Deciphering Cell-Autonomous Mechanisms for Fate Determination

By An-Chi CHANG

Submitted in total fulfilment of the requirements

of the degree of Doctor of Philosophy

2022

The Peter MacCallum Cancer Centre

&

The School of Science, Computing and Engineering Technologies

Swinburne University of Technology

Abstract

Understanding the control of cell fate determination can provide insights for tissue development, immunity and disease evolution. With respect to tissue development, mitosis influences tissue architecture as the daughter cell requires its own space in the tissue microenvironment. The spatial dynamics during mitosis must be well controlled, otherwise the order of tissue homeostasis will break down. Therefore, in this thesis I focused in two chapters on dissecting the mechanism of daughter cell positioning. The results suggested Scribble and E-cadherin provide a platform to stabilise the morphological changes during mitosis, and potentially protect the daughter cells from being extruded. With respect to immunity, T cell development influences a major source of adaptive immunity. However, T cell development is not a straight-forward event of cell differentiation, but is propagated with multiple fate determination events such as survival, death, self-renewal or differentiation. One crucial test for fate determination in T cell development is the β-selection checkpoint. In this thesis, I found that HDAC6 inhibition during T cell development altered the control of β-selection checkpoint, exposing a transitional population we called 'DN3b^{Pre}'. My results suggested a profound fate determination must be coordinated in DN3b^{Pre} to ensure only the most qualified T cell is generated. Needless to mention, generating functional T cells can enhance the chance to combat cancer, but T cell generation *per se* is error-prone, which leads to leukaemia initiation. Therefore, a broad and deep understanding of fate determination during T cell development is urgent. Overall, in this thesis, I demonstrated molecular mechanisms that coordinate cell fate determination.

Unlike many well-proven biological theory that fate determination is guided by extracellular signalling and cell-cell communication, the mechanisms I found are prone to be cell-autonomous. That means fate determination of each cell in part is controlled by the cell itself. This is a mysterious driving force that involves the fingerprints of epigenetics such as HDAC6 activity takes the control of T cell fate. In this thesis I exposed multiple possibilities by which transcriptional control through HDAC6 might govern the cell-autonomous fate development. On the other hand, I found Scribble-E-cadherin complex plays a junctional-independent role, which means the effect was not from neighbouring cells. This is a first time that the protein function of Scribble-E-cadherin is demonstrated as cell-autonomous signalling, and this attribute shapes cell division. Overall, my results contribute to deciphering cell fate determinations with cell-autonomous mechanisms.

Publication and Preprints

Publication and preprints for publication arising from the works described in this thesis:

Anchi S. Chann, Sarah M. Russell; An integrated transcriptional switch at the β-selection checkpoint determines T cell survival, development and leukaemogenesis. Biochem Soc Trans 30 August 2019; 47 (4): 1077–1089. doi: https://doi.org/10.1042/BST20180414

Anchi S. Chann, Ye Chen, Tanja Kinwel, Patrick O. Humbert, Sarah M. Russell; A Scribble-E-cadherin complex controls daughter cell patterning by multiple mechanisms. bioRxiv 2021.04.15.440081; doi: https://doi.org/10.1101/2021.04.15.440081

Anchi S Chann, Mirren Charnley, Lucas Newton, Andrea Newbold, Patrick O Humbert, Ricky W Johnstone, Sarah M Russell; Stepwise progression of β-selection during T cell development as revealed by histone deacetylation inhibition. bioRxiv 2021.10.03.462949; doi: https://doi.org/10.1101/2021.10.03.462949

Declaration

This is to certify that:

i) This thesis comprises only my own original work towards the degree of Doctor of Philosophy, except where indicated and acknowledged in the Preface.

ii) 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

iii) 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

iv) For the publication from this thesis, my name is spelled as Anchi S Chann due to the identification of multiple authors spelled as An-Chi Chang, which is same to my name spelled in English used in my passport.

An-Chi Chang

(Also known as Anchi S Chann)

March, 2022

Preface

I acknowledge the assistance of the following experts who contributed to the efforts described or applied in this thesis:

Professor Patrick Humbert (La Trobe University) for a countless support covering every results chapters, and every Humbert lab members, particularly for: Ms Tanja Kinwel for technical assistance and the cell lines construction which used in Chapter 5 & 6; Mr Lucas Newton for collecting mice fetal livers which used in Chapter 3 & 4.

Professor Ricky Johnstone (Peter MacCallum Cancer Centre) for many generous supports which used in Chapter 3 & 4, and Johnstone lab members, particularly for: Dr Andrea Newbold for the arrangement of pregnant mice and the essential reagents, and Mr Peter Fraser for technical assistance of mice experiments.

Professor Juan Carlos Zuniga Pflucker (University of Toronto, Sunnybrooke Health Sciences Centre), for the OP9 stromal cell lines used in Chapter 3 & 4. Associate Professor Kaylene Simpson (Peter MacCallum Cancer Centre), Dr Sue Haupt (Peter MacCallum Cancer Centre) and Associate Professor Louise Cheng (Peter MacCallum Cancer Centre) for sharing several reagents.

Regarding the facility training, I deeply acknowledge to Dr Sarah Ellis (Peter MacCallum Cancer Centre) for the confocal/ SIM microscopy induction, Dr Mirren Charnley (Swinburne University of Technology) for the flow cytometry induction and the lab maintenance, Dr Ye Chen (Swinburne University of Technology) for the N-STORM microscopy and the idea sharing, Dr Amr Allam (Swinburne University of Technology) for sharing laboratory techniques and discussions, and Dr Callum Dark (Peter MacCallum Cancer Centre) for RNA experiments.

Regarding the reviewing for the thesis writing and the associated manuscripts for publication, I deeply acknowledge to Dr. Karl Poetter, Dr John Lock (University of New South Wales), Associate Professor Elaine Sanij (Peter MacCallum Cancer Centre), Professor Ellis Reinherz (Harvard Medical School) and Dr Jonathan Duke-Cohan (Harvard Medical School) for helpful suggestions.

Acknowledgements

My doctoral training was committed after I had started my industrial career. I clearly remembered at the time I received the offer of PhD scholarship from Swinburne, I was in a meaningful trip at Izu peninsula, where I made a decision of life. Now, after this PhD training, I reconstructed my technical capacity, scientific thinking and future plan to work in science. To this outcome, I can't be more thankful to, who almost solely contributes to my academic transformation, Professor Sarah Russell.

Amid my PhD training, in 2019 there was a 7-month academic visit in Europe, including France, Portugal and Germany. I would like to thank Dr Bassam Hajj (Institut Curie) and Dr Veer Keizer (Institut Curie) for hosting my stay as a research intern, and for mentoring me to accomplish a fantastic project of particle trafficking in cell nucleus. Many thanks to every LOCCO lab members as well for many inspiring discussions. This visit significantly refuelled my momentum to finish the research for my doctoral thesis. The origin of this European tour was extended from the scholarship awarded by Dr Judith Kinnear (Swinburne University), I deeply thank for her generous offer.

I'm finishing my PhD during the pandemic. That means I have been unexpectedly separated from my family for too many years. To all the overcoming over many challenges, I would thank to my family at about 7000 km away from Melbourne. The future to share with you will be much more promising than any past timing.

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).

A

ACD Asymmetric Cell Division
Alpha-MEM Minimal Essential Medium Alpha Modification
AIRE Autoimmune Regulator
AJ Adherens Junction
AP Adaptor protein
APC Adenomatous polyposis coli
aPKC atypical Protein Kinase C
ATM Ataxia Telangiectasia Mutated

B

BCL B-cell lymphoma
β-Pix beta PAK-interacting exchange factor
Baz Bazooka
BM Bone marrow
BSA Bovine Serum Albumin

C

CAR Chimeric antigen receptor
CD Cluster of Differentiation
CFSE Carboxyfluorescein diacetate
CLP Common lymphoid progenitors
CMP Common myeloid progenitor
CNS Central Nervous System
Crb Crumbs
CRISPR Clustered regularly interspaced short palindromic repeats
CSK C-terminal Src kinase

D

DAPI 4′,6-diamidino-2-phenylindole
Dlg Discs Large
DLL1 Delta-like1
DLL4 Delta-like4
DN Double negative
DNA Deoxyribonucleic acid
DNMT DNA methyltransferase
DP Double positive
DSB DNA double-strand break
DMSO Dimethylsulfoxide

E

ECM Extracellular matrix
EDTA Ethylene diamine tetraacetic acid
EGF Epidermal growth factor
EGFP Enhanced green fluorescent protein
ERK Extracellular signal regulated kinase
ETP Early T lineage Precursor

F

FACS Fluorescence-activated cell sorting
FL Fetal liver
FLT Fms-like tyrosine
FITC Fluoroscein isothiocyanate
FSC Forward scatter

G, H

GEF Guanine nucleotide exchange factor
GMP granulocyte-macrophage progenitors
GTPase Guanine tri-phosphatase
HDAC Histone deacetylase
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPV Human Papilloma Virus
HTLV Human T-cell lymphotropic virus
HSC Haematopoietic stem cell

I-L

ICN Intracellular Notch
IL Interleukin
ISP Immature single positive
kDa kiloDalton
LAT linker for activation of T cells
LEF Lymphoid enhancer factor
Lgl Lethal giant larvae
LGN Leucine-Glycine-Asparagine
Lmo LIM-only
LSC Leukemic stem cell

M

MamL Mastermind-like
MAPK Mitogen activated protein kinase
MEP megakaryocyte-erythrocyte progenitors
MFI
MHC Major histoincompatibility complex
miR microRNA
MPP Multipotent progenitor
MRN Mre11-Rad50-Nbs1
MSD Mean Squared Displacement
MTOC Microtubule-organising centre

N

NK Natural Killer
NICD Notch intracellular domain
NOD Non-Obese Diabetic
NuMA Nuclear Mitotic Apparatus

O, P

Par Partitioning defective
PBS Phosphate-buffered saline
PDZ PSD-95/Dlg/ZO-1
PI Propidium Iodide
PI3K Phosphoinositide 3-kinase
PLA Proximity Ligation Assay
PTA Peripheral tissue antigens
PTM Post-translational modification

Q, R

RAG Recombination-activating gene
RhoGEF Rho guanine nucleotide exchange factor
ROCK Rho-associated kinase
RNA Ribonucleic acid
rpm revolutions per minute

S

SCD Symmetric Cell Division

SCF Stem cell factor

SCID Severe Combined Immunodeficiency

Std Stardust

SP Single positive

SSC Side scatter

T-Z

T-ALL T cell acute lymphocytic leukaemia

TCR T Cell Receptor

TCF T-cell factor

TF Transcription factor

Th T helper

TPA Tetradecanoylphorbol-13-acetate

WT Wildtype

Outline of the Thesis

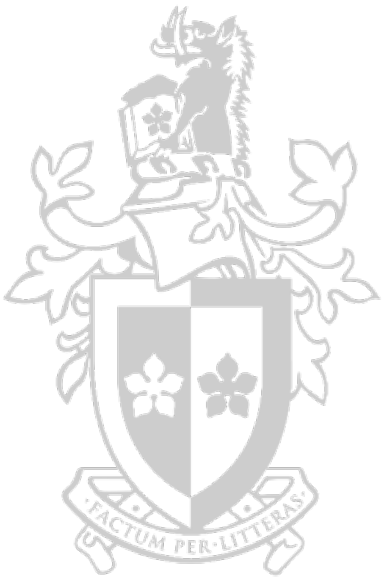
Contents of the Chapters

Chapter 1. Introduction

Chapter 2. Materials & Methods

List of Figures

List of Tables



Chapter 1

Introduction

1.1 Overview of the thesis

1.1.1 A dissertation to study cell fate determination

In any living organism, cell fate determination never stops. The identity of a future cell is determined, or is initiated to be determined, in the current cell (Sagar and Grün, 2020). Fundamental events of fate, for instance, are survival, death, proliferation, differentiation, and self-renewal. Multiple factors are involved to help determine these events of fate, for example, contact-based signals (eg. cell-environment interaction), long-distance effectors (eg. hormone), and cell-autonomous determinants (eg. self-organization of genome) (Chan et al., 2017; Tsuchiya et al., 2020). Here I applied two valuable cell systems (See Section 1.1.2) to study the mechanisms for fate determination, and hope to elucidate cell-autonomous attributes.

1.1.2 Two cell systems were investigated: Immune cells and epithelial cells

Two types of cells were investigated in this study: the first is developing T cell (Chapter 3 and 4) and the second is mammary epithelial cell, MCF10A (Chapter 5 and 6). The motivation to dissect the fate-determining mechanism in developing T cells is because pre-TCR signal serves as a self-examination platform to facilitate a proper T cell selection (Chann and Russell, 2019a), but the molecular sequences that drive fate consequence is not clear. In this study, I used an epigenetic inhibitor ACY1215 to influence the development of DN3 cells, and I proposed novel insights from tracking the changes in fate determination following β-selection.

In the second case, I focused on the process of epithelial cell division. As positioning daughter cells is important for maintaining epithelia homeostasis (Le Bras and Le Borgne, 2014), I studied how mitotic spindle is positioned autonomously, and how two daughters adhere following mitosis. I proposed Scribble and E-cadherin form a complex to guide appropriate positioning of daughter cells. Overall, in this dissertation I used developing T cell and MCF10A cell division to investigate mechanisms for fate determination.

1.1.3 Perspectives

Understanding T cell development is important. At least two contexts are tightly associated with T cell development: T-cell acute lymphoblastic leukemia (T-ALL) and bone marrow transplant. T-ALL is a kind of lethal leukaemia partly driven by the error in T cell development; for instance, Notch1 and Lmo2 promote hyper-proliferation of developing T cell, resulting in the immature T cell bypassing the β-selection checkpoint (Tan et al., 2017).

Many patients with blood malignancies, including T-ALL patients, often require bone marrow transplant (Hamilton et al., 2017). During bone marrow transplantation, the T cell development should be carefully monitored as the new cycles of T cell generation will occur (Moutuou et al., 2018), and the newly imposed haematopoietic progenitors in thymus might induce leukemogenesis as shown in a murine model (Paiva et al., 2021). Immunity re-engineering within the associated procedures such as T cell depletion and 'T-Cell Add-Back' requires a competent thymus (Montero et al., 2006; Moutuou et al., 2018; Sutrave et al., 2017). These concerns of leukemogenesis suggest strong knowledge of T cell development is pivotal.

Another focus of the thesis is fate determination during cell division of breast epithelial cell. Mechanisms of daughter cell positioning were elucidated here, showing the link between spindle orientation and daughter cell positioning in the scale of single mitotic cell. The study addressed the control of spindle positioning checkpoint that can minimise the failure during cytokinesis (Caydasi et al., 2010), therefore illustrated how aneuploidy is avoided (Nicholson et al., 2015). Moreover, as a recent evidence showing cell division contributes to the protrusion of cancer cells (Bonnet et al., 2021), the abnormality of daughter cell positioning likely causes the wrong adaption to the tissue, which leads to a breakdown of tissue homeostasis and the cancer-like dissemination. Thus, understanding of daughter cell positioning can benefit the prevention of cancer initiation.

1.2 An introduction to T cell development

T cell development is pivotal to generate a functional adaptive immune system against infectious disease and cancer. The thymus is the organ to produce T cells. Children born without a thymus because of an inability to form a proper third pharyngeal pouch during embryogenesis (DiGeorge Syndrome) were found to be deficient in T cells (Chinen et al., 2003). Patients of Severe Combined Immune Deficiency (SCID) have a thymus but cannot produce lymphocytes because of defects in enzymes required for somatic recombination (Gennery, 2019). T cell development is greatest during fetal development and before puberty. After puberty the thymus shrinks and T cell production declines; in adult humans, removal of the thymus does not compromise T cell function. Overall, understanding T cell development is important to support primary immune generation and immune regeneration after stem cell transplantation (Krueger et al., 2017). In this section, I introduce T cell development and how functional T cells are selected.

1.2.1 Hematopoietic stem cell differentiates T cell

Hematopoietic stem cells (HSCs) have the potential to derive immune cells (**Figure 1. 1 A**): erythroid, granulocyte and monocyte, megakaryocyte, and lymphocyte. HSCs or hematopoietic progenitors are multipotent, and this multipotency is maintained by self-renewal. Following cell division, hematopoietic progenitors are differentiated to myeloid stem cells or lymphoid stem cells. Lymphoid stem cells then differentiate to T cells (Rothenberg et al., 2010). HSCs grew in a bone marrow niche, surrounded by endothelial cells and mesenchymal stromal cells, and are differentiated to lymphoid progenitors. The lymphoid progenitors in bone marrow migrate through blood vessels then arrive at the thymus, where T cell fate is committed and developed (Shortman and Wu, 1996). The thymus is a multi-lobed organ composed of the cortical and the medullary areas. Early T cell precursors (ETPs) enter the cortical areas, where the cortical epithelial cells form a niche for ETP proliferation (Dzhagalov and Phee, 2012). Following ETP differentiation, cells move from the cortex to the medulla of the thymus, undergoing multiple steps of differentiation to become mature naive T cell. In short, T differentiation in thymus follows the process: Early T-cell precursor (ETP) transits through $CD4^-$ $CD8^-$ double negative (DN) stage to $CD4^+$ $CD8^+$ double positive (DP) stage, then becomes mature $CD4^+$ or $CD8^+$ single positive T cells (**Figure 1. 1 B**). In the progression of DN stage, 4 major steps are defined: First, the potential for non-T cell lineages is progressively lost from DN1 ($CD44^+$, $CD25^-$) to DN2 ($CD44^+$, $CD25^+$) stage. The T-cell fate is committed in DN2 and DN3 ($CD44^-$,

$CD25^+$) stage. DN3 cell undergoes gene recombination to create a TCRβ chain. In the phase that TCRβ gene is recombined, a process called β-selection is implemented to ensure DN3 cell can present a qualified TCRβ chain (see Section 1.2.2). DN3 stage is followed by DN4 ($CD44^-$, $CD25^-$) and DP, where TCRα is recombined. The TCRα chain is then conjugated with the TCRβ chain, forming a αβTCR receptor. The quality of αβTCR at DP cell is then tested by positive selection (see Section 1.2.4) and negative selection (see Section 1.2.5). Because of these three selection steps (β, positive, and negative), most of cells entering thymus were selectively eliminated by apoptosis if the features of T cell aren't correctly developed. The selection mechanism of the four major programs of early T cell selection were introduced in the following Section 1.2.2-1.2.5.

1.2.2 Early T cell selection (1): β-selection

The T cell of αβ lineage is firstly selected by a program called β-selection (Chann and Russell, 2019b). As TCRβ chain is not encoded in the germline, an additional gene rearrangement must be applied. The process to rearrange TCRβ gene and to express a TCRβ chain occurs at the DN3 stage of T cell development. There are two sub-stages in DN3, DN3a (DN3, $CD28^-$) and DN3b (DN3, $CD28^+$). At the DN3a stage, the enzymes to rearrange TCRβ gene, RAG1 and RAG2, are expressed and gene recombination is initiated, with the first step of D-J joining and the second step of V-DJ joining. At the DN3b stage, the recombined TCRβ gene is expressed, together with a pTα chain (a surrogate of the α chain), to form a pre-TCR complex. Pre-TCR signals dictate cell survival and development, therefore determining passage through the β-selection checkpoint to ensure cells without appropriate TCRβ die. (The fate-determining mechanisms at the stage of β-selection are reviewed in the Section 1.4.) Subsequently, once the pre-TCR is formed, cells can pass the β-selection checkpoint, and enter DN4-DP stage. When the cells are at the DP stage, RAG1/2 are re-expressed to assist the generation of the TCRα chain. By replacing the pTα chain, a TCRα chain and a TCRβ chain are paired as a TCR receptor.

1.2.3 Early T cell selection (2): γδ-selection

Although αβ T cell represents a major component of all T cells, there are 0.5–16% γδ T cells that reside in peripheral blood (Deseke and Prinz, 2020). γδ T cells play multiple roles in pathogen clearance, immunosurveillance, cancer, and autoimmune diseases. Being the offspring of lymphoid progenitors, similar to the αβ T cell, the γδ T cell is also specified at DN3a stage and/or in DN2 stage, whose TCR gene recombination is also controlled by RAG1/2 (Parker and Ciofani, 2020). It was reported that γ chain and δ chain are rearranged simultaneously with β chain. Interestingly, δ chain segments of the genome are located

within the regions of the α chain gene sequences. Therefore, it was hypothesised that if δ chain is arranged, the cell is selected to be γδ T cell as the formation of α chain is impossible (Wilson et al., 1996). γδ T cells do not progress to DP stage, and most of γδ T cell remains in a DN surface phenotype (Parker and Ciofani, 2020). Intriguingly, some γδ T cell can be generated in a mouse that lacks thymus, suggesting the general principle above is not completed for the entire γδ-selection (Nonaka et al., 2005).

1.2.4 Early T cell selection (3): Positive selection

For the αβ T cell lineage, a program called positive selection has evolved for DP cells that presents with αβ TCR. The goal of positive selection is to ensure only the DP cell that can interact with MHC can survive, all others will die by neglect within 3-4 days (Klein et al., 2014). Only about 10% of DP cells survive positive selection (Klein et al., 2014). Cortical thymic epithelial cells (cTECs) present a 'private' peptide (also called self-peptide) through a surface MHC class I complex or MHC class II complex. To interact with p-MHC-I or p-MHC-II (p = peptide), a CD8 single positive (CD8-SP) T cell differentiates from DP to bind p-MHC-I, while a CD4 single positive (CD4-SP) T cell differentiates from DP to bind p-MHC-II. The choice of CD4-SP or CD8-SP differentiation is controlled by the transcription factors ThPOK (for CD4) and Runx3 (CD8). ThPOK and Runx3 antagonize each other, enforcing an absolute CD4 or CD8 lineage specification (Taniuchi, 2018). Interestingly, it was suggested that MHC peptide *per se* seems able to influence the choice of CD4/CD8 lineage differentiation (Karimi et al., 2021).

A human immune deficiency called Bare Lymphocyte Syndrome (BLS) is caused by a defect in MHC-II gene expression. CD4 cell production is deficient in BLS patients because of the failure in positive selection. In addition, an inability to express MHC-II means the humoral immunity is also impaired. Overall, the BLS patients often suffer from severe infection, and often die before puberty (DeSandro et al., 2000). HSCs or bone marrow transplantation are the appropriate approach to overcome MHC-II deficiency (Lum et al., 2019). On the other hand, for individuals having dysfunction of MHC-I expression, the development of CD8 T cell is impaired from the stage of positive selection, which lead to disability in cancer cell elimination (Dhatchinamoorthy et al., 2021). Therefore, the error in positive selection is tightly associated with human disease.

1.2.5 Early T cell selection (4): Negative selection

After positive selection, the goal of negative selection is to eliminate T cells that are highly activated by self-antigen. To achieve this, the strong affinity of MHC/self-peptide to TCR

induces apoptosis. This mechanism is important to prevent T cells that attack tissue after they leave the thymus, and enables 'self-tolerance' (Hwang et al., 2012). SP cells that survive positive selection migrate to the thymic cortico-medullary junction where cells interact with mTEC, macrophages and dendritic cells (Inglesfield et al., 2019). Both macrophages and dendritic cells are known to present self-peptide and help negative selection, while a recent study also suggests phagocytosis is also involved in the thymic negative selection (Kurd et al., 2019). Regarding presenting self-peptide, mTEC is a unique cell that can express an enormous diversity of peripheral tissue antigens (PTAs) to filter out the autoreactive SP cells. A master gene, called 'autoimmune regulator' (Aire) gene, controls the expression of PTAs. It is well known that AIRE mutation causes loss of self-tolerance and leads to autoimmune disease (Perniola, 2018).

As the mechanism of positive and negative selection is based on TCR-pMHC adhesion, the threshold of adhesion signals to determine survival or death must differ between positive and negative selection. Otherwise, cells with high affinity that survived in positive selection will die in the negative selection; or cells with low affinity is removed in positive selection, yet that level of affinity is appropriate for negative selection. To explain this phenomenon, a differential avidity model was proposed. This model suggests the same peptide-MHC complex delivers signals for both positive and negative selection; while avidity to positive selection is low, but to negative selection is higher (Ashton-Rickardt et al., 1994). Another model is the differential signalling model. Unlike the differential avidity model, this model suggests different signals, in terms of quality and type, are delivered differently during positive and negative selection. For example, signals of MAPK pathway stimulate some peptide-specific interaction for positive selection but not negative selection (Alberola-Ila et al., 1996).

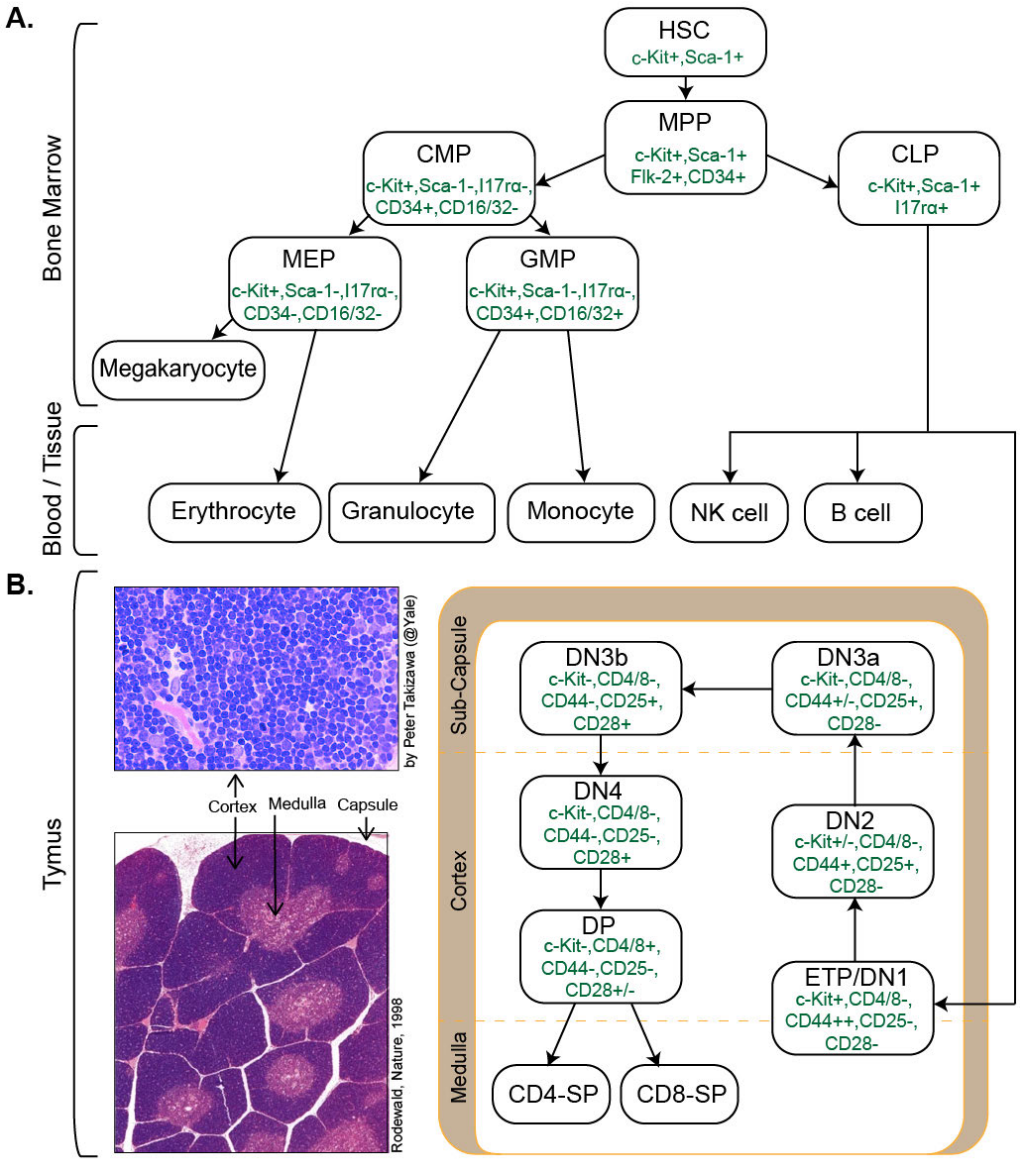


Figure 1. 1 The differentiation from HSC to T cell

(A) The hematopoietic stem cells (HSCs) in bone marrow differentiate to the multipotent progenitor (MPP), which differentiates to the common lymphoid progenitors (CLP) and the common myeloid progenitor (CMP). In myeloid lineage, the megakaryocyte-erythrocyte progenitors (MEP) and the granulocyte-macrophage progenitors (GMP) differentiate from CMP. In lymphoid lineage, T, B and NK cell are differentiated in the blood or other associated tissue. The surface markers (green), adapted from (Challen et al., 2009), indicated mouse HSC differentiation. **(B)** Thymus histology was shown in (Left), adapted from (Rodewald, 1998). The differentiation from the early thymic progenitors (ETP) to CD4-single positive or CD8-single positive cells are illustrated in (Right). The surface markers (green) indicated the differentiation of mouse thymopoiesis from ETP, DN1-4 (double negative 1-4) to DP (double positive).

1.3 An introduction to signalling for thymopoiesis

1.3.1 Signalling in the bone marrow for lymphopoiesis

The bone marrow is a major lymphoid organ not only because it is where hematopoiesis and lymphopoiesis take place, but also because it is a permanent home for primed or naïve T cells (Pabst, 2018). Therefore, understanding the signalling in bone marrow will underpin the investigation of T cell development. For example, many cytokines like SCF (stem cell factor), IL3 (interleukin-3) and IL6 (interleukin-6) are used in the protocol to generate T cell *ex vivo* from HSCs. SCF, also named as Kit ligand (Kitl), is expressed by the cells of a heterogeneous source (eg. fibroblasts and endothelial cells). The major role of SCF is for inducing hematopoiesis. It was recently reported that SCF is synthesised by a type of leptin receptor+ (LepR) stromal cells and endothelial cells in bone marrow to support the growth of c-kit+-restricted hematopoietic progenitors. Interestingly, LepR+ cells are 100 times more numerous than HSCs in bone marrow, creating a ubiquitous niche for the differentiation of c-kit+-restricted progenitors (Comazzetto et al., 2019). It was also shown a bone marrow Osterix+ adipocyte expressed SCF for inducing haematopoiesis (Zengdi et al., 2019). Usually, the effect of haematopoiesis of SCF is synergised through the cooperation with IL-3 and IL-6 (Luskey et al., 1992), but it was recently shown that IL-3 can be alone to stimulate the embryonic hematopoietic specification from the induced pluripotent stem cell (iPSC) (Mania et al., 2020). IL-6 is well demonstrated to support haematopoiesis, probably by means of promoting HSC proliferation (Bhardwaj et al., 2021). It was recently demonstrated that the expression of IL-6 receptor (IL6R) on HSC is triggered by Notch signalling, which lead to HSC proliferation (Tie et al., 2019). On the other hand, the IL-6 expressed by the mesenchymal stromal cell was shown to repress the proliferation of the activated T cell, suggesting an effect of immunosuppression in bone marrow is regulated by IL-6 (Dorronsoro et al., 2020).

1.3.2 Thymic signalling for T cell development

T cell progenitors become T cells in the thymus. T cell development in the thymus is brutal, as a vast majority of developing T cells die in thymus because of a failure in several selection mechanisms (see in Section 1.2.2 – 1.2.5). The thymus is comprised of various thymic epithelial cells, organised in highly specified niche-like microenvironments which trigger different signals such as the Notch and Hippo pathway (Wang et al., 2020; Weerkamp et al., 2006; Yamauchi and Moroishi, 2019). The most explored signal is Notch1. Notch1, with its downstream mediator Hes1, can suppress the differentiation of

non-T lineages, and guide T cell development to at least DN3b stage (**Figure 1.1**) (Souabni et al., 2002). After T cell fate commitment, the expression of Notch1 in developing T cell itself favours αβ fate determination (Ciofani et al., 2006; Wolfer et al., 2002), as Notch1 signalling is also required for suppressing TCRγ and δ expression, termed 'TCRγδ silencing' (Yui et al., 2010). This αβ fate determination is suggested to be a synergy between pre-TCR and Notch signalling (Hayday and Pennington, 2007). In addition, although both Notch1 and Notch3 are upregulated in DN3 stage (as mRNA expression, ImmGen), current research suggests that Notch3 preferentially promotes γδ T cell development (Shi et al., 2011). Based on the clear understanding of Notch1 signalling in T cell development, one of the most popular experimental models for studying T cell development uses a bone marrow-derived cell line (OP9) that expresses the Notch1 ligand Delta-like 1 (DL1) to coculture with hematopoietic stem cells. In this method (ie. OP9-DL1), the HSC can be effectively exposed to Notch1 signalling, and forced to become a T cell.

In OP9-DL1 system, the DP differentiation from DN was impaired if IL-7 was added in the culture media (Balciunaite et al., 2005), suggesting IL-7 signalling negatively regulates the maturation of early αβ T cell development. Through depleting IL-7 in mice, the DN3b cell exhibited this αβ-inhibitory effect when exposed to recombinant IL-7 (Boudil et al., 2015). However, as shown in the same study, this inhibition was not continued at later stages, as DN4 survival and proliferation positively relied on IL-7 signaling, by which stage the IL-7 receptor (IL7R) was depleted (Boudil et al., 2015). This differential requirement can probably be explained by the fact that DN4 population contains a higher heterogeneity of cell type, which might respond to IL7 signalling differently. The best known is the γδ T cell, which is a lineage that required IL-7 signalling to develop (Kang et al., 2001; Parker and Ciofani, 2020). The difference of IL-7 distribution in thymus can also explain the stage-specific effect of IL-7 signalling. In thymic medullar, more IL-7 was expressed than in cortex; this observation might support the observation of DP repression (Hong et al., 2012). Strikingly, in OP9-DL1 system, the developing T cells from the mice engineered with an IL-7 transgene were skewed in development to display certain B cell phenotype and gene profiles. Subsequently, a signal antagonism between Notch1 and IL-7 was proposed (El-Kassar et al., 2012). In summary, IL-7 signal regulates T cell development effectively, with complexity covering the dose-dependent, the lineage-dependent, and the stage-dependent factors.

A tyrosine kinase receptor Flt3 also contributes to T cell development, and the ligand was often supplied for enhancing *ex vivo* T cell generation. The knowledge so far indicated the role of Flt3 is involved in the early stage of thymopoiesis, as Flt3 is expressed in HSC, ETP, DN1 but not after DN2, and Flt3L depletion significantly decreased thymocyte numbers (Sambandam et al., 2005). In different circumstances, ie. after a bone marrow transplant, the newly implanted T cell required Flt3 to generate DN3 and DN4 populations (Wils et al., 2007), suggesting a continued requirement of Flt3 signalling throughout the 4 DN stages to support the T cell development. On the other hand, Flt3 signalling is also required for B lineage development (Ray et al., 1996; Sambandam et al., 2005).

In summary, many cytokines are essential for T cell generation, and were supplied with the OP9-DL1 to induce T cell differentiation. These essential cytokines are SCF, IL-3, IL-6, IL-7 and Flt3L. Their stage-specific effects were illustrated in the schematic below (**Figure 1. 2**).

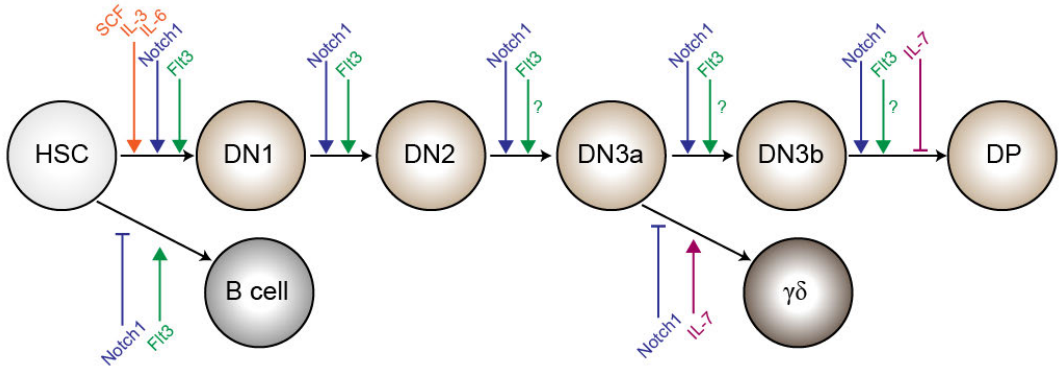


Figure 1. 2 The essential signalling during HSC differentiation to T cell

The schematic shows the stage-specific requirements of cytokines (SCF, IL-3, IL-6, Flt3L, IL-7), and the Notch1 signalling from stromal cells for T cell development, B cell diversion and γδ T cell specification. The arrow represents 'positively regulation', and the bar represents 'negative regulation.'

1.4 A review focusing on fate determination during β-selection

Part of section 1.4 was published in (Chann and Russell, 2019a).

A key attribute of T cell immunity is the expression of a distinct T cell receptor (TCR) on each T cell, which confers individual specificity to different antigens. The diversity of TCR is not encoded in the germline, but is sequentially generated during T cell differentiation. For most T cells, this involves recombination of TCRα and TCRβ genes to generate unique pairs of TCRα and TCRβ chain (hence the name αβ T cell) with diverse specificity (Elhanati et al., 2018). Navigation of genomic recombination during development imposes risks, and many cells fail to produce effective TCRαβ, or become predisposed to leukemic transformation because of errors in genomic recombination. To circumvent or reduce these risks, and to ensure that inappropriately rearranged cells are deleted, the cells must pass through rigorous quality control checks. The first such checkpoint is termed the 'β-selection checkpoint', and occurs in the thymus just after the thymocyte fully commits to T cell development (this stage is therefore termed the β-selection stage). The β-selection checkpoint serves to test whether the cell has appropriately recombined a TCRβ gene. Cells that pass the β-selection checkpoint are permitted to survive, differentiate, proliferate, and therefore proceed to next stage of TCRα recombination (Mallis et al., 2015a).

Changes in cellular activity triggered by the β-selection checkpoint are marked by expression of the cell surface receptors, CD27 and CD28, and mediated by a profound alteration in the transcriptional and protein expression profiles of the cells (Mingueneau et al., 2013b; Taghon et al., 2006; Williams et al., 2005). The role of individual transcription factors at the checkpoint has proven difficult to dissect, in part because they often also play a role at earlier or later stages, and in part because of redundancy and negative feedback loops that are embedded in the system. In this review, we provide an update on the current understanding of how transcriptional control is regulated at the β-selection checkpoint to coordinate survival, apoptosis, proliferation and αβ specification. We also discuss how errors in transcriptional control could override the β-selection checkpoint and trigger leukemogenesis.

1.4.1 The β-selection checkpoint is an essential step in T cell development

The first step in the adoption of antigen specificity by a developing αβ T cell involves the rearrangement of multiple loci (designated as V-(D)-J regions) within the TCRβ gene

segments **(Figure 1.3)**. Most cells will not produce a correct TCRβ chain (for instance, two thirds will be out of frame) (Krueger et al., 2017). Thus an important role of the checkpoint is to allow only those cells with a correct TCRβ to survive. The hallmark of a correctly rearranged TCRβ chain is its capacity to associate with its partner TCRα and relocate to the cell surface in developing T cells, the TCRα will be rearranged only after TCRβ chain is successfully generated. Thus, the newly rearranged TCRβ chain cannot act as a traditional ligand-specific TCR because the cell does not yet have a rearranged partner (TCRα). Instead, a surrogate partner called the pre-TCRα chain, encoded by the pTα gene, is expressed at this developmental stage to partner with the nascent TCRβ. The transient partnership between pre-TCRα chain and TCRβ chain enables pre-TCR signalling allows cells to 'pass the TCRβ checkpoint', and subsequently survive, differentiate and proliferate. It was previously assumed that pre-TCR signalling must be ligand independent, but recent findings suggest the possibility that, at least in some instances, the receptor can bind with low affinity to peptide-MHC complexes presented by adjacent stromal cells (Allam et al., 2021; Li et al., 2021; Mallis et al., 2015b; Yamasaki et al., 2006). Irrespective of the mechanisms of activation, the signals downstream of pre-TCR activation resemble those downstream of the mature αβ TCR (Michie and Zuniga-Pflucker, 2002).

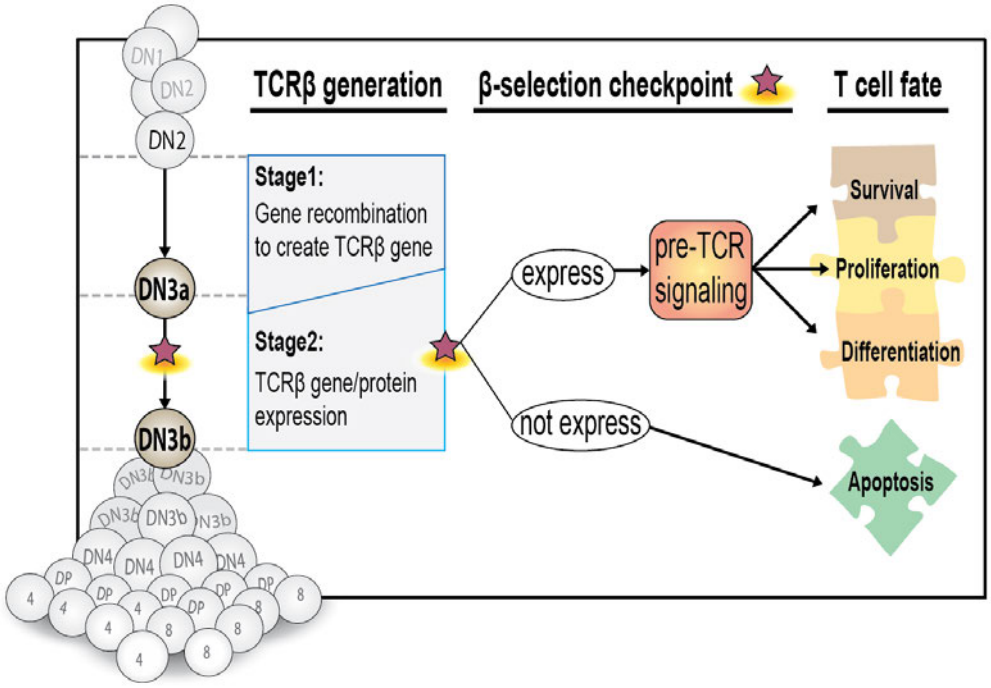


Figure 1. 3 A schematic model that describes the β –selection checkpoint determines T cell fate.

In T cell development, the TCRβ chain is generated as the cell transits from the DN2 (double negative 2) stage to the DN3 (double negative 3) stage. TCRβ gene recombination (Stage 1) results in creation of a functional TCRβ gene, and is completed at the DN3a stage. As a result of expression of a functional TCRβ chain (Stage 2), pre-TCR signalling is triggered during the DN3a to DN3b transition. Cells at the DN3b stage, at which effective pre-TCR signalling is conferred, pass the β-selection checkpoint and are therefore allowed to survive, differentiate, and proliferate. In contrast, cells that do not pass β-selection checkpoint undergo apoptosis.

1.4.2 Control of survival versus death changes over the β-selection checkpoint

The TCRβ checkpoint marks a pivotal stage at which the conditions that determine whether the cell survives or undergoes apoptosis are profoundly altered. These changes occur in two stages. First, the process of genomic recombination to rearrange the TCRβ gene creates challenges for survival that must be managed by the cells (‘Stage 1’ in Figure 1.3). Second, following the recombination, expression of the TCRβ serves as a test by which cells that fail to express an appropriately rearranged TCRβ undergo cell death (Pénit et al., 1995; Shinkai et al., 1993) (‘Stage 2’ in Figure 1.3).

Changes in expression of regulators of survival and apoptosis are required to ensure different criteria for survival in these two stages of differentiation. These changes are primarily mediated by transcriptional regulation (Rothenberg et al., 2016). Several molecules have been implicated in this survival decision, based upon changes in their expression at the checkpoint, or in functional experiments showing that interventions in their expression or function alter cell survival at this differentiation stage. We describe each of these molecules below and discuss how transcriptional regulation influences their activity.

Several mechanisms provide protection from apoptosis throughout T cell development, and will not be detailed here (Zhan et al., 2017), but an additional mechanism comes into play during TCRβ gene recombination and expression. Homologous recombination is initiated by the lymphocyte-specific recombinases, recombination-activating genes (RAG) RAG1 and RAG2, to create DNA double stranded breaks (DSBs) (Helmink and Sleckman, 2012). Homologous recombination requires the Ataxia Telangiectasia Mutated (ATM) serine/threonine kinase and Mre11-Rad50-Nbs1 (MRN) machinery to sense DNA damage, to repair the DSBs and enable recombination of the TCRβ gene (Prochazkova and Loizou, 2016). At this stage, the expression of ATM/MRN, is regulated by the transcription factor, p53 (Prochazkova and Loizou, 2016). p53 is stabilized to slow cycling and therefore enable repair of recombination errors (Guidos et al., 1996; Kastenhuber and Lowe, 2017). p53 is stabilized by the transcription factor c-Myb, which is required for TCRβ V-(D)-J gene recombination and survival of the developing T cell (Bender et al., 2004).

Following successful recombination of the TCRβ gene, the recombinase machinery is downregulated to prevent further DSBs, and p53 activity is inactivated to enable cell survival (Haks et al., 1999). The inactivation of p53 is conditional on pre-TCR signalling, thus ensuring the death of cells that do not create a functional TCRβ chain (Haks et al., 1999). p53 is inactivated by several mechanisms: The BTB-POZ transcription factor, Miz-1 limits p53 expression and antagonises p53-mediated cycling arrest and apoptosis (Miao et al., 2010; Rashkovan et al., 2014; Saba et al., 2011). The zinc finger protein, Yin Yang 1 was recently shown to downregulate p53 in developing T cells to promote survival at the β-selection checkpoint (Chen et al., 2016b). The histone methyltransferase, Ezh2, is critical for passing through antigen receptor checkpoints, and was recently shown to promote degradation of p53 by repressing Cdkn2a gene expression to enable survival following β-selection (Jacobsen et al., 2017).

Both Bcl-2 and Mcl-1 are expressed throughout T cell development and can protect from apoptosis, but a number of publications have led to the consensus that Mcl-1 supersedes Bcl-2 in importance following β-selection (Carrington et al., 2017). One explanation for this switch in roles is that the Mcl-1 protein, by virtue of its short half-life, is more dependent upon the extrinsic signals orchestrated by pre-TCR signalling (Carrington et al., 2017). Before β-selection, Bcl-2 expression requires activation of the transcription factor, nuclear factor of activated T-cells (NFAT) NFATc1 by interleukin-7 (IL-7) signalling (Patra et al., 2013), and pre-TCR expression leads to upregulation of NFATc-1 by altering promotor use, although the impact on apoptosis is not clear (Klein-Hessling et al., 2016) After passing the β-selection checkpoint, pre-TCR signalling induces activation of NF-κB and NFAT5 to induce Bcl-2-related protein A1 (BCL2A1) expression (Aifantis et al., 2001; Berga-Bolaños et al., 2013; Mandal et al., 2005; Voll et al., 2000). Duplication of the Bcl2A1 gene means that knockout experiments are difficult, and it is still not clear how critical a role Bcl2A1 plays (Schenk et al., 2017). Thus, although the picture is not yet clear, the expression of several members of pro- and anti-apoptotic family changes dramatically as cells progress through β-selection and dictates the differential control of apoptosis before, during and after β-selection.

The cell surface receptor and transcriptional regulator, Notch1, plays key roles during T cell development. Notch1 expression peaks at the β-selection checkpoint. The transcriptional response to Notch1 changes dramatically over β-selection (Mingueneau et al., 2013a), but this does not seem to modify its role in protection from apoptosis, as Notch is required for cell survival both before and immediately after the checkpoint, and Notch1 can also ensure the survival of cells without a pre-TCR (Ciofani and Zuniga-Pflucker, 2005; Wolfer et al., 2002). Similar to Notch1, the Wingless (Wnt) effector, β-catenin, is active before and after β-selection, but its role in protection from apoptosis is changed by the process of β-selection. After the checkpoint, productive pre-TCR signalling leads to increased expression of TCF-1 (encoded by the TCF7 gene), which binds β-catenin to mediate cell survival (Dose et al., 2014a; Goux et al., 2005).

Thus, cells traversing this β-selection checkpoint undergo a sharp transition from a state in which they do not express pre-TCR, and do not need it for survival; to a state in which they are absolutely dependent on pre-TCR expression to survive. The transition between these states is enabled by a dramatic change in the expression of regulators of apoptosis in the

cell, including changes in expression of pro- and anti-apoptotic regulators (Tischner et al., 2010).

1.4.3 Gain of proliferation with metabolic activation over the TCRβ checkpoint

A defining characteristic of β-selection is the transition from relative quiescence to rapid proliferation. Cell proliferation is repressed once gene recombination is initiated early in β-selection, and cells are predominantly in the G1 phase of the cell cycle (Hoffman et al., 1996). Besides the effects of p53 described above (Haks et al., 1999), the reduced proliferation is caused by expression of the E-protein transcription factors, E12 and E47 (encoded by the E2A gene) and HEB (encoded by TCF12 gene) (Engel and Murre, 2004; Wojciechowski et al., 2007). E2A restricts proliferation both by inhibiting signalling through the interleukin-7 receptor and by inhibiting expression of genes required for cell proliferation (Miyazaki et al., 2017; Wojciechowski et al., 2007). Once the pre-TCR is expressed and the cell has passed the TCRβ checkpoint, a number of mechanisms combine to release the brake on proliferation. Genes associated with metabolism and proliferation dominate the transcriptional changes that occur during β-selection, reflecting the critical role of the β-selection checkpoint in coordinating proliferation (Mingueneau et al., 2013b). Analysis of transcriptional changes and requirements for individual genes have recently highlighted several examples by which passing through the β-selection checkpoint coordinates with the microenvironment to initiate proliferation.

Notch1 coordinates with pre-TCR signalling to promote and control proliferation and cell metabolism (Taghon et al., 2009) (**Figure 1.4**). pre-TCR signalling triggers expression of c-Myc, which suppresses expression of cell cycle inhibitors such as p27Kip and promotes ribosome biogenesis (Dose et al., 2006). Notch1-mediated transcription of c-Myc, Hes1 and Lef1is enhanced during β-selection by stage-specific expression of the protein inhibitor of activated STAT (PIAS)-like cofactor, Zmiz1 (Wang et al., 2018). The increased nutrient requirement for proliferation also involves coordination between the β-selection checkpoint and the environment. Signalling through the chemokine receptor, C-X-C chemokine receptor type 4 (CXCR-4) and the Interleukin-7 Receptor (IL7R) activates phosphoinositide 3-kinase (PI3K) and the mTORC complex to increase expression of nutrient receptors CD71 and CD98 as the cell passes the β-selection checkpoint (Boudil et al., 2015; Janas et al., 2010; Kelly et al., 2007; Trampont et al., 2010). Proliferation is also fuelled by an increased uptake of glucose and glutamine, which enables protein O-GlcNAcylation and is triggered by cooperation between Notch1 and pre-TCR signals

(Swamy et al., 2016).Further tying together the effects of Notch and pre-TCR signalling, the mechanistic target of the mammalian target of rapamycin complex 1 (mTORC1), was recently found to coordinate effects of pre-TCR and Notch signalling on metabolic activation and proliferation (Yang et al., 2018). Loss of the mTORC subunit, Raptor, arrests development after β-selection, and reduces proliferation by downregulating c-Myc and upregulating protein expression (Yang et al., 2018).

Pre-TCR signalling not only initiates proliferation as described above, but it also directly ensures that this proliferation is self-limited in duration (Yashiro-Ohtani et al., 2009) (Figure 1.4). The self-limiting nature of this cascade ensures that proliferation slows before the next wave of genomic recombination (for the TCRα gene) (Xi et al., 2006). One mechanism for self-limitation involves pre-TCR triggering of expression of transcription factors with different activation and degradation kinetics, which negatively regulate each other. This complex pathway involves initial high levels of Egr3, which induces expression of Id3 to inhibit activity of E-proteins and retinoic acid-related orphan receptor γT (RORγt) expression (Xi et al., 2006). As Egr3 levels decline, RORγt expression increases and leads to a quiescent state required for TCRα gene rearrangement (Guo et al., 2016; Xi et al., 2006). The co-dependency of pre-TCR and Notch1 on proliferation is also rendered self-limiting because pre-TCR-induced E2A inhibits Notch1 expression via Id3 (Yashiro-Ohtani et al., 2009). This effect on Notch expression is amplified by the zinc finger transcriptional regulator, Ikaros, which peaks in expression after β-selection, and represses the transcriptional response to Notch1 (Geimer Le Lay et al., 2014; Kleinmann et al., 2008b), eventually abrogating Notch1 signalling (Mingueneau et al., 2013b)

A further self-limiting mechanism involves the phosphatase and tensin homolog (PTEN), which is expressed in developing T cells but is negatively regulated by Hes1 during and immediately after β-selection (Wong et al., 2012). As Notch signalling is reduced following β-selection, PTEN expression increases and restricts PI3K signalling and c-Myc-induced proliferation (Hagenbeek et al., 2004; Wong et al., 2012). PI3K activity is also limited by pre-TCR-induction of inositol-trisphosphate 3-kinase B (Itpkb). Together, these findings suggest that pre-TCR induces a slow build-up of the expression of PTEN and a reduction in the PI3K/Akt pathway to restrain the proliferation some period of time after the TCRβ checkpoint. Pre-TCR signalling eventually leads to an abrogation of signalling through Notch1. Thus pre-TCR signalling triggers several mechanisms to constrain metabolic

activity and proliferation after the rapid proliferation that follows the β-selection checkpoint (Westernberg et al., 2016).

Recent findings suggest that the transcriptional networks that orchestrate death and proliferation during β-selection can also influence chromatin accessibility (Emmanuel et al., 2018a). For instance, Ikaros reorganises the chromatin environment in DN3 cells, and controls cell cycling at the β-selection checkpoint (Arenzana et al., 2015; Oravecz et al., 2015). The deubiquitinating enzyme, BRCA1-associated protein-1 (BAP1), controls proliferation after the TCRβ checkpoint by a combination of transcriptional and epigenetic influences on the cell cycle (Arenzana et al., 2018). During TCRα recombination, TCF-1 and HEB, cooperatively modulate chromatin accessibility to influence the effects of Notch1 (Emmanuel et al., 2018b). The similarities in control of proliferation during TCRα and TCRβ recombination suggest that a similar role for chromatin accessibility might also apply to β-selection, but this has not yet been tested.

Together, these findings indicate that passing the β-selection checkpoint triggers a complex signalling, transcriptional, and possibly epigenetic response, to regulate proliferation. The response to expression of a functional pre-TCR is coordinated by cues from the external environment. Signalling cascades that enable this proliferation include inbuilt mechanisms to ensure that the proliferation is restricted to only a few cell cycles, and does not last into the next phase of genomic recombination of the TCRα chain.

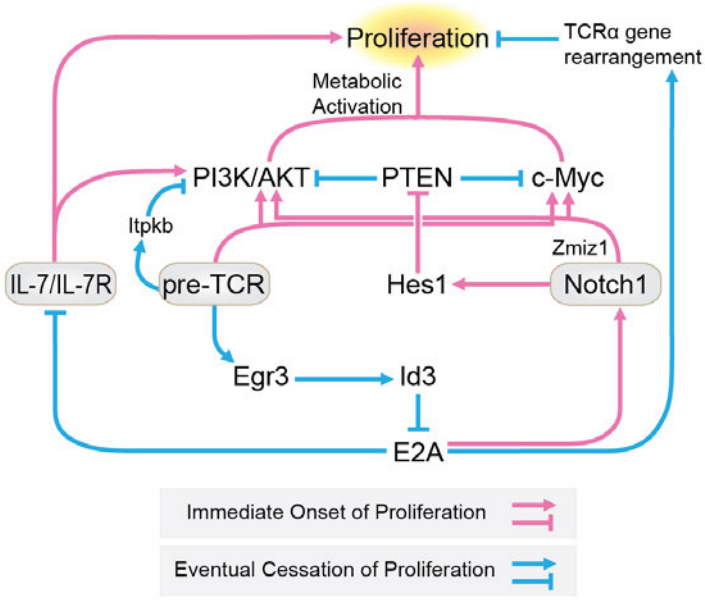


Figure 1. 4 Integration of signals from Notch and pre-TCR enable a self-limiting wave of proliferation after the β-selection checkpoint.

After the β-selection checkpoint, several rounds of cell duplication are initiated and then halted by signals associated with pre-TCR, Notch1 and IL-7 signalling. Pre-TCR signalling orchestrates with Notch1 signalling to contribute proliferation by co-regulating several downstream transcriptional programs for metabolic activation and promotion of the cell cycling.

1.4.4 Control of differentiation versus self-renewal changes over the TCRβ checkpoint

In addition to transcriptional changes, at several stages of differentiation during early T cell development, the epigenome changes dramatically (Isoda et al., 2017; Zhang et al., 2012). Prior to the β-selection checkpoint, the BAF chromatin remodeling complex subunit (Bcl11b) is required to promote T cell identity and prevent differentiation along with alternative pathways (Avram and Califano, 2014; Hosokawa et al., 2018; Isoda et al., 2017). Modification of chromatin is required for the TCRβ-transition, both facilitating access of the RAG recombinase to appropriate regions of the genome, and enabling the proliferative response after the TCRβ checkpoint (Lee et al., 2001). Runx1is essential for TCRβ gene recombination, most likely by binding to enhancer elements upstream of TCRβ and recruiting factors that increase chromatin accessibility (Majumder et al., 2015). Changes in chromatin marks at different sites on the chromatin provide an opportunity for the same

transcription factor to exert different effects at different stages, for instance, enabling GATA3 to influence transcription of different genes before and after the β-selection checkpoint (Zhang et al., 2012).

Passing the TCRβ checkpoint permits the cells to differentiate to new developmental stages that eventually enable recombination of the TCRα receptor gene. This differentiation process is marked by a series of changes in cell surface receptors, starting with downregulation of CD44 (Canté-Barrett et al., 2017), defined as progression from the DN2 to DN3a stage, and coincident with TCRβ recombination, CD28 is then increased (DN3a to DN3b) , and considered the defining hallmark of passage through the TCRβ checkpoint (Teague et al., 2010). Then CD25 is downregulated (DN4), followed by upregulation of the coreceptors CD4 and CD8 (Koch and Radtke, 2011). It is not always easy to dissect the influence of signalling pathways and transcription factors on differentiation along the αβ lineage from their influence on apoptosis or proliferation as described above.

The most valuable information on control of differentiation has come from studies on the mechanisms by which CD4 and CD8, the two co-receptors whose expression marks progression to the DP stage, are switched on. The Ikaros family provides one example of how transcription factors might coordinate with β-selection to influence subsequent differentiation. Ikaros and other family members are expressed throughout lymphocyte development, but seem to be particularly important at stages of sharp transcriptional changes, such as in β-selection (Heizmann et al., 2018). Ikaros expression peaks after the TCRβ checkpoint, and inhibits Notch1 signalling by repressing Hes1 expression (Kleinmann et al., 2008a; Tinsley et al., 2013) This activity, combined with observations that Ikaros is required for CD8 expression, suggests that Ikaros facilitates progression from the β-selection checkpoint to the DP stage (Tinsley et al., 2013) Methylation of chromatin at CpG sites and histones is well established to regulate expression of CD8 (Harland et al., 2018; Issuree et al., 2017). The promotor for CD8 is inaccessible early in T cell development but is remodelled at the DN3 stage, prior to its expression in DP cells, (Harker et al., 2011). This change in gene accessibility seems to be driven by Ikaros-mediated recruitment of the repressive nucleosome remodelling and histone deacetylase (NuRD) complex, which is then evicted in response to pre-TCR signalling (Harker et al., 2011). Similarly, cross-inhibition between Ikaros and the NuRD complex regulates induction of CD4 in DP cells (Naito et al., 2007). The mechanisms by which pre-TCR signalling and Ikaros interact are not yet clear, but these data suggest they coordinate to direct progression

to the DP stage. Changes in chromatin modifications during β-selection also influence the binding specificity and function of the transcription factor, Ets1, enabling it to support differentiation to DP (Cauchy et al., 2016).

It was assumed from the 1970s that thymocytes do not self-renew, but several recent studies have upended this notion: At least one round of proliferation after β-selection is required for the cells to progress through TCRα recombination and to the Double Positive (DP) stage (Kreslavsky et al., 2012). It is now clear that limited self-renewal, defined as the generation of a daughter cell that inherits precisely the phenotype, proliferative and differentiation potential as the parent cell, does occur in the healthy thymus (Peaudecerf et al., 2016). Direct proof that non-transformed thymocytes can self-renew came from evidence that, in the absence of competition from new thymic immigrants, DN3a precursors maintained continuous thymocyte generation and export (Boehm, 2012; Martins et al., 2012; Peaudecerf et al., 2012). The purpose of self-renewal and extent to which self-renewal occurs during normal development is not yet clear. However, emerging evidence suggests that control of self-renewal of DN3 cells is non-cell autonomous. The emerging view is that DN2/3 cells (presumably just before undergoing β-selection) compete for a restricted niche that facilitates self-renewal, and that, under steady state conditions, DN2/3 cells that have recently entered the thymus will expel older DN2/3 cells, thus restricting access to the niche and preventing excessive self-renewal (Paiva et al., 2018). These findings suggest that DN2/3 cells have an inherent capacity for self-renewal, which is controlled by access to the niche (Ballesteros-Arias et al., 2019b; Ginn et al., 2018). It is not yet clear how the capacity to self-renew is functionally linked to β-selection, and whether this relates to changes in transcriptional profile or changes in access to the niche. A possible clue as to mechanism relates to observations in many cell systems that self-renewal is enabled by asymmetric cell division (ACD), which allows one daughter cell to remain identical to its parent, while the other daughter cell progresses through differentiation (Ito and Ito, 2016). We recently found that DN3a cells undergo ACD, to enable the asymmetric inheritance of a regulator of Notch, Numb (Pham et al., 2015a). ACD of DN3a cells requires interaction with stromal cells, raising the possibility that self-renewal during or just prior to β-selection is facilitated by a capacity to divide asymmetrically, conferred by interactions with the thymic niche. This ACD event would potentially allow the daughter cells to adopt different transcriptional profiles and subsequently different cell fates.

Thus, although it is clear that cells progress through defined differentiation steps subsequent to β-selection, many of the processes that have been implicated in differentiation are also known to affect proliferation or death, confounding efforts to assess whether they the impact on differentiation is direct, or a result of the effects on cell expansion. It is now established that not all cells differentiate immediately to the stage required for TCRα gene recombination, as some undergo restricted self-renewal. How self-renewal of DN3 cells is coordinated at the transcriptional level, and how it is integrated with β-selection is not yet clear, but access to a restricted niche plays an important role.

1.4.5 Conclusion and Perspectives

The conditions that dictate whether a cell survives, proliferates and/or differentiates change dramatically once the cell passes the β -selection checkpoint. Much of these changes are cell autonomous, and rely upon the fact that the very process of β-selection triggers a rapid and profound transcriptional response. This then leads to a sequence of changes that alter how the cell responds to its microenvironment, enabling survival, proliferation and differentiation until the cell undergoes its next major challenge – genomic recombination of the TCRα chain.

Interestingly, the induction of self-limiting feedback loops means that the transcriptional changes triggered at the β -selection checkpoint not only control immediate behaviour, but also dictate events several days and generations in the future. Thus, if a cell survives the β -selection checkpoint, it is programmed to increase metabolism and commence proliferation and differentiation. However, the transcription factors that dictate those processes also instigate a more protracted signalling response that dictates a cessation of proliferation once the cell differentiates to the DP stage and undergoes rearrangement of the TCRα gene, This sequence of events ensures that only cells that are fit for purpose can differentiate, and also ensures that precisely the right number of cells with each rearranged TCRβ gene are produced.

Unsurprisingly, errors in such a tightly controlled process not only prevent cells from developing into mature T cells, but also increase the risk of leukemia. The appreciation that increased self-renewal at the β-selection stage is a driver for T-ALL has led to new efforts to characterise this properties of the leukemia initiating cell, highlighting its low proliferation rate (Tremblay et al., 2018b) and importance of CD44, perhaps for enabling interaction with the bone marrow niche for self-renewal (Garcia-Peydro et al., 2018).

Understanding the role of epigenetic modifications in T cell development and T-ALL has led to the suggestion that histone deacetylase inhibitors might provide therapeutic opportunities for the disease (Waibel et al., 2018)

A hallmark of β-selection is a dramatic change in cell mRNA and protein content, with consequent changes in signalling and cell behaviours such as death, proliferation and differentiation. Many of the components of these changes have been identified, particularly transcription factors. How these transcriptional factors are networked has been nicely proposed by several genome-wide computation. Future directions to fill in the gaps in understanding how transcriptional and epigenomic changes in TCRβ selection control normal and oncogenic development will most likely arise from further single cell approaches that are starting to transform our understanding of all development, for example omics sequencing and longitudinal pedigree imaging. Such approaches include barcoding to trace single cells throughout their development, and can combined with optogenetic switch to observe behaviours *in vitro* and *in vivo*.

1.5 The relationship between T cell development and cancer

T cell development can cause cancer. I reviewed two contexts here: The first part (Section 1.5.1-1.5.4) is with respect that the developing T cell *per se* can be transformed to a type of cancer called T-ALL (T-cell acute lymphoblastic leukaemia), the second part (Section 1.5.5) is to discuss the abnormality of the growth of immature T cells in thymomas.

1.5.1 Self-renewal of developing T cells is altered leading to T-ALL transformation

With respect to the oncogenic transformation from the developing T cell. T-ALL is a heterogeneous disease, where leukemic cells arise from the diverse, and poorly defined stages of T cell development (Belver and Ferrando, 2016; Erarslan-Uysal et al., 2020; Vadillo et al., 2018). Some aspects of aetiology are shared with other types of cancer. For example, dysfunction of tumour suppressors such as PTEN, TET2, and BAP1 has also been reported to cause T-ALL (Arenzana et al., 2018; Bensberg et al., 2021; Tottone et al., 2021). Another aspect is the property of self-renewal of T-cell progenitors, which is altered to cause T-ALL initiation. This is extensively demonstrated in a type of T-ALL, early T-cell precursor acute lymphoblastic leukaemia (ETP-ALL). ETP-ALL was accounted for around 15% case in childhood T-ALL and 10~30% in adult T-ALL (Zhang et al., 2020), with the 5-year overall survival rate around 32% compared to 63% of non-ETP-ALL (Morita et al., 2021). Therefore, ETP-ALL remains a significant risk to cause death.

Current treatments for T-ALL/ETP-ALL are often chemotherapies, but the pre-leukemic stem cells (pre-LSC) can survive chemptherapy and cause relapse. Accordingly, the pre-LSCs are less chemo-sensitive due to its self-renewing property and its lower proliferation rate (Gerby et al., 2016; Höpner et al., 2021). Therefore, targeting the self-renewal machinery of pre-LSC is a focus in ETP-ALL therapy development (Gerby et al., 2016). However, targeting self-renewal of pre-LSC isn't easy, because the self-renewal machinery of pre-LSC can be shared with HSC or the hematopoietic precursors (Höpner et al., 2021). For example, a chromatin-binding protein Phf6 restricts HSC self-renewal (Miyagi et al., 2019). Phf6 downregulation during haematopoiesis promotes T cell development (Loontiens et al., 2020). Around 20% T-ALL patients were diagnosed with loss-of-function Phf6 mutations (Wendorff et al., 2019). Loss of function of Phf6 increases chromatin accessibility for genes related to JAK–STAT signalling, and leads to Notch1 activation (Wendorff et al., 2019). Another challenge to targeting self-renewal machinery of ETP-ALL is a lack of knowledge of the leukemic stem cell (LSC) from which T-ALL arises. (Alexander and Mullighan, 2021). The difficulty namely is the heterogeneity of ETP-ALL,

which the clonal evolution is complicated to be modelled (Alexander and Mullighan, 2021). With respect to this challenge, a few recent studies shed light (Martelli et al., 2019). For example, in PTEN-null T-ALL, a small population of LSC was distinguishable from the larger blast population. This LSC uniquely expressed high β-catenin activity, which in turn promotes Spi1 expression and its gene regulatory networks. Subsequently, Spil, a gene that supports ETP development, conferred inappropriate self-renewing ability to LSC, which consequently promotes the growth of PTEN-null T-ALL (Zhu et al., 2018). In another study, Spi1 gene was identified to be fused with TCF7 gene, of which N-terminus β-catenin binding domain was still maintained, and the Spi1-TCF7 fusion promoted LSC production (Van Thillo et al., 2021). The elevation of β-catenin activity also enables colony formation of T-ALL cells, reflecting a LSC property (Zhang et al., 2021b). Therefore, although only a few studies currently addressed the self-renewal for this small fraction of T-ALL stem cell, the switch to control self-renewal in this subset is clearly to be the β-catenin activity. Overall, a trend of therapy development to cure ETP-ALL lies on inhibiting LSC and its self-renewal machinery.

To understand LSC, a recent breakthrough came from a study using single-cell transcriptomics and clonal tracking from 3 patients (Velten et al., 2021): They found the LSC clones are likely associated with genotypes such as DNMT3A mutation and FLT3 upregulation, and can be associated with expression of the surface phenotype like CD96 (Velten et al., 2021). Importantly, by exome sequencing, FLT3 (35%), DNMT3A (16%) and NOTCH1 (14%) are the top three genes that were detected with mutations in ETP-ALL (the frequency indicated the rate of genotype occurrence from the cohort of 68 adult patients) (Neumann et al., 2013a; Neumann et al., 2013b). As these clinical statistics shown, FLT3 signalling is another key to switch ETP-ALL transformation, while FLT3 in development is to support the multipotency of HSC (Stirewalt and Radich, 2003). Thus, these evidence suggests a potential ‘stemness’ of LSC, mediated by FLT3, might be the key to activate T-ALL (Levis, 2017).

1.5.2 Errors at the transcriptional control in β-selection checkpoint lead to T-ALL

As described in above section, T-ALL originates either from mature T cells or developing T cells (Noronha et al., 2019), and ETP-ALL represents less than 30% T-ALL cases (Zhang et al., 2020). Another T cell developmental origin of T-ALL is at the DN3 stage (Curtis and McCormack, 2010b). The translation of cues to cell fate can involve cumulative and

incremental shifts in phenotype, however at critical points during development it can involve a sharp transformation. The 'β-selection checkpoint' of T cell development reflects one such sharp shift. The abruptness of this change in fate maps the temporal and causal sequence of molecular and cellular events that drive fate decisions The importance of β-selection is illustrated by the many layers of regulation involved, and the fact that errors in this process lead to T-ALL. In some contexts, cells from DN3 population can contain pre-leukemic stem cells, which can transform into T-ALL (Tremblay et al., 2018a). In this thesis, I will further review how DN3 goes awry and how the intrathymic environment provides a limited niche, competition for which controls fate of the DN3 cell.

Oncogenes can promote DN3 self-renewal and lead to leukemogenesis (Gerby et al., 2014b; Paiva et al., 2021). Some of these oncogenes implicated in human T-ALL are transcription factors required for β-selection (Girardi et al., 2017). Best characterised is LMO2, of which ectopic expression at the stage of β-selection can trigger T-ALL (McCormack et al., 2010b) (**Figure 1.5**). Normally, LMO2 transcription is sharply downregulated coincident with β-selection, and aberrant LMO2 expression beyond β-selection leads to T-ALL (Curtis and McCormack, 2010a). This can occur when murine T cell precursors are genetically modified to overexpress LMO2, or by chromosomal translocations or deletions that alter transcriptional control of the gene in mice and humans (Belver and Ferrando, 2016; Curtis and McCormack, 2010a; McCormack et al., 2010b). An unexpected finding in humans undergoing gene therapy for X-linked Severe Combined Immunodeficiency (X-SCID) also indicated that retroviruses preferentially insert upstream of the LMO2 gene and can cause T-ALL (Belver and Ferrando, 2016; Hacein-Bey-Abina et al., 2008; Howe et al., 2008; Paiva et al., 2018) . These diverse cases suggested that ectopic LMO2 during human T cell development caused an increase in self-renewal of DN2/3 cells to provide a pool of pre-leukemic cells. These pre-leukemic cells then incur a series of genetic lesions, including many transcription factors implicated in self-renewal and activating mutations in the Notch pathway, that eventually led to T-ALL (Astolfi et al., 2014; Ballesteros-Arias et al., 2019a; Belver and Ferrando, 2016; Curtis and McCormack, 2010a; Ferrando et al., 2002; Gerby et al., 2014a). LMO2 overexpression in murine pre-leukemic cells interacts functionally with the related transcription factor, Lyl1 to cause transcription of genes that have been associated with increased self-renewal (Gerby et al., 2014a; McCormack et al., 2013a; McCormack et al., 2010b). Studies on mice suggest that LMO2 acts as a 'hit-and-run' oncogene, whereby once the cells have accumulated further genetic lesions, LMO2 is no longer needed (García-Ramírez et al., 2018). This 'hit-and-

run' mode can be achieved by activating an essential downstream gene Ldb1 (Li et al., 2020a). These findings led to the prevailing dogma that ectopic LMO2 expression in DN2/3 cells directly drives inappropriate self-renewal as a precursor to T-ALL development.

Emerging evidence in murine models suggests that the capacity for LMO2 to drive T-ALL development at the β-selection stage might require the non-cell autonomous effects on self-renewal of DN2/3 cells highlighted in the section above. In support of this notion, T-ALL arose from mouse models of gene therapy using lentiviral delivery, where insertion upstream of LMO2 did not occur, but did not arise in pre-clinical models of gene therapy to combat an alternative disease (Ginn et al., 2017). Two recent studies suggest that preventing competition for the niche increases the likelihood of leukemia in gene therapy for X-SCID (Ginn et al., 2017; Schiroli et al., 2017). Together, these findings suggest that competition for the niche by DN2/3 cells provides a key restriction of self-renewal, and acts as a tumour suppressor mechanism (Ginn et al., 2018). A general role for competition in tumour suppression during β-selection is further supported by findings that several different conditions that reduce competition for reconstituted T cell precursors all show similar effects of increased self-renewal and T-ALL (Ballesteros-Arias et al., 2019a). How this non-cell autonomous effect cooperates with the transcriptional changes conferred by cell-autonomous oncogenic translocations and mutation is still an open question.

Other pathways that have been implicated in the regulation of β-selection can also go awry in the progression to T-ALL. In particular, Notch1 is hyperactivated in many human and murine tumours including T-ALL, commonly by activating mutations (Belver and Ferrando, 2016). As highlighted by a recent study, DN3-ALL can be Lmo2-dependent, evolving, and Lmo2-independent (Abdulla et al., 2021). The mutants of Notch1 were detected in all these three classifications (Abdulla et al., 2021). This is not surprising, given the role of Notch in proliferation, but further hits are generally required to promote full transformation (Belver and Ferrando, 2016). Key transcriptional regulators that cooperate with Notch in murine models to cause T-ALL include Myc (Herranz et al., 2014) and β-catenin (Dose et al., 2014a; Gekas et al., 2016), but many others show aberrations in human T-ALL (Belver and Ferrando, 2016). Transgenic mice overexpressing hyperactive Notch3 also develop T-ALL (Ferrandino et al., 2018). The role of Ikaros in modifying the transcriptional response to Notch1 positions it to act as a tumour suppressor and this is supported by mouse experiments, although evidence from human T-ALL patients has been elusive (Heizmann et al., 2018). Interestingly, the Lmo2-independent T-ALL was promoted

by Ikaros depletion, and this condition seems not to be associated with Notch1 signalling (Abdulla et al., 2021), suggesting a unique Ikaros dependency should be defined in the future. A recent study provides an important update showing Ikaros has pioneering ability to form a super-enhancer to repress T-ALL induction (Ding et al., 2019). Together these studies indicate that DN3-ALL is induced from multiple sources of error in transcriptional control during β-selection.

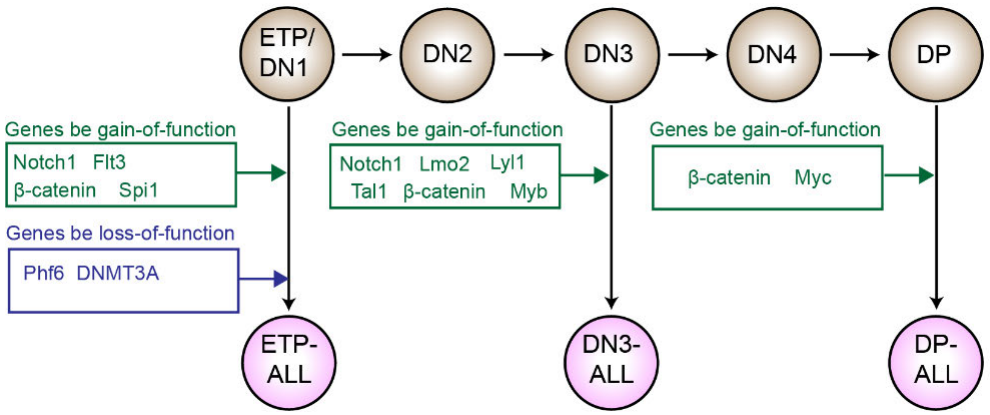


Figure 1. 5 The mutations of gene regulations lead to T-ALL in a stage-specific manner

The schematic shows the mutants of genes, of which been verified both in clinical and in experimental, transform the developing T cell into T-ALL

1.5.3 Errors at the TCRα and β gene recombination lead to genome instability and T-ALL

Genomic recombination during TCRβ selection can directly impact upon the propensity for T-ALL, in part because cells at this stage are, by necessity, tolerant to DNA DSBs, and in part because RAG can enable cytogenetic alterations that facilitate the molecular changes described above (Haines et al., 2006; Helmink et al., 2009; Huang et al., 2007; Matei et al., 2007; Matsumoto et al., 2014; Saidi et al., 2010; Teng et al., 2015). The range of different genomic alterations that can arise in T-ALL was assessed by integrated genomic analysis of 264 human T-ALL cases, suggesting over 100 putative driver genes (Liu et al., 2017). In addition to LMO-2, several other transcription factors that are down-regulated prior to TCRβ gene expression have been implicated in T-ALL, including transcription factors that regulate self-renewal, such as Mycn, Lyl-1, TAL1 (Astolfi et al., 2014; Cleveland et al., 2013; Ferrando et al., 2002; King et al., 2016; McCormack et al., 2013b; McCormack et al., 2010a). Proteins that enable tolerance to DNA damage during β-selection have also been implicated in mouse models of T-ALL, including β-catenin, p57, and Gfi1 (Dose et al., 2014a; Khandanpour et al., 2013; Matsumoto et al., 2014). Similarly, Myb is upregulated in human T-ALL, and is rearranged with the TCRβ gene locus (Bardelli et al., 2021) (Belver and Ferrando, 2016). Myb upregulation is linked to TAL/LMO gene expression enrichment (Bardelli et al., 2021). These studies suggested errors in TCRβ gene rearrangement can trigger oncogene expression. The large number of proteins that both drive or enhance T-ALL and partake in the β-selection checkpoint highlights the delicate balance of events that occur at this differentiation stage. Encouragingly, understanding this process is already starting to enable new therapeutic approaches to be explored. For instance, pharmacologic modification of the epigenetic processes implicated in β-selection have recently shown therapeutic benefit in models of Notch-driven T-ALL (Roderick et al., 2014; Waibel et al., 2018).

As the DSB can activate the spindle assembly checkpoint to arrest mitotic progression, one possible cause of T-ALL could relate to control of the spindle assembly checkpoint. Although no evidence so far has been published in regards to the developing T cell, one report showed overexpression of HTLV-I Tax (Human T-cell lymphotropic virus type 1 Tax) could override the spindle assembly checkpoint in several T-cell lines and result in genome instability (Malu et al., 2019). Another indicated that Phf6 (See section 1.5.1) is enriched at DSB to promote the DNA repair (Warmerdam et al., 2020). This finding might suggest how DSB is prevented in the developing T cell, and how Phf6 mutations can

compromise DSB to lead genome instability and T-ALL induction. Moreover, a recent clinical case study showing hypodiploidy from a T-ALL patient suggested the mutations that lead to aberrant cell cycling (eg. CDK2 mutation and STIL/TAL1 gene rearrangement) seem to be the basis of T-ALL aggressiveness (Stefaniak et al., 2021). These findings provide some support for the notion that disruption of the spindle assembly checkpoint can predispose to T-ALL.

Another mechanism to prevent the cell cycle arrest normally induced by DSB is the oncogene translocation that can subsequently happen after gene recombination. As been described, some oncogenes such as LMO2, TAL1 and TLX1 can be primed or translocated along with TCRβ or TCRδ rearrangement (Larmonie et al., 2014; Nicole et al., 2013). Another possibility to understand the DSBs compromise in DN3 population seem to be governed by the efficient ATM machinery (Bowen et al., 2013; Hathcock et al., 2013). In contrast to TCRβ rearrangement, TCRα gene recombination has been implicated in genome instability and oncogene translocation (Dose et al., 2014b): T-cell lymphoma from the DP stage is associated with RAG-induced DSB, and involves translocations of TCRα to the Myc/Pvt1 locus to induce DP-stage T-cell lymphomas (or DP-ALL?) (Dose et al., 2014b; Guo et al., 2007).

1.5.4 T-ALL cells infiltrate tissue

A recent perspective suggested leukaemia can be defined as a metastatic cancer (Whiteley et al., 2021). This notion comes from the natural ability of T cells migrating in the vascular system, and infiltrating into tissue (Vadillo et al., 2018; Whiteley et al., 2021). T-ALL cells indeed have been detected in skin, CNS, spleen, livers and etc (Whiteley et al., 2021; Yang et al., 2020; Zhang et al., 2021a). The molecular basis of T-ALL-tissue interaction is highly context-specific (Berrazouane et al., 2019), but T-ALL invasion in tissue might share some common mechanisms of cancer dissemination. For example, leukemic T cells infiltrating and migrating across tissue probably depend upon cellular structures such as invadopodia and lamellipodia (Vadillo et al., 2018; Whiteley et al., 2021). Importantly, T-ALL infiltrating in tissue can escape from the drug treatment, which might lead to the poor outcome (Walker et al., 2021).

The expression of CXC chemokine receptor type 4 (CXCR4) is particularly important for T-ALL cell infiltration into tissue, and is regulated by a PFK1-cMyc transcription

activation (Gao et al., 2021; Walker et al., 2021). It's also possible that CXCR4 expression in T-ALL is regulated by β-catenin (Weich et al., 2021; Zhang et al., 2021a), although direct evidence for this notion has not yet been reported. CXCR4 signalling in T-ALL targets multiple substrates, but a pathway to trigger RAS signalling deserves particular attention because RAS activity governs the transformation to metastasis (Hong et al., 2021; Spella et al., 2017). For T-ALL with Spi1-TCF7 fusion, RAS signalling further promoted LSC expansion (Van Thillo et al., 2021). CXCL12 is the ligand to CXCR4. CXCL12 is produced by a type of vascular endothelial cell in bone marrow, attracting the circulating T-ALL cell, which then settles in the bone marrow niche (Pitt et al., 2015). Importantly, CXCL12 is expressed in many organs, but whether CXCL12 can play a similar role as in bone marrow to attract T-ALL infiltration elsewhere is unknown. Other than CXCL12, another novel regulator CD96 has started drawing attention. CD96 is expressed in many tissue-infiltrating T cells to enhance T cell function, and it was also expressed in leukemic stem cell (Velten et al., 2021). These reports suggest understanding how the circulating T-ALL cell become metastatic in tissue, and discovering the solutions for clinical treatment might be important.

1.5.5 The alteration of T cell development by the tumour-derived signals

Besides T-ALL, thymoma is another thymic malignancy that influences T cell development. T-ALL frequently occurs in the childhood, but thymomas are mostly diagnosed in the adult patients as thymoma is a thymic neoplasm transformed from the differentiated thymic epithelium (Den Bakker and Oosterhuis, 2009; Suster, 2006). Thymus is an organ undergoing involution after adolescence, however, thymoma patients show a feature termed thymic recapitulation, a symptom whereby the patient abnormally grows a large number of immature T cells in the mature age when these expanding immature T cells are not normally found (Inoue et al., 1998; Suster, 2006). These thymoma-induced immature T cells are enriched at the thymus cortex, and showed a surface phenotype of $CD34^{+}CD4^{-}CD8^{-}CD3^{-}$ (Inoue et al., 1998). Interestingly, the immature $CD34^{+}CD4^{-}CD8^{-}$ T cells from both normal and thymoma individuals can be induced toward the DP ($CD4^{+}CD8^{+}$) stage by thymoma epithelium co-culture (Inoue et al., 1998), suggesting the thymoma epithelium maintains the capacity to support T cell differentiation. On the other hand, the microenvironment constructed by thymomas epithelium is different than the normal thymic tissue in terms of ECM composition (Wang

et al., 2016), therefore, whether the T cell differentiation is normal in thymomas might need to be revisited. It's also unclear whether the uniqueness of the ECM composition of the thymomas epithelium, for example desmoyokin (Wang et al., 2016), is the basis for rejuvenating the dedifferentiated immature T cell in the thymus. Overall, in my perspective, these studies together suggest a potential signalling platform, by which the thymic tumour derives, can alter the thymocyte fate determination.

A type of T cell, $CD3^+CD^-CD8^-$ (DN) T cell, is enriched upon the stimulation from the tumour, injury and viral infection (Lu et al., 2016; Meng et al., 2019; Torcellan et al., 2017). In light of a study using TCR sequencing, the TCR specificity of intraepithelial DN T cell was distinct from the conventional $CD4^+$ or $CD8^+$ T cell, suggesting that clonal evolution occurs during thymopoiesis to derive a subset of naïve intraepithelial DN T cell (Mayans et al., 2014). In regards to the origin of this $CD3^+$DN T cell, Papiernik and Pontoux show this $CD3^+$DN T cell was derived following the thymic selection, and can be *in vitro* derived from the developing $CD3^-$DN T cell (Papiernik and Pontoux, 1990). These $CD3^-$DN T cells can be transformed from the DP cells in the thymus (Wang et al., 2002), and this observation was later endorsed by examining the post-DP surface phenotype on the intraepithelial DN T cells (Mayans et al., 2014). Although currently much is unknown regarding these $CD3^-$DN T cells, an idea of DNT-cell immunotherapy has been proposed due to its anti-cancer ability (Li et al., 2020b). In short, a small T cell population, showing DN surface phenotype and a unique TCR clonal-type that can be against tumour antigen, might be generated during T cell development in thymus.

The possibility that anti-cancer DNT cells are developed or specified during T cell development is likely challenged by an unknown mechanisms in thymic selection to support DNT cells survival, for two explanations in my speculations. The role of CD4 and CD8 in DNT cell selection would need to be revisited, as the current knowledge posits that positive / negative selection are coordinated by CD4 and CD8. The second is the TCR strength of DNT cell during thymic selection would need to be evaluated. This is particularly important because using a CD2-mediated TCR transgene that recognises tumour antigen, Cui et al found 100% T-ALL induction after exposure to neoantigens without any oncogene alteration, and the cycling of DN2 and DN3 are significantly increased (Cui et al., 2015). The mechanisms behind in this study might suggest this neoantigen-to--TCR (or pre-TCR) signalling uniquely permits the transformation of T-ALL, which in turn needed to be toned or inactivated during normal thymopoiesis. Endorsed by another study using LCK inhibitor to repress TCR/pre-TCR signalling in T-

ALL cell, the cycling of T-ALL cells were subsequently arrested (Yuzhe et al., 2020). On the other hand, currently it's unclear whether the enrichment of the immature T cell in thymomas is benefitted by the neoantigen-TCR signalling or not, or whether the proliferation of the thymomas-associated T cell can be transformed to T-ALL by conducting a neoantigen-TCR signalling. In short, these studies suggest a caveat that tumour signalling (antigen) might cause a high strength of TCR/pre-TCR signalling, which in turn can be a risk for T cell leukemogenesis.

1.5.6 Summary

In this section, I discussed the relationship between T cell development and cancer. First, developing T cell can be transformed to T-ALL, through mechanisms such as altering self-renewal, unrepaired DSB or oncogene translocation. Although many pre-clinical drugs can potentially combat T-ALL, tissue infiltration of T-ALL cells might be a challenge for treatment. Second, immature T cells stored in adult can become thymomas if the proliferation is abnormally induced. With other circumstance such as viral infection (Albano et al., 2019), or thymic inflammation (Cron et al., 2020), T cell development contains inherent opportunities for cancer occurrence.

1.6 The roles of Scribble in cell fate determination

1.6.1 Scribble forms a polarity complex

Scribble is a highly conserved protein that regulates epithelial cell polarity and tissue organisation in *Drosophila*, *C. elegans* and mammal. Scribble forms a complex with Dlg (Discs large) and Lgl (Lethal giant larvae), all are evolutionarily conserved, to regulate multiple functions such as apico-basal polarity and epithelial junction formation (Humbert et al., 2008). In *Drosophila*, loss of either Scribble, Dlg or Lgl results in (1) severe defects in tissue morphology, (2) loss of proliferation control and (3) cancer occurrence (Brumby and Richardson, 2003). A recent study further showed that the Scribble complex regulates polarity in a compensatory manner, as single loss of each of the three (Scrib, Dlg, Lgl) didn't impact PAR polarity in *Drosophila* follicle epithelium, but losing function of any two of the three caused disruption in PAR polarity (Khoury and Bilder, 2020). In mammals, organisation of the Scribble complex is further complicated: Although only one Scribble homologue (hScrib, Scrb1) was identified in mammals, there are two mammalian Lgl homologues (ie. Lgl1 and Lgl2 (Hugl1 and 2)) and four mammalian homologs of Dlg (ie. Dlg1 (hDlg, SAP97), Dlg2 (Chapsyn-110, PSD-93), Dlg3 (NE-Dlg, SAP102), and Dlg4 (PSD-95, SAP90)). Unlike the four Dlg homologs, Dlg5 does not seem to interact with the Scribble complex, but can regulate the Hippo pathway (Kwan et al., 2016). Similar to loss of function of the Scribble complex in *Drosophila*, loss of Scribble (Dow et al., 2003), Dlg (Thomas et al., 1997) and Lgl (Grifoni et al., 2004) in mammals results in defective apical-basal polarity, over-proliferation and tumour growth. Importantly, mammalian Scribble is required for polarity of mature T cells (Ludford-Menting et al., 2005), and in interphase and in asymmetric cell division of developing T cells (Pham et al., 2015b; Pham et al., 2013).

1.6.2 Scribble regulates epithelial tissue organisation

Epithelial tissues are shaped by cell polarity. Taking apical-basolateral polarity as example, the apical membrane of the epithelial cell contains atypical PKC and other proteins of the Crumbs and PAR complexes, while the basolateral domain contains Scribble, Dlg and Lgl complex (Stephens et al., 2018). Close to the apical membrane, the tight junction (eg. Claudin, Occludin and ZO1/2) is formed; while the adherens junctions (eg. E-cadherin, β-catenin, and nectin) is formed beneath the tight junction. The side of apical membrane usually forms lumen, with a surface structure of microvillus that allows molecule of cell signal transport between cells; while the basolateral membrane contains various integrins,

through which extracellular matrix proteins are connected to cytoskeleton. Therefore, apical-basolateral polarity allows for tightly separated intracellular components, which maintains organisation of epithelial tissue. Loss of apical-basolateral polarity is associated with epithelial to mesenchymal transition, which is a sign of oncogenic transformation (Jung et al., 2019).

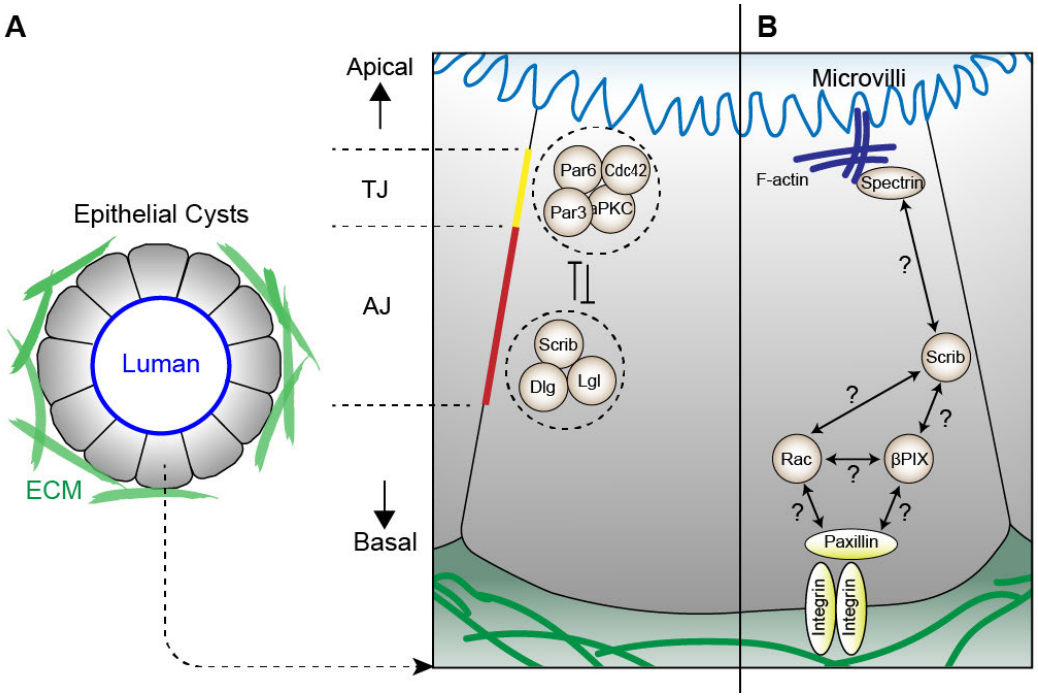


Figure 1. 6 Apical-basolateral polarity in luminal epithelial tissue

(A) The schematic shows the structure of epithelial cysts, where the epithelial cells cluster to form a barrier between luman and ECM. The interface between two epithelial cells was zoomed in to show the localisation of the polarity complex at TJ and AJ. (B) The potential protein-protein interactions linked Scribble to the focal adhesion at basal side, and to the actin networks at the apical side.

Scribble is known to form adherens junction (Bonello and Peifer, 2018). In MDCK cells, Scribble depletion reduced E-cadherin retention at adherens junction, suggesting Scribble is required for the stability of adherens junction (Qin et al., 2005). Scribble seems not to directly interact with E-cadherin, but is rather bridged by p120 or Rho-GEF SGEF (Awadia et al., 2019; Lohia et al., 2012). A recent study suggested Scribble together with Dlg firstly initiated apical-basolateral polarity, then the adherens junction can be positioned and formed (Bonello et al., 2019). This finding provides evidence that epithelial tissue is organised by cellular polarity, which was also endorsed by an almost concurrent study showing Scribble/Dlg formed a complex with SGEF to connect the function of adherent junction and cytoskeleton network (Awadia et al., 2019).

To maintain tissue organisation, an important factor is mitosis, as the cell-cell geometry is changed by the duplication of the cells (Ragkousi and Gibson, 2014). To control the positioning of the mitotic cell, and its daughter cells, spindle orientation is a key mechanism (Ragkousi and Gibson, 2014), because the spindle orientation provides a functional link between the geometric organisation and the fate of the progeny following cell division. Recent studies indicate that several attributes are combined to influence spindle orientation and daughter cell positioning, including intrinsic polarity, the location of adhesions and constraints on the cell shape (Charnley et al., 2013; Dimitracopoulos et al., 2020; Lesman et al., 2014; Li et al., 2019; Li and Burridge, 2019; Matsumura et al., 2016; Mitchison, 1992; Nestor-Bergmann et al., 2019; Niwayama et al., 2019; Petridou and Skourides, 2016; Rizzelli et al., 2020; Thery and Bornens, 2006). Scribble and Dlg are both required for spindle orientation in epithelial tissue (Bergstralh et al., 2013; Godde et al., 2014), suggesting a mechanism controlled by Scribble complex that can organise the tissue is additional to the function of the polarity control as described above.

1.6.3 Scribble acts as tumour suppressor

It is well appreciated that Scribble acts as a tumour suppressor. Some studies suggest the function of tumour suppression might be dependent on the subcellular localisation of Scribble. Scribble is normally localised at the cell cortex beneath the plasma membrane. The cortical localisation of Scribble is maintained by S-palmitoylation, by palmitoyl acyltransferase ZDHHC7, at the evolutionarily conserved N-terminal cysteine residues (Chen et al., 2016a). Taking colorectal epithelia as example, Scribble is co-localised with E-cadherin, ZO-1 and ZO-2 at the basolateral membrane. The LRR domain of Scribble is

crucial to maintain the localisation (Navarro et al., 2005a). A point mutation of P305L at the LRR domain of Scribble mislocalised Scribble at the cytoplasm, resulting in the loss of capacity to suppress Ras-MAPK-induced invasion (Elsum and Humbert, 2013). Overall, Scribble is commonly mislocalised in many human tumours (Elsum and Humbert, 2013). Some mutations of Scribble were detected in patients with prostate and breast cancer with poor outcome, and the mutations projected Scrib mislocalisation. In addition, mutation in Scribble also causes developmental defects, for example, neural tube closure defects, which is driven by Scribble mislocalisation.

The protein level of Scribble is also associated with tumour development. In a clinical study, Scribble protein expression was lost in the majority of invasive lobular breast carcinomas and 50% of ductal invasive carcinomas (Navarro et al., 2005a). The oncoviral protein HPV E6 is reported to cause degradation of Scribble (Banks et al., 2012). Upregulation of Scribble is also common in many cancers such as hepatocellular carcinomas, with high levels of Scribble correlated with reduced patient survival (Savi et al., 2014). Enforced upregulation of a mislocalisation mutant of Scribble in transgenic mice can promote breast cancer progression, suggesting that alterations in localization of Scribble polarity components could contribute to cancer (Feigin et al., 2014a).

The role of Scribble in tumour suppression is associated with by its capacity to maintain adherent junctions (Waghmare and Kango-Singh, 2016). In addition, some studies reveal a functional association with spectrin. β-spectrin (SPTBN1) interacts with SADH motif of Scribble, which supports Scribble cortical stability (Boëda and Etienne-Manneville, 2015). The role of SPTBN1 as a tumor suppressor is mediated through organisation of various signals, such as TGF-β, Wnt/β-catenin, Notch, and NF-κB (Lin et al., 2021; Yang et al., 2021). Scribble is also required for the localisation of many tumour-suppressing proteins, for example PTEN and β−PIX (Feigin et al., 2014a; Lim et al., 2017a). Overall, extensive studies have clearly elucidated how Scribble acts as tumour suppressor.

1.6.4 Scribble scaffolds multiple signalling platforms

Substantial evidence indicates that Scribble, as a scaffold of polarity, also organises a signalling platform. With respect to signalling organisation, protein domains of Scribble include Leucine Rich Repeats (LRR) at the N-terminus and PSD95/DLG/ZO-1 (PDZ) domains at the C-terminus (Santoni et al., 2020). Both LRR and PDZ domains mediate many protein-protein interactions. LRR domains have a 22–28 amino acid motif that has been identified in a number of proteins, and are usually involved in protein-protein

interactions. The crescent shape of LRR domain uniquely creates a long surface of parallel β-strands that can facilitate interactions with other proteins (Santoni et al., 2020). Regarding PDZ domains, protein docking is based on two α-helixes and six β-strands, about in a scale of 80-90aa, to recognise short peptide motifs in its interacting proteins (Lim et al., 2017b). In addition to these two primary domains, two LAPSD domains (ie. LAPSDa and LAPSDb) between LRR and PDZ were identified in Scribble, which leads to Scribble sometimes being categorised as a LAP protein (Santoni et al., 2020). Worth mentioning, ERK binding domains (KIM motifs) (Nagasaka et al., 2010) and spectrin-binding motif (SADH1-3) were identified in Scribble (Boëda and Etienne-Manneville, 2015).

These unique structures allow Scribble to regulate signalling such as the Wnt, PI3K, Hippo and MAPK pathways (Stephens et al., 2018). Interestingly, both in experimental and in structural predictions, most of the signalling interacting domains are the PDZ domains (Bonello and Peifer, 2018). There are at least four major PDZ interacting proteins that dictate cell signalling. First is βPIX (Lim et al., 2017b). βPIX is a Rac1/Cdc42 guanine nucleotide exchange factor (GEF) involved with cytoskeletal remodelling. Second is β-catenin (Yates et al., 2013). β-catenin is a canonical Wnt regulator that plays many roles such as adherens junctions formation and regulating actin dynamics (Yates et al., 2013). The third is VANGL2 (How et al., 2021). VANGL2 is a four-pass transmembrane protein that plays a role in non-canonical Wnt/Planar cell polarity (PCP) maintenance, which can regulate morphogenesis and migration processes. The fourth is PTEN (Feigin et al., 2014b). PTEN (phosphatase and tensin homologue deleted on chromosome ten) is a negative regulator of the PI3K/Akt signalling pathway. Both βPIX and β-catenin bind to Scribble in many cells, for example 293 cells and MCF10A cells, and are both required for roles of Scribble such as wound healing (Dow et al., 2008; Frank et al., 2012). Vangl2 interacts with Scribble to influence neural tube closure and wound healing (How et al., 2021). Scribble interacts with the C-terminus of PTEN through PDZ interactions to affect PI3K pathway (Feigin et al., 2014b).

1.7 Hypothesis and aims of thesis

1.7.1 Using transient HDAC inhibition to study DN3 cell development

I aimed to understand cell fate determination during the development of DN3 cells. As the β-selection is implemented in the DN3 compartment, profound events of fate determination including survival, self-renewal and differentiation take place during DN3 cell development. However, how these fate decisions are coordinated are not clear. In part, DN3 cell survival is underpinned by Notch1 signalling, while self-renewal and differentiation are governed by a cell-autonomous mechanism called pre-TCR signalling (Taghon et al., 2009). Presentation of pre-TCR is achieved through a series of cell-autonomous rearrangement in genome, implying that the status of chromatin might influence the accessibility of recombination machinery to assembly TCR gene. Thus, I hypothesised that the alteration of chromatin state can influence pre-TCR presentation, and therefore change fate determination. To this end, I utilised multiple reagents to influence the chromatin state, and later I focused on using HDAC inhibitors. To identify which HDAC are important in DN3 cell development, expression databases are helpful. I found HDAC4 and HDAC6 are highly expressed, and so likely to be important for T cell development in the DN3 stage (Figure 1.4). To study the role of HDAC4 and HDAC6, I used small molecule inhibitors LMK235 and ACY1215 to target the activity of HDAC4 or HDAC6.

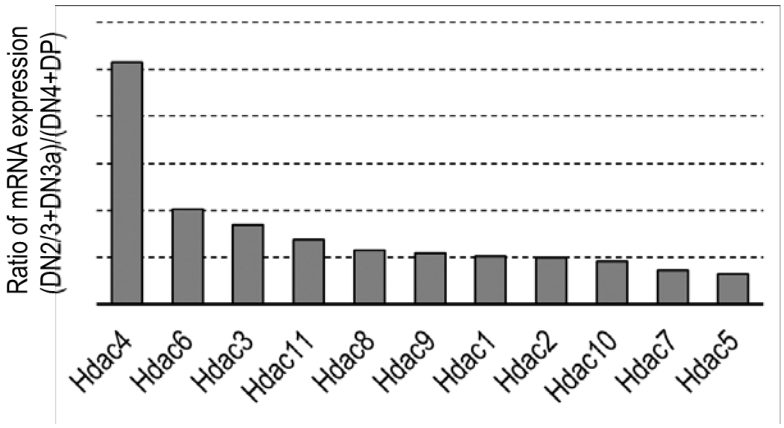


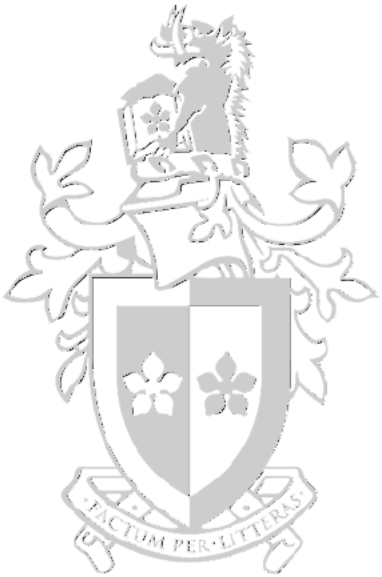
Figure 1. 7 Ratio of mRNA expression of HDACs in T cell development

The chart shows the ratio of mRNA expression (DN2/3+DN3a) / (DN4+DP) of HDACs. The values of mRNA expression were adapted from ImmGen database.

1.7.2 Single cell mitosis to elucidate the mechanism of daughter cell positioning

A crucial event to enable fate determination is cell division. For example, asymmetric cell division or symmetric cell division are pivotal to control levels of fate determinants in stem cell differentiation (Venkei and Yamashita, 2018). There are at least three major mechanisms involved in asymmetric vs. symmetric cell division: (1) environmental cue that guides spindle orientation, (2) cell-autonomous cue such as retraction fibre formation for spindle positioning, (3) differential inheritance of epigenetic modification between daughter (Venkei and Yamashita, 2018). For epithelial cells, the physical positioning of daughter cells is important, as the mitotic process disrupts the order and stability of epithelium. However, how the post-mitotic cells are re-stabilised is unclear. We hypothesised that the re-stabilisation of epithelial daughter cells relied on a mechanism similar to adherence junction (AJ) formation, as AJ is crucial for maintaining cell polarity both in interphase and mitosis (Saadaoui et al., 2014; Tuncay and Ebnet, 2015; Weber et al., 2011). One of the most prominent components to trigger AJ formation is Scribble (Legouis et al., 2000; Vaughen and Igaki, 2016). Therefore we investigated whether and how Scribble is required for daughter cell positioning.

Scribble's enrichment at AJ is highly dependent on E-cadherin. Ectopic expression of E-cad in tumor can re-establish the correct localisation of Scribble (Navarro et al., 2005b). Depletion of Scribble in epithelial cells also down-regulates Ecad and disrupts AJ formation, however the loss of Scribble can't be rescued by Ecad overexpression (Yamben et al., 2013) (Qin et al., 2005). These findings suggest Scribble and E-cad are synergistic in AJ maintenance, but Scribble can be an upstream or be involved with multiple processes that guide AJ formation. Back to the prospect of mitosis, as E-cadherin is required for mitotic spindle orientation through LGN recruitment, we also investigated whether the interaction between Scribble and Ecad is important in mitotic progression. Here, we used a single cell model (MCF10A) to investigate the process of mitosis. Through confocal imaging and live imaging, we elucidated Scribble and E-cadherin coordinate positioning in mitosis. Finally, we revisit whether the principle of AJ formation is applicable in the reattachment of the two daughters.



Chapter 2

Materials and Methods

2.1 Mammalian cell culture

2.1.1 OP9-DL1 stromal cells

OP9-DL1 stromal cells (from Juan Carlos Zúñiga-Pflücker, University of Toronto, Toronto, Canada) was maintained in αMEM (Sigma-Aldrich, M4526) supplemented with 20% (v/v) fetal calf serum, glutamine (1 mM) and penicillin/streptomycin (100 ng/mL).

2.1.2 Hematopoietic stem cells

Hematopoietic stem cells were generated from E14.5 mouse (C57BL/6) fetal liver cells (**Figure 2. 1A**). Briefly, the uteri containing the foetuses were collected from the scarified pregnant mice, and the foetuses were isolated into cold sterile PBS. The livers were then isolated from the connective tissue of the foetus, and then fetal liver cells were collected by forceful pipetting and centrifuge. Fetal liver cells were preserved in fetal calf serum and Dimethyl Sulfoxide (DMSO) (Sigma Aldrich) with a ratio 9:1 and stored in liquid nitrogen.

2.1.3 *Ex vivo* generation of developing T cells

To induce T cell development, hematopoietic stem cells were co-cultured with OP9-DL1 stromal cells at a 1:1 ratio in a 6-well plate (2×10^5). The coculture was maintained in αMEM (Sigma-Aldrich, M4526), supplemented with foetal calf serum (10% v/v), glutamine (1 mM), β-mercaptoethanol (50 μM), sodium pyruvate (1nM), HEPES (10 mM), penicillin/streptomycin (100 ng/mL), mouse interleukin 7 (1 ng/mL) and mouse FMS-like tyrosine kinase 3 (5 ng/ml). When OP9-DL1 stromal cells were confluent after 3-4 days of coculture, primary thymocytes were dissociated from the stromal cells through forceful pipetting, and were harvested with the fresh OP9-DL1 stromal cells.

2.1.4 Primary thymocytes collection and culture

The primary thymocytes were released from 4-5-week C57BL/6 male mouse thymus (**Figure 2. 1B**) for sorting, analysis or further culturing with OP9-DL1 stromal cells under the same culture conditions as the HSC system described above.

2.1.5 Ethics of animal experiments

C57bl/6 mouse E14.5 fetal livers and 4-5-week mouse thymi were collected according to Peter MacCallum Cancer Centre Ethics Committee approval E627 and La Trobe University Animal Ethics Committee approval AEC-20024.

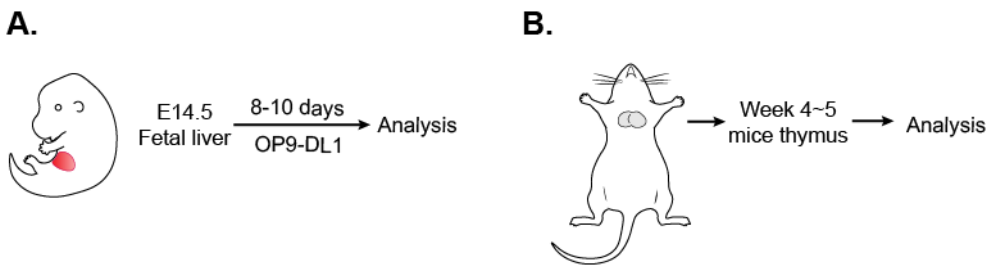


Figure 2. 1 Source of developing T cells

(A) Fetal liver; **(B)** Thymus.

2.1.6 MCF10A cells

MCF10A cells were cultured in DMEM:F12 (Dulbecco's Modified Eagle Medium: F12) media, supplemented with 5% (v/v) horse serum (Gibco), 10 µg/ml insulin (Novo Nordisk) , 0.5 µg/ml hydrocortisone (Sigma-Aldrich), 20 ng/ml human epidermal growth factor (Sigma-Aldrich), 100 ng/ml cholera toxin (List Biological Labs), 100 ng/ml penicillin / streptomycin, 2 mM glutamine, and maintained at 37°C in 5% CO_2. Stable MCF10A cell lines that expressed short hairpin RNA against human Scribble (#7) and the control were created and analysed as described in (Dow et al., 2007). Scribble depletion was re-confirmed by flow cytometry. Briefly, the cells were fixed by IC Fixation Buffer (eBioscience) according to manufacturer's protocol, followed by incubation with anti-Scribble antibody (sc-11049, Santa Cruz Biotechnology), and then labelling with Alexa Fluor 647 conjugated antibody (ab150131, Abcam), and examined by flow cytometry (BD FACSAria III, BD).

2.1.7 HeLa cells

HeLa cells were cultured in DMEM media (Gibco), supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine and 100 ng/ml penicillin / streptomycin, and maintained at 37°C in 5% CO_2.

2.2 Cell-based assays

2.2.1 T cell counting

T cells and OP9-DL1 were seeded at a 1:1 ratio ($4x10^4$) in a 24-well plate. To count T cells from the OP9-DL1 coculture, T cells were dissociated from OP9 cells by forceful pipetting, and OP9 cells were spun down and removed. T cells then were stained with Trypan blue, and were counted using haemocytometer blood counting chamber.

2.2.2 T cell proliferation tracking

T cells were pre-labelled with carboxyfluorescein diacetate (CFSE) (5 μM in PBS, Molecular Probes) for 10 minutes at room temperature and in dark, and then were washed by cell culture media after staining. The T cells stained with CFSE were then co-cultured with the OP9-DL1 cells for the subsequent assay, or were incubated for 1 hour for recording the initial intensity of CFSE. At 1 hour, the T cells stained with CFSE were washed and collected in the FACS buffer, and the fluorescent intensity of CFSE was recorded by flow cytometry.

2.2.3 T cell viability assay

To distinguish dead cells, T cells were labelled with propidium iodide (PI, 92 ng/mL, Sigma Aldrich) in the incubator for 10 minutes. To distinguish apoptotic or pro-apoptotic cells, T cells were labelled with Annexin V and DAPI for 15 minutes in the incubator. After staining, T cells were washed and collected in the FACS buffer for flow cytometry analysis.

2.2.4 MCF10A cell counting

MCF10A cells were collected by using Tryple, and were counted after Trypan blue staining and using haemocytometer blood counting chamber.

2.3 Assays using small-molecule inhibitors

2.3.1 HDAC6 inhibition in T cells

The small-molecule inhibitor ACY1215 (MedChemExpress), also known as Ricolinostat, was used at 1 μM (Laino et al., 2019). ACY1215 was dissolved in DMSO to 5 mM as stock solution, and was diluted to 1 μM with culture media immediately prior to application to cells. ACY1215 is a type of fatty hydroxamic acid-derived histone deacetylase 6 inhibitor, with molecular weight 433.5 (**Figure 2. 2 A**). The IC_{50} of ACY1215 to HDAC6 is around 4.7 nM, which is at least ten-fold more selective to HDAC 1–3 (Xiao and Zhang, 2020). Like most HDAC6 inhibitors, ACY1215 binds to the second catalytic domain of HDAC6, and subsequently inhibits deacetylation to the substrates (**Figure 2. 2 B**). As the simulation shows, ACY1215 is docked in the HDAC6 catalytic domain, and bridged by multiple hydrogen bonds (**Figure 2. 2 C**) (Porter et al., 2017).

2.2.5 Stat5 signalling inhibition in T cells

The small-molecule inhibitor of Stat5, Pimozide (CAS 2062-78-4 Sigma-Aldrich) was used at a final concentration of 10 μM.

2.2.6 Actomyosin depolymerisation in MCF10A cells

Actin depolymerisor Cytochalasin D (Sigma-Aldrich) was used at 5 μM, Myosin II inhibitor Blebbistatin (Sigma-Aldrich) was used at 2.5 μM, and Arp2/3 inhibitor CK-869 (Sigma-Aldrich) was used at 50 μM. The stocks of Cytochalasin D, Blebbistantin and CK-869 were dissolved in DMSO in the titre of 500X-1000X concentrated than the working concentration. The stock solutions were then diluted in the culture media to the working concentration before use. For fixed imaging, the inhibitors were applied for 15 minutes under regular culture condition before fixation. For live-cell imaging, Arp2/3 inhibitor CK-869 was used at 0.1 μM for 2 hours during image acquisition.

2.2.5 E-cadherin blocking in MCF10A cells

Mouse monoclonal anti-E-cadherin antibody HECD-1 (ab1416, Abcam) was used to block the function of surface E-cadherin. For fixed-cell imaging, sparsely plated cells (2×10^3 cells/cm^2) were cultured in culture media containing 5 μg/ml antibody HECD-1 for 1.5 hour before fixation. For live-cell imaging, sparsely plated cells (2×10^3 cells/cm^2) were cultured in HECD-1 containing culture media (5 μg/ml) for one hour prior the time-lapse acquisition, followed with image acquisition for around 2 hours.

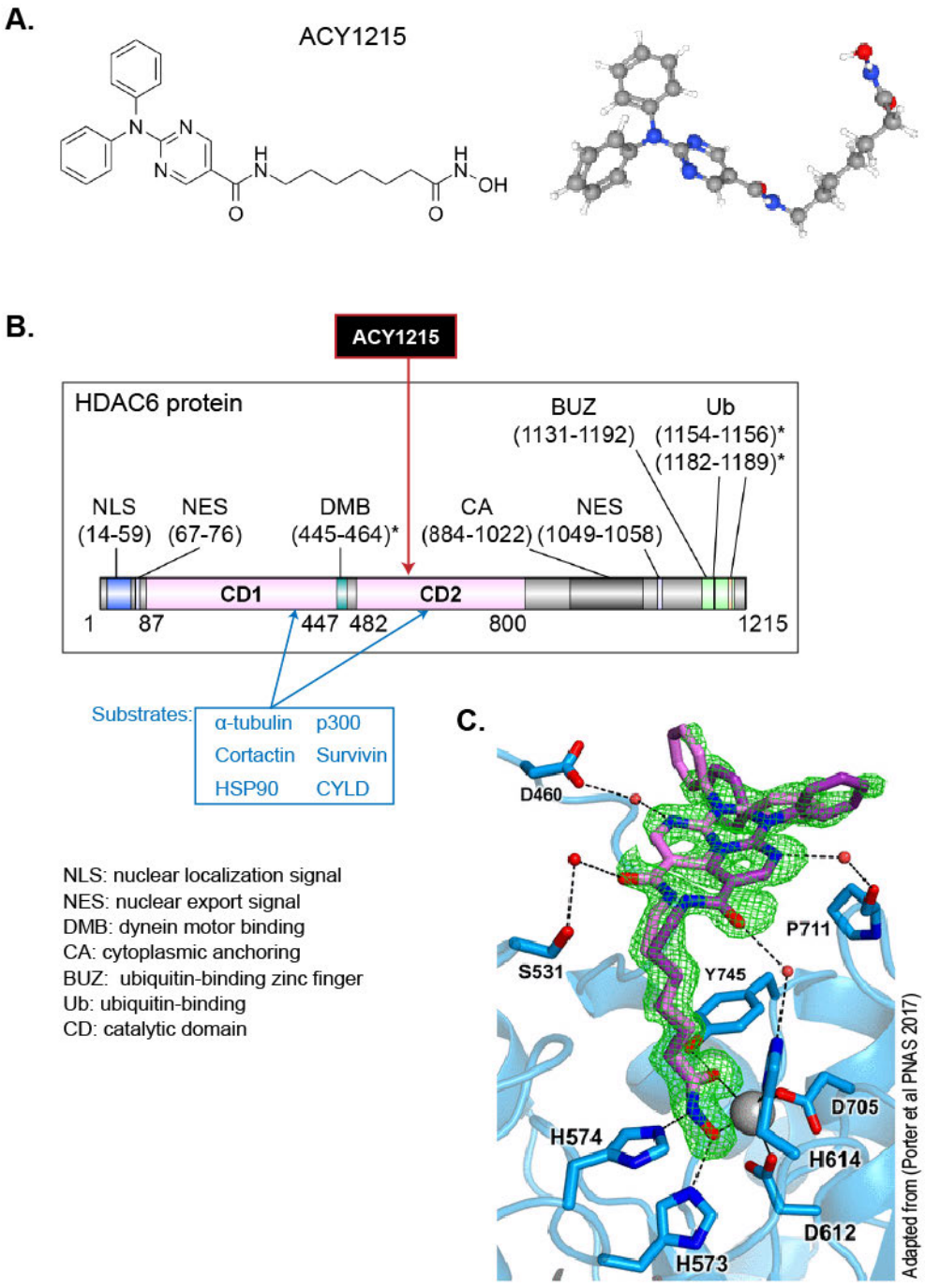


Figure 2. 2 Small-molecule inhibitor ACY1215 targets HDAC6

(A) The chemical structure of ACY1215. (Left) from MedChemExpress database; (Right) from PubChem (1316214-52-4). (B) The functional domains of HDAC6 protein are illustrated according to (Li et al., 2013) and the Uniprot (*). (C) The simulated HDAC6-ACY1215 structure was adapted from (Porter et al., 2017).

2.4 si-RNA silencing

Scribble and E-cadherin was depleted from MCF10A cells and/or HeLa cells by siGENOME SMARTpool si-RNA against human Scribble and/or E-cadherin. si-RNA was carried by DharmaFECT 3 according to the manufacturer's protocol, and was incubated with cells in serum-free cell culture media for transfection. We applied two rounds of si-RNA silencing to increase knockdown efficiency. Briefly, the first round of si-RNA silencing (20 nM of si-RNA) was applied on a 70% confluent monolayer cultured in a well of a 12-well culture plate. After an incubation with si-RNA containing media for 16 hours, the cells were refreshed with regular culture media, and incubated for further 8 hours. We plated these si-RNA-transfected cells on glass-bottomed 8-well chamber (ibidi) for the purpose of subsequent cell imaging, and grew the cells for around 12 hours. A second round of 8-hour si-RNA transfection (10 nM of si-RNA) was applied to these cells, and a subsequent 16-hour incubation under regular culture media. Cells were then fixed for further analysis.

2.5 Flow cytometry

2.5.1 Sorting

To purify T cell populations using flow cytometry, primary thymocytes were dissociated from OP9-DL1 stromal cell, and were resuspended in PBS buffer containing 2% FBS and antibody cocktail for surface staining. Antibody cocktails were comprised of lineage markers (NK-1.1, CD11b, CD45R, Ly6G6C, Ter119), and differentiation markers (CD4, CD8, CD25, CD44, CD28 and CD2; the details about fluorochrome, manufacturer and dilution were summarised at the Table 2.1).

Primary thymocytes or fetal liver cells were incubated with antibody cocktails for 40 minutes before FACS. Flow cytometry (BD Aria III) was used to analyse and sort the populations of developing T cells. As indicated (**Figure 2. 3A**), DN3 cells were identified based on surface expression (Lineage^{-}, CD4^{-}, CD8^{-}, CD25^{HI}, CD44^{LO}). DN3a cells were DN3 cell with CD28^{LO}, and DN3b cells were DN3 cell with CD28^{HI}. DP cells were identified based on surface expression (Lineage^{-}, CD4^{+}, CD8^{+}).

The fetal liver derived DN3a and DN3b cells were generally sorted after 8-10 coculture with OP9-DL1 cells (**Figure 2. 3B**). The primary DN3a and DN3b cells were collected

from adult thymus (**Figure 2. 3C**), and either immediately analysed or were co-cultured with OP9-DL1 stromal cells for further use (**Figure 2. 3D**).

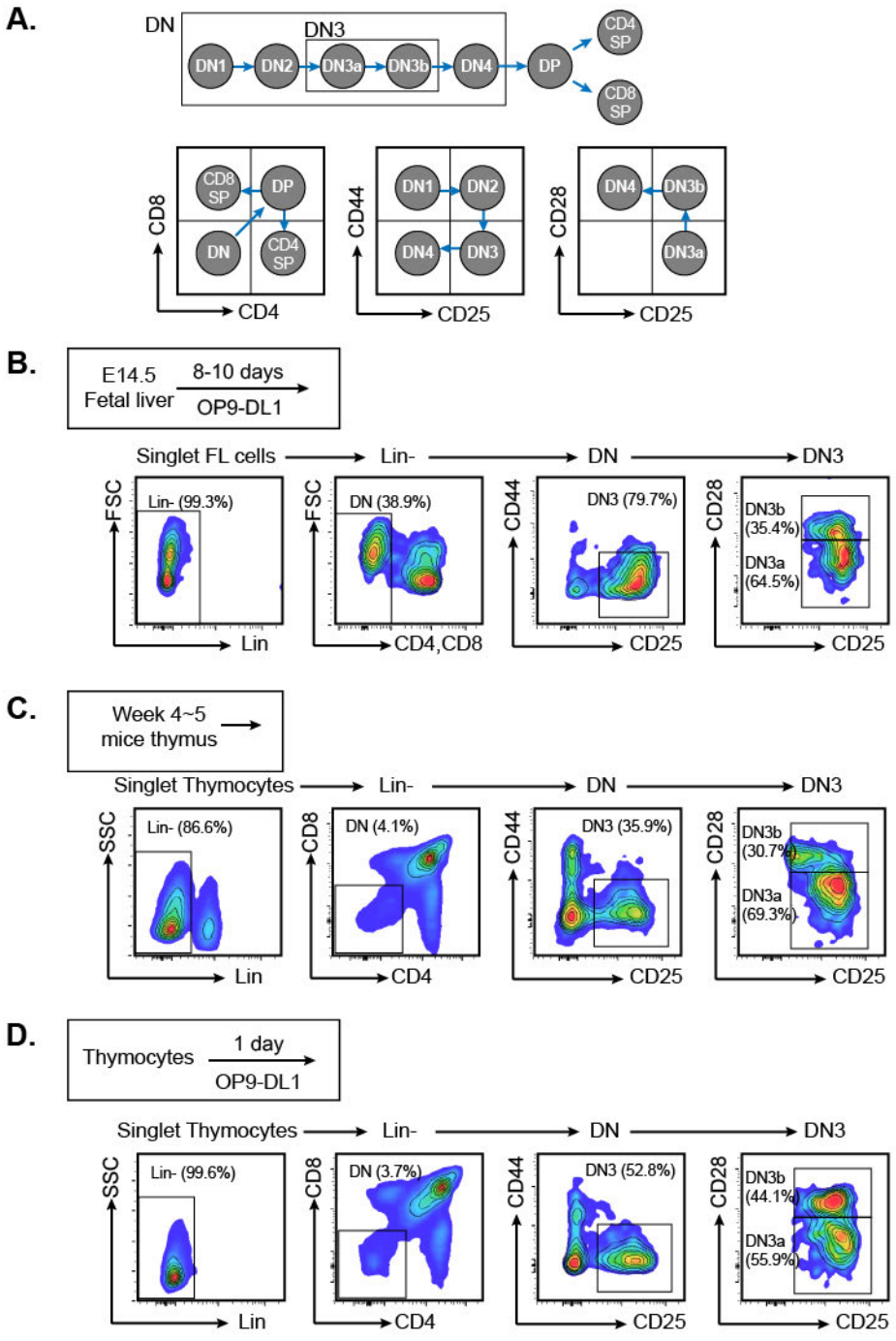


Figure 2. 3 The process of flow cytometry analysis and sorting

(A) The schematic shows the plot of surface phenotypes of developing T cells. (B) The process using fetal livers to generate developing T cells. (C, D) The process using mouse thymocytes.

2.5.2 Intracellular expression analysis using flow cytometry for T cells

To detect intracellular expression of TCRβ, Ki67, Lef1, c-Myc, Notch1, HDAC6 and H3K18ac, the Foxp3/Transcription Factor Staining Buffer Kit (eBioscience) was used according to the manufacturer's instruction. Briefly, the primary thymocytes after surface staining were incubated with fixative for 15 minutes in dark, room temperature, followed with centrifuge and washing out the fixative. To detect expression of acetyl-tubulin, the cells were fixed with IC Fixation Buffer (eBioscience) for 15 minutes in dark, room temperature, followed with centrifuge and washing out the fixative. The fixed cells were then incubated with antibody for flow cytometry analysis.

2.5.3 Intracellular expression analysis using flow cytometry for MCF10A cells

Protein levels of Scribble, E-cadherin and DLG were assessed in unsynchronised and confluent MCF10A cells. To collect the cells from culture dish, 2.5 mM EDTA was applied to the culture media for 20 minutes in incubator before trypsinization. Then, the cells were fixed by IC Fixation Buffer (eBioscience) according to manufacturer's protocol, followed by incubation with primary antibody against Scribble, E-cadherin and DLG respectively. After labelling with Alexa Fluor-conjugated secondary antibody, the cells were examined by flow cytometry (BD FACSAria III, BD).

2.6 Immunofluoescence and fixed image acquisition by confocal microscopy

2.6.1 Fixed imaging for T cells

The sorted DN3a or DN3b were grown on glass-bottomed 8-well chambers (ibidi) with OP9-DL1 stromal cells, and were fixed with 4% formaldehyde for 8 minutes at room temperature followed by 0.1% Triton X-100 permeabilization for 5 minutes. The samples were then incubated with primary antibody overnight at 4°C (details about antibody usage are summarised in Supplementary table 1). After the primary antibody was washed out with PBS, the cells were labelled with fluorochrome-conjugated secondary antibodies (as Table S1), DAPI (Thermo Fisher Scientific), and Phalloidin (Abcam). The samples were examined using a FV3000 confocal microscope (Olympus) and 60X lens (1.30 NA, UPLSAPO, Olympus). The images were processed with maximum intensity projection using Image J. The mean intensity of region of interest was examined in Image J.

2.6.2 Fixed imaging for MCF10A cells

MCF10A cells were grown on glass-bottomed 8-well chambers (ibidi), and fixed with 4% formaldehyde for 8 minutes at room temperature followed by permeabilization with 0.1% Triton X-100 for 5 minutes. The samples were then incubated with primary antibody overnight at 4°C. Next, the samples were washed with PBS, and labelled with fluorochrome-conjugated secondary antibodies, DAPI (Thermo Fisher Scientific), and Phalloidin (Abcam). The samples were examined using a FV3000 confocal microscope (Olympus) and 60X lens (1.30 NA, UPLSAPO, Olympus). To acquire images of the whole cell, scanning of multiple sections with a z distance of 0.5 μm was applied. These sections were either merged or converted to different orthogonal projections using Image J, and were also used in the analysis of spindle orientation. To acquire images of subcellular structures such as retraction fibres or filopodia, scanning of multiple sections with a z distance of 0.2 μm was applied. The images were processed with maximum intensity projection of three continuous stages for subcellular structures such as retraction fibres, filopodia and nascent junctions using Image J. An ImageJ plugin '3D viewer' was used to reconstruct the three-dimensional images.

2.6.3 Proximity Ligation Assays for MCF10A cells

Proximity Ligation Assays (Duolink, Merck) was performed on MCF10A cells according to the instruction of manufacturer. Briefly, cells were stained with primary antibody (from mouse and rabbit respectively) using the immunostaining protocol in 2.5.2. Then, the sample was incubated with Duolink anti-rabbit PLUS and anti-mouse MINUS PLA probes, and followed with DNA ligation. After wash, the ligated DNA template were amplified in situ for detection under microscopy.

2.7 Time lapse imaging

For live-cell imaging, MCF10A cells were sparsely plated at approximately 2×10^3 cells/cm^2 and cultured for 1.5 days. Before image acquisition, the cells were incubated in cell culture media containing 250 nM SiR-DNA (Spirochrome) for 30 minutes to label DNA, which was then replaced with regular cell culture media. The processes of mitosis and cell division were imaged using an Olympus FV3000 confocal microscope (Olympus) and 20X lens (0.75 NA, UPLSAPO, Olympus) in a chamber maintained in 37°C and with 5% CO_2. Mitotic cells were identified by the rounded cell morphology and

the bar shape of condensed chromosomes, and were recorded every 3 minutes for more than 30 minutes after anaphase onset. The z scanning, with 1 μm per z stage, was applied to cover the whole volume of the cell. The images were processed with average intensity projection for both bright field channel and the channel for SiR-DNA by Image J. To quantify the time taken by each daughter to re-adhere to the substrate, the time frame showing each daughter cell flattening was identified as the time of re-adherence, and the time of entry into anaphase onset was subtracted. To quantify the distance between daughters' nucleus labelled by SiR-DNA, we measured the shortest distance between two segregated parts of DNA.

2.8 Data visualisation and statistics

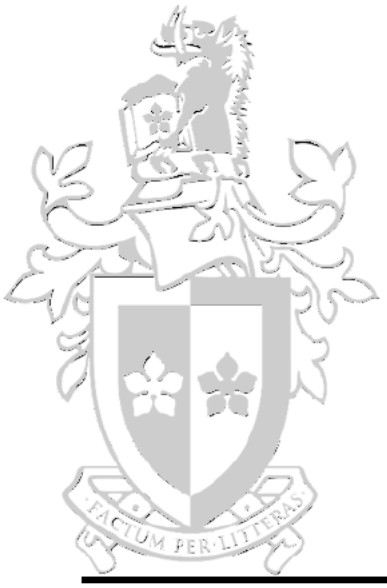
MATLAB (version R2021a) and Python (version 3.8.1) were used to visualise the data. The following algorithms were used in Python for analysis and plotting: matplotlib (version 3.1.3), pandas (version 1.0.1), seaborn (version 0.10.0), numpy (version 1.18.1), and plotly (version 4.14.3). Data of flow cytometry were analysed by FlowJo software (version X10.0.7r2. Tree Star, Inc.). The bar plot was shown as mean ± SEM. More than three repeats were included in the statistics and were colour-coded for each individual repeat. Microsoft Excel (version 2016) was used for t-test. P value were included along with the figures, or were indicated by asterisks "***" (means $p<0.005$), "**" (means $p<0.01$), and "*" means ($p<0.05$); where asterisks were not shown, the p value was greater than 0.05. Confocal images were collected using an Olympus FV3000 confocal microscope and FV31S-SW Viewer software, and were processed using ImageJ (version 1.52p).

Table 2. 1 Antibody used in Chapter 3 and 4

Antibody	Supplier	Clone/Catlog	Usage (titre)	
CD11b	BD Biosciences	M1/70	Flow cytometry	1:1000
NK1-1	BD Biosciences	PK136	Flow cytometry	1:1000
CD45R	Tonbo Biosciences	RA3-6B2	Flow cytometry	1:1000
Ly6G.6C	BD Biosciences	RB6-8C5	Flow cytometry	1:1000
TER119	BioLegend	TER119	Flow cytometry	1:1000
CD4	BioLegend	RM4-5	Flow cytometry	1:400
CD8	BioLegend	53-6.7	Flow cytometry	1:400
CD44	BioLegend	IM7	Flow cytometry	1:300
CD25	BioLegend	PC61	Flow cytometry	1:300
CD28	BioLegend	E18	Flow cytometry	1:200
CD27	BioLegend	LG.3A10	Flow cytometry	1:800
CD2	BioLegend	RM2-5	Flow cytometry	1:500
CD71	BioLegend	RI7217	Flow cytometry	1:400
CD98	BioLegend	4F2	Flow cytometry	1:400
CD5	Tonbo Biosciences	53-7.3	Flow cytometry	1:300
IL7R	BioLegend	SB/199	Flow cytometry	1:200
TCRβ	Tonbo Biosciences	H57-597	Flow cytometry	1:200
TCRγδ	BioLegend	GL3	Flow cytometry	1:400
Ki-67	BioLegend	16A8	Flow cytometry	1:800
c-Myc	Cell Signaling Technology	5605	Flow cytometry	1:250
Lef1	Cell Signaling Technology	2230	Flow cytometry	1:500
Notch1	Abcam	ab27526	Flow cytometry	1:300
			Immunofluorescence	1:200
LAT	Cell Signaling Technology	9166	Immunofluorescence	1:200
Ac-tubulin	Cell Signaling Technology	5335	Flow cytometry	1:500
			Immunofluorescence	1:300
Ac-tubulin	Abcam	Ab24610	Immunofluorescence	1:1000
H3K18ac	Abcam	Ab1191	Flow cytometry	1:1000
HDAC6	Merck	07-732	Flow cytometry	1:500
HDAC6	Novus Biologicals	NBP1-78981	Immunofluorescence	1:500
pStat5	Cell Signaling Technology	4322	Flow cytometry	1:250

Table 2. 2 Antibody used in Chapter 5 and 6

Antibody	Supplier	Catlog	Usage (titre)	
Integrin β1	Abcam	ab30394	Immunofluorescence	1:300
E-cadherin	Abcam	ab1416	Immunofluorescence	1:100
E-cadherin	Cell Signaling Technology	3195	Immunofluorescence	1:250
α-Tubulin	Rockland Immunochemicals	200-301-880	Immunofluorescence	1:1000
α-Tubulin	Rockland Immunochemicals	600-401-880	Immunofluorescence	1:1000
Scribble	Santa Cruz Biotechnology	sc-11049	Immunofluorescence	1:250
Scribble	Upstate	07-643	Immunofluorescence	1:500
NuMA	Abcam	ab109262	Immunofluorescence	1:500
Paxillin	Millipore	05-417	Immunofluorescence	1:400
Arp2	Abcam	ab49674	Immunofluorescence	1:500
Ezrin	BD Biosciences	610602	Immunofluorescence	1:800
Myosin X	Novus Biologicals	NBP1-87748	Immunofluorescence	1:500
β-Catenin	BD Biosciences	610153	Immunofluorescence	1:500
β-PIX	Cell Signaling Technology	4515	Immunofluorescence	1:400
β-PIX	Millipore	07-1450-I	Immunofluorescence	1:500
Myosin IIb	Cell Signaling Technology	8824	Immunofluorescence	1:500



Chapter 3

An investigation of αβ T cell development under histone deacetylase inhibition

3.1 Introduction

T cells are generated in a multistep, highly orchestrated process guided by stromal cells in the thymus. Multipotent hematopoietic progenitors migrate to thymus and commit to T-cell lineage by limiting the potential of non-T lineages such as B-cell lineage or myeloid lineage (Rothenberg, 2011). After T-lineage commitment, two types of T cells, αβ and γδ T cells, are specified in the process of T cell development. The αβ T cell receptor (αβ TCR) serves as an antigen receptor to interact with peptide–MHC complex and mediate an antigen-specific immune response (Wang and Reinherz, 2012). As reviewed in the introduction, the αβ TCR is generated through gene recombination to produce TCRβ and TCRα chains. Unlike αβ T cells, γδ T cells usually use the germline-encoded genome to construct γδ TCR, which only recognise a limited source of antigens (Willcox and Willcox, 2019). The specification of αβ versus γδ T-cell fate happens in the DN3a stage (Taghon et al., 2006) or in the $CD25^+$ DN2 or $CD25^+$ DN3 compartments (Parker and Ciofani, 2020). In most of the cases, αβ TCR and γδ TCR are not co-expressed in T cells, with the exception of a rare subset of T cells expressing αβγδ TCR recently detected in fetal skin (Reitermaier et al., 2021). Overall, understanding the specification between αβ and γδ is an important topic, and can provide the knowledge for T cell generation.

In thymus, Notch1 signalling, through intracellular Delta ligands, is the most explored mechanism to enforce T cell differentiation (Radtke et al., 2010). The Notch1 signalling actives T-cell differentiation by importing the intracellular Notch domain (NICD) into nucleus, and then activating the transcription factor CSL. Interestingly, under the same guidance from Notch1 signalling, αβ T cell and γδ T cell show a distinct transcriptional profile. For example αβ DN3b cells display increased HEB and Aiolos gene expression, while γδ DN3b cells display increased Runx3, Bcl-2, Egr-2, Egr-3, Id3 and Helios (Taghon et al., 2006). This difference suggests some lineage-specific co-factor might guide NICD-CSL complex targeting different genes for transcription. As multiple HDACs play a redundant role as a CSL co-repressor to keep Notch-targeted genes off (Borggrefe and Oswald, 2014), raising the question of whehether selectivity of HDAC controls αβ and γδ fate specification. Here, I used selective HDAC6 inhibitor, ACY1215, to investigate early T cell development.

By using ACY1215, the results lead to a focus on αβ fate specification, as the influence on γδ fate specification was minimal. β-selection is in part determined by stromal cells in the thymic microenvironment, which provide Notch and chemokine signals that promote survival and differentiation (Chann and Russell, 2019; Dutta et al., 2021). Recently, it has become clear that interactions between the pre-TCR and the MHC-peptide on stromal cells also provide an opportunity to test TCR signalling (Allam et al., 2021; Dutta et al., 2021; Li et al., 2021) These interactions trigger a sequence of transcriptional changes and epigenetic modifications that ensure only the cells with an appropriately recombined TCRβ chain survive, proliferate and differentiate. How pre-TCR signalling integrates fate determination including proliferation, differentiation and survival during β-selection is still not understood (Chann and Russell, 2019; Dutta et al., 2021). In this chapter and next chapter, I used ACY1215 to identify novel aspects of β-selection.

3.2 Results

3.2.1 HDAC6 is expressed during T cell development

HDAC6 proteins are abundant in both nucleus and cytoplasm in developing T cells (**Figure 3. 1** and **Figure 3. 2**). HDAC6 activity seems to be associated with epigenetic state in developing T cells as treatment of ACY1215, an HDAC6 selective inhibitor, for two days increased acetylation of H3K18 (**Figure 3. 3**). Moreover, ACY1215 increased tubulin acetylation at MTOC, and presumably the acetylation is not through Arp2/3-dependent mechanisms (Figure 3. 2) (Butler and Cooper, 2009; Shi et al., 2019), but through the mechanism of HDAC6 inhibition. The acetylation increased at both nucleus and cytoplasm reflects the location of endogenous HDAC6. Together, these data suggest HDAC6 is likely required for T cell development.

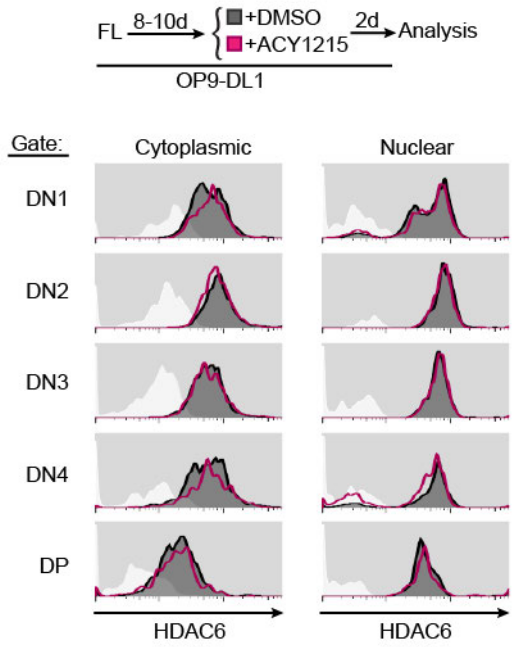


Figure 3. 1 HDAC6 is expressed in developing T cells.

To examine HDAC6 protein expression, developing T cells treated with DMSO or ACY1215 for two days were fixed by cytoplasmic fixative or nuclear fixative, followed by examination of HDAC6 expression.

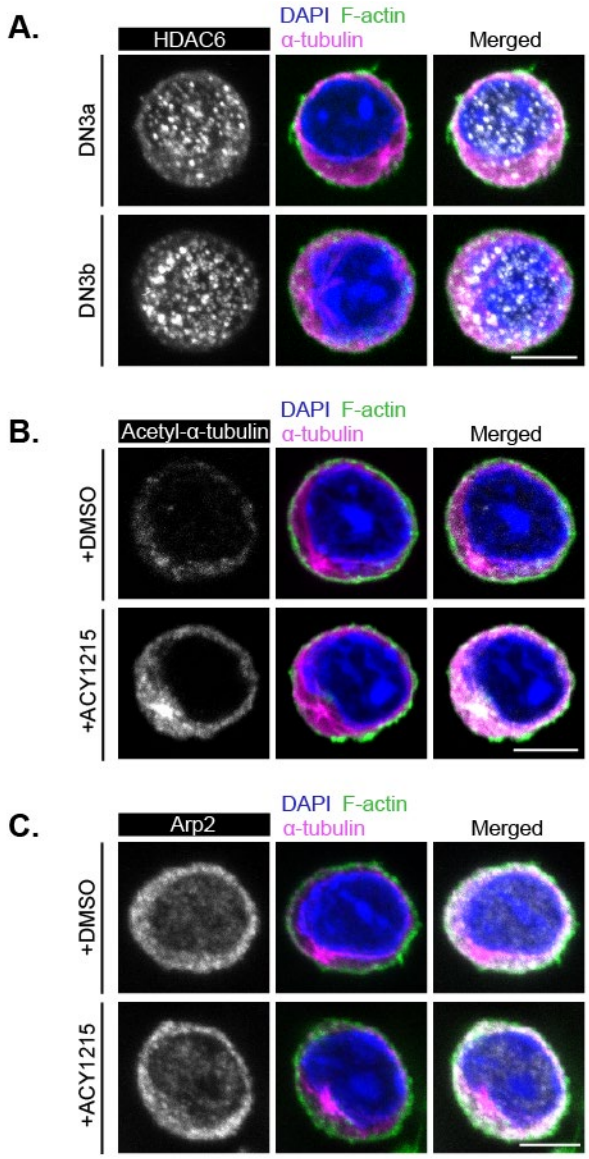


Figure 3. 2 HDAC6 is expressed in nucleus and cytoplasm of developing T cells.

(A) The sorted DN3a or DN3b cells were assessed for HDAC6 expression by immunostaining. **(B, C)** The sorted DN3b cells were treated with ACY1215 or DMSO for 1 day, followed by immunostaining for Arp2, acetyl-α-tubulin (K40), and α-tubulin.

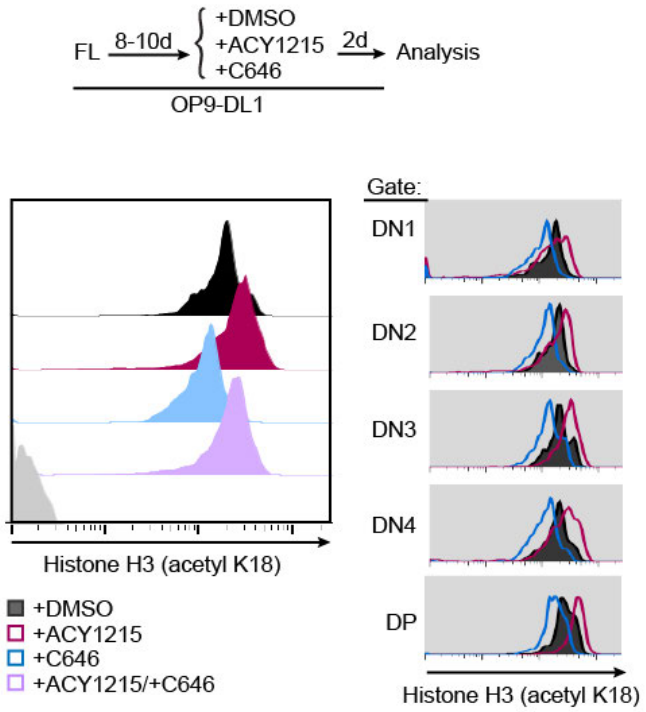


Figure 3. 3 H3K18 acetylation was promoted by ACY1215.

Developing T cells were treated with ACY1215, the selective inhibitor of p300 and CBP, C646, or DMSO for 1 day as indicated, and the expression of H3K18ac were assessed and shown in the histogram.

3.2.2 ACY1215 treatment arrests T cell development at DN3 stage

At Day 8-10 of the co-culture, when the cells were predominantly at the DN3 stage of T cell development, I applied ACY1215 at a concentration previously reported to have no impact on lymphocyte viability but that altered function of mature T cells (Laino et al., 2019a). Compared with DMSO treated cells, cells treated with ACY1215 for two days showed an increased proportion of DN cells, and reduced proportions of the other stages of differentiation: DP, CD4-SP and CD8-SP, suggesting HDAC6 is required for optimal T cell development to the DP stage (**Figure 3. 4A**). This shift in differentiation stages was accompanied by a generalised inhibition of proliferation at several stages of T cell development (**Figure 3. 5**). I analysed CD25 and CD44 expression in the DN cells to compare proportions at different DN stages (**Figure 3. 4B**). An increase in the proportions of DN3 cells, and a commensurate decrease in DN4, suggested that the ACY1215-treated cells were blocked at the DN3 stage.

To define the cell stage at which ACY1215 influences differentiation to DP, I monitored cell expansion and differentiation from sorted DN3a and DN3b that had been treated for four days with ACY1215 or DMSO. By day 4, approximately 25% of the control DN3b cells had progressed to DP (**Figure 3. 6**) and this proportion was reduced to below 5% by ACY1215 treatment. The reduced DP cells was not due to an accumulation of cells at the DN4 stage, since these were also reduced by ACY1215 treatment (Figure 3. 6). I performed a parallel analysis using DN3a cells as a starting population (**Figure 3. 7**) Although the very few DN3a cells had progressed beyond the DN stage by day 4, ACY1215 treatment led to a similar inhibition of progression from DN3a to DP, again with minimal impact on progression through DN4. These data indicate that the major effect of ACY1215 was on the DN3b stage, but did not exclude an additional effect on DN3a cells. To determine whether the shift in proportions indicated alterations in differentiation or proliferation of particular subsets, I assessed the number of cells at each differentiation stage that developed from the sorted DN3b (Figure 3. 6). By day 4, DN3 cells had expanded more than 50 fold, with a negligible reduction caused by treatment ACY1215.DN4 cell numbers were also only slightly reduced by ACY1215 treatment. The most dramatic effect occurred at the DP stage, with a reduction in cell number of over two orders of magnitude. Together, these data indicate that ACY1215 treatment substantially inhibited cells from progressing beyond the DN3b stage.

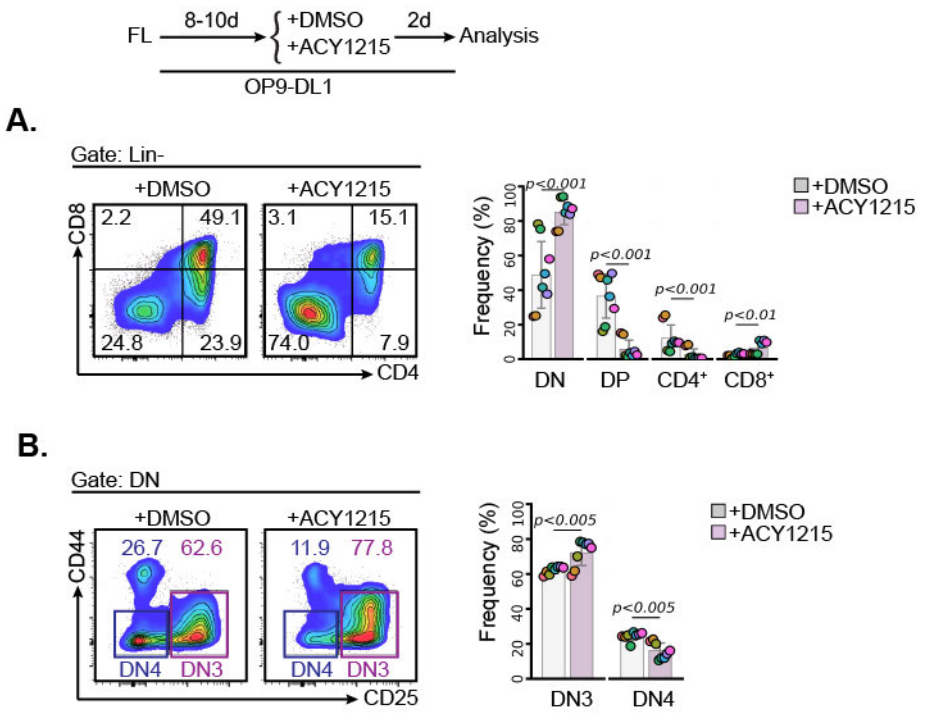


Figure 3. 4 ACY1215 arrests T cell development at DN3 stage

Developing T cells were treated with ACY1215 or DMSO for 2 days as indicated, and the Lin- cells were assessed for CD4 and CD8 expression to determine the proportions of DN, DP, CD8+ and CD4+ populations **(A)**. The DN (Lin-, CD4-, CD8-) cells from (A) were assessed for CD44 and CD25 expression to determine the proportions of DN3 and DN4 populations **(B)**. Individual colours in swarm plot represent independent experiments.

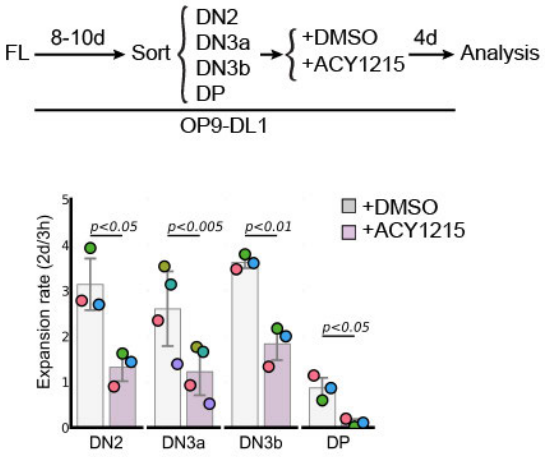


Figure 3. 5 ACY1215 reduced proliferation

Sorted, FL-derived DN2, DN3a, DN3b and DP were cultured for 2 days ACY1215 or DMSO treatment as indicated. Cell numbers were counted at 3 hours and 2 days, and the proliferation rate (2 days / 3 hours) was summarised in swarm plot, with individual colours representing independent experiments.

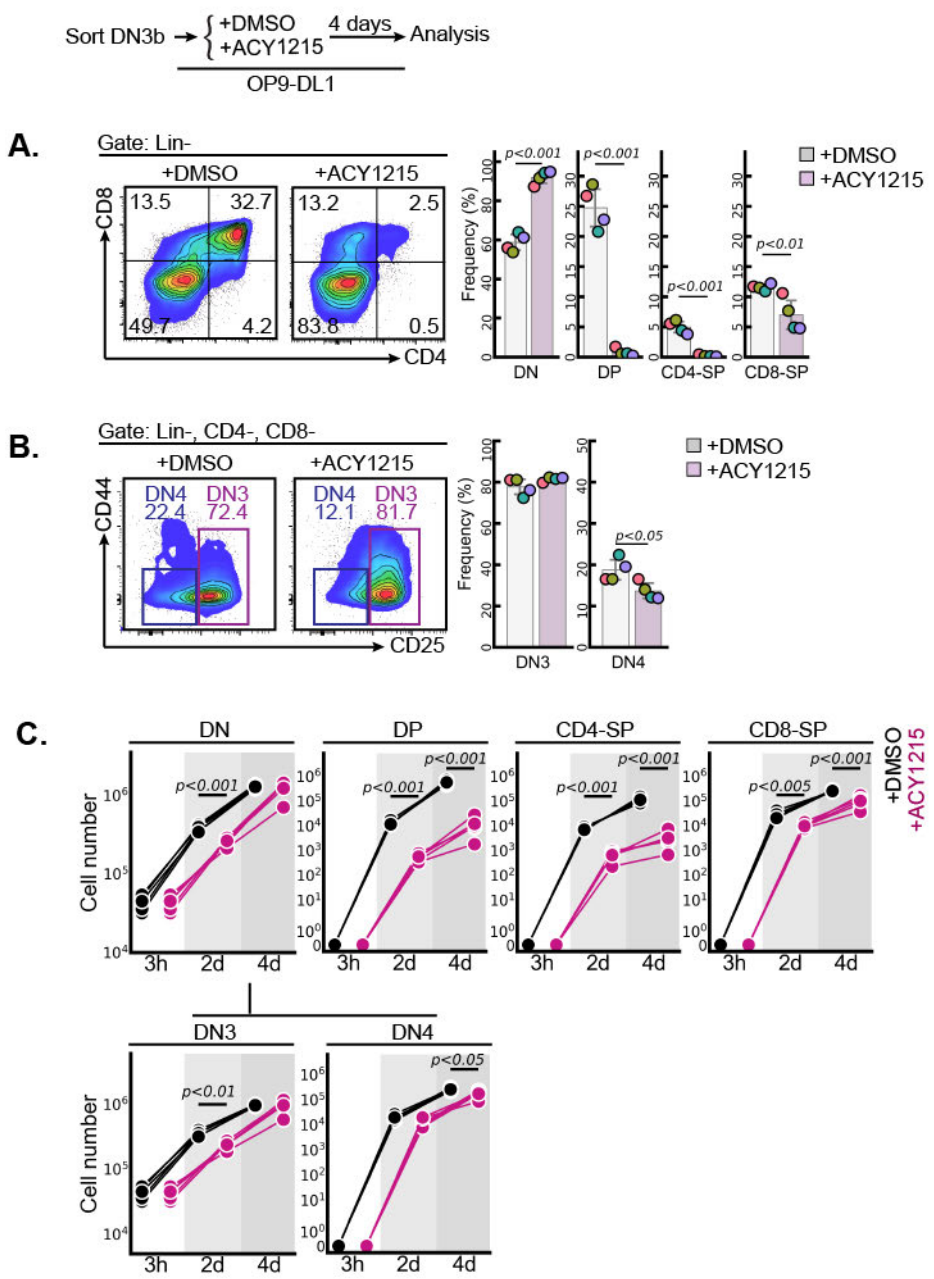


Figure 3. 6 ACY1215 arrests T cell development at DN3 stage

The sorted DN3b cells were cultured for 4 days under ACY1215 or DMSO treatment as indicated, and were analysed by CD4 and CD8 **(A)** and CD44 and CD25 **(B)**. For A and B, the frequency of each population were summarised in swarm plot, with individual colours representing each experiment. Cell numbers per population were estimated by multiplying the total cell number by the frequency and were summarised in the line chart **(C)**.

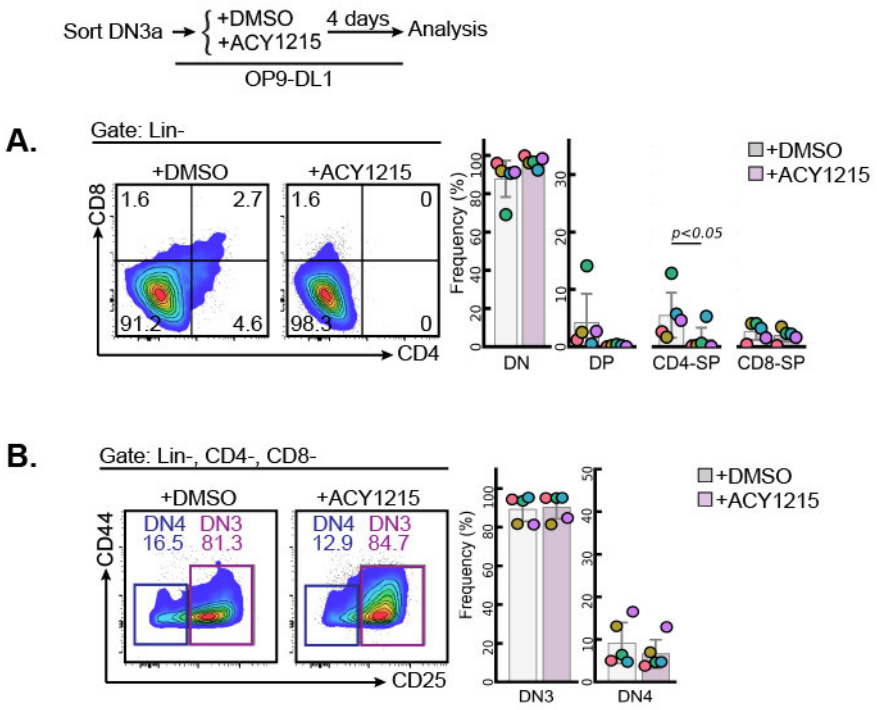


Figure 3. 7 ACY1215 arrests T cell development at DN3 stage

The sorted DN3a were cultured for 4 days under ACY1215 or DMSO treatment as indicated, and were analysed by CD4 and CD8 **(A)** and CD44 and CD25 **(B)**. The frequency of each population was summarised in swarm plot, with individual colours representing independent experiments.

3.2.3 ACY1215 treatment disrupts β-selection

Two T cell lineages arise from DN3 cells, each expressing a different set of T cell receptors (αβ cells and γδ cells) (Hayday and Pennington, 2007). To determine which lineage was altered by ACY1215, I cultured developing T cells for 2 days with and without HDAC6i, and labelled the cells with antibodies specific for these TCRs (**Figure 3. 8**). As expected DN3a cells (which have not undergone genomic rearrangement) expressed neither TCRβ nor TCRγδ. As they progressed through the differentiation DN3b and DN4, untreated cells showed increasing proportions of cells expressing either TCRβ or TCRγδ. By the DP stage, cells predominantly expressed TCRβ as expected. ACY1215 treatment increased the proportion of cells expressing TCRγδ at the DN4 stage, but had no impact at other differentiation stages. This coincided with a reduced proportion of TCRβ–expressing cells at the DN3b and DN4 stages. This reduction was not significantly seen at the DP stage, presumably because these cells had already passed the β-selection checkpoint before ACY1215 was applied. These data are compatible with a specific effect on TCRβ selection, but are difficult to interpret since the drug was acting on a population of mixed differentiation stages. Thus, the impact of ACY1215 on differentiation and TCRβ and TCRγδ expression was assessed on a pure population of DN3b cells (**Figure 3. 9**). After 2 days of culture, the number of cells expressing TCRβ was reduced significantly at DN3, DN4 and DP stages. The number of cells expressing TCRγδ was not substantially reduced by ACY1215 treatment. TCR^- cells showed a small but statistically significant decrease in number at both the DN3 and DN4 stage which might reflect an impact of ACY1215 before the cells had completed β-selection, or might merely reflect TCRβ expression below the level of detection. I confirmed these results with staining for icTCRβ (**Figure 3. 10**), which was expressed on fewer cells at the DN3, DN4 and DP stage by 4 days of ACY1215 treatment. Together, these data indicated that the defect in differentiation to DP was specific to αβ T cells, and likely involved the β-selection checkpoint.

As I found ACY1215 treatment arrests development at DN3 stage, and can be re-accessed as (**Figure 3. 11**), I wondered whether the effect of arrest is lineage-specific. To test this, I demarcated $TCR\beta^+$, $TCR\gamma\delta^+$, and TCR^- cells, and then examined DN3 and DN4 frequency of these three lineages (Figure 3. 11). The results show most of TCR^- cells (from both DMSO and ACY1215) are at DN3 stage, and $TCR\beta^+$ and $TCR\gamma\delta^+$ control cells show the progression to DN4. In contrast, ACY1215 treated $TCR\beta^+$ and $TCR\gamma\delta^+$ cells were both arrested at DN3 stage, suggesting a lineage-independent effect.

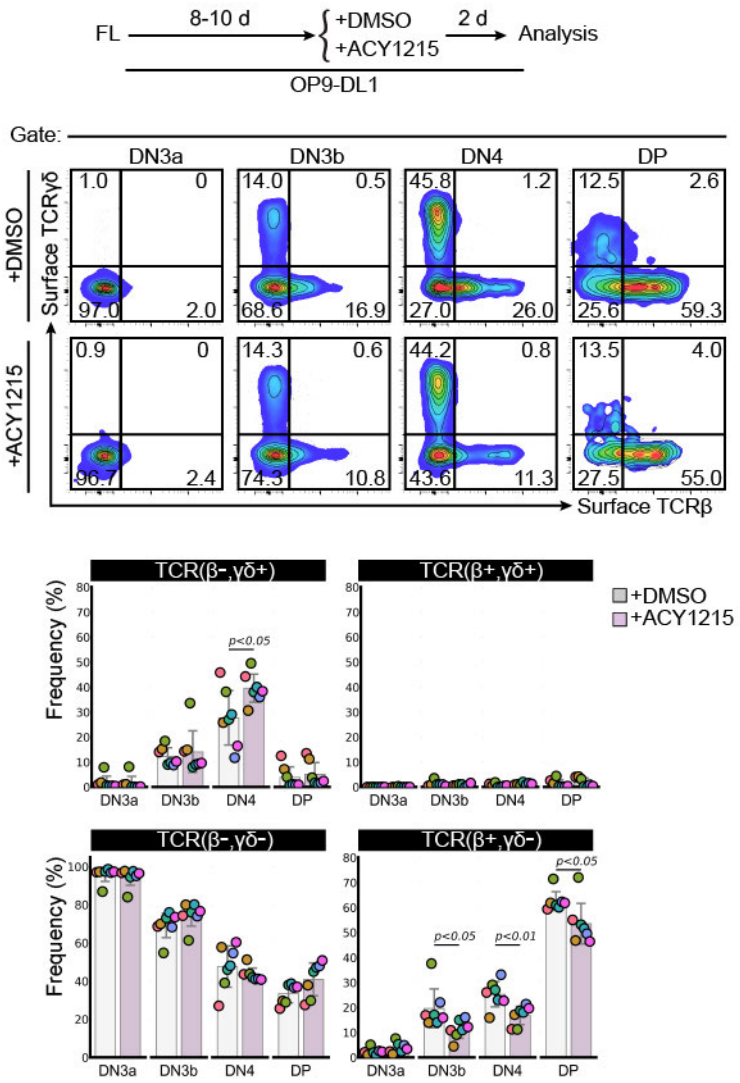


Figure 3. 8 ACY1215 impairs the development of TCRβ lineage

Developing T cells were treated with ACY1215 or DMSO for 2 days as indicated, and DN3a, DN3b, DN4 and DP cells were assessed for surface TCRβ and TCRγδ expression. Individual colours in swarm plot represent independent experiments.

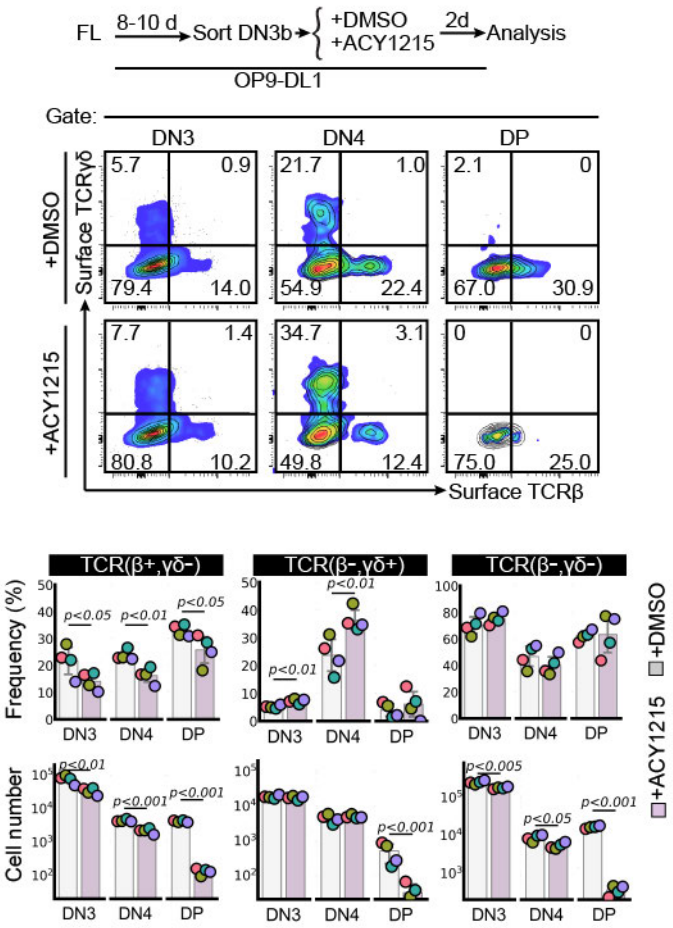


Figure 3. 9 ACY1215 impairs the development of TCRβ lineage

The sorted DN3b cells were cultured for 2 days under ACY1215 or DMSO treatment as indicated, and DN3, DN4 and DP cells were assessed for surface TCRβ and TCRγδ expression. Individual colours in swarm plot represent independent experiments.

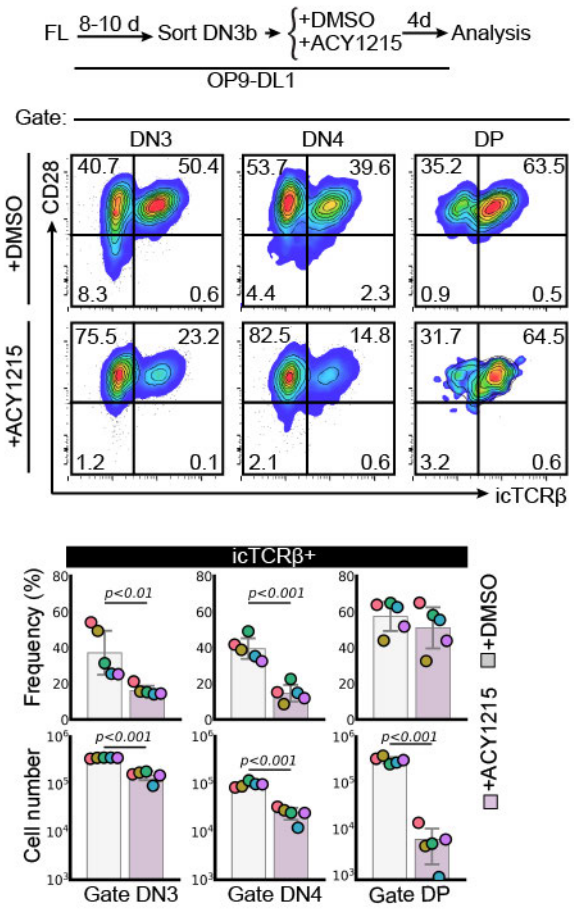


Figure 3. 10 ACY1215 impairs the development of TCRβ lineage

The sorted DN3b cells were cultured for 4 days under ACY1215 or DMSO treatment as indicated, and DN3, DN4 and DP cells were assessed for intracellular TCRβ (icTCRβ) and CD28 expression. The frequency and cell number were summarised in swarm plot, with individual colours representing each experiment.

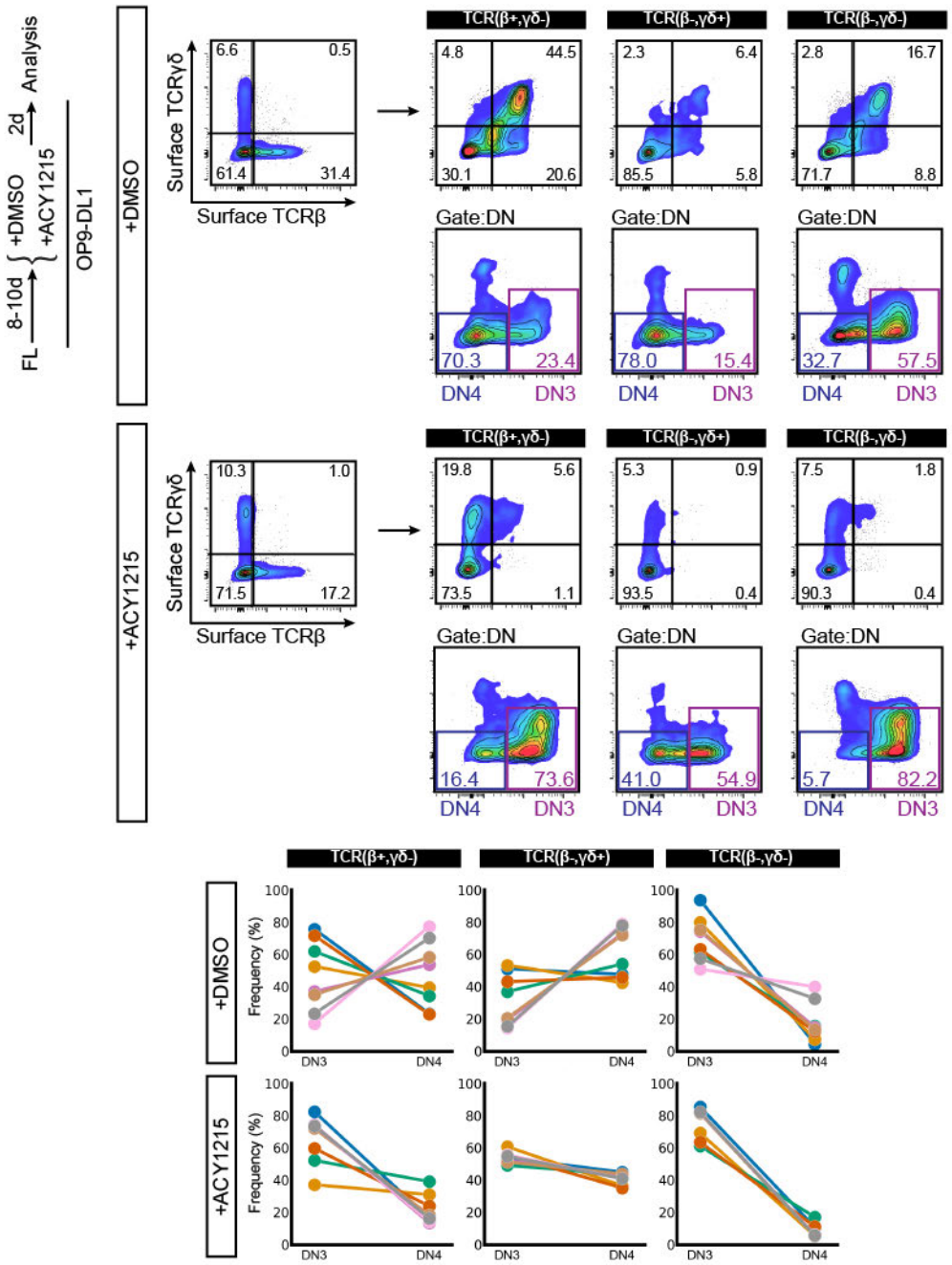


Figure 3. 11 Assessment of DN3 and DN4 progression from αβ or γδ lineage

Developing T cells were treated with DMSO or ACY1215 for 2 days, and the αβ or γδ lineage were accessed for CD4, CD8, CD44 and CD25 expression. The frequency of DN3 and DN4 were summarised in the line plot, with colours representing each experiments.

3.2.4 ACY1215 disrupt DN3a-to-DN3b transition

The passing of the β-selection checkpoint is associated with progression from the DN3a to DN3b stages of differentiation, which is generally defined by expression of CD28 (Teague et al., 2010; Williams et al., 2005). I therefore assessed whether DN3a cells were inhibited from progressing to DN3b (**Figure 3. 12A**). Surprisingly, the reverse was observed. In DMSO control cultures, co-staining with CD25 and CD28 indicated a relatively stable distribution between DN3a ($CD28^{lo}$) and CD3b ($CD28^{hi}$) at 1 and 2 days, but increasingly greater proportions of $CD28^{hi}$ in the ACY1215-treated cells. The conversion from predominantly DN3a in the DMSO-treated cells to predominantly DN3b in the ACY1215-treated cells could reflect either (or both) a reduction in the number of DN3a cells, or an increase in number of DN3b cells, so I calculated total cell numbers in cultures of sorted DN3a cell to address this. The DN3a cells expanded more than 4-fold over 2 days of control DMSO-treated culture. A small fraction of these cells had converted to DN3b cells after 1 day, and both DN3a and DN3b numbers increased substantially in the second day of culture. In contrast, treatment with ACY1215 reduced the number of DN3a cells on Days 1 and 2. The number of DN3b cells produced was not compromised by ACY1215 treatment, and the number of DN4 cells was increased slightly at 1 day of treatment, but this difference was not sustained at 2 days. A similar shift to DN3b and DN4 at the expense of DN3a was observed in thymocytes extracted from an adult mouse and cultured for 1 day on OP9-DL1 (**Figure 3. 12B**), although the relatively low numbers of DN3a in primary thymocytes *ex vivo* made the shift less obvious. Together, these data suggest that ACY1215 treatment of DN3a cells led to a dramatic reduction in DN3a amplification, but little impact upon the number of cells passing through the DN3b stage.

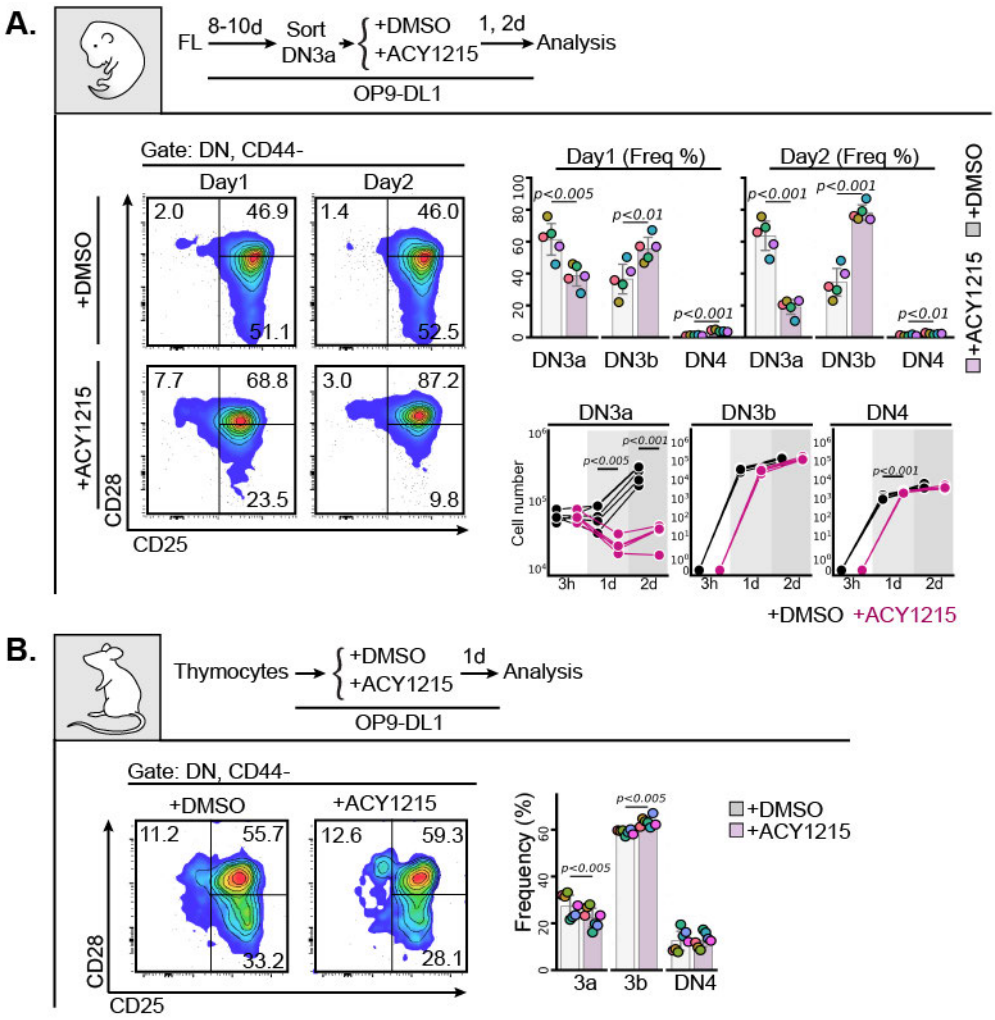


Figure 3. 12 ACY1215 impairs DN3a amplification

The sorted DN3a cells from fetal liver cells **(A)** or thymocyte (B) were cultured under ACY1215 or DMSO treatment for 1 or 2 days as indicated, and the $CD44^-$ DN population (representing DN3 and DN4 stages) was assessed for CD25 and CD28 expression. The frequency of each population were summarised in swarm plots, with individual colours representing each experiment. Cell numbers were summarised in line plots.

3.2.5 ACY1215 treatment represses DN3a self-renewal and proliferation

To account for the reduced numbers of DN3a cells, DN3a cells must either die more frequently, undergo fewer cell divisions, or differentiate into non-DN3a cells, To assess whether the reduction was due to cell death, I stained the cells at 3 and 6 hours with propidium iodide (PI) (**Figure 3. 13**). No differences in cell death was apparent in either DN3a or DN3b cells with ACY1215 compared with DMSO, although the control DP population showed a striking increase in apoptotic cells upon ACY1215 treatment, as did DN3a and DN3b in which Notch ligand had been withdrawn. Similar results were obtained with Annexin V labelling (**Figure 3. 14**). Despite clear induction of apoptosis in both DN3a and DN3b cells upon withdrawal of OP9-Dl1 cells, I saw no significant apoptosis in the sorted DN3a and DN3b after ACY1215 treatment

An alternative explanation for the reduced numbers of DN3a cells is changes in proliferation. This was confirmed with CFSE labelling. I labelled sorted DN3a and DN3b with the fluorescent dye, CFSE, and assessed dilution of the dye as an indicator of the number of cell divisions in the 2 days since labelling (**Figure 3. 15A**). CFSE in thymocytes does not allow for precise allocation of cell divisions that can be achieved with mature T cells, but nonetheless provides a useful comparator between samples. DN3a cells showed extensive cell proliferation as indicated by a reduction in CFSE labelling over time of almost 2 orders of magnitude, and this proliferation was clearly reduced by ACY1215 treatment. The purified DN3b cells showed greater proliferation than DN3a cells as expected, which was again substantially reduced by HDAC6i treatment.

Labelling with the marker of proliferation, Ki-67(Gerdes et al., 1984), one day after treatment showed several interesting findings (**Figure 3. 15B**). First, the relative proportion of cells in the $FSC^{HI}Ki\text{-}67^{HI}$ quadrants (which presumably reflected cells in mitosis) was substantially higher for DN3b than DN3a cells, supporting their greater proliferative rate. Interestingly, of the smaller (low FSC) cells, the Ki-67 staining was much weaker in the presence of ACY1215, suggesting a longer time since their last cell division (Miller et al., 2018). This reduced Ki67 was observed in both DN3a and DN3b cells, but was particularly striking for DN3a cells treated with AY1215. Thus, the reduction in DN3 cell number induced by ACY1215 treatment is at least in part due to direct effects on proliferation of DN3a cells. Another possible explanation for the reduced DN3a numbers is a precocious differentiation of DN3a to DN3b. This is not easy to measure at the population level, but the observation that DN3b cell numbers are

maintained despite a reduction in their proliferation is compatible with the notion that DN3a cells precociously differentiate to DN3b. Together, these data suggest that the cellular basis for reduced DN3a cell numbers upon ACY1215 treatment is caused by reduced proliferation, perhaps precocious differentiation to DN3b, but probably not increased cell death.

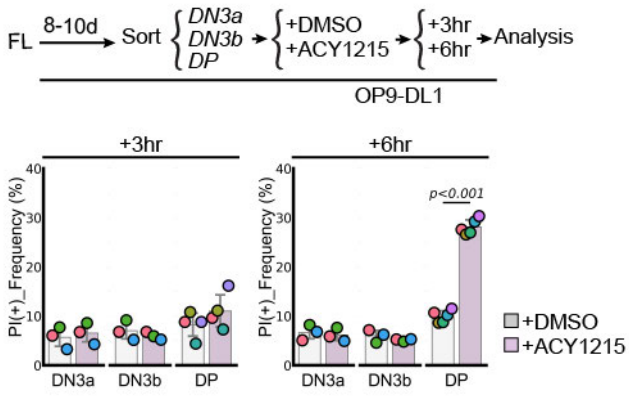


Figure 3. 13 Assessment of death upon ACY1215 treatment

Sorted, FL-derived DN3a, DN3b and DP cells were treated with ACY1215 or DMSO for 3 hours or 6 hours as indicated, followed with propidium iodide (PI) staining to label dead cells. The frequency of PI^+ population were summarised in swarm plot, with individual colours representing each experiment.

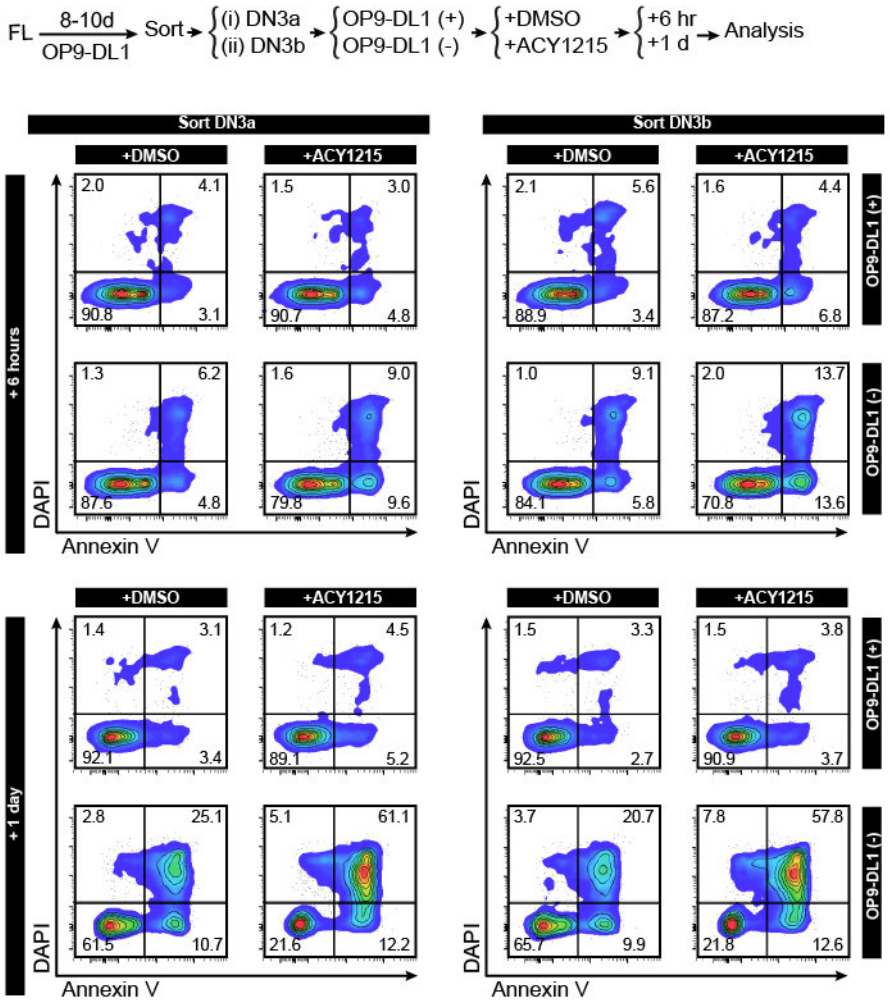


Figure 3. 14 Apoptosis assay for DN3a and DN3b treated with DMSO or ACY1215

The sorted DN3a and DN3b cells were treated with ACY1215 or DMSO for 6 hours or 1 day with or without OP9-DL1 coculture as indicated. The cells were assessed for Annexin V expression and DAPI intensity as shown in the density plot.

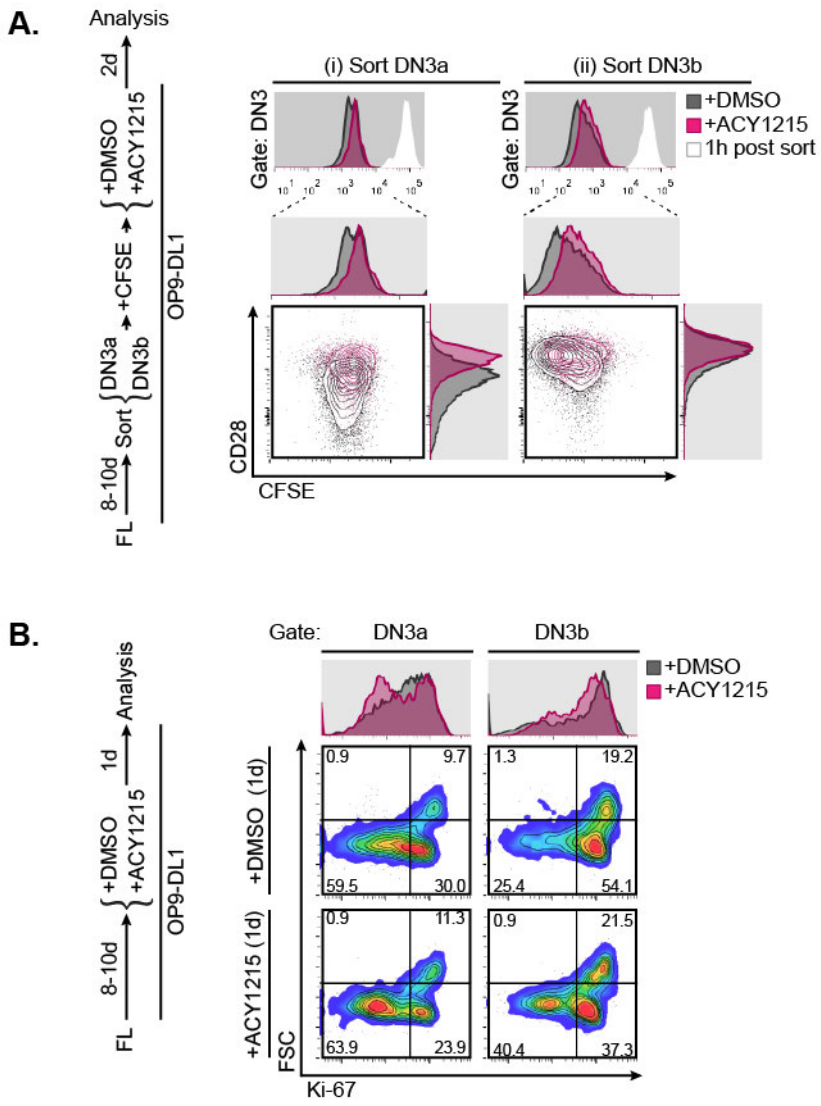


Figure 3. 15 ACY1215 influenced DN3a and DN3b proliferation

(A) Sorted, FL-derived DN3a and DN3b were stained with CFSE after sorting and were cultured with ACY1215 or DMSO for two days as indicated. At one hour post sorting, the intensity of CFSE were recorded, as indicated by the white histogram. At two days, the DN3 cells from the sorted DN3a and DN3b were assessed for CD28 expression and CFSE intensity, as shown in the contour plots and the histograms. **(B)** FL-derived developing T cells were treated with ACY1215 or DMSO for 1 day as indicated, and DN3a and DN3b cells were assessed for FSC and Ki-67 expression.

3.2.6 ACY1215 treatment alters Pre-TCR signalling clustered at MTOC

I explored the possible mechanisms by which ACY1215 might disrupt β-selection. In mature T cells, acetylation of α-tubulin by HDAC6 influences TCR-mediated recruitment of the microtubule-organising centre (MTOC) to the immunological synapse (Serrador et al., 2004). In both DN3a and DN3b cells, HDAC6 was predominantly in the nucleus, but detectable expression in the cytoplasm and near the MTOC was compatible with effects on MTOC modification. I assessed the acetylation of α-tubulin (K40), which can be de-acetylated by HDAC6, on the sorted T cell populations: DN, DP, CD4-SP, CD8-SP, DN1, DN2, DN3a, and DN3b (**Figure 3. 16**). Upon a two-hour treatment with ACY1215, each population showed an increase in acetylated tubulin, suggesting HDAC6 is catalytically active throughout early T cell development.

I have previously found that T cells undergoing β-selection form an immunological synapse, in which MTOC and other TCR-associated signalling proteins move with the MTOC to the site of antigen receptor signalling receptor signalling (Allam et al., 2021). I therefore speculated that an alteration in MTOC organisation has the potential to mediate the effects of ACY1215 on β-selection by impacting the immunological synapse. To begin to address this, I looked at the association of LAT with the MTOC in DN3a cells (**Figure 3. 17**). Acetyl-α-tubulin presented as a single cluster in the site of the MTOC, and was increased in intensity with ACY1215 treatment, compatible with the flow cytometry results. LAT was strongly associated with the MTOC in DMSO treated cells, but altered by ACY1215 treatment, with less intense fluorescence and less clustering at the MTOC. Notch is particularly important during β-selection in mediating assembly of the immunological synapse and transmitting signals downstream of the preTCR signal (Allam et al., 2021; Charnley et al., 2020). I therefore assessed the co-localisation of Notch1 and the MTOC in DN3a cells (**Figure 3. 18A**). In DMSO treated cells, Notch was concentrated at the MTOC, but, similar to LAT was much more diffuse following ACY1215 treatment. Interestingly, the change in localisation of Notch was accompanied by a loss in cell surface Notch expression, and this occurred in DN3a, DN3b and DN4 cells, suggesting a general acceleration of the down-regulation of Notch1 that is a critical component of β-selection (**Figure 3. 18B**). These data suggest that one possible mechanistic basis for the effects of ACY1215 on β-selection is via tubulin acetylation and recruitment by MTOC of Notch and TCR signalling to the immunological synapse.

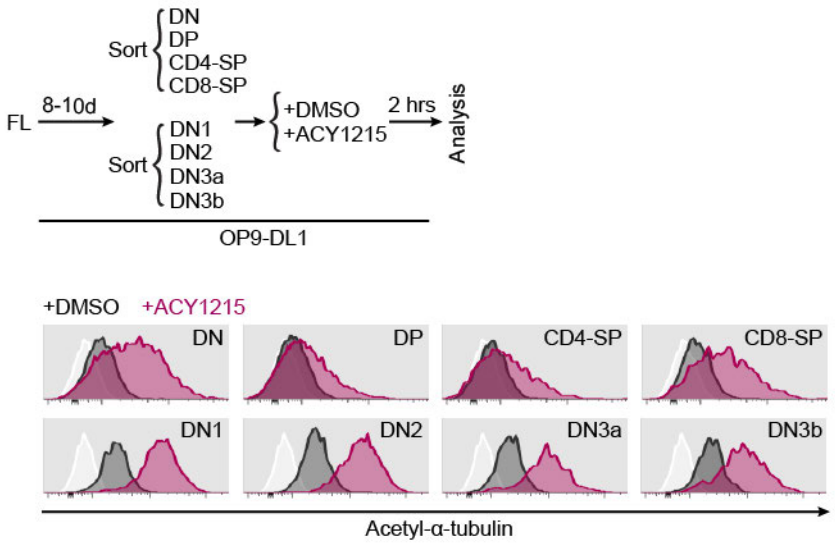


Figure 3. 16 Assessment of tubulin acetylation upon ACY1215 treatment

The sorted DN, DP, CD4-SP, CD8-SP, DN1, DN2, DN3a, DN3b were treated with ACY1215 or DMSO for 2 hours as indicated, and were assessed for acetyl-α-tubulin (K40) as shown in histograms.

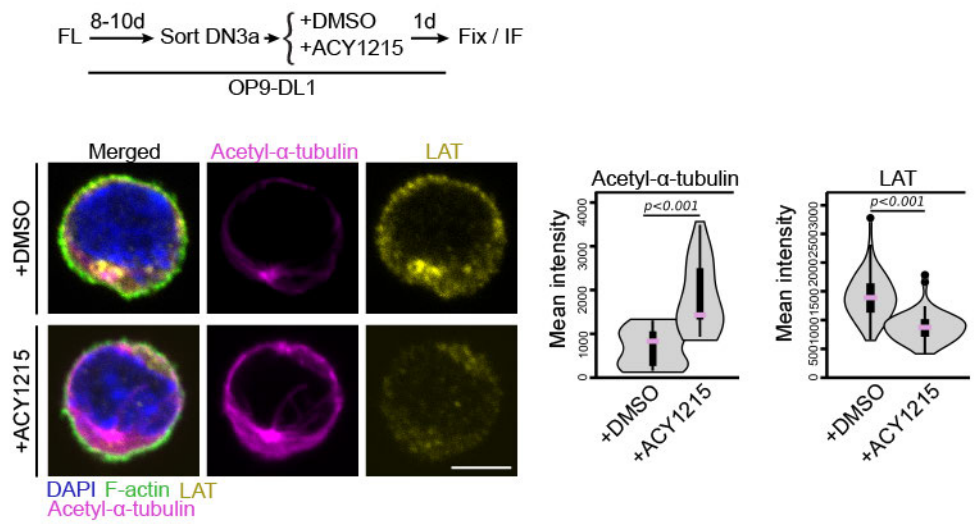


Figure 3. 17 Assessment of LAT expression upon ACY1215 treatment

The sorted DN3a cells were treated with ACY1215 or DMSO for 1 day as indicated, followed by immunostaining for LAT, acetyl-α-tubulin (K40). The representative image was from three individual experiments. The mean intensity of acetyl-α-tubulin, LAT expressed at MTOC versus at nucleus were summarized in violin plots. Cell number for LAT staining: ACY1215 (n=55) and DMSO (n=54); Cell number for acetyl-α-tubulin (K40) staining: ACY1215 (n=30) and DMSO (n=31). The statistics was from three individual experiments.

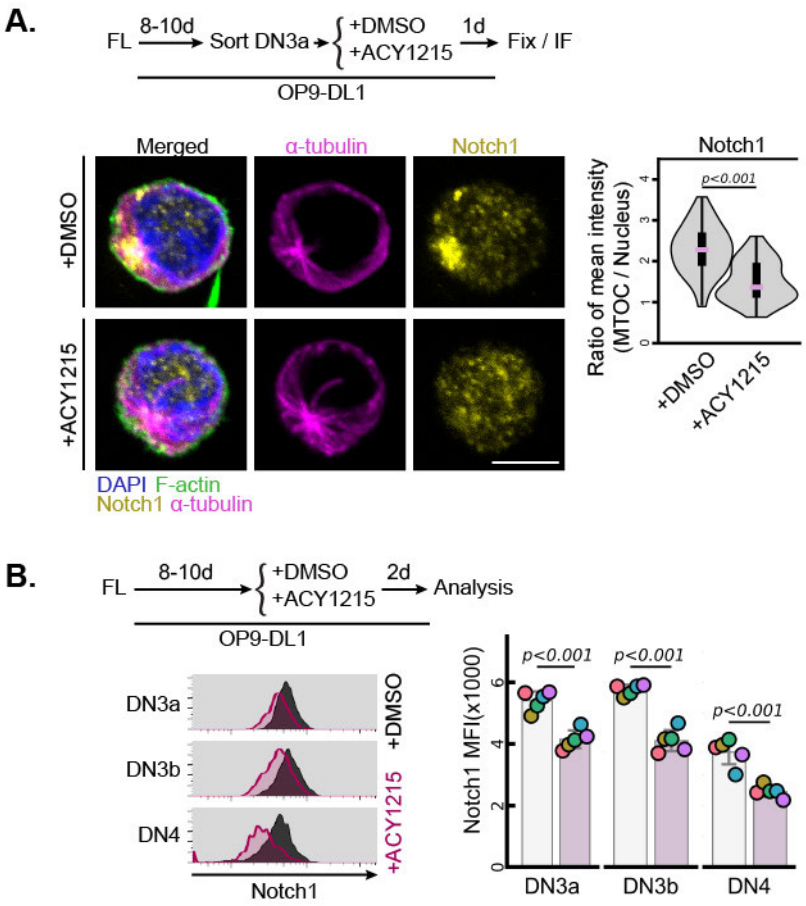


Figure 3. 18 Assessment of Notch1 expression upon ACY1215 treatment

(A) The sorted DN3a cells were treated with ACY1215 or DMSO for 1 day as indicated, followed by immunostaining for Notch1 and α-tubulin. The representative image was from three individual experiments. The ratio of Notch1 expressed at MTOC versus at nucleus were summarized in violin plot. Cell number for Notch1 staining: ACY1215 (n=56) and DMSO (n=55). **(B)** Developing T cells were treated with ACY1215 or DMSO for 2 day, and the DN3a, DN3b and DN4 cells were assessed for Notch1 expression, as summarised in swarm plot, with individual colours representing each experiment. The statistics was from three individual experiments.

3.3 Discussion

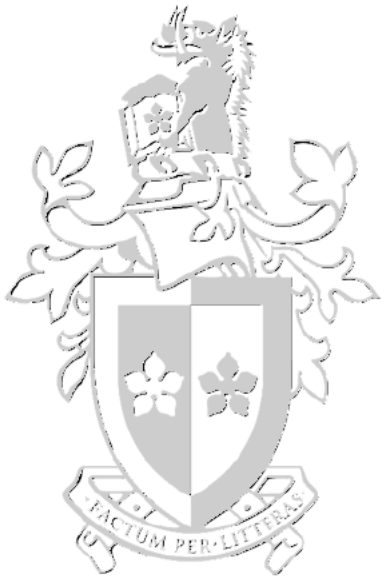
As the hematopoietic progenitors differentiate to T cells, multiple stages of fate commitment, TCR gene recombination and TCR selection are supported by constitutive Notch1 signalling (Deftos and Bevan, 2000). However, Notch1 signalling doesn't trigger only one version of gene expression, but orchestrates multiple waves of transcriptional switches to reach maturation of T cell development (Brandstadter and Maillard, 2019; Weerkamp et al., 2006). Here I found ACY1215 altered Notch1-induced T-cell fate determination.

Unlike HDACs 1, 2, and 3, which are required at multiple stages of T cell development, a role for HDAC6 in T cell development has not been identified (Wang et al., 2020). Mice lacking HDAC6 showed no overt T cell phenotypes (Shapiro and Shapiro, 2020; Zhang et al., 2008) and to our knowledge the effect of acute deletion of HDAC6, or pharmacological inhibition of HDAC6 on T cell development has not been reported (Shapiro and Shapiro, 2020). In this Chapter, I believe the effects from ACY1215 can help to deduce the role of HDAC6 in developing T cells. The known role for HDAC6 in linking tubulin acetylation to the immunological synapse of mature T cells (Serrador et al., 2004), and our finding that acetylation of α-tubulin is increased in DN3 cells, suggested the possibility that the β-selection immunological synapse might mediate the effects of ACY125. Indeed, ACY1215 does alter the organisation of the TCR signalling molecules, LAT, around the MTOC. Similarly, Notch1, which is required to enhance the polarisation of the pre-TCR (Allam et al., 2021; Charnley et al., 2020), is lost from the MTOC following ACY1215 treatment. These findings are particularly interesting in light of a recent report that HDAC6 is required for the formation of aggresomes at the MTOC (Magupalli et al., 2020), and suggest that HDAC6 might play a more general role in coordinating recruitment to the MTOC. An alternative explanation for the effect of ACY1215 on β-selection relates to observations that HDAC inhibitors can induce MHC constituents to promote antigen presentation (Magner et al., 2000). It remains to be determined whether an influence on stromal-presented peptide-MHC plays any role in disrupting β-selection. Interestingly, both CD2 and CD5 can regulate activity of the immunological synapse and connectivity to the MTOC (Brossard et al., 2003; Dustin et al., 1998; Tibaldi et al., 2002) further supporting the possibility that the immunological synapse provides a hub at which deacetylation and pre-TCR signalling converge. Together, these findings suggest that the effect of HDAC6 on β-selection that I see here might occur through disruption of the DN3 immunological synapse.

On the other hand, epigenetic change on H3K18 would highlight an underestimated role of HDAC6 in transcriptional controls. These demonstrations are encouraging because more mechanisms can be provided to the use of ACY1215 in pre-clinical and clinical circumstances. The use of ACY1215 has been described as epigenetic therapy (Hogg et al., 2020), which can synergize with the BET inhibitor, JQ1, to recruit T cell mediated responses to lung adenocarcinoma, enhance T cell responses to *ex vivo* melanoma cultures, impair effector CD8 T cell function during skin inflammation, and alter Treg function (Adeegbe et al., 2017; Laino et al., 2019; Tsuji et al., 2015; Xu et al., 2018; Zhang et al., 2021). As addressed in the Introduction, T cell development can be possible to be transformed toward cancer. For that circumstance, ACY1215 might become a potential approach to regulate therapeutic response. I will globally discuss these possibilities in Chapter 7.

3.4 Conclusion

In this chapter, I assessed the consequences of T cell development upon treatment of HDAC6 selective inhibitor, ACY1215. I found αβ T cell development is disrupted upon treatment, led with aberration, namely the increase of acetylation of α-tubulin and histone tail (H3K18), the decrease of Notch1 and LAT clustered at MTOC. These observation indicated the control of β-selection checkpoint is impaired, which I investigated in the next chapter.



Chapter 4

Stepwise progression of β-selection during T cell development as revealed by histone deacetylation inhibition

4.1 Introduction

In Chapter 3, the composition of DN3 cells was altered by ACY1215, showing reduction in DN3a expansion but less influence on the total amount of DN3b cells. On the other hand, I also demonstrated DN3b-to-DP progression was arrested by ACY1215; however, the change in the molecular basis in DN3b compartments is yet to be determined in Chapter 3. Thus in Chapter 4, the urgent tests are to clarify the property of DN3b under ACY1215, and to assess how the course of β-selection is altered under ACY1215.

In Chapter 4, I will show a striking effect of treatment with the HDAC6-selective inhibitor, ACY1215, on β-selection. This effect allowed us to define several stages of β-selection with high resolution. After the DN3a stage marked by upregulation of the co-receptor, CD28, I observed a transitional stage marked by low levels of the co-receptor, CD2, that I termed $DN3b^{Pre}$. $DN3b^{Pre}$ cells are enriched by ACY1215 treatment. An upregulation of CD2 marks passage through the β-selection checkpoint, to a phase I termed $DN3b^{Post}$. The transcription factor, Lef1 is upregulated in $DN3b^{Pre}$ cells, together with an increasing expression of the reporter of TCR signalling, CD5. These findings implicate a refined model of β-selection in which, following expression of CD27 and CD28, a coordinated increase in expression of pre-TCR, CD5 and Lef1 provides for an escalating test of TCR signalling. Expression of CD2 marks the passing of this test and exit from the β-selection checkpoint. Overall, this chapter updates the understanding regarding the multistep of differentiation across β-selection, of which ACY1215 or HDAC6 activity can influence the process.

4.2 Results

4.2.1 ACY1215 leads to phenotypically aberrant DN3b cells

One possible explanation for this passage to DN3b without optimal subsequent differentiation could be that ACY1215-treated cells express some markers to suggest they have passed β-selection but are not competent precursors of αβ T cells. To assess this, I first characterised several phenotypic markers implicated in progression through the β-selection checkpoint. Co-staining DN3 cells with both CD28 and CD27, both of which can discriminate between DN3a and DN3b cells (Taghon et al., 2006; Teague et al., 2010), clearly discriminated two subpopulations in DN3 cells pre-treated with DMSO (**Figure 4. 1**). A shift from DN3a to DN3b is observed for both fetal liver-derived lymphocytes and *ex vivo* extracted thymocytes cultured on OP9-DL1 stroma. These data confirm the findings above that, at least by the canonical markers, CD27 and CD28, ACY1215 treatment promotes differentiation towards DN3b at the expense of DN3a.

Given that the primary function of this checkpoint is to ensure appropriate TCRβ rearrangement, I assessed the correlation of each marker with TCRβ expression (**Figure 4. 2**). As previously shown (Williams et al., 2005), expression of CD28 was clearly correlated with expression of icTCRβ in cells derived from fetal liver or from the adult thymus. The $CD28^{Hi}$ cells also expressed more of the nutrient transporters, CD71 and CD98, and more surface TCRβ (**Figure 4. 3 A**) indicating that, in general, they had progressed further through β-selection, but the correlation was not absolute. For both cell sources, ACY1215 treatment resulted in less icTCRβ expression in the DN3b (whether measured as $CD28^{Hi}$ or $CD27^{Hi}$ DN3 cells) (Figure 4. 1). To validate the icTCRβ staining, I directly compared it with surface TCRβ (**Figure 4. 3 B, C**). For both cell sources, expression of surface TCRβ was highly correlated with that of icTCRβ and both were reduced by ACY1215 treatment. Thus, as well as depleting DN3a cells, treatment with ACY1215 increases the proportions of a cell population that bears many of the phenotypic markers of passage through β-selection, but these cells appear somewhat defective in expression of TCRβ, and do not progress optimally through the DP stage. I speculated that further characterisation of these cells might yield valuable information about requirements at the β-selection checkpoint.

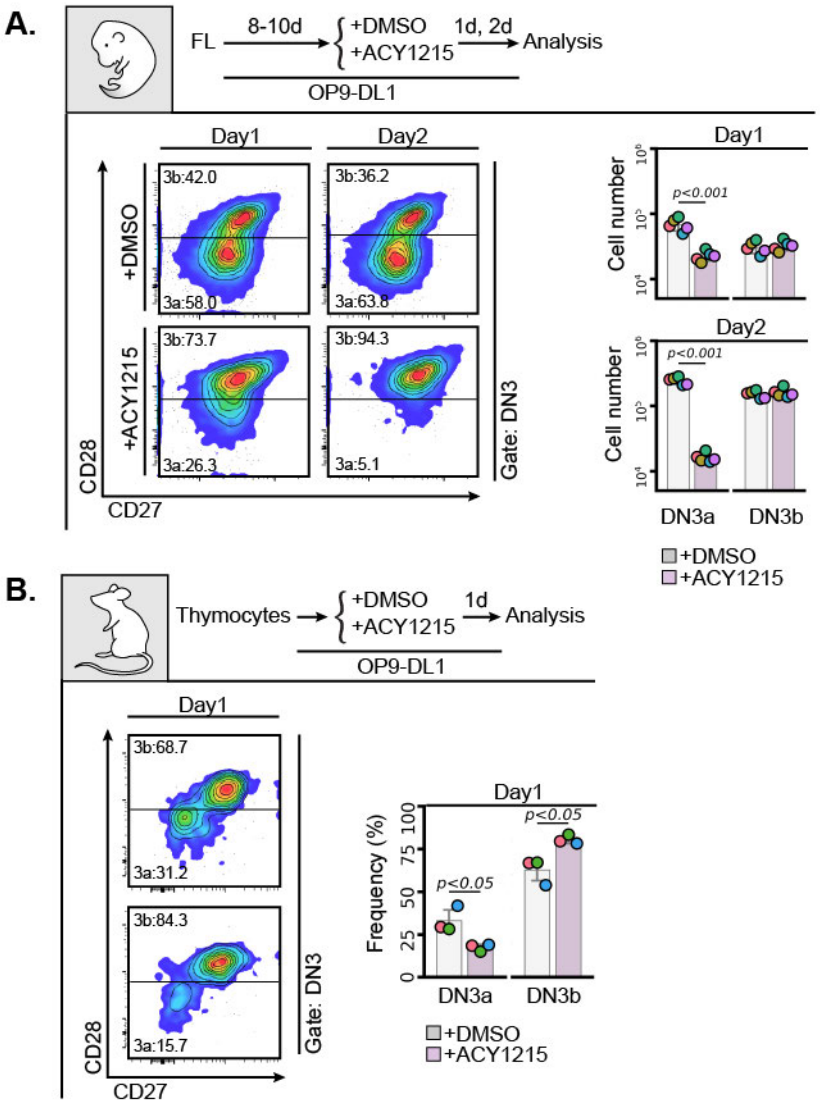


Figure 4. 1 DN3 cells under ACY1215 treatment are skewed toward $CD27^{+}CD28^{+}$.

Developing T cells from FL (A) or the adult thymus (B) were treated with ACY1215 or DMSO for 1 day or 2 days, and the DN3 cells were assessed for CD27 and CD28 expression to discriminate DN3a and DN3b. Cell number or frequency were summarised in the swarm plot, with individual colours representing each experiment.

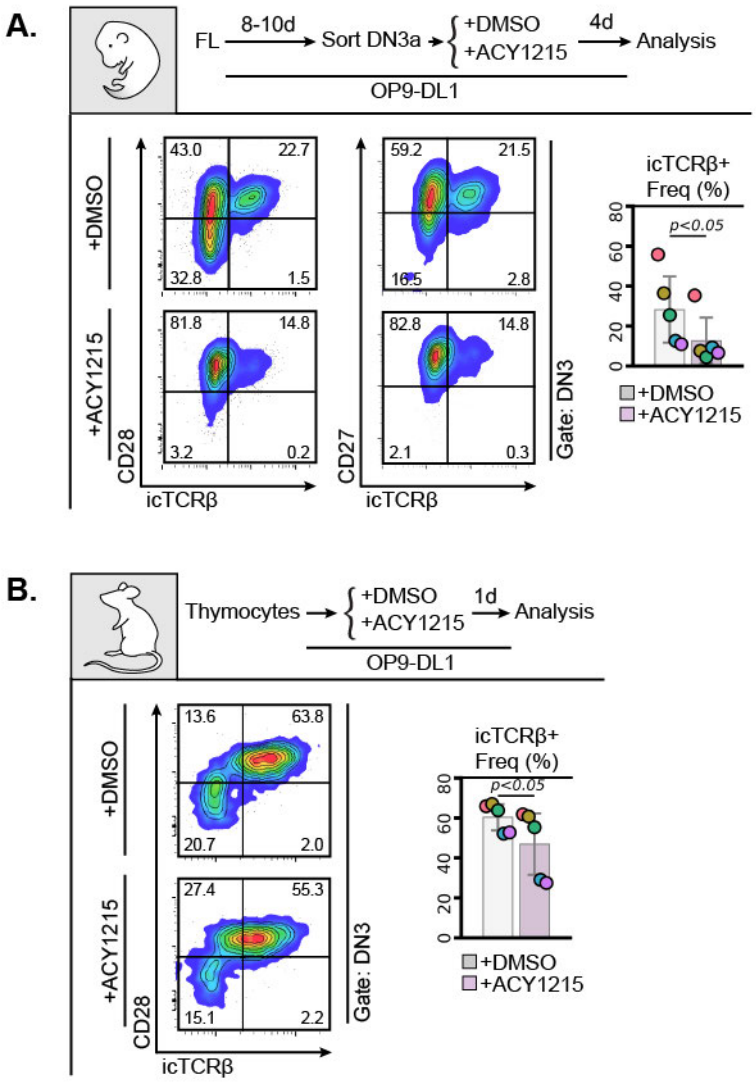


Figure 4. 2 ACY1215 inhibits β-selection.

(A) Sorted, FL-derived DN3a cells were cultured under ACY1215 or DMSO treatment for 4 days as indicated, and DN3 cells were assessed for the expressions of intracellular TCRβ (icTCRβ) versus CD28 or CD27. **(B)** Thymocytes were cultured under ACY1215 or DMSO treatment for 1 day as indicated, and DN3 cells were assessed for the expressions of icTCRβ versus CD28. The frequency of icTCRβ+ cells was summarised in a swarm plot, with individual colours representing each experiment.

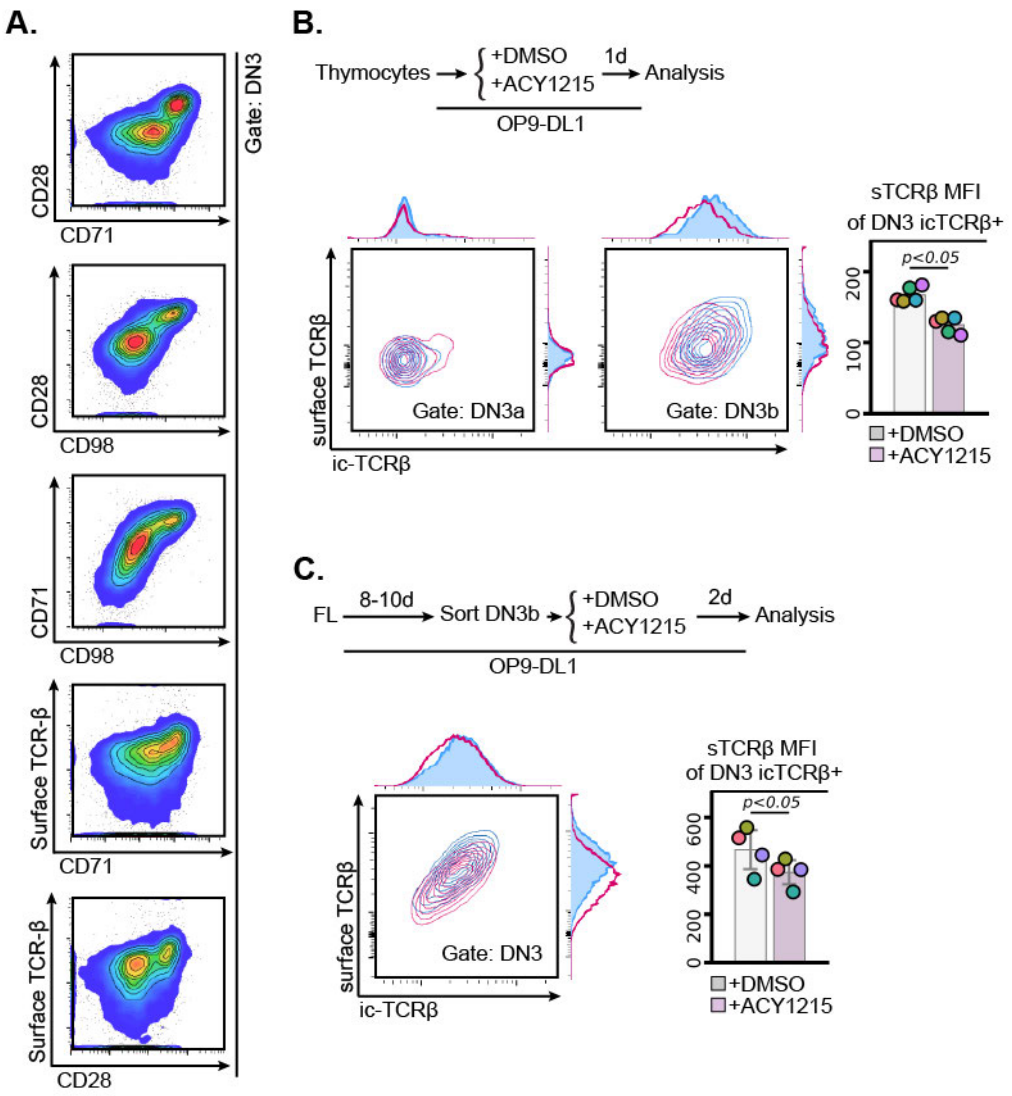


Figure 4. 3 DN3b differentiation is altered upon ACY1215 treatment

(A) CD28 upregulation marks both DN3 cells differentiation and passing β-selection. FL-derived developing T cells at around day 8-10 of coculture with OP9-DL1 stromal cells were assessed for CD28, CD71, CD98, CD27 and surface TCRβ expression. Sorted, FL-derived DN3b **(B)** or thymocytes **(C)** were treated with ACY1215 or DMSO for 1 day or 2 days as indicated, and the DN3 cells were assessed for surface TCRβ and icTCRβ expression. The frequency of icTCRβ^{+} cells was summarised in a swarm plot, with individual colours representing each experiment.

4.2.2 CD2 expression discriminates between DN3b^{Pre} and DN3b^{Post} as two functionally distinct stages in β-selection.

A well-established co-stimulator of TCR signalling is CD2 (Skånland et al., 2014). Expression of CD2 marks TCRβ V(D)J rearrangement and can facilitate pre-TCR signalling for passage through the β-selection checkpoint (Groettrup et al., 1992; Rodewald et al., 1993; Sasada and Reinherz, 2001). In the next series of experiments, I find that CD28 and CD2 reliably define 3 sequential stages of β selection in DN3 cells, which I term DN3a, DN3b^{Pre}, and DN3b^{Pro} to accommodate pre-existing nomenclature (**Figure 4. 4A**). Co-staining of DMSO-treated DN3 cells with CD28 and CD2 showed that all DN3a cells (CD28^{Lo}) were CD2^{Lo}, but DN3b (CD28^{Hi}) cells had two clearly discernible populations of CD2^{Lo} (DN3b^{Pre}) and CD2^{Hi} (DN3b^{Pro}) (Figure 4. 4B). ACY1215 treatment enriched for the DN3b^{Pre}. Cell counts indicated that the numbers of DN3b^{Pre} cells were not substantially affected by ACY1215, but DN3a and DN3b^{Post} numbers were dramatically reduced (Figure 4. 4B). Thus, ACY1215 treatment led to an enrichment in a DN3b^{Pre} population that was low for CD2. The DN3b^{Pre} population **(Figure 4. 4C)** was evident in thymocytes from young adult mice, even without *in vitro* culture, and was again enriched by treatment with ACY1215.

4.2.3 DN3b^{Pre} displays intermediate phenotypes between DN3a and DN3b^{Post}

To confirm that DN3b^{Pre} cells were earlier in β-selection than DN3b^{Post} cells, I assessed the association of CD2 expression with proliferation as measured by CFSE dilution (**Figure 4. 5A**). Two clear populations were evident, with the CD2^{Hi} (DN3b^{Post}) cells having undergone substantially more proliferation than the CD2^{Lo} (DN3b^{Pre}) cells. ACY1215 treatment did not disrupt the association between CD2 and proliferation. Expression of CD27 and surface TCRβ were also increased from DN3a, DN3b^{Pre}, to DN3b^{Post}, with stepwise correlation which was not altered by ACY1215 despite lower surface TCRβ on DN3b^{Post} cells (**Figure 4. 5B**). I then assessed icTCRβ and Ki-67 staining, which should reflect the cell proliferation triggered by successful passage through the β-selection checkpoint (**Figure 4. 6**). Indeed, consistent with previous findings that TCRβ V(D)J recombination is restricted to DN cells positive for CD2 (Rodewald et al., 1993), DN3b^{Pre} cells were intermediate in expression of icTCRβ and Ki67 i.e. between the levels seen on DN3a cells and DN3b^{Post} cells. The correlated, stepwise increase in icTCRβ and Ki-67 was observed in cultures from both fetal liver and adult thymus. This pattern was even more striking in thymocytes from adult mice, where DN3a cells showed no proliferation, DN3b^{Pre}

cells were heterogeneous with respect to proliferation, and proliferation correlated with icTCRβ expression, and DN3b^{Post} cells were all highly proliferative, with minimal impact from ACY1215 treatment. These data suggest that the dramatic reduction in TCRβ expression upon ACY1215 treatment observed in Figure 4. 2 was explained more by the enrichment of DN3b^{Pre} cells, by than a direct effect on TCRβ expression. c-Myc, which mediates the proliferative response to pre-TCR signalling but is not required for differentiation (Dose et al., 2006), showed a similar stepwise increase with these differentiation stages (**Figure 4. 5C**). These data are all compatible with an interim DN3b^{Pre} stage that reflects an early, and pivotal step in β-selection where rearrangement of the TCRβ chain is translated into proliferation.

4.2.4 The CD28^{hi}CD2^{lo} (DN3b^{Pre}) cells are precursors of CD28^{hi}CD2^{hi} (DN3b^{Post})

To formally assess whether the DN3b^{Pre} stage is a transitional stage before DN3b^{Post}, I sorted DN3a, DN3b^{Pre} and DN3b^{Post} cells from adult mice and cultured them for further analysis (**Figure 4. 7**). Analysis of the cells immediately after extraction showed clearly distinct DN3a, DN3b^{Pre} and DN3b^{Post} cells. After two days, DN3b^{Post} cells had produced substantial numbers of DP cells, with fewer produced by DN3b^{Pre}, and fewer still by DN3a cells. Also compatible with unidirectional progression from DN3a to DN3b^{Pre} to DN3b^{Post}, was the proportion of DN4 and DN3 stages from each sorted population. ACY1215 treatment of thymocytes stabilized the DN3b^{Pre} population and blocked progression to DP. Similar data was obtained in the fetal liver-derived cultures (**Figure 4. 8**). These data confirm that DN3b^{Pre} (CD28^{Lo}CD2^{Lo}) is a transitional state between DN3a (CD28^{Hi}CD2^{Lo}) and DN3b^{Post} (CD28^{Hi}CD2^{Hi}), reflecting partial progression through β-selection.

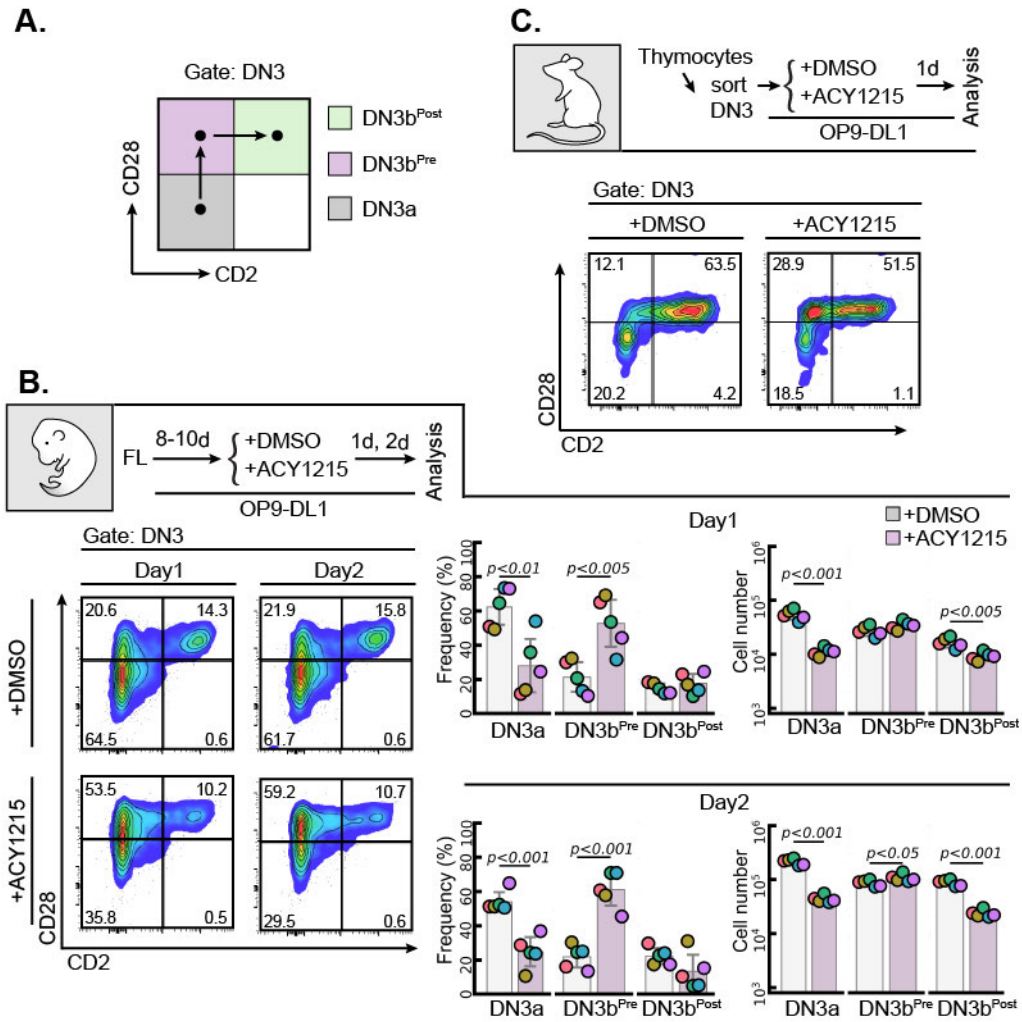


Figure 4. 4 CD2 upregulation discriminates DN3b^{Pre} and DN3b^{Post} populations

(A) The three populations (DN3a, DN3b^{Pre}, and DN3b^{Post}) were indicated in the schematic. FL-derived developing T cells **(B)** or the sorted thymocyte DN3 **(C)** were treated with ACY1215 or DMSO for 1 or 2 days, and the DN3 cells were assessed for CD2 and CD28 expression.

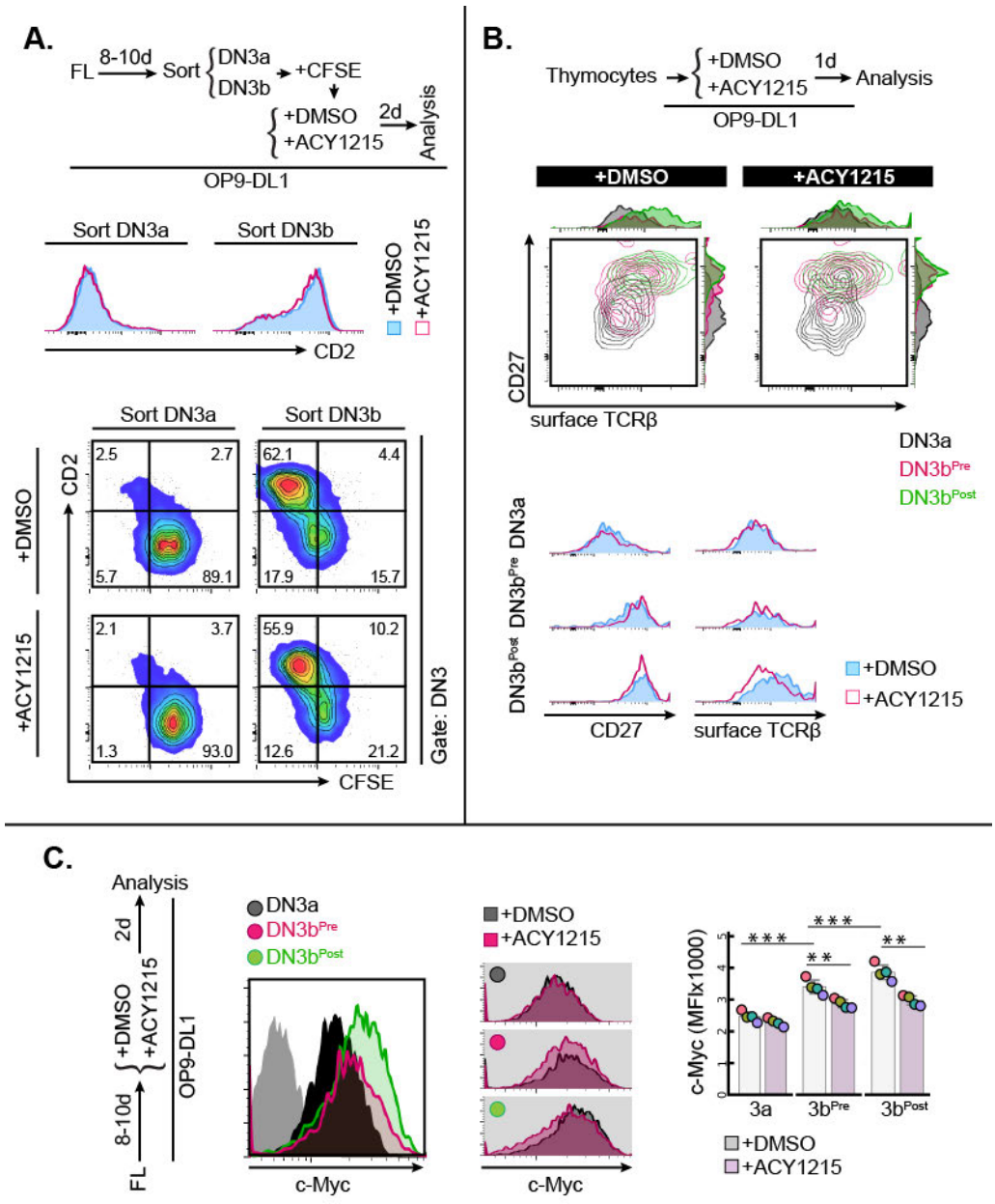


Figure 4. 5 DN3b^{Pre} displays intermediate phenotypes between DN3a and DN3b^{Post}

(A) Sorted, FL-derived DN3a and DN3b were stained with CFSE after sorting and were cultured with ACY1215 or DMSO for two days as indicated. At two days, the DN3 cells from the sorted DN3a and DN3b were assessed for CD2 expression versus CFSE intensity. **(B)** Thymocytes were treated with ACY1215 or DMSO for 2 days, and DN3a, DN3b^{Pre}, and DN3b^{Post} cells were assessed for CD27 and TCRβ expression. **(C)** FL-derived developing T cells were treated with ACY1215 or DMSO for 2 days, and DN3a, DN3b^{Pre}, and DN3b^{Post} cells were assessed for c-Myc expression.

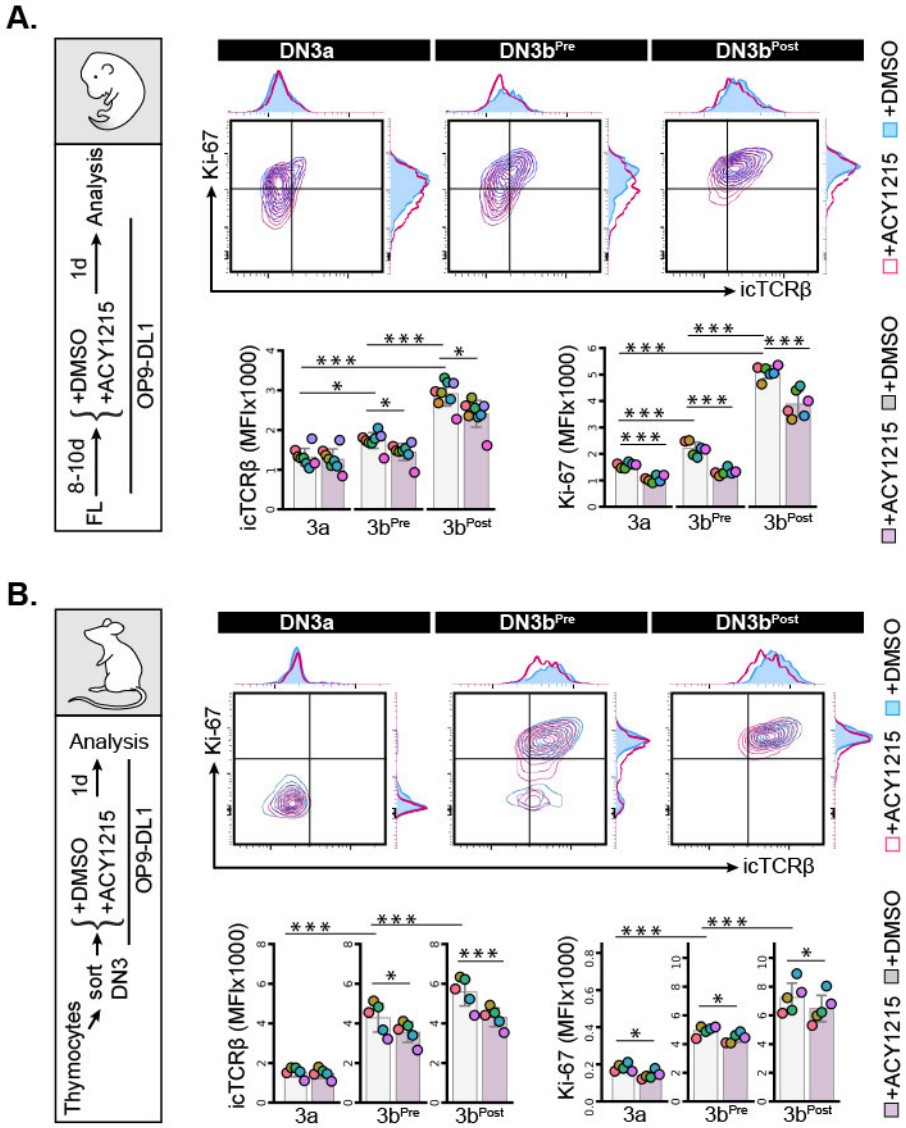


Figure 4. 6 DN3b^{Pre} is intermediate between DN3a and DN3b^{Post}

FL-derived developing T cells **(A)** or the sorted thymus-derived DN3 **(B)** were treated with ACY1215 or DMSO for 1 day, and DN3a, DN3b^{Pre}, and DN3b^{Post} cells were assessed for icTCRβ and Ki-67 expression. MFI were summarised in histogram and swarm plot, with individual colours representing each experiment. (p value < 0.001 = '***', < 0.01 = '**', <0.05 ='*')

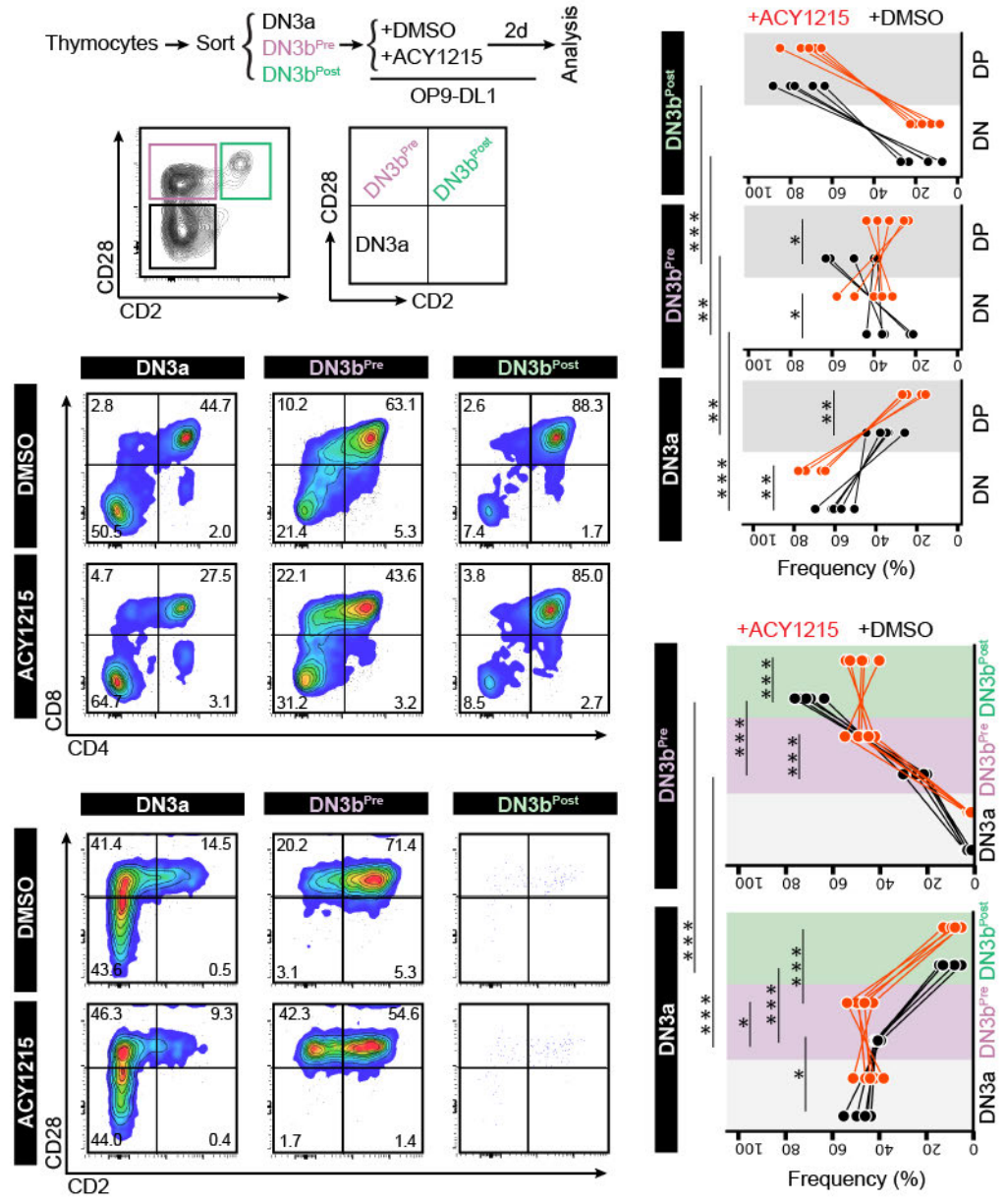


Figure 4. 7 Thymocyte DN3a, DN3b^{Pre} and DN3b^{Post} stages follow a sequential progression.

The DN3a, DN3b^{Pre}, and DN3b^{Post} cells from the adult thymus were sorted as indicated, and were cultured on OP9-DL1. At two day, the cells were analysed by CD4 versus CD8, the DN cells were analysed by CD44 versus CD25, and the DN3 cells were analysed by CD2 versus CD28. The frequencies of each population were summarised in the line plots. (p value < 0.001 = '***', < 0.01 = '**', <0.05 ='*')

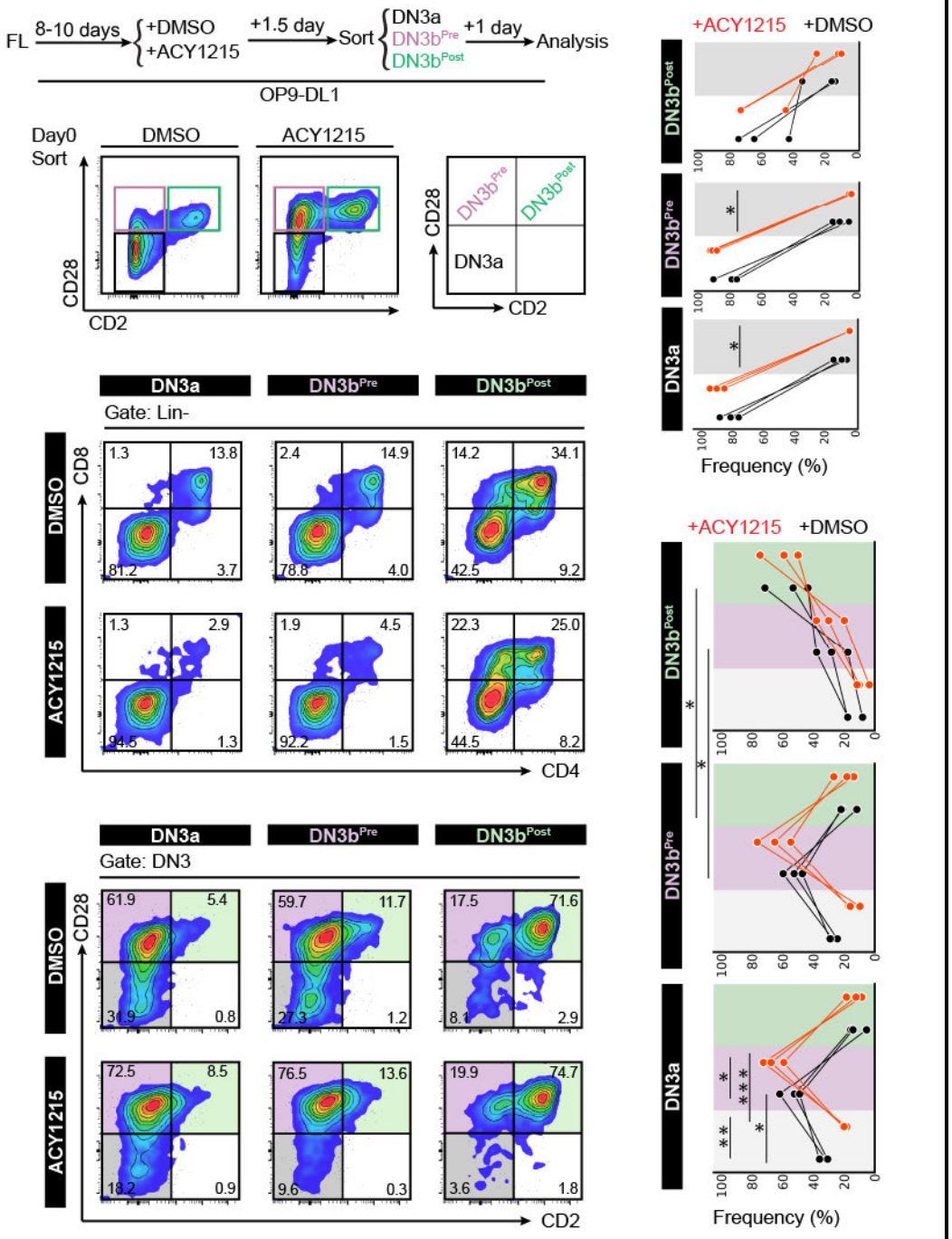


Figure 4. 8 FL-derived DN3a, DN3b^{Pre} and DN3b^{Post} stages follow a sequential progression.

The DN3a, DN3b^{Pre}, and DN3b^{Post} cells from the adult thymus were sorted as indicated, and were cultured on OP9-DL1. At two day, the cells were analysed by CD4 versus CD8, the DN cells were analysed by CD44 versus CD25, and the DN3 cells were analysed by CD2 versus CD28. The frequencies of each population were summarised in the line plots. (p value < 0.001 = '***', < 0.01 = '**', <0.05 ='*')

4.2.5 DN3b^{Pre} cells have commenced but not completed β-selection

How might such a disruption in pre-TCR signalling alter progression from DN3b^{Pre} to DN3b^{Post}? A collaboration between pre-TCR and Wnt signalling is well established, with two core Wnt pathway transcriptional regulators: β-catenin and Lef1/TCF-1 essential to β-selection (Gounari et al., 2001; López-Rodríguez et al., 2015; Staal and Clevers, 2005; Travis et al., 1991; Xu et al., 2009; Zhao et al., 2021). TCF-1 and Lef1 can directly modify histone acetylation, and this function is inhibited by the pharmacological HDAC inhibitors, Tubacin and Vorinostat, (Xing et al., 2016; Zhao et al., 2021). In support of functional interplay between ACY1215 treatment and TCF-1 and Lef1, TCF-1 deficiency leads to a similar phenotype as ACY1215 treatment, with disrupted β-selection and reduced expression of icTCRβ in DN4 cells (Goux et al., 2005), and deletion of both TCF-1 and Lef1 leads to a complete block at DN4 (Yu et al., 2012). I focused on Lef1 since Lef1 is specifically expressed at the β-selection stage (Mingueneau et al., 2013). To assess whether Lef1 might mediate the β-selection decisions at the DN3b^{Pre} stage, I explored how its expression changed with differentiation, and how it was impacted by ACY1215 treatment. Lef1 was minimally expressed in DN3a cells, uniformly bright in DN3b^{Post} cells, but heterogeneous in DN3b^{Pre} cells derived from either fetal liver or adult thymus (**Figure 4. 9**). In the DN3b^{Pre} cells, Lef1-expression was associated with increased expression of TCRβ, albeit with a wide range in TCRβ expression (Figure 4. 9). ACY1215 treatment did not demonstrably alter icTCRβ expression but, prevented the upregulation of Lef1 expression in DN3b^{Pre} cells, suggesting a role in pre-TCR-mediated induction of Lef1.

4.2.6 ACY1215 maintains IL7R expression but attenuates Stat5 phosphorylation

A key feature of progression through β-selection is changes in responsiveness to IL7 signalling (Boudil et al., 2015). IL-7 inhibits expression of Lef-1 and other regulators required for T cell development (Yu et al., 2004). Expression of IL7R is negatively correlated with expression of Lef-1 in DN3b cells (**Figure 4. 10**), compatible with an inhibition of Lef1 expression by IL7 signalling. IL7R expression *per se* is not altered by ACY1215 treatment, but phospho-STAT5 is down-regulated by ACY treatment. These data are compatible with the notion that, as cells enter the DN3b^{Pre} stage of β-selection, a downregulation of IL7 signalling triggers expression of Lef-1.

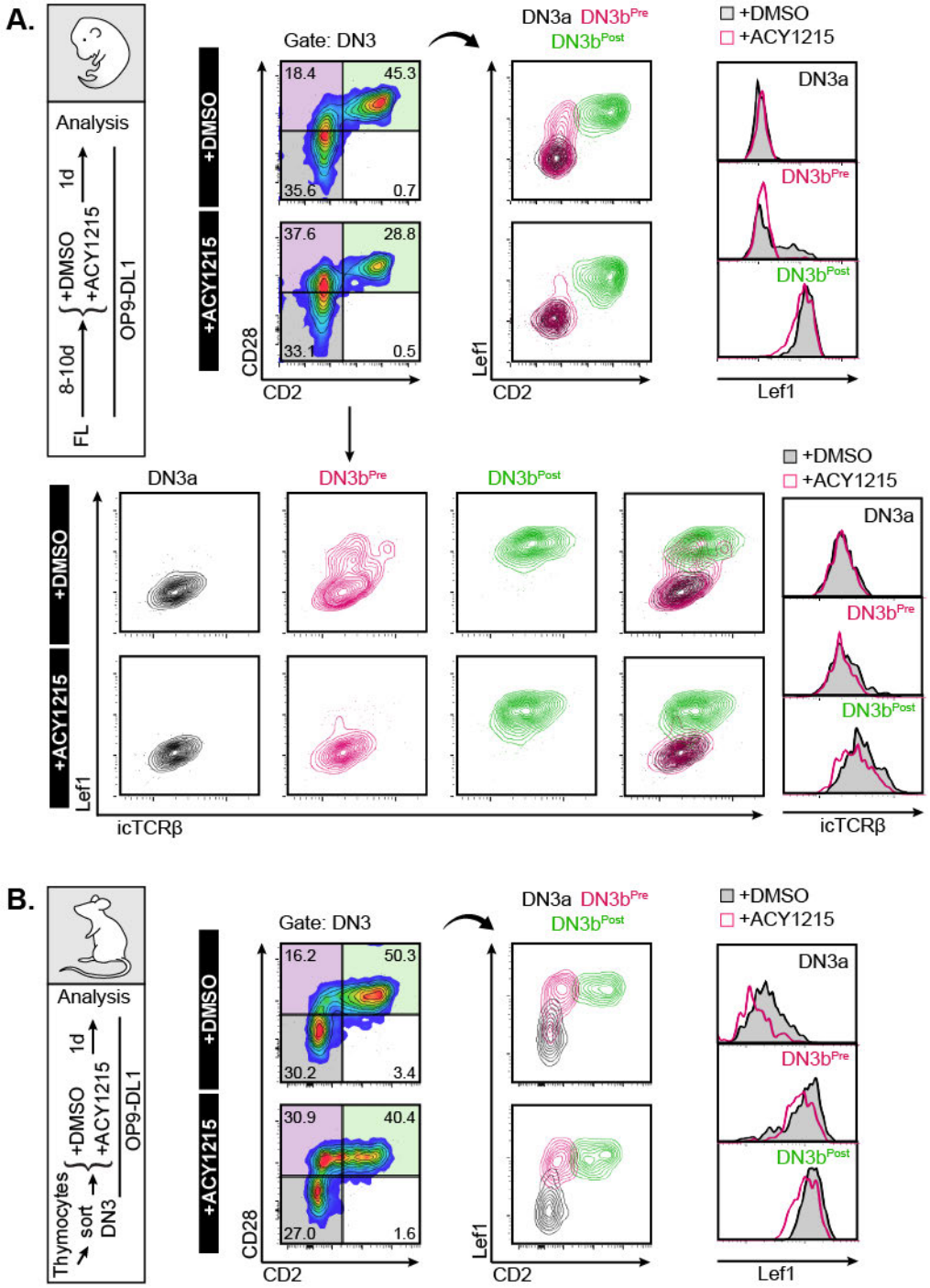


Figure 4. 9 Lef1 is upregulated from DN3b^{pre}

FL-derived developing T cells **(A)** or the sorted thymocyte DN3 **(B)** were treated with ACY1215 or DMSO for 1 day as indicated, and DN3 cells were assessed for CD2 and CD28 expression. The DN3a, DN3b^{Pre}, and DN3b^{Post} cells were assessed for Lef1 expression. DN3a, DN3b^{Pre}, and DN3b^{Post} cells from (A) were assessed for Lef1 and icTCRβ expression.

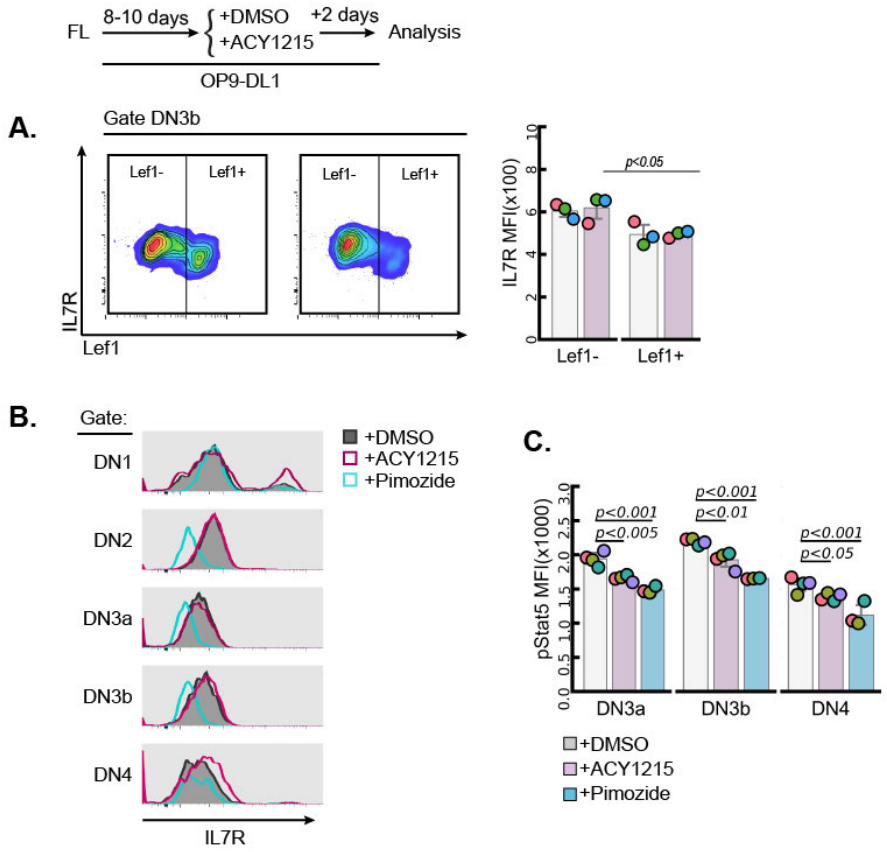


Figure 4. 10 Assessment of IL7R and pStat5 expression upon ACY1215 treatment

(A) Developing T cells were treated with ACY1215 or DMSO for 2 day as indicated, and DN3b cells with Lef1^{HI} and Lef1^{LO} were assessed for IL7R expression. Developing T cells were treated with ACY1215, Pimozide (Stat5 inhibitor) or DMSO for 2 days, and IL7R expression of DN1, DN2, DN3a, DN3b and DN4 cells were shown in the histograms **(B)**, or phosphorylated Stat5 **(C)** of DN3a, DN3b and DN4 cells were summarised the swarm plot, with individual colours representing independent experiments.

4.2.7 ACY1215 impairs CD5 expression in β-selection

The correlation in expression of TCRβ with proliferation and Lef1 expression raised the possibility that the pre-TCR signal might be directly translated into phenotype during the DN3b^{Pre} stage. To explore this, I investigated the TCR-associated receptor, CD5. A role for CD5 has not been demonstrated during β-selection, but during positive selection expression of CD5 reports the strength of TCR signalling and tunes TCR signalling responsiveness, dampening the response of cells to TCR-signalling via the NF-κB pathway (Azzam et al., 2001; Azzam et al., 1998; Tarakhovsky et al., 1995; Voisinne et al., 2018). To explore the dynamics of CD5 expression during β-selection, I stained fetal liver-derived DN3 cells with CD2, Lef1 and CD5 (**Figure 4. 11**). Gating for DN3b^{Pre} with low and high Lef1 expression showed strong correlation of CD5 and Lef1 expression. Interestingly, Lef1 upregulation was retained in the presence of ACY1215, but the correlation with CD5 expression was lost. These data suggest a model in which CD5 expression is downstream of Lef1, and suggest the effects of ACY1215 on progression through β-selection might be mediated by disrupting the functional connection between Lef1 and CD5. Lef1 and CD5 expression were similarly correlated in DN3 cells extracted from the adult thymus, particularly in DN3b^{Pre} cells (**Figure 4. 12**).

To confirm this model, I made use of the fact that progression past DN3 is marked by downregulation of CD25, and a transitional stage between DN3b and DN4 (sometimes termed DN3c) can be identified within the DN3 population (Ananias et al., 2008; Teague et al., 2010). I therefore assessed the relationship between CD2 and CD25 in the DN3b cells. DN3b^{Pre} population was still high for CD25, but DN3b^{post} included CD25^{Lo} cells. (**Figure 4. 13A**). These data are compatible with the notion that DN3b^{Post} marks a later stage in differentiation, that includes, but is not exclusive to, the DN3c population (Ananias et al., 2008). To assess whether the combined expression of CD5 and Lef1 in DN3b cells indicated progression through β-selection, I gated for the two clearly discriminated sub-populations Lef1^{Lo}CD5^{Lo} and Lef1^{Hi}CD5^{Hi}, and compared their CD25 expression (**Figure 4. 13B**). The Lef1^{Hi}CD5^{Hi} cells were substantially lower for CD25, indicating that they had already progressed through β-selection. Although as expected there were far fewer cells in the Lef1^{Hi}CD5^{Hi} population upon ACY1215 treatment, they also exhibited the reduction in CD25, suggesting that ACY1215-treated cells can precociously differentiate beyond β-selection in the absence of CD5 expression.

To confirm the relationship between pre-TCR and CD5 and to assess the impact of ACY121, I cultured DN3a or DN3b cells from adult thymus in the presence of ACY1215

or DMSO for 2 days, gated for DN3b cells, and compared CD5 expression to icTCRβ (**Figure 4. 12B**). Cells from the DN3a starting population were more likely to be dull for both CD5 and icTCRβ, compared with the more mature cells from the DN3b starting population. However, in these mature cells, expression of icTCRβ correlated with expression of CD5, suggesting that, as in other cell types, CD5 expression might report TCR signalling strength during β-selection. In ACY1215-treated cells, expression of surface TCRβ was reduced by the $DN3b^{Post}$ stage, and uncoupled from CD5. Similar findings were obtained using fetal liver cultures and staining for CD5 and icTCRβ (**Figure 4. 14**). The correlation of TCRβ with CD5, and its uncoupling after ACY1215 treatment, was not observed with CD27 and CD28 nor CD2. Together, a functional link between pre-TCR, Lef1and CD5 is coordinated at $DN3b^{Pre}$ stage (**Figure 4. 15**), and is affected by ACY1215.

To assess the functional relationship between TCRβ and CD5, I compared CD5 expression and proliferation in the presence and absence of ACY1215 (**Figure 4. 16**). Proliferation was minimal in most DN3a cells and $DN3b^{Pre}$ cells, but was evident in a portion of $DN3b^{Pre}$, cells from the DN3b starting population, and in $DN3b^{Post}$ cells. This proliferation was clearly correlated with CD5 expression in control cells. However, the correlation between CD5 expression and CFSE dilution was lost in the $DN3b^{Pre}$ cells that had been treated with ACY1215. These data together indicate that CD5 serves as a link between TCRβ expression and proliferation, and that this link is broken by ACY1215 treatment.

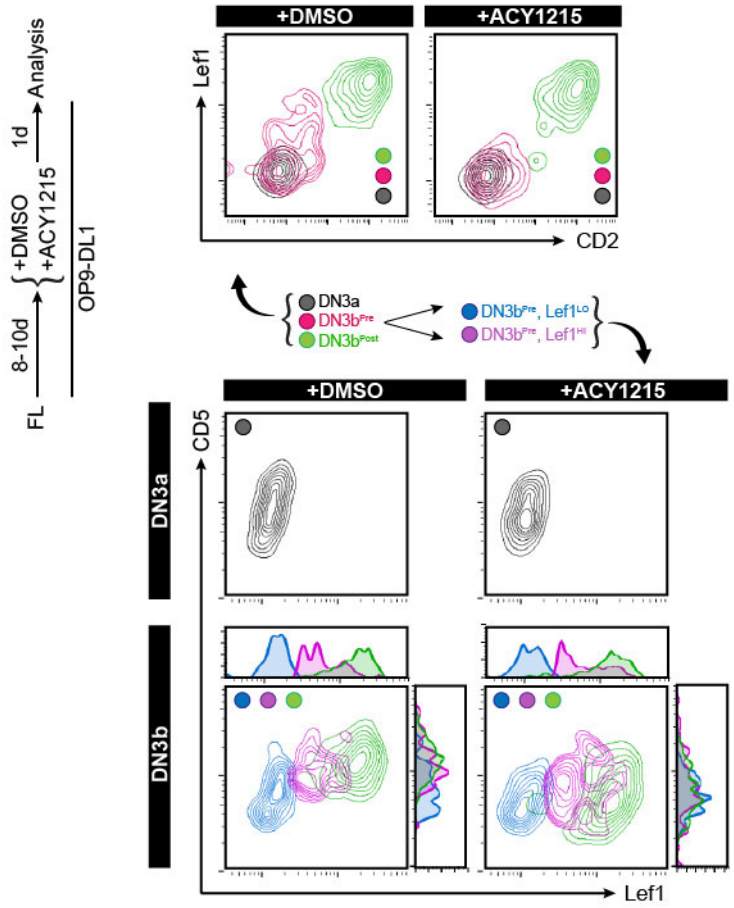


Figure 4. 11 CD5 is upregulated with Lef1 and TCRβ in DN3b^{Post} cells.

FL-derived developing T cells were treated with ACY1215 or DMSO for 1 day as indicated, and DN3a, DN3b^{Pre}, and DN3b^{Post} cells were assessed for Lef1 and CD2 expression. DN3b^{Pre} cells were further characterised by Lef1^{Hi} or Lef1^{Lo}, and were assessed for Lef1 and CD5 expression with DN3a and DN3b^{Post}.

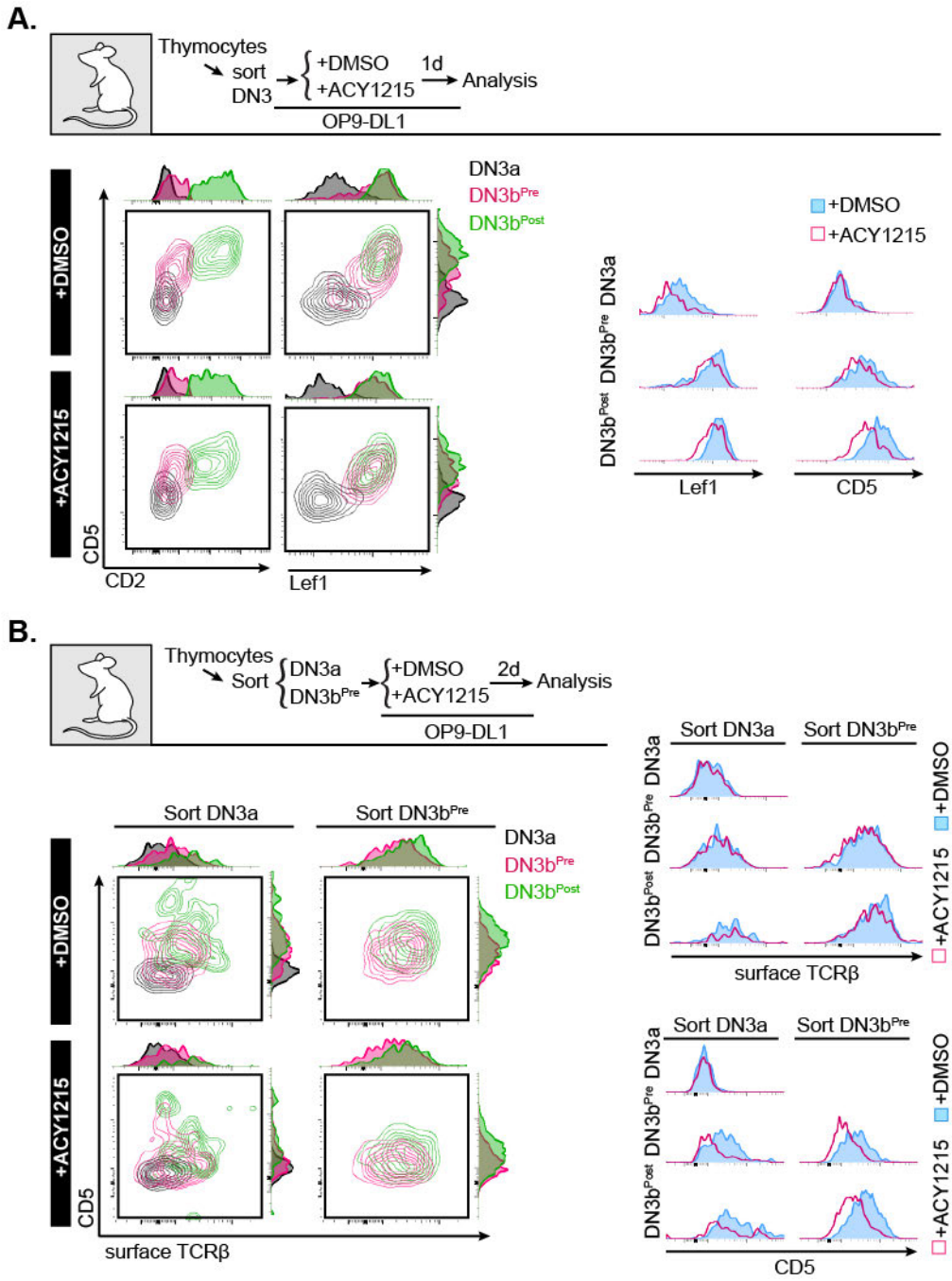


Figure 4. 12 CD5 is upregulated with Lef1 and TCRβ in DN3b^{Post} cells.

(A) Sorted DN3 cells from the adult thymus were treated with ACY1215 or DMSO for 1 day as indicated, and DN3 cells were assessed for CD5, CD2 and Lef1 expression. **(B)** Sorted DN3a and DN3b^{Pre} cells from the adult thymus were treated with ACY1215 or DMSO for 2 day as indicated, and DN3 cells were assessed for CD5 and surface TCRβ expression.

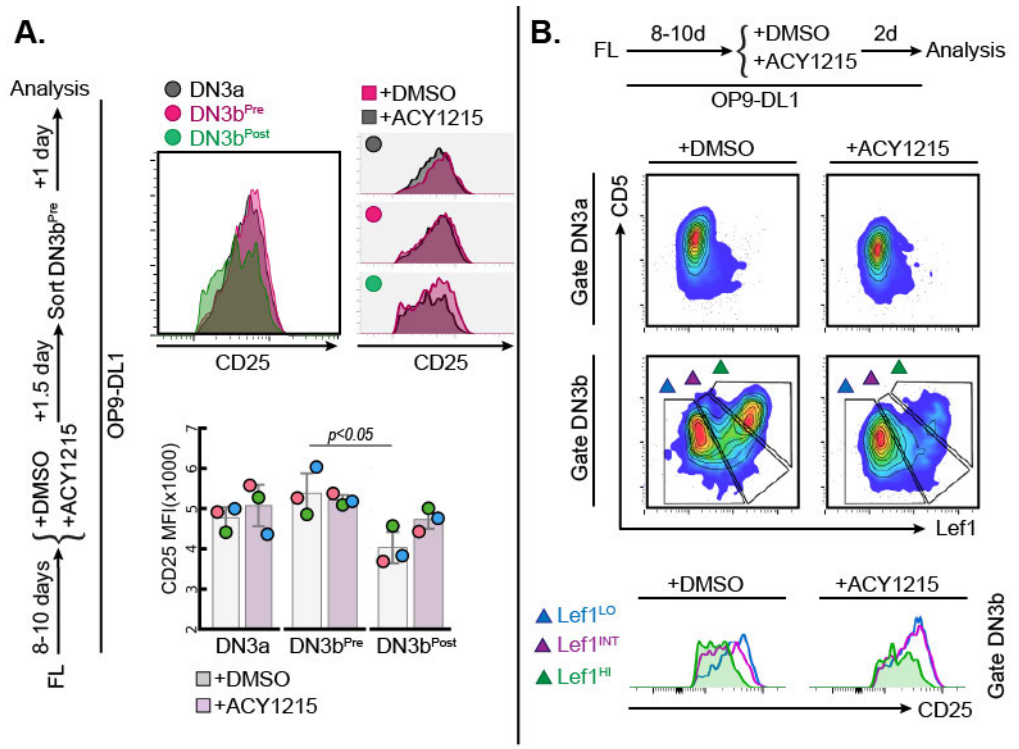


Figure 4. 13 CD25 is downregulated in DN3b^{Post} cells

(A) Developing T cells were treated with ACY1215 or DMSO for 1.5 day, and the DN3b^{Pre} was sorted as indicated. At day1, the three DN3 cells subpopulations were were assessed with CD25 expression, as shown in the contour plots and histograms. (B) Developing T cells were treated with ACY1215 or DMSO for 2 day as indicated, and DN3a and DN3b were assessed for Lef1 and CD5 expression. The expression of CD25 in the DN3b subsets were shown in histogram.

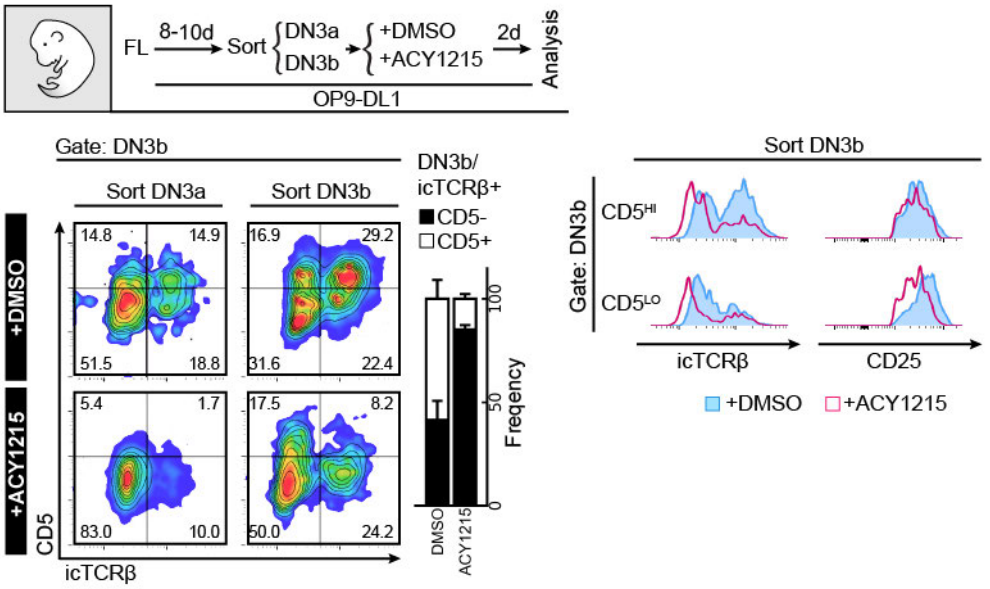


Figure 4. 14 CD5 expression is uncoupled from icTCRβ expression in FL-derived DN3 cells

The sorted DN3a and DN3b cells were treated with ACY1215 or DMSO for 2 days as indicated, and the DN3b cells were assessed for icTCRβ and CD5 expression. For icTCRβ^{+} DN3b cells, the proportions of CD5^{+} or CD5^{-} were summarised in the stacked column. Expression of icTCRβ and CD25 were compared in CD5^{+} or CD5^{-} DN3b cells.

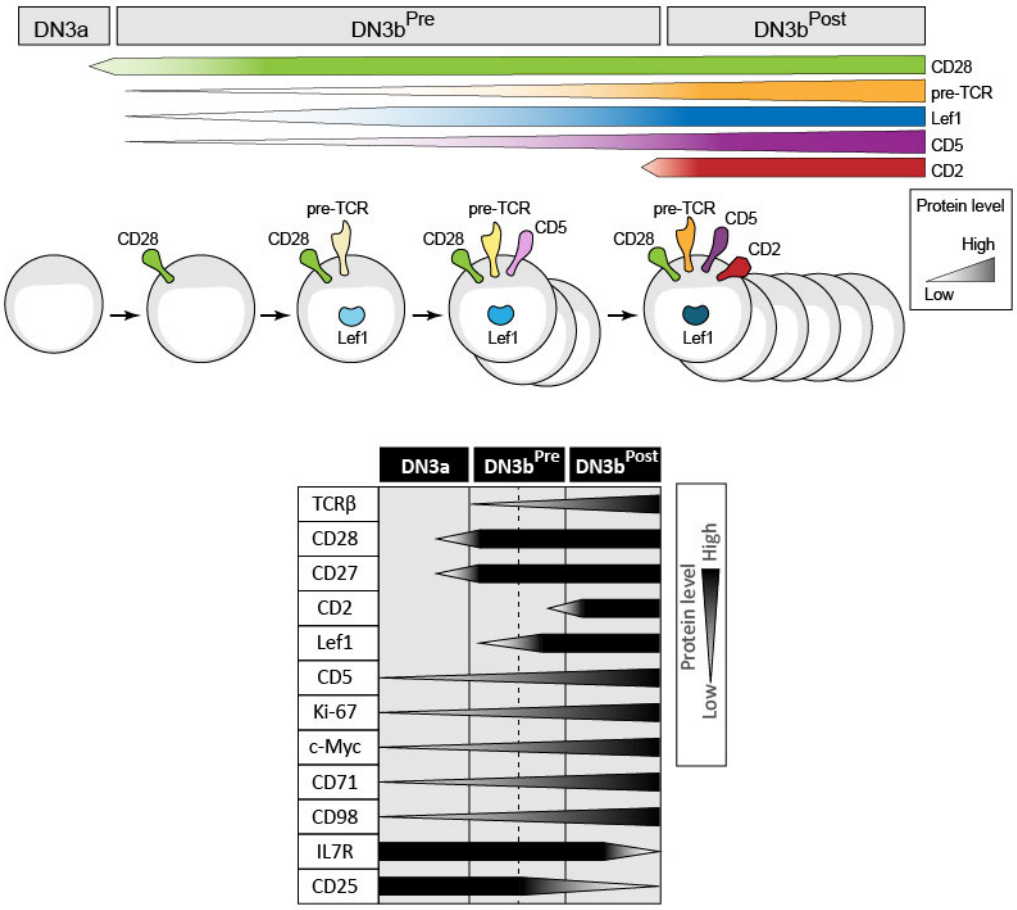


Figure 4. 15 Schematic summary of expression of key markers during β-selection

The upper schematic describes the stepwise expression of TCR co-receptors (CD28, CD5, and CD2) and Lef1 from DN3a, DN3b^{Pre} to DN3b^{Post} stage. A complete profile of markers that were tested in this thesis were listed in the lower schematic.

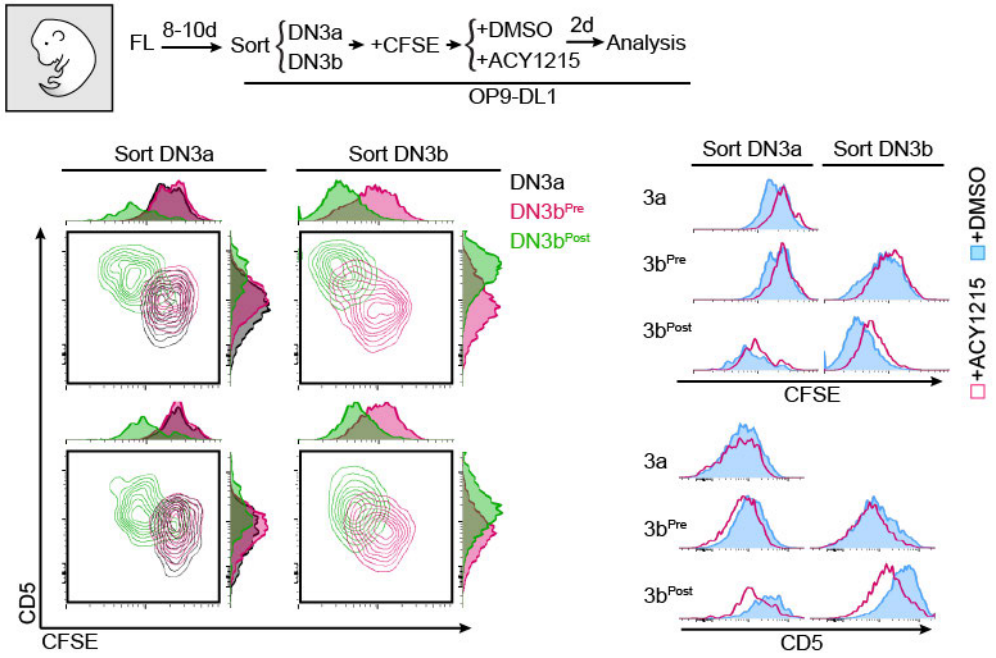


Figure 4. 16 CD5 expression correlates with proliferation

Sorted FL-derived DN3a and DN3b were stained with CFSE after sorting and were cultured with ACY1215 or DMSO for two days as indicated. At two days, the DN3a, DN3b^{Pre}, and DN3b^{Post} cells from the sorted DN3a and DN3b were assessed for CD5 expression versus CFSE intensity.

4.3 Discussion

A critical factor in β-selection is the sequential expression of specific transcriptional regulators, with differential perdurance and cross-inhibition ensuring checks and balances that prevent inappropriate survival and differentiation (Chann and Russell, 2019; Zhao et al., 2021). Many such checks and balances have been identified to play important roles during β-selection, but the stepwise progression of events that dictate fate have been elusive (Chann and Russell, 2019; Zhao et al., 2021). A new working model is suggested by the results described here (**Figure 4. 17**). Once DN3b^{Pre} cells have entered the β-selection checkpoint, they respond to graded levels of TCRβ by expressing Lef1, and then CD5. Proliferation only occurs in cells expressing CD5. Passing of the β-selection checkpoint is then marked by the expression of CD2. The disruption in TCRβ-associated expression of CD5 provides a possible mechanism for the escape of cells lacking an appropriately rearranged TCRβ chain following ACY1215 treatment, although such a role remains to be investigated.

The upregulation of CD27 and CD28 is gradual, but the upregulation of CD2 is more abrupt, making for a convenient demarcation of differentiations stages. Our findings are compatible with previous observations that CD2 expression marks cells with TCRβ gene rearrangement (Rodewald et al., 1993), that knockout of CD2 inhibits progression to the DN4 stage but does not influence TCRβ repertoire (Sasada and Reinherz, 2001), and suggestions of cis-regulation of CD2 and TCRβ expression involving chromatin structure (Kamoun et al., 1995) Together, these findings suggest that expression of CD2 (marking entrance to the DN3b^{Post} stage) reflects a completion of TCRβ recombination and triggers critical fate-determining events that involve progressive upregulation of CD5 and Lef1. CD2 in mature T cells sets quantitative thresholds for activation (Bachmann et al., 1999), suggesting the possibility that a similar process could be involved in β-selection.

A novel finding from this work is that CD5 levels are modified in association with pre-TCR expression, and that treatment with ACY1215 prevents the upregulation of CD5 (but has a lesser effect on Lef1 upregulation) during β-selection. At other stages of T cell development and activation, CD5 reports on the strength of TCR signalling, with increasing expression reflecting a stronger TCR signal (Azzam et al., 1998; Sood et al., 2021; Tarakhovsky et al., 1995). Our findings (the correlation of CD5 expression with TCRβ expression that is lost with ACY1215 treatment, and the correlation of CD5 expression with proliferation that is retained with ACY1215 treatment) now suggest the possibility

that CD5 plays a similar role as a reporter of pre-TCR signalling. Interestingly, it is now emerging that differential CD5 expression also marks the propensity for different fates in thymocytes and $CD4^+$ T cells, and this reflects transcriptional and epigenetic differences between cells with high and low CD5 expression (Rogers et al., 2021). In other cell types the combined action of responding to, and dampening, the TCR signal, allows CD5 to tune the TCR signal and so shift the window and modify the timing of positive selection (Lutes et al., 2021; Matson et al., 2020; Sood et al., 2021; Voisinne et al., 2018). Whether or not such a tuning role might occur for CD5 during β-selection needs further exploration, but a possible role for tuning of the TCRβ repertoire during β selection is made relevant by findings that the pre-TCR can bind with low affinity to MHC-peptide (Li et al., 2021; Mallis et al., 2015). The pre-TCR also forms an immunological synapse with many similarities to the immunological synapse formed by the αβTCR, oriented towards MHC (Allam et al., 2021). More work is needed to determine any functional connection between CD5 and tuning, or epigenetic modifications during β-selection, but these data point to a role for CD5 during the $DN3b^{Pre}$ stage in reporting pre-TCR activity to determine subsequent passage through the β-selection checkpoint.

The molecular basis by which ACY1215 mediates the effects on cell fate I observe here needs more exploration. There are many possible means by which ACY1215 might impact upon transcriptional and epigenetic alterations during β-selection to mediate the phonotypes I observe here. At β-selection, transient activation of β-catenin mediates TCF-1 and Lef1 transcriptional regulation to promote pre-TCR-dependent transition to the DN4 stage (Xu et al., 2009). Ectopic expression of a constitutively active β-catenin allows the cells to bypass pre-TCR signalling, yielding DN4 cells lacking expression of TCRβ (Gounari et al., 2001). The similarities between this phenotype and the apparent bypass of pre-TCR signalling I observe with ACY1215 treatment suggest TCF-1/Lef might mediate the effects of ACY1215. Indeed, HDAC6 can regulate acetylation of β-catenin to coordinate its degradation, providing a possible mechanism for the effects of ACY1215 on TCF-1/Lef activity (Iaconelli et al., 2015). Given the correlation between Lef1 expression and CD5 I observe, CD5 might be downstream of Lef1 during β-selection, perhaps explaining the ACY1215-mediated deregulation of CD5 expression. These findings suggest that the induction of Lef1 at the $DN3b^{Pre}$ stage is coordinated with pre-TCR signalling, in part to promote CD5 expression, and that this activity can be altered by ACY1215 treatment. Whether the effect of ACY1215 relates to HDAC6, an alternative HDAC, or the histone deacetylase activity of Lef1/TCF-1 remains to be determined.

Other molecular mediators downstream of ACY1215 effects are also possible. It was recently shown that the transcriptional repressor, Bcl6, is induced by pre-TCR signalling and required for passage through the β-selection checkpoint (Solanki et al., 2020). Differentiation of T follicular helper cells also requires Bcl6, in this case induced by Lef1 and TCF (Choi et al., 2015). Several HDACs have been shown to complex with and modulate Bcl6 activity to impact on B cell development and function (Wang et al., 2020). Together with our findings, these studies suggest the possibility that ACY1215 might mediate at least some of its effects via Bcl6. HDAC6 has also been implicated in the DNA damage response (Zhang et al., 2019), raising the possibility that ACY1215 effects might relate to the genetic recombination integral to the creation of a TCRβ chain (Miyazaki et al., 2008). In any case, the functional outcome of ACY1215 treatment is to disrupt the connections between pre-TCR signalling and expression of CD5 expression and CD5-associated proliferation. Uncoupling CD5 from TCRβ and Lef1 expression provides a possible mechanism by which ACY1215 allows the wrong cells (perhaps cells without suitable TCRβ) to proliferate and differentiate to $DN3b^{Post}$ cells, but these cells subsequently fail to progress, leading to the block in DP differentiation.

The molecular basis for the effects of ACY1215 on β-selection might therefore by multifaceted, and relate to one or more of direct alteration of the epigenome to influence protein expression, alteration of Lef1 activity, DNA damage response, alteration of pre-TCR signalling via organisation at the MTOC and the immunological synapse. Irrespective of the molecular mechanism, the finding that AY1215 disrupts T cell development has potential implications for the development of clinical applications of small molecule inhibitors of HDAC6, particularly in treatment of young patients, whose T cell repertoire has not yet been established (Cosenza and Pozzi, 2018; Hogg et al., 2020).

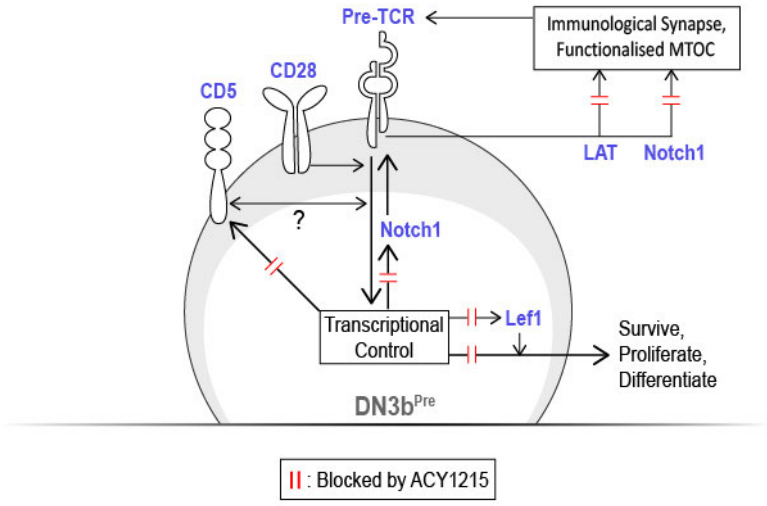
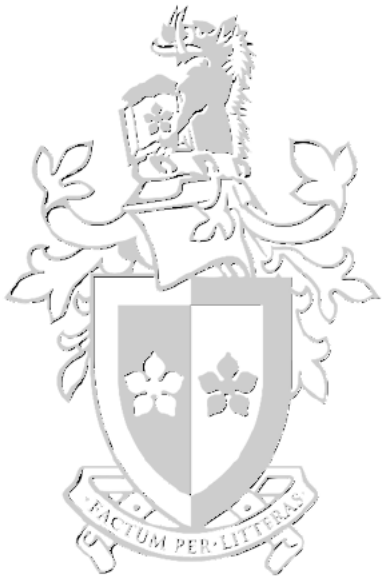


Figure 4. 17 Molecular regulation in DN3b^{Pre}

Schematic summarises the working model of molecular interactions at the DN3b^{Pre} stage. Firstly, CD28 (the defining step in transition from DN3a to DN3b^{Pre}) acts to promote signalling through the nascent pre-TCR. Second, pre-TCR signals induce expression of CD5 (or *vice versa*) and lead to the passing of the β-selection checkpoint. By coordinating transcriptional control, functional MTOC and immunological synapse formation for survival, proliferation and differentiation. Possible molecular events disrupted by ACY1215 treatment to cause these cellular responses are indicated by the red double lines.

4.4 Conclusion

During T cell development, the first step in creating a unique T Cell Receptor (TCR) is the genetic recombination of the TCRβ chain. The quality of the newly recombined TCRβ is assessed at the β-selection checkpoint. Most cells fail this checkpoint and die, but the coordination of the complex events that control fate at the β-selection checkpoint is not yet understood. I shed new light on fate determination during β-selection using a selective inhibitor of histone deacetylase 6, ACY1215, currently in clinical use. ACY1215 disrupted the β-selection checkpoint, progression to the DP stage. Characterisation of the basis for this disruption revealed a new, pivotal stage in β-selection, bookended by the upregulation of the TCR co-receptors, CD28 and CD2 respectively. Within this 'DN3b^{Pre}' stage, CD5 and Lef1 are upregulated to reflect pre-TCR signalling and their expression correlates with proliferation. ACY1215 leads to bypass of the β-selection checkpoint and subsequent failure to progress by disrupting the functional connection from pre-TCR, through CD5 and Lef1, to expansion of cells following β-selection. I propose that the progressive expression of CD28, CD5, then CD2 reports and modulates the pre-TCR signal to orchestrate passage through the β-selection checkpoint. One explanation of these findings suggests a refined model of β-selection in which a coordinated increase in expression of pre-TCR, CD5 and Lef1 provides for an escalating test of TCR signalling strength, and culminates in the expression of CD2 to enable exit from the β-selection checkpoint.



Chapter 5

'SEND' controls spindle positioning in epithelial mitosis

5.1 Introduction

Stable epithelial tissue supports health of multicellular organisms. Some cells that are aging or damaged would be replaced, in part through mitosis. During mitosis, the mitotic cell dissociates from surrounding cells and rounds up with spindle assembly (Walma and Yamada, 2020). After chromosome segregation, daughter cells settle down and integrate their cell shape with surrounding cells by forming cell-cell junctions. An active question in the field is how these cues enable memory of the original position of the parent, and are transmitted to control position and fate of the daughter cells (Lechler and Mapelli, 2021).

Spindle orientation provides a functional link between spatial context and fate of the progeny of a cell division in many contexts. For instance, spindle orientation influences the position, the size, and the fate of the two daughter cells of an epithelial division, impacting upon cell diversification and tissue homeostasis and morphogenesis (Bergstralh et al., 2017; Dewey et al., 2015; di Pietro et al., 2016a; Lu and Johnston, 2013; Seldin and Macara, 2017). Errors in the control of orientation of the mitotic spindle lead to developmental defects and cancer (Bergstralh and St Johnston, 2014; Lu and Johnston, 2013). Recent studies indicate that several attributes combine to influence spindle orientation and daughter cell positioning, including intrinsic polarity, the location of adhesions and constraints on the cell shape (Charnley et al., 2013; Dimitracopoulos et al., 2020; Lesman et al., 2014; Li et al., 2019; Li and Burridge, 2019; Matsumura et al., 2016; Mitchison, 1992; Nestor-Bergmann et al., 2019; Niwayama et al., 2019; Petridou and Skourides, 2016; Rizzelli et al., 2020; Thery and Bornens, 2006). All these cues are transmitted to spindle orientation via positioning of the LGN complex for a final spindle orientation (Lechler and Mapelli, 2021).

Epithelial cells physically interact with the surrounding extracellular matrix via integrins and with neighbouring cells via adherens junctions, providing several possible such molecular means of transmitting cues for spindle orientation (Lock et al., 2018; Walma and Yamada, 2020). As the cell rounds up in metaphase, the protrusion that mediate connections to the substrate are termed retraction fibres (Anastasiou et al., 2020; Finegan and Bergstralh, 2019; Fink et al., 2011; Lam et al., 2020; Petridou and Skourides, 2016; Thery et al., 2005). Characterisation of these fibres suggests a context-dependent molecular composition at the site of tethering to the substratum, which then propagates tensile forces

through the fibre (Kotak and Gonczy, 2013; Nestor-Bergmann et al., 2014). The molecules that provide tensile strength to the retraction fibre, and mediate the functional connection from fibre to LGN, are not yet well understood.

Here, I used a simple model of single MCF10A cells or HeLa cells undergoing mitosis on plastic to explore the minimal requirements for spindle orientation and daughter cell positioning. I made the surprising finding that a complex of E-cadherin and Scribble localises at the base of retraction fibres to dictate spindle orientation, and characterized the molecular basis of that interaction. The findings showed that Scribble traffics dynamically between key regions of the cell during cell division, serving as a mitotic scaffold to host localisation of E-cadherin, DLG and NuMA (I called it 'SEND', which is Scribble, E-cadherin, NuMA and DLG). This SEND coordinates the signals from retraction fibre so as to influence spindle positioning.

5.2 Results

5.2.1 E-cadherin controls cell-autonomous spindle orientation and positioning of daughter cells

I first explored the molecular composition of cell protrusions that connect the mitotic cell to the surface during cell division in the MCF10A human mammary epithelial cell line. MCF10A is a model cell line that is believed to phenotypically recapitulate the physiology of normal breast cells (Qu et al., 2015). During prometaphase, MCF10A cells exhibited long protrusions rich in F-actin and E-cadherin, but with minimal active β1 integrin (**Figure 5. 1**). These protrusions are traditionally termed retraction fibres, and are retained as cells round up for cell division. By the telophase stage, the cell contained shorter protrusions, with more active β1 integrin in the adhesions and less E-cadherin (Figure 5. 1). The enrichment of E-cadherin in prometaphase retraction fibres was a surprise given that E-cadherin is classically considered to mediate cell-cell interactions, rather than cell-matrix interactions (Lecuit and Yap, 2015; Pannekoek et al., 2019). It also does not seem likely that E-cadherin in the retraction fibres is recruited in a ligand-dependent manner, since the substrate was not coated with E-cadherin ligands, and the E-cadherin in fibres was no stronger at the substrate-contacts of the fibres than along their lengths (see XY view of E-cad, Figure. 5.1). Further characterisation indicated that the β1 integrin-associated protein, paxillin, was also not detected in metaphase protrusions, but was clearly at the base of the protrusions by late telophase (**Figure 5. 2**). Of proteins known to mediate E-cadherin functions, β-catenin, but not β-PIX, was detected in protrusions throughout mitosis (**Figure 5. 3**). Together, these findings indicate that the retraction fibres of single MCF10A cells in prometaphase, rather than containing canonical integrin-based adhesions, contain E-cadherin.

I explored the functional implications of E-cadherin during mitosis using a blocking antibody to E-cadherin, HECD-1 that prevents E-cadherin-E-cadherin interactions (Shimoyama et al., 1989; Tomlinson et al., 2001) (**Figure 5. 4**). I first explored the time taken for the daughter cells to re-adhere and spread on the substrate after cell division. In untreated cells, both daughter cells adhered to the substrate within a relatively short time. However, in the presence of HECD-1 treatment, I consistently observed that of two daughter cells produced per division, one would be significantly inhibited in its re-adherence and re-spreading, while the other was unaffected. Thus, E-cadherin in

cytokinesis is not required for daughter spreading *per se*, but is required for optimal positioning and re-adherence of the second daughter cell after division of MCF10A cells.

To determine whether the HECD-1 antibody directly influenced the localisation of E-cadherin, I stained with a second antibody to E-cadherin after HECD-1 treatment (**Figure 5. 5**). Overlays showed strong co-localisation of HECD-1 and anti-E-cadherin, indicating both that the HECD-1 antibody was specific, and that the impact of HECD-1 was not to substantially reorganise the E-cadherin localisation.

One possible explanation for these findings might be that E-cadherin is required for the mitotic spindle to be oriented parallel to the substrate during division, ensuring both daughters have access to substrate for re-adherence following mitosis (den Elzen et al., 2009; Gloerich et al., 2017; Hart et al., 2017; Wang et al., 2018). In some contexts, spindles are randomly oriented in metaphase, and only achieve appropriate orientation in anaphase, but in other contexts, alignment occurs in metaphase (di Pietro et al., 2016b; Kotak, 2019; Machicoane et al., 2014; Yamashita et al., 2003). Single MCF10A cells showed the latter behaviour, with spindles reliably aligned in metaphase, anaphase and telophase (**Figure 5. 6**). However, in cells that had been treated with HECD-1, the spindle was frequently oriented away from the substrate, particularly in metaphase (Figure 5. 6). Thus, inhibiting E-cadherin interactions leads to asymmetric adhesion of the daughter cells, associated with a mis-oriented spindle in the dividing mother cell. Interestingly, this finding contrasts with a previous finding that low focal adhesion connectivity to the substrate led to horizontal spindle orientation of HeLa cells, which was disrupted by increased focal adhesions (Taneja et al., 2016).

To further clarify whether spindle mis-orientation from HECD-1 treatment was not due to E-cadherin stabilisation, I also tested E-cadherin depletion through si-RNA (**Figure 5. 7**). As the results indicated, loss of E-cadherin accompanied with spindle misorientation. This result was similar to a recent study, which showed E-cadherin knockdown disrupts spindle orientation in an immortalized prostate epithelial cell line, RWPE-1 (Wang et al., 2018). Thus, combined with results using HECD-1, I suggested E-cadherin plays an unexpected role in transmitting information about prior adhesions to the substrate to a mitotic cell, influencing daughter cell positioning by controlling the orientation of the mitotic spindle.

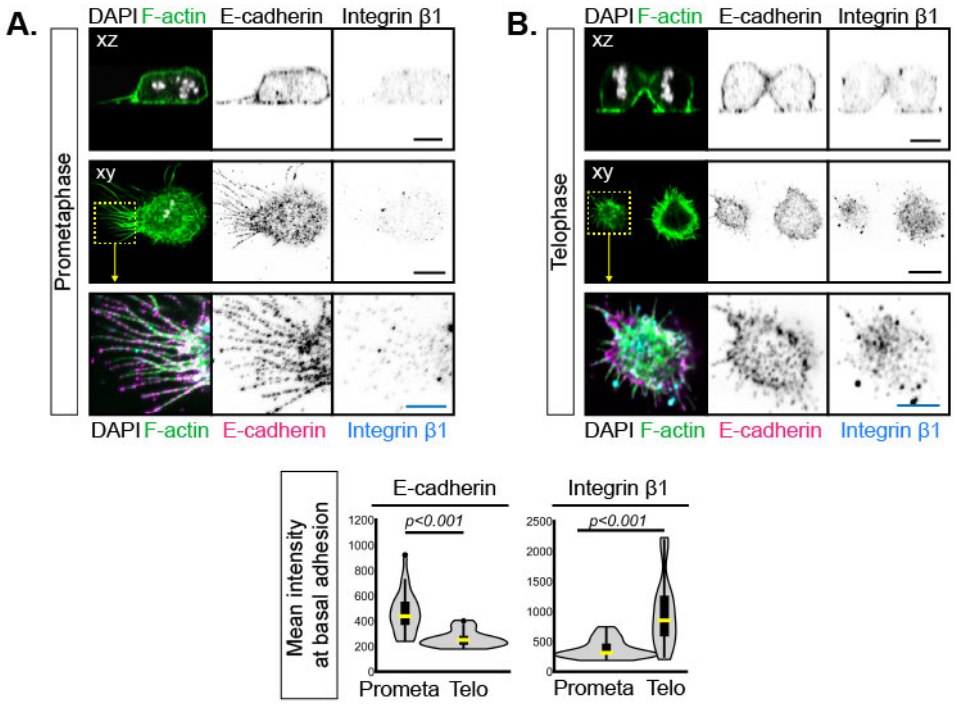


Figure 5. 1 Expression of E-cadherin and activated integrin β1 at basal adhesion at Prometaphase and Telophase

Sparsely plated MCF10A cells were stained with DAPI (white) and phalloidin (green) (pseudo-coloured white and green respectively) to label chromatin DNA and F-actin respectively, and with immunofluorescent antibodies to label E-cadherin (magenta) and active integrin β1 (blue). Representative single cells in prometaphase (A) and telophase (B) are shown in X-Z (left) and in X-Y with two sections of the z-stack shown to represent the centre and in X-Y to represent basal regions. Enlargement of a region of interest (yellow box) in the basal XY slice highlights the localisation of F-actin, E-cadherin and activated integrin β1 (pseudo-coloured according to the protein labels) on the fibres linking the cell to the substrate. Below violin plots quantified the average intensity of E-cadherin (n=23) and activated integrin β1 (n=14) in the basal adhesion region of prometaphase and telophase cells. The median is shown as a yellow bar, the line indicates the first and last quartile, and the box the second and third quartile, p values were calculated using a two-tailed unpaired t test. Scale bar (black) = 10 μm; Scale bar (blue) = 5 μm.

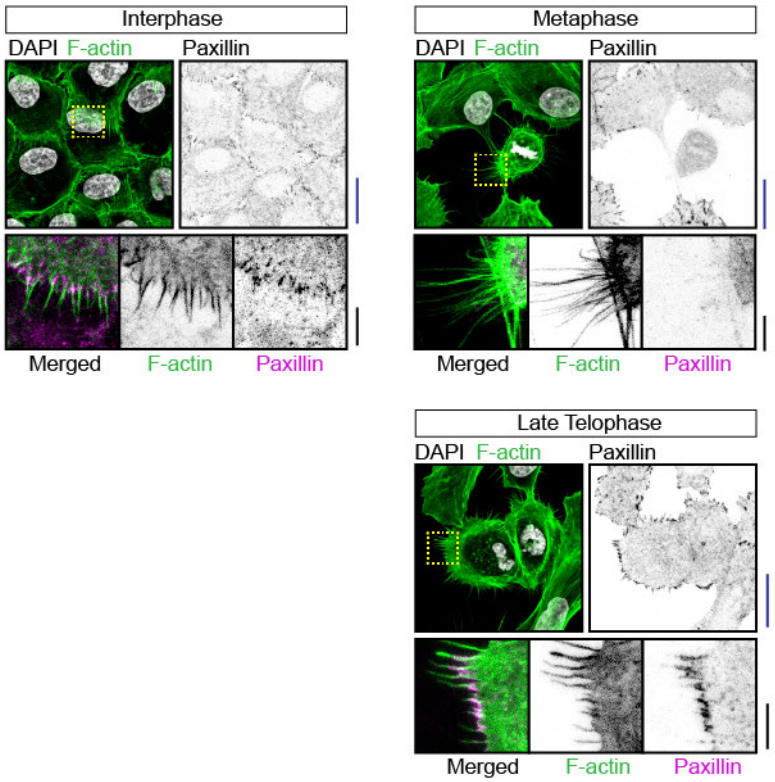


Figure 5. 2 Expression of Paxillin in interphase, metaphase, and late telophase

MCF10A cells at interphase, metaphase and late telophase, labelled with Paxillin, phalloidin (F-actin) and DAPI (DNA), were shown, and with the zoomed-in images (as indicated by the yellow box) showing Paxillin expression in interphase filopodia, mitotic retraction fibre and cytokinetic filopodia. Scale bar (black) = 10 μm; Scale bar (blue) = 5 μm.

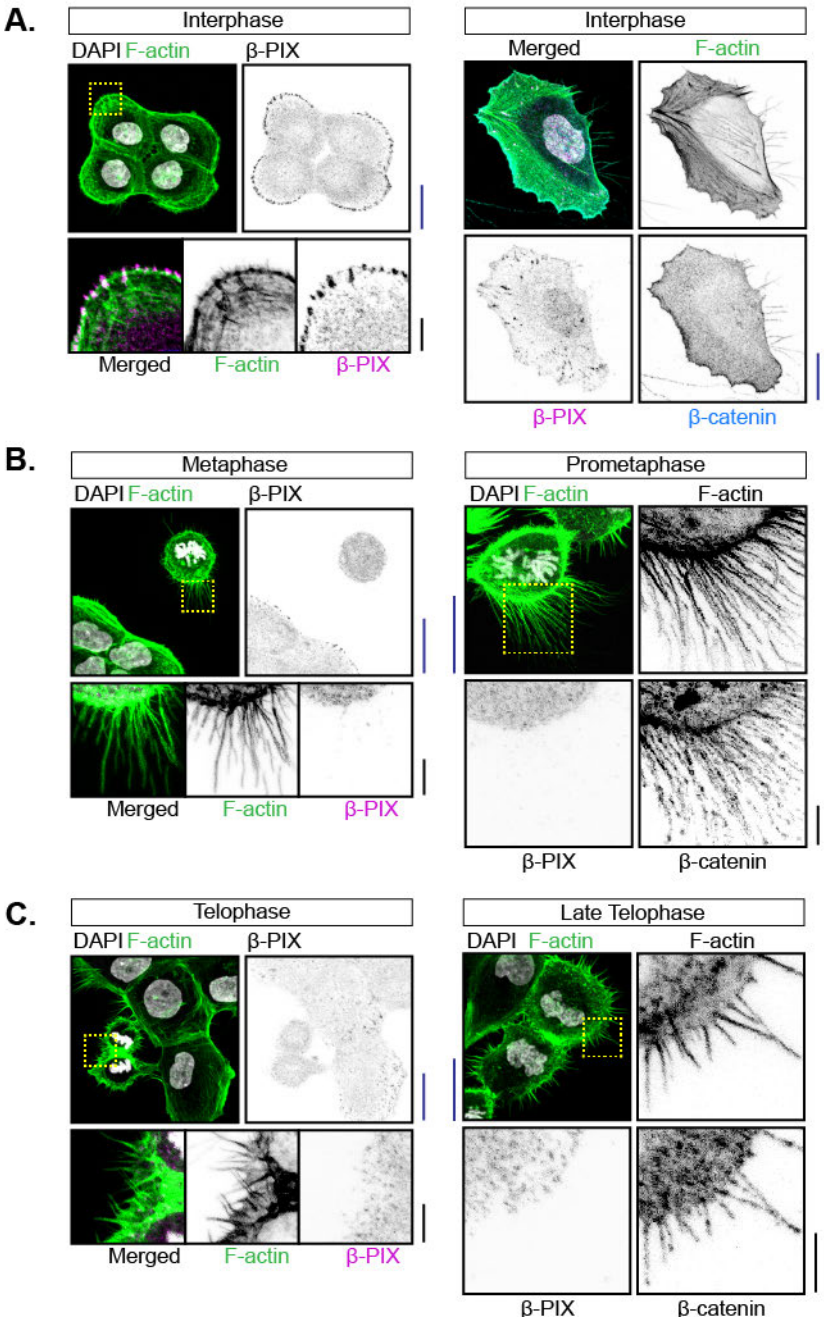


Figure 5. 3 Expression of β-PIX and β-Catenin in different stages of cell cycle

MCF10A cells at interphase **(A)**, prometaphase / metaphase **(B)**, and telophase / late telophase **(C)** were labelled with β-PIX, β-catenin, phalloidin (F-actin) and DAPI (DNA), and were zoomed-in showing protein expression in interphase filopodia, mitotic retraction fibre and cytokinetic filopodia. Scale bar (blue) = 20 μm, scale bar (black) = 5 μm.

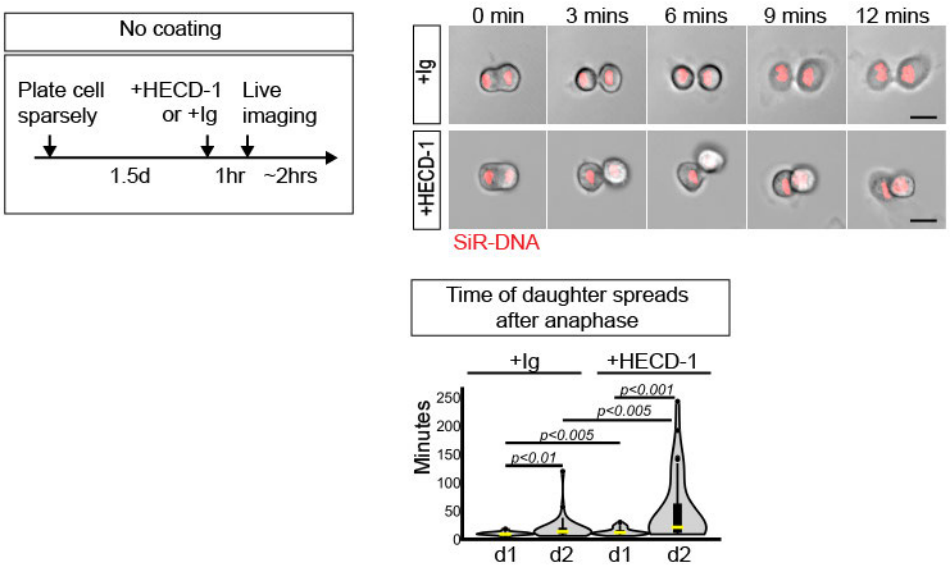


Figure 5. 4 E-cadherin inhibition altered daughter cell attachment

MCF10A cells were labelled with SiR-DNA (red) and treated as indicated in the schematic, and subsequent cell divisions were imaged by time lapse fluorescence microscopy to compare treatments with control antibody ('Ig', n=34) or blocking antibody to E-cadherin ('HECD-1', n=35). The time for each daughter cell to re-adhere to the plastic and spread was recorded as d1 (first daughter to attach) and d2 (second daughter to attach) and shown as violin plots.

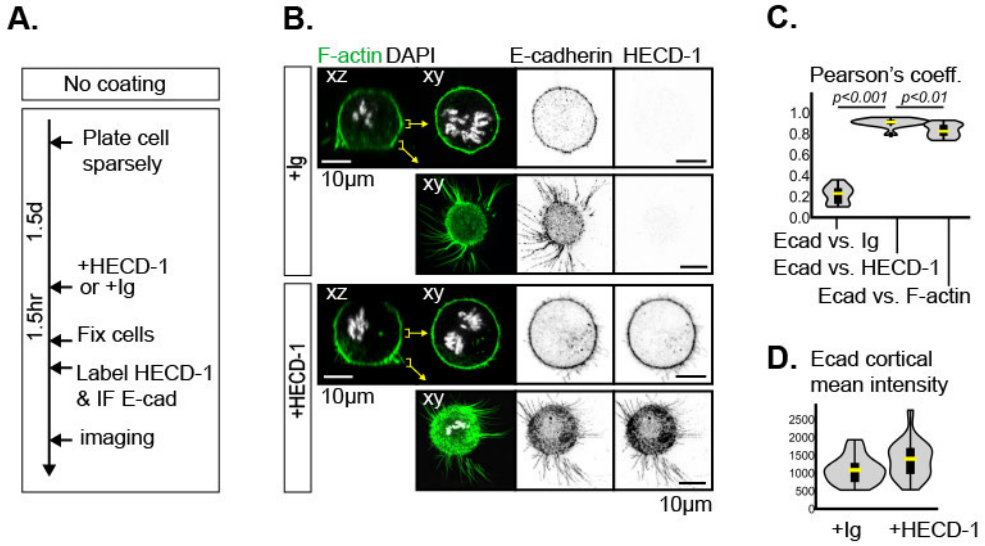


Figure 5. 5 **HECD-1 targeted endogenous E-cadherin in mitosis**

MCF10A cells were treated as indicated in the schematic **(A)** to assess the localisation of HECD-1 and its impact on endogenous E-cadherin by immunefluorescent staining to discriminate between the HECD-1 antibody (anti-mouse Ig) and endogenous E-cadherin **(B)**. Co-localisation of endogenous E-cadherin with control Ig, HECD-1 or F-actin was assessed using Pearson correlation (n = 11, 9, 9) **(C)** and the effect of HECD-1 (n=18) and Ig (n=22) treatment on endogenous E-cadherin intensity at the cortex was measured **(D)**. The p values were calculated using a two-tailed unpaired t test.

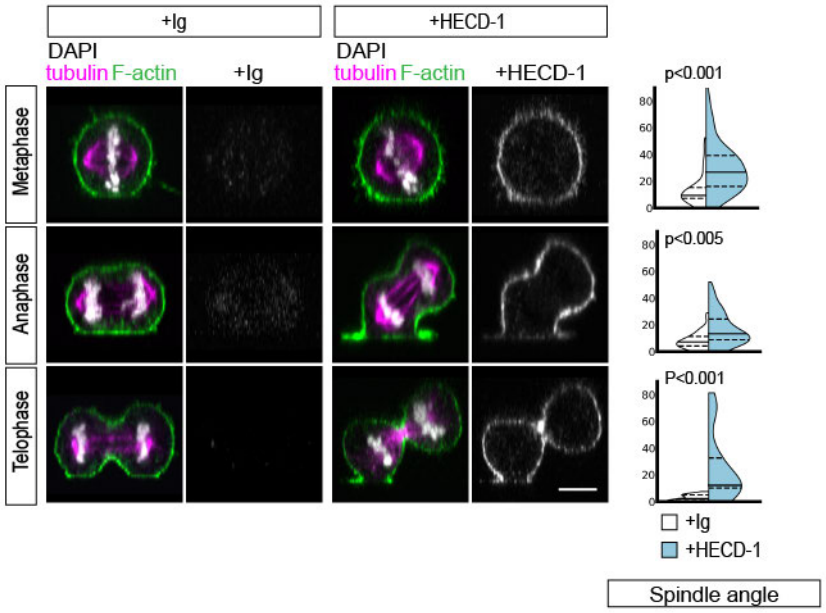


Figure 5. 6 Spindle orientation was disrupted upon E-cadherin inhibition

MCF10A cells were treated with HECD-1 (as in c), and the Ig control as in c(i), si-RNA against E-cadherin, and the si-control, and were stained with antibody against tubulin to measure the mitotic spindle orientation in confocal images (as shown in xz section). Spindle orientation at metaphase, anaphase and telophase were quantified and shown in density plot polar histograms (90° indicates the spindle is perpendicular to the surface). p values were calculated using a one-tailed unpaired t test. Cell number of [metaphase, anaphase and telophase] in Ig treatment is [20, 21, 15], in HECD-1 treatment is [33, 27, 12], in si-Control is [21, 19, 18], and in si-Ecad is [32, 19, 16].

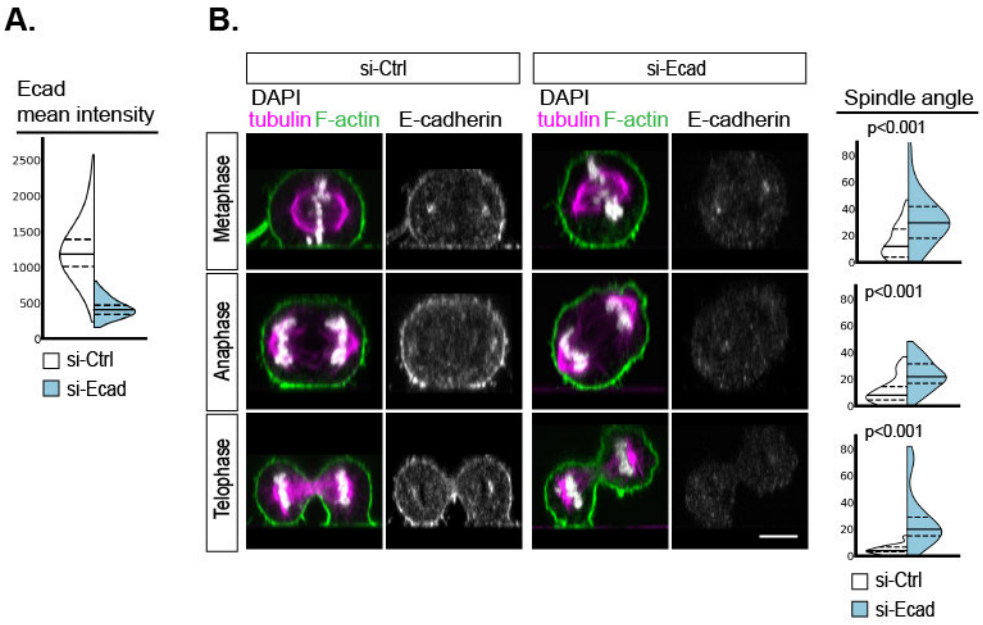


Figure 5. 7 Spindle orientation was disrupted upon E-cadherin depletion

(A), The effect of E-cadherin knockdown on E-cadherin intensity was assessed in violin plot. (B) Spindle orientation upon E-cad depletion was tested. MCF10A cells with si-E-cadherin and si-Control were stained with antibody against E-cadherin and tubulin as shown in the xz section of per metaphase, anaphase and telophase cells. E-cadherin intensity at cortex of si-Ecad (n=32) and si-Control (n=27) was in density plots.

5.2.2 Scribble scaffolds E-cadherin to orient the mitotic spindle

It is well established that stabilization of E-cadherin is key to its function at cell junctions, where E-cadherin stability is promoted by homophilic adhesions (Cavey et al., 2008), but how might E-cadherin influence spindle orientation in this context where intercellular junctions are not available? One possibility is through the polarity protein, Scribble. Scribble functionally interacts with E-cadherin, and is known to orient the mitotic spindle (Bonello and Peifer, 2019; Godde et al., 2014; Nakajima et al., 2019; Nakajima et al., 2013; Qin et al., 2005; Wang et al., 2018). Scribble and E-cad reciprocally regulate each other's positioning and stabilization in a context-dependent manner (Allam et al., 2018; Navarro et al., 2005; Qin et al., 2005). Staining of MCF10A cells undergoing mitosis showed Scribble, E-cadherin and F-actin at the cortex, and in retraction fibres (**Figure 5. 8**). Zooming in on the retraction fibres showed that Scribble and E-cadherin were anti-correlated, with alternating peaks of expression spaced approximately 200 nm apart along the retraction fibres. X-Z views of the dividing cells indicated that cortical Scribble, E-cadherin and F-actin were consistently enriched at the poles of the cells in metaphase (Figure 5. 8). This position is consistent with previously observed localisation of active integrin β1 recruiting the LGN complex to control spindle orientation in single mitotic HeLa cells (Petridou and Skourides, 2016). I also test protein co-localisation through Proximity Ligation Assay (PLA). High spatial proximity between Scribble and E-cadherin were detected at both interphase and mitotic cells (**Figure 5. 9**). In interphase, PLA foci for Scribble and E-cadherin was found between cell-cell junctions, but not for the control pair; Scribble and Paxillin. In mitosis, PLA foci that indicated Scribble-E-cad colocalization were found spread throughout the mitotic cortex. Similarly, PLA foci were fewer between Scribble and Paxillin. Thus, based on the test of colocalization, Scribble might physically interact with E-cadherin in mitosis.

To test whether Scribble impacted E-cadherin stability in either retraction fibres or the cortex, I depleted Scribble in MCF10A cells using two knockdown approaches: si-RNA and sh-RNA. Scribble was uniformly depleted by both knock-down approaches (**Figure 5. 10**). Depletion of Scribble had no impact on adhesion following cell trypsinization (Figure 5. 10), and no impact on the levels of E-cadherin at the cortex of cells in a confluent monolayer (Figure 5. 10). However, Scribble-depleted cells showed almost complete loss of E-cadherin in retraction fibres at prometaphase (**Figure 5. 11**) or at the cortex at metaphase (Figure 5. 11). This, combined with the normal expression of E-cadherin in Scribble-depleted MCF10A cells in interphase, suggests that Scribble is required for the

recruitment or maintenance of E-cadherin in the cortex and retraction fibres during cell division.

All three members of the Scribble complex (Scribble, Discs large and Lethal Giant Larvae) mediate spindle orientation in intact tissues (Allam et al., 2018; Bell et al., 2015; Bonello and Peifer, 2019; Carvalho et al., 2015; Godde et al., 2014; Nakajima et al., 2019; Nakajima et al., 2013; Porter et al., 2019; Qin et al., 2005; Wang et al., 2018; Zigman et al., 2011), so I next investigated whether Scribble is required for spindle orientation in single cells. I assessed spindle orientation in MCF10A cells depleted of Scribble using sh-RNA or si-RNA. In Scribble-depleted cells undergoing cell division, the spindle was mis-oriented (**Figure 5. 12**). This phenotype was similar to that observed with loss of function of E-cadherin (Figure 5. 7). Thus, both Scribble and E-cadherin regulate spindle positioning.

In confluent monolayers, E-cadherin is stabilized at regions of high tension, to which it directly recruits LGN (Gloerich et al., 2017; Hart et al., 2017). LGN then engages with NuMA upon breakdown of the nuclear envelope, which orchestrates pulling on astral microtubules. To assess whether a similar process occurred in single cells, I depleted cells of the canonical spindle regulator, LGN. Depletion of LGN disrupted the spindle orientation (**Figure 5. 13**). Remarkably, depletion of LGN also caused asymmetric adhesion of the daughter cells, with a delay in adherence of the second daughter, albeit less of a delay than seen with E-cadherin inhibition (**Figure 5. 14**). These data together indicate that the asymmetric daughter cell adherence is, at least in part, due to disruption of the spindle orientation. To verify our hypothesis of information flow from E-cadherin and Scribble in retraction fibres, through spindle orientation, to effects on daughter cell positioning, I tracked Scribble-depleted cell using time lapse microscopy (Figure 5. 14). Indeed, Scribble is required for daughter cell positioning, with an asymmetric adherence of one daughter like I had previously seen with E-cadherin inhibition.

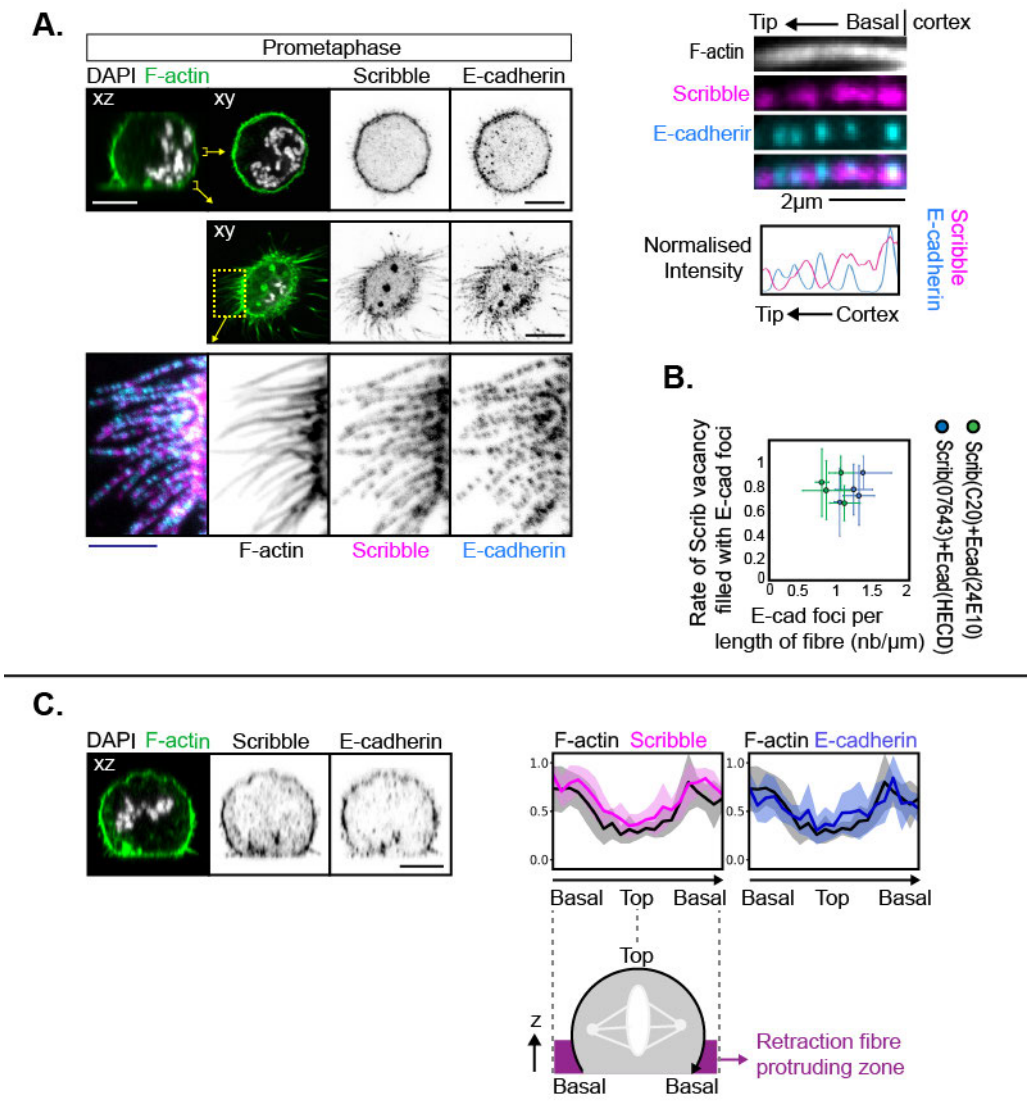


Figure 5. 8 Characterisation of Scribble and E-cadherin colocalization in retraction fibre and mitotic cortex

Sparsely plated MCF10A cell were co-stained with F-actin, E-cadherin, Scribble and DAPI and imaged by confocal microscopy at early stages of mitosis. A representative image at prometaphase **(A)** shows Scribble and E-cadherin co-localised at the cortex (seen in the z-section from the cell centre) and in retraction fibres (in region indicated by the yellow box). Both E-cadherin and Scribble appear punctate and alternating in the fibres, and quantification of the normalised intensity along a fibre demonstrates this alternating pattern. **(B)** To determine the reproducibility of this alternating expression in the retraction fibres, the number of E-cadherin foci per μm along a fibre was plotted against the proportion of Scribble 'vacancies' (patches that did not contain Scribble). **(C)** Cortical distribution in xz section of Scribble, E-cadherin and F-actin were assessed as the schematic indicated, and were shown in the line plot (n=6). Scale bar (black and white) = 10 μm; Scale bar (blue) = 5 μm.

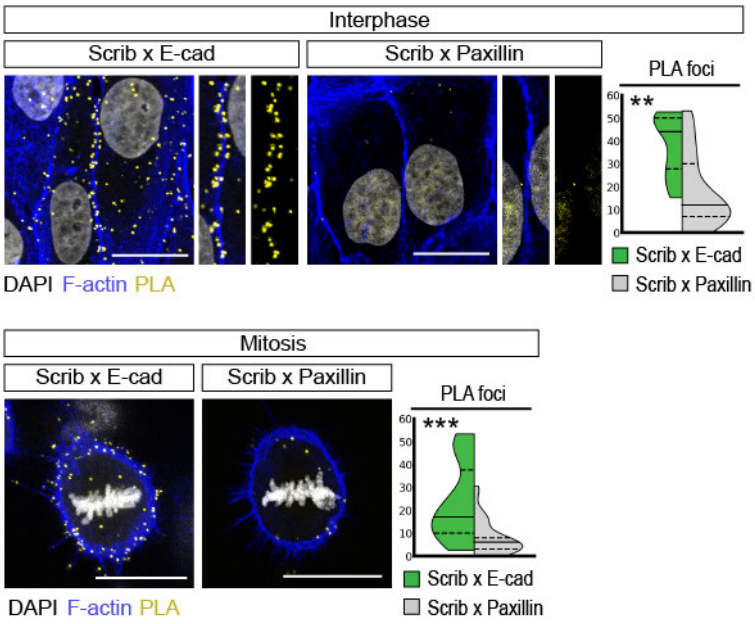


Figure 5. 9 Proximity Ligation Assay to test Scribble interaction with E-cadherin or Paxillin

Proximity ligation assay was applied to determine spatial proximity between Scribble and E-cadherin at interphase (n=11) and mitosis (n=16), and between Scribble and Paxillin at interphase (n=17) and mitosis (n=16). The number of PLA foci was estimated as shown in density plot, with maximum number was counted as 50 if the PLA foci is greater than 50. Scale bar (white) = 20 μm.

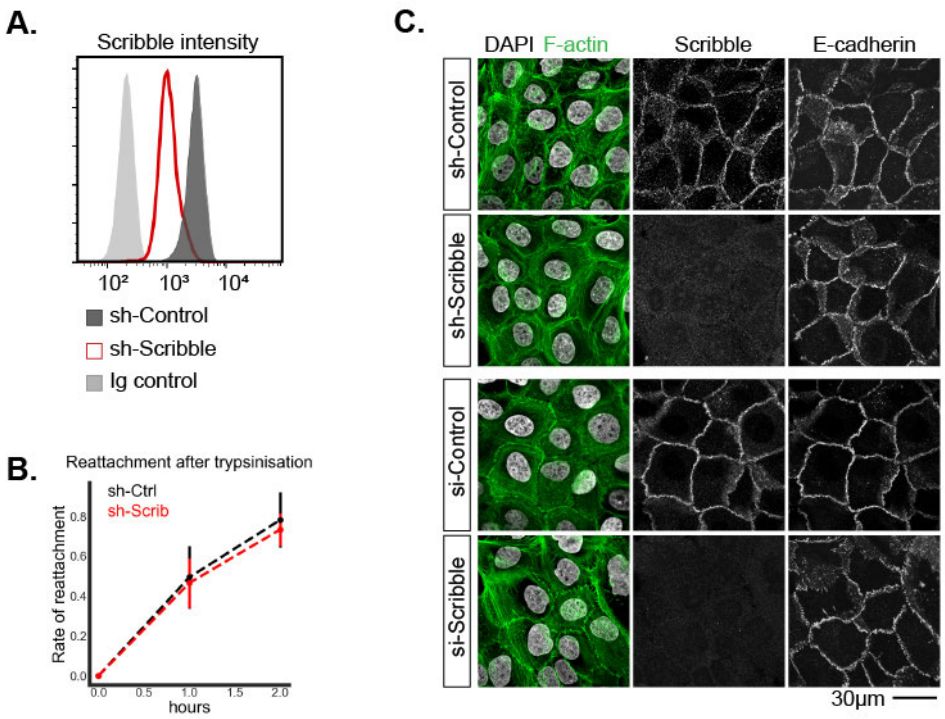


Figure 5. 10 Assessment of Scribble depletion impacts on mitosis-independent physiology

(A), Flow cytometry analysis to examine Scribble depletion by sh-RNA. The cumulative histogram shows the intensity profile of intracellular staining of Scribble of the sh-Scribble cell (red), the sh-control cell (dark grey), and a control of Ig staining (light grey). **(B)** To examine whether Scribble depletion results in a general loss of cell adhesion, the kinetics of reattachment after trypsinisation of sh-Scribble and sh-Control cells were counted. **(C)** Images show adherent junction of interphase monolayer of MCF10A cells (sh-Scribble, sh-Control, si-Scribble and si-Control), labelled with Scribble, E-cadherin, phalloidin (F-actin) and DAPI (DNA). Scale bar = 30 μm.

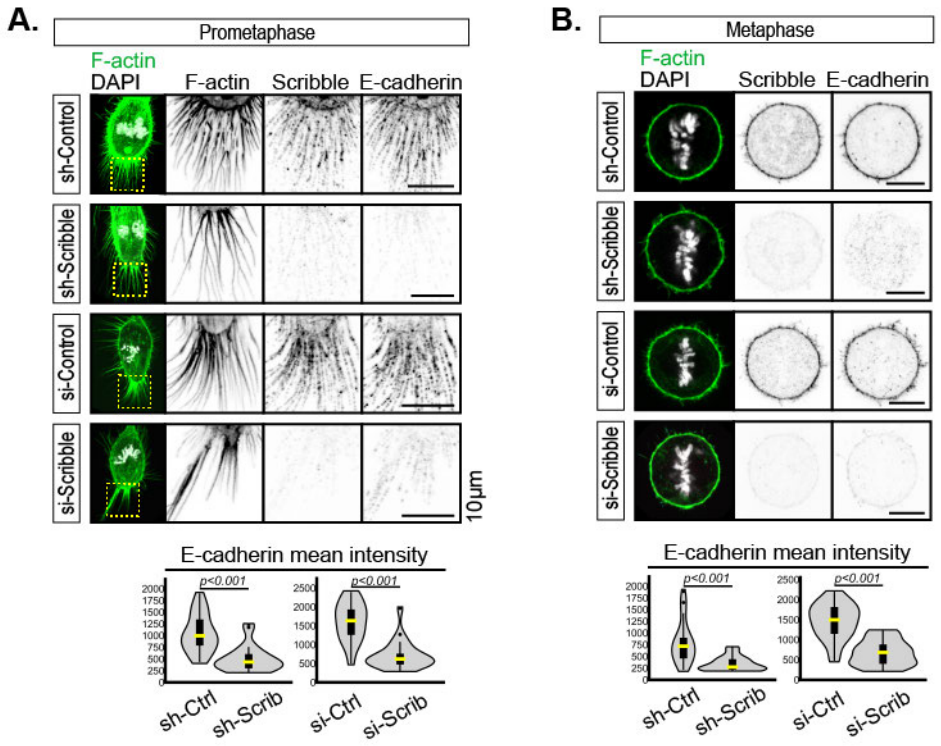


Figure 5. 11 Loss of E-cadherin localisation at retraction fibre and mitotic cortex upon Scribble depletion

(A), Scribble was depleted in MCF10A cells to assess its influence on E-cadherin localisation on the retraction fibre during prometaphase. Cells were treated with sh-Control (n=18), sh-Scribble (n=20), si-Control (n=15), or si-Scribble (n=22), and the mean intensity of E-cadherin in the retraction fibres was shown as density plots overlaid with box plots. **(B)**, Scribble was depleted in MCF10A cells to assess its influence on E-cadherin localisation on the cortex during metaphase. Cells were treated with sh-Control (n=23), sh-Scribble (n=20), si-Control (n=23), or si-Scribble (n=27), and the mean intensity of E-cadherin in the cortex was shown as density plots overlaid with box plots. Scale bar = 10 μm.

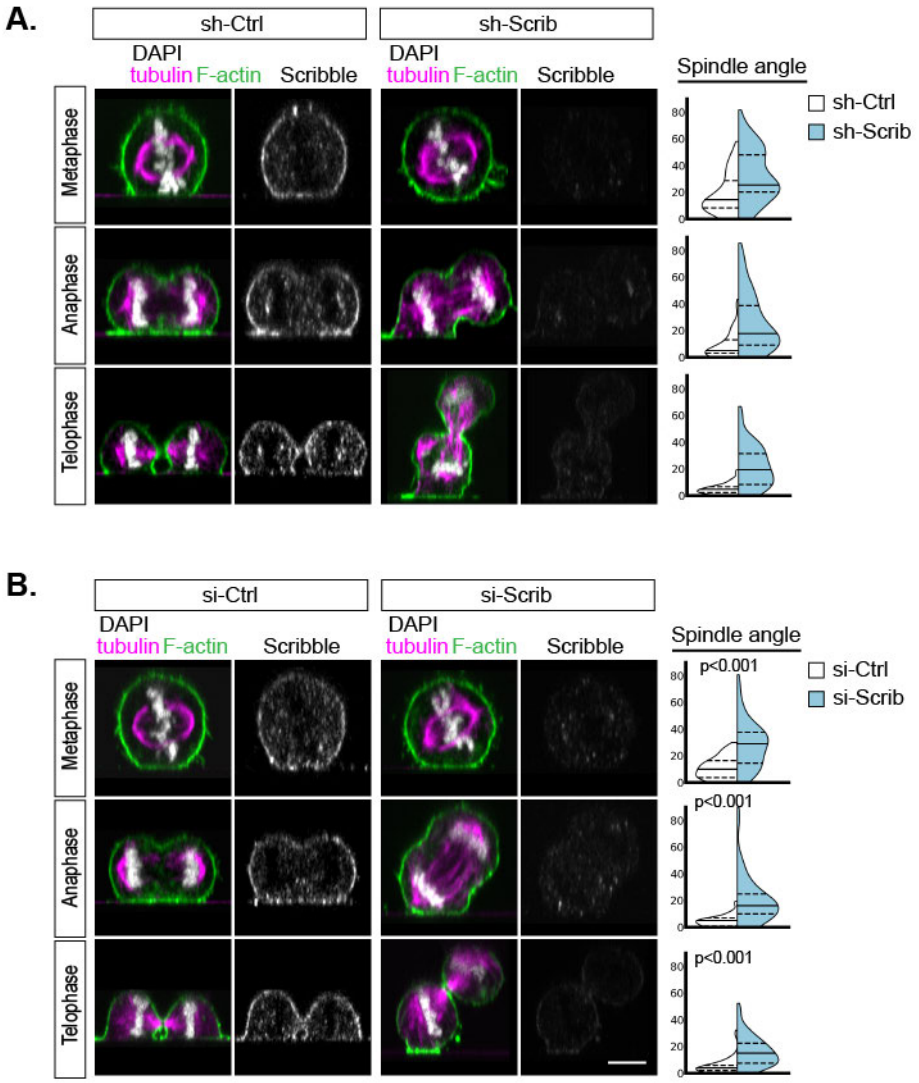


Figure 5. 12 Spindle orientation was disrupted upon Scribble depletion

The effect of Scribble knockdown on spindle orientation was tested. MCF10A cells with **(A)** sh-Scribble and the sh-Control, or with **(B)** si-Scribble and the si-Control were stained with antibody against Scribble and tubulin as shown in the xz section of per metaphase, anaphase and telophase cells to measure the mitotic spindle orientation. Spindle orientation at metaphase, anaphase and telophase were quantified and shown in density plot (90° indicates the spindle is perpendicular to the surface). p values were calculated using a one-tailed unpaired t test. Cell number of [metaphase, anaphase and telophase] in sh-Control is [31, 25, 26], in sh-Scrib is [35, 31, 22], in si-Control is [36, 29, 27], and in si-Scrib is [27, 29, 27]. Scale bar = 10 μm.

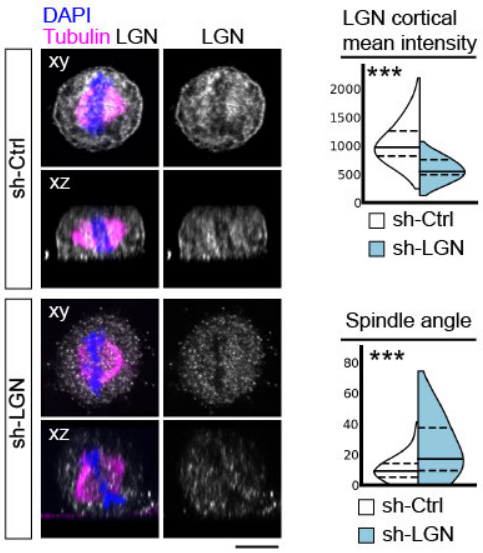


Figure 5. 13 Characterisation of LGN depletion showed spindle misorientation

The effect of LGN knockdown on spindle orientation was tested. MCF10A cells with sh-LGN (n=26) and the sh-Control (n=25) were stained with antibody against LGN and tubulin as shown in metaphase cells. LGN cortical intensity and spindle orientation were analysed as shown in the density plots. Scale bar = 10 μm.

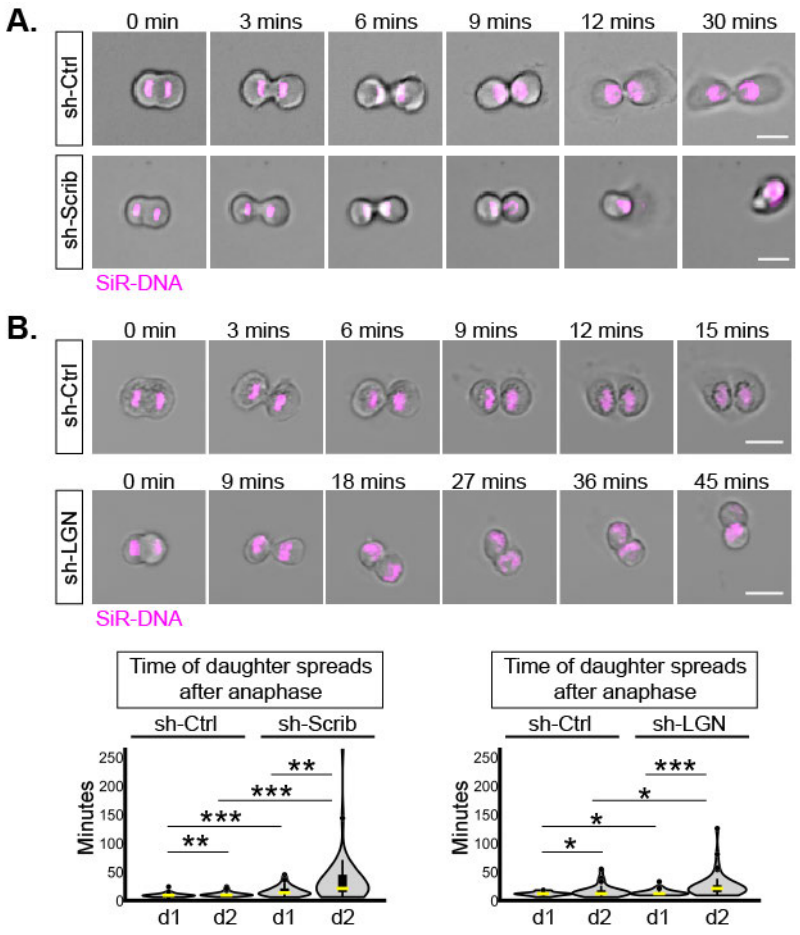


Figure 5. 14 Time-lapse imaging of daughter cell adhesion for Scribble-depleted or LGN-depleted cells

(A), MCF10A cells treated with sh-Control (n=29) and sh-Scribble (n=44) were labelled with SiR-DNA, and subsequent cell divisions were imaged by time lapse fluorescence microscopy. The time for each daughter cell to re-adhere to the plastic and spread was recorded as d1 (first to attach) and d2 (second daughter to attach) and shown as density plots overlaid with box plots. **(B)**, MCF10A cells treated with sh-Control (n=23) and sh-LGN (n=39) were examined as same as in (A). Scale bar = 20 μm.

5.2.3 Scribble coordinates NuMA positioning at the cortical poles for spindle orientation

Mechanisms for controlling spindle orientation have been extensively studied, but it is not yet clear which pathway captures signals from Scribble and E-cadherin. One clue might come from recent observations that in confluent monolayers, E-cadherin recruits LGN (Gloerich et al., 2017; Hart et al., 2017). LGN is known to engage NuMA on astral microtubules then dictates spindle positioning (Gloerich et al., 2017; Hart et al., 2017). However, without such tension from neighbouring junction, I assessed whether Scribble and E-cadherin at these sites might recruit NuMA to orient the mitotic spindle.

In wild-type single MCF10A cells, NuMA indeed localised to spindle poles and weakly at the cortex at metaphase, but not in the retraction fibres (**Figure 5. 15**). This is compatible with previously described cell cycle-dependent influence of LGN on NuMA localisation (Du and Macara, 2004; Zheng et al., 2014). To determine any functional relationship between Scribble and the LGN complex, I assessed NuMA localisation during metaphase in the context of Scribble depletion. Both methods of Scribble depletion reduced the cortical recruitment of NuMA at metaphase (**Figure 5. 16**). The specificity of this effect is demonstrated by our observation that Scribble depletion did not impact the localisation of Myosin IIb (**Figure 5. 17**).

Scribble played similar roles in anaphase as I had observed in metaphase cells. As previously reported (Zheng et al., 2014), NuMA levels are dramatically increased at anaphase (Figure 5. 15), and, similarly to metaphase, at anaphase E-cadherin and Scribble co-localised to the cortical pole (**Figure 5. 18**). Again, depletion of Scribble using either siRNA or shRNA prevented cortical localisation of E-cadherin at anaphase (Figure 5. 18). Similarly, depletion of Scribble reduced cortical NuMA at anaphase (**Figure 5. 19**). These results suggest that cues from retraction fibres are transmitted through Scribble and E-cadherin to recruit NuMA to the cortical pole at the base of the fibre for spindle orientation.

To determine whether the action of Scribble was dependent upon E-cadherin, I tested HeLa cells, which lack E-cadherin expression (**Figure 5. 20**) (Lock and Stow, 2005). Similar to MCF10A cells, NuMA recruitment to the cortex at anaphase was also dependent upon Scribble expression in HeLa cells. The mitotic spindle of HeLa cells on plastic was consistently oriented along the substrate, with a mild loss of control in the absence of Scribble. These data show that Scribble does not require E-cadherin to mediate NuMA

recruitment to the cortex in HeLa cells. I suspect other cadherin might play a role with Scribble, but here the results highlight Scribble is a functionally conserved protein that used by multiple type of cells for controlling spindle positioning.

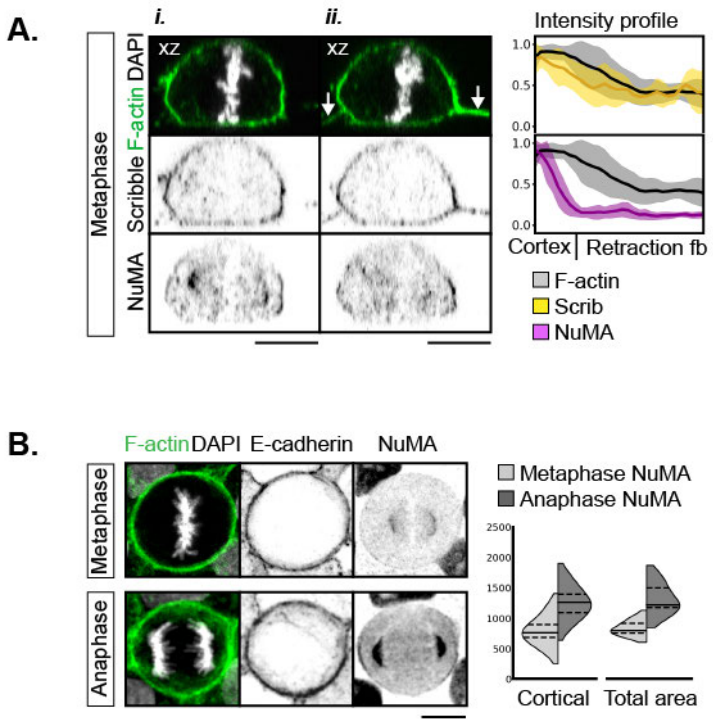


Figure 5. 15 Characterisation of NuMA expression in metaphase and anaphase

(A) To assess whether Scribble and NuMA are co-localized during cell division, sparsely plated MCF10A cells were stained with DAPI (DNA), phalloidin (F-actin) and antibody against Scribble and NuMA. In a representative metaphase cell, two XZ planes are shown. The intensity distributions of Scribble, NuMA and F-actin were examined from cortex to retraction fibre, and were shown in the line plots (n=7). **(B)** The NuMA intensity were analysed in metaphase (n=12) and anaphase (n=11) cells. Scale bar = 10 μm.

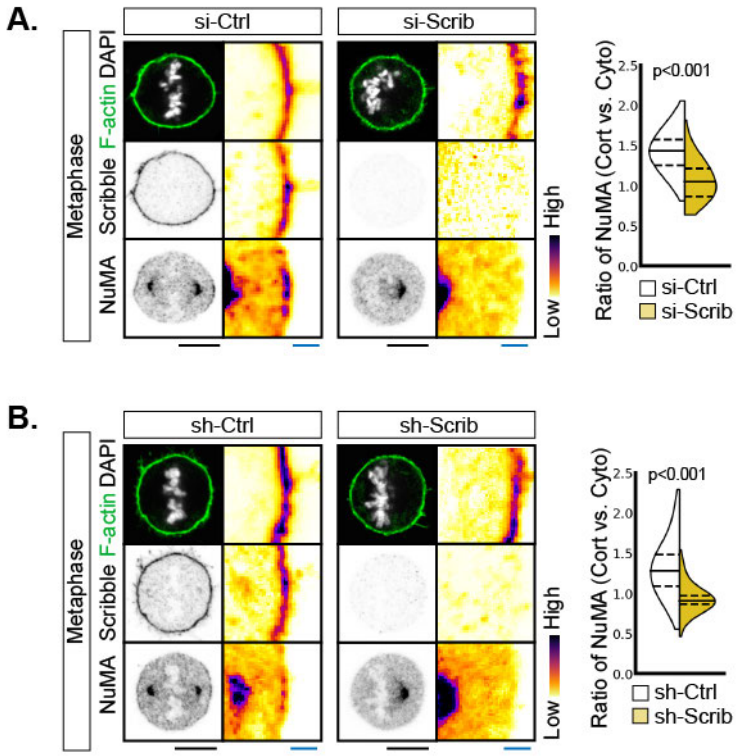


Figure 5. 16 Loss of cortical NuMA at metaphase upon Scribble depletion

MCF10A cells with (A) si-control (n=26), si-Scribble (n=23), sh-control (n=22) and sh-Scribble (n=27) were assessed for NuMA localisation during metaphase. The ratio of cortical to cytoplasmic NuMA was shown as density plots. Scale bar (Black) = 10 µm. Scale bar (Blue) = 2 µm.

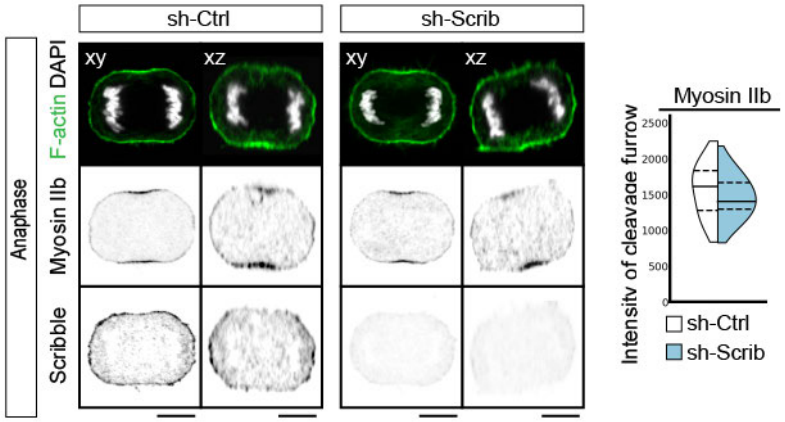


Figure 5. 17 Assessment of Myosin II localisation upon Scribble depletion

MCF10A cells with sh-control (n=6) and sh-Scribble (n=9) were assessed for Myosin IIb at contractile ring, with intensity analysis in density plots. Scale bar = 10 μm.

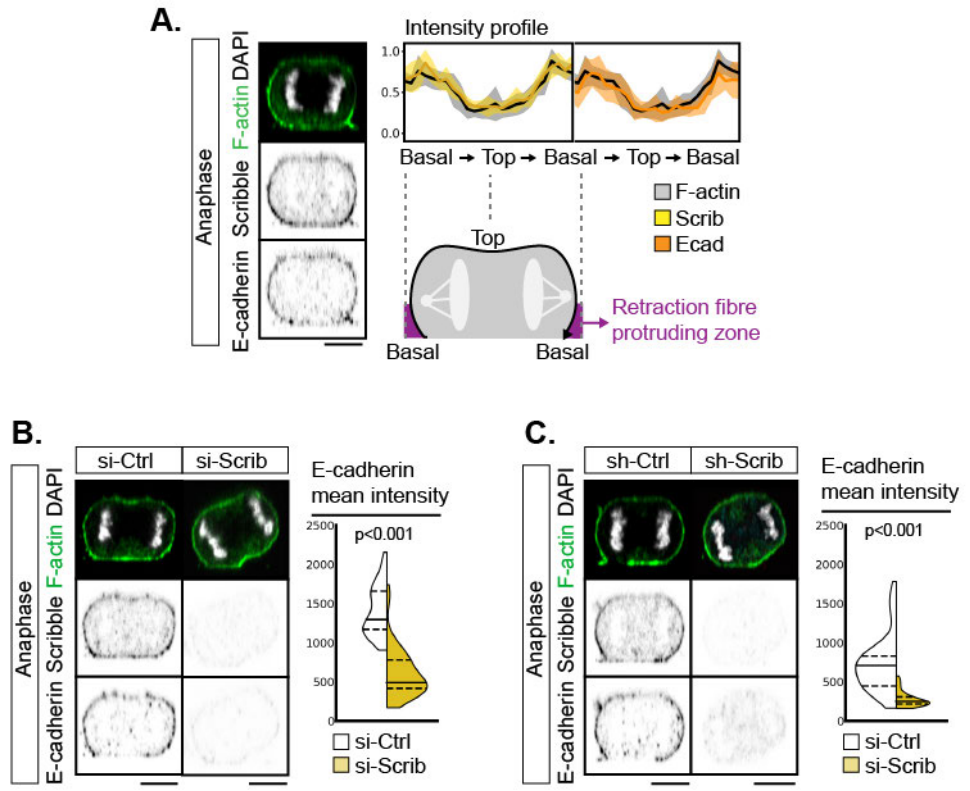


Figure 5. 18 Expression of E-cadherin in anaphase cortex is reduced by Scribble depletion

(A) Both Scribble and E-cadherin are enriched at the retraction fibre attachment region of the anaphase cells. Left, an XZ view of a representative anaphase cell; Right, line intensity profile of Scribble and E-cadherin (n=6). MCF10A cells with **(B)** si-control (n=13), si-Scribble (n=22), and with **(C)** sh-control (n=19) and sh-Scribble (n=15), were assessed for E-cadherin localisation to the anaphase cortex as shown in density plots. Scale bar = 10 μm.

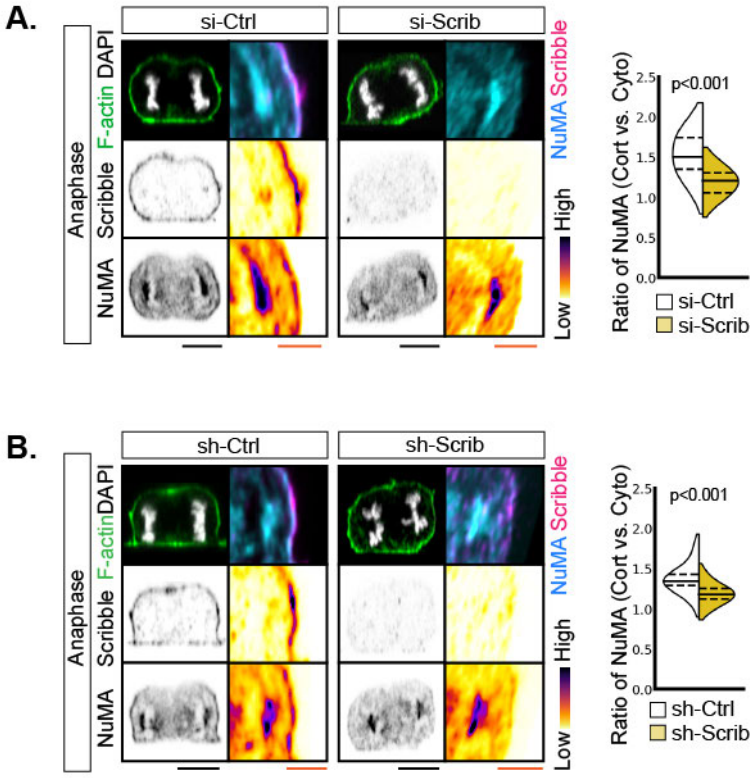


Figure 5. 19 Loss of cortical NuMA at anaphase upon Scribble depletion

MCF10A cells with (A) si-control (n=23), si-Scribble (n=27), and with (B) sh-control (n=18), sh-Scribble (n=18) were assessed for NuMA localisation during anaphase. The ratio of cortical to cytoplasmic NuMA was shown in density plots. Scale bar (Black) = 10 µm. Scale bar (Orange) = 5 µm.

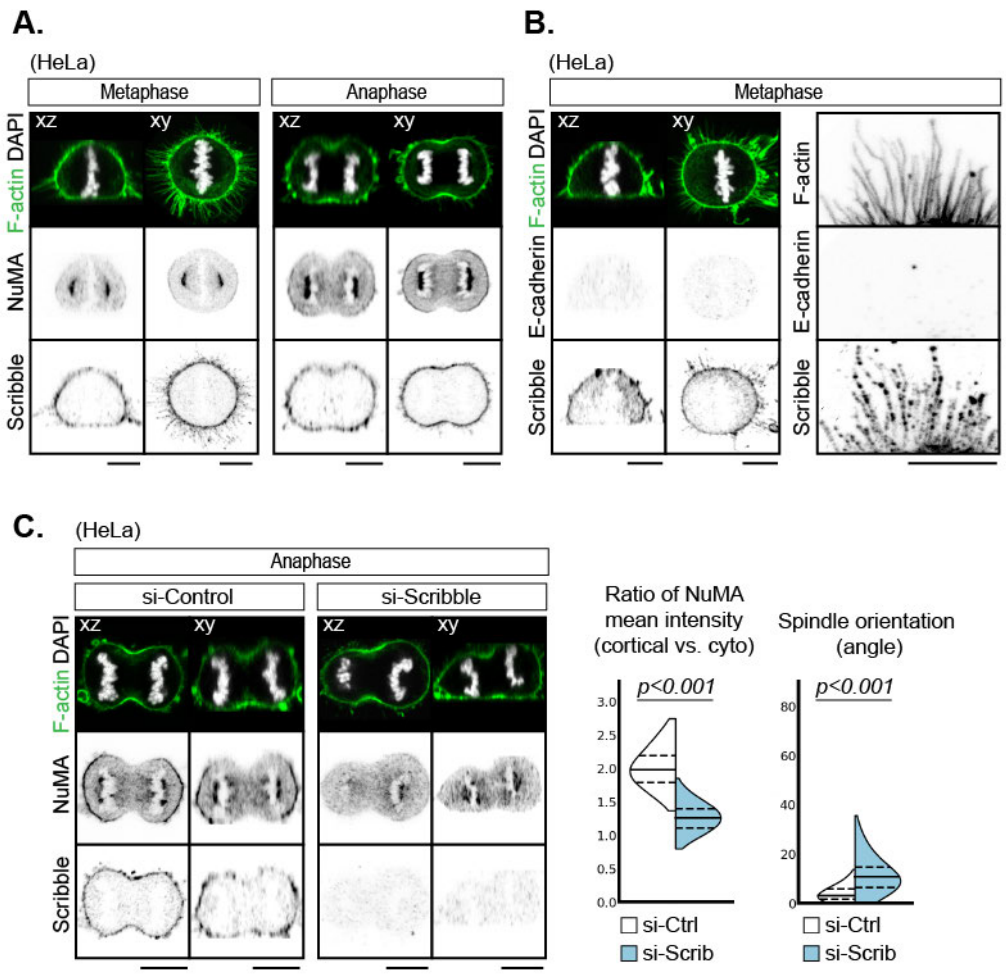


Figure 5. 20 Functional examination of Scribble depletion in HeLa cells

(A) Images show HeLa cells at metaphase and anaphase, labelled with NuMA, E-cadherin, phalloidin (F-actin) and DAPI (DNA). **(B)** Images show HeLa cells at metaphase, labelled with Scribble, E-cadherin, phalloidin (F-actin) and DAPI (DNA). The zoomed-in images show the region of retraction fibre. **(C)** HeLa cells with si-control (n=25) and si-Scribble (n=25) were assessed for the influence on NuMA cortical localisation and spindle orientation as shown in density plot.

5.2.4 Localisation of Dlg in mitosis requires Scribble

These findings above indicate that the presence of E-cadherin and Scribble in retraction fibres at metaphase leads to recruitment of NuMA to the cortical pole, and are reminiscent of previous findings that Dlg, a key functional partner of Scribble (Allam et al., 2018). Dlg can co-localise with Scribble at the spindle pole (Bell et al., 2015), where Dlg recruits the LGN complex to orient the mitotic spindle in several animal models and tissue types (Bergstralh et al., 2013; Johnston et al., 2009; Saadaoui et al., 2014). To assess this, I stained for Dlg in control and Scribble-depleted dividing cells (**Figure 5. 21**). Like Scribble, Dlg was cortical throughout mitosis and clearly present in retraction fibres. Depletion of Scribble clearly reduced cortical Dlg in mitosis. Importantly, Scribble depletion had negligible effect on both Dlg and E-cadherin level in unsynchromised (predominantly interphase) cells (**Figure 5. 22**).

I therefore propose that the E-cadherin and Scribble in retraction fibres establish a 'SEND' complex of Scribble, E-cadherin, NuMA and perhaps Dlg (**Figure 5. 23**). I believe Scribble and E-cadherin utilized the canonical mediators of spindle orientation (di Pietro et al., 2016a), which is dictated by dynein-mediated forces. Dynein-dynactin complex binds NuMA, generating force to pull on astral microtubules emanating from the spindle pole (Kotak, 2019; Thery et al., 2007). The position of the dynein-based motor complex is controlled by the position at the cortex of a complex termed the LGN complex (comprising Gαi, LGN, and NuMA in mammals (di Pietro et al., 2016a; Kiyomitsu, 2019)). Based upon extensive literature on the role of NuMA in LGN-mediated regulation of mitotic spindle orientation, and our findings of defective spindle orientation in metaphase, anaphase and telophase in the absence of Scribble, I propose that SEND, by coordinating pulling forces from the two cortical poles, coordinates stable alignment of the spindle parallel to the substrate.

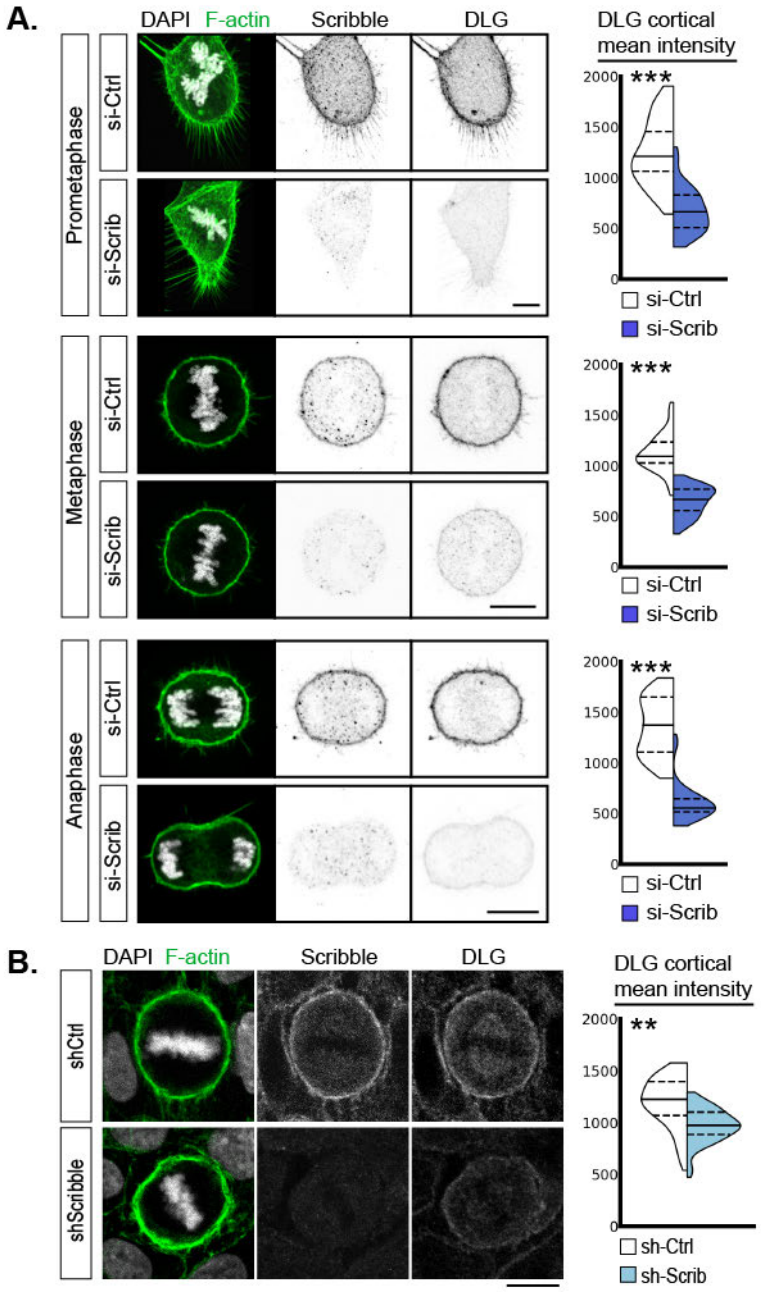


Figure 5. 21 Disruption of Dlg localisation in mitosis by Scribble depletion

(A), MCF10A cells with si-control and si-Scribble were assessed for Dlg localisation, and the impact of si-RNA on cortical intensity were shown in the density plot, with cell number [si-control, si-Scribble] for prometaphase is [19, 15], for metaphase is [16, 17], and for anaphase is [12, 15]. **(B),** MCF10A cells with sh-control (n=18) and sh-Scribble (n=21) were assessed for cortical DLG intensity in metaphase.

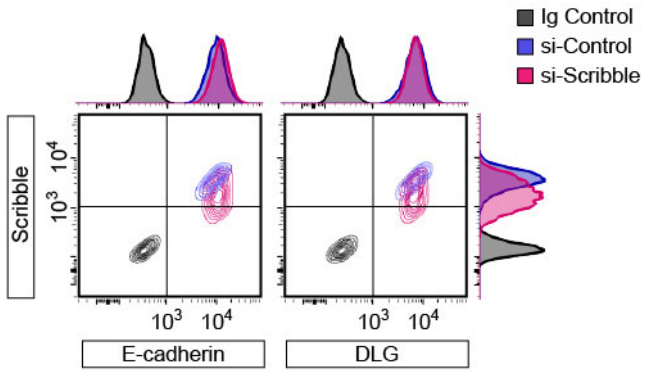


Figure 5. 22 Flow cytometry analysis of total E-cadherin and Dlg expression upon Scribble depletion

Protein level of Scribble, E-cadherin and Dlg of unsynchronised MCF10A cells were assessed by flow cytometry.

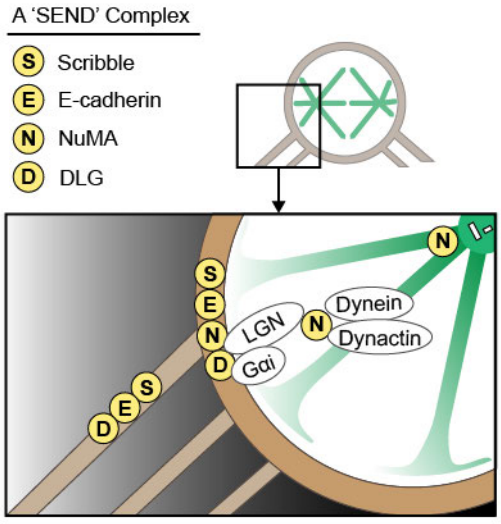


Figure 5. 23 A model illustrates 'SEND'

A schematic to suggest a cohort of proteins 'SEND' (Scribble, E-cadherin, NuMA, Dlg) at the mitotic cortex bridges the signals that passes through retraction fibre to the astral microtubule for spindle positioning.

5.3 Discussion

By exploring MCF10A cells without neighbours, I have identified a novel cell-autonomous mechanism of controlling spindle orientation and daughter cell positioning. Our experiments reveal a ligand-independent role for E-cadherin in transmitting signals from retraction fibres to NuMA of the LGN complex. In contrast to the situation during interphase, the stability and position of E-cadherin is profoundly dependent on Scribble during cell division.

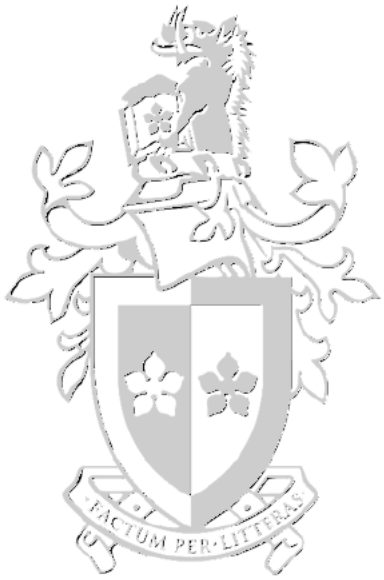
SENDing signals from retraction fibres to orient the mitotic spindle.

E-cadherin is well known to orient the mitotic spindle by transmitting signals from cell neighbours via adherens junctions (Gloerich et al., 2017; Hart et al., 2017). Similarly, integrins can orient the mitotic spindle by transmitting signals from the cell matrix via retraction fibres (Li and Burridge, 2019). In single MCF10A cells without artificial matrix, I was surprised to find E-cadherin, rather than integrin-β1, communicating from the retraction fibres. This finding adds to the growing appreciation that multiple receptors can input signals from the extracellular matrix to control mitotic spindle orientation, including β1-integrin in cells plated on fibronectin (Dix et al., 2018), and β5 integrin in U2OS cells that make their own extracellular matrix(Lock et al., 2018).

The role of Scribble in spindle orientation is well established (Bonello and Peifer, 2019; Godde et al., 2014; Nakajima et al., 2013; Qin et al., 2005; Wang et al., 2018). However, the complexity of the tissues in which this role has been investigated mean that it is still not clear what extrinsic or intrinsic cues are transmitted by Scribble (Godde et al., 2014; Nakajima et al., 2013). Our data suggests a possible effect of Scribble beyond mediating E-cadherin effects, since I observed a role for Scribble in spindle orientation in E-cadherin-deficient cells such as HeLa cells. One explanation for this comes from recent findings of an alternative means of aligning the spindle to the history of pre-mitotic cell shape involves a dynamic, oscillatory feedback between chromosomes and LGN along the short cell axis (Dimitracopoulos et al., 2020). It will be interesting to assess whether Scribble plays a role in this process. Here, I show that Scribble can directly link positional information from retraction fibres to spindle orientation control. In metaphase, this role is clearly via transmission of signals from E-cadherin.

5.4 Conclusion

I identify novel mechanisms of regulation of mitosis positioning in single MCF10A cells, which involve polarity protein, Scribble and Dlg, linking E-cadherin to NuMA. This newly defined protein cohort, SEND, can send signals via retraction fibre for sequential roles in spindle orientation and symmetric daughter positioning. Because the system is coating-free, the results suggest that cell-autonomous initiation of spindle positioning can be controlled by Scribble and E-cadherin.



Chapter 6

'SEAD' controls cytokinetic reshaping for daughter cell positioning

6.1 Introduction

In Chapter 5, I demonstrated that spindle positioning is mediated through a cohort of proteins 'SEND' (Scribble, E-cadherin, NuMA, and DLG). However, to maintain epithelial homeostasis, correct spindle positioning is not sufficient to readapt dividing cell back to the epithelia. On the other hand, there might be some unknown mechanism to correct the error of spindle positioning (Lough et al., 2019). This correcting force might be seen in Chapter 5 (Figure 5.14), as the mis-oriented sh-LGN cells displayed less error in daughter cell positioning than the cases in Scribble or E-cad depletion. This results suggest multiple mechanism are orchestrated to correct spindle orientation, in part by which might involve Scribble and E-cad activity. Thus, I would like to test whether Scribble and E-cadherin play a second role beyond spindle positioning for daughter cell positioning.

Membrane remodelling provides a second critical step to ensuring that daughter cells are appropriately positioned following cell division (Gibson et al., 2006), providing a potential means by which daughter cell positioning could be influenced by a Scribble-Ecad complex. In support of this, Chapter 5 (Figure 5.1) showed E-cad displayed a pattern of enrichment at the basal adhesion that could support a role in cytokinetic reshaping. The mechanisms of cytokinetic reshaping by which daughter cells reclaim the space vacated by their parent involve a complex interplay involving adhesion complexes, tension and geometry (Pinheiro and Bellaiche, 2018). In epithelial monolayers, this process is complicated by the need to maintain epithelial barrier function, which necessitates continual interaction with neighbouring cells (Guillot and Lecuit, 2013). As with spindle orientation, membrane remodelling involves a dynamic relationship between the dividing cell and its neighbours, with physical forces and signalling adhesion proteins such as E-cadherin (Bui et al., 2016; Guillot and Lecuit, 2013), and cell-competition mediator Scribble (Maruyama and Fujita, 2022; Ogawa et al., 2021). New filopodia are formed to facilitate cell spreading and adhesion, and these filopodia are thought to be guided by the retraction fibres that recorded previous positioning of the parent cell (Cramer and Mitchison, 1995; Li and Burridge, 2019). A key focus of research has been on tricellular junctions, which are formed after cytokinesis on either side of the midbody, and can determine whether the daughter cells maintain contact after cell division, or whether these contacts are displaced by neighbouring cells (Higashi and Miller, 2017). The mechanisms of membrane remodelling in the absence of neighbouring cells has been less well studied.

Here, I continued using MCF10A cells dividing on plastic to explore membrane remodelling and daughter cell positioning. I found that E-cadherin and Scribble subsequently relocate to the nascent junction between the two daughter cells, and orchestrate a spreading of that junction to facilitate enduring connection between the daughters. The findings described below show that Scribble and E-cad traffic dynamically between key regions of the cell during cell division, coordinating the E-cadherin-mediated effects on the spindle, and extension of filopodia and the nascent daughter-daughter junction. Importantly, as the force connecting two daughters are primed from daughter cell themselves, the mechanisms proposed here are cell-autonomous.

6.2 Results

6.2.1 Scribble relocates to the daughter-daughter contact to control nascent junction formation

In Chapter 5, the aberrant spindle orientation observed in dividing cells with compromised Scribble or E-cadherin provides one possible explanation for the failure of the second daughter to adhere and spread, but I would like to examine a second possible explanation in this chapter:

In staining the dividing cells I observed that in late telophase, Scribble relocated from the cortical pole to the region between the two nascent daughters. Scribble was not evident at the basal adhesion region between the two daughters where both F-actin and paxillin were enriched, but was strongly expressed at the daughter-daughter contact site above the intracellular bridge (Fededa and Gerlich, 2012; Rathbun et al., 2020) (**Figure 6. 1**). I compared the localisation of Scribble, F-actin and Myosin IIb along regions of furrow ingression and daughter-daughter contact sites at progressive telophase stages (**Figure 6.** 2). This suggested that, although all three proteins were broadly recruited to this region throughout cytokinesis, Scribble was co-enriched with F-Actin at the daughter-daughter contact rather than with Myosin IIb. However, activity of both was required, as treatment with Cytochalasin D and Blebbistatin abrogated recruitment of Scribble to the daughter-daughter interface (**Figure 6. 3**).

I assessed whether the presence of Scribble at the late telophase daughter-daughter contact site might reflect a role in establishing stable connections between the daughters. I found no difference in the intensity of F-actin at the contact site when cells were depleted of Scribble (**Figure 6. 4**). However, Arp2/3, a key mediator of actin branching that enables daughter-daughter adhesions in the context of Drosophila dorsal thorax epithelial cells (Herszterg et al., 2013; Papalazarou and Machesky, 2021) was reduced at the daughter-daughter contact in Scribble-depleted cells (**Figure 6. 5**). This reduction in Arp2/3 was seen in both the F-actin-rich and Myosin IIb-rich regions of the daughter-daughter interface (Figure 6. 5). To confirm the colocalisation, I applied Proximity Ligation Assay to assess the interaction between Scribble and Arp2 (**Figure 6. 6**). The extensive PLA foci found with Scribble and Arp2, but not a control to test the interaction between Scribble and Talin, suggests Scribble works with Arp2. Taken together, these data suggest that Scribble is not

required for recruitment of F-actin to the daughter-daughter interface, but might facilitate the actin branching mediated by Arp2/3, perhaps to stabilize or extend this interface.

A similar role for pushing of Arp2/3-based protrusions for E-cadherin adhesion was recently identified in interphase MDCK cells (Li et al., 2020; Papalazarou and Machesky, 2021), in which showing Arp3 depletion internalised apical junction. Here, I would like to test whether loss of Arp2/3 activity can influence stability of Scribble-mediated adhesion. Thus, I treated MCF10A cells with Arp2/3-specific inhibitor, CK-869. The results indicated that CK-869 reduced both the overall cortical recruitment of Scribble and the proportion of cortical Scribble that was concentrated at the daughter-daughter interface (**Figure 6. 7**). These data, together with Figure 6. 6, indicate that Arp2/3 and Scribble are recruited to the daughter-daughter interface at telophase in a co-dependent manner.

Similarly, Dlg was recruited to the daughter-daughter contact site in late telophase cells (**Figure 6. 8**). Interestingly, the Dlg recruitment to this site around midbody was not dependent upon Scribble expression, even though the cortical recruitment of Scribble was again impacted. These data suggest that Scribble is not dominant for recruitment of F-actin or Dlg to the daughter-daughter interface, but might facilitate the actin branching mediated by Arp2/3, perhaps to stabilize or extend this interface. Here, my results suggest Scribble coordinates an F-actin-mediated event of membrane reconstruction to form nascent daughter-daughter contacts.

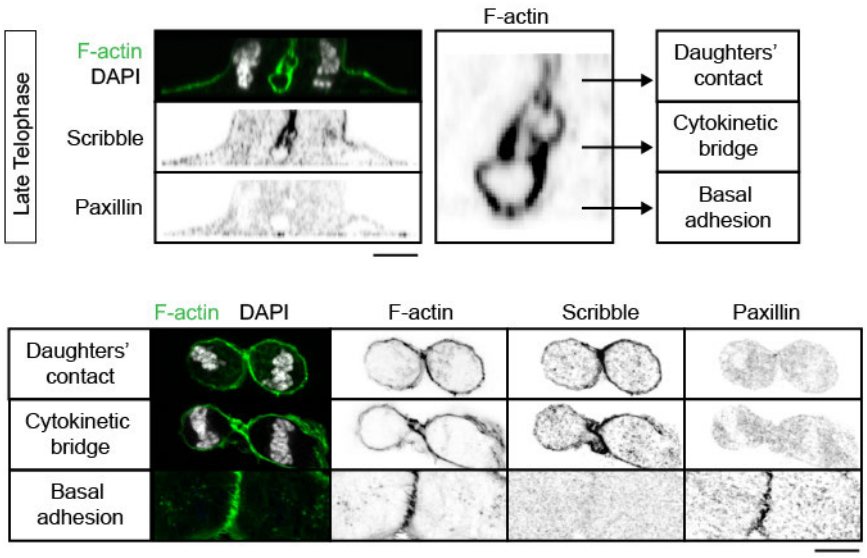


Figure 6. 1 Localisation of Scribble and Paxillin in late telophase

To assess the localisation of Scribble at late telophase, sparsely plated MCF10A cells were stained with DAPI (DNA), phalloidin (F-actin) and antibody against Scribble and Paxillin. The representative image (top) shows an xz view sectioned through a representative cell centre. In the F-actin channel, the regions of each subcellular structure (ie. 'daughters' contact', 'cytokinetic bridge', and 'basal adhesion') were defined. For each region, the images stained with DAPI (DNA), phalloidin (F-actin), Scribble and Paxillin were displayed respectively (bottom). Scale bar = 10μm.

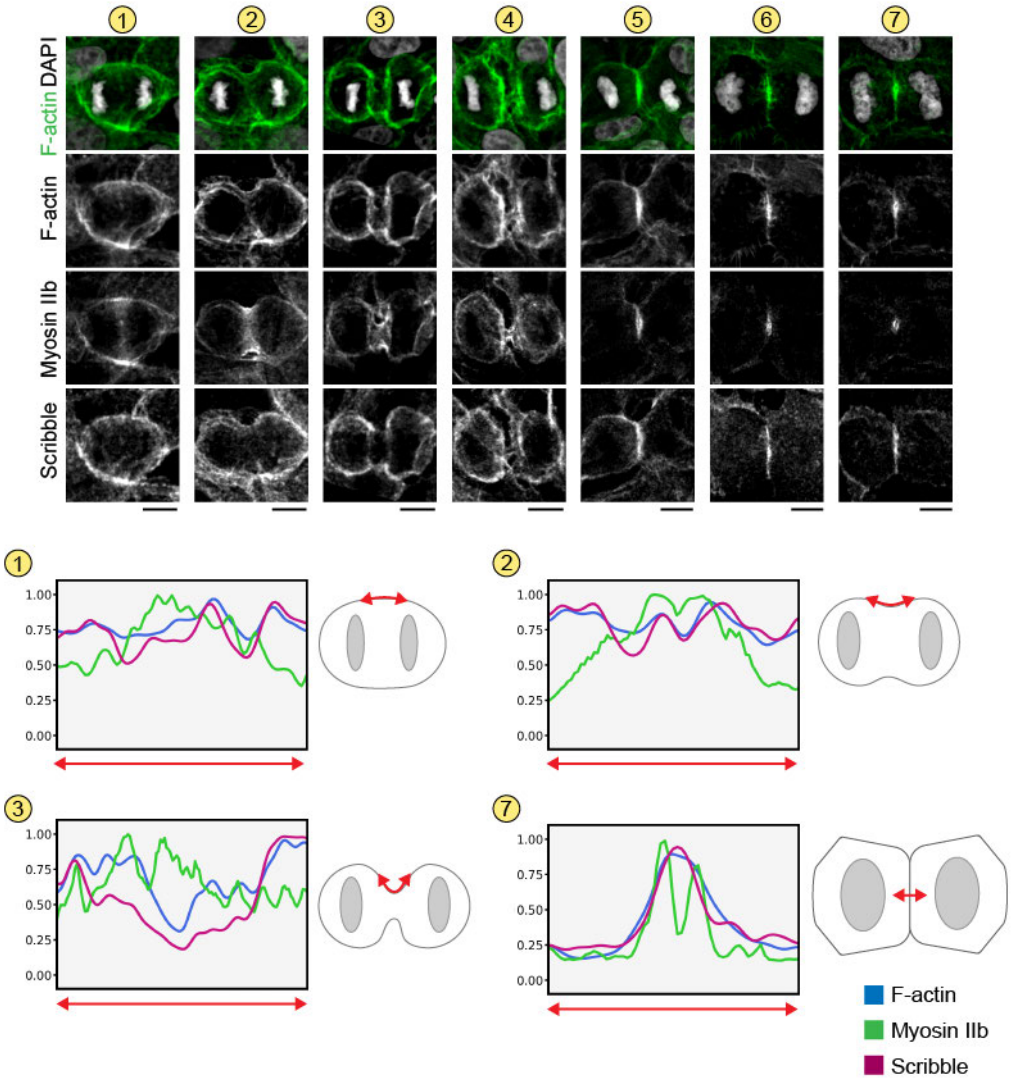


Figure 6. 2 Assessment of Scribble co-localisation with F-actin and Myosin IIb from anaphase to interphase

Characterisation of cortical distribution of Scribble and actomyosin during cell division. Confocal images (1~7) shows representative cells at different stages from anaphase onset. Proteins distribution of Scribble, Myosin IIb, and F-actin (phalloidin) at stage (1, 2, 3, 7) were assessed at the region of interests as the red line marked on the cartoon cells below. Scale bar = 10μm.

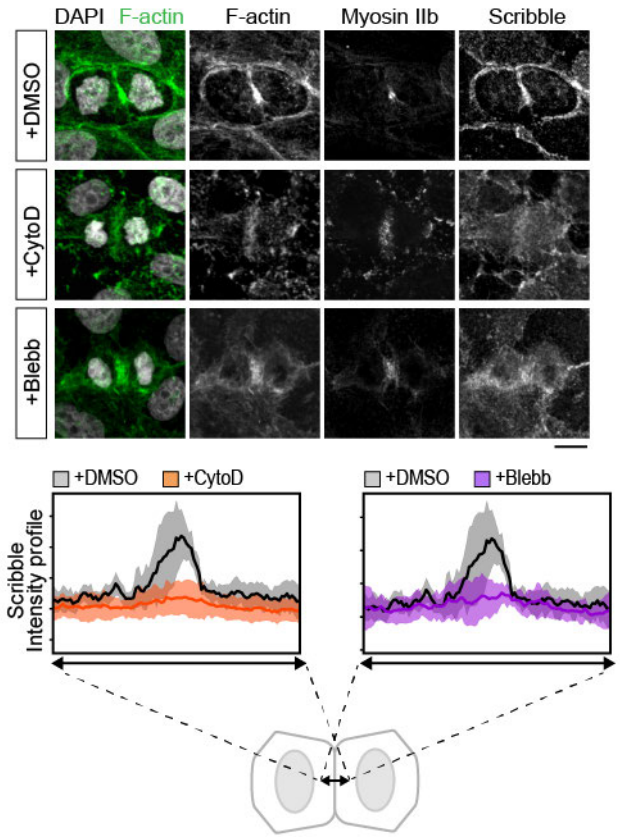


Figure 6. 3 Scribble enrichment at daughter-daughter contact depends on the stability of actomyosin

Treatments of Cytochalasin D and Blebbistatin internalise Scribble from cortex to cytoplasm. MCF10A cells were treated with drugs (DMSO, Cytochalasin D and Blebbistatin, respectively) for 15 minutes, fixed and labelled with Myosin IIb, Scribble, F-actin (phalloidin) and DAPI (DNA). The mean intensity of Scribble of the line crossed through the interface of the daughters were assessed. Cell numbers: Cytochalasin D (n=16), Blebbistatin (n=13), DMSO (n=5). Scale bar = 10μm.

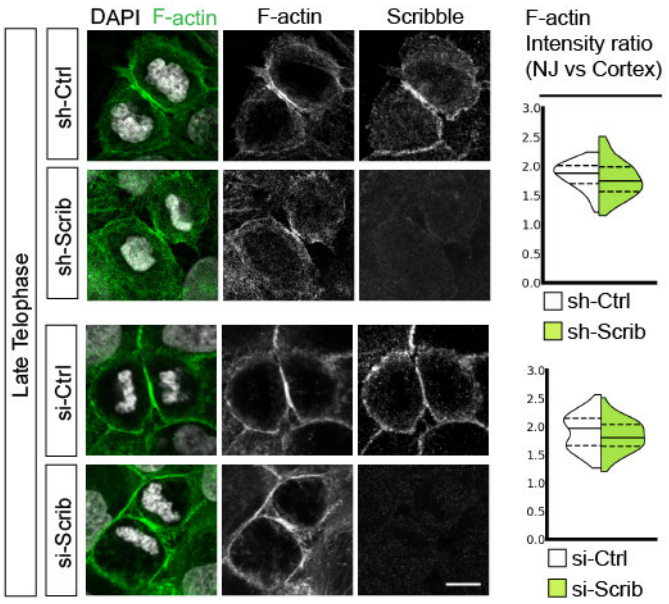


Figure 6. 4 F-actin enrichment at daughter-daughter contact is stable upon Scribble depletion

MCF10A cells with sh-control (n=25), sh-Scribble (n=24), si-control (n=25), and si-Scribble (n=29) were assessed for the ratio of F-actin intensity between nascent junction (NJ) versus the cortex. Scale bar = 10μm.

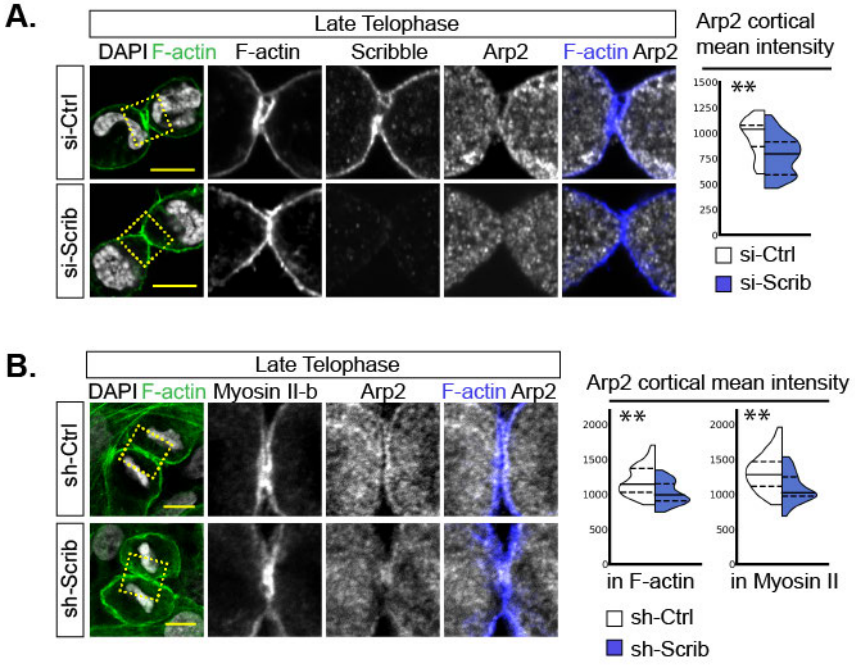


Figure 6. 5 Scribble is required for Arp2/3 localisation at daughter-daughter interface

MCF10A cells with (A) si-control (n=13), si-Scribble (n=19) and (B) sh-control (n=22), sh-Scribble (n=21) were assessed for Arp2 intensity at the cortical region of daughters' contact, as shown in density plots. Scale bar = 10μm.

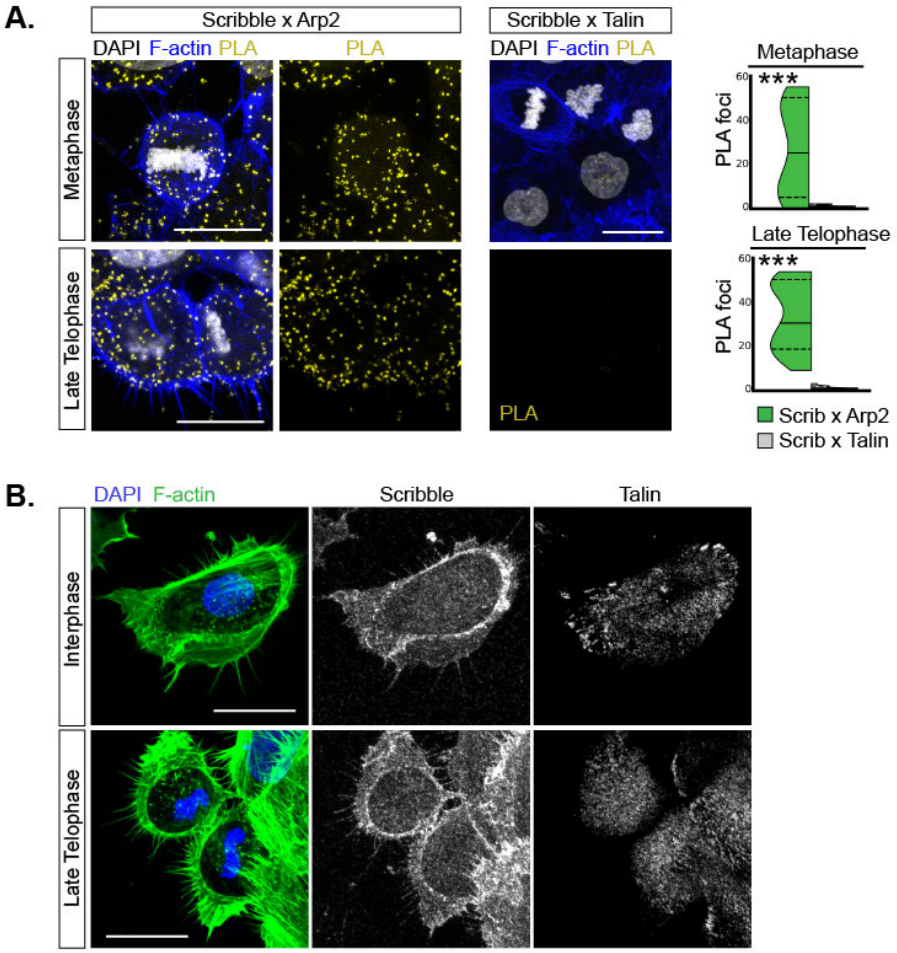


Figure 6. 6 Proximity ligation assay tests Scribble interaction with Arp2 or Talin

(A) Proximity ligation assay was applied to determine spatial proximity between Scribble and Arp2 at metaphase (n=16) and late telophase (n=21), and between Scribble and Talin at metaphase (n=23) and late telophase (n=23). The number of PLA foci was estimated as shown in density plot, with maximum number was counted as 50 if the PLA foci is greater than 50. **(B)** Scribble and Talin were co-stained in MCF10A cells. Scale bar = 20μm.

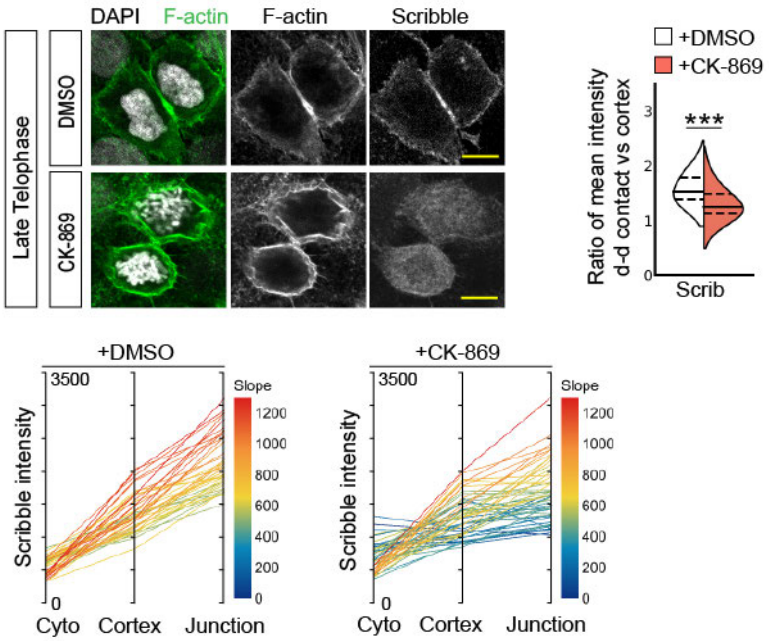


Figure 6. 7 Scribble subcellular localisation is influenced by CK-869 treatment

To investigate the role of Arp2/3, MCF10A cells were treated with Arp2/3 inhibitor CK-869 (50μM, n=26) or DMSO (n=28) for 15 minutes, and stained with DAPI (DNA), phalloidin (F-actin) and antibody against Scribble. A representative image is shown (top-left), and the mean intensity of Scribble at subcellular localisation, including cytoplasm, cortex, and daughters' contact was quantified. For the Scribble intensity analysis in (bottom), the lines connect data points from individual cells, with the colour spectrum highlighting the slope of each line, indexed by red to high slope and blue to low slope. The ratio of Scribble mean intensity at daughters-daughter contact versus cortex were further shown in density plots overlaid with box plots. Scale bar = 10μm.

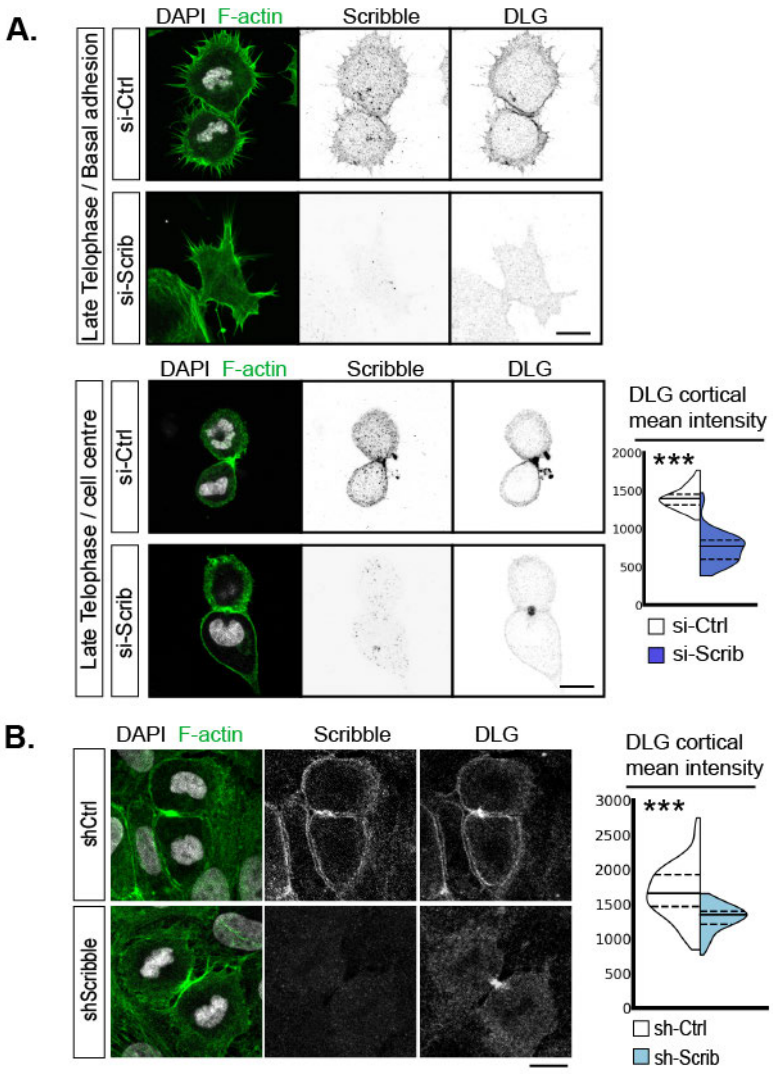


Figure 6. 8 Alteration of DLG localisation at late telophase upon Scribble depletion

MCF10A cells with **(A)** si-control (n=20), si-Scribble (n=21), and **(B)** sh-control (n=24), sh-Scribble (n=20) were assessed for DLG localisation, and the impact of si-RNA on cortical intensity of DLG were shown in the density plot. Scale bar = 10μm.

6.2.2 The nascent daughter-daughter interface impacts upon subsequent positioning of the daughters.

I hypothesized that this recruitment to the interface might influence subsequent daughter-daughter connections. To assess this, I quantified the extent ('width') of the interface, and the shortest distance between the DNA (a surrogate for cell positioning) of each daughter, after Arp2/3 inhibition or Scribble depletion. Inhibition of Arp2/3 and depletion of Scribble dramatically reduced the extent of daughter-daughter contact, but had little or no impact on the cell positioning during telophase (**Figure 6. 9**). These data indicate that actin and myosin-dependent positioning of Arp2/3 and Scribble at the interface between two daughters at telophase enables a stabilization or lengthening of the nascent daughter-daughter contact region.

To assess whether this enrichment of Scribble/Arp2/3 complex at nascent daughter-daughter contact region dictates subsequent positioning of each daughter, I first assessed whether, as with E-cadherin inhibition (Chapter 5), impeding this contact might influence the re-adherence to the substrate of one or both daughters after cell division. Apr2/3 inhibition did not significantly influence the time taken for either daughter to re-adhere (**Figure 6. 10**), but over 30 minutes, the distance between daughter cells diverged, with the distance significantly increased with Arp2/3 inhibition (Figure 6. 10). This finding supports the notion that Arp2/3-mediated expansion of the daughter-daughter adhesion zone might retain the daughters in proximity to each other. As described in Chapter 5, depletion of Scribble led to a slight increase in the time taken for daughter 1 to re-adhere to the substrate, but a dramatic increase for daughter 2. Indeed, some of the second daughters failed to re-adhere before they were lost to the tracking, perhaps because the spindle mis-orientation reduced association with the substrate. These difference between Scribble depletion and Arp2/3 inhibition might be attributed to different interacting proteins between Scribble and Arp2/3. On the other hand, Arp2/3 was prove to facilitate spindle assembly (Plessner et al., 2019), therefore, its role in spindle orientation is impossible to be tested as a regular dose of CK-869 treatment would destroy mitotic spindle. Furthermore, the distance between daughter cells in Scribble-depleted cells could only be quantified for those that adhered within 30 min. However, the lack of effect of Scribble on daughter cell distance should be treated with caution given the issues with delayed re-adherence in Scribble-depleted cells. Overall, these data indicate that the timing and or position of daughter cell re-adhesion after cytokinesis is influenced by both Scribble and Arp2/3 activity.

I further explored the nature of the nascent daughter-daughter junction, and identified intercalated filopodia that were rich in the membrane-cytoskeletal linker, Ezrin, Scribble, and F-actin (**Figure 6. 11**). Ezrin at the interface was dependent upon Arp2/3 activity (Figure 6. 11), similar to the dependence of Scribble on Arp2/3 activity. Together, these experiments suggest a model in which Scribble, Ezrin and Arp2/3 are recruited to interdigitations at the nascent junction, where they enable expansion of the nascent daughter-daughter contact and positioning of one daughter relative to the other.

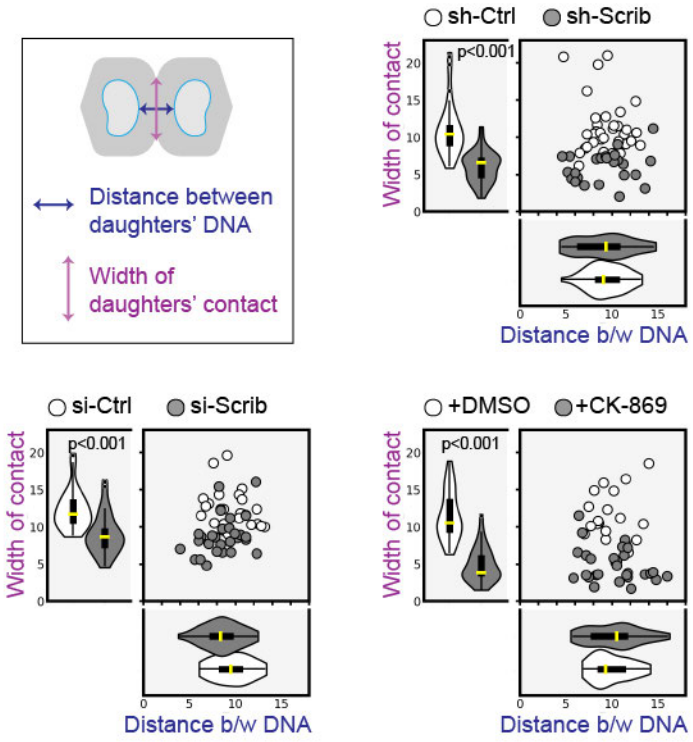


Figure 6. 9 Daughters' coherence depends on activity of Scribble and Arp2/3

Scribble-depleted MCF10A cells were assessed for the width of daughter-daughter contact and the distance between daughter nuclei as the method illustrated, and were shown in the scatter plot and density plots overlaid with box plots. Cell number for sh-Control is 35, for sh-Scribble is 24, for si-Control is 25, and for si-Scribble is 30.MCF10A cells treated with Arp2/3 inhibitor CK-869 (n=29) and DMSO (n=19) were assessed as same as Scribble-depleted MCF10A.

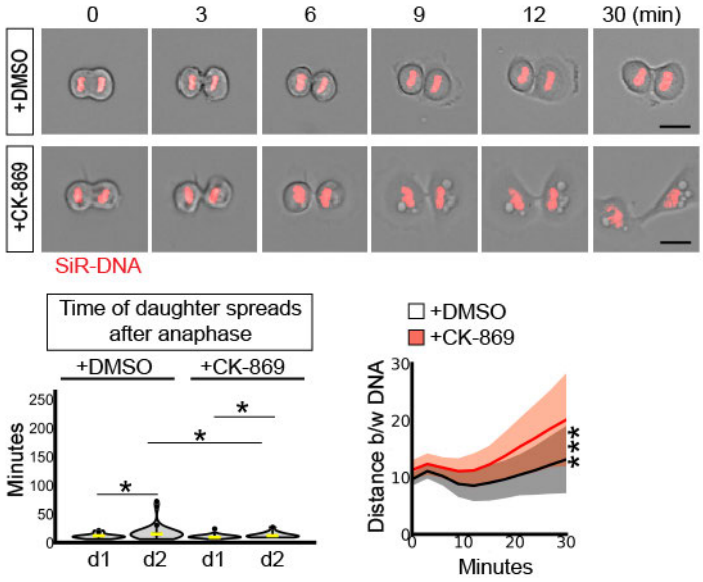


Figure 6. 10 Time-lapse imaging of cell division upon CK-869 treatment

To assess the role of Arp2/3 in daughter cell positioning, MCF10A cells were labelled with SiR-DNA (shown in red), treated with DMSO control (n=29) or inhibitor CK-869 (0.1 µM, n=32), and imaged by time lapse microscopy following division. The time for each daughter cell to re-adhere to the plastic and spread was recorded as d1 (first daughter to attach) and d2 (second daughter to attach) and shown as density plots overlaid with box plots. The distance between daughters' nucleus was measured from the anaphase onset (time = 0 minute) for 30 minutes, and was shown in the line plot, with shadow represented standard deviation. Scale bar = 10µm.

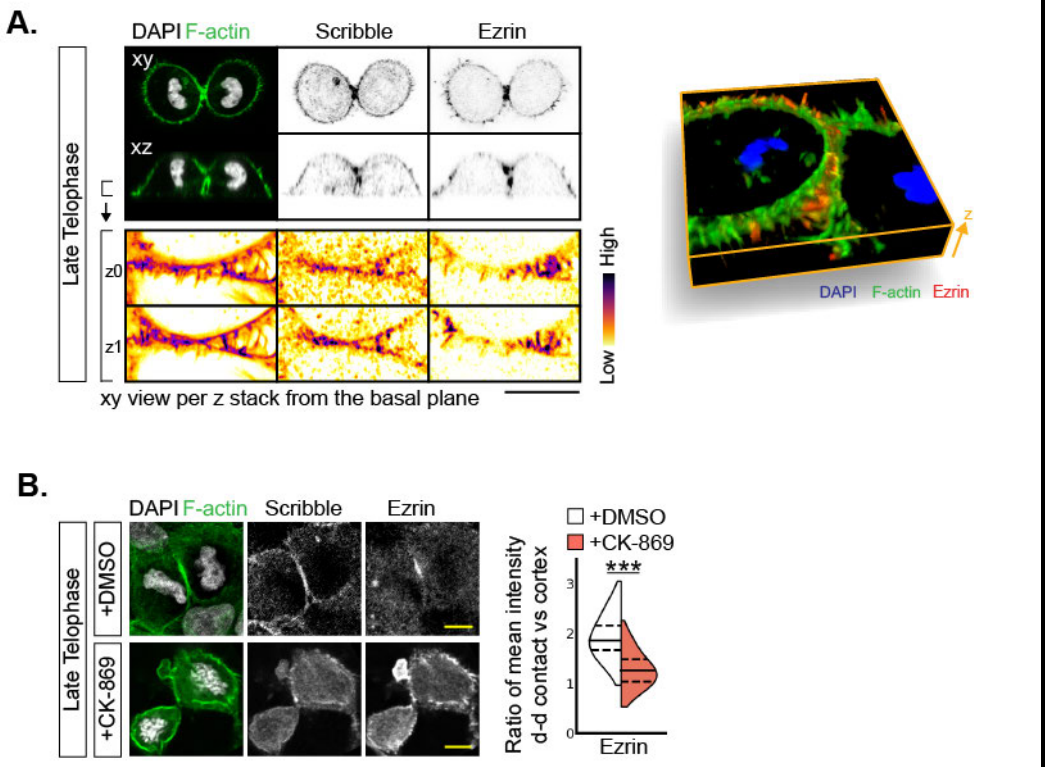


Figure 6. 11 Ezrin subcellular localisation at late telophase is influenced by CK-869 treatment

(A) The protein localisation of Scribble and Ezrin at late telophase was assessed in a sparsely plated MCF10A cell stained with DAPI (DNA), phalloidin (F-actin) and antibody against Scribble and Ezrin. A representative image is shown as sequential Z stacks, and in a 3D reconstructed view. (B) MCF10A cells treated with DMSO (n=22) or CK-869 (n=25) were assessed for Ezrin localisation, and the ratio of daughters-daughter contact versus cortex were shown in density plots. Scale bar = 10μm.

6.2.3 The composition of filopodia evolves throughout cell division, and Scribble influences nascent post-mitotic filopodia

A role of retraction fibres is thought to be the marking of cell position, such that nascent filopodia can reclaim the tracks of past filopodia (Cramer and Mitchison, 1995; Li and Burridge, 2019). To assess any molecular and functional relationship between retraction fibres and post-mitotic filopodia in our system, I first further characterised the filopodia at different stages of cell division. The extensive protrusions observed in prometaphase gradually disappeared, with few observed in anaphase (**Figure 6. 12**). I first explored Myosin X, an unconventional myosin that couples actin-dependent forces from retraction fibres to centrosomes (Kwon et al., 2015), and is recruited to nascent focal adhesions in some contexts (Gallop, 2020; He et al., 2017). Zooming in on individual filopodia indicated that Myosin X was present in discrete puncta along the length of the single MCF10A retraction fibres of prometaphase cells, and these puncta overlapped with Scribble puncta (**Figure 6. 13**). In contrast, Myosin X was predominantly present in at the tips of filopodia during telophase and late telophase, compatible with these representing nascent adhesions. Scribble localisation did not overlap with that of Myosin X in these nascent filopodia. In contrast to Myosin X, the membrane-cytoskeletal adaptor, Ezrin was excluded from the cortex, and highly expressed at the base of prometaphase retraction fibres, and the mid-section of nascent filopodia (Figure 6. 13). The change in composition between the pre-anaphase retraction fibres and the post-anaphase nascent filopodia is described in (Figure 6. 13).

If the components of the retraction fibres are instrumental in production of nascent filopodia, one would expect a functional effect beyond that predicted by the spindle misorientation defects described above. I therefore assessed the number of nascent filopodia in post-mitotic MCF10A cells depleted of Scribble, focussing specifically on the cell divisions where both daughters had re-attached. It has long been appreciated that post-mitotic adhesion is dependent upon filopodia (Cramer and Mitchison, 1993), so I assessed the number of filopodia separately for the daughter that adhered first ('D1') and the daughter that adhered second ('D2'. I examined the cells immediately upon flattening onto the substrate (**Figure 6. 15**). Indeed, both Scribble-depleted daughters exhibited a significant reduction in the number of filopodia at late telophase. A similar reduction in filopodia from both daughters was observed after treatment with the Arp2/3 inhibitor (Figure 6. 15), confirming the importance of Arp2/3 in filopodia remodelling. Interestingly, inhibition of E-cadherin led to reduced nascent filopodia in the second daughter (compatible with

reduced spreading due to mis-oriented spindle) but had a less convincing effect on the number of nascent filopodia in the first daughter. Together, these data suggest the possibility that Scribble, and perhaps E-cadherin, regulate the transition from retraction fibre to nascent filopodia, providing opportunities to reposition the daughter cells according to the position of the cell prior to cytokinesis.

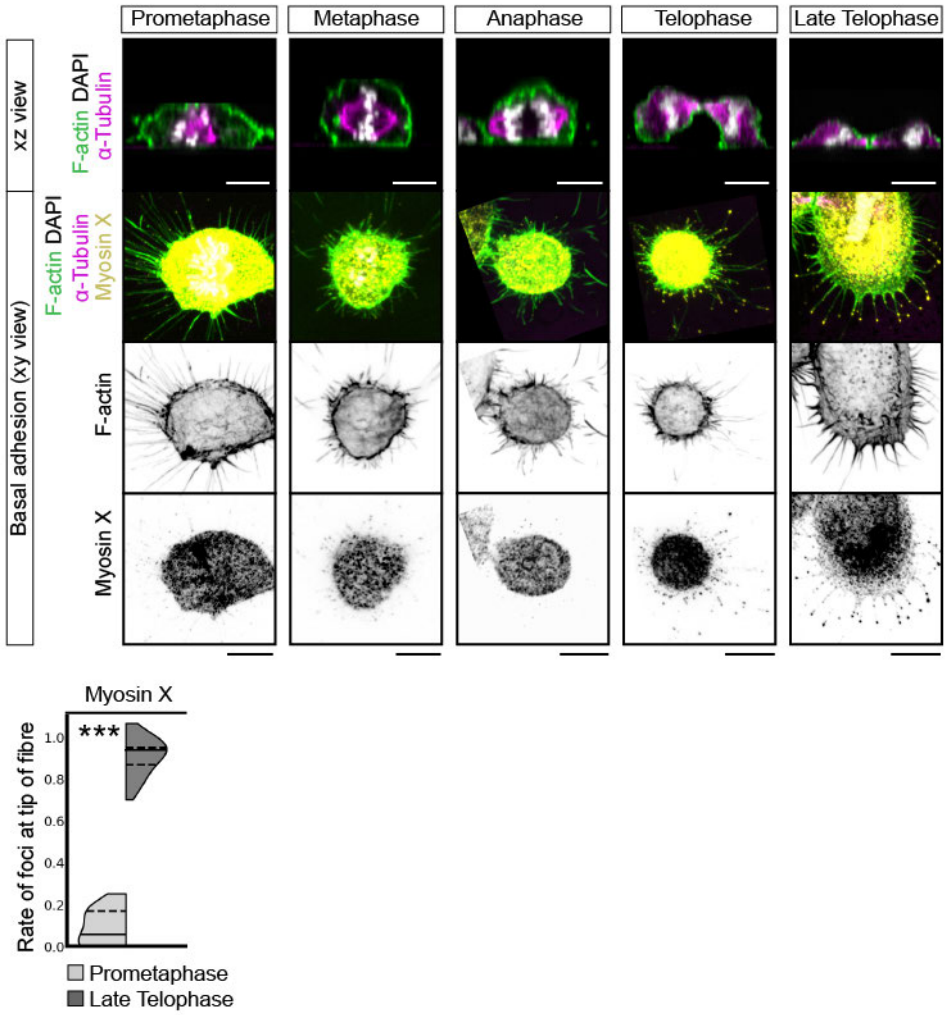


Figure 6. 12 Myosin X localisation during mitosis

MCF10A cells at different stages of mitosis were assessed for Scribble and Myosin X expression. The rate of Myosin X foci concentrated at the tip of fibre was examined as shown in density plot. 0 represents a cell has no fibre that Myosin is concentrated at the tip of fibre; 1 represents a cell has Myosin foci at the tip of every fibre. Scale bar = 10μm.

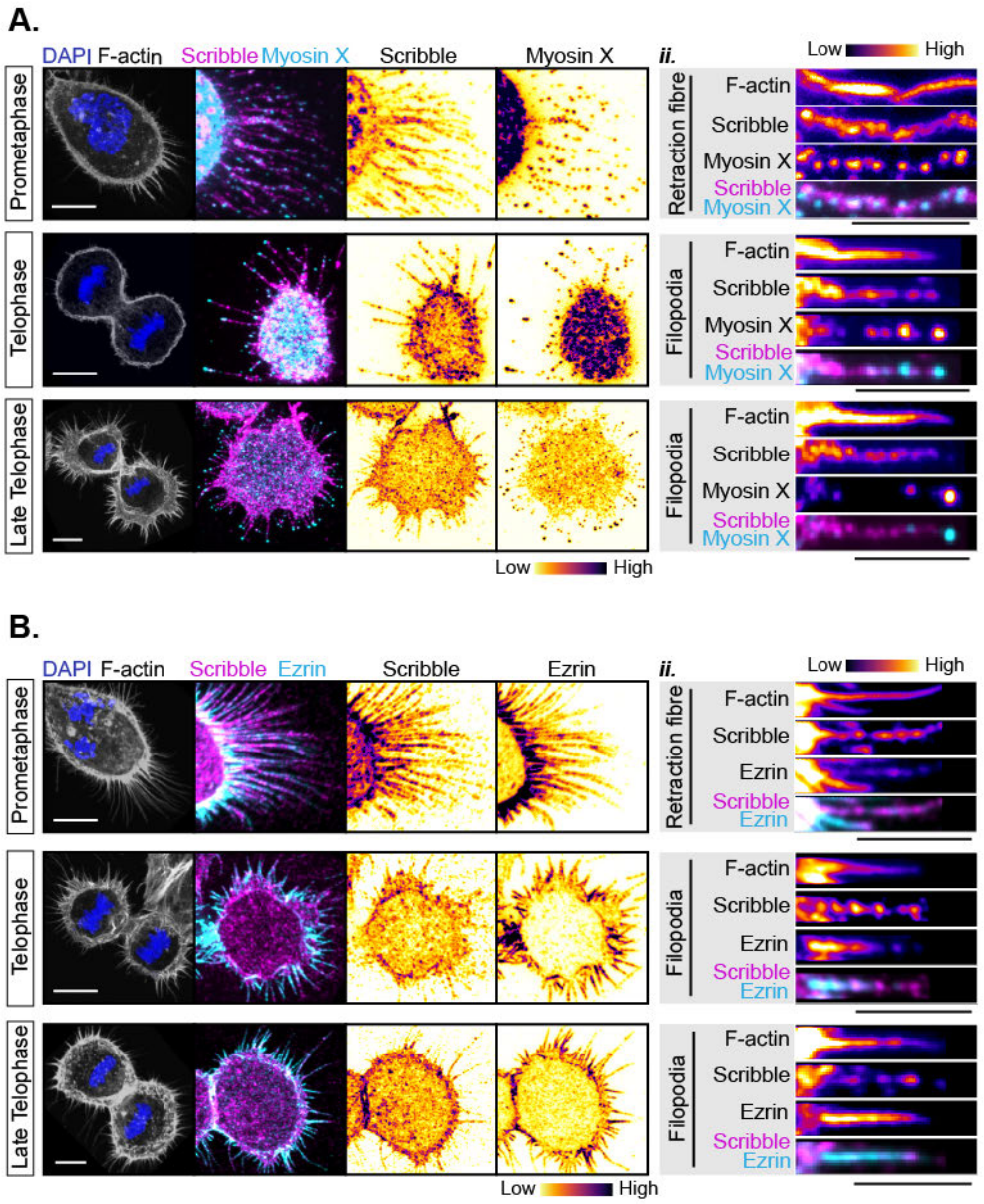


Figure 6. 13 Characterisation of components in the protruded fibre during mitosis

(A), The localisation of Scribble and Myosin X was assessed at prometaphase, telophase and late telophase (Left), with DAPI (DNA), phalloidin (F-actin) co-labelling. The fire coloured images (Right) were the regions of retraction fibre or nascent filopodia cropped from the left images. **(B),** Protein localisation of Scribble and Ezrin at prometaphase, telophase and late telophase (Left), with DAPI (DNA), phalloidin (F-actin) co-labelling. The fire coloured images (Right) were the

regions of retraction fibre or nascent filopodia cropped from the left images. Scale bar (white) = 10 μm; Scale bar (black) = 5 μm.

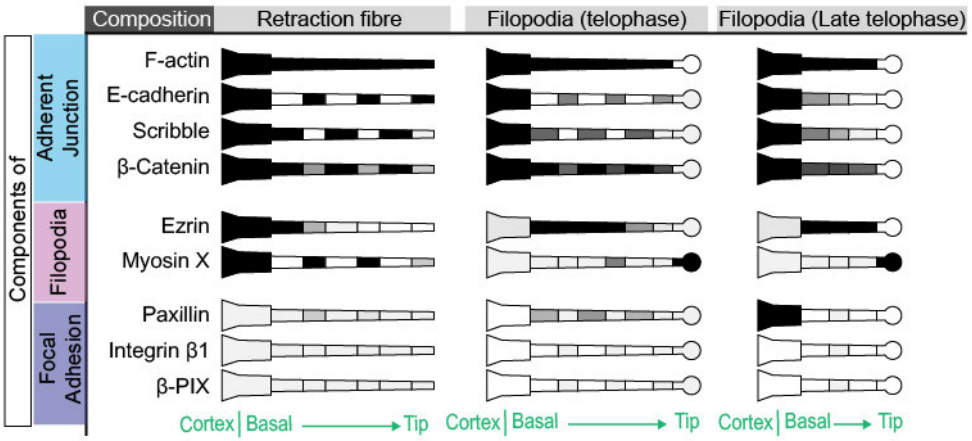


Figure 6. 14 Schematic summary of compositions of mitotic fibres

A schematic to summarise the protein localisation of F-actin, E-cadherin, Scribble, β-catenin, Ezrin, Myosin X, Paxillin, Integrin β1, and β-PIX at retraction fibre and filopodia. The colour of black represents high protein intensity, the white represents the protein intensity was nearly undetectable, and the grey represents the protein intensity is in between the black (high) and the white (none).

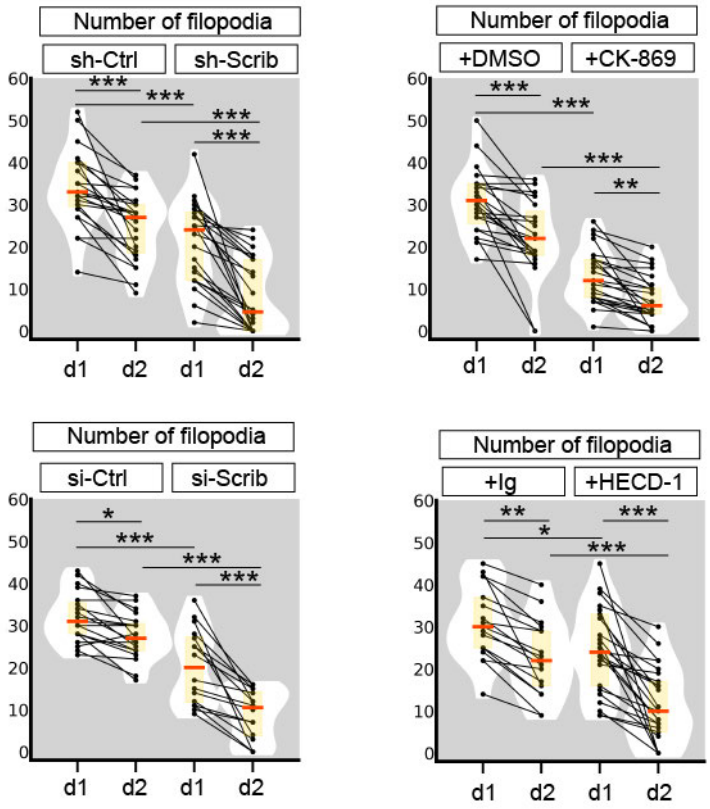


Figure 6. 15 Impacts of loss of function of Scribble, E-cadherin and Arp2/3 on filopodia patterning from telophase

The sparsely plated MCF10A cells, with the treatments of Scribble knockdown ('sh-Scrib' and 'si-Scrib'), knockdown control ('sh-Ctrl' and 'si-Ctrl'), Arp2/3 inhibition ('CK-869', 50μM for 15 minutes), DMSO control ('DMSO'), E-cadherin blockade ('HECD-1') , Ig control ('Ig'), were labelled with F-actin, and the filopodia protruded from the basal adhesion of each daughter cell were counted and shown in the line plot, overlaid with the density plot and the box plot.

6.2.4 Scribble coordinates an E-cadherin-based nascent junction to dictate daughter cell positioning

One area where nascent filopodia were prominent was in the interface between the two daughters (Figure 6. 11). These filopodia were intercalated, providing a possible means of remodelling the nascent daughter-daughter junction. E-cadherin was co-enriched with Scribble at the nascent daughter-daughter junction (**Figure 6. 16**), with high amount of PLA foci indicating the proximity between Scribble and E-cad at this stage (**Figure 6. 17**). I also observed that similar to Scribble, recruitment of E-cadherin and Myosin IIb required Arp2/3 activity (**Figure 6. 18**). In support of a continuing functional interaction between Scribble and E-cadherin, depletion of Scribble resulted in a dramatic loss of E-cadherin, and this was directly correlated with the degree of Scribble depletion (**Figure 6. 19**). As I had previously observed in Chapter 5, the effect of Scribble depletion on E-cadherin expression was not observed in a confluent monolayer of MCF10A cells in interphase, so I now assessed E-cadherin in the nascent junction of cells dividing within a confluent monolayer, using Myosin IIb as a marker of the mature telophase junction (Lecuit and Yap, 2015). Interestingly, like the single cells, E-cadherin was recruited to the nascent daughter-daughter junction within a confluent monolayer, and this recruitment was dependent upon Scribble (**Figure 6. 20**). Given that Dlg generally co-operates with Scribble (Figure 6. 8) (Allam et al., 2018) and has previously been found at nascent daughter-daughter contacts (Li et al., 2018), I propose the acronym SEAD (Scribble, E-cadherin, Arp2/3, and DLG) as a functional complex that mediates expansion of the nascent daughter-daughter contact (**Figure 6. 21**). I propose that this expanded daughter-daughter junction then restricts the movement of the two daughters away from each other.

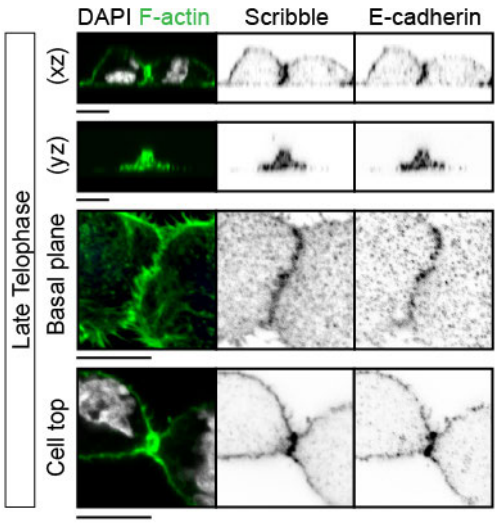


Figure 6. 16 Characterisation of Scribble and E-cadherin localisation at late telophase

Protein localisation of Scribble and E-cadherin at late telophase. Representative image from more than three individual experiments shows a sparsely plated MCF10A cell stained with DAPI (DNA), phalloidin (F-actin) and antibody against Scribble and E-cadherin. The (xz) layout shows the view that sectioned through the cell centre. The (xz) layout shows the view of the interface of daughter-daughter contact. The two (xy) layouts show the view at the basal adhesion and the top of the cell respectively. Scale bar = 10μm.

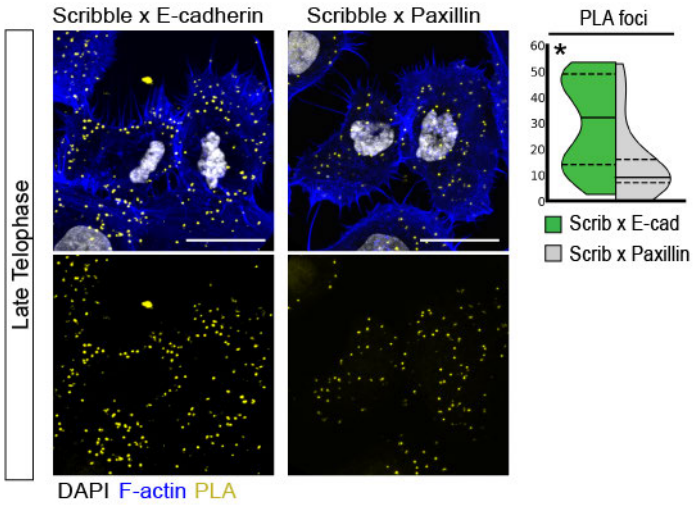


Figure 6. 17 Proximity ligation assay tests Scribble interaction with E-cadherin or Paxillin at late telophase

Proximity ligation assay was applied to determine spatial proximity between Scribble and E-cadherin (n=14), and between Scribble and Paxillin (n=13) at late telophase. The number of PLA foci was estimated as in density plot, with maximum number was counted as 50 if the PLA foci is greater than 50. Scale bar = 20μm.

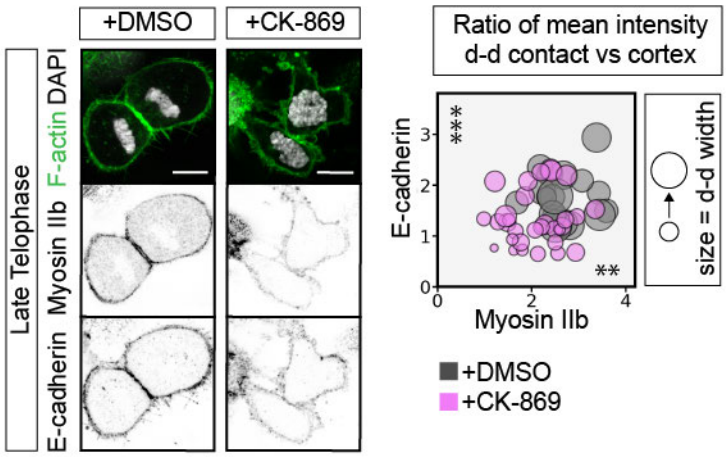


Figure 6. 18 Enrichment of Myosin II and E-cadherin is altered by CK-869 treatment

Inhibitor CK-869 (50μM) was treated on MCF10A cells for 15 minutes. (Left) Representative image shows a MCF10A cell stained with DAPI (DNA), phalloidin (F-actin) and antibody against Myosin IIb and E-cadherin. Ratio of mean intensity of Myosin IIb at cortex versus cytoplasm, and ratio of mean intensity of E-cadherin enriched at daughters' contact versus cortex were co-plotted in a scattering plot (Right), with size of per circle determined by the width of daughters' contact. Scale bar = 10μm.

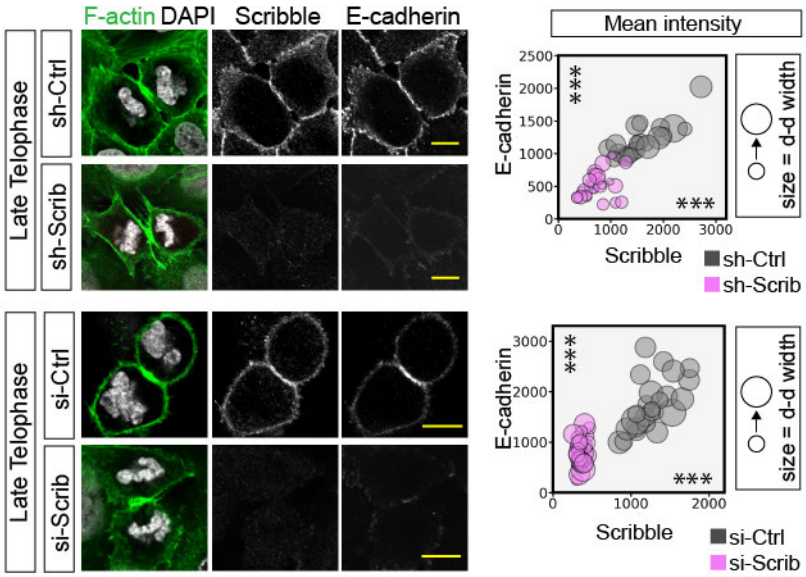


Figure 6. 19 Scribble and E-cadherin enrichment at nascent junction were lost upon Scribble depletion

Scribble was depleted in MCF10A cells by both sh-RNA and si-RNA, and mean intensity of Scribble and E-cadherin were assessed as shown in the scattering plots, with size of per circle determined by the width of daughters' contact. Scale bar = 10μm.

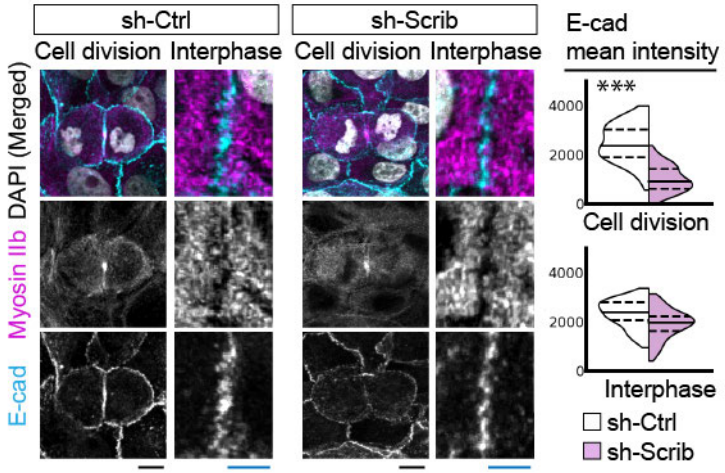


Figure 6. 20 Loss of E-cadherin and Myosin II enrichment at nascent junction upon Scribble depletion

Scribble was depleted in MCF10A cells by sh-RNA, and mean intensity of E-cadherin at the nascent junction between daughters or at the interphase junction was assessed as shown in density plots. Scale bar = 10μm.

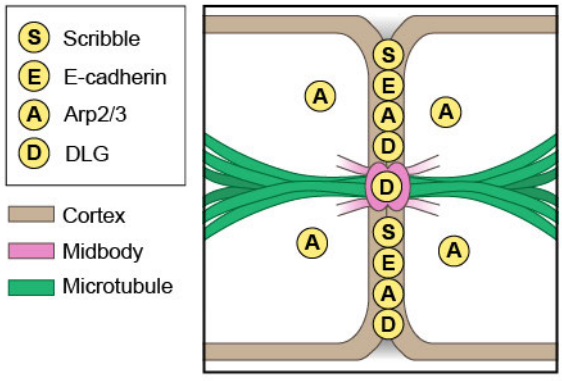


Figure 6. 21 A schematic model illustrates a cohort of proteins 'SEAD'

A schematic to suggest 'SEAD' (Scribble, E-cadherin, Arp2/3, perhaps Dlg) regulates the nascent junction between daughters.

6.3 Discussion

Following the claim of SEND in Chapter 5, which reveal a cell-autonomous role for Scribble-E-cadherin in transmitting signals from retraction fibres to NuMA of the LGN complex. Here I also show that remodelling during cell division leads to further functions for E-cadherin and Scribble, in both the formation of nascent filopodia, and the expansion of a nascent daughter-daughter junction, which controls the positioning of both daughters at and beyond telophase (**Figure 6. 22**).

SEADing information to guide to guide positioning of the daughter cells.

Despite the recognised importance of positioning of the daughter cells following mitosis for tissue organisation during development, metastasis and other pathologies, the means by which adhesive contacts are remodelled following cell division are still unclear (Osswald and Morais-de-Sa, 2019). This understanding is particularly complicated by the need for cells in a monolayer to disengage adherens junctions with neighbouring cells alongside formation of a new adhesive interface (Le Bras and Le Borgne, 2014). As with the spindle orientation, exploring single cells has allowed us to identify novel mechanisms by which the daughter-daughter contact is established, although our findings suggest these mechanisms are also conserved in an intact monolayer. I find that a SEAD of Scribble, E-cadherin and Arp2/3 and perhaps Dlg is recruited to the intracellular bridge that remains in late telophase, where they mediate an expansion of the adhesive interface between the two daughter cells.

Together, these studies identify cell-autonomous roles for Scribble and E-cadherin at several stages in the remodelling of spindle and cell membranes during cell division of a single MCF10A cell. These roles were previously obscured, presumably because of the importance of Scribble and E-cadherin in the junctions of epithelial cells in monolayers. Scribble connects E-cadherin (and perhaps other cues) to spindle orientation machinery, cell protrusions and the nascent daughter-daughter contact. This dynamic repositioning of Scribble to coordinate cell morphology during times when the cell is undergoing rapid shape change has precedence in other biological situations (Allam et al., 2018; Bonello and Peifer, 2019; Ludford-Menting et al., 2005; Osmani et al., 2006). Thus, I propose a dynamic role for Scribble throughout cell division in orchestrating the placement of daughter cells.

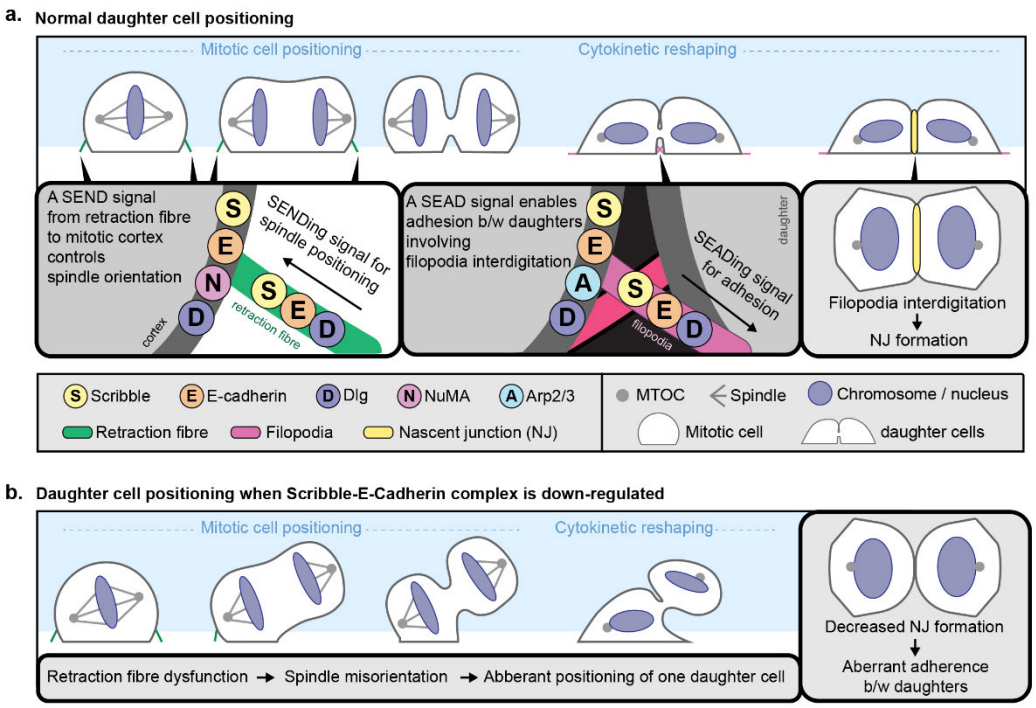
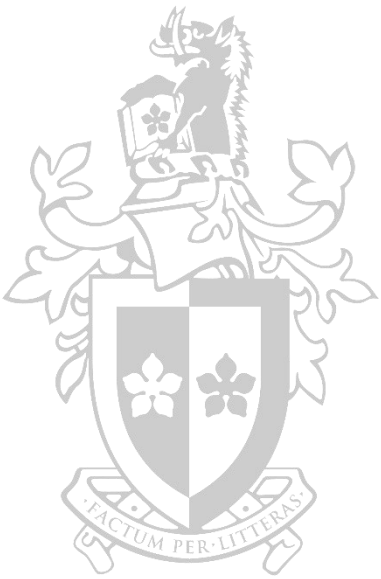


Figure 6. 22 The graphic summary of localisation and function of SEND and SEAD

(A), Cell shape during cell division is patterned by stepwise programs, involving spindle orientation leading to the aberrant position of one daughter cells, followed by formation of a nascent daughter-daughter adhesion. The two programs are controlled by signalling platform involving Scribble and E-cadherin. In the case of spindle positioning, NuMA is also involved (via 'SEND') and for the nascent daughter-daughter adhesion, Arp2/3 is also involved (via 'SEAD'). A normal progression of daughter cell patterning is controlled by SENDing the signal for spindle orientation through retraction fibres, and then by SEADing the drivers for daughter-daughter adhesion. **(B),** Daughter cell patterning in absence of SENDing or SEADing signals are abnormal, such as biased mitotic cell positioning, aberrant daughter-surface adhesion and reduction of daughter-daughter re-adherence.

6.4 Conclusion

The fate of the two daughter cells is intimately connected to their positioning, which is in turn regulated by cell junction remodelling and orientation of the mitotic spindle. How multiple cues are integrated to dictate the ultimate positioning of daughters is not clear. Here, I identify novel mechanisms of regulation of daughter positioning in single MCF10A cells. After anaphase, Scribble re-locates to the junction between the two daughters to allow a new E-cadherin-based-interface to form between them, influencing the width of the nascent daughter-daughter junction, generation of filopodia Thus, E-cadherin and Scribble dynamically relocate to different intracellular sites during cell division to orient the mitotic spindle and control placement of the daughter cells after cell division.



Chapter 7

Discussion and Future Directions

My thesis contributes to knowledge of the cell fate determination process. With respect to T cell development, the experiments described a new sequence of events in the progression of β-selection. As this sequence was unveiled by HDAC6 inhibition, the results expose a possible role of HDAC6 prevents developing T cells from inappropriately bypassing the β-selection checkpoint. With respect to epithelial cell division, I uncovered new roles of Scribble and E-cadherin in regulating daughter cell positioning. In Chapter 6, I will describe possible implications from these findings, and discuss possible next steps.

7.1 Precise waves of phase transition in β-selection allow for better understanding of early T cell selection

Chapter 3 and Chapter 4 have updated a new developmental course: DN3a, DN3b^{Pre} and DN3b^{Post}. Although I haven't yet demonstrated the transcriptional profile for these three stages, here I would like to discuss the possible trends by correlating database and literatures.

The development of T cell is realised through several waves of change in transcriptional control and the surface phenotype. The change of gene expression can be understood through an informative immune genome database called ImmGen (2020; Heng and Painter, 2008). mRNA profile from ImmGen (see **Appendix A** and **Appendix B**) can guide hypotheses as to the functions of many key genes in T cell development (**Figure 7. 1 A**). For example, Notch1, a master transcription factor (TF) that governs T cell fate commitment and initiation of β-selection, reaches a highest expression at the DN3a stage. Notch1 gene expression is then sharply downregulated from DN3b, reflecting a switch of signal reliance to IL7R (Boudil et al., 2015; García-Peydró et al., 2006). This downregulation is probably associated with the termination of genome reorganisation for TCRβ gene recombination, as Notch1 was recently identified to regulate the expression of gene recombination enzymes, RAG1 and RAG2, during β-selection (Dong et al., 2021). A Notch1-targeted gene, Bcl11b, also peaks at the DN3a stage (Figure 7. 1 A). Bcl11b regulates TCRβ gene recombination, therefore can be assumed to collaborate with Notch1 at the DN3a stage. Following the cessation of Notch1 transcription after the DN3a stage, the upregulation of CD28, in both mRNA and protein level, marks the DN3a-DN3b progression (**Figure 7. 1 B, C**). In the ImmGen database, the mRNA expression of Lef1

shows a similar trend to CD28; upregulated from DN3b. The protein level of Lef1 was assessed in this thesis, and was upregulated from DN3b (Figure 7. 1 B, C). In relation to the two new DN3b subpopulations (ie. a transitioning population $DN3b^{Pre}$, and a mature population $DN3b^{Post}$) described in the thesis, I found Lef1 was upregulated from $DN3b^{Pre}$, indicating it was expressed after CD28. In the ImmGen database, both CD2 and CD5 mRNA are increasingly expressed after T cell fate commitment, and peak at the DN4 stage (Figure 7. 1 B, C). In my experiments using the OP9-DL1 coculture system, I found the CD2 and CD5 proteins are upregulated from DN3a to DN3b (Figure 7. 1 B, C). Interestingly, CD2 and CD5 were said to play an opposite role in coordinating TCR signalling, but no systematic analysis of the combined functions of CD2 and CD5 has been reported. In this chapter, I discussed the possibility that CD2 and CD5 can tune pre-TCR signalling, and suggest future studies to explore this possibility.

This study was initiated with a hypothesis that HDACs mediate T cell development, where a focus on HDAC6 was triggered because the profile of HDAC6 peaks at DN2-DN3 stage (Appendix A). My results implied HDAC6 regulates progression through the β-selection checkpoint. As HDAC6 is a unique HDAC among its family, which can modify acetylation of histone and cytoskeleton, in the section 7.6 I would like to discuss the potential role of HDAC6 in T cell development and also to suggest future research about HDAC6.

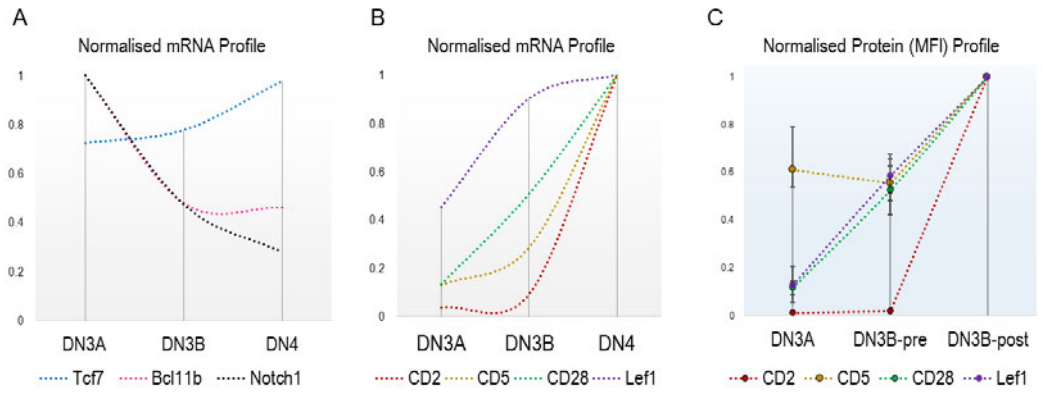


Figure 7. 1 The mRNA profile from ImmGen database and the protein expression profile from this study

(A) Normalised mRNA profile of Tcf7, Bcl11b and Notch1 in DN3A, DN3B and DN4 compartments. To unify the mRNA value into the scale of 0 to 1, the value of mRNA expression of DNx was divided by the value of ETP mRNA expression, followed by the division to the maximum value of DNx/ETP. **(B)** Normalised mRNA profile of CD2, CD5, CD28 and Lef1 in DN3A, DN3B and DN4 compartments. The value was normalised as (A). **(C)** The normalised protein profile of CD2, CD5, CD28 and Lef1 in the DN3a, DN3b^{pre} and DN3b^{post} compartments. The value of MFI of DNx was unified by the division to the MFI of DN3b^{post}.

7.2 Does CD28 regulate metabolism for the passage of β-selection checkpoint?

Since CD28 upregulation was reported to effectively mark the larger cell, namely the cell in the later stage of differentiation in DN3 compartment, labelling for CD28 has become a standard procedure to demarcate DN3b population for immunologists (Zheng et al., 2004). About 15 years ago, two studies respectively using CD28 knockout and CD28 transgene have suggested CD28 supports the growth of the developing T cell. (Williams et al., 2005; Zheng et al., 2004). Likely through promoting metabolism, CD28 supports the proper passage through the β-selection checkpoint. As pre-TCR signalling occurs at the onset of the β-selection checkpoint, the coordination between pre-TCR and CD28 is essential, but the details of this coordination remained elusive (Williams et al., 2005). By contrast, the role of CD28 to promote TCR signalling is well established, and called co-stimulation (Esensten et al., 2016). Once CD28 binds to its ligands, a series of signals are transduced for cell survival, proliferation and differentiation (Esensten et al., 2016). In part, CD28 co-stimulation regulates the PI3K-Akt pathway to promote metabolic activity. In this thesis, I have shown that CD28 upregulation in DN3b population is correlated with signs of PI3K-dependent metabolic activation, such as upregulation of Ki-67, c-Myc, CD71, and CD98 (Kelly et al., 2007). However, direct evidence regarding activation or maintenance of metabolism in DN3b is still lacking. Here, I would discuss the two non-exclusive possible mechanisms that CD28 can regulate metabolism in the developing T cell: (1) PI3K pathway activation and (2) mitochondrial activation:

7.2.1 CD28-PI3K pathway is required for survival, growth and proliferation of developing T cells

CD28 contains an YMNM motif in its intracellular tail. Tyrosine phosphorylation of this motif (ie. pYMNM) enables binding to the two SH2 motifs of PI3-kinase subunit p85 (at the C- and N- terminus respectively) (Prasad et al., 1994). This direct interaction triggers signal transduction through the PI3K pathway, leading to activation of signalling mediators such as Akt and Vav. In the developing T cell, Akt is pivotal for survival, and is required for glucose uptake in DN3 compartments (Juntilla et al., 2007). The PI3K subunit p85 is also required for T cell development (Shiroki et al., 2007) (**Figure 7. 2**). Needless to say, the role of mTOR has been investigated and shown to be critical for several aspects of early T cell development (Werlen et al., 2021). These studies support a hypothesis that the CD28-PI3K pathway is engaged in early T cell development for co-stimulation.

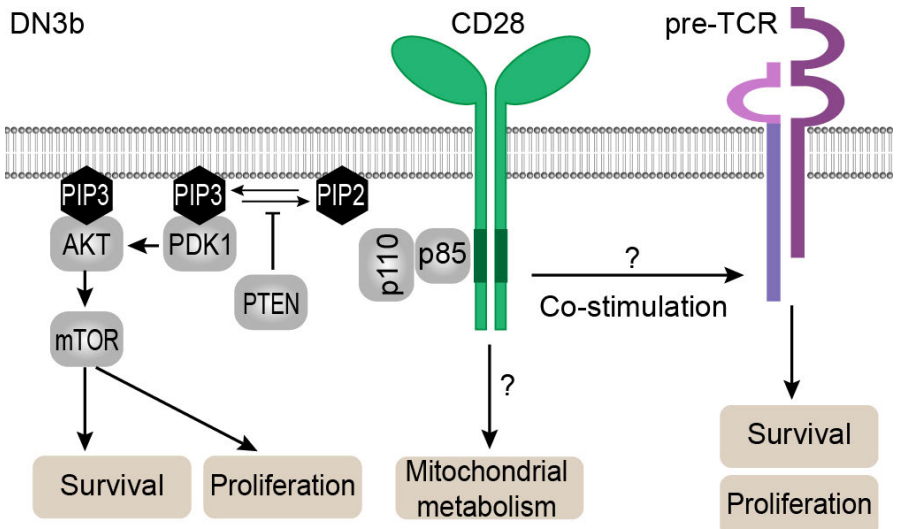


Figure 7. 2 The CD28 pathway in DN3b cells

The schematic shows CD28 signalling in DN3b compartments. In part, CD28 interacts with PI3K complex to activate AKT-mTOR pathway for survival and proliferation. In part, CD28 signalling might orchestrate with pre-TCR signalling to enhance survival and proliferation.

7.2.2 Does CD28 prime mitochondrial activity in developing T cell?

Through *in vitro* CD28 activation assays, Ledderose et al have firstly observed an increase of ATP production, oxidative phosphorylation and mitochondrial synapse accumulation in $CD4^+$ T cells (Ledderose et al., 2014). Priming of mitochondrial activity by CD28 was demonstrated in the context of differentiation of memory $CD8^+$ T cells (Klein Geltink et al., 2017). The research of Geltink et al solved a question of how the long-term antigen response required for differentation is harnessed in $CD8^+$ T cells (Klein Geltink et al., 2017). CD28 co-stimulation inhibits a metabolic regulator TXNIP, which in turn activates Cpt1a. Cpt1a is a mitochondrial enzyme that facilitates Acyl-CoA usage. These mechanisms highlight how CD28 signalling can enhance the mitochondrial capacity for fatty-acid utilisation. In addition to fatty acid oxidation, signalling through CD28 can also increase mitochondrial mass in $CD8^+$ TIL (tumor-infiltrating lymphocytes), coordinating with Glut3 upregulation to increase glycolysis (Beckermann et al., 2020). However, how glycolysis, fatty-acid utilisation and other nutrients uptake are processed in the developing T cells is

unclear. As CD28, CD71 and CD98 are upregulated for the phase of pre-TCR signalling, the entire metabolic pathway, including mitochondrial biochemistry is ripe for study.

7.2.3 CD28 co-stimulation domains are used in CAR T cell for immunotherapy

Nowadays, chimeric antigen receptor (CAR) T cell therapies, using genetic engineering to boost T cell immunity, have been shown to be successful in treating cancer. In the CAR receptor, the CD28 co-stimulation domain is essential to harness the capacity of tumour killing (Cappell and Kochenderfer, 2021). With advantages and disadvantages, CD28 strengthens CAR activity but often accelerates T cell exhaustion (Salter et al., 2018). This feature of exhaustion of CAR T cells has become the limitation in cancer treatment and restricts CAR T cells migrating to tumour (Martinez and Moon, 2019). Interestingly, a recent study showed a design of CD28 mutant in CAR, introducing point mutations to YMNM motif to tune the strength of co-stimulation, that likely improved CAR T cell exhaustion (Boucher et al., 2021). Similarly, the modulation of CD28 signal strength might be a new topic of the pre-clinical research for T-ALL, which will be discussed in Section 7.7.1.

7.2.4 Future directions regarding CD28

(1) The approach of CRISPR-Cas9 to acutely deplete CD28 in developing T cells would be very informative. Through this approach, I can verify whether CD28 regulates metabolism in developing T cells such as glucose uptake, PI3K signalling and mitochondrial activity. Moreover, there are many functional domains in the CD28 intracellular tail that have been identified, such as the N-terminal PxxP motif and the C-terminal PxxP motif in the regulation of tyrosine phosphorylation and the co-stimulatory function of CD28. (Ogawa et al., 2013) I expect the knockout-rescue assay would help to clarify the CD28 pathways in T cell development.

(2) Following CD28 depletion, some analysis that might be informative include: (a) the control of cell size, and (b) the duration of cell cycle. As the state of senescence or quiescence in DN3a is likely to be coordinated with CD28 signalling, it would be worth examining whether CD28 signalling is associated with the cessation of TCRβ gene recombination. The mechanism that controls CD28 expression in DN3a cells is another interesting question.

(3) CD28 co-stimulation relies on the presence of antigen or anti-CD28 antibody. In the thymus, the CD28 antigen, B7, is presented, and likely interacts with CD28 for the development of DN cells. To explore the precise role of CD28 in the developing T cell, manipulating the expression of B7 along with the *ex vivo* expansion could be informative. Particularly, the downstream events of CD28 signalling in developing T cell are not yet mapped, there might be some difference between ligand-dependent and ligand-independent pathways. Moreover, a CD28 superagonist (CD28SA) has been tested clinically, although this treatment led to cytokine storm (Hünig, 2016), the strong effect of CD28SA could be utilised here for mapping the target genes.

(4) The role of other CD28 family members such as CTLA4 and PD-1 could be examed in T cell development. Ctla4 gene expression peaks at the DN3a stage, and Pdcd1 gene expression peaks at the DN4 stage. This will be interesting not only for the context of β-selection, but also in positive selection, because Pdcd1 is highly expressed in a subgroup of $CD69^{+}$ DP cells. I speculate that some self-antigen might partially interact with the CD28 family, and lead to a different autoimmune response in thymus.

7.3 Does CD2 influence TCR gene recombination and pre-TCR co-stimulation?

7.3.1 CD2 modulates TCR co-stimulation, does it do the same for pre-TCR?

CD2 is localised at the T cell immunological synapse to interact with its ligand LFA3 expressed by antigen-presenting cell, then facilitate co-stimulation through the TCR (Binder et al., 2020). In 1992, Turka et al found CD2 was expressed in TCR/CD3$^-$ human T cells (correlated to DN cell in the nomenclature used in this thesis), but the expression level was lower than in unfractionated T cells (mostly DP cell), suggesting CD2 is upregulated when the T cell progresses from the DN to DP stage (Turka et al., 1992). They further tested the consequence of CD2 stimulation. By testing calcium flux and IL2R expression between TCR/CD3$^-$ T cell and the unfractionated T cell, they found TCR/CD3$^-$ T cell did not show significantly increased calcium flux or IL2R expression upon CD2 activation. Does this result imply the DN T cell doesn't rely on CD2 receptor for pre-TCR co-stimulation? To consider this question, the original methods used in the paper matter. Regarding calcium flux, DN cells were shown to have lower calcium signalling compared to DP (Le Borgne et al., 2016), however it's still unclear whether calcium flux is with a functional consequence of pre-TCR signalling. The entire IL2R, except the subunit IL2Rα (CD25), is not expressed at the DN stage, so would not be relevant (Takács et al., 1988). Therefore, the two indicators used in Turka et al can't rule out the potential for pre-TCR co-stimulation from CD2. Thus how CD2 functions in the developing T cell is still unclear.

7.3.2 CD2 expression is coupled with TCRβ gene recombination

In this thesis, I found CD2 is expressed at the DN3b^{Post} stage, a stage presumably with completed TCRβ gene recombination. This finding is consistent with finings of Rodewald et al in 1993: They found CD2$^+$ DN cells had mostly completed with TCRβ gene recombination (Rodewald et al., 1993). On the other hand, an intriguing result was revealed by Turka et al: they found RAG gene expression in the unfractionated T cells (mostly DP cells) was shut down by CD2 activation (Turka et al., 1992). Their result might suggest CD2 signalling negatively controls the process of TCRα gene assembly. But surprisingly RAG expression in DN stage wasn't changed upon CD2 activation. To address this difference, again, the methodology that used anti-CD2 antibody to trigger CD2 activation in DN stage can't lead the interpretation that CD2 signalling is dispensable for TCRβ gene recombination. On the other hand, one important observation from Rodewald et al is that DN cells from RAG deficient mice were mostly positive for CD2 (Rodewald et al., 1993). This is a first functional link between CD2 expression and TCRβ gene recombination, and

the result even implied CD2 expression is downstream of TCRβ gene recombination. The results of Rodewald et al and Turka et al, suggest the possibility CD2 plays a different role in TCRβ gene recombination (DN3) and TCRα gene recombination (DN4-DP). The evidence later provided by Sasada and Reinherz, using CD2-depleted mice, show CD2 is required for DN3 progression, but CD2 depletion didn't broadly influence TCRβ diversity; unlike TCRα diversity and DP progression were significantly impaired by the loss of CD2 (for 6-week mice) (Sasada and Reinherz, 2001). Therefore, a hypothesis suggested here is: Once TCRβ gene recombination is completed, CD2 is triggered to co-stimulate pre-TCR to allow cells to progress from DN3 to DN4. CD2 is later required and is positively associated with TCRα gene recombination, and therefore facilitates the progression from DN4 to DP.

7.3.3 Future direction about CD2

(1) Whether CD2 acts as a co-stimulator with pre-TCR is unclear. It seems using recombinant antibody to trigger CD2 can only succeed in mature T cells, thus a preliminary approach will still be using CRISPR-Cas9 to deplete CD2, or breed CD2 deficient mice (Sasada and Reinherz, 2001). Through CD2 depletion, I can investigate downstream pathways that impact pre-TCR signalling. As IL2R is triggered by CD2 signalling, but the whole IL2R is not presented in T cell development, one focus from here is also to know which, if any, cytokine is cued by CD2 signalling in developing T cell. As IL7R signalling is required for DN3b progression, would CD2 signalling trigger the expression of IL7?

(2) It's important to know whether any CD2 ligands are expressed in the thymus and can interact with CD2 of developing T cells. On the other hand, whether CD2 can act without ligand is not yet clear in developing T cells.

(2) So far, one interesting question is about the unknown relationship between CD2 onset and the completion of TCRβ gene recombination. Whether the transcription of CD2 becomes accessible after TCRβ gene rearrangement is unclear. Together with this question, the identification of the TF for CD2 transcription is likely important. From my result, the first reasonable guess is Lef1.

7.4 Does CD5 dampen pre-TCR signalling?

7.4.1 CD5 negatively regulates TCR signalling

CD5 regulates several signals in T cell in a context-dependent manner. For example, CD5 positively regulates Akt signalling, but negatively regulates Erk signalling (Blaize et al., 2020). In DP and $CD4^+$ T cells, CD5 negatively regulates TCR signalling (Blaize et al., 2020). CD5 attenuates or terminates TCR signalling by recruiting an E3 ligase, c-CBL (Blaize et al., 2020; Voisinne et al., 2016). c-CBL is also recruited by Zap70 to ubiquitinate TCR (O'Leary et al., 2015), but c-CBL-Zap70 seems to be inseparable from CD5-c-CBL complex to tone down TCR signals. Moreover, a TCR negative regulator, CSK, is recruited to the complex of c-CBL and CD5 (Voisinne et al., 2016). A model was proposed that, once p-MHC triggers TCR signalling, Lck phosphorylates CD5 to facilitate the recruitment of CSK to the tail. Subsequently, the CD5-CSK complex attenuates TCR signalling (Voisinne et al., 2018). Therefore, these findings suggested CD5 negatively regulates TCR signalling. CD5 is also involved in the regulation of NF-κB pathway in T cell. The NF-κB pathway is believed to be triggered by TCR/CD28 signalling, which can promote T cell proliferation or differentiation (Paul and Schaefer, 2013). CD5 likely negatively modulates NF-κB signalling, by facilitating the expression of IκBα, a negative regulator of the NF-κB pathway. (Matson et al., 2020). However, it seems still complicated to interpret the study of (Matson et al., 2020), because $CD5^+$ T cells showed localisation of NF-κB suggested to confer a survival advantage, but whether NF-κB signalling was altered or not is less clear. These studies suggested CD5 is a negative regulator to dampen TCR strength.

7.4.2 Potential mechanism that CD5 regulates pre-TCR

The importance of CD5 as a reporter to calibrate pre-TCR signalling strength was perhaps not considered previously because the pre-TCR was not thought to engage MHC-peptide. However, it is now apparent that the pre-TCR can bind with low affinity to MHC-peptide (Li et al., 2021). The pre-TCR also forms an immunological synapse with many similarities to the immunological synapse formed by the αβTCR, oriented towards MHC (Allam et al., 2021). These recent observations support the notion explored above that CD5 could play a similar role in both reporting and handicapping TCR signals. As mentioned in the section of 6.4.1, CD5 can dampen TCR signalling through two possible mechanisms: (1) through CSK recruitment and (2) through modulating NF-κB pathway. To clarify the potential role of CD5 in the context of pre-TCR signalling, I first excluded the involvement of NF-κB pathway, because substantial evidence so far didn't suggest NF-κB pathway participates in

early T cell development (within DN stage) (Liu et al., 2006; Voll et al., 2000; Webb et al., 2016). By contrast, the pathway through CSK seems to be consistent between TCR and pre-TCR (Zikherman et al., 2010). Together with the evidence showing CD5 expression in DN/CD25^{+} T cell relies on Lck (Azzam et al., 1998), I would like to propose a model that CD5 dampens pre-TCR signalling in a manner of: (1) Lck promotes CD5 expression; (2) CD5 recruits CSK to cell membrane close to pre-TCR; (3) CSK inhibits Lck, then represses pre-TCR signalling (**Figure 7. 3**). In addition to this, the recruitment of an E3 ligase is likely to be a mechanism here.

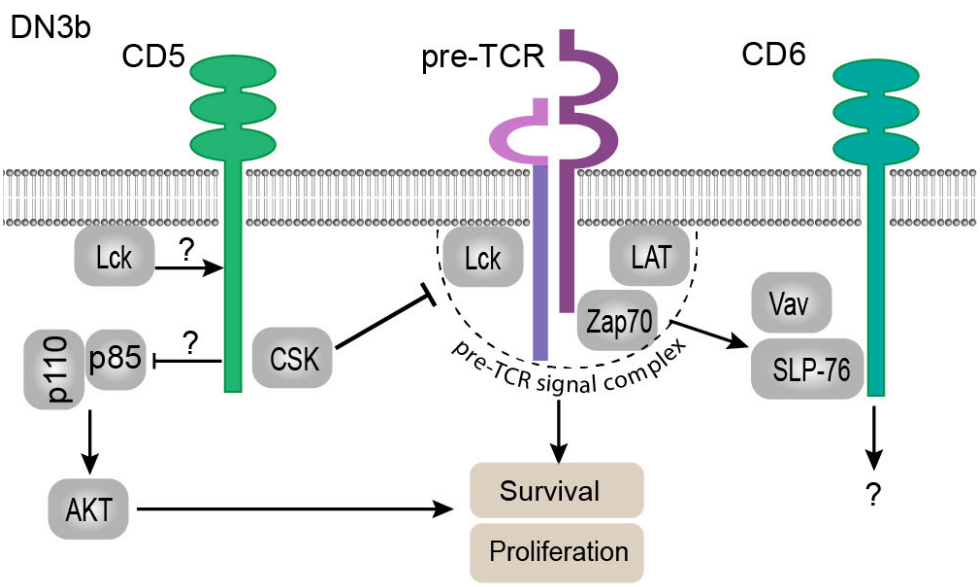


Figure 7. 3 The CD5 and CD6 pathway in DN3b cells

The schematic illustrates the interaction between CD5 and pre-TCR signal complex (including Lck, Zap70 and LAT). In part CD5 is shown to interact with PI3K signalling to restrict survival and proliferation. In part CD5 recruits CSK to dampen pre-TCR signalling. On the other hand, CD6 pathway is illustrated, but the information is yet documented.

7.4.3 Future directions regarding CD5

(1) Whether CD5 dampens pre-TCR is not yet proved by functional assay. To identify which CD5 interacting proteins are the effectors to dampen pre-TCR strengthen, I can consider a BirA-mediated biotinylation assay that can probe CD5 interacting proteins in developing T cells. Through a high-throughput reading by mass spectrometry, I can better know the CD5-interacted proteomics in developing T cells.

(2) If CD5 plays a role to balance pre-TCR strength, the overexpression approach is likely sensible. One option is ectopic overexpression, but since the enhancer of CD5 has been nicely identified, there might be some approach such as 'CRISPERa' (Horlbeck et al., 2016) to promote the endogenous CD5 expression. If the expression level of CD5 can be manipulated, I can next test together with CD28 activation assay to evaluate how CD5 level might tune pre-TCR signalling strength.

(3) Parallel to CD5, the role of CD6 might be worth investigating. The background of CD5-CD6 interaction is: CD6 interacts with ITK, an Lck signal recipient in the LAT complex; while CD5 expression in DN cells relied on ITK (Gonçalves et al., 2018). Less is known for CD6, but its interaction with Vav complex might be a pathway to interact with pre-TCR.

7.5 A novel model and interacting network for β-selection

7.5.1 A novel model of passing β-selection checkpoint

ACY1215 treatment has exposed the DN3b^{Pre} stage of β-selection, encouraging us to identify a critical sequence of events that harmonise cell fate during β-selection. Here I would propose a refined model of β-selection, in which the checkpoint is marked initially by upregulation of CD28. CD28 expression involves a coordinated increase in expression of pre-TCR and CD5, with an escalating requirement for pre-TCR signalling strength. If the culminated pre-TCR signalling strength meets the requirement of β-selection checkpoint, a rapid expression of CD2 indicates exiting from the β-selection checkpoint.

A major finding from this work is that CD5 levels are fine-tuned according to pre-TCR expression, and that treatment with ACY1215 prevents the upregulation of CD5 (but does not disrupt Lef1 upregulation) during β-selection. A role for tuning of the TCRβ repertoire during β selection is suggested by findings that the pre-TCR can bind with low affinity to the complex of MHC-peptide (Li et al., 2021; Mallis et al., 2015). The pre-TCR also forms an immunological synapse with many similarities to the αβTCR-induced immunological synapse(Allam et al., 2021). These recent observations support the notion that CD5 could play a similar role in both reporting and handicapping pre-TCR signals and to coordinate fate determination.

Together with these findings, I have realised that the sequence of expression of CD28, CD5 to CD2 during β-selection is highly similar to the scenario of passing the checkpoint at airports (**Figure 7. 4**). I imagined CD28, which can carry the metabolic fuel, is like the budget that is required for the travel, but can't influence the pass of the checkpoint. As the most essential element, or the pass of the checkpoint itself, is the passport. The passport represents the identity of TCRβ. Only presenting TCRβ can allow a passenger to progress. Then a check of sufficient package in the carry-on is like the function of CD5, which therefore I deemed CD5 is the boarding case. Before landing on the flight, the boarding pass (CD2) is the last check to ensure the appropriateness to fly away to the next destination, which is DP in T cell development.

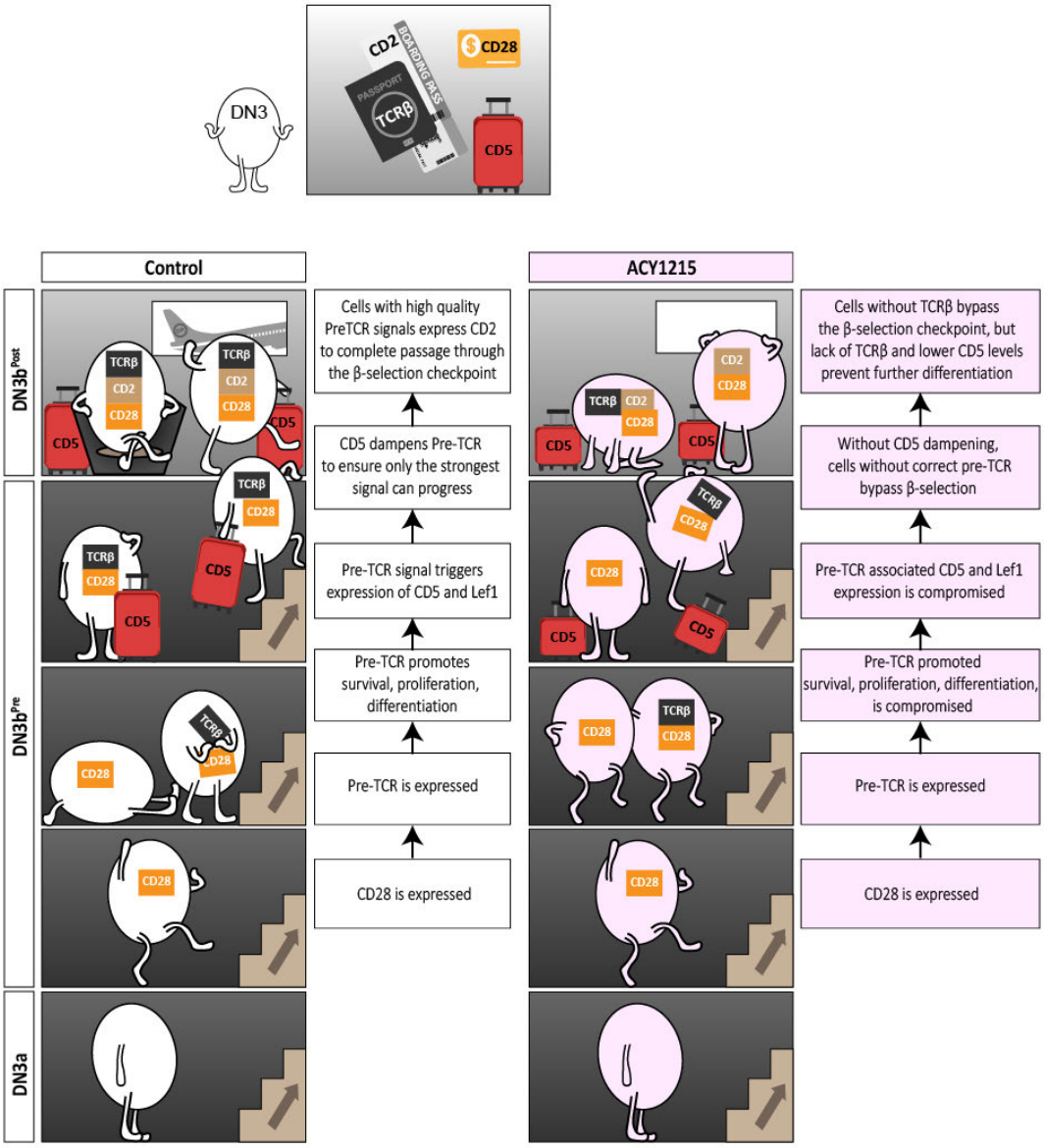


Figure 7. 4 A schematic model of passing β-selection checkpoint

Schematic illustrates how expression of each surface marker (CD28, TCRβ, CD2 and CD5) impacts upon progression through β-selection via DN3a, DN3b^{Pre} to DN3b^{Post} stages. A DN3 cell was imagined as a passenger who passes through the airport. The arrow represents the flow of passing the β-selection. The DN3 cells that treated with ACY1215 were coloured in pink.

7.5.2 An updated signalling schema in the process of β-selection

In this section, I hope to propose an updated signalling network, as shown in (**Figure 7. 5**). First, Notch1 is the upstream TF to drive differentiation of DN3 cells. The genes induced by Notch1 include TCF7 (Germar et al., 2011), CD28 (Chadwick et al., 2009), Bcl11b (Li et al., 2010), and LEF1 (Ross and Kadesch, 2001). In a recent study, RAG1 and RAG2 gene expression were also deemed as the targets of Notch1 (Dong et al., 2021), suggesting a transcriptional regulation for forming pre-TCR. Second, although in the mature T cells, TCF-1 cooperates with LEF1 for gene regulation, it was reported that TCF-1 repressed LEF1 transcription in the developing T cell (Yu et al., 2012). In my opinion, this repression probably contributes to the absence of LEF1 in early T cell development before DN3b. Third, the interaction between LEF1 and CD28 is unclear, but CD28 seems to be upstream of LEF1 expression based on our finding. On the other hand, it's intriguing that a study had distinguished a LEF^{lo} Treg by using anti-CD28 antibody to activate Treg, implying CD28 signalling might be a negative regulator for LEF1 expression. Although it might not be the case in developing T cell, the CD28 signalling is not really mapped in the developing T cell, and the interaction between CD28 and LEF1 is certainly worth studying. Fourth, as CD2 is upregulated after CD28 and LEF1, I explored whether any study suggested either CD28 or LEF1 might activate CD2. Although no such findings have been reported to my knowledge, LEF1 is highly co-upregulated with CD2 in AML (Metzeler et al., 2012), endorsing about the possibility that LEF1 might activate CD2 transcription. To further complete the interacting network, Lck is included. It is unknown what the transcription factor for Lck is, but Lck positively associates with the function of Notch1 (Sade et al., 2004). As mentioned in 6.5.1, an axis of LCK, ITK and CD5 was uncovered, therefore I added this axis in the network accordingly to suggest a negative regulator dampening pre-TCR signalling.

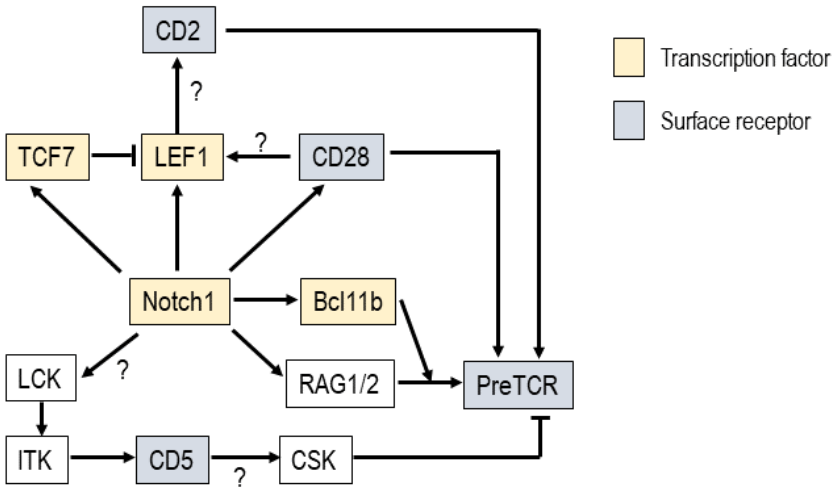


Figure 7. 5 The signalling schema in DN3 compartment

The schematic shows how crucial transcription and crucial surface receptors are looped to pass the β-selection checkpoint. Centred by Notch1, a network linking Tcf7, Lef1, Bcl11b, RAG and Lck are primed. The transcription of Notch1 can influence the expression of CD2, CD5, CD28 and pre-TCR, and therefore control the fate determination of DN3 compartments.

7.5.3 Future directions

(1) As indicated by the multiple question marks in Figure 7. 5, the role of each player in β-selection is still unclear. To further our understanding, one straightforward approach is to profile the gene expression upon acute CD2, CD5 and CD28 depletions.

(2) The signalling schema that I proposed (Figure 7. 5) is focused on β-selection. However, I can't neglect γδ-selection. A first step to bring γδ-selection in the panel might be to consider the transcription control of Notch3 (Van de Walle et al., 2013). Other essential TFs such as Runx1, 2 and 3 should be included.

(3) The regulation of pTα expression is not considered in the current interactome. The transcription of pTα relies on E2A. Bcl11b seems to play some role overlapping with E2A (Longabaugh et al., 2017), but so far little is known on how these and other transcription factors regulate pTα.

7.6 How HDAC6 regulate the progression of β-selection?

7.6.1 HDAC6 might deacetylate the cytoskeleton to influence immunological synapse formation during β-selection

The effect of HDAC6 to reduce tubulin acetylation modulates the functions of immunological synapse of mature T cells (Serrador et al., 2004). My finding here showed that acetylation of α-tubulin was increased in DN3 cells upon ACY1215 treatment, suggesting that the function of immunological synapse during β-selection might be mediated by HDAC6. Indeed, ACY1215 treatment altered the organisation of the pre-TCR signalling complex, LAT, around the MTOC. Similarly, Notch1, which is required to enhance the polarisation of the pre-TCR (Allam et al., 2021; Charnley et al., 2020), was lost from the MTOC following ACY1215 treatment. These findings are interesting in light of a recent report that HDAC6 is required for the formation of aggresomes at the MTOC (Magupalli et al., 2020), where it was suggested that HDAC6 might play a general role in recruiting signalling proteins to MTOC. As well as microtubules, microfilaments can also be modified by HDAC6. The best known is Cortactin. HDAC6 mediates the acetylation level of Cortactin, which in turn facilitates cell migration (Zhang et al., 2007). As some mechanisms of migration are shared with polarity formation, which influences synapse formation, Cortactin might regulate T cell polarity and immunological my work shows that HDAC6 can alter cytoskeleton-dependent polarity in developing T cells, and might subsequently influence the function of immunological synapse during β-selection.

Worth noting, an alternative explanation for the effect of ACY1215 on β-selection relates to observations that HDAC inhibitors can induce MHC constituents to promote antigen presentation (Magner et al., 2000). It remains to be determined whether an influence on stromal-presented peptide-MHC plays any role in disrupting β-selection. Together, these findings suggest that the effect of HDAC6 on β-selection that I see here might occur through disruption of the DN3 immunological synapse.

7.6.2 HDAC6 regulates epigenetic modifications in developing T cells?

In this study, I found ACY1215 treatment increased the acetylation of H3K18, suggesting HDAC6 can regulate the epigenetic status. HDAC6 is predominantly expressed in the DN3 nucleus, but I don't know how HDAC6 mediates epigenetics in developing T cells. To identify the potential HDAC6 mediators, one notion regarding Bcl11b might be worth discussing: First, H3K18 acetylation (which was promoted by ACY1215 treatment in developing T cell) can be mediated by Bcl11b through p300/CBP (Sidwell and Rothenberg,

2021). Second, Bcl11b is reported to interact with HDAC1 and HDAC2 (Fu et al., 2017), which implyies Bcl11b has a broad interaction with other HDACs. Third, HDAC6 and Bcl11b peak at DN2-3 and DN3a respectively (Appendix B). In short, a potential functional relationship between HDAC6 and Bcl11b might be the key to know how HDAC6 influences epigenetic modifications in the developing T cell.

7.6.3 Future directions regarding HDAC6

(1) From the result of this thesis, ACY1215 treatment influences expression of proteins such as CD28 and CD5 during β-selection. To determine whether this indicates a direct role for HDAC6, these results should be verified by acute HDAC6 depletion. Moreover, as some E3 ligases can modulate pre-TCR signalling (Checquolo et al., 2010), the ubiquitination of the CD28 and CD5 proteins upon ACY1215 treatment are the promising avenues for exploration, because the protein degradation by lysine ubiquitination is often prevented by lysine acetylation. Introducing point mutation to prevent ubiquitination in CD5 and CD28 will be sensible to understand the molecular basis for the effects of ACY1215 on T cell development that I show here.

(2) With respect to immunological synapse formation, the contribution from both CD28 and HDAC6 have been addressed separately, but their relationship or the synergistic effect has not been investigated. In particular, a CD28 bound protein, Vav1, can interact with Cdc42; and Cortactin, a HDAC6 substrate, can interact with Cdc42 substrate (Kelley and Weed, 2012). Therefore, to link HDAC6 to CD28, the actin-dependent pathway involved with Vav1-Cdc42-Cortactin might be worth exploring.

7.7 Potential roles of HDAC4 in T cell development

As highlighted at Chapter 1.7.1, HDAC4 mRNA level is very interesting, showing a sharp upregulation at DN3a stage, and followed by an immediate drop at DN3b stage (Appendix A). Although the examinations to understand the function of HDAC4 in T cell development are still ongoing, here I would like to discuss a possible role for HDAC4 through using an inhibitor LMK235. LMK235 was designed to target HDAC4 and HDAC5 (Mazzocchi et al., 2021), although a context-specific influence on HDAC6 activity was reported before (Choi et al., 2019). As shown in Appendix A, HDAC5 transcription was not promoted during T cell development, therefore effects of LMK235 on altering T cell development was unlikely through HDAC5, and most likely through HDAC4.

7.7.1 Preliminary results using LMK235

Through applying LMK235 on day-4 FL which were co-cultured with OP9-DL1 for two days, I found the expression of the surface markers of the non-T lineage (ie. $Lin^{+;}$ CD11b, NK1-1, CD45R and Ly6G6C) was increased (**Appendix C**). Around 40 % of progenitors show Lin^{+} expression, compared to less than 5 % in the control cells. To assess whether LMK235 directly induced expression of any of these markers, I compared the effect of LMK235 treatment on sorted DN3b cells, which were already committed to T cell differentiation and so would not normally express the markers. 2 days of LMK235 treatment on the sorted DN3b cells didn't induce Lin^{+} expression (Appendix C), suggesting LMK235 didn't directly promote Lin^{+} expression. These results together suggested LMK235 skewed differentiation away from the T-lineage. To assess which lineage maker was promoted by LMK235, I tested the lineage markers separately. The solution of lineage markers was a mixture of antibody against NK T cell (NK1.1), B cell (CD45R), and myeloid lineage (CD11b, Ly6G6C. In a concurrent experiments using day-4 FL and the fate-committed DN3b cells at day-16, LMK235 treatment for two days had the most striking effect on CD11b expression on the day-4 FL but had little effect on the other markers, and no effect on CD11b in the control DN3b cells (already committed to T cell fate) (Appendix C). This result probably suggested that the differentiation landscape might be skewed toward a myeloid-like feature. Confirming this would require extensive characterisation of the identity of cells that were changed by LMK235.

Next, I analysed how populations in T cell development were altered with more CD11b expression. From the CD44-CD11b density plot (Appendix C), I found a positive relation

between the expression of CD44 and CD11b. This positive relation probably makes sense because the progressive downregulation of CD44 is a feature of HSC differentiation, and the downregulation of CD11b is one of the features during T cell development. LMK235 treatment didn't alter this positive relation. ETP-like population ($CD44^{++}$) were highly expressed with CD11b from both LMK235 and control FL cells. Interestingly, I found the DN1, DN2 and DN3 compartments from LMK235-treated cells expressed higher CD11b than the control. Overall, these results together might suggest the downregulation of CD11b during Notch1-induced T cell development might require the class II HDAC activity. However, to confirm this hypothesis, CD11b expression upon LMK235 treatment should be assessed in $CD11b^{+}$ myeloid cells

The results of Appendix C showing CD11b was increasingly expressed in the DN1 compartment upon LMK235 treatment, suggesting haematopoiesis was altered. But expression of CD11b in DN3 and DN4 population were intriguing, because it was believed that T-cell commitment is completed at the DN2b stage (Rothenberg, 2011) A recent study show the sorted DN3 (ie. after fate commitment) can re-express CD11b if Tcf7 is depleted (Garcia-Perez et al., 2020), challenging the knowledge of irreversibility after T-cell fate commitment. Thus in part I wonder whether the effect showed in Appendix C was relative to the change of Tcf7 pathway(Xing et al., 2016). Although the methodology to modulate Tcf7 activity is yet to be optimised in the thesis, the results showing CD11b was not increasingly induced in the longer coculture nor in the sorted DN3b (Appendix C) challenged this possibility. Another possibility to explain the preliminary results in Appendix C is the LMK235-treated DN3 and DN4 cells were not the bona-fide population, but were mixed with faulty DN1-DN2 cells in which CD11b was wrongly maintained by LMK235, and then progressed to the DN3-DN4 stage following coculture. Therefore, I would ask whether LMK235 influences DN2 cells development. I applied LMK235 on the developing T cell at day 7 of OP9-DL1 coculture, which is the date that DN2 cells are expanding, for two days. At day 9, the control cells developed DN2, DN3 and DN4 populations; by contrast, the LMK235-treated progenitors were arrested at the DN2 stage (**Appendix D**). To further confirm this, sorted DN2 cells were cultured for 2 days under DMSO or LMK235 treatment (Appendix D). The reduced progression to the DN3 stage was compatible with the notion of a DN2 arrest, with many DN2 characteristics: FSC, SSC, IL7R and the correlation of CD27 with CD25 verified (Appendix D). Taken together, the results suggest the majority of DN2 cells were arrested by LMK235, but a minor population responded differently, which might progress beyond DN2 while expressing CD11b. These

data therefore provide preliminary evidence to suggest that LMK235 disrupts T cell development at DN2 stage.

As LMK235-treated progenitors didn't progress further to DN3, the occurrence of αβ or γδ lineage specification is likely limited. Intriguingly, upon LMK235 treatment, I found an increase of γδ TCR expression in DN1 population (**Appendix E**). This phenomenon was similarly shown in NOD mice that generated γδ T cell in ETP and DN2 populations (Yui et al., 2010). However, it is still unclear how DN1 cells can present γδ TCR. To address this, I took advantage of a report that some γδ T cells, for example IL-17A γδ T cell ($CD25^-CD24^-CD27^-CD44^+$), and IFN-γ γδ T cell ($CD25^-CD24^-CD27^+CD44^+$) (Pang et al., 2012), are $CD25^-CD44^+$, so can be falsely ascribed to the DN1 compartment. To examine this possibility, I checked CD27 expression in the DN1 gate (Appendix E). In the control cells, CD27 is abundant but γδ TCR is not expressed, by contrast, LMK235-treated DN1 cells show an increase of the $CD27^{LO}γδ^+$ cells, suggesting a change of γδ lineage specification was triggered by LMK235.

Overall, LMK235 treatment showed alteration in T cell fate commitment. One possibility is through a change to CD11b-associated pathway, and another possibility is through a lineage alteration toward some type of γδ T cells.

7.7.2 Potential HDAC4 pathway in regulating T cell development

If the results from LMK235 treatment can indicate the role of HDAC4, I would hypothesise HDAC4 is required for initial T cell fate determination. The strength of HDAC4 would be fine-tuned prior to DN3a stage, as a minimum fate plasticity should be maintained at DN2 stage. Once the cell has reached the DN3a stage, a strong HDAC4 expression might ensure to form some T cell clone. The precise comment will be proposed later based on the further identifications listed in the section below (Section 7.7.3).

An important study recently revealed a switch of Runx1 and Runx3 at the DN2a-DN2b transition, showing Runx1 is promoted and Runx3 is repressed (Shin et al., 2021). As HDAC4 was proven to bind Runx2 in bone (Vega et al., 2004), I wonder whether HDAC4 in developing T cell can select which Runx to bind, either to repress or to stabilise, and contribute to fate determination. The Runx specificity of HDAC4 will therefore require further clarification. On the other hand, another HDAC4 regulator, GSK3, has been

demonstrated to regulate T cell development (Wang et al., 2014). As GSK3 can regulate TCF/β-catenin pathway, together with TCF is often regulated by HDACs, it is likely HDAC4 would associate with the switch of TCF usage for T cell fate determination (Johnson et al., 2018).

7.7.3 Future directions regarding HDAC4

(1) It's really important to verify the mRNA profile of HDAC4 (and HDAC5) in developing T cells, to reassure the sharp upregulation at DN3a stage is real. Protein level will be important as well.

(2) Once the HDAC4/5 expression level is clarified, approaches (such as CRISPR-RNP or shRNA) to modulate expression level will be essential.

(3) RNA-Sequencing will be required to understand the effects from LMK235 treatment and HDAC4 depletion, regarding issue of lineage specification and transcriptional control.

(4) To prove whether LMK235 uniquely modulates HDAC4 activity in developing T cells, molecular tests such as *in vitro* acetylation assay should be applied. If that can be reassured, then the assays to understand fate determination as per assessments for ACY1215 (such as cell counts, kinetics and surface phenotyping) should be repeated.

7.8 Clinical epigenetic inhibitors to study T cell development and T-cell leukaemia

Besides the therapy using engineered lymphocytes to treat cancer (Section 6.2.3), using epigenetic-targeted therapy, often in a form of small molecule inhibitor, is also a focus for clinical research and personalised medicine (Cheng et al., 2019). While treating cancer with small molecule inhibitors, one caveat is that other normal tissue might be exposed to the same effect of inhibition. Therefore, the examination of the effect of epigenetic inhibitor on normal tissue or cells is important. In developing T cells, errors in differentiation can lead to T-ALL (Raboso-Gallego et al., 2019). T-ALL is driven by oncogene transcription (Raboso-Gallego et al., 2019). This aetiology leads to an assumption that cells from different T-ALL patients have different epigenetic backgrounds, which might guide the design of personalised epigenetic therapie. Here, I first discuss the link between the key findings in the thesis to the database of mRNA profile of T-ALL patients (Section 6.7.1). Second, based on a comprehensive review that summarized epigenetic regulators in T cell development (Shapiro and Shapiro, 2020), I further discuss the three main branches of epigenetic inhibitors that will have prospects to be used in the research of T cell development and T cell leukaemia (Section 6.7.2-6.7.4).

7.8.1 Surface receptors of DN3 cells are highly expressed in T-ALL patients

Our study of T cell development can contribute to the knowledge of T-ALL. By surveying a database that collected mRNA expression for malignant haematopoiesis, named BloodSpot (Bagger et al., 2018), I found most of key players in DN3 are upregulated in T-ALL patients (**Figure 7. 6**). First, as reported by many researchers, TCF-1 and LEF1 are oncogene for lymphomas (Yu et al., 2012), and are upregulated in T-ALL patients (Figure 7. 6). Second, Notch1, but not Notch3, is upregulated in T-ALL. Correlated with Bcl11b upregulation in T-ALL, these findings together show some T-ALL cells display DN3a phenotype as described previously (Curtis and McCormack, 2010). Interestingly, I also found CD28, but not other family members like PD-1, is upregulated in T-ALL patients. Together with CD2 and CD5 that are also upregulated in some T-ALL cells, these findings suggest some T-ALL cells display a DN3b phenotype. As Lck is also upregulated, I suspect TCR or pre-TCR are presumably activated for a part of T-ALL patients. However, I can't further evaluate whether T-ALL cell of DN3a phenotype is a distinct population from the one of DN3b phenotype.

From my results, I found ACY1215 might have potential to inhibit Notch1, CD5 and possibly Lef1. Therefore the message left for the aspect of therapy is: if a T-ALL patient has high expression of the three (Notch1, CD5, Lef1), then a treatment of ACY1215 might repress the expression. On the other hand, I noticed the CD28 expression in T-ALL patients is variable, meaning some of them are higher than the normal level but some of them are lower. In this case, if a patient has abnormally high expression of CD28, then the treatment of ACY1215 might be concerning because ACY1215 might tend to stabilise CD28, which might strengthen signalling to support leukemic T cell growth (Ecker et al., 2022; Leddon et al., 2020).

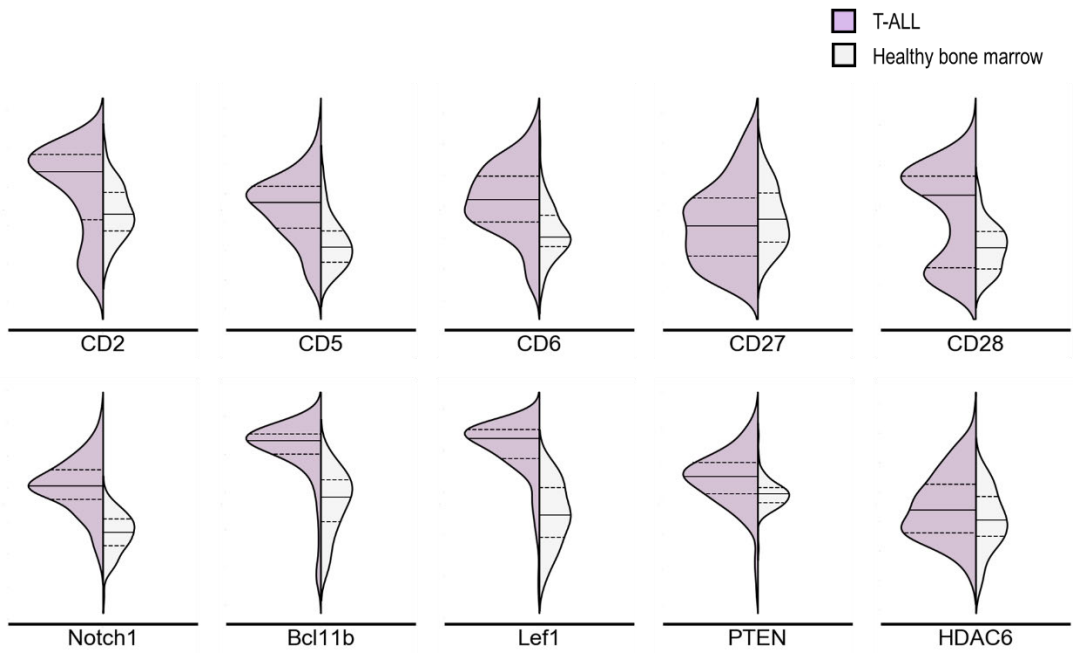


Figure 7. 6 The mRNA expression of CD28, CD2, CD5, Lef1 of T-ALL patients and health individuals

The statistics from Bloodspot were reorganised in the violin plots, showing the comparison between T-ALL patient and healthy individuals. The form of Mean ± SEM was used.

7.8.2 Inhibitors target histone acetylation

Inhibitors of histone deacetylases (HDAC) have shown great promise in mediating clinical antitumor responses, and are also evolving as a possible means of promoting immunity to cancer (Hogg et al., 2020). A deeper understanding of the impact of histone deacetylation on both tumour control and immune regulation is therefore a topic of increasing interest (Hogg et al., 2020).The HDAC6 selective inhibitor ACY1215 (Ricolinostat) can synergize with the BET inhibitor, JQ1, to recruit T cell mediated responses to lung adenocarcinoma, enhance T cell responses to *ex vivo* melanoma cultures, impair effector CD8 T cell function during skin inflammation, and alter Treg function (Adeegbe et al., 2017). (Laino et al., 2019; Tsuji et al., 2015; Xu et al., 2018; Zhang et al., 2021). HDACs 1, , and 3 are required at multiple stages of T cell development, but a role for HDAC6 in T cell development has not been identified (Wang et al., 2020). The treatment of ACY1215 to T-ALL as described in 6.7.1 can be considered for pre-clinical research. Opposite to HDACs, the p300/CBP is a histone acetyltransferase (HAT). CBP depletion impaired T cell development, but p300 depletion didn't (Kasper et al., 2006). There are many promising inhibitors for p300/CBP such as C646 and A-485, which have been demonstrated to influence at least H3K27 acetylation (Lasko et al., 2017). I have tested C646 in developing T cells, but have not identified a conclusive impact upon details. Further analysis using the newly developed A-485 inhibitor might yield more conclusive information regarding a role for p300/CBP in the developing T cell.

7.8.3 Inhibitors target histone methylation

Histone methyltransferase (HMT) inhibitors can be grouped into two categories: (1) to inhibit the methylation on lysines or arginine in the histone tails, and (2) to inhibit methylation on the histone cores. Enhancer of zeste homolog 2 (EZH2) controls gene expression via methylating histone H3 at lysine 27 (H3K27), and is required for DN-DP progression (Shapiro and Shapiro, 2020). A selective inhibitor of EZH2, UNC1999, is shown to preclude TCR activation, therefore it can potentially attenuate T cell–driven autoimmune activity (Dobenecker et al., 2018). This drug has not yet been tested in the context of T cell development, and it might be even more interesting to explore the effect of UNC1999 on positive or negative selection.

7.8.4 Inhibitors target DNA methylation

Most of DNMT enzymes play a role in T cell development (Shapiro and Shapiro, 2020). Some DNA methyltransferase inhibitors (DNMTis) have been approved by the U.S. FDA for the treatment of hematological malignancies, such as decitabine (Dacogen, DAC), and azacytidine (Vidaza, AZA). Decitabine can mediate γδ T cell anti-tumour activity and the immunological synapse formation (Weng et al., 2021). In pediatric T-cell acute lymphoblastic leukemia (T-ALL), azacytidine was shown to reduce hyper-methylation that caused by the loss of TET2 in T-ALL patients. These reagents are not yet used in the study of T cell development or the T-ALL genetics, therefore can be a future direction to investigate.

7.9 Targeting Scribble mis-localisation in cancer by small molecule inhibitors?

In the last part of thesis, the findings suggest Scribble localised at the cell cortex can scaffold a functional complex to maintain proper spindle orientation and to form a nascent daughter-daughter junction. As Scribble in epithelial cells is expressed beneath the cell membrane to support cell-cell junctions, the aberrant localisation of Scribble in the cytoplasm has been observed in many cancer types (see Chapter 1). Scribble is a large scaffold protein that has many post-translational modifications, and several surface chemistry can determine subcellular localisation of Scribble. Therefore, targeting Scribble PTM might influence Scribble localisation, or even rescue Scribble mis-localisation in cancer.

7.9.1 APT2 inhibitor restores Scribble palmitoylation

Scribble membrane localisation relies on palmitoylation on its N-terminus. There are two enzymes that modulate palmitoylation of Scribble: zDHHC protein acyl transferases (PATs), ZDHHC7, adds S-palmitoyl groups on Scribble (Chen et al., 2016); in contrast, Acyl protein thioesterases (APTs), APT2, remove S-palmitoyl groups from Scribble. The APT2 inhibitor ML349 rescued the Snail-induced Scribble mis-localisation (Hernandez et al., 2017). To my knowledge, ML349 has not yet been used in clinical trials. Oncogenic RAS also requires palmitoylation to be functioning (Chen et al., 2019; Dekker et al., 2010), and APT inhibition would likely maintain RAS activity. Because Scribble localisation is impaired by RAS, APT inhibition won't be appealing to the purpose of rescuing Scribble mis-localisation if the background is Ras^{+} tumour.

7.9.2 Using the synergistic effect of RAS/RAF inhibitors to rescue Scribble localisation?

Oncogenic Ras expression in cancer cell is often accompanied with the mis-localisation of Scribble, while Scribble overexpression to maintain cortical proportion seems able to resist a part of Ras-induced cytoplasmic translocation. Therefore, the aim of rescuing Scribble mis-localisation and the aim at targeting Ras activity can likely be seen as a combinatory task. For a long time, Ras is deemed as an undruggable target, but recently, many prominent successes have gradually emerged (Moore et al., 2020). However, Ras inhibitors are diverse, which is selective to different mutant. To my knowledge so far, only G12C inhibitors are profoundly developed, but seems not available for the G12V inhibitor.

Scribble has been shown to supress the functions of cancer signalling such as ERK or Raf (Nagasaka et al., 2010). These cancer signalling proteins are often cytosolic, but not membrane bound. Therefore, in my opinion, it seems Scribble is translocated to cytoplasm to play a suppressing role to these cytosolic cancer signalling. Namely, a hypothesis is Scribble mis-localisation (ie cytoplasmic translocation) is cued by some oncogenic signalling within the cancer cell itself, so as to be in a position to inhibit its cancer signalling. No matter what the driving force for Scribble to translocate to cytoplasm is, the maintenance of Scribble membrane localisation probably could be realised through blocking the activity of these cancerous proteins. To support this notion, many inhibitors of Raf have been developed, some even approved by US FDA. The effect of Raf inhibitor on Scribble localisation is not yet tested, thus my proposition is: by using a cell line such as Ras transformed MCF10A cell, which might show increased cytoplasmic Scribble, I can treat the cell with Raf inhibitor to test whether Raf inhibition can rescue Scribble mis-localisation.

7.10 Conclusion

7.10.1 T cell development

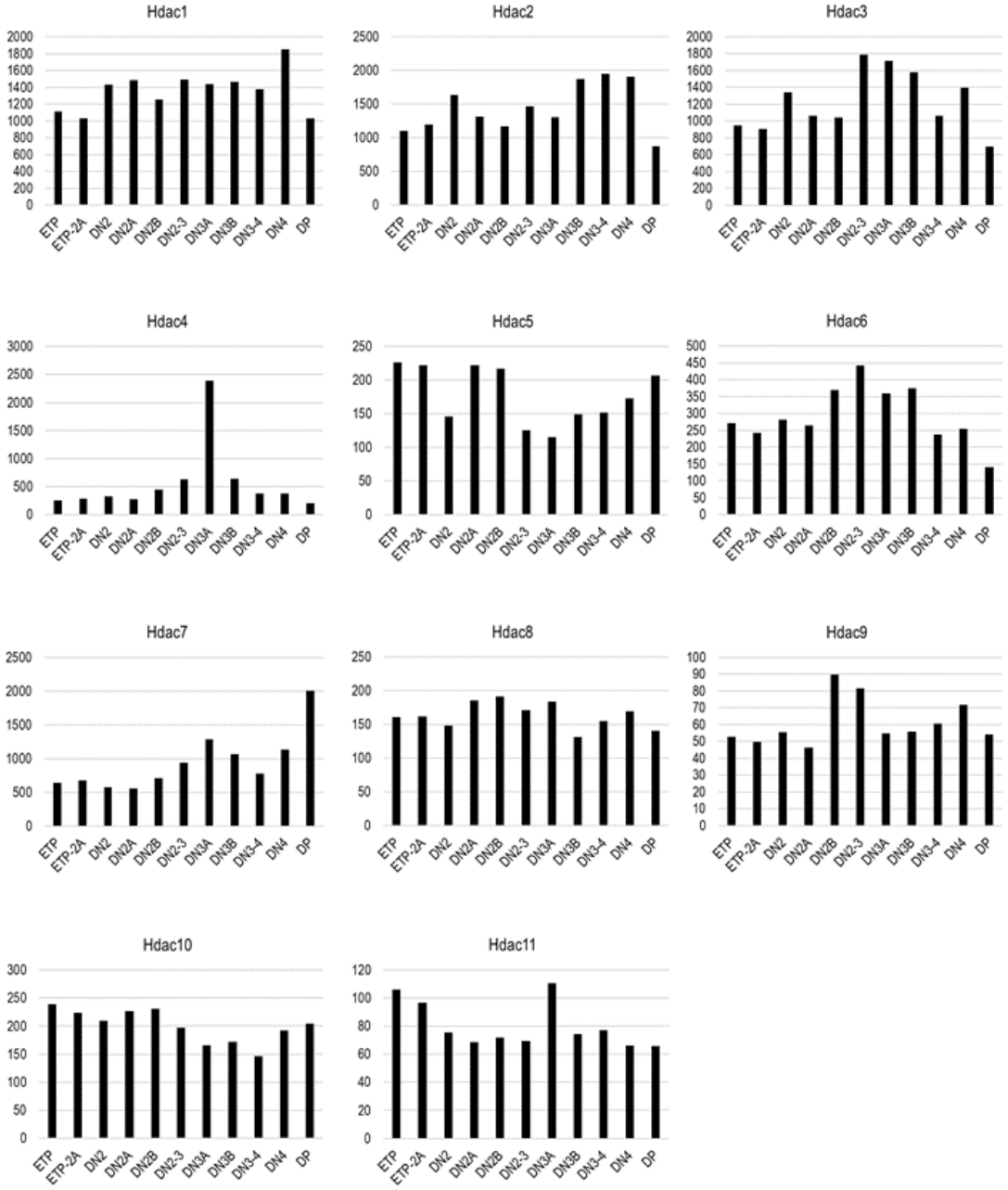
Through treatment with ACY1215, I define with higher resolution several stages of β-selection. First, after the DN3a stage is marked by upregulation of the co-receptor, CD28, I observed a transitional stage marked by low levels of the co-receptor, CD2, that I termed $DN3b^{Pre}$. $DN3b^{Pre}$ cells are enriched by ACY1215 treatment. An upregulation of CD2 marks passage through the β-selection checkpoint, to a phase I termed $DN3b^{Post}$. Second, I show that the transcription factor, Lef1 is upregulated in $DN3b^{Pre}$ cells, together with an increasing expression of the reporter and tuner of TCR signalling, CD5. Treatment with ACY1215 disrupts the functional association between pre-TCR signalling, CD5 and proliferation, enabling cells without TCRβ expression to bypass the β-selection checkpoint, and disrupting T cell development.

7.10.2 Epithelial cell mitosis

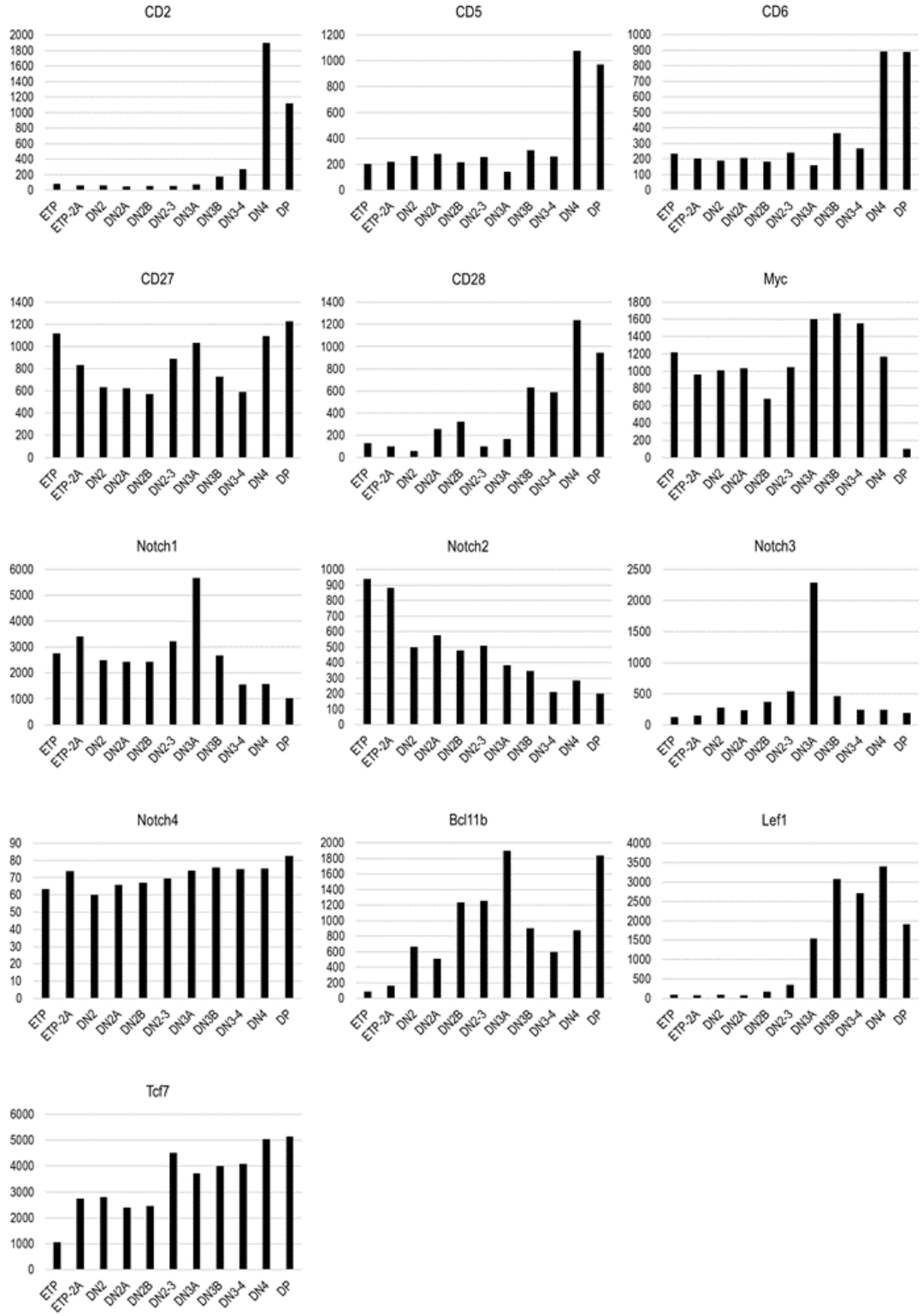
In epithelial cell division, a delicate control of daughter cell positioning is mediated through the compartments of adherent junction, Scribble and E-cadherin. I found Scribble forms is required to form a SEND (Scribble, E-cadherin, NuMA, Dlg) to position dividing cells, followed with a SEAD complex (Scribble, E-cadherin, Arp2/3, and Dlg) to scaffold the nascent junction between daughters.

Appendices

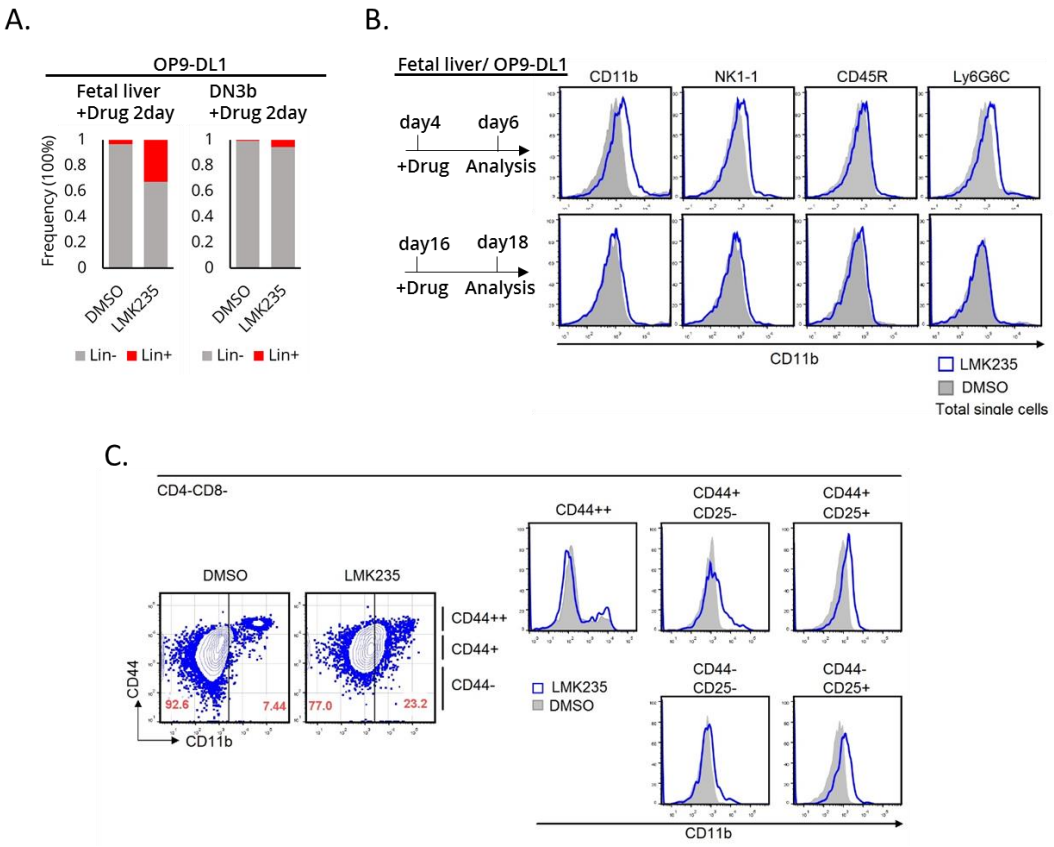
Appendix A


Hdac1
Hdac2
Hdac3
Hdac4
Hdac5
Hdac6
Hdac7
Hdac8
Hdac9
Hdac10
Hdac11
ETP
ETP-2A
DN2
DN2A
DN2B
DN2-3
DN3A
DN3B
DN3-4
DN4
DP

Appendix B


CD2
CD5
CD6
CD27
CD28
Myc
Notch1
Notch2
Notch3
Notch4
Bcl11b
Lef1
Tcf7
ETP
ETP-2A
DN2
DN2A
DN2B
DN2-3
DN3A
DN3B
DN3-4
DN4
DP

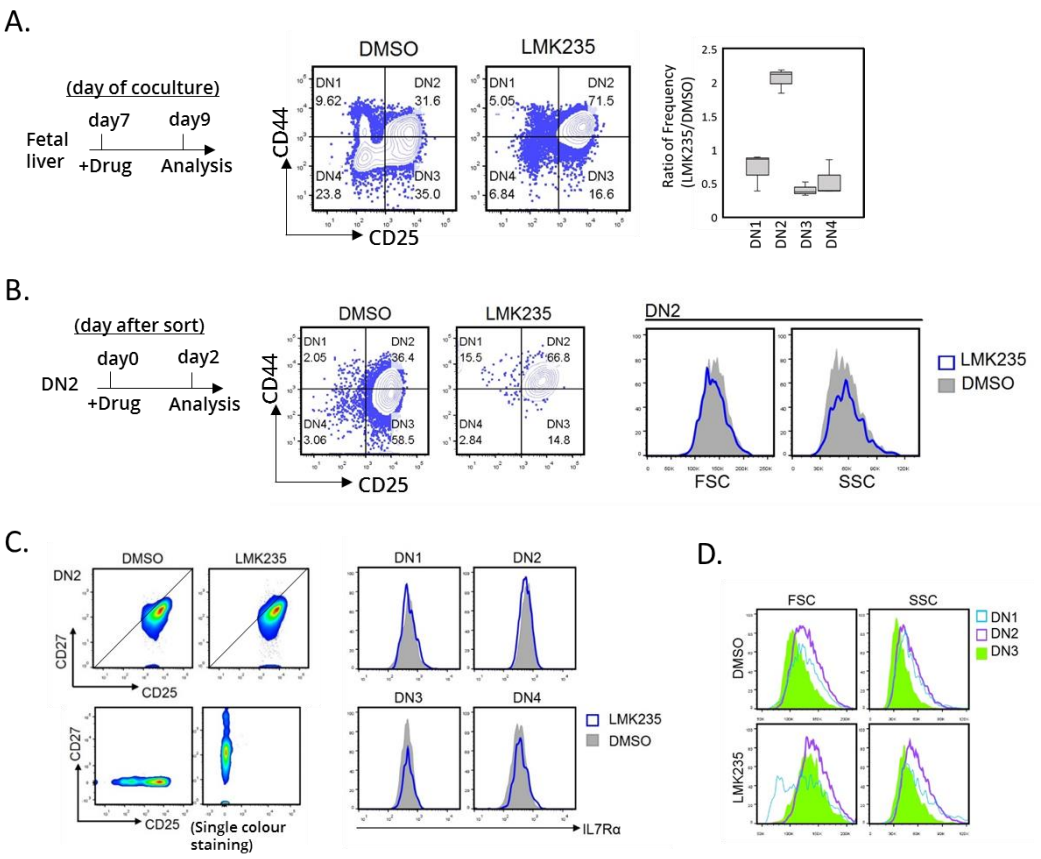
Appendix C



Appendix C. LMK235 alters T-lineage commitment

(A) Fetal liver progenitors and DN3b cells are treated with LMK235 or DMSO for 2 days, and the frequency of lineage+ (Red) and lineage- (Gray) were summarised in the stacked column. **(B)** The 4-day FL progenitor (upper) and the 16-day FL cells (bottom) were treated with LMK235 or DMSO for 2 days. The histograms show the lineage markers of CD11b, NK1-1, CD45R and Ly6G6C respectively, with LMK235 treatment (blue) and DMSO treatment (grey). **(C)** The analysis was extended from the young group of CD11b staining in (B). The expression of CD44 and CD11b of CD4-CD8- cells were assessed in the density plot. The histograms show the intensity of CD11b for the indicated population.

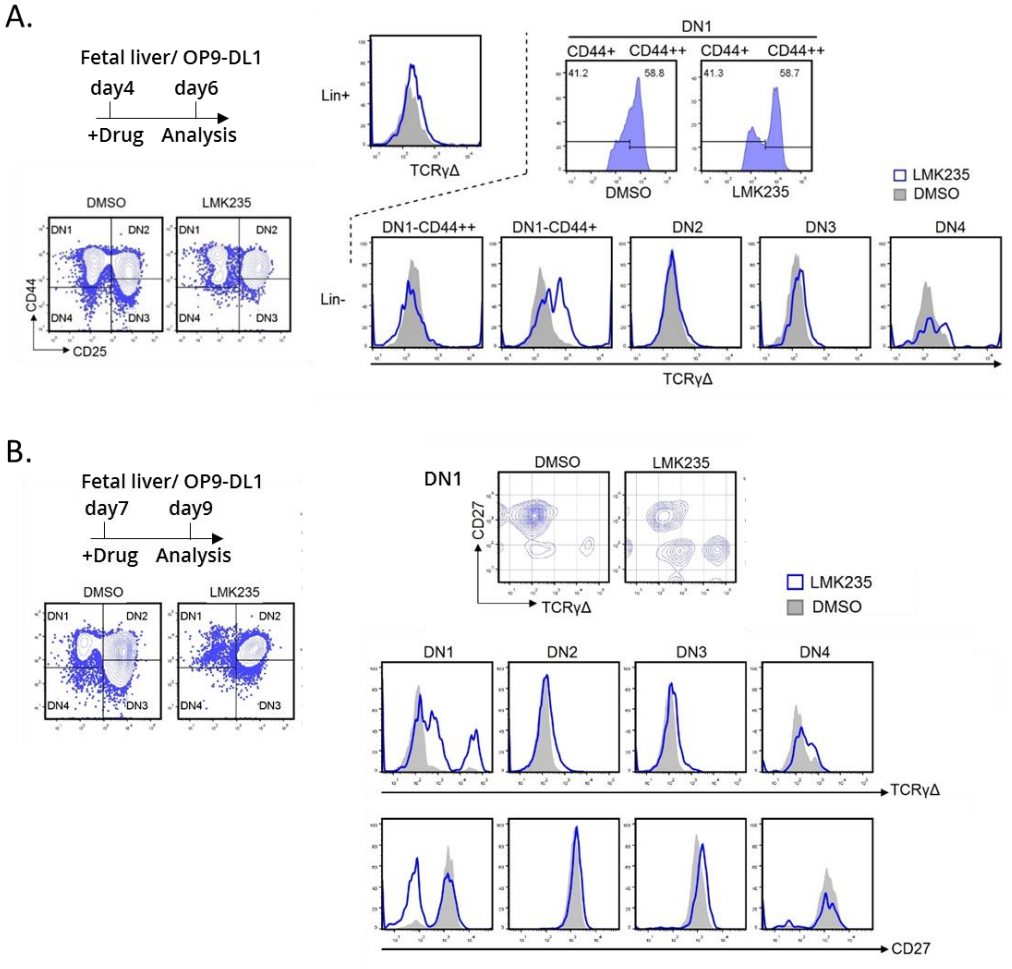
Appendix D



Appendix D. LMK235 disrupts DN2-to-DN3 transition

(A) Fetal liver cells were treated with LMK235 for 2 days as schematic indicated. $Lin^-CD4^-CD8^-$ cells are demarcated into DN1 to DN4 subpopulation. The ratio of frequency of each population between LMK235 and DMSO are summarised in the box plot (n=3). **(B)** The sorted DN2 cells were treated with LMK235 or DMSO for 2 days as schematic indicated. The expression of CD44 and CD25 was assessed and shown in a contour plot. The FSC and SSC of DN2 cells were examined in the histograms. **(C)** The DN2 population from (A) were accessed the expression of CD27 and CD25 (Left). IL7Ra expression of DN1, DN2, DN3 and DN4 were examined in histogram comparing LMK235 (blue line) and control (grey) (Right). **(D)** Morphologic factor FSC and SSC of DN1, DN2 and DN3 from the DMSO and LMK235-treated cells are compared in the histograms.

Appendix E



Appendix E. LMK235 elevates γδ vs αβ fate determination

The surface expression of TCRγδ were compared between LMK235 and DMSO from several development stage, including fetal liver progenitors at day4 **(A)**, and at day 9 **(B)** of OP9-DL1 coculture. (A) After the conventional DN1-4 are demarcated, CD44++ and CD44+ are divided, and then the expression of TCRγδ is compared between DMSO and LMK235 in histogram. (B) After the conventional DN1-4 are demarcated. For DN1 population, the cell is plotted as CD27 versus TCRγδ (right-above). The histogram (right-below) plotted by TCRγδ and CD27 are shown the comparison between DMSO and LMK235.

References

Chapter 1

Abdulla, H., A. Vo, B.J. Shields, T.J. Davies, J.T. Jackson, R. Alserihi, E.M. Viney, T. Wong, F. Yan, N.C. Wong, L. Demoen, D.J. Curtis, W.S. Alexander, P. Van Vlierberghe, R.A. Dickins, and M.P. McCormack. 2021. T-ALL can evolve to oncogene independence. Leukemia 35:2205-2219.

Aifantis, I., F. Gounari, L. Scorrano, C. Borowski, and H. von Boehmer. 2001. Constitutive pre-TCR signaling promotes differentiation through Ca2+ mobilization and activation of NF-kappaB and NFAT. *Nat Immunol* 2:403-409.

Albano, F., E. Vecchio, M. Renna, E. Iaccino, S. Mimmi, C. Caiazza, A. Arcucci, A. Avagliano, V. Pagliara, G. Donato, C. Palmieri, M. Mallardo, I. Quinto, and G. Fiume. 2019. Insights into Thymus Development and Viral Thymic Infections. *Viruses* 11:836.

Alberola-Ila, J., K.A. Hogquist, K.A. Swan, M.J. Bevan, and R.M. Perlmutter. 1996. Positive and negative selection invoke distinct signaling pathways. *J Exp Med* 184:9-18.

Alexander, T.B., and C.G. Mullighan. 2021. Molecular Biology of Childhood Leukemia. *Annual Review of Cancer Biology* 5:95-117.

Allam, A.H., M. Charnley, K. Pham, and S.M. Russell. 2021. Developing T cells form an immunological synapse for passage through the β-selection checkpoint. *Journal of Cell Biology* 220:

Arenzana, T.L., S. Lianoglou, A. Seki, C. Eidenschenk, T. Cheung, D. Seshasayee, T. Hagenbeek, A. Sambandam, R. Noubade, I. Peng, J. Lesch, J. DeVoss, X. Wu, W.P. Lee, P. Caplazi, J. Webster, J. Liu, V.C. Pham, D. Arnott, J.R. Lill, Z. Modrusan, A. Dey, and S. Rutz. 2018. Tumor suppressor BAP1 is essential for thymic development and proliferative responses of T lymphocytes. *Science immunology* 3:

Arenzana, T.L., H. Schjerven, and S.T. Smale. 2015. Regulation of gene expression dynamics during developmental transitions by the Ikaros transcription factor. *Genes Dev* 29:1801-1816.

Ashton-Rickardt, P.G., A. Bandeira, J.R. Delaney, L. Van Kaer, H.P. Pircher, R.M. Zinkernagel, and S. Tonegawa. 1994. Evidence for a differential avidity model of T cell selection in the thymus. *Cell* 76:651-663.

Astolfi, A., F. Vendemini, M. Urbini, F. Melchionda, R. Masetti, M. Franzoni, V. Libri, S. Serravalle, M. Togni, G. Paone, L. Montemurro, D. Bressanin, F. Chiarini, A.M. Martelli, R. Tonelli, and A. Pession. 2014. MYCN is a novel oncogenic target in pediatric T-cell Acute Lymphoblastic Leukemia. *Oncotarget* 5:120-130.

Avram, D., and D. Califano. 2014. The multifaceted roles of Bcl11b in thymic and peripheral T cells: impact on immune diseases. *J Immunol* 193:2059-2065.

Awadia, S., F. Huq, T.R. Arnold, S M. Goicoechea, Y.J. Sun, T. Hou, G. Kreider-Letterman, P. Massimi, L. Banks, E.J. Fuentes, A.L. Miller, and R. Garcia-Mata. 2019. SGEF forms a complex with Scribble and Dlg1 and regulates epithelial junctions and contractility. *J Cell Biol* 218:2699-2725.

Balciunaite, G., R. Ceredig, H.J. Fehling, J.C. Zuniga-Pflucker, and A.G. Rolink. 2005. The role of Notch and IL-7 signaling in early thymocyte proliferation and differentiation. *Eur J Immunol* 35:1292-1300.

Ballesteros-Arias, L., J.G. Silva, R.A. Paiva, B. Carbonetto, P. Faisca, and V.C. Martins. 2019a. T Cell Acute Lymphoblastic Leukemia as a Consequence of Thymus Autonomy. *J Immunol*

Ballesteros-Arias, L., J.G. Silva, R.A. Paiva, B. Carbonetto, P. Faísca, and V.C. Martins. 2019b. T Cell Acute Lymphoblastic Leukemia as a Consequence of Thymus Autonomy. *The Journal of Immunology* ji1801373.

Banks, L., D. Pim, and M. Thomas. 2012. Human tumour viruses and the deregulation of cell polarity in cancer. *Nature Reviews Cancer* 12:877-886.

Bardelli, V., S. Arniani, V. Pierini, T. Pierini, D. Di Giacomo, P. Gorello, M. Moretti, F. Pellanera, L. Elia, A. Vitale, C.T. Storlazzi, D. Tolomeo, E. Mastrodicasa, M. Caniglia, S. Chiaretti, L. Ruggeri, G. Roti, C. Schwab, C.J. Harrison, A. Almeida, T. Pieters, P. Van Vlierberghe, C. Mecucci, and R. La Starza. 2021. MYB rearrangements and over-expression in T-cell acute lymphoblastic leukemia. *Genes, Chromosomes and Cancer* 60:482-488.

Belver, L., and A. Ferrando. 2016. The genetics and mechanisms of T cell acute lymphoblastic leukaemia. *Nature Reviews Cancer* 16:494.

Bender, T.P., C.S. Kremer, M. Kraus, T. Buch, and K. Rajewsky. 2004. Critical functions for c-Myb at three checkpoints during thymocyte development. *Nat Immunol* 5:721-729.

Bensberg, M., O. Rundquist, A. Selimović, C. Lagerwall, M. Benson, M. Gustafsson, H. Vogt, A. Lentini, and C.E. Nestor. 2021. TET2 as a tumor suppressor and therapeutic target in T-cell acute lymphoblastic leukemia. *Proceedings of the National Academy of Sciences* 118:e2110758118.

Berga-Bolaños, R., M. Alberdi, M. Buxadé, J. Aramburu, and C. López-Rodríguez. 2013. NFAT5 induction by the pre–T-cell receptor serves as a selective survival signal in T-lymphocyte development. *Proceedings of the National Academy of Sciences* 110:16091-16096.

Bergstralh, D.T., H.E. Lovegrove, and D. St Johnston. 2013. Discs large links spindle orientation to apical-basal polarity in Drosophila epithelia. *Current biology CB* 23:1707-1712.

Berrazouane, S., M. Boisvert, S. Salti, W. Mourad, R. Al-Daccak, F. Barabé, and F. Aoudjit. 2019. Beta1 integrin blockade overcomes doxorubicin resistance in human T-cell acute lymphoblastic leukemia. *Cell death & disease* 10:357-357.

Bhardwaj, R., L. Kumar, D. Chhabra, N.K. Mehra, A. sharma, S. Mohanty, and V. Kochupillai. 2021. In vitro expansion of fetal liver hematopoietic stem cells. *Scientific Reports* 11:11879.

Boëda, B., and S. Etienne-Manneville. 2015. Spectrin binding motifs regulate Scribble cortical dynamics and polarity function. *eLife* 4:e04726.

Boehm, T. 2012. Self-renewal of thymocytes in the absence of competitive precursor replenishment. *J Exp Med* 209:1397-1400.

Bonello, T.T., W. Choi, and M. Peifer. 2019. Scribble and Discs-large direct initial assembly and positioning of adherens junctions during the establishment of apical-basal polarity. *Development* 146:

Bonello, T.T., and M. Peifer. 2018. Scribble: A master scaffold in polarity, adhesion, synaptogenesis, and proliferation. *Journal of Cell Biology* 218:742-756.

Bonnet, J., L. Rigal, O. Mondesert, R. Morin, G. Corsaut, M. Vigneau, B. Ducommun, and V. Lobjois. 2021. Mitotic arrest affects clustering of tumor cells. *Cell Division* 16:2.

Boudil, A., I.R. Matei, H.Y. Shih, G. Bogdanoski, J.S. Yuan, S.G. Chang, B. Montpellier, P.E. Kowalski, V. Voisin, S. Bashir, G.D. Bader, M.S. Krangel, and C.J. Guidos. 2015. IL-7 coordinates proliferation, differentiation and Tcra recombination during thymocyte beta-selection. *Nat Immunol* 16:397-405.

Bowen, S., D. Wangsa, T. Ried, F. Livak, and R.J. Hodes. 2013. Concurrent V(D)J recombination and DNA end instability increase interchromosomal trans -rearrangements in ATM-deficient thymocytes. *Nucleic acids research* 41:4535-4548.

Brumby, A.M., and H.E. Richardson. 2003. scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in Drosophila. *Embo j* 22:5769-5779.

Canté-Barrett, K., R.D. Mendes, Y. Li, E. Vroegindeweij, K. Pike-Overzet, T. Wabeke, A.W. Langerak, R. Pieters, F.J. Staal, and J.P. Meijerink. 2017. Loss of CD44(dim) Expression from Early Progenitor Cells Marks T-Cell Lineage Commitment in the Human Thymus. *Front Immunol* 8:32.

Carrington, E.M., D.M. Tarlinton, D.H. Gray, N.D. Huntington, Y. Zhan, and A.M. Lew. 2017. The life and death of immune cell types: the role of BCL-2 anti-apoptotic molecules. *Immunology & Cell Biology* 95:870-877.

Cauchy, P., M.A. Maqbool, J. Zacarias-Cabeza, L. Vanhille, F. Koch, R. Fenouil, M. Gut, I. Gut, M.A. Santana, A. Griffon, J. Imbert, C. Moraes-Cabe, J.C. Bories, P. Ferrier, S. Spicuglia, and J.C. Andrau. 2016. Dynamic recruitment of Ets1 to both nucleosome-occupied and -depleted enhancer regions mediates a transcriptional program switch during early T-cell differentiation. *Nucleic acids research* 44:3567-3585.

Caydasi, A.K., B. Ibrahim, and G. Pereira. 2010. Monitoring spindle orientation: Spindle position checkpoint in charge. *Cell Division* 5:28.

Challen, G.A., N. Boles, K.K.-Y. Lin, and M.A. Goodell. 2009. Mouse hematopoietic stem cell identification and analysis. *Cytometry A* 75:14-24.

Chan, C.J., C.P. Heisenberg, and T. Hiiragi. 2017. Coordination of Morphogenesis and Cell-Fate Specification in Development. *Current biology CB* 27:R1024-r1035.

Chann, A.S., and S M. Russell. 2019. An integrated transcriptional switch at the beta-selection checkpoint determines T cell survival, development and leukaemogenesis. *Biochemical Society transactions* 47:1077-1089.

Charnley, M., F. Anderegg, R. Holtackers, M. Textor, and P. Meraldi. 2013. Effect of Cell Shape and Dimensionality on Spindle Orientation and Mitotic Timing. *PLoS One* 8:e66918.

Chen, B., B. Zheng, M. DeRan, G.K. Jarugumilli, J. Fu, Y.S. Brooks, and X. Wu. 2016a. ZDHHC7-mediated S-palmitoylation of Scribble regulates cell polarity. *Nature Chemical Biology* 12:686-693.

Chen, L., D.P. Foreman, D.B. Sant'Angelo, and M.S. Krangel. 2016b. Yin Yang 1 Promotes Thymocyte Survival by Downregulating p53. *The Journal of Immunology* 196:2572-2582.

Chinen, J., H.M. Rosenblatt, E.O. Smith, W.T. Shearer, and L.M. Noroski. 2003. Long-term assessment of T-cell populations in DiGeorge syndrome. *J Allergy Clin Immunol* 111:573-579.

Ciofani, M., G.C. Knowles, D.L. Wiest, H. von Boehmer, and J.C. Zúñiga-Pflücker. 2006. Stage-Specific and Differential Notch Dependency at the αβ and γδ T Lineage Bifurcation. *Immunity* 25:105-116.

Ciofani, M., and J.C. Zuniga-Pflucker. 2005. Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. *Nat Immunol* 6:881-888.

Cleveland, S.M., S. Smith, R. Tripathi, E.M. Mathias, C. Goodings, N. Elliott, D. Peng, W. El-Rifai, D. Yi, X. Chen, L. Li, C. Mullighan, J.R. Downing, P. Love, and U.P. Dave. 2013. Lmo2 induces hematopoietic stem cell-like features in T-cell progenitor cells prior to leukemia. *Stem Cells* 31:882-894.

Comazzetto, S., M.M. Murphy, S. Berto, E. Jeffery, Z. Zhao, and S.J. Morrison. 2019. Restricted Hematopoietic Progenitors and Erythropoiesis Require SCF from Leptin Receptor+ Niche Cells in the Bone Marrow. *Cell stem cell* 24:477-486.e476.

Cron, M.A., É. Guillochon, L. Kusner, and R. Le Panse. 2020. Role of miRNAs in Normal and Myasthenia Gravis Thymus. *Frontiers in Immunology* 11:

Cui, Y., M. Onozawa, H.R. Garber, L. Samsel, Z. Wang, J.P. McCoy, S. Burkett, X. Wu, P.D. Aplan, and C.L. Mackall. 2015. Thymic expression of a T-cell receptor targeting a tumor-associated antigen coexpressed in the thymus induces T-ALL. *Blood* 125:2958-2967.

Curtis, D.J., and M.P. McCormack. 2010a. The Molecular Basis of Lmo2-Induced T-Cell Acute Lymphoblastic Leukemia. *Clinical Cancer Research* 16:5618-5623.

Curtis, D.J., and M.P. McCormack. 2010b. The molecular basis of Lmo2-induced T-cell acute lymphoblastic leukemia. *Clin Cancer Res* 16:5618-5623.

Den Bakker, M.A., and J.W. Oosterhuis. 2009. Tumours and tumour-like conditions of the thymus other than thymoma; a practical approach. *Histopathology* 54:69-89.

DeSandro, A.M., U.M. Nagarajan, and J.M. Boss. 2000. Associations and interactions between bare lymphocyte syndrome factors. *Molecular and cellular biology* 20:6587-6599.

Deseke, M., and I. Prinz. 2020. Ligand recognition by the γδ TCR and discrimination between homeostasis and stress conditions. *Cellular & Molecular Immunology* 17:914-924.

Dhatchinamoorthy, K., J.D. Colbert, and K.L. Rock. 2021. Cancer Immune Evasion Through Loss of MHC Class I Antigen Presentation. *Frontiers in Immunology* 12:

Dimitracopoulos, A., P. Srivastava, A. Chaigne, Z. Win, R. Shlomovitz, O.M. Lancaster, M. Le Berre, M. Piel, K. Franze, G. Salbreux, and B. Baum. 2020. Mechanochemical Crosstalk Produces Cell-Intrinsic Patterning of the Cortex to Orient the Mitotic Spindle. *Curr Biol* 30:3687-3696 e3684.

Ding, Y., B. Zhang, J.L. Payne, C. Song, Z. Ge, C. Gowda, S. Iyer, P.K. Dhanyamraju, G. Dorsam, M.E. Reeves, D. Desai, S. Huang, K.J. Payne, F. Yue, and S. Dovat. 2019. Ikaros tumor suppressor function includes induction of active enhancers and super-enhancers along with pioneering activity. *Leukemia* 33:2720-2731.

Dorronsoro, A., V. Lang, I. Ferrin, J. Fernández-Rueda, L. Zabaleta, E. Pérez-Ruiz, P. Sepúlveda, and C. Trigueros. 2020. Intracellular role of IL-6 in mesenchymal stromal cell immunosuppression and proliferation. *Scientific Reports* 10:21853.

Dose, M., A.O. Emmanuel, J. Chaumeil, J. Zhang, T. Sun, K. Germar, K. Aghajani, E.M. Davis, S. Keerthivasan, A.L. Bredemeyer, B.P. Sleckman, S.T. Rosen, J.A. Skok, M.M. Le Beau, K. Georgopoulos, and F. Gounari. 2014a. beta-Catenin induces T-cell transformation by promoting genomic instability. *Proc Natl Acad Sci U S A* 111:391-396.

Dose, M., A.O. Emmanuel, J. Chaumeil, J. Zhang, T. Sun, K. Germar, K. Aghajani, E.M. Davis, S. Keerthivasan, A.L. Bredemeyer, B.P. Sleckman, S.T. Rosen, J.A. Skok, M.M. Le Beau, K. Georgopoulos, and F. Gounari. 2014b. β-Catenin induces T-cell transformation by promoting genomic instability. *Proceedings of the National Academy of Sciences* 111:391-396.

Dose, M., I. Khan, Z. Guo, D. Kovalovsky, A. Krueger, H. von Boehmer, K. Khazaie, and F. Gounari. 2006. c-Myc mediates pre-TCR-induced proliferation but not developmental progression. *Blood* 108:2669-2677.

Dow, L.E., A.M. Brumby, R. Muratore, M.L. Coombe, K.A. Sedelies, J.A. Trapani, S.M. Russell, H.E. Richardson, and P.O. Humbert. 2003. hScrib is a functional homologue of the Drosophila tumour suppressor Scribble. *Oncogene* 22:9225-9230.

Dow, L.E., I.A. Elsum, C.L. King, K.M. Kinross, H.E. Richardson, and P.O. Humbert. 2008. Loss of human Scribble cooperates with H-Ras to promote cell invasion through deregulation of MAPK signalling. *Oncogene* 27:5988-6001.

Dzhagalov, I., and H. Phee. 2012. How to find your way through the thymus: a practical guide for aspiring T cells. *Cellular and molecular life sciences CMLS* 69:663-682.

El-Kassar, N., F.A. Flomerfelt, B. Choudhury, L.A. Hugar, K.S. Chua, V. Kapoor, P.J. Lucas, and R.E. Gress. 2012. High levels of IL-7 cause dysregulation of thymocyte development. *International Immunology* 24:661-671.

Elhanati, Y., Z. Sethna, C.G. Callan, Jr., T. Mora, and A.M. Walczak. 2018. Predicting the spectrum of TCR repertoire sharing with a data-driven model of recombination. *Immunological reviews* 284:167-179.

Elsum, I.A., and P.O. Humbert. 2013. Localization, Not Important in All Tumor-Suppressing Properties: A Lesson Learnt from Scribble. *Cells Tissues Organs* 198:1-11.

Emmanuel, A.O., S. Arnovitz, L. Haghi, P.S. Mathur, S. Mondal, J. Quandt, M.K. Okoreeh, M. Maienschein-Cline, K. Khazaie, M. Dose, and F. Gounari. 2018a. TCF-1 and HEB cooperate to establish the epigenetic and transcription profiles of CD4(+)CD8(+) thymocytes. *Nat Immunol* 19:1366-1378.

Emmanuel, A.O., S. Arnovitz, L. Haghi, P.S. Mathur, S. Mondal, J. Quandt, M.K. Okoreeh, M. Maienschein-Cline, K. Khazaie, M. Dose, and F. Gounari. 2018b. TCF-1 and HEB cooperate to establish the epigenetic and transcription profiles of CD4+CD8+ thymocytes. *Nature Immunology* 19:1366-1378.

Engel, I., and C. Murre. 2004. E2A proteins enforce a proliferation checkpoint in developing thymocytes. *The EMBO Journal* 23:202-211.

Erarslan-Uysal, B., J.B. Kunz, T. Rausch, P. Richter-Pechańska, I.A. van Belzen, V. Frismantas, B. Bornhauser, D. Ordoñez-Rueada, M. Paulsen, V. Benes, M. Stanulla, M. Schrappe, G. Cario, G. Escherich, K. Bakharevich, R. Kirschner-Schwabe, C. Eckert, T. Loukanov, M. Gorenflo, S.M. Waszak, J.-P. Bourquin, M.U. Muckenthaler, J.O. Korbel, and A.E. Kulozik. 2020. Chromatin accessibility landscape of pediatric T-lymphoblastic leukemia and human T-cell precursors. *EMBO Molecular Medicine* 12:e12104.

Feigin, M.E., S.D. Akshinthala, K. Araki, A.Z. Rosenberg, L.B. Muthuswamy, B. Martin, B.D. Lehmann, H.K. Berman, J.A. Pietenpol, R.D. Cardiff, and S.K. Muthuswamy. 2014a. Mislocalization of the cell polarity protein scribble promotes mammary tumorigenesis and is associated with basal breast cancer. *Cancer Res* 74:3180-3194.

Feigin, M.E., S.D. Akshinthala, K. Araki, A.Z. Rosenberg, L.B. Muthuswamy, B. Martin, B.D. Lehmann, H.K. Berman, J.A. Pietenpol, R.D. Cardiff, and S.K. Muthuswamy. 2014b. Mislocalization of the Cell Polarity Protein Scribble Promotes Mammary Tumorigenesis and Is Associated with Basal Breast Cancer. *Cancer Research* 74:3180-3194.

Ferrandino, F., G. Bernardini, G. Tsaouli, P. Grazioli, A.F. Campese, C. Noce, A. Ciuffetta, A. Vacca, Z.M. Besharat, D. Bellavia, I. Screpanti, and M.P. Felli. 2018. Intrathymic Notch3 and CXCR4 combinatorial interplay facilitates T-cell leukemia propagation. *Oncogene* 37:6285-6298.

Ferrando, A.A., D.S. Neuberg, J. Staunton, M.L. Loh, C. Huard, S.C. Raimondi, F.G. Behm, C.H. Pui, J.R. Downing, D.G. Gilliland, E.S. Lander, T R. Golub, and A.T. Look. 2002. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer cell* 1:75-87.

Frank, S.R., J.H. Bell, M. Frödin, and S.H. Hansen. 2012. A βPIX-PAK2 complex confers protection against Scrib-dependent and cadherin-mediated apoptosis. *Current biology CB* 22:1747-1754.

Gao, X., S. Qin, Y. Wu, C. Chu, B. Jiang, R.H. Johnson, D. Kuang, J. Zhang, X. Wang, A. Mehta, K.D. Tew, G.W. Leone, X.Z. Yu, and H. Wang. 2021. Nuclear PFKP promotes CXCR4-dependent infiltration by T cell acute lymphoblastic leukemia. *J Clin Invest* 131:

Garcia-Peydro, M., P. Fuentes, M. Mosquera, M.J. Garcia-Leon, J. Alcain, A. Rodriguez, P. Garcia de Miguel, P. Menendez, K. Weijer, H. Spits, D.T. Scadden, C. Cuesta-Mateos, C. Munoz-Calleja, F. Sanchez-Madrid, and M.L. Toribio. 2018. The NOTCH1/CD44 axis drives pathogenesis in a T cell acute lymphoblastic leukemia model. *J Clin Invest* 128:2802-2818.

García-Ramírez, I., S. Bhatia, G. Rodríguez-Hernández, I. González-Herrero, C. Walter, S. González de Tena-Dávila, S. Parvin, O. Haas, W. Woessmann, M. Stanulla, M. Schrappe, M. Dugas, Y. Natkunam, A. Orfao, V. Domínguez, B. Pintado, O. Blanco, D. Alonso-López, J. De Las Rivas, A. Martín-Lorenzo, R. Jiménez, F.J. García Criado, M.B. García Cenador, I.S. Lossos, C. Vicente-Dueñas, A. Borkhardt, J. Hauer, and I. Sánchez-García. 2018. Lmo2 expression defines tumor cell identity during T-cell leukemogenesis. *The EMBO Journal* e98783.

Geimer Le Lay, A.S., A. Oravecz, J. Mastio, C. Jung, P. Marchal, C. Ebel, D. Dembele, B. Jost, S. Le Gras, C. Thibault, T. Borggrefe, P. Kastner, and S. Chan. 2014. The tumor suppressor ikaros shapes the repertoire of notch target genes in T cells. *Sci Signal* 7:ra28.

Gekas, C., T. D'Altri, R. Aligué, J. González, L. Espinosa, and A. Bigas. 2016. β-Catenin is required for T-cell leukemia initiation and MYC transcription downstream of Notch1. *Leukemia* 30:2002.

Gennery, A. 2019. Recent advances in understanding RAG deficiencies. *F1000Research* 8:

Gerby, B., C.S. Tremblay, M. Tremblay, S. Rojas-Sutterlin, S. Herblot, J. Hebert, G. Sauvageau, S. Lemieux, E. Lecuyer, D.F. Veiga, and T. Hoang. 2014a. SCL, LMO1 and Notch1 Reprogram Thymocytes into Self-Renewing Cells. *PLoS Genet* 10:e1004768.

Gerby, B., C.S. Tremblay, M. Tremblay, S. Rojas-Sutterlin, S. Herblot, J. Hébert, G. Sauvageau, S. Lemieux, E. Lécuyer, D.F.T. Veiga, and T. Hoang. 2014b. SCL, LMO1 and Notch1 Reprogram Thymocytes into Self-Renewing Cells. *PLOS Genetics* 10:e1004768.

Gerby, B., D.F.T. Veiga, J. Krosl, S. Nourreddine, J. Ouellette, A. Haman, G. Lavoie, I. Fares, M. Tremblay, V. Litalien, E. Ottoni, M. Kosic, D. Geoffrion, J. Ryan, P.S. Maddox, J. Chagraoui, A. Marinier, J. Hébert, G. Sauvageau, B.H. Kwok, P.P. Roux, and T. Hoang. 2016. High-throughput screening in niche-based assay identifies compounds to target preleukemic stem cells. *The Journal of Clinical Investigation* 126:4569-4584.

Ginn, S.L., C.V. Hallwirth, S.H. Liao, E.T. Teber, J.W. Arthur, J. Wu, H.C. Lee, S.S. Tay, M. Hu, R.R. Reddel, M.P. McCormack, A.J. Thrasher, M. Cavazzana, S.I. Alexander, and I.E. Alexander. 2017. Limiting Thymic Precursor Supply Increases the Risk of Lymphoid Malignancy in Murine X-Linked Severe Combined Immunodeficiency. *Molecular therapy. Nucleic acids* 6:1-14.

Ginn, S.L., M.P. McCormack, and I.E. Alexander. 2018. Thymocyte self-renewal and oncogenic risk in immunodeficient mouse models: relevance for human gene therapy clinical trials targeting haematopoietic stem cell populations? *Mamm Genome* 29:771-776.

Girardi, T., C. Vicente, J. Cools, and K. De Keersmaecker. 2017. The genetics and molecular biology of T-ALL. *Blood* blood-2016-2010-706465.

Godde, N.J., J.M. Sheridan, L.K. Smith, H.B. Pearson, K.L. Britt, R.C. Galea, L.L. Yates, J.E. Visvader, and P.O. Humbert. 2014. Scribble Modulates the MAPK/Fra1 Pathway to Disrupt Luminal and Ductal Integrity and Suppress Tumour Formation in the Mammary Gland. *PLOS Genetics* 10:e1004323.

Goux, D., J.D. Coudert, D. Maurice, L. Scarpellino, G. Jeannet, S. Piccolo, K. Weston, J. Huelsken, and W. Held. 2005. Cooperating pre-T-cell receptor and TCF-1-dependent signals ensure thymocyte survival. *Blood* 106:1726-1733.

Grifoni, D., F. Garoia, C.C. Schimanski, G. Schmitz, E. Laurenti, P.R. Galle, A. Pession, S. Cavicchi, and D. Strand. 2004. The human protein Hugl-1 substitutes for Drosophila Lethal giant larvae tumour suppressor function in vivo. *Oncogene* 23:8688-8694.

Guidos, C.J., C.J. Williams, I. Grandal, G. Knowles, M.T. Huang, and J.S. Danska. 1996. V(D)J recombination activates a p53-dependent DNA damage checkpoint in scid lymphocyte precursors. *Genes Dev* 10:2038-2054.

Guo, Y., K.D. MacIsaac, Y. Chen, R.J. Miller, R. Jain, B. Joyce-Shaikh, H. Ferguson, I.M. Wang, R. Cristescu, J. Mudgett, L. Engstrom, K.J. Piers, G.A. Baltus, K. Barr, H. Zhang, H. Mehmet, L.G. Hegde, X. Hu, L.L. Carter, T.D. Aicher, G. Glick, D. Zaller, A. Hawwari, C.C. Correll, D.C. Jones, and D.J. Cua. 2016. Inhibition of RORγT Skews TCRα Gene Rearrangement and Limits T Cell Repertoire Diversity. *Cell Reports* 17:3206-3218.

Guo, Z., M. Dose, D. Kovalovsky, R. Chang, J. O'Neil, A.T. Look, H. von Boehmer, K. Khazaie, and F. Gounari. 2007. β-Catenin stabilization stalls the transition from double-positive to single-positive stage and predisposes thymocytes to malignant transformation. *Blood* 109:5463-5472.

Hacein-Bey-Abina, S., A. Garrigue, G.P. Wang, J. Soulier, A. Lim, E. Morillon, E. Clappier, L. Caccavelli, E. Delabesse, K. Beldjord, V. Asnafi, E. MacIntyre, L. Dal Cortivo, I. Radford, N. Brousse, F. Sigaux, D. Moshous, J. Hauer, A. Borkhardt, B.H. Belohradsky, U. Wintergerst, M.C. Velez, L. Leiva, R. Sorensen, N. Wulffraat, S. Blanche, F.D. Bushman, A. Fischer, and M. Cavazzana-Calvo. 2008. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest* 118:3132-3142.

Hagenbeek, T.J., M. Naspetti, F. Malergue, F. Garçon, J.A. Nunès, K.B. Cleutjens, J. Trapman, P. Krimpenfort, and H. Spits. 2004. The loss of PTEN allows TCR alphabeta lineage thymocytes to bypass IL-7 and Pre-TCR-mediated signaling. *J Exp Med* 200:883-894.

Haines, B B., C.J. Ryu, S. Chang, A. Protopopov, A. Luch, Y.H. Kang, D.D. Draganov, M.F. Fragoso, S.G. Paik, H.J. Hong, R.A. DePinho, and J. Chen. 2006. Block of T cell development in P53-deficient mice accelerates development of lymphomas with characteristic RAG-dependent cytogenetic alterations. *Cancer cell* 9:109-120.

Haks, M.C., P. Krimpenfort, J.H. van den Brakel, and A.M. Kruisbeek. 1999. Pre-TCR signaling and inactivation of p53 induces crucial cell survival pathways in pre-T cells. *Immunity* 11:91-101.

Hamilton, B.K., L. Rybicki, D. Abounader, K. Adekola, A. Advani, I. Aldoss, V. Bachanova, A. Bashey, S. Brown, M. DeLima, S. Devine, C.R. Flowers, S. Ganguly, M. Jagasia, V.E. Kennedy, D.D.H. Kim, J. McGuirk, V. Pullarkat, R. Romee, K. Sandhu, M. Smith, M. Ueda, A. Viswabandya, K. Vu, S. Wall, S.B. Zeichner, M.A. Perales, and N.S. Majhail. 2017. Allogeneic Hematopoietic Cell Transplantation for Adult T Cell Acute Lymphoblastic Leukemia. *Biol Blood Marrow Transplant* 23:1117-1121.

Harker, N., A. Garefalaki, U. Menzel, E. Ktistaki, T. Naito, K. Georgopoulos, and D. Kioussis. 2011. Pre-TCR signaling and CD8 gene bivalent chromatin resolution during thymocyte development. J Immunol 186:6368-6377.

Harland, K.L., A. Fox, S. Nüssing, L. Hensen, K. Kedzierska, S.J. Turner, and A. Kelso. 2018. Limited Phenotypic and Functional Plasticity of Influenza Virus–Specific Memory CD8⁺ T Cells during Activation in an Alternative Cytokine Environment. The Journal of Immunology 201:3282-3293.

Hathcock, K.S., S. Bowen, F. Livak, and R.J. Hodes. 2013. ATM Influences the Efficiency of TCRβ Rearrangement, Subsequent TCRβ-Dependent T Cell Development, and Generation of the Pre-Selection TCRβ CDR3 Repertoire. PLOS ONE 8:e62188.

Hayday, A.C., and D.J. Pennington. 2007. Key factors in the organized chaos of early T cell development. Nat Immunol 8:137-144.

Heizmann, B., P. Kastner, and S. Chan. 2018. The Ikaros family in lymphocyte development. Current Opinion in Immunology 51:14-23.

Helmink, B.A., A.L. Bredemeyer, B.-S. Lee, C.-Y. Huang, G.G. Sharma, L.M. Walker, J.J. Bednarski, W.-L. Lee, T.K. Pandita, C.H. Bassing, and B.P. Sleckman. 2009. MRN complex function in the repair of chromosomal Rag-mediated DNA double-strand breaks. The Journal of Experimental Medicine 206:669-679.

Helmink, B.A., and B.P. Sleckman. 2012. The response to and repair of RAG-mediated DNA double-strand breaks. Annual review of immunology 30:175-202.

Herranz, D., A. Ambesi-Impiombato, T. Palomero, S.A. Schnell, L. Belver, A.A. Wendorff, L. Xu, M. Castillo-Martin, D. Llobet-Navas, C. Cordon-Cardo, E. Clappier, J. Soulier, and A.A. Ferrando. 2014. A NOTCH1-driven MYC enhancer promotes T cell development, transformation and acute lymphoblastic leukemia. Nat Med 20:1130-1137.

Hoffman, E.S., L. Passoni, T. Crompton, T.M. Leu, D.G. Schatz, A. Koff, M.J. Owen, and A.C. Hayday. 1996. Productive T-cell receptor beta-chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. Genes Dev 10:948-962.

Hong, C., M.A. Luckey, and J.-H. Park. 2012. Intrathymic IL-7: the where, when, and why of IL-7 signaling during T cell development. Semin Immunol 24:151-158.

Hong, Z., Z. Wei, T. Xie, L. Fu, J. Sun, F. Zhou, M. Jamal, Q. Zhang, and L. Shao. 2021. Targeting chemokines for acute lymphoblastic leukemia therapy. Journal of Hematology & Oncology 14:48.

Höpner, S.S., A. Raykova, R. Radpour, M.A. Amrein, D. Koller, G.M. Baerlocher, C. Riether, and A.F. Ochsenbein. 2021. LIGHT/LTβR signaling regulates self-renewal and differentiation of hematopoietic and leukemia stem cells. Nature Communications 12:1065.

Hosokawa, H., M. Romero-Wolf, M.A. Yui, J. Ungerbäck, M.L.G. Quiloan, M. Matsumoto, K.I. Nakayama, T. Tanaka, and E.V. Rothenberg. 2018. Bcl11b sets pro-T cell fate by site-specific cofactor recruitment and by repressing Id2 and Zbtb16. Nat Immunol 19:1427-1440.

How, J.Y., R.K. Stephens, K.Y.B. Lim, P.O. Humbert, and M. Kvansakul. 2021. Structural basis of the human Scribble–Vangl2 association in health and disease. Biochemical Journal 478:1321-1332.

Howe, S.J., M.R. Mansour, K. Schwarzwaelder, C. Bartholomae, M. Hubank, H. Kempski, M.H. Brugman, K. Pike-Overzet, S.J. Chatters, D. de Ridder, K.C. Gilmour, S. Adams, S.I. Thornhill, K.L. Parsley, F.J. Staal, R.E. Gale, D.C. Linch, J. Bayford, L. Brown, M. Quaye, C. Kinnon, P. Ancliff, D.K. Webb, M. Schmidt, C. von Kalle, H.B. Gaspar, and A.J. Thrasher. 2008. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J Clin Invest 118:3143-3150.

Huang, C.-Y., G.G. Sharma, L.M. Walker, C.H. Bassing, T.K. Pandita, and B.P. Sleckman. 2007. Defects in coding joint formation in vivo in developing ATM-deficient B and T lymphocytes. The Journal of Experimental Medicine

Humbert, P.O., N.A. Grzeschik, A.M. Brumby, R. Galea, I. Elsum, and H.E. Richardson. 2008. Control of tumourigenesis by the Scribble/Dlg/Lgl polarity module. Oncogene 27:6888-6907.

Hwang, S., K.-D. Song, R. Lesourne, J. Lee, J. Pinkhasov, L. Li, D. El-Khoury, and P.E. Love. 2012. Reduced TCR signaling potential impairs negative selection but does not result in autoimmune disease. Journal of Experimental Medicine 209:1781-1795.

Inglesfield, S., E.J. Cosway, W.E. Jenkinson, and G. Anderson. 2019. Rethinking Thymic Tolerance: Lessons from Mice. Trends Immunol 40:279-291.

Inoue, M., Y. Fujii, M. Okumura, Y. Takeuchi, H. Shiono, S. Miyoshi, H. Matsuda, and R. Shirakura. 1998. Neoplastic thymic epithelial cells of human thymoma support T cell development from CD4-CD8- cells to CD4+CD8+ cells in vitro. Clin Exp Immunol 112:419-426.

Isoda, T., A.J. Moore, Z. He, V. Chandra, M. Aida, M. Denholtz, J. Piet van Hamburg, K.M. Fisch, A.N. Chang, S.P. Fahl, D.L. Wiest, and C. Murre. 2017. Non-coding Transcription Instructs Chromatin Folding and Compartmentalization to Dictate Enhancer-Promoter Communication and T Cell Fate. Cell 171:103-119.e118.

Issuree, P.D., C.P. Ng, and D.R. Littman. 2017. Heritable Gene Regulation in the CD4:CD8 T Cell Lineage Choice. Frontiers in immunology 8:291.

Ito, K., and K. Ito. 2016. Metabolism and the Control of Cell Fate Decisions and Stem Cell Renewal. Annu Rev Cell Dev Biol

Jacobsen, J.A., J. Woodard, M. Mandal, M.R. Clark, E.T. Bartom, M. Sigvardsson, and B.L. Kee. 2017. EZH2 Regulates the Developmental Timing of Effectors of the Pre-Antigen Receptor Checkpoints. The Journal of Immunology 1700319.

Janas, M.L., G. Varano, K. Gudmundsson, M. Noda, T. Nagasawa, and M. Turner. 2010. Thymic development beyond β-selection requires phosphatidylinositol 3-kinase activation by CXCR4. The Journal of Experimental Medicine 207:247-261.

Jung, H.-Y., L. Fattet, J.H. Tsai, T. Kajimoto, Q. Chang, A.C. Newton, and J. Yang. 2019. Apical–basal polarity inhibits epithelial–mesenchymal transition and tumour metastasis by PAR-complex-mediated SNAI1 degradation. Nature Cell Biology 21:359-371.

Kang, J., A. Volkmann, and D.H. Raulet. 2001. Evidence That γδ versus αβ T Cell Fate Determination Is Initiated Independently of T Cell Receptor Signaling. Journal of Experimental Medicine 193:689-698.

Karimi, M.M., Y. Guo, X. Cui, H.A. Pallikonda, V. Horková, Y.-F. Wang, S.R. Gil, G. Rodriguez-Esteban, I. Robles-Rebollo, L. Bruno, R. Georgieva, B. Patel, J. Elliott, M.H. Dore, D. Dauphars, M.S. Krangel, B. Lenhard, H. Heyn, A.G. Fisher, O. Štěpánek, and M. Merkenschlager. 2021. The order and logic of CD4 versus CD8 lineage choice and differentiation in mouse thymus. Nature Communications 12:99.

Kastenhuber, E.R., and S.W. Lowe. 2017. Putting p53 in Context. Cell 170:1062-1078.

Kelly, A.P., D.K. Finlay, H.J. Hinton, R.G. Clarke, E. Fiorini, F. Radtke, and D.A. Cantrell. 2007. Notch-induced T cell development requires phosphoinositide-dependent kinase 1. Embo j 26:3441-3450.

Khandanpour, C., J.D. Phelan, L. Vassen, J. Schutte, R. Chen, S.R. Horman, M.C. Gaudreau, J. Krongold, J. Zhu, W.E. Paul, U. Duhrsen, B. Gottgens, H.L. Grimes, and T. Moroy. 2013. Growth factor independence 1 antagonizes a p53-induced DNA damage response pathway in lymphoblastic leukemia. Cancer cell 23:200-214.

Khoury, M.J., and D. Bilder. 2020. Distinct activities of Scrib module proteins organize epithelial polarity. *Proceedings of the National Academy of Sciences* 117:11531-11540.

King, B., F. Boccalatte, K. Moran-Crusio, E. Wolf, J. Wang, C. Kayembe, C. Lazaris, X. Yu, B. Aranda-Orgilles, A. Lasorella, and I. Aifantis. 2016. The ubiquitin ligase Huwe1 regulates the maintenance and lymphoid commitment of hematopoietic stem cells. *Nature Immunology* 17:1312.

Klein-Hessling, S., R. Rudolf, K. Muhammad, K.P. Knobeloch, M.A. Maqbool, P. Cauchy, J.C. Andrau, A. Avots, C. Talora, V. Ellenrieder, I. Screpanti, E. Serfling, and A.K. Patra. 2016. A threshold level of NFATc1 activity facilitates thymocyte differentiation and opposes notch-driven leukaemia development. *Nature communications* 7:11841.

Klein, L., B. Kyewski, P.M. Allen, and K.A. Hogquist. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nature Reviews Immunology* 14:377-391.

Kleinmann, E., A.-S. Geimer Le Lay, M. Sellars, P. Kastner, and S. Chan. 2008a. Ikaros Represses the Transcriptional Response to Notch Signaling in T-Cell Development. *Molecular and Cellular Biology* 28:7465-7475.

Kleinmann, E., A.S. Geimer Le Lay, M. Sellars, P. Kastner, and S. Chan. 2008b. Ikaros represses the transcriptional response to Notch signaling in T-cell development. *Mol Cell Biol* 28:7465-7475.

Koch, U., and F. Radtke. 2011. Mechanisms of T cell development and transformation. *Annual review of cell and developmental biology* 27:539-562.

Kreslavsky, T., M. Gleimer, M. Miyazaki, Y. Choi, E. Gagnon, C. Murre, P. Sicinski, and H. von Boehmer. 2012. beta-Selection-induced proliferation is required for alphabeta T cell differentiation. *Immunity* 37:840-853.

Krueger, A., N. Ziętara, and M. Łyszkiewicz. 2017. T Cell Development by the Numbers. *Trends Immunol* 38:128-139.

Kurd, N.S., L.K. Lutes, J. Yoon, S.W. Chan, I.L. Dzhagalov, A.R. Hoover, and E.A. Robey. 2019. A role for phagocytosis in inducing cell death during thymocyte negative selection. *eLife* 8:e48097.

Kwan, J., A. Sczaniecka, E. Heidary Arash, L. Nguyen, C.C. Chen, S. Ratkovic, O. Klezovitch, L. Attisano, H. McNeill, A. Emili, and V. Vasioukhin. 2016. DLG5 connects cell polarity and Hippo signaling protein networks by linking PAR-1 with MST1/2. *Genes Dev* 30:2696-2709.

Larmonie, N.S.D., A. van der Spek, A.J.J.C. Bogers, J.J.M. van Dongen, and A.W. Langerak. 2014. Genetic and epigenetic determinants mediate proneness of oncogene breakpoint sites for involvement in TCR translocations. *Genes & Immunity* 15:72-81.

Le Bras, S., and R. Le Borgne. 2014. Epithelial cell division - multiplying without losing touch. *J Cell Sci* 127:5127-5137.

Lee, P.P., D.R. Fitzpatrick, C. Beard, H.K. Jessup, S. Lehar, K.W. Makar, M. Perez-Melgosa, M.T. Sweetser, M.S. Schlissel, S. Nguyen, S.R. Cherry, J.H. Tsai, S.M. Tucker, W.M. Weaver, A. Kelso, R. Jaenisch, and C.B. Wilson. 2001. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 15:763-774.

Legouis, R., A. Gansmuller, S. Sookhareea, J.M. Bosher, D.L. Baillie, and M. Labouesse. 2000. LET-413 is a basolateral protein required for the assembly of adherens junctions in Caenorhabditis elegans. *Nat Cell Biol* 2:415-422. taf/DynaPage.taf?file=/ncb/journal/v412/n417/full/ncb0700_0415.html taf/DynaPage.taf?file=/ncb/journal/v0702/n0707/abs/ncb0700_0415 html.

Lesman, A., J. Notbohm, D.A. Tirrell, and G. Ravichandran. 2014. Contractile forces regulate cell division in three-dimensional environments. *J Cell Biol* 205:155-162.

Levis, M. 2017. FLT3 dancing on the stem cell. *The Journal of experimental medicine* 214:1857-1859.

Li, J., L. Cheng, and H. Jiang. 2019. Cell shape and intercellular adhesion regulate mitotic spindle orientation. *Mol Biol Cell* 30:2458-2468.

Li, L., A. Mitra, K. Cui, B. Zhao, S. Choi, J.Y. Lee, D.B. Stamos, D. El-Khoury, C. Warzecha, K. Pfeifer, J. Hardwick, K. Zhao, B. Venters, U.P. Davé, and P.E. Love. 2020a. Ldb1 is required for Lmo2 oncogene-induced thymocyte self-renewal and T-cell acute lymphoblastic leukemia. *Blood* 135:2252-2265.

Li, X., R. Mizsei, K. Tan, R.J. Mallis, J.S. Duke-Cohan, A. Akitsu, P.W. Tetteh, A. Dubey, W. Hwang, G. Wagner, M.J. Lang, H. Arthanari, J.H. Wang, and E.L. Reinherz. 2021. Pre-T cell receptors topologically sample self-ligands during thymocyte β-selection. *Science* 371:181-185.

Li, Y., and K. Burridge. 2019. Cell-Cycle-Dependent Regulation of Cell Adhesions: Adhering to the Schedule: Three papers reveal unexpected properties of adhesion structures as cells progress through the cell cycle. *Bioessays* 41:e1800165.

Li, Y., K. Dong, X. Fan, J. Xie, M. Wang, S. Fu, and Q. Li. 2020b. DNT Cell-based Immunotherapy: Progress and Applications. *J Cancer* 11:3717-3724.

Lim, K.Y.B., N.J. Gödde, P.O. Humbert, and M. Kvansakul. 2017a. Structural basis for the differential interaction of Scribble PDZ domains with the guanine nucleotide exchange factor β-PIX. *J Biol Chem* 292:20425-20436.

Lim, K.Y.B., N.J. Gödde, P.O. Humbert, and M. Kvansakul. 2017b. Structural basis for the differential interaction of Scribble PDZ domains with the guanine nucleotide exchange factor β-PIX. *Journal of Biological Chemistry* 292:20425-20436.

Lin, L., S. Chen, H. Wang, B. Gao, B. Kallakury, K. Bhuvaneshwar, K. Cahn, Y. Gusev, X. Wang, Y. Wu, J.L. Marshall, X. Zhi, and A.R. He. 2021. SPTBN1 inhibits inflammatory responses and hepatocarcinogenesis via the stabilization of SOCS1 and downregulation of p65 in hepatocellular carcinoma. *Theranostics* 11:4232-4250.

Liu, Y., J. Easton, Y. Shao, J. Maciaszek, Z. Wang, M.R. Wilkinson, K. McCastlain, M. Edmonson, S.B. Pounds, L. Shi, X. Zhou, X. Ma, E. Sioson, Y. Li, M. Rusch, P. Gupta, D. Pei, C. Cheng, M.A. Smith, J.G. Auvil, D.S. Gerhard, M.V. Relling, N.J. Winick, A.J. Carroll, N.A. Heerema, E. Raetz, M. Devidas, C.L. Willman, R.C. Harvey, W.L. Carroll, K.P. Dunsmore, S.S. Winter, B.L. Wood, B.P. Sorrentino, J.R. Downing, M.L. Loh, S.P. Hunger, J. Zhang, and C.G. Mullighan. 2017. The genomic landscape of pediatric and young adult T-lineage acute lymphoblastic leukemia. *Nat Genet* advance online publication:

Lohia, M., Y. Qin, and I.G. Macara. 2012. The Scribble Polarity Protein Stabilizes E-Cadherin/p120-Catenin Binding and Blocks Retrieval of E-Cadherin to the Golgi. *PLOS ONE* 7:e51130.

Loontiens, S., A.-C. Dolens, S. Strubbe, I. Van de Walle, F.E. Moore, L. Depestel, S. Vanhauwaert, F. Matthijssens, D.M. Langenau, F. Speleman, P. Van Vlierberghe, K. Durinck, and T. Taghon. 2020. PHF6 Expression Levels Impact Human Hematopoietic Stem Cell Differentiation. *Frontiers in Cell and Developmental Biology* 8:

Lu, X., B. Su, H. Xia, X. Zhang, Z. Liu, Y. Ji, Z. Yang, L. Dai, L.M. Mayr, C. Moog, H. Wu, X. Huang, and T. Zhang. 2016. Low Double-Negative CD3+CD4−CD8− T Cells Are Associated with Incomplete Restoration of CD4+ T Cells and Higher Immune Activation in HIV-1 Immunological Non-Responders. *Frontiers in Immunology* 7:

Ludford-Menting, M.J., J. Oliaro, F. Sacirbegovic, E.T.Y. Cheah, N. Pedersen, S.J. Thomas, A. Pasam, R. Iazzolino, L.E. Dow, N.J. Waterhouse, A. Murphy, S. Ellis, M.J. Smyth, M.H. Kershaw, P.K. Darcy, P.O. Humbert, and S.M. Russell. 2005. A Network of PDZ-Containing Proteins Regulates T Cell Polarity and Morphology during Migration and Immunological Synapse Formation. *Immunity* 22:737-748.

Lum, S.H., B. Neven, M.A. Slatter, and A.R. Gennery. 2019. Hematopoietic Cell Transplantation for MHC Class II Deficiency. *Frontiers in Pediatrics* 7:

Luskey, B.D., M. Rosenblatt, K. Zsebo, and D.A. Williams. 1992. Stem Cell Factor, Interleukin-3, and Interleukin-6 Promote Retroviral-Mediated Gene Transfer Into Murine Hematopoietic Stem Cells. *Blood* 80:396-402.

Majumder, K., C.H. Bassing, and E.M. Oltz. 2015. Chapter Six - Regulation of Tcrb Gene Assembly by Genetic, Epigenetic, and Topological Mechanisms. In Advances in Immunology. C. Murre, editor Academic Press, 273-306.

Mallis, R.J., K. Bai, H. Arthanari, R.E. Hussey, M. Handley, Z. Li, L. Chingozha, J.S. Duke-Cohan, H. Lu, J.-H. Wang, C. Zhu, G. Wagner, and E.L. Reinherz. 2015a. Pre-TCR ligand binding impacts thymocyte development before αβTCR expression. *Proceedings of the National Academy of Sciences* 112:8373-8378.

Mallis, R.J., K. Bai, H. Arthanari, R.E. Hussey, M. Handley, Z. Li, L. Chingozha, J.S. Duke-Cohan, H. Lu, J.H. Wang, C. Zhu, G. Wagner, and E.L. Reinherz. 2015b. Pre-TCR ligand binding impacts thymocyte development before alphabetaTCR expression. *Proc Natl Acad Sci U S A* 112:8373-8378.

Malu, A., T. Hutchison, L. Yapindi, K. Smith, K. Nelson, R. Bergeson, J. Pope, M. Romeo, C. Harrod, L. Ratner, C. Van Lint, and R. Harrod. 2019. The human T-cell leukemia virus type-1 tax oncoprotein dissociates NF-κB p65RelA-Stathmin complexes and causes catastrophic mitotic spindle damage and genomic instability. *Virology* 535:83-101.

Mandal, M., C. Borowski, T. Palomero, A.A. Ferrando, P. Oberdoerffer, F. Meng, A. Ruiz-Vela, M. Ciofani, J.C. Zuniga-Pflucker, I. Screpanti, A.T. Look, S.J. Korsmeyer, K. Rajewsky, H. von Boehmer, and I. Aifantis. 2005. The BCL2A1 gene as a pre-T cell receptor-induced regulator of thymocyte survival. *J Exp Med* 201:603-614.

Mania, A., H. Kathrin, K. Henning, K. Paul, W. Anna-Carina, H.H.N. Ariane, A. Markus, M. Sylvia, K. Mark Phillip, H. Dorothee, J. Danny, T. Thomas, K. Andreas, D.M. Michael, and L. Nico. 2020. A 3D iPSC-differentiation model identifies interleukin-3 as a regulator of early human hematopoietic specification. *Haematologica* 106:1354-1367.

Martelli, A.M., F. Paganelli, A. Fazio, C. Bazzichetto, F. Conciatori, and J.A. McCubrey. 2019. The Key Roles of PTEN in T-Cell Acute Lymphoblastic Leukemia Development, Progression, and Therapeutic Response. *Cancers* 11:629.

Martins, V.C., E. Ruggiero, S.M. Schlenner, V. Madan, M. Schmidt, P.J. Fink, C. von Kalle, and H.R. Rodewald. 2012. Thymus-autonomous T cell development in the absence of progenitor import. *J Exp Med* 209:1409-1417.

Matei, I.R., R.A. Gladdy, L.M. Nutter, A. Canty, C.J. Guidos, and J.S. Danska. 2007. ATM deficiency disrupts Tcra locus integrity and the maturation of CD4+CD8+ thymocytes. *Blood* 109:1887-1896.

Matsumoto, A., S. Takeishi, and K.I. Nakayama. 2014. p57 regulates T-cell development and prevents lymphomagenesis by balancing p53 activity and pre-TCR signaling. *Blood* 123:3429-3439.

Matsumura, S., T. Kojidani, Y. Kamioka, S. Uchida, T. Haraguchi, A. Kimura, and F. Toyoshima. 2016. Interphase adhesion geometry is transmitted to an internal regulator for spindle orientation via caveolin-1. *Nature communications* 7:ncomms11858.

Mayans, S., D. Stepniak, S. Palida, A. Larange, J. Dreux, B. Arlian, R. Shinnakasu, M. Kronenberg, H. Cheroutre, and F. Lambolez. 2014. αβT cell receptors expressed by CD4(-)CD8αβ(-) intraepithelial T cells drive their fate into a unique lineage with unusual MHC reactivities. *Immunity* 41:207-218.

McCormack, M.P., B.J. Shields, J.T. Jackson, C. Nasa, W. Shi, N.J. Slater, C.S. Tremblay, T.H. Rabbitts, and D.J. Curtis. 2013a. Requirement for Lyl1 in a model of Lmo2-driven early T-cell precursor ALL. *Blood*

McCormack, M.P., B.J. Shields, J.T. Jackson, C. Nasa, W. Shi, N.J. Slater, C.S. Tremblay, T.H. Rabbitts, and D.J. Curtis. 2013b. Requirement for Lyl1 in a model of Lmo2-driven early T-cell precursor ALL. *Blood* 122:2093-2103.

McCormack, M.P., L.F. Young, S. Vasudevan, C.A. de Graaf, R. Codrington, T.H. Rabbitts, S.M. Jane, and D.J. Curtis. 2010a. The Lmo2 Oncogene Initiates Leukemia in Mice by Inducing Thymocyte Self-Renewal. *Science*

McCormack, M.P., L.F. Young, S. Vasudevan, C.A. de Graaf, R. Codrington, T.H. Rabbitts, S.M. Jane, and D.J. Curtis. 2010b. The Lmo2 oncogene initiates leukemia in mice by inducing thymocyte self-renewal. *Science* 327:879-883.

Meng, H., H. Zhao, X. Cao, J. Hao, H. Zhang, Y. Liu, M.-s. Zhu, L. Fan, L. Weng, L. Qian, X. Wang, and Y. Xu. 2019. Double-negative T cells remarkably promote neuroinflammation after ischemic stroke. *Proceedings of the National Academy of Sciences* 116:5558-5563.

Miao, L., Z. Song, L. Jin, Y.M. Zhu, L.P. Wen, and M. Wu. 2010. ARF antagonizes the ability of Miz-1 to inhibit p53-mediated transactivation. *Oncogene* 29:711-722.

Michie, A.M., and J.C. Zuniga-Pflucker. 2002. Regulation of thymocyte differentiation: pre-TCR signals and beta-selection. *Semin Immunol* 14:311-323.

Mingueneau, M., T. Kreslavsky, D. Gray, T. Heng, R. Cruse, J. Ericson, S. Bendall, M.H. Spitzer, G.P. Nolan, K. Kobayashi, H. von Boehmer, D. Mathis, C. Benoist, A.J. Best, J. Knell, A. Goldrath, V. Joic, D. Koller, T. Shay, A. Regev, N. Cohen, P. Brennan, M. Brenner, F. Kim, T. Nageswara Rao, A. Wagers, T. Heng, J. Ericson, K. Rothamel, A. Ortiz-Lopez, D. Mathis, C. Benoist, N.A. Bezman, J.C. Sun, G. Min-Oo, C.C. Kim, L.L. Lanier, J. Miller, B. Brown, M. Merad, E.L. Gautier, C. Jakubzick, G.J. Randolph, P. Monach, D.A. Blair, M.L. Dustin, S.A. Shinton, R.R. Hardy, D. Laidlaw, J. Collins, R. Gazit, D.J. Rossi, N. Malhotra, K. Sylvia, J. Kang, T. Kreslavsky, A. Fletcher, K. Elpek, A. Bellemare-Pelletier, D. Malhotra, and S. Turley. 2013a. The transcriptional landscape of αβ T cell differentiation. *Nat Immunol* 14:619-632.

Mingueneau, M., T. Kreslavsky, D. Gray, T. Heng, R. Cruse, J. Ericson, S. Bendall, M.H. Spitzer, G.P. Nolan, K. Kobayashi, H. von Boehmer, D. Mathis, C. Benoist, C. Immunological Genome, A.J. Best, J. Knell, A. Goldrath, V. Jojic, D. Koller, T. Shay, A. Regev, N. Cohen, P. Brennan, M. Brenner, F. Kim, T.N. Rao, A. Wagers, T. Heng, J. Ericson, K. Rothamel, A. Ortiz-Lopez, D. Mathis, C. Benoist, N.A. Bezman, J.C. Sun, G. Min-Oo, C.C. Kim, L.L. Lanier, J. Miller, B. Brown, M. Merad, E.L. Gautier, C. Jakubzick, G.J. Randolph, P. Monach, D.A. Blair, M.L. Dustin, S.A. Shinton, R.R. Hardy, D. Laidlaw, J. Collins, R. Gazit, D.J. Rossi, N. Malhotra, K. Sylvia, J. Kang, T. Kreslavsky, A. Fletcher, K. Elpek, A. Bellemare-Pelletier, D. Malhotra, and S. Turley. 2013b. The transcriptional landscape of alphabeta T cell differentiation. *Nat Immunol* 14:619-632.

Mitchison, T.J. 1992. Actin based motility on retraction fibers in mitotic PtK2 cells. *Cell Motil Cytoskeleton* 22:135-151.

Miyagi, S , P. Sroczynska, Y. Kato, Y. Nakajima-Takagi, M. Oshima, O. Rizq, N. Takayama, A. Saraya, S. Mizuno, F. Sugiyama, S. Takahashi, Y. Matsuzaki, J. Christensen, K. Helin, and A. Iwama. 2019. The chromatin-binding protein Phf6 restricts the self-renewal of hematopoietic stem cells. *Blood* 133:2495-2506.

Miyazaki, M., K. Miyazaki, K. Chen, Y. Jin, J. Turner, A.J. Moore, R. Saito, K. Yoshida, S. Ogawa, H.R. Rodewald, Y.C. Lin, H. Kawamoto, and C. Murre. 2017. The E-Id Protein Axis Specifies Adaptive Lymphoid Cell Identity and Suppresses Thymic Innate Lymphoid Cell Development. *Immunity* 46:818-834.e814.

Montero, A., B.N. Savani, A. Shenoy, E.J. Read, C.S. Carter, S.F. Leitman, S. Mielke, K. Rezvani, R. Childs, and A.J. Barrett. 2006. T-cell depleted peripheral blood stem cell allotransplantation with T-cell add-back for patients with hematological malignancies: effect of chronic GVHD on outcome. *Biol Blood Marrow Transplant* 12:1318-1325.

Morita, K., N. Jain, H. Kantarjian, K. Takahashi, H. Fang, M. Konopleva, S. El Hussein, F. Wang, N.J. Short, A. Maiti, K. Sasaki, G. Garcia-Manero, S. Konoplev, F. Ravandi, J.D. Khoury, and E. Jabbour. 2021. Outcome of T-cell acute lymphoblastic leukemia/lymphoma: Focus on near-ETP phenotype and differential impact of nelarabine. *American Journal of Hematology* 96:589-598.

Moutuou, M.M., G. Pagé, I. Zaid, S. Lesage, and M. Guimond. 2018. Restoring T Cell Homeostasis After Allogeneic Stem Cell Transplantation; Principal Limitations and Future Challenges. *Frontiers in immunology* 9:1237-1237.

Nagasaka, K., D. Pim, P. Massimi, M. Thomas, V. Tomaić, V.K. Subbaiah, C. Kranjec, S. Nakagawa, T. Yano, Y. Taketani, M. Myers, and L. Banks. 2010. The cell polarity regulator hScrib controls ERK activation through a KIM site-dependent interaction. *Oncogene* 29:5311-5321.

Naito, T., P. Gomez-Del Arco, C.J. Williams, and K. Georgopoulos. 2007. Antagonistic interactions between Ikaros and the chromatin remodeler Mi-2beta determine silencer activity and Cd4 gene expression. *Immunity* 27:723-734.

Navarro, C., S. Nola, S. Audebert, M.-J. Santoni, J.-P. Arsanto, C. Ginestier, S. Marchetto, J. Jacquemier, D. Isnardon, A. Le Bivic, D. Birnbaum, and J.-P. Borg. 2005a. Junctional recruitment of mammalian Scribble relies on E-cadherin engagement. *Oncogene* 24:4330-4339.

Navarro, C., S. Nola, S. Audebert, M.J. Santoni, J.P. Arsanto, C. Ginestier, S. Marchetto, J. Jacquemier, D. Isnardon, A. Le Bivic, D. Birnbaum, and J.P. Borg. 2005b. Junctional recruitment of mammalian Scribble relies on E-cadherin engagement. *Oncogene* 24:4330-4339.

Nestor-Bergmann, A., G.A. Stooke-Vaughan, G.K. Goddard, T. Starborg, O.E. Jensen, and S. Woolner. 2019. Decoupling the Roles of Cell Shape and Mechanical Stress in Orienting and Cueing Epithelial Mitosis. *Cell reports* 26:2088-2100 e2084.

Neumann, M., E. Coskun, L. Fransecky, L.H. Mochmann, I. Bartram, N.F. Sartangi, S. Heesch, N. Gökbuget, S. Schwartz, C. Brandts, C. Schlee, R. Haas, U. Dührsen, M. Griesshammer, H. Döhner, G. Ehninger, T. Burmeister, O. Blau, E. Thiel, D. Hoelzer, W.-K. Hofmann, and C.D. Baldus. 2013a. FLT3 mutations in early T-cell precursor ALL characterize a stem cell like leukemia and imply the clinical use of tyrosine kinase inhibitors. *PloS one* 8:e53190-e53190.

Neumann, M., S. Heesch, C. Schlee, S. Schwartz, N. Gökbuget, D. Hoelzer, N.P. Konstandin, B. Ksienzyk, S. Vosberg, A. Graf, S. Krebs, H. Blum, T. Raff, M. Brüggemann, W.-K. Hofmann, J. Hecht, S.K. Bohlander, P.A. Greif, and C.D. Baldus. 2013b. Whole-exome sequencing in adult ETP-ALL reveals a high rate of DNMT3A mutations. *Blood* 121:4749-4752.

Nicholson, J.M., J.C. Macedo, A.J. Mattingly, D. Wangsa, J. Camps, V. Lima, A.M. Gomes, S. Dória, T. Ried, E. Logarinho, and D. Cimini. 2015. Chromosome mis-segregation and cytokinesis failure in trisomic human cells. *eLife* 4:e05068.

Nicole, S.D.L., A.D. Willem, P.P.M. Jules, H. Irene, J.M.v.D. Jacques, and W.L. Anton. 2013. Breakpoint sites disclose the role of the V(D)J recombination machinery in the formation of T-cell receptor (TCR) and non-TCR associated aberrations in T-cell acute lymphoblastic leukemia. *Haematologica* 98:1173-1184.

Niwayama, R., P. Moghe, Y.-J. Liu, D. Fabrèges, F. Buchholz, M. Piel, and T. Hiiragi. 2019. A Tug-of-War between Cell Shape and Polarity Controls Division Orientation to Ensure Robust Patterning in the Mouse Blastocyst. *Developmental Cell* 51:564-574.e566.

Nonaka, S., T. Naito, H. Chen, M. Yamamoto, K. Moro, H. Kiyono, H. Hamada, and H. Ishikawa. 2005. Intestinal γδ T Cells Develop in Mice Lacking Thymus, All Lymph Nodes, Peyer's Patches, and Isolated Lymphoid Follicles. *The Journal of Immunology* 174:1906-1912.

Noronha, E.P., L.V.C. Marques, F.G. Andrade, L.C.S. Thuler, E. Terra-Granado, M.S. Pombo-de-Oliveira, B.C.S.G.o.A.L. , C. da Paz Zampier, T. da Conceição Barbosa, P. Chagas Neto, G. Dallapicola Brisson, F.V. dos Santos Bueno, I. Cezar Sardou, B. Gonçalves Aguiar, A.C. Silva Dias, P.C. de Brito, G. Pedral Sampaio, R. Antônio Gomes Oliveira, C.T. de Oliveira, C. Casagranda, G. Ramos Vera, G. Ribeiro Neves, I. Maria Quezado Magalhães, J. Carlos Córdoba, J. Teixeira Costa, R. Ferreira Marques, R. Pereira de Souza Barros, R. Sarkis Alves, R. Guedes, and S. Epelman. 2019. The Profile of Immunophenotype and Genotype Aberrations in Subsets of Pediatric T-Cell Acute Lymphoblastic Leukemia. *Frontiers in Oncology* 9:

Oravecz, A., A. Apostolov, K. Polak, B. Jost, S. Le Gras, S. Chan, and P. Kastner. 2015. Ikaros mediates gene silencing in T cells through Polycomb repressive complex 2. *Nat Commun* 6:8823.

Pabst, R. 2018. The bone marrow is not only a primary lymphoid organ: The critical role for T lymphocyte migration and housing of long-term memory plasma cells. *European Journal of Immunology* 48:1096-1100.

Paiva, R.A., C.V. Ramos, and V.C. Martins. 2018. Thymus autonomy as a prelude to leukemia. *The FEBS Journal* 285:4565-4574.

Paiva, R.A., A.G.G. Sousa, C.V. Ramos, M. Ávila, J. Lilue, T. Paixão, and V.C. Martins. 2021. Self-renewal of double-negative 3 early thymocytes enables thymus autonomy but compromises the β-selection checkpoint. *Cell Reports* 35:108967.

Papiernik, M., and C. Pontoux. 1990. In vivo and in vitro repertoire of CD3+CD4−CD8− thymocytes. *International Immunology* 2:407-412.

Parker, M.E., and M. Ciofani. 2020. Regulation of γδ T Cell Effector Diversification in the Thymus. *Frontiers in Immunology* 11:

Patra, A.K., A. Avots, R.P. Zahedi, T. Schuler, A. Sickmann, U. Bommhardt, and E. Serfling. 2013. An alternative NFAT-activation pathway mediated by IL-7 is critical for early thymocyte development. *Nat Immunol* 14:127-135.

Peaudecerf, L., G. Krenn, P. Goncalves, F. Vasseur, and B. Rocha. 2016. Thymocytes self-renewal: a major hope or a major threat? *Immunol Rev* 271:173-184.

Peaudecerf, L., S. Lemos, A. Galgano, G. Krenn, F. Vasseur, J.P. Di Santo, S. Ezine, and B. Rocha. 2012. Thymocytes may persist and differentiate without any input from bone marrow progenitors. *J Exp Med* 209:1401-1408.

Pénit, C., B. Lucas, and F. Vasseur. 1995. Cell expansion and growth arrest phases during the transition from precursor (CD4-8-) to immature (CD4+8+) thymocytes in normal and genetically modified mice. *The Journal of Immunology* 154:5103-5113.

Perniola, R. 2018. Twenty Years of AIRE. *Frontiers in Immunology* 9:

Petridou, N.I., and P.A. Skourides. 2016. A ligand-independent integrin beta1 mechanosensory complex guides spindle orientation. *Nature communications* 7:10899.

Pham, K., R. Shimoni, M. Charnley, M.J. Ludford-Menting, E.D. Hawkins, K. Ramsbottom, J. Oliaro, D. Izon, S.B. Ting, J. Reynolds, G. Lythe, C. Molina-Paris, H. Melichar, E. Robey, P.O. Humbert, M. Gu, and S.M. Russell. 2015a. Asymmetric cell division during T cell development controls downstream fate. *The Journal of Cell Biology* 210:933-950.

Pham, K., R. Shimoni, M. Charnley, M.J. Ludford-Menting, E.D. Hawkins, K. Ramsbottom, J. Oliaro, D. Izon, S.B. Ting, J. Reynolds, G. Lythe, C. Molina-Paris, H. Melichar, E. Robey, P.O. Humbert, M. Gu, and S.M. Russell. 2015b. Asymmetric cell division during T cell development controls downstream fate. *J Cell Biol* 210:933-950.

Pham, K., R. Shimoni, M.J. Ludford-Menting, C.J. Nowell, P. Lobachevsky, Z.e. Bomzon, M. Gu, T.P. Speed, C.J. McGlade, and S.M. Russell. 2013. Divergent lymphocyte signalling revealed by a powerful new tool for analysis of time-lapse microscopy. *Immunology & Cell Biology* 91:70-81.

Pitt, Lauren A., Anastasia N. Tikhonova, H. Hu, T. Trimarchi, B. King, Y. Gong, M. Sanchez-Martin, A. Tsirigos, Dan R. Littman, Adolfo A. Ferrando, Sean J. Morrison, David R. Fooksman, I. Aifantis, and Susan R. Schwab. 2015. CXCL12-Producing Vascular Endothelial Niches Control Acute T Cell Leukemia Maintenance. *Cancer cell* 27:755-768.

Prochazkova, J., and J.I. Loizou. 2016. Programmed DNA breaks in lymphoid cells: repair mechanisms and consequences in human disease. *Immunology* 147:11-20.

Qin, Y., C. Capaldo, B.M. Gumbiner, and I.G. Macara. 2005. The mammalian Scribble polarity protein regulates epithelial cell adhesion and migration through E-cadherin. *J Cell Biol* 171:1061-1071.

Ragkousi, K., and M.C. Gibson. 2014. Cell division and the maintenance of epithelial order. *J Cell Biol* 207:181-188.

Rashkovan, M., C. Vadnais, J. Ross, M. Gigoux, W.-K. Suh, W. Gu, C. Kosan, and T. Möröy. 2014. Miz-1 regulates translation of Trp53 via ribosomal protein L22 in cells undergoing V(D)J recombination. *Proceedings of the National Academy of Sciences* 111:E5411-E5419.

Ray, R.J., C.J. Paige, C. Furlonger, S.D. Lyman, and R. Rottapel. 1996. Flt3 ligand supports the differentiation of early B cell progenitors in the presence of interleukin-11 and interleukin-7. *Eur J Immunol* 26:1504-1510.

Rizzelli, F., M.G. Malabarba, S. Sigismund, and M. Mapelli. 2020. The crosstalk between microtubules, actin and membranes shapes cell division. *Open biology* 10:190314.

Roderick, J.E., J. Tesell, L.D. Shultz, M.A. Brehm, D.L. Greiner, M.H. Harris, L.B. Silverman, S.E. Sallan, A. Gutierrez, A.T. Look, J. Qi, J.E. Bradner, and M.A. Kelliher. 2014. c-Myc inhibition prevents leukemia initiation in mice and impairs the growth of relapsed and induction failure pediatric T-ALL cells. *Blood* 123:1040-1050.

Rodewald, H.-R. 1998. The thymus in the age of retirement. *Nature* 396:630-631.

Rothenberg, E.V., J. Ungerbäck, and A. Champhekar. 2016. Chapter Four - Forging T-Lymphocyte Identity: Intersecting Networks of Transcriptional Control. In Advances in Immunology. F.W. Alt, editor Academic Press, 109-174.

Rothenberg, E.V., J. Zhang, and L. Li. 2010. Multilayered specification of the T-cell lineage fate. *Immunological reviews* 238:150-168.

Saadaoui, M., M. Machicoane, F. di Pietro, F. Etoc, A. Echard, and X. Morin. 2014. Dlg1 controls planar spindle orientation in the neuroepithelium through direct interaction with LGN. *The Journal of cell biology*

Saba, I., C. Kosan, L. Vassen, L. Klein-Hitpass, and T. Möröy. 2011. Miz-1 Is Required To Coordinate the Expression of TCRβ and p53 Effector Genes at the Pre-TCR "β-Selection" Checkpoint. *The Journal of Immunology* 187:2982-2992.

Sagar, and D. Grün. 2020. Deciphering Cell Fate Decision by Integrated Single-Cell Sequencing Analysis. *Annual Review of Biomedical Data Science* 3:1-22.

Saidi, A., T. Li, F. Weih, P. Concannon, and Z.-Q. Wang. 2010. Dual Functions of Nbs1 in the Repair of DNA Breaks and Proliferation Ensure Proper V(D)J Recombination and T-Cell Development. *Molecular and Cellular Biology* 30:5572-5581.

Sambandam, A., I. Maillard, V.P. Zediak, L. Xu, R.M. Gerstein, J.C. Aster, W.S. Pear, and A. Bhandoola. 2005. Notch signaling controls the generation and differentiation of early T lineage progenitors. *Nature Immunology* 6:663-670.

Santoni, M.-J., R. Kashyap, L. Camoin, and J.-P. Borg. 2020. The Scribble family in cancer: twentieth anniversary. *Oncogene* 39:7019-7033.

Savi, F., I. Forno, A. Faversani, A. Luciani, S. Caldiera, S. Gatti, P. Foa, D. Ricca, G. Bulfamante, V. Vaira, and S. Bosari. 2014. miR-296/Scribble axis is deregulated in human breast cancer and miR-296 restoration reduces tumour growth in vivo. *Clinical Science* 127:233-242.

Schenk, R.L., S. Tuzlak, E.M. Carrington, Y. Zhan, S. Heinzel, C.E. Teh, D.H. Gray, L. Tai, A.M. Lew, A. Villunger, A. Strasser, and M.J. Herold. 2017. Characterisation of mice lacking all functional isoforms of the pro-survival BCL-2 family member A1 reveals minor defects in the haematopoietic compartment. *Cell death and differentiation* 24:534-545.

Schiroli, G., S. Ferrari, A. Conway, A. Jacob, V. Capo, L. Albano, T. Plati, M.C. Castiello, F. Sanvito, A.R. Gennery, C. Bovolenta, R. Palchaudhuri, D.T. Scadden, M.C. Holmes, A. Villa, G. Sitia, A. Lombardo, P. Genovese, and L. Naldini. 2017. Preclinical modeling highlights the therapeutic potential of hematopoietic stem cell gene editing for correction of SCID-X1. *Science translational medicine* 9:eaan0820.

Shi, J., M. Fallahi, J.-L. Luo, and H.T. Petrie. 2011. Nonoverlapping functions for Notch1 and Notch3 during murine steady-state thymic lymphopoiesis. *Blood* 118:2511-2519.

Shinkai, Y., S. Koyasu, K. Nakayama, K.M. Murphy, D.Y. Loh, E.L. Reinherz, and F.W. Alt. 1993. Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. *Science* 259:822-825.

Shortman, K., and L. Wu. 1996. Early T Lymphocyte Progenitors. *Annual review of immunology* 14:29-47.

Souabni, A., C. Cobaleda, M. Schebesta, and M. Busslinger. 2002. Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1. *Immunity* 17:781-793.

Spella, M., A. Marazioti, K.A.M. Arendt, and G.T. Stathopoulos. 2017. RAS oncogenes direct metastasis. *Mol Cell Oncol* 4:e1345711.

Stefaniak, M., G. Ręka, J. Zawitkowska, and M. Lejman. 2021. Hypodiploidy in a pediatric patient of T-cell acute lymphoblastic leukemia: a case report. *BMC Medical Genomics* 14:178.

Stephens, R., K. Lim, M. Portela, M. Kvansakul, P.O. Humbert, and H.E. Richardson. 2018. The Scribble Cell Polarity Module in the Regulation of Cell Signaling in Tissue Development and Tumorigenesis. *Journal of Molecular Biology* 430:3585-3612.

Stirewalt, D.L., and J.P. Radich. 2003. The role of FLT3 in haematopoietic malignancies. *Nature Reviews Cancer* 3:650-665.

Suster, S. 2006. Diagnosis of thymoma. *Journal of clinical pathology* 59:1238-1244.

Sutrave, G., E. Blyth, and D.J. Gottlieb. 2017. Cellular therapy for multiple pathogen infections after hematopoietic stem cell transplant. *Cytotherapy* 19:1284-1301.

Swamy, M., S. Pathak, K.M. Grzes, S. Damerow, L.V. Sinclair, D.M. van Aalten, and D.A. Cantrell. 2016. Glucose and glutamine fuel protein O-GlcNAcylation to control T cell self-renewal and malignancy. *Nat Immunol*

Taghon, T., I. Van de Walle, G. De Smet, M. De Smedt, G. Leclercq, B. Vandekerckhove, and J. Plum. 2009. Notch signaling is required for proliferation but not for differentiation at a well-defined beta-selection checkpoint during human T-cell development. *Blood* 113:3254-3263.

Taghon, T., M.A. Yui, R. Pant, R.A. Diamond, and E.V. Rothenberg. 2006. Developmental and molecular characterization of emerging beta- and gammadelta-selected pre-T cells in the adult mouse thymus. *Immunity* 24:53-64.

Tan, S.H., F.C. Bertulfo, and T. Sanda. 2017. Leukemia-Initiating Cells in T-Cell Acute Lymphoblastic Leukemia. *Frontiers in Oncology* 7:

Taniuchi, I. 2018. CD4 Helper and CD8 Cytotoxic T Cell Differentiation. *Annual review of immunology* 36:579-601.

Teague, T.K., C. Tan, J.H. Marino, B.K. Davis, A.A. Taylor, R.W. Huey, and C.J. Van De Wiele. 2010. CD28 expression redefines thymocyte development during the pre-T to DP transition. *International Immunology* 22:387-397.

Teng, G., Y. Maman, W. Resch, M. Kim, A. Yamane, J. Qian, K.R. Kieffer-Kwon, M. Mandal, Y. Ji, E. Meffre, M.R. Clark, L.G. Cowell, R. Casellas, and D.G. Schatz. 2015. RAG Represents a Widespread Threat to the Lymphocyte Genome. *Cell* 162:751-765.

Thery, M., and M. Bornens. 2006. Cell shape and cell division. *Curr Opin Cell Biol* 18:648-657.

Thomas, U., B. Phannavong, B. Müller, C.C. Garner, and E.D. Gundelfinger. 1997. Functional expression of rat synapse-associated proteins SAP97 and SAP102 in Drosophila dlg-1 mutants: effects on tumor suppression and synaptic bouton structure. *Mech Dev* 62:161-174.

Tie, R., H. Li, S. Cai, Z. Liang, W. Shan, B. Wang, Y. Tan, W. Zheng, and H. Huang. 2019. Interleukin-6 signaling regulates hematopoietic stem cell emergence. *Experimental & Molecular Medicine* 51:1-12.

Tinsley, K.W., C. Hong, M.A. Luckey, J.-Y. Park, G.Y. Kim, H.-w. Yoon, H.R. Keller, A.J. Sacks, L. Feigenbaum, and J.-H. Park. 2013. Ikaros is required to survive positive selection and to maintain clonal diversity during T-cell development in the thymus. *Blood* 122:2358-2368.

Tischner, D., C. Woess, E. Ottina, and A. Villunger. 2010. Bcl-2-regulated cell death signalling in the prevention of autoimmunity. *Cell Death &Amp; Disease* 1:e48.

Torcellan, T., H.R. Hampton, J. Bailey, M. Tomura, R. Brink, and T. Chtanova. 2017. In vivo photolabeling of tumor-infiltrating cells reveals highly regulated egress of T-cell subsets from tumors. *Proceedings of the National Academy of Sciences* 114:5677-5682.

Tottone, L., O. Lancho, J.-W. Loh, A. Singh, S. Kimura, J. Roels, A. Kuchmiy, S. Strubbe, M.A. Lawlor, V. da Silva-Diz, S. Luo, S. Gachet, C.A. García-Prieto, R. Hagelaar, M. Esteller, J.P.P. Meijerink, J. Soulier, T. Taghon, P. Van Vlierberghe, C.G. Mullighan, H. Khiabanian, P.P. Rocha, and D. Herranz. 2021. A Tumor Suppressor Enhancer of PTEN in T-cell Development and Leukemia. *Blood Cancer Discovery* 2:92-109.

Trampont, P.C., A.C. Tosello-Trampont, Y. Shen, A.K. Duley, A.E. Sutherland, T.P. Bender, D.R. Littman, and K.S. Ravichandran. 2010. CXCR4 acts as a costimulator during thymic beta-selection. *Nat Immunol* 11:162-170.

Tremblay, C.S., J. Saw, S.K. Chiu, N.C. Wong, K. Tsyganov, S. Ghotb, A.N. Graham, F. Yan, A.A. Guirguis, S.E. Sonderegger, N. Lee, P. Kalitsis, J. Reynolds, S.B. Ting, D.R. Powell, S.M. Jane, and D.J. Curtis. 2018a. Restricted cell cycle is essential for clonal evolution and therapeutic resistance of pre-leukemic stem cells. *Nature Communications* 9:3535.

Tremblay, C.S., J. Saw, S.K. Chiu, N.C. Wong, K. Tsyganov, S. Ghotb, A.N. Graham, F. Yan, A.A. Guirguis, S.E. Sonderegger, N. Lee, P. Kalitsis, J. Reynolds, S.B. Ting, D.R. Powell, S.M. Jane, and D.J. Curtis. 2018b. Restricted cell cycle is essential for clonal evolution and therapeutic resistance of pre-leukemic stem cells. *Nature communications* 9:3535-3535.

Tsuchiya, M., A. Giuliani, and K. Yoshikawa. 2020. Cell-Fate Determination from Embryo to Cancer Development: Genomic Mechanism Elucidated. *International Journal of Molecular Sciences* 21:4581.

Tuncay, H., and K. Ebnet. 2015. Cell adhesion molecule control of planar spindle orientation. *Cellular and molecular life sciences CMLS*

Vadillo, E., E. Dorantes-Acosta, R. Pelayo, and M. Schnoor. 2018. T cell acute lymphoblastic leukemia (T-ALL): New insights into the cellular origins and infiltration mechanisms common and unique among hematologic malignancies. *Blood Reviews* 32:36-51.

Van Thillo, Q., J. De Bie, J.A. Seneviratne, S. Demeyer, S. Omari, A. Balachandran, V. Zhai, W.L. Tam, B. Sweron, E. Geerdens, O. Gielen, S. Provost, H. Segers, N. Boeckx, G.M. Marshall, B.B. Cheung, K. Isobe, I. Kato, J. Takita, T.G. Amos, I.W. Deveson, H. McCalmont, R B. Lock, E.P. Oxley, M.M. Garwood, R.A. Dickins, A. Uyttebroeck, D.R. Carter, J. Cools, and C.E. de Bock. 2021. Oncogenic cooperation between TCF7-SPI1 and NRAS(G12D) requires β-catenin activity to drive T-cell acute lymphoblastic leukemia. *Nature Communications* 12:4164.

Vaughen, J., and T. Igaki. 2016. Slit-Robo Repulsive Signaling Extrudes Tumorigenic Cells from Epithelia. *Developmental cell* 39:683-695.

Velten, L., B.A. Story, P. Hernández-Malmierca, S. Raffel, D.R. Leonce, J. Milbank, M. Paulsen, A. Demir, C. Szu-Tu, R. Frömel, C. Lutz, D. Nowak, J.-C. Jann, C. Pabst, T. Boch, W.-K. Hofmann, C. Müller-Tidow, A. Trumpp, S. Haas, and L.M. Steinmetz. 2021. Identification of leukemic and pre-leukemic stem cells by clonal tracking from single-cell transcriptomics. *Nature Communications* 12:1366.

Venkei, Z.G., and Y.M. Yamashita. 2018. Emerging mechanisms of asymmetric stem cell division. *Journal of Cell Biology* 217:3785-3795.

Voll, R.E., E. Jimi, R.J. Phillips, D.F. Barber, M. Rincon, A.C. Hayday, R.A. Flavell, and S. Ghosh. 2000. NF-kappa B activation by the pre-T cell receptor serves as a selective survival signal in T lymphocyte development. *Immunity* 13:677-689.

Waghmare, I., and M. Kango-Singh. 2016. Loss of Cell Adhesion Increases Tumorigenic Potential of Polarity Deficient Scribble Mutant Cells. *PLOS ONE* 11:e0158081.

Waibel, M., S.J. Vervoort, I.Y. Kong, S. Heinzel, K.M. Ramsbottom, B P. Martin, E.D. Hawkins, and R.W. Johnstone. 2018. Epigenetic targeting of Notch1-driven transcription using the HDACi panobinostat is a potential therapy against T-cell acute lymphoblastic leukemia. *Leukemia* 32:237-241.

Walker, K.L., S.P. Rinella, N.J. Hess, D.P. Turicek, S.A. Kabakov, F. Zhu, M.N. Bouchlaka, S.L. Olson, M.M. Cho, A.E. Quamine, A.S. Feils, T.B. Gavcovich, L. Rui, and C.M. Capitini. 2021. CXCR4 allows T cell acute lymphoblastic leukemia to escape from JAK1/2 and BCL2 inhibition through CNS infiltration. *Leuk Lymphoma* 62:1167-1177.

Wang, H.-X., W. Pan, L. Zheng, X.-P. Zhong, L. Tan, Z. Liang, J. He, P. Feng, Y. Zhao, and Y.-R. Qiu. 2020. Thymic Epithelial Cells Contribute to Thymopoiesis and T Cell Development. *Frontiers in Immunology* 10:

Wang, L., O.E. Branson, K. Shilo, C.L. Hitchcock, and M.A. Freitas. 2016. Proteomic Signatures of Thymomas. *PLOS ONE* 11:e0166494.

Wang, Q., R. Yan, N. Pinnell, A.C. McCarter, Y. Oh, Y. Liu, C. Sha, N.F. Garber, Y. Chen, Q. Wu, C.-J. Ku, I. Tran, A. Serna Alarcon, R. Kuick, J.D. Engel, I. Maillard, T. Cierpicki, and M.Y. Chiang. 2018. Stage-specific roles for Zmiz1 in Notch-dependent steps of early T-cell development. *Blood* 132:1279-1292.

Wang, R., Y. Wang-Zhu, and H. Grey. 2002. Interactions between double positive thymocytes and high affinity ligands presented by cortical epithelial cells generate double negative thymocytes with T cell regulatory activity. *Proc Natl Acad Sci U S A* 99:2181-2186.

Warmerdam, D.O., I. Alonso-de Vega, W.W. Wiegant, B. van den Broek, M.B. Rother, R M. Wolthuis, R. Freire, H. van Attikum, R.H. Medema, and V.A. Smits. 2020. PHF6 promotes non-homologous end joining and G2 checkpoint recovery. *EMBO reports* 21:e48460.

Weber, G.F., M.A. Bjerke, and D.W. DeSimone. 2011. Integrins and cadherins join forces to form adhesive networks. *J Cell Sci* 124:1183-1193.

Weerkamp, F., J.J.M. van Dongen, and F.J.T. Staal. 2006. Notch and Wnt signaling in T-lymphocyte development and acute lymphoblastic leukemia. *Leukemia* 20:1197-1205.

Weich, A., D. Rogoll, S. Gawlas, L. Mayer, W. Weich, J. Pongracz, T. Kudlich, A. Meining, and M. Scheurlen. 2021. Wnt/β-Catenin Signaling Regulates CXCR4 Expression and [(68)Ga] Pentixafor Internalization in Neuroendocrine Tumor Cells. *Diagnostics (Basel)* 11:367.

Wendorff, A.A., S.A. Quinn, M. Rashkovan, C.J. Madubata, A. Ambesi-Impiombato, M.R. Litzow, M.S. Tallman, E. Paietta, M. Paganin, G. Basso, J.M. Gastier-Foster, M.L. Loh, R. Rabadan, P. Van Vlierberghe, and A.A. Ferrando. 2019.

Phf6 Loss Enhances HSC Self-Renewal Driving Tumor Initiation and Leukemia Stem Cell Activity in T-ALL. *Cancer Discovery* 9:436-451.

Westernberg, L., C. Conche, Y.H. Huang, S. Rigaud, Y. Deng, S. Siegemund, S. Mukherjee, L.A. Nosaka, J. Das, and K. Sauer. 2016. Non-canonical antagonism of PI3K by the kinase Itpkb delays thymocyte β-selection and renders it Notch-dependent. *eLife* 5:e10786.

Whiteley, A.E., T.T. Price, G. Cantelli, and D.A. Sipkins. 2021. Leukaemia: a model metastatic disease. *Nature Reviews Cancer* 21:461-475.

Williams, J.A., K.S. Hathcock, D. Klug, Y. Harada, B. Choudhury, J.P. Allison, R. Abe, and R.J. Hodes. 2005. Regulated costimulation in the thymus is critical for T cell development: dysregulated CD28 costimulation can bypass the pre-TCR checkpoint. *J Immunol* 175:4199-4207.

Wils, E.-J., E. Braakman, G.M.G.M. Verjans, E.J.C. Rombouts, A.E.C. Broers, H.G.M. Niesters, G. Wagemaker, F.J.T. Staal, B. Löwenberg, H. Spits, and J.J. Cornelissen. 2007. Flt3 Ligand Expands Lymphoid Progenitors Prior to Recovery of Thymopoiesis and Accelerates T Cell Reconstitution after Bone Marrow Transplantation. *The Journal of Immunology* 178:3551-3557.

Wilson, A., J.P. de Villartay, and H R. MacDonald. 1996. T cell receptor delta gene rearrangement and T early alpha (TEA) expression in immature alpha beta lineage thymocytes: implications for alpha beta/gamma delta lineage commitment. *Immunity* 4:37-45.

Wojciechowski, J., A. Lai, M. Kondo, and Y. Zhuang. 2007. E2A and HEB Are Required to Block Thymocyte Proliferation Prior to Pre-TCR Expression. *The Journal of Immunology* 178:5717-5726.

Wolfer, A., A. Wilson, M. Nemir, H.R. MacDonald, and F. Radtke. 2002. Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes. *Immunity* 16:869-879.

Wong, G.W., G.C. Knowles, T.W. Mak, A.A. Ferrando, and J.C. Zuniga-Pflucker. 2012. HES1 opposes a PTEN-dependent check on survival, differentiation, and proliferation of TCRbeta-selected mouse thymocytes. *Blood* 120:1439-1448.

Xi, H., R. Schwartz, I. Engel, C. Murre, and G.J. Kersh. 2006. Interplay between RORgammat, Egr3, and E proteins controls proliferation in response to pre-TCR signals. *Immunity* 24:813-826.

Yamasaki, S., E. Ishikawa, M. Sakuma, K. Ogata, K. Sakata-Sogawa, M. Hiroshima, D.L. Wiest, M. Tokunaga, and T. Saito. 2006. Mechanistic basis of pre-T cell receptor-mediated autonomous signaling critical for thymocyte development. *Nat Immunol* 7:67-75.

Yamauchi, T., and T. Moroishi. 2019. Hippo Pathway in Mammalian Adaptive Immune System. *Cells* 8:398.

Yamben, I.F., R.A. Rachel, S. Shatadal, N.G. Copeland, N.A. Jenkins, S. Warming, and A.E. Griep. 2013. Scrib is required for epithelial cell identity and prevents epithelial to mesenchymal transition in the mouse. *Dev Biol* 384:41-52.

Yang, F., W. Feng, H. Wang, L. Wang, X. Liu, R. Wang, C. Chen, X. Yang, D. Zhang, Q. Ren, and G. Zheng. 2020. Monocyte-Derived Leukemia-Associated Macrophages Facilitate Extramedullary Distribution of T-cell Acute Lymphoblastic Leukemia Cells. *Cancer Res* 80:3677-3691.

Yang, K., D.B. Blanco, X. Chen, P. Dash, G. Neale, C. Rosencrance, J. Easton, W. Chen, C. Cheng, Y. Dhungana, A. KC, W. Awad, X.-Z.J. Guo, P.G. Thomas, and H. Chi. 2018. Metabolic signaling directs the reciprocal lineage decisions of αβ and γδ T cells. *Science immunology* 3:eaas9818.

Yang, P., Y. Yang, P. Sun, Y. Tian, F. Gao, C. Wang, T. Zong, M. Li, Y. Zhang, T. Yu, and Z. Jiang. 2021. βII spectrin (SPTBN1): biological function and clinical potential in cancer and other diseases. *International Journal of Biological Sciences* 17:32-49.

Yashiro-Ohtani, Y., Y. He, T. Ohtani, M.E. Jones, O. Shestova, L. Xu, T.C. Fang, M.Y. Chiang, A.M. Intlekofer, S.C. Blacklow, Y. Zhuang, and W.S. Pear. 2009. Pre-TCR signaling inactivates Notch1 transcription by antagonizing E2A. *Genes Dev* 23:1665-1676.

Yates, L.L., C. Schnatwinkel, L. Hazelwood, L. Chessum, A. Paudyal, H. Hilton, M.R. Romero, J. Wilde, D. Bogani, J. Sanderson, C. Formstone, J.N. Murdoch, L.A. Niswander, A. Greenfield, and C.H. Dean. 2013. Scribble is required for normal epithelial cell–cell contacts and lumen morphogenesis in the mammalian lung. *Developmental Biology* 373:267-280.

Yui, M.A., N. Feng, and E.V. Rothenberg. 2010. Fine-scale staging of T cell lineage commitment in adult mouse thymus. *J Immunol* 185:284-293.

Yuzhe, S., C.B. Melanie, J.B. Helen, T. Ricky, N. Sirintra, E. Amir, H. Christina, V. Josef, H. Olaf, K.-H. Anja, and W.v.D. Frederik. 2020. Phase II-like murine trial identifies synergy between dexamethasone and dasatinib in T-cell acute lymphoblastic leukemia. *Haematologica* 106:1056-1066.

Zengdi, Z., H. Zan, O. Brianna, S. Chinmayi, Z. Hu, and R. Hai-Bin. 2019. Bone marrow adipose tissue-derived stem cell factor mediates metabolic regulation of hematopoiesis. *Haematologica* 104:1731-1743.

Zhan, Y., E.M. Carrington, Y. Zhang, S. Heinzel, and A.M. Lew. 2017. Life and Death of Activated T Cells: How Are They Different from Naïve T Cells? *Front Immunol* 8:1809.

Zhang, J.A., A. Mortazavi, B.A. Williams, B.J. Wold, and E.V. Rothenberg. 2012. Dynamic transformations of genome-wide epigenetic marking and transcriptional control establish T cell identity. *Cell* 149:467-482.

Zhang, L., J. Wu, Y. Feng, B. Khadka, Z. Fang, J. Gu, B. Tang, R. Xiao, G. Pan, and J.-J. Liu. 2021a. A Regulatory Loop Involving Notch and Wnt Signaling Maintains Leukemia Stem Cells in T-Cell Acute Lymphoblastic Leukemia. *Frontiers in cell and developmental biology* 9:678544-678544.

Zhang, L., J. Wu, Y. Feng, B. Khadka, Z. Fang, J. Gu, B. Tang, R. Xiao, G. Pan, and J.-J. Liu. 2021b. A Regulatory Loop Involving Notch and Wnt Signaling Maintains Leukemia Stem Cells in T-Cell Acute Lymphoblastic Leukemia. *Frontiers in Cell and Developmental Biology* 9:

Zhang, Y., J.-J. Qian, Y.-L. Zhou, X. Huang, J.-H. Li, X.-Y. Li, C.-Y. Li, H.-P. Wang, Y.-J. Lou, H.-T. Meng, W.-J. Yu, H.-Y. Tong, J. Jin, and H.-H. Zhu. 2020. Comparison of Early T-Cell Precursor and Non-ETP Subtypes Among 122 Chinese Adults With Acute Lymphoblastic Leukemia. *Frontiers in Oncology* 10:

Zhu, H., L. Zhang, Y. Wu, B. Dong, W. Guo, M. Wang, L. Yang, X. Fan, Y. Tang, N. Liu, X. Lei, and H. Wu. 2018. T-ALL leukemia stem cell 'stemness' is epigenetically controlled by the master regulator SPI1. *eLife* 7:e38314.

Chapter 2

Dow, L.E., J.S. Kauffman, J. Caddy, K. Zarbalis, A.S. Peterson, S.M. Jane, S.M. Russell, and P.O. Humbert. 2007. The tumour-suppressor Scribble dictates cell polarity during directed epithelial migration: regulation of Rho GTPase recruitment to the leading edge. *Oncogene* 26:2272-2282.

Laino, A.S., B.C. Betts, A. Veerapathran, I. Dolgalev, A. Sarnaik, S.N. Quayle, S.S. Jones, J.S. Weber, and D.M. Woods. 2019. HDAC6 selective inhibition of melanoma patient T-cells augments anti-tumor characteristics. *Journal for ImmunoTherapy of Cancer* 7:33.

Li, Y., D. Shin, and S.H. Kwon. 2013. Histone deacetylase 6 plays a role as a distinct regulator of diverse cellular processes. *The FEBS Journal* 280:775-793.

Porter, N.J., A. Mahendran, R. Breslow, and D.W. Christianson. 2017. Unusual zinc-binding mode of HDAC6-selective hydroxamate inhibitors. *Proceedings of the National Academy of Sciences* 114:13459-13464.

Xiao, Y , and X. Zhang. 2020. Recent advances in small molecular modulators targeting histone deacetylase 6. *Future Drug Discovery* 2:FDD53.

Chapter 3

Adeegbe, D.O., Y. Liu, P.H. Lizotte, Y. Kamihara, A.R. Aref, C. Almonte, R. Dries, Y. Li, S. Liu, X. Wang, T. Warner-Hatten, J. Castrillon, G.C. Yuan, N. Poudel-Neupane, H. Zhang, J.L. Guerriero, S. Han, M.M. Awad, D.A. Barbie, J. Ritz, S.S. Jones, P.S. Hammerman, J. Bradner, S.N. Quayle, and K.K. Wong. 2017. Synergistic Immunostimulatory Effects and Therapeutic Benefit of Combined Histone Deacetylase and Bromodomain Inhibition in Non-Small Cell Lung Cancer. Cancer discovery 7:852-867.

Allam, A.H., M. Charnley, K. Pham, and S.M. Russell. 2021. Developing T cells form an immunological synapse for passage through the beta-selection checkpoint. J Cell Biol 220:

Borggrefe, T., and F. Oswald. 2014. Keeping Notch Target Genes off: A CSL Corepressor Caught in the Act. Structure 22:3-5.

Brandstadter, J.D., and I. Maillard. 2019. Notch signalling in T cell homeostasis and differentiation. Open Biology 9:190187.

Brossard, C., M. Semichon, A. Trautmann, and G. Bismuth. 2003. CD5 inhibits signaling at the immunological synapse without impairing its formation. J Immunol 170:4623-4629.

Butler, B., and J.A. Cooper. 2009. Distinct roles for the actin nucleators Arp2/3 and hDia1 during NK-mediated cytotoxicity. Current biology : CB 19:1886-1896.

Chann, A.S., and S M. Russell. 2019. An integrated transcriptional switch at the beta-selection checkpoint determines T cell survival, development and leukaemogenesis. Biochemical Society transactions 47:1077-1089.

Charnley, M., M. Ludford-Menting, K. Pham, and S.M. Russell. 2020. A new role for Notch in the control of polarity and asymmetric cell division of developing T cells. J Cell Sci 133:jcs235358.

Deftos, M.L., and M.J. Bevan. 2000. Notch signaling in T cell development. Curr Opin Immunol 12:166-172.

Dustin, M.L., M.W. Olszowy, A.D. Holdorf, J. Li, S. Bromley, N. Desai, P. Widder, F. Rosenberger, P.A. van der Merwe, P.M. Allen, and A.S. Shaw. 1998. A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. Cell 94:667-677.

Dutta, A., B. Zhao, and P.E. Love. 2021. New insights into TCR β-selection. Trends in Immunology

Gerdes, J., H. Lemke, H. Baisch, H.H. Wacker, U. Schwab, and H. Stein. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133:1710-1715.

Hayday, A.C., and D.J. Pennington. 2007. Key factors in the organized chaos of early T cell development. Nat Immunol 8:137-144.

Hogg, S.J., P.A. Beavis, M.A. Dawson, and R.W. Johnstone. 2020. Targeting the epigenetic regulation of antitumour immunity. Nature reviews. Drug discovery

Laino, A.S., B.C. Betts, A. Veerapathran, I. Dolgalev, A. Sarnaik, S.N. Quayle, S.S. Jones, J.S. Weber, and D.M. Woods. 2019. HDAC6 selective inhibition of melanoma patient T-cells augments anti-tumor characteristics. Journal for immunotherapy of cancer 7:33.

Li, X., R. Mizsei, K. Tan, R.J. Mallis, J.S. Duke-Cohan, A. Akitsu, P.W. Tetteh, A. Dubey, W. Hwang, G. Wagner, M.J. Lang, H. Arthanari, J.H. Wang, and E.L. Reinherz. 2021. Pre-T cell receptors topologically sample self-ligands during thymocyte beta-selection. Science 371:181-185.

Magner, W.J., A.L. Kazim, C. Stewart, M.A. Romano, G. Catalano, C. Grande, N. Keiser, F. Santaniello, and T.B. Tomasi. 2000. Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. J Immunol 165:7017-7024.

Magupalli, V.G., R. Negro, Y. Tian, A.V. Hauenstein, G. Di Caprio, W. Skillern, Q. Deng, P. Orning, H.B. Alam, Z. Maliga, H. Sharif, J.J. Hu, C.L. Evavold, J.C. Kagan, F.I. Schmidt, K.A. Fitzgerald, T. Kirchhausen, Y. Li, and H. Wu. 2020. HDAC6 mediates an aggresome-like mechanism for NLRP3 and pyrin inflammasome activation. Science 369:eaas8995.

Miller, I., M. Min, C. Yang, C. Tian, S. Gookin, D. Carter, and S.L. Spencer. 2018. Ki67 is a Graded Rather than a Binary Marker of Proliferation versus Quiescence. Cell reports 24:1105-1112.e1105.

Parker, M.E., and M. Ciofani. 2020. Regulation of γδ T Cell Effector Diversification in the Thymus. Frontiers in Immunology 11:

Radtke, F., N. Fasnacht, and H.R. Macdonald. 2010. Notch signaling in the immune system. Immunity 32:14-27.

Reitermaier, R., T. Krausgruber, N. Fortelny, T. Ayub, P.A. Vieyra-Garcia, P. Kienzl, P. Wolf, A. Scharrer, C. Fiala, M. Kölz, M. Hiess, M. Vierhapper, C. Schuster, A. Spittler, C. Worda, W. Weninger, C. Bock, W. Eppel, and A. Elbe-Bürger. 2021. αβγδ T cells play a vital role in fetal human skin development and immunity. Journal of Experimental Medicine 218:

Rothenberg, E.V. 2011. T cell lineage commitment: identity and renunciation. J Immunol 186:6649-6655.

Serrador, J.M , J.R. Cabrero, D. Sancho, M. Mittelbrunn, A. Urzainqui, and F. Sanchez-Madrid. 2004. HDAC6 deacetylase activity links the tubulin cytoskeleton with immune synapse organization. Immunity 20:417-428.

Shapiro, M.J., and V.S. Shapiro. 2020. Chromatin-Modifying Enzymes in T Cell Development. Annu Rev Immunol 38:397-419.

Shi, P., Y. Wang, Y. Huang, C. Zhang, Y. Li, Y. Liu, T. Li, W. Wang, X. Liang, and C. Wu. 2019. Arp2/3-branched actin regulates microtubule acetylation levels and affects mitochondrial distribution. Journal of Cell Science 132:

Taghon, T., M.A. Yui, R. Pant, R.A. Diamond, and E.V. Rothenberg. 2006. Developmental and molecular characterization of emerging beta- and gammadelta-selected pre-T cells in the adult mouse thymus. Immunity 24:53-64.

Teague, T.K., C. Tan, J.H. Marino, B.K. Davis, A.A. Taylor, R.W. Huey, and C.J. Van De Wiele. 2010. CD28 expression redefines thymocyte development during the pre-T to DP transition. Int Immunol 22:387-397.

Tibaldi, E.V., R. Salgia, and E.L. Reinherz. 2002. CD2 molecules redistribute to the uropod during T cell scanning: implications for cellular activation and immune surveillance. Proc Natl Acad Sci U S A 99:7582-7587.

Tsuji, G., N. Okiyama, V.A. Villarroel, and S.I. Katz. 2015. Histone deacetylase 6 inhibition impairs effector CD8 T-cell functions during skin inflammation. J Allergy Clin Immunol 135:1228-1239.

Wang, J.H., and E.L. Reinherz. 2012. The structural basis of αβ T-lineage immune recognition: TCR docking topologies, mechanotransduction, and co-receptor function. Immunological reviews 250:102-119.

Wang, P., Z. Wang, and J. Liu. 2020. Role of HDACs in normal and malignant hematopoiesis. Molecular cancer 19:5.

Weerkamp, F., T.C. Luis, B.A.E. Naber, E.E.L. Koster, L. Jeannotte, J.J.M. van Dongen, and F.J.T. Staal. 2006. Identification of Notch target genes in uncommitted T-cell progenitors: no direct induction of a T-cell specific gene program. Leukemia 20:1967-1977.

Willcox, B.E., and C.R. Willcox. 2019. γδ TCR ligands: the quest to solve a 500-million-year-old mystery. Nature Immunology 20:121-128.

Williams, J.A., K.S. Hathcock, D. Klug, Y. Harada, B. Choudhury, J.P. Allison, R. Abe, and R.J. Hodes. 2005. Regulated costimulation in the thymus is critical for T cell development: dysregulated CD28 costimulation can bypass the pre-TCR checkpoint. J Immunol 175:4199-4207.

Xu, K., W.Y. Yang, G.K. Nanayakkara, Y. Shao, F. Yang, W. Hu, E.T. Choi, H. Wang, and X. Yang. 2018. GATA3, HDAC6, and BCL6 Regulate FOXP3+ Treg Plasticity and Determine Treg Conversion into Either Novel Antigen-Presenting Cell-Like Treg or Th1-Treg. Frontiers in immunology 9:45.

Zhang, X.-H., M. Qin, H.-P. Wu, M.Y. Khamis, Y.-H. Li, L.-Y. Ma, and H.-M. Liu. 2021. A Review of Progress in Histone Deacetylase 6 Inhibitors Research: Structural Specificity and Functional Diversity. Journal of medicinal chemistry 64:1362-1391.

Zhang, Y., S. Kwon, T. Yamaguchi, F. Cubizolles, S. Rousseaux, M. Kneissel, C. Cao, N. Li, H.L. Cheng, K. Chua, D. Lombard, A. Mizeracki, G. Matthias, F.W. Alt, S. Khochbin, and P. Matthias. 2008. Mice lacking histone deacetylase 6 have hyperacetylated tubulin but are viable and develop normally. Mol Cell Biol 28:1688-1701.

Chapter 4

Adeegbe, D.O., Y. Liu, P.H. Lizotte, Y. Kamihara, A.R. Aref, C. Almonte, R. Dries, Y. Li, S. Liu, X. Wang, T. Warner-Hatten, J. Castrillon, G.C. Yuan, N. Poudel-Neupane, H. Zhang, J.L. Guerriero, S. Han, M.M. Awad, D.A. Barbie, J. Ritz, S.S. Jones, P.S. Hammerman, J. Bradner, S.N. Quayle, and K.K. Wong. 2017. Synergistic Immunostimulatory Effects and Therapeutic Benefit of Combined Histone Deacetylase and Bromodomain Inhibition in Non-Small Cell Lung Cancer. Cancer discovery 7:852-867.

Allam, A.H., M. Charnley, K. Pham, and S.M. Russell. 2021. Developing T cells form an immunological synapse for passage through the beta-selection checkpoint. J Cell Biol 220:

Ananias, H.J., I.J. de Jong, R.A. Dierckx, C. van de Wiele, W. Helfrich, and P.H. Elsinga. 2008. Nuclear imaging of prostate cancer with gastrin-releasing-peptide-receptor targeted radiopharmaceuticals. Current pharmaceutical design 14:3033-3047.

Azzam, H.S., J.B. DeJarnette, K. Huang, R. Emmons, C.-S. Park, C.L. Sommers, D. El-Khoury, E.W. Shores, and P.E. Love. 2001. Fine Tuning of TCR Signaling by CD5. The Journal of Immunology 166:5464-5472.

Azzam, H.S., A. Grinberg, K. Lui, H. Shen, E.W. Shores, and P.E. Love. 1998. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. J Exp Med 188:2301-2311.

Bachmann, M.F., M. Barner, and M. Kopf. 1999. CD2 sets quantitative thresholds in T cell activation. J Exp Med 190:1383-1392.

Ballesteros-Arias, L., J.G. Silva, R.A. Paiva, B. Carbonetto, P. Faisca, and V.C. Martins. 2019. T Cell Acute Lymphoblastic Leukemia as a Consequence of Thymus Autonomy. J Immunol

Boudil, A., I.R. Matei, H.Y. Shih, G. Bogdanoski, J.S. Yuan, S.G. Chang, B. Montpellier, P.E. Kowalski, V. Voisin, S. Bashir, G.D. Bader, M.S. Krangel, and C.J. Guidos. 2015. IL-7 coordinates proliferation, differentiation and Tcra recombination during thymocyte beta-selection. Nat Immunol 16:397-405.

Brossard, C., M. Semichon, A. Trautmann, and G. Bismuth. 2003. CD5 inhibits signaling at the immunological synapse without impairing its formation. J Immunol 170:4623-4629.

Chann, A.S., and S M. Russell. 2019. An integrated transcriptional switch at the beta-selection checkpoint determines T cell survival, development and leukaemogenesis. Biochemical Society transactions 47:1077-1089.

Charnley, M., M. Ludford-Menting, K. Pham, and S.M. Russell. 2020. A new role for Notch in the control of polarity and asymmetric cell division of developing T cells. J Cell Sci 133:jcs235358.

Choi, Y.S., J.A. Gullicksrud, S. Xing, Z. Zeng, Q. Shan, F. Li, P.E. Love, W. Peng, H.H. Xue, and S. Crotty. 2015. LEF-1 and TCF-1 orchestrate T differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. Nat Immunol

Cosenza, M., and S. Pozzi. 2018. The Therapeutic Strategy of HDAC6 Inhibitors in Lymphoproliferative Disease. International journal of molecular sciences 19:

Dose, M., I. Khan, Z. Guo, D. Kovalovsky, A. Krueger, H. von Boehmer, K. Khazaie, and F. Gounari. 2006. c-Myc mediates pre-TCR-induced proliferation but not developmental progression. Blood 108:2669-2677.

Douek, D.C., R.A. Vescio, M.R. Betts, J.M. Brenchley, B.J. Hill, L. Zhang, J.R. Berenson, R.H. Collins, and R.A. Koup. 2000. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. Lancet 355:1875-1881.

Dustin, M.L., M.W. Olszowy, A.D. Holdorf, J. Li, S. Bromley, N. Desai, P. Widder, F. Rosenberger, P.A. van der Merwe, P.M. Allen, and A.S. Shaw. 1998. A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. Cell 94:667-677.

Dutta, A., B. Zhao, and P.E. Love. 2021. New insights into TCR β-selection. Trends in Immunology

Gounari, F., I. Aifantis, K. Khazaie, S. Hoeflinger, N. Harada, M.M. Taketo, and H. von Boehmer. 2001. Somatic activation of beta-catenin bypasses pre-TCR signaling and TCR selection in thymocyte development. Nat Immunol 2:863-869.

Goux, D., J.D. Coudert, D. Maurice, L. Scarpellino, G. Jeannet, S. Piccolo, K. Weston, J. Huelsken, and W. Held. 2005. Cooperating pre-T-cell receptor and TCF-1-dependent signals ensure thymocyte survival. Blood 106:1726-1733.

Groettrup, M , A. Baron, G. Griffiths, R. Palacios, and H. von Boehmer. 1992. T cell receptor (TCR) beta chain homodimers on the surface of immature but not mature alpha, gamma, delta chain deficient T cell lines. EMBO J 11:2735-2745.

Hogg, S.J., P.A. Beavis, M.A. Dawson, and R.W. Johnstone. 2020. Targeting the epigenetic regulation of antitumour immunity. Nature reviews. Drug discovery

Iaconelli, J., J.H. Huang, S.S. Berkovitch, S. Chattopadhyay, R. Mazitschek, S.L. Schreiber, S.J. Haggarty, and R. Karmacharya. 2015. HDAC6 inhibitors modulate Lys49 acetylation and membrane localization of beta-catenin in human iPSC-derived neuronal cells. ACS Chem Biol 10:883-890.

Kamoun, M., J.S. Woods, N. Sano, H. Makni, R. Smith, W.B. de Lau, A. van Oers, D. Wotton, M.J. Owen, Y. Hashimoto, and H.C. Clevers. 1995. Analysis of CD2 and TCR-beta gene expression in Jurkat cell mutants suggests a cis regulation of gene transcription. J Immunol 155:3929-3937.

Laino, A.S., B.C. Betts, A. Veerapathran, I. Dolgalev, A. Sarnaik, S.N. Quayle, S.S. Jones, J.S. Weber, and D.M. Woods. 2019. HDAC6 selective inhibition of melanoma patient T-cells augments anti-tumor characteristics. Journal for immunotherapy of cancer 7:33.

Li, X., R. Mizsei, K. Tan, R.J. Mallis, J.S. Duke-Cohan, A. Akitsu, P.W. Tetteh, A. Dubey, W. Hwang, G. Wagner, M.J. Lang, H. Arthanari, J.H. Wang, and E.L. Reinherz. 2021. Pre-T cell receptors topologically sample self-ligands during thymocyte beta-selection. Science 371:181-185.

López-Rodríguez, C., J. Aramburu, and R. Berga-Bolaños. 2015. Transcription factors and target genes of pre-TCR signaling. Cellular and Molecular Life Sciences 72:2305-2321.

Lutes, L.K., Z. Steier, L.L. McIntyre, S. Pandey, J. Kaminski, A.R. Hoover, S. Ariotti, A. Streets, N. Yosef, and E.A. Robey. 2021. T cell self-reactivity during thymic development dictates the timing of positive selection. eLife 10:e65435.

Magner, W.J., A.L. Kazim, C. Stewart, M.A. Romano, G. Catalano, C. Grande, N. Keiser, F. Santaniello, and T.B. Tomasi. 2000. Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. J Immunol 165:7017-7024.

Magupalli, V.G., R. Negro, Y. Tian, A.V. Hauenstein, G. Di Caprio, W. Skillern, Q. Deng, P. Orning, H.B. Alam, Z. Maliga, H. Sharif, J.J. Hu, C.L. Evavold, J.C. Kagan, F.I. Schmidt, K.A. Fitzgerald, T. Kirchhausen, Y. Li, and H. Wu. 2020. HDAC6 mediates an aggresome-like mechanism for NLRP3 and pyrin inflammasome activation. Science 369:eaas8995.

Mallis, R.J., K. Bai, H. Arthanari, R.E. Hussey, M. Handley, Z. Li, L. Chingozha, J.S. Duke-Cohan, H. Lu, J.H. Wang, C. Zhu, G. Wagner, and E.L. Reinherz. 2015. Pre-TCR ligand binding impacts thymocyte development before alphabetaTCR expression. Proc Natl Acad Sci U S A 112:8373-8378.

Matson, C.A., S. Choi, F. Livak, B. Zhao, A. Mitra, P.E. Love, and N.J. Singh. 2020. CD5 dynamically calibrates basal NF-kappaB signaling in T cells during thymic development and peripheral activation. Proc Natl Acad Sci U S A 117:14342-14353.

Mingueneau, M., T. Kreslavsky, D. Gray, T. Heng, R. Cruse, J. Ericson, S. Bendall, M.H. Spitzer, G.P. Nolan, K. Kobayashi, H. von Boehmer, D. Mathis, C. Benoist, C. Immunological Genome, A.J. Best, J. Knell, A. Goldrath, V. Jojic, D. Koller, T. Shay, A. Regev, N. Cohen, P. Brennan, M. Brenner, F. Kim, T.N. Rao, A. Wagers, T. Heng, J. Ericson, K. Rothamel, A. Ortiz-Lopez, D. Mathis, C. Benoist, N.A. Bezman, J.C. Sun, G. Min-Oo, C.C. Kim, L.L. Lanier, J. Miller, B. Brown, M. Merad, E.L. Gautier, C. Jakubzick, G.J. Randolph, P. Monach, D.A. Blair, M.L. Dustin, S.A. Shinton, R.R. Hardy, D. Laidlaw, J. Collins, R. Gazit, D.J. Rossi, N. Malhotra, K. Sylvia, J. Kang, T. Kreslavsky, A. Fletcher, K. Elpek, A. Bellemare-Pelletier, D. Malhotra, and S. Turley. 2013. The transcriptional landscape of alphabeta T cell differentiation. Nat Immunol 14:619-632.

Miyazaki, M., K. Miyazaki, M. Itoi, Y. Katoh, Y. Guo, R. Kanno, Y. Katoh-Fukui, H. Honda, T. Amagai, M. van Lohuizen, H. Kawamoto, and M. Kanno. 2008. Thymocyte proliferation induced by pre-T cell receptor signaling is maintained through polycomb gene product Bmi-1-mediated Cdkn2a repression. Immunity 28:231-245.

Notarangelo, L.D., R. Bacchetta, J.L. Casanova, and H.C. Su. 2020. Human inborn errors of immunity: An expanding universe. Science Immunology 5:eabb1662.

Rodewald, H.R., K. Awad, P. Moingeon, L. D'Adamio, D. Rabinowitz, Y. Shinkai, F.W. Alt, and E.L. Reinherz. 1993. Fc gamma RII/III and CD2 expression mark distinct subpopulations of immature CD4-CD8- murine thymocytes: in vivo developmental kinetics and T cell receptor beta chain rearrangement status. J Exp Med 177:1079-1092.

Rogers, D., A. Sood, H. Wang, J.J.P. van Beek, T.J. Rademaker, P. Artusa, C. Schneider, C. Shen, D.C. Wong, M.-È. Lebel, S.A. Condotta, M.J. Richer, A.J. Martins, J.S. Tsang, L. Barreiro, P. Francois, D. Langlais, H.J. Melichar, J. Textor, and J.N. Mandl. 2021. Pre-existing chromatin accessibility and gene expression differences among naïve CD4⁺ T cells influence effector potential. bioRxiv 2021.2004.2021.440846.

Sasada, T., and E.L. Reinherz. 2001. A critical role for CD2 in both thymic selection events and mature T cell function. J Immunol 166:2394-2403.

Serrador, J.M , J.R. Cabrero, D. Sancho, M. Mittelbrunn, A. Urzainqui, and F. Sanchez-Madrid. 2004. HDAC6 deacetylase activity links the tubulin cytoskeleton with immune synapse organization. Immunity 20:417-428.

Shapiro, M.J., and V.S. Shapiro. 2020. Chromatin-Modifying Enzymes in T Cell Development. Annu Rev Immunol 38:397-419.

Skånland, Sigrid S., K. Moltu, T. Berge, Einar M. Aandahl, and K. Taskén. 2014. T-cell co-stimulation through the CD2 and CD28 co-receptors induces distinct signalling responses. Biochemical Journal 460:399-410.

Solanki, A., D.C. Yánez, C.-I. Lau, J. Rowell, A. Barbarulo, S. Ross, H. Sahni, and T. Crompton. 2020. The transcriptional repressor Bcl6 promotes pre-TCR-induced thymocyte differentiation and attenuates Notch1 activation. Development 147:dev192203.

Sood, A., M.E. Lebel, M. Dong, M. Fournier, S.J. Vobecky, E. Haddad, J.S. Delisle, J.N. Mandl, N. Vrisekoop, and H.J. Melichar. 2021. CD5 levels define functionally heterogeneous populations of naive human CD4(+) T cells. Eur J Immunol 51:1365-1376.

Staal, F.J., and H.C. Clevers. 2005. WNT signalling and haematopoiesis: a WNT-WNT situation. Nat Rev Immunol 5:21-30.

Taghon, T., M.A. Yui, R. Pant, R.A. Diamond, and E.V. Rothenberg. 2006. Developmental and molecular characterization of emerging beta- and gammadelta-selected pre-T cells in the adult mouse thymus. Immunity 24:53-64.

Tarakhovsky, A., S.B. Kanner, J. Hombach, J.A. Ledbetter, W. Muller, N. Killeen, and K. Rajewsky. 1995. A role for CD5 in TCR-mediated signal transduction and thymocyte selection. Science 269:535-537.

Teague, T.K., C. Tan, J.H. Marino, B.K. Davis, A.A. Taylor, R.W. Huey, and C.J. Van De Wiele. 2010. CD28 expression redefines thymocyte development during the pre-T to DP transition. Int Immunol 22:387-397.

Tibaldi, E.V., R. Salgia, and E.L. Reinherz. 2002. CD2 molecules redistribute to the uropod during T cell scanning: implications for cellular activation and immune surveillance. Proc Natl Acad Sci U S A 99:7582-7587.

Travis, A., A. Amsterdam, C. Belanger, and R. Grosschedl. 1991. LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function [corrected]. Genes Dev 5:880-894.

Tsuji, G., N. Okiyama, V.A. Villarroel, and S.I. Katz. 2015. Histone deacetylase 6 inhibition impairs effector CD8 T-cell functions during skin inflammation. J Allergy Clin Immunol 135:1228-1239.

Voisinne, G., A. Gonzalez de Peredo, and R. Roncagalli. 2018. CD5, an Undercover Regulator of TCR Signaling. Frontiers in Immunology 9:

Wang, P., Z. Wang, and J. Liu. 2020. Role of HDACs in normal and malignant hematopoiesis. Molecular cancer 19:5.

Williams, J.A., K.S. Hathcock, D. Klug, Y. Harada, B. Choudhury, J.P. Allison, R. Abe, and R.J. Hodes. 2005. Regulated costimulation in the thymus is critical for T cell development: dysregulated CD28 costimulation can bypass the pre-TCR checkpoint. J Immunol 175:4199-4207.

Xing, S., F. Li, Z. Zeng, Y. Zhao, S. Yu, Q. Shan, Y. Li, F.C. Phillips, P.K. Maina, H.H. Qi, C. Liu, J. Zhu, R.M. Pope, C.A. Musselman, C. Zeng, W. Peng, and H.H. Xue. 2016. Tcf1 and Lef1 transcription factors establish CD8 T cell identity through intrinsic HDAC activity. Nat Immunol

Xu, K., W.Y. Yang, G.K. Nanayakkara, Y. Shao, F. Yang, W. Hu, E.T. Choi, H. Wang, and X. Yang. 2018. GATA3, HDAC6, and BCL6 Regulate FOXP3+ Treg Plasticity and Determine Treg Conversion into Either Novel Antigen-Presenting Cell-Like Treg or Th1-Treg. Frontiers in immunology 9:45.

Xu, M., A. Sharma, D.L. Wiest, and J.M. Sen. 2009. Pre-TCR-induced beta-catenin facilitates traversal through beta-selection. J Immunol 182:751-758.

Yu, Q., B. Erman, J.H. Park, L. Feigenbaum, and A. Singer. 2004. IL-7 receptor signals inhibit expression of transcription factors TCF-1, LEF-1, and RORgammat: impact on thymocyte development. J Exp Med 200:797-803.

Yu, S., X. Zhou, F.C. Steinke, C. Liu, S.C. Chen, O. Zagorodna, X. Jing, Y. Yokota, D.K. Meyerholz, C.G. Mullighan, C.M. Knudson, D.M. Zhao, and H.H. Xue. 2012. The TCF-1 and LEF-1 transcription factors have cooperative and opposing roles in T cell development and malignancy. Immunity 37:813-826.

Zhang, M., C. Hu, N. Moses, J. Haakenson, S. Xiang, D. Quan, B. Fang, Z. Yang, W. Bai, G. Bepler, G.M. Li, and X.M. Zhang. 2019. HDAC6 regulates DNA damage response via deacetylating MLH1. J Biol Chem 294:5813-5826.

Zhang, X.-H., M. Qin, H.-P. Wu, M.Y. Khamis, Y.-H. Li, L.-Y. Ma, and H.-M. Liu. 2021. A Review of Progress in Histone Deacetylase 6 Inhibitors Research: Structural Specificity and Functional Diversity. Journal of medicinal chemistry 64:1362-1391.

Zhang, Y., S. Kwon, T. Yamaguchi, F. Cubizolles, S. Rousseaux, M. Kneissel, C. Cao, N. Li, H.L. Cheng, K. Chua, D. Lombard, A. Mizeracki, G. Matthias, F.W. Alt, S. Khochbin, and P. Matthias. 2008. Mice lacking histone deacetylase 6 have hyperacetylated tubulin but are viable and develop normally. Mol Cell Biol 28:1688-1701.

Zhao, X., Q. Shan, and H.-H. Xue. 2021. TCF1 in T cell immunity: a broadened frontier. Nature Reviews Immunology

Chapter 5

Allam, A.H., M. Charnley, and S.M. Russell. 2018. Context-Specific Mechanisms of Cell Polarity Regulation. J Mol Biol 430:3457-3471.

Anastasiou, O., R. Hadjisavva, and P.A. Skourides. 2020. Mitotic cell responses to substrate topological cues are independent of the molecular nature of adhesion. Science Signaling 13:eaax9940.

Bell, G.P., G.C. Fletcher, R. Brain, and B.J. Thompson. 2015. Aurora Kinases Phosphorylate Lgl to Induce Mitotic Spindle Orientation in Drosophila Epithelia. Curr Biol 25:61-68.

Bergstralh, D.T., N.S. Dawney, and D. St Johnston. 2017. Spindle orientation: a question of complex positioning. Development 144:1137-1145.

Bergstralh, D.T., H.E. Lovegrove, and D. St Johnston. 2013. Discs large links spindle orientation to apical-basal polarity in Drosophila epithelia. Current biology : CB 23:1707-1712.

Bergstralh, D.T., and D. St Johnston. 2014. Spindle orientation: what if it goes wrong? Semin Cell Dev Biol 34:140-145.

Bonello, T.T., and M. Peifer. 2019. Scribble: A master scaffold in polarity, adhesion, synaptogenesis, and proliferation. J Cell Biol 218:742-756.

Carvalho, C.A., S. Moreira, G. Ventura, C.E. Sunkel, and E. Morais-de-Sa. 2015. Aurora A triggers Lgl cortical release during symmetric division to control planar spindle orientation. Curr Biol 25:53-60.

Cavey, M., M. Rauzi, P.F. Lenne, and T. Lecuit. 2008. A two-tiered mechanism for stabilization and immobilization of E-cadherin. Nature 453:751-756.

Charnley, M., F. Anderegg, R. Holtackers, M. Textor, and P. Meraldi. 2013. Effect of Cell Shape and Dimensionality on Spindle Orientation and Mitotic Timing. PLoS One 8:e66918.

den Elzen, N., C.V. Buttery, M.P. Maddugoda, G. Ren, and A.S. Yap. 2009. Cadherin adhesion receptors orient the mitotic spindle during symmetric cell division in mammalian epithelia. Mol Biol Cell 20:3740-3750.

Dewey, E.B., D.T. Taylor, and C.A. Johnston. 2015. Cell Fate Decision Making through Oriented Cell Division. J Dev Biol 3:129-157.

di Pietro, F., A. Echard, and X. Morin. 2016a. Regulation of mitotic spindle orientation: an integrated view. EMBO reports 17:1106-1130.

di Pietro, F., A. Echard, and X. Morin. 2016b. Regulation of mitotic spindle orientation: an integrated view. EMBO Rep 17:1106-1130.

Dimitracopoulos, A., P. Srivastava, A. Chaigne, Z. Win, R. Shlomovitz, O.M. Lancaster, M. Le Berre, M. Piel, K. Franze, G. Salbreux, and B. Baum. 2020. Mechanochemical Crosstalk Produces Cell-Intrinsic Patterning of the Cortex to Orient the Mitotic Spindle. Curr Biol 30:3687-3696 e3684.

Dix, C.L., H.K. Matthews, M. Uroz, S. McLaren, L. Wolf, N. Heatley, Z. Win, P. Almada, R. Henriques, M. Boutros, X. Trepat, and B. Baum. 2018. The Role of Mitotic Cell-Substrate Adhesion Re-modeling in Animal Cell Division. Dev Cell 45:132-145 e133.

Du, Q., and I.G. Macara. 2004. Mammalian Pins is a conformational switch that links NuMA to heterotrimeric G proteins. Cell 119:503-516.

Finegan, T.M., and D.T. Bergstralh. 2019. Division orientation: disentangling shape and mechanical forces. Cell Cycle 18:1187-1198.

Fink, J., N. Carpi, T. Betz, A. Betard, M. Chebah, A. Azioune, M. Bornens, C. Sykes, L. Fetler, D. Cuvelier, and M. Piel. 2011. External forces control mitotic spindle positioning. Nat Cell Biol 13:771-778.

Gloerich, M , J.M. Bianchini, K.A. Siemers, D.J. Cohen, and W.J. Nelson. 2017. Cell division orientation is coupled to cell-cell adhesion by the E-cadherin/LGN complex. Nature communications 8:13996.

Godde, N.J., J.M. Sheridan, L.K. Smith, H.B. Pearson, K.L. Britt, R.C. Galea, L.L. Yates, J.E. Visvader, and P.O. Humbert. 2014. Scribble modulates the MAPK/Fra1 pathway to disrupt luminal and ductal integrity and suppress tumour formation in the mammary gland. PLoS Genet 10:e1004323.

Hart, K.C., J. Tan, K.A. Siemers, J.Y. Sim, B.L. Pruitt, W.J. Nelson, and M. Gloerich. 2017. E-cadherin and LGN align epithelial cell divisions with tissue tension independently of cell shape. Proc Natl Acad Sci U S A 114:E5845-E5853.

Johnston, C.A., K. Hirono, K.E. Prehoda, and C.Q. Doe. 2009. Identification of an Aurora-A/PinsLINKER/Dlg spindle orientation pathway using induced cell polarity in S2 cells. Cell 138:1150-1163.

Kiyomitsu, T. 2019. The cortical force-generating machinery: how cortical spindle-pulling forces are generated. Current Opinion in Cell Biology 60:1-8.

Kotak, S. 2019. Mechanisms of Spindle Positioning: Lessons from Worms and Mammalian Cells. Biomolecules 9:

Kotak, S., and P. Gonczy. 2013. Mechanisms of spindle positioning: cortical force generators in the limelight. Curr Opin Cell Biol

Lam, M.S.Y., A. Lisica, N. Ramkumar, G. Hunter, Y. Mao, G. Charras, and B. Baum. 2020. Isotropic myosin-generated tissue tension is required for the dynamic orientation of the mitotic spindle. Mol Biol Cell 31:1370-1379.

Lechler, T., and M. Mapelli. 2021. Spindle positioning and its impact on vertebrate tissue architecture and cell fate. Nature Reviews Molecular Cell Biology 22:691-708.

Lecuit, T., and A.S. Yap. 2015. E-cadherin junctions as active mechanical integrators in tissue dynamics. Nat Cell Biol 17:533-539.

Lesman, A., J. Notbohm, D.A. Tirrell, and G. Ravichandran. 2014. Contractile forces regulate cell division in three-dimensional environments. J Cell Biol 205:155-162.

Li, J., L. Cheng, and H. Jiang. 2019. Cell shape and intercellular adhesion regulate mitotic spindle orientation. Mol Biol Cell 30:2458-2468.

Li, Y., and K. Burridge. 2019. Cell-Cycle-Dependent Regulation of Cell Adhesions: Adhering to the Schedule: Three papers reveal unexpected properties of adhesion structures as cells progress through the cell cycle. Bioessays 41:e1800165.

Lock, J.G., M.C. Jones, J.A. Askari, X. Gong, A. Oddone, H. Olofsson, S. Goransson, M. Lakadamyali, M.J. Humphries, and S. Stromblad. 2018. Reticular adhesions are a distinct class of cell-matrix adhesions that mediate attachment during mitosis. Nat Cell Biol 20:1290-1302.

Lock, J.G., and J.L. Stow. 2005. Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin. Mol Biol Cell 16:1744-1755.

Lu, M.S., and C.A. Johnston. 2013. Molecular pathways regulating mitotic spindle orientation in animal cells. Development 140:1843-1856.

Machicoane, M., C.A. de Frutos, J. Fink, M. Rocancourt, Y. Lombardi, S. Garel, M. Piel, and A. Echard. 2014. SLK-dependent activation of ERMs controls LGN-NuMA localization and spindle orientation. J Cell Biol 205:791-799.

Matsumura, S., T. Kojidani, Y. Kamioka, S. Uchida, T. Haraguchi, A. Kimura, and F. Toyoshima. 2016. Interphase adhesion geometry is transmitted to an internal regulator for spindle orientation via caveolin-1. Nature communications 7:ncomms11858.

Mitchison, T.J. 1992. Actin based motility on retraction fibers in mitotic PtK2 cells. Cell Motil Cytoskeleton 22:135-151.

Nakajima, Y.-i., Z.T. Lee, S.A. McKinney, S.K. Swanson, L. Florens, and M.C. Gibson. 2019. Junctional tumor suppressors interact with 14-3-3 proteins to control planar spindle alignment. Journal of Cell Biology 218:1824-1838.

Nakajima, Y.I., E.J. Meyer, A. Kroesen, S.A. McKinney, and M.C. Gibson. 2013. Epithelial junctions maintain tissue architecture by directing planar spindle orientation. Nature

Navarro, C., S. Nola, S. Audebert, M.J. Santoni, J.P. Arsanto, C. Ginestier, S. Marchetto, J. Jacquemier, D. Isnardon, A. Le Bivic, D. Birnbaum, and J.P. Borg. 2005. Junctional recruitment of mammalian Scribble relies on E-cadherin engagement. Oncogene 24:4330-4339.

Nestor-Bergmann, A., G. Goddard, and S. Woolner. 2014. Force and the spindle: mechanical cues in mitotic spindle orientation. Semin Cell Dev Biol 34:133-139.

Nestor-Bergmann, A., G.A. Stooke-Vaughan, G.K. Goddard, T. Starborg, O.E. Jensen, and S. Woolner. 2019. Decoupling the Roles of Cell Shape and Mechanical Stress in Orienting and Cueing Epithelial Mitosis. Cell reports 26:2088-2100 e2084.

Niwayama, R., P. Moghe, Y.-J. Liu, D. Fabrèges, F. Buchholz, M. Piel, and T. Hiiragi. 2019. A Tug-of-War between Cell Shape and Polarity Controls Division Orientation to Ensure Robust Patterning in the Mouse Blastocyst. Developmental Cell 51:564-574.e566.

Pannekoek, W.J., J. de Rooij, and M. Gloerich. 2019. Force transduction by cadherin adhesions in morphogenesis. F1000Research 8:

Petridou, N.I., and P.A. Skourides. 2016. A ligand-independent integrin beta1 mechanosensory complex guides spindle orientation. Nature communications 7:10899.

Porter, A.P., G.R.M. White, N.A. Mack, and A. Malliri. 2019. The interaction between CASK and the tumour suppressor Dlg1 regulates mitotic spindle orientation in mammalian epithelia. Journal of Cell Science jcs.230086.

Qin, Y., C. Capaldo, B.M. Gumbiner, and I.G. Macara. 2005. The mammalian Scribble polarity protein regulates epithelial cell adhesion and migration through E-cadherin. J Cell Biol 171:1061-1071.

Qu, Y., B. Han, Y. Yu, W. Yao, S. Bose, B.Y. Karlan, A.E. Giuliano, and X. Cui. 2015. Evaluation of MCF10A as a Reliable Model for Normal Human Mammary Epithelial Cells. PLOS ONE 10:e0131285.

Rizzelli, F., M.G. Malabarba, S. Sigismund, and M. Mapelli. 2020. The crosstalk between microtubules, actin and membranes shapes cell division. Open biology 10:190314.

Saadaoui, M., M. Machicoane, F. di Pietro, F. Etoc, A. Echard, and X. Morin. 2014. Dlg1 controls planar spindle orientation in the neuroepithelium through direct interaction with LGN. J Cell Biol 206:707-717.

Seldin, L., and I. Macara. 2017. Epithelial spindle orientation diversities and uncertainties: recent developments and lingering questions. F1000Research 6:984-984.

Shimoyama, Y., S. Hirohashi, S. Hirano, M. Noguchi, Y. Shimosato, M. Takeichi, and O. Abe. 1989. Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. Cancer Res 49:2128-2133.

Taneja, N., A.M. Fenix, L. Rathbun, B.A. Millis, M.J. Tyska, H. Hehnly, and D.T. Burnette. 2016. Focal adhesions control cleavage furrow shape and spindle tilt during mitosis. Scientific reports 6:29846.

Thery, M., and M. Bornens. 2006. Cell shape and cell division. Curr Opin Cell Biol 18:648-657.

Thery, M., A. Jimenez-Dalmaroni, V. Racine, M. Bornens, and F. Julicher. 2007. Experimental and theoretical study of mitotic spindle orientation. Nature 447:493-496.

Thery, M., V. Racine, A. Pepin, M. Piel, Y. Chen, J.B. Sibarita, and M. Bornens. 2005. The extracellular matrix guides the orientation of the cell division axis. Nat Cell Biol 7:947-953.

Tomlinson, J.S., M.L. Alpaugh, and S.H. Barsky. 2001. An intact overexpressed E-cadherin/alpha,beta-catenin axis characterizes the lymphovascular emboli of inflammatory breast carcinoma. Cancer Res 61:5231-5241.

Walma, D.A.C., and K.M. Yamada. 2020. The extracellular matrix in development. Development 147:dev175596.

Wang, X., B. Dong, K. Zhang, Z. Ji, C. Cheng, H. Zhao, Y. Sheng, X. Li, L. Fan, W. Xue, W.-Q. Gao, and H.H. Zhu. 2018. E-cadherin bridges cell polarity and spindle orientation to ensure prostate epithelial integrity and prevent carcinogenesis in vivo. PLOS Genetics 14:e1007609.

Yamashita, Y.M., D.L. Jones, and M.T. Fuller. 2003. Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. Science 301:1547-1550.

Zheng, Z., Q. Wan, G. Meixiong, and Q. Du. 2014. Cell cycle-regulated membrane binding of NuMA contributes to efficient anaphase chromosome separation. Mol Biol Cell 25:606-619.

Zigman, M., A. Trinh le, S.E. Fraser, and C.B. Moens. 2011. Zebrafish neural tube morphogenesis requires Scribble-dependent oriented cell divisions. Curr Biol 21:79-86.

Chapter 6

Allam, A.H., M. Charnley, and S.M. Russell. 2018. Context-Specific Mechanisms of Cell Polarity Regulation. J Mol Biol 430:3457-3471.

Bonello, T.T., and M. Peifer. 2019. Scribble: A master scaffold in polarity, adhesion, synaptogenesis, and proliferation. J Cell Biol 218:742-756.

Bui, D.A., W. Lee, A.E. White, J.W. Harper, R.C. Schackmann, M. Overholtzer, L.M. Selfors, and J.S. Brugge. 2016. Cytokinesis involves a nontranscriptional function of the Hippo pathway effector YAP. Sci Signal 9:ra23.

Cramer, L., and T.J. Mitchison. 1993. Moving and stationary actin filaments are involved in spreading of postmitotic PtK2 cells. J Cell Biol 122:833-843.

Cramer, L.P., and T.J. Mitchison. 1995. Myosin is involved in postmitotic cell spreading. J Cell Biol 131:179-189.

Fededa, J.P., and D.W. Gerlich. 2012. Molecular control of animal cell cytokinesis. Nat Cell Biol 14:440-447.

Gallop, J.L. 2020. Filopodia and their links with membrane traffic and cell adhesion. Semin Cell Dev Biol 102:81-89.

Gibson, M.C., A.B. Patel, R. Nagpal, and N. Perrimon. 2006. The emergence of geometric order in proliferating metazoan epithelia. Nature 442:1038-1041.

Guillot, C., and T. Lecuit. 2013. Mechanics of epithelial tissue homeostasis and morphogenesis. Science 340:1185-1189.

He, K., T. Sakai, Y. Tsukasaki, T.M. Watanabe, and M. Ikebe. 2017. Myosin X is recruited to nascent focal adhesions at the leading edge and induces multi-cycle filopodial elongation. Scientific reports 7:13685.

Herszterg, S., A. Leibfried, F. Bosveld, C. Martin, and Y. Bellaiche. 2013. Interplay between the dividing cell and its neighbors regulates adherens junction formation during cytokinesis in epithelial tissue. Dev Cell 24:256-270.

Higashi, T., and A.L. Miller. 2017. Tricellular junctions: how to build junctions at the TRICkiest points of epithelial cells. Mol Biol Cell 28:2023-2034.

Kwon, M., M. Bagonis, G. Danuser, and D. Pellman. 2015. Direct Microtubule-Binding by Myosin-10 Orients Centrosomes toward Retraction Fibers and Subcortical Actin Clouds. Developmental cell 34:323-337.

Le Bras, S., and R. Le Borgne. 2014. Epithelial cell division - multiplying without losing touch. J Cell Sci 127:5127-5137.

Lecuit, T., and A.S. Yap. 2015. E-cadherin junctions as active mechanical integrators in tissue dynamics. Nat Cell Biol 17:533-539.

Li, J.X.H., V.W. Tang, and W.M. Brieher. 2020. Actin protrusions push at apical junctions to maintain E-cadherin adhesion. Proc Natl Acad Sci U S A 117:432-438.

Li, Y., and K. Burridge. 2019. Cell-Cycle-Dependent Regulation of Cell Adhesions: Adhering to the Schedule: Three papers reveal unexpected properties of adhesion structures as cells progress through the cell cycle. Bioessays 41:e1800165.

Li, Y., J.A. Junge, C. Arnesano, G.G. Gross, J.H. Miner, R. Moats, R.W. Roberts, D.B. Arnold, and S.E. Fraser. 2018. Discs large 1 controls daughter-cell polarity after cytokinesis in vertebrate morphogenesis. Proc Natl Acad Sci U S A 115:E10859-E10868.

Lough, K.J., K.M. Byrd, C.P. Descovich, D.C. Spitzer, A.J. Bergman, G.M. Beaudoin, 3rd, L.F. Reichardt, and S.E. Williams. 2019. Telophase correction refines division orientation in stratified epithelia. Elife 8:

Ludford-Menting, M.J., J. Oliaro, F. Sacirbegovic, E.T. Cheah, N. Pedersen, S.J. Thomas, A. Pasam, R. Iazzolino, L.E. Dow, N.J. Waterhouse, A. Murphy, S. Ellis, M.J. Smyth, M.H. Kershaw, P.K. Darcy, P.O. Humbert, and S.M. Russell. 2005. A network of PDZ-containing proteins regulates T cell polarity and morphology during migration and immunological synapse formation. Immunity 22:737-748.

Maruyama, T., and Y. Fujita. 2022. Cell competition in vertebrates - a key machinery for tissue homeostasis. Curr Opin Genet Dev 72:15-21.

Ogawa, M., Y. Kawarazaki, Y. Fujita, I. Naguro, and H. Ichijo. 2021. FGF21 Induced by the ASK1-p38 Pathway Promotes Mechanical Cell Competition by Attracting Cells. Current Biology 31:1048-1057.e1045.

Osmani, N., N. Vitale, J.P. Borg, and S. Etienne-Manneville. 2006. Scrib controls Cdc42 localization and activity to promote cell polarization during astrocyte migration. Curr Biol 16:2395-2405.

Osswald, M., and E. Morais-de-Sa. 2019. Dealing with apical-basal polarity and intercellular junctions: a multidimensional challenge for epithelial cell division. Curr Opin Cell Biol 60:75-83.

Papalazarou, V., and L.M. Machesky. 2021. The cell pushes back: The Arp2/3 complex is a key orchestrator of cellular responses to environmental forces. Current Opinion in Cell Biology 68:37-44.

Pinheiro, D., and Y. Bellaiche. 2018. Mechanical Force-Driven Adherens Junction Remodeling and Epithelial Dynamics. Dev Cell 47:3-19.

Plessner, M., J. Knerr, and R. Grosse. 2019. Centrosomal Actin Assembly Is Required for Proper Mitotic Spindle Formation and Chromosome Congression. iScience 15:274-281.

Rathbun, L.I., E.G. Colicino, J. Manikas, J. O'Connell, N. Krishnan, N.S. Reilly, S. Coyne, G. Erdemci-Tandogan, A. Garrastegui, J. Freshour, P. Santra, M.L. Manning, J.D. Amack, and H. Hehnly. 2020. Cytokinetic bridge triggers de novo lumen formation in vivo. Nature communications 11:1269.

Chapter 7

2020. ImmGen at 15. Nat Immunol 21:700-703.

Adeegbe, D.O., Y. Liu, P.H. Lizotte, Y. Kamihara, A.R. Aref, C. Almonte, R. Dries, Y. Li, S. Liu, X. Wang, T. Warner-Hatten, J. Castrillon, G.C. Yuan, N. Poudel-Neupane, H. Zhang, J.L. Guerriero, S. Han, M.M. Awad, D.A. Barbie, J. Ritz, S.S. Jones, P.S. Hammerman, J. Bradner, S.N. Quayle, and K.K. Wong. 2017. Synergistic Immunostimulatory Effects and Therapeutic Benefit of Combined Histone Deacetylase and Bromodomain Inhibition in Non-Small Cell Lung Cancer. Cancer discovery 7:852-867.

Allam, A.H., M. Charnley, K. Pham, and S.M. Russell. 2021. Developing T cells form an immunological synapse for passage through the beta-selection checkpoint. J Cell Biol 220:

Azzam, H.S., A. Grinberg, K. Lui, H. Shen, E.W. Shores, and P.E. Love. 1998. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. J Exp Med 188:2301-2311.

Bagger, F.O., S. Kinalis, and N. Rapin. 2018. BloodSpot: a database of healthy and malignant haematopoiesis updated with purified and single cell mRNA sequencing profiles. Nucleic acids research 47:D881-D885.

Ballesteros-Arias, L., J.G. Silva, R.A. Paiva, B. Carbonetto, P. Faisca, and V.C. Martins. 2019. T Cell Acute Lymphoblastic Leukemia as a Consequence of Thymus Autonomy. J Immunol

Beckermann, K.E., R. Hongo, X. Ye, K. Young, K. Carbonell, D.C.C. Healey, P.J. Siska, S. Barone, C.E. Roe, C.C. Smith, B.G. Vincent, F.M. Mason, J.M. Irish, W.K. Rathmell, and J.C. Rathmell. 2020. CD28 costimulation drives tumor-infiltrating T cell glycolysis to promote inflammation. JCI Insight 5:

Binder, C., F. Cvetkovski, F. Sellberg, S. Berg, H. Paternina Visbal, D.H. Sachs, E. Berglund, and D. Berglund. 2020. CD2 Immunobiology. Frontiers in Immunology 11:

Blaize, G., H. Daniels-Treffandier, M. Aloulou, N. Rouquié, C. Yang, M. Marcellin, M. Gador, M. Benamar, M. Ducatez, K.-d. Song, O. Burlet-Schiltz, A. Saoudi, P.E. Love, N. Fazilleau, A. Gonzalez de Peredo, and R. Lesourne. 2020. CD5 signalosome coordinates antagonist TCR signals to control the generation of Treg cells induced by foreign antigens. Proceedings of the National Academy of Sciences 117:12969-12979.

Boucher, J.C., G. Li, H. Kotani, M.L. Cabral, D. Morrissey, S.B. Lee, K. Spitler, N.J. Beatty, E.V. Cervantes, B. Shrestha, B. Yu, A. Kazi, X. Wang, S.M. Sebti, and M.L. Davila. 2021. CD28 Costimulatory Domain–Targeted Mutations Enhance Chimeric Antigen Receptor T-cell Function. Cancer Immunology Research 9:62-74.

Boudil, A., I.R. Matei, H.Y. Shih, G. Bogdanoski, J.S. Yuan, S.G. Chang, B. Montpellier, P.E. Kowalski, V. Voisin, S. Bashir, G.D. Bader, M.S. Krangel, and C.J. Guidos. 2015. IL-7 coordinates proliferation, differentiation and Tcra recombination during thymocyte beta-selection. Nat Immunol 16:397-405.

Cappell, K.M., and J.N. Kochenderfer. 2021. A comparison of chimeric antigen receptors containing CD28 versus 4-1BB costimulatory domains. Nature Reviews Clinical Oncology

Chadwick, N., L. Zeef, V. Portillo, C. Fennessy, F. Warrander, S. Hoyle, and A.M. Buckle. 2009. Identification of novel Notch target genes in T cell leukaemia. Mol Cancer 8:35.

Chann, A.S., and S M. Russell. 2019. An integrated transcriptional switch at the beta-selection checkpoint determines T cell survival, development and leukaemogenesis. Biochemical Society transactions 47:1077-1089.

Charnley, M., M. Ludford-Menting, K. Pham, and S.M. Russell. 2020. A new role for Notch in the control of polarity and asymmetric cell division of developing T cells. J Cell Sci 133:jcs235358.

Checquolo, S., R. Palermo, S. Cialfi, G. Ferrara, C. Oliviero, C. Talora, D. Bellavia, A. Giovenco, P. Grazioli, L. Frati, A. Gulino, and I. Screpanti. 2010. Differential subcellular localization regulates c-Cbl E3 ligase activity upon Notch3 protein in T-cell leukemia. Oncogene 29:1463-1474.

Chen, B., B. Zheng, M. DeRan, G.K. Jarugumilli, J. Fu, Y.S. Brooks, and X. Wu. 2016. ZDHHC7-mediated S-palmitoylation of Scribble regulates cell polarity. Nature Chemical Biology 12:686-693.

Chen, S., C. Han, X. Miao, X. Li, C. Yin, J. Zou, M. Liu, S. Li, L. Stawski, B. Zhu, Q. Shi, Z.-X. Xu, C. Li, C.R. Goding, J. Zhou, and R. Cui. 2019. Targeting MC1R depalmitoylation to prevent melanomagenesis in redheads. Nature Communications 10:877.

Cheng, Y., C. He, M. Wang, X. Ma, F. Mo, S. Yang, J. Han, and X. Wei. 2019. Targeting epigenetic regulators for cancer therapy: mechanisms and advances in clinical trials. Signal Transduction and Targeted Therapy 4:62.

Choi, S.Y., H.J. Kee, S. Sun, Y.M. Seok, Y. Ryu, G.R. Kim, S.-J. Kee, M. Pflieger, T. Kurz, M.U. Kassack, and M.H. Jeong. 2019. Histone deacetylase inhibitor LMK235 attenuates vascular constriction and aortic remodelling in hypertension. Journal of Cellular and Molecular Medicine 23:2801-2812.

Curtis, D.J., and M.P. McCormack. 2010. The Molecular Basis of Lmo2-Induced T-Cell Acute Lymphoblastic Leukemia. Clinical Cancer Research 16:5618-5623.

Dekker, F.J., O. Rocks, N. Vartak, S. Menninger, C. Hedberg, R. Balamurugan, S. Wetzel, S. Renner, M. Gerauer, B. Schölermann, M. Rusch, J.W. Kramer, D. Rauh, G.W. Coates, L. Brunsveld, P.I.H. Bastiaens, and H. Waldmann. 2010. Small-molecule inhibition of APT1 affects Ras localization and signaling. Nature Chemical Biology 6:449-456.

Dobenecker, M.-W., J.S. Park, J. Marcello, M.T. McCabe, R. Gregory, S.D. Knight, I. Rioja, A.K. Bassil, R.K. Prinjha, and A. Tarakhovsky. 2018. Signaling function of PRC2 is essential for TCR-driven T cell responses. Journal of Experimental Medicine 215:1101-1113.

Dong, Y., H. Guo, D. Wang, R. Tu, G. Qing, and H. Liu. 2021. Genome-Wide Analysis Identifies Rag1 and Rag2 as Novel Notch1 Transcriptional Targets in Thymocytes. Frontiers in Cell and Developmental Biology 9:

Douek, D.C., R.A. Vescio, M.R. Betts, J.M. Brenchley, B.J. Hill, L. Zhang, J.R. Berenson, R.H. Collins, and R.A. Koup. 2000. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. Lancet 355:1875-1881.

Dutta, A., B. Zhao, and P.E. Love. 2021. New insights into TCR β-selection. Trends in Immunology

Ecker, M., R. Schregle, N. Kapoor-Kaushik, P. Rossatti, V.M. Betzler, D. Kempe, M. Biro, N. Ariotti, G.M.I. Redpath, and J. Rossy. 2022. SNX9-induced membrane tubulation regulates CD28 cluster stability and signalling. eLife 11:e67550.

Esensten, Jonathan H., Ynes A. Helou, G. Chopra, A. Weiss, and Jeffrey A. Bluestone. 2016. CD28 Costimulation: From Mechanism to Therapy. Immunity 44:973-988.

Fu, W., S. Yi, L. Qiu, J. Sun, P. Tu, and Y. Wang. 2017. BCL11B-Mediated Epigenetic Repression Is a Crucial Target for Histone Deacetylase Inhibitors in Cutaneous T-Cell Lymphoma. J Invest Dermatol 137:1523-1532.

Garcia-Perez, L., F. Famili, M. Cordes, M. Brugman, M.v. Eggermond, H. Wu, J. Chouaref, D.S.L. Granado, M.M. Tiemessen, K. Pike-Overzet, L. Daxinger, and F.J.T. Staal. 2020. Functional definition of a transcription factor hierarchy regulating T cell lineage commitment. Science Advances 6:eaaw7313.

García-Peydró, M., V.G. de Yébenes, and M.L. Toribio. 2006. Notch1 and IL-7 Receptor Interplay Maintains Proliferation of Human Thymic Progenitors while Suppressing Non-T Cell Fates. The Journal of Immunology 177:3711-3720.

Germar, K., M. Dose, T. Konstantinou, J. Zhang, H. Wang, C. Lobry, K.L. Arnett, S.C. Blacklow, I. Aifantis, J.C. Aster, and F. Gounari. 2011. T-cell factor 1 is a gatekeeper for T-cell specification in response to Notch signaling. Proceedings of the National Academy of Sciences 108:20060-20065.

Gonçalves, C.M., S.N. Henriques, R.F. Santos, and A.M. Carmo. 2018. CD6, a Rheostat-Type Signalosome That Tunes T Cell Activation. Frontiers in Immunology 9:

Heng, T.S., and M.W. Painter. 2008. The Immunological Genome Project: networks of gene expression in immune cells. Nat Immunol 9:1091-1094.

Hernandez, J.L., D. Davda, M. Cheung See Kit, J.D. Majmudar, S.J. Won, M. Gang, S.C. Pasupuleti, A.I. Choi, C.M. Bartkowiak, and B.R. Martin. 2017. APT2 Inhibition Restores Scribble Localization and S-Palmitoylation in Snail-Transformed Cells. Cell Chemical Biology 24:87-97.

Hogg, S.J., P.A. Beavis, M.A. Dawson, and R.W. Johnstone. 2020. Targeting the epigenetic regulation of antitumour immunity. Nature reviews. Drug discovery

Horlbeck, M.A., L.A. Gilbert, J.E. Villalta, B. Adamson, R.A. Pak, Y. Chen, A.P. Fields, C.Y. Park, J E. Corn, M. Kampmann, and J.S. Weissman. 2016. Compact and highly active next-generation libraries for CRISPR-mediated gene repression and activation. eLife 5:e19760.

Hünig, T. 2016. The rise and fall of the CD28 superagonist TGN1412 and its return as TAB08: a personal account. The FEBS Journal 283:3325-3334.

Johnson, J.L., G. Georgakilas, J. Petrovic, M. Kurachi, S. Cai, C. Harly, W.S. Pear, A. Bhandoola, E.J. Wherry, and G. Vahedi. 2018. Lineage-Determining Transcription Factor TCF-1 Initiates the Epigenetic Identity of T Cells. Immunity 48:243-257.e210.

Juntilla, M.M., J.A. Wofford, M.J. Birnbaum, J.C. Rathmell, and G.A. Koretzky. 2007. Akt1 and Akt2 are required for αβ thymocyte survival and differentiation. Proceedings of the National Academy of Sciences 104:12105-12110.

Kasper, L.H., T. Fukuyama, M.A. Biesen, F. Boussouar, C. Tong, A.d. Pauw, P.J. Murray, J M.A.v. Deursen, and P.K. Brindle. 2006. Conditional Knockout Mice Reveal Distinct Functions for the Global Transcriptional Coactivators CBP and p300 in T-Cell Development. Molecular and Cellular Biology 26:789-809.

Kelley, L.C., and S.A. Weed. 2012. Cortactin is a substrate of activated Cdc42-associated kinase 1 (ACK1) during ligand-induced epidermal growth factor receptor downregulation. PLoS One 7:e44363.

Kelly, A.P., D.K. Finlay, H.J. Hinton, R.G. Clarke, E. Fiorini, F. Radtke, and D.A. Cantrell. 2007. Notch-induced T cell development requires phosphoinositide-dependent kinase 1. Embo j 26:3441-3450.

Klein Geltink, R.I., D. O'Sullivan, M. Corrado, A. Bremser, M.D. Buck, J.M. Buescher, E. Firat, X. Zhu, G. Niedermann, G. Caputa, B. Kelly, U. Warthorst, A. Rensing-Ehl, R.L. Kyle, L. Vandersarren, J.D. Curtis, A.E. Patterson, S. Lawless, K. Grzes, J. Qiu, D.E. Sanin, O. Kretz, T.B. Huber, S. Janssens, B.N. Lambrecht, A.S. Rambold, E.J. Pearce, and E.L. Pearce. 2017. Mitochondrial Priming by CD28. Cell 171:385-397.e311.

Laino, A.S., B.C. Betts, A. Veerapathran, I. Dolgalev, A. Sarnaik, S.N. Quayle, S.S. Jones, J.S. Weber, and D.M. Woods. 2019. HDAC6 selective inhibition of melanoma patient T-cells augments anti-tumor characteristics. Journal for immunotherapy of cancer 7:33.

Lasko, L.M., C.G. Jakob, R.P. Edalji, W. Qiu, D. Montgomery, E.L. Digiammarino, T.M. Hansen, R.M. Risi, R. Frey, V. Manaves, B. Shaw, M. Algire, P. Hessler, L.T. Lam, T. Uziel, E. Faivre, D. Ferguson, F.G. Buchanan, R.L. Martin, M. Torrent, G.G. Chiang, K. Karukurichi, J.W. Langston, B.T. Weinert, C. Choudhary, P. de Vries, A.F. Kluge, M.A. Patane, J.H. Van Drie, C. Wang, D. McElligott, E. Kesicki, R. Marmorstein, C. Sun, P.A. Cole, S.H. Rosenberg, M.R. Michaelides, A. Lai, and K.D. Bromberg. 2017. Discovery of a selective catalytic p300/CBP inhibitor that targets lineage-specific tumours. Nature 550:128-132.

Le Borgne, M., S. Raju, B.H. Zinselmeyer, V.T. Le, J. Li, Y. Wang, M.J. Miller, and A.S. Shaw. 2016. Real-Time Analysis of Calcium Signals during the Early Phase of T Cell Activation Using a Genetically Encoded Calcium Biosensor. Journal of immunology (Baltimore, Md. : 1950) 196:1471-1479.

Ledderose, C., Y. Bao, M. Lidicky, J. Zipperle, L. Li, K. Strasser, N.I. Shapiro, and W.G. Junger. 2014. Mitochondria Are Gate-keepers of T Cell Function by Producing the ATP That Drives Purinergic Signaling *. Journal of Biological Chemistry 289:25936-25945.

Leddon, S.A., M.M. Fettis, K. Abramo, R. Kelly, D. Oleksyn, and J. Miller. 2020. The CD28 Transmembrane Domain Contains an Essential Dimerization Motif. Frontiers in Immunology 11:

Li, L., M. Leid, and E.V. Rothenberg. 2010. An early T cell lineage commitment checkpoint dependent on the transcription factor Bcl11b. Science 329:89-93.

Li, X., R. Mizsei, K. Tan, R.J. Mallis, J.S. Duke-Cohan, A. Akitsu, P.W. Tetteh, A. Dubey, W. Hwang, G. Wagner, M.J. Lang, H. Arthanari, J.H. Wang, and E.L. Reinherz. 2021. Pre-T cell receptors topologically sample self-ligands during thymocyte beta-selection. Science 371:181-185.

Liu, H.-H., M. Xie, M.D. Schneider, and Z.J. Chen. 2006. Essential role of TAK1 in thymocyte development and activation. Proceedings of the National Academy of Sciences 103:11677-11682.

Longabaugh, W.J.R., W. Zeng, J.A. Zhang, H. Hosokawa, C.S. Jansen, L. Li, M. Romero-Wolf, P. Liu, H.Y. Kueh, A. Mortazavi, and E.V. Rothenberg. 2017. Bcl11b and combinatorial resolution of cell fate in the T-cell gene regulatory network. Proceedings of the National Academy of Sciences 114:5800-5807.

Magner, W.J., A.L. Kazim, C. Stewart, M.A. Romano, G. Catalano, C. Grande, N. Keiser, F. Santaniello, and T.B. Tomasi. 2000. Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. J Immunol 165:7017-7024.

Magupalli, V.G., R. Negro, Y. Tian, A.V. Hauenstein, G. Di Caprio, W. Skillern, Q. Deng, P. Orning, H.B. Alam, Z. Maliga, H. Sharif, J.J. Hu, C.L. Evavold, J.C. Kagan, F.I. Schmidt, K.A. Fitzgerald, T. Kirchhausen, Y. Li, and H. Wu. 2020. HDAC6 mediates an aggresome-like mechanism for NLRP3 and pyrin inflammasome activation. Science 369:eaas8995.

Mallis, R.J., K. Bai, H. Arthanari, R.E. Hussey, M. Handley, Z. Li, L. Chingozha, J.S. Duke-Cohan, H. Lu, J.H. Wang, C. Zhu, G. Wagner, and E.L. Reinherz. 2015. Pre-TCR ligand binding impacts thymocyte development before alphabetaTCR expression. Proc Natl Acad Sci U S A 112:8373-8378.

Martinez, M., and E.K. Moon. 2019. CAR T Cells for Solid Tumors: New Strategies for Finding, Infiltrating, and Surviving in the Tumor Microenvironment. Frontiers in immunology 10:128-128.

Matson, C.A., S. Choi, F. Livak, B. Zhao, A. Mitra, P.E. Love, and N.J. Singh. 2020. CD5 dynamically calibrates basal NF-κB signaling in T cells during thymic development and peripheral activation. Proceedings of the National Academy of Sciences 117:14342-14353.

Mazzocchi, M., S.R. Goulding, S.L. Wyatt, L.M. Collins, A.M. Sullivan, and G.W. O'Keeffe. 2021. LMK235, a small molecule inhibitor of HDAC4/5, protects dopaminergic neurons against neurotoxin- and α-synuclein-induced degeneration in cellular models of Parkinson's disease. Molecular and Cellular Neuroscience 115:103642.

Metzeler, K.H., B. Heilmeier, K.E. Edmaier, V.P.S. Rawat, A. Dufour, K. Döhner, M. Feuring-Buske, J. Braess, K. Spiekermann, T. Büchner, M.C. Sauerland, H. Döhner, W. Hiddemann, S.K. Bohlander, R.F. Schlenk, L. Bullinger, and C. Buske. 2012. High expression of lymphoid enhancer-binding factor-1 (LEF1) is a novel favorable prognostic factor in cytogenetically normal acute myeloid leukemia. Blood 120:2118-2126.

Moore, A.R., S.C. Rosenberg, F. McCormick, and S. Malek. 2020. RAS-targeted therapies: is the undruggable drugged? Nature Reviews Drug Discovery 19:533-552.

Nagasaka, K., D. Pim, P. Massimi, M. Thomas, V. Tomaić, V.K. Subbaiah, C. Kranjec, S. Nakagawa, T. Yano, Y. Taketani, M. Myers, and L. Banks. 2010. The cell polarity regulator hScrib controls ERK activation through a KIM site-dependent interaction. Oncogene 29:5311-5321.

Notarangelo, L.D., R. Bacchetta, J.L. Casanova, and H.C. Su. 2020. Human inborn errors of immunity: An expanding universe. Science Immunology 5:eabb1662.

O'Leary, C.E., E.L. Lewis, and P.M. Oliver. 2015. Ubiquitylation as a Rheostat for TCR Signaling: From Targeted Approaches Toward Global Profiling. Frontiers in Immunology 6:

Ogawa, S., M. Watanabe, Y. Sakurai, Y. Inutake, S. Watanabe, X. Tai, and R. Abe. 2013. CD28 signaling in primary CD4+ T cells: identification of both tyrosine phosphorylation-dependent and phosphorylation-independent pathways. International Immunology 25:671-681.

Pang, D.J., J.F. Neves, N. Sumaria, and D.J. Pennington. 2012. Understanding the complexity of γδ T-cell subsets in mouse and human. Immunology 136:283-290.

Paul, S., and B.C. Schaefer. 2013. A new look at T cell receptor signaling to nuclear factor-κB. Trends Immunol 34:269-281.

Prasad, K.V., Y.C. Cai, M. Raab, B. Duckworth, L. Cantley, S.E. Shoelson, and C.E. Rudd. 1994. T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. Proceedings of the National Academy of Sciences of the United States of America 91:2834-2838.

Raboso-Gallego, J., A. Casado-García, M. Isidro-Hernández, and C. Vicente-Dueñas. 2019. Epigenetic Priming in Childhood Acute Lymphoblastic Leukemia. Frontiers in Cell and Developmental Biology 7:

Rodewald, H.R., K. Awad, P. Moingeon, L. D'Adamio, D. Rabinowitz, Y. Shinkai, F.W. Alt, and E.L. Reinherz. 1993. Fc gamma RII/III and CD2 expression mark distinct subpopulations of immature CD4-CD8- murine thymocytes: in vivo developmental kinetics and T cell receptor beta chain rearrangement status. J Exp Med 177:1079-1092.

Ross, D.A., and T. Kadesch. 2001. The notch intracellular domain can function as a coactivator for LEF-1. Molecular and cellular biology 21:7537-7544.

Rothenberg, E.V. 2011. T cell lineage commitment: identity and renunciation. J Immunol 186:6649-6655.

Sade, H., S. Krishna, and A. Sarin. 2004. The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. J Biol Chem 279:2937-2944.

Salter, A.I., R.G. Ivey, J.J. Kennedy, V. Voillet, A. Rajan, E.J. Alderman, U.J. Voytovich, C. Lin, D. Sommermeyer, L. Liu, J.R. Whiteaker, R. Gottardo, A.G. Paulovich, and S.R. Riddell. 2018. Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function. Science signaling 11:eaat6753.

Sasada, T., and E.L. Reinherz. 2001. A Critical Role for CD2 in Both Thymic Selection Events and Mature T Cell Function. The Journal of Immunology 166:2394-2403.

Serrador, J.M , J.R. Cabrero, D. Sancho, M. Mittelbrunn, A. Urzainqui, and F. Sanchez-Madrid. 2004. HDAC6 deacetylase activity links the tubulin cytoskeleton with immune synapse organization. Immunity 20:417-428.

Shapiro, M.J., and V.S. Shapiro. 2020. Chromatin-Modifying Enzymes in T Cell Development. Annual review of immunology 38:397-419.

Shin, B., H. Hosokawa, M. Romero-Wolf, W. Zhou, K. Masuhara, V.R. Tobin, D. Levanon, Y. Groner, and E.V. Rothenberg. 2021. Runx1 and Runx3 drive progenitor to T-lineage transcriptome conversion in mouse T cell commitment via dynamic genomic site switching. Proceedings of the National Academy of Sciences 118:e2019655118.

Shiroki, F., S. Matsuda, T. Doi, M. Fujiwara, Y. Mochizuki, T. Kadowaki, H. Suzuki, and S. Koyasu. 2007. The p85α Regulatory Subunit of Class IA Phosphoinositide 3-Kinase Regulates β-Selection in Thymocyte Development. The Journal of Immunology 178:1349-1356.

Sidwell, T., and E.V. Rothenberg. 2021. Epigenetic Dynamics in the Function of T-Lineage Regulatory Factor Bcl11b. Front Immunol 12:669498.

Takács, L., F.W. Ruscetti, E.J. Kovacs, B. Rocha, S. Brocke, T. Diamantstein, and B.J. Mathieson. 1988. Immature, double negative (CD4-,CD8-) rat thymocytes do not express IL-2 receptors. J Immunol 141:3810-3818.

Tsuji, G., N. Okiyama, V.A. Villarroel, and S.I. Katz. 2015. Histone deacetylase 6 inhibition impairs effector CD8 T-cell functions during skin inflammation. J Allergy Clin Immunol 135:1228-1239.

Turka, L.A., M.C. Fletcher, N. Craighead, C.B. Thompson, and C.H. June. 1992. Defective signal transduction by the CD2 molecule in immature T-cell receptor/CD3- thymocytes. Proceedings of the National Academy of Sciences 89:8706-8710.

Van de Walle, I., E. Waegemans, J. De Medts, G. De Smet, M. De Smedt, S. Snauwaert, B. Vandekerckhove, T. Kerre, G. Leclercq, J. Plum, T. Gridley, T. Wang, U. Koch, F. Radtke, and T. Taghon. 2013. Specific Notch receptor–ligand interactions control human TCR-αβ/γδ development by inducing differential Notch signal strength. The Journal of Experimental Medicine

Vega, R.B., K. Matsuda, J. Oh, A.C. Barbosa, X. Yang, E. Meadows, J. McAnally, C. Pomajzl, J.M. Shelton, J.A. Richardson, G. Karsenty, and E.N. Olson. 2004. Histone Deacetylase 4 Controls Chondrocyte Hypertrophy during Skeletogenesis. Cell 119:555-566.

Voisinne, G., A. García-Blesa, K. Chaoui, F. Fiore, E. Bergot, L. Girard, M. Malissen, O. Burlet-Schiltz, A. Gonzalez de Peredo, B. Malissen, and R. Roncagalli. 2016. Co-recruitment analysis of the CBL and CBLB signalosomes in primary T cells identifies CD5 as a key regulator of TCR-induced ubiquitylation. Molecular Systems Biology 12:876.

Voisinne, G., A. Gonzalez de Peredo, and R. Roncagalli. 2018. CD5, an Undercover Regulator of TCR Signaling. Frontiers in Immunology 9:

Voll, R.E., E. Jimi, R.J. Phillips, D.F. Barber, M. Rincon, A.C. Hayday, R A. Flavell, and S. Ghosh. 2000. NF-kappa B activation by the pre-T cell receptor serves as a selective survival signal in T lymphocyte development. Immunity 13:677-689.

Wang, P., Z. Wang, and J. Liu. 2020. Role of HDACs in normal and malignant hematopoiesis. Molecular cancer 19:5.

Wang, Z., G. Qin, and T.C. Zhao. 2014. HDAC4: mechanism of regulation and biological functions. Epigenomics 6:139-150.

Webb, L.V., S.C. Ley, and B. Seddon. 2016. TNF activation of NF-κB is essential for development of single-positive thymocytes. J Exp Med 213:1399-1407.

Weng, R.R., H.-H. Lu, C.-T. Lin, C.-C. Fan, R.-S. Lin, T.-C. Huang, S.-Y. Lin, Y.-J. Huang, Y.-H. Juan, Y.-C. Wu, Z.-C. Hung, C. Liu, X.-H. Lin, W.-C. Hsieh, T.-Y. Chiu, J.-C. Liao, Y.-L. Chiu, S.-Y. Chen, C.-J. Yu, and H.-C. Tsai. 2021. Epigenetic modulation of immune synaptic-cytoskeletal networks potentiates γδ T cell-mediated cytotoxicity in lung cancer. Nature Communications 12:2163.

Werlen, G., R. Jain, and E. Jacinto. 2021. MTOR Signaling and Metabolism in Early T Cell Development. Genes (Basel) 12:

Williams, J.A., K.S. Hathcock, D. Klug, Y. Harada, B. Choudhury, J.P. Allison, R. Abe, and R.J. Hodes. 2005. Regulated costimulation in the thymus is critical for T cell development: dysregulated CD28 costimulation can bypass the pre-TCR checkpoint. Journal of immunology (Baltimore, Md. : 1950) 175:4199-4207.

Xing, S., F. Li, Z. Zeng, Y. Zhao, S. Yu, Q. Shan, Y. Li, F.C. Phillips, P.K. Maina, H.H. Qi, C. Liu, J. Zhu, R.M. Pope, C.A. Musselman, C. Zeng, W. Peng, and H.-H. Xue. 2016. Tcf1 and Lef1 transcription factors establish CD8+ T cell identity through intrinsic HDAC activity. Nature Immunology 17:695-703.

Xu, K., W.Y. Yang, G.K. Nanayakkara, Y. Shao, F. Yang, W. Hu, E.T. Choi, H. Wang, and X. Yang. 2018. GATA3, HDAC6, and BCL6 Regulate FOXP3+ Treg Plasticity and Determine Treg Conversion into Either Novel Antigen-Presenting Cell-Like Treg or Th1-Treg. Frontiers in immunology 9:45.

Yu, S., X. Zhou, Farrah C. Steinke, C. Liu, S.-C. Chen, O. Zagorodna, X. Jing, Y. Yokota, David K. Meyerholz, Charles G. Mullighan, C.M. Knudson, D.-M. Zhao, and H.-H. Xue. 2012. The TCF-1 and LEF-1 Transcription Factors Have Cooperative and Opposing Roles in T Cell Development and Malignancy. Immunity 37:813-826.

Yui, M.A., N. Feng, and E.V. Rothenberg. 2010. Fine-scale staging of T cell lineage commitment in adult mouse thymus. J Immunol 185:284-293.

Zhang, X.-H., M. Qin, H.-P. Wu, M.Y. Khamis, Y.-H. Li, L.-Y. Ma, and H.-M. Liu. 2021. A Review of Progress in Histone Deacetylase 6 Inhibitors Research: Structural Specificity and Functional Diversity. Journal of medicinal chemistry 64:1362-1391.

Zhang, X., Z. Yuan, Y. Zhang, S. Yong, A. Salas-Burgos, J. Koomen, N. Olashaw, J.T. Parsons, X.-J. Yang, S.R. Dent, T.-P. Yao, W.S. Lane, and E. Seto. 2007. HDAC6 modulates cell motility by altering the acetylation level of cortactin. Molecular cell 27:197-213.

Zheng, X., J.-X. Gao, X. Chang, Y. Wang, Y. Liu, J. Wen, H. Zhang, J. Zhang, Y. Liu, and P. Zheng. 2004. B7-CD28 Interaction Promotes Proliferation and Survival but Suppresses Differentiation of CD4[−]CD8[−] T Cells in the Thymus. The Journal of Immunology 173:2253-2261.

Zikherman, J., C. Jenne, S. Watson, K. Doan, W. Raschke, C.C. Goodnow, and A. Weiss. 2010. CD45-Csk phosphatase-kinase titration uncouples basal and inducible T cell receptor signaling during thymic development. Immunity 32:342-354.