Zfp335-Mediated Regulation of T cell Development

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Immunology in the Graduate School
of Duke University

2022

ABSTRACT

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Abstract

T cells are a critical arm of the adaptive immune system which function to coordinate and orchestrate complex immune reactions, as well as, kill damaged and infected cells. Production of a diverse peripheral T cell compartment requires massive expansion of the bone marrow progenitors that seed the thymus. There are two main phases of expansion during T cell development, following T lineage commitment at the DN2 stage and following successful rearrangement and selection for functional TCRβ chains in DN3 thymocytes, which promotes development of DN4 cells to the DP stage. Signals driving expansion of DN2 thymocytes are well studied, however, factors regulating the proliferation and survival of DN4 cells remain poorly understood.

E proteins are transcription factors which have been shown to play essential non-redundant roles throughout T cell development. The functions of E proteins in T cell development include, enforcing T lineage commitment, promoting proper TCR rearrangements, regulating developmental progression and functional checkpoints, and coordinating complex transcriptional networks underpinning developmental progression. Due to the large number of genome-wide binding sites and massive number of genes regulated by E proteins, their numerous functions are poorly understood. The goal of this dissertation is to determine the role of the E protein-regulated transcription factor Zfp335 in T cell development.

We utilized conditional deletion models to determine the role of Zfp335 in early and late stages of conventional and unconventional T cell development. Through these efforts we uncovered we uncover an unexpected link between the transcription factor Zfp335 and control of the cGAS/STING pathway for sensing cytosolic DNA in post-β-selection DN4 thymocytes. The absence of Zfp335 drives cGAS/STING-dependent death of DN4 cells. Zfp335 controls survival by sustaining expression of Ankle2, which in turn regulates the activity of Baf to suppress cGAS/STING-dependent cell death. Additionally, genetic ablation of Zfp335 precludes the development of unconventional iNKT cells due to STING-independent cell death following lineage commitment along with preventing effector differentiation of surviving cells.

Our studies also uncovered an additional cGAS/STING-independent role in the terminal maturation of conventional αβ T cells. The absence of Zfp335 prevents the establishment of a naïve T cell compartment, inhibits differentiation of CD4 T cells and promotes the developmental acquisition of an effector program in CD8 T cells. Using *in vivo* and *ex vivo* genetic manipulation combined with detailed bioinformatic analyses we show that Zfp335 functions to promote T cell development, maturation, and effector differentiation through the regulation of a small but essential set of genes.

To our knowledge, these studies detail the first described role for cGAS/STING in T cell development and strongly suggest a transcriptional mechanism downstream of Zfp335 which coordinates genome-wide alterations to chromatin compaction required

for proper establishment of conventional and unconventional T cell pools. Together, these studies will provide a novel framework for understanding the life and death of developing T cells and may uncover novel pathways for enhancing the efficacy of T cell-based therapeutics.

Dedication

I dedicate this dissertation to Joan, James, and Daniel Ratiu, Pamelia and Louise Fraungruber, Teresa and Gasper Peralta, Lillian and Tom Jennings, my friends, my colleagues, and all the mice that contributed to the work detailed within. All of you helped me in some way during this journey and I am forever grateful! Pamelia E. Fraungruber saw me through thick and thin, before, during, and hopefully for a long time after my graduate education is complete. My parents gave me life, fed my curiosity, and nurtured my inquisitive side. Without you I would not have gotten here.

Contents

Abstract iv

List of Tables xiii

List of Figures xiv

Acknowledgements xvii

1. Introduction 1

1.1 T cells – what are they and what do they do? 3

1.2 T cell development 6

1.2.1 Transcriptional regulation of T cell development 13

1.2.1.1 NOTCH and Bcl11b in T lineage commitment 13

1.2.1.2 E proteins are critical regulators of T cell development 15

1.2.1.3 GATA3 17

1.2.1.4 Runx family transcription factors 18

1.2.1.5 ThPOK 20

1.2.1.6 RORγt 22

1.2.1.7 PLZF – The master transcription factor for iNKT cell development 24

1.3 cGAS/STING cytosolic DNA sensing pathway in T cell-mediated immunity 25

1.3.1 Overview of cGAS/STING pathway 25

1.3.1.1 Cyclic GMP-AMP synthase (cGAS) 26

1.3.1.2 Stimulator of interferon genes (STING) 29

1.3.1.3 Sources of cGAS activating ligand 31

1.3.1.4 cGAS/STING signaling pathway and its outcomes 33

1.3.2 cGAS/STING in T cell responses 34

1.3.2.1 T cell intrinsic roles for cGAS/STING 34

1.3.2.2 T cell extrinsic roles for cGAS/STING 43

1.3.2.3 cGAS/STING in antimicrobial T cell responses 44

1.3.2.4 cGAS/STING in antitumor T cell responses 51

1.3.2.5 cGAS/STING in autoimmunity 57

1.3.2.6 Therapeutic targeting of cGAS/STING pathway 65

1.3.3 Summary and future directions 75

1.4 Zinc Finger Protein 335 (Zfp335) 77

1.4.1 Zfp335 in disease 77

1.4.2 Zfp335 regulates T cell maturation 79

2. Loss of Zfp335 drives cGAS/STING-dependent apoptosis of post-β-selection pre-T cells 81

2.1 Introduction 81

2.2 Results 85

2.2.1 Zfp335, an E-protein target, is critical for T cell development 85

2.2.2 Loss of Zfp335 in DN3 thymocytes does not impair β-selection 91

2.2.3 Zfp335 inhibits apoptosis during the DN-DP transition 94

2.2.4 Ectopic expression of Bcl2 rescues the developmental defect resulting from loss of Zfp335 98

2.2.5 Defining the 'true' DN4 thymocyte population at the single cell level 102

2.2.6 Ankle2 is a critical Zfp335-regulated gene required for survival of DN4 thymocytes 107

2.2.7 Loss of Zfp335 disrupts nuclear envelope architecture and promotes cGAS/STING signaling 112

2.2.8 Disruption of Zfp335/Ankle2/Baf axis drives cGAS/STING-dependent apoptosis of DN4 thymocytes 120

2.2.9 DN4 thymocytes exhibit increased sensitivity to cGAS/STING-mediated cell death 126

2.3 Discussion 129

3. Zfp335 controls the development, maturation, and differentiation of invariant NKT cells and conventional αβ T cells 137

3.1 Introduction 137

3.2 Results 141

3.2.1 Zfp335, an E protein target, is required for iNKT cell development 141

3.2.2 Loss of Zfp335 leads to defective iNKT cell differentiation 143

3.2.3 Bcl2 overexpression rescues total iNKT numbers but not differentiation in the absence of Zfp335 145

3.2.4 Zfp335-deficient thymic iNKT cell developmental defects are cGAS/STING-independent 147

3.2.5 Il13^{YetCre} mediates efficient deletion immediately upon iNKT lineage commitment 148

3.2.6 Loss of Zfp335 following commitment impairs iNKT cell development 151

3.2.7 Zfp335 regulates thymic maturation of conventional $\alpha\beta$ T cells independent of cGAS/STING 153

3.2.8 scRNA-seq identifies Smc2 as a Zfp335 target gene of interest in iNKT cell development and conventional T cell maturation 155

3.2.9 Loss of Zfp335 leads to impaired chromatin condensation in T cells 160

3.2.10 Zfp335 deficiency drives developmentally acquired CD8 T-bet expression. 162

3.3 Discussion 166

4 Methods 170

4.1 Mice 170

4.2 Antibodies 170

4.3 Flow cytometry and cell sorting 171

4.4 ChIP-seq analysis 172

4.5 Bulk RNA-seq 172

4.6 Cell Culture 173

4.7 DN thymocyte enrichment 174

4.8 Retrovirus packaging and transduction 175

4.9 TCRα repertoire analysis 175

4.10 scRNA-seq library preparation 177

4.11 scRNA-seq analysis 177

4.12 Cloning cDNA overexpression vectors 179

4.13 Generation of *Scid.adh.2c2-dCas9*$^{10x\text{-}GCN4}$ CRISPRi cells 179

4.14 Generation of gRNA retroviral vectors 180

4.15 qPCR analysis of gene expression 180

4.16 Proximity Ligation Assay for Baf phosphorylation 181

4.17 Determination of nuclear envelope structure and measurement of cytosolic dsDNA 182

4.18 2′,3′-cGAMP ELISA 184

4.19 *In vivo* H-151 treatment of mice 185

4.20 *In vivo* generation of post-β-selection Rag2$^{-/-}$ thymocytes 185

4.21 Assessment of chromatin condensation 185

4.22 Statistical analysis 186

4.23 Primers 186

5. Future Directions 191

5.1 Does the presence of TCR excision circles (TREC) sensitize DN4 thymocytes to cGAS/STING mediated cell death? 191

5.2 Does survival of DP thymocytes depend upon down-regulation of cGAS and STING? 193

5.3 Is loss of Smc2 expression in Zfp335-deficient thymocytes responsible for differentiation defects observed? 194

5.4 Does genetic ablation of Zfp335 enhance antitumor CD8 T cell responses and can ex vivo genetic manipulation enhance existing engineered T cell-based therapies? 195

References 197

Biography 260

List of Tables

Table 1: Primers used for cloning, genotyping, qRT-PCR, and sequencing library preparation.....186

List of Figures

Figure 1: Post-translational regulation of cGAS/STING signaling 31

Figure 2: Outcomes of cGAS/STING signaling in T cells 39

Figure 3: Mechanisms of exogenous cGAMP uptake/ transfer 41

Figure 4: Viral regulation of cGAS/STING signaling pathway 47

Figure 5: Positive and negative regulatory roles of cGAS/STING pathway in anti-tumor immunity. 56

Figure 6: cGAS/STING-dependent regulation of autoimmune disease. 64

Figure 7: cGAS/STING acts as a rheostat in development of autoimmune disease. 65

Figure 8: Zfp335 is a target of E proteins in developing T cells 87

Figure 9: Zfp335 is critical to αβ T cell development 89

Figure 10: – Zfp335cKO mice exhibit T lymphopenia and reduced peripheral naïve T cells 91

Figure 11: Loss of Zfp335 during DN3 does not impair β-selection 93

Figure 12: Zfp335cKO DN4 thymocytes undergo increased rates of apoptosis 95

Figure 13: Loss of Zfp335 does not impair DP thymocyte survival 97

Figure 14: Bcl2 overexpression rescues Zfp335-deficient thymocytes from apoptosis 99

Figure 15: Bcl2 overexpression fails to rescue thymic differentiation defect and peripheral T lymphopenia in Zfp335-deficient mice 101

Figure 16: Defining the 'true' DN4 thymocyte population at the single cell level 103

Figure 17: Loss of Zfp335 promotes increased type I and II interferon signaling in γδ and maturing αβ thymocytes 105

Figure 18: scRNA-seq identifies 'true' Zfp335 mutant DN4 cells 108

Figure 19: scRNA-seq identifies Ankle2 as a critical Zfp335-regulated gene controlling survival of DN4 thymocytes 110

Figure 20: Disruption of *ANKLE2* or *BANF1* expression leads to abnormal NE architecture in human cells 113

Figure 21: Loss of Zfp335 leads to Baf hyperphosphorylation altered nuclear envelope architecture and accumulation of cytosolic 115

Figure 22: Loss of Zfp335 promotes cGAS/STING signaling in DN4 thymocytes 118

Figure 23: Loss of Zfp335 does not drive mitochondrial membrane deploraization 120

Figure 24: Chemical inhibition of cGAS/STING signaling rescues Zfp335-deficient thymocyte apoptosis 121

Figure 25: Increased rates of Zfp335cKO apoptosis is not dependent upon mTOR or Type I Interferon signaling. 123

Figure 26: The Zfp335/Ankle2/Baf axis functions to suppress cGAS/STING-mediated apoptosis of DN4 thymocytes 125

Figure 27: Cas9-mediated Zfp335 deletion drives cGAS/STING-dependent DN4 apoptosis 126

Figure 28: DN4 thymocytes exhibit increased sensitivity to STING-mediated apoptosis. 128

Figure 29: Schematic diagram of mechanism by which Zfp335 promotes survival of DN4 thymocytes. 129

Figure 30: Loss of Zfp335 severely impairs iNKT cell development 142

Figure 31: Zfp335-deficient mice harbor thymocytes expressing the canonical iNKT TCRα rearrangement. 143

Figure 32: Zfp335-deficient iNKT cells can undergo effector differentiation 144

Figure 33: Bcl2 overexpression rescues Zfp335-deficient iNKT cell numbers but not effector differentiation. 146

Figure 34: cGAS/STING inhibition fails to rescue Zfp335-deficient iNKT cell development 148

Figure 35: scRNA-seq analysis of thymic iNKT cell development 149

Figure 36: *Il13* is a stage 0 iNKT cell-specific gene 151

Figure 37: Loss of Zfp335 following lineage commitment leads to reduced iNKT cell numbers 152

Figure 38: Zfp335 regulates thymic iNKT cell effector differentiation 153

Figure 39: T cell maturation defect resulting from loss of Zfp335 is cGAS/STING-independent 154

Figure 40: scRNA-seq identifies Smc2 as a Zfp335 target of interest in iNKT cell development 156

Figure 41: scRNA-seq analysis of CD8SP maturation identifies Zfp335 target genes of interest 159

Figure 42: Loss of Zfp335 results in reduced chromatin condensation in thymic and peripheral T cells 161

Figure 43: *Tbx21* and *Rorc* loci are dynamically regulated during T cell development 163

Figure 44: Loss of Zfp335 promotes thymic acquisition of T-bet expression in CD8 T cells 165

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1. Introduction

A unifying characteristic of all living things is the ability to mount protective responses in the face of foreign insult; a process collectively referred to as immunity. The ability of an organism to protect itself from biological or non-biological threats is a necessity for life, reproduction, and evolution. Simple single cell organisms such as bacteria evolved intricate systems such as restriction enzymes and the clustered regularly interspersed short palindromic repeat (CRISPR)-Cas system to protect themselves from bacterial viruses known as bacteriophage (Garneau et al., 2010; Horvath & Barrangou, 2010). Multicellular organisms have evolved far more complex and intricate systems to protect themselves from a wider range of threats. In general, immunity in complex multicellular organisms such as mammals, birds and fish can be categorized into innate or adaptive immunity. Innate immune responses are characterized by rapid responses to molecular patterns recognized as potentially threatening. Innate immunity is not antigen-specific and functions as a first line defense should barrier integrity be compromised. Conversely, adaptive immunity is characteristically slow, exhibit a high degree of antigen-specificity, and allows for the generation of long-term durable immunologic memory. A significant degree of interplay between these two systems of immunity is required for efficient and effective protection of the host.

The innate immune system can be broken down into three main categories: barriers, antimicrobial peptides, and cellular responses. Unlike adaptive immunity, all cell types have the capacity to mount some form of innate immune response. Barrier tissues such as the skin and mucosal surfaces are the first and most basic aspect of innate immunity. Barrier tissues function to maintain the separation of the external environment and the body. Should barrier integrity be lost or compromised innate responses ensue. The specific form of innate response is largely context-dependent with significant overlap across types of insult. In the context of bacterial, fungal, viral, or protozoan infection numerous cell types will produce innate effector molecules such as inflammatory cytokines, chemoattractants, antimicrobial peptides, and toxic compounds such as reactive oxygen and reactive nitrogen species. Together, these molecules promote the recruitment and activation of immune cells and can directly kill foreign cells. In its most basic sense, innate immunity functions to suppress propagation of infectious agents and promote adaptive immune responses which ultimately drive clearance of infection.

The adaptive immune system is comprised of two main cell types: B cells and T cells. B cells mediate humoral immunity through the production of antibodies. Collectively, antibodies can recognize virtually any type of organic molecule and exhibit numerous functional capacities. Some critical functions of antibodies in host defense are neutralization, opsonization and complement activation. Antibody-mediated humoral

immunity comprises the cell-extrinsic arm of the adaptive immune system. T cells on the other hand, mediate cellular immunity through the recognition of antigen via the T cell receptor. T cells function to protect the host via orchestration and coordination of responses by other immune cells or directly killing damaged, infected, or cancerous cells.

B cells and T cells, as well as many innate immune cells, originate from a common progenitor, hematopoietic stem cells (HSC). HSCs are long-lived, self-renewing cells typically residing within the bone marrow, and give rise to progenitors which may populate lymphoid or myeloid lineages (Orkin, 1995). B cells and T cells develop from the common lymphoid progenitor in the bone marrow or thymus, respectively. Each cell develops through a complex, yet analogous, series of functionally and phenotypically defined stages. These stages can broadly be defined as commitment to the specific lineage, rearrangement of antigen receptors (AgR), selection for functionally rearranged AgR, and elimination of autoreactivity. Errors in any of these steps often result in disease (Bouis et al., 2019; Buckley et al., 1997; Fischer, 2000). Therefore, understanding the molecular and biochemical underpinnings of these processes is critical to elucidating the causes of immune dysfunction and disease.

1.1 T cells – what are they and what do they do?

T cells are a type of lymphocyte responsible for making up the cellular arm of the adaptive immune system. T cell identity is defined by the expression of somatically

rearranged T cell receptors (TCR) (Arstila et al., 1999; Lieber, 1991). There are two primary types of TCR defined by the TCR chains paired: αβ and γδ. Most αβ T cells are considered conventional T cells. That is, they recognize peptide antigen presented in the context of major histocompatibility complex (MHC) I or II. A minor population of αβ T cells and all γδ T cells are unconventional T cells (Godfrey et al., 2015).

Unconventional T cells are unique in that they exhibit both adaptive and innate characteristics. Unlike conventional T cells, these cells do not require priming and are poised to mount very rapid immune responses. Unconventional αβ typically recognize non-peptide antigens presented via MHC-like molecules (Godfrey & Kronenberg, 2004; Godfrey et al., 2015). The most common among these cell types are natural killer T (NKT) cells (Godfrey et al., 2004) and mucosa-associated invariant T (MAIT) cells (Legoux et al., 2020). In general, unconventional αβ T cells recognize phospholipids or metabolic byproducts. However, in many cases the exact antigen recognized remains unknown (Godfrey et al., 2015). There are numerous types of γδ T cells which may recognize highly diverse antigens in many different contexts (Ribot et al., 2021). Some recognize small molecules presented by MHC-like molecules, lipids, post-translational modifications or conformational changes in host proteins, or even soluble protein (Born et al., 2013; Godfrey et al., 2015). Together, these unconventional T cells function to bridge the gap between foreign insult and initiation of true adaptive responses.

Conventional T cells are exclusively αβ TCR expressing and can be broadly categorized by expression of either CD4 or CD8 co-receptors. CD4 and CD8 T cells recognize peptide antigen presented via MHC-II and MHC-I, respectively. CD4 T cells can be further broken down into helper (Th) or regulatory T cells (Treg) which function to promote immune responses via cytokine and chemokine production or suppress immune responses through a variety of mechanisms, respectively (Luckheeram et al., 2012). CD8 T cells generally are known for their cytotoxic capabilities achieved through production of cytolytic mediators or expression of surface molecules which can induce intrinsic cell death (Taniuchi, 2018).

Specific effector functions of T cells are largely dictated by expression of lineage-defining master transcription factors which are shared across all types of T cells. These transcription factors include: T-bet, GATA3, and RORγt which define type 1, 2 or 17 T cells, respectively (De Obaldia & Bhandoola, 2015). Expression of these transcription factors promotes the production of lineage-specific cytokines and enforce lineage identity. Type 1 T cells primarily function to promote killing or clearance of intracellular pathogens. Type 2 T cells mediate responses to extracellular pathogens such as helminths. Type 17 cells, typically found at mucosal barriers, promote responses to extracellular bacteria and fungi. Additionally, each of these T cell lineages can promote disease (Veldhoen, 2009). Type 1 and 17 T cells contribute to a host of autoimmune diseases including, type 1 diabetes, multiple sclerosis, inflammatory bowel disease and

others. Type 2 T cells contribute to allergy and can promote atopic disease. In many cases, imbalances between these T cell subsets leads to aberrant regulation and inflammation. Interestingly, type 1, 2, 17 lineage identities can be found across virtually all types of T cells (Dong, 2021).

1.2 T cell development

T cells originate from bone marrow-resident HSC, and develop within the thymus prior to egress and population of nearly all bodily tissues. T cells likely first arose in gnathastomes (jawed fish) (Cooper & Alder, 2006). T cells are defined by their expression of characteristic T cell receptors (TCR), which are generated via somatic recombination of TCR gene segments through a process known as V(D)J recombination (Alt et al., 1992). One incredible feature of this process is the immense diversity of possible TCRs that may be generated. It is estimated that recombination of germline-encoded TCR gene segments could yield approximately 10^{15} unique TCR rearrangements; far exceeding the number of cells in the human body (Nikolich-Zugich et al., 2004). This breadth of TCR diversity allows for the generation of T cells specific for virtually any antigen.

T cell development begins when early thymic progenitors (ETP) enter the thymic cortex via the blood. The stages of thymic T cell development are defined by the expression of several surface molecules including: CD4, CD8, CD25, CD44 and CD117.

Upon entering the thymus ETPs become DN1 cells ($CD4^-$ $CD8^-$ $CD25^-$ $CD44^+$ $CD117^+$). DN1 cells then progress to the DN2 stage ($CD4^-$ $CD8^-$ $CD25^+$ $CD44^+$ $CD117^+$) where they commit to the T lineage via NOTCH-driven Bcl11b expression (L. Li et al., 2010). Upon lineage commitment, DN2 cells undergo a brief, but rapid, phase of proliferation which functions to expand the pool of committed progenitors. Following proliferation, these cells down-regulate CD44 and CD117 and progress to DN3 where TCRβ, γ, and δ gene segments are rearranged in a Rag-dependent manner (Dutta et al., 2021). Successful rearrangement of a functional TCRβ chain promotes developmental progression toward the αβ lineage through a process known as β-selection. Should successful rearrangement of both TCRγ and TCRδ chains occur first, cells commit to the γδ lineage, upon which they developmentally acquire effector identity. Following β-selection, cells lose expression of CD25 and progress to the highly proliferative DN4 phase, after which CD8 expression is up-regulated, defining progression to the immature single positive (ISP) stage. From there, CD4 up-regulation defines the transition to double positive (DP), at which point, cell cycle arrest occurs allowing rearrangement of TCRα chains. Successful rearrangement is followed by positive selection via recognition of MHC-I or MHC-II-bound peptides presented by cortical thymic epithelial cells. Following positive selection, cells lose expression of CD4 or CD8 committing to the single-positive (SP) lineage. CD4SP or CD8SP migrate to the thymic medulla where they undergo negative selection to eliminate any autoreactive clones. Should an SP cell survive negative

selection it will undergo final maturation, after which it may exit the thymus via blood and travel to secondary lymphoid organs, such as, lymph nodes or spleen as a naïve T cell.

In total, thymic T cell development is believed to occur over the course of approximately 30-42 days in mice (Krueger et al., 2017). Interestingly, mathematical modeling and experimental evidence suggests that >98% of all developing T cells die within the thymus (Egerton, Scollay, et al., 1990; Egerton, Shortman, et al., 1990; Sawicka et al., 2014). Approximately 65% of pre-DP undergo death by neglect which results from the failure to successfully rearrange a functional TCR or rearrangement of a TCR which is unable to recognize MHC molecules. Estimates suggest that an additional 92% of DP, 9% of CD4SP and 32% of CD8SP thymocytes are lost via negative selection (Sawicka et al., 2014). Therefore, very stringent regulation of the developmental process is required to achieve development of a diverse and functional T cell pool.

T cells are a critical arm of the adaptive immune system with diverse functions ranging from orchestrating tissue development and homeostasis to fighting infections and cancer. These diverse roles are achieved through the immense heterogeneity in mature T cell function and lineage identity. Interestingly, in mammals, T cells represent the only hematopoietic lineage not generated within the bone marrow (Ciofani & Zuniga-Pflucker, 2007). Instead, they primarily develop within the thymus, and possibly within the gut (Kanamori et al., 1996; Lambolez et al., 2002; Saito et al., 1998), from bone

marrow-derived precursor cells. Due to their importance to homeostasis and disease, understanding of T cell development has long been a topic of intense inquiry. Rothenberg and Taghon suggest that T cell development broadly occurs through five distinct stages:

> *"(i) multipotent progenitors enter the T cell pathway and migrate to the thymus; (ii) proliferative expansion and T cell receptor (TCR) gene rearrangement coinciding with T lineage commitment; (iii) β-selection triggered by successful rearrangement of TCRβ V(D)J genes driving pre-TCR signaling and proliferation; (iv) TCR-dependent positive selection; and (v) terminal differentiation of T cells within the periphery*." (Rothenberg & Taghon, 2005)

More granularly, within the thymus T cells undergo a series of phenotypically and functionally defined stages gated by a series of checkpoints to ensure proper rearrangement of TCR genes and elimination of autoreactive clones. These checkpoints are enforced by both positive and negative regulatory functions of numerous transcription factors (Rothenberg & Taghon, 2005). One unifying characteristic across the selection checkpoints surrounding TCR rearrangement is death as a default outcome (Egerton, Scollay, et al., 1990; Stritesky et al., 2013). That is, should any developing T cell fail to receive either pre-TCR signals or pMHC-mediated TCR signaling at the β-selection or positive selection checkpoints, respectively, they will die. It is estimated that throughout these stages of development approximately 90% of T cells will die in this manner (Sawicka et al., 2014). Through the process of positive selection, DP thymocytes will commit to the CD4 or CD8 lineage based on opposing functions of Runx and

ThPOK transcription factors, after which they migrate from the thymic cortex to the medulla (Sato et al., 2005; Sawada et al., 1994; Woolf et al., 2003). Within the medulla they are exposed to nearly all self-antigens presented in the context of MHC class I or II by medulla thymic epithelial cells, dendritic cells, and B cells (Anderson et al., 2002; Herbin et al., 2016; Yamano et al., 2015). Unlike earlier stages, should a single positive thymocyte recognize a self-antigen within sufficient affinity they will die or be diverted to the regulatory T cell lineage (Moran et al., 2011). This negative selection checkpoint is critical to preventing life-threatening autoimmunity (Anderson et al., 2002).

Based on the frequency of developing cells lost at the positive-selecting checkpoints (Sawicka et al., 2014), rearrangement of TCR genes is arguably the most important aspect to successful T cell development. Enforcement of the first (β-selection) checkpoint is inherently cell intrinsic as progression beyond requires pairing of TCRβ chains with pre-Tα produced by the same cell to form the pre-TCR complex. Upon formation of a pre-TCR, pre-Tα mediates spontaneous oligomerization which drives pre-TCR signaling (Yamasaki et al., 2006), repression of RAG-mediated recombination, release from cell cycle arrest and transition from DN3a to DN3b and finally DN4 stage of development (Fehling et al., 1995). Following numerous rounds of division, DN4 cells eventually upregulate CD8 then CD4 and begin to rearrange TCRα genes in a RAG-dependent manner as DP cells (Boudil et al., 2015; Guo et al., 2002; Schatz et al., 1989; Stritesky et al., 2012; Thompson et al., 1990). The DN3b, DN4 and CD8SP immature (ISP)

stages are characterized by rapid proliferation which is thought to serve to expand the population of cells with productive TCRβ rearrangements (Krueger et al., 2017; Trigueros et al., 2003). Due to the extremely transient nature and difficulty in studying stages between β-selection and establishment of DP cells, mechanisms regulating this transition are poorly understood.

Following positive selection developing T cells have three potential fates: CD4SP, CD8SP or death. Death can occur by neglect due to failure to rearrange a functional TCR or through negative selection of self-reactive clones. The decision between CD4 and CD8 lineages is dependent upon MHC restriction, TCR signal strength and transcriptional networks (Singer et al., 2008). Regardless of lineage choice, positively selected thymocytes up-regulate CCR7 expression which facilitates migration to the thymic medulla (Campbell et al., 1999). Once in the medulla, SP thymocytes interact with medullary thymic epithelial cells (mTEC) which facilitate negative selection (Anderson et al., 2002).

mTEC are a unique population of epithelial cells exclusively found within the thymic medulla, endowed with professional antigen presentation abilities. mTEC play an indispensable role in induction of central T cell tolerance through expression and presentation of nearly all self-antigens to single-positive thymocytes. Expression of many of these self-antigens is normally restricted to a specific tissue. Such self-antigens are known as tissue-restricted antigens (TRA). mTEC can either directly present TRA to

developing T cells or transfer these antigens to other medullary antigen presenting cells (APC), typically dendritic cells (DC). Should single-positive thymocytes recognize self-antigen via TCR:MHC interactions with mTEC or DC, T cells are either deleted or selected to become regulatory T cells in an affinity-dependent manner (Kawahata et al., 2002). The importance of these processes underpinning central T cell tolerance is highlighted by the fact that humans and mice harboring mutations in the autoimmune regulator gene (Aire) or Fezf2, which are responsible for expression of roughly 60% of all TRA, exhibit severe systemic autoimmune disease (Anderson et al., 2002; Takaba et al., 2015). Furthermore, the Alymphoplasia (Aly/Aly) mouse with homozygous loss-of-function mutation in NF-κB-inducing kinase (Nik) are characterized by a specific defect in mTEC development and develop severe systemic autoimmunity (Kajiura et al., 2004).

Upon completion of negative selection, surviving SP thymocytes must undergo final maturation prior to exiting the thymus as naïve T cells. Prior to maturation, SP thymocytes express high levels of CD24, are known as semi-mature, and still susceptible to negative selection. Maturation is associated with loss of CD24 expression and sensitivity to negative selection (Kishimoto & Sprent, 1997) and significant phenotypic changes. These changes include up-regulation of MHC-I, Qa2 and CD62L, progressive loss of CD69 and Rag expression, and regained proliferation-competence (Xing et al., 2016). Transcriptionally, these changes are associated with increased E2F, IRF, and NF-κB activity (Xing et al., 2016). Importantly, maturation is also associated with acquisition

of sphingosine 1-phosphate receptor (S1pr1) expression which mediates thymic egress (Allende et al., 2004; Matloubian et al., 2004).

T cell development is controlled by an intricate series of functional and phenotypic changes. Under optimal conditions, these steps facilitate the generation of a robust and diverse pool of T cells which coordinate immune responses to protect the host. Disruptions or dysregulation of these processes often result in disease or immunodeficiency.

1.2.1 Transcriptional regulation of T cell development

T cell development is characterized by numerous layers of regulation. Transcriptional regulation functions to ensure proper timing of developmental progression, as well as, orchestrating the substantial functional changes which occur from one stage to the next. To date, dozens of transcription factors have been identified as contributing to this process. Some play ubiquitous roles while others are highly stage-specific. This section will focus on discussing what is currently known regarding the roles for individual transcription factors in T cell development.

1.2.1.1 NOTCH and Bcl11b in T lineage commitment

Definitive commitment to the T cell lineage occurs at the DN2 stage of development (Yui et al., 2010). Prior to this stage thymocytes exhibit the capacity to differentiate into B cells, myeloid cells, NK cells or ILC. NOTCH is arguably the most important signaling pathway in the generation of T cells due to its absolute requirement

for T cell development (Hozumi et al., 2008). NOTCH promotes survival, proliferation, and developmental progression at least through β-selection (Ciofani & Zuniga-Pflucker, 2006; Yashiro-Ohtani et al., 2010). Engagement of NOTCH ligand induces an autocatalytic cleavage releasing the membrane-bound intracellular signaling domain (ISD). NOTCH signaling functions to promote expression of several key genes in early developing T cells including, *Il2ra, Ptcra, Notch3, and Myc* among others (Romero-Wolf et al., 2020).

Perhaps one of the most important genes induced by NOTCH is Bcl11b. The importance of Bcl11b in T cell development was first recognized in a pair of seminal studies which identified it as critical to T lineage commitment (L. Li et al., 2010) and repression of NK lineage diversion in thymocytes (P. Li et al., 2010). NOTCH-dependent signaling in DN2 thymocytes promotes expression of Bcl11b (P. Li et al., 2010). However, the efficiency of Bcl11b induction is dependent upon the local cytokine environment (Ikawa et al., 2010). Bcl11b drives T lineage commitment through repression of alternative lineage transcriptional programs. This repression is achieved through interaction with chromatin modifying repressor complexes such as the NuRD, Rest, Kdm1a, and Polycomb complexes (Cismasiu et al., 2005; Hosokawa et al., 2018). In addition to repressing alternative lineages, Bcl11b also contributes to proper timing of T cell development through repressing mature T cell transcriptional programs in immature thymocytes (Kastner et al., 2010).

1.2.1.2 E proteins are critical regulators of T cell development

The basic helix-loop-helix (bHLH) E protein family of transcription factors are known to play key roles at nearly every stage of T cell development (Agata et al., 2007; Engel & Murre, 2001, 2004; Jia et al., 2008; Jones-Mason et al., 2012; Jones & Zhuang, 2007, 2009, 2011; Kee et al., 2002; Petersson et al., 2002; Wojciechowski et al., 2007). The E protein family consists of E12 and E47 (encoded by *Tcf3*), HEB and HEBalt (*Tcf12*), and E2-2 (*Tcf4*). Each family member contains basic regions which bind E box (CGNNTG) motifs and HLH domains which mediate dimerization with other HLH-containing proteins. The reliance of E2A and HEB on development is not unique to T cells; these factors are also crucial to B cell development (Lazorchak et al., 2006). Early in T cell development E2A functions to promote developmental progression from DN1 to DN2, and eventually DN3, while repressing NK lineage potency (Heemskerk et al., 1997; Ikawa et al., 2001; Kim et al., 1999). These roles in promoting developmental progression are, at least in part, mediated through IL-7Rα expression, and therefore, IL-7-mediated signaling (Jia et al., 2008; Kee et al., 2002). E box sites are also prominent regulatory elements within the *Rag1/2* and *Tcra/b* loci functioning to promote TCR rearrangement at both the DN3 and DP stages. Beyond the DP stage, E proteins are largely dispensable for T cell development but they have been shown to regulate duration of DP survival through enforcement of RORγt expression (D'Cruz et al., 2010; Jones-Mason et al., 2012; Xi et al., 2006a).

E proteins are negatively regulated by bHLH-containing inhibitor of DNA binding (Id) proteins (Benezra et al., 1990). Id proteins heterodimerize with E proteins via their bHLH motifs thereby preventing DNA binding (Sun et al., 1991). Id2 and Id3 are the primary factors controlling E protein activity in developing thymocytes. Id expression throughout development is, in part, regulated by TCR signaling (Bain et al., 2001). Interestingly, E protein activity also functions to negatively regulate conventional T cell developmental progression in a stage-specific manner. Id2 and Id3 prevent E protein-driven development of innate-like iNKT cells, γδ T cells and γδ NKT cells (Li et al., 2017; Roy et al., 2018; Ueda-Hayakawa et al., 2009; B. Zhang et al., 2018; B. Zhang et al., 2014). Pre-TCR signaling drives expression of Id3 downstream of Egr2, which is necessary to inhibit E protein activity, allowing passage through β-selection (Bain et al., 2001). Failure to upregulate Id proteins through pre-TCR signals prevents RAG down-regulation, driving continued V(D)J recombination which ultimately promotes enhanced development of innate-like γδ T cells. Additionally, E protein activity enforces this checkpoint and further promotes V(D)J recombination through driving G_1 cell cycle arrest (Engel & Murre, 2004). Interestingly, E2A and Id2 have reciprocal functions prior to β-selection where E proteins promote developmental progression while Id proteins slow it.

Due to the immense number of binding sites throughout the genome and functional redundancy, comprehensive understandings of the roles for specific E protein targets in T cell development are far from complete (Roy et al., 2018).

1.2.1.3 GATA3

GATA3 is a zinc-finger transcription factor essential to T cell development (Ting et al., 1996). GATA3 is a GATA-type zinc finger containing transcription factor specifically expressed by T cells and ILCs (Jojic et al., 2013; Mingueneau et al., 2013). GATA3 functions to repress thymic B lineage conversion, enforces T lineage specification and promotes developmental progression throughout T cell development (Rothenberg & Taghon, 2005; Ting et al., 1996). GATA3 is required for the generation of ETPs (Hosoya et al., 2009) and functions to promote proliferation and survival in early thymocytes (Hendriks et al., 1999), as well as, driving TCRβ expression during β-selection (Pai et al., 2003). GATA3 also regulates expression of the TCRδ and TCRγ loci through enhancer binding (Joulin et al., 1991; Ko et al., 1991; Marine & Winoto, 1991). Interestingly, in the absence of NOTCH signals, forced GATA3 expression in DN1 or DN2 thymocytes promotes the development of mast cells (Taghon et al., 2007).

Later in development, GATA3 expression is regulated by TCR signaling and contributes to CD4 vs CD8 lineage decisions at positive selection (Hernandez-Hoyos et al., 2003). GATA3 expression downstream of TCR signaling is induced by c-Myb (Maurice et al., 2007). Interestingly, enforced expression of GATA3 via CD2 promoter

driven transgenic leads to a block in CD8SP cell maturation (Nawijn et al., 2001). It is thought that the magnitude of GATA3 expression downstream of positively selecting TCR signals is partially responsible for the determining CD4 vs CD8 T cell fate (Hernandez-Hoyos et al., 2003). This is supported by the observation that GATA-3 promotes *Zbtb7b* (ThPOK) expression driving CD4 lineage differentiation in ThPOK-dependent and -independent manner (Wang et al., 2008).

In mature CD4 T cells GATA3 serves as the Th2 lineage-defining transcription factor (Zhang et al., 2001) functioning to repress Th1 differentiation (Nawijn et al., 2001; Ouyang et al., 1998) and promote expression of Th2 cytokine genes (Zhang et al., 1997; Zheng & Flavell, 1997) via chromatin remodeling (Lee et al., 2001; Lee et al., 2000; Takemoto et al., 2000). Despite extensive study it still remains unclear exactly which GATA3 target genes are critical to its role in most stages of T cell development (Hosoya et al., 2010).

1.2.1.4 Runx family transcription factors

There are three mammalian Runt domain containing (Runx) family transcription factors: Runx1, Runx2, and Runx3. Runx genes are evolutionarily ancient with at least one homolog found in nearly all metazoan genomes (Rennert et al., 2003). Each Runx family member is broadly and dynamically expressed during T cell development (Mingueneau et al., 2013). Runx genes are not T cell-specific and contribute to the development of multiple immune lineages (Mevel et al., 2019).

In T cell development Runx1 and Runx3 play the most significant roles. Early in T cell development Runx1 supports the progression from DN1-DN3 via regulating the amplitude of Bcl11b expression downstream of NOTCH signaling (Kueh et al., 2016). Together Runx1 and Bcl11b prime developing T cells for lineage specification through inducing ThPOK and Runx3 expression. Early conditional deletion of Runx1 impairs T cell development at the DN3 stage while post-β-selection deletion leads to a severe impairment in positive selection and maturation of CD4SP thymocytes (Egawa et al., 2007; Sato et al., 2003). Conversely, loss of Runx3 severely impairs CD8SP development (Egawa et al., 2007). Interestingly, Runx proteins also function to regulate *Cd4* and *Cd8* expression positively and negatively during development via physical association of the loci (Collins et al., 2011) and controlling enhancer and silencer element activities (Chong et al., 2010; Sawada et al., 1994; Siu et al., 1994). During the DN stages of development Runx1 actively represses expression from both loci whereas Runx3 promotes epigenetic silencing following CD8 lineage commitment (Taniuchi et al., 2002).

In the context of weak positively selecting signals driven via MHC-I: peptide recognition Runx3 promotes CD8 lineage commitment via repressing CD4 and ThPOK expression (Setoguchi et al., 2008; Steinke et al., 2014). CD4 and ThPOK repression is regulated by silencer elements in the first exon of each gene (He et al., 2008; Setoguchi et al., 2008; Taniuchi et al., 2002). Additionally, Runx3 simultaneously functions to enforce CD8 lineage commitment through positively regulating *Cd8* expression via enhancer

binding and activation (Hassan et al., 2011; Kohu et al., 2005). Together Runx1 and Runx3 function to promote early T cell development as well as enforce lineage commitment in CD8 T cells.

1.2.1.5 ThPOK

Zbtb7b encodes the zinc finger and BTB domain-containing transcription factor ThPOK (Galera et al., 1994; Widom et al., 1997). The importance of ThPOK in T cell development was first identified through study of helper T cell deficient (HD) mice. HD mice were found to completely lack peripheral CD4 T cells resulting from an unidentified autosomal recessive mutation which genetically unlinked from MHC-II (Dave et al., 1998). HD mice exhibited significantly expanded $CD4^{+}$ $CD8^{low}$ thymocytes and a near complete loss of mature ($CD24^{-/low}$ $CD62L^{+}$) CD4SP cells. This defect was independent of *Cd4* regulation and the results suggested that CD4 T cell development was blocked at the post-positive selection lineage commitment phase. Subsequent study by the same group determined that the lack of CD4 T cell development in HD mice was not the result of a block in development but instead diversion to the CD8 lineage following positive selection (Keefe et al., 1999). Using the MHC-II-restricted AND TCR transgene crossed to a Rag-deficient HD background and MHC-I-deficient HD mice they showed that only CD8 T cells develop from MHC-II-dependent selection. This finding was further confirmed MHC-II-dependent selection of CD8 T cells in this model by generating AND^{+} $Rag^{-/-}$ $HD^{-/-}$ bone marrow chimeras in MHC-I- or MHC-II-deficient

$Rag^{-/-}$ recipients. Despite these groundbreaking studies it was not until 2005 that the HD mutation was determined to result in a single amino acid substitution in the DNA binding domain of ThPOK as the cause of the phenotype (He et al., 2005). This finding cemented ThPOK as a transcription factor critical to the establishment of the CD4 T cell lineage.

Further study determined that ThPOK enforces CD4 lineage commitment through repression of the master transcription factor for the CD8 lineage, Runx3. Using Runx3 and ThPOK fluorescent reporter mice it was shown that expression of these two factors is largely mutually exclusive in thymocytes (Egawa & Littman, 2008). Furthermore, the authors of this study show that genetic ablation of ThPOK results in acquisition of Runx3 expression in CD4SP cells allowing for upregulation of the CD8 lineage-specific transcriptional program.

In T cell development ThPOK expression and by extension CD4 lineage identity is controlled transcriptionally. Genetic studies identified a distal regulatory element (DRE) in the ThPOK locus with silencer and enhancer functionality (He et al., 2008). Ablation of the dual DRE reduced ThPOK expression in CD4 T cells and resulted in depression of ThPOK in CD8 T cells. Interestingly, *Cd4* expression is controlled in a similar manner by silencer and enhancer activity. Activity of both silencer elements is critical to properly regulate timing of CD4 expression. Prior to selection Myc-associated zinc finger-related factor (MAZR) together with Runx1 drive activity of the ThPOK

silencer, while post-selection silencing is achieved by MAZR-Runx3 (Sakaguchi et al., 2015). Deletion of MAZR and Runx in early thymocytes or peripheral CD8 T cells results in premature acquisition of CD4 expression by DN cells or depression of ThPOK, respectively.

A key aspect of CD4 lineage commitment and maintenance of lineage identity is the requirement to block activity of the *Cd4* and *ThPOK* silencers. ThPOK is directly responsible for this inhibition of silencer activity at both loci (Muroi et al., 2008). This effect is achieved by ThPOK regulating an anti-silencer embedded within the silencer element setting up an autoregulatory loop which sustains its expression and lineage identity (Basu et al., 2021). Critical to the maintenance of this autoregulatory loop is the sustained repression of Runx3 by ThPOK. In the absence of repression Runx3 can interact with Twist2 to drive activation of ThPOK silencer (Hwang et al., 2020). Sustained repression of Runx3 by ThPOK is mediated through induction of SOCS1 expression. In the absence of ThPOK, SOCS1 overexpression is sufficient to drive CD4 T cell development (Luckey et al., 2014).

1.2.1.6 RORγt

Retinoic acid-related orphan receptor (ROR)-γt is best known as the master transcription factor of the type 17 T cell lineages (Ivanov et al., 2006). In addition to regulating the effector identity of these cells RORγt also plays critical roles in the development of T cells and lymphoid organs (He, 2000; Kurebayashi et al., 2000; Sun et

al., 2000). Specifically, in thymocytes RORγt functions to promote survival of DP cells undergoing TCRα rearrangement (Guo et al., 2002; Kurebayashi et al., 2000). RORγt achieves this by promoting and sustaining expression of the anti-apoptotic protein Bcl-xL (Guo et al., 2002; Sun et al., 2000; Xie et al., 2005). The absence of RORγt leads to severely reduced thymic cellularity and skewing of the TCR repertoire towards proximal Vα-Jα pairings due to shortened survival time (Guo et al., 2002). It was shown that induction of RORγt expression by DP thymocytes is dependent upon TCF1 (Wang et al., 2011).

The most significant phenotype observed in RORγt-deficient thymocytes is a very significant reduction in DP cell numbers and a near absence of SP cells (Guo et al., 2002; Kurebayashi et al., 2000; Sun et al., 2000). However, RORγt also plays a key role during the post-β-selection DN-DP transistion where its expression is induced by pre-TCR signaling (Villey et al., 1999). In this context, RORγt functions to promote E protein expression which enforces cell cycle arrest necessary for V(D)J recombination. For cells to progress through this brief phase of proliferation RORγt activity must be repressed via Egr3 down-stream which is also down-stream of pre-TCR signaling (Xi et al., 2006b).

Like the necessity of silencing for progression through β-selection, RORγt must also be silenced for passage of positive selection. This silencing is mediated through HDAC3-dependent epigenetic silencing (Philips et al., 2016). Failure to down-regulate

RORγt during positive selection drives apoptosis due to a failure to up-regulate Bcl2 which can be rescued by ectopic expression of Bcl-xL (Philips et al., 2016).

1.2.1.7 PLZF – The master transcription factor for iNKT cell development

The Promyelocytic Leukemia Zinc Finger Protein (PLZF) encoded by Zbtb16 is a member of the POK (POZ and Kruppel) family of transcriptional repressors. PLZF plays numerous roles in organism-wide developmental processes (Suliman et al., 2012). The role for PLZF in T cell development is however narrow. PLZF functions as the master transcription factor for NKT and γδ NKT cells. While these two populations make up only a small fraction of the total T cell repertoire, they play numerous critical functions including rapid production of cytokines to direct acute immune responses (Crosby & Kronenberg, 2018).

PLZF is one of the transcription factors required for NKT cell development and function (Kovalovsky et al., 2008; Savage et al., 2008). During NKT cell development PLZF expression is dynamically regulated and rapidly induced upon selection within the thymus (Savage et al., 2008). ChIP-seq analysis has shown that PLZF binds to numerous genes critical to iNKT cell differentiation and regulators of effector programs. However, this study found that PLZF does not directly bind to NKT cytokine genes (Mao et al., 2016). Mao et al. also found that PLZF binds to and directly inhibits expression of Bach2 which is a known repressor of effector T cell differentiation. This activity is likely required for iNKT development as these cells undergo effector

differentiation developmentally (Lee et al., 2013). A recent study found that loss of Bcl6 impaired iNKT cell development due to failure to upregulate PLZF expression and loss of Bach2 repression in immediate post-selection stage 0 iNKT cells (Gioulbasani et al., 2020). The importance of PLZF in human iNKT cell development is highlighted by the near complete absence of this population in PLZF-deficient humans (Eidson et al., 2011).

In addition to iNKT cells, PLZF is also expressed by a subset of γδ T cells. In mice, these cells are enriched among Vγ1Vδ6,Vγ5, and Vγ6 expressing cells in mice (Kreslavsky et al., 2009; Lu et al., 2015). Interestingly, Vγ5+ cells do not require PLZF for development while Vγ6+ cells do. However, both require PLZF for effector differentiation (Lu et al., 2015). Vγ1Vδ6, also known as γδ NKT cells, like iNKT cells require PLZF for their development and effector differentiation (Kreslavsky et al., 2009). The molecular mechanisms by which PLZF promotes the development and effector differentiation of these γδ T cell subsets is has yet to be studied in detail and therefore, remains poorly understood.

1.3 cGAS/STING cytosolic DNA sensing pathway in T cell-mediated immunity

1.3.1 Overview of cGAS/STING pathway

The cGAS/STING pathway is an ancient and critical pattern recognition pathway recognizing cytosolic dsDNA. The evolutionarily earliest documented species with a functional cGAS/STING pathway is *Nematostella vectensis*, a sea anemone more than 500

million years diverged from humans (Kranzusch et al., 2015). cGAS/STING is active in numerous cell types and plays critical roles in numerous host defense pathways including responses to infectious agents such as bateria and viruses, antitumor immunity, and maintenance of barrier tissues. While the cGAS/STING pathway is best known for its activity in myeloid and non-immune cells a growing body of literature demonstrates the critical role for this pathway in T cell mediated immunity. This section will focus on what is known regarding cGAS/STING in T cell responses.

1.3.1.1 Cyclic GMP-AMP synthase (cGAS)

Prior to the discovery of cGAS, STING was known to be a critical regulator of type I interferon (IFN-I) production in response to cytosolic dsDNA (Ishikawa & Barber, 2008; Ishikawa et al., 2009). However, the upstream mechanism driving STING-mediated IFN-I production was unknown. The discovery of cGAS began with the identification of cyclic-GMP-AMP (cGAMP) as a critical second messenger driving the production of IFN-I in response to cytosolic dsDNA (Wu et al., 2013). The following year a series of papers were published identifying cGAS as the enzyme responsible for cGAMP production (Ablasser, Goldeck, et al., 2013; Sun et al., 2013) and demonstrating a key role of cGAS in antiviral immunity (D. Gao et al., 2013; Wu et al., 2013). Since its discovery there have been at least 700 publications regarding cGAS.

cGAS is a nucleotidyltransferase encoded by the gene *Mb21d1* with conserved homologs found in bacteria (Cohen et al., 2019; Whiteley et al., 2019). Upon binding to

dsDNA cGAS undergoes homodimerization which is generally required for its catalytic activity and production of cGAMP (Civril et al., 2013; P. Gao et al., 2013; X. Li et al., 2013; X. Zhang et al., 2014). Recognition of dsDNA by cGAS is sequence-independent (P. Gao et al., 2013; Li et al., 2013; Zhang et al., 2014). Due to the necessity for dimerization, strength of cGAS activation is dependent upon dsDNA length. dsDNA shorter than 50 nucleotides (nt) induces minimal cGAS activity and maximal activation requires dsDNA of at least 200nt (Andreeva et al., 2017; Hooy & Sohn, 2018; Luecke et al., 2017).

Interestingly, cGAS is not only a cytosolic protein. A significant proportion of cGAS can be found within the nucleus (Liu et al., 2018). Since cells do not exhibit constitutive IFN-I production, it comes as no surprise that nuclear cGAS is catalytically inactivated via several mechanisms. One such mechanisms is tethering of cGAS to nucleosomes. In 2020, a series of publications determined the structural basis for cGAS inactivation by tethering to the acidic patch of nucleosomes (Boyer et al., 2020; Cao et al., 2020; Kujirai et al., 2020; Michalski et al., 2020; Pathare et al., 2020; B. Zhao et al., 2020). Additional works have shown that cGAS activity must be suppressed during mitosis via chromatin tethering and phosphorylation (T. Li et al., 2021). Insufficient cGAS inactivation upon dissolution of the nuclear envelope (NE) drives mitotic cell death (Zierhut et al., 2019). Additional studies have shown that cGAS activity towards self-DNA is controlled by the DNA-binding protein Barrier-to-Autointegration Factor 1 (Baf

or Banf1) which competitively blocks cGAS activation upon nuclear envelope rupture and promotes repair (Guey et al., 2020; H. Ma et al., 2020).

Naturally cGAS exists in an autoinhibited state in which the catalytic subunit is unable to efficiently interact with its substrates ATP and GTP (Civril et al., 2013; P. Gao et al., 2013; X. Li et al., 2013; X. Zhang et al., 2014). Upon binding dsDNA cGAS undergoes conformational changes in the catalytic pocket allowing optimal interaction with ATP and GTP (Civril et al., 2013; P. Gao et al., 2013; X. Li et al., 2013; X. Zhang et al., 2014). On binding to long dsDNA molecules, cGAS forms oligomeric ladder-like structures which can become phase-separated organelles (Andreeva et al., 2017; Du & Chen, 2018). Following the required conformational changes and binding to GTP and ATP, cGAS catalyzes the formation of a 2′-5′ followed by 3′-5′ linkage between GMP and AMP to produce 2′3′-cGAMP, the functional isomer. 2′3′-cGAMP then activates STING driving downstream signaling (Ablasser, Goldeck, et al., 2013; Sun et al., 2013) which can result in a host of biological outcomes.

Efficient cGAS enzymatic activity requires cationic metal ions, specifically manganese^{2+} (M. Lv et al., 2020; Wang et al., 2018). Upon viral infection, Mn^{2+} is release from organelles and accumulates within the cytosol. This accumulation increases the sensitivity of cGAS to activation by foreign nucleic acids (Wang et al., 2018). Interestingly, it was recently shown that Mn^{2+} is critical to anti-tumor immunity which

can be boosted by Mn^{2+} supplementation and that cGAS/STING is required for the effects of Mn^{2+} supplementation (M. Lv et al., 2020).

1.3.1.2 Stimulator of interferon genes (STING)

STING was first cloned and characterized in 2008 as a key inducer of IFN-I responses (Ishikawa & Barber, 2008). The following year the same group demonstrated the critical role of STING in driving intracellular DNA-mediated IFN-I responses. STING is an endoplasmic reticulum (ER) localized four transmembrane domain-containing signaling adaptor molecule (Ishikawa & Barber, 2008) encoded by the gene *Tmem173*. Binding of cGAMP to the central crevice of the C-terminal domain of dimerized STING induces its activation (Zhang et al., 2013). The mechanisms of signaling downstream of STING will be discussed in a later section. In addition to cGAMP, STING can also bind and be activated by other cyclic dinucleotides (CDN), such as, cyclic di-GMP or cyclic di-AMP produced by bacteria, albeit with much lower affinity (Zhang et al., 2013).

Like many signaling adaptors, STING activity is regulated via post-translational modifications (Figure 1). Of note, the ability of STING to induce downstream signaling requires the phosphorylation of serine 366 (Konno et al., 2013; Liu et al., 2015; Tanaka & Chen, 2012). Interestingly, in addition to facilitating downstream signaling, phosphorylation of STING also promotes its degradation (Konno et al., 2013). This

mechanism of signaling induction and degradation likely functions to prevent excessive signal and deleterious inflammation.

In addition to phosphorylation, STING activity is also regulated by ubiquitination. To date, at least three ubiquitin E3 ligases have been identified as positively regulating STING activity. These include TRIM32 (Zhang et al., 2012), TRIM56 (Tsuchida et al., 2010), and AMFR/INSIG1 (Wang et al., 2014). TRIM32 and TRIM56 promote K63 polyubiquitination while AMFR/INSIG1 drives K27 polyubiquitination. Polyubiquitination via either mechanism promotes recruitment of TBK1 and activation of downstream IFN production. K48 Polyubiquitination by RNF5 and TRIM30a drives proteasomal degradation and thus inhibition of the cGAS/STING pathway (Wang et al., 2015; Zhong et al., 2009).

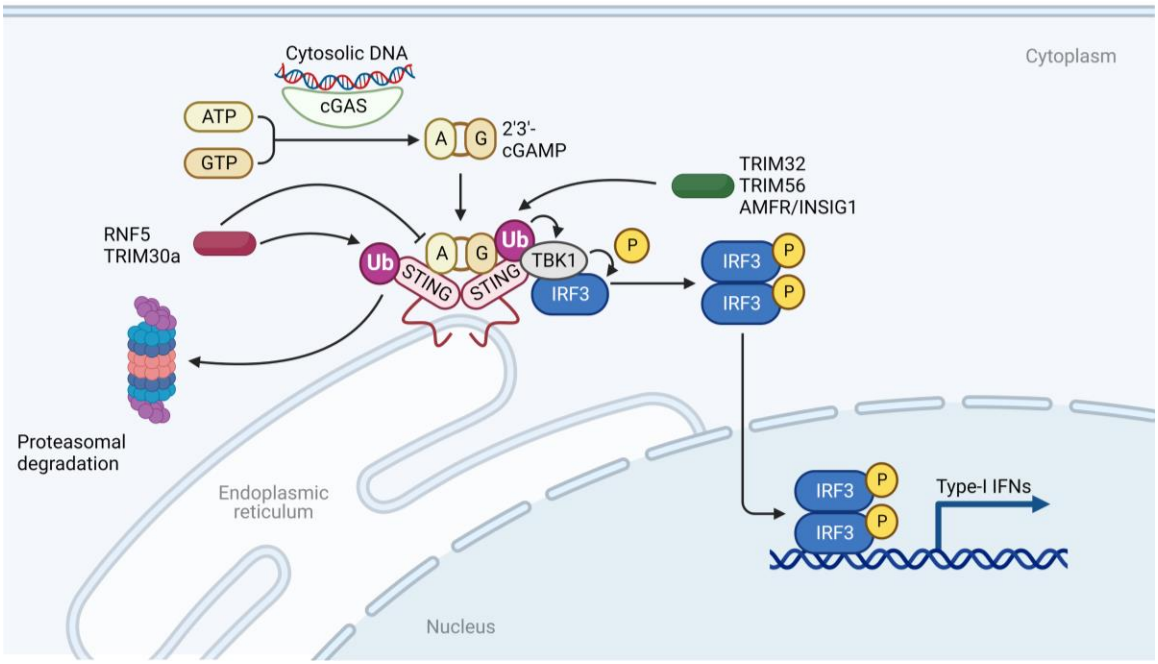

Figure 1: Post-translational regulation of cGAS/STING signaling

Schematic diagram of post-translational regulation of cGAS/STING signaling activity. Downstream signaling through IRF3 is the only pathway shown. However, signaling via NF-κB is regulated in a similar fashion. Image created using Biorender template.

1.3.1.3 Sources of cGAS activating ligand

While there are numerous sources of cGAS-activating ligand they all must be dsDNA and localized to the cytoplasm to drive cGAMP production. Sources of dsDNA may be host- or pathogen-derived. This dsDNA may come from within the cell in which cGAS is activated or from the extracellular space. In this section we will briefly review the numerous sources of cGAS-activating dsDNA.

Early research into cGAS focused on two main sources of activating signal: pathogen-derived dsDNA or interferon stimulating DNA (ISD) introduced

experimentally. Pathogen-derived dsDNA often originates from bacteria, DNA viruses, or retroviruses which utilize a dsDNA intermediate during replication. cGAS activation by obligate extracellular bacteria typically results from uptake of dead bacteria or phagolysosomal leakage of phagocytosed bacterium. ISD is short (45bp) non-CpG methylated dsDNA typically derived from *Listeria monocytogenes*. ISD has been widely used to study several DNA-sensing pathways. In bacteria, phage DNA efficiently activates cGAS-like enzymes (Cohen et al., 2019).

More recently, attention has expanded to exploring host-derived sources of cGAS-activating dsDNA. There are three main sources of host-derived cGAS-stimulating dsDNA: nuclear genomic DNA (gDNA), mitochondrial DNA (mtDNA), and endogenous retrovirus (ERV)-derived dsDNA replication intermediates. gDNA may become cytoplasmic through several mechanisms including, micronuclei formation (Harding et al., 2017; Mackenzie et al., 2017), NE rupture (Denais et al., 2016; Raab et al., 2016), loss of NE membrane integrity (Guey et al., 2020; H. Ma et al., 2020), telomeric loss (Chen et al., 2017; Nassour et al., 2019), mitotic errors (Flynn et al., 2021; Li et al., 2022), or genotoxic stress (Hartlova et al., 2015; Quek et al., 2017; Song et al., 2019). mtDNA may become cytosolic resulting from loss of mitochondrial membrane integrity and efficiently driving cGAS activation (Huang et al., 2020; Willemsen et al., 2021). Additionally, errors in mitophagy, a selective form of autophagy, has recently been shown to drive mtDNA-dependent cGAS activation (Rai et al., 2021; Sliter et al., 2018).

Finally, ERVs are capable of driving cGAS/STING signaling. Activation of cGAS via ERV results from recognition of cytosolic replication intermediates generated following reverse transcription, RNA degradation and second strand synthesis (Lima-Junior et al., 2021; Simon et al., 2019).

1.3.1.4 cGAS/STING signaling pathway and its outcomes

The most common outcome of cGAS/STING signaling is the promotion of pro-inflammatory and antiviral gene expression programs. This typically is achieved through activation of NF-κB and IRF3 signaling pathways. Following cGAS recognition of cytosolic dsDNA cGAMP is produced driving STING activation and trafficking from the ER to the golgi complex. Activated STING recruits TBK1 promoting its phosphorylation and activation and setting of down-stream signaling (Yum et al., 2021). Phosphorylated TBK1 then drives IRF3 phosphorylation. Phosphorylated IRF3 typically dimerizes, translocates to the nucleus, and promotes IFN-I gene expression. Alternatively, phospho-IRF3 may remain cytosolic and dimerize with Bax to promote mitochondrial membrane permeabilization and apoptosis (Zierhut et al., 2019). Additionally, TBK1 activation by interaction with STING promotes NF-κB signaling via activation of IKK complexes and IkBα. Activated NF-κB drives expression of pro-inflammatory cytokines such as TNF and IL-6. Together, activation of IRF3 and NF-κB signaling pathways down-stream of STING synergize to promote the prototypical antiviral and pro-inflammatory profiles associated with cGAS/STING signaling.

In addition to pro-inflammatory program activation, cGAS/STING signaling also drives autophagy to promote clearance of intracellular pathogens and cytosolic dsDNA (Gui et al., 2019). Additionally, autophagy induction downstream of STING functions to limit the activity of this pathway and suppress excessive inflammation (Konno et al., 2013; Prabakaran et al., 2018) via STING degradation. Through this mechanism STING signaling functions to establish an autofeedback loop. Interestingly, activation of STING from the sea anemone *Nematostella vectensis* drives autophagy but not interferon responses suggesting that autophagy induction is the evolutionarily earliest function of STING.

1.3.2 cGAS/STING in T cell responses

1.3.2.1 T cell intrinsic roles for cGAS/STING

A significant amount of work related to the role for cGAS/STING in immune responses has focused on myeloid and non-immune cells. However, a growing body of evidence demonstrates that this pathway is active and plays a significant role within T cells. The first studies exploring the role for cGAS/STING in T cells found that this pathway triggers significantly different responses in these cells compared to others (Wu et al., 2020). While cGAS/STING was widely known to promote expression of pro-inflammatory cytokines and drive IFN-I responses, these studies showed that in T cells cGAS/STING generally drives IFN-independent anti-proliferative responses and cell

death (Cerboni et al., 2017; Gulen et al., 2017; Larkin et al., 2017; Wu et al., 2019; Wu et al., 2020). The pro-apoptotic effects of STING activation in T cells are partially dependent upon the transcriptional activities of p53 and IRF3 (Gulen et al., 2017). However, other studies suggest that STING activation drives more efficient IFN-I production by effector T cells than by innate leukocytes (Imanishi et al., 2019). Interestingly, some pathogens utilize the sensitivity of T cells to cGAS/STING-mediated cell death to escape the immune system. Recently, it was shown that *Listeria*, *Francisella*, and *Legionella* bacterium DNA is packaged into extracellular vesicles and passed to bystander cells activating cGAS/STING which inhibits T cell proliferation and primes them for cell death (Nandakumar et al., 2019).

More recent studies have shown that cGAS/STING also plays important roles in T cell differentiation. The absence of STING precludes the differentiation of Th17 cells within gut-draining lymph nodes (Park et al., 2019). Consistent with this finding, administration of cGAS agonist DNA nanoparticles promoted the expansion of splenic Treg, Th1, and Th17 cells (Lemos et al., 2014). Similarly, under Th polarizing conditions exogenous cGAMP resulted in enhanced differentiation of Th1, Th9, and Th17 effector cells which was partially IFN-I-dependent (Benoit-Lizon et al., 2022). Conversely, in a model of chronic pancreatitis, STING agonism or deletion resulted in reduced or increased accumulation of Th17 cells within the pancreas, respectively (Q. Zhao et al., 2019). However, the validity of the findings from this study have been disputed (Hu et

al., 2020). Thus, in the context of activation and differentiation, cGAS/STING signaling generally promotes efficient acquisition of CD4 T cell effector identity.

In CD8 T cells cGAS/STING signaling may play a more subtle role during differentiation. A recent study found that in mouse models of anti-tumor immunity cGAS/STING signaling promotes the differentiation of stem-like effector memory cells by regulating TCF1 expression and restraining Akt activity. Interestingly, this study was the first to show that cGAS-activating dsDNA in T cells was of an endogenous source (W. Li et al., 2020). Interestingly, STING activity also promotes CD8-mediated disease. Bader et al. found that STING signaling contributes to CD8 T cell-mediated graft-versus-host disease. Genetic ablation of STING reduced CD8-mediated MHC-matched and MHC-mismatched GVHD due to reduced donor T cell activation and enhanced recipient APC survival (Bader et al., 2020).

cGAS/STING signaling plays particularly important roles in regulatory T cells (Treg). Type 1 diabetes (T1D) results from T cell-mediated immune responses directed against insulin-producing pancreatic islets (DiMeglio et al., 2018). In the non-obese diabetic (NOD) mouse model of type 1 diabetes, administration of cGAS-activating DNA nanoparticles was sufficient to protect mice from disease. This disease protection was shown to be IDO-dependent and T cell driven suggesting that activation of the cGAS/STING pathway promotes enhanced Treg effector function (Lemos et al., 2019). Consistent with this finding, a recent study found that STING-deficient NOD mice

develop exacerbated disease and increased accumulation of diabetogenic T cells within their pancreatic islets (Akazawa et al., 2021). Study of the role for cGAS/STING in gut homeostasis revealed that STING-deficient mice are highly susceptible to DSS-induced colitis. Through these studies the authors demonstrated that in the absence of STING Treg development, maintenance, and function within the intestines is severely impaired (Canesso et al., 2018).

cGAS/STING may also play important roles during T cell-intrinsic viral infection. The best studied among such pathogens is human immunodeficiency virus (HIV). There are conflicting reports regarding whether cGAS-dependent responses are driven by HIV infection. An early study showed that human T cells failed to induce IFN-I responses following HIV-1 infection despite recruitment of IFI16, STING, and TBK1 to cytosolic DNA. Interestingly, this inability to respond seemed to be HIV-specific as Sendai Virus infection induced robust IFN-I responses in T cells (Berg et al., 2014). Two years later another group showed that HIV infection drives cGAS-dependent IFN-I responses in CD4 T cells. However, these responses required HIV-1 integration and were simultaneously activated and inhibited by HIV VPR and VPU proteins, respectively (Vermeire et al., 2016). More recently a third group found no measurable IFN-I responses in mouse or human T cells following HIV-1 infection. Importantly, they showed that despite an absence of response to HIV-1, T cells mount efficient cGAS-dependent IFN-I response to mutant herpes simplex virus 1 (HSV-1) infection (Elsner et

al., 2020). Despite the potential defective induction of IFN-I responses within T cells following HIV-1 infection they may still be able to induce these responses in neighboring cells. Previously, it was shown that cGAS can recognize HIV-1 dsDNA in CD4 T cells which then horizontally transfer cGAMP to neighboring myeloid cells via intercellular membrane fusion events driving IFN-I production (Xu et al., 2016). Therefore, T cells are capable of mounting cell-intrinsic cGAS/STING-dependent responses to viral infection. However, the ability of these cells to mount cGAS/STING-dependent responses to HIV-derived dsDNA remains unclear.

While the role for cGAS/STING in T cell development has received little attention there are lines of evidence supporting a role for the pathway. First, humans carrying a gain-of-function mutation in STING which is responsible for the STING-associated vasculopathy with onset in infancy (SAVI) syndrome exhibit severe T lymphopenia (Dai et al., 2020; Liu et al., 2014). Studies of mouse models harboring SAVI-associated STING gain-of-function mutations revealed that T lymphopenia (Motwani et al., 2019) caused by these mutations is due to impaired T cell development which was IFN-I-independent (Bouis et al., 2019; Siedel et al., 2020). Mechanistically, impaired T cell development resulting from STING hyperactivity is due to increased rates of ETP/DN1 thymocyte apoptosis (Bouis et al., 2019). Similarly, mice lacking the exonuclease Trex1 exhibit enhanced cGAS/STING activity and altered T cell development (Morita et al., 2004). Finally, in this dissertation we show that disruption of the nuclear envelope

downstream of Zfp335 deletion triggers cGAS/STING-dependent apoptosis of DN4 thymocytes. Together, these data suggest that cGAS/STING is active within both developing and mature T cells and generally mediate anti-proliferative, pro-apoptotic responses that are IFN-I-independent. Together, these previous studies demonstrate that activation of the cGAS/STING pathway in T cells can positively or negatively impact the ability of these cells to mount effective immune responses depending on the context (Figure 2).

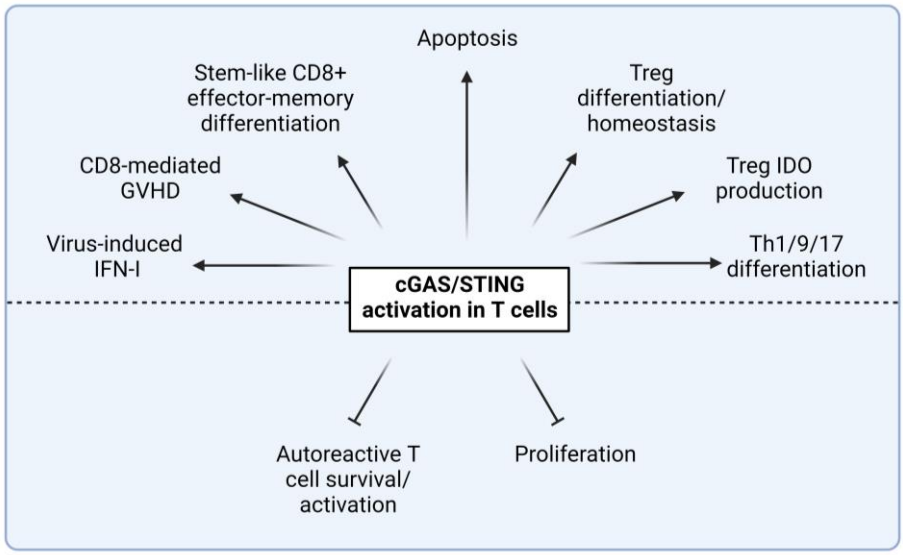

Figure 2: Outcomes of cGAS/STING signaling in T cells

Schematic diagram of the outcomes of cGAS/STING signaling in T cells. Outcomes above or below the dashed line are promoted or inhibited by cGAS/STING signaling in T cells, respectively.

1.3.2.1.1 Mechanisms of exogenous cGAMP uptake to stimulate STING signaling

Production of cGAMP is required for activation of downstream signaling via STING. However, STING activation does not require cGAMP to be produced within the cell in which downstream signaling occurs. To date, at least seven mechanisms (Figure 3) have been identified allowing for horizontal transfer or uptake of cGAMP from the local environment (Ablasser, Schmid-Burgk, et al., 2013; Bridgeman et al., 2015; Concepcion et al., 2022; Cordova et al., 2021; Gentili et al., 2015; Luteijn et al., 2019; Ritchie et al., 2019); some of which may or may not be utilized by T cells. Shortly after the discovery of cGAS as the enzyme responsible for cGAMP production it was shown that cGAMP can be transferred to neighboring cells via gap junctions (Ablasser, Schmid-Burgk, et al., 2013). Additionally, cGAMP has been found to be transferred between cells when packaged into viruses during viral replication (Bridgeman et al., 2015; Gentili et al., 2015).

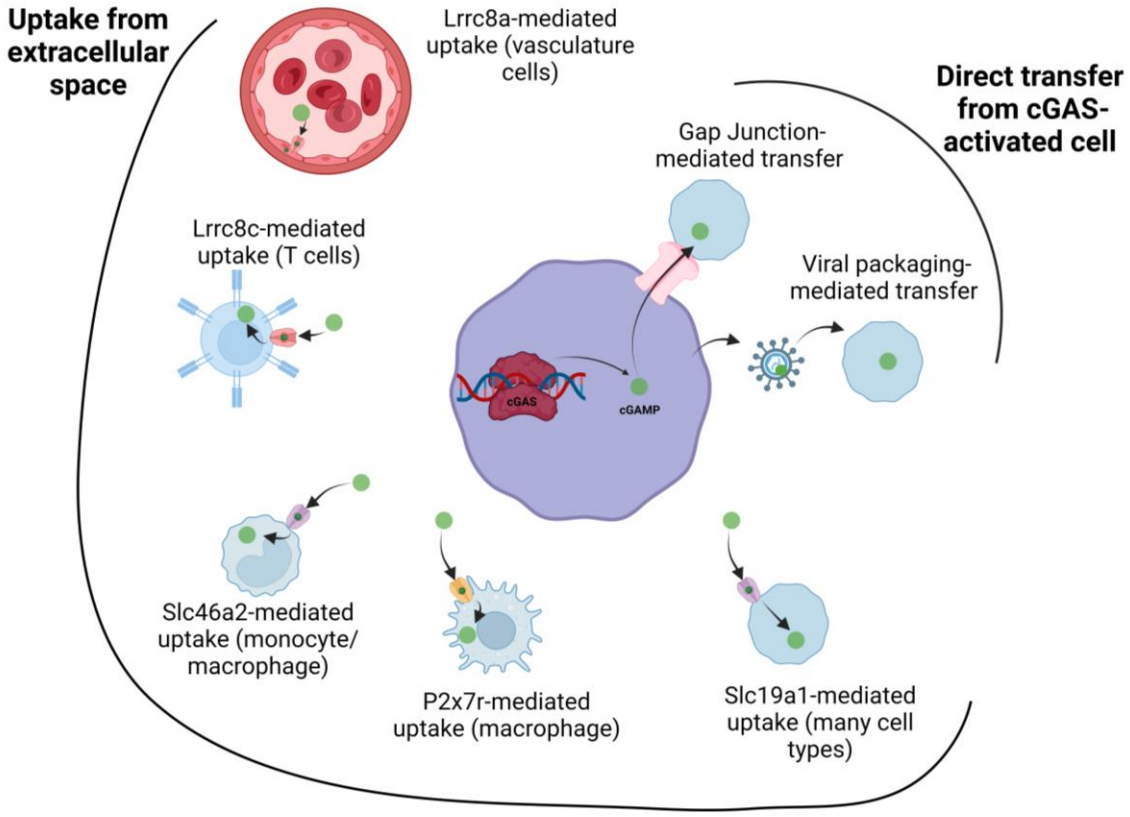

Figure 3: Mechanisms of exogenous cGAMP uptake/ transfer

Schematic diagram of the seven identified mechanisms for transfer or uptake of exogenous cGAMP to promote STING signaling. Diagram generated using Biorender.

cGAMP is not membrane permeable however extracellular cGAMP is capable of driving STING signaling (Ablasser, Goldeck, et al., 2013; Konno et al., 2013; X. Li et al., 2013; Sun et al., 2013). Using CRISPR screens SLC19A1 was identified as the first importer of extracellular cGAMP (Ritchie et al., 2019). However, the utilization of SLC19A1 for cGAMP uptake may be restricted to human cells as STING activity was unaltered in *Slc19a1*-deficient mouse cells in the presence of cGAMP (Luteijn et al., 2019). Later, it was found that tumor-associated macrophage uptake tumor-derived

cGAMP through an ATP-dependent mechanism mediated by purinergic receptor P2X7R (Zhou et al., 2020). It is unclear of the exact mechanism by which P2X7R mediates cGAMP uptake but it may be the result of ATP-dependent pore formation (Karasawa et al., 2017; Steinberg et al., 1987; Surprenant et al., 1996). Recently, SLC46A2 was identified as a second human-specific cGAMP importer but its transporter activity was found to be restricted to monocytes and macrophage (Cordova et al., 2021). Interestingly, this study showed that T cells sense and respond to extracellular cGAMP. However, the authors did not determine if T cell uptake was mediated through SLC46A2 (Cordova et al., 2021). In another CRISPR screen LRRC8A-containing volume regulated anion channels were found to be ubiquitous cGAMP importers in vasculature cells (Lahey et al., 2020). Very recently LRRC8C was identified as the first definitive cGAMP transporter in T cells (Concepcion et al., 2022).

To date, only one means of exogenous cGAMP uptake has been definitively shown in T cells (Concepcion et al., 2022). However, several mechanisms of cGAMP transfer and uptake identified in other cell types may apply to T cells. For instance, P2X7R is expressed by thymocytes (Philips et al., 2019) and signaling through this receptor has been shown to drive thymocyte cell death (Apasov et al., 1997; Zheng et al., 1991). While it is known that activation of P2X7R mediates formation of large membrane pores which may drive cell death (Di Virgilio et al., 2018) it would be interesting to test

whether cGAMP uptake via this mechanism contributes to thymocyte apoptosis resulting from exposure to extracellular ATP.

1.3.2.2 T cell extrinsic roles for cGAS/STING

cGAS/STING has numerous documented T cell extrinsic roles in T cell-mediated immune responses. In general, T cell-extrinsic cGAS/STING activity promotes or enhances T cell mediated immune responses, often through enhancing activation of APCs. However, more recent studies have begun to uncover leukocyte-independent roles for cGAS/STING in promoting T cell responses. Recently, it was shown that STING activity in endothelial cells promotes enhanced T cell migration through increased production of chemokines and cell adhesion molecules (Anastasiou et al., 2021). In keratinocytes, cGAS activity downstream of endogenous retrovirus (ERV) expression was shown to promote type 17 T cell responses as well as homeostatic and inflammatory skin immunity to microbiota (Lima-Junior et al., 2021). Furthermore, numerous studies have shown critical roles for tumor cell-intrinsic cGAS/STING activity in promoting anti-tumor T cell responses through multiple mechanisms which will be described in detail in a later section (Ablasser, Schmid-Burgk, et al., 2013; Demaria et al., 2015; Luo et al., 2020; F. Ma et al., 2020; Marcus et al., 2018; Schadt et al., 2019; Zhou et al., 2020).

Among non-T cell leukocytes cGAS/STING activity is best described in myeloid cells. Activation of this pathway in macrophage or dendritic cells promotes IFN-I-dependent enhancement of T cell priming and activation (Li et al., 2019; M. Lv et al.,

2020; Ma et al., 2021; Schadt et al., 2019; Shmuel-Galia et al., 2021; Si et al., 2022; Woo et al., 2014; H. Zhang et al., 2015). cGAS/STING activation also drives enhanced migration of APC to lymph nodes thereby promoting their interaction and activation of T cells (Park et al., 2019). Interestingly, T cells may also play an active role in promoting activation of APC via this pathway. T cells have been shown to produce extracellular vesicles loaded with mtDNA and gDNA which are transferred to APC during cognate interactions which prime the APC for antiviral immune responses (Torralba et al., 2018).

A major mechanism by which cGAS/STING signaling mediates cell extrinsic enhancement of T cell responses is through enhanced migration of APC to lymph nodes. These effects are largely mediated through the production and signaling by IFN-I which are required for up-regulation of pro-inflammatory cytokines, MHC, and co-stimulatory molecules (Diamond et al., 2011; Woo et al., 2014; Yum et al., 2021).

1.3.2.3 cGAS/STING in antimicrobial T cell responses

Antimicrobial T cell responses are critical to organismal survival. This is highlighted by the significantly higher rates of infection and shortened lifespan of humans with severe combined immunodeficiency (Scid) in the absence of hematopoietic stem cell transplantation (Buckley et al., 1997; Fischer, 2000). Being a key sensor in antiviral immunity, significant effort has been spent studying the role for the cGAS/STING pathway in viral infections. However, this pathway also plays important roles in anti-bacterial responses, as well as, responses to other microbial infections. In

some cases, the pathway is even coopted by pathogens to evade T cell-mediated immune reactions (Nandakumar et al., 2019). Here, I will discuss key roles of the cGAS/STING pathway in antimicrobial immune responses.

A critical role of the cGAS/STING pathway is to promote antiviral immune responses. Inefficient antiviral responses to pathogens, such as, hepatitis B virus (HBV), herpesvirus (HSV), cytomegalovirus (CMV), Epstein-Barr Virus (EBV) and many others, can lead to a host of life long diseases including cancer, organ damage, and neurological problems, and leave the host immunocompromised (Z. Li et al., 2020). cGAS/STING functions to recognize viral DNA and initiate a cascade of antiviral responses including IFN-I and pro-inflammatory cytokine production, autophagy to destroy intracellular viral components, and activation of innate immunity promoting virus control and adaptive immunity. The importance of cGAS/STING in these responses is highlighted by the complete loss of IFN-I production and viral control following dsDNA viral infection in *Mb21d1*$^{-/-}$ or *Tmem173*$^{-/-}$ mice (Ishikawa et al., 2009; Reinert et al., 2016; Sun et al., 2013).

An arms race between virus and host has evolved in which viruses encode proteins capable of inhibiting the cGAS/STING pathway (Figure 4). For instance, HSV has evolved mechanisms to inhibit cGAS activity and dampen antiviral immune responses. HSV-1 UL56 protein promotes viral replication through direct interaction with cGAS, preventing its ability to bind DNA, and therefore, enzymatic activity (Zhou-

Qin Zheng, 2022). Similarly, HSV-1 UL46 protein encoded by the VP11/12 gene promotes STING degradataion and inhibits IFN-I responses (Deschamps & Kalamvoki, 2017). Other means of inhibiting the cGAS/STING pathway have been discovered in viruses. Pseudorabies virus (PRV) UL13 protein inhibits cGAS/STING signaling via IRF3 phosphorylation-mediated signaling inhibition and ubiquitin-dependent STING degradation (Bo et al., 2020; Kong et al., 2022; L. Lv et al., 2020). Procine circovirus 2 (PCV2) infection drives Akt-dependent phosphorylation of cGAS at S278 which promotes K48 poly-ubiquitination and degradation to prevent IFN-I responses (Z. Wang et al., 2021). Herpesviruses such as HSV-1, Kaposi's Sarcoma Associated Herpresvirus (KSHV), CMV and HMV68 also encode several deubiquitinases capable of inhibiting cGAS/STING signaling at multiple steps (Kumari et al., 2017; Ma et al., 2012; Sun et al., 2015). Recently, through a screen of 24 mammalian viruses, it was discovered that poxviruses such as vaccinia encode conserved nucleases, termed poxins, which directly inhibit cGAS/STING signaling through hydrolytic degradation of cGAMP (Eaglesham et al., 2019). Several other mechanisms for viral evasion of the cGAS/STING pathway by inhibition of cGAS or STING activitation, impairing formation of STING-TBK1-IRF3 complexes, and inhibition of downstream transcription factor activation and DNA binding have been described (Stempel et al., 2019).

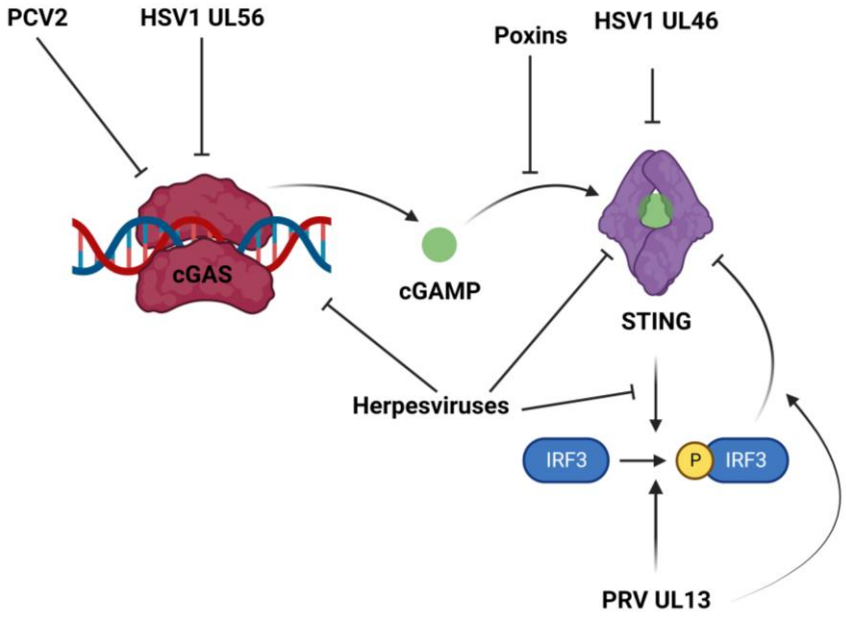

Figure 4: Viral regulation of cGAS/STING signaling pathway

Schematic diagram of virus-mediated inhibition of cGAS/STING signaling pathway. Diagram generated using Biorender.

In addition to viral pathogens, the cGAS/STING pathway also contributes to immunity from bacteria. Interestingly, while activation of the pathway can be beneficial in the context of bacterial infection via IFN-I induction it can also be detrimental. For instance, IFN-I production in response to bacterial infection limits tissue damage and protects from *Streptococcus pyogenes* infection by limiting IL-1 production and hyperinflammation (Castiglia et al., 2016). Conversely, IFN-I production has been shown to increase susceptibility to *Listeria monocytogenes* infection (O'Connell et al., 2004; Stockinger et al., 2002). In this context, cGAS/STING activity may directly impair T cell

responses during *L. monocytogenes* infection by promoting T cell apoptosis and inhibiting proliferation (Nandakumar et al., 2019).

Induction of the cGAS/STING signaling pathway in response to bacterial infection may come from multiple sources. Many bacteria produce CDNs using cGAS-like enzymes (Whiteley et al., 2019) which function as second messengers and are involved in many biological processes including quorum sensing, virulence, metabolism, and motility (Danilchanka & Mekalanos, 2013). CDNs produced by intracellular pathogens may promote clearance through stimulating the cGAS/STING pathway and innate immunity (Dorrington et al., 2021; Liu et al., 2019). Bacterial DNA can also serve as substrate to set off the cGAS/STING signaling cascade (Hansen et al., 2014).

T cells play essential roles in the control and clearance of bacterial infections. In a broad sense IFN-I induction downstream of cGAS/STING may impair the ability of T cells to promote clearance of bacterial pathogens. This is supported by impaired cytokine production by T cells during bacterial infection (Henry et al., 2010), reduced responsiveness of macrophage to IFNγ following IFN-I signaling (Rayamajhi et al., 2010), and improved survival of *Ifnar1*$^{-/-}$ mice with secondary bacterial infection following primary influenza infection compared to WT controls (Shahangian et al., 2009). However, clinical and epidemiological data suggest that despite these impairments in T cell-mediated immune responses that result from cGAS/STING

activity, signaling through the pathway provides protection from bacterial infection. Specifically, it was found that humans with loss-of-function mutations in STING exhibit increased susceptibility to bacterial pneumonia (Ruiz-Moreno et al., 2018). Based on the limited studies directly examining the interplay between cGAS/STING signaling and T cell-mediated anti-bacterial immune responses it is unclear whether the pathway plays a beneficial or detrimental role in T cells during these infections. It is likely that the pathway plays both positive and negative roles depending on the specific context.

Beyond bacterial and viral infections, the cGAS/STING pathway also plays roles in controlling eukaryotic infection. A recent study found that cGAS/STING activity contributes to pathogen control during parasitic infections by malaria-causing *Plasmodium* species. The absence of cGAS/STING signaling did not impair early B or T cell responses to *Plasmodium*. However, by day 14 of infection cGAS-deficient mice exhibited impaired germinal center (GC) formation, reduced production of class-switched antibodies and memory B cells, and reduced numbers of effector and GC Tfh T cells. Together, these impairments resulted in IFN-I-dependent clearance of the parasite (Hahn et al., 2018). Roles for cGAS/STING in controlling other parasitic infections have been demonstrated. Through *in vitro* and *in vivo* mouse models of Toxoplasmosis, the cGAS/STING pathway was shown to enhance host defense allowing clearance and host survival in an IFN-I-dependent manner (Wang et al., 2019).

An area of research ripe for development is the role for cGAS/STING in homeostatic processes. Specifically, the interplay between cGAS/STING-dependent responses and microbiome are emerging as key to tissue homeostasis. To our knowledge, the first documentation of a role for cGAS/STING signaling in tissue homeostasis came from studying the intestinal barrier in STING-deficient mice (Canesso et al., 2018). STING-deficiency led to defective protective mechanisms of intestinal mucosa including reduced goblet cells numbers, mucus production and fecal IgA. These changes also coincided with reduced numbers of intraepithelial lymphocytes and ILC2 with a concomitant increase in ILC1 and ILC3 numbers. Induction of inflammatory responses in the gut of these mice resulted in increased sensitivity to DSS-induced colitis, T cell-induced colitis and *Salmonella* infection, and impaired Treg development and function. These findings were corroborated by recent studies utilizing STING gain-of-function mice (Shmuel-Galia et al., 2021). Constitutive active STING (N153S) mutations causing SAVI in humans resulted in significant intestinal commensal dysbiosis driving ubiquitination and stabilization of STING protein in lamina propria myeloid cells driving chronic colitis, as well as, accumulation of inflammatory leukocytes including T cells.

Interestingly, a recent study demonstrated an elegant mechanism by which microbiota-induced cGAS/STING signaling promotes tissue homeostasis and maintenance of barrier integrity in the skin (Lima-Junior et al., 2021). In this study, the

authors showed that colonization by the mouse commensal *Staphylococcus epidermidis* promotes IFN-I-dependent antiviral transcriptional programs driving type 17 T cell responses. Interestingly, the authors showed that the source of cGAS activating dsDNA was not bacterial in origin but instead was derived from replication intermediates of ERV. They showed that induction of barrier-inducing T cell responses was dependent upon cGAS recognition of ERV-derived dsDNA in keratinocytes. Inhibition of retroviral reverse transcription was sufficient to abrogate these effects. Interestingly, they also showed that this pathway may be involved in inflammatory disease such as psoriasis and high-fat diet induced systemic inflammation.

1.3.2.4 cGAS/STING in antitumor T cell responses

T cells are absolutely required for anti-tumor immunity. $CD8^+$ T cells function to directly kill tumor cells in an MHC-I-dependent manner whereas $CD4^+$ can promote anti-tumor immunity through direct elimination of cancerous cells or modulating the tumor microenvironment in an MHC-II-dependent fashion (Hadrup et al., 2013; Kennedy & Celis, 2008). Over the past decade the cGAS/STING pathway has emerged as a key regulator of antitumor immunity (Jiang et al., 2020; Kwon & Bakhoum, 2020). Here I will discuss the current state of understanding for the role of the cGAS/STING pathway in antitumor T cell responses.

The significance of cGAS/STING signaling in antitumor T cell responses was first appreciated following a study published by Gajewski and colleagues in 2014 (Woo et al.,

2014). In this work, the authors show that the development of tumor-responsive T cells is dependent upon cGAS/STING signaling and IFN-I production. Importantly, they shown that the source of activating ligand was the tumor cells themselves. In the absence of STING APCs failed to upregulate nearly all proinflammatory cytokines, chemokines and do not undergo maturation. These impairments precluded the priming of tumor-specific T cells, and as a result, STING- or IRF3-deficent mice failed to control immunogenic tumor growth.

Interestingly, in 2011 it was shown that radiation-induced antitumor T cell responses are dependent upon IFN-I production (Burnette et al., 2011). In the same issue as Woo et al., 2014, another group showed that the radiation-induced IFN-I response necessary for antitumor responses is mediated through cGAS/STING signaling (Deng et al., 2014). This study was also the first to show that the efficacy of radiotherapy could be enhanced by the addition of exogenous STING agonist. As a result, the use of STING activating ligands has received considerable interest and investment for use in clinical oncology (Decout et al., 2021).

Perhaps one of the most significant breakthroughs in the realm of cancer therapeutics was the development of checkpoint blockade therapy. These drugs function by blocking inhibitory pathways which suppress antitumor T cell responses. While these treatments have been nothing short of revolutionary a significant percentage of patients fail to respond and their cancers progress. The cGAS/STING pathway has also been

found to be essential to the efficacy of checkpoint blockade (Wang et al., 2017). While anti-PD-L1 antibody therapy slowed the growth of melanoma in WT mice, no effect was observed for cGAS-deficient mice receiving the same drug. Additionally, adding exogenous cGAMP further increased anti-PD-L1 efficacy. Mechanistically, cGAS/STING signaling promoted activation of DC and enhanced cross-presentation of tumor antigen to CD8 T cells. These findings have been corroborated by several other studies demonstrating similar results (Corrales et al., 2015; Ji et al., 2021; W. Li et al., 2020; Luo et al., 2020; M. Lv et al., 2020; Ramanjulu et al., 2018; Schadt et al., 2019; Si et al., 2022; Woo et al., 2014; Yum et al., 2021; H. Zhang et al., 2015; Zhou et al., 2020).

Among all the research done regarding the role of cGAS/STING in antitumor immunity a key commonality exists: cGAS/STING signaling and IFN-I production is critical to the induction of T cell responses through the activation and maturation of APC. Interestingly, cGAS/STING activity may also yield pro-tumor effects. Within tumor cells, cGAS can promote tumor growth through inhibiting homologous recombination in chromosomally unstable cancers (Liu et al., 2018). Additionally, accumulation of cytosolic dsDNA in chromosomally unstable cancers can promote survival and metastasis via cGAS/STING-dependent activation of the noncanonical NF-κB pathway (Bakhoum et al., 2018). In brain tumors, gap junctions facilitate the transfer of cGAMP from carcinoma to astrocytes promoting IFN-I and pro-inflammatory

cytokine production setting up a paracrine feedback loop supporting tumor growth, drug resistance and metastasis (Q. Chen et al., 2016).

Continuous production of cGAMP from tumor cells also creates the possibility of activating antitumor immune responses via heightened local concentrations of extracellular cGAMP. Interestingly, some cancers can utilize this potential weakness and turn it into a strength. Hydrolysis of extracellular cGAMP by ENPP1 leads to the production of adenosine, an immunosuppressive purine facilitating metastasis, reduced immune cell infiltration, and resistance to checkpoint blockade (J. Li et al., 2021). Chronic cGAS/STING activation resulting from leakage of genomic DNA downstream of mutagen exposure can also promote inflammation-driven carcinogenesis which is dependent upon STING activity in hematopoietic cells (Ahn et al., 2014). Finally, cGAS/STING activity is also capable of promoting immune evasion of poorly immunogenic tumors via induction of indoleamine 2,3 dioxygenase immunosuppressive activity (Lemos et al., 2016). In this study the authors demonstrate that immunosuppression mediated by this mechanism was only dependent upon STING expression by host cells suggesting that cGAS activity within the tumor drives this response.

Despite the potential for cooption of the cGAS/STING pathway to promote tumor growth and immune evasion, the benefits of this pathway for cancer are context dependent. For instance, cancers derived from cell types sensitive to cGAS/STING-

mediated cell death such as T cells are highly sensitive to apoptosis in response to STING agonists (Gulen et al., 2017). Cancer cells can also transfer cGAMP to tumor infiltrating leukocytes activating STING signaling to drive enhanced APC function and tumor killing (Marcus et al., 2018; Schadt et al., 2019). Additionally, activation of STING signaling in tumor endothelial cells drives cell-intrinsic production of IFN-I promoting T cell recruitment and anti-tumor responses (Demaria et al., 2015).

In the context of tumor immunity T cell intrinsic cGAS/STING signaling may represent a double-edged sword. A recent study found that in mouse models of tumor immunity T cells undergo cGAS/STING signaling because of accumulation of cytosolic dsDNA downstream of TCR signaling (W. Li et al., 2020). While the mechanism driving accumulation of cytosolic dsDNA was not demonstrated the authors showed that cGAS/STING signaling in CD8 T cells was beneficial. These signals drove activated tumor-specific CD8 T cells to a stem-like memory phenotype and prevented exhaustion by restraining Akt activity. Despite this benefit, T cell intrinsic cGAS/STING signaling also comes with risks. In particular, the high sensitivity of T cells to STING-mediated cell death could abrogate the APC-specific enhancement of tumor immunity elicited by cGAS/STING signaling (Cerboni et al., 2017; Gulen et al., 2017; Larkin et al., 2017). Due to this potential risk, delivery vehicles for cGAS/STING-stimulating compounds should be carefully considered to limit negative impacts on anti-tumor T cell responses.

In summary, cGAS/STING exhibits both beneficial and detrimental activities in the context of tumor immunity (Figure 5). On one hand, the pathway can be hijacked by cancer cells to limit local immune responses, promote survival and growth, and drive metastases. Conversely, this pathway also plays a critical role in driving antitumor T cell responses. The balance of these two opposing results may significantly influence overall disease outcomes and pharmaceutical manipulation of the pathway holds the promise of potentially significantly improving the efficacy of our most promising cancer therapeutics.

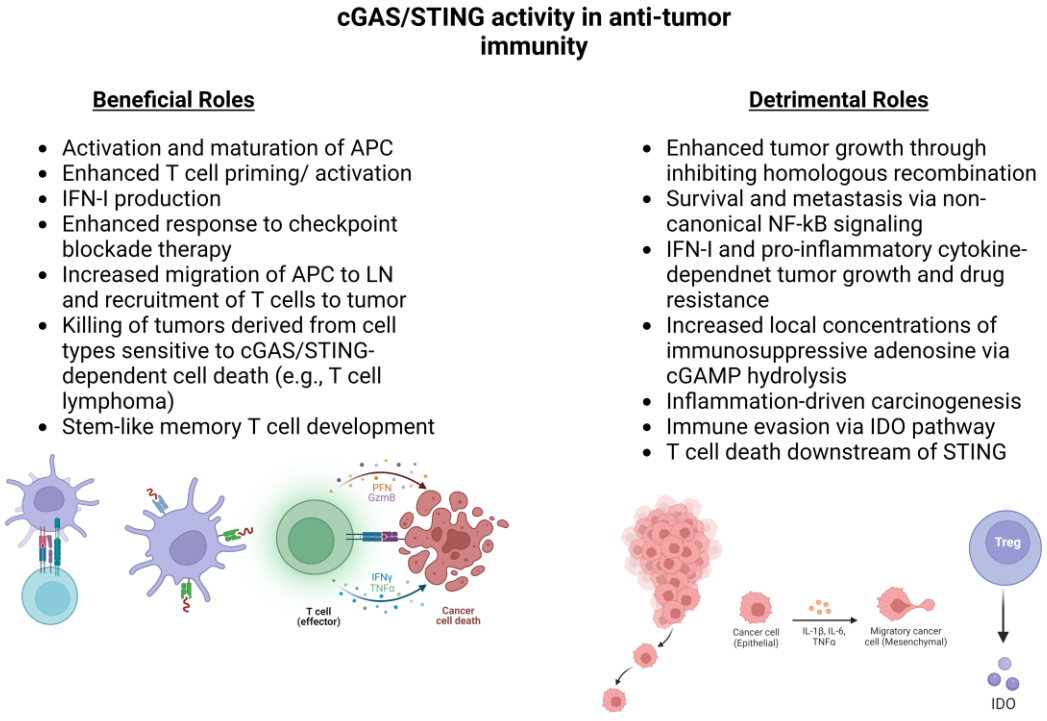

Figure 5: Positive and negative regulatory roles of cGAS/STING pathway in anti-tumor immunity.

Comparison of anti- and pro-tumor effects of cGAS/STING activation. Anti-tumor effects are listed under beneficial roles. Pro-tumor effects are listed under detrimental roles. Diagram generated using Biorender.

1.3.2.5 cGAS/STING in autoimmunity

Autoimmune disease is characterized by aberrant immune responses directed towards host tissues. These responses are often the result of ineffective central tolerance or impaired peripheral tolerance mechanisms. Often, this dysregulation leads to T cells acting as mediators of the disease process. As with many pattern recognition receptors, dysregulation of the cGAS/STING pathway can promote or enhance autoimmune disease. Interestingly, cGAS/STING activity is also protective in several common autoimmune diseases. Here we will discuss the protective and detrimental roles of cGAS/STING in autoimmune processes involving T cell responses.

The first documented role for the cGAS/STING pathway in autoimmune pathology was through the study of Aicardi-Goutieres Syndrome (AGS). The best studied cause of AGS are mutations in the catalytic domain of the 3′-5′ DNA exonuclease TREX1 (Crow, Hayward, et al., 2006; Rice, Newman, et al., 2007; Rice Patrick, et al., 2007). TREX1 deficiency results in the aberrant accumulation of cytosolic dsDNA. Failure to degrade cytosolic dsDNA results in chronic cGAS/STING activation and IFN-I production (Stetson et al., 2008). Ultimately, AGS results in lethal autoimmunity. Further study has identified eight other genes that when mutated can lead to AGS. These genes include, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, ADAR1, IFIH1, LSM11, RNU7-1, (encoding MDA5) (Crow, Leitch, et al., 2006; Lehner et

al., 2006; Rice et al., 2009; Rice et al., 2014; Rice et al., 2012; Uggenti et al., 2020). All these AGS-causing mutations result in enhancement of nucleic acid sensing pathways.

AGS symptoms typically manifest as very early onset (within the first two years of life) severe degenerative encephalopathy leading to progressive microcephaly, calcification of the basal ganglia, chronic cerebrospinal fluid (CSF) lymphcytosis, increased CSF IFNα, thrombocytopenia, hepatosplenomegaly, and elevated liver enzymes. TREX1 mutations are also responsible for a hereditary autoimmune disease known as Familial Chilblains Lupus. AGS patients share many clinical features of systemic lupus erythematosus (SLE). Interestingly, mice carrying the common AGS-associated Trex1 D18N mutation primarily develop multi-organ SLE-like autoimmunity affecting the pancreas, heart, lungs, salivary glands, kidneys, and vasculature (Grieves et al., 2015). These mice also produce large quantities of anti-dsDNA antibodies, a hallmark of SLE. The pathology associated with mouse Trex1 D18N mutations is driven by hematopoietic cells and is STING- and IFN-I-dependent. In this model, IFN-I is largely produced by naïve CD4 T cells and naïve and effector/ memory CD8 T cells (Simpson et al., 2020). T cell from these mice express heightened levels of IRF7, phosphorylated IRF3 and phosphorylated TBK1 and produce IFN-α in response to TCR stimulation. Interestingly, these mice undergo IFN-I-dependent Treg expansion. However, this expansion is not sufficient to protect from autoimmune disease.

Like Trex1 D18N, Trex1-deficient mice exhibit severe systemic autoimmune disease leading to significantly shortened lifespan. Reduced survival is likely due to inflammatory myocarditis which leads to progressive cardiomyopathy and circulatory failure (Morita et al., 2004). In this model, T cells are critical to the development of autoimmunity, however, autoantibodies generated by B cells are the primary cause of death (Gall et al., 2012). The germline deletion model also differs from Trex1 D18N in that IFN-dependent autoimmunity is initiated by IFN-I production by non-hematopoietic cells (Gall et al., 2012). This IFN-I production is dependent upon cGAS/STING activity. Also, like Trex1 D18N, T cells from Trex1-deficient mice are highly activated, express high levels of T-bet, and produce large quantities of IFNγ in a cGAS-dependent manner (Gao et al., 2015). Interestingly, study of these mice also suggests a potential deleterious role for activating cGAS during T cell development. Trex1$^{-/-}$ mice exhibit severely altered thymus histopathology characterized by a near complete absence of the thymic medulla (Morita et al., 2004).

Like Trex1, mutations in other nucleases such as DNase II can result in autoimmune disease. Initial studies of the role for DNase II in mice were hampered by the embryonic/ perinatal lethality of germline deletion due to asphyxiation (Krieser et al., 2002). DNase II-deficient mice also develop lethal anemia due to IFN-β produced by liver macrophage following uptake of undigested erythroid precursor dsDNA (Kawane et al., 2001; Yoshida et al., 2005). This embryonic lethality can be prevented by blocking

IFN-I signaling, however, *Dnase2b*$^{-/-}$ *Ifnar1*$^{-/-}$ mice develop chronic polyarthritis mimicking human rheumatoid arthritis (Kawane et al., 2006). Finally, it was shown that STING was responsible for both the anemia and polyarthritis resulting from DNase II-deficiency (Ahn et al., 2012). While IFN-I production drove anemia in this model, production of other pro-inflammatory cytokines such as, TNFα, IL-1β, and IL-6 likely contributed to polyarthritis.

Like loss-of-function (LOF) mutations in several nucleases, gain-of-function (GOF) mutations, primarily in STING, can drive systemic autoimmunity. STING GOF mutations are responsible for the disease known as STING-associated vaculopathy with onset in infancy (SAVI). As the name suggests, SAVI is characterized by infantile onset autoinflammatory vasculopathy. SAVI exhibits autosomal dominant inheritance (Jeremiah et al., 2014). Consistent with the lupus-like manifestations of AGS, SAVI patients develop skin lesions often affecting the face, ears, nose, and digits. In addition to skin pathology, SAVI patients often develop interstitial lung disease and fibrosis (Jeremiah et al., 2014; Liu et al., 2014). SAVI is refactory to conventional anti-inflammatory drug treatment (Jeremiah et al., 2014), however, *in vitro* studies suggest that JAK inhibition was capable of reducing downstream STAT activation and emeliorate enhanced IFN-I production (Liu et al., 2014). Clinical use of JAK inhibitors and other treatment regimens have yielded inconsistent and often minimal benefit (Y. Wang et al., 2021).

Mouse models carrying SAVI-causing mutations have allowed for mechanistic study of this autoinflammatory disease. Study of STING N153S mice demonstrated that SAVI-associated lung pathology is T cell-mediated and IFN-independent despite T lymphopenia (Luksch et al., 2019; Warner et al., 2017). T cell ablation via Rag1 or TCRβ deletion was sufficient to protect these mice from all lung pathology while B cell ablation had no effect. T cells from STING N153S mice are activated and readily produce IFN-γ and IL-17. Ablation of IFNAR-1, IRF3 and IRF7, or cGAS had no effect on lung pathology in STING N153S mice. In addition to lung pathology, STING N153S initiates commensal dysbiosis and spontaneous T cell-dependent colitis (Shmuel-Galia et al., 2021). Interestingly, gut commensal dysbiosis in STING N153S mice also contributes to lung pathology. Elimination of the gut microbiota with antibiotics was sufficient to protect these mice from lung disease. Similarly, germ-free SAVI mice develop severe lung pathology which can be attenuated by transferring gut microbiota from antibiotic treated colonized mice (Platt et al., 2021).

While SAVI mutations drive T cell-dependent autoimmunity they also result in severe T lymphopenia. STING N153S (Luksch et al., 2019; Siedel et al., 2020) or V154M (Bouis et al., 2019) exhibit significantly reduced peripheral and thymic T cell numbers. This reduction in T cells can be attributed to high rates of apoptosis among ETP and DN1 thymocytes which is IFN-I-independent. While the mechanism mediating thymocyte death in STING N153S mice has not been explored it may be due to

disrupted calcium homeostasis and chronic ER stress (Wu et al., 2019). Interestingly, the T lymphopenia resulting from STING GOF mutations seems to be restricted to αβ T cells as these mice have normal numbers of γδ TCR-expressing cells (Siedel et al., 2020). Therefore, the sensitivity of T cells to STING-mediated cell death may not be universal. Beyond T cell developmental impairments, STING GOF mice also exhibit impaired lymph node organogenesis and lack innate lymphoid cells (Warner et al., 2017).

Activation of the cGAS/STING pathway can promote the development of systemic autoimmune disease. Interestingly, STING activity can play protective roles in several organ-specific autoimmune diseases. IFN-I gene signatures are associated with the incipient stage of T1D development. Interestingly, non-obese diabetic (NOD) mice deficient in granzyme A which activates nucleases to degrade cytosolic DNA develop enhanced disease (Mollah et al., 2017). Study of NOD.Sting$^{-/-}$ mice demonstrated that the cGAS/STING pathway is partially responsible for the elevated IFN-I signature associated with T1D. However, these mice develop exacerbated disease with significantly increased accumulation of autoantigen-specific T cells within islets (Akazawa et al., 2021). This study suggests that cGAS/STING activity functions to limit autoreactive T cell numbers and activities.

Consistent with studies in T1D, the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS) suggests a protective role of the cGAS/STING pathway. Both EAE and MS are demyelinating autoimmune diseases that result from T

cell-mediated destruction of myelin sheaths (Constantinescu et al., 2011). IFN-I has long been known to reduce disease severity in both EAE and MS. In fact, IFNβ was the first disease-modifying treatment approved for use in MS ("Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. I. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial. The IFNB Multiple Sclerosis Study Group" 1993). EAE mice can be protected from disease by administration of DNA nanoparticles in a cGAS/STING/IFN-I-dependent manner (Lemos et al., 2014). Activating the pathway in this manner attenuated effector T cell infiltration into the CNS, suppressed both innate and adaptive immune responses to MOG peptide in the spleen, and induced dominant Treg responses by the cGAS/STING/IFN-I/IDO pathway. Interestingly, treatment of EAE mice with cGAMP-laden microparticles at the peak of disease severity is sufficient to almost completely ameliorate disease symptoms (Johnson et al., 2021). Consistent with these findings, administration of ganciclovir, a STING-dependent inducer of IFN-I production, significantly reduced disease severity in WT but not STING-deficient EAE mice (Mathur et al., 2017). Thus, in summary the cGAS/STING is capable of both suppressing and promoting various autoimmune diseases depending on context (Figure 6).

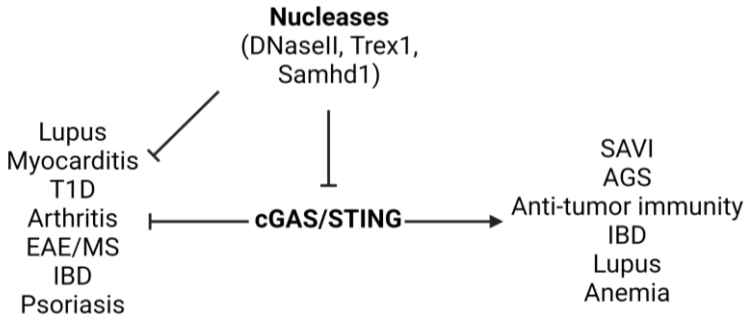

Figure 6: cGAS/STING-dependent regulation of autoimmune disease.

Autoimmune disease positively or negatively regulated by cGAS/STING activity.

One of the most perplexing aspects of the cGAS/STING pathway in T cell-mediated autoimmunity is that the pathway can play both protective and detrimental roles in the same disease. For instance, STING GOF mutations can drive spontaneous colitis (Shmuel-Galia et al., 2021) while STING-deficient mice also exhibit increased sensitivity to T cell-mediated colitis (Canesso et al., 2018). Similarly, STING GOF mutations can drive lupus-like disease but in the MRL/Lpr mouse model of lupus, MRL/Lpr.Sting$^{-/-}$ mice have increased autoantibodies, expanded atypical T cells, and accelerated lupus nephritis and mortality in an IFN-I-independent manner (Sharma et al., 2015). These conflicting findings regarding the cGAS/STING pathway suggest that it may function as a rheostat in autoimmune disease (Figure 7). On one hand, excessive cGAS/STING activity can drive T cell-mediated autoimmune pathologies while, on the other, insufficient activity may break tolerance and allow unrestrained autoreactive T cell destruction of host tissues.

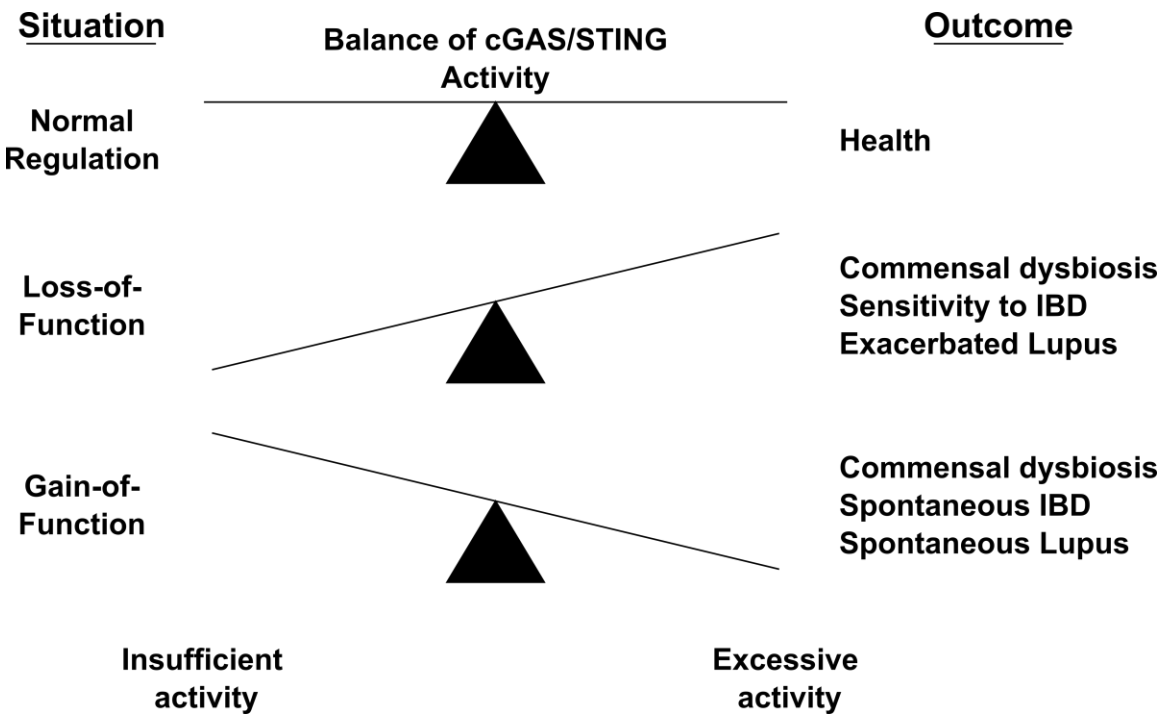

Figure 7: cGAS/STING acts as a rheostat in development of autoimmune disease.

Diagram of the outcomes resulting from imbalance in regulation of cGAS/STING activity resulting from loss-of-function or gain-of-function mutations in cGAS or STING.

1.3.2.6 Therapeutic targeting of cGAS/STING pathway

The cGAS/STING pathway is capable of regulating host immune responses in numerous ways. As described in earlier sections, cGAS/STING activity can promote antimicrobial immunity, is required for antitumor immunity, and can either promote or prevent autoimmune reactions depending on the context. Due to the incredible potential for immunomodulatory capacity of this pathway significant effort has been spent developing compounds to module its activity. Here we will discuss the current state of

development of chemical agents to module cGAS/STING activity with a focus towards diseases in which T cell functionality is key.

1.3.2.6.1 Inhibitors of the cGAS/STING pathway

As previously stated, aberrant or continuous signaling through the cGAS/STING pathway holds the potential to induce numerous autoimmune diseases. As such, several compounds have been developed to chemically inhibit either cGAS or STING. The first cGAS-specific inhibitory compound RU.521 was identified via a high-throughput screen (Vincent et al., 2017). RU.521 was derived from RU.365 and both compounds bind to the active site of cGAS preventing the generation of cGAMP. RU.521 was able to efficiently block IFN-I expression in *Trex1*$^{-/-}$ bone marrow-derived macrophage suggesting it may hold promise as a treatment for AGS. Around the same time, a second group developed the cGAS active-site binding compound PF-06928215 (Hall et al., 2017). Unfortunately, despite its ability to bind to the active site of cGAS, PF-06928215 lacked inhibitory activity in cell-based assays. Recently, a new class of cGAS inhibitors targeting the active site were described. Through these studies the human-specific cGAS inhibitor G150 was developed (Lama et al., 2019). G150 exhibited substantially improved IC-50 values for human cGAS compared to RU.521. An additional new class of human cGAS inhibitors were developed the following year based on earlier work using the compound PF-06928215 which include compound 18, S2 and S3 (W. Zhao et al., 2020). Among these compounds S3 showed the greatest promise as an improved inhibitor of human cGAS.

At the same time a third new class of cGAS inhibiting compounds was generated including CU-32 and CU-76 which exhibit potent inhibitory capacity without affecting RNA sensing (Padilla-Salinas et al., 2020). Additional compounds have been identified with cGAS inhibitory potential but they achieve inhibition through means other than inhibiting catalytic activity (Decout et al., 2021).

In addition to cGAS inhibitors, several STING inhibiting compounds have been identified. Two main approaches have been utilized for small molecule inhibition of STING activity: blocking cGAMP binding to the CDN-binding site or inhibiting palmitoylation near the transmembrane domain to prevent its dimerization. To date, two studies have identified CDN-binding site blocking STING inhibitors. The first identified the natural cyclopeptide isolated from the plant *Aster tataricus*, Astin C, as a potent inhibitor of STING activation via competitive inhibition of cGAMP binding (Li et al., 2018). *In vivo* Treatment with Astin C led to increased susceptibility to HSV-1 and *Listeria* infection in WT mice and prevented STING-mediated autoimmune disease in *Trex1*$^{-/-}$ mice. Shortly after, another study developed a novel class of STING inhibitors with ideal physiochemical properties and potent inhibitory capacity by leveraging a 2:1 binding stoichiometry to occupy and block the large binding pocket of STING (Siu et al., 2019). These studies led to the identification of the tetrahydroisoquinoline, Compound 18, which exhibits potent oral bioavailability and stable binding to the CDN-binding pocket providing functional inhibition of STING-mediated signaling.

Four classes of STING inhibitory compounds have been developed to block palmitoylation near the transmembrane domain. These include nitrofurans, indole ureas, nitro fatty acids, and acrylamides. All classes of compounds utilizing this mode of inhibition bind to, and block, palmitoylation of cysteine 88 or 91 of STING. Nitrofurans and indole ureas were the first identified classes of compounds with this mode of inhibition (Haag et al., 2018). C-176 and C-178 were the first of this class developed and very specifically inhibit mouse, but not human, STING. Analogues of these compounds (C-170 and C-171) were generated which are capable of inhibiting both mouse and human STING. Due to poor solubility of C-178, C-176 was chosen for *in vivo* testing. In *Trex1*$^{-/-}$ mice, C-176 efficiently inhibited STING-mediated pro-inflammatory cytokine production and significantly reduced autoimmune pathology. Based on the promising results of these studies the authors then set out to identify more advanced covalent STING antagonists yielding the compound H-151. H-151 exhibits high bioactivity and is a highly potent and selective mouse and human STING inhibitor ideal for *in vivo* use.

Shortly after the discovery of C-176, C-178, and H-151, another group identified nitro-fatty acids (FAs) as potent inhibitors of STING signaling via blocking palmitoylation (Hansen et al., 2018). The authors of this study used an elegant approach to identify compounds naturally produced during viral infection with capacity to inhibit IFN-I responses. These studies demonstrated that nitro-conjugated linoleic acid (cLA)

and -oleic acid efficiently block palmitoylation at both Cy88 and Cys91 of STING. Importantly, these nitro-FAs efficiently inhibit both mouse and human STING.

Finally, using chemical proteomic activity-based profiling of cysteine reactivity to a library of electrophilic small molecules in T cells, Cravatt and colleagues identified the acrylamides BPK-21 and BPK-25 as STING inhibitors (Vinogradova et al., 2020). Both compounds bind Cys91 thereby preventing palmitoylation and STING signaling. Treatment of cGAMP-stimulated PBMCs demonstrated that BPK-25 efficiently inhibits downstream IFN responses and production of pro-inflammatory cytokines. However, these compounds do not selectively bind STING with each acting as ligands for several proteins. Therefore, the lack of specificity brings into question the utility of these compounds as therapeutics.

1.3.2.6.2 Chemical agonists of the cGAS/STING pathway

T cells exhibit profound abilities to promote tumor control and clearance. Therefore, development of pharmacological agents to block inhibitory pathways or *ex vivo* genetic manipulation of T cells has received considerable interest for several decades. These include therapeutics such as checkpoint blockade, tumor vaccines, and chimeric antigen receptor (CAR) T cell therapy. The primary goal of all T cell-directed cancer therapeutics is to overcome inhibition and unleash anti-tumor T cell responses. While T cell-targeted immunotherapy have yielded revolutionary results, these treatments fail for a significant proportion of patients (Chowell et al., 2022). Therefore,

developing novel approaches to bolster or enhance these treatments is a very active area of research. One such area of development with significant promise is developing cGAS/STING activating compounds to promote anti-tumor T cell responses. Compounds activating this pathway greatly enhance antitumor T cell immunity through disrupting tumor vasculature (Downey et al., 2014), repolarizing suppressive macrophage towards a pro-inflammatory phenotype (Y. Zhang et al., 2018), and enhancing APC-dependent T cell priming (M. Lv et al., 2020; Wang et al., 2017).

Due to the difficulty of delivering dsDNA into cells virtually all compounds developed to date that directly activate this pathway target STING. Several classes of STING-targeting drugs have been developed including, CDN analogues, non-CDN small molecules, nanovaccines, antibody-drug conjugates, bacterial vectors, and engineered exosomes. Due to the very large number of compounds that have been developed to activate this pathway I will not go into detail for each compound but rather discuss the general mode of action for each class of compounds.

CDN analogues are synthetic derivatives of naturally occurring STING ligands and were the first STING-targeting compounds to enter clinical trials. Currently, at least eleven such compounds are in Phase I or Phase II trials (Amouzegar et al., 2021). Mechanistically, these compounds activate STING signaling in a manner identical to cGAMP. In most cases these drugs are delivered intratumorally to limit systemic inflammatory responses. In pre-clinical studies CDNs have shown profound efficacy as

monotherapies or combination therapies with checkpoint blockade (Foote et al., 2017; Francica et al., 2018; Sivick et al., 2018). Interestingly, intratumoral injection of CDNs promotes efficient clearance of uninjected distal tumors (Foote et al., 2017). In clinical trials several of these compounds demonstrate encouraging efficacy statistics and are generally tolerable (Amouzegar et al., 2021).

Non-CDN STING agonists are a larger group of compounds being developed including several chemical families. The first identified STING agonist was the flavonoid, DMXAA (Kim et al., 2013; Prantner et al., 2012). In animal models, DMXAA efficiently induces tumor regression (Corrales et al., 2015; Downey et al., 2014; Weiss et al., 2017). However, the drug failed in clinical trials likely due to its poor binding to human STING (Conlon et al., 2013; Kim et al., 2013). Since the clinical trial failures of DMXAA other flavonoids which bind human STING have been identified and are under active development (Banerjee et al., 2020; Liu et al., 2017; Y. Zhang et al., 2018). These flavonoid compounds bind the C-terminal domain of STING, and therefore, likely induce activation through a mechanism similar to cGAMP (Conlon et al., 2013).

Recently, additional non-CDN STING agonists have been identified. One such compound is kitacinnamycin 8, a cinnamoyl-containing nonribsomal peptide which was discovered through genomic mining of actinobacteria (Shi et al., 2019). *In vitro* kitacinnamycin 8 synergizes with cGAMP to promote enhanced STING signaling. However, the mechanism of action for this compound remains unknown. One of the

more promising non-CDN STING agonists recently developed is di-ABZI, an amide compound, which showed very potent antitumor activity in mouse models and did not require intratumoral delivery (Ramanjulu et al., 2018). di-ABZI exhibits substantially increased STING activating potential relative to cGAMP. Several other amide compounds have been generated with potent STING activating ability and antiviral or antitumor efficacy (Gall et al., 2018; Xi et al., 2020; Zhang et al., 2019). Very recently, two other non-CDN direct STING agonists have been developed, MSA-2 and SR-717 (Chin et al., 2020; Pan et al., 2020). Both compounds bind to STING, inducing a 'closed lid' architecture like that induced by cGAMP. These two compounds are of substantial clinical interest due to their oral bioavailability and high potency.

In addition to small molecules, living therapeutics have been developed to activate the cGAS/STING pathway for the cancer therapy. These types of therapy utilize gut commensal bacteria to induce the desired response. One such living drug is SYNB1891, a strain of the non-pathogenic, probiotic *E. coli* Nissle engineered to produce large quantities of cyclic-di-AMP (Leventhal et al., 2020). SYNB1891 monotherapy efficiently induced protective antitumor CD8 T cell responses and durable memory. Interestingly, oral administration of SYNB1891 yielded improved antitumor immunity over intratumoral injection of c-di-AMP. A recent study identified the probiotic lactobacillus strain *Lactobacillus rhamnosus* GG (LGG) as potent inducers of antitumor T cell responses in tumors refractory to checkpoint blockade (Si et al., 2022).

Mechanistically, LGG promoted antitumor CD8 T cell responses in a cGAS/STING/IFN-I-dependent manner. The specific mechanism by which LGG induces cGAS/STING activation was not elucidated. However, the antitumor effects of LGG colonization were dependent upon active bacterial processes as heat-killed bacteria had no effect on tumor growth.

While cGAS/STING agonists hold significant promise as treatments for several conditions they are not without their drawbacks. Beyond the potential for deadly systemic inflammatory responses, aberrant STING signaling could kill tumor-infiltrating T cells rendering treatment ineffective (Gulen et al., 2017; Larkin et al., 2017). Therefore, targeted delivery of STING agonists may circumvent these deleterious side effects and further enhance efficacy.

Several mechanisms of delivery have been developed in recent years including antibody-drug conjugates (ADC), nanoparticles, and engineered exosomes (Amouzegar et al., 2021). ADCs show great promise as *in vitro* studies suggest that STING agonist ADCs exhibit 150-300-fold increased potency compared to free STING agonists. In mouse models, a single dose of tumor-targeted diABZI ADCs induced durable and complete tumor regression even at doses fifty-fold lower than that used for free diABZI. Importantly, the local delivery of diABZI led to significant reductions in circulating pro-inflammatory cytokines demonstrating a potent local effect for the drug (Bukhalid et al., 2020).

Another mode of delivery for STING agonists is through nanoparticles. Several groups have utilized engineered micro and nanoparticles to deliver STING agonists for cancer therapy, vaccines, and treatment of autoimmune disease (Collier et al., 2018; Gallovic et al., 2022; Gulen et al., 2017; Johnson et al., 2021; Junkins et al., 2018; Luo et al., 2017; Mai et al., 2021; Wilson et al., 2018). These engineered particles also allow an effective means of delivering cGAS-activating dsDNA (Lemos et al., 2014; Lemos et al., 2019; H. Zhang et al., 2015). A key benefit of engineered micro and nanoparticles for delivery of cGAS/STING activating agents is the ability to target these particles to specific cell types such as DC (Gou et al., 2021) or tumor cells (Rao et al., 2020). Additionally, micro or nanoparticles can be loaded with tumor antigen and STING agonist to amplify antitumor T cell immunity (Luo et al., 2017) and overcome checkpoint blockade resistance (Fu et al., 2015).

Like engineered micro or nanoparticles, naturally occurring extracellular vesicles can be loaded with a host of cargo including. In particular, exosomes have emerged as a platform of interest for drug delivery. Exosomes are cell-derived lipid nanoparticles which can facilitate cell-cell communication. In mouse models, exosomes loaded with tumor antigen have been successfully used for delivery of tumor vaccines (Xiao et al., 2017; Xie et al., 2013). Recently, an engineered exosome loaded with CDN STING agonist (ExoSTING) was shown to enhance the potency of CDN signaling and preferentially activate APC within the tumor microenvironment (Jang et al., 2021). Like ADC,

ExoSTING restricted inflammatory responses to the tumor, enhanced local Th1 and CD8 T cell responses, and promoted systemic antitumor immunity.

cGAS/STING modulating compounds show promise for the treatment of many immune-related diseases. cGAS or STING inhibitors may prove effective in the treatment of autoimmune disease that directly result from excessive activation of the pathway, such as, SAVI and AGS. They may also prove useful in the treatment of more complex diseases in which the pathway contributes to disease pathogenesis such as arthritis and lupus. Additionally, agents which activate the pathway may be useful in the treatment of T cell-mediated organ-specific autoimmune diseases such as T1D and MS. Pre-clinical studies suggest that activating STING in T cells can restrain autoreactive clones. However, non-specific activation of the pathway may also drive disease promoting activities in APC. Therefore, effective utilization of these compounds for the treatment of autoimmune disease may require cell type-specific drug delivery. In this regard, ADC may be particularly useful for T cell-specific delivery. Conversely, APC-specific delivery will likely provide the most efficacious treatment for cancer and vaccination strategies.

1.3.3 Summary and future directions

The cGAS/STING pathway is a versatile system for detecting physiological perturbations and inducing effective immune responses. While much of the research

into this pathway has focused on its roles in innate and non-immune cell types mounting evidence clearly demonstrates a critical role for the pathway in adaptive immunity. These lines of evidence include the essential role for cGAS/STING in T cell activation to promote antimicrobial and antitumor immunity, STING signaling-dependent tuning of T cell differentiation, and STING-dependent apoptosis of T cells.

Most studies of the cGAS/STING pathway in T cells and other immune cell types have utilized all-or-nothing approaches. That is, most experiments involve the use of germline or conditional knockouts or stimulation with very high dose agonists. Future work will benefit from studies to fine-tune signaling strength and determine functional outcomes. It is possible that weak signaling below the threshold to induce T cell death may yield very different outcomes from that of strong activation. Furthermore, the source of physiological cGAS ligand in T cells is essentially unknown. While it is likely that the ligand will differ based on context understanding the mechanism of signal initiation may identify targetable pathways to modulate responses.

Chemical modulation of the cGAS/STING pathway has received considerable research interest for several years. Agonism of the pathway has yielded very promising results for the treatment of cancer. Generally, the effects result from disruption of tumor vasculature and promoting APC activation and subsequent T cell priming. However, preclinical studies also suggest that activating the pathway may be beneficial in treating T cell-mediated autoimmune disease. Since global agonism can simultaneously enhance

APC function and limit autoreactive T cell responses there is a risk of selecting for agonist unresponsive T cells and further exacerbating disease. Therefore, generating T cell- or APC-targeted drug delivery vehicles, such as engineered exosomes, antibody-drug conjugates, and nanoparticles, holds the possibility of yielding even more effective means of positively and negatively modulating immune responses.

1.4 Zinc Finger Protein 335 (Zfp335)

The zinc finger protein 335 (*Zfp335*) gene, also known as NRC-interacting factor 1(Nif1), encodes a C2H2 zinc finger transcription factor made up of thirteen C2H2 zinc finger domains. Zfp335 is a highly conserved transcription factor first identified as having significant roles in development when two humans were discovered to carry homozygous or compound heterozygous loss-of-function mutations in the human homolog *ZNF335*. Previous studies have shown that Zfp335 is specifically localized to the nucleus and seems to function exclusively as a transcriptional activator. Little is known regarding the biology and biochemistry of Zfp335; however, it has been shown to interact with nuclear hormone receptors and possibly regulate histone modifications (Yang et al., 2012).

1.4.1 Zfp335 in disease

The functional significance of Zfp335 was first realized in the study of a consanguineous Arab-Isreali family of which seven members presented to the clinic with some of the most severe cases of microcephaly ever documented (Yang et al., 2012).

These patients presented to the clinic with head circumference 9 standard deviations below the mean. Except for one individual all affected family members died within the first year of life. These patients exhibited severe anatomical defects in brain structure and almost no cortical neurons. Genetic studies of this family identified a common homozygous missense mutation in the splice donor of exon 20 in ZNF335 (3332g>a) of all affected family members. This mutation resulted in a significant reduction in ZNF335 protein levels for affected patients and approximately 50% loss for heterozygous parents. Loss of functional ZNF335 resulted in impaired proliferation, differentiation, and migration of neuronal progenitors. The lack of clinical manifestations in heterozygous parents suggests that ZNF335 is haplosufficient. Interestingly, lymphoblastoid cell lines generated from these patients and unaffected family members showed that loss of ZNF335 severely impaired the proliferative capacity of immune cells. The mechanism by which ZNF335 controls neuronal development and differentiation was determined to be through regulation of REST expression downstream of the trithorax complex. REST functions as a master transcription factor in neuronal development. The authors of this study show that ZNF335 directs H3K4me3 deposition by trithorax at the REST promoter.

The specific form of microcephaly resulting from ZNF335 mutations has been termed primary microcephaly-10. More recently, a female Japanese patient from nonconsanguineous parents with severe microcephaly caused by a novel ZNF335

compound heterozygous loss-of-function mutation was identified (Sato et al., 2016). Interestingly, while this patient exhibited severe microcephaly it was much less pronounced (-5.0 SD) than the probands of the Arab-Israeli family (-9.0 SD). Most recently two additional humans were identified with novel ZNF335 mutations but exhibited clinical characteristics unique from the first 8 documented cases (Stouffs et al., 2018).

Study of the role for ZNF335 in cell survival suggests that its requirement extends well beyond neuronal development. Large scale studies of genetic dependency in cancer support a ubiquitous requirement of ZNF335, at least in proliferative cells (Dempster et al., 2019; Ghandi et al., 2019; Pacini et al., 2021), and affirms the haplosufficiency of ZNF335 (McFarland et al., 2018; Tsherniak et al., 2017). Experiments performed through the Cancer Dependency Map Consortium (DepMap) show that of the 1086 cancer cell lines tested via CRISPR screens only 5 did not require ZNF335 for survival while RNAi screens showed that only 0.58% (2/343) of cancer cell lines viability was affected by knock down. In summary, despite our very limited understanding of ZNF335/Zfp335 biology it is clear that this gene plays an essential, non-redundant function in basic cell biology.

1.4.2 Zfp335 regulates T cell maturation

A recent study utilizing ENU mutagenesis screens identified Zfp335 as playing an essential role in the establishment of the naïve T cell compartment. Through these

studies a mouse line with a near absence of naïve T cells was generated (Han et al., 2014). The authors of this study termed this mouse line Bloto (blood low T cells). Exome sequencing of these mice identified a single missense mutation in Zfp335 as the causative genetic change. Detailed characterization of these mice showed normal T cell development up to the stage of semi-mature SP thymocytes. *Zfp335*$^{bloto/bloto}$ mice exhibited severely reduced proportions and numbers of mature SP thymocytes and peripheral T cells along with virtually no naïve T cells. In this study, the transcriptional targets of Zfp335 in thymocytes were described and *Ankle2* was identified as a key Zfp335-regulated gene contributing to the observed phenotype. *Ankle2* encodes an endoplasmic reticulum-restricted lem domain-containing protein (Asencio et al., 2012). Overexpression of Ankle2 via retroviral infection was sufficient to partially rescue the naïve T cell compartment. However, the mechanism by which Ankle2 overexpression achieved this rescue was not elucidated.

An additional study recently identified a role for Zfp335 in early T cell development (Wang et al., 2022). Specifically, this study showed that Zfp335 regulates survival of DN thymocytes and plays an important role in β-selection.

2. Loss of Zfp335 drives cGAS/STING-dependent apoptosis of post-β-selection pre-T cells

All work described in this chapter was adopted from the following manuscript: Ratiu JJ, Barclay W, Lin E, Wang Q, Wellford S, Mehta N, Harnois MJ, DiPalma D, Roy S, Contreras AV, Shinohara ML, Wiest D, Zhuang Y. "Loss of Zfp335 triggers cGAS/STING-dependent apoptosis of post-β-selection pre-T cells". *Nature Communications. Manuscript in review.*

2.1 Introduction

Development of large numbers of T cells with clonally acquired T cell receptor (TCR) in the thymus demands a small number of bone marrow derived progenitors to undergo vigorous expansion prior to each of the sequentially ordered TCR gene rearrangement events. The first major expansion occurs immediately upon T lineage commitment at the DN2 stage prior to rearrangement of any TCR gene (Krueger et al., 2017; L. Li et al., 2010; Tourigny et al., 1997; Wojciechowski et al., 2007). The expanded T cell progenitors enter the DN3 stage where rearrangement at the TCRβ, γ, δ gene loci become permissive. In postnatal thymus, the majority of DN3 cells will choose the αβT cell fate due to the generation of a productively rearranged TCRβ chain. Post β-selection DN3 cells then move to the DN4 stage where the second phase of expansion occurs, typically involving several rounds of rapid proliferation over the course of 2-3 days in mice. The expansion of TCRβ positive cells result in generation of the post mitotic DP

cells, which constitutes 90% of all thymocytes in post-natal mice and humans. DP cells undergo TCRα gene rearrangement and selection, a process resulting in approximately 1% of cells surviving and contributing to the peripheral T cell pool. Therefore, the expansion of post β-selection DN4 cells prior to TCRα gene rearrangement and TCR selection represents a critical amplifier to control the output of $\alpha\beta$T cells from the thymus.

While most stages of T cell development have been subject to extensive genetic and functional characterization, the post-β-selection proliferative phase remains less well understood. Previous studies have shown that proliferation, but not survival, of DN4 cells is dependent upon IL-7R signaling which functions to repress Bcl6 expression (Boudil et al., 2015). Similarly, proliferation during this stage of development also requires the combined activities of NOTCH and pre-TCR signaling (Fehling et al., 1995; Guidos, 2006; Kelly et al., 2007; Yamasaki et al., 2006). This effect is in part the result of induction of Fbxl1 and Fbxl12 which induce polyubiquitination and proteasomal degradation of Cdkn1b ensuring proper cell cycle progression and proliferation (B. Zhao et al., 2019). Survival of proliferating post-β-selection thymocytes was found to require expression of the chromatin associated protein yin yang 1 (Yy1), the absence of which drives p53-dependent apoptosis (L. Chen et al., 2016). Animal models exploring cell death during T cell development have repeatedly shown thymocyte apoptosis, including among DN4 cells, is largely driven by activities of pro-apoptotic Bcl2 family proteins

(Bouillet et al., 2002; Hutcheson & Perlman, 2007; Hutcheson et al., 2005; Ren et al., 2010; Villunger et al., 2003). Pathways controlling the survival and death of early proliferating thymocytes upstream of the Bcl2 family remain largely unexplored.

Underpinning the fate decisions of thymocytes are vast transcriptional networks which coordinate the intricate changes and checkpoint traversals required for proper development (Rothenberg & Taghon, 2005). Numerous transcription factors function at different stages to achieve this result. One transcription factor family of particular importance are the basic helix-loop-helix E proteins, which include E2A, HEB and E2-2. In developing T cells, activities of the E2A and HEB have been shown to regulate nearly all stages of thymopoiesis (Belle & Zhuang, 2014; Sawada & Littman, 1993). These E proteins play critical roles in enforcing the β-selection checkpoint by promoting expression of *Rag1/2* (Hsu et al., 2003) and *pre-Tα* (Herblot et al., 2000), activation of the TCRβ (Jia et al., 2008), TCRγ, and TCRδ loci (Ghosh et al., 2001) and preventing passage of DN cells lacking a functional TCRβ chain from progressing to the DP stage (Engel et al., 2001; Jones & Zhuang, 2011). Additionally, E protein activity has been shown to enforce early T cell lineage commitment (Xu et al., 2013) and promote survival of post-β-selection DP thymocytes undergoing TCR*α* recombination (Jones & Zhuang, 2007). Together, the combined activities of E proteins play critical and indispensable roles in the establishment of a functional T cell repertoire. However, due to the widespread

binding of these factors throughout the genome of developing thymocytes our understanding of their roles in development are far from complete.

The cGAS/STING pathway functions to sense cytosolic DNA and initiate innate immune responses (Kato et al., 2017). Cyclic GMP-AMP (cGAMP) synthase (cGAS) recognizes dsDNA, typically of foreign origin, catalyzing the generation of the cyclic dinucleotide (CDN) second messenger cGAMP which in turn drives STING activation and down-stream signaling (Sun et al., 2013). The cGAS/STING pathway is best known for its functions in non-immune and innate immune cells such as macrophage and dendritic cells in the context of viral or bacterial infections. In these contexts, activation of the pathway typically results in the production of type I interferons and other pro-inflammatory mediators. Recent work has shown that the cGAS/STING pathway is also highly active but functionally divergent within T cells, primarily driving type I interferon-independent responses and apoptosis (Cerboni et al., 2017; Gulen et al., 2017; W. Li et al., 2020; Wu et al., 2020). Under steady-state conditions the cGAS/STING pathway plays a minimal role in T cell development as evidenced by normal thymic T cell subset proportions and overall thymus size in cGAS or STING-deficient C57/BL6 mice (W. Li et al., 2020). However, it remains to be determined whether the cGAS/STING pathway plays a role in sensing and responding to cell intrinsic stresses during thymic T cell development.

In this study we show that loss of Zinc finger transcription factor 335 (Zfp335), triggered cGAS/STING-mediated apoptosis among proliferating DN4 cells. Zfp335 was initially identified from genetic mapping of familial traits that cause a severe form of microcephaly (Yang et al., 2012). Using a conditional knockout mouse model (Park et al., 2010; Yang et al., 2012) we show that loss of Zfp335 promotes cGAS/STING-dependent apoptosis among proliferating post-β-selection DN4 thymocytes, severe reduction in overall thymic cellularity and a near absence of peripheral T cells. Mechanistically, Zfp335 functions to suppress cGAS/STING activation through promoting Ankle2 expression which in turn regulates the cGAS inhibitor Baf (Guey et al., 2020). The importance of cGAS/STING pathway among DN4 thymocytes was further demonstrated by their sensitivity to STING agonist and STING-mediated cell death in wild type mice. Thus, we have uncovered for the first time a role for the cGAS/STING pathway in regulating thymic T cell development and identify the Zfp335/Ankle2/Baf axis as the first transcriptional network functioning to regulate cGAS/STING activity.

2.2 Results

2.2.1 Zfp335, an E-protein target, is critical for T cell development

The E protein family of transcription factors are indispensable regulators of nearly every stage of T cell development (Engel & Murre, 2001, 2004; Jia et al., 2008; Jones-Mason et al., 2012; Jones & Zhuang, 2007, 2011; Rothenberg & Taghon, 2005; Roy et al., 2018; Wojciechowski et al., 2007). E proteins control complex transcriptional

networks which remain incompletely understood. To gain deeper insight into mechanisms by which E proteins regulate T cell development, we previously performed E2A ChIP-seq to identify the genome-wide binding sites during T cell development(Roy et al., 2018). We identified Zfp335 as an E protein target during T cell development (Figure 8A). Analysis of published data showed E protein-deficient thymocytes exhibit significantly reduced *Zfp335* expression (Figure 8B) (Jones-Mason et al., 2012). Additionally, *Zfp335* is ubiquitously expressed among thymocytes (Figure 8C) suggesting that it may play important roles throughout T cell development. Since germline deletion of Zfp335 is non-viable (Yang et al., 2012) we utilized a conditional deletion model in which Cre expression is controlled by the $E8_{III}$ enhancer of *Cd8a* ($E8_{III}$-cre) to allow functional assessment of Zfp335 in post-β-selection thymocytes (Park et al., 2010). There are conflicting reports regarding the deletion kinetics for this Cre (Dashtsoodol et al., 2017; Park et al., 2010), therefore, we began by assessing its activity across T cell development in our system (Figure 8D-E). Consistent with Dashtsoodol *et* al., we found $E8_{III}$-cre is highly active immediately upon entry into DN3a with no recombination activity evident in the preceding DN2 stage. However, deletion does not appear to be complete until the DP stage.

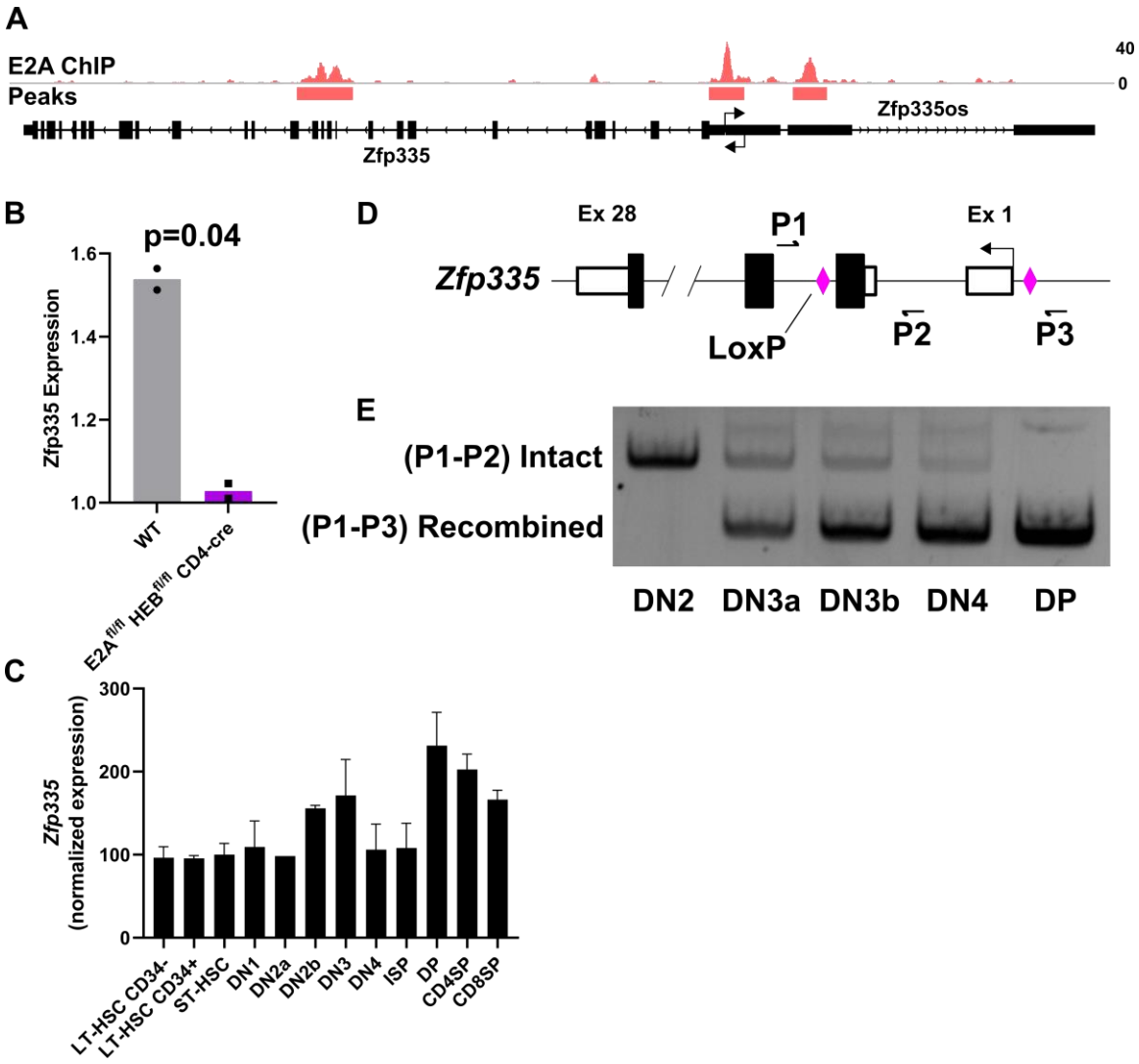

Figure 8: Zfp335 is a target of E proteins in developing T cells

(A) E2A ChIP-seq track for Zfp335 locus in Id2fl/fl Id3fl/fl Lck-cre DP thymocytes (GSE89849). (B) Zfp335 transcript abundance in WT vs. E2A/HEB double knock-out DP thymocytes determine by microarray (GSE9749). (C) Zfp335 expression throughout T cell development determined by RNA-seq (GSE109125). (D) Schematic diagram for PCR-based determination of Zfp335 recombination kinetics. Small arrows indicate approximate positions for primers (P1-3) used for assay. (E) Representative assessment of Zfp335 recombination in sort purified Zfp335fl/fl E8III-cre DN2, DN3a, DN3b, DN4 or DP thymocytes. Data are representative of four individual experiments.

We subsequently assessed *Zfp335*$^{fl/fl}$ E8$_{III}$-cre (Zfp335cKO) mice for thymic T cell development. Deletion of *Zfp335* led to a significant reduction in total thymic cellularity (Figure 9A-B). This reduction in thymic cellularity is likely due to defects in the αβ lineage as numbers of γδ T cells were not altered (Figure 9C-D). Assessment of developmental stages revealed the reduction in thymocyte numbers of Zfp335cKO mice begins at the DN4 stage (Figure 9E-I).

To determine the transcriptional changes resulting from loss of Zfp335 we performed RNA-seq on Zfp335cKO DP thymocytes. DP cells were used as they were the first population exhibiting complete deletion (Figure 8D). We found that loss of Zfp335 results in differential expression of 327 genes (113 down, 214 up; Figure 9K,J). Among the 161 Zfp335 ChIP-seq targets identified in thymocytes (Han et al., 2014), 34 were down-regulated in Zfp335cKO mice (Figure 9K). No Zfp335 target genes were up-regulated in Zfp335cKO samples (Figure 9K) corroborating previous findings that Zfp335 primarily functions as a transcriptional activator(Han et al., 2014; Yang et al., 2012). Consistent with transcriptomic analyses of *Zfp335*^{bloto} mice(Han et al., 2014), gene set enrichment analysis (GSEA) revealed significant enrichment for type I and type II interferon signaling and P53 signaling pathways in Zfp335cKO DP cells (Figure 9L).

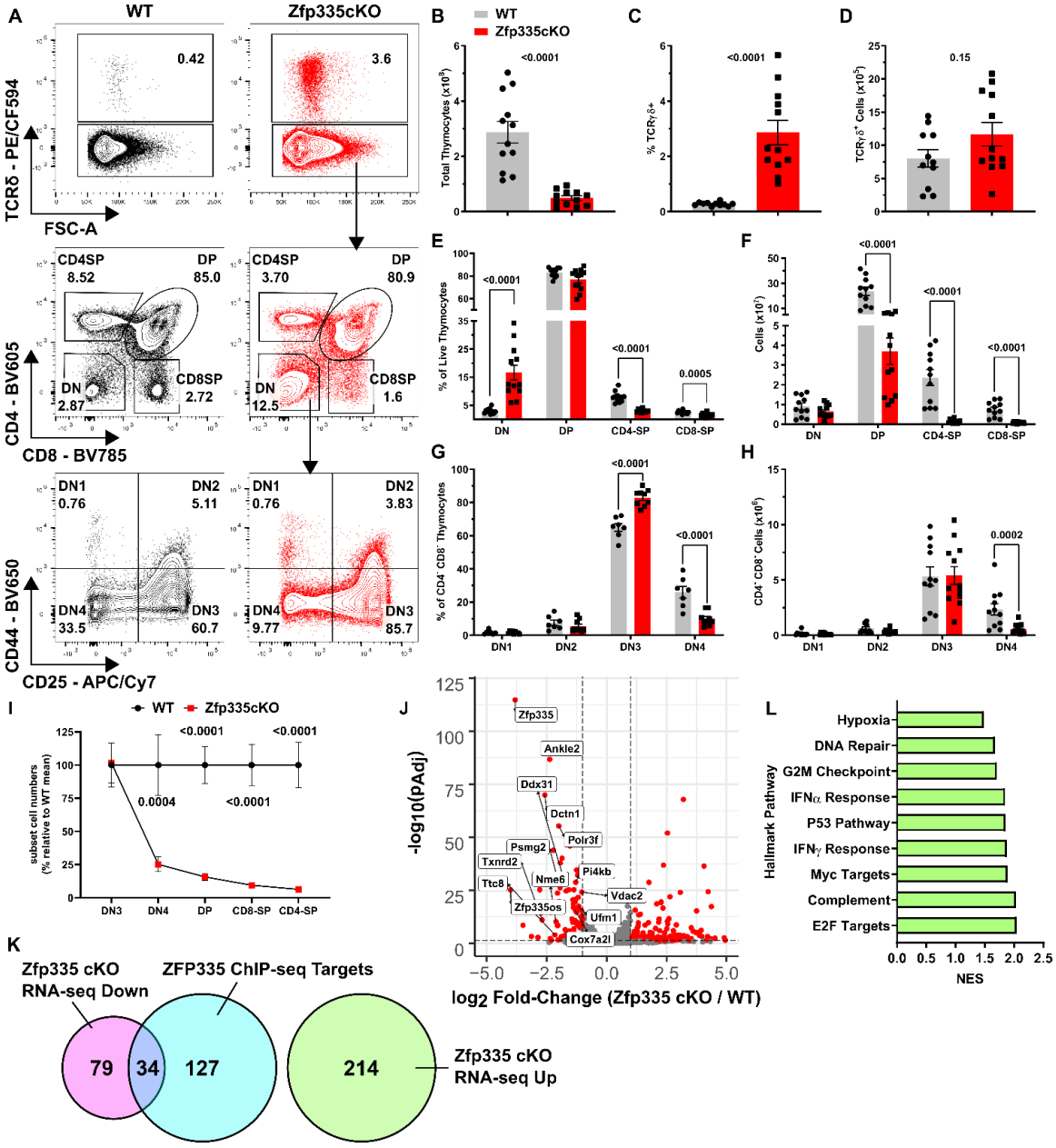

Figure 9: Zfp335 is critical to αβ T cell development

(A) Gating schema for *ex vivo* analysis thymocyte development beginning with live thymocytes (DAPI$^-$ CD90.2$^+$, gating not shown). (B) Total thymic cellularity in WT (Cre-negative) or *Zfp335$^{fl/fl}$ E8$_{III}$-cre* (Zfp335cKO) mice. Total numbers (C) and frequency (D) of TCRγδ$^+$ cells in WT or Zfp335cKO thymuses. Numbers (E) and frequencies (F) of DN, DP, and SP thymocyte subsets in WT or Zfp335cKO thymuses. Numbers (G) and frequencies (H) of early DN1-DN4 thymocyte subsets in WT or Zfp335cKO thymuses. (I)

Relative cells numbers in DN3-SP thymocyte subsets represented as percent of WT mean. (J) Volcano plot of differentially expressed genes between Zfp335cKO and WT by RNA-seq. (K) Overlap between Zfp335 ChIP-seq (GSE58293) and differentially expressed genes in Zfp335cKO and WT DP. (L) Gene Set Enrichment Analysis of differentially expressed genes (K). Positive enrichment scores indicate pathways positively enriched in Zfp335cKO cells. (A-K) Cre-negative WT (n=11) and Zfp335cKO (n=12) 4-5-week-old male and female mice from four independent experiments. *P*-values determined by Two-way ANOVA with *post hoc* Sidak test. (I-K) RNA-seq analysis of *Zfp335*$^{+/+}$ *E8*$_{III}$*-cre* or Zfp335cKO DP thymocytes (n=3 each) of 6-week-old female mice from one experiment. Plots show mean ± sem. Data are compiled from one (J-L) or 5 independent experiments (A-I).

Examination of the peripheral T cell compartment revealed significantly reduced numbers of splenic T cells in Zfp335cKO mice (Figure 10A-G). A previous study identified the hypomorphic *Zfp335*^{bloto} allele as the causative mutation in a unique form of T lymphopenia (Han et al., 2014). Like *Zfp335*^{bloto} mice, we found that peripheral T cells in Zfp335cKO mice were almost exclusively of an effector or memory phenotype (Figure 10H-K) suggesting these mice also exhibit a similar defect in the establishment of the naïve T cell compartment. Together, these findings identify Zfp335 as a key transcription factor regulating T cell development.

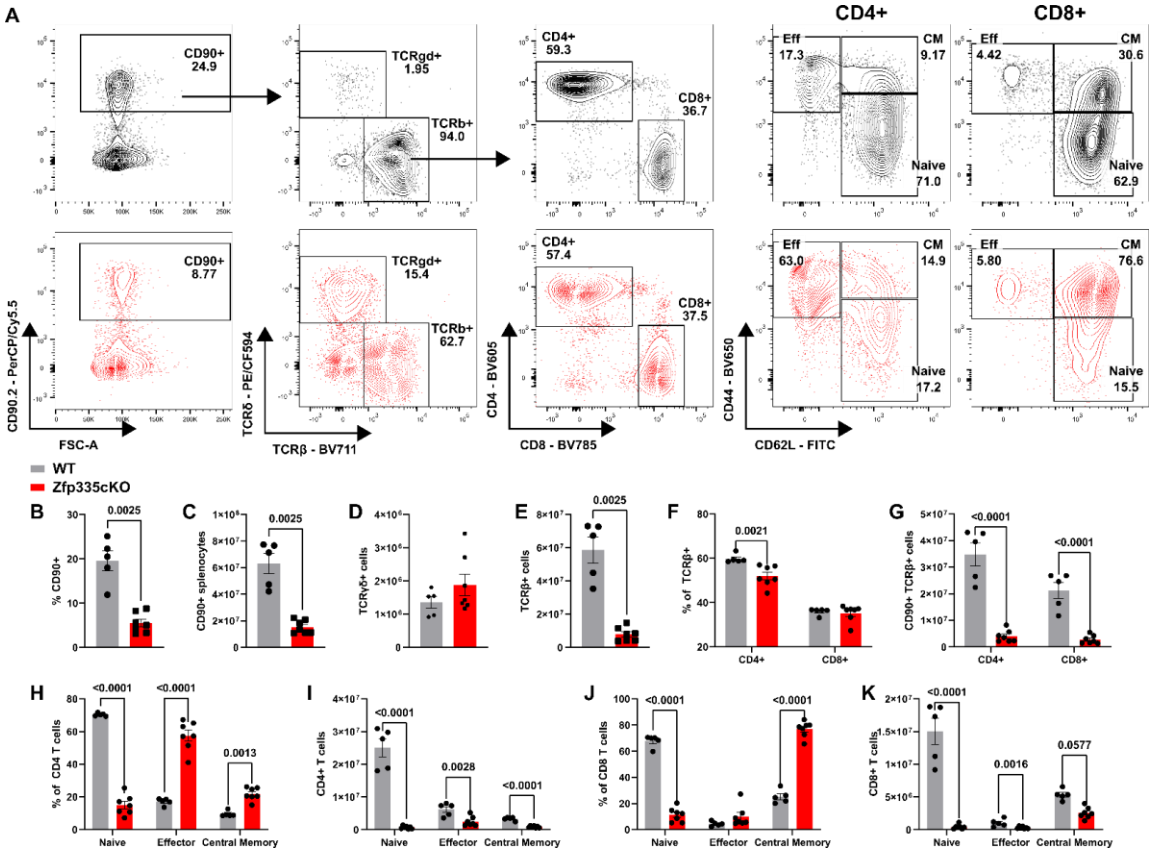

Figure 10: – Zfp335cKO mice exhibit T lymphopenia and reduced peripheral naïve T cells

(A) Gating schema for identification of WT (black) or Zfp335cKO (red) splenic T cell populations beginning with live (DAPI$^-$) splenocytes. Proportion (B) or total numbers (C) of splenic CD90+ cells. Total numbers of TCR$\gamma\delta$+ (D) or TCR$\alpha\beta$+ (E). Proportions (F) and total numbers of CD4+ or CD8+ TCR$\alpha\beta$ cells. Proportions (H, J) and numbers (I, K) of naïve, effector or central memory T cells within the CD4+ or CD8+ compartment. WT (n=5) or Zfp335cKO (n=7) from two separate experiments. *P*-values determined by Mann-Whitney U-test (B-E) or Two-Way ANOVA with *post hoc* Sidak test (F-K). Plots show mean ± sem.

2.2.2 Loss of Zfp335 in DN3 thymocytes does not impair β-selection

Zfp335 deletion results in reduced cell numbers beginning at the DN4 stage, raising the possibility that the inability to rearrange the TCRβ locus could be

responsible. Consequently, we assessed TCRβ rearrangement in DN3 and DN4 thymocytes by intracellular staining. The frequency of icTCRβ$^+$ cells among Zfp335cKO DN3 and DN4 subsets was comparable to that of WT (Figure 11A-C). Therefore, TCRβ rearrangement and subsequent pre-TCR expression are unimpaired in Zfp335cKO mice.

In addition to pre-TCR expression, to successfully traverse the β-selection checkpoint, pre-TCR signals are required for release from cell cycle arrest, survival, and progression to DP (Haks et al., 1999). CD27 surface expression is increased by pre-TCR signals in DN3 thymocytes (Taghon et al., 2006). Zfp335cKO DN3 thymocytes exhibited CD27 upregulation comparable to that of WT (Figure 11D-E) indicating Zfp335-deficiency does not lead to impaired pre-TCR signaling. Together, these results indicate that the observed reduction of DN4 cells in Zfp335cKO mice did not result from failure to produce TCRβ subunits or failure to transduce pre-TCR signals.

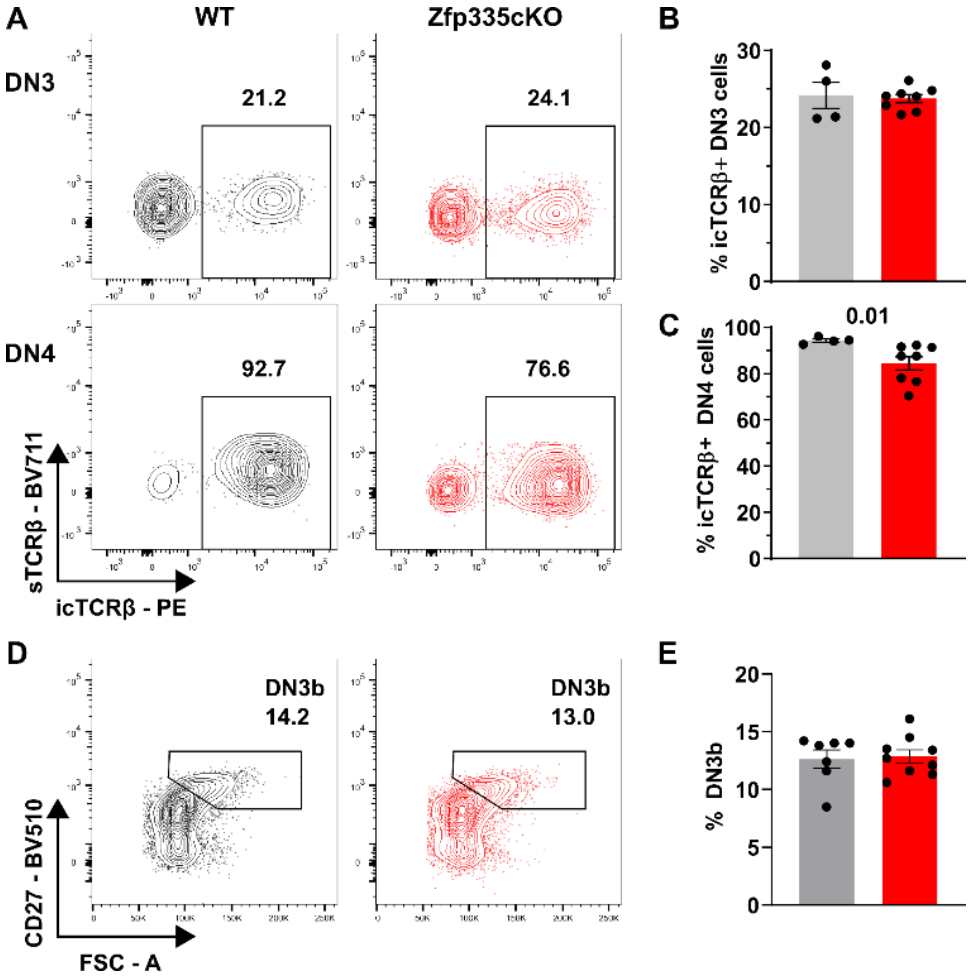

Figure 11: Loss of Zfp335 during DN3 does not impair β-selection

(A) Gating for icTCRβ expression among DN3 (CD90$^+$ TCRδ$^-$ CD4$^-$ CD8$^-$ sTCRβ$^-$ CD44$^-$ CD25$^+$) or DN4 (CD90$^+$ TCRδ$^-$ CD4$^-$ CD8$^-$ sTCRβ$^-$ CD44$^-$ CD25$^-$) thymocytes. Frequency of icTCRβ DN3 (B) or DN4 (C) cells among WT (n=4) or Zfp335cKO (n=8) mice. (D) Flow cytometric gating for identification of WT or Zfp335cKO DN3b thymocytes pre-gated on total DN3 cells. (E) Quantification of DN3b frequency among WT(n=7) or Zfp335cKO (n=9) DN3 thymocytes. *P*-values determined by Two-way ANOVA with *post hoc* Sidak test (B,C) or Mann-Whitney U-Test (E). Plots show mean ± sem.

2.2.3 Zfp335 inhibits apoptosis during the DN-DP transition

Zfp335 deletion during the DN3 stage leads to severe defects in T cell development, likely during the post-β-selection proliferative phase. To determine if Zfp335-deficiency altered either the proliferation or survival of post-β-selection thymocytes, we directly measured these events in OP9-DL1 cultures *in vitro* (Holmes & Zuniga-Pflucker, 2009). Consistent with our *ex vivo* data, Zfp335cKO cells exhibit severely impaired progression to the DP stage (Figure 12A-B). Zfp335cKO cells exhibited modestly reduced proliferation compared to controls (Figure 12C-D). In contrast, Zfp335cKO cells underwent substantially increased rates of apoptosis (Figure 12E-F). Importantly, proliferation tracking (Figure 12G) and assessment of developmental progression (Figure 12H) of apoptotic mutant cells demonstrate they have undergone cell division and largely remain DN.

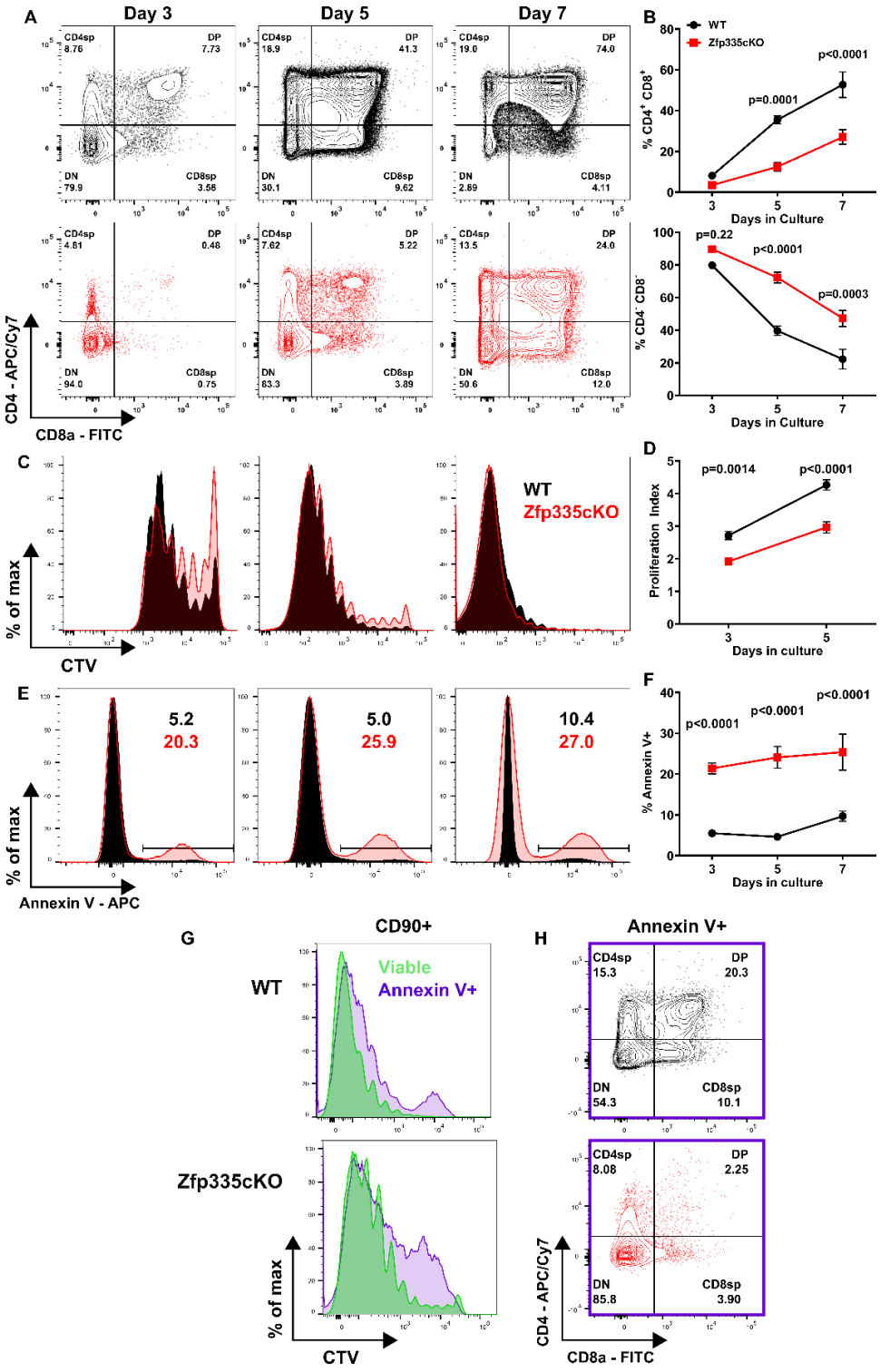

Figure 12: Zfp335cKO DN4 thymocytes undergo increased rates of apoptosis

(A-B) Assessment of developmental progression throughout OP9-DL1 culture. Proliferation assessment (C-D) by Cell Trace Violet (CTV) dilution and apoptosis analysis (E-F) based on Annexin V binding at day3, 5 or 7 of culture. (G) Representative comparison of CTV dilution between Annexin V^+ and viable ($DAPI^-$ Annexin V^-) cells on day 5 of culture. (H) Representative CD4 vs CD8 expression among Annexin V^+ cells on day 5 of culture from (G). n=6 WT or n=5 Zfp335cKO from three independent experiments. *P*-values determined using Two-way Repeated Measures ANOVA with *post hoc* Sidak Test. Plots show mean ± sem.

Next, we sought to determine whether loss of Zfp335 also leads to increased rates of cell death among thymocyte populations other than post-β-selection proliferating cells. Zfp335cKO DP cells generated in OP9-DL1 culture exhibited increased rates of apoptosis compared to controls (Figure 13A). To determine if the same is true *in vivo* we performed TCR*α* repertoire analysis using DP thymocytes. Increased rates of Zfp335cKO DP thymocyte apoptosis would yield skewed *Trac* gene usage towards proximal V and J segments (Carico et al., 2017). However, our analyses showed no such skewing (Figure 13B-C). Furthermore, similar rates of positive selection among DP cells from WT or Zfp335cKO mice were observed (Figure 13D-E). Together, these data suggest that Zfp335cKO cells are dying during the post-β-selection proliferative phase and that Zfp335 activity promotes the survival of DN4 thymocytes.

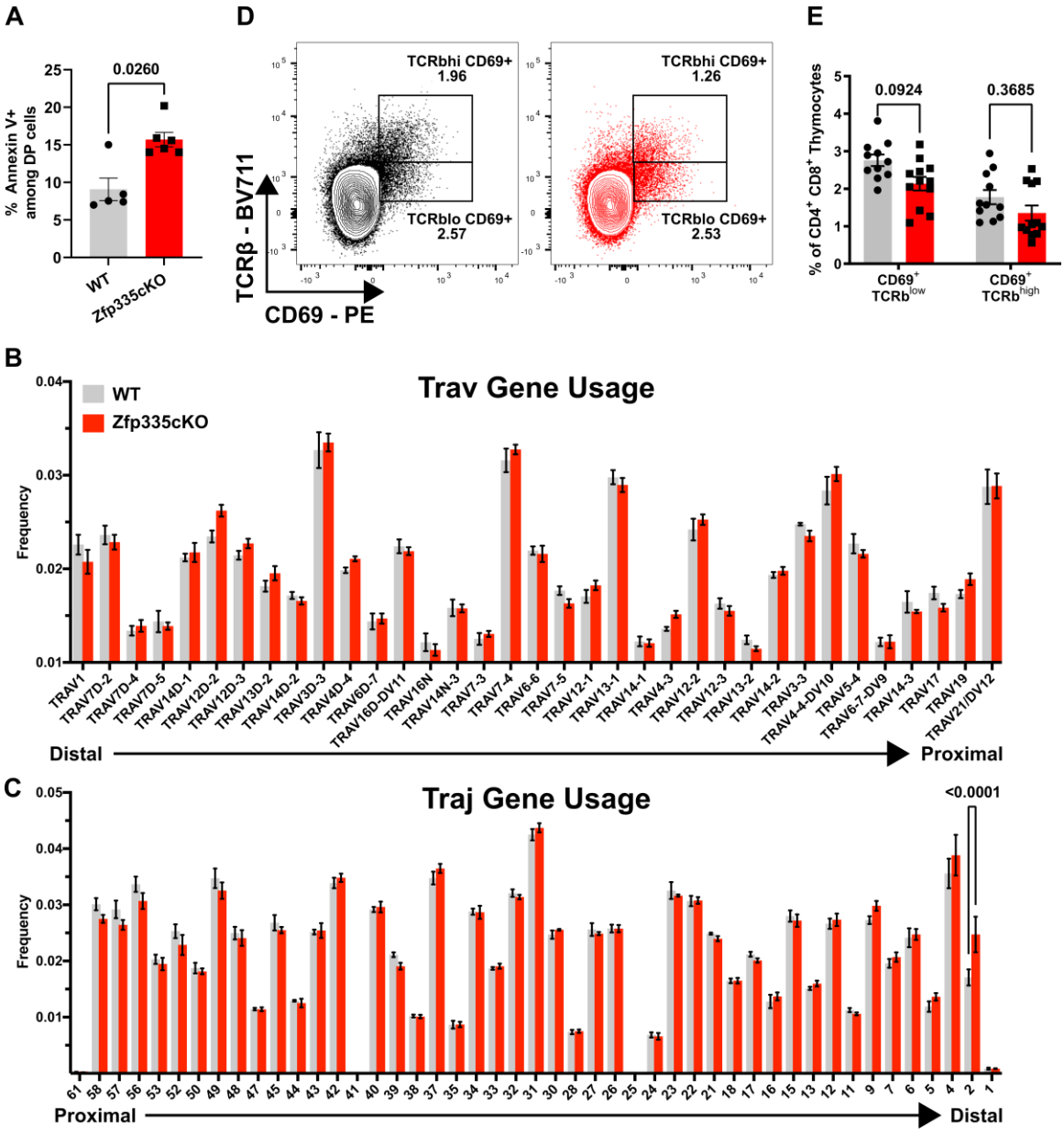

Figure 13: Loss of Zfp335 does not impair DP thymocyte survival

(A) Frequency of Annexin V+ cells among DP thymocytes after culturing DN3/4 thymocytes on OP9-DL1 cells for 3 days (n=5 WT or n=6 Zfp335cKO). Frequency of Trav (B) and Traj (C) gene segment usage in functional *Trac* gene rearrangments in WT (n=3) or Zfp335cKO (n=4) DP thymocytes *ex vivo*. Representative gating (D) and quantification (E) of positive selection among DP thymocytes based on CD69 and TCRβ expression in

WT (n=11) or Zfp335cKO (n=12) . *P*-values determined by Mann-Whitney U-Test (A) or Two-way ANOVA with *post hoc* Sidak test (B-E). Plots show mean ± sem.

2.2.4 Ectopic expression of Bcl2 rescues the developmental defect resulting from loss of Zfp335

Our RNA-seq studies revealed that Zfp335cKO thymocytes exhibit increased expression of the pro-apoptotic Bcl2-family members PUMA (*Bbc3*), NOXA (*Pmaip1*) and *Bax* (Figure 14A), suggesting that these factors may be responsible for the observed increase in apoptosis among Zfp335cKO thymocytes. The function of these proteins can be antagonized by ectopic expression Bcl2. Thus, we asked whether Bcl2 overexpression could rescue Zfp335cKO thymocyte apoptosis. WT or Zfp335cKO DN3/4 thymocytes were transduced with control or Bcl2-expressing retroviruses then grown in the OP9-DL1 culture system. Bcl2 overexpression significantly reduced apoptosis in Zfp335cKO cells, indicating the induction of pro-apoptotic Bcl2 family members was at least partially responsible for the observed increase in apoptosis in Zfp335-deficient thymocytes (Figure 14B-C).

We next sought to test the ability of Bcl2 overexpression to rescue Zfp335-deficient cells from apoptosis *in vivo* through generating Bcl2 conditional transgenic mice (Figure 14D). Intracellular staining revealed that $Zfp335^{fl/fl}$ $R26^{LSL\text{-}Bcl2\text{-}Tg}$ $E8_{III}$-cre (Zfp335cKO Bcl2-Tg) thymocytes exhibited increased Bcl2 protein expression relative to WT (Figure 14E). Phenotypic analysis demonstrated that ectopic Bcl2 expression was able to fully rescue the early developmental defects observed in Zfp335-deficient mice,

restoring traversal of the β-selection checkpoint, transition to the DP stage, and total thymic cellularity (Figure 14F-M).

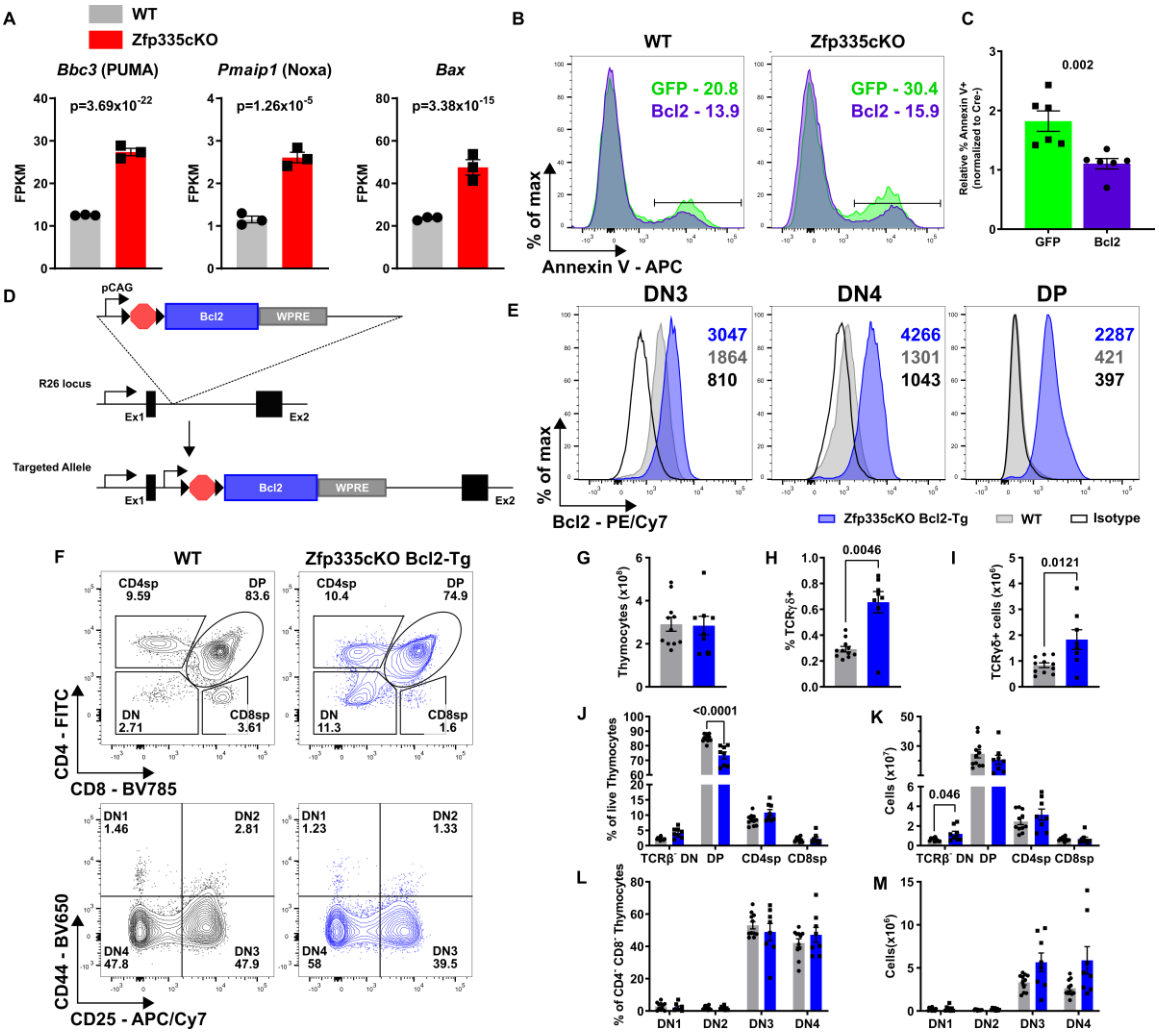

Figure 14: Bcl2 overexpression rescues Zfp335-deficient thymocytes from apoptosis

(A) Expression of pro-apoptotic Bcl2 family genes *Bbc3*, *Pmaip1*, or *Bax* from RNA-seq of control or Zfp335cKO DP thymocytes. Representative gating (B) and quantification (C) of apoptosis among Zfp335cKO thymocytes transduced with Bcl2 or GFP RV after 5 days of OP9-DL1 culture (n=5). (D) Schematic diagram of Rosa26$^{LSL\text{-}Bcl2}$ transgene generation. (E) Representative expression of isotype control (open black) or Bcl2 in WT (grey) or *Zfp335*$^{fl/fl}$ *R26*$^{LSL\text{-}Bcl2}$ *E8*$_{III}$*-cre* (blue) DN3, DN4 or DP thymocytes.

Numbers in histograms indicate representative geometric MFI of Bcl2 expression. (F) Gating for identification of thymocyte subsets in WT WT (grey) or *Zfp335*$^{fl/fl}$ *R26*$^{LSL\text{-}Bcl2}$ *E8*$_{III}$*-cre* (blue) mice. DN1-4 gating pre-gated on TCRβ^{-}. (G) Total thymocyte numbers. Total numbers (H) and proportions (I) of TCRδ^{+} cells. Frequencies (J) and total numbers (K) of DN, DP, CD4-SP and CD8-SP thymocytes. Frequencies (L) and total numbers (M) of DN1-DN4 thymocytes. (F-M) n=11 WT or n=8 *Zfp335*$^{fl/fl}$ *R26*$^{LSL\text{-}Bcl2}$ *E8*$_{III}$*-cre*. Data compiled from one (A), two (B-C) or five (D-L) independent experiments. P-values determined by Wald test (A), Mann-Whitney U-test (C) or Two-way ANOVA with *post hoc* Sidak's test (H-M). Plots show mean ± sem.

Consistent with studies of *Zfp335*^{bloto} mice (Han et al., 2014), Bcl2 overexpression failed to rescue the impairment in final single positive thymocyte maturation (Figure 15A-C) or peripheral T cell compartment numbers (Figure 15D-E) and effector status (Figure 15F-H). Taken together, these data suggest that the early impairment of thymocyte development following loss of Zfp335 expression is due to increased rates of DN4 apoptosis driven by pro-apoptotic Bcl2-family members. However, our *in vivo* studies also revealed an additional, Bcl2-independent late block in terminal T cell differentiation within the thymus.

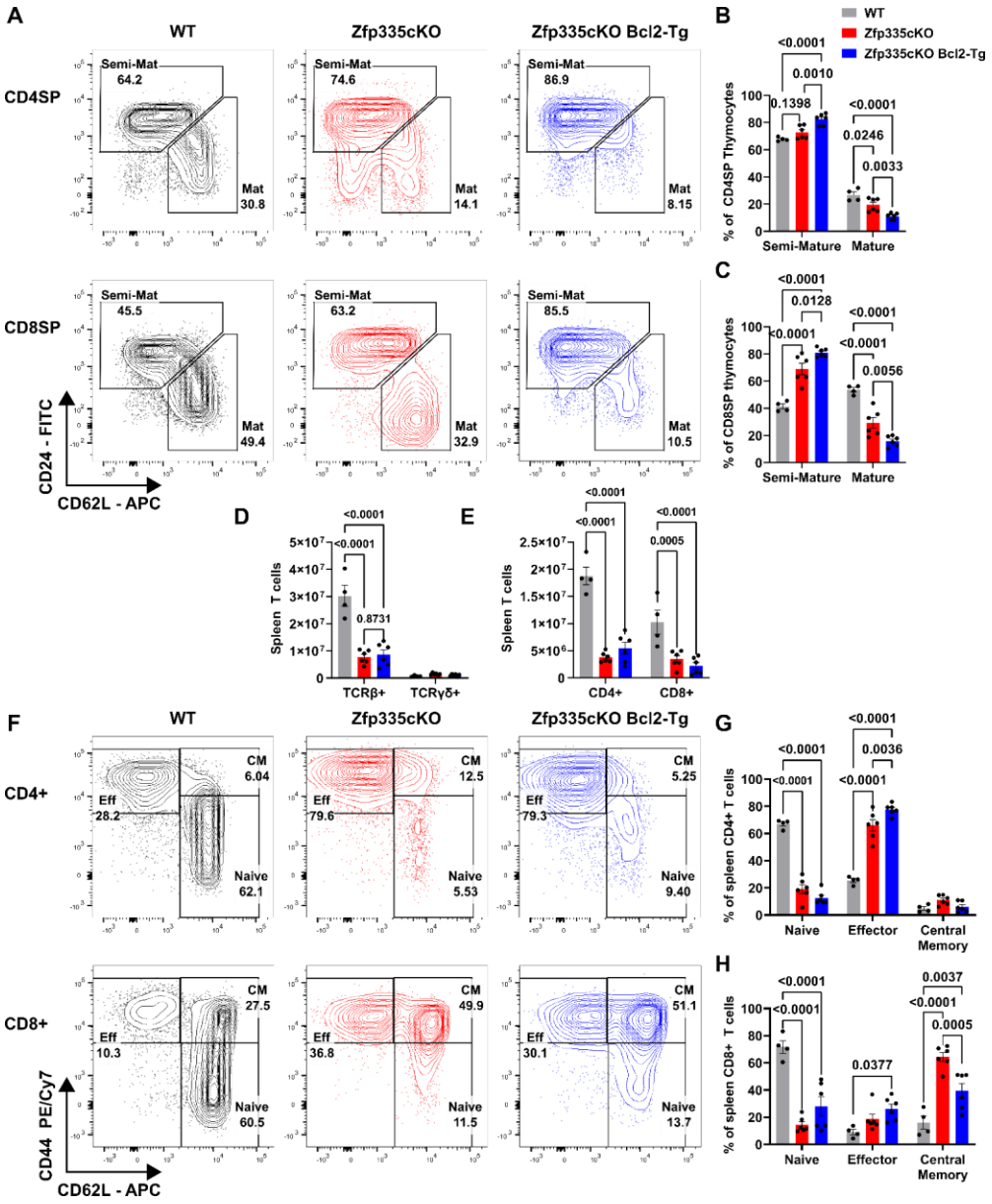

Figure 15: Bcl2 overexpression fails to rescue thymic differentiation defect and peripheral T lymphopenia in Zfp335-deficient mice

Representative gating (A) and quantification of CD4SP (B) or CD8SP (C) thymic maturation. Total splenic TCRβ and TCRγδ (D) T cells. (E) Quantification of total splenic CD4+ or CD8+ TCRβ+ T cells. Representative gating (F) and quantification of splenic CD4+ (G) or CD8+ (H) T cell effector status. n=4 WT, n=6 Zfp335cKO, n=6 Zfp335cKO Bcl2-Tg. Data are compiled from three independent experiments. *P*-values determined by Two-Way ANOVA with *post hoc* Sidak Test. Plots show mean ± sem.

2.2.5 Defining the 'true' DN4 thymocyte population at the single cell level

The DN4 stage of T cell development remains poorly understood and, as a result, poorly defined. DN4 cells are identified by lack of expression of identifying markers associated with any other thymocyte subset. Based on these criteria, it is possible that DN4 cells defined by marker exclusion may not be homogenous. To assess whether there is any heterogeneity in the DN4 compartment exacerbated by Zfp335-deficiency, we performed scRNA-seq of phenotypically defined DN4 cells. After quality control, libraries yielded transcriptome data for 6,537 or 5,392 high-quality cells from WT or Zfp335cKO samples, respectively.

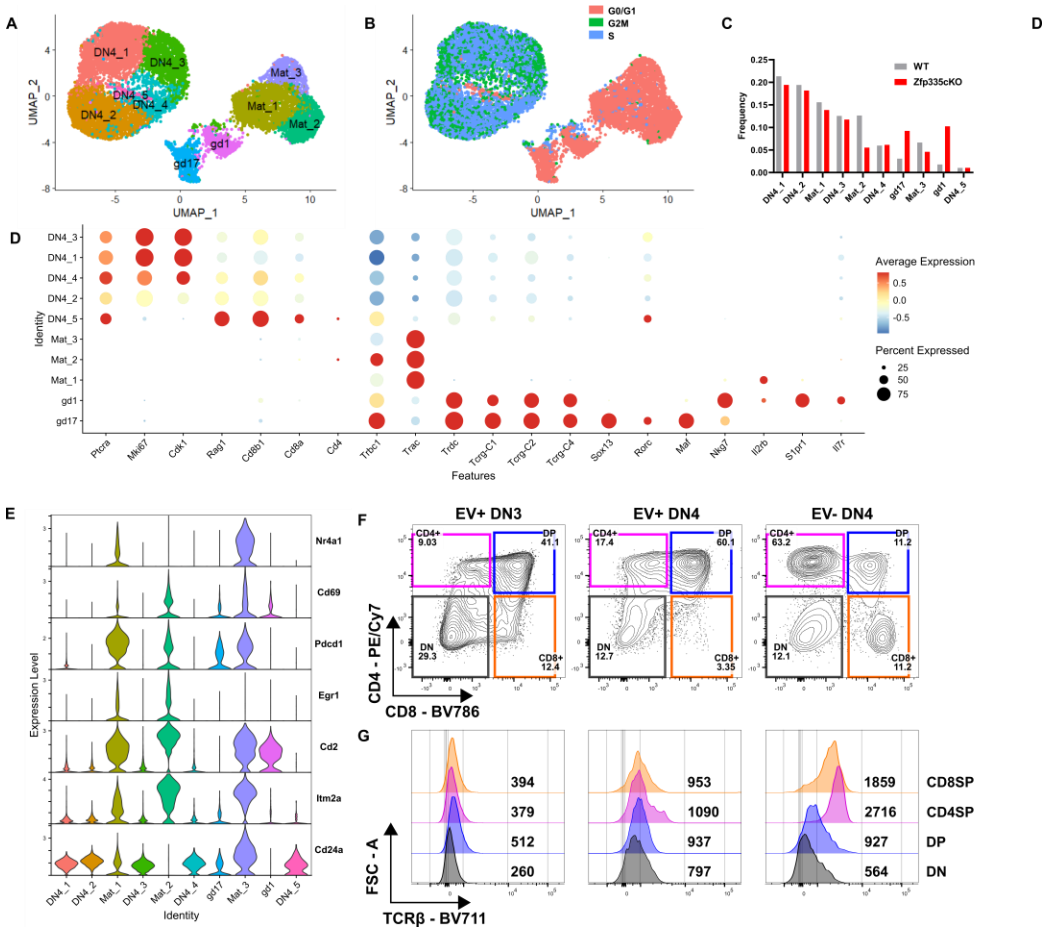

Figure 16: Defining the 'true' DN4 thymocyte population at the single cell level

(A) UMAP projection and identification of 10 clusters identified in full scRNA-seq dataset. (B) UMAP colored by cell cycle phase. Blue or green identify actively cycling cells. (C) Frequency distributions for WT (n=6357) and Zfp335cKO (n=5392) cells across the ten clusters. (D) Dot plot of key cell type-defining genes. (E) Violin plots of positive selection signature genes in thymocytes (Mingueneau et al. 2013). (F) Representative gating for CD4 vs. CD8 expression on day 3 of OP9-DL1 cultures seeded with WT Thy1.1 retrovirus transduced (EV+) DN3 or DN4 cells or non-transduced (EV-) DN4 cells. (G) Representative TCRβ expression among DN, DP, CD4SP or CD8SP cells from (F). Numbers indicate geometric MFI of TCRβ expression. (F-G) Data representative of two independent experiments.

We identified 10 unique cell clusters (Figure 16A-C). Five clusters were largely cycling cells (DN4_1-5; Figure 16A-B) uniquely expressing *Ptcra* (pre-Tα) and proliferation associated genes (*Mki67, Cdk1*) (Figure 16D), representing bona fide DN4 cells. Three clusters (Mat_1-3) expressed high levels of *Trac and Trbc1* transcripts (Figure 16D). Two additional clusters (gd17 and gd1) of γδ T cells were identified. gd17 cells express high levels of *Sox13*, *Rorc* and *Maf*, features of γδ17 while gd1 express *Nkg7*, *Il2rb*, *S1pr1* and *Il7r* associated with cytotoxic γδ T cells (Figure 16D). Based on this clustering, Zfp335-deficiency led to substantial proportional increases and decreases in the γδ T cell clusters and Mat_2 cluster relative to WT control, respectively (Figure 16C). Consistent with our bulk RNA-seq we found increased IFNα and IFNγ signaling activity in Zfp335cKO Mat and the γδ T cell clusters (Figure 17A-B).

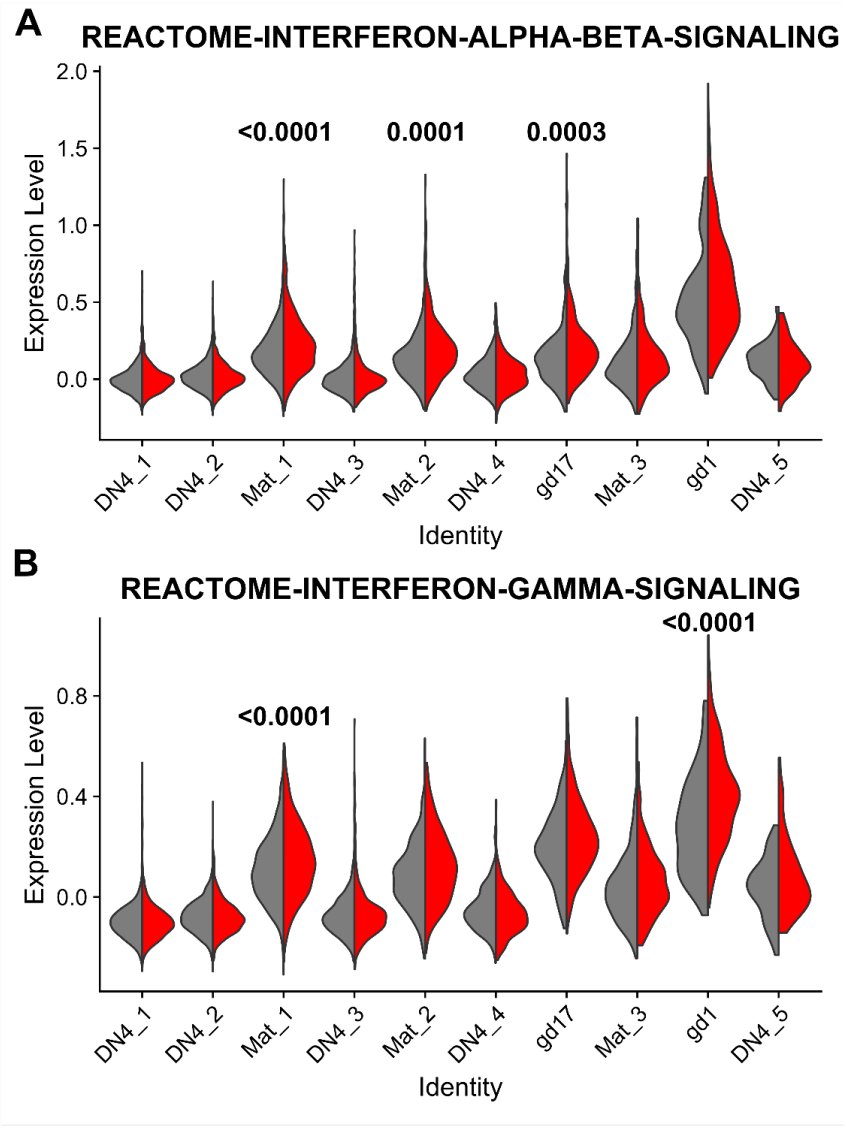

Figure 17: Loss of Zfp335 promotes increased type I and II interferon signaling in γδ and maturing αβ thymocytes

Violin plots showing IFN-I (A) or IFNγ (B) signaling scores for each cluster separated by genotype (WT is grey, Zfp335cKO is red). p-values shown are the adjusted p-values determined by Wilcoxon Rank Sum tests between WT and Zfp335cKO cells within each cluster.

We were surprised to find a large proportion of phenotypically defined DN4 thymocytes expressing *Trac* transcripts and sought to define these populations.

Consistent with their lack of surface CD4 or CD8 these cells uniformly lacked *Cd4*, *Cd8a* and *Cd8b1* transcripts (Figure 16D). We hypothesized that these cells may represent post-positive selection thymocytes that transiently down-regulated surface TCR, CD4 and CD8 expression. Consistent with our hypothesis, we found these cells express high levels of *Nr4a1*, *Cd69*, *Pdcd1*, *Egr1*, *Cd2*, and *Itm2a,* signature genes of positive selection (Mingueneau et al., 2013) (Figure 16E). Based on this profile we define cells from these clusters as maturing αβ T cells. Additionally, we found that Mat_2 cells lack expression of CD24 (Figure 16E) suggesting that this population may represent mature thymocytes explaining the selective reduction in proportions of these cells in Zfp335cKO samples.

Importantly, most cells associated with the maturing αβ or γδ T cell clusters were non-cycling (Figure 16B), and therefore, not 'true' DN4 cells. Retroviral transduction depends on cell cycling (Miller et al., 1990). Therefore, we determined whether 'true' DN4 cells could be separated from contaminating populations *ex vivo* with retroviruses. Virally transduced or non-transduced DN4 cells were placed in OP9-DL1 culture. Non-transduced DN4 cells preferentially give rise to single-positive cells expressing high levels of surface TCR, whereas, transduced DN4 cells become DP (Figure 16F-G). Since OP9-DL1 cells are unable to support positive selection, we conclude that these non-transduced DN4 cells are post-positive selection cells transitioning to SP. Together, these results demonstrate that the phenotypically defined

DN4 compartment is heterogenous and establishes retroviral transduction as a method to isolate DN4 cells for *in vitro* analysis.

2.2.6 Ankle2 is a critical Zfp335-regulated gene required for survival of DN4 thymocytes

Next, we focused our scRNA-seq analyses on determining the transcriptional changes in DN4 cells resulting from loss of Zfp335. Maturing $\alpha\beta$ and $\gamma\delta$ cells were removed leaving only 'true' DN4 cells. Based on recombination kinetics (Figure 8D-E) not all Zfp335cKO DN4 cells have undergone deletion. *Zfp335* expression could not reliably delineate mutant from non-mutant cells due to low detection rate (8% of Zfp335cKO vs 17.7% of WT cells). To identify true mutant DN4 cells in our dataset, we assessed transcription factor activity using gene set scores calculated for each cell based on the expression of the Zfp335 ChIP-seq target genes down-regulated in mutant DP cells (Figure 9J-K). Zfp335cKO cells exhibited a bimodal distribution for the gene set. Using established methods (Trang et al., 2015), cutoff values were determined for the distribution and cells falling below this threshold were considered true mutants (Figure 18A). Cutoffs were confirmed by differential expression analysis between WT and Zfp335cKO targets high or Zfp335cKO targets low cells. Compared to WT, Zfp335cKO targets low cells exhibited differential expression of 80 genes (60 down, 20 up; Figure 18B) whereas Zfp335cKO targets high cells only exhibited differential expression of 7 genes (5 down, 2 up; Figure 18C).

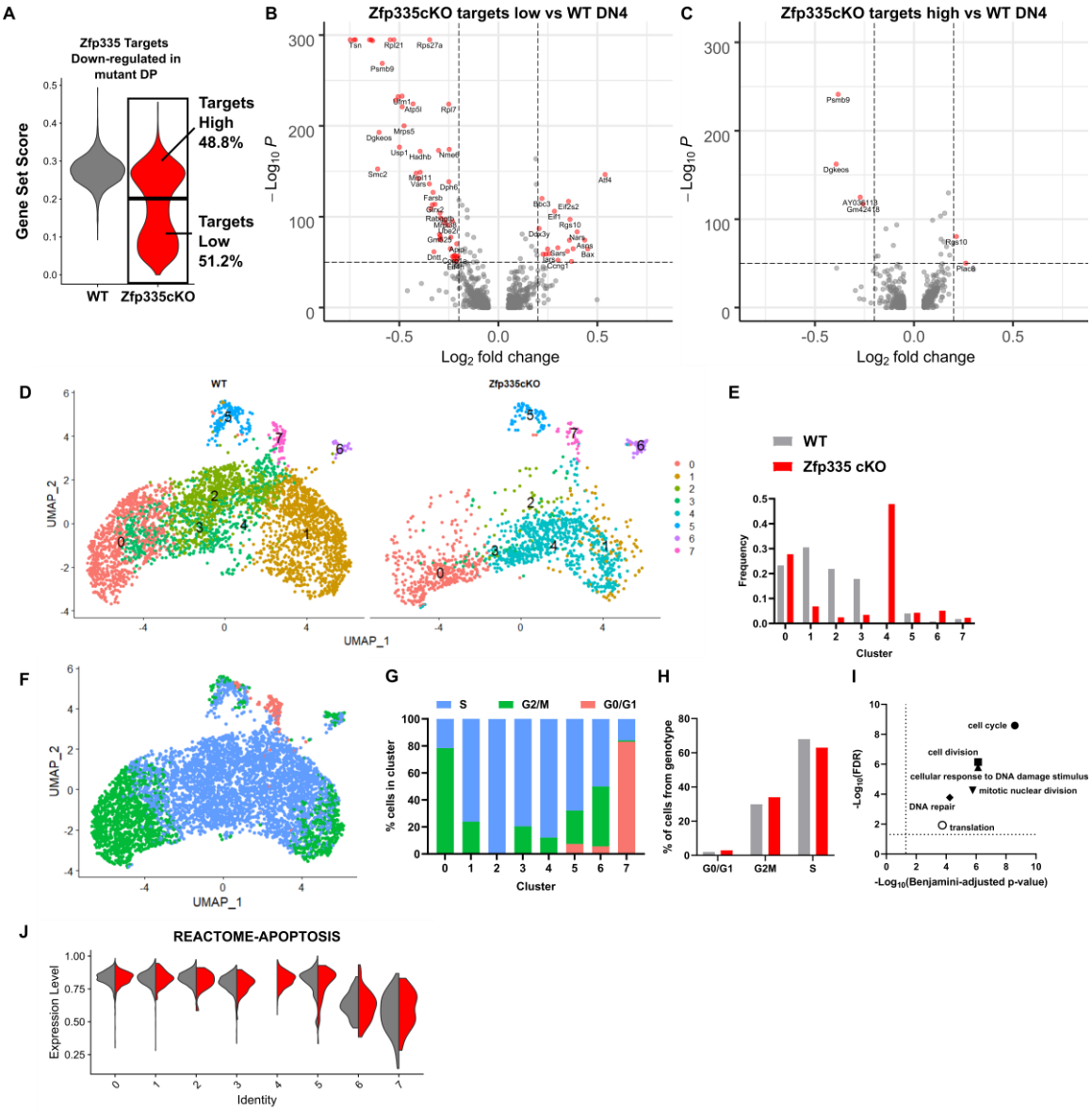

Figure 18: scRNA-seq identifies 'true' Zfp335 mutant DN4 cells

(A) Violin plot of gene set score for Zfp335 target genes down-regulated in mutant DP thymocytes (Figure 1L-M) and cutoff value used to identify 'true' Zfp335 mutant cells with low target score (lower box) and non-mutant cells (upper box). Volcano plots of differentially expressed genes between Zfp335cKO targets low (B) or Zfp335cKO targets high (C) cells compared with WT control. (D) UMAP projections colored by cluster and separated by genotype for WT and true Zfp335 mutant DN4 cells. (E) Frequency of cells found within each cluster. UMAP projection (F) and quantification of cell cycle phase for each cluster (G). (H) Quantification of distribution of cell cycle

phase by genotype. (I) GO analysis of top 25 cluster defining genes for each cluster (dashed lines indicate significance cutoff of $p<0.05$ and FDR<0.05. (J) Violin plot of Reactome-Apoptosis gene signature for WT (grey) or 'true' mutant Zfp335cKO (red) DN4 cells. *P*-values determined by Wilcoxon Rank Sum test (B-C) or Fischer's Exact test with Benjamini-Hochenberg correction (I).

Zfp335cKO cells above the threshold were considered non-mutant, removed and the remaining cells were then reanalyzed identifying 8 unique clusters (Figure 18D). WT and mutant cells were distributed across each cluster. C1-3 were enriched for WT whereas C4 was almost entirely mutant cells (Figure 18E). Despite regression of standard cell cycle-associated genes, clustering was largely dictated by cell cycle (Figure 18F-I). We observed no differences in cell cycle phase distributions between WT and mutant (Figure 18H). Therefore, we chose to compare WT and mutant DN4 cells based on genotype (Figure 19A). Among the 60 down-regulated genes in mutant DN4 cells, 44 are Zfp335 targets by ChIP-seq (Figure 9B)(Han et al., 2014). We hypothesized that reduced expression of one or more of these genes was responsible for the increased rates of apoptosis observed in mutant DN4 cells. Thus, we examined expression of the 12 Zfp335 target genes with experimental evidence demonstrating a negative regulatory role in cell death (Figure 19C-D). Four exhibited reduced expression in mutant DN4 thymocytes (Figure 19C). Examination of expression frequency identified *Ankle2* as having the greatest reduction in percent of mutant cells expression (Figure 19E). Interestingly, pathway analysis did not show any enrichment for apoptosis among Zfp335cKO mutant cells (Figure 18J).

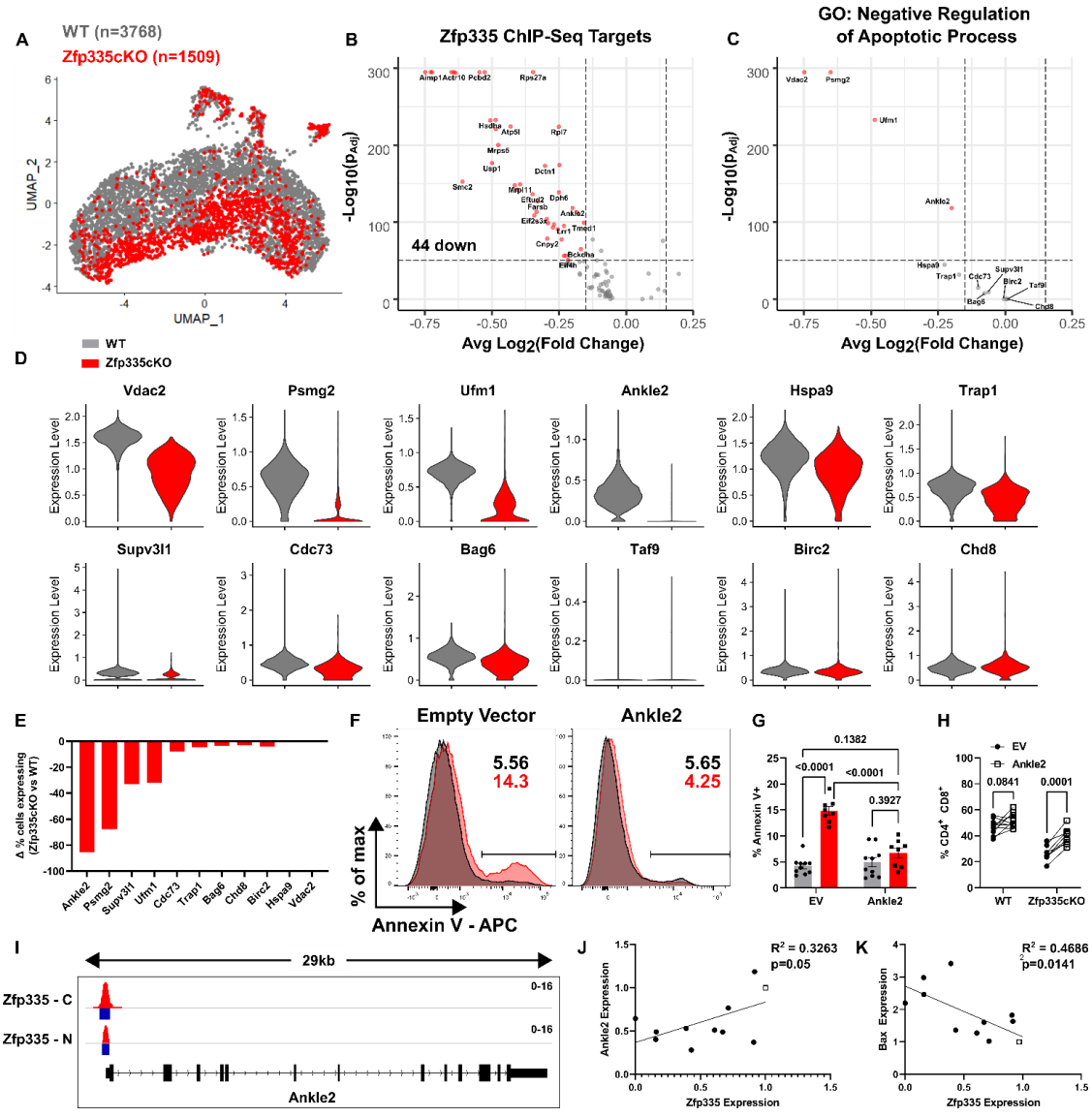

Figure 19: scRNA-seq identifies Ankle2 as a critical Zfp335-regulated gene controlling survival of DN4 thymocytes

(A) UMAP projection of WT and 'true' Zfp335 mutant DN4 cells colored by genotype. Volcano plot of all differentially expressed Zfp335 target genes (B) or those experimentally shown negatively regulate apoptotic processes (C) between Zfp335 mutant and WT cells. (D) Violin plots of anti-apoptotic Zfp335 target gene expression between Zfp335 mutant and WT DN4 cells. (E) Differential proportions of Zfp335 mutant cells expressing anti-apoptotic genes from (C,D) compared to WT cells based on dropout-imputation. Representative gating (F) and quantification of apoptosis (G) or DP cell frequency (H) for EV or Ankle2 retrovirus transduced WT (n=10) or Zfp335cKO

(n=8) DN3 thymocytes cultured on OP9-DL1 cells for 3 days. (I) Zfp335 ChIP-seq track of *Ankle2* locus in WT thymocytes (Zfp335-C or Zfp335-N antibodies, GSE58293). Blue boxes indicate significant binding peaks. Correlation between Ankle2 (J) or Bax (K) and Zfp335 expression in Scid.adh.2c2.SunTag CRISPRi cells expressing non-targeting (open squares, n=2) or Zfp335-targeting (closed circles, n=10) gRNAs. Data are compiled from one (A-E), two (J-K) or three (F-H) independent experiments. *P*-values determined by Wilcoxon Rank Sum test (B-C), two-way ANOVA with *post hoc* Tukey's test for multiple comparisons (G), repeated measures ANOVA with Sidak's test (H) or simple linear regression (J-K). Plots show mean ± sem.

Ankle2 encodes an ER-restricted ankyrin repeat and LEM domain-containing protein (Asencio et al., 2012). *Ankle2* was recently identified as a critical Zfp335-regulated factor in the establishment of the naïve T cell (Han et al., 2014). Therefore, we tested whether *Ankle2* overexpression could rescue Zfp335cKO apoptosis. WT or Zfp335cKO DN3 thymocytes were transduced with EV or Ankle2 retrovirus and cultured on OP9-DL1 cells. Importantly, *Ankle2* overexpression was able to fully rescue Zfp335-deficient thymocytes from increased rates of apoptosis (Figure 19F-G). Moreover, *Ankle2* overexpression led to significantly increased proportions of DP cells among Zfp335cKO samples (Figure 19H).

Next, we sought to confirm that Ankle2 expression is directly regulated by Zfp335 in pre-T cells. Analysis of published ChIP-seq data showed Zfp335 binds the proximal promoter of *Ankle2* in thymocytes (Figure 19I). Like *Zfp335*, *Ankle2* is ubiquitously expressed throughout T cell development (Mingueneau et al., 2013). To examine the relationship between *Zfp335* and *Ankle2* expression we utilize the DN4-like mouse thymocyte cell line *Scid.adh.2c2* (Carleton et al., 1999) for CRISPR-based

transcriptional inhibition (CRISPRi) studies (Tanenbaum et al., 2014). These cells were transduced with retroviruses expressing *Zfp335* promoter-targeting gRNA and anti-GCN4scFv-sfGFP-KRAB fusion construct. *Zfp335*-targeted cells exhibited reduced *Ankle2* expression proportional to the efficiency of *Zfp335* knock-down (KD) (Figure 19J). Additionally, *Zfp335*KD resulted in increased expression of *Bax* like that observed in Zfp335cKO thymocytes (Fig. 19K). Together, these results demonstrate a direct relationship between *Zfp335* and *Ankle2* expression in developing T cells and suggest reduced *Ankle2* expression resulting from loss of Zfp335 drives DN4 apoptosis in Zfp335cKO mice.

2.2.7 Loss of Zfp335 disrupts nuclear envelope architecture and promotes cGAS/STING signaling

Next, we sought to determine the mechanism driving this increase in cell death resulting from reduced *Ankle2* expression. Ankle2 has previously been shown to control nuclear envelope (NE) reassembly and integrity following mitosis through regulation of Barrier to Autointegration Factor 1 (Banf1 or Baf) phosphorylation (Asencio et al., 2012). As previously reported (Kaufmann et al., 2016), disruption of *ANKLE2* or *BANF1* expression in Hela cells led to severe disruptions in NE architecture (Figure 20A).

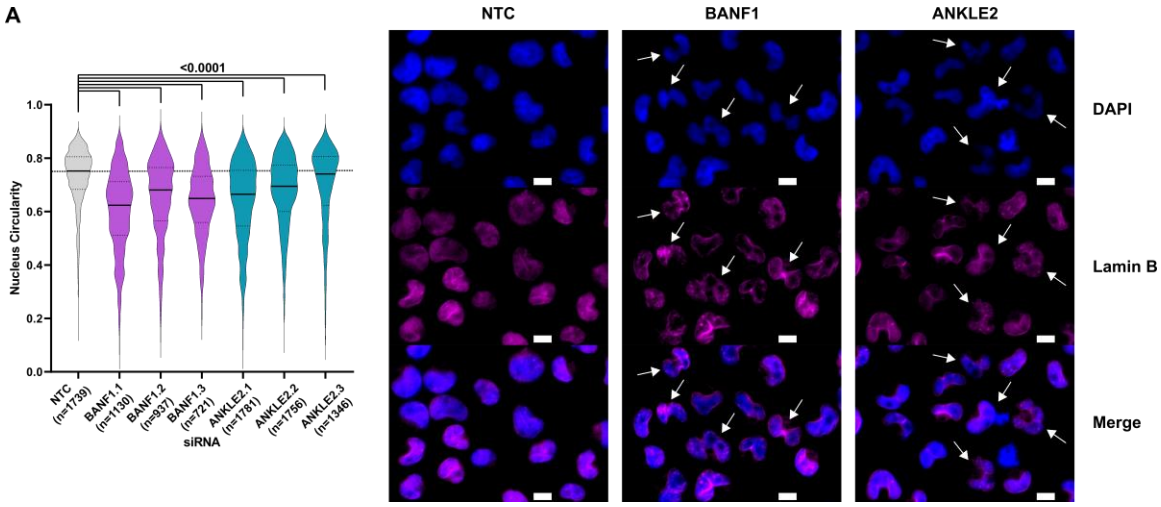

Figure 20: Disruption of *ANKLE2* or *BANF1* expression leads to abnormal NE architecture in human cells

(Left) Quantification of nuclei circularity in Hela cells 48 hours after transfection with non-targeting (NTC), *BANF1* or *ANKLE2*-targeting siRNA. (Right) Representative images of nuclear structure. Arrows indicate cells with abnormal nuclear structure.

To determine if this mechanism applies to Zfp335-deficient DN4 thymocytes we first examined the phosphorylation statues of Baf. Consistent with reduced *Ankle2* expression we observed significant increases in Baf phosphorylation among Zfp335cKO DN4 thymocytes (Figure 21A-C). Next, we sought to determine if loss of Zfp335 results in alterations to the NE *in vivo*. Indeed, Zfp335cKO DN4 thymocytes exhibit significantly altered NE architecture characterized by diffuse Lamin B1 throughout the cytosol, reduced DAPI signal possibly the result of loss of nucleocytosoplasmic compartmentalization and reduced nuclear sphericity (Figure 21D-G). To test whether the observed NE defects result in loss of nucleocytoplasmic compartmentalization we measured the abundance of cytosolic dsDNA in WT or Zfp335cKO thymocytes by

confocal microscopy. Consistent with altered NE architecture, we observed significantly increased abundance of cytoplasmic dsDNA in Zfp335cKO compared to WT thymocytes (Figure 21H-I). Together, these data confirm that loss of Zfp335 leads to significantly altered NE architecture and accumulation of cytoplasmic dsDNA consistent with dysregulation of Ankle2/Baf-mediated NE reassembly and maintenance.

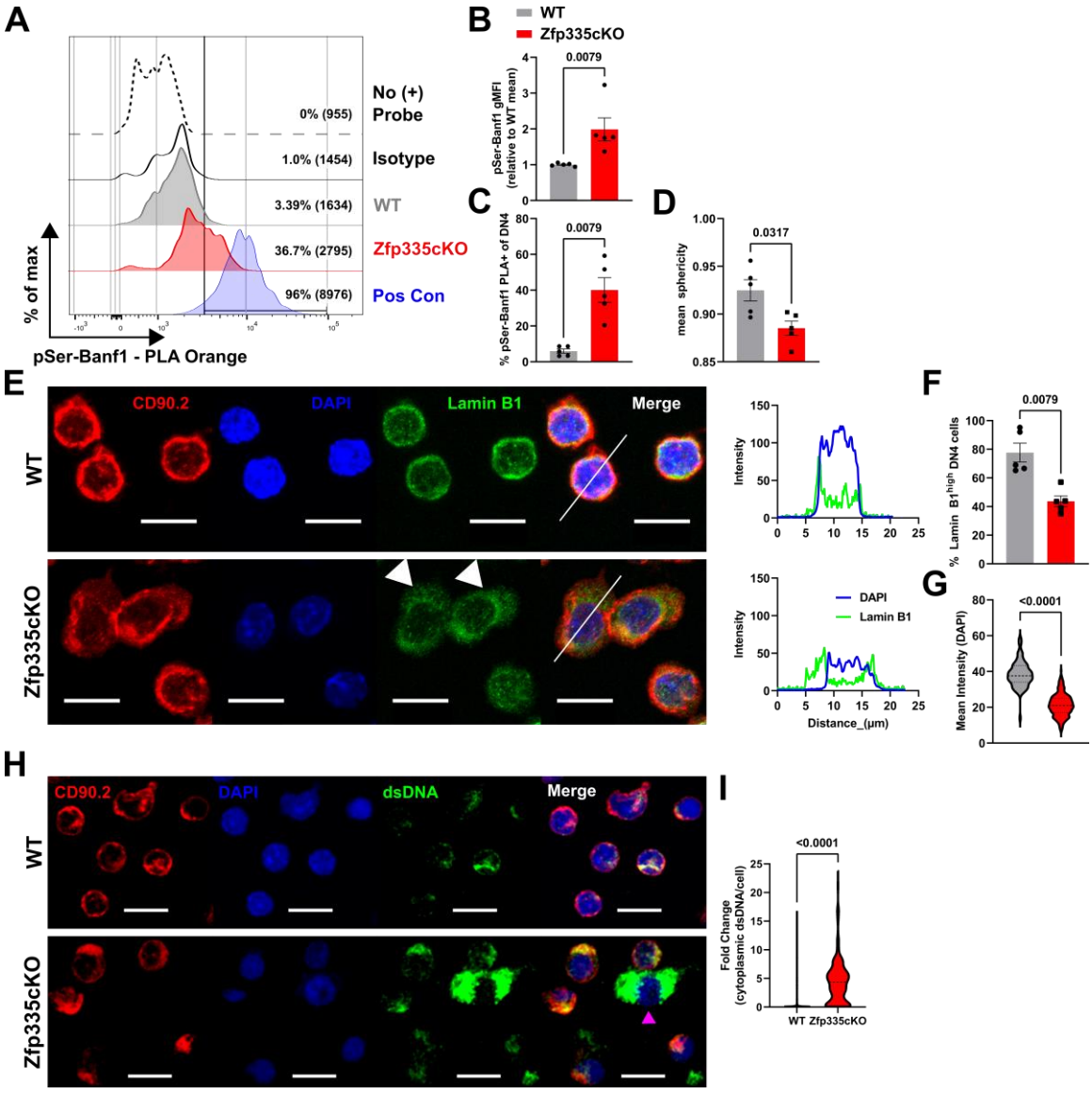

Figure 21: Loss of Zfp335 leads to Baf hyperphosphorylation altered nuclear envelope architecture and accumulation of cytosolic

(A) Representative histograms and gating of Baf phosphorylation as measured by proximity ligation assay (PLA). Percent phosphoserine-Baf and geometric MFI in parenthises are shown. Phosphoserine-Lamin B1 PLA was used as positive control. Quantification of Baf phosphorylation based on geometric MFI (B) or percent positive cells (C). n=5 mice per genotype. (D) Representative immunofluorescence images of full cell thickness maximum intensity projections (left) and profile plots (right) of nuclear envelope staining in *ex vivo* DN4 thymocytes. Profile plots are based on white lines

shown in merged images. Scale bars represent 10μm. Quantification of frequency of cells with high nuclear-associated Lamin B1 (E), mean DAPI pixel intensity (F) or mean nucleus sphericity (G) for *ex vivo* DN4 thymocytes. (E,G) n=5 mice per genotype, (F) n=124 WT and n=490 Zfp335cKO. Representative images (H) and quantification (I) of cytoplasmic dsDNA in WT (n=548 cells, 4 mice) or Zfp335cKO (n=268 cells, 6 mice) thymocytes following 3 days in OP9-DL1 culture. Magenta arrow indicates OP9-DL1 cell. *P*-values determined by Mann Whitney U-test (B-F) or Student's T test (G,I). Data shown are compiled from three (A-G) or two (I) independent experiments. Plots show mean ± sem or mean and interquartile range (G, I).

Accumulation of cytosolic DNA or exposure of nuclear contents to the cytosol via NE disruption have been shown to activate the cGAS/STING pathway (Guey et al., 2020; H. Ma et al., 2020). In T cells, cGAS/STING signaling generally results in anti-proliferative and pro-apoptotic effects (Cerboni et al., 2017; Gulen et al., 2017; Larkin et al., 2017; Wu et al., 2020). Therefore, we hypothesized that NE defects resulting from disruption of the Ankle2-Banf1 pathway downstream of Zfp335 loss drives cGAS/STING activation. Consistent with this hypothesis, GSEA revealed an enrichment for genes upregulated by T cells in response to STING signaling in both our bulk DP and single-cell DN4 datasets (Figure 22A-B). Additionally, we found increased IRF3 activity among mutant cells (Figure 22C). cGAS/STING-mediated death of mature T cells occurs in part, due to increased expression of pro-apoptotic Bcl2 family genes (Gulen et al., 2017). Like our findings from bulk RNA-seq (Figure 14A), we also observed increased expression of Bbc3 (PUMA), Pmaip1 (NOXA), Bcl2l11 (Bid) and Bax among Zfp335cKO DN4 cells in our scRNA-seq dataset (Figure 22D). In agreement with our bioinformatic analyses we observed significantly increased cGAMP levels (Figure 22E),

phosphorylation of STING, TBK1 and IRF3 in Zfp335cKO thymocytes (Figure 22F-G) as well as nuclear translocation of phosphorylated IRF3 (Figure 22H) .

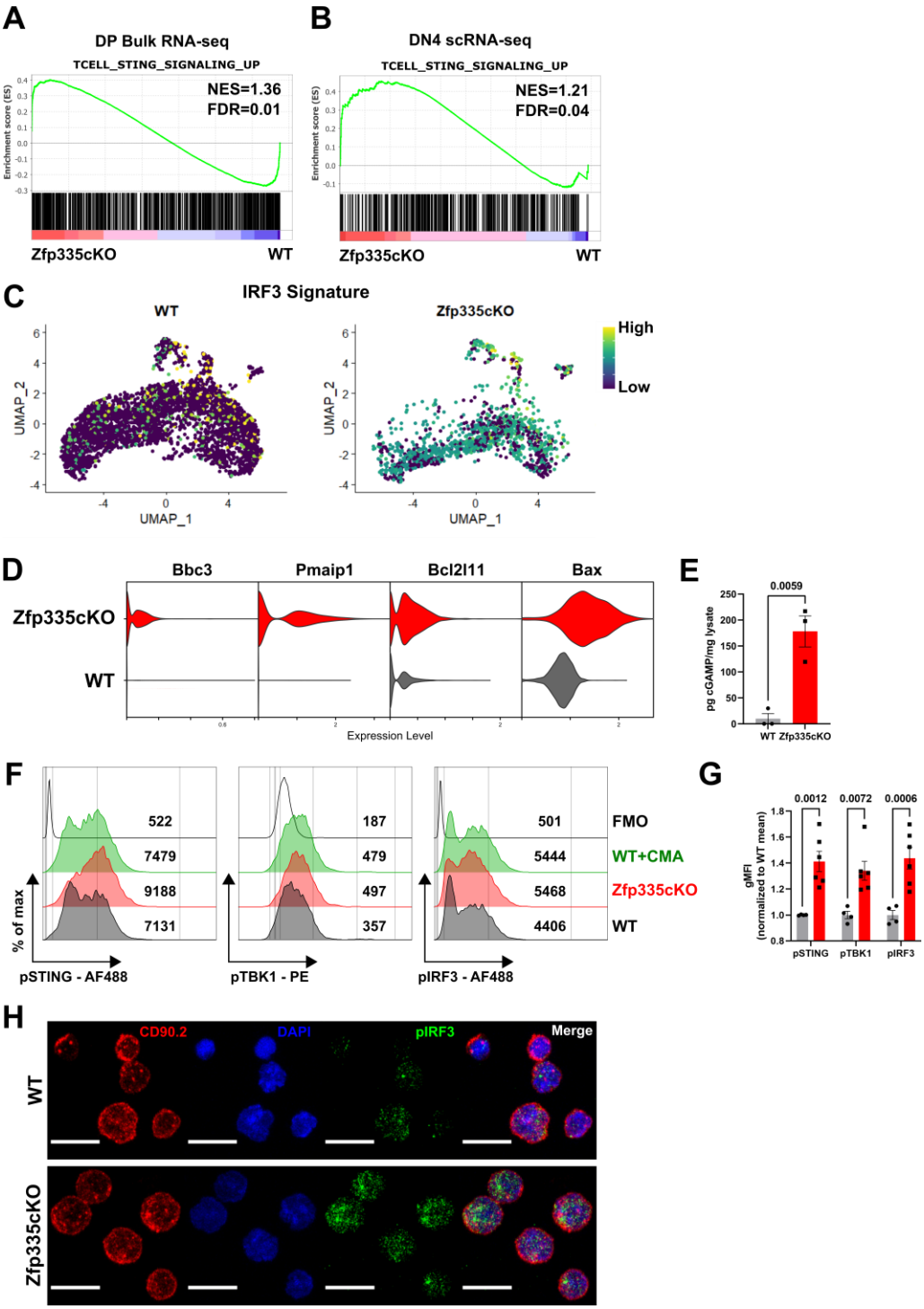

Figure 22: Loss of Zfp335 promotes cGAS/STING signaling in DN4 thymocytes

GSEA enrichment plots for T cell-specific STING signaling gene signature in DP bulk (A) or DN4 scRNA-seq data sets (B). (C) UMAP projection of IRF3 gene signature in WT or Zfp335 mutant DN4 thymocytes. (D) Violin plots of pro-apoptotic Bcl2 gene expression in WT or Zfp335 mutant DN4 thymocytes. (E) Normalized cGAMP concentration for WT (n=3 mice) or Zfp335cKO (n=3 mice) thymocytes. Representative histograms (F) and quantification (G) of phospho-STING, -TBK1, and -IRF3 in WT (n=4 mice) or Zfp335cKO (n=6 mice) thymocytes following 3 days in OP9-DL1 culture. WT thymocytes treated with 250μg/mL cridanimod (WT+CMA) for 2 hours were used as a positive control. (H) Representative images of phospho-IRF3 staining related to Figure 6O-P. *P*-values determined by Mann Whitney U-test (E) or two-way ANOVA with Sidak's test (G). Data shown are compiled from one (A-D) OR two (E-H) independent experiments. Plots show mean ± sem.

In addition to nuclear DNA, mitochondrial DNA (mtDNA) serves as a substrate for cGAS (Zierhut & Funabiki, 2020). mtDNA release requires mitochondrial outer membrane permeabilization resulting in mitochondrial membrane depolarization (McArthur et al., 2018). Examination of mitochondria showed Zfp335cKO thymocytes exhibit normal mitochondrial membrane potential and total mitochondrial mass (Figure 23A-C). Therefore, mtDNA release is unlikely to be driving cGAS/STING-mediated death following loss of Zfp335. Together, these data suggest that exposure of gDNA to cytosolic cGAS resulting from disrupted nuclear envelope architecture is the most likely cause of cGAS/STING signaling resulting from Zfp335 deletion.

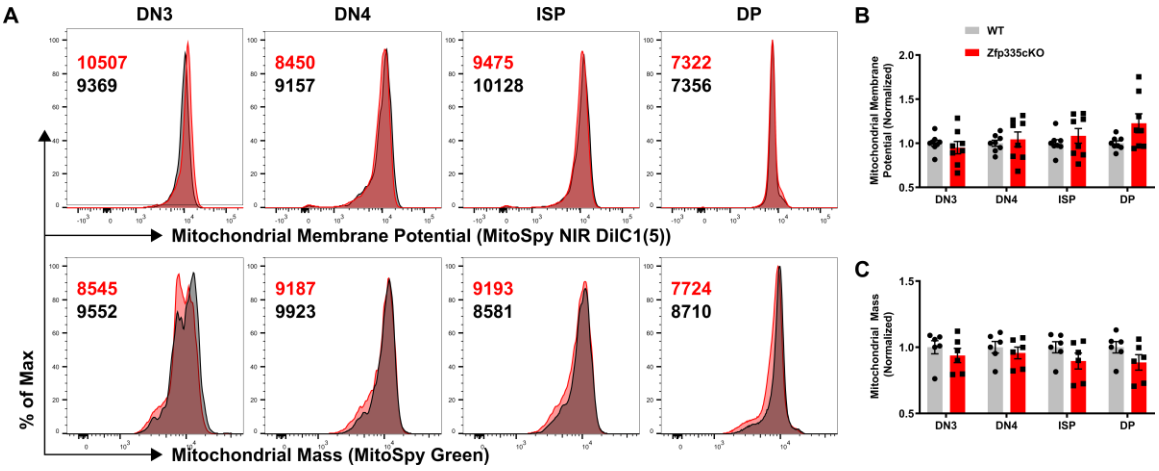

Figure 23: Loss of Zfp335 does not drive mitochondrial membrane deploraization

Representative histograms (A) and compiled data for mitochondrial membrane potential (B) or total mitochondrial mass (C) in WT (n=8 (B) or n=6 (C)) or Zfp335cKO (n=8 (B) or n=6 (C)) thymocyte populations *ex vivo*. Numbers in histograms indicate geometric MFI. Plots show mean ± sem. Data were compiled from two independent experiments.

2.2.8 Disruption of Zfp335/Ankle2/Baf axis drives cGAS/STING-dependent apoptosis of DN4 thymocytes

Next, we sought to determine the functional importance of enhanced cGAS/STING signaling in T cell development following loss of Zfp335. To test this, 'true' DN4 cells were isolated by EV viral transduction then placed in OP9-DL1 culture for 3 days with small molecule inhibitors of cGAS (RU.521) (Vincent et al., 2017) or STING (H-151) (Haag et al., 2018). Chemical inhibition of either cGAS or STING fully rescued Zfp335cKO DN4 cells from death (Figure 24A-C). Additionally, Zfp335cKO mice receiving H-151 for 7 days exhibited significantly increased numbers of total thymocytes compared to vehicle controls (Figure 24D). Importantly, this increase in cellularity was

primarily due to increased DP numbers (Figure 24E-H). Due to the short duration of treatment, we conclude that the increase in DP cells among H-151-treated Zfp335cKO mice is the result of reduced cell death during the preceding proliferative DN4 stage.

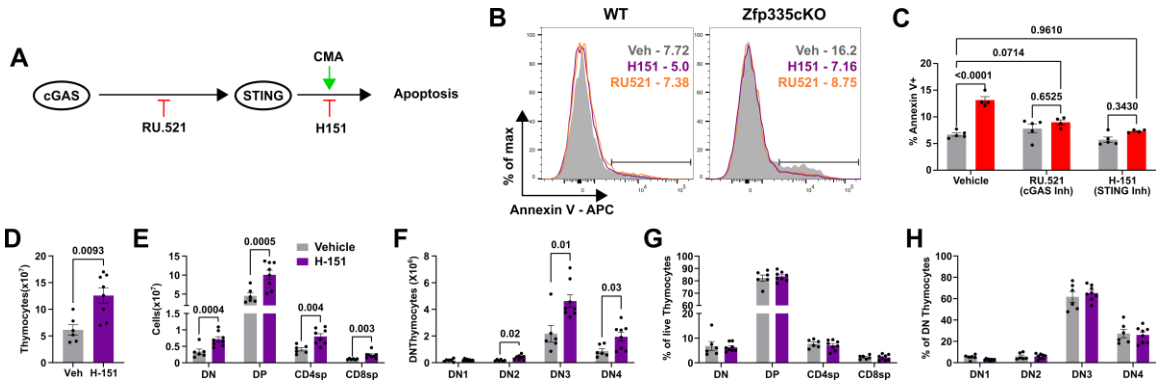

Figure 24: Chemical inhibition of cGAS/STING signaling rescues Zfp335-deficient thymocyte apoptosis

(A) Schematic diagram of inhibitors (RU.521 or H-151) or agonists (CMA) used to study cGAS/STING-dependent apoptosis of DN4 thymocytes. Representative histograms (B) and quantification (C) of Annexin V-binding for WT (n=5 mice) or Zfp335cKO (n=4 mice) DN4 thymoctes treated with cGAS (RU.521) or STING (H-151) inhibitors or vehicle control and cultured on OP9-DL1 stromal cells for 3 days. Total thymocyte (D), DN, DP, CD4SP and CD8SP or DN1-DN4 cell numbers (E,F) or frequencies (G,H) for Zfp335cKO mice treated with H-151 (n=8 mice) or vehicle (n=6 mice) *in vivo* for 7 days. *P*-values determined by Mann Whitney U-test (D) or two-way ANOVA with *post hoc* Tukey's test (C) or Sidak's test (E-H). Data shown are compiled from two (B-C) or five (D-H) independent experiments. Plots show mean ± sem.

cGAS/STING signaling is known to drive type I interferon (IFN-I) responses (Ishikawa et al., 2009) and mTOR activation (Warner et al., 2017). While we did not observe altered transcriptional signatures associated with mTOR activity or IFN-I signaling in our DN4 scRNA-seq dataset (Figure 25A-B) the IFN-I signaling program was significantly enriched among Zfp335cKO DP thymocytes compared to control

(Figure 9L). To test the importance of these pathways in Zfp335cKO cell death we performed OP9-DL1 cultures in the presence of IFNAR-1 blocking antibody or mTOR inhibiting small molecules. Inhibition of either pathway had no impact on rates of Zfp335cKO cell death (Figure 25C-E). Together these data support the conclusion that cGAS/STING signaling resulting from loss of Zfp335 drives apoptosis of DN4 thymocytes independent of IFN-I and mTOR signaling. Instead, cell death is likely driven by induction of pro-apoptotic members of the Bcl2 family.

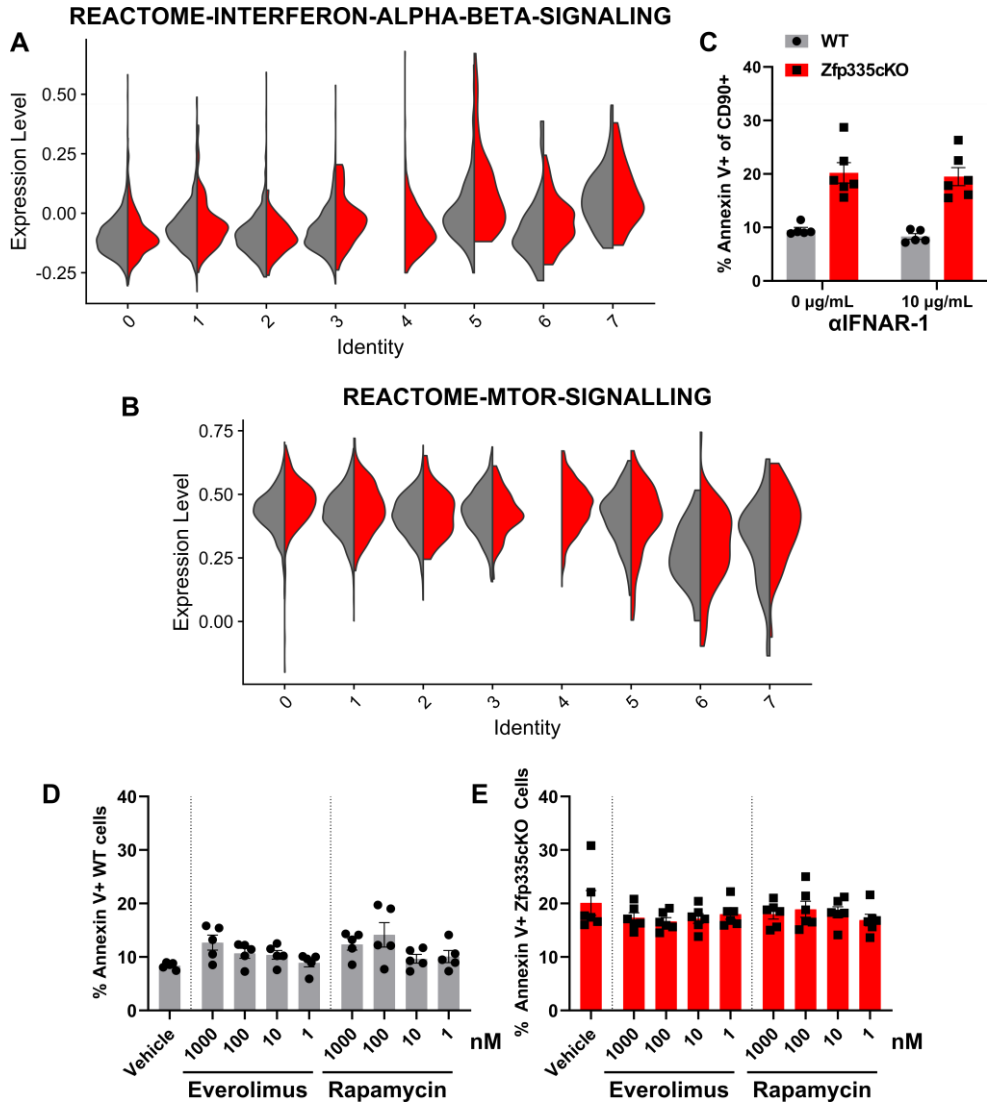

Figure 25: Increased rates of Zfp335cKO apoptosis is not dependent upon mTOR or Type I Interferon signaling.

Gene signature scores for Type I Interferon (A) or mTOR (B) signaling among WT (grey) or 'true' mutant Zfp335cKO (red) DN4 thymocytes separated by cluster. (C) Frequency of Annexin V+ cells among total CD90+ thymocytes derived from DN3/4 cells cultured with OP9-DL1 cells for 3 days in the presence (10µg/mL) or absence (0µg/mL) IFNAR-1 blocking antibody. Frequency of Annexin V+ among total CD90+ thymocytes derived from DN3/4 cells cultured with OP9-DL1 cells for 3 days in the presence of 0, 1, 10, 100, or 1000nM Everolimus or Rapamycin from WT (D) or Zfp335cKO mice (E). (C-E) n=5 WT or n=6 Zfp335cKO. Graphs show mean ± sem. Data are compiled from one (A,B) or two (C-E) independent experiments.

Next, we sought to determine the role of the Zfp335/Ankle2/Baf axis in suppressing the cGAS/STING-mediated apoptosis in DN4 cells. To test this, *R26*$^{LSL\text{-}Cas9}$ *Tcrd*^{CreERT2} DN3/DN4 thymocytes (B. Zhang et al., 2015) were transduced with retroviruses expressing *Zfp335, Ankle2, or Banf1* (encoding Baf) and *Mb21d1* (encoding cGAS) or *Tmem173* (encoding STING)-targeting gRNAs or non-targeting control gRNAs (NTG) then cultured for three days with OP9-DL1 cells in the presence of 4-hydroxytamoxifen. Consistent with conditional deletion, Cas9 targeting of *Zfp335* lead to a substantial increase in DN4 apoptosis (Figure 20). Additionally, targeting of *Ankle2* or *Banf1* similarly lead to increased DN4 apoptosis. Importantly, these increases in apoptosis were cGAS/STING-dependent (Figure 26). Similar results were observed when Cas9 expression was controlled by E8$_{III}$-cre (Figure 27). Together, these results demonstrate that disruption of the Zfp335/Ankle2/Baf axis drives cGAS/STING-mediated apoptosis of post-β-selection DN4 thymocytes.

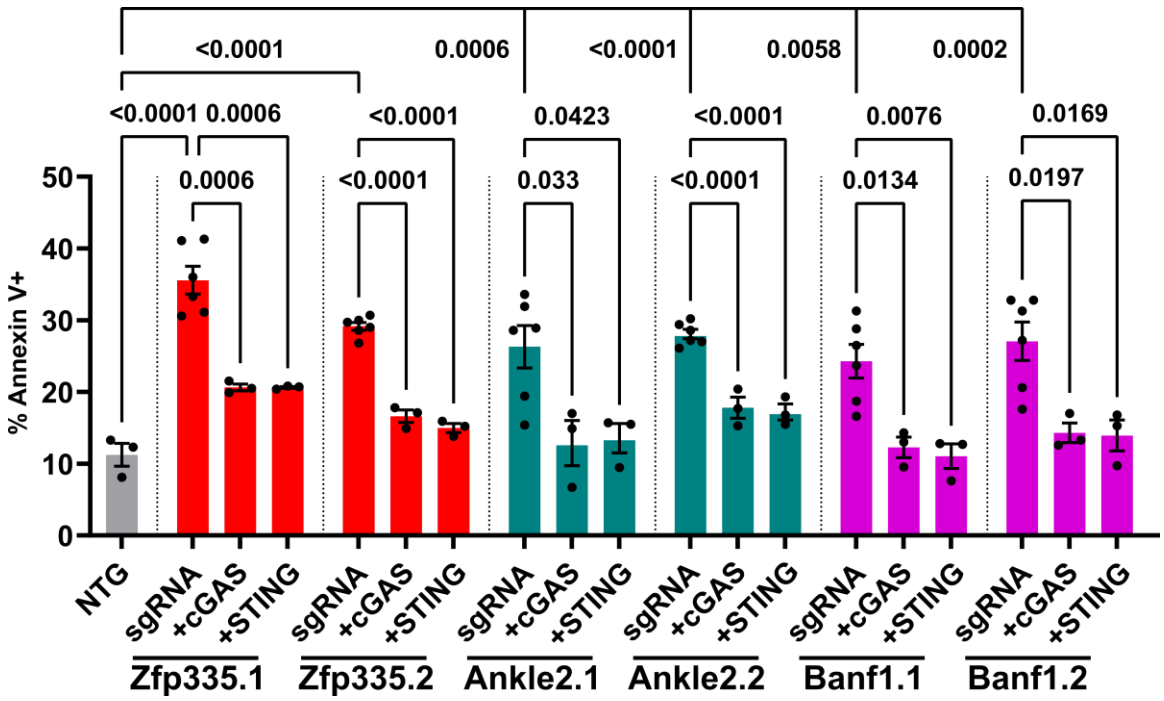

Figure 26: The Zfp335/Ankle2/Baf axis functions to suppress cGAS/STING-mediated apoptosis of DN4 thymocytes

Quantification of rates of apoptosis by Annexin V binding in $Tcrd^{CreERT2}$ $Rosa26^{LSL\text{-}Cas9/LSL\text{-}ZsGreen}$ DN4 thymocytes transduced with guide RNA expressing retroviruses targeting Zfp335, Ankle2, or Baf and cGAS or STING or non-targeting controls (NTG) after 3 days in OP9-DL1 culture. *P*-values determined by One-Way ANOVA with post hoc Dunnett's test for multiple comparisons. Data are compiled from three independent experiments. Each point represents one biological replicate of three pooled mice. Plots show mean ± sem.

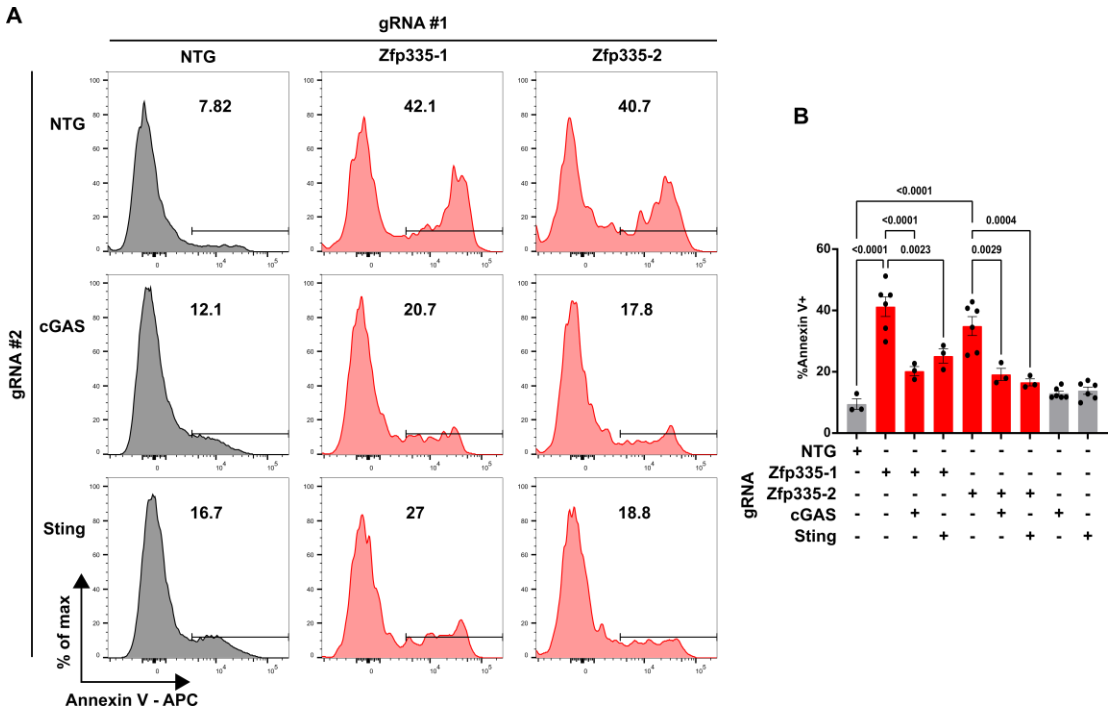

Figure 27: Cas9-mediated Zfp335 deletion drives cGAS/STING-dependent DN4 apoptosis

Representative gating (A) and quantification (B) of Annexin V binding among DN4 cells from *R26*$^{LSL\text{-}Cas9}$ *E8*$_{III}$*-cre* DN3/4 thymocytes transduced with indicated gRNA-expressing retroviruses and cultured for three days on OP9-DL1 cells. n=3 mice for each dual gRNA transduction. P-values calculated using One-Way ANOVA with Dunnett's post hoc test. Plots show mean ± sem. Data are compiled from three independent experiments.

2.2.9 DN4 thymocytes exhibit increased sensitivity to cGAS/STING-mediated cell death

Finally, we sought to determine whether sensitivity to cGAS/STING-driven cell death is a unique feature of Zfp335cKO DN4 cells or a mechanism of the DN4 stage. DN-enriched WT thymocytes were treated with the STING agonist cridanimod (CMA) overnight then assayed for apoptosis. Interestingly, we found DN4 cells are uniquely

sensitive to STING-mediated apoptosis at low concentrations of STING agonist (Figure 28A). However, increasing the STING agonist concentration 10-fold was sufficient to promote apoptosis of all thymocyte subsets (Figure 28B). Additionally, viability of Zfp335cKO Bcl2-Tg thymocytes was not impacted by CMA treatment (Figure 28C) suggesting that induction of pro-apoptotic Bcl2 family members downstream of STING activation are necessary for apoptosis of DN4 thymocytes.

We were surprised at the unique sensitivity of DN4 cells to cGAS/STING-mediated apoptosis. We hypothesized that such a sensitivity may be due to the presence of TCR excision circles (TRECs) generated by V(D)J recombination at the preceding DN3 stage which may be exposed to the cytosol during cell division, thereby, reducing the signaling threshold for STING-mediated cell death. To test this, we generated post-β-selection thymocytes from $Rag2^{-/-}$ mice by intraperitoneal injection of αCD3ε antibody. Following antibody treatment, thymocytes were harvested, subjected to STING agonist treatment *ex vivo* and assessed for apoptosis. Consistent with our hypothesis, Rag-deficient DN4 cells do not exhibit increased sensitivity to STING-mediated cell death compared to vehicle control (Figure 28D). Together, these data demonstrate that activation of the cGAS/STING pathway is a major contributor to Zfp335cKO DN4 apoptosis and that WT DN4 cells exhibit increased sensitivity to cGAS/STING-mediated death, possibly a result of V(D)J recombination at the DN3 stage.

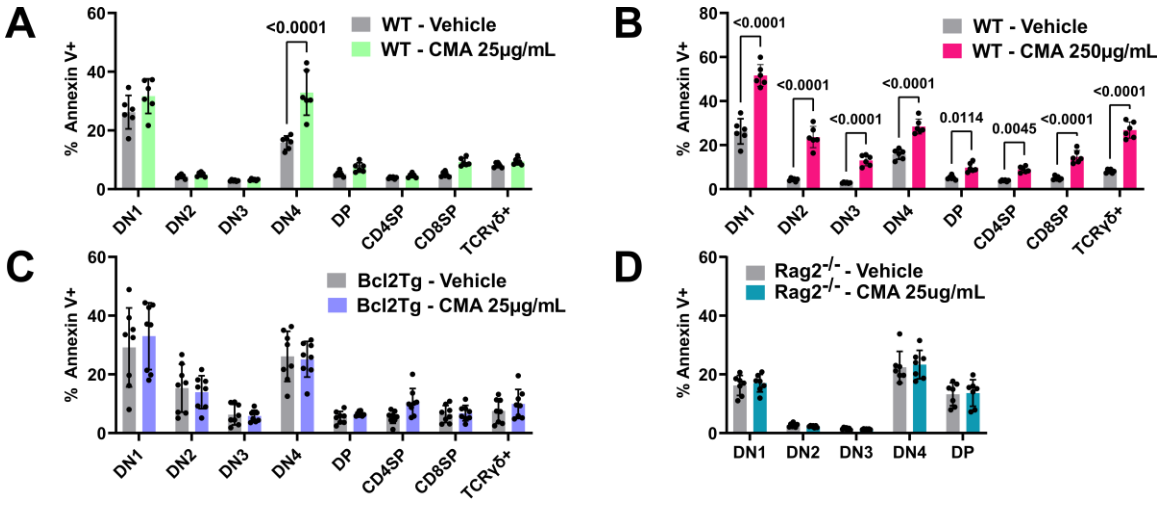

Figure 28: DN4 thymocytes exhibit increased sensitivity to STING-mediated apoptosis.

Quantification of WT thymocyte subset apoptosis following overnight OP9-DL1 culture in the presence of 25 (A) or 250µg/mL CMA. (C) Quantification of apoptosis among Zfp335cKO Bcl2Tg thymocyte subset apoptosis following overnight OP9-DL1 culture in the presence of 25µg/mL CMA. (D) Quantification of $Rag2^{-/-}$ thymocyte subset apoptosis following overnight OP9-DL1 culture in the presence of 25µg/mL CMA. Post β-selection $Rag2^{-/-}$ thymocytes were generated by *in vivo* treatment with αCD3ε antibody via intraperitoneal injection. *P*-values determined using Two-Way ANOVA with post hoc Sidak's test for multiple comparisons. Plots show mean ± sem. Data are compiled from two (A,B,D) or three (C) independent experiments.

Altogether, our studies reveal that loss of Zfp335 leads to defective T cell development resulting from dysregulation of the Zfp335/Ankle2/Baf axis ultimately driving cGAS/STING-mediated DN4 cell death (Figure 29).

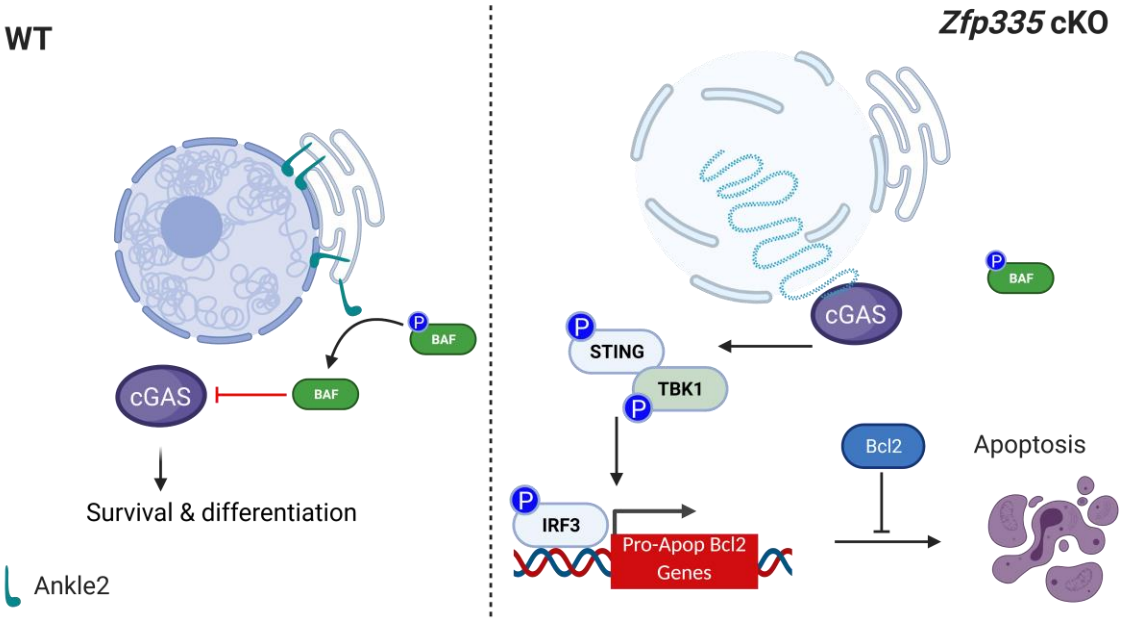

Figure 29: Schematic diagram of mechanism by which Zfp335 promotes survival of DN4 thymocytes.

Normally in WT DN4 thymocytes Zfp335 promotes expression of Ankle2 which, in turn, regulates the dephosphorylation of Baf following mitotic exit. Dephosphorylated Baf supports proper nuclear envelope architecture and inhibits cGAS promoting survival and differentiation to DP. In the absence of Zfp335 (Zfp335cKO), Ankle2 expression is lost driving Baf hyperphosphorylation. Nuclear envelope integrity is lost exposing genomic DNA to cGAS driving downstream STING signaling, induction of pro-apoptotic Bcl2 family genes and ultimately apoptosis.

2.3 Discussion

In this study, we identify Zfp335 as a critical transcription factor regulating early T cell development within the thymus. Specifically, it functions to promote survival of proliferating cells following β-selection. Conditional deletion of Zfp335 led to severe reductions in all T cell populations beginning at the DN4 stage of development. Mechanistically, we show that reduced expression of the Zfp335-regulated gene *Ankle2*

and the resulting disruption of the Zfp335/Ankle2/Baf pathway controlling NE architecture drives cGAS/STING-dependent DN4 cell apoptosis.

Our studies highlight the necessity of sustained Zfp335 and Ankle2 expression to promote DN4 survival and support proper T cell development. Zfp335 is ubiquitously expressed throughout T cell development. Therefore, it is likely that Zfp335 plays numerous roles throughout thymopoiesis. In addition to supporting DN4 survival we show that it is also required for terminal thymocyte maturation. Based on the mechanism uncovered in our work and the high degree of functional similarity between DN4 and DN2 cells it is likely that loss of Zfp335 at the DN1 stage or earlier may block T cell development at the DN2 stage. Such a hypothesis is support by a recent study which found that mice carrying a human disease associated STING mutation (V154M) exhibit severe T lymphopenia likely due to increased rates of early thymic progenitor apoptosis (Bouis et al., 2019). However, to test this hypothesis alternative means of Zfp335 deletion will need to be utilized.

Our studies provide the first comprehensive assessment of the heterogeneity within the DN4 thymocyte compartment at the single cell level. Surprisingly, phenotypically defined DN4 cells consist of cycling cells expressing pre-Tα which represent 'true' DN4 cells and mature or maturing $\alpha\beta$ and $\gamma\delta$ T cells. Positive selection of DP thymocytes induces a slight, transient down-regulation of CD4 and CD8 (Lucas & Germain, 1996), however, the maturing $\alpha\beta$ cells identified in our dataset completely lack

both protein and mRNA expression. The cells we identified expressing TCRα transcripts exhibited expression patterns consistent with positive selection (Mingueneau et al., 2013) and therefore, are likely post positive-selection cells which have transiently lost surface expression of TCR, CD4 and CD8. Alternatively, these cells may have undergone positive selection without ever expressing CD4 or CD8. Regardless, these maturing cells may represent a novel developmental path within the thymus. However, more detailed studies will be needed to fully characterize these cells and determine if they represent a unique lineage or simply a rare differentiation path that can be taken by any positively selected cell.

Han *et al.* recently identified a hypomorph allele of *Zfp335* (*Zfp335*^{bloto}) as the causative mutation leading to reduced total peripheral T cells and an almost complete absence of naïve T cells (Han et al., 2014). They found Ankle2 to be a critical Zfp335-regulated gene controlling late stages of thymic T cell maturation. However, the mechanism by which Ankle2 regulates maturation, and the establishment of the naïve T cell compartment remains unclear. The lack of apparent developmental defects in *Zfp335*$^{blt/blt}$ mice during early T cell development is likely due to their use of a hypomorph allele instead of a conditional knock out as *Zfp335*$^{blt/blt}$ mice exhibited normal expression of Ankle2 during the DN4 stage.

We have shown that Zfp335 is at least partially regulated by E protein activity in developing T cells. E proteins play numerous indispensable roles throughout

organismal development, including T cell development (Agata et al., 2007; Engel & Murre, 2001, 2004; Jia et al., 2008; Jones-Mason et al., 2012; Petersson et al., 2002; Roy et al., 2018; Wang et al., 2006; Wojciechowski et al., 2007). However, due to widespread binding throughout the genome, the roles for transcriptional networks established by E proteins remain incompletely understood (Roy et al., 2018). Our studies identify Zfp335 as a novel transcription factor downstream of E proteins critical to T cell development.

To date, studies of T cell-intrinsic roles for cGAS/STING pathway have largely focused on activation via synthetic STING agonists (Gulen et al., 2017; Larkin et al., 2017; Wu et al., 2020) or expression of constitutive gain-of-function STING mutations (Cerboni et al., 2017). These studies have primarily focused on roles of this pathway in mature peripheral T cells. To our knowledge, this is the first report of a physiological role for cGAS/STING in T cell development. Additionally, our identification of the Zfp335/Ankle2/Baf axis as key in repression of cGAS is the first transcriptional pathway identified which functions to prevent cGAS activation by self-DNA.

Baf was recently identified as a key inhibitor of cGAS sensing of self-DNA through competitive binding (Guey et al., 2020). The ability of Baf to bind DNA is dependent upon its dephosphorylation which has been shown to be controlled by Ankle2 during mitotic exit (Asencio et al., 2012). Therefore, we propose the following mechanism by which loss of Zfp335 drives cGAS/STING-mediated apoptosis of DN4 thymocytes. Loss of Zfp335 results in impaired Ankle2 expression which in turn leads to

the failure of Baf dephosphorylation during division. Baf hyperphosphorylation leads to improper NE reassembly and can drive spontaneous NE rupture exposing nuclear DNA to the cytosol allowing unrestricted cGAS activation and STING-mediated apoptosis.

A surprising finding of our study was the unique sensitivity of DN4 thymocytes to cGAS/STING-mediated apoptosis. We did not observe significant increases in cell death among any other population of thymocytes following low-dose *ex vivo* STING activation. However, 10-fold higher STING agonist concentrations led to significant increases in cell death across all thymocyte subsets. This sensitivity was efficiently abrogated upon Bcl2 overexpression, suggesting a dominant role of pro-apoptotic members of this protein family in driving cell death. Interestingly, we found that preventing V(D)J recombination via deletion of Rag2 was sufficient to protect DN4 cells from STING-mediated cell death suggesting that V(D)J recombination may play a role in this unique sensitivity to cGAS/STING signaling. A reasonable explanation for our finding is that TRECs generated by V(D)J recombination may become localized to the cytoplasm upon cell division at the DN4 stage, thereby providing activating signals for cGAS. This cGAS activity in turn may lower the signaling threshold for STING-mediated cell death. However, this does not explain the lack of DP cell sensitivity to STING agonist which undergo repeated rounds of VJ recombination. We propose that the apparent lack of sensitivity to STING signaling among DP cells is due to loss of cGAS and STING expression during the DN-DP transition (Immgen Data). Should this

be true, it is reasonable to hypothesize that the down regulation of cGAS and STING expression is necessary to facilitate survival of DP thymocytes. Alternatively, the lack of cell division during T cell development beginning at TCRα recombination may prevent exposure of DP-derived TRECs to the cytosol. In either case, further study of this phenomena is warranted.

Consistent with studies of cGAS/STING signaling in mature T cells (Cerboni et al., 2017; Wu et al., 2020), we found cGAS/STING-mediated apoptosis in DN4 cells is independent of IFN-I signaling. Interestingly, we found that Zfp335-deficient DP and DN4-like maturing T cells exhibit increased IFN-I signaling activity compared to controls. Similar transcriptional activity was previously observed in *Zfp335*^{bloto} mice (Han et al., 2014). While tonic IFN-I signaling is critical to normal T cell development (Xing et al., 2016), enhanced signaling has been shown to severely impair thymopoiesis (Lin et al., 1998). The mechanism by which Zfp335 and Ankle2 regulate terminal T cell maturation in the thymus is unclear. However, it is possible that enhanced IFN-I signaling resulting from cGAS/STING activation may contribute to this defect.

Interestingly, in humans, ANKLE2 is a target of Zika virus protein NS4A which antagonizes its activity ultimately leading to microcephaly(Link et al., 2019). Humans carrying homozygous or compound heterozygous mutations in either ZNF335 or ANKLE2 exhibit severe microcephaly like that characteristic of Zika patients (Yamamoto et al., 2014; Yang et al., 2012). Recent studies have demonstrated a critical role for central

nervous system immune cells in regulating neuronal stem cell maintenance and differentiation. Specifically, microglia play a key role in this process (Matsui & Mori, 2018; Ribeiro Xavier et al., 2015; Shigemoto-Mogami et al., 2014). Under conditions which stimulate cGAS activity, microglia and other CNS immune cells preferentially undergo apoptosis (Reinert et al., 2021). Based on the mechanism revealed in this study it is possible that microcephaly resulting from Zika infection or loss of ZNF335 or ANKLE2 may be driven by cGAS/STING-dependent apoptosis of neuronal progenitors and/ or CNS immune cells. Should our mechanism extend to neuronal progenitors or CNS immune cells it may be possible to pharmaceutically prevent microcephaly in these specific instances by inhibition of the cGAS/STING pathway. However, further research will be required to determine the viability of such a therapeutic approach.

Beyond CNS development the mechanism uncovered through or studies may apply to more diverse biological phenomena. Mutations in BAF have previously been shown to drive Nestor-Guillermo Progeria Syndrome, a disease associated with severe pre-mature aging which typically manifest after 2+ years of life (Puente et al., 2011). Interestingly, accumulation of cytosolic DNA is associated with cellular aging and senescence with cGAS/STING implicated in these outcomes (Paul et al., 2021). Whether the accumulation of cytosolic dsDNA and cGAS/STING activity is a cause or result of the aging process is an open question and further study warranted. Additionally, mutations in genes associated with the regulation of nuclear envelope structure and

maintenance (Frost et al., 2016) as well as cGAS/STING (Fryer et al., 2021) have been associated with progression of neurodegenerative diseases such as Parkinson's Disease (Sliter et al., 2018) and prion-mediated neurodegeneration (Nazmi et al., 2019). Therefore, exploration of the role for the Zfp335/Ankle2/Baf pathway uncovered in this study may reveal novel pathways regulating, and potential therapeutic targets to mitigate, neurodegeneration and cellular aging.

3. Zfp335 controls the development, maturation, and differentiation of invariant NKT cells and conventional αβ T cells

3.1 Introduction

The establishment of a robust and diverse peripheral T cell pool requires proper regulation of a complex series of transcriptional changes throughout thymic development. T cell development culminates with the generation of a large number of conventional $CD4^+$ or $CD8^+$ T cells and a relatively small number of unconventional T cells with innate-like properties. Among these unconventional T cells, invariant natural killer T (iNKT) cells are the most abundant.

iNKT cells are a specialized subset of $\alpha\beta$ T cells imbued with innate-like effector functions (Imai et al., 1986; Koseki et al., 1989). iNKT cells develop within the thymus and unlike conventional $\alpha\beta$ T cells their selection and differentiation are tightly linked. That is, developmental selection simultaneously drives effector differentiation. Like conventional T cells, iNKT cell development is defined by surface protein phenotype and can be broken down into four stages, stage 0-3. Upon agonist selection of DP thymocytes expressing the canonical Vα14-Jα18 iNKT TCR (in mice) via CD1d molecules expressed by neighboring thymocytes cells commit to the iNKT lineage . Committed progenitors express surface CD24, lack CD44 or NK1.1 expression, and are known as stage 0 iNKT cells (Benlagha et al., 2002). Stage 0 is characterized by a brief phase of proliferation which functions to expand the pool of committed iNKT cell

progenitors (Benlagha et al., 2002; Dose et al., 2009). Following the brief phase of proliferation iNKT cells lose surface CD24 expression defining stage 1. Next, they upregulate CD44 expression (stage 2) followed by NK1.1 (stage 3) (Benlagha et al., 2002). This linear model of development was originally believed to apply to all iNKT cells. However, several years after this discovery it was realized that fully differentiated effector iNKT cell subsets could be identified across stages 1-3 with a bias in staging between different subsets. Like conventional T cells, iNKT effector subsets are defined by expression of lineage-defining transcription factors. NKT1, NKT2, or NK17 cells express T-bet^{hi} PLZF^{low}, T-bet^{-} PLZF^{hi}, or PLZF^{int} RORγt^{+}, respectively. NKT1 are exclusively found in stage 3, NKT2 consist of stage 1 and stage 2, and NKT17 are uniformly stage 2 (Lee et al., 2013). More recently with the advent of single-cell genomics technologies, it has become apparent that NKT cell developmental progression is associated with loss of alternative effector potential and acquisition of effector identity as cells exit the developmental trajectory (Baranek et al., 2020).

While TCR-mediated antigen recognition was initially recognized as the key event in selection and differentiation of iNKT cells it has become clear that other signals are also necessary. In particular, the roles for signaling through SLAM family receptors (SFR) (Lu et al., 2019) and IL-15R (Ohteki et al., 1997), in addition to several transcriptional regulators, such as Zbtb16 (PLZF) (O'Hagan et al., 2015; Savage et al., 2008), Egr1/2/3 (O'Hagan et al., 2015; Seiler et al., 2012), NOTCH (Oh et al., 2015; Tanaka

et al., 2006), E proteins (D'Cruz et al., 2010) and Id2/3 (Roy et al., 2018) among others, are well documented (Shissler & Webb, 2019). The combination of signal transduction and transcription factor activity establishes the necessary genomic and transcriptional contexts required for proper effector differentiation.

E protein activity, particularly that of HEB, is essential for the development of iNKT cells. Absence of HEB prevents iNKT cell development due to reduced RORγt and Bcl-xL expression and failure to recombine the necessary Vα14-Jα18 TCR common to iNKT cells. Ectopic expression of Bcl-xL or iNKT TCR was sufficient to partially rescue iNKT cell development (D'Cruz et al., 2010). Consistent with this finding, genetic ablation of the E protein inhibitors *Id3* and *Id2* drives expansion of iNKT cells due to increased frequency of Vα14-Jα18 TCR rearrangments (Verykokakis et al., 2013). Thus, to date the described role for E proteins in iNKT cell development is restricted to supporting survival to the point of selection. However, it remains unclear if E protein activity-driven transcriptional networks play additional roles following selection.

In conventional T cells, E proteins are required for proper effector differentiation. The absence of E proteins drives CD8 T cells from a terminal effector to a memory phenotype (Omilusik et al., 2018). Interestingly, excessive E protein activity in Id3-deficient naïve T cells drives the spontaneous developmental acquisition of a differentiated, effector-memory phenotype (Miyazaki et al., 2011). However, the absence of Id2 or Id3 proteins precludes the generation of short-lived or long-lived memory T

cells, respectively (Yang et al., 2011). Therefore, the opposing activities of E and Id proteins functions to maintain a balance required for proper T cell differentiation. However, the specific mechanisms by which Id and E proteins achieve this balance and the downstream transcriptional networks controlling differentiation are largely unknown.

Unlike conventional T cells, iNKT cells often undergo effector differentiation developmentally within the thymus. The transcriptional programming of effector differentiation for both types of T cells is largely shared. We recently uncovered a unique role for the E protein target transcription factor Zfp335 in early stages of T cell development which functions to promote DN4 thymocyte survival by suppressing cGAS/STING activity (Chapter 2). Through these studies we found that loss of Zfp335 also prevents proper T cell maturation and differentiation through a survival-independent mechanism.

In this study, we set out to determine the role for Zfp335 in conventional T cell and iNKT cell development and differentiation. We show that loss of Zfp335 prior to, or immediately following, iNKT selection and commitment severely impairs development due to death of Stage 0 cells and blocks effector differentiation. In conventional T cells, loss of Zfp335 prevents CD4 T cell differentiation and promotes developmental acquisition of T-bet expression in CD8 T cells.

3.2 Results

3.2.1 Zfp335, an E protein target, is required for iNKT cell development

E proteins play indispensable roles for the development and function of iNKT cells (D'Cruz et al., 2014; J. Li et al., 2013; Roy et al., 2018). While the importance of E proteins is known it remains unclear exactly how E protein drive iNKT cell development. Zfp335 is a direct target of E protein activity in developing T cells (Figure A-B). Therefore, we asked whether this E protein target is required for the development iNKT cells. As in our previous studies of the role for Zfp335 in DN4 thymocytes, we examined thymic development of iNKT cells in *Zfp335$^{fl/fl}$E8$_{III}$-cre* (Zfp335cKO) mice. Consistent with impaired conventional T cell development we observed significantly reduced numbers of thymic iNKT cells in Zfp335cKO mice (Figure 30A-C). Zfp335-deficient mice display increased stage 0, reduced stage 2, and normal stage 1 and 3 iNKT frequencies (Figure 30D). The reduction in iNKT cell numbers is first evident at Stage 2 of development (Figure 30E).

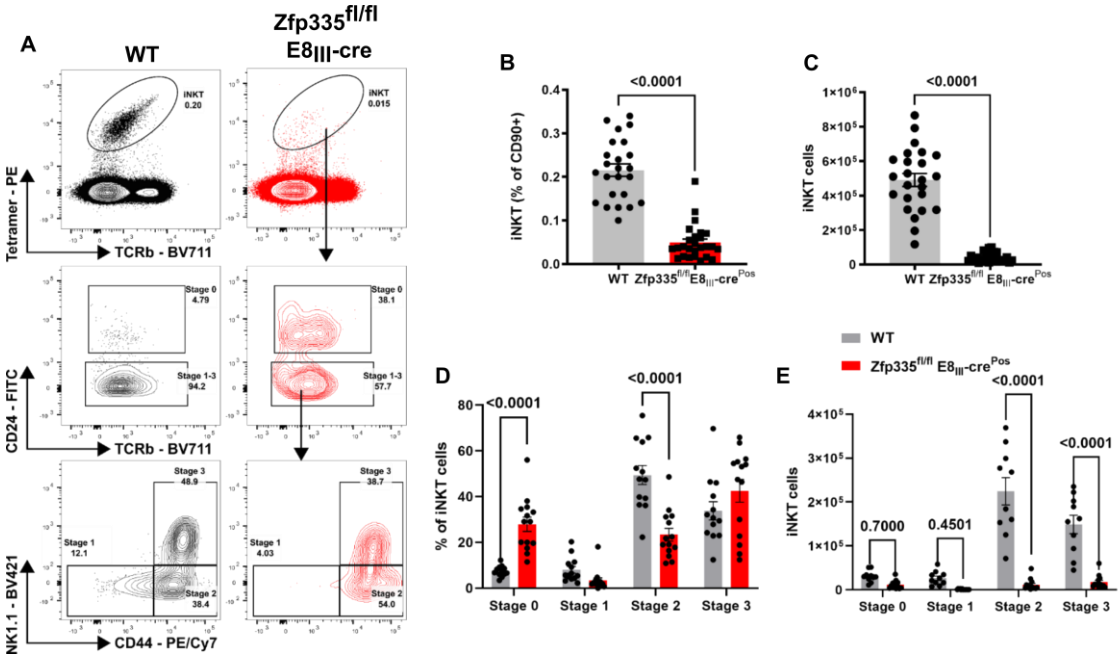

Figure 30: Loss of Zfp335 severely impairs iNKT cell development

(A) Representative gating for developmental staging of thymic iNKT cells; pre-gated on CD90^{+} live singlets. Frequency (B) and total numbers (C) of thymic iNKT cells in WT (n=24) or Zfp335$^{fl/fl}$ E8$_{III}$-cre (n=26) mice. Frequency (D) and total numbers of stage 0 – stage 3 iNKT cells in WT (n=10) or Zfp335cKO (n=14) mice. Data are compiled from eight (B-C) or X (D-E) independent experiments. P-values determined by Mann-Whitney U-Test (B-C) or Two Way ANOVA with post-hoc Sidak's test (D-E). Plots show mean ± sem.

Since the numbers of iNKT cells identified in Zfp335-deficient thymuses were very small we next validated that these cells were, in fact iNKT cells. We examined TCRα repertoire sequencing data derived from DP or CD4SP thymocytes looking for the canonical CDR3 sequence (CVVGDRGSALGRLHF) shared by all type I iNKT cells. While reduced in frequency the canonical iNKT CDR3 rearrangement was found in both DP and CD4SP thymocytes from Zfp335cKO mice (Figure 31A-B).

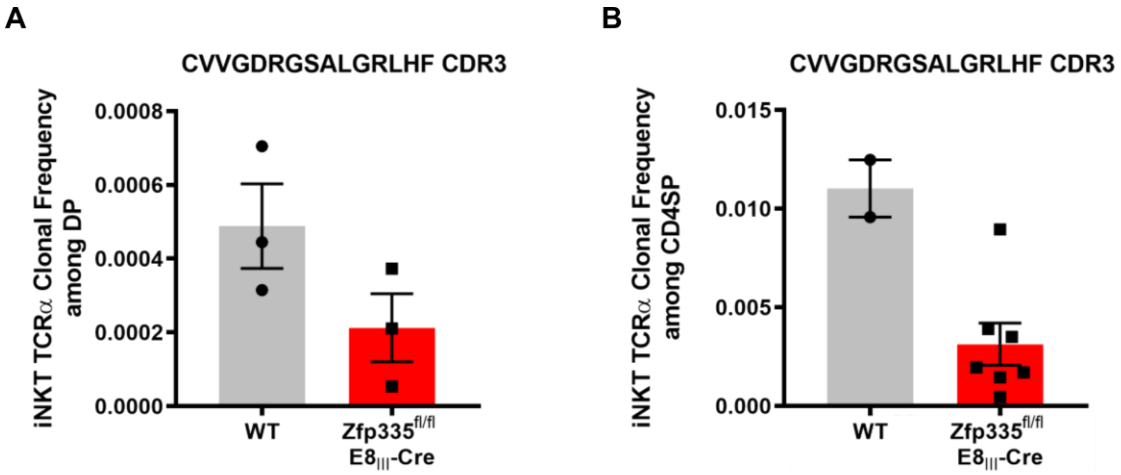

Figure 31: Zfp335-deficient mice harbor thymocytes expressing the canonical iNKT TCRα rearrangement.

Frequency of Vα14-Jα18 CVVGDRGSALGRLHF CDR3 rearrangement among bulk DP (A) or CD4SP (B) thymocytes from WT or Zfp335$^{fl/fl}$ E8$_{III}$-cre mice assessed by TCRα Rep-Seq. Plots show mean ± sem.

Importantly, the impairment of iNKT cell development resulting from loss of Zfp335 cannot be attributed to reduced survival time of DP cells, an inability for these cells to undergo positive selection (Figure 13B-E), or an impairment in the rearrangement of canonical iNKT cell TCR. Therefore, these data demonstrate that Zfp335 is required for the development of iNKT cells.

3.2.2 Loss of Zfp335 leads to defective iNKT cell differentiation

Unlike conventional T cells, iNKT cells undergo effector differentiation developmentally. Effector iNKT cell subsets are defined by expression of PLZF, T-bet, and RORγt. iNKT1 cells express high levels of T-bet and intermediate levels of PLZF. iNKT2 cells are T-bet^{-}PLZF^{+} and iNKT17 cells express RORγt. Therefore, next we sought

to determine whether the iNKT cells which do develop in the absence of Zfp335 are able to undergo differentiation. We examined acquisition of master transcription factor expression in post-commitment ($CD24^-$) WT and $Zfp335^{fl/fl}$ $E8_{III}$-cre thymic iNKT cells. Interestingly, we observed normal frequencies of each iNKT cell effector subset, however, total numbers for each were significantly reduced (Figure 32). Despite normal frequencies of effector iNKT cells among Zfp335cKO mice, a significant increase in proportions of $CD24^+$ cells (Figure 30A,D) suggests that loss of Zfp335 restricts iNKT effector differentiation.

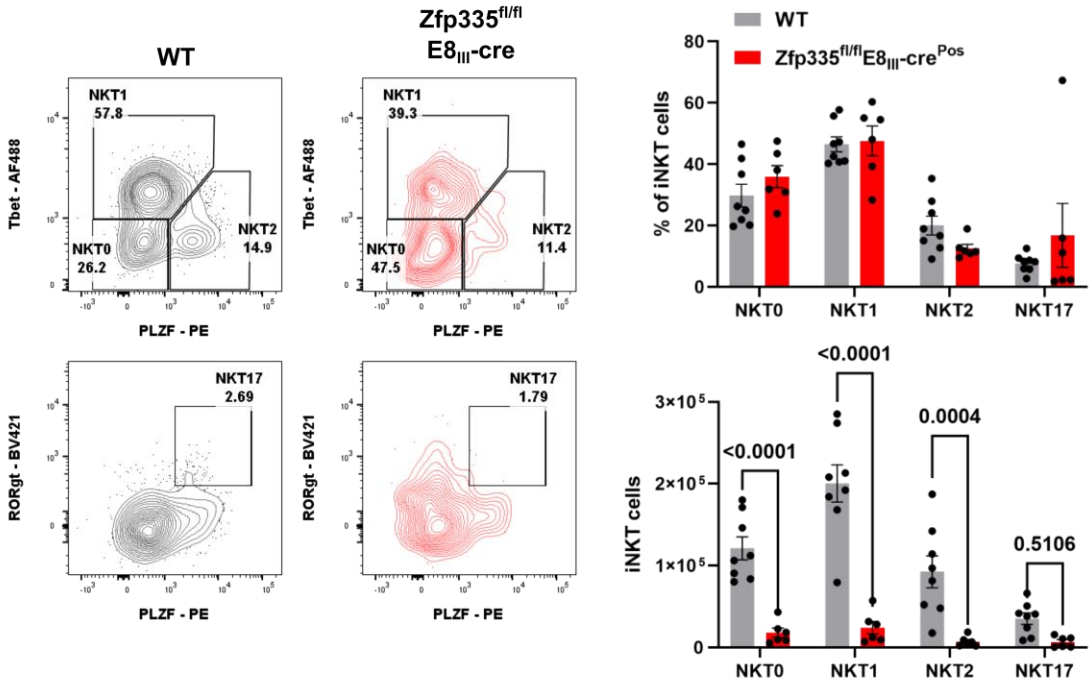

Figure 32: Zfp335-deficient iNKT cells can undergo effector differentiation

Representative gating for thymic iNKT effector subsets (left) pre-gated on $CD90^+$ CD1d-tetramer$^+$ $CD24^-$ live singlets. (Right) Frequency (top) and total numbers (bottom) of thymic iNKT effector subsets in WT (n=8) or $Zfp335^{fl/fl}$ $E8_{III}$-cre (n=6) mice. P-values

calculated by Two Way ANOVA with post-hoc Sidak's test. Data are compiled from three independent experiments. Plots show mean ± sem.

3.2.3 Bcl2 overexpression rescues total iNKT numbers but not differentiation in the absence of Zfp335.

Through our studies of the role for Zfp335 in early T cell development we found that this transcription factor functions to promote survival of post-β-selection, proliferating DN4 cells. The survival defect resulting from loss of Zfp335 in DN4 cells could be rescued by overexpression of the anti-apoptotic factor Bcl2. Interestingly, stage 0 of iNKT development is analogous to the DN4 stage. That is, stage 0 serves as a brief phase of rapid proliferation to expand the pool of iNKT cell progenitors. Zfp335cKO mice exhibit slightly reduced numbers of stage 0 iNKT cells, however, this reduction is not statistically significant.

Therefore, we next sought to determine if the observed iNKT cell defect resulting from loss of Zfp335 was due to cell death. To test this, we examine iNKT cell development in $Zfp335^{fl/fl}$ $R26^{LSL\text{-}Bcl2}$ $E8_{III}$-cre mice. Interestingly, WT and $Zfp335^{fl/fl}$ $R26^{LSL\text{-}Bcl2}$ $E8_{III}$-cre mice had similar numbers of total thymic iNKT cells (Figure 33A-B). However, $Zfp335^{fl/fl}$ $R26^{LSL\text{-}Bcl2}$ $E8_{III}$-cre iNKT cells largely remained stage 0 (Figure 33C-D) mirroring the effects observed for conventional T cell maturation in the absence of Zfp335 (Figure 15A-C). Based on the rescue of overall cell numbers by Bcl2 overexpression the primary defect in iNKT development following loss of Zfp335 is likely due to cell death during the proliferative stage 0 phase. However, blocking cell

death uncovered a secondary defect in the ability of Zfp335-deficient iNKT cells to undergo effector differentiation.

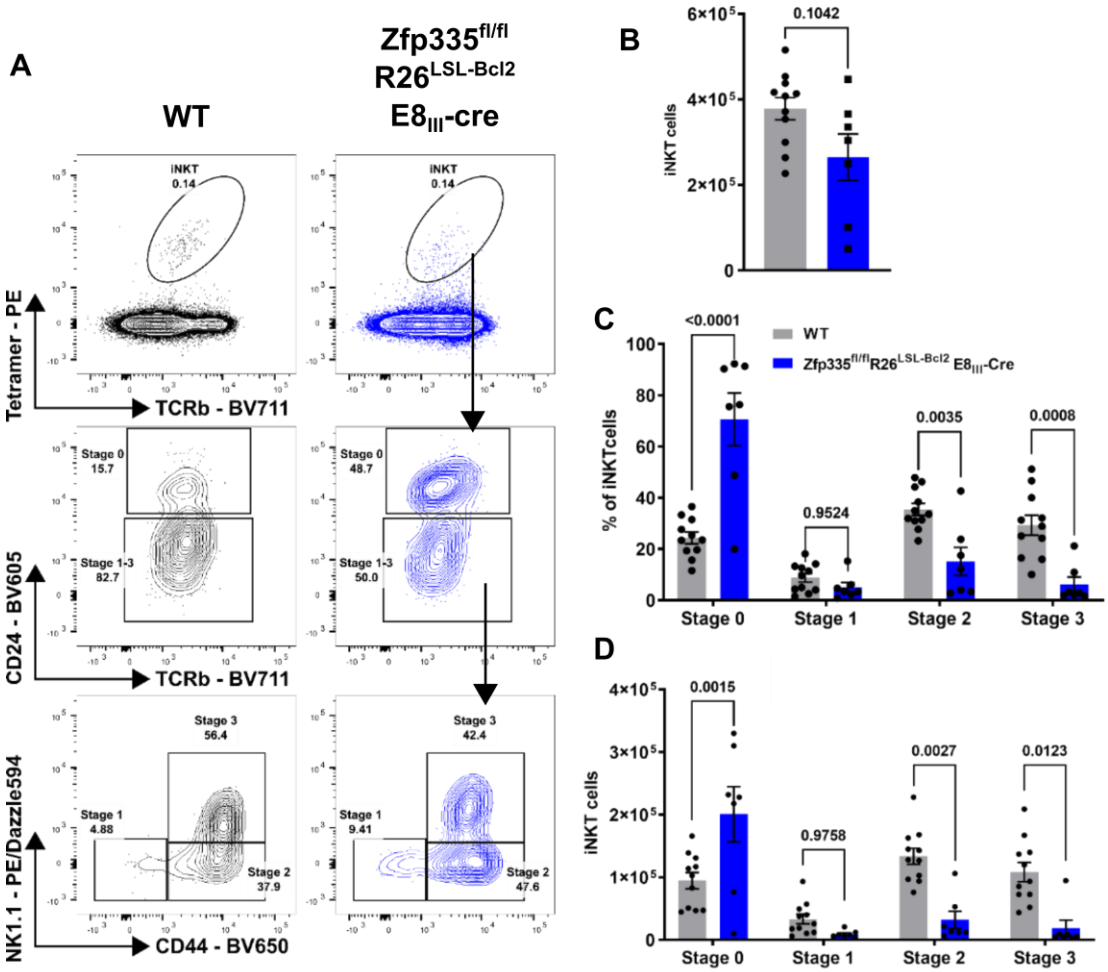

Figure 33: Bcl2 overexpression rescues Zfp335-deficient iNKT cell numbers but not effector differentiation.

Representative gating for identification of iNKT development. (B) Total thymic iNKT cell numbers and frequency (C) or total numbers of iNKT developmental stages in WT (n=11) or Zfp335$^{fl/fl}$ R26$^{LSL-Bcl2}$ E8III-cre (n=7) mice. Data are compiled from four independent experiments. P-values determined by Mann-Whitney U Test (B) or Two Way ANOVA with post-hoc Sidak's test. Plots show mean ± sem.

3.2.4 Zfp335-deficient thymic iNKT cell developmental defects are cGAS/STING-independent

We have established that loss of Zfp335 impairs thymic iNKT cell development through two mechanisms: (1) increased rates of cell death during the proliferative stage 0 and (2) defective effector differentiation. Since stage 0 of iNKT cell development is analogous to the DN4 stage we wondered if a similar mechanism was driving stage 0 iNKT cell death. Specifically, we tested the ability of the STING inhibitor H-151 to rescue iNKT cell numbers in Zfp335cKO mice. We assessed the total numbers and developmental staging of *Zfp335*$^{fl/fl}$ *E8*$_{III}$*-cre* iNKT cells following 7 days of H-151 treatment. Interestingly, unlike in DN4 cells, chemical inhibition of the cGAS/STING pathway had no effect on total numbers or developmental progression of Zfp335-deficient iNKT cells (Figure 34). This lack of developmental rescue suggests that the mechanism driving cell death in Zfp335-deficient iNKT cells is distinct from that of DN4 cells and independent of cGAS/STING.

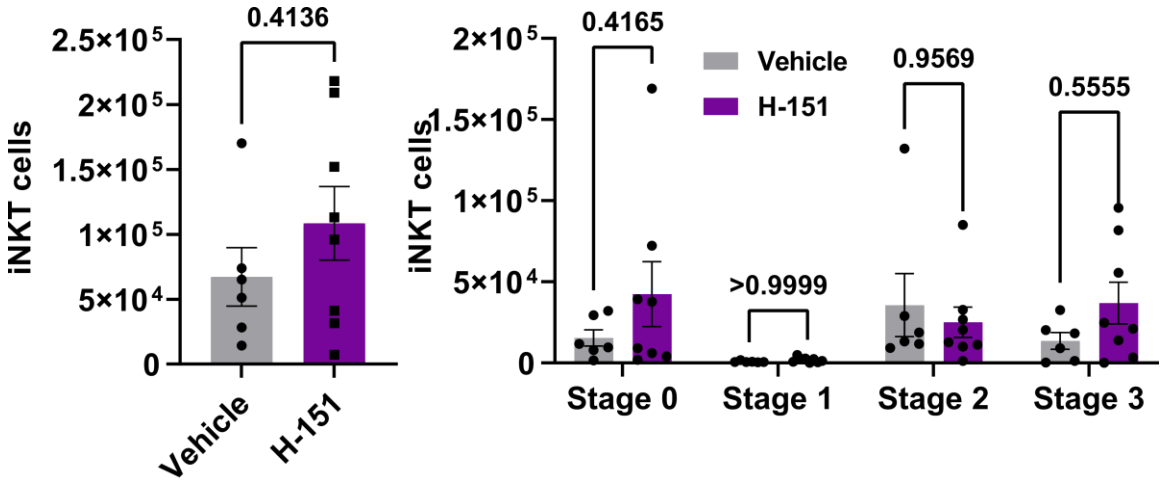

Figure 34: cGAS/STING inhibition fails to rescue Zfp335-deficient iNKT cell development

(Left) Total thymic iNKT cell numbers or stage-specific iNKT cell numbers in Zfp335$^{fl/fl}$ E8III-cre mice following 7 days of treatment with H-151 via intraperitoneal injection. P-values determined by Mann-Whitney U test (left) or Two-Way ANOVA with post-hoc Sidak's test. Plots show mean ± sem.

3.2.5 Il13^{YetCre} mediates efficient deletion immediately upon iNKT lineage commitment

We have established that loss of Zfp335 prior to selection and commitment to the iNKT lineage results in impaired development due cell death during the post-commitment proliferative phase and blocks effector differentiation. However, we could not definitively rule out that these defects were the result of additional effects occurring prior to lineage commitment. Therefore, we sought to identify a means of Zfp335 ablation following selection. To this end, we utilized previously published thymic iNKT cell scRNA-seq from WT mice (Baranek et al., 2020). These data were processed to remove low-quality cells followed by dimensionality reduction and pseudotime analysis. Through these analyses we identified 12 clusters of thymic iNKT cells (Figure

355A). Based on clustering and cell cycle phase (Figure 35B) clusters 3, 5, and 8 likely represent stage 0 iNKT cells. This assignment is supported by high levels of selection-associated expression of Etv5, Nr4a1, Id3, Egr1, Egr2, and CD69, as well as, expression of Cd24 (Figure 35C). The other clusters are a mixture of stage 1, 2, and 3 based on up-regulation of CD44 and Klrb1c (NK1.1) (Figure 35C).

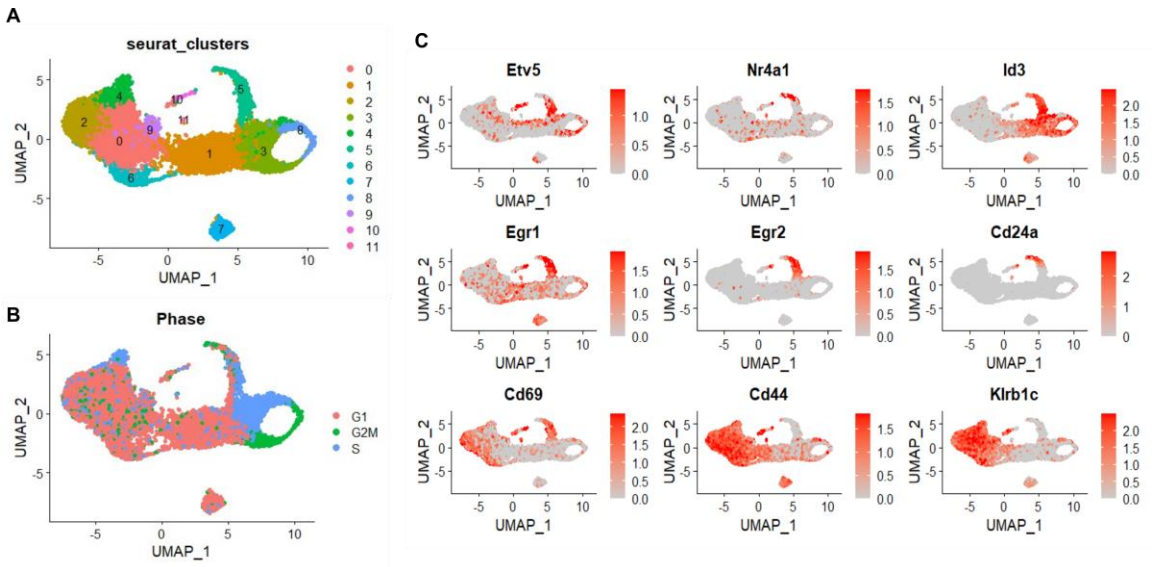

Figure 35: scRNA-seq analysis of thymic iNKT cell development

(A) Uniform manifold approximation projection (UMAP) dimensionality reduction and clustering of thymic iNKT cell scRNA-seq data. (B) UMAP of cell cycle phase. (C) UMAP of expression for key selection and differentiation associated genes. Data are derived from GSE141895.

Next, we focused on identifying gene expression associated with the immediate post-selection proliferative stage 0 phase of development. To this end, pseudotime analysis was performed using Monocle3 (Figure 36A). Interestingly, we found *Il13* expression correlates very well with this window in iNKT cell development (Figure

36B). Specifically, *Il13* expression is restricted to proliferating stage 0 iNKT cells (Figure 36C). Conveniently, an *Il13*-driven Cre mouse line (*Il13*^{YetCre}) was recently generated for lineage tracing studies of Th2 cells (Liang et al., 2011). Importantly, in this knock-in allele, Cre expression is allowed via an internal ribosomal entry site and maintains normal translation of IL-13. To test the utility of this mouse line in studying the role for *Zfp335* following iNKT lineage commitment we generated *Il13*^{YetCre} *R26*$^{LSL\text{-}ZsGreen}$ lineage-tracing reporter mice. Examination of recombination kinetics via flow cytometry showed minimal Cre activity in stage 0 iNKT cells and high levels of activity in stage 1-3 cells (Figure 36D). Therefore, these data suggest that *Il13*^{YetCre} would be an ideal strain for studying the role of Zfp335 in the differentiation of post-proliferation iNKT cells.

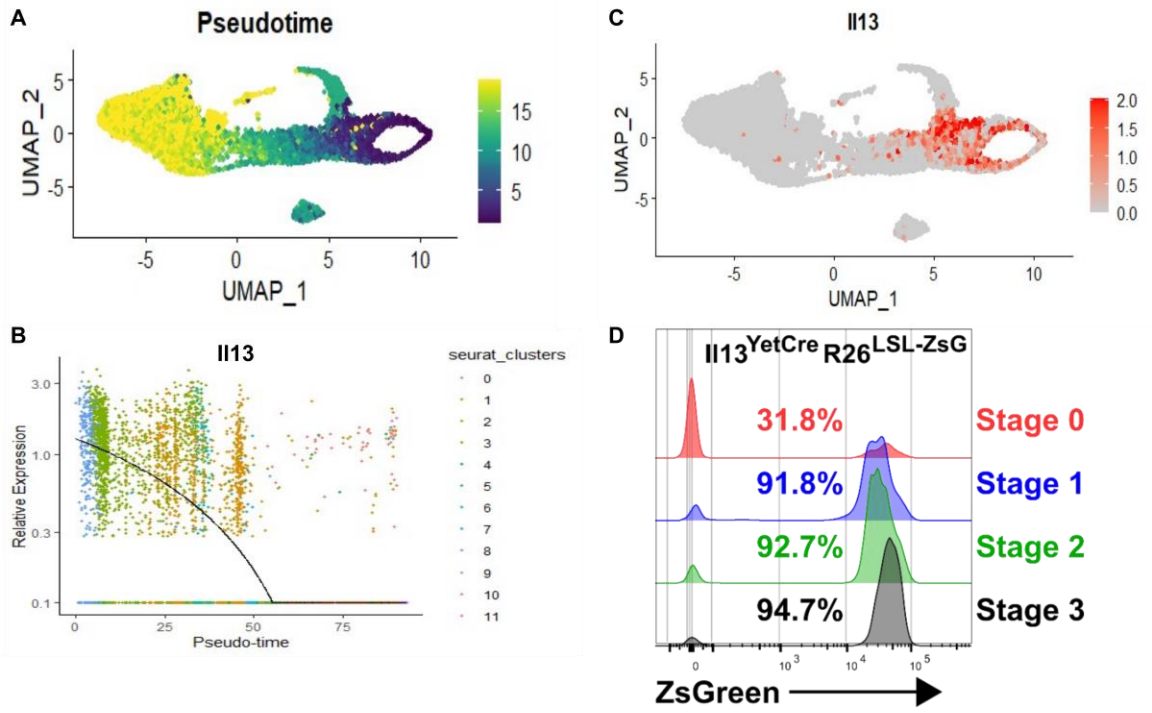

Figure 36: *Il13* is a stage 0 iNKT cell-specific gene

(A) UMAP of pseudotime analysis determined by Monocle3. (B) *Il13* gene expression in pseudotime. (C) UMAP of *Il13* expression in thymic iNKT cell scRNA-seq dataset. (D) Representative histograms for cre-mediated ZsGreen reporter expression across stages of iNKT cell development. (A-C) Data are derived from GSE141895.

3.2.6 Loss of Zfp335 following commitment impairs iNKT cell development

We have shown that loss of Zfp335 prior to iNKT lineage commitment impairs their development and differentiation. To test whether Zfp335 is required post lineage commitment we bred *Zfp335*$^{fl/fl}$ *Il13*$^{YetCre/YetCre}$ mice. Phenotypic characterization revealed a significant reduction in overall thymic iNKT cell numbers in Zfp335$^{fl/fl}$ Il13$^{YetCre/YetCre}$ but not Zfp335$^{fl/+}$ Il13$^{YetCre/YetCre}$ compared to Zfp335$^{+/+}$ Il13$^{YetCre/YetCre}$ mice (Figure 37A-B). These data suggest Zfp335 is haplosufficient in iNKT cell development.

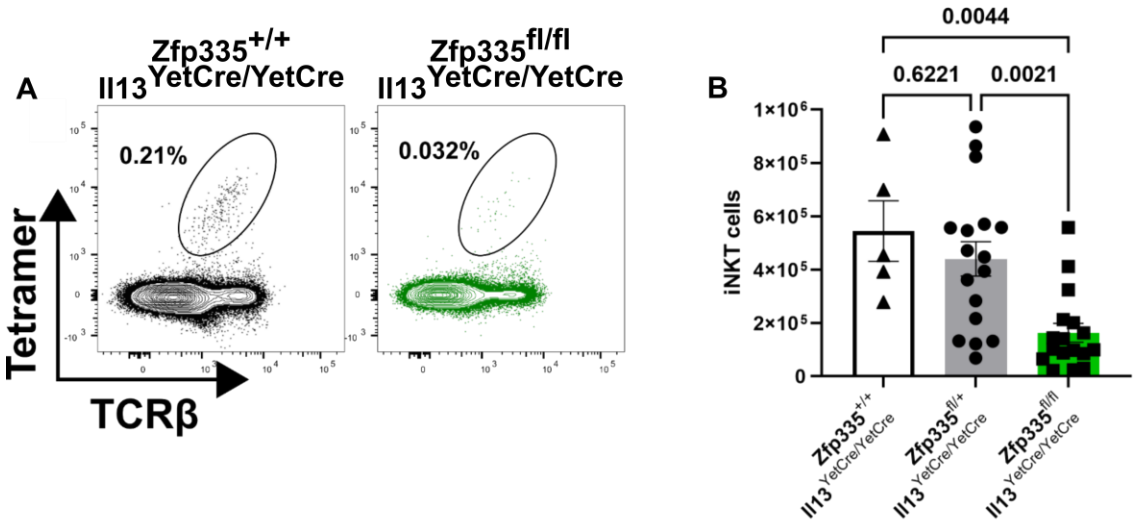

Figure 37: Loss of Zfp335 following lineage commitment leads to reduced iNKT cell numbers

Representative gating (A) and quantification (B) of total thymic iNKT cells in *Zfp335*$^{+/+}$ (n=5), *Zfp335*$^{fl/+}$ (n=16), or *Zfp335*$^{fl/fl}$ *Il13*$^{YetCre/YetCre}$ (n=17) mice. P-values determined by One-Way ANOVA with post-hoc Dunnett's test. Plots show mean ± sem.

Next, we sought to determine the effect of post-lineage commitment deletion of Zfp335 on iNKT cell differentiation. Examination of thymic iNKT cell stages revealed significantly reduced numbers of stage 2 and 3 iNKT cells resulting from loss of Zfp335 (Figure 38A-C). Total numbers and frequency of early iNKT cells (Stage 0-1) were unaffected by the loss of Zfp335 suggesting that it plays an important role in the differentiation process and acquisition of effector identity. To confirm this, we examined expression of lineage-defining transcription factors. Consistent with the hypothesis that Zfp335 regulates acquisition of effector identity we observed significant reductions in iNKT1 and iNKT2 but not iNKT17 or undifferentiated iNKT0 cells (Figure 38D-E). The lack of effect on iNKT17 differentiation may be due to effector lineage-specific roles for

Zfp335 or the very low abundance of iNKT17 cells within the thymus. In either case, these data demonstrate that Zfp335 plays a key role in the post-commitment effector differentiation of iNKT cells.

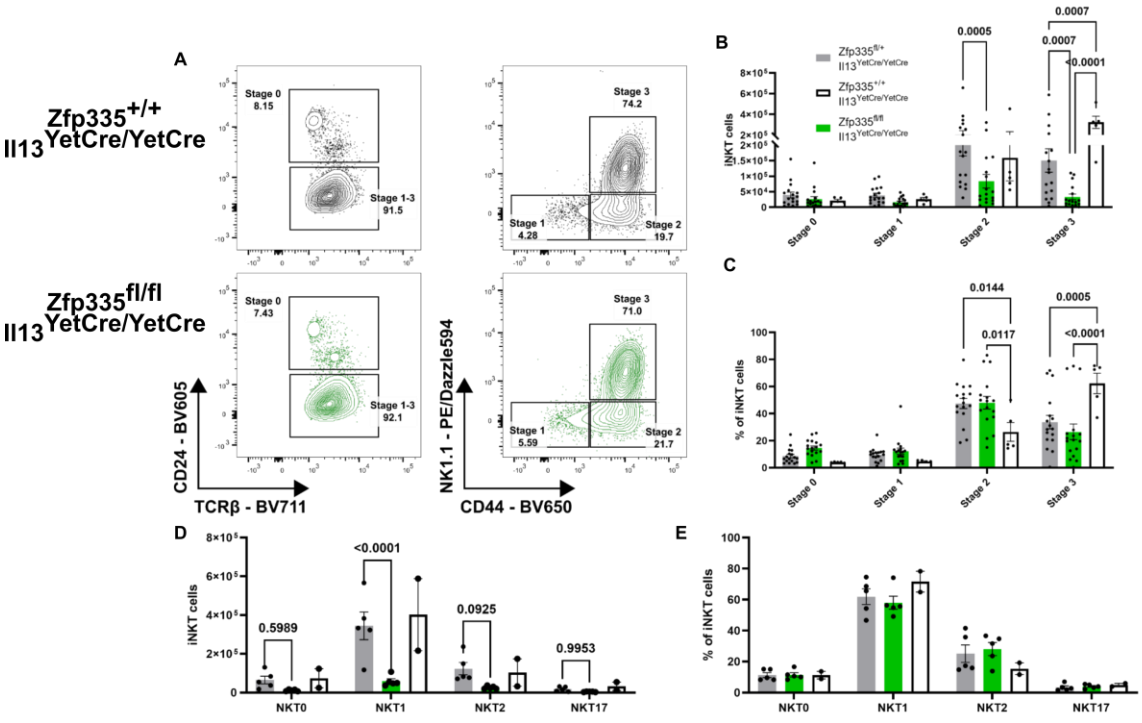

Figure 38: Zfp335 regulates thymic iNKT cell effector differentiation

Representative gating for identification of iNKT cell staging within the thymus. Total numbers (B) and frequency (C) of thymic iNKT cell stages. Total numbers (D) and frequency (E) of iNKT cell effector subsets within the thymus of *Zfp335*$^{+/+}$ (n=5), *Zfp335*$^{fl/+}$ (n=16), or *Zfp335*$^{fl/fl}$ *Il13*$^{YetCre/YetCre}$ (n=17) mice. P-values determined by Two-Way ANOVA with post-hoc Sidak's test. Plots show mean ± sem.

3.2.7 Zfp335 regulates thymic maturation of conventional αβ T cells independent of cGAS/STING

Next, we sought to determine the mechanism by which Zfp335 regulates T cell differentiation. Due to the very small number of iNKT cells in the absence of Zfp335 and

the parallel defect in thymic T cell maturation we hypothesized that a similar mechanism may regulate differentiation and maturation of both populations. We showed that Zfp335 regulates survival of early developing T cells through repression of cGAS/STING via Ankle2/Baf regulation. Therefore, we first sought to confirm that the conventional T cell maturation defect was not the result of cGAS/STING signaling. Mice were given H-151 for seven days then assessed for thymic CD4 and CD8 maturation. Consistent with our data regarding iNKT differentiation chemical inhibition of cGAS/STING signaling had no effect on the T cell maturation defect resulting from loss of Zfp335 (Figure 39).

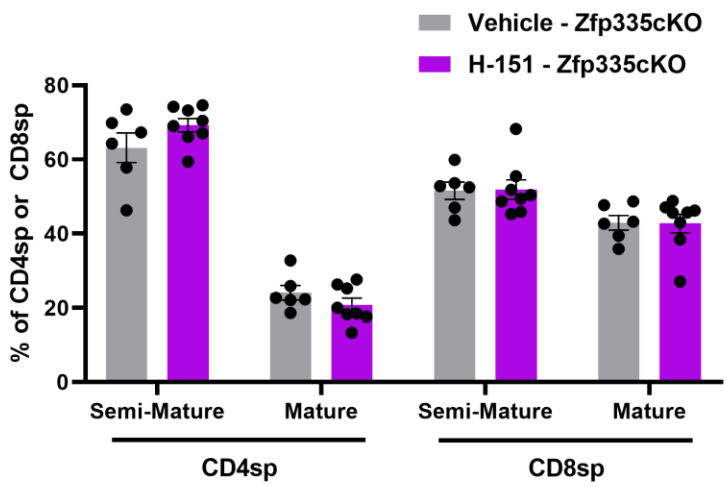

Figure 39: T cell maturation defect resulting from loss of Zfp335 is cGAS/STING-independent

Frequency of semi-mature and mature CD4SP or CD8SP thymocytes in $Zfp335^{fl/fl}$ $E8_{III}$-cre mice following seven days of H-151 treatment.

3.2.8 scRNA-seq identifies Smc2 as a Zfp335 target gene of interest in iNKT cell development and conventional T cell maturation

Since inhibition of cGAS/STING signaling was insufficient to rescue the maturation and differentiation defects in conventional $\alpha\beta$ and iNKT cells, respectively, we sought to identify common Zfp335-regulated gene(s) associated with the proliferative phase of iNKT cell development and conventional T cell maturation. To this end we analyzed previously published thymic iNKT cell and thymic CD8 T cell maturation (Chopp et al., 2020) scRNA-seq datasets. We began by identifying Zfp335-regulated genes differentially expressed between stage 0 and stage 1-3 (Figure 40A) or between cycling and non-cycling iNKT cells (Figure 40B). From this analysis we identified Smc2 as the most highly up-regulated Zfp335 target gene in stage 0 and cycling iNKT cells (Figure 40A-B). Smc2 expression also correlates well with pseudotime analysis (Figure 40 C-E).

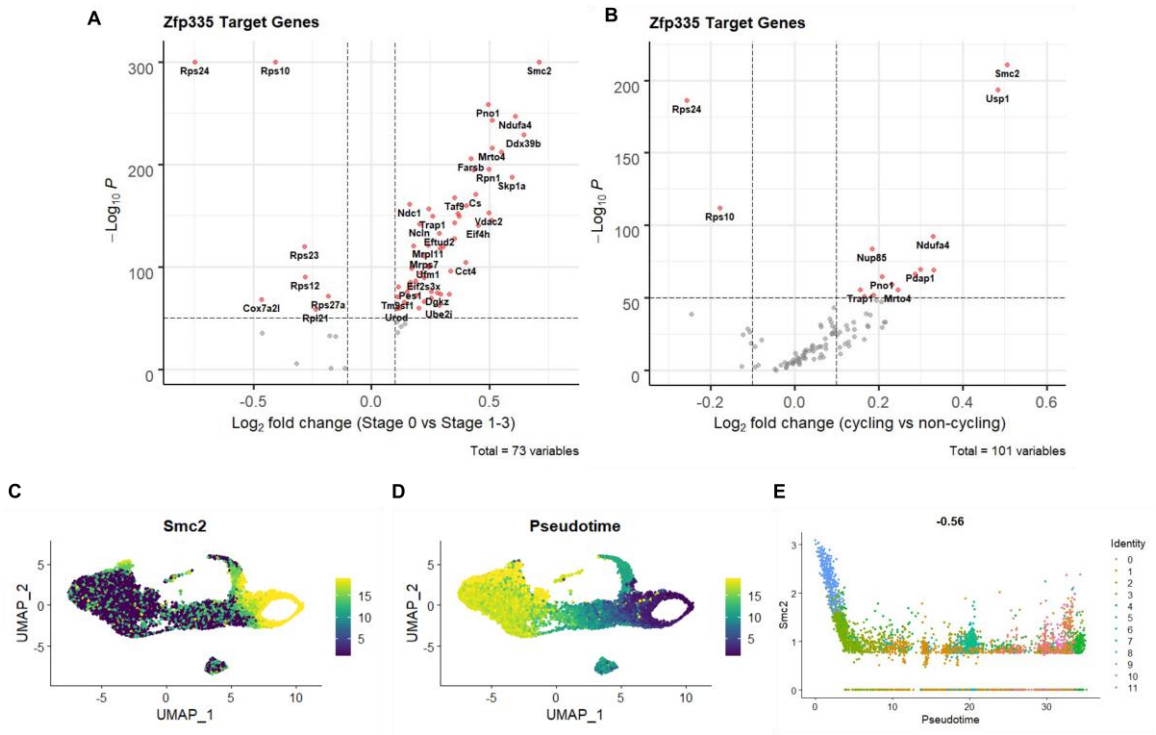

Figure 40: scRNA-seq identifies Smc2 as a Zfp335 target of interest in iNKT cell development

Volcano plots for differential expression of Zfp335 target genes in stage 0 vs stage 1-3 (A) or cycling vs non-cycling iNKT cells. UMAP of Smc2 expression (C) or pseudotime (D) in iNKT cell scRNA-seq data. (E) Scatter plot of Smc2 expression vs pseudotime. Value at top of plot is Pearson correlation. P-values determined by Wilcoxon Rank Sum test (A-B). Data are derived from GSE141895.

Next, we performed similar analyses using a scRNA-seq dataset generated from post-positive selection thymocytes derived from MHC-II-deficient mice (Chopp et al., 2020). We chose to focus our attention on CD8SP thymocyte maturation as the role for Zfp335 in CD4SP maturation has already been studied (Han et al., 2014). Following quality-control and dimensionality reduction we identified 9 unique clusters of thymocytes in various stages of CD8 T cell maturation from pre-selection through

terminal maturation (Figure 41A). Based on unique expression of Rag1 and Rag2, cluster 4 represents the developmentally earliest cells in the dataset (Figure 41B-C). Therefore, we next performed trajectory and pseudotime analysis using cluster 4 as the starting point (Figure 41D). Clusters 5 and 6 still express Cd4 (Figure 41E) but lack Runx3 (Figure 41F) and are therefore, classified as late positive selection DP thymocytes. Two main developmental branches were found by trajectory analysis (Figure 41D). Clusters 0, 1, 7, and 8 likely represent conventionally selected CD8SP cells based on Bcl2 expression (Figure 40G). The second branch leading to clusters 2 and 3 have likely undergone agonist selection based on expression of Bcl2l11 (Figure 41H). These assignments are in agreement with the original publication of this dataset (Chopp et al., 2020).

Next, we focused on exploring transcriptional regulation by Zfp335 in the CD8SP maturation process. Consistent with our observation that terminal T cell maturation is impaired by loss of Zfp335 we found that Zfp335 transcription factor activity increases as CD8 maturation progresses (Figure 41I-J). Based on the trajectory analysis clusters 7 and 8 likely represent two unique populations of mature CD8SP thymocytes. Thus, we next performed differential gene expression analysis to identify Zfp335-regulated genes whose expression is uniquely associated with these two populations. These analyses identified 4 Zfp335 target genes specifically up-regulated during CD8SP terminal maturation: Rps12, Rps24, Vars, and Smc2 (Figure 41K). Interestingly, up-regulation of

Smc2 is associated with both the phase at which iNKT cell development is disrupted and CD8SP maturation is blocked following loss of Zfp335.

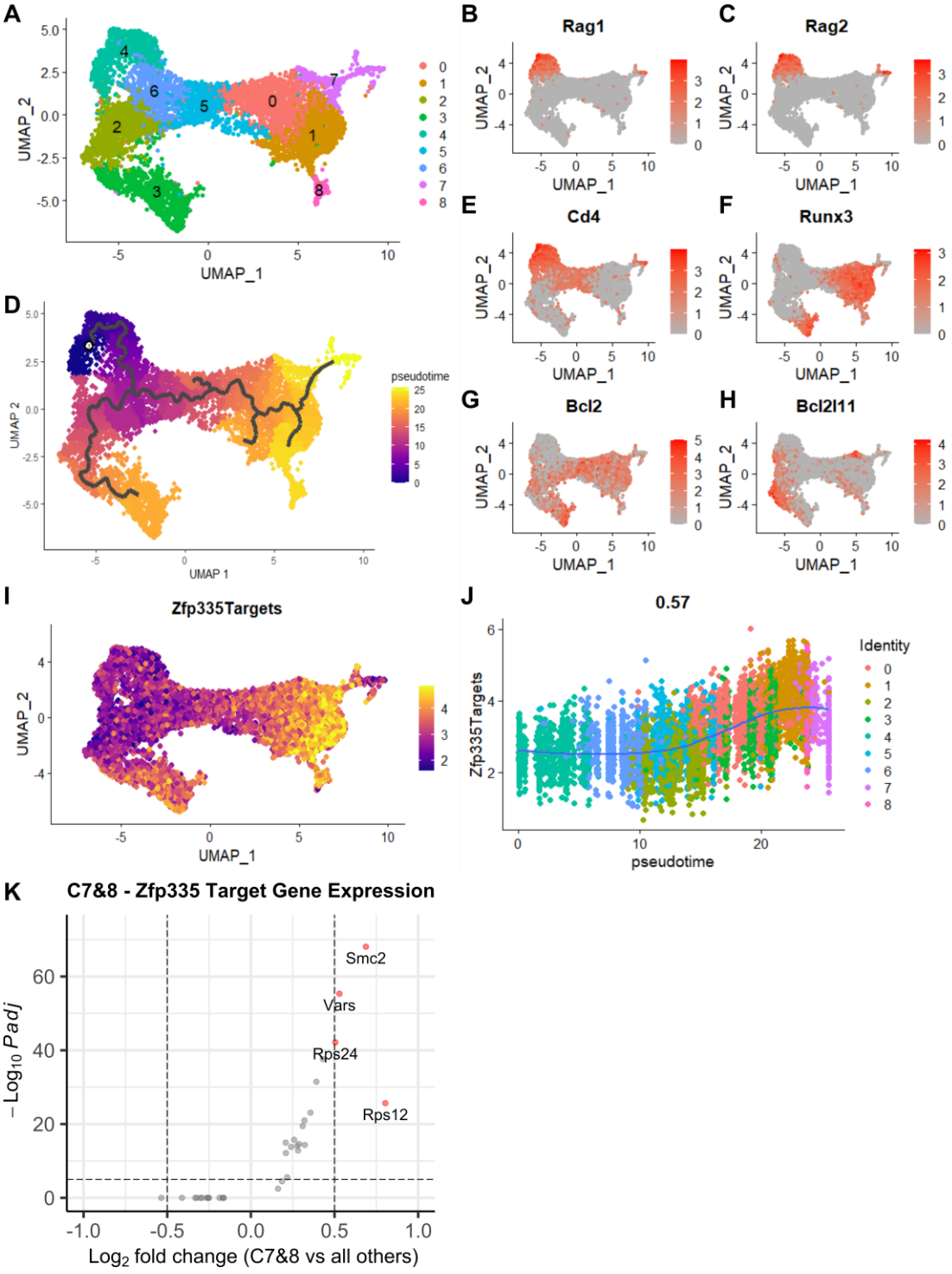

Figure 41: scRNA-seq analysis of CD8SP maturation identifies Zfp335 target genes of interest

(A) UMAP dimensionality reduction and clustering. UMAP projection of Rag1 (B) or Rag2 (C) expression. Pseudotime and trajectory analysis of CD8SP maturation determined by Monocle3. UMAP projection of *Cd4* (E), *Runx3* (F), *Bcl2* (G), or *Bcl2l11* (H). UMAP projection of Zfp335 target gene expression (I) and Zfp335 target gene expression in Pseudotime (J). Volcano plot of Zfp335 target genes differentially expressed in the most mature clusters (7 & 8) compared with all other clusters. Data are derived from GSE148981.

3.2.9 Loss of Zfp335 leads to impaired chromatin condensation in T cells

Smc2 encodes the Structural maintenance of chromosomes protein 2, a member of the cohesin-related condensin complex first identified in *Xenopus* egg extracts (Hirano & Mitchison, 1994). Smc2 and the condensin complex functions to promote chromatin condensation which is required for chromosome segregation during mitosis (Kimura et al., 2001; Strunnikov et al., 1995). Interestingly, while Smc2 has not been directly studied in T cell biology the condensin complex has been shown to play a vital role in T cell development (Gosling et al., 2007). More recently, the condensin complex was also shown to be required for the establishment and maintenance of the naïve T cell compartment and impaired T cell responses (Gosling et al., 2008). Disruption of the condensin complex results in reduced chromatin condensation in T cells (Rawlings et al., 2011).

Based on these previous studies we sought to determine if Zfp335 regulates chromatin condensation in maturing thymocytes. To test this, we utilized the chromatin condensation indicator dye NuclearID-Green which produces green fluorescence in a chromatin density-dependent manner. Consistent with previous findings related to

disruption of the condesin complex, Zfp335-deficient maturing CD8SP thymocytes exhibit significantly reduced chromatin compaction, possibly the result of reduced Smc2 expression (Figure 42A-B). Similarly, we observed significantly reduced chromatin compaction among Zfp335-deficient splenic T cells (Figure 42C). Specifically, compared to control, central memory CD4 and naïve, effector and central memory CD8 T cells exhibit significantly reduced chromatin condensation compared to WT controls (Figure 42D).

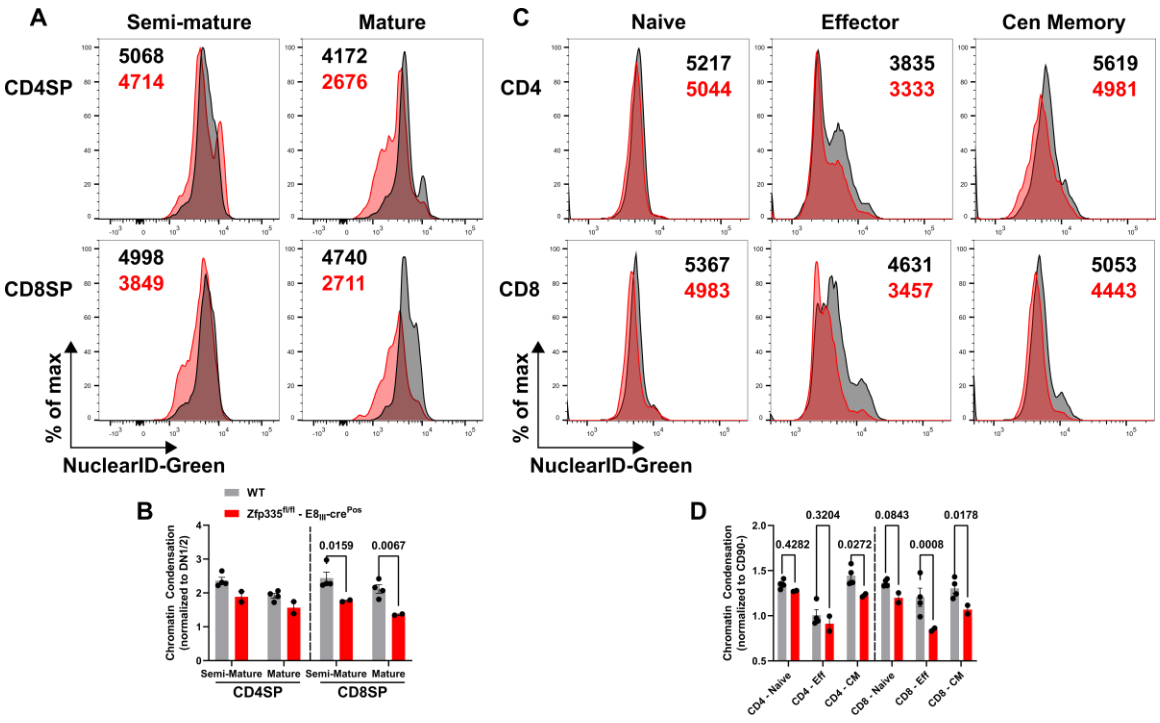

Figure 42: Loss of Zfp335 results in reduced chromatin condensation in thymic and peripheral T cells

Representative histograms (A) and quantification (B) of chromatin condensation for semi-mature and mature CD4SP or CD8SP thymocytes. Representative histograms (C) and quantification (D) of chromatin condensation among splenic CD4 or CD8 T cell

subsets. Data are from one experiment. P-values determined by Two-Way ANOVA with post-hoc Sidak's test. Plots show mean ± sem.

3.2.10 Zfp335 deficiency drives developmentally acquired CD8 T-bet expression

Previous studies have shown that widespread alterations in global chromatin architecture and accessibility are associated with thymocyte developmental progression (Hu et al., 2018). These large-scale changes in chromatin organization likely function to ensure proper regulation of developmentally programmed transcriptional activities required for normal T cell development and establishment of a naïve T cell compartment. Interestingly, TCR-mediated T cell activation promotes global 'relaxing' of chromatin compaction (Rawlings et al., 2011). This 'relaxing' may be required to gain accessibility to lineage-defining transcription factor loci required for effector differentiation.

We reasoned that impaired Smc2 expression resulting from loss of Zfp335 may be responsible for the reduced chromatin compaction observed in Zfp335cKO CD8SP thymocytes. Should this be the case, it is possible that loss of Zfp335 and Smc2 expression in DN4 thymocytes mediated by E8III-cre may lead to a failure to properly limit accessibility to lineage-defining TF loci thereby allowing pre-mature acquisition of effector identity. To test this, we first examined previously published ATAC-seq data (Yoshida et al., 2019) looking for lineage-defining transcription factors with transcription start sites which undergo dynamic accessibility regulation during T cell development. Among the TFs we found that T-bet (*Tbx21*) and RORγt (*Rorc*) loci are dynamically

regulated during the phase at which Zfp335 is lost in our model (Figure 43A-B). Specifically, the *rorc* locus continuously gains accessibility during the DN-DP transition while Tbx21 transiently opens at the DN4 stage. Conversely, Eomes or GATA3 lose or maintain stable accessibility throughout these stages of development (Figure 43C-D).

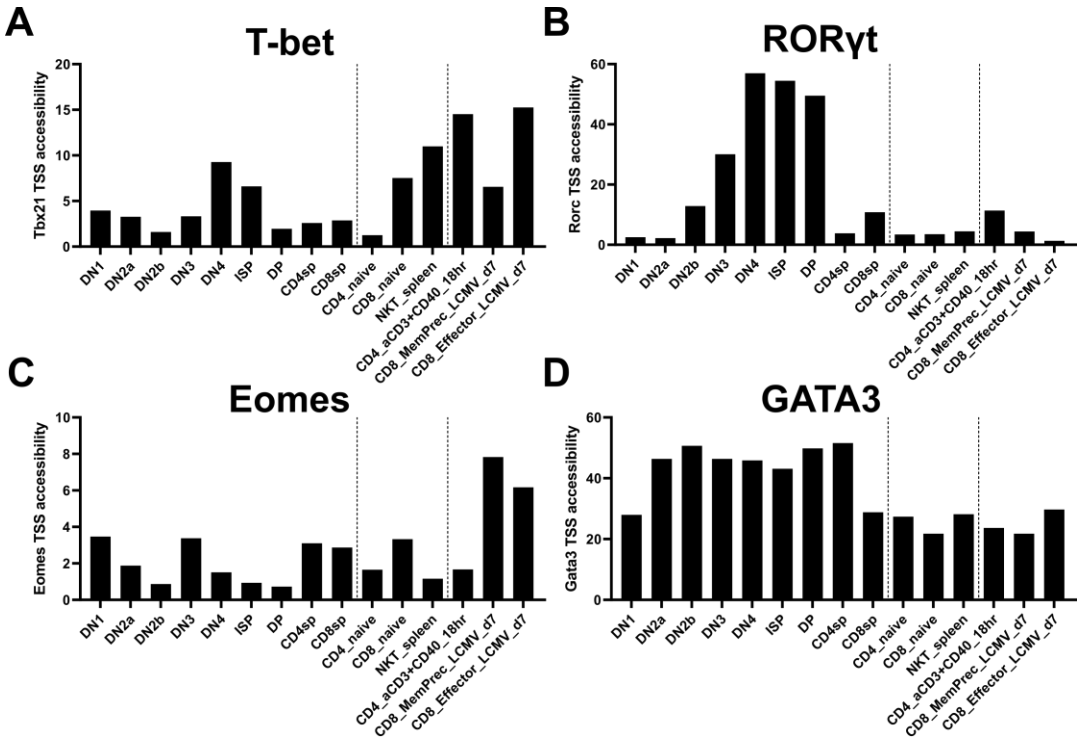

Figure 43: *Tbx21* and *Rorc* loci are dynamically regulated during T cell development

Transcription Start Site (TSS) accessibility for (A) T-bet (*Tbx21*), (B) RORγt (Rorc), (C) Eomes (*Eomes*), and (D) GATA3 (*Gata3*) measured by ATAC-seq across T cell development and in peripheral resting (CD4_naive, CD8_naive, NKT_spleen) or activated (CD4_aCD3+CD40_18hr, CD8_MemPrec_LCMV_d7, CD8_Effector_LCMV_d7) T cells. Data show average accessibility across biological replicates for each population. Data were derived from the Immgen ATAC-seq data browser (GSE100738)

Should our hypothesis be correct loss of Zfp335 and Smc2 would maintain these loci in a poised state allowing acquisition of effector identity through positive selection. Based on previous work, induction of RORγt expression via positive selection would impair CD8 T cell development (Martinez et al., 2015). Therefore, to test our hypothesis we examined expression of T-bet in thymic and peripheral CD8 T cells (Figure 44A). Consistent with our hypothesis, we observed significantly increased T-bet expression among Zfp335-deficient CD8SP thymocytes (Figure 44B-C) and splenic CD8 T cells (Figure 44D-E). Interestingly, T-bet expression among splenic Zfp335-deficient CD4 T cells was largely unaffected by the loss of Zfp335 (Figure 44F-G). Together, these data support our hypothesis that loss of Zfp335 impairs proper CD8 T cell maturation while allowing developmentally acquired effector identity.

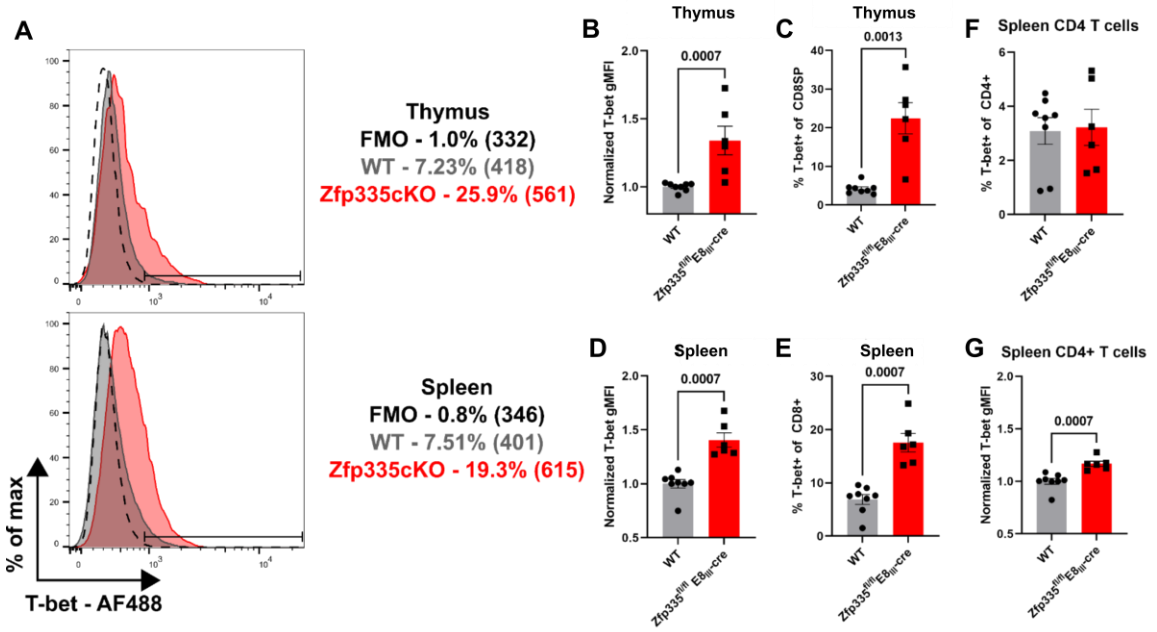

Figure 44: Loss of Zfp335 promotes thymic acquisition of T-bet expression in CD8 T cells

Representative histograms for T-bet expression in thymic CD8SP (top) or splenic CD8 T cells (bottom). Frequency and gMFI of T-bet expression to the right of each. Normalized T-bet expression among total CD8SP thymocytes (B) and frequency of T-bet$^+$ CD8SP thymocytes (C). Normalized T-bet expression among splenic CD8 T cells (D) and frequency of T-bet expressing CD8 T cells (E). Frequency (F) and normalized expression of T-bet (G) among splenic CD4 T cells from WT or *Zfp335$^{fl/fl}$ E8$_{III}$-cre* mice. Data are compiled from two independent experiments. P-values determined by Mann-Whitney U Tests. Plots show mean ± sem.

In summary, we show that Zfp335 regulates the development and maturation of both conventional and unconventional $\alpha\beta$ T cells at multiple levels. Zfp335 is required for iNKT cell survival during the post-commitment expansion phase and subsequent differentiation. Similarly, Zfp335 deletion significantly impairs the proper maturation of CD4 and CD8 conventional T cells and promotes developmental acquisition of an effector-like phenotype which was particularly evident among CD8 T cells. Our

bioinformatic analyses of previously published scRNA-seq data identify several potential Zfp335 target genes contributing to these developmental defects, among which Smc2 is the prime gene of interest.

3.3 Discussion

Here we show that Zfp335 plays in indispensable role in the development and maturation of both conventional and unconventional T cells. This is highlighted by the severe reduction in iNKT cells as well as naïve conventional T cells following genetic ablation of Zfp335. We show that these defects are mechanistically distinct from those observed in DN4 thymocytes (discussed in Chapter 2). We also show that these developmental defects occur at multiple levels including survival and maturation/ differentiation.

Interestingly, while the defects observed in iNKT cell and conventional T cell development share many parallels they are also distinct. Specifically, loss of Zfp335 leads to a severe impairment in iNKT effector differentiation, whereas, the same genetic perturbation promotes pre-mature effector differentiation in CD8 T cells. Through our bioinformatic analyses we identify Smc2 as the most significantly up-regulated Zfp335 target gene associated at the point in which both iNKT and conventional T cell development is blocked following loss of Zfp335. Smc2 is a component of the condensin complex which functions to promote chromatin compaction during cell division and alters global chromatin structure in resting cells. Condensin was previously shown to

play a critical role in T cell maturation and the establishment of a naïve T cell compartment (Gosling et al., 2008; Gosling et al., 2007; Rawlings et al., 2011). Therefore, we propose that Smc2 and the condensin complex functions to establish the proper chromatin context required for development of both T cell lineages. Should our proposed mechanism prove correct we would expect genome-scale alterations to the transcriptome of Zfp335-deficient iNKT and single-positive thymocytes.

Alternatively, loss of ribosomal proteins Rps12 and Rps24 which are regulated by Zfp335 may be responsible for the defects we observed. Ribosomal function is critical to effector differentiation as differentiation involves expression of genes previously silenced. However, recent studies suggest that innate-like and conventional T cells differentially regulate ribosomal gene expression with unconventional T cells expressing these genes at much lower levels (Gutierrez-Arcelus et al., 2019). Therefore, it is unlikely that these genes contribute to the iNKT cell phenotype in Zfp335-deficient mice. Should these Zfp335-regulated genes be important it is more likely that they play a role in conventional T cell development. Mutations in *Rps24* can cause Diamond-Blackfan anemia (DBA), a rare disease characterized by erythroid bone marrow failure (Ulirsch et al., 2018). Analysis of DBA patients suggests that these mutations do not lead to T cell defects (Iskander et al., 2019; Iskander et al., 2021). Additionally, study of Rps12 mutant mice showed no T cell deficits (Folgado-Marco et al., 2021). However, these studies did not thoroughly profile T cell subsets and increased *Rps12* expression is associated with T

cell maturation (Mingueneau et al., 2013). Therefore, further study of the role for these Zfp335-regulated ribosomal proteins in T cell development and maturation is warranted.

A finding of these studies that is of particular interest was the observation that loss of Zfp335 drives acquisition of a type 1 effector program in CD8 T cells. This phenotype is ideal for anti-cancer immune responses. A key aspect underpinning the efficacy of checkpoint blockade therapy is the induction of T-bet by CD8 T cells. T-bet expression facilitates CD8 effector function and trafficking to tumor sites (Berrien-Elliott et al., 2015). Additionally, T-bet functions to repress exhaustion-inducing genes such as PD-1 in CD8 T cells (McLane et al., 2021). Therefore, it will be interesting to test the tumor-killing capacity of Zfp335-deficient CD8 T cells and whether induction of this phenotype requires deletion during development. If loss of Zfp335 in mature CD8 T cells can induce T-bet expression and promote anti-tumor activities then genetic ablation of ZNF335 during CAR-T production may increase the efficacy of treatment and promote resistance to checkpoint inhibition.

In summary, we found that Zfp335 activity is critical to the development, maturation, and effector differentiation of conventional and unconventional $\alpha\beta$ T cells. In conventional CD4 and iNKT cells Zfp335 is required for maturation and effector differentiation, whereas, in CD8 T cells loss of Zfp335 promotes pre-mature acquisition

of effector identity. Zfp335 likely achieves these effects through transcriptional regulation of the condensin complex subunit Smc2.

4 Methods

4.1 Mice

B6.Cg-*Zfp335*^{tm1Caw} (Zfp335$^{fl/fl}$, Stock No. 022413), B6.Cg-*Rag2*$^{tm1.1Cgn}$/J (Rag2$^{-/-}$, Stock No. 8449), and B6J.129(B6N)-*Gt(ROSA)26Sor*$^{tm1(CAG-cas9*,-EGFP)Fezh}$/J (R26$^{LSL-Cas9}$, Stock No. 026175) mice were purchased from The Jackson Laboratory. C57BL/6J-Tg(Cd8a*-cre)B8Asin (E8$_{III}$-cre) mice were generously provided by Jung-Hyun Park (NIH). B6.129S-Tcrd$^{tm1.1(cre/ERT2)Zhu}$ (*Tcrd*^{CreERT2}) have been maintained in our colony since original development. A modified Ai6 targeting vector to drive conditional overexpression of Bcl2 was generated by cloning in mouse *Bcl2* cDNA (Transomic Technologies) using FseI and SfiI restriction sites. R26$^{LSL-Bcl2}$ mice were generated by the Duke University Transgenic Facility using G4 mouse embryonic stem cells. Animals were maintained under specific pathogen-free conditions at the Cancer Center Isolation Facility of Duke University Medical Center. All experimental procedures were approved by the Institutional Animal Care and Use Committee. All mice used in this study were 4-8 weeks old. For all experiments Cre-negative littermate controls were used unless otherwise stated.

4.2 Antibodies

All antibodies used in this study were purchased commercially and have previously been validated. Anti-TCRγδ (GL3) was purchased from BD Biosciences. Anti-TCRγδ (GL3), rabbit anti-Lamin B (10H34L18), rabbit anti-phospho-STING (Cat. PA5-

105674), rabbit anti-phospho-IRF3 (Cat. PA5-36775), mouse anti-dsDNA (AE-2), polyclonal rabbit anti-Banf1 (Cat. PA5-20329) and goat anti-rabbit IgG (H+L)-Alexa Fluor 647 were purchased from ThermoFisher Scientific. Anti-CD16/32 (2.4G2) was purchased from Tonbo Biosciences. Anti-CD90.1 (OX7), anti-CD90.2 (30-H12), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD44 (IM7), anti-CD25 (PC61), anti-CD62L (MEL-14), anti-TCRβ (H57-597), anti-CD27 (LG.3A10), anti-Bcl2 (BCL/10C4), anti-CD24 (M1/69), anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD19 (6D5), anti-Ly6G/Ly6C (RB6-8C5), anti-NK1.1 (PK136), anti-TER119 (TER-119), anti-CD117/c-kit (2B8), anti-Phosphoserine (M380B), mouse IgG1 isotype control (MG1-45), mouse IgG1 isotype control (MOPC-21) and Annexin V were purchased from Biolegend. PE conjugated CD1d-αGalCer tetramer was provided by the NIH Tetramer Core Facility.

4.3 Flow cytometry and cell sorting

Thymus or spleen tissues were harvested from 4-8 week old mice. Tissues were then dissociated in FACS Buffer (PBS supplemented with 2.5% FBS and 2mM EDTA) using a Dounce Homogenizer and filtered through 70μm nylon mesh (Genesee Scientific) to yield single-cell suspensions. For spleen samples, red blood cells were lysed using 1x RBC lysis buffer then resuspended in FACS buffer. 0.5-1x10^7 cells were stained with fluorescently labelled antibodies for 30 minutes at 4°C then washed with excess FACS buffer. Prior to analysis propidium iodide (Sigma-Aldrich, Cat. P4170) or DAPI (Sigma-Aldrich, Cat. D9542) were added to a final concentration of 0.5μg/mL or

100ng/mL, respectively for live/ dead discrimination. Cells were analyzed on a Fortessa X20 (BD Biosciences) or FACSCantoII (BD Biosciences) cytometer. For isolation of thymocyte subsets or virally transduced cells, sorting was performed using a FACSDiva (BD Biosciences) or Astrios (Beckman-Coulter) cell sorter. For sorting of thymocyte subsets *ex vivo*, staining included a lineage dump stain consisting of B220, CD11b, CD11c, CD19, GR-1, NK1.1, TCRβ, TCRγδ and TER119 antibodies. All analyses were performed using FlowJo v10 software (TreeStar). Detailed gating schemes are shown in Supp. Figure 11.

4.4 ChIP-seq analysis

Previously published E2A (Roy et al., 2018) (GSE89849) was generated by our lab or Zfp335 (Han et al., 2014) (GSE58333) ChIP-seq datasets were accessed through the NCBI GEO database. Reads were aligned to the mm9 genome using Bowtie (version 1.1.2, parameters: -chunkmbs 128 -mm -m 1 -best -strata -p 4 -S -q). Peaks were called and Bed and wiggle files generated for visualization using the Integrative Genome Browser using MACS2 (version 1.4.2, default parameters). Peaks were annotated using the NGS: Peak Annotation tool on Nebula.

4.5 Bulk RNA-seq

DP thymocytes (Lin^- $CD4^+$ $CD8^+$) were FACS sorted from total thymus of 7-week-old female $Zfp335^{fl/fl}$ $E8_{III}$-cre or $Zfp335^{+/+}$ $E8_{III}$-cre mice. Purified DP cells were lysed with Trizol and RNA isolated using the DirectZol Micro RNA prep kit (Zymo) according to

manufacturer's recommended protocol. gDNA was eliminated by on-column DNase digestion. Libraries were prepared using standard preparation protocols by BGI Genomics. 150bp paired-end sequencing was performed on the BGISEQ-500 sequencing platform.

Paired-end reads were mapped to the mouse mm10 reference genome using the HiSat2 software and count matrices generated using the featureCounts function of the Subreads software package. Differential expression analysis was performed using DeSeq2 implemented through iDep.91 (http://bioinformatics.sdstate.edu/idep90/). For identification of differentially expressed genes p-values were adjusted based on a 10% false discovery rate and genes with a fold-change of ≥ 2 and an adjusted p-value ≤ 0.05 were considered differentially expressed. Gene-Set Enrichment Analysis (GSEA) was utilized to identify enriched pathways based on differential expression analysis using pre-ranked gene lists with default parameters and the HALLMARKS gene sets (MSigDB). Pre-ranked gene lists were generated by multiplying the $-\log_{10}$(p-value) by the direction of the fold-change.

4.6 Cell Culture

OP9-DL1 cells, kindly provided by Maria Ciofani (Duke University) were cultured in MEMα (Gibco) supplemented with 10% FBS (Atlanta Biologicals) and 1x penicillin/ streptomycin (Gibco). HEK293T cells were cultured in DMEM supplemented with 10% FBS, 1x penicillin/ streptomycin, 1x non-essential amino acids and 1x

GlutaMAX. For OP9-DL1 culture of thymocytes, cultures were additionally supplemented with 5ng/mL recombinant mouse IL-7 (Biolegend). Scid.adh.2c2 cells were cultured in IMDM supplemented with 10% FBS (Hyclone), 1x penicillin/ streptomycin, 1x NEAA, 1x sodium pyruvate, 1x GlutaMAX, and 55μM β-mercaptoethanol. In some OP9-DL1 cultures 0, 1, 10, 100, or 1000 nM rapamycin (Cayman Chemicals), 0, 1, 10, 100, or 1000 nM everolimus (Cayman Chemicals), 10 μg/mL anti-mouse IFNAR-1 antibody (Biolegend), 5 μg/mL RU.521 (Invivogen), 0.5 μg/mL H-151 (Cayman Chemicals) or 25 or 250 μg/mL Cridanimod (Cayman Chemicals) were added. All cultures were maintained at 37^0C with 5% CO_2.

4.7 DN thymocyte enrichment

Total thymocytes were harvested from 4–8-week-old mice. Tissues were dissociated and strained through 30μm nylon mesh (Genesee Scientific). For purification of DN3/4 thymocytes cells were stained with biotinylated antibodies against B220, CD3, CD4, CD8, CD11b, CD11c, CD19, CD44, c-Kit, GR-1, IgM, NK1.1, TCRβ, and TCRγδ. For enrichment of total DN cells CD44 and c-Kit antibodies were excluded. Following antibody staining, cells were incubated with 50μL or 100μL of streptavidin magnetic particles (Spherotech, cat. SVM-40-100) / 10^7 cells at 2 x 10^7 cells/mL in FACS buffer for total DN enrichment or DN3/4 purification, respectively. Particle-bound cells were separated three times on a magnetic rack.

4.8 Retrovirus packaging and transduction

Retrovirus were generated by transfecting HEK293T cells with 1μg/mL each of MSCV transfer and pCL-Eco vectors using Lipofectamine 2000 (Invitrogen) or JetOptimus (Genesee Scientific) according to manufacturer's recommended protocols. Media was changed 24 hours post-transfection and viral supernatants harvested 24 hours later. DN3/4-enriched thymocytes were transduced with fresh viral supernatant via spinfection for 2 hours at 2300 rpm at 30℃ with 6.7μg/mL polybrene (Millipore). Following spinfection cells were transferred to culture on OP9-DL1 stromal cells for overnight culture. 18-24 hours post-infection virally transduced (DsRed+ or Thy1.1+) DN3 (CD25+) or DN4 (CD25-) were isolated by FACS sorting for an additional 3-5 days of culture in the OP9-DL1 culture system. For dual-targeting CRISPR experiments, equal volumes of sgRNA-Thy1.1 and -DsRed viral supernatants were mixed for transduction.

4.9 TCRα repertoire analysis

To prepare TCRα sequencing libraries one million DP or CD4SP thymocytes were sorted from WT or Zfp335cKO thymuses, lysed with Trizol and RNA purified using the Direct-Zol Micro Prep kit (Zymo Research) according to the manufacturer recommended protocol, including on-column DNase digestion. Sequencing libraries were prepared according to previously published methods (Mamedov et al., 2013; Turchaninova et al., 2016). Briefly, 5ng of total RNA was reverse transcribed with Trac-RT primer and SMARTnnnA template switch oligo (IDT) using SmartScribe Reverse

Transcriptase (Takara Bio). Following RT, samples were treated with 5U uracil DNA glycosylase (NEB) for 40 minutes at 37℃. cDNA was purified using Sera-Mag Carboxylate-Modified Magnetic SpeedBeads (GE Healthcare Life Sciences). cDNA was amplified for 18 cycles using Q5 high-fidelity polymerase (NEB) then purified with Sera-Mag SpeedBeads. Amplified cDNA was then dual-indexed by PCR with Q5 polymerase and purified again. Following indexing samples were quality-controlled by agarose gel electrophoresis, pooled at equal concentration and final libraries prepared using the NEBNext Ultra II DNA library preparation kit (NEB). Prior to sequencing libraries were gel extracted using the Zymoclean Gel DNA recovery kit (Zymo Research). All primer sequences can be found in Table 1. 300x300bp sequencing was performed on a MiSeq sequencer (Illumina).

TCRα sequencing analysis was performed as follows. First, raw fastq files were demultiplexed using the Checkout function of Migec v1.2.9 (Shugay et al., 2014) with the following parameters: -cute. Molecular identifier group (MIG) size distributions were determined using the Histogram function of Migec with default parameters. Based on these output UMIs were collapsed and filtered using the AssembleBatch function of Migec with the following parameters: --force-collision-filter –force-overseq 4. Collapsed reads were then merged using MiTools v1.5 (https://github.com/milaboratory/mitools) with the following parameters: -ss -s 0.7. Next, reads were aligned to the mouse TCRα locus using the align function of MiXCR v2.1.1 (Bolotin et al., 2015) with the following

parameters: --loci TRA -s mmu -OvParameters.geneFeatureToAligh=VTranscript. Aligned reads were then used to assemble clonotypes using the assemble function of MiXCR with the following parameters: -OassemblingFeatures=VDJRegion, then exported for downstream analysis using exportClones function of MiXCR. Gene segment usage was determined using the CalcSegmentUsage function of VDJTools v1.2.1 (Shugay et al., 2015) with the following parameters: -u. Gene segment usage was manually sorted based on genomic coordinates.

4.10 scRNA-seq library preparation

For single cell RNA-sequencing, DN4 thymocytes (Live Lin^- $CD4^-$ $CD8^-$ $CD25^-$ $CD44^-$) were sorted from one male and one female mouse pooled for each genotype using an Astrios Sorter. Sorted cells were encapsulated into droplets and libraries were prepared using a Chromium Single Cell 3' Kit using the v3.1 chemistry. 7,000 cells per genotype were targeted. scRNA-seq libraries were pooled and sequenced on a NovaSeq S Prime Flow Cell yielding an average depth of 71,584 or 67,816 reads per cells for Zfp335cKO or WT samples, respectively.

4.11 scRNA-seq analysis

scRNA-seq data were processed using the Cell Ranger pipeline (10x Genomics). FASTQ files were generated from raw base call logs (bcl2fastq, v2.20), aligned to the mouse mm10 (release 93) reference genome (cellranger, v3.1.0; STAR v2.5.3a) to generate raw gene count matrices. To ensure coverage of all relevant genes in our dataset, protein

coding, lincRNA, antisense, and all immunoglobulin and T cell receptor V, D, J and C genes, including pseudogenes were used for generation of the mm10 annotation file.

All downstream analyses were performed using the R software package Seurat (v4.0.0). Data was filtered to exclude cells with < 1,000 genes detected or < 1,000 UMIs. Doublets were excluded by filtering cells with > 60,000 UMIs. Low-quality cells were further filtered by removal of cells with > 7.5% mitochondrial gene expression. Gene expression matrices were then merged using the integration anchor method in Seurat based on the 6,000 most variably expressed genes, log normalized with a scaling factor of 10,000, scaled using variance stabilizing transformation and cell cycle phase determined using the CellCycleScoring function in Seurat with the built in S phase and G2M phase gene lists. Dropouts were imputed using the R package ALRA. Imputed data were only used for determination of frequency of gene expression and not differential expression analysis. Cell cycle phase was regressed, and principal component analysis (PCA) was performed on the 6,000 most variable genes. 35 principal components were selected for downstream analysis based on JackStraw analysis. Nearest neighbor graphs were constructed based on the first 35 principal components using the FindNeighbors functions in Seurat. Clusters were determined using FindClusters with a resolution of 0.5. Dimensionality reduction was performed by Uniform Manifold Approximation and Projection (UMAP) using 35 principal components. Gene expression was visualized by VlnPlot, DotPlot and FeaturePlot

functions in Seurat. Gene signature scores were calculated using SingleCellSignatureExplorer and previously described methods(Yang et al., 2018). Differential expression analysis was performed using the FindAllMarkers or FindMarkers functions in Seurat on the normalized count matrix with Wilcoxon Rank Sum Test (parameters: assay='RNA', logfc.threshold=0).

Previously published scRNA-seq data for thymic iNKT cells (GSE141895) or post-positive selection CD8 lineage cells (GSE148981) were accessed through Gene Expression Omnibus (NCBI). These data were processed according to the methods used in each original manuscript (Baranek et al., 2020; Chopp et al., 2020).

4.12 Cloning cDNA overexpression vectors

Bcl2 overexpression vector was generated by cloning Bcl2 cDNA (Transomic Technologies, Cat. TCM1304) into the pMSCV-loxp-dsRed-loxP-eGFP-puro-WPRE vector (Addgene #32702) using the EcoRI and NsiI restriction sites. Ankle2 cDNA (Transomic Technologies, Cat. TCM1004) was cloned into the MSCV-IRES-Thy1.1 vector using NEBuilder Hifi Assembly (New England Biolabs). All vectors were propagated in Stbl3 cells (ThermoFisher Scientific).

4.13 Generation of *Scid.adh.2c2-dCas9$^{10x\text{-}GCN4}$* CRISPRi cells

dCas9$^{10x\text{-}GCN4}$ (pHRdSV40-dCas9-10xGCN4_v4-P2A-BFP, Addgene #60904) was lentivirally transduced into Scid.adh.2c2 cells, following which BFP+ cells were isolated by flow cytometry. Single cells were then cloned into 96 well plates and screened for

knockdown efficiency using CD25 gRNA retroviral vectors. Clones exhibiting more than 90% CD25 downmodulation were expanded for use in our studies.

4.14 Generation of gRNA retroviral vectors

All gRNAs were designed using the CRISPick (Sanson et al., 2018) gRNA design tool. All gRNAs were cloned into expression vectors by annealing followed by ligation into a BbsI cleavage site. The basic gRNA expression vector used was the MSCV-mU6-sgRNA-hPGK-Thy1.1 (kindly provided by Maria Ciofani). Knock-out gRNAs were first cloned into this Thy1.1 backbone. To generate DsRed expressing vectors for dual targeting, Thy1.1 was removed by digestion with BamHI and EcoRI and replaced with DsRed Express II by NEBuilder Hifi Assembly. The CRISRPi retroviral vector was generated by first cloning the pSV40-scFv-GCN4-sfGFP-VP64-GB1-NLS (Addgene #60904) fusion construct into the MSCV-mU6-sgRNA-hPGK backbone followed by replacement of VP64 with KRAB using NEBuilder.

4.15 qPCR analysis of gene expression

Following viral transduction, Scid.adh.2c2.dCas9$^{10x\text{-}GCN4}$ cells were assessed for transduction efficiency by flow cytometry. For samples exceeding 90% GFP+ 10^6 cells were lysed in Trizol and RNA isolated using the Direct-Zol MicroPrep kit. 500ng of RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) with random hexamers according to the manufacturer's recommended protocol. 5ng of cDNA per 25μL reaction was then used for gene expression analysis with PowerTrack

Sybr Green Master Mix (Applied Biosciences) according to the manufacturer's recommended protocol using fast cycling conditions with an Eppendorf MasterCycler qPCR machine. Relative expression was determined using the ddCt method with Gapdh being used for normalization.

4.16 Proximity Ligation Assay for Baf phosphorylation

Proximity ligation assays were performed using the Duolink® flowPLA Detection Kit – Orange (Millipore Sigma, Cat. DUO94003) according to manufacturers recommended protocol with minor changes. Briefly, total thymocytes were prepared as described in flow cytometry and cell sorting methods section. 10^7 thymocytes were stained with surface antibodies to distinguish all major thymocyte subsets. Next, cells were fixed with 4% paraformaldehyde for 10 minutes, washed and permeabilized for 30 minutes at room temperature. After permeabilization, cells were blocked for 1 hour at 37^0C with 300µl of Duolink® blocking solution then stained overnight at 4^0C with purified mouse anti-Phosphoserine (Biolegend) or purified mouse IgG1 isotype control (clone MG1-45, Biolegend) and purified rabbit anti-Banf1 (ThermoFisher Scientific) or rabbit anti-Lamin B1 (ThermoFisher Scientific) diluted in Duolink® antibody diluent. After each step cells were washed twice with 1mL or Duolink® In Situ wash buffer. Next, cells were incubated for 1 hour at 37^0C with Duolink® In Situ PLA® Probe Anti-Rabbit PLUS (Sigma Millipore, Cat. DUO92002) and Duolink® In Situ PLA® Probe Anti-Mouse MINUS (Sigma Millipore, Cat. DUO92004) diluted in Duolink® antibody diluent.

Additional controls in which individual probes were omitted were also prepared. Following probe incubation, cells were washed then incubated for 30 minutes at 37^{0}C with 1x Duolink® ligation reaction mixture, washed again and incubated for 90 minutes with 1x Duolink® amplification reaction mixture. Following amplification, cells were incubated with 1x Duolink® Detection Solution – Orange for 15 minutes at 37^{0}C. Cells were finally washed, resuspended in PBS and assayed using a FortessaX20 cytometer (BD Biosciences).

4.17 Determination of nuclear envelope structure and measurement of cytosolic dsDNA

5x10^4 Hela cells per well were reverse transfected with 15pmol siRNA using Lipofectamine RNAiMax (ThermoFisher Scientific) in an 8 well chamber slide according to recommended protocols. ANKLE2 and universal non-targeting control siRNAs were purchased from IDT (Design ID: hs.Ri.ANKLE2.13). BANF-targeting siRNAs were purchased from ThermoFisher Scientific (IDs: s16807, s16808, 26065). 48 hours post-transfection cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and permeabilized with permeabilization buffer for 1h at RT temperature. Primary antibody Lamin B (Invitrogen, Cat. 702972) were added for overnight incubation at 4C and washed with 1X PBS for three time. After that, secondary antibody Alexa Fluor 647-conjugated goat anti-rabbit antibody (Invitrogen, Cat. A32733) were added for 12h at 4C in the dark. After washing with 1X PBS for three times, slides were mounted with DAPI-containing mounting media (VECTORLAB, Cat. H-1200). Images

were collected using Zeiss 780 upright confocal. To analyze nuclear structure DAPI channel images were converted to binary with ImageJ. Following binarization, the Watershed function was used to separate touching cells. Circularity was then determined with a minimum threshold of 500 px^2.

For analysis of *ex vivo* DN4 thymocyte nuclear envelope DN4 cells were isolated by magnetic bead-based purification. Following purification, cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature. Cells were then stained overnight with purified rabbit anti-Lamin B1 antibody, followed by incubation with goat anti-rabbit IgG AlexaFluor 488 secondary antibody (ThermoFisher Scientific) for 1 hour. Cells were spun onto slides using the CytoSpin4 centrifuge (ThermoFisher Scientific) and mounted with ProLong™ Gold Antifade Mountant with DAPI (ThermoFisher Scientific). For assessment of cytosolic dsDNA, DN3/4 thymocytes were isolated and expanded in OP9-DL1 culture for 3 days. Cells were harvested from culture, fixed with 4% PFA for 10 minutes at room temperature and permeablized for 5 minutes with 0.01% Triton X-100. Cells were stained overnight with purified mouse anti-dsDNA antibody (Clone AE-2, ThermoFisher Scientific, followed by incubation with donkey anti-mouse IgG AlexaFluor 488 secondary antibody and rat anti-mouse CD90.1 AlexaFluor 647 for 1 hour. Cells were adhered to slides and mounted with ProLong™ Fold Antifade Mountant with DAPI. For assessment of IRF3 translocation cells were prepared identically to the experiments measuring cytosolic dsDNA but stained with

rabbit anti-mouse phosphor-IRF3 (ThermoFisher Scientific) primary and goat anti-rabbit IgG AlexFluor 488 secondary and rat anti-mouse CD90.1 AlexaFluor 647 antibodies.

Microscopy images were acquired on the Zeiss 710 Inverted Laser Scanning Confocal Microscope (Duke University Light Microscopy Core Facility). For quantification, at least 20 cells per animal were imaged as z-stacks using the 63x oil immersion objective. Image quantification was conducted with the Imaris for Neuroscientists Cell Imaging Software v. 9.3.0 (Bitplane) using the Surfaces tool on the acquired DAPI signal to identify nuclei. Identified nuclei were then differentiated by mean fluorescent intensity of Lamin B1, and quantified. The percentage of nuclei identified as Lamin $B1^{high}$ and mean sphericity of all nuclei for each animal was used as a single n for statistical analysis. Additionally, mean DAPI intensity was quantified for individual nuclei, with each nucleus represented as a single data point. For analysis of cytosolic dsDNA, the Surfaces tool was used to define nuclei based on acquired DAPI signal and cell membrane was defined based on acquired CD90 signal. dsDNA was defined using the vesicles tool and cytoplasmic staining defined automatically. Within each experiment dsDNA abundance was determined for each individual cell and normalized to the average of all control cells in the experiment.

4.18 2',3'-cGAMP ELISA

To measure total cellular cGAMP DN3/4 thymocytes were expanded in OP9-DL1 culture for 3 days. Cells were then harvested, washed with PBS and lysed in 100µl

MPER buffer (ThermoFisher Scientific) for 15 minutes at room temperature. Lysates were cleared by centrifugation at 13,000rpm for 15 minutes. Total protein content for lysates were determined using Pierce Detergent-Compatible Bradford Assay (ThermoFisher Scientific) for normalization of cGAMP. cGAMP ELISAs performed using the 2′,3′-cGAMP ELISA (Cayman Chemicals) according to the manufacturer's recommended protocol.

4.19 In vivo H-151 treatment of mice

Mice were administered 750 pmol (210μg) of H-151 (Cayman Chemicals) or vehicle via intraperitoneal injection daily for 7 days beginning at 7 weeks of age. The vehicle for injections was sterile PBS + 10% Tween-80 (VWR).

4.20 In vivo generation of post-β-selection Rag2$^{-/-}$ thymocytes

9-week-old male and female Rag2$^{-/-}$ mice were administered 150μg of ULTRA-LEAF purified anti-CD3 (Biolegend) via intraperitoneal injection. 10 days post-injection thymi were harvested and subjected to overnight stimulation with STING agonist followed by assessment of apoptosis by Annexin V binding.

4.21 Assessment of chromatin condensation

To assess chromatin condensation thymocytes were isolated and stained *ex vivo* with 5μM NUCLEAR-ID Green detection reagent (Enzo Life Sciences) at 4°C for 30 minutes according to the manufacturer's recommended protocol. Cells were then

washed with FACS buffer and stained with surface antibodies as previously described. Data was acquired using a FortessaX20 cytometer.

4.22 Statistical analysis

Statistical tests were performed using GraphPad v9.0.0 (Prism). For graphs with multiple comparisons being made, two-way ANOVA was performed with post-hoc Sidak's test or Tukey's test for multiple comparisons. For comparisons of cell numbers, data was log transformed prior to statistical tests. For all Two-way ANOVA tests normality tests were performed to ensure normalcy assumptions were met. For graphs of single comparisons, a two-tailed Mann-Whitney test was used. All significant p-values are shown in each graph. For all experiments data are reported based on individual animals or biological replicates pooled from multiple animals. No reported data are from repeated measurements of the same samples. No statistical methods were used to predetermine sample size.

4.23 Primers

Table 1: Primers used for cloning, genotyping, qRT-PCR, and sequencing library preparation

Primer	Gene	Sequence	Purpose
Zfp335-F	*Zfp335*	CATGTGGTTTCTGGGAAAAACT	$Zfp335^{fl/fl}$ recombination
Zfp335-ex2F	*Zfp335*	GACCGTCCCAGGATTAAC	$Zfp335^{fl/fl}$ recombination
Zfp335-ex2R	*Zfp335*	CTCTTCCATGATCACTACCC	$Zfp335^{fl/fl}$ recombination
FseI-Kz-Bcl2-F	Bcl2	AAGGCCGGCCGCCGCCACC*ATGG CGCAAGCCGGGA*	Ai6-Bcl2 cloning

SfiI-Bcl2-R	Bcl2	AAGGCCTGTGTGGCC*TCACTTGTG GCCCAGGTATGCAC*	Ai6-Bcl2 cloning
Ankle2-NEB-F	Ankle2	AGATCTCTCGAGATCGATGC*ATGC TGTGGCAGCGGCTG*	MSCV-Ankle2-IRES-Thy1.1 cloning
Ankle2-NEB-R	Ankle2	TATCGGGAATTATCGATGCA*TCAC AGAGAAATGAAGTCCAGGGC*	MSCV-Ankle2-IRES-Thy1.1 cloning
mmGapdh-F	Gapdh	GTCATCCCAGAGCTGAACG	RT-qPCR
mmGapdh-R	Gapdh	TCATACTTGGCAGGTTTCTCC	RT-qPCR
mmAnkle2-F	Ankle2	TTAAACCGGGACCCTTTGAT	RT-qPCR
mmAnkle2-R	Ankle2	ATATGAGGATGGCCCTGTGA	RT-qPCR
mmZfp335-F	Zfp335	CCAGGAACAGACAGTGACCAA	RT-qPCR
mmZfp335-R	Zfp335	CCTTCCTGGACCTGGATATGA	RT-qPCR
mmBax-F	Bax	TGAAGACAGGGGCCTTTTTG	RT-qPCR
mmBax-R	Bax	AATTCGCCGGAGACACTCG	RT-qPCR
Zfp335_iT1	*Zfp335* Promoter	ttgtttGACCTCGTCGATGCCGGAGT	CRISPRi
Zfp335_iT2	*Zfp335* Promoter	ttgtttGCTGTGTCGCTCTCCGACTC	CRISPRi
Zfp335_iT3	*Zfp335* Promoter	ttgtttAGGCTCAGGTTAGCGGCAGC	CRISPRi
Zfp335_iT4	*Zfp335* Promoter	ttgtttCTCAGGTTAGCGGCAGCCGG	CRISPRi
Zfp335_iT5	*Zfp335* Promoter	ttgtttCTGCCGCTAACCTGAGCCTC	CRISPRi
Zfp335_iB1	*Zfp335* Promoter	aaacACTCCGGCATCGACGAGGTCaa	CRISPRi
Zfp335_iB2	*Zfp335* Promoter	aaacGAGTCGGAGAGCGACACAGCa a	CRISPRi
Zfp335_iB3	*Zfp335* Promoter	aaacGCTGCCGCTAACCTGAGCCTaa	CRISPRi
Zfp335_iB4	*Zfp335*	aaacCCGGCTGCCGCTAACCTGAGaa	CRISPRi

	Promoter		
Zfp335_iB5	*Zfp335* Promoter	aaacGAGGCTCAGGTTAGCGGCAGa a	CRISPRi
Zfp335_kT1	*Zfp335* exon 7	ttgtttGTACCCCGAGACCTCGACGG	*Ex vivo* CRISPR KO
Zfp335_kB1	*Zfp335* exon 7	aaacCCGTCGAGGTCTCGGGGTACaa	*Ex vivo* CRISPR KO
Zfp335_kT2	*Zfp335* exon 16	ttgtttACCACAATCATCTACCAGCA	*Ex vivo* CRISPR KO
Zfp335_kB2	*Zfp335* exon 16	aaacTGCTGGTAGATGATTGTGGTaa	*Ex vivo* CRISPR KO
Ankle2_kT 1	*Ankle2* exon 4	ttgtttGCGGAAAGCTGTCGAAAACG	*Ex vivo* CRISPR KO
Ankle2_kB 1	*Ankle2* exon 4	aaacCGTTTTCGACAGCTTTCCGCaa	*Ex vivo* CRISPR KO
Ankle2_kT 2	*Ankle2* exon10	ttgtttGGGAGCTAGCTCATGAGCTG	*Ex vivo* CRISPR KO
Ankle2_kB 2	*Ankle2* exon 10	aaacCAGCTCATGAGCTAGCTCCCaa	*Ex vivo* CRISPR KO
Banf1_kT1	*Banf1* exon 2	ttgtttTTGGTGACGTCCTGAGCAAG	*Ex vivo* CRISPR KO
Banf1_kB1	*Banf1* exon 2	aaacCTTGCTCAGGACGTCACCAAaa	*Ex vivo* CRISPR KO
Banf1_kT2	*Banf1* exon 2	ttgtttACTTCGTGGCAGAGCCCATG	*Ex vivo* CRISPR KO
Banf1_kB2	*Banf1* exon 2	aaacCATGGGCTCTGCCACGAAGTaa	*Ex vivo* CRISPR KO
Mb21d1_kT	*Mb21d1* exon 3	ttgtttTGATAAGAAGTGTTACAGCA	*Ex vivo* CRISPR KO
Mb21d1_kB	*Mb21d1* exon 3	aaacTGCTGTAACACTTCTTATCAaa	*Ex vivo* CRISPR KO
Tmem173_ kT	*Tmem173* exon 6	ttgtttCTACATAACAACATGCTCAG	*Ex vivo* CRISPR KO
Tmem173_ kB	*Tmem173* exon 6	aaacCTGAGCATGTTGTTATGTAGaa	*Ex vivo* CRISPR KO
DsRed-NEB-F	dsRed Express II	CCGACCTCTCTCCCCAGGGGATGG ATAGCACTGAGAAC	Replace Thy1.1 with dsRed in CRISPR KO vectors

DsRed-NEB-R	dsRed Express II	ATAAAATCTTTTATTTTATCGCTACTGGAACAGGTGGTG	Replace Thy1.1 with dsRed in CRISPR KO vectors
Trac-RT	TCRa C region	TTTCGGCACATTGATTTG	TCRa Rep-Seq (RT)
SmartNNNa	N/A	AAGCAGUGGTAUCAACGCAGAGUNNNNUNNNNUNNNNUCTTrGrGrG	TCRa Rep-Seq (RT)
Tcra-n1R	TCRa C region	ATTGGGCAGCCCTGATTGGTGCTGTCCTGAGACCGAG	TCRa Rep-Seq PCR1
M1SS	TSO	AAGCAGTGGTATCAACGCA	TCRa Rep-seq PCR1
M1S-B1	M1SS	NNNNTTGACTCAGTGGTATCAACGCAG	TCRa Rep-seq PCR2 (barcoding)
Z-B1	Tcra-n1R	NNNNGGCCACATTGGGCAGCCCTGATT	TCRa Rep-seq PCR2 (barcoding)
M1S-B2	M1SS	NNNNGGAACTCAGTGGTATCAACGCAG	TCRa Rep-seq PCR2 (barcoding)
Z-B2	Tcra-n1R	NNNNCGAAACATTGGGCAGCCCTGATT	TCRa Rep-seq PCR2 (barcoding)
M1S-B3	M1SS	NNNNTGACATCAGTGGTATCAACGCAG	TCRa Rep-seq PCR2 (barcoding)
Z-B3	Tcra-n1R	NNNNCGTACGATTGGGCAGCCCTGATT	TCRa Rep-seq PCR2 (barcoding)
M1S-B4	M1SS	NNNNGGACGGCAGTGGTATCAACGCAG	TCRa Rep-seq PCR2 (barcoding)
Z-B4	Tcra-n1R	NNNNCCACTCATTGGGCAGCCCTGATT	TCRa Rep-seq PCR2 (barcoding)
M1S-B5	M1SS	NNNNGCGGACCAGTGGTATCAACGCAG	TCRa Rep-seq PCR2 (barcoding)
Z-B5	Tcra-n1R	NNNNATCAGTATTGGGCAGCCCTGATT	TCRa Rep-seq PCR2 (barcoding)
M1S-B6	M1SS	NNNNTTTCACCAGTGGTATCAACGCAG	TCRa Rep-seq PCR2 (barcoding)
Z-B6	Tcra-n1R	NNNNAGGAATATTGGGCAGCCCTGATT	TCRa Rep-seq PCR2 (barcoding)
M1S-B7	M1SS	NNNNCTCTACCAGTGGTATCAACGCAG	TCRa Rep-seq PCR2 (barcoding)

Z-B7	Tcra-n1R	NNNNCTTTTGATTGGGCAGCCCTGATT	TCRa Rep-seq PCR2 (barcoding)

5. Future Directions

Through our studies we have uncovered multiple roles for the E protein target zinc finger transcription factor Zfp335 in the development and maturation of conventional and innate-like T cells. In early stages of conventional T cell development Zfp335 functions to sustain Ankle2 expression and repress cGAS/STING-mediated cell death. Similarly, Zfp335 functions to promote survival and repress apoptosis in immediate post-selection iNKT cells. During maturation, Zfp335 activity promotes acquisition of effector identity in iNKT cells and establishment of the naïve T cells in conventional T cells. Interestingly, these effects are dichotomous in CD4 and CD8 T cells with loss of Zfp335 impairing CD4 differentiation all together and driving developmentally acquired type 1 effector program in CD8 T cells. These discoveries have raised several questions warranting further study.

5.1 Does the presence of TCR excision circles (TREC) sensitize DN4 thymocytes to cGAS/STING mediated cell death?

One of the most surprising findings from our work was the discovery that DN4 thymocytes are specifically sensitive to cGAS/STING-mediated cell death even under weakly stimulating conditions. We showed that under sub-optimal treatment conditions with STING agonist DN4 cells are the only thymocyte subset susceptible to cell death. However, at ten-fold higher agonist concentrations all subsets were susceptible to death with a high degree of variability in sensitivity. We showed that this cell death mediated by this pathway depends on activities of pro-apoptotic Bcl2 family members.

Functionally, DN2 and DN4 cells are highly similar in that they are largely characterized by rapid proliferation. While both cell types are highly susceptible to STING-mediated cell death DN2 cells require significantly stronger signal to drive cell death. We reasoned that this differential sensitivity may be the result of cGAS/STING signaling driven by TRECs generated during TCRβ rearrangements gaining cytoplasmic access during cell division. Consistent with this hypothesis we found that $Rag^{-/-}$ DN4 cells generated by *in vivo* anti-CD3 treatment do not exhibit the heightened sensitivity of WT DN4 cells. Based on this finding it is reasonable to hypothesize that the result is due to TREC-driven cGAS/STING signaling. However, definitive evidence is lacking and warrants further study.

Should TRECs drive cGAS/STING signaling in DN4 cells and sensitize them to cell death this could have significant implications for genetic studies of T cell development. Conditional deletion mouse models utilizing Cre recombinases with activity in DN3 generate circular DNA like TRECs which could hold the potential to activate cGAS/STING in DN4 cells and sensitize them to cell death. Detection of cGAS-bound TRECs would require looking for non-germline encoded junctional sequences. Thus, definitive determination of TREC-driven cGAS signaling is likely to be very technically challenging due to the diversity of junctional sequences that can be generated during V(D)J recombination. However, utilizing a Cre-based recombination model on a Rag-deficient background would allow for simplified detection of cGAS-

bound excision circles compared with TRECs. Since efficiency of cGAS activation is dependent upon the length of dsDNA an ideal approach would involve insertion of a loxP-flanked innocuous DNA sequence several kilobases in length into a safe harbor locus such as Rosa26.

In addition to the implications in genetic studies of T cell development, cGAS recognition of TRECs may have significant implications for mature T cell biology. It was recently shown that TCR-mediated activation of mature peripheral T cells promotes accumulation of cytosolic dsDNA which drives cGAS/STING signaling influencing antitumor responses (W. Li et al., 2020). However, the source of this DNA was not determined. It is possible that TCRα-derived TRECs which are present in naïve T cells could be the source.

5.2 Does survival of DP thymocytes depend upon down-regulation of cGAS and STING?

We show that cGAS/STING signaling drives thymocyte cell death and some aspect of Rag activity sensitizes DN4 thymocytes to cell death. We reason that this sensitivity is the result of TRECs which may become cytoplasmic as a result of cell division. DP thymocytes undergo TCRα recombination which has the potential to generate substantially more TRECs due to the ability of TCRα loci to undergo several rounds of recombination. Previously published work suggests that expression of cGAS and STING is shut off during the DP stage. It is currently unclear why this may be the case. DP cells undergo selection upon functional TCR rearrangement which can result in

positive or negative selection depending on affinity. Since cGAS/STING signaling sensitizes T cells to apoptosis it is possible that transcriptional silencing of these genes is required to prevent excessive negative selection. Alternatively, evidence suggests that nuclear cGAS, while not enzymatically active, can function to block DNA repair (Liu et al., 2018). Therefore, perhaps silencing of cGAS expression is required for efficient repair of DNA damage resulting from V(D)J recombination. In either case, it would be interesting to explore the role for this pathway in stages of T cell development other than what was studied in this dissertation.

5.3 Is loss of Smc2 expression in Zfp335-deficient thymocytes responsible for differentiation defects observed?

In chapters 2 and 3 we show that loss of Zfp335 leads to aberrant and impaired T cell differentiation. In CD4 T cells the absence of Zfp335 prevents their maturation and differentiation whereas in CD8 T cells it promotes pre-mature acquisition of an effector-like phenotype characterized by T-bet expression. Similarly, loss of Zfp335 in iNKT cells precludes their normal thymic effector differentiation. Bioinformatic analyses identified Smc2 as a putative Zfp335 target that may contribute to the phenotypes. Smc2 is a component of the condensin complex which functions to promote chromatin condensation. We found that loss of Zfp335 results in significantly reduced chromatin compaction in maturing thymic and mature peripheral T cells. Interestingly, loss of Zfp335 in our model at the DN4 stage coincides with a brief point in T cell development at which the *Tbx21* locus briefly gains accessibility. We propose that the condensin

complex may be responsible for closing of this locus and that in the absence of Smc2 it remains open from the DN4 stage onward allowing for pre-mature induction of T-bet expression through positive selection.

At the time of writing this dissertation we do not yet have definitive proof for this hypothesis but further study is clearly warranted. Examination of chromatin accessibility, either globally or at the Tbx21 loci specifically, from the DN4 stage through terminal maturation will help determine the role for Zfp335 in regulating these processes. Additionally, retroviral transduction of Smc2 cDNA into HSCs followed by generation of bone marrow chimeras will allow for direct determination of the necessity of Smc2 expression in T cell maturation.

5.4 Does genetic ablation of Zfp335 enhance antitumor CD8 T cell responses and can ex vivo genetic manipulation enhance existing engineered T cell-based therapies?

In chapter 3 we show that impaired T cell maturation downstream of loss of Zfp335 drives developmental acquisition of T-bet expression by CD8SP thymocytes and that expression is maintained in the periphery. Induction of T-bet expression in CD8 T cells is vital to antitumor immunity induced by checkpoint blockade (Berrien-Elliott et al., 2015). Therefore, it would be of interest to determine whether genetic ablation of Zfp335 in mature naïve T cells yields the same effect. Additionally, due to the phenotype of Zfp335-deficient CD8 T cells it would be interesting to assess their ability to mediate antitumor immunity. Based on previous studies, I expect that Zfp335-deficient CD8 T

cells will exhibit enhanced cytotoxic activity. Should this be the case, CRISPR-mediated deletion of ZNF335 during CAR-T production may yield more effective CAR-T cells.

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Biography

Jeremy Ratiu began his research career during his undergraduate studies at Michigan State University in the laboratory of Dr. Jenifer Fenton. In May 2014 he received his Bachelor of Science degree in Genomics and Molecular Genetics from the Lyman Briggs College of Michigan State University.

Upon graduation he moved to the wilds of eastern Maine where he worked as a research assistant in the laboratory of Dr. David Serreze at The Jackson Laboratory (Jax). At Jax, his research focused on studying the role of class switch recombination and somatic hypermutation, MHC-mediated T cell activation, and T cell central tolerance in the development of Type 1 Diabetes. Through these studies Jeremy was involved in publishing seven manuscripts including:

1. Funsten M, Yurkovetskiy L, Kuznetsov A, Hansen C, Senter K, Lee J, **Ratiu J**, Dahal-Koirala S, Reiman D, Antonopoulos D, Dunny G, Sollid L, Serreze D, Khan A, Chervonsky A. "Microbiota-independent attenuation and microbiota-dependent promotion of type 1 diabetes by diet". *Cell*, *Manuscript in review.*
2. Presa M, Racine JJ, Dwyer JR, Lamont DJ, **Ratiu JJ**, Kumar Sarsani V, Chen YG, Geurts A, Schmitz I, Stearns T, Allocco J, Chapman HD, Serreze DV. "A hypomorphic *Nfkbid* allele represents an *Idd7* locus gene contributing to impaired thymic deletion of autoreactive diabetogenic CD8+ T-cells in NOD mice". *Journal of Immunology*. (2018). 201(7):1907-1917.
3. Racine JJ, Stewart I, **Ratiu J**, Christianson G, Lowell E, Helm K, Allocco J, Maser RS, Chen YG, Lutz CM, Roopenian D, Schloss J, DiLorenzo TP, Sereze DV. "Improved Murine MHC-Deficient HLA Transgenic NOD Mouse Models for Type 1 Diabetes Therapy Development." *Diabetes*. 67.5 (2018):923-935.
4. Wang Q, Racine JJ, **Ratiu JJ**, Wang S, Ettinger R, Wasserfall C, Atkinson MA, Serreze DV. "Transient BAFF Blockade Inhibits Type 1 Diabetes Development in Nonobese Diabetic Mice by Enriching Immunoregulatory B Lymphocytes Sensitive to

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5. **Ratiu JJ**[*], Racine JJ[*], Hasham MG[*], Wang Q, Branca JA, Chapman HD, Zhu J, Donghia N, Philip V, Schott WH, Wasserfall C, Atkinson MA, Mills KD, Leeth CM, Serreze DV. "Genetic and Small Molecule Disruption of the AID/RAD51 Axis Similarly Protects Nonobese Diabetic Mice from Type 1 Diabetes through expansion of Regulatory B lymphocytes." *Journal of Immunology*. (2017). 198(11):4255-4267.
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6. Fahey, J, Lyons, B, Olekszak, H, Mourino, A, **Ratiu, J**, Racine, J, Chapman, H, Baker, D, Hendrix, K. "Antibiotic associated manipulation of gut microbiota and phenotypic restoration in NOD mice." *Comparative Medicine*. (2017). 67(4):335-343.
7. Leeth, CM, Racine, J, Chapman, HD, Arpa B, Carrillo, J, Carrascal, J, Wang, Q, **Ratiu, J**, Egia-Mendikute, L, Rosell-Mases, E, Stratmann, T, Verdaguer, J, and Serreze, DV. "B-Lymphocytes Expressing an Ig Specificity Recognizing the Pancreatic β-Cell Autoantigen Peripherin Are Potent Contributors to Type 1 Diabetes Development in NOD Mice." Diabetes 65.7 (2016): 1977-987

Following three years at Jax Jeremy moved on to start his Ph.D. studies at Duke University in 2017. He spent his first four years at Duke in the laboratory of Dr. Yuan Zhuang studying the role of Zfp335 in T cell development and function. His last year of study was spent in the laboratory of Dr. Mari Shinohara where he is concluding his doctoral research. During his time at Duke, Jeremy served as Student Representative for the Department of Immunology and received the Bernard Amos Memorial Award (2021) and Duke Immunology Distinguished Service Award (2021). While at Duke he authored seven additional manuscripts including:

1. Wang Q, Power J, Wang L, Li L, **Ratiu J**, Mihai A, Dong X, Zhuang Y, Li QJ. "SWI/SNF complex regulates esophageal epithelial cell differentiation with mosaic analysis. *Manuscript in preparation*.

2. Liao TC[*], **Ratiu JJ**[*], Shinohara ML. "Early diversity of monocytes in host defense against acute pulmonary infection by *Cryptococcus neoformans* and SARS-CoV2". *Manuscript in preparation*.
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3. Bapoo A, **Ratiu JJ**[ŧ]. "Integrated immune cell-cell communication networks underpinning Rheumatoid Arthritis pathogenesis". *IJHSR. Manuscript submitted.*
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4. Tomaszewski WH, Waibl-Polania J, Chakroborty M, Perera JJ, **Ratiu J**, Miggelbrink A, McDonnell D, Khasraw M, Ashley D, Fecci PE, Racioppi L, Sanchez-Perez L, Gunn MD, Sampson JH. "Stromall CaMKK2 promotes immunosuppression and checkpoint blockade resistance in Glioblastoma". *Nature Communications. Manuscript in review.*

5. **Ratiu JJ**[*,ŧ], Barclay W, Wang Q, Wellford S, Mehta N, Harnois MJ, DiPalma D, Roy S, Contreras AV, Shinohara ML, Wiest D, Zhuang Y. "Loss of Zfp335 triggers cGAS/STING-dependent apoptosis of post-β-selection pre-T cells". *Nature Communications. Manuscript in review.*
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7. Wang Q, Lin Y, Zhang B, Wu J, Roy S, **Ratiu JJ**, Xu Y, Dai M, Hale LP, Li QJ, Zhuang Y. "A mosaic analysis system with Cre or Tomato expression in the mouse". *Proc Natl Acad Sci USA.* (2020). 117(45):28212-28220.