Role of post-transcriptional gene regulation by Roquin in T cell activation and differentiation

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Katharina Marlene Jeltsch

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am Institut für molekulare Immunologie,
Abteilung für molekulare Immunregulation am Helmholtz Zentrum München
und am Institut für Immunologie an der Ludwig-Maximilians-Universität München.

Erster Gutachter: Prof. Dr. Daniel Krappmann
Zweiter Gutachter: Prof. Dr. Elisabeth Weiß

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Eidesstattliche Erklärung

Hiermit versichere ich, Katharina Marlene Jeltsch, an Eides statt, dass die vorgelegte Dissertation mit dem Titel **„Role of post-transcriptional gene regulation by Roquin in T cell activation and differentiation"** von mir selbständig und ohne unerlaubte Hilfsmittel angefertigt worden ist und ich mich nur der ausdrücklich bezeichneten Quellen und Hilfsmittel bedient habe.

Die vorliegende Dissertation wurde weder ganz, noch teilweise bei einer anderen Prüfungskommission vorgelegt und ich habe noch zu keinem früheren Zeitpunkt versucht eine Dissertation einzureichen oder an einer Doktorprüfung teilzunehmen.

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Katharina Jeltsch

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1 LIST OF PUBLICATIONS

The thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-III).

I.

Vogel*, K.U., Edelmann*, S.L., **Jeltsch, K.M.**, Bertossi, A., Heger, K., Heinz, G.A., Zöller, J., Warth, S.C., Hoefig, K.P., Lohs, C., Neff, F., Kremmer, E., Schick, J., Repsilber, D., Geerlof, A., Blum, H., Wurst, W., Heikenwälder, M., Schmidt-Supprian, M., Heissmeyer, V. (2013). Roquin paralogs 1 and 2 redundantly repress the Icos and Ox40 costimulator mRNAs and control follicular helper T cell differentiation. Immunity 38, 655–668. * equal contribution

II.

Jeltsch*, K.M., Hu*, D., Brenner*, S., Zöller, J., Heinz, G.A., Nagel, D., Vogel, K.U., Rehage, N., Warth, S.C., Edelmann, S.L., Gloury, R., Martin, N., Lohs, C., Lech, M., Stehklein, J.E., Geerlof, A., Kremmer, E., Weber, A., Anders, H.J., Schmitz, I., Schmidt-Supprian, M., Fu, M., Holtmann, H., Krappmann, D., Ruland, J., Kallies, A., Heikenwalder, M., Heissmeyer, V. (2014). Cleavage of roquin and regnase-1 by the paracaspase MALT1 releases their cooperatively repressed targets to promote T(H)17 differentiation. Nat. Immunol. *15*, 1079–1089. * equal contribution

III.

Gewies*, A., Gorka*, O., Bergmann, H., Pechloff, K., Petermann, F., **Jeltsch, K.M.**, Rudelius, M., Kriegsmann, M., Weichert, W., Horsch, M., Beckers, J., Wurst, W., Heikenwlader, M., Korn, T., Heissmeyer, V., Ruland, J. (2014). Uncoupling Malt1 threshold function from paracaspase activity results in destructive autoimmune inflammation. Cell Rep *9*, 1292–1305. * equal contribution

2 SUMMARY

Post-transcriptional gene regulation allows an effective and rapid adaption of immune responses to circumstances. Hence, imbalances can lead to inappropriate immune cell fate decisions that have the potential to induce autoimmunity. The post-transcriptional regulators Roquin-1 and Regnase-1 play comparable roles in the repression of target messenger RNAs (mRNAs) via their 3' untranslated regions. *Sanroque* mice with pointmutated Roquin-1 develop autoimmunity resembling human systemic lupus erythematosus that is linked to spontaneous activation of T cells and accumulation of T follicular helper (T_{FH}) cells. Similarly, mice deficient for Regnase-1 show an autoimmune phenotype with spontaneous T cell activation. This thesis aimed at discovering how Roquin-1 and Regnase-1 regulate T cell activity and thus prevent autoinflammatory responses.

First, the role of the Roquin-1 paralog Roquin-2 was addressed and a unique function for mouse survival could be shown. In T cells, however, Roquin-1 and Roquin-2 behaved functionally redundant. Mice with combined deficiency of Roquin-1 and Roquin-2 in T cells exhibited hyperactivated T cells with a tendency towards T_{FH} differentiation but a lupus-like phenotype could not be observed (**Publication I**). Further investigation of these mice unraveled elevated numbers of T_H17 cells in the lymphoid organs, gut and lung, which correlates with gastritis and inflammatory pathological changes in the lung. The T cell-intrinsic T_H17-bias was associated with deregulation of identified Roquin-1/2 target mRNAs coding for the T_H17 promoting factors IL-6, c-Rel, Irf4, IκBζ and IκBNS. Roquin-1 and Regnase-1 were found to cooperate in the repression of these target mRNAs and this required RNA-binding by Roquin-1 and nuclease activity of Regnase-1. Roquin-1/2 and Regnase-1 proteins are regulated during T cell fate decisions as cleavage of Roquin-1/2 and Regnase-1 proteins by the paracaspase Malt1 upon combined T cell receptor and co-stimulatory signaling was detected. This released T cells from Roquin-1- and Regnase-1-mediated post-transcriptional repression. The T cell receptor (TCR) signal strength was translated into graded Roquin-1/2 and Regnase-1 cleavage and differential gene regulation. Only a strong TCR stimulus led to high inactivation of Roquin-1/2 and Regnase-1 proteins and thus T_H17 differentiation (**Publication II**). This pathway bears new possibilities for therapeutical intervention like the use of Malt1 inhibitors specifically blocking its protease activity to pharmacologically control the activity of Roquin-1/2 and Regnase-1. This might prevent overshooting misdirected immune responses. However, analysis of mice with genetic Malt1 protease inactivation revealed an unexpected lethal multi-organ inflammatory syndrome (**Publication III**).

In summary, these discoveries allow a greater understanding of the molecular mechanisms involving Roquin-1/2 and Regnase-1 in the regulation of T cell activation and differentiation.

3 ZUSAMMENFASSUNG

Die posttranskriptionelle Genregulation erlaubt eine effektive und schnelle Anpassung von Immunantworten an die Gegebenheiten. Ein gestörtes Gleichgewicht kann daher zu unangemessenen Differenzierungsentscheidungen von Immunzellen führen, was potentiell Autoimmunität verursacht. Der posttranskriptionelle Regulator Roquin-1 und die Endonuklease Regnase-1 spielen ähnliche Rollen in der Repression von Ziel-Boten-RNAs über deren 3' untranslatierte Regionen. *Sanroque*-Mäuse mit punktmutiertem Roquin-1 entwickeln eine dem humanen systemischen Lupus erythematodes ähnliche Autoimmunität, die in Zusammenhang mit spontaner Aktivierung der T-Zellen und Akkumulation von follikulären T-Helferzellen (T_{FH}) steht. Einen vergleichbaren autoimmunen Phänotyp mit spontaner T-Zellaktivierung zeigen Mäuse, denen Regnase-1 fehlt. Ziel dieser Doktorarbeit war es herauszufinden, wie Roquin-1 und Regnase-1 T-Zellaktivität regulieren und dadurch autoinflammatorische Antworten verhindern.

Zuerst wurde die Rolle des Roquin-1 Paralogs Roquin-2 untersucht und eine einmalige Funktion für das Überleben von Mäusen konnte gezeigt werden. In T-Zellen verhielten sich Roquin-1 und Roquin-2 jedoch funktionell redundant. Mäuse, denen sowohl Roquin-1 als auch Roquin-2 in T-Zellen fehlte, wiesen hyperaktive T-Zellen mit einer Tendenz zur T_{FH} Differenzierung auf, zeigten aber keinen Lupus-ähnlichen Phänotyp (**Publikation I**). Weitere Untersuchungen dieser Mäuse deckten eine erhöhte Anzahl von T_H17-Zellen in den lymphoiden Organen, im Darm und in der Lunge auf. Dies korrelierte mit Gastritis und pathologischer Entzündung der Lunge. Die T-Zell-intrinsische T_H17-Ausrichtung war mit einer Deregulierung von identifizierten Ziel-Boten-mRNAs von Roquin-1 assoziiert. Diese kodieren für Faktoren wie IL-6, c-Rel, Irf4, IκBζ und IκBNS, die das T_H17-Programm fördern. Für die Repression von Ziel-Boten-mRNAs konnte eine Kooperation zwischen Roquin-1/2 und Regnase-1 nachgewiesen werden, die die RNA-Bindungsaktvität von Roquin-1 und die Nukleaseaktivität von Regnase-1 erforderte. Roquin-1/2- und Regnase-1-Proteine werden während der T-Zellimmunantwort reguliert, wie der Nachweis ihrer Spaltung durch die Paracaspase Malt1 nach T-Zellaktivierung in Kombination mit kostimulatorischen Signalen zeigte. Durch die Spaltung wurden T-Zellen von der posttranskriptionellen Repression durch Roquin-1/2 und Regnase-1 befreit. Die T-Zellrezeptor (TZR)-Signalstärke wurde in abgestufte Roquin-1/2- und Regnase-1-Spaltung und differentielle Genregulation übersetzt. Nur ein starker TZR-Stimulus führte zu hoher Inaktivierung der Roquin-1/2- und Regnase-1-Proteine und damit zur T_H17-Differenzierung (**Publikation II**). Dieser Signalweg birgt neue Möglichkeiten für ein therapeutisches Eingreifen wie die den Einsatz von Malt1-Inhibitoren, die speziell dessen Proteasefunktion hemmen. Damit könnte die Aktivität von Roquin-1/2 und

Regnase-1 pharmakologisch kontrolliert und überschießende fehlgeleitete Immunantworten verhindert werden. Die Analyse von Mäusen mit genetischer Inaktivierung der Malt1-Proteaseaktivität führte jedoch zu unerwarteter Letalität mit Entzündungen in multiplen Organen (**Publikation III**).

Zusammenfassend erlauben diese Entdeckungen ein größeres Verständnis der molekularen Mechanismen der von Roquin-1/2- und Regnase-1-vermittelten Regulation von T-Zell-aktivierung und -differenzierung.

4 ABBREVIATIONS

aa	amino acid
ABC-DLBCL	activated B cell diffuse large-B cell lymphoma
AGO	argonaute
AICD	activation-induced cell death
AIRE	autoimmune regulator
AITL	angioimmunoblastic T cell lymphoma
ALPS	autoimmune lymphoproliferative syndrome
AMPK	AMP-activated protein kinase
ANA	anti-nuclear antibody
AP-1	activator protein 1
APC	antigen presenting cell
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy
APS1	autoimmune polyglandular syndrome type 1
ARE	AU-rich elements
ARE-BP	ARE binding proteins
Arg	Arginine
Ascl2	achaete-scute homologue 2
Ask1	apoptosis signaling-regulating kinase 1
AUF1	AU-rich binding factor-1
Bcl6	B cell lymphoma 6
Bcl10	B cell lymphoma 10
BCR	B cell receptor
BM	bone marrow
CARD	caspase-recruitment domain
Carma1	CARD-containing membrane associated guanylate kinase protein 1
CD40L	CD40 ligand
CDE	constitutive decay element
cIAP2	cellular apoptosis inhibitor-2
CNOT6	CCR4-NOT transcription complex subunit 6
CRAC	calcium release-activated calcium
cTEC	cortical thymic epithelial cell
C-term	carboxy-terminus
Ctla-4	cytotoxic T-lymphocyte-associated antigen 4
CUGBP	CUG-binding proteins
CYLD	cylindromatosis
DAG	diacylglycerol
DAMP	damage-associated molecular pattern
DC	dendritic cell
DcpS	scavenger decapping enzyme
DD	death domain
DN	double negative
DP	double positive
DZ	dark zone
EAE	experimental autoimmune encephalomyelitis
Edc	enhancer of decapping

eIF	eukaryotic initiation factor
ELAV	embryonic lethal abnormal vision
ENU	ethylnitrosourea
FasL	Fas ligand
Fig.	Figure
Foxp3	forkhead box protein 3
GAIT	IFN-γ-activated inhibitor of translation
GAPDH	glyceraldehyde 3 phosphate dehydrogenase
GC	germinal center
Glut	glucose transporter
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRE	GU-rich element
HECT	homologous to E6-associated protein C-terminus
Hif-1α	hypoxia inducible factor-1α
HuR	human antigen R
IBP	IFN regulatory factor 4-binding (Irf4-binding) protein
Icos	inducible co-stimulator
IFN	interferon
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
IL-12R	IL-12 receptor
IP3	inositol-1,4,5-triphosphate
IPEX	immuno-dysregulation, polyendocrinopathy an enteropathy, X-linked syndrome
IRE	iron responsive element
Irf4	interferon-regulatory factor 4
IRP	IRE-RNA-binding protein
ITAM	immunoreceptor tyrosine-based activation motifs
ITK	IL2-inducible T cell kinase
iTregs	inducible Tregs
IκBα	inhibitor of NF-κB α
Jnk	c-Jun N-terminal kinase
KSRP	K homology (KH) splicing regulatory protein
LAT	linker for activation of T cells
Lsm	Sm-like protein
LZ	light zone
MAGUK	membrane associated guanylate kinase
MALT	mucosa-associated lymphoid tissue
MALT1	MALT lymphoma-translocation gene 1
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MCPIP1	MIP-1 inducible protein 1
MHC	major histocompatibility complex
miRNA	microRNA
mRNA	messenger RNA

mTEC	medullary thymic epithelial cell
mTOR	mammalian target of rapamycin
N-term	amino-terminus
NF-κB	nuclear factor-κB
NFAT	nuclear factor of activated T cells
NIK	NF-κB-inducing kinase
NMD	nonsense-mediated decay
NMRI	Naval Marine Research Institute
NSD	nonstop decay
nTregs	natural Tregs
Ova	Ovalbumin
P body	processing body
PABP	polyadenylate-binding protein
PAMP	pathogen associated molecular pattern
PAN2/3	PAB-specific ribonulcease 2/3
PARN	poly(A)-specific ribonuclease
PD-1	programmed death-1
PDL	PD-1 ligand
PIN	PilT N-term
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphoinositol-4,5-bisphosphate
PKC	protein kinase C
PLCγ1	phospholipase C-γ1
PM	pointmutant
pri-miRNA	primary miRNA
PRR	pattern-recognition receptor
RAG	recombination-activating gene
RBP	RNA-binding protein
Rc3h1	ring finger and CCCH-type zinc finger domains 1
Regnase-1	regulatory RNase-1
RING	really interesting new gene
RISC	miRNA-induced silencing complex
Rle-1	regulation of longevity by E3
RNase	ribonuclease
RNP	ribonucleoprotein
ROR	retinoic acid-related orphan receptor
Rpl13a	ribosomal protein L13a
SAP	SLAM-associated protein
SG	stress granule
SLAM	signaling lymphocyte activation molecule
SLDE	stem-loop-destabilizing element
SLE	systemic lupus erythematosus
SLP-76	SRC homology 2 (SH2)-domain-containing leukocyte protein of 76 kDa
SP	single positive
STAT	signal transducer and activator of transcription
T-bet	T box expressed in T cells

TAB2	TAK-binding protein 2
TAK1	TGF-β-activated kinase 1
TCA	tricarboxylic acid
TCR	T cell receptor
T_{FH} cells	T follicular helper cells
Tfr	follicular Tregs
TGF-β	transforming growth factor β
T_H cells	T helper cells
TIA-1	T-cell-restricted intracellular antigen-1
TIAR	TIA-1-related protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
Treg	regulatory T cells
TSA	tissue-specific antigens
TSE	translational silencing element
TTP	tristetraprolin
UTR	untranslated region
VEGFA	vascular endothelial growth factor A
WT	wildtype
Xrn1	exoribonuclease 1
ZAP-70	ζ-chain-associated protein of 70 kDa

5 INTRODUCTION

5.1 T cell tolerance and autoimmunity

Our immune system is armed with multiple weapons to defend the body from invading pathogens. A complex interplay of organs, cells and molecules is responsible for defense against aggressions. There are two lines of defense, the innate and the adaptive immune response. The innate immune system is built for general sensing of danger signals and based on cell populations with germline-encoded receptors that recognize invariant pathogen associated molecular patterns (PAMPs). This allows a rapid response to invading pathogens. Effectors of the innate immune system include cells such as macrophages and dendritic cells (DCs). Innate immunity also sets the preconditions for the induction of the following adaptive immune response, which is slower but more efficient and required for the specific recognition of pathogens. The specificity of the adaptive immune system is achieved by the huge repertoire of receptors on B and T lymphocytes. B cells express membrane-bound antibodies as B cell receptors (BCRs) and T cell receptors (TCRs) are displayed on T cells. Millions of different receptors are the result of somatic recombination and mutation of antigen receptor genes (Murphy, 2011). However, the broad range of receptor specificities is random and harbors the danger of recognizing self-structures, which can lead to immune responses against self-tissues. Therefore, a variety of tolerance mechanisms developed to eliminate or neutralize such auto-reactive lymphocytes in order to sustain immunological self-tolerance. A breakdown of these control mechanisms can lead to autoimmune tissue damage and eventually to organ-specific or systemic autoimmune disease (Valdor and Macian, 2013).

5.1.1 Central T cell tolerance

The main concept of self-tolerance is the discrimination between “self” and “non-self”. During T cell maturation in the thymus, positive and negative selection ensure the consequent deletion of self-reactive T cells and the recognition of self-MHC (major histocompatibility complex) molecules via the TCR (Palmer, 2003). More than 95% of thymocytes will be lost during this stringent process (Kyewski and Klein, 2006; Wilkinson et al., 1995). The initial T cell maturation stages take place in the cortex region of the thymus, where expression of TCR, CD4, CD8 and CD3 molecules and TCR rearrangement occur. T cells develop from the double negative stage (DN) via the intermediate stages DN1-4 to $CD4^+CD8^+$ double positive (DP) cells. As thymocytes mature, they migrate from the thymic cortex to the medulla (Murphy, 2011). Positive selection is supposed to take place in the cortex and only those thymocytes with TCRs

capable of binding composites of self-peptides and MHC displayed on the surface of cortical thymic epithelial cells (cTECs) survive. Depending on the interaction with MHC class I or MHC class II molecules, the thymocytes develop into $CD8^+$ or $CD4^+$ single positive (SP) T cells, respectively. The affinity of the TCR is a crucial determinant in the development of T cells in the thymus. Weak binding of self-peptide-MHC allows positive selection. Approximately 90% of the DP thymocytes do not recognize self-MHC molecules or show very low affinity and die due to the lack of survival signals (death by neglect). The surviving SP T cells are tested for their auto-reactivity by negative selection mechanisms. The main mechanism is clonal deletion which eliminates T cells bearing high-affinity self-reactive TCRs whereas anergy and receptor editing play minor roles (Hogquist et al., 2005). T cell anergy turns T cells unresponsive to stimulation signals upon new encounter of MHC-peptides (McCaughtry and Hogquist, 2008). Specialized epithelial cells, so-called medullary thymic epithelial cells (mTECs), and DCs are required for the clonal deletion of T cells in the medulla (Brocker, 1999; Palmer, 2003). mTECs express T cell co-stimulatory molecules like CD80 (B7.1) and CD86 (B7.2), the ligands for T cell co-receptor CD28 and ectopically express peripheral tissue-specific antigens (TSA) to present them to developing T cells (Kyewski and Klein, 2006). TSA, as for example insulin, are normally expressed in peripheral tissues and expressed by mTECs via a special transcription factor known as autoimmune regulator (AIRE) (Anderson et al., 2002; Derbinski et al., 2005, 2001). Although DCs cannot express TSAs themselves, they cross-present self-antigens produced by mTECs (Gallegos and Bevan, 2004). An alternative outcome is that some high-affinity thymocytes are tolerized by negative selection or even selected for (a process termed “agonist selection”), survive and differentiate into a “regulatory” phenotype, including $CD4^+CD25^+$ regulatory T cells (Tregs) (Wing and Sakaguchi, 2010) (see 5.1.2.3.2).

5.1.2 T helper cell activation and differentiation

Mature, naive $CD4^+$ T cells leave the thymus and circulate in the periphery where they encounter their cognate antigen presented by antigen presenting cells (APCs) in secondary lymphoid organs and differentiate to effector cells. Activation is achieved by the specific interaction of the TCR and its co-receptor CD4 with a peptide-MHC complex (signal 1) and signals via co-stimulatory receptors such as CD28 which binds to CD80 or CD86 expressed on APCs (signal 2). Thereupon, T cells proliferate and differentiate into various effector T cell subsets depending on the cytokine milieu (signal 3) in order to orchestrate an adaptive immune response specific for an invading pathogen (Murphy, 2011).

5.1.2.1 T cell receptor signaling

Antigen recognition via the TCR and concomitant co-stimulation induces the formation of an immunological synapse to bring co-receptors and their ligands in close proximity (Chen and Flies, 2013). T cell signaling is initiated by the activation of protein tyrosine kinase Lck of the Src family, which phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) within the TCR complex, and leads to the recruitment and activation of the protein tyrosine kinase ZAP-70 (ζ-chain-associated protein of 70 kDa) of the SYK family kinases (Love and Hayes, 2010). ZAP-70 in turn activates the adaptor proteins LAT (linker for activation of T cells) and SLP-76 (SRC homology 2 (SH2)-domain-containing leukocyte protein of 76 kDa), which associate with the activated TCR complex and act as scaffolds for recruitment of further mediators. Recruited ITK (IL2-inducible T cell kinase) of the Tec kinase family phosphorylates PLCγ1 (Readinger et al., 2009). Active PLCγ1 hydrolyzes the plasma membrane phospholipid phosphoinositol-4,5-bisphosphate (PIP2) into second messengers IP3 (inositol-1,4,5-triphosphate) and DAG (diacylglycerol). In T cells, membrane-associated DAG activates PKCθ (protein kinase C θ) and recruits it to the lipid-raft where subsequent signaling events lead to nuclear factor-κB (NF-κB) activation (Kong and Altman, 2013; Schulze-Luehrmann and Ghosh, 2006) (see 5.1.2.2). Furthermore, DAG activates the GTP-exchange factor RasGRP, which activates the small G protein Ras and initiates the MAPK (mitogen-activated protein kinase) cascade. MAPK signaling ends in the transcription of c-Fos and formation of the transcription factor AP-1 (activator protein 1) by dimerization of c-Fos with constitutively present c-Jun. AP-1 remains inactive until the MAPK c-Jun N-terminal kinase (Jnk) phosphorylates c-Jun (Murphy, 2011). Diffusing IP3 increases intracellular calcium concentration by opening calcium channels of the endoplasmic reticulum, a store depletion that then leads to subsequent opening of CRAC channels (calcium release-activated calcium channels) in the plasma membrane and influx of extracellular calcium (Murphy, 2011). Calcium binds calmodulin, which in turn activates the phosphatase calcineurin to dephosphorylate nuclear factor of activated T cells NFAT that enables its nuclear translocation. The active transcription factors NF-κB, AP-1 and NFAT induce gene transcription of interleukin-2 (IL-2), the hallmark cytokines of activated T cells, as well as of other proteins that drive differentiation, proliferation and effector functions of T cells (Murphy, 2011).

5.1.2.2 The role of Malt1 in T cell activation

Malt1 is a key regulator of antigen signaling and loss-of-function results in immunodeficiency. It was initially identified in human mucosa-associated lymphoid tissue (MALT) B cell lymphomas with a chromosomal translocation that fuses the *cIAP2* (cellular apoptosis inhibitor-2) gene, encoding an inhibitor of apoptosis, in-frame with a novel gene, named

MALT1 (MALT lymphoma-translocation gene 1) (Akagi et al., 1999; Dierlamm et al., 1999; Morgan et al., 1999). The gain-of-function fusion protein cIAP2-MALT1 consisting of the carboxy-terminus (C-term) of MALT1 linked to the amino-terminus (N-term) of cIAP2 is involved in the oncogenesis of MALT lymphoma by mediating constitutive NF-κB activation (Isaacson and Du, 2004; Lucas et al., 2004). Malt1 is involved in both innate and adaptive immune cells downstream of receptors with ITAMs such as TCRs (Thome, 2008). Here, Malt1 acts as a dual regulator of NF-κB signaling. On the one hand Malt1 has an essential scaffold function in the assembly of protein complexes for NF-κB activation and on the other hand it bears a domain with partial homology to caspases and metacaspases indicating a proteolytic activity and therefore Malt1 was named a paracaspase (Uren et al., 2000). The caspase-like domain of Malt1 is preceded by an N-terminal death domain (DD) and two Ig-like domains, and is followed by a third immunoglobulin (Ig)-like domain and a C-terminal end lacking discernible secondary structure (Fig. 1a). As a scaffold, Malt1 constitutively interacts with Bcl10 (B cell lymphoma 10) as a monomer (Lucas et al., 2001; Uren et al., 2000) and is integrated into the so-called CBM complex. In T cells, this complex consists of Carma1 (caspase-recruitment domain (CARD)-containing membrane associated guanylate kinase [MAGUK] protein 1; CARD11), Bcl10 and Malt1, and connects the activity of upstream kinases of TCR signaling to canonical NF-κB activation (Thome, 2008) (Fig 1b). TCR triggering induces formation of the CBM complex by recruitment of Carma1 and PKCθ to the immunological synapse. Carma1 is phosphorylated by activated PKCθ, which induces a conformational change of Carma1 that releases Carma1 from auto-inhibition. Subsequent oligomerization of Carma1 and recruitment of preformed Bcl10-Malt1 complexes (Jiang and Lin, 2012; Rawlings et al., 2006) lead to oligomerization and activation of Malt1 (Rosebeck et al., 2011b). MALT1 oligomers bind to TRAF6 (TNF receptor-associated factor 6), which induces TRAF6 oligomerization, and activates its E3 ligase function (Noels et al., 2007; Sun et al., 2004, p. 6)**.** In addition to auto-ubiquitination, TRAF6 polyubiquitinates Malt1 (Oeckinghaus et al., 2007). Ubiquitination of Bcl10 (Wu and Ashwell, 2008) and Malt1 provides docking sites for the TAK-binding protein 2 (TAB2)-TGF-β-activated kinase 1 (TAK1) complex and the ubiquitin-binding subunit of the IκB kinase (IKK) complex IKKγ. TRAF6 ubiquitinates the regulatory IKKγ subunit and places TAK1 kinase in direct proximity to its substrate IKK. This results in phosphorylation of the IKKβ subunit of the IKK complex and activation of its enzymatic activity. IKK phosphorylates the inhibitor of NF-κB α (IkBα) to target it for ubiquitination and proteasomal degradation resulting in the translocation of NF-κB from the cytoplasm to the nucleus and transcription of NF-κB target genes (Thome, 2004). Malt1 is the only paracaspase known in the mammalian genome and, in contrast to caspases, the proteolytic activity of Malt1 is

arginine-specific and not dependent on internal cleavage. However, full-length Malt1 is not active by itself but requires dimerization (Wiesmann et al., 2012; Yu et al., 2011, p. 1). Malt1-dependent proteolysis was shown for several NF-κB-related factors such as A20, RelB, Bcl10, NIK (NF-κB-inducing kinase) and CYLD (cylindromatosis) and cleavage of these targets is relevant for optimal NF-κB and T cell activation (Coornaert et al., 2008; Hailfinger et al., 2011; Rebeaud et al., 2008; Rosebeck et al., 2011a; Staal et al., 2011).

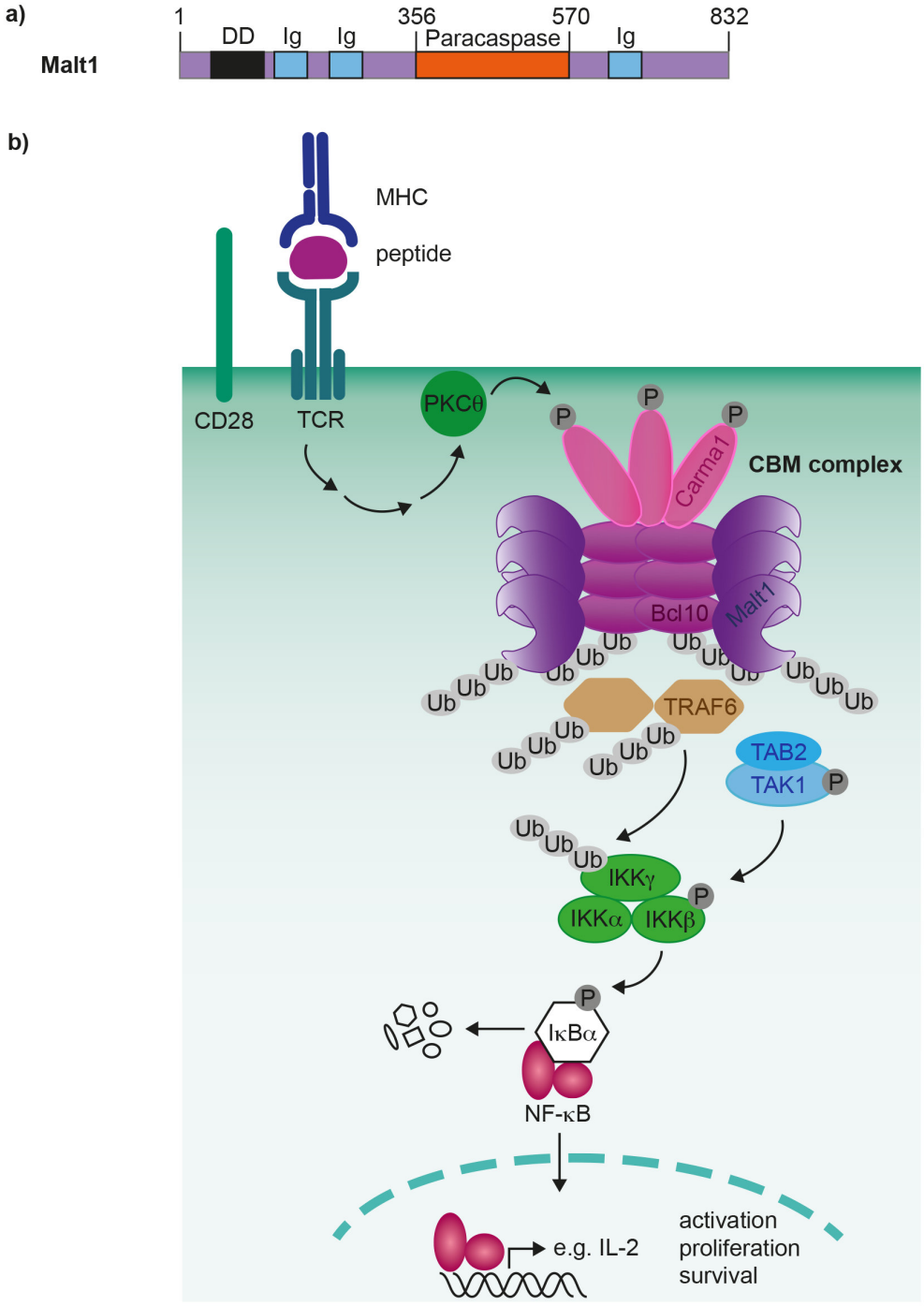

Fig. 1: Malt1 in TCR-induced NF-κB activation

a) Domain organization of Malt1. **b)** Schematic overview illustrating the role of Malt1 linking TCR activation to NF-κB activation (for details, see text 1.1.2.2.). DD, death domain; Ig, Immunoglobulin. The figure was developed based on(Thome, 2008) and (Qiao et al., 2013).

5.1.2.3 T cell differentiation

The expression of subset-defining transcription factors in each $CD4^+$ subset programs the cells for production of subset-specific cytokines that are key for their effector functions (Reiner, 2007; Zhu et al., 2010). Among the established subsets are T_H1, T_H2 (Mosmann et al., 1986) and T_H17 (Harrington et al., 2005; Park et al., 2005) as well as peripheral iTregs (inducible Tregs) (Wilczynski et al., 2008; Yamane and Paul, 2012). Furthermore, T follicular helper cells (T_{FH}) cells have emerged as an important new T_H subset and T_H9 and T_H22 are still discussed as independent subsets (Fig. 2). Despite this classification, there is great plasticity that allows differentiated effector T cells to convert into other effector subtypes under certain conditions (Nakayamada et al., 2012; Wan, 2010; Zhou et al., 2009).

5.1.2.3.1 T_H1 and T_H2 cells

IL-12, a cytokine of activated macrophages and DCs, drives the differentiation of T_H1 cells via a signal transducer and activator of transcription 4 (STAT4)-dependent pathway (Glimcher and Murphy, 2000; Hsieh et al., 1993). T_H1 cells express the transcription factor T-bet (T box expressed in T cells), which in turn induces the expression of the signature T_H1 cytokine, interferon-γ (IFN-γ) (Szabo et al., 2000). At the same time, IFN-γ itself is important for T_H1 differentiation because it induces T-bet expression via STAT1-signaling (Afkarian et al., 2002; Mullen et al., 2001) as well as expression of the β2-chain of the IL-12 receptor (IL-12R). Furthermore, T_H1 cells express TNF and IL-2. T_H2 cells are induced by IL-4, that leads via STAT6 to the expression of the transcription factor GATA3 and subsequent secretion of IL-4, IL-5, and IL-13 (Takeda et al., 1996; Zhang et al., 1997; Zheng and Flavell, 1997). Interferon-regulatory factor 4 (Irf4) was identified as another transcription factor relevant for T_H2 development (Lohoff et al., 2002). Concomitantly to T_H1 induction, IL-12 constrains the generation of T_H2 cells, illustrating a general concept of negative feedback loops on alternative cell-fate differentiation programs in T cells (Zhu and Paul, 2008). In line with this, IL-4 suppresses IL-12 signaling and therefore T_H1 differentiation by inhibition of IL-12Rβ2 expression (Szabo et al., 1997). T_H1 cells help to fight intracellular pathogens such as viruses whereas T_H2 cells are mainly involved in initiating antibody-mediated immune responses against extracellular pathogens and parasites. T_H1 cells are also associated with organ-specific autoimmune disorders and T_H2 cells are involved in the development of allergy and asthma (Zhu et al., 2010).

5.1.2.3.2 T_H17 cells and Tregs

T_H17 cells express the transcription factor RORγt and RORα (retinoic acid-related orphan receptors) (Ivanov et al., 2006; Yang et al., 2008) and are named after the production of their signature cytokines IL-17A and IL-17F. T cell activation in combination with IL-6 and

transforming growth factor β (TGF-β) signals induces T_H17 cells *in vitro* (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006) and is fueled by addition of IL-1β and TNF (Veldhoen et al., 2006). *In vivo*, IL-21, produced by T_H17 cells themselves, amplifies the accumulation of T_H17 cells in a positive feedback loop and IL-21 together with TGF-β is sufficient for their *in vitro* differentiation (Korn et al., 2007). In addition, IL-21 induces the upregulation of the IL-23 receptor (IL-23R) to make T_H17 cells responsive to IL-23, which is secreted by activated DCs (Nurieva et al., 2007; Zhou et al., 2007). IL-23-signaling mediates expansion and survival of T_H17 cells and thus contributes to the maintenance of the T_H17 phenotype (Ivanov et al., 2007). IL-6, IL-21 and IL-23 signal via the STAT3-signaling pathway that is also responsible for inducing IL-17, IL-21 and IL-23R expression (Chen et al., 2007). Moreover, Irf4 is also critical for T_H17 differentiation since T cells from Irf4-deficient mice fail to become T_H17 cells (Brüstle et al., 2007; Huber et al., 2008). T_H17 cells sustain an inflammatory response by inducing the expression of pro-inflammatory cytokines and by recruiting neutrophils to the site of infection (Weaver et al., 2007). These mechanisms are essential for the defense against extracellular bacteria and fungi but the relevance of deregulated T_H17 responses has also been described in autoimmune disorders (Singh et al., 2014) such as multiple sclerosis (Korn et al., 2009). Recently, it was shown that some asthma patients have T_H17-associated infiltrations of neutrophils in the lung even though asthma is normally associated with a T_H2 response and eosinophilic infiltration (Lambrecht and Hammad, 2014).

As mentioned above, T cells with high-affinity TCRs for self-peptides are selected in the thymus and acquire a regulatory phenotype (Sakaguchi, 2004). Apart from these thymus-derived $CD4^+CD25^+$ natural Tregs (nTregs), T cells with a regulatory function also arise in the periphery from naive $CD4^+$ T cells after "tolerogenic" encounters (so-called inducible Tregs (iTregs) (Apostolou and von Boehmer, 2004; Curotto de Lafaille et al., 2004). Both, nTregs and iTregs, express the specific transcription factor forkhead box protein 3 (Foxp3) that is essential for their function (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). In contrast to T_H17 cells, Tregs are crucial in maintenance of peripheral tolerance by controlling other effectors cells and contribute to prevention of autoimmunity (see 5.1.4). Despite their contrary functions, T_H17 cells and iTregs are connected via a reciprocal differentiation program (Bettelli et al., 2006; Zhou et al., 2008) that also involves TGF-β (Chen et al., 2003). TFG-β alone induces the expression of both RORγt and Foxp3 in TCR-stimulated naive $CD4^+$ T cells (Bettelli et al., 2006; Chen et al., 2003; Ivanov et al., 2007). Without pro-inflammatory cytokines, Foxp3 constrains RORγt-induced IL-17 production (Zhou et al., 2008). IL-6 and IL-21

relieve RORγt suppression and promote RORγt expression with simultaneous inhibition of Foxp3 expression and function.

Interestingly, Tregs can coopt the gene expression program of their target cells to restrain a particular effector T cell response type. Expression of canonical T_H cell-associated transcription factors such as T-bet, Irf4 or STAT3 in response to environmental cues equip $Foxp3^+$ Tregs with the necessary migratory ability to co-localize with their target effector cells (Campbell and Koch, 2011; Josefowicz et al., 2012).

5.1.2.3.3 T_{FH} cells and germinal center reaction

T_{FH} cells have been proposed as an individual T_H cell subset characterized by the expression of chemokine receptor CXCR5 and localization to B cell follicles and germinal centers (GC) and are recognized as an independent lineage today (Breitfeld et al., 2000; Schaerli et al., 2000). In the GC reaction, T_{FH} cells provide help to B cells through cell-surface receptors such as CD40 ligand (CD40L) interaction and expression of cytokines such as IL-21 and IL-4. This enables GC B cell differentiation and drives Ig class switch to generate long-lived plasma cells that produce high-affinity antibodies (Crotty, 2011). Bcl6 (B cell lymphoma 6) was discovered as the critical transcription factor for T_{FH} cell differentiation and development of GCs (Johnston et al., 2009; Linterman et al., 2009; Nurieva et al., 2009; Yu et al., 2009) but additional transcription factors like c-Maf and Batf are also involved (Bauquet et al., 2009; Betz et al., 2010; Ise et al., 2011; Kroenke et al., 2012). Furthermore, Irf4-deficient mice have defects in T_{FH} cell differentiation and IL-21 induction indicating a critical contribution of this factor in T_{FH} differentiation (Bollig et al., 2012; Kwon et al., 2009). T_{FH} cell generation is a multistep process that depends on a multitude of signals including TCR and co-stimulation, interaction of T cells with APCs as well as coordinated localization of the T cell to the B cell follicle. In early T_{FH} differentiation, naive $CD4^+$ T cells are primed by DCs and receive signals via IL-6, Icos, IL-2 and TCR signaling independent of B cell contact. The inducible co-stimulator Icos is critical for T_{FH} differentiation (Choi et al., 2011; Nurieva et al., 2008) whereas IL-2 signaling inhibits early T_{FH} differentiation (Ballesteros-Tato et al., 2012; Johnston et al., 2012). Upregulation of CXCR5 and downregulation of CCR7 (chemotactic receptor for T cell zone) on T_{FH} cells leads to co-localization with B cells in the follicle at the T-B border (Ansel et al., 1999; Kim et al., 2001) and is thought to be mediated by the transcription factor Ascl2 (achaete-scute homologue 2) (Liu et al., 2014). At the T-B border, T_{FH} cells interact with B cells via antigenic stimulation, which is required for further T_{FH} cell development and their maintenance (Linterman et al., 2012). Icos ligand expression by follicular bystander B cells drive T cell migration to the B cell follicle (Xu et al., 2013). SAP (signaling lymphocyte activation molecule [SLAM]-associated protein) support recruitment to GC by promoting stable interaction with cognate B cells (Qi et al., 2008).

GC consists of GC T_{FH} cells, GC B cells, follicular DCs, macrophages and stroma cells and a light zone (LZ) and a dark zone (DZ) can be distinguished. In the LZ, B cells encounter their antigen and present it to T_{FH} cells to receive proliferative and survival signals in turn to allow their migration to the DZ. There, they undergo somatic hypermutation, where single nucleotides in BCR genes can be substituted to generate mutant BCR. Upon returning to the LZ, mutated B cells with the highest affinity for antigen are selected by T_{FH} cells (Crotty, 2014). A challenge for T_{FH} cells is to give cognate B cells temporary help without desensitizing due to constant TCR signals or without themselves responding with vigorous proliferation. One way this may be achieved is via high expression of the co-stimulatory molecule PD-1 (programmed cell death 1) that attracts phosphatases and dampens TCR signaling through dephosphorylation of TCR signaling components (Crotty, 2014; Yokosuka et al., 2012). Due to their critical function in effective antibody responses that neutralize pathogens and confer protection upon reinfection, T_{FH} cells are involved in the defense against most pathogens. On the downside this makes T_{FH} cells central players in autoimmunity. Failed deletion of somatically mutated auto-reactive GC B cells can result in autoimmunity similar to human systemic lupus erythematosus (SLE) which is related to the generation of auto-antibodies (Craft, 2012; Linterman et al., 2009; Vinuesa et al., 2009). As a mechanism of GC tolerance, thymus-derived $Foxp3^+$ Tregs can specialize to control the GC reaction and thereby regulate antibody responses. In addition to CD25 and Ctla-4 (cytotoxic T-lymphocyte-associated antigen 4; CD152) expression, these so-called follicular Tregs (Tfr) acquire expression of Bcl6, CXCR5, PD-1 and Icos, which enables them to migrate into GCs (Linterman et al., 2012; Ma et al., 2012). There, Tfr were shown to suppress T_{FH} cells to prevent help of self-reactive T_{FH} cells to B cells or outgrowth of non-antigen-specific B cells and Tfr may also directly eliminate auto-reactive GC B cells (Chung et al., 2011; Linterman et al., 2011).

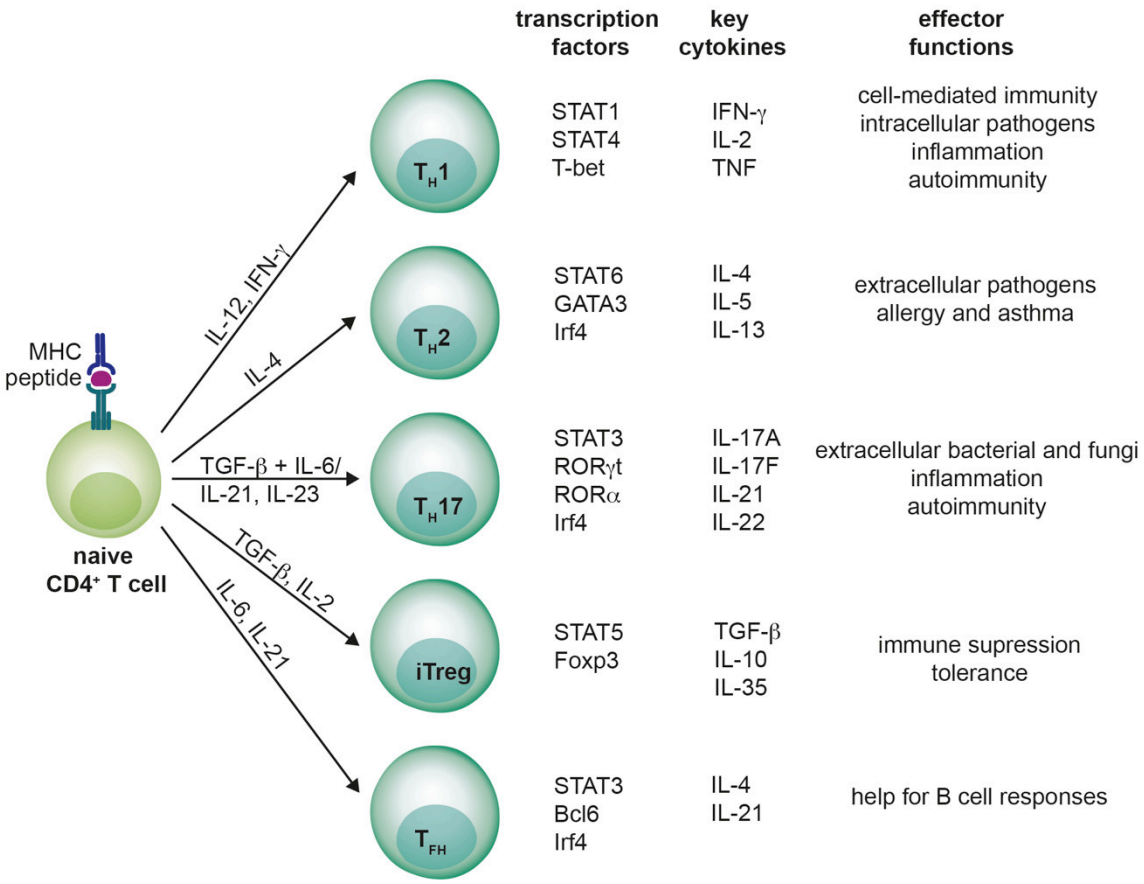

Fig. 2: Schematic representation of CD4$^+$ T cell differentiation

Modes of induction, important transcription factors, key cytokines and effector functions against specific pathogens of CD4$^+$ T cells (for details, see text 1.1.2.3.). iTregs, inducible regulatory T cells; T_{FH}, T follicular helper cells.

5.1.2.4 T cell metabolism

The regulation of metabolic processes is essential for T cell activation, proliferation, differentiation and function by ensuring energy supply and biosynthesis. Hence, changes in the metabolism influence the kind of T cell response and in this manner metabolic signaling pathways are closely connected to immunologic functions. Activation of naive T cells leads to a switch from oxidative metabolism to aerobic glycolysis as the main metabolic program (Gerriets and Rathmell, 2012). This promotes efficient and rapid biosynthesis as metabolic intermediates of glucose degradation can be fed into synthesis of proteins, nucleic acids and lipids. Additionally, some glucose passes through the mitochondria and part of the tricarboxylic acid (TCA) cycle to produce citrate for lipid synthesis. Furthermore, activated T cells increase glutamine oxidation, where glutamine is converted into alpha-ketoglutarate, which goes into the TCA cycle to compensate the diversion of other intermediates used for biosynthesis (MacIver et al., 2013). In contrast, naive T cells oxidate pyruvate, a metabolite of glucose degradation, together with lipids and amino acids via the TCA cycle to efficiently generate energy in the form of ATP (MacIver et al., 2013)(Gerriets and Rathmell, 2012).

Likewise, surviving memory T cells will revert to lipid oxidation and higher energy production at the end of an immune response (MacIver et al., 2013). Metabolic reprogramming and enhanced glycolysis of T cells after TCR ligation are dependent on co-stimulation via CD28 (Frauwirth et al., 2002). Phosphatidylinositol 3-kinase (PI3K)-Akt signaling activate mTOR (mammalian target of rapamycin) and result in localization of the glucose transporter Glut1 to the cell surface to enable higher glucose uptake. Furthermore, the transcription factor c-Myc is induced after T cell activation and promotes the expression of Glut1 and additional genes important for glycolysis and glutaminolysis (MacIver et al., 2013). T cell subsets differ in their metabolic requirements for energy or biosynthesis to support their specific functional needs. Thus, T cell activation under varying conditions not always leads to the same metabolic program in T cells (MacIver et al., 2013). T_H1 and T_H17 cells require active mTORC1 whereas T_H2 cells require active mTORC2 (Delgoffe et al., 2011). *De novo* fatty acid synthesis is a prerequisite for effector T cell proliferation and differentiation (Lochner et al., 2015). In contrast to glucose-dependent effector T cells, Tregs only engage in glycolysis to a small extent and rely on lipid oxidation for energy supply. $CD4^+$ Tregs show increased levels of active, phosphorylated AMP-activated protein kinase (AMPK) that inhibits mTOR signaling and fosters oxidative metabolism in mitochondria and thus lipid oxidation (Lochner et al., 2015). **(MacIver et al., 2013)**

5.1.3 Peripheral T cell tolerance

Although positive and negative selection in central tolerance are efficient mechanisms, some potentially auto-reactive T cells bearing TCRs with intermediate affinity escape into the periphery and have to be kept in check to prevent autoimmunity (Goodnow et al., 2005). (Walker and Abbas, 2002)The mechanisms of peripheral tolerance act either directly in self-reactive T cells (T cell-intrinsic) like anergy or apoptosis or indirectly via additional subsets of cells like tolerogenic DCs or Tregs (T cell-extrinsic) (Walker and Abbas, 2002).

5.1.3.1 T cell-intrinsic mechanisms of peripheral tolerance

One known mechanism of peripheral tolerance is immunological ignorance which describes the simple physical separation of auto-reactive T cells from their antigens, for example by the blood-brain barrier (Mueller, 2010). Yet, even when T cells undergo full activation in response to self-molecules, autoimmune tissue damage can be avoided by skewing T cells to a non-pathogenic phenotype depending on the cytokines and chemokine receptors they express (Walker and Abbas, 2002). Suboptimal activation signals, such as self-antigen presentation (signal 1) in the absence of co-stimulation (signal 2) cannot induce T cell responses and render self-reactive T cells functionally unresponsive (anergy) or induce apoptosis (Kamradt and

Mitchison, 2001). Anergy describes a state of long-term hypo-responsiveness accompanied by impairment of TCR signaling, reduced proliferation and the inability to produce cytokines like IL-2 in response to a second antigen encounter (Schwartz, 2003). Anergy involves the interaction of inhibitory receptors like Ctla-4 or PD-1 with their ligands CD80/86 and PD-1 ligand-1/2 (PDL1/2), respectively (Fife and Bluestone, 2008). Establishment of an anergic state requires an anergy-inducing program of gene expression. This is controlled by a specific set of transcription factors including NFAT. In case of TCR signaling that lacks co-stimulatory signals, DAG fails to properly activate the PKC family members and Ras/MAPK signaling pathways to induce AP-1 formation (Valdor and Macian, 2013). IP3-mediated calcium increase on the other hand requires only TCR engagement and leads to the activation of NFAT proteins, which form homodimers in the absence of AP-1. These induce the expression E3 ubiquitin ligases, proteases, transcriptional regulators and signaling enzymes that inhibit T cell activation on different levels (Baine et al., 2009). Another way of eliminating auto-reactive T cells from the repertoire is apoptosis, a process known as deletion or activation-induced cell death (AICD). Repetitive engagement of the TCR with self-peptide might be followed by the activation of apoptotic signaling pathways like the Fas (CD95) death receptor pathway (Mueller, 2010). Upregulation of FasL (Fas ligand) and subsequent signaling through Fas of the TNF receptor family leads to T cell apoptosis via the caspase-8 proteolytic cascade (Goodnow et al., 2005).

5.1.3.2 T cell-extrinsic mechanisms of peripheral tolerance

Peripheral tolerance involves regulatory cell populations like tolerogenic DCs and Tregs. DCs are considered as the most important APCs that organize the T cell response, mastering the decision between immunity and tolerance. Currently, there are two models to explain how this is achieved. On the one hand, pattern-recognition receptors (PRRs) recognize conserved PAMPs and subsequent signaling leads to maturation of DCs marked by the upregulation of co-stimulatory ligands (e.g. CD80/86, CD40) (signal 2), adhesion molecules, and chemokine receptors on their surface and the release of cytokines (signal 3). This enables DCs to activate T cells with TCRs recognizing the presented antigen (signal 1) and initiates an immune response. The model was proposed by Charles Janeway in 1992 (Janeway, 1992) and confirmed through the identification of Toll-like receptors (TLRs) by Ruslan Medzhitov (Medzhitov et al., 1997). TLRs are PRR recognizing PAMPs like components of the bacterial wall, such as lipopolysaccharide (LPS), which is recognized by TLR4. Upon ligand binding TRLs trigger signaling pathways that lead to the activation of innate immune cells like DCs (Takeuchi and Akira, 2010). A second model neglects the idea of immunologic discrimination between self and non-self and suggests the danger model (Matzinger, 2002). The release of intracellular molecules like heat-shock proteins from attacked, necrotic cells reflect the danger by invading

pathogens and serve as “danger signals” for DCs. These so-called damage-associated molecular patterns (DAMPs) instruct the DC to provide a full stimulus to T cells (Basu et al., 2000; Matzinger, 1994). Constant presentation of self-proteins by DCs in the absence of infection is not linked to co-stimulation and leads to T cell tolerance rather than activation. However, it remains elusive, if any immature DCs can elicit this function or if a dedicated subset of tolerogenic DCs is responsible (Walker and Abbas, 2002). Moreover, peripheral T cell tolerance can be mediated indirectly via the induction of Tregs, which controll or suppress the activity of other effector cells (Curotto de Lafaille and Lafaille, 2009; Sakaguchi, 2004). They are able to suppress the activation, proliferation and IL-2 production of responder T cells as well as the function of APCs. Several ways of suppression are mediated by Tregs and may act synergistically and complementary (Wing and Sakaguchi, 2010). In part, Tregs exert suppression in a cell contact-dependent manner, for example through surface molecules, such as the co-receptor Ctla-4 that delivers a suppressive signal through binding of CD80/CD86 on target T cells (Paust et al., 2004, p. 200; Taylor et al., 2004). Furthermore, Tregs produce immunosuppressive cytokines like IL-35, IL-10 or TGF-β to mediate suppression of T cell responses (Josefowicz et al., 2012). Other proposed mechanisms are modulation of DC maturation and function, outcompeting other T cells for IL-2 survival factor or even direct granzyme- and perforin-dependent killing of target cells (Wing and Sakaguchi, 2010).

5.1.4 Break of tolerance

Loss of tolerance is a multi-factorial process of polygenic nature that leads to autoimmunity. It has not been fully elucidated how genetic predispositions like polymorphisms or environmental factors, such as pathogen exposure or hormone levels, contribute to the diversity of symptoms (Chang, 2014; Rioux and Abbas, 2005). The importance of immunologic tolerance is exemplified by the identification of rare monogenetic causes of autoimmune disorders that provide insights into fundamental mechanisms. One possible scenario to break self-tolerance is a decrease in thymic expression of self-antigens (Rioux and Abbas, 2005). Mutations in the gene encoding AIRE lead to defective clonal deletion of auto-reactive T cells causing the multi-organ syndrome APS1 (autoimmune polyglandular syndrome type 1) formerly known as APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy) in humans (Finnish-German APECED Consortium, 1997; Nagamine et al., 1997; Peterson et al., 1998). Mice deficient for AIRE develop a similar organ-specific autoimmunity (Anderson et al., 2002). Failure of T cell anergy and reduced activation thresholds of self-reactive T cells can also trigger autoimmunity. *Ctla4* knockout mice succumb to lymphoproliferative disease with multi-organ lymphocytic infiltration and tissue destruction

(Tivol et al., 1995; Waterhouse et al., 1995). As stated before, apoptosis is a relevant mechanism for cell death at checkpoints in immunologic tolerance. So it is not surprising that defects in the Fas pathway in humans lead to autoimmune lymphoproliferative syndrome (ALPS) (Fisher et al., 1995; Nagata, 1998). Similarly, MRL/*lpr* and *gld* mice with defects in Fas and FasL, respectively, develop a lymphoproliferative SLE-like syndrome (Sobel et al., 1993; Suda et al., 1993; Watanabe-Fukunaga et al., 1992). Autoimmunity not only results from a failure of tolerance mechanisms but is also attributed to deregulated cell fate decisions of $CD4^+$ T helper cells. Major contributors to the maintenance of peripheral tolerance are $CD4^+CD25^+$ Tregs. Knockout or mutations of their key transcription factor Foxp3 decreases Treg generation and suppressor activity leading to spontaneous systemic autoimmune disease, known as the "scurfy" phenotype in mice (Brunkow et al., 2001; Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003) and early-onset fatal immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in humans (Gambineri et al., 2003; Wildin et al., 2001).

5.2 Post-transcriptional gene regulation

Adaption of immune responses to ever changing intracellular and extracellular stimuli ensures adequate function of the immune system. A rapid and transient response can be achieved through post-transcriptional control mechanisms. Thus, it is no surprise that imbalances between the messenger RNAs (mRNAs) stability and decay of immune effector proteins like inflammatory cytokines contribute to the pathogenesis of autoimmune diseases. An extensive network of post-transcriptional mechanisms evolved based on the interaction of *trans*-acting factors such as RNA-binding proteins (RBPs) and microRNAs (miRNAs) and *cis*-acting sequence elements within protein-coding mRNAs. These elements exist largely in the 5' and 3' untranslated regions (UTRs) of mRNAs. Post-transcriptional RNA regulons present an effective mechanism to turn immune and inflammatory response on and off as required.

5.2.1 Mechanisms of mRNA stability and decay

Typically, mature mRNAs are stabilized by two co-transcriptionally integrated structures, the poly(A) tail, a polymeric stretch of 25-200 adenine nucleotides at the 3' end, and the 5' 7-methylguanosine cap. The eukaryotic initiation factor eIF4E and the polyadenylate-binding protein (PABP) bind to the 5' cap and the poly(A) tail, respectively. Furthermore, the 40S ribosomal subunit binds to the cap via eIF3 and the adaptor molecule eIF4G. The interaction of PABP with eIF4G results in circularization of the mRNA. Thus, the mRNA is protected from degradation by exonucleases and translation initiation is facilitated (Holcik and

Sonenberg, 2005). The balance between transcription and decay determines the half-life of any given mRNA. The cytoplasmic mRNA decay network comprises decapping, 5'-3' exonucleolytic decay, deadenylation, 3'-5' exonucleolytic degradation or endonucleolytic cleavage (Schoenberg and Maquat, 2012).

5.2.1.1 Exonuclease-mediated decay pathways

Most mRNAs are degraded by exonucleases acting at both ends of the molecule (Fig. 3). Exonucleolytic degradation is initiated by shortening of the poly(A) tail by multiprotein complexes containing mRNA deadenylases in a process termed deadenylation (Parker and Song, 2004). Three different deadenylase enzyme complexes are known: the complex of PAB-specific ribonuclease 2 (PAN2) and PAN3, multi-subunit complex CCR4-POP2-NOT1 containing CCR4a (CNOT6), CCR4B (CNOT6L), CAF1A (CNOT7) and POP2 (CNOT8, CAF1B) deadenylases associated with regulatory NOT factors and the deadenylase poly(A)-specific ribonuclease (PARN) (Chen and Shyu, 2011). PAN2-PAN3 nucleases decrease the length of the poly(A) tail to ~50 nucleotides before CNOT6-CAF1-NOT1 further deadenylates the mRNA (Anderson, 2010). In contrast to other deadenylases, PARN function is dependent on the presence of a 5' cap (Garneau et al., 2007). Proteins that are associated with specific mRNAs determine which deadenylase complex is recruited for deadenylation (Ivanov and Anderson, 2013). The first step of deadenylations lead to removal of PABP and allow hydrolysis of the 5' cap through the action of a decapping enzyme consisting of Dcp1A and Dcp2 and 5'-3' degradation by the exoribonuclease 1 (Xrn1) (Schoenberg and Maquat, 2012). Additional factors are necessary for efficient decapping such as the Sm-like (Lsm) proteins. The heteroheptameric Lsm1-7 complex associates with oligoadenylated 3' tails of transcripts and stimulates Dcp1a/Dcp2 decapping (Tharun et al., 2000; Tharun and Parker, 2001). The regulatory Edc (enhancer of decapping) proteins and the helicase Rck also mediate decapping in functional cooperation with Dcp1A and Dcp2 (Coller et al., 2001; Schwartz et al., 2003)(Coller et al., 2001; Coller and Parker, 2005; Schwartz et al., 2003). Alternatively, mRNA can be degraded without prior decapping via the unprotected 3' UTR in 3'-5' direction by the large exosome complex containing multiple exonucleases (Parker and Song, 2004). The remaining cap is removed from short oligonucleotides by the scavenger decapping enzyme DcpS (Parker and Song, 2004). The 3'-5' and the 5'-3' degradation pathways are not mutually exclusive although it is not entirely clear how redundant these mechanisms are (Garneau et al., 2007). mRNA turnover is not only essential in the control of gene expression but also necessary for mRNA quality control to prevent the generation of abnormal proteins. Decay of mRNAs containing premature stop codons is achieved by nonsense-mediated decay (NMD) that acts deadenlylation-independent

and mRNAs that lack termination codons are recognized and degraded via nonstop decay (NSD) in combination with the described decay mechanisms (Schoenberg and Maquat, 2012).

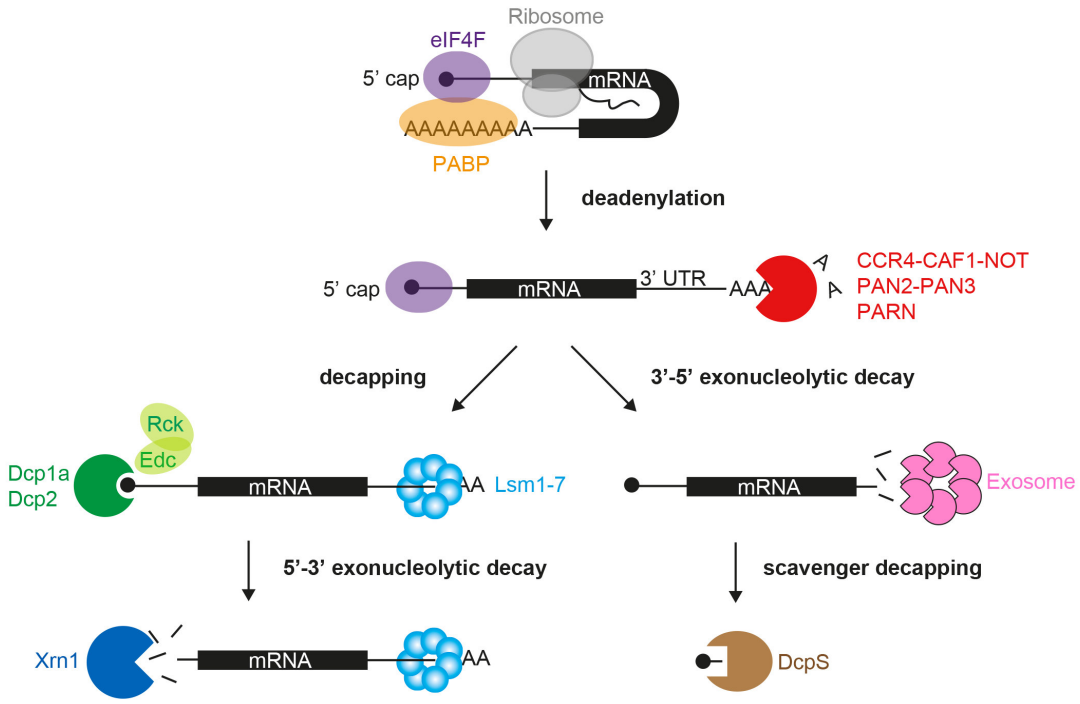

Fig. 3: Scheme of exonuclease-mediated decay pathways

5'-3' and 3'-5' exonucleolytic decay mechanisms (for details, see text 5.2.1.1.). eIF4F, eukaryotic initiation factor 4F; PABP, polyadenylate-binding protein; PAN2/3, PAB-specific ribonulcease 2/3; PARN, poly(A)-specific ribonuclease; Edc, enhancer of decapping ; Xrn1, exoribonuclease 1; Lsm, Sm-like protein; DcpS, scavenger decapping enzyme. The figure was developed based on (Garneau et al., 2007).

5.2.1.2 Endonuclease-mediated decay pathways

In some cases, mRNA degradation is started by site-specific endonuclease cleavage that introduces internal cuts and yields mRNA fragments susceptible to exonucleolytic decay in 5' or 3' direction by Xrn1 and the exosome, respectively (Schoenberg, 2011). The polysome-associated endonuclease PMR1 as well as the endonuclease IRE1 for example target actively translating mRNAs (Garneau et al., 2007). Another endonuclease called regulatory RNase-1 (Regnase-1) is presumed to directly bind its targets mRNAs (Akira, 2013) (see 5.3.2). Likewise, miRNA binding to target mRNAs can result in endonucleolytic degradation by the Argonaute protein-2 (AGO2) (see 5.2.2.3).

5.2.1.3 P bodies and stress granules

The actual sites of mRNA turnover are ribonucleoprotein (RNP) complexes that form granular cytoplasmic foci known as mRNA-processing (P) bodies (Eulalio et al., 2007). P bodies contain specific mRNAs, as well as many RBPs and miRNAs required for mRNA decay, translational silencing and mRNA storage (Franks and Lykke-Andersen, 2008). Cellular stress such as infections or inflammation can also cause formation of discrete cytoplasmic foci termed stress

granules (SGs) (Anderson and Kedersha, 2008). SGs are induced by polysome disassembly and contain polyadenylated mRNAs bound to stalled pre-initiation complexes that block translation (Anderson and Kedersha, 2009). In contrast to P bodies, SGs normally do not incorporate mRNA degradation enzymes but rather serve as storage units for mRNAs awaiting further "decisions" (Anderson and Kedersha, 2009). P bodies and SGs are dynamic structures with constantly changing composition of RBPs and mRNAs (Glisovic et al., 2008) that can merge with one another or spontaneously separate (Kedersha et al., 2005). Hence, P bodies and SGs represent the physical sites where mRNA translation and degradation are regulated.

5.2.2 *Cis*-elements and *trans*-acting factors

The post-transcriptional control of transcripts is based on mRNA regulatory *cis*-elements that are recognized by *trans*-acting factors. The *cis*-elements are mainly located in the non-coding 3' and 5' UTR of mRNAs and comprise sequences, secondary structures (e.g. stem loops, internal bulges) and/or multidomain protein assembly (Kafasla et al., 2014). Interestingly, UTRs are highly conserved through species indicating the need to maintain regulatory elements within these sequences (Matoulkova et al., 2012). *Trans*-acting factors such as RBPs or non-coding RNAs like miRNAs that bind these *cis*-elements can either directly recruit factors of the degradation machinery or induce changes in the overall mRNP composition leading to altered susceptibility of mRNAs to different decay enzymes (Keene, 2007; Parker and Song, 2004). In addition, various aspects of the translation process itself are controlled by t*rans*-acting factors. RBPs bind to RNA via their RNA-recognition domains and carry additional domains that allow protein-protein interactions, direct modifications of RNA or mediate catalytic events (Kafasla et al., 2014; Lunde et al., 2007). Generally, each 3' UTR is composed of more than one regulatory *cis*-element such that a complex network of regulatory factors can bind in cooperation or competition. These *trans*-acting factors can have redundant, additive or antagonistic effects on the mRNA "life".

5.2.2.1 AU-rich elements and their binding proteins

The most studied cis-regulatory RNA elements are AU-rich elements (ARE) and their binding factors, ARE-binding proteins (ARE-BPs). Almost 30 years ago, AUUUA pentamers were found in an adenine und uridine-rich region within the 3' UTR of short-lived cytokine mRNAs (Caput et al., 1986). AREs were first shown to mediate rapid decay of the mRNA encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) (Shaw and Kamen, 1986). As many as 5-10% of the human genes possess AREs in their mRNA sequence with an enrichment of AREs in genes important in immunity (Bakheet et al., 2006). ARE-BPs targeting ARE-containing mRNAs directly modulate mRNA stability or translational efficiency. Generally, they

have multiple functions and auto-regulatory ability, shuttle between the nucleus and the cytoplasm and are regulated themselves via post-transcriptional modifications mainly via kinase signaling pathways (Matoulkova et al., 2012). Examples of such ARE-BPs are AU-rich binding factor-1 (AUF1), tristetraprolin (TTP), K homology (KH) splicing regulatory protein (KSRP), embryonic lethal abnormal vision (ELAV), and T cell-restricted intracellular antigen 1 (TIA-1) and TIA-1-related protein (TIAR) (Garneau et al., 2007; Ivanov and Anderson, 2013). ARE-BPs can directly or indirectly interact with factors of the mRNA-decay machinery to recruit them to the ARE-containing mRNA for their rapid degradation (Garneau et al., 2007). TTP and KSRP for instance, recruit the exosome to induce 3'-5' degradation (Chen et al., 2001; Hau et al., 2007). Moreover, interaction of KSRP with PARN results in deadenylation and decay of the bound target mRNA (Gherzi et al., 2004) and TTP also interacts with decapping enzymes Dcp1 and Dcp2, CNOT6 deadenylase and Xrn1 (Hau et al., 2007; Lykke-Andersen and Wagner, 2005). TIA-1 and TIAR function as transcriptional silencers in the cytoplasm by stalling the pre-initiation complex and sequestering untranslated mRNAs in SGs during the stress-response (Ivanov and Anderson, 2013; López de Silanes et al., 2005). ARE-BPs can also act in a stabilizing manner like HuR (human antigen R; Elav1), a member of the ELAV protein family. HuR was shown to compete with the destabilizing factors AUF1, KSRP and TTP for ARE binding to selected mRNAs and thus prevents mRNA degradation (Lal et al., 2004; Linker et al., 2005). In addition, HuR was described to either promote or inhibit translation (Anderson, 2008; Matoulkova et al., 2012). The complexity of HuR functions arises from its multiple interactions with several different classes of ARE-BPs (Ivanov and Anderson, 2013). TTP was discovered in knockout mice that show autoimmune inflammation accompanied by cachexia, spontaneous arthritis, dermatitis, neutrophilia and auto-antibodies (Taylor et al., 1996)(Carballo et al., 1998; Taylor et al., 1996). Regulation of pro-inflammatory cytokines like TNF (Carballo and Blackshear, 2001; Taylor et al., 1996), GM-CSF (Carballo et al., 2000), IL-1β (Chen et al., 2006), IL-2 (Ogilvie et al., 2005), IFN-γ (Ogilvie et al., 2009) and IL-6 (Sauer et al., 2006) as well as several chemokines makes TTP a critical factor in dampening the inflammatory response.

5.2.2.2 Other *cis*-elements and RNA binding proteins

Like AREs, GU-rich elements (GREs) with overlapping GUUUG pentamers as a basic sequence motif regulate many short-lived transcripts involved in cell activation and development (Vlasova-St Louis and Bohjanen, 2011). GREs are bound by CUG-binding proteins (CUGBP) and share functional similarity with AREs (Ivanov and Anderson, 2013; Moraes et al., 2006). Another example for a *cis*-element is GAIT (IFN-γ-activated inhibitor of translation), a 29-nucleotide hairpin in the 3' UTR of a group of mRNAs encoding e.g. inflammatory mediators as chemokines and chemokine receptors or the vascular endothelial growth

factor A (VEGFA) (Kafasla et al., 2014). The GAIT element is bound by the IFN-γ-induced GAIT complex, a tetramer of four different proteins that also have other cellular functions such as glyceraldehyde 3 phosphate dehydrogenase (GAPDH) and ribosomal protein L13a (Rpl13a). Recruitment of the GAIT complex mediates translational repression by inhibiting translation initiation (Anderson, 2010). The iron responsive elements (IREs) are located in the 3' UTR as well as in the 5' UTR of genes involved in iron metabolism and form stem-loop structures with a hexameric loop and a stem with a small C-bulge. IRE-RNA-binding protein 1 (IRP1) binds IREs in the 5' UTR to block translation initiation whereas IRP2 binds IREs in the 3' UTR to stabilize the target mRNA (Matoulkova et al., 2012). The 3' UTR of the *Tnf* mRNA encodes a constitutive decay element (CDE) downstream of the known ARE. The CDE ensures TNF decay in LPS-stimulated macrophages in conditions where ARE-mediated decay is blocked to prevent excessive TNF production (Stoecklin et al., 2003). Similarly, the stem-loop-destabilizing element (SLDE) was found in addition to the ARE in GM-CSF and accelerates the deadenylation rate, a fail-safe mechanism when ARE-mediated decay is inhibited (Brown et al., 1996; Putland et al., 2002). In contrast to the RBPs introduced above, factors like Regnase-1 and AGO2 can directly cleave their specific target transcripts (Anderson, 2010). The endonuclease Regnase-1 was described to cleave within a putative stem-loop structure that lies upstream of five conserved AREs in the 3' UTR of *Il6* mRNA to initiate its decay (Matsushita et al., 2009; Paschoud et al., 2006).

5.2.2.3 MicroRNAs

Other trans-acting factors in post-transcriptional gene regulation are miRNAs. miRNA-binding sites within 3' UTRs comprise the most common *cis*-elements besides AREs and are overrepresented in immune genes (Asirvatham et al., 2008). Post-transcriptional regulation by miRNAs plays a role in many signal transduction pathways that control differentiation, proliferation, apoptosis or metabolism. miRNAs are single-stranded RNAs with a length of 21 nucleotides and are complementary to miRNA-binding sites in target mRNAs, which they bind by imperfect base pairing. The RNase III enzymes Drosha and Dicer sequentially process miRNAs from longer transcripts called primary miRNAs (pri-miRNAs) to pre- and mature miRNAs. Pri-miRNAs contain characteristic stem-loop structures and are processed into ~65 nucleotide long hairpins in the nucleus by Drosha (Baumjohann and Ansel, 2013). Exportin 5 mediates the export of the resulting pre-miRNAs into the cytoplasm, where they are recognized by Dicer (Hoefig and Heissmeyer, 2008). Dicer cleaves off the loop and thus generates mature miRNAs (Hoefig and Heissmeyer, 2008). Single-strand miRNAs result from segregation of the short miRNA duplex and are bound to AGO proteins as part of the miRNA-induced silencing complex (RISC). The AGO-associated guide RNA strand recognizes

complementary mRNA targets via its “seed sequence” consisting of nucleotides in position 2-8 from the miRNA 5’end with additional contribution from the 3’ end to binding (Baumjohann and Ansel, 2013; Hoefig and Heissmeyer, 2008; Ivanov and Anderson, 2013). Upon binding its target transcript, miRNAs can promote inhibition of mRNA translation but predominantly initiate mRNA degradation (Baek et al., 2008; Hendrickson et al., 2009; Selbach et al., 2008). miRNA-bound mRNAs localize to P bodies where they can be targets of degradation enzymes (Hoefig and Heissmeyer, 2008). In general, miRNA-regulated mRNAs are targeted by several different miRNAs and each miRNA usually targets much more than one mRNA transcript. Similarly to transcription factors, the miRNA repertoire can define a specific cell type, especially hematopoietic cell types. Thus, miRNAs play an important role in processes like differentiation of T cells, class switch recombination in B cells, or the maturation of DCs (Chen et al., 2004). Deregulations in the miRNA system can lead to pathologic phenotypes of the immune system. Dicer-deficient T cells are biased towards the T_H1 subtype and lack Tregs (Cobb et al., 2006, 2005; Muljo et al., 2005). Mice with overexpression of miR-17-92 in lymphocytes suffer from lymphoproliferation and autoimmunity that was associated with auto-antibody production and inappropriate help of T_{FH} cells to self-reactive B cells (Xiao et al., 2008). The miR-19-72 cluster is needed for T_{FH} differentiation due to its repression of PI3K antagonizing phosphatase PTEN which dampens Icos signaling (Baumjohann et al., 2013a; Kang et al., 2013).

5.2.3 Post-transcriptional gene regulation in the immune system

As stated above, autoimmune disorders mainly develop as a result of combined mutations of several genes and display a variety of symptoms and examples for monogenetic disorders are rare. However, mutation of a single RBP can be associated with complex disease phenotypes due to the ability of RBPs to influence a multitude of mRNAs targets. This explains the generalized inflammation seen in TTP-deficient mice due to the role of TTP as a negative feedback regulator of the inflammatory response by destabilizing cytokine mRNAs like *Tnf* (see 5.2.2.1). The importance of post-transcriptional control of *Tnf* mRNA was shown in transgenic mice with deletion of their ARE region. These mice strongly overexpress TNF and develop chronic inflammatory arthritis and Crohn’s-like inflammatory bowel disease (Kontoyiannis et al., 1999; Stamou and Kontoyiannis, 2010). However, *Tnf* mRNA is not only regulated via AREs but also contains a CDE for ARE-independent suppression (see 5.2.2.2). In fact, mRNAs encoding inflammatory mediators like the cytokines TNF, IL-2, IFN-γ, and IL-17 are among the most regulated transcripts. Their mRNAs have a very short half-life, because the corresponding proteins can harm healthy tissue and potentially lead to autoimmune disease if

their expression is not restricted (Seko et al., 2006). CD28 signaling in T cell activation stabilizes *Il2*, *Ifng* and *Tnf* mRNAs among others, as very early work has shown (Lindstein et al., 1989). Furthermore, the progress from primed naive $CD4^+$ T cells to cytokine-secreting effector T cells is tightly controlled. Although priming of $CD4^+$ T cells via the TCR initiates transcription of effector cytokines, for many transcripts protein translation is inhibited without additional TCR stimulation. This two-step process for cytokine production was shown for *Il4* mRNA where transcription and translation are uncoupled by an integrated stress response-like process (Mohrs et al., 2005). After priming, mRNAs are stored in SGs and only antigenic restimulation of the cells results in disassembly of SG, ribosomal mRNA loading and cytokine translation (Scheu et al., 2006).

5.3 Post-transcriptional regulators studied in this work

The post-transcriptional regulators Roquin and Regnase-1 target multiple mRNAs encoding factors relevant for the immune response and play a major role in the maintenance of peripheral tolerance and prevention of autoimmunity.

5.3.1 Roquin

5.3.1.1 Phenotype of Roquin-mutated or -deficient mice

Vinuesa et al. identified a mutation in the gene encoding Roquin as a monogenetic cause of systemic autoimmunity (Vinuesa et al., 2005). The mouse strain was generated by ethylnitrosourea (ENU) mutagenesis and homozygous offspring were screened for high titers of anti-nuclear antibodies (ANAs). Mice, homozygous for a single point mutation in the *Rc3h1* (ring finger and CCCH-type zinc finger domains 1) gene encoding Roquin-1 that introduces one amino acid change, show splenomegaly and enlarged lymph nodes (Vinuesa et al., 2005). Being reminiscent of the bulbonic plague phenotype, the mutant mouse line was named *sanroque* after the patron saint of plague victims. *Sanroque* mice show severe autoimmunity with end-organ pathology that resembles human SLE (Vinuesa et al., 2005). They develop hypergammaglobulinemia, glomerulonephritis with deposition of IgG immune complexes, hepatitis, anemia and autoimmune thrombocytopenia. Further studies revealed an antibody-mediated predisposition for type I diabetes and autoimmune arthritis as well as higher sensitivity to septic shock induction (Pratama et al., 2013; Silva et al., 2011). Even though heterozygous *sanroque* mice do not show lupus-like symptoms, 50% of them develop angioimmunoblastic T cell lymphomas (AITL) with age, driven by T_{FH} cells (Ellyard et al., 2012). The SLE-phenotype is also linked to an accumulation of T_{FH} cells associated with spontaneous formation of GCs with increased numbers of GC B cells and production of high-affinity auto-

antibodies (Vinuesa et al., 2005). $CD4^+$ and $CD8^+$ T cells of *sanroque* mice show a strong activation phenotype with high surface expression of Icos on T cells (Vinuesa et al., 2005). Moreover, short-lived effector cytotoxic $CD8^+$ T cells (SLECs) are increased in *sanroque* mice (Chang et al., 2012). Accumulation of T_{FH} cells has been shown to be a T cell-intrinsic cause for the autoimmune phenotype (Linterman et al., 2009; Vinuesa et al., 2005). Roquin-1 is expressed ubiquitously and a complete loss of Roquin-1 protein by deletion of the *Rc3h1* exons 4-6 leads to perinatal lethality within 6 hours after birth (Bertossi et al., 2011). Newborns show defects in neural tube closure and impaired lung function due to reduced alveolar expansion. Surprisingly, specific deletion of Roquin in the complete hematopoietic system or a B or T cell-specific deletion of Roquin-1 did not result in autoimmunity and only minor immune deregulation was observed in these animals (Bertossi et al., 2011). Despite an increase of effector $CD8^+$ T cells with a SLEC-phenotype, effector $CD4^+$ T cells and T_{FH} cells are normal in these mice (Bertossi et al., 2011). Even though T cells present a moderately increased Icos surface expression, no signs of autoimmunity develop (Bertossi et al., 2011). Similarly, crossing of the Roquin knockout to an outbred strain, which rescued their lethality, did not recapitulate the *sanroque* phenotype (Bertossi et al., 2011).

5.3.1.2 Domain organization of Roquin

Roquin-1 has a paralog, named Roquin-2 that shares a similar domain organization (Fig 4a). Roquin-2, previously named membrane-associated nucleic-acid-binding protein (MNAB) (Siess et al., 2000), is encoded by the *Rc3h2* gene that originates from gene duplication of *Rc3h1* in vertebrates. Homologs of both, *Rc3h1* and *Rc3h2*, can be found from zebrafish to humans whereas Drosophila melanogaster and Caenorhabditis elegans have only one homolog named DmRoquin or Rle-1 (regulation of longevity by E3), respectively. The N-term of Roquin-1 contains a RING (really interesting new gene) finger domain, a newly described ROQ domain and a CCCH-type zinc finger. The C-term appears to be less structured with a proline-rich region followed by sequences enriched for glutamine and asparagine and a coiled-coil domain. The N-terminal part of Roquin-2 is highly conserved whereas the C-term share lower amino acid similarity to Roquin-1 (Athanasopoulos et al., 2010; Pratama et al., 2013). CCCH-type zinc fingers are described to be involved in nucleic acid binding and are found in several post-transcriptional regulators like TTP and Regnase-1 (Lai et al., 1999; Matsushita et al., 2009). Therefore, Roquin function was early on connected to RNA binding although later on the zinc finger was shown to contribute, but was not essential for Roquin-RNA interactions (Athanasopoulos et al., 2010; Glasmacher et al., 2010; Yu et al., 2007). Instead, a novel protein domain termed ROQ domain is critical for mRNA binding and Roquin-1 function as a post-transcriptional regulator (Athanasopoulos et al., 2010; Glasmacher et al., 2010). The ROQ

domain of Roquin-1 is conserved in Roquin-2 and in other species (Vinuesa et al., 2005). Furthermore, it was shown to be required for Roquin localization to SGs (Athanasopoulos et al., 2010). The RING-finger is one of several defining motifs of E3 ubiquitin ligases and indicates a possible E3 ligase activity of Roquin. Whereas E3 ligases with a HECT (homologous to E6-associated protein C-term)-domain have a direct role and form ubiquitin conjugates before ligation, RING E3 ligases act as adaptor-like molecules and mediate the transfer of ubiquitin from E2 to substrate (Ardley and Robinson, 2005). Less is known about the function of the C-term of the Roquin protein but it is required for efficient target mRNA repression and localization of Roquin to P bodies (Glasmacher et al., 2010). The presence of a proline-rich domain suggests a role as platform for complex formation with other proteins.

5.3.1.3 Molecular function of Roquin

Roquin-1 functions as a post-transcriptional regulator and represses protein expression of its target mRNAs. The *Icos* mRNA was the first mRNA identified as a direct target of Roquin-1 that is bound by the ROQ domain via *cis*-elements in its 3' UTR (Athanasopoulos et al., 2010; Glasmacher et al., 2010; Yu et al., 2007). mRNA decay is induced by recruitment of the exonucleolytic machinery (Glasmacher et al., 2010; Leppek et al., 2013). The proline- or glutamine/asparagine-rich region in the C-term is also functionally involved in regulating the *Icos* mRNA (Glasmacher et al., 2010). The molecular requirements in target mRNAs of Roquin-1 were first addressed by Leppek et al. describing a *cis*-regulatory RNA element for Roquin-mediated mRNA decay (Leppek et al., 2013). This RNA-element is located in the previously described CDE in the *Tnf* 3' UTR and forms a hairpin structure (Leppek et al., 2013). The mechanism of Roquin-mediated mRNA decay is not completely understood. Co-immunoprecipitation of Roquin with Edc4 and Rck involved mRNA decapping and degradation from the 5' end (Glasmacher et al., 2010). In addition, interaction of the C-term of Roquin with the CCR4/CAF1-NOT complex was shown, suggesting deadenylation and subsequent 3'-5' degradation or deadenylation-induced decapping and 5'-3' degradation as possible mechanisms (Leppek et al., 2013). Most probably, even more factors of the mRNA degradation machinery play a role here. Consistent with its role in mRNA decay, Roquin-1 and -2 localize to P bodies, but are re-localized to SG after stress induction (Athanasopoulos et al., 2010; Glasmacher et al., 2010; Vinuesa et al., 2005).

An earlier publication on Roquin suggested that miRNAs are required for promotion of *Icos* mRNA decay. Yu et al. proposed that Roquin orchestrates decay of *Icos* mRNA together with miR-101 via a miR-101 recognition motif in the 3' UTR of *Icos* (Yu et al., 2007). Yet, a conclusive mechanism how this cooperation between the miRNA pathway and the RBP Roquin-1 facilitates mRNA decay was not provided. In this regard, a study of our group

identified that Roquin-1 functions independently of the miRNA pathway to repress Icos in Dicer- or AGO1-4-deficient cells (Glasmacher et al., 2010). Nevertheless, a recent publication proposes a role of Roquin in miRNA homeostasis by regulation of miRNA maturation (Srivastava et al., 2015). Srivastava et al. further insinuate involvement of Roquin in miRNA-mediated suppression since Roquin directly binds AGO2, miRNA-146a and one target of miRNA-146a, namely *Icos* mRNA, but further evidence is missing (Srivastava et al., 2015).

Since the RING finger of Roquin is highly conserved among the paralogs and among species, E3 ligase function seems likely. For Roquin-2 interaction with apoptosis signaling-regulating kinase 1 (Ask1; Map3k5) and induction of its poly-ubiquitination has been shown. After cell stress, Ask1 activates Jnk and p38 that mediate for example reactive oxygen species-induced cell death. Roquin prevents apoptosis in response to cell stress by mediating Ask1 degradation (Maruyama et al., 2014). Interestingly, Rle-1, the only Roquin homologue in nematodes, exhibits E3 ubiquitin ligase activity towards its target Daf-16. Rle-1-mediated poly-ubiquitination of Daf-16 induces its proteasomal degradation (Li et al., 2007). Rle-1 deleted worms show an increased life span and higher resistance to cellular stress, which correlates with elevated Daf-16 on the protein but not on the mRNA level (Li et al., 2007). This suggests that Roquin might be involved in ubiquitination and consequent degradation of target proteins in addition to its suppressive function on the mRNA level.

There is some variability to the extent of Roquin-1-mediated downregulation of targets in different cell types suggesting that certain co-factors are required for Roquin function. In addition to the introduced mRNA decay factors, other co-factors may be involved in mRNA target recognition, mRNA binding and recruitment of decay factors or even E3 ligase function.

5.3.2 Regnase-1

The endonuclease Regnase-1, encoded by the gene *Zc3h12a*, is another critical post-trancriptional regulator of immunity, especially of T cell-mediated responses. Regnase-1 shares similarities with Roquin proteins regarding its function, mRNA targets and knockout phenotype (Akira, 2013; Uehata and Akira, 2013). Initially, Regnase-1 was found to be induced in human monocytes treated with monocyte chemoattractant protein-1 (MCP-1; CCL2) and therefore it was previously named MCP-1 inducible protein 1 (MCPIP1) (Zhou et al., 2006). In early studies, nuclear localization was reported and Regnase-1 was implicated in apoptosis and angiogenesis as a novel transcription factor of apoptotic effector proteins and cell surface cadherins 12 and 19, respectively (Niu et al., 2008; Zhou et al., 2006). However, Liang et al. found Regnase-1 proteins predominantly localized in the cytoplasm in granular structures (Liang et al., 2008a), most likely P bodies (Qi et al., 2011), and the role as a transcription factor

was not supported in following studies. Regnase-1 was identified as a gene whose expression is rapidly induced by MyD88-dependent LPS-TLR4-signaling (Liang et al., 2008a). It acts negatively on the expression of inflammatory cytokines such as TNF, IL-1β and IL-6 and thus counteracts macrophage activation (Liang et al., 2008a).

5.3.2.1 Phenotype of Regnase-1-deficient mice

Regnase-1 is critical in prevention of a lethal systemic autoimmune syndrome as Regnase-1-deficient mice resemble the *sanroque* phenotype except for glomerulonephritis (Matsushita et al., 2009; Miao et al., 2013). Regnase-1 knockout mice are born at Mendelian ratios, but most newborns die within 12 weeks after birth and mice that survive weaning show growth retardation (Liang et al., 2010). The animals suffer from anemia, splenomegaly and lymphadenopathy (Matsushita et al., 2009). On a cellular level, spontaneous activation of T cells and accumulation of T cells with an effector or memory phenotype was observed (Matsushita et al., 2009). Knockout animals show an enrichment of plasma cells in lymph nodes and spleen, hypergammaglobulinemia of all Ig isotypes and anti-DNA auto-antibodies as well as ANAs (Matsushita et al., 2009). Plasma cells infiltrate the lung, liver, bile duct and pancreas (Matsushita et al., 2009; Miao et al., 2013) and most of these B cells underwent class switching (Matsushita et al., 2009). The innate immune system is similarly affected since Regnase-1-deficient macrophages show a hyperresponsive phenotype and produce elevated levels of IL-6, IL-12p40 but not TNF as a consequence of increased mRNA stability in response to LPS mediated activation (Matsushita et al., 2009). Furthermore, Regnase-1 knockout animals develop gastritis (Zhou et al., 2013) and are hypersensitive to LPS-induced septic shock (Huang et al., 2013). T cell-specific deletion of Regnase-1 similarly results in autoimmune inflammatory disease characterized by enlarged spleen and lymphnodes, hyperactivated T cells, accumulation of plasma cells, hypergammaglobulinemia and ANAs as well as decreased viability (Uehata et al., 2013).

5.3.2.2 Domain organization and molecular function of Regnase-1

Regnase-1 possesses an intrinsic RNase activity and pre-dominantly targets mRNAs encoding pro-inflammatory cytokines. As mentioned above, mRNA targets of Regnase-1 are endonucleolytically cleaved presumably by recognition of hairpin structures in an ARE-independent pathway (see 5.2.1.2.). Besides the *Il6* mRNA, Regnase-1 destabilizes *Il1b*, *Il12p40* and *Il2* mRNA (Iwasaki et al., 2011; Li et al., 2012; Matsushita et al., 2009; Mizgalska et al., 2009). The mRNA of cRel, Ctla-4, Icos and Ox40 are proposed as additional target mRNAs (Uehata et al., 2013). Regnase-1 belongs to a CCCH-type zinc finger containing family consisting of Regnase-1/2/3 and 4 or MCPIP-1/2/3 and 4 encoded by *Zc3h12a*, *Zc3h12b*,

Zc3h12c and *Zc3h12d*, respectively (Liang et al., 2008a; Xu et al., 2012a). Furthermore, the nuclease function of Regnase-1 is attributed to a characteristic PIN (PilT N-term)-like endoribonuclease domain preceding the zinc finger (Xu et al., 2012a) (Fig. 4b). The CCCH-type zinc finger contributes to mRNA decay since its deletion resulted in slightly lower RNase activity of Regnase-1 (Matsushita et al., 2009). However, the exact contribution of the CCCH-type zinc finger to the function of Regnase-1 as a post-transcriptional destabilizer is unclear. In addition to cytokine mRNAs, several pre-miRNAs serve as substrates for Regnase-1 and are endonucleolytically cleaved in the unpaired terminal loop during mature miRNA generation, which counteracts Dicer activity (Suzuki et al., 2011). Human Regnase-1 was shown to bear anti-viral potential dependent on its intact RNase activity, likely by direct degradation of viral RNAs (Lin et al., 2013, 2014; Liu et al., 2013). Furthermore, a deubiquitinase function via a novel deubiquitinase domain in the N-term overlapping with the RNase domain and an ubiquitination association domain was reported. Deubiquitinase activity negatively regulates Jnk and NF-κB signaling by targeting TRAFs (Liang et al., 2010). Regnase-1 overexpression blocks stress-induced SG formation and thereby sensitizes cells to apoptosis, which relies on its deubiquitinase activity (Qi et al., 2011). The C-term of Regnase-1 contains a proline-rich region, which is believed to be responsible for protein-protein interactions (Xu et al., 2012b).

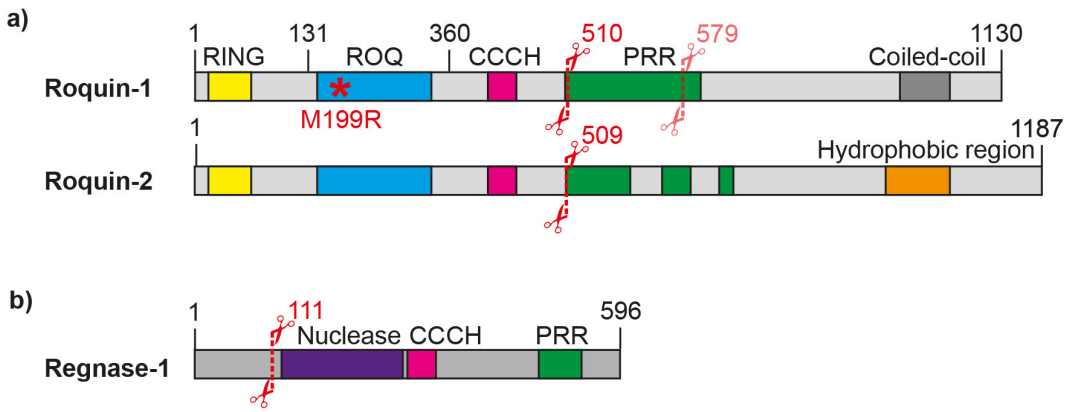

Fig. 4: Domain organization of Roquin-1, Roquin-2 and Regnase-1 proteins

a) Domain organization of Roquin-1 and its paralog Roquin-2. **b)** Domain organization of Regnase-1. Scissors indicate cleavage sites of the paracaspase Malt1 as discussed in 8.3.1. RING, RING-finger; CCCH, CCCH-type zinc finger; PRR, proline-rich region; M199R, *sanroque* pointmutation.

6 AIMS OF THE RESEARCH WORK

The striking difference between pointmutated Roquin-1 expressing *sanroque* mice that develop severe systemic autoimmunity and the relative mild phenotype of Roquin-1 knockout is intriguing. We speculated a possible contribution of the Roquin-1 paralog Roquin-2 to the phenotypes. Hence, the starting point of this research project was to decipher the function of Roquin-2. For this purpose Roquin-2-deficient mice were analyzed to ask whether there are essential roles of Roquin-2 for the survival of mice and to explore whether Roquin-2 has redundant or non-redundant functions to Roquin-1. Functional redundancy of Roquin proteins was further investigated in mice with combined knockout of both Roquin proteins in T cells. From this study conclusions on the role of Roquin proteins in T cell activation and differentiation could be drawn.

Publication I

A comprehensive analysis of the phenotype of mice lacking both Roquin proteins in T cells provided greater insight into the role of Roquin proteins in T cells. To attain a deeper understanding of the underlying mechanisms of T cell immunity, the focus of the following study was on Roquin-mediated post-transcriptional control mechanisms, cooperation of Roquin with additional factors and regulation of Roquin proteins during T cell responses. The identification of new Roquin targets in the pathways of T cell activation and differentiation was an additional aspect of this work. Since the endonuclease Regnase-1 plays a similar role as Roquin for mRNA repression in T cells, and point mutation of Roquin-1 and Regnase-1 deficiency result in similar auto-inflammatory phenotypes with spontaneous activation of T cells, it was addressed whether there is a convergence of Roquin and Regnase-1 function in T cells.

Publication II

Having identified Roquin inactivation through cleavage by the Malt1 paracaspase after TCR activation (Jeltsch et al., 2014) offered the possibility to interfere with Roquin activity by Malt1 inhibitors that selectively abrogate Malt1 protease function. This could allow therapeutic intervention in autoimmune disorders. In order to fathom therapeutic applicability of Malt1 inhibitors, I supported the efforts of Gewies et al. in investigating mice that lack Malt1 proteolytic activity in a collaborative effort. This study allowed conclusions on the consequences and possible side effects of Malt1 protease inhibition on immune responses.

Publication III

7 PUBLICATIONS

In the following, the abstracts of papers I-III are reprinted. For copyright reasons, full versions of the publications are not included in the online version of this thesis. Please download the pdf files directly from the publisher's website.

7.1 Publication I

Vogel*, K.U., Edelmann*, S.L., **Jeltsch, K.M.**, Bertossi, A., Heger, K., Heinz, G.A., Zöller, J., Warth, S.C., Hoefig, K.P., Lohs, C., Neff, F., Kremmer, E., Schick, J., Repsilber, D., Geerlof, A., Blum, H., Wurst, W., Heikenwälder, M., Schmidt-Supprian, M., Heissmeyer, V. * equal contribution

(2013)

Roquin paralogs 1 and 2 redundantly repress the Icos and Ox40 costimulator mRNAs and control follicular helper T cell differentiation.

Immunity 38, 655–668

The Roquin-1 protein binds to messenger RNAs (mRNAs) and regulates gene expression posttranscriptionally. A single point mutation in Roquin-1, but not gene ablation, increases follicular helper T (Tfh) cell numbers and causes lupus-like autoimmune disease in mice. In T cells, we did not identify a unique role for the much lower expressed paralog Roquin-2. However, combined ablation of both genes induced accumulation of T cells with an effector and follicular helper phenotype. We showed that Roquin-1 and Roquin-2 proteins redundantly repressed the mRNA of inducible costimulator (Icos) and identified the Ox40 costimulatory receptor as another shared mRNA target. Combined acute deletion increased Ox40 signaling, as well as Irf4 expression, and imposed Tfh differentiation on $CD4^+$ T cells. These data imply that both proteins maintain tolerance by preventing inappropriate T cell activation and Tfh cell differentiation, and that Roquin-2 compensates in the absence of Roquin-1, but not in the presence of its mutated form.

7.2 Publication II

Jeltsch*, K.M., Hu*, D., Brenner*, S., Zöller, J., Heinz, G.A., Nagel, D., Vogel, K.U., Rehage, N., Warth, S.C., Edelmann, S.L., Gloury, R., Martin, N., Lohs, C., Lech, M., Stehklein, J.E., Geerlof, A., Kremmer, E., Weber, A., Anders, H.J., Schmitz, I., Schmidt-Supprian, M., Fu, M., Holtmann, H., Krappmann, D., Ruland, J., Kallies, A., Heikenwalder, M., Heissmeyer, V. * equal contribution

(2014)

Cleavage of roquin and regnase-1 by the paracaspase MALT1 releases their cooperatively repressed targets to promote T(H)17 differentiation.

Nat. Immunol. *15*, 1079–1089

Humoral autoimmunity paralleled by the accumulation of follicular helper T cells (T_{FH} cells) is linked to mutation of the gene encoding the RNA-binding protein roquin-1. Here we found that T cells lacking roquin caused pathology in the lung and accumulated as cells of the T_H17 subset of helper T cells in the lungs. Roquin inhibited T_H17 cell differentiation and acted together with the endoribonuclease regnase-1 to repress target mRNA encoding the T_H17 cell–promoting factors IL-6, ICOS, c-Rel, IRF4, IκBNS and IκBζ. This cooperation required binding of RNA by roquin and the nuclease activity of regnase-1. Upon recognition of antigen by the T cell antigen receptor (TCR), roquin and regnase-1 proteins were cleaved by the paracaspase MALT1. Thus, this pathway acts as a ‘rheostat’ by translating TCR signal strength via graded inactivation of post-transcriptional repressors and differential derepression of targets to enhance T_H17 differentiation.

7.3 Publication III

Gewies*, A., Gorka*, O., Bergmann, H., Pechloff, K., Petermann, F., **Jeltsch, K.M.**, Rudelius, M., Kriegsmann, M., Weichert, W., Horsch, M., Beckers, J., Wurst, W., Heikenwlader, M., Korn, T., Heissmeyer, V., Ruland, J. * equal contribution

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Uncoupling Malt1 threshold function from paracaspase activity results in destructive autoimmune inflammation.

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The paracaspase Malt1 is a central regulator of antigen receptor signaling that is frequently mutated in human lymphoma. As a scaffold, it assembles protein complexes for NF-κB activation, and its proteolytic domain cleaves negative NF-κB regulators for signal enforcement. Still, the physiological functions of Malt1-protease are unknown. We demonstrate that targeted Malt1-paracaspase inactivation induces a lethal inflammatory syndrome with lymphocyte- dependent neurodegeneration in vivo. Paracaspase activity is essential for regulatory T cell (Treg) and innate-like B cell development, but it is largely dispensable for overcoming Malt1-dependent thresholds for lymphocyte activation. In addition to NF-κB inhibitors, Malt1 cleaves an entire set of mRNA stability regulators, including Roquin-1, Roquin-2, and Regnase-1, and paracaspase inactivation results in excessive interferon gamma (IFNγ) production by effector lymphocytes that drive pathology. Together, our results reveal distinct threshold and modulatory functions of Malt1 that differentially control lymphocyte differentiation and activation pathways and demonstrate that selective paracaspase blockage skews systemic immunity toward destructive autoinflammation.

8 DISCUSSION

8.1 Functional redundancy of Roquin-1 and Roquin-2 in T cells

The mild phenotype of Roquin-1-deficient mice (Bertossi et al., 2011) compared to the phenotype of *sanroque* mice (Vinuesa et al., 2005) suggests that the Roquin-1 paralog Roquin-2 has redundant functions and thus compensates for the loss of Roquin-1. To prove this hypothesis, I investigated the function of Roquin-2.

8.1.1 Phenotype of Roquin-2-deficient mice

In $CD4^+$ T cells, Roquin-2 was about five times less expressed on the protein level than its paralog Roquin-1 and Roquin-1 knockouts and *sanroque* mice ($Rc3h1^{san/san}$) displayed no compensatory upregulation of Roquin-2 (Publication I). To find out if Roquin-1 and Roquin-2 can compensate for each other's loss-of-function despite unequal expression, I first investigated the function of Roquin-2 by analysis of Roquin-2-deficient mice (Publication I). Mice with systemic deletion of Roquin-2 were born at Mendelian ratios but almost all died within threeweeks after birth, most of them in the first days and only 2% survived the weaning period (Publication I). The few survivors were smaller but otherwise appeared healthy. This finding is similar to the perinatal death of Roquin-1 knockout newborns (Bertossi et al., 2011), but Roquin-2-deficient mice showed no obvious neural tube defects in addition to their immature lung phenotype and the exact cause of death remains unknown (Publication I). Crossing of $Rc3h2^{-/-}$ mice on an NMRI (Naval Marine Research Institute) outbred strain rescued their viability and only males still showed growth retardation (Publication I). However, the immune cell homeostasis of these mice was not affected (Publication I). Likewise, conditional deletion of Roquin-2 in T cells did not result in an abnormal immune phenotype. In contrast to $Rc3h1^{fl/fl}$*Cd4*-Cre mice, effector T cells did not accumulate, and even Icos expression was normal in $Rc3h2^{fl/fl}$*Cd4*-Cre mice (Publication I). As a result, deletion of Roquin-2 in T cells has no phenotypic impact, which implies that Roquin-1 can substitute for loss of Roquin-2 in T cells. Despite the potential redundancy of Roquin-1 and Roquin-2 in T cells, the death of mice with systemic deficiency of Roquin-1 or Roquin-2 indicates unique roles of these proteins outside the immune system. This is supported by the detection of Roquin proteins in tissues like lung and brain (Publication I). Considering the malformations in newborns with single knockouts, Roquin-1 and Roquin-2 are probably involved in the regulation of developmental processes in a way that cannot be compensated by the respective paralog. Thus Roquin proteins are essential factors for development and survival.

8.1.2 Organ-specific inflammation of mice with a combined Roquin-1 and Roquin-2 knockout

To confirm the redundancy of Roquin-1 and Roquin-2 in T cells, mice with a combined deficiency of Roquin-1 and Roquin-2 in peripheral T cells (termed *Rc3h1/2*$^{fl/fl}$*Cd4*-Cre or Roquin-deficient) were generated. In contrast to the Roquin-1 single knockout, *Rc3h1/2*$^{fl/fl}$*Cd4*-Cre mice recapitulated some *sanroque* hallmark phenotypes. Besides enlarged spleen and lymph nodes they showed even stronger spontaneous activation of $CD4^+$ and $CD8^+$ T cells than *sanroque* mice and deregulation of T cell differentiation (Publication I). T cells with a T_{FH} phenotype accumulated in the absence of immunization and, as demonstrated by transfer of Roquin-deficient T cells (see 8.3.3), provoked an increased frequency of GC B cells (Publication I). The expression of Icos was strongly increased on the cell surface and was even higher than in *Rc3h1*$^{san/san}$ mice (Publication I). Despite the augmented T_{FH} cell population in mice with Roquin-deficiency in T cells, GCs were not spontaneously formed and ANAs, anti-DNA antibodies or rheumatoid factor were not detected (Publication I; Publication II). Possible reasons for that are discussed below (see 8.4.1). As a consequence the overt lupus-like autoimmunity of *sanroque* mice was not reproduced. Still, *Rc3h1/2*$^{fl/fl}$*Cd4*-Cre mice showed reduced viability in correlation with organ-specific inflammation, namely lung pathology and gastritis (Publication II). The lungs of these mice were infiltrated with hematopoietic cells. Among the T cells, T_H17 cells were prominently detected, and these could also be found in secondary lymphoid organs and in the gut (Publication II). The spontaneous lung inflammation was characterized by the accumulation of neutrophils and symptoms similar to asthma such as increased mucus production by goblet cells (Publication II). Despite greater frequencies of T_H17 cells in the large and small intestine, signs of colitis could not be detected although the presence of T_H17 in the stomach cells correlated with the observed gastritis phenotype (Publication II).

The aggravated phenotype of mice with deletion of both Roquin paralogs in T cells in comparison to Roquin-1 knockouts proved the contribution of Roquin-2 to immune regulation and functional redundancy between Roquin-1 and its paralog Roquin-2 in T cells. (Athanasopoulos et al., 2010b). Further analysis of Roquin-2 protein function revealed an interaction with Edc4 equal to Roquin-1 and binding of the 3' UTR of *ICOS* mRNA in order to suppress ICOS protein expression just as wildtype (WT) Roquin-1 (Publication I). Both, Roquin-1 and Roquin-2, localize to SG and P bodies (Athanasopoulos et al., 2010; Glasmacher et al., 2010; Pratama et al., 2013). In summary, even low levels of Roquin-2 can functionally substitute the loss of Roquin-1. However, the same amount of Roquin-2 in *sanroque* mice is unable to make up for the presence of mutated Roquin-1^{san} protein. The redundant activity of

Roquin-2 seems to be similarly depressed in the WT situation where Roquin-1 is present since loss of Roquin-2 in T cells does not lead to immune deregulation. The mechanism of such a proposed inhibition is unsolved. Competition for shared co-factors, dominant-negative effects of Roquin-1 on Roquin-2 or negative feedback regulation may account for this (Heissmeyer and Vogel, 2013).

A different targeting strategy deleting exon 2 of Roquin-1 and Roquin-2 resulted in alternative transcripts encoding RING-less Roquin-1 and Roquin-2 proteins (Pratama et al., 2013). Mice with a T cell-specific expression of RING-less Roquin-1/2 only exhibited slightly elevated Icos levels on activated T cells and moderately increased frequencies of T_{FH} and GC B cells only upon immunization (Pratama et al., 2013). It is not clear if these effects can be attributed to the absence of the RING-domain since it has not been addressed if protein expression of RING-less Roquin-1 and Roquin-2 are equal to WT levels. Thus, the importance of the E3 ligase activity of Roquin as indicated by evolutional conservation of its RING-finger is still unsolved.

8.2 Cooperation of Roquin and Regnase-1 in post-transcriptional regulation

In search for cooperation partners of Roquin, the striking similarities to the post-transcriptional regulator Regnase-1 caught our attention. This endonuclease plays a comparable role in mRNA repression and Roquin-1 or Regnase-1 deficiencies result in similar auto-inflammatory phenotypes with spontaneous T cell activation (see 5.3.2). For this reason, we hypothesized that Roquin and Regnase-1 have overlapping or shared functions in mRNA regulation of the same mRNA target set.

8.2.1 Roquin and Regnase-1 regulate a shared set of target mRNAs

The similarities of *cis*-elements required for Roquin or Regnase-1 regulation argued for an overlapping mRNA target set. RNA stem-loop structures in the 3' UTRs were suggested for both types of target regulation. Leppek et al. defined a consensus hairpin for Roquin-dependent regulation on the basis of its *Tnf* mRNA target, the so-called CDE (see 5.2.2.2). The CDE consists of a tri-loop of a pyrimidine-purine-pyrimidine sequence and a stem with C-G as closing base pair of the loop followed by two additional U-A base pairs within a 5-8 nucleotide long stem (Leppek et al., 2013). Consensus stem-loop folding was suggested in other potential Roquin target mRNAs in bioinformatic predictions (Leppek et al., 2013). Our group could further increase the spectrum of Roquin *cis*-elements showing that also non-consensus hairpins were accepted for Roquin-mediated post-transcriptional regulation as seen in *ICOS*

and *Ox40* 3' UTRs (Schlundt et al., 2014). Likewise, several mRNAs harboring similar CDE-like stem-loops in their 3' UTR were identified as Regnase-1 targets such as *Il6* and *Il2,* but a consensus sequence or structure for Regnase-1 was not described and formal proof of RNA binding is missing (Iwasaki et al., 2011; Li et al., 2012; Matsushita et al., 2009). *Ox40* mRNA was functionally confirmed as a new post-transcriptional target of Roquin-1 and Roquin-2 (Publication I). In addition to direct binding of *Ox4*0 mRNA by Roquin-1 or Roquin-2, 3' UTR-dependent downregulation of Ox40 expression in response to Roquin-1 or Roquin-2 overexpression was shown (Publication I). Furthermore, regulation of the bona-fide Regnase-1 target *Il6* by Roquin was addressed. Endogenous IL-6 expression after stimulation of fibroblasts with IL-17A and TNF and expression of an ICOS reporter fused to the 3' UTR of *Il6* could be regulated by both Roquin-1 and Regnase-1 (Publication II). Roquin-1 also negatively regulated the known Regnase-1 target mRNAs encoding c-Rel, Ctla-4 and IL-2, and Regnase-1 repressed the Roquin-targeted transcripts *ICOS* and *Ox40* (Publication II and data not shown). Increased Irf4 protein expression in Roquin-1 and -2-deficient T cells was already detected in the study of Vogel et al. (Publication I). At that time we have attributed it to enhanced NF-κB activation downstream of Ox40 signaling as it is known that Irf4 is directly induced by c-Rel in activated lymphocytes (Grumont and Gerondakis, 2000). Our following study then described direct regulation of Irf4 via its 3' UTR by Roquin-1 and Regnase-1 (Publication II). The *Nfkbiz* and *Nfkbid* genes encode for the atypical inhibitors of NF-κB, Iκbζ and IκBNS, and their mRNAs contain one or two predicted CDEs in their 3' UTRs, respectively (Leppek et al., 2013). Furthermore, translational control of IκBζ via a translational silencing element (TSE) with putative stem-loop structures was already described (Dhamija et al., 2010, 2013). Derepression of ICOS fused to the 3' UTRs of *Nfkbiz* and *Nfkbid* after depletion of Roquin-1/2 in mouse embryonic fibroblasts confirmed the encoded IκBζ and IκBNS proteins as Roquin targets (Publication II). Protein levels of IκBNS were elevated in *Rc3h1/2*$^{-/-}$ as well as in *Zc3h12a*$^{-/-}$ fibroblasts (Publication II). According to these findings, both Roquin and Regnase-1 post-transcriptionally regulate the same set of transcripts.

8.2.2 Roquin and Regnase-1 functionally cooperate in target mRNA repression

The relationship between Roquin-1- and Regnase-1-mediated post-transcriptional repression was further explored and potential cooperation investigated. Simultaneous overexpression of Roquin-1 and Regnase-1 in fibroblasts resulted in additive or cooperative repression of *ICOS* mRNA (Publication II). Roquin-1 and Regnase-1 depended on each other's presence since overexpression of Roquin-1 in *Zc3h12a*$^{-/-}$ cells or Regnase-1 in *Rc3h1/2*$^{-/-}$ cells did not lead to downregulation of an ICOS reporter fused to a part of the *TNF* 3' UTR containing the CDE

(Publication II). Structural analyses by our own group and others unraveled that the ROQ domain is sufficient for recognizing the CDE motif (Schlundt et al., 2014; Schuetz et al., 2014; Tan et al., 2014). Mutations of the critical residues in the ROQ domain that reduced RNA affinity also interfered with repression of an ICOS reporter fused to the CDE of *TNF* (Publication II; Schlundt et al., 2014). Hence, RNA-binding by Roquin is required for its repressive function. The RNase activity of Regnase-1 is dependent on a negatively charged pocket in its N-terminal PIN domain and Asp141 was found crucial for catalytic action of Regnase-1 (Matsushita et al., 2009; Xu et al., 2012a). Further analysis endorsed that nuclease but not deubiquitinase function of Regnase-1 is necessary for target regulation (Publication II). Additional insights were generated by combination of the RNA binding activity of Roquin-1 with the RNase activity of Regnase-1 by fusing the ROQ domain of Roquin-1 to the complete open reading frame of Regnase-1 (Publication II). The ROQ-Regnase-1 fusion protein was as active as WT Regnase-1 in Zc3*h12a*$^{-/-}$ cells and functioned independently of cooperation with Roquin in *Rc3h1/2*$^{-/-}$ cells in reporter repression (Publication II). Consequently, Roquin and Regnase-1 cooperate in the repression of at least some or all target mRNAs and this requires the RNA-binding activity of Roquin-1 and the nuclease activity of Regnase-1.

The exact mode of cooperation and mediation of target repression remains elusive. So far no *in vitro* electromobility shift assays or crosslinking and immunoprecipitation assays in cells have been reported that could support the mRNA binding activity of Regnase-1. The publication of Jeltsch et al. suggests that target recognition of Regnase-1 can be conferred by Roquin's RNA binding capacity (Publication II). A direct interaction of Roquin and Regnase-1 proteins could not be demonstrated so far, but the presence of proline-rich regions in both proteins implies potential higher order protein-protein interactions. Indeed, Roquin function depends on its C-term, which contains the proline-rich region (Glasmacher et al., 2010). Two groups reported oligomerization of human REGNASE-1 in relation to its C-terminal proline-rich region and experiments by Suzuki et al. suggested additional interacting proteins (Lin et al., 2013; Suzuki et al., 2011). Deletion of this domain in Regnase-1 resulted in reduced miRNA suppressor activities (Suzuki et al., 2011) or loss of antiviral activity (Lin et al., 2013). The functional cooperation of Roquin and Regnase-1 may cause mRNA repression through the interaction with additional factors such as mRNA decay factors like Edc4, Rck and the CCR4/CAF1-NOT complex whose involvement in Roquin function has already been reported (Glasmacher et al., 2010; Leppek et al., 2013) (see 5.3.1.3). Another point for further research is the possibility that Roquin and Regnase-1 may contribute to target repression by inducing translational inhibition of target mRNAs in addition to mRNA decay. Comparative analyses quantifying the observed reduction in protein expression and mRNA levels of target genes

upon Roquin or Regnase-1 expression will be required to determine the modes and kinetics of mRNA repression.

The question arises if Regnase-1 paralogs can substitute for Regnase-1 in cooperation with Roquin or if they act functionally different. As stated on the outset Regnase-1 family members also contain a CCCH zinc finger and bear a putative nuclease domain as well as a C-terminal proline-rich region (Xu et al., 2012b). In addition, Regnase-2 and Regnase-4 also possess conserved sequences that could serve as Malt1 cleavage sites as it was shown for Regnase-1 (Publication II) (see 8.3.1). Similar to Regnase-1, Regnase-4 was induced by TLR signaling and involved in negative regulation of macrophage activation and additionally decreased cellular ubiquitination by possible action as a deubiquitinating enzyme (Huang et al., 2012). Furthermore, it was localized to P bodies and presumably regulates cytokine mRNAs of IL-2, IL-6, TNF and IL-17A dependent on their 3'UTR (Minagawa et al., 2014). Deletion of Regnase-4 resulted in accumulation of T_H17 cells that exacerbated experimental autoimmune encephalomyelitis (EAE) symptoms (Minagawa et al., 2014). According to these publications Regnase-4 and Regnase-1 share remarkable resemblance and overlapping functions between Regnase-1 paralogs seem likely.

8.3 Function of Roquin and Regnase-1 in T cell activation and differentiation

A complex network of regulatory mechanisms defines the fate of T cells. Notably, half of the changes in protein expression during T cell activation are regulated by post-transcriptional processes, especially regarding the expression of cytokines and chemokines (Cheadle et al., 2005; Hao and Baltimore, 2009). Roquin and Regnase-1 cooperatively regulate a variety of immune factors on the post-transcriptional level as co-stimulatory receptors like Icos and Ox40, cytokines like TNF, IL-2 and IL-6 and transcriptional modulators like c-Rel, IκBNS and IκBζ (see 8.2.1). This work is aimed at understanding the role of Roquin and Regnase-1 proteins in T cell activation and differentiation. The phenotype of mice with deletion of both Roquin-1 and Roquin-2 or Regnase-1 is most likely not the result of a single upregulated protein but rather a summary of combined derepression of several mRNA targets that functionally collaborate to induce inadequate T cell activation and selective differentiation of specific effector types. On the other hand, I speculated that Roquin and Regnase-1 proteins have to be inactivated in physiologic situations like pathogenic threats to allow the onset of a T cell-mediated immune response.

8.3.1 Regulation of Roquin and Regnase-1 by Malt1 cleavage

I became interested in the regulation of Roquin and Regnase-1 protein levels in T cells during T cell activation. So far, little is known about the regulation of Roquin. In a murine T cell line the transcription factors STAT1, STAT3, GATA2 and c-Rel were reported to increase *Rc3h1* transcription via regulatory elements in the *Rc3h1* promoter region in response to IL-10 (Schaefer et al., 2014). Furthermore, IL-10 was proposed to inhibit the expression of 26 miRNAs, among them miR-223, that act as negative regulators of *Rc3h1* mRNA and thus IL-10 has the potential to stabilize Roquin-1 expression (Schaefer et al., 2011). Regulation of Roquin-1 and Roquin-2 on the post-transcriptional level was also suggested by the presence of conserved CDEs in their 3' UTRs (Leppek et al., 2013). Roquin-1 was shown to directly bind *Rc3h1* and *Rc3h2* mRNAs, which implies self-regulation by Roquin-1 in a negative feedback loop (Leppek et al., 2013). In contrast, Regnase-1 expression is known to be dynamically regulated on several levels in innate immune cells. Expression of Regnase-1 in resting cells like for example unstimulated macrophages prevents unwanted production of cytokines in the steady-state situation (Iwasaki et al., 2011). After MyD88-dependent TLR- or IL-1R-signaling, IKK phosphorylates and initiates subsequent proteasomal degradation of Regnase-1 proteins in macrophages (Iwasaki et al., 2011). This may enable short-term release of cytokines in case of infection when the appropriate signals are present. At later phases of stimulation Regnase-1 protein reappears due to transcriptional induction, presumably to shut off the innate response and prevent over-shooting reactions and accumulation of pro-inflammatory molecules that can destroy tissue (Akira, 2013). Regnase-1 is transcriptionally induced by MyD88-dependent signaling (Matsushita et al., 2009; Mizgalska et al., 2009) and the transcription factors NF-κB, Elk-1 and SRF were involved in induction of *Zc3h12a* transcription (Kasza et al., 2010; Skalniak et al., 2009). In addition, Regnase-1 negatively regulates its own transcripts via a critical stem-loop structure in its 3' UTR (Iwasaki et al., 2011; Mizgalska et al., 2009). According to the established cooperation of Roquin and Regnase-1 in target regulation (see 8.2.2), Roquin is most likely also involved in regulation of *Zc3h12a* mRNA. Indeed, deletion of Roquin proteins resulted in Regnase-1 upregulation (Publication II).

In T cells inactivation of Regnase-1 via cleavage by the paracaspase Malt1 was shown after TCR stimulation (Uehata et al., 2013). I similarly detected Malt1-dependent cleavage of Roquin proteins in $CD4^+$ T cells after T cell activation in combination with co-stimulation (Publication II). Malt1 cleaved Roquin-1 after amino acid position Arg510, within an amino acid sequence that is conserved in Roquin-2, and Malt1 also recognized an alternative cleavage site after Arg579 only in Roquin-1 (Publication II) (Fig. 4a). A Malt1-cleavage-resistant mutant of Roquin-1 was more potent in Icos downmodulation in T cells than WT Roquin-1

(Publication II). However, the Roquin-1 cleavage fragment that completely lacks the C-term (amino acids [aa]) 1-510) lost its post-transcriptional repressor activity on *ICOS* mRNA (Publication II). A fragment of similar size with only one amino acid difference, aa 1-509, was found inactive in post-transcriptional repression before (Glasmacher et al., 2010; Publication I). It would be interesting to know if the Roquin-1 cleavage product aa 1-510 still exerts a function since it still has an intact RING domain and full RNA-binding ability through the ROQ domain (Schlundt et al., 2014). The ROQ domain aa 138-337 alone was sufficient to localize the protein to SG (Athanasopoulos et al., 2010), but a Roquin-1 mutant with deleted C-term (aa 1-749) is excluded from P bodies (Glasmacher et al., 2010; Publication I). Moreover, Roquin-1 aa 1-509 could still interact with Rck and Edc4 (Glasmacher et al., 2010). In conclusion, the Roquin-1 cleavage product aa 1-510 might leave the P body structure and guide mRNA transcripts away from the degradation machinery to enable or even facilitate their translation. In a second scenario, prevention of mRNA decay by occupation of binding sites for full-length Roquin in the RNA seems also possible. However, this is pure speculation and needs to be tested. The cleavage site of Regnase-1 at position Arg111 generates a C-terminal Regnase-1 product with an intact RNase domain, which is supposed to be catalytically active but rapidly degraded (Uehata et al., 2013) (Fig. 4b). Malt1-dependent cleavage of Roquin proteins and their functional cooperation partner Regnase-1 released their shared target mRNAs from repression (Publication II). This allowed immediate expression of targeted transcription factors such as Irf4, IκBζ and IκBNS (Publication II). Furthermore, activation of $CD4^+$ T cells extended the half-life of *cRel*, *Ox40* and *Il2* mRNA whereas those mRNAs were destabilized in the presence of Malt1 inhibitor (Uehata et al., 2013).

8.3.2 Role of Roquin and Regnase-1 in T cell activation

Deregulation in the T cell compartment of Roquin- or Regnase-1-deficient mice implies a role of these proteins in T cell activation and various T cell differentiation programs. Normally, activation of naive T cells requires two different signals that converge into productive signaling: recognition of the antigen-MHC complex by the TCR (signal 1) and additional co-stimulatory signals (signal 2). TCR ligation without co-stimulation leads to an unresponsive state termed T cell anergy (see 5.1.3.1). T cells integrate co-stimulatory cues, activation of cytokine receptors and even environmental cues concerning the T cell metabolism to define their fate (Chappert and Schwartz, 2010). The received signals translate into signaling pathways that regulate induction or avoidance of T cell anergy. Thereby, especially post-transcriptional silencing by RBPs and/or miRs accounts for molecular mediation of anergy and plays a role in controlling cytokine expression. According to Villarino et al., there is a

remarkable discrepancy between the amount of cytokine mRNAs and the produced proteins in self-reactive T cells responding to antigen. This was proposed to be achieved by a post-transcriptional block of cytokine mRNA translation due to sequences in their 3' UTR (Villarino et al., 2011).

Rc3h1$^{san/san}$ and *Rc3h1-2*$^{fl/fl}$*Cd4*-Cre as well as *Zc3h12a*$^{-/-}$ and *Zc3h12a*$^{fl/fl}$*Cd4*-Cre T cells were spontaneously activated in the absence of pathogens (Matsushita et al., 2009; Uehata et al., 2013; Vinuesa et al., 2005; Publication I). This was a T cell-intrinsic phenotype of Roquin-deficient T cells since naive CD4$^+$ T cells from *Rc3h1-2*$^{fl/fl}$*Cd4*-Cre mice acquired an activated phenotype upon transfer in WT recipient mice (data not shown). It is not known how this T cell activation is mediated on the molecular level but a number of derepressed targets in Roquin-1 and Roquin-2 double deficient T cells might provide a possible explanation. Deregulated targets with effects on T cell signaling are cytokine-encoding mRNAs such as the Roquin target *Il2* (Publication II). It is long known that IL-2R signaling prevents T cells from becoming anergic (Boussiotis et al., 1994) and that *Il2* mRNA is stabilized upon CD28 engagement (Lindstein et al., 1989). IL-2 has the ability to fully activate the PI3K-Akt pathway that is required for productive T cell activation by upregulation of the metabolic machinery to match the high metabolic demands of activated T cells (Valdor and Macian, 2013; Zheng et al., 2007, 2009). Thus, possible elevation of the IL-2 concentration in *Rc3h1-2*$^{fl/fl}$*Cd4*-Cre mice could be responsible for T cell hyperactivation. Furthermore, the presence of constant inappropriate co-stimulatory signaling may either lead to activation of self-reactive T cells that concomitantly receive signals via antigenic recognition of self-peptides or in a second scenario may overcome the need for higher affinity or tonic TCR signals. Co-stimulation of Roquin- and Regnase-1-mutated T cells is guaranteed by excessive expression of the co-stimulatory receptors Icos and Ox40. This might render T cells independent from cells of the innate immune system, especially DCs that normally have to mature first into co-stimulation-providing cells after pathogen contact. Icos or Ox40 signaling normally expand activated T cells and are critical for the survival of effector T cells (Murata et al., 2002; Simpson et al., 2010; Weinberg, 2010). Ox40 was further involved in the activation of the alternative NF-κB pathway by using agonistic anti-Ox40 antibodies on WT CD4$^+$ T cells (Murray et al., 2011; Publication I). As in WT cells, Ox40 triggering of Roquin-deficient CD4$^+$ T cells resulted in increased processing of p100 to p52 but in *Rc3h1/2*$^{-/-}$ CD4$^+$ T cells canonical NF-κB signaling was also affected as levels of phosphorylated p65 and phosphorylated IκBa were elevated (Publication I). At first glance, it seems surprising that the mRNA for Ctla-4 is placed under the post-transcriptional control of Roquin and Regnase-1 but the increase of Ctla-4 on the surface of activated T cells could serve as a negative feedback mechanism in physiologic situations.

Yet, in Roquin-deficient T cells this dampening mechanism might be outcompeted by constantly high expression of positive co-stimulators like Icos and Ox40.

An unsolved issue concerns the clonality of activated T cell populations. So far it was not addressed if these are oligo- or polyclonal and if they contain self-reactive TCRs. Thymic T cell development seems normal and subset populations are not altered in *Rc3h1-2*$^{fl/fl}$*Cd4*-Cre (Publication I) and *Zc3h12a*$^{fl/fl}$*Cd4*-Cre mice (Uehata et al., 2013). The TCR repertoire of nude mice transplanted with *Rc3h1*$^{-/-}$ fetal thymic epithelial cells (Bertossi et al., 2011) and of *Zc3h12a*$^{fl/fl}$*Lck*-Cre mice (Uehata et al., 2013) was comparable to the respective WT controls, excluding a bias to self-reactive TCRs during development. However, development of angioimmunoblastic T cell lymphomas (AITL) in heterozygous *sanroque* mice is characterized by the presence of oligoclonal T_{FH} cells with a more restricted TCR-βV repertoire, which suggests constant TCR-specific signaling drives tumor development in this case (Ellyard et al., 2012). TCR clonality needs to be tested in mice with T cell-specific deletion of Roquin-1 and Roquin-2. The issue of whether antigenic TCR stimulation is necessary for aberrant activation of Regnase-1-deficient CD4$^+$ T cells was addressed by analysis of *Zc3h12a*$^{fl/fl}$*Cd4*-Cre mice that express the OTII TCR transgene. Indeed, expression of a single OTII TCR recognizing Ovalbumin (Ova) preserved the naive state of most CD4$^+$ T cells and prevented spontaneous development of effector T cells (Uehata et al., 2013). However, immunization with cognate Ova peptide resulted in elevated proliferation and increased effector cytokine expression of Regnase-1-deficient OTII cells (Uehata et al., 2013). Further evaluation is necessary to uncover if Roquin and Regnase-1 suppress excessive T cell activation downstream and independently of the TCR. Adoptive transfer of knockout CD4$^+$ T cells in MHCII-deficient mice would solve the issue of tonic signaling as a requirement. Furthermore, the contribution of individual TCR-induced signaling components needs to be deciphered to allow a complete understanding of Roquin- and Regnase-1-controlled T cell activation.

8.3.3 Role of Roquin and Regnase-1 in T cell differentiation

Presumably, the input of deregulated co-stimulatory receptors, cytokines and transcription factors in Roquin- or Regnase-1-deficient T cells not only lead to their activation but also to T cell differentiation fates like T_{FH} cells that promote antibody production and inflammatory T_H17 cells. Mice with combined deletion of Roquin-1 and Roquin-2 in peripheral T cells accumulated T_{FH} cells similarly to *sanroque* mice (Vinuesa et al., 2005; Publication I). And, like in *sanroque* mice, T cell intrinsic causes accounted for that since adoptive transfer of naive *Rc3h1-2*$^{fl/fl}$*Cd4*-Cre CD4$^+$ T cells resulted in increased T_{FH} differentiation of these T cells (Heissmeyer and Vogel, 2013). Also, adoptively transferred T_H1 cells, that have undergone

acute deletion of Roquin *in vitro*, differentiated into T_{FH} cells in *Icos*$^{-/-}$ recipients (Publication I), whose endogenous T cells are blocked in T_{FH} cell differentiation (Choi et al., 2011). These emerging T_{FH} cells even induced GC B cell differentiation of host B cells (Publication I). In contrast, this effect was not observed with transfer of WT T_H1 cells (Publication I). This demonstrates a plasticity between T_H1 and T_{FH} cells that was observed previously (Lu et al., 2011). In particular, recent data suggest the possibility of re-programming T_H1 into T_{FH} cells via IL-21- and IFN-γ-producing T_H1 cells that simultaneously express T-bet and Bcl6. These cells shared characteristics of both T_{FH} and T_H1 cells, but ultimately T-bet along with IFN-γ signaling antagonized Bcl6 and T_{FH} cell-like features to establish a profound T_H1 phenotype (Nakayamada et al., 2011). Moreover, *in vivo* isolated T_{FH} were shown capable of co-expressing cytokines characteristic of other T_H cell subtypes (Fazilleau et al., 2009; Glatman Zaretsky et al., 2009; Reinhardt et al., 2009). In addition to the preference for T_{FH} cell differentiation, CD4$^+$ T cells deficient for Roquin-1 and -2 displayed a strong bias to the T_H17 cell fate *in vivo* (Publication II) (see 8.1.2). Surprisingly, *sanroque* T cells were prone to become IFN-γ producing T_H1 cells instead (Lee et al., 2012 and own unpublished data). IFN-γ protein concentrations were increased in the serum of *sanroque* mice (Lee et al., 2012 and own unpublished data) but not in the serum of mice with T cell-specific loss of Roquin. Instead, an increase of IL-6 and IL-17 cytokines was observed (Publication II). Under *in vitro* T_H17 culture conditions the frequency of IL-17A$^+$ CD4$^+$ T cells was around twice as high in sorted naive CD4$^+$ T cells of *Rc3h1-2*$^{fl/fl}$*Cd4*-Cre mice as in WT T cells (Publication II). Roquin-deficient CD4$^+$ T cells only showed a tendency towards the T_H1 subtype in *in vitro* differentiation (Publication II). Adenoviral shRNA knockdown of *Zc3h12a* resulting in reduced Regnase-1 protein levels, and upregulation of its target IκBζ, revealed that Regnase-1 plays a similar role in T_H17 and T_H1 differentiation (Publication II). Regnase-1 knockdown led to an increased number of IL-17A- or IFN-γ-producing CD4$^+$ T cells under T_H17- or T_H1 conditions, respectively (Publication II). However, T cells of mice with complete or T cell-specific *Zc3h12a* knockout were reported to incline to a T_H1 phenotype *in vivo* (Akira, 2013; Matsushita et al., 2009; Uehata et al., 2013) even though IL-17 and IL-4 were also upregulated in T cells in addition to elevated IFN-γ levels compared to WT (Miao et al., 2013; Uehata et al., 2013). Analyses of serum of Regnase-1-deficient mice revealed increased protein concentrations of IFN-γ, IL-6, IL-17, IL-4 and IL-2 (Miao et al., 2013). Moreover, enhanced T_{FH} cell development can be assumed on the basis of plasma cell expansion and auto-antibody generation but has not been reported (Akira, 2013; Matsushita et al., 2009; Uehata et al., 2013). In support of this, transfer of Regnase-1-deficient CD4$^+$ T cells into WT mice showed lymphoproliferative behavior with aberrant B cell help of knockout T cells since host B cells differentiated into plasma cells (Uehata et al., 2013).

We focused our research on decoding the molecular network that not only drives T cell activation but also the commitment to the T_{FH} and T_H17 subset. In doing so, several shared mRNA targets of Roquin and Regnase-1 that encode factors promoting T_{FH} and T_H17 cell differentiation were identified (Publication I+II). Icos signaling for example induces c-Maf which regulates IL-21 production in favor of T_{FH} and T_H17 cell development (Bauquet et al., 2009). Constitutive expression of Ox40 ligand on DCs leads to accumulation of activated $CD4^+$ T cells within GCs after immunization (Brocker et al., 1999). Furthermore, Irf4-deficient T cells are strongly impaired in generating T_H17 cells (Brüstle et al., 2007) or T_{FH} cells (Bollig et al., 2012). $Irf4^{-/-}$ mice lack GCs and GC B cells after immunization (Bollig et al., 2012) and are resistant to EAE (Brüstle et al., 2007). Mice that are deficient for Irf4-binding protein (IBP) Def6 develop systemic lupus-like autoimmunity with accumulation of effector/memory T cells and auto-antibody production (Fanzo et al., 2006) due to missing control of Irf4 (Chen et al., 2008). Def6 inhibits the DNA binding and transcriptional activity of Irf4 on genes that encode IL-17 and IL-21 (Chen et al., 2008) that are involved in T_H17 and T_{FH} effector functions. Hence, high levels of Irf4 are likely to promote the generation of T_H17 and T_{FH} cells in mice with T cell-specific Roquin deletion. Mice deficient for both Roquin paralogs combined with deletion of one or two alleles of Irf4 could clarify the contribution of increased Irf4 expression to the pathology of *Rc3h1-2*$^{fl/fl}$*Cd4*-Cre mice. The NF-κB transcription factor c-Rel plays a role in T_{FH} differentiation by inducing IL-21 transcription together with c-Maf (Bauquet et al., 2009; Chen et al., 2010; Nutt and Tarlinton, 2011). Furthermore, it is critical for T_H17 differentiation and mice deficient for c-Rel are resistant to EAE (Chen et al., 2011; Reinhard et al., 2011; Ruan et al., 2011). The contribution of c-Rel in Regnase-1 knockout mice has already been investigated in mice deficient for c-Rel and Regnase-1. According to these findings, c-Rel contributed to T cell activation and accumulation of plasma cells in a dose-dependent manner (Uehata et al., 2013). The atypical inhibitors of NF-κB Iκbζ and IκBNS act as transcriptional modulators. IκBζ was required and sufficient to promote T_H17 differentiation (Okamoto et al., 2010). It affected initial steps of T_H17 differentiation by cooperating with RORα and RORγt to enhance IL-17 expression (Okamoto et al., 2010). IκBNS could modulate transcription of T_H17 effector molecules similar to IκBζ. Furthermore, IκBNS was required for the maturation of $Foxp3^-$ Treg precursors in the thymus where it could cooperate with c-Rel, which might be similarly relevant for T_H17 function (Schuster et al., 2012). I could confirm the relevance of IκBζ as well as newly demonstrate a similar role for IκBNS in T_H17 differentiation by knockdown experiments (Publication II). The crucial role of IκBNS for generation and function of T_H17 cells was confirmed in two recent publications (Annemann et al., 2015; Kobayashi et al., 2014). The Roquin and Regnase-1-target *Il6* encodes a known driver of T_H17 differentiation that

simultaneously suppresses the development of Tregs (Korn et al., 2008). IL-6 also provides important signals for early T_{FH} differentiation mediated by STAT1 and STAT3 to induce Bcl6 and CXCR5 (Choi et al., 2011, 2013). Many cell types produce IL-6 but the main source are stromal cells rather than T cells (Hunter and Jones, 2015). Nonetheless, IL-6 levels in the serum of *Rc3h1-2*$^{fl/fl}$*Cd4*-Cre mice were elevated (Publication II). However, this could be due to concomitantly high levels of TNF and IL-17A in *Rc3h1-2*$^{fl/fl}$*Cd4*-Cre mice, which were shown to further induce IL-6 expression in epithelial cells and fibroblasts (Publication II).

In summary, the combined deregulation of the above mentioned factors contribute to T_{FH} or T_H17 differentiation in mice with a T cell-specific Roquin knockout and also affect phenotypes of Regnase-1-deficient T cells. Roquin and Regnase-1 target mRNAs encoding Icos, Irf4, c-Rel and IL-6 have positive effects on both T_{FH} and T_H17 differentiation, whereas Ox40 involvement was only shown for T_{FH} differentiation and IκBζ and IκBNS have been identified as T_H17-promoting factors. However, possibly not all the target mRNAs shared by Roquin and Regnase-1 have been identified yet. Future work on this aspect is required to unravel the complete molecular network that leads to T cell activation and varying differentiation decisions.

8.3.4 Translation of TCR signal strength into cell fate decisions via Malt1-mediated Roquin cleavage

The molecular mechanisms underlying the translation of antigen dose and signal strength from the TCR into differential T cell effector fates are elusive. Recently, two publications reported that high TCR stimulus correlates with high Irf4 expression in $CD8^+$ T cells and is important for their sustained expansion and effector differentiation (Man et al., 2013; Yao et al., 2013), which has also been shown in an acute virus infection model (Nayar et al., 2014). I could show that TCR signal strength similarly correlated with Irf4 protein expression in $CD4^+$ T cells (Publication II). Malt1-mediated cleavage of Roquin resulted in graded inactivation of Roquin proteins and since *Irf4* mRNA is a direct Roquin target this led to Irf4 expression in a dose-dependent manner (Publication II). Co-culture of OTII T cells with irradiated APCs presenting increasing amounts of Ova peptide or mutated Ova peptides with varying affinities led to graded Irf4 expression (Publication II). The dose of Irf4 expression in response to varying TCR avidity and affinity was connected with T_H17 differentiation frequencies and only a strong TCR stimulus lead to high inactivation of Roquin proteins and thus T_H17 differentiation (Publication II). In summary, I reason that TCR signal strength is translated into graded Roquin and Regnase-1 cleavage and differential target mRNA regulation, which influences the T cell fate (Fig. 5). In this manner, Malt1 paracaspase activity and Roquin are molecular links between TCR strength and the differentiation phenotype of stimulated T cells (Fig. 5). Indeed,

a role of Malt1 in T_H17 differentiation was reported previously (Brüstle et al., 2012) and more recently, Bornancin et al. could show that mice that lack Malt1 protease activity fail to acquire a T_H17 phenotype (Bornancin et al., 2015) (see 8.5). In line, Malt1-deficient mice are resistant to the induction of EAE (Brüstle et al., 2012; Mc Guire et al., 2013).

As described above, T_{FH}, T_H17 and T_H1 cell development represent the main differentiation routes of Roquin-deficient or *sanroque* T cells (see 8.3.3). The question if these cell fates are connected via a common feature arises and physiologic dependence on rather high TCR stimuli would be one possibility. The connection of these cell fates via Irf4 expression is another interesting angle since Irf4 contributes to T_H17 as well as T_{FH} cell development. However, it alo plays a role for T_H2 differentiation (Lohoff et al., 2002) and the role of Irf4 in T_H1 differentiation is not well known (Huber and Lohoff, 2014). It remains to be worked out how T_H1, T_H2 and T_{FH} cells are influenced by graded Irf4 expression in $CD4^+$ T cells. There are few publications on TCR signal strength influencing T cell fate decision and the use of different model systems makes a comparison difficult (Nakayama and Yamashita, 2010; Tubo and Jenkins, 2014). In general, lower antigen doses are believed to induce T_H2 cells and T_{FH} precursors at the T-B border, intermediate peptide doses induce T_H1 cells, and high doses induce T_H2 or GC T_{FH} cells although the discrimination between T_H2 cells and IL-4 producing T_{FH} cells was not always considered (Nakayama and Yamashita, 2010; Tubo and Jenkins, 2014). Increasing the dose of antigen facilitates differentiation of T_H1 and T_{FH} cells until a point where T_H1 cells decreases and GC T_{FH} cells develop, which increase the magnitude of the GC B cell response (Baumjohann et al., 2013b; Tubo et al., 2013). In line with my data, Iezzi et al. could show that T_H17 differentiation requires strong antigenic stimulation but did not provide a molecular mechanism (Iezzi et al., 2009). Furthermore, two publications described that high antigen doses prevented iTreg differentiation and rather supported effector T cell differentiation (Molinero et al., 2011; Turner et al., 2009). But whereas Turner et al. suggested that Akt/mTor signaling, which is known to negatively regulate Foxp3 expression, is the responsible link, Molinero et al. excluded Akt/mTor signaling as an explanation for the inhibitory effect of high TCR stimulation on iTregs (Molinero et al., 2011; Turner et al., 2009). Nevertheless, both groups could show that in turn high TCR stimulus leads to IL-17 cytokine production of T cells (Molinero et al., 2011; Turner et al., 2009). These publications support the view of high TCR stimuli rather leading to T_H1, T_{FH} and T_H17 cell differentiation and we propose that this is translated via our model mechanism (Fig. 5).

Still, there are many open questions regarding translation of TCR strength via Malt1 and Roquin cleavage to T cell fates. How is signal strength translated from the TCR to Malt1 activation to the extent of Roquin cleavage? Recent data suggest that the CBM signalosome is

a helical filamentous assembly in which Carma1 acts as a nucleator to promote Bcl10 polymerization into helical filamentous structures (Qiao et al., 2013). This creates a platform for dimerization of Malt1 which enhances its activity (Qiao et al., 2013). Possibly the magnitude of TCR signaling directly translates at the step of Carma1 recruitment and activation followed by platform creation. Furthermore, it is anticipated that freely diffusing active Malt1 is available after T cell activation to reach its targets Roquin and Regnase-1, which are presumably located in mRNP complexes in P bodies or SG. In support of this, Eitelhuber et al. could show protease activity of Malt1 independently from the CBM complex after initial Malt1 activation (Eitelhuber et al., 2015). How exactly do fine differences in TCR signal strength influence T_{FH}, T_H1 or T_H17 differentiation and what ratio of differentially regulated Roquin targets besides Irf4 are required in each case? According to data from our group, mutants in Roquin weakening its affinity to the CDE have differential effects on different 3' UTRs as shown for *Icos* and *Ox40* mRNA that are less repressed by Roquin compared to the CDE (Schlundt et al., 2014). This could mean that high or low-affinity cis-elements exist in physiologic Roquin targets, which are differently influenced by Roquin levels. Partial Roquin cleavage by Malt1 in response to low TCR strength would de-repress targets with low-affinity cis-elements, whereas those with high-affinity cis-elements are still repressed by low amounts of uncleaved Roquin. This system would add even more complexity to the post-transcriptional regulation of T cell activation and differentiation-determining proteins.

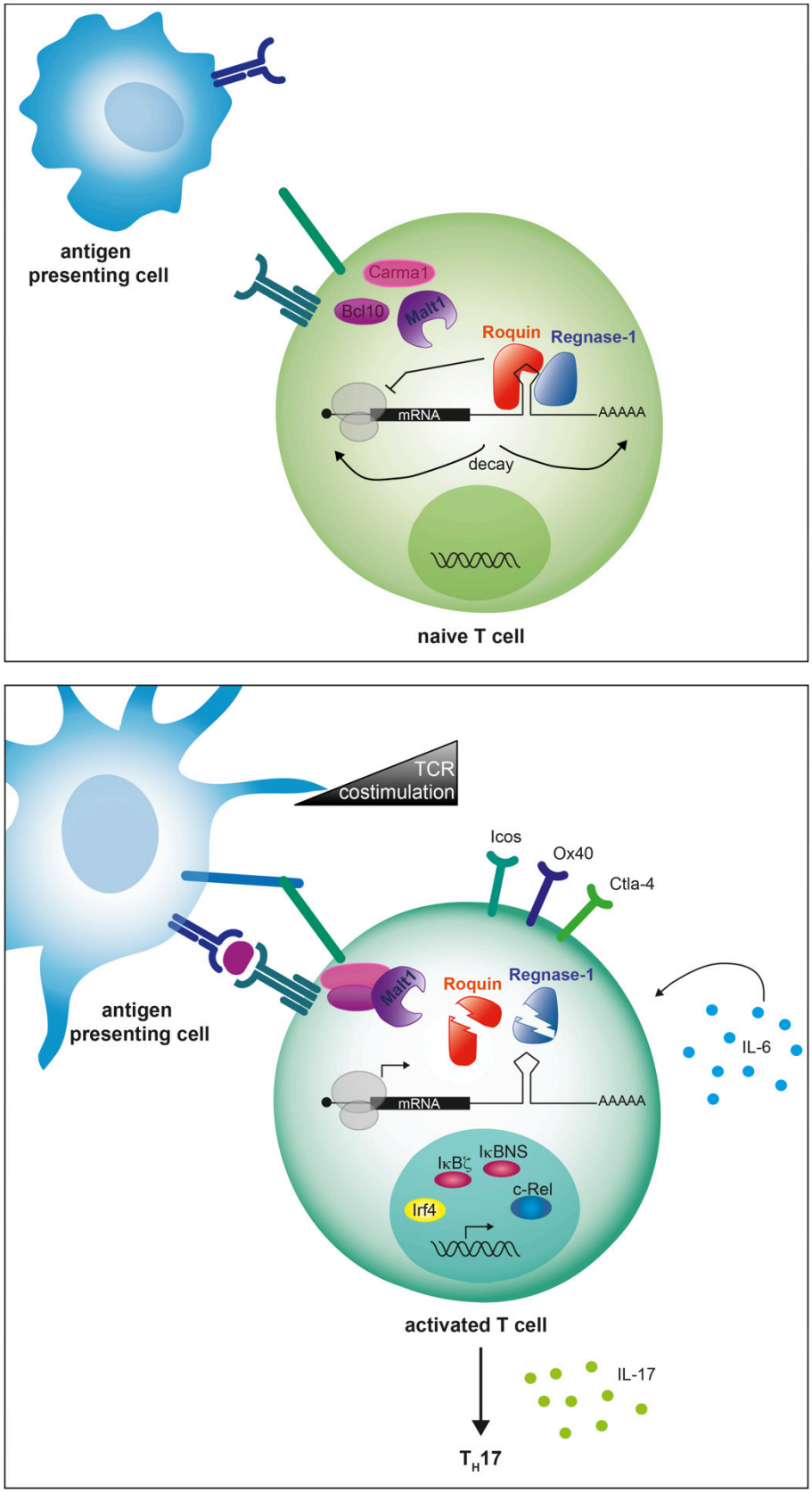

Fig. 5: Current model of Roquin- and Regnase-1-controlled T cell activation

In naive T cells, full-length Roquin and Regnase-1 proteins cooperate to repress target mRNAs encoding proteins such as Icos, Ox40, Ctla-4, IL-6, c-Rel, Irf4, IκBζ and IκBNS and thereby prevent T cell activation and T_H17 differentiation. Combined TCR activation and costimulatory signaling induces cleavage of Roquin-1/2 and Regnase-1 by the paracaspase Malt1 and in this way releases T cells from Roquin-1- and Regnase-1-mediated post-transcriptional repression. This leads to T cell activation and differentiation. The pathway acts as a 'rheostat' by translating TCR signal strength into graded expression of T_H17 promoting factors to enhance T_H17 differentiation.

8.3.5 Possible role of Roquin in T cell metabolism

Metabolic pathways influence cell fate decisions. Thereby, small changes in the equilibrium of energy-consuming and energy-producing pathways define the effector-function of T cells (see 5.1.2.4). It is likely that T cell metabolism is changed upon Roquin mutation or deletion due to deregulation of certain factors and one target candidate would be Irf4. In $CD8^+$ T cells, Irf4 translates TCR strength into transcriptional changes and regulates many genes in the aerobic glycolysis such as Hif-1α (hypoxia inducible factor-1α) to guarantee high glycolytic turnover (Man et al., 2013). In $CD4^+$ T cells, the transcription factor Hif-1α is selectively expressed in differentiated T_H17 cells (Dang et al., 2011; Shi et al., 2011) where it facilitates the expression of glycolytic genes (Gerriets and Rathmell, 2012). Hif-1α-mediated promotion of glycolysis influences the decision between T_H17 and Treg differentiation in favor of T_H17 (Dang et al., 2011; Shi et al., 2011), yet it is dispensable for the switch to glycolysis during initial T cell activation (Wang et al., 2011). Hif1-α collaborates with RORγt to activate transcription of T_H17-promoting genes and concomitantly represses the generation of Tregs by binding Foxp3 and targeting it for proteasomal degradation (Dang et al., 2011; Palazon et al., 2014). Mice deficient for Hif-1α show impaired T_H17 development but increased Treg differentiation and are protected from EAE (Dang et al., 2011; Shi et al., 2011). I hypothesize that Roquin plays an important role in metabolic changes in $CD4^+$ and $CD8^+$ T cells via Roquin-controlled Irf4 expression and subsequent Hif-1α induction. Preliminary data confirmed that Hif-1α expression follows the kinetic of Roquin-cleavage and Irf4 derepression in activated T cells (data not shown), but further experiments are needed to confirm this theory.

In general, metabolic shifts likely contribute to the phenotype of *Rc3h1-2*$^{fl/fl}$*Cd4*-Cre or *sanroque* mice since they can lead to chronic T cell activation and inflammation (Gerriets and Rathmell, 2012; MacIver et al., 2013). In mice with transgenic T cell-specific Glut1 expression, increased glycolysis results in enhanced T cell activation and proliferation with higher cytokine production of IL-2 and IFN-γ. These mice develop lymphadenopathy and autoimmune phenotypes such as high IgG serum concentration and IgG deposition in the kidneys (Jacobs et al., 2008). The E3 ubiquitin ligase Deltex1 promotes the degradation of Hif-1α (Hsiao et al., 2015) and Deltex1-deficiency results in augmented T cell activation and an autoimmune phenotype characterized by auto-antibody production (Hsiao et al., 2009, 2015). The phenotype is attributed to Tregs with impaired suppressor function since high Hif-1α levels are associated with Foxp3 downregulation (Hsiao et al., 2015). High Hif-1α levels due to increased Irf4 expression in $CD4^+$ T cells of mice with T cell-specific deletion of Roquin could therefore be similarly responsible for their autoimmune T_H17-connected phenotype but this needs to be verified in the future.

8.4 Possible reasons for systemic autoimmunity of *sanroque* mice

To our surprise, a T cell-specific knockout of both Roquin paralogs did not result in *sanroque*-like systemic autoimmunity but organ-specific inflammation (Publication II). Mice with complete loss of Roquin in T cells were different from *sanroque* mice in several aspects most strikingly the absence of ANAs and the presence of T_H17-prone $CD4^+$ T cells instead of the T_H1-bias seen in *sanroque* mice (Publication II) (see 8.3.3). In light of this, one wonders how the *sanroque* point-mutation, which introduces one amino acid change at position 199 from methionine to arginine within the ROQ domain (Vinuesa et al., 2005) (Fig. 4), confers SLE-like autoimmunity.

8.4.1 Generation of auto-nuclear antibodies in *sanroque* mice

The absence of ANAs in *Rc3h1-2*$^{fl/fl}$*Cd4*-Cre mice (see 8.1.2) might be explained by the observed destruction of their splenic microarchitecture (Publication I). Scattered cell distribution and unorganized or absent follicles do probably not support efficient T cell-B cell interactions necessary for the GC response and selection of high-affinity self-reactive GC B cells by T_{FH} cells (Publication I). The few GCs present might differ in their qualitative function (Heissmeyer and Vogel, 2013). Architectural disorganization was also observed in secondary lymphoid organs of Regnase-1-deficient mice especially in the spleen, but did not interfere with anti-nuclear auto-antibody generation in this case (Miao et al., 2013). IgG antibodies accumulated in various organs of Regnase-1-deficient mice such as lung and spleen and one could speculate that some of them are tissue-specific (Miao et al., 2013). In contrast, mice with deficiency of Roquin proteins did not develop reactivity to lung antigens, but in general had the potential to generate auto-antibodies as IgG isotype antibodies from their serum reacts for example to pancreatic proteins (Publication II). It needs to be tested if adoptive transfer of TCR-transgenic Roquin-deficient T cells into WT recipients and subsequent immunization lead to effective antibody responses. The cause for the perturbed architecture of the spleen is not clear but a deregulated, so far unknown target of Roquin might be involved. However, auto-antibodies are viewed as primary markers of disease and not as the cause (Crotty, 2014). How are nucleic-specific antibodies selected in *sanroque* mice in contrast to organ-specific antibodies in mice with T cell-specific Roquin-deletion? Potentially, this depends on differences in T cell activation. Maybe self-reactive T cells are selectively activated in *sanroque* mice after receiving signals via self-antigens whereas the activation threshold of Roquin-deficient T cells is generally lowered. In Roquin-deficient T cells activation might take place independently of TCR triggering and thereby independently of

their expressed TCRs. As mentioned before, a thorough comparison of the respective TCR repertoires is necessary (see 8.3.2).

8.4.2 Contribution of non-T cells to overt lupus-like autoimmunity in *sanroque* mice

In general, a driving force for the onset of systemic autoimmunity is the contribution of innate immune cells (Cheng and Anderson, 2012). According to this, non-T cells could be involved in the SLE-like disease of *sanroque* mice. One argument for the role of other immune cells is that ablation of Roquin-1 in the entire hematopoietic system worsend the moderate immune deregulation seen in T cell-specific knockout mice (Bertossi et al., 2011). Activation of $CD4^+$ T cells in addition to activation of $CD8^+$ T cells, higher Icos expression and spontaneous GC formation indicate a trans-effect of non-T cells (Bertossi et al., 2011). However, ANAs or signs of autoimmunity were not detected (Bertossi et al., 2011). To test the contribution of non-T cells to the *sanroque* phenotype one could analyze *Rc3h1*$^{san/fl}$*Cd4*-Cre for ANAs. These animals have a heterozygous expression of Roquin-1^{san} in non-T cells that is compensated by the presence of the WT allele whereas their T cells express only the Roquin-1^{san} protein. What a type of immune cells apart from T cells induces the pathogenic changes in *sanroque* mice, will be subject of future experiments.

8.4.3 Roquin^{san} is a hypomorph that possibly acquires neomorphic functions

The *sanroque* mutation renders the Roquin-1^{san} protein hypomorphic with respect to target mRNA repression as seen for *Icos* (Athanasopoulos et al., 2010; Vinuesa et al., 2005; Yu et al., 2007). However, it is unsolved how this partial loss-of-function triggers systemic autoimmunity. Stability and protein expression levels of Roquin-1^{san} protein were not altered in T cells (Vinuesa et al., 2005; Publication I). Surprisingly, the mutation interfered neither with RNA-binding activity (Athanasopoulos et al., 2010; Leppek et al., 2013; Srivastava et al., 2015) nor with localization of the Roquin-1^{san} protein (Athanasopoulos et al., 2010). Nevertheless, phenotypes that were exacerbated in mice with combined loss of both Roquin proteins in T cells like high Icos expression (Publication I) fit to the presence of a mutated Roquin-1^{san} protein with reduced repressor function in *Rc3h1*$^{san/san}$ mice. Regulation of the same mRNA target set by Roquin-1^{san} with residual activity in comparison to complete deregulation of all targets in the absence of Roquin-1 and -2 could make the difference and rather result in T_H1 than T_H17 differentiation. This is possibly mediated via fine differences in the metabolic status of the respective T cells (see 8.3.5) due to differential expression levels of important metabolism-regulating factors (see 8.3.4). Thorough comparison of expression levels and

kinetics of all known Roquin targets in *sanroque* and Roquin-deficient T cells as well as comparative metabolic studies could shed light on this issue.

In addition to its hypomorphic function, Roquin-1^{san} probably exerts a so far unknown function that is not shared with WT Roquin-1 protein. Unexpectedly, the *sanroque* mutation in the ROQ domain was only associated with minor perturbations in protein conformation (Srivastava et al., 2015; Tan et al., 2014). However, Srivastava et al. suggest that the M199R mutation lead to the exposure of a hydrophobic residue (F234) and imply its capacity for a new interaction potentially with another protein (Srivastava et al., 2015). This could mediate a gain-of-function and potentially involves the Roquin-1^{san} protein in new aspects of post-transcriptional regulation or is associated with its possible E3 ligase activity. Perhaps neomorphic functions can also be attributed to the stable N-terminal cleavage product of Roquin-1^{san} aa 1-510 since, in preliminary experiments, cleavage of Roquin-1^{san} protein upon TCR activation could be detected (data not shown). This might either change an existing role of the cleavage product of Roquin-1 or confer a totally new function to it (see 8.3.1). However, interaction of Roquin-1^{san} or Roquin-1^{san} aa 1-510 with so far unknown factors such as other RBPs could extend the mRNA target repertoire. These so far unknown unique Roquin-1^{san} targets could explain the phenotypes observed in *sanroque* mice. IFN-γ is a possible candidate, which is highly expressed by *sanroque* and not by Roquin-deficient CD4^{+} T cells after *ex vivo* stimulation (data not shown) (see 8.3.3).

8.4.4 Does IFN-γ make the difference?

The cytokine IFN-γ is a major effector molecule of systemic autoimmune diseases, especially SLE (Pollard et al., 2013). Virtually all immune cells express the IFN-γ receptor (IFN-γR) (Szabo et al., 2003), explaining the broad range of IFN-γ effects (Pollard et al., 2013). Among other things, IFN-γ propagates Ig class switching in B cells to more pathogenic auto-antibodies (Billiau and Matthys, 2009; Theofilopoulos et al., 2001). Furthermore, IFN-γ was shown to be necessary for the initiation of lupus pathogenesis by crossing the MRL-*Fas*^{lpr} strain to IFN-γ$^{-/-}$ or IFN-γR$^{-/-}$ mice which reduces disease severity (Balomenos et al., 1998; Hron and Peng, 2004; Lawson et al., 2000; Schwarting et al., 1998). Moreover, most female transgenic mice overexpressing IFN-γ in the epidermis show a lupus-like syndrome by three months of age (Seery et al., 1997). These mice develop ANAs and severe immune complex-mediated glomerulonephritis which is dependent on the presence of T cells (Seery, 2000; Seery et al., 1999). Similarly, chronic low-level expression of IFN-γ due to genetic deletion of the entire AU-rich elements (ARE) region in the 3' UTR of the *Ifng* mRNA leads to a lupus-like autoimmune phenotype with auto-antibody production and glomerulonephritis (Hodge et al., 2014). In line

with this, in *sanroque* mice, deregulation of *Ifng* mRNA half-life leads to constitutively high IFN-γ levels which result in lupus-like autoimmunity (Chang et al., 2012; Lee et al., 2012). IFN-γR deficiency in *sanroque* mice reverts splenomegaly, accumulation of T_{FH} cells and GC formation, and reduces ANAs as well as renal pathology (Lee et al., 2012). In contrast, Icos deficiency cannot prevent the development of autoimmunity in *sanroque* mice (Lee et al., 2012). Prior to this publication, excessive Icos signaling and consequently increased T_{FH} cell expansion were believed to be the reason for the pathogenesis, two features that do not provoke systemic autoimmunity and auto-antibody production in mice deficient for both Roquin paralogs in T cells (Publication I). However, abrogating T_{FH} cell formation generally prevents lupus in *sanroque* mice (Linterman et al., 2009). Lee et al. could show that IFN-γ is directly involved in the promotion of T_{FH} cells via augmentation of Bcl6 expression in a T cell activation-dependent manner (Lee et al., 2012). Potentially, IFN-γ expression by *Rc3h1*$^{san/san}$ T_{FH} cells adds a new quality to their function, which could be missing in *Rc3h1-2*$^{fl/fl}$*Cd4*-Cre T_{FH} cells. In favor of this, crossing of IL-21 deficient mice to *sanroque* mice does not rescue T_{FH} cell frequencies or auto-antibody formation (Linterman et al., 2009) although IL-21 requirement for T_{FH} cell and GC formation was described previously (Nurieva et al., 2008; Vogelzang et al., 2008). As discussed before, there is a phenotypic overlap between T_{FH} and T_H1 cells (Nakayamada et al., 2011) (see 8.3.3). Data of Reinhard et al. supports the idea that IFN-γ-producing T_{FH} cells in the GC regulate isotype class switching and affinity maturation of conjugated B cells and thus shape the antibody repertoire (Reinhardt et al., 2009). In line with this, T_{FH} cells maintain low levels of IFN-γ expression during the course of an LCMV infection (Johnston et al., 2009) and another publication reported T_H1 conversion towards T_{FH} cells in chronic LCMV infection with viral persistence and prolonged TCR stimulation (Fahey et al., 2011).

So far there is no direct evidence for *Ifng* mRNA being a direct target of WT Roquin although Lee et al suggests this possibility by demonstrating increased mRNA half-life in *sanroque* T cells (Lee et al., 2012). Final proof as direct binding of Roquin to *Ifng* mRNA or identification of a hairpin *cis*-element in its 3' UTR, as well as responsiveness of IFN-γ expression to Roquin overexpression or knockdown is at this point missing. Everything so far points towards either indirect regulation of *Ifng* mRNA by Roquin or direct stabilization of *Ifng* mRNA only mediated by the mutated Roquin-1^{san} protein. However, the mRNA of this cytokine underlies intense post-transcriptional regulation. *Ifng* mRNA is negatively regulated by miRNAs like miR-29 (Ma et al., 2011; Steiner et al., 2011) and ARE-BPs such as TTP (Ogilvie et al., 2009) and stabilized by HuR (Wang et al., 2006). A newly described post-transcriptional mechanism involved GAPDH in direct binding of AREs in the *Ifng* mRNA and subsequent suppression (Chang et al.,

2013). The metabolic switch to aerobic glycolysis necessary for T_H1 differentiation (see 5.1.2.4) requires the enzymatic function of GAPDH and reduces its availability for *Ifng* regulation (Chang et al., 2013). The question is how Roquin-1^{san} can be involved in existing post-transcriptional control mechanisms of *Ifng* mRNA. Is a neomorphic function of Roquin-1^{san} responsible or does the *sanroque* mutation interfere with an already present function? A connection between ARE-regulated post-transcriptional mechanisms and Roquin-1^{san} action or the miRNA pathway and Roquin-1^{san} seems possible and requires experimental proof in the future.

In summary, IFN-γ expression by *sanroque* T_{FH} cells could make the difference between the phenotypes observed in *sanroque* mice and mice with combined ablation of Roquin proteins in T cells.

8.5 Mice lacking Malt1 protease activity develop systemic inflammation

Deregulations of Roquin or Regnase-1 function lead to severe immune dysregulation as seen in the respective knockout mice. They might also be responsible for inappropriate immune activation in human autoimmune diseases. There, the regulatory circuit of Malt1 and cleavage of the post-transcriptional repressors Roquin and Regnase-1 could be disturbed which potentially lead to high induction of immune factor expression that are normally targeted by Roquin and Regnase-1. Hence, targeting the proteolytic activity of Malt1 and thereby blocking the cleavage of Roquin and Regnase-1 might be an option to interfere with exaggerated immune responses especially of the T_H17-type. Recently, small-molecule inhibitors of Malt1 paracaspase activity were discovered that show promising pharmacological properties (Fontan et al., 2012; Nagel et al., 2012). The reversible inhibitors mepazine and thioridazine were shown to selectively induce apoptosis of activated B cell diffuse large-B cell lymphomas (ABC-DLBCLs) cell lines, a lymphoma type that is dependent on constitutive anti-apoptotic NF-κB signaling (Nagel et al., 2012). Indeed, further promising results showed attenuation of EAE by treatment of mice with mepazine (Mc Guire et al., 2014). Nonetheless, the consequences of inactivating the catalytic function of Malt1 on the immune response are not clear.

Malt1 knockout mice show an immunodeficient phenotype. These mice lack in addition to the Malt1 protease function also the scaffold function, which is important for NF-κB signaling (see 5.1.2.2). Accordingly, Malt1 paracaspase inhibition was considered to be immunosuppressive. However, analysis of mice with genetic inactivation of the Malt1 protease revealed unforeseen lethal multiorgan inflammatory syndrome in these mice (Bornancin et al., 2015; Jaworski et al., 2014; Yu et al., 2015; Publication III). In all four recent publications Malt1

enzymatic activity was abrogated in mice by genetically introducing a mutation of the active site cysteine 472 to alanine in the paracaspase domain (Bornancin et al., 2015; Jaworski et al., 2014; Yu et al., 2015; Publication III). These mice are called *Malt1*$^{PM/PM}$ (PM for pointmutation) here for simplicity. Jaworski et al. and Bornancin et al. looked at mice homozygous for the pointmutation whereas Gewies et al. examined Malt1$^{PM/-}$ animals after exclusion of dominant-negative effects by mutated Malt1 (Publication III). Malt1^{PM} protein was expressed at WT levels and had no proteolytic function (Bornancin et al., 2015; Jaworski et al., 2014; Yu et al., 2015; Publication III). Hence, *Malt1*$^{PM/-}$ T cells did not show cleaved Roquin and Regnase-1 products upon stimulation (Publication III). Mice lacking Malt1 proteolytic activity were born at Mendelian ratio but lost weight in adulthood and died prematurely due to systemic inflammation (Bornancin et al., 2015; Publication III; Jaworski et al., 2014). According to Gewies et al. *Malt1*$^{PM/-}$ mice developed autoimmune gastritis and neurological defects associated with dystonia and progressive ataxia that started at an age of 3 month (Publication III). The publication of Jaworski et al. agreed on the gastritis phenotype and additionally reported auto-antibodies against the mucosa of the stomach (Jaworski et al., 2014) whereas Bornancin et al. added inflammation of a variety of organs including stomach and peripheral nerves (Bornancin et al., 2015). Surprisingly, the paracaspase activity was largely dispensable for T cell activation and proliferation in contrast to Malt1$^{-/-}$ T cells (Bornancin et al., 2015; Publication III; Jaworski et al., 2014). IKK and Jnk activation were normal in T cells expressing protease-insufficient Malt1 which indicates the dependency of these signaling pathways on the scaffold function of Malt1 (Bornancin et al., 2015; Publication III; Jaworski et al., 2014). Quite contrary to Malt1-deficiency, mice lacking Malt1 protease activity developed lymphadenopathy and had elevated levels of activated T cells (Bornancin et al., 2015; Publication III; Jaworski et al., 2014). These T cells acquired an IFN-γ- or IL-4-producing phenotype (Bornancin et al., 2015; Publication III; Jaworski et al., 2014). Bornancin et al. reported defective *in vitro* T_H17 differentiation of naive *Malt1*$^{PM/PM}$ CD4$^+$ T cells similarly to Malt1-deficient T cells (Bornancin et al., 2015). This fits to our data providing a molecular basis for the dependency of T_H17 differentiation on Malt1 protease activity (Publication II) (Fig. 5). In line with this, Bornancin et al. and Jaworski et al. reported that *Malt1*$^{PM/PM}$ mice were protected from EAE (Bornancin et al., 2015; Jaworski et al., 2014). However, applicability of this disease model even in young mice might be confounded by the severe spontaneous neurodegeneration that Gewies et al. reported in older mice (Publication III). Gewies et al. not only detected T cell infiltration in the brain but also proved their relevance for disease by phenotypic rescue of *Malt1*$^{PM/-}$ mice crossed to a lymphocyte-deficient *Rag1*$^{-/-}$ background (Publication III). Likewise, IFN-γ-deficiency rescued neuropathology as well as weight loss and

gastritis (Publication III). This indicates the importance of IFN-γ-producing T_H1 cells for immunopathology of *Malt1*$^{PM/-}$ mice and exemplifies the role of IFN-γ as a key driver in systemic inflammatory disorders (Publication III). Remarkably, thymic Tregs were absent and peripheral Tregs were substantially reduced in mice lacking Malt1 paracaspase function comparable to Malt1-deficient mice (Bornancin et al., 2015; Publication III; Jaworski et al., 2014). The defect in Treg development was T cell-intrinsic as shown by mixed bone marrow (BM) chimeras of WT and *Malt1*$^{PM/PM}$ BM where Tregs developed only from WT BM in WT recipients (Jaworski et al., 2014). Adoptive transfer of WT Tregs into newborn *Malt1*$^{PM/PM}$ mice could rescue weight loss, T cell activation and increase of IFN-γ$^+$ or IL-4$^+$ CD4$^+$ T cells as well as autoimmune symptoms like gastritis (Jaworski et al., 2014).

In summary, *Malt1*$^{PM/PM}$ T cells obviously receive productive TCR signals *in vivo* via the Malt1 scaffold function although Malt1 protease activity was thought to contribute to optimal activation of the NF-κB pathway by cleavage of important negative regulators such as A20 (see 5.1.2.2). On the other hand, Roquin and Regnase-1 that directly control mRNA repression of T_H17 promoting factors are not cleaved (Publication III). However, activated T cells retain the capacity to generate T_H1 and T_H2 effectors. These effector T cells are not efficiently controlled by Tregs, which could explain the pathology seen in *Malt1*$^{PM/PM}$ mice. Indeed, "scurfy" mice that lack Foxp3$^+$ Tregs similarly develop autoimmune inflammation as described in 5.1.4. Malt1-deficient mice on the contrary display complete abrogation of NF-κB activation resulting in strongly abrogated T cell activation where low Treg numbers do not matter. The reasons for defective Treg development in the thymus and in the periphery in mice expressing pointmutant Malt1 are elusive. As T cells lack Malt1 protease activity they might have problems to differentiate into Tregs due to restricted upregulation of the Roquin- and Regnase-1-controlled proteins c-Rel, IκBNS and probably IL-2 (Publication II), which are essential for Treg development (Ruan et al., 2009; Sakaguchi, 2004; Schuster et al., 2012). As a second possibilty, changes in TCR signaling caused by defective Malt1 proteolytic activity might be responsible for alterations of T cell selection in the thymus. Hypothetically, auto-reactive CD4$^+$ T cells that normally are deleted in the thymus or selected for the nTreg subset are released into the periphery because TCR signaling is probably not high enough when Malt1 proteolytic activity is missing. In the periphery, Malt1 scaffold function seems to be sufficient for the activation of these self-reactive T cells in response to self-antigens and they acquire T_H1 or T_H2 effector function while being impaired in becoming T_H17 cells due to high Roquin and Regnase-1 levels.

These results gained from *Malt1*$^{PM/PM}$ mice raise doubts about therapeutic use of Malt1 inhibitors in treatment of autoimmune and inflammatory diseases. However, if the theory of

absent Tregs in combination with disturbed central tolerance causing disease in mice lacking Malt1 protease activity holds true, treatment with Malt1 inhibitors might still be feasible for Treg-sufficient recipients. Careful studies examining this issue are required in the future to determine potential and beneficial applications of Malt1 inhibitors.

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9 LIST OF FIGURES

10 REFERENCES

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11 DECLARATION OF INDIVIDUAL CONTRIBUTION

I.

Vogel*, K.U., Edelmann*, S.L., **Jeltsch, K.M.**, Bertossi, A., Heger, K., Heinz, G.A., Zöller, J., Warth, S.C., Hoefig, K.P., Lohs, C., Neff, F., Kremmer, E., Schick, J., Repsilber, D., Geerlof, A., Blum, H., Wurst, W., Heikenwälder, M., Schmidt-Supprian, M., Heissmeyer, V. (2013). Roquin paralogs 1 and 2 redundantly repress the Icos and Ox40 costimulator mRNAs and control follicular helper T cell differentiation. Immunity 38, 655–668. * equal contribution

Katharina M. Jeltsch analyzed the postnatal lethality of mice with systemic deletion of Roquin-2 (*Rc3h2*$^{neo/neo}$) (**Fig. 1A, C** and **D** and **Supplemental Fig. S1G**), contributed a Western Blot of T cells with Roquin mutations (**Fig. 3F**) and compared the Icos expression of T cells of *Rc3h1*$^{fl/fl}$*Rc3h2*$^{fl/fl}$*Cd4*-Cre with that in T cells of *Rc3h1*$^{fl/fl}$*Cd4*-Cre and *Rc3h1*$^{san/san}$ mice (**Fig. 4C**). Furthermore, she confirmed Ox40 regulation by Roquin-1 via the *Ox40* 3' UTR in Roquin-1/2-deficient mouse embryonic fibroblasts that were sequentially infected with retroviruses to express Ox40 and Roquin-1 (**Fig. 7E**). In addition, she cloned the isoforms of Roquin-2, that were used in **Fig. 4I** to show Roquin-2-dependent Icos repression in T cells.

II.

Jeltsch*, K.M., Hu*, D., Brenner*, S., Zöller, J., Heinz, G.A., Nagel, D., Vogel, K.U., Rehage, N., Warth, S.C., Edelmann, S.L., Gloury, R., Martin, N., Lohs, C., Lech, M., Stehklein, J.E., Geerlof, A., Kremmer, E., Weber, A., Anders, H.J., Schmitz, I., Schmidt-Supprian, M., Fu, M., Holtmann, H., Krappmann, D., Ruland, J., Kallies, A., Heikenwalder, M., Heissmeyer, V. (2014). Cleavage of roquin and regnase-1 by the paracaspase MALT1 releases their cooperatively repressed targets to promote T(H)17 differentiation. Nat. Immunol. *15*, 1079–1089. * equal contribution

Katharina M. Jeltsch performed most experiments: **Fig. 3** (with initial experiments already performed by Dr. Katharina Vogel and Nina Martin), **Fig. 4** (Fig. 4B in cooperation with Prof. Dr. Jürgen Ruland, Fig. 4E in cooperation with Dr. Daniel Nagel and Prof. Dr. Daniel Krappmann), **Fig. 5B** (together with Dr. Gitta A. Heinz), **Fig. 7** (Fig. 7A-B together with Dr. Sebastian Warth), **Fig. 8** (Fig. 8E-F in cooperation with Renee Gloury and Dr. Axel Kallies), **Fig. S1F** (together with Dr. Katharina Vogel and Dr. Desheng Hu), **Fig. S1G-H** (in cooperation with Prof. Dr. Hans-Joachim Anders) and **Fig. S3** (Fig. S3B together with Claudia Lohs). In these experiments Katharina M. Jeltsch uncovered the role of the paracaspase Malt1 in the regulation of Roquin through cleavage, confirmed the influence of Regnase-1, IκBζ and IκBNS on T_H17 differentiation by knockdown experiments and correlated TCR signal strength with graded Roquin cleavage and graded derepression of Roquin targets which thereby facilitates T_H17 differentiation. Katharina M. Jeltsch analyzed and interpreted data, created figures and wrote part of the manuscript. **Dr. Desheng Hu** performed the in-depth analysis of *Rc3h1*$^{fl/fl}$*Rc3h2*$^{fl/fl}$*Cd4*-Cre mice and described their T_H17-prone lung phenotype in cooperation with Jessica Zöller and Prof. Dr. Mathias Heikenwälder (Fig. 1A-F, Fig. 2, Fig. 5A+C, Fig. S1A-E, Fig. S2) and confirmed the knockdown of Regnase-1, IκBζ and IκBNS by adenoviral shRNAs. Dr. Desheng Hu analyzed and interpreted data, created figures and wrote part of the manuscript. **Dr. Sven Brenner** performed the experiments dissecting the cooperation of Roquin-1 and Regnase-1 in the regulation of shared mRNA targets (Fig. 5E-G, Fig. 6 and Fig. S4F-H), analyzed and interpreted data, created figures and wrote part of the manuscript.

III.

Gewies*, A., Gorka*, O., Bergmann, H., Pechloff, K., Petermann, F., **Jeltsch, K.M.**, Rudelius, M., Kriegsmann, M., Weichert, W., Horsch, M., Beckers, J., Wurst, W., Heikenwlader, M., Korn, T., Heissmeyer, V., Ruland, J. (2014). Uncoupling Malt1 threshold function from paracaspase activity results in destructive autoimmune inflammation. Cell Rep *9*, 1292–1305. * equal contribution

Katharina M. Jeltsch performed the Western Blots of *Malt1*$^{PM/-}$ T cells (**Fig. 5H** and **Fig. S3B**).

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