Polarity during migration & asymmetric cell division in fate determination of thymocytes

By Kim Pham

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&
The Faculty of Engineering and Industrial Sciences
Swinburne University of Technology
Dedicated to my godmother

Elaine Margaret Dellavedova

1937-2012
Abstract

The thymus is the site that generates many of the T cell subsets for our adaptive immunity. The T cells that mediate immune responses are generated in the thymus from thymocytes. Elucidating the mechanisms for thymocyte fate determination has the potential for profound impact upon autoimmunity, vaccination, recovery from immunosuppression during chemotherapy and leukemogenesis.

In solid tissues, fate determination is dictated by asymmetric cell division (ACD). ACD involves the induction and maintenance of polarity during division, resulting in two daughter cells with different molecular composition and fates. Recently, studies have shown that the fate of hematopoietic stem cells and T cells are influenced by ACD, and that the mechanisms of polarity are conserved. It is unknown whether ACD occurs in thymocytes to determine fate outcome.

In this thesis I investigated whether ACD occurs in DN3 thymocytes. Using an in vitro system of T cell development, I established protocols for time lapse imaging of thymocytes, and co-developed a software platform to quantify polarisation in migrating and dividing DN3 thymocytes. A number of proteins previously implicated in ACD polarise in DN3 thymocytes, and excitingly, two of these, Numb and Ap2a2, polarise in DN3 thymocytes during division. These experiments provide the first quantified evidence that DN3 thymocytes undergo ACD.

To investigate the molecular mechanisms and consequences of DN3 ACD, I used several in vitro approaches and a mouse model of T cell leukaemia driven by overexpression of Lmo2. Abrogation of Numb phosphorylation by the polarity protein aPKC prevents Numb polarisation during DN3 division, but not during migration. Disruption of chemokine signalling reduces Ap2a2 polarisation, providing a mechanism through which ACD may be controlled. Using Ap2a2 polarisation as a marker for ACD, I demonstrate that the altered fate of leukaemic DN3 thymocytes is associated with a reduction in ACD. Combinatorial effects of Lmo2 overexpression, inhibition of chemokine signalling and Ap2a2 overexpression correlate with effects on polarity and differentiation of DN3 thymocytes, suggesting that ACD of DN3 thymocytes impacts upon DN3 fate decisions.

These results indicate for the first time that thymocytes undergo ACD, and suggest a novel molecular mechanism for control of fate in thymocytes. Furthermore, they suggest that disruption of ACD is associated with development of leukemia. My preliminary evidence, together with the technological platform that I developed provide a foundation for future experiments to investigate the impact of ACD with thymocyte fate outcome in normal development and leukemogenesis.
Preface

I acknowledge the assistance of the following people who contributed to the work described in this thesis. Professor Juan Carlos Zuniga Pflucker (University of Toronto, Sunnybrooke Health Sciences Centre), for the OP9 stromal cell lines used throughout the thesis. Dr. David Izon (St. Vincents Research Institute), for the colourless OP9-DLL1 stromal cell line that was used throughout the thesis, and for many scientific discussions. Dr. Adam Uldrich (The University of Melbourne), for teaching me adult thymic dissection, thymus flow cytometry analysis and complement killing. Dr. Matthew McCormack (The Walter and Eliza Hall Institute), for providing the CD2-Lmo2 foetal livers as well as helpful discussions on leukaemia, used in Chapters 1, 6 and 7. Professor Ellen Robey and Dr. Heather Melichar (University of California Berkeley), for teaching me foetal thymic lobe dissection and for helpful scientific discussion on thymocyte polarity, used in Chapters 1 and 3. Dr. Daniel Day (Swinburne University of Technology), who microfabricated the cell paddocks shown in Figure 3.1 that was used throughout the thesis. Mr. Raz Shimoni (Swinburne University of Technology), who wrote the TACTICS Graphical User Interface and co-developed the TACTICS software platform, used in Chapters 4, 5 and 6. The Matlab community and Yair Altman, for providing source code for the TACTICS Graphical User Interface. Adam Poetter (work experience high school student), for time lapse data analysis of dividing thymocytes that was used in Chapter 5 and 6. Professor Terry Speed, for insightful advice on stratification of data and statistics; used in Chapter 4. Professor Jane McGlade (University of Toronto, Hospital for Sick Children), who provided the Numb-phosphorylation mutant construct, the phosphorylated Numb antibody and helpful scientific discussion for Chapter 4. Dr. Stephen Ting (Australian Centre for Blood Diseases), for providing the Ap2a2 construct as well as discussion relating to haematopoietic asymmetric cell division, used in Chapters 1 and 5. Ms Mandy Ludford-Menting, for generating the retroviral constructs used in Chapters 4 and 5.
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Declaration

This is to certify that:

i) This thesis contains no material which has been accepted for the award to the candidate of any other degree or diploma, except where due reference is made in the text of the examinable outcome and in the preface

ii) This thesis contains no material previously published or written by another person except where due reference is made in the text of the examinable outcome

iii) Where the work is based on joint research or publications, that the relative contributions of the respective workers or authors are disclosed

Kim Pham
2013
**List of Abbreviations**

Abbreviations for basic biological terms may be found in “Molecular Cloning: A Laboratory Manual” by Sambrook and Russell (1999, Cold Spring Harbour Press).

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACD</td>
<td>Asymmetric Cell Division</td>
</tr>
<tr>
<td>Alpha-MEM</td>
<td>Minimal Essential Medium Alpha Modification</td>
</tr>
<tr>
<td>AP</td>
<td>Adaptor protein</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical Protein Kinase C</td>
</tr>
<tr>
<td>β-ARK</td>
<td>β-adrenergic receptor kinase C-terminal domain</td>
</tr>
<tr>
<td>β-Pix</td>
<td>beta PAK-interacting exchange factor</td>
</tr>
<tr>
<td>Baz</td>
<td>Bazooka</td>
</tr>
<tr>
<td>BISXI</td>
<td>Bisindoylmaleimide XI</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate</td>
</tr>
<tr>
<td>Ch</td>
<td>Cherry</td>
</tr>
<tr>
<td>Crb</td>
<td>Crumbs</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential Interference Contrast</td>
</tr>
<tr>
<td>Dlg</td>
<td>Discs Large</td>
</tr>
<tr>
<td>DLL1</td>
<td>Delta-like1</td>
</tr>
<tr>
<td>DLL4</td>
<td>Delta-like4</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>ETP</td>
<td>Early T lineage Precursor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FTOC</td>
<td>Foetal thymic organ culture</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GMC</td>
<td>Ganglion Mother Cell</td>
</tr>
<tr>
<td>GRP</td>
<td>G protein regulator</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanine triphosphatase</td>
</tr>
<tr>
<td>GUKH</td>
<td>GUK-holder</td>
</tr>
<tr>
<td>Hes1</td>
<td>Hairy enhancer of split 1</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papilloma Virus</td>
</tr>
<tr>
<td>HSC</td>
<td>Haematopoietic stem cell</td>
</tr>
<tr>
<td>ICN</td>
<td>Intracellular Notch</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ISP</td>
<td>Immature single positive</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>LAT</td>
<td>linker for activation of T cells</td>
</tr>
<tr>
<td>Lgl</td>
<td>Lethal giant larvae</td>
</tr>
<tr>
<td>Lmo</td>
<td>LIM-only</td>
</tr>
<tr>
<td>MamL</td>
<td>Mastermind-like</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histoincompatibility complex</td>
</tr>
<tr>
<td>miR</td>
<td>microRNA</td>
</tr>
<tr>
<td>MSD</td>
<td>Mean Squared Displacement</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule-organising centre</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>Par</td>
<td>Partitioning defective</td>
</tr>
<tr>
<td>PBMC</td>
<td>Primary peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95/Dlg/ZO-1</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>Pins</td>
<td>Partner of Inscuteable</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethyl methacrylate</td>
</tr>
<tr>
<td>Pon</td>
<td>Partner of Numb</td>
</tr>
<tr>
<td>PR</td>
<td>Polarisation Ratio</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination-activating gene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RhoGEF</td>
<td>Rho guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated kinase</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RTOC</td>
<td>Reaggregate thymic organ culture</td>
</tr>
<tr>
<td>SCD</td>
<td>Symmetric Cell Division</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>SOP</td>
<td>Sensory organ precursor</td>
</tr>
<tr>
<td>Std</td>
<td>Stardust</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T cell acute lymphocytic leukaemia</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TPA</td>
<td>Tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
</tbody>
</table>
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Chapter 1

Introduction
1.1 PERSPECTIVES. The importance of being a thymus

The primary role of the thymus is to generate T cells, whose name reflects their point of origin. Historically dismissed as a “lymphocyte graveyard”, it was not until 1961 that eminent immunologist Jaques Miller observed a decline in T cells after the surgical removal of the thymus in mice. T cells comprise a major part of our adaptive immune system to protect us from foreign invaders (Miller, 2002; Miller, 2004). Developing T cells, or thymocytes, are provided an optimal environment by the thymus to undergo controlled proliferation, T-cell receptor gene arrangement and differentiation into mature T cells. The thymus is responsible for selecting T cells that will recognize foreign antigens present on invading organisms, be tolerant to self-antigens, and deleting potentially self-reactive thymocytes to avoid autoimmunity. In the young, understanding thymocyte development sheds light on how the immune system copes during prenatal or juvenile conditions. In the elderly, the decline in thymocyte function is a major factor in decreased responses to infection, reduced immunocompetence during autoimmunity and vaccination failures against disease such as HIV. T cell cancer prognosis and therapy also depends greatly on understanding fundamental aspects of thymus biology. The thymus therefore serves as an important site for central tolerance, and is absolutely essential for normal and efficient immune responses.

Anatomically, the thymus is a bilobular organ composed of an outer cortex, and an inner medulla. The thymus is responsible for generating most of our T cells during neonatal and pre-adolescence, reaching its highest activity and growth between birth and puberty (Hollander et al., 2010; Linton and Dorshkind, 2004). In a healthy individual, the thymus generates close to 25 million T cells of differing T cell Receptor (TCR) repertoires (about 2 million clones in mice), including all of our αβ and γδ T cells, CD4+ and CD8+ subsets, regulatory T cells, as well as NK and NKT cells (Arstila et al., 2000; Casrouge et al., 2000; Stutman and Cuttito., 1981). The unique specificity of T cells is orchestrated by massive proliferation of developing thymocytes and death of those with unsuitable TCR. Changes in thymocyte homeostasis have a profound effect on immune development. In the young, thymic hypoplasia due to Down Syndrome, Di George anomaly and Severe Combined Immunodeficiency (SCID), or partial/whole thymectomies during surgery to correct congenital heart defects all result in T cell alterations (Sauce and Appay, 2011). Some of the consequences include lower naïve T cell percentages, skewing of T cell subsets and a significant increase in central memory and terminally differentiated T cells (Gress and Deeks, 2009; Heng et al., 2010; Linton and Dorshkind, 2004; Sauce and Appay, 2011). This highlights the long-term immune consequences of suboptimal thymic activity, and the importance of maintaining the immune system responses throughout life.

By the early teens, the thymus begins to atrophy and is progressively replaced by fatty tissue. Its function decreases at a rate of approximately 3% per year during adulthood (Steinmann, 1986) with little or no function by the age of 60 (Linton and Dorshkind, 2004). The slowing of T cell supply during aging is thought to be a major factor in diminished antibody responses, vaccine failures and increased susceptibility to infections such as the influenza virus, most likely due to the reduced production of naïve T cell subsets and a decline in the ability to respond to novel antigens (Brien et al., 2009; Cicin-Sain et al., 2010; Yager et al., 2008). Diseases of autoimmunity,
where the body attacks ‘self’, can include diseases such as rheumatoid arthritis that spans decades, often resulting in chronic inflammatory stress, morbidity and mortality due to a breakdown of T cell self-tolerance (Weyand et al., 2009). Furthermore, human immunodeficiency virus (HIV), where one of the hallmark symptoms is suppression of thymic activity, particularly CD4 T cells (Dion et al., 2007; Dion et al., 2004), results in inadequate reconstitution of CD4 T cells (Mackall et al., 1995). It is necessary to understand what cellular factors are affected in such developing T cells, and how defects in those cells shape the decisions that contribute to aging, autoimmunity and immunodeficiency.

Much work is currently underway to search for avenues to rejuvenate an ‘old’ thymus. This is particularly relevant in our aging population where close to 20% of our 22.7 million population are 60 years or older (Australian Bureau of Statistics). Strategies to boost thymic function in the aged include administering growth factors or using sex steroids (Goldberg et al., 2010; Heng et al., 2010; Sutherland et al., 2008). Current pioneering work on silicone devices to generate a functional vascularised thymus is underway, although we are not quite at the stage where we can generate an intact thymus for direct implantation into the elderly (Seach et al., 2010). It is clear that we need to have an intimate understanding of how thymus develops and functions in order to develop strategies to regenerate it.

Thymocyte development has important implications for engineering therapy to combat cancer in both the young and elderly. By the age of 85, 1 in 2 Australian men and 1 in 3 women will have been diagnosed with cancer at some stage in their life (Australian Bureau of statistics, AIHW 2010b). Aberrant T cell development is thought to be a major factor in leukaemias such as T cell acute lymphocytic leukaemia (T-ALL), which comprise 15-25% of all ALLs and is the most common cancer in children (Australian Bureau of Statistics and Deschler and Lubbert, 2006; Lichtman and Rowe, 2004; Pieters and Carroll, 2010; Woolthuis et al., 2011). T-ALL often stems from errors during TCR recombination, or from transcription factors placed under the control of TCR loci due to incorrect chromosomal rearrangement (De Keersmaecker et al., 2005). These errors, which result in expression of genes at the wrong place and time, often lead to the uncontrolled proliferation of thymic T cell precursors, allowing further genetic mutations to accumulate and predispose to leukaemia. Studies have begun to map the genetic and phenotypic changes that underscore T-ALL, but we still do not understand how the pre-malignant thymocytes integrate the signals to contribute to the leukaemia.

Depending on a particular disease treatment, a variety of clinical situations can result in serious physiological and pathological deficits in thymic function. Radiation, chemotherapy and immunosuppressive treatments for a wide range of medical conditions can compromise the thymic microenvironment, inducing immunodeficiency, autoimmunity as well as impairing immunosurveillance. Chemotherapeutic agents such as cyclophosphamide and dexamethasome, or immunosuppressants such as cyclosporine can induce a breakdown in self-tolerance (Fletcher et al., 2009). This creates an interesting paradox for treatments that damage the very cells that are crucial for the restoration of our immune system post treatment. Approaches to increase thymic
output show promise in clinical trials, and studying thymocyte development will be paramount to improving current radiation and chemotherapeutic regimens (Dorshkind et al., 2009; Gress and Deeks, 2009; McElhaney and Effros, 2009).

For the immune system to develop properly to protect us from disease and autoimmunity, the checkpoints for proliferation, maturation, survival and death of maturing thymocytes must be closely regulated. It is clearly evident how important thymic regulation is, and how dysregulation impacts on disease state or in response to aging. The following literature review describes the cellular and molecular basis of thymocyte fate determination, with parallels drawn from studies of fate determination of other in/vertebrate systems. In particular the regulation of fate determination in other in/vertebrate systems by asymmetric cell division will be reviewed, and the novel means by which thymocyte fate determination might also be regulated by asymmetric cell division. The regulation of fate determination via asymmetric cell division through several core groups of polarity protein complexes therefore will also be addressed.
1.2 Thymocyte fate determination

Early thymic precursors enter the thymus through blood vessels at the cortico-medullary junction, and then follow a strictly circumscribed route through multiple intrathymic environments that dictate staged interactions with cells, cytokines and other extracellular cues. Fate determination of thymocytes involves tight coordination of overlapping molecular pathways for progressive T lineage commitment at the expense of other alternative fates. Such pathways regulate: (i) expansion of a million fold, (ii) differentiation, (iii) apoptosis, and (iv) limited short term self-renewal. Naïve T cells then exit into the periphery to act in the adaptive arm of the immune system (Ladi et al., 2006; Rothenberg et al., 2008; Takahama, 2006). The following sections describe the key points of T cell development, with a brief overview covering the regulation of T cell specification.

1.2.1 An overview of murine thymocyte development into T cells

The systematic analysis of thymocytes by flow cytometry has identified many of the surface markers that reflect each developmental stage, providing a wealth of information about signalling and transcriptional modules regulating each step of T cell commitment (Garcia-Peydro et al., 2006). In mice, thymocyte differentiation into naïve T cells is generally categorised by the presence of CD4 or CD8 cell surface markers and a functional TCR (Zuniga-Pflucke, 2004). Early thymocytes lack these two cell surface markers and are generally characterised by differential expression of the cell surface molecules CD44 and CD25, in four stages called double negative (DN) 1-4 (CD44+/CD25-, CD44+/CD25+, CD44-/CD25+, CD44-/CD25- respectively, see Figure 1.1). However, analysis of thymocyte development reveals that using CD44 and CD25 alone is insufficient for rigorous purification of particular thymus subsets. In fact only a small population of DN1 cells are early T cell precursors, and DN2 thymocytes seem to be heterogeneous in terms of their potential to adopt a dendritic or NK cell fate. The use of cell surface CD117/KIT expression, a surface receptor protein tyrosine kinase commonly expressed by most haematopoietic cells as well as stem cells, can further differentiate DN1 cells into five subsets, termed DN1a-e, and DN2 cells into two subsets, termed DN2a-b (Allman et al., 2003; Ceredig and Rolink, 2002; Porritt et al., 2004).

DN1 early thymic precursors and DN2 thymocytes proliferate extensively until they reach the DN3 stage, where cell proliferation stops for TCR rearrangement. This process, mediated by the recombination-activating gene (RAG) 1 and 2 complexes, involves clusters of interchangeable DNA fragments encoding TCR genes (TCR-β and TCR-α) that are joined in combination and are randomly mutated to create highly diverse TCR receptors on the surface (Petrie and Zuniga-Pflucke, 2007). DN3 thymocytes are further subdivided before or after β-selection on the basis of CD27 and CD28 expression as well as size into DN3a-b (Hoffman et al., 1996; Taghon et al., 2007; Teague et al., 2010). The first stage of rearrangement is β-selection which occurs at DN3a. β-selection involves rearrangement of TCR-β locus, and a successful rearrangement results in covalent pairing of the TCR-β protein with the preTα chain and subsequent association with CD3δ, ε, γ coreceptors to produce a functional preTCRα complex. Signalling through this
Figure 1.1 Normal intrathymic development

Haematopoietic progenitor cells enter the thymus at the cortico-medullary junction via post capillary venules. Thymocytes development involves stepwise differentiation through the Early T lineage Precursor (ETP)/Double Negative 1 (DN1, $44^{hi}/117^{hi}/25^{hi}$) to DN2a ($44^{hi}/117^{hi}/25^{hi}$), DN2b ($44^{hi}/117^{int}/25^{hi}$), DN3a ($44^{25^{hi}}/27^{hi}/28^{lo}/FSC^{lo}$), DN3b ($44/25^{hi}/27^{lo}/28^{+}/FSC^{hi}$) and DN4 ($44/25^{lo}/28^{+}$) stages. DN3 thymocyte undergo rearrangement for their β- and α-TCR to eventually assemble a functional surface T Cell Receptor. Thymocytes from DN1-DN4 are negative for CD4, CD8, CD11b, CD11c, NK1.1 and TCR. DN4 cells differentiate into Immature Single Positive ($4^{-}/8^{+}/TCR^{lo}/24^{hi}$) thymocytes before upregulating both CD4 and CD8 surface markers to become Double Positive (DP, $4^{+}/8^{+}/TCR^{lo}$) thymocytes. DP thymocytes undergo Positive and Negative selection with CD4+ intermediates ($4^{+}/8^{int}/TCR^{lo}/69^{+}$) or CD8+ intermediates ($4^{lo}/8^{lo}/TCR^{hi}/24^{hi}$), to become mature CD4+ single positive (CD4+SP, $4^{+}/8^{+}/24^{lo}/TCR^{hi}/69^{+}$) and CD8+ single positive (CD8+SP, $4^{lo}/8^{lo}/24^{lo}/TCR^{hi}/69^{+}$) thymocytes, and are exported out of the thymus via post capillary venules. Thymocyte differentiation involves a number of major apoptosis checkpoints (brown), as well as rounds of proliferation (hatched cells).
complex allows survival, proliferation (6-8 rounds) and progression to the DN3b stage (Penit et al., 1995). At DN3b to DN4, rearrangement at the TCR-α locus takes place (Petrie et al., 1993). Successful TCR-α rearrangement leads to the expression of CD4 as well as CD8 and these become double positive (DP) thymocytes. Alternatively, DN3 thymocytes that rearrange TCR-γ and -δ chains instead of -β chains will produce γδ T cells (Aifantis et al., 1998; Bonneville et al., 1989; Haks et al., 2005; Ishida et al., 1990; Taghon and Rothenberg, 2008).

At the DP stage, CD4 and CD8 surface molecules are both upregulated, transiting through an immature single positive (CD4/CD8/TCRβ/CD24hi) stage before positive and negative selection occurs. Positive selection involves appropriate (neither too strong nor too weak) TCR signalling that allows the DP thymocytes to survive and down regulate either one of the cell surface molecules into becoming single CD4 or CD8 positive T cells. Positive selection ensures that emerging T cells can form adequate T cell responses upon encountering a foreign antigen (Aliahmad and Kaye, 2006; Borgulya et al., 1991; Ladi et al., 2006; Petrie and Zuniga-Pflucke, 2007). Negative selection fine tunes positively selected thymocytes, causing death of thymocytes displaying inappropriately strong TCR signals (Surh and Sprent, 1994; Yasutomo et al., 2000). Intermediate TCR signals generate regulatory T cells (Moran et al., 2011). Weak TCR signals results in death of DP thymocytes via apoptosis (Grebe et al., 2004; Minter and Osborne, 2003).

A list of key surface markers during thymocyte fate determination are listed in Table 1.1

### 1.2.2 Systems to study thymocyte fate determination

Several **in vivo** and **in vitro** models have been utilised to study thymocyte development. **In vivo** models include gene modified mice in which thymocyte fate decisions are promoted, halted, or skewed at multiple stages (Crompton et al., 2007; Dose et al., 2006; Takahama, 2006; Guo et al., 2007; Mao et al., 2007). Thymic subsets from these gene modified mice can be directly characterised, or adoptively transferred into recipient hosts to discriminate whether the phenotypes observed are cell autonomous. Other approaches include modifying T cell precursors from normal or gene modified mouse donors and transferring into recipient mice to distinguish possible cell intrinsic defects. Imaging of isolated thymi by intravital microscopy (**in vivo** or thymic explants) has enabled the characterisation of migration defects at a number of thymocyte developmental stages after transfer of sorted thymocytes into recipient mice (Bousso et al., 2002; Chen et al., 2009; Ladi et al., 2008a; Ladi et al., 2008b; Ladi et al., 2006; Witt and Robey, 2005; Yin et al., 2006).

The earliest **in vitro** studies include the use of three dimensional organotypic cultures, namely foetal thymic organ cultures (FTOC) and reaggregate thymic organ cultures (RTOC) that recapitulate many aspects of the thymocyte development (Hare et al., 1999; Ramsdell et al., 2006). FTOC involve dissecting and culturing the foetal thymic lobes of E14 mice to directly analyse the functionality of antigen specific receptors on developing thymocytes, or alternatively, be depleted of resident thymocytes through treatment with deoxyguanosine to provide the necessary
architecture for investigating thymocyte populations of interest (Born et al., 1987; Jenkinson et al., 1982; Takahama, 2000). Similarly, RTOC involve the reconstruction of a three-dimensional thymus using stromal cells from disaggregated thymic lobes and can also be depleted of resident thymocytes through deoxyguanosine treatment (White et al., 2008). These three dimensional organotypic cultures have been useful in investigating the cellular and molecular basis of positive and negative selection events, including the identification of clonal deletion in thymocytes with unsuitable TCR, as well as the ligand efficacy and density necessary to mediate selection (Born et al., 1987; Marrack and Kappler, 1987; Marrack et al., 1987). The use of RTOC and FTOC enable manipulation of the growth conditions, cytokine factors and starting populations of thymocytes to analyse components of thymocyte fate. These experimental approaches have been used for almost half a century (Kamarck and Gottlieb, 1977), despite long preparation times and expensive timed mouse matings to dissect foetal thymic lobes (Schmitt and Zuniga-Pflucke, 2006).

Several stromal cell lines, including those derived from the thymus, were previously tested for their ability to support T cell differentiation in monolayer cultures with little success. However, the identification of Notch signalling as a critical factor for early T cell development has transformed thymocyte development studies in the recent decade (Pui et al., 1999; Radtke et al., 1999). Transduction of the OP9 stromal cell line with the Notch ligand, Delta-like1 or Delta-like 4 (DLL1 or DLL4) can recapitulate nearly all aspects of thymocyte differentiation on monolayer culture from haematopoietic stem cells to DP thymocytes (Ramsdell et al., 2006; Schmitt and Zuniga-Pflucke, 2002). The use of this in vitro system has proven extremely useful in exploring the permissive conditions for thymocyte fate determination. This system allows the inquisition of the effects of gene knockouts/knockdowns, and ectopic expression as well as the addition or subtraction of growth factors. Additionally, the ability to expand cultures from this in vitro system yields exponentially more cell numbers that can be used in downstream in vitro assays or injected back in vivo.

1.2.3 The regulation of thymocyte fate

Progression along the T cell developmental pathway described in Section 1.2.1 involves several levels of regulatory control. These include the expression of restricted homing receptors during chemokine signalling, the expression of surface signalling receptors (namely Notch signalling), and intrinsic transcriptional and translational regulatory elements. Maturing thymocytes commit along the T lineage by positively activating one or several pathways and also silencing pathways needed for differentiation into other lineages.

1.2.3.1 Chemokine signalling

Chemokines and their receptors are responsible for causing T cell precursors to home to the thymus, for specific intrathymic migration of maturing thymocytes to receive developmental cues, and for controlling naïve T cell exit into the peripheral blood. T cell precursors enter the thymus at the cortico-medullary junctions through blood vessels via expression of chemokine receptors CCR9 and/or CCR7, and to a minor extent CXCR4 (chemokines described in more
Table 1.1 Surface markers, chemokines and chemokine receptors during T cell development

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
<th>Surface phenotype</th>
<th>Surface chemokine receptor</th>
<th>Corresponding Chemokine</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP/DN1</td>
<td>Early T lineage Precursor/Double Negative 1</td>
<td>4' 8' 11b' 11c' 19' NK1.1' TCR' 44hi 117hi 25'</td>
<td>CXCR4hi, CCR7, CCR9</td>
<td>CXCL12, CCL19, CCL25</td>
</tr>
<tr>
<td>ETP-DN2A</td>
<td>ETP to DN2a Transitional</td>
<td>4' 8' 11b' 11c' 19' NK1.1' TCR' 44hi 117hi 25int</td>
<td>CXCR4, CCR7, CCR9</td>
<td>CXCL12, CCL19, CCL25</td>
</tr>
<tr>
<td>DN2</td>
<td>Double-Negative 2</td>
<td>4' 8' 11b' 11c' 19' NK1.1' TCR' 44hi 25hi</td>
<td>CCL12, CCL19, CCL25</td>
<td></td>
</tr>
<tr>
<td>DN2 A</td>
<td>Double-Negative 2a</td>
<td>4' 8' 11b' 11c' 19' NK1.1' TCR' 44hi 117hi 25hi</td>
<td>CCL12, CCL19, CCL25</td>
<td></td>
</tr>
<tr>
<td>DN2 B</td>
<td>Double-Negative 2b</td>
<td>4' 8' 11b' 11c' 19' NK1.1' TCR' 44hi 117int 25hi</td>
<td>CCL12, CCL19, CCL25</td>
<td></td>
</tr>
<tr>
<td>DN23 A</td>
<td>DN2 to DN3 transitional</td>
<td>4' 8' 11b' 11c' 19' NK1.1' TCR' 44int 25hi</td>
<td>CCL12, CCL19, CCL25</td>
<td></td>
</tr>
<tr>
<td>DN3</td>
<td>Double-Negative 3</td>
<td>4' 8' 11b' 11c' 19' NK1.1' TCR' 44' 25hi</td>
<td>CCL12, CCL19, CCL25</td>
<td></td>
</tr>
<tr>
<td>DN3 A/E</td>
<td>Double-Negative 3a/e</td>
<td>4' 8' 11b' 11c' 19' NK1.1' TCR' 44' 25hi 27hi 28'</td>
<td>CCL12, CCL19, CCL25</td>
<td></td>
</tr>
<tr>
<td>DN3 B/L</td>
<td>Double Negative 3b/l</td>
<td>4' 8' 11b' 11c' 19' NK1.1' TCR' 44' 25hi 27' 28'</td>
<td>CCL12, CCL19, CCL25</td>
<td></td>
</tr>
<tr>
<td>DN3-4</td>
<td>DN3 to DN4 Transitional</td>
<td>4' 8' 11b' 11c' 19' NK1.1' TCR' 44' 25int 28'</td>
<td>CCL12, CCL19, CCL25</td>
<td></td>
</tr>
<tr>
<td>DN4</td>
<td>Double Negative 4</td>
<td>4' 8' 11b' 11c' 19' NK1.1' TCR' 44' 25' 28'</td>
<td>CCL12, CCL19, CCL25</td>
<td></td>
</tr>
<tr>
<td>ISP</td>
<td>Immature single positive</td>
<td>4' 8' TCR3lo 24hi</td>
<td>CCR7, CCR9</td>
<td>CCL12, CCL25</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive, all</td>
<td>4' 8' TCR4hi 69'</td>
<td>CCR4hi, CCR7, CCR9</td>
<td>CCL12, CCL19, CCL25</td>
</tr>
<tr>
<td>DPbl</td>
<td>Double positive blasts</td>
<td>4' 8' TCR3lo FSC4hi</td>
<td></td>
<td></td>
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<tr>
<td>DPsrm</td>
<td>Double positive small resting</td>
<td>4' 8' TCR3lo FSC4hi</td>
<td></td>
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<tr>
<td>DP69+</td>
<td>Double Positive early positive selection</td>
<td>4' 8' TCR4hi 69'</td>
<td>CCR7, CCR9</td>
<td>CCL12, CCL19, CCL25</td>
</tr>
</tbody>
</table>
Table 1.1  Surface markers, chemokines and chemokine receptors during T cell development (cont.)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
<th>Surface phenotype</th>
<th>Surface chemokine receptor</th>
<th>Corresponding Chemokine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4^+CD8int</td>
<td>Positive selection intermediate</td>
<td>4^+ 8^int TCR^hi 69^+</td>
<td>CCR7</td>
<td>CCL19/CCL21</td>
</tr>
<tr>
<td>CD4intCD8^+</td>
<td>Positive selection intermediate</td>
<td>4^int 8^+ TCR^hi 69^+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 SP 69^+</td>
<td>CD4 Single Positive Intermediate</td>
<td>4^+ 8^ TCR^hi 69^+</td>
<td>CCR7</td>
<td>CCL19/CCL21</td>
</tr>
<tr>
<td>CD4 SP 24int</td>
<td>CD4 Single Positive semimature</td>
<td>4^+ 8^ TCR^hi 24^int</td>
<td>CCR7</td>
<td>CCL19/CCL21</td>
</tr>
<tr>
<td>CD4 SP 24^-</td>
<td>CD4 Single Positive mature</td>
<td>4^- 8^- TCR^hi 24^-/lo</td>
<td>CCR7, SIP1</td>
<td>CCL19/CCL21</td>
</tr>
<tr>
<td>CD8 SP 69^+</td>
<td>CD8 single positive Intermediate</td>
<td>4^- 8^- TCR^hi 69^+</td>
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<tr>
<td>CD8 SP 24int</td>
<td>CD8 single positive semimature</td>
<td>4^- 8^- TCR^hi 24^-/lo</td>
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<tr>
<td>CD8 SP 24^-</td>
<td>CD8 single positive mature</td>
<td>4^- 8^- TCR^hi 24^-/lo</td>
<td>SIP1</td>
<td></td>
</tr>
</tbody>
</table>

Bold- phenotypes frequently described in thesis

Table adapted from Bunting et al, 2011 and www.immgen.org
detail in Section 1.2.4). Single and double mutants of CCR9 and CCR7 demonstrate severely impaired thymus seeding in vivo with reduced thymic cellularity and an almost complete absence of DN1 thymocytes (Krueger et al., 2010; Liu et al., 2006; Zlotoff et al., 2010). Interestingly, some residual T cell precursor recruitment does exist, indicating a role for additional chemoattractive mechanisms, possibly by CXCR4 and CCR5, as simultaneous deletion of CCR5 and blocking of CXCR4 can impair but not fully block recruitment to the thymus (Ara et al., 2003; Nagasawa et al., 1996; Robertson et al., 2006).

Migration of early thymic precursors proceeds from the cortico-medullary junction to the subcapsular zone of the outer cortex (Figure 1.2). The transition from DN1 to DN2 cells coincides with the upregulation of the chemokine receptor CXCR4. CXCR4 may assist in directing maturing DN1 thymocytes away from the medulla, as they accumulate in this region in a conditional cxcr4-/- mouse (Plotkin et al., 2003). Similar to DN2 thymocytes, DN3 thymocytes express CXCR4 but also CCR9 receptors, trafficking in response to their CCL25 and CXCL12 ligands predominantly in the subcapsular region (Kim et al., 1998; Plotkin et al., 2003; Uehara et al., 2006; Wurbel et al., 2006). CXCR4 plays a critical role not only in DN3 trafficking but also in the transduction of survival signals as they undergo β-selection (described in more detail in Section 1.3, Janas et al., 2009; Trampont et al., 2010). The chemokine receptor CCR7 also contributes to retention and survival of DN3 thymocytes, as its absence, or the absence of its ligand results in a decrease in the numbers of DN3 cells in the subcapsular zone (Krueger et al., 2010; Misslitz et al., 2004). The subcapsular zone contains DN4 thymocytes that also express both CXCR4 and CCR9 chemokine receptors and can respond to CXCL12 and CCL25 respectively in vitro (Kim et al., 1998; Plotkin et al., 2003; Wurbel et al., 2006). However, the relevance of these chemokines and their receptors at this stage in vivo remains to be fully investigated.

DP thymocytes are slow moving and express high levels of CCR9, CXCR4 and low levels of CCR7 and CCR8. The slower migration is most likely to aid in interactions with the cortical thymic epithelial cells during positive selection (Carramolino et al., 2001; Davalos-Misslitz et al., 2007; Misslitz et al., 2004; Norment et al., 2000; Schabath et al., 1999; Suzuki et al., 1998; Suzuki et al., 1999; Uehara et al., 2006; Uehara et al., 2002; Ueno et al., 2004; Wurbel et al., 2006; Zingoni et al., 1998). After positive selection there is a profound change in surface chemokine receptor expression, with a steep decrease in CXCR4 and an increase in CCR4, CCR7 and CCR9 in response to TCR stimulation (Davalos-Misslitz et al., 2007; Norment et al., 2000; Suzuki et al., 1999; Ueno et al., 2004). The change in surface chemokine receptors allows positively selected thymocytes to migrate rapidly towards the medulla for negative selection (Ehrlich et al., 2009). The G-coupled Protein receptor sphingosine 1-phosphate 1 (S1P1), is responsible for export of mature CD4 and CD8 SP thymocytes into the periphery via the blood vessels (Drennan et al., 2009). SP thymocytes additionally express low levels of CXCR4, as well as somewhat differential levels of CCR7 and CCR9, although clear cut functions for these chemokine receptors have not been elucidated. However, it is known that human SP thymocytes migrate away from high concentrations of CXCL12 (receptor CXCR4), and in cxcr4-/- mouse foetal thymic organ cultures there is an accumulation of CD4 SP thymocytes (Poznansky et al., 2002; Vianello et al., 2005).
This indicates that chemorepulsion via CXCR4/CXCL12 could also play a role in pushing mature T cells into the periphery.

Important chemokines and their corresponding receptors in the thymus are listed in Table 1.

1.2.3.2 Notch signalling

Notch is a transmembrane receptor involved in cell-to-cell signalling with neighbouring cells possessing the corresponding Notch ligand from the Delta or Serrate/Jagged family. Mammals have four Notch receptors, of which Notch1, Notch2 and Notch3 are expressed in the thymus (Radtke et al., 2004). Notch is the most well studied T-cell lineage regulator to date, stemming from historical discoveries in the analysis of T versus B cell fate decisions by lymphocyte progenitors. Mice with a conditional knockout of Notch (specifically Notch1) demonstrate a severe block in T cell development with the thymus filling up with B cells, as well as the conversion of pro-T cells to plasmacytoid dendritic cells (Feyerabend et al., 2009; Radtke et al., 1999). Conversely, B cell development fails to occur in mice reconstituted with bone marrow progenitors expressing constitutively active Notch. Instead, the bone marrow fills up with developing DP thymocytes (Pui et al., 1999).

Upon engagement and activation of Notch with its ligand (ligands described in more detail in Section 1.2.4) the large intracellular domain of Notch is cleaved (Notch ICN) by two proteases; ADAM10/TACE followed by gamma secretase; and then traffic to the nucleus (Thompson and Zuniga-Pflucke, 2011). Notch ICN recruits the transcription factor recombination site binding protein J, RBPJ (or CSL,-CBF1/Suppressor of Hairless/Lag-1) as well as the Mastermind-like (MamL) proteins (Kovall, 2007; Maillard et al., 2004). This tripartite complex (NotchICN-RPBJ-MamL) activates transcription of a number of downstream genes such as Hairy enhancer of split 1 (Hes1) and Deltex1, as well as T-lineage specific genes including pre TCRα chain and CD25. Notch activity can be modulated by upstream factors such O-fucose glycosylation by the Lunatic-Fringe proteins, as well as E3-ubiquitin ligases which target Notch for degradation, but this is not the focus of this review (Le Bras et al., 2011; Stanley and Guidos, 2009).

Notch signals are essential for several thymocyte fate outcomes including proliferation, rescue from cell death, and differentiation. Notch receptor-ligand interactions regulate thymocyte proliferation and cellular metabolism by activating signal transduction cascades mediated by proteins such as phosphatidylinositol 3 kinase (PI3K) and the serine-threonine kinase Akt. Notch signalling is evident as early as DN1 thymocytes and is required for proliferative waves at the DN2 and DN3 stage. Although cytokines and other growth factors are essential for survival and proliferation of thymocytes, the removal of Notch signals from DN3a cells results in severely decreased trophic function, small cell size and progressive cellular atrophy resulting in rapid apoptosis (Ciofani and Zuniga-Pflucke, 2005). Interestingly, the expression of active Akt can bypass Notch, suggesting that other factors may also activate pathways to promote proliferation or survival (Ciofani and Zuniga-Pflucke, 2005).
Figure 1.2 The supporting thymic stroma

Thymocyte development involves guidance through the thymic milieu comprising of cortical thymic epithelial cells (cTEC), macrophages, subcapsular nurse cells, medullary epithelial cells (mTEC) and dendritic cells. Migration depends on the interaction of chemokine or Notch1 receptor expression on the thymocytes with the corresponding chemokine or Notch ligand expressed by the thymic millieu. Positive and negative selection also involve sampling of peptide antigens (Ag) presented in major histocompatibility (MHC) complexes I or II on dendritic cells. Mature CD4⁺ or CD8⁺ single positive thymocytes are exported from the thymus via post capillary venules through SIP1.

Abbreviations ETP= Early T cell precursor, DN= Double negative, ISP = Immature Single Positive, DP= Double positive, Int= Intermediate, TCR= T Cell Receptor
Notch activity also inhibits transcription factors that promote alternative lineages, down regulating myeloid and NK promoting transcription factors such as PU.1 through up regulating inhibitors of PU.1 activation such as C/EBPα TCF-1 and Gfi1 (described later in Section 1.3). In doing so, Notch makes the thymic environment non-permissive for myeloid, NK and also B cell fates. Even ectopic expression of transcription factors that promote other lineages can be overridden in the presence of Notch (Franco et al., 2006; Schmitt et al., 2004). Thus, Notch acts in concert with several signalling pathways to dictate the lineage choices and the fate outcomes during T cell development.

1.2.3.3 Transcriptional and translational regulatory control of gene expression

Thymocyte fate also depends on the balance of differential gene expression, transcription factors and translational constraints via microRNAs. From DN2a onwards there is a coincident change of at least five major transcription factors that usually confer myeloid versus lymphoid lineage plasticity. These include: 1) the avian erythroblastosis virus E26 oncogene (ETS)- family factor PU.1, 2) the runt related transcription factor 1 RUNX/CBFβ complex, 3) Ikaros, 4) Bcl11b and 5) E protein transcriptional activity (Thompson and Zuniga-Pflucker, 2011). Several major T-cell identity genes are also turned on at the DN2 stage, well before T cell maturation is complete. Genes with key roles during TCR selection such as Cd3ζ, Cd3γ, pre-TCRα chain and Rag1/2 become upregulated, effector genes such as IL-2 become accessible for induction, and there is activation of Lck promoter at DN2b onwards. IL-7Rα is strongly induced from DN1 until after β-selection to sustain proliferation. After TCR rearrangement there is a gradual down regulation of CD117/KIT expression and down regulation of IL-7Rα at DN3b (Rothenberg et al., 1990; Tabrizifard et al., 2004).

PU.1 (encoded by the Sfpi 1 gene) plays a number of roles during lymphoid versus myeloid development. Studies of PU.1 demonstrate that it is a master inductor of myeloid cell lineage, and its overexpression can convert early thymic precursors towards the plasmacytoid dendritic cells or to monocyte lineages (Dakic et al., 2005; Iwasaki et al., 2005). Therefore, the dampening of PU.1 is one of the first transcriptional changes to occur at DN2 (Carotta et al., 2010; Franco et al., 2006; Laiosa et al., 2006a; Laiosa et al., 2006b). The RUNX1/CBFβ complexes are needed for DN3a thymocytes to undergo full TCR-β rearrangement (Grownney et al., 2005; Kawazu et al., 2005; Talebian et al., 2007). Loss of Runx1 is associated with a myeloproliferative defect, but also blocks the generation of DN3 cells and CD4 surface expression (Grownney et al., 2005; Talebian et al., 2007). Ikaros both restrains PU.1 expression and reduces Notch signalling in β-selected thymocytes (Yoshida et al., 2006). In DN4 thymocytes, Ikaros binds to the Hes1 promoters (which is induced by Notch), driving Hes1 downregulation through trimethylation. A reduction in Ikaros activity can also cause T-cell leukaemia, perhaps by collaborating with activated Notch (Kleinmann et al., 2008; Yoshida et al., 2006).

The transcription factor Bcl11b is induced at DN2b and is continuously expressed from then onward, coinciding with loss of NK potential. Bcl11b is the first T cell lineage specific
transcription factor identified to negatively regulate alternative NK cell fate within the thymus (Ikawa et al., 2010; Li et al., 2010a; Li et al., 2010b). Loss of Bcl11b causes a T to NK cell conversion, by permitting the upregulation of NK specific genes such as Zfp105 and transcription factors Nfil3 and Zbtb16 (PLZF) (Ikawa et al., 2010; Li et al., 2010b). Deletion of Bcl11b in ETP cells turns them into NK or NK-like cells (Li et al., 2010b) and deletion at the β-selection checkpoint upregulates a number of cytolytic effector genes to cause premature apoptosis of DP thymocytes (Albu et al., 2007). Bcl11b can also directly repress downstream target genes induced by PU.1 such as Id2 (Albu et al., 2007). Recent work has identified a positive regulator of Bcl11b expression, TCF-1 (Weber et al., 2011; Germar et al, 2011). Interestingly, Notch activity induces TCF-1 expression, which subsequently drives many T lineage promoting genes such as TCR, Gata3 and Bcl11b, but not TCF-7 or CD3γ. Early thymic precursors ectopically expressing TCF-1 can give rise to T lineage cells when cultured in the absence of Notch1 signals on OP9 stromal cells, and are comparable to T lineage cells obtained from equivalent co-cultures on OP9-DLL4 stromal cells. T cell specification therefore involves upregulating pathways that promote T cell fate, while avoiding alternative cell lineages.

E proteins such as E2A and HEB1 upregulate downstream classical Notch target genes such as Deltex1, Hes1, along with Rag1 and preTCRα (Rothenberg et al., 2008). Another E protein, RORγt, is induced by pre-TCR signalling, but its expression does not peak until the DP stage where it promotes DP survival and inhibits proliferation, playing an important role in DP quiescence during selection (Xi et al., 2006). E proteins can therefore promote TCR rearrangement, but also pause proliferation in later developmental stages. The dual function of E proteins from DN3 to DP is not very clear, but it has been suggested that different E proteins can negatively regulate each other. For example, at DN3 the induction of Egr3 induces expression of Id3 (an E protein), and Id3 dimerizes with E2A. E2A normally functions to bind and repress the RORγt promoter, but if bound to Id3 it leaves the RORγt free to be expressed upon pre-TCR signalling, with its protein function peaking at DP (Xi et al., 2006). Several other transcription factors play other minor roles during thymocyte development (Rothenberg, 2011; Thompson and Zuniga-Pflucke, 2011), and more studies will highlight the mosaic nature of thymocyte transcriptional regulation.

Recent studies also implicate microRNAs (miRs), small noncoding RNA approximately 22 nucleotides in length, as important regulators of gene expression. miRs are dynamically expressed throughout thymocyte development but the expression of certain miRs is approximately ten-fold higher in immature DP thymocytes (Neilson et al., 2007). In particular, miR-181a is an important regulator of TCR signalling thresholds during positive and negative selection. miR-181a targets autoreactive cells for apoptosis at the DP stage by repressing genes co-ordinately required for positive selection such as Bcl-2, CD69, and the TCR (Ebert et al., 2009; Li et al., 2007; Neilson et al., 2007). miR181a also aids in labelling DP cells for apoptosis by suppressing a number of phosphatases required for TCR signalling. Phosphatases such as SHP-2, PTPN22, DUSP5 or DUSP6 contain multiple putative miR-181a pairing sites and it has been shown their protein levels can be reduced by miR181a expression (Li et al., 2007). Inhibition of miR-181a results in defective positive and negative selection, permitting the maturation of T cells that react to self
antigen (Ebert et al., 2009; Li et al., 2007). A recent comprehensive profiling of miR expression in human thymocyte subsets also shows an upward trend of miR expression from the DP to the SP stage, but miR150 instead of miR180a seems to be important during positive and negative selection (Ghisi et al., 2011). The multiple action of miRs therefore tune TCR signalling during positive selection, providing an important negative feedback mechanism to prevent the escape of autoreactive T cells.

The transcriptional regulation of thymocyte development is summarized in Figure 1.3.

1.2.4 The thymic stroma - chemokines, Notch ligands, cytokines and antigens

The thymus contains specialised stromal cells that provide molecular cues to support differentiation from thymocytes into various T cells and a small number of other myeloid lineages (Schmitt and Zuniga-Pflucke, 2006). They include 1) subcapsular nurse cells, 2) dendritic cells and 3) the cortical and medullary epithelial cells. As the names suggest, these stromal cells reside in particular anatomical regions of the thymus, either in the inner medulla or the surrounding cortex, and provide a combination of ligands and growth factors to aide in thymocyte trafficking and T cell differentiation. These include 1) chemokines, 2) Notch ligands, 3) cytokines and 4) antigenic molecules for selection/tolerance.

Stromal cells release specific chemokines to guide thymocytes through their differentiation program (Bunting et al., 2011). Recruitment of T cell precursors depends on the expression of CCL19 (receptor CCR7) and CCL25 (receptor CCR9), in the cortical medullary junction and the medullary epithelial cells. The restricted expression of cortical (CCL25, CXCL12) or medullary (CCL19, CCL21, CCL17, CCL22) chemokines contribute to guiding thymocytes through their maturation stages up until the single positive stage (Misslitz et al., 2004; Plotkin et al., 2003; Trampont et al., 2010; Wurbel et al., 2006). Although thymocytes can traverse similar pathways within the thymus at different stages of development, it is the modulation of corresponding chemokine receptors on the cell surface that determines how a thymocyte is influenced by the surrounding thymic environment (Figure 1.2).

Thymic epithelial cells at the cortico-medullary junction also express high levels of the cytokine IL-7, which is important for the proliferative wave during thymocyte development from DN1 to DN2, as well as the survival of DN3 cells (Alves et al., 2009). IL-7 promotes proliferation and survival through direct association with the PI3K pathway in thymocytes possessing the IL-7 receptor (CD127) (Hagenbeek et al., 2004). The survival of early DN1/2 thymocytes also depends on other cytokines released by thymic epithelial cells such as CD117/KIT ligand and Flt3.

As mentioned, Notch signalling endows the thymus with a T cell permissive environment by shutting down differentiation pathways that lead to alternative lineages. Notch signalling is induced by two ligand families expressed by the thymic epithelial cells: Delta-like (DLL1, DLL3 and DLL4) and Jagged (Jagged1 and Jagged 2). DLL1, DLL4 and Jagged2 can all trigger Notch signalling to induce T-lineage differentiation and inhibit both B and myeloid cell fates to
different extents (Ciofani and Zuniga-Pflucke, 2005; Hozumi et al., 2008; Igarashi et al., 2002). Recent studies point to DLL4 being the physiological ligand within the thymus, as DLL4 is a more potent inducer of T-lineage commitment and differentiation than DLL1 (Mohtashami et al., 2010). In limiting dose experiments of DLL1 or DLL4, lower levels of DLL4 are sufficient to induce Notch target gene expression and thymocyte differentiation (Mohtashami et al., 2010). Additional studies show Notch1-DLL4 interactions to be of highest affinit, followed by Notch1-DLL1 (Mohtashami et al., 2010). This is due to the strengthening of Notch1-DLL4 signalling through posttranslational glycosylation of Notch1 by the glycans Lunatic Fringe and Manic Fringe (Stanley and Guidos, 2009). Jagged2 is a comparatively weak Notch1 activator compared to the other Delta-like ligands even though it can still induce T lineage commitment (Van de Walle et al., 2011), revealing a hierarchy of Notch signal access within the thymic milieu (Stanley and Guidos, 2009).

DP thymocytes are much less reliant on Notch signalling. At this developmental stage, one of the most important roles of the thymic stroma is directing thymocytes to distinguish antigens of foreign origin from those derived endogenously. Medullary thymic epithelial cells and antigen presenting cells within the thymus present a diverse set of both foreign and self peptides in the context of major histoincompatibility complexes of two classes (MHC I or II). This allows interaction, recognition, signalling on DP thymocytes for the specification of TCR to become CD4 or CD8 SP cells. There are a number of positive selection models to explain the DP to SP transition (Aliahmad and Kaye, 2006; Petrie and Zuniga-Pflucke, 2007). First is a stochastic model, where CD4 or CD8 coreceptors are randomly repressed to generate CD4 or CD8 SP cells (Robey et al., 1990). Second is an instructive model where CD4 or CD8 is actively repressed depending on whether the thymic epithelial cells express MHC I or MHC II on their cell surface (Borgulya et al., 1991). Third is coreceptor reversal, where the default pathway is loss of CD8 signalling, sustained TCR signalling and CD4 SP fate (Bosselut et al., 2000). However, if loss of CD8 signalling interrupts TCR signalling, CD4 can be reinduced, CD4 is silenced and CD8 SP fate follows (Bunin et al., 2005; Yu et al., 2003). The fourth model describes the strength of signal, where more potent TCR signals induces a CD4 SP fate, and weaker signals allow differentiation into CD8 T cells (Alberola-Ila and Hernandez-Hoyos, 2003). These models are still being debated (Aliahmad and Kaye, 2006; Starr et al., 2003; Wang et al., 2010). In negative selection, the dendritic cell/medullary epithelial cell is responsible for imposing an apoptotic signal to DP or SP thymocytes that express TCR receptors with inappropriately high affinity for their presented self antigens during negative selection (Surh and Sprent, 1994). Thymic epithelial cells are also responsible for displaying a number of self-antigens present in peripheral tissues, regulated by the Aire transcription factor, to endow developing DP thymocytes with non self-reactivity (Anderson et al., 2002).

In summary, the abundance and compartmentalisation of chemokine, cytokines, ligand or antigen within the thymic stroma coordinate to pattern T lineage specification. Migration of thymocytes through different zones within the thymus helps drive T cell differentiation, and the pathways which regulate this have both direct and indirect feedback effects to coordinate development.
Thymocyte development involves gradual shedding of alternative fate choices (B cell, myeloid, NK, αβ or γδ T cell), and involves regulation of Notch signalling as well as transcriptional and translational control of gene expression. Notch1 signalling is enhanced by Lunatic/Manic Fringe O-fucose glycosylation from ETP/DN1 to DN3b stages. At ETP/DN1 B cell fate is switched off through heavy methylation of B fate promoting genes EBF1 and Pax5. At DN2a Notch1 signalling and Bcl11b expression inhibits myeloid and NK cell fate by repressing the activity of several transcriptional factors including PU.1, GATA-3, Zfp105, Nfil3, Zbtb16 and the E protein Id2. Several TCR genes are upregulated at DN2 but are used later at DN3. Total T cell commitment occurs by DN3a, with an extreme dependence on Notch1 signalling that is enhanced by Lunatic/Manic Fringe, and the transcription complexes RUNX/CBFβ and Ikaros. At DN3b there is up regulation of pro-survival or pro-proliferative molecules such c-myc, cyclinA, B, CDK2 and TIS21. TCF-1 positively regulates Bcl11b expression to promote T cell fate. E protein expression Egr3 induces expression of Id3, which represses E2A and relieves its inhibition of RORγt. Low Notch1 signalling favours γδ T cell fate. RORγt expression peaks at the Double Positive (DP) stage where it promotes thymocyte survival. During positive and negative selection, expression of microRNA (miR) 181a targets DP thymocytes to apoptosis with autoreactive T cell receptor (TCR) signalling by inhibiting Bcl-2, CD69 and TCR.
1.2.5 Alternative cell fates within the thymus

Thymic precursors entering the thymus have lost the ability to generate megakaryocytes and erythroid cells. However, they still retain the ability for up to 6 other alternative fates including B cells, myeloid fates like dendritic cells, macrophage or granulocytes, and Natural Killer (NK) fates such as NK and NKT cells.

The potential for B cell fate is thought to be lost at the beginning of thymus precursor entry, although studies during foetal thymic development in mice suggest it is lost even before arriving at the thymus (Harman et al., 2005; Masuda et al., 2005). The early exclusion of B cell fate is due to Notch signalling, although how Notch does this is still unclear. As mentioned, a decrease or absence of Notch signalling results in the thymus filling with B cells, however the induction of Notch signalling happens over the course of days and not immediately at DN1 (Feyerabend et al., 2009; Franco et al., 2006). Work from the Rothenberg laboratory suggest that transcription factors like EBF1 and Pax5, which are essential for B cell development are heavily methylated in early thymic precursors (Rothenberg, 2011). This concurs with a study by Smith and colleagues showing that Notch signalling can interfere with EBF1 functions, indicating that the genes for B cell lineage specification can be silenced epigenetically, and perhaps combined with other transcription factors to restrain the B lineage programme along with Notch (Smith et al., 2005).

The specificity for myeloid fate exists until the DN2 stage, and if thymocyte development continues undisturbed the normal outcome is a population in which T cells predominate, with some dendritic cells, macrophages, granulocytes and NK cells. The potential for NK fate disappears between the DN2a (c-Kit++ CD44+ CD25+) and DN2b (c-Kit+ CD44+ CD25+) stages, and this is due to induction of T-lineage specific transcription factor Bcl11b as mentioned in Section 1.2.3.3. Bcl11b deletion can induce NK development from DN2 thymocytes (Balciunaite et al., 2005; Ikawa et al., 1999) or de-differentiation of T cell committed thymocytes back into DN2a thymocytes, thereby controlling pathways which negatively regulate NK cell fate (Ikawa et al., 2010; Li et al., 2010a; Li et al., 2010b).

Alternative fates within the thymus are complicated by the fact that similar regulatory pathways are accessed by a number of lineages for specification. Early thymic precursors, DN2a, B cells and myeloid cells share certain transcription factors such the bZIP transcription factor C/EBPα and the (ETS)-family factor PU.1 (Laiosa et al., 2006b). Furthermore, PU.1 is essential for dendritic cell development (Carotta et al., 2010). T and B cells both use transcription factors Ikaros, Runx1/CFBβ, as well as the Myb and E proteins to regulate their fate (Mansson et al., 2007; Miyamoto et al., 2002). The commitment to the T cell lineage is then fully launched at the DN3 stage to generate either γδ or αβ T cells. For the purposes of this review the focus will be on T cell fate decisions during αβ maturation of thymocytes, particularly at DN3.
1.3 A focus on DN3 fate determination

A number of fate choices are available for DN3 thymocytes including quiescence, self-renewal, proliferation, differentiation and death, all of which involve complex coordination of factors. The following describes in detail the critical fate choices and their regulation at DN3.

1.3.1 DN3 TCR-β rearrangement triggers fate choices such as quiescence

Following the extensive proliferation and steady increase in T cell commitment at the DN2 stage, thymocytes enter the DN3a phase (originally termed DN3e) and are small in cell size with low surface CD27/CD28 expression (Hoffman et al., 1996; Teague et al., 2010). DN3a thymocytes arrest at G0/G1, and this is necessary to enable rearrangement of the TCR-β, -γ and -δ chains. Quiescence is imposed by TIS21, a pan-cell cycle regulator from the antiproliferative gene family (Ikematsu et al., 1999; Rouault et al., 1992). Interestingly, ectopic expression of TIS21 has an antiproliferative affect in competitive co-cultures of sorted DN1 thymocytes on OP9-DLL1 stromal cells, but does not affect differentiation. However, when the same experiments are repeated with sorted DN3 thymocytes there is decreased proliferation, increased death and reduced differentiation towards the DP stage (Konrad and Zuniga-Pflucke, 2005). This indicates that TIS21 affects fate choices differently at DN1 compared to the DN3 stage.

1.3.2 Selected DN3 thymocytes undergo bursts of proliferation and includes self-renewal

DN3a cells are thought to undergo limited self-renewal, 4 rounds of division, due to prolonged Notch1-Delta ligand interactions facilitated by Lunatic Fringe as a mechanism for expansion of early β-rearrangement events (Kreslavsky et al., 2012; Penit et al., 1995; Petrie et al., 2000; Yokota et al., 2006; Yuan et al., 2011). Incorrectly TCR-β arranged DN3a thymocytes die, but if the new TCR-β is in-frame and produces a pre-TCR signalling complex, these DN3a thymocytes differentiate through DN3b (originally termed DN3l), characterised by increased CD27/CD28, reduced CD25, and large cell size (Hoffman et al., 1996). DN3b thymocytes then undergo a burst of proliferation, at least 6-8 rounds of divisions causing a 100-fold expansion before initiation of TCR-α rearrangement (David-Fung et al., 2006). Notably, self-renewal of DN3 thymocytes is not unlimited. Continuous seeding of precursors into the thymus is necessary for the generation of T cells, and studies have shown intrathymic or intravenous transfer of thymocytes and bone marrow cells give rise to transient thymopoiesis, but this is not sustained (Donskoy and Goldschneider, 1992; Goldschneider et al., 1986; Scollay et al., 1986). This led to the idea that the thymus does not provide a suitable environment for self-renewal. However, we now know that limited self-renewal of DN3 and DN4 thymocytes does occur and is necessary to provide enough DP thymocytes for positive and negative selection where up to 97% DP thymocytes will die.
1.3.3 DN3 thymocytes absolutely rely on Notch signalling for survival

From DN2b to DN3a there is an abrupt transition to extreme Notch dependence (Ciofani et al., 2004; Ciofani and Zuniga-Pflucke, 2005). Notch promotes survival of pre-T cells by regulating cellular metabolism and this is acutely so during DN3 β-selection. Notch also cooperates with pre-TCR signals, as conditional inactivation of Notch using the lck promoter results in a partial arrest at DN3a and impairments in overall TCR rearrangement (Wolfer et al., 2002). The involvement of Notch at DN3 stages has been well studied using Rag2−/− thymocytes where thymic development is paused at DN3a prior to β-selection. DN3a thymocytes from Rag2−/− mice cannot maintain glycolysis and undergo accelerated cell death in the absence of Notch and TCR-β signalling, displaying increased features of apoptosis such as loss of mitochondrial potential and caspase 3 activation (Ciofani and Zuniga-Pflucke, 2005). The expression of pro-survival proteins such as Bcl-2 cannot rescue this defect in proliferation and accelerated apoptosis. However, isolated DN3a thymocytes from Rag2−/− deficient mice transduced with TCR-β can maintain glycolysis and survive, developing into CD4−CD8− DP stage upon culturing on the OP9-DL1 stromal cells (Ciofani et al., 2004). As mentioned in Section 1.2.3.2, ectopic expression of downstream Notch pathways such as active Akt can restore β-selection in the absence of Notch signals. Notch also acts through PI3K and the oncogene Myc (Ciofani and Zuniga-Pflucke, 2005; Palomero et al., 2006; Weng et al., 2006) and it will be interesting if these signalling pathways can also substitute for Notch. These data suggests that Notch engagement and signalling facilitates TCR rearrangement by aiding the survival of DN3 thymocytes through TCR.

1.3.4 Transcriptional change at DN3

A number of transcription factors maintain the exclusion of myeloid cell fates within the thymus through the modulation of each other, or the modulation of other transcriptional elements to promote T lineage specification at DN3. For example, the Runx/CBFβ complexes are in part regulated by Notch signalling to maintain the repression of myeloid fate from DN2 to DN3 (Georgescu et al., 2008). Also, the transcription factor GATA-3 negatively regulates PU.1 expression, which is needed at high levels for myeloid and B cell development (Taghon et al., 2007). In addition to TIS21, quiescence can also be imposed at the DN3 stage through the expression of E proteins such as E2A and HEB1. These two E proteins induce the expression of suppressor of cytokine signalling 1 and 3 (SOCS1 and SOCS3). SOCS1 and SOCS3 temporarily uncouple DN3a surface IL-7 receptors from the proliferative machinery as they undergo TCR-β rearrangement, resulting in cell cycle arrest and hence quiescence (Schwartz et al., 2006). Transcriptionally, the “winning combination” mediating T cell differentiation from DN3 seems to be strong expression of Ikaros and Gfi, moderate expression of C/EBPα, Egr2, or Id2, repression of PU.1 by GATA-3, Runx1, Gfi1 and TCF-1 along with the continuous activity of E proteins (Rothenberg et al., 2010).
1.3.5 CXCR4 signalling synergizes with pre-TCR and Notch at DN3

The importance of chemokine signalling at the DN3 stage is apparent from two recent studies of CXCR4. Trampont and colleagues use of a conditional deletion of cxcr4−/− in the adult thymus to demonstrate that CXCR4 is necessary for differentiation and survival of DN3 thymocytes at the β-selection checkpoint. Loss of CXCR4 results in an accumulation of DN2/3 thymocytes in the mid cortex, and decreased downstream differentiation when sorted cxcr4−/− DN3 thymocytes are cultured on OP9-DLL1 stromal cells (Trampont et al., 2010). These thymocytes also undergo higher levels of apoptosis due to a reduction of the prosurvival protein Bcl2-A1, which is induced in response to CXCR4 signalling. Interestingly, DN3a thymocytes from Rag2−/− mice also display very little migration in response to the CXCR4 ligand, CXCL12, similar to cxcr4−/− DN3a thymocytes. In parallel studies, Janas and colleagues identify p110δ and p110γ as critical catalytic subunits of the PI3K pathway downstream of CXCR4 signalling (Janas et al., 2009). These subunits are required for pre-TCR-mediated Akt phosphorylation, and as mentioned previously are also activated in response to Notch, indicating crosstalk between CXCR4, pre-TCR and Notch.

1.3.6 DN3 thymocytes can differentiate into γδ and αβ T cells

Depending upon the rearrangement outcome, most cells will either differentiate further to become an αβ T cell, or die if no intact TCR complex is produced (Taghon and Rothenberg, 2008). A minority will produce in-frame γ and δ chains and differentiate into a γδ T cell. Deletion of the Notch-recruited factor RBPj results in an increase of γδ T cells, and expression of TCR-γδ alone in Rag2−/− DN3a cells can also divert them to the γδ lineage (Tanigaki et al., 2004; Wong and Zuniga-Pflucke, 2010). This indicates that γδ thymocytes are less dependent on Notch signalling whereas for αβ thymocytes, Notch signalling is indispensable (Ciofani and Zuniga-Pflucke, 2010). Current studies also highlight E protein regulation for αβ versus γδ T lineage divergence. High levels of the E protein Id3 and strong TCR signals are sufficient to induce γδ T cell development, whereas both Id3 and Notch signalling are required for αβ T cell specification (Lauritsen et al., 2009; Ueda-Hayakawa et al., 2009). Therefore, DN3 could serve as a critical lineage stage for a choice between αβ versus γδ fate. Strong TCR/pre-TCR signals will give rise to γδ T cells, whereas weak/intermediate TCR/pre-TCR signalling favours αβ T cell development (Hayes et al., 2005; Hayes and Love, 2006).

Taken together, it can be seen that Notch, CXCR4 and the pre-TCR are three critical regulators at the DN3 checkpoint. The combined effects of these three signals enable DN3 cells to pass the β-selection checkpoint, avoid apoptosis and differentiate into DP cells. The various choices at the DN3 stage are summarised in Figure 1.4.
Figure 1.4 Regulation of fate choices at DN3

DN3a thymocytes paused at G0/G1 undergo TCR-β rearrangement, and for each successful rearrangement a new population of DN3b thymocytes arise, which undergo cell cycle arrest for TCR-α recombination. Signals (mediated by pre-TCR, CXCR4 and Notch) include alterations in expression or activity as shown (up/down arrows) to determine allelic exclusion, proliferation (when and for how many generations), differentiation (αβ and perhaps γδ), death, and limited self-renewal.
1.4 Deregulation of thymocyte fate in T cell leukaemia

Given the many fate choices during the DN3 stage, alterations in these choices can contribute to T cell leukaemia. With respect to leukaemia, fate choices at DN3 have major clinical relevance as altered expression of a number of genes and transcription factors which impact on fate, can result in malignant transformation. T cell acute lymphocytic leukaemia (T-ALL) is an aggressive malignancy characterised by high numbers of undifferentiated thymocytes in the bone marrow, lymph nodes and often the central nervous system. Molecular analysis of T-ALL cases reveals that the alteration of least four pathways contributes to thymocyte malignancy with many involving aberrant DN3 development. These include genes that deregulate i) self-renewal capacity, ii) the cell cycle, iii) proliferation, and iv) differentiation.

Genes expressed at DN3 such as TCR-β, -α, and -δ feature frequently in chromosomal aberrations in T-ALL. This is due to interchromosomal translocations rather than the intrachromosomal translocations required during proper TCR rearrangement, possibly through aberrant activity of RAG recombinase (Boehm et al., 1988). The partner genes misexpressed in these chromosomal translocations include Notch1. Adult human T-ALL patients commonly present with t7:9 chromosomal translocations placing Notch1 under enhancer-promoter elements of the TCR-β gene (Ellisen et al., 1991). As mentioned, Notch is critical for DN3 proliferation and survival, but whether it is also important for regulating stem cell maintenance through self-renewal has been more controversial. Many gain-of-function studies implicate an important role for Notch for haematopoietic stem cell expansion, however deletion of Notch1, Notch/Notch2 or RBP-J does not affect haematopoietic stem cells in the mouse, nor does preventing transcriptional activation of all four Notch receptors through transgenic expression of dominant negative MamL (Karanu et al., 2000; Maillard et al., 2008; Stier et al., 2002; Varnum-Finney et al., 2000; Wu et al., 2007). This suggests that T-ALL translocations or activating mutations including Notch also give unlimited expansion, and a second hit may be necessary to induce self-renewal potential in leukaemic thymocytes. Activating mutations in Notch1 contributes to over 50% of paediatric T-ALL, with 44% of these cases containing Notch1 mutations that increase Notch ICN production rates without the need for ligand stimulation (Weng et al., 2004). Another 30% of these Notch1 mutations extend Notch ICN half-life, which is normally short (between 1-3 hours) due to ubiquitination and degradation (Gupta-Rossi et al., 2001; Schweisguth, 2004). Most of these Notch mutations still require cleavage activity by gamma-secretase activity to generate downstream signalling, making it an attractive candidate for therapy. However, long term treatment using gamma-secretase inhibitors in Alzheimers patients result in defects in gut epithelial differentiation, as well as decreased thymic cellularity and impaired DN2/3 differentiation (Wong et al., 2004).

The LYL1, TAL1, Lmo1 and Lmo2 transcription factor genes also partner frequently in TCR-β, -α, and -δ chromosomal translocations (De Keersmaecker et al., 2005). These transcription factors are important for early hematopoietic development during foetal stages (Dear et al., 1995; Roberts et al., 1994). Ectopic expression of LYL1 and TAL1 results in a developmental block of subcapsular thymic cells consistent with the anatomical region for DN3 thymocytes, and correlates with unfavourable clinical outcome in paediatric T-ALL (Ferrando et al., 2002). As
LYL1 and TAL1 can both directly bind E proteins such as E2A (Hsu et al., 1991; Hsu et al., 1994; Miyamoto et al., 1996), this would suggest that constant expression of these transcription factors inhibits E protein activity, deregulating DN3 fate by allowing incorrect TCR rearrangements to pass checkpoints, predisposing to T-ALL.

The LIM-only proteins 1 and 2, Lmo1 and Lmo2 (previously RBTN1/2 or TTG1/2), are transcription factors with functions in early erythropoiesis and angiogenesis. Null mutations in mice lead to failure of yolk sac erythropoiesis, extensive vascular disorganisation and embryonic lethality at E10.5 (Mead et al., 2001; Warren et al., 1994). In particular, Lmo2 is found as a complex with other DNA binding proteins such as GATA1, LDB1, E47 and TAL1 in normal erythroid cells (Osada et al., 1995; Wadman et al., 1994; Wadman et al., 1997). However, a different complex assembles in leukemic thymocytes of CD2-Lmo2 transgenic mice, in which Lmo2 is ectopically expressed from the CD2 T cell promoter (Larson et al., 1996). In this complex GATA1 is replaced by another TAL1 molecule (Grutz et al., 1998; Larson et al., 1996). CD2-Lmo2 mice display developmental blocks at the DN3 stage, indicating that Lmo2 either induces genes that promote DN3 proliferation/self-renewal, or represses genes required for differentiation. Indeed, McCormack and colleagues have compared gene expression profiles of CD2-wildtype and CD2-Lmo2 thymocytes to show that Lmo2 upregulates the expression of stem cell associated genes such as Hhex and Lyl1, whilst repressing T-cell developmental genes such as TCR-β, pre-TCRα and CD8 (McCormack et al., 2010). They identify the DN3 population as the self-renewing population, as isolation and transfer of these thymocytes in primary, secondary, tertiary and quaternary transplants all give rise to T-ALL (McCormack et al., 2010). There is a long latency period of up to 10 months, indicating that additional mutations must accumulate for overt tumour formation. In agreement, all currently studied CD2-Lmo2 tumours display diverse surface TCR, as well as additional mutations in TAL1 and LYL1 (Aplan et al., 1990; Bash et al., 1995; Larson et al., 1996; McCormack et al., 2010). Collectively, expression and mutation of these transcriptional factors results in impairment of differentiation and excessive self-renewal of thymocytes, highlighting DN3 as the oncogenic pool.

I have described broadly how thymocyte differentiation occurs, and how commitment of early thymic precursors involves the gradual relinquishment of alternative fate decisions. T cell fates are directed by the thymic epithelium that provides potent cues such as Notch and chemokine signalling. Although many of the molecular players during this fate determination process have been identified, we still do not yet understand how these signals are integrated to co-ordinate such fate decisions, or how these processes are dysregulated to cause T cell leukaemia. How does a thymocyte know when to proliferate? To undergo quiescence? To self-renew? Or die? What extent are these processes reversible? How can self-renewal occur in DN3 thymocytes, if all thymocytes are destined to differentiate and die? Similar fate decisions are, in fact, made by many different cells during development, and the principles governing their fate choices may also apply to thymocyte fate. Fate determination has been extremely well-studied in a number of multicellular organisms such as in worm and fl, and studies are also beginning to emerge for mammalian development which will be described in the next section.
1.5 Asymmetric cell division and its regulation during fate determination

Fate determination is critical for organogenesis in all multicellular organisms. In progenitor cells of the developing nematode and fl, fate determination is dictated by a type of cell division known as asymmetric cell division (ACD). ACD involves the partitioning of information differently into daughters of dividing cells including protein, mRNA, microRNA or other cellular constituents. The result of ACD imparts different fates to those daughters which include: self-renewal, proliferation, apoptosis and differentiation. Popular models used to study ACD, how it regulates different fate decisions and outcomes include the nematode *Caenorhabditis elegans* zygote formation, the fruit fly *Drosophila melanogaster* neuroblast and sensory organ precursor, as well as more recent studies of gut, skin and neuronal development in mammals.

1.5.1 ACD is a type of cell division

Cell division, or mitosis, is a fundamental process for cellular expansion as well as other processes such as tissue growth and wound healing. Cell division includes five stages to complete, beginning with prophase, metaphase, anaphase and telophase, and ending with the separation of newly formed daughter cells at cytokinesis. At prophase, the chromosomes duplicate and are released into the cytoplasm following the breakdown of the nuclear envelope. During metaphase, the chromosomes are sorted and tethered to the mitotic spindle, a transient structure whose function is to capture the duplicated chromosomes as well as other cell factors, and pull them into each daughter cell. In anaphase, the sister chromatids are pulled to opposite poles, to which the nuclear envelope reforms at telophase and a cleavage furrow forms to separate the daughter cells via cytokinesis.

Historically, mitosis has been considered a process for eukaryotes to partition genetic information equally between daughter cells that can differentiate afterwards if required. We now know that cellular diversity not only can be generated subsequent to cells dividing into two identical daughters with subsequent differentiation, symmetrically, but they can also divide asymmetrically to produce two daughter cells with inherently distinct fates (Figure 1.5).

1.5.2 ACD mediates self-renewal for tissue maintenance

To ensure that a population of parent cells is maintained, ACD often controls self-renewal to generate a daughter cell identical to its parent, as well as another daughter that is programmed to proliferate, differentiate, or do both. Self-renewal is a common outcome for ACD to maintain the blood, muscle, gut and skin stem cells (Giebel, 2008; Knoblich, 2010; Potten et al., 1997). Furthermore, the cells that come from this type of ACD are multipotent and are capable of undergoing further ACD to generate the downstream cell types as required.
In the vinegar fruitfly *Drosophila melanogaster*, self-renewal through ACD is the primary mechanism for generating the cells of their central nervous system. During the initial development of the larval nervous system, parent cells called neuroblasts delaminate from the neuroepithelium to undergo up to twenty rounds of ACD, each round involving self-renewal of the neuroblast as well as differentiation into neural cells. After being quiescent during pupation, the existing neuroblasts re-enter the cell cycle, re-initiate ACD, and complete the generation of the adult fly brain (Ito and Hotta, 1992; Knoblich, 2010). Type 1 neuroblasts (most common) and Type II neuroblasts (dorsoposterior region) both undergo self-renewal and differentiation by ACD to generate two lineages of neurons that finally comprise the nervous system.

### 1.5.3 ACD regulates proliferation

ACD not only controls self-renewal, but it also influences the expansion of each daughter cell. The small nematode *Caenorhabditis elegans* is an excellent model to study fate outcome including proliferation during ACD, as the fate of every single one of its 959 (adult hermaphrodite) or 1031 cells (adult male) is known (Sulston and Horvitz, 1977). Furthermore, the developmental program of *C. elegans* is relatively invariable and highly reproducible, from the timing of its divisions to the cell fates that arise. In *C. elegans*, ACD starts at the first embryonic division at the one cell P0 stage to produce an anterior AB cell and a posterior P1 cell (Figure 1.6). A series of four asymmetric cell divisions follow; each producing one daughter that contributes only to soma and the other only to the germline. Embryogenesis is then followed by four larval stages (L1-4) and an adult stage and each stage involves ACD. ACD during embryogenesis produces 558 nuclei, after which a period of quiescence takes place. The larval stage uses 53 of its somatic blast cells to generate the final 959 or 1031 somatic nuclei of the adult organism (Sulston et al., 1983). No further somatic divisions take place during the adult stage.

Although the fate of every *D. melanogaster* neuroblast has not been mapped, proliferation is also evident during neuroblast ACD, as the origin of thousands adult neurons in the central nervous system derive from a limited set of neuroblasts. As previously mentioned, larval neuroblasts undergo numerous rounds of ACD, whose daughter cells self-renew and undergo further ACD, expanding the neural pool. This is also seen during neurogenesis in the mammalian brain, which begins with a burst of symmetric cell divisions to increase the progenitor pool, then a series of ACD that balance self-renewal with differentiation of cells committed to the neuronal lineage. Thus, cell division can switch from symmetric to asymmetric where either mode can selectively expand specific cellular pools, or generate more differentiated cell types as the need arises.

### 1.5.4 ACD regulates apoptosis

ACD also controls downstream fate by preferentially inducing apoptosis of one daughter cell. Peripheral nervous system formation in *D. melanogaster* is a good example of ACD regulation of apoptosis (Figure 1.7). The development of the external sensory organ for the peripheral nervous system derives from asymmetrically dividing sensory organ precursor (SOP) cells to generate the
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Figure 1.5 Symmetric versus asymmetric cell division

Cellular expansion and diversity occurs through two types of division modes: A) symmetric cell division where information is packaged equally into daughter cells and equal fates are imparted. Subsequent diversity would be due to extrinsic factors. An alternative mode of division is B) asymmetric cell division where a cue (in this case another cell) results in information differentially packaged into one or the other daughter. This imparts different fate potentials including different capacities for proliferation, quiescence, apoptosis, differentiation and self-renewal.
initial pIIa and pIIb daughters which divide further to give rise to the external and internal sensory organs respectively. These pIIa and pIIb cells contain different fate potential in subsequent ACDs. All of the pIIa descendants give rise to a variety of accessory cells in the sensory organ. pIIb descendants will undergo an ACD to generate a neuron, but also a glia cell that always undergoes apoptosis and does not become part of the sensory organ (Gho et al., 1999; Lai and Orgogozo, 2004). ACD regulation of apoptosis in the pIIb daughters repeats over again until the peripheral nervous system is fully developed, possibly as a way to balance self-renewal and proliferation.

1.5.5 ACD regulates differentiation

The differentiation of cells can be due to many external or internal influences, and as mentioned earlier ACD is one method to generate the diverse cells types during animal development. ACD might be a ‘cost effective’ method to generate diversity, self-renewal and apoptosis, in one single division.

Again, the development of the D. melanogaster peripheral nervous system provides a good illustration of how ACD regulates differentiation. Subsequent to SOP ACD, the initial pIIa daughters undergoes a further ACD, giving rise to accessory cells such as the hair and socket cells in the external sensory structure, whereas the pIIb daughters divide further to give rise to neurons, and glia cells that undergo apoptosis as mentioned previously (Gho et al., 1999) (Figure 1.7). It is currently thought that Notch and its ligands are present in both pIIa and pIIb daughters, but the presence of other cell fate determinants such as Numb represses Notch signalling in the pIIb daughter through targeted degradation via endosomal pathways (Berdnik et al., 2002). It is possible that pIIb daughters preferentially die because of repressed Notch signalling, although this remains to be demonstrated. The absence of essential proteins and cell fate determinants to regulate ACD can also drastically affect SOP differentiation, producing the incorrect cell (Betschinger and Knoblich, 2004; Betschinger et al., 2006; Rhyu et al., 1994; Uemura et al., 1989). This demonstrates that ACD of SOP cells can dictate the fates of the daughters as well as affect the downstream differentiation of the granddaughter cells.

Another example of ACD regulation of differentiation is the initiation of the sex-specific program in C. elegans, which derives from establishing a proximal-distal axis of asymmetrically dividing somatic gonadal precursor cells during the L1 larval stage. Depending on whether the embryo is XX (hermaphrodite) or XO (male), these somatic gonadal precursor cells undergo ACD to generate a double armed ovotestis (hermaphrodite), or a single-armed tesis (male). The differentiation of daughter cells to produce these two sex organs relies on two major pathways of regulation in subsequent ACDs. The first pathway involves the Wnt/MAPK pathway. This pathway is responsible for asymmetrically distributing transcriptional activators to specify the number of distal or proximal daughter cells that will go onto to differentiate into either sex organ. The second pathway induces sexual differentiation during distal or proximal daughter ACD, and involves the asymmetric inheritance of transcription factors such as FKH-6 for male specification, or TRA-1 in hermaphrodites (Hodgkin and Brenner, 1977; Mathies et al., 2004; Tilmann and...
Figure 1.6 Asymmetric cell division during *C. elegans* embryogenesis to adulthood.

The development of *C. elegans* nematode starts with asymmetrical cell division during the first cleavage of the egg cell (P0) to produce the somatic founder cells AB, MS, E, C and D, and the germline precursor P4 (red). Asymmetric cell division during embryogenesis produces 558 nuclei, and during the four larval sages (L1-4), 53 somatic blast cells are used to generate the final 959 (male) or 1031 (hermaphrodite) nuclei that comprise the final tissues of the adult organism. Nomenclature system as defined by Sulston and Horvitz, 1977. Adapted from Sulston et al., 1983.
Kimble, 2005). ACD therefore specifies *C. elegans* sexual fate through differential inheritance of pathways and factors that act on cell number position and cell differentiation.

Evidence of ACD in mammals also exists during the development and differentiation of the brain, muscle, gut, mammary glands, skin or cells of the blood system (Faubert et al., 2004; Klezovitch et al., 2004; Kuang et al., 2008; Lechler and Fuchs, 2005; Wu et al., 2007). All of these processes involve ACD, or balances between symmetric and asymmetrical divisions to control different downstream fates. For example, the human colon crypt is a high turnover tissue where $10^{10}$ cells are replenished using a balance of symmetric and asymmetrical division (Potten et al., 1997). Within the folds of epithelium lining the colon, crypt cells undergo ACD to self-renew and to generate a proliferative daughter cell to populate the migrating compartment. These migrating daughters also undergo differentiation towards the crypt surface. Once there they die, shed into the lumen and are replaced by the new generation of daughter cells asymmetrically dividing from the crypt. The mechanisms guiding these decisions in mammals are not well understood, however, many molecular players have been identified in seminal studies from *C. elegans* and *D. melanogaster*, and preliminary evidence suggests high conservation in mammals. The regulation of ACD and the role of those molecular players shall be described in the next section on polarity proteins.
D. melanogaster sensory organ precursor cells undergo asymmetric cell division to produce the pIIa and pIIb daughter cells. The pIIa daughter undergoes asymmetric cell division to produce the external socket and hair cells of the external sensory organ. The pIIb daughter cell undergoes asymmetric cell division to produce a neuron and a glial cell that usually undergoes apoptosis. In some cases the glial cell survives and undergoes limited proliferation. This process iterates until the peripheral nervous system is fully developed.
1.6 Polarity proteins and ACD

Developmental studies of both *C. elegans* and *D. melanogaster* in the past two decades show that ACD is largely regulated by an evolutionary conserved group of polarity proteins. These polarity proteins exist in complexes and are responsible for organising a cell during division as well as many other contexts. The localisation of polarity proteins depends on cell positioning, such as maintaining contact within a particular microenvironment or neighbouring cell, which can be critical for sending polarity signals to affect cell-fate choice. Cell positioning also determines how a cell is attracted to a particular niche via chemokine and other paracrine signals. These signals in turn provide polarity cues to establish an orientation axis for cell division which can be regulated by polarity proteins. The axis of polarity during division also induces major cytoskeletal reorganisation, mitotic spindle alignment and asymmetric proportioning of molecules for ACD. The net result is the bifurcation of cell fate determinants to impart different cell fates on the daughters. To illustrate the involvement of the polarity protein complexes during ACD, I will first describe initial studies in polarised epithelial cells. The involvement of the polarity protein complexes during the regulation of ACD shall then be discussed.

1.6.1 The polarity protein complexes: its discovery and early studies in polarised epithelial cells

Cell polarity is the asymmetrical distribution of cellular constituents, such as proteins, carbohydrates and lipids, to particular regions within a cell or group of cells (Muth and Caplan, 2003). In epithelial cells, polarity can be broadly categorised into 5 forms including: 1. Apical-basal (top-and-bottom) polarity 2. Planar (side-by-side) polarity 3. Migratory (front-back) polarity, 4. Cell-specific polarity and 5. Asymmetric cell division (ACD). Genetic analysis of polarised epithelial cells in *D. melanogaster* has identified three protein complexes which interact to establish cell polarity: (1) Bazooka (Baz) / Par6 / atypical protein kinase C (aPKC), (2) Crumbs (Crb) / Stardust (Sdt) and (3) the Scribble / Discs large (Dlg) / Lethal giant larvae (Lgl) group. Mutations in any one of these genes results in loss of epithelial cell polarity, disruption of tissue architecture and neoplastic tumours, leading to the designation of the genes as cell polarity genes and tumour suppressors (Wodarz, 2000).

The following sections provide a brief description of each of the proteins in each complex. A list of the *C. elegans* and mammalian homologues, general function and involvement in ACD is summarised in Table 1.2.

1.6.1.1 Bazooka (Baz) or Par3 / Par6 / atypical protein kinase C (aPKC)

The Baz, Par6 and aPKC proteins were first discovered to regulate polarity in studies of *C elegans* blastocyst cleavage patterns, followed by studies of *D. melanogaster* embryogenesis during epithelial apical-basal polarity establishment (Hutterer et al., 2004; Kemphues et al., 1988; Tabuse et al., 1998). Baz and Par6 contain PDZ (PSD95/Dlg/ZO-1) protein interacting domains that
enable Baz and Par6 to tether proteins and signalling molecules into complexes, whereas aPKC is a serine/threonine kinase that acts by phosphorylating proteins to restrict localisation of itself or other proteins (Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Romero et al., 2011; Suzuki et al., 2001). These polarity proteins are localised to C. elegans blastomeres, the apical and lateral cell membrane in polarised D. melanogaster epithelial and neuroblast cells, as well as mammalian epithelia (Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Joberty et al., 2000; Tabuse et al., 1998). The Baz/Par3/Par6 polarity complex is responsible for establishing the apical identity of each epithelial cell during embryonic invagination, as mutations or loss of Baz, Par6 or aPKC results in loss of the apical domain. Two aPKC homologues (PKCι/λ and PKCζ), three Par6 homologues (Par6α, β and γ) and one Baz homologue (Par3) have been identified in mammals. To date it has emerged that modulation of aPKC kinase activity is largely conserved and central to consequent polarity output by the cells. For example, Par6 has been shown to regulate the spatio-temporal activity of aPKC through direct interaction with GDP-bound but not GTP-bound Cdc42 (Cau and Hall, 2005). aPKC is also known to phosphorylate and polarise the endosomal protein Numb, a cell fate protein required for binary cell-fate decisions during D. melanogaster nervous system development (Smith et al., 2004). aPKC phosphorylation of Numb restricts its subcellular localisation in both D. melanogaster and mammalian cells, which can be prevented through mutation of aPKC at serine 7 and serine 295 (Dho et al., 2006; Nishimura and Kaibuchi, 2007; Smith et al., 2007). Polarity proteins therefore function to segregate proteins differentially during various cell functions, which can include preferential packaging of information into daughters to regulate asymmetric fates.

1.6.1.2 Crumbs (Crb)/ Stardust (Std)

The function of the transmembrane proteins Crb and Sdt is two-fold: junctional assembly at the apical epithelial membrane, and polarity maintenance once those junctions have been formed. Crb function depends on binding of its highly conserved C-terminal domain with Stardust (Roh et al., 2003). D. melanogaster Crb has three mammalian homologues, of which only Crumbs3 (CRB3) is widely expressed in mammals. The mammalian homologue to Stardust is Protein associated with Lin7 (Pals1). In both mammals and flies, the interaction between CRB3 and Pals1 is required to regulate the formation of apical (tight) junctions and in mammalian epithelial cells, aPKC phosphorylates CRB3. Pals1 also binds to the PDZ domains of Par6 along with Par3, stabilising aPKC activity which is repressed by Par6 (Hurd et al., 2003; Lemmers et al., 2004).

1.6.1.3 Scribble/ Discs large (Dlg) / Lethal giant larvae (Lgl)

Similar to studies of the Par and Crb complexes, loss of Scribble, Dlg and Lgl results in disruption of apical-basal epithelial cell polarity (Bilder et al., 2000). Members of this protein complex are rich in protein-protein interaction domains necessary for tethering and docking of partner proteins to mediate downstream cellular polarity. Scribble is a scaffolding protein from the LAP family, containing four PDZ domains and sixteen leucine rich repeats (Bilder et al., 2000). Dlg contains one Src homology domain (SH3), three PDZ domains and a guanylate kinase-like domain and
Table 1.2. The polarity protein complexes

<table>
<thead>
<tr>
<th>C elegans</th>
<th>D melanogaster</th>
<th>Mammals</th>
<th>Described function</th>
<th>Involvement in ACD?</th>
<th>References</th>
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<tbody>
<tr>
<td>Par Complex</td>
<td></td>
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<tr>
<td>PAR-3</td>
<td>Bazooka</td>
<td>Par3</td>
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<tr>
<td>Dlg-1</td>
<td>Discs Large</td>
<td>Dlg 1-4</td>
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<td>Yes</td>
<td>Woods and Bryant, 1989, Bossinger et al 2001, Albertson and Doe, 2003</td>
</tr>
</tbody>
</table>
Lgl contains four W40 repeats (Strand et al., 1994; Woods et al., 1996). Scribble indirectly associates with Dlg through mutual binding of a protein termed GUK-holder (GUKH) (Mathew et al., 2002). Lgl is a cytoplasmic protein that binds directly to the LRR domains of Scribble, but also can interact with members of the Par complex (Kallay et al., 2006). The Par and Scribble complexes exert polarity through antagonistic effects on each other, and this may occur due to phosphorylation events between aPKC and Lgl (Humbert et al., 2003). In D. melanogaster loss of either one of these genes drastically alters the polarity and downstream fate of cells throughout the organism. Maternal/zygotic mutation of scribble/dlg/lgl in D. melanogaster either results in embryonic lethality, pupation failure and overgrowth of tissues. In these cases the mutant contains up to five times as many cells as wildtype, and the overgrown larvae fail to pupate. Polarity proteins are therefore critical for proper cell fate.

In mammals there is only one homologue for Scribble, whereas Dlg has four (Dlg1-4) and Lgl has two (Lgl1-2) homologues. This makes mammalian studies for null or mutant alleles for these genes difficult due to genetic redundancy between polarity gene homologues. Both mammalian Lgl1 and Lgl2 isoforms can still both bind to Par6 and serve as substrates for aPKC dependent phosphorylation, and because of these redundancies are frequently referred to as Lgl collectively for both isoforms (Plant et al., 2003; Yamanaka et al., 2006). In mammalian epithelial cells, interactions between Lgl, Par6 and aPKC can suppress the kinase activity of aPKC (Yamanaka et al., 2006), and aPKC itself can suppress Lgl activity in the apical domain via phosphorylation of central conserved serine residues, restricting its cortical localisation to the basal region of the cell (Musch et al., 2002). This shows polarity protein function is conserved to some extent throughout evolution, and that the cooperation between polarity protein complexes could be regulated by similar pathways. A diagrammatic summary of the polarity proteins and their interplay is shown in Figure 1.8.

### 1.6.2 The maintenance of polarity by Rho GTPases

Much of what is known about the maintenance of polarity derives from studying epithelial cells. The establishment and maintenance of polarity relies largely on the recruitment and activity of small Rho GTPases which direct multiple signal transduction pathways that impact on cytoskeletal organisation during shape change, adhesion, migration, membrane trafficking and division (Citi et al., 2011). Well studied Rho GTPases include RhoA, Rac1, and Cdc42. Studies in astrocytes and fibroblasts shows that migration primarily involves the action of RhoA, which triggers contractility of the cytoskeleton to enable forward propulsion (Hall, 2005). Rac1 and Cdc42 are key Rho GTPases responsible for stabilising aspects of junctional assembly between epithelial cells through interaction with Par6, as well as polarising the microtubule organising centre during forward migration and division (Aceto et al., 2006; Hall, 2005; Joberty et al., 2000; Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006).

Rho GTPases are activated by Rho guanine nucleotide exchange factors (RhoGEFs). The activity of Rac1 and Cdc42 in leading membrane ruffles during forward extensions relies on the Rho GEF
Bazooka (Baz), Par6 and aPKC are recruited to the apical region of the cell where the GTP-bound Rho GAP Cdc42 activates aPKC kinase activity through direct interaction with Par6. aPKC phosphorylates Crumbs (Crb) and Lethal Giant Larvae (Lgl). Phosphorylated Crumbs is recruited to the apical domain where it interacts with Stardust. Phosphorylation of Lgl restricts its activity to the basal membrane where it interacts directly with Scribble (Scrib), and indirectly with Discs Large (Dlg). The Scrib/Dlg/Lgl complex and Baz/Par6/aPKC complexes maintain mutually exclusive localisation through an as yet unidentified antagonistic repression mechanism. Antagonistic repression also exists between the Scrib/Dlg/Lgl and Crumbs/Stardust complexes. Scrib also directly interacts with the Rho GEF β-pix. This interaction can translocate Scrib and Dlg to the cell membrane to redirect microtubule reorganisation during migration or T cell immunological synapse formation. GTP-bound Cdc42 also activates Pak, β-pix, and the Rho GEF Rac1 to mediate dynamic microtubule and actin reorganisation. Indicated names are as for *D. melanogaster* proteins. See Table 2 for equivalent *C. elegans* and mammalian homologues.
β-pix (Audebert et al., 2004; Wittmann and Waterman-Storer, 2001). Knockdown of β-pix results in a decrease of actin-based protrusions and migration, demonstrating it is required to recruit and activate Rac1, whereas Cdc42 activates β-pix through interaction with Pak (Cau and Hall, 2005; Gomes et al., 2005; ten Klooster et al., 2006). Scribble binds directly to β-pix through its PDZ domains, thereby linking the polarity protein complexes to downstream cell polarity via the Rho GTPases (Figure 1.8, and Audebert et al., 2004). A study by Cau and colleagues in primary rat fibroblasts show Cdc42 regulates the repositioning of the nucleus and the microtubule organising centre through activation of Par6/aPKC (Cau and Hall, 2005). Hence, the establishment and maintenance of polarity also involves cross-talk between Rho GTPases and polarity proteins.

Maintenance of polarity through Rho GTPases also includes crosstalk with other signalling pathways controlling microtubule orientation. When the microtubule apparatus is altered through disruption of G-protein coupled signalling, germ cells in zebrafish fail to undergo polarised migration to directional cues (Xu et al., 2012). Instead, the germ cells display increases in Rac1 activity, developing multiple cell protrusions in random locations (Xu et al., 2012). Taken together, it can be seen that the establishment and maintenance of polarity relies on temporal and spatially restricted activation of Rho GTPases. Their action and connection of the polarity proteins with other signalling networks ensure cell junctions are constrained and that cells move in the right direction. Rho GTPases also link actin microtubule changes during mitosis, from cell rounding to the extension of the mitotic spindle, to contractile ring formations and cleavage furrowing during cytokinesis (Canman et al., 2008; Maddox and Burridge, 2003; Yoshida et al., 2009). Rho GTPases are therefore also important for organising the cell cycle machinery and polarity proteins during ACD.

1.6.3 Conserved mechanisms of polarity and ACD during worm, fly and mammalian development

The polarity protein complexes play important roles in the regulation of fate determination. This includes dorsal closure during gastrulation, organ specification, neurogenesis in brain and gut formation. Disruption of these processes can result in defects in fate determination during organogenesis, and is one of the major factors contributing to the invasiveness of metastatic cancers. This next section describes the regulatory processes of polarity underscoring ACD with examples in several organisms, and how this might impact on cancer.

The regulation of ACD involves three parts. First, a polarity cue to align the cells along an axis, second, the interplay of polarity and cytoskeletal proteins to sustain polarity of cell fate determinants along that axis and third, the coordination of the mitotic spindle with the polarity axis to ensure asymmetric inheritance into each daughter cell and different fates (see Box 1).
Chapter 1: Introduction

Polarity cue

**Extracellular matrix:** provides the necessary scaffold to position a cell's polarity axis prior to asymmetric cell division. This can include collagen, fibronectin, vitronectin, or laminin. Cells would have to possess the corresponding adhesion receptors such as integrins, cadherins, selectins and syndecans.

**Cellular cues:** direct cellular cues such as cell-to-cell contact, or indirect cues such as exogenous cytokines, hormones and chemokine gradients can act to position and polarise a cell prior to asymmetric cell division.

Maintain the axis of polarity

**Polarity proteins:** mutual antagonistic, signaling and tethering activity of the Scrib/Dlg/Lgl, Par3/Par6/aPKC and Crb/Sdt polarity protein complexes can maintain the asymmetric distribution of intracellular constituents as well as the polarity axis during division.

Coordinate the mitotic spindle

**Microtubule structures:** basal bodies and centrosomes are microtubule organisation centre (MTOC) structures that align the mitotic spindle. The mitotic spindle is derived from astral microtubules attached to two centrosomes. Alignment of the mitotic spindle is coordinated by polarity proteins, RhoGTPases and cytoskeletal proteins.

**RhoGTPases:** localised activation of RhoGTPases such as RhoA, Rac1 and Cdc42 by a variety of RhoGEFs direct multiple signal transduction pathways that organise the cellular cytoskeleton during shape change including asymmetric cell division.

**Cytoskeletal proteins:** include actin, dynein, EB1 and APC. Spatial regulation of the mitotic spindle by these cytoskeletal proteins as well as RhoGTPases and polarity proteins assist in coordinating the mitotic spindle for asymmetric cell division.

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**Box 1.1 The checkpoints for asymmetric cell division**

<table>
<thead>
<tr>
<th>Polarity cue</th>
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<tbody>
<tr>
<td><strong>Extracellular matrix:</strong> provides the necessary scaffold to position a cell's polarity axis prior to asymmetric cell division. This can include collagen, fibronectin, vitronectin, or laminin. Cells would have to possess the corresponding adhesion receptors such as integrins, cadherins, selectins and syndecans.</td>
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1.6.3.1 Polarity Cues

a. The extracellular matrix (ECM).

The tissue architecture provides cells with the necessary structural scaffold to position a polarity axis during migration or ACD. The surrounding extracellular matrix (ECM); rich in collagen, fibronectin, vitronectin and laminin polymers, are involved in interactions to generate functional polarised tissues (Klinowska et al., 1999; Schuger et al., 1998). Cellular niches can contain local combinations and concentrations of these ECM components, sending signals to instruct dividing cells to proliferate and differentiate. The niche can therefore act as a polarity cue to dictate the initial events of ACD. In turn, to receive and transduce these signals a dividing cell would need to contain the appropriate cell adhesion receptors for these ECM components. These receptors include the classic integrins, cadherins, selectins and syndecans (Klinowska et al., 1999; Schuger et al., 1998). Immune cells can also interact with the ECM through the immunoglobulin superfamily of cell adhesion molecules to mediate cell-matrix communication which is important during the immune response (Russell, 2008).

b. Cellular cues.

Aside from the ECM, cellular cues can also initiate the axis of polarity prior to a cell undergoing ACD. Direct cellular cues can occur between the binding of adhesion receptors between two cells to signal a polarity cue. This is particularly important in maintaining polarity in epithelial cell sheets, and also applies to ACD. Direct cellular interaction that depends on cell positioning
during colon gut formation or dermal skin cell maturation is critical for setting up a polarity axis (Achilleos et al., 2010; Bloor and Kiehart, 2002; Schneider et al., 2010). Another example is the position of sperm entry into an oocyte during fertilisation, which sets in motion the axis of polarity and downstream ACD events (described in Section 1.6.3). Indirect cellular cues include factors released by cells including growth/inflammatory signals such as cytokines, hormones and chemokine gradients. These signals can act on cells just prior to division, polarising their surface receptors (adhesion or chemokine), or at division, polarising the delivery and recycling of intracellular endocytic vesicles. Cellular cues can initiate the downstream signalling events that coordinate the polarity axis during ACD (Ivaska et al., 2005; Palacios et al., 2001).

1.6.3.2 Polarity and cytoskeletal proteins

While the transduction of external signals can initiate the axis of polarity, polarity must be coordinated and maintained throughout the cell during ACD. As mentioned in Section 1.6.1, functional studies of the polarity proteins in D. melanogastor and its mammalian homologues show some conservation in function. The current model for polarity assumes that asymmetric distribution of intracellular constituents is achieved through mutual antagonistic activity of localisation between each of the Scribble/Dlg/Lgl, Par3/Par6/aPKC and Crb/Sdt protein complexes. In both D. melanogaster and mammalian epithelial cells, the Par and Crb complexes are responsible for restricting the Scribble polarity proteins to the apical domain, and the Scribble polarity protein complex is thought to repress the apical activity of the Par and Crb complexes (Tepass et al., 1996; Uemura et al., 1996). The function of these polarity proteins is also conserved across species to some extent during ACD, with evidence of crosstalk with cytoskeletal proteins such as dynein to control cellular morphology as well as maintaining the axis of polarity of the mitotic spindle.

1.6.3.3 The mitotic spindle and centrosomes

Microtubules play a key role to maintain the axis of polarity during ACD particularly at telophase. As part of the cytoskeletal network, microtubules nucleate and are organised by microtubule-organising centres (MTOCs) such as centrosomes and basal bodies. Microtubule structures take part in many functions such as generating propulsion during migration, moving intracellular organelles, as well as setting up a polarity axis during mitosis. During ACD, the mitotic spindle derives from astral microtubules attached to two centrosomes. Polarisation of the mitotic spindle depends on tight spatial restriction of many signalling components including the polarity protein complexes, Rho GTPases, as well as cytoskeletal proteins such as actin, dynein, EB1 and APC (Cau and Hall, 2005). Several studies have shown that polarity proteins can affect the centrosomes, and in turn the centrosome can dictate the localisation of those proteins (de Forges et al., 2012; Hong et al., 2010; Manneville et al., 2010; Motegi et al., 2011; Yuan et al.).
1.6.4 Examples of ACD during development

The following three examples describe how the requirements for ACD are incorporated to generate cellular diversity (summarised in Figure 1.9).

1.6.4.1 Example 1- C. elegans zygote formation for embryogenesis at P0

The large size and transparent nature of the C. elegans zygote allows the easy viewing of the first P0 division, generating two daughters of unequal size, a large anterior AB cell and a smaller posterior P1 cell (Figure 1.9 A). Prior to fertilisation the C. elegans oocyte is essentially unpolarised, apart from dispersed actomyosin contractions that presumably drive local membrane invaginations. The position of the sperm entry upon fertilisation at P0 is the external cue that determines the cleavage plane, as well as the axis of polarity during ACD. The movement of the sperm MTOC towards the oocyte cell cortex triggers a cessation of local contractility resulting in asymmetrical cortical flows and marks the posterior end of the developing embryo. It is still unclear how the sperm acts as a posterior polarity cue, although studies point to the rapid discharge of internal mechanical stress to induce a cortical flow, the movement of chromosomes or the redirection of actin-dependent endosome movement (Li et al., 2008; Munro and Bowerman, 2009; Taunton et al., 2000; van der Gucht and Sykes, 2009). The change in cortical flows within the vicinity of the sperm cue is regulated by a sustained gradient of Rho-1 and Cdc42 activity. This enables the transport of Par-3/Par-6/aPKC polarity proteins towards the anterior pole, and the cell fate determinant proteins Par-1, Par-2 and P granules to the posterior pole (Jenkins et al., 2006; Kemphues, 2000; Motegi and Sugimoto, 2006; Schonegg and Hyman, 2006; Watts et al., 2000). In turn, Par-1 regulates the diffusion and segregation of other cellular determinants such as MEX-5 and PIE-1 for somatic versus germline cell fates through differential phosphorylation events and localised modifications of the actin cytoskeleton (Daniels et al., 2009; Tenlen et al., 2008).

In the first asymmetric division at P0, regulatory feedback between the distribution of Par-3/Par-6/aPKC proteins and the other cell fate determining molecules maintain the anterior and posterior boundaries after asymmetrical contraction ceases. Mutation of the anterior Par-3/Par-6/aPKC proteins results in a posterior shift of the boundary, whereas par-1 mutants display an anterior shift (Cuenca et al., 2003; Kirby et al., 1990; Munro et al., 2004; Velarde et al., 2007). The magnitude and extent of cortical flow also seems to regulate the position of the anterior-posterior boundary during the establishment of polarity. For example, increasing Rho activity results in stronger contractile forces that cause an anterior shift, and when contractility is inhibited a posterior shift occurs (Schmutz et al., 2007; Schonegg et al., 2007; Shelton et al., 1999). However, considering the sperm triggers a local cessation of contractility in the posterior end, this alone cannot explain the opposing forces existing at the anterior-posterior boundary. There is speculation that differences in cytoplasmic viscosity at the posterior end exists as an opposing force to maintain the anterior-posterior boundary (Munro and Bowerman, 2009).

The P0 stage coincides with the expansion of the microtubule spindle coming from the sperm aster and maternal centrosome, indicating that they are required to establish and maintain polarity...
Figure 1.9 Models of asymmetric cell division in C. elegans, D. melanogaster and mammals.

A) Asymmetric cell division in the P0 division in C. elegans. The site of sperm entry serves as a polarity cue for the asymmetric distribution of polarity and cell fate determining proteins as well as spindle asymmetry. Wnt signalling in the emerging posterior AB daughter cell induces POP-1 export into the cytoplasm from the nucleus, thereby specifying all future posterior or soma fates. The anterior daughter does not export POP-1 from the nucleus and becomes the P1 cell, and all future germline cells of C. elegans. B) In D. melanogaster neuroblasts, neuronal precursors delaminate from the neuroepithelium to undergo asymmetric cell division. The polarity cue is the apical crescent, and duplicated centrosomes rotate 90 degrees to create the distinct apical and basal sides. Asymmetric distribution of polarity and cell fate determinants causes spindle asymmetry to result in a large self-renewing neuroblast cell and a smaller Ganglion Mother Cell (GMC). C) Neuronal precursor asymmetric division in mammals. The polarity cue is contact through N-cadherin interactions between the neuronal precursors and the apical side of the neuroepithelium. Neuronal precursors rotate the emerging basal daughter by 45 degrees, asymmetrically distributing Notch and potentially Numb signalling which is restricted to the lateral membrane. Par3/aPKC are localised to the apical region of neuronal precursors, along with Lgl. The first asymmetric cell division produces a neuron and an Intermediate Neuronal Precursor (INO), which undergoes a symmetric division to produce two neurons. Hatched lines indicate the axis of division, for the names of distributed proteins refer to the coloured key below each diagram.
Chapter 1: Introduction

C. elegans 
- Early division: P0 division
- Late division: AB cell soma fate

D. melanogaster 
- Neuroblast division
- Apical crescent

Mammals 
- Neuronal precursor division
- Apical contact

Proteins
- Par-3/Par-6/PKC3
- LIN-5/Gα
- POP-1
- Par-3/Par-6/PKC3
- Rho1/Cdc42

Cell fate
- AB cell soma fate
- PI cell germinal fate

Polarity cues
- Basal
- Apical

Proteins
- Baz/Par-6/PKC3
- Inscuteable/Pins/Gai/Mud
- Numb/Brat/Prospero
- Miranda/Pon/Notch
- Scribble/Dlg/Lgl
- N-cadherin/Notch
- Par3/aPKC
- GMR-1/GRP-2
- GMR-1/GRP-2
- POP-1

- GRP-1/GRP-2
- Lgl
- Notch
prior to the first cell division. The difference in size between the AB and P1 daughter cells after
the first ACD is thought to derive from asymmetric pulling forces exerted on the mitotic spindle
by the PINS homologue G protein regulator 1 and 2 (GRP-1 and 2) (Gonczy, 2008). Proteins that
provide an attachment site for the plus end of microtubules such as LIN-5 and LIS-1 are recruited
by Gα bound GRP-1 and -2. Because the concentration of bound GRP-1 and -2 is higher in
the posterior end, an uneven pulling force is exerted on the mitotic spindle, causing an unequal
mitotic spindle, and therefore unequal daughter size during the first ACD.

*C. elegans* mutants that fail to establish polarity correlate with the loss of maternally supplied
proteins controlling centrosome assembly and maturation (Cowan and Hyman, 2004; Hamill et
al., 2002). Conversely, where meiosis fails or is slowed there is mislocalisation of the Par proteins,
actomyosin and some (but not all) cell fate determinants (Cowan and Hyman, 2006; O’Connell,
2000; Sonneville and Gonczy, 2004; Tsai and Ahringer, 2007; Wallenfang and Matunis, 2003). In
studies by Tsai and Ahringer, zygotes with smaller mitotic asters take longer to establish polarity,
suggesting that the spindle and/or centrosomes are responsible for transmitting polarity signals
during ACD. Interestingly, if the centrosomes are ablated after establishment of the anterior-
posterior boundary, it shifts posteriorly. If disrupted before stabilisation, the anterior-posterior
boundary shifted slightly anteriorly compared to wildtype embryos (Tsai and Ahringer, 2007).
This implies the regulatory networks governing the establishment of zygote polarity must be
maintained, with constant feedback between the polarity proteins and cytoskeletal dynamics.
Further studies are needed to understand the full interplay between the Par proteins and Rho
GTPases, how contractile force is achieved to control the anterior-posterior boundary and how
cortical flow generates force during intracellular contractions

After the first P0 ACD, the posterior blastomere produces a Wnt ligand that specifies the bifurcation
of anterior versus posterior cell fate at P0. Posterior daughters receiving the Wnt signal promote
nuclear export of the DNA-binding protein POP-1, a phenomenon known as POP-1 asymmetry.
Mutants of Wnt upstream components such as sys-1/wrm1 result in nuclear POP-1 symmetry
between daughters and extra proximal cells (Herman, 2001; Miskowski et al., 2001; Siegfried et
al., 2004; Siegfried and Kimble, 2002). Gonadal sex-specification (described in Section 1.5.4)
also utilises the Wnt pathway, and gonadal cell precursors with excess SYS-1/WRM-1 result in
extra distal cells (Kidd et al., 2005). This suggests the asymmetric localisation of downstream
Wnt regulators determines asymmetric fates in post-embryonic cells and in this instance establish
the anterior-posterior axes.

1.6.4.2 Example 2- regulation of *D. melanogaster* neuroblast division

In *D. melanogaster* neuroblasts, polarity proteins and other cell fate determinants maintain
polarity just prior to and during ACD. Asymmetrically dividing neuroblasts align their mitotic
spindle along a pseudo “apical/basal” axis to regenerate an apically positioned neuroblast, and a
basally positioned ganglion mother cell (GMC). The spindle orientation is perpendicular relative
to the overlaying ectoderm of the apical crescent (*Figure 1.9 B*). Dividing neuroblasts do not
contain junctional complexes like adherens junctions, and disruption of junctional components like APC1/2 do not perturb polarity nor mitotic spindle orientation (Akong et al., 2002; Lu et al., 2001). What then, is the cue for polarity?

The answer lies in the apical crescent. The division of the first embryonic neuroblast after delamination determines the apical-basal axis, as well as the local phosphorylation events which occur subsequent to the establishment of this axis. Time lapse microscopy of *D. melanogaster* neuroblasts show the mitotic spindle is parallel to the embryonic surface, but then rotates 90 degrees to assume its apical-basal position (Kaltschmidt et al., 2000). Every subsequent division is then aligned along the same apical-basal axis. After this rotation there is enrichment of several proteins at the apical crescent, namely the Par complex. Apical activation of the kinase Aurora A results in the phosphorylation of Par6, which in turn activates aPKC. aPKC then exits the complex after phosphorylating Lgl. Loss-of-function studies of the apical complex proteins, of *baz* and *par6* result in randomisation of the mitotic spindle (Kuchinke et al., 1998) (Petronczki and Knoblich, 2001; Wodarz et al., 1999). This indicates that the initiation and establishment of polarity does not involve a linear sequence of events of cue, the triggering of polarity and then mitotic axis formation, but in fact a crosstalk between all these factors for ACD.

Similar to the *C. elegans* zygote, Baz, Par6 and aPKC also regulate segregation of cell fate determinants such as Prospero, Miranda, Numb, and partner of Numb (Pon). In *D. melanogaster* zygotic mutants of *apkc* mislocalise Lgl and Miranda to the cortical cell membrane with only weak staining at the basal or apical ends (Rolls et al., 2003). However, in contrast to the *C. elegans* zygote the Baz-Par6-aPKC complex in *D. melanogaster* neuroblasts is held in check at the apical membrane during division by the basally located adaptor protein Inscuteable (Insc) as well as the recruitment of Pins (a receptor-independent regulator of Ga<sub>i</sub>). Mutation of these genes leads to loss of spindle reorientation, primarily because the apical aster loses its microtubule-nucleating activity and starts to migrate basally (Rebollo et al., 2007). The net action of the Baz, Par-6 and the Pins/Gai complex thereby provides another polarity cue to coordinate the orientation of the mitotic spindle along an apical-basal axis, the targeting of different cell fate determinants and the strong daughter size asymmetry upon division. Interestingly, the neuroblasts are still centred on the apical crescent, indicating that aPKC is not required for alignment of the mitotic spindle along the divisional axis of polarity. However, aPKC is required for neuroblast cellular proliferation as the adult alpha and beta lobes contain about a quarter the number of neurons in *apkc* dominant negative mutants compared to wildtype counterparts (Rolls et al., 2003). This is not due to increases in cell death, but due to both daughters inheriting Miranda, Prospero, Numb or other cell fate determinants that induce quiescence and a halting of further division. These studies contrasts several aspects of polarity regulation during ACD in *C. elegans* and *D. melanogaster*. The Par3 proteins in *C. elegans* are responsible for establishing the anterior and posterior boundaries during ACD, whereas in the *D. melanogaster* neuroblast, they not only regulate the segregation of fate determinant but also control the orientation and position of the mitotic spindle, as well as daughter cell size.
Biochemical and genetic studies show that aPKC phosphorylates Lgl protein locally, causing its dissociation from the apical cortex. In a study by Albertson and colleagues, GFP-Lgl is enriched apically during early neuroblast division at metaphase along with myosin-II. This dissociation results in non-muscle myosin II filament extension to inhibit the cell fate determinant Miranda (Betschinger et al., 2006). Consistent with this observation, myosin II is also concentrated apically in neuroblasts, and when inhibited by mutation or chemical inhibitors of Rho-associated kinase (ROCK), mislocalise Numb and Miranda (Barros et al., 2003). Crosstalk between the apical complexes, the polarity proteins and the actin cytoskeleton is thought to act similarly for the other cell fate determinants.

Like the Par polarity complex, the Scribble/Dlg/Lgl polarity proteins localise at the apical side during early neuroblast division and disperse cortically at telophase (Albertson and Doe, 2003). Mutations in scrbibe or lgl do not affect Dlg polarisation, but Dlg is required for the cortical recruitment and polarisation of both Scribble and Lgl (Albertson and Doe, 2003). Furthermore, Dlg and Lgl are required for the localisation of Numb and Miranda in basal crescents, but interestingly are not required for apical localisation of the Baz/Par-6/aPKC complex, suggesting they function in parallel or downstream (Peng et al., 2000). The function of the Scribble complex extends to more than regulation of its own complex proteins. Scribble and Dlg act to dock and target basal proteins to the plasma membrane. The LRR and PDZ domains of Scribble are required for this targeting, although expression of the LRR domain alone can partially rescue the loss of polarity exhibited in scribble mutant embryos (Albertson et al., 2004; Zeitler et al., 2004). Lgl is similarly responsible for basolateral targeting of proteins but occurs through active phosphorylation by aPKC. Lgl phosphorylation by aPKC is essential to restrict its localisation to the basal cortex of the cells. It is thought that restricting Lgl to the basal cortex prevents it from associating with the plasma membrane to establish basal identity with Scribble and Dlg. Interestingly, expression of a non-phosphorylatable form of Lgl (Lgl-3A) does not disrupt polarity in epithelial cells as it does in neuroblasts, indicating that the function of the polarity proteins complexes overlap and depend on the cell type (Peng et al., 2000).

Neuroblasts expressing dominant negative forms of apkc do not display defects in apical localisation of Par3, Insucutable or Pins and this is similarly observed in single, double and triple mutants of scribble, dlg and lgl. However, in the scribble, dlg and lgl mutants, Miranda and Prospero mislocalise to the spindle microtubules, centrosomes and the cytoplasm (Humbert et al., 2003). The mislocalisation of these fate determinants both limits cellular proliferation, and reduces the size of the apical spindle cortex resulting in a smaller neuroblast cell compared to the budding ganglion mother cell. This inverted cell division derives from the dysregulation of the polarity network, where instead of spindle poles of asymmetrical length, the distance from centrosome to the cortex equalises in both hemispheres (Albertson and Doe, 2003). Given that Lgl is known to interact with myosin, and vertebrate orthologues of Dlg physically interact with microtubule-binding proteins, it is possible that apical Scribble/Dlg/Lgl actively regulate and promote apical spindle growth (Hanada et al., 2000; Matsumine et al., 1996; Niethammer et al., 1998). In summary, the polarity proteins Scribble, Dlg and Lgl can regulate mitotic spindle length.
for downstream daughter cell size. Par6 and aPKC function is responsible for anchoring the apical end of a dividing neuroblasts to transduce downstream signals controlling cell proliferation, possibly through the transcription factor Miranda, but do not affect the orientation of the mitotic spindle.

Along with the action of polarity proteins, Notch signalling in *D. melanogaster* neuroblasts and sensory organ precursor cells regulates fate. Notch is present in both daughters after neuroblast and sensory organ precursor ACD, but downstream signalling of Notch is regulated by the cell fate determinants Numb and Neuralised (Betschinger and Knoblich, 2004). Numb is an endocytic protein that localises to the basal crescent in asymmetrically dividing neuroblasts through phosphorylation by apical aPKC. Genetic and biochemical assays show Numb directly binds to the intracellular domain of Notch, acting as a negative regulator possibly by mediating Notch degradation via endocytic pathways mediated by α-Adaptin, a component of the AP-2 complex which targets proteins for endocytosis (Berdnik et al., 2002; Takei and Haucke, 2001). During sensory organ precursor ACD, Notch signalling induces pIIa cell fate, but the presence of Numb in the other daughter prevents signal transduction and results in a pIIb cell fate. As Numb polarises the distribution of α-Adaptin into the pIIb daughter cell, this suggests that Numb represses Notch signalling by targeting it for endocytosis and degradation. Interestingly, loss of α-adaptin leads to phenotypes similar to loss of Numb during sensory organ precursor ACD, where neurons and glia are transformed into socket and hair cells (Berdnik et al., 2002). Of note, the same phenotypes are observed with loss of *lgl*, and epistasis experiments place *lgl* downstream or parallel to *notch* and *numb* mutations. Neuralised regulates Notch activity in two ways to specify sensory organ precursor pIIa and pIIb fate. First, Neuralised targets the Notch ligand Delta for ubiquitination in the pIIb cells thereby preventing Notch activation, and secondly it also facilitates cleavage of Notch in pIIa cells to activate signalling (Le Borgne and Schweisguth, 2003; Li and Baker, 2004; Parks et al., 2000). Numb and Neuralised therefore act on Notch to generate a bias in Notch signalling for specifying neuroblast and sensory organ precursor cell fate.

### 1.6.4.3 Example 3- conserved mechanisms of polarity and ACD in mammals

Studies of the invertebrate models like *C. elegans* and *D. melanogaster* have been fundamental to identifying the molecular players involved in regulating ACD to mediate fate decisions. Only in recent years have we begun to investigate whether they are conserved in vertebrates such as mammals. Many of the polarity, cytoskeletal and cell fate proteins that regulate ACD in these studies are also expressed and conserved to some extent in mammals, with some existing as spliced variant homologues. The function of mammalian polarity homologues are also conserved in terms of regulating epithelial polarity (described in [Section 1.6.1](#)). In addition to the polarity proteins, Gpsm2/LGN (Gpsm2/LGN is Pins in *D. melanogaster*) and Numb are all expressed and are also involved in the orientation of the mitotic apparatus as well as differential inheritance of fates.
Neural progenitor differentiation is the best studied example of mammalian ACD (Figure 1.9C). Neural progenitor cells on the apical side of neuroepithelium in the mammalian brain undergo ACD to produce a daughter that is a copy of the parent (self-renewal), as well as a daughter that will undergo further division and differentiation to generate a neuron, or an intermediate neural progenitor which undergoes symmetric cell division to generate two neurons. Neuroepithelial progenitors must retain apical contact via N-cadherin to set up a polarity axis and therefore could serve as the polarity cue (Marthiens and ffrench-Constant, 2009). Studies by Marthiens and colleagues show that N-cadherin is asymmetrically inherited in almost 50% of cases, but interestingly no correlations of cell fate with these differences have been identified (Marthiens and ffrench-Constant, 2009). Polarity proteins such as aPKC, Par3 and Lgl localise on the apical region of apical neural progenitors, and Numb concentrates on the apical adherens junctions and on the lateral membrane (Siller and Doe, 2009). ACD is thought to occur when the apical progenitor rotates approximately 45 degrees, inactivating Numb in one of the two daughter cells. As it is known that Par3 promotes Numb phosphorylation by aPKC in neuroblasts, the inactivation of Numb stems from differential phosphorylation by aPKC in one of the daughter cells after ACD. Interestingly, altering spindle orientation through cre-lox deletion of Gpsm2 (the mammalian homologue of Pins) causes a randomisation of spindle orientation during ACD (Konno et al., 2008). This results in neural progenitor migration deeper into the brain, but overall neural development remains normal. Only when downstream components of Gpsm2 signalling pathway are disrupted do both mitotic spindle orientation and daughter neural fate alter. For example, when the β-adrenergic receptor kinase C-terminal domain (β-ARK) is overexpressed, sequestering of Gβγ proteins disrupt G-coupled protein signalling, causing a shift in spindle orientation and hyper differentiation of neural progenitors that would otherwise undergo ACD (Sanada and Tsai, 2005). It is possible that other upstream polarity components aside from Gpsm2 act on G-coupled protein signalling to alter neural fate through spindle orientation. However, how the polarity proteins are involved to regulate ACD in these situations is still largely unknown. More studies are needed to dissect the localisation and interplay of other polarity protein players such as aPKC, and other cell fate determinants.

1.6.5 Dysregulated polarity and ACD during cancer

Aberrant cell polarity is a frequent observation in cancers, and the connection between perturbed polarity, ACD and tumourogenesis has changed the way we look at fate determination. For example greater than 90% of diagnosed human cancers originate in epithelial tissues such as lung, colon, breast or prostate, and while each cancer type is distinct in its aetiology, disruption to normal cell polarity and fate is frequently observed (Parkin et al., 2005). It is possible that these cancers arise from defects in ACD that distribute cell fate determinants incorrectly into daughter cells. In these cases daughters usually targeted by ACD for apoptosis may instead inherit factors that allow rapid proliferation, self-renewal, or both. Mounting evidence described in studies of oncogenesis below have begun to suggest that tumours are derived from defects in ACD and this idea is rapidly gaining momentum (Gonzalez, 2007; Hawkins and Russell, 2008; Humbert et al., 2008; Klezovitch et al., 2004).
Several lines of evidence demonstrate a link between dysregulated polarity, fate determination and cancer. Firstly, Dlg and Scribble are both targets for degradation by the E6 oncoprotein from high risk human papilloma viruses HPV16 and HPV18, which are causally linked in more than 90% of cervical cancers (Burger et al., 1996; Gardiol et al., 1999; Nakagawa and Huibregtse, 2000; zur Hausen, 2000). More importantly, the E6 protein from these viruses binds to the PDZ domains of Dlg and Scribble among other proteins that lead to cellular transformation. Mutants lacking these domains cannot transform rodent cells or induce epithelial hyperplasia even though they still bind and inactivate p53 (Kiyono et al., 1997; Nguyen et al., 2003). Furthermore, other oncogenic viruses such as HTLV-1, Tax and Ad9 E4 ORF1 can also bind Dlg at its PDZ-binding sites, increasing the activity of the tumour suppressor protein Adenomatous polyposis coli (APC) and leading to uncontrolled cell proliferation (Suzuki et al., 1999). In these cases the skewing of polarity deregulates the normal fate of cells, predisposing to cancer, possibly through defects in ACD.

Secondly, both Scribble and Dlg protein expression are dysregulated in a variety of tumours in both mouse and human cancers. For example, Dlg and Scribble are both reduced in late-stage invasive tumours, and Scribble expression is reduced or increased in tumour associated with HPV infection, breast and colon cancer (Gardiol et al., 2006; Massimi et al., 2004; Nakagawa et al., 2004; Navarro et al., 2005). cDNA microarray analysis reveal Dlg1 and Dlg4 correlate with invasive diffuse gastric cancers, as they are down regulated in comparison to normal gastric tissues (Boussioutas et al., 2003). These studies show that the invasiveness of cancers correlate with a deregulation of polarity.

Thirdly, in D. melanogaster, genetic screens have identified mutant alleles of scribble, dlg and lgl as negative regulators of Cyclin E, the protein responsible for S-phase entry during mitosis (Brumby et al., 2004). In particular, loss of scribble and with oncogenic ras and notch cooperate to drive invasive cancer by deregulating the cell cycle (Brumby and Richardson, 2003). Furthermore, in brat mutants (a translation inhibitor which is asymmetrically inherited), the neuroblast cells display prolonged cell cycle blocks, followed by rapid indefinite proliferation and tumour formation (Bello et al., 2006; Betschinger et al., 2006). Cumulatively, these studies show that defects in polarity can cause the formation of tumours comprising of cells lacking the appropriate cell cycle checkpoints. It also shows that asymmetrically segregating determinants like polarity proteins act as tumour suppressors, at least in D. melanogaster.

Finally, several mammalian studies highlight links between cancer and perturbed fate determination and polarity. Investigation of in vitro derived mammalian breast stem cells as mammospheres that overexpress the oncogene erythroblastosis B2 (ERBB2), has been important for connecting mislocalisation of cell fate determinants with tumour formation (Dontu et al., 2003). In wild type tissue, only the cells containing PKH26 (a dye that marks active proliferation) can create secondary mammospheres, and these localise Numb asymmetrically. In this case one cell retains the dye after single mammosphere isolation, indicating the initial division was asymmetric. In contrast, mammospheres that have been transformed with ERBB2 all proliferate to contribute to
tumour growth. When isolated and cultured, none of the cells localise Numb nor are they marked with PKH26, indicating the initial division was symmetric. Similar observations have been made for p53-mutant mice, the master regulator of cell cycle and survival. It is also known that p53 degradation is regulated by Numb (Cicalese et al., 2009; Colaluca et al., 2008), strengthening the link between cancer and aberrant fate determination.

In addition to the neoplastic phenotypes observed in mammospheres, loss of lgl results in tumour phenotypes in both the D. melanogaster and mammalian brain (Klezovitch et al., 2004; Rolls et al., 2003). In D. melanogaster, aPKC and Lgl genetically and functionally interact, as reduced levels of aPKC can suppress the lgl- brain tumour phenotype and also suppress the lgl- epithelial cell polarity defect. This implies that the coordinated regulation of Lgl phosphorylation is essential for the establishment of polarity and therefore cell fate during neuroblast ACD. Interestingly, lgl- mice display the formation of neuroectodermal tumours and severe disruption to the brain architecture (Klezovitch et al., 2004). In the same mice, staining of brain sections show mislocalisation of the apically localised fate determinant Numb. This suggests dysregulation of fate determination (via Numb) occurs in the absence of a classic tumour suppressor (Lgl) that is also a polarity protein. These studies indicate that cell proliferation and maintenance of polarity are interlinked, and when polarity is absent the brakes on cell checks lift to allow cell transformation. This transformation may need additional mutations in order for oncogenic transformation. For example, the depletion of Scribble along with the addition of mutations in c-myc cooperate to induces dysplasia in vivo after a long latency period (Zhan et al., 2008). In an elegant study of murine prostate cancer by Pearson and colleagues using conditional scribble- knockout mice, they demonstrate that Scribble depletion alone is a poor initiator of prostate cancer. However, the addition of activating K-ras mutations cooperates with scribble- to accelerate prostate tumour initiation and progression, therefore supporting a ‘multihit’ hypothesis to cancer (Pearson et al., 2011).

To directly test how ACD is altered during cancer will be difficult because ACD itself potentially determines all fates such as differentiation, apoptosis, self-renewal and quiescence. Furthermore, regulators of ACD such as polarity proteins are reduced or expression is lost in some cancers, but are increased in other cancers. Polarity proteins also function in other physiological processes, and depending on the cell type and context their dysregulation may impact on ACD in entirely different ways. It may also depend on the species, and while evidence of ACD exists across species, not all processes may be conserved due to genetic redundancies. Sophisticated experimental approaches will be necessary to determine if ACD functions during oncogenesis. These can include the development of genetically modified mice for conditional expression of molecules, genomic screens to assess epigenetic modifications, or the advancement of imaging technology to track daughter fates during the onset of cancer. Such approaches will enable molecular dissection to determine of ACD and how it is involved in cancer.
1.6.6 Summary

The early studies of polarity in *C. elegans* and *D. melanogaster* have contributed enormously to the current understanding of ACD, its involvement and regulation during fate determination. Direct or indirect polarity cues regulate localised phosphorylation events that are orchestrated by the polarity proteins, and in turn these pulling and pushing forces govern the fundamental basis of ACD. If polarity proteins and its downstream effectors are inherited asymmetrically, the daughters each contain a different capacity to operate the cell cycle machinery. This provides mechanistic explanation how differences in downstream fates would be linked on a molecular level. Disruption of any of the three requirements; be it the cue, polarity proteins or the mitotic axis can all influence cell fate, and therefore extend to affecting tissue homeostasis, aging, wound healing, or disease.

ACD is complicated because its regulators have multiple functions in many physiological processes across species, and these studies highlight several non-overlapping aspects of polarity regulation during ACD in *C. elegans* and *D. melanogaster*. The Par complex proteins in *C. elegans* are responsible for establishing the anterior and posterior boundaries during ACD, whereas in the *D. melanogaster* neuroblast, they control the orientation and position of the mitotic spindle, as well as daughter cell size. There is also emerging evidence showing that centrioles inherited during ACD are not identical, and that the self-renewing daughters preferentially inherit the mother centriole (Spradling and Zheng, 2007). The ECM, niche, and polarity cues may dictate a plane of division, but may not necessarily translate to a cell dividing symmetrically or asymmetrically. Indeed, *D. melanogaster* neuroblasts undergo ACD along an apical-basal plane, whereas *D. melanogaster* sensory organ precursors undergo ACD along an anterior-posterior axis. ACD can be utilised in various manners by the cell depending on the time and context.

There are other questions that remain. Do defects in ACD pave the way for tumourogenesis? The studies described here point to an essential role for polarity in cell fate outcome where the loss of cell polarity in addition to accumulations of oncogenic mutations initiates tumour formation. These lines of evidence suggest that loss of either of these polarity proteins may contribute to oncogenic progression by altering ACD, resulting in the increased aggressiveness of the cancer.
1.7 ACD and polarity in cells of the haematopoietic system

The function of the polarity protein complexes have largely been addressed through studies of invertebrate systems such as *C. elegans, D. melanogaster*, as well as epithelial cells in all of these systems including mammals. However, it is clear that several of the same proteins also function in many other cell types, including those of the haematopoietic system. The haematopoietic system is responsible for generating a vast array of myeloid and lymphoid cells that circulate throughout our blood. Recent studies have highlighted that fate determination in haematopoietic stem cells, as well as the B and T cells of lymphoid lineage can be influenced by ACD (summarised in Figure 1.10).

1.7.1 ACD in haematopoietic stem cell differentiation

Studying polarity during ACD in stem cells of the hematopoietic system is a challenge because unlike polarised epithelial cells which have distinct apical and basolateral sides, haematopoietic stem cell (HSC) polarisation is not so morphologically obvious. From the description of fate determination in invertebrate systems, it is apparent that ACD function includes regulation of stem cell fate and involves stem cell-like components (Hawkins and Russell, 2008; Morrison and Kimble, 2006). Therefore, there is growing thought that HSCs may also undergo ACD to regulate fate choices. In our blood the stem cell pool is maintained through a balance of divisions giving rise to differentiated blood cells whilst still maintaining stem cell fate through self-renewal. Several studies illustrate that HSCs undergo ACD, and that it is integral to the process of self-renewal. However, relatively little is known about the involvement of the polarity protein complexes during early and adult haematopoiesis and whether they play a role in ACD.

HSCs are often observed interacting with cells present in the niche of various organs which could provide polarity cues to mediate ACD to govern its fate. HSCs localise in the bone marrow, in foetal liver and in the peripheral blood (Giebel, 2008). In the bone marrow and foetal liver, HSCs interact intimately with endothelial cells surrounding the blood vessels, osteoblasts (bone marrow), or sinusoids in extramedullary sites (Morrison and Spradling, 2008). The cells in these niches could provide direct cues via cellular interaction or indirect cues through chemoattractive mechanisms for setting up a polarity axis. For example, the osteoblasts in bone marrow can express Notch ligands such as Jagged-1, adhesion molecules such as ICAM-1/LFA, L-selectins, and CD44, and also express chemokines such as CXCL12 (Dar et al., 2005) (Figure 1.10 A).

Although HSCs do not contain definitive apical or basal polarisation, they do exhibit morphological changes when dividing or migrating. Dividing cells are round and relatively immotile. Migrating cells display a hand mirror shape with a leading edge rich in adhesion molecules and a protrusion at the rear termed the uropod (Brummendorf et al., 1998; Peled et al., 2000). While several surface molecules have been identified to localise to either leading edge or uropod, it remains to be shown whether HSCs express and localise the various polarity proteins. Interestingly, HSCs express Numb, however, Numb polarisation is only observed in HSCs of young and not older mice (Giebel, 2008).
Figure 1.10. Models of asymmetric cell division in haematopoietic stem cells, B cells, T cells and DN3 thymocytes.

Multiple polarity cues might dictate ACD of haematopoietic stem cells (HSCs). A) HSCs migrating in a stem cell niche in the bone marrow can receive adhesion, Notch or chemokine cues from surrounding endothelial, osteoblast or sinusoidal cells, resulting in asymmetric distribution of cell fate determinants such as Notch and Numb (during attachment with the interacting cell, or separately) to produce a self-renewing haematopoietic stem cell and a haematopoietic progenitor cell which will go on to differentiate. In B) B cells and C) T cells the polarity cue could be provided through interaction with macrophages, T follicular cells and antigen presenting cells such as dendritic cells via ICAM-1 or an as yet unidentified molecule. ICAM-1 sets up an axis of division and asymmetric distribution of several surface molecules, antigen polarity and cell fate determinants. In B cells daughters proximal to the interacting cells favour memory B cell fate, as well as more potent T cell activators and proliferators. Distal B cell daughters may favour antibody secreting cell fate, with moderate T cell activating and proliferative capabilities. In the absence of ICAM-1, it appears that memory B cell fate is favoured at the expense of antibody secreting B cell fate. Once study suggests that proximal T cell daughters favour memory T cell fate, whereas distal T cell daughters favour effector T cell fates. In C) DN3 thymocytes, polarity cues could be delivered to the thymocyte via Notch, Chemokine of pre-TCR signallling to set up a polarity axis and resulting in an asymmetric distribution of protein and/or mRNA to impart different fates.
Chapter 1: Introduction

A. Haematopoietic stem cell

- Polarity cue
- Proximal
- Distal

B. B Cell

- Polarity cue
- Proximal
- Distal

C. T cell

- Polarity cue
- Proximal
- Distal

D. Thymocyte (DN3)

- Polarity cue
- Proximal
- Distal
The laboratory of Reya and colleagues contribute two findings of HSC fate determination during ACD. First, by using a GFP-Notch reporter system and timelapse imaging of paired daughters, they show HSCs undergo ACD to create signalling differences related to self-renewal of one daughter cell. Secondly, they show that asymmetric distribution of Numb is influenced during coculture with different stromal cells (Wu et al., 2007). This asymmetric distribution of Numb is a feature reminiscent of studies of *D. melanogaster* neuroblasts as well as sensory organ precursors. The same group also demonstrate that ectopic expression of Numb inhibits the progression of chronic myeloid leukaemia, indicating that Numb expression can affect HSC fate in the context of leukaemia (Wu et al., 2007). The exact role of Notch in HSCs has been more controversial. Signalling pathways such as Wnt have been shown to upregulate Notch target genes, and addition of soluble Notch ligands or constitutive Notch activation can expand long term HSC populations (Butler et al., 2010; Calvi et al., 2003; Kunisato et al., 2003; Varnum-Finney et al., 2000). However, Notch inactivation through dominant negative repression or combined Notch knockouts does not appear to impact on steady state adult HSC populations, despite low levels of Notch transcriptional targets such as Hes1 and Deltex1 (Maillard et al., 2008). This does question the observations made by Reya and colleagues that differences in Notch signalling impacts on self-renewal, however, not all the downstream targets of Notch have been identified. Notch signalling is definitely important for survival and expansion fates, but the evidence is less compelling for self-renewal, and currently it is not yet known how the polarity proteins are involved.

Recent work from the Sauvageau laboratory using gain-of-function *in vitro* and *in vivo* assays identify Ap2a2, a component of the endosomal AP-2 complex, as a protein that endows *in vivo* proliferative advantage and an increase in *in vitro* HSC maintenance (Ting et al., 2011). Given that AP-2 is also important for ACD in *D. melanogaster* neuroblasts and sensory organ precursors, it suggests that mechanisms of fate determination through ACD could be evolutionarily conserved in HSCs. Time lapse imaging of these HSCs containing fluorescently-tagged Ap2a2 show asymmetric inheritance in approximately 50% of HSC divisions. Interestingly, knockdown of Ap2a2 does not affect HSC proliferation, differentiation, homing or apoptosis, and there is no co-localisation of Ap2a2 and Numb as both proteins seem to be localised to separate vesicles. Therefore, these studies indicate that the AP-2 complex could function to mediate downstream changes in HSC fate, but it may not perform these functions through Numb-Notch signalling as previously observed in *D. melanogaster*. In fact, haematopoiesis seems completely normal in double *numb-numblike* conditional mutants, as well as in double knockouts of *PKCζ-PKCιδ*. (Sengupta et al., 2011; Wilson et al., 2007).

Thus, the evidence is suggestive rather than definitive that ACD may control aspects of HSC self-renewal and differentiation. This highlights the difficulty in linking ACD to HSC daughter cell fate because of the heterogeneity of the haematopoietic system as well as the redundant or pleiotropic functions of cell fate and polarity proteins. ACD in mammalian HSCs may involve as yet unidentified cell fate determinants or molecules to govern fate, and ongoing studies in our laboratory and others are currently being performed.
1.7.2 ACD in B cells

B cells are important for generating efficient antibody responses in the humoral immune system. Deriving from the lymphoid arm of haematopoiesis, B cells migrate from the bone marrow into the lymph node, and mature within a lymph node structures known as germinal centres. Similar to thymocyte development, this process involves prolonged interactions with surrounding cells which impose positive and negative selection signals during B cell receptor rearrangement. B cell development involves fate choices such as proliferation, self-renewal and differentiation to result in the formation of memory B cells, and plasma cells that produce antibodies of unique specificity (Rajewsky, 1996). The majority of B cell selection and activation occurs during antigen presentation in the germinal centre (via interactions with dendritic cells and macrophages) or at the B-T cell border (with follicular T helper cells) in lymph nodes. How these different fates are orchestrated and the signalling pathways involved during selection are poorly understood. There is growing thought that B cells could receive instructional cues through engagement with dendritic, macrophage or T helper cells to dictate downstream fates via ACD.

Three recent studies provide conflicting views on the possibility that B cells may undergo ACD. The first study by Barnett and colleagues demonstrates that dividing B cells within the germinal centre asymmetrically localise the transcription factor Bcl6, the receptor for the cytokine IL-21, and the polarity protein aPKC (Barnett et al., 2012). To maintain asymmetry of these proteins during division requires constant signalling through contact with antigen presenting cells, possibly by LFA-1/ICAM1. In Icam1−/− deficient mice, B cells do not efficiently polarise Bcl6 or aPKC, nor do they produce sufficient numbers of antibody secreting plasma cells. These data indicate that adhesion defects deplete necessary polarity cues for B cells to undergo ACD and as such affect downstream differentiation.

In a separate study, multi-photon microscopy of explanted lymph nodes shows that B cells acquire antigen from macrophages in a polarised manner in vivo, and that the acquired antigen accumulates preferentially to one daughter cell after B cell division (Thaunat et al., 2012). Antigen asymmetry persists for up to three rounds after B cell division, and, using this feature statistical modelling predicts that up to 25% of B cells undergo asymmetric inheritance of antigen. Ex vivo isolated B cells containing low or high amounts of antigen possess different capacities to present surface antigen complexed with MHC as well as a difference in the ability to activate T cells. Furthermore, the B cells containing high amounts of antigen proliferate robustly in comparison to B cells that have low levels of antigen. Interestingly, these authors observe asymmetry of aPKC up until division but this is not maintained as B cells progress through metaphase and anaphase. Taken together, these two studies raise the possibility that B cell diversity may arise from ACD, which may or may not include evolutionary conserved mechanisms through polarity proteins such as aPKC (Figure 1.10 B).

In a parallel to these studies, Duffy and colleagues embark on statistical approach to model a large timelapse screen of in vitro B cell activation (Duffy et al., 2012). The analysis of up to 1500 cells over 7 generations shows that daughters from B cell divisions largely undergo symmetrical
fates. High correlation exists between differentiation and death fates of single B cell daughters, as well as the time taken to reach these fates. The same observations are seen when daughter sibling fates are compared to each other, fitting in with a stochastic competitive statistical model of cell fate. Interestingly, a small proportion of B cell divisions display asymmetric cell fates when one B cell daughter dies whereas the other one survives. However, the time for this to occur is the same for all asymmetrical B cell divisions. These authors demonstrate that B cell daughter fates are largely symmetrical no matter what the fate is, and B cells undergoing asymmetric fates do so with similar kinetics. A point to consider in this study is that the large population of symmetric B cell fates observed arise from the addition of soluble activating factors freely diffusing through the media. These factors are not presented in the context of a polarity cue on antigen presenting cells and as such, the B cells could be biased towards symmetrical fates. Currently it remains to be shown whether B cells express polarity proteins, and whether polarity proteins are involved in regulating B cell fate. Until more research is done, ACD is one possible method by which B cell diversity is regulated during humoural responses.

1.7.3 Polarity and ACD in T cells

T cells are an important part of our immune system, surveying, aiding and targeting foreign cells to protect us from infection. Polarity plays an integral role during these T cell functions including scanning, migration, activation and killing (Ludford-Menting et al., 2005; Round et al., 2005; Russell, 2008; Xavier et al., 2004). Similarly to HSCs, scanning and migration involve cytoskeletal changes within a T cell resulting in a leading edge rich in chemokine receptors responsible for directing its migration, and orientation of the MTOC at the rear in the uropod for forward propulsion. T cells also express and polarise polarity proteins. Both Scribble and Dlg are essential for T cell uropod formation, and knockdowns of scribble or blocking of Dlg4 in a T cell line both abrogate random migration, disrupt the ability for T cells to form stable immunological synapses with antigen presenting cells, and cause mislocalisation of several TCR signalling molecules such as CD3 and PKCθ (Ludford-Menting et al., 2005; Round et al., 2005; Xavier et al., 2004). The Par complex protein, aPKC, has been shown to antagonize aspects of T cell polarity mediated by the Scribble complex, as well as regulate several forms of T cell behaviour such as homing, migrations and scanning (Giagulli et al., 2004; Real et al., 2007). Par3 controls T cell polarity during chemokine stimulation by regulating the Rho GTPases, Rap1 and Rac1 (Bertrand et al., 2010; Gerard et al., 2007; Real et al., 2007). Polarity proteins therefore play a role in almost every facet of T cell function from scanning and migration, to activation and killing.

During the course of an infection, the activation of CD8+ T cells involves engagement of the TCR with an antigen presenting cell in the context of MHC I. The binding of TCR with cognate antigen complexed with MHC I triggers polarisation of local signalling clusters at the proximal interface between the T cell and the antigen presenting cell to form the immunological synapse (Cemerski and Shaw, 2006; Krummel and Macara, 2006). Polarisation of TCR signalling molecules, polarity proteins (Scribble, Dlg1-4, CRB3) and Rho GTPases (Rac1, Cdc42, RhoA) to the synapse initiates downstream signalling to induces changes in cell shape and well as proliferation to produce
effector T cells for killing infected cells, and memory T cells should the infection arise once more (Fung et al., 2012; Ludford-Menting et al., 2005). CD4⁺ T cells undergo similar recognition and signal transduction but bind to cognate antigen in the context of MHC II. Upon activation, CD4⁺ T cells proliferate to produce helper T cells which do not have killing ability but instead function in modulating and aiding immune cell responses through cytokine secretion, induction of peripheral CD8 T cell memory, and the generation of antibody by B cells.

Current theories as to how T cells acquire different fates centre on the prevailing dogma that after activation, T cell division occurs separately from the antigen presenting cell. The current hypothesis is that T cell fate results from stochastic changes inside the cell, random differences in certain cytokines or chemokines, or chance interactions with costimulatory molecules and other signalling proteins on other antigen presenting cells (Russell, 2008). However, our laboratory and others have observed T cells remain in contact with the antigen presenting cell over long time periods, even while dividing (Chang et al., 2007; Oliaro et al., 2010; Stoll et al., 2002). It is possible that a single T cell could undergo ACD, unequally polarising information to each daughter cell and causing them to inherit CD4⁺ or CD8⁺ T cell fates.

A study by Chang and colleagues contribute to mounting evidence that demonstrates T cells may also undergo ACD (Chang et al., 2007). The isolation of mitotic CD8⁺ T cells at the time of their first division following Listeria infection demonstrate clear asymmetric polarisation of several polarity proteins. These include Scribble and aPKC, the cell fate determinant Numb, as well as surface molecules important for T cell function such as CD8. When populations of daughter T cells from the first division are sorted on the basis of differential CD8 expression and injected into Listeria-infected mice, functional analysis show those mice receiving daughter cells with higher surface CD8 clear the infection more efficiently. This suggests that ACD can confer different effector capacities in CD8 T cells. The same laboratory also demonstrates asymmetric distribution of the transcription factor T-bet in CD4⁺ T cells. T-bet is a T cell fate determining protein important for promoting effector over memory CD8⁺ T cell fates, as well as T helper 1 (Th1) over Th2 and Th17 fates in CD4⁺ cells (Intlekofer et al., 2005; Joshi et al., 2007). The preferential localisation of T-bet in one T cell daughter is concordant with the inverse localisation of the immunoproteosome, which possibly functions to degrade T-bet. Both T-bet and the immunoproteosome mislocalise when components of the polarity network such as aPKC are disrupted, however it remains to be proven whether this translates to distinct helper T cell fates. Nevertheless, these two studies suggest upon activation, the first division of either CD4⁺ or CD8⁺ T cell could be capable of producing daughters of heterogeneous fates by ACD.

Our laboratory also demonstrated that during fixed staining of OT-1 CD8⁺ T cell immunological synapses, T cells remain attached and polarised during division from an antigen presenting cell (Oliaro et al., 2010). Similar to Chang and colleagues, we quantify several polarity proteins as asymmetric upon division, including proteins of the Par complex aPKC and Par3, and also of the Scribble complex such as Scribble and the Dlg family members. Interestingly, we find that T cell ACD requires sustained contact with the antigen presenting cell but not a sustained
immunological synapse. T cell ACD also requires correct spindle orientation and appears to utilise the same Gpsm2(Pins)/Gzo1 protein module described in *D. melanogaster* neuroblast ACD. The classic cell fate determinant Numb also localises asymmetrically. The disruption of mitotic spindle orientation through targeting Gpsm2 signalling mislocalises Numb and alters memory and effector T cell fate ratios. Collectively, these studies provide the first convincing *ex vivo* and *in vitro* correlative evidence that following ACD, molecular differences between the two daughters T cells result in different fates and functional abilities. Furthermore, these disruptions of ACD coincided with alterations in T cell fate.

A model for T cell ACD fate could be as follows (Figure 1.10 C). The polarity cue could derive from interaction between the T cell and the antigen presenting cell. A sustained immunological synapse may not be needed, but other molecules such as Notch, chemokines or adhesion molecules working with TCR on the antigen presenting cell may serve as possible polarity cues. The stable recruitment of the MTOC to the interface with the antigen presenting cell implies that the polarity cue is translated to the polarity network via microtubules and induces changes in the actin cytoskeleton (Yamashita and Fuller, 2008). Scribble and Dlg are transiently recruited to the immunological synapse (Ludford-Menting et al., 2005). As it is known that Scribble directly interacts with the GEF β-Pix in epithelial cells, Scribble polarisation to the immunological synapse may also recruit β-Pix to activate Rac1 and Cdc42 locally (Audebert et al., 2004; Ivetic and Ridley, 2004). TCR signalling also induces the expression of the GEF Vav which further activates Cdc42 (Miletic et al., 2009). The alignment of the mitotic spindle is maintained by intact Gpsm2 signalling (Oliaro et al., 2010). Upon division key polarity proteins are asymmetrically inherited along with classic cell fate determinants like Numb as well as T cell determining proteins such as CD8, T-bet and the immunoproteosome. The maintenance of polarity and the unequal segregation of these proteins impart either memory or effector T cell fates. This model suggests that T cells utilise several evolutionary conserved mechanisms in order to achieve ACD.

### 1.7.4 Thymocytes may undergo ACD

The notion that thymocytes might undergo ACD has been suggested previously by Don Metcalf in the 1960’s (Metcalf and Wiadrowski, 1966). Thymocytes labelled with tritiated thymidine undergo division to produce daughters of different size as well as daughters with asymmetric amounts of tritiated thymidine label. Don Metcalf postulated that these daughters arose from ACD, however, the difficulties in quantification and functional correlations prohibited advances at that time. Asymmetry of the fate determinant, Numb, has been proposed in one recent paper, but without definitive quantification and no correlation in differences in daughter cell fate (Aguado et al., 2010).

Advances in new imaging technologies makes it possible to determine how polarity could orchestrate signals for thymocyte cell fate decisions. The first tantalizing evidence of polarity has come from 2-photon microscopy of thymocytes within the thymus in the Robey laboratory. They revealed that transitions between polarised shapes either related to antigen presentation
and migration dictate the outcome of thymocyte development. In addition, they demonstrate that thymocytes undergoing selection can form both transient and stable synapses with thymic epithelial and dendritic cells (Bousso et al., 2002). Other studies also show that thymocyte TCR engagement signals through many of the same receptors and pathways as mature T cells (Dustin, 2009; Richie et al., 2002). Hence, the dynamic changes in cell shape and conservation of TCR signalling suggest that components regulating these processes in T cells such as the polarity network may also function in thymocyte development.

Only very recently has direct evidence surfaced to connect polarity proteins with thymocyte function. *In vitro* analysis of immunological synapse kinetics between thymocyte and dendritic cells demonstrate that Dlg4 is polarised to the synapse in a rapid manner just prior to calcium release following TCR activation (Affaticati et al., 2010). As Dlg4 is also recruited to the immunological synapse following mature T cell activation to assist in assembly of signalling proteins, it is possible that Dlg4 functions similarly to recruit signalling proteins during thymocyte synapse formation (Round et al., 2005). Another recent *in vitro* analysis of T cell differentiation by Pike and colleagues demonstrate that proper DN3 thymocyte development requires Scribble (Pike et al., 2011). Knockdown of Scribble in T cell precursors leads to an accumulation of DN3 thymocytes on OP9-DLL1 co-cultures, and inefficient DP generation in FTOC cultures. Interestingly, depletion of Scribble does not affect migration as seen in mature T cells but does affect DN3 thymocyte clustering through limiting the polarisation of the integrin ICAM-1/LFA-1 (Ludford-Menting et al., 2005; Pike et al., 2011). Taken together, these studies provide the first hints that polarity proteins are important for aspects of thymocyte development but if depleted (such as with Scribble), can affect downstream fate choices. Data from gene expression databases illustrate thymocytes express various polarity and cell fate proteins in a stage specific manner (*Appendix A*). Whether these proteins are polarised in thymocytes and have other functions, remains to be shown.

A number of stages in thymocyte development involve links between proliferation and fates such as differentiation, sensitivity to apoptosis, and limited self-renewal. The proliferation of thymocytes combined with these numerous cell fate choices during their development suggests ACD may act to regulate these fates. T cell precursors arriving from the bone marrow develop in stages within the thymus by interacting with the stromal cells of the thymus. This stromal network provides a vast array of developmental cues for thymocyte fate determination, and in turn could provide key polarity cues for thymocyte ACD. As with mature T cell ACD, it is possible that thymocyte development depends on divisions requiring the axis of polarity to be maintained, in this case through constant interaction with a stromal cell. Such divisions could result in daughters inheriting an asymmetric distribution of proteins (polarity or signalling), microRNAs or epigenetic factors to affect downstream fate. ACD could explain the different types of cells that can arise from the thymus, namely the classic CD4 and CD8 T cells, regulatory T cells, NK, NKT cells and γδ T cells.
Chapter 1: Introduction

1.8 Hypothesis and aims of thesis

There are at least three possible checkpoints where ACD could exert influence during thymocyte development: alternative NK/myeloid fate specification, DN3 β-selection and positive selection. These processes involve checkpoints that prevent proliferation and trigger death unless distinct molecular conditions are satisfied. For example, alternative myeloid or NK fates could depend on the asymmetric segregation of transcriptional machinery to favour one cell fate over the other. During positive selection, the initiation of TCR signalling, the type of presenting cell, the quality of antigenic peptide or the context that it is presented in could all mediate differential downstream signalling events to impart differential thymocyte fates.

In this thesis I propose the hypothesis that polarity and ACD regulate fate decisions during DN3 thymocyte development. My studies focus on DN3 β-selection, and whether polarity and/or ACD plays a role during migration or cell division during DN3 fate determination (Figure 1.10 D). ACD at the DN3 stage may involve segregation of determinants for proliferation with determinants for susceptibility to apoptosis, thereby controlling the net numbers of DN3 progeny.

In chapter 3 I examine whether several candidate polarity and cell fate determining proteins are polarised in DN3 thymocytes during interphase. I utilise an in vitro system that enables the observation of these candidate proteins, and establish a time lapse method to monitor protein trafficking in migrating and dividing DN3 thymocytes. In Chapter 4 I describe custom image analysis software that is developed and validated to quantify protein distribution in migrating T cells and thymocytes. Using this software I explore whether a mechanism for Numb polarity by aPKC phosphorylation is conserved in T cells and thymocytes compared to epithelial cells, and discover that is not conserved during migration. In Chapter 5, I demonstrate that DN3 thymocytes undergo ACD. I identify that the endosomal proteins Numb and Ap2a2 are asymmetrically localised during DN3 thymocyte division, and establish that the mechanisms for Numb polarisation are conserved during DN3 division. In Chapter 6, I pursue Ap2a2 to investigate the polarity cues that regulate Ap2a2 asymmetry. Using Ap2a2 as a marker I elucidate how DN3 fate is perturbed using a mouse model of T cell acute lymphocytic leukaemia, and identify a possible role for Ap2a2 during this leukaemia. This investigation reveals that ACD does occur during DN3 fate specification, and highlights the physiological context by which ACD can occur. Understanding the integration of polarity cues during ACD at the DN3 stage highlights a step forward in understanding thymocyte fate determination.
Chapter 2

Materials & Methods
2.1 Mammalian cell culture

2.1.1 Peripheral blood mononuclear cells
Primary peripheral blood mononuclear cells (PBMC) were isolated from buffy coats using Ficoll gradient and stimulated with mouse/human anti-CD3 (0.2 mg/ml, clone OKT3) + rIL-2 (50 IU/ml, Chiron, Emeryville, CA) then cultured in RPMI medium 1640 with glutamine (1 mM, GIBCO-BRL), foetal-calf serum (10% v/v), sodium pyruvate (1 nM, GIBCO-BRL) and non-essential amino acids (100 nM, GIBCO-BRL) for 7 days at 37°C, 5% CO₂.

2.1.2 MLA and Phoenix E cells
The T cell leukaemic gibbon line MLA-144 (ATCC) and Phoenix E cells (provided by Gary Nolan) were maintained at 37°C, 10% CO₂ in Dulbecco’s Minimal Essential Medium supplemented with foetal calf serum (10% v/v) and L-glutamine (1 mM) until needed.

2.1.3 Dendritic Cells
Freshly isolated bone marrow cells were isolated from hind limbs of C57BL/6 mice cultured at 37°C, 5% CO₂ in RPMI medium 1640 supplemented with granulocyte macrophage colony-stimulating factor (10 ng/ml, Peprotech), IL-4 (5 ng/ml, Peprotech), glutamine (1mM, GIBCO-BRL) and foetal-calf serum (10 % v/v) for 6 days to generate immature dendritic cells (CD11c⁺/CD86low/MHCIIlow) for use as antigen presenting cells.

2.1.4 Primary OT1 T cells
Naïve OT-1 CD8+ T cells were isolated from single cell suspensions of OT-1 mice using MACS negative selection and magnetic beads. The OT-1 mice were transgenic for a TCR recognising the ovalbumin peptide SIINFEKL in the context of H-2Kb. T cells were maintained in RPMI medium 1640 with glutamine (1 mM, GIBCO-BRL), foetal-calf serum (10%v/v), sodium pyruvate (1 nM, GIBCO-BRL) and non-essential amino acids (100 nM, GIBCO-BRL) at 37°C, 5% CO₂.
Dendritic cells were incubated with SIINFEKL peptide (1 hour, 37°C), washed once in full media and incubated with naïve CD8+ T cells for various assays (described below in long term T cell imaging).

2.1.5 OP9-DL stromal cells
OP9, OP9-DLL1 and OP9-DLL4 stromal cells (from Juan Carlos Zuniga Pflucker) were maintained at 37°C, 10% CO₂ in Minimal Essential Medium Alpha Modification (SAFC Biosciences, Sigma Aldrich) supplemented with 20% (v/v) fetal calf serum, glutamine (1 mM, GIBCO-BRL) and 100 ng/mL penicillin/streptomycin.
2.1.6 Primary thymocyte co-culture

E14.5 mouse foetal liver cells or bone marrow cells isolated from mouse hind legs/pelvis bones (C57Bl/6, CD2-Lmo2, Lgl^-/- or Scribble^-/- background) were used as haematopoietic progenitors and seeded onto OP9-DLL1 stromal cells at a 1:1 ratio in a 6 well plate (2x10^5), 12-well plate (1x10^6) or 24-well plate (4x10^5) for differentiation of foetal liver cells into thymocytes. Co-cultures were grown at 37°C, 10% CO₂ in Minimal Essential Medium Alpha Modification (SAFC Biosciences, Sigma Aldrich) supplemented with foetal calf serum (10% v/v), glutamine (1 mM), β-mercaptoethanol (50 μM, Calbiochem), sodium pyruvate (1mM, GIBCO-BRL), HEPES (10 mM, GIBCO-BRL), 100 ng/mL penicillin/streptomycin, 1 ng/mL mouse interleukin 7 (Peprotech) and 5 ng/mL mouse FMS-like tyrosine kinase 3 (Peprotech). Thymocytes were harvested via forceful pipetting and co-cultured on freshly seeded OP9-DLL1 stromal cells every 3-8 days.

2.2 Cell-based assays

2.2.1 Cumulative thymocyte cell counts

Thymocyte and OP9-DL co-cultures were seeded at a 1:1 ratio (4x10^4) in a 24-well plate and allowed to proliferate in primary thymocyte co-culture media. Thymocytes were harvested by forceful pipetting, spun for 10 seconds at 400 G to remove residual stromal cells, counted by trypan blue exclusion, and re-plated on freshly seeded OP9-DL cells at the same density. Co-cultures were counted every 4 days for 32 days and values calculated as cumulative fold proliferation over the culture period.

2.2.2 T cell proliferation (CFSE) and viability assay

To test proliferation and viability of PBMCs the cell paddocks (Dr Daniel Day, Swinburne University) were cut out and adhered into a 96-well plate so that the entire floor of each well was covered in PDMS. PBMCs were pre-labelled with carboxyfluorescein diacetate (CFSE) (5 μM, Molecular Probes) for 7 mins at RT before washing in serum free media, and were seeded at 5 x 10^6 per well with or without PDMS. At 3 hours, 4 days and 6 days cells were harvested and labelled with propidium iodide (PI, 92 ng/mL, Sigma Aldrich) for analysis by flow cytometry. For viability of T cells and thymocytes cells were labelled with Fluorogold and measured by Fluorogold exclusion (0.0025%w/v saline, Biotum Inc.)

2.2.3 PKC inhibition and activation

Cell lysates were prepared from cultured MLA-144 cells cultured in T125 flasks. Following one wash in 0.1% BSA/PBS, cells were resuspended in either 0.1-5 μM PMA (Sigma) for PKC activation, 10 μM Bisindoylmaleimide XI (Sigma) for PKC inhibition, or an equivalent DMSO diluent at 37°C at the indicated time points. Cells were then washed in ice-cold 0.1%w/v BSA/PBS, spun and lysed for SDS-PAGE and Western Blotting.
2.2.4 CXCR4 inhibition assay
Sorted DN3 thymocytes (C57BL/6 or CD2-Lmo2) co-cultured on OP9-DLL1 stromal cells, were treated in the presence or absence of AMD3100 (2mg/ml, Sigma-Aldrich) in thymocyte culture media for 5 to 8 days before harvesting and assessment of proliferation and differentiation by flow cytometric analysis.

2.2.5 Transwell Chemotaxis assay
24-well transwell plates and 5 μm pore size inserts (Corning) were coated in 500 μl (plate) and 100 μl (insert) 0.1% BSA/PBS overnight at 4°C. Plates and inserts were dried at room temperature prior to adding CXCL12/SDF1a (40 μg/ml, Peprotech) or thymocyte media + 1% BSA. 1x10⁶ sorted thymocytes (in 100 μl) were overlayed on top of each transwell and allowed to migrate for 2 hours at 37°C, 10% CO₂, to which the % migrated (output) was calculated as a proportion and fold change from the original input numbers of sorted thymocytes.

2.3 Transfection, virus collection and viral transduction
Calcium phosphate transfection was performed on Phoenix E cells with 5μg of the following pMSCV retroviral constructs- Cherry, GFP, Cherry-tubulin, GFP-Numb, GFP-Numb2a, Cherry-Ap2α2, GFP-Scribble, GFP-Dlg4, GFP-LAT and GFP-Carma (courtesy of Mandy Ludford-Menting and Stephen Ting, Peter MacCallum Cancer Centre) in 10cm dishes (Corning). Viral supernatant (6 mls) was harvest 48 hours post transfection and concentrated to 1 ml for 10 mins, 3000 G using an Amicon concentrating column (100K/100,00 MWCO, Millipore, Ireland). Concentrated virus was placed in a 6 well-plate (Cat. No. 351146, Falcon, BD Pharmingen) that was pre-coated in 15 μg/ml RetroNectin/r-Fibronectin (Takara), blocked in 2% bovine serum albumin/phosphate buffer saline and then spun at 2000 G, 1 hour. 6-well plates were incubated 4 hours 37°C. 1x10⁶ MLA-144 T cells, 1-4x10⁶ bone marrow/E4.5 foetal liver cells or 1-4x10⁶ thymocytes (day 6-8 co-culture) were added and plates were spun for 1 hour, 1200 G. Transduced cells were rested in appropriate cell culture media or in IL-7 (20 ng/ml) and stem cell factor enriched Minimal Essential Medium Alpha Modification for 24-72 hours, and sorted by flow cytometry for live imaging, re-coculture, or reconstitution into sublethally irradiated B6-Ptpcrα or OT-1 mice (C57BL/6 background).

2.4 Flow cytometry (sorting and analysis)
All antibodies were purchased from BD Pharmingen unless otherwise specified. Retrovirally transduced cells were sorted on the basis of GFP or Cherry fluorescence on the BD FACS Vantage (BD Pharmingen). DN3 thymocytes were sorted for GFP or Cherry fluorescence (if transduced) as well as CD25⁻/CD44⁺/CD4⁻/CD8⁻, lineage negative: Mac1⁻/CD3⁻/RB6-8C5⁻/NK1.1⁻/Ter119⁻. Bulk CD2-Lmo2 thymocytes were sorted by CD45⁻/CD4⁻/CD8⁻/CD3⁺. Analysis of proliferation
and viability (CFSE/Fluorogold or PI) or thymus subsets (DN1-4: CD44 (e-bioscience)/CD25/CD28/TCRβ/CXCR4/CD4/CD8/lineage negative, αβ versus γδ T cell: TCRβ/TCRγ/lineage negative, DN-SP: CD4/CD8/CD24/TCRβ/CXCR4/lineage negative) was performed on the LSR. Prior to antibody staining thymocyte Fc receptors were blocked by a 15 min incubation with 24G.2 (). Notch ligand expression (Delta 1, Delta 4, Jagged 1, Jagged 2, supplied by Hideo Yagita Juntendo University, Tokyo, Japan) on tested OP9-DL stromal cells were also analysed on the LSR. Refer to Table 2.1 and Table 2.2 for the list of all antibodies used during flow cytometry sorting and analysis.

2.5 Immunofluorescence and fixed image acquisition by confocal microscopy

1-5x10^4 cells MLA-144 T cells were cytospun using the Cytospin3 (Shandon) onto microscope slides at 250 rpm, 10 mins. For thymocyte staining, 7x10^3 OP9 or OP9-DLL1 stromal cells were seeded in each well of an 8-well chamber slide (NUNC Nalgene) and left to adhere overnight. 1-4x10^4 thymocytes were added with fresh media and co-cultured for 1-2 days. Cells were then fixed with 3.7% (w/v) paraformaldehyde in 100 mM PIPES, 5 mM MgSO_4, 10 mM EGTA and 2 mM DTT (10 min, RT), washed twice, and then permeabilised in 0.1% Triton X-100 in PBS without MgCl_2 or CaCl_2 (5 min, RT). Thymocytes were treated with 24G.2 to block binding of Fc receptors for 15 min incubation at 4ºC. All cells were then labelled with primary and secondary antibodies (Table 2.1 and Table 2.2) and mounted in Prolong Gold antifade (Molecular Probes). The slides were examined at room temperature using a FluoView FV1000 BX61 confocal microscope (Olympus) using a UIS2PlanApoN 60x oil immersion objective (NA 1.42). 3D images of the cells were acquired with a Z distance of 0.5μm. Maximum intensity projections of Z sections spanning the entire cell were used for all analyses using the FluoView software (Version 1.7b).

2.6 Cell paddock fabrication and modification

Cell paddocks were produced and co-developed with Dr. Daniel Day (Swinburne University). Briefly, cell paddocks were made by fabricating a mould from which the microgrids could be replicated. The mould was made of polymethyl methacrylate (PMMA) and was fabricated by etching out the inverse image of the final cell paddock structure using an amplified femtosecond pulse laser (Spitfire, Spectra Physics). After fabrication, the mould was cleaned in an ultrasonic bath and rinsed with isopropanol and dried with nitrogen. Polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning) was then poured over the mould. A second substrate, a glass or plastic slide, was then placed on top of the mould and heated on a hotplate to 85°C for at least 20 mins to allow the PDMS to cure to its final state. After curing the slide with the cell paddocks were peeled off the mould for use in imaging experiments as described in the next section.
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Table 2.1. List of primary antibodies used in this thesis (cont...)

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<th>Isotype</th>
<th>Supplier</th>
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Table 2.1. List of primary antibodies used in this thesis (cont...)

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<th>Antigen</th>
<th>Cat. No</th>
<th>Antibody (clone)</th>
<th>Reactivity</th>
<th>Source</th>
<th>Isotype</th>
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2.7 Time lapse imaging

2.7.1 Long term imaging of OT-1 T cells

Individual 125 x 125 µm cell paddocks were peeled off a slide and adhered into an 8 well microscope chamber slide (Nunc Nalgene) or pre-sterilised 3 cm coverslip-bottomed dish (Matek, Ashland, MA, USA). The chamber was given a 100% ethanol wash for sterilisation and 5 washes in warm media afterwards to dilute the ethanol. This ensured that the cell paddock was always covered in liquid to prevent air bubbles from forming inside the individual grids. Dendritic cells (4x10^5) were seeded on top of the microgrids and left for 24 hours to adhere. Dendritic cells were then incubated with 1 µM SIINFEKL (1 h, 37°C), washed once in full media and 8x10^4 naive CD8^+ T cell were overlayed on top. The co-cultures were left for 40 hours before imaging. Naive T cell imaging was performed on an Olympus IX-81 epifluorescence microscope fitted with a Mercury arc lamp and a temperature controlled chamber maintained at 37°C (Solent Scientific, UK) and 5% CO_2. Multiple stage positions (up to 20) controlled by a computerised XY stage (Prior Proscan, MA) were taken, captured by an ORCA-ER CCD camera (Hamamatsu, Japan) with either a 20x OBJ (NA 0.4) or 40x OBJ (NA 0.6). Mirror units for capturing green fluorescence images included an Olympus wide band U-MWIBA2 filter cube, a band pass excitation filter from 460-550 nm, emission filter BA-510-550 and a 505 nm dichroic mirror. Mirror units for capturing red fluorescence images included an Olympus wideband U-MWlY2 filter cube, a band pass excitation filter from 343 nm – 580 nm, emission filter BA610F and a 600 nm dichroic mirror.

2.7.2 Long term imaging of MLA-144 T cells

Individual 250 x 250 µm cell paddocks were peeled, sterilised as above and adhered into an 8 well microscope chamber slide (Nunc Nalgene and IBIDI). 1.7 x 10^4 MLA-144 cells untransduced or retrovirally transduced were seeded into the chamber for long term DIC or fluorescence imaging. Long term time-lapse DIC imaging of the MLA-144 line on the Olympus IX81epifluorescence microscope was taken every 4 mins binned at 2x2, with DIC exposure time of 30 ms for up to 22 hours. Long term timelapse fluorescent and DIC imaging of MLA-144 transduced with Cherry-tubulin were taken every 10 mins binned at 4x4, with DIC and Texas Red exposure times of 15 ms and 50 ms respectively for up to 22 hours. Time lapse imaging of MLA cells was also performed on the Leica TCS SP5 multispectral commercial Confocal Laser Scanning Microscopy (CLSM) (Leica Microsystems CMS GmbH, Germany) fitted with a temperature controlled chamber at 37°C and 10% CO_2. Images were acquired using a HCXPLApo x63 glycerol immersion objective (NA 1.3). Multiple stage positions controlled by LAS AF v2.0 software interface were captured every 2 mins for 1-24 hours with line averaging of 2, and 5 z sections of range 7.5µm-15µm, corresponding to optical distance of 2.5-3µm between each section. To look at Numb mobilisation cells were treated with 0.1-5 μM TPA (Sigma) for 20 mins, 37°C, 10% CO_2 prior to imaging.
2.7.3 Long term imaging of thymocytes and stromal cells

Thymocytes were sorted for the DN3 population (CD25\textsuperscript{+}/CD44\textsuperscript{lo}, lineage negative: CD8\textsuperscript{-}/CD4\textsuperscript{-}/CD3\textsuperscript{-}/RB6-8C5\textsuperscript{-}/NK1.1\textsuperscript{-}/Ter119\textsuperscript{-}) and seeded at a 1:1 ratio onto stromal cells (2 x 10\textsuperscript{4} cells) in a 8-well chamber slide containing 250 x 250 \(\mu\)m cell paddock pre-washed with 100% ethanol and warm media as described previously. Multiple stage positions were captured on the Olympus IX81 with DIC every 6 mins for 40 hours, binning 2x2, with an exposure time of 30 ms. Time lapse imaging of thymocytes on the Leica TCS SP5 CLSM was as for the MLA-144 T cells.

2.8 Imaging processing

2.8.1 Metamorph processing

Time lapse image analysis performed on the Olympus IX81 epifluorescence microscope were analysed and processed using MetaMorph Imaging Series (Version 7.7.4, Universal Imaging Corporation, USA). For time lapse imaging acquired by the a Leica TCS SP5 CLSM, sequential multiparametric (x, y, z, time and multi-fluorescent wavelengths), 2-D images of 512x512 pixels were saved as .lif files (Leica LAS AF v2.0 support format) and exported as individual 16-bit tiffs and stacks using an automated and memory handling journal “Lif to tiff Adaptable with Projections” written in MetaMorph Image Series 7.7.4 software (Journal by Cameron Nowell, Ludwig Centre for Cancer Research, available upon request). Subsequent image processing was then performed using custom TACTICS software

2.8.2 Custom TACTICS software

Tiff images from experiments were arranged into folders by position number and merged in the z-direction (all 5 slices) by their average fluorescence using Matlab (MATLABR2010a version 7.10, The MathWorks, Inc). Experiment files (.dat format) were created for each separate experiment, or collated experiments acquired over several time lapse imaging sessions. For each protein investigated, approximately 40-80 positions were analysed from T cells and thymocytes which resulted in roughly 500GB of data processed (per protein). Calculations were performed on an HP Z400 workstation equipped with a 3.3 GHz Intel Xeon W3580 Quad processor and 16GB of RAM working under a Windows 7 64-bit operation system. The TACTICS platform was co-designed with, and written by Raz Shimoni (Swinburne University). TACTICS was divided into five modules, briefly described bel .

Module 1: TACTICS_F

i) Filtering

A number of standard and morphological filter options enabled custom filtering to obtain minimal background fluorescence interference. First, for each stack, the 5 focal sections were projected into a 2D plane by averaging each pixel over its value in each stack. These projected images were then smoothed using a median filter (3x3) and morphological close opening filter (3x3 disk structuring
element). A copy of each raw tiff image was made upon filtering, to maintain preservation of the
original data. Filter settings were saved for each experiment so that they could be applied to other
experiments captured in the same manner.

ii) Cell segmentation and identification
Cells from time lapse images were segmented in two steps. First, Otsu’s method was used to
automatically choose a threshold that gave minimum interclass variance of the high and low
fluorescence intensity (Otsu, 1979). As this method overestimated the perimeter of the cell, a
second segmentation procedure was then used for refinement of the cell perimeter by ubiquitous
Cherry, GFP or Cherry-tubulin expression. In this case, an iterative thresholding procedure was
performed on the best section (automatically defined as the section with the largest grey-level
variance. Touching cells were split into individual cells, using cell classifier parameters. Objects
such as background noise, spectral bleed-through, dust and dead cells, were filtered out based
upon geometric criteria such as area and circularity. Gaps in the remaining objects were filled
using the Matlab imfill function, labelled and the centroid (geometric centre) determined for each
identified cell.

Module 2: TACTICS_C
Cell tracking and manual registration of cells
To link cells at each consecutive frame, Matlab code for particle tracking based upon John C.
Crocker was utilised to associate the points of the centroid to create the cell trajectories, and
a bounding box labelled each tracked cell over time. The specific code used was written by D.
Blair (Georgetown University, Washington DC) and E. Dufresne (Yale University, New Haven,
CT) and was downloaded from http://www.physics.emory.edu/~weeks/idl/, and the Hungarian
algorithm was used to associate bipartite centroids (written by Yi Cao, Hungarian Algorithm
V2.3.A). TACTICS software enabled manual corrections. False detections were removed and
poorly segmented cells were either improved or discarded. Undesired tracks were filtered out
using parameters such as velocity and length. Manual identification of dividing cells was marked
by a mouse click (denoted by an X) for future analysis.

Cell alignment
To align cells (migrating or dividing) along an X or Y axis, an ellipse was fitted to the cell
borders to determine the morphological major axis. Cells were rotated so that the axis could be
determined as minor (short) or major (long), and the images were rotated to align the major axis
with the y-axis. The rotation was performed around the centroid of the cell. Nearest-neighbour
interpolation was used for the rotation. Once the cell was rotated a new major and minor axis
was found and the minor axis was used as a border that split the cell into two (in this case not
necessarily equal) parts. For migration studies, cell images were rotated so the uropod faced the
centre of the bottom of the image.
Module 3: TACTICS_M

Quantification of cellular and morphological parameters was made available in TACTICS_M for export as figures and/or export into Microsoft Excel or GraphPad Prism for graphical representation. The main extractable data parameters were broadly typed under three categories: i) morphological and geometric descriptors (e.g. area, circularity, major/minor axis length, perimeter, ii) parameters describing the motion of the cell including: apparent velocity, Mean Square Displacement (MSD) and turning angle and iii) A reconstructed cell image within a bounding box. Series of reconstructed images (migrating or dividing cells) were assembled into montage sequences. Individual bounded images of migrating cells, or dividing cells (specifically parent and daughter cell post division) were also created (SEQtages). For the SEQtages, images were rotated along the major axis (for migrating cells the uropod was orientated at the bottom). Spectral bleed-through between channels was also corrected for by estimating that the total emission in each channel contributed by each fluorophore, and then subtracting the relative proportions of each band in the unmixed output. Extraction of other parameters were performed on multispectral channels. For example, for migration analysis a specific channel was chosen to generate velocity, mean squared displacements and centroids (usually GFP, Cherry, or Cherry-tubulin), and another fluorescent channel enabled generation of an axis for cell alignment, or vectors to display proximity to other cells of interest. A third channel enabled ratiometric fluorescence quantification of a particular protein of interest. To calculate the Polarisation Ratio (PR) in migrating and dividing cells respectively, the difference in total fluorescence of each hemisphere or daughter divided by the sum of total fluorescence from both hemispheres or daughters.

Module 4: TACTICS_A

After processing by TACTICS_M there were two interactive Graphic User Interfaces (GUIs) for visual inspection of individual or populations of cells (migrating or dividing), or of data derived from migrating or dividing cells.

Interactive visualisation of data during T cell and thymocyte migration

Relationships between data outputs relating to PR values (major or minor axis) with parameter descriptors of morphology and motion of the cell were explored by plotting in dotplot format enabling comparisons of two parameters at any one time (plotted X or Y axis). Each dot on each dot plot was also linked to time lapse movies and images of individual cells for qualitative inspection. Gating of populations of datapoints were performed for reprocessing and inspection as required.

Parameter optimisation during thymocyte division

PR values one frame after division were then converted to a single PR pixel and subjected to increasing threshold increments (0.1% increment, 0-100%). Heat maps were created for each control and candidate protein during division, and a 25% threshold line (based on the Cherry Control Protein) was used to create scatterplots of PR values. To differentiate between symmetric cell divisions (SCD) and asymmetric cell divisions (ACD) a cutoff was drawn based on the highest
Module 5: TACTICS_R

Robust tool for multi position plotting

Iterative analysis procedures from TACTICS_F, T, C and M were performed using TACTICS_R. Pipelines were created by importing settings saved from the desired TACTICS modules, and applied iteratively to multiple positions within experiments as well as multiple experiments.

2.9 Nucleic acid preparation

2.9.1 Bacterial transformation

Retroviral plasmids (described in Section 2.3) were transformed into chemically competent Top10 or DH5α bacterial cells by heat shock at 42°C for 90 secs. Transformed cells were selected on Luria Broth Agar plates containing ampicillin or kanamycin (35-100μg/ml) overnight. Colonies were picked for generation of glycerol/Luria Broth (50% v/v) stocks for DNA plasmid maxipreps.

2.9.2 DNA plasmid maxiprep

Plasmid DNA purification from 200 mL overnight bacterial culture was performed using Qiagen DNA or Nuclebond Xtra Maxi Macherey-Nagel maxi columns and buffers, according to manufacturers instructions (Qiagen, Norway or Macherey-Nagel GmbH & Co., Germany). Following cell lysis, before column purification, the DNA in solution was filtered through two layers of Kimtech low lint plus science wipes (Cat. No. 4103, Kimberly-Clark Professional) to remove contaminating cell debris when using the Qiagen DNA Maxi prep kit. DNA concentration was determined by UV absorption spectrophotometry at 260nm/280nm.

2.9.3 Polymerase Chain Reaction (PCR) for mouse genotyping

Tail/ear clips of mice or E14.5 foetuses were snap frozen until needed, or digested for 1 hour-overnight in lysis buffer (10 %v/v Modified Gitschier Buffer, 5 %v/v Triton X, 1 % BME, 2 %v/v Proteinase K), denatured at 100°C for 5 mins and then subjected to polymerase chain reaction performed on the Biorad iCycler. The PCR conditions were: 96°C for 2 mins; denaturation 96°C for 30 secs; annealing and extension 55°C for 30 secs (x 30 cycles); 72°C for 1 min.

Primers for genotyping

LMO2 Fwd (5’-3’) TGG ATG AGG TGC TGC
LMO2 Rev (5’-3’) GGA TGC ACA GAG ACC
2.10 RNA extraction and quantitative real-time polymerase chain reaction (PCR)

Total RNA was harvested by pelleting cells using Qiagen RNeasy Mini Kit according to manufacturers instructions (Qiagen, Norway) with the alteration of adding 10μl BME/1ml RLT at the cell lysis step. Alternatively, pelleted cells were extracted using Trizol reagent and purified by chloroform extraction and ethanol precipitation according to the manufacturers instructions (Invitrogen). Pelleted RNA was resuspended in 20μL RNase-free DEPC treated water. The concentration and purity of RNA was quantitated by UV spectrophotometry/Nanodrop machine (260nm/280nm).

For quantitative analysis of mRNA levels, cDNA was created from 2μg total RNA using random primers and Superscript III reverse Transcriptase (Invitrogen) on the BioRad iCycler. Quantitative polymerase chain reaction was performed with the primers (listed below) on the 7900HT (Applied Biosystems) in triplicate with Fast SYBR Green® dye detection method in a 384-well MicroAmp plate (Cat. 4309849, Applied Biosystems, Foster City, CA) as per manufacturers specifications. cDNA PCR conditions were: cDNA denaturation 65°C for 5 mins, 4°C hold, then 25°C for 5 mins, 50°C for 60 mins, 70°C for 15 mins and 4°C hold.

Q-PCR conditions were 95°C for 10 mins followed by 40 cycles of 95°C for 15 secs and 60°C for 1 min. Loading per sample was normalised by comparing CT of sample to CT of G6PD (deltaCT). DeltaCT was used to calculate relative abundance of mRNA (1000/2deltaCT).

Quantitative real-time PCR primer sequences:

House keeping genes
G6PD Fwd (5’-3’) GGG TCA GCT TCA GTC AAA GCA CA
G6PD Rev (5’-3’) TAG TTG CCG CTG CCA AAC AC

Gpsm2/PINS primers
Gpsm2/Pins Fwd (5’-3’) CTG CAG CGG TTC CTT GTT
Gpsm2/Pins Rev (5’-3’) TAG TTG CCG CTG CCA AAC AC

Ap2a2 (sequencing primers)
Ap2a2 Fwd (5’-3’) CTG TCT CGC TAG CTG TCT CG
Ap2a2 Rev (5’-3’) CTC GAA GAT CTG TTG ATG CAG A

2.11 Protein manipulation

2.11.1 Immunoprecipitation

Cells were grown in T250 flasks prior to rinsing in 0.1% BSA/PBS and lysis in either NETN lysis buffer (0.5% NP40, 1 mM EDTA, 20 mM Tric, 100 mM NaCl) or SDS total lysis buffer (30%
glycerol, 10% SDS, 250 mM Tris-HCl) with MiniComplete protease (Roche) and PhosphoSTOP inhibitors (Roche). 1.5 mg of total protein was subjected to immunoprecipitation using 4μg of α-Numb rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., USA,) or purified rabbit IgG (as control). Antibody and lysates were incubated at 4 °C for 2 hours followed by 1 hour with 35μl of a 50:50 Protein G Fast Flow sepharose beads (GE Healthcare). Antibody bound sepharose was centrifuged at 3000 rpm and washed 10 times with lysis buffer. After spinning at 6000 rpm, 1 min, the supernatants were subjected to SDS-PAGE and Western blotting.

2.11.2 SDS-PAGE and Western Blotting

Cell pellets were lysed in either NETN or SDS total lysis buffer (see above for recipe). Lysates were then boiled for 5 mins, 95°C. Proteins were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membrane (Immobilon-P), and immunoblotted with primary antibodies overnight at 4°C, or for 30 mins at room temperature. Bound antibodies were visualised using anti-rabbit or anti-mouse horseradish peroxidase conjugated protein A (Bio-Rad) in conjunction with the ECL system (Amersham Pharmacia Biotech).

2.12 Animal husbandry

C57BL/6, B6-Ptprca, OT-1 (C57BL/6 background), CD2-Lmo2 (C57BL/6 background), Lgl\(^{-}/^{-}\) (OT-1 C57BL/6 background), Scribble\(^{floxflo}\) (C57BL/6 background) 6-30 weeks were used. All mice were maintained in a specific pathogen-free environment with food and water freely available. C57Bl/6 mice were obtained from the Walter and Eliza Hall Institute animal breeding facility and CD2-Lmo2 mice from Matthew McCormack (WEHI, Parkville, Australia). All experiments on mice were performed in accordance with the Animal Experimentation Ethics Committee of the Peter MacCallum Cancer Centre (AEEC #1349, Appendix B).

2.13 Statistics

All data from flow cytometry, immunofluorescence staining, time lapse microscopy and mouse data were assumed to have normal distributions (using a normality test in GraphPad Prism). To derive statistics, un-paired students T-tests were performed with the exception of Chapter 5, where paired T-tests were performed between control and test protein polarisation ratios at the time of division. “n” specified two types of data and always described in the text. First, “n” was referred to as the number of individual data observation points. For example, the number of cells analysed by immunofluorescence or time lapse microscopy. In such cases statistics were not derived. Second, for most cases “n” represented the number of individual experiments performed, with statistics derived from averages of replicates (at least 3 for each experiment), or from the replicates of a representative experiment. For example, statistical tests were performed on replicate averages from different experiments for most immunofluorescence, time lapse microscopy (Figure
4.9C), flow cytometry and mouse experiments. For flow cytometric analysis of DN3 thymocyte differentiation, statistics were derived from replicates of representative experiments. Due to slight variations in media conditions or harvest times, the stage of thymocyte differentiation on a population level may have been different between each experiment. As such, pooling the average values of replicates between experiments would obscure interpretation of results. Care was taken to ensure that the same trend in differentiation was similar between flow cytometric experiments (for example, analysing effects of ectopic protein expression on DN3 differentiation), before choosing a representative experiment and applying an un-paired T test.
Chapter 3

Development of a time lapse method to investigate thymocyte polarity during interphase and mitosis
3.1 Introduction

Although we have identified many of the molecular players during DN3 thymocyte development, it is not known how these players coordinate DN3 differentiation. This is due to current studies being (i) generally population-based, leaving little opportunity to elucidate the behaviour of minority cells, or (ii) focussed on measuring an endpoint, rather than a process that leads up to that endpoint. For example, measuring the numbers of αβ vs γδ T cells produced under different conditions does not indicate whether these numbers come from altered fate choices such as death, proliferation and differentiation. Several studies have proposed that thymocytes may undergo ACD, however, the approaches involved description of fixed, static imaging of thymic sections with limited resolution and no dynamic correlation with fate (Aguado et al., 2010; Metcalf and Wiadrowski, 1966; Sugimoto and Yasuda, 1983). To resolve these issues, cells must be monitored by time lapse microscopy to see if asymmetry exists at division, and leads to different fate outcomes. Thymocytes must also be visualised at a sufficient spatial resolution to determine whether the same molecular players responsible for ACD in other systems are also polarised during DN3 decisions.

Time lapse imaging has revolutionised the elucidation of molecular processes underlying how many cellular activities are performed. The molecular processes governing ACD have largely been revealed through high resolution time lapse imaging of slow moving, large adherent cells such as epithelial and neuronal cells. Unlike these slow moving cells, thymocytes are smaller (5-10μm), migrate rapidly (up to 16μm/min) and tend to clump on other thymic stromal cells (Dzhagalov and Phee, 2012; Richie et al., 2002). Two-photon imaging enables for cataloguing the migratory and interactive behaviours of thymocytes, such as during two-photon laser scanning microscopy of thymocytes in RTOCs, FTOCs, thymic slices or explants (Bhakta and Lewis, 2005; Bousso et al., 2002; Ladi et al., 2008a; Richie et al., 2002; Robey and Bousso, 2003; Witt and Robey, 2005). However, the rapid migration and limited spatial resolution of intracellular molecular processes of in vivo imaging means that an in vitro system capable of recapitulating thymocyte development is needed to accurately visualize protein trafficking in differentiating DN3 cells, to monitor DN3 cell fate over multiple generations for pedigree analysis, and to correlate the localisation of proteins with different DN3 fates.

In this chapter I utilise the OP9-DL system to develop a method whereby thymocyte protein localisation and fate can be monitored during interphase and division. The OP9-DL system can recapitulate almost all aspects of thymocyte development up to the DP stage and provides a robust system to determine whether ACD is involved during thymocyte development. I validate that DN3 thymocytes undergo several rounds of division before differentiating into DP thymocytes, and establish that they express and localise polarity and cell fate proteins.
3.2 Results

3.2.1 Fine tuning the in vitro OP9-DL system for time lapse imaging

The in vitro model system developed by the laboratory of Zuniga-Pflucker (Schmitt and Zuniga-Pflucke, 2002) involves culturing hematopoietic stem cells with a stromal cell line (OP9) transfected with the Notch ligand Delta-like 1 or 4 (OP9-DLL1 or 4). Therefore with the appropriate cytokines, OP9-DLL enable full development through to the DP thymocyte stage. Although this system has been used and validated by many laboratories in the past 10 years, currently no studies have used this system for time lapse imaging over multiple generations.

Firstly, I obtained OP9 stromal cell lines from the laboratory of Zuniga-Pflucker: OP9 (no Notch ligand control), OP9-DLL1 (containing the Notch ligand Delta Like 1, DLL1) and OP9-DLL4 (containing the Notch ligand Delta Like 4, DLL4 ligand). I confirmed that the OP9 line, two OP9-DLL1 lines (GFP and YFP labelled), and one OP9-DLL4 line (GFP labelled) expressed the appropriate Notch ligands (Figure 3.1). All lines also expressed low levels of the Notch ligand Jagged 1, and the OP9-DLL4 line expressed detectable levels of Jagged 2. E14.5 foetal liver haematopoietic progenitors were then co-cultured on the different OP9-DL stromal lines in alpha-MEM media enriched with 1 ng/ml IL-7 + 5 ng/ml Flt-3, and were harvested every 4 days. All OP9-DL lines supported robust proliferation (Figure 3.2) and differentiation along the T cell developmental pathway, with the OP9-DLL1 line displaying the highest differentiation toward the DP stage (Figure 3.3). These results are interesting considering that the physiological Notch ligand for thymocytes is known to be DLL4 (Feyerabend et al., 2009; Mohtashami et al., 2010). All future experiments were then carried out using the OP9-DLL1 stromal cell line.

To investigate whether different haematopoietic progenitor sources could initiate thymocyte differentiation in the OP9-DLL1 in vitro system to the same capacity, I co-cultured either isolated E14.5 foetal liver cells or adult (12 week) bone marrow (BM) progenitor cells on the various OP9-DLL stromal cells (Figure 3.4). E14.5 foetal liver co-cultures were superior to adult BM as a source of T cell precursors displaying greater differentiation and proliferation. Notably, in this experiment the adult BM cells did not differentiate to DP thymocytes compared to E14.5 FL cells in these experiments, although some limited differentiation to DP was observed in other co-culture experiments (data not shown). Suboptimal BM differentiation on OP9-DLL1 cells was most likely due to BM cells being more sensitized to IL-7 levels in the media, which, at higher levels, is known inhibit differentiation (Wang et al., 2006). There was no differentiation of either foetal liver or BM cells along the T lineage pathway when co-cultured on the OP9 control cell line (Figure 3.4), although foetal liver cells did proliferate similarly to BM cells when cultured on OP9-DLL1 (Figure 3.5). These cells were later confirmed to be B cells, dendritic and macrophages (data not shown). DN1 thymocytes from foetal and adult thymic lobes (E15 and 12 weeks respectively) were also compared (data not shown), but were not chosen as a haematopoietic source because of low starting cell numbers. In summary, E14.5 foetal livers displayed the most reliable differentiation to DP thymocyte stage using the OP9-DLL1 co-culture system.
Figure 3.1 Notch ligand expression on OP9-DL stromal cells

Expression of the Notch ligands Delta 1/4 and Jagged 1/2 on OP9 stromal lines utilised in this study (Grey solid lines: isotype control, blue lines: Staining with specific antibodies for the proteins indicated).
Figure 3.2 Fold proliferation on OP9-DL stromal cells

C57 Bl/6 E14.5 foetal liver cells were co-cultured on OP9-DLL1 (GFP), OP9-DLL1 (YFP) and OP9-DLL4 (GFP) stromal lines and counted over 4, 8, 12, 16, 20, 28 and 32 days. The cumulative fold proliferation indices are shown.
Figure 3.3 T lineage differentiation on OP9-DL stromal cells

Differentiation of C57 Bl/6 E14.5 foetal liver cells through the A) DN1-4 stages (CD44/CD25 staining) and B) the DN to SP stages (CD4/CD8 staining) on OP9-DLL1 (GFP), OP9-DLL1 (YFP) and OP9-DLL4 (GFP) stromal lines over 4, 12, and 20 days.
Adult (4-8 weeks) bone marrow (BM) and E14.5 foetal liver (FL) cells were co-cultured on OP9-DLL1 stromal cells compared to the OP9 stromal cell control with no Notch ligand. Harvesting was performed at day 4, 8, 12 and 16 of co-culture and stained for the DN1-4 stages (CD44/CD25 staining) through to the DP stages (CD4/CD8 staining). Note that the BM on OP9 shows dramatically reduced proliferation in Figure 3.5, perhaps influencing the profiles observed here.
Figure 3.5 Comparing T lineage fold proliferation on OP9-DL stromal cells using foetal liver and bone marrow cells

Adult (4-8 weeks) bone marrow (BM) and E14.5 foetal liver (FL) cells were co-cultured on OP9-DLL1 stromal cells compared to the OP9 stromal cell control with no Notch ligand. Harvesting was performed at day 4, 8, 12 and 16 of co-culture and cell counts were performed. The cumulative fold proliferation from each harvest to the next is shown as a line graph. Experimental replicates shown.
I next asked whether it would be feasible to analyse the division of DN3 thymocytes using the OP9-DLL1 system. During co-culture, I observed a large proportion of DN3 thymocytes between day 8 and 20 co-culture, demonstrating that there was a time period of 12 days where DN3 divisions could be visualised. To confirm this, I labelled sorted day 9 DN3 thymocytes with carboxyfluorescein succinimidyl ester (CFSE), a dye that dilutes in daughter cells such that the fluorescence halves every time a cell divides (Lyons and Parish, 1994). Indeed, by analysing CFSE dilution through flow cytometry this demonstrated that DN3 thymocytes underwent several rounds of division before differentiation into DP thymocytes (Figure 3.6).

### 3.2.2 Thymocytes express and localise polarity and cell fate proteins

Online databases such as the Immunological Genome Project “Immgen” (www.Immgen.org) provide a wealth of gene expression data from myeloid and lymphoid cells directly isolated ex vivo, forming a great platform for in silico analysis of gene expression in thymocytes during T lineage specification for cell fate proteins. I used this resource to determine whether mRNA for genes of polarity proteins from the Scribble, Par, and Crumbs complexes, shown to operate in a conserved manner in both T cells and epithelial cells, were expressed in thymocytes (Humbert et al., 2003). I next investigated whether thymocytes expressed a variety of these cell fate and polarity proteins by mining the Immgen gene expression database (Appendix A). Immgen employs highly standardised procedures for isolation of thymocyte subsets through different stages of differentiation. Expression analysis of polarity and cell fate proteins in the DN1-DN4 and DP stages could possibly highlight a role for thymocyte development, particularly if up-/down-regulated in a stage specific manner. From data mining of Immgen, a number of proteins implicated for cell fate, polarity complex, and spindle orientation were shown to be expressed by thymocytes from DN1-SP (Appendix A). Compared to a baseline expression level (50 units was considered little or no expression), the cell fate determinant Notch1 was highly expressed (up to 5850 units) in early developmental thymocytes (DN1-DN3), and expression dropped sharply after DN4 (650 units). The cell fate determinant Numb displayed fairly low expression across all thymocyte subsets (approximately 150 units). Of the polarity proteins in the Par complex, Par3 gene expression was negligible across all thymocytes subsets (mean, 53 units), followed by Par6 (mean, 123 units) and aPKC (mean, 244 units). Interestingly, there was an increase in aPKC gene expression from the ISP (100 units) to DP (440 units) thymocyte stage. There was also a peak in Scribble expression at the DN3a to DN3b stage (215 to 395 units), concomitant with a reduction of Dlg4 (435 to 220 units). Notably, the gene critical for proper spindle orientation during D. melanogaster and mammalian neuronal precursor division Gpsm2, jumped almost 5 fold in gene expression from the DN3 to DP stage (125 to 600 units). These data indicate that the major components of ACD regulation are expressed in thymocytes.

In mature T cells, the presence of a microtubule organising centre to one side of the T cell, for example, at the interface during immunological synapse formation, is indicative of intracellular polarity (Ludford-Menting et al., 2005). To confirm gene expression data with endogenous protein expression and localisation, immunofluorescent microscopy of fixed in vitro derived DN3
Figure 3.6 DN3 thymocytes undergo several rounds of proliferation before differentiation into DP thymocytes

Purified, sorted DN3 thymocytes from *in vitro* OP9-DLL1 co-cultures were labelled with CFSE (Day 0 CFSE label) and re-cultured on OP9-DLL1 stromal cells for a further 4 days (Day 4 CFSE label). Thymocytes were then harvested, re-stained for the DN1-4 (CD44/CD25 staining) and DN-SP (CD4/CD8 staining) markers, and the CFSE levels of DN3 (red) and DP (blue) thymocytes were plotted.
thymocytes was performed. Firstly, sorted DN3 thymocytes were stained with alpha-tubulin. Many DN3 thymocytes possessed a tightly clustered microtubule organising centre (MTOC) to one side, indicating that there was intracellular polarity during interphase (Figure 3.7). Thymocytes were then co-stained for several polarity and cell fate proteins, and DN3 thymocytes with a polarised MTOC were scored for protein localisation relative to the MTOC. Three localisation descriptors were used for scoring, “not polarised” (NP), “polarised with MTOC” (P) or “polarised distal to the MTOC” (DP) (Figure 3.7A). Proteins of the Par polarity complex (Par3, Par6 and aPKC) and Scrib complex (Scribble, Dlg4) displayed a variety of all three types of localisation patterns, although Scribble was striking in showing the most clear polarisation to the distal pole (Figure 3.7B). A variety of localisation patterns were also observed for the TCR-associated molecule Carma and PKCθ (Figure 3.8, top two panels). The cell fate determinants Notch1, Numb, and the spindle protein Gpsm2 appeared to be strongly polarised to the thymocyte interface in the majority of conjugates, and the clathrin associated endocytic molecule Ap2a2 showed a variety of localisations (Figure 3.8, bottom four panels). Collectively these results demonstrate that DN3 thymocytes possess intracellular polarity, expressing and distributing cell fate determinants and polarity proteins during interphase in a localised manner. The polarisation patterns were mixed, perhaps reflecting different stages of interaction with stromal cells.

3.2.3 Development of a timelapse method to monitor protein trafficking in migrating and dividing DN3 thymocytes

Studying lymphocytes such as thymocytes, mature T cells and mature B cells through time lapse imaging has been a challenge because of their tendency to clump or migrate rapidly. Given the recent discovery that T and B cells may undergo ACD to dictate their cell fate (Barnett et al., 2012; Chang et al., 2011; Chang et al., 2007; Oliaro et al., 2010; Thaunat et al., 2012), there has been an impetus to develop robust in vitro time lapse imaging to capture long term lymphocyte behaviours over hours or days. Methods to overcome the problems of motility and clumping include the coating of coverslips with extracellular matrix poly-L-lysine which would bind lymphocytes to the surface through their surface proteins (Potter et al., 2001). However, adhesion usually involves centrifugation of cells directly onto the coated coverslips such that the normal process of interaction between is prevented and could affect subsequent downstream signalling. Another approach is to embed cells of interest, for example dendritic cells and T cells in an extracellular matrix to facilitate the observations of molecular signalling. However, such an environment may not be physiologically relevant, local cytokines and growth factors cannot freely diffuse through the media, and the physical constraints applied may affect downstream signalling (Dustin et al., 2001). An alternative approach is to create a planar bilayer containing the molecules that activate signalling in lymphocytes (DeMond and Groves, 2007). The advantage is that molecules are free to diffuse between cells, and the layers can be built with increasing complexity to address aspects of signalling in lymphocytes. However, using lymphocytes with their cognate activating cells provides a more physiological context for such observations. What is needed is a system that enables long term observations of thymocytes and stromal cells and their interactions in an environment that would still enable exchange of fluid and cytokines.

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Figure 3.7 DN3 thymocytes express and polarise proteins from the Par and Scribble complex

Purified, sorted DN3 thymocytes from in vitro OP9-DLL1 co-cultures were fixed and stained for alpha-tubulin and a protein of interest by immunofluorescence. Thymocytes selected for analysis all contained tubulin at the interface with the stromal cell, and localisation descriptors were assigned to the protein of interest as A) not polarised (NP), polarised with the MTOC/stromal interface (P) or polarised distal to the MTOC (DP). B) Immunofluorescence staining of DN3 thymocytes with alpha-tubulin with the Par complex proteins (Par3, Par6, aPKC) and the Scribble complex proteins (Scribble, Dlg4). Localisation scores are shown to the right of each panel.
The normal thymus is populated by thymocytes and supporting stromal that are in close proximity. However, having such large numbers would confound observations during time lapse imaging. To address this issue, I titrated different densities of DN3 thymocytes with OP9-DLL1 stromal cells (Figure 3.9A). Low titration of thymocytes and OP9-DLL1 stromal cells did not adversely affect differentiation or proliferation when compared to co-cultures seeded at high density. Both seeding conditions had less than a two-fold difference in proliferation (Figure 3.9B), although a slight enhancement of DN4 differentiation was observed when low density OP9-DLL1 stromal cells were co-cultured with either low or high numbers of sorted DN3 thymocytes. This did not impact on proliferation (Figure 3.9B). Hence, co-culture at low densities of thymocytes and stromal cells did not adversely affect proliferation and differentiation when compared to high densities.

When Differential Interference Contrast (DIC) imaging of DN3 thymocytes and OP9-DLL1 stromal cells seeded at low density was performed, cell clumping was still observed as well as rapid migration of thymocytes and stromal cells out of the imaging field (Figure 3.10 A). To this end, I sought to isolate single thymocytes and stromal cells in individual chambers. Microfabricated grids, or “cell paddocks” were engineered from polydimethylsiloxane (PDMS) that was set in a polymethyl methacrylate (PMMA) mould in arrays of 250 mm² chambers to fit the width of the OP9-DLL1 stromal cell and 60 μm high walls which was enough to confine thymocytes but also enable exchange of culture medium (Figure 3.10 B). I tested the viability of proliferation of a range of lymphocytes including peripheral blood mononuclear cells (PBMCs) within the cell paddocks and observed no detrimental effects of viability or proliferation over 6 days (Figure 3.11). Imaging of antigen presenting dendritic cells with T cells, the T cell lines MLA-144 or PBMC’s were easily contained within the field of view during time lapse imaging up to 12 hours (data not shown). Interestingly, dendritic cells but not OP9-DLL1 stromal could adhere to the cell paddocks, perhaps due to the high hydrophobicity of the PDMS. To overcome this, prior coating with the extracellular matrix fibronectin was needed (Figure 3.12 A and B), and OP9-DLL1 stromal cells were irradiated at 20 Gy to inhibit excessive stromal cell division and migration but still serve as feeder cells for the differentiating thymocytes. Irradiation of OP9-DLL1 stromal cells (GFP or colourless) did not affect their ability to initiate differentiation and proliferation of DN3 thymocytes to the DP stages (Figure 3.12 C). DIC imaging at 2 min intervals enabled easy identification of thymocyte division from OP9-DLL1 stromal cells within the microfabricated grids (Figure 3.13). Fluorescent imaging was confirmed to be compatible with the microgrids, and DN3 thymocytes were retrovirally transduced with two constructs (either GFP or Cherry) to enable quantification of intracellular candidate protein localisation against an internal ubiquitous fluorescent control. Using this approach, long term thymocyte stromal interactions could be monitored for up to 48 hrs without adverse effects on survival or proliferation. The approach I developed took into account effects such as photobleaching, by including a fluorescent control in addition to the fluorescently tagged candidate protein when using post quantification procedures. The optimization of the live imaging procedure is summarised in Box 3.1.
Figure 3.8 DN3 thymocytes express and polarise proteins related to the TCR machinery, cell fate and spindle orientation

Purified, sorted DN3 thymocytes from *in vitro* OP9-DLL1 co-cultures were fixed and stained for alpha-tubulin and co-stained for T cell Receptor associated proteins (Carma, PKCtheta), cell fate determinant proteins (Notch, Numb), the clathrin endocytic protein subunit Ap2a2, and the protein important for spindle orientation, LGN. Localisation scores and numbers of cell analysed are shown to the right of each panel.
Figure 3.9 Low titration of stromal cells and thymocytes does not substantially affect proliferation and differentiation.

Day 9 thymocytes (Th) were sorted and re-cultured on OP9-DLL1 stromal (St) cells at high (2x10^5) or low (7x10^4) confluency in a 6-well plate and 4 days later assessed for A) differentiation by CD44 versus CD25 staining and B) fold proliferation. n= 1 independant experiment.
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Figure 3.10 Formation of microfabricated cell paddocks for time lapse imaging

A) Thymocyte and stromal cells disappear from the imaging field, indicating that containment procedures are needed. B) The procedure for microfabrication of cell paddocks. 1: A mould is created by etching an inverse cell paddock array into a polymethyl methacrylate (PMMA) substrate using a femtosecond pulsed laser. 2: Polydimethylsiloxane (PDMS, blue) is poured into the mould and 3: a glass slide is placed over the PDMS. Curing at 80°C for 20 minutes solidifies the PDMS. 4: After the PDMS has solidified the cell paddocks are removed from the mould and are ready to use.
Figure 3.11 Primary cell culture is unaffected in the presence of PDMS

Viability (PI staining) and proliferation (CFSE dilution) of primary peripheral blood mononuclear cells (PBMCs) show no differences in the presence or absence of PDMS at 8 hours, 24 hours, days and 6 days.
Figure 3.12 OP9-DL stromal cells require fibronectin to adhere to PDMS

A) OP9-DLL1 stromal cells (GFP/green) were cells seeded on cut PDMS sheets (P, bottom half) placed in plastic IBIDI 8-well chambers (CH, top half). Prior to seeding chambers were given either no prior treatment, coated with 1% Poly-L-lysine (Poly-L) or 10μg/ml fibronectin (FN). B) Fibronectin coated cell paddocks and irradiation prevents OP9-DLL1 migration and division from the cell paddock. C) Irradiation or use of colourless stromal cells does not alter differentiation of thymocyte co-cultures on OP9-DL1 stromal cells.
Figure 3.13 Thymocytes and stromal cells imaged at low density in 250μm² PDMS fabricated cell paddocks

A) Representative Differential Interference Contrast (DIC) timelapse images taken at 2 min intervals on OP9-DLL1 stromal cells enables the long term tracking of migrating (black arrows) and dividing (white arrows) thymocytes. B) DIC and fluorescent confocal time lapse imaging of thymocytes expressing GFP (green) and a Cherry-tagged protein X (red) on OP9-DLL1 stromal cells (which are colourless).

Scale bar 10μm.
### Box 3.1 Summary of optimisation procedures performed for time lapse imaging

<table>
<thead>
<tr>
<th>Problem</th>
<th>Optimisation</th>
<th>Solved?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cannot image many cells in one field</td>
<td>Reductionist culture of stromal and thymocyte cells</td>
<td>Yes</td>
</tr>
<tr>
<td>Thymocytes and stromal cells move out of field</td>
<td>Contain cells in cell paddocks</td>
<td>Yes</td>
</tr>
<tr>
<td>Stromal cells do not adhere to cell paddocks</td>
<td>Coat cell paddocks with fibronectin</td>
<td>Yes</td>
</tr>
<tr>
<td>Excessive stromal migration and division</td>
<td>Irradiate stromal cells (20Gy)</td>
<td>Yes</td>
</tr>
<tr>
<td>Need to visualise intracellular protein distribution</td>
<td>Retrovirally transduce thymocytes with two fluorescent constructs that are GFP- /Cherry-tagged to serve as control and candidate protein of interest</td>
<td>Yes</td>
</tr>
</tbody>
</table>
| Not enough time and depth resolution in live cell movies              | *Time:* trial 5, 3, and 2 minute intervals  
*Depth:* trial z-sections at 3, 4 and 5 slices over 10-15µm thickness | Yes. 2 minute intervals with 5 z-sections over a depth of 10-15µm is sufficient to assess protein movement |
3.3 Discussion

3.3.1 Thymocytes demonstrate polarity

It is now clear that many of the proteins that regulate epithelial polarity are also expressed in lymphocytes, and can influence lymphocyte polarity (Ludford-Menting et al., 2005; Pike et al., 2011; Round et al., 2007; Round et al., 2005). The notion that polarity proteins function in thymocytes has been relatively unexplored. Studies in the Robey laboratory have described changes in thymocyte morphology during the interaction with stromal cells which suggest that thymocytes can be polarised (Bhakta and Lewis, 2005; Bousso et al., 2002; Ladi et al., 2008a; Robey and Bousso, 2003; Witt and Robey, 2005). For example, the absence or overexpression of the chemokine receptors CCR7 on these thymocytes, or the ligand quality of TCR selecting peptides on the stromal cells can affect whether thymocytes engage in stable or highly dynamic cell contacts, whether the thymocytes migrate slowly or rapidly, or whether thymocytes exhibit round versus elongated morphology (Bousso et al., 2002; Ladi et al., 2008b). While this demonstrates morphological polarity, there have been some recent publications suggesting the polarity network could be responsible for these morphology changes. A study by Melichar and colleagues has shown that aPKC is polarised in the uropod in migrating DP thymocytes undergoing selection, and that both mature T cells and thymocytes polarise Dlg4 at the synapse with dendritic cells just prior to calcium release following TCR activation (Affaticati et al., 2010; Melichar et al., 2011; Round et al., 2005). Furthermore, Pike and colleagues demonstrate that DN3 thymocytes require Scribble, possibly to aid in cell-cell clustering (Pike et al., 2011). These observations, along with my data showing that Scribble and Dlg4 adopt a variety of polarisations in DN3 thymocytes, provides the first hint that polarity proteins are directly involved during thymocyte function. In other studies, Scribble and Dlg family members also polarise preferentially to the proximal daughter cell during T cell ACD (Oliaro et al., 2010). It will be interesting to investigate in future experiments whether polarity proteins such as Scribble and Dlg localise during DN3 division.

Using the OP9-DLL1 in vitro co-culture system I demonstrate that DN3 thymocyte express and localise several key cell fate and polarity proteins previously shown to be important during ACD. This observation provides direct evidence that the molecular players of ACD are both expressed and localised in DN3 thymocytes. As mentioned, only one other publication has illustrated localisation of the polarity protein Dlg4, however, similar to the observations of aPKC polarisation this was only shown in DP thymocytes during interactions with antigen presenting dendritic cells (Affaticati et al., 2010). A range of localisations during fixed staining of DN3 thymocytes was observed for each of the polarity proteins and cell fate proteins analysed. From the localisation scores, it appeared the polarity proteins Par3, Scribble, and Dlg4 displayed polarisation with the MTOC to the stromal cell interface. However, not all of the polarity proteins were strongly polarised, including Par6 and aPKC. The cell fate proteins Notch1 and Numb displayed high polarisation with the MTOC to the stromal interface. Whilst it would be tempting to speculate that polarity proteins with low polarisation scores were not polarised at all, this may not be true. What these observations suggest is that polarity and cell fate protein localisation may be dynamic, polarising in a temporal manner, perhaps representing a particular stage in fate such as
differentiation, survival or apoptosis.

3.3.2 High resolution thymocyte stromal interactions can be monitored over long periods of time

Early microscopic evidence dating back over 40 years ago provided the first suggestions of asymmetry in dividing thymocytes (Metcalf and Wiadrowski, 1966; Sugimoto and Yasuda, 1983). However, the difficulty in obtaining quantitative data and functional correlations has prohibited research into the area until quite recently. The advent of new technologies such as time lapse imaging, and utilisation of fluorescently tagged proteins in a variety of spectra, have enabled not only the observation of molecular asymmetry in real time in dividing cells, but also correlation of this asymmetry with differential fates of daughter cells. Indeed, a great deal has been gleaned from time lapse imaging of dividing C. elegans blastocysts as well as D. melanogaster neuroblasts and sensory organ precursor cells to uncover the molecular requirements for ACD (Aceto et al., 2006; Gonczy, 2008; Mayer et al., 2005; Munro et al., 2004). Ideally, in vivo imaging of thymic explants or 3D thymic slices enable the most faithful approach for understanding thymocyte behaviours in their normal physiological environment. However, the lack of spatial resolution on a subcellular level has hindered the ability to resolve the finer details within thymocytes, and limited studies largely to the kinetics of migration during positive and negative selection (Bhakta and Lewis, 2005; Ueno et al., 2004; Witt and Robey, 2005). I have developed an in vitro imaging approach using the OP9-DL system in which thymocytes and stromal cells are contained within a field of observation during long term time lapse imaging without compromise of proliferation and differentiation. Thymocytes were shown to undergo both intermittent and prolonged interactions with stromal cells, and some thymocytes even appear to be engulfed by the stromal cells, perhaps by emperipoiesis (Pezzano et al., 2001). The ability to functionalise the surface of the cell paddocks with extracellular matrix such as fibronectin means other surface modifications may be added to investigate many other types of cellular interactions. This system provides an excellent platform to investigate the kinetics of thymocyte stromal interactions in a contained environment, and to quantify and characterise subcellular protein movement within thymocytes during migration and division.

3.3.3 Summary

The results described in this chapter validate that the OP9-DL in vitro system is a robust system in which thymocyte intracellular polarisation during migration, division and impacts on fate determination can be assessed. Thymocytes express and localise a number of polarity and cell fate proteins that have been demonstrated to be involved in ACD in other cell systems. Given that a range of localisations are observed, and that DN3 thymocytes undergo several divisions before differentiation into DP thymocytes, the next steps will be to investigate that these candidate proteins localise during DN3 division. The time lapse method developed enables long term observations between thymocyte and the stromal cells. It provides the platform to formally test
these questions, and the experiments are described in the following chapters. Aspects of this work have been published (Day et al., 2008), and have been applied to studies of other non-adherent lymphocytes such as mature T cells and B cells (Day et al., 2008; Duffy et al., 2012; Oliaro et al., 2010).
Chapter 4

The investigation of Numb polarity during T cell and thymocyte migration reveals divergent mechanisms of signalling
4.1 Introduction

Cell polarity is the asymmetric distribution of proteins to opposite ends of the cell, and controls many cellular activities such as migration and fate determination (Martin-Belmonte and Perez-Moreno, 2012; St Johnston and Ahringer, 2010). Cell polarity is known to be regulated by polarity proteins and has been best studied in cells of solid tissues such as neurons and epithelial cells. Recent studies have shown many of the proteins that regulate epithelial polarity are also expressed in lymphocytes and can influence lymphocyte polarity (Fung et al., 2012; Krummel and Macara, 2006; Lin et al., 2009; Ludford-Menting et al., 2005; Pike et al., 2011; Round et al., 2007; Round et al., 2005; Zanin-Zhorov et al., 2012). The role of polarity proteins in thymocytes has been less explored.

One such important polarity protein is atypical Protein Kinase C (aPKC), a serine threonine kinase protein that regulates polarity in many cell types during migration and ACD (Grzeschik et al., 2010; Guilgur et al., 2012; Hao et al., 2010). aPKC is polarised during ACD of T cells, and localises to the trailing uropod in migrating thymocytes as well as DN3 thymocytes (Chang et al., 2011; Chang et al., 2007; Ciocca et al., 2012; Melichar et al., 2011; Oliaro et al., 2010; Real et al., 2007) and previous Chapter). However, the role of aPKC in lymphocytes remains unclear because of possible redundancies between the two mammalian aPKC homologues PKCζ and PKCζ. In particular, the deletion of aPKC does not appear to impact on T cell development or naïve T cell function, and haematopoiesis appears normal in double knockouts of PKCζ and PKCζ (Sengupta et al., 2011; Soloff et al., 2004). However, deletion of PKCζ has been shown to affect mature T cell IL-4 signalling and the differentiation of Th2 T cells (Duran et al., 2004; Martin et al., 2005). Studies by Real and colleagues using overexpression of kinase-dead mutants of aPKC have shown that it reduces the motility of human peripheral blood T cells (Real et al., 2007). It remains unclear how aPKC functions in these lymphocytes.

In epithelial cells and neuronal precursors, aPKC co-polarises with the endocytic protein Numb (Smith et al., 2007; Wirtz-Peitz et al., 2008). aPKC is responsible for localised phosphorylation of membrane bound Numb, resulting in phosphorylated Numb movement from the membrane to the cytosol. Numb is polarised in dividing C. elegans blastocysts, D. melanogaster neuroblasts, haematopoietic stem cells, mature T cells and also in thymocytes (Aguado et al., 2010; Bhakta and Lewis, 2005; Chang et al., 2007; Gonczy, 2008). Mechanistically, Numb serves as a substrate for aPKC phosphorylation at serine residues 7 and 295 (Smith et al., 2007). In asymmetrically dividing D. melanogaster neuroblasts, mutation of two serine residues in Numb prevents aPKC phosphorylation, Numb mislocalisation and altered neuroblast fate (Smith et al., 2007). Mutation of these two serine residues in Numb is also sufficient to disrupt basolateral polarity in Madin Darby Kidney Cells (MDCK) cells, and prevents cytoplasmic Numb relocalisation from the plasma membrane in Hela cells (Nishimura and Kaibuchi, 2007). In asymmetrically dividing mature T cells, disrupting aPKC alters Numb polarisation (Oliaro et al., 2010). From my results in the previous chapter, I also demonstrate that Numb is polarised in DN3 thymocytes during interphase. Given that DN3 thymocytes depend heavily on Notch signalling and therefore are likely to be influenced by Numb (Anderson et al., 2005; Ciofani and Zuniga-Pflucke, 2005),
I investigated whether the mechanism of Numb polarisation by aPKC was conserved during DN3 thymocyte migration. If this was true, then intracellular Numb polarity during thymocyte migration could be disrupted by the same two serine mutations.

In the previous chapter I developed a time lapse method that enables visual assessment of polarity of fluorescent tagged proteins in thymocytes at high resolution. A challenge arising from this time lapse imaging approach is that sophisticated methods of analysis are required for the study of thymocyte polarity. Currently, most quantification approaches in timelapse imaging assess only a few to tens of cells that have been carefully selected as a representative cohort, or a large population that has not undergone selection but overlooks biologically relevant criteria. The latter approach clearly has more statistical power and avoids the possibility of user-bias, but noise in the data can often obscure the results.

In the following chapter I describe a new software platform called TACTICS that enables the interactive visualisation of data parameters from time lapse imaging of single cells in the context of large data sets. By adapting similar approaches of flow cytometry, populations of data and time lapse images are connected through a mouse click, enabling interactive analysis on a single to population level. Subsets of population data can be ‘gated’ on to visualise and explore relevant parameters of interest. Because of difficulties of obtaining large numbers of dividing thymocytes, I utilise TACTICS to firstly investigate whether the mechanism of Numb polarisation by aPKC is conserved between T lymphocytes and epithelial cells during migration. I demonstrate that Numb polarises differently in T cells and thymocytes during migration to epithelial cells. Mutation of two serine residues in Numb is not sufficient to abrogate intracellular polarity, and rather surprisingly the intracellular polarisation of Numb can still be altered during hyperactivation of aPKC. These studies reveal that Numb polarisation is not conserved in T cells or thymocytes during migration, and the mechanism of Numb intracellular polarisation most likely differs to what is currently known in epithelial cells.

4.2 Results

4.2.1 GFP-Numb traffics similarly to endogenous Numb in T lymphocytes

In polarised epithelial cells, Numb is enriched to both the apical and lateral membrane. Localised phosphorylation via aPKC regulates Numb recycling between the apical membrane and the cytoplasm (Smith et al., 2007). In migrating polarised epithelial cells, Numb is localised to both the trailing and leading edge, where aPKC phosphorylation of Numb mediates integrin endocytosis during forward propulsion (Nishimura and Kaibuchi, 2007). To compare and contrast these observations of Numb polarisation in lymphocytes, I first investigated the localisation of endogenous Numb in a highly polarised T cell line, MLA-144 (MLA) (Rabin et al., 1981). These MLA cells were fixed and stained by immunofluorescence for Numb (Figure 4.1 A). In round MLA cells, Numb appeared to be localised to the cell membrane, with some vesicular staining in the cytoplasm (data not shown). In contrast, MLA cells with a uropod showed enrichment of Numb
**Figure 4.1 GFP Numb traffics similarly to endogenous Numb in ML T cells**

Fixed, Z-projected confocal images of A) Untransduced MLA cells or B) MLA cells ectopically expressing GFP-Numb, stained for GFP and Numb. Merge indicates the overlay between GFP (green) and Numb (red). Representative images obtained over 3 independent experiments. White scale bar 10μm.
localisation to the uropod, with some staining at the leading edge (Figure 4.1 B). To quantify the polarisation of Numb, MLA cells were transduced with GFP-Numb. Immunofluorescence staining of transduced MLA cells with an antibody to GFP also showed a similar pattern of staining as observed for Numb (Figure 4.1 B). Similarly, staining with an antibody to Numb confirmed that the localisation of ectopically expressed GFP-Numb was similar to that of endogenous Numb. Therefore, both Numb and GFP-Numb appeared polarised to the uropod. The next step was to quantify GFP-Numb intracellular polarisation.

4.2.2 TACTICS quantification platform to analyse Numb intracellular polarity in migrating T lymphocytes

Using the imaging method developed in Chapter 3, MLA cells were then transduced with GFP-Numb and Cherry-tubulin. Tubulin was used as a positive marker of the uropod in polarised migrating T cells (Ratner et al., 1997). MLA cells were subjected to time-lapse fluorescence confocal microscopy in cell paddocks. For high throughput quantification of GFP-Numb polarisation in MLA cells, our laboratory developed a dedicated computational toolbox for analysis of time lapse imaging. Called TACTICS, this toolbox comprised several collaborative modules that applied a variety of image processing algorithms for interactive analysis of time lapse data from individual or populations of cells. Each module had a user-friendly interface with interactive scroll tables, menus, buttons and slide bars. A TACTICS pipeline was then developed to analyse GFP-Numb polarisation in T lymphocytes as follows (Figure 4.2).

First, confocal time lapse images of MLA cells expressing GFP-Numb and Cherry-tubulin were inputted into TACTICS and then subjected to standard algorithms for image filtering, segmentation and cell tracking (Figure 4.2A). Cherry-tubulin fluorescence was used to mark both the MLA cell borders for segmentation, as well as the uropod where the microtubule organising centre (MTOC) was concentrated. The time frame of each MLA cell was treated as a separate independent data point to avoid possible confounding results when using MLA cells tracked at different lengths, and a total of 5366 data points from 94 tracked MLA cells expressing GFP-Numb + Cherry-tubulin were assessed. Second, analytical features relating to morphology, migration and fluorescence intensity were extracted and compiled in a user interface that would enable interactive analysis of linked features from single data points or populations (Figure 4.2B). Third, the tracked cells were assigned a minor (short) or major (long) axes for qualitative and quantitative exploration of GFP-Numb and Cherry-tubulin polarisation (Figure 4.2C).

To qualitatively inspect GFP-Numb polarisation, I manually selected MLA cells which were elongated and contained a clear uropod. An axis was ascribed to automatically align MLA cells along their major or minor axis, and then vertically averaged along a single intensity scaled line that was one pixel in width (Figure 4.2Di). Each vertical line represented one independent data point so populations of MLA cell projections could be displayed or aligned side-by-side. This approach facilitated a qualitative assessment of fluorescence distribution specifically in the major or minor axis. Projections of uropodic MLA cells orientated along their major axis showed a
strong distribution of Cherry-tubulin fluorescence distribution to the bottom of each projected line, indicating strong polarisation to the uropod in the population of MLA cells tracked (Figure 4.2Di, first row). This was expected as it was a positive marker of the MTOC that localised to the uropod. Similarly, projections of MLA cells orientated along their major axis showed GFP-Numb largely concentrated in fluorescence to the bottom half, also indicating polarisation to the uropod, although not as strongly as Cherry-tubulin (Figure 4.2Di, third row). The same MLA cells were then orientated along the minor axis to serve as negative controls for polarisation and indeed, the fluorescence distributions did not appear localised for GFP-Numb nor Cherry-tubulin (Figure 4.2Di, second and bottom row). These data suggest that Cherry-tubulin and GFP-Numb were polarised along the major axis (to the uropod) but not the minor axis.

An advantage of TACTICS is the ability to extract several analytical parameters on populations of cells and then gate on single or subsets of that population for further exploration. To quantitate the extent of GFP-Numb intracellular polarisation in MLA cells, a ratiometric formula previously used to measure differences in fluorescence intensity during ACD of mature T cells was applied (Oliaro et al., 2010) to calculate the Polarisation Ratio (PR) (Figure 4.2 Dii):

\[
PR = \frac{(\sum H1 - \sum H2)}{(\sum H1 + \sum H2)}
\]

where the sum of intensity in H1 (Hemisphere 1) subtracted by the sum of intensity in H2 (Hemisphere 2), over the sum of intensities in both H1 and H2 hemispheres. PR values ranging from -1 to +1 were obtained, where 0 meant no polarisation and -1 or +1 meant high polarisation. PR values were plotted onto a scatter graph (Figure 4.2Dii, middle panel). There was a large concentration of scatter points around 0 for GFP-Numb in MLA cells orientated along the minor axis, compared to a slightly larger spread of ratios for GFP-Numb in MLA cells orientation along the major axis. The larger spread of scatter indicated that GFP-Numb may be weakly polarised. Scatter plots of GFP-Numb on the major/minor axis were then translated into a frequency histogram. The histogram for GFP-Numb on the minor axis (Figure 4.2Dii, right panel, left histogram, red dotted lines) peaked at 0, whereas the histogram for GFP-Numb MLA cells rotated along the major axis also peaked at 0 but had a slightly larger spread (Figure 4.2Dii, right panel, left histogram, red solid lines). These spreads did not change when the absolute PR frequencies were plotted (Figure 4.2Cii, right panel, right histogram), with a shift in the median PR values of 0.07 to 0.11 when MLA cell were rotated along the minor versus major axis respectively. The slight difference in spread suggested that GFP-Numb was weakly polarised in MLA cells, but this was not compelling.

### 4.2.3 Numb is polarised in migrating T cells

I next considered that the whole population of tracked MLA cells were a heterogeneous population; containing MLA cells undergoing migration, division, death or getting stuck on the corner of the cell paddocks. This would obscure interpreting the PR values for GFP-Numb in migrating...
Figure 4.2 TACTICS pipeline to analyse Numb polarisation

A) Time lapse images of GFP-Numb+Cherry-tubulin MLA cells in cell paddocks are inputted into TACTICS where standard algorithms of filtering, segmentation and tracking are applied to identify the cells (for details see Materials and Methods). B) Identified cells are then analysed for a number of selected cellular based features and these data parameters are extracted, and are also C) assigned a major or minor axis. Manually selected uropod containing T cells were then D)i viewed as vertically projected images to inspect the data qualitatively. Each tracked T cell data point (rotated along the major or minor axis) is vertically projected and averaged along a single pixel, and populations of the MLA cells were lined side-by-side. Depicted are the vertical projections line side-by-side for Cherry-tubulin and GFP-Numb in tracked MLA cells rotated by the major or minor axis. Note the fluorescence concentration below the hatched line (drawn halfway across each projection) for Cherry-Tubulin and GFP-Numb when the MLA cells are orientated along the major axis, which indicates concentration of polarisation to the uropod. D)ii. Rotated GFP-Numb MLA cells are bisected according to their major or minor axis, and the Polarisation Ratio (PR) for GFP-Numb is calculated, shown as scatterplots (minor axis and major axis PR values shown) and frequency histograms (minor axis and major axis, signed or absolute PR values shown).
Chapter 4: Results

A) Image processing

Filter
Segment
Track

B) Extract data points linked to tracks

Selected analytical features

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<thead>
<tr>
<th>Feature</th>
<th>Data Points Linked to Tracks</th>
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<tr>
<td>Cell Area</td>
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<td>Distance travelled</td>
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<td>2D projection</td>
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C) Define axis

LEADING EDGE
Major axis
Minor axis
UROPOD

D) Qualitative exploration of population data

D)i Polarisation ratio, PR

\[
PR = \frac{(\Sigma H1 - \Sigma H2)}{(\Sigma H1 + \Sigma H2)}
\]

Normalised Counts

-1.0 0 1.0
Signed PR

-1.0 -0.5 0 0.5 1.0
Absolute PR

Minor axis
Major axis

PR, minor: 0.07
PR, major: 0.11
MLA cells, which would so far indicate only weak polarisation. To this end, I used TACTICS to explore and interact with data parameters linked from the single to the population level like flow cytometric data. Parameters extracted from the tracked MLA cells could be plotted as two dimensional scatterplots, and access information corresponding to cell tracks, time lapse movies and other data parameters extracted could be accessed by clicking on single data points.

To compare tubulin and Numb polarisation, PR values for Cherry-tubulin (major axis) were plotted against PR values for GFP-Numb (also major axis) (Figure 4.3). A strong positive Pearson’s correlation coefficient of $R=0.87$ indicated that at a population level Cherry-tubulin and GFP-Numb were co-polarised. Exploring individual data points on this dot plot revealed that MLA cells with both high Cherry-tubulin and GFP-Numb PR values were elongated MLA cells migrating in a straight direction (Figure 4.3, right top row). This was in contrast to clicking on data points with low Cherry-tubulin and GFP-Numb ratios, which usually corresponded to still, rounded MLA cells (Figure 4.3, right bottom row). This data suggested that Numb was polarised in T cells with polarised tubulin, and that the unimpressive PR values previously observed in Figure Dii might be, in part, due to many T cells in this population that were not polarised.

Based on the observation that tubulin and Numb polarisation were positively correlated, MLA cells that were polarised (represented by high Cherry-tubulin PR values) were gated on and inspected for Numb polarisation (Figure 4.4). A dot plot of GFP-Numb PR values (major versus minor axis) illustrated that ungated cells showed an even distribution along both axes (Figure 4.4, left dot plot, grey dots), and that the gated MLA cells were clearly polarised along the major axis (Figure 4.4, left dot plot, black dots). When the cumulative frequency histograms of GFP Numb PR values were plotted, the median PR (major axis) shifted to the right from 0.11 to 0.20 when compared to un-gated MLA cells (Figure 4.4 B, top and bottom histograms). In comparison to ungated and gated MLA cells, a median PR of 0.15 was observed when MLA cells were manually selected as containing uropods (Figure 4.4 B, middle histogram). These data combined illustrate the advantage of interactive interrogation of data from large populations of cells at the individual cell level, and indicate that GFP-Numb is polarised in migrating T cells.

4.2.4 Mutation of the aPKC phosphorylation sites in Numb prevents phosphorylation, but does not abrogate polarity in lymphocytes

The current model for aPKC regulation of Numb polarisation in epithelial cells suggests that Numb is normally localised to cortical regions of the cell (Nishimura and Kaibuchi, 2007). Areas enriched for aPKC such as the apical membrane enable direct phosphorylation of Numb at two critical serine residues 7 and 295 (Dho et al., 2006; Nishimura and Kaibuchi, 2007; Smith et al., 2007). Mutation of these residues from serines to alanines (Numb2A) prevents aPKC phosphorylation, and Numb2A is unable to re-polarise to the basolateral membrane (Smith et al., 2007). To investigate whether this mechanism of aPKC-regulated polarisation of Numb was conserved in lymphocytes, MLA cells were transduced with GFP-Numb2A and Cherry-tubulin. If this mechanism of polarity was conserved then GFP-Numb2A would not be polarised to the
Figure 4.3 GFP-Numb and Cherry-tubulin are positively correlated with migration

PR values of GFP-Numb plotted against Cherry-tubulin (major axis). A positive linear Pearsons correlation coefficient of R=0.87 is shown (red line) between GFP-Numb and Cherry-tubulin. Each coloured dot on the plot represents a separate data point with the same colours representing one cell tracked over time and is linked to cells movies, montages and cell tracks. i. A data point selected with high GFP-Numb and Cherry-tubulin polarisation ratios indicate this MLA cell is migrating in a straight line whereas ii. A T cell selected with low GFP-Numb and Cherry-tubulin polarisation ratios is relatively immotile and unpolarised. Cell tracks (red) and montage for each selected MLA cell shown.
Figure 4.4 Image cytometry reveals GFP-Numb is polarised to the uropod in T cells

A) Multicoloured plots of polarisation ratios for Cherry-tubulin (major versus minor axis). Gates were drawn on high Cherry-tubulin ratios (major maxis) and the GFP-Numb polarisation ratios (major versus minor axis) are plotted (grey= ungated, black= gated). B). Polarisation ratios of GFP-Numb calculated on ungated data points, manually selected uropod containing cells and MLA cells gated on high Cherry-tubulin polarisation ratios (major axis).
uropod in migrating MLA cells. Fixed immunofluorescence staining of untransduced, GFP-Numb and GFP-Numb2A MLA cells was performed for Numb and GFP, and indicated a broadly similar distribution between endogenous Numb and GFP-Numb (Figure 4.5 A top panels). This demonstrated that GFP-Numb2A was also polarised in uropod-containing MLA cells. MLA cells with Cherry-tubulin and GFP-Numb2A were then subjected to time lapse imaging and analysis using TACTICS to quantify the polarisation of GFP-Numb2A using the approach described for GFP-Numb in Figure 4.2. Remarkably, Numb2A was highly polarised in MLA cells gated for Cherry-tubulin polarisation (Figure 4.5 Bi left histogram; blue lines; median PR: 0.33 for major axis, 0.09 for minor axis), showing even higher polarisation than GFP-Numb (Figure 4.5 Bi left histogram; red lines; median PR: 0.20 for major axis, 0.05 for minor axis). Therefore, the mutation of the two serine residues did not abrogate polarisation of Numb in T cells, and instead resulted in increased polarisation to the uropod.

In previous work by Smith and colleagues, hyperactivation of all PKC isoforms through addition of the phorbol ester TPA (Tetradecanoylphorbol-13-acetate) induced rapid loss of Numb polarisation, whereas GFP-Numb2A was insensitive to TPA treatment (Smith et al., 2007). Based on this, I hypothesised that the polarisation of wildtype GFP-Numb but not mutant GFP-Numb2A would reduce in response to aPKC hyperactivation by TPA treatment. Quantification of the median polarisation ratios of GFP-Numb MLA cells treated with TPA during time lapse imaging demonstrated Numb polarisation had decreased (Figure 4.5 Bi, right histogram; red lines; median PR: 0.20 for untreated and median PR: 0.18 for TPA treated). However, when GFP-Numb2A MLA cells were treated with TPA, there was also a decrease in polarisation (Figure 4.5 Bi, right histogram; blue lines; median PR: 0.33 for untreated and median PR: 0.24 for TPA treated) similar to the level of wild type GFP-Numb. Mutation of the aPKC phosphorylation sites in Numb prevents phosphorylation in T cells as it does in epithelial cells, but does not abrogate polarity in T cells as previously shown in the epithelial studies. Together, these data show that the influence of these two phosphorylation sites of Numb differs between T cells and epithelial cells.

It was possible that in MLA cells, Numb2A was not phosphorylated and therefore was insensitive to TPA treatment. To compare whether GFP-Numb and GFP-Numb2A was phosphorylated by aPKC, immunoblotting was performed using an antibody specific for Numb phosphorylated at Ser7 (Figure 4.5 C). Indeed, GFP-Numb but not GFP-Numb2A was phosphorylated at Ser7. The phosphorylation of GFP-Numb Ser7 was also reduced upon addition of the PKC inhibitor Bisindolylmaleimide XI (Figure 4.5 C WT, +B lane) indicating that Ser7 of Numb was phosphorylated by aPKC in MLA cells. Immunoblotting of GFP-Numb showed that TPA treatment did not lead to an increase phosphorylation of Ser7 at the time point analysed, indicating that Numb was possibly already phosphorylated on other serine residues (Figure 4.5 C WT, +T lane).

In epithelial cells, Numb is excluded from cortical regions rich in aPKC (Smith et al., 2007), and to determine whether similar spatial antagonism occurs in MLA cells, I co-stained GFP-Numb and GFP-Numb2A expressing cells with an antibody specific for aPKC (Figure 4.5 D). Both Numb and aPKC were observed in the uropod, irrespective of whether the cells expressed wild-type
Numb or Numb2A. Together, the data suggest that while Numb is localised and phosphorylated by aPKC in T cells similarly to epithelial cells, its localisation is regulated differently. Mutation of the two serine residues is insufficient to abrogate polarity of Numb in T cells but is also insufficient to abolish Numb mobilisation in response to hyperactivation of all PKC isoforms.

4.2.5 Numb in thymocytes is polarised to the uropod and does not require aPKC phosphorylation, similar to T cells

I next assessed whether the regulation of Numb polarisation observed in the MLA cells was similar in DN3 thymocytes. E14.5 foetal liver cells were co-cultured on OP9-DLL1 stromal cells to differentiate along the T cell development pathway. Day 8 co-cultures (mainly DN2 and DN3 thymocytes) were harvested and transduced with GFP-Numb/GFP-Numb2A and Cherry. Cherry was used in this instance to identify the borders of the thymocyte and to facilitate analysis of polarisation during division by TACTICS (Chapter 5). DN3 thymocytes positive for both GFP-Numb/GFP-Numb2A and Cherry were sorted and re-cultured on OP9-DLL1 stromal cells for flow cytometric analysis of differentiation and proliferation. Time lapse imaging was also performed in parallel. Flow cytometric analysis of sorted DN3 thymocytes 6 days post co-culture indicated that the ectopic expression of wild type GFP-Numb did not perturb proliferation or differentiation through the DN1, 2, 3, 4 (Figure 4.6 A) and DP stages (Figure 4.6 B). A subtle block in differentiation from DN to DP was observed with the ectopic expression of GFP-Numb2A in comparison to Cherry only transduced thymocytes (Figure 4.6 B). There appeared to be no impact on the DN3 thymocytes subset (my broad interest for this thesis) and so these were sorted for further analysis.

DN3 thymocytes that had been sorted for GFP-Numb/GFP-Numb2A +Cherry fluorescence were cultured with OP9-DLL1 stromal cells in cell paddocks, subjected to time lapse imaging, and analysed using TACTICS to quantify GFP-Numb polarisation. In this instance, rather than Cherry-tubulin as a positive control for polarisation, I used unfused Cherry for tracking and segmentation, and to control for any possible artefacts of shape differences between the two hemispheres of the cell. Each frame was treated as a single data point, with 51349 data points analysed from 1159 tracked cells for GFP-Numb thymocytes, and 77137 data points analysed from 2093 tracked cells for GFP-Numb2A thymocytes. Similarly to T cells, when uropod-containing thymocytes were manually selected, GFP-Numb appeared weakly polarised to the uropod but mutant GFP-Numb2A appeared strongly localised to the uropod (Figure 4.7 A) and this was also seen using vertical projections of thymocytes rotated by the major or minor axis (Figure 4.7 B). In all cases (montages and vertical projections), Cherry fluorescence was spread fairly evenly throughout the thymocyte and would serve as a negative control for polarisation.

To determine whether ectopic expression of wildtype or mutant Numb impacted on thymocyte migration, the migration characteristics were investigated. There were no obvious differences between wildtype and mutant Numb when the displacements from the origin were plotted, as both GFP-Numb and GFP-Numb2A thymocytes appeared to migrate and turn similarly (Figure 4.8 A).
Figure 4.5 Mutation of aPKC serine residues does not abrogate Numb polarisation in MLA T cells

A) MLA cells transduced with wild-type GFP-Numb and GFP-Numb2A were cytospun, stained for immunofluorescence with antibody to Numb (red), and imaged for GFP-fluorescence (left, pseudocoloured green) and Numb staining (middle panels, red). Merge (right panels) indicates overlay of GFP fluorescence and Numb staining. Representative images obtained from three independent experiments. White scale bar 10 μm. Images represent Z-projections of confocal sections. Bi) Polarisation ratios were derived from time lapse imaging of migrating MLA cells transduced with Cherry-tubulin and either wild-type GFP-Numb (red lines) and GFP-Numb-2A (blue lines). Events were gated for polarised MLA cells based upon Cherry-tubulin fluorescence, and Cherry-tubulin PR values along the major axis (solid lines) were compared with PR values along the minor axis (dotted lines). Bii) PR values from Cherry-tubulin-polarised MLA cells in Bi were compared with ratios after TPA treatment (5 μM, added 30 mins before imaging commenced). Only polarisation along the major axis is shown, untreated cells are indicated by the solid line, and TPA-treated cells by the dotted lines. Events analysed: wild-type GFP-Numb untreated n=1691, TPA treated n=2724; GFP-Numb2A untreated n=1186; TPA-treated n=2783. C). Wildtype Numb is phosphorylated at serine 7, and is sensitive to PKC inhibition but not hyperactivation by TPA treatment. T cells (Parental, WT or 2A) were treated with 5 μM TPA PMA(+T) (+P lanes), 10μM Bisindoylmaleimide XI BIS XI (+B lanes) or DMSO control (- lanes) for 30 mins before lysis and Western blot analysis. Blots are representative of 4 independent experiments. D) aPKC localisation is coincident with Numb in MLA cells to the uropod. MLA cells transduced with wild-type GFP-Numb and GFP-Numb2A were cytospun, stained for immunofluorescence with antibody to aPKC (pseudocoloured green) and Numb (red). Merge (right panels) indicates overlay of aPKC and Numb staining. Representative images obtained from three independent experiments. White scale bar 10 μm. All images represent Z-projections of confocal sections.
Figure 4.6 Ectopic expression of GFP-Numb and GFP-Numb2A in thymocytes

Flow cytometry profiles of sorted DN3 thymocytes transduced with GFP-Numb + Cherry (second column) or GFP-Numb2 + Cherry (fourth column) Respective Cherry only transduction shown to the left of each Numb sample panel. A) DN1 (CD44+/CD25-), DN2 (CD44+/CD25+), DN3 (CD44-/CD25+), DN4 (CD44-/CD25-) flow cytometry profiles of Day 6 sorted transduced DN thymocytes with fold proliferation and DN subpopulation statistics B) DN (CD4-/CD8-), DP (CD4+/CD8+) flow cytometry profiles of the same transduced thymocytes with summarised graphs below each profile. An unpaired students T-test was performed between Cherry and GFP-Numb2A DN subpopulations (P<0.001) and DP subpopulations (P<0.0005). Data is representative of n=3 independent experiments.
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Figure 4.7 Numb polarisation in thymocyte appears similar to T cells

A) DN3 thymocytes were identified, tracked, and assembled into montage libraries by TACTICS. Three representative examples of wildtype GFP-Numb (WT) and mutant GFP-Numb2A (2A) are shown in the DIC (grey), Cherry (red) and GFP (green) spectral channels, rotated along the major axis. Scale bar 10µm.

B) Kymograph-like vertical projections of manually selected uropod containing thymocytes rotated along the major axis. The cherry (control) and GFP (WT or 2A) intensity distributions are shown for each wildtype GFP-numb (n= 1965) mutant GFP-Numb2A (n=2093) thymocytes. Below the hatched line drawn across each kymograph indicates the uropod.
A). However, mutant compared to wildtype Numb thymocytes migrated on average significantly further (722.8 μm compared to 612.5 μm total distance) and at faster speed (17.7 μm/min compared to 14.9 μm/min) respectively (Figure 4.8 B). Mutations of the aPKC sites appeared to impact on how far and fast the DN3 thymocytes migrated.

Because the thymocytes expressed Cherry and not Cherry-tubulin, the DN3 thymocytes could not be selected on the basis of being polarised. Using TACTICS, an alternative gating method was explored to quantify Numb intracellular polarisation of DN3 thymocytes. The whole population of tracked DN3 thymocytes was gated by migration speeds and aspect ratio. Thymocytes migrating above 2 μm/min were chosen, as well as those with aspect ratios less than 0.6 (Figure 4.9A, green boxes). Aspect ratio is the width divided by the length of the cell, where rounded cells would have a high aspect ratio (close to 1) and elongated cells would have small aspect ratios. The PR values (major axis) were then calculated. Although not as high as the MLA cell PR values, this gating procedure confirmed that GFP-Numb was polarised (Figure 4.9Ai, median PR: 0.12), and that there was increased polarisation of mutant GFP-Numb2A DN3 thymocytes (Figure 4.9Ai, median PR: 0.21). Rounded thymocytes, regardless of their migration speed did not polarise GFP-Numb or GFP-Numb2A and displayed median PR values very similar to the Cherry control in the same populations (Figure 4.9A and ii, red boxes, histogram shown in ii).

To determine whether there was variation between experiments, the population of tracked DN3 thymocytes were divided into 9 experiments that were performed in order to obtain the time lapse data, and the polarisation of GFP-Numb was re-checked and quantified along the major or minor axis (Figure 4.10). Ungated GFP-Numb DN3 thymocytes displayed no hints of polarisation along the major axis compared to when rotated along the minor axis (Figure 4.10 A, X is minor axis, Y is major axis). GFP-Numb DN3 thymocytes were then gated by migration speeds over 2μm/min and aspect ratios less than 0.6 (Figure 4.10 B). This gating procedure confirmed across all 9 experiments that GFP-Numb was polarised in DN3 thymocytes similarly to T cells during migration, with a significant increase in median PR of GFP-Numb from across all 9 experiments from PR: 0.07 (ungated) to PR: 0.14 (gated) (Figure 4.10 C, P<0.0001). These data indicate that GFP-Numb and GFP-Numb2A polarised similarly in thymocytes compared to T cells, and this was not due to interexperimental variation.
Figure 4.8 GFP-Numb2A thymocytes migrate further and faster.

A) Representative spidergrams of tracked GFP-Numb (WT) and GFP-Numb2A (2A) thymocyte displacements from the origin (scale in µm). N= 30 representative thymocytes chosen randomly for WT and 2A. B) Graphs of total distance travelled (µm) and average speed (µm/sec). n= 600 thymocytes for each of WT and 2A, with at least 30 tracked points per thymocyte. An unpaired Students T-test was performed for total distance travelled and average speed (P<0.005).
Figure 4.9 Mutation of aPKC serine residues does not abrogate polarity in DN3 thymocytes

GFP-Numb (WT) and GFP-Numb2A (2A) DN3 thymocytes were gated on speeds larger than 2μm/min and aspect ratios smaller than 0.6 (green gate, “faster, elongated cells”) or aspect ratios higher than 0.8 (red gate, “round cells”). The corresponding polarisation ratios for i. faster elongated cells or ii. round cells are plotted as a frequency histogram. GFP-Numb n= 51349 data points, 1159 tracked cells; GFP-Numb2A, n=77137 data points 2731 tracked cells.
Chapter 4: Results

Figure 4.8

Gate faster, elongated cells

Gate round cells

i

ii

GFP-NumbWT

GFP-Numb2A

Normalized counts

Normalized counts

- WT, Ch PR=0.05
- 2A, Ch PR=0.06
- WT, GFP-Nb PR=0.12
- 2A, GFP-Nb2A PR=0.21

- WT, Ch PR=0.04
- 2A, Ch PR=0.04
- WT, GFP-Nb PR=0.07
- 2A, GFP-Nb2A PR=0.06
**Figure 4.10 Interexperimental variation of GFP-Numb polarisation ratios in thymocytes over 9 experiments**

A) Raw ungated GFP-Numb thymocyte populations rotated along the major (blue shades, denoted by “X”) or minor axis or (red shades, denoted by “Y”), B) GFP-Numb thymocytes gated on faster and elongated cells, also rotated along the major or minor axis. C) Comparison of median PR values over 9 experiments of ungated or gated GFP-Numb thymocyte populations. Number of datapoints analysed are shown. An unpaired Students T-test was performed between gated GFP-Numb thymocytes rotated along the minor versus major axis (P<0.0001).
Figure 4.9

A) Ungated GFP-Numb thymocytes

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B) Gated GFP-Numb thymocytes (migrating/elongated)

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C) P<0.0001

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4.3 Discussion

4.3.1 A new tool for analysis of intracellular polarity

The analysis and quantification of time lapse microscopy data remains the bottleneck for many biologists. To quantify data, biologists often manually draw regions of interest to compare measurements (Mayer et al., 2005; Oliaro et al., 2010; Sasawatari et al., 2010). However, monitoring changes in cell organelle distribution and measuring the intracellular localisation of proteins of interest within rapidly moving, migrating cells are prime examples of complex phenotypes that are not amenable to simply drawing regions. Moreover, the large datasets generated from such time lapse experiments usually cannot be handled by openly available image analysis software.

The TACTICS approach used in this chapter to quantify Numb polarisation utilises the ability to visually explore and interact with time lapse data. One of the advantages of having an interactive platform is that exploration of datasets can suggest the formulation of more useful questions in which to probe the same data (Tweedie and Spence, 1998). In biology, the advantage of a flow cytometric platform is that relationships between different parameters for thousands of events can be displayed with ease, and potential relationships between those parameters can be unveiled through gating strategies. A similar approach to image quantification is available in a plugin called Paranorama for the image analysis program CellProfile, where imaging data can be processed multiple times using different parameters, and subsets of the data can be reprocessed as required (Pretorius et al., 2011). However, Paranorama focuses on how changes in parameter values influence the outcomes, rather than on what the relationships between parameters could mean. The commercially available Imaris Vantage (BitPlane Scientific Software) produces multidimensional scatterplots to determine relationships between different parameters, but unlike TACTICS, Imaris scatterplots do not have gating capabilities for further exploration. DISC software (developed by an immunology laboratory) applies flow cytometry like phenotypic analysis to intravital multiphoton imaging, but does not have the streamlined capability of image processing prior to the phenotypic analysis (Moreau et al., 2012). The TACTICS approach is perhaps most similar to the commercially available MetaXpress/AcuityXpress software (Molecular Devices) and the recently developed ImageRail software, which stores fluorescence and localisation data in semantically typed hypercubes (SD Cubes) for interactive analysis (Millard et al., 2011). ImageRail allows dynamic linking of extracted data to source images, and allows gating for further analysis (Millard et al., 2011). TACTICS achieves all these criteria but also supports time lapse microscopy, enabling the quantification of all these parameters over time, and allowing for the history of the cells to be used in gating strategies. Using this features, the relationship between the Numb and tubulin polarisation were discovered to be positively correlated, along with Numb polarisation in migrating T cells over hundreds of time points. I also demonstrated that time lapse data could be “noisy” where no apparent polarisation could initially be observed in both T cells and thymocytes. However, by simply gating for cells that were polarised for tubulin, or moving at a reasonable speed, Numb polarisation patterns became clear.
4.3.2 Numb may be involved in endocytic pathways that regulate thymocyte migration

In differentiating thymocytes, Numb co-localises with various proteins of the T cell receptor (TCR) signalling machinery (Anderson et al., 2005; Chang et al., 2011; Oliaro et al., 2006), suggesting that Numb could be involved functionally during TCR rearrangement. It is also possible that Numb may involved in other DN3 behaviours such as migration or proliferation. This study reveals that DN3 migration may be connected to Numb, and that mutation of two serine residues may alter migratory characteristics.

These results are interesting in light of the observations that epithelial cells also rely on Numb for proper migration. In epithelial cells, Numb controls integrin mediated endocytosis for polarised migration with aPKC and PAR-3 (Nishimura and Kaibuchi, 2007). The adhesive strength of cell contact during this migration depends partly on the amount of surface E-cadherin. Numb depletion induces E-Cadherin to relocalise from the lateral epithelial membrane to the apical membrane, indicating aPKC phosphorylates Numb and negatively regulates Numb-mediated endocytosis of E-cadherin similar to integrin (Sato et al., 2011). Additionally, the perturbation of Numb activity through hairpin RNA interference has been shown to increase F-actin polymerisation, decrease cell adhesion and increase migration during wound healing (Wang and Li, 2010). It has been postulated that this enhanced migration centres on altering the ability for Numb to regulate the trafficking of critical small Rho GTPases essential for cytoskeletal reorganisation such as Cdc42, whose deletion alters localisation of aPKC, Par6 and Numb in the neuroepithelium (Chen et al., 2006). While Numb polarisation may be regulated differentially in thymocytes, the downstream migration pathways affected by Numb may be conserved between epithelial cells and thymocytes. This could also explain why mutant Numb thymocytes are slower to transit from DN to the DP on OP9-DLL1 stromal cells. With decreased adhesive strength, a DN thymocyte may not be able to efficiently engage in prolonged stromal interactions necessary to signal their differentiation. Before any conclusions are drawn, the migration characteristics of GFP only transduced DN3 thymocytes will need to be performed and compared to GFP-Numb and GFP-Numb2A DN3 thymocytes in future experiments. Ideally, a knockin mouse for either GFP-Numb or GFP-Numb2A would have been superior to investigate the mechanisms of Numb polarity in T lymphocytes. However, these were not available during my thesis studies and the next best system was ectopic expression of the fluorescent constructs.

4.3.3 aPKC regulation of Numb polarisation is not conserved in T cells and DN3 thymocytes

In both epithelial cells and asymmetrically dividing neuroblasts, Numb is phosphorylated by aPKC to remove it from the cell membrane and maintain polarisation of Numb. If the mechanisms of polarisation in lymphocytes were conserved, the polarisation of Numb to the T cell or thymocyte uropod would be expected to be abrogated by mutation of the two serine residues. The results observed in this chapter indicate that the mutation of two serine residues does not abrogate polarity...
to the uropod and in fact results in increased polarisation. However, Numb2A still responds to aPKC hyperactivation. The mechanisms of Numb polarisation in DN3 thymocyte differentiation therefore contradict what is known in epithelial cells.

There are two reasons accounting for these unexpected results. The first is the possibility that some but not all polarity complex proteins show similar localisation characteristics from epithelia to T lymphocytes. The Scribble polarity complex (Scrib, Dlg and Lgl) are known to co-localise to the basolateral end of polarised epithelia, and work from our laboratory has described that the same polarity proteins co-localise to the uropod in T cells (Ludford-Menting et al., 2005). Components of the Par complex (Crumbs, Par3 and Par6) are co-localised in the apical side of epithelial cells, and we have observed these proteins to co-localise to the leading edge of T lymphocytes (Ludford-Menting et al., 2005). aPKC is a Par complex protein that is localised to the apical side in epithelial cells (Smith et al., 2007). Therefore, it would be tempting to speculate that aPKC should also localise to the leading edge in T cells. In contrast, aPKC was localised to the uropod in T cells where it co-localised with Numb. Co-localisation of the various polarity complex proteins may overlap from epithelia to lymphocyte cells but it is apparent that they are not identical. Other studies have also highlighted these dissimilarities of polarity protein localisation. Depending on the cellular context in D. melanogaster neuroblasts and embryonic epithelial cells, Par3 apical localisation is independent of aPKC, while in mammalian epithelial cells, Par3 is not apical but is associated with tight junctions (Harris and Peifer, 2007; Joberty et al., 2000). These differences in localisation by the polarity proteins could result in differential regulation of Numb localisation in T cells and DN3 thymocytes compared to epithelial cell in other systems.

If aPKC localisation is not identical in lymphocytes, then a second reason for the differences in Numb polarisation could stem from differential aPKC phosphorylation patterns. Five serine residues (7, 52, 276 and 295) have been shown to be conserved aPKC phosphorylation sites across several species, with serine 7 and 295 as critical residues for Numb regulated localisation in mammalian epithelial cells and D. melanogaster neuroblast development (Nishimura and Kaibuchi, 2007; Smith et al., 2007). However, presently up to 40 potential aPKC phosphorylation sites have been identified for Numb, all of which could be phosphorylated in lymphocytes by aPKC or by other unidentified kinases (Smith et al., 2007). Numb therefore may be subjected to different levels of aPKC phosphorylation, or phosphorylation of another unidentified kinase for its polarisation in T cells and DN3 thymocytes. Whereas aPKC is responsible for positively regulating Numb localisation in D. melongaster neuroblasts and mammalian epithelial cells (Smith et al., 2007), by contrast, aPKC phosphorylation might actually negatively influence polarisation of Numb to the uropod in T cells and thymocytes. In the absence of the two aPKC serine residues, Numb polarisation to the uropod increases during migration. Collectively these data clearly indicate that the mechanisms previously defined for Numb polarisation in mammalian epithelial cells and D melanogaster sensory organ precursors are divergent in T cells and DN3 thymocytes.
4.3.4 Summary

This study provides the first measure of whether a mechanism of Numb polarisation is conserved in T cells and thymocytes as they are in other mammalian epithelial cells. Using the novel, user-friendly interactive analysis platform that supports quantification of fluorescent time lapse microscopy, TACTICS, Numb is shown to polarise to the uropod in migrating T cells and thymocytes. The mutation of two conserved serine residues does not abrogate polarity, however, aPKC hyperactivation can still induce Numb2A depolarisation from the uropod, suggesting unidentified serine residues on Numb are regulated by aPKC phosphorylation. TACTICS is a novel computational platform that enables robust quantification of intracellular polarisation, and this approach can now be applied to address the quantification of protein distribution during DN3 thymocyte division, which is described in the next chapter.

The data for this chapter has been published (Pham et. al., 2013).
Chapter 5

Robust ratiometric quantification of protein distribution reveals that ACD occurs in DN3 thymocytes
5.1 Introduction

Approaches to study asymmetric cell division (ACD) range from purely qualitative descriptions such as size or staining variation of proliferating thymocytes (Metcalf and Wiadrowski, 1966; Sugimoto and Yasuda, 1983), to quantitative measurements of asymmetry that ascribe measurable differences in distributions between two dividing daughter cells. Quantitative approaches, such as directly comparing the percentage of staining intensity from drawn regions around stained daughter cells have proven enormously useful in characterising the distribution of various cell fate determinants and polarity proteins during ACD of *C. elegans* blastocysts and murine T cells (Chang et al., 2011; Takeshita and Sawa, 2005). However, some of the approaches used in these studies can be improved. For example, T cell divisions have been characterised as ACD if one daughter inherits at least 1.5 times more stained protein compared to the other daughter cell (Chang et al., 2011). However, this approach is an arbitrary measure of ACD with no symmetric control protein to compare against out-of-focus cells. Another quantitative approach to measure protein distribution has involved directly comparing changes in fluorescence intensity of tagged-proteins in specific regions of interest over time, such as the anterior cortex of asymmetrically dividing mammalian skin cells (Lechler and Fuchs, 2005). However, one caveat is that direct comparisons are not normalised to the total area of the cell or intensity in daughter cells, which can obscure results.

One emerging quantitative approach that overcomes these caveats is ratiometric analysis of protein distribution during division that enables deriving a ratio of fluorescence intensity distributions (stained or tagged-protein) of daughter halves that speaks to the extent of asymmetry. Studies assessing the distribution of the polarity and cell fate proteins during mammalian radial glial cell division, *D. melanogaster* sensory organ precursor division, as well as studies in our laboratory during mature T cell ACD have used this approach to quantify protein asymmetry (Bultje et al., 2009; Mayer et al., 2005; Oliaro et al., 2010). In each study, regions are manually drawn to define the boundaries of the daughter cells to compare the ratiometric distribution of candidate proteins against total area, or a negative control that is ubiquitously expressed. In Chapter 4, I have developed an automated approach for ratiometric quantification that works well for measuring protein distributions in both T cells and DN thymocytes during migration. In this chapter, I adopt the same approach to investigate whether ACD occurs during DN3 division.

From the studies of ACD in non-adherent cells, such as hematopoietic stem cells, mature T cells and B cells, there are a number of proteins of interest to investigate (Chang et al., 2011; Chang et al., 2007; Oliaro et al., 2010; Thaunat et al., 2012; Ting et al., 2011; Wu et al., 2007). The first obvious candidate is the classic cell fate determinant Numb which is polarised during division in *C. elegans* blastocysts, *D. melanogaster* neuroblasts, and a variety of mammalian cells including T cells and thymocytes (Aguado et al., 2010; Bhakta and Lewis, 2005; Chang et al., 2007; Gonczy, 2008; Wakamatsu et al., 1999; Zhong et al., 1996). Second, a number of T cell receptor (TCR) signalling molecules and polarity proteins in naive T cells have been demonstrated to be spatially compartmentalised during division (Chang et al., 2011; Chang et al., 2007; Ciocca et al., 2012; Oliaro et al., 2010). Third, endocytic molecules responsible for dictating cell fate,
such as Ap2a2, share some overlapping functional features with Numb during fate determination in D. melanogaster (Berdnik et al., 2002). These proteins have been verified in Chapter 3 to be localised in DN3 thymocytes during interphase and therefore are all possible candidates for ACD of DN3 thymocytes.

In this Chapter I utilise TACTICS for robust ratiometric quantification of fluorescent protein distribution during DN3 thymocytes division. I perform a time lapse screen of a selected number of cell fate, TCR associated, polarity and endocytic proteins that have been verified in Chapter 3 to be polarised in DN3 thymocytes. I develop a TACTICS pipeline to extract polarisation ratios of control and candidate proteins in dividing DN3 thymocytes, and use these values to distinguish symmetric cell division (SCD) and ACD. This selected screen quantifies a number of proteins as symmetrically distributed during DN3 division including the TCR-associated protein LAT and polarity protein Scribble. In contrast, the cell fate protein Numb, polarity protein Dlg4 and the endocytic molecule Ap2a2 are asymmetrically distributed in a proportion of DN3 thymocyte division. This study demonstrates that DN3 thymocytes can undergo ACD.

5.2 Results

5.2.1 The cell fate determinant Numb is distributed symmetrically and asymmetrically upon DN3 thymocyte division

Numb is polarised in asymmetrically dividing C. elegans blastocysts, D. melanogaster neuroblasts, as well as mammalian neuroepithelia cells, haematopoietic stem cells, mature T cells (Bhakta and Lewis, 2005; Chang et al., 2007; Gonczy, 2008; Wakamatsu et al., 1999; Zhong et al., 1996). There is also some evidence that Numb may polarise during thymocyte division (Aguado et al., 2010). In many of these systems the polarisation of Numb is key to regulating Notch signalling during ACD. Given that DN3 thymocytes depend acutely on Notch signalling at this stage, and that Numb is polarised to the uropod during interphase and thymocyte migration (Chapters 3 and 4), Numb was the first candidate I investigated to assess and quantify polarisation during DN3 division using TACTICS.

To investigate possible effects of ectopic expression on proliferation or differentiation, thymocytes from OP9-DLL1 cultures were retrovirally transduced with GFP-Numb + Cherry with further culture of sorted DN3 thymocytes on OP9-DLL1 stromal cells. In the previous chapter (Chapter 4), I demonstrated that ectopic expression of GFP-Numb did not affect DN3 thymocyte differentiation into DP thymocytes and so moved onto time lapse imaging.

To facilitate the assessment of GFP-Numb + Cherry in dividing DN3 thymocytes, the following TACTICS pipeline was developed (Figure 5.1). Time lapse images from the movies were subjected to standard algorithms of filtering and segmentation followed by manual identification of divisions (Figure 5.1 A, marked by an ‘X’). In this case, segmentation for defining the cell
borders and the identification of division was performed on the Cherry fluorescence channel. Numbers were then assigned by TACTICS to the parent cell (prior to division) and daughter cells (post division), to which the associated daughter cells were tracked (Figure 5.1 B). Images of the parent cell and daughters were then utilised to create montages orientated along the major axis to enable qualitative assessment of Cherry and GFP-Numb fluorescence distribution (Figure 5.1 C, left panel). Inspection of the montages showed that Cherry fluorescence was dispersed throughout the cytoplasm in parent and paired daughters, whereas GFP-Numb fluorescence appeared predominantly cell membrane in localisation in the same cells during division.

To quantify GFP-Numb and Cherry in all divisions, TACTICS automatically defined the area of parental and daughter DN3 thymocytes using the Cherry channel. Images of all the defined parent cells and daughter cells were separated by a bounded box, spectrally unmixed, and aligned along the major axis to create “sequence montages” (SEQtages) (Figure 5.1 C, right panel). This enabled extraction of both Cherry and GFP-Numb fluorescence intensities within each designated area for ratiometric comparisons and quantification. Finally, the “Polarisation Ratio” (PR) could be calculated (Figure 5.1 D):

\[
PR = \frac{\sum H_1 - \sum H_2}{\sum H_1 + \sum H_2}
\]

where the difference in total intensity between Daughter 1 (H1) and Daughter 2 (H2) was divided by the sum of intensities in both Daughter 2 (H1) and Daughter 2 (H2) one frame after division. This approach had been previously applied by our laboratory to calculate protein polarisation during ACD of naïve T cells (Oliaro et al., 2010) and in Chapter 4 during my studies of polarisation during T lymphocyte migration. One key difference in this study to that of naïve T cell ACD was that it was unknown which daughter thymocytes was H1 or H2 (i.e. proximal or distal). Therefore, absolute PR values were taken to obtain PR values between 0 (completely symmetric) and 1 (completely asymmetric). A second key difference was the presence of a Cherry control that was not available in the fixed T cell staining ACD study. The Cherry control protein was crucial for live imaging because of the increased likelihood of differences in the focal plane for H1 and H2. The PR values of GFP-Numb and Cherry control was calculated for 63 DN3 thymocyte divisions.

Surprisingly, the PR values obtained for GFP-Numb and the Cherry control across 63 divisions were both low and similar (Figure 5.1 Ei, top row, 0% threshold, dark blue). My first conclusion was that GFP-Numb was symmetrically localised upon division, similar to the Cherry control. However, ratiometric measurements of fluorescence microscopy images should always be treated with caution, as different background subtraction adjustments can affect end results. In fact, using subtraction methods such as threshold has been shown to alter the obtained size measurements of dividing bacteria (Guberman et al., 2008). To this end I applied a systematic approach to study the effect of threshold on the obtained PRs for both Cherry and GFP-Numb in all 63 divisions. As expected, increasing the threshold level corresponded to an increase in the absolute PR values for
**Figure 5.1** The TACTICS pipeline to quantify GFP-Numb polarisation reveals it is both symmetrically and asymmetrically localised during DN3 thymocyte division.

A) Time lapse images of DN3 thymocytes expressing both GFP-Numb + Cherry were subjected to filtering for background subtraction and segmentation based on Cherry control to identify borders of cells. Dividing cells were identified manually (marked by an X, red) usually one frame before daughter separation.

B) Daughters cells were tracked (at least 20 frames) to create C) montage images of parent and paired daughters, and also sequences montages (SEQtages) that had additional spectral unmixing and alignment (along major axis) applied to the parent and tracked daughter cells. From the same data as from which the SEQtages were produced, the D) Polarisation Ratio (PR) was calculated for both Cherry and GFP-Numb fluorescence intensities in each daughter cell one frame after division, and absolute PR values were taken (0= completely symmetric; 1= completely asymmetric). TACTICS can also average PR values over multiple frames post division but this was not used in the final analysis. E) i. SEQtage of daughters cells one frame after division were then converted to a single PR pixel, where the colour of the PR pixel reflected the magnitude of asymmetry (dark blue= completely symmetric, red= completely asymmetric). Heat maps were created for the Cherry and GFP-Numb in dividing DN3 thymocytes, derived by applying an increased threshold level (0-100%, 0.1% increments, shown along Y axis) to SEQtage images one frame after division before calculating the PR. Each PR value obtained after thresholding was lined underneath each other from 0-100% threshold for each division (n=63, X-axis). The dashed line indicates the 25% threshold line used to ii. Create scatterplots of Cherry control and GFP-Numb DPR values. To differentiate between symmetric cell divisions (SCD) and asymmetric cell divisions (ACD) a cutoff was drawn based on the highest PR value for the Cherry control (PR=0.27). Divisions above the cutoff were classified as ACD and below the cutoff SCD (greyed area). iii. An example of a SEQtage classified as “SCD” or ACD” according to the PR=0.27 cutoff, and F) the percentages of SCD and ACD events summarised as a column graph for both Cherry-control and GFP-Numb in the 63 divisions. G) Graph of paired PR values of Cherry and GFP-Numb DN3 divisions. For statistics, a paired Students T-test was performed. Cherry vs GFP-Numb: P<0.0001. The difference between each Cherry control and GFP-Numb pair are plotted on the scatterplot to the right, with the mean difference and 95% confidence interval shown.
Chapter 5: Results

A) Input images into TACTICS
   Identify dividing DN3 thymocytes

B) Track daughters of dividing thymocytes

C) Create Montage and SEQtage images

D) Calculate Polarisation Ratio (PR)
   \[ PR = \frac{(\sum H_1 - \sum H_2)}{(\sum H_1 + \sum H_2)} \]

E)i Assign threshold cutoff

E)ii Assign ACD cutoff

E)iii Qualitative exploration

F) Plot % of symmetrical and asymmetrical divisions

G) Paired T-test
   Mean = 0.07
   95% CI: -0.10 to -0.04
both Cherry and GFP-Numb, however, to different extents (Figure 5.1 Ei, 0% - 100% threshold). The PR values for Cherry control only started to increase (ie. became more asymmetric) when a value of above 25% threshold was applied. As Cherry was a negative symmetry control for thymocyte division, this indicated using a threshold value above 25% would introduce artificially high PR values. In contrast to Cherry, at 25% threshold, there was a mixture of low and high PR values for GFP-Numb in the same 63 divisions. These results suggest that at one frame after division using a 25% threshold, Cherry is symmetric in all DN3 divisions. In contrast, GFP-Numb is distributed both symmetrically and asymmetrically.

To visualise the spread of PR values at 25% threshold, PR scatterplots were constructed for both Cherry and GFP-Numb (Figure 5.1 Eii). Analysis of the PR scatter plots showed that for the 63 divisions, the PRs for Cherry control were fairly low and grouped together, whereas there appeared to be a spread of PRs for GFP-Numb. To assign whether a GFP-Numb DN3 division was symmetric or asymmetric, I next assigned a cut off (PR= 0.27) based on the highest division polarisation value obtained for Cherry, the symmetric control (Figure 5.1 Eii, dashed line). Indeed, visual exploration of SEQtages of daughters below PR=0.27 supported symmetrical distribution of both Cherry and GFP-Numb (Figure 5.1 Eiii left panel), whereas divisions above PR=0.27 demonstrated asymmetric GFP-Numb distribution but still symmetrical distribution of Cherry control (Figure 5.1 Eiii right panel). The percentages of Cherry and GFP-Numb SCD and ACD were then plotted (Figure 5.1 F), demonstrating that GFP-Numb was distributed both symmetrically (85.7%, 42/63 divisions) and asymmetrically (14.3% 9/63 divisions) during division. The difference between the control PR values with its paired test PR values for each division were also plotted and a paired T-test indicated that these differences were significant across the 63 divisions (Figure 5.1 G, Cherry vs GFP-Numb, P<0.0001). This data suggested that GFP-Numb was consistently inherited more asymmetrically than Cherry during DN3 thymocyte division. Taken together, DN3 thymocytes undergo both symmetric and asymmetric division of Numb.

5.2.2 Mutation of phosphorylated serine reduces Numb asymmetry during DN3 thymocyte division

In asymmetrical dividing D. melanogaster sensory organ precursor cells, Numb localises to the apical cortex, opposite aPKC (Bellaiche et al., 2001; Roegiers et al., 2001). To sustain Numb polarisation two serine residues serves as substrates for aPKC phosphorylation. Mutations of these serine residues result in Numb mislocalisation and spreading throughout the cell cortex instead of being restricted to the apical cortex (Smith et al., 2007). aPKC regulation of Numb polarisation is also important in asymmetrically dividing T cells, where interfering with aPKC signalling alters Numb polarisation (Oliaro et al., 2010). Given that I demonstrated these serine mutations did not abrogate but increased Numb polarity during migration (Chapter 4), I next investigated whether mutation of the same serine residues would alter Numb polarisation during DN3 division. If these Numb mutations resulted in an increase in its polarity to the uropod during migration, it was possible that the same mutations would increase Numb polarisation and hence
asymmetry during DN3 thymocytes division.

DN3 thymocytes transduced with GFP-Numb2A + Cherry were sorted and cultured on OP9-DLL1 stromal cells. Previously, I demonstrated in Chapter 4 that ectopic expression of GFP-Numb2A did not affect fold proliferation of differentiating thymocytes, but did result in a subtle block in differentiation from DN to DP (Figure 4.6, P<0.001). Using the same TACTICS pipeline developed to measure GFP-Numb (Section 5.2.1), I quantified GFP-Numb2A distribution in 50 DN3 divisions and observed that mutation of the aPKC sites did not increase asymmetry during division. In fact, only 6% of divisions were classified as ACD, compared to 14.3% of GFP-Numb (Figure 5.2 A and Aii), and the differences between paired Cherry and GFP-Numb PRs were not significant (Figure 5.2 Aiii). Interestingly, 6% of Cherry control in dividing thymocytes was also classified as ACD. I manually checked the GFP-Numb2A and Cherry SEQtage images that were classified as SCD or ACD divisions, and observed that the classification of “ACD” to these divisions tended to be dividing cells with dim fluorescence, possibly due to suboptimal imaging conditions and unable to be distinguished by TACTICS (Figure 5.2 Ai). Instead of increasing Numb polarisation as previously observed during DN3 migration, mutation of the aPKC serine residues reduced Numb polarisation during DN3 division.

5.2.3 The T Cell Receptor molecule LAT is distributed symmetrically during DN3 division

Both mature T cells and DN thymocytes signal through similar signalling pathways and one such TCR molecule is LAT (linker for activation of T cells), a bridging molecule responsible for linking TCR components to downstream signalling events (Horejsi et al., 2004; Samelson, 2002). LAT is indispensable for thymocyte development, with a complete lack of peripheral T cells in LAT-/- mice due to a block at the DN3 thymocyte stage (Finco et al., 1998; Zhang et al., 1999). Mice expressing a mutant form of LAT (Y136F) have disrupted phospholipase C-gamma1 activation and reduced calcium flux in both thymocytes and peripheral T cells, resulting in severe lymphoproliferative disease (Sommers et al., 2005). This is also micked when LAT is deleted after the DN3 stage using Cre-excision, although T cell impairment is not as severe compared to the LATY136F mice (Shen et al., 2009). Collectively these studies highlight that LAT is crucial to linking DN3 thymocytes with differentiation, but the mechanism by which this occurs is not fully understood. It is possible that LAT polarisation during DN3 division could create signalling differences that would affect downstream fate and differentiation. I hypothesised that DN3 thymocytes would asymmetrically localise LAT as a possible mechanism to generate these TCR signalling differences.

The same experimental procedures as for Numb and Numb2A were performed, and sorted DN3 thymocytes expressing GFP-LAT + Cherry were cultured on OP9-DLL1 stromal cells for flow cytometric analysis of differentiation and proliferation, as well as time lapse imaging in cell paddocks. After 7 days of sorted DN3 co-culture on OP9-DLL1 stromal cells, thymocyte counts revealed ectopic expression of GFP-LAT resulted in an increase in fold proliferation, from
Figure 5.2 Mutation of phosphorylated serine reduces Numb asymmetry during DN3 thymocyte division

A) Scatterplots of Cherry control and GFP-Numb2A PRs in divided DN3 daughters after a 25% threshold. Divisions above PR=0.27 cutoff were classified as ACD and below as SCD (greyed area, dashed line) i. An example of Cherry and GFP-Numb2A SEQtage classified as SCD or ACD at division. ii. The percentages of SCD and ACD events for both Cherry and GFP-Numb2A summarised as a column graph. iii. Graph of paired PR values of Cherry and GFP-Numb2A DN3 divisions. For statistics, a paired Students T-test was performed. Cherry vs GFP-Numb2A: P=0.5, n.s. The difference between each Cherry control and GFP-Numb2A pair were plotted on the scatterplot to the right, with the mean difference and 95% confidence interval shown. Data is from n=50 divisions accumulated from 4 independent experiments.
Figure 5.3 Ectopic expression of the TCR signalling molecule LAT subtly alters thymocyte proliferation or differentiation

A) Fold proliferation graph of sorted DN3 thymocytes transduced with Cherry only (Control) or GFP-LAT + Cherry (GFP-LAT) after 6 days culture on OP9-DLL1 stromal cells. At 6 days thymocytes were also analysed by flow cytometry to assess impacts on B) DN1-DN4 differentiation (CD44/CD25 staining) and C) DN –SP differentiation (CD4/CD8 staining). Representative plots shown, with statistics of pooled replicates shown in the right column. An unpaired Students T-test was performed between Control and GFP-LAT fold proliferation (P<0.05) and DN subpopulations (DN3, DN4, P<0.01). Data is representative of 3 independent experiments.
approximately 4.2 fold compared to 7.1 fold proliferation (Figure 5.3 A, P<0.05). Moreover, after 7 days there were significantly more GFP-LAT DN3 thymocytes that had reached the DN4 stage compared to the Cherry control (Figure 5.3 B, P<0.01), although the overall proportion of DN and DP thymocyte populations did not change (Figure 5.3 C). Ectopic expression of LAT in DN3 thymocytes therefore appeared to promote proliferation (or decrease apoptosis), as well as promote differentiation. To quantify GFP-LAT distribution during DN3 division, time lapse imaging and TACTICS quantification was performed. During the initial inspection of time lapse movies GFP-LAT appeared cortically localised with no polarisation to either the leading edge or uropod (data not shown). When GFP-LAT fluorescence distribution during DN3 division was quantified and visually assessed GFP-LAT was symmetrically distributed to both DN3 daughters in all divisions, and the paired differences between Cherry control and GFP-LAT were not significant (Figure 5.4 A, Ai Aii and Aiii). Therefore, GFP-LAT does not asymmetrically localise during DN3 thymocyte division.

5.2.4 The polarity protein Dlg4 but not Scribble may be asymmetrically localised during DN3 division

Polarity proteins play multiple roles in both mature and immature T cell function, from migration and TCR signalling, to ACD. In particular, the polarity proteins Scribble and Dlg4 (Discs Large 4) are essential for efficient T cell migration, and knockdown of Scribble or blocking of Dlg4 in a T cell line reduces migration, disrupts immunological synapse formation with antigen presenting cells, and causes mislocalisation of several TCR signalling molecules (Ludford-Menting et al., 2005; Round et al., 2005; Xavier et al., 2004). Recent studies in thymocytes also show that DN3 thymocytes require Scribble, possibly to aid in cell-cell clustering to sustain signalling during differentiation (Pike et al., 2011). In other studies, both mature T cells and thymocytes polarise Dlg4 at the synapse with dendritic cells just prior to calcium release following TCR activation, perhaps to recruit co-activating proteins for downstream signalling (Affaticati et al., 2010; Round et al., 2005). Scribble and Dlg family members also polarise preferentially to the proximal daughter cell during T cell ACD (Oliaro et al., 2010). Whether Scribble and Dlg4 polarise during DN3 thymocyte division has not been explored.

I first investigated whether ectopic expression of GFP-Scribble and GFP-Dlg4 impacted on proliferation or differentiation of DN3 thymocytes during culture on OP9-DLL1 stromal cells (Figure 5.5 A, B, C). Thymopoiesis from the DN3 to the DP stages was grossly unaffected, except for a subtle difference in differentiation from DN3 to DN4 when GFP-Scribble was ectopically expressed. Time lapse imaging was then performed. Using the TACTICS pipeline, 7.6% of DN3 thymocytes asymmetrically localised GFP-Scribble (8/105 divisions) compared to 12.1% of DN3 thymocytes that displayed asymmetric localisation of GFP-Dlg4 (4/33 divisions) (Figure 5.6 A, Aii and B, Bii). Interestingly, similar to what was observed for GFP-Numb2A during DN3 division, visual inspection of GFP-Scribble SEQtages classified as “ACD” contained very dim GFP-Scribble fluorescence (Figure 5.6 Ai). It was possible that these divisions were artificially classified as ACD because of variations in background fluorescence or suboptimal imaging, and
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Figure 5.4 The TCR molecule LAT is distributed symmetrically during DN3 division

A) Scatterplots of Cherry control and GFP-LAT PRs in divided DN3 daughters after a 25% threshold. Divisions above PR=0.27 cutoff were classified as ACD and below as SCD (greyed area, dashed line) i. An example of Cherry and GFP-LAT SEQtage classified as SCD at division ii. The percentages of SCD events for both Cherry and GFP-LAT summarised as a column graph in 27 divisions accumulated from 2 independent experiments. iii. Graph of paired PR values of Cherry and GFP-LAT DN3 divisions. For statistics, a paired Students T-test was performed. Cherry vs GFP-LAT: P=0.9, n.s. The difference between each Cherry control and GFP-LAT pairs were plotted on the scatterplot to the right, with the mean difference and 95% confidence interval shown. Data is from 27 divisions accumulated from 2 independent experiments.
Figure 5.5 Ectopic expression of polarity proteins Scribble and Dlg4 does not substantially alter thymocyte proliferation or differentiation

A) Fold proliferation graph of sorted DN3 thymocytes transduced with Cherry only (Control) and either GFP-Scribble (GFP-Scribble, left column) or GFP-Dlg4 (right column) after 5 and 7 days culture on OP9-DLL1 stromal cells respectively. B) At the same time point thymocytes were also analysed by flow cytometry to assess impacts on DN1-DN4 differentiation (CD44/CD25 staining) and C) DN–SP differentiation (CD4/CD8 staining). Representative plots shown, with statistics of replicates shown below each plot using an unpaired Students T-test performed between Control and GFP-Scribble (DN3, P< 0.02, DN4 P< 0.02). Data is representative of 3 and 2 independent experiments for GFP-Scrib and GFP-Dlg4 respectively.
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Figure 5.5

A) 

GFP-Scribble

GFP-Dlg4

Fold proliferation

Control GFP-Scribble

Control GFP-Dlg4

B) 

Cherry control

GFP-Scribble (+Cherry)

Cherry control

GFP-Dlg4 (+Cherry)

P<0.02

P<0.02

% Control GFP-Scribble

% Control GFP-Dlg4

C) 

CD4

CD8

% Control GFP-Scribble

% Control GFP-Dlg4

DN DP

DN DP

DN DP

DN DP
Figure 5.6 Scribble is distributed symmetrically, whereas Dlg4 is both symmetric and asymmetric upon DN3 thymocyte division

A) Scatterplots of Cherry control and GFP-Scribble in divided DN3 daughters. i. An example of a Cherry and GFP-Scribble SEQtage classified as SCD or ACD. Divisions above PR=0.27 were classified as ACD and below PR=0.27 as SCD (cutoff is shown by the dotted line). ii. The percentages of SCD and ACD events for Cherry and GFP-Scribble. iii. Graph of paired PR values of Cherry and GFP-Scribble DN3 divisions. Paired Students T-test, Cherry vs GFP-Scribble: P=0.09, n.s. From this graph the difference between each Cherry control and GFP-Scribble pair were plotted as the scatterplot, with the mean difference and 95% confidence interval shown. Data is from n=101 divisions from 6 independent experiments. B) Scatterplots of Cherry control and GFP-Dlg4 PR values in divided DN3 daughters. Divisions above PR=0.27 were classified as ACD and below PR=0.27 as SCD (cutoff is shown by the dotted line). i. An example of Cherry and GFP-Dlg4 SEQtage classified as SCD or ACD at division. ii. The percentages of SCD and ACD events for both Cherry and GFP-Dlg4 summarised as a column graph. iii. Graph of paired PR values of Cherry and GFP-Dlg4 DN3 divisions. Paired Students T-test, Cherry vs GFP-Dlg4: P<0.05. From this graph the difference between each Cherry control and GFP-Dlg4 pair were plotted as the scatterplot, with the mean difference and 95% confidence interval shown. n=33 divisions from 4 independent experiments.
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A) i

n=101

SCD
CherryGFP-Scribble

Absolute PR
cutoff (0.27)

ACD
Cherry
GFP-Scribble

PR= 0.01
PR= 0.11

PR= 0.04
PR= 0.33

ii

96.2% 3.8% 92.4% 7.6%

94% 6% 87.9% 12.1%

iii

Paired T-test

Mean = -0.02
95% CI: -0.04 to 0.00

B) i

n=33

SCD
CherryGFP-Dlg4

Absolute PR
cutoff (0.27)

ACD
Cherry
GFP-Dlg4

PR= 0.04
PR= 0.04

PR= 0.04
PR= 0.33

ii

96.2% 3.8% 92.4% 7.6%

94% 6% 87.9% 12.1%

iii

Paired T-test

Mean = -0.05
95% CI: -0.09 to 0.00

P<0.05

95% CI: -0.09 to 0.00

Figure 5.6

GFP-ScribbleCherry
control

Absolute PR

n.s

Difference

Mean = -0.02
95% CI: -0.04 to 0.00

Paired T-test
that GFP-Scribble may not be asymmetrically localised during division. Fewer divisions were identified for GFP-Dlg4, although DN3 thymocytes that were classified as ACD showed clearer asymmetric distribution of GFP-Dlg4 to one of the daughter cells (Figure 5.6 Bi). Indeed, the paired differences of the Cherry control PRs with the GFP-Dlg4 PRs were significant (Figure 5.6 Bi) but the paired differences of the Cherry control PRs with the GFP-Scribble PRs were not significant (Figure 5.6 Aii). Taken together, GFP-Scribble appeared symmetrically localised during DN3 division, and GFP-Dlg4 was predominantly symmetrical with some instances of asymmetry.

5.2.5 The endocytic molecule Ap2a2 is distributed both symmetrically and asymmetrically upon DN3 thymocyte division

The adaptor protein (AP) complexes regulate the formation of clathrin-coated vesicles, promoting the endocytosis of cell surface receptors for downstream signalling or degradation (Boehm and Bonifacino, 2001; Nakatsu and Ohno, 2003; Rappoport, 2008). There are four types of AP complexes that are involved in distinct vesicular transport pathways, but each complex consists of four subunits, two large chains (α, γ, δ, or ε and β), one medium chain (μ), and a small chain (σ) (Bonifacino and Weissman, 1998; Hirst and Robinson, 1998; Ohno, 2006). Cell surface receptors are brought into clathrin-coated pits by direct interaction with the adaptor complex AP-2, or through binding to other adaptor proteins which in turn interacts with the AP-2 (Boehm and Bonifacino, 2001). In asymmetrically dividing D. melanogaster sensory organ precursor cells, Numb physically binds and polarises with the α-adaptin subunit of AP-2. Numb preferentially polarises AP-2 to the pIIb daughter cell where it targets the endocytosis of Notch for degradation, hence generating a bias in signalling for specifying pIIb cell fate (Berdnik et al., 2002). Ap2a2, which is part of the α-adaptin subunit of AP-2, has been identified by members of our laboratory to induce haematopoietic stem cell self-renewal both in vitro and in vivo, and is segregated asymmetrically in approximately 50% of dividing haematopoietic stem cells using time lapse imaging (Ting et al., 2011). In Chapter 1, I also demonstrated that AP-2 (and indirectly Ap2a2) displayed a variety of localisations during interphase in DN3 thymocytes. Given the link between Numb and Ap2a2, and that Numb asymmetrically localised during DN3 division, I hypothesised that Ap2a2 could serve as a marker of SCD versus ACD.

5.2.5.1 Ectopic expression of Ap2a2 alters DN3 thymocyte proliferation differentiation in vitro

Given that Ap2a2 was initially identified in an overexpression screen to increase haematopoietic stem cell self-renewal (Ting et al., 2011), I first investigated whether ectopic expression of Cherry-Ap2a2 affected proliferation or differentiation of DN3 thymocytes during culture on OP9-DLL1 stromal cells. Sorted DN3 thymocytes expressing both GFP and Cherry-Ap2a2 were cultured on OP9-DLL1 stromal cells for 5 days then harvested for counting to assess proliferation as well as flow cytometric analysis of differentiation. Cell counts revealed that ectopic Ap2a2 expression slightly increased thymocyte proliferation (Figure 5.7 A), and it also increased the proportion of
Figure 5.7 Ectopic expression of the endocytic molecule Ap2a2 alters proliferation and differentiation of DN3 thymocytes

A) Fold proliferation graph of sorted DN3 thymocytes transduced with GFP only (Control) or Cherry-Ap2a2 + GFP (Cherry-Ap2a2) after 5 days culture on OP9-DLL1 stromal cells (Control vs Cherry-Ap2a2, P<0.05). At 5 days thymocytes were also analysed by flow cytometry to assess impacts on B) DN1-DN4 differentiation by CD44/CD25 staining (Control vs Cherry-Ap2a2 DN3, P<0.05) and C) DN–SP differentiation by CD4/CD8 staining (Control vs Cherry-Ap2a2, DN P<0.005, DP P<0.005). Representative plots shown. Statistics derived by using an unpaired Students T-test performed between Control and Cherry-Ap2a2. Data is representative of 4 independent experiments.
thymocytes that differentiated to the DP stage (Figure 5.7 B and C). These results indicated that ectopic expression of Ap2a2 may enhance thymocyte differentiation.

### 5.2.5.2 Ap2a2 is asymmetric in a proportion of DN3 thymocyte divisions

I next performed time lapse imaging and quantitative analysis of Cherry-Ap2a2 + GFP during DN3 division. Remarkably, of the 52 dividing DN3 thymocytes, 23% (12/52 divisions) asymmetrically localised Cherry-Ap2a2 during division compared to 3.8% (2/52) for the GFP-control (Figure 5.8 A and Aii). Not only were the paired differences in GFP control and Cherry-Ap2a2 PR values significant (Figure 5.8 Aiii, P<0.0005) but visual inspection of SEQtages of PR values in the scatterplots classified as SCD clearly showed symmetrical distribution of Cherry-Ap2a2 into each daughter cell (Figure 5.8 Ai, left panel), and clearly showed asymmetrical distribution of Ap2a2 in ACD DN3 divisions (Figure 5.8 Ai, right panel). Given the known interaction between Numb and Ap2a2 polarisation in D. melanogaster to regulate SOP fate decisions, these results are extremely interesting considering that Numb also distributes asymmetrically during DN3 division. Ap2a2 is both symmetrically and asymmetrically distributed during DN3 division. As Ap2a2 had the clearest asymmetry, I only considered Ap2a2 in all subsequent experiments.

A summary of all candidate proteins quantified during DN3 division and the percentages of ACD for each are summarised in Figure 5.9. For summaries of all heatmaps see Appendix C, and for graphs of descriptive differences from the T-tests see Appendix D.

### 5.2.6 Ap2a2 asymmetry is maintained over time during DN3 thymocyte division

To investigate whether the polarisation of Ap2a2 was only at the time of division or was sustained after division, I utilised another feature of TACTICS. TACTICS could project each SEQtage frame of the tracked parent and daughter cells (orientated along the major axis) along a single vertical line, and then line them side-by-side as a kymograph to visually assess the distribution of either GFP control or Cherry-Ap2a2 over time (Figure 5.10 A for schematic representation). Analysis of the heatmaps of DN3 thymocytes classified as ACD showed that the GFP-control protein was evenly distributed prior to and after DN3 division (Figure 5.10 Bi). In contrast to GFP, Cherry-Ap2a2 fluorescence in the same division displayed dynamic polarisation prior to division, with preferential segregation into one daughter cell up until the daughters could no longer be tracked (Figure 5.10 C, representative of at least 3 tracked divisions). Ap2a2 therefore could polarise prior to, during and after DN3 division.

### 5.2.7 Endogenous Ap2a2 is asymmetric during DN3 division

To control for any possible artefacts of Ap2a2 protein localisation due to fusion to Cherry or ectopic expression, I next assessed the distribution of endogenous Ap2a2 during early and late DN3 mitosis. Untransduced sorted DN3 thymocytes on OP9-DLL1 stromal cells were cultured overnight, then fixed and stained for endogenous Ap2a2, tubulin and DNA (Figure 5.11). Dividing DN3 thymocytes were identified on the basis of nuclear shape and the presence of a mitotic spindle, and then the distribution of Ap2a2 was blindly scored as asymmetric or symmetric. Of
Figure 5.8 Ap2a2 is distributed both symmetrically and asymmetrically during DN3 division

A) Scatterplots of GFP control and Cherry-Ap2a2 PR values in divided DN3 daughters after a 25% threshold. Divisions above PR=0.27 cutoff were classified as ACD and below as SCD (greyed area, dashed line) i. An example of GFP and Cherry-Ap2a2 SEQtage classified as SCD or ACD at division. ii. The percentages of SCD and ACD events for both GFP and Cherry-Ap2a2 summarised as a column graph. iii. Graph of paired PR values of GFP + Cherry-Ap2a2 DN3 divisions. For statistics, a paired Students T-test was peformed. GFP vs Cherry-Ap2a2: P<0.0005. The difference between each GFP control and Cherry-Ap2a2 pair were plotted on the scatterplot to the right, with the mean difference and 95% confidence interval shown. Data is from 52 divisions accumulated from 9 independent experiments.
Figure 5.9. Summary of quantified ratiometric protein distribution during DN3 thymocyte division

A) Scatterplots of PRs for all test proteins (blue dots) and their control proteins (red dots, shown to the left of each test protein) quantified during DN3 thymocyte division. Using a cutoff of PR = 0.27, B) the percentage of ACD divisions were plotted for each test protein and corresponding control.
Figure 5.9 Summary of quantified ratiometric protein distribution during DN3 thymocyte division (cont.)

C) Graphs of paired PR values for all test proteins (blue dots) and their control proteins (red dots, shown to the left of each test protein). For statistics a paired Students T-test was applied between paired control PR and test protein PR values (Control vs Numb P<0.0001, Control vs Numb2A P=0.5, Control vs LAT P=0.9, Control vs Scribble P=0.09, Control vs Dlg4 P<0.05, Control vs Ap2a2 P<0.0005). Refer to Appendix 3 for a summary of the PR differences.
Figure 5.10 Ap2a2 asymmetry is maintained in DN3 daughters post division

A) Schema to construct 2-dimensional (2D) kymographs of DN3 thymocytes prior and during division. The image of each time point (GFP or Cherry-Ap2a2) was projected and averaged along the single vertical line, which could be lined side-by-side to create a kymograph. B) Kymographs of GFP and Cherry-Ap2a2 in a dividing DN3 thymocyte. Dotted line indicates the point of division.
Figure 5.11. Endogenous Ap2a2 is both symmetric and asymmetric upon DN3 division

Sorted in vitro derived DN3 thymocytes were cultured on OP9-DLL1 stromal cell overnight, then fixed and stained with DAPI to mark the nucleus (blue), tubulin (red) and Ap2a2 (green). Dividing DN3 thymocytes were identified on the basis of nuclear shape and the presence of a mitotic spindle and then scored for Ap2a2 polarisation as symmetric or asymmetric. Representative examples of maximum projected confocal images for A) symmetric or B) asymmetric Ap2a2 polarisation during early and late mitosis. C) Summary of total SCD and ACD Ap2a2 events scored. n= 102 from 4 independent experiments. Scale bar, 5μm.
102 DN3 divisions scored, 17.6% (18/102) asymmetrically localised Ap2a2 score during late or early mitosis, while in the rest of the divisions endogenous Ap2a2 were symmetric (Figure 5.11 B and A respectively, and C). This value was slightly lower to 23% Cherry-Ap2a2 asymmetry quantified by TACTICS during DN3 division. Although there may be subtle effects of ectopic Ap2a2 expression, Ap2a2 appears to be a useful marker for asymmetrically dividing DN3 thymocytes.

5.3 Discussion

In this chapter I have demonstrated that DN3 thymocytes undergo both SCD and ACD using quantified time lapse imaging of a selected number of candidate proteins. The TCR associated protein LAT is not asymmetrically localised during DN3 division and neither is the polarity protein Scribble. The polarity protein Dlg4 might be asymmetric in a low proportion of DN3 divisions. Excitingly, the cell fate protein Numb undergoes asymmetry in a number of DN3 divisions, and this proportion of asymmetry is reduced if Numb is mutated at putative aPKC phosphorylation sites. Most strikingly, the subunit of the clathrin endocytic protein AP-2, Ap2a2, is most frequently asymmetrically distributed during DN3 division of all proteins investigated.

5.3.1 Is there a quantifiable definition of asymmetr

In this chapter I demonstrate that TACTICS provides robust quantification of fluorescent protein distribution in dividing thymocytes, creating montaged images and kymographs for visual quality control, extracting PR values in a high throughput manner, and enabling downstream quantification processes for classification of SCD and ACD. This multitude of data highlights an important question- is there a truly quantifiable definition of ACD? If so, is the binarisation of ACD and SCD appropriate, or is the P value comparing extent of asymmetry between test and control proteins a more accurate indicator? Clearly, a correlation of ACD and SCD with functionally distinct fates will ultimately be required to resolve this issue. In the meantime, we need to establish a best-practise approach to generate a framework with which to assess functional correlates of the asymmetry that I have identified in this chapter.

5.3.1.1 Background subtraction methods can alter apparent protein distributions in dividing cells

During ACD of C. elegans blastocysts, D. melanogaster neuroblasts and sensory organ precursors, the asymmetry of cell fate and polarity proteins are often so obvious that it is not necessary to quantify protein distributions to ascribe SCD versus ACD. Perhaps, due to the small size, high motility or more complex regulation of immune cells, the localisation of proteins in dividing lymphocytes is less obvious, requiring a more quantified approach to delineate ratio differences in protein distributions. This raises two concerns about defining ACD on the basis of protein distributions. One concern is that image processing such as thresholding to subtract background fluorescence alters the PR values. In this chapter, I show that increasing threshold levels also
artificially increased the PR values (Figure 5.1 D, and Appendix C for all PR heatmaps). Adding to this concern is that the extent of background subtraction performed is often undisclosed in studies that have identified lymphocytes as undergoing ACD. For example, dividing T cells have been classified as ACD if one daughter inherits 1.5 times more staining intensity compared to its sister cell (Chang et al., 2011), but no mention of image processing procedures (if any) are disclosed that could alter the classification of ACD. A second concern is that SCD or ACD protein distributions should be appropriately distinguished by comparisons with a baseline control at exactly the same time and space in the dividing cell. The analysis that I have performed of protein distribution during DN3 division addresses both these concerns where the test protein (Numb, LAT, Scribble, Dlg4, Ap2a2) in one fluorescent channel is compared directly against a ubiquitously expressed protein (Cherry or GFP) that is always symmetric upon division (baseline). Using the control protein as a baseline, it is possible to identify the most appropriate level of background subtraction that does not drastically alter the PR values, and apply this level of subtraction to the test protein (see Appendix C for all PR heatmaps). The scatterplots of PR values that follow then provide a platform for one to decide how to differentiate between SCD or ACD. In this chapter I used a PR of 0.27 to differentiate between SCD and ACD. This was based on the highest PR value for the Cherry control protein in my first study quantifying Cherry and GFP-Numb distribution during DN3 division. Although based on the control protein, I note that ascribing a line at PR=0.27 to differentiate between SCD and ACD is subjective. For example, in Figure 5.9A where I show the summary of all PR scatterplots for each protein, it becomes apparent that the spread of PR values differs slightly for each protein. However, only one universal cutoff used. Binarisation of the scatterplots using a cutoff is necessary to separate ACD from SCD but depending on the value chosen, will almost certainly produce false positive or negative ACD events. To some extent this is answered by the paired T-test graphs to support whether the difference in paired control PR and test protein PR values are significant. Obviously, degrees of asymmetry will eventually need to be taken into consideration. A fully objective definition of what is ACD is needed, and perhaps another statistical approach that measures the probability of obtaining a particular PR value for either control and test protein could be formulated. Such a statistical approach would need to consider that each protein has its own biological constraints, localisations, functions and regulatory inputs. More importantly, once ACD has been defined, that asymmetry needs to correlate with different cell fates.

5.3.1.2 The expression patterns of fluorescently-tagged proteins alter the probability of its distribution during DN3 division

In addition to background subtraction methods altering PR values, the pattern of fluorescent protein intensity in space within a dividing thymocyte can also alter the probability of its inheritance into two daughter cells. The largely symmetric divisions quantified for LAT, Scribble, Dlg4, as well as Numb2A during division could be due to occupying either disperse localisations throughout the cytoplasm or membrane within a cell (bright or dim). This pattern of localisation increases the probability of symmetric inheritance during division. By contrast, protein distributions that do not occupy a lot of space within a cell have different probability characteristics, and the
likelihood of its asymmetry therefore will increase during division. Given that Ap2a2 and Numb are both endocytic molecules, vesicular in localisation and occupying less space within a cell, these conditions increase the probability of their asymmetric inheritance during division. The TACTICS platform has the potential to provide several insightful analysis capabilities for this purpose, however, not all of these approaches could be fully developed and tested within the time frame of my thesis studies. This includes comparing PR values of dividing thymocytes rotated along the minor instead of major axis to confirm asymmetry. Such an approach is similar to Chapter 4, where I confirmed Numb polarisation by rotating images of T lymphocytes along both minor and major axis. It will also be important to compare the polarisation ratios at division, or averaged over multiple frames. There is still a long way to go, however, my work has certainly both improved the quantitation of ACD and provided exciting evidence of which proteins can be asymmetric in dividing DN3 thymocytes.

5.3.2 DN3 thymocytes undergo both symmetric and asymmetric cell division

Given not all the proteins in this study localise during division, and for those that do localise, only do so in a proportion of divisions, how could SCD and ACD impact on DN3 fate decisions?

5.3.2.1 The TCR associated molecule LAT is important for DN3 function but does not asymmetrically localise during DN3 division

Time lapse imaging of GFP-LAT in mature activated T cells within lymph nodes show that LAT polarises to the uropod, as well as the site of contact with B cells (Azar et al., 2010). This fits in with known functions of LAT as a co-activator protein of TCR signalling at least during immunological synapse formation (Horejsi et al., 2004; Samelson, 2002). Given the similarities in TCR signalling between T cells and thymocytes, LAT polarisation may also be required to sustain TCR signalling throughout division. However, the lack of LAT polarisation during DN3 division suggests that LAT polarisation is not required because up until this point in differentiation there is no TCR signalling during DN3 TCR rearrangement. In this case, LAT could serve as a symmetric protein control for future studies to look for ACD proteins. LAT function is certainly not unimportant, as its ectopic expression promotes proliferation and differentiation of DN3 thymocytes during in vitro culture (Figure 5.3) in contrast to the blocks at DN3 in Lat-/- knockout mice (Finco et al., 1998; Zhang et al., 1999). However, LAT function most likely acts on downstream TCR signalling pathways and not by asymmetry during division to regulate DN3 fates.

5.3.2.2 A potential role for polarity proteins during thymocyte fate determination?

The polarity proteins Scribble and Dlg4 are largely symmetric during DN3 division. This data is interesting as it contrasts with previous data from our laboratory demonstrating that asymmetrically dividing T cells subtlety polarise Scribble and Dlg family members to the proximal daughter cell (Oliaro et al., 2010). On the one hand, I have used a rather conservative cutoff to define ACD, and more positive ACD events would have been observed had I used a more lenient cutoff, particularly
for Dlg4. On the other hand, this data fits in with previous observations that Scribble and Dlg4 polarise in dividing *D. melanogaster* neuroblasts at early mitosis but disperse throughout the cortical membrane during late mitosis (Albertson and Doe, 2003). In neuroblasts, Scribble and Dlg are therefore only required to set up but not maintain the axis of division. Thymocytes may have conserved these features of Scribble and Dlg polarity throughout evolution, or, Scribble and Dlg may have other functions during DN3 fate determination that is different to T cells. For example, DN3 thymocytes show some dependency on Scribble when it is depleted by shRNA *in vitro*, showing defects in cell clustering and differentiation (Pike et al., 2011). Also, there are currently four identified mammalian Dlg family members, of which only Dlg4 was quantified during DN3 division. To dissect the true requirement of polarity during thymocyte development, future work would need knockouts for each of the polarity proteins, alone and in combination.

Fortunately in the laboratory we currently have mouse knockouts of three polarity proteins, Scribble, Dlg1 and Lgl. Overall, peripheral T cell development appears normal in *scribble*\(^{-/-}\) *dlg1*\(^{-/-}\) and *lgl*\(^{-/-}\) mice on the C57/Bl6 strain (manuscript submitted). Preliminary experiments show that *in vitro* thymopoiesis on OP9-DLL1 stromal cells is normal when using *lgl*\(^{-/-}\) bone marrows as a stem cell source, however, *scribble*\(^{-/-}\) bone marrow cells do not initiate *in vitro* thymopoiesis as efficiently as WT bone marrow cells, with defects in proliferation, differentiation and migration (data not shown). Other preliminary analyses of thymii from mice deficient in *lgl* (OT-1 background) have shown an imbalance in the DN3-DN4 compartment (data not shown). This developmental block at DN3 in OT-1 *lgl*\(^{-/-}\) mice is compatible with an increased propensity to develop leukaemia (such as in the Lmo2 model), and this agrees with the role of Lgl as tumour suppressors. Experiments performed by other members in the laboratory show that *lgl*\(^{-/-}\) (OT-1 background) mice have enhanced mature CD8 T cell effector function (manuscript submitted). These preliminary studies are exciting observations which indicate efficient thymopoiesis requires polarity proteins. Closer dissection to differentiate between polarity defects in stem cell populations that contribute to the T cell pathway, or intrinsic defects in polarity during thymopoiesis will need to be investigated in future experiments.

5.3.3 DN3 thymocytes undergo ACD - conserved and divergent mechanisms of polarity

For the first time, my investigation identifies that Numb as well as Ap2a2 polarise during mitosis, demonstrating that DN3 thymocytes indeed undergo ACD. One hypothesis is that Numb and Ap2a2 asymmetry during DN3 division impact on fate decisions.

5.3.3.1 The known mechanisms of Numb polarity during migration and division share complementary and contradictory phenotypes in DN3 thymocytes

Much of what is known about how polarity regulates fate determination processes comes from studying epithelial cells. In asymmetrically dividing *D. melanogaster* neuronal precursors Numb is recruited to the plasma membrane via positively charged amino acids in its amino terminus. Mutation of two phosphorylation sites by aPKC abrogates the polarisation of Numb, suggesting aPKC-mediated phosphorylation neutralises the charges on the amino terminus of Numb, thereby
preventing its membrane association (Smith et al., 2007; Wirtz-Peitz et al., 2008). In this chapter I also demonstrated that Numb was associated with the plasma membrane during DN3 thymocyte division, and that mutation of the aPKC sites in Numb reduced its asymmetry. Studies from our laboratory have also shown that disruption of aPKC function results in loss of Numb asymmetry during T cell division and function (Oliaro et al., 2010). These results indicate that that aPKC regulation of Numb polarity during DN3 ACD is conserved throughout evolution.

However, there are also some clear differences in the types of polarity adopted by thymocytes from epithelial cells, and the cues that can initiate that polarity. Thymocytes are exposed to different chemokines and extracellular matrix, and their polarisation depends on the stage of development and interaction with the thymic stromal cells. While aPKC regulation of Numb polarity is conserved during DN3 ACD through Ser7 and Ser295 phosphorylation, I also demonstrate that it is not conserved during DN3 migration (Chapter 4). Other mechanisms of polarity are therefore likely to be imposed during migration. To identify and characterise these divergent mechanisms, TACTICS could be used to quantify Numb and Numb2A polarisation with either tubulin and aPKC polarisation during thymocyte division and migration. It would also be important to measure the changes in polarisation ratios for each protein change as a function of time (division or migration), orientation (division or migration) and speed (migration).

Some other interesting and conflicting observations have been made of Numb function during fate determination. During D. melanogaster sensory organ precursor ACD, Numb segregates unequally into the anterior daughter cell via Ap2a2 binding, promoting the endocytosis of Notch for degradation and eventual specification of pIIb fate (Berdnik et al., 2002; Guo et al., 1996; Rhyu et al., 1994; Santolini et al., 2000). Deletion of Numb in D. melanogaster result in defects in sensory organ precursor ACD in the peripheral nervous system that is associated with hyperactivation of Notch receptor signalling (Berdnik et al., 2002). However in mice, haematopoiesis and lymphopoiesis appears completely normal in double knockouts of numb and numb-like (Wilson et al., 2007). Interestingly, numb/numb-like knockout mice do display mild impediments in differentiation at DN3, and in those studies, the mx-Cre deletion may not have been complete. Indeed, as little as 5-10% Numb expression is enough to enable normal ACD in mammalian neuronal precursors (Petersen et al., 2006; Petersen et al., 2002; Petersen et al., 2004).

In a study by Aguado and colleagues the transgenic expression of wildtype or dominant negative forms of Numb result in altered DN3 pre-TCR signalling, proliferation and differentiation (Aguado et al., 2010). Asymmetry of Numb was also proposed by this group as a mechanism for these signalling and fate differences. However, there was a lack of quality of Numb staining (very dim), a small number of divisions analysed (10 telophases in total), the method of quantifying ACD was not normalised for focal plane (there was no control fluorophore), and there was no disclosure of image processing during quantification (which would affect the ACD values). As mentioned previously, in asymmetrically dividing T cells the alteration of Numb polarisation by disrupting aPKC signalling results in conversion of T cell fate and function (Oliaro et al., 2010). Collectively, this suggests that Numb instigates fate decisions not only by regulating Notch but also TCR, and is regulated by polarity proteins. These results indicate that proper Numb polarisation may be
important for inducing signalling differences that impact on DN3 fate decisions during ACD. Cleaner knockouts of Numb/Numb-like will be required to dissect its function during thymocyte fate determination, and tracking of fate of daughters that have inherited Numb asymmetrically over time will be required to determine the role of Numb asymmetry at division.

5.3.3.2 Is Ap2a2 a carrier molecule during ACD?

Ap2a2 is part of the ear domain of α-adaptin, one of the four subunits of the AP-2 complex that recruit clathrin for the formation of pits to endocytose a wide variety of cell surface molecules (Mayor and Pagano, 2007). Mutations that specifically delete or inactivate Ap2a2 function in *D. melanogastor* result in defects in the peripheral nervous system identical to null mutations of Numb (Berdnik et al., 2002). These studies highlight that the major contributor to this coordinated process of cell fate determination is the cellular endocytic machinery that asymmetrically localises Numb protein during mitosis.

Currently, very little is known about Ap2a2 (and more broadly endocytosis) during ACD in haematopoietic cells, and its role in thymocytes has not been explored. However, recently Ap2a2 has been identified to distribute asymmetrically in haematopoietic stem cells, and its ectopic expression results in increased stem cell maintenance and proliferation *in vitro* and *in vivo* respectively (Ting et al., 2011). While these two observations of asymmetry and fate appear to go hand-in-hand, it is not proven that Ap2a2 asymmetry correlates with differences in self-renewal or proliferative fates in haematopoietic stem cells. In my hands, ectopic expression Ap2a2 expression appears to promote *in vitro* proliferation and differentiation of DN3 thymocytes, but I still have not linked Ap2a2 asymmetry with fate, even though it is sustained in DN3 daughters. Based on current literature I have discussed for Numb so far, it is tempting to suggest Ap2a2 polarises Numb by endocytosis during division of DN3 thymocyte to create downstream signalling differences that impact on fate decisions. Future experiments will require monitoring the daughters of divided DN3 thymocytes over two or three generations in order to correlate asymmetry with fate.

5.3.4 Summary

Although it is well known that invertebrates system use ACD to generate cellular diversity, only in the past 5 years has it emerged that cells of the lymphoid system such as mature T cells also undergo ACD, polarising a group of functionally conserved proteins during division to generate fate differences in an immune response. In this chapter I investigated whether ACD also occurs at the precursor stage of T cell development in DN3 thymocytes. I use a computational platform to quantify protein distributions of well-known regulators of ACD in invertebrates, and demonstrate that DN3 thymocytes undergo ACD. The cell fate protein Numb and endocytic protein Ap2a2 are both symmetric and asymmetric upon DN3 division, whereas the TCR associated molecule LAT and polarity proteins Scribble and Dlg are largely symmetric upon DN3 division. Mutation of aPKC phosphorylation sites on Numb disrupts its asymmetry during DN3 division, suggesting
that unlike in migrating T lymphocytes (Chapter 4), Numb polarisation might be regulated by conserved mechanisms. Ap2a2 is not only asymmetric during division, but its asymmetry is sustained after division, suggesting that it could be a marker for asymmetrically dividing thymocytes. Ap2a2 may also be a biologically relevant molecule of ACD that is involved during DN3 fate specification, as its ectopic expression alters DN3 differentiation and proliferation \textit{in vitro}. As Ap2a2 had the highest percentage of asymmetry during DN3 division, the possible roles of Ap2a2 during ACD will be addressed in the next chapter.
Chapter 6

Using Ap2a2 to explore the role of ACD in thymocyte fate determination
6.1 Introduction

In the previous chapter I have shown that DN3 thymocytes undergo ACD, demonstrating that the α-adaptin subunit Ap2a2 asymmetrically polarises in a proportion of dividing DN3 thymocytes. Moreover, the ectopic expression of Ap2a2 can promote DN3 differentiation into DP thymocytes. In light of this data several key questions remain. Firstly, what is the mechanism that cues Ap2a2 polarisation? Secondly, can Ap2a2 polarisation during division be a surrogate readout of ACD in situations of aberrant DN3 fates such as leukaemia? And thirdly, does ACD impinge on DN3 thymocyte fate decisions?

In this final chapter of results, I investigate whether chemokine receptor signalling could provide a polarity cue for Ap2a2 polarisation. Chemokine signalling can induce polarity in many cell types including T cells, B cells, neutrophils, thymocytes (Bunting et al., 2011; Cane et al., 2012; Dzhagalov and Phee, 2012; Harkin and Bignold, 1994; Sanchez-Madrid and del Pozo, 1999). Studies by the laboratories of Ravichandran and Turner indicate that the CXCR4 chemokine ligand produced by stromal cells, CXCL12, is essential for the β-selection checkpoint in DN3 thymocytes (Janas et al., 2009; Trampont et al., 2010). The chemokine receptor CXCR4 is also internalised via AP-2 mediated trafficking for its downstream signalling (Schwartz et al., 2012; Sorkin and von Zastrow, 2009; Venkatesan et al., 2003). The interaction of CXCL12 on the stromal cells with its receptor CXCR4 on DN3 thymocytes could therefore cue Ap2a2 polarisation during ACD of DN3 thymocytes.

Next, I use the findings of Ap2a2 asymmetry to discern whether ACD might be involved in the murine CD2-Lmo2 model of T cell Acute Lymphoblastic Leukaemia (T-ALL). The LIM-only domain protein Lmo2 (previously called RBTN or TTG), is a master regulator of haematopoiesis. In Lmo2 null mice, death of embryos occurs early in utero due to failure of yolk sac erythropoiesis as well as angiogenesis, and adult chimeras containing Lmo2 null ES cells fail to contribute to any haematopoietic lineage (Warren et al., 1994; Yamada et al., 1998). In cases of acute human paediatric T cell leukaemias, approximately 50% of these neoplasms comprise reciprocal chromosomal translocations of Lmo2 (Boehm et al., 1988a; Look, 1997; McGuire et al., 1989; Nam and Rabbitts, 2006). These translocations with Lmo2 commonly occur with promoter or enhancer components of the TCR machinery, causing a breakdown in TCR rearrangement or aberrant RAG recombinase activity in DN thymocytes (Boehm et al., 1988b; Finger et al., 1986; Garcia et al., 1991). Lmo2 is therefore important during fate determination during haematopoiesis but when incorrectly switched on, contributes to T cell leukaemia. In addition to paediatric T-ALL, four cases of X-linked SCID showed that Lmo2 was activated by retroviral insertion, with the leukaemia occurring almost 3 years after gene therapy (Hacein-Bey-Abina et al., 2003; Nam and Rabbitts, 2006; Rabbitts, 1998). The long latency observed in these cases suggested that the cell of origin must have possessed the property of self-renewal. Recent studies now show that the constitutive expression of Lmo2 does induce continual self-renewal of DN3 thymocytes, suggesting that their altered fate provides the preleukaemic pool that contributes to overt leukaemia (McCormack et al., 2010). This enhancement of self-renewal could be due to alterations in ACD that control DN3 fate.
In this chapter I demonstrate that disruption of chemokine signalling with an inhibitor to the chemokine receptor CXCR4 reduces Ap2a2 polarisation in DN3 thymocytes prior to division, providing a mechanism through which Ap2a2 polarity may be controlled. By using Ap2a2 as a marker and quantifying Ap2a2 polarisation during division, I demonstrate that CD2-Lmo2 DN3 thymocytes undergo an increase number of SCDs at the expense of ACD. In CD2-Lmo2 DN3 thymocytes the mechanism for Ap2a2 polarisation via CXCR4 signalling is also conserved. Remarkably, the ectopic expression of Ap2a2 promotes a differentiation transition that is usually impeded in the DN3 thymocytes of CD2-Lmo2 mice. These data combined imply that Ap2a2 is both a marker and a functionally relevant molecule during DN3 ACD. More importantly, correlations between polarity and differentiation provide exciting preliminary data to suggest that ACD impacts upon fate decisions of DN3 thymocytes.

6.2 Results

6.2.1 Stromal cells and chemokine signalling provide a cue for ACD

Let us briefly revisit the three requirements for ACD (Figure 6.1). The requirements are; first, that an anchor is present to provide a cue for polarity in the dividing thymocyte; second, that division occurs along that axis of polarity where the mitotic spindle aligns perpendicular to the anchor; and third, that polarity is maintained through division. The third requirement has been fulfilled and is provided by my observations of Ap2a2 asymmetry. The fact that Ap2a2 asymmetry results from coordination of the spindle angle satisfies the second requirement. However, the polarity cue has not yet been defined. One way to understand ACD of DN3 thymocytes is to use Ap2a2 as a marker of polarity to discern the polarity cue.

6.2.1.1 Asymmetrically dividing DN3 thymocytes preferentially divide perpendicular to the stromal cell

In naive T cells the polarity during ACD is dictated by contact with the antigen presenting cell, and by a perpendicular division to the antigen presenting cell that defines both proximal and distal daughter T cells (Oliaro et al., 2010). From these results, it is possible that thymocyte contact with OP9-DLL1 stromal cells could provide a similar anchor. To address whether thymocytes also divide while attached to, and perpendicularly to the stromal cell, in vitro derived DN3 thymocytes were transduced with Cherry-tubulin and cultured with OP9-DLL1 stromal cells for visual assessment of stromal contact and orientation of the mitotic spindle during division (marked by Cherry-tubulin). Interestingly, 79.5% (62/78 divisions) of DN3 divisions divided while attached to the stromal cells, suggesting that contact was necessary for division (Figure 6.2 A). However, the DN3 thymocytes appeared to divide in a wide variety of orientations, with a fairly even proportion (25.5-32.1%) of horizontal and perpendicular DN3 thymocytes, as defined by orientation of the mitotic spindle. Thus, DN3 thymocytes need a stromal cell anchor for division, but do not always align perpendicularly to the stromal cell during division.
Figure 6.1 The three requirements for ACD

For control of progeny proliferation, death and differentiation during ACD three requirements must be fulfilled. 1) An anchor to dictate the axis of polarity, in this case a stromal cell; 2) the dividing cell is aligned along the axis of division, usually perpendicular to the anchor (perpendicular orientation shown by the alignment of mitotic spindle, red) and 3) that polarity of the protein (green) is maintained throughout division.
Figure 6.2 DN3 thymocytes divide in a variety of orientations, but asymmetrically dividing DN3 thymocytes favour perpendicular divisions.

A) Sorted DN3 thymocytes transduced with Cherry-Tubulin (red) and seeded onto OP9-DLL1 stromal cells (GFP, green) at low density for time lapse microscopy. Divisions were identified where the orientation of division (horizontal, perpendicular, burrowed or alone) in relation to the stromal cell was assessed. n=78 divisions. B) Column graph grouping the percentage of asymmetrical (ACD) or symmetrical (SCD) Ap2a2 divisions (of sorted DN3 thymocytes expression GFP+Cherry-Ap2a2) by orientation (horizontal, perpendicular, or burrowed). The percentage of ACDs are indicated. n= 62 divisions.
I next re-analysed timelapse movies of DN3 thymocytes transduced with GFP + Cherry-Ap2a2, this time focussing on characterising the orientation of thymocytes undergoing Ap2a2 SCD versus ACD. As these thymocytes expressed Cherry and not Cherry-tubulin, the orientation of the dividing thymocyte was determined by viewing the Differential Interference Contrast (DIC) time lapse images during division. Remarkably, 53.6% of thymocytes undergoing ACD divided perpendicularly to the stromal cell, whereas only 17.9% of thymocytes undergoing SCD divided perpendicularly to the stromal cell (Figure 6.2 B). Although DN3 thymocytes divide in a variety of orientations, asymmetrically dividing DN3 thymocytes favour a perpendicular orientation whereas symmetrically dividing thymocytes favour horizontal orientation. These data suggest that interaction with the stromal cell might influence the coordination of polarity with spindle orientation in dividing DN3 thymocytes.

6.2.1.2 Notch1, pre-TCR and CXCR4 receptors localise in DN3 thymocytes during interphase and serve as possible polarity cues

The triggers that promote survival and proliferation of DN3a thymocytes prior to β-selection are Notch1, pre-TCR and CXCR4 signalling. Therefore, all three are possible cues for Ap2a2 polarisation at the DN3 stage (Figure 6.3). For Notch1, the receptor-ligand interaction between Notch1 and DLL1/DLL4 could polarise Ap2a2 to mediate its internalisation and signalling (Figure 6.3 A). Another possibility is pre-TCR that is expressed on the surface of DN3 thymocytes after successful TCR-β rearrangement (Figure 6.3 B). It is known that thymocytes share components of the TCR machinery whose polarisation is necessary for downstream signalling in T cells, and so pre-TCR may polarise also Ap2a2 (Balagopalan et al., 2009). Chemokine signalling through CXCR4 receptor may also provide a polarity cue as its signalling is essential for the β-selection checkpoint in DN3 thymocytes (Janas et al., 2009; Trampont et al., 2010). The interaction of CXCL12 on the stromal cells with its chemokine receptor CXCR4 on DN3 thymocytes could therefore also regulate Ap2a2 polarisation (Figure 6.3 C).

I hypothesised that the polarity cue would need to localise at the thymocyte-stromal interface to orchestrate Ap2a2 polarisation, at least prior to division. I next investigated the localisation for each potential polarity cue (Notch1, pre-TCR, CXCR4) in DN3 thymocytes during interphase. In vitro derived sorted DN3 thymocytes cultured on OP9-DLL1 stromal cells were fixed and stained for tubulin with either Notch1, pTα (part of the pre-TCR complex) or CXCR4 (Figure 6.4). Selecting on DN3 thymocytes that polarised their tubulin during interaction with stromal cells (shown by the presence of MTOC at the stromal-thymocyte interface), I next scored the localisation of each potential cue as either with MTOC, cytoplasmic, membrane or distal to the MTOC. Similar to my previous data in Chapter 3 (Figure 3.8), Notch1 was again highly polarised with MTOC at the thymocyte-stromal interface (Figure 6.4 A), whereas pTα and CXCR4 displayed a variety of localisations (Figure 6.4 B and C). Overall, the range of localisations indicated that Notch1, pre-TCR and CXCR4 could all be potential polarity cues.
Figure 6.3 Schema of possible cues controlling DN3 polarity

There are three possible molecular cues that sets up the axis of polarity prior to DN3 ACD. A) Notch1: receptor-ligand interaction between Notch1 on the DN3 thymocytes and DLL1 on the stromal cells, B) Pre-TCR, which acts downstream of the β-selection checkpoint in thymocytes, or C) Chemokines: CXCL12/ SDF-1α from stromal can engage the CXCR4 receptor that could set up an axis of polarity the DN3 thymocytes. For each case the requirement would be that 1) an anchor dictates the axis of polarity, in this case a stromal cell which provides the polarity cue 2) that the mitotic spindle is aligned along the axis of division (perpendicular to the anchor) and 3) that polarity is maintained throughout division.
Figure 6.4 Notch1, pre-TCR and CXCR4 are polarised in DN3 thymocytes during interphase

Sorted *in vitro* derived DN3 thymocytes were cultured on OP9-DLL1 stromal cell overnight, then fixed and stained with DAPI (blue), tubulin (red) and either A) Notch1 (n=62), B) pTα (n=31) or C) CXCR4 (n=84) (shown in green). DN3 thymocytes were selected upon the basis of a polarised microtubule organising centre (MTOC) at the thymocyte-stromal interface, where a localisation descriptor was assigned (with MTOC, cytoplasmic, membrane or distal). Representative maximum Z-projected confocal images shown of each molecular cue polarised with MTOC at the thymocyte-stromal interface. Scale bar, 5μm.
To identify the most likely polarity cue, one needs to remove or inhibit the cue and investigate the impact on Ap2a2 polarisation. To investigate Notch1 as a polarity cue, sorted DN3 thymocytes were cultured on OP9 stromal cells that did not express the Notch ligand DLL1. Rapid death and down regulation of CD25 within 24 hrs was observed (data not shown). Therefore, while Notch1 had the highest polarisation between the thymocyte-stromal interface, it would be experimentally difficult to dissect alterations in Ap2a2 polarity in the absence of Notch signalling. It would be equally difficult to address the impact of Ap2a2 polarisation in the absence of pre-TCR signalling, as RAG-/- mice (which have no TCR rearrangement) are blocked at the DN3a stage (Yokota et al., 2006). However, it is known that CXCR4 signalling induces polarisation in thymocytes, and, in the absence of signalling impedes but does not completely block DN3 differentiation (Bunting et al., 2011; Janas et al., 2009; Trampont et al., 2010). The chemokine CXCR4 was therefore pursued as a potential polarity cue for Ap2a2 polarisation.

6.2.1.3 In vitro thymopoiesis from DN3 to DP depends on intact CXCR4 signalling

In cxcr4-/- deficient thymocytes there is a defect in differentiation from DN3 to DN4, associated with increased cell death and reduced proliferation due to a reduction in PI3K signalling and reduction in expression of pro-survival proteins (Janas et al., 2009; Trampont et al., 2010). The defect in differentiation from DN3 to DN4 is also mimicked in vitro when DN3 thymocytes from WT mice are cultured on OP9-DLL1 stromal cells in the presence of a CXCR4 specific inhibitor, AMD3100 (Janas et al., 2009). To investigate CXCR4 as a polarity cue for Ap2a2 polarisation, I first investigated whether I could recapitulate the defects in differentiation due to disrupted CXCR4 signalling using in vitro rather than in vivo derived DN3 thymocytes. Indeed, in vitro derived DN3 thymocytes cultured in the presence of AMD3100 (2 μg/ml) displayed reduced proliferation (Figure 6.5 A), increased cell death (Figure 6.5 B) and abrogated differentiation from the DN3 to DP stage at the time points analysed (Figure 6.5 C). In line with published data, in vitro derived DN3 thymocytes rely on intact CXCR4 signalling, but in contrast to the absence of Notch1 signalling, do not result in rapid death or downregulation of DN3 surface molecules. CXCR4 is therefore amenable for investigation as a potential polarity cue for Ap2a2 polarisation.

6.2.1.4 Intact CXCR4 signalling cues Ap2a2 polarisation in WT DN3 thymocytes during interphase

To investigate the role of CXCR4 as a polarity cue, sorted in vitro derived WT DN3 thymocytes were cultured overnight on OP9-DLL1 stromal cells in the presence or absence of AMD3100, then fixed and stained for endogenous tubulin and Ap2a2. Notably, there was no difference in the polarisation of MTOC between WT DN3 thymocytes to the thymocyte-stromal interface in the presence or absence of CXCR4 signalling (Figure 6.6 A). However, the proportion of Ap2a2 polarisation with MTOC at the thymocyte-stromal interface in interphase significantly decreased in the absence of CXCR4 signalling (Figure 6.6 B, P<0.005). This data indicates that intact CXCR4 signalling cues Ap2a2 polarity prior to division, suggesting the possibility that it is at least part of the polarity cue for ACD.
Figure 6.5 *In vitro* thymopoiesis requires intact CXCR4 signalling during culture on OP9-DLL1 stromal cells

C57Bl/6 WT E14.5 foetal liver cells were cultured on OP9-DLL1 stromal cells with cytokines and in the presence or absence of 2 μg/ml AMD3100. Thymocytes were then harvested A) 5 and 8 days later and assessed for proliferation (cell counts); B) Percentage death (after 8 days), as well as C) DN1-DN4 (CD44/CD25 staining) and DN-SP (CD4/CD8 staining) differentiation subpopulations (after 8 days, column graphs shown). An un-paired Students T-test was performed between No treat and AMD3100 populations (% Death P<0.001, DN3 and DN3 P<0.005, DN P<0.0001, DP P<0.001). Representative replicates from 2 independent experiments are shown.
Figure 6.6 Intact CXCR4 signalling provides the cue for proper Ap2a2 polarisation prior to division

Sorted in vitro derived Day 9 C57 Bl/6 wildtype (WT) DN3 thymocytes were re-cultured on OP9-DLL1 stromal cell overnight with or without 2 μg/ml AMD3100, then fixed and stained with DAPI (blue) tubulin (red) and Ap2a2 (green). A) All WT DN3 thymocytes from randomly chosen fields of view were assessed for MTOC polarisation and thymocyte-stromal interaction. Thymocytes that polarised MTOC at the thymocyte-stromal interface (white star) were then scored for B) Ap2a2 localisation (with MTOC, cytoplasmic, membrane or distal). Representative maximum projected confocal images show for No treat or AMD3100 treated WT DN3 thymocytes. n=150, from three independent blinded experiments. For statistics an un-paired Students T-test was performed (With MTOC P<0.005). White scale bar, 5μm.
6.2.2 Leukaemic CD2-Lmo2 DN3 thymocytes undergo altered ACD

In CD2-Lmo2 mice, McCormack and colleagues have identified that DN3 thymocytes are the cellular origin of T-ALL (McCormack et al., 2010). CD2-Lmo2 DN3 thymocytes repopulate the thymus in secondary, tertiary and quaternary transplants with eventual leukaemia at 6-10 months (Larson et al., 1994; McCormack et al., 2010). When compared to WT, the thymi of 8-week old CD2-Lmo2 mice have higher percentages of DN3 thymocytes at the expense of DP thymocytes, indicating a possible defect in differentiation \textit{in vivo}. Expression profiles of these CD2-Lmo2 thymocytes also show that genes normally expressed in haematopoietic stem cells are upregulated, whereas genes important for T cell development are downregulated (McCormack et al., 2010). Given the role of Lmo2 in early haematopoiesis, this suggests that Lmo2 alters the fate of DN3 thymocytes by promoting self-renewal. I hypothesized that the enhancement of DN3 self-renewal was due to an alteration in the proportion of SCD versus ACD fates, and would use Ap2a2 as a marker for ACD to investigate this.

I first investigated whether the differences in CD2-Lmo2 thymopoiesis observed \textit{in vivo} could be observed \textit{in vitro}. Wildtype (WT) or CD2-Lmo2 E14.5 foetal liver cells were co-cultured with OP9-DLL1 stromal cells along the T cell differentiation pathway. At Day 11, differentiating WT thymocytes were mainly at the DN3 stage of differentiation compared to CD2-Lmo2 thymocytes that were mainly at DN2 (\textbf{Figure 6.7 A}). This indicated that the kinetics of T lineage specification was slower in CD2-Lmo2 compared to WT thymocytes \textit{in vitro}. The same Day 11 cultures (WT and CD2-Lmo2) were then sorted for DN3 and re-cultured on OP9-DLL1 stromal cells to look at the transition point from DN3 to DP. Interestingly, flow cytometric analysis of sorted DN3 thymocytes 6 days post culture indicated again, reduced T cell differentiation of CD2-Lmo2 thymocytes, with a lower proportion of sorted CD2-Lmo2 DN3 thymocytes reaching the DN4 and DP stage compared to WT DN3 thymocytes (\textbf{Figure 6.7 B}). Interestingly, a large proportion of DN2 thymocytes were present in CD2-Lmo2 cultures, although only DN3 thymocytes were sorted. CD4 single positive and CD8 single positive cells were observed and comparable between WT and CD2-Lmo2 thymocytes. Overall, this demonstrated that all DN to SP subpopulations could be produced \textit{in vitro} from CD2-Lmo2 mice. CD2-Lmo2 thymopoiesis can be observed \textit{in vitro} just as it can \textit{in vivo} (McCormack et al., 2010), and also appears to occur with slower kinetics compared to WT thymopoiesis, most likely due to the altered transcriptional program that promotes self-renewal.

WT and CD2-Lmo2 \textit{in vitro} thymocyte cultures were then transduced with Cherry-Ap2a2 + GFP in which DN3 thymocytes positive for both constructs were sorted and seeded onto OP9-DLL1 stromal cells for time lapse imaging in cell paddocks. Using Cherry-Ap2a2 as a marker for asymmetry, TACTICS quantification of Ap2a2 distribution in 45 WT DN3 divisions indicated a similar proportion of ACD (\textbf{Figure 6.8 A} and \textbf{B}, 22.2%, 10/45 divisions) compared to the initial screen of Ap2a2 in Chapter 5 (23%, 12/52 divisions, Chapter 5, \textbf{Figure 5.7}). In contrast, quantification of Ap2a2 polarisation in dividing CD2-Lmo2 DN3 thymocytes showed that Ap2a2 asymmetry was reduced (\textbf{Figure 6.8 A} and \textbf{B}, 6.5%, 3/45 divisions). I next assessed the paired differences of WT GFP and WT Cherry-Ap2a2 PR values, and similar to my initial study in
Figure 6.7 Defects in CD2-Lmo2 thymopoeisis can be observed *in vitro*

A) Wildtype (WT) and CD2-Lmo2 E14.5 foetal liver cells were co-cultured on OP9-DLL1 stromal cells with cytokines to initiate thymopoiesis, harvested at Day 11 and assessed by flow cytometry for the DN1-DN4 subpopulations (CD44/CD25 staining) and DN-SP subpopulations (CD4/CD8 staining). B) From the cells in A), WT or LMO2 DM3 thymocytes were sorted, re-cultured on OP9-DLL1 stromal cells, and harvested 6 days later. Representative plots of DN1-4 and DP-SP subpopulations 6 days post sort shown, and graphs below. An unpaired Students T test was performed for statistics comparing WT and CD2-Lmo2 subpopulations (DN2, DN4 P<0.0001; DN P<0.05, DP P<0.005). Data is representative of n=2 independent experiments.
Chapter 6: Results

A)

WT

CD2-Lmo2

11 days on OP9-DLL1

sort DN3 culture 6 days on OP9-DLL1

B)

Figure 6.1

CD25

CD44

CD8

CD4
Figure 6.8 ACD as defined by Ap2a2 asymmetry is reduced during Lmo2 DN3 division

A) Scatterplot of absolute Polarisation Ratios (Absolute PRs) of GFP control and Cherry-Ap2a2 in either WT and CD2-LMO2 DN3 divisions. Below the PR cutoff of 0.27 (greyed area, bellow dashed line) indicates symmetrical cell division (SCD), and above the PR cutoff indicates asymmetrical cells division (ACD).
B) Percentage ACD observed for GFP-Control or Cherry-Ap2a2 in WT and CD2-Lmo2 DN3 divisions.
C) Graph of paired PR values of GFP + Cherry-Ap2a2 DN3 divisions (WT and CD2-Lmo2). For statistics a paired Students T-test was performed. GFP paired with Cherry-Ap2a2: WT P<0.0001, CD2-Lmo2 P<0.05. Below each graph is the difference between each GFP control and Cherry-Ap2a2 pair plotted as a scatterplot with the mean difference and 95% confidence interval shown. D) Examples of a WT or CD2-Lmo2 SEQtag showing either Cherry-Ap2a2 SCD or ACD.

WT n= 45 divisions, CD2-Lmo2 n= 45 divisions.
Chapter 6: Results

A) Absolute PR

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<td>n=45</td>
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B) % ACD

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<th>Cherry Ap2a2</th>
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<td>4.4%</td>
<td>6.5%</td>
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<td>6.5%</td>
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C) WT

P<0.0001

Mean = -0.1

95% CI: -0.14 to -0.05

LMO2

P<0.05

Mean = -0.04

95% CI: -0.07 to 0.00

D) WT

CD2-Lmo2

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Chapter 5 these paired differences were significant, further supporting Ap2a2 as a marker for ACD (Figure 6.8 C, P<0.0001). The paired differences of CD2-Lmo2 GFP control and Cherry-Ap2a2 PR values were also significant (Figure 6.8 C, P<0.05), although not to the same extent as WT, suggesting that ACD was reduced but not eliminated (Figure 6.8 D). The finding that ACD is reduced in Lmo2 transgenic thymocytes raises the possibility alterations in ACD might provide at least part of the mechanism by which Lmo2 influences T cell leukaemia.

6.2.3 Intact CXCR4 signalling also promotes differentiation and cues Ap2a2 polarisation in CD2-Lmo2 DN3 thymocytes

So far, the data in this chapter suggests that the promotion of DN3 to DP differentiation depends on CXCR4 signalling, and might (at least in part), be mediated by its effects on Ap2a2 polarity. Both Lmo2 overexpression and CXCR4 inhibition result in reduced differentiation and reduced polarisation of Ap2a2, suggesting the possibility that they might operate through similar pathways. To address this, I tested whether Lmo2 overexpression and CXCR4 inhibition could cooperate in regulating differentiation and polarity. Firstly, I cultured in vitro derived CD2-Lmo2 DN3 thymocytes on OP9-DLL1 stromal cells in the presence or absence of AMD3100. The ratio of DN:DP was further reduced by AMD3100, similar the previous observations of WT DN3 thymocytes (Figure 6.9, compare to Figure 6.6). CD2-Lmo2 DN3 thymocytes therefore also require intact CXCR4 signalling for progression from the DN3 to DP. When Lmo2 overexpression and CXCR4 inhibition are combined, this contributes to further defects in DN3 to DP differentiation. The additive effects of CXCR4 inhibition and Lmo2 overexpression suggests the possibility of functional interactions between the two signalling pathways.

To determine whether this functional cooperation coincided with effects on polarity, I next investigated the role of CXCR4 as a polarity cue for Ap2a2 polarisation in CD2-Lmo2 DN3 thymocytes. Sorted in vitro derived CD2-Lmo2 DN3 thymocytes were cultured overnight on OP9-DLL1 stromal cells in the presence or absence of AMD3100, then fixed and stained for endogenous tubulin and Ap2a2. Similarly to WT, there was no difference in the polarisation of MTOC to the thymocyte-stromal interface in the presence or absence of CXCR4 signalling (Figure 6.10 A, compare with Figure 6.6 A). A similar proportion of Ap2a2 polarity with MTOC at the thymocyte-stromal interface in interphase was scored in CD2-Lmo2 DN3 thymocytes, as well as a similar reduction in Ap2a2 polarisation in the absence of CXCR4 signalling compared to WT (Figure 6.10 B, compare with Figure 6.6 A). One interesting observation was that WT and CD2-Lmo2 thymocytes treated with AMD3100 tended to cap Ap2a2 polarisation distal to the MTOC (Figure 6.6 B and Figure 6.10 B). Therefore, despite the abrogation of Ap2a2 polarisation at division in CD2-Lmo2 DN3 thymocytes, Ap2a2 polarises normally towards the interface with the stromal cell during interphase. However, in CD2-Lmo2 DN3 thymocytes Ap2a2 polarisation to the interface with stromal cells depends on CXCR4 signalling similarly to WT. Collectively, these data indicate that CXCR4 cues Ap2a2 polarity, at least prior to division in both WT and leukaemic contexts. The cooperation between Lmo2 and CXCR4 in both differentiation and polarity regulation, suggests the possibility that both molecular interventions impact on ACD to
Figure 6.9 *In vitro* CD2-Lmo2 thymopoiesis requires intact CXCR4 signalling during culture on OP9-DLL1 stromal cells

CD2-Lmo2 E14.5 foetal liver cells were cultured on OP9-DLL1 stromal cells with cytokines, and in the presence or absence of 2 µg/ml AMD3100. Thymocytes were then harvested 8 days later and analysed by flow cytometry. Representative plots of DN1-DN4 (CD44/CD25) and DN-SP (CD4/CD8) differentiation (statistics in column graphs) are shown. An un-paired Students T-test was performed between No treat and AMD3100 populations for statistics (DN3 and DN4 P<0.005; DN P<0.0001; DP P<0.0001). Representative plots of replicate pools from 3 independent experiments.
Sorted in vitro derived Day 9 CD2-Lmo2 DN3 thymocytes were re-cultured on OP9-DLL1 stromal cell overnight with or without 2 μg/ml AMD3100, then fixed and stained with DAPI (blue) tubulin (red) and Ap2α2 (green). A) All CD2-Lmo2 DN3 thymocytes from randomly chosen fields of view were assessed for MTOC polarisation and thymocyte-stromal interaction. Thymocytes that polarised MTOC at the thymocyte-stromal interface (white star) were then scored for B) Ap2α2 localisation (with MTOC, cytoplasmic, membrane or distal). Representative maximum projected confocal images show for No treat or AMD3100 treated CD2-Lmo2 DN3 thymocytes. n=150, from three independent blinded experiments. For statistics an un-paired Students T-test was performed (With MTOC P<0.02, Distal P<0.05). White scale bar, 5μm.
control thymocyte fate.

6.2.4 Altering the balances of Lmo2, CXCR4 and Ap2a2 influences fate outcome of DN3 thymocytes

Although I have made use of Ap2a2 a marker for ACD of DN3 thymocytes, it is clear that its overexpression has functional consequences that result with enhanced DN3 thymocyte differentiation towards the DP lineage (Chapter 5, Figure 5.10). It has previously been speculated that Ap2a2 overexpression might enhance asymmetry during division (Ting et al., 2011), but my data do not exclude the possibility that Ap2a2 might act through an alternative pathway, such as mediating endocytic trafficking. In this chapter I showed that both Lmo2 overexpression and CXCR4 inhibition reduced polarity and reduced differentiation. To further assess whether alterations in polarity correlate with alterations in differentiation, I took advantage of these findings. I hypothesised that Lmo2 overexpression could cooperate with CXCR4 inhibition and counterbalance Ap2a2 overexpression in controlling polarity. If polarity and differentiation were causally linked, I should see the same pattern of cooperation and antagonism in both polarity and DN3 differentiation.

6.2.4.1 Ap2a2 overexpression in vitro promotes differentiation of CD2-Lmo2 DN3 thymocytes

I first investigated whether ectopic expression of Ap2a2 might rescue the defect in differentiation of CD2-Lmo2 DN3 thymocytes. Untransduced and/or ectopically transduced GFP (Control) and GFP + Cherry-Ap2a2 WT and CD2-Lmo2 DN3 thymocytes were cultured on OP9-DLL1 stromal cell and harvested after 8 days for flow cytometric analysis. At this time point, the differentiation of WT or CD2-Lmo2 DN3 to the DN4 stage did not change regardless of whether Ap2a2 was ectopically expressed (Figure 6.11 A). The CD2-Lmo2 thymocytes still displayed a defect in DN2/DN3 differentiation compared to WT. However, it was possible the lack of DN3 and DN4 differences might relate to the rapid transit of differentiation from DN4 to DP that may have been missed after 8 days culture. The proportion of bulk DN and DP subpopulations were then analysed. As previously observed, control CD2-Lmo2 thymocytes displayed impeded differentiation to the DP stage compared to WT (Figure 6.11 B, WT Control vs CD2-Lmo2 Control, P<0.0001). Remarkably however, ectopic expression of Ap2a2 normalised both CD2-Lmo2 bulk DN and DP subpopulations to near WT levels (Figure 6.11 B WT Control vs CD2-Lmo2 Cherry-Ap2a2, P value not significant). These data suggests that overexpression of Ap2a2 promotes differentiation of DN3 thymocytes that normalises CD2-Lmo2 differentiation to WT levels.

6.2.4.2 Overexpression of Ap2a2 partially rescues DN to DP transition in the absence of CXCR4 signalling in both WT and CD2-Lmo2 thymocytes

To determine whether Ap2a2 and CXCR4 function were causally linked, sorted in vitro derived WT DN3 thymocytes expressing Cherry-Ap2a2+GFP or untransduced/GFP alone were co-cultured with OP9-DL1 stromal cells with or without AMD3100 to inhibit CXCR4 signalling, and harvested 6 days later for flow cytometric analysis (Figure 6.12 and 6.13). As observed
Figure 6.11 Overexpression of Ap2a2 does not alter WT or CD2-Lmo2 DN3 differentiation to DN4 ...

A) Sorted *in vitro* derived WT and CD2-Lmo2 DN3 thymocytes (“Control”: Untransduced and GFP transduced; “Cherry-Ap2a2”: Cherry-Ap2a2 and GFP+Cherry-Ap2a2 transduced) were re-cultured on OP9-DLL1 stromal cells for 8 days then harvested for flow cytometry. Representative plots of DN1-4 subpopulations (CD44/CD25 staining).
Figure 6.11 (cont..) ... but does rescue differentiation to DP thymocytes after 8 days culture on OP9-DLL1

B) Representative plots of DN to SP subpopulations (CD4 vs CD8 staining). An un-paired Students T-test was performed to compare DN-SP subpopulations (WT control DN vs LMO2 control DN P<0.0001; WT control DN vs LMO2 Cherry-Ap2a2 DN P is not significant; WT control DP vs LMO2 control DP P<0.0001; WT control DP vs LMO2 Cherry-Ap2a2 DP P value is not significant). Data are representative replicates of 4 independent experiments.
A) WT Control No Treat WT Cherry-Ap2a2 No treat WT Control +AMD3100 WT Cherry-Ap2a2 +AMD3100

B) WT Control No Treat WT Cherry-Ap2a2 No treat WT Control +AMD3100 WT Cherry-Ap2a2 +AMD3100

Figure 6.12 Overexpression of Ap2a2 partially rescues blocks in WT DN3 differentiation in the absence of CXCR4 signalling

Sorted in vitro derived WT DN3 thymocytes (“Control”: Untransduced and GFP transduced; “Cherry-Ap2a2”: Cherry-Ap2a2 and GFP+Cherry-Ap2a2 transduced) were re-cultured on OP9-DLL1 stromal cells for 6 days in the presence or absence of 2 μg/ml AMD3100, then harvested for flow cytometry. The A) DN1 to DN4 subpopulations (CD44/CD25 staining) and B) bulk DN, DP and SP subpopulations (CD4/CD8 staining) were assessed. An un-paired Students T-test was performed to compare WT Control +AMD3100 vs WT Cherry-Ap2a2 +AMD3100 (DN and DP P<0.0001). Data are representative replicates of 4 independent experiments.
Figure 6.13 Ectopic expression of Ap2a2 partially rescues blocks in CD2-Lmo2 DN3 differentiation in the absence of CXCR4 signalling

Sorted in vitro derived CD2-Lmo2 DN3 thymocytes ("Control": Untransduced and GFP transduced; "Cherry-Ap2a2": Cherry-Ap2a2 and GFP+Cherry-Ap2a2 transduced) were re-cultured on OP9-DLL1 stromal cells for 6 days in the presence or absence of 2 μg/ml AMD3100, then harvested for flow cytometry. The A) DN1 to DN4 subpopulations (CD44/CD25 staining) and B) bulk DN, DP and SP subpopulations (CD4/CD8 staining) were assessed. An un-paired Students T-test was performed to compare CD2-Lmo2 Control +AMD3100 vs CD2-Lmo2 Cherry-Ap2a2 +AMD3100 (DN and DP P<0.0001). Data are representative replicates of 4 independent experiments.
previously in WT DN subpopulations, the inhibition of CXCR4 signalling impeded DN3 to DN4 differentiation (Figure 6.12 A Control No treat versus Control +AMD3100), and the ectopic expression of Ap2a2 did not alter the defect in DN3 to DN4 differentiation (Figure 6.12 A Control No treat versus Control +AMD3100). However, although inhibition of CXCR4 signalling impeded the transition from DN to DP, the ectopic expression of Ap2a2 partially rescued the differentiation defect, significantly increasing the proportion of DN3 thymocytes reaching the DP stage (Figure 6.12 B DP group, Control +AMD3100 versus Cherry-Ap2a2 +AMD3100, P<0.0001, and also DN group, Control +AMD3100 versus Cherry-Ap2a2 +AMD3100, P<0.0001). Similar results were observed in differentiating CD2-Lmo2 DN3 thymocytes. CD2-Lmo2 DN thymocytes still displayed defective DN3 to DN4 differentiation compared to WT, with an accumulation of DN2 thymocytes regardless of whether intact CXCR4 signalling was present (Figure 6.13 A Control No treat bar versus Control +AMD3100), or when Ap2a2 was ectopically expressed (Figure 6.13 A Control No treat versus Control +AMD3100). However, similarly to WT, in the absence of intact CXCR4 signalling the ectopic expression of Ap2a2 partially rescued the DN to DP defect in differentiation, significantly increasing the population of DN3 thymocytes reaching the DP stage (Figure 6.13 B Control +AMD3100 versus Cherry-Ap2a2 +AMD3100, P<0.0001). Collectively, the data suggests that Ap2a2 and CXCR4 functionally interact at the level of both polarisation and differentiation or survival. CXCR4 appears to function upstream of Ap2a2 because it cues Ap2a2 polarisation prior to division in both WT and CD2-Lmo2 thymocytes.

6.3 Discussion

In this chapter I identify that intact CXCR4 signalling, facilitated by stromal cell interaction, is a polarity cue for Apa2 polarisation. Using Ap2a2 as a marker of polarity I demonstrate that ACD is reduced in Lmo2-induced leukaemic thymocytes. Ap2a2 overexpression, Lmo2 overexpression and CXCR4 inhibition can all coordinate to influence polarity during ACD and DN3 fate outcome.

6.3.1 Changes in polarity that occur in Lmo2-mediated leukaemogenesis might be in part a reflection of altered polarity during thymocyte ACD

It is well-accepted that alterations in polarity pave the way for cellular transformation and oncogenesis for many cancers (described in Chapter 1) (Gardiol et al., 2006; Massimi et al., 2004; Nakagawa et al., 2004; Navarro et al., 2005; Bello et al., 2006; Betschinger et al., 2006; Brumby et al., 2004). Traditionally thought in many of these cases is that the disruption of polarity impinges directly on pathways that control cell adhesion, endocytosis, and signalling, leading to defects in how a cell senses and responds to its extracellular environment (Humbert et al., 2008; Lambert et al., 2012; Lu and Bilder, 2005; Vaccari and Bilder, 2005). However, it is now well established that a major mechanism by which loss of polarity impacts upon oncogenesis is by altering ACD and subsequent cell fate determination (Graham et al., 2010; Knoblich, 2010; Lee and Vasioukhin, 2008; Martin-Belmonte and Perez-Moreno, 2012; McCaffrey and Macara, 2011; Neumuller and Knoblich, 2009). In fact, many of the key regulators of ACD also act as tumour
suppressors (Betschinger et al., 2006; Caussinus and Gonzalez, 2005; Gonzalez, 2007; Lee et al., 2006; Wang and Li, 2010).

6.3.1.1 Can disruption of polarity during ACD lead to leukaemogenesis?

The notion that disruption of polarity during ACD impacts on oncogenesis dates back to the initial studies of nervous system development in D. melanogaster. Loss of basal polarisation of cell fate determinants such as Numb, Prospero, Miranda and Pons all result in excess neuroblast formation, decreased neuronal differentiation and the formation of tumours (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006; Wang et al., 2007). In mammals, neuronal precursors that lack the tumour suppressor and polarity protein Lgl mislocalise the cell fate determinant Numb during division, and this is correlated with the formation of neural ectodermal tumours in the brain (Klezovitch et al., 2004; Rolls et al., 2003). Because ACD often balances the inheritance of fate determinants such as the capacity for self-renewal and proliferation with quiescence over many generations (as seen in stem cells), it has been speculated that disruption of polarity during ACD might lead to cancer by incorrectly combining cell fate determinants such as proliferative capacity with longevity (Florian and Geiger, 2010; Hawkins and Russell, 2008; Powell et al., 2010).

The lessons learnt from studying polarity in epithelial oncogenesis suggest how altered polarity might impact on leukaemogenesis. Based on my finding that Ap2a2 was polarised in a proportion of dividing DN3 thymocytes (Chapter 5), I used Ap2a2 as a marker for ACD. This study revealed that CD2-Lmo2 DN3 thymocyte displayed increased SCD, or a reduction in the degree of Ap2a2 polarisation. This data is exciting as it indicates that these leukaemic DN3 thymocytes have altered ACD that might relate to changes in the differentiation or self-renewal phenotype. One hypothesis is that loss of polarity alters ACD in CD2-Lmo2 thymocytes, and that the subsequent increase in SCD might actually reflect the promotion of self-renewal and proliferation. Therefore, in addition to upregulating stem cell like genes at the expense of T cell development genes, one possibility is that Lmo2 DN3 thymocytes possess a modified transcriptional program that impinges on pathways controlling polarisation during ACD.

6.3.1.2 Does polarity and endocytosis play a role in differential cell fate decisions following ACD?

One possible way that alterations in the polarity can lead to differences in fate outcome and leukaemogenesis is by impacting on endocytic pathways. In this chapter I showed that altering the activity of CXCR4 or the expression level of Ap2a2 can respectively impede or enhance DN3 thymocyte differentiation. This data is interesting in light of the notion that cell surface receptors such as chemokines including CXCR4, have been shown to access clathrin mediated endocytosis pathway via AP-2 for downstream degradation or signalling (Garcia-Regalado et al., 2008; Sorkin and von Zastrow, 2009). Whether the chemokine receptor CXCR4 interacts with the same AP-2 mediated pathways in DN3 thymocytes, is currently unknown. Given that both Ap2a2 and CXCR4 are polarised in DN3 thymocytes and both have been implicated in the regulation of endocytosis (Berdnik et al., 2002; Garcia-Regalado et al., 2008), the polarisation of
both molecules might impact on thymocyte fate following division.

The idea that polarisation and signalling of endocytic molecules are important for cell fate is supported by a study from Couturier and colleagues that demonstrate that Numb generates biases in Notch signalling and fate outcome through regulating endocytosis (Couturier et al., 2012). In asymmetrically dividing *D. melanogaster* sensory organ precursors, Numb interacts both with a membrane protein called Sanpodo as well as α-adaptin (of which Ap2a2 is part of in the AP-2 complex) (Berdnik et al., 2002; Couturier et al., 2012; Hutterer and Knoblich, 2005; Tong et al., 2010). In the absence of either Numb, Sanpodo or α-adaptin, reduced or symmetric amounts of Notch are present in both p11a and p11b cells, resulting in symmetric activation of Notch and loss of ACD (Couturier et al., 2012). This suggests that the molecular interactions of Numb with Sanpodo-Notch complexes and the AP-2 endocytic machinery alter the polarisation of Notch to create signalling differences for mediating fate outcomes.

From these observations, it could be suggested that asymmetrically dividing thymocytes polarise Ap2a2 and Numb that translates into signalling differences to mediate different fate outcome. As the overexpression of Ap2a2 rescues the differentiation defects of both CXCR4 inhibition and the CD2-Lmo2 transgene, it can be suggested that CXCR4 inhibition and Lmo2 expression alter polarisation of endocytic pathways controlled by Ap2a2 for signalling and regulation of DN3 fate. This indicates that the regulation of vesicle trafficking may be an important process to direct DN3 signalling and fate decisions, and that mechanisms of polarity could alter these pathways to impact on leukaemogenesis. It will be important in future work to elucidate whether loss of ACD during division of CD2-Lmo2 DN3 thymocytes is due to defects in the polarisation of endocytic pathways, and identity the proteins that might access these pathways.

### 6.3.2 A possible role of DN3 ACD in fate control

In this chapter I demonstrate that Ap2a2 polarisation to the thymocyte-stromal interface relies on intact CXCR4 signalling, suggesting that chemokine signalling provides the polarity cue that enables DN3 ACD.

My findings provide the beginnings of a molecular mechanism for thymocyte ACD (Figure 6.14). Thymocyte-stromal interactions provide the necessary polarity cues via CXCR4 to polarise Ap2a2, satisfying the requirements that initiate ACD for proper DN3 fate outcome. However, any alterations in the balance between CXCR4 signalling, Ap2a2 overexpression or Lmo2 overexpression results in alterations in DN3 polarity and differentiation. For example, the loss of Ap2a2 polarisation during CD2-Lmo2 DN3 division suggests that there is a concomitant loss in the ability to respond to the polarity cue provided by chemokine (in this case CXCL12) released by the stromal cell. Lmo2 overexpression also reduces DN3 differentiation, and during inhibition in CXCR4 signalling there is a loss in differentiation in addition to Ap2a2 polarisation. However, the overexpression of Ap2a2 can promote differentiation of thymocytes in both wildtype and leukaemic contexts as well as in the absence of CXCR4 signalling. This indicates that overexpression of Ap2a2 might also promote polarity to facilitate ACD, perhaps as a carrier...
Chapter 6: Results

**Figure 6.14 The balance between ACD and SCD in DN3 thymocytes**

In this model, DN3-stromal interactions via CXCR4 enables polarisation of Ap2a2 (red vesicles) that promotes ACD. The overexpression of Ap2a2 might also promote polarity and differentiation via ACD. Conversely, expression of Lmo2 alters thymocyte polarity (directly or indirectly) to promote SCD instead of ACD. This model describes that altering the balance of these molecular components (alone or in combination) may alter polarity, the propensity for ACD, and fate outcome (summarised in the table).

* Can not directly compare to no Ap2a2 overexpression.

( ) Assumption only.
protein (presumably Numb or an as yet unidentified molecule). These studies highlight the importance in maintaining the proper balance of the molecular components that facilitate polarity during ACD of DN3 thymocytes for correct fate outcome.

On a side note, the fact that chemokine receptor and stromal cell interactions are important for Ap2a2 polarisation in thymocytes is somewhat counterintuitive because chemokines are usually gradients. Given that CXCL12 is secreted by OP9-DLL1 (Janas et al., 2009), one hypothesis could be that stromal-derived CXCL12 recruits DN3 thymocytes to the stromal cells and subsequent interactions dictate polarity. However, the inhibition of CXCR4 did not impact upon the interactions between thymocytes and stromal cells per se, but did prevent polarisation of thymocytes in contact with stromal cells. This suggests that CXCR4 directly influences polarity only at the interface between the stromal cell and the thymocyte. Thymocytes engaged with stromal cells with polarised CXCR4 may also signal to impact on ACD, downstream differentiation and also survival. For future experiments, it will be important to isolate DN3 daughter thymocytes containing less or more Ap2a2 to reveal differences in the exact molecular properties linking ACD with DN3 fate in normal and leukaemic contexts. Timelapse imaging would also be key to directly correlate Ap2a2 polarisation with thymocyte fate in the absence/presence of intact CXCR4 signalling, and in both WT and leukaemic contexts. Current movies of WT and Lmo2 thymocytes could also be re-analysed to quantify whether there are differences in interaction with the OP9-DLL1 stromal cells. Thymii from WT and CD2-Lmo2 mice will need to be isolated, sectioned and stained to identify if Lmo2 DN3 thymocytes reside in a thymic niche in vivo, and whether these niches correspond to high or low CXCL12 expression. It is also likely that CXCR4 is not the only cue, given that Notch1 was highly polarised during thymocyte interaction with stromal cells. In addition to CXCR4, it will be interesting to investigate whether other polarity cues regulate DN3 polarisation during ACD.

6.3.3 Summary

My studies show exciting quantitative and preliminary functional evidence that ACD occurs in DN3 thymocytes. I provide the first indication that chemokine signalling through CXCR4 constitutes a molecular requirement for DN3 polarisation, serving as a polarity cue that localises the endocytic marker Ap2a2 prior to DN3 division. Using Ap2a2 as an ACD marker, I demonstrate that approximately 20% of DN3 thymocytes undergo ACD, and that Lmo2 DN3 thymocytes display a loss of ACD, thereby linking the cellular origin of this leukaemia to possible alterations in ACD. Finally, I observe correlations between polarity and fate that strongly suggests ACD regulates DN3 fate choices. Together, my results highlight the physiological context by which ACD occurs in DN3 thymocytes, and how pathways regulating ACD alter in the context of leukaemia.
Chapter 7

Discussion & Future Directions
Described in this thesis is the investigation of whether DN3 thymocytes undergo ACD, the molecular components that could regulate this process, and possible functional consequences of DN3 ACD. Through development of a timelapse technique and a computational platform, this technology is combined with a number of in vitro experiments that support the hypothesis that ACD and polarity regulate fate decisions during DN3 thymocyte development. In addition, I show the beginnings of a molecular mechanism that could regulate DN3 ACD, and demonstrate how a T cell leukaemia might be a consequence of deregulated polarity during DN3 ACD. The implications for these findings will be discussed here.

7.1 Imaging systems facilitate the study of thymocyte fate determination

7.1.1 Cell paddocks enable analysing long term behaviours of motile lymphocytes at high spatial and temporal resolution

In Chapter 3, I developed a time lapse imaging methodology to contain thymocytes and stromal cells in culture for long term observation. Using this method, I have found that thymocytes leave and return to the stromal cell intermittently during migration, prefer to divide while in contact with the stromal cell, and divide in a variety of orientations. This time lapse method with the cell paddocks provides an excellent platform to investigate the kinetics of thymocyte behaviour during migration and division in a contained environment, and can be applied to studying any type of non-adherent immune cell. Clearly, some very interesting interactions between thymocytes and stromal cells merit further exploration for future studies.

In some other experiments I have used this methodology to image mature naïve T cells and antigen presenting dendritic cells in cell paddocks. Naïve T cells also appear to undergo prolonged interactions (up to 25 hrs) with dendritic cells, possibly to receive signals prior to undergoing division (data not shown). It is interesting that while dendritic cells can adhere to the cell paddocks, OP9-DLL1 stromal cells require fibronectin to adhere on the floors of the paddock. The ability to functionalise the surface of the cell paddocks with extracellular matrix such as fibronectin means that other surface modifications may be added to the paddocks to investigate many other types of cellular interactions. For example, members in our laboratory have customised the cell paddocks by coating with specific antibodies to control activation of lymphocytes such as T cells without the use of antigen presenting cells (unpublished data). With the added ability to fabricate cell paddocks of different height or width dimensions, it will be possible to image and contain many different types of non-adherent immune cells for long term analysis over multiple generations. Other future applications could be adapting a feeder free system for thymocyte development (Ikawa et al., 2010), where the Notch ligands DLL1 or DLL4 can be immobilized to the bottom of the cell paddocks. This would enable dissection of the molecular requirements for ACD and its downstream consequences on fate at high temporal and spatial resolution.
7.1.2 Quantitative microscopy that elucidates lymphocyte behaviours is an interactive process

Only in the last decade has serious effort been made to provide quantitative measurements about immune processes, including the cellular dynamics of lymphocytes within a lymph node, the induction of humoral immunity, or the tracking of immunosuppression (Azar et al., 2010; Bouso et al., 2002; Germain et al., 2012; Moreau et al., 2012; Stoll et al., 2002). The transition of imaging practices within immunology into more quantitative techniques, and the emergence of more software tools demonstrates a realisation that computational solutions provide far better objective and reproducible analysis (de Chaumont et al., 2012; Matis et al., 2012; Rajaram et al., 2012; Schindelin et al., 2012; Wahlby et al., 2012; Zhong et al., 2012). In Chapters 4 and 5, I co-developed and used the TACTICS platform to demonstrate the first robust measure of protein polarisation during DN3 thymocyte migration and division. Using TACTICS, I was able to measure Numb localisation in both migrating T cell and thymocyte populations, and I extracted polarisation ratios under several parameter optimisations procedures to demonstrate that DN3 thymocyte underwent ACD.

The quantification approach I have employed is based upon a long-standing notion that analysis of complex data requires a more interactive approach. For a good image analysis platform one must work side-by-side with a software developer. This is an interactive and iterative process. It would not have been possible to provide a priori instructions to the software developer to create an analytical platform that would neatly answer all of the questions in this thesis. Ongoing intellectual input was needed to construct a working pipeline, from hardware to required software, presentation tools, data presentation tools and high throughput quantification. For example, to test whether speed or aspect ratio could be used to gate on polarised thymocytes (Chapter 4), I worked with the software developer to enable measurements that facilitated gating as well as allowed parameters such as speed and aspect ratio. Another example was the quantification of protein polarisation for ACD (Chapters 5 and 6). To investigate whether there was ACD, TACTICS needed to be able to process polarisation ratios under different threshold parameters, a feature that would be extremely time-consuming to perform manually for all divisions identified during this study. My interaction with the software developer enabled a means with which to optimize and standardize imaging quantification so that symmetric or asymmetric divisions could be defined, measured, and compared between control and test protein.

7.1.3 TACTICS enables objective, quantitative analysis of ACD, with room for further improvements

As previously discussed, TACTICS provides a platform with which parameter optimization enabled SCD and ACD to be defined during DN3 division. In Chapters 5 and 6, I used two cutoffs—first, from the threshold heatmaps, and second, from the PR scatterplots. For the purposes of analysis I chose a 25% threshold cutoff, and ascribed high PR values as ‘ACD’, and low to zero PR values as ‘SCD’. This was done for pragmatic reasons to generate a number with which to compare proteins or conditions, as is the standard in the field of quantifying ACD so far. The decision for the two cutoffs to define ACD and SCD was only made after careful iterative
processing and quality control. After being tested, TACTICS could then be applied to compare polarisation in polarity mutants like Numb2A, and in T-ALL leukaemia in the CD2-Lmo2 DN3 thymocytes. This approach to quantifying ACD is by far the most objective for any biological system to date. It surpasses the quantification of ACD in solid tissues that still mostly use subjective scoring, greatly improves the approach employed by the Reiner laboratory to describe T cell ACD, and progresses our laboratory’s current approach to measuring T cell ACD (Barnett et al., 2012; Chang et al., 2011; Chang et al., 2007; Oliaro et al., 2010). With the added potential for TACTICS to track polarisation prior to, during, and after division, this will enable assessment of the impact in fate during normal and aberrant DN3 development.

That being said, even with carefully considered comparisons of parameters between control and test proteins, the cutoffs used in this thesis to define ACD versus SCD are unlikely to be so black and white. It will only be possible to determine what degree of polarisation is functionally relevant by imaging over multiple generations to correlate polarity and fate (for example, the time to the second division and the time to differentiate or die). This will be made possible if the time lapse system that I have developed is extended. More information can be also gained from both sets of data with which the cutoffs were derived. From the first set of data (the heat maps), analysis of the shape and spread of PR values over different thresholds could inform us of the magnitude of asymmetry for a test protein (Appendix 2). In part, this is achieved by the T-tests (done on the first threshold cutoff from the heatmaps), where the confidence in the difference between the paired control PR and test protein PR values are determined (Appendix 3). From these graphs the magnitude and direction of these paired differences of asymmetry could describe the type of asymmetry observed. From the second set of data (the PR scatterplots), analysis of the differences in the spread and shape of the PR data points may inform a pattern that could indicate ACD or SCD, and this might be different for each test protein. For example, the PR scatterplots of Dlg4 clearly show two groups of PR values that are suggestive of two modes of division (ACD and SCD, Figure 5.6), whereas for LAT the majority of the PR values are grouped near zero (only SCD, Figure 5.9). Using a single cutoff for all the PR scatterplots supports the idea that LAT is symmetric, however, this also labels a number of SCD events for Dlg4 that might instead be ACD. A universal secondary cutoff therefore most certainly introduces false negative and positive ACD events. In order to properly define ACD, perhaps the expression levels for each protein should be automatically taken into account to set a base requirement for subsequent quantification. Future work will also require fine tuning of this analysis to exploit the paired nature of all this data.

7.2 DN3 thymocytes undergo ACD- the implications for downstream fate

These thesis studies demonstrate a number of key findings about polarity and ACD of DN3 thymocytes. First, during interphase, a number of cell fate determinants (Notch1, Gpsm2), polarity (aPKC, Dlg4, Par3) and TCR signalling molecules (pre-TCR, Carma) are polarised in DN3 thymocytes. Second, during division Numb and Ap2a2 are polarised in DN3 thymocytes. The TCR signalling molecule LAT is not polarised during DN3 division, and the polarity proteins...
Scribble and Dlg4 show little polarisation. Third, mutation of two aPKC phosphorylation sites in Numb is sufficient to abrogate Numb polarisation during DN3 division but not during migration. Fourth, CXCR4 signalling provides a polarity cue for Ap2a2 polarisation. Fifth, the leukaemogenesis induced by Lmo2 abrogates or severely reduces Ap2a2 polarisation during DN3 division. A summary of polarising molecules during interphase and division of DN3 thymocytes is summarised in Figure 7.1. Collectively, these studies indicate that DN3 thymocytes are polarised and can undergo ACD.

7.2.1 Asymmetry must correlate with downstream DN3 fate decisions

In Chapter 5 I demonstrate that DN3 thymocytes asymmetrically localise Numb and Ap2a2 during division. While the unequal distribution of these proteins upon division shows that DN3 thymocytes can undergo ACD, it does not show that ACD regulates DN3 fate. What is fate? In this context, fate of DN3 thymocytes can encompass death, proliferation, differentiation or self-renewal. In order to analyse whether downstream DN3 divisions have altered fates due to an initial ACD event, two successive DN3 divisions need to be analysed. The time lapse movies performed in this thesis were approximately 24 hrs in length which was enough to capture DN3 divisions that occurred once in every 10-15 hrs (data not shown). A longer time lapse imaging period, most likely up to 40 hrs, will be required to capture two successive generations. Tracking of the thymocyte daughters will also need to be improved to enable long term monitoring, characterisation and quantification of thymocyte divisions without disrupting proliferation or differentiation. Cell divisions and death of thymocyte daughters undergoing Numb or Ap2a2 asymmetry could be visualised in time lapse movies without the need for additional tools. To assess differentiation, I have developed other protocols during my thesis studies to add low concentrations of fluorescently tagged antibodies (non-blocking and non-activating) to surface molecules that mark particular thymocyte differentiation choices or stages (data not shown). Fluorescently tagged antibodies to CD28 could mark differentiation from DN3a to DN3b, whereas CD8 marks the DN4 transition to ISP before the DP stage (Matsumoto et al., 1991; Teague et al., 2010). In all of these cases, fluorescence will only be visible on thymocytes when it differentiates and expresses the specific molecule on its surface. Members in the laboratory are working on TACTICS to display lineage mapping of tracked thymocyte progeny undergoing ACD over multiple generations. Eventually, it will be possible to map lineage fate analysis by surface acquisition of molecules and to construct lineage trees depicting downstream fates of asymmetrically dividing DN3 cells.

7.2.2 Can DN3 thymocytes switch between SCD and ACD as part of their fate determining process?

My studies have shown that DN3 thymocytes are capable of both SCD and ACD, and so it is possible that DN3 thymocytes could switch between these two modes of division. How might switching be regulated, and what might it influence? Precedents to switching include studies of embryogenesis-to-adult development in C. elegans, as well as peripheral nervous system development in D. melanogaster. In C. elegans, ACD predominates in the initial stages of embryogenesis to generate the key germline and somatic cell populations (Sulston and Horvitz, 1977; Sulston et al., 1983). During the larval stages, 53 somatic nuclei blasts then switch from
During DN3 interphase, endocytic molecules (Ap2a2, Numb, Numb in Ap2a2 vesicles, Notch1), TCR signalling molecules (pre-TCR, Carma), polarity proteins (Scribble, aPKC, Dlg4, Par3) and regulators of polarity (Gpsm2) are localised to the thymocyte-stromal interface with the microtubule organising centre (MTOC). Scribble can also polarise to the distal pole in DN3 thymocytes. During DN3 division, CXCR4 engagement in the DN3 thymocyte with the stromal cells provides the polarity cue for Ap2a2 and perhaps also Numb polarisation. Numb polarisation during division is also regulated by phosphorylation by aPKC. The polarisation of Ap2a2/Numb during division results in daughters with less or more Ap2a2/Numb, and possible different fates.

**Figure 7.1 A summary of polarising molecules during interphase and division of DN3 thymocytes**
ACD and undergo SCD to generate the final 959 or 1031 somatic nuclei of the adult organism (Sulston et al., 1983). In *D. melanogaster*, the peripheral nervous system is comprised of thousands of neurons. However, these neurons are derived from a limited set of sensory organ precursors. Sensory organ precursors undergo different types of ACD to generate the initial p11a and p11b populations, and the daughters from these divisions either undergo further ACD for differentiation and self-renewal, or switch to SCD for selective expansion of cells that eventually comprise the sensory organ (Gho et al., 1999; Lai and Orgogozo, 2004). The switching between ACD and SCD in *C. elegans* and *D. melanogaster* regulates fate outcomes of proliferation, quiescence, apoptosis and differentiation. Similar mechanisms have been proposed for HSCs, and DN3 thymocytes may also switch between SCD and ACD depending on the cues that are satisfied, such as being on or off a stromal cell, or the particular phase of differentiation, such as DN3a versus DN3b.

### 7.2.3 Does ACD occur at DN3a or DN3b?

In addition to switching between ACD and SCD, the mode of DN3 division might depend on the stage of differentiation. In the time lapse experiments I have performed, all DN3 thymocytes are purified and so divisions of all DN3 generations are observed. Given that ACD is only observed in a proportion of DN3 divisions (for example, approximately 14% undergo Numb ACD, 23% undergo Ap2a2 ACD), it is possible that ACD occurs preferentially at the DN3a or DN3b stage. Our laboratory has obtained a transgenic mouse with inducible expression of the TCR-β chain (Tet-O-Beta). When crossed onto the *Rag1*−/− background (preventing rearrangement of endogenous TCR-β), Tetβ-LTH-*Rag1*−/−, thymocytes can only form a functional pre-TCR complex when expression of the exogenous TCR is induced by removal of tetracycline (Dose et al., 2006). DN3 thymocytes from these mice are therefore halted at the DN3a stage. In order to determine the stage in which ACD occurs, DN3a thymocytes transduced with fluorescent-tagged constructs to Numb or Ap2a2 could be isolated from Tetβ-LTH-*Rag1*−/− mice, then imaged in tetracycline-free media and assessed for protein distribution during division. With the use of other surface markers such as CD28, whose surface expression flanks the β-selection checkpoint (Teague et al., 2010), the first division imaged will be the one that follows β-selection and upregulates CD28 surface expression. This will enable us to distinguish whether the DN3a or DN3b (or both) thymocytes undergo ACD and provide a detailed characterisation of the events occurring at the β-selection checkpoint. Importantly, identifying the stage at which ACD occurs in high proportions will facilitate future experiments to define the mechanisms and consequences of ACD.

### 7.3 Disruptions in DN3 ACD during leukaemia provides insights about the normal regulation of DN3 fate determination

Given that the technology for long term time lapse imaging of thymocytes is not yet mature, I have adopted an alternate strategy to assess the mechanisms and consequences of ACD. In Chapter 6, I made use of Ap2a2 as a marker for ACD to show that leukaemic DN3 thymocytes
Chapter 7: Discussion

with enhanced self-renewal phenotypes undergo altered ACD. I demonstrated that while WT DN3 thymocytes undergo a similar proportion of ACD as was previously observed during my first quantification of Ap2a2 asymmetry, CD2-Lmo2 DN3 thymocytes lost the capability for ACD and predominantly underwent SCD. One hypothesis could be that the increase in SCD seen in leukaemic DN3 thymocytes may correlate with fates that result in self-renewal.

7.3.1 Does an increase in SCD relate to DN3 thymocyte self-renewal?

Self-renewal during ACD classically relates to the production of a more differentiated cell, as well as an exact copy of a stem cell-like parent for maintenance. However, in studies of CD2-Lmo2 mice the DN3 thymocytes are described as undergoing continual self-renewal, suggesting that self-renewal in this context refers to an unlimited expansion of DN3 parents at the expense of differentiated cells, and SCD instead of ACD (McCormack et al., 2010 and Chapter 6). How can self-renewal occur in DN3 thymocytes, when the current dogma indicates that the thymus requires continual seeding from bone marrow progenitors for T lineage specification? (Donskoy and Goldschneider, 1992; Goldschneider et al., 1986; Scollay et al., 1986).

There is now a growing body of evidence demonstrating that thymocytes have the capacity to undergo moderate self-renewal, either at DN2/DN3 stage to facilitate the expansion of early β-rearrangement events, or at the DP stage to expand clones that pass selection (Berzins et al., 1998; Martins et al., 2012; Peauderferf et al., 2012; Penit et al., 1995; Petrie et al., 2000; Yokota et al., 2006; Yuan et al., 2011). Recent studies from the Rocha and Roderwald groups show that under certain conditions, thymocytes can persist, self-renew, and differentiate without any input from bone marrow progenitors (Martins et al., 2012; Peauderferf et al., 2012). In some elegant studies, both groups transplant neonatal WT thymii into lymphopenic hosts (Rag2−/−IL-7R− or Rag2γc−, these mice lack all thymocyte subsets) to investigate the reconstitution of peripheral T cell populations. They demonstrate that these transplanted WT thymii can maintain a continuous process of thymocyte self-renewal, proliferation, and differentiation to reconstitute the full peripheral T cell repertoire. Notably, in the Roderwald study the peripheral T cell repertoires are not as large as that observed by the Rocha laboratory. It is proposed that the limited T cell repertoire is from early expansion of early clones from similar cohorts of DP cells, most likely self-renewal. In contrast, the Rocha study show an increase in DN2 cells in reconstituted lymphopenic hosts and propose that it is the DN2 thymocytes that undergoes self-renewal. It is debatable from these studies what exactly the self-renewing populations are, but it is clear that in spite of current dogma, thymocytes do have the capacity for some self-renewal. Interestingly, in the Rocha study the transplanted thymocytes show signs of contraction after 3-4 months, and in the Roderwald study approximately half of the reconstituted mice go on to develop T cell leukaemias later in life. This indicates that self-renewal is not an unlimited feature of thymocytes, however, in the absence of regulatory checkpoints that would otherwise control self-renewal, the extended life of these thymocytes is unveiled with consequences that could predispose for genetic mutations that result in T cell leukaemia. In light of my thesis findings, this suggests that CD2-Lmo2 DN3 thymocytes undergo an increase in SCD due to enforced Lmo2 expression that could extend or promote the self-renewal capacity of DN3. The extension of DN3 self-renewal thus provides the ultimate
predisposition to accumulate additional genetic mutations, predisposing the cells to leukaemic transformation. The next key step will be to determine whether the reduction in ACD is causal in altering DN3 fate and leukaemogenesis in the CD2-Lmo2 mice.

7.3.2 Models for DN3 fate determination during normal and leukaemic development

As discussed in Chapter 1, ACD regulates the balance in the proportion of cell fate determinants that are inherited by each daughter cell for different downstream fates. This has led to the growing thought that disruptions in ACD might lead to leukaemia by combining longevity with determinants such as proliferation and self-renewal (Florian and Geiger, 2010; Hawkins and Russell, 2008; Powell et al., 2010). In doing so, the cells from such divisions are given a chance to accumulate additional mutations that may result in leukaemogenesis. Several models may be proposed to explain how disruptions in the balance of DN3 SCD and ACD can contribute to leukaemia.

During normal DN3 fate determination, let us assume DN3a thymocytes are the population that undergoes ACD (Figure 7.2, Model 1). These DN3a thymocytes have low proliferative potential and are beginning to undergo TCR-β recombination. Classically, ACD involves generating two daughters that differentially adopt the properties of self-renewal or some degree of differentiation that is combined with rapid proliferation and a finite number of divisions (Hawkins and Russell, 2008). In this case ACD of DN3a thymocytes might generate another DN3a thymocyte for self-renewal or re-entering TCR-β recombination, but also a DN3b thymocyte in which TCR-β recombination has been successfully achieved. These DN3b thymocytes then switch to SCD to expand, and after a finite number of divisions would be compelled to differentiate to DN4 and onwards to DP. At all times the distinct molecular requirements, such as the proper balance of fate determinants must be fulfilled. It is also possible that expanded DN3b cells might switch from SCD back to ACD to generate daughters with different capacities for self-renewal and proliferation. This means that ACD of DN3b thymocytes might generate a DN3b and a DN4 thymocyte, or that a DN3a thymocyte in addition to a DN3b thymocyte is produced. This idea is supported by previous work showing that DN3b thymocytes may possess bidirectional differentiation back to DN3a, and that DN4 thymocytes can differentiate to either DN3 or DP (Taghon et al., 2006; Teague et al., 2010). During DN3 fate determination, both ACD and SCD balance self-renewal with differentiation and proliferation, but might also provide the ability to regenerate self-renewal capacity from a more differentiated cell.

In the case of CD2-Lmo2 T-ALL, there is loss or at least reduction in ACD. Following on from Model 1, instead of a DN3a thymocyte producing one daughter that self-renews and another DN3b daughter with the capacity to proliferate and differentiate, two DN3a or two DN3b daughters might be generated, both possessing self-renewal properties and the capacity to proliferate rapidly with little differentiation (Figure 7.2, Model 2a). Another possibility is that the molecular changes due to Lmo2 transgene expression induces a rapid DN3a to DN3b transition so there is no opportunity for ACD, and only SCD of DN3b are enabled (Figure 7.2, Model 2b). Given the reduction in ACD and the increase in SCD, switching from SCD to ACD is unlikely to occur, or is substantially reduced.
Figure 7.2 Possible models describing the role of ACD in DN3 fate

During normal ACD (Model 1), DN3a thymocytes (low proliferative potential, beginning to undergo TCR-β recombination) undergo ACD that generates another DN3a thymocyte for self-renewal/re-entering TCR-β recombination, as well as DN3b thymocyte in which TCR-β recombination has been successfully achieved. The DN3b thymocytes then switch to SCD to expand, and after a certain number of divisions would be compelled to differentiate to DP and SP. DN3a thymocytes arising from the first ACD event may also undergo further ACD for self-renewal and the generation of DN3b thymocytes. It is also possible that DN3b cells might switch from SCD back to ACD to generate daughters of different capacities for self-renewal and proliferation. ACD of DN3b thymocytes might therefore generate a DN3a thymocyte in addition to a DN3b thymocyte. In CD2-Lmo2 leukaemia the loss or reduction of ACD may be explained by two models. In Model 2a, due to enforced expression of Lmo2, DN3a thymocytes undergo SCD instead of ACD, producing two DN3a/b daughters that possess enhanced self-renewal properties combined with an unlimited capacity to proliferate. Alternatively, the loss or reduction of ACD could be explained by Model 2b, where molecular changes due to Lmo2 transgene expression induces a rapid DN3a to DN3b transition so there is no opportunity for ACD, and only SCD of DN3b are enabled.
Interestingly, analysis of thymi in CD2-Lmo2 mice show that similar to WT DN3b, the majority of these DN3b thymocytes are blasted, large in size and primed for division. However, unlike WT, most of the CD2-Lmo2 DN3b thymocytes possess an unusually high proportion of intracellular TCR-β, indicating a defect in differentiation to DN4 (Matt McCormack, personal communication). If these altered DN3b thymocytes derive from increased SCD DN3a events then Model 2a would be supported. If these altered DN3b thymocytes arise due to rapid transit of DN3a that prevents proper TCR-β rearrangement then Model 2b would be likely. To formulate any definitive model, the fates of WT and CD2-Lmo2 DN3 divisions would need to be tracked for at least 2 generations to correlate SCD and ACD with fate decisions. If CD2-Lmo2 mice are crossed with Tetβ-LTH-Rag1-/- mice to pause the thymocytes at DN3a, it may be possible to identify the exact stage at which ACD is altered. However, past studies by Drynan and colleagues have shown that mice from CD2-Lmo2 and Rag-/- crosses still develop leukaemia, despite there being no TCR rearrangement (Drynan et al., 2001). CD2-Lmo2 DN3 thymocytes therefore display increased SCD which correlates with self-renewal, but this may either enable or contribute to further transformations that bypass the β-selection checkpoint for leukaemogenesis.

7.4 Cues and conserved mechanisms of polarity and its regulation of ACD

To understand how ACD occurs in thymocytes, we first need to elucidate the cue and the molecular components that initiate and maintain polarity during ACD. In Chapter 6 I demonstrated that CXCR4 is an important polarity cue for Ap2a2 polarisation. However, is CXCR4 the sole polarity cue for ACD? One hypothesis is that in addition to CXCR4, other polarity cues exist for ACD of DN3 thymocytes.

7.4.1 Is Notch1 a potential polarity cue?

In Chapters 3 and 6 I show that Notch1 is recruited to the interface in an overwhelming proportion of DN3 thymocytes during interaction with stromal cells at interphase compared to CXCR4 (up to 70-80%, compared to 25% for CXCR4, Figures 3.8 and 6.6). Presumably these are DN3a thymocytes as Notch1 is needed for DN2-DN3a survival, and its expression drops at DN3b. It is currently unknown whether Notch1 polarisation has any relevance to DN3 ACD, or whether it interacts with other molecules such as Numb to coordinate downstream fate decisions as observed in other models of ACD (Bhakta and Lewis, 2005; Chang et al., 2007; Gonczy, 2008; Wakamatsu et al., 1999; Zhong et al., 1996). However, components of Notch signalling are consistently asymmetric in the daughters of an ACD in other cell systems that are crucial for determining their differential fate (Knoblich, 2008). Moreover, exposure to Notch ligands can prevent HSC differentiation, causing them to maintain a stem cell-like state (Wu et al., 2007). Given that deregulated Notch signalling often results in leukaemia in a variety of animal studies (Hawkins and Russell, 2008), it is possible that polarisation of Notch signalling during ACD is an essential component of leukaemic suppression. In Chapter 6, I was unsuccessful in investigating Notch1 as a polarity cue due to the rapid death that followed when Notch signals were taken away from DN3 thymocytes. However, it may be possible to use low doses of Notch inhibitors such as gamma-
secretase inhibitors to achieve a graded inhibition that would facilitate studying the possible impacts on Numb or Ap2a2 polarisation during DN3 division and subsequent downstream fates. Structurally diverse gamma-secretase inhibitors have been previously used to demonstrate that lower concentration of potent inhibitors impair but not block differentiation of DN thymocytes (Doerfler et al., 2001). Garbe and colleagues have also used graded concentrations of gamma-secretase inhibitors to investigate the requirement of Notch for the proliferation and development of DN3 thymocytes to the DP stages (Garbe et al., 2006). These future studies will indicate whether the inhibition of Notch signalling represents an additional means of deregulating polarity during ACD.

7.4.2 E-cadherin could serve as a possible polarity cue

Other polarity cues may also be provided by the thymic stroma, and one such cue could be the adhesion molecule E-cadherin. E-cadherin is considered essential for the cell-to-cell contacts present in cohesive epithelial tissues, and is known to cooperatively regulate apical-basal polarity in epithelial cells along with Scribble and Dlg family members (Navarro et al., 2005). More importantly, E-cadherin regulates ACD and acts as a polarity cue for spindle orientation in dividing D. melanogaster and murine epithelial cells (den Elzen et al., 2009; Le Borgne et al., 2002), D. melanogaster male germline stem cells (Yamashita et al., 2003), and mammalian neural stem cells (Karpowicz et al., 2009). E-cadherin is expressed by thymic epithelial cells and thymocytes (Lee et al., 1994), and its expression jumps almost 3-fold in DN3a thymocytes compared to any other DN, DP or SP thymocyte subpopulation (Appendix 4). In studies by Muller and colleagues, addition of a monoclonal antibody that blocks homotypic E-cadherin interaction between stromal cells and thymocytes severely reduces DN differentiation to DP stage (Muller et al., 1997). In this light, E-cadherin mediated interactions between DN3 thymocytes and stromal cells could be critical for proper fate decisions. With the aid of the time lapse imaging procedure and TACTICS software platform, it will be important to investigate whether chemokine signalling is the only polarity cue, or if other molecules such as Notch1 or E-cadherin contribute to the cue that enables ACD in DN3 thymocytes.

7.4.3 Could Gpsm2 translate the polarity cues into an axis of polarity during DN3 ACD?

Once the polarity cues have been identified, the next step would be to understand how the cues are translated into an axis of polarity during DN3 division. This would involve identifying the molecular components regulating spindle orientation. In D. melanogaster, polarity during ACD of neuroblasts is held in check by an adaptor protein called Inscuteable and a regulator of Gαi signalling called Pins. Mutations in either of these genes results in loss of spindle orientation, loss of daughter size asymmetry and mistargeting of cell fate determinants (Guilgur et al., 2012; Rebollo et al., 2007; Siegrist and Doe, 2005). In the brain of gpsi−/− mice (Gpsm2/LGN is the mammalian homologue of D. melanogaster Pins), neuroepithelial precursors are unable to undergo ACD and show randomized orientation of normally planar divisions (Konno et al., 2008). In Chapter 3, I show that Gpsm2 is recruited to the stromal site in a high proportion of thymocytes (almost 80%, similar to Notch1 Figure 3.8). Given the known functions of Gpsm2
in regulating spindle orientation and polarity in other systems of ACD, there is a possibility that Gpsm2 is also involved in translating the polarity cues during DN3 ACD. This idea is supported by gene expression analysis of HSCs where overexpression of Ap2a2 correlates with up regulation of Gpsm2 expression (Ting et al., 2011). As Gpsm2 is also responsive to G-protein signalling, it might act downstream of the polarity cue provided by CXCR4 (a G-protein). Future work will require the dissection of this potential pathway to reveal if a hierarchy in signalling exists. Work is currently being undertaken in the laboratory to analyse the thymocyte compartment in gpsm2−/− mice.

7.5 A new player in mediating thymocyte fate - implications for immunity and leukaemia

Fate determination in thymocytes is a complex process, involving many intersecting regulatory inputs, pathways and players. In this thesis I show that DN3 thymocytes undergo ACD as part of their fate determining process, and that this correlates with fate differences in differentiation and survival. Elucidating the molecular components for thymocyte ACD, from the polarity cues, to the markers and the fate determinants involved, has major implications for immune development. Identifying what molecular components for ACD are integrated for thymocyte fate outcome will enable approaches to control thymocyte proliferation, death, differentiation and self-renewal. This will facilitate well-formulated strategies to boost thymic function and immune cell responses in the elderly, to improve current radiation and chemotherapeutic regimens that damage thymic function, and to promote self-renewal of thymocytes following their transplantation into immunocompromised patients.

A particularly interesting finding is that thymocyte ACD is altered in T cell leukaemia. Currently, it is not known how pre-malignant thymocytes integrate signals that result in uncontrolled proliferation, the lack of differentiation, or the enhancement of self-renewal. These thesis studies suggests that aberrations in thymocyte ACD may result from defects in polarity, and that such defects enable the ideal predisposition for leukaemic transformation. The finding that CXCR4 provides the polarity cue, and that polarity might be altered Lmo2 induced leukaemia fits with other studies of T-ALLs, where altered PI3K signalling frequently underpins the transformed phenotype of these cells. Together, with evidence that CXCR4 cooperates with pre-TCR and Notch, this paves the way for examining the responses of Lmo2 induced T-ALL to AMD3100 alone or in combination with other drugs to suggest a new therapeutic potential.

Equally interesting is that Ap2a2 overexpression can promote differentiation that is otherwise impeded in Lmo2 induced leukaemia. It will be important to elucidate how changes in Ap2a2 levels impinge on differentiation, or identify the determinants that are trafficked in Ap2a2 pathways during ACD that impact on thymocyte differentiation. Identifying the components that interact with, or are targets of Ap2a2 during thymocyte ACD may provide a basis for improving T cell leukaemia prognosis and therapy. Given knockouts of any AP-2 subunit results in embryonic
lethality, knockdowns or a conditional knockout of Ap2a2 may be beneficial to understanding the functional requirement of Ap2a2 during thymocyte ACD. It will also be important to investigate whether Ap2a2 transduced Lmo2 thymocytes develop T-ALL with a longer latency period compared to control transduced Lmo2 thymocytes when they are injected into irradiated recipients. This thesis presents an ideal framework for which future in vivo analysis of thymocyte ACD and its consequences can be investigated, and indeed, compared with pathologies associated with leukaemia.

7.6 Conclusions

Studies in the past two decades have established that ACD is an evolutionary conserved process regulating fate determination in many invertebrate organisms. ACD is mediated through interplay of many molecular processes, involving proteins that assist to initiate the axis of polarity, maintain that polarity, and to impart differences in fate decisions. In the past 5 years it emerged that cells of the haematopoietic system; stem cells, B cells and T cells also underwent ACD. In this thesis I describe the first quantitative evidence that DN3 thymocytes undergo ACD, and that situations of aberrant fates such as leukaemia correlate with alterations in ACD. Understanding the mechanisms of polarity regulation represents an important step in our knowledge of DN3 thymocyte behaviour during migration and division, and I show that while some aspects of polarity regulation are conserved, there are other features of polarity that may be regulated entirely differently. The advantage of using an in vitro system paired with time lapse imaging and analytical software is that the molecular mechanisms of polarity during DN3 migration or ACD can be teased apart, and many relationships between polarity cues and signalling on fate outcome can be elucidated at the cellular level. This thesis contributes to new knowledge about polarity during DN3 thymocyte fate determination, and of polarity cues provided by the stromal cells that regulate ACD.
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References


Appendix A  Gene expression profiles of selected ACD regulators in thymocyte subsets. Adapted from www.Immgen.org

Notch1

Numb

Par3

Par6

aPKC
Appendix A Gene expression profiles of selected ACD regulators in thymocyte subsets. Adapted from www.Immgen.org
Appendices

Appendix B  Animal ethics approval

The Peter MacCallum Cancer Institute Animal Experimental Ethics Committee (AAEC) has approved animal use on above project from 28th February 2008 to 28th February 2011.

Approval is subject to:


2. Submission of satisfactory Investigator Annual Report to the AAEC and Bureau of Animal Welfare Animal Use Report for each calendar year. Please note: a final report is to be submitted to the AAEC within six months of completion of the project.

3. Immediate reporting of any adverse occurrences which impact on the welfare of the animals OR unexpected mortality OR breaches of protocol relating to animal use within 48 hours.

4. Seeking approval from the AAEC using a "Application for Minor Amendment to Approved Project" form:
   a. Change in animal housing or research location
   b. Change in Procedures
   c. Change in animal numbers or species
   d. Extension of project

5. Seeking approval from the AAEC for additional co-investigators using a "Application for Additional Co-Investigator" form.

6. Compliance with any special conditions specified by the AAEC: No.

7. Special responsibilities by Animal Facility Staff: NO

The investigators are to be reminded to use extreme care with monitoring and handling mice used in this project.

Approved by,

Handwritten Signature

Dr Phillip Darcy
Chairperson
Animal Experimental Ethics Committee
Peter MacCallum Cancer Centre

Document Version 1: 20/02/2008
ANIMAL EXPERIMENTAL ETHICS COMMITTEE

Notification of Project Approval

To
Dr Patrick Humbert, Chief Investigator
Dr Sarah Russell, Chief Investigator

From
Dr Phil Darcy, Chairperson, Animal Experimental Ethics Committee (AECC)

Cc
Jo Phipps-Nelson, AECC Secretary/Research Governance Officer
Brent Fullerton, Animal House Manager

Notice of approval of AECC application
1349
Investigating the role of polarity regulators in development, immune function, wound healing and cancer
28th February 2008

The Peter MacCallum Cancer Institute Animal Experimental Ethics Committee (AECC) has approved animal use on above project from 28th February 2008 to 28th February 2011.

Approved by,

[Signature]

Dr Phillip Darcy
Chairperson
Animal Experimental Ethics Committee
Peter MacCallum Cancer Centre

Document Version 1: 20/02/2008
Appendix C  Polarisation ratio heatmaps of proteins investigated in the ACD study

Polarisation ratios converted to single pixels with increasing threshold (0-100%) for dividing DN3 thymocytes expressing control (left) and candidate protein (right). The 25% threshold cutoff is shown on each heatmap (dashed line).
Appendix D  Summary of ACD study. T-test graphs and scatterplots of paired differences between control PR and test protein Polarisation Ratios (PR)

T-test graphs and scatterplots of paired differences between control PR and test protein Polarisation Ratios (PR)

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<th>Control</th>
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