RNA decay machinery safeguards immune cell development and immunological responses

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Highlight Box

- Disruption of some RNA decay pathways provokes inflammatory diseases and tumor progression in humans and mice

- Exonuclease RNA decay pathways mediated by ZFP36L family proteins and the CCR4-NOT complex regulate quiescence, V(D)J recombination, and apoptosis in early differentiation of lymphocytes.

- RNA binding protein ZFP36 family, ROQUIN family, and REGNASE family are non-redundantly required to suppress inappropriate activation of peripheral T cells

- Epigenetic N6-adenosine methylation of mRNA regulates T cell homeostasis and differentiation, and may link transcription with mRNA decay.

Abstract

mRNA decay systems control mRNA abundance by counterbalancing transcription. Several recent studies show that mRNA decay pathways are crucial to conventional T and B cell development in vertebrates, in addition to suppressing autoimmunity and excessive inflammatory responses. Selective mRNA degradation triggered by the CCR4-NOT deadenylase complex appears to be required in lymphocyte development, cell quiescence, V(D)J (variable-diversity-joining) recombination, and prevention of inappropriate apoptosis in mice. Moreover, a recent study suggests that mRNA decay may be involved in preventing human hyperinflammatory disease. These findings imply that mRNA decay pathways in humans and mice do not simply maintain mRNA homeostatic turnover, but can also precisely regulate immune development and immunological responses by selectively targeting mRNAs.

RNA decay machinery is critical for immune development and immunological functions

Cytoplasmic mRNA abundance is a key determinant of protein expression, and regulation of mRNA transcription has been widely studied in mammalian immune cells. In addition to transcriptional regulation, mRNA expression is controlled during several post-transcriptional steps (i.e. capping, mRNA splicing, polyA tail addition, RNA editing). mRNA decay is a post-transcriptional mechanism for reducing mRNA abundance, counterbalancing transcription, and thereby maintaining or altering mRNA expression.

RNA decay is not only necessary for homeostatic turnover and mRNA quality control, but it also actively regulates mRNA abundance in immune cells. For instance, recent studies indicate that mRNA decay is indispensable for passing checkpoints in early differentiation process of B and T cells in mice [1-4]. In addition to development, requirements for RNA decay pathways in suppressing aberrant immune responses have been demonstrated in mice [5-13]. This concept has recently been implicated in onset of human inflammatory diseases [14], in addition to T cell lymphoma [15] by whole exome sequencing analysis to identify causal mutations of these diseases. Notably, a mouse model study suggested that modification of RNA decay could be utilized in **cancer immunotherapy** [16], impelling further research on immune system regulation via RNA decay.

In this review, we describe roles of RNA decay machinery in immune development and immunological responses. Moreover, implications of RNA decay systems for human disease onset and therapy are described. Since a recent review focused on post-transcriptional regulation of CD8 T cell functions [17], here we focus on immunological roles of RNA decay systems that actively regulate mRNA stability.

General mechanisms inducing cytoplasmic mRNA decay

Cytoplasmic mRNA decay processes comprise two classes that differ functionally [18]. The first is to control mRNA quality, thereby preventing production of potentially toxic proteins. **Non-sense mediated mRNA decay** (NMD) occurs during early rounds of translation and rapidly degrades mRNAs that prematurely terminate translation [18-20]. Additionally, **no-go mRNA decay** or **non-stop mRNA decay** act when translation is accidentally blocked or fails to terminate, respectively [21].

Second, mRNA decay systems actively regulate stability of coding mRNAs, thereby dynamically modulating amounts of functional proteins [18, 22]. This mechanism involves the exonuclease and endonuclease decay pathways [18, 22] (Figure 1). Here, we focus mainly on mechanisms of RNA decay machinery that actively regulate mRNA abundance, and consequently affect immunological functions.

The exonuclease pathway is typically initiated by removing polyA tails from mRNAs (Fig. 1). Deadenylation of polyA tails is the rate-limiting step for RNA decay and is catalyzed by three deadenylases, the **CCR4-NOT complex**, **Pan2-Pan3**, and **PARN** [18, 22], all of which are conserved in eukaryotes. Of these, the CCR4-NOT complex is the predominant deadenylase affecting polyA tail lengths of mRNAs in human cell lines [23]. The CCR4-NOT complex is a large protein complex consisting of 9 protein subunits in humans and mice [24]. Deadenylase activity is mediated by the CNOT6/6L and CNOT7/8 subunits [25-29]. CNOT1, the largest subunit of the CCR4-NOT complex, acts as a scaffold to assemble the other subunits, and also recruits RNA-binding proteins (RBPs) [30-32]. Other CNOT subunits (CNOT2, 3, 9,10, and 11) do not possess deadenylase activity, but appear to modulate functions of the intact complex [33-41]. Following deadenylation, the de-capping complex (Dcp1/Dcp2) is recruited and removes an mRNA's 5'-cap, which promotes complete mRNA decay [42]. Finally, exonucleases XRN1 and 2 degrade mRNAs from their 5'-ends [42]. Degradation by exosomes from the 3'-end also occurs after deadenylation [22]. Although in cell lines, CNOT8 was reported to bind to SMG7, a subunit of the NMD complex [43], the physiological role of the CCR4-NOT complex on NMD is unclear.

In contrast, the endonuclease decay pathway begins by cleaving mRNAs internally (Fig. 1). This cleavage generates unprotected 5'- and 3'-ends, thereby enabling exonuclease-dependent degradation of mRNA fragments. These endonucleases recognize primary and secondary sequences of target mRNAs [44].

- In addition to degradation of aberrant mRNAs, NMD maintains an appropriate level of gene expression by regulating physiological mRNAs [45]. For instance, siRNA-mediated depletion of RENT1, a trans-effector of NMD [46], up-regulated 4.9% of transcripts in HeLa cells [45]. Moreover, NMD is involved in embryonic development and tissue-specific cell differentiation [20]. However, no physiological mRNA targets of NMD have been identified in immune responses.

RNA-decay pathways regulating lymphocyte development and immunological responses

B cells

Cellular mechanisms of B cell development have been widely studied in humans and mice [47] (Box 1). Several studies have suggested that the RNA decay exonuclease pathway is triggered by the CCR4-NOT complex in early B cell development in mice [1-3].

Two studies showed that B cell-specific depletion of the CNOT3 subunit blocks the transition from pro-B cells to pre-B cells [1, 2] (Box 1, Fig. 2). Relative to wild-type B cells, *Cnot3* gene deletion in mice caused

up-regulation of *Trp53* mRNA encoding P53 protein and its target genes in B cells [1]. Moreover, deletion of *Trp53* gene in *Cnot3*-deficient mice partially rescued developmental defects of B cells caused by CNOT3 deficiency [1]. Thus, the CCR4-NOT complex was deemed to promote the pro-B cell to pre-B cell transition by degrading *Trp53* mRNA. Another study showed that the CCR4-NOT complex interacts with transcription factor EBF1 [2], which is crucial for establishment of B-cell lineages [47, 48]. Interestingly, interactions between the CCR4-NOT complex and EBF1 both enhance transcription and destabilize mRNAs. Although both studies revealed defects of pro-B cell development, there are some inconsistencies between the two studies. For instance, *Trp53* mRNA reduction was not observed in the latter study. This apparent discrepancy may be ascribed to the Cre deletion mice used in each study. For instance, *mb1*-Cre may have caused genotoxic stress in pro-B cells [49].

Cellular quiescence is needed for **V(D)J (variable-diversity-joining) recombination** because **RAG2 protein** expression is limited in the G0-G1 phases of the cell cycle [50-53]. ZFP36L1 and ZFP36L2 are RBPs that recognize specific sequences in 3'-untranslated regions (UTRs) of vertebrate mRNAs[54]. In mice, lack of both ZFP36L1 and ZFP36L2 in early B cells reduces populations of pre-B cells and later stages of B cells, delays variable-V(D)J recombination, and causes failure at the pre-BCR checkpoint [3]. ZFP36L1 and ZFP36L2 promote quiescence of murine pro-B cells by promoting decay of mRNAs involved in the transition into S phase of the cell cycle [3]. Notably, ZFP36L1 and ZFP36L2 recruit the CCR4-NOT complex, thereby inducing exonuclease-dependent mRNA decay [55, 56]. Consistently, B cell-specific deletion of the CNOT3 subunit of the CCR4-NOT complex in mice also impaired V(D)J recombination in pro-B cells [1, 2]. Additionally, putative target mRNAs of ZFP36L1, such as *Cdkn1a*, are upregulated in *Cnot3*-deficient pro-B cells, indicating functional interaction between the CCR4-NOT complex and ZFP36L1.These findings suggest that RNA decay, involving ZFP36L1, ZFP36L2, and the CCR4-NOT complex, can coordinate cell cycle entry and exit to ensure progression of V(D)J recombination [1-3].

In addition to early B cell development, a recent study showed that B cell-specific deletion of ZFP36L1, but not *ZFP36L2*, causes improper localization and impaired survival of **marginal zone B cells** in murine spleen, most likely by down-regulating *Klf2* and *Irf8* mRNAs [57]. However, a possible role of the CCR4-NOT complex in marginal zone B cell maintenance remains to be demonstrated.

Overall, these data suggest that active RNA decay regulates development of B cells in relatively early stages. ZFP36 family mediate decay of specific target mRNAs (Box 2, Figure 4), most likely by recruiting the CCR4-NOT complex.

T cells

From another angle, several studies have suggested that early T cell development (Box 1) is also controlled by active mRNA decay.

For instance, mice deficient in both *Zfp36l1* and *Zfp36l2* genes in early T cell progenitor cells developed **T cell acute lymphoblastic leukemia (T-All)** [58]. T lymphoblasts in mutant mice expressed cell surface CD8 instead of TCRβ. Moreover, DN thymocytes in mutant mice lacked intracellular TCRβ expression. These results suggest that DN thymocytes in mutant mice aberrantly passed the **β-selection** checkpoint without expression of TCRβ. Consistently, V(D)J recombination was defective in DN thymocytes of *Zfp36l1* and *Zfp36l2*-double deficient mice [59]. Moreover, gene sets involved in **DNA damage-response** signaling and cell cycle progression were up-regulated in DN thymocytes of these mutant mice compared to wild-type mice [59]. These data suggest that although double-stranded DNA breakage during V(D)J recombination can up-regulate a DNA damage-responsive (DDR) gene set that may cause cell death, the ZFP36L1 and ZFP36L2-inducing RNA decay pathway appears to inhibit this DDR pathway to ensure cell survival. In addition, ZFP36L1 and ZFP36L2 can suppress cell proliferation by destabilizing cell cycle-promoting gene expression. This suppression appears crucial to induce cellular quiescence required for V(D)J recombination in DN thymocytes. Thus, ZFP36L and ZFP36L2-mediated gene suppression is essential for appropriate differentiation of DN thymocytes without development of T cell leukemia. In addition to thymocytes, ZFP36L2 also binds to the AU rich element in the 3'-UTR of *Ifng* mRNA in human and murine peripheral T cells [60]. In this case, ZFP36L2 blocked binding of ribosomes to *Ifng* mRNA 3'-UTR, and thereby inhibited its translation.

Our recent study revealed that the CCR4-NOT complex participates in positive selection [4] (Fig. 3, Box 1). Specifically, positive selection of DP thymocytes was impaired by T cell-specific deletion of *Cnot3* in mice, which inhibited generation of mature $CD4^+$ and $CD8^+$ T cells [4]. In thymocytes of *Cnot3*-deficient mice, *Dap2ip* mRNA was up-regulated in the early stage of positive selection ($CD3^{int}$ $CD69^{hi}$ thymocytes). In contrast, *Bbc3* mRNA was up-regulated in the later stage of positive selection ($CD3^{high}$ $CD69^{high}$ thymocytes) in *Cnot3*-deficient thymocytes. Thus, the CCR4-NOT complex inhibited up-regulation of pro-apoptotic *Bbc3* and *Dab2ip* mRNAs in different stages of positive selection (Fig. 3). Failure of positive selection caused by CNOT3 deficiency was counteracted by forced expression of anti-apoptotic BCL-2 in hematopoietic progenitors. Thus, the CCR4-NOT complex ensures DP positive selection by down-regulating pro-apoptotic molecules.

ROQUIN-1 and ROQUIN-2 (ROQUIN family) attach to 3'-UTRs of various mRNAs and recruit the CCR4-NOT complex in mammalian cells [61-63]. By stabilizing *Icos* (inducible T-cell co-stimulator) mRNA, mice with a missense mutation in *Rc3h1* (encoding ROQUIN-1) spontaneously developed **systemic lupus erythematosus**-like autoimmunity with enhanced proliferation of **follicular helper T cells** (Tfh) and impaired negative selection of autoimmune germinal center B cells relative to control wild-type mice [5-8]. Moreover, T-cell specific deletion of both *Rc3h1* and *Rc3h2* (encoding ROQUIN-2) genes in mice caused enhanced helper T cell activation and Tfh cell differentiation with spontaneous inflammation [9, 10]. Thus, ROQUIN family proteins inhibit inappropriate T cell activation and differentiation. The phenotype of point mutant mice differed in part from those of *Rc3h1* and *Rc3h2*-doubly deficient mice. A possible explanation is that another cell population was involved in the phenotype of point mutant mice. Still another possibility may be that mutant ROQUIN-1 protein may form a dysfunctional multiprotein complex, which cannot be substituted by ROQUIN-2.

As with ZFP36L1 and ZFP36L2, ZFP36 protein preferentially recognizes AREs in 3'-UTRs, and limits expression of target mRNAs [64]. T cell activation and anti-viral immunity were enhanced in *Zfp36*-deficient mice compared to wild-type mice [65]. Moreover, TCR-signaling-dependent expression of activation marker genes and proliferation increased in T cells in the absence of ZFP36. Thus, ZFP36 also inhibits aberrant T cell activation.

ZFP36 family (ZFP36, ZFP36L1 and ZFP36L2) and ROQUIN family recruit the CCR4-NOT complex [66]. However, as described, phenotypes of T cells in mice deficient in these were different from one another and also from *Cnot3*-deficient mice. This apparent inconsistency should be partly due to the Cre deleter used in each study. For instance, *Zfp36l1* and *Zfp36l2* genes were deleted from DN stages whereas the *Cnot3* gene was deleted from DP stages [58]. Moreover, because T cell-specific deletion of *Cnot3* severely impaired development of mature T cells in the thymus [4], the phenotype in the later stage of T cells remained unclear. Therefore, it is important to determine the differentiation stage at which RBPs initiate their function by recruiting the CCR4-NOT complex. It is also possible that other decay systems beside the CCR4-NOT complex may function downstream of the ZFP36L and ROQUIN families [67-69]. Alternatively, coupling of transcription and mRNA decay by the CCR4-NOT complex may explain why phenotypes of *Cnot3*-deficient mice differ from those of RBPs [2] [70]. For instance, in yeast, translocation of cytoplasm RNA-decay factors into the nucleus occurred in a degradation-dependent manner, and then activated mRNA transcription in the nucleus [70]. A similar mechanism may robustly maintain total mRNA levels against perturbation by eliminating the CNOT3 subunit.

Epigenetic modification of mRNA is central to RNA metabolism [71]. For instance, N6-methyl adenosine (m6A) modification in RNA is relatively well characterized [72, 73]. mRNA containing m6A binds to YT521-B homology Domain-containing Family (YTHDF) 2, which recruits the CCR4-NOT complex in eukaryotes[74]. Of note, m6A modification contributes to a link between mRNA transcription and decay. Thus, when transcription rate of specific genes is decelerated, their mRNAs can undergo m6A modification and subsequent decay. METTL3 and METTL14 methyltransferase complexes catalyze m6A modification [75]. Lack of METTL3 suppressed homeostatic proliferation of murine T cells when they were transferred into lymphopenic mice [76]. Interleukin (IL)-7-induced degradation of *Socs* mRNA, which is crucial for proliferation and differentiation of naïve T cells, was impaired in *Mettl3*-deficient T cells [76]. Moreover, **regulatory T cell** (Treg)-specific deletion of *Mettl3* in mice impaired immune suppressive function of Tregs [77]. T cell-specific deletion of *Mettl14* induced spontaneous colitis in mice, which was due to Treg dysfunction because transfer of wild-type Tregs ameliorated the colitis. These suggest that m6A modification is critical for IL-7-dependent homeostatic proliferation of T cells and Treg functions

Last, REGNASE-1 is an endonuclease that cleaves translationally active mRNAs through recognition of a **stem-loop RNA structure** in the **polysomal fraction** [13, 44]. T cell-specific deletion of the *Zc3h12a* gene encoding REGNASE-1 in mice spontaneously provoked autoimmune phenotypes with increased expression of several cytokines, surface co-stimulatory molecules, and transcription factors in T cells [78]. Notably, TCR-signaling cleaves REGNASE-1 in a paracaspase MALT1-dependent manner [78, 79], which turns off REGNASE-1-dependent decay of target mRNAs. Thus, RNA decay via REGNASE-1 pathways precisely regulates peripheral T cell function and maintenance. One study recently reported a negative role of REGNASE-1 in anti-tumor responses of $CD8^+$ T cells in mice [16]. Abolishment of *Zc3h12a* gene in $CD8^+$ T cells by CRISPR-Cas9 technology improved their therapeutic efficiency against tumors in mouse models [16]. This finding suggests the possible manipulation of REGNASE-1 gene to enhance cancer therapy.

Overall, these finding indicate that thymic T cell differentiation and selection may be regulated by several endonuclease-dependent RNA decay modules (Box 2, Figure 4). In addition, both endonuclease- and exonuclease-inducing RNA decay machinery control proper T cell activation and function in peripheral tissues. Moreover, the artificial conversion of T cell function for cancer therapy may be possible by manipulating RNA decay machinery.

Innate lymphoid cells

Group 2 innate lymphoid cells (ILC2s) produce type 2 cytokines and are critical for immune responses such as protection against helminth infection [80] and allergic pulmonary inflammation [81, 82]. ILC2 from mice

with a degradation-resistance mutation in REGNASE-1 showed reduced expression of IL-33-inducing mRNAs, compared to wild-type ILC2 [83]. Moreover, bleomycin-induced pulmonary fibrosis in lymphocyte-deficient mice was more severe when ILC2 from *Zc3h12a*-deficient mice was transferred, compared with wild-type ILC2 transfer [84]. Interestingly, *Zc3h12a* expression was negatively correlated with cellularity of ILC2 in bronchoalveolar lavage fluid of humans [84]. These data imply a possible link between REGNASE-1-dependent RNA decay and ILC2-inducing lung inflammatory diseases such as idiopathic pulmonary fibrosis.

RNA decay pathways regulating myeloid cell functions

Signaling from **Toll-like receptors** (TLRs) initiates innate immune responses and inflammation. TLR ligands induce transcription of *Zc3h12a* mRNA in murine macrophages [13]. The absence of REGNASE-1 caused inflammation in mice and over-production of IL-6 in response to lipopolysaccharide (LPS) in macrophages, by stabilizing *Il6* mRNAs. Upon IL-1β and TLR stimulation [13], REGNASE-1 is degraded via ubiquitin-proteasome machinery [85], which can contribute to rapid elevation of cytokine concentrations in innate immune responses, and thereby precisely regulates inflammation.

In another example of RNA decay, mice deficient in the *Zfp36* gene spontaneously develop progressive and lethal inflammation due to aberrant up-regulation of tumor necrosis factor (TNF) and IL-23 [11, 12]. Moreover, myeloid-specific deletion of *Zfp36* in mice results in hypersensitivity to low doses of LPS, accompanied by increased serum TNF compared to wild-type mice, although these mice do not completely manifest severe spontaneous inflammation [86, 87], intimating involvement of ZFP36 in non-myeloid cells in inflammation onset in *Zfp36*-deficient mice. In addition to TNF, ZFP36-dependent inhibition of IL-1 protein expression is critical for in suppression of spontaneous inflammation [88, 89]. Thus, LPS-inducing expression of *Il1a* and *Il1b* mRNAs increased in bone marrow-derived dendritic cells (BMDCs) prepared from *Zfp36*-deficient mice relative to wild-type BMDCs [89]. Notably, spontaneous inflammation occurring in *Zfp36*-deficient mice was considerably ameliorated by deletion of the *Il1r1* gene [89], revealing a critical role of the IL-1 signaling. Overall, these studies show that ZFP36 inhibits inflammation by regulating multiple target genes.

A recent study revealed that m6A modification is involved in innate immune responses of cells [90]. *IFNB* mRNA, encoding type I interferon IFN-β, was modified by m6A and was more stable in METTL3-depleted cells than in wild-type cells [90]. Moreover, deletion of METTL3 and YTHDF2 incrementally induced interferon-stimulated genes in cell lines [90]. Thus, m6A modification of *IFNB* mRNA negatively regulates type I interferon responses to infections.

When mice with a missense mutation in *Rc3h1* were crossed with *Rag1*-deficient mice (which lack T and B cells), the offspring developed severe arthritis induced by **arthritogenic serum injection** relative to wild-type mice [91, 92]. This suggested that ROQUIN-1 activity in nonlymphoid cells may help prevent excessive inflammatory responses [91].

Overall, these studies revealed that REGNASE-1 and ZFP36 regulate TLR signaling-dependent expression of inflammatory cytokines. On the other hand, m6A modification recruitment of YTHDF2 is critical for type I interferon responses against viral infection. However, although ZFP36 and YTHDF2 recruit the CCR4-NOT complex for endonuclease-dependent RNA degradation, genetic evidence of CCR4-NOT complex recruitment in these innate immune responses has not been reported, and might be a fruitful area of future investigation.

RNA decay pathways in non-immune cell functions

Recent studies have shown that RNA decay regulators in non-immune cells can regulate immune responses. A recent study found that epithelial cell-specific deletion of the *Zfp36* gene provokes spontaneous skin inflammation with **psoriasis**-like lesions, and **dactylitis** in mice [93], similar to the pathology of conventional *Zfp36*-deficient mice. The authors further showed that inflammatory pathology of epithelial-specific *Zfp36*-deficient mice was alleviated by TNF deletion in skin epithelial cells [93]. Thus, it is likely that ZFP36 inhibits inappropriate TNF expression in skin epithelial cells.

Moreover, immunological roles of REGNASE-1 in nonimmune cells have also been reported in lung and colon epithelial cells. In murine lung, REGNASE-1 is down-regulated by administration of heat-killed *Pseudomonas aeruginosa* [94]. In contrast to wild-type mice, lung epithelial-specific deletion of the *Zc3h12a* gene augmented innate immune responses, and enhanced IgA secretion and **Th17 cell** accumulation in the lung after *Pseudomonas aeruginosa* infection in mice [94]. Colon epithelium-specific deletion of *Zc3h12a* conferred resistance to dextran sodium sulfate-inducing colon inflammation relative to wild-type [95]. Mechanistically, REGNASE-1 could regulate mTOR signaling and purine metabolism under inflammatory conditions in the colon [95]. These findings demonstrate critical roles of REGNASE-1 in immunological barrier cells to inhibit excess inflammatory responses.

In contrast to these RBPs, involvement of the CCR4-NOT complex in immunological functions of epithelial barriers remains unclear. However, deletion of *Cnot3* or *Cnot1* in pancreatic β-cells [96], hepatocytes [97,

98], and adipocytes [99, 100] augmented expression of inflammation-related genes, which may imply involvement of the CCR4-NOT complex in immunological barrier functions.

Although involvement of RNA degradation machinery in these processes has not been clearly demonstrated, these findings suggest that RNA-decay systems may be important not only in immune cells, but also in cells interacting with the immune microenvironment in various tissues, functions that certainly merit further investigation.

RNA decay systems in human disease onset

A previous study showed that CNOT3 mutation is associated with the development of human T-All [15], and also that reduced expression of *Not3* promotes tumor development in a *Drosophila sp* tumor model [101]. As mentioned, considering that deletion of the ZFP36L1 and ZFP36L2 causes T-All in a mouse model [58], this finding is consistent with the hypothesis that mRNA decay mediated via ZFP36L1/2 - CCR4-NOT signaling ordinarily inhibits onset of T-All in humans, a hypothesis that remains to be tested [15].

From another perspective, a recent study suggested the involvement of ROQUIN-1 in human **hyperinflammatory syndrome**. Indeed, a homozygous nonsense mutation in *RC3H1* was reported in a patient presenting with hyperinflammatory syndrome [14]. Consistent with the expectation that this mutation would yield truncated, dysfunctional ROQUIN-1 protein, $CD4^+$ T cells from the patient displayed increased expression of both ICOS and OX40, similar to what has been observed in an *Rc3h1* point-mutant mouse model [9]. Notably, the truncated ROQUIN-1 protein generated by the human mutation was incapable of interacting with the CCR4-NOT complex, delaying deadenylation of the target *ICOS* mRNA. Thus, it is reasonable to speculate that RNA decay via the Roquin-1-CCR4 NOT pathway might inhibit human hyperinflammatory syndrome, but this remains to be investigated.

Recent studies revealed the involvement of REGNASE-1 in human ulcerative colitis (UC) development [102, 103]. Whole exosome sequencing of crypts [103] and colon organoids [102] from UC patients revealed somatic mutations in the DSGxxS motif of REGNASE-1. Mutations in this motif reportedly cause stabilization of REGNASE-1 protein in murine macrophages, thereby enhancing degradation of target mRNAs [85]. Consistently, human colon organoid and cell lines expressing the mutant proteins reduced expression of target mRNAs, such as NFKBIZ, compared to wild-type protein [102, 103].

Taken together, inflammatory diseases and tumor incidents might be provoked by the dysregulation of the RNA decay machineries. Further researches should be necessary to address possible involvement of the RNA decay pathway in other human immune diseases.

What are the benefits of RNA-decay systems in immune cell development and immunological responses?

As described, RNA decay pathways function in inflammatory responses, cell survival, and differentiation. During these cellular processes, in many cases, transcription of mRNAs encoding functional proteins is induced rapidly and simultaneously. On the other hand, over-production of these proteins needs to be prevented to avoid aberrant cellular responses. mRNA decay mechanisms diminish protein abundance more rapidly than transcriptional suppression. However, once proteins are generated, indirect reduction of protein concentrations by RNA decay mechanisms should be slower than direct protein degradation. Accordingly, we hypothesize that RNA decay-dependent regulation of protein concentrations may function most effectively when gene expression is induced by signaling, and also before protein translation is completed. Therefore, RNA decay-dependent regulation may occur when gene expression is dynamically induced, such as during inflammatory responses, cell survival, and differentiation, a hypothesis that merits further attention.

One advantage of mRNA decay may involve energy constraints. Theoretical calculation of energy necessary to maintain cellular mRNA and protein amounts reveals that protein synthesis consumes more than 90% of the energy whereas less than 10% is necessary for transcription [104]. Therefore, mRNA turnover may conserve energy in regulating protein abundance, which may also represent another interesting area for future investigation, with relevance for immune cell, as well as non-immune cell functional outcomes.

Concluding Remarks

RNA decay systems regulate lymphocyte development. Given that lymphocyte development proceeds via multiple steps, RNA decay pathway functions in other differentiation stages and unconventional lymphocyte development should be addressed (see outstanding questions). Moreover, beside REGNASE-1, functions of RNA decay machinery in nonlymphoid cells during inflammatory responses also need to be clarified. In terms of mechanisms, it remains largely unclear how RNA decay pathways choose their target genes. Thus, selective mRNA decay appears to be regulated by not only RNA-binding molecules, but also by epigenetic modification, cross-talk with transcriptional machinery, and localization in the cell. Complex combinations and interactions of regulatory mechanisms seem to be involved in determining mRNA half-lives. Trans-omics and mathematical analysis may help reveal a complete picture of regulatory mechanisms underlying

selective mRNA decay in immune responses and development. Finally, involvement of mRNA decay pathways in human physiology and pathology should be further investigated. Such information would be beneficial in developing therapies targeting mRNA decay pathways.

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Declaration of Interests

The authors declare that they have no competing interests related to this work.

Figure Legends

Figure 1. Exonuclease- and endonuclease-dependent mRNA decay pathways in vertebrates. Some mRNA decay systems modulate amounts of proteins dynamically in order to regulate mRNA stability. Such "active" RNA decay pathways mainly comprise the exonuclease and endonuclease pathways [18, 22]. In the exonuclease pathway (a),deadenylase complexes such as the CCR4-NOT complex, the PAN2-PAN3 complex, and PARN (here only the CCR4-NOT complex is shown) are recruited to mRNAs to shorten their poly A tails. After polyA tails are shortened, the Lsm1–7/Pat1 complex interacts with 3′-terminal adenosine extensions and recruits de-capping enzymes DCP2 and NUDT16 (here only DCP2 is exhibited). After de-capping, mRNAs undergo degradation by exonuclease XRN1 from their 5'-ends and exosomes from their 3'-ends. (b) Endonuclease recruited to mRNAs generates unprotected 5'- and 3'-ends. These unprotected ends are recognized and undergo degradation by XRN1 and exosomes.

Figure 2. CCR4-NOT complex-dependent mRNA decay participates in the transition from pro-B to pre-B cells in mice. V(D)J recombination in B cells is mediated by the RAG protein complex, consisting of RAG1 and RAG2. RAG2 protein expression is restricted in the G0-G1 phases of the cell cycle [50-53]. ZFP36L1 or ZFP36L2 binds to mRNAs encoding cell cycle entry genes via recognition of the AU rich element (ARE) in the 3'-UTR in mammalian cells [3] [54]. After binding to the ARE, ZFP36L1 and ZFP36L2 recruit the CCR4-NOT complex. That complex, in turn, initiates degradation of poly A tails via the exonuclease pathway (see Fig. 1a), which finally causes complete degradation of target mRNAs encoding cell-cycle entry proteins. Accordingly, this mRNA decay pathway should be crucial to induce quiescence of Pro-B cells to maintain expression of RAG2 protein. Similar events may occur in the DN3 stage of thymocyte development.

Figure 3. Control of thymic positive selection by the CCR4-NOT complex in mice. Positive selection of $CD4^{+}CD8^{+}$ thymocytes proceeds through $CD69^{low}CD3^{int}$, $CD69^{high}CD3^{int,}$ $CD69^{high}CD3^{high}$, and $CD69^{lo}CD3^{high}$ stages in the thymus [105]. During this process, the CCR4-NOT complex purportedly inhibits inappropriate apoptosis [4]. Expression of some subunits of the CCR4-NOT complex is up-regulated in $CD3^{int}CD69^{lo}$ thymocytes from DN thymocytes. Thymocytes receive self-peptide-MHC complex signaling from cortical thymic epithelia cells (cTECs), and induce CD69 expression on the cell surface. In $CD69^{high}CD3^{int}$ thymocytes, although transcription of *Dap2ip* mRNA, encoding anti-apoptotic DAP2IP, is up-regulated, it undergoes degradation by the CCR4-NOT complex. After this process, $CD69^{hi}CD3^{int}$ thymocytes differentiate into $CD69^{hi}CD3^{int}$ thymocytes where the CCR4-NOT complex inhibits up-regulation of *Bbc3* mRNA, encoding pro-apoptotic BBC3 proteins in $CD3^{hi}CD69^{lo}$ thymocytes.

Figure 4. Possible mechanisms regulating target selection of mRNA decay pathways in eukaryotes. Cis-regulatory elements in mRNA should be determinants of selection of target mRNAs via binding of RBPs and non-coding RNAs (i.e., miRNA) [106-108]. miRNAs form complexes with Ago family proteins on their target sequences. This complex recruits RNA decay machinery, such as the CCR4-NOT complex [93]. m6A modification occurs in the nucleus and is determined by the rate of transcription. Thus, "slow" transcription may tend to permit mRNA to undergo m6A modification [85]. m6A modification is recognized by YTHDF2, which in turn recruits the CCR4-NOT complex for exonuclease degradation. In addition, localization of mRNAs may influence selection of target mRNAs [44]. In addition to its deadenylase activity, the CCR4-NOT complex also functions as a transcriptional regulator [24], although it is unclear how these two functions are correlated.

Box 1. B and T cell development in humans and mice.

In adult bone marrow, lymphoid progenitor cells differentiate into pro-B cells in vertebrates[47]. Recombination of immunoglobulin heavy chain genes (**V(D)J recombination**) occurs in pro-B cells, which differentiate into pre-B cells that express pre-B cell receptors. Pre-B cells undergo further differentiation, giving rise to immature B cells that express B-cell antigen receptors.

Conventional T cell development occurs mainly in the vertebrate thymus [105]. Briefly, progenitor cells from bone marrow enter the thymic cortex and differentiate into $CD4^{-}CD8^{-}$ (DN) thymocytes. Through expression of CD25 and CD44 (CD117), DN thymocytes differentiate into DN1 → DN2 → DN3 → DN4. DN4 gives rise to $CD4^{+}CD8^{+}$ (DP) thymocytes [105]. Recombination of the T cell antigen receptor (*TCR*) gene occurs during the DN3 and DP thymocyte stages [105]. In the thymic cortex, DP thymocytes interacting with self-antigen on MHCs of cortical thymic epithelial cells are selected (positive selection) and further differentiate into CD4 or CD8 single-positive T cells [109]. After further elimination of self-reactive T cells in the thymic medulla (negative selection), T cells egress from the thymus for immune responses in peripheral tissues.

Box 2. Mechanisms that determine targets for RNA decay

Mechanisms triggering selective RNA decay should depend on *cis*-acting mRNA sequence elements, in addition to regulatory factors (Fig. 4) that recruit RNA-degradative enzymes to appropriate targets. In addition, target selection may depend on other factors, such as transcription rate and RNA modification (Fig. 4).

The RNA-binding protein family regulates RNA decay by binding specific sequences and structures. Although members of the ZFP36 family interact with AREs [64], AU-rich sequences are estimated to occur in 3'-UTRs of more than 8% of all mRNAs [110]. However, target genes actually regulated by ARE-binding proteins appear to be limited. Many ARE-binding proteins function as multimers and are phosphorylated by different kinases [22]. These up-stream regulatory mechanisms should contribute to selective degradation of target mRNAs in a cell- and response-dependent manner. Moreover, cooperative and competitive interaction among RBPs may be involved in specifying target mRNAs. Of note, REGNASE-1 and ROQUIN recognize a common, conserved, stem-loop motif [44] [111]. However, REGNASE-1 and ROQUIN function in endoplasmic reticulum and processing-body/stress granules, respectively [44]. Therefore, in eukaryotes they control decay of common mRNAs in distinct spatiotemporal processes, depending on translational activity of mRNAs [44]. Thus, targeting of mRNAs for decay may be coupled with translation. For another instance, ZFP36 and ROQUIN family bind to AREs and stem-loop forming elements in 3'-UTRs of target mRNAs [44, 61, 112, 113], respectively. Because 3'-UTRs of *Tnfα* and *Il-6* mRNAs contain both AREs and stem-loop forming elements [44, 61, 112-114], there may be some functional interactions between ZFP36 and ROQUIN family. However, whether ZFP36, ROQUIN1/2 and m6A modification promote degradation of such mRNAs additively, synergistically, or exclusively has not been determined.

Beside RBPs, non-coding RNAs should be candidates to determine targets for RNA decay in immune regulation [106-108]. MicroRNAs (miRNAs) can influence the rate of mRNA decay directly by precluding binding of mRNA decay factors. Whereas each miRNA has many target genes, one mRNA may be regulated by many different miRNAs simultaneously. Thus, a combination of expressed miRNAs may also contribute to target mRNA selection. Moreover, miRNAs form complexes with Ago family proteins on their target sequences, thereby recruiting the CCR4-NOT complex or the PAN2-PAN3 complex in eukaryotes [115]. In addition to Ago2, complex formation of other RBPs with miRNA may also be involved in specifying target mRNAs.

RNA epigenomic modification confers target selectivity for mRNA decay. Epigenomic RNA modification is regulated by balancing methyltransferases, demethylases and its binding proteins [73].These up-stream regulators may function depending on cellular responses, thereby specifying mRNA targets for decay. a recent study showed that m6A modification is determined by the rate of transcription in mammalian cell lines [116]. Slow transcription and subsequent modification may thus allow enough time for mRNA to undergo m6A modification. This suggests that the rate of transcription is coupled to RNA decay mediated by the CCR4-NOT complex by m6A modification.

The CCR4-NOT complex controls transcription in addition to deadenylation of mRNAs [24], suggesting that the CCR4-NOT complex may contribute to coupling between transcription and mRNA degradation. Indeed, the CCR4-NOT complex binds to EBF1 and modifies both transcription and RNA degradation in B cell development [2]. Additionally, some mechanisms for coupling transcription and RNA decay have been reported in yeast [117] [118] [119]. Thus, a similar mechanism may be involved in selection of target mRNAs in RNA decay systems.

Finally, it is possible that defects in the general mRNA decay pathway and the subsequent increase of global mRNA expression could trigger cellular responses [20]. In this case, cellular responses would be independent of protein functions encoded by target mRNAs.

Overall, several mechanisms appear to be involved and to interact with each other in selection of target mRNAs for RNA decay machinery. Detail mathematical modeling and trans-omics studies may help to resolve this complexity.

Glossary

Non-sense-mediated RNA decay: An mRNA decay mechanism that degrades mRNAs containing premature translation-termination codons.

No-go mRNA decay: An mRNA decay mechanism that eliminates mRNAs containing stalled ribosomes

Non-stop mRNA decay: An mRNA decay mechanism that eliminates mRNAs lacking stop codons.

CCR4-NOT complex: Carbon catabolite repressor 4 (CCR4)- Negative on TATA(Not) is a multi-protein complex having the dominant role in catalysis of mRNA deadenylation in eukaryotes.

PAN2-PAN3: A protein complex consisting of the poly A nuclease (PAN) 2 and PAN3. PAN2 has exonuclease activity.

PARN: polyA-specific ribonuclease (PARN) is a polyA-specific 3- exonuclease.

RAG2 protein: a subunit of the RAG recombinase complex that induces V(D)J recombination

Exosome: A ribonuclease complex that processes degradation of RNA in the 3'-to-5' direction

Marginal zone B cells: An innate B-like lymphocyte subset that resides in the marginal zone of the spleen. Marginal zone B cells rapidly respond to antigens.

T cell acute lymphoblastic leukemia: An aggressive hematologic malignancy that results from transformation of immature T-cell progenitors.

β-selection: A mechanism eliminating thymocytes that fail rearrangement of the functional T-cell antigen receptor beta chain.

DNA damage responses: Cellular processes including DNA-damage sensing, DNA-lesion repair, and damage tolerance.

V(D)J (variable-diversity-joining) recombination: The somatic rearrangement of respective V(variable), D(diversity) and J(joining) gene segments of T-cell antigen receptors (TCR) and immunoglobulin genes to produce a diverse repertoire.

Cellular quiescence: A cell state of reversible proliferative arrest. Quiescent cells are able to reenter the cell cycle upon receiving an appropriate stimulus.

Systemic lupus erythematosus: A chronic autoimmune disorder that has various manifestations in multiple organs.

Follicular helper T cells: A subset of CD4(+) T cells that promote differentiation of B cells into plasma cells and memory B cells.

Germinal center B cells: A subset of B-cells in dark zones of the germinal center, which is specialized for various B cell responses depending on T cells. Germinal center B cells divide rapidly and undergo somatic hypermutation of their immunoglobulin (Ig) variable domains.

Stem-loop RNA structure: Structure of single-stranded RNA and DNA, consisting of a stem, double helix, and a loop.

Psoriasis: An immune-related chronic skin disease that causes red, flaky, crusty patches of skin.

Th17: A helper T cell subset express IL-17A and the lineage-defining transcription factor RORγt. Th17 is crucial for clearance of extracellular bacterial and fungal infections, but also promotes inappropriate inflammation and autoimmune disorders.

Polysomal fraction: A fraction containing a complex of mRNA and several ribosomes that corresponds to active mRNA translation.

Cancer immunotherapy: A therapy used to treat cancer patients that uses the immune system to prevent and eliminate cancer.

Arthritogenic serum injection: A murine model for rheumatoid arthritis and other arthritides. In this mouse model, serum from transgenic K/BxN mice is transferred to naive mice.

Dactylitis: Inflammation of a digit (either finger or toe)

Regulatory T cells: A T cell subset that maintain self-tolerance and immune homeostasis by preventing excessive immune activation.

Hyperinflammatory syndrome: Severe inflammatory diseases provoked by dysregulated immune cell activation and cytokine expression in the body.

Toll-like receptors: Membrane-bound receptors that initiate the early immune response by recognizing conserved molecular patterns of pathogens.

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Outstanding Question Box

Do RNA decay systems regulate other differentiation and selection stages of lymphocytes? Other types of lymphocyte selection, such as negative selection and agonistic selection could be regulated by RNA decay mechanisms, and their disruption could cause immune-related diseases.

Are RNA decay pathways involved in responses of other immune cell types, including unconventional lymphocytes or other innate lymphoid cells beside ILC2? Precise regulation of inflammation and immune responses may be achieved by the RNA decay machinery in some minor cell populations.

What mechanisms identify target mRNAs in RNA decay systems in immune cells? The complexity of RNA decay regulation should be clarified for understanding global and dynamic regulation of mRNA abundance in the cells. Trans-omics and mathematical modeling analysis should be useful to address this issue.

How does RNA decay machinery influence nonimmune cells that create the microenvironment for immune cell development and responses?

Is the RNA decay system involved in the onset of other immune-related diseases and tumors in humans? Dysregulation of RNA decay machinery might provoke human diseases such as ulcerative colitis and leukemia. It may also influence onsets of other inflammatory diseases and tumor incidence.

Can manipulation of RNA decay systems benefit other immune-related diseases, as well as cancer immunotherapy? Manipulation of RNA decay pathways may be useful for precise control of some human diseases.