Aus dem Institut für Immunologie im Biomedizinischen Centrum
der Ludwig-Maximilians-Universität München
Direktor: Prof. Dr. rer. nat. Thomas Brocker

A global definition of Roquin-mediated regulation of mRNA expression and translation uncovers its impact on PI3K-mTOR signaling and T cell differentiation

-Dissertation-
zum Erwerb des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)
an der Medizinischen Fakultät
der Ludwig-Maximilians-Universität München

vorgelegt von
Katharina Alice Maria Essig
aus Heidelberg

München, 2018

Gedruckt mit Genehmigung der Medizinischen Fakultät der Ludwig-Maximilians-Universität München

Betreuer:	Prof. Dr. rer. nat. Vigo Heissmeyer
Zweitgutachter:	Priv. Doz. Dr. Dietmar Martin
Dekan:	Prof. Dr. med. dent. Reinhard Hickel

Tag der mündlichen Prüfung: 27.02.2019

Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt,
dass ich die vorliegende Dissertation mit dem Thema

„**A global definition of Roquin-mediated regulation of mRNA expression and translation uncovers its impact on PI3K-mTOR signaling and T cell differentiation.”**

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, den 10.03.2019 Katharina Essig

„Wenn das die Lösung ist, will ich mein Problem zurück!“

Autor unbekannt

Publications of the thesis

Publication I

Essig K, Kronbeck N, Guimaraes JC, Lohs C, Schlundt A, Behrens G, Hoffmann A, Brenner S, Kowalska J, Lopez-Rodriguez C, Jemielity J, Holtmann H, Reiche K, Hackermüller J, Sattler M, Zavolan M and Heissmeyer V., **Nat Commun. 2018** Sep 19; 9(1):3810.

DOI https://doi.org/10.1038/s41467-018-06184-3

Publication II

Essig K, Hu D, Guimaraes JC, Alterauge D, Edelmann S, Raj T, Kranich J, Behrens G, Heiseke A, Floess S, Klein J, Maiser A, Marschall S, Hrabě de Angelis M, Leonhardt H, Calkhoven CF, Noessner E, Brocker T, Huehn J, Krug AB, Zavolan M, Baumjohann D, Heissmeyer V., **Immunity. 2017** Dec 19; 47(6):1067-1082.e12.

DOI https://doi.org/10.1016/j.immuni.2017.11.008

Summary

$CD4^+$ T cells comprise effector and regulatory subsets, which exhibit unique functions and properties and protect against different pathogens. Several molecular cues that define the differentiation into the distinct T cell subsets have already been identified. One determinant is the RNA-binding protein Roquin that post-transcriptionally regulates gene expression and prevents autoimmunity. It is therefore crucial to precisely understand how Roquin controls $CD4^+$ T cell fate decisions on the molecular level. In this thesis, we proved that Roquin acts on different layers of regulation to direct T cell differentiation. On the one hand by destabilizing but also by inhibiting the translation of its mRNA targets, and on the other hand by suppressing the PI3K-mTOR signaling pathway. In **publication I,** we investigated whether translational regulation is a further post-transcriptional mechanism by which Roquin controls its targets. By performing PAR-CLIP, mRNA sequencing and ribosome profiling on MEF cells, we globally determined that Roquin does not only reduce the stability of its mRNA targets, but also, at least for a smaller subset, inhibits their translation. These data sets also enabled us to identify a new linear binding element (LBE) of Roquin that is highly abundant in its targets and is recognized by its ROQ and Znf domains. Furthermore, we revealed that Roquin-mediated translational regulation of its targets depends on the number of binding sites in their 3′ untranslated regions (UTRs). Among the translationally-repressed Roquin targets we found the T cell relevant genes *Nfat5 and Nfkbid*. Through further analysis of the 3′ UTR of *Nfkbid* we have identified a minimal response element, which consists of six stem loop structures and is indispensable for its post-transcriptional regulation by Roquin. In **publication II** we examined the function of Roquin in Treg cells by analyzing mice, which lacked Roquin expression specifically in these cells. We found that Roquin-deficient Treg cells are dysfunctional and failed to inhibit the induction of colitis in a T cell transfer model. Furthermore, we observed that they lose the expression of the IL-2 receptor α chain (CD25) and adopt a functional follicular Treg (Tfr) phenotype. Mechanistically, we elucidated that Roquin suppresses the PI3K-Akt-mTOR-Foxo1 signaling pathway in Treg and conventional T cells by controlling two novel Roquin-targeted mRNAs, the PI3K antagonist Pten and the Foxo1-specific E3 ubiquitin ligase Itch. Roquin interferes with miR-17~92 binding to an overlapping cis-element in the PTEN 3′ UTR thereby upregulating Pten expression. In addition, it represses Itch expression leading to increased levels and nuclear localization of Foxo1. Finally, we showed that inhibition of PI3K or mTOR rectifies the aberrant frequencies of Roquin-deficient Th17 and iTreg cells *in vitro* and Tfh cells *in vivo*. My contribution to these two studies was to globally define a Roquin-bound mRNA set and its mode of post-transcriptional repression. These investigations also enabled me to elucidate Roquin-mediated regulation of the PI3K-mTOR signaling pathway, thereby controlling Treg cell function and differentiation of T helper cells.

Zusammenfassung

CD4$^+$ T Zellen umfassen Effektor- und regulatorische Untergruppen mit spezifischen Funktionen und Eigenschaften, die vor Infektionen mit Pathogenen schützen. Zahlreiche molekulare Signale, die die Differenzierung in die unterschiedlichen T-Zell-Untergruppen steuern, wurden bereits identifiziert. Das RNA-bindende Protein Roquin ist einer dieser entscheidenden Faktoren, der die Genexpression post-transkriptionell reguliert und die Entstehung von Autoimmunerkrankungen verhindert. Deshalb ist es entscheidend, ein genaues Verständnis darüber zu erlangen, wie Roquin Schicksalsentscheidungen von CD4$^+$ T Zellen auf molekularer Ebene kontrolliert. In der vorliegenden Arbeit haben wir bewiesen, dass Roquin auf verschiedenen regulatorischen Ebenen T-Zell-Differenzierung lenkt, zum einen, indem es sowohl die Stabilität als auch die Translation seiner mRNA Zielmolekülen verringert, und zum anderen durch Hemmung des PI3K-mTOR Signalwegs. In **Publikation I** haben wir untersucht, ob translationale Regulation einen weiteren post-transkriptionellen Mechanismus darstellt, durch den Roquin seine Zielmoleküle kontrolliert. Mit Hilfe der Methoden PAR-CLIP, mRNA Sequenzierung und Ribosome Profiling konnten wir global in MEF Zellen feststellen, dass Roquin nicht nur die Stabilität seiner mRNA Zielmoleküle senkt, sondern auch die Translation einiger weniger Zielmolekülen hemmt. Diese Datensätze ermöglichten es uns zudem, ein neues lineares Bindungselement (LBE) von Roquin zu identifizieren, das in seinen Zielemolekülen sehr häufig vorkommt und von seinen ROQ und Znf Domänen erkannt wird. Weiterhin zeigten wir, dass die durch Roquin-vermittelte translationale Regulation seiner Zielmoleküle von der Anzahl der Bindungsstellen innerhalb ihrer 3′ untranslatierten Region (UTR) abhängt. Unter den translational reprimierten mRNAs fanden wir die T-Zell relevanten Gene *Nfkbid* und *Nfat5*. Durch weitere Analysen des 3′ UTRs von *Nfkbid* haben wir ein minimales Response-Element identifiziert, das aus sechs Haarnadelstrukturen besteht und für die post-transkriptionelle Regulation durch Roquin notwendig ist. In **Publikation II** haben wir die Funktion von Roquin in Treg Zellen erforscht und dazu Mäuse untersucht, denen spezifisch in diesem Zelltyp die Expression von Roquin fehlt. Hier zeigte sich, dass Roquin-defiziente Treg Zellen dysfunktional sind und somit nicht in der Lage waren, das Einsetzen von Colitis in einem T Zell Transfer Modell zu verhindern. Weiterhin konnte beobachtet werden, dass diese Zellen die Expression der alpha-Kette des IL-2 Rezeptors (CD25) verlieren und einen funktionellen follikulären regulatorischen T Zell (Tfr) Phänotyp annehmen. Mechanistisch konnten wir zeigen, dass Roquin den PI3K-Akt-mTOR-Foxo1 Signalweg in Treg und Effektor-T-Zellen hemmt, indem es zwei neue Roquin Zielmoleküle, den PI3K Antagonist Pten und die Foxo1-spezifische E3 Ubiquitin Ligase Itch, kontrolliert. Roquin verhindert die Bindung von miR-17~92 an ein überlappendes cis-Element im 3′ UTR von Pten, wodurch dessen Expression erhöht wird. Außerdem verringert Roquin die Expression von Itch, was zu einem höheren Proteinlevel und zur nuklearen Lokalisation

von Foxo1 führt. Abschließend zeigten wir, dass die Inhibierung von PI3K oder mTOR die abnormale Entstehung von Roquin-defizienten Th17 und iTreg Zellen *in vitro*, sowie die von Tfh Zellen *in vivo*, auf Wildtyp Niveau korrigiert. Mein Beitrag zu diesen zwei Veröffentlichungen war es, global alle Roquin-gebundenen mRNAs sowie ihre durch Roquin-ausgelöste post-transkriptionelle Hemmung aufzuklären. Des Weiteren ermöglichten es mir diese Untersuchungen aufzudecken, dass Roquin den PI3K-mTOR Signalweg reguliert, um dadurch die Funktion und Differenzierung von Treg und T Helferzellen zu steuern.

Table of Contents

List of abbreviations

ADE	Alternative decay element
Ago	Argonaute
BCR	B cell receptor
CD	Cluster of differentiation
CD25	α-chain of the IL-2 receptor
CDE	Constitutive decay element
DKO	Double knockout
Foxo1	Forkhead box O1
GC	Germinal center
ICOS	Inducible T cell co-stimulator
IL	Interleukin
IFN-γ	Interferon-gamma
LBE	Linear binding element
MEF	Mouse embryonic fibroblasts
miRNA or miR	microRNA
mRNA	messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
mTORC1/mTORC2	mTOR complex 1 or 2
NFAT5	Nuclear factor of activated T cells 5
PAR-CLIP	Photoactivatable-Ribonucleoside-Enhanced Cross-linking and Immunoprecipitation
PI3K	Phosphatidylinositol-3-kinase
Pten	Phosphatase and tensin homologue
RISC	RNA-induced silencing complex
SGK1	Serum- and glucocorticoid-induced protein kinase 1
TCR	T cell receptor
Teff	T effector cells
TGF-β	Transforming growth factor
Th	T helper
Tfh	Follicular T helper cells
Tfr	Follicular regulatory T cells
TOP	Terminal oligopyrimidine tract
iTreg	induced regulatory T cells
tTreg	thymic regulatory T cells
UTR	Untranslated region
Znf	Zinc finger

1 Introduction

The immune system can be divided into two components, the innate and the adaptive immune system. Both are essential for combating various pathogens, but they differ in their function, specificity and complexity. The innate immune system forms the first protective barrier against pathogens. This type of immune reaction comprises the activation of macrophages, dendritic cells and natural killer cells and is triggered directly after the infection by the recognition of several conserved molecular structures of bacteria and viruses. Nevertheless, certain types of microbes and viruses are capable to evade these mechanisms. Therefore, the adaptive immune system with its immense diversity of antigen-specific receptors is additionally activated and can eliminate the respective pathogens after a certain induction phase. The central cellular components herein are B and T lymphocytes, which recognize a broad variety of antigens via their B (BCR) and T cell receptor (TCR). B cells derive from the bone marrow and can differentiate into plasma cells, which produce large amounts of antibodies. T cells develop in the thymus where they differentiate into two different subsets: cytotoxic $CD8^+$ T cells, which kill cells infected with viruses or intracellular pathogens, and $CD4^+$ T cells. Basically, $CD4^+$ T cells can also be subdivided into two groups. On the one hand, there are $CD4^+$ T effector (Teff) cells like T helper (Th) cells including Th1, Th2, Th17 and follicular T helper (Tfh) cells, and on the other hand regulatory T cells. The latter can also be categorized into three subsets, thymus-derived Treg (tTreg), peripherally induced Treg (iTreg) and follicular regulatory T (Tfr) cells. The different $CD4^+$ T cell subsets, with the exception of tTreg and Tfr cells, differentiate from naive $CD4^+$ T cells upon TCR activation in an appropriate cytokine milieu. Each subset has distinct immunological functions, requires and secretes different cytokines and is characterized by specific transcription factors (**Figure 1**). The major function of Teff cells is to protect the organism from various infections, whereas Treg cells suppress self-reactive immune responses to prevent autoimmune diseases. In a healthy organism, Treg and Teff cells are in a balance to maintain self-tolerance and in parallel to avoid inflammations.

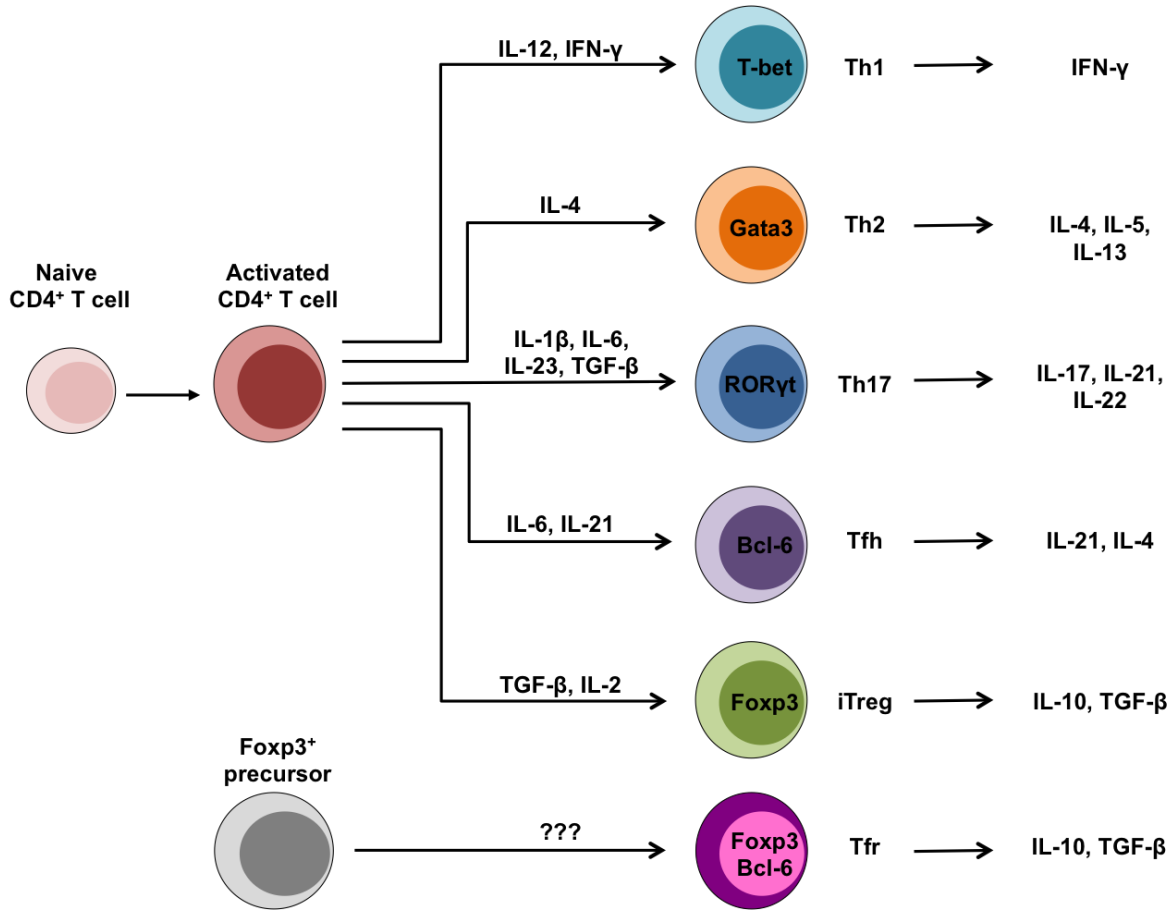

Figure 1 **CD4⁺ T cell subsets.**

The different CD4⁺ T cells subsets including Th1, Th2, Th17, Tfh and iTreg cells differentiate from naive CD4⁺ T cells upon T cell activation in the presence of a different cytokine milieu. Tfr cells develop from thymus-derived Foxp3⁺ precursors. Each CD4⁺ T cell subset is characterized by specific transcription factors and secretes different types of interleukins (IL).

However, when the differentiation of naive T cells into the different CD4⁺ T cell subsets is dysregulated and the balance between Treg and Teff cells is tilted towards a strong Teff response, this can lead to dysregulated and exaggerated immune responses, hence to severe autoimmune diseases. For this reason, it is of great importance for the organism to maintain the immune homeostasis, and that the naive T cells make the “right“ decision to become either a Treg or Teff cell. An arising question is what influences a T cell to make the “right” decision? There are several critical determinants that affect T cell fate decisions: environmental cues e.g. diet and stress, signal-induced ubiquitylation and protein degradation, epigenetics, TCR signal strength, transcription, post-transcriptional gene regulation, metabolism, and signaling pathways (**Figure 2**). The complex interplay between these factors and their sensitivity towards minor changes can lead to drastic effects on T cell homeostasis and function.

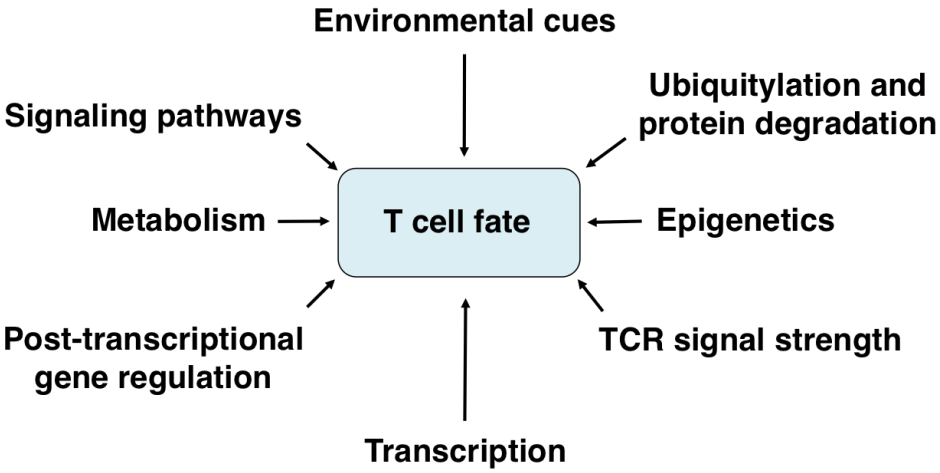

Figure 2 **Determinants of T cell fate.**

Environmental factors, protein stability, epigenetics, signaling, transcription, post-transcriptional gene regulation and metabolism can influence T cell fate decisions.

This introduction will focus on one determinant of T cell fate decision, the RNA-binding protein Roquin that post-transcriptionally regulates gene expression to control $CD4^+$ T cell differentiation and protects against autoimmunity.

The importance of Roquin in the prevention of autoimmune diseases was discovered in an ethylnitrosourea (ENU) mutagenesis screen in mice. It identified a single point mutation in the *Rc3h1* gene, encoding the Roquin-1 protein that causes a lupus-like autoimmune disease in homozygous so-called sanroque mice (Vinuesa et al., 2005). These mice carry a M199R mutation in the ROQ domain of Roquin-1 and are characterized by an accumulation of Tfh cells, which promote the spontaneous formation of germinal centers (GC) where they provide inappropriate help to B cells, stimulating the generation of GC B cells and the development of high-affinity anti-nuclear autoantibodies (Linterman et al., 2009; Vinuesa et al., 2005). Further investigations of Roquin-1 and its paralog Roquin-2 have also shown that combined deletion of Roquin-1 and Roquin-2 encoding genes in $CD4^+$ T cells (DKO^T mice) causes autoinflammatory diseases and leads to spontaneous activation of $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells in this mouse model adopt a pro-inflammatory Th17 phenotype and accumulate in the lung and are more prone to become Tfh cells. Both subsets are likely to contribute to the pathology of the DKO^T mice (Jeltsch et al., 2014; Vogel et al., 2013). Currently, the pronounced T cell phenotype of the sanroque and the DKO^T mouse is explained by the post-transcriptional derepression of several Roquin-targeted mRNAs, leading to an increase in their expression and thereby influencing T cell differentiation. In particular elevated mRNA and protein levels of the co-stimulatory receptors ICOS and OX40 and the Th17 promoting factors IκBNS and IκBzeta, encoded by *Nfkbid* and *Nfkbiz*, respectively, were found to be involved in the manifestation of the phenotype (Jeltsch et al.,

2014; Pratama et al., 2013; Vinuesa et al., 2005; Vogel et al., 2013; Yu et al., 2007). On the molecular level, several publications have demonstrated that Roquin post-transcriptionally controls its targets by recruiting the mRNA decay machinery (Glasmacher et al., 2010; Leppek et al., 2013; Murakawa et al., 2015; Sgromo et al., 2017). So far, Roquin function was only investigated with respect to its function in mRNA degradation. However, it remained elusive if Roquin uses other regulatory mechanisms to control its targets and thereby regulates $CD4^+$ T cell differentiation. Furthermore, it is of great interest to identify new Roquin-targeted mRNAs to obtain closer insights how Roquin influences T cell fate decisions by targeting different mRNAs. Taken together, the focus of this study was centered on investigations of new post-transcriptional mechanisms and targets by which Roquin can maintain T cell homeostasis and prevent autoimmune and autoinflammatory diseases.

The first aim of my work was to investigate whether translational regulation is a further mechanism that is exerted by Roquin to regulate its targets. The idea of this analysis arose from some pre-experiments performed in our laboratory, which hinted to a possible translational regulation of Roquin-targeted mRNAs. To test this hypothesis on a global scale, I established two state-of-the art methods, namely PAR-CLIP (Photoactivatable ribonucleoside crosslinking and immunoprecipitation) and ribosome profiling in mouse embryonic fibroblast (MEF) cells. PAR-CLIP was the ideal method to identify transcriptome-wide binding sites of Roquin and hence to define a Roquin-bound mRNA target set. Furthermore, this analysis resulted in the discovery of the first linear sequence element, the so-called LBE, that is directly bound via the ROQ and zinc finger (Znf) domains of Roquin (**Publication I**). This finding was unexpected, because several studies proved that Roquin mainly binds to stem-loop structures in the 3′ untranslated region (UTR) of its targets. These hairpins consist of tri- or hexa-loops, also known as constitutive (CDE) or alternative decay elements (ADE), respectively, which are recognized by the ROQ domain in a more structure- and less sequence-dependent manner. In principle, CDEs are characterized by a pyrimidine-purine-pyrimidine (Py-Pu-Py) sequence, and ADEs have uridine-rich sequences in their loops (Codutti et al., 2015; Janowski et al., 2016; Leppek et al., 2013; Murakawa et al., 2015; Sakurai et al., 2015; Schlundt et al., 2014; Schuetz et al., 2014; Tan et al., 2014). Interestingly, the core of the LBE sequence is also composed of a Py-Pu-Py order that is exclusively recognized by the ROQ domain, whereas the Znf domain recognizes the flanking regions preferentially enriched in uridines. However, it has not yet been clarified if the recognition of the LBE contributes to the regulatory function of Roquin (**Publication I**). Moreover, we also detected a lower binding affinity of Roquin to the LBE compared to stem-loop structures. Nevertheless, the LBE is highly abundant in Roquin-targeted mRNAs and possibly sterically more accessible for Roquin, suggesting that the LBE could function as a stabilizing cis-element for the potentially less stable but high-affinity stem-loops.

Using the defined Roquin mRNA target set and combining it with the ribosome profiling and mRNA sequencing data in MEF cells, we were the first to globally prove that Roquin not only destabilizes its mRNA targets, but can also regulate them at the translational level. In fact, we identified 96 out of 974 targets, which are translationally repressed by Roquin. Remarkably, we found that these targets typically have four or more Roquin-binding sites in their 3′ UTRs (**Publication I**). With relevance for $CD4^+$ T cell differentiation I found two novel direct Roquin targets in our PAR-CLIP dataset: the kinase SGK1 (serum- and glucocorticoid-induced protein kinase 1), and the transcription factor NFAT5 (nuclear factor of activated T cells 5) (**Publication I**). Both targets play a critical role in Th17 cell differentiation (Alberdi et al., 2016; Kleinewietfeld et al., 2013; Wu et al., 2013) and might contribute to the Th17 phenotype of the DKO^T mouse. Interestingly, we found that *Nfat5* is a translationally regulated Roquin target and contains ten Roquin binding sites in its 3′ UTR. Furthermore, we focused our analysis on the previously described Roquin target *Nfkbid*, which is also essential for Th17 cell generation and function, but also for the development of Treg cells in the thymus (Annemann et al., 2015; Kobayashi et al., 2014; Schuster et al., 2012). However, because *Nfkbid* is lowly expressed in MEF cells, I performed ribosome profiling with a reporter system containing its 3′ UTR, thereby identifying *Nfkbid* as a further potential translationally regulated Roquin target. Additionally, polysome profiles of endogenous *Nfkbid* substantiated this finding. Moreover, we identified six stem-loop structures within the 3′ UTR of *Nfkbid*, which are crucial for its post-transcriptional regulation by Roquin (**Publication I**).

Surprisingly, through further exploration of the ribosome profiling data, I discovered a strong downregulation of the translation efficiency of mRNAs encoding for components of the translation machinery, such as ribosomal proteins, elongation factors as well as several initiation factors. These mRNAs are also known as 5′ TOP mRNAs, that are defined by an oligopyrimidine tract at their 5′ termini, the 5′ TOP motif, which is crucial for their translational control (Avni et al., 1994; Levy et al., 1991). In addition, we revealed that Roquin also negatively impacts on global protein synthesis. This type of regulation was a completely new aspect of Roquin-mediated post-transcriptional gene regulation that I did not only find in MEF cells, but also in $CD4^+$ T cells (**Publication II**). Previous studies have already shown that primary $CD4^+$ $Foxp3^-$ and $CD4^+$ $Foxp3^+$ T cells largely differ in their translational activity to determine their cell fate (Bjur et al., 2013). These investigations led us to the assumption that Roquin, in addition to regulating individual targets, might also control the overall translational machinery to direct $CD4^+$ T cell differentiation. To date, there is only one study showing that the translational regulation of TOP mRNAs can affect $CD8^+$ T cell differentiation (Araki et al., 2017). Nevertheless, we first wanted to investigate how Roquin post-transcriptionally regulates TOP mRNAs and initially thought that Roquin directly binds to the TOP motif.

However, the PAR-CLIP data did not indicate any Roquin binding sites in the TOP mRNAs, suggesting that Roquin indirectly controls the translation of TOP mRNAs. However, I found that the ROQ domain and therefore Roquin binding is indispensable for the inhibitory effect on protein synthesis (**Publication II**). A closer look into the literature pointed out that the translation of 5′ TOP mRNAs is mainly controlled by the mechanistic target of rapamycin (mTOR) (Hsieh et al., 2012; Thoreen et al., 2012), and this fact prompted me to investigate whether Roquin impacts the PI3K-Akt-mTOR signaling pathway (**Publication II**).

mTOR is a serine/threonine kinase that regulates various cellular processes like growth, survival, proliferation, metabolism, translation, and cell differentiation. Especially in T cells, mTOR integrates a variety of environmental cues such as growth factors, nutrients, cytokines, co-stimulation, and TCR signals to translate them into distinct T cell fate decisions (Chapman and Chi, 2015; Chi, 2012; Pollizzi and Powell, 2015; Saxton and Sabatini, 2017). Over the last few years, several publications have revealed the importance of the PI3K-Akt-mTOR signaling pathway for the regulation of $CD4^+$ T cell differentiation into the different subsets (Delgoffe et al., 2009; Delgoffe et al., 2011; Kurebayashi et al., 2012; Lee et al., 2010; Ray et al., 2015; Sauer et al., 2008; Xu et al., 2017; Yang et al., 2016b; Yang et al., 2013; Zeng et al., 2016; Zeng et al., 2013). The results of these studies lead us to speculate that Roquin might regulate this pathway to control $CD4^+$ T cell differentiation. To address this issue, I first investigated the phosphorylation levels of important downstream substrates of the mTOR complexes, mTORC1 and mTORC2, and disclosed that their activities are increased in Roquin-deficient $CD4^+$ T cells (**Publication II**). Interestingly, in the PAR-CLIP data set from publication I, I found the phosphatase Pten as a novel direct Roquin target, which functions upstream of mTORC1 and mTORC2 and inhibits the activation of the PI3K-Akt-mTOR pathway. Moreover, it is described that the loss of Pten in T cells results in constitutive mTOR activation, thereby increasing Tfh cell differentiation (Zeng et al., 2016) and causing dysfunction and instability of Treg cells (Huynh et al., 2015; Shrestha et al., 2015). Remarkably, the lack of Roquin in $CD4^+$ T cells elicited a reduction of the mRNA and protein levels of Pten, an effect that has not yet been observed for any known direct Roquin target. In addition, it is known that Pten is also a target of the miR-17~92 cluster, which has an important role in the survival, proliferation, differentiation, and function of $CD4^+$ T cells (Baumjohann et al., 2013; de Kouchkovsky et al., 2013; Jiang et al., 2011; Kang et al., 2013; Liu et al., 2014; Montoya et al., 2017; Simpson et al., 2014; Wu et al., 2015; Xiao et al., 2008; Yang et al., 2016a). For this reason, we hypothesized that Roquin might interfere with the post-transcriptional repression of Pten by the miR-17~92 cluster, thereby increasing its expression. With the help of our PAR-CLIP dataset, we found that Roquin recognizes a predicted RNA-stem-loop in the Pten 3′ UTR that overlaps with a binding site of the miR-17~92 cluster (**Publication II**). Therefore, we supposed that direct binding of Roquin to Pten

induces a structural switch within its 3′ UTR, and thus impedes the binding of the miR-17~92 that is loaded into an RNA-induced silencing complex (RISC) (**Figure 3**). This structural change might then prevent miRNA-mediated mRNA decay.

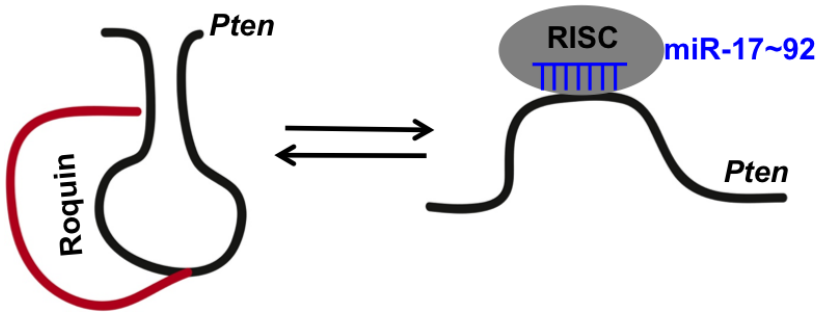

Figure 3 **Model of post-transcriptional regulation of Pten by Roquin.**
Roquin competes with the miR-17~92 RISC complex to prevent miRNA-mediated decay of the *Pten* mRNA.

To prove this model, I performed an Argonaute 2 (Ago2) immunopreciptation in $CD4^+$ T cells and analyzed the amount of *Pten* mRNA associated with Ago2 via quantitative RT-PCR. Here, we detected a stronger association of Ago2 with *Pten* in extracts of Roquin-deficient $CD4^+$ T cells compared to control T cells, demonstrating that the RISC complex might bind more efficiently to the *Pten* mRNA when Roquin is not present (**Publication II**). We have thereby uncovered a novel function of Roquin in modulating miRNA-mediated gene expression regulation by competing with a miRNA for a common mRNA target. To date the existence of such a mechanism could only be shown for one other RNA-binding protein named Pumilio, which also induces structural changes in its targeted 3′ UTR, but in this case to facilitate miRNA-mediated gene silencing (Kedde et al., 2010).

Since we discovered decreased Pten levels in Roquin-deficient $CD4^+$ T cells, we asked whether further downstream substrates of the PI3K-mTOR signaling pathway are affected. One substrate of particular interest for us was the transcription factor Foxo1, which is an essential regulator for $CD4^+$ T cell differentiation. It positively controls the development and function of iTreg cells, but restrains the formation of Tfh and Th17 cells. Upon activation of the PI3K-Akt-mTOR signaling pathway Foxo1 is phosphorylated by the kinase Akt and exported to the cytoplasm, where it can no longer regulate the transcription of Treg-, Tfh- and Th17-related genes (Fabre et al., 2005; Kerdiles et al., 2010; Lainé et al., 2015; Ouyang et al., 2010; Ouyang et al., 2012; Stone et al., 2015). In the cytoplasm Foxo1 can be ubiquitylated by the E3 ligase Itch promoting its degradation (Xiao et al., 2014). Concomitantly with reduced Pten expression in Roquin-deficient $CD4^+$ T cells, we found increased activation of Akt, and therefore investigated Foxo1 localization. In fact, we observed a stronger translocation of Foxo1 to the cytoplasm. Additionally, we detected

increased Foxo1 levels, which coincided with a strong decrease in Itch levels (**Publication II**). Surprisingly, Itch was found in our PAR-CLIP dataset in MEF cells, and in further validation experiments I confirmed Itch as a novel direct Roquin target in $CD4^+$ T cells. Roquin might repress Itch to enable Foxo1 to relocalize into the nucleus, thereby blocking Tfh and Th17 cell differentiation and promoting the generation and function of Treg cells.

At this point, the question arose whether Roquin indeed regulates the PI3K-Akt-mTOR-Foxo1 signaling pathway to regulate $CD4^+$ T cell differentiation. In order to shed light on this issue, we examined iTreg and Th17 cell differentiation *in vitro* and Tfh cell differentiation *in vivo,* using different inhibitors against PI3K or mTOR. Strikingly, we obtained a rectification of the aberrant frequencies of the three T cell subsets from Roquin-deficient mice to levels found in wild-type controls (**Publication II**). These results clearly demonstrate that Roquin suppresses this signaling pathway to control $CD4^+$ T cell differentiation.

While a dominant role of Roquin in Th17 and Tfh cell differentiation *in vivo* was established, its effect on other $CD4^+$ T cell subsets remained unclear. Based on the fact that Treg cells have a pivotal role in the suppression of autoreactive T cells, and that both, the sanroque and the DKO^T mouse, exhibit signs of autoimmunity and autoinflammation, we hypothesized that Roquin might also be involved in Treg cell differentiation and function. Hence, it was of great interest to analyze the role of Roquin in Treg cells to obtain a closer insight into the mechanism how Roquin protects against autoimmunity. For this analysis, we examined Treg cells from mice with a specific deletion of Roquin-1 and Roquin-2 in these cells (DKO^{Treg} mice) and revealed that they lose their suppressive activity on Teff cells (**Publication II**). Based on these findings, my aim was to elucidate the molecular mechanism how Roquin controls Treg cell function. Interestingly, the extensive investigation of the phenotype of the DKO^{Treg} mice showed a strong overlap with the phenotype of mice with a Treg-specific deletion of *Pten* (Pten KO^{Treg} mice). Both phenotypes exhibited increased numbers and frequencies of Treg cells, presumably due to a stronger proliferation, enhanced activation of $CD4^+$ and $CD8^+$ T cells, loss of Treg function *in vivo* and development of splenomegaly and lymphadenopathy. The phenotype of the Pten KO^{Treg} mouse was explained by a strong PI3K-mTORC2 activity in Treg cells (Huynh et al., 2015; Shrestha et al., 2015). Moreover, previous studies have demonstrated that both mTOR complexes negatively impact Treg cell differentiation and indicated that both complexes must be inactive to allow Treg cell differentiation (Delgoffe et al., 2009; Delgoffe et al., 2011; Sauer et al., 2008). However, a further study showed that the suppressive activity of Treg cells mainly relies on mTORC1 signaling (Zeng et al., 2013). Taken together, these findings convinced me to investigate the PI3K-Akt-mTOR signaling pathway in Treg cells. Firstly, I analyzed again mTORC1 and mTORC2 activity by examining the phosphorylation levels of their downstream substrates, and herein I detected increased activity of both complexes in

Roquin-deficient Treg cells. In addition, as already shown for $CD4^+$ T cells, Pten levels were downregulated and Itch levels were upregulated in Roquin-deficient Treg cells demonstrating that the regulation of the PI3K-Akt-mTOR signaling pathway is a central mechanism that Roquin also uses to control Treg cell differentiation and function (**Publication II**).

Interestingly, when we analyzed the DKO^{Treg} mice, we observed a strong accumulation of Tfr cells. This $CD4^+$ T cell subset was initially discovered a few years ago, and since then several studies have focused on the elucidation of their development and function, which have not yet been fully clarified. So far, it is known that Tfr cells develop from thymus-derived $Foxp3^+$ Treg precursor cells (**Figure 1**) and resemble Tfh and Treg cells phenotypically by the coexpression of Tfh and Treg signature markers. However, in contrast to Tfh cells, Tfr cells suppress GC responses by inhibiting Tfh and GC B cells (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011). Furthermore, differentiation and function of Tfr cells require mTORC1 activity (Xu et al., 2017). First of all, we were interested if the arising Tfr cells in the DKO^{Treg} mice are functional in inhibiting GC reactions. Therefore, I immunized wild-type and DKO^{Treg} mice with an antigen to induce an immune response and found that only a few antigen-specific GC B cells were formed in the DKO^{Treg} compared to wild-type mice (**Publication II**). Furthermore, we observed that Roquin-deficient Treg cells strongly down-regulated the expression of the α-chain of the IL-2 receptor (CD25). In general, IL-2 is essential for Treg cell development, maintenance and function (Cheng et al., 2013; Chinen et al., 2016; de la Rosa et al., 2004; Fontenot et al., 2005). To elucidate the mechanism of Roquin in restraining CD25 expression on Treg cells on the molecular level, I performed mRNA sequencing to compare mRNA expression of $CD25^+$ and $CD25^-$ Roquin-deficient Treg cells. Surprisingly, we found that $CD25^-$ Roquin-deficient Treg cells strongly express Tfh signature genes, whereas $CD25^+$ Roquin-deficient Treg cells still express Treg signature genes (**Publication II**). From these results we concluded that Roquin-deficient Treg cells that lose CD25 are no longer sensitive to IL-2 and can therefore convert into Tfr cells and suppress GC immune responses. During the revision of our publication two other studies also came up with the finding that Tfr cells do not express CD25 because ongoing IL-2 signaling limits their differentiation (Botta et al., 2017; Wing et al., 2017). Nevertheless, we were the first to connect increased mTORC1 and mTORC2 activity in Treg cells to CD25 downregulation, and thereby to the generation of Tfr cells in a Roquin-dependent manner.

In conclusion, in this thesis I identified novel post-transcriptional mechanisms that Roquin exerts to maintain T cell homeostasis and to prevent autoimmune and autoinflammatory diseases. In particular I elucidated that Roquin does not only destabilize its mRNA targets but also represses their translation. Furthermore, I proved that Roquin suppresses the PI3K-Akt-mTOR-Foxo1 pathway to control $CD4^+$ T cell differentiation. The multiple layers on which Roquin mediates its regulatory effect on differentiation are good

examples for the complexity of T cell fate decisions and its fragile nature. The absence of a functional Roquin protein in both the sanroque and the DKO^{T} mouse results in a tilted immune balance towards inflammation. A broader understanding of the determinants of T cell fate decisions *in vivo* is required to understand how dysregulated immune responses arise and how they could be controlled.

2 References

Alberdi, M., Iglesias, M., Tejedor, S., Merino, R., López-Rodríguez, C., and Aramburu, J. (2016). Context-dependent regulation of Th17-associated genes and IFNγ expression by the transcription factor NFAT5. Immunology And Cell Biology *95*, 56.

Annemann, M., Wang, Z., Plaza-Sirvent, C., Glauben, R., Schuster, M., Ewald Sander, F., Mamareli, P., Kühl, A.A., Siegmund, B., Lochner, M*., et al.* (2015). IκBNS Regulates Murine Th17 Differentiation during Gut Inflammation and Infection. The Journal of Immunology.

Araki, K., Morita, M., Bederman, A.G., Konieczny, B.T., Kissick, H.T., Sonenberg, N., and Ahmed, R. (2017). Translation is actively regulated during the differentiation of CD8(+) effector T cells. Nature immunology *18*, 1046-1057.

Avni, D., Shama, S., Loreni, F., and Meyuhas, O. (1994). Vertebrate mRNAs with a 5'-terminal pyrimidine tract are candidates for translational repression in quiescent cells: characterization of the translational cis-regulatory element. Molecular and cellular biology *14*, 3822-3833.

Baumjohann, D., Kageyama, R., Clingan, J.M., Morar, M.M., Patel, S., De Kouchkovsky, D., Bannard, O., Bluestone, J.A., Matloubian, M., Ansel, K.M*., et al.* (2013). The microRNA cluster miR-17∼92 promotes T FH cell differentiation and represses subset-inappropriate gene expression. Nature Immunology *14*, 840-848.

Bjur, E., Larsson, O., Yurchenko, E., Zheng, L., Gandin, V., Topisirovic, I., Li, S., Wagner, C.R., Sonenberg, N., and Piccirillo, C.A. (2013). Distinct translational control in CD4+ T cell subsets. PLoS genetics *9*, e1003494-e1003494.

Botta, D., Fuller, M.J., Marquez-Lago, T.T., Bachus, H., Bradley, J.E., Weinmann, A.S., Zajac, A.J., Randall, T.D., Lund, F.E., León, B*., et al.* (2017). Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. Nature Immunology *18*, 1249.

Chapman, N.M., and Chi, H. (2015). mTOR links environmental signals to T cell fate decisions. Frontiers in Immunology *6*, 1-11.

Cheng, G., Yu, A., Dee, M.J., and Malek, T.R. (2013). IL-2R Signaling Is Essential for Functional Maturation of Regulatory T Cells during Thymic Development. The Journal of Immunology *190*, 1567.

Chi, H. (2012). Regulation and function of mTOR signalling in T cell fate decisions. Nature Reviews Immunology *12*, 325-338.

Chinen, T., Kannan, A.K., Levine, A.G., Fan, X., Klein, U., Zheng, Y., Gasteiger, G., Feng, Y., Fontenot, J.D., and Rudensky, A.Y. (2016). An essential role for the IL-2 receptor in Treg cell function. Nature Immunology *17*, 1322.

Chung, Y., Tanaka, S., Chu, F., Nurieva, R.I., Martinez, G.J., Rawal, S., Wang, Y.-H., Lim, H., Reynolds, J.M., Zhou, X.-h*., et al.* (2011). Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nature Medicine *17*, 983.

Codutti, L., Leppek, K., Zálešák, J., Windeisen, V., Masiewicz, P., Stoecklin, G., and Carlomagno, T. (2015). A Distinct, Sequence-Induced Conformation Is Required for Recognition of the Constitutive Decay Element RNA by Roquin. Structure *23*, 1437-1447.

de Kouchkovsky, D., Esensten, J.H., Rosenthal, W.L., Morar, M.M., Bluestone, J.A., and Jeker, L.T. (2013). microRNA-17-92 Regulates IL-10 Production by Regulatory T Cells and Control of Experimental Autoimmune Encephalomyelitis. The Journal of Immunology *191*, 1594-1605.

de la Rosa, M., Rutz, S., Dorninger, H., and Scheffold, A. (2004). Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. European Journal of Immunology *34*, 2480-2488.

Delgoffe, G.M., Kole, T.P., Zheng, Y., Zarek, P.E., Matthews, K.L., Xiao, B., Worley, P.F., Kozma, S.C., and Powell, J.D. (2009). The mTOR Kinase Differentially Regulates Effector and Regulatory T Cell Lineage Commitment. Immunity *30*, 832-844.

Delgoffe, G.M., Pollizzi, K.N., Waickman, A.T., Heikamp, E., Meyers, D.J., Horton, M.R., Xiao, B., Worley, P.F., and Powell, J.D. (2011). The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. Nature Immunology *12*, 295-304.

Fabre, S., Lang, V., Harriague, J., Jobart, A., Unterman, T.G., Trautmann, A., and Bismuth, G. (2005). Stable Activation of Phosphatidylinositol 3-Kinase in the T Cell Immunological Synapse Stimulates Akt Signaling to FoxO1 Nuclear Exclusion and Cell Growth Control. The Journal of Immunology *174*, 4161-4171.

Fontenot, J.D., Rasmussen, J.P., Gavin, M.A., and Rudensky, A.Y. (2005). A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nature Immunology *6*, 1142.

Glasmacher, E., Hoefig, K.P., Vogel, K.U., Rath, N., Du, L., Wolf, C., Kremmer, E., Wang, X., and Heissmeyer, V. (2010). Roquin binds inducible costimulator mRNA and effectors of mRNA decay to induce microRNA-independent post-transcriptional repression. Nature immunology *11*, 725-733.

Hsieh, A.C., Liu, Y., Edlind, M.P., Ingolia, N.T., Janes, M.R., Sher, A., Shi, E.Y., Stumpf, C.R., Christensen, C., Bonham, M.J.*, et al.* (2012). The translational landscape of mTOR signalling steers cancer initiation and metastasis. Nature *485*, 55-61.

Huynh, A., Dupage, M., Priyadharshini, B., Sage, P.T., Quiros, J., Borges, C.M., Townamchai, N., Gerriets, V.A., Rathmell, J.C., Sharpe, A.H.*, et al.* (2015). Control of PI(3) kinase in Tregcells maintains homeostasis and lineage stability. Nature Immunology *16*, 188-196.

Janowski, R., Heinz, G.A., Schlundt, A., Wommelsdorf, N., Brenner, S., Gruber, A.R., Blank, M., Buch, T., Buhmann, R., Zavolan, M.*, et al.* (2016). Roquin recognizes a non-canonical hexaloop structure in the 3′-UTR of Ox40. Nature Communications *7*, 1-13.

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.*, et al.* (2014). Cleavage of roquin and regnase-1 by the paracaspase MALT1 releases their cooperatively repressed targets to promote TH17 differentiation. Nature Immunology *15*, 1079-1089.

Jiang, S., Li, C., Olive, V., Lykken, E., Feng, F., Sevilla, J., Wan, Y., He, L., and Li, Q.-J. (2011). Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation. Blood *118*, 5487-5497.

Kang, S.G., Liu, W.H., Lu, P., Jin, H.Y., Lim, H.W., Shepherd, J., Fremgen, D., Verdin, E., Oldstone, M.B.A., Qi, H.*, et al.* (2013). MicroRNAs of the miR-17∼92 family are critical regulators of T FH differentiation. Nature Immunology *14*, 849-857.

Kedde, M., van Kouwenhove, M., Zwart, W., Oude Vrielink, J.A.F., Elkon, R., and Agami, R. (2010). A Pumilio-induced RNA structure switch in p27-3′ UTR controls miR-221 and miR-222 accessibility. Nature Cell Biology *12*, 1014.

Kerdiles, Y.M., Stone, E.L., Beisner, D.L., McGargill, M.A., Ch'en, I.L., Stockmann, C., Katayama, C.D., and Hedrick, S.M. (2010). Foxo Transcription Factors Control Regulatory T Cell Development and Function. Immunity *33*, 890-904.

Kleinewietfeld, M., Manzel, A., Titze, J., Kvakan, H., Yosef, N., Linker, R.A., Muller, D.N., and Hafler, D.A. (2013). Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. Nature *496*, 518-522.

Kobayashi, S., Hara, A., Isagawa, T., Manabe, I., Takeda, K., and MaruYama, T. (2014). The Nuclear IκB Family Protein IκBNS Influences the Susceptibility to Experimental Autoimmune Encephalomyelitis in a Murine Model. PLOS ONE *9*, e110838.

Kurebayashi, Y., Nagai, S., Ikejiri, A., Ohtani, M., Ichiyama, K., Baba, Y., Yamada, T., Egami, S., Hoshii, T., Hirao, A.*, et al.* (2012). PI3K-Akt-mTORC1-S6K1/2 Axis Controls Th17 Differentiation by Regulating Gfi1 Expression and Nuclear Translocation of RORγ. Cell Reports *1*, 360-373.

Lainé, A., Martin, B., Luka, M., Mir, L., Auffray, C., Lucas, B., Bismuth, G., and Charvet, C. (2015). Foxo1 Is a T Cell-Intrinsic Inhibitor of the RORγt-Th17 Program. Journal of immunology (Baltimore, Md : 1950) *195*, 1791-1803.

Lee, K., Gudapati, P., Dragovic, S., Spencer, C., Joyce, S., Killeen, N., Magnuson, M.A., and Boothby, M. (2010). Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways. Immunity *32*, 743-753.

Leppek, K., Schott, J., Reitter, S., Poetz, F., Hammond, M.C., and Stoecklin, G. (2013). Roquin promotes constitutive mRNA decay via a conserved class of stem-loop recognition motifs. Cell *153*, 869-881.

Levy, S., Avni, D., Hariharan, N., Perry, R.P., and Meyuhas, O. (1991). Oligopyrimidine tract at the 5' end of mammalian ribosomal protein mRNAs is required for their translational control, pp. 3319-3323.

Linterman, M.A., Pierson, W., Lee, S.K., Kallies, A., Kawamoto, S., Rayner, T.F., Srivastava, M., Divekar, D.P., Beaton, L., Hogan, J.J.*, et al.* (2011). Foxp3+ follicular regulatory T cells control the germinal center response. Nature Medicine *17*, 975.

Linterman, M.A., Rigby, R.J., Wong, R.K., Yu, D., Brink, R., Cannons, J.L., Schwartzberg, P.L., Cook, M.C., Walters, G.D., and Vinuesa, C.G. (2009). Follicular helper T cells are required for systemic autoimmunity. The Journal of Experimental Medicine *206*, 561.

Liu, S.Q., Jiang, S., Li, C., Zhang, B., and Li, Q.J. (2014). Mir-17-92 cluster targets phosphatase and tensin homology and ikaros family zinc finger 4 to promote th17-mediated inflammation. Journal of Biological Chemistry *289*, 12446-12456.

Montoya, M.M., Maul, J., Singh, P.B., Pua, H.H., Dahlström, F., Wu, N., Huang, X., Ansel, K.M., and Baumjohann, D. (2017). A Distinct Inhibitory Function for miR-18a in Th17 Cell Differentiation. The Journal of Immunology *199*, 559.

Murakawa, Y., Hinz, M., Mothes, J., Schuetz, A., Uhl, M., Wyler, E., Yasuda, T., Mastrobuoni, G., Friedel, C.C., Dölken, L.*, et al.* (2015). RC3H1 post-transcriptionally regulates A20 mRNA and modulates the activity of the IKK/NF-κ B pathway. Nature Communications *6*.

Ouyang, W., Beckett, O., Ma, Q., Paik, J.-h., DePinho, R.A., and Li, M.O. (2010). Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells. Nature immunology *11*, 618-627.

Ouyang, W., Liao, W., Luo, C.T., Yin, N., Huse, M., Kim, M.V., Peng, M., Chan, P., Ma, Q., Mo, Y.*, et al.* (2012). Novel Foxo1-dependent transcriptional programs control T reg cell function. Nature *491*, 554-559.

Pollizzi, K.N., and Powell, J.D. (2015). Regulation of T cells by mTOR: The known knowns and the known unknowns. Trends in Immunology *36*, 13-20.

Pratama, A., Ramiscal, R.R., Silva, D.G., Das, S.K., Athanasopoulos, V., Fitch, J., Botelho, N.K., Chang, P.-P., Hu, X., Hogan, J.J.*, et al.* (2013). Roquin-2 Shares Functions with Its Paralog Roquin-1 in the Repression of mRNAs Controlling T Follicular Helper Cells and Systemic Inflammation. Immunity *38*, 669-680.

Ray, J.P., Staron, M.M., Shyer, J.A., Ho, P.C., Marshall, H.D., Gray, S.M., Laidlaw, B.J., Araki, K., Ahmed, R., Kaech, S.M.*, et al.* (2015). The Interleukin-2-mTORc1 Kinase Axis Defines the Signaling, Differentiation, and Metabolism of T Helper 1 and Follicular B Helper T Cells. Immunity *43*, 690-702.

Sakurai, S., Ohto, U., and Shimizu, T. (2015). Structure of human Roquin-2 and its complex with constitutive-decay element RNA. Acta Crystallographica Section F, Structural Biology Communications *71*, 1048-1054.

Sauer, S., Bruno, L., Hertweck, A., Finlay, D., Leleu, M., Spivakov, M., Knight, Z.A., Cobb, B.S., Cantrell, D., O'Connor, E.*, et al.* (2008). T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. Proceedings of the National Academy of Sciences *105*, 7797-7802.

Saxton, R.A., and Sabatini, D.M. (2017). mTOR Signaling in Growth, Metabolism, and Disease. Cell *168*, 960-976.

Schlundt, A., Heinz, G.A., Janowski, R., Geerlof, A., Stehle, R., Heissmeyer, V., Niessing, D., and Sattler, M. (2014). Structural basis for RNA recognition in roquin-mediated post-transcriptional gene regulation. Nature Structural & Molecular Biology *21*, 671-678.

Schuetz, A., Murakawa, Y., Rosenbaum, E., Landthaler, M., and Heinemann, U. (2014). Roquin binding to target mRNAs involves a winged helix-turn-helix motif. Nature communications *5*, 5701-5701.

Schuster, M., Glauben, R., Plaza-Sirvent, C., Schreiber, L., Annemann, M., Floess, S., Kühl, A.a., Clayton, L.K., Sparwasser, T., Schulze-Osthoff, K.*, et al.* (2012). IκB(NS) protein mediates regulatory T cell development via induction of the Foxp3 transcription factor. Immunity *37*, 998-1008.

Sgromo, A., Raisch, T., Bawankar, P., Bhandari, D., Chen, Y., Kuzuoğlu-Öztürk, D., Weichenrieder, O., and Izaurralde, E. (2017). A CAF40-binding motif facilitates recruitment of the CCR4-NOT complex to mRNAs targeted by Drosophila Roquin. Nature Communications *8*, 14307-14307.

Shrestha, S., Yang, K., Guy, C., Vogel, P., Neale, G., and Chi, H. (2015). Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. Nature immunology *16*, 178-187.

Simpson, L.J., Patel, S., Bhakta, N.R., Choy, D.F., Brightbill, H.D., Ren, X., Wang, Y., Pua, H.H., Baumjohann, D., Montoya, M.M.*, et al.* (2014). A miRNA upregulated in asthma airway T cells promotes T(H)2 cytokine production. Nature immunology *15*, 1162-1170.

Stone, E.L., Pepper, M., Katayama, C.D., Kerdiles, Y.M., Lai, C.Y., Emslie, E., Lin, Y.C., Yang, E., Goldrath, A.W., Li, M.O.*, et al.* (2015). ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation. Immunity *42*, 239-251.

Tan, D., Zhou, M., Kiledjian, M., and Tong, L. (2014). The ROQ domain of Roquin recognizes mRNA constitutive-decay element and double-stranded RNA. Nature structural & molecular biology *21*, 679-685.

Thoreen, C.C., Chantranupong, L., Keys, H.R., Wang, T., Gray, N.S., and Sabatini, D.M. (2012). A unifying model for mTORC1-mediated regulation of mRNA translation. Nature *485*, 109-113.

Vinuesa, C.G., Cook, M.C., Angelucci, C., Athanasopoulos, V., Rui, L., Hill, K.M., Yu, D., Domaschenz, H., Whittle, B., Lambe, T.*, et al.* (2005). A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. Nature *435*, 452-458.

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.*, et al.* (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.

Wing, J.B., Kitagawa, Y., Locci, M., Hume, H., Tay, C., Morita, T., Kidani, Y., Matsuda, K., Inoue, T., Kurosaki, T.*, et al.* (2017). A distinct subpopulation of CD25- T-follicular regulatory cells localizes in the germinal centers. Proceedings of the National Academy of Sciences *114*, E6400-E6409.

Wollenberg, I., Agua-Doce, A., Hernández, A., Almeida, C., Oliveira, V.G., Faro, J., and Graca, L. (2011). Regulation of the Germinal Center Reaction by Foxp3+ Follicular Regulatory T Cells. The Journal of Immunology *187*, 4553.

Wu, C., Yosef, N., Thalhamer, T., Zhu, C., Xiao, S., Kishi, Y., Regev, A., and Kuchroo, V.K. (2013). Induction of pathogenic TH 17 cells by inducible salt-sensing kinase SGK1. Nature *496*, 513-517.

Wu, T., Wieland, A., Lee, J., Hale, J.S., Han, J.-H., Xu, X., and Ahmed, R. (2015). miR-17-92 is required for both CD4 Th1 and T(FH) responses during viral infection(). Journal of immunology (Baltimore, Md : 1950) *195*, 2515-2519.

Xiao, C., Srinivasan, L., Calado, D.P., Patterson, H.C., Zhang, B., Wang, J., Henderson, J.M., Kutok, J.L., and Rajewsky, K. (2008). Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. Nature Immunology *9*, 405-414.

Xiao, N., Eto, D., Elly, C., Peng, G., Crotty, S., and Liu, Y.C. (2014). The E3 ubiquitin ligase Itch is required for the differentiation of follicular helper T cells. Nature Immunology *15*, 657-666.

Xu, L., Huang, Q., Wang, H., Hao, Y., Bai, Q., Hu, J., Li, Y., Wang, P., Chen, X., He, R.*, et al.* (2017). The Kinase mTORC1 Promotes the Generation and Suppressive Function of Follicular Regulatory T Cells. Immunity *47*, 538-551.e535.

Yang, H.-Y., Barbi, J., Wu, C.-Y., Zheng, Y., Vignali, Paolo D.A., Wu, X., Tao, J.-H., Park, Benjamin V., Bandara, S., Novack, L.*, et al.* (2016a). MicroRNA-17 Modulates Regulatory T Cell Function by Targeting Co-regulators of the Foxp3 Transcription Factor. Immunity *45*, 83-93.

Yang, J., Lin, X., Pan, Y., Wang, J., Chen, P., Huang, H., Xue, H.H., Gao, J., and Zhong, X.P. (2016b). Critical roles of mTOR complex 1 and 2 for t follicular helper cell differentiation and germinal center responses. eLife *5*, 1-22.

Yang, K., Shrestha, S., Zeng, H., Karmaus, P.W.F., Neale, G., Vogel, P., Guertin, D.A., Lamb, R.F., and Chi, H. (2013). T Cell Exit from Quiescence and Differentiation into Th2 Cells Depend on Raptor-mTORC1-Mediated Metabolic Reprogramming. Immunity *39*, 1043-1056.

Yu, D., Tan, A.H.-M., Hu, X., Athanasopoulos, V., Simpson, N., Silva, D.G., Hutloff, A., Giles, K.M., Leedman, P.J., Lam, K.P.*, et al.* (2007). Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. Nature *450*, 299-303.

Zeng, H., Cohen, S., Guy, C., Shrestha, S., Neale, G., Brown, S.A., Cloer, C., Kishton, R.J., Gao, X., Youngblood, B.*, et al.* (2016). mTORC1 and mTORC2 Kinase Signaling and Glucose Metabolism Drive Follicular Helper T Cell Differentiation. Immunity *45*, 540-554.

Zeng, H., Yang, K., Cloer, C., Neale, G., Vogel, P., and Chi, H. (2013). MTORC1 couples immune signals and metabolic programming to establish T reg-cell function. Nature *499*, 485-490.

3 Publication I

DOI https://doi.org/10.1038/s41467-018-06184-3

Roquin targets mRNAs in a 3′-UTR-specific manner by different modes of regulation

Katharina Essig, Nina Kronbeck, Joao C. Guimaraes, Claudia Lohs, Andreas Schlundt, Anne Hoffmann, Gesine Behrens, Sven Brenner, Joanna Kowalska, Cristina Lopez-Rodriguez, Jacek Jemielity, Helmut Holtmann, Kristin Reiche, Jörg Hackermüller, Michael Sattler, Mihaela Zavolan, and Vigo Heissmeyer

3.1 Contribution to the publication

As a first author of this publication, I was deeply involved in the conception of this study, performed the main body of the experimental work and wrote the manuscript together with the two corresponding authors Mihaela Zavolan and Vigo Heissmeyer. A central part of my work was to establish and perform two state-of-the art methods: PAR-CLIP and ribosome profiling. The data sets from these techniques enabled us to define a Roquin target mRNA set in MEF cells (Fig. 1a-d and Fig. S1), and by combining them with mRNA sequencing data to globally dissolve translational regulation of Roquin-targeted mRNAs (Fig. 7d-e). The bioinformatic analysis was performed by Joao Guimaraes. Additionally, I investigated the regulation of the Roquin targeted 3′ UTR of *Nfkbid* in ribosome profiles of MEF cells (Fig.2d and Fig.6a) and analyzed this 3′ UTR as well as mutant forms in degradation kinetic studies and polysome profiles using a β-globin reporter system in HeLa cells (Fig. 7 a-c and Supplementary S6 i). Furthermore, I reanalyzed the polysome profiling data generated by our collaboration partners and compared subpolysomal with polysomal fractions to prove translational regulation of endogenous *Nfkbid* by Roquin in MEF and $CD4^+$ T cells (Fig. 6c, e and Fig. S6g). To examine Roquin-mediated mRNA decay in more detail, I also generated and tested a MEF cell line expressing a doxycycline-inducible shRNA against the core subunit of the deadenylase complex cNOT1 (Fig. 5k). Finally, I identified two novel Roquin targets, *Sgk1* and *Nfat5*, and validated these targets by investigating Roquin-dependent regulation in reporter assays as well as on the mRNA and protein level (Fig. 8d-j and Fig. S7d-e).

4 Publication II

DOI https://doi.org/10.1016/j.immuni.2017.11.008

Roquin suppresses the PI3K-mTOR signaling pathway to inhibit T helper cell differentiation and conversion of Treg to Tfr cells

Katharina Essig, Desheng Hu, Joao C. Guimaraes, Dominik Alterauge, Stephanie Edelmann, Timsse Raj, Jan Kranich, Gesine Behrens, Alexander Heiseke, Stefan Floess, Juliane Klein, Andreas Maiser, Susan Marschall, Martin Hrabě de Angelis, Heinrich Leonhardt, Cornelis F. Calkhoven, Elfriede Noessner, Thomas Brocker, Jochen Huehn, Anne B. Krug, Mihaela Zavolan, Dirk Baumjohann, and Vigo Heissmeyer

4.1 Contribution to the publication

As a first author of this publication, I made major contributions to the experimental work as well as to the conception of the study. Furthermore, I wrote the manuscript together with the two corresponding authors Desheng Hu and Vigo Heissmeyer. Initially, through a thorough analysis of the mRNA-sequencing and ribosome profiling data from publication I, I found that Roquin indirectly downregulates the translation of 5′ TOP mRNAs and thereby restricts the overall protein synthesis in MEF and $CD4^+$ T cells (Fig. 5A-C and Fig. S5A-D). According to the literature it is known that the translation of 5′ TOP mRNAs is mainly controlled by the kinase mTOR, and this finding prompted me to investigate the impact of Roquin on the PI3K-mTOR signaling pathway (Fig. 5D-F and Fig. S6A-C). Herein, I disclosed that Roquin suppresses this pathway by regulating its direct targets PTEN and Itch in conventional T and Treg cells (Fig. 5I-K, Fig. 6B-E and Fig. S6D-E, G). In addition, I elucidated the mechanism how Roquin post-transcriptionally regulates *Pten* mRNA expression (Fig. 6H-I). Moreover, I had the idea to investigate whether the aberrant numbers of Th17, Treg and Tfh cells in mice with a T cell specific deletion of Roquin-1 and Roquin-2 can be corrected to levels found in wild-type counterparts through the inhibition of PI3K or mTOR *in vitro* and *in vivo.* To show this, I performed several experiments by myself (Fig. 7B,D and Fig. S7A-C, E) and I also strongly supported my colleagues for their analyses (Fig. 7G-M and Fig. S7F-M). Furthermore, I investigated the function of Tfr cells in suppressing germinal center responses (Fig. 2G-I and Fig. S1I-J) and examined the conversion of Treg into Tfr cells on the mRNA and protein level (Fig. 3C, E-F and Fig. S4F-G). Additionally, I contributed to the experiments performed by our collaboration partners. I provided and prepared cells for the analysis of cytokines (Fig. S4A-E) and Foxo1 localization (Fig. 5G-H, $CD4^+$ T cells), immunized mice and collected blood samples to study antibody affinity maturation (Fig. 2J-M), and sorted Treg cells for the *in vitro* suppression assay (Fig. S2A-B).

5 Acknowledgement

In conclusion to my thesis, I would like to take the opportunity to thank all people supporting me during the last four years of my PhD.

First, I would like to thank *Prof. Dr. Vigo Heissmeyer* for his valued support, permanent trust, and challenging input to advance my PhD project, which enabled me to publish my two main projects in top tier journals. Moreover, I appreciate his dedicated commitment in gaining important cooperation partners.

At this point, I would like to express my deep gratefulness to all collaboration partners who accompanied me during the years of my studies and the publication process.
A special thank you goes to *Dr. Joao Guimaraes* and *Prof. Dr. Mihaela Zavolan* for their great bioinformatics analyses and their permanent and valuable support. Their data have contributed substantially to the two publications. Also, many thanks to *Dr. Andreas Schlundt* and *Prof. Dr. Michael Sattler* for their detailed investigations of the Roquin sequence motif and their very fast and reliable work for this study.

My thanks are extended to the whole Heissmeyer group for the collegial atmosphere and helpfulness. Especially, I would like to thank *Nina Kronbeck* and *Dr. Gesine Behrens* for their great help, advices and scientific discussions. My thanks should also go to *Temssy Raj* for her generous support during the revision of the immunity paper, and to *Désirée Argiriu* for introducing me into the lab and for making my lab life easier.

My heartfelt thanks I would like to express to *my parents* for their never-ending and enormous support. Thank you so much for your encouragements even in difficult times of my PhD studies and your great interest in my scientific career. Many thanks also to my brother, *Dr. Sebastian Essig*, who is always interested in my scientific work, for his encouraging words that kept me going through my PhD.

I am sincerely grateful to *Dominik Alterauge* for his wonderful, understanding support and motivation during this time. Without your unconditional help I would have never finished my PhD work. Thank you so much for your belief in me and my scientific abilities, as well as for the many scientific conversations and discussions. I wish you all the best for your PhD and career!

Finally, I do not want to miss this opportunity to thank *all members of my doctoral committee* who gave time and interest to my project.

6 List of publications

- Essig K, Kronbeck N, Guimaraes JC, Lohs C, Schlundt A, Behrens G, Hoffmann A, Brenner S, Kowalska J, Lopez-Rodriguez C, Jemielity J, Holtmann H, Reiche K, Hackermüller J, Sattler M, Zavolan M and Heissmeyer V., **Nat Commun**. **2018** Sep 19; 9(1):3810.

- Essig K, Hu D, Guimaraes JC, Alterauge D, Edelmann S, Raj T, Kranich J, Behrens G, Heiseke A, Floess S, Klein J, Maiser A, Marschall S, Hrabě de Angelis M, Leonhardt H, Calkhoven CF, Noessner E, Brocker T, Huehn J, Krug AB, Zavolan M, Baumjohann D, Heissmeyer V., **Immunity. 2017** Dec 19; 47(6):1067-1082.e12.

- Baejen C, Torkler P, Gressel S, Essig K, Söding J, Cramer P., **Mol Cell. 2014** Sep 4; 55(5):745-57.

- Teplova M, Hafner M, Teplov D, Essig K, Tuschl T, Patel DJ., **Genes Dev. 2013** Apr 15; 27(8):928-40.

- Bayer H, Essig K, Stanzel S, Frank M, Gildersleeve JC, Berger MR, Voss C., **J Biol Chem. 2012** Oct 19; 287(43):35873-86. 7