



RIG-I-like receptors: their regulation and roles in RNA sensing

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Abstract | Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are key sensors of virus infection, mediating the transcriptional induction of type I interferons and other genes that collectively establish an antiviral host response. Recent studies have revealed that both viral and host-derived RNAs can trigger RLR activation; this can lead to an effective antiviral response but also immunopathology if RLR activities are uncontrolled. In this Review, we discuss recent advances in our understanding of the types of RNA sensed by RLRs in the contexts of viral infection, malignancies and autoimmune diseases. We further describe how the activity of RLRs is controlled by host regulatory mechanisms, including RLR-interacting proteins, post-translational modifications and non-coding RNAs. Finally, we discuss key outstanding questions in the RLR field, including how our knowledge of RLR biology could be translated into new therapeutics.

Toll-like receptors (TLRs). A family of membrane-bound innate immune receptors that recognize various bacterial or virus-derived pathogen-associated molecular patterns. These sensors are crucial for eliciting an early innate immune response and for modulating adaptive immunity.

Type I interferons are highly potent cytokines that were initially identified for their essential role in antiviral defence^{1,2}. They shape both innate and adaptive immune responses and induce the expression of restriction factors, which are proteins that directly interfere with a step in the life cycle of a virus³. Both beneficial and detrimental roles for type I interferons have been described in the context of bacterial infections and cancer^{2,4–6}, and these cytokines are also linked to the development of several autoinflammatory and autoimmune diseases⁷ and metabolic syndromes⁸. Therefore, the study of the biology of type I interferons has many implications for human health and disease.

The human genome encodes 17 different type I interferons, including 13 types of interferon- α (IFN α), as well as IFN β , IFN ϵ , IFN κ and IFN ω . Type I interferons are secreted and then signal in an autocrine and paracrine manner by binding to the type I interferon receptor (interferon- α/β receptor (IFNAR)) that activates the intracellular Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway and activator of transcription (JAK–STAT) pathway, particularly STAT1 and STAT2 (REFS^{9,10}). Upon phosphorylation, STAT1 and STAT2 combine with interferon regulatory factor 9 (IRF9) to form the interferon-stimulated gene factor 3 (ISGF3) transcriptional complex¹¹. ISGF3 binds to interferon-stimulated response elements in type I interferon-dependent gene promoters and thereby drives the expression of hundreds of type I interferon-stimulated genes (ISGs). The proteins encoded by ISGs mediate the effects of type I interferons; for example, some ISGs encode virus restriction factors.

Under homeostatic conditions, type I interferons are expressed at very low and often undetectable levels.

Their expression is rapidly induced by several innate immune signalling pathways. These signalling events are typically initiated by proteins called nucleic acid sensors that monitor cells for unusual nucleic acids. For example, it is an indispensable step in the life cycle of any virus to introduce a genome consisting of DNA or RNA into the infected cell. Nucleic acid sensors survey different subcellular compartments and include the endosomal Toll-like receptors (TLRs) TLR3, TLR7, TLR8 and TLR9; the cytosolic DNA sensor cyclic GMP–AMP synthase (cGAS); and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs)^{12,13}. Once activated, these and other sensors engage converging signalling cascades that lead to transcriptional induction of the genes encoding type I interferons and other immune genes (FIG. 1).

RLRs are RNA sensors localized in the cytosol^{12,13}. This protein family encompasses three members: RIG-I, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). All RLRs have a central helicase domain and a so-called carboxy-terminal domain (CTD) (FIG. 2a). These two domains work together to detect immunostimulatory RNAs. RIG-I and MDA5 additionally have two amino-terminal caspase activation and recruitment domains (CARDs), which mediate downstream signal transduction. LGP2 lacks the CARDs and is widely believed to regulate RIG-I and MDA5 (BOX 1). It is now over 15 years since RIG-I and MDA5 were discovered in overexpression studies to induce type I interferons^{14,15}. Soon after their description, knockout studies in mice and mouse-derived cells established that RIG-I and MDA5 are essential for antiviral defence and type I interferon induction in virus infection models^{16,17}. It is now

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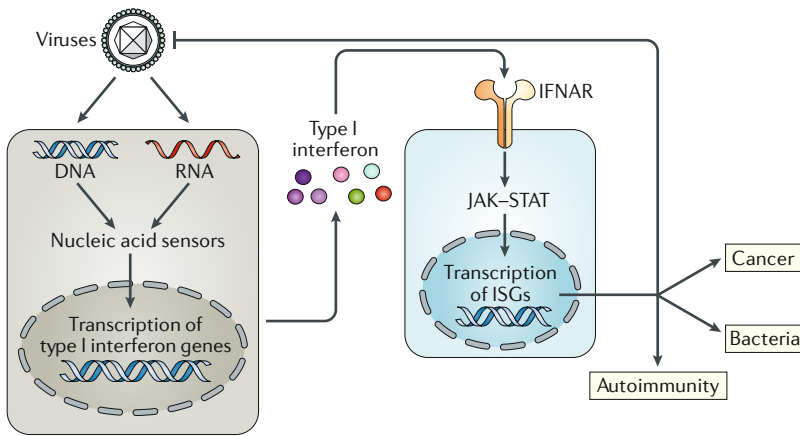


Fig. 1 | The type I interferon system. Nucleic acid sensors of the innate immune system recognize unusual DNA and RNA molecules, for example, viral genomes. This results in the triggering of an intracellular signalling cascade that transcriptionally induces the genes encoding type I interferons, which are subsequently secreted. Type I interferons act in an autocrine (not shown) and paracrine manner by binding to their receptor, interferon- α/β receptor (IFNAR), which activates the Janus kinases (JAKs). These in turn activate the transcription factors signal transducer and activator of transcription 1 (STAT1) and STAT2, leading to expression of interferon-stimulated genes (ISGs). Several ISGs have direct antiviral effects. Type I interferons and ISGs also play important roles in bacterial infections and cancer; these effects can be both beneficial and detrimental, depending on the setting. Finally, production of type I interferons and ISGs over extended periods of time can lead to autoinflammatory and autoimmune diseases.

clear that infections with almost all major virus families are recognized by RLRs (TABLE 1).

Much work has also been done to delineate the structural biology of RLR activation. The structure of full-length RIG-I without RNA ligand showed that RIG-I is in a signalling-repressed, closed conformation that is mediated by specific contacts of CARD2 with an inserting domain located between the two RecA-like helicase domains of RIG-I¹⁸. When bound to RNA ligand, the helicase domain of RIG-I tightly wraps around the RNA in a C-clamp-like fashion. ‘Pincer’ helical elements found in the helicase of RIG-I connect to the CTD and are believed to trigger RNA binding-dependent conformational changes in RIG-I that expose the CARDS for signalling. The crystal structure of 2CARD-deleted RIG-I, or the isolated CTD, bound to RNA revealed specific contacts between the CTD and the 5’-terminal region of the RNA^{18–21}. The current model is that RIG-I binds to RNA in its monomeric form; however, signalling of RIG-I via its CARDS requires RIG-I oligomers that are nucleated and stabilized by non-degradative K63-polyubiquitin chains (as discussed in detail below). In contrast to RIG-I, MDA5 is believed to adopt a more open conformation in the absence of RNA ligand. In the presence of long double-stranded RNA (dsRNA), MDA5 assembles into helical ‘filaments’, which are MDA5 polymers^{22–24}.

Upon RNA binding and oligomerization^{24–26}, RLRs interact with the CARD domain found in mitochondrial antiviral-signalling protein (MAVS), which serves as the essential adaptor protein for RLR signal transduction. MAVS then activates TANK-binding kinase 1 (TBK1) and I κ B kinase- ϵ (IKK ϵ), which in turn activate IRF3 and IRF7; these, together with nuclear factor- κ B (NF- κ B),

then induce transcription of the genes encoding type I interferons and other antiviral or immunoregulatory genes^{12,13} (FIG. 2b).

In this Review, we focus on recent developments in our understanding of the mechanisms by which RLRs are activated. First, we discuss the types and properties of RNA molecules that trigger RIG-I and MDA5 activation. Interestingly, these proteins not only detect viral RNA but also cellular RNAs that are unusual, mislocalized or misprocessed. Such disturbances of cellular RNA homeostasis can be an indirect molecular signature of infection or occur in sterile inflammatory pathologies. In the second part of the article, we highlight the regulation of RLR activity by host mechanisms such as post-translational modifications (PTMs), interacting proteins and cellular non-coding RNAs. Tight control of RLRs is important both to prevent detrimental type I interferon responses⁷ in the absence of infection and to allow rapid and sensitive recognition of pathogens.

RLR recognition of immunostimulatory RNA

RLRs are expressed in most cell types and are primarily located in the cytosol, although recent studies showed that RIG-I may also localize to the cell nucleus^{27,28}. They induce type I interferon responses upon recognition of RNA molecules that are usually not present in mammalian cells, for example, the genomes of RNA viruses. In this section, we discuss the properties that mark RNA molecules as immunostimulatory and the principles explaining why RLRs are not activated by the abundant RNA content of the cytosol of healthy cells. The mechanisms by which RLRs distinguish between normal RNAs found in cells under homeostatic conditions and immunostimulatory RNAs are important for our understanding of how type I interferon responses are initiated during virus infection and in sterile pathologies. Furthermore, fundamental insights into RLR activation have led to the development of synthetic RLR agonists, which may hold therapeutic value for viral and malignant diseases, as well as vaccination (BOX 2).

Activation of RIG-I by viral RNAs. Since the discovery of RIG-I, much research has been done to unravel the molecular and structural requirements for RIG-I activation by immunostimulatory RNAs. Collectively, this work shows that RIG-I monitors the 5’ ends of RNA molecules for several biochemical features, a process involving both the CTD and helicase domain (FIG. 2c). The first breakthrough was the finding that RNA molecules bearing a triphosphate (PPP) group at their 5’ end activate RIG-I^{29,30}. This observation was subsequently extended to include additional properties of RNA 5’ ends that determine RIG-I activation. First, base pairing of the 5’-PPP-containing RNA molecule to a complementary strand of RNA is required for RIG-I activation, with the most potent RNA agonists forming a blunt end^{31,32}. Second, RNAs with uncapped diphosphate (PP) groups at the 5’ end activate RIG-I, much like RNAs with 5’-PPP moieties³³. Third, the 5’-terminal nucleotide needs to be unmethylated at its 2’-O position to allow RNA recognition by RIG-I³⁴. These functional data are nicely underpinned by structural studies of RIG-I.

Cyclic GMP-AMP synthase (cGAS). A key intracellular innate immune receptor that recognizes DNA from invading pathogens or cellular DNA that is mislocalized inside the cell. When activated, cGAS enzymatically synthesizes cyclic GMP-AMP, which acts as a second messenger to trigger type I interferon induction via stimulator of interferon genes (STING).

Negative-sense RNA viruses

Viruses containing single-stranded RNA in negative polarity as their genomes. Viral RNA-dependent RNA polymerases copy these genomes into reverse-complementary, positive-sense RNA, which is then translated into viral proteins.

The CTD has a pocket binding specifically to 5'-PPP or 5'-PP groups and also contacts the unmethylated 2'-O group of the first nucleotide^{20,35–37}. The base-paired portion of the RNA forms contacts with the helicase domain in RIG-I^{18,19,21}. The helicase domain has ATPase activity, and ATP hydrolysis determines the dissociation rate of RIG-I from RNA^{37–40}. Stable RIG-I–RNA interaction displaces the CARDS, which in the inactive RIG-I conformation interact with parts of the helicase domain¹⁸. As a consequence, the CARDS from multiple RIG-I proteins oligomerize and become accessible for interaction with MAVS, resulting in downstream signalling (reviewed elsewhere⁴¹).

Importantly, the molecular features of RIG-I-stimulatory RNAs (5'-(P)PP, duplex structure, no ribose 2'-O-methylation) are found in several viral RNA

molecules but are typically absent in the vast majority of host cytosolic RNAs. For example, cellular mRNAs acquire a cap structure involving 2'-O-methylation (that is, cap 1 or cap 2) in the cell nucleus following transcription, before export to the cytoplasm. Similarly, 28S and 18S ribosomal RNAs (rRNAs) and tRNAs are processed in the cell nucleus and their mature, cytosolic forms typically only have a single phosphate at the 5' end. The 5S rRNA retains a 5'-PPP moiety but is methylated and extensively complexed with proteins. Therefore, such RNAs do not activate RIG-I; in fact, 5'-monophosphate RNAs have recently been suggested to block RIG-I⁴².

In contrast to cytosolic cellular RNAs, several viral RNAs have features characteristic of RIG-I agonists. The genomes of many negative-sense RNA viruses contain partially self-complementary 5' and 3' ends, which base pair to form duplex structures called 'pan-handles'³¹. In addition, such viral RNAs often have an unmethylated 5' end that bears a PPP group, derived from the ribonucleoside triphosphate used by viral polymerases during initiation of genome replication. Probably the best studied example of this is influenza A virus (IAV), and work by several groups has shown that RIG-I detects IAV genomes in infected cells^{43–46}. More recently, small aberrant viral RNAs produced by the IAV polymerase were shown to also activate RIG-I; these RNAs — much like full-length genomes — have a 5'-PPP group and form a duplex structure⁴⁷. In addition to negative-sense RNA viruses, infections with some positive-sense RNA viruses are also detected by RIG-I. For example, these infections include dengue and Zika viruses of the Flaviviridae family, and involve recognition of nascent viral genomes containing 5'-PPP groups prior to capping^{48,49}. Activation of RIG-I by viral RNAs not containing the typical features of RIG-I agonists has also been proposed; for example, a uridine-rich stretch in the hepatitis C virus (HCV) RNA genome located at a distance from the 5' end was shown to activate RIG-I⁵⁰. It is noteworthy that the presence of a 5'-PPP group on viral RNAs not only makes these 'visible' to RIG-I but also allows restriction factors, including IFIT family proteins, to target these RNAs (reviewed elsewhere⁵¹).

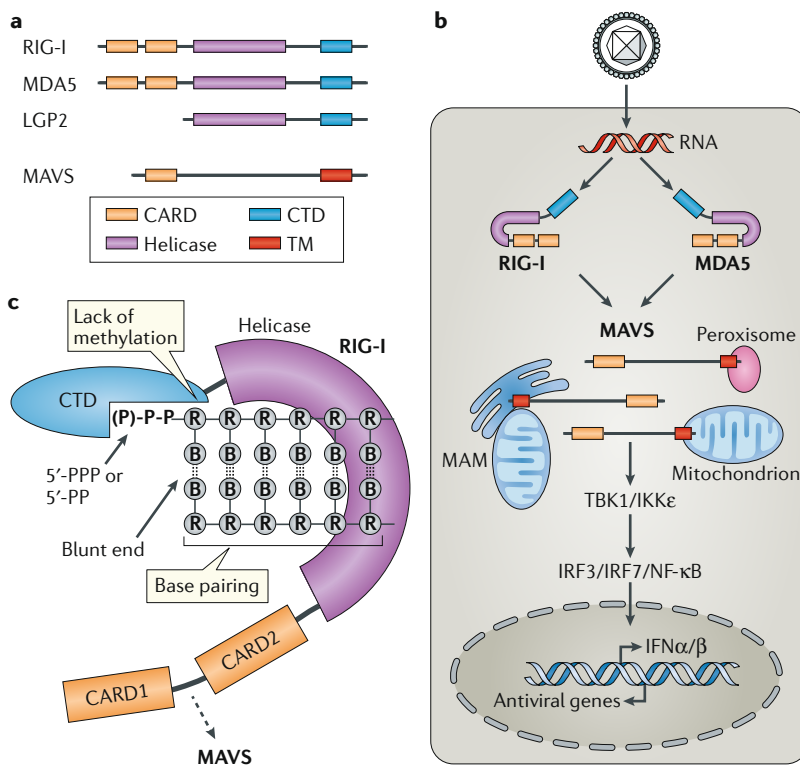


Fig. 2 | RIG-I-like receptors. a | Domain architecture of retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and mitochondrial antiviral-signalling protein (MAVS). The three members of the RLR family have a central helicase domain and form a subfamily of SF2 helicases. An additional RNA-binding fold, called the carboxy-terminal domain (CTD), is also found in all RLRs. RIG-I and melanoma differentiation-associated protein 5 (MDA5) contain two caspase activation and recruitment domains (CARDS) in tandem orientation at their N termini. The signalling adaptor protein MAVS also has a CARD as well as a C-terminal transmembrane domain (TM). **b** | RLR signalling pathway. RIG-I and MDA5 are activated by immunostimulatory RNA, for example, viral RNAs. They then undergo conformational changes that expose and multimerize their CARDS (not shown for simplicity), which allows homotypic CARD–CARD interactions with MAVS. MAVS is anchored with its TM into mitochondria, mitochondrial-associated membranes (MAMs) and peroxisomes, and relays the signal to TANK-binding kinase 1 (TBK1) and IκB kinase-ε (IKKε). These in turn activate interferon regulatory factor 3 (IRF3) and IRF7, which together with the transcription factor nuclear factor-κB (NF-κB) induce the expression of type I interferons and other genes. **c** | RIG-I is shown in its active conformation, bound to an immunostimulatory RNA. The molecular features of RIG-I-stimulatory RNAs are also illustrated; please see text for details. B, nucleobase; IFNα/β, interferon-α/β; LGP2, laboratory of genetics and physiology 2; P, phosphate; PP, diphosphate; PPP, triphosphate; R, ribose.

Activation of RIG-I by host RNAs. Recent work has revealed that virus infection can also result in RIG-I activation by host cellular RNAs, instead of viral RNAs^{52,53}. These studies set out to explain earlier observations that cells infected with herpesviruses, a family of double-stranded DNA viruses, induce type I interferon and pro-inflammatory cytokine responses partially dependent on RLRs. RIG-I immunoprecipitation from cells infected with herpes simplex virus type 1 (HSV-1) or Kaposi's sarcoma-associated herpesvirus (KSHV), followed by high-throughput sequencing of co-purifying RNAs, revealed the association of cellular non-coding RNAs with RIG-I^{52,53}. In the case of HSV-1, 5S rRNA pseudogene transcripts, and particularly *RNA5SP141*, were most highly enriched in RIG-I immunoprecipitations⁵². In contrast to the parental 5S rRNA, the biological functions of these 5S rRNA pseudogene transcripts, which have 5'-PPP groups and self-complementary regions, are poorly understood.

Box 1 | The immunoregulatory roles of LGP2

Similarly to retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), laboratory of genetics and physiology 2 (LGP2) — the third RIG-I-like receptor (RLR) family member — has a central DECH-box helicase domain with ATP hydrolysis activity and a carboxy-terminal domain, both involved in RNA binding and conformational rearrangements. However, LGP2 lacks the amino-terminal tandem caspase activation and recruitment domain, and thus lacks independent signal-transducing activity. A series of studies showed that LGP2 regulates the activities of RIG-I and MDA5 in a positive and negative manner (reviewed in detail elsewhere¹⁶⁸). LGP2 can function as a feedback inhibitor of RIG-I signalling, likely through various mechanisms that include sequestration of immunostimulatory RNA, prevention of RIG-I binding to mitochondrial antiviral-signalling protein (MAVS), and inhibition of RIG-I ubiquitylation by the E3 ligase tripartite motif-containing 25 (TRIM25)^{169,170}. On the other hand, LGP2 promotes MDA5-mediated antiviral interferon responses. Mice lacking LGP2 were shown to be more susceptible to certain RNA viruses (for example, encephalomyocarditis virus (EMCV)) that are mainly recognized by MDA5 (REF.¹⁷¹). In accord, cells derived from LGP2-deficient mice failed to trigger an effective interferon response upon EMCV infection. Structural and functional studies showed that LGP2, through cooperative binding, promotes the nucleation of MDA5 oligomerization on double-stranded RNA (dsRNA) and, thereby, MDA5 signalling^{172,173}. In addition to its role in RLR-mediated immunity, LGP2 has been implicated in the inhibition of TNF receptor associated factor (TRAF) proteins¹⁷⁴ and the regulation of T cell function during viral infection¹⁷⁵. Moreover, a recent study showed that LGP2 suppresses antiviral RNA interference in mammals by preventing Dicer-mediated processing of long dsRNA¹⁷⁶.

HSV-1 infection unmasks these cellular non-coding RNAs for RIG-I recognition in two ways. First, 5S rRNA pseudogene transcripts mislocalize to the cytoplasm in HSV-1-infected cells. Second, HSV-1-induced host shut-off leads to downregulation of the mRNAs that encode specific binding proteins of *RNA5SP141*; these are TST and MRPL18 (REF.⁵²). Silencing of *RNA5SP141* by small interfering RNAs or antisense oligonucleotides diminished cytokine responses during infection by HSV-1 and also upon reactivation of Epstein–Barr virus, another herpesvirus. It is noteworthy that the type I interferon response to IAV, which replicates in the cell nucleus, is partially dependent on *RNA5SP141* (REF.⁵²), suggesting that both viral RNA and cellular *RNA5SP141* contribute to full RIG-I activation during IAV infection.

RNA5SP141 is transcribed by the cellular RNA polymerase III (Pol III) and an independent set of studies reported that Pol III also transcribes AT-rich viral DNA sequences into 5'-PPP-containing, RIG-I-stimulatory RNAs^{54–56}. In the case of lytic KSHV infection, vault RNAs (vtRNAs) were highly enriched in RIG-I precipitates⁵³. vtRNAs are conserved and participate in the formation of cytoplasmic ribonucleoprotein particles called vaults⁵⁷. vtRNAs are highly self-complementary, are transcribed in the cell nucleus and have a 5'-PPP group from the initiating ribonucleotide. However, dual specificity phosphatase 11 (DUSP11) usually removes two phosphates from the 5' end of vtRNAs, creating 5'-monophosphate ends⁵⁸. Lytic KSHV infection results in reduced levels of DUSP11 and in the accumulation of 5'-PPP-bearing vtRNAs that then activate RIG-I⁵³. Interestingly, DUSP11 levels are not affected during latent infection⁵³. Given that RIG-I activation prevents lytic KSHV infection⁵³, it is possible that a pathway involving DUSP11, vtRNAs, RIG-I and type I interferons determines the balance between lytic and latent

infection. It is noteworthy that HIV-1 may also down-regulate DUSP11 by means of the viral Vpr protein⁵⁹. Concomitantly, non-coding cellular Y-RNAs were found to accumulate in their 5'-PPP-containing form and to bind to RIG-I in HIV-1-infected cells⁵⁹. Moreover, endogenous RNAs lacking the canonical features of RIG-I agonists, including RNAs cleaved by RNase L⁶⁰ and microRNAs (miRNAs)⁶¹, have been suggested to activate RIG-I in virus-infected cells. Finally, endogenous retroviral elements were shown to activate RIG-I via de-SUMOylation of the host transcriptional repressor tripartite motif-containing 28 (TRIM28; also known as KAP1)⁶².

Activation of MDA5 during viral infection. How MDA5 is activated during virus infection is less well understood and is only briefly described here in light of a recent review on the subject⁶³. Infection with many different types of virus, including RNA viruses and DNA viruses, results in the triggering of MDA5 signalling. MDA5 is essential for type I interferon induction during infection with picornaviruses^{16,17}, a family of positive-sense RNA viruses. For many other viruses, MDA5 appears either partially redundant with other sensors of infection or acts in a temporally distinct manner⁶³. In many instances, infection with viruses that activate MDA5 results in the accumulation of dsRNA in the infected cell, which has been proposed to activate MDA5. However, studies analysing RNAs bound by MDA5 in infected cells reported that MDA5 interacts predominantly with one RNA strand of viral RNA^{64,65}. Additionally, surrogate LGP2 immunoprecipitation identified encephalomyocarditis virus (EMCV) L-region antisense RNA as an MDA5 ligand⁶⁶. Future work, perhaps using iCLIP (individual-nucleotide resolution ultraviolet crosslinking and immunoprecipitation) and other techniques to identify MDA5-associated RNAs, will be required to understand whether virus infection generates a specific 'RNA molecular pattern' for MDA5 recognition, as seen for RIG-I.

Activation of RLRs in sterile conditions. RLR activation has been reported in several autoinflammatory and autoimmune diseases, as well as in cancer. This activation can occur via two distinct mechanisms. In one mechanism, RLR mutations result in spontaneous signalling in the absence of virus infection. Alternatively, alterations in RNA metabolism can generate RLR-stimulatory RNAs; for example, mislocalization or defects in RNA processing can result in the detection of endogenous RNAs.

We start by discussing the first setting, which is illustrated by two rare genetic diseases: Singleton–Merten syndrome and Aicardi–Goutières syndrome. Patients affected by these diseases produce type I interferon over extended periods of time⁷. Activating mutations in the genes encoding RIG-I and MDA5 (*DDX58* and *IFIH1*, respectively) have been identified in some patients with Singleton–Merten syndrome or Aicardi–Goutières syndrome^{67–70}. Some of these mutations may change the conformation of RLRs in such a way that spontaneous signalling to MAVS occurs in an RNA-independent manner^{69,71}. Furthermore, RLR mutations may result

Host shut-off

A process by which viruses prevent expression of cellular proteins. This may involve degradation of cellular transcripts or disruption of protein translation by viral factors. Host shut-off is believed to facilitate viral gene expression.

Latent infection

An often lifelong form of herpesvirus infection during which no infectious virus is produced. Viral DNA is maintained in the nucleus of infected cells and a small set of viral RNAs and proteins may be expressed.

Y-RNAs

Small non-coding, structured RNAs that are components of the Ro60 ribonucleoprotein complex and play roles in DNA replication.

MicroRNAs

(miRNAs). Cellular non-coding, single-stranded RNAs of 17–24 nucleotides that are found in most organisms and that silence gene expression via direct targeting of complementary messenger RNAs.

iCLIP

(Individual-nucleotide resolution ultraviolet crosslinking and immunoprecipitation). A method used to identify and map RNA–protein interactions in which ultraviolet light induces covalent bonds between the protein and RNA binding partners.

***Alu* elements**

Endogenous retroelements found in more than 1 million copies in the human genome. *Alu* elements rarely retrotranspose but can be transcribed. Adjacent *Alu* elements in inverted orientation can give rise to long duplex RNA structures.

Single-nucleotide polymorphisms

Substitutions of a single nucleotide at a specific position compared with the reference genome. Single-nucleotide polymorphisms may affect the expression level or amino acid sequence of a protein.

in aberrant detection of normal cellular RNAs^{38,39,70,72}. For example, long hairpin RNAs derived from inverted repeat *Alu* elements have been suggested to activate mutant forms of MDA5 found in Aicardi–Goutières syndrome⁷². In addition to RLR mutations in rare diseases, *IFIH1* single-nucleotide polymorphisms have been associated with many common, inflammatory diseases, including type 1 diabetes mellitus^{73–79}. These *IFIH1* single-nucleotide polymorphisms are likely to induce heightened type I interferon levels, but whether this involves constitutive activation or increased responses to infections remains to be determined.

Much progress has been made over the past few years connecting changes in RNA metabolism to RLR activation. For example, mislocalization of mitochondrial RNA (mtRNA) into the cytosol leads to MDA5 activation⁸⁰. This occurs when the function of SUV3 and PNPase, mitochondrial enzymes involved in the degradation of mtRNA, is compromised and results in chronic type I interferon production, including in patients with mutations in the gene encoding PNPase⁸⁰. The mechanism by which mtRNA escapes the mitochondria and gains access to the cytosol in this setting remains to be determined. In addition to mislocalization of cellular RNAs, defects in A-to-I RNA editing result in activation of MDA5. Indeed, adenosine deaminase acting on RNA 1 (ADAR1) deficiency in humans and mice causes chronic type I interferon production and inflammatory pathology^{81,82}. These effects require MDA5, suggesting that editing of endogenous RNAs by ADAR1 prevents engagement of MDA5 (REFS^{83–85}). Unedited duplex RNAs derived from inverted repeat *Alu* elements

have been proposed to be a major MDA5 stimulus in ADAR1-deficient cells⁷². Interestingly, some types of tumour are dependent on ADAR1: loss of ADAR1 in transformed cells can sensitize to immunogenic cell death, which involves MDA5-driven type I interferon production and protein kinase R (PKR)-mediated effects on cell viability^{86–88}. Finally, in contrast to the cell-autonomous scenarios discussed above, RLR activation by endogenous RNAs can also occur in a cell non-autonomous manner. In a breast cancer model, the tumour microenvironment results in increased production of the non-coding, 5′-PPP-containing *RN7SL1* RNA in stromal cells, followed by transfer of this RNA via exosomes to cancer cells, resulting in RIG-I activation⁸⁹. Of note, in this specific setting, RIG-I activation and type I interferon induction were shown to promote cancer progression⁸⁹.

RLR regulation

Accurate recognition of immunostimulatory RNA and rapid signal activation by RLRs are crucial steps in the initiation of innate immunity. As such, the sensing and signalling activities of RIG-I and MDA5 need to be tightly controlled to allow for an effective antiviral defence when needed, while maintaining immune homeostasis under normal physiological conditions. The activity of RLRs is controlled by several mechanisms that include PTMs mediated by regulatory enzymes; regulation by interacting proteins (for example, co-receptor molecules); post-transcriptional mechanisms, in particular cellular non-coding RNAs; and autophagy.

Regulation by PTMs. Over the past decade it has become clear that the activities of RIG-I and MDA5 are dynamically controlled by several types of PTM, including ubiquitylation (both non-degradative and degradative ubiquitin-linkage types), conjugation of ubiquitin-like (UBL) proteins, phosphorylation, acetylation and deamidation (FIG. 3). Accordingly, several enzymes catalysing these PTMs have been identified and their roles in RLR-mediated innate immunity have been characterized in vitro and, for many, also in vivo.

The first PTM identified for RLRs was covalent K63-linked polyubiquitylation of RIG-I (at K172, the major site, as well as five other residues located in the tandem CARD)⁹⁰. Biochemical and functional studies have established that K63-linked ubiquitylation of RIG-I at the CARDS is important for MAVS binding and antiviral cytokine responses⁹⁰. Structural analysis showed that K63-linked polyubiquitin stabilizes the RIG-I CARDS in a signalling-active, oligomeric, ‘lock-washer’ conformation in which the ubiquitin chains are bound along the outer rim of the 2CARD tetramer. Both covalently attached and unanchored (meaning, non-covalently bound) K63-linked ubiquitin chains contribute to effective RIG-I oligomerization and signal transduction^{91,92}. TRIM25, from the tripartite motif (TRIM) protein family (BOX 3), has been shown to mediate RIG-I CARD K63-linked ubiquitylation, and studies in cells in which TRIM25 was silenced or knocked out showed impaired cytokine responses to diverse stimuli (for example, infection with Sendai virus, IAV or human papillomavirus 16,

Table 1 | Selected viral infections detected by RLRs

Baltimore classification	Virus family (examples)	RLR	Key refs
I dsDNA	Herpesviridae (herpes simplex virus type 1; Kaposi’s sarcoma-associated herpesvirus; Epstein–Barr virus)	RIG-I and MDA5	52–54,206–210
	Poxviridae (vaccinia virus)	RIG-I and MDA5	211–213
	Adenoviridae (adenovirus)	RIG-I	214,215
II ssDNA	No known examples that activate RLRs	NA	NA
III dsRNA	Reoviridae (rotavirus)	RIG-I and MDA5	33,181,216–218
IV ssRNA (+)	Picornaviridae (encephalomyocarditis virus; rhinovirus; coxsackie B virus)	RIG-I and MDA5	16,17,219–221
	Flaviviridae (West Nile virus; hepatitis C virus; Zika virus)	RIG-I and MDA5	49,222–224
	Coronaviridae (SARS coronavirus)	RIG-I and MDA5	225–227
V ssRNA (–)	Orthomyxoviridae (influenza A virus)	RIG-I	16,43,44
	Paramyxoviridae (measles virus)	RIG-I and MDA5	228–230
	Filoviridae (Ebola virus, Marburg virus)	RIG-I and MDA5	231,232
VI ssRNA (RT)	Retroviridae (human immunodeficiency virus)	RIG-I and MDA5	233–235
VII dsDNA (RT)	Hepadnaviridae (hepatitis B virus)	RIG-I and MDA5	236,237

dsDNA, double-stranded DNA; dsRNA, double-stranded RNA; MDA5, melanoma differentiation-associated protein 5; NA, not applicable; RIG-I, retinoic acid-inducible gene I; RLR, RIG-I-like receptor; RT, reverse transcribing; SARS, severe acute respiratory syndrome; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA.

A-to-I RNA editing

A process that converts adenosine to inosine (A-to-I) in RNA. A-to-I editing is catalysed by adenosine deaminases acting on RNA (ADARs) and can change RNA structure and stability. A-to-I editing can also introduce mutations into proteins as 'I' bases are read as guanines instead of adenosines during translation.

Autophagy

A lysosome-dependent recycling and degradation process used to remove unnecessary or dysfunctional cellular material, such as long-lived proteins or organelles. Autophagy is also used by the cell to eliminate microorganisms, such as certain bacterial and viral pathogens.

Deamidation

A process catalysed by aminotransferases in which an amide functional moiety is removed from a glutamine or asparagine residue in the substrate protein. Deamidation can have diverse outcomes for the target protein, including degradation and activation of its signal-transducing activity.

or transfection with poly(I:C) or RNA from HCV)^{90,93–97}. Given the important role of TRIM25 in RIG-I-mediated antiviral gene induction, several viral pathogens have evolved antagonists that directly inhibit TRIM25 activity, including NS1 from IAV^{96,98}, NS1 from respiratory syncytial virus⁹⁹, E6 from human papilloma virus⁹⁷ and the BPLF1 protein from Epstein–Barr virus¹⁰⁰, or the subgenomic RNA of dengue virus¹⁰¹ (reviewed in detail elsewhere^{102,103}). Moreover, in recent years several cellular proteins (for example, nuclear dbf2-related 2 (NDR2), zinc finger CCHC-type-containing 3 (ZCCHC3), NLR family pyrin domain-containing 12 (NLRP12) and caspase 12) that specifically regulate TRIM25's function in RIG-I K63-linked ubiquitylation have been identified (as described below). Structural studies revealed the molecular details of RIG-I signalosome assembly through a mechanism of TRIM25 dimerization^{104,105}.

Given that K63-linked ubiquitylation is a key activation step in RIG-I signalling, it is not surprising that partial redundancy exists. Besides TRIM25, Riplet (also known as REUL or RNF135) has been shown to mediate RIG-I K63-linked ubiquitylation, thereby facilitating RIG-I signal transduction¹⁰⁶. Of note, although several studies showed that Riplet modifies the CTD of RIG-I and thereby acts cooperatively with TRIM25 (REFS^{106–108}), other studies suggested that Riplet can also modify the RIG-I CARDS^{109,110}. Interestingly, Riplet has recently been implicated in an IRF3-mediated antiviral innate response that is independent of the RIG-I–MAVS signalling pathway¹¹¹, which raises the question of whether the effect of Riplet deficiency on antiviral cytokine induction is primarily due to Riplet's role in RIG-I activation or its independent signalling function downstream of it. Finally, the ubiquitin ligases TRIM4 and Mex-3 RNA binding family member C (MEX3C) also reportedly promote RIG-I signal activation through K63-linked polyubiquitylation of multiple residues in the CARDS¹¹², and thus a model of sequential and hierarchical RIG-I CARD ubiquitylation has been proposed¹¹³.

Conversely, RIG-I stability is regulated by classical degradative K48-linked polyubiquitylation mediated by at least three E3 ligases — Cbl, ring finger protein 122 (RNF122) and RNF125 — whereas ubiquitylation mediated by linear ubiquitin chain assembly complex (LUBAC) negatively affects the stability of TRIM25 and its interaction with RIG-I^{114–117} (FIG. 3). Ubiquitylation events are reversible processes, and, accordingly, several deubiquitylating enzymes, in particular ubiquitin specific peptidase 3 (USP3), USP21 and CYLD lysine 63 deubiquitinase (CYLD), modulate RIG-I signalling by removing K63-polyubiquitin chains, although with unique kinetics (reviewed elsewhere^{112,118}). On the other hand, USP4 and USP15 enhance the stability of RIG-I and TRIM25, respectively, by proteolytically cleaving K48-linked ubiquitylation from these molecules^{119,120}.

Besides multisite ubiquitylation, phosphorylation, acetylation and deamidation are critical for regulating RIG-I signalling or sensing activity. Phosphorylation of RIG-I at both the CARDS (at S8 and T170) and the CTD (at T770 and S854/855) keeps the sensor in an autorepressed state, preventing premature signalling before infection^{121–123}. Phosphomimetic mutants of RIG-I at the CARD sites exhibited abolished TRIM25 binding, K63-linked ubiquitylation and downstream signalling ability, whereas mutants of RIG-I, which cannot be phosphorylated, showed higher levels of K63-linked ubiquitylation and, accordingly, increased signalling activity compared with wild-type RIG-I. Conventional isoenzymes of PKC (PKCα and PKCβ), as well as casein kinase II (CKII), were shown to phosphorylate RIG-I at the CARDS and the CTD, respectively^{123,124}, whereas protein phosphatase 1 (PP1) is responsible for removing the phosphorylations in the CARDS following virus infection¹²⁵.

Similarly to RIG-I, aberrant activation of MDA5 in uninfected cells is prevented by phosphorylation (at S88 and S828)^{125,126}. Upon virus infection, MDA5 is activated by removal of these phosphorylation events by PP1α/γ^{125,127}. In contrast to RIG-I, MDA5 regulation by K63-polyubiquitin is less well established. Although studies on MDA5 CARD K63-linked ubiquitylation are conflicting^{90,92,94,128}, it was recently shown that the helicase domain of MDA5 (specifically K743) is modified with K63-linked ubiquitin chains synthesized by TRIM65, which activates the sensor molecule by promoting its oligomerization¹²⁹.

Recently, SUMOylation of MDA5 and also RIG-I (at several residues in their CARDS and CTD) has emerged as a critical regulatory mechanism of their activity¹³⁰. SUMOylation of RIG-I and MDA5 by TRIM38 in uninfected cells, or at early times after viral infection, prevents their K48-polyubiquitin-dependent degradation, stabilizing the sensor proteins. During the late phase of infection, de-SUMOylation of RLRs by sentrin/SUMO-specific protease 2 (SEN2) triggers their proteasomal degradation, aiding in termination of the pro-inflammatory response once the virus has been cleared¹³⁰.

Non-covalent binding of RIG-I to FAT10, a less well-characterized UBL protein, was reported to dampen the RIG-I-mediated antiviral response by sequestering signalling-active RIG-I from mitochondria¹³¹. Moreover,

Box 2 | Therapeutic approaches and synthetic RLR agonists

Building on the increasing knowledge of RIG-I-like receptor (RLR) activation by RNA agonists that occur naturally, the field is exploring therapeutic approaches based on RLR stimulation. A particular focus has been on cancer (reviewed elsewhere¹⁷⁷). One example is the finding that DNA-demethylating agents such as 5-azacytidine — used in the clinic to treat leukaemia — activate melanoma differentiation-associated protein 5 (MDA5)^{178,179}. This may involve the recognition of transcripts derived from endogenous retroviruses^{178,179}. Moreover, RLRs have been reported to be activated by cellular non-coding RNAs during chemotherapy or after treatment with ionizing radiation¹⁸⁰. Several synthetic RLR agonists have also been developed. These include poly(I:C), which activates retinoic acid-inducible gene I (RIG-I) and MDA5 (REFS^{33,181}), and several chemically synthesized 5'-triphosphate (5'-PPP) duplex RNAs that engage RIG-I, such as 3pRNA, and a sequence-optimized (uridine-rich) hairpin 5'-PPP RNA called M8 (REFS^{31,182}). These showed promising results in several cancer models^{183–185}, as well as in augmenting immunity^{186–188}. Additionally, activation of RIG-I signalling by 5'-PPP RNA was shown to facilitate tumour responsiveness to checkpoint inhibitors^{189,190}. Furthermore, in vitro synthesized circular RNAs have been proposed to activate RIG-I, and such RNA preparations induce antitumour responses in an in vivo model^{191,192}. Circular RNAs lack a 5' end and whether RIG-I activation is mediated directly by circular RNAs or by other contaminating RNAs is controversial^{192,193}. Finally, stimulation of RLRs is of therapeutic use in vaccination models, which may be related to their role in modulating immune cell functions^{186,194–197}.

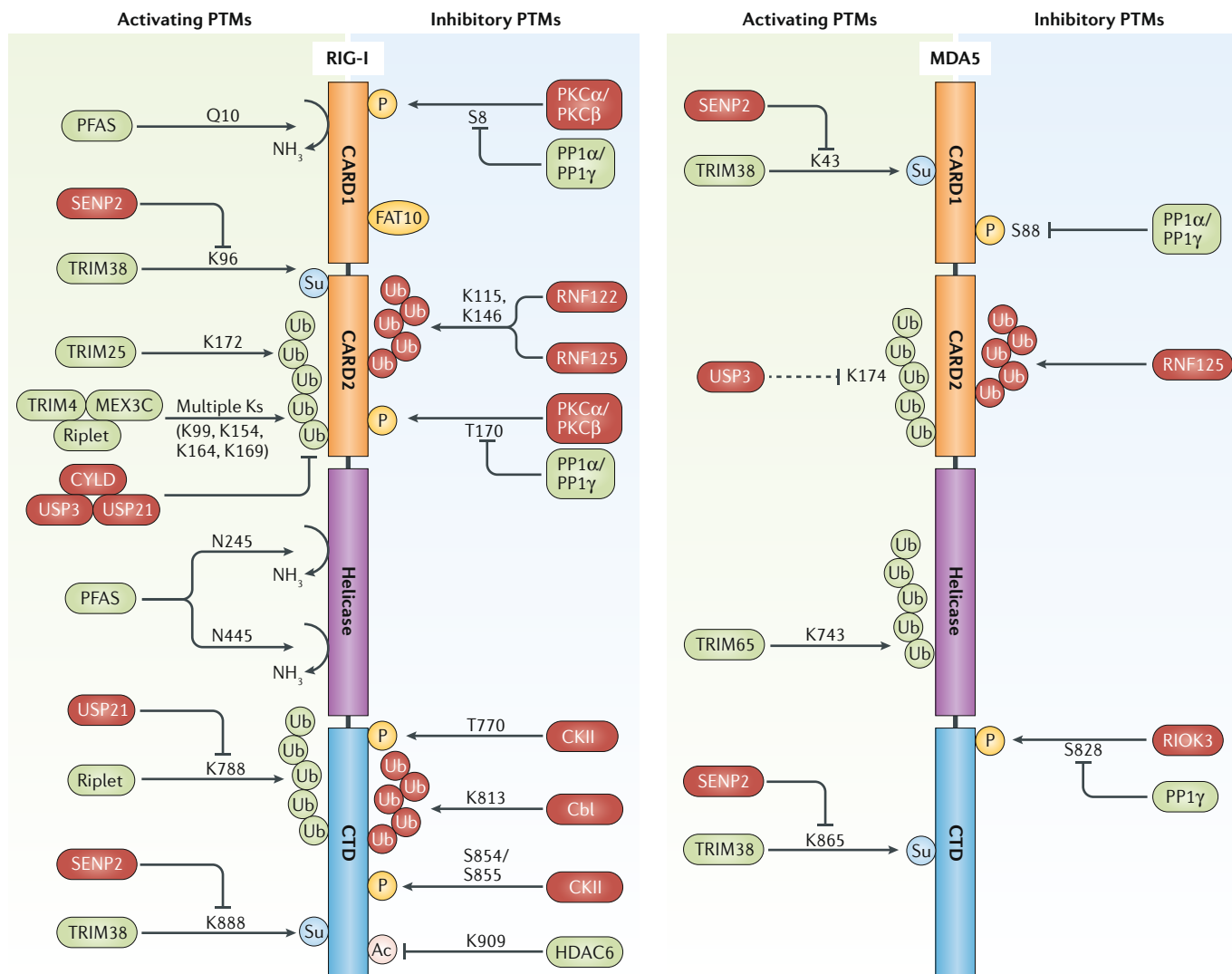


Fig. 3 | Post-translational modifications of RIG-I and MDA5 and responsible enzymes. RIG-I (retinoic acid-inducible gene I) and MDA5 (melanoma differentiation-associated protein 5) are regulated by acetylation, deamidation, phosphorylation, SUMOylation, modification with FAT10 and several types of polyubiquitylation, in particular K63-linked and K48-linked polyubiquitin-linkage types (illustrated in green and red, respectively). Activating regulatory post-translational modifications (PTMs) are illustrated on the left, and those that inhibit RIG-I-like receptor (RLR) activation are depicted on the right, as well as the respective enzymes that synthesize, or remove, these PTMs. Enzymes that ultimately lead to RLR activation are shown in green; those that lead to RLR signal inhibition are shown in red. Furthermore, key modified amino acid residues in human RIG-I and MDA5 are illustrated, if known. Ac, acetylation; CARD, caspase activation and recruitment domain; CKII, casein kinase II; CTD, carboxy-terminal domain; CYLD, CYLD lysine 63 deubiquitinase; HDAC6, histone deacetylase 6; MEX3C, Mex-3 RNA binding family member C; P, phosphorylation; PFAS, phosphoribosyl-formylglycinamide synthase; PKC, protein kinase C; PP1, protein phosphatase 1; RIOK3, RIO kinase 3; RNF, ring finger protein; SENP2, sentrin/SUMO-specific protease 2; Su, SUMOylation; TRIM, tripartite motif; Ub, ubiquitin; USP, ubiquitin specific peptidase.

Tripartite motif (TRIM) protein family

A family of ~80 proteins in humans that is structurally defined by the tripartite motif (also known as the RBCC motif) comprising a RING-finger E3 ligase, B-box and coiled-coil domain. In addition, TRIM proteins encode numerous different carboxy-terminal domains (for example, SPRY) that allow for the recruitment of specific proteins or have other functions.

it was recently shown that RIG-I is regulated by deamidation, which is an emerging PTM in innate immunity¹³². Deamidation of RIG-I (at residues Q10, N245 and N445) by cellular phosphoribosyl-formylglycinamide synthase (PFAS) triggers RIG-I activation specifically during herpesvirus infection, and, intriguingly, this activation event is usurped by these viruses for their own replication¹³².

Whereas all the above described PTMs regulate the signal-transducing activity of RLRs, reversible acetylation was shown to modulate the ability of RIG-I to bind to viral RNA^{133,134} (FIG. 3). Acetylation

of RIG-I in its CTD (at K909 and also other lysines) prevents RIG-I from binding viral RNA and assembling into a signalling-competent oligomeric form. Inversely, CTD deacetylation induced by histone deacetylase 6 (HDAC6) allows for efficient RIG-I sensing and signalling. Consistent with this observation, HDAC6-deficient cells and mice were more permissive to several RNA viruses, such as IAV, vesicular stomatitis virus (VSV) and certain flaviviruses^{133,134}. It is noteworthy that, in contrast to the extensive characterization of PTMs regulating RIG-I and MDA5, regulation of LGP2 by PTMs remains elusive.

Deubiquitylating enzymes
Proteases that cleave the (iso) peptide bond between ubiquitin, or ubiquitin-like molecules, and the modified substrate. The human genome encodes ~100 deubiquitylating enzymes, some of which regulate pathogen recognition and signalling in innate immunity.

Regulation by interacting proteins. Recent studies identified several RLR-binding proteins that regulate RLR activity by modulating several processes: dsRNA binding to RLRs (for example, co-receptor proteins); RLR oligomerization; TRIM25-mediated K63-linked ubiquitylation of RLRs; or subcellular localization of RLRs (FIG. 4).

Numerous dsRNA-binding proteins, which are involved in many aspects of RNA biology, have been shown to regulate RLRs. One of the first dsRNA-binding proteins reported to regulate RLRs was PACT. PACT was shown to interact with the CTD of RIG-I and to stimulate the ATPase activity of RIG-I^{135,136}. Interestingly, recent studies showed that PACT also promotes MDA5 activation by facilitating its ability to oligomerize¹³⁷. In addition to PACT, the third RLR member, LGP2, positively and negatively regulates RLR activities through multiple mechanisms (BOX 1).

Whereas the concept of a receptor/co-receptor mechanism is well known in adaptive immunity, it is less well established in innate immunity. The zinc-finger protein ZCCHC3 has recently been shown to function as a co-receptor for both RIG-I and MDA5 (REF.⁹⁴). Co-immunoprecipitation and cell fractionation experiments showed that ZCCHC3 binds with its C-terminal zinc finger domains to RNAs that activate RLRs (including 5'-PPP RNA and high molecular weight poly(I:C)), whereas its N-terminal domain interacts with the helicase and CTD of RIG-I and MDA5. This interaction mode enhanced RNA binding by RLRs, as well as the K63-linked ubiquitylation of RLRs by TRIM25, which was also found to be in complex with ZCCHC3 (of note, Riplet, TRIM4 and TRIM65 were not found to be associated with ZCCHC3). Functional studies in ZCCHC3-deficient cells and mice demonstrated that ZCCHC3 was required for efficient RLR-mediated cytokine responses to several RNA

viruses. Of note, the phenotype of ZCCHC3 knock-out was comparable with that of TRIM25 gene editing using CRISPR, which also markedly reduced Sendai virus-triggered cytokine responses⁹⁴. Moreover, several DExD/H-box helicases may regulate RLR signalling (FIG. 4). For example, DEAH-box helicase 15 (DHX15) was shown to bind dsRNA and to facilitate the ability of RIG-I to hydrolyse ATP and to signal downstream¹³⁸. In accord, cells depleted of DHX15 were more susceptible to infection by RNA viruses from several families, including paramyxoviruses and rhabdoviruses. DExD/H-box helicase 60 (DDX60) has been suggested to promote RIG-I-dependent (and potentially also MDA5-dependent) antiviral interferon and ISG induction, although conflicting results have been reported in DDX60-knockout cells and mice^{139–141}. Riplet, which is one of the E3 ligases mediating RIG-I K63-linked ubiquitylation, was recently shown to promote RIG-I signalling in a ubiquitylation-independent manner. It was suggested that by forming a dimer and by crossbridging RIG-I filaments on longer RNA molecules, Riplet induces higher-assembly aggregates of RIG-I¹⁰.

Recently, several regulatory proteins for TRIM25 have been identified and functionally characterized. The kinase NDR2 (also known as serine/threonine-protein kinase 38-like (STK38L)) was shown to be crucial for TRIM25-mediated and RIG-I-mediated antiviral interferon responses, evidenced by the impairment of antiviral cytokine induction when NDR2 was gene-edited in cells (in particular, macrophages) and in mice. Interestingly, the kinase activity of NDR2 was shown to be dispensable for its positive effect on RIG-I signalling. Using cell-based assays and in vitro experiments with recombinant RIG-I and TRIM25 proteins, this study showed that NDR2, through interaction with both RIG-I and TRIM25, stabilized their complex formation and promoted TRIM25-induced K63-linked ubiquitylation of the RIG-I CARDs¹⁴². Ting and colleagues recently unveiled a novel regulatory mechanism that interconnects the RLR and NOD-like receptor (NLR) pathways. The pyrin-containing NLR member, NLRP12, which is expressed predominantly in dendritic cells and neutrophils, was shown to dampen cytokine responses by RIG-I following RNA virus infection or 5'-PPP RNA stimulation, evidenced by higher innate immune signalling in NLRP12-deficient bone marrow-derived dendritic cells. This study further showed that, under normal conditions, NLRP12 interacts with TRIM25, blocking its ability to ubiquitylate and activate RIG-I. At the same time, NLRP12 enhanced the K48-linked ubiquitylation of RIG-I by RNF125, thereby destabilizing RIG-I. During the course of viral infection, however, NLRP12 protein abundance is gradually reduced, relieving the TRIM25-inhibitory mechanism and, at the same time, stabilizing RIG-I¹⁴³. Caspase 12, a negative regulator of inflammasome signalling, was shown to be required for type I interferon induction in response to West Nile virus infection in vitro and in vivo by interacting with RIG-I and regulating its K63-linked ubiquitylation by TRIM25 (REF.¹⁴⁴). In addition to these TRIM25-regulatory proteins, several cellular RNAs have been discovered to bind to TRIM25

Box 3 | TRIM proteins function as key regulators of innate immune signalling

Members of the tripartite motif (TRIM) protein family have emerged as important regulators of cytokine induction elicited by retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and other innate immune receptors such as Toll-like receptors (TLRs) and cyclic GMP-AMP synthase (cGAS)^{198–200}. Moreover, some TRIM proteins modulate signal transduction downstream of the interferon- α/β receptor (IFNAR) and, thereby, the expression of interferon-stimulated genes (ISGs)¹⁹⁸. Conceptually, the amino-terminal RING domain of TRIM proteins confers E3 ligase activity for ubiquitin or ubiquitin-like proteins (for example, SUMO or ISG15), allowing modification of either innate sensors or their downstream signalling proteins. Many TRIM proteins, including TRIM25 and TRIM56, modify innate sensor or signalling proteins with non-degradative polyubiquitin types, in particular K63-linked polyubiquitylation^{112,201}. K63-polyubiquitin can prompt or stabilize the multimeric, active form of innate signalling proteins, and/or facilitate the recruitment of other proteins into the complex, ultimately stimulating antiviral signalling and cytokine induction^{92,128}. Other TRIM proteins have been shown to dampen pattern-recognition receptor-mediated signalling through catalysis of classical K48-linked polyubiquitin, which triggers substrate degradation by the proteasome¹⁹⁸. Of note, the expression of many TRIM proteins in humans is transcriptionally induced by interferons²⁰², indicating that these proteins are part of a positive feedforward regulatory circuit. Degradative and non-degradative ubiquitylation by TRIM proteins is reversed by specific deubiquitylating enzymes, which is ultimately crucial for immune homeostasis. Lastly, many TRIM proteins fulfil dual roles in antiviral immunity by regulating cytokine/ISG production and by directly antagonizing viral replication through various mechanisms that include sequestration or degradation of viral components and modulation of autophagy^{203–205}.

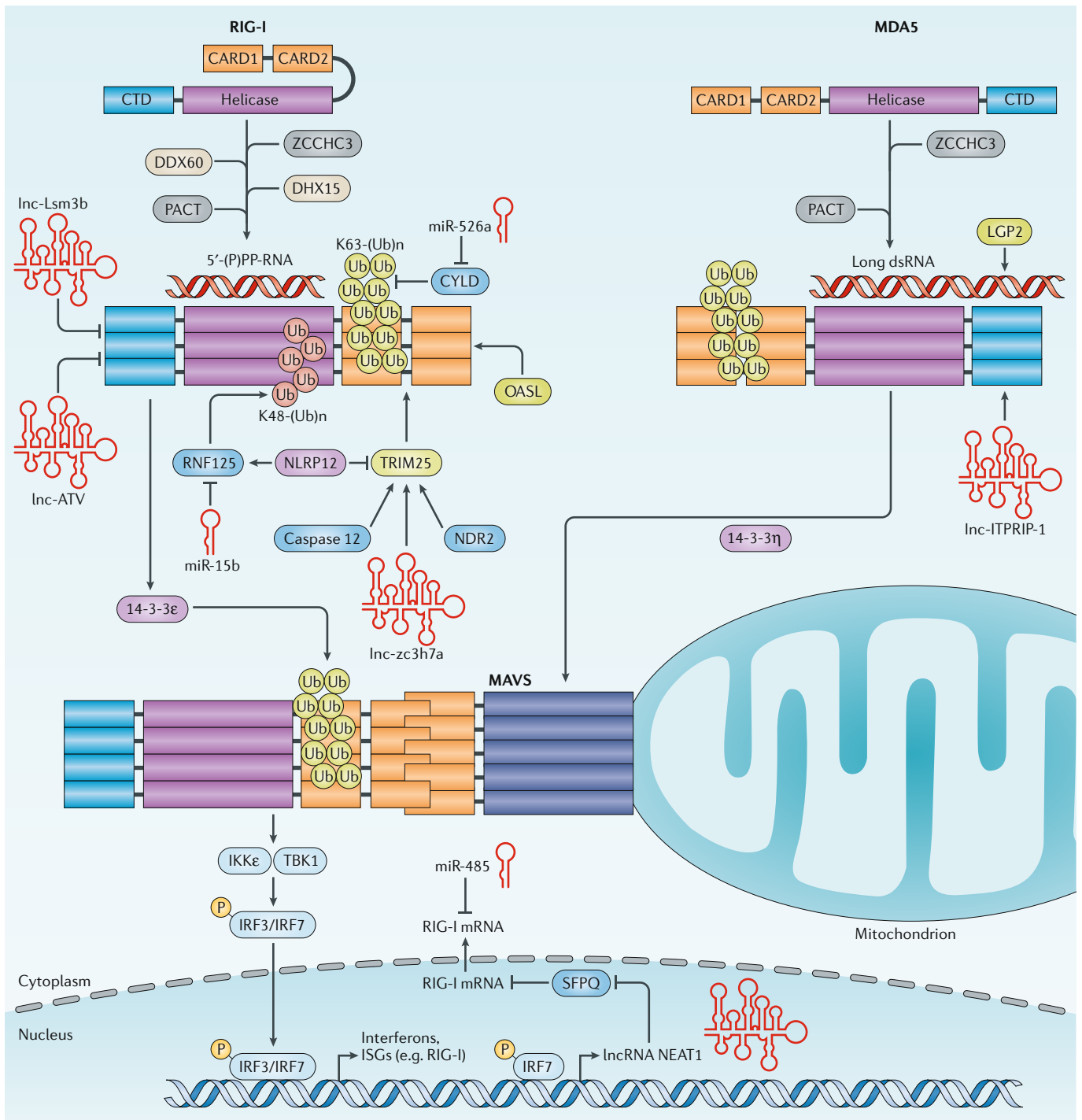


Fig. 4 | Regulation of RIG-I and MDA5 activity by interacting proteins and non-coding RNAs. The activities of retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) are regulated by interacting proteins that modulate their RNA-binding ability, oligomerization, tripartite motif-containing 25 (TRIM25)-mediated K63-linked ubiquitylation or subcellular localization. Furthermore, several cellular long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) regulate RIG-I-mediated and MDA5-mediated innate immune signalling. Conceptually, these RNAs promote or diminish RIG-I-like receptor (RLR) signalling by regulating the gene expression of these sensors or regulatory proteins in the RLR pathway, or they modulate the activity of RLRs or TRIM25 through a direct physical interaction. CARD, caspase activation and recruitment domain; CTD, carboxy-terminal domain; CYLD, CYLD lysine 63 deubiquitinase; DDX60, DEXD/H-box helicase 60; DHX15, DEAH-box helicase 15; dsRNA, double-stranded RNA; IKKε, IκB kinase-ε; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; K48-(Ub)n, K48-linked ubiquitylation; K63-(Ub)n, K63-linked ubiquitylation; LGP2, laboratory of genetics and physiology 2; lncRNA, long non-coding RNA; MAVS, mitochondrial antiviral-signalling protein; NDR2, nuclear dbf2-related 2; NLRP12, NLR family pyrin domain-containing 12; OASL, oligoadenylate synthetase-like; P, phosphorylation; (P)PP, (tri)diphosphate; RNF, ring finger protein; SFPQ, splicing factor proline and glutamine rich; TBK1, TANK-binding kinase 1; Ub, ubiquitin; ZCCHC3, zinc finger CCHC-type-containing 3.

Long non-coding RNAs (lncRNAs). A class of host RNAs longer than 200 nucleotides that do not encode a protein. lncRNAs, which are often only expressed in certain cell types or tissues, regulate many cellular processes through modulation of gene expression or post-transcriptional mechanisms.

RNA interference

A process in which long double-stranded RNA is cut into RNA fragments of ~21 nucleotides long by the enzyme Dicer. These RNA fragments, when further processed, can bind to complementary messenger RNA target sequences and, thereby, silence gene expression.

and regulate its E3 ligase activity (as described below). RIG-I reportedly can also be activated in a K63-linked polyubiquitin-independent manner by oligoadenylate synthetase-like (OASL), which is a unique member of the OAS family in that it is devoid of 2′–5′ oligoadenylate synthetase activity and possesses two UBL domains. It has been proposed that upon transcriptional induction of OASL and its binding to RIG-I, the UBL domains of OASL mimic the presence of K63-polyubiquitin chains, leading to RIG-I signalling¹⁴⁵.

Besides viral RNA binding, oligomerization and modification by various enzymes, RLRs need to relocate from areas of RNA sensing to mitochondria and mitochondrial-associated membranes, where further downstream signalling occurs via MAVS. Members of the 14-3-3 protein family, which comprises seven isoforms in human, have emerged as critical regulators of the cytosol-to-mitochondria translocation step of RLRs. Gale and colleagues showed that 14-3-3 ϵ forms a complex with endogenous RIG-I and TRIM25, stabilizing their interaction and mediating their translocation to MAVS on mitochondrial-associated membranes⁹³. Accordingly, silencing of 14-3-3 ϵ diminished TRIM25-induced and RIG-I-induced signalling to Sendai virus infection or HCV RNA stimulation. Similarly, relocalization of MDA5 was recently shown to be mediated by 14-3-3 η , indicating that specific 14-3-3 isoforms function as ‘RLR translocon’ proteins¹⁴⁶.

Regulation by non-coding RNAs. Cellular non-coding RNAs, in particular long non-coding RNAs (lncRNAs) and miRNAs, have emerged as important regulators of RLR signal transduction. In most cases, viral infection and/or interferon stimulation triggers the expression of these non-coding RNAs, often in a cell type-specific or tissue-specific manner. Upon induction, some lncRNAs and miRNAs promote or dampen RLR signalling by regulating the gene expression of RLRs or other key molecules in the RLR pathway. In addition, some lncRNAs were shown to directly bind to the RLR sensor protein (in particular RIG-I), or its activator TRIM25, which itself has RNA-binding capacity^{101,147,148} (FIG. 4). Lastly, miRNAs and lncRNAs modulate interferon-dependent and ISG-dependent innate immune responses by multiple pattern-recognition receptors, including RLRs, through broader mechanisms, such as chromatin remodelling (reviewed in detail elsewhere¹⁴⁹).

Several miRNAs and lncRNAs have been shown to regulate the gene expression of RLRs, or other molecules in the interferon induction pathway, thereby creating feedback or feedforward regulatory loops. lncRNA NEAT1, which is induced upon hantavirus infection, promotes the expression of RIG-I through sequestration of the splicing factor and transcriptional repressor protein splicing factor proline and glutamine rich (SFPQ)¹⁵⁰. Inversely, miR-485 targets the RIG-I mRNA for degradation, thereby dampening the antiviral type I interferon response¹⁵¹. miRNAs also regulate the expression of RLR-regulatory enzymes. miR-526a downregulates the expression of the deubiquitylating enzyme CYLD, which ultimately promotes RIG-I K63-linked ubiquitylation and interferon responses¹⁵². miR-15b

negatively regulates gene expression of the E3 ligase RNF125, thereby controlling RIG-I protein stability during Japanese encephalitis virus infection¹⁵³. Using an RIG-I affinity purification–RNA immunoprecipitation approach combined with ultraviolet crosslinking, Cao and colleagues identified that, in murine macrophages, several lncRNAs were enriched with RIG-I following VSV infection¹⁵⁴. Individual silencing of RIG-I-bound lncRNAs showed that lnc-Lsm3b, when depleted, increased IFN β production upon infection with RNA viruses (for example, Sendai virus and VSV), but not the DNA virus HSV-1. Furthermore, lnc-Lsm3b was transcriptionally upregulated during the course of RNA virus infection, suggesting that lnc-Lsm3b could function as a negative-feedback regulator of RIG-I signalling, maintaining immune homeostasis. Indeed, lnc-Lsm3b-deficient mice challenged with VSV produced higher amounts of type I interferons and, correlated to this, showed reduced viral burden and disease symptoms compared with infected control mice. Biochemical characterization showed that lnc-Lsm3b interacts with the CTD of RIG-I, competing with RNA agonists for RIG-I binding. Furthermore, lnc-Lsm3b stabilized the intramolecular bond between the N-terminal CARDs and the central helicase domain, maintaining RIG-I in an autorepressed, monomeric state. By contrast, lnc-Lsm3b deficiency promoted the interaction of RIG-I with TRIM25, TRIM25-induced K63-linked ubiquitylation and, consequently, RIG-I oligomerization following VSV infection (FIG. 4). It is important to note that lnc-Lsm3b is specific to mice; as such, exploring human lncRNAs that act as feedback inhibitors, similarly to lnc-Lsm3b, will be an exciting new avenue of research.

Indeed, a recent study showed that lnc-ATV, which is a previously uncharacterized, human-specific lncRNA, dampens RIG-I signalling¹⁵⁵. lnc-ATV was identified by high-throughput transcriptome analysis as one of 272 lncRNAs that were significantly upregulated in response to stimulation by type I or III interferons. Functional RNA interference screening showed that lnc-ATV promoted the replication of several RNA viruses, specifically HCV, Zika virus, Newcastle disease virus and Sendai virus, hinting at the possibility that lnc-ATV promotes an antiviral host pathway commonly triggered by these viruses. Functional studies indicated that lnc-ATV suppresses the RIG-I-mediated antiviral response as knockdown of lnc-ATV promoted TBK1-dependent and IRF3-dependent interferon responses to RNA virus infection. lnc-ATV was shown to bind to RIG-I, although more detailed studies are needed to define the precise mechanism by which lnc-ATV inhibits RIG-I activity.

Although RIG-I is well known to be a bona fide RNA sensor, its activator, TRIM25, has recently been shown to also have RNA-binding capacity^{101,147,148}. Biochemical and functional studies showed that the ability of TRIM25 to bind RNA is important for its E3 ligase activity and, thus, its positive effect on RIG-I antiviral signalling¹⁴⁸. However, which type (or types) of host or viral RNAs are bound by TRIM25 in physiological contexts, such as virus infection, has remained unknown until recently. Lin et al. identified that a previously uncharacterized lncRNA, called lnczc3h7a, directly binds to TRIM25,

stabilizing the interaction of the E3 ligase with RIG-I, which ultimately promotes RIG-I signalling¹⁵⁶. Silencing or gene-editing of *Lnczc3h7a* significantly decreased interferon and ISG expression in peritoneal macrophages infected with viruses that predominantly trigger RIG-I (VSV and Sendai virus). However, silencing of *Lnczc3h7a* had no effect on pro-inflammatory responses to non-RIG-I stimuli (for example, EMCV or lipopolysaccharide (LPS)). *Lnczc3h7a* is transcriptionally induced by interferon stimulation or viral infection, and gene expression profiling showed that *Lnczc3h7a* is expressed in many immune cell types. *Lnczc3h7a*-knockout mice showed defective cytokine responses, enhanced virus replication and more severe pathology following VSV infection compared with control mice¹⁵⁶. Through the iCLIP assay, the authors showed that *Lnczc3h7a* not only interacts with TRIM25 but also, through a short RNA stem-loop structure, with the helicase domain of RIG-I, suggesting that *Lnczc3h7a* acts as a molecular scaffold to enhance RIG-I–TRIM25 complex formation; this promoted TRIM25-mediated K63-linked ubiquitylation of RIG-I and, ultimately, its ability to form signalling-active oligomers early during infection. With regard to MDA5 activation, *lnc-ITPRIP-1* was shown to interact with the sensor, stabilizing its binding to viral RNAs and promoting its oligomeric signalling-competent form¹⁵⁷. Finally, besides the described lncRNAs and miRNAs that directly regulate RLR activities, others act downstream of RLRs or broadly regulate inflammatory gene or ISG expression^{158–160}.

Regulation by autophagy. Accumulating evidence indicates that autophagy regulates RLR signalling through diverse mechanisms that include autophagy-dependent degradation of RLRs or downstream innate signalling molecules; direct interaction between autophagic proteins and RLRs; and RLR signal amplification via reactive oxygen species (ROS) production regulated by autophagy (reviewed elsewhere¹⁶¹). In most cases, an absence of autophagy promotes the RLR-mediated cytokine responses, indicating that autophagy has a dampening effect on RLR signalling. The LRR-containing protein leucine rich repeat-containing 25 (LRRC25) recognizes RIG-I ‘earmarked’ with ISG15, inducing its degradation by p62-mediated selective autophagy¹⁶². The autophagy-related protein conjugate, ATG5–ATG12, suppresses *IFN* gene expression through direct binding to both RIG-I and MAVS, preventing their CARD–CARD interaction¹⁶³. NLR family member X1 (NLRX1) together with its binding partner, the mitochondrial Tu translation elongation factor (TUFM),

dually regulates virus-induced type I interferon induction and autophagy¹⁶⁴. Complex formation of NLRX1, TUFM, ATG5–ATG12 and ATG16L1 negatively controlled RIG-I–MAVS binding during infection while, at the same time, promoting virus-induced autophagy^{164,165}. Furthermore, in autophagy-defective cells, RLR signalling has been found to be more effective owing to overzealous production of ROS due to the accumulation of dysfunctional mitochondria¹⁶¹. Interestingly, RLR activation also induces autophagy^{166,167}, showing an intricate relationship between these two host pathways that, on the one hand, could aid in terminating cytokine responses, while, at the same time, promoting direct virus restriction by autophagy.

Conclusions and future directions

Significant progress has been made in dissecting the mechanisms that lead to RLR activation and, equally importantly, on what keeps RLRs controlled in uninfected conditions. Although much has been learned about the molecular features of viral RNAs that induce RIG-I activation, significantly less is known concerning the circumstances in which host-derived RNAs activate RLRs and the mechanistic basis of this. Recent work highlighted the importance of mislocalized, misprocessed or unmasked cellular RNAs (mostly non-coding RNAs) in RLR-driven autoimmunity or antiviral responses, which is expected to stimulate future studies to determine the physiological relevance of such RNAs in disease contexts. In particular, the recognition of DNA virus infection by RLRs via sensing of non-coding RNAs is an emerging theme. It will be important to test whether this concept applies not only to herpesviruses but generally to DNA viruses and to other virus classes detected by RLRs (TABLE 1). The recent advance in the characterization of RLR-mediated immune sensing of nucleic acid ligands can now be utilized for the development of novel RLR agonist or antagonist-based therapies for autoimmunity, infectious diseases or cancer. With regard to the regulatory mechanisms of RLR function by various enzymes, it remains to be determined how these regulators themselves are activated during viral infection and what their cell type-specific and species-specific roles are. Moreover, the role of miRNAs and lncRNAs in RLR signal modulation has just begun to be elucidated, and future work may further expand the repertoire of non-coding RNAs implicated in the RLR regulatory circuit, which will be critical information for potential therapeutic purposes.

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