murine WT and XT spleen cryosections stained with goat anti-Shh antibody (Santa Cruz) and a donkey secondary Alexa488-conjugated antibody (Jackson Immune Research), with or without DAPI (left and right respectively). DAPI is a nuclear counter-stain. Bottom left: immunofluorescence image of a WT spleen section stained with secondary-only Alexa488 antibody alone as a negative control. Image representative of one out of two WT/XT pairs stained for Shh. B. Bar charts show two relative transcription of Dhh in splenocytes isolated from two WT/XT pairs under steady-state conditions. Each pair was measured in an independent RT-PCR experiment. The scale shows expression normalized to the levels of the housekeeping gene HPRT. Error bars represent ±SEM.
Figure 6.5 A shows that Shh is more highly expressed in the spleens of XT mice compared to WT, supporting our hypothesis that the Gli3 model represents increased Shh signalling in the spleen. The secondary antibody only negative control confirmed that the Shh expression that was visualised represented true expression. On the other hand, Figure 6.5. B showed that in both analysed WT and XT pairs, Dhh expression is reduced in the XT spleen compared to the WT. This disproves our hypothesis that Dhh is also increased in the Gli3+/- context, in a similar manner to Shh. This suggests that under wild-type physiological conditions, Gli3 may not act directly on Dhh.

**6.3.4. Splenic erythroblast differentiation is increased in the BM but not in the spleen of XT mice**

Having assessed the expression levels of Shh and Dhh in the spleens of Gli3+/- mice under steady-state conditions, additional mice of this genotype in parallel to WT were irradiated to study erythroblast recovery kinetics. To this end, mice were sublethally irradiated at a dose of 6 Grays. As Gli3 heterozygosity causes decreased levels of Dhh and increased levels of Shh in the spleen under steady-state conditions, we hypothesised that this expression pattern is maintained between both genotypes, even though most likely amplified (as shown in chapter 5, section 5.3.3 in the PHZ-treated WT condition), during haematopoietic recovery. As studies on Dhh-/- mice also showed similar trends in erythropoiesis in the BM and spleen, we speculated that such Dhh expression profiles in the Gli3+/- context also apply to the BM. In line with this, we wanted to test the hypothesis that erythroblast differentiation is increased in this context during steady state and stress-induced erythropoiesis, in the presence of potentially lower Dhh expression in Gli3+/- mice. In doing so, we also wanted to investigate the effect of increased levels of Shh on Dhh in terms of erythroblast differentiation.

Full recovery of erythropoiesis was measured in this experiment over a period of 31 days post irradiation, with the 31-day time-point considered a full haematopoiesis recovery time-point. This included measuring all progenitors, erythroblasts, reticulocytes, as well as white pulp and red pulp morphological histology in the spleen. BM and splenic progenitors from WT and XT mice were measured at 13, and 31 days post-irradiation, in parallel to non-irradiated WT and XT control mice. H&E staining of WT and XT spleens to assess RP and
WP areas, as well as splenic and BM erythroblasts, were measured on days 7, 13 and 31 post-irradiation, in addition to no-irradiation controls.
Figure 6.6. CMP progenitors are significantly increased in the BM after post-irradiation recovery, but not in the spleen. A. The top and bottom graphs represent the average percentages of MEP, GMP, and CMP progenitors, as well as the average ratios between MEP and CMP of WT and XT mice at days 13 and 31 post-irradiation, with no irradiation controls, in the BM (top) and spleen (bottom) respectively. The results are representative of n = 3 WT/XT pairs for all time-points including the no irradiation controls, except for the Day 13 WT data, that only includes two WT mice. B. FACS plots representative of one WT/XT pair per condition showing the percentages of the three progenitor populations (MEP, GMP and CMP) in the BM and spleens of WT and XT mice that are non-irradiated, or 13 and 31 days post-irradiation. *p<0.05. Error bars represent ±SEM.

Figure 6.6 shows that no significant changes in BM MEP progenitors were found between WT and XT mice post-irradiation during haematopoietic recovery. When looking at MEP/CMP values, which indicate the differentiation rate that occurs between CMP and MEP progenitors, no differences were observed either during recovery or under steady-state conditions, despite the fact that elevated CMP progenitor values in XT mice compared to WT on day 31 are significant.
In the spleen, a decrease in MEP progenitors and a raise in GMP progenitors was observed at day 13 post-irradiation, during recovery of haematopoiesis in XT mice compared to WT, but this was not significant. No significant differences in MEP/CMP ratios were observed either at all time points between XT and WT mice, suggesting that the differentiation rate between MEP and CMP is not significantly changed in the XT context. It is interesting however to observe that overall, splenic MEP progenitors are reduced at day 13-post irradiation compared to no irradiation values, suggesting a time lag before which splenic progenitors are produced again to contribute to splenic stress erythropoiesis, compared to BM MEP progenitors, which already fall within the no irradiation range by day 13.
Figure 6.7. Stress erythropoiesis temporarily occurs in the BM early in irradiation-induced haematopoietic recovery, before being shifted to the spleen. Erythroblast differentiation is accelerated in the BM of Gli3+/- (XT) mice during irradiation-induced haematopoietic recovery, but not in the spleen. A. The top and bottom graphs represent the average percentages of erythroblast populations II to IV, as well as Ter119+ve cells in the BM and spleen respectively, of WT and XT mice, culled after 7, 13, and 31 days post-irradiation. No irradiation controls were analysed in parallel. Averages are representative of three mice per genotype per time point, including the no irradiation controls, except for day 7 where one WT/XT pair only was stained. B. Kinetics of recovery of all four erythroblast populations after nonlethal irradiation in the BM and spleen at 7 and 13 days after irradiation, with a non-irradiated WT as a control. Dot plots are representative of one out of three WT/Gli3+/- pairs analysed per time point (except for day 7). *p<0.05. Error bars represent ±SEM.
Figure 6.7 shows the erythroblast flow cytometry data that was obtained in this irradiation experiment. In panel A, only erythroblast populations II to IV are shown, as population I represents a very small percentage. Interestingly, one striking observation from this data is that stress erythropoiesis in the BM does take place very early on during irradiation-induced haematopoietic recovery by day 7. Out of erythroblast populations II to IV, the most mature erythroblast population IV is the one that is expanded on day 7 post-irradiation, up to 97.83% for the WT and 99.63% for the XT mouse, while the more immature populations II and III are low, and fall within the no-irradiation (control) range, at the same time point. This suggests that existing erythroblast differentiation is temporarily accelerated in the BM, before returning to no irradiation levels by day 13, when stress erythropoiesis is shifted to the spleen. This is consistent with splenic erythroblast data in the same Figure, which shows that splenic erythroblast population II and III are significantly raised, well above no irradiation values, on day 13 compared to day 7 post-irradiation. Additionally, erythroblast population IV percentages on day 13 fall within the no irradiation range, showing that a fresh wave of stress-induced erythroblast differentiation occurs in the spleen.

When combining these findings with the progenitor data shown in Figure 6.6, these erythroblast observations may explain why by day 13, BM MEP progenitors fall within the no-irradiation progenitor range. This is likely the result of temporary stress induced erythropoiesis that occurs in the BM around day 7. Indeed, BM MEP progenitors fall below no-irradiation values by day 31 as a result of the shift of stress erythropoiesis to the spleen. Although BM stress erythropoiesis has already shifted by day 13 to the spleen, BM MEP progenitors may remain raised up till day 13 as they infiltrate to the spleen, to initiate splenic stress erythropoiesis. This possibility is consistent with the finding that splenic MEP progenitors are strikingly low compared to no-irradiation values on day 13, but are raised by day 31.

In terms of genotype differences, erythroblast differentiation is significantly increased in the BM of XT mice compared to WT during post-irradiation haematopoietic recovery at day 13 in terms of Ter119+ subset analysis, although BM MEP progenitors were not raised in XT mice compared to WT at this time-point. When looking at the steady state conditions however, XT mice do not show differences in the Ter119+ subset compared to WT in the BM.
In the spleen, no significant differences in Ter119+ erythroblasts were observed under steady state and stress-induced conditions, as for the MEP erythroid progenitors, although opposing trends were observed between both cell populations. Reticulocytes were also measured in WT and XT irradiated mice culled after 13, 24, and 31 days of treatment, in parallel to no-irradiation controls, but no differences were observed between genotypes (data not shown).

To understand further the impact of Gli3-mutation in the spleen during post-irradiation recovery, we sought to determine whether spleen morphology in terms of red pulp (site of erythropoiesis) and white pulp histology reflects the dynamics of erythropoiesis and/or erythroid progenitors measured by flow cytometry. Spleen sections produced from the same irradiation experiment were sent for standard Haematoxylin & Eosin (H&E) processing. H&E analysis was carried out on sections from irradiated WT and XT mice culled at 7, 13, and 31 days after irradiation, in addition to no-irradiation controls, as shown in Figure 6.8.
Figure 6.8. XT spleens exhibit changes in red and white pulp ratios compared to WT during post-irradiation haematopoietic recovery. A. Haematoxylin & eosin (H&E) histology showing RP and WP areas in WT and XT (Gli3+/-) spleens, after induction of stress-induced erythropoiesis at 7, 13 and 31 days post sublethal irradiation, with a no-irradiation pair as a control. Paraffin-embedded spleen sections were stained with haematoxylin and eosin to identify WP areas, which stained deeper purple, and RP areas, which stained pink. Typical WP and RP are illustrated by staining of the labelled non-irradiated pair. Images represent one of two pairs stained for H&E per time point, including the no-irradiation control. B. Ratios of red pulp to white pulp measured in WT and XT mice at days 13 and 31 post-irradiation, with no irradiation controls, calculated from H&E images (A) using ImageJ software. Ratios were calculated from average values representative of two pairs per time point, including the no-irradiation condition. C. Photographs represent typical spleens from a 13-day (top left) and 31-day (top right) post-irradiation WT/XT pair, with a non-irradiation pair (bottom) in parallel for both time points. Ruler scale is in cm.

H&E spleen analysis shown in Figure 6.8 shows representative images of one out of two irradiated pairs per time point, including a no-irradiation pair. The ratio of red pulp to white pulp was calculated using ImageJ by measuring the surface area of the individual white pulp areas, calculating their sum and subtracting this from the total surface area of the spleen in question, to obtain the red pulp surface area. The red pulp surface area was then divided by the total white pulp surface area to obtain a ratio. Based on this analysis, the average ratio of RP to WP is shown to be decreased in XT spleens (2.55) compared to WT (3.16) at day 13 post-irradiation, but this trend is inverted after recovery at day 31 (1.94 in WT and 2.16...
in XT), and in the no-irradiation controls (2.33 in WT and 2.98 in XT). Interestingly, this trend is consistent with the recovery splenic progenitor data shown in Figure 6.6, where day 13 splenic MEP progenitors are decreased, although this was not significant. Figure 6.8 shows that during and after recovery and under steady-state conditions, the spleen sizes of XT mice are consistently smaller compared to WT, suggesting that a given subpopulation may be significantly reduced to account for this size difference, in addition to the subsets mentioned above.

6.3.5. Mice heterozygote for Shh do not exhibit changes in erythroblast differentiation post-irradiation and in the steady state

As an additional study to measure the potential effect of Shh on erythropoiesis, we carried out a sublethal irradiation experiment of 6 Grays on 6-8 week old WT and Shh-heterozygote (Shh+/-) mice. Mice were culled at 7, 13 and 22 days post-irradiation, and stained for erythroblasts, B cells, T cell, progenitors and reticulocytes, alongside no-irradiation (steady state) control pairs, using flow cytometry. H&E morphological staining was also carried out on spleen sections isolated from mice culled at all time-points, to assess potential differences in red pulp (RP) and white pulp (WP) histology between both genotypes.

Bone marrow erythroblasts

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<th>Percentage within live bone marrow cells</th>
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<tr>
<td>II</td>
<td>Day 13 WT</td>
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<td></td>
<td>Day 22 WT</td>
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<td></td>
<td>NO IRR WT</td>
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<tr>
<td>III</td>
<td>Day 13 Shh+/-</td>
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<td>Day 22 Shh+/-</td>
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<td>NO IRR Shh+/-</td>
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Figure 6.9. Erythroblast kinetics are not significantly different in the BM and spleen of Shh+/- deficient mice. The top and bottom graphs represent the average percentages of erythroblast populations II to IV, as well as Ter119+ve cells in the BM and spleen respectively, of WT and Shh+/- mice, culled after 7, 13, and 22 days post-irradiation. No irradiation controls were analysed in parallel. Averages are representative of three mice per genotype per time point, including the no irradiation controls.

In this Shh+-/- study, no concrete trends or genotype differences were observed in the data obtained, suggesting that Shh heterozygosity does not exhibit a direct effect on erythropoiesis during steady state conditions and anaemic stress. It is noteworthy however that this model represents the loss of only of one Shh copy, and is therefore not representative of full Shh deficiency. Figure 6.9 shows two graphs of erythroblast kinetics that were analysed in this experiment in the BM and spleen, showing no significant differences between both genotypes. In this Figure, only erythroblast populations II to IV are shown, as population I represents a very small percentage.

6.4. Discussion

This section discusses the results obtained in various irradiation experiments carried out on Dhh-/-, Shh-/- and Gli3+/- mice, in addition to WT and Dhh-/- GBS-GFP experiments to assess Gli-binding activity in erythroblasts under steady state (WT and Dhh-/-) and anaemia-induced conditions (WT only). In all cases, steady state data is shown as a
baseline control. These experiments were inspired by the irradiation and phenylhydrazine experiments carried out by Lau et al. on Dhh-/- mice, with the aim of further elucidating the role of Hedgehog signalling, and in particular Dhh and its interactions with other Hedgehog proteins, in erythropoiesis.

6.4.1. Reticulocytes and splenic progenitors are raised in Dhh-/- mice during irradiation-induced haematopoietic recovery

One of our objectives was to further identify the dynamics of erythroblast differentiation in Dhh-/- mice during irradiation-induced haematopoietic recovery, as a continuation to Lau et al.’s study (Lau et al., 2012). As the frequencies of progenitors and reticulocytes had not been previously documented in Dhh-/- mice in the irradiation context, these two cell populations were analysed to test the hypothesis that they are increased after sublethal irradiation, as described in section 6.3.1. The irradiation progenitor data that was obtained ties in with published progenitor phenylhydrazine data, which shows that splenic MEP progenitors are also elevated in the spleens of Dhh-/- mice, compared to WT (Lau et al., 2012). Indeed, we found that splenic MEP progenitors increased compared to WT at day 13 post-irradiation. This supports the view that increased erythroid progenitor infiltration from the BM to the spleen occurs in Dhh-/- mice compared to WT, in line with published findings showing that erythropoietic differentiation is increased in Dhh-/- spleens under stress conditions (Lau et al., 2012). Under steady-state conditions, we found that splenic MEP progenitors also remain elevated in Dhh-/- mice compared to WT, which also ties in with published data depicting heightened erythropoiesis in Dhh-/- mice spleens, as well as the BM in the steady-state, due to the lack of negative regulation by Dhh (Lau et al., 2012). Further experiments would be required to study erythroid BM progenitors in this irradiation context to determine whether they are reduced in Dhh-/- mice during recovery, and therefore follow this increased splenic infiltration model during recovery, in the absence of Dhh.

In terms of our reticulocyte analysis, reticulocyte percentages between WT and Dhh-/- remained similar for the first 13 days after irradiation, prior to the reticulocyte surge that took place at day 13 when the erythropoiesis niche had been sufficiently replenished. After this surge, genotype differences were clearer, as the scale of reticulocytes produced rapidly increased. Dhh-/- reticulocytes remained consistently higher than WT reticulocytes after day
13 in response to increased erythroblast precursors in Dhh-/- mice compared to WT, prior to reticulocyte maturation in the blood, supporting our hypothesis. Our progenitor and reticulocyte data, together with the spleen sizes, corroborate Dhh as a negative regulator of erythropoiesis, as it ties in with the erythropoiesis dynamics observed in Dhh-/- mice compared to WT during irradiation-induced recovery. This confirmation provided further rationale for the production of an mCherry reporter for Dhh (described in chapter 3), to further study the important role of Dhh in the context of erythropoiesis.

6.4.2. Gli-binding activity is reduced in Dhh-/- mice under steady state conditions, and raised under PHZ-induced stress conditions in WT mice

The results obtained from the GBS-GFP transgenic reporter model (which measures total Gli binding activity) in the steady state in the context of Dhh deficiency discussed in section 6.3.2, suggests that Dhh is responsible for a significant portion of Hh signalling activity in erythroblasts. To make full use of this GBS-GFP model, we decided to undertake a PHZ experiment on WT GFP-positive mice. The results shown in Figure 6.4 suggest that the increase in GFP expression observed after 10 days of PHZ treatment, compared to the no PHZ control, is in line with the rapid expansion of erythroblasts (known to exhibit high Gli-binding activity as shown in Figure 6.4), that takes place during anaemic recovery in the spleen by day 7 onwards. This data supports PHZ-induced anaemia as a faithful model to study erythropoiesis recovery in mice in the context of Hedgehog signalling.

6.4.3. Shh is elevated and Dhh is decreased in XT mice under steady state conditions

To address the potential role of other Hedgehog factors in our Gli3+/- model, the expression of Shh and Dhh were measured in WT and XT mice under steady-state conditions, as shown in section 6.3.3. Shh expression was clearly increased in the spleens of XT mice compared to WT, confirming that our Gli3+/- model effectively induces higher hedgehog signalling. Based on published data describing Gli3 as a repressor of the Shh response in embryo studies, in which the amino-terminal region of Gli3 is directly responsible for this antagonism (Meyer & Roelink, 2003), this finding supported our hypothesis that this mechanism is also true for the spleen. Indeed, when a copy of Gli3 is lost, this antagonistic activity is diminished, causing raised Shh signalling. As the effect of Gli3 on Dhh is not
known in the spleen, two independent RT-PCR experiments were carried out on WT and XT mice in the steady state. Based on the finding that Dhh is reduced in the Gli3+/- spleen compared to the WT spleen, we suggest that Gli3 may not be acting as a direct repressor of the Dhh gene, as it does for Shh. It is noteworthy that a future cross between the mCherry reporter for Dhh and Gli3+/- would provide Gli3+/- mCherry reporter mice, from which Dhh protein expression in the spleen could also be evaluated, as a more direct comparison to the Shh immunofluorescence data mentioned above.

Based on this steady state data, we speculated that Shh and Dhh maintain their expression profiles in the Gli3+-/ context during post-irradiation haematopoietic recovery as well, although future experiments would be required to confirm this. Having now measured Shh and Dhh in the steady-state spleen, we investigated the kinetics of erythropoiesis in the context of partial Gli3 deficiency during steady state and post-irradiation haematopoietic recovery in the BM and spleen, discussed below. We hypothesised that erythropoiesis may be increased in this context due to lowered Dhh expression and therefore a negative regulatory effect on erythropoiesis in the spleen, although the potential effect of Shh on Dhh was not known at this stage. As erythroblast kinetics showed similar trends between the BM and spleen in the published irradiation study on Dhh-/- mice, we also hypothesised that increased splenic erythropoiesis also occurs in the BM in the Gli3+/- context.

6.4.4. Splenic erythroblast differentiation is increased in the BM but not in the spleen of XT mice

Our irradiation experiment on the Gli3+/- mouse line, a mutant that has previously been shown to exhibit increased Hedgehog signalling during limb development and in the embryonic thymus, produced interesting findings. Taken together, our XT irradiation data showed significant differences in erythropoiesis kinetics in the BM only, where erythroblasts were raised in XT mice during post-irradiation recovery. A significant increase in the CMP progenitor subset was also observed in the BM after recovery, suggesting that granulocytes and or macrophages may be significantly changed in this context. This data supports our hypothesis that erythroblast differentiation is elevated in the BM, possibly due to reduced levels of Dhh during recovery, and therefore a negative regulatory effect on erythropoiesis (based on the steady state splenic RT-PCR data). However as Dhh expression levels were only analysed in the spleen and under steady state conditions, further experiments would be
required to confirm that Dhh is indeed reduced in XT mice during post-irradiation recovery in the BM.

Interestingly, no significant differences in erythroblast kinetics or other immune populations were observed in the spleen under steady state and stress-induced conditions, despite the reduced RP/WP ratio observed in XT spleens alongside reduced spleen sizes during recovery, suggesting lowered erythropoiesis activity in XT mice.

The inverse trends observed here between the BM and spleen are very different to the trends observed in Dhh-/− mice during recovery, where BM and splenic erythroblast II populations were both consistently raised throughout irradiation recovery in Dhh-/− mice (Lau et al., 2012). These findings suggest that other factors may be occurring in the unique BM and spleen microenvironments of XT irradiated mice, which are creating these differences in erythroblast kinetics in the Gli3+/− context. It is also possible that Gli3 itself modulates other erythropoiesis-related genes unrelated to Hedgehog. Furthermore, it is interesting to note that the significant expansion in erythropoiesis in the BM in XT mice occurs during irradiation recovery and not in the steady state, suggesting that irradiation may expand/alter other erythropoiesis-relevant factors. Future experiments on expression profiles of the Hh proteins during recovery would allow a clearer image to be painted as to the potential roles of the individual proteins (individually and in combination) in erythroblast kinetics.

Interestingly, our experiments also indicate that a portion of stress erythropoiesis may take place in the BM very early on during irradiation-induced haematopoietic recovery. Indeed, existing erythroblast differentiation appears temporarily accelerated in the BM by day 7, before returning to no irradiation levels by day 13, when stress erythropoiesis is shifted to the spleen, consistent with splenic erythroblast analysis.

6.4.5. Mice partially deficient for Shh do not exhibit changes in erythroblast differentiation post-irradiation and in the steady state

Having found that Shh is significantly elevated in the spleens of XT mice, and may have therefore also contributed to the unique dynamics of erythropoiesis previously observed in the spleen and BM during recovery, we chose to analyse this morphogen further. To this
end, an irradiation experiment was carried out on adult Shh+/− mice (dose; 6 Grays). The results obtained were very variable, where no clear trends were observed compared to WT controls both under steady state and stress-induced conditions, in terms of progenitor, erythroblast and reticulocyte populations as well as splenic H&E analysis. As a full adult Shh deficiency model cannot be analysed, because Shh−/− mice are embryonic lethal (Chiang et al., 1996), it is difficult to determine whether these results truly reflect the lack of a potential role of Shh in erythropoiesis. Indeed, partial Shh deficiency may also be affecting other Hedgehog components that in turn contribute to erythropoietic recovery.

The role of Ihh expression in the spleen and BM would also require further analysis in the context of haematopoietic recovery, as it has already been implicated as a positive regulator in erythropoiesis in embryogenesis studies. These trends however were not replicated in steady-state Ihh+/− adult mouse models studied by our lab, although once again, partial Ihh deficiency may not fully (indirectly) reflect the potential role of Ihh in adult erythropoiesis (Cridland et al., 2009).

6.5. Conclusion

When studying irradiated Dhh−/− mice, we found that splenic MEP progenitors and reticulocytes were increased in Dhh−/− mice compared to WT during haematopoietic recovery. This supported our hypothesis that splenic progenitor and reticulocyte trends mimic the progenitor and reticulocyte trends observed in Dhh−/− mice under PHZ treatment. This fits in with the concept that Dhh−/− BM progenitors infiltrate the spleen to become ‘stress progenitors’ at a higher rate than WT, due to the lack of a negative regulatory mechanism that would normally be orchestrated by Dhh (Lau et al., 2012; Lenox et al., 2005). Our work on the GBS-GFP reporter model, which measures total Gli-binding activity, also provides further evidence for the importance of Dhh in erythropoiesis. Under steady-state conditions, Dhh−/− mice were shown to have significantly less Gli-binding activity compared to WT, especially in the BM, where the majority of steady-state erythropoiesis occurs. These observations further support an important functional role for Dhh in erythropoiesis. Taken together, this data further corroborates Dhh as a negative regulator which, in the context of sublethal irradiation treatment, acts to control rapid erythropoiesis expansion in the first days of haematopoietic recovery (Lau et al., 2012; Perry et al., 2009). Additionally, our findings on PHZ-treated WT GBS-GFP spleens showed that PHZ-induced
anaemia is an effective model to study haematopoietic recovery, as shown by the increase in GFP expression as a result of haematopoietic cell expansion.

Our analysis of steady state Gli3+/- mice suggests that Gli3 is a direct repressor of Shh in the spleen but does not have this effect on Dhh, as Shh was found to be elevated but Dhh reduced in the Gli3+/- spleen. The analysis of sublethally irradiated Gli3+/- mice showed that erythropoiesis is significantly increased during recovery in the BM of this genotype, compared to WT. This supports our hypothesis that Dhh may be reduced in the BM (as in the XT steady-state spleen) during recovery and therefore may have a reduced negative regulatory effect on erythropoiesis in partial Gli3 deficiency, compared to WT. Interestingly, erythropoiesis was not significantly expanded in the spleen, and H&E histological analysis suggested a reduction in red blood cell activity in the spleens of XT mice compared to WT, which was also supported by the significant size reduction observed in the spleens of XT mice. Changes in the frequencies of other cell populations such as splenic stromal cells may also be contributing to the reduction in spleen size in XT mice.

These findings suggest that if Dhh is decreased in the spleen as seen in the steady-state condition, and therefore exhibiting reduced negative regulatory activity, there may be other factors such as Shh that may be dampening down erythropoiesis. Additionally, the possibility that Gli3 modulates other erythropoiesis-related genes not associated with Hedgehog signalling cannot be excluded. Interestingly, the data also indicates that stress erythropoiesis may take place early on in the BM during irradiation-induced haematopoietic recovery before shifting to the spleen, the main site of stress-induced erythropoiesis. Taken together, our data also suggests that the BM and spleen microenvironments are unique and highlights the complexity of the role of hedgehog signalling pathway in erythropoiesis, which may be greater than originally believed. The results also highlight the potential existence of other factors within the hedgehog signalling pathway and their interactions with Dhh, still undefined, which may be taking place under the umbrella of erythropoiesis regulation. Further experiments on various Hedgehog-related models, including potential crosses between Dhh mCherry mice and other Hedgehog-related mouse lines, may help to paint a clearer picture of the intricacies of the Hedgehog signalling pathway, and its regulatory role in erythropoiesis under physiological conditions.
Chapter Seven: Conclusions and future directions

7.1. Conclusion

In conclusion, an mCherry reporter for Desert Hedgehog has been successfully designed, created and applied during this PhD, as described in chapter 3. Validation experiments using tissues where Dhh expression is well documented (testes, brain and thymus) described in chapter 4, supported our hypothesis that the new mCherry transgenic is a faithful reporter for murine physiological expression of Desert Hedgehog. This validated BAC technology and recombineering as a time-effective and efficient cloning system of choice for the creation of this mouse line (Shizuya & Kouros-Mehr, 2001). Choosing mCherry as our fluorescent protein tag also allowed for successful endogenous Dhh detection by flow cytometry, as well as by immunofluorescence using an anti-mCherry antibody. As part of this validation, Dhh was efficiently detected in the Sertoli cells of the testes, the brain and thymic epithelial cells (TECs) of the thymus. In the latter cell subset, validation experiments also showed that Dhh is expressed in mTECs, and not cTECs. This data is consistent with the published Dhh expression profile of mTECs versus cTECs of the murine thymus, available in public microarray and RNA-Seq studies (Zuklys et al., 2012, GSE44945). This work paves the way for further experimentation to identify the functional significance of Dhh expression in the thymus.

Our in-depth analysis of Dhh in the spleen, as described in chapter 5, also validated our mCherry line as a true reporter of Dhh expression in splenic tissue using flow cytometry. We found that Dhh is expressed by a fraction of all known ICAM-1-positive stromal subsets, particularly RPFs and FRCs, but not ICAM-1-negative vascular endothelial cells in the spleen in the steady state, in line with immunofluorescence data showing mCherry is scattered throughout the spleen. This disproved our hypothesis that Dhh is more highly expressed in steady state RPFs compared to other stromal cells. Interestingly, Dhh was detected in a higher fraction of haematopoietic cells than stromal cells, which include both APCs (DCs, B cells and possibly macrophages) and non-APCs (T cells and possibly other subsets to be evaluated) in the steady state. Under stress-induced conditions by PHZ treatment, Dhh was also significantly upregulated in APCs and non-APCs, as well as FDCs.
and MRCs, but not RPFs, despite the latter subset till expressing the highest number of mCherry+ stromal cells overall. This supported our hypothesis that Dhh is expressed more highly in RPFs under stress-induced conditions. Considering both the haematopoietic and stromal cell data together, it can be speculated that Dhh may play a role in the formation of secondary immune responses in the white pulp of the spleen, in addition to its known role in erythropoiesis as a negative regulator.

To further our understanding of the Hedgehog signalling pathway in erythropoiesis, we carried out various sublethal irradiation and PHZ experiments on established Hedgehog-related mouse lines, with baseline controls at all times to also study steady state conditions. Our finding that splenic erythroid progenitors and reticulocytes are increased during post-irradiation recovery in Dhh-/- mice further supports Dhh as an important negative regulator of erythropoiesis (Lau et al., 2012; Lenox et al., 2005). Furthermore, our finding that Gli binding activity is significantly reduced in BM and splenic erythroblasts of Dhh-/- mice using the GBS-GFP reporter line under steady-state conditions corroborates the importance of Dhh signalling to erythroblasts, during erythropoietic regulation. Using the same model, the significant increase in GFP expression observed within WT spleens upon anaemia-inducing PHZ treatment supports this treatment shows that Hh pathway activation is increased in the recovering spleen.

Our study on Gli3+/- splenic tissue supported our hypothesis that Shh is elevated in this model under steady state conditions, suggesting that Gli3 acts as a direct repressor of Shh. Dhh however was found to be decreased in Gli3+/- tissue under the same conditions. Having established this, our hypothesis that erythroblast differentiation is expanded in the BM and spleen during haematopoietic recovery due to potential decreased levels of Dhh was supported in the BM, but not in the spleen. Interestingly, H&E histological analysis even suggests a reduction in erythrocyte activity in the spleens of XT mice compared to WT, in line with the significant size reduction observed in the spleens of XT mice, during recovery. Taken together this data highlights the difference in BM and spleen microenvironments, and the intricacy of the Hedgehog signalling pathway and its role in erythropoiesis, in which several other undefined factors may be involved as a direct or indirect effect of Gli3. Interestingly, this data also indicates that a portion of stress erythropoiesis may take place in the BM very early on during irradiation-induced haematopoietic recovery before shifting to the spleen, which is the main site of stress-induced erythropoiesis.
7.2. Future work

The newly made mCherry reporter mouse line, which has been validated as a faithful reporter for Dhh, is now an established model that can be used in a plethora of future experiments. The finding that splenic white pulp stromal cells produce Dhh, as well as haematopoietic cells including APCs, raises the question as to the potential function of Dhh in the white pulp, in addition to its known role in erythropoiesis as a result of red pulp fibroblast and macrophage production (Immgen). The white pulp being a complex microenvironment consisting of three distinct compartments involved in initiating immune responses to blood-borne antigens, Dhh can potentially play one of many roles. It is also possible that potential Dhh function in the white pulp is associated with Shh, which is known to be expressed by FDCs to provide survival signals to GC (germinal centre) B cells (Sacedón et al., 2005). Ihh, which is also expressed in the spleen, may also be involved although it has not so far been functionally implicated in the spleen (Perry et al., 2009). This avenue of research would therefore be worthwhile to pursue.

In doing so, it would be informative to carry out further experiments in continuation to the steady state and anaemia-induced experiments that were carried out on stromal subsets of the spleen, using the mCherry reporter line. Sublethal irradiation (which suppresses haematopoiesis) could be used as an additional model to study Dhh production by stromal subsets of the spleen during recovery. In such mCherry experiments, the use of additional stroma surface antibodies targeted against receptors such as CD16 and RANKL, would allow the individual analysis of Dhh production by follicular dendritic cells (FDCs) and marginal reticular cells (MRCs), respectively (Mueller & Germain, 2009). Combining such stroma-specific antibodies with other antibodies targeted against B cells, macrophages, DCs and T cells would paint the phenotypic picture of Dhh expression in the various compartments of the white pulp. The expression of Dhh by red pulp fibroblasts and macrophages could also be further studied in combination with erythrocyte and macrophage markers involved in the red pulp of the spleen.

Additionally, injecting mCherry mice with an antigen complex such as Prevenar 13 (Pfizer), a pneumococcal vaccine representative of thirteen of the most common Streptococcus pneumoniae bacterial strains, may provide a functional platform to study the role of Dhh in the white pulp of the spleen (Siggins et al., 2015). Such a vaccine would induce reactions in
germinal centres (comprised of B cells, FDCs, tingible body macrophages and some T cells where B cells undergo clonal expansion and selection for memory B cell and plasma cell production) in the spleen without causing disease (Sacedón et al., 2005). Dhh expression could then be measured by flow cytometry in different cell subsets at various time points during the generation of secondary immune responses in conjunction with B cell, T cell and DC markers, to investigate a potential white pulp role for Dhh. Furthermore, the study of the expression of Dhh in haematopoietic cells and mesenchymal stromal subsets of the BM in the steady state, and during recovery, would be interesting to pursue. Such analysis could help further delineate the potential roles of Dhh in the BM and spleen, other than erythropoiesis, in view of the potentially distinct functions the protein may have in both tissues, as alluded to in chapter 5, section 5.4.1 (Anjos-Afonso & Bonnet, 2008).

Once a clearer picture of Dhh expression and its potential role in the BM and spleen (other than erythropoiesis) is drawn, it would also be useful to cross the mCherry line with other available Hedgehog-related lines. Such lines may include mice that are deficient for Shh and Ihh. As both Shh and Ihh are embryonic lethal, it may be more beneficial to study the dynamics of Dhh by mCherry in the context of full Shh or Ihh deficiency in embryos (Chiang et al., 1996; Cridland et al., 2009). In this manner, full Hedgehog deficiency may be studied in the context of mCherry, which would in turn allow a clearer understanding of the implications of possible Hedgehog proteins interactions in different cellular processes, such as erythropoiesis. In such a study it would be important to consider potential differences in the mechanisms studied between adult mice and embryos. Crossing our mCherry line with Gli3+/− mice would also allow the identification of Dhh protein expression profiles in the spleen and BM of Gli3+/− mice, which could provide an additional measure to interpret the differences in erythropoiesis visualised in Gli3+/− BM and spleen during recovery (chapter 6).

Finally, our mCherry reporter also offers the possibility to visualise mCherry (Dhh) and GFP-expressing cells of a gene of interest in combination owing to their distinctive emission spectra (Doherty et al., 2010). One application of this may be to cross the mCherry reporter line with the GBS-GFP reporter line that measures Gli-binding activity. Such crosses should help to improve our understanding of the complexity of the Hedgehog signalling pathway, and its regulatory role in erythropoiesis under physiological conditions.
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