RNA-binding proteins and RNA methylation in myeloid cells

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Summary

RNA-binding proteins (RBPs) regulate all aspects of the life of mRNA transcripts. They are critically important in regulating immune responses, most notably by restraining excessive inflammation that can potentially lead to tissue damage. RBPs are also crucial for pathogen sensing, for instance for the recognition of viral nucleic acids. Concordant with these central regulatory roles, the dysregulated activity of many RBPs can give rise to disease. The expression and function of RBPs are therefore highly controlled by an elaborate network of transcriptional, post-transcriptional and post-translational mechanisms, including the ability of different RBPs to cross-regulate each other's expression. With an emphasis on macrophages and mast cells, we review current knowledge on the role of selected RBPs that have been shown to directly impact the expression of inflammatory transcripts. By focusing specifically on proteins of the Regnase and ZFP36 family, as well as on factors involved in N^6-methyladenosine (m^6A) deposition and recognition, we discuss mechanism of action, regulatory feedback, and impact of these selected proteins on immune responses. Finally, we include examples of the role of m^6A and RBPs in the recognition of viral RNAs. Overall, we provide a general overview of the impact of selected RBPs on the myeloid compartment, followed by a discussion of outstanding questions and challenges for the future.

Running Title

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Keywords

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Introduction

Throughout their lifetime, mRNA molecules associate with RNA-binding proteins (RBPs) that regulate every aspect of their life cycle. The maturation, stability and translation of mRNA transcripts are all controlled by a large number of co-transcriptional and post-transcriptional processes occurring both in the cell nucleus (5'-capping, splicing, poly-A tail addition, RNA editing, RNA methylation) and in the cytoplasm (regulation of nuclear export, quality control, modulation of stability, translation and turnover). The association of mRNAs with regulatory RBPs was hypothesized shortly after the initial discovery and characterization of mRNAs [1,2]. By studying fish embryos, it was observed that some of the newly synthesized mRNAs were not immediately translated, but they were rather maintained for a time in an inactive form as part of non-ribosomal ribonucleoprotein complexes [3]. Such ribonucleoproteins were defined "informosomes", since they somehow carried the information relative to mRNA translation and they represented a new layer of post-transcriptional regulation [3]. Indeed, it later became clear that even after maturation, splicing and nuclear export, the mature, cytoplasmic transcripts are at the center of an RNA-protein interactome containing a variety of RBPs that recognize RNA modifications, sequence motifs and secondary structures. Such extensive RBP network modulates mRNA translation and stability in the cytoplasm, and a substantial part of the regulatory mechanisms occurs within the 3'-untranslated region (UTR) of mRNAs. Here, we focus primarily on selected RBPs that have a role in modulating inflammatory responses, acting as regulators of inflammatory mRNA expression and decay. Specifically, we discuss the mechanism of action and regulation of the Regnase, ZFP36 and YTHDF families of RBPs, the latter being important in the recognition of the N^6-methyladenosine (m^6A) modification.

Regnase and ZFP36 proteins in inflammation

The Regnase and ZFP36 families of RBPs represent two examples of the variety of mechanisms of action and regulation that can be observed among the many RBPs that modulate gene expression in the immune

system, and their importance is highlighted by the severity of the phenotype of mice lacking these factors (*Fig. 1*).

Tristetraprolin (TTP, encoded by the *Zfp36* gene) is one of the best characterized destabilizing RBPs in the immune system. TTP binds to mRNA targets containing sequence motifs known as AU-rich elements (AREs), which can be found within the 3'UTR of many inflammatory mRNAs. Indeed, TTP is a crucial factor in restraining excessive inflammation, by limiting the production of highly inflammatory cytokines such as TNF. The physiological importance of TTP in post-transcriptional regulation was revealed by the phenotype of *Zfp36*-deficient mice [4]. These mice develop a complex inflammatory syndrome, in addition to autoimmunity and myeloid hyperplasia, that was primarily mediated by the enhanced expression of TNF. Indeed, TTP was shown to accelerate degradation of the *Tnf* mRNA via direct binding to the ARE in its 3'UTR. Myeloid-specific deletion of the *Zfp36* gene led to high susceptibility to endotoxin challenge and grossly elevated levels of TNF in the serum, although this phenotype did not completely phenocopy the full TTP deficiency, highlighting the importance of TTP expression in multiple immune cell types [5]. Indeed, ablation of TTP in neutrophils led to their increased accumulation at sites of infection, and the direct comparison of differential gene expression in macrophages *vs.* neutrophils lacking *Zfp36* revealed that the effect of TTP on the transcriptome was dependent on the cell type [6]. One prominent example of such differential effect is provided by the inflammatory cytokine *Il6*, whose expression was decreased in neutrophils lacking TTP while it was increased in macrophages [6]. The mechanism underlying this differential regulation remains to be understood. As for its mode of action, TTP was shown to interact with the CCR4-NOT deadenylation complex, which plays a central role in mRNA decay mediated by TTP[7].

The ZFP36 family includes two additional family members, structurally related to TTP, ZFP36L1 and ZFP36L2. Despite the similarities, specialized functions for these two family members have been reported primarily in B and T lymphocytes, and the full extent of redundancy and target overlap of these proteins in different cell types and conditions is still incompletely explored [8,9].

The Regnase family of proteins represents another example of RBPs with an important function in restraining inflammation. These proteins are characterized by an intrinsic RNase enzymatic activity, and recognize stem-loop structures in target inflammatory mRNAs, leading to their direct degradation. Within the immune system, the most prominently expressed family member is Regnase-1, that in macrophages regulates the expression of a number of inflammatory transcripts including *Il6, Il12b* and *Ptgs2* [10,11]. Regnase-1 preferentially degrades transcriptionally active mRNAs, after they have undergone at least one round of translation [10,12], and in this process it requires the helicase UPF1, which contributes to Regnase-1-dependent target degradation by unwinding the stem-loop structure [10,12]. Indeed, RNA-immunoprecipitation and sequencing (RIP-seq) analysis of CBP80- and eIF4E-associated mRNAs (identifying mRNA undergoing the first or subsequent rounds of translation, respectively), in wild-type and Regnase-1-deficient cells, showed that Regnase-1 bound to and affected the stability specifically of CBP80-associated inflammatory mRNAs [12]. This observation suggests a mechanism permissive of fast expression of inflammatory transcripts in response to stimulation followed by rapid degradation, therefore potentially contributing to restrain excessive inflammation. Within the immune system, Regnase-1 is expressed in both myeloid and lymphoid cells [13], while the other Regnase family members show more restricted patterns of expression. For example, Regnase-3 appears to function mostly in a myeloid-specific manner and to be highly expressed especially in macrophages [14]. Mice lacking Regnase-3 in macrophages developed lymphadenopathy linked to excessive interferon (IFN)-γ expression and dysregulated IFN signaling, although the exact mechanism underlying this effect remains to be fully understood [14]. This observation suggests that RBPs of the same family, despite sharing the same functional domains and mode of action, may have unique functions linked to their levels of expression and cell type specificity.

Regulation of RBP activity

Remarkably little is known about how RBPs are themselves regulated, although there are increasing examples of RBPs being regulated by phosphorylation, subcellular localization, proteolytic cleavage and proteasomal degradation [15] (*Fig. 2*). From a transcriptional point of view, different members of the same RBP family can be regulated by different transcription factors, indicating that even when their functions appear to be fully redundant *in vitro*, the expression kinetics or response to specific signals may differ widely, leading therefore to distinct functional outcomes *in vivo*. For example, the transcription factor BHLHE40 regulates the expression of *ZC3H12D* (encoding for Regnase-4) but not *ZC3H12A* (Regnase-1) in human T lymphocytes [16]. In macrophages, IFN signaling, and in particular the transcription factor IRF7, was shown to induce Regnase-3, but not Regnase-1 expression, which was instead under the control of NF-kB activation [14]. Different RBPs can also strongly regulate their own expression and the expression of other members of the same family. For instance, Regnase-1 targets its own mRNA [17], and it is also regulated post-transcriptionally by direct binding of Regnase-3 to its 3'UTR, at least in macrophages [14]. How this cross-regulation impacts the immunomodulatory functions of these proteins was revealed by a study showing that Regnase-3 promotes skin inflammation by favoring expression of TNF by macrophages while at the same time repressing IL-6 expression by plasmacytoid dendritic cells (pDCs). While reduced IL-6 expression was due to direct binding of Regnase-3 to the *Il6* mRNA, followed by its degradation, Regnase-3 indirectly promoted *Tnf* expression by degrading the transcript encoding for Regnase-1, which in turn acts as a negative regulator of TNF production [18].

One key aspect of regulation in post-transcriptional networks is indeed represented by the fact that different RBPs often regulate their own expression and that of other regulatory proteins, increasing the complexity of dissecting the impact of a given RBP on gene expression. Apart from the cross-regulation of Regnase factors mentioned above, another example in this direction is provided by the interaction between the RBP HuR, microRNAs (miRNAs) and TTP. Specifically, HuR (*ELAVL1*) is an ARE-binding protein involved in the regulation of the stability of inflammatory transcripts. Overexpression of HuR in

transgenic mice initially revealed a role as a negative regulator of inflammatory responses [19], and mice lacking HuR in myeloid cells displayed an exacerbated inflammatory profile [20]. Relevant to the interplay of HuR functions with other RBPs, transcriptome-wide analysis of HuR and miRNA binding sites in murine macrophages revealed that the proximity of HuR sites attenuated miRNA binding to the same target [21]. Interestingly, among the genes prominently affected by this competition between HuR and miRNA binding was *Zfp36*. Upon stimulation with lipopolysaccharide (LPS), HuR binding to the 3'UTR of *Zfp36* attenuated miR-27b-mediated suppression of TTP expression, leading to the upregulation of TTP itself [21]. Further highlighting the extent of cross- and co-regulation between RBPs, a recent analysis across 150 different RBPs showed that RBP binding to the 3'UTR generally facilitated miRNA binding through increased accessibility of mRNA secondary structures [22]. How this interplay between regulatory factors impacts mRNA translation and cell responses remains to be understood.

The expression and activity of different RBPs are also controlled at the level of protein stability and post-translational modifications, some of which are summarized in *Fig. 2*. In general, phosphorylation of RBPs can influence RNA-binding affinity, protein stability and subcellular localization. For instance, phosphorylation of HuR within its RNA-recognition domains leads to reduced RNA affinity, while phosphorylation within a hinge region between the different domains alters its nuclear transport [23-25]. HuR is also methylated on arginines located in the hinge region, a modification that occurs in macrophages following LPS stimulation, and that may contribute to stabilization of mRNA transcripts mediated by HuR [26]. Another prominent example of regulation of RBP activity by phosphorylation is provided by TTP, which becomes phosphorylated in macrophages upon LPS stimulation, leading to TTP inactivation and to the stabilization of inflammatory transcripts. Accordingly, the mutation of these phosphorylation sites gave rise to a version of TTP with increased destabilizing capacity, leading to attenuated systemic responses to LPS in mice [27]. Finally, upon activation of macrophages with LPS, Regnase-1 is rapidly phosphorylated by the IKK complex, followed by proteasomal degradation. Degradation of the Regnase-1 protein likely contributes to releasing a 'brake' on the expression of IL-6 and other inflammatory

mediators [17]. Proteasomal degradation of Regnase-1 in macrophages has also been shown to be in part initiated by the paracaspase MALT1 [28]. In fact, MALT1-mediated proteolytic cleavage of Regnase-1 has been extensively described in stimulated T lymphocytes [13]. Because the activity of RBPs can be modulated by post-translational modifications that may be difficult to recapitulate in an experimental setting, it is possible that the effect of a given RBP *in vivo* may differ from its overall targeting capabilities observed *in vitro*. For instance, Regnase-3 was shown to be able to bind mRNA targets *in vitro*, but to be unable to modulate them *in vivo*, most likely because of the requirement for specific post-translational modifications or protein partners that are still to be uncovered [14]. The existence of multiple post-translational mechanisms, which may also be cell type- and stimulus-dependent, ensures tight control of RBP expression and function.

RNA methylation

Post-transcriptional modifications regulate the fate of RNA molecules by modulating the binding of dedicated RBPs. This can occur either directly, for instance through RBPs that recognize specifically the RNA modification, or indirectly, since RNA modifications can affect RNA secondary structures and consequently the binding of proteins recognizing such structures [29]. The N^6-methylation of adenosines is one of the most abundant modifications identified in mRNAs and is mainly located near stop codons and within the 3'UTRs.

The methyl group of m^6A is specifically recognized by a specialized YTH protein domain, conserved from yeast to human, and such interaction influences many aspects of the mRNA life cycle, including splicing, RNA degradation and translation [30]. Among the described YTH-containing proteins, YTHDF1, YTHDF2 and YTHDF3 are paralogues, and they probably possess fully redundant functions, although their impact on specific mRNA targets may also depend on their relative level of expression in different cells and tissues [31,32]. Differently from the YTHDF proteins, YTHDC1 is nuclear and is involved in the

silencing of retrotransposons [33,34], while YTHDC2 (which despite the name is not a paralogue of YTHDC1) has primarily germ cell-associated functions [35,36].

At least when considering cytoplasmic mRNA, the most common and established outcome of m^6A methylation is the destabilization of the targeted transcript, although other effects, for instance on mRNA translation rates, have been observed (reviewed in [37]). The m^6A modification is deposited on nascent mRNAs in the nucleus in a process that appears to be associated with the rate of transcription, thereby coupling transcription regulation to mRNA stability. Indeed, 'slow' gene-specific transcription results in increased m^6A deposition, leading to shortening of the poly-A tail and enhanced mRNA decay mediated by the recruitment of the CCR4-NOT complex [38], suggesting a central role for m^6A methylation in the crosstalk between transcription and translation.

The methyltransferase complex that deposits m^6A on mRNA molecules contains the catalytic subunit METTL3 and several additional components, most notably METTL14 and WTAP [37]. The methyl group can also be "erased" from mRNA molecules through either direct removal or iterative oxidation mediated by ALKBH5 and FTO [39], respectively, although the general physiological relevance of such process remains to be fully understood. A recent CRISPR-Cas9 screen focused specifically on RBPs identified *Mettl3* as a prominent player in the regulation of macrophage activation in response to LPS. Indeed, mice lacking *Mettl3* specifically in macrophages had increased susceptibility to sepsis and reduced ability to control tumor growth [40]. Interestingly, although dysregulated in macrophages lacking *Mettl3*, the transcripts encoding for cytokines like *Tnf* and *Il6* contained no detectable m^6A peaks, suggesting that they were affected indirectly by the absence of *Mettl3*. Mechanistically, increased LPS responses in *Mettl3-/-* cells were linked to the increased stability of transcripts encoding for negative regulators of TLR4-signaling such as IRAKM, which are normally heavily m^6A-modified, confirming a prominent role for m^6A in regulating the stability of mature mRNAs in the cytoplasm.

As highlighted in the example above, one of the key questions when studying mRNA methylation is to understand if a particular mRNA is indeed methylated and where. Moreover, whether the same transcripts

are similarly modified (and thereby similarly regulated) across multiple cell types remains to be established. While the experimental analysis of m^{6}A in the system under investigation remains crucial, databases and m^{6}A target prediction tools are now becoming more common and accessible and are briefly summarized in *Box 1*.

Box 1. m^{6}A RNA-methylation: databases and online tools

In the past few years, the ability to map RNA modifications transcriptome-wide through next-generation sequencing technologies revolutionized our understanding of the biology and function of m^{6}A. Adenosine methylation occurs in the context of a rather common and promiscuous consensus sequence, although the proportion of sites that are actually methylated represents only a fraction of the overall number of consensus sequences available. Furthermore, these sites may be methylated with variable frequency, indicating the some aspects of the regulatory logic underlying m^{6}A deposition remain poorly understood [37]. To date, several high-throughput sequencing methods have been developed to detect m^{6}A across the entire transcriptome. Consequently, a large amount of datasets profiling m^{6}A and other RNA modifications in different system and organisms has been generated. This increased production of data led to the development of databases and bioinformatics interfaces that aim at collecting, organizing and sharing RNA-modification data within the scientific community. Methylated RNA immunoprecipitation and sequencing (MeRIP-seq) [41,42] was the first method developed to profile m^{6}A across the transcriptome. Despite being less accurate compared to more recent protocols that achieved single nucleotide resolution [43-45], it requires less starting material and produces higher coverage [46]. Therefore, it is not surprising that the vast majority of the m^{6}A-related databases available online comprises transcriptome-wide m^{6}A peaks obtained from published MeRIP-seq data. Among these, MethylTranscriptome DataBase (MeT-DB) is a comprehensive database designed for m^{6}A in mammalian cells. This database collects m^{6}A peaks identified by MeRIP-seq performed in samples from 7 species (including human and mouse) and includes m^{6}A sites detected also in non-coding RNAs such as lncRNAs and miRNA [47]. All this information can be

easily navigated through a genome browser platform. The RNA modification Base (RMBase) database is another interesting tool that, on top of collecting MeRIP-seq data about m^6A, integrates sequencing data for other different mRNA modifications (m^6A, m^1A, m^5C, Ψ, 2'O-Me and others) [48]. Compared to MeT-DB this database provides a more comprehensive overview of the epitranscriptomic landscape and allows the user the possibility to display and download modified sites identified in experimentally validated datasets. Moreover, it also grants the possibility to display cell-specific position weight matrices (PWMs) which were *de novo* identified by re-analyzing public dataset. Finally, the RNA EPItranscriptome Collection (REPIC) database [49] integrates m^6A data with public data from Gene Expression Omnibus (GEO) and ENCODE, allowing the navigation of potential interactions between m^6A modification and histone marks or chromatin accessibility. All these databases provide user-friendly platforms to explore m^6A data in experimentally validated datasets. However, despite providing valuable information about a given site being potentially methylated, Met-DB, RMBase and REPIC do not consider tissue or cell specificity. Towards this goal, the CVm6A database provides a cell line-dependent collection of m^6A patterns, including information relative to the abundance of the m^6A modification and to the subcellular localization of transcripts [50]. At least for the time being, CVm6A displays only data relative to specific cell lines, which can be sometimes difficult to translate to primary cells or tissues given the context-specific nature of RNA modifications. To date, the only database providing quantitative, condition-specific information of RNA modified site in tissues, cells and cell lines is m^6A-Atlas [51]. This database collects the profiling datasets for m^6A deriving only from single base-resolution technologies, therefore increasing the accuracy of the annotated sites. Of note, m^6A-Atlas also provides data about conservation of m^6A sites across several species and the annotation of several modifications in 10 viral strains, although the lack of a light genome browser makes this very promising database at times difficult to navigate. Moreover, probably because RNA modification profiling methods at single base resolution are still less used compared to non-base-resolution techniques, the majority of the data on this platform are related to human samples.

Beside databases for m^6A visualization, several bioinformatics tools based on machine learning have been developed to predict m^6A sites in specific query sequences. M6APred-EL and sequence-based RNA adenosine methylation site predictor (SRAMP) are two simple online platforms that allow the prediction of m^6A site in input RNA or cDNA sequences, something that can be very useful to identify putative m^6A sites on specific RNAs of interest [52,53]. However, these platforms do not allow a list of RNAs as input, making them suitable primarily for investigating a few transcripts at a time. Finally, it is worth mentioning the existence of the RNAmod web-based platform [54], which allows the analysis and functional annotation of several mRNA modifications. This web tool uses BED input files containing chromosomal locations of RNA modifications obtained from sequencing data and allows even the users that are less familiar with coding language to perform a comprehensive annotation and visualization of the distribution of specific mRNA modifications in sequenced samples of interest.

RNA-binding proteins and RNA modifications as roadblocks against parasitic nucleic acids

Since the half-life of an mRNA molecule is usually in the range of minutes to hours, it would seem more efficient to primarily regulate gene expression at the level of transcription, and to translate the mature mRNA for the duration of its relatively short life, followed by degradation [55]. Why then so many post-transcriptional "roadblocks" to protein synthesis? There are several possible answers that we can envision. First, like the complexity that is observed in the regulation of transcription, the regulation of mRNA translation contributes to the spatial and temporal regulation of gene expression that is required during the development and evolution of complex multicellular organisms, as seen in the case of the fish embryos mentioned above [3]. Second, post-transcriptional regulation fine-tunes responses to environmental signals. For example, regulation of the stability and translation of pre-formed mRNAs is especially important in the context of the immune system, ensuring fast responses to an invading pathogen [56]. Finally, such complexity in the mechanism of mRNA regulation creates an additional

opportunity to discriminate cellular, 'self' nucleic acids while at the same time fending off the invasion of parasitic nucleic acids, like in the case of viral infections [55]. Indeed, several RBPs, located both in the cytosol and in endosomes, act as cellular sensors against viral infections, and their engagement leads to the initiation of anti-viral responses including RNA degradation, production of type I IFNs and inhibition of protein synthesis. These include Toll-like receptors (TLRs) 3, 7 and 8, the serine/threonine kinase PKR and the RIG-I-like receptors RIG-I and MDA5. These RNA sensors are activated by unusual RNA products derived from viral replication, such as long double-stranded (ds) RNA or triphosphate 5' ends, allowing the discrimination of host and viral RNAs [55,57]. For instance, recognition of long dsRNAs activates PKR to phosphorylate the translation initiation factor elF2α, leading to the direct inhibition of mRNA translation. Both TLR3 and TLR7 are primarily localized in endosomes, and while TLR3 detects long dsRNA, TLR7 is preferentially activated by G and U rich sequences in single-stranded RNAs [58]. Viral RNAs containing 5'-triphosphates instead of the 5'-cap, as well as dsRNAs are recognized in the cytoplasm by RIG-I, which in turn triggers the production of type I IFNs. MDA5 recognizes complex, structured RNAs, including dsRNAs and branched RNAs that are likely to be intermediates of viral replication [57].

Importantly, incorporation of modified nucleosides into viral RNAs can negatively affect recognition by nucleic acid sensors, resulting in an ablated inflammatory response [59]. For example, the m^6A modification can act as a molecular marker contributing to the ability of the cells to discriminate self *vs*. non-self RNA, since non-methylated RNA can more easily be recognized by viral sensors. The fact that m^6A deposition is crucially involved in modulating the recognition of self-nucleic acids was remarkably shown by the deletion of *Mettl3* in the hematopoietic compartment. The overall outcome of such deletion was bone marrow failure and embryonic lethality. Mechanistically, loss of m^6A resulted in the accumulation of aberrant endogenous dsRNAs, which in turn led to the unwarranted activation of an innate IFN response, contributing to the compromised hematopoietic output [60]. How the presence of m^6A is normally able to suppress the formation of endogenous dsRNAs remains to be uncovered.

RNA methylation is also exploited by some RNA viruses, including SARS-CoV-2, to mimic cellular RNA and evade recognition by the cytoplasmic RNA sensor RIG-I [61-63] (*Fig. 3*). The m^{6}A modification can also critically alter the outcome of infections by affecting not only viral, but also host mRNAs involved in antiviral responses. Indeed, the mRNA transcripts encoding for type I IFNs are m^{6}A-modified, and depletion of components of the m^{6}A machinery increased the abundance of IFN-α/β, leading to reduced viral replication [64].

Expression of RBPs in mast cells and other innate immune cells

A comparative analysis of the expression of Regnase, TTP, HuR and m^{6}A-related proteins across different innate immune cells based on RNA-sequencing data from the Immunological Genome Project (www.immgen.org) [65], revealed some level of both cell type and tissue specificity in the expression of several of these factors (*Fig. 4*). For example, among the different ZFP36 family members, *Zfp36* and *Zfp36l2* are expressed by most innate cells (including dendritic cells, macrophages, neutrophils, and mast cells), but *Zfp36l2* is especially high in basophils and eosinophils, although the reason for this difference and to what extent the different *Zfp36* family members have unique or overlapping functions remains to be fully understood. On the other hand, the genes encoding the m^{6}A readers *Ythdf1*, *Ythdf2*, and *Ythdf3* showed more similar and consistent expression across different cell types and conditions, except for generally lower expression of *Ythdf1* compared to the other family members (*Fig. 4*). However, the different paralogues of the YTHDF family are likely to act in a fully redundant manner [31,32], so it remains unclear at this stage if differences in the expression of one family member may have a biologically relevant impact.

More specifically about mast cells, these are innate immune cells with key effector functions in allergy and asthma. In response to a large variety of signals, these cells produce an array of mediators, including cytokines, chemokines and proteases that can influence the functionality of the surrounding tissues, the

recruitment and activation of other immune cell types, and the permeability of blood vessels [66]. Importantly, mast cells are exclusively tissue-resident cells, and as such they are not easily accessible for experimental purposes, a limitation that in part explains the many open questions that still remain about their physiological functions and regulation. Recent efforts to study directly the transcriptome and proteome of tissue-resident mast cells in mouse and human revealed that these cells appear to be rather dissimilar from other immune cell types, and to be suited to interact with the tissue microenvironment through the expression of many receptors, including for example adhesion molecules (CD312, SIGLEC6 and others) and molecules involved in the interaction with neurons, most notably MRGPRX2, which mediates mast cell degranulation in response to neuropeptides [67,68]. At least when compared to immune cells circulating in the blood, mast cells represent a very distinct cell population, both at transcriptome and proteome levels [67,68], although to what extent mast cells acquire distinct phenotypes in response to the tissue microenvironment remains to be fully understood.

As for the expression and function of RBPs in mast cells, browsing published transcriptome data revealed that resting, unstimulated murine mast cells derived from different tissues express high levels of all the Zfp36 family members (*Fig. 5*). Among the Regnase family members, *Zc3h12a* and *Zc3h12c* (Regnase-1 and Regnase-3) are detectable at variable extent depending on the tissue, while expression of *Zc3h12b* and *Zc3h12d* (Regnase-2 and Regnase-4) is negligeable. Most of the genes involved in m^6A metabolism and recognition are also well-expressed. Finally, apart from RIG-I (*Ddx58*), most RNA sensors had very low expression in mast cells [68], a finding mostly consistent with the proteome profiling of human mast cells from skin and fat, that could not identify expression of any RNA sensor except for RIG-I [67]. No major difference in the expression of the RBPs indicated in *Fig. 5* was observed when comparing inducible bone marrow–derived mucosal mast cells and constitutive, embryonic-derived connective tissue mast cells in the mouse, except for *Zfp36* that appeared to be more highly expressed in the constitutive subpopulation of mast cells [69]. While the pattern of expression of the abovementioned RBPs may provide some initial insights about their role in mast cells, their function remains for the most part unknown in

this specific cell type. At least some of the general mechanisms of regulation may probably be inferred from studies performed on other cell types. For instance, the paracaspase MALT1 was shown to inactivate Regnase-1 in T lymphocytes in response to TCR signaling [13]. Since activation of mast cells through the high-affinity IgE receptor (FcεRI) also requires MALT1 expression [70], it is probably not unreasonable to hypothesize that the MALT1-mediated inactivation of Regnase-1 might be important in mast cells to license full expression of inflammatory mediators upon IgE crosslinking. Another example is provided by TTP, whose expression was induced in mast cells by IL-4 signaling. This in turn limited TNF production in an ARE-dependent manner upon acute activation [71], suggesting that TTP may exert its negative regulatory effects on TNF in all cells in which both of these factors are expressed. However, the impact of TTP on mRNA expression was also shown to be at least in part dependent on the cell type, as revealed by the direct comparison of differential gene expression in macrophages and neutrophils lacking TTP [6], suggesting that the fine regulation of the different RBPs and their impact on myeloid cell functions requires further studies.

Concluding remarks and outstanding questions

Overall, extensive research in recent years has uncovered crucial roles of RNA methylation and RBPs in mast cells, macrophages and other myeloid cells and in the regulation of the immune system as a whole. However, the emerging complexity in the post-transcriptional regulation of gene expression also highlights the limitations in our ability to distinguish and dissect causes from consequences when it comes to mechanistic, molecular analyses. As exemplified by the instances in which different RBPs (and miRNAs) cross-regulate each other's expression, perturbation in the expression of one single factor may determine an abundance of both direct and indirect effects that may be very difficult to untangle.

One key question that remains to be understood for many RBPs belonging to the same family is the issue of how unique or redundant the functions of the different family members might be. For instance, while

proteins belonging to the YTHDF family were shown to act in a redundant, compensatory manner [31,32], the different mechanisms of transcriptional, post-transcriptional and post-translational regulation of proteins of the Regnase family suggest that the different family members may have at least some unique functions. For example, Regnase-1 and Regnase-3 were shown to localize in different subcellular compartment in macrophages, with Regnase-1 being predominantly associated with P bodies and the endoplasmic reticulum, while Regnase-3 was predominantly associated with endosomes [14]. The mechanism leading to such different localization and the functional outcome of such compartmentalized expression are not known, although one possibility that was put forward is that Regnase-3 may be involved in the degradation of foreign nucleic acids [14]. Another outstanding question regarding Regnase proteins is the issue of binding *vs.* activity on particular transcripts. For instance, Regnase-3 was shown to bind the 3'UTR of the *Ifng* mRNA, despite being unable of regulating its expression [14]. Such discrepancy between binding and activity was observed only for some targets, and how widespread this effect is remains to be investigated. Nevertheless, these results suggest that Regnase-3 may require additional signals to activate its enzymatic functions or that alternatively, it requires interactions with specific co-regulators that still require identification.

An additional complication is provided by the fact that recent large RNA-protein interaction studies have uncovered hundreds of additional RBPs with unknown functions and mechanism of action. Many of these proteins also lack conventional RNA-binding domains, indicating that the logic behind probably the majority of RNA-protein interactions in the immune system still remains to be unraveled. Many of these RBPs bind RNA through intrinsically disordered protein regions, characterized by low sequence complexity, a low proportion of bulky hydrophobic amino acids and a high proportion of polar and charged amino acids [72]. Interestingly, due to their physical properties, intrinsically disordered proteins frequently interact with many other partners and may represent "hubs" of promiscuous protein-protein and protein-nucleic acid interaction networks that makes studying their functions particularly challenging [73]. In the future, the study of these proteins in the context of immune responses will undoubtedly provide

a better understanding of the mechanisms underlying the regulation of immune responses, potentially opening new possibilities of immune-modulation in disease.

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Conflict of Interest Statement

Nothing to declare

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Figure Legends

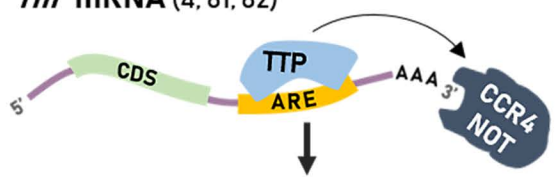
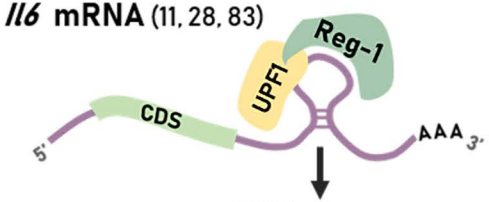
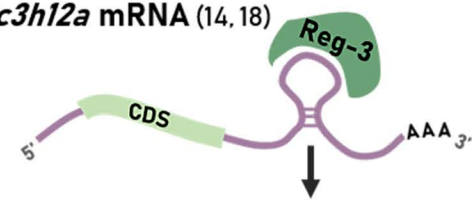
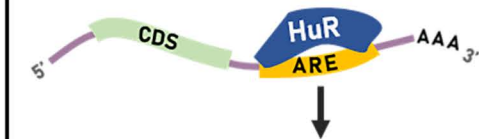
Figure 1. Role selected RBPs in macrophages. Summary of the phenotype resulting from deleting the indicated RBPs either globally in all mouse tissues or selectively in myeloid cells using the indicated Cre transgenes. The mechanism of action on selected mRNA targets is also summarized.

Figure 2. Post-translation regulation of selected RBPs. Example of mechanisms that contribute to modulate RBP activity at a post-translational level. Phosphorylation of TTP, Regnase-1 and HuR (top, clock-wise) was shown to impact protein activity, stability and subcellular localization. Methylation of HuR may be linked to increased stability of the HuR-bound mRNAs, although this remains to be formally demonstrated (hence the question mark), while Regnase-1 undergoes proteolytic cleavage mediated by the paracaspase MALT1.

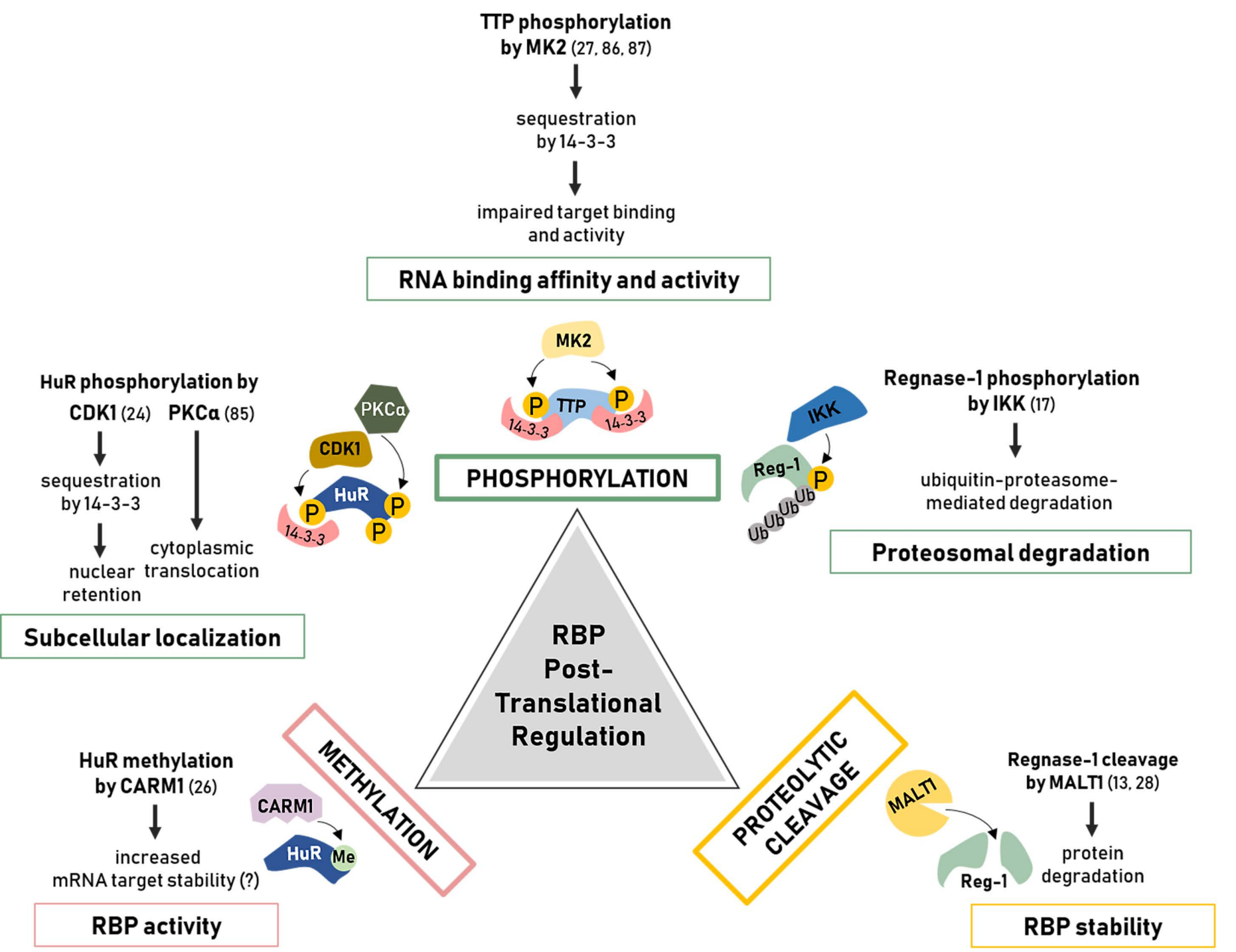
Figure 3. m^6A modification and RNA sensing. A methyltransferase complex minimally composed of METTL3 and METTL14 leads to adenosine methylation in mRNAs, while enzymes such as FTO and ALKBH5 can function as m^6A demethylases. Although not depicted, the deposition of m^6A occurs primarily in the cell nucleus. The presence of m^6A in viral RNAs can impede recognition by the RNA sensor RIG-I, thereby allowing viral escape from an innate immune response.

Figure 4. Expression of mRNAs encoding selected RBPs across the myeloid compartment. The heatmap shows the relative expression of selected RBPs across myeloid cell types obtained from different tissues as indicated. Expression data were obtained from the Immunological Genome Project.

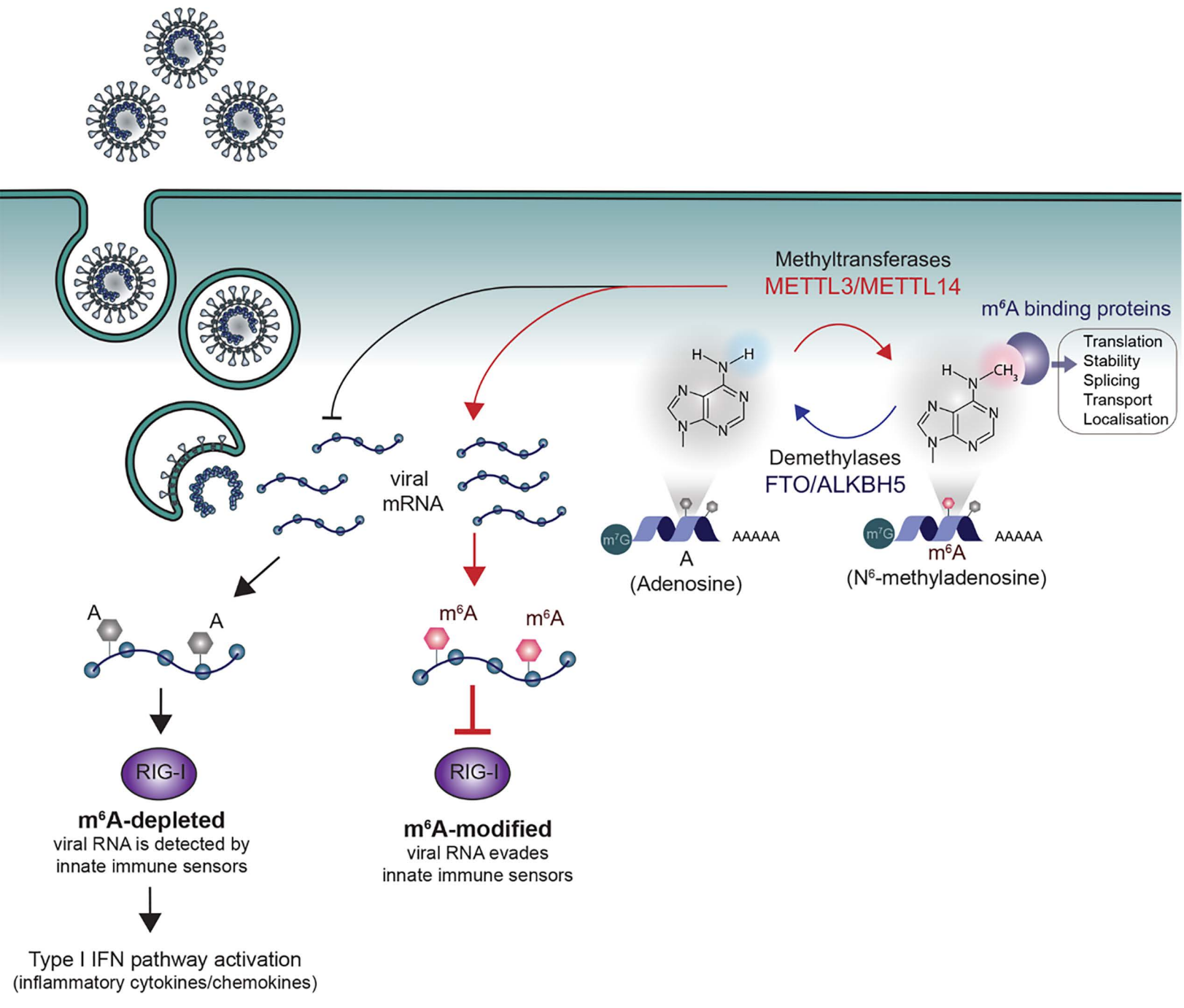
Figure 5. Expression of mRNAs encoding selected RBPs in mast cells. Expression data are from Ref. [68], and only skin mast cells are shown. *Eif2ak2* encodes for PKR and *Ifih1* for MDA5. Other genes are defined in the text.

Immunomodulatory RBPs	TTP	Regnase-1	Regnase-3	HuR
Global KO mouse phenotype	Early-onset severe inflammatory phenotype (arthritis, dermatitis, conjunctivitis), cachexia, autoimmunity, myeloid hyperplasia (4)	Embryonic lethality, severe anemia, splenomegaly, lymphadenopathy, elevated cytokine production, multi-organ inflammation (11)	Severe lymphadenopathy, systemic increase in IFN signaling, suppressed follicle and germinal center formation (14)	Embryonic lethality, atrophy of immune and hematopoietic organs, disruption of intestinal integrity (74, 75)
Myeloid cell-specific KO phenotype (LysM-Cre + *RBP gene*$^{fl/fl}$)	LysM-Cre+*Zfp36*$^{fl/fl}$ Extreme susceptibility to low-dose endotoxin challenge, elevated TNF levels (5)	LysM-Cre+*Mcpip1*$^{fl/fl}$ Late-onset spontaneous inflammatory syndromes, extreme susceptibility to endotoxin-induced inflammation and lung injury, premature death (28)	LysM-Cre+*Regnase-3*$^{fl/fl}$ Lymphadenopathy (14)	LysM-Cre+*Elav1*$^{fl/fl}$ Systemic pathologic inflammation (endotoxemia, exacerbated inflammatory profile, colitis, and colitis-associated cancer) (20)
Mechanism of action	***Tnf* mRNA** (4, 81, 82)  **mRNA degradation** Via recruitment of CCR4-NOT complex	***Il6* mRNA** (11, 28, 83)  **mRNA cleavage** Via intrinsic RNase activity	***Zc3h12a* mRNA** (14, 18)  **mRNA cleavage** Via intrinsic RNase activity	***Tnf* mRNA** (19, 20, 79, 80)  **mRNA stabilization**

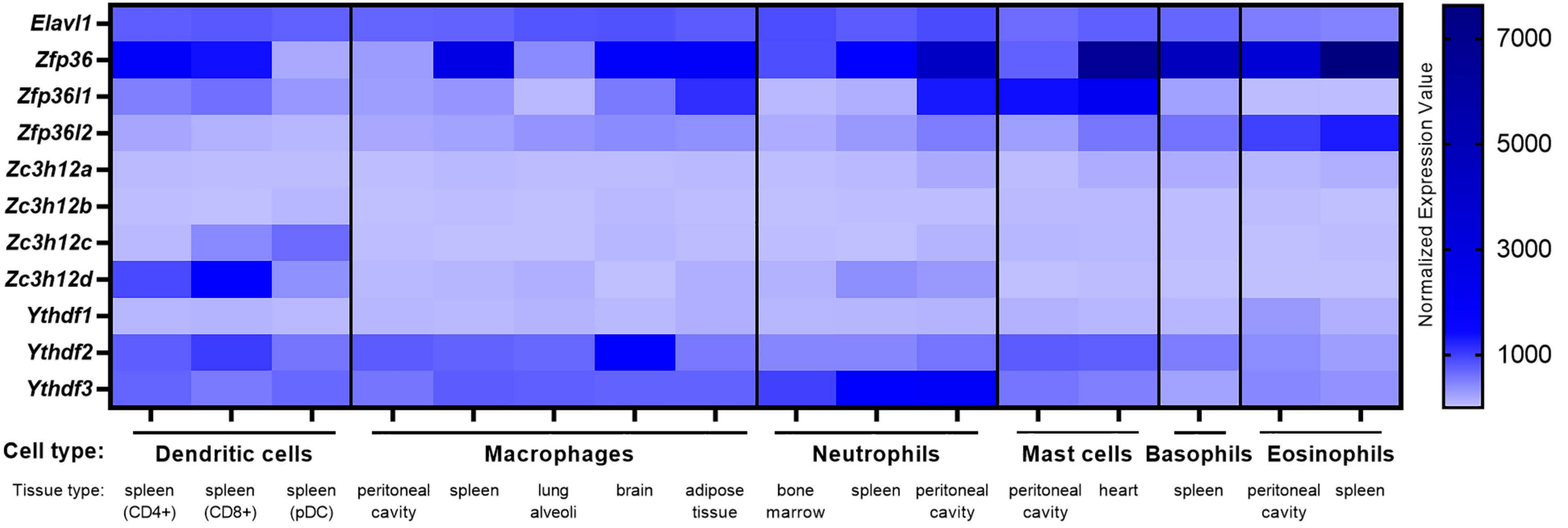
Bataclan et al., Figure 1



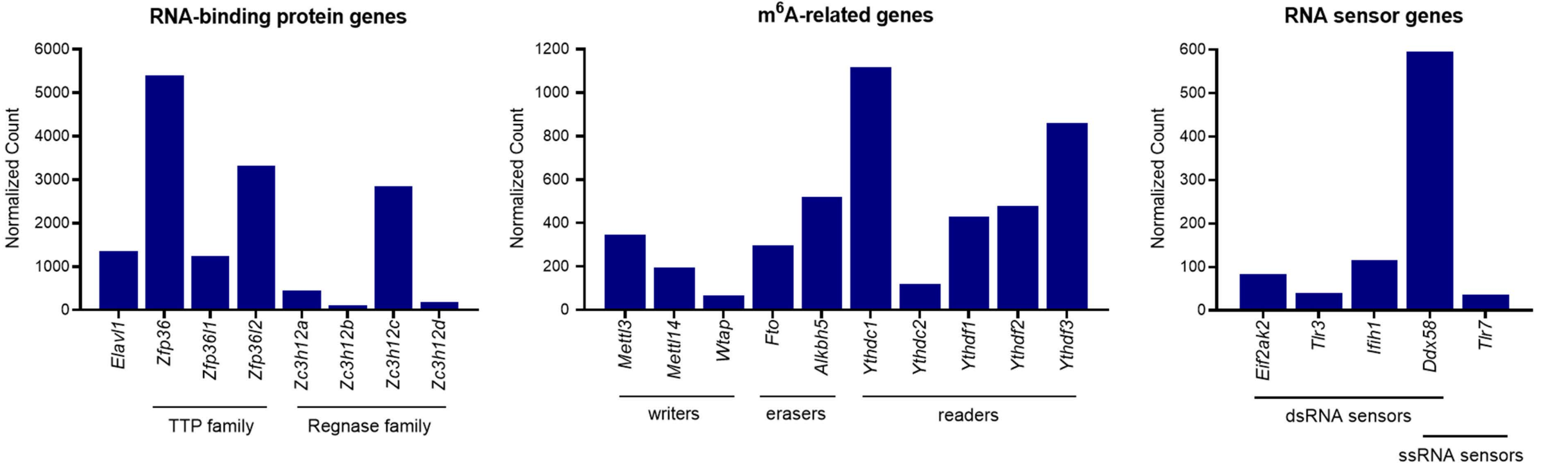
Bataclan et al., Figure 2



Bataclan et al., Figure 3



Bataclan et al., Figure 4



Bataclan et al., Figure 5