The role of the transcription factor Gli3 and Hedgehog signalling in fetal B cell and thymocyte development

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A thesis submitted to University College London for the Degree of Doctor of Philosophy

September 2017

Great Ormond Street Institute of Child Health
Infection, Immunity and Inflammation
University College London
Declaration of Originality

With reference to UCL Academic Regulations and Guidelines for Research Degree Students 2016/17, I, Anisha Solanki confirm that the work presented in this thesis is my own unless otherwise clearly stated.

Anisha Solanki

September 2017

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Abstract

Gli3 is a Hedgehog (Hh) responsive transcription factor that can function as a transcriptional repressor or activator. Here, I show that Gli3 is required for both fetal B cell and thymocyte development. Gli3 represses Sonic hedgehog (Shh) in the fetal liver and thymus. Gli3 deficiency shows both Gli3-dependent-Shh-independent and Shh-dependent changes.

Before birth B-cells develop in the fetal liver (FL). Our results show that Gli3 activity in the FL stroma is required for B-cell development. In the Gli3-deficient FL B-cell development is reduced at multiple stages, while the Shh-deficient FL shows an increased B-cell development, and Gli3 functions to repress Shh transcription. Using a transgenic Hedgehog (Hh)-reporter mouse, I show that Shh signals directly to developing B-cells, and Hh pathway activation is increased in developing B-cells from the Gli3-deficient FL. RNA-Sequencing confirmed that Hh-mediated transcription was increased in B-lineage cells from Gli3-deficient FL, and showed that these cells expressed reduced levels of B-lineage transcription factors and BCR/pre-BCR-signalling genes. Expression of the master regulators of B-cell development, Ebf1 and Pax5, was reduced in developing B-cells from Gli3-deficient FL and increased in Shh-deficient FL, and in vitro Shh-treatment or neutralisation repressed or induced their expression respectively.
In the fetal thymus (FT), Gli3 activity in thymic epithelial cells (TEC) is required for differentiation from CD4+CD8+ to CD4+ single positive (SP4) cell and Gli3 represses Shh. Constitutive deletion of Gli3, and conditional deletion of Gli3 from TEC, reduced differentiation to SP4, whereas conditional deletion of Gli3 from thymocytes did not. Conditional deletion of Shh from TEC increased differentiation and expression of Shh was upregulated in the Gli3-deficient thymus. Use of a transgenic Hh-reporter showed that the Hh pathway was active in thymocytes, and increased in the Gli3-deficient FT. Neutralisation of endogenous Hh proteins in the Gli3-mutant thymus restored SP4 differentiation, indicating that Gli3 in TEC promotes SP4 differentiation by repression of Shh. Transcriptome analysis showed that Hh-mediated transcription was increased but TCR-mediated transcription decreased in Gli3-/- thymocytes.
Acknowledgements

Firstly, I would like to thank my family for instilling the importance of education in me. Your support and encouragement has allowed me to accomplish many great successes and will continue to do so in the future. I am forever grateful to you and dedicate this thesis to your unconditional love and encouragement.

I am indebted to my super-amazing supervisor Tessa, without whom this journey would not be possible. Thank you Tessa for your invaluable guidance, support and motivation. Your very intelligent yet humble nature inspires me, and I am very fortunate to have had this opportunity of working with you. Thank you 😊

I would also like to thank my excellent team (Rain, Diana, Eleftheria, Sonia, Kostas, Hemant, Jiawei, Jose, Sue, Anna, Martino and Masahiro) for their incredible support. From teaching me various lab and computational techniques to answering all my trivial questions, I thank you all for your patience and motivation through this journey.

Finally, I express my sincere gratitude towards the Great Ormond Street Hospital Children’s Charity (GOSHCC) for supporting me through this PhD.
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My Publications

First Author Publications


- Solanki A, Saldana JI, Ross S, Lau CI, Yanez DC and Crompton T. 2017. The transcription factor Gli3 promotes differentiation from double positive to CD4 single positive thymocyte by repression of Shh (Positively reviewed by Development, Pending Resubmission).


Co-Authored Publications


Publications in progress


Abbreviations

-/- Knockout or deficient
+/- Heterozygous
+/- Wildtype
µg Microgram
µl Microlitre
µH Pre-BCR heavy chain
AGM Aorta-gonad-mesonephros
Au Arbitrary unit
B-ALL B-cell Acute Lymphoblastic Leukemia
BMP Bone Morphogenetic Protein
BM Bone Marrow
BSA Bovine Serum Albumin
cAMP Cyclic adenosine monophosphate
CBP CREB binding protein
CCA Canonical correspondence analysis
CD Cluster of Differentiation
cDNA Complementary DNA
Ci Cubitus interruptus
Cos2 Costal2
Co-Smad Common-mediator Smads
CREB cAMP response element-binding protein
Dhh Desert hedgehog
DEG Differentially Expressed Genes
DN Double Negative
DNA Deoxyribonucleic acid
dNTPs Deoxynucleotide triphosphates
DP Double Positive
E Embryonic day
eBayes Empirical Bayes
EBF Early B Cell Factor
EDTA Ethylenediaminetetraacetic acid
Egr1-4 Early growth response family members 1-4
ELISA Enzyme-Linked Immunosorbent Assay
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>ETP</td>
<td>Early thymic progenitor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FL</td>
<td>Fetal liver</td>
</tr>
<tr>
<td>fl/</td>
<td>Floxed</td>
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<td>FLOC</td>
<td>Fetal liver organ cultures</td>
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<tr>
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<td>forward scatter/ side scatter</td>
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<td>FTOCs</td>
<td>Fetal thymic organ cultures</td>
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<td>Fused</td>
</tr>
<tr>
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<td>GATA binding protein 3</td>
</tr>
<tr>
<td>GBS</td>
<td>Gli Binding Site</td>
</tr>
<tr>
<td>Gli</td>
<td>Glioma associated oncogene family</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen Synthase Kinase 3</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog family</td>
</tr>
<tr>
<td>Hhip</td>
<td>Hedgehog-interacting protein</td>
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<tr>
<td>HPRT</td>
<td>Hypoxanthine Guanine Phosphoribosyl Transferase</td>
</tr>
<tr>
<td>HSA</td>
<td>Heat Stable Antigen</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
</tr>
<tr>
<td>HhSC</td>
<td>Hh signalling complex</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>Ihh</td>
<td>Indian Hedgehog</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ISP</td>
<td>CD8+ Immature single-positive cells</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base</td>
</tr>
<tr>
<td>Lck</td>
<td>Lymphocyte-specific protein tyrosine kinase</td>
</tr>
<tr>
<td>Lin-</td>
<td>Lineage negative (CD71-,Ter119-,Gr-1-,CD11b-,CD3-)</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphoid Enhancer Factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LSK</td>
<td>Lineage negative Sca1+cKit+ cells</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Flourescence Intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MoFlo</td>
<td>Modular Flow Cytometer</td>
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<tr>
<td>Mutant</td>
<td>+/- (Heterozygote) and -/- (Knockout)</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
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<td>Nuclear factor-kB</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer cell</td>
</tr>
<tr>
<td>p</td>
<td>p-value</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
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<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PCs</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>pre-TCR</td>
<td>Pre-T cell receptor complex</td>
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<tr>
<td>pTa</td>
<td>pre-T alpha chain</td>
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<td>Ptch</td>
<td>Patched</td>
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<tr>
<td>RAG</td>
<td>Recombinase activating gene</td>
</tr>
<tr>
<td>rShh</td>
<td>Recombinant Shh</td>
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<tr>
<td>rHhip</td>
<td>Recombinant Hh inhibing protein</td>
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<tr>
<td>Q-RT-PCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
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<td>Runx3</td>
<td>Runt-related transcription factor 3</td>
</tr>
<tr>
<td>S1P1</td>
<td>Sphingosine-1-Phosphate receptor 1</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>Smo</td>
<td>Smoothened</td>
</tr>
<tr>
<td>SP</td>
<td>Single Positive</td>
</tr>
<tr>
<td>Su(fu)</td>
<td>Suppressor of Fused</td>
</tr>
<tr>
<td>TCF</td>
<td>T cell factor</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEC</td>
<td>Thymic Epithelial Cell</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
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<tr>
<td>Th-POK</td>
<td>T-helper-inducing POK/Kruppel like factor</td>
</tr>
<tr>
<td>Tox</td>
<td>Thymus high mobility group box protein</td>
</tr>
<tr>
<td>V(D)J</td>
<td>Variable (Diversity) joining</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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Chapter 1
Chapter 1. Introduction

1.1 Murine Embryo Hematopoiesis

Hematopoiesis is defined as the continuous production or renewal of all mature functional blood cell types such as lymphocytes, erythrocytes, monocytes and granulocytes from a common self-renewing multi-potent hematopoietic stem cell (HSC).

It is very important to understand the pathways regulating hematopoiesis, particularly lymphocyte development, as the dysregulation of many of these pathways have been shown to be key contributors of autoimmunity and cancer. In addition, understanding lymphocyte development will also help identify targets that can enhance immunity.

Hematopoiesis takes place sequentially in various tissues of the embryo. It begins at around embryonic day 7 (E7) in the yolk sac, then moves on to the aorta-gonad-mesonephros (AGM) region between E9-10 and continues in the fetal liver until birth, after which it occurs in the adult bone marrow (Medvinsky and Dzierzak 1996, Tober, Koniski et al. 2007, Orkin and Zon 2008, Kissa and Herbomel 2010). The fetal liver is the main site of hematopoiesis during the fetal stages of life while hematopoiesis in adults mainly occurs in the bone marrow (Orkin and Zon 2008).
During embryonic development, hematopoiesis occurs in two sequential waves. The initial phase is known as the primitive hematopoiesis, which promotes the production of red blood cells (RBCs) to allow oxygen transport to various embryonic tissues, which in turn allows rapid growth of the embryo (Orkin and Zon 2008). The primitive phase is later replaced by definitive hematopoiesis, which begins in the AGM region. Hematopoiesis in the AGM region is transient and although the first HSCs are formed in the AGM, they expand, reside and further differentiate in the fetal liver until birth (Medvinsky and Dzierzak 1996, Orkin and Zon 2008, Ciriza, Thompson et al. 2013).

The fetal liver is the main site of HSC expansion and differentiation into the myeloid-erythroid and lymphoid lineages. HSC are defined by their surface expression of c-Kit (CD117). These c-Kit positive cells differentiate further to express the cell surface marker Sca-1 and are known as LSK (Lineage negative Sca1+cKit+). The LSK cells differentiate to either express CD34 and are defined as common myeloid-erythroid progenitor (CMEP) cells, or express the IL-7Rα (CD127) and are defined as common lymphoid progenitor (CLP) cells (Figure 1.1). Historically, it was believed that pluripotent HSCs could either give rise to a common myeloid-erythroid progenitor (CMEP) or a common lymphoid progenitor (CLP) (Figure 1.1). The CMEP could only give rise to myeloid cells including megakaryocytes, granulocytes, monocytes and erythrocytes, while the CLP could only give rise to T and B cells (Figure 1.1) (Kawamoto, Wada et al. 2010) (Janeway et al., 2009). However,
recent studies have proposed a myeloid-based model where the CMEPs can give rise to progenitors which can commit to T or B cells (Figure 1.1). For example, the CMEP can give rise to a myeloid-T cell (MT) progenitor that specifies commitment to either a myeloid or a T cell. Interestingly, the CMEP can also give rise to a myeloid-B cell (MB) progenitor that can only commit to a myeloid or a B cell (Kawamoto, Ohmura et al. 1997, Kawamoto, Ikawa et al. 2000, Kawamoto 2006, Kawamoto, Ikawa et al. 2010). The early thymic progenitor (ETPs) of the thymus, which were always thought to give rise to T cells are capable of generating myeloid cells as well (Bell and Bhandoola 2008). Therefore, the lineage separation between the myeloid and lymphoid lineages is not stringent but rather flexible, so that myeloid progenitors can give rise to T and B lymphocytes and early lymphoid progenitors can produce myeloid cells. However, studies have highlighted that CLPs are more like to develop into lymphoid lineages than myeloid. CLPs are defined by their expression of the FMS-like tyrosine kinase 3 (FLT3) and the IL-7 receptor (IL-7R), which are both vital for development of B cells at this stage (Sitnicka, Brakebusch et al. 2003).

HSCs from the fetal liver migrate to various other fetal tissues including the fetal thymus, fetal spleen and the fetal bone marrow. Progenitor cells from the fetal liver start seeding the fetal thymus at E11 giving rise to early thymic progenitors (ETPs) which differentiate into mature T cells (Scollay, Smith et al. 1986). The fetal spleen is seeded on E15 and active hematopoiesis occurs in the fetal spleen until E17.5 (Christensen,
Wright et al. 2004, Ciriza, Thompson et al. 2013). HSCs from the fetal liver migrate to the bone marrow during late stages of embryonic life. The first HSCs are found in fetal bone marrow on E17.5, and postnatal hematopoiesis continues there throughout life (Christensen, Wright et al. 2004, Ciriza, Thompson et al. 2013).

1.2 B cell development

1.2.1 B cell development in adults

There are two main populations of B cells: B-1 and B-2 cells. B-1 cells account for ~5% of the total adult B cell population in mice and are found in the spleen, intestine, pleural and peritoneal cavities (Kroese, Ammerlaan et al. 1992, Kantor and Herzenberg 1993). They are subdivided into B1a (sIgM+CD11b+CD5+) and B1b (sIgM+CD11b+CD5-) and mediate innate responses by recognizing self-antigens and producing immunoglobulins (Igs) against specific pathogens (Montecino-Rodriguez and Dorshkind 2012). Recently, B-1 cells have also been defined in humans as cells expressing the surface markers CD20+CD27+CD43+ and are found in both the umbilical cord blood and peripheral blood (Rothstein, Griffin et al. 2013).

B-2 cells are generated in the bone marrow and mainly reside in the spleen. They mature into a predominant population of follicular cells and smaller marginal zone population. B-2 cells undergo class-switching and differentiate into memory cells or effector cells, which are both
crucial for mediating adaptive immunity (Dorshkind and Montecino-Rodriguez 2007, Hardy, Kincade et al. 2007, Montecino-Rodriguez and Dorshkind 2012). An important way to distinguish B-1 and B-2 progenitors is by examining their response to TSLP, B-1 cells proliferate in response to TSLP (Thymic Stromal Lymphopoeitin) while B-2 cells do not (Dorshkind and Montecino-Rodriguez 2007, Barber, Montecino-Rodriguez et al. 2011).

Recently, many studies have shown that B-1 cells are mainly present in the fetal liver, fetal spleen and fetal bone marrow while B-2 cells predominantly exist in the adult bone marrow (Ghosn, Yamamoto et al. 2012, Montecino-Rodriguez and Dorshkind 2012).

Although B cell development begins in fetal liver, after birth, the bone marrow takes over the regeneration of all hematopoietic cells, including B cell development and maturation. B cells undergo many stages of differentiation and maturation in the bone marrow. The earliest common lymphoid progenitor cell (CLP), first gains the expression of the surface marker B220 (CD45R) and becomes restricted to the B lineage. These pre-pro-B cells mature to express CD19 at intermediate levels (B220+CD19\textsuperscript{int}) known as pro-B cells and then mature to express high levels of CD19 (B220+CD19\textsuperscript{hi}) to be known as pre-B cells. The pre-B cells undergo heavy chain rearrangements and express the pre-BCR. Following light chain rearrangement and expression they start expressing a functional B cell receptor (BCR) detected by the expression of cell surface IgM. This mature BCR can bind to specific soluble
antigens (molecules) and internalise them by receptor mediated endocytosis. If the antigen contains a protein component the B cell will process it into peptide fragments and display these peptides as peptide-MHC class II complexes. These peptide-MHC class II complexes are recognised by the TCR of antigen specific CD4-T cells and induce further differentiation and activation of the B cells (Murphy, Travers et al. 2012).

1.2.2 Fetal B cell development

Unlike adult B cell development in the bone marrow, during embryonic stages of life, B cell development occurs in three distinct waves. The first wave occurs at around embryonic day 9 (E9) in the yolk sac (YS) and in the intra-embryonic para-aortic splanchnopleura (PAS) (Palis and Yoder 2001). These tissues have been observed to give rise to CD93+CD19+B220lo/neg B-1 progenitor cells, which mature into B-1 cells (Yoshimoto, Montecino-Rodriguez et al. 2011). The second wave occurs in the fetal liver, spleen and bone marrow during mid-gestation (Ghosn, Yamamoto et al. 2012). In the fetal liver, B cell development is initiated on E11 where c-kit+CD93+CD19+Pax5+ B cell restricted progenitors differentiate into mature B cells (de Andres, Gonzalo et al. 2002). Finally, the third wave of development occurs in the adult bone marrow where development of B-1 progenitors declines and the developmental program of the B-2 progenitors takes over (Montecino-Rodriguez and Dorshkind 2012).
The B cell development from the second and third wave is believed to occur from HSC in the fetal liver, which give rise to the CLP. These further differentiate into either the B-1 or the B-2 cells. Commitment to the B cell lineage first occurs on E12.5, as cells mature to initially express CD19 or B220 and are defined as either B-1 (CD19+B220lo-neg) or B-2 (CD19-B220+) lineage cells (Egawa, Kawabata et al. 2001, Dorshkind and Montecino-Rodriguez 2007, Montecino-Rodriguez and Dorshkind 2012).

There are many controversies regarding B-1 and B-2 fetal cell development. Some studies suggest that commitment into either lineage occurs at the CLP stage, while others suggest that the CLP population is heterogeneous containing cells already defined to differentiate into either B-1 or B-2 lineage (Barber, Montecino-Rodriguez et al. 2011).

Most B-1 cells are derived from the HSCs in the fetal liver and their numbers peak at around E16-17, and decrease after birth as B-2 cell development in the bone marrow takes over (Montecino-Rodriguez and Dorshkind 2006).

Both B-1 and B-2 progenitors mature into B220+CD19+ double positive cells, which undergo immunoglobulin heavy chain gene rearrangement to give rise to the first cells that express cell surface uH (pre-BCR) (Dorshkind and Montecino-Rodriguez 2007, Montecino-Rodriguez and Dorshkind 2012). This pre-B population can also be identified by cell surface expression of BP-1, prior to rearrangement of the light chain
locus and cell surface expression of IgM (Hardy and Hayakawa 2001, Dorshkind and Montecino-Rodriguez 2007, Montecino-Rodriguez and Dorshkind 2012). A schematic of fetal liver B cell development is shown in Figure 1.3.

1.2.3 Pre-BCR and BCR formation and signalling

The presence of a functional BCR is a key identity of a mature B cell. In mammals, a diverse repertoire of BCRs is formed through the rearrangement of the V, D and J gene segments of the Immunoglobulin (Ig) heavy chain and by the rearrangement of the V and J genes of the light chain. The diversity is necessary to allow the BCRs to recognise a vast repertoire of foreign antigens.

Pre-BCR rearrangement occurs before a functional BCR is expressed on the cell surface. The Pre-BCR rearrangement process is initiated through the expression of the Recombinase Activating Genes 1 and 2 (Rag1/2), which are important for joining the D and J_L segment at the heavy chain locus. In order to prevent the B cell from having two different receptors of antigen specificities, a process known as allelic exclusion occurs, where only one of the two heavy chain alleles is successfully rearranged (Murphy, Travers et al. 2012).

Once the heavy chain is successfully rearranged, the two surrogate light chain components, λ5 and VpreB, and the Ig accessory proteins Igα and Igβ associate with the rearranged heavy chain to form the pre-BCR.

This pre-BCR signalling complex is translocated to the cell membrane, to transduce downstream cytoplasmic signalling. Recent studies suggest that the ligand-independent pre-BCR oligomerisation can also transduce signalling (LeBien and Tedder 2008), however more studies are needed to evaluate this.

Signalling through the pre-BCR causes phosphorylation of the signal transduction molecules ITAM (Immunoreceptor Tyrosine Activation Motifs) present on the Igα and Igβ. This phosphorylation transduces a cytoplasmic signalling cascade that arrest the heavy chain recombination and allows clonal expansion of these recombination arrested large Pre-B cells. At this stage and Pre-B cells with an incompetent pre-BCR will not be able to signal effectively, thus limiting their survival (Karasuyama, Rolink et al. 1996, Hardy and Hayakawa 2001).

Following clonal expansion, the Rag1/2 genes are re-expressed and the expanded cells undergo light chain rearrangement, where the $V_L$ and $J_L$ segment join on one of light chain containing chromosomes. There are two different of light chains, the kappa ($\kappa$) and the lambda ($\lambda$) loci. Light gene rearrangement begins $\kappa$-locus, if rearrangement of the $\kappa$-chain fails then $\lambda$-chain rearrangement takes place. Once one of the light chain has successfully synthesised, it pairs with the heavy chain to form the mature BCR.
The mature BCR is screened for self-reactivity and BCRs strongly recognizing multivalent self-antigens either undergo receptor editing or clonal deletion. BCRs clones possessing weak reactivity to self or recognizing soluble antigens are capable of reaching peripheral B cell compartments, but these cells are anergic (non-functional) (Cyster, Hartley et al. 1994). Therefore a very small percentage (~10%) of B cell clones can successfully transit through negative selection and leave the bone marrow to migrate to the spleen (Rolink, Andersson et al. 1998, Hardy and Hayakawa 2001, Jung, Giallourakis et al. 2006).

1.3 T cell development in the fetal thymus

1.3.1 Early Thymocyte development

As discussed in the section 1.1, progenitors from the fetal liver emigrate to the fetal thymus on E11 and generate early thymic progenitor cells which differentiate into mature thymocytes (Scollay, Smith et al. 1986). In addition to thymocyte development, the thymus itself, including its structural components the cortex and medulla are also developing during embryogenesis. The structure of the thymus develops as developing thymocytes travel around the thymus and interact with various developing stromal cells at different positions in the fetal thymus. The lympho-stromal cross-interactions dictate the structural positioning of the thymic stromal cells relative to the developing thymocytes to allow the formation of the structural
partitions of the thymus, such as the thymic subcapsule, cortex and the medullary regions (Takahama 2006, Love and Bhandoola 2011).

The different micro-environments of the thymus are essential for sequentially regulating the development of the ETP into a non-self reactive mature T cell (Petrie and Zuniga-Pflucker 2007). Thymocyte development begins with the immature Double Negative (DN, CD4-CD8-) population differentiating to the semi-mature Double Positive (DP, CD4+CD8+) stage to then give rise to the most mature Single Positive (SP, CD4+CD8- or CD4-CD8+) cell. Each distinct stage of development is characterized by the presence of different cell surface markers including c-kit, CD44 (cell-surface glycoprotein), CD25 (alpha chain of the IL-2 receptor), and the T cell co-receptors CD4 and CD8. A schematic of the different stages of thymocyte development is shown in Figure 1.5.

The immature DN stage is divided into four sequential stages of maturation from the most immature DN1 to the DN4 population. DN1 cells are multipotent and can develop into other lineages apart from T cells, such as granulocytes, NK, thymic dendritic cells (DC) and macrophages (Mϕ). DN1 cells are defined by cell surface expression of c-kit and CD44. They begin migrating deeper into the thymic cortex from the subcapsular zone to interact with the cortical thymic epithelial cells (cTECs) which allow their differentiation into DN2 cells (Koch and Radtke 2011).
DN2 cells gain cell surface expression of CD25 and are defined as c-kit+CD44+CD25+ cells. The DN2 stage can be divided into early (DN2a) and late (DN2b) stages. The differentiation potential of early DN2a cells is more restricted compared to DN1, but, they can still differentiate into NK cells or thymic dendritic cells (Wu, Li et al. 1996). In contrast, late DN2b cells are committed to developing into T lineage cells only. The DN2 stage is important for initiating the rearrangement of T cell receptor chains (TCRβ, TCRδ and TCRγ), which is completed at the DN3 stage (Petrie, Livak et al. 1995, Naito, Tanaka et al. 2011). DN2 cells differentiate into DN3 as they lose cell surface expression of c-kit and CD44. Cells at the DN3 stage have to undergo the first developmental checkpoint known as β-selection, a process where their rearranged TCRβ chain is assessed for its functionality. Only cells that express a functional TCRβ chain can proceed from the DN3 to DN4 stage (Mallick, Dudley et al. 1993, Dudley, Petrie et al. 1994). At the DN4 stage cell lose the expression of CD25 and start moving back towards the thymic medulla (Koch and Radtke, 2011), where they proliferate, expand and gain cell-surface expression of the co-receptor CD8 to become immature CD8+ single positive intermediate (ISP). The ISP is a transient rapidly cycling population and most cells immediately express the co-receptor CD4 (MacDonald, Budd et al. 1988). Therefore most DN4 cells differentiate into CD4+CD8+ DP cells. Further differentiation and maturation of DP thymocytes will be discussed in the subsequent sections.
1.3.2 Repertoire Selection and Lineage Commitment

A second developmental checkpoint occurs at the DP stage where cells rearrange their TCRα chain and generate an αβ TCR which is examined for its specificity and affinity to recognise self-peptides. A diverse TCR population is generated by temporally regulating DP proliferation and the Rag2 protein level. DP thymocytes first undergo rapid proliferation and the Rag2 gene is only elevated once proliferation is terminated. This allows each clone of cells with a single TCRβ chain to be available for α chain rearrangement, and therefore increases the chances of generating different combinations of TCRαβ. This random TCR germ-line rearrangement can generate more than $10^{15}$ different αβ receptors, which forms a diverse TCR repertoire (Robey and Fowlkes 1994). This diverse repertoire can recognize, processed self and foreign antigens associated with self and non-self MHC molecules and self MHC molecules alone (Starr, Jameson et al. 2003).

It is essential to eliminate the TCRs recognizing self-antigens as they can lead to autoimmunity. TCRs with a very low or a very strong affinity for the self-MHC molecules and peptide are usually self-reactive. Therefore, DP cells undergo negative selection where these self-recognising TCRs are eliminated through apoptosis in the thymic medulla (Starr, Jameson et al. 2003, Koch and Radtke 2011).

Negative selection usually occurs after positive selection. During positive selection TCRs capable of binding to self-MHCs with
appropriate affinity are selected to allow for Major Histocompatibility Complex (MHC) restriction. These are the only $\alpha\beta$ TCRs that would be able to recognise and bind to self-MHC molecules with the processed antigen. DP thymocytes with such TCRs with an intermediate affinity receptor for self MHC molecules are positively selected for further maturation while the rest undergo negative selection related apoptosis (Starr, Jameson et al. 2003). This stringent selective process of TCR selection results in approximately 1-3% of DP thymocytes completing maturation and migrating out of the thymus (Goldrath and Bevan 1999).

Positively selected DP cells differentiate to either lose their CD8 co-receptor to become a mature CD4+CD8- (SP4) cell, or lose CD4 co-receptor to become a mature CD4-CD8+ (SP8) cell (Koch and Radtke 2011). The decision to commit to either a SP4 or a SP8 cell is influenced by the class of MHC restricted on the thymocyte during positive selection. DP thymocytes recognising a MHC Class II will commit to becoming SP4 cells while DPs recognising MHC Class I will commit to the SP8 lineage (Teh, Kisielow et al. 1988). Studies have suggested that lineage committed SP4 or SP8 thymocytes with non-matching MHC specificities do not survive to mature further (Chan, Cosgrove et al. 1993, Itano, Kioussis et al. 1994, Leung, Thomson et al. 2001).

The decision of committing from the DP to SP stage is mainly determined by the TCR affinity and specificity, as discussed above. However, the choice of committing to either a SP4 or a SP8 is still under intense debate and studies have proposed various factors influencing
the SP4/8 lineage commitment. Some of these include the TCR signal strength, duration of the TCR signal and the selection of correctly matched MHC-restricted co-receptors (Singer, Adoro et al. 2008).

The engagement of the $\alpha\beta$ TCR to a peptide-MHC (a MHC molecule presenting a processed antigen peptide) generates a TCR signal. Interestingly, TCR engagement to a MHC class II peptide complex generates a stronger signal than engagement to a MHC class I peptide complex. The co-receptor CD4 binds to more Lck on its intracellular cytoplasmic tail compared to CD8, therefore transducing stronger TCR signals (Shaw, Amrein et al. 1989). This differential signalling allows the DP thymocyte to terminate the expression of one of its co-receptors as stronger TCR signals favour commitment towards the SP4, while weaker signals allow SP8 lineage differentiation (Seong, Chamberlain et al. 1992, Itano, Salmon et al. 1996).

In addition to the TCR signal strength, the duration of the TCR signal also influences SP4/SP8 the commitment. TCR signals of longer durations favour SP4 commitment by causing transcriptional termination of the CD8 co-receptor. In contrast, TCR signals of shorter duration arrest CD4 transcription, allowing SP8 lineage commitment (Yasutomo, Doyle et al. 2000). The duration of the TCR signal is regulated by IL-7 signalling. Continuous TCR signalling downregulates IL-7 signal transduction allowing the formation of SP4 cells by transcriptionally terminating CD8 co-receptor surface expression. In contrast, a disruption in TCR signalling allows continuous IL-7

Interestingly, there are approximately four times more SP4s emigrating from the thymus compared to SP8. A recent study has proposed that this 4:1 bias may be due to a higher apoptosis rate in the MHC Class I restricted DP TCR^{int}CD5^{hi} thymocytes. Therefore, despite a similar level of initial development of both lineages, the increased survival MHC Class II restricted cells allows the commitment and emigration of a greater number of SP4s compared to SP8s (Sinclair, Bains et al. 2013).

1.3.3 Transcriptional regulation during Lineage Commitment

In addition to the requirement for TCR signalling as a regulator of SP4/SP8 lineage choice, various studies have highlighted the role of lineage determining transcription factors such as Runx3, Th-POK, Gata3 and Tox (Naito, Tanaka et al. 2011). Runx3 promotes commitment towards SP8 by binding to the enhancer element of the CD8 gene, thus mediating CD8 transcription and silencing CD4 transcription by binding to the CD4 silencing element (Taniuchi, Osato et al. 2002, Grueter, Petter et al. 2005, Sato, Ohno et al. 2005). Runx3 also binds to and downregulates the expression of the key SP4 lineage committing gene, Th-POK, therefore promoting the maximal number of SP8 cell
commitment (Setoguchi, Tachibana et al. 2008). Th-POK is an essential SP4 lineage commitment gene discovered by two independent studies (He, He et al. 2005, Sun, Liu et al. 2005). Enforced Th-POK expression was shown to promote all positively selected thymocytes towards the SP4 lineage, while loss of Th-POK diminished SP4 cell generation. Interestingly, Th-POK can suppress the enhancer activity on the CD8 gene leading to a decrease in CD8 surface expression. In addition, Th-POK prevents the binding of CD4 silencing genes such as Runx3, thus maintaining CD4 surface expression and allowing maximal SP4 cell commitment (Jenkinson, Intlekofer et al. 2007). This antagonistic relationship between Th-POK and Runx3 is central to the transcriptional regulation of SP4/8 lineage commitment.

Th-POK expression is enhanced by the transcription factor Gata3, which is upregulated before Th-POK and Runx3 in DP thymocytes. Studies have shown that loss of Gata3 decreases both Th-POK expression and differentiation to SP4 (Pai, Truitt et al. 2003, Wang, Wildt et al. 2008). However, enhanced Gata3 expression does not direct DPs towards the SP4 lineage, suggesting an indirect regulatory role of the protein in directing SP4 commitment (Hernandez-Hoyos, Anderson et al. 2003). Another key transcription factor promoting SP4 lineage commitment is Tox. Tox deficient DP thymocytes lack Th-POK expression and fail to differentiate into SP4 cells (Aliahmad, O'Flaherty et al. 2004, Aliahmad and Kaye 2008, Aliahmad, Kadavallore et al. 2011).
1.3.4 Thymic Emigration

Thymocytes travel through and exit from the thymus by receiving chemokine signals from the thymic microenvironment and regulating their surface chemokine receptors. For example, the chemokines Cxcl12, Ccl19, Ccl21, Ccl25 and upregulation of their ligands Cxcr4, Ccr7 and Ccr9 lead to thymocyte accumulation at the cortico-medullary junction (Ara, Itoi et al. 2003, Misslitz, Pabst et al. 2004, Takahama 2006), while the expression of the sphingosine-1-phosphate receptor 1 (S1P1) on SP thymocytes allows their exit from the thymus (Chaffin and Perlmutter 1991, Allende, Dreier et al. 2004, Matloubian, Lo et al. 2004).

In addition to chemokine receptor S1P1, thymic egress is also controlled by cell surface receptors such as Qa2, CD62L, HSA and CD69. Once positive selection occurs, newly selected thymocytes have a semi-mature phenotype defined as QA2loCD62LhighHSAloCD69lo. This phenotype is susceptible to apoptosis. These cells differentiate further to change their surface phenotype to QA2highCD62LhighHSAloCD69lo. The upregulation of Qa2, CD62L, and S1P1 allows the SP cell to emigrate from the thymus to the periphery (Weinreich and Hogquist 2008).

1.3.5 Morphogen Signalling in thymocyte development

Morphogens play an important role in regulating thymocyte development, as they act in a concentration dependent manner and can
thereby determine final thymocyte fate. In addition, morphogens also regulate cell fate in other self-renewing tissues including the hematopoietic system. There are three important morphogen families: The Wnt family, Bmp2/4 (Bone Morphogenetic Proteins) and Hedgehog family (Hh).

Wnt proteins signal to developing thymocytes by inducing the nuclear translocation of β-catenin, which acts via T cell factors (Tcf1-3) and Lymphoid enhancer factor (Lef1) to activate target genes that encode key regulators of thymocyte development such as Gata3 and Bcl11b (Moon, Bowerman et al. 2002). Wnt signalling promotes thymocyte development by regulating thymocyte survival and inducing DN cell proliferation (Hsu, Shakya et al. 2001, Staal, Meeldijk et al. 2001, Varas, Hager-Theodorides et al. 2003).

In addition to Wnt, two important Bmp family members Bmp2 and Bmp4 regulate many different stages of thymocyte development. Bmp2 and Bmp4 are secreted by the thymic epithelium and their receptors are expressed on thymocytes and thymic stromal cells (Hager-Theodorides, Outram et al. 2002, Tsai, Lee et al. 2003, Hager-Theodorides, Ross et al. 2014). They are members of the TGF-β family and signal to developing thymocytes through hetero-dimerization of their serine-threonine kinase receptors. This hetero-dimerization causes the phosphorylation of R-Smads: (Smad-1, Smad-5 and Smad-8) which allows them to recruit their Co-Smad/Smad4 and move to the nucleus to
form complexes with several transcription factors and activate Bmp target genes (Wall and Hogan 1994, Miyazono, Kusanagi et al. 2001).

Treatment of fetal thymic organ cultures (FTOCs) with Bmp4 inhibits thymocyte differentiation at the DN1 stage before T cell lineage commitment (Hager-Theodorides, Outram et al. 2002, Hager-Theodorides, Ross et al. 2014) and negatively regulates the DN to DP transition (Graf, Nethisinghe et al. 2002, Hager-Theodorides, Ross et al. 2014). This regulation is thought to be important for controlling thymocyte numbers and maintaining a pluripotent precursor population (Varas, Hager-Theodorides et al. 2003).

The third morphogen family, the Hedgehog (Hh) proteins, and its signalling will be discussed in the next section.

1.4 Hedgehog Signalling

1.4.1 Discovery of Hedgehog

The Hedgehog (Hh) protein was first discovered in studies investigating the segmental patterning of *Drosophila melanogaster* larvae. The spiky appearance of the protein coined its name as Hedgehog (Nusslein-Volhard and Wieschaus 1980). The protein was later identified in vertebrates (Echelard, Epstein et al. 1993, Riddle, Johnson et al. 1993).

In mammals, three homologues of the Hh protein sharing around 90% homology to each other were discovered. These are, Sonic Hedgehog
(Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh) (Bitgood and McMahon 1995, Shimeld 1999, Ingham and McMahon 2001). Although, the three Hh homologues share the same signalling pathway, they have different temporal and tissue specific expression patterns, and therefore their roles in embryonic patterning and tissue development are distinct and non-overlapping (Ingham and McMahon 2001, Ruiz i Altaba, Sanchez et al. 2002).

In mice, homozygous Shh and Ihh mutants are embryonic lethal (Chiang, Litingtung et al. 1996, St-Jacques, Hammerschmidt et al. 1999), while Dhh mutants survive as adults but males are sterile (Bitgood, Shen et al. 1996).

### 1.4.2 The Hedgehog signalling pathway

The Hedgehog signalling pathway is regulated by two key transmembrane proteins, Patched (Ptch), a 12-span transmembrane protein and Smoothened (Smo), a 7-span transmembrane protein. Ptch has two mammalian homologues Ptch1 and Ptch2 which regulate the Hh signal through the Hh signal transducer Smo (Goodrich, Johnson et al. 1996, Stone, Hynes et al. 1996, Motoyama, Heng et al. 1998). In the absence of the Hh ligand, Ptch inhibits Smo, a central transducer of Hh signalling and activity (Mullor and Guerrero 2000). The inhibition of Smo by Ptch is not due to direct interaction of Smo and Ptch but rather indirect, where Ptch inhibits the synthesis of activator of Smo, Phosphatidylinositol-4-phosphate, which limits Smo activation (Yavari,
Nagaraj et al. 2010). Ptch also reduces the expression of Smo on the plasma membrane by transporting a small molecule inhibitor that binds to Smo and alters its localization (Denef, Neubuser et al. 2000, Incardona, Gruenberg et al. 2002, Taipale, Cooper et al. 2002).

In the presence of the Hh ligands (Shh, Ihh or Dhh), Hh binds to Ptch, relieving Ptch’s inhibitory mechanisms on Smo and allowing maximal translocation of Smo to the plasma membrane. Smo is responsible for the downstream activation of Glioma associated family members including Gli1, Gli2 and Gli3 in mammals (Sasaki, Hui et al. 1997, Sasaki, Nishizaki et al. 1999) and the Drosophila Gli gene Cubitus interruptus (Ci) (Alexandre, Jacinto et al. 1996). The Gli genes are the most crucial effectors of the Hh signalling pathway and will be discussed later in section 1.4.3.

Once Smo is translocated to the plasma membrane, it is phosphorylated by various protein kinases including Protein Kinase-A (PKA), Glycogen Synthase Kinase-3 (GSK3) and Casein Kinase-I (CKI). These protein kinases together with the Ci gene are recruited together by the motor protein Costal2 (Cos2) to form the Hh signalling Complex (HhSC). Phosphorylation of Smo causes a change in its confirmation allowing it to interact with Cos2, which is further phosphorylated by the kinase Fused (Fu) (Liu, Cao et al. 2007). Fu also phosphorylates the kinase Suppressor of Fused (Sufu), which together with Cos2 phosphorylation causes Ci to be dissociated from the HhSC and translocated to the nucleus to promote transcriptional activation of Hh target genes (Lum,
Zhang et al. 2003, Ruel, Rodriguez et al. 2003). A summary figure showing the Hh signalling pathway is shown in Figure 1.6.

In mammals, the Hh signalling pathway is very similar, however, in mammals Hh signal transduction usually requires localization and transport of Smo and the Gli proteins in the primary cilia. In addition, the Cos2 orthologue is known as Kif7 in mammals and Kif7 regulates the activity three mammalian Gli proteins (Gli1, Gli2 and Gli3) instead of one Gli protein in Drosophila (Robbins, Nybakken et al. 1997, Sisson, Ho et al. 1997).

1.4.2 Glioma associated family members

In mammals, there are three Glioma associated transcription factors, Gli1, Gli2 and Gli3, which are the key regulators of the Hh signalling activity and pathway (Ingham and McMahon 2001). All three Gli proteins have a C-terminal activatory domain and as Gli2 and Gli3 have an additional repressive N-terminal domain. Gli proteins bind defined DNA consensus sequences, with Gli1 and Gli3 recognising GACCACCCA and Gli2 recognising GAAACCACCCA (Vortkamp, Gessler et al. 1995, Tanimura, Dan et al. 1998).

Gli1 is itself a Hh-target gene, and only encodes an activator of transcription as it lacks the N-terminal repressive domain (Park, Bai et al. 2000), while Gli2 and Gli3 are bi-functional and can be processed to function as transcriptional activators (Gli2A/Gli3A, in the presence of Hh pathway activation) or transcriptional repressors (Gli2R/Gli3R, in the
absence of Hh pathway activation), once they undergo cleavage of their C-terminal activatory domain (Sasaki, Nishizaki et al. 1999). Gli2 is required to initiate the Hh signal, and functions largely as a transcriptional activator \textit{in vivo} (Park, Bai et al. 2000, Bai, Auerbach et al. 2002). In contrast, Gli3 functions predominantly as a transcriptional repressor \textit{in vivo} (Wang, Fallon et al. 2000).

The three Gli proteins have partially overlapping functions. The Gli1 homozygous mutants survive as adults (Park, Bai et al. 2000), however, Gli2 deficiency and Gli3 deficiency are embryonic lethal (Hui and Joyner 1993, Mo, Freer et al. 1997). The Gli2 homozygotes are small in size and have craniofacial abnormalities, while Gli3 homozygous mutants have polysyndactyly (extra toes) and severe skeletal defects. Gli1 and Gli2 or Gli3 and Gli2 double knockouts have more pronounced defects than single mutant homozygotes suggesting functional redundancies between the three Gli proteins, and expression of Gli1 under the control of a Gli2 promoter can rescue several defects in the Gli2 mutant homozygote (Bai and Joyner 2001).

\textbf{1.4.3 Regulation of the Hh signal}

Since Hedgehog is a morphogen, its activity needs to be crucially regulated in order to control both its site specific concentration and its temporal expression pattern. Both, the Hh protein concentration and the duration of its signal can influence the outcome of Hh pathway activation in developing cell. For example, during the development of
the vertebrate nervous system, Shh acts in a gradient dependent manner to define the development of different neural progenitor cells (Briscoe and Ericson 1999). Cell fate is determined by the concentration of Hh protein exposed to the cells, which is dependent on the location of the cell relative to the source of Shh and the duration of the Hh signal, which is dependent on the Hh signal regulation (Briscoe and Ericson 1999, Harfe, Scherz et al. 2004, Varjosalo and Taipale 2008).

The Hh signal is regulated by multiple positive and negative feedback mechanisms, and *Ptch1* is itself a Hh-target gene, functioning to sequester Hh proteins and limit activation of the pathway (Ingham, Nakano et al. 2011). In addition, the cell surface receptor Hh interacting protein (Hhip) sequesters Hh ligands to decrease Hh pathway activation (Beachy, Hymowitz et al. 2010). In the absence of Hh signalling, the activatory C-terminals of the Gli proteins are phosphorylated and marked for deubiquitination catalysed by the Btrcp protein, which leads to C-terminal degradation thus limiting Hh pathway activation (Jia, Zhang et al. 2005, Tempe, Casas et al. 2006). Gli protein regulation also occurs at the transcriptional level where, the Hh target Mim/Beg4 binds to and upregulates the expression and transcriptional activity of both Gli1 and Gli2 (Callahan, Ofstad et al. 2004, Gonzalez-Quevedo, Shoffer et al. 2005), and the kinase Drk1 maintains Gli1 expression in nucleus thus enhancing its activity (Mao, Maye et al. 2002).
Additionally, the amount of Hh signal dependent transcription in a cell is directly correlated to concentration of Gli proteins in the cell. The ratio of the Gli Activator forms (GliA) to the Gli Repressor forms (GliR) is crucial interpreting the Hh signal in a cell (Stamataki, Ulloa et al. 2005). This ratio is affected by the amount of Hh signalling a cell receives and the position of the cell relative to the source of the Hh protein (Stamataki, Ulloa et al. 2005). A positive feedback loop exists, where cells closer to the source of Hh have a higher level of GliA compared to GliR, which augments the Hh signal within the cell. In contrast, cells located further away from the Hh source have a higher level of the GliR compared to GliA, therefore have a lower level of Hh activity. This balance of the GliA/GliR activity within each cell allows it to differentiate into a specific cell type.

1.4.4 Developmental defects associated with the Hh pathway

The Hedgehog signalling pathway is involved in various developmental processes including cell cycle regulation, cell differentiation and survival and cell fate determination, ultimately leading to tissue development and maintenance (Varjosalo and Taipale 2008). Therefore defects in the pathway can lead to various developmental defects in different tissues and can also promote oncogenesis through cell cycle dysregulation.
Shh is the most broadly expressed Hh ligand in mammals. Therefore, abberations in Shh signalling can cause serious developmental defects in many tissues, as it regulates patterning in the spinal cord, axial skeleton and the vertebrate limb and also regulates the development of the heart, brain, eye, ear and kidneys. Thus, Shh homozygous mutant are embryonic lethal (Marigo, Johnson et al. 1996, Marigo, Laufer et al. 1996). In humans, loss of Shh can lead to severe developmental abnormalities including cyclopia and Shh deficiency leads to embryonic lethality (Chiang, Litingtung et al. 1996).

The expression of Ihh is more restricted than Shh. However, Ihh homozygotes are also embryonic lethal due to a poorly developed yolk salk (Dyer, Farrington et al. 2001) and homozygous mutants can be identified by their distinct truncated limbs, as Ihh is essential for long bone formation. Defective Ihh signalling reduced the proliferation and development of the bone forming cells osteoblasts and chondrocytes, thus leading to the truncation of long bones (St-Jacques, Hammerschmidt et al. 1999, Razzaque, Soegiarto et al. 2005).

Dhh is important for the proliferation of the male germ-line cells. Although, Dhh-/- mice are viable they have severe defects in spermatogenesis, Schwann cell function (Mirsky, Parmantier et al. 1999, Parmantier, Lynn et al. 1999, Clark, Garland et al. 2000) and dysregulated erythropoiesis (Lau, Outram et al. 2012).

In addition to the Hh ligands, the loss of Gli proteins can also lead to defective development. The defects in Gli mutant mice were discussed
previously in section 1.4.3. In humans, Gli2 deficiency causes severe defects in the anterior pituitary formation and pan-hypopituitarism with or without cleft-palate (Roessler, Du et al. 2003), while mutations in Gli3 can lead to severe skeletal and lung abnormalities, including Greig’s Cephalopolysyndactyly (Hui and Joyner 1993, Vortkamp, Gessler et al. 1995).

Since Hh signalling regulates cell cycle proliferation, differentiation and survival, defective signalling can lead to oncogenesis. Dysregulation of Hh signalling occurs in many malignant tumours such as pancreatic, stomach, lung and prostate cancers (Thayer, di Magliano et al. 2003, Karhadkar, Bova et al. 2004, Chi, Huang et al. 2006). In humans, Ptch mutations have been identified in childhood cancers including medulloblastoma (Wolter, Reifenberger et al. 1997), rhabdomyosarcoma (Hahn, Wojnowski et al. 1998) and the Gorlin syndrome which predisposes children to Basal Cell Carcinoma (BCC)(Gorlin 1995).

1.4.5. The importance of Hh signalling in hematopoiesis

Many Studies have highlighted the role of Hh signalling in hematopoiesis. Detmer and colleagues (2000) have shown that inhibiting Hh signalling in vitro using the Hh inhibitor cyclopamine, significantly reduced erythroid maturation and the number of erythroid colonies generated by bone marrow hematopoietic progenitors. In contrast, the addition of recombinant Shh (rShh) to hematopoietic
progenitors increased their commitment to erythrocytes, granulocytes and monocytes, visibly observed by the increased size colonies for each population in culture. Further, Gli1 deficient HSCs have defects in differentiating to the myeloid lineage and have a reduced number of granulocytes, while Ptch1 deficient mice, which exhibit higher Hh signalling, show an expanded HSC pool. In addition, Ptch+/- fetal liver cells exhibit enhanced self-renewing and regenerating abilities compared to their WT counterparts while, Smo deficiency impairs the self-renewing abilities of HSCs. In humans, studies have shown that treating human primitive hematopoietic progenitors with soluble forms of Dhh or Shh significantly increases their proliferation.

In humans, Bhardwaj and Colleagues (2001) have shown that human cord blood HSCs express the Hh signalling components SMO and PTCH1 and the downstream Gli transcription factors, GLI1, GLI2 and GLI3 (Bhardwaj, Murdoch et al. 2001) and treatment with human recombinant SHH and DHH significantly increased the proliferation of these primitive human HSCs (Bhardwaj, Murdoch et al. 2001).

Recently, the importance of Ihh and Dhh has been highlighted in erythropoiesis. Ihh and Dhh are highly expressed during development in the yolk sac, and loss of Ihh in yolk sac, results in defective blood island formation and vasculogenesis, causing partial embryonic lethality. Although, Ihh deficient HSCs are generated in normal numbers in vivo, their ability to differentiate into terminal erythroid lineage cells is defective, leading to fatal anemia during mid-gestation and thus
embryonic death (Cridland, Keys et al. 2009). Ex vivo, Ihh has been shown to promote hematopoiesis and Ihh treatment of HSCs in stroma-dependent cultures allowed them to expand by increasing their proliferation.

Unlike Ihh, Dhh-deficiency promotes erythroid differentiation. Loss of Dhh, biases the differentiation of the common myeloid progenitor to the erythroid lineage rather than the granulocyte lineage (Lau, Outram et al. 2012). Therefore, the proportion of mature erythroblasts is higher in the Dhh-/- mice, while the granulocyte population is reduced. The Dhh deficient spleen is significantly larger in size and Dhh-deficiency allows a faster recovery of erythrocytes following acute anemia, suggesting that Dhh also acts as negative regulator of stress erythropoiesis (Lau, Outram et al. 2012).

Although, many studies have shown the importance of Hh signalling in HSC development and differentiation, some studies are inconsistent with this role. Studies have shown that the conditional deletion of Smo from the hematopoietic lineage did not affect self-renewal of HSCs or their ability to proliferate and differentiate. These studies suggested that the loss of Smo does not affect adult definitive hematopoiesis.

In addition to the upstream components of the Hh pathway and the Hh ligands themselves, HSC differentiation and human pluripotent stem cell development has been shown to be regulated by the Gli transcription factors, Gli1 and Gli3.
1.4.6. The importance of Hh signalling in thymocyte and Thymic Epithelial Cell (TEC) development

1.4.6.1. Expression of Hh signalling components in the fetal and adult thymus

Our lab has worked extensively on understanding Hh signalling in thymocyte development. The first study published by our lab in 2000, showed the presence of the Hh pathway family members including the Hh ligands, Shh and Ihh, the Hh receptor and signal transducer, Ptch and Smo, and the downstream Hh targets Gli1, Gli2 and Gli3 in the adult and fetal thymus (Outram, Varas et al. 2000). This study also showed that Hh signalling regulated thymocyte development from the DN to DP stage (Outram, Varas et al. 2000).

The location of various Hh ligands were visualised using immunohistochemistry in the adult thymus (Outram, Varas et al. 2000, Sacedon, Varas et al. 2003, El Andaloussi, Graves et al. 2006). All three Hh ligands, Shh, Ihh and Dhh were observed the adult thymus. Shh and Dhh were mainly present in the thymic stroma, where they were produced by the thymic epithelial cells in the medulla, sub-capsular region and in the cortico-medullary region while Ihh was mainly the present in the DP thymocytes (Outram, Varas et al. 2000, El Andaloussi, Graves et al. 2006, Outram, Hager-Theodorides et al. 2009). Later studies showed the differential expression of Hh pathway transducers, Smo and Ptch1, and its downstream targets, the Gli transcription factors.
family members (Gli1, Gli2 and Gli3) in both the adult and fetal thymocytes (Hager-Theodorides, Dessens et al. 2005, El Andaloussi, Graves et al. 2006, Crompton, Outram et al. 2007, Rowbotham, Hager-Theodorides et al. 2007, Hager-Theodorides, Furmanski et al. 2009, Rowbotham, Hager-Theodorides et al. 2009, Drakopoulou, Outram et al. 2010, Barbarulo, Lau et al. 2016). In adults, Smo is highly expressed during the immature DN stage and its expression decreases as thymocytes mature to the DP and SP stage (Outram, Varas et al. 2000). Similarly, in fetal thymocytes, Smo is highly expressed at the immature DN2 stage and gradually decreases at the more mature DN3 and DN4 stages (Outram, Varas et al. 2000, Barbarulo, Lau et al. 2016). Smo expression is very low as cells mature to the DP and SP stages, while Ptch1 is expression is maintained through the DN, DP, and SP stages of maturation in both adult and fetal thymocyte development, with its highest expression at the DN2 stage (Outram, Varas et al. 2000, Crompton, Outram et al. 2007, Furmanski, Saldana et al. 2012).

In addition to Ptch and Smo activity, the Gli transcription factors Gli1, Gli2 and Gli3 are all expressed in the adult and fetal thymus. During fetal thymocyte development, Gli genes are differentially expressed through the different thymocyte development stages. Gli1 and Gli2 are both expressed at the DN1 and DN2 stages; however, the expression of Gli2 is higher at both stages. At the DN3 stage, just before pre-TCR signalling, Gli2 expression decreases while Gli1 expression increases and as pre-TCR signalling is induced, the expression of Gli1 decreases,

In addition to developing thymocytes, Hh signalling components are present in the thymic epithelial stromal cells of the embryonic thymus. Both the medullary epithelial cells (mTEC) and cortical epithelial cells (cTEC) express the three Hh ligands, Ptch1 and Smo and the Gli transcription factors, with a very high expression of Gli3 in both subsets (Barbarulo, Lau et al. 2016, Saldana, Solanki et al. 2016). The importance of Hh signalling in TEC will be discussed later in section 1.4.6.2.4.

The Hh pathway family members, SHH, DHH, IHH, SMO, PTC, GLI1, GLI2 and GLI3 are also expressed in the human thymus with SHH mainly expressed in the medullary and subcapsullary regions (Sacedon, Varas et al. 2003, Saldana, Solanki et al. 2016).
1.4.6.2. The role of Hh signalling in thymocyte development

The expression of various Hh signalling pathway components and downstream Hh targets during different stages of thymocyte maturation suggests that Hh signalling regulates multiple stages of thymocyte development. Several studies have highlighted the importance of Hh signalling during three stages of development including the earliest developmental DN1 to DN2 transition; pre-TCR induced differentiation to DP and finally the maturation of DP to SP4/8 thymocyte.

1.4.6.2.1. The importance of Hh signalling in DN1 to DN2 transition

Murine knockout studies have shown that Shh, Gli2 and Gli3 are required for the transition of cells from the DN1 to DN2 stage. Shh deficient thymi had 10 times fewer thymocytes than their WT littermates and a severely defective DN1 to DN2 transition, hence a reduced number of DN2 cells (Shah, Hager-Theodorides et al. 2004). Similarly, Gli2 deficient thymi were smaller than their WT littermates and showed a development arrest at the DN1 to DN2 transition, leading to an accumulation of cells at the DN1 stage (Rowbotham, Hager-Theodorides et al. 2009). In addition to Gli2, Gli3 was also essential for the progression of DN1 cells to DN2 (Hager-Theodorides, Dessens et al. 2005). However, the transition was not affected Gli1-deficient thymi, suggesting that Gli1 is not required for this transition in development (Drakopoulou, Outram et al. 2010). This suggests that the Gli family
member play non-redundant roles during thymocyte development. Interestingly, Gli3 is dominantly a repressor of Hh signalling in many different tissues with Shh and Gli3 deficiencies showing opposite phenotypes (discussed in section 1.4.7). However, at the DN1 to DN2 transition both Shh and Gli3 mutants arrest the transition to DN2. This suggests that Gli3 can act as an activator or a repressor.

1.4.6.2.2. The role of Hh signalling in pre-TCR induced transition of DN3 to DP

The transition from DN3 to DP stage is regulated by the β-selection checkpoint as discussed previously. DN3 thymocytes that fail to rearrange their TCRβ chain undergo apoptosis at the DN4 stage. Various studies have shown that Hh signalling is a negative regulator of pre-TCR-induced differentiation to DP from the DN stage, in both mice and humans.

In humans, Gutierrez-Frias and Colleagues (2004) showed that the differentiation of CD34+ human precursors to DP cells was accelerated by treatment with anti-SHH neutralizing antibody in human thymus re-aggregation cultures, and arrested on treatment with recombinant SHH (Gutierrez-Frias, Sacedon et al. 2004).

The first study that highlighted the importance of Hh signalling at the transition from DN to DP transitional development was by our lab in 2000 (Outram, Varas et al. 2000). This study showed that the neutralisation of Hh signalling was essential for thymocytes to mature
from the DN to DP stage in both adult and fetal murine thymocytes (Outram, Varas et al. 2000). Shh treated murine FTOCs showed a developmental arrest at the DN3 stage, which was overcome by the addition of Shh neutralizing antibody (5E1) allowing thymocytes to differentiate into DP cells (Outram, Varas et al. 2000). The authors used Recombination-activating gene 1 (Rag1)-/ FTOCs to study the importance of Hh signalling during and after the pre-TCR signal. The addition of anti-CD3 to Rag1-/ FTOCs mimics the pre-TCR and artificially induces differentiation from DN3 to DP. The addition of anti-Shh mAb (5E1) to neutralize Hh in the anti-CD3 treated Rag1-/ FTOCs enhanced thymocyte differentiation to the DP stage, while treatment with rShh arrested it, confirming that Shh acts as a negative regulator of the DN3 to DP transition (Outram, Varas et al. 2000).

In contrast to the in vitro results, analysis of Shh deficient embryos ex vivo showed that the loss of Shh decreases the number and proportion of DP cells (Shah, Hager-Theodorides et al. 2004). However, this decrease in DP thymocytes was due to a higher apoptotic rate at the DN4 stage (Shah, Hager-Theodorides et al. 2004, Barbarulo, Lau et al. 2016).

The negative regulatory role of Shh at the DN to DP transition was analysed in vivo using Gli2 and Gli3 deficient embryos. Although, Gli2 deficient E13.5-E15.5 thymi were smaller compared to their WT littermates, as development to the DP stage was initiated at E16.5-17.5, the Gli2-/ thymi had twice as many thymocytes compared to their WT
counterparts, revealing an acceleration in differentiation to the DP stage (Rowbotham, Hager-Theodorides et al. 2009). This was consistent to the expression pattern of Gli2 in the fetal thymus, where Gli2 expression rises from the DN3 to DN4 stage as pre-TCR signalling is initiated (Crompton, Outram et al. 2007, Rowbotham, Hager-Theodorides et al. 2009, Barbarulo, Lau et al. 2016). Anti-CD3-treated Rag1-/-Gli2-/- FTOCs, expanded and differentiated to DP faster compared to their anti-CD3 treated Rag1-/-Gli2WT counterparts (Rowbotham, Hager-Theodorides et al. 2009). These results indicated that Gli2 negatively regulates fetal thymocyte expansion and progression to DP after pre-TCR signal transduction.

In contrast, anti-CD3 treated Rag1-/-Gli3-/- FTOCs showed a defective differentiation to DP cell after the pre-TCR signal. The E16.5 Gli3-/- thymus also showed a partial arrest at the DN to DP transition, suggesting that Gli3 acts as a repressor of Hh signalling at this stage of thymocyte development, and Gli3 is required for differentiation to the DP stage (Hager-Theodorides, Dessens et al. 2005). Taken together, these findings suggested that two main roles of Shh: positive regulation at the DN1 to DN2 transition and negative regulation the after the pre-TCR signal to the DP transition.

Since Hh signalling components and the Gli transcription factors are expressed by both the thymocytes and thymus stroma, it is difficult to attribute the changes seen in the Hh and Gli mutants to direct Hh signalling in the thymocytes as Hh signalling to the thymic stromal
cells, can induce differences in TEC development or signalling which lead to the developmental differences in thymocytes seen in these mutants. Therefore, our lab generated transgenic mice with differential Hh signalling specifically in T-lineage cells. The Gli2ΔN₂ transgenic mice had increased Hh signalling due to the constitutive expression of the transcriptional-activator forms of Gli2, while the Gli2ΔC₂ had a constitutive inhibition of Hh signalling as they expressed the transcriptional-repressor form of Gli2 (Rowbotham, Hager-Theodorides et al. 2007, Rowbotham, Furmanski et al. 2008). In addition, the transgenic adult mice were viable and therefore could be used to study the importance of Hh signalling in many different T-lineage specific biological processes including development of T cells in different organs, T cell proliferation, apoptosis and differentiation (Rowbotham, Hager-Theodorides et al. 2007, Rowbotham, Furmanski et al. 2008, Rowbotham, Hager-Theodorides et al. 2009, Furmanski, Saldana et al. 2012).

Analysis of adult thymocytes in the Gli2ΔC₂ showed increased differentiation to DP while the Gli2ΔN₂ showed a decreased proportion of DP (Rowbotham, Furmanski et al. 2008, Rowbotham, Hager-Theodorides et al. 2009). Interestingly, anti-CD3-treated Rag1-/-Gli2ΔC₂ FTOCs differentiated quicker to the DP stage compared to anti-CD3-treated Rag-/-Gli2ΔN₂ FTOCs (Rowbotham, Hager-Theodorides et al. 2009). This suggested that Hh signalling in developing thymocytes negatively regulates pre-TCR-mediated differentiation to DP.
In addition to the regulatory role of Shh and the Gli transcription factors during the DN to DP development, Ihh is crucial for the homeostasis and control of the DP population. Ihh is the most highly expressed Hh ligand in DP thymocytes, and E16.5 Ihh deficient thymi have a decreased number of thymocytes and a reduced proportion of DP cells compared to their WT counterparts, suggesting that Ihh promotes DP thymocyte development (Outram, Hager-Theodorides et al. 2009). However, the loss of one copy of Ihh in the Ihh+/- E16.5 thymi, promotes DP development and the mutant thymus has more than twice the number of DP cells compared to the WT, suggesting that Ihh can also negatively regulate DP cell development. Further analysis of the Ihh+/- DN population showed that more DN cells were in cell cycle (Outram, Hager-Theodorides et al. 2009). This suggests that Ihh from the DP cells may signal to the DN3 population to regulate DN3 expansion and differentiation into DP, thus maintaining the DP thymocyte cell numbers and overall thymocyte homeostasis (Outram, Hager-Theodorides et al. 2009, Barbarulo, Lau et al. 2016).

1.4.6.2.3. The role of Hh signalling at the DP to SP transition and TCR repertoire selection

In addition to regulating the DN to DP transition, the analysis of Shh (Shh-/-) and Gli mutants (Gli1-/-, Gli2-/-, Gli2ΔN2, and Gli2ΔC2) show that Hh signalling negatively regulates the DP to SP transition. In addition, Hh signalling modulates the TCR signal by weakening it, thus decreasing commitment to the SP4 lineage. As discussed in section
1.3.2, the commitment to SP4 or SP8 from the DP stage is determined by TCR signal strength and duration, which are modulated by co-receptor signalling, cytokine signalling and the transcriptional outcome of the TCR. Studies use the cell surface CD5 intensity as an indicator of TCR signal strength on thymocytes. Higher cell surface CD5 expression reflects a stronger TCR signal while lower CD5 expression represents a weaker TCR signal (Azzam, DeJarnette et al. 2001).

Ex vivo analysis of the Shh-/- thymus showed a higher SP:DP ratio, reflecting a faster rate of differentiation from DP to SP transition (Rowbotham, Hager-Theodorides et al. 2007). The Shh-/- FTOCs had an increased SP4:SP8 ratio and a higher percentage of mature SP4 cells compared to their WT littermates. Similarly, Gli2ΔC2 FTOCs, in which Hh-mediated transcription is specifically inhibited in thymocytes, showed a higher SP4:SP8 ratio and an increased CD5 expression on SP4 cells and doubling the C2 transgene copy number further increased the CD5 intensity, SP4:SP8 ratio and the mature SP4 cells (Furmanski, Saldana et al. 2012). In contrast, treatment of WT FTOCs with rShh reversed these changes, showing a decreased SP4:SP8 ratio and a decreased cell surface CD5 expression, marking a weaker TCR signal (Rowbotham, Hager-Theodorides et al. 2007). Interestingly, loss of the Gli transcription factors, Gli1-/- and Gli2-/- in FTOCs showed an increased DP to SP transition with an increased commitment to SP4 over SP8 (Rowbotham, Hager-Theodorides et al. 2007, Drakopoulou, Outram et al. 2010). Additionally, the transgenic Gli2ΔN2 thymocyte analysis
showed a decreased cell surface CD5 intensity and a decreased commitment to CD4, while the Gli2ΔC2 transgenic showed the reverse (Rowbotham, Hager-Theodorides et al. 2007, Rowbotham, Hager-Theodorides et al. 2009, Furmanski, Saldana et al. 2012). Taken together, the results suggested that an increased level of Hh signalling weakens the TCR signal strength, leading to decreased DP to SP4 lineage commitment and a reduced SP4:SP8 ratio.

In addition to modulating the TCR signal strength, Hh signalling reduces Gata3 expression, which further decreases SP4 lineage commitment (Furmanski, Saldana et al. 2012). As discussed previously, Gata3 is one of the crucial transcription factors for SP4 lineage commitment and strong TCR signals increase the expression of Gata3, driving differentiation towards SP4 (Hernandez-Hoyos, Anderson et al. 2003). Using WT FTOCs to induce or repress Shh signalling by adding rShh or anti-Shh mAb (5E1) respectively, Furmanski and Colleagues (2012), have shown that rShh treatment decreases the proportion of Gata3 expression in DP and SP cells, while neutralising Shh signalling increases Gata3 expression and intensity on the DP and SP cells (Furmanski, Saldana et al. 2012). These results suggests that Shh signalling weakens the TCR, which decreases Gata3 expression, and both the low Gata3 expression and the weakened TCR signal lead to defective SP4 commitment.

Since TCR signal strength influences both the TCR repertoire and CD4/8 lineage commitment, the influence of Shh on the TCR signal strength
may alter both of these. Shh-expressing Thymic Epithelial Cells (TECs) are scattered around the medulla and the cortico-medullary junction, so their influence on the TCR signal strength and the outcome of TCR ligation (i.e. positive or negative selection and CD4/8 lineage decision) for each cell is dependent on the location of the cell relative to the source of Shh. Therefore, Shh-expressing TECs may have specialised functions such as induction of positive selection and commitment to the CD8 lineage. Thus, the nature and strength of Hh signal a thymocyte receives depends on its position in the thymus, which is further modulated by its signal receiving machinery.

1.4.6.2.4. The importance of Hh signalling in Thymic Epithelial Cell (TEC) development

Thymic Epithelial Cells (TECs) are essential for specification of T cell fate and selection of the TCR repertoire and peripheral tolerance and by regulating positive and negative selection. Thus the presence of Hh signalling components in TEC suggests that Hh signalling may play a role in determining T cell fate, the TCR repertoire and regulating peripheral tolerance. Hh signalling also regulates TEC development.

There are two types of TEC, cortical (c)TEC, which present MHC-peptide ligands for positive selection and specify T cell fate, while medullary (m)TEC are mainly specialised for inducing negative selection. cTEC express antigen presentation genes such as *Cathepsin-L*, *Prss16*, and β5t, while mTEC express *Aire* and *Cathepsin-S*, that facilitate the expression and presentation of Tissue Restricted Antigens (TRA) for the

Recent studies have highlighted the expression of Hh signalling pathway components and Hh ligands in both cTECs and mTECs. I reported the presence of active Hh signalling in both embryo (E14.5-E18.5), neonate (at birth) and adult TEC populations using the Gli Binding Site (GBS)-Green Fluorescent Protein (GFP) transgenic embryos, which express GFP when activator forms of Gli bind to GFP construct (Saldana, Solanki et al. 2016). This suggests that Hh is a crucial regulator of TEC development and may also influence the functions of TECs including positive and negative thymocyte selection.

As discussed previously, the Shh deficient thymus is smaller in size than WT and has a decreased number of thymocytes. Interestingly, our recent study showed that the number of TEC were significantly lower in the Shh-/- thymus compared to its WT littermates (Saldana, Solanki et al. 2016). The development of mTEC was more severely affected than cTEC but cell surface expression of MHCII was higher on both cTEC and mTEC, which may influence positive and negative selection in the Shh-/- thymus (Saldana, Solanki et al. 2016). Although fewer TECs were present in the Shh-/- thymus, each TEC expressed more MHCII-peptide complexes.

Interestingly, neutralising Shh by treating WT FTOCs with recombinant Hhip (rHhip), (which binds to endogenous Hh proteins and neutralises their effect in culture) also showed an overall decrease in TEC numbers
with a significant decrease in the proportion of mTEC and increased cell surface MHC-II expression on mTEC. This confirmed that the changes in TEC development were due to loss of Shh signalling. In contrast, the loss of Gli3 in the Gli3-/− thymus showed an increase in overall TEC numbers and a decrease in cell surface expression of MHC-II in both cTEC and mTEC (Saldana, Solanki et al. 2016). The opposite phenotypes shown by the Shh and Gli3 mutants suggests that Gli3 is acting as a repressor of Hh signalling in the embryonic thymus. Therefore loss of Gli3 in the embryonic thymus can lead to Hh dependent changes or Hh-independent changes due to loss of Gli3 itself as discussed in the subsequent section 1.4.7.

1.4.7. The Hh-dependent and Hh-independent roles of Gli3 during development

Gli3 is dominantly known to repress the Hh signalling pathway. However, Gli3 can be processed to function as activator (Gli3A) or a repressor (Gli3R). Therefore the outcome of its function in various tissues is dependent on the balance between its Gli3A and Gli3R forms.

In addition, Gli3 can have both Hh-independent and Hh-dependent functions during tissue development (te Welscher, Fernandez-Teran et al. 2002, Hager-Theodorides, Dessens et al. 2005). Gli3R functions to limit Hh pathway activation in many tissues including patterning during limb development; DN to DP transition in the thymus; TEC development and maturation in the thymus (Wang, Fallon et al. 2000, Ahn and Joyner
There are at least two distinct mechanisms by which Gli3R can limit Hh signalling: it may repress expression of Hh genes in the Hh-producing cell via repression of Hh activating genes, thus limiting Hh protein concentration in the tissue. For example, during pre-patterning of the limb bud, Gli3R spatially limits the expression of dHand, an activator of \textit{Shh} gene expression (te Welscher, Fernandez-Teran et al. 2002). Alternatively, when Gli3 is expressed in the signal receiving cell, the concentration of Gli3R in a given cell increases the further away the cell is located from the Hh secreting source, resulting in correspondingly increased repression of Hh target genes (Wang, Fallon et al. 2000, te Welscher, Fernandez-Teran et al. 2002). In fact, in many tissues, Shh and Gli3 have opposing functions, with Shh-deficiency and Gli3-deficiency giving opposite phenotypes (Wang, Fallon et al. 2000, Shah, Hager-Theodorides et al. 2004, Hager-Theodorides, Dessens et al. 2005, Hager-Theodorides, Furmanski et al. 2009, Saldana, Solanki et al. 2016).

In the fetal thymus, Gli3 has been shown to be a dominant repressor of Hh signalling. The Gli3-/- thymus has an increased level of Gli1 (an activator of Hh signalling) in both the thymocytes and fetal thymus stroma and treatment with the Shh neutralising mAb (5E1) decreased Gli1 expression (Hager-Theodorides, Furmanski et al. 2009). This suggests that increased levels of Shh signalling were present in the
Gli3−/− thymus. Therefore the changes in the Gli3−/− thymus can either be due to loss of Gli3 or due to increased Shh signalling.

The Gli3+/− thymus can be used to determine whether changes in the Gli3−/− thymus are due to the loss of Gli3 or due to the increased Hh signalling. For example, Nos2 is a Gli3 target gene, which was downregulated in both the Gli3−/− and Gli3+/− thymus. However, the treatment of Gli3+/− FTOCs with anti-Shh mAb recovered Nos2 expression to physiological levels, but the neutralisation of Shh in the Gli3−/− FTOCs did not change the Nos2 expression, suggesting the Nos2 requires Gli3 for its normal expression (Hager-Theodorides, Furmanski et al. 2009).

Interestingly, Nos2 is an important gene regulating negative selection related apoptosis. It causes the production of nitric oxide (NO), a potent pro-apoptotic agent, which promotes apoptosis of non-selected autoreactive thymocytes (Tai, Toyo-oka et al. 1997). The treatment of WT and Gli3−/− FTOCs with anti-CD3e to mimic negative selection, showed that the Gli3−/− thymus DP cells had a decreased intracellular active caspase-3 expression, correlating with a lower level of apoptosis compared to WT. This suggests that negative selection related apoptosis of DP thymocytes is attenuated in the Gli3 mutant thymus, partly due to decreased Nos2 expression (Hager-Theodorides, Furmanski et al. 2009). This defective negative selection can lead to the survival of autoreactive thymocytes, a change in a TCR repertoire and an increased susceptibility to autoimmune diseases.
Further studies are needed to evaluate whether other proapoptotic genes work together with Nos2 leading to the defective negative selection in the Gli3-/ thymus. Additionally, the Gli3-/ thymus has a higher level of Hh signalling, therefore Gli3 independent Hh dependent transcriptional targets could also be involved in regulating negative selection.

1.5 Thesis Objectives

Since Gli3 can have both Hh-dependent and Hh-independent effects, I decided to use the Gli3 mutant model to understand the role of both Gli3 and Hh signalling during T and B cell development. Hh signalling has not previously been explored during embryonic fetal liver B cell development. Here, I test the hypothesis that Gli3 and Shh regulate B cell development in the fetal liver. I investigate the role of Hh signalling from the earliest CLP stage to the most mature heavy chain expressing (B220+CD19+IgM+) cell. In addition, I explore the transcriptional mechanism through which Hh alters fetal liver B cell development.

Additionally, I investigate the role of Gli3 and Hh signalling during the DP to SP transition and TCR repertoire selection. Here, I test the hypothesis that Gli3 and Shh regulate thymocyte development from the DP to SP stage of maturation. Gli3 and Hh signalling have both been previously been shown to be important during the DN to DP transition, however, the role of Gli3 during DP to SP, and further SP maturation has
not yet been investigated. I aim to understand both the Hh-dependent and Hh-independent effects of Gli3 during the DP to SP differentiation and further SP maturation. I also use the Gli3 mutants to understand the transcriptional mechanisms regulating both positive and negative regulation downstream of the TCR.
Figure 1.1. Embryonic hematopoiesis through development

Figure 1.1 shows the different organs where hematopoiesis takes place during embryo development. Early primitive hematopoiesis occurs in the yolk sac from embryonic day 7 (E7) to E10. Primitive hematopoiesis is essential for red blood cell production to allow oxygen transport and early embryo growth. Primitive hematopoiesis is taken over by definitive hematopoiesis from E10 until birth. Definitive hematopoiesis begins in the AGM region after E10. HSCs from the AGM travel to the fetal liver, where they expand and differentiate. HSCs from the fetal liver also seed organs including the fetal thymus, fetal spleen and bone marrow.

HSC = Hematopoietic Stem Cell, AGM = Aorta-gonads-mesonephros
Figure 1.2. The historically known Classical model and the newly proposed myeloid-based model

Figure 1.2 shows the classical and myeloid-based model. The main difference between the classical and myeloid-based model is that the myeloid lineage is not restricted to the CMEP lineage but rather myeloid cells can differentiate from the MPP, CMEP and CMLP as well.

MPP, multipotent progenitor; CMEP, common myeloid/erythroid progenitor; CLP, common lymphoid progenitor; CMLP, common myeloid/lymphoid progenitor; E, M, T, B represent erythroid, myeloid, T and B cells, respectively. Modified from (Kawamoto, Ikawa et al. 2010)
Figure 1.3. The stages of B cell development and the markers defining each stage of development in the fetal liver

Figure 1.3 shows the different stages of B cell development in the fetal liver. The Common Lymphoid Progenitor cells (CLP) defined by the cell surface expression of IL-7Ra (CD127) and c-Kit (CD117) differentiate into either B-1 (B220lo/-CD19+) or B-2 (B220+CD19-) progenitors and then mature into B220+CD19+ cells. The heavy chain is then rearranged and the B220+CD19+ cells start expressing µH and BP-1 prior to light chain rearrangement.
Figure 1.4: Pre-BCR and BCR rearrangement and clonal selection

Figure 1.4 shows the B cell developmental checkpoints during pre-BCR and BCR formation. Successful Pre-BCR is formed once the rearranged heavy chain (μH) assembles with the surrogate light chain and Igα and Igβ. Immature B cells that fail to successfully rearrange the heavy chain or fail to assemble and translocate pre-BCR components to the cell surface undergo cell death via apoptosis.

Once the heavy chain is rearranged and pre-BCR formed, B cell undergo the next stage of development where they rearrange their light chain. Once the light chain is rearranged, the mature BCR is tested for its functionality. If the BCR can strongly crosslink with self-antigens, the cell either undergoes apoptosis or undergoes receptor editing, where the second light chain is rearranged. The cell is further tested for self-antigen reactivity. Cells with BCRs that are non-self reactive are allowed to mature further and migrate to the periphery. Figure modified from (Murphy, Travers et al. 2012). BCR = B cell receptor
Figure 1.5. The different stages of thymocyte maturation

Figure 1.5 shows that thymocyte development begins in the thymus once the Early Thymic Progenitor (ETP) cell moves into the thymus from the fetal liver. Thymocytes differentiate from the DN1 to DN4 and further mature in DP cells, which undergo selection to commit into either SP4 or SP8 lineage.

There are four DN stages of T cell development: DN1 to DN4. These stages are characterised by the cell surface expression pattern of CD25 (alpha chain of the IL-2 receptor), CD44 (cell-surface glycoprotein involved in cell–cell interactions) and CD117 (cytokine receptor expressed on the surface of hematopoietic stem cells).

The three checkpoints of thymocyte development are shown numbered 1, 2 and 3 on figure. The first checkpoint is important for restrict the commitment of DN1/2 cell to the T cell lineage. The second checkpoint is the B-selection checkpoint to assess the rearrangement of the pre-TCR alpha chain and the final checkpoint represents negative and positive selection in thymus, where cells with a non-self reactive TCR can proceed through further maturation and emigrate from the thymus to the periphery.

Figure modified from (Koch and Radtke 2011)
Figure 1.6 shows the different components of the mammalian Hedgehog signalling pathway. In the absence of Hh, Ptch inhibits Smo, allowing Kif7 to recruit Fused, Sufu and the Gli proteins. The kinase PKA phosphorylates Gli2 and Gli3 to generate their repressor forms thus inhibiting the transcription via Gli proteins. In the presence of Hh, Hh binds to Ptch relieving the inhibition of Ptch on Smo. Downstream Smo signalling causes the phosphorylation of Kif7 allowing Gli proteins to dissociate from the Kif7, Fused and Sufu complex. Gli2 and Gli3 are not phosphorylated thus they translocate to the nucleus with the help of CBP in their active forms and cause the transcription of Shh target genes.

The two blue lines at the top represent the cell membrane and the dotted grey circle shows the nucleus. P in a circle indicates phosphorylated molecule. A blunt ended line indicates inhibition and an arrow indicates activation.
Shh, Sonic Hedgehog; N-Shh, N-terminal-cholesterol-bearing Shh; Ptc, Patched; Smo, Smoothened; PKA, Protein Kinase A; Cos2, Costal 2 protein; Fu, Fused; Su(fu), Suppressor of Fused; CBP, Cyclic adenosine monophosphate response element (CREB)-Binding Protein. Modified from (Ingham and McMahon 2001, Varas, Hager-Theodorides et al. 2003).
Chapter 2
Chapter 2: Materials and Methods

2.1 Mice

All mice studies were reviewed and approved by the United Kingdom Home Office. Mice were bred and maintained at the University College London (UCL) Biological Services Unit. Mice given as gifts were backcrossed onto C57BL/6 for at least 16 generations before being used for experiments. Mice strains and their origin are listed in Table 2.1 below.

Table 2.1: A List of all mice strains used and their origin

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>B&amp;K Universal (UK)</td>
</tr>
<tr>
<td>Shh(^+/−)</td>
<td>Gift from Philip Beachy (Chiang, Litingtung et al. 1996)</td>
</tr>
<tr>
<td>Gli3(^+/−)</td>
<td>Purchased from Jackson Laboratories (USA)</td>
</tr>
<tr>
<td>Gli3fl/fl</td>
<td>Purchased from Jackson Laboratories (USA)</td>
</tr>
<tr>
<td>ShhfI/fl</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>FoxN1-iCre</td>
<td>Gift from George Hollander (Zuklys, Gill et al. 2009)</td>
</tr>
<tr>
<td>Vav-iCre</td>
<td>Gift from Dimitris Kioussis (de Boer, Williams et al. 2003)</td>
</tr>
<tr>
<td>CD4-Cre</td>
<td>Purchased from Jackson Laboratories (USA)</td>
</tr>
</tbody>
</table>

Adult mice were used between 4-8 weeks old for experiments. The Shh and Gli3 knockout mice are not viable as adults, therefore I analysed embryo tissues from these mice. For Embryo analyses, timed mates
were performed by mating a male with two females and the day the plug was found was counted as embryonic day 0.5 (E0.5).

2.2 Mice Genotyping

The genotype of all adult and embryo mice was determined by extracting DNA from either 2mm tail tips or ear biopsies and subsequently performing a PCR with relevant primers (Table 2.2). The final PCR products were resolved using Gel Electrophoresis.

2.2.1 DNA extraction for Genotyping

DNA was extracted by adding 100µl of tissue digesting buffer containing 50mM KCL, 10mM Tris HCL (pH 8.5), 1.5 mM MgCl₂, 0.01% gelatin, 0.45% Noident P-40 and 0.45% Tween20) and 0.5ug/ml of Proteinase K (Sigma-Aldrich) in ultra pure water (Life Technologies) to each tail sample or ear biopsy. The samples were left to shake at 56°C overnight on the shaker at 600rpm to allow the enzymatic and mechanical disruption of the tissue in order to release DNA.

2.2.2 Polymerase Chain Reaction (PCR) for Genotyping

Once the samples were digested overnight they were spun at 13000rpm in a micro-centrifuge for 5mins to help separate the DNA from the impurities. 1µL of the sample supernatant containing ~1µg of DNA was
used as template for the PCR reaction. Each PCR reaction was made-up of a 20 µl mix containing 1 µl of sample template DNA, 50% 2x GreenTaq DNA Polymerase (Sigma-Aldrich) and 10µM of each relevant forward (Fw) and reverse (Rv) primer (described in Table 2B) made up with ultra pure water (Life Technologies). PCR was carried out on a Stratagene Robocycler (Stratagene, US) following the steps below:

Common Step 1: 5 minutes at 94°C (For polymerase activation)

Step 2: This step was different because each set of primers had different primer-annealing temperatures as shown below:

- **For Shh, Gli3 and CD4-Cre**: 35 cycles for 90 sec at 94°C, 60 sec at 58°C, 80 sec at 72°C
- **For Vav-iCre and FoxN1-iCre**: 32 cycles for 90 sec at 94°C, 60 sec at 58°C, 80 sec at 72°C
- **For Shh (Shhfl/fl) and Gli3 (Gli3fl/fl) floxed genes**: 36 cycles for 90 sec at 94°C, 60 sec at 58°C, 80 sec at 72°C

Common Step 3: 10 minutes at 72°C (For PCR completion by product elongation)

**Table 2.2 Forward and reverse Primer Sequences for the different Genes**

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primer Sequence</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Shh Mutant gene</strong></td>
<td>Fw: CTG TGC TCG ACG TTG TAC TG Rv: AAG CCC GAG ACT TGT GTG GA</td>
<td>1300</td>
</tr>
<tr>
<td><strong>Gli3 Mutant gene</strong></td>
<td>Fw: GGC CCA AAC ATC TAC CAA CAC AT Rv: GTT GGC TGC TGC ATG AAG ACT GAC</td>
<td>580</td>
</tr>
<tr>
<td><strong>Gli3fl/fl WT and LoxP gene</strong></td>
<td>Fw: CTG GAT GAA CCA AGC TTT CCA TC Rv: CTG CTC AGT GCT CTG GGC TCC</td>
<td>WT: 195 LoxP: 500</td>
</tr>
</tbody>
</table>
Table 2.2 shows the Forward and Reverse primer sequences for all the different genes used for the genotyping PCR.

### 2.2.3 Gel Electrophoresis

The final PCR products were resolved using a 2% agarose gel made from agarose (Sigma-Aldrich), 1× TAE buffer (Life Technologies) and stained with Gel Red (Biotium), which binds to DNA and fluoresces under UV light. HyperLadderII (Bioline) ranging from 50bp to 2000bp was as a marker used to estimate the PCR product band size. After electrophoresis, the gel was analysed under ultraviolet light (Herolab, Germany) to reveal the PCR products and the results were photographed (Sony).

### 2.2.4 GBS-GFP Mice genotyping

The GBS-GFP mice were genotyped for the presence of the GFP construct by assessing the presence of GFP in the blood of the mice. A drop of blood was taken in 1ml of 1xPBS and acquired on the Accuri C6 flow cytometer (Becton Dickinson) to detect presence of GFP by assessing fluorescence in Fluorescence Channel 1 (FITC).
2.3 Fetal Organ Cultures set up

Each embryo thymus and liver was carefully micro-dissected using sharp tweezers (Tweezerman) under the light microscope. All organs were kept in sterile conditions at all times.

2.3.1 Fetal Thymus Organ Cultures (FTOC)

Each dissected E17.5 fetal thymus lobe was placed on a 0.8µm Millipore filters (Millipore) on 1ml of AIM-V (Invitrogen) in a 24-well culture plate. Since each thymus has 2 lobes, 1 lobes was used as a control and the other was treated with either mouse recombinant (r) Hhip (Sigma-Aldrich) or mouse recombinant (r) Shh (R&D) at 1µg/ml. The thymi were kept in culture for 2 to 4 days in the incubator at 37°C and 5% CO₂ before analysis.

2.3.2 Fetal Liver Organ Cultures (FLOC)

Each dissected E17.5 fetal liver was cut into ~1mm cubes and cultured on 0.8µm Millipore filters (Millipore) on 1ml of RPMI (Life Technologies) supplemented with 10⁻⁵M b-mercaptoethanol (Sigma-Aldrich), 2% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Sigma-Aldrich) in a 24-well culture plate for two to four days at 37°C and 5% CO₂ before analysis. In some experiments, rHhip (R&D), rShh (R&D) were added at 1µg/ml and rIL-7 (R&D) was added at 25ng/ml. The activity of rHhip was measured by its ability to inhibit Sonic Hedgehog (Shh) induction of alkaline phosphatase production in C3H10T1/2 mouse embryonic
fibroblast (MEF) cells. The activity of rShh was measured by its ability to induce alkaline phosphatase production by C3H10T1/2 MEF cells and the activity of rIL-7 was measured in a cell proliferation assay using PHA-activated human peripheral blood lymphocytes (PBL).

2.4 Cell Isolation and Counting

2.4.1 Fetal Thymus, spleen and liver cell isolation

Once the fetal thymus, spleen and liver were dissected, each organ was crushed between the frosted ends of two glass slides and washed using FACS buffer containing 1mM EDTA (Invitrogen), 2% Fetal Calf Serum (FCS, Gibco) and 0.01% Sodium Azide (Severn Biotech) in 1x PBS (Sigma Aldrich). This cell suspension was filtered through a 70-µm cell strainer to eliminate any large tissue chunks and resuspended in 5ml of FACS buffer. Next, cells were counted using the Accuri C6 flow cytometer (section 2.4.2) and subsequently stained with the relevant antibodies (section 2.5).

2.4.2 Cell Counting

A sample of cells from each cell suspension was diluted 1:50 using FACS buffer and acquired and counted using the Accuri C6 flow cytometer (Becton Dickinson). Live cells were gated using the Forward Scatter (FSC) and Side Scatter (SSC) axes. The FSC determines the cell size while the SSC determines the granularity of the cell. Total cell
counts for each organ were calculated based on the live cell gate percentage.

2.5 Flow Cytometry and Antibody staining

A sample of cells from each organ was stained using different fluorochrome conjugated antibodies (Table 2.3) and acquired on the Accuri C6 flow cytometer (Becton Dickinson) or the or LSRII (BD Pharmingen) flow cytometer.

2.5.1 Surface antibody staining

Cells were surface stained with combinations of different fluorochrome conjugated antibodies from BD Pharmingen (BD), Biolegend (BL) and Ebioscience (EB) (Table 2.3). The flow cytometry antibodies used to analyse fetal thymocytes, liver B cells, liver progenitors and liver stromal cells are listed in Table 2.3.

Table 2.3 Surface fluorochrome conjugated antibodies

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>Fluorochrome</th>
<th>Fetal Cells Stained</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>FITC/PE</td>
<td>Thymocytes</td>
<td>EB</td>
</tr>
<tr>
<td>CD5</td>
<td>FITC/PE</td>
<td>Thymocytes</td>
<td>EB</td>
</tr>
<tr>
<td>CD4</td>
<td>PCPCy5.5/ACP</td>
<td>Thymocytes</td>
<td>EB/BL</td>
</tr>
<tr>
<td>CD8</td>
<td>PCPCy5.5/ACP</td>
<td>Thymocytes</td>
<td>EB/BL</td>
</tr>
<tr>
<td>CD24</td>
<td>PE</td>
<td>Thymocytes</td>
<td>EB</td>
</tr>
<tr>
<td>QA2</td>
<td>FITC</td>
<td>Thymocytes</td>
<td>EB</td>
</tr>
<tr>
<td>CD69</td>
<td>PCPCy5.5/PE</td>
<td>Thymocytes</td>
<td>EB/BL</td>
</tr>
<tr>
<td>CD25</td>
<td>PE/APC</td>
<td>Thymocytes/Liver B cells</td>
<td>EB</td>
</tr>
</tbody>
</table>
Table 2.3. Antibodies used for surface staining of the cells from the different fetal organs.

A 50\(\mu l\) sample of the cell suspension was used for surface antibody staining. Firstly, a cocktail of different relevant fluorochrome conjugated antibodies was made in FACS buffer, where each antibody was diluted 1:100. Subsequently, 50\(\mu l\) of the antibody cocktail was added to the 50 \(\mu l\) of the cell sample and the sample remained staining at 4°C for 30mins in the dark. The stained sample was then washed twice with FACS buffer at 1400 rpm for 5 minutes and acquired on either the Accuri C6 (BD) or LSRII (BD Pharmingen). A minimum of \(10^5\) events were acquired within the live gate defined using the forward scatter (FSC) and side scatter (SSC). The compensation for a multicolour stained panel was defined using an unstained samples and single
stained controls. All flow cytometry experiments were analysed using Flowjo 7.5 (Tree Star, US).

### 2.5.2. Annexin-V apoptosis assay

Surface stained cells were sometimes used for Annexin-V staining to detect apoptotic cells. Once the surface staining procedure was completed the Annexin-V-FITC apoptosis detection kit (BD Pharmingen) was used according to manufacturer’s instruction.

### 2.5.3. Intracellular Staining

Surface stained cells were sometimes stained for intracellular markers including anti-Ebf1 (PE) anti-Pax5 (APC) and anti-Ki67 (FITC). The intracellular staining procedure was performed using the intracellular staining kit from Ebioscience according to manufacturer’s instruction. 100µl of the Fix/Perm working solution (made by adding 1 part of the Fix/Perm Concentrate to 3 parts of the Fix/Perm diluent) was added to each surface stained sample and incubated for 30minutes in the dark at 4°C. The sample was then washed with 1xPermeabilization buffer at 1400rpm for 5minutes. The antibody cocktail for 1:100 dilutions of the relevant antibodies was then added to the sample and incubated again for 30minutes in the dark at 4°C. The stained sample was further washed twice with FACS buffer and then acquired on the flow cytometer.
2.6 Cell sorting and Purification

Fetal thymus and liver cells were stained using the relevant antibodies diluted in 1:50 of FACS buffer. The stained cells were incubated for 30 minutes at 4°C in the dark and then washed twice with FACS buffer after staining. The stained samples were further sorted at the ICH/GOSH Flow Cytometry Core Facility using either the BD FACS Ariall (BD Pharmingen) or the Modular Flow Cytometer (MoFlo XDP, Beckman Coulter). Only gated live cells as assessed by the forward scatter/ side scatter (FSC/SSC) were collected.

In some experiments the CD19+ population was purified from the fetal liver cell suspension using the EasySep Biotin Positive Selection Kit (StemCell Technologies) according to manufacturers instructions.

The relevant sorted or purified cell populations were spun at 1400rpm for 5 minutes for pelleting the cells. Once the supernatant was aspirated the cell pellet was lysed using the cell extraction buffer from the Arctus Picopure RNA extraction kit (Applied Biosystems) and lysed cells were kept at 42°C for 30 minutes after which they were stored at -80°C for later RNA extraction.

2.7 RNA extraction and cDNA conversion

RNA was extracted using the Arctus Picopure RNA isolation kit (Applied Biosystems) according to manufacturer’s instructions. RNA concentration (ng/µl) and quality (260/280) was evaluated using the
The extracted RNA was either used for RNA-Sequencing, Microarray or Quantitative Real time PCR (Q-RT-PCR) analysis.

cDNA was normally converted when the sample was used for Q-RT-PCR analysis. In order to convert up to 2\(\mu\)g of RNA into cDNA, the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used according to the manufacturer's protocol. The cDNA concentration (ng/\(\mu\)l) was determined using the Nanodrop (ND-1000).

2.8. Quantitative Real-Time Polymerase Chain Reaction (Q-RT-PCR)

Q-RT-PCR of each cDNA sample was performed on the CFX Connect (Biorad) in triplicate using the iTaq Universal SYBR Green Supermix (Bio-Rad) and Quantitect primers (Qiagen), according to manufacturer's instructions. Each PCR reaction mix contained approximately 10ng of sample cDNA, 0.3M forward and reverse primers combined, 10\(\mu\)l SYBR Green 2X Supermix (containing 100mM KCl, 40mM Tris-HCl, pH8.4, 0.4mM of each dNTP (dATP, dCTP, dGTP, dTTP), iTaq\(^{\text{TM}}\) DNA polymerase (50 units/ml), 6mM MgCl2, SYBR Green 1, 20nM Fluorescein and stabilizers) made up to 20\(\mu\)l with HPLC grade water. Hypoxanthine Guanine Phosphoribosyl Transferase (\(HPRT\)) was used as a housekeeping gene for normalisation of each sample cDNA. The \(HPRT\) transcript was quantified using a dilution series from an embryo head.
cDNA. Each unknown sample cDNA was compared to this dilution series to allow its precise quantification. In each experiment a melt curve was generated using the manufacturers programme to check the melting temperature of the products produced and to ensure that the right size product was produced and not primer-dimers. The transcripts detected by Q-RT-PCR are listed in Table 2.4 below.

**Table 2.4 showing the transcripts analysed by Q-RT-PCR**

<table>
<thead>
<tr>
<th>Transcript Name</th>
<th>Fetal Cell Type Analysed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shh</td>
<td>Liver</td>
</tr>
<tr>
<td>Gli3</td>
<td>Liver</td>
</tr>
<tr>
<td>Gli1</td>
<td>Liver</td>
</tr>
<tr>
<td>Gli2</td>
<td>Liver</td>
</tr>
<tr>
<td>Ptc1</td>
<td>Liver</td>
</tr>
<tr>
<td>Smo</td>
<td>Liver</td>
</tr>
<tr>
<td>Pax5</td>
<td>Liver</td>
</tr>
<tr>
<td>Ebf1</td>
<td>Liver</td>
</tr>
<tr>
<td>Egr2</td>
<td>Thymus</td>
</tr>
<tr>
<td>Tox</td>
<td>Thymus</td>
</tr>
</tbody>
</table>

Table 2.4 shows the transcripts analysed by Q-RT-PCR. Quantitect primers were used for all the transcript analysis.

### 2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

Fetal Thymi were crushed between the frosted ends of two glass slides and washed in 200μl of AIM-V media. The cell suspension was then
spun at 4000rpm in a micro-centrifuge for 10mins and 50ul of the supernatant was used to perform the Shh ELISA assay using Shh N-Terminus Quantikine ELISA Kit (R&D) according to the manufacturers protocol.

2.10. Experimental Data Analysis

Relative analysis was used to analyse the different cell populations in every embryo litter. For each litter, the average WT percentage of a particular population, was compared to the percentage of the same population in each embryo within the litter, and a relative value was obtained for each embryo in the litter. These relative numbers were then compared using a paired two-tailed student’s t test to test the significance of differences between the wildtype when compared to the Heterozygote and Knockout littermates. A value of p<0.05 was considered significant.

2.11. RNA Sequencing and Normalisation

RNA from CD19+B220+ fetal liver cells was sequenced by UCL Genomics on the Illumina Next Seq 500. The RNA-Seq dataset was processed and standardised using the Bioconductor package DESeq2. DESeq2 was also used to generate normalised estimates of transcript abundance.
2.12. Microarray and Normalisation

RNA for Fetal Thymus CD4+CD8- (SP4), CD69-DP, CD69+DP cells was provided to UCL Genomics to perform microarrays on the Affymetrix Mouse Gene 2.0 ST Platform using standard Ambion (Invitrogen, US) chemistry. The Affy package from Bioconductor was used to assess the quality of each microarray by analyzing the probe intensity images and probe intensity distributions to check whether any artifacts or probe saturation was present respectively. Once quality checked, all the microarrays were normalised using log-scale Robust Multi-Array Average (RMA), which eliminates optical background noise and non-specific binding.

2.13. RNA-Sequencing and Microarray analysis

Once the datasets are normalised further analysis including Differentially Expressed Gene (DEG) analysis, Principal Component Analysis (PCA) and Canonical correspondence Analysis (CCA) were performed.

2.13.1 Differentially Expressed Gene Analysis

Differentially Expressed Genes (DEG) were selected out using the Bioconductor Package Limma (Ritchie, Phipson et al. 2015). Ebayes
statistic was used to select out genes that was significantly different (p<0.05) between the WT and mutant datasets. The DEG list was intersected with the PCA gene list (describe in section 2.12.2) to create gene expression heat maps where red represents high expression and green showed low expression.

### 2.13.2 Principal Component Analysis

Principal Component Analysis (PCA) was carried out using the CRAN package ade4. PCA is an exploratory multivariate statistical approach, where datasets are assessed to project most of the variation from the original data in the first principal component (PC1) followed by the other principal components. Mathematically each principal component (PC) axes represents the percentage of variation in the dataset, however principal components can also represent a biological variable e.g. a genotype or an experimental condition. The scores for genes on each PC axis can help determine their relationship to a particular genotype/experimental condition. For examples, if a genotype segregates on the PC1 axis where the WT is towards the negative end of the axis and the KO is towards the positive end. I can analyse the genes at each end of the axis to help reveal their importance towards a particular genotype.

### 2.13.3 Canonical Component Analysis

CCA is another multivariate statistical method used to analyse and explain a given dataset (i.e. the main dataset using an explanatory
dataset i.e. the environmental dataset). CCA was carried out using the CRAN package vegan. The explanatory dataset determines the relationships to be examined in the main dataset. For example, an explanatory dataset containing two different experimental conditions or genotypes (A and B) can define a gene expression gradient axis based on the selected significantly DEG between datasets A and B. These genes can then be extracted from the main dataset, and using CCA one can determine whether these selected genes exhibit similar differences in the main dataset. Hence, CCA can visualise correlations between genes and cell subsets with different experimental conditions/phenotypes, by constraining the main dataset using the interpretable explanatory dataset such that the most interpretable part of the main dataset is used to identify relationships (Ono, Tanaka et al. 2013).

CCA first regresses the main dataset onto the explanatory dataset, and thereby constrains the former dataset to the one that can be explained by the latter dataset (GreenAcre, 2010). The constrained data (the part of data explained by the explanatory dataset) further undergoes Correspondence Analysis (CA) analysis for dimensional reduction and visualisation. CCA is known as a variant of CA because the search for the principle axes determining variation in data is restricted to a constrained space defined by the explanatory dataset (GreenAcre, 2010). A summary of CCA is described in five steps below:
1. Firstly, the explanatory dataset, which is used to explain the main dataset, was chosen. Quality control and normalisation were then performed on both datasets.

2. Next, a number of significant genes were selected from the explanatory dataset using Ebayes analysis (p<0.05).

3. These genes were then selected out of the main experimental Th-dataset and the explanatory dataset.

4. CCA was then performed on the new datasets with the selected genes only. CCA linearly regresses the main dataset onto the explanatory dataset and performs a singular value decomposition of the main constrained dataset, assigning numerical values to samples and genes to allow maximum data dispersion and hence obtain various axes describing the variance in the whole dataset.

5. Finally, the data is visualised by plotting the constrained main data and the explanatory variables (explanatory dataset).
Chapter 3. The role of Gli3 and Shh in Fetal Liver B cell development

3.1 Introduction

The fetal liver is the predominant site of B cell development during mid-gestation. Here, B cell development begins at embryonic day 11 (E11) and cells continue to differentiate and mature into heavy chain expressing cells. During mid to late gestation, cells from the FL migrate to the fetal spleen and fetal bone marrow (de Andres, Gonzalo et al. 2002, Montecino-Rodriguez and Dorshkind 2012).

Fetal liver HSCs differentiate into more restricted multipotent progenitors known as common lymphoid progenitors (CLPs). CLPs are thought to be fully committed to B lineage once they express the surface marker AA4.1 together with either B220 or CD19 (Hardy and Hayakawa 2001). Recent studies have shown that cells that express CD19 before B220 are committed to mature into B-1 (CD19+B220<sub>lo-neg</sub>) cells, while cells that express B220 before CD19 become B-2 (CD19-B220+) lineage cells (Egawa, Kawabata et al. 2001, Dorshkind and Montecino-Rodriguez 2007, Montecino-Rodriguez and Dorshkind 2012).

Interestingly, both B-1 and B-2 cells mature into double positive B220+CD19+ cells which further mature to undergo immunoglobulin heavy chain gene rearrangement to give rise to the first cells that
express cell surface µH (pre-BCR). These cells are defined by the cell surface expression of BP-1 (Hardy and Hayakawa 2001, Montecino-Rodriguez and Dorshkind 2012).

Since, these various stages of B cell development are clearly defined by the expression of different surface markers, I can examine influence of the loss of various genes at the different stages of B cell development. In this study, I investigate the role of the Hh responsive transcription factor Gli3 at each stage of FL B cell development. The role of Gli3 during fetal liver B cell development has not to our knowledge been previously studied. However, Gli3 has been shown to negatively regulate early thymocyte development and patterning of the fetal limb(Ahn and Joyner 2004, Hager-Theodorides, Dessens et al. 2005). These previous roles of Gli3 as a regulator of embryonic development led us to test the hypothesis that Gli3 may influence embryonic B cell development.

As discussed previously, Gli3 is usually a repressor of Hh signalling in vivo, therefore using a Gli3-/- model to study changes in B cell development will allow us to study both, the Gli3 dependent and Gli3-independent-Shh-dependent effects.

Interestingly, Gli3 and Shh have been known to be involved in several B cell leukemias and Hh activity inhibitors have limited the self-renewal potential of several B cell acute lymphocytic leukemias (B-ALL) (Lin, Wang et al. 2010, Ramirez, Singh et al. 2012). Hence, it is important to understand the role of Gli3 in B cell development, as this may reveal the
transcriptional targets of Gli3 and Shh, which may shed light on their function in B cell leukemias.

3.2 Chapter Objectives

In this chapter, I aim to test the hypothesis that Gli3 regulates B cell development in the fetal liver. I will carry out an analysis of the phenotypic changes in Gli3 mutant B cells as they differentiate from the earliest common lymphoid progenitor stage to the mature heavy chain expressing stage. Since Gli3 is a repressor of Hh signalling, the Gli3 mutant will be used to test both Gli3-dependent-Hh-independent changes and the Hh-dependent changes during FL B cell development.

I will first examine the expression of the Hh signalling pathway members in the Gli3 mutant FL. Subsequently, I will assess the Hh-dependent changes in FL B cell development using Fetal Liver Organ Cultures (FLOC), treated with either Shh or the Hh-neutraliser Hhip. The changes in vitro in FLOCs, will be confirmed by the in vivo analysis of FL B cell development in the Shh mutant.
3.3 Results

3.3.1 Expression of Gli3 and Hh pathway members in fetal liver

Fetal liver is the main site of hematopoeisis during mid to late gestation. During embryo stages of life, B cells mainly develop in the fetal liver. However, postnatal B cell development continues throughout life in the bone marrow. Recently, Cridland and Colleagues (2009) have shown that the Gli transcription factors Gli1, Gli2 and Gli3; and the Hh signalling components Ptch1 and Smo are expressed in the FL (Cridland, Keys et al. 2009). I analysed Microarray data from the Immgen database (Heng, Painter et al. 2008) to investigate transcript levels of the Hh ligands (Shh, Ihh and Dhh), Hh responsive Gli transcription factors (Gli1, Gli2 and Gli3), and Hh signal transducers Ptch1 and Smo in embryonic day (E)15 fetal liver HSCs, CLPs, pro-B (CD19+ µH-) and pre-B (CD19+ µH+) cells (Figure 3.1A-E). Since, Gli3 and the Hh family members are expressed at the different stages during FL B cell development, I decided to study the role of Gli3 and importance of Hh signalling during B cell development.

3.3.2 Genotyping of Gli3 mutant embryos

The Gli3/- mice are embryonic lethal, therefore our study was limited to understanding the role of Gli3 in during fetal B cell development and I was unable to follow the changes in the adult bone marrow. In order
to obtain Gli3−/−, Gli3+/− and Gli3+/+ (Wild Type, WT) embryo littermates, I set up time-mates by crossing a Gli3+/− male with a Gli3+/− female and the day when the plug was found was counted as 0.5.

Gli3 mutant embryos were genotyped visually by assessing for their limb digit formation; Gli3 heterozygotes (Gli3+/−) had an extra toe on one end of the limb, while the Gli3 deficiency (Gli3−/−) resulted in a distinct polysyndactly phenotype (Figure 3.2) (Schimmang, Lemaistre et al. 1992).

### 3.3.3 Loss of Gli3 leads to defective FL B cell development

I analysed FL B cell populations at different stages of B cell development including E14.5, E17.5 and E18.5. Firstly, I assessed changes in the FL cell numbers between the WT, Gli3+/− and Gli3−/− and found no significant differences in the number of FL cells. I then used flow cytometry to analyse B lineage surface markers (CD19, B220, μH), which marked for B1-progenitors (CD19+B220−), B2-progenitors (CD19−B220+), the semi-mature CD19+B220+ cells and the mature surface heavy chain expressing CD19+B220+μH cells. I observed a statistically significant gene-dose dependent decrease in the proportions of the CD19+ cells, B220+ cells, and CD19+B220+ cells in the E18.5 Gli3-deficient FL (Figure 3.3A-D). The Gli3−/− FL showed a significant decrease in the proportion of CD19-B220+ B2-progenitor cells but I did
not observe any significant changes in the CD19+B220− B1-progenitor cell population (Figure 3.3C and 3.3D).

I further characterised early B-cell development, by staining against the B-lineage marker, CD93, and then subdividing the CD93+ population by expression of C-Kit, CD127, HSA, CD43 and BP-1, in addition to CD19 and μH expression, to identify four fractions of increasing maturity (Figure 3.3E-F). The overall proportion of CD93+ cells (B-lineage committed) was significantly reduced in the Gli3−/− FL compared to WT (Figure 3.3E). Gating on these CD93+ cells, the proportion of the early c-Kit+CD127+ population (Fraction A) was also significantly reduced in the Gli3−/−FL compared to WT, as were the later CD43+CD19+HSA+BP-1+ population (Fraction D) and the CD19+HSA+μH+ population (Fraction E) (Figure 3.3F). In addition, I analysed the proportion of CD19+ cells expressed the cell surface heavy chain μH and observed a significantly reduced in the Gli3 mutant FL compared to WT (Figure 3.4A-B).

3.3.4 Gli3 activity in the hematopoietic compartment of the fetal liver is not required for B cell development

Previous studies by Cridland and Colleagues and the Immgen dataset analysis show that Gli3 is expressed in both the FL stroma and in the hematopoietic B cells (Figure 3.1). Therefore, I decided to investigate whether the reduction in B cell development in the Gli3 mutant FL is due to cell intrinsic Gli3 activity in the hematopoietic compartment or due to
Gli3 activity in the non-hematopoietic compartment (FL stroma). In many tissues stromal-cell interactions determine final cell fate. For example, in the thymus, the thymic stromal cells send either maturation signals to allow thymocyte survival or apoptotic signals, which cause thymocyte death. In the fetal liver, the stromal-cell interactions allow hematopoietic cells to differentiate, proliferate and mature.

Here, I used the Cre-loxP system to conditionally delete Gli3 from the hematopoietic lineage and generated a Gli3fl/fl,VavCre+ (Gli3coKO) mice to investigate the role of Gli3 in the hematopoietic cells rather than in the stroma. I time-mated the Gli3fl/fl,VavCre+ female adult mice to Gli3fl/fl,VavCre- male and analysed the FL B cell populations of the E17.5 Gli3coKO and control (Cre-negative) embryos.

I found no significant difference in the different stages of B cell development between the control (Cre-negative) and Gli3coKO FL. The proportion of cells that expressed CD19, B220 and BP-1 (a pre-BCR stage marker) were not different between control and Gli3coKO (Figure 3.5A-E). In addition, the proportions of B1 and B2 progenitor populations were not different between the control and the Gli3coKO FL (Figure 3.5C-D). Hence I suggest that Gli3 activity in the FL stroma, rather than hematopoietic cell intrinsic activity regulates B cell differentiation.
3.3.5. The Gli3 mutant fetal liver has increased Hh signalling

As Gli3 is a repressor of the Hh pathway in vivo in many different tissues, I tested if the Gli3-mutant FL had increased levels of Hh pathway activation. I used Q-RT-PCR to measure the transcription of various Hh pathway components and target genes in the Gli3 mutant FL (Figure 3.6A-D). Our results showed that the Hh-receptor Ptch1 and the Hh responsive transcription factors Gli1 and Gli2 were increased in the Gli3-/- FL compared to WT (Figure 3.6A-C). In addition, the transcription of the Hh ligand Shh was also higher in Gli3-/- FL compared to WT (Figure 3.6D). This increase in Shh expression in the Gli3-/- FL was consistent with Gli3 functioning to repress Shh in the FL.

I then Facs-sorted CD19+ (B-lineage) and non-hematopoietic CD45- cells (stroma) from Gli3-/- and littermate WT FL and examined the expression of Hh pathway components and target genes (Figure 3.7A-D). The expression of Gli1 was higher in non-hematopoietic WT stroma cells compared to the CD19+ population. Interestingly, its expression increased in the Gli3-deficient populations, with a greater increase in the non-hematopoietic (stromal) Gli3-deficient compartment (Figure 3.7A). Gli2 expression was approximately five-fold higher in the Gli3-deficient stromal cells compared to WT, and was relatively very low in the CD19+ fraction (Figure 3.7B). In contrast, expression of Ptch1 was increased in both populations sorted from Gli3-/- compared to WT FL (Figure 3.7C). Expression of Shh was greatly upregulated in the non-
hematopoietic (stromal) component of the Gli3-/- FL compared to WT, consistent with Gli3 functioning to repress Shh expression in the FL stroma (Figure 3.7D). This result was consistent and with a previous study, where expression of Shh was confirmed in Dlk(+) hepatoblasts (Hirose, Itoh et al. 2009).

Given that loss of Gli3 increased the transcription of various Hh pathway genes and Shh, while it decreased the development of the B cell progenitor populations, I tested if developing B cells and stromal cells are undergoing active Hh signalling in the FL. I used Gli binding site (GBS)-GFP transgenic reporter mice, which have eight concatamerized binding sites for Gli transcription factors regulate GFP expression. GFP is expressed when activator forms of Gli proteins bind to the GBS in the transgene and therefore active Hh-dependent transcription can be measured in various tissues of these transgenic mice (Balaskas, Ribeiro et al. 2012).

Our results showed that approximately 4% of CD19+ cells expressed GFP in the WT FL (Figure 3.8A) and a higher level of GFP expression ~8% and ~9% was observed in the CD19+B220- (B-1 progenitors) and CD19-B220+ (B-2 progenitors) respectively (Figure 3.8B and 3.8C). This suggests that a higher level of Hh signalling is present in cells transitioning from the immature B1 and B2 progenitor stages towards the semi-mature CD19+B220+ stage.

Interestingly, the proportion of GFP expressing CD19+, CD19+B220- and CD19-B220+ cells was significantly increased in the Gli3-/-
compared to WT (Figure 3.8A-D). However, there was no significant difference in the proportion of GFP+ cells between Gli3+/− and WT (Figure 3.8D). The mean fluorescence intensity (MFI) of the CD19+GFP+ cells significantly increased from WT to Gli3+/− and from WT to Gli3−/−, indicating higher Hh-dependent transcription in individual cells (Figure 3.8E).

A high percentage of non-hematopoietic (CD45−) stromal FL cells (~57%) and of the Epcam+ subset of CD45− FL cells (~53%) expressed GFP, indicating that Hh signalling is also active in the FL stroma (Figure 3.9A-B). These results were consistent with our Q-RT-PCR data, which indicated that Hh signalling is also active in the non-hematopoietic stromal compartment (Figure 3.7A and C). This suggests that Hh signalling may regulate the stromal-B cell interactions, which lead to changes in B cell development. Overall, our results indicate that the Gli3−/− FL has an increased level of Hh activity in both the FL stromal cells and B lineage cells.

3.3.6. Hh signalling negatively regulates FL B cell development in vitro

Since, the Gli3−/− FL has reduced FL B cell development but increased expression of Hh pathway genes, the Hh ligand Shh and upregulated Hh activity in developing B cells, I decided to test whether the increased Hh signalling in the FL reduces B cell development in vitro.
I assessed the various B cell populations after 4 days culture in WT E17.5 FL Organ Cultures (FLOC) treated with recombinant (r)Shh alone, with rHhip (to bind and neutralize endogenous Hh proteins in the cultures) alone, or treated with both rShh and rHhip, compared to control non-treated FLOC (Figure 3.10A-B). Treatment with rShh significantly reduced the proportion of CD19+ cells from 26% in control cultures to 19.6% in rShh-treated FLOC, whereas neutralization of endogenous Hh proteins by treatment with rHhip significantly increased the proportion of CD19+ cells to 31.3% (Figure 3.10A-B). To confirm the specificity of the reagents, and that the inhibitory effect of rShh was not the result of non-specific toxicity, I added both reagents together, and found that the proportion of CD19+ cells was not significantly different from control cultures. A similar result was observed in the proportion of the B220+CD19+ and CD19+µH+ populations, where both populations significantly decreased by rShh treatment and increased with rHhip treatment when compared to their respective untreated controls in FLOCs (Figure 3.10B and 3.11A-B).

Subsequently, to test if the reduction in B cell development in the Gli3-/- FL is due to an increase in Hh proteins, I treated the Gli3-/- FLOC with rHhip to neutralise endogenous Hh proteins. Treatment of Gli3-/- FLOC by rHhip significantly increased the proportion of CD19+ cells compared to the untreated Gli3-/- control (Figure 3.12). This suggests that the reduction in B cell development in the Gli3 mutant was largely
due to an increase in Hh proteins in the FL, and that Hh signalling negatively regulates B cell development in vitro.

3.3.5 Loss of Shh accelerates in vivo FL B cell development

In order to confirm our in vitro results showing that Shh negatively regulates FL B cell development, I examined FL B cell development in Shh mutant embryos. Most Shh-/− embryos die before E16, so I analysed the E14.5 FL.

Our results showed that the loss of one copy of Shh in the heterozygote (Shh+/−) and the complete loss of Shh in the Shh-/- embryos significantly increased the percentages of CD19+ cells, B220+ cells, and of the semimature CD19+B220+ populations. Both B1 (CD19+B220-) and B2 (CD19-B220+) progenitor populations significantly increased compared to WT, with the heterozygote showing intermediate proportions (Figure 3.13A-B). The proportion of B-lineage committed CD93+ cells and the proportion of CD93+ cells that were CD19+ were also significantly increased in the Shh-/- FL compared to WT (Figure 3.13C).

I also analysed the E14.5 Gli3-mutant FL in order to allow comparison of FL B cell development of both genotypes at a similar stage in embryogenesis. Interestingly, the Gli3 mutant FL showed the opposite phenotype to the Shh mutant FL, with significantly decreased CD19+, B220+, CD19+B220+ and CD19+B220- B1-progenitor populations.
compared to WT littermates (Figure 3.14A-B). However, I did not observe any significant changes in the B2-progenitor population in the E14.5 Gli3-/ FL.

I then analysed B cell development at the pre-BCR stage, marked by surface expression of BP-1 and found that the proportion of CD19+BP1+ (Pre-B) cells was significantly increased in the Shh-/ E14.5 FL compared to WT, while the reverse was seen in the Gli3-/ E14.5 FL (Figure 3.15A-B). Additionally, the treatment of E14.5 Shh-/ FLOC with rShh for 4 days reduced the proportion of CD19+ cells compared to the untreated control FLOC (Figure 3.16), confirming that Shh negatively regulates B cell development.

Since Shh regulates B cell development from the earliest B1 and B2 progenitor cell population to the pre-BCR stage, I wanted to investigate whether Shh impacts commitment to the B lineage as well. I analysed the proportion of CLP, defined by the surface markers Lin-CD117+CD127+. CLPs are the earliest lymphoid committed progenitors and they are still multipotent having the ability to differentiate into T, B or myeloid lineages.

The Shh-deficient FL showed a significant increase in the proportion of the CLP population relative to WT (Figure 3.17). In contrast, the Gli3-deficient FL had a significantly decreased proportion of CLP compared to the WT (Figure 3.17). Therefore, taken together, our experiments indicate that Gli3 and Shh influence B cell development from the CLP populations through to the CD19+B220+pre-BCR+ stage of
development. Loss of Gli3 leads to a decrease in B cell development, possibly through increased Hh signalling, as loss of Shh promotes B cell development.

### 3.4 Discussion

Here I showed that Gli3, expressed by the stromal compartment, is required for B cell development in the FL. Mutation of Gli3 led to an overall reduction in B-lineage committed cells, reduction in the proportion of pre-B cells, and reductions in CLP and both the CD19+B220- B1-progenitor population on E14.5 and to the CD19-B220+ B2-progenitor population on E17.5. The changes in B cell differentiation in the Gli3 mutants could be due to Hh-dependent or Hh-independent effects. I found that loss of Gli3 led to increased Shh expression and overall increased Hh signalling in the murine FL. Gli3 was thus acting as a repressor of the Hh pathway in the FL, as observed in the development of other Gli3 deficient cells and tissues, such as the neural stem cells, vertebrate limb bud and thymus (te Welscher, Fernandez-Teran et al. 2002, Hager-Theodorides, Dessens et al. 2005, Hager-Theodorides, Furmanski et al. 2009, Petrova, Garcia et al. 2013, Saldana, Solanki et al. 2016). Treatment of Gli3-deficient FLOCs with rHhip (to neutralise endogenous Hh proteins) increased B cell development, and therefore the reduction in B cell development in the Gli3 mutants was Hh-dependent and due to increased Shh-expression in the Gli3-/- FL.
In contrast, the Shh-deficient FL had increased B-lineage commitment and B cell differentiation, demonstrating that Shh negatively regulates B cell development in vivo. In addition, Shh treatment in in vitro FLOCs decreased B cell development while neutralising Hh signalling using rHhip increased B cell differentiation. I showed that both Shh transcription and Hh signalling to B-lineage cells is increased in the Gli3-deficient FL and that Shh negatively regulates B cell development by signalling directly to developing hematopoietic cells, as Hh target genes were upregulated in the Gli3-/- CD19+B220+ population, and the Hh-reporter transgenic showed increased GFP expression in the Gli3-/- CD19+ population.

Shh signalling from follicular dendritic cells to B cells in the adult spleen has been shown to promote B cell survival and function (Sacedon, Diez et al. 2005), and in the adult bone marrow, components of the Hh signalling pathway are expressed in developing B cells (Heng, Painter et al. 2008, Cooper, Hardy et al. 2012). Interestingly, the conditional deletion of Smo from the B cell lineage did not influence B cell development in the adult bone marrow. However, the deletion of Smo from stromal cells impaired differentiation of hematopoietic progenitors towards the B lineage in the adult bone marrow (Cooper, Hardy et al. 2012).

Our study demonstrates that the Hh signalling pathway is active in developing fetal B cells and regulates B cell development in the FL. Therefore, there may be tissue-specific or life-stage specific differences
in the function of Hh signalling between FL and adult bone marrow. Alternatively, it is possible that Hh pathway activation is non-canonical (Smo-independent) in B lineage cells, which do not have primary cilia or that, a balance between canonical and non-canonical signalling may exist, as described in osteoblast differentiation (Yuan, Cao et al. 2016).

3.5 Future Work

Since many of the Gli family members have partially overlapping functions, it would be interesting to investigate the role of the other Gli proteins (Gli1 and Gli2) and Hh ligands, Ihh and Dhh, in FL B cell development. Although, I would not be able to study adult B cell differentiation in the adult bone marrow of Gli3 or Shh deficient mice as they are embryonic lethal, I would like to study B cell differentiation in the Gli3 and Shh heterozygote adult mice and observe whether the fetal differences during B cell development correlate with the adult B cell differentiation differences. It would also be important to understand the transcriptional changes underlying the phenotype changes in Gli3 mutant B cells. These will be analysed using RNA-Sequencing of the CD19+B220+ WT and Gli3 mutant B cell population in the next chapter four.
3.6 Summary

In this chapter, I show that Gli3 activity in the FL stroma is required for normal B cell development. The Gli3-/- FL has an increased Shh expression and an increased Hh activity, which leads to decreased B cell development. Shh signals directly to B lineage cells and negatively regulates their development as attenuation of Hh signalling in in vitro FLOC and loss of Shh in vivo increased B lineage commitment and development.
Figure 3.1. Expression of Hh ligands and Hh signalling pathway components in the different B cell fetal liver populations from Immgen database.

Figure 3.1 shows transcript expression from the Immgen database for Hh molecules (Shh, Ihh, Dhh) and Hh pathway components including Gli1, Gli2, Gli3, Ptc1 and Smo in (A) Common Lymphoid Progenitors (CLP) (AA4+Kit+IL7ra+B220-), (B) Fr.A (AA4+Kit+IL7ra+B220+), (C) Fr.B/C (AA4+IgM-CD19+CD43+HSA+), (D) Fr.D (AA4+IgM-CD19+CD43-HSA+), (E) Fr.E (AA4+IgM+CD19+HSA+). Triplicates with mean ±SEM shown for each gene. Data from GSE15907 (Heng, Painter et al. 2008).
Figure 3.2 The fetal limb phenotype of the Gli3 mutants

Gli3+/+  Gli3+-/  Gli3-/-

Figure 3.2 shows the WT (Gli3+/+) and Gli3 mutant forelimb phenotype. The Gli3 heterozygote (Gli3+-/) has an extra toe on the anterior forelimb, while the Gli3-/- forelimb shows polysyndactly.
Figure 3.3. B cell development in E18.5 and E17.5 Gli3+/+, Gli3+/- and Gli3-/- fetal liver.

A

Gli3+/+  Gli3+/-  Gli3-/-

B

C

D

<table>
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<tr>
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<th>Gli3+/+</th>
<th>Gli3+/-</th>
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<td>A</td>
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<td>7.83%</td>
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<td>B</td>
<td>15.4%</td>
<td>13.2%</td>
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<tr>
<td>C</td>
<td>0.384%</td>
<td>0.486%</td>
<td>0.265%</td>
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Percentage in E17.5 - 18.5 Fetal Liver (relative to WT)

*  **  **  *
Figure 3.3 shows (A-C) Flow cytometry profile of E18.5 FL from Gli3+/+ (WT), Gli3+- and Gli3-/- littermates following lysis of red blood cells from a representative experiment. Dotplots show (A) SSC against CD19 (B) SSC against B220 and (C) CD19 versus B220. (D) Bar charts show mean percentage of FL populations, relative to mean of WT littermates ±SEM, giving statistical significance by student’s t-test compared to WT littermate FL for: B220+ (Gli3-/- p=0.02), CD19+ (Gli3+-p=0.03, Gli3-/-p=0.002), CD19+B220+ (Gli3+-p=0.05, Gli3-/-p=0.001) and CD19-B220+ (Gli3-/- p=0.02), CD19+B220-, from E17.5 and E18.5: Gli3-/- (n=25), Gli3-/- (n=14), and Gli3+/+ (n=15). (E-F) Bar chart: mean ±SEM of FL populations, relative to mean of WT littermates, giving statistical significance by student’s t-test for Gli3-/- (n=4) and WT (n=3), where (E) shows % of CD93+ cells (Gli3-/- p=0.008) and (F) shows four populations, gated on CD93+, for Fraction (Fr.) A: Gr1-, Mac1-, Ter119-, CD71-, c-Kit+, CD127+ (p=0.03); Fr.B/C: CD19+, HSA+, CD43+, BP-1-; Fr.D: CD19+, HSA+, CD43+, BP-1+ (p=0.03); Fr.E: CD19+HSA+µH+ (p=0.002).
Figure 3.4 Percentage of $\mu$H positive cells E17.5 Gli3+/+, Gli3+-/- and Gli3--/ fetal liver.

Figure 3.4 shows (A) SSC against $\mu$H gated on CD19+ cells and (B) $\mu$H gated on CD19+ cells (Gli3+-/-p=0.05, Gli3--/-p=0.001), from E17.5 and E18.5: Gli3+-/- (n=25), Gli3--/- (n=14), and Gli3++/+ (n=15).
Figure 3.5. B cell development in Gli3 conditional mutant (Gli3fl/fl, VavCre+)

A

Gli3fl/flVavCre-  Gli3fl/flVavCre+

B

Gli3fl/flVavCre-  Gli3fl/flVavCre+

C

Gli3fl/flVavCre-  Gli3fl/flVavCre+

D

Percentage in E17.5 Fetal Liver (relative to WT)

B220+  CD19+  CD19+B220+  CD19-B220+  CD19+B220-

E

Percentage of BP-1+ Cells Gated on CD19 (relative to WT)

BP-1+
Figure 3.5 shows flow cytometry profile of E17.5 FL from control (Gli3fl/fl, VavCre) and Gli3coKo (Gli3fl/fl, VavCre+) littermate embryos from a representative experiment. Dotplots show (A) SSC against CD19 (B) SSC against B220 and (C) CD19 versus B220. (D) Bar charts show mean percentage, relative to mean of WT littermates ±SEM of B220+, CD19+, CD19+B220+, CD19-B220+, CD19+B220- and (E) BP-1+ gated on CD19+ cells, from Gli3coKO (Gli3fl/fl, VavCre+) (n=8) and control (Gli3fl/fl, VavCre-) (n=8) littermates. There were no significant differences by student’s t-test.
Figure 3.6. Expression of Hh pathway components in E14.5 and E17.5 WT and Gli3 mutant fetal liver

Figure 3.6 shows transcript expression of Gli1, Gli2, Ptch and Shh. (A-D) Bar charts: representative experiment showing mean ± SEM (n = 3) of Q-RT-PCR analysis of the FL from WT, Gli3+/− and Gli3−/− littermates, normalized relative to HPRT for (A) Gli1, (B) Gli2, (C) Ptch1 on E17.5 and (D) Shh on E14.5 fetal liver.
Figure 3.7. Expression of Hh pathway components in CD19+ and CD45- (stroma) cells in the E17.5 WT and Gli3-/- fetal liver.

Figure 3.7 shows transcript expression of Gli1, Gli2, Ptch and Shh in facs sorted CD19+ and CD45- (stroma) cells. (A-D) Bar charts: representative experiment showing mean ± SEM (n = 3) of Q-RT-PCR analysis of the FL from WT and Gli3-/- littermates, normalized relative to HPRT for (A) Gli1, (B) Gli2, (C) Ptch1 and Shh on E17.5 fetal liver.
Figure 3.8. Presence of active Hh signalling in E14.5 and E17.5 WT and Gli3 mutant fetal liver.

A


GFP (Gated on CD19)

B

Gli3 +/+  Gli3 −/−

GFP (Gated on CD19+B220-)

C

Gli3 +/+  Gli3 −/−

GFP (Gated on CD19-B220+)

D

% of GFP+ and MFI of GFP+ cells Gated on CD19
(Relative to WT)

GFP+  MFI of GFP+

**  *
Figure 3.8 shows (A-D) Flow cytometric analysis of GFP expression in GBS-GFP-reporter transgenic E17.5 FL from WT, Gli3+/− and Gli3−/− littermates. (A) Dotplots show SSC v GFP-fluorescence, gated on CD19+ cells. (B-C) Histograms show expression of GFP on (B) CD19+B220− and (C) CD19−B220+ in the Gli3+/+ and Gli3−/− E17.5 FL. (D) Bar charts show mean percentage and mean MFI, ±SEM, of GFP-fluorescence in CD19+ cells, relative to the mean of WT. (n=4 Gli3+/−; n=3 Gli3−/− and n=3 WT). Differences are statistically significant by student’s t-test, compared to WT, for % of GFP+ in the CD19+ cells, for Gli3−/− p=0.001, and for the MFI of GFP on the CD19+ cells, for Gli3+/− p=0.03, and for Gli3−/−, p=0.04.

Figure 3.9. Presence of active Hh signalling in E14.5 WT fetal liver stroma

Figure 3.9 shows (A) Histogram shows expression of GFP in the E14.5 WT FL stroma (CD45−) cells (B) Histogram shows expression of GFP in Epcam+ subset of CD45− FL cells (Epcam+CD45−CD19−).
Figure 3.10 Modulation of B cell development by rShh and rHhip treatment of WT FLOCs

Figure 3.10 shows (A-B) WT E17.5 FLOC treated with recombinant proteins for 4 days and analysed by flow cytometry (n=4). (A) Dot plots show SSC versus CD19 staining in control (untreated) and treated with rShh, rHhip and rShh + rHhip respectively. (B) Bar charts show the mean ±SEM, relative to the mean of control untreated FLOC from littermates, showing statistical significance by student’s t-test compared to untreated for CD19+ cells (left hand chart) (rShh treatment p=0.001, rHhip treatment p=0.04), and CD19+B220+ cells (right hand chart) (rShh treatment p=0.002, rHhip treatment = p=0.04). The dotted line represents the mean of the WT untreated control.
Figure 3.11 Modulation of Pre-B cell development by rShh and rHhip treatment of WT FLOCs

Figure 3.11 shows (A-B) WT E17.5 FLOC treated with recombinant proteins for 4 days and analysed for the CD19+ μH+ population by flow cytometry (n=4). (A) Dot plots showing μH+ gated on the CD19+ population in control (untreated) and treated with rShh, rHhip and with rShh and rHhip together. (B) Bar chart showing the mean ±SEM, relative to the mean of control untreated FLOC showing statistical significance by student’s t-test compared to untreated for μH+ gated on the CD19+ population (rShh treatment p=0.03).
Figure 3.12 Modulation of B cell development by rHhip treatment of Gli3-/- FLOCs

Figure 3.12 shows Gli3-/- FLOC treated with rHhip and control untreated Gli3-/- FLOC (n=4) cultured for 4 days. Dot plots show SSC versus CD19 staining. Difference in the mean percentages were statistically significant between rHhip treatment and control untreated, p=0.02 for CD19+ cells and p=0.03 for CD19+B220+ cells.
Figure 3.13. B cell development in the E14.5 Shh-deficient fetal liver.
Figure 3.13 shows (A-C) Flow cytometry analysis of E14.5 FL from Shh+/+ (WT), Shh+- and Shh-/- littermates. (A) Dotplots show SSC against CD19 staining (upper panel) and CD19 staining against B220 staining (lower panel). (B) Bar charts show the relative mean percentage ±SEM, relative to the mean of WT littermates of the populations stated, showing statistical significance compared to WT for CD19+ (Shh+/- p=0.03, Shh-/- p=0.002), B220+ (Shh+/- p=0.02, Shh-/-p=0.02), CD19+B220+ (Shh+/- p=0.02, Shh-/-p=0.001), CD19-B220+ (Shh-/-p=0.02), CD19+B220- (Shh+/-p=0.02, Shh-/-p=0.001) cells. For Shh+/-, n=10; Shh-/-, n=4; Shh+/+, n=5. (C) Dot plot: SSC against CD93 staining (upper panel); Histogram: CD19 staining, gated on CD93, both populations are statistically significant, relative to the WT (p=0.05 and p=0.03, respectively, for Shh-/-, n=4)
Figure 3.14. B cell development in the E14.5 Gli3-deficient fetal liver.

**Graph A**
- **Gli3+/+**
  - B220
  - CD19
  - Percentage: 0.471%

- **Gli3+/−**
  - B220
  - CD19
  - Percentage: 0.300%

- **Gli3−/−**
  - B220
  - CD19
  - Percentage: 0.220%

**Graph B**
- B220
- CD19
- B220+CD19
- CD19-B220
- CD19+B220

- **Gli3+/+**
- **Gli3+/−**
- **Gli3−/−**
Figure 3.14 shows flow cytometry analysis of E14.5 FL from Gli3+/+ (WT), Gli3+- and Gli3-- littermates. (A) Dotplots show SSC against CD19 staining (upper panel) and CD19 staining against B220 staining (lower panel). (B) Bar charts show the relative mean percentage ±SEM, relative to the mean of WT littermates of the populations stated, showing statistical significance relative to WT for B220+ (Gli3-- p=0.04), CD19+ (Gli3+- p=0.04, Gli3-- p=0.001), CD19+B220+ (Gli3-- p=0.003), CD19-B220+ (not significant), CD19+B220- (Gli3+- p=0.03, Gli3-- p=0.003). For Gli3+-, n=10; Gli3--, n=9; Gli3+/+, n=7.
Figure 3.15. Pre-B cell development in E14.5 Gli3-/- fetal liver

Figure 3.15 shows (A) Histograms show BP-1 staining, gated on CD19+ cells from E14.5 Shh-/- FL and WT littermate (left hand plots) and E14.5 Gli3-/- FL and WT littermate (right hand plots). (B) Bar charts show the mean percentage ±SEM of BP-1+ cells, gated on CD19+, relative to the mean of their WT littermates for Gli3-/- (n=9, p=0.03) and Shh-/- (n=7, p=0.001) FL.
Figure 3.16. B cell development in rShh treated Shh-/- FLOC

Figure 3.16 shows Shh-/- FLOC treated with rShh for 4 days. Bar chart shows the mean (relative to mean of control cultures) percentage of CD19+ cells. The difference was statistically significant by student’s t-test (p=0.008, n=4).
Figure 3.17. CLP development in Shh and Gli3-/- fetal liver

Figure 3.17 shows Bar charts showing mean percentage ±SEM of CD117+CD127+ Common Lymphoid Progenitor (CLP) cells in E17.5 Gli3-/- (n=9, p=0.02) and E14.5 Shh-/- (n=4, p=0.001) FL, relative to their respective WT littermates. The shaded bars are knockout and unfilled bars are WT.
Chapter 4
Chapter 4. The transcriptional mechanisms regulating B cell development in the Gli3 mutant fetal liver

4.1. Introduction

The results from the previous chapter showed that Gli3-deficiency decreased B cell development from the CLP to the mature heavy chain expressing stage. Here, I explore the transcriptional changes underlying the decreased B cell development in the Gli3 mutant.

4.1.1 Transcriptional Control of B cell development

Transcription factors regulate many biological processes by binding to specific DNA sequences that can either promote or inhibit target gene transcription. These changes in gene expression occur dynamically in differentiating progenitors as they transit from the HSCs into a mature lineage. The key genes regulating early B lineage differentiation, commitment and maturation include *IL-7R*, *Pu.1*, *Ebf1*, *E2a* and *Pax5*. Mice deficient in any of these lack B cells.

The earliest commitment to the lymphoid lineage is induced by the transcription factor Ikaros. Ikaros deficiency arrests B cell development at the earliest CLP stage but the production of T cells is not affected
Ikaros positively regulates the key CLP and B cell expansion gene flt3 (Sitnicka, Brakebusch et al. 2003, Yoshida, Ng et al. 2006). Following flt3 expression, IL-7R expression is induced by the transcription factors Ikaros, Pu.1 and other ETS family members (DeKoter, Lee et al. 2002). IL-7R signalling provides survival signals to B cells and induces Ebf1 expression by upregulating Stat5 (Kikuchi, Kasai et al. 2008). The induction of IL-7R on the cell surface marks entry into the CLP pool.

Ebf1 is a master regulator of B cell differentiation from the earliest CLP stage to the mature stage of B cell development. Pu.1, E2A and IL-7R collaborate to induce Ebf1 expression. Ebf1 and E2A act synergistically and collaborate at many gene loci to induce target gene expression. Ebf1 promotes its own as well as E2A expression (Zhuang, Jackson et al. 2004). A positive feedback loop exists between Ebf1, E2A and Pax5, where E2A upregulates Ebf1 and vice versa and Ebf1 upregulates itself as well as Pax5 (Figure 4.1).

Since Ebf1 positively regulates its own expression and that of Pax5 and E2a, its expression is maintained throughout B cell development and it plays a central role in B cell commitment and maturation. Studies have shown that Ebf1 expression can partially recover B cell development in Pu.1, E2a, Ikaros and IL-7R deficient mice (Seet, Brumbaugh et al. 2004, Dias, Silva et al. 2005, Reynaud, Demarco et al. 2008). However, induction of Pax5 alone is not sufficient to recover B cell development in these mutants.
Pax5 is an important target of Ebf1 during B cell development. Interestingly, B cell development in Pax5 deficient mice proceeds further than in Ebf1 or E2a null mice. Pax5 maintains B cell identity and prevents pro-B cells from reacquiring a multi-lineage potential. Pax5 is essential for B cell development after the CLP stage until the final B cell maturation stages. Pax5 deficiency causes mature B cells to acquire T cell potential \textit{in vivo} (Mikkola, Heavey et al. 2002). Studies have shown that Pax5 transcriptionally represses Notch1 and Csfr1 to prevent B cells from acquiring an alternative lineage (Souabni, Cobaleda et al. 2002).

A positive feedback loop between Pax5 and Ebf1, maintains the expression of both these transcriptional factors allowing commitment, differentiation and maturation of B cells from the earliest stem cell stage (Roessler, Gyory et al. 2007).

Many other transcription factors regulate B cell development by regulating the expression of Pax5 and Ebf1. For example, Runx1 activity decreases the expression of Ebf1, E2a and Pax5, thus blocking B cell development (Kuo, Gerstein et al. 2008). However, whether these interactions are direct is not currently known. The transcription factor Id2 is also known to inhibit E2a expression; therefore its downregulation is necessary to allow B cell development (Ji, Li et al. 2008).

Since B cell development can be regulated transcriptionally through interactions of many B lineage determining transcription factors, l
examined whether the phenotype change in Gli3 mutant correlated to changes in master regulatory transcription factors of B cell development.

4.2. Chapter Objectives

In this chapter, I will test the hypothesis that Gli3 and Shh transcriptionally regulate B cell development in the fetal liver. In order to understand the mechanism though which Gli3 directly, or indirectly via increased Hh signalling, influences B cell development, I will examine the genome wide transcriptional changes in developing B cells in the Gli3 mutant FL. I will use RNA-Sequencing (RNA-Seq) to analyse the genome wide transcriptional changes in facs sorted Gli3 mutant CD19+B220+ FL B cells and investigate changes in key master regulators of FL B cell development. Subsequently, since Gli3 mutant FL has increased Shh signalling in vivo, I will test whether Shh regulates these RNA-Seq identified master regulators of FL B cell development and propose a mechanism through which the Gli3 and Shh regulate FL B cell development.
4.3. Results

4.3.1. Loss of Gli3 inhibits transcription of B cell lineage commitment, signalling and maturation genes.

I used Principal Component Analysis (PCA) and Differentially Expressed Gene (DEG) analysis together with Ebayes statistics to analyse the transcriptional changes in WT and Gli3 mutant CD19+B220+ FL datasets. These analyses revealed many transcriptional targets of both Gli3 and Shh that are important during FL B cell development.

First, I analysed the dataset in an unbiased manner using Principal Component analysis (PCA). The dataset segregated by genotype on both Principal Component axis 1 (PC1) and Principal Component axis 3 (PC3) (Figure 4.2A). PC1, the axis contributing to the largest differences in the dataset (60% of variability), separated the WT from the Gli3 mutants (Gli3+/- and Gli3-/-), while PC3 showed differences between Gli3+/− and Gli3-/- (Figure 4.2A).

Further analysis of the genes with high positive and negative scores on PC1 showed that PC1 reflected differences in genes associated with Hh signalling and genes associated with B cell signalling and differentiation. Key Hh signalling and target genes including Gli1, Hdac3, Smo had positive PC1 scores indicating that the expression of these is higher in the Gli3 mutant. In contrast, genes that were lower in the Gli3 mutants had high negative PC1 scores and were mainly B cell
signalling and lineage commitment genes. Thus, not only did Gli3-mutation reduce the proportion of CD19+B220+ B-committed cells in the FL, but also within that sorted population, expression of genes required for B cell differentiation was reduced.

In order to understand better the genome-wide differences in the dataset, I intersected the differentially expressed genes identified by Ebayes statistics with the genes identified by PCA. I selected 3000 genes (highest and lowest scorers) that contributed most to the PC1 axis and intersected these with the 3000 most significantly differentially expressed genes between the Gli3-/- and WT by Ebayes statistics (Table S1). The resulting 1122 genes were clustered and a heatmap showing their gene expression was drawn (Figure 4.2B and 4.1C). This intersection highlighted genes that have been previously shown to be Shh target genes in other tissues, such as Stmn1, Hmgb1, Hmgb2, Hoxa4, Cul4a and Bmi1, which were all upregulated in the Gli3-/- compared to WT (Figure 5C) (Itou, Taniguchi et al. 2011, Wang, Venugopal et al. 2012, Lu, Li et al. 2015, Yang, Ni et al. 2015). In contrast, master regulators of early B cell development; Ebf1 and Foxo1 were downregulated in the Gli3-/- compared to WT, and several other B lineage differentiation and maturation genes including Klf13, Egr1, Irf1, Irf4 and Cd69 were also lower in the Gli3-/- (Gururajan, Simmons et al. 2008, Lu 2008, Outram, Gordon et al. 2008). B cell activation and signalling genes, including genes involved in modulating MapKinase signalling such as Dusp1, Dusp2, Map3k3, MapKapk2 (Lang, Hammer et
al. 2006), and canonical NfκB signalling genes (Nfkbid, Nfkbiz and Tnfaip3), were all downregulated in the Gli3-/-. In addition, the AP1 components Fosb, Jun and Junb required for pre-BCR signal transduction (de Gorter, Vos et al. 2007), and Klf2, an essential late target gene of the pre-BCR, were also lower in the Gli3-/- compared to WT (Winkelmann, Sandrock et al. 2014).

As both pre-BCR/BCR signalling and IL-7 signalling are important regulators of B cell development, I compared expression levels of genes that encode downstream components of these signalling pathways, or are their immediate transcriptional targets (Figure 4.2D-E). Expression levels of many genes involved in pre-BCR/BCR signal transduction (Dusp1-3, Tnfaip3, Nfkbid, and the AP-1 components Junb and Fosb) were lower in the Gli3-/- datasets than WT, as were its immediate transcriptional targets, CD69 and Egr1 (Figure 4.2D). I observed no difference in expression in Tcf3 (E2A) between genotypes (Figure 4.2D) or in IL7r, Il2rg, Stat5a, Stat5b, and Pik3ca (components of the IL7/TSLP signalling pathway) or in Myb, Bcl2 or Bcl2l1 (transcriptional targets of IL7 signalling in B-lineage cells) (Figure 4.2E).

I did not detect Shh expression in any dataset (Figure 4.2E), consistent with the Q-RT-PCR showing Shh upregulation in Gli3-/- FL being restricted to stromal cells and not B-lineage cells (Figure 3.7D).

Taken together, these analyses indicate that the Gli3 mutant B220+CD19+ population has increased Hh-dependent transcription, consistent with our previous results showing increased Shh expression
and increased Hh signalling in the Gli3 mutant FL and Gli3-/- B-lineage cells (Figure 3.5 and 3.6 Chapter 3). Gli3-deficiency also decreased transcription of genes required for B cell differentiation, maturation and signalling within the sorted B220+CD19+ population; Thus, increased Shh signalling to hematopoietic progenitor cells repressed the transcription of regulators of B lineage commitment and differentiation. I therefore investigated whether Shh-treatment can directly downregulate transcription of the key B-lineage transcription factors, Ebf1 and Pax5.

### 4.3.2 Shh signalling inhibits B lineage development through Ebf1 and Pax5

Many studies have shown that Shh signalling interacts with and regulates various Pax family members, which in turn regulate the development of different tissues (Macdonald, Barth et al. 1995, Torres, Gómez-Pardo et al. 1996, Borycki, Mendham et al. 1998). For example, Shh signalling directly represses *Pax6* during neuronal development, which prevents Pax6 from functioning normally during neuron development (Ericson, Rashbass et al. 1997, Chi and Epstein 2002). This interaction of Shh with many Pax family members together with the differential expression of Ebf1, a key transcriptional regulator of Pax5, led us to investigate the relationship between Shh, Pax5 and Ebf1.

Ebf1 and Pax5 are master regulators of B lineage commitment and B cell development. B cell lineage commitment from the CLP stage is
regulated by Ebf1 (Zhang, Cotta et al. 2003), which promotes its own transcription as well as Pax5 transcription. Pax5 further increases Ebf1 transcription (Roessler, Gyory et al. 2007) creating a positive feedback loop between itself and Ebf1. This mechanism allows Ebf1 and Pax5 to regulate B cell development and maturation. Since Ebf1 is downregulated in the Gli3-/- FL and Shh may interact with and regulate Pax5, which could further regulate Ebf1, I tested whether the increase in Shh in the Gli3-/- drives the reduction in B cell development by repressing Ebf1 and Pax5.

I first measured overall Ebf1 and Pax5 transcription in the Gli3-mutant E17.5 FL, and found a reduction in their transcript expression in both Gli3+/− and Gli3-/- relative to WT (Figure 4.3A-B), consistent with the RNA-Seq data, and the reduction in B cell development. In contrast, I found an increase in the expression of both transcription factors in the Shh+/- and Shh-/- E14.5 FL, consistent with increased commitment to the B lineage in the absence of Shh (Figure 4.4A-B).

Since the Gli3 mutant has increased Shh signalling and rShh treatment decreased the CD19+ population in FLOC, I tested if I could influence both Pax5 and Ebf1 transcription and protein expression in vitro by modulating Hh signalling in FLOCs by treatment with rShh or rHhip, over a 4-day culture period. I measured the expression of intracellular Pax5 and Ebf1 by facs analysis to investigate protein expression in single cells. On day1 after treatment, there were no significant differences in the proportion of Ebf1+Pax5- and Ebf1+Pax5+ cells.
However, a significant reduction in the proportion of Ebf1+Pax5+ cells was seen on day2 and day4 of rShh treatment (Figure 4.5A). This Ebf1+Pax5+ population was significantly increased and sustained in the rHhip-treated cultures, whereas the cultures in which both rShh and rHhip were added together were not different to the control cultures (Figure 4.5A). Comparison of the ratio of Ebf1+Pax5+ cells: Ebf1+Pax5- cells, showed that rHhip treatment increased the ratio by two-fold by day four in culture, indicating that inhibition of Hh-signalling accelerated the transition from Ebf1+Pax5- cell to Ebf1+Pax5+ cell (Figure 4.5B). In contrast, rShh treatment reduced this ratio on both day2 and day4 of culture, suggesting that Shh signalling repressed the induction of Pax5 and the transition to Ebf1+Pax5+ cell (Figure 4.5B). The changes in the Ebf1 and Pax5 proteins were consistent with the changes in transcript expression of Ebf1 and Pax5 on day4 (Figure 4.5B-C).

Interestingly, the proportion of Ebf1+Pax5+ cells was reduced in the Gli3-/- FL compared to WT, consistent with the Q-RT-PCR data (Figure 4.6 and 4.2 respectively). I then facs sorted CD19+ cells from Gli3-/- and WT littermates and analysed the expression of Ebf1 and Pax5. I observed a decrease in both Ebf1 and Pax5 transcription in purified CD19+ cells from the Gli3 mutant FL compared to WT (Figure 4.7). The decrease in expression of both Ebf1 and Pax5 in the sorted Gli3-/- CD19+ cells was more pronounced than the reduction in transcript expression in the unsorted FL, and was also greater than the
proportional change in the CD19+ population in the Gli3-/ FL compared to WT. This was consistent with the action of Shh to reduce B cell development in FLOC, in which the magnitude of the reduction in Pax5 and Ebf1 transcription was greater than the change in the Ebf1+Pax5+ population (Figure 4.5A and 4.4C, respectively), and also greater than the magnitude of the change in the overall proportion of B lineage cells caused by Shh treatment (Figure 3.10A Chapter 3). Thus, Shh treatment in vitro, or Gli3-deficiency in vivo, not only reduced the proportion of CD19 cells that were present, but also repressed transcription of the key B lineage master regulators Ebf1 and Pax5 within the B lineage committed population, presumably by signalling to upregulate the transcription of an intermediate transcriptional repressor of Ebf1 and/or Pax5.

In order to investigate the relationship between Hh-mediated transcription and repression of Ebf1 and Pax5 I measured intracellular Ebf1 and Pax5 expression in rShh treated and control untreated GBS-GFP-transgenic FLOC. I found that >62% of Ebf1+Pax5- cells expressed high levels of GFP, indicating that this population actively mediates Hh-dependent transcription, which is therefore compatible with Ebf1 protein expression (Figure 4.8). These cells are the earliest B cell population that differentiate from the CLP stage (Egawa, Kawabata et al. 2001, Mebius, Miyamoto et al. 2001). Interestingly, GFP expression was reduced to <0.3% in the later Ebf1+Pax5+ population (Figure 4.8), indicating that Hh signalling decreases as cells become more mature,
and that once Pax5 is expressed very few cells are undergoing Hh-mediated transcription. This is consistent with Shh signalling acting to inhibit Pax5 transcription. The Ebf1+Pax5- population were Hh-responsive, as expression of GFP in the Ebf1+Pax5- population was increased on rShh treatment, with the proportion of GFP-negative cells decreasing from ~38% to ~28%. In contrast, GFP was not induced by rShh treatment in the Pax5+Ebf1+ population during the two day culture, and the proportion of GFP-negative cells remained >99.7% under both conditions.

4.3. Discussion

Our genome wide RNA-Sequencing data from the sorted CD19+B220+ population in the Gli3 mutants revealed many differentially expressed genes, between Gli3-/- and WT. Many Hh target genes (e.g. Stmn1, Hdac3, Hoxa4, Hmgb2, Bmi1 and Cul4a) were upregulated in the Gli3 mutants, consistent with the increased Hh-mediated transcription measured using the GBS-GFP reporter, and confirming that Shh signals directly to developing B cells.

In contrast, many B cell signalling pathway genes involved in NFkB (Nfkbid, Nfkbiz, Tnfaip3) activity, MAPK (Mapkapk2, Map3k3) signalling and members of the AP-1 family (Junb, Fosb) were decreased in the Gli3 mutant. These pathways are required for pre-BCR and BCR signalling, and survival and expansion during development (Feng, Cheng et al.
2004, de Gorter, Vos et al. 2007). In addition to regulating NF-kB activity, *Tnfaip3* has been shown to regulate marginal zone and B1 cell development in the adult (Chu, Vahl et al. 2011), and I observed a reduction in B1 progenitor cells in the E14.5 Gli3−/− FL. Interestingly, Hh-mediated transcription in developing and mature T cells also represses expression of genes that regulate activity of NF-kB, MAPK and AP1, leading to reduced pre-TCR and TCR signalling (Rowbotham, Hager-Theodorides et al. 2007, Rowbotham, Hager-Theodorides et al. 2009, Furmanski, Saldana et al. 2012, Furmanski, Saldana et al. 2013, Furmanski, Barbarulo et al. 2015).

In addition to the decreased transcription of genes associated with pre-BCR and BCR signalling, the RNA-Seq revealed a significant decrease in key transcriptional regulators of B cell differentiation including *Ebf1*, *Foxo1*, *Runx1* and *Irf4* (Dengler, Baracho et al. 2008, Niebuhr, Kriebitzsch et al. 2013). *Ebf1* is required from the early CLP stage to the late mature stages of B cell development (Roessler, Gyory et al. 2007, Nechanitzky, Akbas et al. 2013), and importantly activates transcription of another key master regulator of B cell development, Pax5. Pax5 then promotes Ebf1 transcription, creating a positive feedback loop, which supports all stages of B cell development (Roessler, Gyory et al. 2007).

Expression of the master regulators of B cell development, Ebf1 and Pax5 was reduced in the Gli3-deficient FL but increased in the Shh-deficient FL, and treatment of WT FLOC with Hhip to neutralise endogenous Hh molecules in the cultures increased the proportion of
Ebf1+Pax5+ cells and increased transcription of both Ebf1 and Pax5 within the CD19+ population, whereas rShh-treatment had the opposite effect. The Ebf1+Pax5- population showed high Hh-mediated transcription in the Hh-reporter transgenic FL, and I therefore proposed that Shh signalling within the Ebf1+Pax5- cells repressed expression of Pax5, thereby also reducing the Pax5-dependent induction of Ebf1 expression, leading to an overall reduction in B cell development.

I found high Hh pathway activity (measured by GFP expression in the Hh-reporter embryo) in Ebf1+Pax5- cells, but GFP expression ceased in the following Ebf1+Pax5+ population. Both Ebf1 and Pax5 protein and gene expression were inhibited by rShh-treatment and increased by neutralisation of Hh proteins by rHhip-treatment in FLOC, and manipulation of Hh signalling influenced the transition from Ebf1+Pax5- to Ebf1+Pax5+ cell. I therefore propose that Shh signalling to developing B cells functions to suppress Pax5 expression, which then breaks the positive feed-back loop, leading to reduction in Ebf1 expression. Interestingly, Shh signalling has been shown to interact with and regulate other Pax family members, in the development of other tissues (Chi and Epstein 2002, Blake and Ziman 2014).

Dysregulated Hh pathway activation is involved in some B cell malignancies (Dierks, Grbic et al. 2007, Lin, Wang et al. 2010), including B-ALL (Ramirez, Singh et al. 2012, Qu, Liu et al. 2013), a relatively common cancer of early childhood. An understanding of the function of Hh signalling in normal fetal B cell development, and its
effect on Pax5 and Ebf1 expression, will be important to our understanding of its role in B-ALL and other B cell malignancies. In the future it will be important to investigate how dysregulated Hh signalling influences Pax5 and Ebf1 activity in B-ALL.

4.4. Future Work

In the future, it would be important to investigate whether Shh binds to the Pax5 or Ebf1 to directly regulate their activity. I would like to test whether ectopic induction of Pax5 or Ebf1 increases FL B cell development in the Gli3-/- FL and reverse is seen when Pax5 or Ebf1 expression is repressed in the Shh-/- FL.

From the results, I propose that Shh acts via an intermediary to repress Pax5 or Ebf1. Therefore, it is important to investigate this intermediate gene and assess whether this intermediary is a direct target of Shh.

I would also like to examine whether Shh regulates adult bone marrow B cell development through the same transcriptional mechanisms as in the fetal liver. Finally, it will be important to study the role of Shh during human B cell development.
4.5 Summary

Taken together, I show that the Gli3 deficiency results in transcriptionally increased Hh signalling, which leads to decreased B cell development. Furthermore, loss of Shh in vitro and in vivo led to increased B lineage commitment and development transcription factors Ebf1 and Pax5. I propose that Shh signalling represses Pax5 (most likely by transcriptional activation of a transcriptional repressor of Pax5) and this leads to loss of Pax5’s induction of Ebf1, reduction in both Pax5 and Ebf1 expression, and to the negative regulation of B lineage development.
Figure 4.1 shows the positive feedback loop between E2a, Ebf1 and Pax5. The arrows show the positive upregulation of a transcription factor by its respective transcription factor. E2a upregulates Ebf1 and Ebf1 upregulates E2a. Ebf1 also upregulates itself and Pax5 and Pax5 upregulates Ebf1.
Figure 4.2. RNA sequencing detects transcriptional differences in Hh signalling genes and B cell differentiation and signalling genes between the WT and Gli3 mutant CD19+B220+ cells.
Figure 4.2 shows (A) PCA showing sample relationships in PC1 and PC3 for WT (n=2) and Gli3−/− (n=2) CD19+B220+ population from E17.5 FL. (B) Venn diagram: 1122 genes intersect out of the 3000 genes that contributed most to PC1 (highest and lowest scoring genes), and the 3000 most significant genes by Ebayes statistics. (C) Gene expression heatmap showing Hh signalling genes in blue and B cell differentiation and signalling genes in black. Normalised expression signals are represented on a log scale where green is lower expression and red higher expression levels. (D) Transcript expression (reads per million) of Pre-BCR/BCR signalling and target genes and (E) Shh, IL-7 and IL-7/TSLP signalling and target genes in the Gli3−/− (green) and WT (blue) RNA sequencing datasets.
Figure 4.3: Transcript expression of *Pax5* and *Ebf1* in the WT and Gli3 mutant fetal liver

Figure 4.3 shows representative experiment showing mean ± SEM (n = 3) of transcript expression by Q-RT-PCR of (A) *Pax5* and (B) *Ebf1* in Gli3 mutants (Gli3+/- and Gli3-/-) compared to WT littermates in the FL. All transcripts expression levels were normalised to *HPRT*.
Figure 4.4. Transcript expression of *Pax5* and *Ebf1* in the WT and Shh mutant fetal liver

Figure 4.4 shows representative experiment showing mean ± SEM ($n = 3$) of transcript expression by Q-RT-PCR of (A) *Pax5* and (B) *Ebf1* in Shh mutants (Shh+/− and Shh−/−) compared to WT littermates in the FL. All transcripts expression levels were normalised to *HPRT*. 
Figure 4.5. Expression of Pax5 and Ebf1 in rShh and rHhip treated WT FLOC

A

Day 2

WT Control  +Shh  +Hhip  Shh+Hhip

Day 4

WT Control  +Shh  +Hhip  Shh+Hhip

B

Day 2

Day 4

Ratio of Ebf1+Pax5+ : Ebf1+:Pax5-
Figure 4.5 shows a representative WT FLOCs treated with rShh, rHhip and rShh+rHhip for 2 and 4 days and compared to untreated control cultures, analysed by flow cytometry and Q-RT-PCR (n=3). (A) Dot plots show intracellular anti-Ebf1 and anti-Pax5 staining on day2 (upper panel) and day4 (lower panel). (B) Charts show the ratio of Ebf1+Pax5+:Ebf1+Pax5- cell in the different culture conditions as stated on day2 (upper chart) and day4 (lower chart). (C) Bar charts show Q-RT-PCR analysis of Pax5 and Ebf1 transcripts on day4 of WT FLOCs treated with rShh, or rHhip and control untreated.
Figure 4.6. Expression of Pax5 and Ebf1 in the Gli3-/- fetal liver

Figure 4.6 shows flow cytometry dot plots profile of intracellular anti-Pax5 and anti-Ebf1 staining in Gli3+/+ and Gli3-/- littermate FL cells.
Figure 4.7. Transcript expression of *Pax5* and *Ebf1* in WT and Gli3-/- cells purified CD19+ cells.

Figure 4.7 shows a representative experiment showing mean ± SEM (*n* = 3) of transcript expression of *Pax5* and *Ebf1* by Q-RT-PCR in purified CD19+ cells from Gli3+/+ and Gli3-/- E17.5 FL. All transcripts expression levels were normalised relative to *HPRT*. 
Figure 4.8. Presence of Hh signalling in WT and rShh treated Ebf1 and Pax5 expressing FL cells.

Figure 4.8 shows histograms showing GFP expression in Ebf1+Pax5- (top panel) and Ebf1+Pax5+ (bottom panel) cells in the E17.5 GBS-GFP-transgenic FLOC, control (solid lines) and rShh treated (dotted lines) FL, cultured for 2 days.
Chapter 5
Chapter 5. The role of Gli3 and Shh during DP to SP4 thymocyte development

5.1. Introduction

5.1.1 Thymocyte development at the DP to SP transition

Immature CD4-CD8- double negative (DN) thymocytes, migrate through the thymus acquiring signals for maturation to co-express the surface markers CD4 and CD8 to become Double Positive cells. DP cells differentiate and mature into either single positive (SP), SP4 (CD4+CD8-) or SP8 (CD4-CD8+). Maturation from DP to SP follows successful rearrangement of the TCRα locus, and requires TCR signalling: positive selection results in appropriate MHC-restriction of SP cells, followed by negative selection of potentially self-reactive clones (Starr, Jameson et al. 2003, Klein, Kyewski et al. 2014). Many models have been proposed to describe how DP thymocytes commit to SP4 and SP8 lineages, and how positive selection ensures that selected SP4 and SP8 populations express TCR appropriately restricted by MHCII and MHCI respectively (Starr, Jameson et al. 2003, Carpenter and Bosselut 2010). The strength and duration of the TCR signal that a developing cell receives broadly determine its
fate, with the strongest signals leading to negative selection, usually at the SP stage in the medulla (of TCR recognizing self antigens), intermediate signals leading to positive selection, and weaker signals or lack of TCR signalling leading to cell death by neglect (Singer, Adoro et al. 2008).

For DP thymocytes undergoing positive selection, again TCR signal strength and duration influence CD4 and CD8 fate decision, with those cells receiving stronger TCR signals tending towards the CD4 fate, while weaker/more transient signals favoring differentiation to CD8 SP (Starr, Jameson et al. 2003, Bosselut 2004, Klein, Kyewski et al. 2014). TCR signal strength and duration are dependent on avidity of the TCR for its ligand (and therefore on the TCR sequence), but may also be modulated by other factors, such as coreceptor signalling, and intracellular or extracellular influences on TCR signal transduction. Thus, local thymic stromal influences, including cytokine, Notch and morphogen signalling may also influence SP lineage choice and selection (Brugnera, Bhandoola et al. 2000, Takahama 2006, Crompton, Outram et al. 2007, Laky and Fowlkes 2008, Park, Adoro et al. 2010). In addition, several transcription factors are required for the SP4/SP8 lineage decision, including Th-POK, Gata3, Runx1, Runx3, and Sil4 (Carpenter and Bosselut 2010, Naito, Tanaka et al. 2011).
5.1.2 The transcription factor Gli3 and thymocyte development

As discussed in Chapter 1, the transcription factor Gli3 can act as a repressor of transcription (Gli3R) in the absence of Hh signalling, or an activator (Gli3A) on Hh signal transduction (Sasaki, Nishizaki et al. 1999). In the fetal thymus, Gli3 mainly acts a repressor of Hh signalling \textit{in vivo} (Hager-Theodorides, Dessens et al. 2005). Gli3R can repress the Hh signal transduction by decreasing the expression of \textit{Hh} genes in the Hh-secreting cell, therefore decreasing the overall Hh protein concentration. This decreased Hh protein concentration allows Gli3 in the signal-receiving cell to be processed into Gli3R, which further transcriptionally represses Hh target genes (te Welscher, Zuniga et al. 2002, Hager-Theodorides, Dessens et al. 2005).

In the thymus stroma, Gli3 has both Hh-independent and Hh-dependent functions, and Gli3-deficiency leads to Hh-dependent upregulation of the Hh-target gene, \textit{Gli1} (Hager-Theodorides, Furmanski et al. 2009). In the fetal thymus (FT), Gli3-deficiency and Shh-deficiency both lead to reduced differentiation from DN1 to DN2 cell, suggesting that Gli3A is downstream of Shh at this developmental stage (Shah, Hager-Theodorides et al. 2004, Hager-Theodorides, Dessens et al. 2005). However at the pre-TCR induced transition from DN to DP, Gli3-deficiency results in reduced differentiation, whereas Shh-deficiency leads to the opposite phenotype (Hager-Theodorides, Dessens et al. 2005, Rowbotham, Hager-Theodorides et al. 2009).
Previous studies have shown that Gli3 is expressed in thymic epithelial cells (TEC) and fetal thymocytes, and is required for pre-TCR induced differentiation from DN to DP cell, but the impact of Gli3-deficiency on differentiation from DP to SP cell is not known (Hager-Theodorides, Dessens et al. 2005, Saldana, Solanki et al. 2016). Here I investigate the function of Gli3 during T cell development in the fetal thymus at the transition from CD4+CD8+ double positive (DP) to CD4 single positive (SP4) and CD8 single positive (SP8) cell.

**5.2. Chapter Objectives**

In this chapter, I aim to test the hypothesis that Gli3 regulates thymocyte differentiation at the DP to SP transition. I will assess the changes in different cell surface markers of thymocyte maturity in the Gli3 mutant thymus. Since Gli3 is a repressor of Hh signalling, the Gli3 mutant thymus will be used to test both the Hh-independent-Gli3-dependent and the Hh-dependent changes during thymocyte development. I will first assess the presence of the Hh activity in both the WT and Gli3 mutant thymocytes and then assess Hh-dependent changes in the Gli3 mutant by neutralising Hh signalling in FTOCs.

I will also test whether Gli3 activity in the thymic epithelial cells (TEC) or thymocyte-intrinsic Gli3 activity regulates thymocyte differentiation and maturation. I will use conditional knockouts to specifically delete Gli3 from the hematopoietic lineage in the Gli3fl/fl,VavCre+ mice, from CD4
positive cells in the Gli3fl/fl,CD4Cre+ mice, and from TEC in the Gli3fl/fl,FoxN1Cre+. To assess the Hh-dependent changes in the Gli3 conditional mutant, I will neutralise Hh in Gli3fl/fl,FoxN1Cre+ FTOCs. I will also use Shhfl/fl,FoxN1Cre+, where Shh is conditionally deleted from the TEC, to assess Shh-dependent changes in vivo and examine whether the Shhfl/fl,FoxN1Cre+ shows an opposite phenotype to the Gli3fl/fl,FoxN1Cre+ conditional mutant.

5.3. Results

5.3.1 Impaired development of mature SP4 T-cells in the Gli3-mutant thymus

Our group has previously shown that Gli3-deficiency decreases the pre-TCR induced transition from DN to DP (Hager-Theodorides, Dessens et al. 2005). Here, I investigate the role of Gli3 at the next stage of thymocyte differentiation, at the DP to SP transition. Since the Gli3 deficiency is embryonic lethal, I used fetal thymus organ cultures (FTOC) to analyse changes in the thymocyte development in the Gli3 mutant fetal thymus. I cultured WT and Gli3-mutant E17.5 FTOC for 4 days and then assessed changes in developmentally regulated cell surface markers. This culture period enabled us to measure the rate of differentiation of the mature SP populations, as they are first produced. I observed a significant gene-dose dependent decrease in the proportion of SP4 cells and in the SP4:SP8 ratio in the Gli3-mutant FTOC
compared to WT, and the proportion of CD4-CD8+ cells was significantly increased (Fig 5.1A-B). This suggests that efficient differentiation from DP to SP4 cells required Gli3, and that Gli3-deficiency favoured lineage commitment to SP8 over SP4. However, as the fetal CD4-CD8+ population also contains immature single positive cells (ISP), I gated on the CD3hi population, and analysed the distribution of DP and SP thymocytes. Gating on CD3hi, confirmed the requirement for Gli3 for normal differentiation from CD3hiDP to SP4 cell, as the proportion of DP cells was significantly increased, and proportion of SP4 cells was significantly decreased, but showed that the proportion of the CD3hiSP8 population was not significantly different between the three genotypes of embryo (Fig 5.2A-B).

In order to dissect further the stages of maturity affected by Gli3, I stained FT against the surface markers CD69, HSA and Qa2. DP thymocytes express high levels of HSA and then acquire CD69 expression as a result of TCR signalling for positive selection (Ge and Chen 1999). Newly positively-selected SP thymocytes also express high levels of HSA and CD69, and as they mature they down-regulate HSA and CD69 and gain expression of Qa2 (Ge and Chen 1999, Weinreich and Hogquist 2008).

The proportions of mature HSA-Qa2+ cells in the SP4 and the SP8 populations were significantly decreased in Gli3-/- compared to WT (Fig 5.3A-B). Thus, the Gli3-/- FTOC contained overall fewer SP4 cells, and a lower proportion of mature HSA-Qa2+ SP4 cells, and although the
overall proportion of CD3$^{hi}$SP8 cells was not decreased, the SP8 population was less mature.

CD69 expression was significantly decreased on DP, SP8 and SP4 thymocytes in the Gli3$^{-/-}$ FT compared to WT (Fig 5.4A-B), indicating that fewer DP cells were undergoing positive selection, and consistent with the overall reduction in SP maturation.

Positive and negative selection and SP4/8 lineage commitment are determined by many factors including transcriptional regulators of TCR signal transduction and the TCR signal strength itself. A stronger TCR signal strength promotes differentiation towards SP4, while a weaker signal favours SP8 (Bosselut 2004). Since Gli3 deficiency suppressed lineage commitment towards SP4 cells and biased the SP4:SP8 ratio I investigated whether the TCR signal strength was affected in the Gli3 mutant FTOC. High cell surface CD5 expression correlates with a stronger TCR signal and vice versa (Azzam, DeJarnette et al. 2001). I therefore analysed the mean fluorescence intensity (MFI) of anti-CD5 staining on both DP and SP cells. The MFI of CD5 on the DP, SP4 and SP8 cells and the mature CD3$^{hi}$ DP and SP cells was significantly decreased in the Gli3$^{-/-}$ FT compared to WT (Fig 5.5A-C). This suggested that reduced TCR signal strength may be one factor responsible for the decreased commitment to SP4 in the Gli3-mutant.
5.3.2 Increased Shh signalling in Gli3-mutant thymocytes

I next wanted to assess whether the changes in the Gli3 mutant FT were due to the increased Hh signalling. Our group has previously shown that the Gli3 mutant FT has increased expression of the Hh-target gene Gli1 in stroma and thymocytes (Hager-Theodorides, Furmanski et al. 2009, Drakopoulou, Outram et al. 2010), indicating that overall Gli3 acts as a repressor of Hh pathway activation in the FT. Since Gli3 can repress Shh expression by repression of an intermediate transcriptional activator of Shh in other tissues (te Welscher, Fernandez-Teran et al. 2002, Ahn and Joyner 2004), and Shh is the key Hh protein expressed by TEC (Outram, Varas et al. 2000, Shah, Hager-Theodorides et al. 2004, Rowbotham, Hager-Theodorides et al. 2007, Saldana, Solanki et al. 2016), I tested whether more Shh protein was present in the Gli3-deficient FT compared to WT by ELISA. Our results show that Shh protein was significantly increased in Gli3-/- FT compared to WT (Fig 5.6).

Subsequently, to test if Shh was signalling directly to developing T-cells, I used Gli binding site (GBS)-GFP transgenic (tg) reporter mice to measure active Hh-dependent transcription in DP, SP4 and SP8 populations in the Gli3-mutant FT (Fig 2B-C). The GBS-GFP-tg express GFP when activator forms of Gli proteins bind to the GBS in the transgene (Balaskas, Ribeiro et al. 2012). I observed an increase in GFP expression in DP, SP4 and SP8/ISP populations in GBS-GFP-tgGli3-/- and
+/- compared to GBS-GFP-tgGli3+/+ (Fig 5.7A-B). The MFI of GFP on the SP4, DP and SP8/ISP populations was also significantly higher in Gli3-/- FT compared to WT (Fig 5.7C). Gating on CD3hi thymocytes, the proportion of GFP-positive mature CD3hiSP4 cells was significantly higher in the Gli3-mutant FT compared to Gli3+/+, and the MFI of GFP in CD3hiDP and ISP/SP8 cells was also significantly higher (Fig 5.7D-E). This increase in GFP expression in Gli3-mutant thymocytes showed that Hh pathway activation is increased in the developing thymocytes of the Gli3-mutant thymus and that the greater Shh protein level is signalling directly to developing T-cells.

5.3.3 Attenuation of Hh signalling in Gli3-mutant FT reverses the decrease in SP4 T cell maturation

Since the Gli3-deficient FT has increased Hh signalling, loss of Gli3 in the thymus could cause changes that are directly dependent on the increase in the Hh signal, or alternatively that are dependent on Gli3 but independent of the increase in Hh pathway activation. Therefore, to investigate whether the differences in the Gli3-mutant FT were directly due to increased Hh signalling, I attenuated Hh signalling by using the Hh binding protein rHhip to neutralise endogenous Hh proteins in FTOC. rHhip-treated WT FTOC had a higher proportion of SP4 and SP8 cells but a decreased percentage of DP than untreated controls (Fig 5.8A-B). rHhip-treated Gli3-/- FTOC had a significantly higher proportion of SP4 cells (Fig 5.8A-B). The mature CD3hiSP4 and CD3hiSP8 populations were significantly increased and the CD3hiDP population
decreased in the rHhip-treated WT FTOC relative to their controls, whereas in the rHhip-treated Gli3-/- FTOC, although the mean proportional change in both CD3hiSP4 and CD3hiSP8 populations was greater than in the WT, only the increase in the CD3hiSP4 was significant, because of greater variation in the CD3hiSP8 population (Fig 5.9A-B). This increased variability in response to Hh-neutralisation suggested that Gli3 may be required for normal interpretation of changes in the Hh signal.

Neutralisation of Hh proteins also increased cell surface CD5 expression in WT FTOC (Fig 5.10A-C). As expected, highest cell surface CD5 expression was observed in the SP4 population in all cultures, and gating on CD3hi cells showed that the CD3hiDP populations expressed intermediate levels of cell surface CD5 between that of the CD3hiSP4 and CD3hiSP8 populations (Fig 5.10A). Interestingly, rHhip-treatment significantly increased the MFI of CD5 on the CD3hiDP populations in the Gli3-/-FTOC, whereas in WT FTOC MFI of CD3hiSP4 and CD3hiSP8 populations were significantly increased (Fig 5.10C).

These experiments suggest that the decrease in SP4 differentiation in the Gli3-/- FTOC at this developmental transition is the direct result of the increase in Shh, but that Gli3 may also be required to respond to changes in the Shh signal. Consistent with this, previous studies showed that Shh-treatment of WT FTOC decreases the SP4 population, the SP4:SP8 ratio and cell surface CD5 expression, most likely by decreasing TCR signal strength, and in mature T cells constitutive
activation of Gli2-mediated transcription reduces TCR signal transduction (Rowbotham, Hager-Theodorides et al. 2007, Furmanski, Saldana et al. 2012, Furmanski, Barbarulo et al. 2015). In contrast, loss of Shh, Gli1 or Gli2 from the FT increases differentiation from the DP to SP stage (Rowbotham, Hager-Theodorides et al. 2007, Drakopoulou, Outram et al. 2010).

5.3.4 Gli3 expression in TEC plays a key role in T-cell development

Gli3 is expressed in fetal TEC and fetal thymocytes, but not adult thymocytes (Hager-Theodorides, Dessens et al. 2005, Barbarulo, Lau et al. 2016, Saldana, Solanki et al. 2016). Shh is expressed in TEC, which also transduce Hh signals (Barbarulo, Lau et al. 2016, Saldana, Solanki et al. 2016). Therefore, I tested if the changes in thymocyte selection and maturation were the result of the activity of Gli3 expressed in TEC, rather than cell-intrinsic Gli3 activity in developing thymocytes. I analysed thymocyte development in four conditional knockouts: Gli3fl/flFoxN1Cre+, in which Gli3 is conditionally deleted from TEC; Gli3fl/flVavCre+, in which Gli3 is specifically deleted from all hematopoietic cells including all thymocytes; Gli3fl/flCD4Cre+, in which Gli3 is conditionally deleted from the CD4+ thymocytes (from DP stage onwards); and Shhfl/flFoxN1Cre, in which Shh is conditionally deleted from TEC. Interestingly, most of the changes observed in the Gli3-deficient thymus were due to loss of Gli3 expression from TEC. The conditional deletion of thymocyte intrinsic Gli3 in the Gli3fl/flVavCre+
and Gli3fl/flCD4Cre+ did not result in significant changes in differentiation from DP to SP4 or SP maturation (Fig 5.11 and Fig 5.12). However, conditional deletion of Gli3 from TEC in Gli3fl/flFoxN1Cre+ mice resulted in a significant decrease in the proportion of SP4 cells and the SP4:SP8 ratio, while the proportion of DP cells significantly increased compared to the Cre- littermate control (Fig 5.13A-B). The proportion of mature CD3<sup>hi</sup> SP4 and the CD3<sup>hi</sup> SP4:SP8 ratio was also significantly decreased in the Gli3fl/flFoxN1Cre+ (Fig 5.13A and 5.13C), and the cell surface CD5 expression significantly decreased in the DP population from Gli3fl/flFoxN1Cre+ FTOC compared to Cre- littermate (Fig 5.13D).

Since constitutive loss of Gli3 in the FT led to Hh-dependent changes in thymocyte differentiation and maturation, I tested whether the changes that resulted from conditional deletion of Gli3 specifically from TEC were also Hh-dependent. I treated the Gli3fl/flFoxN1Cre+ FTOC with rHhip and observed a significant increase in the SP4 population and a significant decrease in the DN population compared to untreated FTOC (Fig 5.14A-B). Gating on CD3<sup>hi</sup> cells also showed that the rHhip treated Gli3fl/flFoxN1Cre+ FTOC had an increased proportion of SP4 and a decreased proportion of CD3<sup>hi</sup>DP (Fig 5.14A). The MFI of CD5 on SP4 and DP cells in the rHhip treated Gli3fl/flFoxN1Cre+ FTOC was significantly increased compared to untreated control (Fig 5.15A-B). This was in contrast to the effect of rHhip treatment on constitutive Gli3-/- FTOC, in which although rHhip-treatment increased the
proportion of SP4 cells (Fig 5.8), it only increased the MFI of anti-CD5 staining in the CD3<sup>hi</sup>DP population (Fig 5.10C). As thymocytes in the Gli3fl/flFoxN1Cre+ FTOC express Gli3, whereas those in the Gli3+/− FTOC do not, this difference indicates that Gli3 activity in developing fetal thymocytes is also required for interpretation of the changes in the Shh signal, when the high Shh signal caused by Gli3-deficiency is neutralised by rHhip-treatment.

Interestingly, conditional deletion of Shh from TEC (Shhfl/flFoxN1Cre+) in vivo caused a significant increase in the proportion of SP4 cells and the SP4:SP8 ratio, but a significant decrease in the DP population compared to Cre- control FTOC (Fig 5.16A-B). The proportion of mature CD3<sup>hi</sup>SP4 was also significantly higher while the proportion of CD3<sup>hi</sup> DP was significantly lower in Shhfl/flFoxN1Cre+ FTOC compared to Cre-controls (Fig 5.16A and 5.16C). Treatment of Shhfl/flFoxN1Cre+ FTOC with rShh reversed this effect and reduced differentiation to SP4 and CD3<sup>hi</sup>SP8 but increased the proportion of CD3<sup>hi</sup>DP cells (Fig 5.17A-C).

Taken together, these experiments showed that most of the changes in the Gli3-deficient thymus are due to the increase in Shh and that Gli3 expression in TEC is necessary for normal physiological levels of Shh. Neutralisation of Shh by rHhip-treatment in the Gli3fl/flFoxN1Cre+ thymus reversed the phenotype at the DP to SP4 transition, consistent with the effect of conditional deletion of Shh from TEC in the Shhfl/fl, FoxN1Cre+ thymus. This indicates that Gli3 expression in TEC is
necessary for positive selection and maturation of SP4 thymocytes and that it acts by repression of Shh expression.

5.4 Discussion

Here I showed that expression of the transcription factor Gli3 in TEC is necessary for normal differentiation from DP to mature SP4 thymocyte in the FT. Constitutive deletion of Gli3 from the FT reduced differentiation and maturation of SP4 T cells. This reduction in SP4 differentiation was also seen when Gli3 was conditionally deleted from TEC only, but not when Gli3 was conditionally deleted from thymocytes. Gli3 repressed expression of Shh and analysis of the Hh-reporter line showed that the Hh signalling pathway was active in developing thymocytes, and that activation of the pathway in thymocytes was increased when Gli3 was deleted. DP and SP thymocyte populations from the Gli3-deficient FT had reduced levels of cell-surface CD5, indicative of lower TCR signalling, and consistent with the fact that rShh-treatment of WT FTOC and constitutive activation of Hh-mediated transcription both reduced cell surface CD5 expression (Rowbotham, Hager-Theodorides et al. 2007, Furmanski, Saldana et al. 2012). Taken together with experiments showing that conditional deletion of Shh from TEC increased differentiation from DP to SP, and that differentiation from DP to SP4 in the Gli3-mutant FT was restored by neutralisation of endogenous Hh proteins, these findings support the model that Gli3 activity in TEC promotes SP4 T-cell development by
repression of Shh, which signals directly to developing T cells to reduce TCR signal strength.

Gli3 is expressed in fetal but not adult thymocytes (Hager-Theodorides, Dessens et al. 2005), and although conditional deletion of Gli3 from thymocytes did not significantly influence the proportions of DP and SP populations, our experiments revealed that Gli3 activity in thymocytes is required for normal interpretation of changes in the Hh signal.

In the constitutive Gli3-/- thymus where increased Shh signalling to developing thymocytes reduced differentiation from DP to SP, although neutralisation of the Hh signal with recombinant Hhip-treatment was able to restore the proportion of the SP4 population, it only significantly increased the cell surface expression of CD5 in the CD3hiDP population. In contrast to the constitutive Gli3-/- thymus, in Gli3fl/flFoxN1-Cre+ FTOC rHhip-treatment increased cell surface CD5 expression on SP4 and CD3hiDP populations, suggesting that the ability of developing thymocytes to respond to the decrease in the Shh signal on rHhip-treatment requires thymocyte intrinsic Gli3 activity.

Interestingly, the conditional deletion of Shh from TEC (Shhfl/flFoxN1-Cre+ FTOC) showed opposite results to Gli3fl/flFoxN1-Cre+ FTOC. The Shhfl/flFoxN1-Cre+ FTOC had an accelerated DP to SP4 transition, and the mature CD3hi SP4 was significantly higher. These changes were reversed when the Shhfl/flFoxN1-Cre+ FTOC was treated with Shh, suggesting that most of these changes in Gli3fl/flFoxN1-Cre+ FTOC were due to the increase in Shh. I suggest that Gli3 activity in the TEC
regulates positive selection and maturation of SP4 thymocytes by repressing of the expression of Shh.

5.5 Future Work

Our results show that the Gli3 regulates the TCR signal and affects differentiation and selection to the SP4 lineage. I would like to investigate whether changes in thymic selection in the Gli3 mutant thymus also affect the final TCR repertoire in the Gli3 mutants. It would also be interesting to sort thymocytes undergoing Hh signalling during the DP to SP4 transition and perform an RNA-Sequencing analysis on these to identify Shh regulated transcription factors. I would also like to investigate importance of Gli3 and Shh in human thymocyte development.

5.5 Summary

Here, I showed that Gli3 is a transcription factor expressed by TEC that is required for normal SP4 T-cell development in the fetal thymus. Gli3 deficiency increases Shh expression and Shh activity in the thymus, which negatively regulates thymocyte differentiation from the DP to SP4 transition. The neutralisation of Shh in the Gli3 mutant thymus reverses this negative regulation, suggesting that Gli3 is essential for the DP to SP4 maturation.
Figure 5.1. DP to SP thymocyte development in Gli3+/+, Gli3+/− and Gli3−/− E17.5+4 Days FTOCs

Figure 5.1 shows flow cytometry analysis of E17.5 FTOCs for 4 days from Gli3+/+, Gli3+/− and Gli3−/−. (A) Dotplots showing CD8 against CD4. (B) Bar chart showing mean percentage of FT populations, relative to mean of WT littermates ±SEM, giving significance by student’s t-test compared to WT for: (B) SP4 (Gli3+/− p<0.03; Gli3−/− p<0.003); SP8+ (p<0.03); SP4:SP8 ratio (Gli3+/− p<0.02; Gli3−/− p<0.0006) for Gli3+/− (n=25), Gli3−/− (n=14) Gli3+/+ (n=15).
Figure 5.2. Mature CD3+ DP to SP thymocyte development in Gli3+/+, Gli3+/- and Gli3-/- E17.5+4 Days FTOCs

Figure 5.2 shows flow cytometry analysis of E17.5 FTOCs for 4 days from Gli3+/+, Gli3+/- and Gli3-/- (n=25), Gli3-/-(n=14) Gli3+/+(n=15).

(A) Dotplots showing CD8 against CD4 gated on CD3 High. (B) Bar chart showing mean percentage of FT populations, relative to mean of WT littermates ±SEM, giving significance by student’s t-test compared to WT for: (D) CD4 and CD8 gated on CD3^hi, CD3^hiSP4 (Gli3+/- p<0.002, Gli3-/- p<0.004); CD3^hiSP4:SP8 ratio (Gli3-/- p<0.002); CD3^hiDP (Gli3-/- p<0.05, Gli3/- p<0.02) for Gli3+/- (n=25), Gli3-/- (n=14) Gli3+/+ (n=15).
Figure 5.3. Percentage of HSA and Qa2 on SP thymocytes in Gli3+/+, Gli3+-/ and Gli3-/- E17.5+4 Days FTOCs

Figure 5.3 shows (A) Dotplots show HSA and Qa2 expression gated on SP8 and SP4 cells from Gli3+/+ and Gli3-/-.

(B) Bar chart showing relative mean percentage of FT populations ±SEM, giving significance by student’s t-test compared to WT for: HSA-QA2+ in SP8 (p<0.0007); HSA-QA2+ in SP4 (p<0.04); For Gli3-/-(n=14) and Gli3+/+(n=15).
Figure 5.4. Percentage of DP and SP CD69+ thymocytes in E17.5 Gli3+/+, Gli3+-/ and Gli3-/-

Figure 5.4 shows (A) Histogram: CD69 expression on SP4, DP and SP8 from E18.5 Gli3+/+ and Gli3+-/ FT. (B) Bar chart: mean percentage of CD69+ cells in FT populations, relative to mean of WT ±SEM, for SP4 (p<0.07); DP (p<0.04); SP8 (p<0.04): For Gli3+/+ (n=3); Gli3-/- (n=3).

A

Gli3 +/+  Gli3 -/-

SP4

41.1%  20.4%

DP

7.16%  3.87%

SP8

2.54%  1.79%

CD69

B

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Gli3+/+, Gli3+-/ and Gli3-/-
Figure 5.5. Mean Flourescence Intensity of CD5 on DP and SP thymocytes in in Gli3+/+, Gli3+-/ and Gli3--/ E17.5+4 Days FTOCs
Figure 5.5 shows (A) Histograms: anti-CD5 staining on E17.5 DP, SP4, and SP8 populations, showing MFIs. Solid line: Gli3+/+; Dashed line: Gli3−/−. Barcharts: (B) Relative mean MFI of CD5 on SP4 cells (Gli3+/− p<0.007; Gli3−/− p<0.004); DP cells (p<0.001); SP8 cells (p<0.004). (C) Relative mean MFI of CD5 gated on CD3hi SP4 cells (Gli3−/− p<0.04); CD3hiDP cells (Gli3−/− p<0.004); CD3hiSP8 cells (Gli3−/− p<0.01) from E17.5: Gli3+/− (n=25), Gli3−/− (n=14) Gli3+/+ (n=15).
Figure 5.6 Shh protein concentration in WT and Gli3-/- E17.5 fetal thymus

Figure 5.6 shows bar chart showing Shh protein measured by ELISA in Gli3+/+ and Gli3-/- E17.5 FT (p<0.003; Gli3+/+ n=3, Gli3-/- n=3).
Figure 5.7. Presence of active Hh signalling in the DP and SP thymocytes

A

Gli3+/+  Gli3+-  Gli3-/-

SSC

GFP on CD4+CD8-

2.74%  4.38%  5.93%

GFP on CD4+CD8+

1.15%  1.94%  1.82%

GFP on CD4-CD8+

2.50%  3.82%  3.13%

B

Percentage of GFP+ thymocytes (Relative to Control)

* *

SP4  DP  SP8

1.5  3  3.5
Figure 5.7 shows (A-E) Flow cytometry analysis of GFP expression in GBS-GFPtg E17.5 FTOC + 4 days from Gli3+/+, +/- and -/- littermates. (A) Dotplots: SSC v GFP, gated on SP4, DP and SP8 cells Gli3+/+ (left), Gli3+/− (middle) and Gli3−/− littermates (right). (B-E) Bar charts: relative mean percentage ±SEM. (B) Mean percentage of GFP+ in SP4, DP (Gli3+/− p<0.02, Gli3−/− p<0.04); SP8 (Gli3+/− p<0.05, Gli3−/− p<0.05). (C) Mean of MFI of GFP in SP4 (Gli3+/− p<0.02 and Gli3−/− p<0.04); DP (Gli3−/− p<0.04); SP8 (Gli3−/− p<0.02). (D) Relative mean percentage of GFP+ cells for CD3hiSP4 (Gli3+/− p<0.04, Gli3−/− p<0.05); CD3hiDP; CD3hiSP8. (E) Relative mean of MFI of GFP in SP4, DP (Gli3−/− p<0.02); SP8 (Gli3+/− p<0.02, Gli3−/− p<0.04) gated on CD3hi from n=4 Gli3+/−; n=3 Gli3−/−; n=3 Gli3+/+. 
Figure 5.8. Changes in DP and SP thymocytes through the attenuation of Hh signalling by rHhip-treatment of WT and Gli3-/- FTOCs

A

B

SP4

SP8

DP

WT

Gli3-/-
Figure 5.8 shows (A-B) WT and Gli3-/- E17.5 FTOC treated with rHhip for 4 days analysed by flow cytometry (WT n=4 and Gli3-/- n=6). (A) Dotplots showing CD8 versus CD4 in control (untreated) and rHhip-treated FTOC (upper panel=WT, lower panel=Gli3-/-). (B) Bar charts: mean ±SEM, relative to mean of control untreated FTOC from same thymus (one lobe treated, and one lobe untreated), showing significance by student’s t-test for rHhip treated SP4 (WT p<0.05, Gli3-/-p<0.03); SP8 (WT p<0.002); DP (WT<0.006).
Figure 5.9. Changes in mature CD3\textsuperscript{hi} DP and SP thymocytes through the attenuation of Hh signalling by rHhip treatment of WT and Gli3\textsuperscript{-/-} FTOCs
Figure 5.9 shows (A-B) WT and Gli3-/- E17.5 FTOC treated with rHhip for 4 days analysed by flow cytometry (WT n=4 and Gli3-/- n=6). (A) Dotplots showing CD8 versus CD4 gated on CD3^hi in control (untreated) and rHhip-treated FTOC (upper panel=WT, lower panel=Gli3-/-). (B) Bar charts: mean ±SEM, relative to mean of control untreated FTOC from same thymus (one lobe treated, and one lobe untreated), showing significance by student’s t-test for rHhip treated in CD3^hiSP4 (WT p<0.04); CD3^hiSP8 (p<0.05); CD3^hiDP (p<0.05).
Figure 5.10. Changes in the MFI of CD5 in DP and SP thymocytes through the attenuation of Hh signalling by rHhip-treatment of WT and Gli3-/- FTOCs
Figure 5.10 shows (A-C) WT and Gli3-/- E17.5 FTOC treated with rHhip for 4 days analysed by flow cytometry (WT n=4 and Gli3-/- n=6). (A) Scatter plots: MFI of CD5 on SP4, DP and SP8 cells and in same populations gated on CD3^hi from a representative experiment. Shaded circles: WT control. Open circles: rHhip-treated WT. Shaded triangles: Gli3-/- control. Open triangles: rHhip-treated Gli3-/. (B-C) Bar charts: mean ±SEM, relative to mean of control untreated FTOC, showing significance by student’s t-test for rHhip-treated FTOC. (F) MFI of CD5 on SP4 (WT p<0.05); SP8; DP cells. (G) MFI of CD5 on CD3^hiSP4 (WT p<0.002); CD3^hiSP8 (WT p<0.0001); CD3^hiDP (Gli3-/- p<0.05).
Figure 5.11. Conditional deletion of thymocyte-intrinsic Gli3 does not affect SP4 development.

Figure 5.11 shows flow cytometry of E17.5 FTOC + 4 days from Gli3fl/flVavCre- and Gli3fl/flVavCre+ littermates. There were no significant differences by student’s t-test in SP4, SP8, DP and the SP4:SP8 ratio between Cre- (n=7) and Cre+ (n=6).
Figure 5.12. Conditional deletion of Gli3 from CD4 thymocytes does not affect DP to SP4 development.

Figure 5.12 shows flow cytometry of E17.5 FTOC + 4 days from Gli3fl/flCD4Cre- and Gli3fl/flCD4Cre+ littermates. Dotplots: Upper panel shows CD8 against CD4; Lower panel shows CD8 against CD4 gated on CD3^{hi}. There were no significant differences by student's t-test in SP4, SP8, DP and the SP4:SP8 ratio between Cre- (n=7) and Cre+ (n=6).
Figure 5.13. Conditional deletion of Gli3 from TEC decreases SP4 differentiation

A

Gli3fl/flFoxN1Cre-  Gli3fl/flFoxN1Cre+

B

Percentage of thymocytes (Relative to Control)

SP4  DP  SP8  SP4:SP8

*  **  *

197
Figure 5.13 shows flow cytometry analysis of E17.5 FTOC + 4 days from Gli3fl/flFoxN1Cre- and Gli3fl/flFoxN1Cre+ littermates. Dot plots: (A) Upper Panel = CD8 against CD4; Lower Panel = CD8 against CD4 gated on CD3<sup>hi</sup>. (B-C) Bar charts: mean percentage of FT populations, relative to mean of WT littermates ±SEM, giving significance by student’s t-test compared to WT littermate for Upper Panel = SP4 (p<0.02); DP (p<0.007); SP4:SP8 ratio (p<0.05); Lower Panel = CD4 and CD8 for CD3<sup>hi</sup>SP4 (p<0.008); CD3<sup>hi</sup>SP4:SP8 ratio (p<0.001). (C) Relative mean MFI of CD5 on SP4, DP (p<0.007) and SP8 cells for Cre- (n=7) and Cre+ (n=12).
Figure 5.14. Attenuation of Hh signalling in the Gli3fl/fl, FoxN1Cre conditional mutant increases SP4 development.
Figure 5.14 shows flow cytometry analysis of Gli3fl/flFoxN1Cre+ E17.5 FTOC treated with rHhip for 4 days. (A) Dotplot: Upper Panel = CD8 against CD4; Lower Panel = As in H but gated on CD3^hi. (B) Bar chart showing mean ±SEM, relative to the mean of control untreated FTOC from Gli3fl/flFoxN1Cre+ (n=8) FTOC +4 days, showing significance by student's t-test for rHhip-treated versus control untreated: (I) SP4 (p<0.02), DP, SP8; DN (p<0.006).
Figure 5.15. Attenuation of Hh signalling in the Gli3fl/fl, FoxN1Cre conditional mutant increases mean fluorescence intensity of CD5

Figure 5.15 shows flow cytometry analysis of Gli3fl/flFoxN1Cre+ E17.5 FTOC treated with rHhip for 4 days. Bar chart showing mean ±SEM, relative to the mean of control untreated FTOC from Gli3fl/flFoxN1Cre+ (n=8) FTOC +4 days, showing significance by student’s t-test for rHhip-treated versus control untreated: (A) Relative mean of MFI of rHhip-treated SP4 (p<0.02); DP (p<0.007); SP8 cells. (B) Gated on CD3^hi, relative mean of MFI of rHhip-treated CD3^hiSP4 (p<0.01); CD3^hiDP (p<0.05); CD3^hiSP8.
Figure 5.16. Conditional deletion of Shh from TEC increases SP4 differentiation
Figure 5.16 shows flow cytometry analysis of E17.5 FTOC + 4 days from Shhfl/flFoxN1Cre- and Shhfl/flFoxN1Cre+ littermates. Dotplots: (A) CD8 against CD4 (upper panel); gated on CD3^{hi} (lower panel). (B-C) Bar charts: mean percentage of FT populations, relative to mean of WT littermates ±SEM, giving significance by student’s t-test compared to WT littermate for: (B) SP4 (p<0.02); DP (p<0.04); SP4:SP8 ratio (p<0.05). (C) CD4 and CD8 gated on CD3^{hi}; CD3^{hi}SP4 (p<0.003); CD3^{hi}DP (p<0.002) for Cre- (n=4) and Cre+ (n=6).
Figure 5.17. Addition of rShh to Shhfl/fl, FoxN1Cre mutant decreases SP4 differentiation

A

<table>
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<th>Shhfl/fl FoxN1Cre- Control</th>
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<td>27.1%</td>
<td>64.6%</td>
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<td>4.26%</td>
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B

Percentage of thymocytes treated with Shh (Relative to Untreated Control)

- SP4
- DP
- SP8

* Shhfl/fl FoxN1Cre+ Control
* Shh treated Shhfl/fl FoxN1Cre+
Figure 5.17 shows flow cytometry analysis of E17.5 Shhfl/flFoxN1Cre+ FTOC treated with 1μg/ml rShh for 4 days compared to control untreated Shhfl/flFoxN1Cre+ (n=6). Dot plots (A) CD8 against CD4 staining of FT populations (upper panel); gated on CD3\textsuperscript{hi} (lower panel). (E) Bar chart: mean relative proportion of thymocyte populations SP4 (p<0.04), DP and SP8 in rShh treated (shaded bars) and control untreated (open bars). (F) Bar chart: mean relative percentage, gated on CD3\textsuperscript{hi}, for DP (p<0.02) and SP8 (p<0.007) populations from rShh-treated (shaded bars) and control (open bars).
Chapter 6
Chapter 6. The transcriptional mechanisms regulating DP to SP4 thymocyte development in the Gli3 mutant thymus

6.1. Introduction

In the previous chapter, I show that Gli3 expressed in thymic epithelial cells is important in regulating the differentiation from the DP to SP4 stage. The developmental transition from DP to SP is an important determinant of the final TCR repertoire. During this transition DP thymocytes undergo positive and negative selection to select for functional TCRs, which are not self-reactive, thus importantly regulating autoimmunity. Positive and negative selection are regulated by a network of transcriptional interactions, which also determine lineage commitment to either SP4 or SP8.

Key lineage commitment transcription factors Th-POK, Gata3, Tox and Runx3 regulate the induction and repression of the CD4 and CD8 genes on DP thymocytes. Runx3 allows CD8 transcription and silences CD4 gene transcription (Taniuchi, Osato et al. 2002, Grueter, Petter et al. 2005). In contrast, Th-POK suppresses CD8 gene transcription and allows continuous CD4 transcription by preventing Runx3 from silencing the CD4 gene (Jenkinson, Intlekofer et al. 2007). Interestingly,
the transcription factors Gata3 and Tox enhance Th-POK expression to further enhance SP4 lineage commitment (Hernandez-Hoyos, Anderson et al. 2003, Aliahmad, Kadavallore et al. 2011).

The Egr transcription factor family members (Egr1-3) regulate both positive and negative selection in the thymus. The expression of all three members is upregulated after TCR stimulation (Shao, Kono et al. 1997). Egr2 promotes the positive selection of both SP4 and SP8 cells, by upregulating the thymocyte survival molecule Bcl2 during positive selection, thus allowing DP cells to mature into SP cells (Lauritsen, Kurella et al. 2008).

In addition, the Nr4a family (Nr4a1-3) are crucial in regulating the TCR signal and also modulate positive selection. The expression of Nr4a1 on thymocytes correlates to the TCR signal strength (Moran, Holzapfel et al. 2011). Higher levels of Nr4a1 represent a strong TCR signal and vice versa. Nr4a1 is very important for the negative selection of thymocytes (Rajpal, Cho et al. 2003) and it has recently been shown to regulate SP8 development and downstream SP8 effector functions (Nowyhed, Huynh et al. 2015).

Given the importance of transcriptional interactions during DP to SP differentiation and lineage commitment, it was important to understand the genome wide transcriptional changes in the Gli3 mutant during selection and commitment to SP4.
6.2. Chapter Objectives

In this chapter, I aim to test the hypothesis that Gli3 in TEC transcriptionally regulates thymocyte differentiation from the DP to SP4 stage. In order to understand the transcriptional mechanisms that regulate the DP to SP4 transition and thymic selection in the Gli3-mutant FT, I performed microarrays on Facs sorted CD69-DP, CD69+DP and SP4 thymocytes from the WT and Gli3-deficient E18.5 thymus. This analysis will help identify transcription factors involved in positive and negative selection and SP4 maturation. Next, I will also test whether these thymic selection and SP4 lineage commitment differentially expressed transcription factors are regulated directly through Gli3 or independent of Gli3 through Shh.

6.3. Results

6.3.1 Transcriptional mechanisms at the DP to SP transition in the Gli3-deficient thymus

I used Differentially Expressed Gene (DEG) analysis, Principal Component Analysis (PCA) and Canonical Correspondence Analysis (CCA) to understand the changes between the WT and Gli3 mutant in the three different thymus populations DP, CD69+DP and SP4. First, I analysed the datasets for each thymocyte population in an unbiased manner using Principal Component Analysis (PCA) (Fig 6.1A-C). The
CD69-DP dataset segregated by genotype on Principal Component axis 2 (PC2), which accounted for 18% of variability (Fig 6.1A). Genes with a negative PC2 score (genes that contributed to the mutant phenotype) included many Hh signalling and target genes, such as *Hoxd13, Hoxa13, Hoxd8, Smoc2, H19, S100A9* (Mandhan, Quan et al. 2006, Pazin and Albrecht 2009, Chan, Wang et al. 2014, Lu, Li et al. 2015) and the Hh family member *Ihh*, which is known to be expressed by DP cells (Outram, Hager-Theodorides et al. 2009). In addition to Hh signalling genes, *Cd53*, which is induced upon a lower affinity TCR-MHC interaction, and *Runx3* and *Notch1*, which both promote SP8 differentiation over SP4, had negative PC2 scores reflecting higher expression in the Gli3 mutant (Fowlkes and Robey 2002, Puls, Hogquist et al. 2002, Bosselut 2004). Genes with a positive PC2 score were downregulated in the Gli3-mutant, and included genes important in thymic selection and SP4 maturation such as *Pten* and *Fas*, which are regulators of apoptosis during thymic selection, and *Mapk1*, which is required for positive selection, and maturation towards SP4 (Castro, Listman et al. 1996, Germain 2002, Xue, Chiang et al. 2008) (Fig 6.1A).

I selected the 2000 most significant differentially expressed genes (DEG) by Ebayes and intersected these with the most significantly PC axis contributing genes (2000 PC genes with the highest positive and 2000 genes with most negative score for the appropriate PC axis for each subset). These intersected genes are shown as heatmaps for each subset (Fig 6.1A-C). The intersection for the CD69-DP subset revealed
the Hh target genes *Fgf1* and *Kif24*, the Wnt pathway activator *Bcl9* (Martin 1998, Sarkar, Li et al. 2010) and *Cd53*. Interestingly, genes involved in TCR signalling and apoptosis during repertoire selection, including, *Fas*, *Tcerg1*, *Ltbr*, *Pten*, *CamK4*, and *Ctcf* (Krebs, Wilson et al. 1997, Heath, Ribeiro de Almeida et al. 2008, Zhu, Brown et al. 2010, Montes, Coiras et al. 2015) were downregulated in the CD69-DP cells from the Gli3-/-.

PCA of the datasets from the CD69+DP population, which are cells that have received the TCR signal for positive selection (Ge and Chen 1999) segregated the Gli3-mutant and WT datasets by genotype on PC3 contributing to 18% variability (Fig 6.1B). Genes with a negative PC3 score were upregulated in the Gli3-/-, and revealed Hh signalling and target genes such as *Foxa1*, *Kif3a*, *Hdac2* and *Tgfb2* (Katoh and Katoh 2009, Furmanski, Saldana et al. 2013); Fig 6.1B). Genes that were lower in the Gli3-mutant had high PC3 positive scores, and included genes involved in selection and SP4 commitment, including, *Egr1* and *Egr2* which are downstream of TCR signalling and involved in positive selection (Lauritsen, Kurella et al. 2008); *Itk*, a tyrosine kinase downstream of the TCR required for positive selection and the SP4:SP8 lineage decision regulators (Lucas, Miller et al. 2003); *Gata3*, a key SP4 commitment gene (Hernandez-Hoyos, Anderson et al. 2003). The intersection analysis of CD69+DP datasets revealed 1150 genes (Fig 6.1B). Some upregulated genes were known Hh targets, such as *Hoxa7*, *Bmp2*, *Tulp3*, *Itgav* and *H19* (Chan, Wang et al. 2014), while many
downregulated genes were crucial for thymic selection, including *Itk* and *Egr1*; *Socs1*, whose deficiency promotes CD8 differentiation (Ilangumaran, Gagnon et al. 2010); *Anxa5*, which promotes apoptosis during negative selection (Rosenbaum, Kreft et al. 2011); and *Cd6*, a costimulatory molecule which interacts with its ligand on TEC to promote differentiation to SP (Singer, Fox et al. 2002).

PCA on the SP4 datasets showed that the Gli3-/- SP4 data segregated from the WT on PC2, which accounted for 22% of variability (Fig 6.1C). Intersection with DEG revealed 1105 genes. Genes downregulated in the Gli3-/- SP4 cells included *Egr2* and *Nab2*, required for selection, and *Anxa6*, which regulates selection related apoptosis (Collins, Wolfraim et al. 2006, Rosenbaum, Kreft et al. 2011). *Nr4a1*, *Nr4a3* and *Cd5* were also lower in the Gli3-/-.. These genes are transcriptional targets of TCR signal transduction whose level of expression correlates with TCR signal strength (Moran, Holzapfel et al. 2011, Sekiya, Kashiwagi et al. 2013). Genes that promote SP4 lineage commitment and maturation such as *Lef1* and *Tox*, were lower in the mutant (Aliahmad, Kadavallore et al. 2011, Steinke, Yu et al. 2014), whereas various Hh target genes, such as *Itgav*, *Stmn1*, *Itga6* and *Nedd9* were upregulated in the Gli3-/- SP4 subset (Aquino, Lallemend et al. 2009, Lu, Li et al. 2015), consistent with increased Hh signalling in the SP4 cells from the Gli3-/- FT (Fig 6.1C).
6.3.2 Hh dependent transcriptional regulators at the DP to SP transition in the Gli3-deficient thymus

Since Hh target genes were upregulated in the Gli3-/- CD69+DP population (which is undergoing positive selection) and the FTOC experiments suggested that the reduced DP to SP4 transition in the Gli3-/- thymus was due to increased Hh signalling, I tested if the transcription of genes required for differentiation to SP cell could be modulated by rShh-treatment. After 2 days rShh-treatment of WT E17.5 FTOC levels of Egr2 and Tox were reduced compared to untreated control (Fig 6.2A and 6.2B, respectively). Interestingly, the microarray transcript expression of both Egr2 and Tox were low and very similar between the WT and Gli3-/- in the CD69-DP dataset (Fig 6.3A and 6.3B, respectively). Both transcripts, however, were upregulated in the CD69+DP population and were significantly lower in the Gli3-/- CD69+DP and the SP4 datasets, which contain cells undergoing selection (Fig 6.3A and 6.3B). I observed the same pattern of expression in target genes of TCR signal transduction, Nr4a1 and CD5 (Fig 6.3C and 6.3D, respectively), which were low in CD69-DP cells, upregulated in the CD69+DP population, and again in the SP4 population, but significantly lower in the Gli3-/- CD69+DP and SP4 datasets than WT. Thus, the differences in expression in these genes became apparent after TCR signalling for positive selection, in support of the idea that Gli3-mutation influences SP4 T-cell development by increasing Shh
which signals to DP thymocytes to dampen TCR signal transduction during positive selection.

6.3.4 CCA confirms that the Gli3 mutant SP4 datasets have a weaker TCR signal strength compared to the WT

The transcriptome data analysis suggests that cells undergoing selection in the Gli3-/- thymus have a lower average strength of TCR signal. To test this I used Canonical Correspondence Analysis (CCA) to compare the patterns of gene expression in our SP4 datasets to transcriptome data from publically available datasets that were prepared from thymocytes which were receiving different strengths of TCR signals during selection (GSE38909; (Lo, Donermeyer et al. 2012)). I selected the 1500 most significant DEG genes from the GSE38909 dataset and used these to generate a scale of strong TCR signalling to weak TCR signalling and plotted our SP4 datasets against this scale. This analysis showed that the SP4 Gli3-/- samples have the transcriptional signature of thymocytes receiving a lower TCR signal than those of WT, whereas the transcriptome of the SP4 population from the Gli3+/- thymus showed an intermediate transcriptional signature (Fig 6.4). The CCA therefore confirms that Gli3 activity in TEC is required for selection and differentiation from DP to SP4, and is consistent with higher Shh expression in TEC dampening the TCR signal during repertoire selection in the Gli3-/- thymus.
6.5 Discussion

Here, I show that the transcriptome data were consistent with the mechanism of action of Gli3 being that Gli3 activity in TEC promotes SP4 T-cell development by repression of Shh, which signals directly to developing T cells to reduce TCR signal strength. PCA segregated the transcriptome data from each sorted thymocyte population by genotype, with known target genes of the Hh signalling pathway contributing to the Gli3-/- score on the relevant PC axis, and genes which are known to be associated with TCR signalling contributing to the WT score. The intersection of the PCA and DEG analysis showed a downregulation of negative selection, SP4 lineage commitment and TCR signalling genes in the Gli3-/- thymus, while the Hh target genes showed an increased expression compared to the WT. Interestingly, CCA confirmed that WT SP4 cells had the transcriptional signature of cells that had undergone stronger TCR signalling than either Gli3+/- or Gli3-/-.

Additionally, examination of expression levels of the direct transcriptional targets of TCR signalling Nr4a1 and CD5 (Azzam, DeJarnette et al. 2001, Moran, Holzapfel et al. 2011) in the three sorted populations showed that in the CD69+DP population, which are cells that have undergone TCR signalling for positive selection, expression levels were lower in Gli3-/- compared to WT, whereas prior to positive selection in the CD69-DP population, expression of these genes was low and similar in WT and Gli3-/- thymus. A similar pattern of expression was seen for the SP4 lineage commitment genes Egr2 and Tox. Both
genes had a low expression in the Gli3-/− DP dataset, however, their expression was upregulated during selection in CD69+DP and SP4 population, and the Gli3-/− had a lower expression than the WT. Taken together, our results suggests that the Gli3-/− thymus has increased Hh signalling, which decreases the TCR signal strength and downregulates SP4 lineage commitment genes.

6.6 Future Work

In the future, it will be important to investigate whether the SP4 lineage commitment gene Tox and Egr2 are directly repressed by Shh, or via an intermediate repressor that is upregulated by Shh.

It would also be interesting to perform a genome wide transcriptional analysis of the Shh mutant FTOCs and compare the changes to this analysis and to help identify genes that are solely Shh dependent.

The decreased TCR signal strength and the defective selection towards CD4 indicates that the selected TCR repertoire may be different in the Gli3-/−. Hence, it would be interesting to carry out a TCR repertoire analysis on the DP and SP4 cells.
6.7 Summary

In summary, I showed that Gli3 is a transcription factor is required for normal DP to SP4 T-cell development in the FT. Gli3 deficiency reveals both Hh dependent and Hh-independent Gli3 dependent changes during positive and negative selection in the thymus and SP4 lineage commitment. Hh signalling transduces changes in thymic selection genes downstream of the TCR.
Figure 6.1. Microarray dataset analysis showing WT and Gli3-mutant E18.5 FT sorted CD69-DP, CD69+DP and SP4 populations.
Figure 6.1 shows Microarray datasets showing transcriptional differences in Hh signalling genes and thymic selection genes between the WT (n=2) and Gli3-/-(n=2) E18.5 FT sorted CD69-DP, CD69+DP and SP4 populations. (A) Left panel: PCA Axis 2 (PC2) for WT and Gli3-/- Facs sorted CD69-DP from E18.5 FT, showing genes contributing to negative (Gli3-/-) and positive (WT) PC2 axis. Right panel: Heatmap of DEG between WT and Gli3-/- CD69-DP. (B) Left panel: PC3 for WT and Gli3-/- CD69+DP, showing genes contributing to the negative (Gli3-/-) and positive (WT) axis. Right panel: Heatmap of DEG between WT and Gli3-/- CD69+DP. (C) Left panel: PC2 for WT and Gli3-/- SP4, showing genes contributing to the negative axis (WT) and positive axis (Gli3-/-). Right panel: Heatmap of DEG between WT and Gli3-/- SP4.
Figure 6.2. Q-RT-PCR analysis of Egr2 and Tox transcripts in Shh treated E17.5 FT.

Figure 6.2 shows Q-RT-PCR analysis of a representative experiment showing mean ± SEM \((n = 3)\) of the transcript expression of (A) Egr2 and (B) Tox in the WT Control and rShh-treated E17.5 FTOCs for 2 days. Differences were significant: Egr2 \((p<0.02)\); Tox \((p<0.07)\). The transcripts were normalised relative to HPRT.
Figure 6.3. Microarray datasets analysis of transcriptional differences in thymic selection and TCR signal determining genes.

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</tr>
<tr>
<td>Nr4a1</td>
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<td>Cd5</td>
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A. Egr2 Gene Expression

B. Tox Gene Expression

C. Nr4a1 Gene

D. Cd5 Gene Expression
Figure 6.3 shows microarray transcript expression of thymic selection genes (A) *Egr2*, (B) *Tox*, and TCR signal determinants (C) *Nr4a1* and (D) *Cd5* in each of the CD69- DP, CD69+ DP and SP4 from WT (n=2) and Gli3-/- (n=2) datasets.

Figure 6.4. Canonical Correspondence Analysis (CCA) of WT, Gli3+/- and Gli3-/- SP4 microarray datasets.

Figure 6.4 shows CCA showing the separation of the WT, Gli3+/- (HET) and Gli3-/- (KO) SP4 on a scale of strong to weak TCR stimulation, which was generated by using the datasets from GSE38909 (Lo, Donermeyer et al. 2012).
Chapter 7. Summary and Future directions

7.1 The importance of the transcription factor Gli3 in fetal liver B cell development

Here I show that the transcription factor Gli3 is required for fetal liver B cell development. Gli3 mainly acts as a repressor of Hh signalling in the developing fetal liver and therefore loss of Gli3 results in changes, which are either Hh-dependent or Hh-independent.

Our results show that the loss of Gli3 in the fetal liver results in increased Hh activity in fetal liver. Hh signalling is present in both the developing B cells and at higher levels in fetal liver stroma. Therefore Hh activity regulates B cell development by influencing the stromal-B cell interactions.

Interestingly, neutralisation of endogenous Hh proteins in the Gli3-/- fetal liver increases B cell development and *in vitro* modulation of the WT FLOCs with rShh addition or neutralisation decreases or increases B cell development respectively. Loss of Shh *in vivo* also increases fetal liver B cell development, suggesting that Shh negatively regulates fetal liver B cell development.

Analysis of our genome wide RNA-Sequencing data from sorted CD19+B220+ fetal liver B cells in the Gli3 mutants revealed increased
expression of Hh target genes, while important B cell signalling, lineage commitment and maturation genes were downregulated. This correlated with flow cytometry data showing increased Hh activity and a decreased B cell development in the Gli3 mutant fetal liver.

Interestingly, the key master regulators of B cell differentiation Ebf1 and its downstream target Pax5, which form positive feedback loop to govern B cell differentiation, were significantly downregulated in the Gli3−/− fetal liver but increased in the Shh deficient fetal liver. In addition, modulating Hh signalling in vitro showed that rShh treatment decreased the expression of both transcription factors, while rHhip (a Hh neutraliser) upregulated their expression.

Interestingly, Hh pathway activity is increased in the Ebf1+Pax5− early committed B cells, but Hh signalling ceases in the subsequent more mature Ebf1+Pax5+ population. This suggests that Shh signalling functions to suppress Pax5 expression in developing B cells, which then hinders the positive feedback loop between Ebf1 and Pax5, leading to reduction in Ebf1 expression. However, whether Shh directly binds to Ebf1 to directly repress its transcription, or upregulates an intermediary repressor of Ebf1 expression would be important to investigate in the future.

Since B cell development continues in the bone marrow after birth, it would be interesting to investigate the importance of Gli3 inactivation and Shh inactivation during B cell development in the adult bone marrow. In addition, it would be important to examine the role of other
Gli transcription factors including Gli1 and Gli2 in B cell differentiation to understand whether the Gli family have distinct or redundant roles during fetal and adult B cell development.

Dysregulated Gli3 and Hh signalling is known to be involve in many B cell malignancies including B cell leukemias (Ramirez, Singh et al. 2012, Qu, Liu et al. 2013), hence it is essential to investigate whether the Hh related B cell malignancies also have defects in Pax5 or Ebf1 activity and whether Hh inhibitors could decrease Pax5 and Ebf1 activity. It would also be important to investigate whether key targets of Hh are involved in initiating or maintaining B cell malignancies.

7.2 The importance of the transcription factor Gli3 in DP to SP fetal thymocyte development

Similar to B cell development, Gli3 is required for the differentiation of DP to SP fetal thymocytes. Our group has previously shown that the Hh target gene Gli1 is upregulated in the Gli3 mutant fetal thymus (Hager-Theodorides, Furmanski et al. 2009, Drakopoulou, Outram et al. 2010), and here I show the presence of increased Hh activity in developing Gli3 mutant fetal thymocytes. Therefore, Gli3 is acting as a repressor of Hh signalling during the DP to SP thymocyte differentiation stage. The Gli3 mutant thymus has both Hh-dependent and Hh-independent changes.
Our results show that the Gli3-/- fetal thymus has decreased differentiation to the SP4 lineage from the DP stage. This was similar to the action of Gli3 on the pre-TCR, where Gli3 deficiency decreases the development of DP thymocytes from the DN stage (Hager-Theodorides, Dessens et al. 2005).

The Gli3-/- thymocytes are less mature expressing a lower level of cell surface maturity markers CD3, CD69 and Qa2. In addition, Gli3 deficiency decreases cell-surface CD5 expression, reflecting a lower TCR signalling strength. TCR signal strength is an important determinant of SP lineage commitment. A strong TCR signal skews commitment to the SP4 lineage while weaker signals favour SP8 commitment. Interestingly, attenuation of Hh signalling using the Hh neutraliser rHhip increases cell surface CD5 expression, consistent with results from previous studies which show that rShh-treatment decreases cell surface CD5 expression (Rowbotham, Hager-Theodorides et al. 2007, Furmanski, Saldana et al. 2012).

Taken together, our results show that loss of Gli3 increases Hh activity in thymocytes leading to weaker TCR signalling, which decreases DP thymocyte differentiation and commitment to SP4 lineage.

Here I also show that the specific deletion of Gli3 from the TEC (Gli3fl/fl, FoxN1Cre+), rather than hematopoietic cell intrinsic Gli3 deletion (Gli3fl/fl, VavCre+), was important for normal SP4 differentiation and maturation. This was consistent with the expression of Gli3 in the fetal thymus stroma (Hager-Theodorides, Furmanski et al.
However, Gli3 activity in the fetal thymocytes is also important for interpreting the changes of Hh signal, as the neutralisation of Hh in the Gli3-deficient thymus did not increase the cell surface expression of CD5 on the SP4 cells, but when Hh was neutralised in the Gli3fl/fl, FoxN1Cre+ thymus, cell surface CD5 expression was increased. Therefore, thymocyte intrinsic Gli3 was required to respond to the changes in the Shh signalling in the fetal thymus.

The conditional deletion of Gli3 from TEC decreased SP4 differentiation and maturation while attenuation of Hh signalling in the Gli3fl/fl, FoxN1Cre+ increased differentiation and maturation to the SP4 lineage. Consistent with this, conditional deletion of Shh increased differentiation and maturation of SP4 cells which was reversed with rShh treatment. Taken together, these results suggest that Gli3 in TEC, represses Shh in fetal thymus, regulating positive selection and increases the TCR signal strength to promote SP4 differentiation and maturation.

Consistent with the phenotype results from the Gli3 mutants, the genome wide transcriptional analysis of the Gli3 mutant CD69-DP, CD69+DP and SP4 populations showed that the Gli3 mutant thymocytes have increased Hh activity but decreased positive selection and SP4 lineage commitment. Interestingly, I observed that Shh signalling mainly acts downstream of TCR, as most positive selection, SP4 lineage commitment (Egr2 and Tox) and TCR signal strength regulating genes (Nr4a1 and CD5) were significantly downregulated in the Gli3-/-. 
CD69+DP and SP4 populations which had undergone TCR signalling, rather than the CD69-DP population. Additionally, our CCA results showed that the Gli3 mutant SP4 thymocytes had a transcriptional signature of cells that had undergone a stronger TCR signalling compared to WT. This confirmed that a dose-dependent loss of Gli3 was important in regulating the TCR signal accordingly. In addition, the transcription factors Egr2 and Tox were also regulated by Shh rather than Gli3, as the Shh treated fetal thymus showed a significantly decreased expression of Egr2 and Tox. However, I have not yet investigated how Shh suppresses the expression of Egr2 and Tox, or whether Shh upregulates a repressor of Egr2 and Tox in fetal thymus. In the future, it would be important to better understand the direct mechanisms through which Shh decreases SP4 lineage commitment.

Given that the Gli3 mutant thymocytes have decreased TCR signalling and show a transcriptional signature similar to that of thymocytes having a weaker TCR signal strength, the Gli3 mutant thymocytes may have a different TCR repertoire and it would be very interesting to analyse the TCR repertoire of the Gli3 mutant thymocytes.

Dysregulated Hh-signalling is involved in T-cell acute lymphoblastic leukemia (T-ALL) (Irvine and Copland 2012, Ok, Singh et al. 2012), therefore it is very important to understand how Hh signalling influences the environment of the thymus, where T-ALL arises.
Chapter 8


Macdonald, R., K. A. Barth, Q. Xu, N. Holder and I. Mikkola (1995). "Midline signalling is required for Pax gene regulation and patterning of the eyes." ...


Appendix
The transcription factor Gli3 promotes B cell development in fetal liver through repression of Shh

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Before birth, B cells develop in the fetal liver (FL). In this study, we show that Gli3 activity in the FL stroma is required for B cell development. In the Gli3-deficient FL, B cell development was reduced at multiple stages, whereas the Sonic hedgehog (Hh [Shh])–deficient FL showed increased B cell development, and Gli3 functioned to repress Shh transcription. Use of a transgenic Hh-reporter mouse showed that Shh signals directly to developing B cells and that Hh pathway activation was increased in developing B cells from Gli3-deficient FLs. RNA sequencing confirmed that Hh-mediated transcription is increased in B-lineage cells from Gli3-deficient FL and showed that these cells expressed reduced levels of B-lineage transcription factors and B cell receptor (BCR)/pre-BCR–signaling genes. Expression of the master regulators of B cell development Ebf1 and Pax5 was reduced in developing B cells from Gli3-deficient FL but increased in Shh-deficient FL, and in vitro Shh treatment or neutralization reduced or increased their expression, respectively.

INTRODUCTION
During B cell development in the fetal liver (FL), hematopoietic stem cells, defined as lineage-negative Sca-1+ c-Kit+ cells, mature to gain expression of IL-7Rα (CD127) and are known as common lymphoid progenitor (CLP) cells (Egawa et al., 2001; Mebius et al., 2001). However, this population is multipotent and still contains cells with potential for both lymphoid and myeloid lineages (Mebius et al., 2001). Commitment to the B cell lineage first occurs on embryonic day 12.5 (E12.5), as cells mature to initially express CD19 or B220 and are defined as either B-1 (CD19+B220lo-neg) or B-2 (CD19+B220hi) lineage cells (Egawa et al., 2001; Dorshkind and Montecino-Rodriguez, 2007; Montecino-Rodriguez and Dorshkind, 2012). B-1 cell development is more prominent in the FL and fetal BM, whereas B-2 cells are mainly produced in the adult BM (Montecino-Rodriguez and Dorshkind, 2012).

Both B-1 and B-2 progenitors mature into B220+CD19+ double-positive cells, which undergo immunoglobulin heavy chain gene rearrangement to give rise to the first cells that express cell surface μH (pre-BCR; Dorshkind and Montecino-Rodriguez, 2007; Montecino-Rodriguez and Dorshkind, 2012). This pre-B cell population can also be identified by cell surface expression of BP-1, before rearrangement of the light chain locus and cell surface expression of IgM (Hardy and Hayakawa, 2001; Dorshkind and Montecino-Rodriguez, 2007; Montecino-Rodriguez and Dorshkind, 2012).

Here, we investigate the role of Sonic hedgehog (Hh [Shh]) and the transcription factor Gli3 in the regulation of B cell development in the FL. Shh is one of three mammalian Hh proteins (Shh, Indian Hh [Ihh], and Desert Hh [Dhh]) that share a common signaling pathway (Ingham et al., 2011; Ramsbottom and Pownall, 2016). Hh proteins signal by binding to their cell surface receptor Patched1 (Ptc1), thereby releasing Ptc1’s repression of Smoothened (Smo), allowing Smo to transduce the Hh signal. At the end of the signaling pathway are the Hh-responsive transcription factors Gli1, Gli2, and Gli3 (Ingham et al., 2011). Gli1 is itself an Hh-target gene and encodes an activator of transcription (Park et al., 2000), whereas Gli2 and Gli3 can be processed to function as transcriptional activators (Gli2A/Gli3A, in the presence of Hh pathway activation) or transcriptional repressors (Gli2R/ Gli3R, in the absence of Hh pathway activation; Sasaki et al., 1999). Gli2 is required to initiate the Hh signal and functions largely as a transcriptional activator in vivo (Park et al., 2000; Bai et al., 2002). In contrast, Gli3 functions predominantly as a transcriptional repressor in vivo (Wang et al., 2000). The pathway has multiple positive and negative feedback mechanisms, and Ptc1 is itself an Hh-target gene, functioning to sequester Hh proteins and limit activation of the pathway (Ingham et al., 2011).

Gli3 can have both Hh-independent and Hh-dependent functions (te Welscher et al., 2002; Hager-Theodorides et al., 2009). Gli3R functions to limit Hh pathway activation in many tissues (Wang et al., 2000; Ahn and Joyner, 2004; Hager-Theodorides et al., 2009). There are at least two distinct mechanisms by which Gli3R can limit Hh signaling: it may...
repress expression of Hh genes in the Hh-producing cell via repression of Hh-activating genes, thus limiting Hh protein concentration in the tissue. For example, during prepattern- ing of the limb bud, Gli3R spatially limits the expression of dHand, an activator of Shh gene expression (te Welscher et al., 2002). Alternatively, when Gli3 is expressed in the signal-receiving cell, the concentration of Gli3R in a given cell increases the further away the cell is located from the Hh-secreting source, resulting in correspondingly increased repression of Hh-target genes (Wang et al., 2000; te Welscher et al., 2002). In fact, in many tissues, such as thymus and limb bud, Shh and Gli3 have opposing functions, with Shh deficiency and Gli3 deficiency giving opposite phenotypes (Wang et al., 2000; Shah et al., 2004; Hager-Theodorides et al., 2005, 2009; Barbarulo et al., 2016). Here, we show that Gli3 expressed in the FL stroma promotes B-lineage commitment of hematopoietic progenitor cells and B cell development by suppression of Shh signaling.

RESULTS

Impaired B-lineage commitment in the Gli3-deficient FL

The Hh-responsive transcription factor Gli3 is expressed in mouse FL (Cridland et al., 2009) and human FL and BM (Fig. S1 A; Su et al., 2004). Microarray expression profiles from the Imagen database show expression of Smo, Ptc1, and the Gli transcription factors in mouse E15 FL hematopoietic stem cells, CLPs, and pro–B (CD19+µH+) and pre–B (CD19+µH−) cells (Fig. S1, B–F; Heng et al., 2008). Therefore, to investigate the role of Gli3 in fetal B cell development, we analyzed B-lineage markers in Gli3−−/−, Gli3+/−, and Gli3+/+ (WT) littermate E18.5 FL. We found statistically significant gene dose–dependent decreases in the proportions of the CD19+ cells, B220+ cells, and CD19+B220+ cells in the E18.5 Gli3-mutant FL (Fig. 1, A–E). The Gli3−−/− FL also showed a significant decrease in the proportion of CD19+B220+ B2 progenitor cells (Fig. 1, C and E). Additionally, the proportion of CD19− cells that expressed the cell surface heavy chain µH was significantly reduced in the Gli3−−/− FL compared with WT (Fig. 1, D and F).

We further characterized early B cell development by staining against the B-lineage marker CD93 and then subdividing the CD93+ population by expression of ckit, CD127, heat stable antigen (HSA), CD43, and BP-1, in addition to CD19 and µH expression, to identify four fractions of increasing maturity (Fig. 1, G and H). The overall proportion of CD93+ cells (B lineage committed) was significantly reduced in the Gli3−−/− FL compared with WT (Fig. 1 G). Gating on these CD93+ cells, the proportion of the early ckit−CD127+ population (fraction A) was also significantly reduced in the Gli3−−/− FL compared with WT, as were the later CD43−/CD19−HSA+B−P-1− (fraction D) and CD19−HSA+µH+ populations (fraction E; Fig. 1 H).

As Gli3 deficiency influenced early B cell maturation and reduced the proportion of µH+ B-lineage cells, we used quantitative RT-PCR (Q-RT-PCR) to test whether heavy chain rearrangements were reduced in FACS-sorted CD19+ cells from Gli3−−/− and WT littermates. We quantified rearrangements between two different VH to JH segments and found no evidence for reduced gene rearrangement in developing B cells from the Gli3-deficient FL (Fig. 1 I).

Gli3 activity is not required for B cell development in the hematopoietic compartment of the FL

To investigate whether the reduction in B cell development in the Gli3− mutant FL is caused by cell-intrinsic Gli3 activity in the hematopoietic compartment or caused by Gli3 activity in the nonhematopoietic compartment (stroma), we used the Cre-loxP system to conditionally delete Gli3 from the hematopoietic lineage in Gli3fl/flVavCre+ (Gli3coKO) embryos. We found no significant difference in B cell differentiation between the control (Cre negative) and Gli3coKO FL, and the proportion of cells that expressed CD19, B220, and BP-1 was not different between control and Gli3coKO (Fig. 1, J–L). Therefore, Gli3 activity in the FL stroma, rather than hematopoietic cell–intrinsic activity, regulates B cell differentiation.

Increased Hh signaling in the Gli3-mutant FL

As Gli3 can have Hh-independent and Hh-dependent functions and can act to limit Hh pathway activation, we tested whether the Gli3− mutant FL had increased levels of Hh pathway activation by measuring the transcription of Hh pathway components and target genes by Q-RT-PCR from the tissue (Fig. 2, A–D). The Hh-target genes, the transcription factor Gli1, and the Hh receptor Ptc1 were increased in the Gli3−−/− FL compared with WT, as was the Hh-responsive transcription factor Gli2 (Fig. 2, A–C). Transcription of Shh was also increased, consistent with Gli3 functioning to repress Shh expression (Fig. 2 D). Then, we FACS-sorted CD45+CD19+ (B lineage) and nonhematopoietic CD45+ (stroma) cells from Gli3−−/− and littermate WT FL and compared expression of Hh pathway components and target genes (Fig. 2, E–H). Expression of Gli1 was higher in the nonhematopoietic WT cells than in the CD19+ population, and its expression was increased in the Gli3-deficient populations, with greater increase in the nonhematopoietic (stromal) Gli3-deficient compartment (Fig. 2 E). Gli2 expression was approximately fivefold higher in the stromal cells of the Gli3-deficient FL compared with WT and was relatively very low in the CD19+ fraction (Fig. 2 F). In contrast, expression of Ptc1 was increased in both populations sorted from Gli3−−/− compared with WT FL (Fig. 2 G). Expression of Shh was greatly up-regulated in the nonhematopoietic (stromal) component of the Gli3−−/− FL compared with WT, consistent with Gli3 functioning to repress Shh expression in the FL stroma (Fig. 2 H) and with a previous study on expression of Shh by Dlk+ hepatoblasts (Hirose et al., 2009). Although Gli3 and Gli2 can have overlapping or redundant functions in some tissues, we found no evidence for redundancy between Gli3 and Gli2 in repression of Shh expression in the stroma; in the absence of Gli3, Shh was up-regulated.
Figure 1. B lineage development in E18.5 and E17.5 Gli3^{+/+}, Gli3^{+-}, and Gli3^{-/-} FL. (A–F) Flow cytometry profile of E18.5 FL from Gli3^{+/+} (WT), Gli3^{+-}, and Gli3^{-/-} littermates after red blood cell lysis from a representative experiment. (A–D) Dot plots: SSC against CD19 (A), SSC against B220 (B), CD19 versus B220 (C), and SSC against μH gated on CD19^{+} cells (D). (E and F) Bar charts: mean percentage of FL populations, relative...
Given that transcription of Hh-target genes and Shh were up-regulated in the absence of Gli3, whereas the B cell progenitor populations were reduced, we tested whether developing B cells and stromal cells are undergoing active Hh signaling in the FL. We used Gli binding site (GBS)–GFP-transgenic reporter mice, in which GFP is expressed when activator forms of Gli proteins bind to the GBS in the transgene, to measure active Hh–dependent transcription (Balasak et al., 2012). Approximately 4% of CD19+ cells expressed GFP in the WT FL (Fig. 2 I), and a higher level of GFP expression of ~8% and ~9% was observed in the CD19+B220− (B-1 progenitors) and CD19+B220+ (B-2 progenitors), respectively (Fig. 2, J–K), suggesting that Hh signaling is higher in cells transitioning from the immature B1 and B2 progenitor stages toward the CD19+B220+ stage. High proportions of nonhematopoietic (CD45+) stromal FL cells (~57%) and of the epithelial cell adhesion molecule+ subset of CD45+ FL cells (~53%) expressed GFP (Fig. 2 L), confirming the Q-RT-PCR data, which indicated that Hh signaling is also active in the nonhematopoietic stromal compartment (Fig. 2, E and G). The proportion of GFP–expressing CD19+, CD19+B220−, and CD19+B220+ cells was significantly increased in the Gli3+−/− compared with WT (Fig. 2, I–K and M). However, there was no significant difference in the proportion of GFP+ cells between Gli3+−/− and WT (Fig. 2 M). The mean fluorescence intensity (MFI) of the CD19+GFP+ cells significantly increased from WT to Gli3+−/− and from WT to Gli3+−/−, indicating higher Hh-dependent transcription in individual cells (Fig. 2 M).

Hh signaling is a negative regulator of fetal B cell development

The Gli3+−/− FL had increased Shh transcription, increased expression of Hh–target genes, and increased GFP expression in developing B cells in the GBS-GFP reporter–transgenic embryos (Fig. 2). Thus, we tested whether increased Hh signaling reduces B cell development in vitro. We assessed B cell populations after 4 d in WT E17.5 FL organ cultures (FLOCs) treated with recombinant Shh (rShh) alone, with recombinant Hh–interacting protein (Hhip [rHhip]; to bind the Shh/scm toxicity, we added both reagents together and found that the proportion of CD19+ cells was significantly increased compared with the untreated Gli3+−/− control (Fig. 3 E). This suggests that the reduction in B cell development in the Gli3 mutant was largely caused by an increase in Hh proteins in the FL and that Hh signaling negatively regulates B cell development in vitro.

B cell development in the Shh−/− fetus

To test whether Shh negatively regulates B lineage development in vivo, we assessed B cell development in Shh−/− FLs. Most Shh−/− embryos die before E16, so we analyzed the E14.5 FL. Both Shh−/− and Shh+/− had significantly increased percentages of CD19+ cells, B220− cells, and of the CD19+B220−, CD19+B220+, and CD19+B220+ populations compared with WT, with the heterozygote showing intermediate proportions (Fig. 4, A and B). The proportion of B lineage–committed CD93+ cells and the proportion of CD93− cells that were CD19+ were significantly increased in the Shh+/− FL compared with WT (Fig. 4 C). The proportion of CD19+BP1+ (Pre-B) cells was also significantly increased in the Shh+/− E14.5 FL compared with WT (Fig. 4 D).

As expected, the E14.5 Gli3−/− mutant FL showed the opposite phenotype, with significantly decreased CD19+, B220−, CD19′B220+, CD19′B220−, and CD19′BP1+ populations compared with WT littermates (Fig. 4, E–G). Treatment of E14.5 Shh+/− FLOCs with rShh for 4 d reduced
Figure 2. Expression of Hh pathway components and active Hh signaling in E14.5 and E17.5 WT and Gli3-mutant FL. (A–H) Bar charts: Representative experiments show mean ± SEM (n = 3) of Q-RT-PCR analysis from WT, Gli3+/−, and Gli3−/− littermates for whole FL Gli1 (A), Gli2 (B), and Ptc1 (C) on E17.5 and Shh on E14.5 (D) and for FACS-sorted CD19+ and CD45− (stromal) cells for Gli1 (E), Gli2 (F), Ptc1 (G), and Shh (H). au, arbitrary units. (I–M)
the proportion of CD19\(^+\) cells compared with the untreated control FLOCs (Fig. 4 H), confirming that Shh negatively regulates B cell development.

As Shh inhibited B cell development in B-committed populations, we investigated the impact of Shh deletion and Gli3 deletion on the development of CLPs. We analyzed the proportion of CLPs, defined by the surface markers Lin\(^{-}\)CD117\(^{+}\)CD127\(^{−}\), and found a significant increase in the CLP population in the Shh-deficient FL and decrease in the Gli3-deficient FL, relative to their WT littermates (Fig. 4 I). Collectively, our experiments indicate that Gli3 and Shh influence B cell development from the CLP populations through to the CD19\(^{+}\)B220\(^{+}\)pre-BCR\(^{+}\) stage of development.

**Conditional deletion of Shh from the hematopoietic compartment of the FL does not increase B cell development**

To investigate whether the increase in B cell development in the E14.5 Shh\(^{+/−}\) FL is caused by cell-intrinsic loss of Shh expression in the hematopoietic compartment or caused by loss of Shh secretion by the nonhematopoietic compartment (stroma), we used the Cre-loxP system to conditionally delete Shh from the hematopoietic lineage in Shh\(^{fl}\)VavCre\(^+\) (ShhcoKO) embryos. We found no significant differences in B cell differentiation between the control (Cre negative) and ShhcoKO E14.5 FL and no significant differences in the proportions of cells that expressed CD19, B220, and B2− and in the proportion of CLPs between control and ShhcoKO (Fig. 5, A–C). We likewise found no significant differences in B cell populations defined by cell surface expression of CD19, B220, and m\(^{H}\) on E16.5 and E18.5 and no significant difference in the proportion of CLPs on E18.5 between ShhcoKO and WT (Fig. 5, D–H). Therefore, it is Shh expression by the FL stroma, rather than hematopoietic cell–intrinsic Shh expression, that regulates B cell differentiation. This is consistent with the increase in Shh expression observed in the Gli3\(^{−/−}\) FL stroma compared with WT (Fig. 2 H) and with the fact that conditional deletion of Gli3 from the hematopoietic compartment has no influence on B cell differentiation (Fig. 1, J–L). Collectively, these experiments indicate that Gli3 activity in the FL stroma promotes B cell development by repression of Shh expression in the stroma.

**Gli3 mutation inhibits transcription of B cell–lineage commitment, signaling, and maturation genes**

To investigate the mechanisms of action of Gli3 on B cell development, we measured transcription in developing B cells in the Gli3-mutant FL. We used RNA sequencing to analyze whole-genome expression in FACS-sorted CD19\(^{+}\)B220\(^{+}\) FL B cells in the WT and Gli3 mutants. First, we analyzed the dataset in an unbiased manner using principal component analysis (PCA). The dataset segregated by genotype on both principal component axis 1 (PC1) and PC3 (Fig. 6 A). PC1, the axis attributing to the largest differences in the dataset (60% variability), separated the WT from the Gli3 mutants (Gli3\(^{+/−}\) and Gli3\(^{−/−}\)), whereas PC3 showed differences between Gli3\(^{+/−}\) and Gli3\(^{−/−}\) (Fig. 6 A).

Further analysis of the genes with high positive and negative scores on PC1 showed that PC1 reflected differences in genes associated with HH signaling and genes associated with B cell signaling and differentiation. Key HH signaling and target genes, including Gli1, Hdac3, and Smo, had positive PC1 scores, indicating that the expression of these is higher in the Gli3 mutant. In contrast, genes that were lower in the Gli3 mutants had high negative PC1 scores and were mainly B cell–signaling and –lineage commitment genes. Thus, not only did Gli3 mutation reduce the proportion of CD19\(^{+}\)B220\(^{+}\) B-committed cells in the FL, but also, within that sorted population, expression of genes required for B cell differentiation was reduced.

To understand better the genome-wide differences in the dataset, we intersected the differentially expressed genes identified by Ebayes statistics with the genes identified by PCA. We selected the 3,000 genes (highest and lowest scorers) that contributed most to the PC1 axis and intersected these with the 3,000 most significantly differentially expressed genes by Ebayes statistics (Table S1). The resulting 1,122 genes were drawn (Fig. 6 A). Further analysis of the genes with high positive and negative scores identified by Ebayes statistics with the genes identified by PCA. We selected the 3,000 genes (highest and lowest scorers) that contributed most to the PC1 axis and intersected these with the 3,000 most significantly differentially expressed genes by Ebayes statistics (Table S1). The resulting 1,122 genes were drawn (Fig. 6 B and C). This intersection highlighted genes that have been previously shown to be Shh–target genes in other tissues, such as Serpin1, Hngb1, Hngh2, Hoxa4, Cull4a, and Bmi1, which were all up-regulated in the Gli3 mutant (Fig. 6 C; Itou et al., 2011; Wang et al., 2012; Lu et al., 2015; Yang et al., 2015). In contrast, master regulators of early B cell development Ebf1 and Foxo1 were down-regulated in the Gli3 mutants compared with WT, and several other B lineage differentiation and maturation genes, including Ifit3, Egr1, Irf1, Irf4, and Cd69, were also lower in the Gli3 mutant (Gururajan et al., 2008; Lu, 2008; Outram et al., 2008). B cell activation and signaling genes including genes involved in modulating MAPK signaling such as Dusp1, Dusp2, Map3k3, Mapkapk2 (Lang et al., 2006), and canonical NF-κB signaling genes (Nikbid, Nikbiz, and Ttaip3) were all down-regulated in the Gli3 mutant. In addition, the AP1...
components *Fosb, Jun*, and *Junb*, required for pre-BCR signal transduction (de Gorter et al., 2007), and *Klf2*, an essential late target gene of the pre-BCR, were also lower in the *Gli3* mutant (Winkelmann et al., 2014).

As both pre-BCR/BCR signaling and IL-7 signaling are important regulators of B cell development, we compared expression levels of genes that encode downstream components of these signaling pathways or are their immediate tran-
Figure 4. B cell development in the E14.5 Shh-deficient and Gli3-deficient FL and Shh−/− FLOC. (A–D) Flow cytometry of E14.5 FLs from Shh+/+ (WT), Shh+/−, and Shh−/− littermates. (A) Dot plots: SSC against CD19 staining (top) and CD19 staining against B220 staining (bottom). (B) Bar charts: relative mean percentage ± SEM, relative to the mean of WT littermates for Shh−/− (n = 7), Shh+/− (n = 10), and WT (n = 4) of populations stated, showing
scriptional targets (Fig. 6 D). Expression levels of many genes involved in pre-BCR/BCR signal transduction (Dusp1-3, Tnafip3, Nkx2d, and the AP-1 components Junb and Fosb) were lower in the Gli3\(^{-/-}\) datasets than WT, as were its immediate transcriptional targets CD69 and Egr1 (Fig. 6 D). We observed no difference in expression in Tcfl (E2A) between genotypes (Fig. 6 D) or in Il7r, Il2rg, Stat5a, Stat5b, and Pik3ca (components of the IL-7/Thymic stromal lymphopoietin signaling pathway) or in Myb, Bcl2, or Bcl2L1 (transcriptional targets of IL-7 signaling in B-lineage cells; Fig. 6 E).

We did not detect Shh expression in any dataset (Fig. 6 E), consistent with the Q-RT-PCR, showing Shh up-regulation in Gli3\(^{-/-}\) FLs being restricted to stromal cells and not B-lineage cells (Fig. 2 H) and with the fact that conditional deletion of Shh from B-lineage cells had no impact on their development (Fig. 5).

Together these analyses indicate that the Gli3-mutant B220\(^{+}\)CD19\(^{+}\) population has increased Hh-dependent transcription, consistent with our previous results showing increased Shh transcription in the Gli3-mutant FL stroma (Fig. 2 H) and increased Hh signaling in Gli3\(^{-/-}\) B-lineage cells (Fig. 2, G, I–K, and M). Gli3 deficiency also decreased transcription of genes required for B cell differentiation, maturation, and signaling within the sorted B220\(^{+}\)CD19\(^{+}\) population. Thus, increased Shh signaling reduced transcription of regulators of B lineage commitment and differentiation. Therefore, we investigated whether Shh treatment can directly down-regulate transcription of the key B-lineage transcription factors Ebf1 and Pax5 in vitro.

Shh signaling leads to reduced Ebf1 and Pax5 expression during B lineage development

Ebf1 and Pax5 are master regulators of B-lineage commitment and B cell development. B cell–lineage commitment from the CLP stage is regulated by Ebf1 (Zhang et al., 2003), which promotes its own transcription as well as Pax5 transcription. Pax5 further increases Ebf1 transcription (Roessler et al., 2007) creating a positive feedback loop between itself and Ebf1. This mechanism allows Ebf1 and Pax5 to regulate B cell development and maturation. Therefore, we tested whether the increase in Shh in the Gli3-mutant influences Ebf1 and Pax5 expression during B cell development.

First, we measured overall Ebf1 and Pax5 transcription in the Gli3-mutant E17.5 FL and found a reduction in both Gli3\(^{-/-}\) and Gli3\(^{-/-}\) relative to WT (Fig. 7 A), consistent with the RNA sequencing data and the reduction in B cell development. In contrast, we found an increase in the expression of both transcription factors in the Shh\(^{+/-}\) and Shh\(^{-/-}\) E14.5 FL, consistent with increased commitment to the B lineage in the absence of Shh (Fig. 7 A).

Because the Gli3 mutant has increased Shh signaling and rShh treatment decreased the CD19\(^{+}\) population in FLOCs, we tested whether we could influence both Pax5 and Ebf1 transcription and protein expression in vitro by modulating Hh signaling in FLOCs by treatment with rShh or rHhip over a 4-d culture period. We measured the expression of intracellular Pax5 and Ebf1 by FACS analysis to investigate protein expression in single cells. On day 1 after treatment, there were no significant differences in the proportions of Ebf1\(^{-}\)Pax5\(^{-}\) and Ebf1\(^{-}\)Pax5\(^{+}\) cells (not depicted). However, a significant reduction in the proportion of Ebf1\(^{-}\)Pax5\(^{-}\) cells was seen on days 2 and 4 of rShh treatment (Fig. 7 B). This Ebf1\(^{-}\)Pax5\(^{-}\) population significantly increased and was sustained in the rHhip-treated cultures, whereas the cultures in which both rShh and rHhip were added together were not different from the control cultures (Fig. 7 B). Comparison of the ratio of Ebf1\(^{-}\)Pax5\(^{-}\) to Ebf1\(^{-}\)Pax5\(^{+}\) cells showed that rHhip treatment increased the ratio by twofold by day 4 in culture, indicating that inhibition of Hh signaling accelerated the transition from Ebf1\(^{-}\)Pax5\(^{-}\) cell to Ebf1\(^{-}\)Pax5\(^{+}\) cell. In contrast, rShh treatment reduced this ratio on both days 2 and 4 of culture, suggesting that Shh signaling repressed the induction of Pax5 and the transition to Ebf1\(^{-}\)Pax5\(^{-}\) cell.

The changes in the Ebf1 and Pax5 proteins were consistent with the changes in transcription of Ebf1 and Pax5 on day 4 (Fig. 7 C). In the Gli3\(^{-/-}\) FL, the proportion of Ebf1\(^{-}\)Pax5\(^{+}\) cells was reduced compared with WT, consistent with the Q-RT-PCR data (Fig. 7 D).

Then, we FACS sorted CD19\(^{+}\) cells from Gli3\(^{-/-}\) and WT littermates and analyzed the expression of Ebf1 and Pax5. We observed a decrease in both Ebf1 and Pax5 transcription in purified CD19\(^{+}\) cells from the Gli3-mutant FL compared with WT (Fig. 7 E). Interestingly, the decrease in expression...
Figure 5.  

**B cell development in the Shh<sup>fl/fl</sup>VavCre FL.**  (A–H) Flow cytometry of the E14.5 FL (A–C), E16.5 FL (D and E), and E18.5 FL (F–H) from Shh<sup>fl/fl</sup>VavCre<sup>−</sup> (E14.5, n = 5; E16.5, n = 3; E18.5, n = 5) and Shh<sup>fl/fl</sup>VavCre<sup>+</sup> (E14.5, n = 3; E16.5, n = 5; and E18.5, n = 3) littermates. (A, D, and F) Dot plots: SSC against CD19 staining (left), CD19 staining against B220 staining (middle), and µH staining for D and F (right). (B, E, and G) Bar charts: mean percentage.
of both Ebf1 and Pax5 in the sorted Gli3<sup>−/−</sup> CD19<sup>+</sup> cells was more pronounced than the reduction in transcript expression in the unsorted FL and was also greater than the proportional change in the CD19<sup>+</sup> population in the Gli3<sup>−/−</sup> FL compared with WT. This was consistent with the action of Shh to reduce B cell development in FLOCs, in which the magnitude of the reduction in Pax5 and Ebf1 transcription was greater than the change in the Ebf1<sup>−/−</sup>Pax5<sup>−/−</sup> population (Fig. 7, B and C) and also greater than the magnitude of the change in the overall proportion of B-lineage cells caused by Shh treatment (Fig. 3 A). Thus, Shh treatment in vitro or Gli3 deficiency in vivo not only reduced the proportion of CD19 cells that were present, but also led to a reduction in transcription of the key B-lineage master regulators Ebf1 and Pax5 within the B lineage–committed population, most likely by signaling to up-regulate the transcription of an intermediate transcriptional repressor of Ebf1 and/or Pax5.

To investigate the relationship between Hh-mediated transcription and the reduction in transcription of Ebf1 and Pax5, we measured intracellular Ebf1 and Pax5 expression in rShh-treated and control untreated GBS-GFP–transgenic FLOCs. We found that >62% of Ebf1<sup>−/−</sup>Pax5<sup>−/−</sup> cells expressed high levels of GFP, indicating that this population actively mediates Hh-dependent transcription, which is therefore compatible with Ebf1 protein expression (Fig. 7 F). These cells are the earliest B cell population that differentiates from the CLP stage (Egawa et al., 2001; Mebius et al., 2001). Interestingly, GFP expression was reduced to <0.3% in the later Ebf1<sup>−/−</sup>Pax5<sup>−/−</sup> population (Fig. 7 F), indicating that Hh signaling decreases as cells become more mature and that, once Pax5 is expressed, very few cells are undergoing Hh-mediated transcription. This is consistent with Shh signaling acting directly or indirectly to inhibit Pax5 transcription. The Ebf1<sup>−/−</sup>Pax5<sup>−/−</sup> population was Hh responsive, as expression of GFP in the Ebf1<sup>−/−</sup>Pax5<sup>−/−</sup> population was increased on rShh treatment, with the proportion of GFP-negative cells decreasing from ∼38% to ∼28%. In contrast, GFP was not induced by rShh treatment in the Pax5<sup>−/−</sup>Ebf1<sup>−/−</sup> population during the 2-d culture, and the proportion of GFP-negative cells remained >99.7% under both conditions.

Collectively, our experiments indicate that the Gli3-mutant FL had increased Hh signaling resulting in decreased B cell development. Furthermore, loss of Shh in vitro and in vivo led to increased B-lineage commitment and development. We propose that Shh signaling either directly or indirectly represses Pax5 expression (most likely by transcriptional activation of a transcriptional repressor of Pax5) and that this leads to loss of Pax5’s induction of Ebf1, reduction in both Pax5 and Ebf1 expression, and therefore to the negative regulation of the B lineage development observed.

**DISCUSSION**

Here, we showed that Gli3, expressed by the stromal compartment, is required for B cell development in the FL. Mutation of Gli3 led to an overall reduction in B lineage–committed cells, reduction in the proportion of pre–B cells, and reductions in CLPs and both the CD19<sup>+</sup>B220<sup>−</sup> B1 progenitor population on E14.5 and the CD19<sup>+</sup>B220<sup>−</sup> B2 progenitor population on E17.5. Gli3 mutation additionally reduced expression of B lineage–specifying and –signaling genes within the B220<sup>−</sup>CD19<sup>+</sup> population, indicating that the effect of Gli3 mutation is not entirely caused by its influence on CLPs and the earliest B cell progenitors but that it continues to influence the more mature B lineage–committed population. The changes in B cell differentiation in the Gli3-mutant FL could be caused by Hh–dependent or Hh–independent effects. We found that loss of Gli3 led to increased Shh expression and overall increased Hh signaling in the mouse FL. Thus, Gli3 was acting as a repressor of the Hh pathway in the FL, as observed in the development of other cells and tissues, such as the neural stem cells, vertebrate limb bud, and thymus (te Welscher et al., 2002; Hager-Theodorides et al., 2009; Petrova et al., 2013; Saldaña et al., 2016). Treatment of Gli3-deficient FLOCs with rHhip (to neutralize endogenous Hh proteins) increased B cell development, and therefore, the reduction in B cell development in the Gli3 mutants was Hh dependent and caused by increased Shh expression in the Gli3<sup>−/−</sup> FL.

In contrast, the Shh–deficient FL had increased B lineage commitment and B cell differentiation, demonstrating that Shh negatively regulates B cell development in vivo. We showed that both Shh transcription in FL stroma and Hh signaling to B-lineage cells are increased in the Gli3–deficient FL and that Shh negatively regulates B cell development by signaling directly to developing hematopoietic cells, as Hh-target genes were up-regulated in the Gli3<sup>−/−</sup> CD19<sup>+</sup>B220<sup>+</sup> population, and the Hh-reporter transgenic FL showed increased GFP expression in the Gli3<sup>−/−</sup> CD19<sup>+</sup> population.

Expression of the master regulators of B cell development, Ebf1 and Pax5, was reduced in the Gli3–deficient FL but increased in the Shh–deficient FL, and treatment of WT FLOCs with Hhip to neutralize endogenous Hh molecules in the cultures increased the proportion of Ebf1<sup>−/−</sup>Pax5<sup>−/−</sup> cells and increased transcription of both Ebf1 and Pax5 within the CD19<sup>+</sup> population, whereas rShh treatment had the opposite effect. The Ebf1<sup>−/−</sup>Pax5<sup>−/−</sup> population showed high Hh-mediated transcription in the Hh-reporter transgenic FL, and therefore, we proposed that Shh signaling within the Ebf1<sup>−/−</sup>Pax5<sup>−/−</sup> cells reduced expression of Pax5, thereby also reducing the Pax5–dependent induction of Ebf1 expression, leading to an overall reduction in B cell development.
RNA sequencing detects transcriptional differences in Hh signaling genes and B cell differentiation and signaling genes between the WT and Gli3-mutant CD19^+B220^+ cells. (A) PCA showing sample relationships in PC1 and PC3 for WT (n = 2), Gli3^+/− (n = 2), and Gli3^−/− (n = 2) CD19^+B220^+ populations from E17.5 FLs. (B) Venn diagram: 1,122 genes intersect out of the 3,000 genes that contributed most to PC1 (highest and lowest scoring genes) and the 3,000 most significantly expressed genes by Ebayes statistics. (C) Gene expression heat map showing Hh signaling genes in blue and B cell differentiation and signaling genes in black. Normalized expression signals are represented as a z score where green is lower expression and red is higher expression levels. (D and E) Transcript expression (reads per million kilobases) of pre-BCR/BCR signaling and target genes (D) and Shh, IL-7, and IL-7/thymic stromal lymphopoietin (TSLP) signaling and target genes (E) in the Gli3^−/− (green; n = 2) and WT (blue; n = 2) RNA sequencing datasets.
Figure 7. Expression of Pax5 and Ebf1 in the FL. (A) Representative experiment showing mean ± SEM (n = 3) of Q-RT-PCR for Pax5 and Ebf1 in FLs from Gli3 and Shh mutants compared with WT littermates. au, arbitrary units. (B and C) WT FLOCs (n = 3) were treated with rShh, rHhip, and rShh + rHhip for 2 and 4 d, compared with untreated control cultures, and analyzed by flow cytometry and Q-RT-PCR. (B) Dot plots: anti-Ebf1 and anti-Pax5 staining on day 2 (top) and day 4 (bottom). Charts: ratio of Ebf1+Pax5+ to Ebf1+Pax5− cells in the different culture conditions on day 2 (top) and day 4 (bottom).
Shh signaling from follicular dendritic cells to B cells in the adult spleen has been shown to promote B cell survival and function (Sacedón et al., 2005), and in the adult BM, components of the Hh signaling pathway are expressed in developing B cells (Heng et al., 2008; Cooper et al., 2012). However, conditional deletion of Smo from the B cell lineage did not influence B cell development in the adult BM (Cooper et al., 2012). Our study demonstrates that the Hh signaling pathway is active in developing fetal B cells and regulates B cell development in the FL. Therefore, there may be tissue- or life stage–specific differences in the function of Hh signaling between FL and adult BM. Alternatively, it is possible that Hh pathway activation is noncanonical (Smo independent) in B-lineage cells, which do not have primary cilia, or that a balance between canonical and noncanonical signaling may exist, as described in osteoblast differentiation (Yuan et al., 2016).

Our genome-wide RNA sequencing data from the sorted CD19+B220+ population in the Glil3 mutants revealed many differentially expressed genes between Glil3+/− and WT. Many Hh-target genes (e.g., Smrun1, Hdc3, Hoxa4, Hngb2, Bmi1, and Cul4a) were up-regulated in the Glil3 mutants, consistent with the increased Hh-mediated transcription measured using the GBS-GFP reporter and confirming that Shh signals directly to developing B cells.

In contrast, many B cell signaling pathway genes involved in NF-κB activity (Nikbid, Ntkbuz, and Tnaiip3), MAPK signaling (Mapkapk2 and Map3k3), and components of AP-1 (Junb and Fosb) were decreased in the Glil3 mutant. These pathways are required for pre-BCR and BCR signaling (Feng et al., 2004; de Gorter et al., 2007). In addition, Tnaiip3 regulates marginal zone and B1 cell development in the adult (Chu et al., 2011), and we observed a reduction in B1 progenitor cells in the E14.5 Glil3+/− FL. Interestingly, Hh-mediated transcription in developing and mature T cells also represses expression of genes that regulate activity of NF-κB, MAPK, and AP1, leading to reduced pre-TCR and TCR signaling (Rowbotham et al., 2007, 2009; Furmanski et al., 2012, 2015; Barbarulo et al., 2016).

In addition to the decreased transcription of genes associated with pre-BCR and BCR signaling, the RNA sequencing revealed a significant decrease in key transcriptional regulators of B cell differentiation including Ebf1, Foxo1, Runx1, and Irf4 (Dengler et al., 2008; Niebuhr et al., 2013). Ebf1 is required from the early CLP stage to the late mature stages of B cell development (Roessler et al., 2007; Nechanitzky et al., 2013) and, importantly, activates transcription of another key master regulator of B cell development, Pax5. Then, Pax5 promotes Ebf1 transcription, creating a positive feedback loop, which supports all stages of B cell development (Roessler et al., 2007).

We found high Hh pathway activity (measured by GFP expression in the Hh-reporter embryo) in Ebf1+/Pax5− cells, but GFP expression ceased in the next Ebf1+/Pax5− population. Both Ebf1 and Pax5 protein and gene expression were reduced by rShh treatment and increased by neutralization of Hh proteins by rHhip treatment in FLOCs, and manipulation of Hh signaling influenced the transition from Ebf1+/Pax5− to Ebf1+/Pax5+ cell. Therefore, we propose that Shh signaling to developing B cells functions to reduce Pax5 expression, which then breaks the positive feedback loop, leading to reduction in Ebf1 expression. Interestingly, Shh signaling has been shown to interact with and regulate other Pax family members in the development of other tissues (Chi and Epstein, 2002; Blake and Ziman, 2014).

Dysregulated Hh pathway activation is involved in some B cell malignancies (Dierks et al., 2007; Lin et al., 2010), including B cell acute lymphoblastic leukemia (B-ALL; Ramirez et al., 2012; Qu et al., 2013), a common cancer of early childhood, and microarray expression profiles show that Hh pathway components are expressed in human FL and BM (Fig. S1 A; Su et al., 2004). Understanding the function of Hh signaling in normal fetal B cell development and its effect on Pax5 and Ebf1 expression will be important to our understanding of its role in B-ALL. In the future, it will be important to investigate how dysregulated Hh signaling influences Pax5 and Ebf1 activity in B-ALL.

In summary, we show that Glil3 activity in the FL stroma is required for normal B cell development. We showed that Shh signaling directly to B-lineage cells negatively regulates their development.

**MATERIALS AND METHODS**

**Mice**

C57BL/6 mice were purchased from Envigo. GBS–GFP–transgenic (GBS–GFP-tg) mice were provided by J. Briscoe (Crick Institute, London, England, UK; Balaskas et al., 2012), Vav-iCre-tg by D. Kioussis (National Institute for Medical Research, London, England, UK; de Boer et al., 2003), and Shh+/− mice by P. Beachy (Stanford University School of Medicine, Palo Alto, California; Chiang et al., 1996) and were backcrossed for >12 generations on C57BL/6 mice. Gli3+/− mutants revealed Glil3+/−/+ and Glil3+/−/+ mice on day 4 of WT FLOCs treated with rShh or rHhip and control untreated. (D) Dot plots: anti-Pax5 and anti-Ebf1 staining in Glil3+/− and Glil3−/− littermate FLs. (E) Representative experiment showing mean ± SEM (n = 3) of Q-RT-PCR for Pax5 and Ebf1 on day 4 of WT FLOCs treated with rShh or rHhip and control untreated. (D) Dot plots: anti-Pax5 and anti-Ebf1 staining in Glil3+/− and Glil3−/− littermate FLs. All transcript expression levels were normalized relative to HPRT. (F) Histograms: GFP expression in Ebf1+/Pax5− and Ebf1+/Pax5+ cells in the E17.5 GBS–GFP–transgenic FLOCs, control (continuous lines), and rShh treated (dotted lines) FLs, cultured for 2 d.
Flow cytometry, antibodies, and cell purification

FL cell suspensions were made by crushing each FL between two frosted slides. Where stated, red blood cells were lysed using 1× RBC lysis buffer (eBioscience) according to the manufacturer's instructions. Cells were stained as described previously (Hager-Theodorides et al., 2005) using directly conjugated antibodies from BD, BioLegend, and eBioscience. Data were acquired on a C6 Accuri flow cytometer (BD) and analyzed using FlowJo software (Tree Star). Live cells were gated by forward scatter and side scatter (SSC) profiles. The data represent at least three experiments. In some experiments, CD19+ cells were purified using the EasySep biotin magnetic bead positive selection kit (STEMCELL Technologies) according to the manufacturer's instructions, and RNA was extracted for Q-RT-PCR analysis.

FLOCs

FLs were extracted from embryos at different stages of development. They were cut into ~1-mm cubes and cultured on 0.8-µm filters (EMD Millipore) in 1 ml of AIM-V serum-free medium (Invitrogen) in 24-well plates for up to 4-d at 37°C and 5% CO2 before analysis. In some experiments, rHhip (Sigma-Aldrich) or rShh (R&D Systems) was added at 1 µg/ml. To allow comparison between litters for statistical analysis, relative numbers or percentages for each genotype or treatment were calculated by dividing by the mean of controls from the same litter (untreated control or WT littermates).

RNA sequencing and data analysis

FL from WT, Gli3−/−, and Gli3+/− embryos (n = 2) were dissected on E17.5 and crushed between two frosted slides. The cell suspension was stained with the antibodies CD19-APC and B220-PerCP-Cy7, and the double-positive B220+/CD19+ population was FACs sorted. RNA from this population was extracted using an Arcturus PicoPure RNA isolation kit (Applied Biosystems), and quantity and quality were determined by a Bioanalyzer 2100 (Agilent Technologies).

RNA was sequenced by University College London Genomics on a NextSeq 500 system (Illumina). The sequenced data are publicly available in the GEO database under accession no. GSE81467. The RNA sequencing dataset was processed and standardized using the Bioconductor package DESeq2. The Bioconductor package DESeq2 was used to generate normalized estimates of transcript abundance, expressed as RPKM (reads per kilobase of transcript per million mapped reads). Differentially expressed genes were determined using the moderated EBayes t statistic P < 0.05 from the limma package in Bioconductor. PCA was performed using the CRAN package ade4.

Q-RT-PCR

RNA extraction and cDNA synthesis were performed as described previously (Sahni et al., 2015). We used the Quantitect primers for Gli1, Gli2, Shh, Hhip, Gli3, Sno, Ptc1, Ebf1, and Pax5 from Qiagen. The cDNA samples were prepared using the iQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions and run on a iCycler system (Bio-Rad Laboratories). Gene transcript levels were normalized relative to HPRT.

For quantification of VH to JH rearrangements, we prepared RNA from FACS-sorted CD19+ cells from Gli3−/− and WT E17.5 FL and followed the protocol described by Braikia et al. (2014), using the primers combinations: VH7183-Fw and JH1R; VH558-Fw and JH1R; and HS5-Fw1 and HS5-R1 for normalization.

PCR analysis for genotyping

DNA for PCR analysis was extracted from tissues by digesting in lysis buffer containing 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris HCl, pH 8.5, 0.01% gelatin, 0.45% Nonidet P-40, 0.45% Tween 20, and 0.5 µg/ml proteinase K (Sigma-Aldrich) in water. Approximately 1 µg DNA was used as a template in each PCR reaction, using primers for Shh+/−, Shh+/−/−, and Shh−/−/− as described by Shah et al. (2004). Gli3+/−, Gli3+/−, and Gli3−/− were distinguished phenotypically (Johnson, 1967), and genotype was confirmed by PCR as previously described (Hager-Theodorides et al., 2005). Gli3+/−, Shh+/−/−, and Vav-iCre-tg mice were genotyped as previously described (Saldaña et al., 2016).

Statistical analysis

Statistical analysis was performed using unpaired two-tailed Student’s t tests, and probabilities were considered significant if P ≤ 0.05 (*), P ≤ 0.01 (**), or P ≤ 0.001 (***)

Online supplemental material

Fig. S1 represents the transcript expression of Hh pathway members (GLI1, GLI2, GLI3, PTC1H1, SMO, and SHH) in the human BM and FL and shows transcript expression of Hh molecules (Shh, Ihh, and Dhh) and pathway components Gli1, Gli2, Gli3, Ptc1, and Sno from the ImmGen database in mouse FL. Table S1 is available as an Excel file and contains a list of 3,000 differentially expressed genes, significant by EBayes statistics, 1,500 genes with high positive PC1 scores, and 1,500 genes with high negative PC1 scores.

ACKNOWLEDGMENTS

This work was funded by the Medical Research Council (grants G090016/1 and MR/P000843/1), Biotechnology and Biological Sciences Research Council (grant BB/I026324/1), Wellcome Trust (grant WT094255MF), and Great Ormond Street Hospital Children’s Charity and supported by the National Institute for Health Research Bio-medical Research Centre (ormbr-2012-1) at Great Ormond Street Hospital for Children NHS Foundation Trust and University College London.

The authors declare no competing financial interests.
REFERENCES


SUPPLEMENTAL MATERIAL

Solanki et al., https://doi.org/10.1084/jem.20160852
Figure S1. Expression of Hh ligands and Hh signaling pathway components in human FL and BM and mouse B cell FL populations. (A) Transcript expression of human Hh pathway members (GLI1, GLI2, GLI3, PTCH1, SMO, and SHH) in the human BM (circles) and FL (triangles) for duplicate biologically independent microarrays from the publically available GEO database (accession no. GSE1133). (B–F) Transcript expression of (Shh, Ihh, Dhh, and Gli1, Gli2, Gli3, Ptc1, and Smo) in CLP (AA4+Kit+IL-7a+B220−; B), fraction A (Fr.A; AA4+Kit+IL-7a+B220−; C), fraction B/C (AA4+IgM−CD19+CD43+HSA+; D), fraction D (AA4+IgM−CD19+CD43+HSA−; E), and fraction E (AA4+IgM+CD19+HSA+; F). Triplicates are from microarrays from independent biological replicates from the Immgen database (GEO database accession no. GSE15907). Line shows mean normalized expression ± SEM for each gene.
Hedgehog Signalling in the Embryonic Mouse Thymus

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Academic Editors: Henk Roelink and Simon J. Conway

Received: 14 June 2016; Accepted: 14 July 2016; Published: 16 July 2016

Abstract: T cells develop in the thymus, which provides an essential environment for T cell fate specification, and for the differentiation of multipotent progenitor cells into major histocompatibility complex (MHC)-restricted, non-autoreactive T cells. Here we review the role of the Hedgehog signalling pathway in T cell development, thymic epithelial cell (TEC) development, and thymocyte–TEC cross-talk in the embryonic mouse thymus during the last week of gestation.

Keywords: thymus; Hedgehog; Shh; Ihh; T cell development; thymic epithelial cell (TEC); Gli3; Gli2; Gli1

1. Introduction

T cells develop in the thymus, which provides an essential environment for T cell fate specification, and for the differentiation of multipotent progenitor cells into major histocompatibility complex (MHC)-restricted, non-autoreactive T cells. Here we review the role of the Hedgehog (Hh) signalling pathway in T cell development, thymic epithelial cell (TEC) development, and thymocyte–TEC cross-talk in the embryonic thymus.

The thymus is an epithelial organ surrounded by a mesenchymal capsule. In the mouse embryo, it develops from the third pharyngeal pouches, which also gives rise to the parathyroids [1]. By embryonic day (E)12.5, the thymus and parathyroid have become distinct organs, and the thymus first starts to be seeded by haematopoietic progenitor cells that migrate from the foetal liver. Progenitor cells enter the thymus from the outside through the capsule, and migrate towards the center of the thymus as they differentiate (illustrated in Figure 1). This is in contrast to the adult thymus, which is seeded by progenitors that enter through blood vessels at the corticomedullary junction.

During embryonic life, mature T cells are first produced on ~E18.5, which then start to leave the thymus and move to peripheral lymphoid organs by birth. In addition, TECs develop into two distinct populations—medullary(m) TEC and cortical(c) TEC—with different functions and locations within the thymus [2]. Haematopoietic progenitor cells are dependent on cTEC for T cell fate specification and positive selection of the T cell receptor repertoire (TCR), while mTEC are necessary for tolerance induction (negative selection of the TCR repertoire and differentiation of regulatory T cells) [3]. At the same time, TECs require signals from thymocytes for their development, and thymocyte–TEC cross talk in the embryonic thymus has been shown to be essential for establishment of the mature mTEC population and the architecture of the mature thymus, while its role in cTEC differentiation is less well-defined [2,4,5].
Figure 1. Hedgehog (Hh) expression in different microenvironments as thymocytes migrate through the embryonic thymus. The cartoon illustrates the stages of T cell development in the embryonic thymus, as developing thymocytes move through different thymus microenvironments and receive different amounts of Hh signal. Molecules of Indian hedgehog (Ihh, expressed by double positive (DP) thymocytes) are shown in green, and molecules of Sonic hedgehog (Shh, expressed by TEC in the subcapsular region and medulla) are shown in yellow. Progenitor cells first enter the embryonic thymus through the capsule on ~E12.5, and migrate towards the centre of the thymus as they differentiate. DP cells first appear on E16.5 and are located in the cortex. Mature single positive (SP) T cells and mature medullary TEC (mTEC) and cortical TEC (cTEC) populations are present by birth.

During T cell development, thymocytes pass through a series of stages that have been defined by the expression of cell surface markers as they migrate through the thymus [6] (see Figure 1). In brief, the earliest thymocytes—termed CD4−CD8− double negative (DN)—rearrange the TCRβ chain locus and express the pre-TCR in order to differentiate to the CD4+CD8+ double positive (DP) stage. They then rearrange the TCRα locus and must signal through the αβTCR to differentiate to mature CD4+ single positive (SP) or CD8+ SP T cell. The DN population has been further subdivided by cell surface expression of CD44 and CD25. The earliest DN1 populations are CD44+CD25−; these then acquire CD25 expression (DN2), lose CD44 expression (DN3), and finally become CD44−CD25− (DN4) cells before differentiating to DP cell, often via a CD8+ immature single positive (ISP) intermediate.

The development of TEC in the embryonic thymus is less well understood, but both lineages of TEC originate from a common CD45−Epcam-1+CD40lowCD205low progenitor cell population [7,8]. As development proceeds, these cells gain greater density of CD40 and CD205 expression, resulting in transitional progenitors which have the potential to differentiate into two populations—cTEC (CD40intCD205highLy51+) or mTEC (CD40highCD205lowUEA-1+) [9,10].

2. Expression of Hedgehog (Hh) Proteins and Pathway Components in the Embryonic Thymus

There are three mammalian Hh proteins (Sonic Hh, Shh; Indian Hh, Ihh; and Desert Hh, Dhh), which share a common signalling pathway [11]. When Hh proteins bind to their cell surface receptor Patched1 (Ptc1), the inhibition of Ptc1 on the signal transducer molecule Smoothened (Smo) is relieved, and Smo transduces the Hh signal. At the end of the signalling pathway are the Gli family of transcription factors (Gli1, Gli2 and Gli3). Gli1 is an activator of transcription only, whereas Gli2 and Gli3 can be processed (cleavage and post-translational modification) to function as
We carried out microarray analysis of gene expression in fluorescence activated cell sorting (Facs)-sorted cTEC and mTEC purified from E14.5 foetal thymus organ culture (FTOC) after 7 days in culture. TEC were isolated as described [21,23–25]. We carried out microarray analysis of gene expression in fluorescence activated cell sorting (Facs)-sorted cTEC and mTEC purified from E14.5 foetal thymus organ culture (FTOC) after 7 days in culture. TEC were isolated as described [21,23–25].

Hh pathway components are also expressed by TEC in the embryonic thymus [21,22,27–29]. Reverse transcription (RT)-PCR has shown that developing foetal thymocytes express the Gli transcription factors, Ptc1, Smo, and Ihh, but not Shh; however, TEC express Shh [21–25]. Quantitative (Q)RT-PCR from sorted thymocyte subsets on E16.5 showed that the Gli genes are differentially expressed during T cell development, with highest expression of Gli2 in the DN1 and DN2 populations, whereas Gli1 expression is highest in the DN3 population, and Gli3 in the DN4 population [21,23–25]. In contrast, Ihh expression is undetectable in the earliest DN thymocyte subsets, and is most highly expressed in the DP population (Figures 1 and 2). Flow cytometry, RT-PCR and QRT-PCR have demonstrated that Smo is highly expressed in DN thymocytes, and down-regulated following pre-TCR signalling, whereas Ptc1 expression is maintained in DP cells [22]. Expression levels of Smo reduce with maturity in the DP population, but DP cells can transduce Hh signals [26].

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Figure 2. Expression of components of the Hh signalling pathway in embryonic thymocytes and TEC. (A) Graphs illustrate the relative expression of Gli1, Gli2, Gli3, and Ihh in sorted DN1, DN2, DN3, DN4, and DP thymocytes from E16.5 thymus, determined by quantitative reverse transcription polymerase chain reaction (QRT-PCR); (B) Plots show the normalised expression of Aire, Ptc1, Smo, Shh, Ihh, Dhh, Gli1, Gli2, and Gli3 by microarray analysis (GSE81433) from fluorescence activated cell sorting (Facs)-sorted cTEC (CD45–EpCam1+Ly51+UEA-1–, left-hand plot), and mTEC (CD45–EpCam1+Ly51–UEA-1+, right-hand plot); populations prepared from E14.5 WT (C57BL/6) foetal thymus organ culture (FTOC) after 7 days in culture. TEC were isolated as described [29].
3. Regulation of T Cell Development by Hh Signalling

Analysis of T cell development in the foetal thymus of multiple Hh pathway mutants has shown that Hh signalling is an important regulator of T cell development in the embryo, which affects several stages of thymocyte development (Figure 3). We will review the experimental evidence showing that Hh signalling influences three key check-points in embryonic αβ T cell development: the transition from DN1 to DN2 cell; pre-TCR induced differentiation to DP cell; and the transition from DP to mature SP cell.

![Figure 3](image)

**Figure 3.** Hh signalling regulates multiple stages of thymocyte development. The cartoon illustrates the different stages of thymocyte differentiation that are regulated by Hh signalling in the embryonic thymus. (TCR: T cell receptor.)

3.1. Hedgehog Signalling in DN1 and DN2 Cells

As developing thymocytes differentiate from the earliest DN1 progenitors to express cell surface CD25 and become DN2 cells, they specify to the T cell fate, and initiate recombination of their TCRβ gene locus [6]. At this stage, Shh, Gli2 and Gli3 are required for differentiation and expansion of the DN2 population. Thymi from E13.5 Shh−/− embryos are much smaller than those of wild type (WT) littermates and have ~10 times fewer thymocytes and a reduction in the DN2 population [19]. Reduced differentiation to DN2 cell has also been observed in E13.5 Gli2−/− and Gli3−/− thymus [23,25]. In contrast, analysis of Gli1-deficient foetal thymus has shown that Gli1 is dispensable for the DN1 to DN2 transition [24]. In many tissues, Gli3 functions to repress Hh pathway activation, and Gli3-deficiency has the opposite phenotype to Shh-deficiency, so the fact that mutants of both Shh and Gli3 show reduced early thymocyte differentiation might seem surprising. However, the expression pattern of the Gli transcription factors in the DN populations is consistent with the findings from the Gli-mutants in early DN cell development. Gli2 and Gli3 are both highly expressed in DN1 and DN2 cells, while expression of Gli1 in early DN subsets is relatively low (Figure 2).

The role of Ihh in early thymocyte development has also been studied [21]. Analysis of E13.5 Ihh−/− thymus shows that deletion of Ihh alone has no influence on development at the DN1 to DN2
transition. However, analysis of the double-mutant Shh+/−/Ihh−/− foetal thymus showed a reduction in the DN2 population, indicating that Shh and Ihh have redundant functions at the DN1 to DN2 transition [21].

3.2. The Transition from DN to DP Cell and Pre-TCR Signalling

Differentiation from DN3 cell through to DP cell requires successful rearrangement of the TCRβ gene locus and formation of the pre-TCR complex [6]. Signalling through the pre-TCR complex results in a complex transcriptional programme, leading to expansion, survival, allelic exclusion of the TCRβ gene locus, and differentiation to DP cell [30,31]. In foetal thymocyte development, differentiation from DN3 to DN4 cell seems to be somewhat uncoupled from pre-TCR signal transduction and TCRβ chain expression [32], and thymocytes that have failed to rearrange their TCRβ gene locus die at the DN4 stage [32,33].

Hedgehog signalling to DN thymocytes negatively regulates pre-TCR-induced differentiation to DP cell in both mouse and human [22,25,34]. The first evidence for this came from in vitro studies which demonstrated that Hh signalling negatively regulates DN to DP differentiation in foetal thymus organ cultures (FTOCs) that were treated with recombinant (r) Shh [22]. Thymocyte development was arrested at the DN3 stage, whereas FTOCs treated with a neutralizing monoclonal antibody (mAb) against Shh showed increased differentiation from DN to DP cell. Thymocytes from Recombination-activating gene 1 (Rag1)−/− FTOCs can be induced to differentiate from DN3 to DP cell by treatment with anti-CD3 mAb, which mimics the pre-TCR signal [31,35]. Addition of the anti-Shh mAb to anti-CD3 treated Rag1−/− FTOC enhanced thymocyte differentiation to the DP stage, whereas treatment with rShh arrested it, confirming the negative regulatory role of Shh at this developmental stage.

In the embryo, DP cells first appear on E16.5, allowing observation of the transition from DN to DP population in a synchronized manner. Analysis of the E16.5 Shh−/− foetal thymus unexpectedly showed that the transition from DN to DP cell was reduced compared to WT, in contrast to the in vitro studies [19]. However, the E16.5 Shh−/− thymus also had increased apoptosis in the DN4 population compared to WT, consistent with increased death of cells that had failed to rearrange the TCRβ locus. This suggested that the requirement for Shh before pre-TCR signalling might impact the production of DP cells on E16.5 [19].

In vivo evidence for the negative regulatory role of Hh signalling at this developmental stage came from analysis of foetal thymocyte development in embryos mutant for Gli2 and Gli3 [23,25]. From E13.5 to E15.5 Gli2−/− thymi were smaller than that of WT littermates, whereas the E17.5 Gli2−/− thymus contained over twice as many thymocytes as WT. The expansion of the Gli2−/− thymus on the embryonic day after DP cells first appear, together with the expression pattern of Gli2 in the foetal thymus which shows three-fold upregulation in DN4 compared to DN3 cells, suggested that Gli2 plays a negative role in thymocyte expansion and progression to DP after pre-TCR signal transduction during foetal development. This was confirmed in anti-CD3-treated Rag1−/−/Gli2−/− FTOCs, which expanded and differentiated to DP faster than their anti-CD3 treated Rag1−/−/Gli2+/+ counterparts. Interestingly, anti-CD3-treated Rag1−/−/Shh−/− FTOC also differentiated faster than anti-CD3-treated Rag1−/−/Shh+/+ FTOC, confirming that Gli2 is downstream of Shh as a negative regulator of differentiation after pre-TCR signalling [25]. In contrast, analysis of anti-CD3-treated Rag1−/−/Gli3−/− FTOCs revealed a partial arrest at the DN-to-DP stage after pre-TCR signalling [23]. The same result was observed in E16.5 Gli3−/− thymus, indicating that Gli3 functions as a transcriptional repressor of the Hh pathway at this developmental stage, and that Gli3 activity is required for normal differentiation from DN to DP cell.

Given that the Gli transcription factors are expressed by both stromal and thymocyte compartments of the foetal thymus (Figure 2), these experiments with constitutive knockouts did not test whether or not Shh was directly signalling to thymocytes to control their differentiation, or whether the impact on T cell development was an indirect effect due to Hh signalling to TEC or
another stromal cell population. Therefore, the impact of Hh-mediated transcription in developing thymocytes on their differentiation and function was investigated by the production of transgenic (tg) mice which over-expressed activator (Gli2A-tg (Gli2ΔN2-tg)), or repressor (Gli2R-tg (Gli2ΔC2-tg)) forms of Gli2 in T lineage cells only (driven by the lck promoter) [36,37]. Anti-CD3-treated Rag1−/− Gli2A-tg FTOC differentiated more slowly to DP than their non-transgenic counterparts, whereas anti-CD3-treated Rag1−/− Gli2R-tg FTOC—in which physiological Hh-mediated transcription is repressed—differentiated more quickly [25]. Thus, Hh pathway activation in developing thymocytes negatively regulates pre-TCR-mediated differentiation to DP cell.

3.3. Ihh at the Transition from DN to DP Cell and Its Role in Homeostasis and the Control of DP Cell Number

Ihh is produced by both the foetal thymus stroma and developing thymocytes with a six-fold higher expression in the DP population compared to DN cells and stroma, which show similar Ihh expression levels [21] (Figure 2). The DP population expresses low levels of the target gene Gli1, suggesting that Ihh is not signalling to the DP population in an autocrine manner, but feeding back to signal to the DN3 population, which express the highest levels of Gli1.

In the E16.5 Ihh−/− embryo, both thymocyte numbers and the proportion of DP cells were reduced, indicating that Ihh promotes T cell development. Interestingly, however, the E16.5 Ihh+/− thymus contained 2.4 times more DP cells than WT, implying that Ihh also negatively regulates thymocyte development. Ihh+/+ Rag1−/− FTOCs contained more cells than their Ihh+/− Rag1−/− counterparts, but after anti-CD3 treatment for 5 days, the Ihh+/− Rag1−/− FTOCs differentiated faster than the Ihh+/+ Rag1−/− FTOC, confirming that Ihh promotes early DN thymocyte development before the pre-TCR signal, but negatively regulates the pre-TCR-induced transition to the DP stage.

In the Ihh+/− E16.5 thymus, the sorted CD25+ DN population contained significantly more cells in cell cycle (S/G2+M) than its WT counterpart. Taken together, these data indicated that Ihh produced by the DP population feeds back to negatively regulate the differentiation and expansion of the DN3 population after pre-TCR signal transduction. Thus, as the total amount of Ihh protein present in the thymus largely depends on the size of the DP population, this negative feedback loop may be thought of as a counting system to regulate thymocyte number and maintain thymocyte homeostasis [21].

3.4. Hh Signalling in TCR Repertoire Selection and the Transition from DP to SP Cell

Hh signalling also plays an important role during the maturation of DP to CD4SP and CD8SP cell in the embryonic thymus. Maturation from DP to SP cell occurs following successful rearrangement of the TCRα gene locus, and requires TCR signalling; positive selection ensures appropriate MHC-restriction of SP cells, followed by negative selection of potentially self-reactive clones [3,6]. Many models have been proposed to describe how DP thymocytes commit to the CD4 and CD8 lineages, and to explain how positive selection ensures that selected CD4 and CD8 SP populations express TCR appropriately restricted by MHCII and MHCI, respectively [38]. Strength and duration of the TCR signal that a developing cell receives broadly determine its fate, with the strongest signals leading to negative selection, usually at the SP stage in the medulla (of TCR recognizing self-antigens)—intermediate signals leading to positive selection, and weaker signals or lack of TCR signalling leading to cell death by neglect. For DP thymocytes undergoing positive selection, TCR signal strength and duration again influence CD4 and CD8 fate decision, with those cells receiving stronger TCR signals tending to be biased towards the CD4 fate, and weaker/more transient signals tending to favour differentiation to CD8 SP. TCR signal strength and duration are dependent on the avidity of the TCR for its ligand (and therefore on the TCR sequence), but may also be modulated by other factors, such as coreceptor signalling, intracellular or extracellular influences on TCR signal transduction, and integration of the transcriptional outcome of TCR signalling with the transcriptional context of the cell.
Analysis of embryonic thymus from Shh−/−, Gli1−/−, Gli2−/−, Gli2A-transgenic (tg) and Gli2R-tg, and in vitro FTOC experiments have all shown that Shh negatively regulates the DP to SP transition, most likely by lowering TCR signal strength (see Figure 4) [24,26,36].

Figure 4. Hh signalling at the transition from DP to SP thymocyte. (A) The cartoon illustrates the influence of Shh on positive selection. Positive selection is increased in the Shh−/− thymus and the proportion of CD4SP cells is increased. Cell surface expression of major histocompatibility complex class II (MHCII) is increased on Shh−/− TEC compared to wild type (WT) TEC. (B) The influence of Gli3 on negative selection. In the Gli3−/− thymus MHCII expression is decreased in TEC and nitric oxide (NO) activity is decreased. This may allow SP thymocytes to escape from negative selection.

The Shh−/− embryonic thymus is smaller than WT but has a higher SP:DP ratio, reflecting an accelerated rate of differentiation from DP to SP [36]. Shh−/− FTOCs had a greater proportion of mature CD4SP cells and an increased CD4SP:CD8SP ratio than WT (Figure 4A). Likewise, Gli2−/− and Gli1−/− FTOCs showed increased differentiation from DP to SP cells and increased CD4SP:CD8SP ratio [24]. The increased differentiation towards the CD4SP lineage might reflect an increase in TCR signal strength, and both positive and negative selection of transgenic TCR were increased in Shh−/−, Gli2−/−, and Gli1−/− FTOC compared to control [24,36]. Analysis of the Gli2A-tg and Gli2R-tg embryonic thymus confirmed that physiological Hh-mediated transcription in thymocytes negatively regulates the DP-to-SP transition and TCR repertoire selection [26].

Cell surface CD5 intensity is an indicator of TCR signal strength on thymocytes, with higher CD5 expression reflecting a stronger TCR signal and vice versa [39,40]. Gli2R-tg FTOCs, in which physiological Hh-mediated transcription is inhibited, showed increased differentiation to CD4SP,
higher CD5 expression on the CD4SP population, and a higher CD4SP:CD8SP ratio than WT [26]. Doubling the Gli2R-transgene copy number further increased the proportion of CD4SP cells, their CD5 cell surface expression, and the CD4SP:CD8SP ratio [26]. Interestingly, these dose-dependent phenotypic changes were reversed with rShh treatment of WT FTOC. Decreased cell surface CD5 expression and a lower CD4SP:CD8SP ratio were observed when WT FTOC were treated with rShh [36]. Therefore, an increased level of Shh signalling appeared to give rise to weaker TCR signal strength, leading to reduced selection from DP to CD4SP; whereas selection to the CD8 lineage, which is believed to require lower strength and duration of TCR signal transduction, was less affected.

Gata3 is a key regulator of CD4 lineage commitment, and strong TCR signals increase expression of Gata3, which drives differentiation towards the CD4 lineage [41]. Interestingly, treatment of WT FTOCs with rShh decreased the proportion of Gata3-expressing DP and SP cells, while attenuating Shh signalling by treatment with the neutralising anti-Shh mAb increased the intensity and expression of Gata3 on the DP and SP4 populations [26]. Thus, the influence of modulation of Shh signalling on Gata3 expression is consistent with the effect of Shh on CD4/CD8 lineage commitment, and with its influence on TCR signal strength [36,42].

TCR signal strength is a crucial determinant of the TCR repertoire and CD4/8 lineage commitment and the influence of Shh on TCR signal strength may thus alter both of these: Since Shh-expressing TECs are scattered around the medulla and the cortico-medullary junction, their influence on TCR signal strength and the outcome of TCR ligation (i.e., positive or negative selection and CD4/8 lineage decision) for each cell is dependent on the location of the cell relative to the source of Shh. It is therefore possible that Shh-expressing TEC may have specialised functions in the thymus, such as induction of positive selection or commitment to the CD8 lineage.

3.5. Gli3 Regulates Nos2 Expression during Negative Selection

Analysis of the Gli3 mutant thymus showed that Gli3 acts as a repressor of Hh signalling in the foetal thymus and that Gli3 mutation may also influence TCR repertoire selection in developing thymocytes [23,27]. These experiments were carried out using constitutive Gli3 knock-outs, so that the lineage-specific requirements for Gli3 are not known, and both the Gli3−/− foetal thymus stroma and foetal thymocytes had increased expression of the transcriptional target of Hh-signalling, Gli1 [24,27]. Treatment of Gli3−/− FTOC with a neutralising anti-Shh mAb reduced Gli1 expression in the stroma [27], demonstrating that the increase in Gli1 expression was a result of increased Hh signalling.

PCR array analysis of gene expression in the Gli3−/− foetal thymus stroma compared to WT identified Gli3-target genes in the stroma, and revealed that Nos2 is a Gli3 target gene, which was significantly down regulated in the Gli3−/− and Gli3+/− stroma [27]. Nos2 expression recovered to WT levels when Gli3+/− FTOC was treated with an anti-Hh neutralising mAb, but was not affected when Gli3−/− FTOC were treated with an anti-Hh mAb, suggesting that Nos2 required Gli3 for its physiological levels of expression (most likely through Gli3R’s repression of an intermediate transcriptional repressor of Nos2) [27].

Nos2 is induced in the thymic stroma during negative selection. It causes the production of nitric oxide (NO), a potent pro-apoptotic agent, which promotes apoptosis of autoreactive thymocytes [43]. Treatment of WT and Gli3−/− FTOC with anti-CD3 mAb to mimic negative selection, showed that the Gli3−/− DP cells had decreased intracellular active caspase-3 expression, correlating with a lower level of apoptosis compared to WT. This suggests that negative selection-related apoptosis of DP thymocytes is attenuated in the Gli3−/− thymus and that this may be due to reduced Nos2 expression in the thymic stroma. (Figure 4B). It is likely that this would lead to a change in the TCR repertoire and survival of autoreactive thymocytes in the embryonic thymus. Indeed, negative selection of an MHCI-restricted transgenic TCR is reduced in the adult Gli3+/− thymus [27], although the impact of Gli3-mutation on negative selection of a transgenic TCR has not been investigated in the embryonic thymus.
4. Hedgehog Signalling in Foetal Thymic Epithelial Cell (TEC) Development and Function

The role of the Hh pathway in embryonic TEC development has been less extensively investigated than its function in T cell development, although Shh is involved early in thymus organogenesis during patterning of the pharyngeal pouches [44]. In this section, we will briefly discuss the early role of Shh in pharyngeal patterning and thymus organogenesis before reviewing recent experiments demonstrating that Hh signalling is required for normal TEC development in the embryonic thymus during the last week of gestation.

Shh is involved in the formation and patterning of the entire pharyngeal apparatus and affects the third pouch and the thymic anlage [44]. In Shh−/− embryos, the development of the third pharyngeal pouch is defective and the thymic rudiment fails to bud off entirely from the pharyngeal endoderm [45]. However, once the Shh−/− thymus is formed, although it is small, it is capable of supporting T cell development; however, there are defects in normal TEC development [19,21].

There are two types of TEC, which are derived from a common progenitor, and which have distinct functions, localisations within the thymus, and cell surface markers [2,4,5,46]. Cortical(c) TECs provide DL4 for T cell fate specification, and present MHC+peptide ligands for positive selection. These cTECs are defined as EpCam1+, CD205+, Ly51+ and MHCII+, and express genes for antigen presentation, including Cathepsin-L, Prs16, and β5t. Medullary(m) TECs are specialised for induction of negative selection, are defined as cell surface EpCam1+, CD40+, CD205−, Ly51− and MHCII−, and bind the lectin UEA-1. They express the Aire gene and Cathepsin-S, facilitating the expression and presentation of Tissue Restricted Antigens (TRA) for induction of tolerance. During embryonic development, the cell surface markers CD40 and CD205 can be used to map TEC development. CD40lowCD205low progenitor cells gain cell surface CD40 and CD205 expression, resulting in transitional progenitors which have the potential to differentiate into the two mature populations: cTEC (CD40intCD205highLy51+) and mTEC (CD40highCD205lowUEA1+) [9,10].

Embryonic TECs express components of the Hh signalling pathway as well as the genes encoding the Hh ligands (Figure 2). Therefore, to investigate if TECs are transducing Hh signals in vivo in the embryonic thymus, Gli Binding Site (GBS)-Green Fluorescent Protein (GFP) transgenic embryos—which report Hh-mediated transcription by expression of GFP—were analysed [27]. Hh pathway activation was observed in the TEC populations of embryos from E14.5 through to birth (neonate). The greatest intensity of GFP-fluorescence was observed on E14.5, and fluorescence was seen TEC development and the regulation of mTEC/cTEC lineage choice [29]. In the Shh−/− foetal thymus, there was a reduction in thymus size, overall cell number, and number of thymocytes and of TECs (CD45−EpCam1+) compared to WT littermates [19,21,29]. The numbers of both mature cTECs (CD45−EpCam1+Ly51+UEA1−) and mTECs (CD45−EpCam1+Ly51−UEA1+) were reduced in Shh−/− compared to WT, but the mTEC lineage was particularly affected. The proportion of mTECs generated in Shh−/− FTOC fell from 27.4% to 13.2%, compared to WT FTOC, with a concomitant increase in the proportion of mature cTECs [29]. Interestingly, although the number of TECs was reduced in the Shh−/− FTOC, cell surface expression of MHCII was increased in both cTEC and mTEC (Figure 5) [29]. Expression of MHCII is essential on cTECs to provide ligands for positive selection for differentiation from DP to CD4SP, and on mTECs for negative selection of MHCII-restricted thymocytes. The increase in MHCII expression on cTECs and mTECs in the Shh−/− foetal thymus could therefore potentially influence the outcome of positive and negative selection of thymocytes (Figures 3–5) [29]. Although there are overall fewer TECs in the Shh-deficient thymus, on each individual TEC, more MHCII-plus-peptide complexes for positive and negative selection will be
available to developing thymocytes, and so it is possible that this may affect the outcome of TCR repertoire selection to the CD4 T cell lineage by changing MHCII-restricted TCR antigen dwell time or avidity [47] (see Figures 4 and 5).

The influence of Shh on foetal TEC development was confirmed when WT FTOC were treated with recombinant Hhip, a Hh-binding protein that neutralizes endogenous Hh proteins in the cultures. Hhip-treatment led to an overall decrease in TEC cell numbers and a significant decrease in the proportion of mature mTECs. The proportion of mTECs that expressed Aire protein was also reduced, but there was an increase in cell surface MHC-II on the mTEC population [27].

The opposite was observed in Gli3+/− foetal thymus (Figure 5). There was an increase in overall TEC numbers but a decrease in cell surface expression of MHC-II in both cTEC and mTEC populations (Figures 4B and 5) [29]. This is consistent with Gli3 acting as a suppressor of Shh in the foetal thymus [27].

5. Conclusions and Future Directions

Hedgehog signalling influences multiple stages of T cell development, and also regulates foetal TEC differentiation (Figures 3 and 5). Very few pathways or secreted signalling molecules have been identified that regulate foetal TEC development or cTEC/mTEC lineage choice, so it will also be important to identify the mechanisms of Shh’s regulation of TEC differentiation in the embryonic thymus. It will be interesting to identify Hh target genes in different subsets of TEC, and test the impact of mutation of these genes on TEC development and function, and on the ability of TEC to support T cell development.

Hh signalling influences three key check-points in embryonic αβT cell development: the transition from DN1 to DN2 cell; pre-TCR-induced differentiation to DP cell; and the transition from DP to mature SP cell. The different mechanisms that account for these different developmental-stage specific outcomes of Hh pathway activation in developing T cells are as yet unknown, and Hh target genes have not been identified in thymocyte subsets.
In the future, it will also be important to investigate the interactions between Hh signalling and other pathways that interact with Hh signalling in other tissues, and also regulate foetal thymocyte development—such as BMP2/4 signalling [48–50]. BMP2/4 signalling is also involved in early thymus organogenesis and is required for early TEC development, so it will also be interesting to assess interactions between BMP and Shh signalling in TEC development [51,52].

Acknowledgments: This work was funded by grants from the MRC (G0900161/1), BBSRC (BB/I026324/1), Wellcome Trust (090233/Z/09/Z) and supported by the National Institute for Health Research Biomedical Research Centre at Great Ormond Street Hospital for Children NHS Foundation Trust and University College London. AB was funded by a fellowship from the Istituto Pasteur/Cenci Bolognetti Foundation. AS and TC were funded by Great Ormond Street Hospital Children’s Charity.

Author Contributions: A.B., C.-I.L., K.M., S.R. and A.S. contributed equally to the preparation of this review. J.I.S. carried out the microarray analysis, which was analysed by A.S.. T.C. Contributed to preparation of this review.

Conflicts of Interest: The authors declare no conflict of interest.

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