

REVIEW



## The immunomodulatory role of Regnase family RNA-binding proteins

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### ABSTRACT

RNA-binding proteins regulate RNA fate and govern post-transcriptional gene regulation. A new family of RNA-binding proteins is represented by regulatory RNases (Regnase, also known as Zc3h12 or MCPIP), which have emerged as important players in immune homeostasis. Four members, Regnase1-4, have been identified to date. Here we summarize recent findings on the role of Regnase in the regulation of RNA biology and its consequences for cell functions and inflammatory processes.

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### Introduction

In recent years, RNAs have emerged as modulators of cellular functions. Their fate is closely associated with that of RNA-binding proteins (RBPs), which bind, process, and degrade respective target RNAs. By these means, RBPs modulate biological processes and can shape both innate and adaptive immune responses [1,2,3]. Growing evidence supports their role in a wide variety of diseases and clinical conditions, including autoimmune disease and also sterile inflammation of the heart and other organs [4, 5, 6, 7]. To date, four members of the RegnaseRBP family have been identified. They are also known as Zc3h12a-d or MCPIP1-4. All members share two conserved domains: a CCCH-type zinc finger domain and a Pilt-N-terminus (PIN) like domain that recognizes and binds RNA [4,8,9]. Thereby, they can modulate cellular functions and immune responses. In this review, we summarize recent publications on the four members of the Regnase family and give insight into their contribution, mode of action, and modulatory role in inflammation.

### Molecular function of RNA-binding proteins

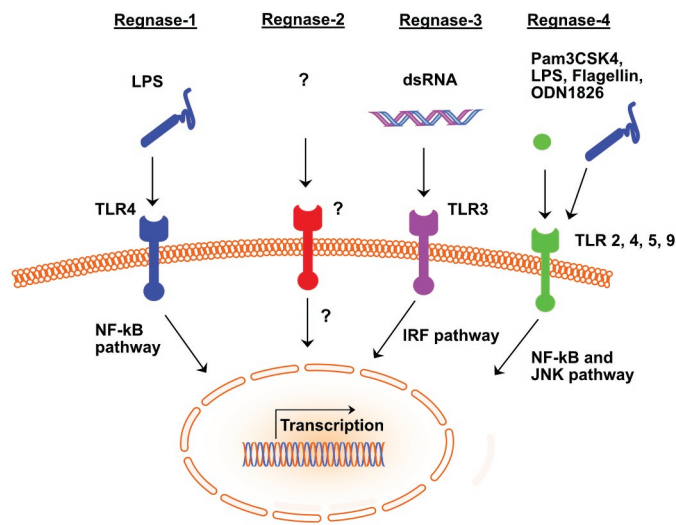
The RBP–RNA interaction is mediated mainly by *cis*-regulatory elements present in the 3'-untranslated region (UTR) of the respective RNA and the function of *trans*-acting RNA-binding protein [7, 10, 11]. AU rich elements (ARE) and stem-loop structures present in the *cis*-regulatory elements facilitate the anchoring of RNA-binding proteins. The stem-loop structures represent a three-dimensional structure for the interaction of the RNA-binding proteins and the respective RNA. They comprise a pyrimidine-purine-pyrimidine tri-loop sequence in the 3' UTR of inflammatory mRNA that is recognized and bound by RBP, such as Regnase-1 [12]. Regnase-1 acts as an endoribonuclease, directly degrading target cytokine mRNA and thereby modulating the local and systemic immune response. Regnase-1 contains

a CCCH zinc finger domain and a PIN-like RNase domain (see Figure 2). The PIN domain harbours the catalytic center responsible for the endonuclease function. For instance, Regnase-1 destabilizes interleukin-6 (IL-6)mRNA via the conserved *cis*-regulatory element present in its 3'UTR.

### Regnase-1 in the innate immune system

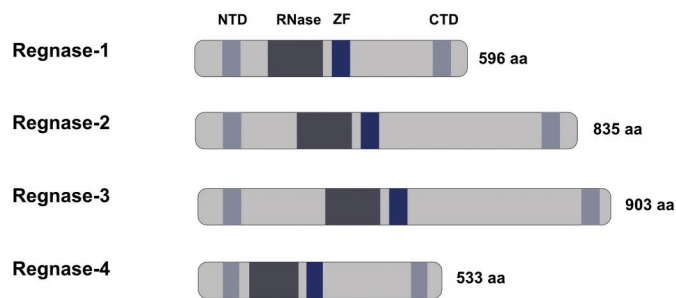
Regnase-1 is a well-characterized member of this RNA-binding protein family. It is of importance in both adaptive and innate immune responses as well as in haematopoiesis. Mice with global deficiency in Regnase-1 die around 12 weeks of age, presenting with anaemia, elevated serum levels of immunoglobulins, and autoantibody production with increased numbers of plasma cells [13]. In the following, we highlight specific functions of Regnase-1 in different immune cell populations and tissues.

In the innate immune system, biological functions of Regnase-1 have mainly been identified in macrophages, splenocytes, epithelial cells, and epidermal keratinocytes. Macrophages from Regnase-1 global knockout mice show highly increased production of interleukin-6 (IL-6) and IL-12b in response to toll-like receptor (TLR) ligands. While activation of the TLR pathway is normal, also the *Il-6* messenger RNA decay is severely impaired in Regnase-1 deficient macrophages (see Figure 1). Overexpression of Regnase-1 enhances *Il-6* mRNA degradation and regulates further RNAs such as *Il-12b* and *calcitonin receptor (Calcr)* mRNA [13]. Also, macrophages and splenocytes from Regnase-1 deficient mice display an elevated expression of inflammatory genes and increased *JNK* and *IkBkinase* activation [14]. A specific immunomodulatory role of Regnase-1 has been identified in alveolar macrophages. Elevated protein levels of Regnase-1 mediate prolonged survival of rats in a model of acute lung injury. Through activation of the *JNK/c-Myc* pathway, macrophages alter their polarization from pro-inflammatory M1-like to the anti-inflammatory M2-like phenotype in this setting [15].



**Figure 1.** Activation of Regnase 1 to 4 by ligands and their intracellular pathways.

Regnase-1 expression is mainly induced by LPS, and the TLR4 mediated activation of the NF- $\kappa$ B pathway. The mode of activation, the receptor, and the intracellular signalling pathway for Regnase-2 is still unknown. The transcription of Regnase-3 is induced by dsRNA and through the TLR3 and the interferon regulatory factor 3 pathway. The expression of Regnase-4 is induced by several TLR ligands and leads downstream to the activation of JNK and NF- $\kappa$ B signal pathways.



**Figure 2.** Schematic representation of the Regnase family members structure.

Regnase-1 to 4 comprise of a Pilt N-terminal-like RNase domain followed by a CCCH-type zinc finger domain (ZF). A helical domain located at the C-terminus (CTD). The N-terminal domain (NTD) harbours a domain similar to ubiquitin-associated protein.

Further, Regnase-1 is expressed in airway epithelial cells, which are essential for the innate immune defence against inhaled pathogens. Regnase-1 coordinates innate and adaptive immune responses against inhaled *Pseudomonas aeruginosa* infections. Deletion of Regnase-1 augments innate defence pathways by sustaining Regnase-1-dependent inflammatory genes. This enhances the secretion of pseudomonas-specific immunoglobulins and T cell accumulation in the lung, culminating in significant resistance against *P. pseudomonas* re-infection in vivo [16]. Likewise, an immune-modulatory role of Regnase-1 has also been described in epidermal keratinocytes, where it mediates decreased skin inflammation. In this context, Regnase-1 limits the Il-36 mediated inflammation in epidermal cells [17].

### Regnase-1 in the adaptive immune system

The modulatory role of Regnase-1 in the adaptive immune response is primarily mediated by T cells. In these cells, Regnase-

1 regulates sets of genes, including *c-Rel*, *Ox40*, and *Interleukin-2 (Il-2)*. Mechanistically, T cell receptor stimulation leads to cleavage of Regnase-1 by Malt1/paracaspase and thereby liberates T cells from Regnase-1 mediated suppression of inflammatory mRNA. The Malt1 protease activity is also critical for controlling the stability of mRNA of T-cell effector genes, thereby regulating their abundance in the cytosol [18].

Roquin also represents an immune-related RNA-binding protein that coordinates the stability of inflammatory mRNA by recognizing stem-loop structures in target mRNA. Roquin mutation in mice is implicated in the development of systemic lupus erythematosus (SLE)-like pathology (reviewed by [2]). Regnase-1 and Roquin are closely intertwined in regulating adaptive immune homeostasis. Defects in both Regnase-1 and Roquin result in a substantial increase in their target mRNAs [12]. Although deficiency of either Regnase-1 or Roquin leads to enhanced T-cell activation, their functional relationship has not been fully elucidated yet, because of lethality in mouse models with a double genetic knockdown. Cui and Takeuchi tackled this question by using a *Regnase-1* conditional allele model, in which mutations of both *Regnase-1* and *Roquin* in T-cells lead to strong lymphocyte activation. Mutation of either of them affected T cell activation to a lesser extent compared to the double mutation [5]. These double mutants suffered from severe autoimmune inflammation and early fibrotic tissue turnover, especially in the heart. This is accompanied by an increased expression of *Interferon  $\gamma$* , while *Il-4* and *Il-17a* expression remain unaltered. Consistently, mutation of both *Regnase-1* and *Roquin* lead to an increase in T helper cells (Th) 1, but not Th2 or Th17 populations, in the spleen compared to the single knockouts. Regnase-1 and Roquin repress the expression of mRNAs encoding for proteins involved in the Th1 differentiation, such as *Furin* (encoding for a proprotein convertase) and *Il12rb1* (encoding for the subunit of the IL-12 receptor). Regnase-1 is also capable of repressing *Roquin* mRNA, which may lead to a cross-regulated synergistic control of T cell activation. Recently, the role of Regnase-1 in adoptive cell therapy in effector T cells was elucidated by using an in vivo CRISPR platform [19]. Regnase-1 deficient CD8<sup>+</sup>T cells display a new therapeutic approach with high efficacy against mouse melanoma and leukaemia. Further, the Basic leucine zipper ATF-like transcription factor (BATF) was discovered as a key target of Regnase-1 in T cells. Engineered Regnase-1 deficient T cells could, therefore, provide a new tool in cancer therapy.

### Regnase-1 in viral infections

Regnase-1, apart from its immunomodulatory role in innate immune cells, exhibits direct effector functions in immune defence by degrading genomic nucleic acids of positive-sense, negative-sense RNA and DNA viruses [20, 21]. Hereby, RNA-binding proteins act as sensors for viral RNA and DNA [22, 23]. Regnase-1 expression can be induced by hepatitis C virus (HCV) infection in Huh7.5 hepatoma cells, and Regnase-3 expression is higher in liver tissue samples from patients with chronic HCV infection compared to those without chronic disease. The knockdown of Regnase-1 increases HCV replication and HCV-mediated expression of inflammatory cytokines. Overexpression

of Regnase-1 leads to a significant reduction in HCV replication and pro-inflammatory cytokine expression.

This effect of Regnase-1 has also been observed in other viral infections, e.g., the Japanese encephalitis virus and dengue virus replication [21]. Further mechanistic analysis revealed that RNA-binding, RNA degradation (RNase), and oligomerization, but not deubiquitinases are required for proper functioning of Regnase-1 in this context. Noteworthy, even positive-sense RNA viruses (namely sindbis virus), encephalitis virus, negative-sense RNA virus (e.g., influenza virus), and DNA viruses (e.g., adenovirus), can be blocked by the RNase activity of Regnase-1 [21].

### Regnase-1 in haematopoietic homeostasis

The role of Regnase-1 in haematopoietic homeostasis involves modulation of *Gata2* and *Tal1* mRNA levels in haematopoietic stem- and progenitor cells [24]. Dysfunction of Regnase-1 is associated with the accumulation of immature haematopoietic stem and progenitor cells, and therefore Regnase-1 may as well play a role as a suppressor of abnormal haematopoiesis, potentially leading to leukaemia.

### Intracellular regulatory mechanisms in Regnase-1 biology

Mechanistically the IKK complex phosphorylates not only I $\kappa$ B  $\alpha$ , thereby activating transcription, but also Regnase-1, and thus releases the 'brake' on *Il-6* mRNA expression [25]. Furthermore, the IKK complex controls the phosphorylation of Regnase-1 that undergoes ubiquitination and degradation in the proteasome. Phosphorylated Regnase-1 is released from the endoplasmic reticulum into the cytosol, thereby losing its mRNA degrading function, which leads to expression of *Il-17* associated genes [26]. In the respective study, two knock-in mice were generated with blocked IKK phosphorylation sites in Regnase-1 as well as with deletion of the C-terminal portion of Regnase-1, resulting in a lack of phosphorylation of the RNase. The phosphorylation of Regnase-1 and its release from the endoplasmic reticulum is sufficient to suppress the function of Regnase-1. Mechanistically, Regnase-1 mediated degradation of mRNA seems to be dependent on the IKK complex [27].

Binding of various ubiquitin molecules to the same target protein is called ubiquitination. It represents a vital signal for degradation of respective protein in the proteasome. In addition to its post-transcriptional effects, Regnase-1 exhibits also post-translational potential through deubiquitination. Regnase-1 also promotes the removal of ubiquitin marker proteins from TNF receptor-associated factors (TRAF2, TRAF3, and TRAF6) and thereby interacts with JNK and NF- $\kappa$ B signalling pathways. It is still controversial if deubiquitination is performed by Regnase-1 itself or it merely acts as a scaffold for other deubiquitinases such as USP10 [28]. Nevertheless, the interplay of cytokine production and degradation results in fatal inflammatory responses in Regnase-1 knockout mice [14].

### Regnase-2

The expression and function of Regnase-2 across tissues is incompletely understood. Regnase-2 is expressed in the brain, thymus, and testis [29]. In a gene expression analysis, mRNA levels of *Regnase-2* are highest in the human brain, and the neuroblastoma-derived cell line SH-SY5Y [30]. On structural analysis, Regnase-2 carries the conserved and membrane-associated as-like GTPase domain at the C-terminus, suggesting its potential involvement in signal transduction [29]. Notably, Regnase-2 is not inducible by LPS and IFN $\gamma$  stimulation in Raw264.7 cells, which is in contrast to Regnase-1 or Regnase-3, representing an unusual feature of this RNA-binding protein family. The biological function of Regnase-2 is depended on an intact NYN/PIN (PiLT N-terminal) RNase domain for substrate recognition [30]. Regnase-2 binds endogenous *Il-6* mRNA and regulates its turnover upon stimulation with *Il-1 $\beta$* . Furthermore, Regnase-2 interacts with other mRNAs, namely the *immediate early response 3 gene (IER3)*, possibly protecting from TNF $\alpha$  induced apoptosis. In summary, the current literature paints an incomplete picture of gene expression, cellular distribution, and function of Regnase-2. Data on the characterization of this RNA-binding protein in the mice models are missing entirely. Thus, further research with global and conditional deficiency models in mammals are needed to understand the nature of Regnase-2.

### Regnase-3

To date, only sparse data give insight into the modulatory role of Regnase-3 in endothelial and myeloid cell lines. In studies using human umbilical vein endothelial cells (HUVEC), Regnase-3 inhibits the endothelial inflammatory response [31]. The overexpression of Regnase-3 significantly attenuates TNF $\alpha$  induced expression of chemokines and adhesive molecules, and reduces monocyte adherence to HUVECs. In the first publication of the myeloid Regnase-3 of Liang et al. in 2008, LPS stimulated macrophages upregulated Regnase-1 and Regnase-3, which is in line with the upregulation of macrophage expression patterns by inflammatory signals such as TNF $\alpha$ , *MCP-1*, *Il-1 $\beta$*  and *Il-6* [9].

After a decade of this first analysis of the expression pattern of myeloid Regnase-3, the first characterization of the function of the Regnase-3 in mouse models has been published using global and conditional Cre-induced knockouts. Global Regnase-3 deficiency in mice promotes systemic increased interferon (IFN) signalling and suppression of germinal centre formation. Nevertheless, deficient animals did not display manifestations towards autoimmune disease. Sequencing analysis revealed 12 significantly up-regulated genes that are part of the interferon response genes. This phenotype is observed in the absence of Regnase-3, as well as in mice with conditional Regnase-3 deficiency in myeloid cells. The abundance of Regnase-3 is increased explicitly in macrophages upon TLR3 activation and is regulated by IRF3/IRF7 signalling (see Figure 2). This stays in contrast to Regnase-1, which is highly expressed in the lymphoid cell line and interacts via NF- $\kappa$ B [32].

Regnase-1 and Regnase-3 share a high homology (97%) for the PIN domain and zinc finger domain that are thought to

act as RNA sensors. In vitro reporter experiments confirmed that Regnase-1 UTR is a target for Regnase-3 in macrophages. By now, it remains unclear which other RNAs are targeted by Regnase-3 and whether Regnase-3 degrades intracellular mRNAs or incoming RNA from cell debris or viruses.

Nevertheless, Regnase-3 is a new potent player in immune homeostasis that warrants further studies on the mode of action. This first in mouse study supports observations from genome-wide associations identifying loci polymorphisms in Regnase-3 that are associated with human inflammatory diseases, such as psoriasis [33].

## Regnase-4

In the field of neuroinflammatory disorders of the central nervous system (CNS), a gene-ontology pathway analysis in white matter alterations (leukoaraiosis) revealed significant inflammatory changes to be associated with Regnase-4 gene- and mRNA expression. This could implicate potential molecular mechanisms in cerebro-inflammatory diseases [34]. When tested in an autoimmune encephalitis animal model, Regnase-4 seems to play a role in T cell effector function by inhibiting the synthesis of cytokines such as *Il-2*, *Il-6*, *Il-10*, *TNF $\alpha$* , and *Il-17a* [35].

Besides the neurological research, Liang et al. conducted a genome-wide survey and expression profiling of CCHC Zinc finger families that revealed high expression of Regnase-4 in organs with abundance of macrophages such as thymus, spleen, lung, intestine and adipose tissue [9]. This distribution pattern is supported by expression analysis of Regnase-4 being enriched in the spleen, lung, and lymph nodes and, to a lesser extent, in the heart, liver, intestine, thymus, and kidney [36]. Their experiments showed that Regnase-4 negatively regulates TLR signalling and macrophage activation. In a model of acute lung injury, Regnase-4 attenuated the inflammatory response by reducing mRNA stability of pro-inflammatory genes [37]. Regnase-4 levels are decreased in airway immune cells in LPS-induced inflammation. The overexpression of the RNA-binding protein inhibits the expression of inflammatory genes, e.g., *NF-kB subunit RelA* (p65) and cytokines. In line with these results, Regnase-4 reduces the mRNA stability of cytokines (*Il-6*, *Il-1 $\beta$* , and *TNF $\alpha$* ), *NF-kB p65*, and *c-fos*.

In macrophages, the expression of Regnase-4 is induced by TLR ligands through JNK and NF- $\kappa$ B signal pathways. The overexpression of Regnase-4 inhibits TLR2 and TLR4 activation-induced JNK, ERK and NF- $\kappa$ B signalling as well as macrophage inflammation. Interestingly, similar to Regnase-1, Regnase-4 decreases the global cellular protein ubiquitination in vitro. Thereby, Regnase-4 could act as a novel negative feedback regulator of TLR signalling and macrophage activation in inflammation.

Furthermore, Regnase-4 interacts with Regnase-1 by forming a complex which regulates mRNA degradation [38]. Regnase-4 is identified as an interaction partner of Regnase-1 by mass-spec analysis and co-immunoprecipitation. Immunofluorescence staining showed that Regnase-4 co-localized with Regnase-1 in the processing bodies, which play a fundamental role in mRNA decay. However, the localization of these RNA-binding proteins

is still controversial and further characterization is necessary to clarify the cellular localization and interaction between Regnase-1 and Regnase-4 [12,35,39,40]. In vitro, both Regnase family members can degrade *Il-6* mRNA, with an enhanced effect when both RBPs are present. Similarly to Regnase-1, the Regnase-4 participates in the 3' UTR-dependent regulation of the turnover of *Il-6*, *TNF $\alpha$* , and *immediate early response 3 gene (IER3)* in an NYN/PIN-like domain-dependent mechanism [41]. In summary, deciphering the immune-modulatory role of Regnase-4 has only scratched the surface. The purpose of Regnase-4 in multiple inflammatory conditions and models remains to be elucidated by further experiments, which could lay the foundation for target-specific modulation of inflammatory diseases.

## Role of Regnase family members in inflammatory diseases

The purpose of the Regnase family members in immune homeostasis is evident for both adaptive and innate immune responses. Uncontrolled inflammation in autoimmune-related diseases such as psoriasis or Crohn's disease are characterized by the breakdown of self-tolerance towards antigens, and the imbalance between activation and repression of immune cells. There are nowadays established treatment regimens harnessing antibodies as immune modifying agents, such as adalimumab or infliximab. Regarding intestinal inflammation, mutation of Regnase-1 is associated with the pathogenesis of ulcerative colitis [42,43]. Whole-exome genome sequencing data from human colon tissue identified mutations in Regnase-1 and genes related to IL-17 signalling in the inflamed epithelium. Regnase-1 deficiency in intestinal epithelial cells does not increase inflammation at steady state, but colitis induced by dextran sulphate sodium administration is blunted in the knockout mice [44]. Even iron homeostasis in duodenal enterocytes is affected by this RNA-binding protein [45]. The research group of Osamu Takeuchi identified a destabilizing role of Regnase-1 for transferrin receptor 1 mRNA, and that lack of duodenal Regnase-1 leads to iron deficiency anaemia in vivo.

In cardiovascular diseases, inflammation plays a significant role not only in inflammatory conditions like myocarditis but also in atherosclerosis and even more so in cardiac remodelling after myocardial infarction. Here the inflammatory processes are a two-edged sword, on the one hand being essential for, e.g., clearing of dead cell debris, but on the other hand, through overshooting inflammation, enhancing ischaemia-induced tissue damage. Novel therapeutic approaches concerning such inflammatory responses are already been tested. The CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study) trial invested the interleukin-1 $\beta$  targeting monoclonal antibody, which in the setting of human atherosclerosis reduced the rate of cardiovascular events such as myocardial infarction [46]. Other studies are ongoing, that test the effect of anti-interleukin-6 receptor antibodies in the context of atherosclerosis [47]. The ability of Regnase-1 to interact and modulate the amount of pro-inflammatory mRNA such as *Il-6* and *Il-12b* could thereby lead to new treatment approaches. In viral-induced diseases, overexpression of Regnase-1 suppresses the



replication of the hepatitis c virus, Japanese encephalitis virus, and dengue virus infection. Enhancing the function of Regnase-1 might, therefore, represent a new therapeutic strategy.

## Conclusion

The Regnase protein family has been identified as a new player in mammalian immune homeostasis. However, a significant gap of knowledge exists regarding their precise functions and modes of molecular actions, especially regarding Regnases-2, 3, and 4. Further research in animals and in vitro experiments is needed to validate their targeted mechanism of mRNA degradation and to address their interacting partners. Elucidating the inflammatory RNA modifying potential of Regnase family members could lay the foundation for modulating immune cell functions and shaping immune-related pathways.

## Disclosure of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest; Friedrich-Baur-Stiftung; DZHK Standortprojekt [81Z0600204]; DZHK Shared Expertise [81X2600256]; DFG [project number MA 2186/14-1].

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