

The Role of Chromodomain Helicase DNA Binding Protein 6 (CHD6) in Thymic Epithelial Cell Development and Function



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II. Abstract

Thymic epithelial cells (TECs) uniquely have broad transcriptional ability to express over 90% of all protein-coding genes, including those which are tissue-restricted, through a process referred to as promiscuous gene expression (PGE). PGE is in part under the control of the autoimmune regulator (AIRE) in TECs, although the precise molecular mechanisms by which AIRE operates to enable PGE, are not fully understood. Chromodomain helicase DNA binding protein 6 (CHD6) is a DNA-dependent ATPase, which plays a role in chromatin remodeling, and it has been proposed as one of the AIRE partners. The overall aim of this doctoral thesis is to define and understand the role of CHD6 in thymus biology. A mouse model, which is conditionally deficient of CHD6 in TECs (designated as *Chd6*^{TEC^{-/-}}), was generated to investigate the proposed objective. Phenotypic analyses of *Chd6*^{TEC^{-/-}} mice demonstrated no differences in the qualitative and quantitative measures of TECs, thymocytes and peripheral T cells compartment, indicating non-essential role of CHD6 in the development of thymic epithelial cells and thymocytes maturation in general. Interestingly, genome-wide transcriptomic data and analysis of tissue-specific-antigen in TECs revealed that CHD6 is a positive regulator of PGE, predominantly among genes decorated with repressive histone marks. CHD6 is proposed to regulate PGE by: i) localising and recruiting AIRE protein complexes to specific loci possessing DNA strand breaks and decorated with specific repressive histone marks, and ii) changing the chromatin accessibility of the particular loci. Confocal imaging confirmed the physical proximity of CHD6 and AIRE in medullary TEC which corroborates the proposed functional interaction. Additionally, *Chd6*^{TEC^{-/-}} mice are observed to be more susceptible to organ-specific-autoimmunity. In summary, this study has demonstrated CHD6 as a novel regulator of PGE and its crucial role in central tolerance induction.

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VI. Abbreviations

AADC	Aromatic L-amino acid decarboxylase
AIRE	Autoimmune regulator
APC	Antigen-presenting cells
APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy syndrome
APS1	Autoimmune polyendocrinopathy syndrome type 1
ATAC-seq	Assay for transposase-accessible chromatin, followed by deep sequencing
BCR	B cell receptor
BRK	<i>Brahma</i> and <i>Kismet</i>
CARD	Caspase-activation recruitment domain
cDNA	Complementary DNA
CDR	Complementarity-determining regions
CFTR	Cystic fibrosis transmembrane conductance regulator
CHD	Chromodomain-helicase-DNA-binding
ChIC	Chromatin immunocleavage
ChIP	Chromatin Immunoprecipitation
CMJ	Corticomedullary junction
cTEC	Cortical TEC
CUT&RUN	Cleavage Under Targets and Release Using Nuclease
DAPI	4'6-Diamidino-2-Phenylindole, Dihydrochloride
DC	Dendritic cells
DDR	DNA damage response
DEG	Differentially expressed genes
DN	Double-negative
DNA-PK	DNA-activated protein kinase
dNTP	Deoxynucleotides
DP	Double-positive
DSB	Double strand breaks
DUF	Domains of unknown functions
E #	Embryonic day #
ETP	Early thymic progenitor
FDR	False Discovery Rate
FEZF2	Forebrain-expressed zinc finger 2
FFPE	Formalin-fixed paraffin embedded
FRET	Fluorescence resonance energy transfer
GAD	Glutamic acid decarboxylase
gMFI	Geometric mean fluorescence intensity
GP2	Glycoprotein2
GWAS	Genome-wide association study
H&E	Haematoxylin and Eosin
H2AX	H2A histone family member X
HA	Human influenza hemagglutinin
IM	Immature
INO80	Inositol requiring 80
IRBP	Interphotoreceptor retinoid-binding protein
ISWI	Imitation switch
ITGB4	Integrin Subunit Beta 4
iT _{reg}	Induce T _{reg}
K #	Cytokeratin #
L1CAM	L1 Cell Adhesion Molecule
LMPP	Lymphoid-primed multipotent progenitor
M	Mature
MHCI	MHC Class I
MHCII	MHC Class II
mTEC	Medullary TEC

NLS	Nuclear-localisation signal
NQO1	NAD(P)H-quinone oxidoreductase
nT _{reg}	Natural T _{reg}
PALM	Fluorescence photo-activated localisation microscopy
PARP1	Poly- (ADP-ribose) polymerase 1
PC	Principal component
PCA	Protein-fragment complementation assays
PCC	Pearson's correlation coefficient
PGE	Promiscuous gene expression
PHD	Plant homeodomain
PLA	Proximity ligation assay
PLIC	Proximity ligation imaging cytometry
PMA	Phorbol 12-Myristate 13-Acetate
PRRs	Pattern-recognition receptors
PTM	Post-translational histone modifications
qRT-PCR	Real Time Quantitative PCR
RNAPII	RNA-Polymerase II
ROI	Region of interest
SM	Semimature
SP	Single positive
STED	Stimulated emission depletion
STORM	Stochastic optical reconstruction microscopy
SWI/SNF	Mating-type switching/sucrose non-fermenting
TCR	T cell receptor
TEC	Thymic epithelial cell
TES	Transcription end site
T _{FH}	T-follicular helper cells
TOP	Topoisomerases
TPM	Transcripts Per Million
TRA	Tissue-restricted antigen
T _{reg}	Regulatory T cells
TRG	Tissue-restricted gene
TSP	Thymic seeding progenitor
TSPAN8	Tetraspanin-8
TSS	Transcriptional start site
UEA1	Ulex Europeus Agglutinin 1

Chapter 1 Introduction

1.1. Immune system

A comprehensive and effective immune system is fundamental for the survival of humankind against multiple disease-causing pathogens and malignant transformed cells. Whilst it is crucial to be broadly comprehensive in detecting a wide range of invading pathogens, our immune system needs to be specifically discriminative in differentiating between our self and non-self antigens to prevent the development of autoimmune diseases ^{1,2}. It is therefore critical for our immune system to orchestrate a broad scope of protective approaches to enable effective recognition and removal of pathogens, whilst preventing devastation of its tissues as a consequence. This is possible through a network of physical barriers, multiple chemical mediators and diverse types of cellular interactions ².

Our immune system can be classified into two effector types namely the innate and adaptive immunity^{1,3,4}. They are distinctively classified based on the speed and specificity of their immunological response against the invading pathogen. Despite differences in their cellular components and mechanisms of action, both the innate and adaptive immune systems are synergistically cooperating in ensuring an effective elimination of pathogens whilst maintaining integrity of the host.

1.1.1. Innate immune system

The innate immunity is an evolutionarily conserved protection mechanism present within multicellular organisms and acts as the first line of defence. The innate immune system generally consists of three main elements which are: i) mechanical barriers; ii) various chemical mediators, and iii) multiple effector cells ⁴⁻⁶. The skin and mucosal lining present in the gastrointestinal, respiratory and genitourinary tracts, are the main mechanical barriers preventing the entrance of intruding pathogens ⁶.

When the host mechanical barriers fail to prevent pathogen invasion, the other elements of innate immune response are rapidly triggered, inducing multiple cascades of

immune responses. This results in the secretion of chemokines and cytokines which are crucial for recruitment of inflammatory cells ⁷. These effector cells include phagocytes, dendritic cells, mast cells, basophils, eosinophils, natural killers and innate lymphoid cells, all of which are essential in the eradication of the intruding pathogens ⁴⁻⁶.

The rapid detection of foreign pathogens by activation of the innate immune response relies mainly on germline-encoded molecules, known as pattern-recognition receptors (PRRs) ⁸. PRRs are evolutionarily conserved receptors, which are able to identify pathogens through the invariant molecular patterns that are present in most pathogens and characteristically distinguishable and absent from healthy host cells ^{4,5,7,9}. Additionally, PRRs are also able to recognise damage-associated molecular patterns which are released from damaged cells that trigger and strengthen the immune response ⁷⁻⁹. Although the innate immune system has the capacity to rapidly recognise and eradicate different types of pathogens by possessing different PRRs, microorganisms are continuously mutating and evolving their evasion mechanisms to avoid recognition by PRRs. Innate immune responses are also not specific to a particular pathogen and lack the immunologic memory to recognise previously encountered pathogens. Both of these limitations are characteristic features of the innate immune system that are complemented by the adaptive immune system.

1.1.2. Adaptive immune system

The development of adaptive immunity is in cooperation with the activities of the innate immune system and comes into play when innate immunity is incapable of eradicating intruding pathogens or the invasion by malignant tumour cells. The adaptive immune system is only present in vertebrates and consists of two lymphocyte subsets: the T lymphocytes and antibody-producing cells known as B lymphocytes ¹⁰⁻¹².

The ability of pathogens to mutate and avoid innate host detection requires both the B and T cells to develop a sophisticated detection system to recognise the pathogens in order to eliminate them effectively. In comparison to the limited germline-encoded PRRs of the innate immune system, adaptive immune responses rely on the recognition

power of the receptor which is generated via rearrangement of different gene segments, known as VDJ-somatic recombination^{10,12,13}. The unique gene rearrangement process allows the adaptive immunity to possess a vast repertoire of individually selected receptors, which can recognise seemingly antigenic peptide that identifies the invading pathogens or the malignant cells. Recognition of the antigenic epitope leads to the expansion of the specific T and B cells, thus generating an immune response against the particular threat.

While an extensive gene rearrangement ensures generation of a broad repertoire of antigen receptors, it is conceivable that the randomly rearranged receptors also generate specificities that are reactive to host self-antigen. The inability of the adaptive immune system to distinguish "self" and "non-self" antigens can lead to destructive immune responses against host tissues, manifesting as systemic or organ-specific autoimmune diseases. To prevent this pathology from happening, the development of the adaptive immune system's function is to ensure that receptors with self-specificity are rearranged or deleted. Another defining hallmark of adaptive immunity is the capacity for immunological memory, which can mount a faster and more competent immune response upon subsequent encounters with the pathogenic antigens¹⁰⁻¹². This unique competence is the principle for the effectiveness of vaccines.

1.1.2.1. B cells and antibody mediated-immunity

B cells are derived from hematopoietic progenitors that are initially detected in the fetal liver and later evident in the bone marrow. During the development of B cells, somatic VDJ-recombination processes generate the membrane-bound antigen-specific receptors known as B cell receptors (BCRs)¹⁴. BCR binding to its specific cognate antigen triggers the internalisation of the BCR complex and the subsequent presentation of the antigen peptide on MHC-II molecules.

Antigen-specific CD4 helper T cells recognise peptide MHC-II complexes, which in turn provide the B cells with the additional stimulation to clonally expand and differentiate into antibody-secreting plasma cells^{15,16}. A subset of differentiated CD4 helper T cells known as T-follicular helper cells (T_{FH}), are crucial in assisting B cells to produce and secrete antigen specific antibodies required for an effective humoral

immune response¹⁷. T_{FH} do not just furnish signals that allow plasma cell differentiation but also: i) provide survival signals to B cells via the production of IL4; ii) stimulate their proliferation via CD40L-CD40 interactions; and iii) facilitate somatic hypermutation and antibody-isotype class-switching by inducing expression of activation-induced deaminase enzyme^{16,18}.

The secreted antibodies directly bind with the extracellular pathogens, both neutralising and marking them for destruction by phagocytes and the complement system. Antibodies play a vital function in limiting the invasion and proliferation of pathogen during the acute phase of an infection^{19,20}. However, antibody-mediated immunity is not proficient in the eradication of the pathogen, and requires participation of cell-mediated immune responses by T cells in elimination of intracellular pathogens^{1,2,10}.

1.1.2.2. T cells and cell-mediated immunity

Cell-mediated immunity is fundamental in the elimination of cancer, virus-infected cells and intracellular pathogens that are able to survive and persist in the cellular environment^{10,21}. T cells are the primary effector cells of cell-mediated immunity^{22,23}. As for B cells, T cells have a broad array of antigen-binding receptors on their membrane, known as the T cell receptors (TCRs), which are generated via gene arrangement during the cells' development in the thymus²⁴.

Specialised antigen-presenting cells (APC), such as dendritic cells and B cells, are required to process and present the antigenic peptides²⁵. For an immune response to be initiated, naïve T cells need to engage their TCR with cognate antigen presented by the APC. This in part triggers the production of interleukin-2²⁶, which result in proliferation of the activated T cells and in the cell's differentiation into different phenotypes according to the condition of stimulation. Consequently, activated T cells migrate to inflammatory sites to facilitate pathogen and cancerous cells eradication via the production of cytokines and the cells' own cytotoxic activity²⁶⁻²⁸.

The effector responses in the context of cell-mediated immunity depend on the function of T cells which can correlate with a specific phenotype, being simply spoken either

cytotoxic (CD8 T cells) or providing help (CD4 T cells) ²⁹. CD8 cytotoxic T cells are characteristically involved in the destruction of infected or malignant transformed host cells recognised by their antigen expression ²⁸. Their activation is induced by the engagement of the TCR with endogenous peptides presented within the groove of MHC Class I molecules, generated from the components of the malignant transformed cell or the intruding pathogens ^{30,31}. Activated CD8 T cells perform their function by three mechanisms: i) the production and the release of cytotoxic granules; ii) the elimination of infected cells via Fas/FasL interaction; and iii) the production and secretion of anti-tumour TNF- α and anti-viral function of IFN- γ cytokines ^{32–35}. Consequently, innate phagocytic cells such as macrophages are responsible for clearing the apoptotic cell debris ^{34,36}. Activated effector cells have a short half-life, with the exception of those cells that have been committed to become memory cells, which contribute to sustained long-term immunity ^{37,38}. The ability of memory T cells to rapidly and effectively recall previous immunological responses is essential during re-exposure to the specific invading pathogen.

CD4 helper T cells, on the other hand, do not possess cytotoxic activity, hence are not able to directly kill infected cells. Their function is to “help” orchestrate and enhance the immune response by regulating and signalling other effector cells which are involved in cellular and humoral immune responses ^{16,39,40}. CD4 T cells are activated via their TCR recognition of exogenous antigenic peptide bound by MHC class II molecules, processed and presented on the cell surface of APC ^{12,28}. CD4 T cells can be broadly classified into six different effector subsets based on their functions: i) T_H1 cells produce IFN- γ that activates macrophages and monocytes for the defence against intracellular pathogens; ii) T_H2 cells produce IL-4, IL-5, IL-13 which activate eosinophils, basophils and mast cells, in the defence mechanism against extracellular parasites; iii) T_H9 cells produce IL-9 that is important in cancer regulation and mucosal immunity; iv) T_{FH} cells produce IL-21 that is crucial in activating B cells, to stimulate and enhance antibody production; v) T_H17 cells produce IL-17 and IL-22, which are crucial in neutrophil production and recruitment; and vi) regulatory T cells or T_{reg} ^{40–43}.

T_{reg} cells are distinct as they function by limiting immune activity, which is fundamental to control aberrant responses against host tissue, thus preventing the development of autoimmune diseases ⁴³. The immunoregulatory mechanisms utilised

by T_{reg} include: i) the production of cytokines that dampen inflammatory responses such as IL-10, IL-35 and TGF- β ; ii) the killing of other effector T cells by perforin-granzyme-dependent pathway; and iii) the up-regulation of intracellular cyclic AMP in both CD8 and CD4 T cells, which disrupts IL-2 secretion resulting in inhibition of T cell proliferation⁴⁴⁻⁴⁶. Activated naïve CD4 T cells stimulated by TGF- β and IL-2, in the absence of IL-6, induces conversion towards induced T_{reg} phenotype (iT_{reg})^{47,48}. However, T_{reg} are not exclusively induced within the periphery and can naturally be generated in the thymus, known as natural T_{reg} (nT_{reg})^{49,50}.

1.2. Thymus as a primary lymphoid organ

The role of thymus in immunity was first highlighted by Jacques Miller, who elegantly documented the thymus as the organ responsible for the development of immunocompetent cells that constitute a specific cell population of thymus-dependent cells, later referred to as T-lymphocytes^{51,52}. This concept was drawn from the observation that thymomectomy in neonatal mice resulted in the absence of these cells, revealing the crucial role of the thymus in T cell development during murine fetal life⁵¹.

The thymus is a complex organ formed by developing T cells or thymocytes, thymic epithelial cells (TECs), dendritic cells, macrophages, endothelium cells and fibroblasts, forming a three-dimensional cellular network⁵³. In the mouse, the thymus primordium starts to form at embryonic day (E) 10.5⁵⁴, originating from endodermal cells of the ventral lining of the third pharyngeal pouch. This anlage starts to grow before eventually being separated from the pharyngeal pouch structure at around E11.5, further dividing to form the thymus and the parathyroid, with the thymus migrating downward to its final position above the heart^{55,56}.

Expression of the Foxn1 transcription factor at E11.25 in the thymus primordium is essential in initiating the differentiation of epithelial cells into proficient TECs^{53,55,57,58}. Colonisation of haematopoietic precursors in the thymus at E11.5 is another crucial factor for a successful thymic epithelium development⁵⁹. The thymus can be anatomically and functionally divided into two separate compartments, namely the

cortex and the medulla. Cortical TEC (cTEC) and medullary TEC (mTEC) are the respective cellular components in each of these two compartments. During embryonic and neonatal period, a common TEC progenitor gives rise to both the cTEC and mTEC lineages⁶⁰. In mice, cTEC is defined by the expression of cytokeratin 8 (K8), cytokeratin 18 (K18), Ly51 (also known as BP1/CD249) and CD205 (also known as DEC-205)⁶¹. On the other hand, mTEC in mice are identified by the expression of cytokeratin 5 (K5), cytokeratin 14 (K14) and also shown to selectively bind to the lectin *Ulex Europaeus* Agglutinin 1 (UEA1)^{62,63}.

cTECs are responsible for attraction of pre-T cells from the blood to the thymus, supporting early thymocyte development and orchestrate selection of thymocyte, known as thymocyte positive and negative selections^{64,65}. cTECs process and present peptides derived from self-antigen in the context of MHC molecules (designated pMHC). These complexes are required for the aforementioned selection processes. The protein degradation process of cTEC is distinct: these cells express the β 5t-containing thymoproteasomes which form a unique repertoire of self-peptides for MHC Class I presentation, and several proteases, including cathepsin-L, which generate peptides for MHC class II-associated presentation⁶⁶⁻⁷⁰. cTEC is also in part, involved in the first part of thymocyte negative selection process in the cortex, referred to as the wave 1 thymocyte negative selection.

The mTEC microenvironment continues to expand during postnatal life in mouse, fostered by crosstalk between TEC and mature thymocytes⁷¹⁻⁷³. mTEC controls the later stages of thymopoiesis and are essential in the second wave of thymocyte negative selection⁶⁴, which is contingent upon its unique ability to collectively express nearly all peripheral tissue antigens. The inherent ability of mTEC to perform this function is via a process known as promiscuous gene expression (PGE), which is partly dependent on the expression of the transcriptional facilitator Autoimmune regulator or AIRE (to be reviewed in section 1.2.3.1).

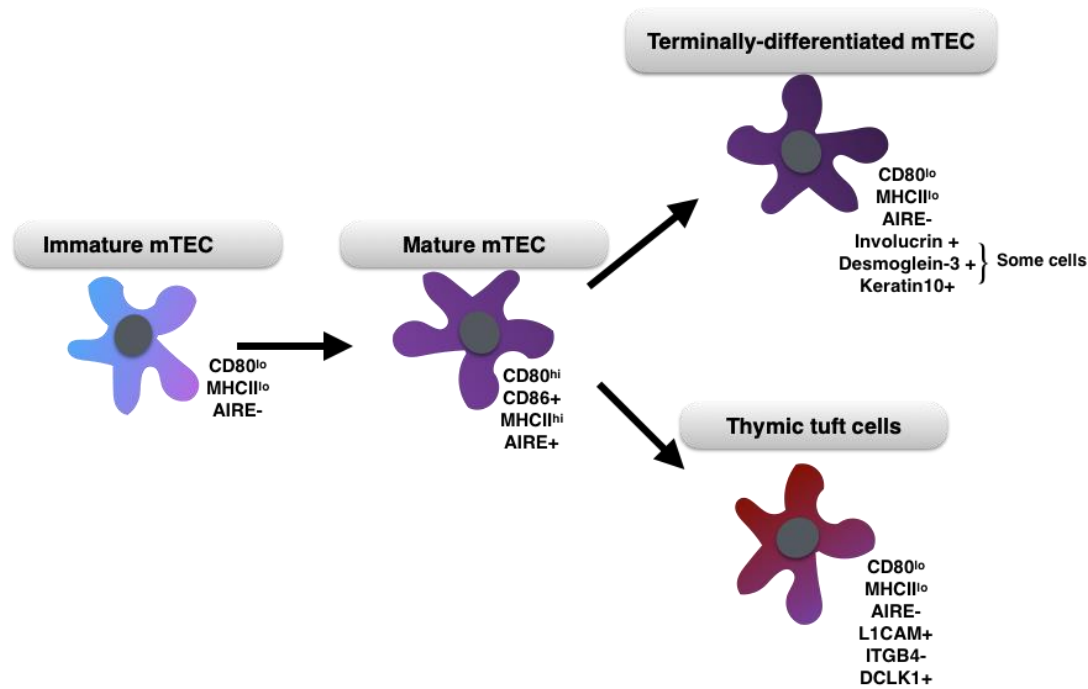


Figure 1.1 Schematic diagram of mTEC differentiation

Immature mTECs serve as precursor to TEC population with a more mature phenotype and subsequently terminally-differentiated mTEC phenotype. Thymic tuft cells are a subset mTEC that express tuft cell-like features and are phenotypically different from the terminally-differentiated mTEC. Most thymic tuft cells pass through an AIRE-expressing stage, thus are considered as a subset of post-AIRE mTEC. However, there is evidence to suggest the existence of an AIRE-independent development pathway. Shown for each mTEC subset, are protein markers utilised to phenotypically identify and differentiate the four mTEC subsets.

The thymus medulla is composed of a heterogeneous group of different mTEC subtypes, which distinguish themselves by their phenotype, maturational stage and function. mTEC were initially classified into what was believed to be two sequential maturational stages, termed as $mTEC^{lo}$ and $mTEC^{hi}$ (termed as such based on their expression levels of MHC Class-II) with the latter differentially expressing a higher level of CD80 and MHC Class-II on their surface⁷⁴. $mTEC^{lo}$ were previously defining the immature pre-AIRE mTEC, while $mTEC^{hi}$ define mature thymic epithelia with up-regulation of AIRE, which in turn regulates the expression of tissue-restricted antigens (TRA) in TEC. The mTEC maturation is reflected clearly in $mTEC^{lo}$ transiting to $mTEC^{hi}$ signals via different TNF receptor superfamily members including RANK and CD40⁷².

Recent findings based on the use of novel reporter mice and single-cell transcriptomics revealed a large degree of TEC heterogeneity, especially within the population of $mTEC^{lo}$ as defined by flow cytometer analysis^{75–77}. This phenotype represents not only

both several immature TEC stages but also functionally mature and specialised subtypes e.g. post-AIRE terminally differentiated mTEC⁷⁵⁻⁷⁸. mTEC subsets are presently classified by cell surface features via FACS into four different phenotypes: i) pre-AIRE immature mTEC; ii) mature AIRE-expressing mTEC; iii) post-AIRE terminally differentiated mTEC; and iv) tuft cells-like TEC^{76,77} (Figure 1.1). Pre-AIRE immature mTEC are the highly proliferative mTEC, while mature AIRE-expressing mTEC belongs to the population of mTEC^{hi}, which collectively expressing around 98% of all the TRG⁷⁵⁻⁷⁷. The post-AIRE terminally differentiated mTEC in human are claimed to be part of Hassall's corpuscles, and are characterised by the expression of terminally differentiated epithelial cell markers such as Involucrin, Cytokeratin 10 and Desmoglein-3^{75,77}. This mTEC subset expresses a moderate number of TRG including AIRE-dependent TRG, despite AIRE no longer being expressed at this later developmental stage. Thymic tuft cells constitute another TEC subpopulation, characteristically expresses transcription factor POU2F3 and broad array of type II taste receptor family members that may enable these cells to sense microenvironmental cues^{76,77}. These thymic tuft cells are shown to be particularly distinct to their intestinal counterpart, as they are uniquely able to express MHC class II for antigen presentation, thus suggesting their additional role in inducing tolerance to tuft cell-restricted antigens⁷⁶.

The thymus is the first organ that shows the effect of ageing whereby it undergoes the process of gradual involution that disrupts the tissue architecture, leading to less efficient T cell development⁷⁹⁻⁸¹. In human, growth of the thymus is maximal during fetal life and continues until birth, after which thymic involution commences, and thymus size and T cell output starts to decline⁸². Therefore, the function of the thymus during fetal development and early postnatal life in human is critical in producing and educating a wide-ranging T cell repertoire to defend the host from foreign pathogens and dangers for the rest of its life^{79,80,83}.

1.2.1. Thymic T cell development

Hematopoietic stem cells differentiate into non-self-renewing multipotent progenitors and eventually give rise to lymphoid-primed multipotent progenitors (LMPPs)^{84, 85}. LMPPs are considered the earliest lymphocyte progenitors but are yet to be T cell committed as they maintain the multipotential for B cell and myeloid development^{86,87}. LMPPs subsequently give rise to T cell lineage by the expression of IL-7R α , defining the earliest thymic seeding progenitors (TSPs)^{84,87}. For TSPs to progress to the next developmental stage, they need to enter the thymus, which requires an orchestrated chemokine-receptor signalling involving CCR7: CCL21C/CL19, CCR9: CCL25 and CXCR4-CXCL12^{88,89}. TSPs enter the thymus via blood vessels at the corticomedullary junction (CMJ) and are subsequently referred to as early thymic progenitors (ETPs)^{86,90,91} (Figure 1.2).

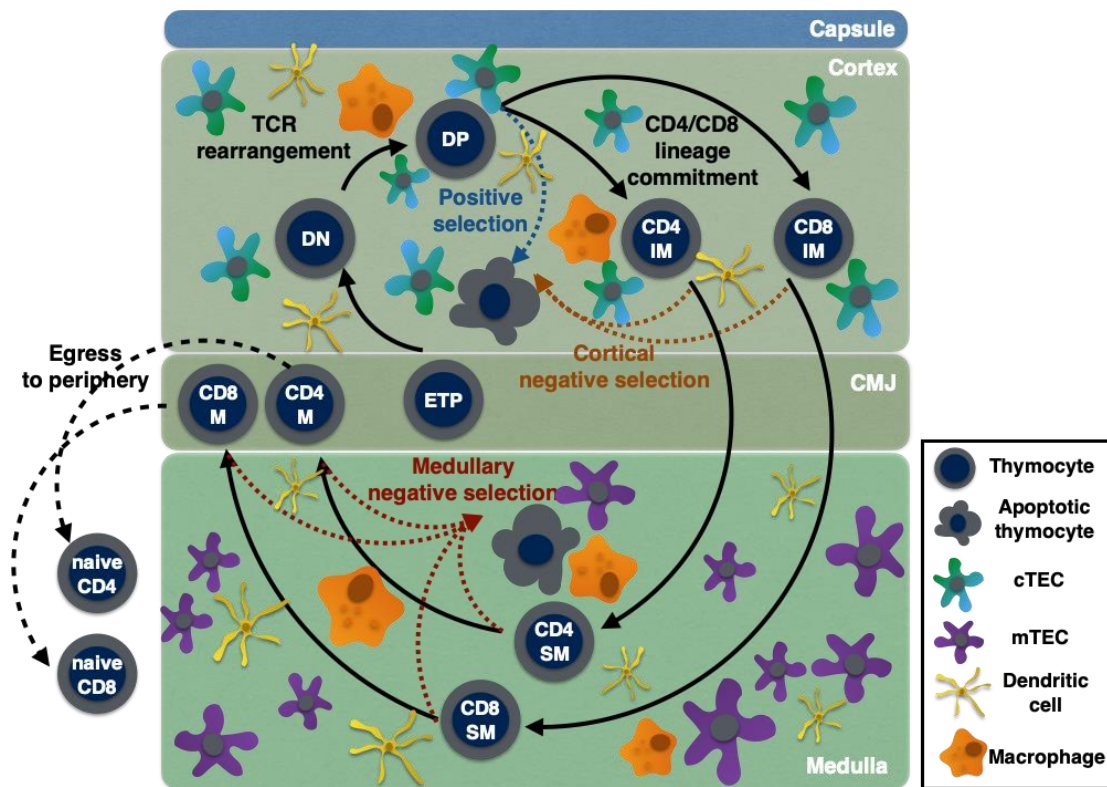


Figure 1.2 Schematic diagram of T cell development in the thymus

Early T cell progenitors (ETPs) enter the thymus at the corticomedullary junction (CMJ) and are characterised as double-negative (DN) thymocytes. DN move from the CMJ to the subcapsular region and initiate the rearrangement of the TCR, undergo positive selection and express both CD4 and CD8 co-receptors thus adopt double-positive (DP) phenotype. Subsequently, DP thymocytes differentiate into single positive immature (IM) CD4 or CD8 thymocytes and undergo negative selection. Thymocytes that survive express CCR7 and migrate to the medulla. In the medulla, thymocytes undergo further maturation (from semi mature (SM) to mature (M) phenotype) and medullary negative selection processes, before egressing to the periphery.

The initial stages of intrathymic T cell development lack the expression of both CD4 and CD8 co-receptors, and are therefore referred to as double-negative thymocytes (DN)⁸⁶. The DN development can be divided into four sequential developmental stages from DN1 to DN4, based on differential surface expression of CD44 and CD25 (Figure 1.3)⁸⁸

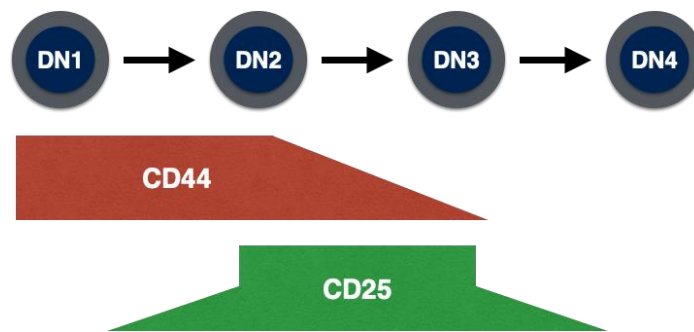


Figure 1.3 Schematic diagram of different DN stages based on CD44 and CD25 expressions

ETP are negative for the expression of both co-receptors CD8 and CD4, designated as double negative (DN). DN thymocytes progress through different sequential maturational stages defined by the surface expression of CD25 and CD44: DN1 (CD44+ CD25-), DN2 (CD44+ CD25+), DN3 (CD44- CD25+), DN4 (CD44- CD25-).

As the development progresses, the DN1 thymocytes (CD4- CD8- CD44+ CD25-) migrate towards the subcapsular zone and differentiate further into DN2 stage (CD4- CD8- CD44+ CD25+) where the rearrangement of the TCR γ , TCR δ and TCR β loci occurs⁹². Thymocytes differentiation continues within the subcapsular zone giving rise to DN3 (CD4- CD8- CD44- CD25+), where they are committed towards $\alpha\beta$ T cell fate. During this stage, the thymocytes' newly-arranged TCR β associate with an invariant pre-TCR α chain to form a pre-TCR receptor. The capability for this pre-TCR receptor to adequately signal is examined through a process known as β -selection, the success of which allow the thymocytes progression to DN4 stage. Upon acquiring DN4 phenotype, rearrangement of the TCR α simultaneously occurs to generate the $\alpha\beta$ TCR⁹³. Successful TCR signaling is crucial to initiate the expression of CD8 and CD4 co-receptor on their surface, thus adopting a double-positive (DP) phenotype^{94,95}.

Engagement of $\alpha\beta$ TCR to pMHC with sufficient high-affinity provides the DP thymocytes with the vital intracellular signals to escape the phenomenon known as death by neglect^{96,97}. This process is known as the thymocyte positive selection mediated by cTECs in the cortex^{98,97,99,100}. DP cells which have successfully

undergone positive selection subsequently differentiate into single positive CD4 or CD8 thymocytes, depending on the specificity of their TCR for MHC Class II (MHCII) or MHC Class I (MHCI) presented peptides, respectively. The CD4 and CD8 co-receptor engagement with the cognate binding sites on MHCII and MHCI molecules and the time of engagement provides the necessary signaling cues that terminate the expression of the converse co-receptor and thus initiates lineage-specific gene expressions^{29,101}. The thymocytes also express chemokine receptor CCR7, which enable them to migrate to the medulla, where its ligands CCL19 and CCL2 are produced by mTEC^{102,103}. Although the commitment to CD4 and CD8 lineages is independent of a migration to the medulla, both events occur in parallel.

The thymocytes also undergo a second selection process, in which their $\alpha\beta$ TCR are probed for autoreactivity, where highly self-reactive thymocyte clones are eliminated through apoptosis. This second selection, which is referred to as thymocyte negative selection, occurs both in the cortex and medulla^{100,104}. The process of thymic negative selection identifies thymocytes with high-affinity $\alpha\beta$ TCR signalling towards self-peptide MHC complexes on cTECs, specialised thymic dendritic cells and mTECs in order to eliminate these self-reactive thymocytes via programmed cell death¹⁰⁰. For the process of negative selection to be comprehensive, it is dependent on the ability of the thymus to successfully present numerous repertoire of “self”-peptide to the developing thymocytes¹⁰⁵. Although not exclusively involved in thymocyte negative selection, mTECs are essential to screen against self-reactive T cells comprehensively and remains a crucial fundamental player in the optimal establishment of central tolerance. Uniquely, mTECs as a whole, are able to present nearly all the self-antigens in the body through a process known as promiscuous gene expression^{100,106,107}, which will be discussed in greater detail in section 1.2.3. mTECs are also responsible to cross-present the processed antigen to their thymic dendritic cells (DC) counterparts, expanding the cellular base where negative selection can occur, thus enhancing the self-tolerance induction.

The lineage-committed CD4 and CD8 thymocytes are initially immature (termed as CD4-immature (CD4-IM) or CD8-immature (CD8-IM), respectively) and required to endure several rounds of post-selectional maturation stages. This late stages of thymocytes maturation involve crucial NF- κ B and tonic type I interferon signalling in

the thymus ¹⁰⁸. Activation of NF- κ B in the developing thymocytes is crucial to protect the thymocyte from complex-II mediated death downstream of TNF-R1, thus allowing survival of these post-selected thymocytes to become CD4-semimature (CD4-SM) and CD8-semimature (CD8-SM). Proliferation competence is acquired subsequently, and only thymocytes that are able to adequately respond will progress to become mature thymocytes (CD4-M and CD8-M). Finally, the mature thymocytes exit from the thymus require expression of sphingosine-1 phosphate receptor-1 (S1PR1) that mediates thymic egress to the peripheral blood ^{108,109} to become antigen unexperienced naïve T cells.

1.2.2. Generation of a diverse T cell antigen receptor repertoire

The T cell antigen receptor (TCR) is a heterodimer of two glycoprotein chains, and forms a complex with the invariant CD3 chain molecules ^{110–112}. Based on the TCR chain used, two separate types of TCRs are defined $\alpha\beta$ or $\gamma\delta$ TCRs. In humans and mice, the vast majority of T cells express the $\alpha\beta$ heterodimer (thus referred to as $\alpha\beta$ T cells) whilst the remainder of T cells express a $\gamma\delta$ (thus referred to as $\gamma\delta$ T cells)^{113,114}.

Each TCR glycoprotein chain is comprised of two extracellular domains, the variable (V) domain, which is the antigen-binding portion of the receptor, and the constant (C) domain, which is the transmembrane part of the receptor that transduces the activating signals ¹¹¹. Three hypervariable complementarity-determining regions (CDR) are located within the variable domain, with each having a different function ^{112,115}. The third CDR is the most hypervariable region (designated CDR3) and largely responsible for the recognition of the pMHC complex ^{115–117}.

The diversity of TCR CDR3 region arises from a genetic recombination of individual exons V (variable), D (diversity), and J (joining) gene segments, a process termed as V(D)J ^{14,118}. This recombination utilises the recombinases RAG1 and RAG2, and occurs in an orderly fashion, with recombination of D to J gene segments happening first before a V gene segment is ligated to the recombined DJ gene segments ^{14,118}. Additionally, the repertoire of the TCR generated is broadened by a junctional diversity achieved by the addition of random non-templated nucleotides at the splice sites of

recombined gene segments. This addition is mediated by terminal deoxynucleotide transferase enzyme or TdT¹¹⁹.

Utilising mathematical modelling to quantify TCR diversity of the developing T cells, the thymus is theoretically able to generate approximately 10^{12} different TCR combinations¹²⁰. The molecular mechanisms described above are the basis for the generation of a diverse TCR repertoire, which is able to respond to every possible foreign antigen independent of subtle in structure. However, this capacity holds the risk that self-antigen can be recognised which can lead to autoimmunity.

1.2.3. Promiscuous gene expression by thymic epithelial cells

PGE enables transcription of approximately 92% of all protein-coding genes in the mTEC^{121–123}. From approximately 22,000 protein-coding genes, a fraction of these genes (around 8000) are designated tissue-restricted genes (TRGs) as they encode peripheral TRAs^{106,121}. TRG expressions in mTEC are essential components of the molecular mechanism that establishes central tolerance to self-antigen. With almost all proteins being represented by the PGE process in the mTEC, it is imaginable that the molecular atlas of the body's self-antigens is mirrored in the thymus for the sole purpose of thymocyte negative selection to be comprehensive^{99,122,124}.

Balancing between a comprehensive molecular representation of the body's self-antigen and expression of adequately high densities of self-pMHC, each mature mTEC is only expressing up to 3% of the total TRG at any given time⁷⁵. PGE of a particular self-antigen is also spatially distributed in a random manner within the medullary region to increase the likelihood of each single thymocyte to encounter that particular antigen within a limited migration area⁷⁵. Self-peptide generated by mTEC and presented by thymic DC after antigen transfer, further enhances thymocyte negative selection^{104,125}.

PGE is partly regulated by the transcription facilitator AIRE, which is typically expressed in a subpopulation of mTEC^{hi} cells¹²⁶. There are also AIRE-independent mechanisms that control PGE, as AIRE is only responsible for the expression of 60% of TRG¹⁰⁶. Transcription factor forebrain-expressed zinc finger 2 (FEZF2) is one of

the factors that also regulate PGE^{100,127}. However, the proclaimed function of FEZF2 remains to be confirmed¹²⁸. Additionally, transcription factor PRDM1 and ATP-dependent chromatin remodeler BRG1 are recently discovered factors that appear to regulate PGE, although the molecular mechanisms of their respective actions have yet to be explored^{129,130}.

1.2.3.1. AIRE in promiscuous gene expression

Mutations in the gene encoding AIRE result in a complex syndrome of multi-organ autoimmunity (known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy syndrome, APECED or autoimmune polyendocrinopathy syndrome type 1, APS1)¹³¹. The syndrome is classically characterised by a triad of chronic candidiasis, hypoparathyroidism and adrenal insufficiency. Other autoimmune manifestations include hypergonadotropic hypogonadism, ovarian failure, insulin-dependent diabetes mellitus, autoimmune hepatitis, vitiligo, hypothyroidism and other organ-specific pathologies. The detection of autoantibodies against multiple tissue-specific antigens is the hallmark of the disease and serves as both diagnostic and predictive biomarkers for disease progression. AIRE deficiency in mice also results in a similar, albeit milder multi-organ autoimmune pathology^{132–135}.

AIRE expression in thymus is restricted to a subset of the mTEC^{hi}, presenting around 60% of these cells^{136–139}. The expression of AIRE in mature mTEC establishes a comprehensive repertoire of peptides used to instruct central tolerance^{105,140,141}. AIRE deficiency results in a compromised capacity to affect negative selection of self-reactive T cells due to a decrease in TRG expression, and also impairs the generation of regulatory T cells^{100,142–144}. Outside of the thymus, AIRE is also expressed in secondary lymphoid organs, within a small subset of cells known as extrathymic AIRE-expressing cells (eTAC)^{145–148}. However, AIRE expression in eTACs are at a lower level of expression when compared to mature mTEC, and their functional role, with regard to peripheral tolerance is not yet confirmed^{146–148}.

AIRE possesses the following structural features: i) the caspase-activation recruitment domain (CARD) region; ii) a nuclear localisation signal (NLS); iii) a SAND domain (SP100, AIRE, Nuc p41/75, DEAF); iv) four leucine-rich LXXLL motifs; and v) two

plant homeodomain (PHD) zinc fingers²³. Nuclear AIRE forms distinctive speckle-like structure known as AIRE nuclear bodies, which are bound to the nuclear matrix^{142,149}. AIRE affects transcriptional indirectly via chromatin changes^{149–151}. The CARD and SAND domains are involved in homodimerization of AIRE proteins into dimers or tetramers^{152–154}. AIRE's PHD1 interacts with unmethylated histone 3 marks, namely lysine at position 4 (H3K4me), and as a result, mediates the transcriptional activation of genes with that distinct epigenetic mark^{155–158}. Although AIRE's PHD2 domain structurally resembles the PHD1 domain, the surface of the PHD2 domain is positively charged, making it incapable of interacting with histones¹⁵⁶. The AIRE protein also has an uncharacterised proline-rich region between the PHD zinc fingers and four LXXLL motifs, which are parts of many regulatory proteins in the nucleus and are required for protein-protein interactions, for example, with nuclear receptors¹⁵⁹.

Given that AIRE controls the expression of a wide variety of TRG, it is imperative to fully decipher how this is achieved. AIRE is not an orthodox transcription factor, as it does not bind directly to any DNA motif of the target gene inferring the requirement of a functional and possibly physical relationship with other molecular partners that may facilitate the recognition of nucleic acids and/or chromatin structures²³. Indeed, proteomic screen by mass spectrometry reveals that AIRE is part of a large complex with more than 40 proteins¹⁵⁰. These partners can be broadly classified into four functional groups: i) DNA-damage response proteins; ii) proteins involved in chromatin structure and remodelling; iii) proteins related in transcription regulation; and iv) proteins required in mRNA splicing and nuclear transport^{150,160}.

AIRE-controlled gene transcription is suggested to be initiated upon DNA cleavage by type 1 and 2 topoisomerases (TOP1 and TOP2 respectively), two AIRE interacting partners^{161,162}. The double-stranded DNA breaks consequently recruit DNA-activated protein kinase (DNA-PK) and poly- (ADP-ribose) polymerase 1 (PARP1) among other AIRE partners, which are involved in the DNA break repair mechanism using non-homologous end joining^{140,150}. The transcription of the target gene is parallel with the changes in chromatin, notably the accumulation of γ H2AX and the exchange of histone H1 with HMGB1 at the promoter site^{161,162}. Inhibition of TOP2 and TOP1 re-ligation activity by etoposide and camptothecin, respectively, increases frequency of unresolved DNA double-strand breaks, which in turn enhances AIRE-mediated

transcription¹⁶¹. Together with the DNA break and repair mechanism, chromatin remodelling enzymes, such as BRG1, BRD4 and CHD6 are proposed to be responsible for removing DNA supercoils which resulted in a local relaxation of the chromatin, rendering this part of the chromosome more accessible to the transcriptional machinery^{140,150}. CREP-binding protein is crucial in stabilising the sub-localisation of AIRE to the stalled RNA-Polymerase (RNAPII), which usually initiates transcript^{163–165}. Moreover, several AIRE partners are involved in transcript elongation (e.g. positive transcription elongation factor, p-TEFb) and pre-mRNA processing (e.g. RNA helicases and the splicing factor such as SFRS2)^{150,166}. Recruitment of p-TEFb to the site of the complex releases the stalled RNAPII, facilitates transcription and eventually allows splicing factors to process the pre-mRNA into mature transcripts¹⁶⁶.

Although the mechanism by which AIRE operates is now better understood, the questions on how specific genes are targeted by AIRE still remain unanswered. In this respect, the epigenetic mechanism, including specific histone modifications, have been linked to AIREs' ability in recognition of the specific gene loci and the regulation of PGE process^{106,141,167,168}. Indeed, ChIP analyses reveal distinct histone PTM at the promoter of AIRE-regulated genes; specifically permissive histone marks (i.e. H3K4me3) are reduced, whereas repressive marks (i.e. H3K27me3, H3K9me3) are enriched^{106,169,170}. It is important to note that AIRE does not bind with H3K27me3¹⁶⁹. Moreover, although the PHD1 domain of AIRE was experimentally shown to recognise H3K4me0 directly^{157,171}, the disruption of AIRE binding to H3K4me0 *in vivo* failed to disrupt AIRE-controlled PGE, suggesting that AIRE-H3K4me0 interaction on its own, does not fully responsible for AIRE-controlled loci targeting¹⁷². Hence, an additional AIRE binding would need to recognise H3K27me3, H3K9me3 marks and the lack of H3K4me3 for the transcription of the targeted loci to occur. Interaction of AIRE with CHD molecules, especially CHD6, may account for such an interaction, which can recruit AIRE to target loci, and subsequently facilitate transcription by overriding the repressive chromatin state^{150,160,173,174}.

1.3. Chromatin remodelling enzymes and their mechanism

Chromatin is a highly dynamic complex of DNA and proteins, which packages cell's DNA into chromosomes. The core component of chromatin is the nucleosome, which consists of 147 bp of DNA-wrapped around an octomer of histone proteins^{175,176}. The primary mechanisms by which the chromatin structure is modified to impact gene expression are DNA methylation via the catalytic activity of methyltransferase and the modification of nucleosome proteins via the activity of chromatin remodelling enzymes^{177,178}. There are two different classes of chromatin remodelling enzymes: i) those that modify histone proteins; and ii) those that utilise adenosine-5'-triphosphate (ATP) to alter the DNA-nucleosome contacts via their disruption¹⁷⁹⁻¹⁸¹. The position of the nucleosome on DNA is transient as dictated by chromatin-modifying enzymes, which facilitate “sliding” of the nucleosome along DNA. This phenomenon changes the access of transcription factors and silencers to DNA, resulting in the regulation of gene expression¹⁷⁹⁻¹⁸².

Four types of chromatin remodelling enzymes are known to date: i) SWI/SNF (mating-type switching/sucrose non-fermenting); ii) ISWI (imitation switch); iii) INO80 (inositol requiring 80); and iv) CHD (chromodomain-helicase-DNA-binding) (Figure 1.4). These chromatin remodelling enzymes have four common features, namely: i) a preference to localise within the nucleosome; ii) a domain that identifies different histone modifications; iii) the utilisation of the energy generated from the highly conserved SNF2 ATPase/helicase domain¹⁸³; and iv) a domain enabling the interface with other chromatin components or proteins involved in transcription.^{179,184} The CHD family of proteins is different from the ISWI, SWI/SNF or the INO80 families due to the possession of the tandem chromodomains in the N-terminal region of the proteins^{185,186}.

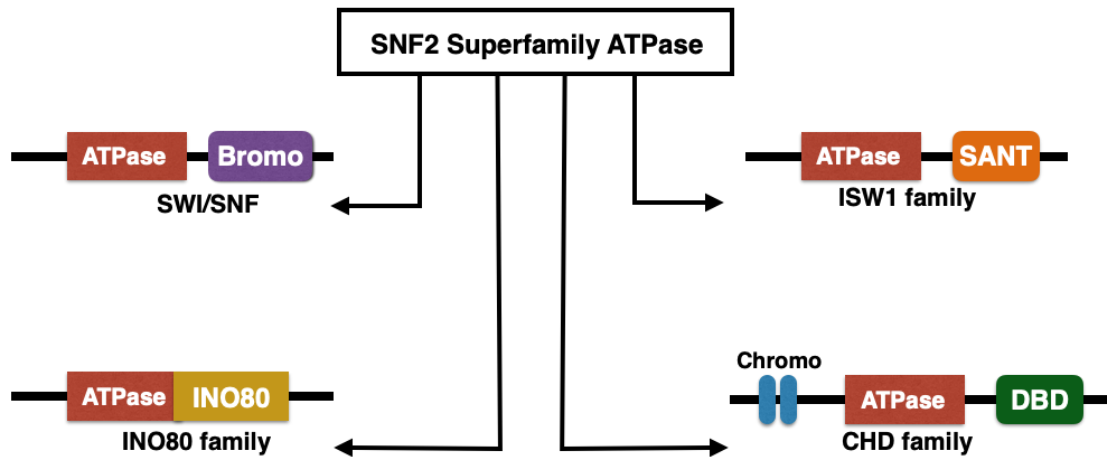


Figure 1.4 Four families of ATP-Dependent SNF2 Chromatin remodelling enzymes

ATP-dependent SNF2-related chromatin remodelling complexes: SWI/SNF, ISWI, CHD and INO80. Each family is defined by the presence of a distinct ATPase containing signature structural domains and a unique subunit composition: Bromodomain in SWI/SNF, SANT domain in ISWI, and tandem chromodomain in CHD. An extended split in their ATPase domain characterizes INO80.

1.3.1. CHD family of chromatin remodelling enzymes

The CHD families in human and mice consist of nine different enzymes. Each of them shares a highly conserved SNF2 ATPase/helicase domain, a nuclear localisation signal placed in the N-terminal aspect of the molecule and two chromodomains in tandem. The nine CHD enzymes are further divided into three subfamilies based on the presence of a specific protein^{187,185,186} (Figure 1.5). CHD1 and 2 enzymes form subfamily I, possess a well- characterised SANT-SLIDE DNA-binding domain at the C-terminal to their ATPase-Helicase region^{188,189}. CHD3, CHD4 and CHD5 from subfamily II, which are characterised by the presence of two PHD fingers in the molecule's 5' regions. In addition, CHDs of this subfamily have two additional domains of unknown functions (DUF) in their C-terminus (Figure 1.5). Both CHD3 and CHD4 had been shown to preferentially bind to histone H3K9me and unmodified H3K4 marks through their PHD domains^{190,191}. Inactivation of CHD4 targeted to TEC disrupts the normal thymic architecture and impaired its thymopoeitic (F. Dhalla, unpublished). The third subfamily of CHD proteins consist of CHD 6, 7, 8 and 9, are the least well-characterised CHD molecules. They share the *Brahma* and *Kismet* (BRK) domain and also possess additional domains in the C-terminus which distinguish one with another^{192–194} (Figure 1.5).

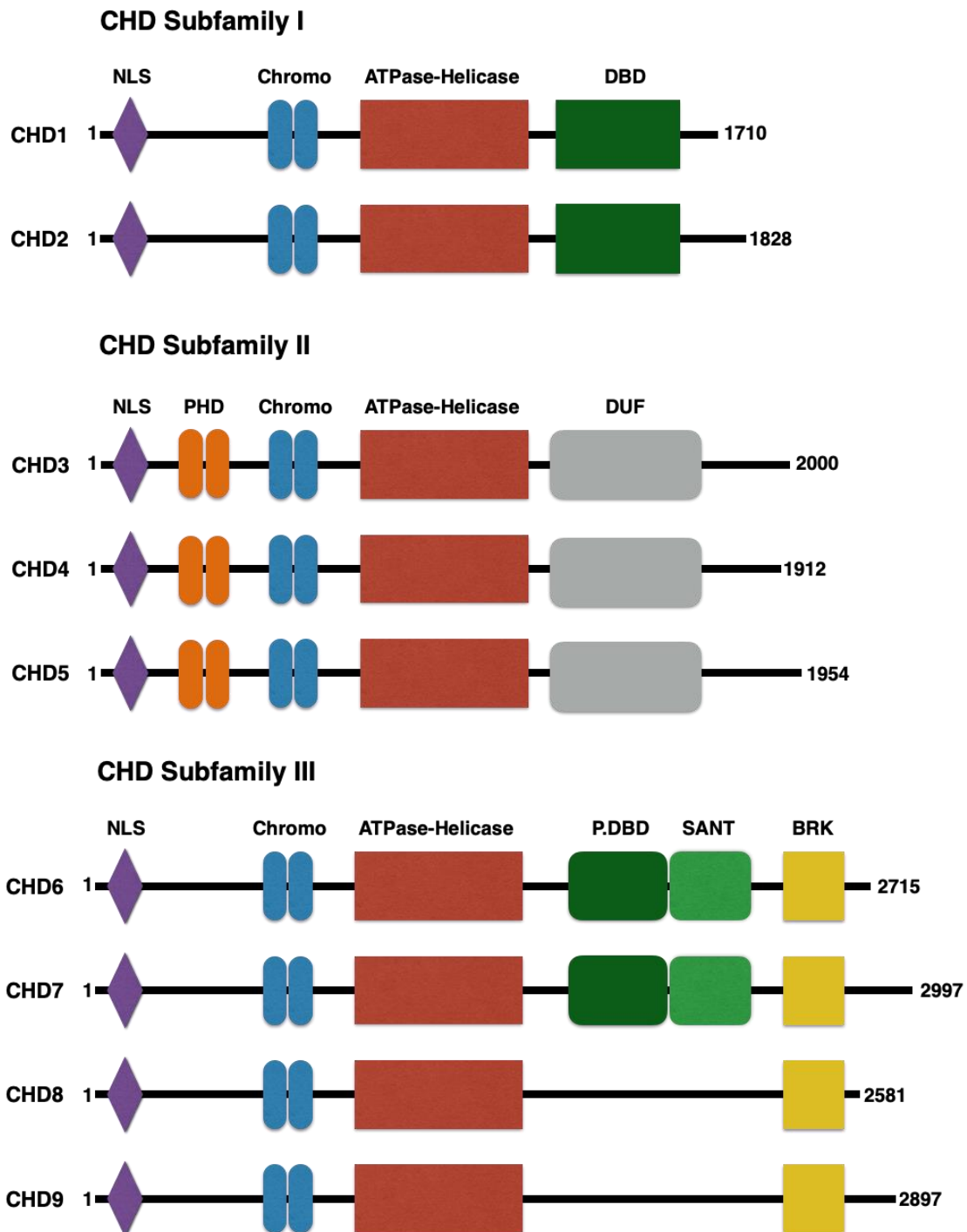


Figure 1.5 Schematic domain representations of CHD family enzymes

All CHD enzymes contain a putative nuclear-localisation signal (NLS) in the N-terminus, a double-chromodomain (chromo), and a SWI/SNF ATPase-Helicase catalytic core. The CHD subfamily I members contain a C-terminal SANT-SLIDE DNA-binding domain (DBD). CHD subfamily II members possess tandem PHD domains in the N-terminus and a domain of unknown function (DUF) in their C-terminus. The CHD subfamily III contains a C-terminal BRK domain while both CHD6 and CHD7 have a putative DNA binding region (P.DBD) and the SANT domain.

1.3.1.1. Chromodomain-helicase-DNA-binding 6 (CHD6)

CHD6 is a ubiquitously expressed and the gene is located on chromosome 20 (at position 20q12) in human and on chromosome 2 in the mouse genome ^{195,196}. Mice with a deletion of exon 12 of *Chd6* which encodes the conserved portion of the ATPase domain (designated as *Chd6* exon 12-/- mouse) have not been analysed for their immune system ¹⁹⁷. CHD6 is a nuclear protein and characteristically part of a large complex with a predicted molecular weight of 305.4 kDA¹⁹⁶. CHD6 co-localises with RNAPII during the preinitiation and elongation stages of RNA synthesis, possibly via facilitating the transcription of target genes ¹⁹⁶. In agreement with this function, KIS-L protein, the CHD subfamily III homolog in *Drosophila*, co-localises together to RNAPII protein and is involved in early stages of transcription ¹⁹⁸. CHD6 interacts in mammalian cells with the transcription factor Nrf2 and is required for the transcription of the NAD(P)H-quinone oxidoreductase (*NQO1*), an enzyme that maintains cellular redox homeostasis ^{199,200}. Other than facilitating in transcriptional activity, CHD6 also functions in remodelling the chromatin structure, such as in the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene locus, where CHD6 acts to enhance the chromatin accessibility for transcriptional factor CTCF to bind and continue to maintain the integrity of CTCF recruitment to the *CFTR* locus ²⁰¹. Additionally, a recent study has described the role of CHD6 as a novel responder to oxidative-stress-induced DNA damage ²⁰². PAR-dependent CHD6 recruitment to the sites of DNA damage, is required for restructuring of the chromatin nucleosome configuration ²⁰².

1.3.1.2. CHD6 as partners of AIRE in PGE

Mass spectrometric analysis of AIRE-enriched nuclear extracts identified more than 40 proteins, which interact with AIRE, one of which is CHD6 ¹⁵⁰. The mechanism of CHD6 in exerting its functions, is in line with the proposed machinery by which AIRE promotes PGE ^{201,203}. Moreover, CHD6 binds with several histone marks, including the H3K27me3 via its tandem chromodomain, ^{202,204}, which is in keeping with the observation that AIRE gene targets are marked by the repressive H3K27me3 modification. Finally, AIRE also hijacks the DNA damage machinery to affect PGE, which matches with CHD6's involvement in the DNA damage response.

1.4. Hypothesis and aim of the doctoral project

CHD6 is a chromatin remodelling enzyme that preferentially binds to repressive histone marks and this facilitate gene transcription by changing chromatin accessibility¹⁵⁰. In addition, CHD6 is also involved in DNA damage response^{173,205}. Both features of CHD6 are relevant to how AIRE affects PGE. To formally test a functional role for CHD6 in TEC biology in general and AIRE-dependent PGE in particular, I have generated mice with a CHD6 deficiency targeted to TEC (see section 2.1.4). I will therefore address the following key questions:

1. What is the precise role of CHD6 in thymus organogenesis and development?
2. Is CHD6 expression in thymic epithelial cells important for thymocyte differentiation?
3. Does CHD6 play an essential role in promiscuous gene expression?

To answer these questions, I first probed the biological properties of CHD6 for the differentiation of thymic epithelial cells and their ability to form a regular epithelial scaffold with a normal cortex:medulla segregation (Chapter 4). Next, I tested whether CHD6 interacts with AIRE protein (Chapter 3). The capacity of TEC for PGE in the absence of CHD6 is investigated and related to thymocyte development (Chapter 4 and Chapter 5). Finally, I tested whether central T cell tolerance induction by TEC requires CHD6 expression (Chapter 6). Taken together, these experiments provide a comprehensive analysis of the roles for CHD6 in TEC biology.

Chapter 2 Materials and Methods

2.1. Mice

Mice work was performed in compliance with the UK Home Office regulations. Mouse colonies were kept at the University of Oxford Biomedical Science facilities. The Administrative Panel of Laboratory Animal Care of the Research Compliance Office of the University of Oxford approves the animal protocols. All experiments were performed with littermate wild-type controls (denoted as *Chd6*^{TEC^{+/+}}). *Chd6*^{TEC^{+/+}} was verified to be phenotypically wild-type C57BL/6, which represent a normal, conventional phenotype, of non-genetically, modified C57BL/6 background mouse strain. All control mice were sex and age-matched in all experiments. Mice were culled using Schedule 1 methods and all procedures were conducted via specified methods as documented on the project license.

2.1.1. C57BL/6

C57BL/6J black inbred wild-type mice were obtained from the in-house colony maintained by the University of Oxford.

2.1.2. ActFlpe

ActFlpe mice (known as B6;SJL-Tg(ACTFLPe)9205Dym/J) were purchased from The Jackson Laboratory. This transgenic strain expresses a variant of the *Saccharomyces cerevisiae* FLP1 recombinase gene under the direction of the human ACTB promoter (3 kb flanking sequence, 78-bp 5' untranslated region, 832-bp of exon 1)²⁰⁶. The line was maintained as heterozygous for the transgenic allele. This deleter strains was bred to *Chd6*^{tm1a (KOMP) Wtsi} to delete the Frt sites that flank the selection cassette to generate *Chd6*^{tm1c} mice (*Chd6*^{TEC flx/flx}).

2.1.3. Foxn1^{Cre}

Foxn1^{Cre} mice were obtained from Saulius Žuklys²⁰⁷. The Foxn1^{Cre} transgenic mice were previously generated by insertion of iCre coding sequence into a Foxn1 PAC clone, which was subsequently introduced into the pronuclei of fertilised B6D2F1 oocytes, to produce the transgenic line B6;D2-Tg(Foxn1-Cre)8Ghr. As the homozygous Foxn1^{Cre} mice are infertile, the mice line was maintained as a heterozygous Foxn1^{Cre+/-} colony. Foxn1^{Cre+/-} mice were bred to *Chd6*^{TEC flx/flx} mice to delete the loxP sites to generate mice conditionally deficient for CHD6 expression in Foxn1-expressing cells.

2.1.4. Mouse model exhibiting TEC-targeted loss of *Chd6* (*Chd6*^{TEC-/-})

Mice conditionally deficient in CHD6 expression in TEC (*Foxn1-Cre::Chd6*^{fl/fl}; designated *Chd6*^{TEC -/-}) were generated to determine the biological role of CHD6 in TEC development and function. *Chd6*^{TEC -/-} mice were established by the deletion of *Chd6* exon 3 that led to a frameshift mutation, resulting in premature stop codon in *Chd6* exon 4.

2.1.4.1. *Chd6*^{TEC-/-} mouse construct

The conditional deletion of exon 3 of the mouse *Chd6* gene in Chromosome 2 (*Chd6*^{tm1a (KOMP) Wtsi}) utilised a targeting vector construct in JM8.F6 parental cell line, which contained a Frt/loxP flanked lacZ-neomycin cassette, positioned upstream to exon 3 with the downstream loxP site inserted 302bp after exon 3 (Figure 2.1). Targeting vector constructs utilised were produced as part of the Knockout Mouse Project (3U01HG004080) by the Children's Hospital Oakland Research Institute and the Wellcome Trust Sanger Institute.



Figure 2.1 *Chd6*^{tm1a (KOMP) Wtsi}

2.1.4.2. Embryonic stem cell and chimera development

Two JM8.F6-derived *Chd6*^{tm1a (KOMP) Wtsi} targeted embryonic stem (ES) cell clones (EPD0034_3_A11 & EPD0034_3_D11) were obtained from the KOMP Repository (www.komp.org). The targeted ES cell clones were thawed, passaged and cultured on murine embryonic fibroblast. Subsequently, these prepared ES cells clones were injected into blastocysts from BALB/c donor to generate chimeric mice by Mouse Transgenic Core Facility, MRC Weatherall Institute of Molecular Medicine, University of Oxford. Briefly, donor blastocysts were harvested following successful mating of superovulated donor BALB/c females with wild-type stud male. 10-15 targeted ES cell clones were microinjected into each donor blastocyst and injected blastocysts were place into the pre-equilibrated KOSM-AA drops and incubated in a 5% CO₂ at 37°C. Successively, 6 to 7 of the microinjected blastocysts were transferred into the uterine horn of 2.5 day post-coitum pseudopregnant recipient female mice. Two chimeras from the EPD0034_3_D11 clone had successful germline transmission of the correctly targeted allele and were used for onward breeding.

2.1.4.3. *Chd6*^{TEC-/-} mice breeding strategy

Mice with a correctly targeted allele in the germline were crossed with C57BL/6 mice expressing Flipase (ActFlp) to generate the conditional (*Chd6*^{TEC flx/flx}) allele mice and subsequently to Foxn1 driven Cre-expressing C57BL/6 mice (Foxn1^{Cre}) to obtain the conditional knocked out mouse (*Chd6*^{TEC-/-}) (Figure 2.2).

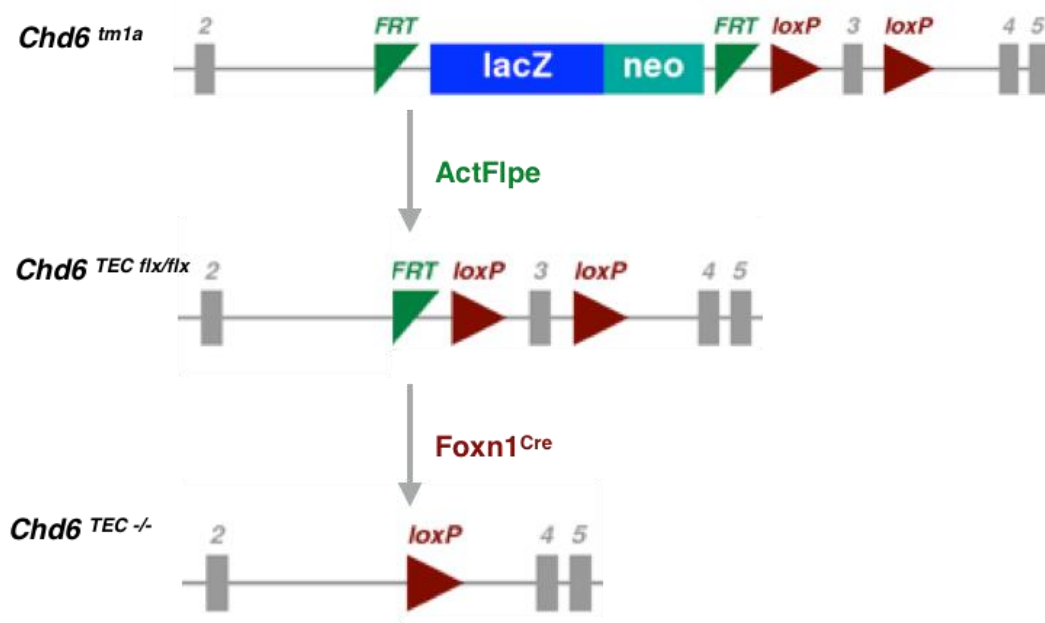


Figure 2.2 Breeding strategy to generate TEC devoid of *Chd6* expression.

Actin-Flipase –mediated (ActFlp) site-specific excision was used to remove Frt-sites flanking the lacZ-Neo cassette from tm1a allele (*Chd6*^{tm1a}). This recombination produced allele tm1c (*Chd6*^{TEC flx/flx}), which could then be further engineered by Foxn1-Cre-mediated (Foxn1^{Cre}) to remove exon 3 of *Chd6* (designated *Chd6*^{TEC -/-}) tm1d allele in TEC.

2.1.4.4. Deletion efficiency and tissue specificity *Chd6*^{TEC -/-} mice

The deletion efficiency and tissue specificity of the generated *Chd6*^{TEC -/-} mice were assessed both at the genomic, RNA and protein levels by PCR genotyping, quantitative PCR (qRT-PCR) and immunofluorescent staining of TEC respectively as described below.

2.1.4.4.1. Deletion of *Chd6* Exon3 gene locus in TEC of *Chd6*^{TEC -/-} mice

Assessment of *Chd6* Exon 3 gene locus in *Chd6*^{TEC -/-} mice was performed to confirm the genomic excision of *Chd6* Exon 3 gene locus in the thymic epithelial cells of *Chd6*^{TEC -/-}. Briefly, TECs were isolated from the thymic tissue after enzymatic tissue disruption with Liberase and DNaseI, followed by magnetic-activated cell sorting by AutoMACS and fluorescence-activated cell sorting by FACS AriaIII (BD Bioscience). The following TECs subpopulations were sorted based on their cell surface markers' expression: CD45 (CD45⁺, EpCAM⁻), cTEC (CD45⁻, EpCAM⁺, Ly51⁺, UEA1

negativity), mTEC^{lo} (CD45-, EpCAM+, Ly51-, UEA1 positivity, MHCII^{lo}, CD80^{lo}) and mTEC^{hi} (CD45-, EpCAM+, Ly51-, UEA1 positivity, MHCII^{hi}, CD80^{hi}).

The *Chd6* gene locus was analysed by PCR, using genomic DNA and the following primers: *Chd6*-In2F (GCTCCTGTGACACGCTTCTG) and *Chd6*-In3R (GGCCAGGAAGTAAAATAGGGTTC). The genomic locations of these primers are shown in Figure 2.3a. The PCR conditions were as follows: initial activation of the polymerase for 5 minutes at 94°C, followed by 40 cycles of 30 seconds denaturation at 94°C, annealing for 45 seconds at 60°C and extension for 1 minute at 72°C, and finally 8 minutes fill in steps at 72°C. A band size of 1538 bp is diagnostic for the conditional allele, and a band size of 467 bp for the deleted allele (Figure 2.3b). Conditionally targeted *Chd6* mice were crossed with Foxn1^{Cre} animals to generate a thymic epithelial cell-specific knockout for *Chd6*.

Additionally, qRT-PCR spanning *Chd6* Exon 1 to Exon 3 transcript in *Chd6*^{TEC-/-} mice was also performed to further confirm the predicted absence of *Chd6* Exon 3 transcript in the thymic epithelial cells of *Chd6*^{TEC-/-}. Briefly, total RNA was extracted from sorted TECs (as per TEC subpopulation described above) using the RNeasy Micro Kit (Qiagen) according to the manufacturers' protocols. Complementary DNA (cDNA) was prepared using the SensiFAST kit (Bioline). cDNA was used for qRT-PCR using SensiFAST SYBR Hi-Rox Kit (Bioline) and StepOnePlus (Applied Biosystems), with the following primers: *Chd6* Exon 1-3 and *Chd6* Exon 5-7 (Table 2.4). Different *Chd6* exon regions were analysed as shown in Figure 2.3c. Reactions were carried out in triplicates and expression values were normalised to *Gapdh* expression using comparative C_τ ($\Delta\Delta C_{\tau}$) methods, where the relative expression was calculated as $2^{-\Delta\Delta C_{\tau}}$, where C_τ represents the threshold cycle. RNAase free water was used as negative technical control.

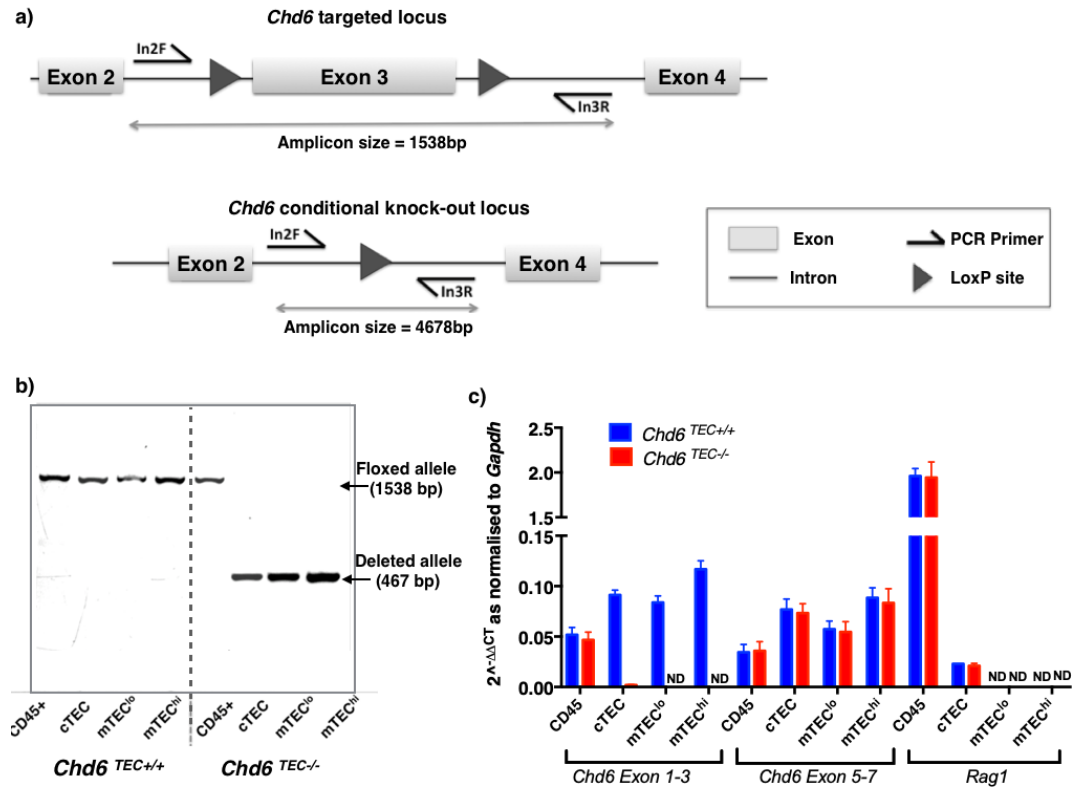


Figure 2.3 Deletion of *Chd6* Exon 3 region in TEC of *Chd6* ^{TEC-/-} mice

a) The mouse *Chd6* gene locus consists of 37 exons; exon 2 contains the translational initiation site. Exon 3 was targeted and flanked by loxP sites. The conditional knockout allele was generated by in vivo Cre-mediated deletion of exon 3, resulting in a frameshift mutation. A band size of 1538 bp is diagnostic for the floxed allele, and a band size of 467 bp for the deleted allele. b) Genotyping of FACS sorted CD45⁺, cTEC, mTEC^{lo} and mTEC^{hi} cells of the *Chd6* ^{TEC-/-} and *Chd6* ^{TEC+/+} control mice are shown. A band size of 1538 bp denotes the floxed allele and a band size of 467 bp the deleted allele. c) qRT-PCR analysis of different *Chd6* exon regions in CD45⁺, cTEC, mTEC^{lo} and mTEC^{hi} isolated from *Chd6* ^{TEC-/-} mouse (n=3, 3 female, 4.5 weeks old) and wild-type control *Chd6* ^{TEC+/+} mouse (n=3, 3 female, 4.5 weeks old). qRT-PCR of *Rag1* was performed in cTEC to document thymocyte contamination in the sorted cTEC (from thymic nurse cells). ND= not detected.

2.1.4.4.2. CHD6 staining in thymus tissue of *Chd6* ^{TEC-/-} mice

Absence of CHD6 proteins in TECs was further verified by immunofluorescent microscopy (Figure 2.4). Briefly, thymic tissues from 4.5 week old *Chd6* ^{TEC-/-} (n=6, 3 males, 3 females) and wild-type control *Chd6* ^{TEC+/+} mouse (n=6, 3 males, 3 females) mice were prepared for immunofluorescent staining, embedded in Tissue-Tek OCT compound and sectioned (8μm thickness) with a cryostat. Prepared tissue slides were fixed, permeabilised, incubated with primary antibodies (for an hour at 37°C) before being counter-stained with relevant secondary antibodies and DAPI (Vectashield) (antibodies as in Table 2.6). Slides were viewed using Confocal Microscopy 780 Zeiss

Inverted microscope, at 63 times magnification and assessed for the presence of CHD6 proteins.

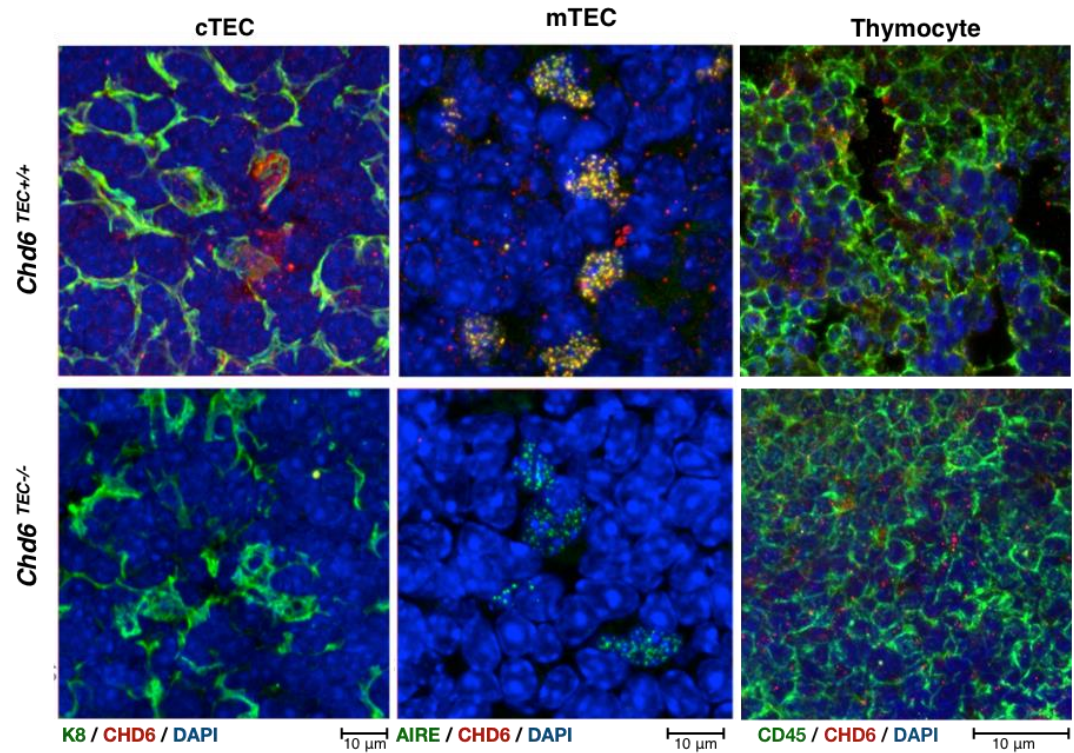


Figure 2.4 Loss of CHD6 protein detection in the TECs of *Chd6*^{TEC/-} mice

Presence of CHD6 protein was assessed by immunofluorescent confocal microscopy of frozen thymic tissue from *Chd6*^{TEC/-} mouse (n=6, 3 male & 3 females, 4.5 weeks old) and wild-type control *Chd6*^{TEC+/+} mouse (n=6, 3 male & 3 females, 4.5 weeks old). Thymic tissue sections were stained with antibodies against CHD6 (red), and additionally were stained with: i) antibodies against K8 (green) to stain for cTEC (left panel), ii) antibodies against AIRE (green) to stain for mTEC (middle panel) and iii) antibodies against CD45 (green) to stain thymocyte (right panel). Yellow represents merging signal of AIRE and CHD6. Nuclei were counterstained with DAPI.

2.2. General reagents

Listed are standard buffer/media used and made in-house with the composition and final concentrations shown (Table 2.1 and Table 2.2).

Table 2.1 Standard buffers

Buffer	Ingredients	Company	Working Concentrations
Fluorescence-Activated Cell Sorting (FACS) Buffer	Phosphate-Buffered Saline (PBS)	Gibco	1X
	Fetal Calf Serum (FCS)	Sigma	2%
	PBS	Gibco	1X

Liberase Digestion Buffer	Liberase	Roche	2.5 mg/mL
	DNase	Roche	10 mg/mL
Lysis Buffer	Tris	Merck	100mM
	EDTA	Sigma	5mM
	Sodium Dodecyl Sulfate (SDS)	Sigma	0.2%
	NaCl	VWR	200mM
	Proteinase K	Roche	100 µg/mL
Magnetic-Activated Cell Sorting (MACS) Buffer	PBS	Gibco	1X
	EDTA	Sigma	5mM
	Filtered Bovine Serum Albumin (BSA)	Sigma	0.2%
Red Blood Cell (RBC) Lysis Buffer	Ammonium Chloride (NH ₄ Cl)	Sigma	1.66%
	PBS	Gibco	1X
Tris-EDTA (TE) Buffer	Tris	Merck	10mM
	EDTA	Sigma	1mM
ATAC-Seq Lysis Buffer	Tris-Cl	Merck	10mM (pH7.4)
	NaCl	VWR	10mM
	MgCl ₂	VWR	3mM
	Igepal CA-630	Merck	0.1%

Table 2.2 Standard medias

Media	Ingredients	Company	Working Concentrations
HEK293T Media	DMEM	Gibco	1X
	Heat-Inactivated FCS (FCS-HI)	HyClone	10%
	Pen-Strep	Sigma	1X
TEC Media	IMDM	Gibco	1X
	2-Mercaptoethanol	Sigma	50 µM
	FCS-HI	HyClone	10%
T Cell Media	RPMI	Sigma	1X
	FCS-HI	HyClone	10%
	Pen-Strep	Sigma	1X
	2-Mercaptoethanol	Sigma	50 µM

2.3. Genotyping

2.3.1. Isolation and amplification of genomic DNA

Tissue samples from ear clips or sorted cells samples were digested in 600 µl lysis buffer incubated at 55°C for a minimum of 5 hours. Debris was pelleted and removed, and DNA precipitated with 600 µl isopropanol (VWR) and subsequently washed with 600 µl 70% ethanol (VWR). The supernatant was discarded and the DNA pellet air dried then resuspended in 50 µl TE buffer. Thereafter, genomic DNA was amplified through polymerase chain reaction (PCR) under the Thermocycler conditions (Applied Biosystems) as documented below (Table 2.3). Each reaction was performed in 25 µl volume containing 2µl of amplified genomic DNA, 0.2 µM each of the relevant forward and reverse primers (Table 2.4), 1X Taq buffer, 200 µM deoxynucleotides (dNTPs) (Sigma), 0.02 u/µl Taq polymerase (Sigma). The amplification temperature (T_a) was calculated as minus 5°C from the lowest melting temperature (T_m) of the primer pair ($T_m - 5^\circ\text{C}$).

Table 2.3 Thermocycler conditions for standard genotyping PCR

Step	Cycles	Temperature	Duration
1	1	94°C	4 minutes
2	34	94°C	30 seconds
		T_a	50 seconds
		72°C	1 minute
3	1	72°C	8 minutes
		4°C	Hold

Table 2.4 List of primers for genotyping and qRT-PCR analysis**(All primers were synthesised by Sigma)**

Target	Forward Primer	Reverse Primer
<i>ActFlp</i>	CACTGATATTGTAAGTAGTTTGC	TACTTCTTTAGCGCAAGGGGTAG
<i>Foxn1-Cre</i>	TCTGATGAAGTCAGGAAGAACC	GAGATGTCCTTCACTCTGATTC
<i>Chd6</i> In2-In3	GCTCCTGTGACACGCTTCTG	GGCCAGGAAGTAAAATAGGGTTC
<i>Chd6</i> Exon 1-3	GCTGTTTGTATTTTGGCTGCT	AAGCTCTTAACTTGGACAACCTGC
<i>Chd6</i> Exon 5-7	CAAGACTGTCCAGGAGGTTCA	CATCTGGGCCTGTTTGTTC
<i>Gapdh</i>	GGTGAAGGTCGGTGTGAACG	ACGACTGTCCAGGAGGTTCCG
<i>Rag1</i>	TGCAGACATTCTAGCACTCTGG	TTCAAAGGATCTCACCTAAACAG
<i>Aire</i>	TGTGCCACGACGGAGGTGAG	GGTCTGTGGACTCTGCCCTG
<i>Chd4</i>	GAGGTGGAGTGTGGCAGA	CCGCTCTGACATCTGTAACCTC
Human-CHD6	CAGGACTGAGGGGGTTTCTTC	GAGCCTTCAGGAGTTCGCTC
<i>Cyt11</i>	GCTGAGAGACTTCGTGGCTT	AGAGGAATACCAAGTCCCGC
<i>Svs4</i>	TCAAAGAGAAAGAAGAGCCGGT	ATGGCTCCAGCATATTGGTCC
<i>Ppy</i>	CAGGCCTAGGTATGGGAAGA	AGAGAGGCTGCAAGTCCATT
<i>Gp2</i>	CAAGAACAGATGCCAAACCAA	AATGGCTGGTCTACTACTGCG
<i>Tspan8</i>	TTCAGTCGGAGTTCAAGTGCT	AACGCCAGTCCAAAAGCAA
<i>Tff3</i>	TACGTTGGCCTGTCTCCAAG	CAGGGCACATTTGGGATACT
<i>Apoh</i>	GGACCCGATTGCCAGAA	CGGCAAGAAGGACCAAGTT
<i>Crp</i>	TGGTGGGAGACATCGGAGAT	GCCCGCCAGTTCAAAACATT
<i>Upk1a</i>	CAGGCAACTTCATCCCCATC	GGCAAAGCCAAACCACGATA
<i>Spink7</i>	CCATCCCTTGCCCCATTGTA	GGATTTTCCATTGCTTCTCAAGAT
<i>Ctsj</i>	ACGAAGGAAAAGACGGACCC	AAAGCTCATTCGGTGGGAGG
<i>Spink12</i>	TCTGTAGATGCTGTGAGCCAA	GCAGAATGCACAGCGGTTTT
<i>Fabp2</i>	TGGAAAGGAGCTGATTGCTGT	TCCTTCATATGTGTAGGTCTGGAT
<i>Tff1</i>	TAAATTGTGGCTTCCCCGGT	GGGACATTCTTCTTCTGAGTGTTT
<i>Reg1</i>	CCCCAAAGGAATCGTCGCTG	CCCCAAAGGAATCGTCGCTG
<i>Sec1</i>	GACGACGACGCTGAAACCT	CTTCTCTGGCTACACACCTGA
<i>Gh</i>	TTCGAGCGTGCCTACATTCC	CGAAGCAATTCCATGTCGGTT
<i>Sult1c2</i>	ACAAATCGTTCTACGGCCCC	CCAATCACCCACAGTTCCTTTTC
<i>Itln1</i>	TTGCTACCAGAGGTTGCACT	AGACCATCTTGTGCCTTTGTGT
<i>Angptl7</i>	ACTGTTCTTCCCTGTACCAGA	TCACAGAACACCTCTAGCTCA
<i>Ca4</i>	TCCAAACCCATTCAACCTCT	TACATTTGGTGCTCGGGGG
<i>Slc13a2</i>	CTCTTCTGGGTGTCAACTCC	TAGAGAGAGGATGAGCACGG
<i>My19</i>	TTTGGGGAGAAGCTGAACGG	TCCTCGTGGATGAAGCCTGAG

2.3.2. Agarose gel electrophoresis

Agarose gels were prepared at 1-2% agarose concentration based on the predicted PCR product size by melting UltraPure Agarose (Invitrogen) in 1X Tris-Borate-EDTA (TBE) buffer (Sigma) and 1:20,000 GelRed dye (Biotium). Each amplified PCR product was mixed with 6X gel loading dye at 5:1 dilution (Thermo Fisher Scientific), and 10 µl of the mixed product was loaded into each well and run in a gel tank. 3µl of 1 kb Plus DNA ladder (NEB) was loaded for each gel and run simultaneously for size estimation of the PCR product. Gel Doc XR+ system (Bio-Rad) was used to visualise the gel.

2.4. *In-vitro* experiments

2.4.1. Plasmids

Human CHD6 plasmid (pCDNA3 Human HA-CHD6 [HA-CHD6] (designated Hu-CHD6) was a gift from Amelia Nieto, in which the full length *CHD6* cDNA carrying a N-terminal triple HA-tag was cloned into vector pCDNA3 (Invitrogen)¹⁹⁶. Human AIRE plasmid (Plasmid pSV-40 Human-AIRE [pAP4]) (designated Hu-AIRE) was a gift from Bergithe Oftedal, in which the full length *AIRE* cDNA was cloned into vector pSV-40¹⁵².

2.4.2. Bacterial transformation, selection and plasmid purification

The plasmids were transformed into *Escherichia coli* TOP10 One Shot competent cells (Invitrogen), following the manufacturer's guideline. Purification of plasmids was performed using Plasmid Mini Kit (Qiagen) as per manufacturer's protocol. The transformed plasmids were propagated in LB liquid culture and centrifuged to pellet the bacterial cells. Supernatants were removed and bacterial cell pellets were sequentially washed using several supplied buffers. Plasmids were selectively adsorbed by silica membrane by the supplied QIAprep column (Qiagen) and eluted directly from the column in EB buffer (supplied by the manufacturer).

2.4.3. Cell line preparation

HEK293T was commercially purchased, while the mTEC4D6 human thymic epithelial cell line was a kind gift from Bergithe Oftedal. Related cell line work, including freezing, thawing, passaging and expansion were performed in a sterile Class II laminar flow hood, and conducted in accordance with standard tissue culture guidelines. Cells were passaged at 85-95% confluency and harvested after trypsinisation using 0.25% Trypsin-EDTA (Gibco) at 37°C for 10 minutes. Counting of cells was performed by haemocytometer (Neubauer) and Trypan blue (Thermo Fisher Scientific). HEK293T were cultured with HEK293T media and the human mTEC4D6 cell line with TEC media.

2.4.4. Mammalian cell transfection

HEK293T or human mTEC4D6 cells were seeded in 6-well plates at 50-60% confluency in the appropriate media and kept at 37°C, 5% CO² in a humidified atmosphere. Transfections were performed within 24 hours of cell plating, with the plasmid(s) of interest using Lipofectamine 3000 (Thermo Fisher Scientific) as per manufacturer's recommendation. Transfected cells were incubated for 48 hours, following that the cells were harvested by trypsinisation with 0.25% Trypsin-EDTA (Gibco), washed, pelleted and stored at -20°C.

2.4.5. Co-immunoprecipitation and Western blot

HEK293T cells and human mTEC4D6 were co-transfected with the plasmids pSV-40 Human-AIRE (Hu-AIRE) and pCDNA3 Human HA-CHD6 (Hu-CHD6) with Lipofectamine 3000 (Thermo Fisher Scientific) as previously described (see section 2.4.4). 8 X 10⁶/cells of co-transfected HEK293T and mTEC4D6 samples were harvested after 48 hours of transfection for preparation of protein lysates. Protein lysates were prepared using RIPA lysis buffer [25mM Tris-HCl (pH7.5), 150mM NaCl, 1% NP-40, 0.1% SDS, 10% glycerol, 1mM DTT, 0.2mM PMSF, plus complete protease inhibitor mixture (Roche) and incubated on ice for 20 minutes. Protein lysate extracts from co-transfected cells were incubated with Protein-G Magnetic Dynabeads,

coupled with rabbit anti-HA antibodies for 2 hours with rotation at 4°C. Bound proteins were eluted by boiling, separated by SDS-PAGE, and electro-transferred to PVDF membranes, which were incubated with mouse anti-Human AIRE antibody and rabbit anti-HA antibody, respectively.

2.4.6. *In-vitro* mitogen proliferation assay

In-vitro mitogen proliferation assay was performed in mature CD4 thymocytes (CD4 M2) and naïve T cells (CD4 and CD8).

2.4.6.1. Mitogenic stimulation with anti-CD3 and anti-CD28

Lymph nodes or thymi were harvested under sterile conditions and T cells or thymocytes were released by gentle mechanical disruption using frosted glass slides as described (see section 2.5.2). Cells were counted, suspended in 200uL of FACS buffer and stained using appropriate antibodies. Cells were acquired using a FACS AriaIII and naïve non T_{reg} CD4 cells(TCRβ⁺ B220⁻ CD4⁺ CD8⁻ CD44⁻ CD62L⁺), naïve CD8 T cells (TCRβ⁺ B220⁻ CD8⁺ CD4⁻ CD44⁻ CD62L⁺) or CD4 mature thymocytes (TCRβ⁺ CD4⁺ CD8⁻ CD25⁻ CD69⁻ MHCI⁺) were sorted in 1.5mL 100% FCS solution in 15mL centrifuge tube respectively (see section 2.5.3). 96-well flat bottom plates were coated with a range of concentrations of purified anti-mouse CD3 antibody (145-2C11, Biolegend at 1.25, 2.5, 5.0 and 10.0μg/mL respectively) at 4°C overnight, then washed with 1X PBS to remove excess antibody. Sorted cells were stained with 5μM CellTrace™ Violet for 20 minutes at 37°C, followed by quenching with pre-warmed complete RPMI for 5 minutes at 37°C (C34557, Life Technologies). Stained cells were washed, and 150,000 naïve T cells or 50,000 mature CD4 thymocytes were plated per well into a 96-well plate pre-coated with anti-CD3 as previously described. Plated cells were incubated in culture media supplemented with anti-CD28 (1.0 μg/ml, 37.51, Biolegend) and incubated in 5% CO₂ for three days. On Day 3, stimulated cells were harvested by gentle pipetting and stained with Near-Infrared Live/Dead staining. CellTrace Violet dilution was assessed by flow cytometry, and subsequently analysed using FlowJo v 10 Software Proliferation Wizard Platform (FlowJo, LLC). Briefly, after sequentially gating on Singlets, Live cells, and CellTrace Violet-positive cells,

two parameters of responding (dividing) cells relative to the input was obtained i.e. proliferative index and expansion index using the provided software algorithm.

2.4.6.2. Mitogenic stimulation with PMA and Ionomycin

Naïve CD4 or CD8 T cells were sorted using flow cytometry (see section 2.5.3) in 1.5mL 100% FCS solution a 15mL centrifuge tube. Sorted cells were counted and stained with 5µM CellTrace™ Violet for 20 minutes at 37°C, followed by quenching with pre-warmed complete RPMI for 5 minutes at 37°C (C34557, Life Technologies). Stained cells were washed, and 150,000 cells were plated per well into a 96-well plate. Plated cells were supplied with culture media supplemented with different dilutions of PMA Ionomycin eBioscience™ Cell Stimulation cocktail (0, 1:2000, 1:1000 and 1:500, stock concentration: Phorbol 12-Myristate 13-Acetate (40.5 µM), Ionomycin (670 µM) in Ethanol (500X)) and incubated in 5% CO₂ for three days. On Day 3, stimulated cells were harvested by gentle pipetting and stained with Near-Infrared Live/Dead staining. CellTrace Violet dilution was assessed by flow cytometry and subsequently analysed using FlowJo v 10 Software Proliferation Wizard Platform (FlowJo, LLC.) Briefly, after sequentially gating on Singlets, Live cells and CellTrace Violet-positive cells, two parameters of responding (dividing) cells relative to the input was obtained i.e. proliferative index and expansion index using the provided software algorithm.

2.4.7. TNFα production assay

Thymi were harvested under sterile conditions and thymocytes were released by gentle mechanical disruption using frosted glass slides as described previously (see section 2.5.2). Cells were counted and 5 X 10⁶ thymocytes were cultured for 4 hours with Brefeldin-A GolgiPlug™ (1.0 µl/ml, 555029, BD) and anti-CD28 (1.0 µg/ml, 37.51, Biolegend) in a 12-well plate coated with anti-CD3 (5µg/ml, 145-2C11, Biolegend). Thymocytes were then assessed by flow cytometry using the cell surface staining and intracellular TNFα staining.

2.5. Primary murine cell isolation

2.5.1. Thymic epithelial cells isolation

Thymi were harvested under sterile environment and cleaned off connective tissues and remaining fats. TECs were then isolated by exposing the thymi to sequential enzymatic digestion by incubation at 37°C in Liberase digestion buffer. 1.5 ml of digestion buffer was used to digest two thymi lobes and gentle pipetting disintegrated the lobes. The sample was subsequently incubated for 10 minutes, and supernatant was collected into a 50 ml centrifuge tube containing 40 ml of cold FACS buffer. The described steps were performed a total of three rounds, with 1 ml of fresh digestion buffer used for the second round and 0.8ml for the third round of digestion. The pooled supernatant was centrifuged and the cell pellet was suspended as single cell suspension. Cells were counted and incubated with α -CD45 beads (Miltenyi Biotec) at a concentration of 5 μ l per 10×10^6 cells for 15 minutes at 4°C. Subsequently, cells were washed with FACS buffer, incubated with 100 μ g/ml DNase I (Roche) and filtered through a 30 μ m filter. Cells were then resuspended at a concentration of 100×10^6 cells/ml in FACS buffer and enriched for TEC by depletion of CD45 using the “Depletes” program on the AutoMACS separator (Miltenyi Biotec). Enriched TECs were washed and counted before being prepared for analysis via flow cytometry.

2.5.2. T cell isolation

Thymi, spleen or lymph nodes were harvested under sterile conditions and T cells were released by gentle mechanical disruption using two frosted glass slides (Thermo Scientific). For spleen, isolated splenocytes were incubated 1:4 with RBC lysis buffer (1.66% NH₄Cl) for 5 minutes at room temperature to remove erythrocytes. Cells were then washed with cold FACS buffer and filtered through a 70 μ m filter, followed by resuspension as a single cell suspension. Cells were counted before being prepared for further analysis.

2.5.3. Naïve CD4 and Naïve CD8 sorting

Lymph nodes were harvested under sterile conditions and T cells were released by gentle mechanical disruption using frosted glass slides. Cells were then washed with cold FACS buffer (2% fetal calf serum) and filtered through a 70µm filter followed by resuspension as a single cell suspension. Cells were then counted, resuspended in 200uL of FACS buffer and sample were stained using the appropriate antibodies. Cells were acquired using a FACS AriaIII and naïve non T_{reg} CD4 (TCRβ⁺ B220⁻ CD4⁺ CD8⁻ CD44⁻ CD62L⁺) and naïve CD8 T cells (TCRβ⁺ B220⁻ CD8⁺ CD4⁻ CD44⁻ CD62L⁺) were sorted in 1.5mL 100% FCS solution in 15mL centrifuge tube on ice. Sorted cells were then washed with ice-cold PBS and counted before further analysis.

2.6. Flow cytometry analysis

2.6.1. Flow cytometry antibodies

The antibodies/lectin listed below (Table 2.5) were utilised for flow cytometry analysis to phenotypically differentiate specific cell types and populations.

Table 2.5 FACS antibodies/Lectin

Antigen	Clone	Isotype	Conjugate	Source
Aire	5H12	Rat IgG2c, κ	Alexa Fluor 488	Invitrogen
B220	RA3-6B2	Rat IgG2a, κ	BV605, PE	BD Biosciences
CCR6	29-2L17	Armenian Hamster IgG	PE	BioLegend
CCR7	4B12	Rat IgG2a, κ	BV421, PE	BioLegend
CD103	2E7	Armenian Hamster IgG	Alexa Fluor 647	BioLegend
CD11b	M1/70	Rat IgG2b, κ	Biotin	BioLegend
CD11c	N418	Armenian Hamster IgG	Biotin	BioLegend
CD19	1D3	Rat IgG2a, κ	Biotin	Invitrogen
CD24	M1/69	Rat IgG2b, κ	APC, FITC, PerCP-eF710	BioLegend
CD25	PC61	Rat IgG1, 1	Biotin, PerCP-Cy5.5, BV605	BioLegend
CD25	PC61.5	Rat IgG1, 1	eF450	eBioscience

CD3e	145-2C11	Armenian Hamster IgG	PerCP-Cy5.5	Invitrogen
CD4	GK1.5	Rat IgG2b, κ	PE-eF610	BioLegend
CD4	RM4.5	Rat IgG2a, κ	Pe-eF610	Invitrogen
CD44	IM7	Rat IgG2b, κ	PE, PE-Cy7, PE-eF610	eBioscience
CD45	30-F11	Rat IgG2b, κ	Alexa Fluor 700	BioLegend
CD5	53-7.3	Rat IgG2a, κ	PE-Cy7, PerCP-Cy5.5, PE-Cy5	BioLegend
CD62L	MEL-14	Rat IgG2a, κ	PE-Cy7	BioLegend
CD69	H1.2F3	Armenian Hamster IgG	PE-Cy5	BioLegend
CD73	TY/11.8	Rat IgG1, κ	PE	BioLegend
CD80	16-10A1	Armenian Hamster IgG	PE-Cy5	BioLegend
CD86	GL-1	Rat IgG2a, κ	PE-Cy7	BioLegend
CD8a	53-6.7	Rat IgG2a, κ	Alexa Fluor 700, Alexa Fluor 647	BioLegend
Desmoglein-3	DA1E	Mouse IgG1	-	AbCam
EpCAM	G8.8	Rat IgG2a, κ	PERCP-Cy5.5	BioLegend
F4/80	BM8	Rat IgG2a, κ	Biotin	BioLegend
Fc Block	2.4G2	Rat IgG2b, κ	-	BD Biosciences
Foxp3	FJK-16s	Rat IgG2a, κ	FITC, PE-eF610	eBioscience
FR4	12A5	Rat WI IgG1	APC	BD Biosciences
Gp2	2F11-C3	Rat IgG2a, κ	Alexa Fluor 488	MBL Int Corp
Gr-1	RB6-8C5	Rat IgG2b, κ	Biotin	BioLegend
Helios	22F6	Armenian Hamster IgG	PE, BV421	BioLegend
ICOS	15F9	Syrian Hamster IgG	PE-Cy5	BioLegend
ICOS	C398.4A	Armenian Hamster IgG	PE, Biotin	BioLegend
Isotype	RTK2071	Rat IgG1, κ	Alexa Fluor 647, APC	BioLegend
Isotype	MOPC-21	Mouse IgG1, κ	APC-Cy7	BioLegend
Isotype	DA1E	Rabbit IgG	Alexa Fluor 488, PE	Cell Signalling
Itgb4	346-11A	Rat IgG2a, κ	FITC	BioLegend
L1cam	555	Rat IgG2a, κ	Alexa Fluor 700	R&D System
Ly51	6C3	Rat IgG2a, κ	PE, PE-C7	BioLegend
MHC II (I-A/ I-E)	M5/114.15.2	Rat IgG2b, κ	BV421, FITC	BioLegend
MHC-I (H-2Dk)	28-14-8	Rat IgG2a, κ	PE	eBioscience
Mouse IgG1	RMG1-1	Rat IgG1, κ	APC-Cy7	BioLegend
NK1.1	PK136	Mouse IgG2a, κ	Biotin	BioLegend
PD-1	29F.1A12	Rat IgG2a, κ	APC	BioLegend
Qa-2	695H19-9	Mouse IgG2a, κ	Biotin	BioLegend

Streptavidin	-	-	APC, APC-Cy7, BV605, FITC, PE-Cy7, PerCP-Cy5.5	BioLegend
TCR β	H57-597	Armenian Hamster IgG	APC-Cy7, FITC, PE, PE-Cy5	BioLegend
TCR $\gamma\delta$	GL3	Armenian Hamster IgG	Biotin	eBioscience
Ter-119	TER-119	Rat IgG2b, κ	Biotin	BioLegend
Tspan8	657909	Rat IgG2b, κ	APC	R&D System
TNF α	MP6-XT22	Rat IgG1, κ	Alexa Fluor 647	BioLegend
UEA-1 (Lectin-Ulex europaeus agglutinin 1)			Cy5, Biotin	Vector Laboratories

2.6.2. Live/Dead Staining

The Live/Dead Fixable Aqua staining (ThermoFisher Scientific) was performed by adding 1 μ l of the stain into a total sample of up to 10 million cells in 1 ml, incubated for 30 minutes at room temperature in the dark. For some FACS panel analysis, DAPI staining was utilised to phenotypically distinguish between live and dead cells, and this was performed by adding 1 μ l of DAPI (1:363 of 5mg/mL, Sigma) per 100 μ l of sample volume and gently vortexing it just before acquisition of the sample.

2.6.3. CCR7 surface staining

The CCR7 surface staining was performed by adding 0.5 μ l of the antibody into a total sample of up to 10 million cells in 0.1ml, incubated for 30 minutes in 37°C waterbath, in the dark.

2.6.4. Extracellular surface staining

Extracellular antibody surface stainings were performed for 20 minutes at 4°C in the dark. Concentrations of antibodies used were determined based on the titrated volumes, and range between 0.25-1.5 μ g/100 μ l (for up to 10 million cells).

2.6.5. Intracellular staining

For intracellular antibody staining, extracellular antibody surface staining needs to be performed before the samples were fixed and permeabilised using reagents from the Foxp3 staining buffer set (eBioscience) for 30 minutes at 4°C in the dark. Fixed and permeabilised cells were then stained with the appropriate intracellular antibodies for an hour at 4°C in the dark.

2.6.6. FACS analysis and sorting

The FACS AriaIII (BD Biosciences) was utilised for phenotypic analysis and sorting of the cells. The prepared stained samples were resuspended in appropriate volume of FACS buffer (ranging between 0.2 to 1 ml) and vortexed gently before acquisition. Cells were sorted directly into 1.5ml microcentrifuge tube or 15ml centrifuge tubes pre-coated with 100% cold FCS. Sorting was performed using the BD 4-way purity mask, with a documented purity of more than 99%. Data analysis was performed with FACS Diva (BD) and FlowJo (TreeStar).

2.7. Imaging

2.7.1. Tissue frozen section processing

Relevant organs were freshly isolated under sterile condition with residual fats, blood and remaining connective tissues removed. For fresh frozen samples, cleaned organs were immersed in Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and frozen in cryomolds (Leica) using mixture of dry ice and isopropanol. Frozen tissue samples were stored at -20°C. When needed, frozen tissue blocks were sectioned at 8µm thickness with cryostat (CM3050 S, Leica, Deerfield, IL), carefully placed onto charged SuperFrost Plus precleaned microscope slides (VWR), air-dried for 30 minutes, and stored at -20°C until use.

2.7.2. Tissue Formalin-fixed paraffin embedded (FFPE) processing

Relevant organs were freshly isolated under sterile condition with residual fats, blood remaining connective tissues removed. For paraffin embedded samples, harvested organs were fixed immediately after dissection in 10% formalin for 24 hours. The samples were subsequently dehydrated gently by immersion in increasing concentrations of ethanol (70, 80, 90 and 100%) over two days. The dehydrated samples were incubated in xylene before paraffin embedding and the embedded samples were allowed to harden overnight. Finally, the samples were sectioned at 8µm thickness with microtome (Leica RM2255 Fully Automated Rotary Microtome) and stored at room temperature.

2.7.3. Haematoxylin and Eosin (H&E) staining and microscopy

Prepared FFPE slides were stained in filtered haematoxylin (Sigma) for 1 minute then rinsed with running distilled water. Slides were then stained with eosin (Thermo Scientific) for 2 minutes and rinsed in distilled water for 30 seconds. Stained slides were then sequentially dehydrated with increasing ethanol concentrations solutions (70, 80, 90 and 100%) for 30 seconds each. Slides were left to dry before mounting with Hydromount (National Diagnostics) and coverslips sealed using nail polish. Prepared H&E stained slides were visualised using the ICC50 HD microscope (Leica). Captured images were corrected for colour contrast and tile images were stitched together using Adobe Photoshop (Adobe).

2.7.4. High Resolution and Superresolution Confocal Microscopy

Prepared frozen tissue slides were fixed in 3.6% paraformaldehyde (Sigma Aldrich) for 20 minutes, followed by methanol for 5 minutes. Tissue were then permeabilised with 0.3% Triton X-100 in PBS for 10 minutes and were washed with PBS. Permeabilised tissue slides were blocked with goat serum before incubation in relevant primary antibodies (prepared in 0.3% Triton-X PBS at suggested antibodies dilution)

in the dark for 1 hour at 37°C. Tissue slides were washed in PBS, stained with relevant secondary antibodies (prepared in 10% goat serum in PBS) in the dark for 30 minutes at 37°C. Subsequently, tissue slides were washed and stained with 4'6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, Vectashield), mounted and sealed. Antibodies or lectin and reagents used are listed in Table 2.6. Slides were viewed using Confocal Microscopy on a 780 Zeiss Inverted microscope, at 63 times magnification. For superresolution microscopy, slides were viewed using Confocal Microscopy 880 Zeiss Inverted with AiryScan at maximum 100 times magnification. Images were further analysed using ImageJ (v2.0.0) for Z-stack and co-localisation analyses using Coloc2 plugin.

Table 2.6 Antibodies/Reagent used for immunofluorescence

Species	Antibody/Reagent	Antibody Specificity	Company	Dilution
Rabbit	CHD6	Human-CHD6	Bethyl Laboratories	1:500
Rat	AIRE	Mouse-AIRE	eBioScience	1:500
Rat	K8	Mouse-Keratin 8	eBioScience	1:500
Rat	CD45 FITC	Mouse-CD45	BioLegend	1:500
-	UEA1 Cy5 (Lectin)	-	Vector Laboratories	1:250
Goat	Goat anti-rabbit AF555 conjugated	Rabbit Fc Receptor	Life Technologies	1:500
Goat	Goat anti-rat AF488 conjugated	Rat Fc Receptor	Life Technologies	1:500

2.7.5. DNA double strand break analysis in TECs

Thymic tissues from 4.5 week old *Chd6*^{TEC-/-} (n=5) and *Chd6*^{TEC+/+} (n=5) mice were prepared for immunofluorescent staining: embedded in Tissue-Tek OCT compound and sectioned (8µm thickness) with a cryostat. Prepared tissue slides were fixed, permeabilised, incubated with primary antibodies (an hour at 37°C) before counter-stained with relevant secondary antibodies and DAPI (Vectashield) (antibodies and reagents used as in Table 2.7). Slides were viewed using Confocal Microscopy 780 Zeiss Inverted, at 63 times magnification. Images were further analysed using Zen 2 (Blue

edition) for using colocalisation analyses which parameters include: area of nucleus analysed, specific-pixel quantity and intensity, pixel-merging analysis and correlated co-efficient of both AIRE and γ H2AX merging signals.

Table 2.7 Antibodies/Reagent used for DNA double strand break analysis

Species	Antibody/Reagent	Antibody Specificity	Company	Dilution
Rabbit	γ H2AX	Human- γ H2AX	Cell Signalling	1:200
Rat	AIRE	Mouse-AIRE	eBioScience	1:500
Goat	Goat anti-rabbit AF555 conjugated	Rabbit Fc Receptor	Life Technologies	1:500
Goat	Goat anti-rat AF488 conjugated	Rat Fc Receptor	Life Technologies	1:500

2.8. Molecular biology and transcriptomic analysis

2.8.1. RNA extraction and complementary DNA synthesis

RNA was extracted using the RNeasy Plus Micro Kit (Qiagen) according to the manufacturer's recommendation. Briefly, cells were lysed using the supplied RLT buffer and homogenised by centrifugation using the supplied QIAshredder column (Qiagen). RNA was precipitated using an equal volume of 70% ethanol and the precipitation was subsequently loaded onto the RNeasy MinElute spin columns (Qiagen). Loaded columns underwent several washing steps and treated with RNase-free DNase (Qiagen) to dissolve contaminated DNA from the column. Isolated RNA samples were eluted using 12 μ l nuclease-free water and utilised immediately or stored at -20°C until subsequent use. RNA concentrations were measured using the NanoDrop instrument (Thermo Scientific). Complementary DNA (cDNA) was synthesised using the SensiFAST cDNA kit (Bioline). Extracted RNA, 4 μ l 5X TransAmp Buffer, 1 μ l reverse transcriptase (Bioline) made up to 20 μ l with nuclease-free water were mixed gently and incubated in a thermocycler for 10 minutes at 25°C, 15 minutes at 42°C and 5 minutes at 85°C. Prepared samples were used immediately or stored at -80°C.

2.8.2. Real Time Quantitative PCR (qRT-PCR)

Total RNA was extracted using the RNeasy Plus Micro Kit (Qiagen) followed by cDNA preparation using the SensiFAST kit (Bioline) according to the manufacturers' protocols as described previously (see section 2.8.1). cDNA was used for quantitative PCR (qRT-PCR) with the relevant forward and reverse primers (Sigma), using SensiMix SYBR Hi-Rox Kit (Bioline) and StepOnePlus Real-Time PCR system (Applied Biosystems). 0.5-2 µl of template cDNA were used per 10 µl reactions containing 1 X SensiMix SYBR (Bioline) and 100 nM of each relevant primer pair (Table 2.4) and reactions were run as per thermocycler condition listed below (Table 2.8). For a standard experiment analysis, at least three biological replicates for each sample were performed, unless otherwise specified. Each biological reaction was carried out in technical triplicates and expression values were normalised to *Gapdh* expression and calculated as $2^{-\Delta C_{\tau}}$ to demonstrate fold change, where C_{τ} represents the threshold cycle.

Table 2.8 Thermocycler conditions for qPCR

Step	Procedure	Cycles	Temperature	Duration
1	Polymerase Activation	1	94°C	4 minutes
2	Amplification	40-50	95°C	15 seconds
			60°C	15 seconds
			72°C	15 seconds
3	Melt Curve	Variable	60-95°C	Variable

2.8.3. Bulk RNA-Sequencing and Principal component analysis

Mice with homozygous TEC conditional deletion of *Chd6* (*Chd6*^{TEC^{-/-}}, n=3; female) were analysed at 4.5 week old alongside littermate controls (*Chd6*^{TEC^{+/+}}, n=3; 3 female). TECs were isolated from thymic tissue after enzymatic tissue disruption with Liberase and DNaseI (see section 2.5.1), followed by magnetic-activated cell sorting by AutoMACS and fluorescence-activated cell sorting by FACS AriaIII (BD Bioscience) as described previously (see section 2.6.6). The following were the different TECs subpopulations sorted based on their cell surface markers expressions: i) cTEC: CD45⁻, EpCAM⁺, Ly51⁺, UEA1 low positivity; ii) mTEC^{lo}: CD45⁻

EpCAM⁺, Ly51⁻, UEA1 high positivity, CD80^{lo}, MHCII^{lo}; and iii) mTEC^{hi}: CD45-EpCAM⁺, Ly51⁻, UEA1 high positivity, CD80^{hi}, MHCII^{hi}. 3000 cells of each different TECs subpopulations were directly sorted into 75µL of RLT RNeasy plus lysis buffer followed by RNA extraction using RNeasy Plus Micro kit (Qiagen) as previously described (see section 2.8.1). RNA quality was assessed using Agilent RNA High Sensitivity TapeStation 2200 as per company's protocol. RNA-sequencing libraries were prepared using the Illumina Smartseq2 kit as per company's protocol. Paired end sequencing (75 bp read length) using the HiSeq4000 platform (Illumina) was conducted for prepared libraries over one lane of a flow cell (Oxford Genomics Centre). Bioinformatics analyses were done in collaboration with Dr Kevin Rue Albert from Kennedy Institute, University of Oxford. Briefly, QC metrics were generated by the FastQC package and all samples passed QC. Read alignment, quantification and post-mapping QC was performed using pipeline_scrnaseq.py (<https://github.com/snsansom/scseq>) (Kevin Rue Albert, University of Oxford). Sequence reads were mapped by hisat2 using a two-pass strategy and the number of reads mapping to gene models quantitated with the feature Counts program²⁰⁸. Per-gene Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values were estimated using Cufflinks²⁰⁹, and read counts were converted to upper-quartile normalised Transcripts Per Million (TPM) values using Salmon²¹⁰.

One of the statistical analyses employed to analyse and robustly exploring the generated genome-wide transcriptomic dataset, was Principal component analysis (PCA). Briefly, PCA allows the reduction in the dimensionality of the large dataset by recognising the principal direction, referred to as “principal component”, in which the data varies^{211–213}. This analysis is based on the assumption that the direction with the largest data variances is most important and is considered to be the main principal component^{211,212,214}. This main principal direction is termed as “Principal Component 1” or PC1, with the subsequent directions then be projected based on its degree of relationship to the main principal. The “Principal component 2” or PC2, as such, is the second most important direction and shown as an axis orthogonal to the PC1 axis. The proportion of variation accounted by each principal component will be given by its eigenvalue. For example, an eigenvalue of 5.0 in PC1, relay to us that 50% of the variation in the dataset is explained by this first principal component. Employing an unsupervised PCA analysis in the transcriptomic dataset, it allows: (i) exploration of

the relatedness and identification of the correlated variables between the different samples; (ii) reduction dimensionality of the large dataset by removing redundancy and noise; and (iii) discovery of potential patterns in a large dataset in a robust and unbiased manner.

2.9. Total IgG measurement

Sera were obtained from aged (8 months) female *Chd6*^{TEC^{-/-}} mice (n=8) and their sex and age-matched wild-type controls (n=8). The IgG concentration of these sera was measured using commercially available mouse IgG ELISA kit following the manufacturer's instruction (Thermo ScientificTM Easy-TiterTM Mouse IgG Assay Kit). Briefly, sera were diluted to a 1: 40,000 dilution in commercially supplied Dilution Buffer. Diluted sera were plated in 96-well plate in triplicates, mixed with supplied sensitised beads and incubated at room temperature for 5 minutes on plate mixer at 700 rpm. Blocking buffer was subsequently added and samples were incubated for 5 minutes at room temperature on plate mixer at 700 rpm. The IgG concentrations were determined using SPECTROstar^{Nano} microplate reader at 405nm. Standard curves were constructed using the mouse IgG supplied with the ELISA kit, and this was used to interpolate the IgG concentrations in the samples expressed as the µg concentration per mL of serum.

2.10. Inter-rater reliability assessment

Inter-rater reliability was conducted on the three independent observers for the evaluation of: i) serum autoantibodies detection and ii) lymphocytic organ infiltration. To perform this assessment, forty slides were prepared and scored by the three selected observers for: i) lymphocytic infiltrations (using the previously described scoring system¹³³) and ii) autoantibodies detection, respectively. The reliability of scoring between the three observers was measured using Fleiss' kappa, κ ²¹⁵. κ value ranges from ≤ 0 to 1, with $\kappa = 1$ indicates a perfect agreement and $\kappa \leq 0$ indicates a poor agreement between the observers^{215,216}.

2.11. Serum autoantibodies detection

Tissue-specific autoantibodies were assessed in a set of 8 female *Chd6*^{TEC-/-} mice and their gender- and aged-matched controls at 8 months of age. 8 μ m frozen tissue sections of eye, ovary, intraorbital lacrimal gland, salivary gland, stomach, lung, liver, and pancreas were prepared from 6 week old B6 *Rag2*-deficient mouse. Whole blood from the aged *Chd6*^{TEC-/-} mice and wild-type controls were collected via cardiac puncture. Sera were obtained by allowing the blood to clot at room temperature for 30 minutes, supernatant was collected after centrifuging at 2000G for 10 minutes. Sera collected were stored at -20°C. Prepared *Rag*-deficient organ slides were incubated with a 1:20 serum dilution in sterile PBS and autoantibodies were revealed using FITC-conjugated goat anti-mouse IgG (1:500). Stained slides were analysed by Zeiss 780 Inverted confocal immunofluorescence microscopy.

2.12. Lymphocytic Organ Infiltration

Lymphocytic organ infiltrations were assessed in a set of 8 female *Chd6*^{TEC-/-} mice and their gender- and aged-matched controls at 8 months of age. 8 µm of paraffin-embedded tissue sections of eye, ovary, intra-orbital lacrimal gland, salivary gland, stomach, lung, liver, and pancreas were prepared and stained for H&E (see section 2.7.3). Lymphocytic infiltrations were evaluated using ICC50 HD microscope (Leica). Scoring was performed based on the previously described scoring system¹³³. In general scores, 0, 0.5, 1, 2, 3, and 4 indicate no, trace, mild, moderate, or severe lymphocytic infiltrations, and complete destruction, respectively. For retinal infiltrations, score of 1 was given when the lesion affecting less than half of the photoreceptor layer; score of 2 was given when more than half of the photoreceptor layer was involved; score of 3 was given when the entire photoreceptor layer was destroyed but not effecting the outer nuclear layer while the score of 4 was given if the entire photoreceptor layer and the outer nuclear layer was destructed.

2.13. Statistical analysis

Graphs show mean values and error bars represent standard error of mean, calculated by the software Prism (Graphpad). Statistical analysis was conducted using Graphpad prism. Probability values were calculated with Student's t-test (unpaired, two-tailed, normal distribution) unless otherwise stated. Significance is represented as * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$.

Chapter 3 Biological properties of CHD6 in thymic epithelial cells

3.1. Aim

CHD6 is a nuclear protein and exists in a large complex with a predicted molecular weight of 305.4 kDA¹⁹⁶. Lutz et.al had successfully detected CHD6 using Western blot of different human derived cell lines (HEK293, HeLa) and primary mouse tissues (liver, heart, kidney, spleen, lung)¹⁹⁶. However, no publication had commented specifically on the expression of CHD6 in different murine TECs, either at transcriptomic or protein level. As I aim to study the role of CHD6 in the thymus, I first established the expression of *Chd6* in murine TECs. I then further deciphered the biological properties of CHD6 expression in different TECs.

3.2. *Chd6* gene expression in TEC

Initially, using the TEC-specific human and murine cell lines (mTEC4D6 and mTEC1C6, respectively), I confirmed the gene expression of *Chd6* by qRT-PCR (Figure 3.1).

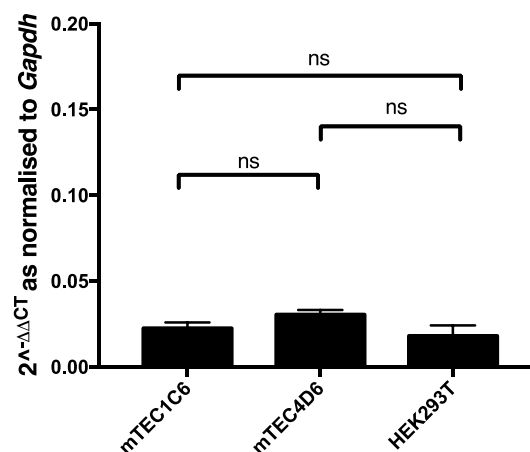


Figure 3.1 *Chd6* mRNA expression in different cell lines

The relative expression of *Chd6* was measured by qPCR in murine mTEC1C6, human mTEC4D6 and human HEK293T cell lines. Values were normalised to *Gapdh* expression. Graph shown data of three biological replicates of each cell type sample (mean with S.D. shown). p-values were determined by Student's t test; ns = not significant.

I then determined the *Chd6* gene expression in different murine TECs subpopulations using qRT-PCR, as shown in Figure 3.2. I was able to detect the expression of *Chd6* at the transcript level in both cTEC and mTECs. The expression levels were 10 to 15-fold lower when compared to the expression of *Gapdh* in both mTECs and cTEC respectively (Figure 3.2a). Nonetheless, the low expression was comparable to the expression of *Chd4* transcript when quantified in the same cell type, with *Chd6* transcripts noted to be expressed significantly higher in both analysed mTEC subsets. *Chd4* expression was used as a reference, as both CHD6 and CHD4 are from the same CHD remodeling enzymes family, and earlier study from our lab had confirmed and documented *Chd4* expressions in murine TEC (F. Dhalla, unpublished). Moreover, as I am interested to study the role of CHD6 in relation to AIRE functions, the expression of *Chd6* and *Aire* transcripts are of equivalent level when assessed by qRT-PCR analysis (Figure 3.2b). Further analysis also revealed a higher expression of *Chd6* in the mature mTEC (designated mTEC^{hi}) when compared to cTEC and immature mTEC (designated mTEC^{lo}) (Figure 3.2).

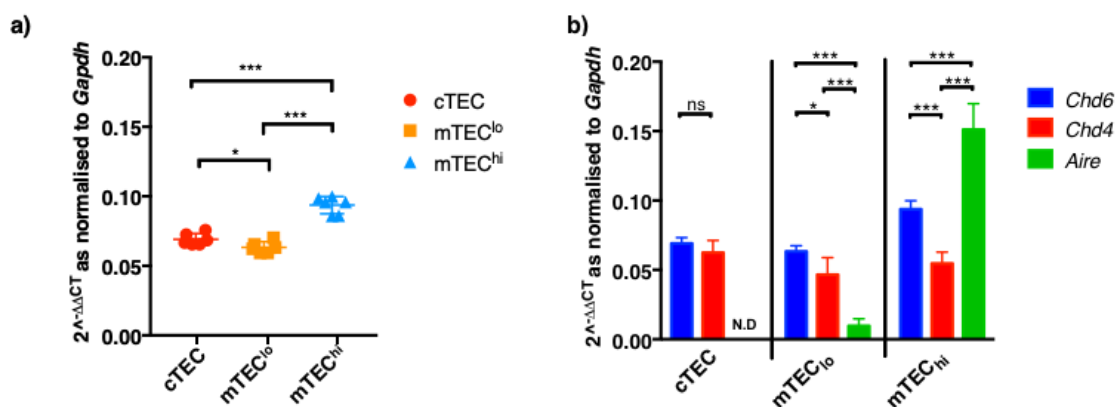


Figure 3.2 *Chd6* mRNA expression in murine TEC

a) qRT-PCR analysis of *Chd6* expression in different TECs subsets isolated from wild-type C57BL/6 mice. Mice were analysed at 4.5 weeks of age. The graph shown with each data point from a single mouse, mean and S.D are displayed. Two independent experiments (with n=3 for each independent experiment, total experiment size, n=6) were conducted and pooled, and values normalised to *Gapdh* expression. P-values were determined by Student's t test; ns = not significant, i.e. p>0.05, * = p<0.05, ** = p<0.005, *** = p<0.001.

b) qRT-PCR analysis of *Chd6*, *Chd4* and *Aire* expression in different TECs subsets isolated from wild-type C57BL/6 mice. Mice were analysed at 4.5 weeks of age. Bar graph showing the mean with the whisker plot showing the S.D. with N.D. designated not-detected. Two independent experiments (with n=3 for each independent experiment, total experiment size, n=6) were conducted and pooled, and values normalised to *Gapdh* expression. P-values were determined by Student's t test; ns = not significant, i.e. p>0.05, * = p<0.05, ** = p<0.005, *** = p<0.001.

3.3. CHD6 protein expression in TEC

As I had established the *Chd6* gene expression in murine TECs, I next determined the CHD6 protein expression level in the same primary TEC subpopulations. For this purpose, I first validated the utility of the commercial polyclonal rabbit anti-human CHD6 antibody, which is raised against synthetic peptide corresponding to a region between amino acid 2665-2715 of the human CHD6 (Bethyl Laboratories). The epitope used to generate the antibody against CHD6 is 80.1% identical to the corresponding mouse sequence, which made the antibody likely to cross-react with the mouse protein. Indeed, the immunopurified antibody recognised a protein of the expected molecular weight as human CHD6 and had been used previously to demonstrate CHD6 expression in different mouse tissues namely liver, heart, kidney, spleen and lung¹⁹⁶.

Based on data displayed in Figure 3.3, the rabbit polyclonal anti-human CHD6 antibody is suitable to detect CHD6 in murine TEC using flow cytometry. At an antibody dilution of 1:100, 99.7% of TEC (EpCAM+ cells) analysed stained positively (Figure 3.3b) for CHD6. The frequency of positive cells decreased with a higher dilution (or lower concentration) of the primary antibody (1:250: 98.1%; 1:500: 85.5%). I therefore concluded that a 1:100 dilution is the best for detection and quantification of CHD6 expression by FACS analysis and have used this concentration for CHD6 detection in TEC. The gating was determined based on the clearly demarcated CHD6 negative subpopulation in EpCAM positive cells in *Chd6*^{TEC-/-} mice, which equally detect >99.0% of the EpCAM positive cells in wild-type mice to be positive for CHD6 expression. It is important to note that the gating strategy detected a low frequency (<5%) of the *Chd6*^{TEC-/-} EpCAM positive cells to be positively stained for FITC-conjugated CHD6. This observation is most likely due to the combination of non-specific binding of both the primary rabbit polyclonal anti-human CHD6 and the secondary antibodies used. Hence, frequency of CHD6-positive population less than 5% among TEC of *Chd6*^{TEC-/-} mice was accepted as a background staining (Figure 3.3a). The possibility of CHD6 is still being expressed in TEC of *Chd6*^{TEC-/-} mice due the “leakiness” in the deletion efficiency of the Foxn1^{Cre} system used is unlikely based on

my earlier assessment of *Chd6*^{TEC-/-} mice showing a complete absence of CHD6 protein in TEC as assessed by confocal immunofluorescence microscopy (see section 2.1.4.4).

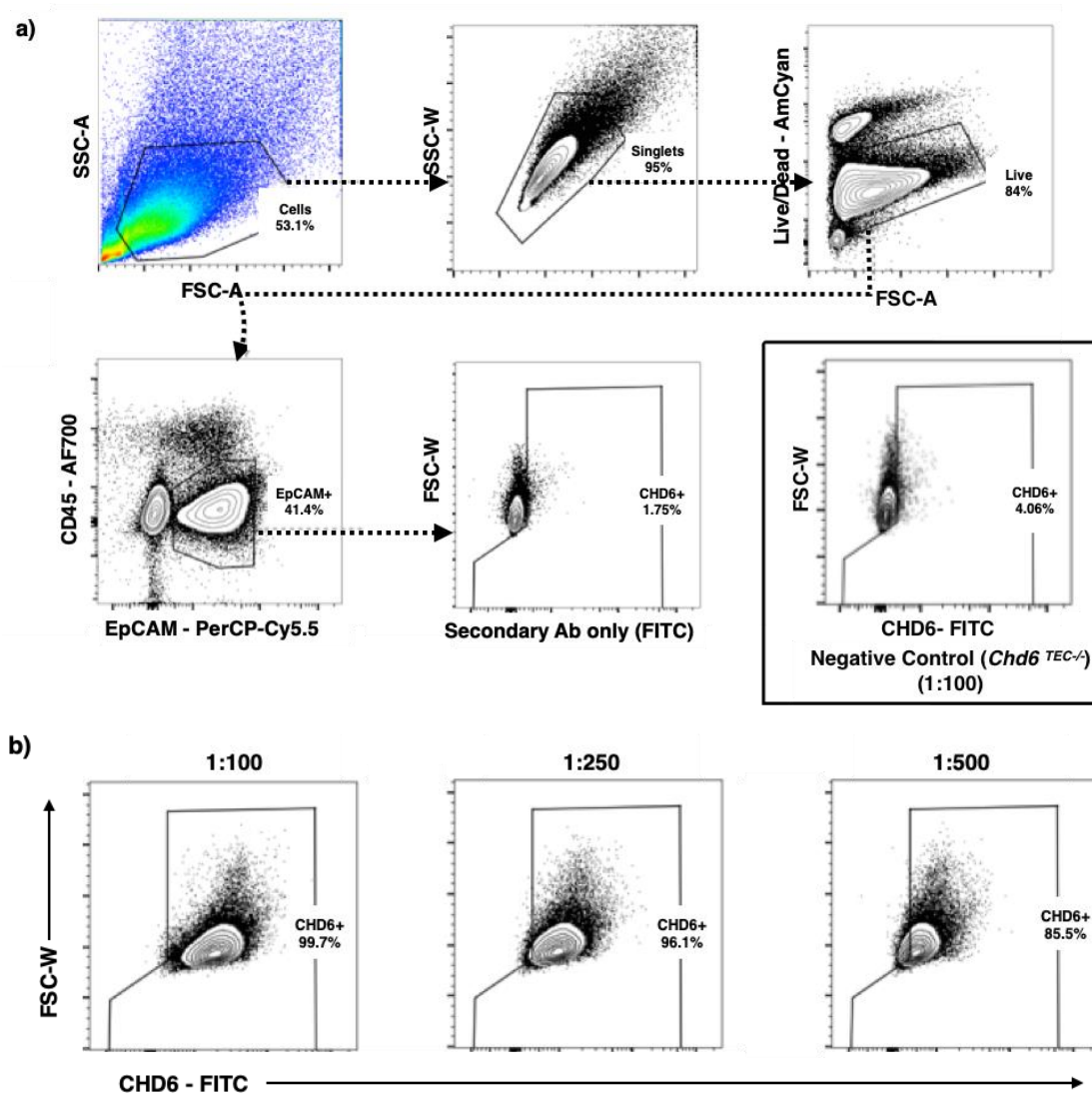


Figure 3.3 Validation and optimisation of CHD6 antibody for FACS analysis

a) Gating strategy to determine CHD6 expression in thymic epithelial cells (EpCAM+ CD45-). Wild-type TECs from 4.5 weeks old female mice (*Chd6*^{TEC+/+}) were stained only with a secondary antibody (anti-rabbit IgG FITC) to determine background staining (frequency noted as 1.75%). *Chd6*^{TEC-/-} TECs were used as negative control for verification of antibody specificity and set the gating for CHD6 positivity (as shown in the box, using 1:100 dilutions of anti-CHD6 antibody). Data shown is a representative analysis of three independent experiments.

b) Frequencies of CHD6 positive-TEC (as determined by EpCAM positivity) using the indicated dilutions of anti-CHD6 antibody. Gating for CHD6 positivity was based on the gating shown in a).

Using the mentioned antibody, I determined the CHD6 protein expressions in murine TEC (CD45- EpCAM+) and their different subpopulations. As predicted, CHD6 is ubiquitously expressed in TEC populations (Figure 3.3). Subsequently, I quantified the expression level of CHD6 proteins by comparing the values of the geometric mean fluorescence intensity (gMFI) of different TEC subpopulations namely: i) cTEC (CD45-, EpCAM+, Ly51+, UEA1-non-reactive), ii) mTEC^{lo} (CD45-, EpCAM+, Ly51-, UEA1-reactive, CD80^{lo}, MHCII^{lo}) and iii) mTEC^{hi} (CD45-, EpCAM+, Ly51+, UEA-reactive, CD80^{hi}, MHCII^{hi}). gMFI value represents the expression level of a particular marker per cell by calculating the logarithmic fluorescent intensity of the marker specific fluorophore, dividing it with the number of events detected within the particular channel. I observed a higher level of CHD6 expression in mature mTEC (mTEC^{hi}) when compared to either cTEC or mTEC^{lo} (Figure 3.4). This finding corroborates the previous observation of the differential *Chd6* expression among the different TEC subpopulations (see section 3.2).

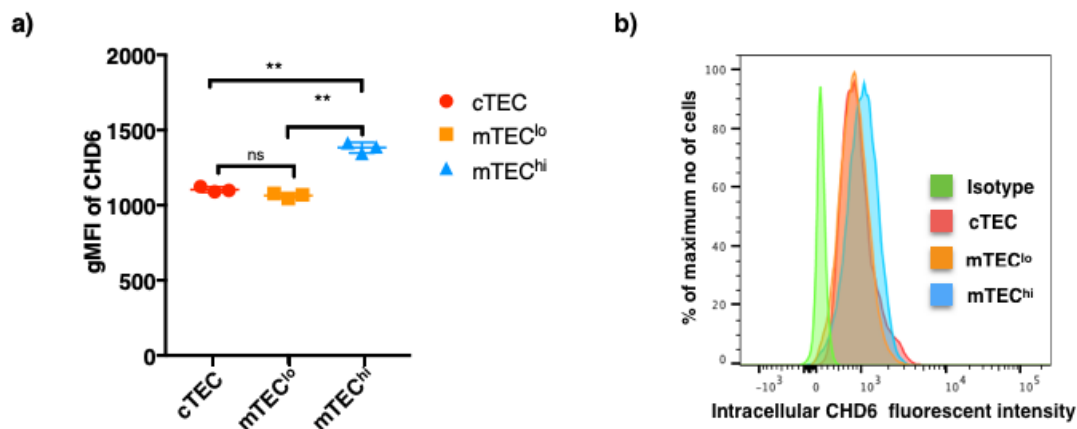


Figure 3.4 CHD6 expressions in murine TECs

a) gMFI of CHD6 was determined in different TECs subsets isolated from female wild-type C57BL/6 mice. Mice were analysed at 4.5 weeks of age. The graph shown is from a single experiment (n=3) and is representative of two independent experiments performed (total experiment size, n=6) with each data points represents an individual mouse. P-values were determined by Student's t-test; ns=not significant, i.e. $p>0.05$, * = $p<0.05$, ** = $p<0.005$, *** = $p<0.001$.

b) Histogram showing CHD6 expression in different TEC subsets with isotype matched control antibody was used to determine the background of the stain. Data shown is from single mouse, representative from a total of 6 female wild-type C57BL/6 mice analysed.

3.4. CHD6 localisation in thymic epithelial cells

CHD6 as a chromatin remodelling enzyme is described to be exclusively present in the nucleoplasm^{173,196}. Thus, I determined the expression pattern of CHD6 in thymic epithelial cells in order to provide a complete understanding of CHD6 expression landscape in different TECs. To address this, confocal immunofluorescence microscopy was performed to illustrate the intracellular localisation of CHD6 in different TEC subsets. Murine thymic tissues from 4.5 week old C57BL/6 mice were stained for CHD6 expression and different TEC surface markers (see section 2.7.4). Additionally, as CHD6 had been specifically proposed to be a protein partner of AIRE, its localisation and interaction with AIRE proteins in mature mTEC were further explored.

In cTEC (identified by Keratin-8 (K8) expression), CHD6 was evenly distributed in the cell's nucleus in a fine granular pattern, although the protein could also be detected in the cytoplasm (Figure 3.5a). In contrast, CHD6 was exclusively located in the nucleus of mature mTEC as identified by the expression of AIRE proteins (Figure 3.5b(ii)). There, the expression of CHD6 was detected as distinctive nuclear speckles. Co-staining for AIRE revealed strong co-localisation of the separate signals (CHD6 and AIRE) providing for both identical patterns, which suggested physical proximity (Figure 3.5b(ii)). This discreet speckled pattern was only noted in the presence of AIRE as CHD6 expressions in AIRE-negative mTEC were distributed in a fine granular pattern both in the nucleus and cytoplasm, similar to the observation in cTEC (Figure 3.5b(i)). Collectively, these findings are in support of the proposed partnership between CHD6 and AIRE as: i) CHD6 complete localisation to the nucleus only occurs when AIRE is present in the mTEC, and ii) the strong co-localisation of these two factors in a distinctive nuclear speckles in mature mTEC. CHD6 localisation into the nucleus is crucial for its function in remodelling the chromatin and the presence of nuclear speckles is associated with active chromatin regions^{196,201}. Hence, the detection of CHD6 in the cytoplasm and the lack of the CHD6 nuclear speckles in cTEC and AIRE-negative mTEC, maybe in disagreement with the active role of CHD6 in these TEC subtypes.

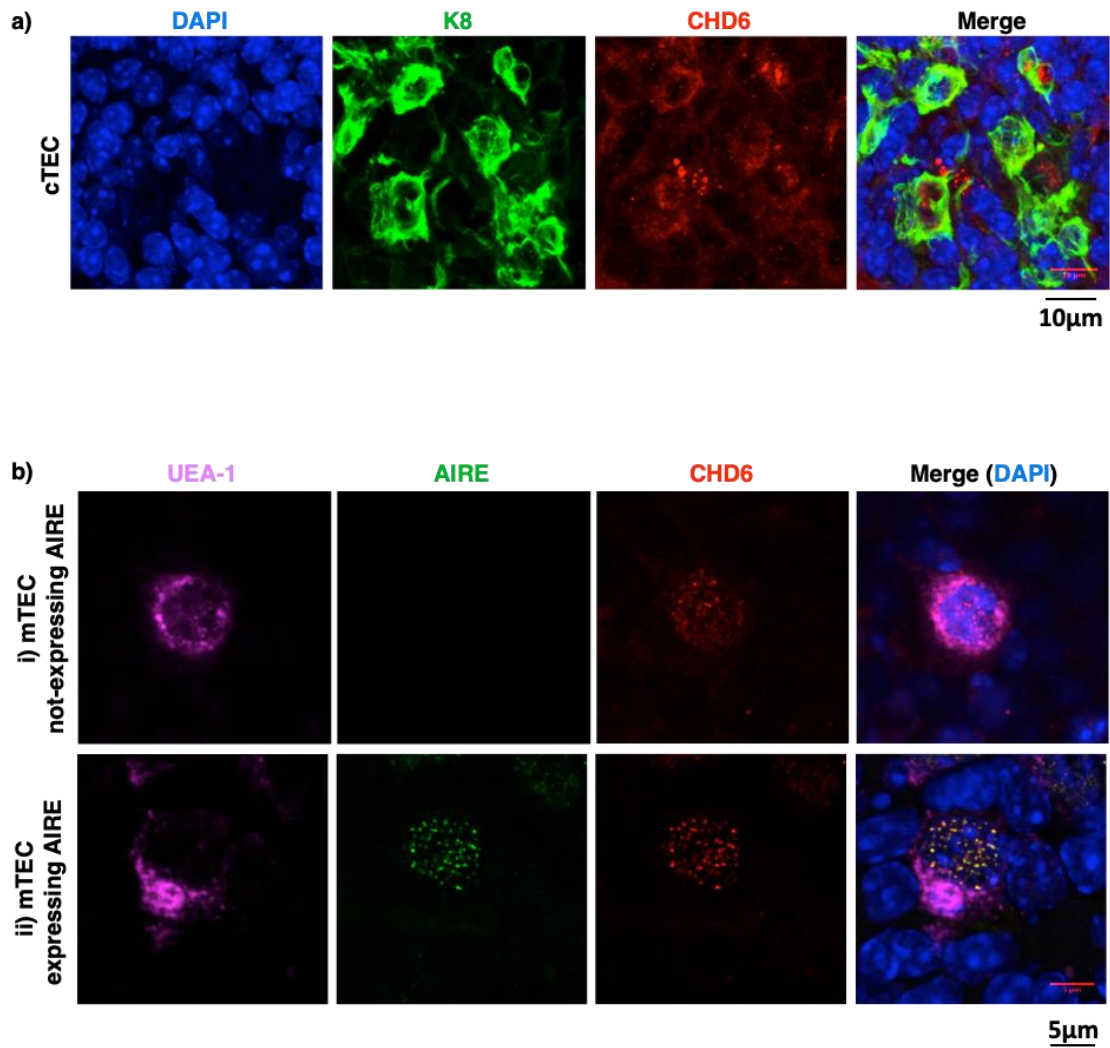


Figure 3.5 CHD6 expressions in murine thymic tissue

a) Confocal microscopy of CHD6 expression in cTEC. Frozen thymic tissue sections (8µm thickness) from 4.5 week old female wild-type C57BL/6 mice were stained with anti-keratin 8 (K8, green) and anti-CHD6 (red) antibodies. Nuclei were counterstained with DAPI (blue).

b) Confocal microscopy showing expression pattern and localisation in mTEC either: **(i)** negative (upper panel) or **(ii)** positive for the expression of AIRE (lower panel). Frozen thymic tissue sections (8µm thickness) from 4.5 week old female wild-type C57BL/6 mice were stained with reactivity to UEA-1 (purple), anti-AIRE (green) and anti-CHD6 (red). Nuclei were counterstained with DAPI (blue).

The photomicrographs are representatives from five independent experiments, involving samples prepared from five 4.5 weeks old female wild-type C57BL/6 mice. For each mouse, at least four thymic tissue sections were analysed.

3.5. CHD6 does not physically interact with AIRE

Since the confocal imaging data had indicated that CHD6 and AIRE co-localise in mTEC, I explored the possibility for further physical association. For this purpose, co-immunoprecipitation (co-IP) studies were undertaken using HEK293T and mTEC4D6 cells transfected to express both AIRE and CHD6 (see section 2.4.5). This experimental approach was chosen because none of the TEC lines established for *in-vitro* growth endogenously express AIRE (Figure 3.6). HEK293T cell line was selected in addition to the human mTEC4D6, as HEK293T displays high transfection efficiency and it had been previously used to study AIRE protein functions in other publications.

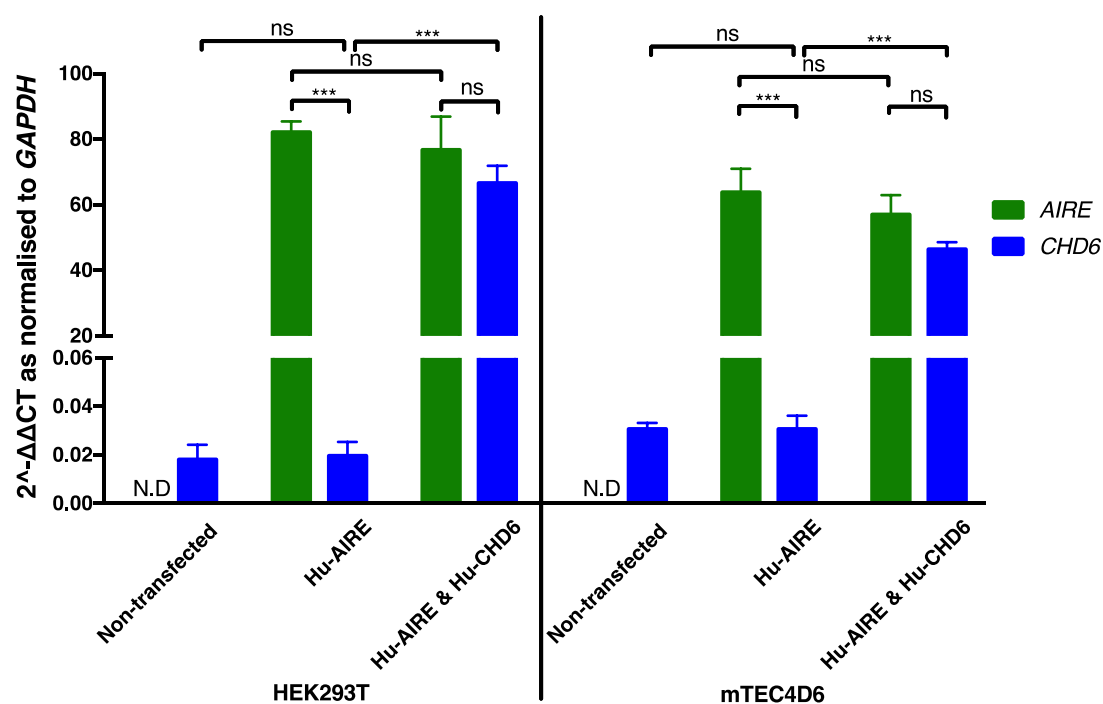


Figure 3.6 *CHD6* and *AIRE* expressions in HEK293T and mTEC4D6 expressing Human AIRE or/and Human CHD6

The relative expressions of *CHD6* (blue) and *AIRE* (green) were determined by qRT-PCR in human HEK293T (left panel) and human mTEC4D6 (right panel) cell lines either non-transfected or transfected with Human-AIRE (Hu-AIRE) or/and Human-CHD6 (Hu-CHD6), respectively. Values were normalised to *GAPDH* expression. Graph shown represents data of three biological replicates of each cell type samples (mean with standard deviation shown). N.D.=not detected. P-values were determined by Student's t-test; ns=not significant, i.e. $p>0.05$, * = $p<0.05$, ** = $p<0.005$, *** = $p<0.001$.

Using a recombinant expression vector, designated Hu-AIRE (see section 2.4.1), HEK293T and mTEC4D6 were successfully transfected to express human AIRE (see

section 2.4.4) (Figure 3.6). The overexpression of AIRE in these two cell lines did not cause any change in the endogenous expression of *CHD6*, indicating that the transfection procedure did not interfere with the transcription regulation of *CHD6*. Following transfection, *AIRE* expression was noted to be more than 10,000-fold and 2,500-fold higher than the endogenous *CHD6* expression in transfected HEK293T and mTEC4D6, respectively (Figure 3.6). Given the lower expression of endogenous *CHD6* when compared to the *AIRE* expression in both cell lines transfected to over-express AIRE, it was likely that CHD6 binding with AIRE, as revealed by anti-AIRE immunoprecipitation would only be detected in cells which also over-express CHD6. For this purpose, I used a recombinant plasmid that allows the expression of human influenza hemagglutinin (HA)-tagged, human-CHD6 plasmid, designated Hu-CHD6 (see section 2.4.1). The expressed human CHD6 protein is fused to HA epitope tag, which is conveniently ideal for the intended co-IP studies as it will facilitate easier detection of CHD6 via Western blot. As shown in Figure 3.6, transfection of HEK293T and mTEC4D6 cells to co-express both human AIRE and human CHD6 revealed comparable level of *AIRE* and *CHD6* expressions.

Using these co-transfected cell lines, I proceeded to perform the intended co-IP studies, followed by a Western blot, to verify the interaction between AIRE and CHD6 (see section 2.4.5). It is important to note that Abramson et al., who initially proposed the partnership between CHD6 and AIRE, had previously failed to verify the physical interaction between AIRE and CHD6 using the similar approach¹⁵⁰. In their attempt, the HEK293T cells used were transfected to over-express mouse AIRE and human CHD6¹⁵⁰. Despite the fact that AIRE is a highly conserved protein across various species, the amino acid identity and amino acid similarity between the mouse and human AIRE proteins is only around 73% and 76%, respectively²¹⁷. Therefore, given that HEK293T cells are human derived cells and human CHD6 were over-expressed¹⁵⁰, failure to observe an interaction between AIRE and CHD6 in the previous study was possibly due to the different properties of mouse AIRE in comparison to its human homolog. Based on that assumption, the current experimental approach utilising both over-expression of human AIRE and human CHD6 in HEK293T cells is believed to be more appropriate. However, my observation revealed that under these experimental conditions, a physical interaction between AIRE and CHD6 could not be confirmed

(Figure 3.7a). As the interaction between AIRE and CHD6 might only occur in thymic epithelial cells, I proceeded to probe the physical interaction between AIRE and CHD6 in TEC-specific cell line. Human mTEC4D6 cell was chosen for this purpose as it has been widely used for the biochemical and functional studies of AIRE in TEC-specific cell lines and both of the plasmids were based on human gene sequence. However, repeated Co-IP experiment using co-transfected mTEC4D6 cells expressing Human AIRE and Human CHD6, still failed to verify the physical interaction between AIRE and CHD6 (Figure 3.7b).

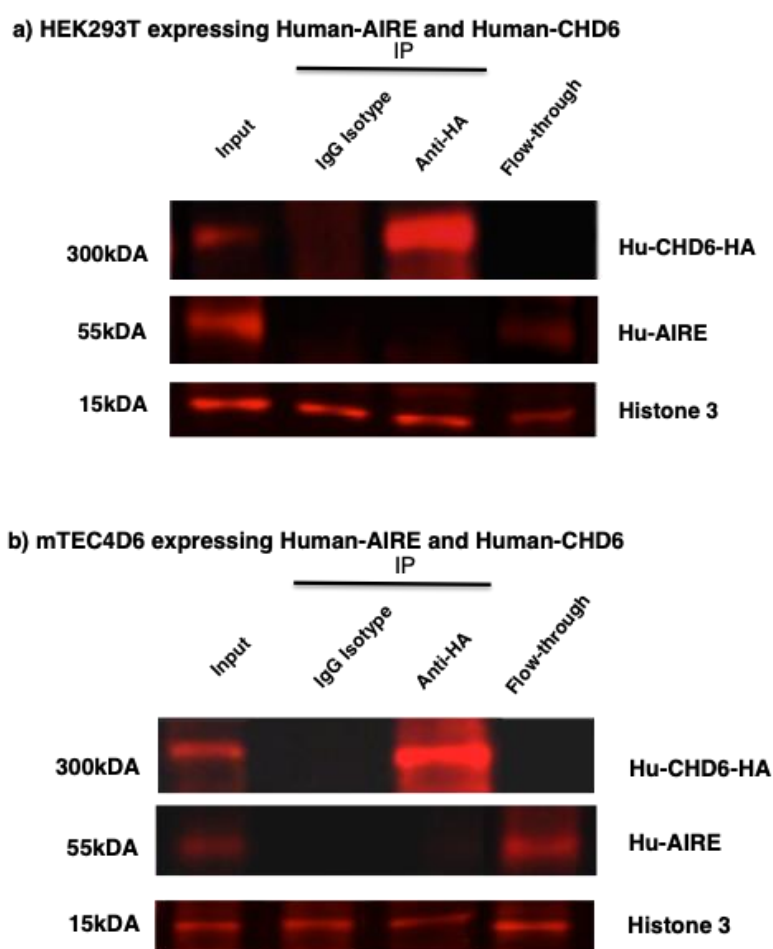


Figure 3.7 Co-IP study in HEK293T and mTEC4D6 cells expressing Human-AIRE and Human-CHD6
Western blot analysis of lysates from **a)** HEK293T and **b)** mTEC4D6 cells; both were co-transfected to express AIRE and HA-CHD6. Immunoprecipitation of HA-CHD6 (upper panel) followed by detection of AIRE (middle panel). The expected molecular sizes for CHD6 and AIRE are 304 kDA and 55 kDA, respectively. Histone H3 (expected molecular size is 15 kDA) was used as loading control (lower panel). The data are representatives of three independent experiments. Note: the differences in loading control observed in the flow-through in comparison to other samples. Flow-through was loaded as the collected solution and was not standardized in their concentrations to other samples.

3.6. CHD6 co-localises with AIRE in mature mTEC

To further verify a physical interaction between CHD6 and AIRE proteins in murine mTEC, I opted to utilise the super-resolution confocal imaging based on the pinhole-plane imaging concept. Airyscan is a 32-channel area super-resolution detector, which allows improvement in resolution by the factor of 2.4 and enhances the signal-to-noise ratio by up 8 times in comparison to the conventional confocal imaging technique ²¹⁸. As this innovation is solely relying on the advancement of the detector component of otherwise a standard confocal microscopy, there is no issue of photobleaching and phototoxicity which may occur with other ultra-high resolution microscopy such as in stimulated emission depletion (STED) or fluorescence photo-activated localisation microscopy/stochastic optical reconstruction microscopy (PALM/STORM) ²¹⁸. Additionally, sample preparation is uncomplicated as it is similar to those utilised in the conventional confocal microscopy.

Using frozen thymic tissue sections, CHD6 and AIRE were stained with immunofluorescent-labelled antibodies (see section 2.7.4) and visualised using Confocal Microscopy 880 Zeiss Inverted with Airyscan. The observation revealed that CHD6 in AIRE-expressing mTEC were predominantly expressed in the nucleoplasm and distributed uniformly in a discreet speckled pattern. AIRE expression was exclusively and evenly dispersed in a similar pattern throughout the nucleoplasm (Figure 3.8a). Merging analysis of these two signals visually revealed a complete overlapping of both CHD6 and AIRE signals (Figure 3.8a), verifying the earlier findings from the confocal microscopy performed (see section 3.4). Yet, overlapping in fluorescence signals in 2-dimension images does not directly indicate a true co-localisation between these two factors as: i) it does not consider the spatial distribution of these two factors in the cellular structure, and ii) it might have coincidentally overlapped by chance. With an improved resolution in all three spatial dimensions, live 3D-reconstruction of the acquired images partly addressed this issue. As shown in 3D-reconstructed images (Figure 3.8b), AIRE and CHD6 were observed to co-distribute spatially in proportion to one another within the nucleus, thus further confirming their close proximity. Overlapping between the two signals was also repeatedly observed in multiple mTEC analysed from five biological replicates, thus increasing the certainty that both AIRE

and CHD6 occupy the same region.

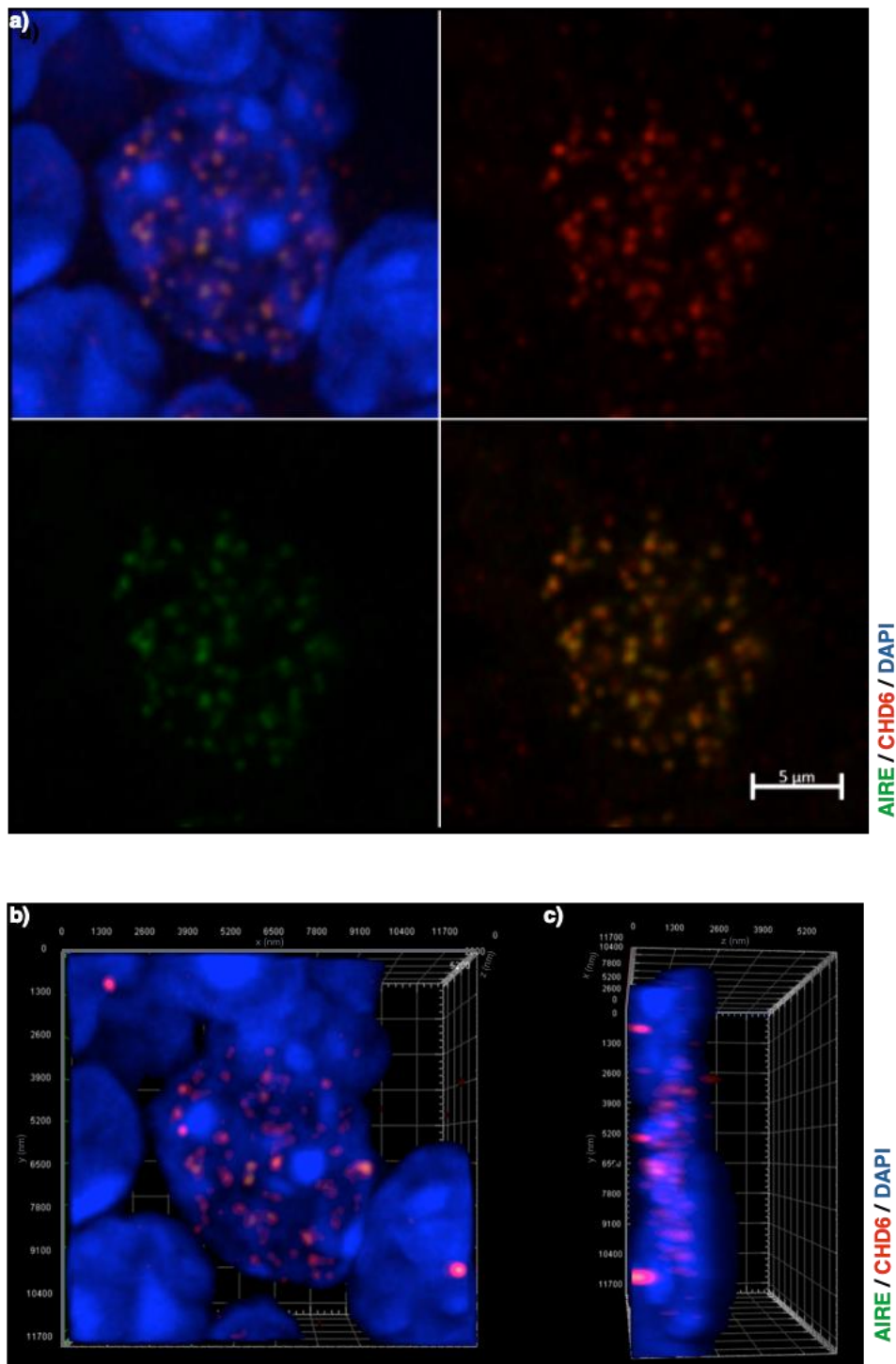


Figure 3.8 CHD6 expressions in murine thymic tissue using superresolution microscopy

Superresolution microscopy with Airyscan of CHD6 and AIRE in mTEC shown in **a)** 2D-superresolution processing mode and 3D-reconstructed images projected on **b)** x-and y-axes and **c)** y- and z-axes. Frozen thymic tissues (8μm thickness) were stained for the expression of CHD6 (red) and AIRE (green). Nuclei were counterstained with DAPI (blue). Merging overlapping signals of both CHD6 and AIRE are displayed in yellow. The photomicrograph shown is representative of five independent experiments involving samples prepared from five 4.5 weeks old female wild-type C57BL/6 mice.

To further confirm the co-localisation observed, I determined the degree of co-localisation between CHD6 and AIRE in mTEC. To perform this, 2D-super-resolution images were analysed using Coloc 2, a co-localisation analysis plugin of ImageJ software (see section 2.7.4). A total of 50 AIRE-expressing mTECs were analysed with the nuclei set as the region of interest (ROI) (Figure 3.9a). Co-localisation was calculated using Pearson's correlation coefficient (PCC) algorithm, represented as r , which determine the degree of AIRE-CHD6 spatially overlapping signals relate to each other over the ROI (Figure 3.9b). r value ranges from -1 to 1, with value of 1 or -1 indicates a perfect co-localisation, while value of 0 indicate no correlation between the two factors. The analysis revealed an average calculated r value of 0.89 ± 0.06 (Figure 3.9c) indicating a high degree of correlation between CHD6 and AIRE, thus supporting the proposed partnership between these two factors.

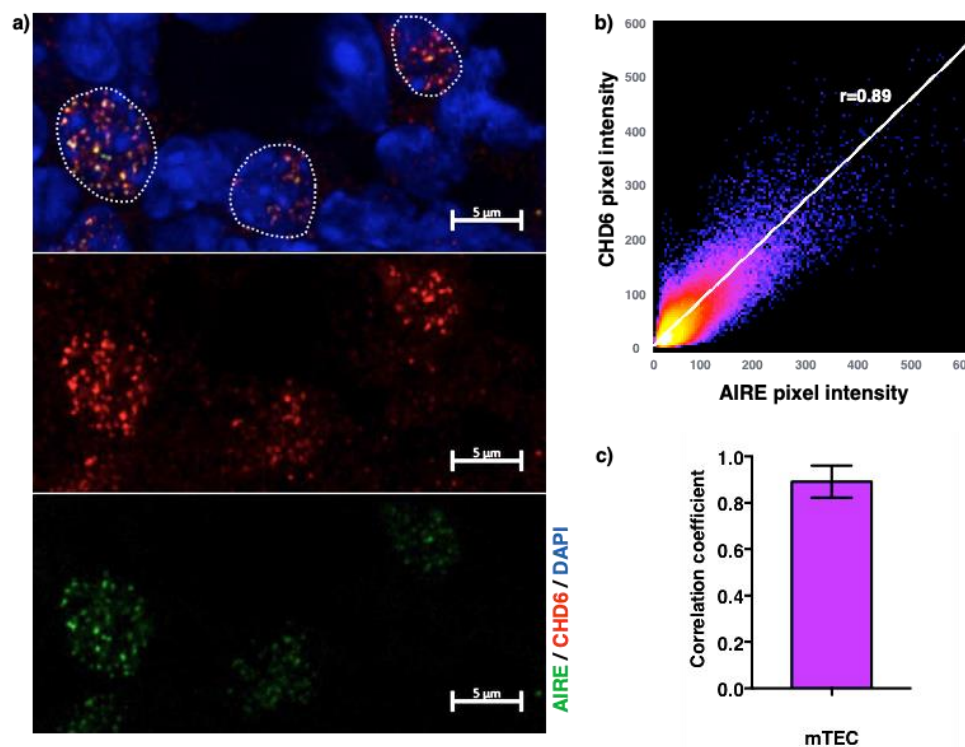


Figure 3.9 Co-localisation analysis of CHD6 with AIRE in murine mTEC

a) Representative 2D-superresolution confocal images utilised for the co-localisation analysis with CHD6 signals (red), AIRE signals (green) and nuclei counterstained with DAPI (blue). Dotted grey line delineates the ROI. **b)** Representative scatterplot of AIRE and CHD6 pixel intensities from the analysis. White line represents the calculated Pearson's correlation coefficient (PCC), r . A total of 50 AIRE-expressing mTEC were analysed from five 4.5 weeks old female wild-type C57BL/6 mice (10 nuclei were randomly selected from each biological sample). **c)** Graph shows r value from co-localisation analysis of 50 mTEC-expressing AIRE nuclei (mean with S.D. shown).

3.7. Discussion and Summary

The findings in this chapter have established and quantified the expression level of CHD6 at both transcriptomic and protein levels in different subsets of murine TECs. CHD6 is ubiquitously expressed in TECs with more than 99% of the analysed EpCAM⁺ thymic epithelial cells were expressing CHD6. However, the expression level of *Chd6* is low when compared to the housekeeping gene tested. This observation is consistent with previous publications reporting low levels of *Chd6* mRNA expression in various tissues of mouse origin, denoting that CHD6 is only needed in small quantities to exude its biological function^{195,196}. Interestingly, I observed that the expression level of CHD6 is significantly higher in mTEC^{hi} subsets when compared to other TEC subsets. Whether the greater expression of CHD6 in the mTEC^{hi} has any connection to mTEC developmental stages and CHD6 partnership with AIRE, this will be of interest and is going to be explored further in the following chapter.

Using confocal imaging, I documented the expression pattern and localisation of CHD6 proteins in the different TEC subsets. CHD6 is expressed in an evenly distributed granular pattern in the cTEC and AIRE-negative mTEC, both in the nucleus and the cytoplasm. These findings were not surprising, as this cytoplasmic description of CHD6 expression had been documented in several parts of murine fetal and adult brain tissues²¹⁹. Despite the fact that CHD6 was predicted to have nuclear localisation (based on the PSORTII nuclear localisation signal (NLS) score of 11.97¹⁹⁶), this observation together with the previous description in the brain indicate that CHD6 is also present in other cellular compartments²¹⁹. CHD6 as a chromatin remodelling factor needs to be transported into the nucleus to perform its function. Visualisation of cytoplasmic CHD6 expression in AIRE-negative TECs was not due to overload of the nuclear import as: i) the nuclear expression of CHD6 in these TEC subsets were not saturated (Figure 3.5), and ii) the total CHD6 expressions in these TEC subsets were lower than the expression in AIRE-expressing mTEC (mTEC^{hi}) (Figure 3.4). The absence of the nuclear-speckles pattern in CHD6 expressed in the nucleus of these AIRE-negative TECs (Figure 3.5), further indicate that the expressed CHD6 do not associate with active chromatin regions. Therefore, the presence of CHD6 in the

cytoplasmic compartment of these TEC subsets may either signify: i) a lack of CHD6 role in that specific subtype of cells, or ii) a yet to be discovered role of CHD6 in the cytoplasm¹⁹⁶. Most of the evidences shown in this chapter were pointing towards the lack of CHD6 roles in these TEC subsets, and indeed findings in the following chapters explored and further supported this assumption.

Fascinatingly, while examining the spatial and pattern of CHD6's expression in different thymic epithelial cells, I observed that the distribution pattern of CHD6 is distinctively different in mature AIRE-expressing mTEC in comparison to other TEC subtypes and exceptionally similar to the expression pattern of AIRE (Figure 3.5). Indeed, using a superresolution microscopy, I was able to verify the overlapping of both AIRE and CHD6 signals at an improved resolution, both at the lateral and axial stages (Figure 3.8). Co-localisation analysis calculated using PCC, revealed a high degree of co-localisation between AIRE and CHD6 in the mTEC (Figure 3.9), thus supporting the possible interactions between the two proteins. AIRE is known to interact with different proteins to exert its function. One of these proteins has been shown experimentally to be CHD6, a finding that is clearly supported by this data^{106,150}.

Although my results conclude that CHD6 and AIRE are co-localised as demonstrated by superresolution microscopy, I cannot demonstrate a stable physical interaction between the two proteins using immunoprecipitation (Figure 3.7). The lack of co-precipitation using an antibody directed against CHD6 may be explained by the physical interaction of CHD6 and AIRE being either weak, transient or involving intermediate partners to form the entire protein complex. Because CHD6 is a large protein, AIRE interactions with CHD6 possibly account for only a small fraction of the overall CHD6 protein complex interactions and transiently occur in the specific tissue microenvironment. Another plausible explanation could be that CHD6 can interact with, or is recruited to AIRE complex by another intermediate protein, which is functionally associated with both AIRE and CHD6. As the interaction between AIRE and CHD6 is probably dynamic, transient, likely to be unstable and may require a third protein to play a bridge role; the performed co-immunoprecipitation study is limited in detecting this proposed interaction; thus, technically explaining the lack of interaction observed.

Other possible cause for the lack of a detection of physical association between AIRE and CHD6 concerns the *in-vitro* system used. Over-expression of both AIRE and CHD6 may functionally change the properties of the proteins from their physiological state, either by: i) the massive abundance may prevent most of the over-expressed protein to occupy the limited specific niche it physiologically resides to exert its function, or ii) the post-translational modifications required for its localisation and function may not be able to occur. In addition to that, as I proposed intermediate proteins as possible links to mediate the interaction between AIRE and CHD6, the lower endogenous availability of these intermediate proteins in comparison to the level of over-expressed CHD6 and AIRE in the *in-vitro* system, may not allow the interaction of AIRE and CHD6 to be revealed.

Another potential failure of the performed *in-vitro* co-immunoprecipitation study was the possible structural interference of the fused HA epitope tag that was recombinantly expressed on the transfected human CHD6 protein. HA-peptide tag fused to CHD6 protein was used to allow easier detection and precipitation of CHD6 in the *in-vitro* system used. However, the peptide tag may disrupt the CHD6 protein folding and interrupt its protein interactions.

With this limitation, I explored the possibility of using other methods to verify CHD6 and AIRE interactions including the use of: i) fluorescence resonance energy transfer (FRET), ii) protein-fragment complementation assays (PCA), iii) proximity ligation assay (PLA), iv) STED and v) PALM/STORM²²⁰. Although many methods can be used in studying protein interactions in living cells, the limitations in applying either of these techniques in my study include: i) the detection markers may prevent required conformational changes of the proteins of interest (in the case of FRET, PLA and PCA); or ii) most of the methods are dependent on distance, whereby the conformational disorientation of the detection markers may lead to false-negative/positive results (in the case of FRET, PLA and PCA); or iii) the need for genetic modification or over-expression of the protein of interest (in the case of PCA), or iv) the requirement of external light source which may induce photobleaching and/or phototoxicity that will affect the detection and sample integrity (in the case of FRET,

STED and PALM/STORM)²²⁰. Therefore, due to the above reasons, none of these techniques are suitable for my study purpose.

Recently, proximity ligation imaging cytometry (PLIC) was developed and had been suggested as a technique which would enable a high-resolution detection and quantification of protein-protein interaction in thymic epithelial cells at a single-cell level²²¹. PLIC is based on PLA, where it utilises a pair of oligonucleotide labelled antibodies as detection markers that binds together in close proximity (30-40nm apart). Combining PLA with imaging flow cytometry, PLIC, have an advantage whereby it allows multi-parametric quantitative fluorescent analysis of single cells in an accurate and robust manner. Hence, PLIC is an attractive method to further verify AIRE and CHD6 interaction and we are currently optimising the protocol for the intended purpose in our laboratory. Analysis of AIRE and CHD6 interaction using PLIC in primary murine mTEC is part of the future experiment that will be conducted (see section 7.2.3).

In addition, covalently locking the interacting proteins *in-situ* during their momentary interactions using a biocompatible chemical crosslinker, may enable the identification of the proposed low affinity and transient interactions between CHD6 and AIRE²²²⁻²²⁴. One of the attractive methods which has been recently developed is Genetically Encoded Chemical Cross-linking of proteins coupled with Mass Spectrometry (GECX-MS)²²². This method utilises a genetically encoded unnatural amino acid into the bait protein, which reacts with a specific residue of the interacting protein, thus covalently cross-linking and capturing the interacting proteins²²². Mass spectrometric analysis of the cross-linked interacting protein complex will then identify the interacting protein partners and pinpoint the interaction regions between proteins, allowing physiologically relevant understanding of the given protein-protein interaction. Beside enabling the identification of the weak and/or transient interactions between proteins, GECX-MS also circumvents the need to choose a high affinity specific antibody of the interacting proteins and allows more robust assessment of all the interacting partners *in-situ*.

Another fascinating finding was the observation that CHD6 was differentially expressed in the distinctive speckled pattern in the nucleus only upon the presence of AIRE in the mTEC (Figure 3.8). Expression of chromatin-associated protein in a speckled pattern is an indication of the protein active state as it positively correlated with gene expression up-regulation^{225,226}. Nuclear speckles are dynamic structures which are intimately involved, mainly in highly active transcription activity, pre-mRNA processing and mRNA transport^{226,227}. The formation of nuclear speckles usually constitute: (i) nuclear speckle resident protein, one of such is AIRE; (ii) multiple RNA splicing factors; (iii) proteins crucial for epigenetic regulation and chromatin organization; (iv) proteins involved with DNA repair and RNA modification^{226,227}. AIRE is classically described to be present in speckled nuclear structure, which is contributed via its CARD domains^{153,228}. These AIRE speckled nuclear bodies are shown to be the centres where regulatory machinery for gene transcription is dynamically linked to AIRE-dependent loci, through modification of the chromatin structure via looping and remodelling^{140,229}. In line with this, most of the nuclear speckles-associated proteins are also proteins which have been shown to be protein partners of AIRE, among those include DNA topoisomerase II (TOP2), LaminB1, DNA-dependent protein kinase (DNA-PK), Cohesin complex and Minichromosome maintenance (MCM) complex^{140,150}. The observation of the discreet nuclear speckles co-localisation of both AIRE and CHD6 do not just propose their possible partnership, but also suggest an active transcription regulation of this region mediated by both proteins. CHD6, as an ATP-dependent chromatin remodelling protein, fits perfectly to the requirement of this highly dynamic nuclear speckle-hub where active chromatin restructuring occurs in facilitating gene transcription.

As observed, CHD6 localisation to the nuclear and its functional activation appears to be associated and/or maybe dependent on AIRE. Additionally, the expression of CHD6 was also higher in mTEC^{hi} in comparison to other TEC subtypes, thus questioning whether AIRE up-regulates the expression of CHD6 in TEC? To partly address the question, over-expression of AIRE using recombinant protein vector as performed in both HEK293T and mTEC4D6, did not lead to any change in the endogenous expression of *CHD6*, thus arguing against the notion that AIRE is involved in transcription regulation of CHD6 (Figure 3.6).

However, the higher expression of CHD6 in mTEC^{hi} if it is not due to the up-regulation by AIRE, it must have been due to an increase in the requirement of CHD6 specific function in this mTEC developmental stage. For this, two explanations may justify the observations. Firstly, mTEC^{hi} is a transcriptionally active TEC subtype, which is dependent mainly on the dynamic changes in chromatin structures^{106,230}. Indeed, mTEC^{hi} were shown to possess significantly more open chromatin structures in comparison to mTEC^{lo} and cTEC¹²⁹. CHD6 as a chromatin remodeller, is perhaps responsible in modifying the chromatin accessibility of some loci in this mature mTEC. Attractively, this is one of the proposed mechanisms on how AIRE regulates the promiscuous expressions of multiple different tissue-restricted genes^{129,150}. Therefore, CHD6 which has been suggested to be a partner of AIRE, may mediate the chromatin structure changes in these loci.

Secondly, CHD6, as an early responder of DNA damage repair, is involved in localise DNA repair complex to the site of DNA damage^{199,202,205}. Some studies have indicated that mature mTEC contains more DNA DSB than the other TECs subtype^{150,161}. This is largely contributed to the generation of multiple DNA DSB breaks to allow transcription of the loci to occur as part of the PGE process regulated by AIRE^{150,161}. Although CHD6 is largely associated with DNA DSB caused by oxidative damage²⁰², it may function differently in mTEC^{hi} as these cells are highly active in inducing multiple DNA strand breaks, demanding fast and efficient repair responses. In this environment of mature mTEC, CHD6 conceivably involves in ensuring the rapid and efficient recruitment of DNA repair complex to these DNA DSB site. Although AIRE and CHD6 might not be physically interacting together, both of the proposed explanations well justify the co-localisation of CHD6 and AIRE in the distinctive nuclear pattern and their functional involvement without the need to directly interact. Additionally, RNAPII, DNA-PK and TOP2A, to name a few, are among the proteins that constitute the large CHD6 multiprotein complex¹⁹⁶, and also among the known AIRE partners. CHD6 has previously being shown to co-localise with RNAP-II and DNA-PK and engaged in transcription preinitiation and elongation and DNA repair machinery, respectively^{196,202}. As AIRE does not have a dedicated DNA recognition motif it might hijack CHD6's ability to localise to an exclusive set of tissue restricted genes and CHD6 machinery in DNA DSB repair for the transcription of AIRE regulated genes.

Taken together, in this chapter, I have presented data to describe the expression of CHD6, both at the transcript and protein levels in murine TECs. Additionally, I have documented the nuclear and cytoplasmic localisation, and the differential expression pattern of CHD6 in murine TEC subsets. Interestingly, I was able to unravel a discreet and near perfect co-localisation of both CHD6 and AIRE in mature mTEC. In summary, these observations corroborate previous findings suggesting CHD6 to be a partner of AIRE and indicate that CHD6 may play a crucial role in thymus biology specifically through its interaction with AIRE in the mature mTEC.

Chapter 4 The role of CHD6 in thymic epithelial cells development

4.1. Aim

CHD proteins are functionally important during early embryogenesis in the context of cell lineage commitment and terminal differentiation ²³¹. For example, the TEC-specific inactivation of CHD4 expression impairs the cells' normal development and function (F. Dhalla, unpublished data). In the previous chapter, CHD6 was shown to be expressed in both cTEC and mTEC, and observed to be associated with AIRE. The aim of this chapter is to investigate the role of CHD6 in thymus organogenesis and function. To address this, mice with a TEC-targeted loss of CHD6 have been generated and investigated.

4.2. Mouse model with a TEC-specific deletion of CHD6 (designated *Chd6*^{TEC-/-})

To determine the functional role of CHD6 in TEC biology, mice with a TEC-targeted deletion of CHD6 were generated. To this end, mice with a conditional *Chd6* locus where *Chd6* exon 3 is floxed by loxP sites were crossed to animals expressing the recombinant Cre under the transcriptional control of the *Foxn1* locus (see section 2.1.4). The recombinant animals, designated as *Chd6*^{TEC-/-} were first probed for the efficient deletion of CHD6 expression using qRT-PCR and immunofluorescent staining (see section 2.1.4.4). All experiments were performed with littermate wild-type controls, which do not express Cre but possessed the *Chd6* gene targeted locus (denoted as *Chd6*^{TEC+/+}). *Chd6*^{TEC+/+} was verified to be phenotypically wild-type C57BL/6, which represent a normal, conventional phenotype, of non-genetically, modified C57BL/6 background mouse strain. All control mice were sex and age-matched in all experiments.

4.3. TEC with a deficiency in CHD6

4.3.1. Loss of CHD6 does not affect thymus cellularity and tissue architecture.

Chd6^{TEC-/-} mice were viable, fertile and born with expected frequencies of the different genotypes. There were no visible differences in the appearance of *Chd6*^{TEC-/-} mice when compared to the wild-type (Figure 4.1).

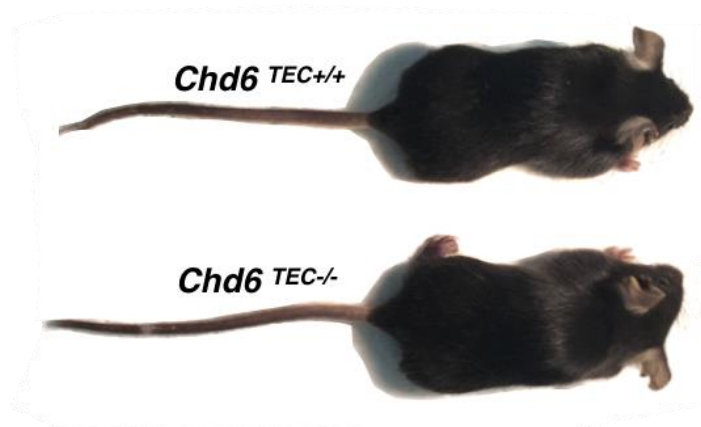


Figure 4.1 Phenotype of *Chd6*^{TEC-/-}

The appearance of female *Chd6*^{TEC-/-} mouse shown in the lower panel in comparison to a female wild-type control (*Chd6*^{TEC+/+}) as observed at 4.5 weeks of age.

The consequence of a loss of CHD6 expression in TEC on the thymus biology was investigated. Thymus anatomy, position and cellularity were normal in both male and female 4.5 week old *Chd6*^{TEC-/-} mice and comparable to that of wild-type animals (Figure 4.2a,b). H&E staining of *Chd6*^{TEC-/-} tissue sections histology revealed an intact corticomedullary organisation (Figure 4.2c). Further assessment of the TEC compartment using immunofluorescence microscopy with antibodies directed against the cTEC marker K8 and the mTEC marker K5, respectively, showed a clear corticomedullary demarcation with the typical presence of a low number of double-positive (i.e. K5/K8 staining) (Figure 4.2d). Taken together, these findings demonstrated that *Chd6*^{TEC-/-} mice have a normal-sized and-structured thymus.

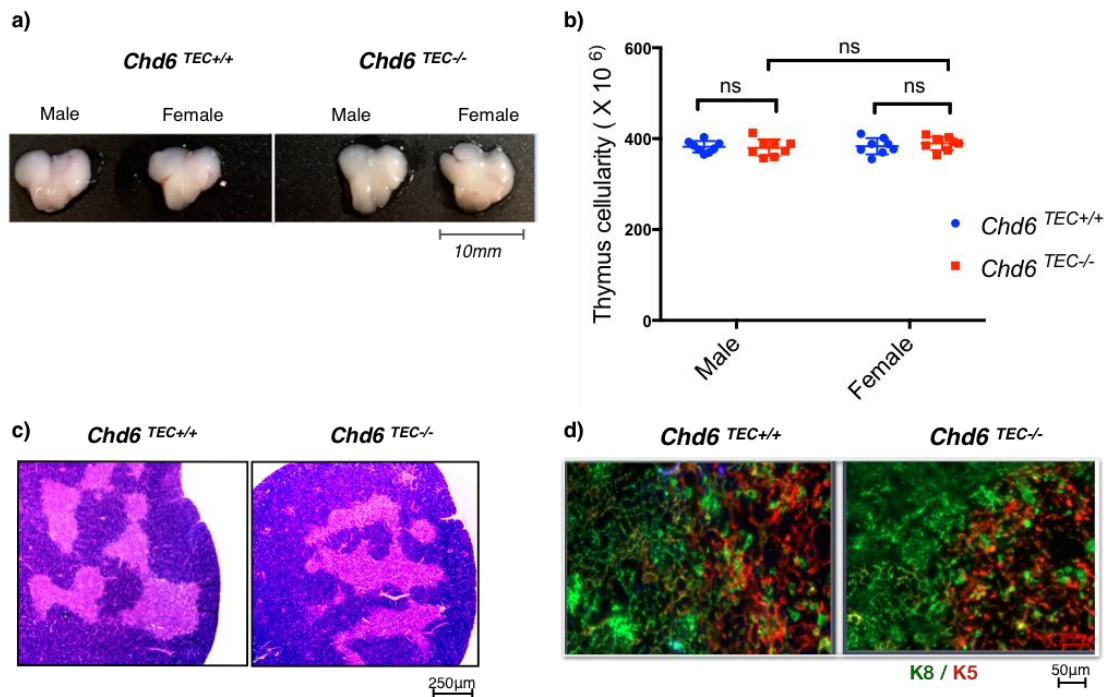


Figure 4.2 Thymus morphology, cellularity and thymic architecture in *Chd6*^{TEC-/-} mice

a) Thymi from male and female 4.5 week old *Chd6*^{TEC-/-} mice compared to gender- and age-matched wild-type animals. **b)** The thymus cellularity was determined in single-cell suspensions of thymic lobes generated by enzymatic digestion. The mice analysed were at 4.5 weeks of age. Each data point represents an individual mouse. The data shown are the combination of two-independent experiments, display mean values and standard deviations. p-values were determined by Student's t-test; ns=not significant. For each independent experiment, 16 mice were used (8 *Chd6*^{TEC-/-} and 8 *Chd6*^{TEC+/+}, Total experiment size: n=32). **c)** Figure shows a representative frozen thymus tissue sections (8μm-thickness) stained with H&E from wild-type on the left and *Chd6*^{TEC-/-} mice on the right stained. Cortical region was stained in dark purple and medullary region was stained in pink. **d)** Figure shows a representative immunofluorescence staining of frozen thymus tissue section (8μm-thickness) from wild-type on the left and *Chd6*^{TEC-/-} mice on the right. Antibodies directed against cytokeratin-8 (K8-green) and cytokeratin-5 (K5-red) were used to identify cTEC and mTEC respectively. Mice were analysed at 4.5 weeks of age. For both H&E and immunofluorescence staining, 5 female *Chd6*^{TEC-/-} mice and 5 female wild-type mice were analysed and for each animals four thymus sections were prepared.

4.3.2. Total TEC cellularity is unchanged in *Chd6*^{TEC-/-} mice

Thymic epithelia were operationally defined as EpCAM positive and CD45 negative cells (Figure 4.3, gating strategy shown in Figure 4.4). Total TEC cellularity of *Chd6*^{TEC-/-} mice was comparable to the wild-type control (Figure 4.5).

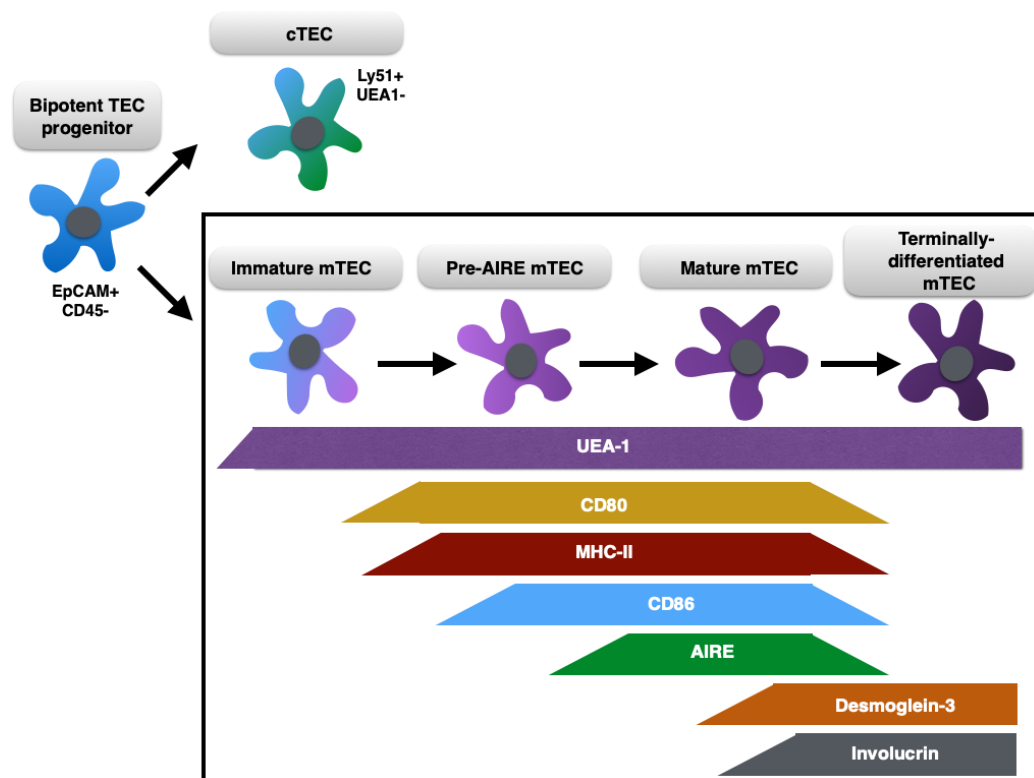


Figure 4.3 Schematic of the phenotypes of TEC differentiation and maturation

Cortical TEC (cTEC) and medullary TEC (mTEC) originate from a common bipotent TEC progenitor. cTECs express Ly51 whereas mTECs are identified by their reactivity with the lectin UEA1. mTEC lineage differentiation is marked by the sequential expression and loss of several molecular markers, including MHCII, CD80, CD86, AIRE, Desmoglein-3 and Involucrin.

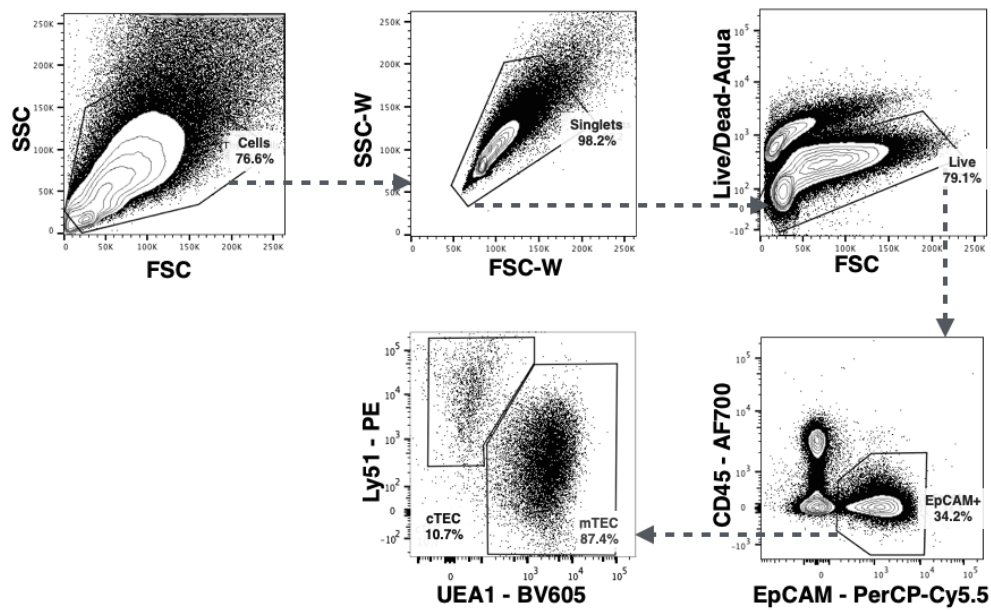


Figure 4.4 Flow cytometric gating strategy for TEC analysis

TEC were first characterized by their size as assessed by their Forward Scatter (FSC). Doublets and dead cells were excluded from downstream analyses using gating for Side Scatter (SSC) and positive live/dead aqua staining, respectively. TECs were selected by positive expression of EpCAM and negative expression of CD45. cTECs and mTECs were identified by the differential expression of Ly51 and UEA1.

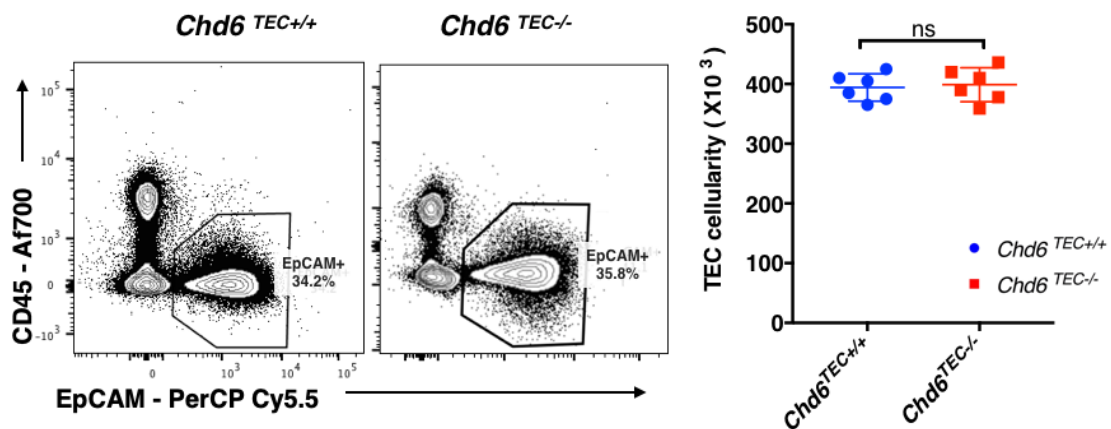


Figure 4.5 Total TEC cellularity in *Chd6*^{TEC-/-} mice

TEC (EpCAM⁺, CD45⁻) cellularity was determined by flow cytometry, representative analyses shown in the left panels. Mice were analysed at 4.5 weeks of age and compared to the wild-type controls. Total TEC cellularity was calculated and data shown on the right is representative of two independent experiments. Each data point represents the value of an individual mouse. p-values were determined by Student's t-test; ns signifies not significant, i.e. $p > 0.05$. For each independent experiment, 3 *Chd6*^{TEC+/+} and 3 *Chd6*^{TEC-/-} were analysed. Total experiment size $n=12$: *Chd6*^{TEC+/+} $n=6$, *Chd6*^{TEC-/-} $n=6$.

4.3.3. Loss of CHD6 does not affect cTEC to mTEC ratio

The frequency of cTEC (Ly51+ UEA1-) and mTEC (Ly51- UEA1+) was next determined employing a gating strategy shown in Figure 4.4. The frequencies of both cTEC and mTEC at 4.5 week old were similar for both gene-targeted mutants and control animals (Figure 4.6a, b). cTEC to mTEC ratio was lowest at 4 to 8 week old mice, with more than 85% of the TEC have a medullary phenotype. The calculated cTEC to mTEC ratio in 4.5 week old *Chd6*^{TEC-/-} mice was of the expected ratio for that age and similar to that of age-matched controls (Figure 4.6c).

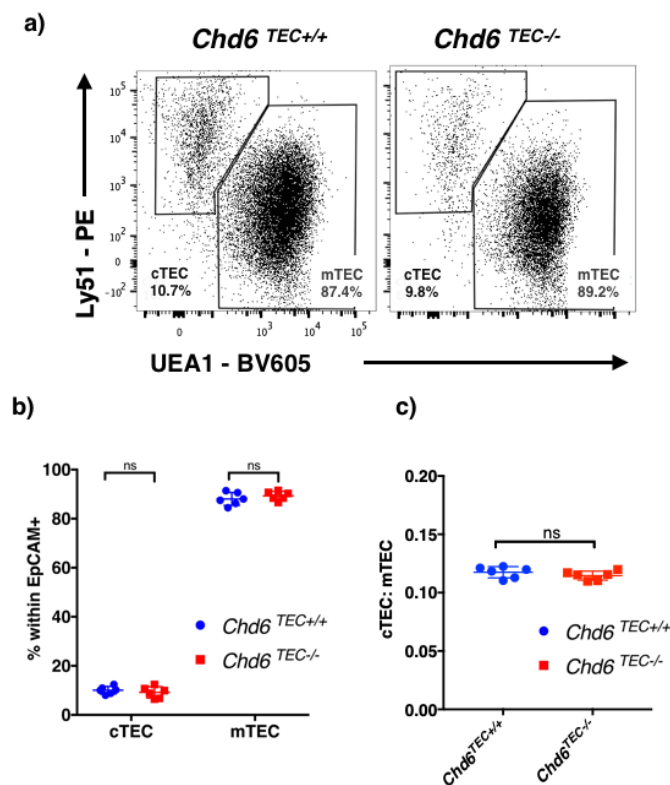


Figure 4.6 Relative frequency and cTEC to mTEC ratio in *Chd6*^{TEC-/-} mice

a) Flow cytometric data showing the frequency of cTECs (Ly51+, UEA1-) and mTECs (Ly51-, UEA1+) within the TEC population (EpCAM+ CD45-), data shown is representative of two independent experiments. **b)** Relative frequency of cTECs and mTECs presented as percentage within the TEC compartment (EpCAM+ CD45-). **c)** Graph showing the calculated cTEC to mTEC ratio. Mice were analysed at 4.5 weeks of age and compared to the wild-type controls. Each data point represents the value of an individual mouse. p-values were determined by Student's t-test; ns signifies not significant, i.e. $p > 0.05$. For each independent experiment, 3 *Chd6*^{TEC+/+} and 3 *Chd6*^{TEC-/-} were analysed. Total experiment size $n=12$: *Chd6*^{TEC+/+} $n=6$, *Chd6*^{TEC-/-} $n=6$.

4.3.4. Loss of CHD6 does not affect mTEC maturation.

mTEC maturation can be resolved into distinct developmental stages using the differentiated expression of CD80, CD86, MHCII, CCL21, AIRE and Involucrin^{65,82,128,232} (Figure 4.3, using a gating strategy as shown in Figure 4.7). The deletion of CHD6 in TEC did not affect the normal mTEC differentiation from immature to terminally differentiated TEC (Figure 4.8). The mTEC compartment is heterogeneous with the terminally differentiated mTEC lose their expression of AIRE, down-regulate the expression of CD80, CD86 and MHCII and become positive for markers such as Involucrin and Desmoglein-3^{74,75,78,136,233,234}. I attempted to further characterise the mTEC maturation into six different stages based on the differential expression of CD80, MHCII, CD86 and Desmoglein-3 (the gating strategy for this analysis is shown in Figure 4.9a). mTEC maturation based on these separate stages was not affected by deletion of CHD6 expression (Figure 4.9b).

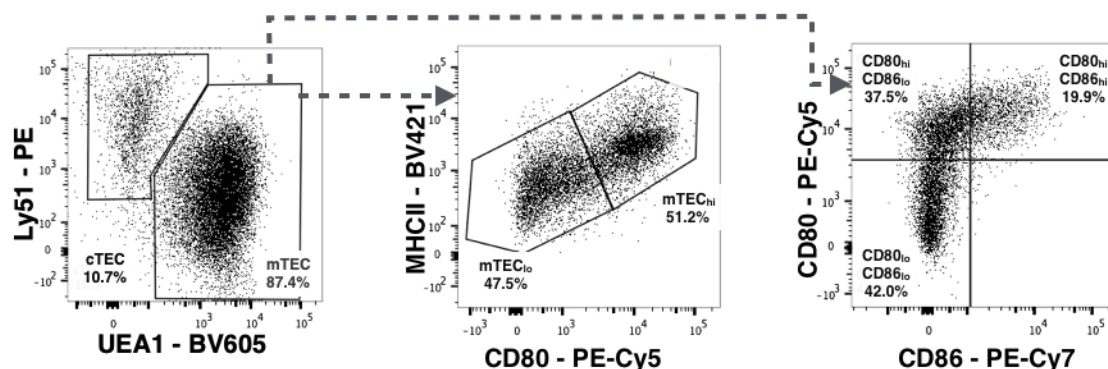


Figure 4.7 Flow cytometric gating strategy for mTEC maturation stages based on the combination of i) CD80 and MHCII, and ii) CD80 and CD86 expressions

mTECs (EpCAM+ UEA1+ Ly51-) as previously defined, were further characterised based on: i) CD80 and MHCII expressions into mTEC_{lo} and mTEC_{hi} (middle panel) and ii) three different maturation stages based on differential expressions of CD80, and CD86 (right panel) : 1) CD80_{lo} CD86_{lo} (lower left quadrant), 2) CD80_{hi} CD86_{lo} (upper left quadrant) and 3) CD80_{hi} CD86_{hi} (upper right quadrant).

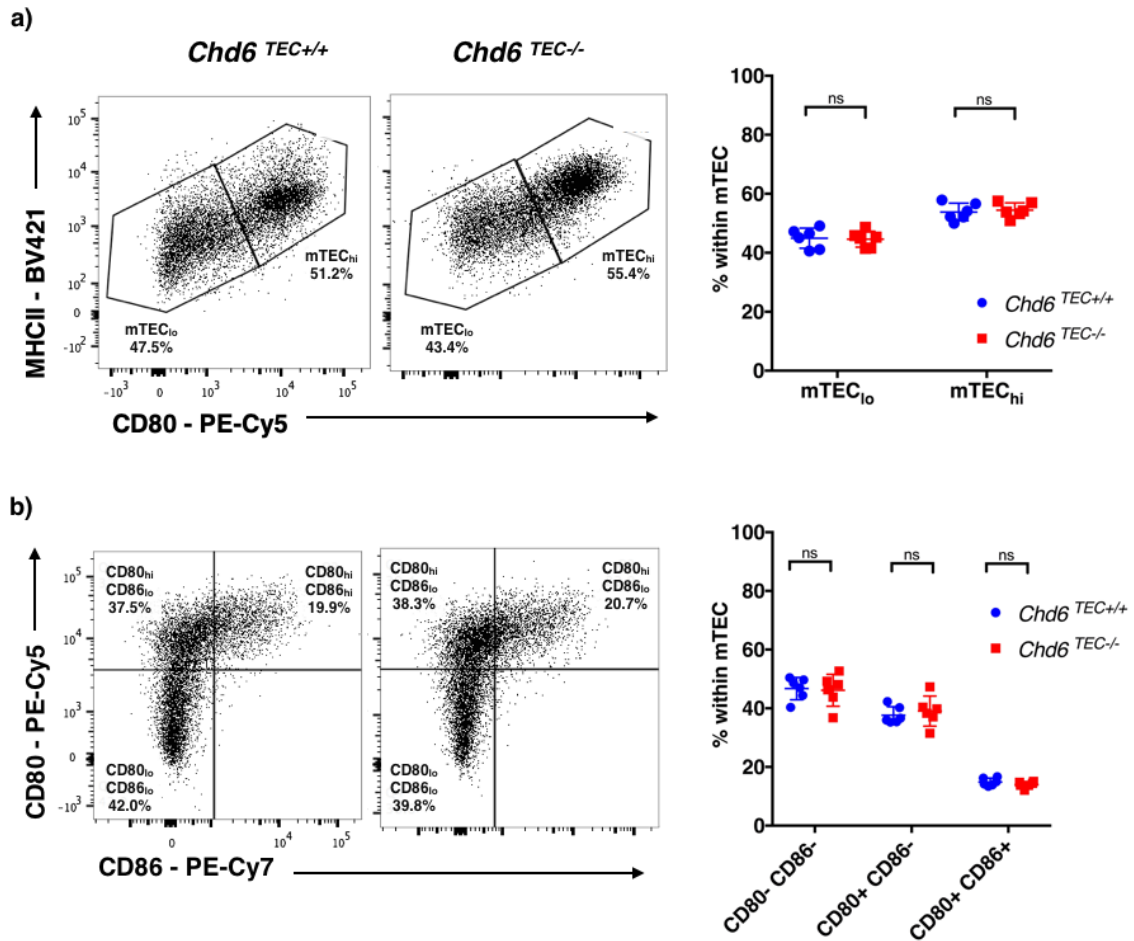


Figure 4.8 Relative frequency of individual mTEC subpopulations based on the CD80, CD86 and MHCII expressions

mTECs were phenotyped for their expression of **a)** CD80 and MHCII into mTEC^{lo} (CD80^{lo} MHCII^{lo}) and mTEC^{hi} (CD80^{hi} MHCII^{hi}), and **b)** mTECs were phenotyped for their expressions of CD80 and CD86 into three different subpopulations **i)** CD80⁻ CD86⁻ **ii)** CD80⁺ CD86⁻ and **iii)** CD80⁺ CD86⁺. Mice were analysed at 4.5 weeks of age and compared to wild-type control animals. Flow cytometric data shown on the left is representative of two independent experiments with frequencies shown on the right. Each data point represents the value of an individual mouse. p-values were determined by Student's t-test; ns=not significant, i.e. $p > 0.05$. For each independent experiment, 3 *Chd6*^{TEC+/+} and 3 *Chd6*^{TEC-/-} were analysed. Total experiment size n=12: *Chd6*^{TEC+/+} n=6, *Chd6*^{TEC-/-} n=6.

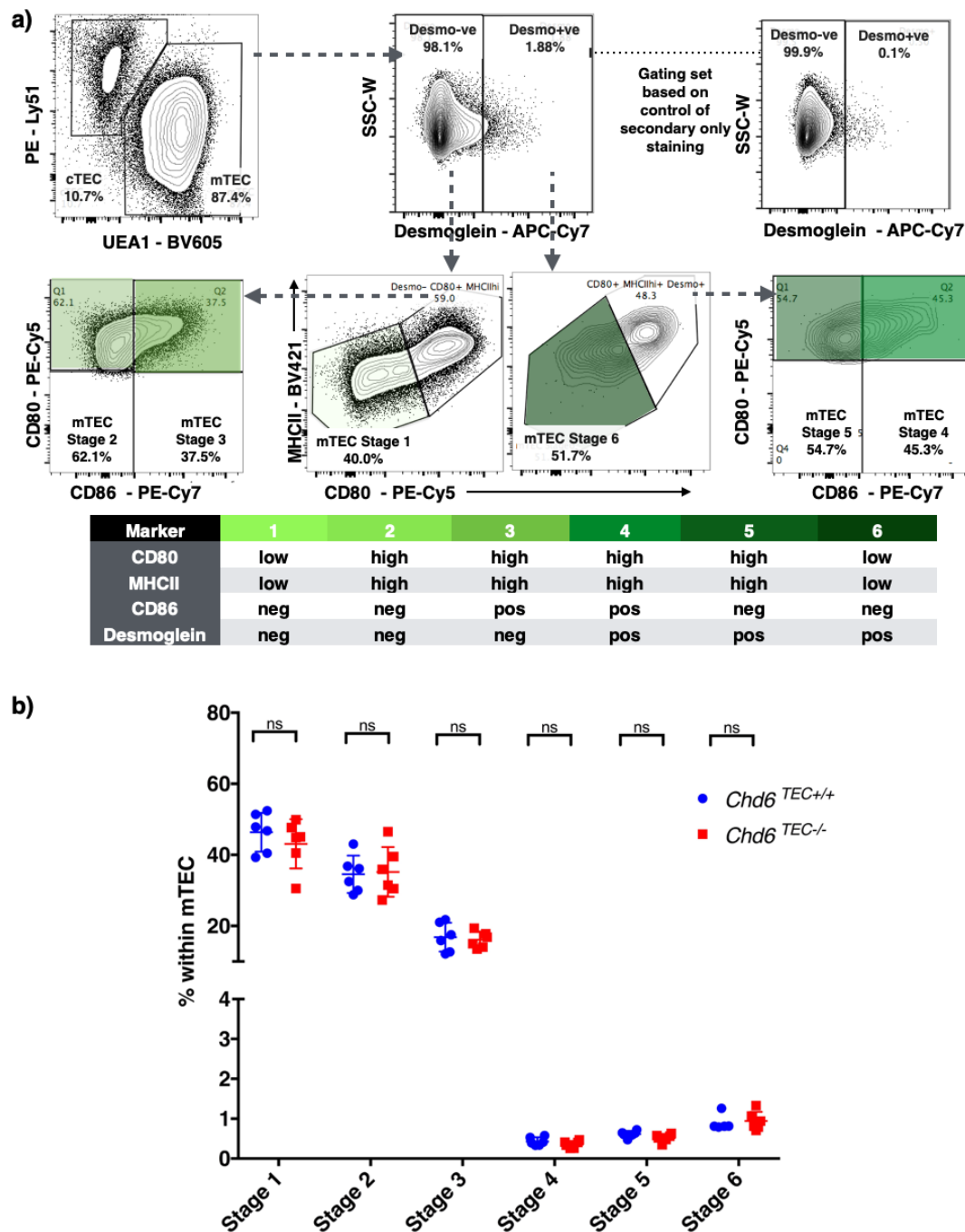


Figure 4.9 mTEC maturation stages based on CD80, MHCII, CD86 and Desmoglein expressions

a) Different mTEC maturation stages (Stage 1 to 6) were gated based on differential expressions of CD80, MHCII, CD86 and Desmoglein. Positive gating for Desmoglein was set based on secondary antibody staining, and gating for CD80 and CD86 subpopulations were set based on cTEC subpopulation. For Stage 1 and Stage 6, relative frequencies were based on the value given by the expression of MHCII and CD80 gateings. **b)** Relative frequencies of different mTEC maturation stages were determined by their expressions of CD80, MHCII, CD86 and Desmoglein. Mice were analysed at 4.5 weeks of age and compared to wild-type control animals. Each data point represents the value of an individual mouse. p-values were determined by Student's t-test; ns=not significant, i.e. $p > 0.05$. For each independent experiment, 3 *Chd6*^{TEC+/+} and 3 *Chd6*^{TEC-/-} were analysed. Total experiment size $n=12$: *Chd6*^{TEC+/+} $n=6$, *Chd6*^{TEC-/-} $n=6$.

4.3.5. Loss of CHD6 does not change the AIRE expression level.

AIRE is expressed in the specific mTEC cell population that expresses high levels of MHCII and CD80 co-stimulatory markers, or mTEC^{hi} 233,235. I had shown previously that deletion of CHD6 in TEC does not interfere with mTEC differentiation, particularly the mTEC^{hi} subset (Figure 4.8). Determination of AIRE expression in mTEC showed that CHD6 deletion does not interfere with the relative frequency of mTEC-expressing AIRE (Figure 4.10a). The expression level of AIRE within the mTEC-expressing AIRE as analysed by the gMFI was also not affected by the deletion of CHD6 (Figure 4.10b).

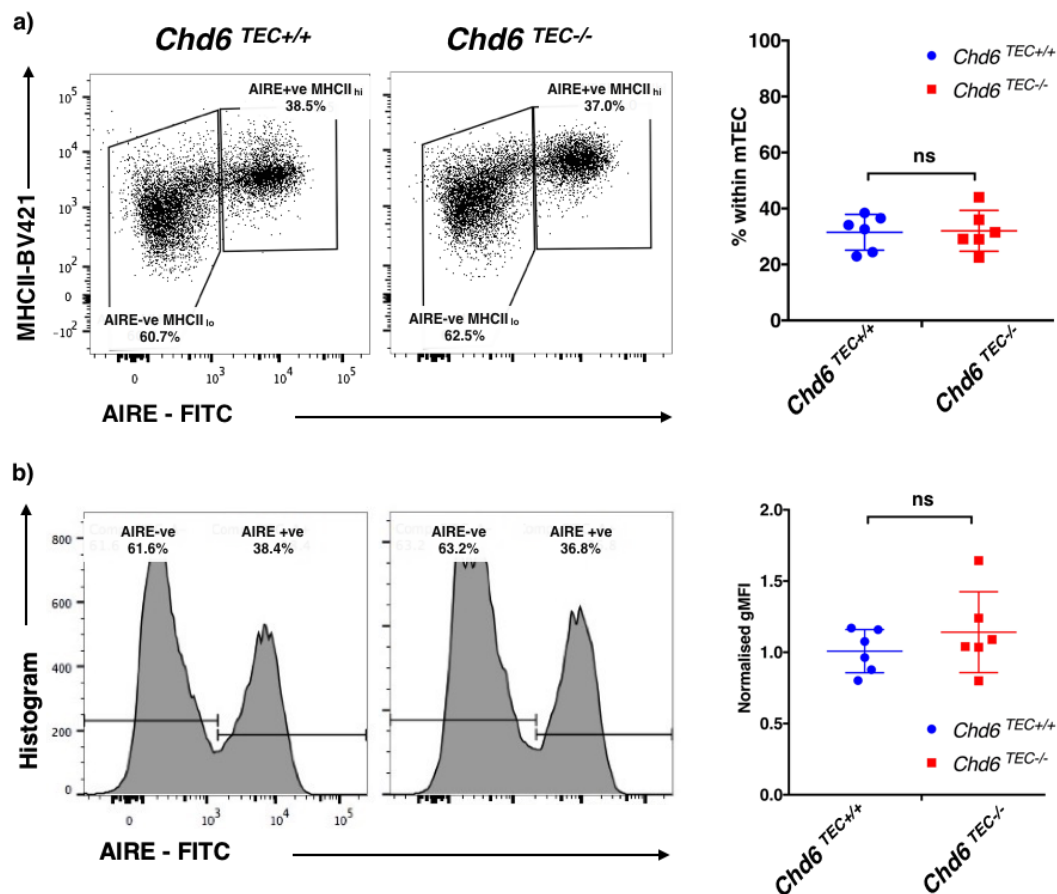


Figure 4.10 Relative frequency and gMFI of mTEC-expressing AIRE in *Chd6* ^{TEC-/-} mice

a) mTECs (EpCAM+ UEA1+ Ly51-) were phenotyped for their expression of AIRE and MHCII, with flow cytometric data shown on the left is representative of two independent experiments with frequencies of mTEC expressing AIRE shown on the right. **b)** Histogram showing AIRE expression within mTEC on the left is representative of two independent experiments and gMFI of mTEC-expressing AIRE were shown on the right. Mice were analysed at 4.5 weeks of age and compared to wild-type control animals. Each data point represents the value of an individual mouse. p-values were determined by Student's t-test; ns=not significant, i.e. $p > 0.05$. For each independent experiment, 3 *Chd6* ^{TEC+/+} and 3 *Chd6* ^{TEC-/-} were analysed. Total experiment size $n=12$: *Chd6* ^{TEC+/+} $n=6$, *Chd6* ^{TEC-/-} $n=6$.

4.3.6. Loss of CHD6 does not affect the frequency of thymic tuft cells

Two recent studies had reported the presence of another TEC subset with resemblance to intestinal tuft cells, term as ‘thymic tuft cells’^{76,77}. These thymic tuft cells are defined as non-AIRE expressing mTEC that is highly expressing L1 Cell Adhesion Molecule (L1CAM) with reduced expression of Integrin Subunit Beta 4 (ITGB4)⁷⁷. Thymic tuft cells (defined as EpCAM+ Ly51- UEA1+ CD80^{lo} ITGB4^{lo} L1CAM^{hi}) in *Chd6*^{TEC-/-} mice and their wild-type control were identified using the gating strategy as shown in Figure 4.11. The frequency of thymic tuft cells was not affected by the loss of CHD6 expression (Figure 4.12).

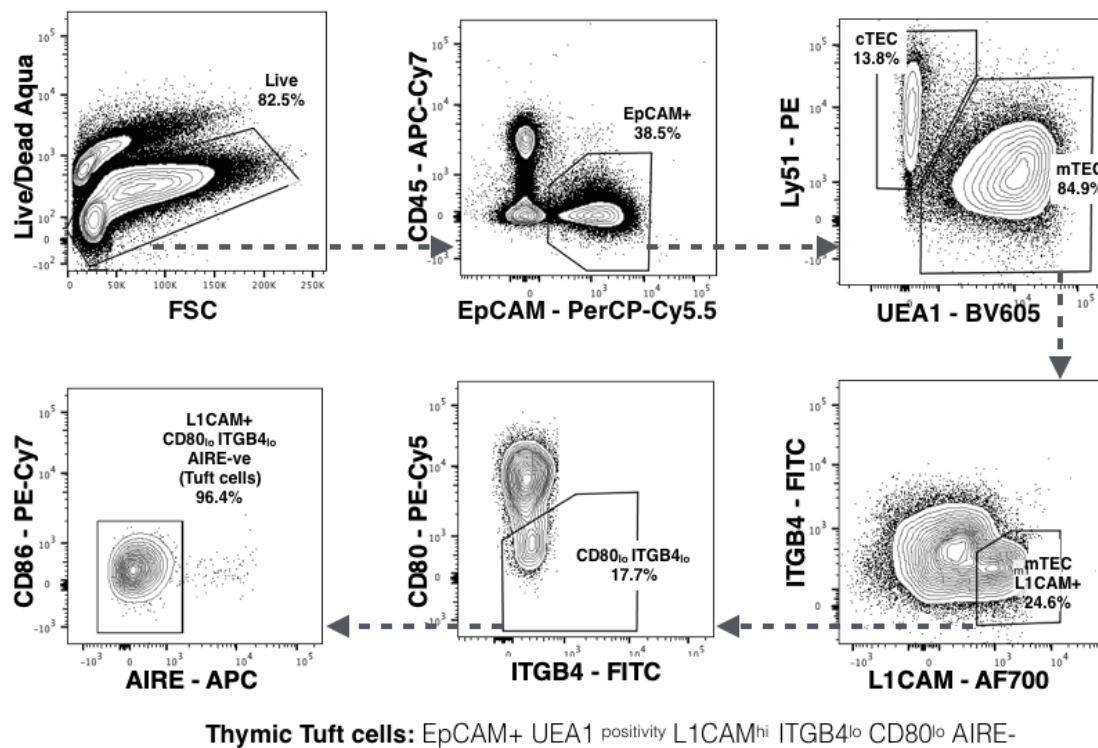


Figure 4.11 Flow cytometric gating strategy for identification of thymic tuft cells

TECs (EpCAM+ CD45-) were selected after exclusion of doublets and dead cells as previously performed (as shown in Figure 4.4). mTECs (EpCAM+ Ly51- UEA1+) were further selected and subsequently gated based on differential expressions of L1CAM, ITGB4, CD80, CD86 and AIRE. Thymic tuft cells were defined as mTEC cells that have low expressions of ITGB4 and CD80, while highly expressing L1CAM. The defined thymic tuft cells were further verified to have low expression of CD86 and did not express AIRE.

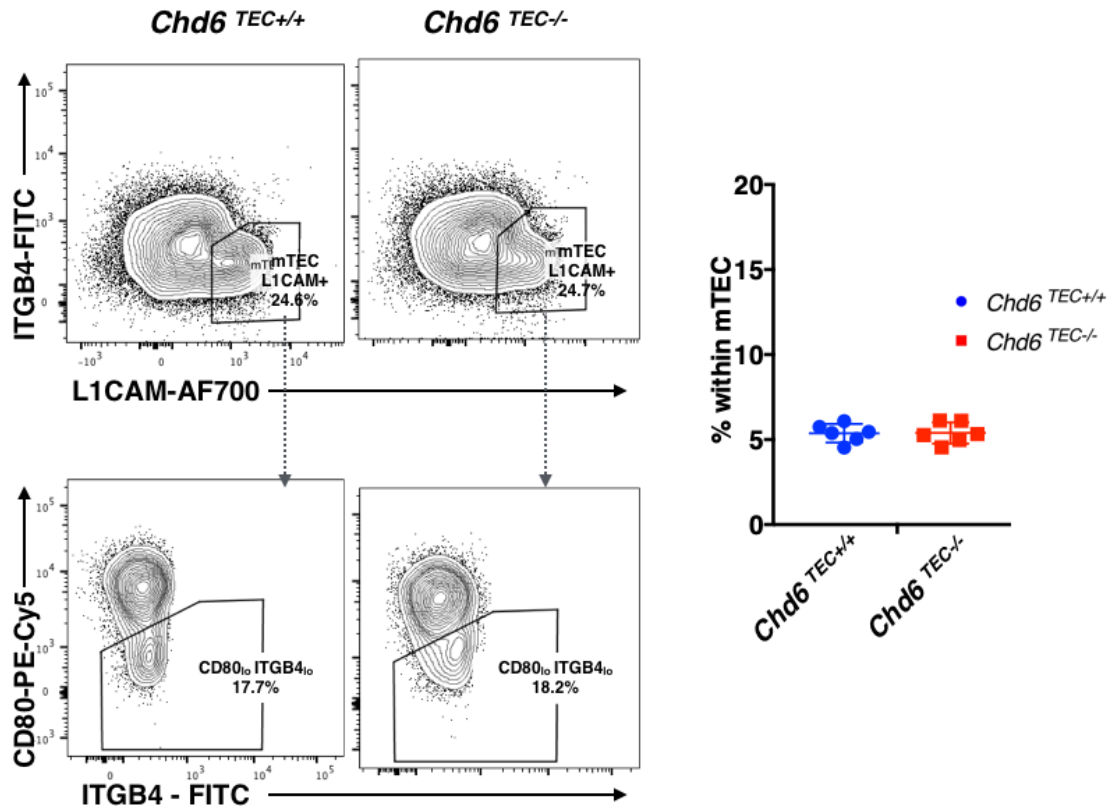


Figure 4.12 Relative frequency of thymic tuft cells in *Chd6* ^{TEC-/-} mice
mTECs (EpCAM⁺ Ly51⁻ UEA1⁺) were phenotyped for their expressions of ITGB4, L1CAM and CD80, with representative flow cytometric data on the left and frequency of thymic tuft cells (CD80^{lo}, ITGB4^{lo}, L1CAM^{hi}) shown on the right. Mice were analysed at 4.5 weeks of age and compared to wild-type control animals. Each data point represents the value of an individual mouse. p-values were determined by Student's t-test; ns=not significant, i.e. p>0.05. For each independent experiment, 3 *Chd6* ^{TEC+/+} and 3 *Chd6* ^{TEC-/-} were analysed. Total experiment size n=12: *Chd6* ^{TEC+/+} n=6, *Chd6* ^{TEC-/-} n=6.

4.3.7. Loss of CHD6 affects the mTEC expressions of GP2 and TSPAN8.

Up until this stage, analysis of the *Chd6* ^{TEC-/-} mice reveals that the loss of CHD6 in TEC does not impact: i) thymic cellularity and their spatial organization; ii) the cTEC and mTEC compartments; and iii) the expression of AIRE in the thymus. The suggested function of CHD6 in the thymus was based on the proposal that CHD6 may facilitates AIRE in modulating PGE in mTEC. I continued to evaluate the effect of the loss of CHD6 in TEC by specifically looking at its impact on the expression of TRAs that are regulated by AIRE. Glycoprotein2 (GP2) and Tetraspanin-8 (TSPAN8) are

among thousands of TRAs, which are promiscuously expressed in the thymus through the regulation of AIRE^{75,126}. Using flow cytometric analysis, the expressions of both these TRAs in the mTEC of CHD6 mutant mice were determined. Indeed, significant reductions in the expressions of both TSPAN8 (3.381% $\pm$ 0.6639 vs. 6.945 % $\pm$ 0.6507, $p < 0.05$) and GP2 (1.94% $\pm$ 0.06455 vs. 2.643% $\pm$ 0.1229, $p < 0.001$) were observed in *Chd6*^{TEC-/-} mice when compared to their wild-type controls (Figure 4.13).

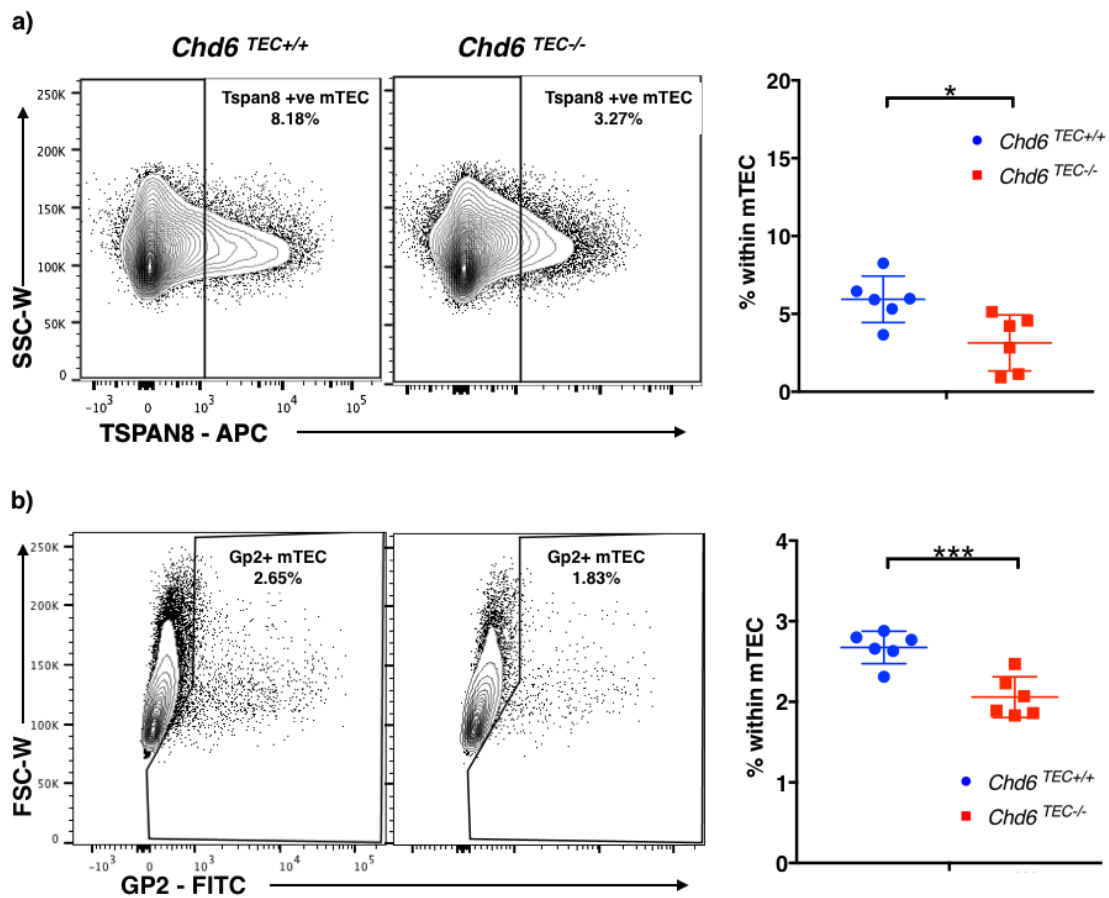


Figure 4.13 Relative frequency of TSPAN8+ and GP2+ mTEC in *Chd6*^{TEC-/-} mice

mTECs were phenotyped for their expression of **a)** TSPAN8 and **b)** GP2. Representative flow cytometric data on the left is representative of two independent experiments and frequencies shown on the right. Mice were analysed at 4.5 weeks of age and compared to wild-type control animals. Positive gating for both TSPAN8+ and GP2+ were set based on specific fluorophore-isotype control. Each data point represents the value of an individual mouse. p-values were determined by Student's t-test; * = $p < 0.05$, *** $p < 0.001$. For each independent experiment, 3 *Chd6*^{TEC+/+} and 3 *Chd6*^{TEC-/-} were analysed. Total experiment size $n = 12$: *Chd6*^{TEC+/+} $n = 6$, *Chd6*^{TEC-/-} $n = 6$.

4.4. Transcriptional changes in TEC lacking CHD6

Detailed characterisation of the thymus revealed that the loss of CHD6 in TEC interferes with the expressions of GP2 and TSPAN8 on mTEC without compromising the overall development of the TEC lineages and consequently of obvious thymus function as measured by cellularity and morphology. I further verified the role played by CHD6 in facilitating the ectopic expression of *Aire*-dependent and *Aire*-independent genes in mTEC^{hi}. This was achieved by analysing the mRNA expression of selected tissue-restricted genes (TRG) by qRT-PCR. The TRGs were selected based on their robust gene expressions in wild-type murine mTEC^{hi} based on previously generated single cell RNA-Seq dataset¹⁰⁶.

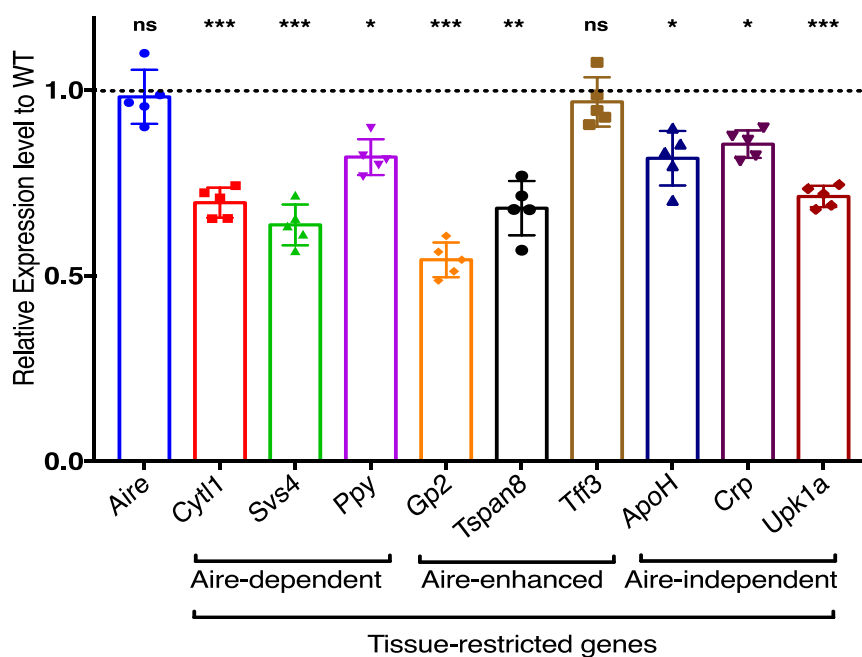


Figure 4.14 *Aire* and TRGs expression in mTEC^{hi} of *Chd6*^{TEC-/-} mice

The expressions of *Aire*, *Aire*-dependent, *Aire*-enhanced and *Aire*-independent TRGs were measured by qRT-PCR in FACS-sorted mTEC^{hi} cells (EpCAM+ Ly51- UEA1+ CD80+ MHCII^{hi}) from 4.5 week old *Chd6* mutant mice (*Chd6*^{TEC-/-}, n=5, female) and wild-type control mice (*Chd6*^{TEC+/+}, n=5, female). Values were normalised to *Gapdh* expression and those in the wild-type control were set to 1 (dotted line). Each individual value plot represents the mean of 5 biological replicates with standard deviation. p-values were determined by Student's test; ns=not significant, * = p<0.05, ** = p<0.005, *** = p<0.0001.

This analysis revealed that transcripts for most of the selected TRGs to be analysed were down-regulated in *Chd6*^{TEC-/-} mice without affecting the expression of *Aire* mRNA (Figure 4.14). This latter finding concurred with the immunophenotyping results

that showed an uncompromised detection of AIRE in mTEC deficient for CHD6 when compared to wild-type cells (Figure 4.10). Notably, both genes dependent and independent of AIRE for their promiscuous expressions were affected by the absence of CHD6 (Figure 4.14). This finding implies that CHD6 plays an important role in PGE for a number of loci whether their promiscuous expression is dependent or independent of AIRE. Taken together, the data presented strongly suggests that changes in PGE in *Chd6*^{TEC-/-} mice were most likely the direct consequence of a loss of CHD6 function and not the result of other genes involved in this unusual form of transcriptional control, including AIRE.

As previously discussed, AIRE promotes PGE in a subset of mTEC but does not directly bind to DNA, suggesting the need of other associated molecules to allow the localisation of AIRE to DNA and its control on transcription initiation. CHD6 has been identified as one of the protein partners that is associated with AIRE¹⁵⁰. CHD6 as a chromatin remodelling enzyme, functions via its binding to several histone modifications^{202,204}. It is therefore plausible that CHD6 acts as a mediator “linking” AIRE to sequences to be promiscuously expressed in mTEC in the context of PGE.

To expand my qRT-PCR analysis of TRA expression by TEC deficient for CHD6 (Figure 4.14), I next carried out a population-based genome-wide RNA-Seq analysis of TEC isolated from *Chd6*^{TEC-/-} and wild-type mice. This analysis was designed to address specifically two questions: i) how does a loss of CHD6 expression in TEC impact on the capacity of mTEC^{hi} to effect PGE; and ii) what are the molecular features of genes that require the presence of CHD6 for their promiscuous expression in mTEC^{hi} cells? To this end, cTEC, mTEC^{lo} and mTEC^{hi} subpopulations were separately sorted from three female 4.5 week old *Chd6*^{TEC-/-} mice and three gender- and age-matched wild-type animals as controls (designated *Chd6*^{TEC+/+}). RNA was isolated from the individual samples and libraries were generated using the SmartSeq2 amplification method (see section 2.8.3). The bioinformatic analysis was performed in collaboration with Dr Kevin Rue-Albrecht and Dr Stephen Samson (Kennedy Institute of Rheumatology, University of Oxford).

4.4.1. Validation and exploratory analysis of *Chd6*^{TEC-/-} RNA-Seq data

All samples taken from wild-type control mice showed the inclusion of exon 3 in their mature CHD6 transcripts. Two of the three *Chd6*^{TEC-/-} samples demonstrated a complete absence of exon 3 as an expected consequence of the Cre-mediated recombination of the *Chd6* locus which has loxP sites positioned adjacent to this exon (Figure 4.15) allowing for the Cre-controlled removal of the intervening DNA sequence. However, one of the *Chd6*^{TEC-/-} mice (KO2) displayed evidence of inclusion of exon 3 at least in cTEC and mTEC^{lo} cell types (Figure 4.15).

The presence of *Chd6* exon3 in one of the cTEC samples (KO2) may have resulted from a contamination with thymocytes captured by nurse cells²³⁶. This is *per se* not unexpected, as I had also observed in this sample transcripts for both *Rag1* and *Rag2* suggesting contributions of transcripts originating from thymocytes, which would be compatible with the nature of cortical nurse cells and the known difficulty to release thymocytes from these epithelia during the preparation of these TEC. However, this justification does not explain the detection of *Chd6* exon 3 in the mTEC^{lo} sample from KO2 sample. Upon revisiting the sample and library preparations for this experiment, I noted contamination of the mutant sample with wild-type cells, which occurred during thymic digestion steps. Given these reasons, I decided to exclude the contaminated KO2 sample from further downstream analyses.

I further explored the overall profile and established the relatedness between the individual samples using a principal component analysis (see section 2.8.3, Figure 4.16). Notably, principal components (PC) 1 and 2 primarily separated the three individual cell subtypes independent of their genotype, accounting for 67% of the variance in the data set (Figure 4.16). To explore the relationship between samples from the two genotypes within each group of identical cell types, I also applied PCA separately to the individual samples of the different cell populations defined by their specific phenotypes, after having removing the KO2 sample for reasons explained above (Figure 4.17).

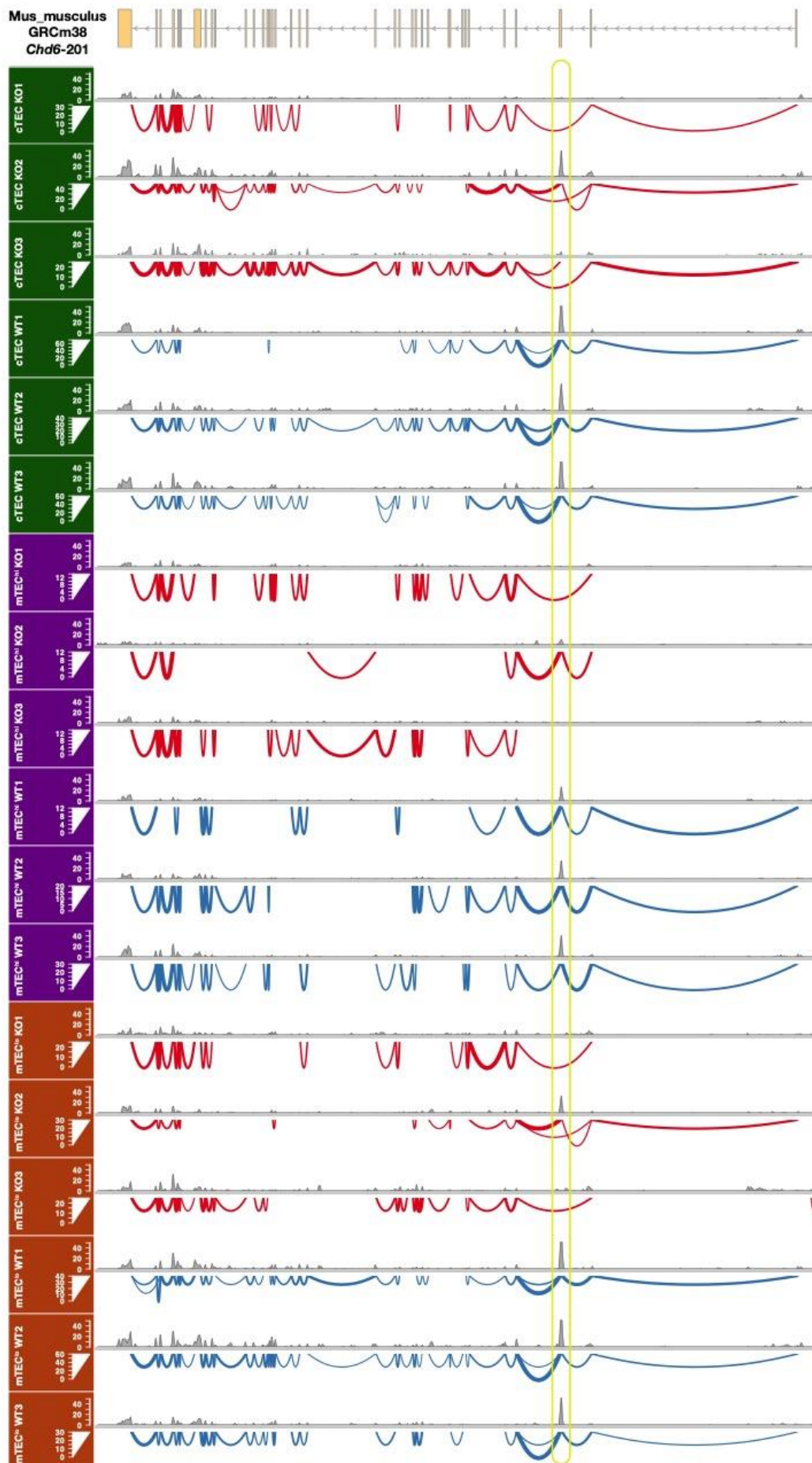


Figure 4.15 Coverage and splicing events for the *Chd6* locus.

Figure 4.15 Coverage and splicing events for the *Chd6* locus.

The sashimi plots were generated by mapping the processed sequencing reads on the y-axis with their genomic coordinates on the x-axis. Wild-type samples (WT: *Chd6*^{TEC+/+}) are shown in blue, and CHD6 conditional knockout samples (KO: *Chd6*^{TEC-/-}) are shown in red. From top to bottom are samples from cTEC (in green), mTEC^{hi} (in purple) and mTEC^{lo} (in orange), respectively. The *Chd6* gene model annotation is shown on the upper panel (*Mus musculus* genome assembly GRCm38 (mm10) from Genome Reference Consortium with the main *Chd6* isoform; *Chd6*-201 depicted). Sashimi plots for the exons are shown with the genomic reads converted into read densities (per-base expression on y-axis), while junction reads are plotted as arcs. The width corresponding to the number of sequenced reads that span each exon junction. The yellow box highlights the differential splicing of *Chd6* exon 3, which were included in all the wild-type samples but mostly excluded in the knockout samples except for KO2 sample.

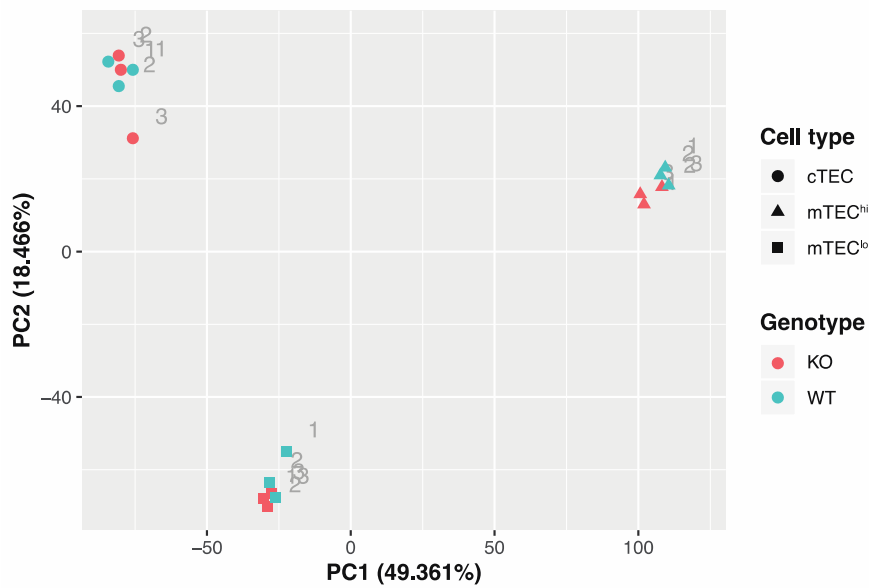


Figure 4.16 Principal component (PC) analysis showing clustering based on different cell types

PC analysis is shown for samples based on genes detected above the 25th expression quartile in at least three biological samples (n=34,039). Wild-type samples (WT: *Chd6*^{TEC+/+}) are shown in green, and CHD6 conditional knockout samples (KO: *Chd6*^{TEC-/-}) are shown in red. The first two PCs; PC1 and PC2 explained 49.361% and 18.466% of the variance, respectively, separating the datasets into distinct clusters based on their cell types (cTEC vs. mTEC^{hi} vs. mTEC^{lo}). Other remaining PCs (data not shown), only contribute to small variances in the dataset.

Samples from both genotypes (excluding KO2) clearly separated by the PC 1 alone (in cTEC and mTEC^{hi}) or a combination of PC 1 and PC 2 (in mTEC^{lo})(Figure 4.17). It is worthwhile to note that the variance accounting for the difference between the two genotypes in cTEC and mTEC^{lo} datasets were low in comparison to the variance observed in mTEC^{hi} (Figure 4.17). As expected, analysis of all samples (i.e. including KO2) failed to clearly separate the genotypes in cTEC and mTEC^{lo} (data not shown).

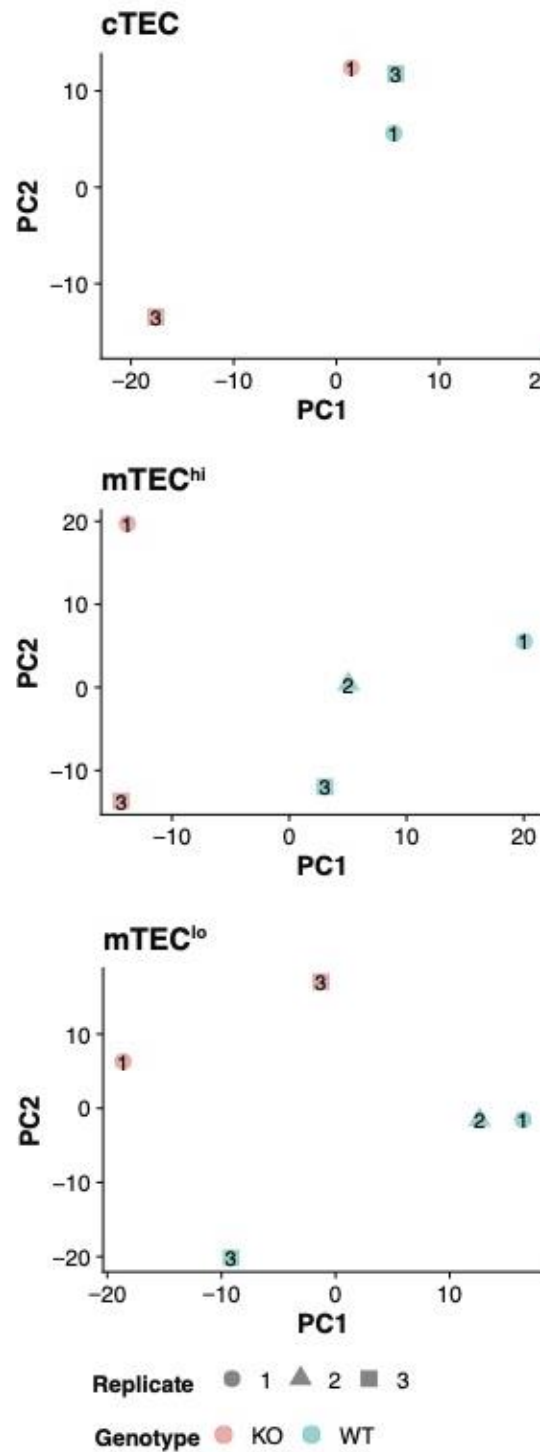


Figure 4.17 PC analysis of different cell types showing separation of datasets based on genotype
 PC analyses are shown for samples based on all genes (n=53,557) excluding KO2 sample. Wild-type samples (WT: *Chd6*^{TEC+/+}) are shown in blue, and CHD6 conditional knockout samples (KO: *Chd6*^{TEC-/-}) are shown in red, with replicate depicted with different shapes. Exclusion of KO2 sample from the PCA analysis managed to separate the KO and WT samples into distinct clusters, despite a smaller variance in mTEC^{lo} and cTEC, compared to the variance seen in mTEC^{hi}.

4.4.2. The expression of protein coding TRG transcripts is reduced in CHD6-deficient mTEC^{hi}

I next enumerated the genes for which transcripts could be detected in bulk RNASeq analyses of the samples from different TEC subpopulations using HISAT2 and featureCounts. Figure 4.18a displays the number of genes detected above the various upper-quartile normalised Transcripts Per Million (TPM) value. Additionally, I determined the number of TRG transcripts, which can be detected within the protein coding genes. The tissue specificity of genes were calculated using Tau method, in which the expression of the gene in the each tissue is compared over the maximal expression of the gene across all tissues and the number of different tissues analysed²³⁷. Using the calculated Tau value, TRGs were defined as protein coding genes with a Tau value greater than 0.7 based on mouse ENCODE data (unpublished data processed by Dr. Kathrin Jansen and Dr Kevin Rue-Albrecht, University of Oxford) (Figure 4.18b). As expected, more total gene transcripts as well as TRG transcripts were detected in mTEC^{hi} when compared to mTEC^{lo} and, again, fewer transcripts were successfully recovered from cTEC in comparison to the two other samples (Figure 4.18b).

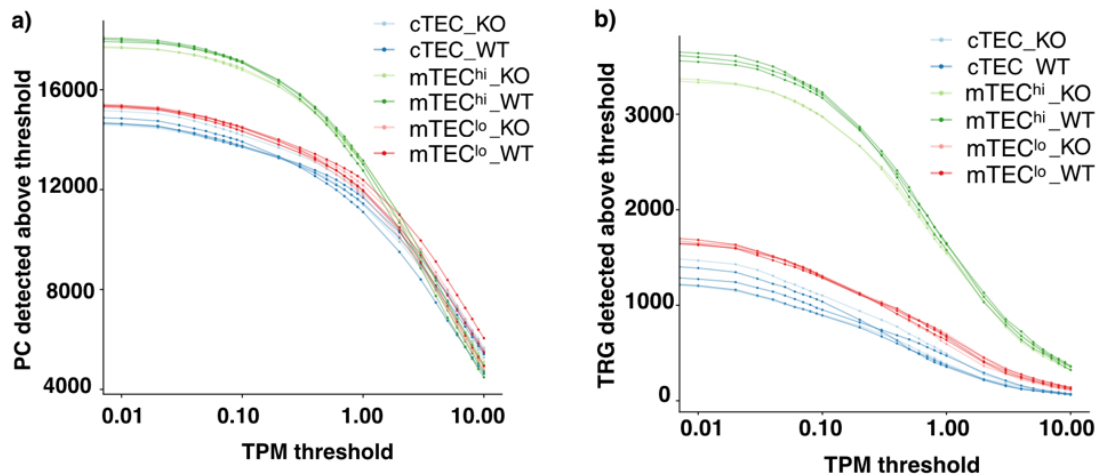


Figure 4.18 Number of protein coding (PC) and tissue-restricted genes (TRG) detected at various normalised Transcript Per Million (TPM) values.

a) Number of protein coding genes detected at various upper-quartile normalised TPM thresholds. Numbers of genes shown were value detected above the given threshold. **b)** Protein coding genes were further classified into tissue-restricted genes (TRG) and non-TRG. Number of TRG shown was value detected at various-upper quartile normalised TPM threshold.

Using a TPM value of 0.1 (the TPM value was determined as cut-off threshold at which genes were detectable at local False Discovery Rate (FDR) of less than 5%), I

compared the number of genes expressed and detected in different TEC populations of both CHD6 mutant mice and the wild-type controls. The number of protein coding genes whose transcripts could be detected above a TPM value of 0.1 was not significantly different when comparing the three TEC subsets from each genotype (representative TEC dataset shown in Figure 4.19a was the calculated value in mTEC^{hi} subset). Focusing my analysis on detecting TRG transcripts above the same TPM cut-off value (TPM value of 0.1), a significant reduction in the number of TRG could be detected only in the mTEC^{hi} of mutant mice in comparison to wild-type control (3412 ± 41.02 vs 2925 ± 31 , $p < 0.005$) (Figure 4.19b). The numbers of TRG differentially detected in either the mTEC^{lo} or cTEC subsets were not significantly different, implying that PGE in these TEC subpopulations, which do not express AIRE, was less affected and thus, does not require CHD6 (data not shown). As CHD6 is proposed to be specifically involved in enabling PGE together with AIRE, this finding was not unexpected. Additionally, this finding fittingly explained and correlated well with the overall smaller variance observed in mTEC^{lo} and cTEC samples between the wild-type and KO based on the PCA analyses performed earlier (Figure 4.17).

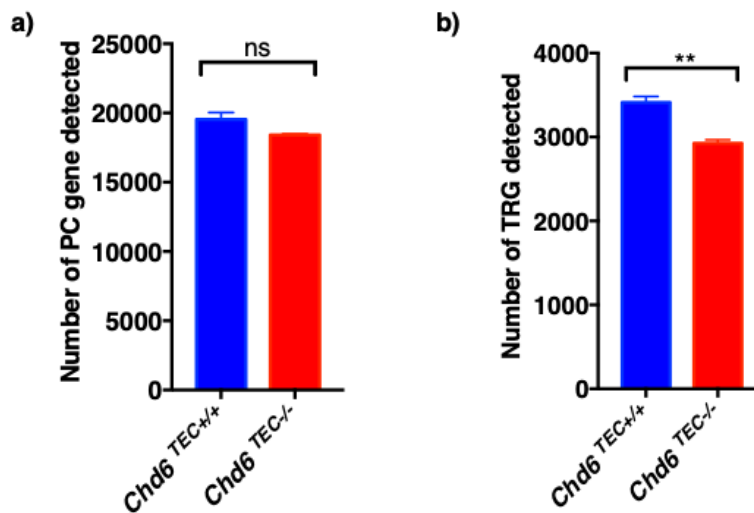


Figure 4.19 Numbers of protein coding and TRG detected in mTEC^{hi} subsets.

a) Comparison between numbers of protein coding (PC) genes detected in mTEC^{hi} samples from *Chd6* TEC^{-/-} mice and wild-type control animals (at TPM value of 0.1). p-values were determined by Student's t-test, ns= not significant i.e. $p > 0.05$. **b)** Comparison between number of TRG detected in mTEC^{hi} samples from *Chd6* TEC^{-/-} mice and wild-type control (at TPM value of 0.1). p-values were determined by Student's t-test where ** = $p < 0.005$.

4.4.3. Genes not detected in CHD6-deficient mTEC^{hi} are enriched for *Aire*-regulated TRGs

Since significantly fewer TRGs were detected in mTEC^{hi} isolated from *Chd6*^{TEC^{-/-}} mice, I further investigated whether their transcriptions require the presence of AIRE. I therefore categorised the non-detected genes in *Chd6*^{TEC^{-/-}} mice according to their dependence on AIRE: i) *Aire*-dependent genes, i.e. genes whose expression is absent in mice deficient for AIRE; ii) *Aire*-enhanced genes, i.e. genes that are reduced by two-fold or more but still transcribed despite an absence of AIRE; and iii) genes whose promiscuous expression is not dependent on AIRE²³⁸. I noted that a large fraction of the genes not expressed in the absence of CHD6 belong to the loci that require AIRE for their PGE (80% *Aire*-regulated loci; with 6.0% *Aire*-dependent and 74.0% *Aire*-enhanced). Since only 49% of TRG being *Aire*-regulated loci in the wild-type, this finding suggests that CHD6-controlled genes mainly consist of TRGs that are regulated by AIRE. This shift towards *Aire*-regulated genes was statistically significant, indicating that CHD6 preferentially is required for the genes that need AIRE for their PGE (Figure 4.20a). Of note, the absence of CHD6 in mTEC^{hi} compromised the expression of approximately 10% of the total *Aire*-regulated TRGs (11.8% of *Aire*-enhanced TRG and 5.8% of *Aire*-dependent TRG) (Figure 4.20b).

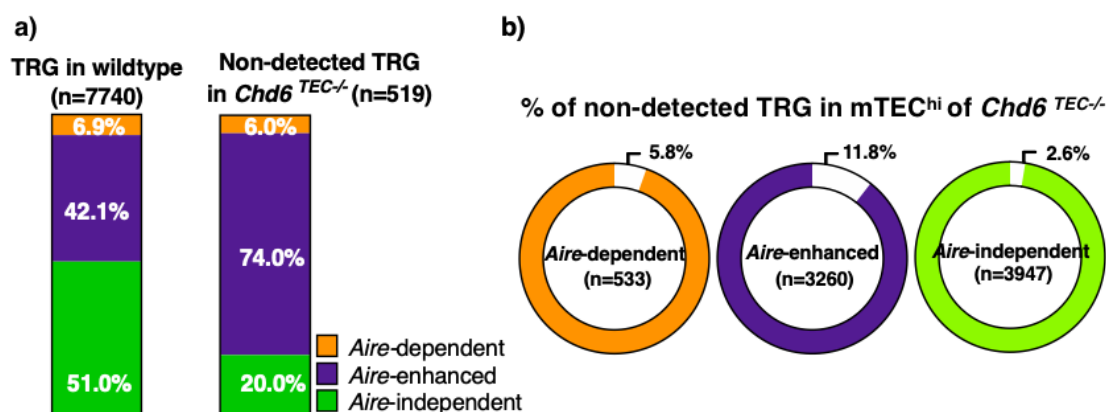


Figure 4.20 Characterisation of non-detected TRG in mTEC^{hi} of *Chd6*^{TEC^{-/-}} mice based on AIRE-dependency.

a) Fraction of non-detected TRG in mTEC^{hi} of *Chd6*^{TEC^{-/-}} mice (right bar graph) was categorised based on AIRE-dependency as previously described¹⁰⁶, shown as calculated percentage over the non-detected TRG (n=519). For comparison, TRG as expressed in wild-type mouse (n=7740) observed in TEC-population level as previously documented¹⁰⁶, were similarly categorised and presented (left bar graph). **b)** Non-detected TRG in mTEC^{hi} of *Chd6*^{TEC^{-/-}} mice as categorised based on the AIRE-dependency, were further calculated as percentage over the expected expression observed in TEC population-level for each category as previously documented¹⁰⁶.

4.4.4. Differentially expressed genes (DEG) in CHD6-deficient TEC

I next determined the number of genes differentially expressed when comparing CHD6 proficient and deficient cTEC, mTEC^{lo}, and mTEC^{hi}. For this analysis, the transcriptome of wild-type, age- and gender-matched TEC was used as the reference, which means that positive log fold-change values indicate a higher expression in the mutant cells. I noted a ratio of 2:1 for genes that were differentially down- and up-regulated in mTEC^{hi} (Figure 4.21) and mTEC^{lo} (Figure 4.22), when compared to wild-type cells. This result indicates that the numbers of genes differentially down-regulated were more than genes that were differently up-regulated upon deletion of CHD6 in both mTEC subsets. In contrast, this ratio was 1:1 indicating a comparable number of genes was up- and down-regulated in cTEC in the absence of CHD6 and when compared to wild-type cTEC (Figure 4.23). Subsequently, genes differentially expressed between CHD6-deficient and -proficient TEC were further characterised whether they are: a) a known target of a defined autoimmune response; b) similar in their biological function according to gene ontology; c) dependent on AIRE for their promiscuous expression (see section 4.4.5); d) marked by H3K27me3, H3K4me3 and/or H3K9ac post-translational histone modifications (analysed for DEG in mTEC^{hi} and mTEC^{lo}, see section 4.5).

We observed 107 down- and 63 up-regulated genes in mTEC^{hi} isolated from *Chd6*^{TEC-/-} mice and compared to wild-type cells of the same phenotype (Figure 4.21). The number of differentially expressed genes was relatively small, suggesting that the impact of a loss of CHD6 only affected a small, yet possibly specific set of loci. The expression of *Ctsj*, encoding Cathepsin-J protein, was most differential when comparing mTEC^{hi} from wild-type and mutant mice. In the context of mTEC as the main cellular player in thymocyte selection process, *Ctsj* is a promiscuously expressed genes and the reduction in its expression implied the role of CHD6 in positively controlling the machinery of PGE in mature mTEC. In line with that notion, of the genes down-regulated in mTEC^{hi} in the absence of CHD6, a significantly large fraction of it encoded tissue-restricted antigens. I therefore explored these genes in detail and presented it the following sections (see section 4.4.5 and 6.3).

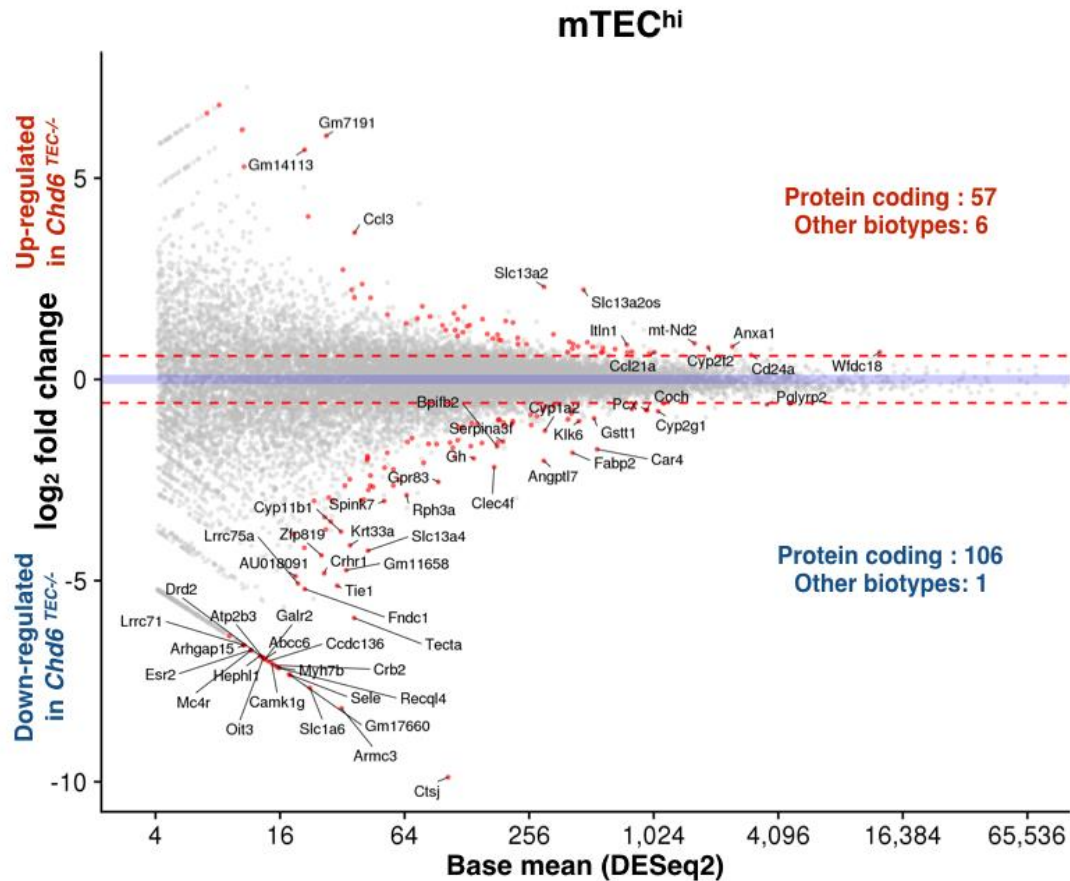


Figure 4.21 MA plots highlighting differentially expressed genes in mTEC^{hi} of *Chd6*^{TEC-/-} mice
Wild-type mTEC^{hi} serve as a reference for differential gene expression in CHD6-deficient TEC. Positive log fold change value indicates a higher expression in *Chd6*^{TEC-/-}. Differential expression analyses were performed using DESeq2. Highlighted in red dots are genes with absolute fold change >1.5, adjusted p-value <0.05.

Among the genes up-regulated upon deletion of CHD6 in mTEC^{hi} are *Anxa1*, *Ccl13* and *Slc13a2* (Figure 4.21). CCL13 (encodes by *Ccl13*) binds to several chemokine receptors (CCR1, CCR2 and CCR3) acting as chemo-attractant in recruitment of monocytes and lymphocytes including T cells to inflamed tissues²³⁹. Annexin-A1 (encodes by *Anxa1*) is a crucial modulator of thymocyte activation, particularly in regulating the strength of TCR signalling during thymocyte positive and negative selections²⁴⁰. However, the role of both CCL13 and Annexin-A1 in TECs are yet to be explored. *Slc13a2* is an upstream gene of *Foxn1*, and previous study had suggested that the regulatory element which is partly responsible for *Foxn1* expression is located between the first coding exon of *Foxn1* and *Slc13a2*²⁴¹. However, although *Slc13a2* was up-regulated in mTEC^{hi} of *Chd6*^{TEC-/-} mice, *Foxn1* expression remains unchanged in any of the TEC subsets of the mutant CHD6 mice. Additionally, the specific role of

sodium-dependent dicarboxylate transporter member 2 (Slc13a2) in TECs had never been described.

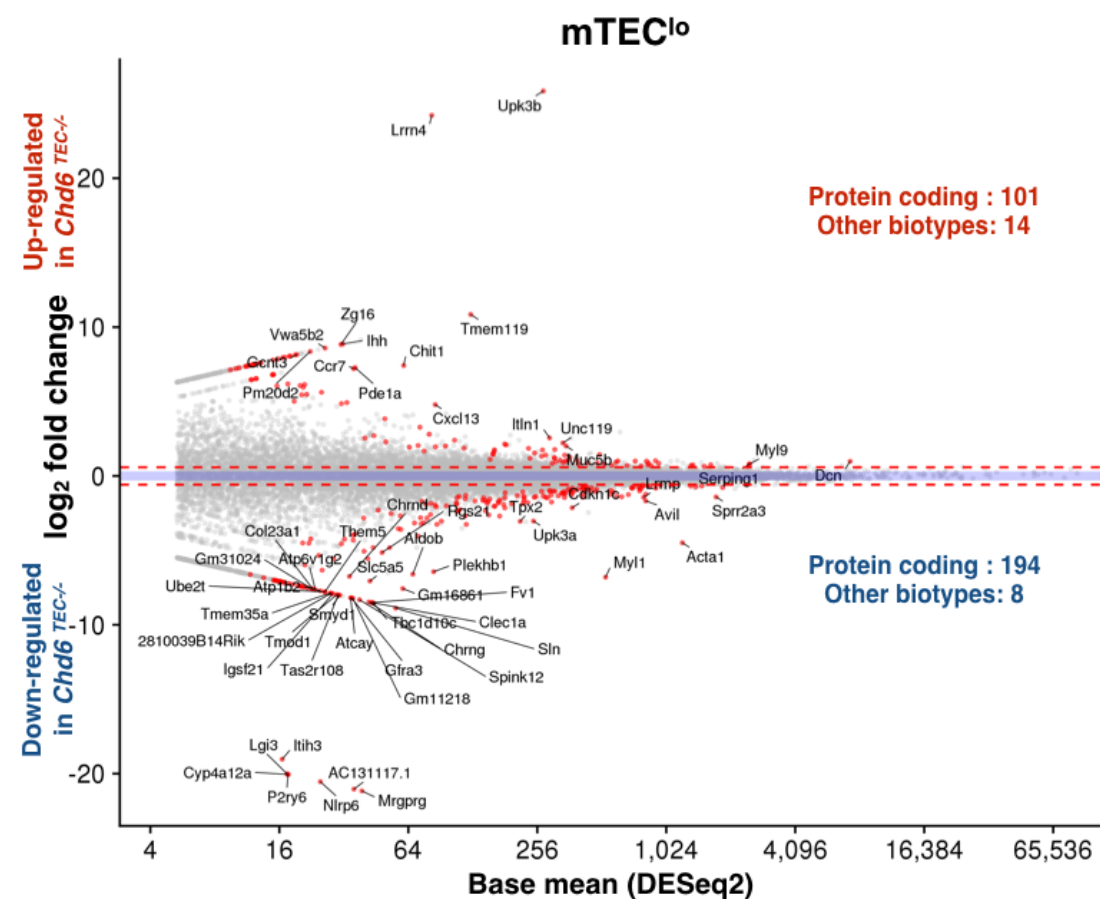


Figure 4.22 MA plots highlighting differentially expressed genes in mTEC^{lo} of *Chd6*^{TEC-/-} mice
Wild-type mTEC^{lo} serve as a reference for differential gene expression in CHD6-deficient TEC. Positive log fold change value indicates a higher expression in *Chd6*^{TEC-/-}. Differential expression analyses were performed using DESeq2. Highlighted in red dots are genes with absolute fold change >1.5, adjusted p-value <0.05.

mTEC^{lo} deficient in CHD6 expression had 202 genes down- and 115 genes up-regulated in comparison to CHD-proficient mTEC^{lo} (Figure 4.22). Notably, there were 9 loci that had a reduced expression of a log₂ fold-change greater than 20 (Figure 4.22), including *Itih3*, *Cyp4a12a* and *Lgi3*. Among the highly down-regulated genes were loci that encode antigens known to act as targets of clinically relevant autoimmune responses. For example, *Itih3* and *Cyp4a12a* encode autoantigens relevant for the pathogenesis of Sjogren's syndrome and autoimmune hepatitis, respectively. Another gene highly down-regulated was *Lgi3* which encodes the LG1 protein previously

linked to the autoimmune epilepsy syndrome ^{242,243}. Interestingly, all these three genes are also TRGs that are regulated by AIRE in their expressions in mTEC^{hi}. As the group of cells with an mTEC^{lo} phenotype also includes cells that have matured from mTEC^{hi} to a post-AIRE terminally differentiated stage, the absence in expression of these self-antigens may reflect the dependence of specific loci on CHD6 for their promiscuous expression in mTEC^{hi}. Indeed, these TRGs were also expressed at a reduced level in the mTEC^{hi} of *Chd6*^{TEC-/-} mice (Figure 4.21), therefore is in agreement with the proposed notion that this observation is due to the changes in the post-AIRE terminally-differentiated mTEC subset.

Thymic tuft cells are post-Aire mTEC that possess broad array of type II taste receptor family members in order to sense microenvironmental cues ^{76,77}. In keeping with their perceived function, thymic tuft cells typically express *Tas2r108* and *Tas2r105* ⁷⁶, two genes encoding type II taste receptors. The expression of *Tas2r108* and *Tas2r105* were noted to be reduced in mTEC^{lo} as a result of an absence of CHD6 (Figure 4.22). Interestingly, other genes characteristically associated with the transcriptome of thymic tuft cells were also noted to be down-regulated in mTEC^{lo} in the absence of CHD6, namely *Avil* (log2FC= -1.67, adj p<0.0001), *Lrmp* (log2FC= -1.43, adj p<0.0001), *Gnat3* (log2FC= -1.377, adj p<0.05) and *Trpm5* (log2FC= -1.000, adj p<0.05^{76,77}). Alternatively, a reduced expression of tuft cell associated transcripts in the transcriptome of bulk mTEC^{lo} may also come about by a numerical reduction of tuft cells in that mTEC^{lo} population. However, the cellularity of tuft cells was not reduced in *Chd6*^{TEC-/-} mice (Figure 4.12), which renders this possibility practically impossible. Hence, CHD6 is not required for thymic tuft cells development, yet this molecule still appears to play a role in the transcription of genes that are seemingly important for the cells' function. Additionally, *Tas2r105*, *Tas2r108* and *Gnat3* are also classified as TRGs. Hence, this further implies that thymic tuft cells may also play a role in promiscuously expressing tissue-restrictive antigens and is partly dependent on CHD6 to do so. Conversely, among the genes up-regulated as a consequence of a loss of CHD6 expression in mTEC^{lo} were *Lrrn4* and *Upk3b* (Figure 4.22), which serve as markers of primary mesothelial cells ²⁴⁴. However, the role of these two genes in TEC biology has yet to be identified.

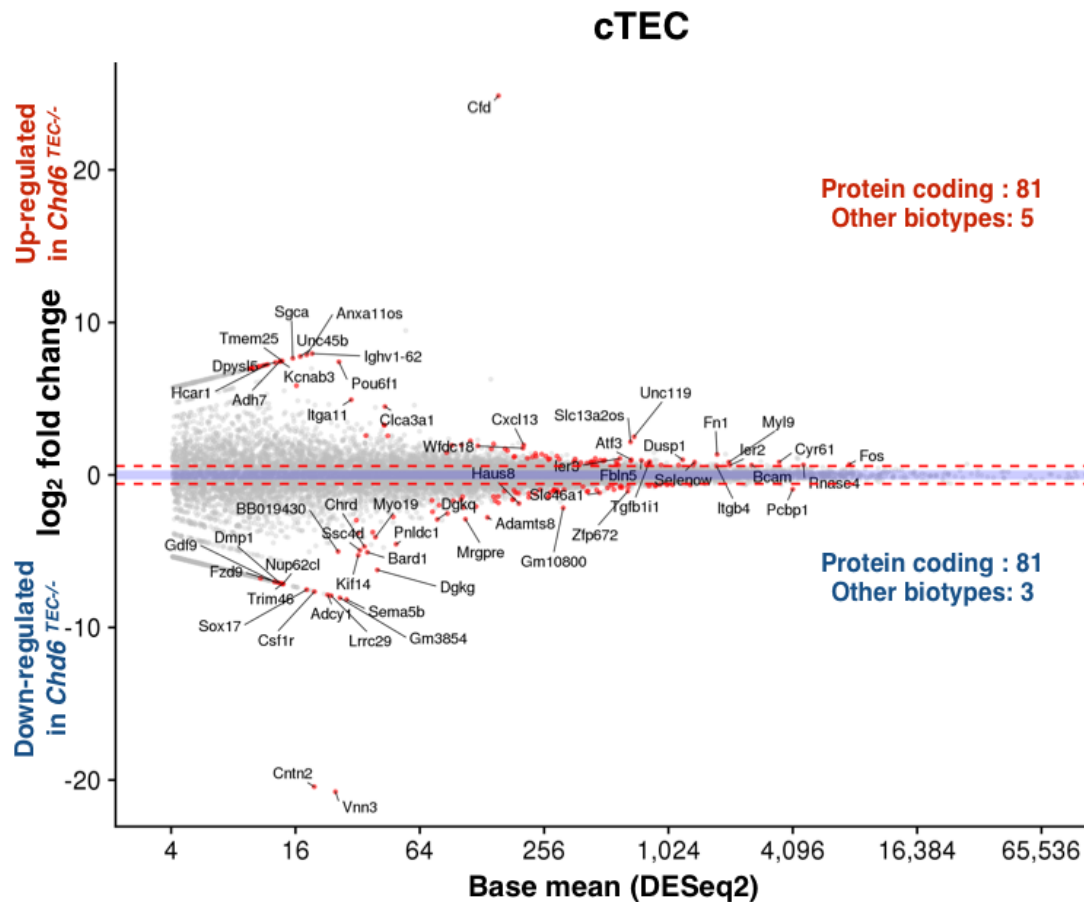


Figure 4.23 MA plots highlighting differentially expressed genes in cTEC of *Chd6*^{TEC-/-} mice
 Wild-type cTEC serve as a reference for differential gene expression in CHD6-deficient TEC. Positive log fold change value indicates a higher expression in *Chd6*^{TEC-/-}. Differential expression analyses were performed using DESeq2. Highlighted in red dots are genes with absolute fold change >1.5, adjusted p-value <0.05.

In cTEC devoid of CHD6, I observed that the expression of *Cntn2* and *Vnn3* was down-regulated by an absolute log₂ fold-change value of greater than 20 (Figure 4.23). The AIRE-independent *Cntn2* encodes contactin-2 which is an auto-antigen relevant in the pathogenesis of multiple sclerosis¹⁰⁵. Vanin-3, encoded by *Vnn3*, is expressed in differentiated epidermal cells, where it has been identified as an autoantigen in the context of psoriasis and atopic dermatitis²⁴⁵. Unpublished data from the lab has demonstrated that the population of cells with a cTEC phenotype (as defined by flow cytometry) also encompasses TEC that have recently been transcriptionally identified as tuft cells. As thymocyte negative selection does occur in the cortex¹⁰⁴ and thymic tuft cells are shown to act as antigen-presenting cells^{76,77}, the role of Contactin-2 and Vanin-3 as part of the TRAs generated and presented by cTEC, is a noteworthy concept to explain the observations.

I next analysed the genes differentially expressed in CHD6-deficient TEC whether these belonged to any specific pathways using gene ontology analysis. For the positive identification of specific pathways, the following criteria needed to be met: i) more than five genes needed to be enriched in a specific pathway; ii) the calculated odds ratio needed to be greater than 2; and iii) the adjusted p-value needed to be less than 0.05. Given these conditions, the ontology analysis of differentially expressed genes failed to identify for cTEC and mTEC^{hi} any enrichment of genes belonging to a particular pathway. This result may be reflective of the relatively low number of differentially expressed genes in these cells. Furthermore, it was not unexpected as a large fraction of the genes down-regulated in CHD6-deficient mTEC^{hi} encode tissue specific antigens and thus per se do not belong to a particular biochemical pathway. In contrast, the ontology analysis of genes down-regulated in mTEC^{lo} as a result of a lack of CHD6 revealed an enrichment of genes associated with two separate biological processes, i.e. “response to nicotine” and “skeletal muscle contraction”. On further probing, the two biological pathways shared three common genes, namely *Chrnbl*, *Chrnd* and *Chrng*, which encode different subtypes of nicotinic acetylcholine receptors. Autoantibodies against these nicotinic acetylcholine receptors lead to autoimmune channellopathies at neuromuscular junctions, clinically manifesting as early onset myasthenia gravis ²⁴⁶. Another gene of the “skeletal muscle contraction” pathway is *Tnni2*, which encodes Troponin I. This muscle protein serves as an autoantigen serving as a myocardial target for severe autoimmune inflammation ^{247,248}.

The observed alterations in the gene expression of mTEC^{hi} that occur as a result of the loss of CHD6 were independently confirmed using qRT-PCR. Figure 4.24 demonstrates that the set of genes chosen for this analysis, behaved as expected in that some were up- and others down-regulated in their expression independent of their reliance on AIRE. Hence, the findings obtained by RNAseq were verified by an alternative method resulting in an identical conclusion, namely that some genes require for their transcription in TEC the expression of CHD6.

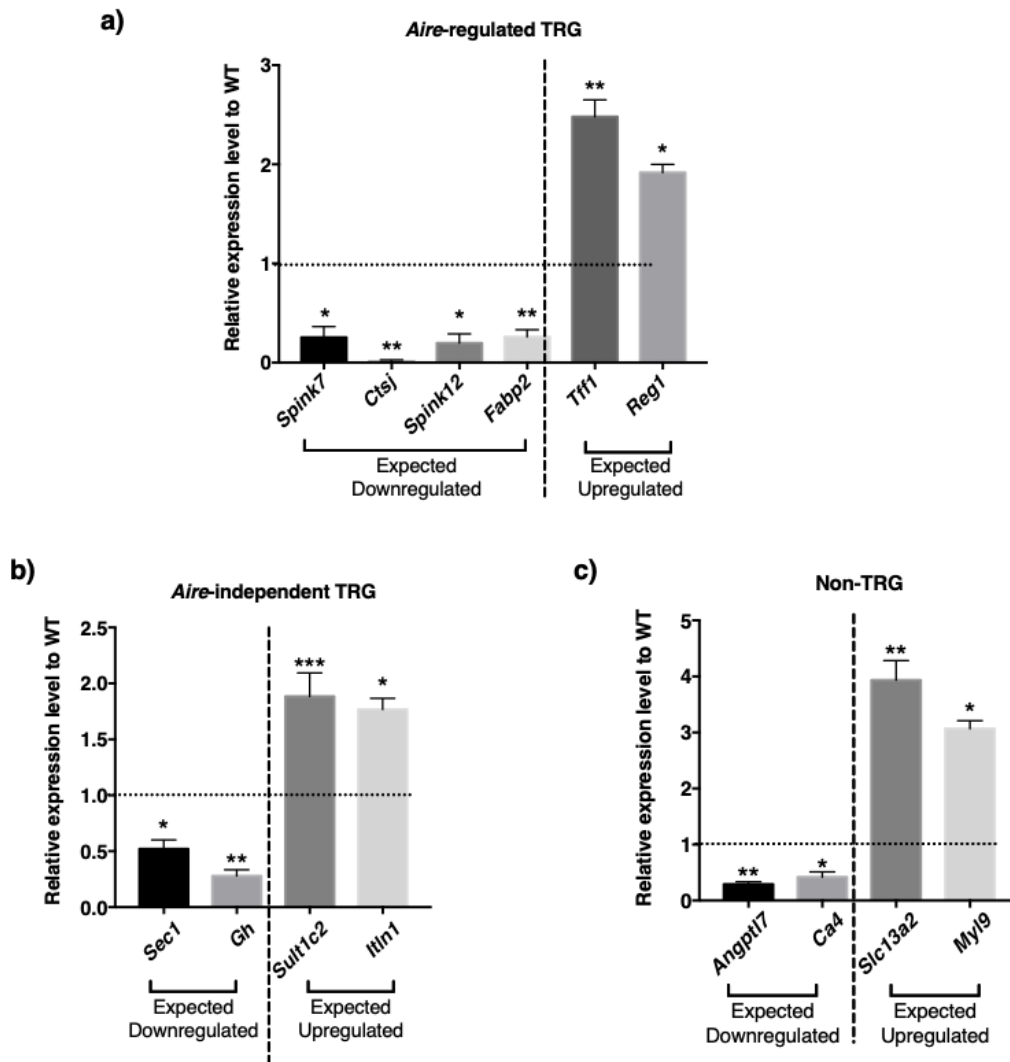


Figure 4.24 Differential gene expression assessed by qRT-PCR in mTEC^{hi} of *Chd6*^{TEC-/-} mice

The expression of a selected set of genes: **a)** *Aire*-regulated TRG; **b)** *Aire*-independent TRG; and **c)** Non-TRG. Transcripts from FAC-sorted mTEC^{hi} isolated from 4.5 week old *Chd6*^{TEC-/-} mice (n=3, female) and wild-type control mice (*Chd6*^{TEC+/+}, n=3, female) were detected and quantified using qRT-PCR. Values were normalised to *Gapdh* expression and values from wild-type control were arbitrarily set to 1 (dotted line). Each bar graph represents the mean of three biological replicates and, in addition, displays the standard deviation. The expression patterns (down- vs up-regulated) based on the bulk RNAseq data set, are provided for each panel.

4.4.5. Genes down-regulated in CHD6-deficient mTEC^{hi} are enriched for TRGs, particularly those that depend on AIRE for their expression

I next grouped the protein-coding genes that were differentially expressed in mTEC^{hi} into TRG or non-TRG (e.g. housekeeping genes). The TRGs were further categorised based on the dependency for AIRE to be transcribed¹⁰⁶. I observed that approximately 75% of the down-regulated genes were represented by TRGs, of which almost 90% were either *Aire*-enhanced or *Aire*-dependent (Figure 4.25).

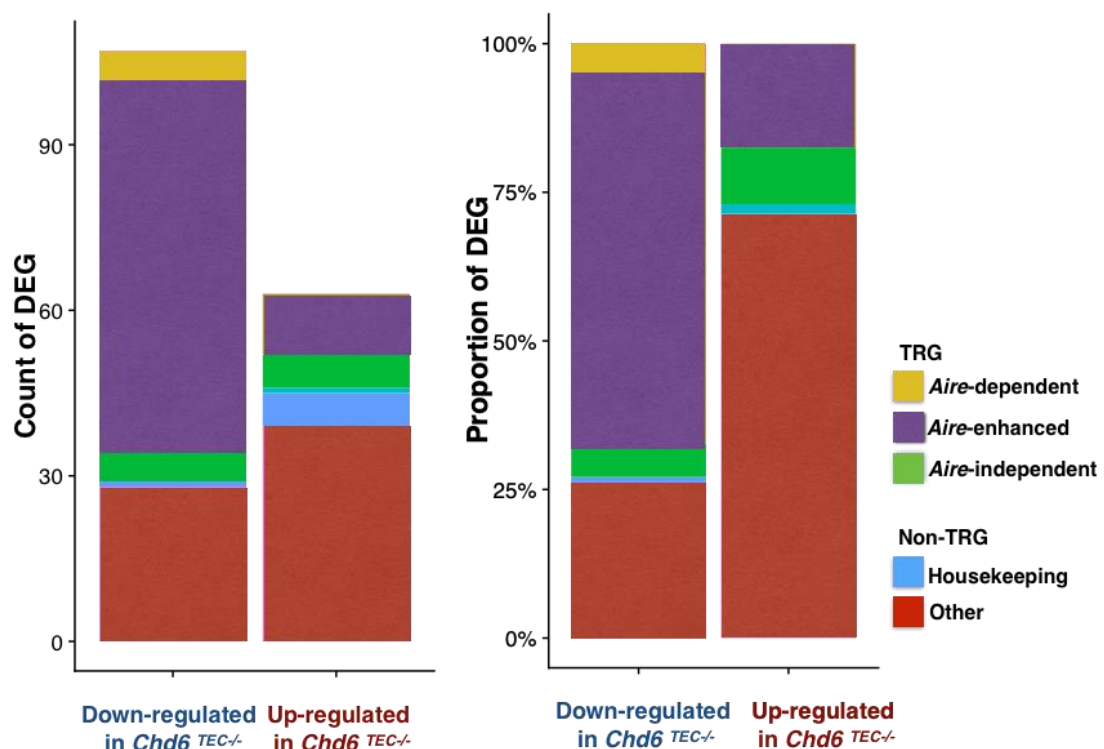


Figure 4.25 Expression of protein coding genes among DEG in mTEC^{hi} of *Chd6*^{TEC-/-} mice
 Count (left) and percentage (right) of differentially expressed genes (DEG) in mTEC^{hi} of *Chd6*^{TEC-/-} mice when compared to CHD6-proficient mTEC^{hi}. Genes are categorised on AIRE-dependency as previously described¹⁰⁶. DEG were determined by DESeq2 analysis with absolute fold change >1.5, adjusted p-value <0.05. Total down-regulated DEG genes in *Chd6*^{TEC-/-} mice = 107 genes, total up-regulated DEG genes in *Chd6*^{TEC-/-} mice = 63 genes.

The strong enrichment of genes that are AIRE-controlled in their expression among the down-regulated transcripts in CHD6-deficient mTEC^{hi} highlights the importance of CHD6 for the promiscuous expression of some loci but not others. I therefore verified whether the observed enrichment was expected by chance or indeed reflected a mechanistic link between the necessity for CHD6 expression and the transcription of

specific genes. To this end, the differential gene expression in CHD6-deficient mTEC^{hi} was compared to that of CHD6-proficient mTEC^{hi}, and tested for significance of their respective overlap with a set of protein-coding TRGs obtained from the mouse ENCODE RNASeq of peripheral tissues database. I found that the presence of CHD6 is related to the positive regulation of protein coding TRGs, as apparent by the calculated odds ratio (i.e. a highly significant adjusted p-value; Figure 4.26). Notably, the odds ratio was nearly as high as the odds of AIRE in regulating the expression of TRGs (Figure 4.26). It is however important to note that the calculation of this odds ratio was based on a relatively small number of genes (57 in *Chd6*^{TEC-/-} mice compared to 2787 gene in *Aire*-deficient mice), clearly indicating that the observed effect is limited to a restricted subset of TRGs (Figure 4.26). Taken together, the data presented concludes a role for CHD6 in the promiscuous transcription of tissue-restricted antigens, which are characteristically controlled in their expression by AIRE.

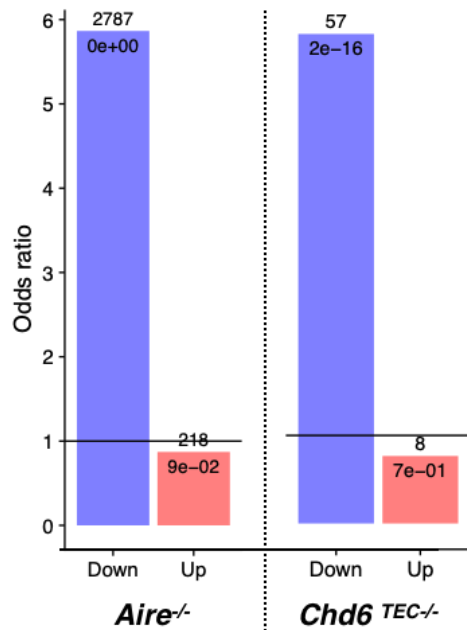


Figure 4.26 Odds ratio overlap between the expressions of tissue restricted genes in mTEC^{hi} of *Aire*-deficient mice and *Chd6*^{TEC-/-} mice

An odds ratio greater than 1 indicates an overlap in gene expression between the two conditions that is greater than what can be expected by chance whereas an odds ratio of 1 or less indicates no association between the differentially expressed genes observed for the two experimental conditions. The numbers of genes overlapping are shown above each bar. Adjusted p-values are shown in the bar as calculated by Benjamini-Hochberg correction.

4.5. Histone landscape of differentially expressed genes

The transcriptomic data analyses indicated that CHD6 is specifically involved in facilitating the promiscuous expression of some genes expressed by mTEC, especially loci regulated by AIRE. Indeed, approximately 10% of TRA that require the presence of AIRE for their expression are down-regulated in mTEC^{hi} due to the absence of CHD6. It was previously shown that *Aire*-regulated loci are characteristically marked by the presence of histone 3 that is tri-methylated at lysine in position 27 (H3K27me3)^{106,169}. Further experimental evidence also strongly suggests that AIRE is unable to bind this histone mark directly¹⁵⁷. Therefore, CHD6 via the recognition of and binding to H3K27me3 marks, may aid in positioning a multi-molecular complex containing AIRE to regulatory sequences that control the promiscuous expression of specific TRGs. I therefore tested whether genes that are down-regulated in their expression as a result of a lack of CHD6 are marked in mTEC^{hi} by repressive histone marks, in particular H3K27me3. Together with Dr Kevin Rue-Albrecht and Dr Stephen Samson (Kennedy Institute of Rheumatology, University of Oxford), we detailed the presence of H3K27me3, H3K4me3 and H3K9me3 at loci encoding genes that are differentially expressed between CHD6-proficient and -deficient mTEC^{hi} and mTEC^{lo}. A detailed analysis of the histone landscape had previously been established by the Holländer lab^{106,170}. The reads from this database were trimmed to a common 50bp length and mapped to the mouse GRCm38/mm10 reference genome²⁴⁹. For the H3K4me3 and H3K9me3 analysis, promoters were defined by the -500bp to +500bp region around the transcriptional start site (TSS). As H3K27me3 histone modifications are lowly expressed and spread over the gene body, the median enrichment over the gene bodies scaled to -3kb to +6kb region around the TSS were used for the H3K27me3 analysis.

4.5.1. Genes induced by CHD6 in mTEC^{hi} are epigenetically marked by H3K27me3

Genes down-regulated in CHD6-deficient mTEC^{hi} were associated with significantly higher detection of H3K27me3 in their promoter regions when compared to corresponding sequences of loci encoding genes that were either not-differentially expressed or up-regulated (Figure 4.27 left panel). Conversely, the two permissive histone

marks tested, H3K4me3 and H3K9ac, were reduced among genes that were down-regulated as a consequence of a loss of CHD6 expression (Figure 4.27 middle, right panel). This observation is in accordance with the notion that CHD6 facilitates the transcription of genes that are enriched for H3K27me3 and depleted of H3K4me3, as previously noted for AIRE-regulated TRGs^{158,169}.

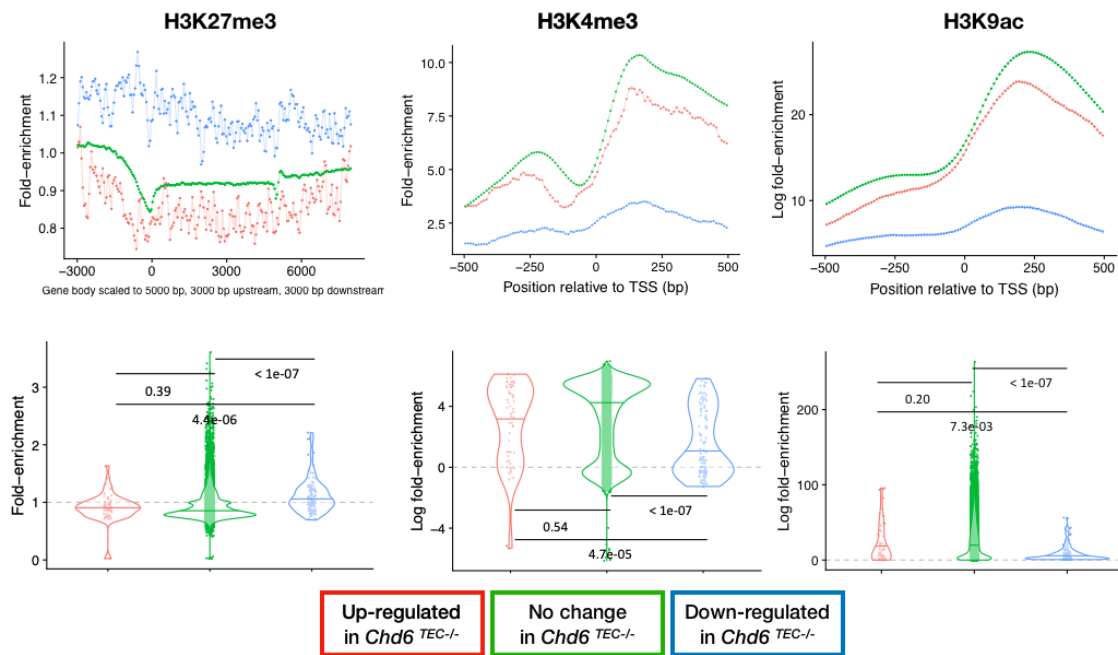


Figure 4.27 Histone landscape at the TSS of genes that depend on the presence of CHD6 for their expression in mTEC^{hi}

H3K27me3 (left), H3K4me3 (middle) and H3K9ac (right) marks of TSS of genes differentially expressed between CHD6-proficient and -deficient mTEC^{hi} as initially established in wild-type control cells using ChIP-Seq.^{106,170} **Upper panel:** left: Enrichment of H3K27me3 histone marks over the entire gene body; middle and right: Enrichment of the H3K4me3 and H3K9ac marks, respectively, at the TSS; **Lower panel:** violin plots visualizing the distribution of enrichment for the selected histone modifications H3K27me3, H3K4me3, and H3K9ac. Genes were categorised according to their differential expression into up-regulated (red), unchanged (green) and down-regulated (blue) in mTEC^{hi} of *Chd6*^{TEC-/-}. The enrichment for each locus was calculated as the median enrichment (over input) using Wilcoxon signed-rank test, with the p-values shown in the graph. For the analysis of histone marks over the entire gene body, the calculation considered 10bp “bins” of sequence ranging from the TSS to the transcription end site (TES). For the analysis of sequences 5’ and 3’ of the TSS, the calculation was performed over 1kb region (500bp upstream and downstream of the TSS).

4.5.2. In mTEC^{lo}, genes controlled in their expression by CHD6 are epigenetically marked by H3K4me3 and H3K9ac

I further extended the analysis of mTEC^{hi} cells to the other major mTEC population, namely mTEC^{lo}, which largely (but not uniquely) encompasses immature cells giving rise to mature mTEC. In contrast to the behaviour observed in mTEC^{hi}, genes which

were down-regulated in mTEC^{lo} due to the absence of CHD6 were depleted of H3K27me3 marks but enriched for H3K4me3 and H3K9ac marks (Figure 4.28). Taking both the findings together, the results implied that loci controlled by CHD6 do associate with different histone PTMs at different maturation stages in the same type of cells. Loci-positively controlled by CHD6 in mTEC^{lo} are generally permissive while in mTEC^{hi}, CHD6 appeared to be controlling the transcription of inactive gene loci.

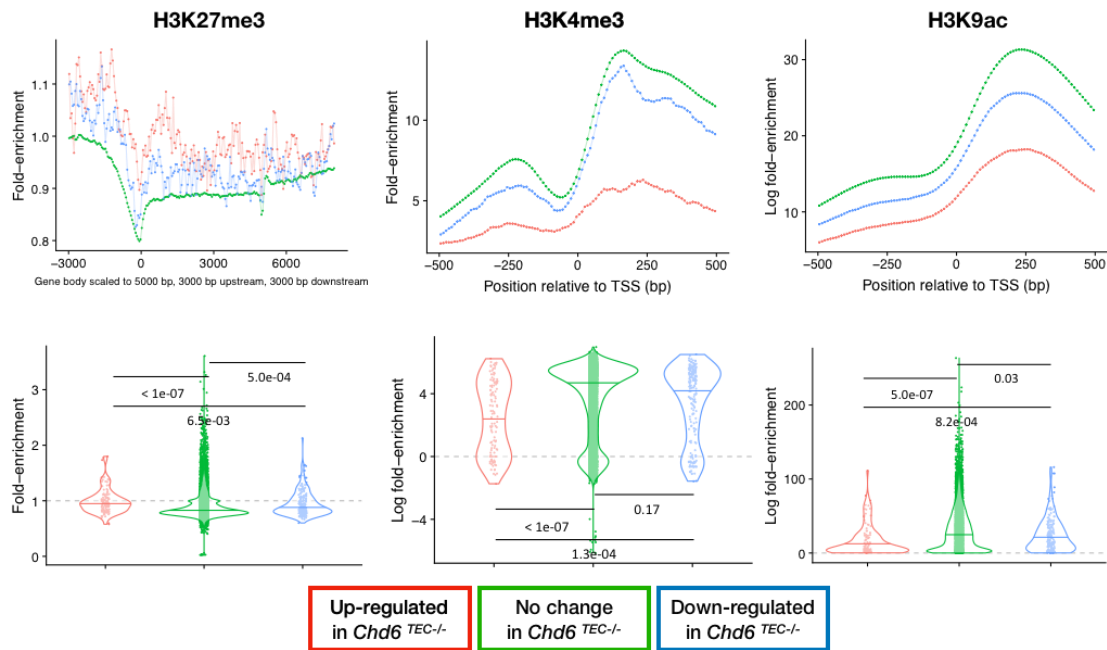


Figure 4.28 Histone landscape at the TSS of genes that depend on the presence of CHD6 for their expression in mTEC^{lo}

H3K27me3 (left), H3K4me3 (middle) and H3K9ac (right) marks of TSS of genes differentially expressed between CHD6-proficient and -deficient mTEC^{lo} as initially established in wild-type control cells using ChIP-Seq.^{106,170} **Upper panel:** left: Enrichment of H3K27me3 histone marks over the entire gene body; middle and right: Enrichment of the H3K4me3 and H3K9ac marks, respectively, at the TSS; **Lower panel:** violin plots visualizing the distribution of enrichment for the selected histone modifications H3K27me3, H3K4me3, and H3K9ac. Genes were categorised according to their differential expression into up-regulated (red), unchanged (green) and down-regulated (blue) in mTEC^{lo} of *Chd6*^{TEC} ^{-/-}. The enrichment for each locus was calculated as the median enrichment (over input) using Wilcoxon signed-rank test, with the p-values shown in the graph. For the analysis of histone marks over the entire gene body, the calculation considered 10bp “bins” of sequence ranging from the TSS to the TES. For the analysis of sequences 5’ and 3’ of the TSS, the calculation was performed over 1kb region (500bp upstream and downstream of the TSS).

4.6. Assessment of DNA damage response in *Chd6*^{TEC-/-} mice

Based on results so far presented, CHD6 facilitates the promiscuous expression of a subpopulation of TRGs that mostly require AIRE for their efficient transcription in the context of mTEC and that are typically decorated in these cells by H3K27me3. Direct physical interaction between AIRE and CHD6 is, however, not proven (see section 3.5) but may exist, possibly via the involvement of other molecules that in aggregate form a multi-molecular complex. Hence, additional proteins may partner with CHD6 and AIRE whereby CHD6 is essential to place the complex to specific epigenetically modified sequences. *In-vitro* studies had shown that CHD6 is crucial in recruiting DNA repair complex to the damage site^{202,205}, and truncation of different regions of CHD6 proteins interfered with the retention of CHD6 to the DNA breaks²⁰². Additionally, CHD6 partners include TOP2A, H2A histone family member X (H2AX) and PARPs, all are proteins involve in DNA damage response (DDR), thus supporting the definite role of CHD6 as a vital component of DDR^{201,202}. Therefore, CHD6 may indirectly recruit together the CHD6-AIRE multi-protein complexes to loci with DNA strand breaks and/or undergoing DNA damage repair. This concept is in keeping with a previously claimed mechanism postulating DNA DSB repair to be linked to the mechanisms by which AIRE promiscuously controls TRG transcription¹⁵⁰. Indeed, proteins that interact with AIRE have also been functionally proven to be involved in the DDR, including TOPs and DNA-PKs¹⁵⁰. It is therefore likely that CHD6 and consequently also AIRE are localised to sites of DNA DSB as part of the mechanism underpinning PGE in mTEC^{hi} as I have initially proposed (see section 3.7). Consequently, in the absence of CHD6, AIRE may not be able to localise to DNA DSB, a scenario that would impair PGE.

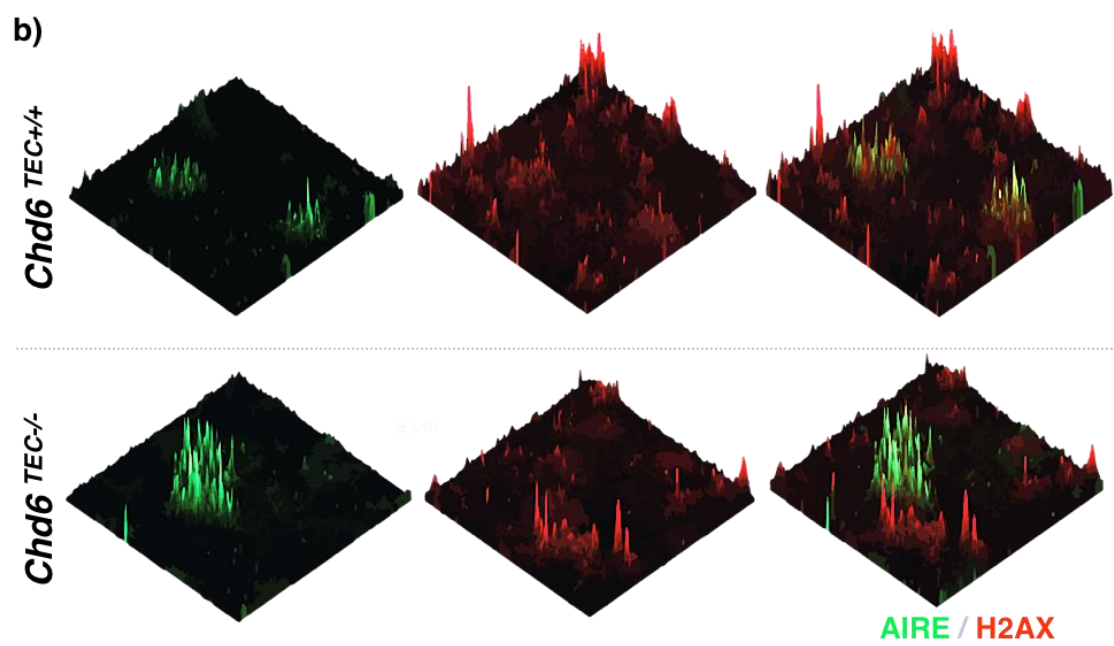
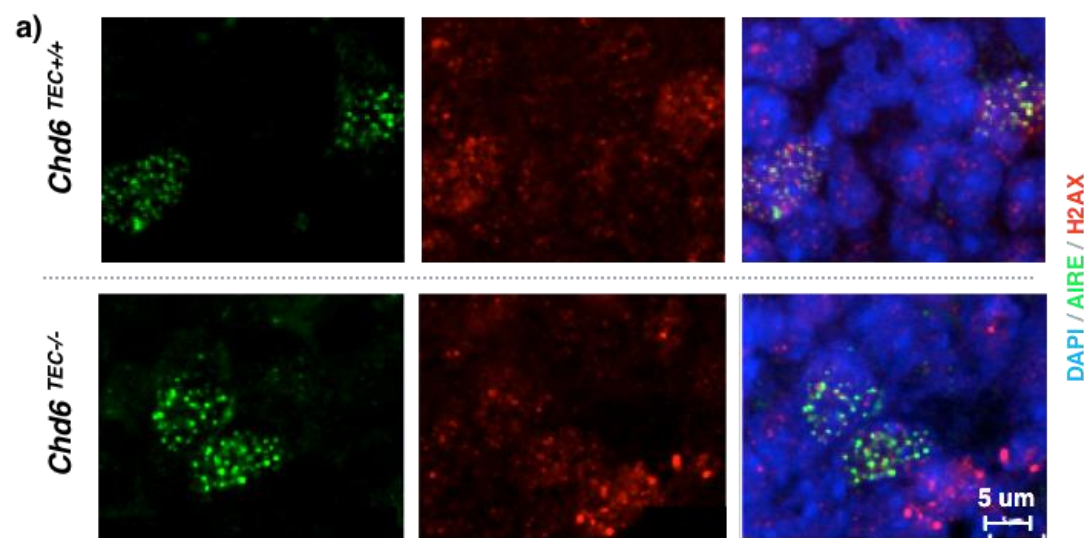
To probe this scenario of CHD6 localising AIRE to site of DNA DSB, I determine the nuclear co-localisation of AIRE with molecular features typical for DNA DSB, such as γ H2AX protein. **Phosphorylation** of the Ser-139 residue of the histone variant H2AX to generate γ H2AX, is a vital stage in the signalling and initiation of DNA DSB repair mechanism^{250–252}. Although the γ H2AX marker represents an indirect detection of DNA DSB, it has been shown to be a highly sensitive and reliable method in detecting

and quantifying DNA DSBs based on these advantages: (i) quantification of γ H2AX foci is considered as a measure of the number of DNA DSB, with each DSB break being documented to correlate to one γ H2AX foci^{253,254}; (ii) immunofluorescence imaging combines with the quantification of the number of γ H2AX foci in-situ, allows spatial assessment of DSB^{255,256}; and (iii) the rapid H2AX phosphorylation and gradual decline within hours, is reflecting the true kinetic of DNA DSB repair²⁵⁷. Thus, the detection of γ H2AX foci using confocal immunofluorescence imaging in-situ, offers a sensitive and accurate spatial assessment of DNA DSB. Thymic tissue sections from 4.5 week old *Chd6*^{TEC-/-} and *Chd6*^{TEC+/+} mice were therefore prepared and stained with directly-labelled antibodies against AIRE and γ H2AX. High-resolution immunofluorescence confocal microscopy was used to visualise a possible co-localisation of AIRE and γ H2AX in TEC nuclei (see section 2.7.5).

4.6.1. Loss of CHD6 in mTEC^{hi} does not impair γ H2AX and AIRE expressions

γ H2AX protein marking DNA DSB were detected comparably in CHD6-proficient and –deficient mTEC-expressing AIRE (Figure 4.29). This staining therefore implied that the absence of CHD6 does not interfere with the expression of γ H2AX and its molecular response to DNA DSB. AIRE expressions in mTEC were also observed to be comparable in CHD6-proficient and –deficient mTEC, both in their frequencies and the expression patterns (Figure 4.29), in keeping with the earlier findings that revealed the lack of CHD6 in TEC do not interfere with the expression of AIRE.

Additionally, in CHD6-proficient mTEC^{hi}, AIRE were observed to express frequently together with γ H2AX, indicating that both factors were localised at the same loci (Figure 4.29). This observation is in agreement with the notion that DNA repair processes are part of the mechanisms how AIRE regulates the promiscuous expression of different TRGs. Interestingly, the concomitant staining for AIRE revealed that its co-localisation with γ H2AX in CHD6-deficient mTEC^{hi} appeared to be reduced when compared to CHD6-proficient mTEC^{hi} (Figure 4.29). To objectively investigate this, I next quantified the nuclear expression of AIRE and γ H2AX as pixel signals in CHD6-proficient and –deficient, AIRE-positive mTEC^{hi} utilizing Zen Blue imaging software.



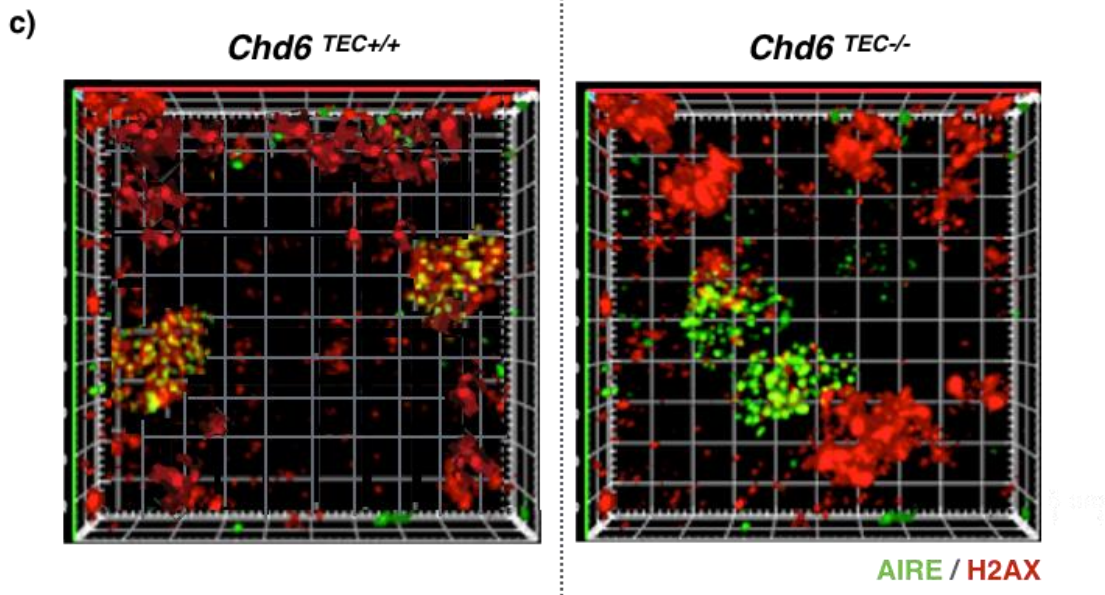


Figure 4.29 Expression and co-localisation of γ H2AX and AIRE in CHD6-proficient and –deficient mTEC^{hi}

a) Representative confocal images of frozen thymus tissue sections stained for AIRE (green) and γ H2AX (red) in wild-type animal (*Chd6*^{TEC+/+}) and CHD6 mutant mice (*Chd6*^{TEC-/-}). Nuclei were counterstained with DAPI. **b)** 3D graphical projection of the representative confocal images showing the expression of AIRE (green) and γ H2AX (red) expressions. **c)** Composite Z-slices of the representative confocal images showing AIRE (Green) and γ H2AX (red) expressions. Yellow signals identify the co-localisation of the two proteins. Five female *Chd6*^{TEC-/-} mice and five gender- and age-matched wild-type control mice (*Chd6*^{TEC+/+}) each at 4.5 weeks of age were analysed, whereby four separate thymus tissue sections were prepared and analysed for an individual animal.

A total of 35 nuclei were analysed and the signal was related to the cross-sectional area of each nucleus analysed, albeit this value was comparable for mutant and wild-type cells (Figure 4.30a). The number of sites of DNA DSB (as assessed by the detection of γ H2AX) and the signal of AIRE was not affected by the absence of CHD6 expression, further confirming that this loss does not affect the frequency of DNA DSB and/or the detection of AIRE (Figure 4.30b,c,d). Hence, these findings render it feasible to quantify the co-localisation of γ H2AX and AIRE without the need to account for changes brought about by the absence of CHD6 and affecting the expression of either of those two molecules.

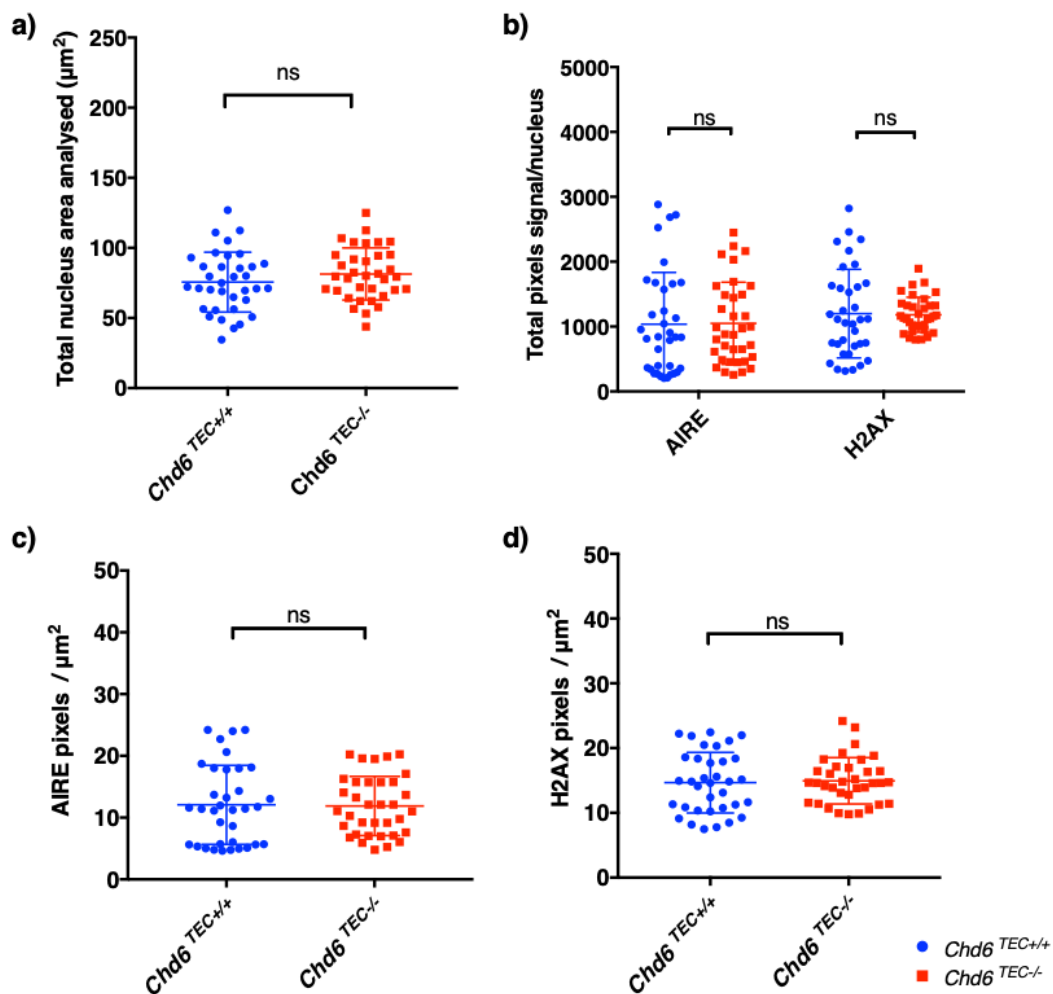


Figure 4.30 Area of individual nuclei and the number of pixels for the detection of γ H2AX and AIRE
a) Total AIRE-positive mTEC nucleus area (μm^2) analysed. **b)** Total AIRE and γ H2AX pixels expression per nucleus analysed. **c)** AIRE pixels per nucleus area. **d)** H2AX pixels per nucleus area. Total of 35 AIRE positive mTECs were analysed. Tissue sections were taken from 4.5 week old female *Chd6* ^{TEC-/-} (n=5) and wild-type (*Chd6* ^{TEC+/+}, n=5) mice. Each data point displays the pixel value from a single nucleus analysed. p-values were determined by Student's t-test, where ns=not significant.

4.6.2. Quantification of co-localisation of AIRE and γ H2AX in TEC deficient or proficient for CHD6

Signals of AIRE and γ H2AX co-localising in mTEC were next quantified in a total of 35 nuclei. A weighted co-localisation coefficient was calculated using the colocalisation algorithm in ZenBlue imaging software²⁵⁸. This analysis demonstrated that AIRE co-localised with γ H2AX at a higher frequency in cells that were CHD6-proficient when compared to cells lacking this molecule ($60.76\% \pm 18.25$ vs $32.75\% \pm$

14.70, $p < 0.001$) (Figure 4.31). This finding indicates that in the absence of CHD6 in mTEC^{hi}, AIRE localisation to DNA damage sites is compromised. The failure of AIRE to localise to DNA DSB may affect the PGE of that loci, therefore partly contributed to the lower expression of TRGs as observed in mTEC^{hi} of CHD6 mutant mice.

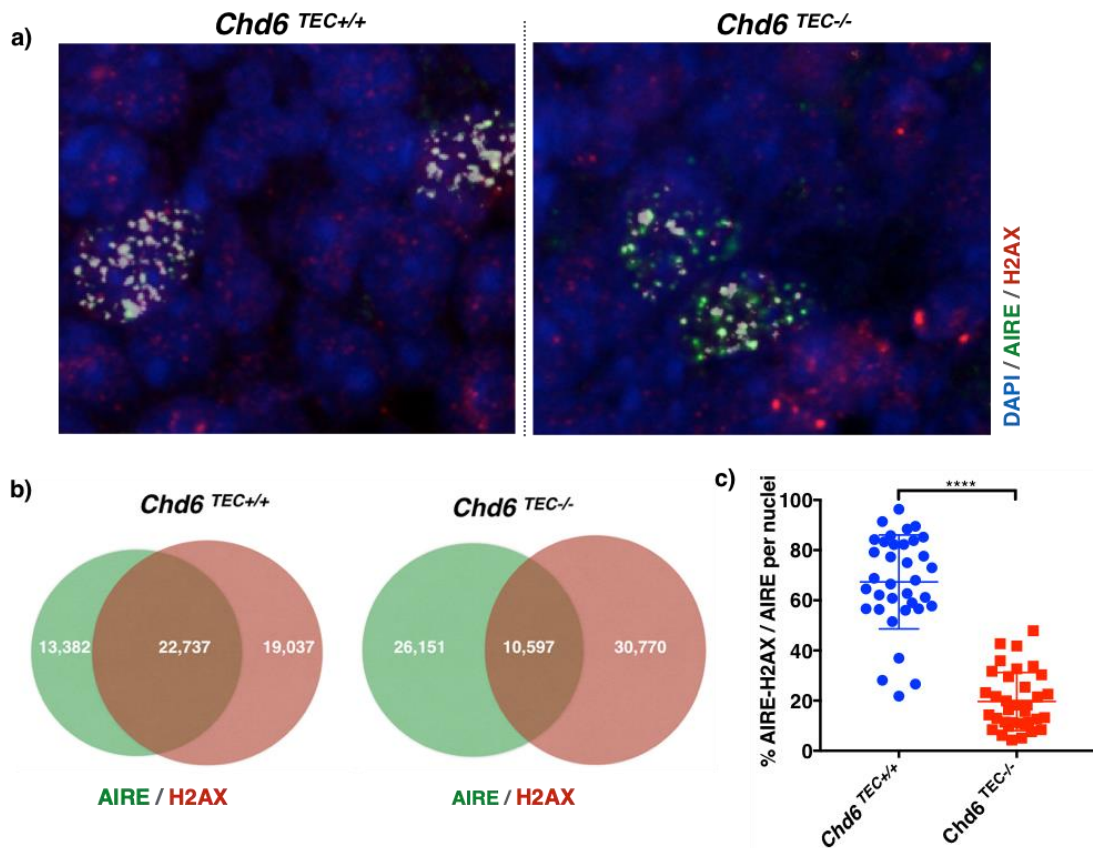


Figure 4.31 Co-localisation of γH2AX and AIRE in CHD6-proficient and -deficient mTEC

Total of 35 AIRE positive mTECs were analysed. Tissue sections were taken from 4.5 week old female *Chd6*^{TEC-/-} (n=5) and wild-type (*Chd6*^{TEC+/+}, n=5) mice. **a)** Representative confocal microscopy images displaying the detection of γH2AX (red) and AIRE (green). Overlapping signals are shown in white. **b)** Venn diagram displays the number of AIRE only (green), γH2AX only (red) and co-localised signals. The number in the overlap green-red area represents the co-localised pixels. **c)** Percentage of AIRE signals co-localising with γH2AX signals over total AIRE signals. Each data point shows the pixel value from a single nucleus analysed. p-values were determined by Student's t-test, where *** = $p < 0.001$.

4.7. Aging TEC with a deficiency in CHD6

As cumulative DNA damage increases whilst the mice aged, CHD6 which serves as a DNA repair protein may play a different role as the thymus increase in age^{196,202}. I therefore investigated whether the process of TEC senescence was normally progressing in the absence of CHD6. To this end, I comparatively analysed 8 month old wild-type and *Chd6*^{TEC-/-} mice using extensive phenotyping of the epithelial stromal compartment.

4.7.1. Loss of CHD6 expression does not affect thymic stromal architecture, TEC cellularity and TEC phenotype in the aging thymus

Histology and flow cytometry were used to detail the thymus of 8 month old female *Chd6*^{TEC-/-} and wild-type mice. Irrespective of the deletion of CHD6, both size and total cellularity were reduced to the same extent in these mice when compared to young mice (4.5 week old) of either genotype (Figure 4.32). Moreover, gross anatomy and the segregation into cortex and medulla were unaffected by the loss of CHD6 (Figure 4.33).

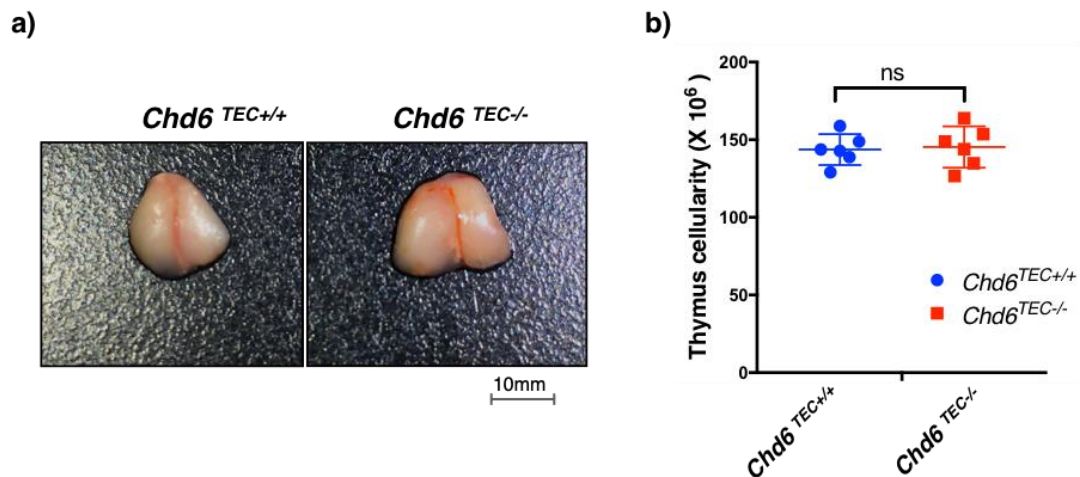


Figure 4.32 Thymus morphology and total cellularity of 8 month old female *Chd6*^{TEC-/-} mice

a) Gross anatomy of thymic tissue isolated from *Chd6*^{TEC-/-} and *Chd6*^{TEC+/+} mice. **b)** Total thymus cellularity. Each data point represents the total cellularity of an individual mouse obtained after enzymatic disruption of thymic lobes. The data shown is a combination of two independent experiments and displays the mean and standard deviations with a total of 8 mice for each genetic condition. p-values were determined by Student's t-test; ns=not significant.

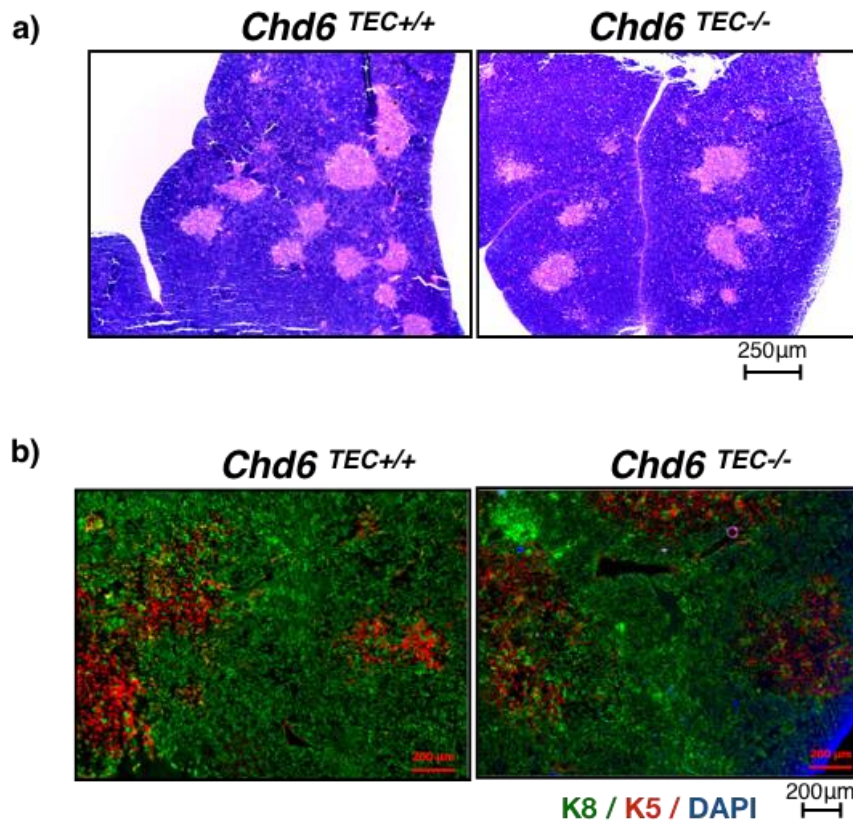


Figure 4.33 Thymic architecture in 8 month old female *Chd6*^{TEC-/-} mice

a) H&E staining of thymus sections. The data shown is representative of 4 sections per thymi from a total of 8 mice analysed per genotype. **b)** Immunofluorescent staining of thymus sections for the expression of cytokeratin-8 (K8, green) and cytokeratin-5 (K5, red). The data shown is representative of 6 sections per thymi from a total of 8 mice analysed per genotype.

The frequencies of both cTECs and mTECs at 8 months of age were comparable between *Chd6*^{TEC-/-} and wild-type mice (Figure 4.34a). The developmental progression from immature to mature mTEC phenotypes appeared unaffected by the loss of CHD6 as the acquisition of high level expression of CD80 and MHCII was identical for TEC from both strains of mice (Figure 4.34b). Finally, a lack of CHD6 expression did not interfere with the expression of AIRE (Figure 4.34c).

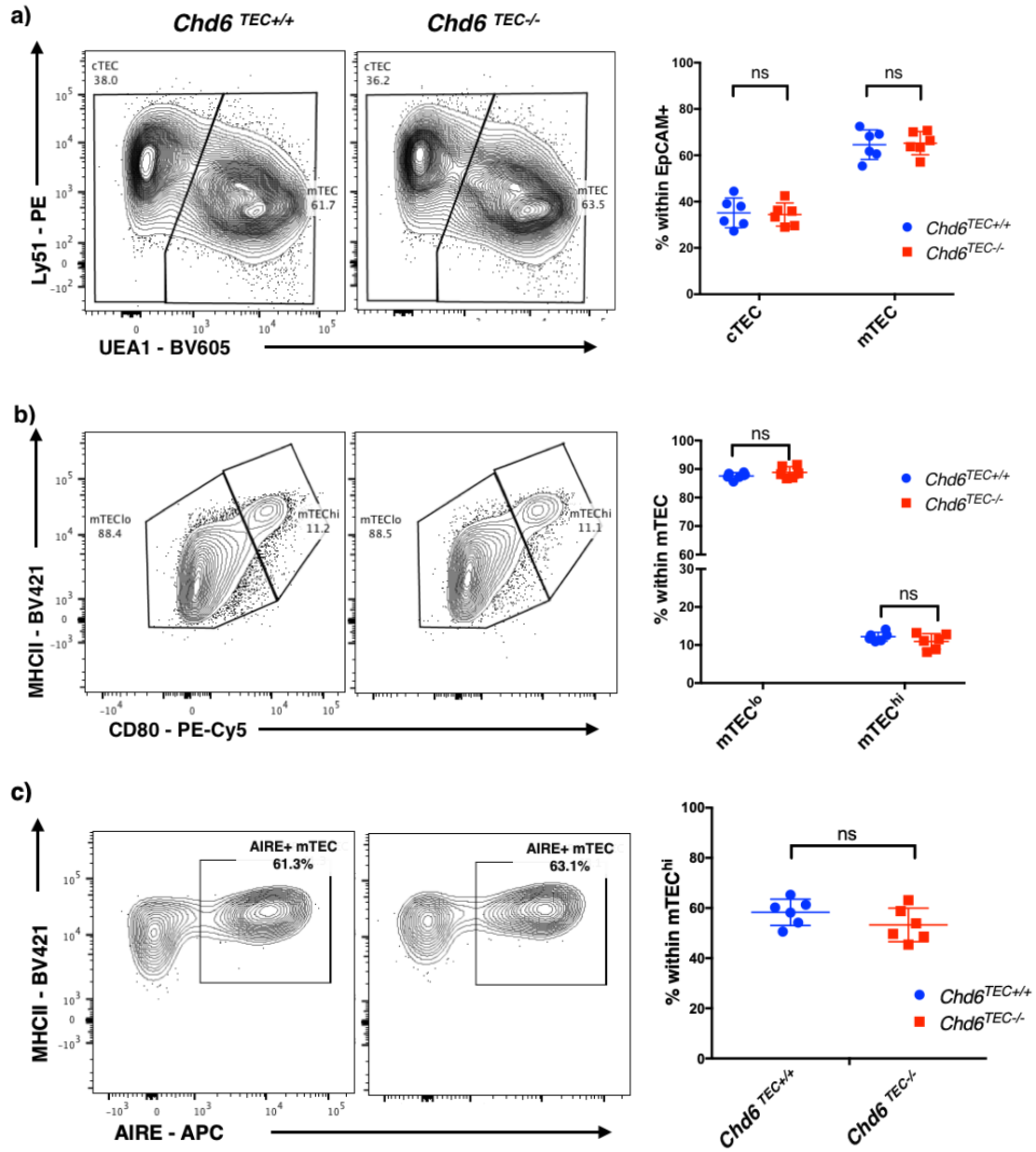


Figure 4.34 Relative frequencies of cTECs, mTEC subsets and mTEC^{hi}-expressing AIRE in 8 month old female *Chd6*^{TEC-/-} mice

Relative frequency of **a)** cTECs (Ly51⁺, UEA1⁻) and mTECs (Ly51⁻, UEA1⁺) within the epithelial (EpCAM⁺ CD45⁻) compartment, **b)** immature (mTEC^{lo}: CD80^{lo} MHCII^{lo}) and mature (mTEC^{hi}: CD80^{hi} MHCII^{hi}) mTEC and **c)** mTEC^{hi}-expressing AIRE (CD80^{hi} MHCII^{hi} AIRE⁺). Left panels: representative zebra plots; right: the dot graph displays the relative frequency of the indicated TEC phenotypes. A total of 6 mice were analysed for each genotype in two independent experiments. The gating was according to the strategy displayed in Figure 4.7. Each data point represents the value of an individual mouse. p-values were determined by Student's t-test; ns=not significant, i.e. p>0.

4.7.2. Loss of CHD6 in aging TEC does compromise PGE

The loss of CHD6 expression did not impair TEC lineage development and maturation in older mice. Given its function in PGE in younger mice, I determined whether CHD6 would play an identical role in mTEC^{hi} of aged *Chd6*^{TEC-/-} mice. I therefore compared the transcriptome of mTEC^{hi} isolated from 8 month old female *Chd6*^{TEC-/-} and wild-type mice using qRT-PCR. As anticipated, based on the data from 4.5 week old mice, the absence of CHD6 also impaired the promiscuous expression of a selected group of TRGs whose transcription is characteristically controlled by AIRE (Figure 4.35). The observed decrease in transcript abundance as a consequence of a lack of CHD6 expression was however smaller than what had been observed in young mice (Figure 4.14). This difference observed between the young and aged *Chd6*^{TEC-/-} mice was not unexpected. This was partly due to the reduced expression of TRGs in the aged mice, which can also be seen in the older wild-type mice (Figure 4.36). Independent of this variance in the abundance of promiscuously expressed genes, the importance of CHD6 expression in the process of AIRE-dependent PGE was confirmed in older mice.

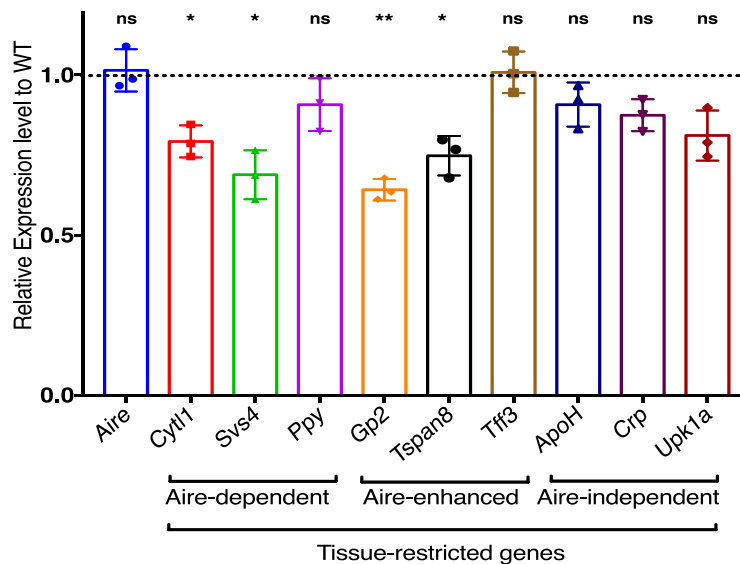


Figure 4.35 Expression of *Aire* and the promiscuous transcription of a selection of TRGs in mTEC^{hi} of 8 month old female *Chd6*^{TEC-/-} mice

Expression of the indicated genes in FAC-sorted mTEC^{hi} (EpCAM⁺, Ly51⁻, UEA1⁺, CD80⁺, MHCII^{hi}) was measured by qRT-PCR comparing cells from *Chd6*^{TEC-/-} (n=5) and wild-type control mice (*Chd6*^{TEC+/+}, n=5). Values were normalised to *Gapdh* expression and those in wild-type control were arbitrarily set to a value of 1 (shown as a dotted line). Each individual value represents the mean and standard deviation from five biological replicates. p-values were determined by Student's t-test; ns=not significant, * = p<0.05, ** = p<0.005, *** = p<0.0001.

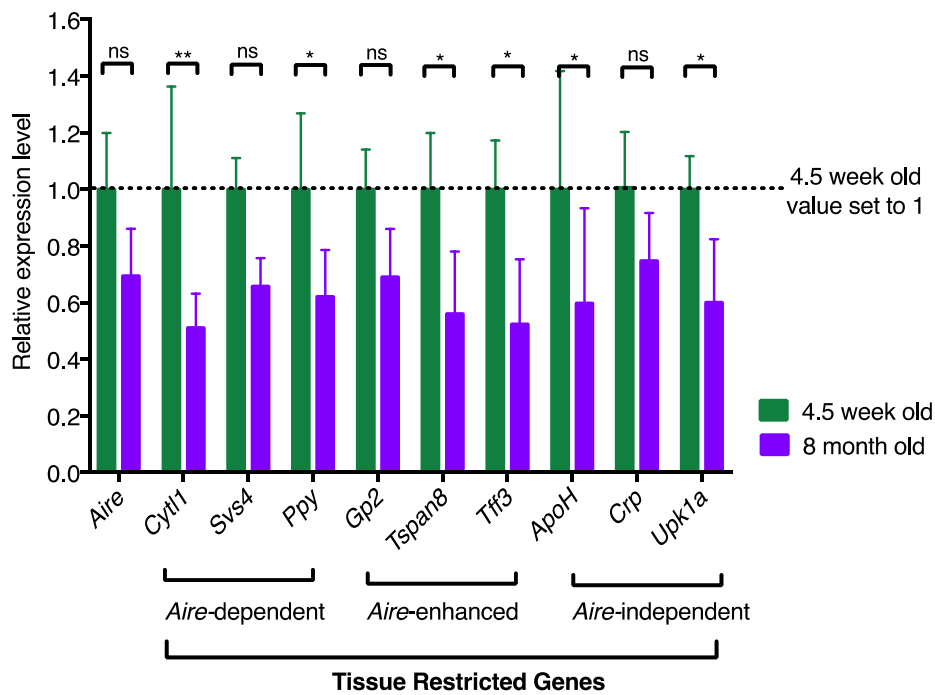


Figure 4.36 Expression of *Aire* and the promiscuous transcription of a selection of TRGs in mTEC^{hi} of 8 month old wild-type mice in comparison to the 4.5 week old wild-type mice

Expression of the indicated genes in FAC-sorted mTEC^{hi} (EpCAM⁺, Ly51⁻, UEA1⁺, CD80⁺, MHCII^{hi}) was measured by qRT-PCR comparing cells from 8 month old (n=4) and 4.5 week old (n=5) female wild-type B6 mice. Values were normalised to *Gapdh* expression and those in 4.5 week old mice were arbitrarily set to a value of 1 (shown as a dotted line). Each individual value represents the mean and standard deviation from five biological replicates. p-values were determined by Student's test; ns=not significant, * = p<0.05, ** = p<0.005.

4.8. Summary and Discussion

The mouse model with a TEC-specific loss of CHD6 (*Chd6*^{TEC^{-/-}}) was generated via genetic recombination mediated by Cre expressed under the transcriptional control of *Foxn1*. Since the expression of *Foxn1* in mice is detected: i) as early as embryonic day 10.5 in thymus rudimentary structure; and ii) specific to the thymic epithelial cells (other than the skin), *Chd6*^{TEC^{-/-}} provides the perfect mouse model to study the impact of the loss of CHD6 on thymus biology.

Histology and detailed immunophenotyping by flow cytometric analyses were employed to assess the impact of CHD6 deletion in TEC development. Morphology, histology, and total thymus cellularity were unchanged in *Chd6*^{TEC^{-/-}} mice (Figure 4.2 and Figure 4.5). No statistical differences were observed between *Chd6*^{TEC^{-/-}} mice and the wild-type control in cTECs, mTECs, and different mTEC maturation stages (Figure 4.6,

Figure 4.8, Figure 4.9). In addition, the frequency of thymic tuft cells was unaffected upon deletion of CHD6 in TEC (Figure 4.12). Parallel to the lower expression of CHD6 in both cTEC and mTEC^{lo} subsets (Figure 3.4), the lack of changes observed in the phenotype and transcriptomic profile of cTEC and mTEC^{lo} upon CHD6 deletion, further supporting the lack of CHD6's function in these cells. In summary, the findings suggest that CHD6 does not play a role in TEC development and differentiations. This was further supported by the population-based gene profiling of different TEC subpopulations (cTEC, mTEC^{lo} and mTEC^{hi}) in *Chd6*^{TEC-/-} mice, as none of the DEG showed any enrichment involving developmental pathway of TEC. In contrast to these findings, unpublished data from our group shows a reduction of thymic cellularity and drastic disruption of corticomedullary organization and TEC differentiation when CHD4 function is inactivated in TEC (F.Dhalla, unpublished). This further implies the specific function of different CHD proteins in thymus development.

In addition, AIRE protein expression levels within the compartment of mature mTECs cells remained unaffected by the deletion of CHD6 (Figure 4.10). I also observed that nuclear localisation of AIRE was intact in *Chd6*^{TEC-/-} mice thymus, suggesting that CHD6 did not regulate the intracellular expression of AIRE and its nuclear localisation (Figure 2.4). In contrast, the expression of TSPAN8 and GP2 were observed to be significantly reduced in the mTEC of *Chd6*^{TEC-/-} mice (Figure 4.13). As both TSPAN8 and GP2 are TRAs that are regulated by AIRE as part of PGE, this suggests the functional cooperation between CHD6 and AIRE in PGE. In agreement to the above finding, genome-wide transcriptomic analyses revealed a strong enrichment of *Aire*-regulated genes among the down-regulated genes in mTEC^{hi} deficient in CHD6 (Figure 4.25). This highlights the importance of CHD6 for PGE, thus supporting my initial hypothesis.

Despite the definite role of CHD6 in facilitating PGE of *Aire*-regulated TRGs, the extent of CHD6 involvement seems to be limited to a small number of genes (affecting around 10% of *Aire*-regulated TRGs, Figure 4.20). There are several reasons that could explain this observation. One of the major technical limitations was contributed by the characteristic of genes affected in their transcription in the absence of CHD6 in TEC. As CHD6 almost exclusively impacts transcription of TRGs, assessment of TRGs expression by population-based transcriptomic analysis need to consider two important

points. The first point is that a single TRG is expressed at lower frequency within the mature mTEC subset accounting generally 3-5% of the total mTEC compartment. Secondly, TRGs are expressed at relatively lower levels compared to other housekeeping or tissue-specific genes. Thus, to comprehensively assess TRGs transcription at population level, a large amount of cell is required and a deeper transcriptome phenotyping need to be undertaken. Due to the limited number of mature mTECs that can be sorted from a single mouse, this drawback needs to be considered during analysis of the data. Indeed, when a more liberal cut-off was employed in the differential expression analysis ($\log_2$ fold change of >0.5 instead of >1.5), more DEGs were observed to be down-regulated in the mTEC^{hi} of *Chd6*^{TEC^{-/-}} mice. Importantly, I persistently observed a strong enrichment of TRGs specifically of *Aire*-regulated genes within the down-regulated DEG in mTEC^{hi} of *Chd6*^{TEC^{-/-}} at this lower $\log_2$ fold change cut-off value ($\log_2$ fold change of >0.5). Hence, if a more sensitive and specific technique is employed (i.e. single cell transcriptomic analysis of mTEC^{hi}), it is possible to determine the extent of CHD6 functions in regulating PGE in TEC.

The biological reasons for the limited effect observed towards *Aire*-regulated PGE in *Chd6*^{TEC^{-/-}} were also investigated. However, I was unable to identify any enrichment of DEG in *Chd6*^{TEC^{-/-}} mice: i) within a particular pathway by gene ontology analysis; ii) as being part of a specific tissue-pathology; or iii) within a particular chromosomal location. This indicates that the role of CHD6 in TEC is not be related to any specific pathway or tissue-pathology, and not confined to any particular chromosome.

Several epigenetic mechanisms including specific histone modifications have been previously linked to the phenomenon of PGE. My results showed that TSS of the genes down-regulated in the mTEC^{hi} of *Chd6*^{TEC^{-/-}} mice were enriched for H3K27me3 and conversely depleted of H3K4me3 and H3K9ac (Figure 4.27). These observations are consistent with the previously described epigenetic landscape of genes regulated by AIRE in mTEC^{hi}. Additionally, the genes differentially expressed in mTEC^{lo} of *Chd6*^{TEC^{-/-}} mice were associated with the opposite histone PTMs pattern, being enriched with H3K4me3 and H3K9ac, whilst depleted of H3K27me3 (Figure 4.28). Therefore, genes-positively controlled by CHD6 in mTEC^{lo} consist of physiologically permissive genes, whilst in mTEC^{hi}, the genes-positively controlled by CHD6 are mainly repressive genes. Histone PTMs architecture in mTEC was previously shown to be largely similar

in mTEC^{lo} and mTEC^{hi} ²³⁰. Additionally, previous work has documented that CHD6 do not have the ability to further modify the different histone marks²⁰². Therefore, this contrasting behaviour of the associated histone PTMs with the CHD6-controlled genes between mTEC^{hi} and mTEC^{lo}, implied that another key regulator protein, which is specific to mTEC^{hi}, is required to co-regulate the transcription of these physiologically repressive loci. In this context, AIRE is the perfect candidate which fits the description of the implied regulator protein. Hence, it is conceivable that the positive effect on the transcription of DEGs in mTEC^{hi} can be attributed to the cooperation between CHD6 and AIRE, as opposed to the autonomous role of CHD6.

Assessment of DNA DSB in *Chd6*^{TEC-/-} mice demonstrated that CHD6 was partly responsible in the localisation of AIRE protein complex to DNA DSB (Figure 4.31). As previously suggested, AIRE requires the DNA breaks to allow transcription of the regulated genes to occur. PGE regulated by AIRE had been shown to be initiated upon DNA breaks, one of the mechanisms suggested is through the enzymatic action of TOP1 and TOP2 ^{161,162}. The resulting DNA breaks subsequently employed a number of DNA damage response factors which include DNA-PK PARP1 and Ku80, all are known as AIRE protein partners ^{140,162,229}. CHD6 had been shown to be promptly recruited to the DNA damage sites upon PARP1 activities ^{202,205}. As shown by my data in this chapter, the recruitment of AIRE to the DNA damage sites is in part contributed by the action of CHD6 at DNA DSB region. The presence of AIRE at these DNA DSBs had been suggested to stabilise the DSBs, thus permitting AIRE to hijack the DNA-damage response mechanism, this allows for transcription of the AIRE-regulated loci to occur ^{161,162}. AIRE in this context, utilises its protein partners which are involved in gene transcription activities and splicing machineries namely in: (i) transcription initiation such as CREP-binding protein and RNAPII, (ii) transcription elongation such as p-TEFb; and (iii) pre-mRNA processing such as SFRS2 ^{140,150}. Hence, failure to localise AIRE protein complex to the DNA DSB compromises transcription of the genes, which fittingly explained the reduced expression of *Aire*-regulated transcript in mature mTECs of *Chd6*^{TEC-/-} mice.

From the results presented in this chapter, it is evident that CHD6 is involved specifically in PGE, and acts as a positive facilitator for the expression of *Aire*-regulated genes. The proposed mechanisms by which CHD6 acts on PGE are through:

1. CHD6 ability to read and bind to loci marked with repressive histone mark namely H3K27me3 and depleted of permissive histone marks H3K4me3 and H3K9ac
2. Localising AIRE-protein complex to loci with DNA DSB break
3. Increasing chromatin accessibility of loci regulated by AIRE, thus allowing gene transcription

The first two proposed mechanisms are supported by my findings as presented in this chapter, while the third proposed mechanism would be carried out as a future work (see section 7.2.2). In the following chapter, the impact of CHD6 deletion in TEC towards T cell development in the thymus and its composition and function in the periphery will be explored.

Chapter 5 The role of CHD6 in thymocyte development and maturation

5.1. Aim

Thymic epithelial cells are responsible in supporting the survival, differentiation, proliferation and selection of developing thymocytes (as reviewed in section 1.2.1). How this essential function is maintained in the absence of CHD6 expression in TEC remains unknown, thus will be the focus of this chapter. Here, I will provide a description of mouse strain whose TEC have been rendered CHD6 deficient (referred to as *Chd6*^{TEC-/-}), in regard to thymocyte development and the establishment of peripheral T cell compartment and functions. The deletion of CHD6 targeted to TEC neither disrupts the normal thymic epithelial scaffold nor the differentiation of the distinct TEC lineages (Chapter 4). However, PGE was impaired in *Chd6*^{TEC-/-} and how this specifically affects negative selection of thymocytes will be explored. Consequently, the T cell antigen receptor repertoire of peripheral T cells is seemingly altered, giving rise to autoimmunity.

5.2. Thymocyte maturation and function in 4.5 week old *Chd6*^{TEC-/-} mice

5.2.1. Total thymocyte counts are unchanged in *Chd6*^{TEC-/-} mice

The total thymocyte counts were comparable for *Chd6*^{TEC-/-} mice and their age- and gender-matched controls (Figure 5.1). Hence, the absence of CHD6 did not influence the gross anatomical appearance and size of the mouse thymus.

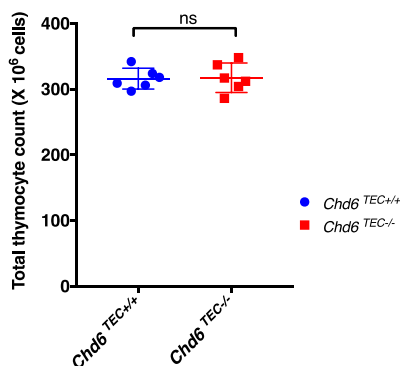


Figure 5.1 Total thymocyte count of 4.5 week old female *Chd6*^{TEC-/-} mice

Each data point represents the total thymus cellularity of an individual mouse after mechanical disruption of both thymic lobes. The data shown is a combination of two independent experiments and displays the mean and standard deviations of a total of 6 mice for each genetic condition. p-values were determined by Student's t-test; ns=not significant.

5.2.2. Early thymocytes development and positive selection are unchanged in *Chd6^{TEC-/-}* mice

Early thymocyte development is marked by a phenotype that lacks the expression of the TCR co-receptors CD4 and CD8 (hence referred to as double negative, DN). The development of DN thymocytes is phenotypically characterised by four sequential stages which are defined by the cell surface expressions of CD44 and CD25: CD44^{hi} CD25⁻ (DN1) have not yet exclusively committed to a T cell lineage and give rise to a transient population of CD44^{hi} CD25⁺ thymocytes (DN2) which progress to CD44^{lo} CD25⁺ cells (DN3) that rearrange their TCR β chain and progress to CD44^{lo} CD25⁻ (DN4) thymocytes under the condition that this rearrangement has been productive and a functional pre-TCR was generated ²⁵⁹. The gating strategy to identify these four DN subpopulations is displayed in Figure 5.2a. The absence of CHD6 expression in TEC did not disrupt the differentiation of DN1 to DN4 stage (Figure 5.2b) as the absolute and relative cellularity of these individual subpopulations of early thymocytes were identical for both mutant and control mice.

Thymocytes that have successfully passed β -selection and transiently attained the DN4 phenotype will acquire the expression of CD4 and CD8 (hence adopting a double positive (DP) phenotype). The DP thymocytes also express a complete $\alpha\beta$ TCR on their surface which will need to be probed for its individual usefulness employing a series of selection stages. The final stages of intrathymic maturation are marked by the appearance of cells that are single positive for either CD4 (CD4 SP) or CD8 (CD8 SP), which are, undergoing further post-selectional maturation before exiting to the periphery. The gating strategy to identify the different thymocyte subpopulations is displayed in Figure 5.2a. The comparative analysis of mice with CHD6-proficient and –deficient TEC did not reveal any significant difference. This implied that early intrathymic maturation, at least as judged by the serial progression from DN to DP and SP thymocytes, does not require the expression of CHD6 in thymic epithelia (Figure 5.2c). This conclusion is compatible with an overall unchanged total cellularity in *Chd6^{TEC-/-}* mice when compared to wild-type mice.

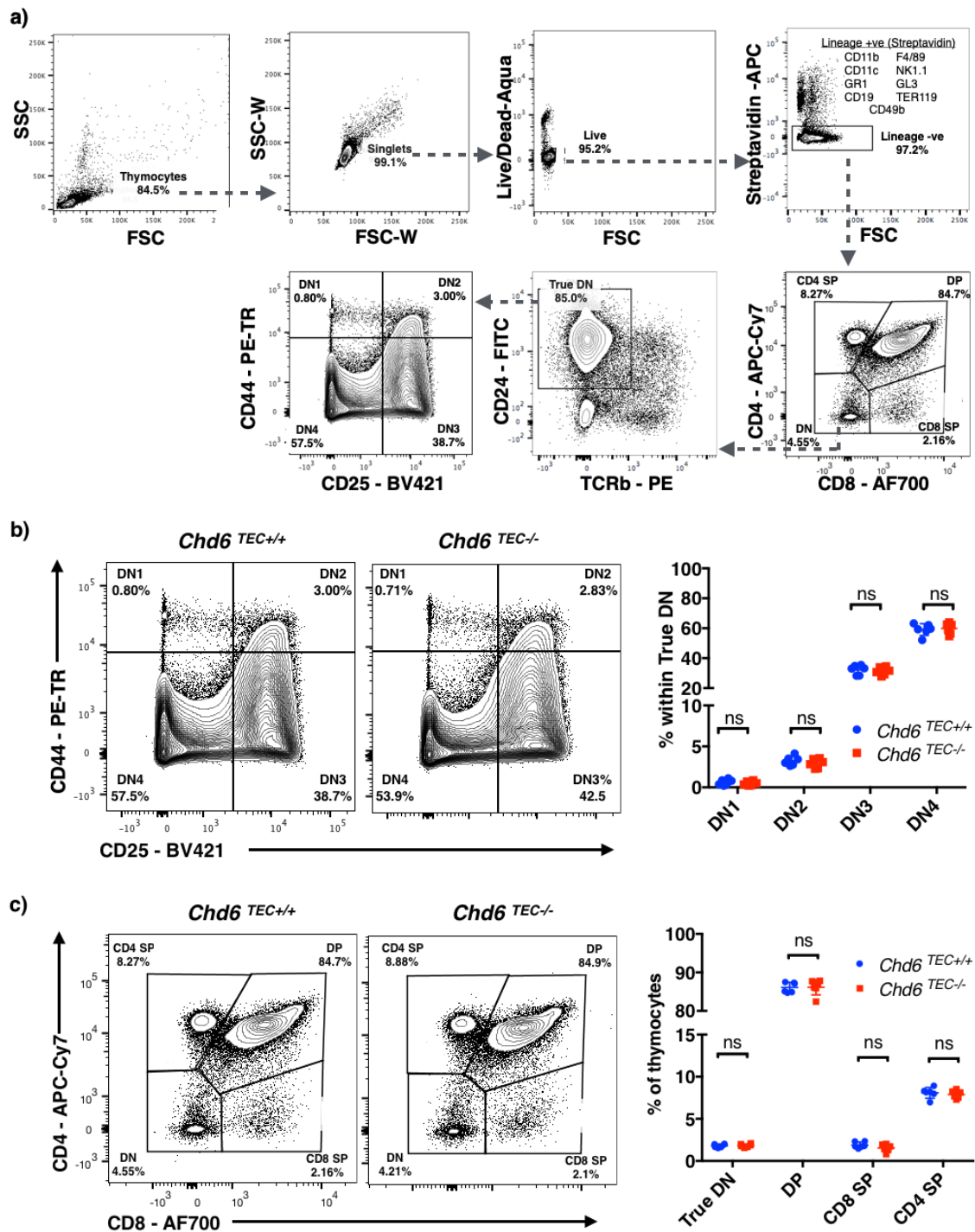


Figure 5.2 Early thymocyte maturation in 4.5 week old female *Chd6*^{TEC-/-} mice

a) Representative FACS plots showing the gating strategy for defining DN, DP, CD4 SP, CD8 SP thymocytes. The cells were initially pre-gated (lineage -ve) to exclude myeloid, NK, B cells and $\gamma\delta$ T cells. DN thymocytes were further defined into four different maturational stages (DN1 to DN4). **b)** Relative frequency of DN stages. Left: representative zebra plots; right: relative frequency of indicated DN subpopulations. **c)** Relative frequency of different thymocyte subpopulations. Left: representative zebra plots; right: relative frequency of the indicated thymocyte subpopulations. A total of 6 mice were analysed for each genotype in two independent experiments. Each data point represents an individual mouse. The data displays the mean values and standard deviations. p-values were determined by Student's t-test; ns=not significant.

The successful acquisition of a DP phenotype is, as already alluded to, dependent on a productive rearrangement of the TCR β chain. Once the DP cell stage is reached, a rearrangement of the TCR α chain allows the formation of the complete hetero-dimeric TCR associated with the CD3 complex, a functional receptor unit that can interact with peptide-MHC complexes (pMHC). Only TCRs that interact with a sufficiently high affinity with “their” cognate self-pMHC complex presented on cTEC, are able to transmit the necessary signal strength to rescue DP thymocytes from a pre-programmed cell death, a process known as thymocyte positive selection²⁶⁰. In contrast, the inability to engage with the self-pMHC complex or to bind with an affinity above a particular threshold, will not generate the required survival signal. Consequently, thymocytes that bear a TCR with such a feature will undergo apoptosis after 3-4 days, a phenomenon known as “death by neglect”²⁶⁰.

Thymocytes having received signals via their TCR during positive selection are identified by the concomitant expression of the activation marker CD69 and a high cell surface expression of the TCR²⁶¹. This process is classified into several sequential stages (gating strategy as in Figure 5.3a), starting with the pre-selection stage namely Stage 1(S-1: TCR β - CD69-) followed by several selection stages: stage 2 (S-2) characterized by a TCR β^{int} CD69- phenotype, whereas stage 3 (S-3) is marked by a TCR $\beta^{\text{int/hi}}$ CD69+ phenotype. This latter progresses to post-selection stage, stage 4 (S-4) that is typified by a TCR β^{hi} CD69+ phenotype. After that stage, positively selected thymocytes will eventually down-regulate the expressions of CD69 into the mature stage, stage 5 (S-5: TCR β^{hi} CD69-). Positive selection based on these different stages occurred normally in *Chd6*^{TEC-/-} mice when compared to control mice, thus indicating that CHD6 expression was not essential to endow cTEC with this unique capacity (Figure 5.3b).

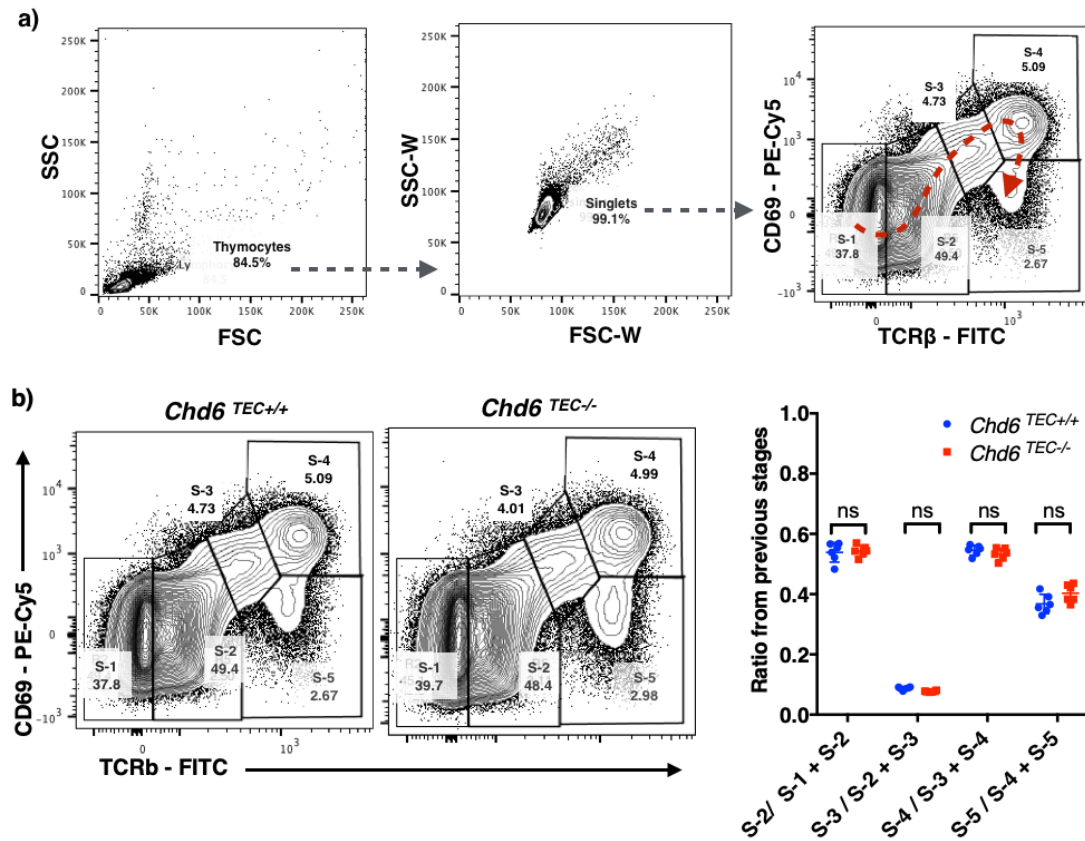


Figure 5.3 Thymocyte positive selection in 4.5 week old female *Chd6*^{TEC-/-} mice

a) Representative FACS plots showing the gating strategy to define the indicated thymocyte subpopulations representing individual stages, before, during and after T cell receptor signaling in the context of thymocyte positive selection. The red dotted line represents the direction of the developmental trajectory from stage 1 (S-1:TCRβ⁻ CD69⁻) → stage 2 (S-2: TCRβ^{int} CD69⁻) → stage 3 (S-3: TCRβ^{int} CD69⁺) → stage 4 (S-4: TCRβ^{hi} CD69⁺) → stage 5 (S-5:TCRβ^{hi} CD69⁻).

b) TCRβ and CD69 expression by thymocytes of isolated from *Chd6*^{TEC+/+} and *Chd6*^{TEC-/-} mice. Left panels: Representative zebra plots; Right panel: developmental stage progression during TCR-mediated signalling. The graph displays the calculated ratio of the distinct stages during the developmental maturation whereby the relative frequency of cells at a given stage is related to the sum of frequencies of these cells and their immediately preceding cell population (i.e. B/A+B). Each data point shown represents a ratio as observed for the indicated developmental stage detected in an individual mouse. The graph shows the composite of data from two independent experiments with 3 mice each per group and displays the mean values and standard deviations. p-values were determined by Student's t-test; ns=not significant.

5.2.3. Medullary thymocyte negative selection is impaired in *Chd6*^{TEC-/-} mice

Upon positive selection, DP thymocytes down-regulate the expression of either CD4 or CD8 according to the TCR specificity for MHC class II and I, respectively. Positively selected DP and lineage-restricted CD4 and CD8 are subsequently subject to further selection and post-selectional maturation in order to acquire a fully mature phenotype and the ability to egress to the periphery. After positive selection, the emerging TCR repertoire is further shaped by sequential selection events, known as negative selection which intended to physically remove cells whose antigen receptor displays a high affinity for self-antigens presented by MHC^{100,260}. Negative selection is a multi-step, dynamic process first initiated in the cortex where first DP and then lineage-committed cortical SP thymocytes are probed for their TCR specificity. Cells that survived the cortical selection are again probed for their antigen specificity but this second wave of negative selection now occurs in the medulla. I next tested whether this essential step in thymocyte maturation requires the expression of CHD6 by TEC. The individual, sequential stages of negative selection are identified by a characteristic combination of cell surface markers: Cortical DP thymocytes undergoing negative selection are identified as CD4+CD8+CCR7-PD1+Helios+ cells (wave 1a) whereas CD4 SP thymocytes subjected to negative selection will bear a phenotype of CD69+CD24+CCR7- PD1+Helios+ (wave 1b). Thymocytes that access the medulla express the chemokine receptor CCR7 and are again scrutinized for the specificity of their TCR. Separate phenotypes identify thymocytes negatively selected in the medulla: the wave 2a stage is characterized by the CD4+CD69+CD24+CCR7+ Helios+ phenotype whereas cells subject to wave 2b are marked by a CD69-CD24- Helios+ phenotype (It is of note that the phenotypic analysis of single positive thymocytes subject to negative selection has only been verified for the CD4 lineage²⁶², it is for this reason that only SP4 cells are analyzed here). A schematic representation of negative selection and its phenotypes are depicted in Figure 5.4.

A lack of CHD6 expression in TEC did not alter the frequencies and absolute cellularity of thymocytes with a single CD4 or CD8 positive phenotype (Figure 5.2c). This finding was in agreement with the result that cortical thymocyte selection (i.e. wave 1) was unaffected by the loss of CHD6 expression in cTEC (Figure 5.5a). In contrast, negative

selection in the medulla (wave 2) was compromised by the absence of CHD6 expression in mTEC in that marginally but statistically significantly fewer CD4SP expressed the phenotypic markers of cells subjected to negative selection (Figure 5.5b). Hence the absence of CHD6 expression in thymic epithelial cells had a differential impact on negative selection; cTEC maintained their ability to enforce negative selection normally as measured by the frequency of thymocytes being marked for negative selection. Whereas, fewer SPCD4 thymocytes present in the medulla bore markers for negative selection suggesting that late negative selection (wave 2) was affected in the absence of CHD6 expression by mTEC.

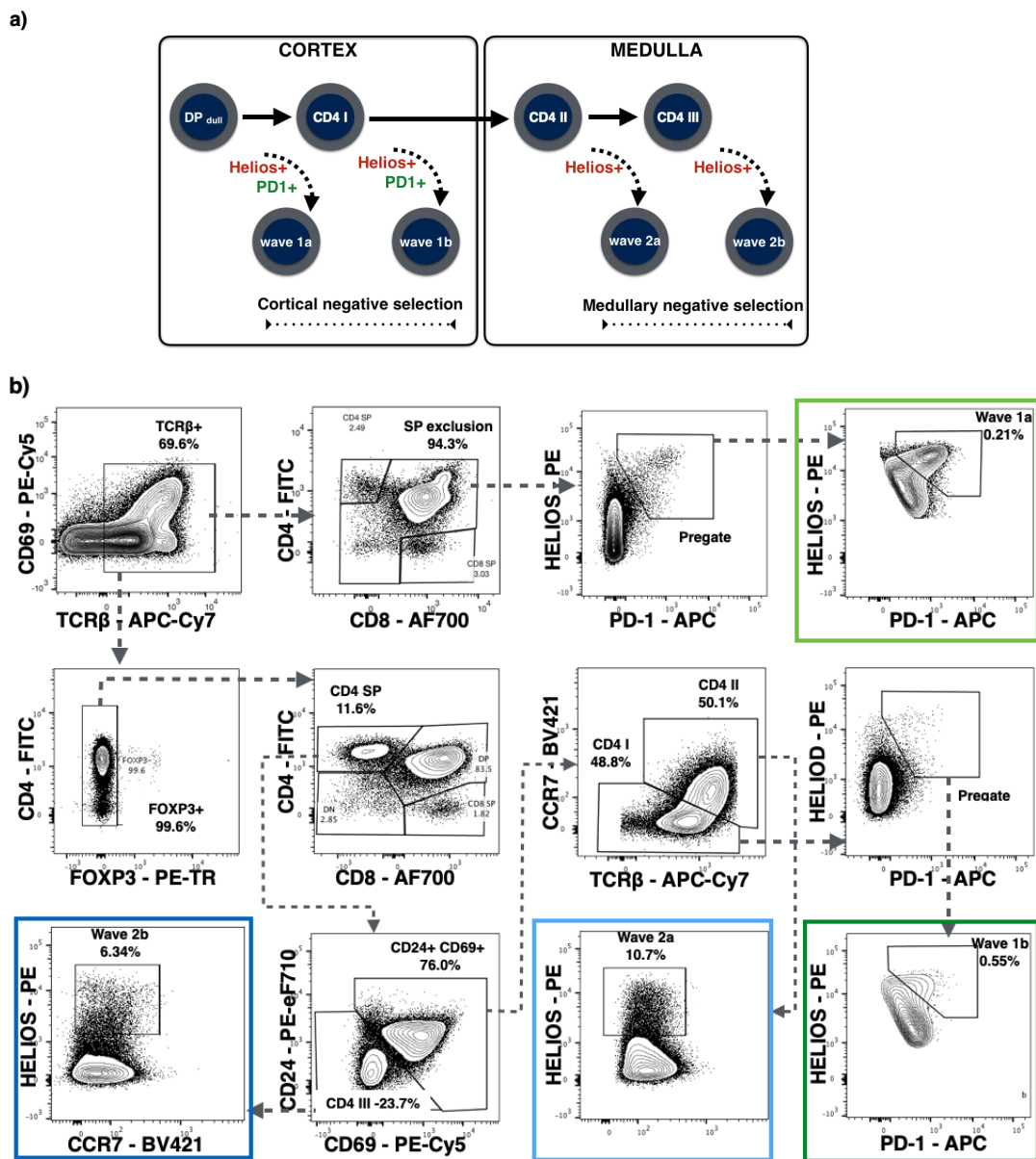
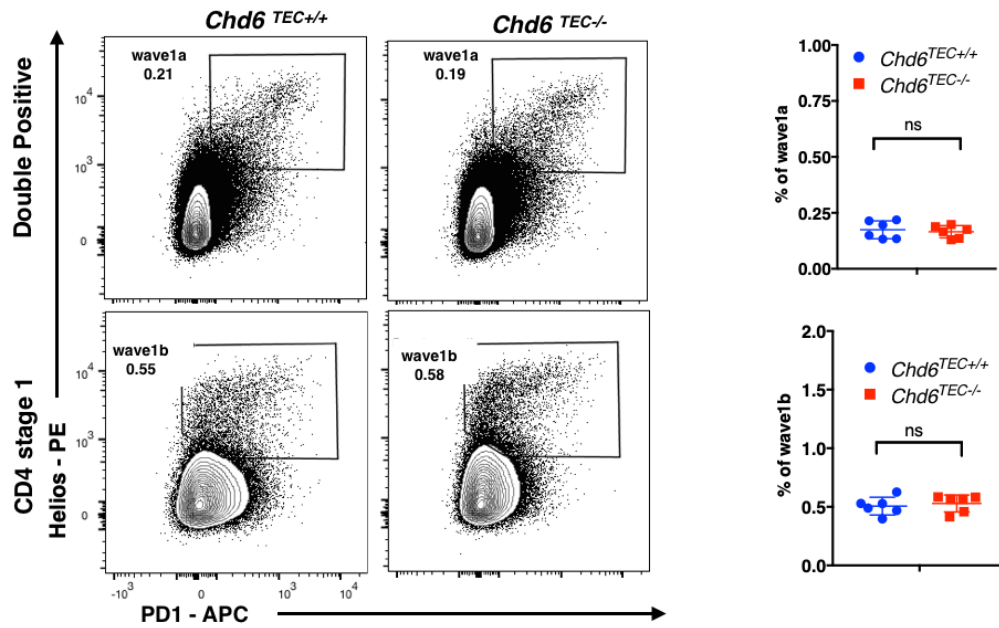


Figure 5.4 Analysis of thymocyte negative selection

a) Stages of thymocyte negative selection in the cortex (designated wave 1) and medulla (wave 2) affecting distinct developmental stages. b) Gating strategy to identify the frequency of thymocytes subjected to distinct events in thymocyte negative selection.

a) Cortical thymocyte negative selection



b) Medullary thymocyte negative selection

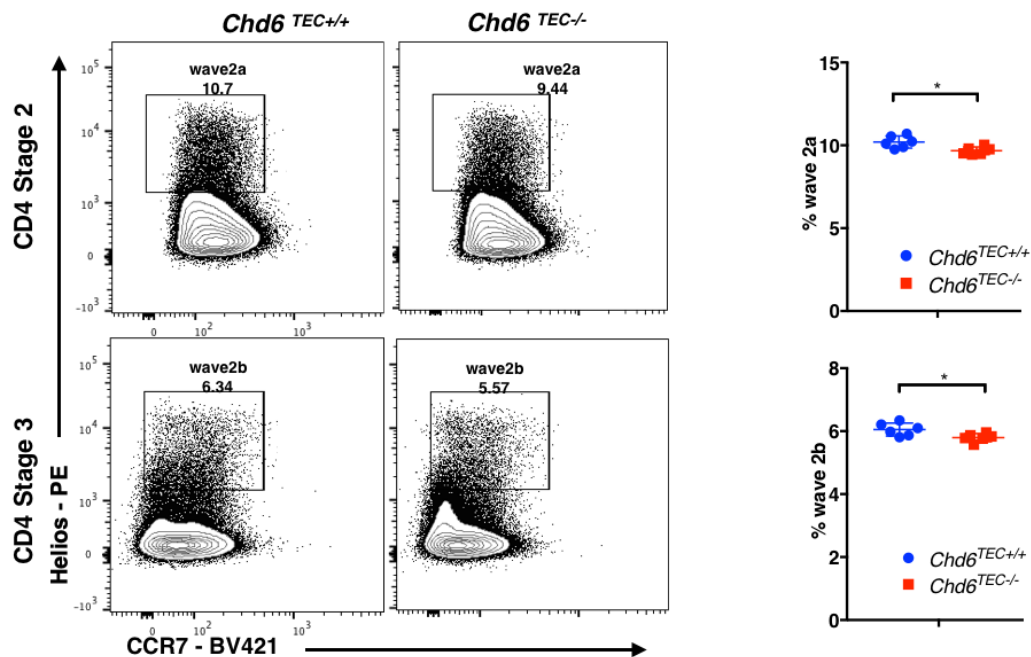


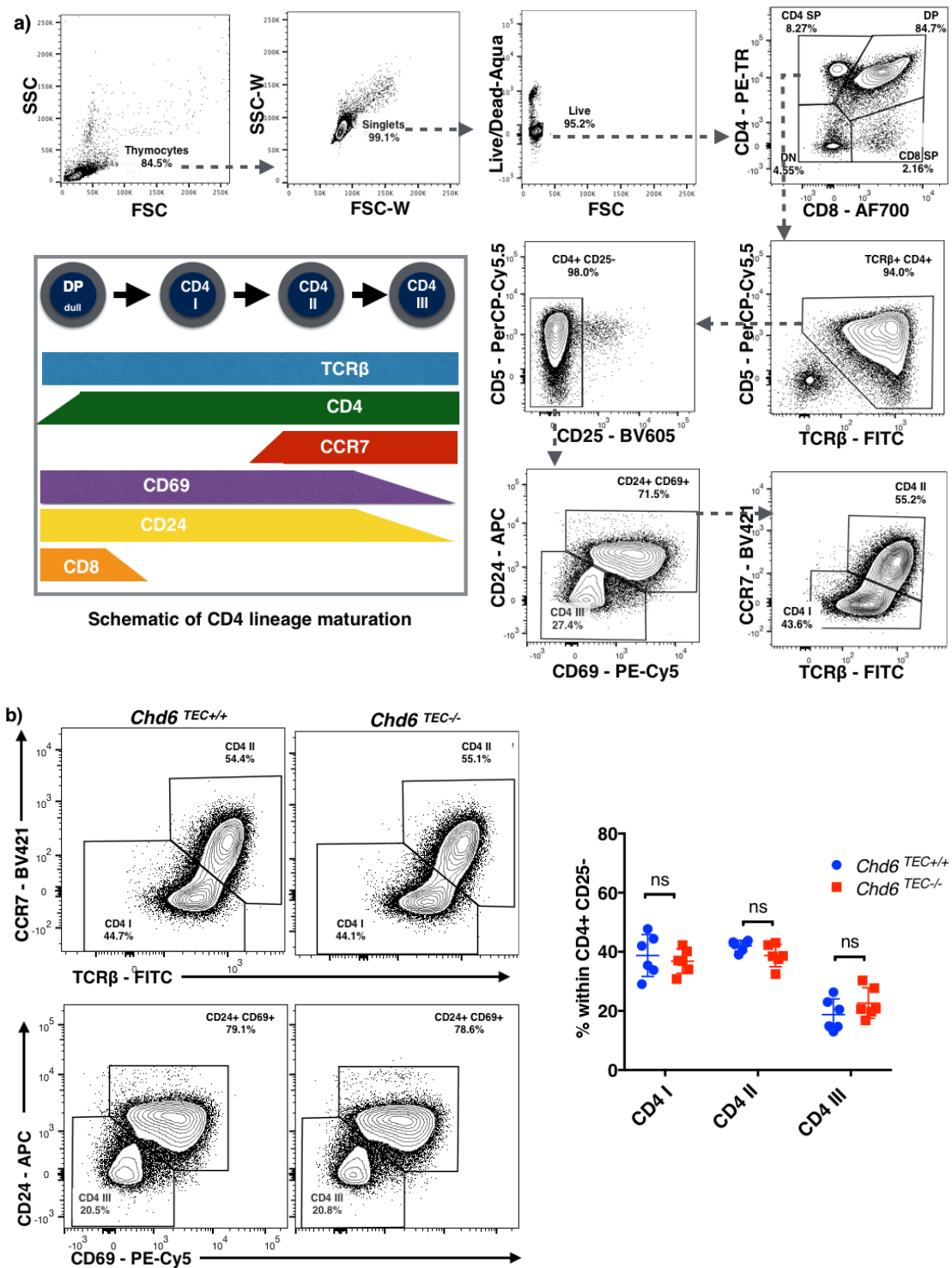
Figure 5.5 Wave 1 and 2 thymocyte negative selections in 4.5 week old female *Chd6*^{TEC-/-} mice

a) Relative frequency of thymocyte of indicated phenotypes that are subject to wave-1 negative selection. Left panels: Representative zebra plots; Right panels: The dot graph displays the relative frequency of thymocytes at the indicated developmental stages. Each data point shown represents data from an individual mouse. **b)** Relative frequency of thymocyte of indicated phenotypes that are subject to wave-2 negative selection. Left panels: Representative zebra plots; Right panels: The dot graph displays the relative frequency of thymocytes at the indicated developmental stages. Each data point shown represents data from an individual mouse. p-values were determined by Student's t-test; ns = not significant, i.e. $p > 0.05$, * = $p < 0.05$.

5.2.4. Lineage maturation of single CD4 and CD8 thymocytes is unchanged in *Chd6^{TEC-/-}* mice

CD4⁺ and CD8⁺ thymocytes lineage maturation can be phenotypically characterised into distinct stages using the detection of several cell surface markers including CD24, CD69 and CCR7²⁶¹, as schematically represented in Figure 5.6a and Figure 5.7a. CD69 and CD24 remain up-regulated after positive selection until the final maturation stage whereas CCR7 expression enables cortical thymocytes to migrate to the medulla for their further selection and maturation but is eventually down-regulated in the medulla. Hence the differential expression of these markers on TCR⁺ cells committed to the CD4 and CD8 lineages allows the identification of discrete developmental stages: stage I: CD24⁺CD69⁺ CCR7⁻; stage II: CD24⁺CD69⁺ CCR7⁺; stage III: CD24⁻CD69⁻ CCR7⁻ for either lineage.

The absence of CHD6 in TEC did not impair the maturation of the thymocytes in either the CD4 or the CD8 lineage as assessed by the developmental stages defined by the differential expression of CD24, CD69 and CCR7 (Figure 5.6b and Figure 5.7b). These results support the notion that the absence of CHD6 does not impair the maturation of thymocytes following positive selection.



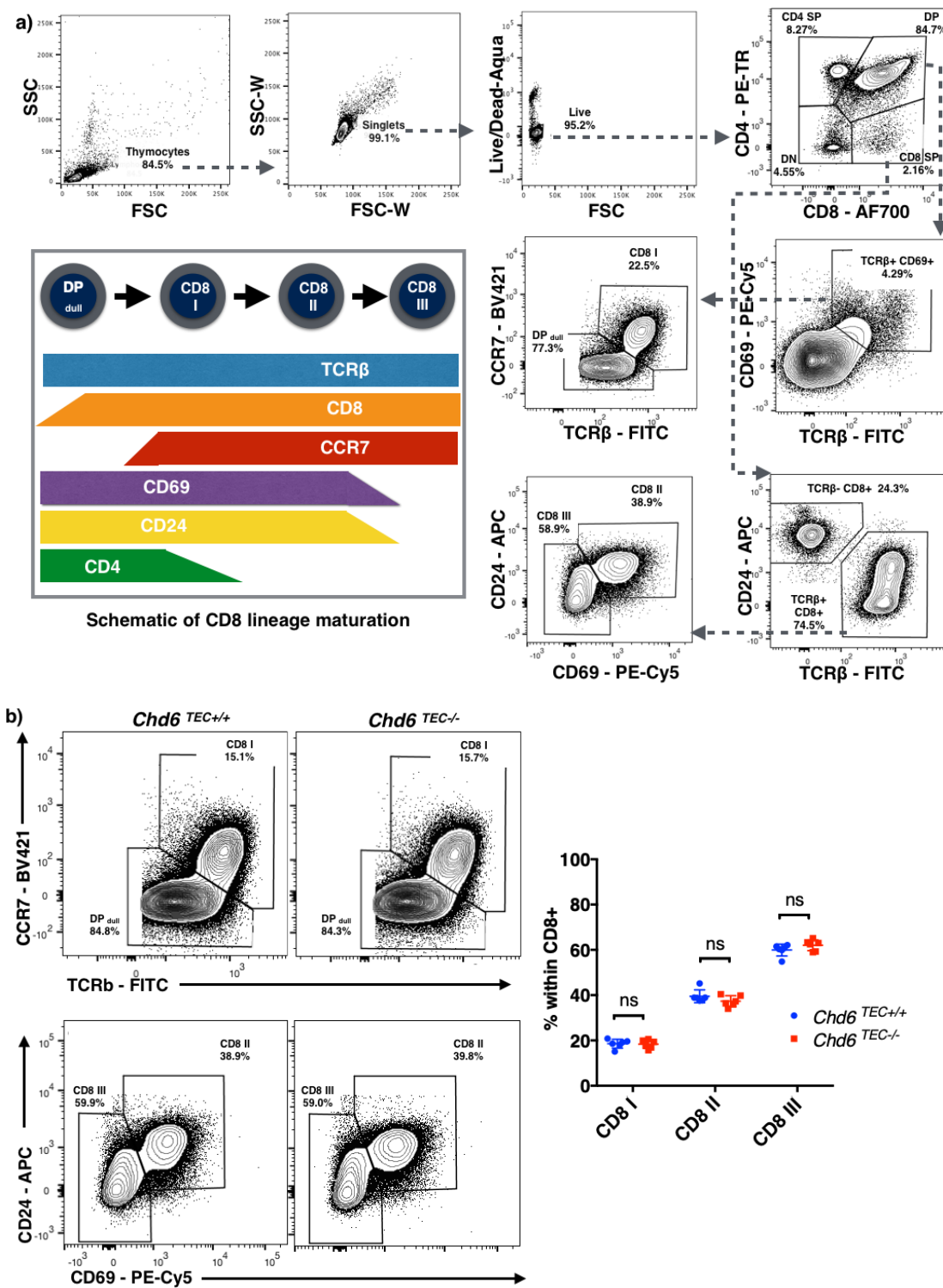


Figure 5.7 Single CD8⁺ thymocyte lineage maturation in 4.5 week old female *Chd6* *TEC*^{-/-} mice
a) FACS gating strategy and a schematic of the lineage maturational stages of CD4⁺CD8⁺ and CD4⁻CD8⁺ thymocytes based on their expression of CCR7, CD24 and CD69. **b)** Relative frequencies of CD4⁻CD8⁺ thymocytes at the indicated lineage maturational stages. Left panels: representative zebra plots; Right panel: The dot graph displays the relative frequency of thymocytes at the indicated developmental stages. Each data point shown represents data from an individual mouse. The graph shows the composite of data from two independent experiments with 3 mice each per group and displays the mean values and standard deviations. p-values were determined by Student's t-test; ns=not significant.

5.2.5. The generation of thymic T_{reg} is unaffected in *Chd6*^{TEC-/-} mice

I next determined whether an absence of CHD6 expression in TEC would also affect the generation and detection of thymic T_{reg}. For this purpose, Lin⁻ CD4⁺CD8⁻ thymocytes were analysed for the cell surface expression of CD25 and the nuclear expression of Foxp3. The former is the alpha chain of the IL-2 receptor whereas the latter is a transcription factor essential for the development of thymic T_{reg}^{47,49}. In the mouse, the development of thymic (also known as natural) T_{reg} is marked by the sequential acquisition of these two molecules whereby intermediate stages leading to CD25⁺Foxp3⁺ thymocytes can either be CD25⁺Foxp3⁻ or CD25⁻Foxp3⁺^{47,263} (gating strategy as in Figure 5.8).

The comparative analysis of *Chd6*^{TEC+/+} and *Chd6*^{TEC-/-} mice demonstrated comparable frequency of thymic T_{reg}, thus demonstrating that CHD6 expression in TEC was dispensable for the generation a normal frequency of T_{reg}. (Figure 5.9a). Moreover, the frequencies of thymic T_{reg} that had recirculated from the periphery back to the thymus (as identified by the co-expression of CCR6 and CD44) were also comparable for both mouse strains (Figure 5.9b). Hence, CHD6 was also not required to provide the adequate conditions for the “return” of natural T_{reg} back to the thymic microenvironment.

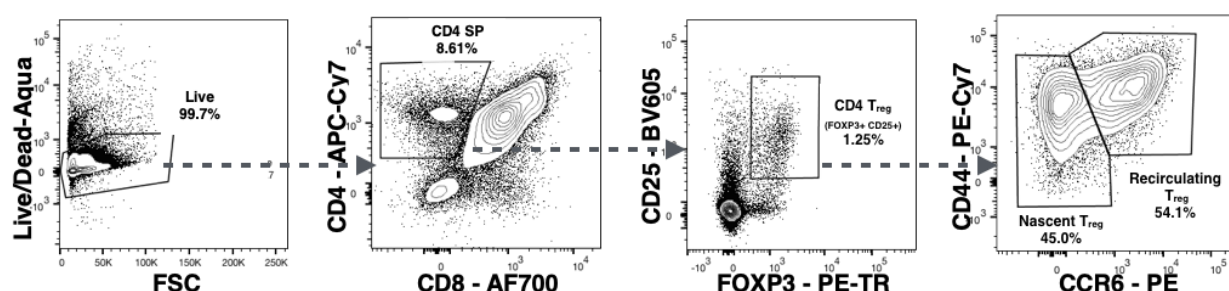


Figure 5.8 Flow cytometric gating strategy for characterisation of thymic T_{reg}

Representative FACS plots demonstrating the gating strategy to identify thymic T_{reg}. Thymocytes excluding doublets and dead cells were gated for CD4⁺ CD8⁻ cells and subsequently analysed for the expression of CD25 and FOXP3. These cells were further differentiated into nascent thymic T_{reg} (CCR6^{lo} CD44^{lo}) and T_{reg} (CCR6^{hi} CD44^{hi}) recirculating from the periphery.

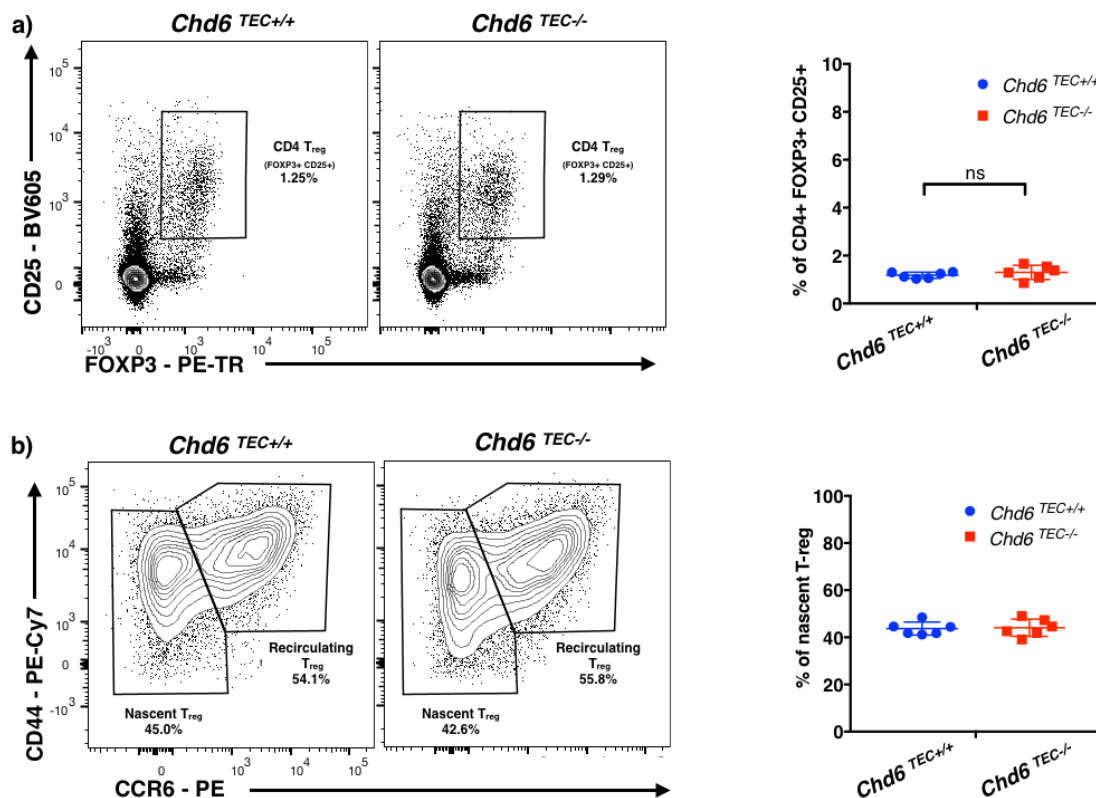


Figure 5.9 Relative frequency of thymic T_{reg} in 4.5 week old female *Chd6*^{TEC-/-} mice

a) Thymic T_{reg} cells (CD4⁺CD25⁺Foxp3⁺). Left panels: Representative zebra plots; Right panels: The dot graph displays the relative frequency of T_{reg} cells. Each point in the graph represents data from an individual mouse. The graph shows the composite of data from two independent experiments with 3 mice each per group and displays the mean values and standard deviations. p-values were determined by Student's t-test; ns=not significant. **b)** Nascent and recirculating T_{reg} in the thymus of *Chd6*^{TEC+/+} and *Chd6*^{TEC-/-} mice according to the absence or presence of CD44 and CCR6 expression, respectively. Each point in the graph represents data from an individual mouse. The graph shows the composite of data from two independent experiments with 3 mice each per group and displays the mean values and standard deviations. p-values were determined by Student's t-test; ns=not significant.

5.2.6. CD5 expression in different thymocyte subpopulations in *Chd6*^{TEC-/-} mice

The overall avidity of the TCR expressed by thymocytes for their cognate pMHC complex directly correlates with the surface expression of CD5 (as measured by gMF)²⁶⁴. I therefore tested whether the TCR avidity of thymocytes undergoing negative selection instructed by TEC would change if the latter failed to express CHD6. Thus, I measured the gMFI for CD5 for different thymocytes before and after positive selection and when subjected to the four distinct events of negative selection (Figure 5.10). These analyses demonstrated that thymocytes developing beyond the positive

selection stage expressed lower CD5 gMFI values if they had matured in the presence of TEC devoid of CHD6 expression. This finding therefore suggested that in the absence of CHD6 expression in TEC, thymocytes – whether subject to negative TCR selections or surviving these checkpoints – expressed antigen receptors with a lower avidity for pMCH complexes.

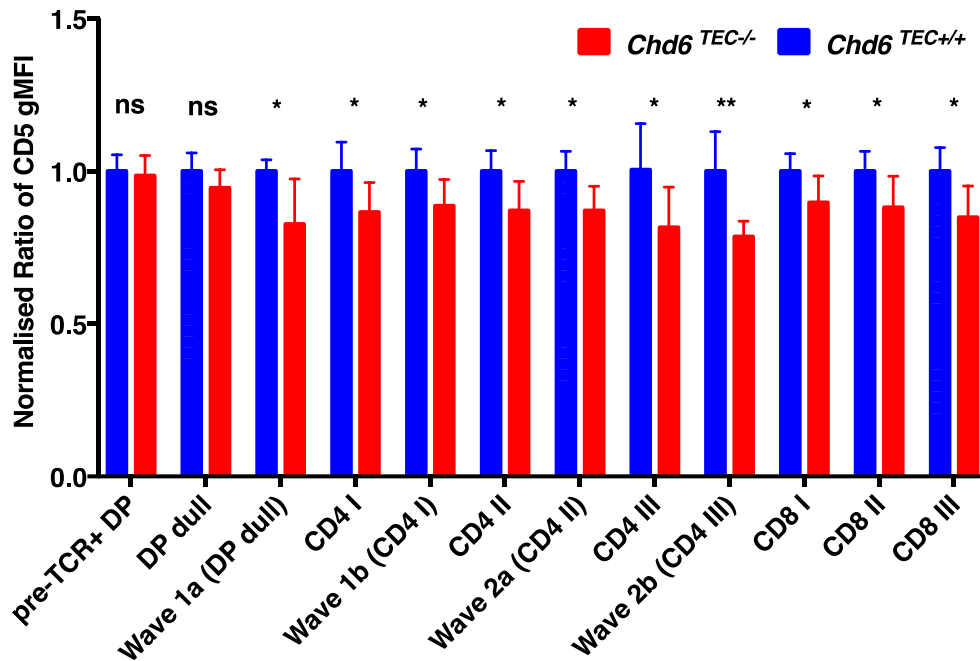


Figure 5.10 Normalised gMFI ratio of CD5 expression.

CD5 gMFI values for the indicated cell stages in *Chd6*^{TEC-/-} were normalized to those of the corresponding thymocyte phenotype of *Chd6*^{TEC+/+} mice whereby these latter ones were arbitrarily set to a value of “1”. Each bar graph represents the pooled mean value of normalized gMFI from two independent experiments with each 3 mice and displays the standard deviation. Statistical comparison was done with two-tailed unpaired Student t-tests with correction for multiple comparison; ns = not significant, *=p<0.05, **=p<0.005.

5.2.7. Terminal maturation of thymocytes in *Chd6*^{TEC-/-} mice is unchanged.

Thymocytes deficient in CD5 expression display an enhanced proliferative response to anti-CD3 mitogenic activation in comparison to wild-type mice²⁶⁵. Because thymocytes of *Chd6*^{TEC-/-} mice express lower CD5 cell surface concentrations when compared to wild-type mice, I next investigated whether their proliferative behaviour and the acquisition of features characteristic for terminally differentiated thymocytes was unperturbed.

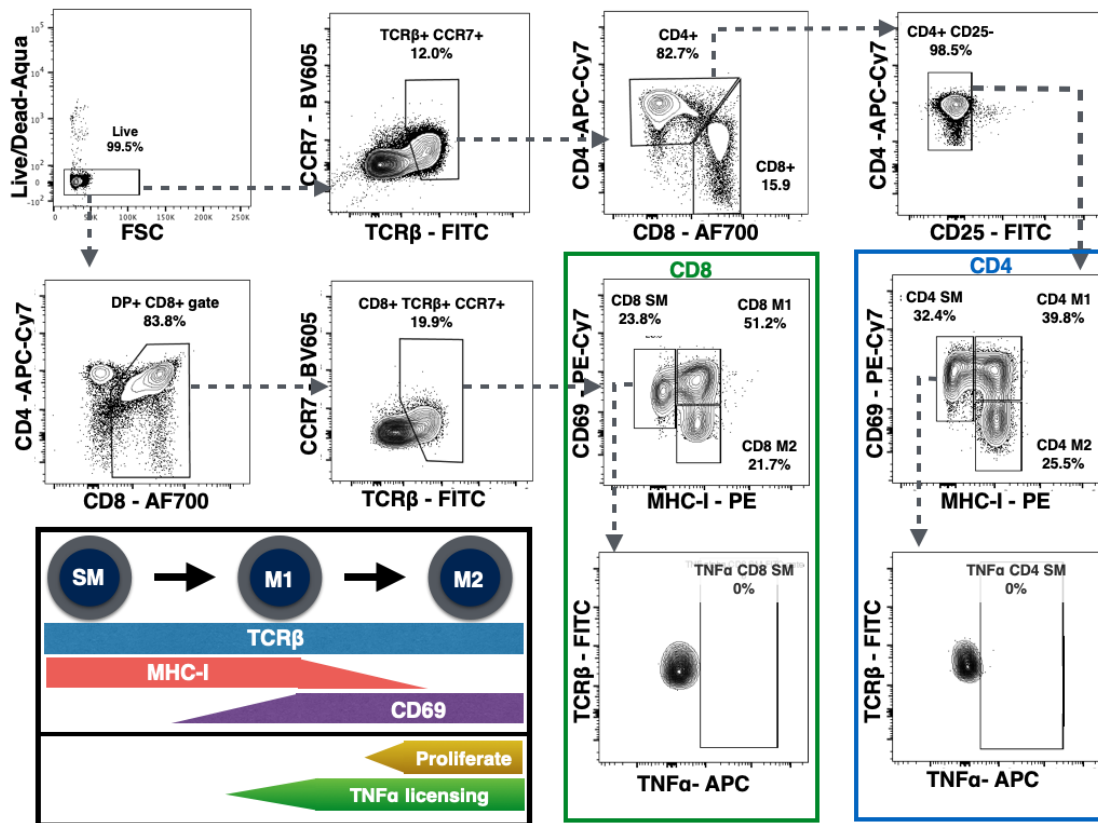


Figure 5.11 Intrathymic terminal thymocyte maturation

Lower left box: Scheme of the terminal differentiation stages of thymocytes and their designation as semi-mature (SM: CD69- MHC^{hi}), mature-1 (M1: CD69+ MHC^{hi}), and mature 2 (M2: CD69+ MHC^{lo})²⁶⁶. Gating strategy to identify the distinct stages of thymocyte terminal differentiation using several cell surface markers as indicated. Detection of TNFα production in stimulated semi-mature thymocytes of the CD4 and the CD8 lineages are shown, as pre-set gating for evaluation of TNFα production in M2 thymocytes (see section 5.2.9).

Positively selected thymocytes were categorised based on their differential CD69 and MHC I surface expression into three separate populations: i) semi-mature (CD69-MHC I-), ii) mature stage 1 (CD69+MHC I+) and iii) mature stage 2 (CD69-MHC I+) thymocytes²⁶⁶. The flow cytometric gating strategy to identify these separate maturational stages is displayed in Figure 5.11. As shown in Figure 5.12, *Chd6*^{TEC+/+} and *Chd6*^{TEC-/-} mice displayed the same frequencies for thymocytes at these distinct developmental stages. These results unequivocally demonstrate that CHD6 was dispensable for TEC to instruct a normal phenotypic maturational progression of thymocytes within both the CD4 and CD8 T cell lineages.

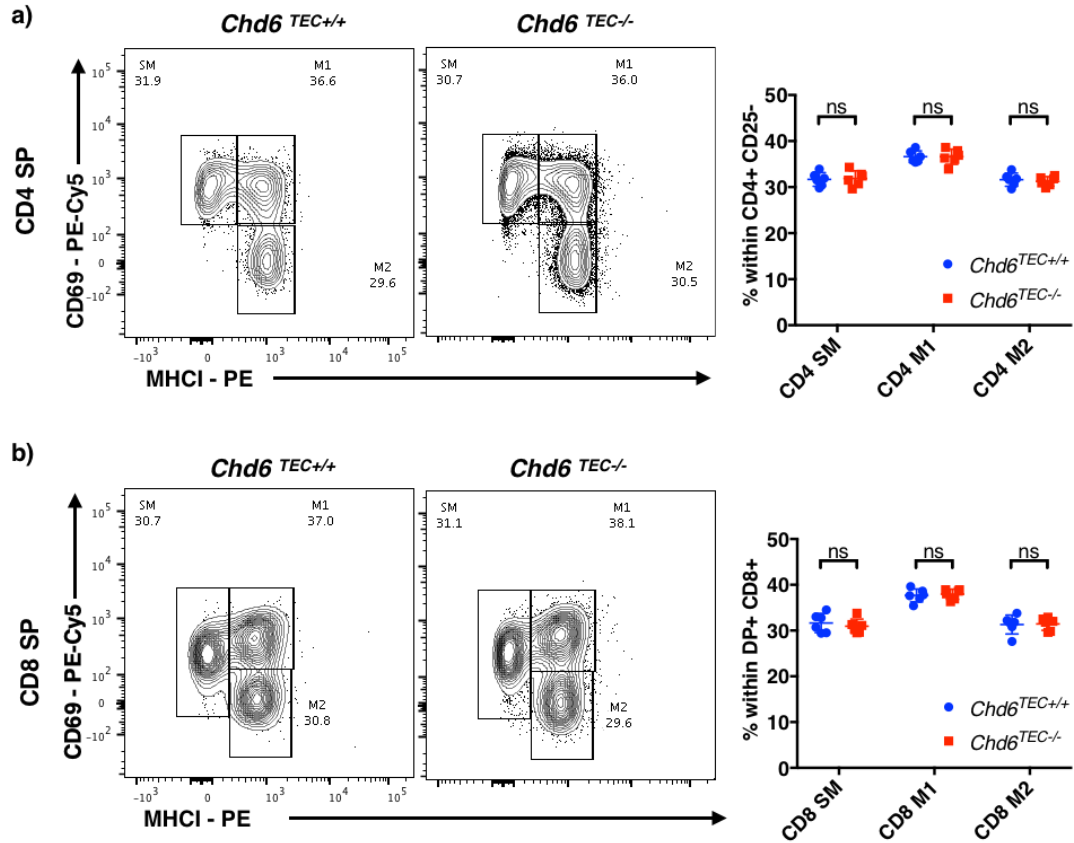


Figure 5.12 Intrathymic terminal thymocyte maturation in 4.5 week old female *Chd6* ^{TEC-/-} mice

a) Frequency of semi-mature (SM), mature stage 1(M1) and mature stage 2 (M2) single CD4+ thymocytes. Left panels: Representative zebra plots; Right panel: The graph displays the relative frequency of thymocytes at the indicated developmental stages. Each point represents data from an individual mouse. The graph shows the composite of data from two independent experiments with 3 mice each per group and displays the mean values and standard deviations. p-values were determined by Student's t-test; ns=not significant. **b)** Frequency of semi-mature (SM), mature stage 1(M1) and mature stage 2 (M2) of the CD8+ thymocytes lineage. Left panels: Representative zebra plots; Right panel. The graph displays the relative frequency of thymocytes at the indicated developmental stages. Each point represents data from an individual mouse. The graph shows the composite of data from two independent experiments with 3 mice each per group and displays the mean values and standard deviations. p-values were determined by Student's t-test; ns=not significant, * = p<0.05.

5.2.8. Proliferative capacity of mature CD4 SP from *Chd6*^{TEC-/-} mice is unchanged

Terminally differentiated thymocytes when stimulated through their antigen receptor are able to signal via NF- κ B to express TNF α ¹⁰⁸. This competence is important to protect SM thymocytes from complex-II mediated death downstream of TNF-R1 signalling, thus enabling their survival during post-selectional maturation¹⁰⁸. The competency to proliferate is subsequently acquired and can be observed in thymocytes at the terminal maturation stage 2 (M2: CD69-, MHC^{hi}) and is a pre-requisite to be able to fully differentiate¹⁰⁸.

I next tested the response of terminally mature (i.e. M2) CD4 SP thymocytes from *Chd6*^{TEC+/+} and *Chd6*^{TEC-/-} mice to respond to different concentrations of anti-CD3 mitogenic stimuli in the presence of anti-CD28 antibodies²⁶⁷. The proliferative response was quantified using the dilution of the cell membrane dye CellTrace Violet, which linearly reduced with each cell division. Proliferation and expansion indices were calculated for each of the different experimental conditions (i.e. anti-CD3 concentrations used) (*nota bene*, the proliferation index assesses the number of cell divisions elicited among responding cells, whereas the expansion index measures the fold expansion of stimulated cells). As shown in Figure 5.13, the ability to proliferate in response to a mitogenic stimuli of different strength were identical for M2 CD4 SP thymocytes irrespective whether they had differentiated in *Chd6*^{TEC+/+} and *Chd6*^{TEC-/-} mice. Taken together, this result confirmed previous phenotypic findings that the absence of CHD6 expression in TEC did not disrupt normal intra-thymic maturation within the CD4 lineage.

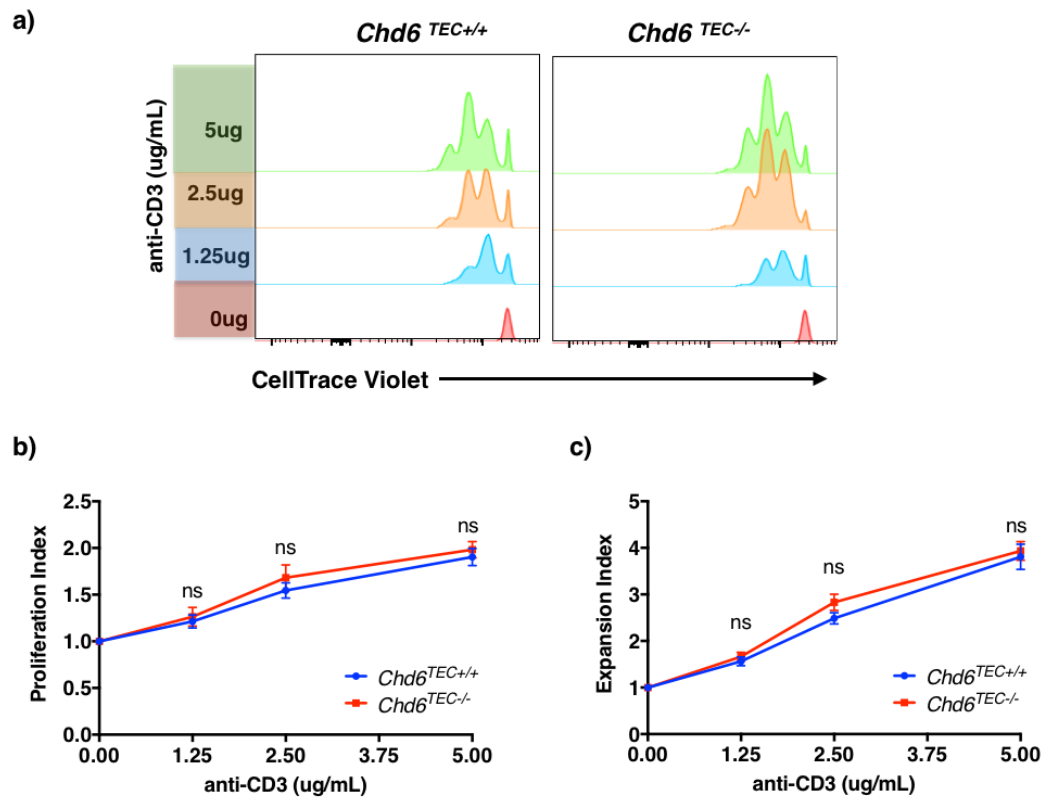


Figure 5.13 Mitogen responsiveness of M2 CD4 thymocytes in 4.5 week old female *Chd6*^{TEC-/-} mice
a) Representative Cell Trace Violet stains of sorted M2 CD4 SP thymocytes stimulated with the indicated anti-CD3 concentrations in the presence of anti-CD28 (1 ug/ml). **b)** Proliferation and **c)** expansion indices of M2 CD4 SP thymocytes stimulated with the indicated anti-CD3 concentrations in the presence of anti-CD28 antibodies (1 ug/ml). Each data point is representative of 50,000 cells stimulated as indicated for three days. The graph shows the composite of data from two independent experiments with 3 mice each per group and displays the mean values and standard deviations. p-values were determined by two-tailed unpaired Student t-tests with correction for multiple comparison; ns = not significant, i.e. $p > 0.05$.

5.2.9. TNF α licensing of CD4 SP and CD8 SP thymocytes in the absence of CHD6 expression by TEC remains unchanged.

Terminally differentiating thymocytes gain the ability to produce TNF α under experimental conditions in response to mitogenic activation¹⁰⁸. While semi-mature (SM) thymocytes exhibit only a poor competence to do so, thymocytes at the final stage of intrathymic maturation (i.e. M2) are fully licensed to produce TNF α ¹⁰⁸. I therefore evaluated whether M2 thymocytes isolated from *Chd6*^{TEC-/-} mice acquired the capacity to produce TNF α in a manner comparable to that of thymocytes from control animals. The TNF α production - quantified by flow cytometry - was comparable for M2 thymocytes isolated from both mouse strains, thus further confirming that the absence

of CHD6 expression in TEC did not interfere with the maturational cues that license thymocytes at the late stages of their development (i.e. M1 and M2) to express TNF α in response to (*in-vitro* mitogenic) cell activation (Figure 5.14).

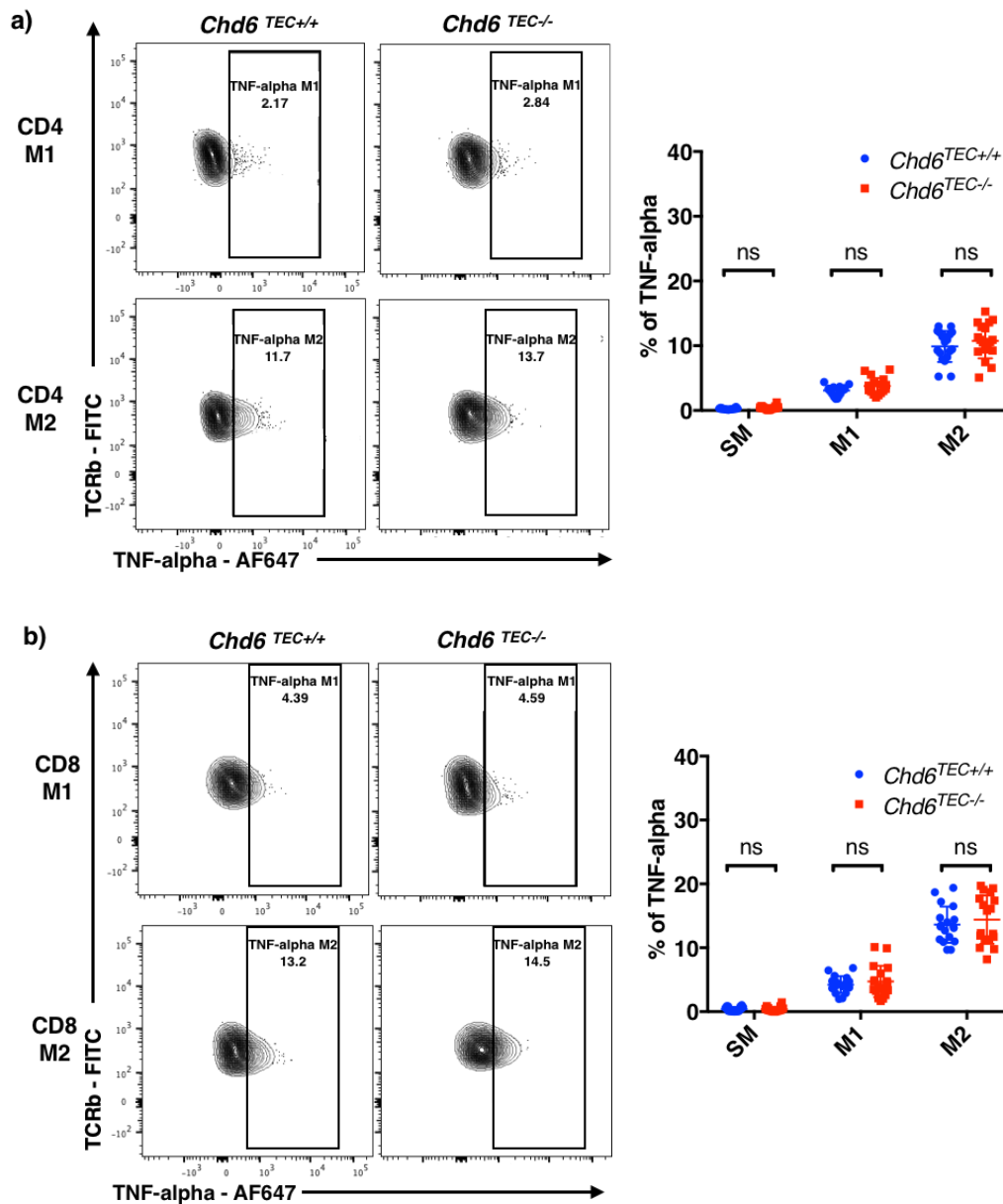


Figure 5.14 TNF α production by late stage CD4 SP and CD8 SP thymocytes isolated from 4.5 week old female *Chd6*^{TEC-/-} mice

TNF α production by **a)** M1 and M2 CD4 SP thymocytes and **b)** M1 and M2 CD8 SP thymocytes. Left panels: representative zebra plots, Right panels: the relative frequency of thymocytes expressing TNF α at the indicated developmental stages. Each point represents data from an individual mouse. Total thymocytes were stimulated with plate-bound anti-CD3 plus anti-CD28 for 4 hours in the presence of GolgiPlug, before being fixed and stained for intracellular TNF α and cell surface molecules that allowed their categorization in to SM, M1 and M2 stages. The graph shows the composite of data from three independent experiments with 4 mice each per group and displays the mean values and standard deviations. p-values were determined by Student's t-test; ns=not significant.

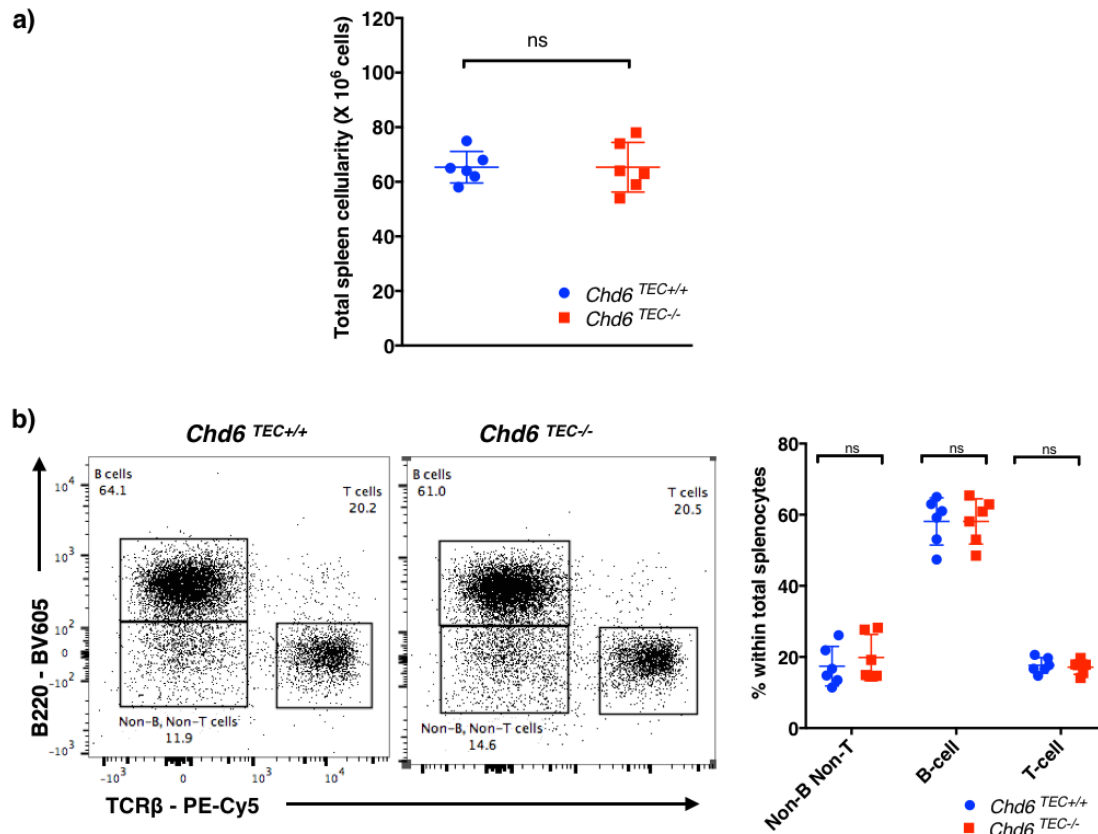


Figure 5.16 Total and relative cellularity of splenic T, B and non-T/B cells in 4.5 week old female *Chd6*^{TEC-/-} mice

a) Splenic cells were isolated by mechanical disruption from spleens of 4.5 week old female *Chd6*^{TEC-/-} mice and gender- and aged-matched wild-type animals. Each data point in the graph represents the splenic cellularity of an individual mouse. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean and standard deviations. p-values were determined by Student's t-test; ns=not significant. **b)** Relative frequency of splenic T, B and non-T/B cells, with representative dot plot analyses (left panel) and frequencies of indicated lymphocyte subpopulations (right panel). Each data point in the graph represents frequency in an individual mouse. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean and standard deviations. p-values were determined by Student's t-test; ns=not significant.

5.3.2. Peripheral CD4 and CD8 T cells are unchanged in *Chd6*^{TEC-/-} mice

Peripheral CD4⁺ and CD8⁺ were further classified as naïve (CD44⁻ and CD62L⁺) and memory T cells (CD44⁺ CD62L⁻); the gating strategy is displayed in Figure 5.15. T cell development in a thymic microenvironment that lacks CHD6 expression in TEC did not affect T cell commitment into the separate CD4 or CD8 lineages nor did it alter the balance of T cells with a naïve and an antigen-experienced phenotype within each of these lineages (Figure 5.17).

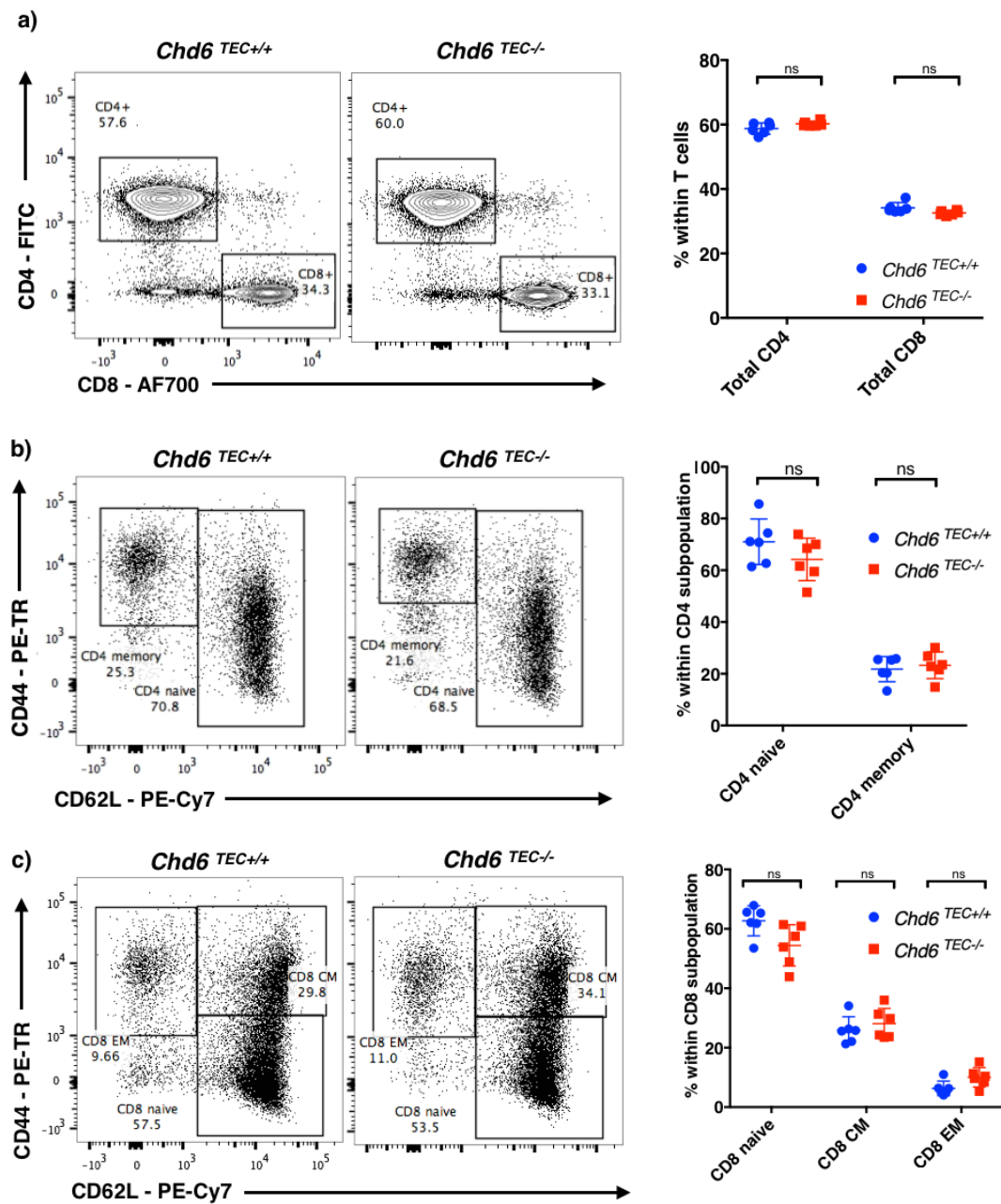


Figure 5.17 Relative frequency of CD4 and CD8 T cells and their subpopulations in 4.5 week old female *Chd6*^{TEC-/-} mice

a) Single CD4⁺ and CD8⁺ T cells. **b)** Naïve and memory CD4⁺ T cells (CD4⁺ CD25⁻ CD8⁻ TCRβ⁺ B220⁻). **c)** Naïve, central memory (CM, CD44^{hi} CD62L^{hi}) and effector memory (EM, CD44^{hi} CD62L^{lo}) CD8⁺ T cells (CD8⁺ CD4⁻ TCRβ⁺ B220⁻). Representative dot plots (left) and dot graph displaying the cells' relative frequencies (right). Each data point shown in the dot graphs represents the results of an individual mouse. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean and standard deviations. p-values were determined by Student's t-test; ns=not significant.

5.3.3. Peripheral T_{reg} cells are unchanged in *Chd6*^{TEC-/-} mice

Several distinctive markers can be utilised to further discriminate peripheral T_{reg} cells with different functions. The expression of inducible costimulator (ICOS) and CD103 are among these surface markers by which T_{reg} cells with high suppressive potency cells are identified²⁶⁸. The gating strategy to recognize peripheral T_{reg} subpopulations is displayed in Figure 5.18. The frequency of peripheral T_{reg} (CD3+ CD4+ CD25+ Foxp3+, Figure 5.19a) and subset of CD103+ ICOS+ T_{reg} and CD103- ICOS- T_{reg} (Figure 5.19b) were comparable for both mutant and wild-type mice thus demonstrating that an absence of CHD6 expression in TEC did not impact on the peripheral T_{reg} compartment and its subsets.

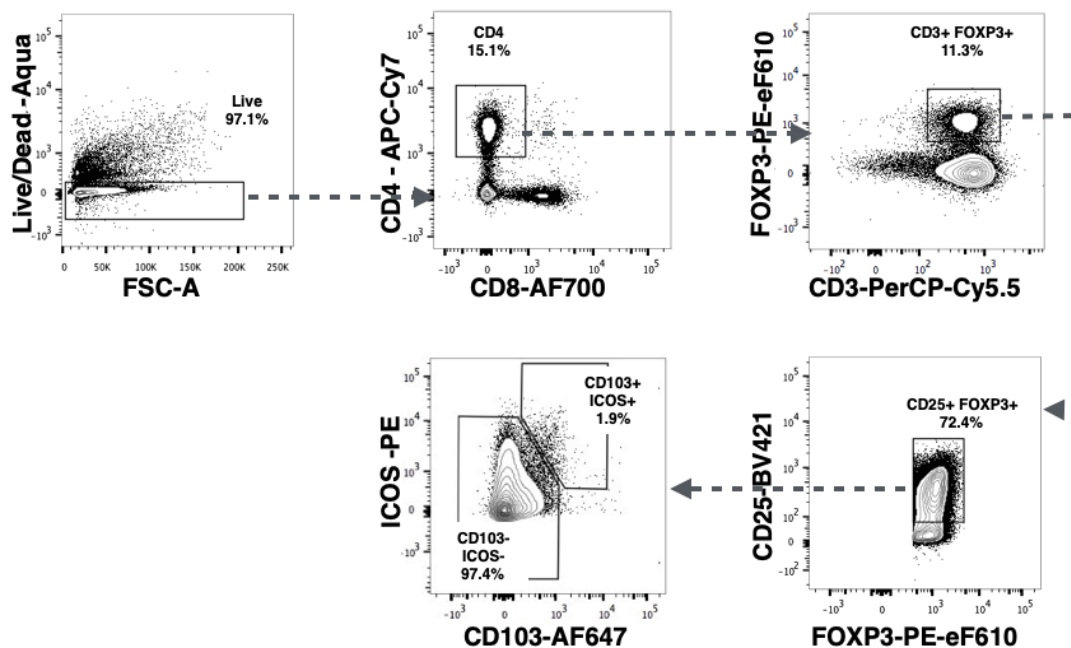


Figure 5.18 Flow cytometric gating strategy for the identification of peripheral T_{reg}

Peripheral lymphocytes (excluding doublets and dead cells) were first gated for CD4 T cells (CD3+ CD4+ CD8-), subsequently analysed for the presence of T_{reg} as defined as FOXP3+ CD25+ CD4 T cells, and finally categorized as CD103+ ICOS+ T_{reg} and CD103- ICOS- T_{reg}.

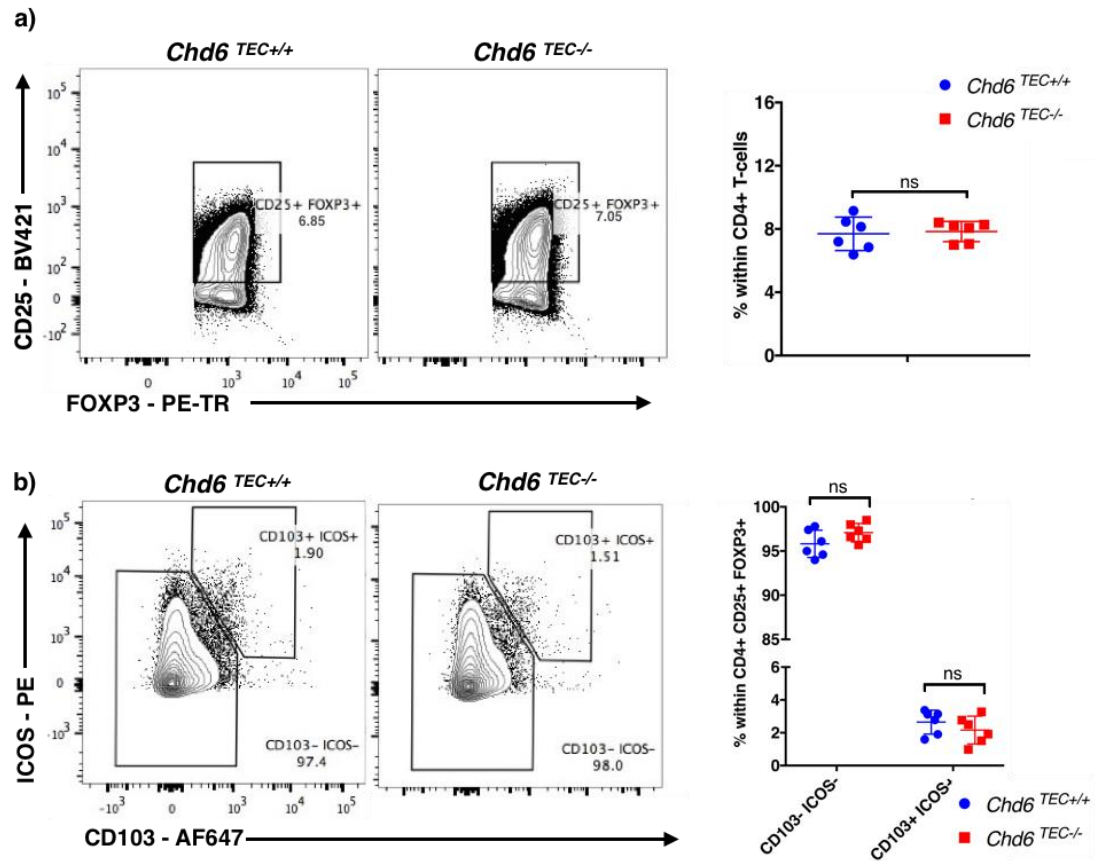


Figure 5.19 The relative frequency of T_{reg} in 4.5 week old female *Chd6* *TEC*^{-/-} mice

a) Splenic frequency of T_{reg} (CD4⁺ CD25⁺ FOXP3⁺) and **b)** its subsets of CD103⁺ ICOS⁺ T_{reg} and CD103⁻ ICOS⁻ T_{reg}. Representative dot plots (left) and dot graph displaying the cells' relative frequencies (right). Each data point shown in the dot graphs represents the results of an individual mouse. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean and standard deviations. p-values were determined by Student's t-test; ns=not significant.

5.3.4. Unchanged frequency of anergic CD4 T cells in *Chd6* *TEC*^{-/-} mice

The peripheral induction of T cell anergy constitutes an other mechanism by which immunological tolerance is achieved despite the physical presence of autoreactive T cells in the periphery that have escaped thymic negative selection²⁶⁹. This state of non-responsiveness is, for example, enforced under conditions of chronic exposure to self- and non-self antigens in the absence of co-stimulatory signals necessary for the complete activation of T cells and typically provided in the context of inflammation²⁶⁹. In light of my findings that *Chd6* *TEC*^{-/-} mice are mildly defective in thymocyte negative selection, I investigated next whether this deficiency is met by a higher frequency of anergic T cell as defined by a phenotype of high CD44, CD73 and folate

receptor 4 (FR4) expression ²⁶⁹. The gating strategy to identify anergic CD4 T cells is displayed in Figure 5.15. A comparative analysis of *Chd6*^{TEC-/-} and wild-type mice identified a comparable frequency of anergic CD4 T cells in each of these mouse strains (Figure 5.20). Hence, the deletion of CHD6 targeted to TEC and the subsequent disruption of normal thymocyte negative selection did not apparently lead to a change in the frequency of anergic CD4 T cells.

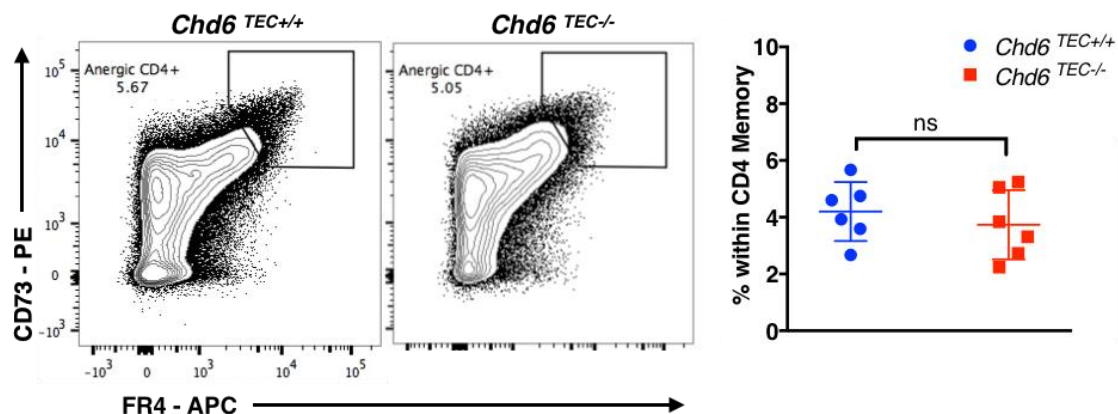


Figure 5.20 The relative frequency of anergic cells in 4.5 week old female *Chd6*^{TEC-/-} mice CD4 memory T cells (CD4⁺ CD44^{hi} CD62L^{lo}) were phenotype for presence of anergic cells as marked by the high expression of FR4 and CD73. Representative dot plots (left) and dot graph displaying the cells' relative frequencies (right). Each data point shown in the dot graphs represents the results of an individual mouse. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean and standard deviations. p-values were determined by Student's t-test; ns=not significant.

5.3.5. Naïve CD4 and CD8 T cells from *Chd6*^{TEC-/-} mice display an enhanced proliferative response to mitogenic, TCR-mediated stimulation.

I next determined whether naïve CD4 and CD8 T cells isolated from *Chd6*^{TEC-/-} mice could be normally activated *in-vitro* by a mitogenic stimulus using a combination of anti-CD3 and anti-CD28 antibodies. T cells isolated from *Chd6*^{TEC-/-} mice displayed a mildly greater proliferative response to the stimulus over a range of different anti-CD3 concentrations (Figure 5.21 and Figure 5.22).

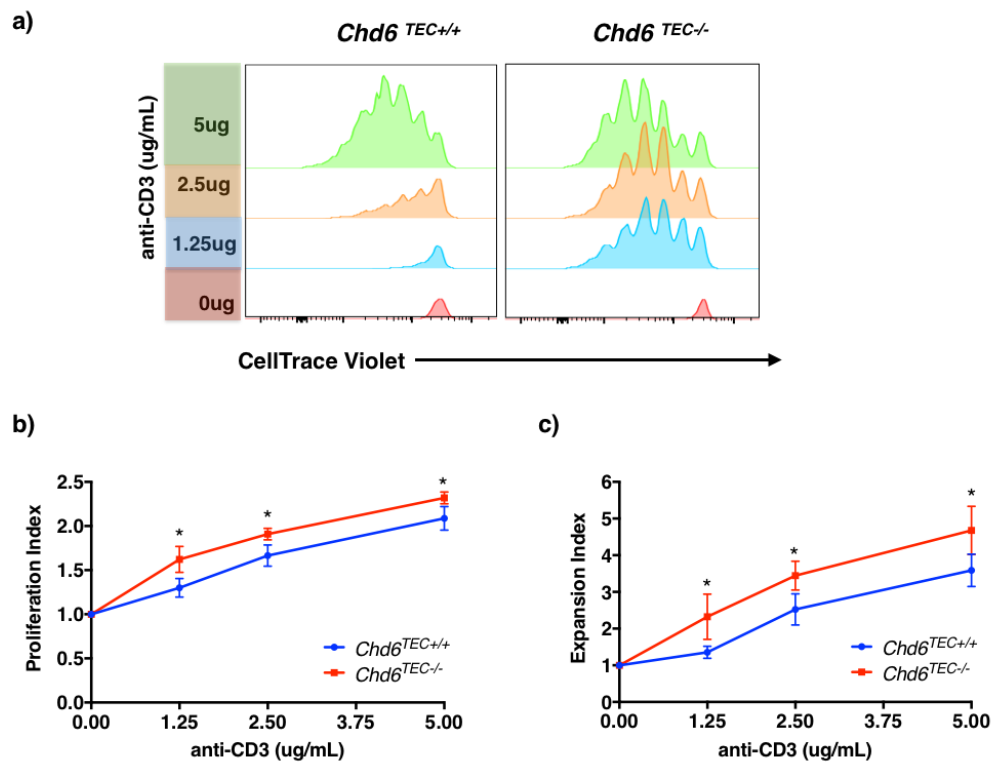


Figure 5.21 CD3-mediated mitogenic response of naïve CD4 T cells from 4.5 week old female *Chd6*^{TEC-/-} mice

a) Representative Cell Trace Violet tracks of naïve CD4 T cells stimulated with the indicated doses of anti-CD3 concentrations in the presence of anti-CD28 (1 µg/ml). **b)** Proliferation and **c)** expansion indices. Each data point represents 150,000 cells initially stimulated. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean values and standard deviations. p-values were determined by two-tailed unpaired Student t-tests with correction for multiple comparison; ns = not significant, i.e. $p > 0.05$, * = $p < 0.05$.

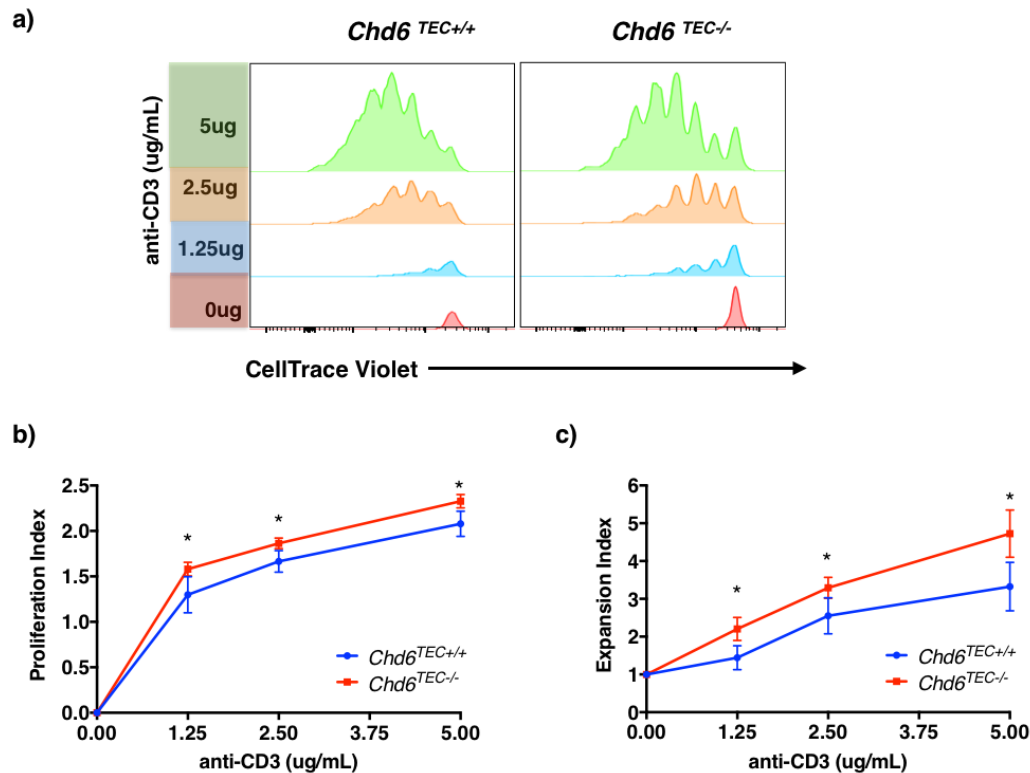


Figure 5.22 CD3-mediated mitogenic response of naïve CD8 T cells from 4.5 week old female *Chd6*^{TEC-/-} mice

a) Representative Cell Trace Violet tracks of naïve CD8 T cells stimulated with the indicated doses of anti-CD3 concentrations in the presence of anti-CD28 (1 ug/ml). **b)** Proliferation and **c)** expansion indices. Each data point represents 150,000 cells initially stimulated. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean values and standard deviations. p-values were determined by two-tailed unpaired Student t-tests with correction for multiple comparison; ns = not significant, i.e. p>0.05, * = p<0.05.

I next verified whether the enhanced proliferation was directly related to the TCR/CD3 complex on the cell's surface or whether differences in the signalling pathways downstream of this complex accounted for the heightened response. Naïve CD4 and CD8 T cells were therefore exposed to Phorbol 12-myristate 13-acetate (PMA) and ionomycin as chemical mitogenics, thus circumventing the need to activate the TCR/CD3 complex directly. PMA is an organic allosteric activator of protein kinase-C and inositol phosphates, which trigger an intracellular calcium release that is further enhanced by Ionomycin, a calcium ionophor. Calcium release is a prerequisite for downstream NFAT signalling leading to full T cell activation. Cell Trace Violet-labelled T cells from *Chd6*^{TEC-/-} mice and wild-type controls were stimulated *in-vitro* with different PMA and ionomycin concentrations (see section 2.4.6.2 for detail) and the proliferation and expansion indices were calculated based on the dilution of the

membrane dye. The chemical activation with PMA and Ionomycin resulted in a of CD4⁺ and CD8⁺ T cell response that was identical irrespective whether the cells had been isolated from mutant or wild-type animals (Figure 5.23 and Figure 5.24).

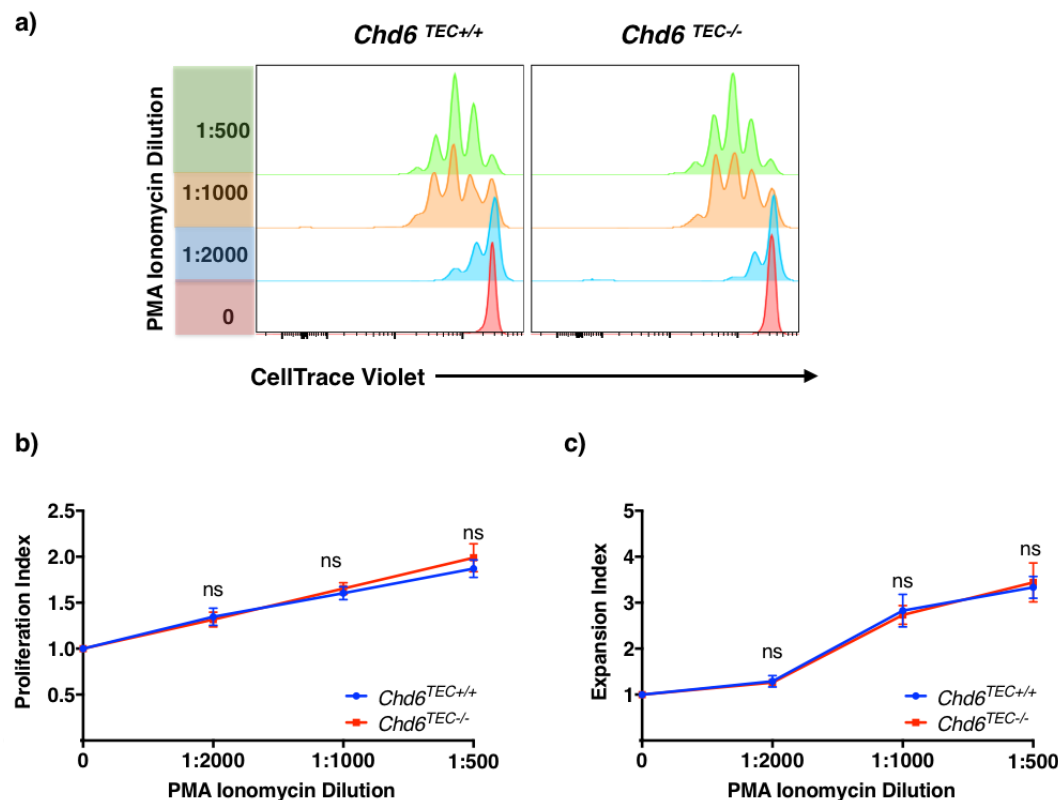


Figure 5.23 Proliferative response of naïve CD4 T cells from 4.5 week old female *Chd6*^{TEC-/-} mice to PMA and Ionomycin

a) Representative Cell Trace Violet traces of naïve CD4 T cells stimulated with the indicated PMA and Ionomycin dilutions (for detail see section 2.4.6.2) **b)** Proliferation and **c)** expansion indices. Each data point represents 150,000 cells initially stimulated. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean values and standard deviations. p-values were determined by two-tailed unpaired Student t-tests with correction for multiple comparison; ns = not significant, i.e. p>0.05.

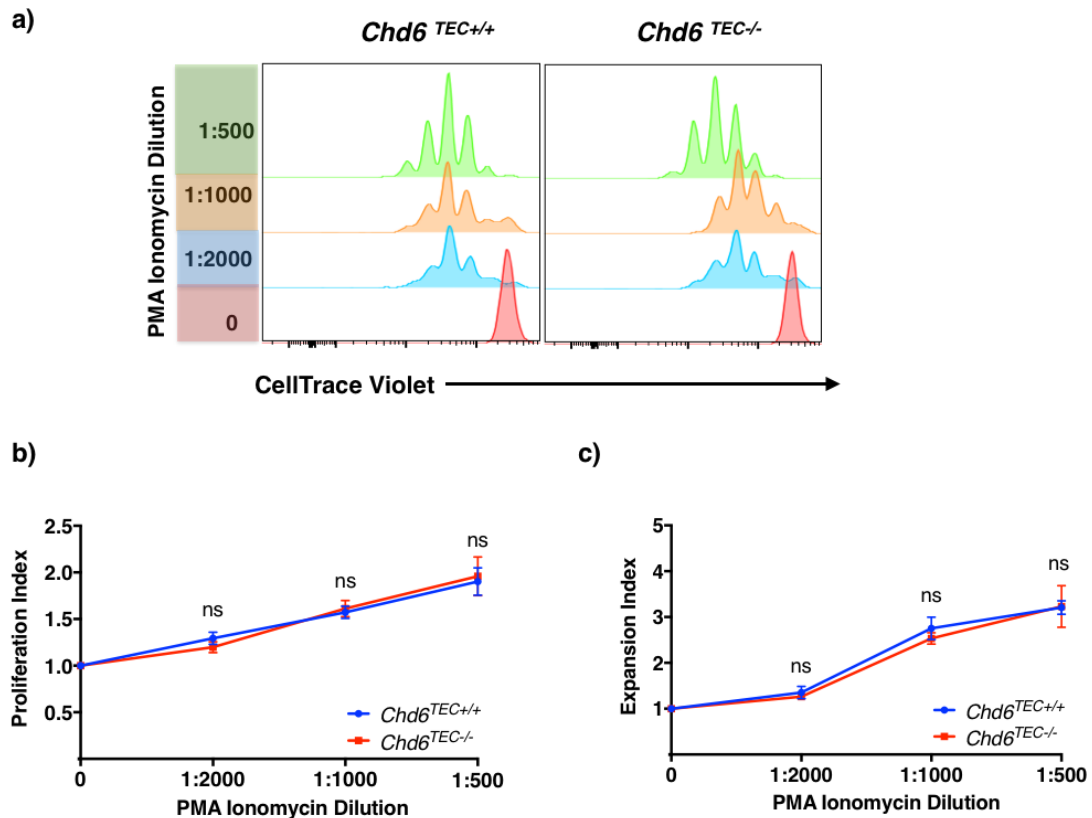


Figure 5.24 Proliferative response of naïve CD8 T cells from 4.5 week old female *Chd6*^{TEC-/-} mice to PMA and Ionomycin

a) Representative Cell Trace Violet traces of naïve CD8 T cells stimulated with the indicated PMA and Ionomycin dilutions (for detail see section 2.4.6.2). **b)** Proliferation and **c)** expansion indices. Each data point represents 150,000 cells initially stimulated. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean values and standard deviations. p-values were determined by two-tailed unpaired Student t-tests with correction for multiple comparison; ns = not significant, i.e. p>0.05.

These results therefore suggest that events at the level of the TCR/CD3 complex and cell surface co-receptors but not the down-stream positioned signalling cascade, were responsible for the observed difference in proliferation of T cells activated by anti-CD3/CD28 vs. PMA/Ionomycin. It is important to note that TCR/CD3 complex expression on CD4 and CD8 T cells of CHD6 mutant mice were unchanged (see next section, 5.3.6, and Figure 5.27), suggesting that the greater proliferation were not caused by the differences in the availability of TCR/CD3 complex on T cells of *Chd6*^{TEC-/-} mice. Taken together, these results suggest that the greater proliferative potential observed in T cells generated in the thymus devoid of CHD6 is TCR-dependent and not T cell autonomous.

5.3.6. Normal frequency of activated T cells in *Chd6*^{TEC-/-} mice.

I next investigated whether the enhanced proliferation of peripheral T cells in response to mitogenic, TCR-mediated stimuli correlated with changes in the pre-activation state of these cells when isolated from *Chd6*^{TEC-/-} mice. CD69 and CD25 are T cell activation markers whereby CD69 is up-regulated within hours and down-regulated after 48 hours of TCR-mediated activation²⁷⁰. CD25, the alpha chain of IL-2R, is up-regulated after 24 hours and remains detectable more than a week after TCR activation²⁷. The frequency of naïve and memory CD4⁺ and CD8⁺ T cells expressing CD69 and CD25 and their cell surface concentration (as quantified by gMFI) were comparable for CHD6 mutant and wild-type mice (Figure 5.25 and Figure 5.26).

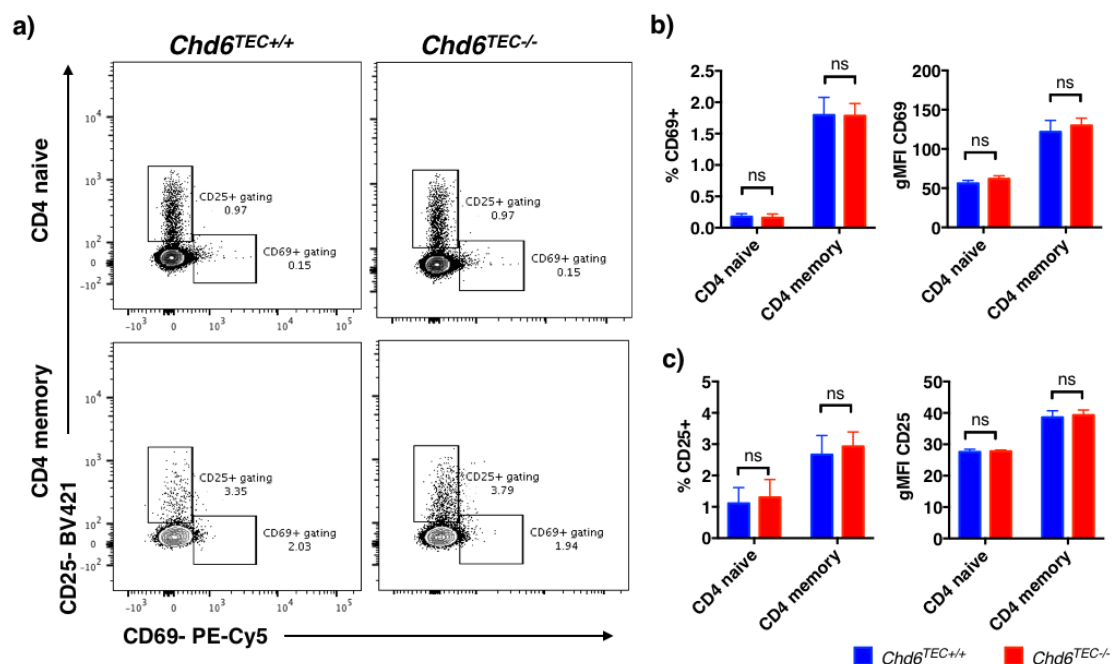


Figure 5.25 Expressions of CD69 and CD25 on CD4 T cell subpopulations isolated from 4.5 week old female *Chd6*^{TEC-/-} mice

a) Expression of CD25 and CD69 on naïve (upper panel) and memory CD4 T cells (lower panel). Plots show representative data sets of two independent experiments. Bar graphs report frequencies of cells positive for and the cell surface concentration of **b)** CD69 and **c)** CD25 on the indicated CD4 subpopulations. The bar graphs show the mean and standard deviation and display an aggregate of two independent experiments with three mice each per group (n=6/group). p-values were determined by Student's t-test; ns=not significant.

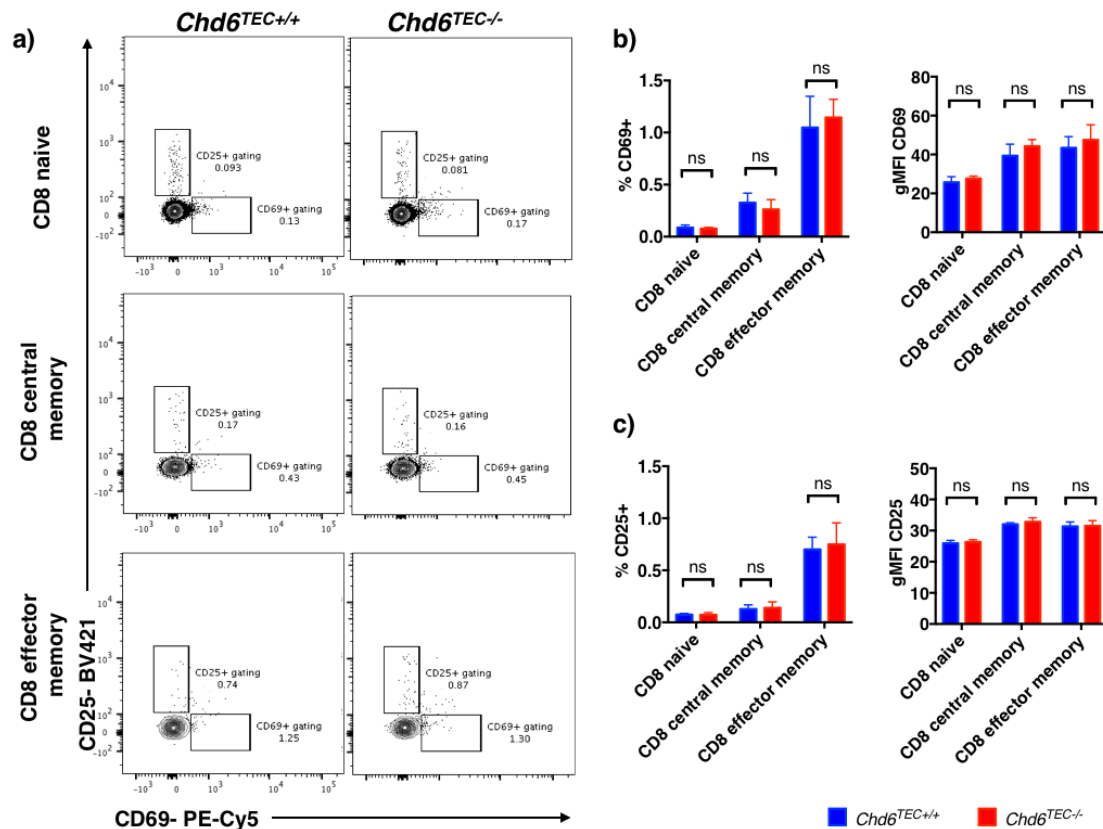


Figure 5.26 Expressions of CD69 and CD25 on CD8 T cell subpopulations isolated from 4.5 week old female *Chd6^{TEC-/-}* mice

a) Naïve (upper panel), central memory (middle panel) and effector memory (lower panel) CD8 T cells were assessed for their expression of CD25 and CD69. Plots shown representative data sets of two independent experiments. Bar graphs show frequencies of cells positive for and cell surface concentration of **b)** CD69 and **c)** CD25 on the indicated CD8 subpopulations. The bar graphs show the mean and standard deviation and display an aggregate of two independent experiments with three mice each per group (n=6/group). p-values were determined by Student's t-test; ns=not significant.

The level of CD5 expression on mature, peripheral T cells not only directly corresponds to the avidity of its TCR for the cognate pMHC complex but is also continuously tuned²⁷¹. In contrast to a lower level of CD5 expression level on developing thymocytes, peripheral T cells from *Chd6^{TEC-/-}* mice expressed CD5 cell surface concentrations that were identical to those observed in wild-type mice (Figure 5.27a).

The expression of CD24, a heat-stable antigen, is low on quiescent T cells but rapidly up-regulated following activation via the TCR-CD3 complex²⁷². In keeping with the expression pattern of CD3 and MHCI, the phenotype of T cells isolated from *Chd6^{TEC-/-}* mice also did not differ in CD24 expression when compared to that of wild-type

animals (Figure 5.27b,c,d). Taken together, there was no appreciable differences between T cells from mutant and wild-type mice regarding both the frequency of cells positive for and the level of expression of CD69, CD25, CD5, CD24, MHCI and CD3, all are markers that have previously been established to be essential for T cell function.

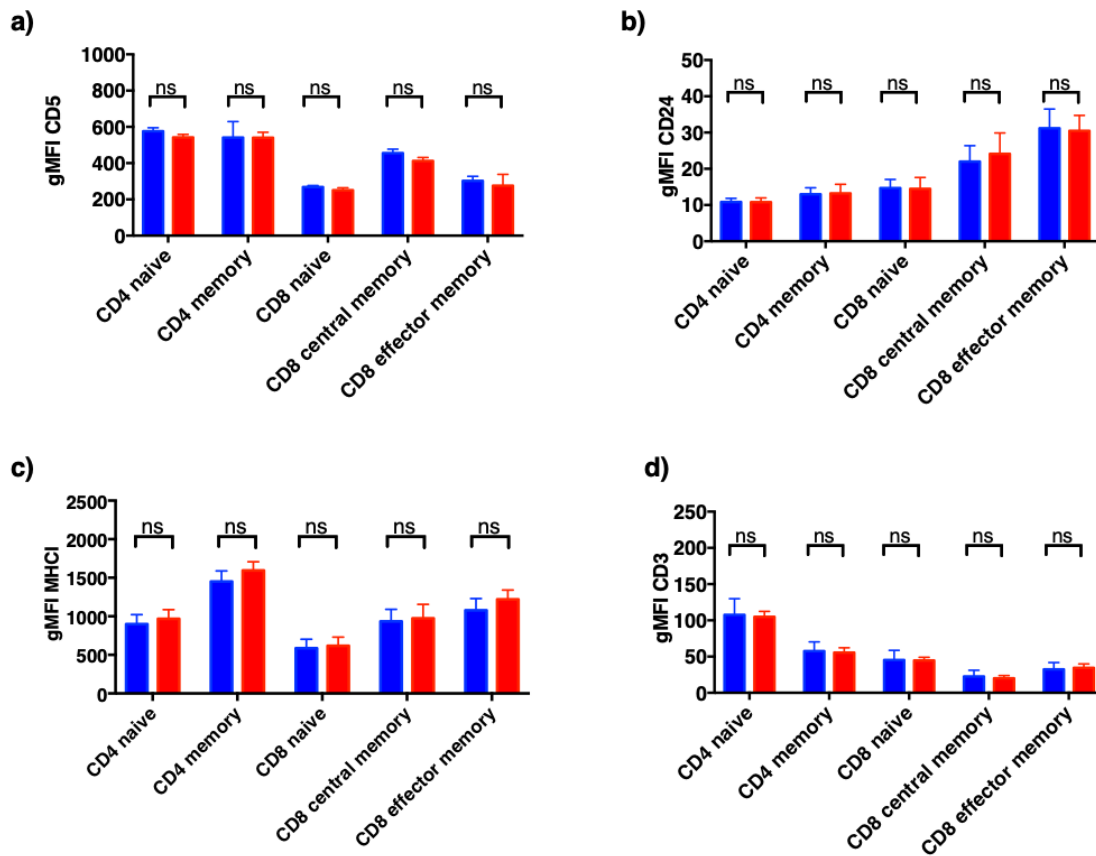


Figure 5.27 CD5, CD24, MHCI and CD3 expression levels on different CD4 and CD8 T cells subpopulations of 4.5 week old female *Chd6*^{TEC-/-} mice

gMFI values of **a)** CD5, **b)** CD24, **c)** MHCI and **d)** CD3 expression on the indicated CD4 and CD8 T cells subpopulations of wild-type (blue) and *Chd6*^{TEC-/-} mice (red). The bar graphs show the mean and standard deviation and display an aggregate of two independent experiments with three mice each per group (n=6/group). p-values were determined by Student's t-test; ns=not significant.

5.4. Thymocyte maturation and peripheral T cells in 8 month old *Chd6*^{TEC-/-} mice

The analyses of TEC compartment in 8 month old *Chd6*^{TEC-/-} mice revealed a defect in PGE in the mature mTECs (see section 4.7). Since aging leads to progressive decrease in thymopoiesis activity, I therefore conducted a comparative analyses of (i) thymocytes, (ii) peripheral T cell phenotype and (iii) peripheral T cell function from 8 month old *Chd6*^{TEC-/-} and wild-type mice to get a sense whether CHD6-deficient TEC age differently than CHD6-proficient animals.

5.4.1. Impaired thymocyte negative selection in the medulla of 8 month old female *Chd6*^{TEC-/-} mice.

The extent of the age-related decrease in total thymocyte cellularity was comparable for 8 month old *Chd6*^{TEC-/-} and wild-type mice (Figure 5.28a). This result mirrored normal early thymocyte development (Figure 5.28b and Figure 5.28c), regular positive selection (Figure 5.28d), and unperturbed CD4+ and CD8+ lineage commitment and differentiation including T_{reg} differentiation in *Chd6*^{TEC-/-} mice (Figure 5.30 and Figure 5.31). Identical to findings in young *Chd6*^{TEC-/-} mice (see Figure 5.5b), the two steps of medullary thymocyte negative selection (designated wave 2a and b) were, however, impaired in aged *Chd6*^{TEC-/-} mice (Figure 5.29b). Hence, the absence of CHD6 expression in TEC did not modify the process of thymus senescence, as thymocyte cellularity, differentiation and selection – with the notable exception of thymocyte negative selection in the medulla - remained normal.

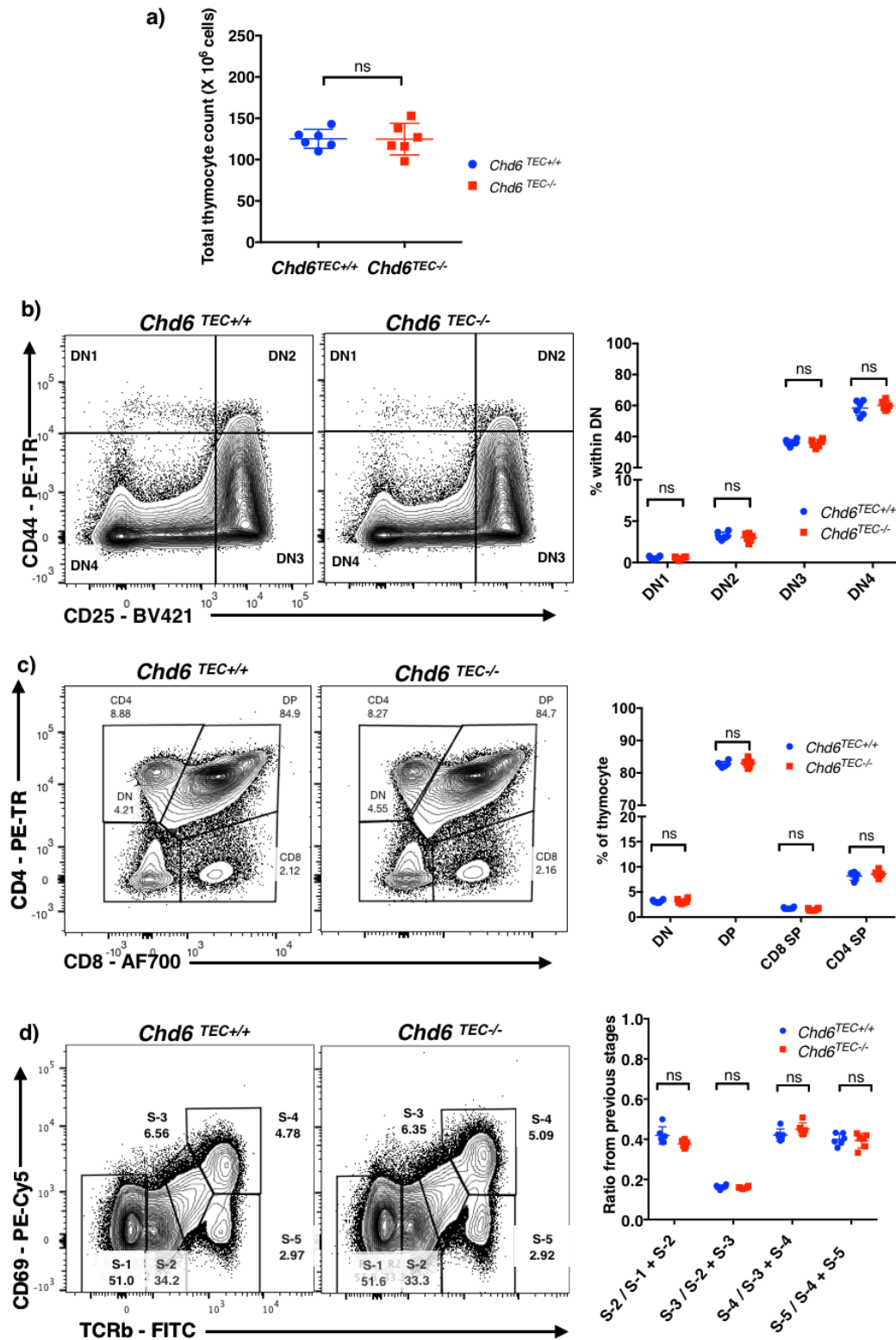
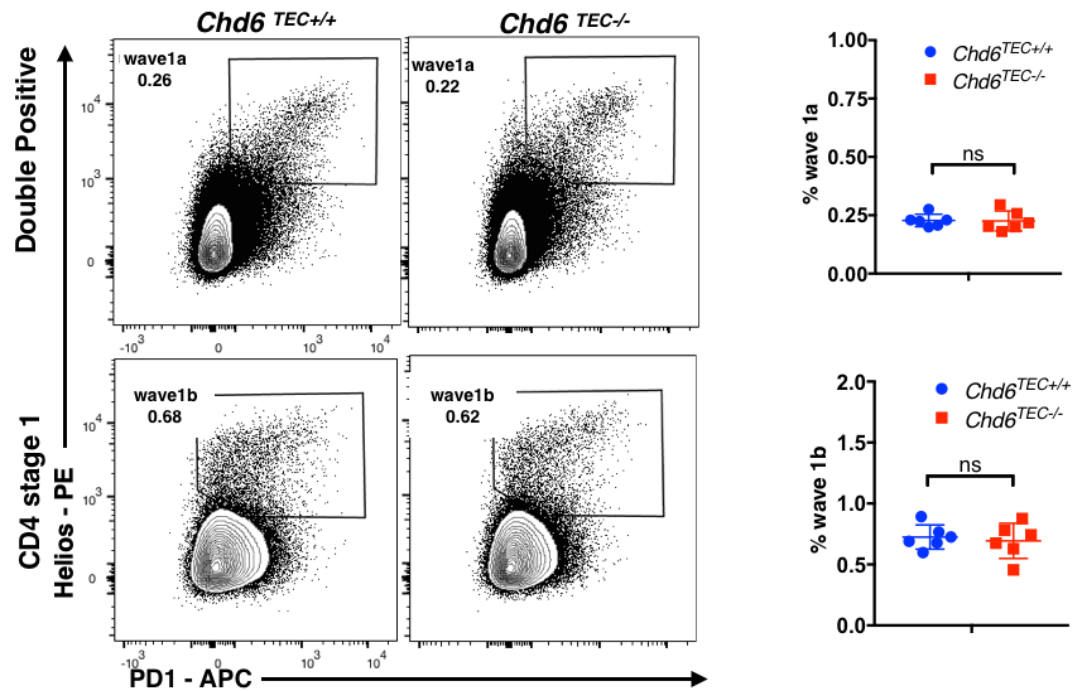


Figure 5.28 Total count and early thymocyte development in 8 month old female $Chd6^{TEC-/-}$ mice
 Analysis of **a)** total thymocyte count **b)** individual DN stages, **c)** different thymocyte subpopulations, and **d)** stages following TCR-mediated activation of thymocytes. Gating and nomenclature according to Figure 5.2a and Figure 5.3. Representative dot plots (left) and dot graph displaying the cells' relative frequencies (right). Each data point shown in the dot graphs represents the results of an individual mouse. The data represents an aggregate of two independent experiments with three mice each per group ($n=6/\text{group}$) and displays the mean and standard deviations. p-values were determined by Student's t-test; ns=not significant.

a) Cortical thymocyte negative selection in 8 month old female mice



b) Medullary thymocyte negative selection in 8 month old female mice

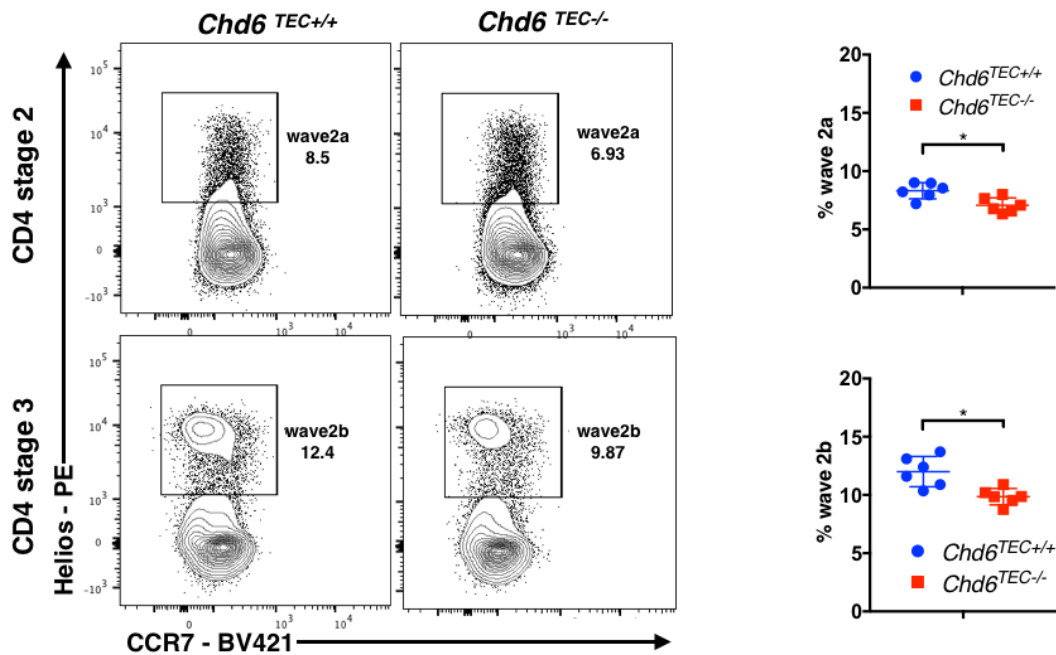
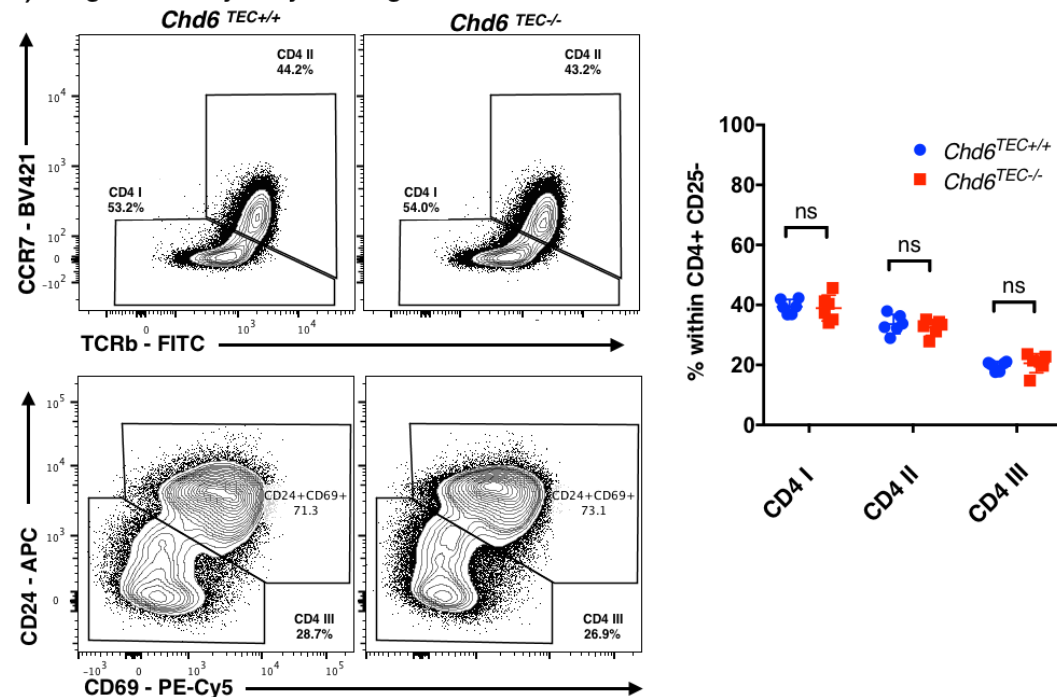


Figure 5.29 Thymocyte negative selection in 8 month old female *Chd6*^{TEC-/-} mice

Analysis of a) wave 1 and b) wave 2 thymocyte negative selection. Gating and nomenclature according to Figure 5.4b. Representative dot plots (left) and dot graph displaying the cells' relative frequencies (right). Each data point shown in the dot graphs represents the results of an individual mouse. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean and standard deviations. p-values were determined by Student's t-test; ns=not significant, * = p<0.05.

a) Single CD4+ thymocyte lineage maturation in 8 month old female mice



b) Single CD8+ thymocyte lineage maturation in 8 month old female mice

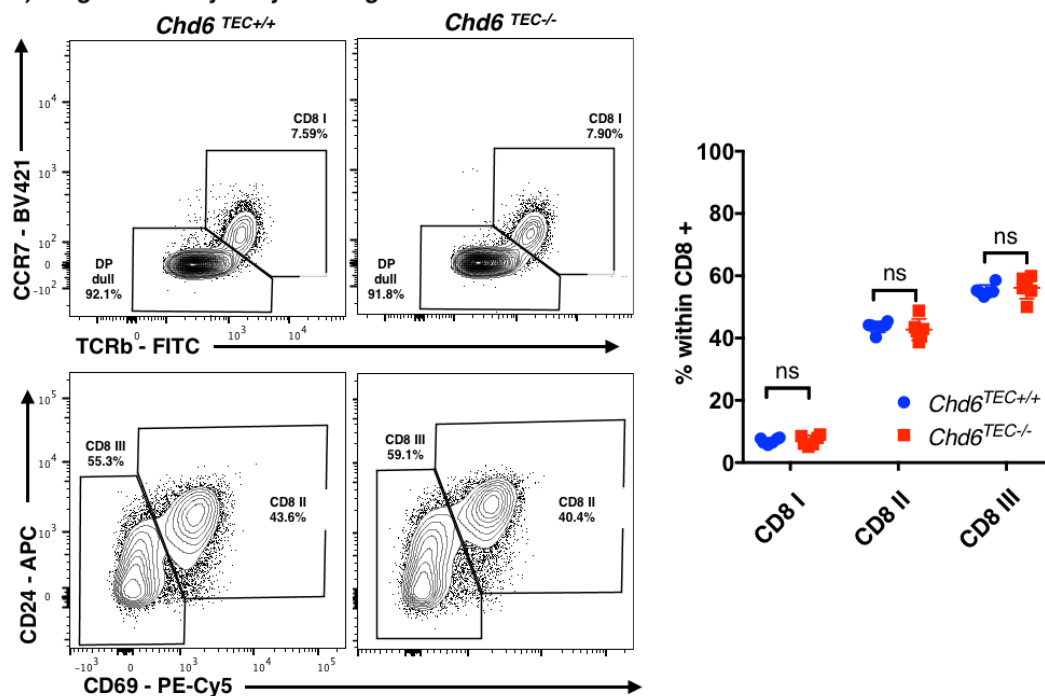


Figure 5.30 Thymocyte lineage maturation in 8 month old female *Chd6*^{TEC-/-} mice

Analysis of a) CD4+ and b) CD8+ thymocytes lineage maturation. Gating and nomenclature according to Figure 5.6a and Figure 5.7a. Representative dot plots (left) and dot graph displaying the cells' relative frequencies (right). Each data point shown in the dot graphs represents the results of an individual mouse. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean and standard deviations. p-values were determined by Student's t-test; ns=not significant.

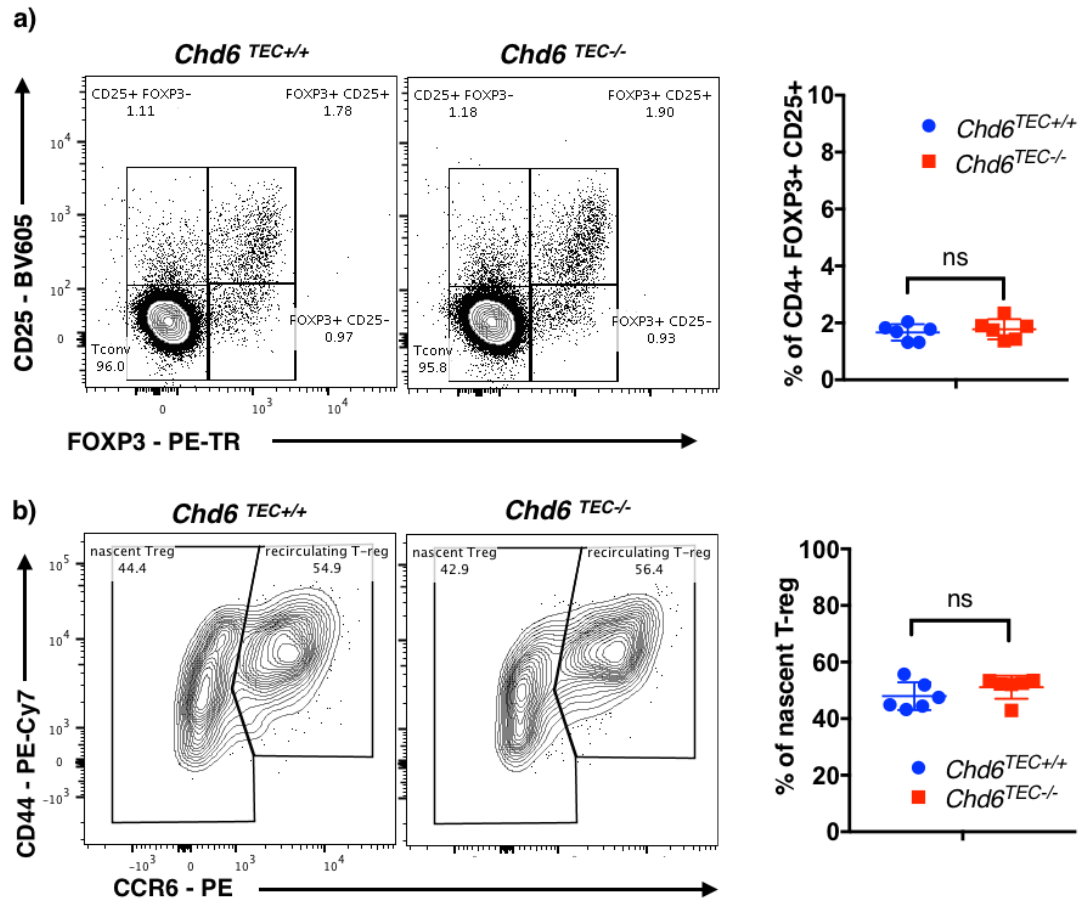


Figure 5.31 Relative frequency of thymic T_{reg} in 8 month old female *Chd6*^{TEC-/-} mice

Analysis of **a)** thymic T_{reg} and **b)** nascent and recirculating T_{reg} cells. Representative dot plots (left) and dot graph displaying the cells' relative frequencies (right). Each data point shown in the dot graphs represents the results of an individual mouse. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean and standard deviations. p-values were determined by Student's t-test; ns=not significant.

5.4.2. Peripheral T cell subpopulations are unchanged in 8 month old *Chd6*^{TEC-/-} mice

Peripheral T cell functions are equally influenced and shaped by the exposure of different pathogen and environmental factors^{81,273}. Although, the thymus and thymocyte developments in aged *Chd6*^{TEC-/-} mice were assessed to be comparatively similar to the wild-type control animals, numerous studies had presented evidence of profound changes in T cell function in aged animals and human alike^{80,81,273,274}. Thus, I next characterised peripheral T cells in both *Chd6*^{TEC-/-} and wild-type, 8 months old female mice. Total splenic cellularity was comparable for the two groups of mice (Figure 5.32a) and the relative frequencies of total T cells (Figure 5.32b), CD4 and CD8 T cell

subsets (Figure 5.33a), as well as their respective subpopulations were normal (Figure 5.33b and Figure 5.33c). Moreover, the frequencies of anergic CD4 T cells (Figure 5.34a) and peripheral T_{reg} (Figure 5.34b and Figure 5.34c) were unchanged in 8 month old *Chd6*^{TEC-/-} mice compared to control animals. Thus, the lack of CHD6 expression in TEC of 8 month old mice did not impair the representation of the different T cell subpopulations in the spleen.

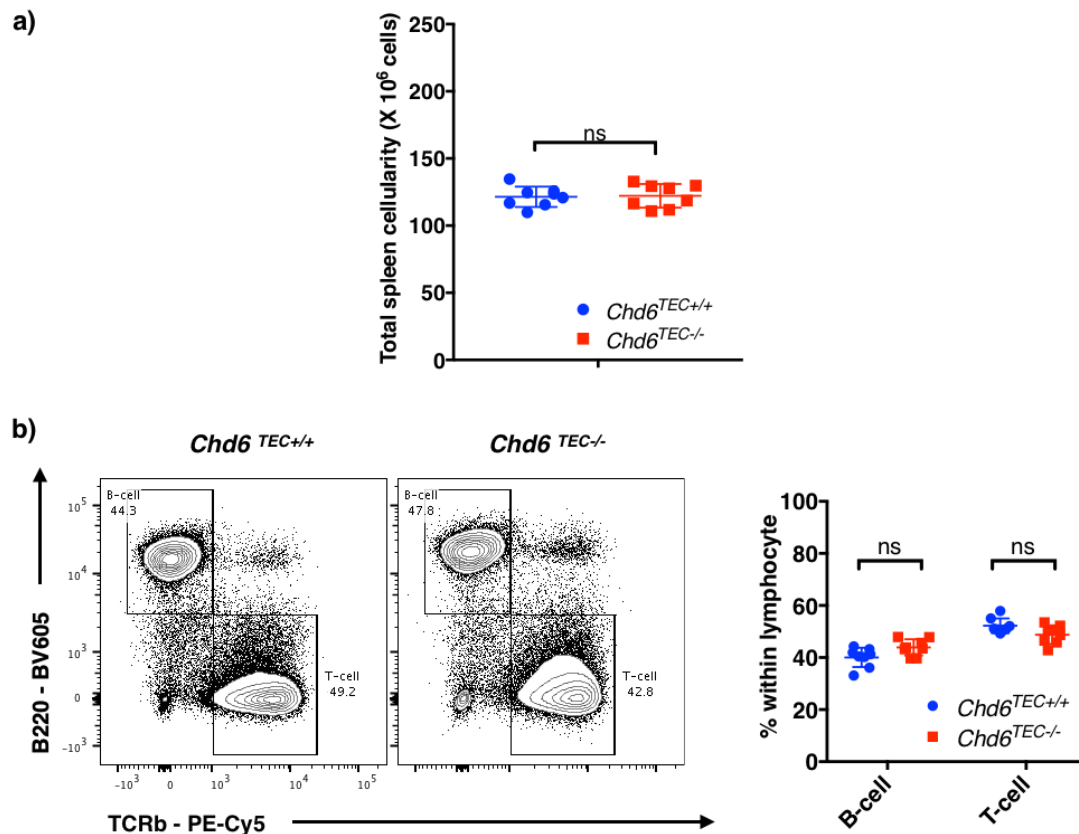


Figure 5.32 Total splenic cellularity and relative frequencies of T and B cells in 8 month old female *Chd6*^{TEC-/-} mice

Analysis of **a)** the total number of splenocytes, and **b)** the frequencies of splenic B and T cells. The lower panels display on the left representative dot plots and on the right a dot graph demonstrating the cells' relative frequencies. Each data point shown in the dot graphs represents the results of an individual mouse. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean and standard deviations. p-values were determined by Student's t-test; ns=not significant.

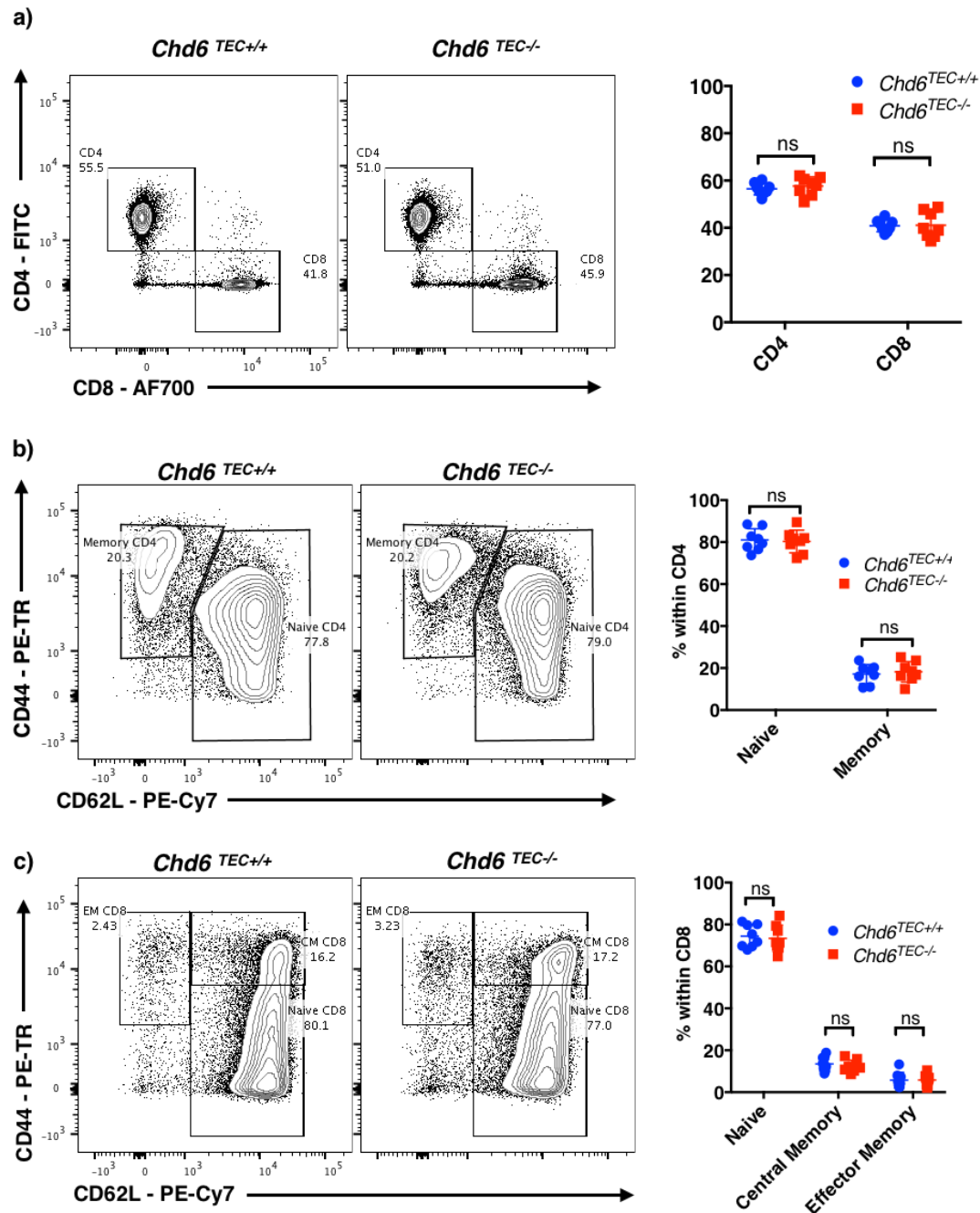


Figure 5.33 Relative frequencies of CD4 and CD8 subpopulations in 8 months old female *Chd6* ^{TEC-/-} mice

Analysis of **a)** single CD4 and CD8 T cells, **b)** CD4 naïve and memory subpopulations, and **c)** CD8 naïve, central memory and effector memory T cells. Panels on the left display representative dot plots and the dot graphs on the right demonstrate the cells' relative frequencies. Each data point shown in the dot graphs represents the results of an individual mouse. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean and standard deviations. p-values were determined by Student's t-test; ns=not significant.

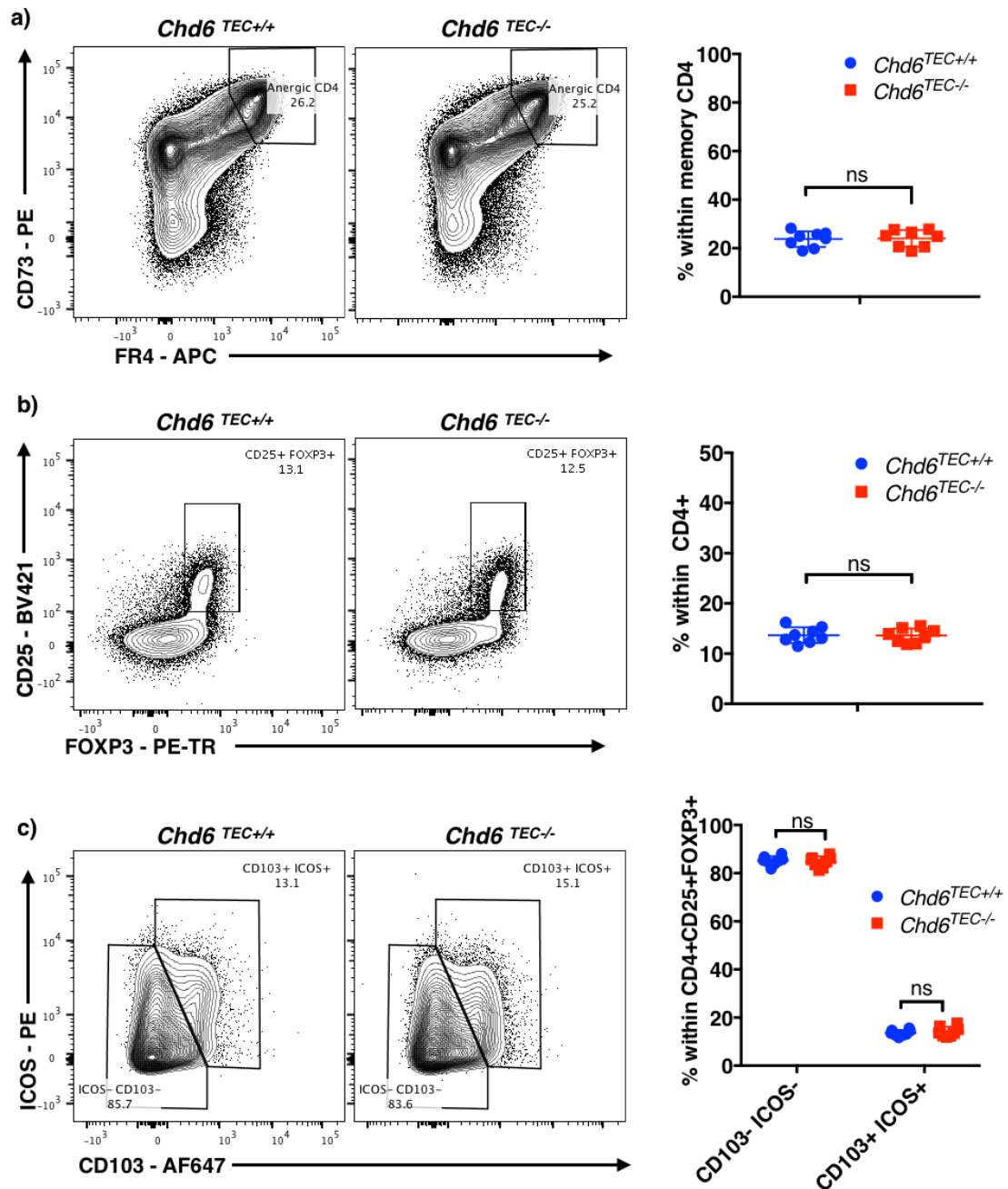


Figure 5.34 Relative frequency of anergic CD4 T cells and T_{reg} in the spleen of 8 month old female *Chd6* ^{TEC-/-} mice

Analysis of the relative frequency of **a)** anergic CD4 T cells, **b)** T_{reg} cells, and **c)** CD103⁺ ICOS⁺ T_{reg} and CD103⁻ ICOS⁻ T_{reg} in the spleen. Panels on the left display representative dot plots and the dot graphs on the right demonstrate the cells' relative frequencies. Each data point shown in the dot graphs represents the results of an individual mouse. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean and standard deviations. p-values were determined by Student's t-test; ns=not significant.

5.4.3. Activated naïve CD4 and CD8 T cells isolated from 8 month old *Chd6*^{TEC-/-} mice respond differently to mitogenic stimulate in comparison to wild-type mice.

Naïve CD4 and CD8 T cells from 4.5 week old female *Chd6*^{TEC-/-} mice showed an enhanced proliferative response to anti-CD3-mediated TCR cross-linking in the presence of anti-CD8 while such a response was not observed when using PMA and Ionomycin (see section 5.3.5). To test whether this pattern of response remained unchanged in older mice, I next exposed separately naïve CD4 and CD8 T cells isolated from 8 month old *Chd6*^{TEC-/-} mice to anti-CD3 and -28 antibodies. T cells from both lineages demonstrated an expansion index that was mildly (CD4 T cells) to robustly (CD8 T cells) increased whereas the proliferation index was identical (CD4 T cells) or minimally heightened (CD8 T cells) when compared to naïve wild-type T cells (Figure 5.35 and Figure 5.36 respectively). Proliferative response in T cell from *Chd6*^{TEC-/-} mice was predominantly dominated by the expansion of the activated T cells rather than in the increase of proliferation cycle. Since T cells from aged mice were shown to be unable to sustain proliferation in a similar manner as their younger counterparts²⁷⁵ (shown by the lower proliferation index), to overcome that inadequacy, it was possible that more cells may need to be expanded (shown by the higher expansion index) in order to mount an adequate response. Additionally, CD8 T cells were shown to be more robust in their proliferative response when compared to CD4 T cells. It is unclear to what may contribute to these differences, as the frequencies of CD4 to CD8 in aged CHD6 mutant mice were relatively similar to their aged wild-type counterparts. Despite some differences in the proliferative responses, more importantly, aged T cells generated in thymus devoid of CHD6, remains hyperproliferative upon anti-CD3 TCR mediated activation, similar to the findings observed in their younger counterparts.

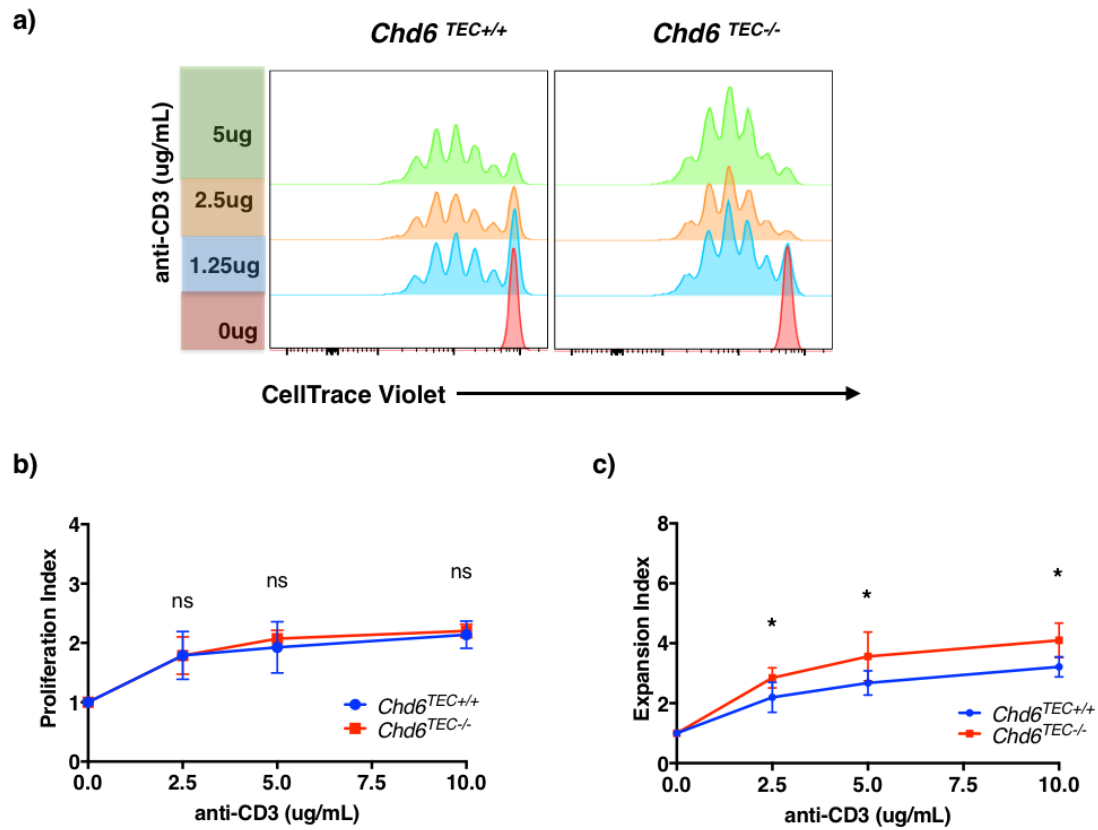


Figure 5.35 TCR-mediated mitogenic response of naïve CD4 T cells from 8 month old female *Chd6*^{TEC-/-} mice

a) Representative Cell Trace Violet tracks of naïve CD4 T cells stimulated with the indicated doses of anti-CD3 concentrations in the presence of anti-CD28 (1 ug/ml). **b)** Proliferation and **c)** expansion indices. Each data point represents 150,000 cells initially stimulated. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean values and standard deviations. p-values were determined by two-tailed unpaired Student t-tests with correction for multiple comparison; ns = not significant, i.e. $p > 0.05$, * = $p < 0.05$.

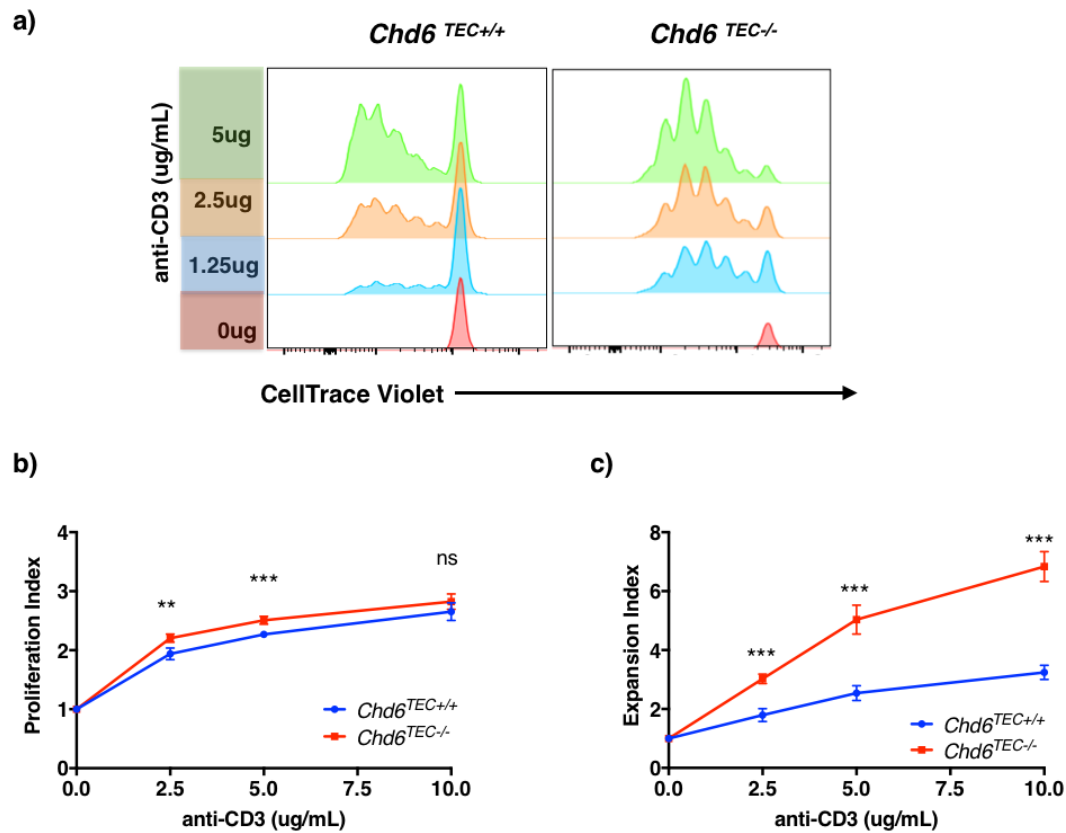


Figure 5.36 TCR-mediated mitogenic response of naïve CD8 T cells from 8 month old female *Chd6*^{TEC-/-} mice

a) Representative Cell Trace Violet tracks of naïve CD8 T cells stimulated with the indicated doses of anti-CD3 concentrations in the presence of anti-CD28 (1 ug/ml). **b)** Proliferation and **c)** expansion indices. Each data point represents 150,000 cells initially stimulated. The data represents an aggregate of two independent experiments with three mice each per group (n=6/group) and displays the mean values and standard deviations. p-values were determined by two-tailed unpaired Student t-tests with correction for multiple comparison; ns = not significant, i.e. $p > 0.05$, * = $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

5.5. Summary and Discussion

Comparable cellularities, frequencies and phenotypic features of different thymocyte maturational stages were found between the *Chd6*^{TEC^{-/-}} mice and the wild-type controls (*Chd6*^{TEC^{+/+}}) in both the young (at 4.5 week old) and the aged mice (at 8 month old). The crucial distinction of thymopoiesis in *Chd6*^{TEC^{-/-}} mice is the specific defect in the second waves of thymocyte negative selection that occur in the medulla as assessed by the expression of Helios. With the particular PGE impairment in the mTECs of *Chd6*^{TEC^{-/-}} mice as documented in the earlier chapter, this observation subsequently delineates the impact of PGE changes on thymocyte negative selection processes. One of the technical constraints in evaluating thymocyte negative selection using the markers for Helios and PD-1, involves the difficulty in determining the rate by which apoptotic cells are cleared by thymus-resident phagocytic cells. The frequencies of thymocyte negative selection based on my assessment here were much lower than the real value, and this was technically expected²⁷⁶. As the apoptotic cells clearance is exceptionally efficient in the thymus, assessment of negative selection by Helios and PD-1 staining will only identify a small fraction of negatively selected cells that has yet to be scavenged²⁷⁶. It is therefore plausible that the value may not adequately represent the negative selection process. Determination of thymic clonal deletion using cleaved caspase 3 had recently been described²⁷⁷, yet this technique is still limited in identifying only a small fraction of negatively selected thymocytes. Although the frequency of thymocyte negative selection as evaluated by Helios might be lower than the “true” value, the reduction in the number of thymocytes assessed to be negatively selected was persistently significant and shown to be affected in both the younger and aged *Chd6*^{TEC^{-/-}} mice. The differences in the negative selected thymocyte were small in the context of relative percentages and this raises a few discussion points. Firstly, this difference is likely reflecting the limited effect of CHD6 deletion on the PGE in mTECs, thus the impact towards thymocyte negative selection was expected to be small. Secondly, there is an issue on whether this small difference has any biological significance in the generation of autoreactive T cells and subsequently, to the development of autoimmunity in the mice. Thus, assessment for autoimmune manifestation in *Chd6*^{TEC^{-/-}} mice were carried out and presented in Chapter 6.

I also noted lower CD5 expression level in the positively and negatively selected thymocytes in *Chd6*^{TEC-/-} mice. This observation was appealing considering that previous publications had reported that the developmental regulation of CD5 expression level on single positive thymocytes reflects the signalling avidity of the selecting TCR–pMHC interaction. Thus, I postulated that the level of CD5 expression in the thymocytes, correlates with the number of TCR–pMHC interactions during both positive and negative selection processes^{264,271}. As thymocytes migrate through the thymus, they continuously scan pMHC complex presented by TECs and other thymic APC via their TCRs. Lower CD5 expression in *Chd6*^{TEC-/-} thymocytes may reflect reduced selection efficiency due to a lower TCR–pMHC density to be selected. This is possibly caused by the reduction of the number of TRAs expressed on the mature mTECs in *Chd6*^{TEC-/-} mice. Following TCR stimulation, the cytoplasmic domain of CD5 becomes rapidly phosphorylated and this resulted in the recruitment of other signalling molecules and represents a mechanism for fine-tuning thymic selection²⁷⁸. CD5 is known to negatively regulates antigen-receptor mediated signalling in thymocytes, where in the absence of CD5 expression, thymocyte becomes hyperresponsive to TCR stimulation. This effect can be observed in T cells from Cd5 deficient mice exhibiting an enhanced proliferation upon TCR stimulation^{265,279}. In aggregate, this observation supports the concept wherein: i) thymocytes in *Chd6*^{TEC-/-} mice had reduced avidity of their TCR for its selecting ligand due to reduced expression of TRAs to be selected on, and ii) the reduced CD5 expression level will lead to enhance TCR responsiveness making it likely to be more reactive upon cognate antigen stimulation.

Although CHD6 deletion in TECs impairs PGE and subsequently affects thymocyte negative selection process in the medulla, I did not detect any functional defect in the ability of thymocytes to either produce TNF α or proliferate upon mitogenic stimulation. I initially postulated that impairment of thymocyte negative selection might have a detrimental effect on the thymocyte maturation and functional acquisition. However, data available to date and the findings as presented in this chapter suggest otherwise. Defect in thymic tolerance induction does not impair the thymocyte maturational acquisition to proliferate and produce cytokine, as shown in mice lacking of organised medullary environment¹⁰³. In fact, the thymocyte phenotypic maturation and acquisition of proliferation competence is hugely driven by

NF- κ B activation¹⁰⁸, in which this signaling pathway is unaffected in *Chd6*^{TEC-/-} mice (as verified by the transcriptomic data in section 4.4)

Induction of central tolerance in the thymus *per se*, is not adequate to ensure immunological self-tolerance. In this context, peripheral immune tolerance and homeostasis are additionally maintained by the functional differentiation within the T_{reg} population^{50,280}. Data analysis of *Chd6*^{TEC-/-} mice showed that, although there was a defect in the medullary negative selections, the generation of both the thymic Treg and the peripheral induced T_{reg}, were unchanged. Based on this finding, several points can be deduced, i.e. (i) CHD6 does not play a direct role in thymic T_{reg} generation, (ii) defect in PGE in the mTEC is not caused by the deletion of CHD6 and does not impact the induction of thymic or peripheral induced T_{reg}. This observation suggests that there is a separate mechanism which governs the induction of central tolerance through regulation of PGE, and those responsible to maintain peripheral immune tolerance.

The establishment of T cell populations in the periphery, as assessed by T cell cellularity and detailed immunophenotyping of *Chd6*^{TEC-/-} mice, were indistinguishable from that of wild-type control mice. However, I observed an increased proliferative capacity in the naïve CD4 and CD8 T cells as they egress to the periphery. As this greater functional competence was not found in the mature CD4 thymocyte (CD4 M2); either in their proliferative competence or ability to license cytokine production, this proliferative advantage must be acquired upon the T cell residency in the periphery. Moreover, this greater proliferative potential was specific upon stimulation of the TCR, as post-stimulation with PMA and Ionomycin on the naïve CD4 and CD8 T cells did not lead to enhance proliferation, indicating that it was not due to cell-intrinsic component. Considering all these findings, greater proliferative potential observed in *Chd6*^{TEC-/-} mice may suggest activation of non-deleted self-reactive T cells by peripheral self-antigens via TCR stimulation. It is likely that naïve T cells with autoreactive potential require the peripheral lymphoid organ environments to encounter their “cognate” self-antigen, which provides the priming signals for the “discreet” autoreactive T cell to become functionally autoreactive. Indeed, abnormal presence of autoreactive lymphocytes resulted in lymphoproliferative disorder and autoimmune manifestations^{281–283}. However, if the autoreactive T cells require “priming” of their TCR to peripheral self-antigen, why do the “primed” autoreactive T

cells still require additional TCR activation to manifest their hyperproliferative response? This further proposes that the activation of the autoreactive T cells may involve two steps interactions. Firstly, the “priming signal” from antigen-TCR interaction to partly unravel the activation pathway and secondly, the “activating signal” of the “primed” antigen-TCR pathway to unleash the immunogenic downstream responses. Nevertheless, it remains to be fully tested to what extent this greater proliferative potential in T cells of CHD6 mutant mice were indeed due to the proposed notion.

Experiments to clarify whether the reduction in thymocyte negative selection in *Chd6*^{TEC^{-/-}} mice can truly result in the escape and increase of autoreactive T cells repertoire in the peripheral blood were attempted. As there is lack of specific markers to identify autoreactive T cells *in vivo*, I decided to determine the expression of different activation markers in peripheral T cells, in the anticipation of identifying differences in the physiological state of T cells in the *Chd6*^{TEC^{-/-}} mice. However, no significance differences in the expression of these activation markers were detected in different T cells subpopulation in mutant CHD6 mice. This is not without surprise as the markers analysed were non-specific and consist of general markers identifying "activated" T cell instead being truly specific to identify autoreactive T cell. Identification of specific autoreactive T cells in a disease-specific mouse model or patients with autoimmune diseases is usually performed by using tetramer reagents to detect autoreactive T cells specific to the tissue-specific antigen. Thymic-specific peptide epitopes of the tissue-specific antigen need to be identified for me to utilise this method. To the best of my knowledge, interphotoreceptor retinoid-binding protein (IRBP) is the only tissue-specific antigen where the thymic-specific peptide have been described, and specific tetramer reagents had been established²⁸⁴. However, defect in the medullary PGE in *Chd6*^{TEC^{-/-}} mice (assessed by transcriptomic analysis of *Chd6*^{TEC^{-/-}} RNA-Seq data and qRT-PCR analysis) did not affect the IRBP gene. Furthermore, I did not observe any sign of autoimmunity in the retina of aged CHD6 mutant mice (details in 6.2) making it improbable to have specific autoreactive T cells against IRBP peptide in *Chd6*^{TEC^{-/-}} mice model. While the notion that the TCR-dependent proliferative advantage in the naïve T cells as the consequence of a loss of CHD6 in TEC was supported by the defect observed in medullary thymocyte negative selection, it nevertheless cannot be concluded with certainty. A definite solution to the interrogation of whether this

increased proliferative capacity is linked directly to the defect in negative selection of thymic-specific antigen would be possible if: i) similar phenomenon could be observed against a thymic-specific peptide of CHD6-dependent tissue-specific antigen or ii) antigen-specific autoreactive T cell is identified.

Additionally, the other main question which arises from the result presented here is, how might the result of the decreased medullary thymocyte negative selection which was observed in *Chd6*^{TEC^{-/-}} mouse model (see section 5.2.3) translate to a primary human inborn error of immunity? One main limitation in answering this with certainty, is that, currently, there is no known report of human patient with homozygous mutation involving CHD6, either as primary human inborn error of immunity or with any other clinical presentation. Hence, to partly address this question, reviewing the well-established and studied mouse model with the defect in promiscuous gene expression, namely the *Aire*-knock out mouse model, may throw some light to this question.

The *Aire*-knock out mouse model was first genetically developed back in 2002, in an attempt to develop a mouse model for the human disease, APS-1, whereby the patients suffered mutation in their AIRE gene¹³⁴. This mouse model, known as *Aire*^{-/-}, demonstrated signs of multiple-organ autoimmunity as evidenced by lymphocyte organ infiltration and presence of specific circulating autoantibodies¹³⁴. Nonetheless, although the phenotype observed in the *Aire*^{-/-} mouse model was mimicking the human disease, further detailed assessments showed some differences in their phenotype, especially within the context of the type of autoantigens involved²⁸⁵. Despite a possible similarity in the type of organs affected between mouse and human AIRE-deficient counterparts, molecular assessments have shown different autoantigens were affected upon AIRE/*Aire* deletion in these two species. Since then, there have been other different *Aire*-knock out mouse models which had been generated using different mouse genetic background and utilising different type of *Aire* mutations^{135,286,287}. In general, all the *Aire*-knock out mouse models, shared one similarity with their human AIRE-deficient counterpart, that is, all of them showed an impairment in the PGE and thymocyte negative selection to some extent^{134,135,285,288}. Yet, all of these mouse models showed variable penetration in their autoimmunity phenotype. Hence, based on this knowledge, it is imperative to deduce that a defect in medullary thymocyte negative selection shown here in my *Chd6*^{TEC^{-/-}} mice, may contribute to some degree

of autoimmunity phenotype in human. To further determine this, I have dissected my *Chd6*^{TEC^{-/-}} mice model for features of autoimmunity and this will be presented in the following chapter.

Overall, in this chapter, I have documented in detail the immunophenotyping and functional analyses of both thymocytes and peripheral T cells in *Chd6*^{TEC^{-/-}} mice, both in the young and older mice. The data demonstrated the direct effects of a TEC-specific loss of CHD6 expression on thymocyte maturation and its contribution to the peripheral T cell homeostasis. Cumulatively, the results presented here are consistent with the findings described in the previous chapter showing that the loss of CHD6 exclusively disrupts PGE in the mTECs, leading to a specific impairment in thymocyte negative selection in the medulla. This further emphasises CHD6's precise role as one of the regulatory partners of AIRE, in enabling the complex mechanism of PGE in the thymus.

Chapter 6 The consequences of CHD6 deletion in TEC on central tolerance induction

6.1. Aim

Impairment of PGE leads to the development of autoimmunity. This is evident by the immunopathology observed in APECED (aka APS1, OMIM#240300) and its related experimental models where cellular inflammatory infiltrates in multiple organs and the tissue-specific autoantibodies are detected secondary to a loss of AIRE expression^{132,134,135,285–287}. In this chapter, I hypothesise that the consequences of lacking of CHD6 expression in TEC results in deficient central T cell tolerance induction leading to a repertoire of incorrectly selected T cells, with the capacity to elicit organ-specific autoimmunity. I also propose that these autoimmune manifestations are involving tissues characteristically affected by AIRE deletion. To verify my postulation, *Chd6*^{TEC^{-/-}} mice were assessed for signs of spontaneous autoimmunity through the detection of serum autoantibodies and lymphocytic organ infiltrations.

6.2. Autoimmunity assessments in aged *Chd6*^{TEC^{-/-}} mice

Spontaneous autoimmunity was assessed in a set of 8 female *Chd6*^{TEC^{-/-}} mice and their gender- and age-matched wild-type (*Chd6*^{TEC^{+/+}}) controls at 8 months of age to increase chances of identifying autoimmunity features. Two approaches were conducted which include: i) detection of tissue-specific autoantibodies, and ii) evaluation of lymphocytic organ infiltrations (Figure 6.1). For detection of tissue-specific autoantibodies, sera collected via cardiac puncture in CHD6 mutant mice and their wild-type control were assessed for presence of autoantibodies indirectly using organ tissue sections of B cell deficient tissues followed by indirect immunofluorescence (see section 2.11). For detection of lymphocytic tissue infiltrates, organs were collected from aged wild-type and CHD6 mutant animals and analysed by histology using paraffin tissue embedding and H&E staining (see section 2.12).

The following organs were chosen and analysed: Liver, eyes, lungs, stomach, extra-orbital lacrimal gland, salivary gland, pancreas and ovaries (Figure 6.1). These organs were selected based on the previous studies which showed the presence of autoimmunity in these organs in different *Aire* knockout mouse models¹³³. Tissue infiltrates were scored according to a published system¹³³. In general, scores of 0, 0.5, 1, 2, 3, and 4 indicate no, trace, mild, moderate, or severe lymphocytic infiltration, and complete destruction, respectively¹³³. Both antibody reactivity and lymphocytic infiltrations were analysed by three independent observers who have no prior knowledge of the samples allocated to them. Inter-reader reliability assessments were initially performed using Fleiss' kappa, κ , where κ value range from ≤ 0 to 1, with $\kappa = 1$ indicates a perfect agreement and $\kappa \leq 0$ indicates a poor agreement between the observers (see section 2.10)²¹⁵. Inter-reader reliability, κ for detection of tissue-specific autoantibodies and lymphocytic organ infiltrations were 0.91 and 0.89, respectively, indicating a high degree of agreement between individual assessors.

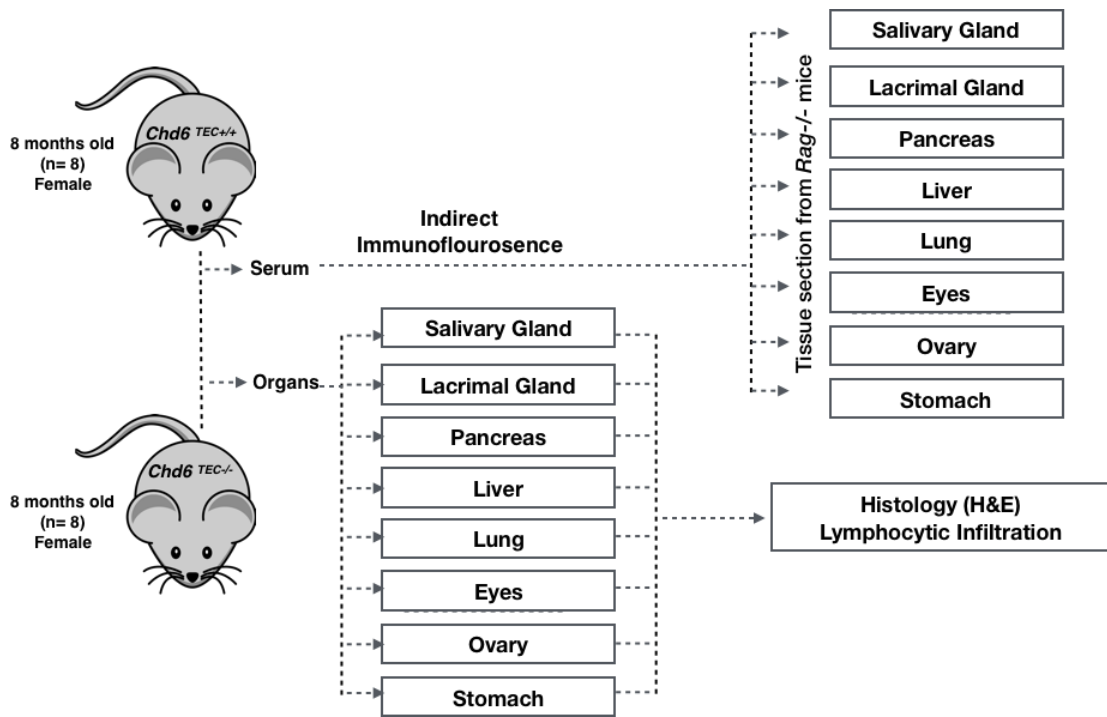


Figure 6.1 Spontaneous autoimmunity assessments in aged *Chd6* ^{TEC}^{-/-} mice

For histological assessment, collected organs from aged experimental mice (*Chd6* ^{TEC}^{-/-}) and their wild-type controls (*Chd6* ^{TEC}^{+/+}) were paraffin-embedded and HE-stained. Scoring of the extent of lymphocytic organ infiltrates were performed based on the previously described scoring system¹³³. For serum autoantibodies detection, pre-prepared slides with *Rag*-deficient organ tissue sections were incubated with diluted serum (1:20) obtained from aged experimental mice and their wild-type control. Antibody reactivity was revealed using immunofluorescence microscopy.

6.2.1. Loss of CHD6 in TEC leads to production of tissue-specific autoantibodies

Experiment for the assessment of organ-specific autoantibodies was conducted as outlined in the scheme above (Figure 6.1). Briefly, diluted sera (1:20) collected from the experimental cohort and wild-type mice were tested for the presence of autoantibodies on pre-prepared *Rag*-deficient organ tissue sections by immunofluorescence microscopy. Each mouse serum was incubated on three different tissue sections per organ, and the antibody reactivity was analysed by three independent observers (see section 2.11). As a prerequisite, serum IgG concentrations in the aged *Chd6*^{TEC-/-} mice in comparison to the wild-type control were determined using ELISA (see section 2.10). The data revealed that total IgG in aged *Chd6*^{TEC-/-} mice was comparable to the wild-type control (Figure 6.2a).

Autoantibodies against salivary glands were detected in 7/8 (87.5%) and 1/8 (12.5%) of the *Chd6*^{TEC-/-} and wild-type mice sera, respectively (Figure 6.2b). Five out of eight sera (62.5%) sera obtained from *Chd6*^{TEC-/-} contained autoantibodies with specificities against lacrimal gland, lung and/or stomach, where as the frequency of 25% (2 out of 8 *Chd6*^{TEC-/-} mice sera) were positive for autoantibodies against hepatocytes (Figure 6.2b). In comparison, autoantibodies against lacrimal gland and lung were detected only in one of the wild-type samples (Figure 6.2b). Collectively, autoantibodies were detected in all *Chd6*^{TEC-/-} mice, whilst only two out of the eight control animals were positive for these kind of autoantibodies. Additionally, comparison between individual CHD6 mutant animals also showed that the number of affected organs could vary considerably from one to five organs, indicating individual diversity (or variation) even in mice with similar genetic background (Figure 6.2b).

I also observed that the autoantibodies were confined mostly to specific anatomical structures within each of the organs affected, which is expected with the organ-specific autoimmunity in lieu of systemic autoimmunity. Positive reactivity was observed around the peribronchiolar structures of the lung, while in the salivary and lacrimal glands, the binding of the autoantibodies seem to be more prominent around the perivascular and the ductal epithelial regions (Figure 6.2c). Autoantibodies were also

detected in the periportal and gastric parietal cells in the liver and stomach, respectively (Figure 6.2c). The organ substructures of which these autoantibodies were reactive to, are identical to the substructures affected in AIRE knock-out mice, suggesting that seemingly comparable specificities of autoantibodies were generated in *Chd6*^{TEC^{-/-}} mice^{132–134}.

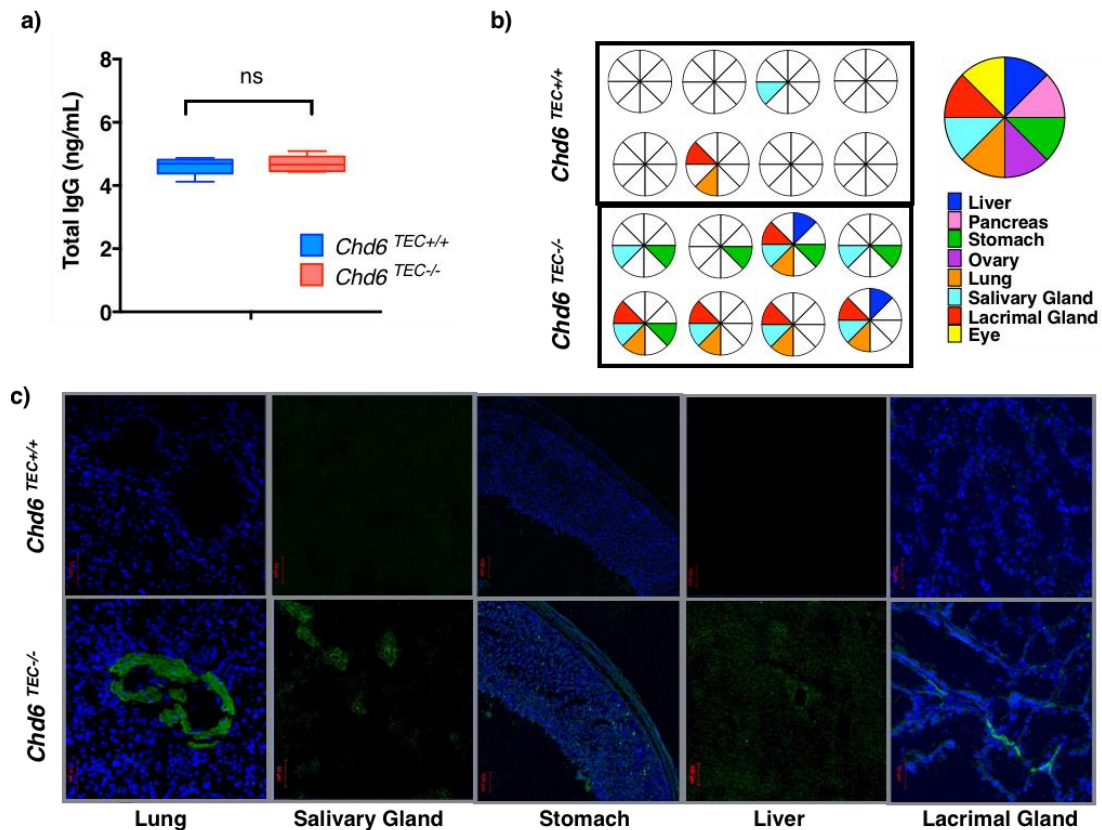


Figure 6.2 Total IgG concentrations and tissue-specific-autoantibodies and in aged *Chd6*^{TEC^{-/-}} mice
a) IgG concentrations as determined by ELISA in aged *Chd6*^{TEC^{-/-}} and wild-type (*Chd6*^{TEC^{+/+}}) mice. The box centres are medians; the box limits the 25th and 75th percentiles, the whiskers represent the minimum and maximum values. P-values were determined by Mann-Whitney U; ns signifies not significant, i.e. $p > 0.05$. Total experiment size: $n = 16$ (*Chd6*^{TEC^{+/+}} $n = 8$, *Chd6*^{TEC^{-/-}} $n = 8$). **b)** Detected tissue-specific autoantibodies in aged *Chd6*^{TEC^{-/-}} mice and their wild-type control. Each circle represents individual mice, with colour filled segments corresponding to the organs indicated in the key filled. **c)** Autoantibodies as revealed by FITC-labelled anti-mouse IgG (green) staining. *Rag*-deficient mouse tissues sections of lung, salivary gland, stomach, liver and lacrimal gland pre-incubated with serum from individual 8 month old *Chd6*^{TEC^{-/-}} mice and their wild-type control (*Chd6*^{TEC^{+/+}}) mice. Nuclei were counterstained with DAPI (blue). Shown are representative images of stained individual tissues sections using sera from 8 mice for each genotype.

6.2.2. Lymphocytic organ infiltrations detected in *Chd6*^{TEC-/-} mice

Assessment for organ lymphocytic infiltrations was conducted as outlined (Figure 6.1). In short, organs from aged *Chd6*^{TEC-/-} wild-type mice were harvested, paraffin-embedded and analyzed by H&E staining whereby for each organ at least four sections were prepared and scored. Sections were scored for lymphocytic infiltrations by three “blinded” observers (see section 2.12). The scores shown here (Figure 6.3below) represent the average value of three separate assessments of a given section using the previously published scoring system¹³³.

Lymphocytic infiltrations were observed in all of the eight analysed *Chd6*^{TEC-/-} mice either in the salivary glands, lacrimal glands, lungs and/or livers whereas such changes could only be detected in the salivary gland of a single wild-type mouse (Figure 6.3). Coincidentally, it was the same wild-type mouse that had exhibited autoantibodies to the salivary gland (Figure 6.4b). As observed in the organ-specific autoantibodies assessment, the numbers of affected organs differed between individual mutant mice. All mutant mice (8/8) demonstrated salivary gland infiltrations of different extent, 5/8 (62.5%) had demonstrated changes in their lacrimal glands and in 3/8 (37.5%) infiltrates in the liver and/or lung were detected (Figure 6.3). The lymphocytic infiltrates were generally mild (average histology scores were between 1-2), except in the salivary glands where moderate infiltrations (histology score of 3) were observed (Figure 6.3below).

The lymphocytic infiltrates were confined to certain particular organ anatomical structures rather than being evenly distributed throughout the organs, similar to the observation in organ-specific autoantibodies (Figure 6.3top). In the salivary and lacrimal glands, infiltrates were typically detected perivascularly and periductally which are sites where inflammatory changes occurred in *Aire*-deficient mice and patients with primary Sjogren’s syndrome^{132,133}. The pathology in the lung was concentrated around the small airway, consistent with the features observed in mouse models of autoimmune lung disease^{135,285,289}. The infiltrations observed in the liver were accumulated around the periportal region (Figure 6.3top), compatible with changes observed in autoimmune hepatitis manifesting in *Aire*-deficient mice and APECED patients^{132,134,135,285}.

Not all organs which were affected in *Aire*-deficient mice models showed positive infiltrations in the mutant CHD6 mice¹³⁵. This was not unexpected, given the fact that CHD6 is only regulating a fraction of TRGs expression that is classically regulated by AIRE. For example, lymphocytic infiltrates of the retina which is a typical autoimmune feature in *Aire*-deficient mice, was not observed in *Chd6*^{TEC-/-} mice^{132–135}. This complication in *Aire*-deficient mice is the consequence of a reduction in the AIRE-dependent expression of IRBP²⁸⁴. The absence of a retinal pathology in *Chd6*^{TEC-/-} mice is in line with the finding that transcriptions of *Irbp* were unchanged in both wild-type and CHD6 mutant mature mTEC.

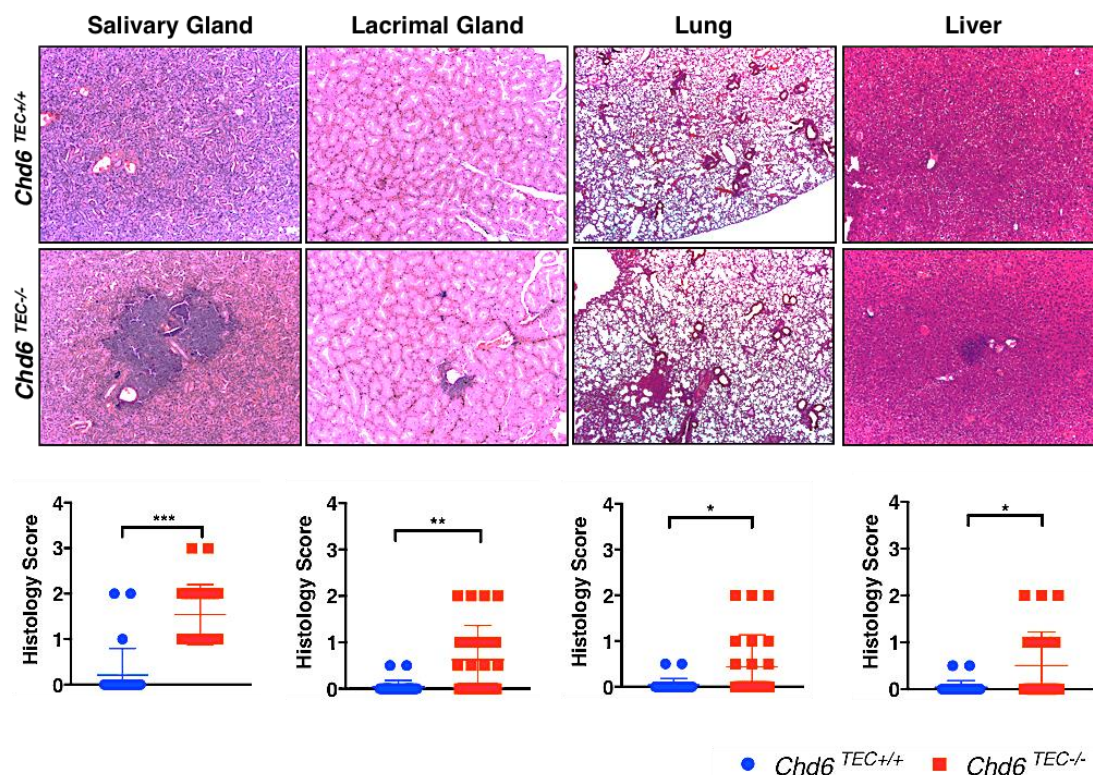


Figure 6.3 Multi-organ lymphocytic infiltrations in aged *Chd6*^{TEC-/-} mice

Top: H&E staining of salivary gland, lacrimal gland, lung and liver tissue sections for the detection of mononuclear infiltrates in 8-month-old *Chd6*^{TEC-/-} and control mice. Histological images are representative for analyses of each genotype. Histological scoring of the lymphocytic organ infiltrations was performed based on the previously described scoring system. **Below:** The whisker plots displays the mean values and standard deviations of the histological scores of the lymphocytic infiltrations for each organ. Each score of an individual tissue is represented as a single symbol where by the average score is shown of three separate assessments each provided by three individual observers. *p*-values were calculated by Student-t-test, where * = *p* < 0.05, ** = *p* < 0.01 and *** = *p* < 0.001.

6.3. CHD6 positively-controlled genes encode auto-antigen in APECED and *Aire*-deficient mice

The loss of expression of tissue-specific antigens in mTEC is correlated with the development of autoimmunity^{105,284,285,287}. To determine whether CHD6 controlled genes also encode the auto-antigens in APECED patients and/or *Aire*-deficient mice, gene transcripts from the RNA-Seq data were analysed. List of described auto-antigen in APECED patients and *Aire*-deficient mice were compiled and genes encoding these auto-antigen were analysed using transcriptomic data of *Chd6*^{TEC-/-} mice. Only genes that showed more than 1.2-fold reduction in *Chd6*^{TEC-/-} were included in the analysis and presented (Table 6.1).

From the analysis, results showed that the *Cyp1a2*, *Itih3*, *Klk1b22*, *Atp4*, *Sox19*, *Trp1* and *Trim38* were significantly down-regulated in mTEC^{hi} of *Chd6*^{TEC-/-} (Table 6.1). The significant differential expression of 8.7-fold reduction in *Trim38* was observed (Table 6.1). *Trim38* encodes E3-ubiquitin-protein ligase, which is the target of autoimmunity in Sjogren's syndrome. The presence of anti-TRIM38 antibodies in Sjogren's patients was closely associated with the increased severity of dry eye as results of extensive dacryoadenitis²⁹⁰. Inter- α -inhibitor H3 chain (encoded by *Itih3*) and Kallikrein 1-related peptidase b22 (*Klk1b22*) are also auto-antigens present in both salivary and lacrimal glands which resulted in autoimmune manifestations as observed in *Aire*-deficient mice and also patients with Sjogren's syndrome^{132,291}.

Cytochrome P450 1A2 or CYP1A2 (encoded by *Cyp1a2*), a member of the cytochrome P450 superfamily, was discovered as liver autoantigen in APECED patients^{292,293}. Deletion of CHD6 in mTEC also down-regulated the expression of *Atp4* which encodes gastric specific-proton pump and is a target autoantigen in APECED patients with autoimmune gastritis²⁹⁴. Sox10 and Trp1, both indicated as autoantigens responsible for development of vitiligo in APECED patients, were also significantly impaired in their expressions in mTEC of *Chd6*^{TEC-/-} mice^{288,295}. Apart from skin-related autoimmunity which was not assessed as part of the work presented here, the auto-antigens described here were correlated with organ-specific autoimmune pathology observed in aged *Chd6*^{TEC-/-} mice.

Autoreactive T-lymphocytes from *Aire*-deficient mice responded to vomeromodulin, an auto-antigen of the respiratory tract, which may cause interstitial pneumopathy²⁸⁹. I observed more than 2-fold reduction in the number of transcripts for vomeromodulin (encoded by *Bpifb9a*) in *Chd6*^{TEC-/-} mice, albeit statistically not significant. Genes encoding pancreas-specific auto-antigens, such as *Gad* and *Ins2* were mildly affected (with the exception of *Ica1*) with none of them showing statistical significance. This clearly correlates to the absence of autoimmunity features in the pancreas of mutant CHD6 mice²⁹⁵. Genes expressing auto-antigens in kidney and thyroid were also found to be down-regulated in the deletion of CHD6 in mTEC, despite being at a lower degree and without statistical significance. Collectively, these findings suggest that defects in TRG expression in mice lacking CHD6 expression in TEC resulted in the comprehensive lack of central T cell tolerance induction.

Table 6.1 Selected expression of AIRE-controlled loci in mature mTEC of 4.5 week old *Chd6*^{TEC-/-} mTEC compared to wild-type cells[±]

Autoantigen	Gene	Organs affected (Associated Disease)*	Fold-reduction	Adjusted p-value
Cytochrome P450 1A2 ^{^§}	<i>Cyp1a2</i>	Liver (AIH)	2.42	<0.001
Inter- α -inhibitor H3 chain [^]	<i>Itih3</i>	Salivary (SS), Liver (AIH)	2.52	0.002
Kallikrein 1-related peptidase b22 ^{^§}	<i>Klk1b22</i>	Lacrimal, Salivary (SS)	1.72	0.006
Gastric ATPase H+/K+ subunit α ^{^§}	<i>Atp4</i>	Stomach (AIG)	1.67	0.009
SRY-Box 10 [^]	<i>Sox10</i>	Skin (Vitiligo)	6.80	0.034
Tyrosinase-related protein 1 ^{^§}	<i>Trp1</i>	Skin (Vitiligo)	1.76	0.046
E3 ubiquitin-protein ligase ^{^§}	<i>Trim38</i>	Lacrimal, Salivary (SS)	8.70	0.05
Islet cell autoantigen 1 ^{^§}	<i>Ica1</i>	Pancreas (T1DM)	4.13	0.074
Interleukin-17F [^]	<i>Il17f</i>	Inflammatory AID	4.00	0.177
Lgp10 [§]	<i>Lgp10</i>	Lacrimal (DA)	3.00	0.280
Vomeromodulin [§]	<i>Bpifb9a</i>	Lung (ILD)	2.28	0.321
α -1-microglobulin bikunin [§]	<i>Ambp</i>	Liver, kidney	1.52	0.146
Tyrosine Phosphatase ^{^§}	<i>Ptprn</i>	Pancreas (T1DM)	1.45	0.347
T cell Ig mucin-domain protein 2 [§]	<i>Timd2</i>	Liver, kidney	1.37	0.406
Preproinsulin-II [§]	<i>Ins2</i>	Pancreas (T1DM)	1.34	0.333
Glutamate Decarboxylase 2 ^{^§}	<i>Gad2</i>	Pancreas (T1DM)	1.31	0.773
α -fodrin ^{^§}	<i>Sptan1</i>	Lacrimal (DA)	1.25	0.178
Glutamate Decarboxylase 1 [^]	<i>Gad1</i>	Pancreas (T1DM)	1.24	0.433
Tyrosine Hydroxylase [^]	<i>Th</i>	Scalp (Alopecia)	1.24	0.398
Kallikrein-1 [§]	<i>Klk1</i>	Liver, kidney	1.24	0.486
Thyroid Peroxidase ^{^§}	<i>Tpo</i>	Thyroid (HT)	1.23	0.559

[±] Gene expression (from bulk RNA-Seq in mature mTEC of 4.5 week old *Chd6*^{TEC-/-} mice) are presented according to their fold-reduction calculated in reference to corresponding wild-type value. p-values calculated by Benjamini-Hochberg correction are shown. Genes with more than 1.2-fold reduction yet do not achieve statistical significance are in blue shaded area.

Autoantigen described as targets of autoimmunity in humans (^) and mice (§)

*SS=Sjogren's syndrome, AIH=Autoimmune Hepatitis, ILD= Interstitial lung disease, AIG=Autoimmune Gastritis, DA=Dacryoadenitis, T1DM =Type 1 Diabetes Mellitus, HT=hypothyroid

6.4. Summary and Discussion

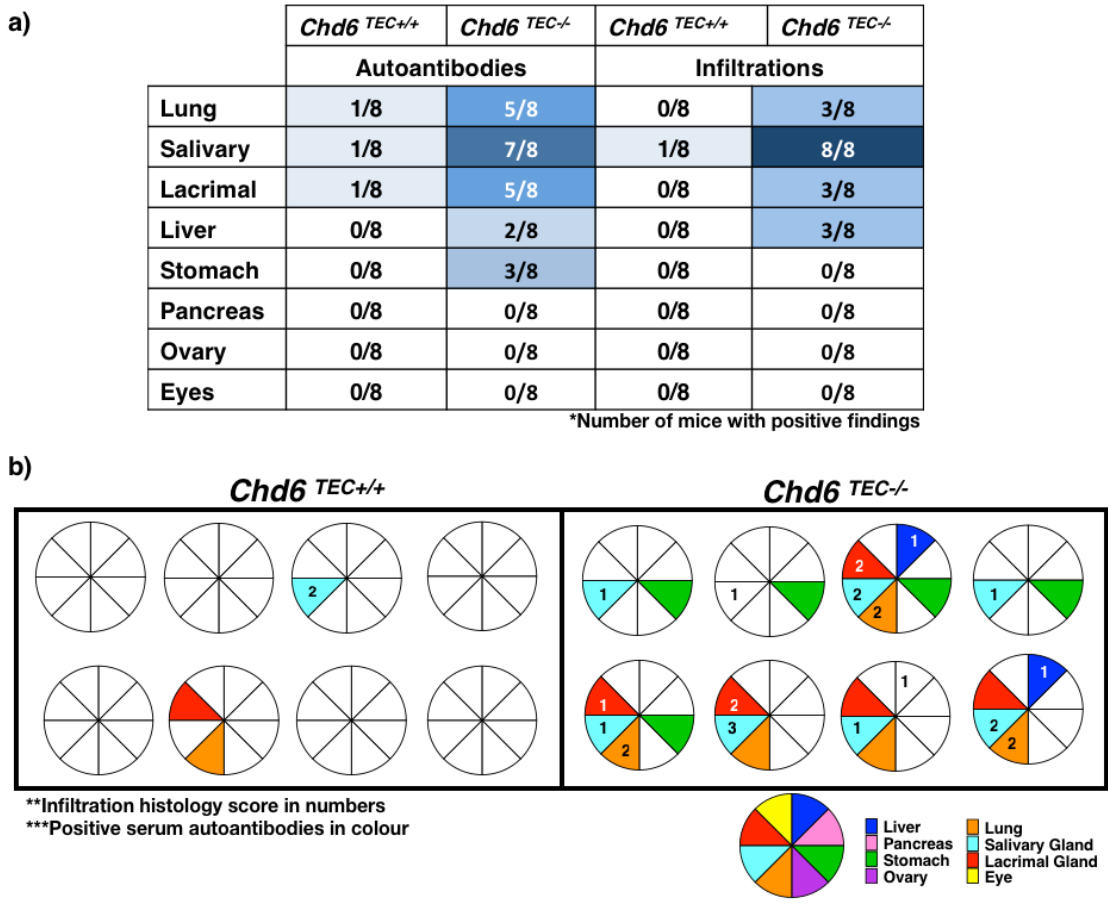


Figure 6.4 Summary of spontaneous autoimmunity findings in aged *Chd6*^{TEC-/-} mice
a) Frequency of wild-type and mutant mice with autoantibodies and mononuclear tissue infiltrates of indicated organs. **b)** Summary of positive autoantibodies and histopathology from aged *Chd6*^{TEC-/-} mice and their wild-type control. Each circle represents individual mouse, with colour filled segments corresponding to the positive serum autoantibodies and the numbers indicating histopathology scoring for organs indicated in the key filled.

Older female animals were studied for the occurrence of spontaneous autoimmunity as the number of organs and pathology severity increase with age, especially in female animals^{133–135}. Moreover, our transcriptomic data sets for comparative analysis of wild-type and AIRE-deficient mice have exclusively been established for female mice. It is however to be assumed that fundamentally comparable results will be obtained studying male animals.

Serological analysis of all the tested *Chd6*^{TEC-/-} sera contained at least one autoantibody; with more than 85% (7 out of 8 sera tested) exhibit reactivity to two or more of the eight target organs tested (Figure 6.4). In contrast, none of the wild-type

control mice showed autoreactivity to more than one organ (Figure 6.4). Development of autoantibodies requires cognate B cell/ CD4 T cell interactions to initiate the production of immunoglobulin^{296,297}. Tissue-specific autoantibodies in autoimmune diseases are shown to be derived through CD4 T cell dependent pathways, as evidence by the presence of autoreactive CD4 T cell driving the loss of B cell tolerance²⁹⁸. Since I have shown that negative selection in CD4 thymocytes was defective in *Chd6*^{TEC^{-/-}} mice (see section 5.2.3 and 5.4.1), it is plausible that the generation of organ-specific autoantibodies in the CHD6 mutant mice is driven by the autoreactive CD4-T cell.

Additional autoimmune assessment by lymphocytic organ infiltrations revealed a similar pattern, with more organs being affected in *Chd6*^{TEC^{-/-}} mice (Figure 6.4). As autoimmune organ infiltration is mediated by autoreactive CD8 and CD4 T cell, it is reasonable to imply that a similar defect in negative selection of CD8 thymocytes may be responsible for the autoimmune phenotype. There was a clear correlation between organs manifesting lymphocytic infiltrates and those targeted by autoantibodies in *Chd6*^{TEC^{-/-}} mice (Figure 6.4b), indicating a defect in the induction of tolerance towards the same tissue-specific antigens. Taken together, the findings imply that both the negative selection of CD4 and CD8 thymocytes were affected in similar degree. However, due to the paucity of tools available to assess negative selection of CD8 thymocytes, this hypothesis needs to be verified independently.

Most of the APECED patients develop reactivity towards auto-antigen present in Langerhans islets, particularly against glutamic acid decarboxylase (GAD) and the aromatic L-amino acid decarboxylase (AADC), which are the causative auto-antigens in diabetes^{288,295,299}. However, analysis of the transcriptomic data revealed that the expression of the genes encoding GAD and AADC were intact in CHD6-deficient TEC. Indeed, this correlated well with the absence of autoimmunity manifestation involving the pancreas in mutant CHD6 mice (Figure 6.4).

As mentioned earlier, I noted that the autoimmunity features were confined within specific organ anatomical structures in *Chd6*^{TEC^{-/-}} mice. Moreover, the autoantibodies and the lymphocyte infiltrates in the same organ exhibit comparable target distribution (Figure 6.2c and Figure 6.3top). APECED and *Aire*-deficient mice are known to develop autoimmunity against specific-tissue antigens, and these auto-antigens located

specifically in these substructure in the organ where the reactivity took place^{132,134,135,285}. In agreement with this, analysis of the transcriptomic data revealed that TRGs encoding auto-antigens in the affected substructures were down-regulated in mutant CHD6 mice (Table 6.1). The similarity of substructures affected in the *Chd6*^{TEC^{-/-}} mice to the ones reported in APECED patients and *Aire*-deficient mice models was mainly due to the consequence that CHD6 is affecting the same loci regulated by AIRE.

The mouse genetic background strongly influences the penetration of higher susceptibility for autoimmunity. For example, *Aire*-deficient mice in a NOD background display severe autoimmunity when compared to *Aire* knockout mice generated on B6 background¹³⁵. Therefore, it is conceivable that the mild autoimmune manifestations exhibited in *Chd6*^{TEC^{-/-}} mice were partly due to the B6 background of the mice used for these experiments. Since CHD6 is utmost only involved in 10% of *Aire*-regulated TRGs, the resultant disruption in central tolerance induction is expected to be limited to only a fraction of tissue-restricted antigens. Although AIRE is primarily expressed in the thymus, detection of AIRE in several peripheral tissues including B cells, has been documented^{145,146}. Nonetheless, the functional property of this extra-thymic AIRE cells on induction tolerance has been inconclusive^{145,146}. As I specifically deleted CHD6 in *Foxn1*-expressing cells, the functional interaction between AIRE and CHD6 in these other AIRE-expressing organs cannot be excluded. If extra-thymic AIRE contributes to the induction of tolerance, the interaction of CHD6 with AIRE in these extra-thymic organs could possibly compensates the functional disturbance of PGE in the thymus.

In summary, the occurrence of spontaneous autoimmunity in older *Chd6*^{TEC^{-/-}} mice is compatible with a defect in central T cell tolerance induction. As observed in *Aire*-deficient mice, the *Chd6*^{TEC^{-/-}} mice model shows multiorgan autoimmunity as marked by lymphocytic infiltrates and recognition by serum autoantibodies despite a milder phenotype. The number of organs affected by the absence of CHD6 is restricted because of the breadth of genes requiring CHD6 for their promiscuous expression is also significantly smaller when compared to that of *Aire*-controlled loci.

To respond to a theoretical question that may arise, if ever there was a 100% fidelity between *Chd6*^{TEC^{-/-}} mice and a human disease, what would the expected human phenotype be? Learning closely from a number of widely studied *Aire*-knock out

mouse models which have been generated, firstly, I am well aware that the fidelity between human and mouse phenotype is not 100%^{134,135,286,288,300}. Extensive molecular studies done on different *Aire* -knock out mouse models and their human APS-1 counterpart, have shown that different autoantigens were affected^{135,286,301}. However, although different autoantigens were affected, it was clearly shown that the deletion of *Aire* gene in mouse, phenocopy human APS-1 disease in certain extent, with generally a milder phenotype in the mouse model^{134,135,285,287,300}. Hence, deriving from *Chd6*^{TEC}^{-/-} mouse system that I have studied, I predicted that human lacking the CHD6 gene would be expected to be presented with features of autoimmunity involving namely the: (i) lung; (ii) liver; (iii) stomach; (iv) salivary; and (v) lacrimal glands. The human clinical phenotype, based on my prediction, will be presenting with features of Sjogren's syndrome, autoimmune gastritis, autoimmune hepatitis and/or interstitial lung disease, either in combination or as a single clinical entity.

To the best of my knowledge, there is no existing literature documenting a naturally occurring homozygous mutation of CHD6 which causes significant disease in humans. Up to this date, only a single case of a female patient, with severe mental retardation and brachydactyly of the toes who had been implicated due to haploinsufficiency of CHD6³⁰². In this reported case, a balanced translocation in intron 27 of CHD6 at 20q12, was shown to reduce the CHD6 mRNA expression up to 50% in the patient's lymphoblastoid cells³⁰². Additionally, whole genome analysis of numerous cancer tissues and tumour cell lines have reported numbers of point mutations within CHD6 region and gene copy number variations^{205,303,304}. However, these reported mutations and gene copy number variations were occurring at low frequencies, hence unlikely to be the primary contributor to the development of these cancers.

Although there is a lack of available data to implicate CHD6 mutations in the development of autoimmune diseases, my current study suggests a possibility of CHD6 as a driver for autoimmunity. Hence, it will be important to study the presence of CHD6 polymorphism or hypomorphism among patients with autoimmune diseases or autoimmunity features. *Chd6*^{TEC}^{-/-} mouse model had been shown in my study, to be closely mirroring *Aire*-knock out mouse model albeit a milder phenotype. As *Aire*-knockout mouse model phenocopy autoimmunity changes found in human with APS-1, mining of the available genome-wide association study (GWAS) databases,

particularly among cohort of patients with autoimmune features involving organs which were documented to have autoimmunity changes in *Chd6*^{TEC^{-/-}} mouse, will be an appropriate starting point. This proposed GWAS database exploration, may unravel single nucleotide variations or copy number variations involving CHD6, which may suggest the role of CHD6 in the development of autoimmunity in human.

Chapter 7 Conclusion and future work

7.1. Conclusion

This thesis documents the work that was conducted in understanding the role of CHD6 in TEC biology. Most of the work presented here was dissected using *Chd6*^{TEC^{-/-}} mouse model, a newly established conditional knock-out mice with a TEC specific deficiency of CHD6. In this setting, *Chd6*^{TEC^{-/-}} mice served as a perfect tool to investigate the role of CHD6 in TEC and its impact on thymopoiesis, and subsequently the integrity of adaptive immune function. *In-vitro* works utilising transfected TEC-specific cell line were also performed to provide additional understanding in the interaction between CHD6 and AIRE; one of the proteins of interest in thymus biology. To date, this is the first-ever study to look at the specific function of CHD6 in thymus biology.

The main finding of the study revealed that the targeted loss of CHD6 in TEC contributed to specific defect in PGE, in particular of AIRE-regulated TRGs. Although CHD6 seems to be impacting the transcription of only a fraction of these TRGs (around 12% of the total AIRE-regulated TRGs), the associated partnership between CHD6 and AIRE is indeed true and strong. Parallel to this notion, CHD6 is observed to discreetly localise with AIRE in mTEC. This finding is in agreement with the earlier proposal suggesting CHD6 as one of the protein partners of AIRE. CHD6 also appeared to be associated with loci decorated with H3K27me3 in mature mTEC (mTEC^{hi}). AIRE-regulated loci in mTEC^{hi} are shown to be associated with similar histone modifications changes and previous publications had indicated that these histone PTMs are the key mechanisms utilised by AIRE in enabling PGE in the large number of different loci. However, as AIRE is not able to directly bind with H3K27me3, this signifies the role of CHD6 as the mediator binding partner of both H3K27me3 and AIRE, thus allowing the transcription regulation of AIRE to the loci decorated with this histone PTM.

Although the findings seem to be largely in keeping with the proposed hypothesis of CHD6 as the protein partner of AIRE, one particular question which needs to be addressed is: If CHD6 identifies H3K27me3 and relocates AIRE protein complex to that particular loci, why was the observed defect in PGE limited to a certain number of

genes when the decorated loci with H3K27me3 are numerous. Thus, the mechanisms on how CHD6 decides which loci to bind and localise the AIRE protein complexes were studied. In this context, other than the technical limitations, which were discussed earlier (see section 4.8) one finding partly revealed and explained the phenomenon. The loci positively controlled by CHD6 did not just possess enrichment of H3K27me3 but were equally depleted of active histone marks namely H3K9ac and H3K4me3. This histone PTMs combination appears to be physiologically associated with an inactive locus in other cell type. Thus, it is plausible that in the context of PGE in mTEC, loci with this specific histone PTMs combination require CHD6 to change their accessibility to allow gene transcription machinery to take place.

To date, very little information has been published on CHD6 in regards to its property and function. Nonetheless, in the context of DNA damage response, CHD6 was reported to play a crucial role in localising and recruiting the DNA repair complexes to the DNA damage sites. In line with that finding, my study showed that the lack of CHD6 in TEC disrupted the localisation of AIRE on the site of DNA break in mTEC. As DNA break repair is one of the earlier steps in the mechanism of PGE by AIRE, the inability of AIRE to relocate to this DNA break site will interfere with the transcription of these loci. Taken together, this implies that CHD6 is required in recruiting AIRE protein complexes to the loci: i) undergoing DNA damage response and ii) possessing specific histone PTMs combination namely the enrichment of H3K27me3, and depletion of H3K4me3 and H3K9ac (Figure 7.1).

In addition, CHD6 as a chromatin remodeler had been shown to modify the chromatin accessibility of the loci it binds to^{201–203}. As most of the TRGs exist physiologically in heterochromatin, it is imperative that in the context of PGE, upon binding to the loci with the specific histone PTMs combination, CHD6 changes the chromatin landscape to be more accessible for transcription to occur (Figure 7.1). Currently, experimental work is in progress to verify this notion (see section 7.2.2). Based on these studies and the proposed mechanisms, CHD6, to the best of my knowledge, is the only protein, which is suggested to mediate PGE in such a novel approach.

Consequently, the impairment of PGE in *Chd6*^{TEC-/-} was shown to be directly and specifically affecting the thymocyte negative selection in thymic medulla, potentially

resulting in the development of autoreactive T cells. The current thought is that the escape of these undeleted autoreactive T cells from the thymus may induce autoimmunity in the host tissues. Supporting this notion, aged *Chd6*^{TEC-/-} mice had been shown to significantly exhibit multiple organ-specific autoimmunity, albeit a milder phenotype than those documented in AIRE knock-out mouse.

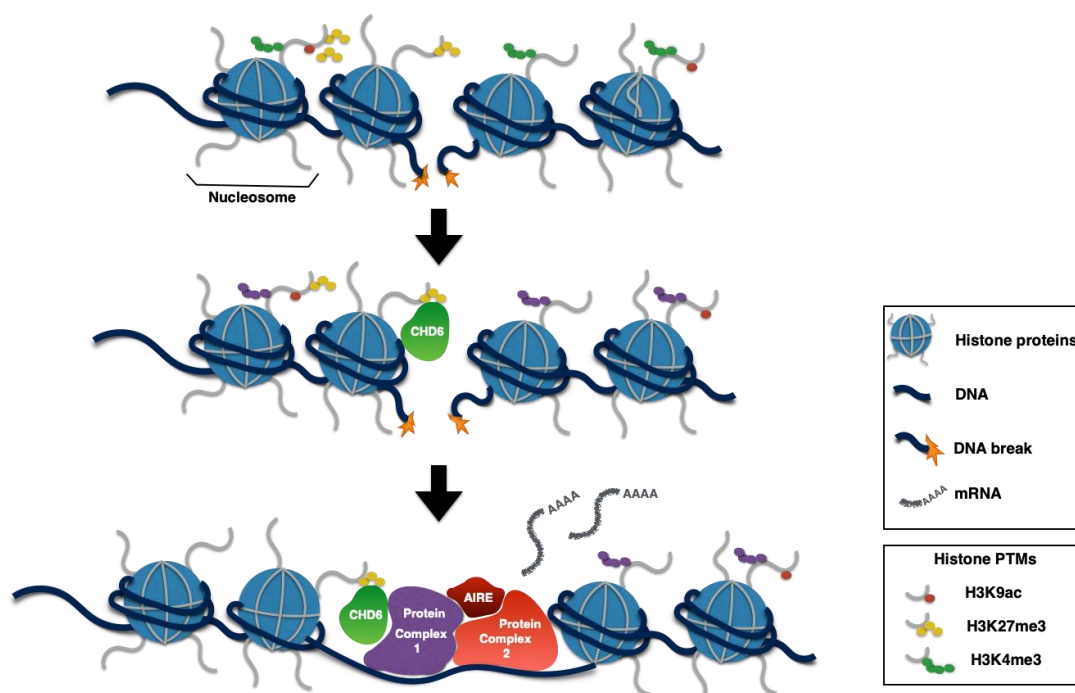


Figure 7.1 Proposed molecular mechanism of CHD6 in enabling PGE in TEC

In mature mTEC, loci positively controlled by CHD6 are mainly TRGs, which are: i) enriched with H3K27me3 and ii) depleted of H3K9ac and H3K4me3, indicating that CHD6-dependent loci consist of inactive genes that generally exist as heterochromatin in the nucleus. CHD6 via its double chromodomain region is able to bind specifically to H3K27me3. CHD6 also identifies and localises itself to DNA region possessing double strand break through its partnership with PAR protein. Upon localisation to these loci, CHD6 recruits its protein partners (visualised as Protein complex 1) to these DNA damage sites, which, among others, includes molecules that are involved in DNA damage repair such as topoisomerases and DNA-PKs. Some of these proteins are epistatic to AIRE and also attract AIRE to the loci. The presence of AIRE within the large protein complex, combined with the specific histone PTMs combination (enrichment of H3K27me3, depletion of H3K9ac and H3K4me3), induces CHD6 to change the existing chromatin landscape to be more accessible. The change in the chromatin accessibility by CHD6 is crucial in allowing other AIRE protein partners (visualise as protein complex 2) to be recruited to the loci and permitting transcription to occur.

Histology and immunophenotypic analyses in both young (at 4.5 week old) and older mice (at 8 month old) *Chd6*^{TEC-/-} mice demonstrated no significant differences in thymic size, thymic architecture and the frequency of total TECs, cTECs or mTECs.

Furthermore, no significant changes were observed in the number of total thymocytes and their frequencies at different maturational stages between *Chd6*^{TEC+/+} and *Chd6*^{TEC-/-} mice. This strongly suggests that CHD6 is not essential in the development of TEC. These observations are not unexpected as: i) CHD6 is yet to be implicated in any tissue developmental process, and ii) studies with conventionally generated CHD6 knockout mouse model reported to be developmentally normal¹⁹⁷. In contrast, inactivation of CHD4 in mouse TEC had been shown to disrupt the formation of a normal thymus (F.Dhalla, unpublished), implying the non-redundant role of different CHD proteins in modulating the changes in chromatin landscape needed for a specific cellular requirement. The lack of changes in TEC and thymopoiesis activities except for the very specific defect in PGE displayed by *Chd6*^{TEC-/-} mouse, makes it a perfect mouse model to unleash the molecular mechanism that allows PGE to occur in the thymus. Hence, in the context of thymus biology, CHD6 is a remarkable chromatin remodeling protein, as it appears to be exceptionally specific in its function to enable PGE in TEC via its partnership with AIRE.

7.2. Future work

Further research should focus on: i) determining the involvement of CHD6 at the controlled loci, ii) elucidating its role in causing the epigenetic changes needed for its function, iii) verifying the interaction between CHD6 and AIRE, and iv) identifying other CHD6 partner(s) specifically in TECs. Some of these works are already in progress.

7.2.1. Chromatin immunocleavage (ChIC)-sequencing of CHD6 proficient mTEC^{hi}

ChIC of CHD6 proficient mTEC^{hi} cells obtained from wild-type mice, coupled with deep sequencing will be used to investigate whether CHD6 binds to transcriptional start sites of the controlled loci. This information will allow us to document the mechanism CHD6 taken in enabling the expression of the controlled genes. The conventional ChIP requires a large number of cells or in the case of low cell number

ChIP, the investigated protein needs to be in abundance. As the expression of CHD6 is low in mTEC, the conventional ChIP approach requires pooling of cells from a large number of mice to provide the needed biological samples for this study. Cleavage Under Targets and Release Using Nuclease (CUT&RUN) is a ChIC antibody-nucleases approach in chromatin profiling that allows quantitative high resolution chromatin mapping *in-situ*. The main advantage in utilising CUT&RUN in comparison to the conventional ChIP is that, the chromatin cleavage strategy only occurs around the protein binding sites *in-situ* contributing to lower background sequencing. Hence, this allows a significantly lower number of cells needed to adequately prepare the sample for the subsequent sequencing analysis. This CUT&RUN technique is currently being optimised for primary murine TEC in our laboratory. For the mentioned aim, at least three biological samples of female wild-type mice at 4.5 week old will be used for this ChIC-Seq analyses.

7.2.2. Assessment of chromatin accessibility in mTEC^{hi} of *Chd6*^{TEC-/-} mice by assay for transposase-accessible chromatin, followed by deep sequencing (ATAC-seq).

I hypothesise that CHD6 promotes chromatin accessibility at AIRE-regulated tissue restricted loci. To further elucidate the CHD6-dependent mechanisms which poise tissue specific genes in mature mTEC (mTEC^{hi}), the chromatin accessibility to determine whether tissue-specific loci positively/or negatively controlled by CHD6 to become accessible will be examined. Using ATAC-seq of mTEC^{hi} in *Chd6*^{TEC-/-} mice and wild-type control at 4.5 week old, chromatin accessibility landscape in relation to the transcriptomes for tissue specific genes can be defined (using the generated RNA-Seq data). However, this approach using the conventional bulk ATAC-Seq may not be suitable to specifically validate the proposed hypothesis. Loci which are positively controlled by CHD6 consist mainly of TRGs. Therefore, the frequency of these loci being transcribed at the population level are generally low (around 3-5% from the whole mTEC population). Hence, the analysis of genome-wide chromatin accessibility using bulk preparation of cells will not be able to provide the resolution required to define the chromatin accessibility of these CHD6-controlled loci. In order to prove this hypothesis, the combined single cell ATAC-Seq and single cell RNA-Seq (scATAC-plus-RNA-Seq) need to be performed concurrently in the same cell to permit detailed

understanding of gene expression profile in relation to their chromatin accessibility. This scATAC-plus-RNA-Seq approach has been described, yet the application in primary cell is very limited and still requires further optimisation, which is currently underway before it can be utilised for the purpose of future study.

7.2.3. Proximity ligation imaging cytometry (PLIC) of CHD6 and AIRE in mTEC^{hi} of wild-type mice

Results presented in Chapter 3 have shown the proximity of CHD6 to AIRE proteins in mature mTEC by using high-resolution microscopy. However, I was unable to prove the physical interaction of AIRE and CHD6 using the conventional methods. Hence, other methods should be explored to verify and ascertain these protein-protein interactions. As previously discussed (see section 3.7), PLIC is a feasible and robust method that will be used to further study the interactions between AIRE and CHD6 in murine thymus. This study is currently in progress.

7.2.4. Identification of CHD6-associated proteins in murine mTEC

CHD6 is associated with multiple chromatin-modifying and -remodeling complexes. Large scale immunoaffinity chromatography and mass spectrophotometry studies in the human embryonic carcinoma cell line NTera-2 revealed a network of proteins involves in nucleosome remodeling, chromosome organization, RNAPII-mediated transcription and histone modification. Interestingly, most of the proteins associated with CHD6 also overlapped with those that are proposed to be protein partners of AIRE. Identifying CHD6-associated proteins in murine mTEC will enable identification of the different mechanistic pathways, which involve CHD6 in facilitating PGE in the mTEC. To gain insight into molecular partners associated with CHD6 in the mTEC, I propose to perform mass spectrometry of proteins eluted by immunoprecipitation of nuclear enriched CHD6 complex from mTEC^{hi} cells sorted from wild-type mice.

7.3. Final remarks

Collectively, the findings shown in this thesis demonstrate the essential role of CHD6 in the machinery of PGE in the thymus. The knowledge gained from the present works provides further insight into understanding the molecular mechanism of PGE processes in the thymus. I have presented here a comprehensive immunophenotypic description of *Chd6*^{TEC^{-/-}} mouse, a newly generated mouse model with a lack of CHD6 expression specifically in TECs. The *Chd6*^{TEC^{-/-}} mouse represents a solid mouse model to document a specific defect in the PGE, which leads to development of autoimmunity, without compromising TEC composition. Indeed, it is a valuable murine system, in addition to the AIRE-knockout mouse model, to further dissect the machinery of PGE in the thymus. Importantly, the data proposed CHD6 as a potential key target for pharmacological intervention to specifically enhance or attenuate PGE in TEC without compromising other vital thymus biological functions. Lastly, the work presented in this thesis has established CHD6 as a novel epigenetic co-activator of PGE in the thymus and its specific and significant impact on the induction of central tolerance.

VII. References

1. Marshall, J. S., Warrington, R., Watson, W. & Kim, H. L. An introduction to immunology and immunopathology. *Allergy Asthma. Clin. Immunol.* **14**, 49 (2018).
2. Chaplin, D. D. Overview of the immune response. *J. Allergy Clin. Immunol.* (2010) doi:10.1016/j.jaci.2009.12.980.
3. Küppers, R. Overview of the immune system. in *The Lymphoid Neoplasms 3ed* (2010). doi:10.1201/b13424.
4. Janeway, C. A. & Medzhitov, R. INNATE IMMUNE RECOGNITION. *Annu. Rev. Immunol.* (2002) doi:10.1146/annurev.immunol.20.083001.084359.
5. Artis, D. & Spits, H. The biology of innate lymphoid cells. *Nature* (2015) doi:10.1038/nature14189.
6. Beutler, B. Innate immunity: An overview. *Mol. Immunol.* (2004) doi:10.1016/j.molimm.2003.10.005.
7. Mogensen, T. H. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews* (2009) doi:10.1128/CMR.00046-08.
8. Kumar, H., Kawai, T. & Akira, S. Pathogen recognition by the innate immune system. *Int. Rev. Immunol.* (2011) doi:10.3109/08830185.2010.529976.
9. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* (2006) doi:10.1016/j.cell.2006.02.015.
10. Bonilla, F. A. & Oettgen, H. C. Adaptive immunity. *J. Allergy Clin. Immunol.* (2010) doi:10.1016/j.jaci.2009.09.017.
11. Cooper, M. D. & Alder, M. N. The evolution of adaptive immune systems. *Cell* (2006) doi:10.1016/j.cell.2006.02.001.
12. Vanguri, V. K. The Adaptive Immune System. in *Pathobiology of Human Disease: A Dynamic Encyclopedia of Disease Mechanisms* (2014). doi:10.1016/B978-0-12-386456-7.01101-1.
13. Litman, G. W., Rast, J. P. & Fugmann, S. D. The origins of vertebrate adaptive immunity. *Nature Reviews Immunology* (2010) doi:10.1038/nri2807.
14. Jackson, K. J. L., Kidd, M. J., Wang, Y. & Collins, A. M. The shape of the lymphocyte receptor repertoire: Lessons from the B cell receptor. *Frontiers in Immunology* (2013) doi:10.3389/fimmu.2013.00263.
15. Deenick, E. K. & Ma, C. S. The regulation and role of T follicular helper cells in immunity. *Immunology* (2011) doi:10.1111/j.1365-2567.2011.03487.x.
16. Parker, D. C. T Cell-Dependent B Cell Activation. in *Encyclopedia of Immunobiology* (2016). doi:10.1016/B978-0-12-374279-7.09010-X.
17. Johnston, R. J. *et al.* Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* (80-.). (2009) doi:10.1126/science.1175870.
18. Crotty, S. A brief history of T cell help to B cells. *Nat. Rev. Immunol.* (2015) doi:10.1038/nri3803.
19. Mauri, C. & Bosma, A. Immune Regulatory Function of B Cells. *Annu. Rev. Immunol.* (2012) doi:10.1146/annurev-immunol-020711-074934.
20. Rajewsky, K. Clonal selection and learning in the antibody system. *Nature* (1996) doi:10.1038/381751a0.

21. Vojdani, A. & Lambert, J. Cell-mediated immunity. in *Neuroimmunity and the Brain-Gut Connection* (2016).
22. Marrack, P. & Kappler, J. The T cell receptor. *Science* (80-.). (1987) doi:10.1126/science.3317824.
23. Perniola, R. & Musco, G. The biophysical and biochemical properties of the autoimmune regulator (AIRE) protein. *Biochim. Biophys. Acta* **1842**, 326–37 (2014).
24. Marchalonis, J. J., Schluter, S. F. & Edmundson, A. B. The T-cell receptor as immunoglobulin: Paradigm regained. *Proceedings of the Society for Experimental Biology and Medicine* (1997).
25. Lin, J. & Weiss, A. T cell receptor signalling. *Journal of Cell Science* (2001).
26. Malissen, B., Grégoire, C., Malissen, M. & Roncagalli, R. Integrative biology of T cell activation. *Nature Immunology* (2014) doi:10.1038/ni.2959.
27. Izquierdo, M. & Cantrell, D. A. T-cell activation. *Trends Cell Biol.* (1992) doi:10.1016/0962-8924(92)90199-W.
28. Koretzky, G. A. Multiple Roles of CD4 and CD8 in T Cell Activation. *J. Immunol.* (2010) doi:10.4049/jimmunol.1090076.
29. Germain, R. N. t-cell development and the CD4-CD8 lineage decision. *Nature Reviews Immunology* (2002) doi:10.1038/nri798.
30. Bevan, M. J. Helping the CD8+ T-cell response. *Nature Reviews Immunology* (2004) doi:10.1038/nri1413.
31. Kerkvliet, N. & Lawrence, B. P. Cytotoxic T Cells. in *Comprehensive Toxicology, Second Edition* (2010). doi:10.1016/B978-0-08-046884-6.00606-0.
32. Topham, D. J., Tripp, R. A. & Doherty, P. C. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. *J. Immunol.* (1997).
33. Abusamra, A. J. *et al.* Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis. *Blood Cells, Mol. Dis.* (2005) doi:10.1016/j.bcmd.2005.07.001.
34. Groscurth, P. & Filgueira, L. Killing mechanisms of cytotoxic T lymphocytes. *News Physiol. Sci.* (1998).
35. Cao, X. *et al.* Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance. *Immunity* (2007) doi:10.1016/j.immuni.2007.08.014.
36. Hoebe, K., Janssen, E. & Beutler, B. The interface between innate and adaptive immunity. *Nature Immunology* (2004) doi:10.1038/ni1004-971.
37. Harty, J. T. & Badovinac, V. P. Shaping and reshaping CD8+ T-cell memory. *Nature Reviews Immunology* (2008) doi:10.1038/nri2251.
38. Obar, J. J. & Lefrançois, L. Memory CD8+ T cell differentiation. *Annals of the New York Academy of Sciences* (2010) doi:10.1111/j.1749-6632.2009.05126.x.
39. Zhou, L., Chong, M. M. W. & Littman, D. R. Plasticity of CD4+ T Cell Lineage Differentiation. *Immunity* (2009) doi:10.1016/j.immuni.2009.05.001.
40. Luckheeram, R. V., Zhou, R., Verma, A. D. & Xia, B. CD4 +T cells: Differentiation and functions. *Clinical and Developmental Immunology* (2012) doi:10.1155/2012/925135.
41. Zhu, J., Yamane, H. & Paul, W. E. Differentiation of Effector CD4 T Cell Populations. *Annu. Rev. Immunol.*

- (2010) doi:10.1146/annurev-immunol-030409-101212.
42. Taniuchi, I. CD4 Helper and CD8 Cytotoxic T Cell Differentiation. *Annu. Rev. Immunol.* (2018) doi:10.1146/annurev-immunol-042617-053411.
 43. Miyara, M. *et al.* Functional Delineation and Differentiation Dynamics of Human CD4+ T Cells Expressing the FoxP3 Transcription Factor. *Immunity* (2009) doi:10.1016/j.immuni.2009.03.019.
 44. Maynard, C. L. *et al.* Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nat. Immunol.* (2007) doi:10.1038/ni1504.
 45. Turnis, M. E. *et al.* Interleukin-35 Limits Anti-Tumor Immunity. *Immunity* (2016) doi:10.1016/j.immuni.2016.01.013.
 46. Lazarski, C. A., Hughson, A., Sojka, D. K. & Fowell, D. J. Regulating Treg Cells at Sites of Inflammation. *Immunity* (2008) doi:10.1016/j.immuni.2008.09.012.
 47. Chen, W. J. *et al.* Conversion of Peripheral CD4 + CD25 - Naive T Cells to CD4 + CD25 + Regulatory T Cells by TGF- β Induction of Transcription Factor Foxp3. *J. Exp. Med.* (2003) doi:10.1084/jem.20030152.
 48. Schmitt, E. G. & Williams, C. B. Generation and function of induced regulatory T cells. *Front. Immunol.* (2013) doi:10.3389/fimmu.2013.00152.
 49. Lio, C. W. J. & Hsieh, C. S. A Two-Step Process for Thymic Regulatory T Cell Development. *Immunity* (2008) doi:10.1016/j.immuni.2007.11.021.
 50. Lee, H. M., Bautista, J. L. & Hsieh, C. S. Thymic and Peripheral Differentiation of Regulatory T Cells. in *Advances in Immunology* (2011). doi:10.1016/B978-0-12-387827-4.00002-4.
 51. MILLER, J. F. Immunological function of the thymus. *Lancet (London, England)* **2**, 748–9 (1961).
 52. Miller, J. F. A. P. The thymus and the development of immunologic responsiveness. *Science (80-.)*. (1964) doi:10.1126/science.144.3626.1544.
 53. Rodewald, H.-R. Thymus Organogenesis. *Annu. Rev. Immunol.* **26**, 355–388 (2008).
 54. Gordon, J., Bennett, A. R., Blackburn, C. C. & Manley, N. R. Gcm2 and Foxn1 mark early parathyroid- and thymus-specific domains in the developing third pharyngeal pouch. *Mech. Dev.* (2001) doi:10.1016/S0925-4773(01)00333-1.
 55. Manley, N. R. & Condie, B. G. Transcriptional regulation of thymus organogenesis and thymic epithelial cell differentiation. in *Progress in Molecular Biology and Translational Science* (2010). doi:10.1016/S1877-1173(10)92005-X.
 56. Rodewald, H.-R. Thymus organogenesis. *Annu. Rev. Immunol.* **26**, 355–88 (2008).
 57. Vaidya, H. J., Briones Leon, A. & Blackburn, C. C. FOXP1 in thymus organogenesis and development. *European Journal of Immunology* (2016) doi:10.1002/eji.201545814.
 58. Balciunaite, G. *et al.* Wnt glycoproteins regulate the expression of FoxN1, the genes defective in nude mice. *Nat. Immunol.* (2002) doi:10.1038/ni850.
 59. Itoi, M., Kawamoto, H., Katsura, Y. & Amagai, T. Two distinct steps of immigration of hematopoietic progenitors into the early thymus anlage. *Int. Immunol.* (2001) doi:10.1093/intimm/13.9.1203.
 60. Anderson, G., Jenkinson, E. J. & Rodewald, H. R. A roadmap for thymic epithelial cell development.

- European Journal of Immunology* (2009) doi:10.1002/eji.200939379.
61. Ohigashi, I., Kozai, M. & Takahama, Y. Development and developmental potential of cortical thymic epithelial cells. *Immunol. Rev.* (2016) doi:10.1111/imr.12404.
 62. Farr, A. G. & Anderson, S. K. Epithelial heterogeneity in the murine thymus: fucose-specific lectins bind medullary epithelial cells. *J. Immunol.* (1985).
 63. Klug, D. B. *et al.* Interdependence of cortical thymic epithelial cell differentiation and T-lineage commitment. *Proc. Natl. Acad. Sci. U. S. A.* (1998) doi:10.1073/pnas.95.20.11822.
 64. Anderson, G. & Takahama, Y. Thymic epithelial cells: Working class heroes for T cell development and repertoire selection. *Trends in Immunology* (2012) doi:10.1016/j.it.2012.03.005.
 65. Alves, N. L., Huntington, N. D., Rodewald, H.-R. & Di Santo, J. P. Thymic epithelial cells: the multi-tasking framework of the T cell “cradle”. *Trends Immunol.* **30**, 468–474 (2009).
 66. Tomaru, U. *et al.* Exclusive expression of proteasome subunit $\beta 5t$ in the human thymic cortex. *Blood* (2009) doi:10.1182/blood-2008-11-187633.
 67. Tomaru, U. *et al.* Restricted Expression of the Thymoproteasome Is Required for Thymic Selection and Peripheral Homeostasis of CD8⁺ T Cells. *Cell Rep.* (2019) doi:10.1016/j.celrep.2018.12.078.
 68. Sasaki, K. *et al.* Thymoproteasomes produce unique peptide motifs for positive selection of CD8⁺ T cells. *Nat. Commun.* (2015) doi:10.1038/ncomms8484.
 69. Nakagawa, T. *et al.* Cathepsin L: Critical role in Ii degradation and CD4 T cell selection in the thymus. *Science* (80-.). (1998) doi:10.1126/science.280.5362.450.
 70. Honey, K., Nakagawa, T., Peters, C. & Rudensky, A. Cathepsin L regulates CD4⁺ T cell selection independently of its effect on invariant chain: A role in the generation of positively selecting peptide ligands. *J. Exp. Med.* (2002) doi:10.1084/jem.20011904.
 71. White, A. J. *et al.* Lymphotoxin Signals from Positively Selected Thymocytes Regulate the Terminal Differentiation of Medullary Thymic Epithelial Cells. *J. Immunol.* (2010) doi:10.4049/jimmunol.1002151.
 72. Akiyama, T. *et al.* The Tumor Necrosis Factor Family Receptors RANK and CD40 Cooperatively Establish the Thymic Medullary Microenvironment and Self-Tolerance. *Immunity* (2008) doi:10.1016/j.immuni.2008.06.015.
 73. Rossi, S. W. *et al.* RANK signals from CD4⁺3⁻ inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. *J. Exp. Med.* (2007) doi:10.1084/jem.20062497.
 74. Wang, X. *et al.* Post-Aire maturation of thymic medullary epithelial cells involves selective expression of keratinocyte-specific autoantigens. *Front. Immunol.* (2012) doi:10.3389/fimmu.2012.00019.
 75. Dhalla, F. *et al.* Biologically indeterminate yet ordered promiscuous gene expression in single medullary thymic epithelial cells. *bioRxiv* (2019) doi:10.1101/554899.
 76. Miller, C. N. *et al.* Thymic tuft cells promote an IL-4-enriched medulla and shape thymocyte development. *Nature* **559**, 627–631 (2018).
 77. Bornstein, C. *et al.* Single-cell mapping of the thymic stroma identifies IL-25-producing tuft epithelial cells. *Nature* **559**, 622–626 (2018).

78. Metzger, T. C. *et al.* Lineage Tracing and Cell Ablation Identify a Post-Aire-Expressing Thymic Epithelial Cell Population. *Cell Rep.* (2013) doi:10.1016/j.celrep.2013.08.038.
79. Palmer, D. B. The effect of age on thymic function. *Frontiers in Immunology* (2013) doi:10.3389/fimmu.2013.00316.
80. Lynch, H. E. *et al.* Thymic involution and immune reconstitution. *Trends in Immunology* (2009) doi:10.1016/j.it.2009.04.003.
81. Aspinall, R. & Andrew, D. Thymic involution in aging. *Journal of Clinical Immunology* (2000) doi:10.1023/A:1006611518223.
82. Gray, D. H. D. *et al.* Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. *Blood* (2006) doi:10.1182/blood-2006-02-004531.
83. Miller, J. F. A. P. The discovery of thymus function and of thymus-derived lymphocytes. *Immunological Reviews* (2002) doi:10.1034/j.1600-065X.2002.18502.x.
84. Månsson, R. *et al.* Molecular Evidence for Hierarchical Transcriptional Lineage Priming in Fetal and Adult Stem Cells and Multipotent Progenitors. *Immunity* (2007) doi:10.1016/j.immuni.2007.02.013.
85. Sitnicka, E. *et al.* Critical role of FLT3 ligand in IL-7 receptor-independent T lymphopoiesis and regulation of lymphoid-primed multipotent progenitors. *Blood* (2007) doi:10.1182/blood-2006-10-054726.
86. Rothenberg, E. V., Moore, J. E. & Yui, M. A. Launching the T-cell-lineage developmental programme. *Nature Reviews Immunology* (2008) doi:10.1038/nri2232.
87. Schlenner, S. M. *et al.* Fate Mapping Reveals Separate Origins of T Cells and Myeloid Lineages in the Thymus. *Immunity* (2010) doi:10.1016/j.immuni.2010.03.005.
88. Takahama, Y. Journey through the thymus: Stromal guides for T-cell development and selection. *Nature Reviews Immunology* (2006) doi:10.1038/nri1781.
89. Porritt, H. E., Gordon, K. & Petrie, H. T. Kinetics of steady-state differentiation and mapping of intrathymic-signaling environments by stem cell transplantation in nonirradiated mice. *J. Exp. Med.* (2003) doi:10.1084/jem.20030837.
90. Carlyle, J. R. *et al.* Identification of a novel developmental stage marking lineage commitment of progenitor thymocytes. *J. Exp. Med.* (1997) doi:10.1084/jem.186.2.173.
91. Rossi, F. M. V *et al.* Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nat. Immunol.* (2005) doi:10.1038/ni1203.
92. Oettinger, M. A., Schatz, D. G., Gorka, C. & Baltimore, D. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science* (80-.). (1990) doi:10.1126/science.2360047.
93. Baldwin, T. A., Sandau, M. M., Jameson, S. C. & Hogquist, K. A. The timing of TCR α expression critically influences T cell development and selection. *J. Exp. Med.* (2005) doi:10.1084/jem.20050359.
94. Yamasaki, S. *et al.* Mechanistic basis of pre-T cell receptor-mediated autonomous signaling critical for thymocyte development. *Nat. Immunol.* (2006) doi:10.1038/ni1290.
95. Yamasaki, S. & Saito, T. Molecular basis for pre-TCR-mediated autonomous signaling. *Trends in Immunology* (2007) doi:10.1016/j.it.2006.11.006.

96. Fu, G. *et al.* Fine-tuning T cell receptor signaling to control T cell development. *Trends in Immunology* (2014) doi:10.1016/j.it.2014.05.003.
97. Hogquist, K. A. *et al.* T cell receptor antagonist peptides induce positive selection. *Cell* (1994) doi:10.1016/0092-8674(94)90169-4.
98. Kisielow, P., Teh, H. S., Blüthmann, H. & Von Boehmer, H. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature* (1988) doi:10.1038/335730a0.
99. Derbinski, J. & Kyewski, B. How thymic antigen presenting cells sample the body's self-antigens. *Curr. Opin. Immunol.* **22**, 592–600 (2010).
100. Takaba, H. & Takayanagi, H. The Mechanisms of T Cell Selection in the Thymus. *Trends Immunol.* **38**, 805–816 (2017).
101. Campbell, I. K. *et al.* Autoimmune regulator controls T cell help for pathogenetic autoantibody production in collagen-induced arthritis. *Arthritis Rheum.* **60**, 1683–1693 (2009).
102. Ueno, T. *et al.* CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *J. Exp. Med.* (2004) doi:10.1084/jem.20040643.
103. Kurobe, H. *et al.* CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance. *Immunity* (2006) doi:10.1016/j.immuni.2005.12.011.
104. McCaughy, T. M., Baldwin, T. A., Wilken, M. S. & Hogquist, K. A. Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla. *J. Exp. Med.* (2008) doi:10.1084/jem.20080866.
105. Alvarez, I. *et al.* Central T cell tolerance: Identification of tissue-restricted autoantigens in the thymus HLA-DR peptidome. *J. Autoimmun.* **60**, 12–9 (2015).
106. Sansom, S. N. *et al.* Population and single-cell genomics reveal the Aire dependency, relief from Polycomb silencing, and distribution of self-antigen expression in thymic epithelia. *Genome Res.* **24**, 1918–31 (2014).
107. Chan, A. Y. & Anderson, M. S. Central tolerance to self revealed by the autoimmune regulator. *Ann. N. Y. Acad. Sci.* (2015) doi:10.1111/nyas.12960.
108. Xing, Y., Wang, X., Jameson, S. C. & Hogquist, K. A. Late stages of T cell maturation in the thymus involve NF- κ B and tonic type I interferon signaling. *Nat. Immunol.* **17**, 565–573 (2016).
109. Resop, R. S. *et al.* Sphingosine-1-phosphate/sphingosine-1-phosphate receptor 1 signaling is required for migration of naive human T cells from the thymus to the periphery. *J. Allergy Clin. Immunol.* (2016) doi:10.1016/j.jaci.2015.12.1339.
110. Birnbaum, M. E. *et al.* Molecular architecture of the $\alpha\beta$ T cell receptor-CD3 complex. *Proc. Natl. Acad. Sci. U. S. A.* (2014) doi:10.1073/pnas.1420936111.
111. Rojo, J. M., Bello, R. & Portolés, P. T-cell receptor. *Advances in Experimental Medicine and Biology* (2008) doi:10.1007/978-0-387-09789-3_1.
112. Garcia, K. C., Teyton, L. & Wilson, I. A. STRUCTURAL BASIS OF T CELL RECOGNITION. *Annu. Rev. Immunol.* (1999) doi:10.1146/annurev.immunol.17.1.369.
113. Allison, J. P. & Havran, W. L. The Immunobiology of T Cells with Invariant gammadelta Antigen Receptors. *Annu. Rev. Immunol.* (1991) doi:10.1146/annurev.iy.09.040191.003335.

114. Haas, W., Pereira, P. & Tonegawa, S. Gamma/Delta Cells. *Annu. Rev. Immunol.* (1993) doi:10.1146/annurev.iy.11.040193.003225.
115. Katayama, C. D., Eidelman, F. J., Duncan, A., Hooshmand, F. & Hedrick, S. M. Predicted complementarity determining regions of the T cell antigen receptor determine antigen specificity. *EMBO J.* (1995) doi:10.1002/j.1460-2075.1995.tb07074.x.
116. Garcia, K. C. *et al.* Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen. *Science* (80-.). (1998) doi:10.1126/science.279.5354.1166.
117. Davis, M. M. *et al.* LIGAND RECOGNITION BY $\alpha\beta$ T CELL RECEPTORS. *Annu. Rev. Immunol.* (1998) doi:10.1146/annurev.immunol.16.1.523.
118. Alt, F. W. *et al.* VDJ recombination. *Immunology Today* (1992) doi:10.1016/0167-5699(92)90043-7.
119. Nikolich-Zugich, J., Slifka, M. K. & Messaoudi, I. The many important facets of T-cell repertoire diversity. *Nature Reviews Immunology* (2004) doi:10.1038/nri1292.
120. Mora, T. & Walczak, A. M. Quantifying lymphocyte receptor diversity. in *Systems Immunology* (2018). doi:10.1201/9781315119847-11.
121. Derbinski, J., Schulte, A., Kyewski, B. & Klein, L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat. Immunol.* **2**, 1032–9 (2001).
122. Gäbler, J., Arnold, J. & Kyewski, B. Promiscuous gene expression and the developmental dynamics of medullary thymic epithelial cells. *Eur. J. Immunol.* **37**, 3363–72 (2007).
123. Derbinski, J., Schulte, A., Kyewski, B. & Klein, L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *J. Immunol.* (2016) doi:10.1038/ni723.
124. Magalhães, D. A. R. *et al.* Promiscuous gene expression in the thymus: the root of central tolerance. *Clin. Dev. Immunol.* **13**, 81–99.
125. Hubert, F. X. *et al.* Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance. *Blood* (2011) doi:10.1182/blood-2010-06-286393.
126. Rattay, K., Meyer, H. V., Herrmann, C., Brors, B. & Kyewski, B. Evolutionary conserved gene co-expression drives generation of self-antigen diversity in medullary thymic epithelial cells. *J. Autoimmun.* **67**, 65–75 (2016).
127. Takaba, H. *et al.* Fezf2 Orchestrates a Thymic Program of Self-Antigen Expression for Immune Tolerance. *Cell* **163**, 975–987 (2015).
128. Abramson, J. & Anderson, G. Thymic Epithelial Cells. *Annu. Rev. Immunol.* (2017) doi:10.1146/annurev-immunol-051116-052320.
129. Koh, A. S. *et al.* Rapid chromatin repression by Aire provides precise control of immune tolerance article. *Nat. Immunol.* (2018) doi:10.1038/s41590-017-0032-8.
130. Roberts, N. A. *et al.* Prdm1 Regulates Thymic Epithelial Function To Prevent Autoimmunity. *J. Immunol.* (2017) doi:10.4049/jimmunol.1600941.
131. Aaltonen, J. *et al.* An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat. Genet.* (1997) doi:10.1038/ng1297-399.

132. Anderson, M. S. Projection of an Immunological Self Shadow Within the Thymus by the Aire Protein. *Science* (80-.). **298**, 1395–1401 (2002).
133. Jiang, W., Anderson, M. S., Bronson, R., Mathis, D. & Benoist, C. Modifier loci condition autoimmunity provoked by Aire deficiency. *J. Exp. Med.* **202**, 805–815 (2005).
134. Ramsey, C. Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. *Hum. Mol. Genet.* **11**, 397–409 (2002).
135. Hubert, F.-X. *et al.* Aire-Deficient C57BL/6 Mice Mimicking the Common Human 13-Base Pair Deletion Mutation Present with Only a Mild Autoimmune Phenotype. *J. Immunol.* (2009) doi:10.4049/jimmunol.0802124.
136. Nishikawa, Y. *et al.* Biphasic Aire expression in early embryos and in medullary thymic epithelial cells before end-stage terminal differentiation. *J. Exp. Med.* **207**, 963–71 (2010).
137. Herzig, Y. *et al.* Transcriptional programs that control expression of the autoimmune regulator gene Aire. *Nat. Immunol.* **18**, 161–172 (2017).
138. Kawano, H. *et al.* Aire Expression Is Inherent to Most Medullary Thymic Epithelial Cells during Their Differentiation Program. *J. Immunol.* (2015) doi:10.4049/jimmunol.1501000.
139. Michel, C. *et al.* Revisiting the Road Map of Medullary Thymic Epithelial Cell Differentiation. *J. Immunol.* **199**, 3488–3503 (2017).
140. Abramson, J. & Goldfarb, Y. AIRE: From promiscuous molecular partnerships to promiscuous gene expression. *Eur. J. Immunol.* **46**, 22–33 (2016).
141. Villaseñor, J., Besse, W., Benoist, C. & Mathis, D. Ectopic expression of peripheral-tissue antigens in the thymic epithelium: Probabilistic, monoallelic, misinitiated. *Proc. Natl. Acad. Sci. U. S. A.* (2008) doi:10.1073/pnas.0808069105.
142. Heino, M. *et al.* Autoimmune regulator is expressed in the cells regulating immune tolerance in thymus medulla. *Biochem. Biophys. Res. Commun.* **257**, 821–5 (1999).
143. Passos, G. A., Speck-Hernandez, C. A., Assis, A. F. & Mendes-da-Cruz, D. A. Update on Aire and thymic negative selection. *Immunology* **153**, 10–20 (2018).
144. Peterson, P., Org, T. & Rebane, A. Transcriptional regulation by AIRE: Molecular mechanisms of central tolerance. *Nature Reviews Immunology* (2008) doi:10.1038/nri2450.
145. Gardner, J. M. *et al.* Deletional Tolerance Mediated by Extrathymic Aire-Expressing Cells. *Science* (80-.). **321**, 843–847 (2008).
146. Taniguchi, R. T. & Anderson, M. S. The role of Aire in clonal selection. *Immunology and Cell Biology* (2011) doi:10.1038/icb.2010.132.
147. Gardner, J. M. *et al.* Extrathymic aire-expressing cells are a distinct bone marrow-derived population that induce functional inactivation of CD4+ T cells. *Immunity* (2013) doi:10.1016/j.immuni.2013.08.005.
148. Metzger, T. C. & Anderson, M. S. Control of central and peripheral tolerance by Aire. *Immunol. Rev.* (2011) doi:10.1111/j.1600-065X.2011.01008.x.
149. Akiyoshi, H. *et al.* Subcellular Expression of Autoimmune Regulator Is Organized in a Spatiotemporal

- Manner. *J. Biol. Chem.* **279**, 33984–33991 (2004).
150. Abramson, J., Giraud, M., Benoist, C. & Mathis, D. Aire's partners in the molecular control of immunological tolerance. *Cell* **140**, 123–35 (2010).
 151. Tao, Y. *et al.* AIRE recruits multiple transcriptional components to specific genomic regions through tethering to nuclear matrix. *Mol. Immunol.* **43**, 335–45 (2006).
 152. Halonen, M. *et al.* APECED-causing mutations in AIRE reveal the functional domains of the protein. *Hum. Mutat.* **23**, 245–257 (2004).
 153. Ferguson, B. J. *et al.* AIRE's CARD revealed, a new structure for central tolerance provokes transcriptional plasticity. *J. Biol. Chem.* (2008) doi:10.1074/jbc.M707211200.
 154. Sparks, A. E., Chen, C., Breslin, M. B. & Lan, M. S. Functional domains of autoimmune regulator (AIRE) modulate INS-VNTR transcription in human thymic epithelial cells. *J. Biol. Chem.* (2016) doi:10.1074/jbc.M116.722488.
 155. Yang, S., Bansal, K., Lopes, J., Benoist, C. & Mathis, D. Aire's plant homeodomain(PHD)-2 is critical for induction of immunological tolerance. *Proc. Natl. Acad. Sci.* **110**, 1833 LP – 1838 (2013).
 156. Musco, G. & Peterson, P. PHD finger of autoimmune regulator: An epigenetic link between the histone modifications and tissue-specific antigen expression in thymus. *Epigenetics* **3**, 310–314 (2008).
 157. Org, T. *et al.* AIRE activated tissue specific genes have histone modifications associated with inactive chromatin. *Hum. Mol. Genet.* **18**, 4699–4710 (2009).
 158. Koh, A. S. *et al.* Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity. *Proc. Natl. Acad. Sci.* **105**, 15878–15883 (2008).
 159. Plevin, M. J., Mills, M. M. & Ikura, M. The LxxLL motif: a multifunctional binding sequence in transcriptional regulation. *Trends Biochem. Sci.* **30**, 66–9 (2005).
 160. Gaetani, M. *et al.* AIRE-PHD fingers are structural hubs to maintain the integrity of chromatin-associated interactome. *Nucleic Acids Res.* **40**, 11756–11768 (2012).
 161. Guha, M. *et al.* DNA breaks and chromatin structural changes enhance the transcription of autoimmune regulator target genes. *J. Biol. Chem.* **292**, 6542–6554 (2017).
 162. Bansal, K., Yoshida, H., Benoist, C. & Mathis, D. The transcriptional regulator Aire binds to and activates super-enhancers. *Nat. Immunol.* **18**, 263–273 (2017).
 163. M., S., A., R. & P., P. Autoimmune regulator (AIRE) is acetylated by transcriptional co-activator p300. *Eur. J. Immunol.* (2009) doi:10.1002/eji.200990184 LK - <http://limo.libis.be/resolver?&sid=EMBASE&issn=00142980&id=doi:10.1002%2Feji.200990184&atitle=Autoimmune+regulator+%28AIRE%29+is+acetylated+by+transcriptional+co-activator+p300&stitle=Eur.+J.+Immunol.&title=European+Journal+of+Immunology&volume=39&issue=&spage=S402&epage=&aulast=Saare&aufirst=M.&aunit=M.&aufull=Saare+M.&coden=&isbn=&pages=S402-&date=2009&aunit1=M&aunitm=>.
 164. Boija, A. *et al.* CBP Regulates Recruitment and Release of Promoter-Proximal RNA Polymerase II. *Mol. Cell* (2017) doi:10.1016/j.molcel.2017.09.031.

165. Giraud, M. *et al.* Aire unleashes stalled RNA polymerase to induce ectopic gene expression in thymic epithelial cells. *Proc. Natl. Acad. Sci. U. S. A.* (2012) doi:10.1073/pnas.1119351109.
166. Oven, I. *et al.* AIRE Recruits P-TEFb for Transcriptional Elongation of Target Genes in Medullary Thymic Epithelial Cells. *Mol. Cell. Biol.* (2007) doi:10.1128/mcb.01085-07.
167. Guerau-de-Arellano, M., Mathis, D. & Benoist, C. Transcriptional impact of Aire varies with cell type. *Proc. Natl. Acad. Sci. U. S. A.* (2008) doi:10.1073/pnas.0806616105.
168. Ucar, O. & Rattay, K. Promiscuous Gene Expression in the Thymus: A Matter of Epigenetics, miRNA, and More? *Front. Immunol.* **6**, 93 (2015).
169. Org, T. *et al.* AIRE activated tissue specific genes have histone modifications associated with inactive chromatin. *Hum. Mol. Genet.* **18**, 4699–710 (2009).
170. Handel, A. E. *et al.* Comprehensively Profiling the Chromatin Architecture of Tissue Restricted Antigen Expression in Thymic Epithelial Cells Over Development. *Front. Immunol.* **9**, (2018).
171. Koh, A. S. *et al.* Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity. *Proc. Natl. Acad. Sci. U. S. A.* (2008) doi:10.1073/pnas.0808470105.
172. Koh, A. S., Kingston, R. E., Benoist, C. & Mathis, D. Global relevance of Aire binding to hypomethylated lysine-4 of histone-3. *Proc. Natl. Acad. Sci. U. S. A.* (2010) doi:10.1073/pnas.1004436107.
173. Alfonso, R. *et al.* CHD6 chromatin remodeler is a negative modulator of influenza virus replication that relocates to inactive chromatin upon infection. *Cell. Microbiol.* **13**, 1894–906 (2011).
174. Mansfield, R. E. *et al.* Plant homeodomain (PHD) fingers of CHD4 are histone H3-binding modules with preference for unmodified H3K4 and methylated H3K9. *J. Biol. Chem.* (2011) doi:10.1074/jbc.M110.208207.
175. Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F. & Richmond, T. J. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* (1997) doi:10.1038/38444.
176. Richmond, T. J. & Davey, C. A. The structure of DNA in the nucleosome core. *Nature* (2003) doi:10.1038/nature01595.
177. Li, G. & Reinberg, D. Chromatin higher-order structures and gene regulation. *Current Opinion in Genetics and Development* (2011) doi:10.1016/j.gde.2011.01.022.
178. Talbert, P. B. & Henikoff, S. Histone variants on the move: Substrates for chromatin dynamics. *Nature Reviews Molecular Cell Biology* (2017) doi:10.1038/nrm.2016.148.
179. Flaus, A. & Owen-Hughes, T. Mechanisms for ATP-dependent chromatin remodelling: The means to the end. *FEBS Journal* (2011) doi:10.1111/j.1742-4658.2011.08281.x.
180. Yodh, J. ATP-dependent chromatin remodeling. *Adv. Exp. Med. Biol.* (2013) doi:10.1007/978-1-4614-5037-5_13.
181. Hargreaves, D. C. & Crabtree, G. R. ATP-dependent chromatin remodeling: Genetics, genomics and mechanisms. *Cell Res.* (2011) doi:10.1038/cr.2011.32.
182. Narlikar, G. J., Sundaramoorthy, R. & Owen-Hughes, T. Mechanisms and functions of ATP-dependent

- chromatin-remodeling enzymes. *Cell* (2013) doi:10.1016/j.cell.2013.07.011.
183. Flaus, A., Martin, D. M. A., Barton, G. J. & Owen-Hughes, T. Identification of multiple distinct Snf2 subfamilies with conserved structural motifs. *Nucleic Acids Res.* (2006) doi:10.1093/nar/gkl295.
 184. Ryan, D. P. & Owen-Hughes, T. Snf2-family proteins: Chromatin remodellers for any occasion. *Current Opinion in Chemical Biology* (2011) doi:10.1016/j.cbpa.2011.07.022.
 185. Marfella, C. G. A. & Imbalzano, A. N. The Chd family of chromatin remodelers. *Mutat. Res. - Fundam. Mol. Mech. Mutagen.* (2007) doi:10.1016/j.mrfmmm.2006.07.012.
 186. Sims, J. K. & Wade, P. A. SnapShot: Chromatin remodeling: CHD. *Cell* (2011) doi:10.1016/j.cell.2011.02.019.
 187. Delmas, V., Stokes, D. G. & Perry, R. P. A mammalian DNA-binding protein that contains a chromodomain and an SNF2/SWI2-like helicase domain. *Proc. Natl. Acad. Sci.* **90**, 2414–2418 (1993).
 188. Ryan, D. P., Sundaramoorthy, R., Martin, D., Singh, V. & Owen-Hughes, T. The DNA-binding domain of the Chd1 chromatin-remodelling enzyme contains SANT and SLIDE domains. *EMBO J.* (2011) doi:10.1038/emboj.2011.166.
 189. Liu, J. C., Ferreira, C. G. & Yusufzai, T. Human CHD2 is a chromatin assembly ATPase regulated by its chromo- And DNA-binding domains. *J. Biol. Chem.* (2015) doi:10.1074/jbc.M114.609156.
 190. Hoffmeister, H. *et al.* CHD3 and CHD4 form distinct NuRD complexes with different yet overlapping functionality. *Nucleic Acids Res.* (2017) doi:10.1093/nar/gkx711.
 191. Xue, Y. *et al.* NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. *Mol. Cell* (1998) doi:10.1016/S1097-2765(00)80299-3.
 192. Boyer, L. A. *et al.* Essential role for the SANT domain in the functioning of multiple chromatin remodeling enzymes. *Mol. Cell* (2002) doi:10.1016/S1097-2765(02)00634-2.
 193. Mo, X., Kowenz-Leutz, E., Laumonier, Y., Xu, H. & Leutz, A. Histone H3 tail positioning and acetylation by the c-Myb but not the v-Myb DNA-binding SANT domain. *Genes Dev.* (2005) doi:10.1101/gad.355405.
 194. Daubresse, G. *et al.* The Drosophila kismet gene is related to chromatin-remodeling factors and is required for both segmentation and segment identity. *Development* (1999).
 195. Schuster, E. F. & Stöger, R. CHD5 defines a new subfamily of chromodomain-SWI2/SNF2-like helicases. *Mamm. Genome* **13**, 117–119 (2002).
 196. Lutz, T., Stöger, R. & Nieto, A. CHD6 is a DNA-dependent ATPase and localizes at nuclear sites of mRNA synthesis. *FEBS Lett.* **580**, 5851–5857 (2006).
 197. Lathrop, M. J. *et al.* Deletion of the Chd6 exon 12 affects motor coordination. *Mamm. Genome* **21**, 130–42 (2010).
 198. Srinivasan, S. The Drosophila trithorax group protein Kismet facilitates an early step in transcriptional elongation by RNA Polymerase II. *Development* **132**, 1623–1635 (2005).
 199. Hayes, J. D. & Dinkova-Kostova, A. T. Epigenetic Control of NRF2-Directed Cellular Antioxidant Status in Dictating Life-Death Decisions. *Mol. Cell* **68**, 5–7 (2017).
 200. Nioi, P., Nguyen, T., Sherratt, P. J. & Pickett, C. B. The carboxy-terminal Neh3 domain of Nrf2 is required

- for transcriptional activation. *Mol. Cell. Biol.* **25**, 10895–906 (2005).
201. Sancho, A. *et al.* CHD6 regulates the topological arrangement of the CFTR locus. *Hum. Mol. Genet.* **24**, 2724–32 (2015).
 202. Moore, S. *et al.* The CHD6 chromatin remodeler is an oxidative DNA damage response factor. *Nat. Commun.* **10**, 241 (2019).
 203. Manning, B. J. & Yusufzai, T. The ATP-dependent chromatin remodeling enzymes CHD6, CHD7, and CHD8 exhibit distinct nucleosome binding and remodeling activities. *J. Biol. Chem.* **292**, 11927–11936 (2017).
 204. Alfonso, R., Rodriguez, A., Rodriguez, P., Lutz, T. & Nieto, A. CHD6, a cellular repressor of influenza virus replication, is degraded in human alveolar epithelial cells and mice lungs during infection. *J. Virol.* **87**, 4534–44 (2013).
 205. Stanley, F. K. T., Moore, S. & Goodarzi, A. A. CHD chromatin remodelling enzymes and the DNA damage response. *Mutat. Res.* **750**, 31–44 (2013).
 206. Rodríguez, C. I. *et al.* High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nat. Genet.* **25**, 139–40 (2000).
 207. Zuklys, S. *et al.* Stabilized β -Catenin in Thymic Epithelial Cells Blocks Thymus Development and Function. *J. Immunol.* **182**, 2997 LP – 3007 (2009).
 208. Kim, D., Langmead, B. & Salzberg, S. L. HISAT: A fast spliced aligner with low memory requirements. *Nat. Methods* (2015) doi:10.1038/nmeth.3317.
 209. Trapnell, C. *et al.* Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* (2010) doi:10.1038/nbt.1621.
 210. Patro, R., Duggal, G., Love, M. I., Irizarry, R. A. & Kingsford, C. Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods* (2017) doi:10.1038/nmeth.4197.
 211. Vidal, R., Ma, Y. & Sastry, S. S. Principal component analysis. in *Interdisciplinary Applied Mathematics* (2016). doi:10.1007/978-0-387-87811-9_2.
 212. Jolliffe, I. T. & Cadima, J. Principal component analysis: A review and recent developments. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences* (2016) doi:10.1098/rsta.2015.0202.
 213. Abdi, H. & Williams, L. J. Principal component analysis. *Wiley Interdisciplinary Reviews: Computational Statistics* (2010) doi:10.1002/wics.101.
 214. Groth, D., Hartmann, S., Klie, S. & Selbig, J. Principal components analysis. *Methods Mol. Biol.* (2013) doi:10.1007/978-1-62703-059-5_22.
 215. McHugh, M. L. Interrater reliability: the kappa statistic. *Biochem. medica* **22**, 276–82 (2012).
 216. Landis, J. R. & Koch, G. G. The Measurement of Observer Agreement for Categorical Data. *Biometrics* (1977) doi:10.2307/2529310.
 217. Blechschmidt, K. *et al.* The mouse Aire gene: comparative genomic sequencing, gene organization, and expression. *Genome Res.* **9**, 158–66 (1999).

218. Huff, J. The Airyscan detector from ZEISS: confocal imaging with improved signal-to-noise ratio and super-resolution. *Nat. Methods* (2015) doi:10.1038/nmeth.f.388.
219. NadejdaTsankova, B. N. Characterizing Intracellular Localization and Chromatin Remodeling Role of CHD6 in Human Fetal Development and in Glioblastoma. *Columbia Undergrad. Sci. J.* **10**, 10–16 (2016).
220. Xing, S., Wallmeroth, N., Berendzen, K. W. & Grefen, C. Techniques for the analysis of protein-protein interactions in vivo. *Plant Physiol.* pp.00470.2016 (2016) doi:10.1104/pp.16.00470.
221. Avin, A., Levy, M., Porat, Z. & Abramson, J. Quantitative analysis of protein-protein interactions and post-translational modifications in rare immune populations. *Nat. Commun.* **8**, 1524 (2017).
222. Yang, B. *et al.* Spontaneous and specific chemical cross-linking in live cells to capture and identify protein interactions. *Nat. Commun.* (2017) doi:10.1038/s41467-017-02409-z.
223. Kluger, R. & Alagic, A. Chemical cross-linking and protein-protein interactions-a review with illustrative protocols. *Bioorganic Chemistry* (2004) doi:10.1016/j.bioorg.2004.08.002.
224. Tang, X. & Bruce, J. E. Chemical cross-linking for protein-protein interaction studies. *Methods Mol. Biol.* (2009) doi:10.1007/978-1-59745-493-3_17.
225. Galganski, L., Urbanek, M. O. & Krzyzosiak, W. J. Nuclear speckles: Molecular organization, biological function and role in disease. *Nucleic Acids Research* (2017) doi:10.1093/nar/gkx759.
226. Spector, D. L. & Lamond, A. I. Nuclear speckles. *Cold Spring Harb. Perspect. Biol.* (2011) doi:10.1101/cshperspect.a000646.
227. Galganski, L., Urbanek, M. O. & Krzyzosiak, W. J. Nuclear speckles: Molecular organization, biological function and role in disease. *Nucleic Acids Research* (2017) doi:10.1093/nar/gkx759.
228. Ferguson, B. J. *et al.* AIRE's CARD revealed, a new structure for central tolerance provokes transcriptional plasticity. *J. Biol. Chem.* (2008) doi:10.1074/jbc.M707211200.
229. Abramson, J., Giraud, M., Benoist, C. & Mathis, D. Aire's Partners in the Molecular Control of Immunological Tolerance. *Cell* (2010) doi:10.1016/j.cell.2009.12.030.
230. Handel, A. E. *et al.* Comprehensively Profiling the Chromatin Architecture of Tissue Restricted Antigen Expression in Thymic Epithelial Cells Over Development. *Front. Immunol.* (2018) doi:10.3389/fimmu.2018.02120.
231. Hota, S. K. & Bruneau, B. G. ATP-dependent chromatin remodeling during mammalian development. *Development* **143**, 2882 LP – 2897 (2016).
232. Nishikawa, Y. *et al.* Biphasic Aire expression in early embryos and in medullary thymic epithelial cells before end-stage terminal differentiation. *J. Exp. Med.* (2010) doi:10.1084/jem.20092144.
233. Yano, M. *et al.* Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance. *J. Exp. Med.* (2008) doi:10.1084/jem.20080046.
234. Thompson, P. M., Gotoh, T., Kok, M., White, P. S. & Brodeur, G. M. CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system. *Oncogene* (2003) doi:10.1038/sj.onc.1206211.
235. Anderson, M. S. & Su, M. A. Aire and T cell development. *Current Opinion in Immunology* (2011)

doi:10.1016/j.coi.2010.11.007.

236. Nakagawa, Y. *et al.* Thymic nurse cells provide microenvironment for secondary T cell receptor α rearrangement in cortical thymocytes. *Proc. Natl. Acad. Sci. U. S. A.* (2012) doi:10.1073/pnas.1213069109.
237. Kryuchkova-Mostacci, N. & Robinson-Rechavi, M. A benchmark of gene expression tissue-specificity metrics. *Brief. Bioinform.* bbw008 (2016) doi:10.1093/bib/bbw008.
238. Sansom, S. N. *et al.* Population and single-cell genomics reveal the Aire dependency, relief from Polycomb silencing, and distribution of self-antigen expression in thymic epithelia. *Genome Res.* (2014) doi:10.1101/gr.171645.113.
239. Mendez-Enriquez, E. & García-Zepeda, E. A. The multiple faces of CCL13 in immunity and inflammation. *Inflammopharmacology* **21**, 397–406 (2013).
240. Paschalidis, N. *et al.* Role of endogenous annexin-A1 in the regulation of thymocyte positive and negative selection. *Cell Cycle* (2010) doi:10.4161/cc.9.4.10673.
241. Larsen, B. M. *et al.* Identification of an Intronic Regulatory Element Necessary for Tissue-Specific Expression of Foxn1 in Thymic Epithelial Cells. *J. Immunol.* (2019) doi:10.4049/jimmunol.1801540.
242. Watanabe, O. Autoimmune Associated Encephalitis and Dementia. *Brain and nerve = Shinkei kenkyū no shinpo* (2016) doi:10.11477/mf.1416200403.
243. Ohkawa, T. *et al.* Autoantibodies to epilepsy-related LGI1 in limbic encephalitis neutralize LGI1-ADAM22 interaction and reduce synaptic AMPA receptors. *J. Neurosci.* (2013) doi:10.1523/JNEUROSCI.3506-13.2013.
244. Kanamori-Katayama, M. *et al.* LRRN4 and UPK3B are markers of primary mesothelial cells. *PLoS One* (2011) doi:10.1371/journal.pone.0025391.
245. Jansen, P. A. M. *et al.* Expression of the vanin gene family in normal and inflamed human skin: Induction by proinflammatory cytokines. *J. Invest. Dermatol.* (2009) doi:10.1038/jid.2009.67.
246. Huang, K., Luo, Y.-B. & Yang, H. Autoimmune Channelopathies at Neuromuscular Junction. *Front. Neurol.* **10**, (2019).
247. Kaya, Z., Katus, H. A. & Rose, N. R. Cardiac troponins and autoimmunity: Their role in the pathogenesis of myocarditis and of heart failure. *Clinical Immunology* (2010) doi:10.1016/j.clim.2009.04.008.
248. Göser, S. *et al.* Cardiac troponin I but not cardiac troponin T induces severe autoimmune inflammation in the myocardium. *Circulation* (2006) doi:10.1161/CIRCULATIONAHA.106.635664.
249. Genome Reference Consortium. GRCh38.p12. *NCBI* (2017).
250. Firsanov, D. V., Solovjeva, L. V. & Svetlova, M. P. H2AX phosphorylation at the sites of DNA double-strand breaks in cultivated mammalian cells and tissues. *Clinical Epigenetics* (2011) doi:10.1007/s13148-011-0044-4.
251. Scully, R. & Xie, A. Double strand break repair functions of histone H2AX. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis* (2013) doi:10.1016/j.mrfmmm.2013.07.007.
252. Kinner, A., Wu, W., Staudt, C. & Iliakis, G. Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic acids research* (2008) doi:10.1093/nar/gkn550.

253. Rogakou, E. P., Boon, C., Redon, C. & Bonner, W. M. Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J. Cell Biol.* (1999) doi:10.1083/jcb.146.5.905.
254. Sedelnikova, O. A., Rogakou, E. P., Panyutin, I. G. & Bonner, W. M. Quantitative detection of 125idU-induced DNA double-strand breaks with γ -H2AX antibody. *Radiat. Res.* (2002) doi:10.1667/0033-7587(2002)158[0486:QDOIID]2.0.CO;2.
255. Cai, Z., Vallis, K. A. & Reilly, R. M. Computational analysis of the number, area and density of γ -H2AX foci in breast cancer cells exposed to 111In-DTPA-hEGF or γ -rays using Image-J software. *Int. J. Radiat. Biol.* (2009) doi:10.1080/09553000902748757.
256. Cornelissen, B. *et al.* Imaging DNA damage in vivo using γ H2AX-targeted immunoconjugates. *Cancer Res.* (2011) doi:10.1158/0008-5472.CAN-10-4587.
257. Sharma, A., Singh, K. & Almasan, A. Histone H2AX phosphorylation: A marker for DNA damage. *Methods Mol. Biol.* (2012) doi:10.1007/978-1-61779-998-3_40.
258. Zinchuk, V. & Zinchuk, O. Quantitative colocalization analysis of confocal fluorescence microscopy images. *Curr. Protoc. Cell Biol.* (2008) doi:10.1002/0471143030.cb0419s39.
259. Ceredig, R. & Rolink, T. A positive look at double-negative thymocytes. *Nature Reviews Immunology* (2002) doi:10.1038/nri937.
260. Klein, L., Kyewski, B., Allen, P. M. & Hogquist, K. A. Positive and negative selection of the T cell repertoire: What thymocytes see (and don't see). *Nature Reviews Immunology* (2014) doi:10.1038/nri3667.
261. Van De Wiele, C. J. *et al.* Thymocytes between the β -Selection and Positive Selection Checkpoints Are Nonresponsive to IL-7 as Assessed by STAT-5 Phosphorylation. *J. Immunol.* (2004) doi:10.4049/jimmunol.172.7.4235.
262. Daley, S. R., Hu, D. Y. & Goodnow, C. C. Helios marks strongly autoreactive CD4⁺ T cells in two major waves of thymic deletion distinguished by induction of PD-1 or NF- κ B. *J. Exp. Med.* **210**, 269–85 (2013).
263. Cabarrocas, J. *et al.* Foxp3⁺ CD25⁺ regulatory T cells specific for a neo-self-antigen develop at the double-positive thymic stage. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 8453–8 (2006).
264. Azzam, H. S. *et al.* CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *J. Exp. Med.* (1998) doi:10.1084/jem.188.12.2301.
265. Perez-Villar, J. J. *et al.* CD5 Negatively Regulates the T-Cell Antigen Receptor Signal Transduction Pathway: Involvement of SH2-Containing Phosphotyrosine Phosphatase SHP-1. *Mol. Cell. Biol.* (1999) doi:10.1128/mcb.19.4.2903.
266. Hogquist, K. A., Xing, Y., Hsu, F.-C. & Shapiro, V. S. T Cell Adolescence: Maturation Events Beyond Positive Selection. *J. Immunol.* **195**, 1351–1357 (2015).
267. Frauwirth, K. A. & Thompson, C. B. Activation and inhibition of lymphocytes by costimulation. *Journal of Clinical Investigation* (2002) doi:10.1172/JCI0214941.
268. Barthlott, T. *et al.* A subpopulation of CD103^{pos} ICOS^{pos} Treg cells occurs at high frequency in lymphopenic mice and represents a lymph node specific differentiation stage. *Eur. J. Immunol.* **45**, 1760–1771 (2015).

269. Kalekar, L. A. *et al.* CD4(+) T cell anergy prevents autoimmunity and generates regulatory T cell precursors. *Nat. Immunol.* **17**, 304–14 (2016).
270. Simms, P. E. & Ellis, T. M. Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation. *Clin. Diagn. Lab. Immunol.* (1996).
271. Azzam, H. S. *et al.* Fine Tuning of TCR Signaling by CD5. *J. Immunol.* (2001) doi:10.4049/jimmunol.166.9.5464.
272. Ayre, D. C. & Christian, S. L. CD24: A rheostat that modulates cell surface receptor signaling of diverse receptors. *Frontiers in Cell and Developmental Biology* (2016) doi:10.3389/fcell.2016.00146.
273. Minato, N., Hattori, M. & Hamazaki, Y. Physiology and pathology of T-cell aging. *Int. Immunol.* (2020) doi:10.1093/intimm/dxaa006.
274. Gui, J., Mustachio, L. M., Su, D. M. & Craig, R. W. Thymus size and age-related thymic involution: Early programming, sexual dimorphism, progenitors and stroma. *Aging and Disease* (2012).
275. Moretto, M. M., Lawlor, E. M. & Khan, I. A. Aging mice exhibit a functional defect in mucosal dendritic cell response against an intracellular pathogen. *J. Immunol.* **181**, 7977–84 (2008).
276. Stritesky, G. L. *et al.* Murine thymic selection quantified using a unique method to capture deleted T cells. *Proc. Natl. Acad. Sci. U. S. A.* (2013) doi:10.1073/pnas.1217532110.
277. Breed, E. R., Watanabe, M. & Hogquist, K. A. Measuring Thymic Clonal Deletion at the Population Level. *J. Immunol.* (2019) doi:10.4049/jimmunol.1900191.
278. Tarakhovsky, A. *et al.* A role for CD5 in TCR-mediated signal transduction and thymocyte selection. *Science* (80-.). (1995) doi:10.1126/science.7542801.
279. Dong, B. *et al.* CD5-mediated inhibition of TCR signaling proceeds normally in the absence of SHP-1. *Int. J. Mol. Med.* (2016) doi:10.3892/ijmm.2016.2592.
280. Abbas, A. K., Lohr, J., Knoechel, B. & Nagabhushanam, V. T cell tolerance and autoimmunity. *Autoimmunity Reviews* (2004) doi:10.1016/j.autrev.2004.07.004.
281. Linnerbauer, S. *et al.* Virus and Autoantigen-Specific CD4+ T Cells Are Key Effectors in a SCID Mouse Model of EBV-Associated Post-Transplant Lymphoproliferative Disorders. *PLoS Pathog.* (2014) doi:10.1371/journal.ppat.1004068.
282. Jain, N. *et al.* CD28 and ITK signals regulate autoreactive T cell trafficking. *Nat. Med.* (2013) doi:10.1038/nm.3393.
283. Lenardo, M. *et al.* MATURE T LYMPHOCYTE APOPTOSIS—Immune Regulation in a Dynamic and Unpredictable Antigenic Environment. *Annu. Rev. Immunol.* **17**, 221–253 (1999).
284. Taniguchi, R. T. *et al.* Detection of an autoreactive T-cell population within the polyclonal repertoire that undergoes distinct autoimmune regulator (Aire)-mediated selection. *Proc. Natl. Acad. Sci. U. S. A.* (2012) doi:10.1073/pnas.1120607109.
285. Jiang, W., Anderson, M. S., Bronson, R., Mathis, D. & Benoist, C. Modifier loci condition autoimmunity provoked by Aire deficiency. *J. Exp. Med.* (2005) doi:10.1084/jem.20050693.
286. Pöntynen, N. *et al.* Aire deficient mice do not develop the same profile of tissue-specific autoantibodies as

- APECED patients. *J. Autoimmun.* (2006) doi:10.1016/j.jaut.2006.06.001.
287. Hässler, S. *et al.* Aire-deficient mice develop hematopoietic irregularities and marginal zone B-cell lymphoma. *Blood* (2006) doi:10.1182/blood-2006-04-019679.
 288. Kisand, K., Peterson, P. & Laan, M. Lymphopenia-Induced Proliferation in Aire-Deficient Mice Helps to Explain Their Autoimmunity and Differences from Human Patients. *Front. Immunol.* **5**, (2014).
 289. Shum, A. K. *et al.* Identification of an Autoantigen Demonstrates a Link Between Interstitial Lung Disease and a Defect in Central Tolerance. *Sci. Transl. Med.* **1**, 9ra20-9ra20 (2009).
 290. Rybakowska, P. *et al.* Multiple TRIM proteins are targets of autoimmune response in lupus and Sjogren's syndrome. (HUM7P.308). *J. Immunol.* **192**, 184.17 LP-184.17 (2014).
 291. Jiang, G. *et al.* A New Model of Experimental Autoimmune Keratoconjunctivitis Sicca (KCS) Induced in Lewis Rat by the Autoantigen Klk1b22. *Investig. Ophthalmology Vis. Sci.* **50**, 2245 (2009).
 292. Clemente, M. G. *et al.* Cytochrome P450 1A2 is a hepatic autoantigen in autoimmune polyglandular syndrome type 1. *J. Clin. Endocrinol. Metab.* (1997) doi:10.1210/jc.82.5.1353.
 293. Zachou, K., Rigopoulou, E. & Dalekos, G. N. Autoantibodies and autoantigens in autoimmune hepatitis: Important tools in clinical practice and to study pathogenesis of the disease. *Journal of Autoimmune Diseases* (2004) doi:10.1186/1740-2557-1-2.
 294. D'elios, M. M. *et al.* H⁺,K⁺-ATPase (proton pump) is the target autoantigen of Th1-type cytotoxic T cells in autoimmune gastritis. *Gastroenterology* **120**, 377–386 (2001).
 295. Kisand, K. *et al.* Mucocutaneous candidiasis and autoimmunity against cytokines in APECED and thymoma patients: Clinical and pathogenetic implications. *European Journal of Immunology* (2011) doi:10.1002/eji.201041253.
 296. Ma, C. S., Deenick, E. K., Batten, M. & Tangye, S. G. The origins, function, and regulation of T follicular helper cells. *Journal of Experimental Medicine* (2012) doi:10.1084/jem.20120994.
 297. Vinuesa, C. G., Linterman, M. A., Goodnow, C. C. & Randall, K. L. T cells and follicular dendritic cells in germinal center B-cell formation and selection. *Immunological Reviews* (2010) doi:10.1111/j.1600-065X.2010.00937.x.
 298. Busser, B. W., Adair, B. S., Erikson, J. & Laufer, T. M. Activation of diverse repertoires of autoreactive T cells enhances the loss of anti-dsDNA B cell tolerance. *J. Clin. Invest.* (2003) doi:10.1172/JCI200318310.
 299. Bosticardo, M., Peterson, P. & Villa, A. Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED). in *Encyclopedia of Immunobiology* (2016). doi:10.1016/B978-0-12-374279-7.18009-9.
 300. Kärner, J. *et al.* Anti-cytokine autoantibodies suggest pathogenetic links with autoimmune regulator deficiency in humans and mice. *Clin. Exp. Immunol.* (2013) doi:10.1111/cei.12024.
 301. Bruserud, Ø., Oftedal, B. E., Wolff, A. B. & Husebye, E. S. AIRE-mutations and autoimmune disease. *Current Opinion in Immunology* (2016) doi:10.1016/j.coi.2016.07.003.
 302. Yamada, K. *et al.* Characterization of a de novo balanced t(4;20)(q33;q12) translocation in a patient with mental retardation. *Am. J. Med. Genet. Part A* (2010) doi:10.1002/ajmg.a.33174.

303. Gui, Y. *et al.* Frequent mutations of chromatin remodeling genes in transitional cell carcinoma of the bladder. *Nat. Genet.* **43**, 875–8 (2011).
304. Lathrop, M., Rhodes, C. H. & Fiering, S. CHD6 (chromodomain helicase DNA binding protein 6). *Atlas Genet. Cytogenet. Oncol. Haematol.* **19**, 379–382 (2017).