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REVIEW

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Tricks and threats of RNA viruses – towards understanding the fate of viral RNA

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ABSTRACT

Human innate cellular defence pathways have evolved to sense and eliminate pathogens, of which, viruses are considered one of the most dangerous. Their relatively simple structure makes the identification of viral invasion a difficult task for cells. In the course of evolution, viral nucleic acids have become one of the strongest and most reliable early identifiers of infection. When considering RNA virus recognition, RNA sensing is the central mechanism in human innate immunity, and effectiveness of this sensing is crucial for triggering an appropriate antiviral response. Although human cells are armed with a variety of highly specialized receptors designed to respond only to pathogenic viral RNA, RNA viruses have developed an array of mechanisms to avoid being recognized by human interferon-mediated cellular defence systems. The repertoire of viral evasion strategies is extremely wide, ranging from masking pathogenic RNA through end modification, to utilizing sophisticated techniques to deceive host cellular RNA degrading enzymes, and hijacking the most basic metabolic pathways in host cells. In this review, we aim to dissect human RNA sensing mechanisms crucial for antiviral immune defences, as well as the strategies adopted by RNA viruses to avoid detection and degradation by host cells. We believe that understanding the fate of viral RNA upon infection, and detailing the molecular mechanisms behind virus-host interactions, may be helpful for developing more effective antiviral strategies; which are urgently needed to prevent the far-reaching consequences of widespread, highly pathogenic viral infections.

In light of recent outbreaks of severe acute respiratory syndrome (SARS) and chicken or pig flu, viruses have been observed to pose a threat, not only to the health of infected individuals, but more importantly, as a cause of pandemic. Such outbreaks may also result in serious problems for healthcare systems and economies worldwide.

Viruses are composed of a genetic material core, either single- (ss) or double-stranded (ds) DNA or RNA, and an outer shell. The genome of ssRNA viruses may be either positive-sense, the (+) strand, or negative-sense, the (-) strand. Upon infection, the genome of positive-strand RNA viruses, for example hepatitis C virus (HCV), SARS virus, and an alphavirus-like superfamily of viruses, is immediately ready to be translated into viral proteins. On the other hand, negative-strand RNA viruses, for example influenza or Ebola viruses, and dsRNA viruses, for example reoviruses, genomes are unreadable by ribosomes; thus requiring transcription by viral RNA (vRNA)-dependent RNA polymerase (RdRp) into positive-sense RNA strands prior to initiating viral gene expression [1].

Interestingly, highly pathogenic RNA viruses are responsible for zoonotic and epidemic diseases such as coronavirus disease (COVID-19) and flu, outbreaks of haemorrhagic fever such as yellow fever, Dengue fever, and Ebola disease, or encephalitis such as Japanese

only on this particular group of viral pathogens, namely RNA viruses, and their interaction with human cell response pathways upon infection.

After only 24–48 hours after viral infection, up to 25% of all RNA molecules present in host cells are pathogenic vRNA [2], including viral messenger RNA (mRNA), encoding viral proteins, viral genomic RNA (gRNA), and ds replication intermediates (dsRNA). To prevent such a takeover of the host metabolism, the innate immune system protects cells, with adequate inflammatory and antiviral responses strongly relying on the proper detection of vRNAs. Viral nucleic acids are one of the strongest pathogen-associated molecular patterns (PAMPs), molecules causing particular immune system reactions. PAMPs are recognized by numerous pattern recognition receptors (PRRs) scattered across almost every part of the cell, where they sense the presence of viral particles. Among specific vRNA sensors, retinoic acid-inducible gene I (RIG-I)–like receptors (RLRs), Toll-like receptors (TLRs), 2′-5′-oligoadenylate synthetase (OAS), dsRNA-dependent protein kinase R (PKR), and interferon (IFN)-induced proteins with tetratricopeptide repeats (IFITs) can be found [3]. Upon detection of foreign RNA, type 1 IFN gene expression, as well as other inflammatory cytokines and chemokines, are stimulated to fight the infection. IFNs act in both an autocrine and paracrine manner, causing an effect on the cells from which the IFNs were produced or on nearby cells, respectively; thus

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activating IFN-alpha/beta receptors (IFNAR) and resulting in the expression of antiviral IFN-stimulated genes (ISGs) that have diverse antiviral and immunoregulatory functions.

In this review, we provide a comprehensive overview of the RNA sensing mechanisms involved in antiviral immune defence. Moreover, we discuss what countermeasures are implemented by RNA viruses to avoid being identified, as well as the cell strategies involved in diminishing the amount of vRNA upon its recognition. Understanding the RNA-sensing system and its detailed molecular mechanisms will help to better plan antiviral strategies, as more effective approaches are urgently needed to prevent the far-reaching consequences of highly pathogenic viral infections.

RNA sensing

During infection, vRNA is introduced into the cell. Foreign nucleic acid present in the cytosol is one of the most potent PAMPs; the host cell recognizes vRNA through the independent activation of several types of innate immune receptors in different cellular compartments (Fig. 1). In the cytoplasm, viral gRNA and its transcriptional intermediates are recognized mostly by RLRs, RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2), which belong to the DExD/H-box helicase family. In endolysosomes, the major RNA sensors that recognize either ds or ss nucleic acid are TLRs 3, 7, and 8. Since newly RNA binding properties of IFITs were discovered, they have been started to be considered as the important ISGs responsible for foreign RNA recognition [2]. Moreover, very recently one member of NOD-like family of proteins, which is the best recognized as key players in activation of

immune response during microbial infections [4], was identify as viral dsRNA sensor [5].

For activation of any RNA sensor, detecting an abnormal molecular RNA pattern, not present under normal conditions, is obligatory. These patterns may be some chemical modification of RNA, or the absence of such one, specific secondary or tertiary RNA structure, particular sequence, or annealed dsRNA intermediates occurring during viral propagation [6].

Cellular RNA is usually unable to activate the immune response, with several modifications protecting self-RNA from being recognized by pattern recognition receptors in the cytoplasm, under the normal conditions described. Recent studies have revealed that discriminating between self and non-self RNA depends on the 5′ end of the molecule [7,8]. In the nucleus, RNA polymerases synthesize nascent transcripts possessing a 5′ triphosphate group (ppp-RNA) at the 5′ end. In the case of premature mRNA, 5′ ends of nascent transcripts undergo co-transcriptional modification – the addition of a cap structure, meaning N7-methylated guanosine is joined to the first transcribed nucleotide through a 5′-5′ triphosphate bridge, forming a cap-0 structure (Fig. 2). The roles of cap-0 in stability, splicing, polyadenylation, mRNA export, and translation have been well defined and characterized for many years [9,10]. Importantly, in humans, like in other higher eukaryotes, cap-0 is further modified by nuclear cap-specific 2′-O-RNA methyltransferase, where the 2′-O-position of the ribose of the first transcribed nucleotide is methylated. Furthermore, after mRNAs are exported to the cytoplasm, their cap-1 may be subjected to one more methylation, where the ribose of the second transcribed nucleotide may be methylated at position 2′-O, forming cap-2[10]. Recent studies revealed that cap-1 plays a crucial role in distinguishing between self and non-self RNA [7,8], while the role of cap-
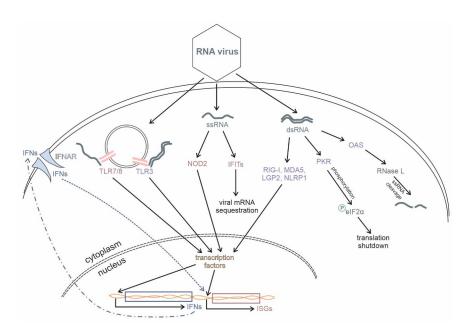

Figure 1. Cell signalling pathways that respond to viral RNA. During viral infection vRNA, single- or double-stranded, is introduced into the cell. This RNA is recognized by cellular sensors, RIG-I, MDA5, LGP2, PKR, OAS, TLR3/7/8, NLRP1, NOD2, and IFITs. Upon vRNA sensing, type I interferon response, as well as the production of antiviral IFN-stimulated genes (ISGs), is activated. Moreover, some vRNA sensors exert their function directly on viral RNA either by sequestrating viral transcripts from the pool of translationally active mRNAs (IFIT proteins) or by stimulating RNase L to degrade RNAs (OAS). Activation of PKR leads to phosphorylation of eukaryotic initiation factor eIF2α what subsequently results in global translation shutdown.
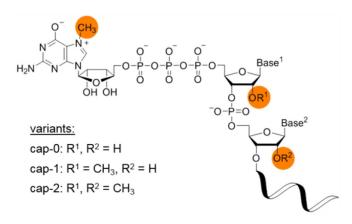
Figure 2. Structures of 7-methylguanosine RNA caps.

2 is still elusive. Nevertheless, some other classes of cellular RNAs, for example transfer RNA (tRNA) and ribosomal RNA (rRNA), are subjected to specialized processing pathways through which mature 5′ monophosphorylated molecules are generated. Interestingly, a fraction of small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) undergo another modification in which a trimethylguanosine cap (TMG cap) is incorporated at their 5′ end [9]. Among the four nucleotides comprising cellular RNAs, namely adenosine triphosphate, cytidine triphosphate, uridine triphosphate, and guanosine triphosphate, some may be found chemically modified in a post-transcriptional manner. To date, more than 140 of these modifications have been characterized [11]. Interestingly, the presence of some post-transcriptionally modified nucleotides was shown to also modulate innate immune responses [11–13]. Finally, what also protects endogenous RNA from being recognized by RNA sensors is the association of nucleic acids with different proteins; as RNA rarely fulfils its function alone, rather operating as part of the ribonucleotide complex (RNP).

RIG-I

RIG-I, with a multi-domain structure typical for proteins from the RLR group, is the first known member of the RLR family. RIG-I is composed of N- and C-terminal domains, with its central part known as a core. The core, a DExD/H-box helicase with an ATP-binding motif, is formed by subdomains Hel1 and Hel2, which are separated with a third subdomain, Hel2i. Hel1 and Hel2 bind ssRNA through several conserved motifs, named V and Ib. Hel2i has a regulatory function [31], also supposedly playing a role in dsRNA recognition [32,33]. Side RIG-I domains are the ss/dsRNA-binding C-terminal domain (CTD) and two N-terminal (caspase)-recruiting (CARD)-like domains. CARD domains stay repressed until the ligand is bound through the central DExD/H-box helicase core. Upon ligand binding, CTD releases the CARD domains, allowing them to bind to other CARD-containing proteins; this ability also makes CTD a repressor domain (RD) [34]. Interestingly, Hel2i can be mutated to produce constitutively active phenotypes, where the protein cannot be observed in its repressed form [31].

Interactions between the CARD domain and the Hel2i subdomain serve as receptor activation regulators, also governing ligand selection [35]. Activated RIG-I interacts with mitochondrial signalling protein (MAVS) at the cytosolic face of the outer mitochondrial membrane in order to trigger the signalling cascade [36]. However, to stimulate MAVS, CARD domain release is not sufficient; RIG-I must additionally undergo oligomerization [37].

The RIG-I CTD domain focuses on the 5′ end of RNA, recognizing molecules terminated with 5′-triphosphate or 5′-diphosphate groups [36,38,39] (Table 1). Therefore, distinguishing between self and non-self RNA occurs with 5′ end modification. Thanks to the presence of a cap structure on their 5′ end, endogenous mRNAs cannot be bound by RIG-I CTD [40]. Upon viral infection, short dsRNAs, 30–300 base pair (bp) molecules, bearing two to three phosphate groups at their 5′ ends, the most potent RIG-I activators, appear in the host cell cytoplasm. Nevertheless, RNAs up to 2 kbp have also been reported as RIG-I ligands [41,42]. Interestingly, dsRNA carrying cap-0 binds to RIG-I with similar affinity to ppp-dsRNA, with only the presence of cap-1 abrogating the interaction between dsRNA and this sensor protein [43]. Likewise, already mentioned RIG-I ligands, such as ssRNAs with a polyuridine-rich sequence and RNA bearing a 3′-phosphoryl group, can also activate this sensor protein [44,45]. Recently, Lu et al. described N6-methyladenosine (m^6A) RNA modification as an additional marker for enabling discrimination between self and non-self RNAs. It was shown that human metapneumovirus RNAs are m^6A methylated, guaranteeing deception of RIG-I [46].

MDA5

Similarly to RIG-I, MDA5, the second member of the RLR family, is composed of CARD, CTD, and helicase domains. However, in contrast to RIG-I, MDA5 does not recognize specific chemical groups at the 5′ end of RNA, rather discriminating for dsRNA based on molecular length [37,38]. Polyriboinosinic:polyribocytidylic acid (poly I:C), a synthetic analogue of dsRNA, was shown to, when long enough, behave as an MDA5 agonist; however, when successively digested to a length below 2 kbp, poly I:C became a RIG-I agonist [38]. Viral dsRNAs in the range of ~0.5–7 kbp are also reportedly recognized by MDA5[53]. Differences in substrate specificity between MDA5 and RIG-I result from discrepancies in their C-terminal domain structures. MDA5 CTD lacks a positive charge in its binding pocket, making it unable to recognize RNAs based on their negatively charged di/triphosphorylated 5′ ends. The RNA binding surface of MDA5 is also flatter than in RIG-I. Moreover, MDA5 CTD has a lower affinity for dsRNA, compared to the RIG-I C-terminal domain [47]. However, CTD is necessary for MDA5 to bind dsRNA with positive cooperativity (the longer the dsRNA, the more MDA5 particles can bind to the nucleic acid chain, with each attached MDA5 molecule making it easier for the next MDA5 protein to recognize the transcript), and when this domain is deleted [48] or replaced with RIG-I CTD, cooperative binding is abolished [47,48]. Structural studies have further revealed that, along dsRNA, MDA5 forms filaments

Table 1. Types of viruses and viral PAMPs to be recognized by various RNA sensing mechanisms.

Sensor	Virus	Viral genome	Viral PAMP
RIG-I	Orthomyxoviruses (Influenza A virus – IAV) [37,38]	Negative-strand RNA	ppp-ssRNA/dsRNA
	Paramyxovirus (measles, mumps, Sendai virus) [14,37,38]		cap-0-ssRNA/dsRNA
	Flaviviruses (Hepatitis C Virus – HCV, Japanese encephalitis virus) [15]	Positive-strand RNA	
MDA5	Picornaviruses (poliovirus, encephalomyocarditis virus – EMCV, Theiler's virus, and Mengo virus and rotavirus) [16,17,19-21,37,38]	Positive-strand RNA	dsRNAs
	Flaviviruses (Dengue virus) [74]		
	Paramyxoviruses (Sendai virus) [20]	Negative-strand RNA	
	Rhabdoviruses (Rabies virus) [17]		
LGP2	Orthomyxoviruses (IAV) [21]	Negative-strand RNA	non direct binding
	Paramyxoviruses (Sendai virus, Newcastle disease virus) [52]		
	Flaviviruses (HCV) [34]	Positive-strand RNA	
TLR3	Pneumoviruses (Respiratory Syncytial Virus – RSV) [22]	Negative-strand RNA	dsRNA
	Flaviviruses (West Nile virus) [22]		
	Orthomyxoviruses (IAV) [23]		
	Picornaviruses (poliovirus) [59]	Positive-strand RNA	dsRNA
TLR7	Orthomyxoviruses (IAV) [24]	Negative-strand RNA	ssRNA
	Rhabdoviruses (Rabies virus, Vesicular stomatitis virus – VSV) [25]		
	Paramyxoviruses (Sendai virus) [82]		
	Flaviviruses (Dengue virus) [74]	Positive-strand RNA	ssRNA
TLR8	Flaviviruses (HCV) [76]	Positive-strand RNA	ssRNA
PKR	Rhabdoviruses (VSV) [26]	Negative-strand RNA	dsRNA
IFIT1	Flaviviruses (Japanese encephalitis virus) [27]	Positive-strand RNA	ppp-ssRNA,
	Rhabdoviruses (VSV) [28]	Negative-strand RNA	cap-0-ssRNA
IFIT2	Rhabdoviruses (Rabies virus, VSV) [29]	Negative-strand RNA	ppp-ssRNA, cap-0-ssRNA
IFIT3	Orthomyxovirus (IAV) [28]	Negative-strand RNA	ppp-ssRNA,
	Phenuiviruses (Rift Valley virus) [28]	Negative-strand RNA	cap-0-ssRNA
IFIT5	Orthomyxovirus (IAV) [30]	Negative-strand RNA	ppp-ssRNA
	Rhabdoviruses (VSV) [30]		
NLRP1	Togaviruses (Semliki Forest virus) [5]	Negative-strand RNA	dsRNA
NOD2	Orthomyxovirus (IAV) [106]	Negative-strand RNA	ssRNA
	Pneumoviruses (RSV) [106]		

consisting of ring-structured elements occupying ~14 bp each [48]. Consistent with these studies is the finding that the level of antiviral response positively correlates with the amount of MDA5 protein bound to dsRNA; the longer filaments are, the longer target is recognized [47,49]. Longer filaments formed by MDA5 are also more stable when bound to dsRNA, explaining the dsRNA-length-dependence of MDA5 signalling activity [49]. Similarly to RIG-I, after binding to target RNA, MDA5 exposes a CARD domain and initiates cytokine and type I IFN production via MAVS signalling[50]. Although some viruses can be recognized by both MDA5 and RIG-I, these receptors predominantly recognize nonoverlapping RNA targets, implying that they possess distinct virus specificities (Table 1).

LGP2

Another RLR family member, LGP2, was originally found as a highly expressed gene in mammary tumours[34], where it was considered to be a potential negative regulator of RLR signalling[51]. Later, LGP2 expression appeared to also be induced by the presence of dsRNA, IFN treatment, or viral infection [52]. For instance, LGP2 protein overproduction inhibits both Sendai virus and Newcastle disease virus signalling[52]. Compared to other members of the RLR family, LGP2 lacks functional CARD domains and therefore cannot induce downstream signalling on its own [53]. Historically, as it was shown to act as both an activator and repressor of the immune response, the role of LGP2 has been disputed. Three models describing LGP2 involvement in viral dsRNA recognition have been proposed: 1) The first assumes LGP2 direct binding to vRNA, preventing RIG-I- and MDA5-mediated

recognition [52]; 2) In the second, LGP2 supposedly inhibits oligomerization of RIG-I and its subsequent interaction with MAVS via the LPG2 repressor domain [34]; 3) The last model describes LGP2 as a competing factor of Iκβ kinase I (IKK-I) in the recruitment of MAVS, thereby suppressing RLR signalling. However, in light of recent data, the aforementioned models must be questioned; since LGP2 appears to be an important regulator of RIG-I- and MDA5-mediated immune responses [54]. LGP2 facilitates the sensing of some viruses, greatly enhancing, or even enabling, viral dsRNA recognition [51] (Table 1). Most current data suggest that LGP2, through the DExD/H helicase domain, supports vRNA recognition via RIG-I and MDA5[57]. Recently, Takahashi et al [55]. described a new LGP2 function, suggesting that RIG-I, but not MDA5, indirectly interacts with the TAR-RNA binding protein (TRBP) through LGP2, regulating endogenous microRNA-mediated RNA silencing. TRBP is a dsRNA binding protein that binds to the HIV type 1 trans-activation response (TAR) element and functions as an RNA silencing enhancer [56].

Other DEXD/H box helicases

Recently, several additional DEXD/H box helicases have been reported to sense non-self dsRNAs. Zhang et al. showed that DDX1, DDX21, and DHX36 form a complex, binding to foreign dsRNA, and activating the type I IFN response [57]. DDX60, an orthologue of the yeast exosome cofactor Ski2, whose expression increases during a viral infection, is another example [58]. Unlike the previously mentioned three helicases, DDX60 does not act independently to sense dsRNA; instead binding to RIG-I-like receptors and promoting an anti-viral response [58]. DDX60 can also act independent of

RLRs, for example in HCV RNA degradation [59]. Furthermore, a growing body of evidence suggests that other DEXD/H box helicases may exhibit multiple functions in innate immunity. Just to mention that, for example DDX3 plays an antiviral role against a broad range of RNA viruses, including HCV [60], Dengue virus [61], Japanese encephalitis virus [62], and West Nile virus of the *Flaviviridae* family [63] and influenza of the *Orthomyxoviridae* family [64].

TLRs

TLRs are transmembrane glycoprotein receptors with an extracellular N-terminal part, responsible for PAMP-binding, and an intracellular C-terminal region, also known as a Toll/IL-1 R homology (TIR) domain, which takes part in downstream signalling. Characteristic features of this membrane-anchored receptor family include multiple leucine-rich repeats (LRRs), which form a horseshoe structure in the extracellular ectodomain, similarity in the TLR C-terminal region to the intracellular domain of the interleukin-1 receptor (IL-1 R) [65], and proteolytic pre-activation by specific digestion of the ectodomain in order to allow ligand binding [66,67]. In humans, this family consists of 10 members, of which four, TLR3, 7, 8, and 9, are responsible for sensing foreign nucleic acids. Sequence homology has revealed that TLR7, TLR8, and TLR9 exist in one evolutionarily conserved cluster within the TLR family [68]. TLR9 recognizes unmethylated deoxycytidyl-phosphate-deoxyguanosine (CpG) motifs commonly present in bacterial and viral genomes [69], whereas TLR3 and TLR7 and 8 sense ds- and ssRNAs, respectively [70,71]. In addition, TLR7 and 8 can recognize uridine-containing ssRNA [71] and synthetic guanosine-like compounds, such as resiquimod (R-848) [72], as well as, in the case of TLR7, short interfering RNAs (siRNAs) [73]. The most potent TLR7 agonists are vRNAs 0.8–2 kb long, while molecules exceeding 11 kb generally exhibit less potency [74]. Although TLR7 and TLR8 play a similar biological role, their expression varies among different cell types. TLR7 is present in plasmacytoid dendritic cells, while TLR8, which has been associated with enhanced human papillomavirus infection clearance and recognition of other viral ssRNA (Table 1) [75], is characteristic in monocytes and macrophages [76]. TLR7 and TLR8 [77] both possess two different binding sites. The first binding site, which is well conserved among TLR proteins, recognizes small chemical components and is localized in the sensor dimerization interface; upon ligand binding TLRs dimerize. The second binding site, which is responsible for ssRNA recognition, has different localization between the two TLRs; in TLR7 it is found in the dimerization region, while in TLR8 it localizes beyond the dimerization interface. The first binding site preferentially recognizes guanosine, while the second binds uridine moieties in ssRNA specifically. Additionally, this second binding site has slightly different sequence specificity for particular proteins; TLR7 preferentially binds polyU 3-mer, while TLR8 senses UG or UUG oligoribonucleotides [78]. In turn, it seems that TLR3 does not have any sequence specificity, with the minimum length of dsRNA required for its cooperative binding 40–45 bp [79,80]. It recognizes the dsRNA of rotaviruses, as well as viral replication intermediates of ssRNA viruses rich in guanosine and uridine [81] (Table 1). Availability of target RNA to TLRs depends largely on endocytosis of extracellular viral particles and on autophagy, which transports cytosolic viral replication intermediates into the lysosome [82]. Lysosomal localization is also beneficial as TLRs exhibit pH dependence of RNA binding [83]. Reduced pH of the endosome/lysosome promotes protonation of histidine residues, thereby enhancing RNA phosphate backbone binding affinity for TLRs. Interestingly, Wang et al. showed that the influenza virus activates TLRs at a lower pH than Dengue virus [74].

PKR

The antiviral response is also mediated through kinases, among which, PKR can be distinguished. PKR is a powerful factor that is activated upon foreign dsRNA binding, leading to a global translation shutdown [84]. It is composed of two distinct domains, an N-terminal regulatory domain for binding dsRNA (dsRBD), and a C-terminal catalytic domain (kinase domain). Under normal conditions, PKR is maintained as an inactive monomer. The primary function of dsRBD is to promote protein dimerization and activation [85]. This domain responds to both viral and synthetic dsRNA particles [86] (Table 1). PKR requires only 20–80 bp of dsRNA to optimally bind and activate an immune response [87]. Additionally, it may be activated directly or indirectly by other stimuli such as oxidative stress, growth factors, cytokines, and cellular proteins, such as PKR-associated activators (PACTs), or following TLR stimulation [88,89]. PKR activation disrupts the autoinhibitory state of this protein, enabling its homodimerization and autophosphorylation, thereby promoting phosphorylation of the eukaryotic initiation factor 2 alpha subunit (eIF2α) [89]; which results in blocked translation initiation and accumulation of stalled ribosomal pre-initiation complexes. Importantly, as most RNA viruses require the participation of eIF2α in mRNA translation, the ability to cause global arrest of not only cellular, but also viral protein synthesis, can lead to apoptosis in response to viral infection [84,88], making PKR a prominent antiviral agent. Viral infection and translation inhibition results in the appearance of specific structures called stress granules (SGs), which contain transiently repressed mRNAs, initiation factors, small ribosomal subunits, and RNA-binding proteins, of which, one is PKR [88].

Recently, Pham et al. reported an additional function of PKR, which is independent of translation inhibition [90]. In the aforementioned study, PKR was shown to be critical for IFN production downstream of MDA5, but not RIG-I. In addition, MDA5 was required for stimulation of PKR catalytic activity occurring in response to infection by an MDA5-restricted virus, but not in response to a RIG-I-sensed virus. These findings identified the previously uncharacterized role of PKR catalytic action; which cooperates with MDA5 signalling and highlights MDA5 unexpected role in stimulating PKR enzymatic activity [90]. Therefore, it is not surprising that viruses have developed mechanisms to counteract the

antiviral functionality of PKR. To avoid the immune response, viral proteins either bind viral dsRNA to prevent PKR pathogenic nucleic acid recognition, for example non-structural protein 3 (NSP3) from rotaviruses [91], or interact directly with PKR to prevent its activation, for example NSP1 from influenza viruses [92]. Similarly, the Japanese encephalitis virus uses its NSP2A to modulate PKR activity [93]. Among the multitude of evasion mechanisms that the HCV has evolved, inhibition of PKR through internal ribosome entry sites (IRESs) of gRNA has been distinguished. The HCV IRES has a complex secondary structure that efficiently competes with dsRNA for PKR binding, thus preventing kinase activation [94]. Moreover, HCV uses its NSP5A and E2 [95] proteins to directly bind and inhibit PKR [96], although the exact role of these proteins in this mechanism is not well established [97]. Interestingly, other viruses such as poliovirus and enterovirus induce rapid degradation of PKR [98,99].

IFITs

Among ISGs, the most important contributors to facilitating the immune response to exogenous RNA are IFITs [2] (Table 1). The human genome encodes five different IFIT proteins, namely IFIT1, IFIT1B, IFIT2, IFIT3, and IFIT5 [100,101,102]. Although IFITs, in contrast to RLRs, recognize ssRNA and bind directly to non-self RNA to inhibit their translation or replication, only IFIT1 and IFIT5 are able to interact directly with RNA 5′ end. IFIT1 and IFIT5 discriminate RNA on the basis of the 5′ end of the transcript, with IFIT5 binding only ssRNAs with a triphosphate group at this position, while ligands of IFIT1 may possess a triphosphate group or a cap-0 [2,101,102]. Although crystallographic studies have revealed the presence of a similar positively charged tunnel through which IFIT1 and IFIT5 interact with the phosphate backbone of bound RNA at the 5′ end, only the former possesses a large hydrophobic cavity, accommodating the cap structure, at the rear of the tunnel [103]. The finding that IFIT1 could directly bind capped RNA supports a model in which it competes with eukaryotic translation initiation factor 4E (eIF4E) to bind to the N7-methylguanosine triphosphate cap at the 5′ end of vRNA [2]. Endogenous mRNAs are protected from IFIT1 recognition via the presence of cap-1. Interestingly, with the exception of IFIT5 which exists exclusively as a monomer, IFIT proteins have the propensity to homo- and heterooligomerize [100]. IFIT1 is able to form complexes of different composition, interacting with both, or one, of IFIT2 and IFIT3 [102,103]. In each case, complex formation strengthens the interaction between IFIT1 and RNA. Furthermore, it occurred that IFIT2 specifically binds to AU-rich RNAs irrespective of modifications present at transcript 5′ end [86]. Tran et al. revelled that IFIT2 binding enhances translation efficiency of cellular mRNAs including interferon stimulated genes in order to stimulate antiviral response [104]. Interestingly, influenza mRNAs also contain AU-rich sequences and thus influenza exploits IFIT2 to stimulate its own gene expression and replication.

NOD-like receptors

NOD-like receptor (NLR) is a large family of pattern recognition receptors responsible for immune system activation during microbial infection, cellular injury or stress [4]. NLR members share common structural features, they all are composed of N-terminal effector, central nucleotide binding domain (NBD) and C-terminal leucine-rich repeat (LRR) domain. Some of them, e.g. NLRP1 and NLRP3, were acknowledged to form multi-protein complexes known as inflammasomes, which are molecular platform responsible for signal transduction. However, other NLR members, e.g NOD1 and NOD2, can act in immune response in an inflammasome-independent manner. Interestingly, besides their well-established role in immune response to bacterial infection, it occurred that some NLR members are engaged in antiviral response, among them NOD2 and NLRP3 could be distinguished. It is known that NOD2 is involved in the restriction of respiratory syncytial virus and influenza virus [105], whereas NLRP3 is important for immune response upon Sendai virus and influenza infection [5,106]. Both proteins induce interferon production via MAVS signalling, however only NOD2 was described as direct RNA sensor (Table 1). Very recently, unexpectedly Bauernfried et al. presented that another member of NLR, namely NLRP1, which antiviral activity was well documented in rodents [107], is engaged in immune response in human cells [5]. It occurred that NLRP1 is a direct sensor for RNA binding double-stranded molecules [5], while NOD2 recognizes ssRNAs [105]. Moreover, NLRP3, like MDA5, activates immune response upon recognition long dsRNAs [5,37,38]. This observation puts NLRP1 together with other pattern recognition receptors such as MDA5 and RIG-I as genuine dsRNA sensor.

Viral evasion and host shutoff

Although human cells have formed various pathways for the detection of viral particles, the multitude of our defence systems have forced viruses to develop sophisticated mechanisms through which to deceive host cells (Fig. 3). Several methods through which RNA viruses hide, shield, or modify their own genetic material to avoid recognition are discussed below. We also present some clever methods that viruses utilize to modulate cell RNA metabolism, and finally lead to host shutoff.

Replication in specialized bodies

During replication, RNA viruses produce several RNA species normally absent in uninfected cells, such as dsRNAs and ppp-RNAs [108]. Typically in host cells, RNA is not copied from RNA templates, thus, these unusual intermediate RNA molecules are recognized by innate immune sensors; however, viruses have learned how to circumvent and/or suppress these intracellular antiviral responses. One of the primary strategies that (+)ssRNA viruses utilize is to 'hide' highly recognizable replication intermediates from host sensors e.g. RIG-I and MDA5. Upon entry, coronaviruses and rhinoviruses elaborately modify intracellular membrane organelles, such as endoplasmic
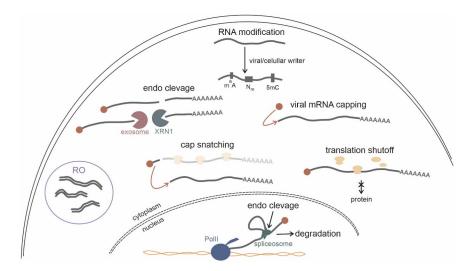
Figure 3. RNA viruses strategies either to avoid recognition by host cell immune system or to modulate cellular translation. Viruses use several methods to hide their genetic material from cellular RNA sensors. In order to do that viral RNA can undergo post-transcriptional modifications such as N6-methylation of adenosine (m^{6}A), internal 2′-O-methylation (N$_m$), and cytosine-5-methylation (5mC). Moreover, to evade recognition by anti-viral immune system, on 5′ end of viral mRNA cap structure can be installed either by viral capping enzyme or through the 'cap snatching'. RNA intermediates of viral replication can be hidden from host RNA sensors in specialized bodies called replication organelles (ROs). RNA viruses also learned how to lower the translation rate of cellular transcripts either by directly interfere with the biosynthesis of host proteins or by modulating RNA metabolism what in consequence leads to degradation of cellular transcripts.

reticulum (ER) and mitochondria, to form cytosol isolated organ-like spaces for vRNA replication, also called replication organelles (ROs) [109–111]. RO structures concentrate the viral replication machinery and replication byproducts inside membrane-bound vesicles or invaginations, making them undetectable by host cell cytosolic innate immune sensors. ROs can be divided into two types: the invaginated vesicle/spherule, and the double-membrane vesicle [112]. Although the exact method of RO formation is not known, invaginated vesicle/spherule ROs are usually formed upon infection with *Flaviviridae*, such as Dengue or Zika virus, while double-membrane vesicle ROs are established during infection with *Picornaviridae*, such as poliovirus or coxsackievirus B3, or *Coronaviridae*, such as severe acute respiratory syndrome coronavirus (SARS-CoV, also named SARS-1) or middle east respiratory syndrome coronavirus (MERS-CoV). Aside from the number of layers composing the specific types of replication organelles, the main difference between ROs is whether their content is connected to the cytosol. Only invaginated vesicle/spherule ROs can freely exchange their content with the cytosol. Interestingly, infection of cells with HCV leads to formation of membranous web, structure which is combination of single layer invaginated vesicle/spherule and double layer double-membrane vesicle [113]. Very little is known about how the number of ROs during infection correlates with infection severity in animal models or real hosts. Al-Mulla et al. showed that during infection with coronavirus mutants, those able to produce only half of the original number of ROs, no effect on replication or fitness of these viruses was observed [114]; although it may be arguable that viral replication occurs outside ROs in the case of these mutants. Interestingly, the presence of ROs in (-)ssRNA viruses infected cells is questionable; although, respiratory syncytial

virus reportedly has replication enzymes associated with cytosolic occluded structures, here named inclusion bodies [115].

Capping of vRNA

Although replication organelles serve as a spatial barrier enabling viral replication intermediates to hide from cell immune sensors, in some cases, this is not sufficient protection against host RNA receptor recognition. As mentioned above, some sensors rely on recognizing 5′ end RNA structures; therefore, many RNA viruses have developed additional protection strategies, including protection of vRNA 5′ ends through cap addition or structure imitation. A 5′ cap not only masks viral molecules from detection, but also provides efficient translation of invader proteins [116]. Poliovirus encodes a specialized cap mimicking the VPq protein, attaching it to the 5′ end of vRNAs, thus protecting these nucleic acids from recognition [117,118]. Interestingly, although these viruses do not require a cap structure for translation, since their vRNAs undergo cap-independent IRES-mediated translation [119,120], most viruses with an ssRNA genome synthesize a 5′ cap using their own set of enzymes [121,122]. After adding guanosine to the 5′ end of vRNA, through a triphosphate bridge, this newly formed cap is methylated at positions N7 and 2′-O by either one bi-functional N7/2′-O methyltransferase, like in flaviviruses [123], or by two separate enzymes, like in coronaviruses [124]. These two methylation steps enable formation of the vRNA cap-1 structure, a molecular signature of endogenous 'self' RNA in human cells. Lack of 2′-O-methylation, and in consequence formation of cap-0, leads to activation of the host defence system. Viral mRNAs bearing only cap-0 are sensed by IFIT proteins and excluded from the pool of translationally active

mRNAs [8]. Daffis et al. demonstrated that IFIT proteins recognized vRNAs produced by a mutated strain of the flavivirus, West Nile virus, which lacked 2′-O-methyltransferase activity [101]. Interestingly, cap 2′-O-methylation not only masks vRNAs from IFIT protein recognition, but also impacts MDA5 and TLR7 viral transcripts sensing. Züst et al. showed that in wild-type mice, 2′-O-methyltransferase deficient coronaviruses were non-pathogenic, although their replication and spread was restored in murine strains lacking MDA5 and TLR7 to the same level as in IFNAR mice [125].

Interestingly, viruses from the *Togaviridae* family, such as alphaviruses and rubiviruses, synthesize RNA 5′ cap structure lacking 2′-O-methylation at the first transcribed nucleotide [121,122,126,127]. However, despite bearing only a cap-0, alphavirus transcripts evade host recognition as non-self RNAs. This is possibly due to secondary structures within the 5′ untranslated region (UTR) of alphavirus RNA, which protects it from being sensed by IFIT proteins [128].

A different approach to protecting vRNAs is presented by (-)ssRNA viruses belonging to *Arenaviridae*, *Bunyaviridae*, and *Orthomyxoviridae* families [121,122]. Although these pathogens do not have their own cap synthesizing machinery, they are able to steal caps from endogenous cellular mRNAs to prime replication of their own vRNAs, a process known as 'cap snatching'. Upon recognizing a hosts mRNA cap, the transcript is cleaved by viral endonucleases usually 10–20 bases downstream of this structure [129]. These short-capped molecules are then used as primers for the synthesis of vRNAs via RNA-dependent RNA polymerase (RdRp). Interestingly, influenza carries out 'cap snatching' in the host nucleus, while most other viruses perform this action in the cell cytoplasm [129–131]. Following endonucleolytic cleavage, the remaining host mRNA is subjected to degradation via cellular RNA degradation machinery, for example enzyme XRN1. Limited studies investigate how 'cap snatching' affects host cell functioning, however this phenomenon likely plays a role in viral-host interplay.

Post-transcriptional viral RNA modifications

To date, more than 140 RNA post-transcriptional modifications have been reported in humans [11]. Inserting, deleting, substituting, or chemically modifying nucleotides within nascent RNA are not the only ways through which to manipulate protein expression, although these are convenient tactics to avoid activating the immune response via self-RNA molecules. Several major RNA modifications and editing events also occur in the case of vRNA, which have an influence on viral gene expression similarly to cellular RNA [40,46,132,133]. These include N6-methylation of adenosine, internal 2′-O-methylation, cytosine-5-methylation, isomerization of uridine to pseudo-uridine, and deamination of adenosine to inosine (A-to-I). These RNA modification/editing events have distinct consequences on viral infection, with the presence of post-transcriptional modifications in viral genomes potentially an evolutionary adaptation for immune evasion.

The best-studied, and likely the most abundant vRNA modification, post-transcriptional modifications of cellular RNAs is m^6A [134,135]. Three types of enzymes are related to m^6A modification processing, namely writer, eraser, and reader enzymes. Writers, methyltransferase-like enzymes, such as METTL3 and METTL14, together with Wilms tumour 1-associated protein (WTAP) and KIAA1429, deposit this modification onto RNA [136]. Erasers, such as the demethylase fat mass and obesity-associated protein (FTO) and α-ketoglutarate-dependent dioxygenase AlkB homolog 5 (ALKBH5), are responsible for removing the methyl group from adenosine [137,138]. The YTH domain family of reader proteins, including YTHDC1, YTHDC2, YTHDF1, YTHDF3, and YTHDF3, direct m^6A post-transcriptional functions [136]. Upon binding, YTHDF1 promotes translation of mRNAs, while YTHDF2 targets transcripts for degradation. YTHDF3 has been found to play a dual role, promoting protein biosynthesis in synergy with YTHDF1, and also affecting methylated mRNA decay mediated by YTHDF2 [139]. RNA genomes of HCV, Zika virus, Dengue virus, yellow fever virus, and West Nil virus of the *Flaviviridae* family contain such m^6A modifications [140–142]. The main reported role of m^6A refers to its actions in viral gene expression [134,135,143]. This type of methylation is considered a negative regulator of both HCV and Zika virus life cycles [140,142]. Manipulating writer and eraser amounts results in significant changes to intracellular HCV protein levels, as well as the amount of HCV RNA in the extracellular environment [140]. Most recently, human metapneumovirus RNA was shown to be m^6A methylated in order to avoid RIG-I recognition [46].

For cellular RNA, pseudouridine is the most abundant post-transcriptional modifications; although there are only a few reports that confirm its presence in vRNAs [144,145]. In human cells, as in other eukaryotes, isomerization of uridine to form 5-ribosyl uracil (pseudouridine) is catalysed by pseudouridine synthases (PUS) [145]. Although, in yeast RNA, pseudouridine is suggested to play a role on transcript stability [133], its function in regulating viral gene expression remains largely unexplored. Recently, PUS proteins were identified in a CRISPR-based screen designed to reveal host factors facilitating flavivirus replication [146]. Interestingly, pseudouridine, when incorporated into a synthetic transcript for therapeutic purposes, guaranteed both higher mRNA stability and protein biosynthesis yield, also helping modified molecules to escape immune recognition [147,148].

The next most common modification found in different human RNA species is cytosine-5-methylation (5mC). This is present in highly abundant tRNA and rRNA, supposedly having an impact on nucleic acid structure and function [132]. Although this modification was also identified in mRNA, its role in messenger molecule metabolism is still enigmatic. Currently, it is known that the position of 5mC within mRNA has differential effects on transcript fate [149–152]. Enzymes responsible for 5mC belong either to DNMT2 or to NOL1/NOP2/sun (Nsun) methyltransferase subgroups [153,154]. Unfortunately, far less is known about 5mC effector proteins. Recently, 5mC was shown to be specifically recognized by the mRNA export adaptor ALYREF, whose downregulation in HeLa cells resulted in increased nuclear retention of 5mC-containing transcripts [150]. Infection with Dengue virus, Zika virus, HCV, or poliovirus changes the 5mC RNA landscape in infected

cells, suggesting its potential role in the viral replication cycle. Moreover, data show that 5mC is present in both vRNA isolated from cells and in released viral particles [155,156].

Interestingly, some viruses that cap their own viral mRNA with cap-1, using 2′-O- methyltransferase, can utilize the same enzyme to introduce internal modifications, for example, Dengue virus employs its NSP5 protein to modify internal adenosines in vRNA [157]. 2′-O-methylated nucleotides, present in short vRNA sequences, repress cytokine and IFN production; a result of activating the endosomal immune sensor TLR7 [158]. It is possible that internal 2′-O-methylated adenosines also limit innate sensing during viral entry, although such a conclusion requires verification [159].

In human cells, enzymes called adenosine deaminases act on RNA (ADAR) 1 and 2, catalysing the deamination of position C6 in adenine (A) to produce inosine (I) [160]. These A-to-I editing events occur in dsRNA. This conversion may have significant biological implications such as changing the coding capacity of ribosomes or viral RNA dependent RNA polymerases, and altering the RNA structure, as I behaves similarly to guanosine (G). Interestingly, in human cells, ADARs have been shown to edit genomes of paramyxoviruses, such as human respiratory syncytial virus, parainfluenza virus 5, and human metapneumovirus [111,161,162]. Additionally, I has also been found in (+)ssRNA flaviviruses, for example HCV, Zika virus, and Dengue virus, and picornaviruses, namely poliovirus [155,163]. Taylor et al. showed that this type of post-transcriptional modifications successfully reduced the presence of HCV proteins, most likely due to IFN-mediated decreases in cap-independent translation [163]. In contrast, installation of I also masks vRNA from MDA5 sensor detection, and prevents IFN and other inflammatory protein synthesis [164,165]. Thus, A-to-I editing in vRNA may potentially suppress the innate immune response [166].

Viral endonucleases

One of the most intriguing ways through which several viruses avoid recognition is endonuclease action on their own vRNA. Though it may seem counter-intuitive to destroy one's own RNA, this has proven to be a useful strategy in evading immune recognition. For instance, the SARS-CoV genome encodes for NSP15, an endoribonuclease [167]. Although NSP15 does not degrade viral or host RNA in a non-specific manner, it is responsible for cleaving the poly-(U) tail, generated at the 3′ end of transcripts during viral replication, from vRNAs. Eliminating this tail limits PAMP formation, hampering MDA5s ability to activate the innate immune response to viral infection [168].

The polymerase acidic protein (PA) of influenza also possesses endoribonuclease activity. PA, together with subunits PB1 and PB2, forms a viral polymerase complex [130]. The PA factor participates in endonucleolytic cleavage of host mRNA during the 'cap-snatching' process [131]. However, the alternative products of the *PA* gene, called PA-X, and its truncated variant PAXΔC20, were recently shown to be responsible for host shutoff in influenza infected cells [169–172]. Although PA-X preferentially targets ssRNA transcribed by host RNA polymerase II (Pol II) only, it also efficiently cleaves dsRNA [173]. Interestingly, the PAXΔC20 protein is characterized by severely reduced endonuclease activity. Recently, Gaucherand et al. revealed that PA-X targets majority of host cell mRNA for degradation [170]. Following endonucleolytic cleavage, resulting RNA fragments are eliminated via host RNA degradation machinery. Additionally, PA-X selective cleavage of endogenous Pol II transcripts is tightly connected with RNA splicing. These data suggest distinct cellular roles for both PA-X and PAXΔC20 proteins in viral infection. Taken together, influenza viruses possess a unique host shutoff mechanism that contributes to viral infection efficiency. Moreover, this is a remarkable example of how viruses hinder host cell immunity while promoting the expression of their own viral mRNA.

Hijacking host translation

General host shutoff (GHS) is a broadly termed consequence of all viral actions, eventually halting overall protein expression in the infected cell. Such a strategy allows viruses to take full capacity advantage of cellular translation machinery. The lower total protein expression rate means that fewer RNA receptors are produced by the host cell, allowing more viral transcripts to be efficiently processed into viral proteins. Various viruses handle expression hijacking differently. Coronaviruses, for example SARS-CoV and MERS-CoV, cause host shutoff at the post-transcriptional level; where the process is mediated by NSP1, which binds to cellular translation factors, preventing mRNA translation. SARS-CoV NSP1 blocks the 40S ribosomal subunit, effectively repressing the translation of both viral and host transcripts [174–176]. Data by Lokugamage et al. revealed that, depending on the mechanism of initiation operating on the mRNA template, NSP1 is able to inhibit translation at this stage [175]. Furthermore, MERS-CoV NSP1 utilizes yet another mechanism, through which it is able to differentiate between cellular mRNA produced in the nucleus and viral mRNA synthesized in the cytosol. SARS-CoV NSP1 is exclusively localized in the cytoplasm, stably binding to the 40S ribosomal subunit, while MERS-CoV NSP1 is present not only in the cytoplasm, but also in the cell nucleus, with a less stable interaction occurring with 40S. These data suggest that MERS-CoV NSP1 targets host mRNA in the nucleus, exporting it together with host transcripts to the cytoplasm, thus sparing viral mRNAs of cytoplasmic origin [177].

Viruses of the family *Picornaviridae*, including poliovirus, human rhinoviruses, and coxsackievirus, use a yet another tactic to inhibit cap-dependent translation of host mRNA [178–180]. Although picornaviruses use 2A proteinase to cleave the translation initiation factor elF4G, viral protein biosynthesis is unaffected since translation from viral mRNA depends on IRES [181]. Recent work by Aumayr et al. indicated that, in order to efficiently cleave eIF4G, human rhinovirus 2A proteases must bind to another translation initiation factor, elF4E [182].

In the case of human respiratory syncytial virus, from the *Paramyxoviridae* family, it is difficult to identify a single mechanism responsible for host shutoff. Bruce et al. suggested

that human respiratory syncytial virus may specifically alter the expression of surfactant protein A (SP-A) in human pulmonary epithelial cells by interfering with protein translation [183]. The role of SP-A proteins is to bind directly to human respiratory syncytial virus, marking the virus for degradation by the host defence system. Moreover, the human respiratory syncytial virus genome encodes two unique nonstructural proteins, NSP1 and NSP2; the presence of which should lead to an effective induction of host shutoff. Production of these proteins strongly reduces IFN response activation upon infection with human respiratory syncytial virus [184]. Furthermore, both NSP1 and 2 individually and cooperatively target proteins from the IFN signalling cascade, including RNA sensors, such as RIG-I, MDA5 and OAS, and ISGs, such as IFIT1 [185–188]. Therefore, to better understand the impact of NS1 and NS2 on the innate immune system, Goswami et al. performed a series of experiments suggesting that these proteins are part of a multiprotein complex known as the NS degradosome [189]. Another layer of complexity to how human respiratory syncytial virus may gain advantage over host cells is through stress granule formation, a phenomenon benefited by viral replication [190,191].

Viral RNA degradation

Recognition of vRNA is a crucial step in the host cells defence against invading viruses. To efficiently eradicate an infection, prompt degradation of recognized vRNA is vital. Regular RNA decay enzymes digest vRNA from the 5′ to 3′ end, and vice versa; however, cells have also developed specialized factors dedicated to eliminating vRNA particles [192–194]. To combat this, viruses have again gained specific mechanisms to counteract these cellular RNA degradation systems.

XRN1

The 5′-3′ exonuclease, XRN1, is a key player in RNA turnover and surveillance pathways, catalysing mRNA degradation [195]. XRN1-dependent mRNA degradation regulates the proteome by controlling the mRNA population available for translation, which also serves a significant role in shaping the cell response to viral infection. RNA molecules are protected from XRN1 digestion when their 5′ end is capped; with the decapping process strictly controlled by a complex net of interacting proteins. Elimination of this cap structure predestinates endogenous mRNAs, and vRNA alike, for degradation by XRN1. Thus, viral pathogens have evolved means through which to manipulate the fate of their mRNA [195]. RNA viruses present different strategies via which to circumvent XRN1 action, thereby protecting their genomic integrity. Those which have m^{7}GTP-capped mRNA simply avoid the exonucleolytic activity of XRN1[196]. As mentioned above, capped host transcripts must first undergo decapping, a reaction performed by the DCP1-DCP2 complex, which leads to generation of 7-methylguanosine diphosphate (m^{7}GDP) and a 5′-monophosphorylated mRNA exposed to 5′-exonucleolytic degradation. Additionally, viral capped RNAs are the perfect substrates for this decapping complex,

the action of which would leave pathogenic molecules exposed to XRN1 degradation.

Therefore, it is not surprising that viruses attempt to reduce XRN1 availability in the host cytoplasm in order to accumulate their own RNAs. Rotaviruses decrease the level of cytoplasmic XRN1, along with DCP1, by relocating these proteins to the cell nucleus [196]. Moreover, rotavirus NSP1 protein has been shown to target another component of the RNA degradation machinery, PAN3, a component of the deadenylation complex [196]. Furthermore, poliovirus also targets this same set of enzymes, although upon viral infection, all three RNA decay factors, XRN1, DCP1, and PAN3, undergo accelerated degradation [197].

A different approach to XRN1 targeting is exploited by flaviviruses such as Zika or Dengue virus. Although, initially, XRN1 appears to act as an antiviral agent, degrading pathogenic gRNA, presence of highly structured sequences in its 3′ UTR causes XRN1 stalling. This results in accumulation of subgenomic flavivirus RNA (sfRNA), which is toxic to the cell [198–200]. Accumulated sfRNAs facilitate infection with flavivirus by interacting with many cellular proteins responsible for the modulation of host RNA interference, RNA decay, and IFN activation [201]. Moreover, XRN1 stalling on sfRNAs results in repression of XRN1 activity and accumulation of uncapped cellular mRNAs [200,202]. A slightly different strategy to repress XRN1 is applied by other members of the *Flaviviridae* family, such as HCV and bovine viral diarrhoea virus. In this case, the region where XRN1 stalls is located in the 5′ UTR of gRNA, leading to repression of XRN1 activity [203]. This dysregulation causes stabilization of normally short-lived mRNAs, including mRNAs encoding immune regulators and oncogenes. Therefore, this finding contributes a new layer of evidence to HCV pathogenesis, including for hepatocellular carcinoma.

The mechanisms through which host RNA degradation machinery exerts its function in order to eliminate vRNAs remain elusive. Recently, Ng et al. shed some light on this process, suggesting that XRN1 and DCP2 repress replication of the Newcastle disease virus, from the *Paramyxoviridae* family, as well as the encephalomyocarditis virus, from the *Picornaviridae* family, inside discrete cytoplasmic foci, distinct from SGs and processing bodies (P-bodies) [204]. Interestingly, this finding indicates that the function of both XRN1 and DCP2 could be tightly regulated to specifically target vRNAs, separate to host transcripts.

Exosome complex

Another major component of RNA degradation machinery is a multisubunit exosome complex, which predominantly behaves as a 3′-5′ exoribonuclease [205]. It is composed of catalytic proteins and nine core subunits, six of which, Rrp41, Rrp45, Mtr3, Rrp42, Rrp43, and Rrp46, form a ring-like structure capped by a trimer of Rrp4, Rrp40, and Csl4. Interestingly, although each hexameric ring subunit possesses an RNase PH-like domain, the exosome core is catalytically inactive. Therefore, to fulfill its duties, the exosome core in the cytoplasm of human cells associates with other subunits such as RRP44, a 3′-5′ exoribonuclease also named DIS3,

which can also act as an endonuclease [206], or the 3′-5′ exoribonuclease DIS3L. Additionally, the RRP6 exoribonuclease supports the exosome with its 3′-5′ activity [207,208]. Moreover, productive exosome action requires interactions with several cofactors, which target the exosome to different RNA substrates. Many of these cofactors were described in budding yeast, including the Ski (super killing) complex, composed of Ski2/Ski3/Ski8, which is responsible for feeding RNAs to the exosome in the cytoplasm [209,210]. Historically, Ski genes were named for their dsRNA virus 'super killing' ability; pathogens which would have otherwise been lethal for yeast strains deficient in these genes [209]. Importantly, orthologues of all three Ski genes are also present in higher eukaryotes, although their substrate specificity is still unknown [211,212]. Recently, Eckard et al. showed that SKIV2L, the human orthologue of yeast Ski2, engaged in the regulatory mechanism responsible for limiting RIG-I activation. Cells depleted in SKIV2L were found to accumulate cleavage products of the inositol-requiring enzyme 1 (IRE-1) endonuclease, leading to RIG-I activation. Moreover, patients with SKIV2L deficiency show a potent IFN signature in their peripheral blood cells [213].

In humans, the nuclear exosome associates with at least three different cofactors, including NEXT (MTR4, ZCCHC8, and RBM7), PAX (MTR4, ZFC3H1, and PABPN1), and hTRAMP (MTR4, ZCCHC7 and PAPD5 [205]), in which only RNA helicase MTR4 is a common component. Recently, it has been demonstrated that, upon infection with an ssRNA virus, normally nuclear proteins, MTR4 and ZCCHC7, shuttle to the cytoplasm to form cytoplasmic complexes that specifically recognize and induce degradation of viral mRNA [214]; however, the exact mechanism of viral transcript degradation is yet to be established.

OAS and RNase L

RNase L is a ubiquitous cellular endoribonuclease whose expression in humans, as in most vertebrates, is induced upon recognition of vRNA. IFNs transcriptionally induce OAS proteins that, by binding dsRNA, are activated to produce, from cellular ATP, higher oligomers of 2′-5′-oligoadenylates, which activate RNase L. Upon binding these 2′-5′-oligoadenylates, RNase L forms either homo- or oligomers, thus becoming an active enzyme that cuts, without distinguishing, both viral and cellular ssRNA [45,89]. RNase L cleavage products are recognized by RIG-I and MDA5, further increasing IFN production and the antiviral response. It is still unclear how cells coordinate RNA sensing via signalling and IFN production. Recently, RNase L was shown to participate in the formation of antiviral SGs (avSGs) through its dsRNA cleavage products. These dsRNAs activate PKR and recruit other antiviral proteins, including RIG-I, PKR, and avSGs [89,215–217].

RNP assemblies formed in the cytoplasm in response to diverse stress signals are called SGs [215]. As the main role of SGs is to prevent unnecessary protein generation by pausing translation upon cellular stress, SG composition depends on the nature of the stress signal. Different proteins are recruited to non-membranous complexes, for example protein kinases such as PKR, general control non-derepressible 2 (GCN2), or PKR-like ER kinase (PERK), in response to specific types of stimuli, including heat, oxidation, hypoxia, and osmotic pressure [215,218,219]. Transcripts accumulated in SGs are either subjected to degradation, or translation is re-initiated.

avSGs are formed in response to viral infections and can be compared to typical SGs formed as a response to canonical stress signals. SGs, whose main role is signalling, are RNP assemblies in the cytoplasm [89,220]. The production of IFNs during several viral infections was shown to require avSGs for formation. Thus, avSGs have been described as an important platform for sensing vRNAs during viral infection.

ISG20

The protein of the IFN-stimulated gene of 20 kDa (ISG20) was first described as an IFN-induced protein associated with pathological structures of promyelocytic leukaemia (PML) [221,222]. ISG20 is a member of the DnaQ-like 3′-5′ exonuclease superfamily, with 3′-5′ activity towards RNA. Its catalytic property has been associated with replication inhibition in a broad range of RNA viruses, including *Flaviviridae* (yellow fever virus, West Nile virus, Dengue virus, and HCV) [223–226] and *Orthomyxoviridae* (influenza) [227].

Supposedly, the main antiviral mechanism of ISG20 is direct 3′-5′ exonucleolytic degradation of vRNA; as suggested by its potent exonuclease function *in vitro* and the observed loss of antiviral effects upon its mutation at residues of the death effector domain (DEDD) [222,228,229]. However, several studies also reported ISG20 viral inhibition in the absence of vRNA degradation [225,230], raising the question whether alternative mechanisms may be involved. ISG20 was recently showed to inhibit the vesicular stomatitis virus through translational blockage. Data by Wu et al. revealed that ISG20 exhibited the ability to act as a general translational modulator of exogenous transcripts [231]. Interestingly, the authors also showed that it appears to spare self RNA [231], although its exact mechanism of RNA discrimination remains unknown. Nonetheless, researchers excluded the idea that ISG20 affects mRNA nucleocytoplasmic transport and translation initiation [231]. Furthermore, specific co- or post-transcriptional modifications, such as m^6A, may contribute to RNA discrimination.

ZAP

Identification of vRNA by the host innate immune system could be performed not only based on vRNAs characteristic features or the presence of a triphosphate group at the 5′ end, but also on more genome-wide attributes of RNA viruses, such as nucleotide composition. The zinc-finger antiviral protein (ZAP) eliminates cytoplasmic RNAs recognized as non-self, due to their elevated CpG dinucleotide content, compared with endogenous mRNAs. Therefore, it is not surprising that the replication of high CpG content-RNA viruses, such as alphaviruses, influenza viruses, and flaviviruses, is controlled by ZAP [226,232–236]. The ZAP protein contains several domains, among which the N-terminal RNA-binding domain, composed of four CCCH zinc fingers, can be

distinguished. This RNB domain has the ability to directly bind to CpG motifs in the RNA sequence, making it necessary for the protein to fulfill its function [237]. Interestingly, RNA viruses with an increased frequency of UpA also undergo ZAP-mediated attenuation [238,239]. In line with these results is the observation that a reduction in CpG or UpA frequencies leads to an increase in viral replication rate [239].

The ZAP protein binds directly to specific vRNA, recruiting the exosome complex and degrading targeted transcripts [234,240,241]. On the other hand, vRNAs recognized by ZAP could undergo decapping and would thus be subjected to XRN1 degradation [240,241]; however, whether viral transcripts are eliminated via this alternative pathway is still unclear [234]. Upon alphavirus infection, ZAP was also shown to repress viral mRNA cap-dependent translation by interfering with translational initiation factors eIF4G and eIF4A [242]. Moreover, Odon et al. unexpectedly revealed that sequences with a high CpG or UpA content may be recognized via OAS and be further degraded by RNase L [238]. However, further work is needed to investigate the potential crosstalk between ZAP-, OAS- and RNase L-mediated antiviral pathways.

RNA interference

In canonical RNAi pathway dsRNA is cleaved by Dicer endoribonuclease producing 20–24 nt long RNA duplexes – short interfering RNAs (siRNAs) [243]. When separated, one strand from siRNA molecule is incorporated into RNA-Induced Silencing Complex (RISC) and serves as a guide RNA for finding a complementary mRNA. Target mRNA and RISC binding induces transcript cleavage mediated by Argonaute protein, component of RISC, what leads to particular gene silencing. In course of the studies it occurred that RNAi pathway is also an important antiviral mechanism. Dicer could digest viral dsRNA intermediates what would lead to production of virus-derived siRNAs (vsiRNAs) that facilitate antiviral response. Presence of vsiRNAs in infected cells was well documented in plants and in invertebrates, such as fruit fly or nematode. Thus, it is also possible that the same antiviral mechanism could be present in mammalian cells. Several studies dedicated to find vsiRNAs in mammalian cells failed what put into question possibility of generating these siRNA species upon viral infection in mammals [244,245]. Interestingly, an exception arises in the case of murine oocytes and embryonic stem cells [246–248]. These observations led to conclusion that RNAi pathway could play a role in antiviral response only in cells that are undifferentiated somatic cells. This discrepancy between undifferentiated and differentiated cells could be due to differences in Dicer domain composition, somatic Dicer is full length protein, while in embryonic stem cells truncated form is present [249]. It was shown that only amino-terminally truncated Dicer was able to process dsRNAs and turn these into functional siRNAs in murine and human cells [249,250]. However, situation changed completely in 2013, when Mallard et al. and Li et al. proved independently that vsiRNAs are also present in virus infected somatic mammalian cells [251,252]. Previous inability to capture the presence of vsiRNAs in viral infected cells could be explained in several ways. Differentiated cells are able to induce potent interferon response upon virus infection, which inhibits Dicer activity [55,253,254]. Moreover, many RNA viruses, like Nodamura virus, influenza, and Dengue virus, actively antagonize host immunity by the production of viral suppressors of RNAi: B2, NS1 and 2A protein, respectively, to hamper Dicer activity [251,252,255]. Thus, infection of mammalian cells with viral strains deficient in aforementioned proteins enabled vsiRNA detection.

Based on already carried out studies it is certain that RNAi pathway helps to eradicate viruses from mammalian cells but from the other hand this strategy is not the main mechanism to fight viral infection. Further studies are needed to learn to what extent RNAi contributes to antiviral response.

Concluding remarks and future perspectives

Significant progress has been made in elucidating which mechanisms lead to induction of host cell antiviral defences. Although much is known about the main molecular features of virally-derived RNAs responsible for inducing innate immune system receptors, significantly less is known about how viruses countermeasure a host defence mechanisms. Recent work has highlighted the importance of masking vRNAs through various 5′ end modifications, as well as processes of mimicking cellular RNAs via 'cap snatching' or even direct blocking of receptor proteins, thus preventing recognition. Among the vast repertoire of viral tricks are m^{6}A methylation to avoid RIG-I recognition, 2′-O-methylation preventing IFIT, MDA5, and TLR7 recognition, forming complex secondary RNA structures which interfere with PKR sensing. Interestingly, majority of RNA viruses use multiple mechanisms to avoid recognition by the host cell. For instance, coronaviruses instal a cap-1 structure on their mRNA, as well as hide their replication in cytosol isolated organ-like structures, known as ROs. In addition to reducing vRNA visibility, viruses are able to harness those host cellular mechanisms dedicated to eliminating them, for their own benefit. Flaviviruses exploit the 5′-3′ exonuclease, XRN1, to generate, from their genomic nucleic acids, sfRNAs, which are toxic to the host cell.

Despite our knowledge of well-known viral evasion mechanisms, our understanding of exactly how host RNA degradation machinery exerts its function to eliminate vRNAs remains unclear. Thus, further studies are needed to fully understand the interplay between viruses and host cells. Moreover, an in-depth understanding of the molecular mechanisms between viral-host relationships would allow for the design of appropriate antiviral drugs.

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