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An evolving arsenal: viral RNA detection by RIG-I-like receptors
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RIG-I-like receptors (RLRs) utilize a specialized, multi-domain architecture to detect and respond to invasion by a diverse set of viruses. Structural similarities among these receptors provide a general mechanism for double strand RNA recognition and signal transduction. However, each RLR has developed unique strategies for sensing the specific molecular determinants on subgroups of viral RNAs. As a means to circumvent the antiviral response, viruses escape RLR detection by degrading, or sequestering or modifying their RNA. Patterns of variation in RLR sequence reveal a continuous evolution of the protein domains that contribute to RNA recognition and signaling.

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In mammalian cells, pattern recognition receptors (PRRs) protect against host infection by recognizing specific pathogen associated molecular patterns (PAMPs) and eliciting signals that initiate an immune response [1–4]. A structurally related group of PRRs that include Retinoic acid-Inducible Gene 1 (RIG-I), Melanoma Differentiation-Associated Gene 5 (MDA5) and Laboratory of Genetics and Physiology 2 (LGP2), sensitively detect structural variations among viral RNA molecules [5–7]. These proteins, which are collectively called the RIG-I like receptors (RLRs) play a key role in vertebrate response to viral infection.

RLR proteins are specialized RNA-dependent ATPases that have evolved around a DExD/H-box ATPase core that contains two RecA-like folds (Hel1 and Hel2) and a specialized insertion domain (Hel2i) that promotes recognition of double-stranded RNA (dsRNA) [8,9,10,11,12]. To facilitate the detection of a broad range of non-self RNA targets, each RLR contains a similar but divergent C-terminal domain (CTD) that mediates RLR-specific interactions between bound nucleic acids or neighboring protein partners [13,14]. The CTD is mechanically coupled to the ATPase core and Hel2i via a pincer domain that transduces information on molecular interactions throughout the protein [9,10,11,12]. In addition to these specialized target recognition domains, RIG-I and MDA5 contain a pair of tandem caspase activation and recruitment domains (CARDs) that engage the innate immune response by activating the downstream adaptor protein MAVS [15,16]. The importance of all these components in RNA detection and signaling is evident from mutational analyses and recent studies of RLR evolution (vide infra).

RNA recognition by RLR proteins
Both RIG-I and MDA5 bind dsRNA through interactions mediated by the CTD, the ATPase core, and the insertion domain [12]; however, differences in the modes of target recognition at the CTD cause the determinants for non-self RNA recognition to vary considerably between the two proteins. In RIG-I, the CTD has evolved to engage in a high affinity interaction with a triphosphate group [13,17,18**] present at the dsRNA termini of deposited genomic RNA [19,20] or replicative intermediates [21] of target viruses (Figure 1 Detection). In addition to recognizing this chemical moiety, the CTD of RIG-I caps the duplex terminus through stacking interactions with terminal base pairs [9,10,11,22]. Once bound to its target RNA, RIG-I becomes competent for ATP hydrolysis and downstream signaling.

In contrast to RIG-I, target recognition by MDA5 is chemically more general, and relies on the presence of long, accessible RNA duplexes [23]. The CTD of MDA5 contains a shallow binding surface that forms sequence-independent interactions with the minor groove of target RNAs and accommodates a continuous RNA backbone. Further, the CTD of one MDA5 molecule can form a cooperative binding interface with the insertion domain of a neighbor [7]. Multiple cooperative binding interactions result in a propensity for oligomerization, causing MDA5 to form long filaments along RNA duplexes [24].
Activation and evasion of RLRs by viral RNA in different cellular compartments. Detection: Viral RNA is differentially detected by RIG-I-like receptors (RLRs). RIG-I specifically recognizes and binds to the 5’ triphosphorylated, blunt-end termini of viral RNA targets. MDA5 recognizes long, double-strand RNA and forms cooperative filaments along internal regions of target duplexes. Once bound to RNA, these proteins can initiate an immune response through activation of the mitochondrial adaptor protein MAVS. In addition to monitoring the cytoplasm for viral RNA, RIG-I has been observed in the perinuclear space, stress granules, and at membrane boundaries between the endoplasmic reticulum and mitochondria, potentiating interactions with viruses that localize to subcellular compartments. Evasion: Viral strategies to avoid detection. Many viruses have developed strategies to evade RLR recognition, including removing or occluding the triphosphate moiety, 5’ end capping their RNA, and masking duplex regions via binding of viral proteins. RIG-I is shown in red, MDA5 in green, MAVS in cyan, viral proteins in purple, and an endogenous RNA cap structure in pink. Phosphates are represented by yellow circles. The area within the dashed circle represents a stress granule.

(Figure 1 Detection). These filaments may cause clustering of the CARDs of MDA5, making them competent to engage in immune signaling.

The role of cellular compartmentalization in recognition by RLR proteins
Specific subcellular localization of the RLRs plays a key role in their ability to detect pathogenic RNA and activate the anti-viral response. The RLRs are generally thought to localize within the cytoplasm, where they can detect genomic viral RNA and RNA replication intermediates [19–21]. While cytoplasmic localization is likely to play a major role in RLR targeting and signal transduction, recent work suggests that variation in subcellular localization may contribute to RNA recognition and function.

In a recent study aimed at visualizing RLR localization during viral infection, a mutant form of influenza A was used to infect live cells [25**]. The mutant form of the influenza virus lacks the nonstructural protein 1 (NS1) gene, which is a potent inhibitor of RIG-I signaling [26]. In cells infected with the influenza A virus lacking the NS1 gene, the RLRs localize to speckle-like structures known as stress granules [25**]. Stress granule formation...
is induced by genomic RNA of the influenza virus [25**, indicating that there is a specific mechanism by which the cell compartmentalizes pathogenic RNA. Furthermore, the appearance of stress granules correlates with the RIG-I mediated interferon response in the cell [25**]. These results indicate that the cytoplasmic stress granules are compartments containing viral RNAs that are accessible to the RLRs during the anti-viral response.

Distribution of RIG-I to the perinuclear region has been shown to be important for MAVs-dependent anti-viral signaling. Specifically, cellular fractionation and fluorescence microscopy experiments using cells infected with Sendai virus provided new insights into RIG-I localization [27**]. Analysis of the cytoplasmic and membrane fractions of the cell revealed the presence of RIG-I in the membrane during acute infection while RIG-I was strictly observed in the cytoplasm in mock treated cells. Additional experiments monitoring fluorescently tagged RIG-I revealed that it is localized to the perinuclear region of the cell, which is near the site of mitochondria-associated membranes (MAMs) [27**,28]. Concomitant with localization to the membranes, RIG-I associates with the ubiquitin ligase, TRIM25, and the adapter protein, 14-3-3e [27**]. These protein associations are necessary for localization of RIG-I to the membrane [27**]. Recruitment of RIG-I to the MAMs allows for interaction with MAVs and initiation of anti-viral signaling [27**].

In the absence of viral infection, RIG-I is distributed among various cellular compartments. RIG-I associates with the actin cytoskeleton and localizes to actin-enriched membrane ruffles [29]. The CARDs mediate these interactions, revealing a novel role for the RIG-I signaling domains in cytoskeleton associations [29]. Close inspection of confocal micrographs of fluorescently tagged RIG-I [25**,27**,29] reveals its distribution throughout the cytoplasm and slightly in the nucleus. In support of this observation, an amino-terminal variant of MDA-5 can also localize to the nucleus after stimulation with pro-apoptotic factors that induce cleavage of the CARDs [30]. These observations suggest that the RLRs are multi-functional proteins that carry out their roles in diverse compartments within the cell.

Viral strategies for RLR evasion: tricking the RNA binding machinery

Because RIG-I, MDA-5 and the TLRs use different strategies to bind RNA ligands, the vertebrate PRR proteins are collectively able to detect a wide variety of RNA and DNA viruses [31,32]. Nonetheless, viruses are able to ultimately infect and replicate in host cells by circumventing the machinery of the innate immune response [33]. Because viral lethality and pathogenicity are partly determined by the strength of the response mounted in early infection [34], viruses evade RLRs by preventing RNA binding [35,36,37–39]. Strategies employed by viruses to escape detection by RIG-I and MDA-5 include modification, masking and degradation of the viral RNA (Figure 1 Evasion), along with alteration of its cellular compartmentalization.

A major (if not surprising) mechanism for viral evasion is the incorporation of RNA modifications that prevent RLR recognition. The 5’ triphosphate is an important determinant for RIG-I recognition, so the removal or modification of this moiety is an effective strategy for evading RIG-I detection. For example, Hantaan virus (HTNV) and Crimean-Congo hemorrhagic fever virus (CCHV), which belong to the Bunyaviridae family of negative strand RNA viruses, do not trigger interferon production [38]. In contrast, viruses from the same family, such as Rift Valley fever virus (RVFV) and LaCross virus (LACV), trigger a RIG-I-dependent innate immune response via RIG-I binding to the 5’ triphosphorylated viral genomes [20,38]. To evade detection by RIG-I, the 5’ triphosphates of the HTNV and CCHV genomes are removed during a ‘prime and realign’ process whereby the first nucleotide is cleaved by an exonuclease, leaving a 5’ monophosphate [40]. Compounding this effect, negative strand viruses such as HTNV and CCHV do not produce substantial amounts of dsRNA [40,41], thereby eluding MDA-5 detection.

Viruses also modify the 5’ termini of their RNA by capping and 2’-O-methylation. Cytoplasmic eukaryotic mRNAs, which are not generally detected by RLRs, are 5’ end-capped with methylations on the first and second nucleotides of the ribose-2’-O position, thereby conferring stability and preventing degradation [42]. It is assumed that neither RIG-I nor MDA-5 recognize these cap structures, which are comprised of a 7-methylguanosine linked to the first nucleotide through a 5’–5’ triphosphate bridge. Viruses that replicate in the cytoplasm have evolved machinery that is designed to cap and methylate viral RNA in order to appear like host RNA and thereby evade receptor recognition. In these cases, viral RNA is capped using cellular machinery, viral encoded cap machinery, or by a cap snatching mechanism [42]. For example coronavirus strains with an inactive cap-methylating enzyme (2’-O-methyltransferase) induce higher levels of type I interferon production compared to wild-type virus in human macrophages and in mice, in an MDA-5 dependent manner [43**]. The 2’-O-methylation at the 5’ cap appears to be a molecular feature that is critical for the distinction of self and non-self RNA by MDA-5, and it is readily exploited by the coronavirus.

In addition to altering deposited or replicative RNA, viruses have also evolved strategies for masking the determinants of RLR-mediated detection. This is often achieved by encoding viral proteins that bind the RNA 5’ terminus or duplex stems of viral RNA in order to
out-compete binding by RIG-I or MDA5. For example, filoviruses, like Ebola virus and Marburg virus, encode viral proteins (VP) that antagonize RLRs through RNA binding. Recent crystal structures of Marburg virus VP35 and Ebola virus VP35 reveal interactions with the RNA backbone and duplex ends, which effectively sequester the RNA and preclude detection by both RIG-I and MDA-5 [35,36*,44].

Degradation of RNA represents another way that viruses avoid detection. For example, Lassa fever virus, belonging to the Arenaviridae family, encodes a multi-functional nucleoprotein (NP) that acts as a 5′–3′ exoribonuclease on dsRNA. NP suppresses interferon induction via its exoribonuclease function, presumably by creating ssRNA regions and thus removing the dsRNA PAMP [37].

Viruses also evade recognition by RIG-I and MDA-5 by altering the subcellular compartmentalization of dsRNA. Coronaviruses, like most RNA viruses, typically replicate in the cytoplasm, where dsRNA replicative intermediates are readily detected by RLRs. However, SARS-coronavirus (SARS-CoV) has evolved a mechanism to sequester the dsRNA. SARS-CoV induces formation of double-membrane vesicles on an altered endoplasmic reticulum, as revealed by electron tomography images of infected cells [45]. Duplex RNA localizes to the interior of these vesicles, suggesting a mechanism by which host cell membranes are reorganized by SARS-CoV to conceal viral RNAs from cytoplasmic RLRs. Although some viruses may effectively avoid an immune response by sequestering dsRNA, RLRs may combat similar evasion mechanisms by localizing to various compartments in the cell.

**Evolution of RLRs provides insight into viral recognition strategies**

Studies on the evolutionary origin and variation of RLRs provide important insights into the molecular targets of these proteins. Previous work attempting to establish the common ancestor and evolutionary heritage of RLR proteins did not reach consensus [46,47], likely because the studies were conducted prior to the availability of structural information and boundaries between protein domains were not well understood.

However, recent studies of sequence variation among populations have revealed much about the evolutionary history of RLRs, their individual target specificities, and the protein domains that adapt to accommodate different viral subtypes. For example, the first reported studies of genetic variation in human RLRs [48] show that patterns of variation within the protein domains differ greatly among the three RLRs, supporting the notion that they each recognize significantly different PAMPs and are under different levels and types of selection. Intriguingly, diversity of RIG-I is the most strictly constrained, consistent with increasing evidence that it has other functions in the cell [25**,27**,29,30]. Patterns of sequence variation within the individual RLRs provide a window into the diversification of RNA binding modes. For example, geographically distinct human populations display variation within RNA binding domains of RIG-I, particularly at amino acids of the CTD and Hel2i that directly contact the 5′-triphosphate and duplex region of RNA PAMPs, respectively, suggesting that RIG-I has been tuned to recognize subtle alterations in viral RNA structures.

An intriguing analysis of cause and effect between RIG-I variation and viral susceptibility is provided by studies of RLR variation in rabbits [49*], and its conclusions are supported by larger studies of mammalian populations in general [50**]. Myxomatosis (MYXV) is lethal for some rabbits (European rabbits), while others (American cottontails and brush rabbits) are not highly susceptible. Given the role of RLRs in defense against MYXV infection, investigators examined patterns of RLR sequence variation and found important differences between European rabbits and others. For RIG-I, the most pronounced variation occurs at the terminus of pincer, and in the linker that connects pincer to the CTD, and at the connection between Hel1 and CARD2. These protein regions are essential for transmitting information about RNA binding and ATP hydrolysis to the CARD domains, suggesting that European rabbits have a defective connection between viral RNA binding and signaling.

An elegant structure-based analysis of RIG-I variation among diverse mammals also suggests that the pincer domain plays an important role in coupling RNA binding to signaling, and perhaps in tuning specificity for various RNAs [50**]. Alternatively, the pincer variations may indicate sites of interaction with other proteins, which may allosterically regulate the RLR signaling apparatus [50**]. Intriguingly, multiple studies [48,50**] have noted sequence variation along the outer surface of Hel2i as it appears in recent crystal structures. While this has been attributed to a potential interaction interface between Hel2i and other proteins, the same amino acids are actually located at the intramolecular interface between Hel2i and CARD2, which has been crystallographically visualized in truncated versions of the RIG-I protein [10*]. Prior to binding of RNA and ATP, the CARDS can dock against Hel2i [12,14], and therefore any sequence variation at this position will affect release of the CARDS and downstream signaling.

**Conclusion**

New insights into RLR sensing are emerging from diverse fields, in studies ranging from structural biology and immunology to evolutionary analysis. Together,
these findings are revealing a complex network of RNA sensors that sensitively detect and adapt to viral RNA molecules within the environment, providing insights into the continuous interplay between viruses and their hosts.

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