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THE RNA-BINDING PROTEINS REGNASE-1, REGNASE-4 AND PDAP1 MODULATE HUMAN T HELPER LYMPHOCYTE CYTOKINE PRODUCTION AND PROLIFERATION

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"Du hast dich selber überwunden: aber warum zeigst du dich mir nur als den Überwundenen?
Ich will den Siegreichen sehen: wirf Rosen in den Abgrund und sprich: 'Hier
mein Dank dem Unthiere, dafür daß es mich nicht zu verschlingen wußte!'"
(Friedrich Wilhelm Nietzsche: Fragmente Juli 1882 bis Herbst 1885)

Abstract

T helper (T_H) lymphocytes are essential cells of the immune system that produce cytokines which tailor the immune response to be most efficient against a specific pathogen. Gene expression of T_H lymphocytes must be carefully and rapidly regulated, since they have to mount fast and specific responses upon encountering an antigen, while at the same time limiting excessive inflammation and tissue damage. Post-transcriptional regulation plays a key role in the function of T_H lymphocytes by modulating the stability and degradation of mature mRNAs, including those encoding for cytokines and other immune-relevant genes. This level of regulation is tightly controlled by a complex network of RNA-binding proteins (RBPs) in cooperation with microRNAs (miRNAs). RBPs often have multiple functions, paralogues and dynamic expressions which is why their function remains partially elusive, while for other RBPs the functions are completely unknown.

In the work presented in this thesis, I aimed to characterize the role of RBPs in modulating the function of T_H lymphocytes. Specifically, in the first part of the study, I investigated the redundancy of two RBP paralogues (Regnase-1 and Regnase-4), which have some known common and different features, as seen on the mouse knockout (KO) phenotypes. Regnases are RBPs with intrinsic ribonuclease activity that directly degrade mRNAs of immune-related genes. By using CRISPR-Cas9, I identified that Regnase-4 does not have unique mRNA targets, however, the deletion of both Regnases lead to a combined derepression of multiple known Regnase targets. Interestingly, despite high homology, Regnase-1 and Regnase-4 have differing expression dynamics upon T cell activation. Therefore, the observed functional differences between Regnase-1 and Regnase-4 can be attributed to their different expression levels and gene dosage.

In the second part of the study, I described how we identified and functionally characterized another RBP, namely PDAP1. We identified that RFX transcription factors modulate the expression of miR-150 which directly negatively regulates the expression of PDAP1. RNA-immunoprecipitation and sequencing (RIP-seq) of PDAP1 revealed directly bound mRNA targets that are crucial for T-cell activation, differentiation, and proliferation. Analysis of PDAP1 KO clones suggested that PDAP1 increases the stability of at least a subset of direct mRNA targets. The abrupt downregulation of miR-150 upon T cell activation releases PDAP1 from the negative brake enabling it to promote proliferation of T_H lymphocytes.

Overall, this study contributed to our understanding of the function of RBP paralogues and a novel RBP in modulating T cell function and proliferation.

Zusammenfassung

T-Helferzellen (T_H-Zellen) sind essenzielle Zellen des Immunsystems, die durch die Produktion von Zytokinen die Immunantwort so anpassen, dass sie gegen einen bestimmten Krankheitserreger am wirksamsten ist. Die Genexpression von T_H-Zellen muss sorgfältig und schnell reguliert werden, da sie beim Auftreffen eines Antigens prompt eine spezifische Reaktion dar geben müssen, die aber nicht durch eine übermässige Reaktion gesundem Gewebe schadet. Die posttranskriptionelle Regulation spielt eine Schlüsselrolle in der Funktion von T_H-Zellen, indem sie die Stabilität und den Abbau von mRNAs moduliert, einschliesslich derjenigen, die Zytokine und andere immunrelevante Gene codieren. Dieser Grad der Regulierung wird durch ein komplexes Netzwerk aus RNA-bindenden Proteinen (RBP) in Zusammenarbeit mit microRNAs (miRNAs) streng kontrolliert. RBPs haben oft mehrere Funktionen, Paraloge und dynamische Expressionen, weshalb die Funktion gewisser RBPs teilweise unklar bis zu völlig unbekannt sein kann.

In der Arbeit, die in dieser Dissertation vorgestellt wird, war mein Ziel, die Rolle von RBPs bei der Modulation der Funktion von T_H-Zellen zu charakterisieren. Im ersten Teil der Studie habe ich insbesondere die Redundanz zweier RBP-Paraloge (Regnase-1 und Regnase-4) untersucht, die einige gemeinsame und unterschiedliche Merkmale aufweisen, wie sie bei den Maus-Knockout-Phänotypen (KO) zu sehen sind. Regnasen sind RBPs mit intrinsischer Ribonuklease-Aktivität, die mRNAs immunbezogener Gene direkt abbauen. Durch die Verwendung von CRISPR-Cas9 habe ich festgestellt, dass Regnase-4 keine spezifische Ziel-mRNA hat, jedoch führt die Gendeletion beider Regnasen zu einer kombinierten Derepression mehrerer bekannter Regnase-Ziel-mRNAs. Trotz hoher Homologie haben Regnase-1 und Regnase-4 eine unterschiedliche Expressionsdynamik nach der T-Zell-Aktivation auf. Daher können die beobachteten funktionellen Unterschiede zwischen Regnase-1 und Regnase-4 auf ihre unterschiedlichen Expressionen und der Gendosierung zurückgeführt werden. Im zweiten Teil dieser Dissertation habe ich beschrieben, wie wir ein weiteres RBP, nämlich PDAP1, identifiziert und funktionell charakterisiert haben. Wir haben festgestellt, dass RFX-Transkriptionsfaktoren die Expression von miR-150 modulieren, welche die Expression von PDAP1 direkt negativ reguliert. Die RNA-Immunpräzipitation und Sequenzierung (RIP-seq) von PDAP1 ergaben direkte Ziel-mRNAs, die für die Aktivierung, Differenzierung und Proliferation von T_H-Zellen von entscheidender Bedeutung sind. Die Analyse von PDAP1-KO-Klonen legte nahe, dass PDAP1 direkt die Stabilität einer Gruppe der Ziel-mRNAs erhöht. Die abrupte Herabregulierung von miR-150 während der T-Zell-Aktivierung löst PDAP1 von der Expressionsbremse ab, was die Proliferation von T_H-Zellen fördert. Insgesamt trug diese Studie zum Verständnis von RBP-Paralogen und einem neuem RBP bei, die die Funktion und Proliferation von T_H-Zellen modulieren.

List of abbreviations

A KO	*ZC3H12A* knockout
A+D KO	*ZC3H12A* + *ZC3H12D* knockout
AGO	Argonaute proteins
AIDS	Acquired immunodeficiency syndrome
AHR	Aryl hydrocarbon receptor
AP-1	Activator protein-1
APC	Antigen-presenting cell
ARE	Adenosine uridine (AU)-rich elements
ARID5A	Adenosine thymidine (AT)-rich interactive domain-containing protein 5A
ATAC-seq	Assay for transposase-accessible chromatin sequencing
ATF	Activating transcription factor
BCL	B cell lymphoma
BHLHE40	Basic helix-loop-helix family member E40
BIM	B cell lymphoma-2 interacting mediator
BrdU	Bromodeoxyuridine
β-TrCP	β-transducin repeats-containing protein
C111A	Cysteine 111 to alanine mutant
cAMP	Cyclic adenosine monophosphate
CAR	Chimeric antigen receptor
CARMA1	Caspase activation and recruitment domains-containing MAGUK protein 1
Cas9	CRISPR-associated protein 9
CBM complex	CARMA1-BCL10-MALT1 complex
CCCH	Cysteine-cysteine-cysteine-histidine
CCL	Cysteine-cysteine motif chemokine ligand
CCR	Cysteine-cysteine motif chemokine receptor
CCR4-NOT	Carbon catabolite repression 4 - negative on TATA-less
CD	Cluster of differentiation
cDNA	Complementary DNA
CELF1	CUGBP Elav-like family member 1
CFSE	Carboxyfluorescein succinimidyl ester
ChIP-qPCR	Chromatin immunoprecipitation and quantitative PCR
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Cysteine-X-cysteine motif chemokine ligand
CXCR	Cysteine-X-cysteine motif chemokine receptor
D KO	*ZC3H12D* knockout
D141N	Aspartic acid 141 to asparagine mutant
D95N	Aspartic acid 95 to asparagine mutant
DAG	Diacylglycerol
DAMP	Damage-associated molecular pattern
DBD	DNA-binding domain
DCP2	Decapping mRNA 2
DEG	Differentially expressed gene
DSGXXS motif	Aspartic acid-serine-glycine-X-X-serine motif
EAE	Experimental autoimmune encephalomyelitis

EDC4	Enhancer of mRNA-decapping protein 4
ELK1	ETS like 1
ERK1/2	Extracellular-signal regulated kinase 1/2
FOXP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS motif	γ-interferon-activated sequence motif
GATA3	GATA-binding protein 3
gDNA	Genomic DNA
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRE	Guanosine uridine (GU)-rich element
GWAS	Genome-wide associated study
HA	Hemagglutinin
HEK293T	Human embryonic kidney 293T cells
HIV	Human immunodeficiency virus
HuR	Human antigen R
ICOS	Inducible costimulator
IDP	Intrinsically disordered protein
IDR	Intrinsically disordered region
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IκB	Inhibitor of nuclear factor κ B
IKK	IκB kinase
IL	Interleukin
IP3	Inositol-3-phosphate
IPEX syndrome	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IRAK1	Interleukin 1 receptor associated kinase 1
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
IVT	*in vitro* transcribed
IκB	Inhibitor of κB
JAK	Janus kinase
JIA	Juvenile idiopathic arthritis
KO	knockout
LCK	lymphocyte-specific tyrosine kinase
LPS	Lipopolysaccharides
LSM1-7	Like-Sm protein 1
LT-α	Lymphotoxin α
m^6A	N^6-methyladenosine
m^7G	N^7-methylguanosine
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK	Mitogen-activated kinases
MBS	miRNA binding site
MCPIP1-4	Monocyte chemoattractant protein induced protein 1-4
MEK1/2	Mitogen-activated protein kinase kinase 1/2
MFI	Mean fluorescence intensity
MHC I/II	Major histocompatibility complex I/II
miRNA	Micro RNA

MRE	miRNA response element
mRNA	Messenger RNA
MS	Multiple sclerosis
NF90	Nuclear factor 90
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NTD	N-terminal domain
OOPS	Orthogonal organic phase separation
PAMP	Pathogen-associated molecular pattern
PARN	Poly(A)-specific ribonuclease
PAT1	Protein associated with topoisomerase II 1
PBMCs	Peripheral blood mononuclear cells
P-bodies	Processing bodies
PD-1	Programmed cell death 1
PDAP1	Platelet-derived growth factor A associated protein 1
PD-L1/2	Programmed cell death ligand 1/2
PEI	Polyethylenimine
PHA	Phytohemagglutinin
PIN domain	Pili twitching motility (PilT) N-terminal domain
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLCγ1	Phospholipase C γ1
PMA	Phorbol myristate acetate
Poly(A) tail	Polyadenylic acid tail
PRD	Proline-rich domain
qRT-PCR	Quantitative reverse transcription-PCR
RA	Rheumatoid arthritis
RAF	Rapidly accelerated fibrosarcoma
RAG	Recombination activating gene
RAS	Rat sarcoma GTPase
RBD	RNA-binding domain
RBP	RNA-binding protein
Regnase	Regulatory ribonuclease
RFX	Regulatory factor X
RIP-seq	RNA immunoprecipitation and sequencing
RISC	RNA-induced silencing complex
RNase	Ribonuclease
RORγt	Retinoic acid receptor-related orphan receptor γ T
sgRNA	Single guide RNA
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
SLO	Secondary lymphoid organ
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
T1D	Type 1 diabetes
T_{CM}	T central memory cells
TCR	T cell receptor
T_{EM}	T effector memory cells
T_{FH}	T follicular helper cells
TFL	Transformed follicular lymphoma

TGF-β	Transforming growth factor β
T_H	T helper cells
TIGIT	T cell immunoreceptor with immunoglobulin and ITIM domains
Tmem	T memory cells
T_N	T naïve cells
TNF-α	Tumor necrosis factor α
TNFRSF	Tumor necrosis factor receptor superfamily
tracrRNA	Trans-activating CRISPR RNA
TRAF	Tumor necrosis factor receptor associated factor
Treg	T regulatory cells
T_{RM}	Tissue-resident memory T cells
T_{SCM}	Stem cell memory T cells
TSS	Transcription start site
TTP	Tristetraprolin
UPF1	Up frameshift 1
UTR	Untranslated region
XRN1	Exoribonuclease 1
XRNAX	Protein-crosslinked RNA extraction
ZAP-70	ζ-chain-associated protein kinase 70
ZC3H12A-D	Zinc finger CCCH-type containing 12A-D
ZF	Zinc finger
ZFP36	Zinc finger protein 36
ZFP36L1/2	Zinc finger protein 36 like 1/2

1 The immune system

The immune system is a complex network of specialized cells that enables an organism to defend itself against pathogens and cancer. The key feature of the immune system is the recognition of antigens which leads to the activation of immune cells with the aim of eliminating pathogens by different mechanisms. Antigens are expressed by pathogens, such as bacteria and parasites, as well as by cancer cells. The main mechanisms used by the immune system to fight pathogens can be divided into cell-mediated immunity and humoral immunity. Cell-mediated immunity describes the activity of immune cells, such as macrophages, monocytes, neutrophils, natural killer cells or cytotoxic T lymphocytes, that phagocytose or induce apoptosis of the pathogens thereby neutralizing them. Humoral immunity encompasses immune responses that are mediated by secreted proteins present in the blood and lymph such as antibodies and complement proteins that neutralize extracellular microbes or toxins. Elie Metchnikoff, who coined the term *immunology*, is often deemed the father of innate immunity for his landmark description of phagocytosis. For his findings, he shared the Nobel prize in 1908 with Paul Ehrlich, who in turn described antibodies and antibody-mediated immunity (Kaufmann, 2008).

The technological development of the 20th and 21st century allowed the exponential growth of knowledge about the complexity and diversity of the immune mechanisms exerted by leukocytes which are the main agents of the immune system. Apart from leukocytes, other cell types can also act as members of the immune system such as epithelial cells, Kupffer cells, and microglia.

1.1 Innate and adaptive immunity

The immune system can be divided into the innate (natural, native) and adaptive (acquired) system based on their specificity and ability of immunological memory.

The innate immune system, as the name implies, is the inborn, broad-based defense mechanism that provides an immediate response to disease-inducing agents. It does not confer long-lasting immunity to specific pathogens and consists of physical epithelial barriers, namely the skin and mucosal membranes, as well as cellular and molecular components. Innate immune cells include phagocytes (macrophages and neutrophils), monocytes, natural killer cells, dendritic cells, mast cells, basophils, eosinophils, and innate lymphoid cells. These cells recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) with specialized receptors that initiate cellular effector functions (Seong & Matzinger, 2004). PAMPs are often

conserved molecular structures found in pathogens, e.g., bacterial cell wall components such as lipopolysaccharides, viral double-stranded RNA, and fungal β-glucans. On the contrary, DAMPs are molecules produced by the host itself due to tissue damage or cell death caused by different reasons including infections, physical trauma, burns, hemorrhagic shock or chemical toxins (Seong et al., 2022). The complement system is the non-cellular compartment of the innate system which consists of plasma proteins that opsonize and neutralize microbes either directly or by recruiting phagocytes. The recruitment of phagocytes and other leukocytes to the site of a pathogen is called inflammation (Dunkelberger & Song, 2010).

The adaptive immune system, in contrast to the innate, is a specialized defense mechanism characterized by its ability to recognize a highly diverse set of antigens and to remember them specifically. This allows the adaptive immune system to mount a targeted response to pathogens upon repeated exposure, so-called *immunological memory*. B and T lymphocytes constitute the cells of the adaptive immune system which have distinct surface receptors and immune functions. B lymphocytes have a B cell receptor, mature in the bone marrow and are a part of the humoral immunity by secreting antibodies (or immunoglobulins (Ig)) upon antigen recognition. Igs bind to antigens in their unprocessed native form causing different downstream responses such as phagocytosis, activation of the complement system, activation of basophils and mast cells based on the Ig class bound to the pathogen (IgA, IgD, IgE, IgG or IgM) (LeBien & Tedder, 2008; Raff, 1973).

T lymphocytes have a T cell receptor (TCR), mature in the thymus, and are a part of the cell-mediated immunity because they either directly kill pathogens and cancer cells or activate other cells to do so. In contrast to B lymphocytes, T lymphocytes recognize only processed antigens that are presented as short peptides on the major histocompatibility complex (MHC) molecules on other cells. Peptides deriving from cytosolic proteins are presented on MHC class I (MHC I) molecules which are expressed on the surface of all nucleated cells and platelets. On the other hand, if the peptide derives from phagocytosed pathogens, it is presented on MHC class II (MHC II) molecules expressed only on professional antigen-presenting cells (APCs) which are dendritic cells, macrophages and B lymphocytes. Peptides presented on the different MHC classes are recognized by different types of T lymphocytes. The MHC I-peptide complex is recognized by T lymphocytes that express the cluster of differentiation (CD) 4, while the MHC II-peptide complex is recognized by CD8-expressing T lymphocytes.

$CD4^+$ and $CD8^+$ T lymphocytes have distinct immunological functions: $CD8^+$ T lymphocytes have the ability to directly kill microorganisms and cells which is why they are also called cytotoxic T cells. Conversely, $CD4^+$ T lymphocytes are called T helper (T_H) lymphocytes because they secrete cytokines that activate or "help" other immune cells to kill microbes and tumor cells. T_H lymphocytes are essential components of the immune system and their disfunctions are the cause of different health disorders. For example, immunodeficiencies of T_H lymphocytes can be lethal as seen in the example of the acquired immunodeficiency syndrome (AIDS). AIDS is caused by the human immunodeficiency virus (HIV) which infects and destroys $CD4^+$ T lymphocytes. If left untreated, severe $CD4^+$ T cell depletion leads to lethal opportunistic secondary infections and an increased susceptibility to cancers (Gallo & Montagnier, 2003). On the other hand, hyperactive T_H lymphocytes are the drivers of many autoimmune disorders such as multiple sclerosis (MS), rheumatoid arthritis (RA), and psoriasis.

The innate and adaptive immune systems are closely intertwined, and their direct cooperation leads to the efficient clearance of a pathogen. For example, when an extracellular pathogen crosses the epithelial barrier, it is phagocytosed by dendritic cells. The pathogen-derived peptides presented on MHC II are recognized by the cognate $CD4^+$ T lymphocytes that become activated and start to proliferate and secrete cytokines such as interleukin 17A (IL-17A). The secreted cytokines stimulate the recruitment and activation of neutrophils to phagocytose the pathogen. Upon pathogen clearance, the majority of the activated T_H lymphocytes die. However, some cells survive and form a pool of long-lived memory T (Tmem) lymphocytes. Tmem lymphocytes have undergone epigenetic and transcriptional changes that enable them to recognize and respond to the same antigen upon re-exposure more rapidly and efficiently.

In summary, T_H lymphocytes are essential cells of the immune system that amplify and shape the immune reaction and generate immunological memory. In this study, I investigated their function, and the subsequent chapters will be focused on them.

1.2 Development of T helper cells

All blood cells, including T_H lymphocytes, arise from hematopoietic stem cells in the bone marrow. The progenitor cells of T lymphocytes migrate from the bone marrow to the thymus which contains specialized epithelial cells, antigen-presenting cells, specific chemokines, and cytokines that drive and shape the T lymphocyte maturation. During maturation, the developing T lymphocytes, also called thymocytes, undergo different developmental stages characterized by the expression of the

co-receptors CD4 and CD8 and the TCR. Thymocytes that have recently reached the thymus from the bone marrow are deemed as double-negative ($CD4^-CD8^-$) since they do not express neither CD4 nor CD8, and they also lack the TCR complex. At this stage, proteins Recombination activating gene-1/2 (RAG-1/2) rearrange the locus encoding the variable domain of the TCR-β chain. The β chain then pairs with an invariant TCR-α chain and associates with CD3 chains to form a pre-TCR complex. This receptor complex is functional and transduces essential signals for the further development of T lymphocytes; survival, strong proliferative expansion, genetic recombination of the TCR-α chain locus and the expression of the CD4 and CD8 molecules, making them double positive ($CD4^+CD8^+$) thymocytes with a fully functional αβ TCR (Germain, 2002).

The highly diverse double positive αβ thymocytes undergo an important developmental checkpoint in the thymus to ensure that only T lymphocytes with tolerance to self-antigens reach the periphery. A prominent role in this process is played by the epithelial and antigen-presenting cells in the thymus. They allow thymocytes to be exposed to self-antigens bound to MHC I and II. If the TCR interacts with the MHC with low avidity, the thymocyte is positively selected and receives positive survival signals. If the TCR interacts with high avidity to self-antigens, the thymocyte is negatively selected and undergoes apoptosis or differentiates into regulatory T cells (discussed in chapter **1.5 Effector T cell subsets**) (von Boehmer et al., 1989). Most double positive thymocytes (~98%) interact very weakly with the MHC molecules and do not receive survival signals leading to cell death (so-called death by neglect) (Sprent & Surh, 2011). During these selection processes thymocytes become single positive (either $CD4^+$ or $CD8^+$). If they recognize peptides bound to MHC I, they become $CD8^+CD4^-$ cytotoxic T lymphocytes, while those that recognize MHC II-bound peptides become $CD4^+CD8^-$ T_H lymphocytes.

In addition to TCR stimulation and selection, the thymus provides an essential cytokine and chemokine milieu for the survival of T lymphocytes. The best-known function of chemokines is chemotaxis, but they also have functions in cell-cell adhesion, survival, proliferation, and differentiation (Lancaster et al., 2018). Among the essential cytokines and chemokines for thymocyte development are IL-7, cysteine-cysteine motif chemokine ligand (CCL) 19 (CCL19), CCL21, CCL25 and cysteine-cysteine motif chemokine ligand (CXCL) 12 (CXCL12) (von Freeden-Jeffry et al., 1995). IL-7 promotes the survival and proliferation of T lymphocytes by upregulating the expression of anti-apoptotic molecules of the B cell lymphoma 2 (BCL2) family and downregulating the pro-apoptotic molecule B cell lymphoma 2 interacting mediator (BIM) (Koenen et al., 2013; Z.-H. Liu et al., 2014). The chemokine CCL25 and the cysteine-cysteine motif chemokine receptor (CCR) 9 (CCR9)

are responsible for the chemotaxis of progenitor cells into the thymus (Zlotoff et al., 2010). CCL19 and CCL21, ligands for the chemokine receptor CCR7, are responsible for thymic entry, intrathymic migration, TCR signaling, formation of the immunological synapse, as well as homing to lymphoid tissues (Gollmer et al., 2009; Laufer et al., 2019; Misslitz et al., 2004; Zlotoff et al., 2010). The chemokine CXCL12, which is the ligand for cysteine-X-cysteine motif chemokine receptor (CXCR) 4 (CXCR4), is important for the survival and localization of double negative thymocytes and provides also a costimulatory signal for the pre-TCR to mediate thymocyte survival and differentiation (Lancaster et al., 2018; Trampont et al., 2010).

T lymphocytes that have not yet encountered the cognate antigen for their specific TCR are called naïve T (T_N) lymphocytes. Mature, self-tolerant T_N lymphocytes leave the thymus and migrate to secondary lymphoid organs through vascular and lymphatic vessels. T_N lymphocytes that egress from the thymus are characterized by the high expression of the lymph node-homing receptors CD62L and CCR7 as well as CD45RA which is involved in proximal TCR signaling (Courtney et al., 2019; Sallusto et al., 2000; Sprent & Surh, 2011). In the periphery, these cells receive survival signals from IL-7 and from weak TCR stimulation by interacting with MHC II molecules presenting self-peptides. This tonic TCR stimulation of T_N lymphocytes is below the threshold necessary for a full activation (Sprent & Surh, 2011). Secondary lymphoid organs, which are comprised of lymph nodes, the spleen, and lymphoid follicles in mucosa-associated lymphoid tissues, have anatomical structures that facilitate the interaction of T lymphocytes and antigens. Upon recognition of the cognate antigen with high affinity and co-stimulation provided by antigen-presenting cells, T_N lymphocytes initiate proliferation and assume effector functions by secreting cytokines that shape the immune response to efficiently clear the pathogen.

1.3 TCR structure and proximal signaling

The surface expression of the TCR is the defining feature of all T lymphocytes. The TCR is formed by covalently linked polypeptide chains TCRα, TCRβ, TCRγ, and TCRδ that form two distinct heterodimers: TCRαβ and TCRγδ. Majority of T lymphocytes express the TCRαβ heterodimer and are referred to as αβ T cells, while a small portion (~4%) of circulating T lymphocytes are γδ T cells (Groh et al., 1989). γδ T cells are often found in mucosal tissues where they exert local immunosurveillance (Deusch et al., 1991; Girardi et al., 2002). These cells are different from αβ T lymphocytes because they can recognize lipids and non-peptidic phosphorylated molecules as antigens (Constant et al., 1994; Russano et al., 2007). In this study, I focused on αβ T cells and will refer to them in the following chapters unless otherwise specified.

The TCR chains consist of an extracellular region, transmembrane region and a short cytoplasmic region with no signaling capacity. The extracellular region contains a variable domain, a constant domain and a connecting peptide. The variable domain forms the antigen recognition site and as the name suggests, it undergoes genetic rearrangements during T cell development that lead to a vast TCR repertoire. The variability of this domain is the basis for the exceptional target diversity of T lymphocytes. An individual person can have 10^5 to 10^8 unique TCR sequences, although theoretically it can be more than 10^{15} (Francis Elliott et al., 1988; Nikolich-Žugich et al., 2004). The constant domains of the TCR chains associate non-covalently with CD3 chains δ, ε, γ, and ζ, that unlike the TCR chains, contain a total of 10 immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic regions that are able to conduct the TCR signal downstream into the cell. T lymphocyte activation is a complex sequence of events involving multiple receptors that are often for practical purposes categorized as the first, second and third signal.

The first signal in the activation of T lymphocytes is the recognition by the TCR of the cognate antigen presented on the MHC on APCs. $CD8^+$ T lymphocytes and $CD4^+$ T lymphocytes recognize antigens presented on MHC class I and II receptors, respectively. This cellular contact is stabilized by adhesion molecules that form a so called "immunological synapse" together with the MHC-TCR complex, coreceptors and costimulatory receptors (Dustin & Cooper, 2000). The coreceptors CD4 and CD8 have the Lymphocyte-specific tyrosine kinase (LCK) bound to the cytoplasmic domains (Veillette et al., 1988). By binding to constant regions of MHC molecules, CD4 and CD8 bring LCK in close proximity to the ITAMs in the CD3 chains leading to a cascade of phosphorylations and conformational changes involving a network of enzymes, adaptor proteins, and secondary messengers that activate distinct signaling pathways eventually leading to the nuclear translocation of transcription factors. One of the first steps is the recruitment of ζ-chain-associated protein kinase 70 (ZAP-70) which is subsequently phosphorylated by LCK. In turn, ZAP-70 activates Phospholipase C γ1 (PLCγ1) which hydrolyzes the membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol-3-phosphate (IP3). Both DAG and IP3 are essential secondary messengers for multiple downstream signaling cascades (**Figure 1**).

Costimulatory receptors provide the second signal to lymphocytes which is necessary for complete activation and differentiation into effector cells. Without this second signal, T lymphocytes become unresponsive, or anergic, and undergo apoptosis. T lymphocytes have multiple costimulatory and coinhibitory receptors. CD28, which binds to CD80 or CD86 on APCs, is the principal costimulatory receptor that strongly enhances the TCR signal and induces the expression of the major survival and

proliferation factor IL-2 and of other costimulatory receptors. Inducible costimulator (ICOS) is another costimulatory receptor that belongs to the CD28 family. Although it activates similar signaling pathways as CD28, the main difference from CD28 is that its expression is rapidly induced upon T cell activation and it does not upregulate IL-2 (Hutloff et al., 1999). OX40 (Tumor necrosis factor receptor superfamily, member 4 (TNFRSF4), also known as CD134) is costimulatory receptor that is induced upon activation and it does not belong to the CD28 family. Upon binding to the OX40 ligand (OX40L), several anti-apoptotic factors of the BCL2 family are induced extending the survival of T lymphocytes without directly promoting proliferation (Rogers et al., 2001). Unlike CD28 and ICOS that directly associate with protein kinases, OX40 is involved in downstream signaling through the TNF-receptor associated factor (TRAF) family of adaptor proteins (Smith-Garvin et al., 2009).

The Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), on the other hand, is a coinhibitory receptor that competes with CD28 with the binding to CD80/CD86, thereby inhibiting the CD28 signaling (van der Merwe et al., 1997). Another example of a coinhibitory receptor is Programmed cell death protein-1 (PD-1) which binds to Programmed cell death-ligands 1 and 2 (PD-L1/2). PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic region which attenuate the downstream TCR signaling by recruiting phosphatases (Chemnitz et al., 2004; Okazaki et al., 2001). T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) is a coinhibitory receptor that binds to Nectins. The engagement of TIGIT also inhibits downstream signaling pathways through the recruitment of phosphatases and by promoting the secretion of the anti-inflammatory cytokine IL-10 (Joller et al., 2011).

The third signal is a broad term used to describe all other signals that T lymphocytes are exposed to that shape the differentiation to effector cells. The main players of the third signal are cytokines whose role in the differentiation of T lymphocytes will be discussed in chapter **1.5 Effector T cell subsets**.

1.4 Distal TCR signaling and transcription factors

Upon activation, T lymphocytes increase in size, proliferate, secrete effector molecules, and undergo a metabolic transition from oxidative phosphorylation to glycolysis to meet the higher energy demand (van der Windt & Pearce, 2012). The massive proliferation, also called clonal expansion, which is primarily driven by the autocrine and paracrine effect of IL-2, is a critical step in the defense process to amplify the immune reaction against the specific invading pathogen. These metabolic and

functional changes are driven by the activity of transcription factors. There are approximately 400 transcription factors that are expressed during different stages of T lymphocyte development and differentiation (J. A. Zhang et al., 2012). Based on the inducing stimulus, they can be grouped as TCR-induced transcription factors and cytokine induced transcription factors.

TCR-induced transcription factors combine the first, second and third signal of T cell activation and are responsible for the immediate T cell changes. These transcription factors include Activator protein 1 (AP-1), Nuclear factor of activated T cells (NFAT), and Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) (**Figure 1)**.

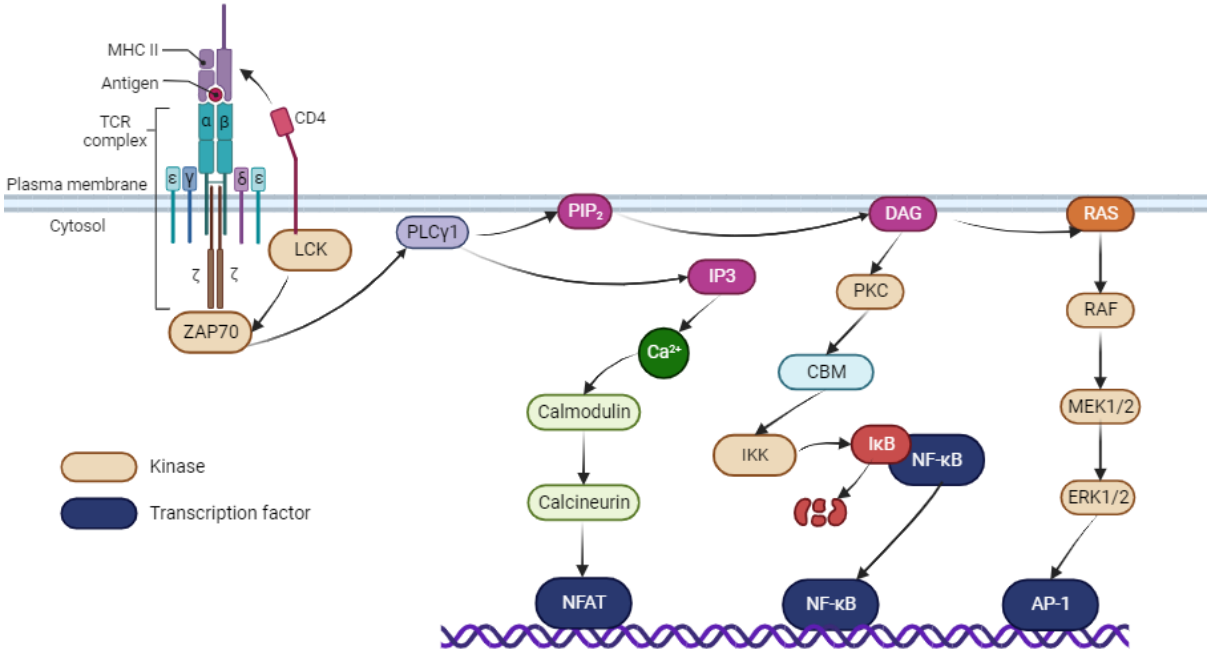

Figure 1. Schematic representation of TCR-induced transcription factors. Upon TCR activation, several signaling cascades which include kinases, adaptor molecules, phosphatases, and other signaling messengers are triggered which lead to the nuclear translocation of transcription factors. LCK - Lymphocyte-specific tyrosine kinase, ZAP70 - ζ-chain-associated protein kinase 70, PLCγ1 – Phospholipase C γ1, PIP2 - phosphatidylinositol 4,5-bisphosphate, IP3 - inositol-3-phosphate, DAG – diacylglycerol, PKC –Pprotein kinase C, CBM - CARMA1-BCL10-MALT1 complex, IKK - IκB kinase, RAS – Rat sarcoma GTPase, RAF – Rapidly accelerated fibrosarcoma kinase, MEK1/2 – Mitogen-activated protein kinase kinase 1/2, ERK1/2 – Extracellular signal-Regulated Kinase, NFAT – Nuclear Factor of Activated T Cells, NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells, AP-1, Activator protein-1. Adapted from Shah et al., 2021. Created with BioRender.com.

AP-1 is a group of transcription factors consisting of four subfamilies: Jun, Fos, Maf, and the Activating transcription factor (ATF) protein families. The family members form homo- and heterodimers that bind to common AP-1-binding site in the DNA (Karin et al., 1997). Mitogen-activated kinases (MAPK) are the essential components of the signaling cascade that leads to the activation of AP-1 members. PLCγ1 and DAG induce the activation of Rat sarcoma GTPase (RAS), a

small G protein that initiates the RAS-MAPK signaling cascade by activating the kinase Rapidly accelerated fibrosarcoma (RAF). RAF, in turn, phosphorylates Mitogen-activated protein kinase kinase 1/2 (MEK1/2), which then phosphorylates Extracellular-signal regulated kinase 1/2 (ERK1/2) (Hashimoto et al., 1998; Whitmarsh & Davis, 1996). Downstream of ERKs, transcription factors induce the expression of the of AP-1 members which form dimers with different target genes and abilities to induce transcription. For example, Jun/Fos heterodimers are stronger transcriptional inducers than other dimers (Atsaves et al., 2019).

NFAT family of transcription factors expressed in T lymphocytes (NFAT1, NFAT2, NFAT4) are required for the expression of many important genes including cytokines such as IL-2, Tumor necrosis factor α (TNF-α), Lymphotoxin-α (LT-α), and transcription factors such as Forkhead box P3 (FOXP3) (Macian, 2005; Vaeth et al., 2012). The binding of IP3 to the endoplasmic reticulum causes the release of Ca^{2+} into the cytosol. The increased cytosolic Ca^{2+} binds to the calcium-binding protein Calmodulin which in turn binds and activates the phosphatase Calcineurin that dephosphorylates NFAT eventually revealing the nuclear localization signal. In the nucleus, NFAT forms a complex with AP-1 and induces the expression of various effector molecules responsible for T cell activation. The composite DNA binding sites for NFAT and AP-1 have been identified in the promoter regions of many genes encoding proteins important for the function of T cells including IL-2, IL-4, IL-8, IL-13, Interferon γ (IFN-γ), TNF-α, Granulocyte-macrophage colony-stimulating factor (GM-CSF), Cyclooxygenase-2, and the transcription factor members of NFAT/AP-1 (Macián et al., 2001). Importantly, in the absence of AP-1, NFAT alone activates genes that are responsible for T cell anergy. Thus, NFAT controls opposite T lymphocyte functions: activation and anergy (Shah et al., 2021).

NF-κB. This family, which is composed of five members, NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel, is essential for the survival, proliferation, and differentiation of T lymphocytes. They form various hetero- and homodimers and are bound by inhibitors of κB (IκB) in the cytoplasm in an inactive form (T. Liu et al., 2017). Typical IκBs are IκBα, IκBβ, IκBε, which inhibit NF-κB by covering nuclear localization signals that prevents nuclear translocation (Beg et al., 1992). TCR engagement initiates the canonical pathway of NF-κB activation which consists of the phosphorylation, ubiquitination, and subsequent degradation of the IκB by the IκB kinase (IKK) complex. Protein kinase C (PKC) phosphorylates the adaptor proteins that form a trimer complex consisting of Caspase activation and recruitment domains-containing MAGUK protein 1 (CARMA1), B cell lymphoma 10 (BCL10) and Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) (CBM complex), that in turn recruits TRAF6 (Smith-Garvin et al., 2009). TRAFs are important adaptor

proteins that convey signals from receptors of the TNF receptor superfamily downstream to NF-κB. One of their main functions in signal transduction is mediated by their the E3 ubiquitin ligase activity (H. H. Park, 2018). TRAF6 ubiquitinates the IKK-γ subunit which allows the phosphorylation of IκB by the IKK catalytic subunits and subsequent IκB degradation (Smith-Garvin et al., 2009). This releases NF-κB from its inhibited state in the cytoplasm which leads to the nuclear translocation and its binding to κB DNA biding sites in the promoter region of many pro-inflammatory genes. (Annemann et al., 2016). However, there are also atypical IκBs, i.e. BCL3, IκBNS, IκBζ, IκBη, which do not act only as inhibitors but also as inducers of transcription by recruiting NF-κB to promoter regions (Hildebrand et al., 2013). IL-2, the most important survival and proliferation factor of T lymphocytes requires the coinciding binding of NFAT, AP-1 and NF-κB to its promoter region (X.-Y. Zhou et al., 2002).

Cytokine induced transcription factors. Signal transducer and activator of transcription (STAT) are a family of 7 transcription factors that convey the signaling of over 50 ligands (cytokines, hormones, and growth factors) through tyrosine phosphorylation-mediated activation, primarily mediated by Janus kinases (JAKs) associated to the receptors (Villarino et al., 2017). Upon binding of the ligand, the receptor dimerizes and JAKs are trans-activated due to the close proximity. The JAKs form a docking site for the recruitment of cytoplasmic STATs where they become phosphorylated. Once phosphorylated, STATs form dimers, leave the docking site and translocate to the nucleus where they bind to γ-interferon-activated sequence (GAS) motif within the promoter region of target genes (Awasthi et al., 2021; Seif et al., 2017). The GAS motifs have minimal sequence differences, and all STATs can engage with each other's "preferred" binding site and yet, the phenotypes of mice deficient in specific STATs exhibit redundant as well as non-redundant functions (Akira, 1999; Villarino et al., 2017). STATs play a critical role in the induction of lineage-specific transcription factors which will be discussed in the context of each specific subset in the following chapter.

Many other transcription factors are important for T lymphocyte development, control of proliferation, survival, and other processes, and are not necessarily involved in immediate responses upon recognition of a pathogen. Some of these factors are involved in the differentiation of specialized T cell subsets and will be discussed in chapter **1.5 Effector T cell subsets.**

Among the many transcription factors with a role of T lymphocyte biology I will just mention the Regulatory factor X (RFX) family, which will be discussed in **Manuscript [1].** This family comprises 8 members (RFX1-8) that share a highly conserved winged-helix DNA-binding domain (DBD) that binds

to X-box motifs (Aftab et al., 2008; Sugiaman-Trapman et al., 2018). The RFX family members have in their promoter regions binding sites for at least 19 other transcription factors which underlines their involvement in diverse biological processes. They are involved in regulating the cell cycle progression, cell proliferation, differentiation, and apoptosis by targeting integrins, chemokines, cytokines, receptors, and transcription factors (Sugiaman-Trapman et al., 2018). In immunity, RFX5 has a well-established role in the expression of MHC molecules which are fundamental for the development and function of T lymphocytes. Loss-of-function mutations of RFX5 result in complete lack of MHC II expression which leads to a severe primary immunodeficiency known as bare lymphocyte syndrome (Steimle et al., 1995). RFX1 has also been implicated in the function of T cells and the progression of systemic lupus erythematosus (SLE). RFX1 inhibits the expression of IL-17A by modulating chromatin modifications of the cytokine locus. Repressed expression of RFX1 in T_H lymphocytes promotes the unchecked expressed of IL-17A, exacerbating the disease (Zhao et al., 2018).

1.5 Effector T cell subsets

Activated T lymphocytes can assume different effector phenotypes depending on multiple factors: TCR affinity, duration of antigenic stimulation, concentration of the antigen, presence of costimulatory factors and the molecular milieu, including the presence of cytokines, chemokines and metabolites deriving from pathogens and cancer cells. The assumed phenotypes can be classified based on the expression of chemokine receptors, signature cytokines and transcription factors into subsets (**Figure 2**). Seminal work by Mosmann et *al.* in the 1980s formed the basis of our understanding of the subsets T_H1 and T_H2 (Mosmann et al., 1986) Further research and the development of novel technologies identified other subsets: T_H17, T_H22, regulatory T lymphocytes and follicular T_H lymphocytes. This topic of cellular immunology is a matter of active research and is being expanded with evidence of additional subsets such as T_H1^* ($T_H1/17$), T_H9 and $T_{GM\text{-}CSF}$ (Annunziato et al., 2007; Noster et al., 2014; Veldhoen et al., 2008). In the following paragraphs, I will focus on the most well described effector subsets.

T_H1. During infections by intracellular pathogens, natural killer cells and dendritic cells create an environment rich in IFN-γ and IL-12. These cytokines activate STAT1 and STAT4 in T_H lymphocytes, respectively, leading to the activation of T-bet which is the lineage-defining transcription factor of the T_H1 subset. This transcription factor directly regulates the expression of IFN-γ and the tissue homing receptors CXCR3 and CCR5 (Jacobson et al., 1995; Szabo et al., 2000). The auto/paracrine production of IFN-γ promotes further activation of STAT1 and T-bet in a positive feedback loop,

enhancing the phenotype and differentiation of other T_N cells in the vicinity (Amsen et al., 2009). T_H1 cells produce high levels of IFN-γ, TNF-α, LT-α which activate monocytes and macrophages to initiate a cell-mediated response and phagocytic clearance of an intracellular pathogen. B lymphocytes also receive stimuli from T_H1 for antibody class-switching to promote the production of opsonizing IgG antibodies (Caza & Landas, 2015; Smith et al., 2000). T_H1 have been implicated in the development of autoimmune diseases such as RA, celiac disease, type 1 diabetes (T1D), and Hashimoto's thyroiditis (Luo et al., 2022; Nilsen et al., 1998; Phenekos et al., 2004; L. S. K. Walker & von Herrath, 2015).

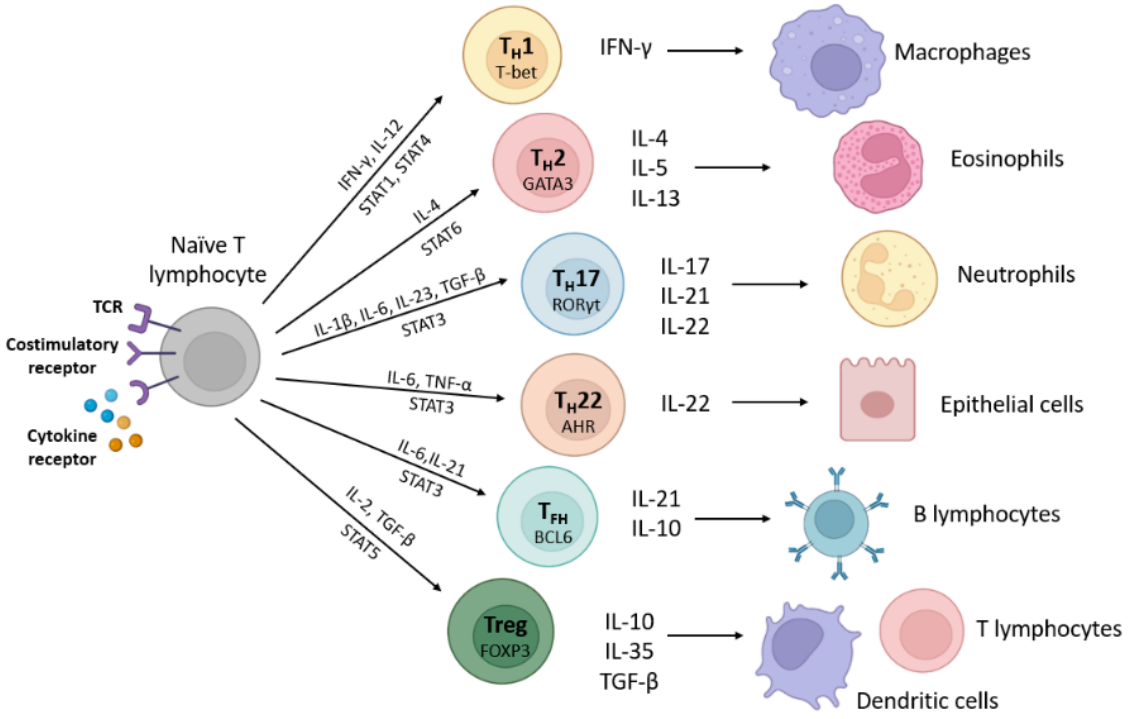

Figure 2. Differentiation of T_H subsets. Upon activation, T_N lymphocytes can acquire different phenotypes based on the activation signals and the cytokine milieu. The phenotypes are specialized to activate other immune cells in clearing the source of the antigen. Adapted from Leung et al., 2010; Loo et al., 2018; Sallusto, 2016. Created with www.BioRender.com

T_H2. A critical factor in the differentiation of T_H2 is the presence of IL-4 and absence of IFN-γ and IL-12. IL-4 activates STAT6, which in turn induces the lineage-defining transcription factor GATA-binding protein 3 (GATA3). There is evidence that STAT6 might be dispensable for T_H2 differentiation, however, GATA3 is essential for the Th2 phenotype and can also be induced through other mechanisms such as through signaling by CD40L and the Notch ligand Jagged 1 (Amsen et al., 2009; J. A. Walker & McKenzie, 2018). T_H2 cells are characterized by the expression of the receptors CCR3, CCR4 and CRT_H2 and the production IL-4, IL-5 and IL-13 (Cosmi et al., 2000; Sallusto et al., 1998; W. Zheng & Flavell, 1997). T_H2 cells initiate a humoral response mainly against extracellular

microbes and intestinal helminths, mediated by the activation of B lymphocytes, eosinophils, basophils, and mast cells. IL-4 causes B lymphocytes to switch to IgE secretion which in turn leads to the release of histamine, serotonin, prostaglandins and leukotrienes by mast cells, eosinophils, and basophils. These molecules orchestrate contractions of smooth muscles in the intestine and lungs which helps in the expelling of parasites. T_H2 cells are the main drivers of allergies, asthma and hepatic fibrosis (Chiaramonte et al., 1999; Robinson et al., 1992).

T_H17. This subset was first described in 2005 as a distinct pro-inflammatory T_H subset with IL-17A as the signature cytokine (Harrington et al., 2005; H. Park et al., 2005). Later, they were defined by the expression of $CXCR3^{-}CCR4^{+}CCR6^{+}$ and the transcription factor Retinoic acid receptor-related orphan receptor γ t (RORγt) (Acosta-Rodriguez, Rivino, et al., 2007; X. O. Yang et al., 2007). Several members of the NF-κB pathway have been shown to be important in exerting a T_H17 phenotype (Annemann et al., 2016). In particular, mice lacking IκBζ (encoded by *NFKBIZ*) showed an almost complete absence specifically of T_H17 cells and were resistant to the induction of experimental autoimmune encephalomyelitis (EAE) (Okamoto et al., 2010). IL-6 and IL-23 activate STAT3 which further activates RORγt resulting in a T_H17 phenotype (X. O. Yang et al., 2007). IL-1β and Transforming growth factor β (TGF-β) have also been shown to induce a T_H17 phenotype (Acosta-Rodriguez, Napolitani, et al., 2007; Veldhoen et al., 2006; L. Yang et al., 2008). In contrast to T_H1 and T_H2 cells, T_H17 cells exert a higher plasticity and can assume pro-inflammatory and anti-inflammatory properties characterized by the co-expression of IFN-γ and IL-10, respectively (Wu et al., 2018; Zielinski et al., 2012). Another difference in respect to T_H1 and T_H2 subsets is that T_H17 cells do not amplify further T_H17 differentiation in positive feedback, at least not directly, due to the lack of IL-6 production. However, they can indirectly promote T_H17 differentiation by secreting IL-21, another STAT3 activator (Yamane & Paul, 2012). T_H17 cells play a critical role in the protection against bacteria and fungi at mucosal barriers which they achieve by secreting pro-inflammatory cytokines IL-17A, IL-17F, IL-21, IL-22 and GM-CSF resulting in the recruitment and activation of neutrophils. The hyperactivity of T_H17 cells has been implicated in the development of a number of autoimmune disorders such as MS, RA and psoriasis (Stockinger & Veldhoen, 2007), while a deficiency results in chronic and recurrent infections with *Candida albicans* and *Staphylococcus aureus* (McDonald, 2012).

T_H22 are phenotypically and functionally similar to T_H17 cells. They participate in the protection against infections at epithelial barriers, express IL-22, CCR6 and CCR4, but in contrast to T_H17, completely lack the expression of IL-17A and express CCR10 (Duhen et al., 2009; Eyerich et al., 2009; Fujita et al., 2009; Trifari et al., 2009). Their differentiation is promoted by IL-6 and TNF-α which

activate STAT3 and the expression of the master transcription factor Aryl hydrocarbon receptor (AHR) (Trifari et al., 2009.; Yeste et al., 2014). Apart from cytokines IL-22, IL-13 and TNF-α, this subset also produces fibroblast growth factors important for wound healing, tissue repair, tissue regeneration, and fibrosis (Eyerich et al., 2009). The cytokine IL-22 affects important aspects of keratinocyte function: differentiation, proliferation, mobility, wound healing and the production of antimicrobial peptides (Wolk et al., 2006). Hyperactive T_H22 cells have a prominent role in psoriasis but have also been implicated in numerous other pathologies including RA, SLE, MS, immune thrombocytopenia, myasthenia gravis, Grave's disease and Hashimoto's thyroiditis (Hossein-Khannazer et al., 2021; Jia & Wu, 2014; Jiang et al., 2021).

Regulatory T (Treg) lymphocytes express the lineage-specific transcription factor FOXP3 and have a function in immunosuppression and the maintenance of self-tolerance (Hori et al., 2003; Sakaguchi et al., 1995). They can develop directly from thymocytes that recognize a self-antigen with high avidity (also called "natural Tregs") (Jordan et al., 2001), or can be induced in the periphery from T_N cells (also called "induced Tregs") (W. Chen et al., 2003). FOXP3 expression is induced and maintained in response to TGF-β and IL-2 signaling (Loo et al., 2018). In line with this, Tregs have the highest expression of CD25, the high affinity IL-2 receptor α chain, among all resting $CD4^+$ lymphocytes (Schmiedel et al., 2018). These cells can be distinguished from other $CD4^+$ lymphocytes by the high expression of CD25 and low expression of CD127, the IL-7 receptor (W. Liu et al., 2006). Tregs exert multiple mechanisms of immunosuppression. The main mechanism is mediated by the activity of anti-inflammatory cytokines IL-10, TGF-β and IL-35 (Collison et al., 2007; Moore et al., 2001; Nakamura et al., 2001). These cytokines suppress the activity of other immune cells to prevent excessive inflammation and tissue damage. Tregs also have the ability of inhibition through cell-to-cell contact. They express surface markers CTLA-4, PD-1, and TIGIT that interact with receptors on other immune cells, thereby inhibiting their immune functions (Fallarino et al., 2003; Francisco et al., 2009; Joller et al., 2014). Additionally, Treg lymphocytes can also influence the metabolic activity of other immune cells, for example by depriving them of IL-2 (deemed also as "IL-2 sink") or by secreting cyclic adenosine monophosphate (cAMP) (Bopp et al., 2007; Höfer et al., 2012). There is also evidence that Tregs are capable of killing conventional T lymphocytes in a granzyme- and perforin-dependent manner (Tang & Bluestone, 2008; Workman et al., 2009). A functional deficiency of Tregs has been associated with several immune diseases including T1D, SLE, and immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome which is characterized by multisystem autoimmunity with clinical manifestations raging from food allergies, eczema to endocrinopathies (Barreto et al., 2009; Chatila et al., 2000; Ferraro et al., 2011).

Follicular T_H (T_{FH}) lymphocytes. Central memory T lymphocytes that express CXCR5 have been defined as T_{FH} lymphocytes because of their predominant localization within B lymphocytes follicles in secondary lymphoid organs (Breitfeld et al., 2000; C. H. Kim et al., 2001). The lineage-defining transcription factor B cell lymphoma 6 (BCL6) and STAT3 are essential for establishing and maintaining their phenotype (Johnston et al., 2009; Ma et al., 2012; Nurieva et al., 2009; Yu et al., 2009). The costimulatory receptor ICOS is critical for the development of T_{FH} lymphocytes (Akiba et al., 2005). The most important molecules they express are IL-10, IL-21, and CD40L which support B lymphocytes in the production of antibodies and the formation of germinal centers (Breitfeld et al., 2000; Crotty, 2014). Immunodeficiencies of T_{FH} cells lead to an impaired humoral immune response due to defects in generating memory B cells and immunoglobulin isotype switching (Ma et al., 2014).

1.6 Generation of Memory T lymphocytes

Most activated T_H lymphocytes are short-lived and die upon clearance due to the lack of the activating stimulus. However, some cells survive and become long-lived with the ability to respond quickly and more potently upon recognizing the same antigen. These cells that "memorize" their cognate antigen are called memory T (Tmem) lymphocytes and comprise the majority of the T lymphocyte compartment in the peripheral blood of adults (Saule et al., 2006). The threshold of activation of Tmem lymphocytes is lower than in T_N lymphocytes and are less dependent on costimulation and cytokines (Croft et al., 1994). In respect to their homing potential, localization, and functional capacity, Tmem lymphocytes can be broadly classified as central memory (T_{CM}) ($CD45RA^-CCR7^+CD62L^+$) and effector memory T lymphocytes (T_{EM}) ($CD45RA^-CCR7^-$) (Sallusto et al., 1999). The constitutive expression of CCR7 and CD62L allows T_{CM} to extravasate through high endothelial venules and enter secondary lymphoid organs. For this reason, T_{CM} are enriched in secondary lymphoid organs such as lymph nodes and tonsils. Upon TCR activation, they produce mainly IL-2, but can eventually differentiate into effector cells and produce effector cytokines such as IFN-γ and IL-4. On the other hand, T_{EM} cells do not express CCR7 but tissue-specific chemokines and adhesion molecules required for homing to non-lymphoid tissues such as the gut, lung and liver (Sallusto et al., 2004). T_{CM} have a higher proliferative capacity, while T_{EM} cells assume effector functions very rapidly upon restimulation (Lefrançois & Marzo, 2006). More recently, a rare subset of stem cell memory T lymphocytes (T_{SCM}) ($CD45RA^+CCR7^+CD95^+CD122^+$) have been described with high proliferative and self-renewal capacity, but without effector functions (Gattinoni et al., 2011). Tmem lymphocytes that leave the blood and lymph and migrate to non-lymphoid tissues are so called tissue-resident memory T cells (T_{RM}). They reside in tissues such as the lung, skin, gut, reproductive organs where they confer long-lasting localized immunity. Instead of CCR7 and CD62L,

they express integrins and chemokine receptors specific for entering and homing to these tissues where they serve the function of local immunosurveillance (Geberhardt et al., 2009; Iijima & Iwasaki, 2014; M. Z. M. Zheng & Wakim, 2022).

It is still not fully understood how Tmem lymphocytes develop from T_N lymphocytes. Multiple models have been proposed to explain their genesis. One model suggests a divergent pathway with an asymmetric division of a T_N lymphocyte that gives rise to daughter cells with different phenotypes: one effector-like and one memory-like daughter cells. In this model, memory lymphocytes develop directly from T_N lymphocytes bypassing an effector stage (Kaech et al., 2002) A progressive model proposes that T lymphocytes accumulate signals from the TCR, costimulatory and cytokine receptors at different intensities, qualities, and durations, eventually leading to different levels of differentiation. After removal of the antigen, activated cells are selected for their ability to survive in the presence of homeostatic cytokines. This ensures that only the “fittest” activated T lymphocytes enter the memory pool. According to this model, cells at intermediates stages of the differentiation process form a T_{CM} pool (Lanzavecchia & Sallusto, 2002).

In summary, T_H lymphocytes are essential players of the immune system. They have a central role in regulating functions of innate immune cells, the epithelium, cytotoxic T lymphocytes and B lymphocytes. The thymus is the main site of their development where they undergo different stages of maturation and TCR selection to ensure survival of cells that weakly recognize self-antigens. Mature T_N lymphocytes, upon recognition of the cognate antigen, can become Tmem lymphocytes and assume different effector phenotypes depending on a variety of conditions present during activation. The different phenotypes are specialized in supporting the removal of a wide range of pathogens ranging from intra- and extracellular bacteria, viruses, fungi to helminths.

2 Post-transcriptional gene regulation in T helper lymphocytes

Upon antigen recognition, T_H lymphocytes undergo extensive changes including a switch to glycolysis, proliferation, and acquire a new phenotype by changing the expression of surface receptors and secretion of effector molecules. These dynamic changes happen within hours and days upon activation, and therefore need to be rapidly and tightly regulated to assume an effective phenotype to fight against the specific pathogen. Furthermore, the initiation, duration and resolution of the immune response has to be regulated to prevent a pathological, unrestrained, overreaction that can cause damage to healthy tissue. Cytokines, the main effector molecules of T_H lymphocytes, are not stored in subcellular vesicles and their secretion depends on the translation of transcripts generated by AP-1, NFAT, NF-κB and other transcription factors explained in chapter **1.4 Distal TCR signaling and transcription factors**.

However, transcriptional regulation only partially explains the regulation of the protein output. In fact, the transcribed RNA undergoes different processing steps at the post-transcriptional level that represents another, more immediate, checkpoint of protein production. During the transcription in the nucleus, the pre-messenger RNA (pre-mRNA) is already being modified by RNA-binding proteins (RBPs) that mediate splicing, capping (adding 7-methylguanosine (m^7G) cap at the 5' end) and polyadenylation (adding a polyadenylic acid (poly(A)) tail at the 3' end) of the transcript. These modifications render the mature mRNA stable, which is then actively transported into the cytoplasm where translation can be initiated. However, not all mRNAs serve as a template for translation. Some transcripts generate aggregates in the cytoplasm forming processing bodies (P-bodies) or stress granules or get actively degraded by RBPs. Therefore, the amount of mRNA is governed by the balance of mRNA transcription and degradation (Akira & Maeda, 2021). This post-transcriptional level of gene expression regulation in T_H lymphocytes is exceedingly important because it allows a fast, fine-tuned adjustment of protein output. In fact, it was proposed that up to 50% of changes in mRNA abundance upon activation are due to changes in stability of mature mRNA as opposed to synthesis of new mRNA transcripts (Cheadle et al., 2005). The main mechanism of decreased transcript stability is through mRNA decay (Raghavan et al., 2002). The regulation of mRNA stability is governed by the interaction of *cis*-acting factors found in the 5' and 3' untranslated regions (UTRs) of mRNA with *trans*-acting factors that recognize and bind them. The binding of *trans*-acting factors to the UTRs leads to the recruitment of other factors that regulate the half-life of the mRNA transcript. The molecular mechanisms affecting the decay of mature mRNA will be the focus of the following chapters.

2.1 *Cis*- and *trans*-acting factors

Post-transcriptional regulation is a complex network of interactions between RNA and proteins that can be classified into two major molecular components: *cis*-acting regulatory sequence elements (*cis*-elements) and *trans*-acting factors. *Cis*-elements are sequences contained in the 5' and 3' UTRs, introns, and coding regions of precursor and mature mRNAs. These regulatory sequences can form secondary structures, so called stem loops or hairpin loops. The 3'UTR of cytokines, which are often longer than the coding sequence, contain many *cis*-elements allowing the interaction with potentially hundreds of trans-acting factors (S. Kim et al., 2021). *Cis*-elements are recognized by *trans*-acting factors microRNAs (miRNAs) and RBPs. The mechanism of recognition by miRNAs is based on partial sequence complementarity, while for many RBPs it is mediated by RNA-binding domains (RBDs). In the next paragraphs, I will explain in more detail the *cis*-elements and *trans*-acting factors relevant for T cell biology.

Adenosine uridine (AU)-rich elements (AREs) are most frequent *cis*-element in the human genome, with 5-8% of all genes containing them (Bakheet et al., 2001). They are also the best studied ever since their first description as conserved stretches of adenosine and uridine in the 3'UTR of *TNF* (Caput et al., 1986). Since then, AREs have been identified as crucial half-life regulators of many cytokines and immune-related genes including *IL1B, IL2, IL3, IL6, IL10, IL17A, CSF2, FOS* and *JUN* (C.-Y. A. Chen & Shyu, 1995; Gratacós & Brewer, 2010; Lee et al., 2012; Stoecklin et al., 2008). There is no single consensus sequence of AREs, but most often they include several repeats of the pentamer AUUUA ranging from 50 to 150 nucleotides (C.-Y. A. Chen & Shyu, 1995; Peng et al., 1996). The importance of AREs was demonstrated with the mouse model lacking ARE in the 3'UTR of *Tnf* (TNF-ΔARE). These mice suffered from chronic inflammatory arthritis and Crohn's-like inflammatory bowel disease due to the inability of the RBP Tristetraprolin (TTP) to initiate an ARE-dependent degradation of *Tnf* (Kontoyiannis et al., 1999; Taylor et al., 1996). The mRNA *HBB* (encoding β-globin) which is under normal conditions a stable transcript, became unstable when AREs were introduced in the 3'UTR (Shaw & Kamen, 1986). For these reasons, AREs are generally considered as destabilizing factors, however, some RBPs stabilize ARE-containing transcripts such as the RBP human antigen R (HuR) (Fan & Steitz, 1998).

Stem loops or hairpin loops are secondary structures generated by complementary base-pairing of RNA that forms a short double-stranded stem capped with a loop. They can also assume a more complex structure with multiple stems and bulges at regions with unpaired sequences. In the UTRs of mRNAs, they serve as binding sites for proteins and act as substrates for enzymes. Stem loops are

involved in diverse processes in the life of mRNA including subcellular localization, regulation of translation and stability (Svoboda & Cara, 2006). In the context of immunity, stem loops are present in the 3'UTR regulating the stability of cytokines and immune-related genes. The recognition of stem loops is not based on sequence specificity but on the stem loop shape. However, RBPs such as Roquin-1, Roquin-2 and Regulatory ribonuclease 1 (Regnase-1) do require a pyrimidine–purine–pyrimidine loop (Schlundt et al., 2014).

Guanosine uridine (GU)-rich elements (GREs) are another type of *cis*-elements that are less investigated in comparison to AREs and stem loops. They have been described to be functionally similar to AREs by destabilizing transcripts in human T lymphocytes and are overrepresented in the 3'UTR of short-lived transcripts in human T cells, such as *TNFR2* and *JUN*. The RBPs CUG triplet repeat, RNA binding protein 1 (CUGPB1) and CUGBP Elav-like family member 1 (CELF1) recognize them and recruit the Poly(A)-specific ribonuclease (PARN) for degradation (Moraes et al., 2006; Vlasova et al., 2008).

miRNAs are short (~22 nucleotides) non-coding RNAs that act as *trans*-acting factors by binding to miRNA-binding sites (MBS), also called miRNA response element (MRE), mainly located in the 3'UTR of target mRNA. It is estimated that ~30% of the human protein-coding genome (~8000 genes) contains MBS, implying an essential role in multiple biological processes. Interestingly, they are more frequently present in immune-related genes in comparison to the rest of the genome, predominantly regulating transcription (co)factors, signaling pathways and chromatin regulators (Asirvatham et al., 2008). Their binding usually results in a negative regulation of the target expression. Genes encoding miRNAs are transcribed as longer primary transcripts that are processed by the enzymes Drosha and Dicer. The resulting short double-stranded RNA is incorporated into the RNA-induced silencing complex (RISC) through the binding to Argonaute proteins (AGOs). The RISC, guided by the miRNA, binds to complementary sequences in the target mRNA and causes inhibition of mRNA translation or induction of mRNA degradation (Ivanov & Anderson, 2013). In rare cases, miRNA binding can induce translation such as the binding of miRNA-10a in the 5'UTR of mRNA encoding ribosomal proteins (Ørom et al., 2008). Genetic ablation of the main players of miRNA biogenesis, Drosha and Dicer, resulted in defective T lymphocyte development, function, and lymphopenia in mice due to a reduction in miRNA expression (Chong et al., 2008; Muljo et al., 2005). miRNAs can potentially regulate multiple targets because of their short sequences and mismatched pairing to MBS. For example, miR-181a, which has a critical role in T lymphocyte development, has ~600 predicted target genes (Asirvatham et al., 2008; Q.-J. Li et al., 2007). One of the most

abundantly expressed miRNAs in $CD4^+$ and $CD8^+$ lymphocytes is miR-150, and accordingly, it regulates multiple processes in T cell biology. Specifically, miR-150 has been shown to be involved in the differentiation of effector and memory T lymphocytes, regulation of T cell activation, proliferation, and survival by targeting several genes involved in signaling pathways, including *MYB*, *FOXP1* and the Roquin-1 encoding gene *RC3H1* (Ménoret et al., 2023; Xia et al., 2022).

RBPs are essential *trans*-acting factors and mediators of post-transcriptional regulation. They recognize *cis*-elements and N^6-methyladenosine (m^6A) modification in the RNA, thereby regulating all aspects of RNA life, including splicing, capping, polyadenylation, methylation, editing, nuclear export, localization, and decay (Akira & Maeda, 2021; Newman et al., 2016). An integrative approach based on multiple methods predicted 4200 putative RBPs in all human cell types, which represents one fifth of the protein-coding genome and exceeds the number of transcription factors, further underlying the importance of post-transcriptional regulation (Gebauer et al., 2021). Human T_H lymphocytes express 800-1250 identified RBPs that bind to mature polyadenylated mRNA, with an overlap of ~70% with murine T_H lymphocytes (Hoefig et al., 2021). The functions and mechanisms of action of many RBPs have been difficult to unravel, partly due to complex pleiotropic, redundant functions and the presence of multiple paralogues. Interestingly, a number of RBPs have been identified to have multiple functions that are not related to post-transcriptional control. For example, the housekeeping enzyme Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which plays a key role in glycolysis and multiple other cellular processes, is an RBP that in T lymphocytes is involved in the RNA stability and translation efficiency of *IFNG* and *IL2* (Chang et al., 2013; White & Garcin, 2016). As mentioned earlier, resting T lymphocytes rely on oxidative phosphorylation. This enables GAPDH to bind to the 3'UTRs of cytokine mRNAs decreasing their translation. Upon activation, T cells switch to glycolysis where GAPDH is recruited thereby releasing the cytokine mRNAs from the negative regulation (Chang et al., 2013).

There are around 30 RBDs described that interact with RNAs which include the RNA recognition motif, cysteine-cysteine-cysteine-histidine (CCCH) zinc finger (ZF) domain, K homology, ROQ domain and YTH domain (Gebauer et al., 2021). Crucial regulators of cytokine mRNA regulation in T lymphocytes contain a CCCH ZF domain such as TTP, Roquin and Regnase family of RBPs (Fu & Blackshear, 2017). However, unconventional RBPs have also been identified and they have no discernable RBDs but intrinsically disordered regions that engage with RNA (Castello et al., 2016; Gebauer et al., 2021).

2.2 Mechanisms of RBP-mediated mRNA decay

Cytoplasmic turnover of mRNA plays a central role in protein output. Mature mRNA contains an m^7G cap at the 5′ ends and a poly(A) tail at the 3′ ends which render the transcript stable. Therefore, many decay mechanisms typically involve the generation of bare 5′ and 3′ ends. There are several protein-RNA interactions important for mRNA decay that can be roughly classified into exonucleolytic and endonucleolytic decay mechanisms. There are also other mRNA degradation mechanisms that act as a surveillance mechanism for RNA quality which are triggered when an aberrant translational status is detected, including nonsense-mediated decay, no-go decay, and nonstop decay (Akiyama et al., 2021; J. Cheng et al., 2017). For the regulation of cytokine production, exonucleolytic and endonucleolytic decay are the main mechanisms of establishing an adjusted immune response (**Figure 3**). RBPs such as TTP and Roquin recognize AREs and stem loops in the 3′UTR, respectively, and recruit the Carbon catabolite repressor 4–negative on TATA (CCR4-NOT) deadenylase complex to remove the poly(A) tail. The shortened poly(A) tail is then targeted by the LSM1–7/PAT1 (Like-Sm protein 1/Protein associated with topoisomerase II 1) complex which recruits the decapping enzyme DCP2 (Decapping protein 2) that removes the m^7G cap, allowing the 5′→3′ exonucleolytic cleavage by Exoribonuclease 1 (XRN1) (Akiyama et al., 2021; C.-Y. Chen et al., 2001; Fenger-Grøn et al., 2005; Hsu & Stevens, 1993; Leppek et al., 2013; Sandler et al., 2011). The endonucleolytic cleavage is initiated by RBPs with intrinsic ribonuclease (RNase) activity, which generate a bare 5′ and 3′ ends rendering the transcript susceptible to the above-mentioned exonucleolytic enzymes (Akiyama et al., 2021; Yoshinaga & Takeuchi, 2019).

The expression levels, competition, and cooperation between RBPs govern the extent of mRNA decay. For example, in unstimulated T lymphocytes, ARE in the 3′ UTR of the *IL2* mRNA mediates rapid degradation by negative regulators such as TTP (Ogilvie et al., 2005). Upon activation, *IL2* is stabilized by the binding of the RBP Nuclear factor 90 (NF90) to the ARE, slowing down the degradation of the mRNA (Shim et al., 2002). *IL2* also contains stem loops in the 3′UTR that are targeted for degradation by Regnase-1 (M. Li et al., 2012). Upon activation, Regnase-1 expression is strongly reduced, releasing the negative brake from IL-2 production (Uehata et al., 2013). Alternatively, the access of Regnase-1 to its target can be blocked by the RBP AT-rich interactive domain-containing protein 5A (ARID5A), which protects transcripts from degradation by binding to stem loops (Masuda et al., 2013). RBPs can potentially synergistically regulate mRNA decay. There is strong evidence that Regnase-1 and Roquin-1 (and its redundant paralogue Roquin-2) cooperatively regulate a common set of target genes through the same stem loops (Mino et al., 2015). A disrupted

interaction between Regnase-1 and Roquin-1 led to a pronounced pro-inflammatory phenotype (Behrens et al., 2021).

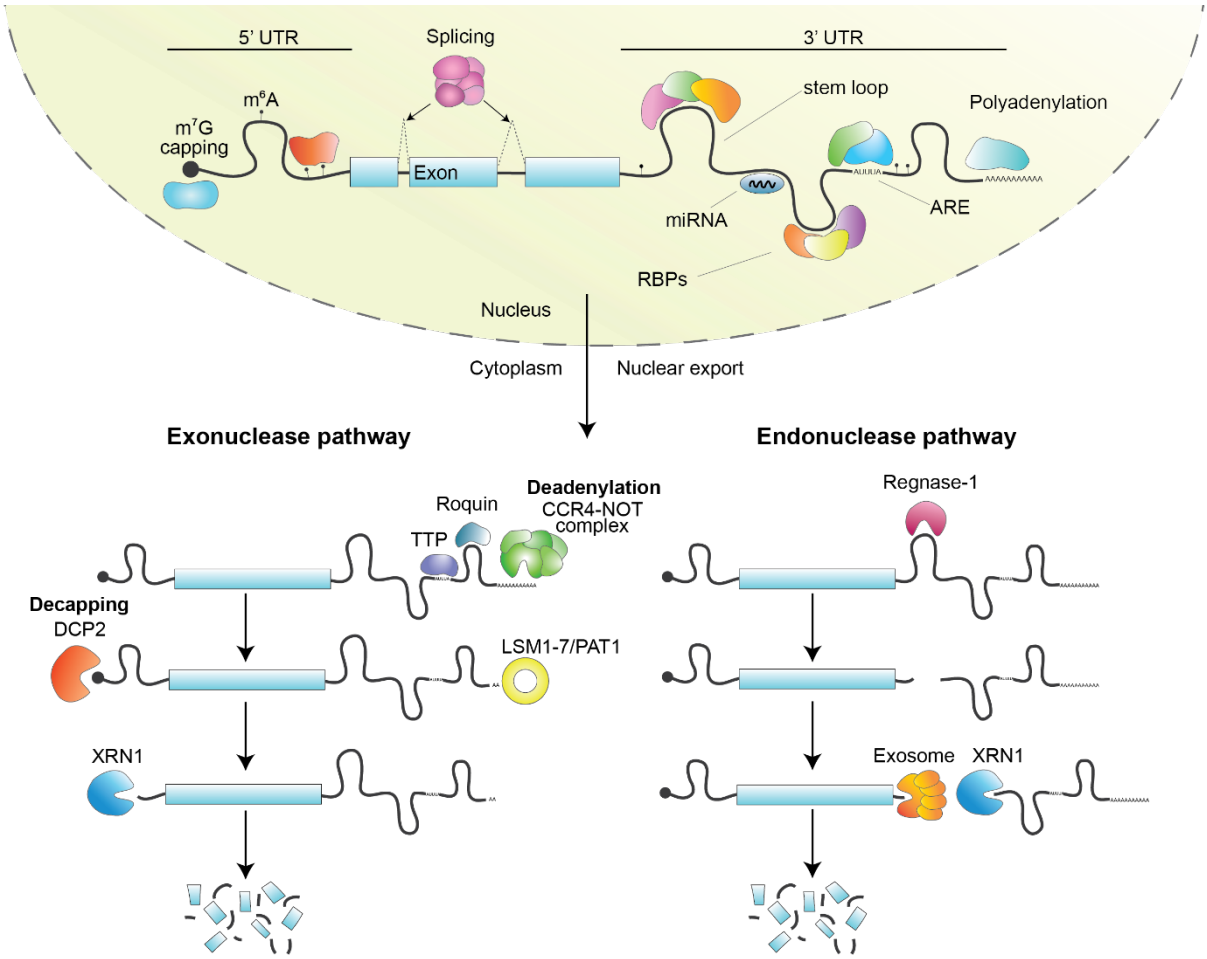

Figure 3. Schematic overview of post-transcriptional regulation of mRNA. During and immediately after transcription, mRNA is bound by RBPs and miRNAs that regulate every aspect of its expression and function through splicing, capping, polyadenylation, editing, stability, and nuclear export. Transferred in the cytoplasm, its stability is regulated by RBPs that can induce the mRNA degradation through different mechanisms. ARE – AU-rich element, CCR4-NOT – Carbon catabolite repression 4-negative on TATA-less, DCP2 – Decapping mRNA 2, LSM1-7 – Like-Sm protein 1-7, m^6A – N^6-methyladenosie, m^7G – N^7-methylguanosine, miRNA – microRNA; PAT1 – Protein associated with topoisomerase II, RBP – RNA-binding protein, TTP – Tristetraprolin, UTR – untranslated region, XRN1 – Exoribonuclease 1. Adapted from Akiyama et al., 2021.

In summary, miRNAs and RBPs form an exceedingly complex network of positive and negative regulators of mRNA expression at the post-transcriptional level that fine-tunes the expression of immune-related genes at different stages of T cell activation. A factor that makes the investigation of this network more challenging is the fact that many RBPs have paralogues. For example, TTP (encoded by *ZFP36* – Zinc finger protein 36) has paralogues ZFP36 like 1 and 2 (ZFP36L1 and ZFP36L2) in human and murine T lymphocytes with mostly redundant functions, but ZFP36L1 and

ZFP36L2 are specifically required for antigen-specific T cell clonal expansion, at least in the murine system (Cook et al., 2022). Similarly, Roquin-1 and Roquin-2 are fully redundant and Roquin-2, which is expressed at 5 times lower levels than Roquin-1, can compensate for the lack of Roquin-1 (Vogel et al., 2013). Regnase-1 is also part of a family with 4 paralogues which are much less investigated than the Roquin family.

Part of this study was focused on elucidating the role of Regnases, whose known functions and mechanisms I will explain in the following chapter.

2.3 Regnases – a family of destabilizing RBPs

Regnases are a family of four RNases encoded by the genes zinc finger cysteine-cysteine-cysteine-histidine 12A-D (*ZC3H12A-D*). The family namesake Regnase-1 (*ZC3H12A*, also called MCPIP1 – monocyte chemoattractant protein induced protein 1) was first described as a protein induced by treating monocytes with monocyte chemoattractant protein-1 (MCP-1; CCL2), IL-1β and lipopolysaccharides (LPS) (Mizgalska et al., 2009; L. Zhou et al., 2006). For this reason, the earliest data on the importance of Regnase-1 in inflammation comes from data based on murine monocytes. Of note, Regnases exhibit a high level of conservation within multiple species, and the homology between human and mouse Regnases varies from 81 to 92% (Liang et al., 2008). In macrophages, Regnase-1 is induced by different signals, suggesting the importance of this protein in keeping the cells in a quiescent state. Because of a putative nuclear localization sequence arginine-lysine-lysine-proline (RKKP), a ZF motif and an *in vitro* transactivation assay, Regnase-1 was considered to be a transcription factor that induces the expression of pro-apoptotic genes (Niu et al., 2008; L. Zhou et al., 2006). The landmark work by Mastushita *et al.*, described Regnase-1 as an RNase with a pili twitching motility N-terminal (PIN) domain that contains an aspartic acid residue 141 (D141) which was essential for degrading *Il6* in a 3'UTR-dependent manner (Matsushita et al., 2009). The catalytic RNase site in the PIN domain was later confirmed to be a negatively charged pocket formed by several conserved acidic residues where D141, D225, D226 and D244 were the most important residues (Xu et al., 2012). A mutation of the amino acid D141 completely abrogated the RNase activity (Matsushita et al., 2009).

Although both the ZF domain and the PIN domain can bind to RNA, these domains exist and function independently without interacting with each other (Yokogawa et al., 2016). Further investigations revealed the importance of domain-domain interactions of Regnase-1 for exhibiting the RNase activity *in vitro*. More precisely, it was identified through *in vitro* cleavage assays and electrophoretic

mobility shift assays with *Il6* mRNA that the presence of the N-terminal domain (NTD) strongly increased the RNase activity of the PIN domain, despite not directly binding to RNA. It is further suggested that the NTD/PIN interaction changed the conformation of the PIN domain allowing the binding of Mg^{2+} which is necessary for the RNase activity (Xu et al., 2012; Yokogawa et al., 2016). Additionally, Regnase-1 formed dimers through PIN-PIN interactions that are required for *in vitro* RNase activity, since the monomeric mutants (P212A, R214A, D278R) displayed no RNase activity (Yokogawa et al., 2016). The model of the enzymatic activity proposed by Yokogawa *et al.* suggests that in the absence of a target mRNA, the PIN domain forms dimers. However, only when the NTD of one of the Regnase-1 molecules binds to its PIN domain, a fully functional dimer is generated (Yokogawa et al., 2016).

Interestingly, Regnase-1 is also involved at the translational level of regulation by removing ubiquitin moieties from TRAF2, TRAF3 and TRAF6 proteins thereby inhibiting the NF-κB pathway. The mutation of cysteine 157 (C157) abrogates the deubiquitinase activity, at least *in vitro* (Liang et al., 2010). Therefore, Regnase-1 has different enzymatic activities that are mechanistically not fully elucidated, but it is clear that complex intermolecular and intramolecular interactions tightly control the protein's activity and therefore the immune response.

The global Regnase-1 deficiency in mice lead to death within 12 weeks after birth due to a severe lupus-like auto-inflammatory disease that included severe anemia, splenomegaly, lymphadenopathy, increased numbers of plasma cells, increased levels of anti-nuclear antibodies and anti-double-stranded-DNA antibodies, increased secretion of pro-inflammatory cytokines by macrophages and T lymphocytes (Matsushita et al., 2009). The $CD4^{+}$-specific deletion of Regnase-1 recapitulated the global knockout (KO), underlining the importance of Regnase-1 in restraining T cell activation for the prevention of auto-inflammatory disorders (Uehata et al., 2013). Most of the T cells of the KO mouse were of the effector/memory phenotype ($CD62L^{-}CD44^{hi}$) with increased proliferation and secretion of IFN-γ, IL-17A and IL-4 upon stimulation, suggesting that Regnase-1 KO T cells were polarized into specialized subsets. Subsequent studies in a number of immune cell types found a multitude of additional genes that are directly or indirectly negatively regulated by Regnase-1 such as *REL, NFKBIZ, NFKBID, CTLA4, ICOS, IL1B, IL2, IL6, IL12, PTGS2*, *TNFSF4* (OX40), and the list is steadily being expanded (M. Li et al., 2012; Mino et al., 2015; Uehata et al., 2013; Uehata & Akira, 2013; Wei et al., 2019).

Regnase-1 has multiple levels of regulation. Early on it was identified that stimulating monocytes with MCP-1 and IL-1β promoted the activation of the transcription factor ETS Like-1 (ELK1), which in turn binds to the promoter of *ZC3H12A* (Kasza et al., 2010). Regnase-1 is in a negative regulatory feedback loop with the NF-κB pathway: Regnase-1 expression is induced by four κB sites, while Regnase-1 itself degrades *NFKBID* and *NFKBIZ*, negatively regulating NF-κB (Mino et al., 2015; Mino et al., 2019; Skalniak et al., 2009) Upon TCR activation, the paracaspase MALT1 cleaves Regnase-1 at the arginine residue 111 (R111) inactivating it (Jeltsch et al., 2014). A possible mechanism of the inactivation following the cleavage by MALT1 is the removal of the NTD which *in vitro* greatly decreased the RNase activity (Yokogawa et al., 2016). It was shown in murine monocytes that the IKK complex and the Interleukin 1 receptor associated kinase 1 (IRAK1) phosphorylate Regnase-1 at the serine residues in the aspartic acid-serine-glycine-X-X-serine motif (DSGXXS motif), marking it for ubiquitination by the E3 ligase β-transducin repeats-containing protein (β-TrCP) and subsequent proteasomal degradation (Iwasaki et al., 2011). The association of Regnase-1 with β-TrCP was also observed in HeLa cells upon stimulation with IL-1β (Akaki et al., 2021). Interestingly, the 14-3-3 protein family members were also induced upon stimulation of HeLa cells, and they competed with β-TrCP for the binding to the same serine residues in the DSGXXS motif (Akaki et al., 2021). The binding of 14-3-3 family members protected Regnase-1 from the β-TrCP-mediated proteasomal degradation. However, the 14-3-3 complex functionally inhibited Regnase-1 from degrading mRNA and it sequestered it in the cytoplasm by preventing nuclear import (Akaki et al., 2021).

At the mRNA level, *ZC3H12A* is negatively regulated by Roquin-1 and Regnase-1 itself (Iwasaki et al., 2011). Also, miRNAs miRNA-9, miR-139, and miR-27a-5P have been identified to initiate degradation of the Regnase-1-econding mRNA (Y. Cheng et al., 2015; Makki & Haqqi, 2015; L. Yang et al., 2013).

The targeted *cis*-elements by Regnase-1 are stem loops. No common stem sequence has been identified among the targets, but the loops itself contain a pyrimidine-purine-pyrimidine sequence (UAU and UGU) (**Figure 4**) (Mino et al., 2015). The mechanism of stem loop recognition and subsequent mRNA degradation is not fully understood. There is evidence that the ribosomal protein up frameshift 1 (UPF1) associates with Regnase-1, and its helicase activity is critical for Regnase-1-mediated decay by unwinding the stem loop of actively transcribed mRNAs (Mino et al., 2019). In accordance with this, Regnase-1 was identified to associate with mostly ribosomal proteins in unstimulated HeLa cells (Iwasaki et al., 2011).

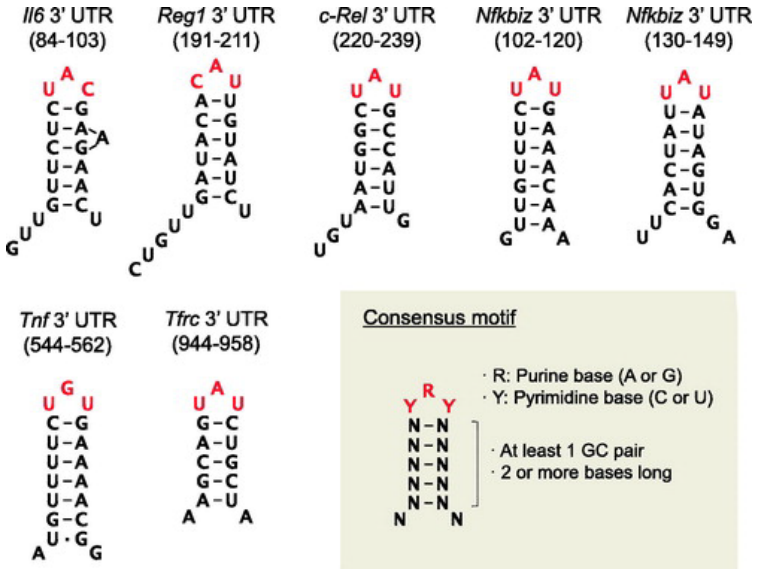

Figure 4. *Cis*-elements targeted by Regnase-1. Regnase-1 targets stem-loops in the 3'UTR without sequence specificity of the stem, but with a specificity to a loop with a final pyrimidine-purine-pyrimidine loop (Yoshinaga & Takeuchi, 2019).

Regnase-1 shares functional similarity with the RBP Roquin-1. Both RBPs degrade pro-inflammatory mRNAs and are degraded by MALT1 upon TCR activation (Jeltsch et al., 2014). Initial results suggested that these RBPs downregulate a common set of genes through distinct spatiotemporal mechanisms (Mino et al., 2015). However, more recent data in T lymphocytes showed that Regnase-1 and Roquin-1 functionally and physically cooperate (Behrens et al., 2021; Jeltsch et al., 2014). It was identified that Regnase-1 and Roquin-1 form a ternary complex with the *Zc3h12a* mRNA *in vitro*, suggesting a cooperative negative regulation of common targets. Furthermore, the disruption of their interaction by mutating amino acid residues in Roquin-1 at the binding interface with Regnase-1 lead to a higher frequency of activated T lymphocytes, increased proliferation, increased IFN-γ, and induced the accumulation of effector memory $CD8^+$ in mouse models (Behrens et al., 2021) Therefore, the interaction of Regnase-1 with Roquin-1 is important for keeping cells in a quiescent state. This further suggested that the disruption of this interaction might be used as an approach for improving cancer immunotherapy which showed promising preclinical results (Behrens et al., 2021)

Regnase-1 has three paralogues: Regnase-2, Regnase-3 and Regnase-4 (**Figure 5**). The paralog Regnase-4 (*ZC3H12D,* also called Transformed follicular lymphoma (TFL), and MCPIP4) was initially described as a tumor suppressor gene in follicular lymphoma and lung cancer (Minagawa et al., 2007; Wang et al., 2007). It has a high sequence homology to Regnase-1 and shares the protein domains. Same as Regnase-1, the overexpression of Regnase-4 inhibits proliferation and induces apoptosis (Minagawa et al., 2009, 2014).

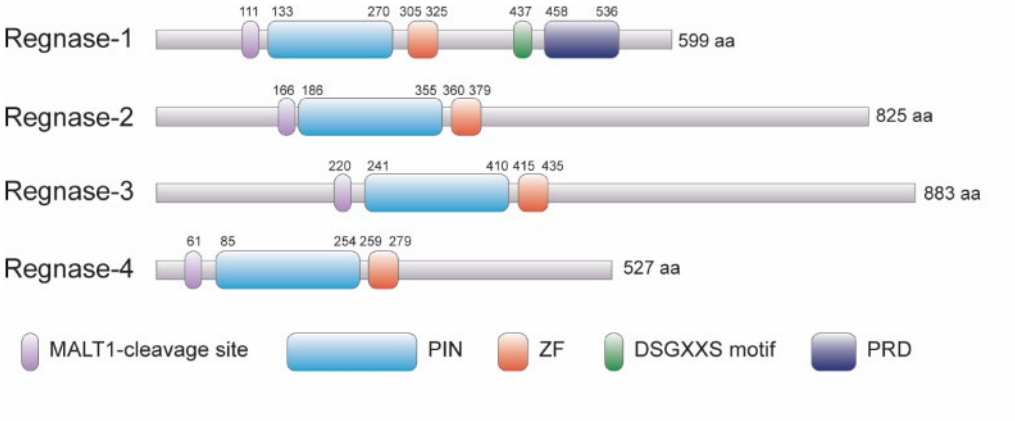

Figure 5. Schematic structures of Regnase family members. MALT1 – Mucosa-associated lymphoid tissue lymphoma translocation protein 1, PIN-(PilT) N-terminal domain, ZF-zinc finger, DSGXXS motif – aspartic acid-serine-glycine-X-X-serine motif, PRD-proline rich domain.

In contrast to Regnase-1, knocking out Regnase-4 in mice is not lethal. In fact, the KO mice lived up to 2 years under steady-state conditions. However, with the induction of EAE, the KO mice had a more severe and prolonged paralysis with T lymphocytes sorted from the brain having a higher expression of T_H17-related genes *IL17A, IL23RA and IL23RB* (Minagawa et al., 2014). Further studies identified that Regnase-4 negatively regulates mRNAs *IL1B, IL2, IL6, IL10, TNF, IL17A* and *ZC3H12A* via their 3'UTRs in an RNase-dependent manner (Iwasaki et al., 2011; Minagawa et al., 2014; Mizgalska et al., 2009; H. Zhang et al., 2015). Mutating the conserved aspartic acid residue 95 (D95) abrogates the RNase activity (Wawro et al., 2017). Importantly, Regnase-1 and Regnase-4 contribute additively, but independently, to the negative regulation of at least one mRNA, namely *Il6*, as seen in activated RAW264.7 cells, a murine macrophage cell line (S. Huang et al., 2015). Furthermore, co-immunoprecipitation and confocal microscopy have confirmed that Regnase-1 and Regnase-4 directly interact with each other (S. Huang et al., 2015). The two RBPs co-localize with P- bodies in HeLa cells, which contain protein machineries for RNA degradation (S. Huang et al., 2015).

Despite the similarity with Regnase-1 as a negative regulator of pro-inflammatory genes, one difference between Regnase-1 and Regnase-4 has been identified concerning their transcriptional regulation. The transcriptional repressor Basic helix-loop-helix family member E40 (BHLHE40) has been identified to downregulate the expression of *ZC3H12D* but not of *ZC3H12A* in primary human T cells (Emming et al., 2020).

The other two Regnase family members, Regnase-2 and Regnase-3, are expressed at very low levels in human T lymphocytes (Emming et al., 2020; Schmiedel et al., 2018). Their function in other cell types is largely unknown, although there have been some studies in recent years. Regnase proteins

are highly conserved, therefore it is expected that they exert similar functions. Indeed, overexpression of Regnase-2 suppressed the 3'UTR of *IL6* mRNA in HeLa cells, but its role *in vivo* is still not characterized (Wawro et al., 2019). Regnase-3 is highly expressed in myeloid cells. Regnase-3-deficient mice develop lymphadenopathy, most likely due to overproduction of IFN-γ in macrophages, although the direct targets of Regnase-3 *in vivo* remain unknown (von Gamm et al., 2019). The overexpression of Regnase-2, Regnase-3 and Regnase-4 in murine T lymphocytes lacking endogenous Regnase-1 was able to downregulate the expression of ICOS, a known Regnase-1 target, further suggesting the redundancy of the Regnase paralogues (Behrens et al., 2021).

2.4 Intrinsically disordered regions in RBPs

Novel RBPs are identified by different biochemical assays, such as RNA-immunoprecipitation and sequencing (RIP-seq), orthogonal organic phase separation (OOPS), protein-crosslinked RNA extraction (XRNAX), and mRNA-oligo-deoxythymidine (dT) pull-down coupled with mass-spectrometry (Baltz et al., 2012; Castello et al., 2012, 2016; Hoefig et al., 2021; Trendel et al., 2019). Because RBPs are highly conserved, their putative RBDs can be predicted computationally, at least in some cases. However, in a superset of 3470 RBPs analyzed genes, only 25% had a predicted RBD (Gebauer et al., 2021). This underlines the many unknowns in the molecular mechanisms exhibited by RBPs. In particular, many RBPs are characterized by intrinsically disordered regions (IDRs) which are natively flexible and lack a stable three-dimensional structure (Castello et al., 2012). Intrinsically disordered proteins (IDPs) frequently have multiple functions through multiple interaction interfaces and thereby are involved in a multitude of regulatory functions including transcription, RNA folding, RNA export, RNA granule formation, RNA degradation, translation and signaling pathways (Castello et al., 2012; Hautbergue et al., 2008; Järvelin et al., 2016; Vandelli et al., 2022; Wright & Dyson, 2015). IDRs can be considered as non-canonical RBDs because in 170 RBPs these regions were the sole detectable RNA-binding site (Castello et al., 2016). Importantly, RBPs that specifically interact with mRNAs are highly enriched in IDRs in comparison to the complete human proteome (Castello et al., 2012). Thus, IDRs represent a multifunctional RNA-binding module.

One example of an IDR-containing RBP is Platelet-derived growth factor A associated protein-1 (PDAP1) which has been shown to protect B lymphocytes from cell death and to promote antibody gene diversification (Delgado-Benito et al., 2020). It was originally described as a mitogen-inducing protein with no significant homology to any known class of protein in a rat neural retina cell line (Fischer & Schubert, 2002). The roles of PDAP1 in cell survival, apoptosis resistance, proliferation, and metastasis were confirmed in cancer cell lines and mouse models (Cui et al., 2022; Sharma et al.,

2016; Weston et al., 2018). Due to the high and broad expression levels across many tissues, including the brain, gastrointestinal tract, pancreas, bone marrow and lymphoid tissues, it is expected that also in these tissues PDAP1 might have a range of similar functions (Karlsson et al., 2021; Uhlén et al., 2015; Uhlen et al., 2019).

2.5 RBPs in health and disease

As described in the previous chapters, RBPs have an important role in fine-tuning the function of T_H lymphocytes by regulating the stability and decay of pro-inflammatory mRNA transcripts. Thus, the dysregulation of RBP expression is implicated in the development of inflammatory diseases in humans. In mouse models, a TTP deficiency leads to a TNF-α-mediated severe inflammatory phenotype with erosive arthritis, cachexia, and autoimmunity (Taylor et al., 1996). In humans, genome-wide associated studies (GWAS) have connected single nucleotide polymorphisms (SNPs) in the *ZFP36* gene and its paralogues *ZFP36L1* and *ZP36L2* to pathological conditions. The studies have identified multiple SNPs in the loci of these genes in patients with RA, juvenile idiopathic arthritis, psoriasis, and MS (Carrick et al., 2006; Hinks et al., 2013; Suzuki et al., 2008). In addition, *ZFP36L2* is identified as a susceptibility gene of MS, and its expression is decreased in MS patients (Parnell et al., 2014). Therefore, the variants of *ZFP36*, *ZFP36L1*, and *ZFP36L2* or their dysregulated expressions may be the underlying cause of the development of autoimmune disorders.

Regnase-1 has also been implicated in some autoimmune and inflammatory disorders. A frameshift mutation in *ZC3H12A* caused an absence of the full-length Regnase-1 in one patient which caused a primary immunodeficiency (Hashim, 2017). The patient had increased levels of (auto)-antibodies, IL-6 and suffered from autoimmune hepatitis, anemia, thrombocytopenia, and recurrent respiratory infections. Similarly, a dysregulated Regnase-1 expression dependent on IL-17A has been identified in the skin of psoriasis patients (Ruiz-Romeu et al., 2016). Furthermore, studies of intestinal inflamed epithelium identified mutations in Regnase-1 to be associated with ulcerative colitis (Kakiuchi et al., 2020; Nanki et al., 2020). In contrast, human studies of Regnase-4 are much more limited. The only available study, with a total number of 6 patients, described a significant change in the methylation of the promoter of *ZC3H12D* in patients with leukoaraiosis which is a neuroimaging abnormality of the cerebral white matter (Huang et al., 2018).

Unraveling the molecular mechanisms of RBP-dependent diseases allows the development of possible therapeutical treatments, even though these are still at a pre-clinical stage. One potential mechanism is disrupting the self-regulation of *ZC3H12A* by Regnase-1 itself. Indeed, when antisense

oligonucleotides were used to target the 3'UTR of *ZC3H12A*, it blocked the negative self-regulation of Regnase-1, eventually causing a higher Regnase-1 expression. This impeded the development of EAE due to a lower expression of pro-inflammatory cytokines and chemokines (Tse et al., 2022). Another field where Regnase-1 modulation can be used is in cancer immunotherapies. The adoptive transfer of Regnase-1-deficient $CD8^+$ T cells resulted in a better anti-tumor response against both solid and blood cancers by reprogramming the tumor-infiltrating KO $CD8^+$ cells to long-lived effector cells (Wei et al., 2019). This anti-tumor effect *in vivo* was further confirmed and enhanced by adoptively transferring human chimeric antigen receptor $CD8^+$ T (CAR-T) cells lacking both Regnase-1 and Roquin-1 (Mai et al., 2023).

To summarize this chapter, post-transcriptional regulation is exceedingly important in fine-tuning the immune response of T_H lymphocytes. This is mainly mediated by RBPs that regulate the stability of mRNAs encoding cytokines and other immune-related genes. RBPs usually have paralogues such as the Roquin, ZFP36 and Regnase families, while other RBPs do not have a discernible classical RBD. As RBPs are prominent modulators of the immune response, attempts have been made to harness their function for medical treatment with promising pre-clinical results.

3 Hypothesis and aim of the thesis

T_H lymphocytes are at the center of the immune network by secreting cytokines that affect all other immune cells and neighboring tissues. The gene expression of T_H lymphocytes must be carefully and rapidly regulated, since they have to mount fast and specific responses upon encountering an antigen, while at the same time limiting excessive inflammation and tissue damage. Post-transcriptional regulation plays a key role in the function of T_H lymphocytes by modulating the stability and translation of mRNAs encoding for cytokines and other immune-relevant genes. RBPs, in cooperation with miRNAs, are essential for the adjusted and fine-tuned reaction of T_H lymphocytes. The fact that many RBPs have paralogues renders their functional investigation more challenging. For example, the Regnase family has two members, Regnase-1 and Regnase-4, expressed in T_H lymphocytes that seemingly have similar, but not identical functions and characteristics, with unknown levels of cross-interaction, cooperation and redundancy. Furthermore, many RBPs remain with completely unknown functions in T_H lymphocytes, such as PDAP1.

The aim of my thesis is the comparison of RBPs within the same family, as well as the characterization of a novel RBP, in the context of human T_H lymphocyte function.

In the first part of my studies, I compared the role of Regnase-1 and Regnase-4 in the repression of target mRNAs. The results of this study are included in **Chapter 4. Functional analysis of Regnase-1 and Regnase-4 in human T helper lymphocytes.**

In the second part, I contributed to the functional characterization of the RBP PDAP1. The results of this study are described in **Manuscript [1]**, of which I am the joint first author.

Additionally, I am the joint first author of a review article (**Manuscript [2]**) where I contributed to describing the role of RBPs in restraining the pro-inflammatory phenotype of T_H lymphocytes.

4 Functional analysis of Regnase-1 and Regnase-4 in human T helper lymphocytes

4.1 Rationale of the study

Two members of the Regnase RBP family, namely Regnase-1 and Regnase-4, have been described to be negative regulators of inflammatory genes in T_H lymphocytes. They share a high degree of homology and have overlapping mRNA targets; however, some important differences have been identified. For example, a T-cell specific deletion of Regnase-1 is lethal within 12 weeks after birth, while Regnase-4 can live for up to 2 years but exhibit a worse paralysis upon EAE induction (Matsushita et al., 2009; (Minagawa et al., 2014). Also, the transcription factor BHLHE40 negatively regulates *ZC3H12D*, but not *ZC3H12A* expression (Emming et al., 2020). For these reasons, I aimed to functionally compare Regnase-1 and Regnase-4 in order to identify unique, redundant or compensatory roles in human T_H lymphocytes.

4.2 Material and methods

T cell separation. Buffy coats from healthy donors were obtained from the Swiss Blood Donation Centers of Basel and Lugano (Switzerland) with informed consent from the Swiss Red Cross and authorization number CE 3428 from the Comitato Etico Canton Ticino. Peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation with Ficoll-Paque Plus (GE Healthcare), followed by positive selection for $CD4^+$ cells using magnetic microbeads (Miltenyi Biotec). T cell populations were further sorted (FACSaria and FACSymphony A6, BD Biosciences) based on the expression of surface markers as follows:

T_N cells: $CD4^+$ $CD25^-$ $CD45RA^+$ $CCR7^+$,

Tmem cells: $CD4^+$ $CD25^-$ $CD45RA^-$ $CCR7^{+/-}$,

T_H1 cells: $CD4^+$ $CD25^-$ $CD45RA^-$ $CXCR3^+$ $CCR4^-$ $CCR6^-$,

T_H2 cells: $CD4^+$ $CD25^-$ $CD45RA^-$ $CXCR3^-$ $CCR4^+$ $CCR6^-$,

T_H17 cells: $CD4^+$ $CD25^-$ $CD45RA^-$ $CXCR3^-$ $CCR4^+$ $CCR6^+$ $CCR10^-$,

T_H22 cells: $CD4^+$ $CD25^-$ $CD45RA^-$ $CXCR3^-$ $CCR4^+$ $CCR6^+$ $CCR10^+$ and

Treg: $CD4^+$ $CD25^{hi}$ $CD127^{lo}$.

Sorting strategies are shown in **Figure 4.1** and antibodies used in this study are listed in **Table 4.1**.

Culturing of T cells. Sorted T cell populations were activated for two days with plate-bound anti-CD3 (clone TR66 (Lanzavecchia & Scheidegger, 1987), recombinant, made in-house, a kind gift from

Federica Sallusto and Antonio Lanzavecchia) and anti-CD28 (BD Pharmingen) in Nunc MaxiSorp (Thermo Fisher Scientific) 96-well plates in complete medium consisting of RPMI-1640 medium (Gibco) supplemented with 5% human serum (Swiss Blood Center), 1% non-essential amino acids, 1% sodium pyruvate, 1% glutamate, 1% penicillin/streptomycin (all from Gibco) and 50 µM β-mercaptoethanol (considered as complete medium). After two days of activation, cells were moved into round-bottom 96-well plates. For cultures longer than 5 days, 50-200 U/ml human IL-2 (recombinant, made in-house) was added to the medium and the cells were split every 3-4 days 1:2.

Culturing of T cell clones. T memory cells were single cell cloned and activated as previously described (Messi et al., 2003). In brief, 0.8 cells per well were seeded in 384-well plates in complete medium containing 500 U/ml IL-2, 1 µg/ml phytohemagglutinin (PHA) (Thermo Fisher Scientific) and 2.6×10^4 irradiated (45 Gy) PBMCs. After 14 days, individual clones were transferred into round-bottom 96-well plates and further expanded with complete medium containing 500 U/ml IL-2 up to day 21 when functional analysis was performed. Similarly, sorted T_H17 cells were single cell cloned and screened for the production of IL-17 on day 20 post-activation. On day 28, a single IL-17^+ clone was transfected with CRISPR-Cas9 ribonucleoparticle (RNP) and single cell cloned again on day 30. Single clones were moved to round-bottom 96-well plates and used for functional analysis on day 56. Clones used for Western blotting were expanded by stimulating 100'000 cells of each clone on day 28 post-activation in 1 ml complete medium containing 500 U/ml IL-2, 1 µg/ml, 1 million allogeneic irradiated PBMCs until day 44 when they were harvested for protein extraction.

CRISPR-Cas9 gene editing. CRISPR-Cas9 gene editing was performed as previously described (Emming 2020; Leoni, Bianchi, 2021). In brief, CRISPR RNAs (crRNAs) and trans-activating CRISPR RNAs (tracrRNAs) (Dharmacon, IDT) were mixed at a final concentration of 80 µM in 10 µl of nuclease-free duplex buffer (Dharmacon, IDT), followed by boiling for 5 minutes at 95°C and cooled down at room temperature for 10 minutes to generate single guide RNAs (sgRNAs). The crRNAs are listed in **Table 4.2**. RNPs were generated by combining 1.5 µl (120 pmol) of sgRNA with 1.5 µl (46.19 pmol) of TrueCut Cas9 Protein v2 (Thermo Fisher Scientific) in a volume of 3 µl. 1.2 µl of 100 mg/ml poly-L-glutamic acid sodium salt (Sigma) was added to the RNP to a final volume of 4.2 µl. The RNPs were transfected into 0.8-1 $\times 10^6$ cells either by using the 10 µl Neon Transfection System Kit (Thermo Fisher Scientific) (20 ms, 1 pulse, 1800V for resting cells, 2200V for activated cells) or 20 µl P3 Primary Cell 4D-Nucleofector Kit (Lonza) (code EH-115). After transfection, cells were left to recover in complete medium without antibiotics with 200 U/ml IL-2 for 36-48h prior to activation.

Analysis of CRISPR-Cas9 deletion efficiency of T cell clones. In the case of the gene *ZC3H12A*, sgRNAs were transfected in the combinations of sgRNAs_1+3 or sgRNAs_2+3 (A KO) interchangeably where both strategies produced a similar deletion of ~5.7 kilobase pairs (kb) (**Figure 4.3B**). In the case of the gene *ZC3H12D*, two KO strategies were also applied. The first strategy consisted of transfecting sgRNAs_1+2 (D KO) that were designed to produce a deletion of 150 base pairs (bp) (**Figure 4.3C**). The second strategy consisted of transfecting sgRNAs_1+3 (D KO_1+3) which produced a deletion of ~17.7 kb (**Figure 4.3F**). The presence of deletions was assessed by PCR of the genomic DNA (gDNA). The gDNA was isolated using the QIAamp DNA Micro Kit (Qiagen) or extracted with the QuickExtract DNA extraction solution (Lucigen) following the manufacturer's protocol. The extracted gDNA was used as a template for a PCR with KOD Hot Start DNA Polymerase (Sigma) and analyzed by gel electrophoresis. To screen for the presence of genomic deletions, PCRs were designed to span the regions of double-stranded breaks to distinguish between unmodified (wild type, WT) and KO clones. The screening primers are listed in **Table 4.3**. For selected clones, the existence of a KO was confirmed by Western blot.

Quantitative reverse transcription-PCR (qRT-PCR). Total RNA was extracted using the TRI Reagent and the Direct-zol RNA Kit (Zymo Research) according to manufacturer's protocol. cDNA was retrotranscribed with the qScript cDNA SuperMix (QuantaBio). PCR reactions were performed with PerfeCTa SYBR green FastMix (QuantaBio), 2-10 ng cDNA and 0.2 mM primers and run on QuantStudio 3 (Thermo Fisher Scientific). All primers used for qRT-PCR are listed in **Table 4.4**.

Nanostring transcriptome profiling. Total RNA was extracted same as for qRT-PCR. 40-100 ng of total RNA of pools of KO clones was hybridized and probed with the nCounter Human Immunology v2 Panel (Nanostring) and ran on an nCounter PRO or nCounter SPRINT instrument (Nanostring). Data were normalized to housekeeping genes expressed above the expression threshold. The data was analyzed with the nSolver 3.0 software.

Intracellular and surface staining. For IL-17A detection, cells were stimulated for 5 h with 200 nM of phorbol myristate acetate (PMA) and 1 µg/ml ionomycin. For the last 2.5 h of stimulation, 10 µg/ml of brefeldin A was added to the culture medium. Prior to fixation, cells were stained with the LIVE/DEAD Fixable Stain Kit (Thermo Fisher Scientific). Cells were fixed with 4% paraformaldehyde, followed by the permeabilization with 0.5% BSA and 0.1% saponin in PBS and staining with anti-IL-17A-e660 (APC) antibody (BioLegend). For intracellular staining of hemagglutinin (HA), Regnase-1 and Regnase-4, cells were fixed and stained by using the FOXP3/Transcription Factor Staining Buffer

Set (Thermo Fisher Scientific) following the manufacturer's protocol. For the surface staining of ICOS, cells were incubated on ice for 15 minutes with ICOS-FITC (Invitrogen). Samples were acquired on a Fortessa or FACSymphony A5 flow-cytometer (BD Biosciences) and analyzed with the FlowJo software (BD Biosciences).

Plasmids. Plasmids were generated and modified using standard molecular cloning techniques. For luciferase reporter assay, the 3'UTRs of *NFKBIZ* and *IL17A* was amplified by PCR and cloned into the pmirGLO plasmid (Promega). Human *ZC3H12A* was subcloned into pLVX-EF1α-IRES-ZsGreen (Clontech). pReceiver-Lv165-ZC3H12D was obtained from Genecopoeia. For *in vitro* mRNA transcription, a ZsGreen reporter and the genes of interest were cloned into pUC57mini. Site-directed mutagenesis was performed using the Quick Change II kit (Agilent) or Q5 Site-Directed Mutagenesis Kit (New England BioLabs) according to manufacturers' instructions. All plasmids were verified by Sanger sequencing. Cloning primers and plasmids are listed in **Table 4.5** and **Table 4.6**.

***In vitro* mRNA transcription and transfection.** DNA templates for *in vitro* transcription (IVT) were generated by linearization of the pUC57mini plasmids (**Table 4.6**). The plasmids were designed to have the 5'UTR of *HBB*, followed by the ZsGreen coding sequence, P2A, and FLAG-HA-tagged Regnase contructs followed by two repeats of *HBB* 3'UTR. The plasmids were linearized by digesting with SpeI immediately downstream of the last 3'UTR and purified with the E.Z.N.A. Gel Extraction Kit (Omega Bio-tek). mRNA was generated using the HiScribe T7 ARCA mRNA Kit with tailing (New England BioLabs) according to manufacturer's protocol. In brief, 0.5-1 µg of DNA template and 1.25 mM pseudo-UTP (Jena BioSciences) was used for transcription for 1 h followed by 30 minutes of DNase treatment. Poly(A) tailing was performed for 60 minutes. mRNA was purified by adding 400 µl of TRI Reagent to the reaction mix followed by using the Direct-zol RNA Kit. 0.5 pmol of *in vitro*-transcribed mRNA was transfected into $CD4^+$ Tmem cells on day 3 of activation using the 10 µl Neon Transfection System Kit and the setting of 1800 V 20 ms 1 pulse. Upon transfection, the cells were moved into antibiotic-free complete medium with 200 U/ml IL-2. Cells were used for RNA extraction and intracellular staining 4h post-transfection.

Luciferase reporter assay. Human embryonic kidney 293T (HEK293T) cells were transfected in 6-well plates with a mix containing 3.5 µg of the pmirGLO plasmid, 1 µg of Regnase-expressing vector and 27 µg polyethylenimine (PEI) in 0.5 ml OptiMEM (Gibco) per well. After 36 h, cells were lysed and analyzed with a GloMax luminometer (Promega) using the Dual-luciferase reporter assay kit (Promega). Plasmids used for transfection are listed in **Table 4.6**.

Western blotting. Cells were lysed in RIPA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) supplemented with a cocktail of protease inhibitors (Sigma). Protein extracts were cleared and quantified with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). 40-100 µg of protein extract per sample were separated on a 8-12% SDS-PAGE. After electrophoresis, proteins were blotted to an Immobilon-P PVDF membrane (Millipore) using a wet transfer system. Blocking was done for 1 h at room temperature with 5% milk in TBST (5 mM Tris pH 7.3, 150 mM NaCl, 0.1% Tween-20). Blots were incubated with 1 µg/ml primary antibodies anti-ZC3H12D (Sigma), anti-MCPIP1/ZC3H12A (BioTechne), or anti-GAPDH (Sigma) in 5% bovine serum albumin, 0.02% sodium azide in TBST. After washing with TBST, blots were incubated with 0.1-0.4 µg/ml secondary HRP-conjugated antibodies for 1 h at room temperature in 2.5-5% milk in TBST. Blots were developed with the Clarity Western ECL Substrate (Bio-Rad Laboratories) and detected with the Fusion FX Edge (Vilber). The quantification of signals was done with ImageJ.

Statistical analysis. nSolver 4.0 was used for statistical analysis of Nanostring profiling. All other statistical analyses were performed using GraphPad Prism 8. GraphPad Prism 8 software was used for generating graphs. The comparison between two means was evaluated by an unpaired *t*-test if the two populations compared were normally distributed or by the Wilcoxon-Mann-Whitney test in case the populations were not normally distributed. Distributions were tested using the Kolmogorov Smirnov test. Welch's correction *t*-test was applied in the analysis of Nanostring transcriptome analysis. Comparisons among three or more sample means were made by ANOVA.

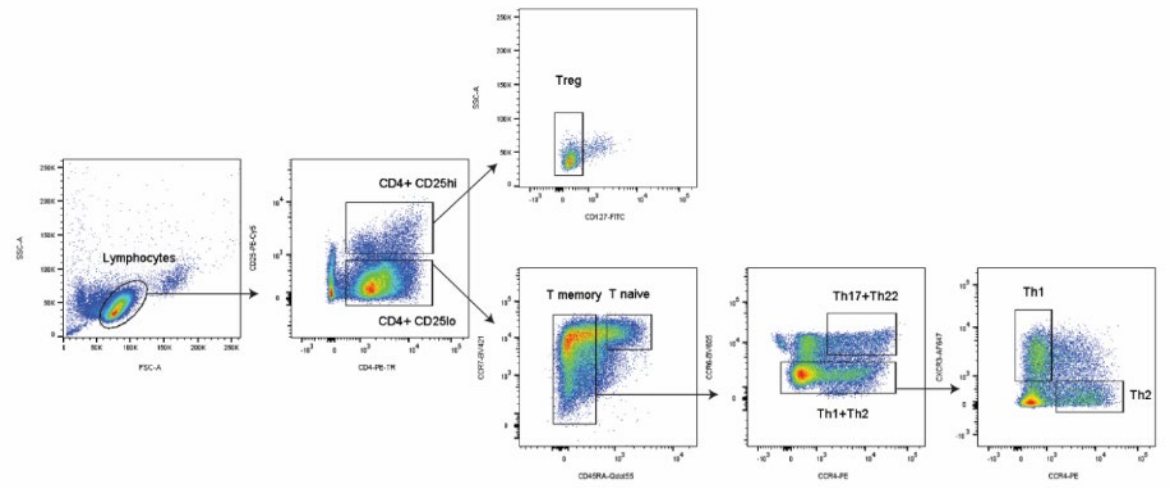

Figure 4.1. Sorting strategy of different CD4⁺ cell populations. The T_H17 + T_H22 population was further stained for CCR10 and sorted as $CCR10^-$ T_H17 and $CCR10^+$ T_H22.

Table 4.1. List of antibodies		
Name	**Company**	**Product number**
anti-CCR4-PE	Biolegend	359411
anti-CCR10-PerCP-Cy5.5	BD Pharmingen	564772
anti-CCR4-PE-Cy7	BD Pharmingen	557864
anti-CCR6-BV605	Biolegend	353419
anti-CCR6-QD605	Biolegend	353420
anti-CCR7-BV421	Biolegend	353208
anti-CD127-FITC	BD Pharmingen	561697
anti-CD25-PE	Biolegend	356103
anti-CD25-PE-Cy5	Beckman Coulter	IM2646
anti-CD4-FITC	Beckman Coulter	A07750
anti-CD4-PE-TR	Invitrogen	MHCD0417
anti-CD45RA-QD655	Invitrogen	Q10069
anti-CXCR3-AF647	Biolegend	353711
anti-CXCR3-APC	BD Pharmingen	550967
Anti-IL-17A-eFluor660	Life Technologies	50-7179-42
Anti-ICOS-FITC	Invitrogen	11-9948-42
anti-HA.11	Novus Biologicals	901501
Anti-MCPIP1/ZC3H12A	Novus Biologicals	MAB7875
Anti-ZC3H12D	Sigma	HPA036897
Anti-GAPDH	Sigma	G9545
Anti-rabbit-AF647	Sigma	SAB4600352
Anti-mouse-AF594	Life Technologies	A11005
anti-mouse-HRP	Southern Biotech	1031-05
anti-rabbit-HRP	Sigma	A0545

Table 4.2 List of sgRNAs		
Name	**Sequence**	**Comment**
Scramble control	GGTTCTTGACTACCGTAATT	(Mocciaro et al., 2018)
ZC3H12A_sgRNA_1_AD	AACACGGGACAGCCACCGAG	Exon 2
ZC3H12A_sgRNA_2_AO	GAGACCAGTGGTCATCGATG	Exon 2
ZC3H12A_sgRNA_3_AA	TTCACACCATCACGACGCGT	Exon 4
ZC3H12D_sgRNA_1_AB	CCTGGTCAACGACGTGCTGC	Exon 2
ZC3H12D_sgRNA_2_AO	CACTATGGGTCGCAGAGAAC	Exon 2
ZC3H12D_sgRNA_3_AA	TCGTCTCCAACGACAACTAC	Exon 4

Table 4.3. List of screening PCR primers and screening strategies			
Gene	**FW**	**RV**	**Amplicon**
ZC3H12A	CTCATCCTGCTGGATGT GGTTTTGG	CATACATCCCCTCATAAG TGCTACGG	WT = 6719 bp sgRNAs 1+3 KO = ~810 bp sgRNAs 2+3 KO = ~855 bp
ZC3H12D	AGTCTGAGAAACAAGA AACCTGTGT	GTTAGGGACAGACTCCC AACAAG	WT = 1098 bp sgRNAs 1+2 KO = ~981 bp
ZC3H12D	AGTCTGAGAAACAAGA AACCTGTGT	AGTGGTGCCCTAAACAC ATGCTT	WT = 18542 bp sgRNAs 1+3 KO = ~840 bp

Table 4.4. List of qRT-PCR primers		
Gene	**Forward sequence**	**Reverse sequence**
ICOS	GAAGTCAGGCCTCTGGTATTTC	TATTTGCCCCCCTTTCAGCAAC
IL17A	CCACCTCACCTTGGAATCTC	TGGTAGTCCACGTTCCCATC
LTA	AAACCTGCTGCTCACCTCATT	ACCTGGGAGTAGACGAAGTAGATG
NFKBIZ	CAAAGGATGCAGATGGTGACAC	AAGGCACTCTGTCCATTGTGCT
ZC3H12A	CCACTCCCAGAAGAGGAAAA	CAGGAGAAGACCTCCTTGTT
ZC3H12B	CAGAGAGATTGCAAGCCCTGAA	CCACAGCAAGTTGTATTCCTCTGC
ZC3H12C	GATGGCAGCAATGTGGCAAT	TGTCTTTGTGGCCTCTTTCC
ZC3H12D	CCTGGAAGAGGACTTCAGAACC	GTCAACAGCCAGCTTGATTCCC
Housekeeping genes		
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC
UBE2D2	GATCACAGTGGTCTCCAGCA	CGAGCAATCTCAGGCACTAA

Table 4.5. List of cloning and mutation primers	
Name	**Sequence**
ZC3H12A_ FW	AAAACTAGTCTAGAGAACCCACTGCTTACT
ZC3H12A _RV	TTAAACTTAAGCTAGATCTGCGGCCG
ZC3H12A_D141N_FW	CGTTGCTCCCATTAATGACCACTGGTCTCAGGTCGC
ZC3H12A_D141N_RV	GCGACCTGAGACCAGTGGTCATTAATGGGAGCAACG
ZC3H12D_D95N_FW	GCGACCCATAGTGATTAATGGCAGCAACGTGGCGATG
ZC3H12A_D95N_RV	CATCGCCACGTTGCTGCCATTAATCACTATGGGTCGC
ZC3H12D_C111_FW	AGCCAGCTTGATTCCTCGAGCAGAGAAGGTTTCTTTATTTCCATGGCTCA
ZC3H12D_C111_RV	TGAGCCATGGAAATAAAGAAACCTTCTCTGCTCGAGGAATCAAGCTGGCT
FLAGHA_FW	AATAAGAATTCGCCACCATGGACTACAAGGAC
FLAGHA_RV	AATAAGAATTCTCCGGCGTAGTCGGGCAC
NFKBIZ-3'UTR _FW	AACGGAATTCCTCCATTAGCTTGGAGCCT
NFKBIZ-3'UTR _RV	TTGCCTCGAGCTAGGGAAATAAGGCACTAGG
IL-17A 3'UTR_FW	ATCGAATTCCTCTGGGGAGCCCACACTC
IL-17A 3'UTR_RV	ACTTCTAGAGGGCGAAAATGGTTACGATGT

Table 4.6. List of plasmids	
Name	**Comment**
EF1α-Empty-IRES-ZsGreen	Clontech (product # 631982)
EF1α-FLAG-HA-ZC3H12A-WT-IRES-ZsGreen	This thesis
EF1α-FLAG-HA-ZC3H12A-D141N-IRES-ZsGreen	This thesis
EF1α-FLAG-HA-ZC3H12D-WT-IRES-eGFP	Genecopoeia (product # EX-Y1201-Lv165)
EF1α-FLAG-HA-ZC3H12D-D95N-IRES-eGFP	This thesis
EF1α-FLAG-HA-ZC3H12D-C111A-IRES-eGFP	This thesis
pmiRGLO-MCS	Multi-cloning site generated in-house starting from Addgene 78131 plasmid
pmiRGLO-NFKBIZ-3'UTR	This thesis
pmiRGLO-IL17A-3'UTR	This thesis
pUC57mini-HBB-5UTR-ZsGreen-MCS-2xHBB3UTR (pUC57mini-Empty)	In-house designed
pUC57mini-ZsGreen-P2A-FLAG-HA-ZC3H12A-WT	This thesis
pUC57mini-ZsGreen-P2A-FLAG-HA-ZC3H12A-D141N	This thesis
pUC57mini-ZsGreen-P2A-FLAG-HA-ZC3H12D-WT	This thesis
pUC57mini-ZsGreen-P2A-FLAG-HA-ZC3H12D-D95N	This thesis

4.3 Results

4.3.1 Regnase-1 and Regnase-4 expression kinetics in human T_H lymphocytes

To determine the expression kinetics of the genes encoding the different Regnase family members, I activated T_N and Tmem primary human $CD4^+$ T lymphocytes with plate-bound anti-CD3 and anti-CD28 antibodies for up to 5 days and measured the expression of the *ZC3H12A-D* mRNAs by qRT-PCR. At the resting state, Tmem and T_N cells expressed relatively high levels of *ZC3H12A* and *ZC3H12D*, while *ZC3H12B* and *ZC3H12C* were expressed at low levels (**Figure 4.2A**), which is consistent with available data (Emming et al., 2020; Schmiedel et al., 2018). The TCR activation of both Tmem and T_N cell populations further downregulated the *ZC3H12B* transcript, while *ZC3H12C* was significantly increased by day 5, although remaining at comparatively low levels. Interestingly, the expression of *ZC3H12A* dropped significantly within 1 day after activation, while the expression of *ZC3H12D* remained overall relatively stable.

Next, I proceeded to determine the expression of Regnase-1 and Regnase-4 at the protein level, and both mirrored the mRNA expression levels. Regnase-1 was highly expressed by $CD4^+$ Tmem lymphocytes at the resting state but was rapidly downregulated upon T cell activation and remained low up to 8 days, most likely due to a combination of reduced mRNA expression and proteolytic cleavage (**Figure 4.2B**). In fact, the predicted cleaved product was detected rapidly upon activation, which has been described earlier to be caused by the paracaspase MALT1 at the site of the arginine residue R111 (Uehata et al., 2013). The protein expression of Regnase-4 also followed the pattern of the expression of its transcript and remained relatively stable over time with an increased trend at days 3/4 post activation. The perceived low levels at later stages of activation (days 7/8) can be attributed to the increased expression of the of the housekeeping control GAPDH. Regnase-4 also contains the conserved putative cleavage site at R61, however it is not possible to detect the predicted cleaved product since the antibody used for Regnase-4 does not bind to the C-terminal cleavage product. To see if Regnase-4 can be downregulated with a stronger stimulation, I treated Tmem lymphocytes on day 6 post-TCR-activation with PMA/ionomycin for 0.5h and 3h (**Figure 4.2C**). Surprisingly, Regnase-4 was rapidly downregulated after 0.5h of treatment. This suggests that Regnase-4 might also be proteolytically regulated.

To investigate if there is a difference of expression across specialized T cell subsets, I measured the expression of *ZC3H12A* and *ZC3H12D* in *ex vivo* sorted cells. The analysis showed that *ZC3H12A* was relatively constant across the different subsets, while *ZC3H12D* showed preferential expression in T_H17 and Treg cells compared to the T_H1 and T_H2 subsets (**Figure 4.2D**).

4.3.2 CRISPR-Cas9 KO of *ZC3H12A* and *ZC3H12D* in memory T lymphocytes

To functionally compare Regnase-1 and Regnase-4, I deleted *ZC3H12A* (A KO) and *ZC3H12D* (D KO) in human $CD4^+$ Tmem lymphocytes by CRISPR-Cas9, as previously described (Emming et al., 2020; Leoni et al., 2021) (**Figure 4.3A**). In brief, freshly isolated cells were transfected with RNPs targeting the genes of interest. To delete each gene, two RNPs were used to generate two double-stranded breaks. After activation, cells were single cell cloned by limiting dilution and cultured for 21 days. Individual clones were screened for genomic deletions by PCR (**Figure 4.3B-C**). qRT-PCR analyses confirmed ablated expression of the targeted genes in selected clones (**Figure 4.3D**). The ablation at the protein level was confirmed at the level of populations (**Figure 4.3E**). Interestingly, the ablation of Regnase-4 caused a significant increase in Regnase-1 expression at the population level, while the expression of Regnase-1 in Regnase-4 KO populations was variable.

The genetic deletion in A KO is 5.7 kb long, while for the D KO it is much shorter with 150 bp, leaving the possibility of generating a truncated functional protein, as detected by Western blotting (**Figure 4.3E)**. To confirm the results of the D KO with a longer genetic deletion, I used a second KO strategy that generates a deletion of 18.5 kb (D KO_1+3) (**Figure 4.3F**). I confirmed the downregulation of Regnase-1 and Regnase-4 in individual clones by Western blotting (**Figure 4.3G**). Interestingly, also here I could observe a limited level of cross-regulation between Regnase-1 and Regnase-4. Pilot experiments comparing the two D KO strategies showed no significant differences, suggesting that the shorter genetic deletion is enough to ablate a functional Regnase-4.

Next, 4-10 A KO or D KO and control clones from two different donors were pooled, and the expression of inflammatory genes was measured by Nanostring profiling. Out of 242 expressed genes, I found that upon deletion of *ZC3H12A* and *ZC3H12D* 78 and 91 genes, respectively, were differentially expressed (log_2 ratio higher or lower of 0.5 compared to control group). Most of the differentially expressed genes (DEGs) in the KO experimental groups were upregulated (**Figure 4.3H**), consistent with the established role of Regnase-1 and Regnase-4 as negative regulators of transcript stability. Analysis of the DEGs revealed that while a large proportion (49.5%) of the genes was upregulated upon deletion of both *ZC3H12A* and *ZC3H12D* (for instance, *ICOS, IL17A, IL17F, TNF*), other targets appeared to be targeted preferentially by Regnase-1 (*NFKBIZ, IL1A, IL5, PTGS2*) or Regnase-4 (*IL2, IL22, LTA*) (**Figure 4.3I**). Previous RIP-seq analysis of HeLa cells treated with IL-1β identified 68 direct targets of Regnase-1 (Mino et al., 2015), and 10 of those genes are included in the Nanostring immunology panel. 5 of these genes were upregulated in A KO (*IL1A, IL6, NFKBIZ, PTGS2, CCL20*), suggesting that at least for these targets KO effect is direct, while only one of these genes (*CCL20*) was differentially upregulated in the D KO. The downregulated DEGs were very few (19 total) and are likely to represent indirect effects (**Figure 4.3J**). Among the most differentially expressed genes are known targets such as *NFKBIZ, ICOS, PTGS2*, and *IL17A* (**Figure 4.3K**). In both KO groups, several top DEGs were linked to T_H17 cell functions (*IL17A, IL17F, CCL20, RORC*).

4.3.3 Double knockout in T_H17 subset

Because of the established importance of Regnase-1 in the T_H17 pathway (Jeltsch et al., 2014; Minagawa et al., 2014) and the most DEGs in KO clones being associated with the T_H17 phenotype (**Figure 4.2I-K**), I aimed to determine the relative role of Regnase-1 and Regnase-4 specifically in human T_H17 cells. Therefore, I enriched Th17 cells from PBMCs and single cell cloned them by limiting dilution. On day 20, I screened the clones for IL-17A production and transfected with

CRISPR-Cas9 one individual IL-17$^+$ clone to delete *ZC3H12A* and *ZC3H12D*, alone or in combination (A+D KO), followed by a second round of single cell cloning (**Figure 4.4A**). After the second cloning, individual clones were screened by PCR to detect the expected genomic deletion same as **Figure 4.3B-C**. qRT-PCR analysis confirmed ablated expression of the targeted gene(s) in selected clones (**Figure 4.4B**). Analysis of 51 A KO clones, 46 D KO clones, 36 A+D KO clones and 72 control clones by intracellular staining revealed significant derepression of IL-17A production by clones lacking *ZC3H12A* alone or both *ZC3H12A* and *ZC3H12D*, although I could not detect significant derepression of IL-17A protein production in T_H17 clone lacking only *ZC3H12D* (**Figure 4.4C**). To determine the ability of Regnase-1 and -4 to target directly the *IL17A* 3'UTR, I performed luciferase reporter assays. The co-transfection of the *IL17A* 3'UTR reporter with a Regnase-1 expression plasmid led to a strong significant reduction of the luciferase signal, confirming the ability of Regnase-1 to directly modulate IL-17A expression (**Figure 4.4D**). This effect was completely abrogated by mutating a single amino acid (D141N) that abrogates RNase enzymatic activity (Matsushita et al., 2009). Interestingly, expression of Regnase-4 was also able to limit luciferase expression from the *IL17A* 3'UTR reporter, but to a much lesser extent. Same as for Regnase-1, this was fully abrogated when co-transfected with the RNase-inactive mutant D95N (Wawro et al., 2017), but not by the mutation of cysteine 111 (C111A) that potentially affects the deubiquitination activity. Next, I performed a Nanostring transcriptome profiling of the clones from **Figure 4.4B-C** that were stimulated for 3h with PMA/ionomycin (**Figure 4.4E**). Out of 219 expressed genes, I found that upon deletion of *ZC3H12A and ZC3H12D* 47 and 16 genes, respectively, were differentially upregulated (log_2 ratio higher than 0.5 compared to control group) (**Figure 4.4F**). Analysis of the DEGs in the different experimental groups confirmed that *NFKBIZ, IL1A, IL-5* and *PTGS2* were affected exclusively by the *ZC3H12A* deletion either alone or in combination. Targets including *ICOS, IL6* and *LCP2* transcripts were instead affected in all experimental conditions, indicating that they are sensitive to the activity of both Regnases. Interestingly, I could not identify targets that were exclusively affected by the Regnase-4 deletion, except *ARHGDIB* and *CDKN1A* that had a low log_2 ratio of 0.6. Taking also into consideration that *CDKN1A* is upregulated during the DNA damage response, this gene might be an indirect CRISPR-Cas9 KO effect (Dulić et al., 1994; Schiroli et al., 2019). On the other hand, the upregulation of genes in the double KO was striking with 84 differentially expressed genes. There is an apparent combined effect in the double KO on multiple targets including *NFKBIZ* and *ICOS*, while other genes such as *LTA* did not have a combined derepression suggesting an indirect effect of upregulation on this gene (**Figure 4.4G**). I confirmed by qRT-PCR on clones from independent donors that *NFKBIZ* is significantly derepressed only in the single or combined deletion of *ZC3H12A* (**Figure 4.4H**). *ICOS* had a similar result by having the highest expression in the double KO, while *LTA* had a

variable expression with no combined KO effect, further suggesting that this gene is likely not a target of either Regnase. I was able to confirm the upregulation of 8 direct targets that were identified by RIP-seq of HeLa Cells (Mino et al., 2015) in at least one experimental group, namely *IL1A, NFKBIZ and CXCL2* at the intersection of the A KO and A+D KO*, CCL20, CXCL1, IL8* and *PTGS2* only in the A+D KO, and *IL6* at the intersection of all three KO groups (targets in bold in **Figure 4.4F**). Analysis of downregulated genes showed only very few targets in the A KO and A+D KO (6 genes each) that in general have low expression in T_H17 cells, such as *PRF1, IL10* and *GZMB*, and therefore this result might be attributed to indirect effects (**Figure 4.4I**). However, D KO showed 26 downregulated genes. Some of these genes are usually expressed at low levels in T_H17 cells (*IFNG*, *IL13)*, but several other genes are known targets of Regnase-1 (*CTLA4, CXCL1, PTGS2*), suggesting that this result might be a secondary effect of an induced Regnase-1 expression, although it remains to be confirmed. *NFKBIZ* did not show any effect when *ZC3H12D* was deleted individually, but the combined effect in the double KO indicates that also Regnase-4 might be a negative regulator of *NFKBIZ*. To investigate this, I performed a luciferase reporter assay on the *NFKBIZ* 3'UTR (**Figure 4.4J**). The co-transfection of the *NFKBIZ* 3'UTR reporter with a Regnase-1 expression plasmid significantly reduced the luciferase signal, confirming the direct negative regulation by Regnase-1 in an RNase-dependent manner. Surprisingly, the Regnase-4-expressing plasmids with intact RNase domains (WT and C111A constructs) also strongly reduced the luciferase signal. This effect was completely abrogated and even significantly increased by the RNase-inactive D95N mutant. These results suggest that Regnase-4 *in vitro* can also bind to *NFKBIZ* 3'UTR and negatively regulate it in an RNase-dependent manner.

Finally, to confirm that some selected mRNA targets were indeed targeted by one or both proteins in T cells, I established an *in vitro* transcription (IVT) system for mRNA transfection into primary T cells. Tmem lymphocytes were transfected 3 days post-activation with anti-CD3/anti-CD28 antibodies with IVT mRNA encoding the tagged wild-type and RNase-inactive mutants of Regnase-1 (Z3H12A-WT and ZC3H12A-D141N) and Regnase-4 (ZC3H12D-WT and ZC3H12D-D95N). 4h after transfection, the viability in all experimental groups was over 80% and the overexpression of Regnases was over 40% in all Regnase-overexpressing groups, as assessed by intracellular staining followed by flow-cytometry (**Figure 4.5A**). The mRNA expression of *NFKBIZ* was reduced in cells transfected with ZC3H12A-WT, although it did not reach statistical significance (**Figure 4.5B**). *NFKBIZ* was not affected by the transfection with ZC3H12D-WT, which is in line with the CRISPR-Cas9 KO data where an individual deletion of *ZC3H12D* did not impact the *NFKBIZ* expression. Interestingly, ZC3H12D-D95N expression tended to increase the *NFKBIZ* expression, same as in the luciferase reporter assay

(**Figure 4.4J**), although the mechanism behind this effect remains to be understood. In the case of *ICOS*, ZC3H12A-WT significantly reduced the expression, and this effect was completely abrogated when transfected with ZC3H12A-D141N. ZC3H12D-WT and ZC3H12D-D95N mirrored the same results, but due to the donor-to-donor viability it did not reach statistical significance. I measured also the expression of *LTA* due to its variable upregulation in the Regnase KOs based on the Nanostring and qRT-PCR data. *LTA* mRNA expression was unaffected by the overexpression of Regnase constructs, further suggesting that it does not represent a target and that the upregulation in the KO is a false positive.

Overall, I found that Regnase-1 and Regnase-4 possess mostly redundant functions in primary human T lymphocytes and selected transcripts such as *ICOS* are targeted by both enzymes, although some inflammatory transcripts, such as *NFKBIZ* appear to be more sensitive to Regnase-1 activity. Furthermore, I could not identify a target that was regulated uniquely by Regnase-4. Thus, Regnase-4 does not have unique targets, Regnase-1 is a stronger modulator, and their combined deletion leads to a combined derepressive effect on inflammatory transcripts.

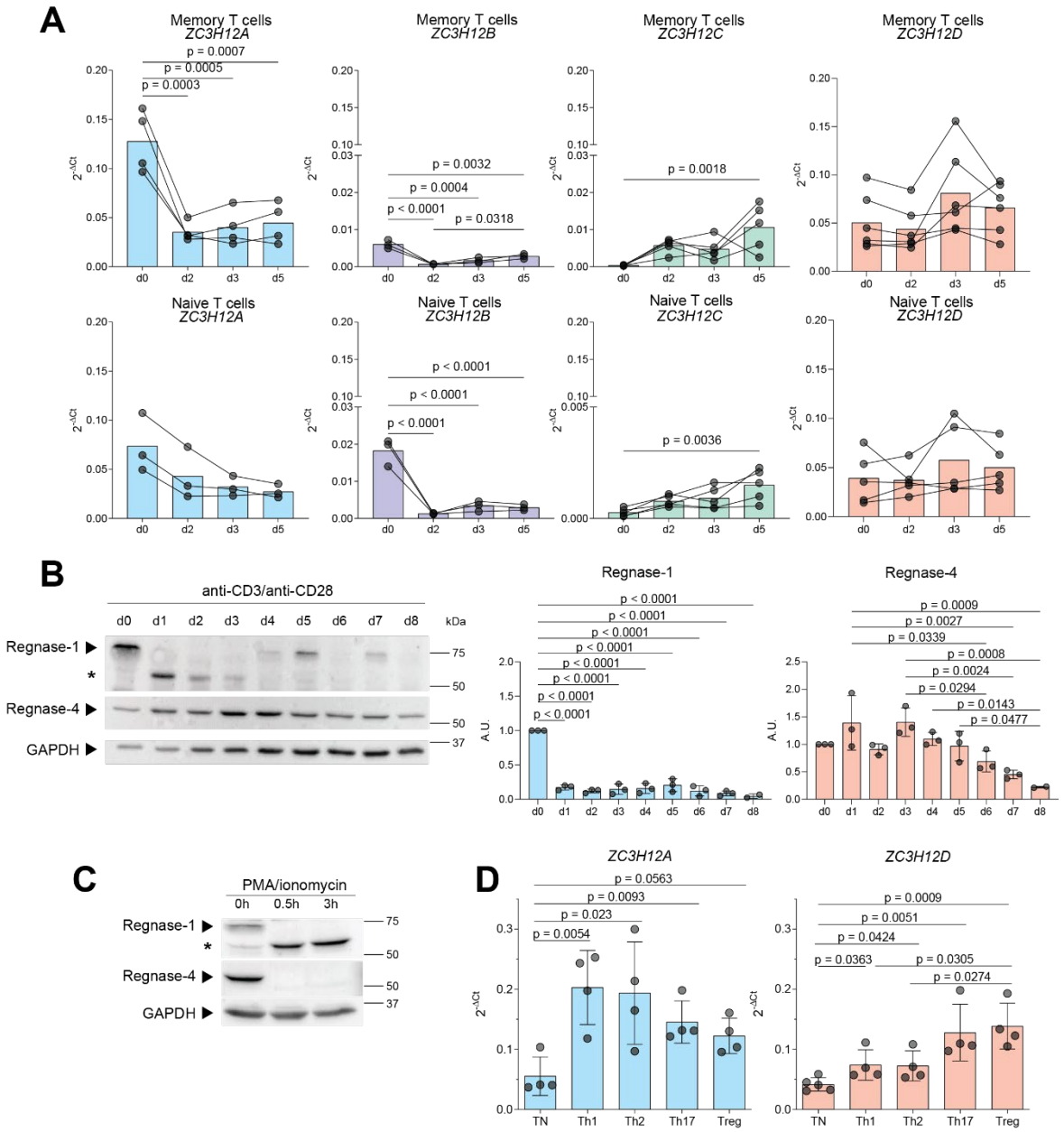

Figure 4.2. Regnase-1 and Regnase-4 expression in human T_H lymphocytes. (A). Expression of *ZC3H12A, ZC3H12B, ZC3H12C* and *ZC3H12D* was measured by qRT-PCR in Tmem and T_N lymphocytes at different days post-activation with anti-CD3/anti-CD28, normalized to *UBE2D2*. N≥3 independent donors, one-way ANOVA. **(B)** Expression of Regnase-1 and Regnase-4 was measured by Western blotting in Tmem lymphocytes at different days post-activation with anti-CD3/anti-CD28. The quantification is shown on the right, N≥2 independent donors, mean±SD, one-way ANOVA, * - cleaved product. **(C)** Expression of Regnase-1 and Regnase-4 was measured by Western blotting in Tmem lymphocytes on day 6 post activation with anti-CD3/anti-CD28 activation and PMA/ionomycin stimulation, N=1. **(D)** Expression of *ZC3H12A* and *ZC3H12D* mRNAs was measured by qRT-PCR in different subsets of $CD4^+$ T lymphocytes isolated *ex vivo* from peripheral blood, normalized to *UBE2D2*. N≥3 independent donors, mean±SD, unpaired *t*-test. A.U. – arbitrary units.

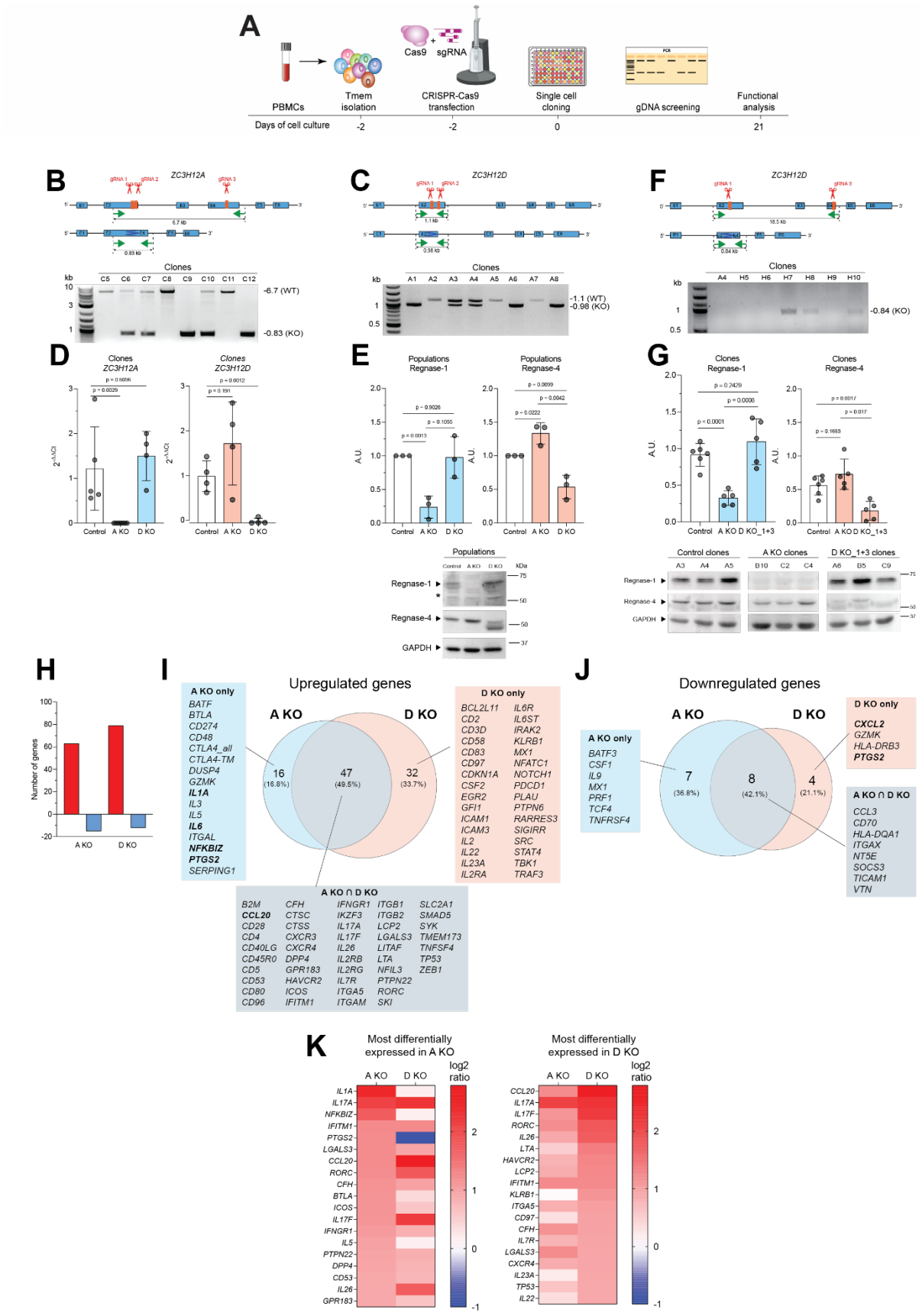

Figure 4.3. CRISPR-Cas9-mediated deletion of *ZC3H12A* or *ZC3H12D* leads to the derepression of target mRNAs in memory T cells. (A). Schematic representation of the experimental design to generate T lymphocyte KO clones by CRISPR-Cas9. **(B)** Top: Schematic representation of the *ZC3H12A* genes, the location of the sgRNAs and the KO screening strategies. A PCR product of 0.8 kb is indicative of a genetic deletion in *ZC3H12A*. Bottom: Example screening of individual KO clones

by PCR. **(C)** Top: Schematic representation of the *ZC3H12D* genes, the location of the sgRNAs and the KO screening strategies. A PCR product of 0.98 kb is indicative of a genetic deletion in *ZC3H12D*. Bottom: Example screening of individual KO clones by PCR. **(D)** mRNA expression measured by qRT-PCR of *ZC3H12A* and *ZC3H12D* in individual clones cultured for 21 days and treated 3h with PMA/ionomycin, normalized to *UBE2D2*. N≥4 individual clones, mean±SD, unpaired *t*-test. **(E)** Protein expression of Regnase-1 and Regnase-4 in Tmem lymphocyte populations transfected with CRISPR-Cas9 and activated for 13 days. The quantification is shown on the right. N=3 independent donors, mean±SD, unpaired *t*-test, * - cleaved product. **(F)** Top: Schematic representation of the *ZC3H12D* gene, the location of the sgRNAs that generate a longer deletion and the KO screening strategy of D KO_1+3. A PCR product of 0.84 kb is indicative of a genetic deletion in *ZC3H12D*. Bottom: Example screening of individual KO clones by PCR. **(G)** Protein expression of Regnase-1 and Regnase-4 in independent single KO clones transfected with CRISPR-Cas9 and cultured for 44 days. N≥5 individual clones mean±SD, unpaired *t*-test. **(H)** Identified KO and control clones from two donors were stimulated 3h with PMA/ionomycin and pooled as follows: control pool 1 = 9 clones, control pool 2 = 10 clones, A KO pool 1 = 5 clones, A KO pool 2 = 9, A KO pool 3 = 4, D KO pool 1 = 7, D KO pool 2 = 7, D KO pool 3 = 10, and D KO pool 4 = 5. Pooled RNA was analyzed with nCounter SPRINT. The number of differentially upregulated (red) and downregulated (blue) expressed genes (log_2 ratio <0.5<) compared to control group is shown. **(I)** Venn diagram of differentially upregulated (log_2 ratio>0.5) genes. **(J)** Venn diagram of differentially downregulated (log_2 ratio<0.5) genes. Venn diagrams were generated with www.deepvenn.com. Genes in bold are identified direct targets obtained from RIP-seq data in HeLa cells (Mino et al., 2015). **(K)** Heatmaps of most differentially expressed genes of KO clones. A.U. – arbitrary units.

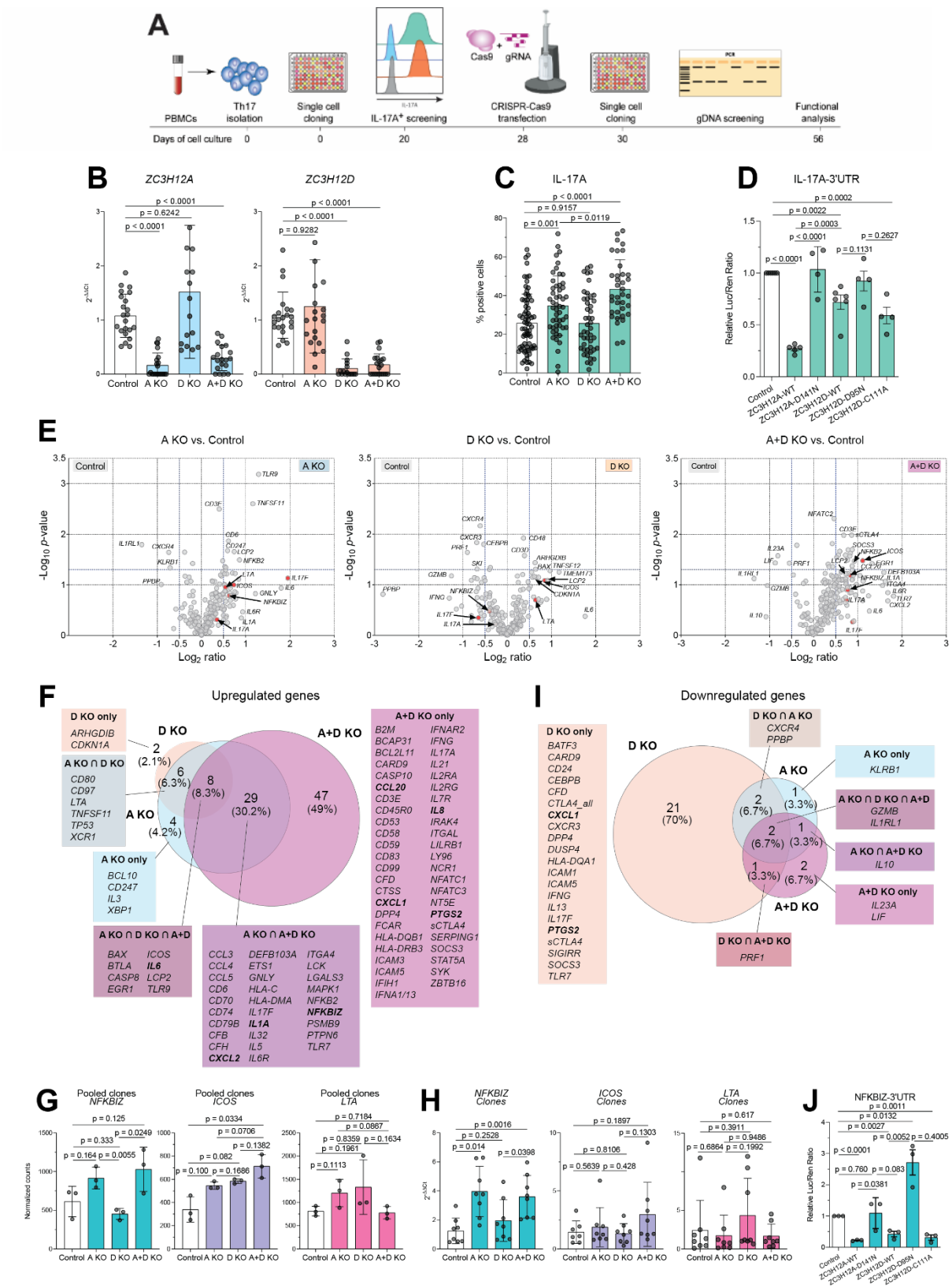

Figure 4.4. Combined deletion of *ZC3H12A* and *ZC3H12D* leads to a stronger derepression than individual KOs. (A). Schematic representation of the experimental design to generate T_H17 KO clones by CRISPR-Cas9. **(B)** mRNA expression measured by qRT-PCR of *ZC3H12A* and *ZC3H12D* in individual clones cultured for 56 days and treated 3h with PMA/ionomycin, normalized to *UBE2D2*. N≥16 individual clones, mean±SD, Mann-Whitney test. **(C)** Intracellular staining

for IL-17A in individual clones activated for 5h with PMA/ionomycin, N≥36 individual clones, mean±SD, Mann-Whitney test. **(D)** Luciferase reporter assay in HEK293T cells using an *IL17A* 3'UTR reporter, co-transfected with vectors expressing either wild-type or mutant versions of Regnase-1 and Regnase-4. mean±SEM, unpaired *t*-test **(E)** Clones from (B) and (C) were stimulated for 3h with PMA/ionomycin, and the RNA was extracted and pooled as follows: 3 A KO pools with each 7 clones, 3 D KO pools with each 5 clones, 3 A+D KO pools with each 5 clones, 3 pools of control clones with each 6 clones followed by analysis with Nanostring nCounter PRO. Red dots = selected genes of interest (*NFKBIZ, ICOS, IL17A, IL17F, LTA*) **(F)** Venn diagram of differentially upregulated (log_2 ratio>0.5) genes. Venn diagrams were generated with www.deepvenn.com. Genes in bold are identified direct targets obtained from RIP-seq data in HeLa cells (Mino et al., 2015). **(G)** Expression of *NFKBIZ*, *ICOS* and *LTA* from (F), dot = pool of clones, mean±SD, Welch's *t*-test. **(H)** mRNA expression of *NFKBIZ*, *ICOS* and *LTA* was confirmed by qRT-PCR on independent individual clones, N≥7 individual clones, mean±SD, unpaired *t*-test. **(I)** Venn diagram of differentially downregulated (log_2 ratio<0.5) genes **(J)** Luciferase reporter assay using an *NFKBIZ* 3'UTR reporter, co-transfected with vectors expressing either wild-type or mutant versions of Regnase-1 and Regnase-4. mean±SEM, unpaired *t*-test.

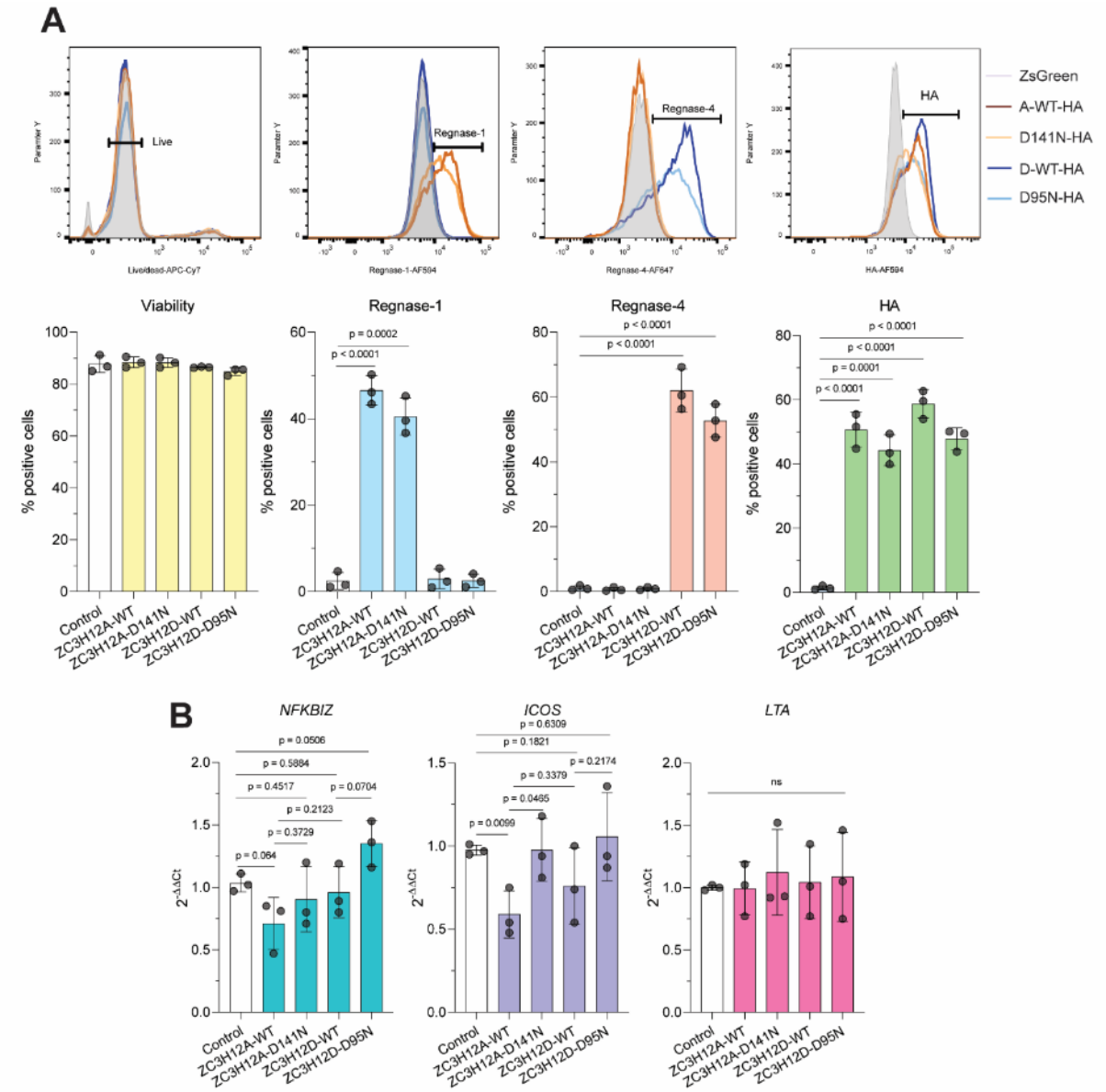

Figure 4.5. IVT mRNA overexpression allows overexpression of Regnases and reduced expression of selected Regnase targets. (A) Tmem lymphocytes were transfected with IVT mRNA encoding tagged wild-type or mutant Regnase-1 and Regnase-4 or GFP control on day 3 post-activation with anti-CD3/anti-CD28 antibodies. 4h post-transfection, cells were stained for Regnase-1, Regnase-4 and hemagglutinin (HA) and analyzed by flow-cytometry to determine the viability and level of overexpression. N=3 individual donors, mean±SD, unpaired *t*-test. **(B)** qRT-PCR mRNA expression of *NFKBIZ, ICOS,* and *LTA* in samples from (A), normalized to *GAPDH*, N=3 individual donors, mean±SD, unpaired *t*-test.

4.4 Discussion

With this study, I identified some common and unique features of Regnase-1 and Regnase-4. First, I identified that the activation of T cells has opposite effects on the expression of the two Regnases. Regnase-1 is completely cleaved within one day of activation, while Regnase-4 expression at the protein and mRNA levels is more stable with a slightly increasing trend. The Regnase-4 antibody does not allow the detection of the predicted proteolytically cleaved Regnase-4 and needs to be investigated with further experiments.

The CRISPR-Cas9-generated Tmem KO clones of *ZC3H12A* and *ZC3H12D* showed more upregulated than downregulated genes in the panel of 594 tested inflammatory genes with the Nanostring technology. This confirmed both Regnase-1 and Regnase-4 as negative inflammatory regulators. In fact, the majority of the upregulated genes were common in the two experimental groups, including the T_H17-specific genes which were among the most differentially expressed. Generally, T_H17 effector cells are a rare population among Tmem cells, and there was the possibility that the results were skewed due to clonal variability. Because of this, I generated T_H17 clones with a combined (A+D KO) or individual deletions of *ZC3H12A* and *ZC3H12D*. The T_H17 KO clones did not reveal the existence of any unique target of Regnase-4. However, it showed that the A+D KO had a combined derepressive effect on many targets including *NFKBIZ*, ICOS, *IL17A, IL6* and *CCL20,* suggesting that Regnase-4 also negatively regulates these targets, to some extent.

IL-17A was derepressed at the protein and mRNA level only in the groups A KO and A+D KO, while the D KO had the same expression levels as the control groups. Similar results were obtained for *NFKBIZ* where the individual deletion of *ZC3H12D* had no effect, but it increased the *NFKBIZ* expression in the combined KO. The luciferase reporter assay confirmed that Regnase-4 overexpression *in vitro* can strongly negatively regulate the *NFKBIZ* transcript, but *in vivo* in Tmem cells it did not have any detectable effect. Interestingly, the overexpression of the RNase inactive ZC3H12D-D95N in Tmem cells increased the expression of *NFKBIZ*, similar to the luciferase reporter assay results. One possible explanation for this observation is that ZC3H12D-D95N binds to the *NFKBIZ* 3'UTR without degrading it, thereby competing with degradation-inducing RBPs, but this needs further investigation.

Available RIP-seq data identified 68 direct mRNA targets of Regnase-1 (Mino et al., 2015) of which the immunology Nanostring panel includes 10 of these genes. 8 of these mRNAs were upregulated in at least one KO experimental group (*IL1A, IL6, IL8, CCL20, CXCL1, CXCL2, PTGS2*), suggesting that the

upregulation of at least these genes is a direct effect. On the other hand, the upregulation of *LTA* in Tmem and T_H17 KO clones is most likely a false positive because it didn't show any effect upon IVT mRNA overexpression.

Regnase-1 and Regnase-4 have been shown to negatively regulate *ZC3H12A* 3'UTR *in vitro* (Iwasaki et al., 2011; Wawro et al., 2017). Also in this study, I identified that the ablation of Regnase-1 significantly increases the Regnase-4 expression, and that Regnase-4 ablation tends to increase Regnase-1, although not significantly. Therefore, there is a possibility that an increased Regnase-1 expression in the D KO fully compensates for the lack of Regnase-4 thereby keeping the *IL17A* expression unchanged, but this needs further investigation. Of note, Regnase-4 cannot compensate for the lack of Regnase-1 as seen on the effects on *NFKBIZ*, *IL1* and *IL5*.

The overexpression of Regnase-1 is known to induce apoptosis (Qi et al., 2011; L. Zhou et al., 2006) which makes it technically challenging to overexpress Regnases in T cells with classical methods such as plasmid transfection or lentiviral transduction. Here, I established a method to short-term overexpress Regnase-1 and Regnase-4 in a precise and highly efficient manner by transfecting Regnase-encoding mRNAs. With this method, I was able to identify a condition in which the viability of transfected T cells allowed the assessment of Regnase overexpression of certain mRNA targets. The overexpression of Regnase-1 showed that it can negatively regulate *ICOS* expression in an RNase-dependent manner, while for Regnase-4 a similar trend was also detected. RNA-immunoprecipitation could confirm that the putative targets are directly regulated by Regnases in T_H cells, however, the experimental procedures for this come with some challenges. For one, immunoprecipitation of endogenous proteins is limited by the availability and specificity of antibodies. Furthermore, the RNase activity of the proteins might degrade and omit *bona fide* targets from the analysis. This could be solved by transfecting cells with catalytically dead constructs, such as in the available RIP-seq data on HeLa cells which was performed with a tagged RNase-inactive (D141N) mutant (Mino et al., 2015). However, primary T lymphocytes are notoriously difficult to transfect. Another option is the transfection of T cell lines (e.g. Jurkat cells, HUT78 cells), but they do not express the targets of interest including *NFKBIZ*, *IL-17A*, and *ICOS*. Therefore, such experiments require extensive optimization to give robust results.

Previous work in murine and human cell lines showed that Regnase-1 and Regnase-4 colocalize, directly interact with each other and additively negatively regulate the *Il6* mRNA (S. Huang et al., 2015). In accordance with this, *IL6* mRNA was upregulated in the individual and combined KO of

Regnase-1 and Regnase-4 (**Figure 4.4E-F**). However, it is unknown if this effect in human T cells is the result of the same intermolecular interactions. Since Regnases exhibit a high level of conservation across species and within the protein family, it is more likely than not that Regnase-1 and Regnase-4 form dimers and oligomers, as it has been described for Regnase-1 (Yokogawa et al., 2016). Further investigation is required to confirm this interaction in human T lymphocytes. On the same line, it is important to consider the possibility of molecular structures composed of Regnase-1, Regnase-4, and other proteins in a specific cellular context that could potentially shape the affinity towards certain mRNA targets in distinct cell types.

In summary, this study identified that Regnase-1 and Regnase-4 have different expression dynamics upon activation and that Regnase-4 does not have unique targets and cannot compensate for the lack of Regnase-1. Also, both Regnase-1 and Regnase-4 can negatively regulate a common set of targets, but some of them seem to be more sensitive to Regnase-1 activity, such as *NFKBIZ*. Hence, the observed differences in the role of Regnases can be explained by the combined effect of different expression dynamics, compensation, and potential cross-regulation within the same family.

5 Manuscript [1]

RFX transcription factors control a miR-150/PDAP1 axis that restrains proliferation of primary human T lymphocytes

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☯ Equal contribution

miRNAs are versatile post-transcriptional regulators that affect a large number of genes across all cell types. In this study, we aimed to investigate the expression and relevance of the top expressed miRNAs in T_H lymphocytes.

Nanostring transcriptomics revealed that miR-150 was the most expressed of more than 800 tested miRNAs. To identify direct targets of this miRNA, my colleagues performed a biotinylated miR-150 pull-down and sequencing, which retrieved several bound targets. The analysis identified PDAP1 to be a novel direct target of miR-150. RIP-seq analysis revealed that PDAP1 is an RBP that binds to mRNAs important for T cell activation and proliferation. I generated CRISPR-Cas9 KO clones and confirmed that PDAP1 positively regulates the expression of *CBL*, *BCL9L* and other transcripts encoding genes relevant in the regulation of proliferation. Furthermore, I deleted the predicted miR-150 binding sites in the 3'UTR of PDAP1 and confirmed that mir-150 directly negatively regulates PDAP1 expression in T_H lymphocytes *in vivo*. Finally, my colleagues performed bioinformatical and experimental analysis, including assay for transposase-accessible chromatin and sequencing (ATAC-seq) and chromatin immunoprecipitation and quantitative PCR (chIP-qPCR), and identified that the RFX transcription factor family is necessary for sustaining miR-150 expression in resting T_H lymphocytes.

I actively contributed to this work by generating and analyzing *PDAP1* CRISPR-Cas9 KO clones (Fig. 4D-E, S5B, S6B), by generating deletions of miR-150 binding sites in the *PDAP1* 3'UTR (Fig. S2), and by contributing to the preparation of the ATAC-seq samples (Fig. 6, 7A, S8).

5.1 Abstract

Within the immune system, miRNAs exert key regulatory functions. However, what are the mRNA targets regulated by miRNAs and how miRNAs are transcriptionally regulated themselves remains for the most part unknown. We found that in primary human memory T helper lymphocytes, miR-150 was the most abundantly expressed miRNA, and its expression decreased drastically upon activation, suggesting regulatory roles. Constitutive *MIR150* gene expression required the RFX family of transcription factors, and its activation-induced downregulation was linked to their reduced expression. By performing miRNA pull-down and sequencing experiments, we identified PDAP1 (PDGF-associated protein 1) as one main target of miR-150 in human T lymphocytes. PDAP1 acted as an RNA-binding protein, and its CRISPR-Cas9-mediated deletion revealed that it prominently contributed to the regulation of T cell proliferation. Overall, using an integrated approach involving quantitative analysis, unbiased genomics and genome editing, we identified RFX factors, miR-150 and the PDAP1 RNA-binding protein as the components of a regulatory axis that restrains proliferation of primary human T lymphocytes.

5.2 Introduction

Through their ability to target a variety of mRNAs and regulate their translation and stability, microRNAs (miRNAs) modulate all aspects of the biology of T lymphocytes, including cell differentiation, activation and proliferation (1, 2). The effect of any given miRNA is dependent on its expression level relative to that of its targets (3, 4), and also on the specific context and cell-specific usage of target sites in the 3' untranslated region (3'UTR) of mRNAs (5), resembling the cell type-specific regulation of gene expression mediated by transcription factors. The quantitative analysis of miRNA expression in different T cell subsets and in response to T cell receptor (TCR) triggering may thus provide clues on the functional impact of individual miRNAs on T cell responses. Abundant miRNAs that are downregulated after stimulation may be involved in restraining T cell activation, as shown in the case of miR-125b, which is required to maintain the naïve state of human T cells (6). By contrast, miRNAs that are expressed at very low levels are highly unlikely to reach the concentrations required to exert biological functions (4, 7). Finally, modestly expressed but inducible miRNAs may dynamically reach intracellular concentrations relevant in the modulation of T cell activation. Examples in this group include miR-155 (8, 9) and miR-146a (10) which are responsible for enhancing and attenuating T cell responses, respectively.

In this study, we took advantage of an integrated approach combining quantitative miRNA analysis, unbiased genomics and genome editing to identify miRNAs highly expressed in primary human T lymphocytes, analyze the regulatory logic underpinning their expression, and finally characterize mRNA target regulation and their functional impact.

Specifically, we focused on the single miRNA accounting for almost 50% of miRNAs constitutively expressed in human T cells, miR-150-5p (hereafter miR-150). This miRNA is abundantly expressed in both T and B lymphocytes (11), and its deletion in mouse models revealed that it modulates B lymphocyte and $CD8^+$ T cell differentiation (12-15). To identify the mechanisms controlling constitutive miR-150 expression and its activation-induced downregulation, we used an unbiased genomic approach to map the *cis*-regulatory elements in the *MIR150* locus that controlled its expression, leading to the identification of RFX (Regulatory Factor X) transcription factors as crucial regulators of constitutive miR-150 expression in resting cells and stimulus-induced downregulation. Finally, we used miRNA pull-down and sequencing to identify the mRNAs specifically targeted by miR-150 in human T lymphocytes. MiR-150 targeted modulators of T cell proliferation, including the transcription factor MYB and a previously unidentified target, PDAP1 (PDGFA associated protein 1), that we characterized as an RNA-binding protein (RBP). Deletion of *MYB*, *PDAP1* or *MIR150* itself by

CRISPR/Cas9-mediated gene editing in primary human T lymphocytes revealed the contribution of each of these factors to the regulation of T cell proliferation in response to activating signals. Overall, our data identified a miRNA-regulated network involved in restraining proliferative responses of circulating resting T lymphocytes.

5.3 Materials and methods

Isolation, culture and activation of human CD4$^+$ peripheral T lymphocytes. Buffy coats from healthy donors were obtained from the Swiss Blood Donation Center of Basel and Lugano (Switzerland), with informed consent from the Swiss Red Cross and authorization number CE 3428 from the Comitato Etico Canton Ticino. Leukocytes were separated by gradient centrifugation (Ficoll-Paque Plus; GE Healthcare), then CD4$^+$ T cells were isolated by magnetic microbeads and LS columns (Miltenyi Biotec). Naïve and memory T cell subsets were then sorted using FACSaria (BD Biosciences) based on the expression of the following surface markers: naïve T cells: CD4$^+$CD25$^-$CD45RA$^+$CCR7$^+$; total memory T cells: CD4$^+$CD25$^-$CD45RA$^-$CCR7$^{+/-}$; T_{CM} cells: CD4$^+$CD25$^-$CD45RA$^-$CCR7$^+$; T_{EM} cells: CD4$^+$CD25$^-$CD45RA$^-$CCR7$^-$. Other T cell subsets were separated from CD4$^+$ T cells by sorting for the following surface markers: T_H1 cells: CD4$^+$CD25$^-$CD45RA$^-$CXCR3$^+$CCR4$^-$CCR6$^-$; T_H2 cells: CD4$^+$CD25$^-$CD45RA$^-$CXCR3$^-$CCR4$^+$CCR6$^-$; T_H17 cells: CD4$^+$CD25$^-$CD45RA$^-$CXCR3$^-$CCR4$^+$CCR6$^+$; T_H22 cells: CD4$^+$CD25$^-$CD45RA$^-$CCR4$^+$CCR6$^+$CCR10$^+$. When needed, cells were cultured in RPMI-1640 medium supplemented with 5% human serum, 1% non-essential amino acids, 1%, sodium pyruvate, 1% glutamine, penicillin, streptomycin and 50 μM β-mercaptoethanol (complete medium). Cells were activated in Nunc MaxiSorp flat 96-well plates with plate-bound anti-CD3 (recombinant TR66 clone, in house production) and anti-CD28 (1 μg/mL) antibodies. Cells were removed from stimuli and placed in a round bottom plate after 48 h. When needed, cultures were supplemented with IL-2 at the concentration of 60 U/mL after the initial 5 days of activation.

Transfection of primary T cells and cell lines. Primary T cells were transfected with NEON nucleofector (Invitrogen) at 1800-2200 V, 20 ms, 1 pulse, using the provided buffer T. A total of 1 x 10^6 cells was used with the 10 μl tip transfections or 2.5 x 10^6 cells with the 100 μl tip. LNAs and siRNAs (listed in **Table S8**) were used at the concentration of 2 μM. Primary cells were cultured for up to 48 h post-transfection in pre-warmed complete medium without antibiotics. About 2.5 x 10^6 Jurkat cells were nucleofected at 1325 V, 10 ms, 3 pulses using buffer R, and were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 1% glutamine, penicillin, streptomycin, kanamycin and 50 μM β-mercaptoethanol. HEK293 cells were

transfected with polyethylenimine (PEI) using standard protocols and cultured in DMEM with 4.5 g/L D-glucose supplemented with 10% FBS, 1% sodium pyruvate, penicillin, streptomycin and 50 µM β-mercaptoethanol.

Lentivirus production and cell transduction. Lentiviral particles were purified from the supernatant of transfected HEK293 cells by sucrose gradient (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 25% sucrose) and ultracentrifugation (2.5 h, 100'000 x *g*, 4°C). For some experiments, lentivirus particles were concentrated using a PEG-8000 solution (63) (80 g PEG-8000, 14 g NaCl in 200 ml of PBS, pH 7.2) followed by centrifugation at 1,600 x *g*, 1 h at 4°C. For primary T cells, 5 µl of lentiviral concentrate were mixed with 150,000 resting cells in a flat-bottom 96-well-plate. After 24-48 h, cells were transferred to a Nunc plate coated with anti-CD3 and anti-CD28 antibodies for 48 h, and finally transferred to a round bottom 96-well plate with until day 5-7. Jurkat T cells were transduced with 5 µl of lentivirus-containing medium in a 96-well plate, 150,000 cells/well. After 48-72 h, transduced cells were selected by the addition of puromycin (2 µg/ml) for 72 h or sorted for GFP expression, depending on the selection marker.

Plasmids and cloning. Plasmids were constructed and modified by standard molecular cloning techniques. The miR-150 lentiviral vector pLL3.7_hsa-miR-150 (#25792) and the empty backbone pLL3.7 (#11795) were obtained from Addgene. The RFX3- and RFX5-expressing lentiviral vectors were obtained from Genecopoeia. For dual luciferase assays, regions of ~600 bp containing the putative miR-150 sites were amplified by PCR from the 3'UTRs of the *MYB*, *PDAP1*, *HNRNPAB* and *PIK3R1* genes, and were cloned into the pmirGLO plasmid using the NheI/XbaI restriction sites. The *PDAP1*-3'UTR-containing plasmid was further mutated to abrogate the miR-150 binding sites by site-directed mutagenesis using the Quick Change II kit (Agilent) according to manufacturer's instructions. All plasmids were verified by Sanger sequencing.

Luciferase Assay. HEK cells were transfected in a 96-well plates with 25 ng of pmirGLO plasmid (containing part of the 3'UTR of the candidate target downstream to luciferase gene) and 1 µM of miR-150 mimic or control oligonucleotide using a standard PEI (polyethylenimine) protocol. After 24 h, cells were lysed and analyzed using the Dual-luciferase reporter assay system (Promega) and a GloMax luminometer (Promega).

Nanostring SPRINT profiling. Total RNA was isolated using TRI reagent RT (MRC) and ZymoSpin columns and eluted in nuclease-free water. When needed, RNA was concentrated with a speed vac.

100 ng of total RNA at a concentration of 33 ng/□l were used for each experimental condition and probed with the Human miRNA v3 assay, according to manufacturer's instructions. Data were normalized to the 25 most highly expressed hits and p-values and ratios were calculated using the n-Solver 3.0 software.

Quantitative RT-PCR. Total RNA was isolated using TRI reagent RT (MRC) and ZymoSpin columns and eluted in nuclease-free water. For gene expression analysis, RNA was retrotranscribed with qScript cDNA SuperMix (Quanta Biosciences) and PCR performed with PerfeCTa SYBR green FastMix (Quanta Bioscience) using primers listed in **Table S8**. For miRNA expression, TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems) were used. Taqman probes are listed in **Table S8**. PCR reactions were run on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) or using the Quant Studio 3 Real-Time PCR System (ThermoFisher). Data were normalized to the *UBE2D2* housekeeping gene for SYBR-based qRT-PCRs or on RNU48 for Taqman reactions.

Cell proliferation. For CSFE dilution, memory T cells were resuspended in PBS with 2% human serum and incubated with CFSE at the final concentration of 5 µg/ml for 8 min at 37°C, followed by quenching with complete medium and extensive washing prior to activation with plate-bound anti-CD3 and anti-CD28 antibodies. For BrdU incorporation, 100,000 primary T cells were plated over-night in 1 ml of complete medium in a 48-well plate. BrdU was incorporated at the concentration of 3 µg/ml for either 1 h (day 3-stimulated primary T cells), 5.5 h (day 5-stimulated cells) or 24 h (day 6 or later time points after stimulation). After incorporation, cells were assayed using the APC BrdU Flow Kit by Pharmingen. For Jurkat cells, BrdU was incorporated for 5 h.

Western blotting. For protein extraction, T cells were washed with PBS and lysed in RIPA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) supplemented with a cocktail of protease inhibitors (Sigma, P8340). Protein concentration was measured using a BCA assay (Thermo Fisher Scientific, Pierce BCA Protein Assay Kit) and samples were either frozen or directly loaded onto 8-12% polyacrylamide gels. About 40 µg of total protein extract was used per sample. After electrophoresis, blotting on a PVDF membrane was performed using a wet transfer system and a methanol-based transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol). Blocking was performed with 5% milk in TBST (5 mM Tris pH 7.3, 150 mM NaCl, 0.1% Tween-20) for 60 min at RT with gentle shaking. Blots were incubated with primary antibodies over-night at 4°C or 1 h at room temperature, followed by washing and incubation with an HRP-

conjugated secondary antibody. Blot development was performed using the ECL Prime Western Blotting Detection Reagent (Amersham) and immediately analyzed with a blot imager (GE, Amersham Imager 680).

Biotinylated miRNA:targets pull-down and sequencing. The biotinylated miRNA pull-down was performed as described (50) with optimization for primary human T helper cells. Briefly, 20 x 10^6 memory T cells were stimulated with anti-CD3 and anti-CD28 antibodies and cultured for 5-6 days in complete medium. About 80 x 10^6 cells were then transfected with 50 nM of biotinylated miRNA mimic or control oligonucleotide (Exiqon) using the 100 µl kit for NEON nucleofector (Thermo Fisher Scientific) in multiple transfections of 2.5 x 10^6 cells each (2200 V, 20 ms, 1 pulse). Immediately after transfection, cells were incubated in pre-warmed complete medium without antibiotics supplemented with 60 U/mL of recombinant human IL-2. After 24 h, cells were collected, washed with MACS buffer (PBS, 0.5% BSA, 2 mM EDTA), and lysed in 500 µl of Lysis Buffer (20 mM TRIS pH 8.0, NaCl 70 mM, KCl 150 mM, NP-40 0.5%, DTT 1 mM, glycerol 10%, EDTA 2 mM, RNAsin inhibitor (Promega), and protease inhibitor cocktail (Sigma)). Lysed cells were left 15 min on ice and then transferred at -80°C to ensure complete lysis. After thawing, the cell lysate was cleared of cell debris by centrifugation at 4°C for 20 min. 30 µl of the cleared lysate were set aside and mixed with 90 µl of TRI-reagent for RNA extraction of the "input" fraction. The protein content in the remaining lysate was quantified with a BCA kit. 100 µl of streptavidin-agarose resin (Sigma) were washed twice with Lysis Buffer and subsequently incubated in Blocking Buffer (Lysis Buffer containing 1 mg/ml BSA, 100 µg/ml ssDNA Salmon Testis (Sigma), 500 µg/ml yeast tRNA (Thermo Fisher Scientific)) for at least 1 h at 4°C on a wheel. All centrifugation steps with agarose resin were performed at 11,000 x *g* for 11 sec. After 1 h, the streptavidin-agarose resin was washed 3 times with 500 µl of Lysis buffer and incubated over-night at 4°C on a spinning wheel with 1 mg of protein extract freshly supplemented with protein and RNAse inhibitors. The minimum volume of extract used for the incubation was 250 µl, at a final protein concentration of 1-3 mg/ml. The following day, samples were spun at 11,000 x *g* for 11 sec. The agarose beads were washed 4 times with 1 ml of Lysis buffer, then incubated with 400 µl of TRI-reagent, 15 min at room temperature and 1 h 4°C. After centrifugation to remove the beads, the supernatants contained the "pull-down" fraction. Total RNA was extracted from the pull-down and input fractions using Zymo-Spin IC columns and quantified using a Qubit fluorometer. Sequencing was performed at the Next Generation Sequencing platform at the University of Bern (Switzerland), using an Illumina HiSeq 3000 (for miR-150, 1x 100bp reads) or a NovaSeq 6000 (for miR-146a, 2x 50bp reads). For library preparation, the Takara SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian was used. Fastq files were analyzed using Linux and R. Quality control

was performed with FastQC and FastQScreen. Reads were trimmed of overrepresented sequences with Cutadapt and mapped to the human GRCh37 - hg19 assembly with Hisat2. Counts were generated using featureCounts and differential expression analysis performed with DESeq2.

MiRNA seed analysis. MiRWalk 2.0 (21) was used to predict miRNA targets. Predictions were done on 3'UTR, CDS and 5'UTR. Afterwards, the presence of at least a 6-mer was verified manually for each pull-down target.

ATAC-seq library preparation. ATAC-seq was performed on $1x10^5$ sorted $CD4^+$ memory and naive T cells obtained from four independent donors. After isolation, cells were either left resting or activated with plate-bound anti-CD3 and anti-CD28 antibodies in complete medium. Cells were processed on day 0 (resting condition), as well one and three days after activation. Briefly, cells were resuspended in 50 µl of lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 0.1% Igepal CA-630) and incubated on ice for 3 minutes followed by centrifugation (500 × *g*, 4°C, 20 min). Nuclei were then resuspended in 50 µl of tagmentation buffer (10 mM Tris-HCl, 25 mM $MgCl_2$ and 1 µl of adaptor-loaded Tn5 transposase (produced in-house)) and incubated for 1 h at 37°C. The cleanup of the tagmented DNA was performed by adding 10 µl of clean-up buffer (900 mM NaCl, 30 mM EDTA), 2 µl of 10% SDS, 6 µl of milliQ water and 2 µl of Proteinase K (20 µg/µl) followed by incubation at 40°C for 30 minutes. Tagmented DNA was isolated using 2× AMPure XP beads and amplified by PCR with barcoded primers using 14 cycles of PCR. Finally, fragments smaller than 500 bp were purified with 0,65× AMPure XP beads and primers were removed by purification with 1,8× AMPure XP beads. Libraries were sequenced paired-end on an Illumina NextSeq500 platform.

ATAC-seq analysis. Paired end reads were adapter trimmed using BBDuk in pair-end mode (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/ - last modified version November 7, 2019). Reads were subsequently trimmed with trimmomatic in pair-end mode (version 0.39 flags: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:25). Resulting reads were mapped to hg38 using bowtie2 (version 2.3.5.1; with flags: --very-sensitive -k 2 -t --phred33 -p 4 -q). Resulting bam files were then filtered using samtools (version 1.9) in order to remove unmapped reads, failing quality, mpping to mitochondrial chromosome or with their mate unmapped (flag: samtools view -b -f 3 -F 524). Reads mapping to ENCODE black-list regions (https://github.com/Boyle-Lab/Blacklist) were also removed using bedtools pairToBed (version 2.29.2 flag: -type neither). PCR duplicated reads were removed using samtools markdup as described in the reference manual (http://www.htslib.org/doc/samtools-markdup.html). ATAC reads were

then shifted as reported previously (64) using deepTools alignmentSieve (version 3.4.1; flag: --ATACshift). Peak calling was performed using MACS2 callpeak (version 2.2.6; options: –nomodel --format=BAMPE -B -g hs --call-summits). A reference set of peaks was created by selecting peaks called in each samples/replicate with qvalue≤10^{-10} and being consistent between replicates (e.g. having an overlapping area of at least 50% between replicates). The resulting set of peaks was used to count reads in each sample using the R/Bioconductor package GenomicRanges and GenomicAlignment. Sample normalization was achieved by selecting invariant ATAC peaks across samples (for sample normalization strategy see Ref. (65)). Differentially regulated peaks were selected using DESEq2 (R/Bioconductor package version 1.26.0; R version 3.6.2). Peak clustering was performed in R implementing a strategy similar to the one described by Dorrity et al. 2020 (PMC7093466). Transcription factor motif enrichment analysis was performed for each identified cluster using GimmeMotifs (40) using all accessible sites as background.

CRISPR-Cas9 gene editing and single-cell cloning. CRISPR-Cas9 ribonucleoproteins (crRNPs) were delivered to primary $CD4^+$ T memory cells by NEON transfection exactly as described (36). Briefly, crRNAs and fluorescently labelled tracrRNAs (Dharmacon, IDT) were mixed at a final concentration of 80 µM in 10 µl of Nuclease Free Duplex buffer (Dharmacon, IDT). The solution was then incubated for 5 min at 95°C and left at room temperature for 20 min for annealing. The RNP complex was prepared immediately before transfection by mixing 7.5 µg of recombinant TrueCut Cas9 Protein v2 (Thermo Fisher Scientific) with 1.5 µl of the crRNA/tracrRNA duplex mix in a total volume of 3 µl followed by incubation for 20 min at room temperature. To increase transfection efficiency, Alt-R Electroporation enhancer (IDT) was added at a final concentration of 1.7 µM to the transfection mix (exclusively for primary cells). Transfection was performed with the 10 µl Neon Transfection System kit. One million memory T cells or Jurkat cells were resuspended in Neon Buffer T or Buffer R respectively and added to the electroporation solution. Cells were then electroporated at 2200 V, 20 ms, 1 pulse for primary cells and 1600 V, 10 ms, 3 pulses for Jurkat cells. After transfection, individual T cells were seeded in 384-well plates at 0.4-0.65 cells per well in complete medium in the presence of recombinant IL-2 (500 U/ml, produced in house), 1 µg/ml of phytohaemagglutinin (PHA) and 25,000 irradiated (45 Gy) allogeneic feeder cells (PBMCs) per well (36, 66). After 2 weeks, individual clones were transferred into round-bottom 96-well plates and further expanded for another 10 days in presence of IL-2, 500 U/mL.

Analysis of CRISPR/Cas9 deletion efficiency in T cell clones. Genomic DNA (gDNA) from individual clones (~1 x 10^5 cells) derived from primary human T cells was isolated using the QIAamp DNA Micro

Kit and DNeasy Blood & Tissue kit (Qiagen) or extracted with QuickExtract DNA extraction solution (Lucigen) following manufacturer's protocol. To screen for the presence of deletions a simple PCR spanning the region of interest was used. In the case of the *MIR150* gene, the two sgRNAs were designed to produce a deletion of ~200 bp, and clones were screened for the presence of this deletion by PCR, using external primers. To screen for the presence of mutations, a T7 endonuclease I cleavage assay was used (67, 68). Briefly, primers were designed to amplify a ~600-1000 bp region surrounding the targeted area. PCR amplification was performed using the high fidelity KOD Hot Start DNA polymerase and 20-100 ng of gDNA template in 30 µl. Because the T7 endonuclease I cleaves mismatched heteroduplex DNA, 15 µl of each PCR product were denatured and re-annealed to produce potential heteroduplexes of wild-type and mutated DNA strands. Five units of T7 endonuclease I (New England Biolabs) were added directly to the annealed PCR product and incubated at 37°C for 15 min. As control, parallel reactions without T7 endonuclease were performed. After resolution of the DNA bands on a 1% agarose gel, band intensities were quantified with ImageJ and the percentage of cleavage efficiency was calculated by dividing the density of the cut product by that of the uncut. We considered as "modified" any clone presenting a cleavage efficiency higher than the average background generated by the control clones.

Chromatin immunoprecipitation. About 60 million freshly isolated $CD4^+$ T cells were resuspended in 36 ml of PBS and 1 mL of formaldehyde solution 37% (Sigma) to a final concentration of ~1%. After 10 min, the reaction was quenched with glycine at a final concentration of 0.125 M. After washing, cell pellets were flash-frozen in liquid nitrogen. Frozen cells were thawed and lysed in 3 mL of RIPA lysis buffer (Tris-HCl pH 8.0 10 mM, EDTA pH 8.0 1 mM, NaCl 140 mM, SDS 0.1%, deoxycholic acid 0.1%, phenylmethylsulfonyl fluoride 2 mM, 1× Sigma protease inhibitor) for 30 min on ice, followed by sonication using a Diagenode Bioruptor Plus, 4°C, 45 cycles 30 sec on/60 sec off, leading to DNA fragments of ~200 bp in size. At the end of the sonication process, chromatin was cleared by centrifugation and addition of 1% Triton-X. 20 µl of cleared chromatin were set aside as input. To assess the extent of shearing, part of the chromatin (100 µl) was de-crosslinked by incubation with 5 µg of proteinase K overnight at 65°C and column-purified before visual assessment on an agarose gel. For immunoprecipitation, 75 µl of magnetic Dynabeads protein G (Thermo Fisher Scientific) were washed twice in Binding Buffer (PBS, BSA 0.5%, Tween-20 0.5%) and incubated with 10 µg of mouse anti-human RFX5 antibody (Santa Cruz Biotechnology sc-271756-X) in 300 µL of Binding Buffer for 2 h at room temperature on a rotating platform. After washing, the beads were finally incubated with 1 ml of cleared chromatin overnight at 4°C on a rotating platform. After removal of the supernatant, the beads were washed five times with RIPA buffer, 2 times with Tris-HCl pH 8.0, 10

mM, EDTA pH 8.0 1 mM, NaCl 500 mM, SDS 0.1%, deoxycholic acid 0.1%, Triton-x-100 1%, 2 times with LiCl buffer (Tris-HCl pH 8.0 10 mM, EDTA pH 8.0 1 mM, LiCl 250 mM, NP-40 0.5%, deoxycholic acid 0.5%), and once in Tris-EDTA buffer. Beads were then resuspended in 50 µL of Direct Elution Buffer (Tris-HCl pH 8.0 10 mM, EDTA pH 8.0 5 mM, NaCl 300 mM, SDS 0.5%), and treated with 5 µg of RNse A, 37°C for 30 min. Glycogen 1 µl and 2.5 µl Proteinase K (20 mg/ml) were added and samples were incubated at 37°C for additional 2 h, shaking. Samples were finally reverse-crosslinked by incubation at 65°C for 6-18 h. Beads' supernatants were transferred to new tubes and 132 µl of SPRI magnetic beads (Mag-Bind RxnPure Plus, Omega Bio-tek) were added to each sample and incubated 5 min at room temperature, followed by two washes with 500 µl of ethanol 70%, while leaving the tubes on the magnetic rack. After drying, the DNA was eluted with 30-60 µl of Tris-HCl pH 8.0 and DNA samples were quantified by Qubit fluorometric quantification (Invitrogen). Finally, 600 pg of immunoprecipitated DNA were used for qPCR, in a final volume of 10 µl.

RNA immunoprecipitation. Twenty million memory T cells were isolated from healthy donors and activated with plate-bound anti-CD3 and anti-CD28 antibodies for 5 days. Cells from two distinct donors were then pooled together for a total of ~80×10^6 cells. The cells were resuspended in ice-cold PBS at the density of 20-30 $\times$ 10^6 cells/ml, and half of them were irradiated twice with 254nm UV light at 0.2J using a UV Stratalinker. Each irradiation cycle was conducted on ice with two-minutes shaking intervals. After centrifugation, the cells were lysed in RIPA buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, 140mM NaCl) with recombinant protease inhibitors (Sigma) and RNAse inhibitor (1 U/µl, Promega). Protein content was quantified using a BCA kit (Thermo Fisher Scientific), and 1mg of cell extract was incubated on a rotating wheel ~16 h at 4°C using 6 µg of antibody (anti-PDAP1 Bethyl A304-651A or Proteintech anti-Tubulin 66240-1-Ig). Upon addition of 60 µl of protein-G dynabeads (Thermo Fisher Scientific) and incubation for 4 h at 4°C, beads were washed twice with RIPA buffer and two more times with RIPA-500 (same as RIPA, except for NaCl 500 mM). Washed beads were treated with proteinase K (60 mg in 60 µl of RIPA buffer containing RNAse inhibitor) at 37°C for 30 min, followed by RNA extraction using 400 µl of TRI-reagent (MRC) and Zymo spin RNA low-quantity IC columns. RNA was retrotranscribed using Quanta Bio master mix and qPCR was performed using SYBR green reagents (Quanta Bio). For sequencing, total RNA was quantified using a Qubit fluorometer, followed by library preparation and sequencing at the Next Generation Sequencing platform of the University of Bern (Switzerland), using an Illumina NovaSeq 6000. For library preparation, the CORALL Total RNA-Seq Library Prep Kit was used. Quality control was performed with FastQC (v. 0.11.5) and RSeQC (v. 2.6.4). PCR duplicates were removed using UMI-Tools v1.1.1 and the resulting reads were

mapped to the human GRCh38.104 assembly with Hisat2 (v. 2.1.0). Counts were generated using featureCounts (v. 1.6.0) and differential expression analysis performed with DESeq2. Only protein-coding transcripts were considered for further analysis.

Poly-A RNA pull-down. mRNA pull-down was performed using the Dynabeads Oligo(dT)25 kit (Thermo Fisher Scientific) according to manufacturer's instructions. Briefly, 60-80 $\times$ 10^6 T cells were isolated and irradiated as described for RNA immunoprecipitation. Cells were then lysed directly in 1 ml of Lysis/Binding buffer and crude extracts were passed 20 times through a 21-gauge needle to decrease viscosity, and then incubated directly with 100 µl of Dynabeads-oligo(dT) for 3 h at 4°C on a rotating wheel. After extensive washing, beads were resuspended in 60 µl of Laemmli buffer and boiled at 95°C for 5 min. Samples were then used for SDS-page and blotted with an anti-PDAP1 antibody (1:500 dilution).

Immunofluorescence. 1x10^5 memory T cells were fixed using 3.7% formaldehyde in PBS and then spun on cytospin slides (5 min at 500 rpm). The cells were then permeabilised with 0.1% TritonX in PBS and blocking was performed using 10% goat serum in PBS. Slides were incubated with primary antibodies for 1 h at room temperature, followed by washing and incubation with an secondary antibodies for 30 min at room temperature. Nuclei were counterstained with DAPI. Images were acquired with a Leica TCS SP5 laser-scanning confocal microscope (LSCM), using a 63x/NA 1.4 PL APO CS Oil objective with a XY pixel size of 55 nm and pinhole 1 AU. Fluorescence was excited with 594 nm He:Ne laser and collected in range 600-700nm. Quantification of the mean intensity of the signals in individual cells was performed using ImageJ software.

Statistical analyses. Graphs were created with GraphPad Prism 8 software. FACS plots were analyzed by FlowJo 10 software. Heatmaps were made by R software using ggplot2, viridis and reshape2 libraries. Statistical analyses were performed using GraphPad Prism 8. The comparison between two means was evaluated by parametric *t*-test if the two populations compared were normally distributed or by non-parametric (Wilcoxon-Mann-Whitney) test in case the populations were not normally distributed. Distributions were tested using the Kolmogorov Smirnov test. Welch's correction to *t*-test was applied in case the two populations were heteroscedastic (distribution of populations was evaluated with an F-test). Comparisons among three or more sample means were made by ANOVA.

Data availability. All relevant data are within the paper and its Supporting Information files, and are available at GEO with accession number GSE161100. The numerical data used in all figures are included in Data S1.

5.4 Results

miR-150 is the most highly expressed miRNA in human T cells and is downregulated by activation. To identify and accurately quantify miRNAs that are expressed by *ex vivo*-isolated primary human T cells, we performed Nanostring digital profiling of $CD4^+$ naïve, central memory (T_{CM}) and effector memory (T_{EM}) T cell subsets isolated from four independent donors. Among the 827 miRNAs quantified, only 48 were detectable in these subsets (**Table S1)**. The levels of expression of these miRNAs differed widely, with the combined expression of only two of them (miR-150 and miR-142) representing >70% of the overall miRNA content in all the T cell subsets analyzed (**Fig. 1A**). MiR-150 was the most highly expressed miRNA, with an average number of ~110,000 molecules per 100 ng of total RNA (**Fig. S1A**). While miR-150 expression was substantially similar among subsets, a few moderately expressed miRNAs (such as miR-222) were preferentially expressed in memory T cells (both T_{CM} and T_{EM}) compared to naïve cells, while miR-181a was instead preferentially expressed in naïve compared to memory T lymphocytes (**Fig. S1B**). No significant differences were observed between T_{CM} and T_{EM} cells (**Fig. S1B**).

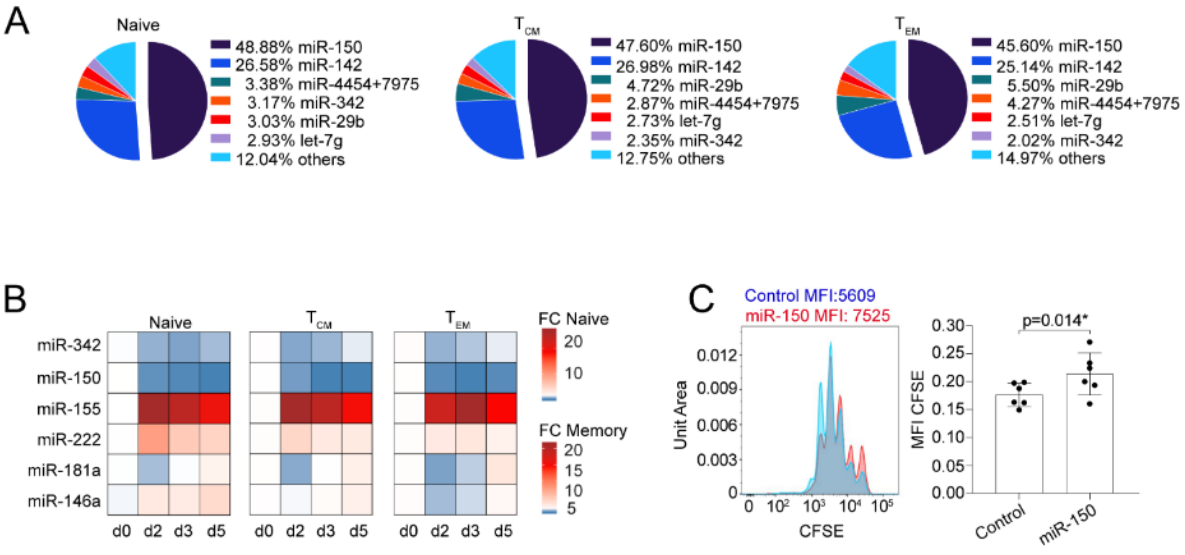


Figure 1. miRNA expression in human $CD4^+$ T cell subsets. (A) Total RNA was extracted from freshly isolated $CD4^+$ naïve, T_{CM} and T_{EM} T cell subsets, and miRNA expression was measured by Nanostring Sprint profiling. The most highly expressed miRNAs are shown, and data are expressed as percentage of normalized counts over the total. N=3

independent donors. **(B)** Total RNA was extracted from the indicated T cell subsets freshly isolated from peripheral blood. miRNA expression was measured by qRT-PCR and data are expression as $2^{-\Delta Ct}$. N=3 independent donors. **(C)** Freshly isolated memory T lymphocytes were loaded with CFSE, transfected with either a miR-150 mimic or a control oligonucleotide and activated with anti-CD3 and anti-CD28 antibodies. The extent of cell proliferation was measured 3 days after activation. Data in the bar graph were normalized to the overall baseline signal on day 0, prior to stimulation, to compensate from experimental differences in basal CFSE loading. N=6 independent experiments. Mean ± SD. Student's *t*-test, two-tailed, paired.

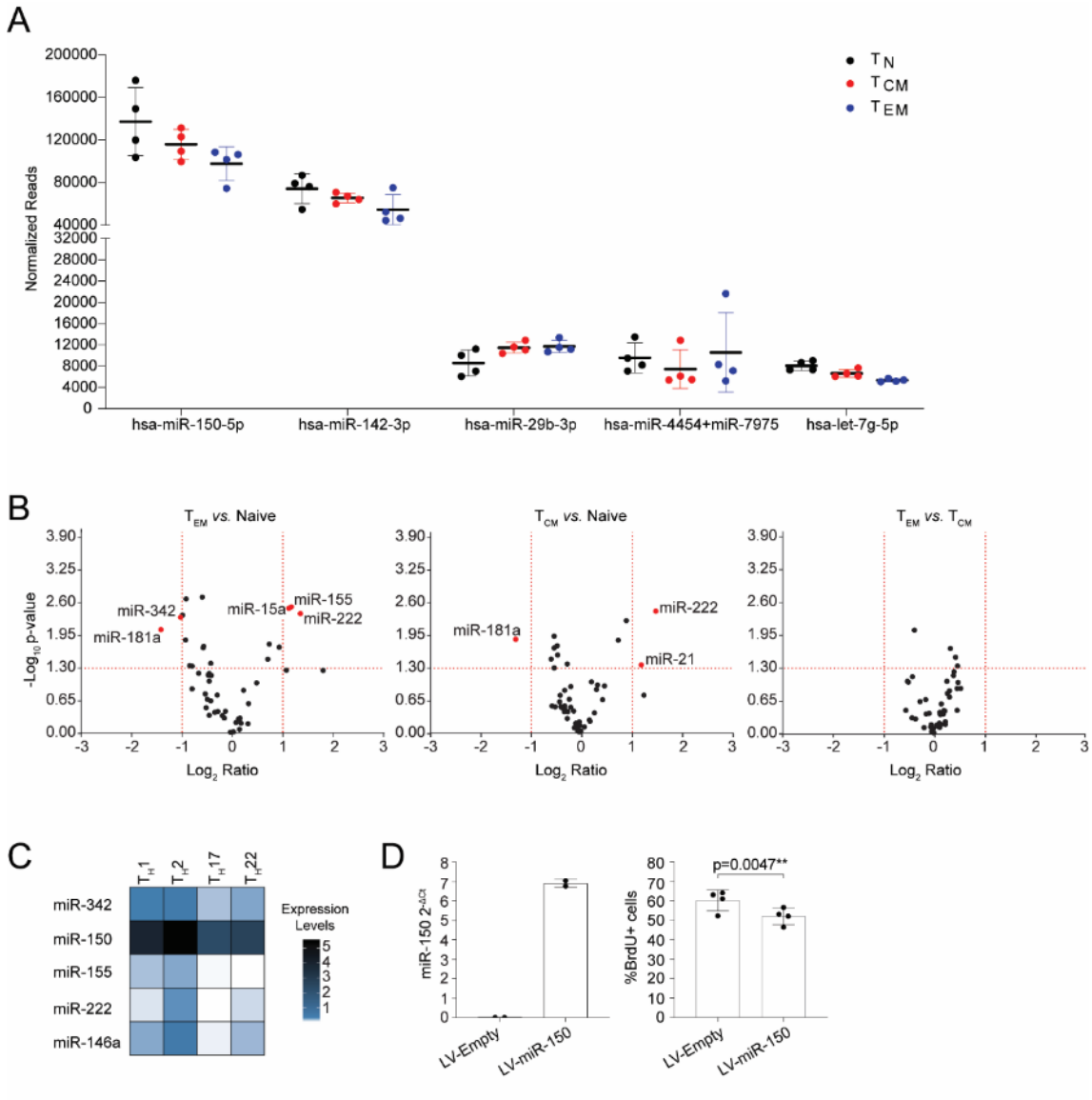


Figure S1. miRNA expression in primary human T lymphocytes. (A) Naïve, T_{CM} and T_{EM} lymphocytes were freshly separated from the peripheral blood of four independent donors. 100 ng of total RNA were used for the analysis. Raw data were normalized to the top 25 most expressed miRNAs and considered as expressed if following thresholds applied: at least one sample with more than 125 normalized reads and no more than one sample containing less than 100 normalized reads. Mean ± SD. Each dot represents an independent donor. **(B)** Volcano plot representations of the same data an in (A), to compare miRNA expression across subsets. Differentially expressed miRNAs (Log_2 ratio ≥ 1 and ≤

-1; Log_{10} p-value $\geq$ 1.3) are shown in red. N=3 independent donors. **(C)** Naïve, T_{CM} and T_{EM} cell subsets were isolated from peripheral blood and were either left resting or activated with plate-bound anti-CD3 and anti-CD28 antibodies for the indicated times. Total RNA was extracted and the expression of the indicated miRNAs measured by qRT-PCR. Data are shown as Fold Change (FC) compared to resting day 0 (d0) cells. N=3 independent donors. **(D)** Jurkat T cells were transduced with a lentiviral vector (LV) to force miR-150 expression. Expression of miR-150 compared to control samples was measured by qRT-PCR (*left*), and cell proliferation was measured by BrdU incorporation assay (*right*). N=4 independent experiments. Mean $\pm$ SD. Student's *t*-test, two-tailed, paired.

Next, we selected some of the highly expressed or differentially expressed miRNAs to assess their regulation in response to T cell activation. T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies and miRNA expression was measured by qRT-PCR over time (**Fig. 1B**). Some of the miRNAs expressed at moderate levels in resting lymphocytes (miR-155, miR-222, miR-146a) were substantially induced upon TCR stimulation, especially in naïve cells. Abundant miRNAs such as miR-150 and miR-342 were instead markedly reduced after two days of activation, while miR-181a had a more variable pattern of expression across the different subsets and time-points. We further measured the expression of these highly abundant or inducible miRNAs in different *ex vivo* isolated effector subsets, namely T_H1, T_H2, T_H17 and T_H22 cells. We observed quantitatively modest and non-significant differences, concordant with differential miRNA expression being limited primarily to naïve *vs.* memory cells (**Fig. S1C**). The dynamic regulation of miR-150 upon activation together with its high levels of expression in resting cells pointed towards its possible role in the regulation of T cell responses upon TCR triggering.

To determine the functional role of miR-150 in human T cells, we transfected freshly isolated memory T lymphocytes with either a miR-150 mimic or a control oligonucleotide and we measured cell proliferation over time by CFSE dilution. We found that in the presence of miR-150, T cell proliferation was significantly affected after three days of anti-CD3 and anti-CD28 stimulation, as shown by the reduced dilution of CFSE, leading to higher mean fluorescence intensity (MFI) (**Fig. 1C**). Proliferation (measured by BrdU incorporation) was similarly reduced in Jurkat T cells stably transduced with a miR-150-expressing lentivirus (**Fig. S1D**). Overall, miR-150 was the most highly expressed miRNA in human T lymphocytes, in which it controlled proliferation in response to stimuli.

Identification of miR-150 targets in human T cells. Cellular context-dependent regulation is a crucial aspect of miRNA-mediated regulation that is mainly based on the relative abundance of a miRNA and its targets within a specific cell type or activation state (5, 16). Such context-dependent regulation mediated by miRNAs cannot be predicted by the available databases and can only be experimentally explored. To identify the mRNAs that are directly and specifically regulated by miR-

150 in T lymphocytes, we transfected activated memory T cells from three independent donors with either a biotinylated version of a miR-150 mimic or a control oligonucleotide, followed by streptavidin-agarose pull-down and sequencing (17-20). As a control of target specificity, we performed the same experiment using biotinylated miR-146a. We found that the pull-down of both miR-150 and miR-146a recovered established targets for these miRNAs, namely *MYB* for miR-150 and *IRAK1* and *TRAF6* for miR-146a, thus confirming target specificity (**Fig. 2A, Table S2, S3**).

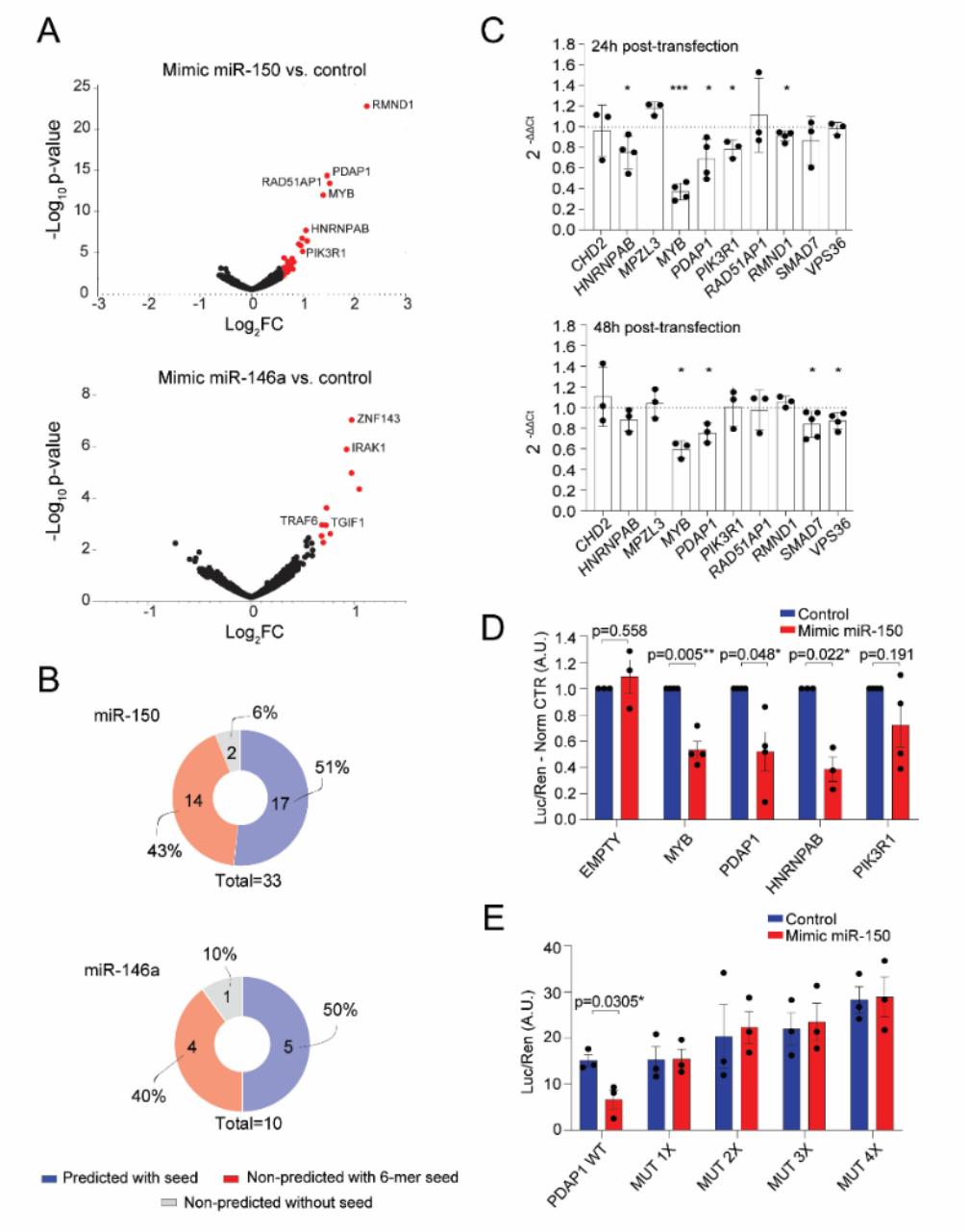


Figure 2. Identification of miR-150 targets. (A) Volcano plot of differentially expressed genes between the indicated miRNA mimic and control oligonucleotides. Genes in red were considered significantly differentially expressed when $Log_2FC \geq 0.6$, $-Log_{10}$ p-value ≥ 2. **(B)** The list of genes obtained from (A) was intersected with several prediction

databases using Mirwalk 2.0. Both the 3'UTRs and the CDS were manually searched for the presence of at least a 6-mer miR-150 or miR-146a binding site. **(C)** Activated memory cells were transfected with miR-150 mimic or control oligonucleotide. 24 h or 48 h after transfection, the expression of the indicated genes was measured by qRT-PCR. N=3-5 independent donors. Mean ± SD. Student's *t*-test, two-tailed, paired. **(D)** The 3'UTR of the indicated genes was cloned in a dual-luciferase reporter vector and transfected into HEK cells together with either a miR-150 mimic or a control oligonucleotide. Luciferase reads were normalized to the renilla ones. N=3-4 independent experiments. Mean ± SEM. Student's *t*-test, two-tailed, paired. **(E)** Same as in (D), except that the four putative miR-150 binding sites identified in the *PDAP1* 3'UTR were mutated by site-directed mutagenesis. N=3 independent experiments. Mean ± SEM. Student's *t*-test, two-tailed, paired. A.U.: arbitrary units.

Further analysis of the recovered targets showed that 31 out of the 33 miR-150 putative targets contained at least one 6-mer seed within either the 3'UTR, 5'UTR or the coding sequence (CDS), and ~50% (17 out of 33) of these were predicted miR-150 targets by the miRWalk 2.0 database (21) (**Fig. 2B**). Our results are in line with previous observations, reported by other groups, showing that about half of bound miRNA sites are noncanonical, and that most noncanonical sites are bound and functional in a cell-type-specific manner (5, 22, 23). To which extent these noncanonical sites (that are efficiently bound *in vivo*) mediate effective target repression remains to be fully understood (24). Similar results were obtained for miR-146a (**Fig. 2B**). Next, as validation of these pull-down data we selected 10 putative miR-150 targets and tested the effects of a miR-150 mimic on their expression in T cells from an independent set of donors. Memory T cells were transfected with either the miR-150 mimic or control oligonucleotide, and mRNA expression was analyzed 24 h or 48 h later (**Fig. 2C**). For some of the targets (*HNRNPAB*, *MYB*, *PDAP1*, *PIK3R1*, *RMND1*), a suppressive effect of miR-150 was already observed after 24 h, while for others (*SMAD7* and *VPS36*) a significant reduction was observed only after 48 h, most likely due to varying mRNA stability and turnover. To determine whether the observed downregulation of these putative miR-150 targets was mediated by a direct activity of miR-150 on their 3'UTRs, we cloned either the entire 3'UTR or the regions containing the predicted miR-150 binding site(s) in a reporter vector. Co-transfection of these plasmids with a miR-150 mimic oligonucleotide led to significantly reduced luciferase expression for three out of four targets tested, namely *MYB*, *PDAP1* and *HNRNPAB*, which were therefore the highest confidence targets, while the effect on *PIK3R1* appeared to be more variable (**Fig. 2D**). The *PDAP1* 3'UTR contains five putative miR-150 binding sites predicted by TargetScan 7.2 (25), four of which are clustered in the distal region of the 3'UTR. We cloned the region containing the four clustered sites and we evaluated the impact of mutating these sites on miR-150-mediated repression. We found that mutation of only one site was sufficient to abrogate repression by miR-150, suggesting that all four clustered sites are required for full miR-150 activity on the *PDAP1* 3'UTR (**Fig. 2E**), although this effect may be different *in vivo*. To further investigate the relationship between PDAP1 and miR-150

in a more physiological setting, we deleted one or three clustered miR-150 binding sites from the 3'UTR of the *PDAP1* gene in primary human T lymphocytes, using CRISPR-Cas9 editing. We found that deletion of one single site was insufficient to completely abrogate miR-150 activity, while the partial deletion of three sites reduced miR-150 responsiveness (**Fig. S2**). Overall, our target analysis in primary human T lymphocytes recovered established targets of miR-150, such as *MYB*, and identified additional ones, such as *PDAP1*, as direct miR-150 targets in human T cells.

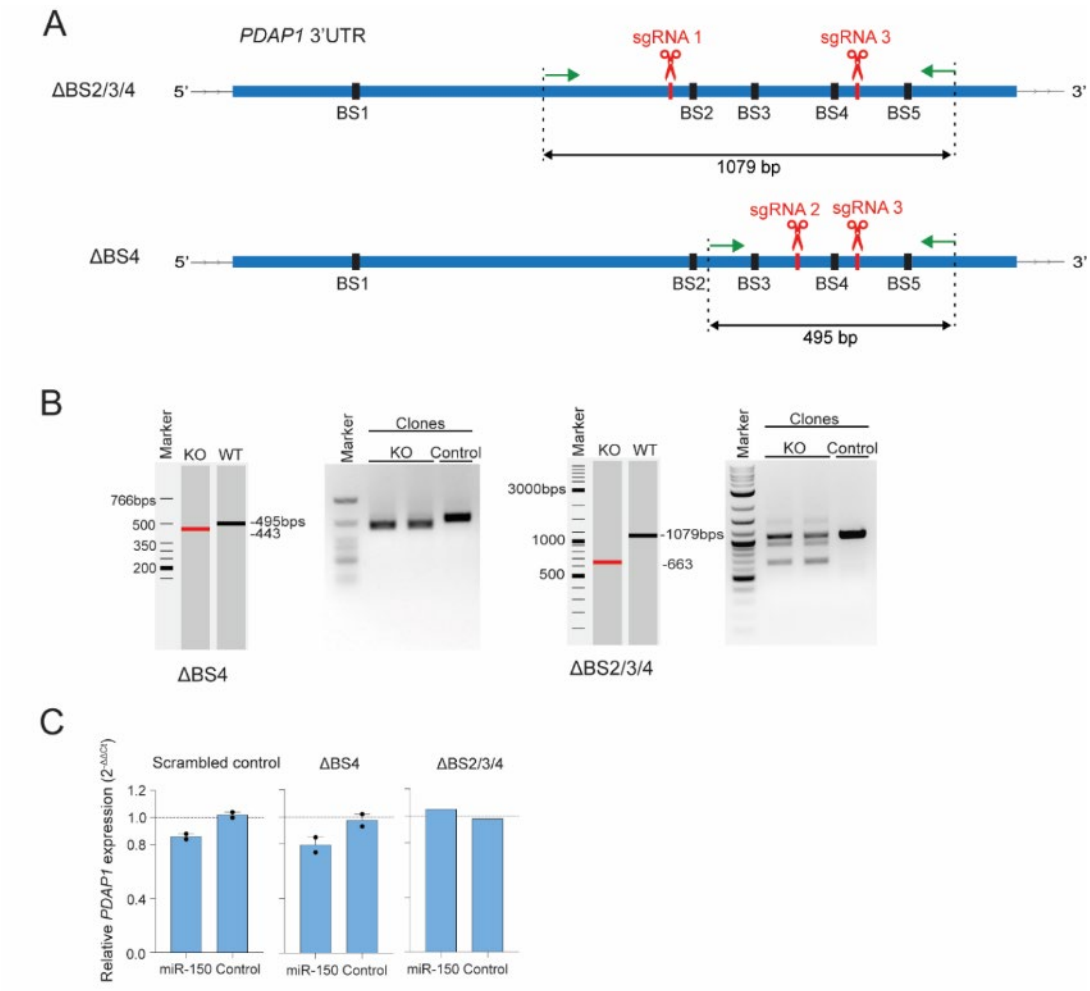


Figure S2. Genomic deletion of three miR-150 binding sites in the PDAP1 3'UTR abrogates miR-150 regulation. (A) Schematic representation of the *PDAP1* 3'UTR with indicated the locations of the predicted miR-150 binding sites (BS, black), the sgRNAs (red) and the primers used for gDNA screening (green arrows) of cells and clones lacking three or one single miR-150 site (DBS2/3/4 and DBS4, respectively). **(B)** Example of gDNA screening for single clones DBS4 (*left*) and DBS2/3/4 (*right*). We could identify only two clones with a partial deletion of DBS2/3/4. **(C)** Individual clones with the desired genomic modifications in the 3'UTR of the *PDAP1* gene (or control clones, transfected with a scrambled sgRNA sequence) were pooled and transfected with either a miR-150 mimic or control oligonucleotide. 24h after transfection, expression of *PDAP1* was measured by RT-qPCR (Scrambled control clones: N=8 pooled clones, 2 experiments; DBS4: N=9 pooled clones, 2 experiments; DBS2/3/4: N=2 pooled clones).

PDAP1 acts as an RNA-binding protein. Consistent with the fact that *MYB* is a target of miR-150 (12), we found that in the presence of miR-150, endogenous *MYB* mRNA expression was significantly reduced compared to the baseline in both transiently transfected primary memory T lymphocytes and transduced Jurkat cells (**Fig. S3A**). As expected, the down-modulation of *MYB* expression using siRNAs was sufficient to limit human T cell proliferation (**Fig. S3B**).

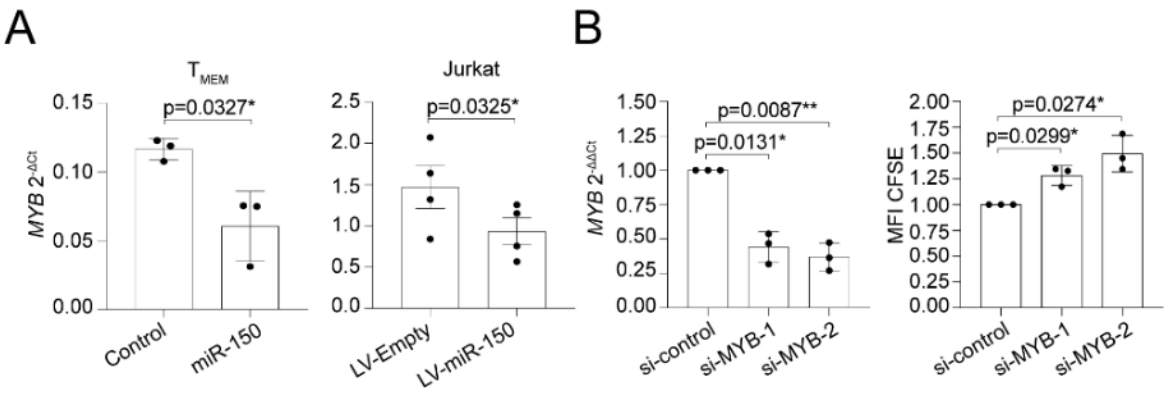


Figure S3. miR-150 and MYB modulate T cell proliferation. (A) Primary memory T cells and Jurkat cells were transfected with an miRNA mimic or transduced with a control or miR-150-expressing lentivirus. Seven days after transduction *MYB* expression was measured by RT-qPCR. N=3 (primary T) or N=4 (Jurkat) independent experiments. Mean ± SD (primary T) or SEM (Jurkat). Student's *t*-test, two-tailed, paired. **(B)** Memory T lymphocytes were loaded with CFSE, transfected with either siRNAs against *MYB* or a control oligonucleotide and activated with anti-CD3 and anti-CD28 antibodies. The extent of *MYB* downregulation was measured by RT-qPCR (*left*), while cell proliferation was measured by CFSE dilution 4 days after activation (*right*). N=3 independent experiments. Mean ± SD. Student's *t*-test, two-tailed, paired.

Next, we investigated the role of additional miR-150 targets in the regulation of T cell proliferation. We focused on *PDAP1* as it was the second most affected target in T cells transfected with miR-150. PDAP1 is a highly conserved protein whose precise functional role and mechanism of action is largely unknown. PDAP1 was found associated with different types of cancers (26, 27), hinting at a role in the regulation of cell proliferation. Indeed, T cell tumors also show high expression of PDAP1 (28). First, we transfected memory T lymphocytes with a miR-150 mimic oligonucleotide, which led to a significant reduction in endogenous PDAP1 protein expression (**Fig. 3A**). Next, we investigated the impact of PDAP1 on memory T cell proliferation using transfection of siRNAs. Compared to cells transfected with a control oligonucleotide, downregulation of *MYB* or *PDAP1*, or transfection of the miR-150 mimic oligonucleotide, all led to a significant reduction in T cell proliferation, as assessed by BrdU incorporation assay (**Fig. 3B**). Consistent with a positive role of PDAP1 in controlling T cell proliferation, we found that its expression increased, both at the mRNA and protein level, upon activation of T lymphocytes (**Fig. 3C-D**). We also found that PDAP1 remained strictly cytoplasmic in both resting and activated cells, pointing towards a role in signaling and/ or mRNA translation (**Fig. 3D**).

The mechanisms by which PDAP1 regulates proliferation is incompletely understood, although it was recovered as an RBP in several RNA–protein interactome studies (29-32), and it was also described as an RNA-dependent protein, namely a protein able to engage in larger, yet uncharacterized, complexes only in the presence of RNA (33). Indeed, PDAP1 was reproducibly recovered after RNA pull-down in different human cell types, including human T lymphocytes (29-32), pointing towards a crucial function of this protein as an RBP (**Fig. 4A, Table S4**). To experimentally determine whether PDAP1 can indeed act as an RBP in human T lymphocytes, we performed an oligo-dT pull-down of total mRNA in memory T cells. Western blot analysis of these samples revealed the presence of PDAP1, which was preferentially enriched in samples that underwent UV crosslinking (**Fig. 4B,** and raw data in **Fig. S4**), suggesting that this protein is indeed capable of RNA binding in T cells, either directly or as part of a larger RNA-binding complex, yet to be determined.

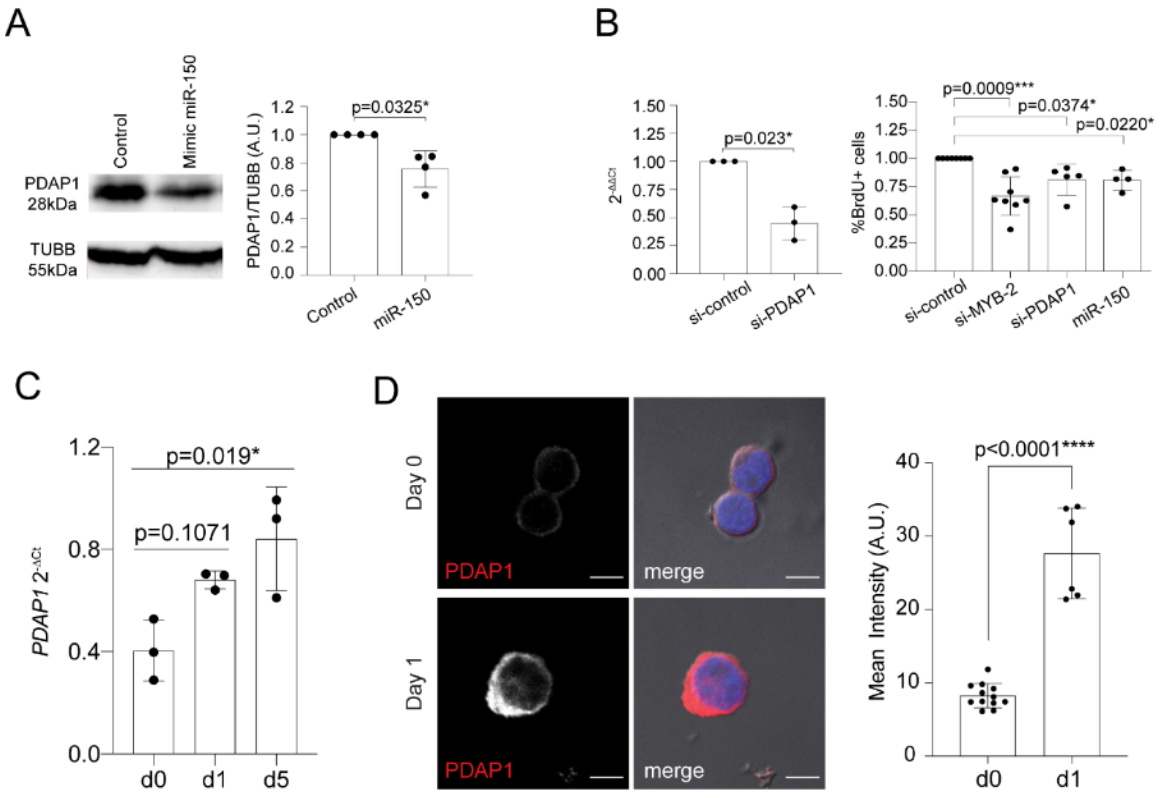


Figure 3. PDAP1 regulates T cell proliferation and is upregulated upon activation. (A) Memory T lymphocytes were transfected with either a miR-150 mimic or control oligonucleotide. 24 h after transfection, expression of PDAP1 was assessed by western blot. Tubulin was used as loading control. A representative western blot is shown on the left, while the densitometric quantification of independent experiments in shown on the right. N=4. Mean ± SD. Student's *t*-test, two-tailed, paired. **(B)** Memory T lymphocytes were transfected with siRNAs targeting either *MYB* or *PDAP1*, or with a miR-150 mimic oligonucleotide. The extent of *PDAP1* downregulation was measured by RT-qPCR (*left*), while cell proliferation was measured by BrdU incorporation assay. N=3-8 independent experiments (each dot represents one donor). Mean ± SD. Student's *t*-test, two-tailed, paired. **(C)** Memory T lymphocytes were stimulated with plate-bound

anti-CD3 and anti-CD28 for the indicated days, followed by RT-qPCR analysis of *PDAP1* expression. N=3, each dot represents one donor. Mean ± SD. One-way ANOVA. **(D)** Memory T lymphocytes were stimulated with plate-bound anti-CD3 and anti-CD28 for the indicated days, followed by immunofluorescence for PDAP1. The bar corresponds to 5mm. Representative of N=2 experiments. Right: Quantification of the mean intensity of the PDAP1 signal in individual cells. Mean ± SD. Unpaired *t*-test, two-tailed.

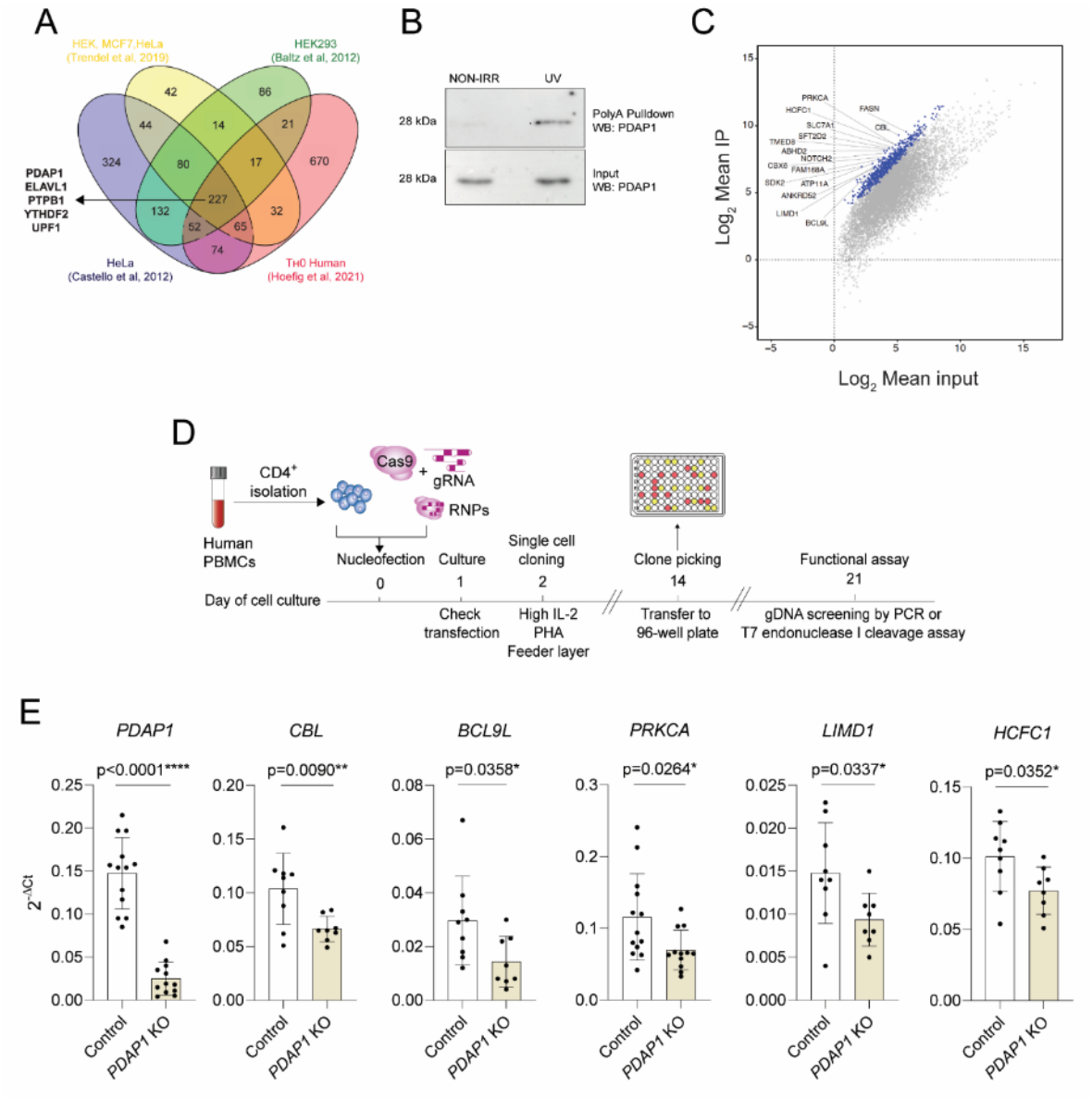


Figure 4. PDAP1 acts as an RNA-binding protein in primary human T lymphocytes. (A) Venn diagram showing the extent of overlap between RBPs identified in the indicated studies and cell types. **(B)** Activated memory T lymphocytes were UV-irradiated and lysed prior to poly-A mRNA pull-down and western blot. Non-irradiated samples were also used. Representative of N=2 independent experiments. **(C)** Activated memory T lymphocytes were UV-crosslinked and lysed prior to immunoprecipitation using either an anti-PDAP1 or an anti-tubulin antibody as irrelevant control. The extent of enrichment of the indicated mRNA targets was measured by RT-qPCR. N=3 independent experiments. Mean ± SD. (**D**) Sequencing of RIP samples as in in (**C**).

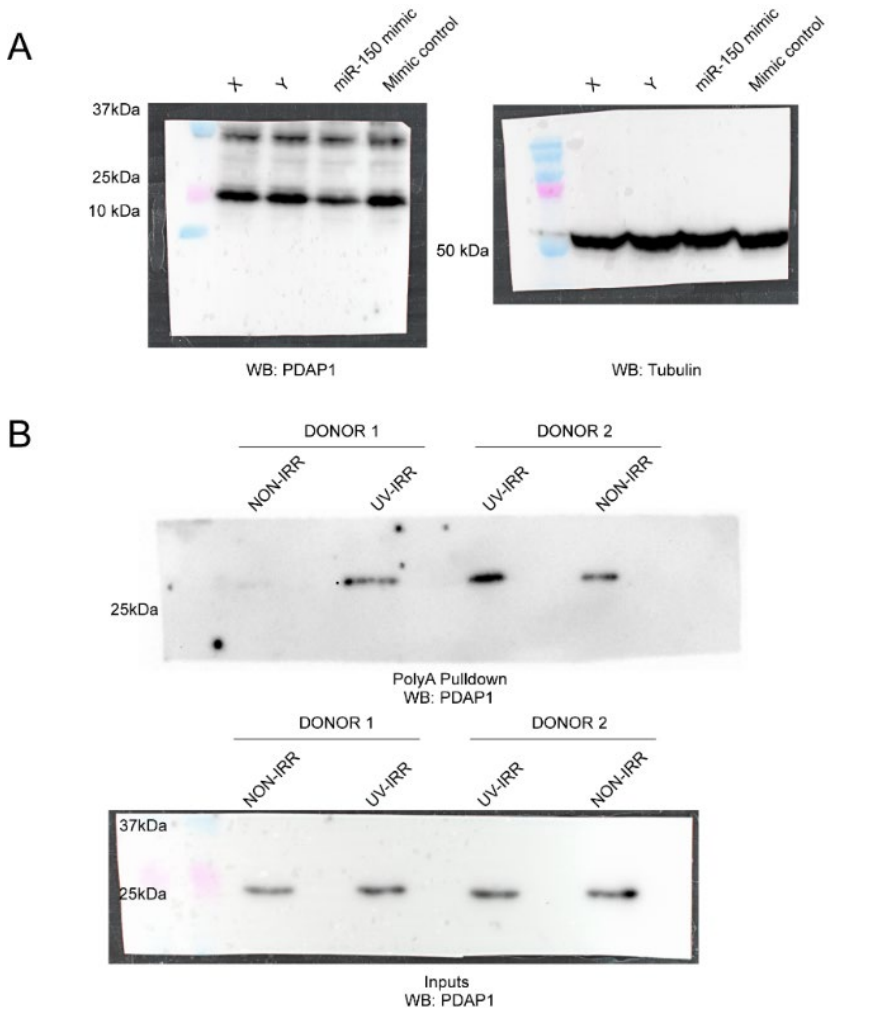
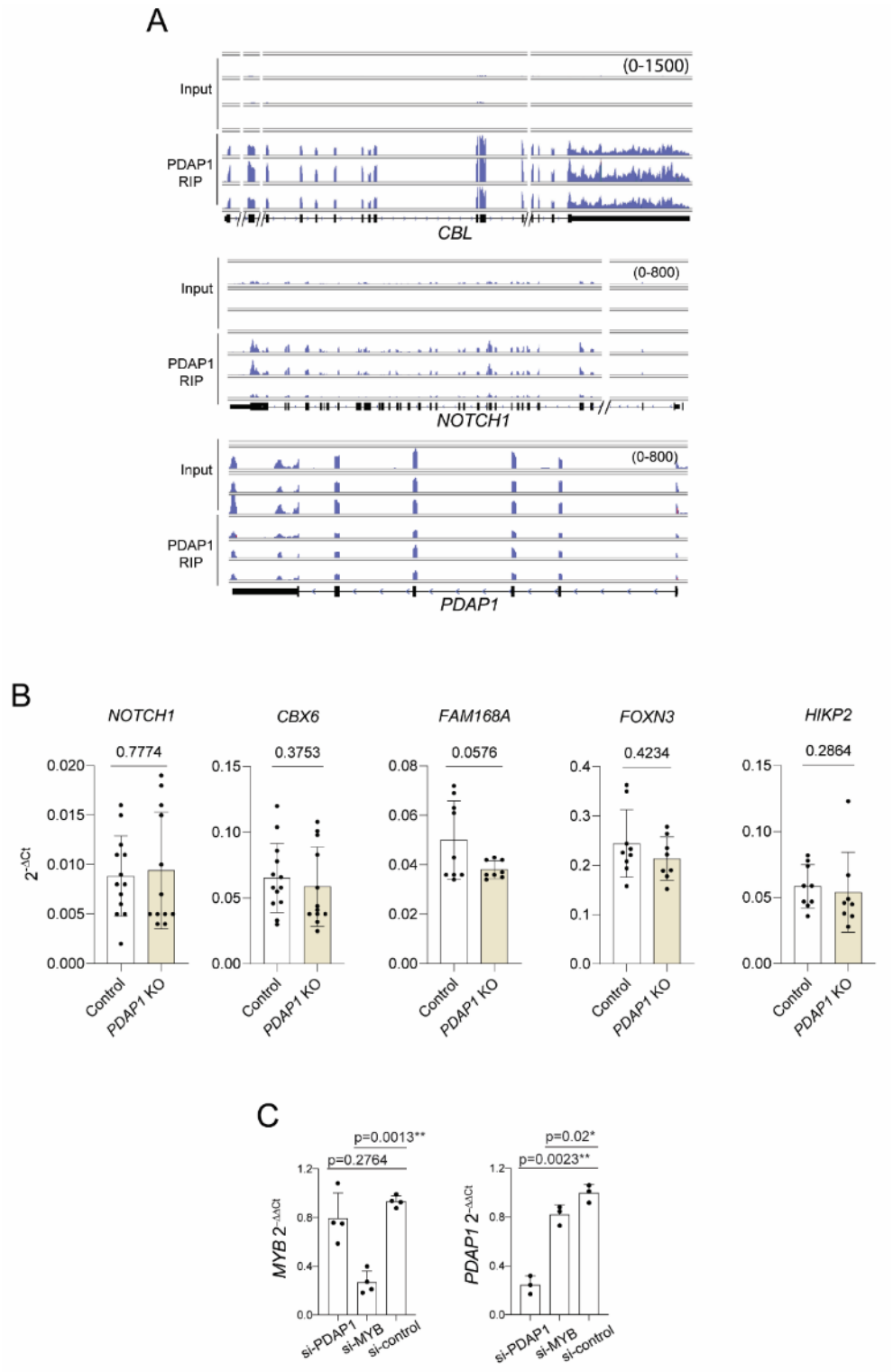


Figure S4. Poly-A mRNA pull-down recovers the PDAP1 protein raw data. (A) Complete western blot images of the images shown in Fig. 3a and **(B)** Fig 4b.

Next, we searched for candidate targets that might be regulated by this protein. We initially focused our attention on AKT1 and PDK1 (phosphoinositide-dependent kinase-1), two selected candidates that were previously shown to be impacted both at the mRNA and protein level by PDAP1 deletion in glioma cells (34). RNA-immunoprecipitation (RIP) experiments in UV-crosslinked memory T lymphocytes using an anti-PDAP1 antibody revealed that both transcripts for *AKT1* and *PDK1* were enriched in crosslinked samples, as opposed to the control mRNA *UBE2D2* (**Fig. 4C**). Importantly, RNA-sequencing of the immunoprecipitated samples identified other crucial factors in T cell biology that were bound by PDAP1, most notably the key regulators of T cell activation, differentiation and functions *CBL*, *NOTCH1* and *NOTCH2* (**Fig. 4D**, **S5A, Table S5**). Many of the most highly significantly enriched transcripts (indicated in blue in the figure) were expressed at moderate to high levels in human T cells as shown in the DICE database (35), except for *SDK2* that was lowly expressed.



Fugre S5. (A) RIP-seq for PDAP1 in memory T lymphocytes. Examples of snapshots of the sequencing tracks for selected genes. **(B)** Memory T lymphocytes were transfected with recombinant Cas9 and sgRNAs against PDAP1 or scrambled controls. After cloning, expansion and selection of PDAP1 KO clones, n=7 individual clones were pooled and the expression of the indicated mRNA transcripts was measured by RT-qPCR. **(C)** Memory T lymphocytes were transfected with siRNAs against *PDAP1* or *MYB*. After 24h, RNA was extracted and the expression of *MYB* and *PDAP1* was measured by RT-qPCR. N=3 independent donors. Mean ± SD. Student's *t*-test, two-tailed, paired.

The function of most of these factors in T lymphocytes remains to be established. Other regulators of T cell functions bound by PDAP1 included the *RUNX* family of transcription factors as well as some of the *AGO* mRNAs (**Table S5**). Factors that were instead not significantly enriched by PDAP1 immunoprecipitation included *RORC*, Drosha, as well as *PDAP1* itself and *MYB*. Importantly, deletion of PDAP1 in primary human T cells by CRISPR-Cas9 editing reduced the expression of *CBL* and *NOTCH1*, while *NOTCH2* was less affected (Fig. **S5B**), suggesting a role for PDAP1 in modulating mRNA stability. Interestingly, knockdown of *PDAP1* also modestly affected the expression of *MYB* (**Fig. S5C**), although this could be an indirect effect due for instance to the altered availability of miR-150 upon removal of one its abundantly expressed primary targets. Even though the mechanistic underpinning of PDAP1 function and regulation on mRNA stability or translation remains to be understood, these results further indicate that PDAP1 is an RBP capable to modulate T cell proliferation at least in part by affecting the expression of factors that are central to T cell activation and metabolism.

Deletion of PDAP1 limits lymphocyte proliferation. To further assess the role of miR-150 and its targets in T cell proliferation, we performed CRISPR/Cas9-mediated deletion of either the *PDAP1* or *MYB* gene. The workflow of the overall experimental design includes transfecting primary human memory T lymphocytes with two sgRNAs for each gene together with recombinant Cas9 protein, followed by single-cell cloning, expansion, selection of gene-modified clones and functional analyses (**Fig. 5A**) (36, 37). After transfection, primary memory T cells were cloned in 384-well plates by limiting dilution, after which individual clones were screened for the presence of insertions/ deletions (indels) or mutations in the genomic region of interest by PCR and T7 endonuclease I cleavage assay (Fig. **S6A-C**). We found that the cloning efficiency in two independent donors was on average 16% for PDAP1 and 15% for MYB, compared to 24% for the control clones (transfected with sgRNAs targeting an irrelevant, non-expressed gene), suggesting that the targeted genes affected the ability of the clones to expand. Cell proliferation of individual clones was therefore measured by BrdU incorporation. By analyzing 12 *MYB*-KO and 25 *PDAP1*-KO clones, we found that T cell proliferation was significantly decreased for both (**Fig. 5B**). A similarly reduced proliferation was observed also in Jurkat cells transfected with sgRNAs against either *MYB* or *PDAP1* (**Fig. S6D**). Further highlighting the role of PDAP1 in modulating lymphocyte proliferation, we found that in a panel of B-cell lymphoma cell lines, PDAP1 expression was often increased compared to primary B lymphocytes, and CRISPR-Cas9 deletion of PDAP1 in these cell lines led to significantly reduced proliferation (**Fig. S7**). Finally, to unequivocally determine whether miR-150 expression was sufficient to restrain T cell proliferation, we transfected memory T cells with two sgRNAs targeting

the *MIR150* gene (**Fig. S6C**), which led to high deletion efficiency (67%). We found that upon targeting the *MIR150* locus, T cells from two independent donors proliferated significantly more (~37% increase) compared to control clones (**Fig. 5C**). Overall, the experimental identification of miR-150 targets coupled to their functional validation revealed that PDAP1 is a crucial regulator of T cell proliferation whose activity is restrained in the resting state by high levels of miR-150.

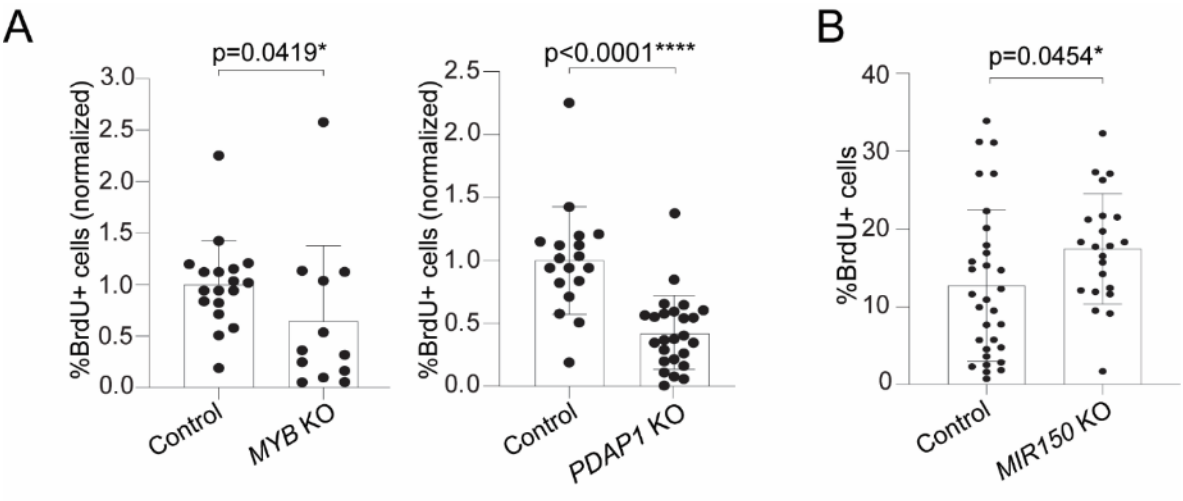


Figure 5. miR-150 restrains T cell proliferation through MYB and PDAP1. (A) Schematic representation of the experimental workflow to generate primary T lymphocytes knock-out for the factors and miRNA of interest. **(B)** Primary memory T cells were transfected with Cas9 RNPs to delete either *MYB* (*left*) or *PDAP1* (*right*), followed by single-cell cloning. Individual clones were selected based on the presence of a large genomic deletion in the gene of interest, and proliferation was measured by BrdU incorporation assay. For the *MYB* gene, N=18 control clones and N=12 *MYB*-edited clones, from two independent donors. For the *PDAP1* gene, N=18 control clones and N=25 *PDAP1*-edited clones, from two independent donors. Mean ± SD. Mann-Whitney test. **(C)** Memory T cells were transfected with Cas9 RNP complexes containing two different sgRNAs targeting the *MIR150* gene. Individual clones were selected based on the presence of a genomic deletion overlapping the *MIR150* sequence, and proliferation was measured by BrdU incorporation assay. N=31 control clones and N=22 *MIR150*-edited clones, from two independent donors. Mean ± SD. Welch's *t*-test, two-tailed.

RFX family transcription factors modulate miR-150 expression. High levels of miR-150 in basal conditions were coupled with its strong reduction upon T cell activation. In memory T cells, the reduced abundance of miR-150 was significant already at 24 h of stimulation with anti-CD3 and anti-CD28 antibodies (**Fig. 6A**), namely before cells started to proliferate (38), thus ruling out a role of passive dilution of the mature miRNA. To investigate potential mechanisms of miR-150 downregulation, we first measured the expression of the primary (*pri-miR-150*) transcript. We found that its expression was almost completely abrogated 15 h after activation (**Fig. 6B**), pointing toward the rapid downregulation of *MIR150* gene transcription upon T cell activation.

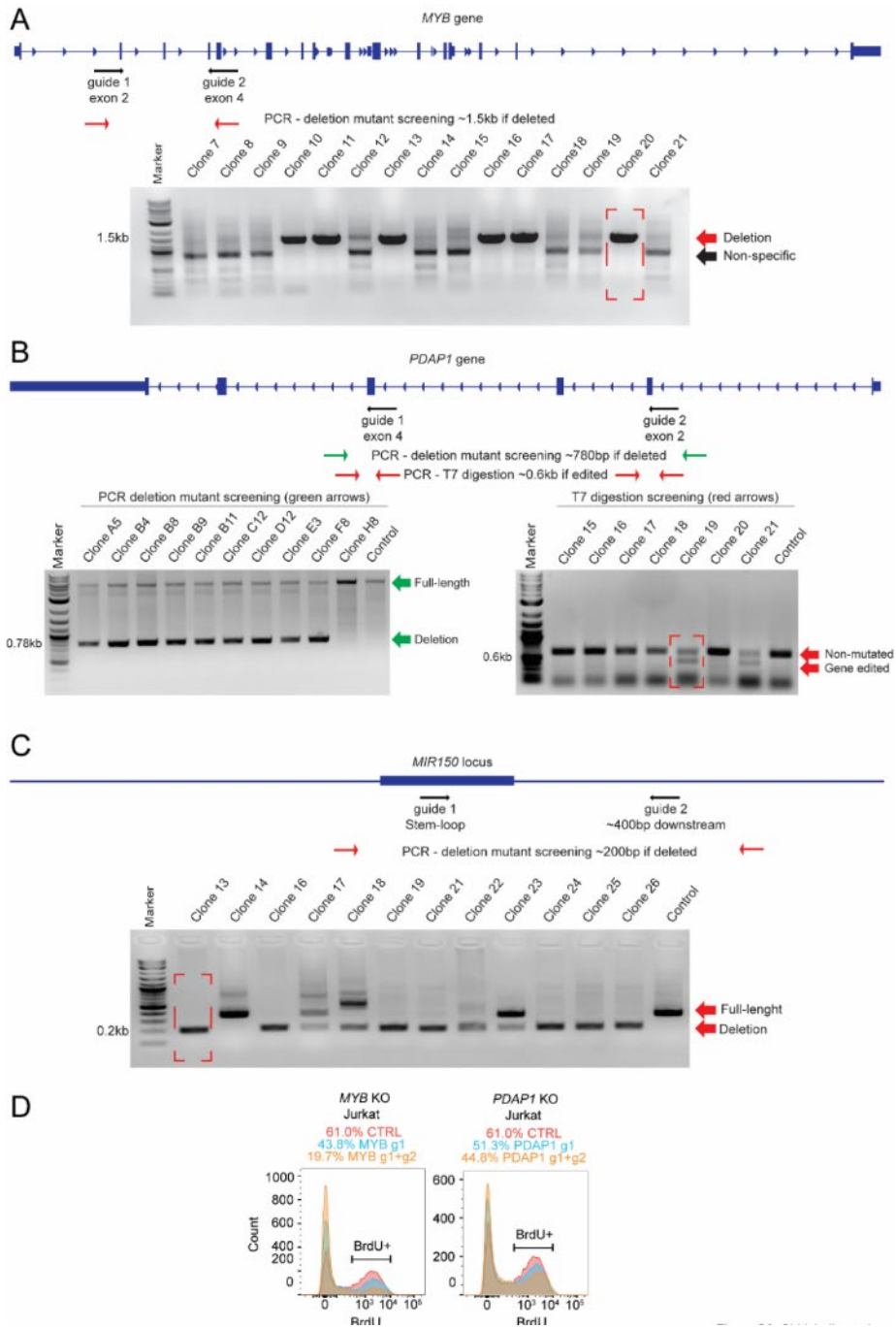


Figure S6. Screening strategies for CRISPR-Cas9-targeted primary T cell clones. (A) Clones targeted in the *MYB* gene were screened for deletion mutants by using PCR primers located externally to the sgRNAs. **(B)** Clones targeted in the *PDAP1* gene were identified by PCR followed by T7 endonuclease I digestion. When digested by T7 endonuclease I, the PCR product of ~600 bp is digested into two segments of 200 and 400 bp if a mutation or small indel is present in this region. **(C)** Clones targeted in the *MIR150* gene were screened for the presence of a deletion across the miR-150 sequence by PCR, using primers located externally to the two sgRNAs. In each case, the red dashed square highlights an example of deleted or mutated clone that was used for further analyses. **(D)** Jurkat T cells were transfected with Cas9 RNPs to delete either *MYB* (left) or *PDAP1* (right). Cells were transfected with either one (blue line) or two (yellow line) sgRNAs at the same time. Cell proliferation was measured 4 days after transfection by BrdU incorporation assay.

To identify in an unbiased manner the transcriptional mediators of miR-150 downregulation in activated primary T lymphocytes, we first set out to identify the genomic *cis*-regulatory elements in the *MIR150* locus that were deactivated upon stimulation.

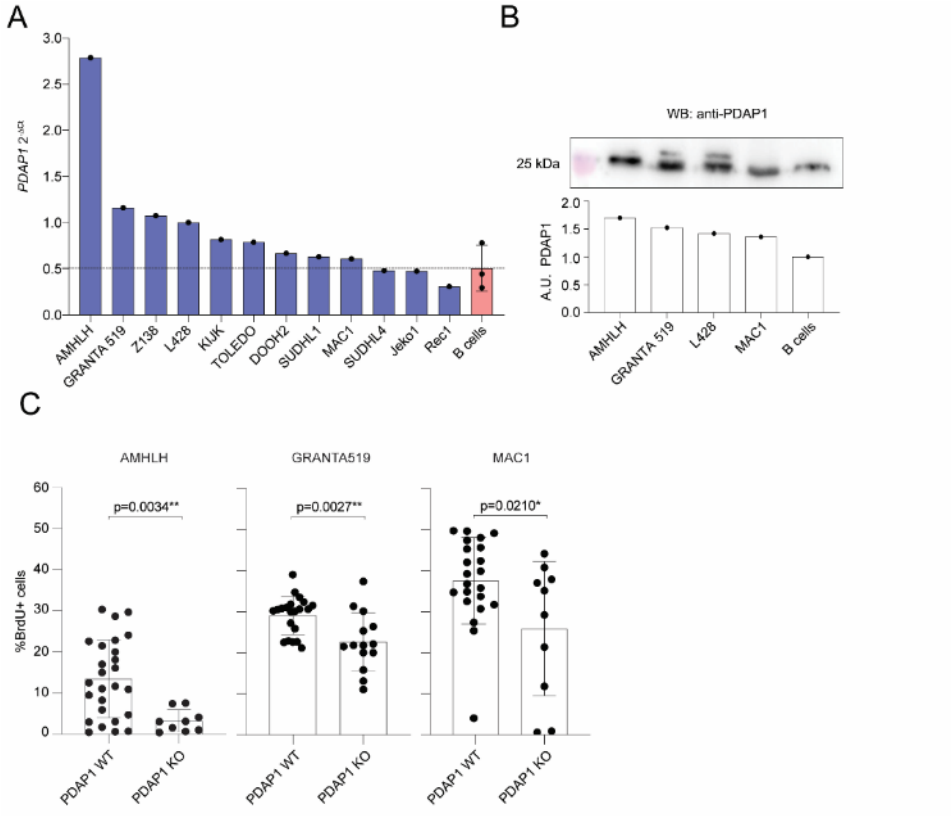


Figure S7. Deletion of the PDAP1 gene in B cell lymphoma cell lines led to reduced cell proliferation. **(A)** RT-qPCR and **(B)** western blot analyses for PDAP1 expression in the indicated cell lines and in freshly isolated primary human $CD19^+$ B lymphocytes. **(C)** The *PDAP1* gene was deleted by CRISPR-Cas9 in the indicated cell lines. After single-cell cloning, selection and expansion, cell proliferation of the individual clones was measured by BrdU incorporation. Each dot represents one clone. Mean ± SD. Student's *t*-test, two-tailed, unpaired.

To this aim, we performed Assay for Transposase Accessible Chromatin and Sequencing (ATAC-seq) in primary human naïve and memory T cells, either resting or activated with anti-CD3 and anti-CD28 antibodies for one or three days. In memory T cells, 747 peaks were already significantly reduced after one day of activation ($\log_2$ fold change $\leq$ -1 and adjusted p-value $\leq 10^{-5}$). Of these, 103 peaks could be associated to a transcription start site (TSS). In the same samples, 6,237 peaks instead increased after activation. Naïve T cells showed similar results, with 923 ATAC-seq peaks reduced after activation with 124 of them matching a TSS, and 6,616 induced peaks (**Table S6**).

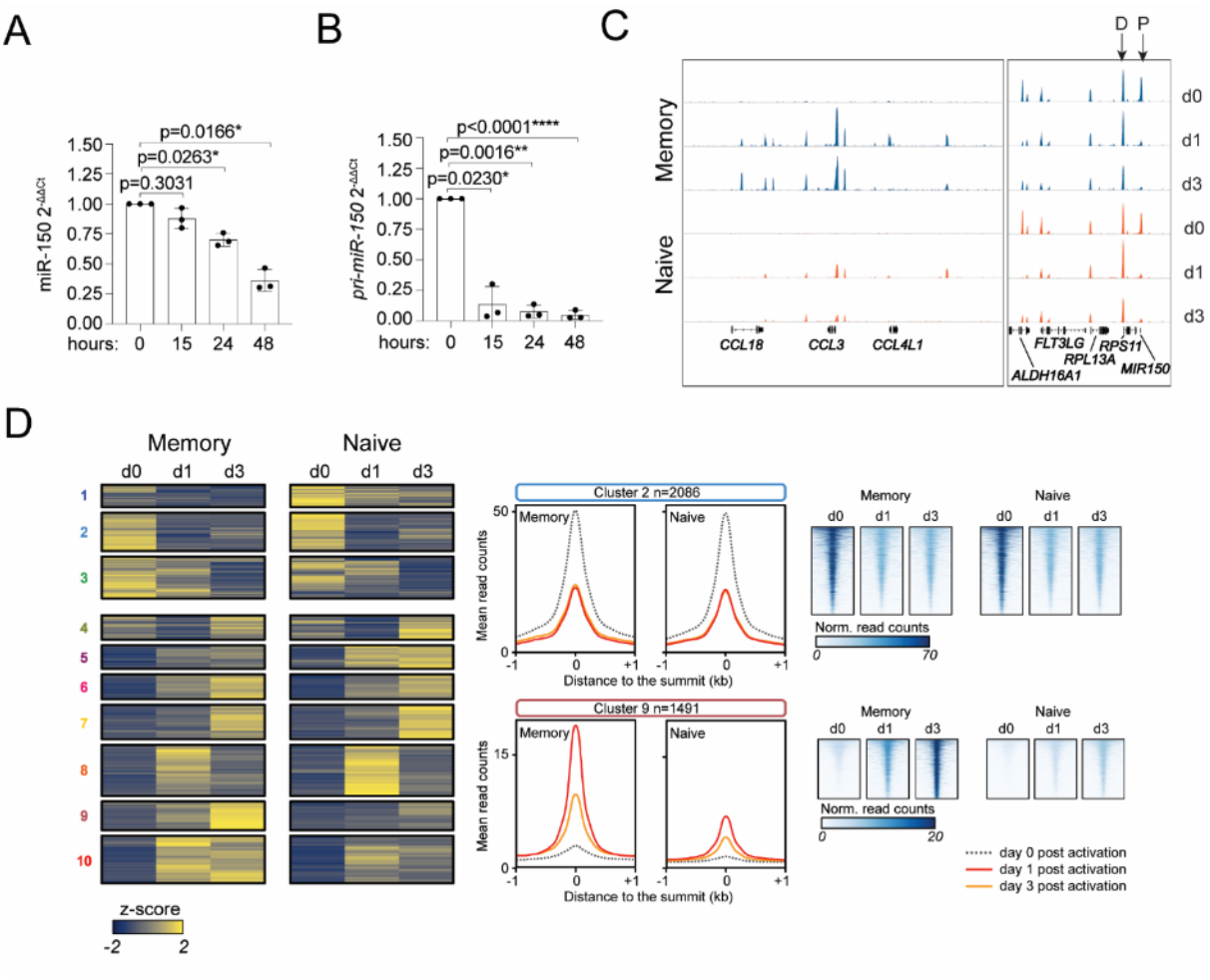


Figure 6. ATAC-seq analysis in human CD4⁺ T cell subsets. (A) Memory T cells were sorted from peripheral blood and were either left resting or activated with plate-bound anti-CD3 and anti-CD28 antibodies for the indicated number of hours. Total RNA was extracted and miR-150 expression was measured by qRT-PCR. N=3 independent donors (each dot represents one donor). Mean ± SD. One-way ANOVA. **(B)** Same as (A), except that the expression of *pri-miR-150* was measured. N=3. Mean ± SD. One-way ANOVA. **(C)** Naïve and memory T cells were freshly isolated from three independent donors and were either left resting or were stimulated with anti-CD3 and anti-CD28 antibodies for one or three days, before tagmentation and sequencing for ATAC-seq analysis. Representative snapshots of the sequencing tracks. Arrows indicate the distal (D) and proximal (P) peaks relative to *MIR150*. **(D)** Clustering analysis including all ATAC-seq peaks that were significantly affected after 24 h or 72 h of activation in either naïve or memory T cells, with different representations of cluster 2 and 9 (middle and right panels).

Visual inspection of the data revealed two prominent ATAC-seq peaks in the proximity of the *MIR150* gene (**Fig. 6C**, right panel): the distal one (D) was located upstream of *MIR150* and was likely to be involved in the regulation of the adjacent gene *RPS11*, but a role in the control of *MIR150* itself cannot be ruled out since the promoter and transcription start site of the full *pri-miR-150* gene remain undetermined. This peak showed modest, if any, changes during T cell activation and corresponded to a region bound by the transcription factor XBP1 in T lymphocytes (39).

The second, proximal peak (P) almost perfectly coincided with the miR-150 sequence (**Fig. 6C**, right panel), and while it was very prominent in both naïve and memory resting T lymphocytes, it quickly and almost completely disappeared in both cell subsets 24 h after stimulation and it was not regained at a later time point, suggesting a possible role in the direct control of *MIR150* expression.
In a clustering analysis including all ATAC-seq peaks that were significantly affected after 24 h or 72 h of activation in either naïve or memory T cells (n = 16,697 sites), the downregulated peak overlapping *MIR150* belonged to a large cluster (cluster 2, **Fig. 6D**) that included all peaks that lost accessibility after 1 day of stimulation in both naïve and memory T cells, hinting at a shared regulation associated with activation. Other clusters included peaks that were more gradually reduced over time (clusters 1-4), or those that were variably induced by activation (clusters 5-10) (**Fig. S8**). Several peaks, like those in cluster 9, were more strongly affected in memory compared to naïve T cells, pointing towards regulatory regions and genes likely to be more active in one of the subsets (**Fig. 6D**).

We subsequently focused on cluster 2, containing the ATAC-seq peak coinciding with *MIR150*. In order to identify transcription factor DNA binding motifs associated with the accessible regions in this cluster, we performed transcription factor motif enrichment analysis (40). To this aim, peaks in cluster 2 were compared to all accessible sites detected. The DNA binding motifs recognized by the RFX family of transcription factors were consistently the sites most over-represented in cluster 2 in both naïve and memory cells (**Fig. 7A** and **Table S7**).

Among the eight members of the RFX family, RFX4, 6 and 8 showed low-to-undetectable expression in T lymphocytes according to both the Human Protein Atlas (41) and the Database of Immune Cells (DICE) (35). RFX2 was also lowly expressed. We therefore assessed the expression of the remaining RFX family members in resting and activated naïve and memory T cells. We found that *RFX7* expression was diminished in naïve T cells upon activation but induced in memory cells (**Fig. S9A**), while expression of *RFX1* did not change significantly upon T cell activation, and *RFX2* expression increased over time (**Fig. 7B**, **Fig. S9B**), all patterns that were not consistent with the rapid downregulation of miR-150 expression in both naïve and memory T cells. Conversely, expression of both *RFX3* and *RFX5* strongly diminished upon activation (**Fig. 7B, Fig. S9B**), hinting at their potential involvement in the regulation of miR-150 expression.

To determine the functional impact of RFX3 and RFX5 on miR-150 expression we performed RNAi experiments in primary resting memory T cells. We found that downregulation of either *RFX3* or

RFX5, as determined by qRT-PCR, reduced the expression of *pri-miR-150* and mature miR-150 (**Fig. 7C**). In these experimental conditions the levels of miR-150 remain however overall very high, therefore no significant effect on *MYB* and *PDAP1* expression could be measured (**Fig. S9C**).

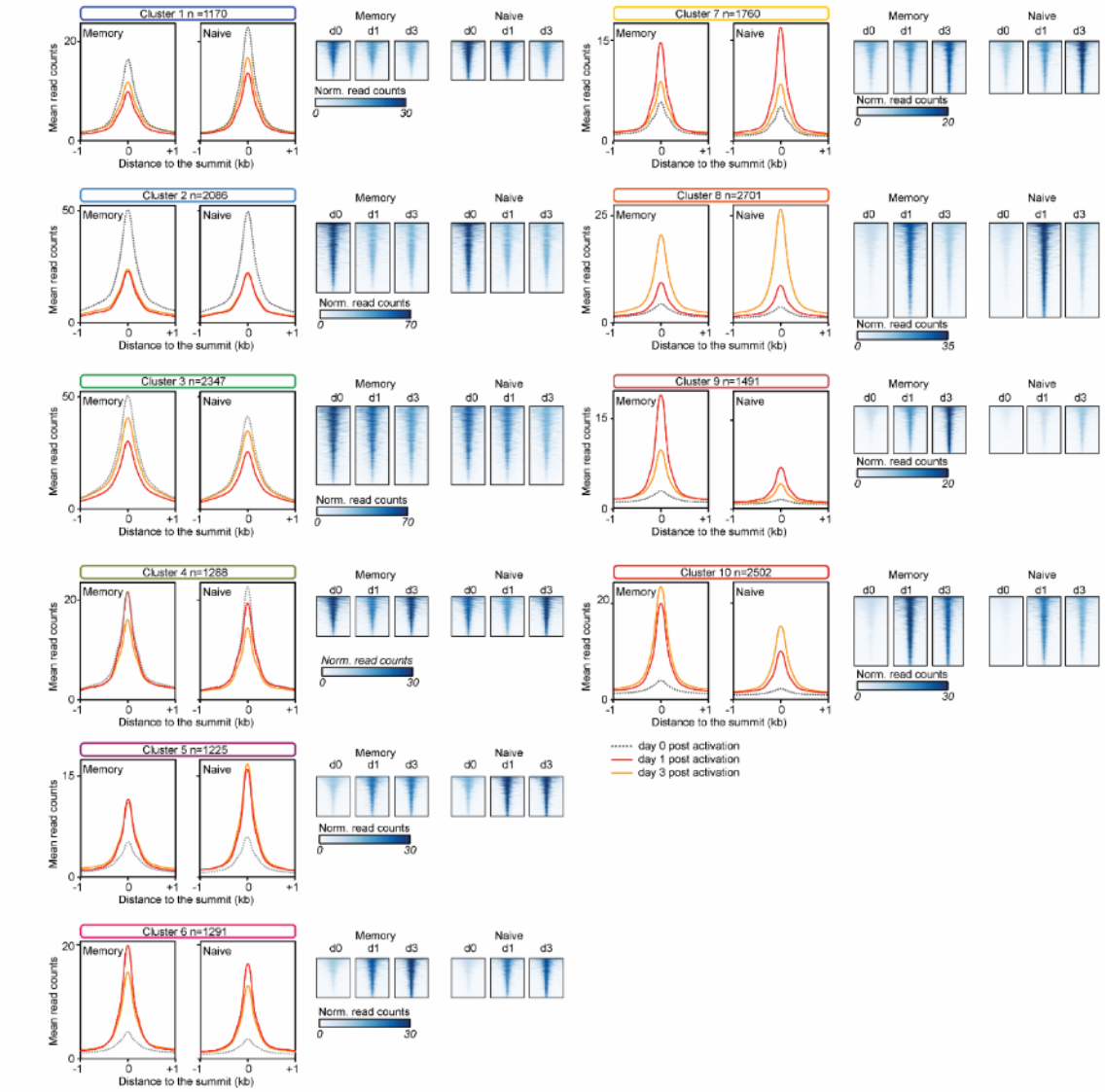


Figure S8. ATAC-seq analysis in primary T cells. Clustering analysis including all ATAC-seq peaks that were significantly affected after 24 h or 72 h of activation in either naïve or memory T cells.

Finally, overexpression of *RFX3* or *RFX5* in T lymphocytes before activation did not significantly affect miR-150 expression or T cell proliferation after five days of activation (**Fig. S10**). Therefore, RFX3 and 5 are both required to maintain basal miR-150 expression in resting T cells, but they are not sufficient to avoid the drastic reduction in miR-150 expression that occurs upon TCR activation.

Next, we explored whether such regulation was due to direct RFX factor binding to the *MIR150* locus. Browsing of the ChIP-Atlas (chip-atlas.org, (42)) identified an RFX5 ChIP-seq peak in B

lymphocytes upstream of the miR-150 sequence (GM12878 ENCODE, (43)), within the ATAC-seq peak identified in our dataset (**Fig. 7D**).

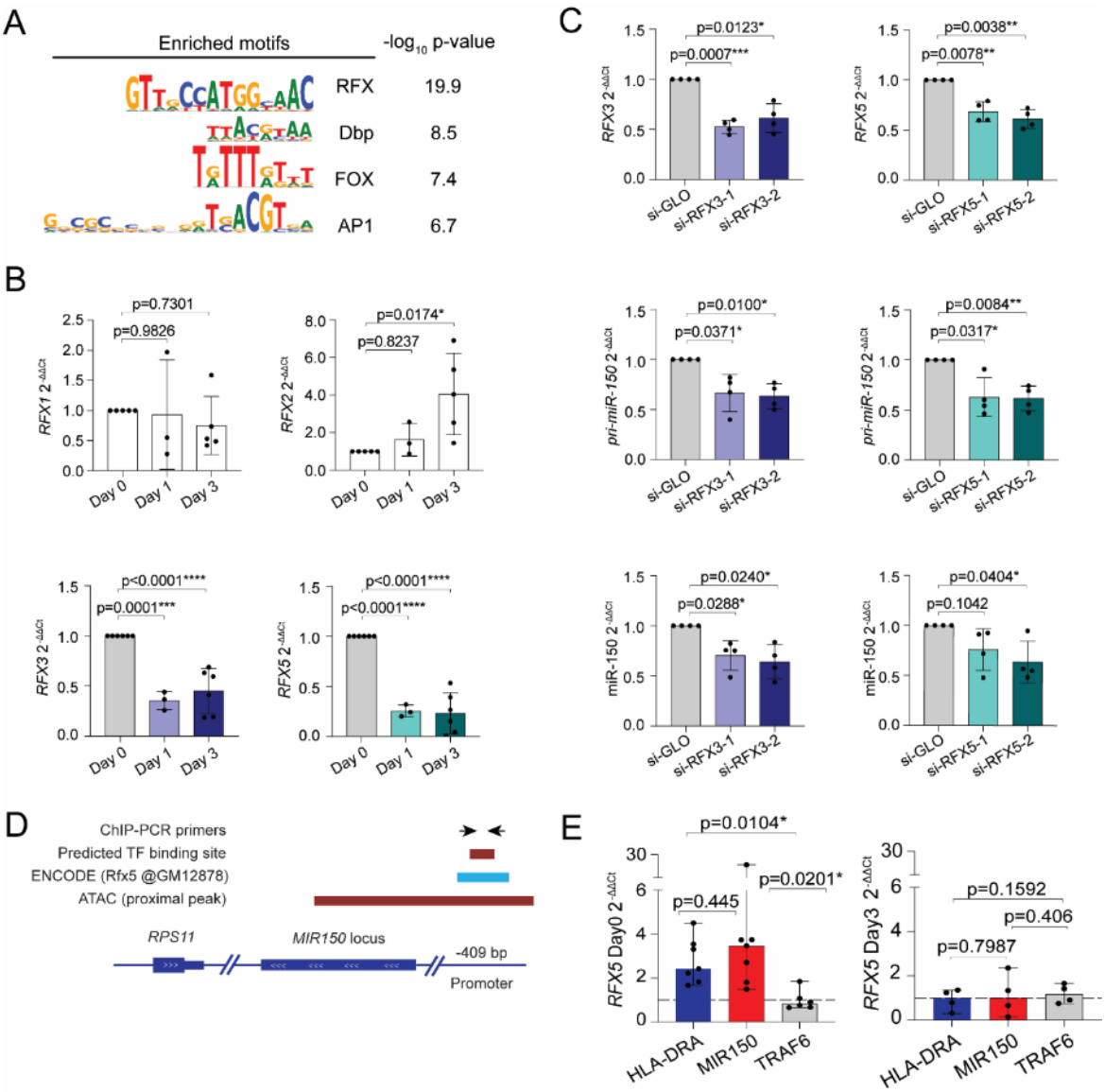


Figure 7. RFX factors regulate miR-150 expression. (A) Transcription factor motifs enrichment analysis. Peaks in cluster 2 were compared to all accessible sites detected. **(B)** Memory T lymphocytes were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for the indicated times. Total RNA was extracted and the expression of the different *RFX* mRNAs was measured by qRT-PCR. N=3-6 independent donors (each dot represents one donor). Mean ± SD. One-way ANOVA. **(C)** Resting memory T lymphocytes were transfected with siRNAs targeting either *RFX3* (left) or *RFX5* (right). 24 h after transfection, total RNA was extracted and the expression of the indicated genes measure by qRT-PCR. N=4 independent donors (each dot represents one donor). Mean ± SD. Paired *t*-test, two-tailed. **(D)** Schematic representation of the *MIR150* locus, with overlapping ATAC peak and the location of an RFX5 ChIP-seq peak in the human B lymphocyte cell line GM12878 as described by the ENCODE project (ChIP-Atlas). The location of PCR primers for ChIP analysis of RFX5 binding is also indicated. **(E)** RFX5 binding at the indicated genomic loci was determined by ChIP-qPCR in resting (day 0) and activated (day 3) memory T lymphocytes. Data were normalized on the input and a control immunoprecipitation with an irrelevant antibody for each target (dashed line y=1). Target genes exceeding the dashed line threshold were considered to be bound

by RFX5. Data are shown as median with 95% confidence interval; at least N=4 independent human donors. Each dot represents one experiment. Ratio paired *t*-test, two-tailed.

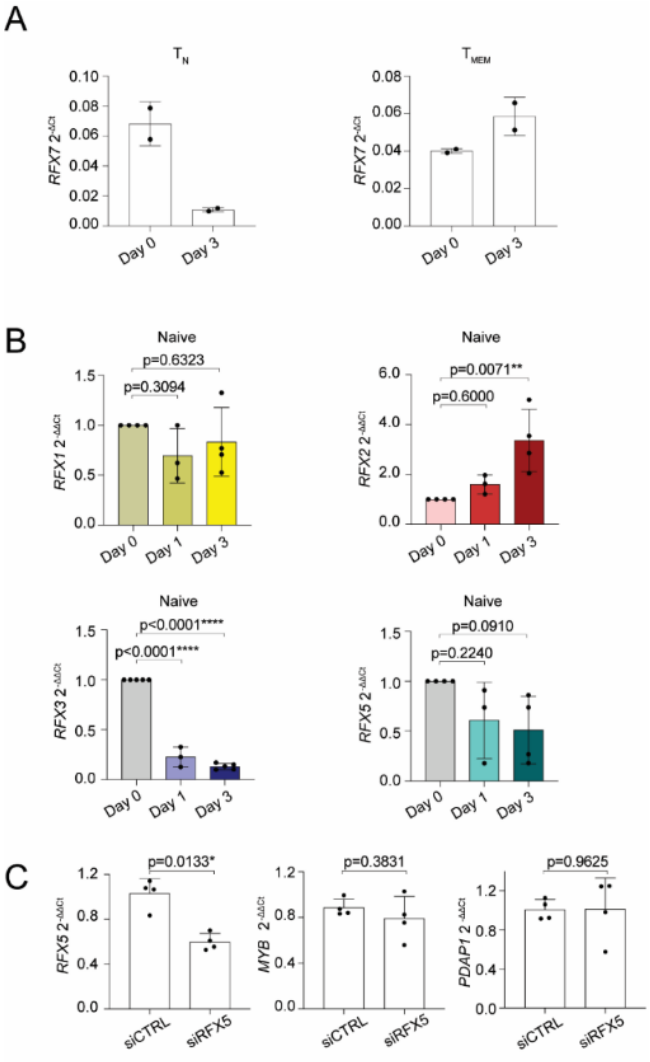


Figure S9. *RFX7* expression in human T lymphocytes. (A) Naïve and memory T lymphocytes were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for three days. Total RNA was extracted and the expression of *RFX7* was measured by RT-qPCR. N=2 independent donors (each dot represents one donor). Mean ± SD. This experimental setup, with two data-points (average of techical duplicates for each donor), precludes a statistical assessment of the differences observed. **(B)** Naïve T lymphocytes were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for the indicated times. Total RNA was extracted and the expression of the different *RFX* mRNAs was measured by RT-qPCR. N=3-5 independent donors (each dot represents one donor). Mean ± SD. One-way ANOVA. **(C)** Resting primary memory $CD4^+$ T lymphocytes were transfected with siRNAs against RFX5. Total RNA was extracted 24h after transfection, followed by RT-qPCR analyses of the indicated genes. Each dot represents one donor/ experiment. Mean ± SD. Paired *t*-test, two tailed.

To assess whether RFX5 directly bound the *MIR150* locus in T lymphocytes, we performed RFX5 ChIP-qPCR in primary human CD4$^+$ T lymphocytes either resting or activated with anti-CD3 and anti-CD28 antibodies for three days. As a positive control for RFX5 binding, we used a region of the *HLA-DRA* gene containing an RFX5 binding site (44). We found that in resting, but not in activated T cells, the region containing the *MIR150* locus was enriched in the RFX5-bound DNA fraction, while no enrichment was detected for the non-target gene *TRAF6* (**Fig. 7E**). Thus, RFX5 regulated basal miR-150 expression in resting T cells by binding directly to the *MIR150* locus.

Overall, we identified a regulatory network required to restrain lymphocyte proliferation, composed by an RFX-miR-150 axis required to limit the activity of factors important for T cell proliferation, most notably the RBP PDAP1.

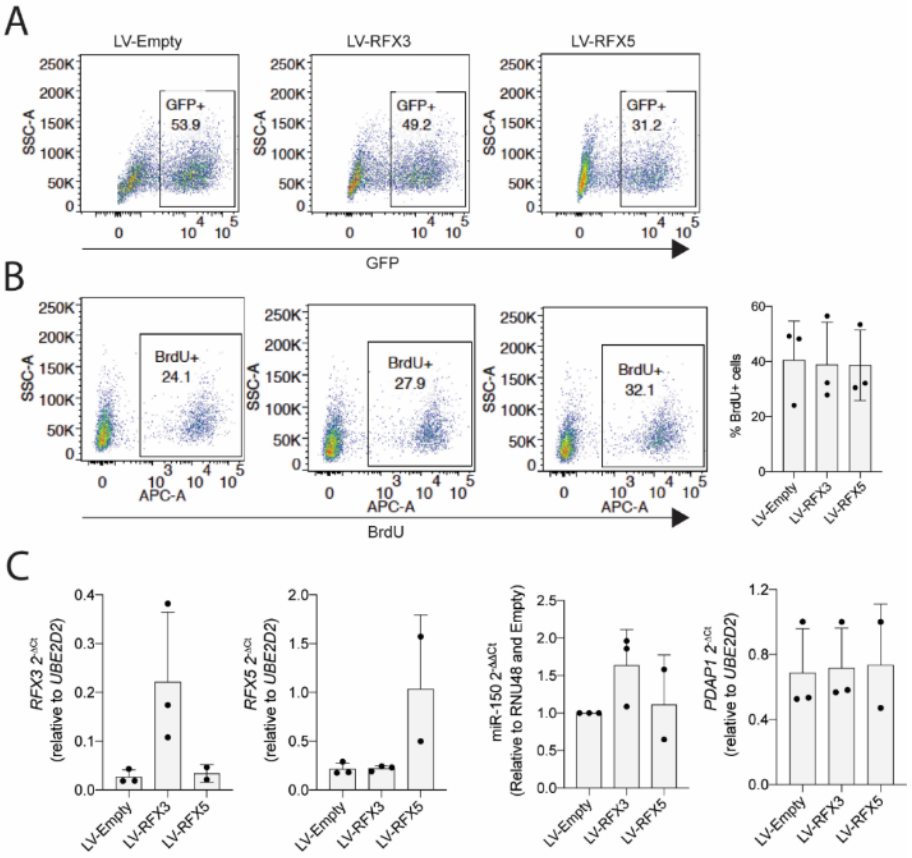


Figure S10. Overexpression of RFX factors in activated cells is not sufficient to influence miR-150 expression and T cell proliferation. (**A**) Resting memory T lymphocytes were transduced with the indicated plasmid (containing an IRES-GFP), followed by activation with plate-bound anti-CD3 and anti-CD28. Representative example of the efficiency of cell transduction. (**B**) GFP$^+$ cells were sorted after 5 days and proliferation was measured by BrdU incorporation assay. The results of N=3 independent experiment is shown on the right. Each dot represents one donor. Mean ± SD. (**C**) A portion of the cells was used to measure the indicated transcripts by RT-qPCR. Each dot represents one donor. Mean ± SD.

5.5 Discussion

In this work, we identified RFX family members as factors involved in maintaining basal miR-150 expression in resting cells, which in turn restrained proliferation by targeting *MYB* and *PDAP1*. The role of miR-150 in the control of cell proliferation is suggested by several pieces of evidence. First, it is significantly downregulated in different types of T cell lymphoma, including peripheral T cell lymphomas (PTCL) and advanced cutaneous T cell lymphoma (CTCL) (45, 46). Second, reduced miR-150 expression was associated with invasion and metastasis in mouse models, suggesting that in these cells miR-150 acts as a crucial tumor suppressor.

While our analyses identified MYB and PDAP1 as direct miR-150 targets in activated human T lymphocytes, a few other targets previously identified in $CD4^+$ T cells were not detected in our study. For instance, miR-150 was shown to cooperate with miR-99 to repress mTOR expression and to promote Treg differentiation in the mouse. Interestingly, miR-150 could only exert its repressing activity on this target in the presence of miR-99, pointing towards cooperativity between different miRNA binding sites (47). Other reported miR-150 targets included *AKT3* (48) and *SLC2A1* (GLUT1), which was targeted by miR-150 in regulatory Th1 cells stimulated with anti-CD3 and anti-CD46 antibodies (49). The fact that these genes were not identified in our experiments might be linked to the different T cell subsets under consideration, or to the conditions of T cell culture and stimulation. Moreover, for most other reported targets the strength of a direct *in vivo* association with miR-150 was not assessed. In this respect, the pull-down approach that we used to identify T cell-specific targets may also be limited by the necessity to balance stringency (to reduce false positives) and sensitivity, which might lead to the predominant enrichment of abundant targets that are most strongly regulated by miR-150 (17, 19, 50).

Apart from *MYB*, we identified *PDAP1* as a direct miR-150 target implicated in regulating T cell proliferation. PDAP1 (also known as PAP, HAP28) is a 28 kDa phosphoprotein that was originally identified as a modulator of mitosis in association with PDGFA and PDGFB in rat neural retina cells (51). Browsing of the Human Protein Atlas (41) revealed the broad tissue expression of PDAP1 and mainly cytoplasmic and plasma membrane-associated expression. However, very little is known about the physiological functions of this protein, although a genetic association with Mendelian diseases of the nervous system was identified (52). In T lymphocytes, RBPs such as the Roquin, Regnases and the TTP family of proteins have an important role in post-transcriptional gene regulation, being key actors in modulating T cell activation and functions, for instance through the

regulation of cytokine mRNA expression and stability (36, 53). These proteins often contain defined RNA-binding domains able to recognize specific features on the transcripts, such as AU-rich elements and stem-loop structures (53). Although our own data and data from RNA-interactome studies clearly revealed that PDAP1 can act as an RBP, it contains no recognizable RNA-binding domains (D2P2 Database of Disordered Protein Predictions) (54), an observation compatible with the intrinsically disordered regions often observed in an abundant and understudied class of noncanonical RBPs (29, 30, 32, 55). Interestingly, although the *PDAP1* mRNA contains a coding sequence that measures only 546 nucleotides in length, its 3'UTR is instead much larger (more than 2 kb), pointing towards a highly regulated expression for this protein mediated both by miRNAs as shown in this study, and potentially also through extensive cross-regulation with other RBPs, as shown for many other instances of RBP regulation (53). In murine B lymphocytes, PDAP1 was shown to protect mature B cells from stress and to favor antibody diversification, although no clear mechanism of action could emerge, most likely due to the high number of genes that were affected both positively and negatively in the absence of PDAP1 (56). Such large changes in the transcriptome of cells lacking PDAP1 are likely to be the result of a complex pattern of direct and indirect effects. What is the exact mechanism of action of PDAP1 and to what extent its RNA-binding capacity is relevant to its functions remains to be understood and it will be the subject of future studies. At this stage, we also have no evidence for any direct relationship between MYB and PDAP1, apart from both of these factors being targeted by miR-150 and being involved in regulating T cell proliferation.

One other intriguing observation of our study is the very rapid reduction of miR-150 levels after T cell activation. Other miRNAs, like let-7, were also reported to be downregulated upon T cell activation, at least in murine $CD8^+$ T cells (57). While we found that a large component of miR-150 downregulation was transcriptional, some post-transcriptional mechanisms may also be at play, and would probably contribute to explain the observed reduction of miR-150 expression even in the absence of cell division. For instance, miR-150 was shown to be degraded by the inositol-requiring enzyme 1α (IRE1α), which possesses endoribonuclease activity towards cellular mRNAs, that was shown to directly cleave selected miRNAs, including miR-17a and miR-150 (58, 59). Finally, miR-150 is highly abundant in extracellular vesicles derived from activated primary human T lymphocytes (60, 61), a process that may also be important to achieve the rapid elimination from the cytoplasm of negative regulators of lymphocyte activation (60, 62). Apart from the abrupt loss of transcription described in our study, these post-transcriptional mechanisms may also variably contribute to the reduction of mature miR-150 in the cytoplasm, thereby allowing full-blown T cell activation.

Acknowledgments

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Authors Contributions

M.C., N.B., E.D., E.F. and A.K. performed experiments and analyzed data. F.G. and G.N. analyzed data. S.M. overviewed the project, analyzed data and wrote the manuscript with input from all authors.

Declaration of Interests

The authors declare no competing interests.

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6 Manuscript [2]

Cell-intrinsic mechanisms to restrain inflammatory responses in T lymphocytes

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Unrestrained Inflammatory responses by T lymphocytes are at the base of many immunological pathologies, however, an overly restrained activation promotes the development of tumors. In this manuscript, we reviewed the current opinions on how T lymphocytes balance the extent of the immune response. In the first part, we discussed the role of the most prominent transcription factors in establishing T cell phenotypes. In the second part, we described the mechanisms of selected RBPs in adjusting the immune reaction in cooperation with miRNAs, and in the last part we discussed the roles of these regulatory checkpoints in the development of autoimmunity and antitumoral responses.

I actively contributed to this work by writing the manuscript in collaboration with the other authors and by generating Figure 3 and Table 1.

6.1 Abstract

Activation of T lymphocytes induces robust immune responses that in most cases lead to the complete eradication of invading pathogens or tumor cells. At the same time, however, such responses must be both highly controlled in magnitude and limited in time to avoid unnecessary damage. To achieve such sophisticated level of control, T lymphocytes have at their disposal an array of transcriptional and post-transcriptional regulatory mechanisms that ensure the acquisition of a phenotype that is tailored to the incoming stimulus while restraining unwarranted activation, eventually leading to the resolution of the inflammatory response. Here, we will discuss some of these cell-intrinsic mechanisms that control T cell responses and involve transcription factors, microRNAs, and RNA-binding proteins. We will also explore how the same mechanisms can be involved both in anti-tumor responses and in autoimmunity. A mechanistic understanding of the regulatory circuits that control the effector responses of memory T helper lymphocytes, and in particular their ability to produce pro-inflammatory cytokines, may lead to effective therapeutic interventions in all immune-related diseases.

6.2 Introduction

Initially thought to be a passive process, the resolution of inflammatory responses to an invading pathogen or noxious agent is now understood to be actively controlled, involving the upregulation of pathways with anti-inflammatory and reparatory functions, including anti-inflammatory cytokines, microRNAs (miRNAs) and inhibitory molecules and enzymes. Defects in these regulatory pathways can lead to sterile inflammatory processes and autoimmunity. Despite their importance in maintaining immune homeostasis, the mechanisms that restrain or dampen pro-inflammatory responses in T lymphocytes are not completely understood, and the therapeutic stimulation of resolution of inflammation may become attractive in the treatment of chronic inflammatory disorders such as multiple sclerosis and other autoimmune diseases. A number of cell-intrinsic factors can modulate T cell responses to an antigen, both at a transcriptional level through epigenetic mechanisms and transcription factors, and at a post-transcriptional level, for example through the action of miRNAs and RNA-binding proteins (RBPs) that collectively regulate mRNA stability and translation (**Fig. 1**).

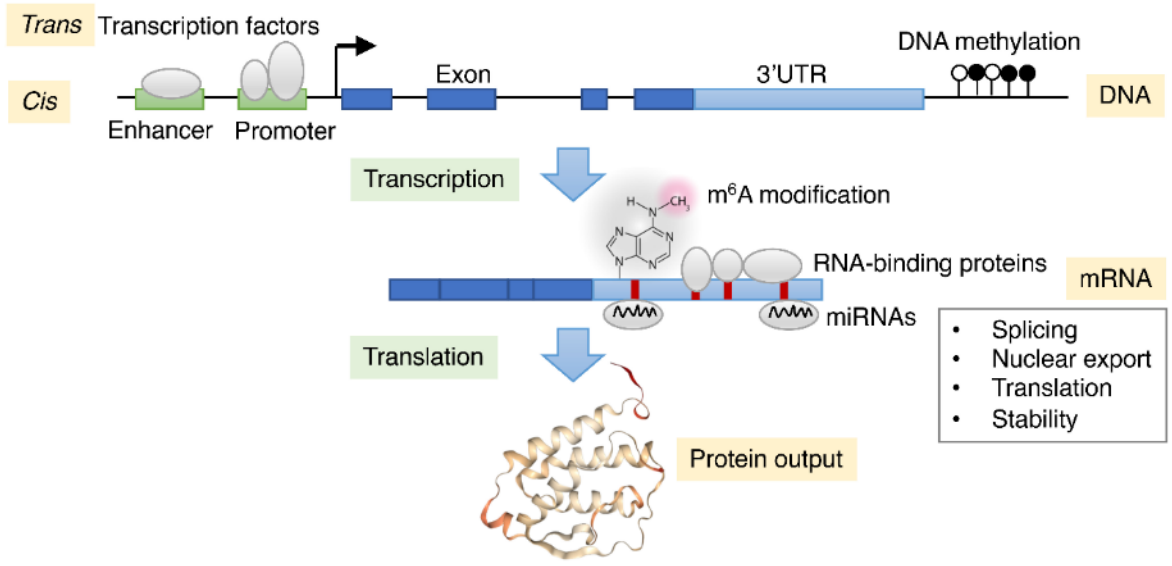

Figure 1. An integrated regulatory network in the transcriptional and post-transcriptional control of gene expression. Gene expression is a complex process with many regulatory layers. Transcription is controlled by *trans*-acting elements (e.g. transcription factors) binding to *cis* regulatory regions on the DNA (e.g. promoters and enhancers), but also by DNA methylation, histone modifications (not depicted) and in general all the mechanisms that influence chromatin topology and DNA accessibility. Once an mRNA transcript is generated, its maturation, intracellular localization, stability and translation are regulated by an array of RBPs, possibly acting in a cooperative or antagonistic, redundant or unique manner, and potentially also with modes of action that are defined in time and space by environmental triggers. All of these mechanisms (and more that are not depicted for simplicity), contribute to modulate the final output on protein synthesis. Arrow: transcriptional start site; open and closed circles, unmethylated and methylated cytosines in DNA; red vertical lines: RBP binding regions; the protein shown is human GM-CSF (image from the RCSB PDB (rcsb.org), PDB ID 2GMF (158)).

An integrated network in which multiple layers of regulation cooperate in modulating the rapidity, intensity and length of an immune reaction is particularly important to achieve responses that are swift (for instance by maintaining 'pre-stored' mRNAs in a repressed state until they are needed), and at the same time sufficiently dynamic that they can be turned off before the onset of excessive damage.

Here, we will discuss how some of these mechanisms may intrinsically regulate the pro-inflammatory phenotype of T lymphocytes. We will focus primarily on regulatory networks that may influence the phenotype of inflammatory memory $CD4^+$ T helper (Th) lymphocytes, while for more in-depth discussions on the regulation of T regulatory (Treg) cells and the initial naïve-to-memory cell transition and differentiation, we refer the reader to some outstanding reviews on the topic (1-3).

6.3 Transcription factors

T helper lymphocytes have a central role in the immune system thanks to their main effector function of secreting cytokines that affect the activity of other innate and adaptive immune cells. Upon activation, T lymphocytes undergo widespread changes in gene expression, allowing them to differentiate into effector phenotypes able to elicit efficient responses against invading pathogens. Many transcriptional regulators contribute to the induction and maintenance of a broad range of T cell phenotypes, from highly pro-inflammatory and potentially tissue damaging to regulatory or exhausted, the latter being associated with reduced effector functions (**Fig. 2**). Among the transcription factors that are clearly associated with a pro-inflammatory phenotype, T-BET and RORγt are primarily expressed by the IFN-γ-producing and IL-17-producing subsets of T lymphocytes (Th1 and Th17 cells, respectively), contributing to their cellular identity and acting as master regulators of cytokine production. For instance, T-BET induces directly the expression of IFN-γ in Th1 cells, but it also limits the acquisition of the alternative IL-4-producing Th2 phenotype by sequestering GATA3 and cooperating with Runx-3 to silence IL-4 production (4-6). RORγt expression instead characterizes the highly pro-inflammatory Th17 and Th1* subsets, the latter being defined by a dual Th1/17 phenotype (7-11). Concordant with their role in the regulation of inflammatory cells, these transcription factors are also involved in autoimmunity (12, 13). Among the factors that are now emerging as consistently associated with a high cytokine-producing and pro-inflammatory T cell signature is also BHLHE40, a transcription factor that appears to regulate gene expression acting primarily as a repressor of transcription (14-18). In both mouse and human T cells, expression of this transcription factor was associated with a pathogenic signature (14, 19), and its deletion led to

reduced expression of several pro-inflammatory cytokines, most notably GM-CSF (14, 19-24). In human T cells, it was highly expressed by inflammatory Th1* cells (14), and *Bhlhe40-/-* mice were protected from experimental autoimmune encephalomyelitis (EAE) (19, 21, 22), pointing towards a crucial pro-inflammatory role for this transcription factor. Emphasizing the complex interplay between regulatory factors that can either favor or limit the acquisition of a pro-inflammatory phenotype by T lymphocytes, we found that BHLHE40 could bind to the promoter region of the *ZC3H12D* gene, encoding for Regnase-4, a negative regulator of cytokine mRNA stability and translation. BHLHE40-mediated regulation reduced *ZC3H12D* expression, ultimately leading to increased cytokine production (14).

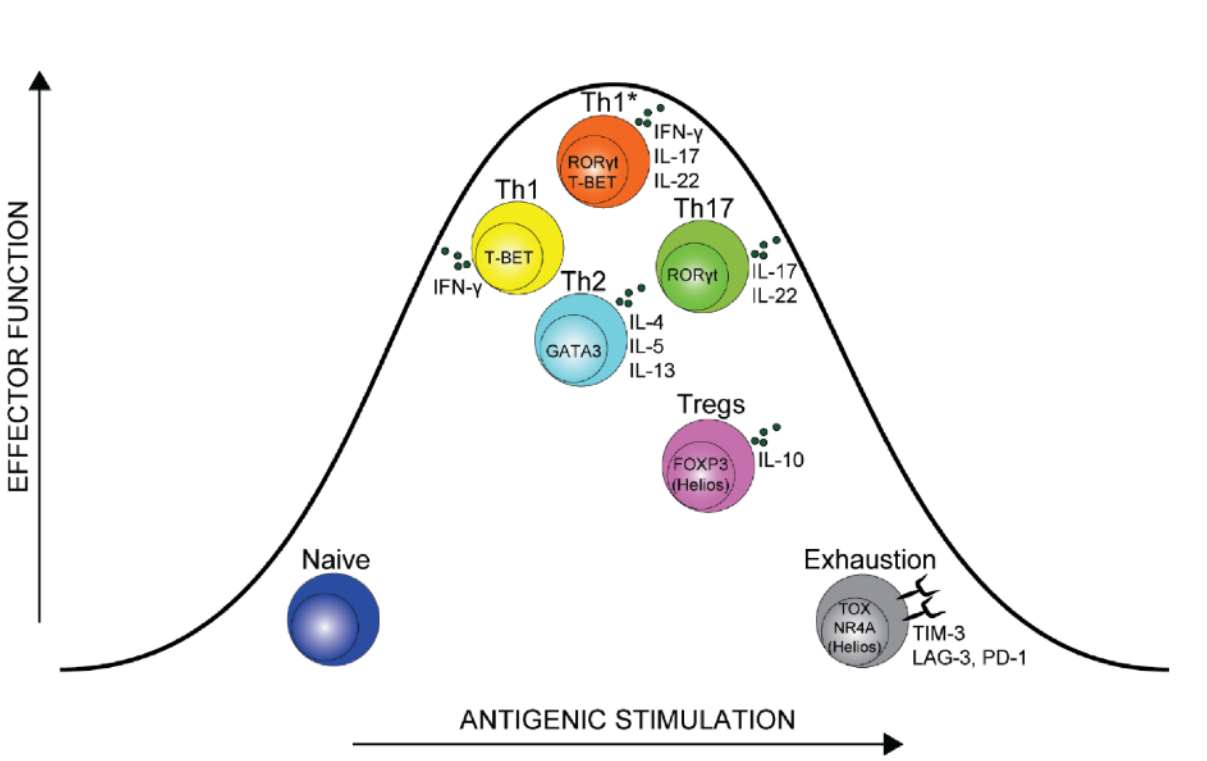

Figure 2. T helper lymphocyte differentiation upon antigenic stimulation. Following TCR engagement, T lymphocytes differentiate toward effector subsets characterized by specific functions, determined by a finely tuned transcriptional profile. Specifically, Th1, Th17 and Th1* T cell subsets display a more inflammatory profile, characterized by the expression, among others, of the transcription factors T-BET and RORγt and the cytokines IFN-γ, IL-17 and IL-22. Similarly, Th2 cells are characterized by GATA3 and IL-4 expression, while the hallmark of Treg cells is the expression of the transcription factor FOXP3. However, chronic TCR stimulation may lead to the loss of functional effector responses and to the induction of inhibitory surface receptors such as PD-1, LAG-3 and TIM-3. The transcription factors TOX and NR4A recently emerged as key factors associated with the exhausted phenotype, while Helios expression was associated to a subset of Treg cells and chronically activated $CD4^+$, but not $CD8^+$ T cells.

While these and other factors have been consistently associated with an inflammatory phenotype, other transcription regulators are primarily linked to a regulatory or exhausted phenotype and

contribute to restraining T cell responses. In this regard, FOXP3 represents a prototypical example. This transcription factor orchestrates the genetic program regulating the differentiation and maintenance of Treg cells, and its deficiency in both mouse and human results in severe, often fatal multiorgan autoimmunity (25). However, human (but not mouse) conventional T cells also transiently upregulate FOXP3 expression upon activation (26). Although such transient FOXP3 expression is not sufficient to induce a full Treg differentiation program, for which stable and robust expression is required (27), this observation suggests that at least in human cells, limited FOXP3 expression may represent a contributing factor in reducing the activity of effector T lymphocytes in a cell-intrinsic manner. Expression of the transcription factor Helios (encoded by the *IKZF2* gene) also characterizes a subset of Treg cells, and at least in humans, loss of *IKZF2* expression resulted in impaired Treg functions (28, 29). Whether these factors also effectively contribute to the termination of the response in conventional T lymphocytes remains to be fully understood.

Other transcription factors, such as TOX, are instead primarily associated with T cell exhaustion, defined as a state of reduced T cell responsiveness in both $CD4^+$ and $CD8^+$ T lymphocytes (30-34). Following chronic antigenic stimulation, TOX promoted the acquisition of a dysfunctional phenotype by inducing widespread epigenetic and gene-expression changes in $CD8^+$ T cell (30-34). TOX expression was induced by activation of NFAT (30, 31, 33), a transcription factor with a crucial role in the regulation of T cell effector functions and in the induction of an anergic state (35). Importantly, NFAT activation also led to the induction of the NR4A transcription factor, a member of a family of nuclear receptors which is also critical for T cell exhaustion (33). Indeed, chimeric antigen receptor (CAR) T cells lacking all three members of the NR4A family (NR4A1-3) showed highly efficient tumor killing capacity and led to prolonged mouse survival compared to wild-type CAR T cells (36). While these results clearly identified TOX as a regulator of a dysfunctional program in T lymphocytes, its expression may not be exclusively associated with exhaustion. For example, circulating TOX^+ memory $CD8^+$ T cells were recently detected in humans. These cells targeted persistent viruses, while retaining the ability to produce high levels of effector molecules, such as perforin and granzyme B (37), suggesting that some aspects of the regulation of the dysfunctional state may somewhat diverge in T cells from human and mouse origins. Further regulatory differences may also occur between $CD8^+$ and $CD4^+$ T lymphocytes. For instance, transcriptional profiling of $CD4^+$ and $CD8^+$ T cells during chronic viral infections identified both shared and unique factors potentially involved in regulating the hypofunctional state of these cells following chronic stimulation (38). Among these, expression of *Ikzf2* was specifically associated with exhausted conventional $CD4^+$, but not $CD8^+$ T

cells, pointing towards some unique transcriptional aspects defining the dysfunctional state in these two cell populations (38).

Overall, a number of transcription factors and networks are emerging as key regulators of the phenotype of inflammatory T cells. Although some of these transcription factors are primarily associated to a particular T cell phenotype or state (for instance, Treg or exhausted cells), they might also more generally contribute to either restraining or favoring responses of conventional effector and memory cells. Adding to the possible combinatorial complexity in transcriptional regulation, many key factors, including for example FOXP3, form multi-protein complexes by interacting with a variety of binding partners. Depending on the recruited interacting partner, each factor can therefore influence transcription positively or negatively, potentially leading to very different transcriptional landscapes and cellular states (39).

6.4 RNA-binding proteins

Throughout the process of T cell activation and differentiation, tight control of cytokine production is necessary to orchestrate appropriate responses to incoming signals while avoiding excessive damage to healthy tissues. The expression of cytokines and other immune-relevant genes is therefore controlled not only at a transcriptional, but also at a post-transcriptional level, through the action of RBPs that modulate the stability and translation of target mRNAs (3, 40-42). For instance, the 3'-untranslated regions (3'UTRs) of many cytokine mRNAs contain multiple regulatory *cis*-acting elements, including stem-loop structures and adenine and uridine (AU)-rich elements (AREs) that influence mRNA stability and translation (43, 44). These *cis* elements are recognized in *trans* by RBPs that can positively or negatively affect mRNA decay and protein synthesis. Other factors that may strongly influence the stability and functionality of a given mRNA include miRNAs, which exert their functions in the context of miRNA-containing RNA silencing complexes (45), as well as the YTH domain-containing family of proteins YTHDF1-3, which recognize the N^6-methyladenosine (m^6A) modification in target transcripts (46). Therefore, although each RBP can individually affect mRNA translation or decay, mRNAs may contain binding sites for a multitude of factors (ARE and stem-loop binding proteins, miRNAs, m^6A-binding proteins), all potentially binding in a cooperative or competitive manner, and contributing to the final outcome on mRNA half-life and protein output. For instance, the cytokine transcripts *CSF1* (encoding G-CSF), *IL2* and *IL6* were shown to contain both ARE and stem-loop elements (47). Further increasing the complexity of studying the impact of post-transcriptional mechanisms on regulation of gene expression, each RBP-encoding mRNA can be subjected to cross-regulation by other RBPs, as illustrated by the fact that Regnase-1 is capable of

repressing Roquin expression by targeting its 3'UTR (48). Because of these complexities and the number of possible regulatory combinations, here we will focus on specific examples of RBPs that have been shown to impact primarily the pro-inflammatory phenotype of T helper lymphocytes, including tristetraprolin (TTP), Roquin and Regnase family members (**Table 1**).

Table 1. Comparison of selected RBPs with a regulatory role in T lymphocytes.

	Anti-inflammatory RBPs						**Pro-inflammatory RBPs**		
	Regnase-1	**Regnase-2**	**Regnase-3**	**Regnase-4**	**Roquin-1/2**	**TTP**	**HuR**		**ARID5A**
Encoding gene	*ZC3H12A*	*ZC3H12B*	*ZC3H12C*	*ZC3H12D*	*RC3H1/2*	*ZFP36*	*ELAVL1*		*ARID5A*
Mechanism of action	RNase activity Catalyzed by four aspartic acid residues (77, 120) Deubiquitinase activity catalyzed by at least one cysteine residue (76)	Probable RNase, aspartic acid residues conserved (121) Deubiquitinase activity unknown, cysteine residue conserved	Unknown, aspartic acid and cysteine residues conserved	Probable RNase, aspartic acid residues conserved (122, 123) Deubiquitinase activity unknown, cysteine residue conserved	Recruitment of CCR4-NOT and EDC4 for deadenylation and decapping (73, 124) Ubiquitination of targets (125, 126)	Recruitment of CCR4-NOT and DCP1/2 for deadeny-lation and decapping, recruitment of exosome and RISC (55, 56, 127-129)	Promotes translation by binding to 5'UTR mRNA (130) Blocks binding of the silencing complex RISC (131) Recruits translational inhibitor TIA-1 (132)		Competes with Regnase-1 and Roquin-1/2 for *cis*-element (133)
Cis-acting element	Stem loop (74)	Stem loop (121)	Not described	Stem loop (123)	Stem loop (73, 134)	ARE (135)	ARE (136)		Stem loop (137)
Targeted mRNAs	*IL1B, IL12B, IL2, IL6, ICOS, INOS, TNF, TNFRSF4 (OX40), CTLA4, CREL, CCL2, NFKBID, NFKBIZ, IRF4, TFRC, PTGS2 (COX2), ZC3H12A* (74, 75, 120, 138)	*IL6, IER3, ZC3H12A* (121)	*VCAM1, ICAM1, LECAM2, IL-8, CCL2, ZC3H12A* (79, 121)	*CFOS, IL1B, IL10, IL17A, IL2, IL2, IL6, IER3, INOS, NFKBIZ, TNF, ZC3H12A* (75, 80, 122, 123, 139)	*ICOS, NKFBIZ, NKFBID, TNF, TNFRSF4, PTGS2* (67, 73, 134)	*IL1B, L2, IL3, L6, IL10, IL27, TNF, TTP, IL17A, CSF2, CXCL1, IFNG, PTGS2, ZPF36* (67, 140, 141)	*CDKN1B, IGF1R, THBD, WNT5A, MYC* (142)	*CFOS, IL6, ELAVL1, CSF2, TGFB, TNF, TLR4, PTGS2* (143-148)	*IL6, TBX21, TNFRSF4, STAT3, CXCL1, CXCL5, NFKBIZ* (112, 149)

	Regnase-1	**Regnase-2**	**Regnase-3**	**Regnase-4**	**Roquin-1/2**	**TTP**	**HuR**	***ARID5A***
Regulation	Cleaved by MALT1 paracaspase at site of one arginine residue (69, 75) Proteasomal degradation upon phosphorylation of DSGXXS motif (150)	Unknown; arginine residue conserved, DSGXXS motif not conserved	Proteasome degradation, but mechanism unknown, DSGXXS motif not conserved, arginine residue conserved (79)	Unknown; arginine residue conserved, DSGXXS motif not conserved	Cleaved by MALT1 paracaspase at site of one arginine residue (69)	Proteasomal degradation upon interaction with pyruvate kinase M2 (151, 152)	Cleaved by caspase-3/8 (153) Proteasomal degradation mediated by E3-ubiquitin-ligase TRIM13 (154)	Proteasomal degradation upon phosphor-rylation by MAPK (155)
Knockout mouse phenotype	Systemic autoimmunity lethal within 12 weeks after birth (74, 75)	Unknown	Macrophage-dependent sublethal lympha-denopathy (79)	No effect under steady state; severe paralysis with induced EAE (75)	Perinatal lethality (68)	Systemic inflam-mation, TNFα-mediated cachexia dermatitis arthritis (57)	Embryonic lethality; atrophy of bone marrow, thymus (156, 157)	Resistant to EAE induction (133)

In T cells, the importance of post-transcriptional regulation of cytokine mRNAs is highlighted by the recognition that CD28-mediated co-stimulation led to the stabilization of a number of transcripts, including *IL2*, *IFNG*, *TNF* and *CSF2* (encoding GM-CSF) (49), thereby ensuring that T lymphocytes produced cytokines shortly after activation, but only for a limited time. Interestingly, some of these regulatory mechanisms are cytokine-specific, and guarantee that individual cytokines are expressed at the appropriate time after activation. For instance, in murine effector and memory $CD8^+$ T cells, the immediate production of TNF-α was shown to be intense but transient, and primarily linked to the translation of pre-synthesized mRNAs, while IL-2 expression was dependent on *de novo* transcription upon antigen stimulation (50). Production of IFN-γ instead required both the initial translation of pre-formed mRNAs and *de novo* transcription, highlighting the importance of appropriate expression kinetics for each individual cytokine. IFN-γ expression was regulated post-transcriptionally via AREs present in the 3'UTR of the *Ifng* mRNA, and germline deletion of these elements led to uncontrolled, chronic cytokine production and tissue pathology with an autoimmune phenotype (51).

AREs are often found in short-lived mRNAs and usually confer rapid decay in a process known as ARE-mediated decay. There are several different domains, found in a variety of proteins, that were shown to bind AREs, although some of them may also recognize stem-loops (43). TTP (encoded by the *Zfp36* gene) represents one well-studied example of a destabilizing protein that recognizes ARE-containing inflammatory transcripts, such as *TNF*, *IL6*, *CSF2*, *IFNG*, *IL2*, but also the anti-inflammatory cytokine mRNA *IL10*, suggesting that RBPs contribute to modulating both the amplitude and duration of the initial inflammatory response, and also its decline and resolution (43, 52). TTP binds directly to target mRNAs (53), and induces their destabilization through deadenylation-dependent decay (54), by recruiting the CCR4-CAF1-NOT deadenylase complex and the exosome for RNA degradation (55, 56). Mice lacking TTP developed a severe and complex autoimmune syndrome (57), while stabilization of TTP expression protected mice against severe forms of inflammatory pathologies such as collagen-induced arthritis, imiquimod-induced dermatitis and EAE (58). To achieve such protection from a number of inflammatory diseases, it was sufficient to remove an ARE-containing region in the *Zfp36* mRNA that is responsible for TTP binding and destabilization of its own transcript. These results underscore the importance of tight post-transcriptional control in modulating immune responses.

Another RBP with key functions in immune cells is human antigen R (HuR, encoded by the *ELAVL1* gene). HuR also has preference for ARE sequences (59, 60), and its predominant role in the regulation of gene expression is linked to mRNA stabilization (61), although the final outcome on the phenotype in *in vivo* settings may be more complex (62, 63). Both TTP and HuR contribute to coordinated regulation of mRNA stability, as shown for example for *TNF*. Specifically, stimulation of macrophages by lipopolysaccharide induced phosphorylation of TTP, reducing its affinity for ARE sites in the *TNF* 3'UTR. This in turn allowed HuR binding and transcript stabilization, leading to increased translation and protein synthesis (64). Interestingly, since the *Zfp36* mRNA (encoding TTP) is under the same feedback control mediated by TTP itself, this mechanism may create a window of opportunity for HuR-mediated stabilization and translation of *TNF*, which would however be limited in time due, at least in part, to the concomitant stabilization of *Zfp36* (64).

Stem-loop-binding proteins strongly involved in the regulation of T cell functions and inflammation include the Roquin family members Roquin-1 and -2 (encoded by the *Rc3h1* and *Rc3h2* genes) (65), and the Regnase family members Regnase 1-4 (encoded by the *Zc3h12a-d* genes) (66). Roquin-1 and -2 are E3 ubiquitin ligase enzymes essential during development, as shown by the early postnatal lethality of both *Rc3h1* and *Rc3h2* deletions (67, 68). In T lymphocytes, the combined deletion of

both enzymes led to hyperactivation of both $CD4^+$ and $CD8^+$ T cells, with development of splenomegaly and lymphadenopathy, pointing towards compensatory functions for these RBPs that are key to restrain effector cell functions (67, 69). Roquin ablation led to the aberrant differentiation of Th17 and T follicular helper cells, and also affected the phenotype and functionality of Treg cells, which became defective in their ability to suppress the activation of conventional T lymphocytes (70). The RNA-binding ROQ domain of Roquin proteins recognizes stem-loop structures (called constitutive decay elements or CDEs) characterized by a short stem and a 3-nucleotides loop (71-73). Similar to ARE-mediated mRNA decay, Roquin interacts with the CCR4-CAF1-NOT deadenylase complex, leading to mRNA decay upon deadenylation and decapping (73).

Regnase-1 was first shown to be involved in the direct destabilization and degradation of the *Il6* and *Il12* mRNAs (74). Such destabilization was dependent on the presence of a conserved region in the target 3'UTRs, and on the RNase, endoribonuclease activity of the Regnase-1 enzyme (74). Regnase-1 acts therefore as a strong negative regulator of inflammation. In its absence, mice developed a severe autoimmune pathology and died within 12 weeks after birth with severe splenomegaly, lymphadenopathy and hyperimmunoglobulinemia (74). Underlining the key role of Regnase-1 expression in T cells, deletion of this enzyme only in the $CD4^+$ T cell compartment was sufficient to induce an autoimmune phenotype similar to full *Zc3h12a*-deleted mice (75). Perhaps unsurprisingly, the expression of this potent modulator of inflammation is also regulated by multiple mechanisms. First, Regnase-1 is capable of degrading its own mRNA in a negative feedback loop. Second, the paracaspase MALT1 cleaves Regnase-1 (and also Roquin-1 and -2) protein upon T cell receptor (TCR) activation, essentially removing the "inflammation brake" which subsequently leads to a high production of cytokines (69, 75).

All members of the Regnase family share a conserved region containing RNase and deubiquitinase catalytic domains (76, 77). Regnase-2 (*ZC3H12B*) and Regnase-3 (*ZC3H12C*) are expressed at very low levels in human T lymphocytes (78), and they are therefore unlikely to have a relevant functional impact in these cells. Concordant with this observation, deletion of mouse *Zc3h12c* led to some lymphocyte abnormalities that were not cell-autonomous, but rather secondary to defects in the myeloid lineage (79). Both Regnase-1 and Regnase-4 were shown to degrade an overlapping set of mRNAs, such as *IL2*, *IL6*, *IL10*, and *TNF* in an RNase-dependent manner via the targets' 3'UTRs (**Table 1**). This observation raises the question about protein redundancy, and specifically whether these two enzymes may have fully overlapping or also unique functions in T cells. Pointing towards the possibility that these two proteins may not be fully redundant is the observation that the phenotype

of mice lacking either *Zc3h12a* or *Zc3h12d* is different. Specifically, in contrast to Regnase-1, deletion of Regnase-4 did not lead to macroscopic changes in immune functions under steady state conditions. However, in models of EAE, these mice showed exacerbation of symptoms both at the peak of disease as well as in the resolution phase (80). These findings suggest that these two enzymes may in fact have functions that are not fully redundant, with Regnase-1 being potentially primarily involved in regulating the initial activation of T lymphocytes, while Regnase-4 might be more important during the resolution phase of inflammation. Concordant with this hypothesis, the expression of Regnase-4 protein in mouse splenocytes was increased after three days of TCR stimulation with anti-CD3 antibodies, suggesting a role at later stages of activation (80), while *ZC3H12A* was most highly expressed in resting lymphocytes, suggesting a role in restraining initial T cell activation (14, 78) (**Fig. 3**). An alternate explanation for these findings is that the apparent functional differences between these two proteins are actually due primarily to their relative expression levels, since Regnase-1 is more abundantly expressed than Regnase-4 in T lymphocytes. This might also explain the more severe phenotype observed upon Regnase-1 ablation, that could not be fully compensated by the more moderately expressed Regnase-4.

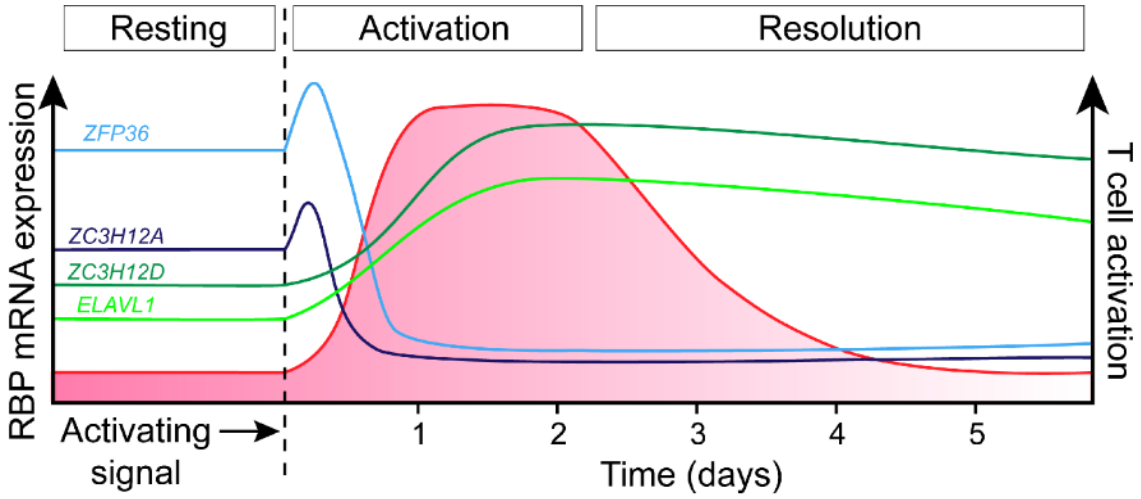

Figure 3. Dynamic expression of specific RBPs at different stages of T cell activation. Following T cell activation, the expression of selected RBPs relevant for the regulation of inflammation exhibit dynamic changes in mRNA expression. Specifically, based on our own data (14) and on the Database of Immune Cells (DICE) (78), *ZFP36* (TTP) was the most highly expressed RBP in resting lymphocytes, followed by *ZC3H12A* (Regnase-1), *ZC3H12D* (Regnase-4) and *ELAVL1* (HuR). Upon stimulation, *ZFP36* and *ZC3H12A* are transiently upregulated, at least at the mRNA level, followed by a strong downregulation, thereby potentially enabling cells to produce high levels of cytokines. In contrast, *ZC3H12D* and *ELAVL1* are gradually induced during the course of activation. Expression data for activated T cells were based on microarray data published in (118), as well as our own unpublished data. Similar changes in expression upon activation (downregulated TTP and Regnase-1, upregulated Regnase-4 and HuR) were reported also at protein level (75, 80, 119).

The relevance of the m^{6}A modification in T cell biology was revealed by studies showing that conditional deletion of the *Mettl3* gene (encoding for the m^{6}A methyltransferase enzyme Mettl3) in murine T lymphocytes compromised Treg cell functions (81) and led to the inability of naïve T cells to proliferate in response to cytokine signalling (82). Functional consequences of the m^{6}A marks on RNA are mediated by m^{6}A-binding proteins, including primarily members of the YTH family. Within the immune system, YTHDF1 impacted anti-tumor responses, and its ablation led to improved immunity and prolonged survival (83). Recent efforts to understand the level of redundancy between the different paralogs of the YTH family (YTHDF1-3) of m^{6}A-binding proteins recently reconciled apparently divergent results by showing that within individual cells, the three paralogs can fully compensate for each other in a gene dosage-dependent manner to mediate degradation of m^{6}A-containing mRNAs (84, 85). However, the phenotypes of the individual knock-out mice revealed differences due to the varying levels of expression of the YTH proteins across cell types (85). In other words, even though two proteins may be in reality fully redundant, deletion of a lowly expressed family member may have subtle consequences, for instance by affecting a subset of mRNAs highly sensitive to gene-dosage effects, while the deletion of a more highly expressed family member would affect mRNA stability or translation in a more widespread manner, leading to overall different phenotypes (84). Similar to the YTH proteins example, more studies investigating the details of Regnase-1 and -4 regulation and their mechanism of action will shed light on the level of functional overlap between these two enzymes, and specifically whether some of the differences observed when deleting individual proteins are due to unique functions and regulatory mechanisms, or they reflect instead hypomorphic phenotypes.

Overall, the overarching results paint a picture of a tightly controlled network of RBPs that regulate immune cell functions and responses both at resting state, and during the initiation and resolution phases of inflammation. However, mRNAs are subjected to regulation (and cross-regulation) by a large number of factors binding to a variety of sites in a cooperative or competitive manner, for which the regulatory logic remains for the most part to be examined.

6.5 The interplay between miRNAs and RBPs in the regulation of T lymphocytes

Through their ability to target a variety of mRNAs and limit their translation, miRNAs strongly influence T cell responses by modulating T cell differentiation, activation and proliferation (86, 87). When investigating the role of miRNAs in T lymphocytes, the level of expression and the kinetic of miRNA expression in response to TCR triggering may already provide some information about their potential functional role. First, highly abundant miRNAs that are rapidly downregulated upon

stimulation may be involved in restraining 'spurious' T cell activation that may occur in the absence of a genuine threat for the organism. One such example is provided by miR-125b, that was shown to be involved in the maintenance of the naïve state in human T cells (88). On the other hand, very lowly expressed miRNAs are very unlikely to achieve thresholds of expression that can lead to biologically relevant effects beyond stochastic noise (89, 90), if not in specific conditions of a few, very high-affinity target sites (potentially in cooperation with other miRNAs) and/ or in specialized T cell subsets. For instance, despite being an overall moderately expressed miRNA, miR-181a was expressed at relatively high levels in the Th17 subset of human T lymphocytes, where it contributed to define the threshold of TCR activation in these cells (91). Finally, modestly expressed miRNAs in resting cells that are strongly induced upon acute stimulation may achieve during this process an intracellular concentration sufficient to modulate the expression of mRNA targets important during T cell activation. Depending upon the kinetics of induction, categories of 'inducible miRNAs' might be divided into 'early' miRNAs (hours), potentially involved in favoring T cell activation and proliferation (e.g. miR-155 (92, 93)), and 'late' miRNAs (days), likely involved in the resolution of inflammation. A representative example of the latter category is provided by miR-146a, a negative regulator of NF-kB activation (94), whose deletion led to defective resolution of inflammation and development of T cell-associated autoimmunity (95).

Similar to protein-coding genes, miRNA expression is regulated at the transcriptional level by transcription factors. In contrast, there are not many examples in the literature regarding interactions between miRNAs and RBPs. However, one can easily envision various situations in which a) miRNA expression is regulated by RBPs; b) RBP transcripts are targeted and regulated by miRNAs; and c) both miRNAs and RBPs contribute to the regulation of the same target mRNA, in a cooperative, competitive or antagonist manner. The concept that miRNA expression is regulated post-transcriptionally by RBPs is exemplified by miR-146a, which was shown to be regulated by Roquin. In the absence of Roquin, miR-146a levels increased in T lymphocytes, due to the augmented stability of the mature miRNA (96). This stabilization was associated with enhanced ability of the Dicer enzyme to process the precursor pre-miR-146a (96). MiRNAs are also abundantly found in extracellular vesicles derived from many cell types, including activated primary human T lymphocytes (97, 98), which may have a role in cell-to-cell communication and also in the process of rapid 'elimination' of cytoplasmic factors that may hinder rapid T cell activation upon recognition of a specific antigen (99). Interestingly, hnRNPA2B1, a ubiquitously expressed RBP, was shown to be involved in the selection and loading of specific miRNAs into the exosomes of activated T cells,

pointing towards an active sorting process in the loading of the exosomes' cargo and a further layer of interplay between RBPs and miRNAs (100).

As for examples of 'classic' miRNA regulation of RBP expression, miR-27b was shown to modulate TTP levels in macrophages, in a complex interplay with HuR binding (101). In addition, both miRNAs and RBPs may target the same site on a given mRNA, as shown for example by *Pten*. This mRNA is regulated both by miR-17~92 and by Roquin, although in this case Roquin was shown to limit the access to the miR-17~92 site through competitive binding at an overlapping site (70). A further example is provided by HuR, which was shown to modulate miRNA-mediated mRNA targeting genome-wide in macrophages (101). Specifically, the presence of HuR-binding sites in the proximity of miRNA sites antagonized and attenuated miRNA activity resulting in increased gene expression, highlighting once more the complex interaction between different players in regulating mRNA stability and translation. In general, it is increasingly clear that both miRNAs and RBPs have a substantial impact of the regulation of gene expression during immune responses. However, the interplay between these different factors and their potential for cross-regulation remains less understood.

6.6 From autoimmunity to anti-tumor responses

Several of the molecular mechanisms discussed so far that affect lymphocyte responses are crucial in the context of their ability to limit T cell responses in autoimmunity, as shown by the many instances in which deletion of a miRNA or RBP led to spontaneous immune cell activation and pathology. However, the reverse can also be true, that unleashing the activity of these same factors might enhance T cell functions in the context of anti-tumor responses. Indeed, immune-related adverse effects such as autoimmunity also arise during immune cancer therapy, reflecting the removal of inhibitory brakes to T cell function (102). Understanding the mechanisms that regulate the balance between inflammation and tissue damage may be beneficial for both autoimmunity (where reduced inflammation is a desired outcome) and cancer immunotherapy, which instead would benefit of enhanced inflammatory and cytotoxic T cell responses.

The advent of immunotherapy has revolutionized prospects for cancer treatment, opening the possibility of inducing or reactivating anti-tumor immunity. Despite its effectiveness in many instances, the outcomes of immune checkpoint therapy are overall still highly heterogenous, and the basic biological knowledge that would reveal mechanistic insights about efficacy (or lack of thereof) in inducing anti-tumor responses is still lagging behind (103). The presence at tumor sites of

infiltrating T lymphocytes retaining effector functions usually correlates with more favorable outcomes (104). However, tumor-infiltrating lymphocytes are very heterogenous regarding gene expression and functional properties, and especially cells of the $CD8^+$ subset can acquire a functionally impaired state that limits their ability to control tumor growth (104). Critical differences between human and mouse models also hinder the interpretation of exhausted states. For instance, markers of T cell exhaustion such as the transcription factor TOX are associated with dysfunction in the mouse but not in human cells, suggesting underlying mechanistic differences (37).

Several factors discussed so far that were associated with a cytokine-producing, potentially pathogenic T cell phenotype were also shown to impact T cell responses to tumors. For example, T cell-specific ablation of Regnase-1 in mouse models caused pathogenic activation of T lymphocytes, aberrant cytokine production and spontaneous autoimmune disease (75). However, Regnase-1-deficient $CD8^+$ T lymphocytes also showed markedly increased efficacy in models of cancer immunotherapy (105). Similarly, while the transcription factor BHLHE40 was associated to a pathogenic cytokine signature in both human and mouse (14, 19), a T cell subset defined by BHLHE40 expression was specifically expanded in colorectal cancer patients with favorable responses to treatment with immune-checkpoint therapy (106). These findings highlight how these (and probably many other) factors may be involved in 'tipping the balance' of T cell responses, from beneficial for cancer therapy to pathogenic in autoimmunity.

One caveat to the apparently simple idea that anti-tumor responses should activate pathways that should instead be dampened in autoimmunity, is represented by the fact that the association between the two diseases is in reality bidirectional, and patients with autoimmune diseases are often also at increased risk of developing malignancies, at least in part because of the chronically altered inflammatory milieu (107). For instance, while the transcription factor TOX was clearly implicated in the acquisition of a dysfunctional, exhaustion program in tumor-specific $CD8^+$ T cells (30-34), it was also shown to promote $CD8^+$ T cell-mediated autoimmunity (108). These observations critically emphasize the importance of gaining a more comprehensive understanding of the intricacies of immune cell regulation.

One example of a potent pro-inflammatory cytokine at the crossroad between autoimmunity and cancer is provided by IL-17A. This cytokine plays a key role in the responses against infections with extracellular bacteria and fungi. However, its dysregulated expression is also strongly associated with autoimmunity, as shown by the effectiveness of neutralizing antibodies against IL-17A in the

treatment of psoriasis (109). Dysregulated, chronic production of this cytokine is now emerging also as a player in tumorigenesis (110). Interestingly, IL-17-signaling was found to strongly affect the stability of different mRNAs, in part by inducing the expression of mRNA stabilizers such as HuR and ARID5A (111). ARID5A in particular binds to the 3'UTR of target mRNAs at stem-loop structures overlapping with Regnase-1 binding sites, thereby counteracting Regnase-mediated degradation and promoting mRNA translation (112). In T lymphocytes, ARID5A favored differentiation of naïve T cells towards the inflammatory Th17 subsets through the stabilization of the *Stat3* mRNA, which is required for Th17 cell differentiation (112). The *IL17A* mRNA is also itself a target of post-transcriptional regulation, as shown for instance by the reduced IL-17 expression observed in the absence of HuR in Th17 cells, and the consequent amelioration of EAE development (113). Whether some of these mechanisms may be harnessed in the context of anti-cancer therapies remains to be investigated. Overall, it is becoming clear that many of the abovementioned regulatory factors act at the interface between anti-tumor responses and autoimmunity, underlying a role that is most likely primarily linked to the maintenance of balanced immune responses.

6.7 Outstanding questions

Many questions remain open about the role of specific transcription factors in regulating T cell functions. Most notably, it will be important to better understand whether some of the factors that have been implicated primarily in the regulation of Treg cells or chronically activated, exhausted cells may actually have important physiological functions in conventional T lymphocytes. Such factors could for instance contribute to the attenuation of the response once a pathogen has been successfully eliminated. Other key issues that will have to be better understood in the future are related to potential regulatory differences between $CD4^+$ and $CD8^+$ T lymphocytes, and also whether some of the important findings that have been described using mouse models can be now recapitulated in humans.

Functional studies have shown that the dysregulation of miRNA expression can be causative in various diseases, leading to an interest in the development of therapeutics to harness miRNAs for clinical benefit (114). One of the key issues in targeting these molecules to modulate immune cell functions is whether delivery systems can be designed that target efficiently and specifically the desired cell type, and at the same time can deliver sufficient quantities of miRNA mimic or antagonist that can reach biologically relevant intracellular concentrations. Moreover, the chemical modifications that are introduced in miRNAs to improve their stability *in vivo* can interfere with loading onto the silencing complex or with mRNA targeting (114). As a result, only few miRNA mimic

molecules progressed to some initial clinical testing. An interesting development in this direction was recently described for a miR-146a mimic oligonucleotide conjugated to a Toll-like receptor 9 agonist. This conjugated molecule efficiently reached the cytoplasm of myeloid and leukemic cells and was effective in reducing NF-kB activation, in limiting leukemia progression and in dampening excessive inflammation in models of cytokine release syndrome. Whether this or similar systems can be exploited therapeutically will undoubtedly be explored in the near future (115).

Likewise, in the case of RBPs, and despite their emerging importance in the regulation of immune responses, a number of outstanding questions remain to be addressed. Specifically: which cytokines and genes are affected by the expression of individual RBPs? What is the extent of target overlap, if any, between RBPs belonging to the same family, such as Regnase-1 and Regnase-4? How are these rheostats of inflammation themselves regulated in T lymphocytes? And finally, what is the precise kinetic of events in the regulation of inflammatory mRNA stability and translation in response to environmental cues? Understanding the temporal relationship between different RBPs and mRNA expression may lead to a better understanding of the impact of many of these post-transcriptional regulators on the initiation and persistence of inflammatory responses, as well as their resolution.

6.8 Concluding Remarks

If not appropriately controlled, the transcriptional program induced by pro-inflammatory stimuli or noxious agents has the potential to inflict significant damage to healthy tissues. The control of RNA stability and decay acts therefore in concert with transcription to delimit the amplitude and duration of an inflammatory response. Potentially, a combined approach that inhibits pro-inflammatory, pathogenic T cells and at the same time actively promotes the resolution of inflammation and tissue repair may become an attractive answer in the treatment of autoimmunity (116). On the other hand, the enhancement of some of these pathways, at least temporarily or targeted to antigen-specific cells (117), may improve strategies for cancer immunotherapy, highlighting the importance of advancing our understanding of the molecular and functional bases for immune activation and regulation. Many of the factors discussed heretofore (transcription factors, RBPs, miRNAs) act in a concerted, regulated manner to modulate the interface between insufficiently protective and excessively damaging immune responses, and may eventually become valuable targets for immunomodulation.

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Conflict of Interest

The authors have no conflict of interest to declare.

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7 Discussion

T_H lymphocytes undergo remarkable metabolic and functional changes throughout their lifetime. These changes must happen very rapidly upon antigen recognition to ensure an appropriate immune reaction and clearance of the pathogen or tumor cells. Our understanding of the importance of RBPs in establishing the immune response has become more appreciated since the first serious studies on TTP in the 1990s. In T lymphocytes, the most important role of RBPs is the regulation of the stability of target mRNA through different decay mechanisms. Newly developed technologies keep expanding the list and currently the number of predicted RBPs in human T_H lymphocytes that bind to mature mRNA is as high as 1250 (Hoefig et al., 2021). The dysregulation of RBP expression leads to aberrant cytokine production and immune pathologies which warrants the investigation of their molecular mechanisms.

In **Chapter 4. Functional analysis of Regnase-1 and Regnase-4 in human T helper lymphocytes** I aimed to describe the unique and redundant function of the Regnase family members. I confirmed that Regnase-1 and Regnase-4 are expressed and functional in T_H lymphocytes, while Regnase-2 and Regnase-3 expression was very low or below the expression threshold. We noticed an increase of Regnase-3 mRNA expression during activation, but it did not reach the expression levels of *ZC3H12A* and *ZC3H12D*, as measured by qRT-PCR. Previous data demonstrated that a T cell-specific Regnase-3 KO mouse model showed no effect on the number and phenotype of T cells, while the macrophage-specific Regnase-3 KO mouse model led to an excessive IFN-γ expression (von Gamm et al., 2019). These results suggest that RBPs of the same family may have unique functions dependent on the cell-specific expression level and cellular context. Of note, Roquin-2 can compensate for the lack of Roquin-1 despite its 5 times lower expression (Vogel et al., 2013), and therefore there is a possibility that Regnase-3 at least partially compensates for the lack of Regnase-1 and Regnase-4. I was able to identify the redundant and compensatory effects of Regnase-1 and Regnase-4 by generating a double KO that led to a striking increase of upregulated pro-inflammatory genes. Apart from the Roquin family, the redundancy of paralogues has also been described in other RBP families such as the *ZFP36* family that has 3 members (*ZFP36* (TTP)*, ZFP36L1, ZFP36L2*) expressed in human and mouse T cells. The T-cell specific triple KO mouse model is lethal within 6 weeks after birth due to a severe systemic inflammatory syndrome. However, the expression of any of the three members fully prevented the lethality suggesting a complete functional redundancy within the family (Cook et al., 2022). Unexpectedly, the double KO of *ZFP36L1* and *ZFP36L2* rendered mice resistant to the induction of EAE due to a failed priming of antigen-specific T_H cells. Thus, members of the same RBP family can have mostly redundant functions, but also specific additional non-redundant roles.

An interesting finding of this study were the different expression kinetics of Regnase-1 and Regnase-4 across activation stages. I confirmed previous results that Regnase-1 becomes abruptly cleaved upon activation. However, Regnase-4 did not show a reduction of expression, in fact, it had an increasing trend up to day 3/4 post-activation. The antibody used for Regnase-4 detection does not recognize the C-terminus, as in the case of the Regnase-1 antibody, and for this reason it cannot detect the putative cleaved product. However, the recognition site by the paracaspase MALT1 is conserved between Regnases, and one would expect the same regulatory checkpoint. To confirm this, further experiments are needed. It is important to note that it is computationally predicted that the RNase catalytic site is still present in the cleaved product of Regnase-1 and Regnase-4. Therefore, it might be possible that the cleaved products still exhibit RNase activity, albeit this is unlikely since the presence of the NTD is necessary for these RBPs to be functional, as previously described (Xu et al., 2012; Yokogawa et al., 2016). Additionally, there is evidence that Regnase-1 forms functional dimers with itself (Yokogawa et al., 2016). Due to the structural similarity between Regnase-1 and Regnase-4, and their previously described colocalization in HeLa cells (S. Huang et al., 2015) , there is a possibility that the two RBPs form dimers with each other in T lymphocytes, which requires further investigation.

The dysregulated expression of immunomodulatory RBPs is a double-edged sword. On one hand, their reduced expression leads to pro-inflammatory disorders, on the other hand, this can have tumor suppressive functions. For example, an adoptive cell therapy of Regnase-1-KO $CD8^+$ T cells in mice greatly improved the anti-tumor response thanks to a stronger and more persistent effector phenotype of the engineered cells (Wei et al., 2019). The effect was further confirmed and enhanced with human CAR-T cells lacking both Regnase-1 and Roquin-1 (Mai et al., 2023). Thus, the results of this study, which showed a strong pro-inflammatory phenotype of the double KO of Regnase-1 and Regnase-4, warrants further investigation and a potential of Regnase-4 as well in cancer immunotherapies. In summary, studying the function of RBPs and their paralogues is important to unravel possible molecular and cellular mechanisms to advance medical treatments.

In **Manuscript [1]**, we identified an axis comprising the RFX transcription factors, miR-150 and the RBP PDAP1 that regulates the proliferation of T_H lymphocytes. miR-150 is highly expressed in resting T cells, and it is strongly reduced upon activation. We showed that miR-150 directly negatively regulates the expression of PDAP1 by binding to MBS in the 3'UTR of *PDAP1 in vivo* in human T_H lymphocytes and in *in vitro* reporter assays. Therefore, the abrupt downregulation of miR-150 releases the negative brake from PDAP1 allowing it to exert its pro-proliferative function. Our data

suggests that PDAP1 either directly stabilizes certain transcripts (most prominently *CBL*) while other targets might be regulated at the level of translation regulation. PDAP1 has been retrieved in RBP databases as an IDP that functions in B lymphocytes and cancer cell lines to promote proliferation and cell cycle progression. Here, we described PDAP1 to have a similar role in primary human T lymphocytes. IDPs have fluctuating tertiary structures which potentially interact with many partner proteins and mRNAs (Wright & Dyson, 2015). Indeed, IDPs frequently act as hubs in protein interaction networks (Dunker et al., 2005). The exact mode of mRNA recognition by IDRs is still a matter of ongoing investigations, but they are certainly very important regions for post-transcriptional control as seen by the fact that RBPs that interact with mRNAs are enriched with IDRs (Castello et al., 2012). IDPs can directly interact with RNA through the IDR itself, but they can also form larger complexes (so-called *fuzzy complexes* because of the disordered structure) that form functional units (Castello et al., 2016; Tompa & Fuxreiter, 2008) Thus, IDPs can potentially regulate the stability of mRNA transcripts through different mechanisms, which we also propose to be possible for PDAP1. It is feasible to inhibit the function of IDPs by targeting with small molecules (Santofimia-Castaño et al., 2020). Considering that PDAP1 promotes the proliferation of B and T lymphocytes and cancer cell lines, it might be a potential target for developing anti-proliferative cancer treatments.

The study of RBPs in T lymphocytes is challenging for multiple reasons. For one, the intrinsic instability of mRNA transcripts as well as intrinsic RNase activity of RBPs as is the case of Regnases. Also, RBPs usually have paralogues with compensatory and redundant effects among the family members and even non-family members. Another issue comes with the investigation of intrinsically disordered RBPs which give no structural hints to possible co-factors, pathways, or putative functional mutants. Finally, the dynamic expression changes during activation underlines that each RBP network has to be studied longitudinally at the global level. However, investigating these molecular mechanisms, despite the challenges, is essential due to the great potential they hold for the development of future autoimmune and cancer therapies.

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