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Interactions between Regnase-1 and Roquin Regulate Helper T Cell Polarization

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Abstract

Post-transcriptional regulation is critical for the control of both innate and adaptive immunity. Many immune-related mRNAs have short half-lives because of conserved *cis*-elements, including AU-rich elements and stem-loop structures in their 3' UTRs. Regnase-1 (also known as MCPIP1 and Zc3h12a) and Roquin-1/2 (known as Rc3h1/2) are RNA binding proteins that are important for degradation of inflammatory mRNAs by recognizing stem loops and maintenance of immune homeostasis.

Deficiency of either of the proteins leads to enhanced T cell activation and massive production of cytokines. However, due to lethality upon mutation of both Regnase-1 and Roquin, their mechanistic relationship in T cells has yet to be clarified. By using a Regnase-1 conditional allele, I show that deficiency of both Regnase-1 and Roquin in T cells leads to the high increase of Th1 subtype T helper cells. Regnase-1 and Roquin are capable of repressing the expression of a group of mRNAs encoding factors involved in Th1 differentiation, thus suppressing Th1 differentiation. Furthermore, early formation of fibrosis was discovered in the cardiac region of these mice, which might have led to heart failure and subsequently, earlier death. Collectively, my results reveal the essential role of Regnase-1 and Roquin in T cell differentiation and a complex relationship between these two proteins.

Abbreviation

c-Rel, *NF*- κ B family subunit

CTLA-4, cytotoxic T-lymphocyte-associated protein 4

IL-6, interleukin-6

MFI, mean fluorescence intensity

qPCR, quantitative PCR

TAC, transverse aortic constriction

TLR, Toll-like receptor

Th, helper T cell

Tfh, follicular helper T cell

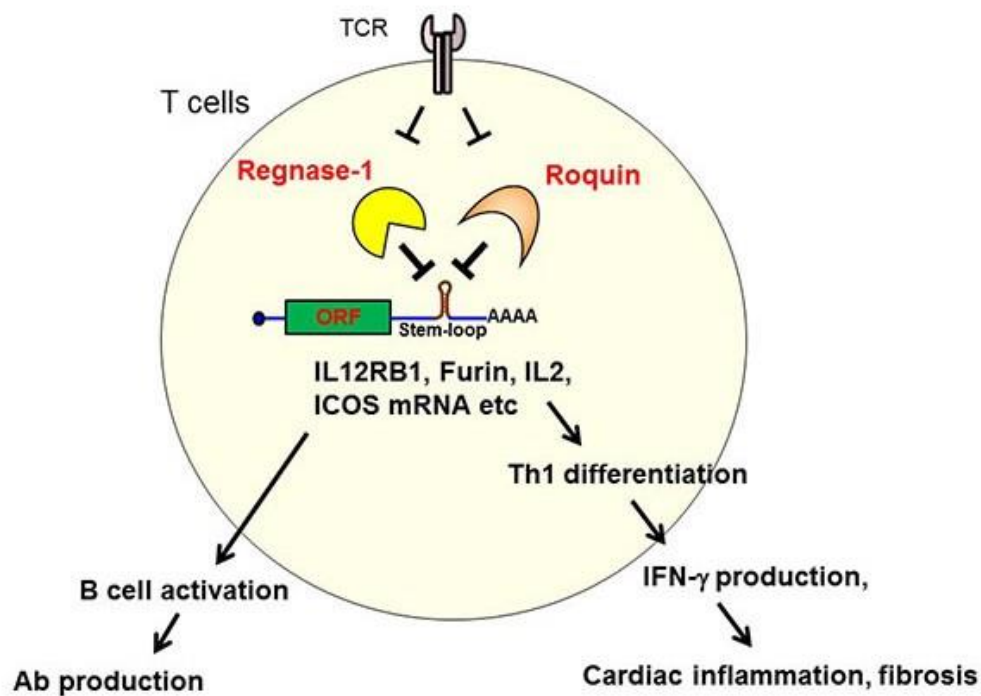
TNF, tumor necrosis factor

UPF1, up-frameshift 1

UTR, untranslated region

Chapter 1

Introduction



Schematic Diagram indicating the roles of Regnase-1 and Roquin in regulating T cell polarization.

Post-transcriptional regulation is critical for controlling gene expression in immune system¹. Many immune-related mRNAs have short half-lives because of conserved *cis*-elements, including AU-rich elements (AREs) and stem-loop (SL) structures in their 3' UTRs^{2,3}. Two major mRNA decay factors, Regnase-1 (also known as MCPIP1 and Zc3h12a) and Roquin-1/2 (also known as Rc3h1/2) are known to be immune regulators that act on mRNA stability⁴⁻⁷. Previous studies show that these factors destabilize mRNAs via distinct SL-mediated mechanisms⁸⁻¹². Regnase-1 targets translationally

active mRNA via its endoribonuclease activity. Regnase-1-mediated decay requires the helicase activity of UPF1, a NMD regulator⁹. In contrast, Roquin, comprised of Roquin-1 and -2, controls translationally inactive mRNA independently of UPF1. Roquin localizes to P-bodies where senescent mRNAs accumulate and recruit the CCR4-NOT deadenylase complex, thereby promoting subsequent degradation of mRNA^{7,12}. Despite the distinct decay mechanisms, these decay factors regulate a set of common target mRNAs including *Nfkbiz* and *Ptgs2*, suggesting that Regnase-1 and Roquin spatiotemporally regulate common target mRNA to maintain immune homeostasis^{7,13}.

Regnase-1 and Roquins also have similarity in vivo in terms of systemic inflammation and autoimmunity^{7,10,13}. It was previously reported that mice lacking Regnase-1 specifically in T cells showed spontaneous T cell activation leading to lymphocyte infiltration into various organs, especially in the lung and liver^{10,13}. Regnase-1-deficient T cells produce elevated amounts of Th signature cytokines, including IFN- γ , IL-17 and IL-4^{13,14}. Additionally, B cells differentiate into plasma cells producing all subclasses of immunoglobulin. In contrast, mice harboring a point mutation in Roquin-1

(Sanroque) develop autoimmunity accompanied with an increased follicular helper T cell population^{6,15,16}. This is explained in part by Roquin-1 and Roquin-2 redundantly regulating T cell activation by targeting shared mRNAs including *Icos* and *Ox40*. In addition, dysfunction of Roquin preferentially skews differentiation towards Th1 and Th17, but not Th2, cells, suggesting that Roquin-1/2 control T cell activation and polarization^{17,18}. Additionally, accumulation of Th17 cells was observed in the lung of mice lacking Roquin-1/2 in T cells, suggesting a direct connection between accumulation of Th17 cells and lung pathology¹⁸.

Interestingly, upon TCR engagement, both Regnase-1 and Roquin-1/2 are rapidly degraded by paracaspase Malt1, releasing their repression on targets, subsequently facilitating T cell activation^{13,18,19}. In T cells, these proteins regulate mRNAs encoding a series of activation molecules and differentiation factors such as OX40 (Tnfr-superfamily member 4), ICOS and CTLA-4^{13,17,18,20–22}. It was proposed that Regnase-1 and Roquin cooperate to suppress *Icos* expression by expressing a Roquin-Regnase-1 fusion protein in T cells¹⁸. However, due to perinatal lethality upon mutation of both Regnase-1 and Roquin-1/2, their relationship in the context of T cell

activation remains unclear.

Cardiac tissue is composed of various cellular and extracellular compartments^{23–25}. The cellular compartment is mainly comprised of cardiomyocytes, capillary endothelial cells, resident fibroblasts, monocytic cells and vascular cells. In between cardiomyocytes, fibroblasts synthesize collagen type 1 and type 3, which build up to the main components of the extracellular matrix²³. Dysregulation of the synthesis process of the extracellular matrix leads to severe cardiac dysfunction or heart failure; one of the major cause of hospitalization, morbidity and mortality^{23,25,26}. One form of cardiomyopathy, known as inflammatory cardiomyopathies, is caused by autoimmune or immune response to infection. Recent studies have revealed that heart failure induced by hemodynamic overload also involves a significant inflammatory component²⁴. This inflammation is thought to be induced by infiltration of immune cell in the myocardium and upregulation of inflammatory cytokines, such as IL-1 β , IL-6 and TNF^{23,25–27}. Among these cytokines, IL-6 is reported to be sufficient in inducing the process leading to pathological cardiac hypertrophy²⁷. Nevertheless, recent studies show that Th1 effector T cells are capable of inducing cardiac fibroblast transition to

myofibroblasts, through direct cross-talks between Th1 cell and fibroblasts^{28–30}. As Th1 effector T cells are also known to accelerate the proliferation and secretion of profibrogenic markers of other cell types, such as hepatic stellate cells through the interferon gamma (IFN- γ)/STAT1 pathway, these cells can be another factor which plays a critical role in the establishment of inflammatory cardiomyopathies.

In this study, I investigate the role of Regnase-1 and Roquin-1/2 in T cells. I show that while either T-cell specific Regnase-1 knockout mice or Sanroque show pathogenesis at a later time point, mice lacking both functional Regnase-1 and Roquin in CD4 T cells display severe inflammation at very early stage, subsequently succumbing to the effects of inflammation by seven weeks after birth. Notably, the loss of function of Regnase-1 and Roquin mice showed a high incidence of heart fibrosis, which was not observed in mice lacking Regnase-1 in T cells or Sanroque mice. This could be in part due to an enhanced population of IFN- γ -producing cell upon the inactivation of Regnase-1 and Roquin in CD4 T cells, as evidenced at both molecular and genetic levels. Taken together, my results demonstrate a non-redundant control of Regnase-1 and Roquin-1/2 in Th1 differentiation,

and a rather complex regulation between these proteins.

Chapter 2

Methods

2-1 Mice

C57BL/6J mice were purchased from CLEA Japan, Inc. (1-2-7, Higashiyama, Meguro-ku, Tokyo 153-8533, Japan). Generation of *Regnase-1*^{-/-}, *Regnase-1*^{flox/flox}, CD4-Cre transgenic, Sanroque mice have been described in previous publications^{4,5,13}. All mice were raised under specific pathogen-free condition except for the infection experiments. All protocols of animal experiments were approved by the Kyoto University Animal Experimentation Committee.

2-2 Histological analysis

Mice were sacrificed around 5 weeks after birth. Organs were fixed in 10% buffered formalin, processed and embedded in paraffin. Sections were stained according to standard H&E or AZ techniques.

2-3 Sample collection from mice

Mice were sacrificed around 5 weeks after birth. For the analysis of protein, the heart samples were homogenized immediately after the dissection in RIPA buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA, 1%

Nonidet-P40, 0.1% SDS, 1% sodium deoxycholate, and Complete Mini Protease Inhibitor Cocktail without EDTA (F. Hoffmann-La Roche Ltd., Basel, Switzerland)), then centrifuged with 15,000 rpm for 10 min at 4°C, and the supernatant was collected. Protein concentration was determined by BCA protein assay (Thermo Fisher Scientific Inc., 168 Third Avenue, Waltham, MA 02451, USA) and adjusted for Western blotting. For the analysis of RNA, the heart, kidney and lung samples were preserved in RNeasy lysis solution (Qiagen, Hilden, Germany), followed by homogenization in TRIzol® (Invitrogen Corp., Carlsbad, CA 92008, USA).

2-4 Plasmids and reagents

The plasmids expressing Regnsae-1 and Roquin have been described in previous publications^{4,9,13}. Full-length 3'UTR of *Furin*, *Il12rb1*, *Il18r*, *Roquin-1*, *Roquin-2*, *Regnase-1* and *OX40* (1027-1048), *Regnase-1* (191-210), *Regnase-1* (244-379), *Regnase-1* (378-392), *Regnase-1* (400-731) sequences were inserted in the pGL3 vector (Promega, WI, USA). PMA and ionomycin were from Sigma-Aldrich.

2-5 Generation of Regnase-1, Roquin-1 and Roquin-2 deficient Jurkat cell line

Depletion of Regnase-1, Roquin-1 and Roquin-2 gene from the Jurkat cell line was performed by using the CRISPR/ Cas9 system. Briefly, pX330-GFP plasmid, which was a generous gift from Toru Okamoto (Department of molecular virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) was digested with Bbs1 (New England Biolabs, Inc., Beverly, MA 01915, USA), and insert double strand DNA with sequences of 5'-aaggaggtcttctctgccg-3' (Regnase-1), 5'-tgggcagcagtaagggctag-3' (Roquin-1) and 5'-tgggccgctgtcagggctcg-3' (Roquin-2) was ligated. The plasmid was transfected into Jurkat cells via the Neon transfection system (Invitrogen Corp., Carlsbad, CA 92008, USA). Individual cells were then separated and cultured. To screen cells lacking expression of Regnase-1 and Roquin-1/2 protein, western blot analysis was applied. Direct sequencing was performed to further confirm the disruption of the Regnase-1 and/Roquin-1/2 gene in both alleles. To generate Regnase-1; Roquin-1/2-deficient cell line, Regnase-1 editing plasmid was introduced into the Roquin-1/2-deficient Jurkat cell line. Clones with successful

depletion were used for further analysis as a Regnase-1 and/or Roquin-1/2-deficient cell line.

2-6 Cells and transfection

Hela cells were maintained in DMEM (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% FBS, 50 μ M β -mercaptoethanol (Nacalai Tesque, Inc., Kyoto, Japan). Jurkat cells were maintained in RPMI-1640 (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% FBS. Plasmids were transfected into cells with the use of Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA 92008, USA), or the Neon transfection system according to the manufacturer's instruction. Jurkat cells were transiently transfected with expression plasmids by electroporation (Neon; Invitrogen), according to manufacturer's protocol. After 24 hrs culture, cells were used for further experiments.

2-7 Luciferase assay

HeLa cells were transfected with luciferase reporter plasmid pGL3 containing the 3'UTR of indicated genes, together with expression plasmid

for Regnase-1, Roquin or empty plasmid (as control). After 24 hrs of cultivation, cells were lysed and luciferase activity in lysates was determined with the Dual-luciferase Reporter Assay system (Promega). The gene encoding Renilla luciferase was transfected simultaneously as an internal control.

2-8 Quantitative RT-PCR analysis

Cells or tissues were lysed with TRIzol® reagent (Invitrogen Corp., Carlsbad, CA 92008, USA), and total RNA was extracted. Reverse transcription was performed by using ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo CO., LTD., Japan) according to the manufacturer's instruction. The cDNA fragments were amplified with SYBR® Green PCR Master Mix (Applied Biosystems), and fluorescence was detected by a StepOne Real-Time PCR System (Applied Biosystems). Human *GAPDH* or murine *Gapdh* mRNA abundance was utilized for the normalization.

2-9 Western blot

Cell lysate was prepared with RIPA buffer. Protein concentration was

measured by BCA Protein Assay. Immunoblot was performed as described in previous publications⁹. The following antibodies were used: anti-mouse Roquin and anti-mouse/ human β -actin (sc-1615; Santa Cruz). Rabbit anti-mouse/ human Regnase-1 antibody has been described in previous publications¹⁰. Luminescence was detected with a luminescent image analyzer (ImageQuant LAS 4000; GE Healthcare), and then analyzed via the ImageJ software (NIH, Bethesda, Maryland).

2-10 Flow Cytometry

Antibodies for flow cytometry were purchased from Biolegend: anti-CD3(145-2C11), anti-CD4(RM4-5), anti-CD8(53-6.7), anti-CD44(IM7), anti-CD62L(MEL-14), anti-69(H1.2F3), anti-CD138(281-2), anti-CD19(6D5) (Biolegend, Inc., San Diego, CA 92121, USA). Antibodies for flow cytometry were purchased from BD Bioscience: anti-IL-4(11B11), anti-IL-17A(TC11-18H10.1), anti- IFN- γ (XMG1.2) (Becton, Dickinson and Company, 1 Becton Drive, Franklin Lakes, NJ 07417-1880, USA). Antibody for flow cytometry were purchased from eBioscience: anti-Foxp3(FJK-16s) (Thermo Fisher Scientific Inc., MA, USA). For surface staining, cells were

maintained in the dark at 4°C throughout. Cells were washed in ice-cold FACS buffer (2% BSA in PBS), then incubated with each antibody for 15 min and washed twice with FACS buffer. For intracellular cytokines, splenocytes were stimulated for 2 hrs with PMA-ionomycin and treated with GolgiStop in complete media for an additional 3 hr. Cells were stained with anti-CD4 antibody and anti-TCR β antibody. Then the cells were permeabilized and stained using Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (Becton, Dickinson and Company, 1 Becton Drive, NJ, USA) following the manufacturer's instructions. Data were acquired on a FACS Aria (Becton, Dickinson and Company, NJ, USA), and analyzed using FlowJo (FlowJo, LLC., USA).

2-11 ELISA

IFN- γ concentrations in culture supernatants (Thermo Fisher Scientific Inc., MA, USA), and mouse anti-nuclear antibody level in serum (Alpha Diagnostic International, Inc., TX, USA), were measured by ELISA following manufacturer's protocols. Concentrations of mouse IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA in the sera were measured as described in previous

publication³¹.

2-12 RNA sequencing and transcriptome analysis

CD4⁺ cells from mice spleen were sorted via the SONY Cell Sorter SH800 and lysed with TRIzol. Total RNA was extracted as described above. RNA integrity number for each sample were confirmed to be higher than 9.0, before sequencing on a HiSeq 2000 system (Illumina, Inc., San Diego, CA 92122, USA) according to the manufacturer's instruction. Identified reads were mapped to the murine genome (mm10). Genes without annotations as well as genes whose RPKM values were less than 1 were removed. Difference in levels of expression of each gene in *CD4^{cre}Regnase-1^{fl/fl}*, *Roquin^{San/San}* and *CD4^{cre}Regnase-1^{fl/fl}; Roquin^{San/San}* with respect to the control was evaluated by using a custom R script and edgeR.

2-13 Statistical Analysis

All data are presented as mean and SD. Statistical analysis was performed with the Student's t-test. Statistical significance was defined as a $p < 0.05$ (*) $p < 0.01$ (**), or $p < 0.001$ (***).

Chapter 3

Results

3-1 Lack of Functional Regnase-1 and Roquin Causes Postnatal Death

To investigate the complex role of Regnase-1 and Roquin in T cells, mice lacking functional Roquin (Sanroque) together with a conditional Zc3h12a allele in CD4 T cells were generated. Although *CD4^{cre}Regnase-1^{fl/fl}; Roquin^{San/San}* (*CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}*) mice were born in a Mendelian ratio, they showed severe growth retardation and most of the mice died spontaneously within 7 weeks of birth. In comparison, less than 20% of *CD4^{cre}Regnase-1^{fl/fl}* (*CD4^{cre}Reg-1^{fl/fl}*) mice and less than 10% of *Roquin^{San/San}* (*Roq^{San/San}*) died by 8 weeks of age (Figure 1C, 1E). Additionally, the spleen of *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice were larger compared to either *CD4^{cre}Reg-1^{fl/fl}* or *Roq^{San/San}* mice (Figure 1A and 1B). As a result of spleen hypertrophy, the amount of splenocytes were also largely increased in the *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice (Figure 1F). These mice had smaller body sizes and decreased body mass compared with the control, *CD4^{cre}Reg-1^{fl/fl}* or *Roq^{San/San}* littermates (Figure 1D, Figure 1G).

Figure 1

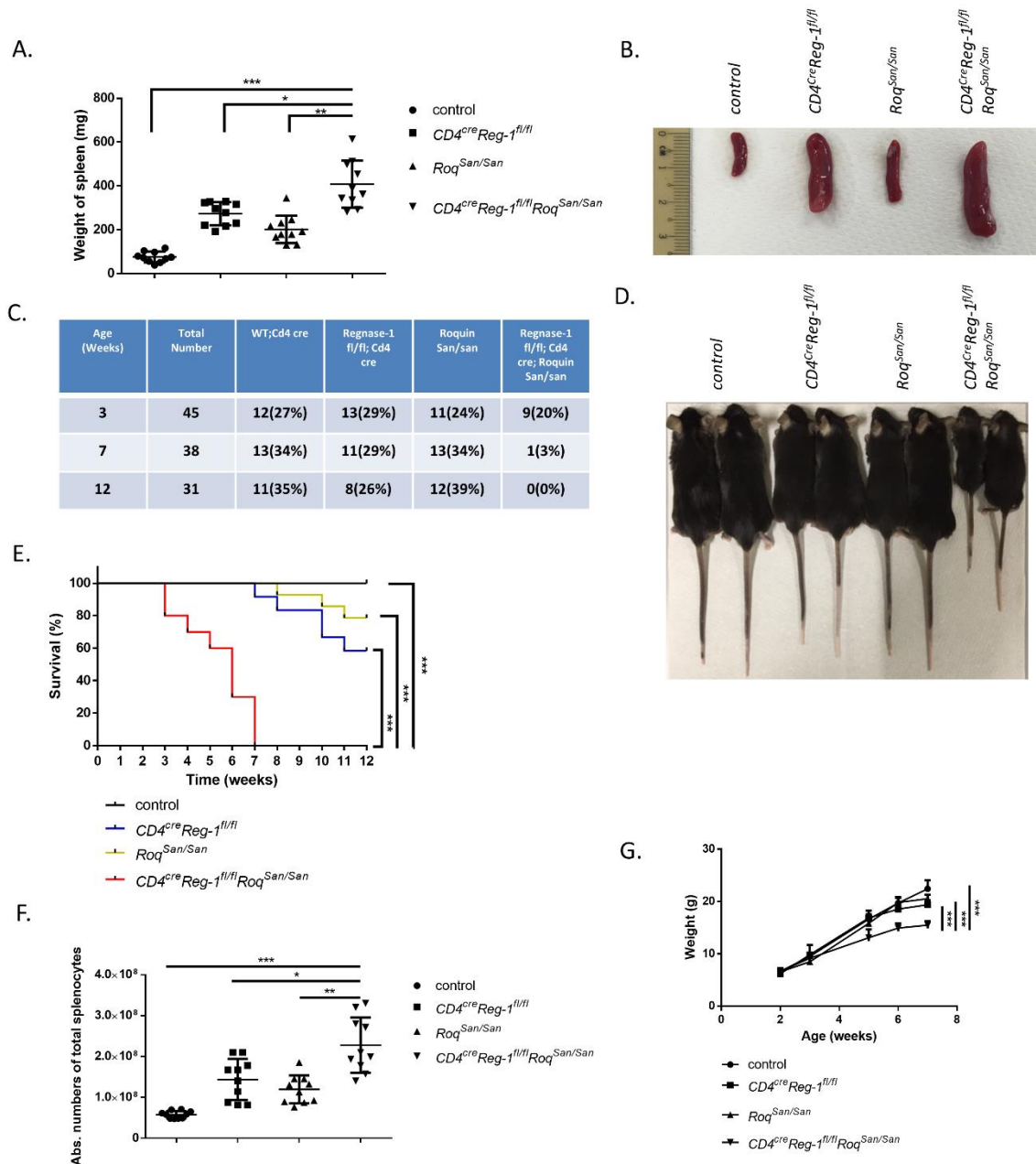


Figure 1.

- (A) Spleen weight for control, $CD4^{cre}Reg-1^{fl/fl}$, $Roq^{San/San}$ and $CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}$ mice at 6 week post birth (n=10).
- (B) Gross appearance of spleens from control, $CD4^{cre}Reg-1^{fl/fl}$, $Roq^{San/San}$ and $CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}$ mice at 6 week post birth.
- (C) Genotype frequency of control, $CD4^{cre}Reg-1^{fl/fl}$, $Roq^{San/San}$ and $CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}$ mice.

- (D) Body size of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.
 - (E) Survival rates of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice at indicated time periods (n=10).
 - (F) Absolute number of total splenocytes obtained from whole spleen from control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice at 6 week post birth (n=10).
 - (G) Body weight curves of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice until 7 weeks post birth.
- Genotypes are indicated in the figure, and statistical significances were calculated by Student's t-test. Data are represented as mean $\pm$ SD.

3-2 Deletion of Both Regnase-1 and Roquin in CD4⁺ T Cells Induces T and B Cell Activation

The lymphoid organs of moribund *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice were examined and compared with those of the age-matched control, *CD4^{cre}Reg-1^{fl/fl}* or *Roq^{San/San}* mice. Generally, apart from swollen spleen, other lymphoid organs such as the thymus or lymph nodes were found to be significantly enlarged. Like the splenocytes counts, the amount of either thymocytes or lymphocytes from lymph nodes were greatly increased (data not shown).

Next, the expression of CD44, CD62L and CD69 on T cells from the cell population mentioned was examined above to determine their activation status. The expression of CD69 on CD4⁺ T cells and CD8⁺ was significantly

increased in $CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}$ mice (Figure 2A, Figure 2B). Transition of naive T cell into memory T cell population in both $CD4^{cre}Reg-1^{fl/fl}$ and $Roq^{San/San}$ models were observed. This phenomenon was intensified in $CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}$ mice as little CD62L expression was detected on either CD4⁺ or CD8⁺ T cells (Figure 2C). Nevertheless, expression of ICOS, a previously reported T cell activation marker was also highly upregulated (Figure 2D, 2E). The Treg cell population however, was not significantly upregulated (Figure 2F). Analyses therefore reveal that deletion of both Regnase-1 and Roquin in CD4⁺ T cells led to the hyperactivation of T cells.

Figure 2

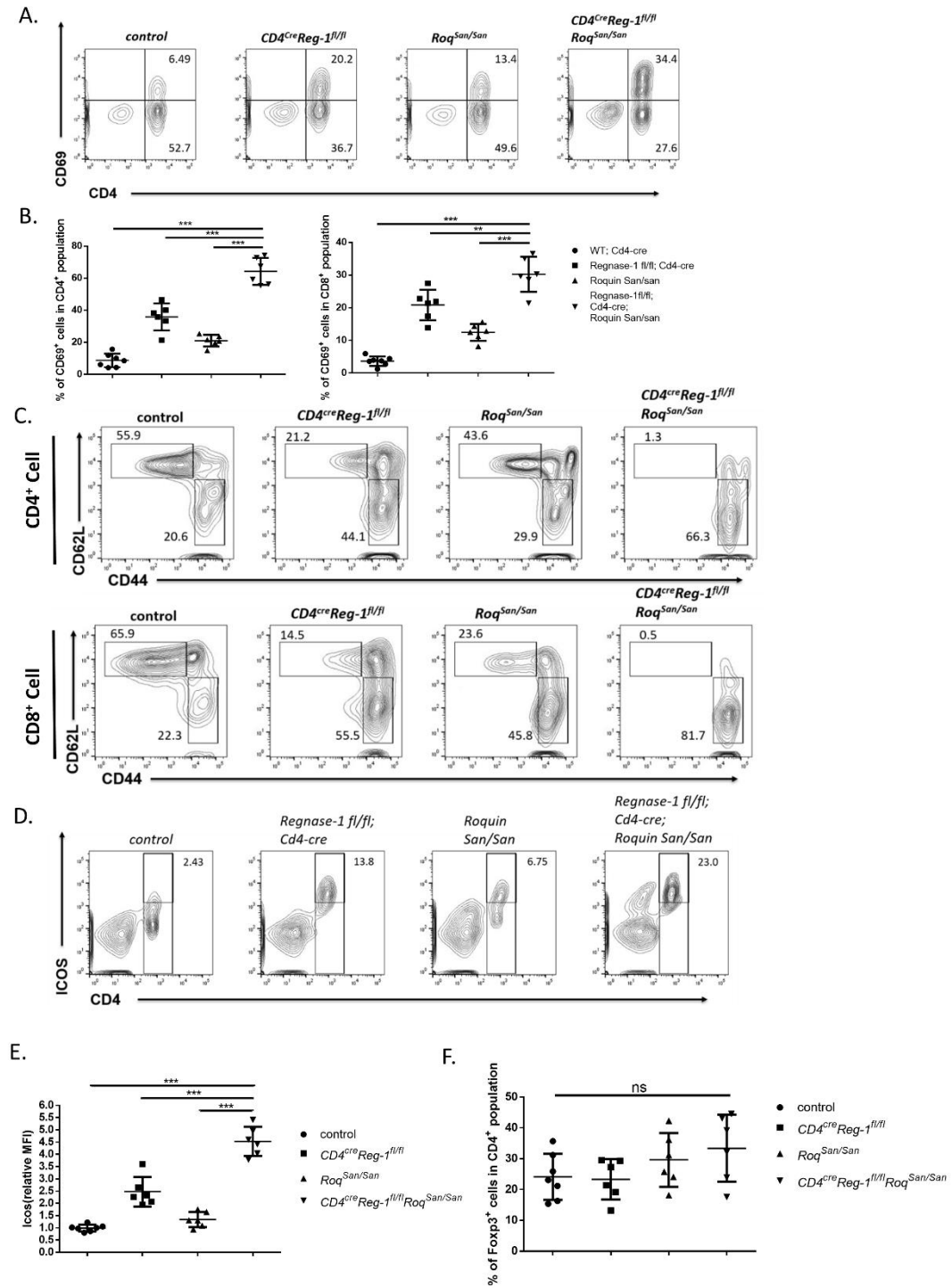


Figure 2.

(A) Flow cytometric analysis of expression of CD69 on CD4⁺ cells from spleen.

- (B) Percentage of CD69 positive population of splenic CD4⁺ or CD8⁺ cells of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.
 - (C) Flow cytometric analysis of expression of CD62L and CD44 on splenic CD4 and CD8 T cells.
 - (D) Flow cytometric analysis of expression of ICOS on splenic CD4 T cells.
 - (E) Mean fluorescence intensity of ICOS expression on splenic CD4 T cells of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.
 - (F) Percentage of Foxp3 positive population of splenic CD4⁺ cells from control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.
- Genotypes are indicated in the figure, and statistical significances were calculated by Student's t-test. Data are represented as mean ± SD.

Consistent with previous research, CD19^{dull} CD138^{hi} plasma cells accumulated in both *CD4^{cre}Reg-1^{fl/fl}* and *Roq^{San/San}* mice upon T cell activation. This population was further enhanced in the *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice (Figure 3A). A significantly higher level of anti-nuclear antibody in the sera of these mice was detected (Figure 3B). All subtypes of immunoglobulins were also greatly upregulated in *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice (Figure 3C). In conclusion, deletion of both Regnase-1 and Roquin in CD4⁺ T cells indirectly caused B cell hyperactivation leading to the development of severe autoimmune inflammation.

Figure 3

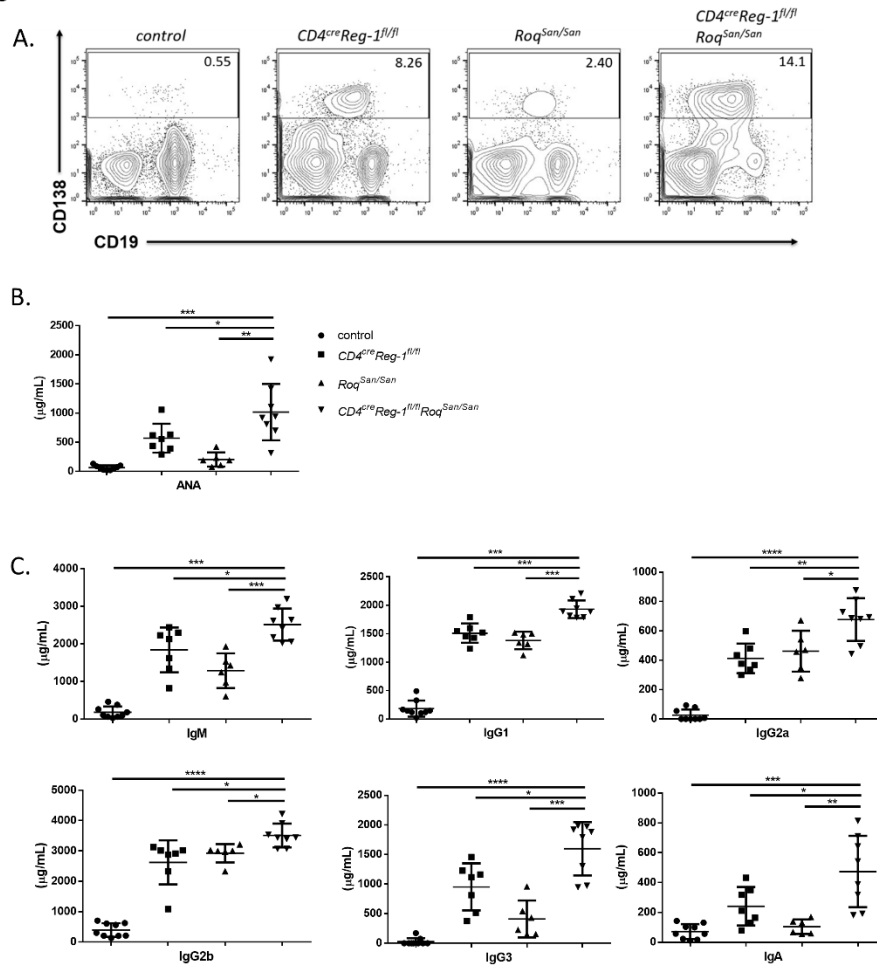


Figure 3.

- (A) Flow cytometric analysis of expression of CD138 on CD19 positive cells from spleen.
- (B) Production in antinuclear antibodies (ANA) in control, $CD4^{cre}Reg-1^{fl/fl}$, $Roq^{San/San}$ and $CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}$ mice.
- (C) Hypergammaglobulinemia in control, $CD4^{cre}Reg-1^{fl/fl}$, $Roq^{San/San}$ and $CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}$ mice. Serum immunoglobulin levels were measured.
- Genotypes are indicated in the figure, and statistical significances were calculated by Student's t-test. Data are represented as mean $\pm$ SD.

3-3 A Set of Th1 Related Genes Is Highly Upregulated in CD4⁺ T Cells Lacking Both Regnase-1 and Roquin

Given that T cell differentiation is tightly controlled by Regnase-1 and Roquin, the enhanced population of T helper cell subtypes was accessed. To address this, splenic T cells from control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice were sorted. After PMA-ionomycin stimulation, a significantly higher concentration of IFN- γ in *CD4^{cre}Reg-1^{fl/fl}*; *Roq^{San/San}* mice was detected (Figure 4A). FACS analysis data also showed that *CD4^{cre}Reg-1^{fl/fl}*; *Roq^{San/San}* mice had an elevated population of Th2 and Th17 cells, which was comparable to that of the *CD4^{cre}Reg-1^{fl/fl}* mice. However, the Th1 population was significantly increased compared with either the *CD4^{cre}Reg-1^{fl/fl}* or *Roq^{San/San}* mice (Figure 4B). Collectively, these results suggest that the lack of both functional Regnase-1 and Roquin could lead to enhanced Th1 differentiation.

Figure 4

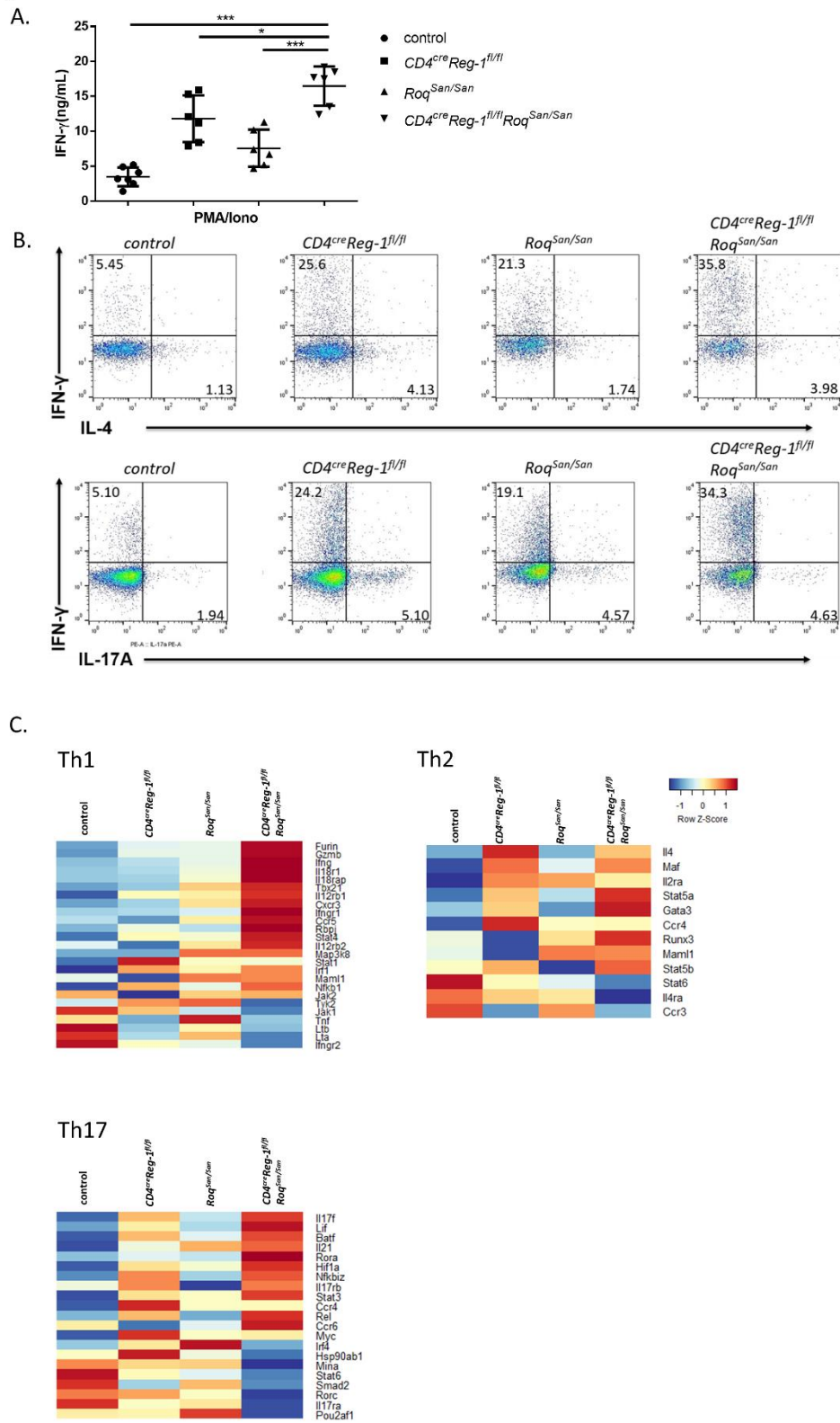


Figure 4.

- (A) Production of IFN- γ in response to PMA plus ionomycin in splenic T cells.
- (B) Intracellular staining of IFN- γ , IL-4 and IL-17 in splenic CD4⁺ T cells after stimulation with PMA plus ionomycin.
- (C) Heatmaps of effector T cell subtypes in control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice and their associated signature genes. Genotypes are indicated in the figure, and statistical significances were calculated by Student's t-test. Data are represented as mean $\pm$ SD. Row Z-scores are indicated in the figure.

To further analyze the molecular basis for Th1 upregulation, gene expression profiles in CD4⁺ T cells from control were examined in the *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice. Signature genes which were associated with the major T cell subtypes (Th1, Th2, Th17 and Treg) were selected and grouped according to their respective groups, based on Kyoto Encyclopedia³² of Genes and Genomes database and recent literature^{33–36}. Signature markers of Th1 cells were prominent and exclusive to the *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice whereas no such pattern was observed in either *CD4^{cre}Reg-1^{fl/fl}* or *Roq^{San/San}* mice. No obvious clustering was observed for the Th2 or Th17 group of genes, unlike that of the Th1 group (Figure 4C). These results were in agreement with the FACS results, indicating an increase in the Th1 population in *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.

Among genes that were exclusively upregulated in *CD4^{cre} Reg-1^{fl/fl}*; *Roq^{San/San}* mice, three mRNAs, *Furin*, *Il12rb1* and *Il18r*, which play an essential role in Th1 subtype T helper cell differentiation were investigated^{33,35,37}. By testing individual luciferase constructs harboring the 3'UTRs of the respective genes, it was discovered that Regnase-1 and Roquin targets the 3'UTR of both *Furin* and *Il12rb1* whereas the UTR of *Il18r* could only be targeted by Regnase-1 (Figure 5).

Figure 5

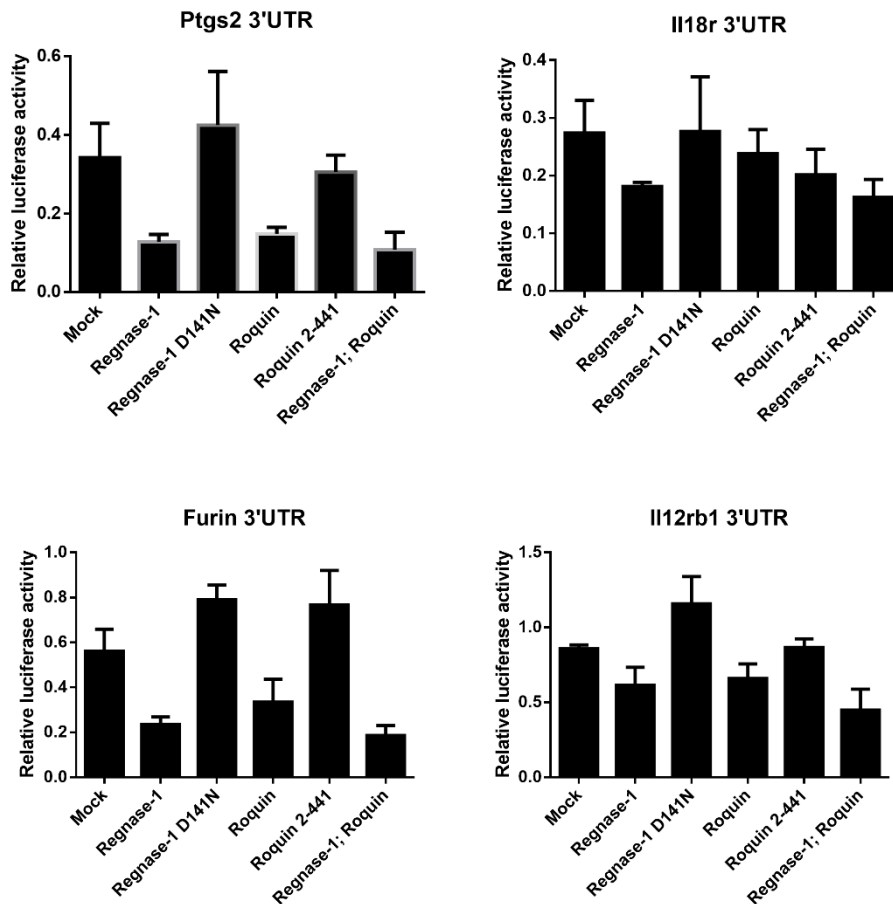


Figure 5

Luciferase activity of HeLa cells transfected with indicated reporter, Regnase-1 and Roquin expression plasmids.

Data are represented as mean $\pm$ SD.

3-4 Cooperative, Non-redundant and Complex Relationship between Regnase-1 and Roquin

To further confirm the effects of the lack of both Regnase-1 and Roquin in T cells, Jurkat cells with deficiency of Regnase-1, Roquin-1/2 or both Regnase-1 and Roquin-1/2 were generated (Figure 6A). An upregulated expression level of *Furin* and *Il12rb1* from Regnase-1 knock-out and Roquin-1/2 knock-out cell mRNAs was detected. This level was further increased in Regnase-1; Roquin-1/2 KO cells, suggesting that *Furin* and *Il12rb1* are cooperatively regulated by both Regnase-1 and Roquin (Figure 6B). In the meantime, similar mRNA expression level of *Ifng* was uncovered (Figure 6B). Furthermore, changes in mRNA expression of these cell line in response to PMA and ionomycin stimulation was studied. *Rel*, a known target of Regnase-1, was increased in Regnase-1 KO and Regnase-1; Roquin-1/2 mRNAs. Interestingly, *Il2* mRNA expression levels in all three KO cell lines were found to be upregulated. In contrast, *Nfkb1a* mRNA level was

comparably induced between WT and KO Jurkat cells (Figure 6C). Reciprocally, overexpression of Regnase-1, Roquin or both Regnase-1 and Roquin in Jurkat cells resulted in the downregulation of *IL2* mRNA (Figure 6D). Taken together, these results demonstrate that Regnase-1 and Roquin can control T helper cell differentiation towards Th1 subtype by cooperatively repressing the expression of Th1 promoting factors and associated molecules such as *Furin* and *IL12rb1*.

Figure 6

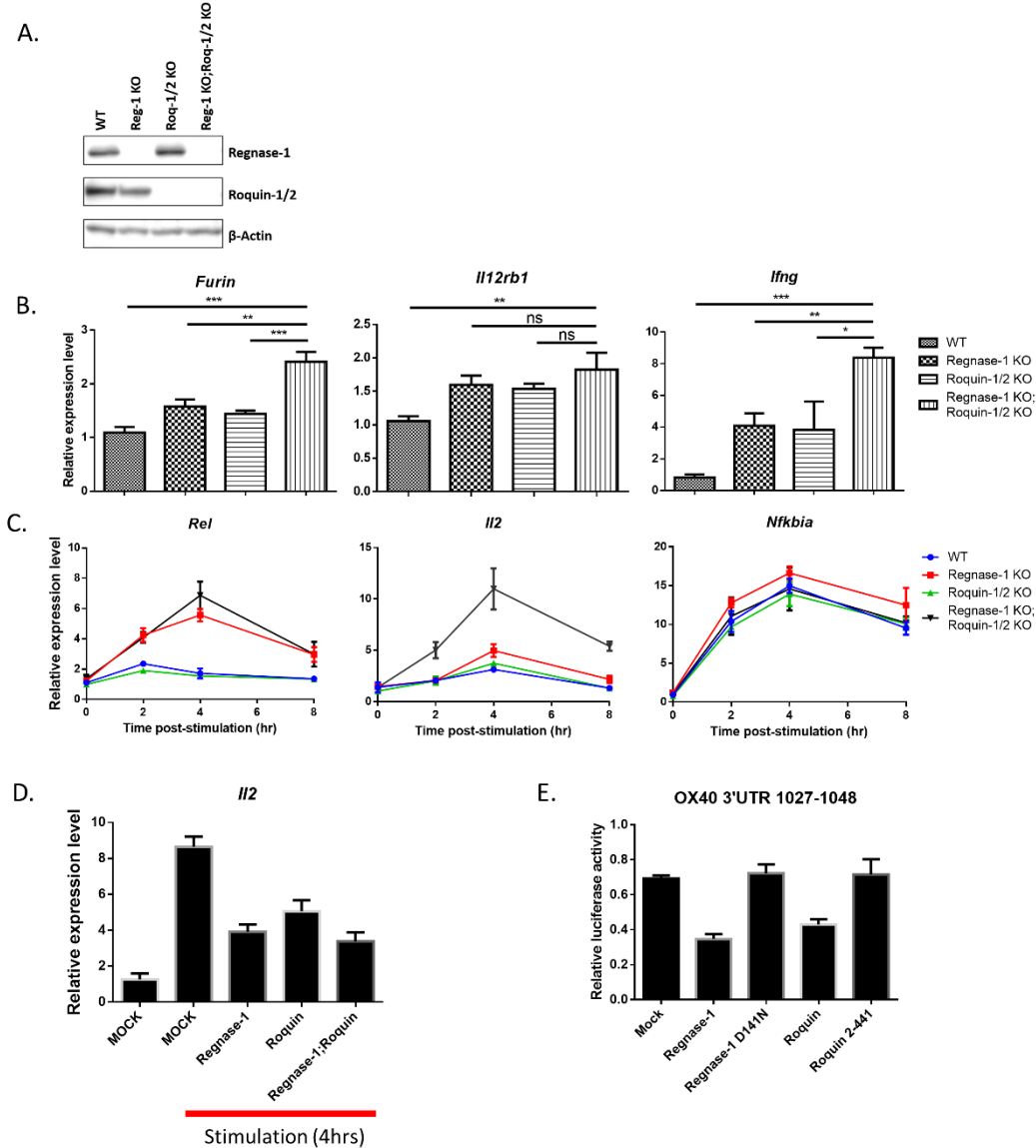


Figure 6.

- (A) Western blot of Regnase-1 and Roquin in WT, Regnase-1 knock-out, Roquin-1/2 knock-out or Regnase-1; Roquin-1/2 knock-out Jurkat cells.
- (B) Quantitative PCR analysis of mRNAs encoding *Furin*, *Il12rb1* and *Ifng* in WT, Regnase-1 knock-out, Roquin-1/2 knock-out or Regnase-1; Roquin-1/2 knock-out Jurkat cells.
- (C) Quantitative PCR analysis of mRNAs encoding *Rel*, *Il2* and *Nfkb1a* in WT, Regnase-1 knock-out, Roquin-1/2 knock-out or Regnase-1; Roquin-1/2 knock-out Jurkat cells and stimulated with PMA plus ionomycin for 0-8 hr.
- (D) Quantitative PCR analysis of mRNAs encoding *il2* in WT, Regnase-1 knock-out,

Roquin-1/2 knock-out or Regnase-1; Roquin-1/2 knock-out Jurkat cells that electroporated with Regnase-1, Roquin or both expression plasmids and then stimulated with PMA plus ionomycin for 4 hr.

(E) Luciferase activity of HeLa cells transfected with indicated reporter, Regnase-1 and Roquin expression plasmids.

Statistical significances were calculated by Student's t-test and data are represented as mean $\pm$ SD.

It was previously reported that Regnase-1 and Roquin can recognize overlapping target mRNAs via the same stem loop (SL) structure in their 3'UTR⁹. One recent report suggested that Roquin recognizes a hex-loop structure in the *OX40* mRNA 3'UTR, in addition to the tri-loop structure²². Regnase-1 was shown to be capable of recognizing the same hex-loop structure in *OX40* mRNA 3'UTR (Figure 6E).

Figure 7

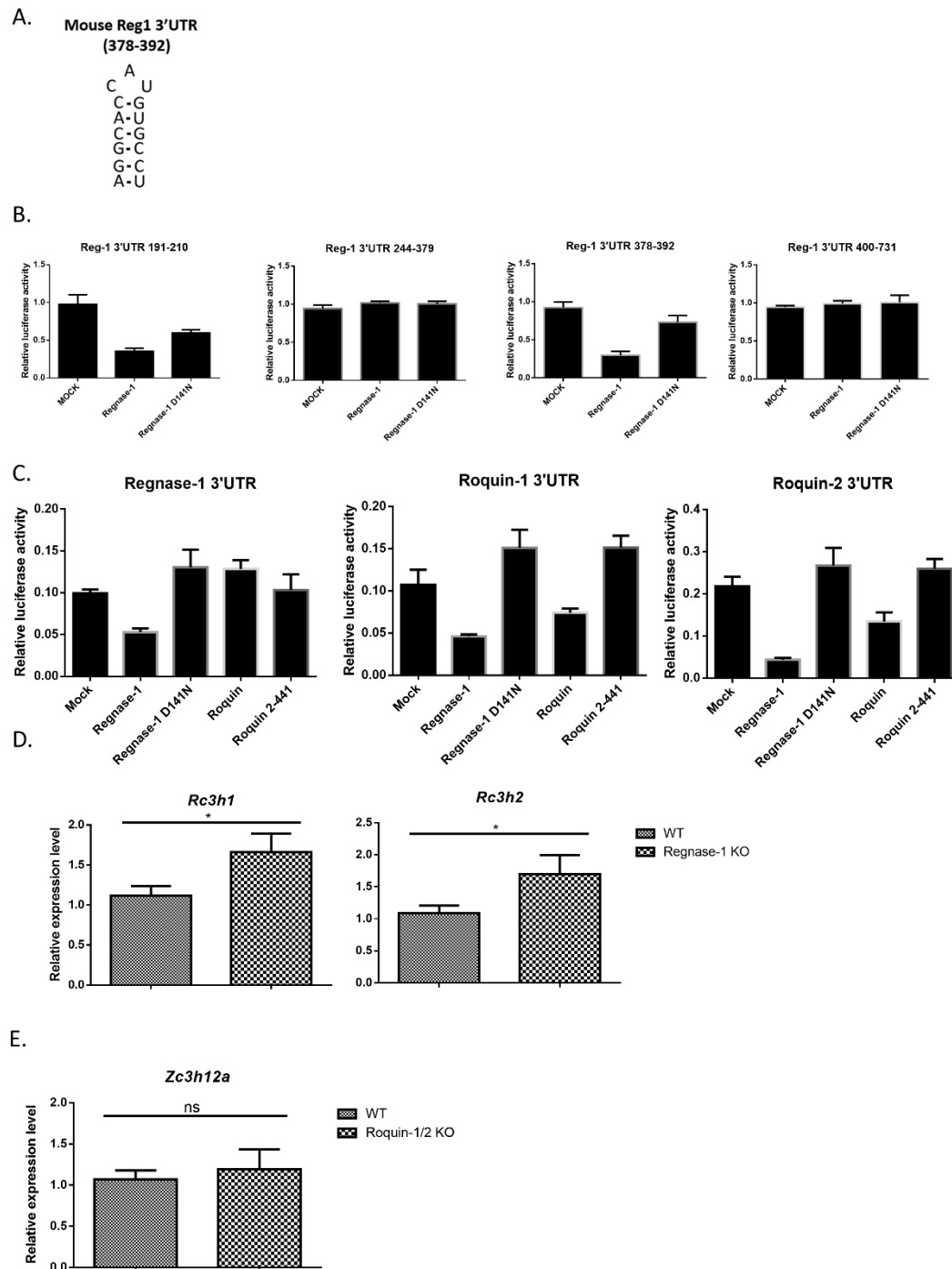


Figure 7.
Complex Relationship between Regnase-1 and Roquin in T cells

- (A) Predicted stem-loop structure of the Regnase-1 responsive element in the 3'UTR of Regnase-1 mRNA.
 - (B) Luciferase activity of HeLa cells transfected with indicated reporter, Regnase-1 expression plasmids.
 - (C) Luciferase activity of HeLa cells transfected with indicated reporter, Regnase-1 and Roquin expression plasmids.
 - (D) Quantitative PCR analysis of mRNAs encoding *Rc3h1* and *Rc3h2* in WT and Regnase-1 knock-out Jurkat cells.
 - (E) Quantitative PCR analysis of mRNAs encoding *Regnase-1* in WT and Roquin-1/2 knock-out Jurkat cells.
- Statistical significances were calculated by Student's t-test and data was represented as mean $\pm$ SD.

Besides targeting itself, Regnase-1 was reported to degrade a set of mRNAs such as IL-6. Based on Regnase-1's recognition motif, another SL, targetable by Regnase-1, in Regnase-1 mRNA 3'UTR (378-392) was discovered in addition to a previously reported SL at 191-210 (Figure 7A, 7B). As Regnase-1 shares a common target structure with Roquin, the ability of Regnase-1 and Roquin in repressing each other's mRNA structure was investigated. A set of luciferase constructs harboring full length 3'UTR of Regnase-1 and Roquin-1/2 was generated. As the result, Regnase-1 3'UTR, Roquin-1/2 3'UTR sequences were significantly inhibited by Regnase-1 overexpression, whereas Roquin overexpression only repressed Roquin-1/2 3'UTR sequences (Figure 7C). Moreover, an upregulation of *Rc3h1* and

Rc3h2 mRNA (encode Roquin-1 and Roquin-2) levels in Regnase-1 KO cells was discovered (Figure 7D). In contrast, expression of *Zc3h12a* mRNA (encoding Regnase-1) was not affected (Figure 7E). These results demonstrate that both Regnase-1 and Roquin can recognize their respective mRNA and repress their individual expression. Additionally, Regnase-1 can down-regulate Roquin mRNA expression.

3-5 Early Heart Pathology in Mice Lacking Functional Regnase-1 and Roquin in T cells

From the histology examination, massive infiltration of lymphocytes in heart region of *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice was discovered. This phenomenon was not observed in *CD4^{cre}Reg-1^{fl/fl}* and *Roq^{San/San}* mice (Figure 8A). Azan's collagen staining revealed the presence of fibrosis in the heart of *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice (Figure 8B). The presence of soluble and cellular immune mediators within the myocardium of these mice was accessed via quantitative PCR 5 weeks after birth. A significant upregulation of *Il6* and *Tnfa* in *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* was detected. Expression of *Tgfb1*, an earlier fibrosis indicator was also upregulated. Interestingly, *Ifng*

was significantly induced (Figure 8C). In contrast, transcripts of cytokines that characterize Th2 and Th17 responses, such as *Il4* and *Il17* were not significantly altered (Figure 9A). In addition, *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice produced increased amount of IgG against its own myocardium region. However, the levels of such antibodies were comparable with *CD4^{cre}Reg-1^{fl/fl}* mice (Figure 9B).

[illegible]

Figure 8.

- (A) Microscopy of heart tissue sections from control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice, stained with hematoxylin and eosin (original magnification $\times 100$).
 - (B) Azan's trichrome collagen staining (blue) of heart from control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.
 - (C) Quantitative PCR analysis of mRNAs encoding *Il6*, *Il1b*, *Tnf*, *Ifng*, *Tgfb* and *Cxcl10* from left ventricle tissues of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.
 - (D) Quantitative PCR analysis of mRNAs encoding *Col1a1*, *Col1a2* and *Col3a1* from left ventricle tissues of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.
 - (E) Quantitative PCR analysis of mRNAs encoding *Acta2* from left ventricle tissues of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.
- Genotypes are indicated in the figure, and statistical significances were calculated by Student's t-test. Data are represented as mean $\pm$ SD.

Next the expression of collagen type 1 and type 3, main components of the extracellular matrix which are synthesized by fibroblasts was investigated. The expression level of these collagens was significantly upregulated in the myocardium of *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice (Figure 8D). *Acta2*, which encodes smooth muscle actin and plays an essential role in fibrosis formation, was also highly induced (Figure 8E).

In addition to the heart, the lung and kidney of these mice were examined. An increased expression level of various cytokines was observed. However, the expression level of collagen type 1 and type 3 were comparable

between $CD4^{\text{cre}}Reg-1^{\text{fl/fl}}$, $Roq^{\text{San/San}}$ and $CD4^{\text{cre}}Reg-1^{\text{fl/fl}}$; $Roq^{\text{San/San}}$ mice (Figure 9C, 9D, 9E, 9F). In agreement with the qPCR results, formation of fibrosis in such organs was not detected (data not shown).

Figure 9

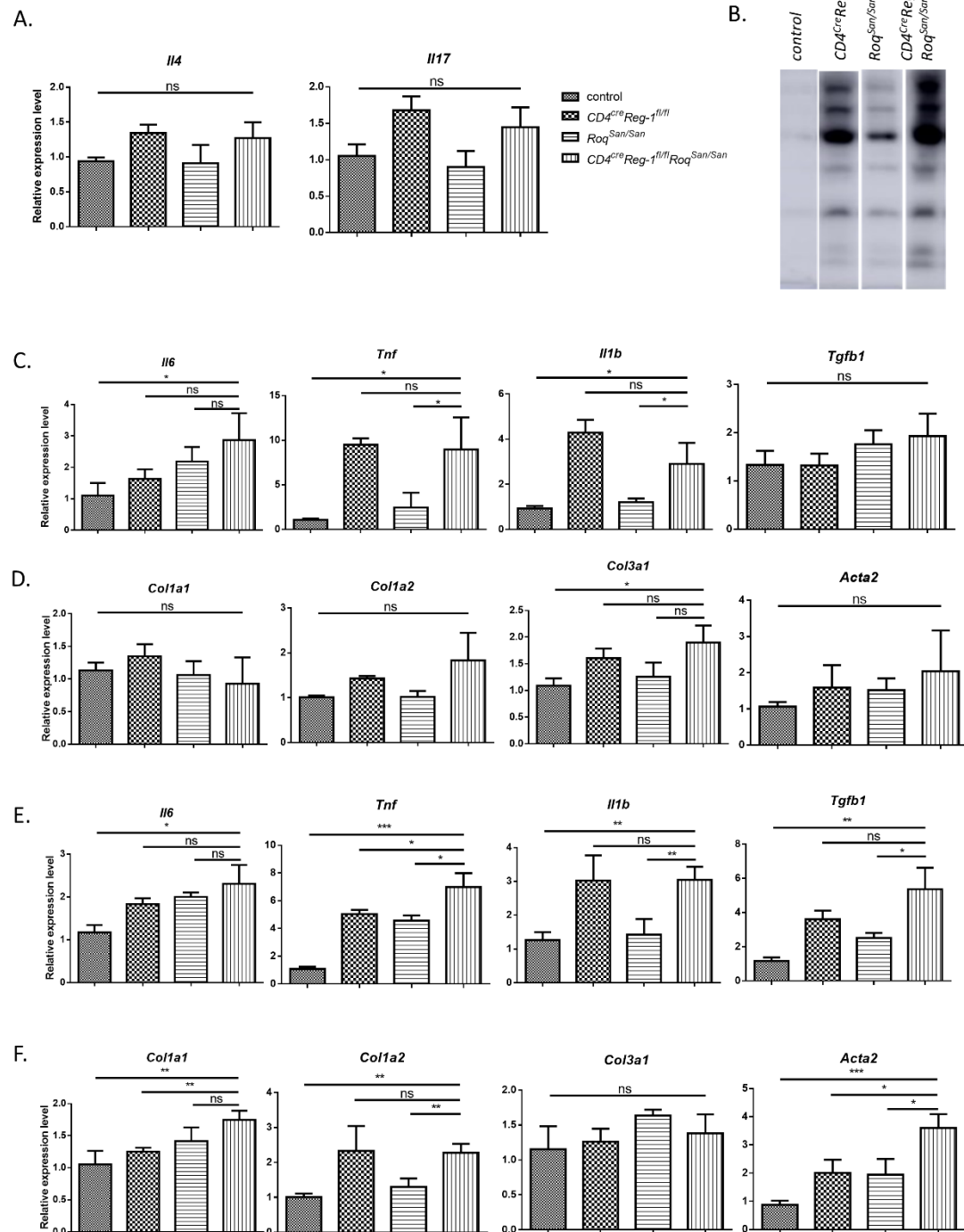


Figure 9

(A) Quantitative PCR analysis of mRNAs encoding *Il4* and *Il17* from left ventricle tissues of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.

(B) Representative immunoblot analysis of extract from heart tissue of RAG-2 deficient

mice, probed with serum of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice as indicated.

- (C) Quantitative PCR analysis of mRNAs encoding *Il6*, *Tnf*, *Il1b* and *Tgfb1* from lung tissues of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.
- (D) Quantitative PCR analysis of mRNAs encoding *Col1a1*, *Col1a2*, *Col3a1* and *Acta2* from lung tissues of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.
- (E) Quantitative PCR analysis of mRNAs encoding *Il6*, *Tnf*, *Il1b* and *Tgfb1* from kidney tissues of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.
- (F) Quantitative PCR analysis of mRNAs encoding *Col1a1*, *Col1a2*, *Col3a1* and *Acta2* from kidney tissues of control, *CD4^{cre}Reg-1^{fl/fl}*, *Roq^{San/San}* and *CD4^{cre}Reg-1^{fl/fl}; Roq^{San/San}* mice.

Genotypes are indicated in the figure, and statistical significances were calculated by Student's t-test. Data are represented as mean $\pm$ SD.

These results, taken together, suggest that the lack of functional Regnase-1 and Roquin may induce early heart pathology in mice via significantly increased population of Th1 cells.

Chapter 4

Discussion

In this study, mice lacking both functional Regnase-1 and Roquin in T cells were successfully generated. These mice suffered from massive inflammation and died at an early age. By analyzing these mice model and comparing with either conditional Regnase-1 knockout mice or Sanroque mice, a huge increase of T cell activation and cytokine production, followed by B cell over-maturation and enormous production of antibodies was discovered. This indicates that Regnase-1 and Roquin play a critical role in resting cells, preventing unwanted production of cytokines and controlling T cell activation.

Regnase-1 and Roquin negatively regulate activation of T cells as well as the control of inflammatory gene expression. Previous research by Jeltsch *et al.* suggests that Regnase-1 and Roquin function cooperatively during T cell activation and polarization¹⁸. The contribution of the nuclease activity of Regnase-1 together with the RNA-binding activity of Roquin was required to cooperatively repress target mRNAs. On the other hand, it was showed that Regnase-1 and Roquin function to degrade common inflammatory mRNAs in a distinct fashion in innate immune cells and HeLa cells⁹. The deficiency of Regnase-1 or Roquin leads to the upregulation of

different gene sets related to T cell activation and polarization. Highly increased production of IFN- γ under both Regnase-1 and Roquin deficiency indicate a non-redundant control of Th1 cell differentiation by Regnase-1 and Roquin, instead of a cooperative relationship suggested by Jeltsch *et al.*¹⁸.

In this study, a high incidence of early development of heart inflammation and fibrosis in mice harboring mutations in both Regnase-1 and Roquin was observed. The TGF- β receptor, which is highly expressed in cardiomyocytes, can form a complex with TGF- β and trigger a downstream TAK1 response³⁸. Together with activation of the Rho-associated kinase pathway, this can lead to an increased expression of smooth muscle actin and extracellular matrix protein, making the heart a primary organ afflicted by inflammation³⁹. Moreover, smooth muscles cells which can be found primarily in the heart, express a relatively high level of ICAM1, a critical binding molecule for inflammatory cell infiltration⁴⁰. However, the heart is not always a primary organ that is affected during inflammatory disease progression. As inflammatory cell infiltration is an intrinsically complicated process involving multiple organs, cell-to-cell interactions and cross-talk, further analysis is required to establish a comprehensive answer with

respect to the inflammation of the heart.

In this study, *Furin* and *Il12rb1* mRNAs have been shown to be commonly degraded by Regnase-1 and Roquin. Furin was previously reported to be preferentially expressed in Th1 cells, possessing an essential function in proteolytic processing of the inactive pro-TGF- β 1 precursor into its bioactive form^{41,42}. Additionally, it has been reported that Furin is able to regulate IFN- γ production, further inducing the STAT1-Tbet-IFN- γ amplification mechanism during Th1 differentiation^{35,41,43}. Additionally, the expression of Furin is regulated in an IL-12/Stat4-dependent manner and its inhibition in human Jurkat cell lines results in decreased IL-2 production³⁵. IL-12R β 1 itself is a critical receptor during early stage of Th1 cell differentiation with IL-12r β 1-deficient naive T cells failing to produce IFN- γ after IL-12 treatment^{44–46}. Additionally, the formation of the IL-18R complex, where the expression of IL-18R is controlled by Regnase-1, further induces IFN- γ through AP-1-dependent transcription^{47,48}. Given that these mRNAs are highly upregulated in Regnase-1 and Roquin TKO cells, it is plausible that non-redundant regulation of Th1 related genes by Regnase-1 and Roquin plays an essential role in Th1 differentiation in a T cell-intrinsic

fashion.

Regnase-1 destabilizes its own mRNA, possibly to prevent excessive translation of Regnase-1 and degradation of cytokine-encoding mRNAs. To achieve this, Regnase-1 recognizes a target SL containing a Py-Pu-Py loop sequence within its own 3'UTR. Mutation of such loop sequences results in Regnase-1 unresponsiveness, which is a rule shared by Roquin^{9,12}. Following this rule, another target loop sequence that is highly conserved in *homo sapiens* and *mus musculus* within Regnase-1 mRNA 3'UTR was discovered and validated, in addition to the previously reported SL at 191-210¹⁰. Recognition of either of these two SLs leads to degradation of its own mRNA. However, the reason for the presence of two target SLs on the Regnase-1 mRNA 3'UTR remains unclear. One possible explanation is that the presence of double SLs may attract other trans-activating factors that act cooperatively to control Regnase-1 expression through posttranscriptional pathways of gene regulation.

Roquin was recently reported to be able to recognize a non-canonical hexaloop structure in the 3'UTR of OX40 mRNA²². In this study, Regnase-1 could also interact the same structure, suggesting Regnase-1 and Roquin

may have a wide range of overlapping targets. Although Regnase-1 and Roquin share the common target recognition rules, *in vitro* experiments showed that Roquin failed to repress Regnase-1 3'UTR. This suggests Roquin may have stricter recognition rules. In addition to the Py-Pu-Py loop sequence, specific stem sequences are required for Roquin recognition^{11,49–52}.

Figure 10

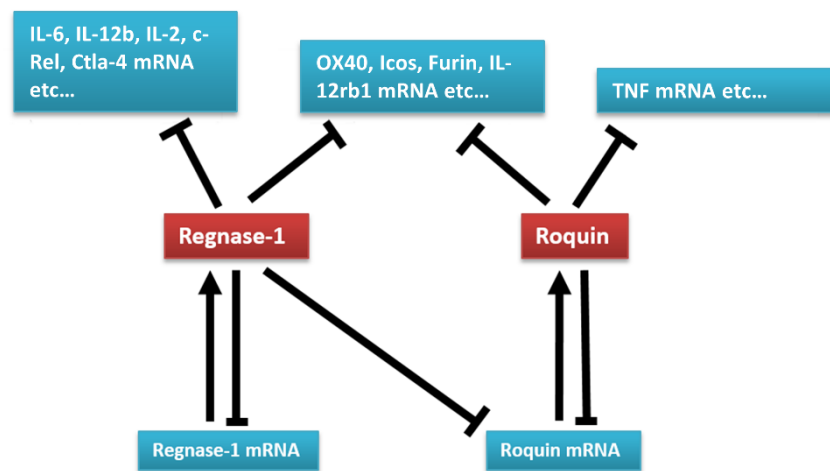


Figure 10

Summary of the targets of Regnase-1 and Roquin.

Thus far, Regnase-1 is known to negatively regulate the expression of *Il6*, *Il12b*, *Il2*, *Icos*, *Rel*, *Ctla4* and *Ox40* mRNA^{4,10,13}. Roquin is known to negatively regulate the expression of *Tnf*, *Icos* and *Ox40* mRNA^{6,7,12,17}. In

this study, *Furin* and *Il12rb1* mRNA which encode factors essential for Th1 differentiation, have been shown to be commonly regulated by both Regnase-1 and Roquin. Similar to Regnase-1, Roquin can destabilize its own mRNA as shown in this study. It was discovered that Regnase-1 also destabilizes both Roquin-1 and Roquin-2 mRNA, possibly through similar target SLs as Roquin. Taken together, my results show that Regnase-1 and Roquin possess complex and crucial roles in homeostasis of the immune system (Figure 10). It was previously reported that Regnase-1 tends to specifically degrade translationally active mRNAs at an early phase of inflammation together with the helicase activity of UPF1 whereas Roquin cleaves translationally inactive mRNAs at a rather late phase of inflammation, independent of UPF1⁹. Therefore, a possible reason for this cross-regulation is that both Regnase-1 and Roquin are degraded upon T cell stimulation, thus directing T cell activation and differentiation in a different direction⁵³. Meanwhile, cleavage of Regnase-1 reduces its repression on Roquin mRNA expression, thus relatively retaining its repression on Th1 and Th17 differentiation, maintaining immune homeostasis. However, as both Regnase-1 and Roquin have feedback regulating mechanisms, it is

insufficient to discuss their relationship solely at the mRNA level. Systematic analysis of both Regnase-1 and Roquin transcripts and protein levels may provide deeper insights into their roles during T cell activation/polarization.

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Regnase-1 and Roquin non-redundantly regulate Th1 differentiation causing cardiac inflammation and fibrosis

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