**Title: Post-transcriptional regulation of immunological responses by Regnase-1-related RNases**

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**Abstract**

Regulation of messenger RNA (mRNA) decay plays a crucial role in the control of gene expression. Canonical mRNA decay pathways are initiated by deadenylation and decapping, and are followed by exonucleolytic degradation. However, recent studies revealed that endoribonucleolytic cleavage also mediates mRNA decay, and both exoribonucleolytic and endoribonucleolytic decay pathways are important for the regulation of immune responses. Regnase-1 functions as an endoribonuclease to control immunity by damping mRNAs. Particularly, Regnase-1 controls cytokines and other inflammatory mediators by recognizing their mRNAs via stem–loop structures present in the 3′ untranslated regions. Regnase-1 was found to be critical for human inflammatory diseases such as ulcerative colitis and idiopathic pulmonary fibrosis. Furthermore, a set of Regnase-1-related RNases contribute to immune regulation as well as antiviral host defense. In this review, we provide an overview of recent findings as to immune-related RNA-binding proteins (RBPs) with an emphasis on stem–loop-mediated mRNA decay via Regnase-1 and related RNases and discuss how the function of these RBPs is regulated and contributes to inflammatory disorders.

**Introduction**

Post-transcriptional regulation is crucial for fine-tuning gene expression and consequently the regulation of protein production. RNA binding proteins (RBPs) have a key role in the RNA life-cycle from synthesis to degradation, and dictate messenger RNA (mRNA) turnover. The ribonucleoprotein (RNP) complex acts as a functional unit and determine the RNA fate. RNPs continuously undergo remodeling depending on cellular conditions and influence the rate of mRNA decay. mRNA decay mechanisms are known to be involved in diverse biological processes, including cell survival, differentiation, stress responses and inflammation in immune cells. mRNA degradation is mediated by two distinct mechanisms: exoribonucleolytic and endoribonucleolytic pathways. The exoribonucleolytic mRNA decay is initiated by deadenylation and decapping, followed by exonucleolytic degradation. In contrast, endoribonucleases such as Regnase-1 directly cleave and digest specific sets of mRNAs.

mRNAs harbor *cis*-acting elements within 3′ untranslated regions (UTRs) that are encoded in the genome and are closely involved in mRNA stability. In particular, the mRNAs encoding inflammatory cytokines, such as *Tnf* and *Il6*, are rapidly degraded due to the presence of destabilizing sequences (1). Adenine and uridine-rich elements (ARE) are a well-studied *cis*-element (2), which is recognized by ARE-binding proteins (ARE-BPs) such as tristetraprolin, ZFP36L1 and ZFP36L2. ARE-BPs bind to ARE sequences (e.g. AUUUA) and regulate mRNA stability by either inducing or inhibiting deadenylation. A stem–loop structure is another *cis*-acting element where the specificity of RBP recognition is determined by both the RNA sequence and secondary structures. Roquin is a stem–loop binding protein that destabilizes mRNAs through deadenylation, decapping or recruitment of micro-RNAs (miRNAs) (Fig. 1b) (3).

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Dysregulation of these RBPs causes deleterious outcomes, such as autoimmunity. On the other hand, the stem–loop structure serves as the *cis*-element for recognition by Regnase-1, an endoribonuclease critical for the maintenance of immune homeostasis. In this review, we will discuss the post-transcriptional regulatory mechanisms of immune responses by particularly focusing on Regnase-1 and its related ribonucleases.

## Roles of Regnase-1-related endoribonucleases in immune regulation

### *Regnase-1, a central regulator of immune cell activation*

Regnase-1 (also known as Zc3h12a) harbors a PIN-like domain (that contains the RNase catalytic center) and a CCCH-type zinc-finger domain and acts as an endoribonuclease. This protein is highly expressed in mature immune cells, including lymphocytes and macrophages. The roles of Regnase-1 in immune regulation have been demonstrated by the analysis of *Regnase-1*-deficient mice, which spontaneously develop autoimmune diseases characterized by a massive infiltration of lymphocytes to various organs and also by the production of autoantibodies (4).

Macrophages are one of the key players that initiate and promote inflammatory responses. Upon engagement of Toll-like receptors (TLRs), *Regnase-1*-deficient macrophages show increased expression of inflammatory cytokine and chemokine genes, such as *Il6*, *Il12p40* and *Il1b*. Nevertheless, genetic deletion of *Il6* or *Il12p40* does not rescue the inflammatory phenotype observed in *Regnase-1*-deficient mice. Rather, T cell-specific deletion of Regnase-1 almost phenocopied the sign of systemic inflammation observed in *Regnase-1*-deficient mice, though there is a delay in the onset of the inflammatory disease (5). *Regnase-1*-deficient T cells display spontaneous differentiation into effector cells and expansion through T cell antigen-receptor (TCR) stimulation triggered by recognition of either endogenous or exogenous antigens. Antibiotic treatment ameliorates the inflammatory condition in *Regnase-1*-deficient mice (6), which suggests the involvement of the microbiota in the intestine or other mucosal tissues.

Regnase-1 regulates a variety of mRNAs encoding *Il2*, *Icos*, *Ox40* and *Rel* in T cells. *Rel* encodes c-Rel protein, which belongs to the NF-κB family, is critical for type 1 immunity and is associated with autoimmune diseases (7). Genetic deletion of Rel ameliorated T cell activation and improved autoimmunity

in *Regnase-1*-deficient mice (5). Regnase-1 expressed in B cells was also found to be important for maintaining immune homeostasis (8). B cell-specific deletion of *Regnase-1* augmented the germinal center reaction, class-switching and differentiation into plasma cells, culminating in increased production of antigen-specific antibodies. Recent studies revealed that Regnase-1 functions even in nonhematopoietic cells. Mice lacking Regnase-1 in airway epithelial cells are resistant to *Pseudomonas aeruginosa* infection by enhancing innate responses and producing *P. aeruginosa*-specific IgA (9).

In addition to the control of inflammatory responses, Regnase-1 regulates iron homeostasis. *Regnase-1*-deficient mice show severe anemia accompanied by decreased iron storage (10,11). The anemia observed in these mice can be rescued by iron supplementation or in combination with vitamin B12. In intestinal epithelial cells, Regnase-1 modulates iron uptake by degrading *Phd3* mRNA (11). Further, it has been shown that Regnase-1 is implicated with the pathogenesis of many other diseases, ranging from cancer, allergy, fibrosis and ischemia to adipogenesis (12-18).

*The Regnase family and other Regnase-1-related RNases*

In addition to Regnase-1, there are three other Regnase-family proteins characterized by the conservation of the RNase and the adjacent zinc-finger domains. Accordingly, these Regnase proteins are shown to induce RNA degradation through their RNase activity targeting inflammatory genes, although further studies are required to verify if they are endoribonucleases (19-21). Therefore, the other Regnase family members are also thought to be critical for the control of inflammation, although there are only limited numbers of studies on their roles *in vivo*.

Regnase-3 (also known as Zc3h12c) is highly expressed in myeloid lineage cells, such as macrophages and dendritic cells. *Regnase-3*-deficient mice are reported to show slowly progressive inflammation and develop lymphadenopathy (21). Systemic inflammation observed in *Regnase-3*-deficient mice is characterized by activation of the interferon γ (IFN-γ) signaling pathway but mice lacking *Regnase-3* specifically in T or B lymphocytes do not show any phenotype. In macrophages stimulated with lipopolysaccharide (LPS), *Regnase-3* deficiency does not affect inflammatory responses such as *Il6* expression, probably because of compensatory Regnase-1 upregulation in the absence of Regnase-3. Regnase-3 is localized to endosomes or phagosomes, which suggests its involvement in endocytosis or phagocytosis pathways in macrophages (21). Nevertheless, it remains unclear how Regnase-3 regulates immune responses.

Regnase-4 (also known as Zc3h12d or TFL) is highly expressed in lymphocytes, with much lower expression in macrophages, at least at steady state. *Regnase-4* deficiency does not cause spontaneous immune cell activation. However, *Regnase-4*-deficient mice show high susceptibility to experimental autoimmune encephalomyelitis (EAE) (19), which suggests that Regnase-4 plays an important role to suppress T cell-mediated disease development. Regnase-4 localizes to P bodies where untranslated mRNAs undergo degradation (22). An *in vitro* study reported that Regnase-1 interacted with Regnase-4 and both proteins can suppress *Il6* mRNA following overexpression (22). However, TLR-induced expression of *Il6* was not increased in *Regnase-4*-deficient cells (unpublished observation). Thus it is likely that Regnase-4

has an mRNA decay mechanism distinct from Regnase-1 in terms of cofactors and target specificity. In contrast to Regnase-3 and -4, there is little information on the physiological roles of Regnase-2 in immune responses.

In addition to Regnase family proteins, the Regnase-1-related RNase domain is present in another class of proteins that includes N4BP1, KHNYN and NYNRIN. These proteins harbor two K-homology (KH) domains in the N-terminal region besides the C-terminal RNase domain.

Among them, N4BP1 is highly expressed in various organs including lymphoid tissues, skin and brain, and is inducible in response to type I interferons. The role of N4BP1 was first described in its function in antiviral immunity (23). N4BP1 restricts human immunodeficiency virus 1 (HIV-1) replication in T cells and macrophages. Mechanistically, N4BP1 directly degrades HIV-1 viral RNA through its nuclease activity, although the sequence specificity remains unclear. Additionally, recent work revealed that N4BP1 negatively regulates host innate immune reactions. *N4bp1*-deficient macrophages produced more inflammatory cytokines upon activation of MyD88-dependent TLR signaling pathways (24,25). *N4BP1*-deficient mice show mild inflammation as they age, characterized by lymphocyte infiltration in the lung. Psoriasis induced by imiquimod (TLR7 ligand) was exacerbated in *N4BP1*-deficient mice (26). N4BP1 was found to be a substrate of caspase-8 in macrophages stimulated via TLR3 or TLR4 (24,25). Whereas a study demonstrated that N4BP1 functions to suppress NF-κB activation through inhibition of NEMO oligomerization independent of RNase activity (25), another study claims that N4BP1 destabilizes mature mRNAs coding *JunB* and *FosB* in keratinocytes (26). Further studies are necessary to understand how N4BP1 controls immune cell activation.

KHNYN was also reported as an antiviral protein that degrades viral RNA (27,28). KHNYN interacts with ZAP (also known as zinc-finger CCCH-type containing antiviral 1, ZC3HAV1) and acts as a sensor for the recognition of target viral sequences. ZAP directly binds to CpG dinucleotides, and CG dinucleotide-rich viruses are targeted by ZAP. Nevertheless, the sequence or virus specificity of KHNYN-mediated viral RNA decay needs further investigation.

**Molecular mechanisms for mRNA decay by Regnase-1-related RNases**

Regnase-1 preferentially recognizes stem–loop structures with a pyrimidine–purine–pyrimidine loop sequence (29). Regnase-1 localizes in close proximity to the endoplasmic reticulum (ER). Regnase-1 associates with ribosomes and degrades actively translated mRNAs, in particular following the pioneer rounds of translation. Regnase-1-mediated mRNA decay requires the unwinding of target stem–loop structures by UPF1, an RNA helicase that is also critical for non-sense-mediated mRNA decay (NMD) (Fig. 1a) (29). The phosphorylation of UPF1 by SMG1 kinase that is triggered following translation induces association between Regnase-1 and UPF1. In accordance with this finding, structural analysis of Regnase-3, whose RNase and zinc-finger domains are highly similar to those of Regnase-1, revealed that the RNase and zinc finger were co-crystalized with a linear AU-rich RNA heptamer (30). Thus, it is suggested that RNA cleavage happens to single-strand RNA after unwinding of the target stem–loop structure.

The stem–loop structure is commonly recognized by another RBP, Roquin, via the ROQ domain (31,32). In contrast to Regnase-1, Roquin proteins recruit the CCR4–NOT (carbon catabolite repression 4 – negative on TATA-less) complex, or alternatively associates with an RNA helicase Rck (also known as DDX6) and the enhancer of decapping 4 (Edc4) (Fig. 1b), thereby inducing exoribonucleolytic mRNA degradation via deadenylation or decapping (33,34). Roquin localizes to stress-granules and P bodies and degrades translationally inactive mRNAs. Since Regnase-1 and Roquin share target stem–loop structures, the presence of two distinct regulatory mechanisms enables tight regulation of immune-related mRNAs and immune reactions (29,35). Also, Arid5a directly binds to the stem–loop structures and antagonizes Regnase-1-mediated mRNA destabilization (36). Indeed, Arid5a is required for the promotion of IL-6 production in macrophages and the development of EAE (36).

As demonstrated, mRNAs that are related to immune responses are generally regulated by more than one *cis*-regulatory element, such as AREs and stem–loop structures, which are recognized by multiple RBPs. For example, *Il6* and *Tnf* transcripts are regulated strictly by Roquin and Regnase-1 as well as ARE-binding proteins. Thus, the complex interactions between RBPs and RNA build up a higher-order dependencies in the post-transcriptional regulatory network.

**Post-translational regulation of Regnase-1 and Regnase-1-related RNases**

For timely induction and resolution of inflammation, the expression of immune-related RBPs is tightly regulated during the course of immune cell activation. Regnase-1 is known to undergo post-translational modifications such as phosphorylation and protein cleavage depending on external stimuli (Fig. 2). First, proteasomal degradation of Regnase-1 is induced by the activation of TLR/IL-1R signaling pathways (Fig. 2a) (37). This is mediated by IκB kinase (IKK)-induced phosphorylation of Regnase-1 at the DSGXXS motif that is conserved among species. Mutations at these two serine residues result in massive accumulation of Regnase-1 protein (38). Regnase-1 degradation depends on activation of MyD88-dependent signaling pathways, which are triggered by TLRs except TLR3, and a set of cytokine receptors for IL-1β, IL-18, IL-33, IL-17 and IL-25 (5,16,37,38). Despite the engagement of the IKK complex, TNF does not induce any change of Regnase-1 protein expression (37). Knock-in mice harboring double mutations at S435 and S439 present in the DSGXXS motif display resistance to the propagation of inflammation (38). Regnase-3 protein also undergoes degradation in response to LPS (TLR4 ligand) through the proteasomal machinery (21).

Another mode of phosphorylation modulates Regnase-1 protein expression. Activation of the IL-17R signaling pathway induces phosphorylation at S513, which is mediated by the TBK1/IKK-i/ε complex (38). Act1 augments this phosphorylation through its interaction via the C-terminal region of Regnase-1. It was reported that substitution of S513 for Regnase-1 with alanine or deletion of the C-terminal region containing this residue results in accumulation of the unphosphorylated form, which leads to downregulated cytokine expression. The S513 phosphorylation was reported to promote translocation of Regnase-1 from the endoplasmic reticulum to the cytosol (38).

Another type of Regnase-1 post-translational modification is its cleavage via the protease function of MALT1 upon engagement of the TCR or BCR (Fig. 2b) (5,8). MALT1 plays a central role in the antigen-receptor signaling as a component of the CARMA–BCL10–MALT1 (CBM) complex. MALT1 acts as a scaffold molecule and as a protease for the activation of downstream signaling molecules. As for the protease function, MALT1 is an arginine-specific protease that cleaves its substrates after arginine residues (39,40). Regnase-1 is cleaved at the position of R111 located prior to the RNase domains (5). The large fragment of Regnase-1 generated by MALT1-mediated cleavage, which still harbors an intact RNase domain, loses its nuclease activity, because of disruption of the binding region for UPF1. MALT1 also cleaves Roquin and N4BP1 upon TCR stimulation (23,41).

Thus, the control of RBPs that are critical for immune cell activation is closely related to immune signaling pathways. On the other hand, Regnase-1 expression is also regulated at the post-transcriptional level. Regnase-1 destabilizes its own transcript through the binding to its target stem–loop structures (37). This self-regulatory system acts as a negative feedback to prevent unintended mRNA degradation. Given the importance of Regnase-1 in the control of inflammatory responses, these mechanisms would be therapeutic targets for autoimmune diseases and chronic inflammatory diseases.

**Implications for inflammatory diseases**

Regnase-1 is involved in the pathogenesis of diverse diseases in humans. Inflammatory bowel diseases, such as ulcerative colitis (UC), are characterized by chronic inflammation where the damage and regeneration of mucosal tissue are repeated. The exome sequencing analysis of crypts from UC patients revealed that, comparing with normal crypts, *REGNASE-1* mutations were positively selected in UC-derived crypts (42-44). Interestingly, these mutations turned out to be enriched in the DSGXXS motif that was identified as the IKK-mediated phosphorylation site for proteasomal degradation. The *REGNASE-1* mutants were resistant against IL-17A-mediated degradation, and thereby they are gain-of-function mutations that suppress inflammation in the crypts. In addition, loss-of function mutations of *NFKBIZ* were significantly enriched in the UC epithelium (42,43). Given that NFKBIZ plays an important role in responses against IL-17A stimulation, mutations that inhibit IL-17A-induced responses are increased in UC patients to allow adaptation of colon epithelial cells under inflammatory conditions.

However, it should be noted that mutations of both *REGNASE-1* and *NFKBIZ* are under-represented in colitis-associated cancer samples (42). This finding indicates that colorectal carcinogenesis may be generated from crypts that fail to adapt to intestinal inflammation. Another GWAS study with psoriasis patients identified 15 new susceptibility loci, including *ZC3H12C* (Regnase-3) (45). The *ZC3H12C* mutation was found upstream of *ZC3H12C* but it leads in the direction of upregulation. Like the *REGNASE-1* mutation in UC crypts, upregulation of ZC3H12C may be explained by mechanisms for adaptation to skin inflammation. Nevertheless, the role of ZC3H12C is largely unknown, and further investigation will be necessary.

Idiopathic pulmonary fibrosis (IPF) is another therapeutic-target disease that may be controlled by Regnase-1 expression. Type 2 immunity, such as that mediated by IL-4 and IL-13, has been shown to contribute to

IPF pathogenesis but the clinical trials that evaluated the efficacy of therapies that block type 2-related cytokines in IPF patients showed negative outcomes (46,47). Nevertheless, the emerging role of type 2 innate lymphoid cells (ILC2s) in IPF pathogenesis brought type 2-related cytokines into the limelight again (48,49). We demonstrated that ILC2s play a key role in the fibrotic process under the control of Regnase-1 (50). Our finding provides a potential therapeutic target for IPF development. Yet, further investigation will be necessary to elucidate how the expression and function of Regnase-1 in lung-resident ILC2s are regulated in fibrotic lungs.

## Conclusion

As discussed, Regnase-1 and its related RNases play essential roles in the control of inflammatory responses in a variety of immune cells. The expression, function or both of immune-related RBPs are altered by immune signaling pathways, and dysregulation of these RBPs leads to disruption of immune homeostasis. On the other hand, RNP assembly undergoes remodeling throughout the RNA life-cycle. Accordingly, RNA secondary structures are thought to be altered, but the regulatory mechanisms for RNA structures are largely unknown. The helicase UPF1 is key to change stem–loop structures for RNA degradation by Regnase-1. However, it remains unclear if other Regnase proteins also requires helicase activity. Besides, the RNA-binding specificity of individual RBPs may be more complex. RNA modifications can affect the sequence specificity of RBPs. To overcome this issue, a comprehensive understanding of the complex RNP assembly is necessary.

Protein-centric technological advances have enabled us to obtain a significant insight into the molecular basis of protein–RNA interaction occurring in living cells. In future research, integration of cross-linking and immunoprecipitation (CLIP) sequencing data with RNA-centric approaches, such as RNA interactome capture and RNA proximity protein labelling, will help us to understand how RNA molecules organize RNP assembly. This approach will not only allow us to gain a full picture of RNP organization but also may give us a valuable opportunity to decipher novel therapeutic targets for inflammatory diseases.

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**[Au: I added page-numbers, etc. for References #16, 18, 26, 27, 46 and 49; please check these.]**

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**Fig. 1.** mRNA fate is determined by RNP complexes. During or soon after transcription, RBPs bind to RNA, which forms RNP complexes. Nuclear RNPs govern early steps of RNA processing, including mRNA splicing, modification and nuclear export. In the cytoplasm, mRNAs associate with the translational machinery and undergo translation. Constituents of RNP complexes alter, depending on the cellular context or external signals, which guides mRNAs to their subcellular localization. (a–c) Representative mRNA decay pathways: (a) and (b) stem–loop-mediated decay and (c) ARE-mediated decay. (a) Regnase-1 targets translationally active mRNAs and recognizes stem–loop structures in their 3′UTR. Upon termination of translation, the helicase UPF1 unwinds the stem–loop structure and subsequently mRNA is cleaved by Regnase-1 endonucleolytic cleavage. The resulting fragments undergo further degradation by 5′–3′ and 3′–5′ exonucleolytic decay pathways. (b) Roquin also recognizes stem–loop structures in the 3′UTR. In contrast to Regnase-1, Roquin targets translationally inactive mRNAs that localize to stress granules or P bodies. Roquin recruits the deadenylase CCR4–NOT complex to remove the poly(A) tail. Alternatively, Roquin enhances the decapping enzymes DCP1 and DCP2 through the recruitment of Rck and Edc4. (c) ARE-BPs, such as tristetraprolin and its paralogs ZFP36L1 and ZFP36L2, bind to AREs. ARE-BPs also induce deadenylation or decapping in a similar manner to Roquin. Subsequently, the mRNA body undergoes further degradation via the 5′–3′ and/or 3′–5′ exonucleolytic decay pathways.

**Fig. 2**. Post-translational regulation of Regnase-1

(a) Triggering of MyD88-dependent signaling pathways by the ligation of TLRs except TLR3 or IL-1R induces phosphorylation of Regnase-1 at the positions of Ser 435 and 439 by the IKK complex. Once phosphorylated, Regnase-1 undergoes polyubiquitination, followed by the proteasomal degradation. (b) Upon antigen recognition by T cells, the CBM complex consisting of CARMA1, BCL10, and MALT1 is activated by PKC. MALT1 acts as a protease and directly cleaves a set of RNA decay proteins, including N4BP1 and Roquin as well as Regnase-1.

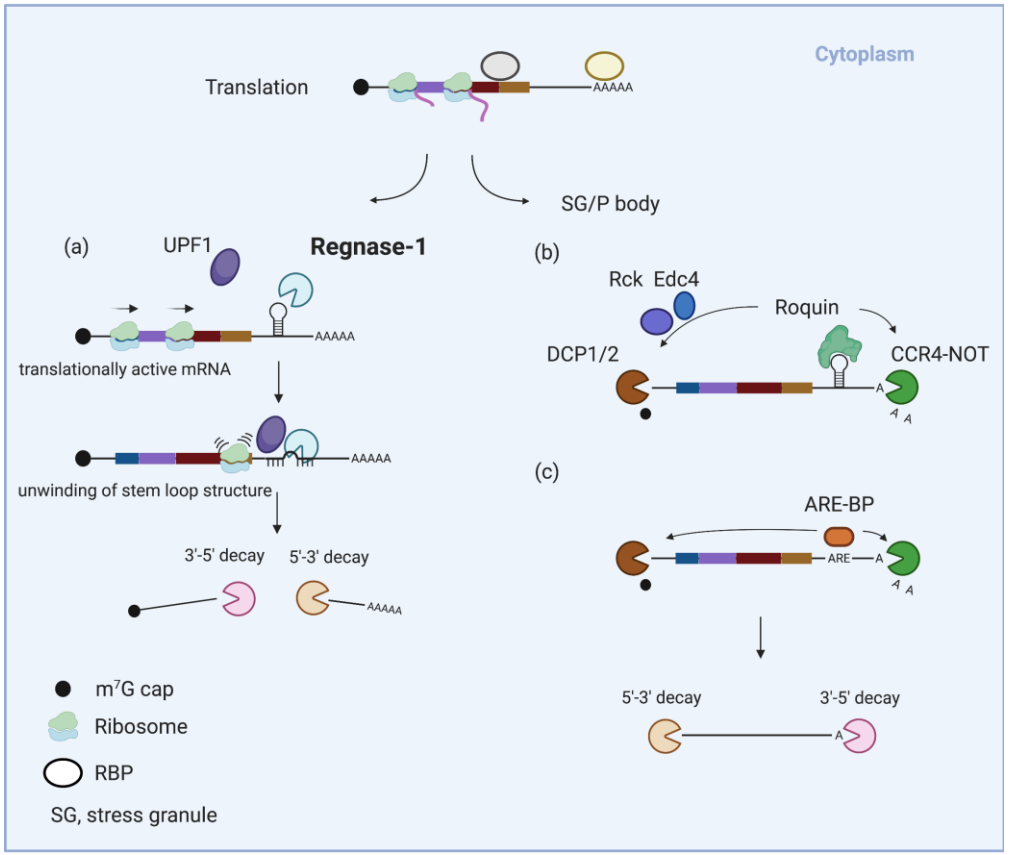


Figure 1 mRNA fate is determined by RNP complexes.
During or soon after transcription, RBPs bind to RNA, which forms RNP complexes. Nuclear RNPs govern early steps of RNA processing, including mRNA splicing, modification, and nuclear export. In the cytoplasm, mRNAs associate with translational machinery and undergo translation. Constituents of RNPs complexes alter, depending on cellular context or external signals, which guides mRNAs to subcellular localization. (a-c) Representative mRNA decay pathways: (a) and (b) stem loop-mediated decay and (c) AU-rich element (ARE)-mediated decay. (a) Regnase-1 targets translationally-active mRNAs and recognizes stem loop structures in their 3'UTR. Upon termination of translation, the helicase UPF1 unwinds the stem loop structure, and subsequently mRNA is cleaved by Regnase-1 endonucleolytic cleavage. The resulting fragments undergo further degradation by 5'-3' and 3'-5' exonucleolytic decay pathways. (b) Roquin also recognizes stem loop structures in 3'UTR. In contrast to Regnase-1, Roquin targets translationally-inactive mRNAs that localizes to stress granules or P bodies. Roquin recruits the deadenylase CCR4-NOT complex to remove poly(A) tail. Alternatively, Roquin enhances the decapping enzymes DCP1 and DCP2 through the recruitment of Rck and Edc4. (c) ARE-binding proteins (ARE-BPs), such as tristetraprolin and its paralogs ZFP36L1 and ZFP36L2, bind to AREs. ARE-BPs also induce deadenylation or decapping in a similar manner to Roquin. Subsequently, the mRNA body undergo further degradation via the 5'-3' and/or 3'-5' exonucleolytic decay pathways.

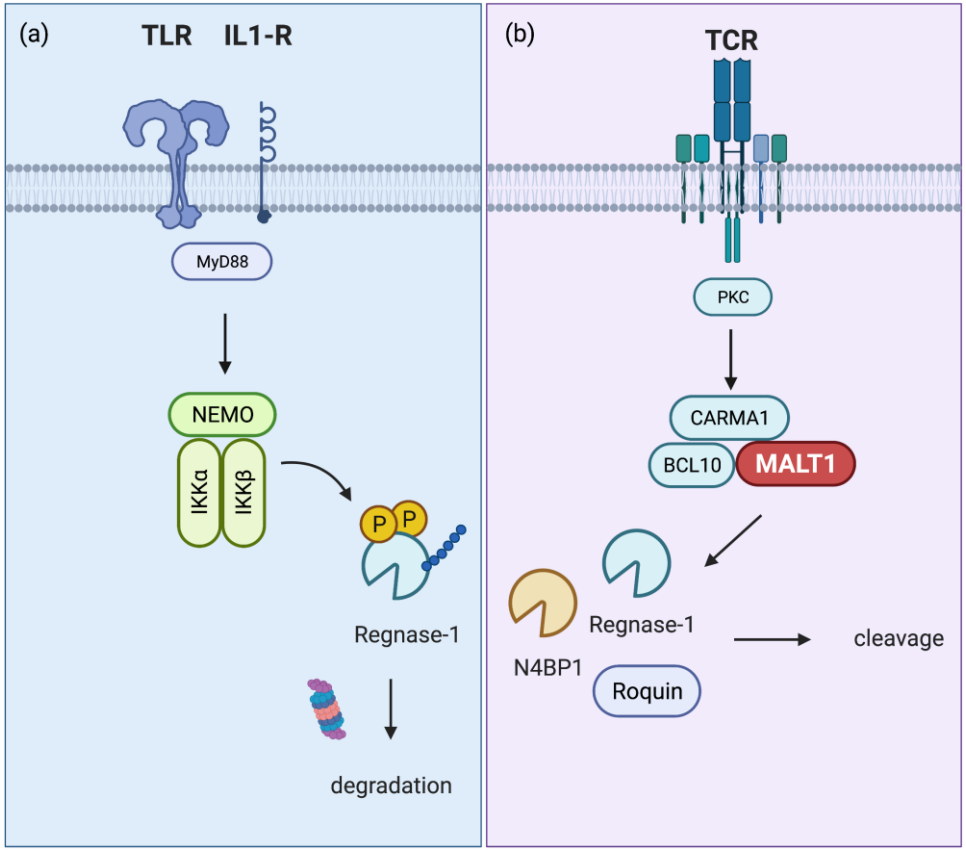


Figure 2 Post-translational regulation of Regnase-1
(a) Upon triggering of MyD88-dependent signaling pathways such as TLRs (except TLR3) and IL1-R, Regnase-1 is phosphorylated at the positions of Ser 435 and 439, which is induced by IKK complex. Once phosphorylated, Regnase-1 protein undergoes ubiquitination, followed by proteasomal degradation. (b) Upon antigen recognition by lymphocytes, such as T cells, the CBM complex consisting of CARMA1, BCL10, and MALT1 is activated by PKC. MALT1 acts as a protease and directly cleaves a set of RNA decay proteins, including N4BP1 and Roquin as well as Regnase-1.