

## Review

## A Balancing Act: MDA5 in Antiviral Immunity and Autoinflammation

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**Induction of interferons during viral infection is mediated by cellular proteins that recognise viral nucleic acids. MDA5 is one such sensor of virus presence and is activated by RNA. MDA5 is required for immunity against several classes of viruses, including picornaviruses. Recent work showed that mutations in the *IFIH1* gene, encoding MDA5, lead to interferon-driven autoinflammatory diseases. Together with observations made in cancer cells, this suggests that MDA5 detects cellular RNAs in addition to viral RNAs. It is therefore important to understand the properties of the RNAs which activate MDA5. New data indicate that RNA length and secondary structure are features sensed by MDA5. We review these developments and discuss how MDA5 strikes a balance between antiviral immunity and autoinflammation.**

**MDA5, a Key RNA Sensor**

Mammalian cells use pattern-recognition receptors (PRRs) to detect the presence of infectious microorganisms [1]. These receptors are activated by pathogen-associated molecular patterns (PAMPs). In the case of virus infection, PAMPs are often nucleic acids [2,3]. For example, viral RNAs trigger PRRs, including the endosomal toll-like receptors (TLRs) 3 and 7 and the cytosolic RIG-I-like receptors (RLRs) [2,3]. The RLR family comprises three members: retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) [2,3]. These proteins are ubiquitously expressed at low levels. All RLRs contain a DExD/H-box RNA helicase domain and a C terminal domain (CTD), both responsible for RNA binding [4]. In addition, RIG-I and MDA5 have two N terminal caspase activation and recruitment domains (CARDs). Upon activation of RLRs by RNA binding, the CARDs interact with the adaptor mitochondrial antiviral signalling protein (MAVS), ultimately leading to the transcription of the genes encoding type I **interferons (IFNs)** (see [Glossary](#)) [2–4] ([Figure 1](#)). Autocrine and paracrine IFN stimulation subsequently induces transcription of hundreds of IFN-stimulated genes (ISGs), several of which encode proteins with direct antiviral functions [5]. RLRs themselves are encoded by ISGs, constituting a feed-forward loop. Type I IFNs further coordinate cellular immune responses to virus infection and are thus essential for antiviral immunity [6]. In addition to inducing type I IFNs, RLRs and MAVS also activate apoptosis, leading to the elimination of the infected cell [7].

Given the abundance of nucleic acids in healthy cells, a key question is to understand the mechanisms by which nucleic acid-sensing PRRs become activated specifically following virus infection. The features of RIG-I-stimulatory RNAs are well understood and include the presence of two or three phosphate groups at the 5'-end, the absence of 5'-cap methylation, and base-pairing adjacent to the 5'-end [8–15]. These features are characteristic of viral RNAs produced by some viruses, such as influenza A virus or reovirus [8,9,12], but are not typically found in cellular RNAs present in the cytosol of healthy cells, explaining selective activation of RIG-I in virus-infected cells. It is also possible that, in some settings, mislocalised cellular RNAs activate

**Highlights**

MDA5 is a pattern-recognition receptor for RNA and induces a type I interferon response.

MDA5 is activated in a variety of clinically relevant settings. This includes infection with ssRNA, dsRNA, and dsDNA viruses; several autoimmune and autoinflammatory diseases, such as type 1 diabetes and Aicardi-Goutières syndrome; and some forms of cancer treatment.

Synthetic, viral, and cellular RNAs can all activate MDA5. The latter may include transcripts from endogenous retroelements such as *Alu* repeats.

Length and secondary structure are important features that determine whether an RNA molecule is detected by MDA5. Indeed, long, base-paired RNA molecules potentially activate MDA5 in the test tube.

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RIG-I and other nucleic acid sensors of the innate immune system [16,17]. Although RIG-I and MDA5 share similar domains, they detect different viral infections [18]. For example, RIG-I detects infection with orthomyxoviruses, such as influenza A virus, while MDA5 senses picornavirus infection. In contrast to RIG-I, the mechanisms that allow MDA5 to recognise viral RNAs while avoiding cellular RNAs are less well understood. Biochemical and structural work using recombinant MDA5 protein demonstrated that MDA5 senses RNA length and secondary structure [19–21]. Understanding molecular features of MDA5-stimulatory RNAs is important for another reason: in addition to its protective role in antiviral defence, MDA5 has been implicated in autoimmune and autoinflammatory diseases such as type 1 diabetes (T1D), **systemic lupus erythematosus (SLE)** and **Aicardi-Goutières syndrome (AGS)** [2,3,22]. Moreover, new data indicate that MDA5 is activated during some forms of cancer treatment [23,24] or in settings where mitochondrial RNA degradation is compromised [17]. These observations highlight that, in some circumstances, cellular RNAs trigger MDA5. Indeed, transcripts from repetitive genome segments, including **endogenous retroelements**, such as **Alu elements**, and mitochondrial RNA have been suggested to bind and activate MDA5 [17,23–26]. Here, we review RNA sensing by MDA5 in the context of antiviral immunity and autoinflammation. We discuss how important recent developments in this area set the stage for future exploration of MDA5-RNA interactions in living cell systems, including models of authentic virus infection and autoinflammation.

### The Discovery of MDA5

MDA5 was first described in 2002 as a helicase protein in mouse and human cells [27,28]. Interestingly, these initial reports suggested that MDA5 is involved in the execution of apoptosis. In 2004, Rick Randall's group found that overexpression of MDA5 alone induces the expression of IFN- $\beta$ , a type I IFN [29]. Furthermore, this study showed that the IFN- $\beta$  response of cells stimulated by transfection of polyriboinosinic:polyribocytidylic acid (**poly I:C**), a synthetic RNA, is greatly enhanced by MDA5 overexpression [29]. Shortly afterwards, knockouts of the mouse *Iflh1* gene encoding MDA5 demonstrated that MDA5 is essential for the type I IFN response to poly I:C [18,30]. Together, these landmark studies established MDA5 as an RNA sensor inducing type I IFN (Figure 1).

### Activation of MDA5 by Synthetic RNAs

Poly I:C is often used as a synthetic mimic of double-stranded RNA (dsRNA). This led to the hypothesis that the PAMP recognised by MDA5 is the double-stranded conformation of RNA. Indeed, structural studies of MDA5 show that the protein adopts a ring-like conformation around dsRNA [21]. Contacts between MDA5 and dsRNA are along the phosphodiester backbone, suggestive of sequence-nonspecific binding [21]. Experiments in the test tube using recombinant MDA5 and *in vitro* transcribed dsRNAs established that MDA5 forms filaments along dsRNA [19,31]. These filaments are particularly stable on long dsRNA molecules and are mediated by both protein–RNA and protein–protein interactions [20,21]. These biophysical studies used filament formation and ATP hydrolysis by the helicase domain of MDA5 as surrogates for its activation. Consistent with these data is the finding in cells that the IFN response to transfected poly I:C depends on length: long poly I:C molecules preferentially trigger MDA5, whereas short poly I:C activates RIG-I [32]. Together, these observations have led to the widely held notion that MDA5 detects long dsRNA. However, other observations suggest that dsRNA – that is, two complementary RNA molecules annealed to form a helical A-form duplex – may not always be sufficient to explain activation of MDA5. For example, IFN induction is particularly strong in response to poly I:C, while other dsRNAs such as poly A:U, trigger no response [33,34]. It should nevertheless be noted that, compared with G:C and I:C

### Glossary

#### **Aicardi-Goutières syndrome**

**(AGS):** this progressive and severe brain disease is characterised by spontaneous production of type I IFNs that are detectable in the patients' blood and cerebrospinal fluid [22]. AGS can manifest at birth and has some phenotypic overlap with congenital virus infection as well as with SLE. Mutations in any one of seven genes cause AGS. The proteins encoded by these genes are all enzymes involved in nucleic acid or nucleotide metabolism.

**Alu elements:** endogenous retroelements of the short interspersed nuclear element (SINE) class that are ca. 300 nt in length. They are present in the human genome in more than one million copies, and some *Alu* elements are arranged as inverted repeats [118]. The vast majority of *Alu* elements do not complete retrotransposition, and *Alu* elements are absent in mice.

**Endogenous retroelements:** a large proportion of mammalian genomes consists of endogenous retroelements, which are subdivided into endogenous retroviruses and retrotransposons [78]. These heritable DNA sequences resemble extant infectious retroviruses: retroelements can be transcribed into RNA that is reverse transcribed into a cDNA copy, which then reintegrates into the genome. These activities are usually silenced efficiently by cells to maintain genome integrity and to avoid innate immune activation.

**Interferons (IFNs):** secreted proteins that play pivotal roles in antiviral immune responses and are divided into types I, II, and III IFNs [6]. Humans have 17 different genes encoding type I IFNs (13 subtypes of IFN- $\alpha$  as well as IFN- $\beta$ , - $\epsilon$ , - $\kappa$ , and - $\omega$ ). All type I IFNs signal via the ubiquitously expressed type I IFN receptor to induce the expression of hundreds of genes, several of which have antiviral functions. The single type II IFN, IFN- $\gamma$ , plays important roles in cellular immune responses. Type III IFNs (also known as IFN- $\lambda$ s) have similar effects as type I IFNs but act primarily on mucosal tissues.

**Poly I:C:** a synthetic RNA prepared by annealing inosine and cytidine homopolymers. Poly I:C is frequently

duplexes, A:U duplexes have a lower stability, which could explain their lower signalling activity. It is also noteworthy that poly I:C consists of annealed inosine and cytidine homopolymers. These are produced enzymatically from ribonucleoside diphosphates, using polynucleotide phosphorylase, and are heterogeneous in length [35]. Annealing thus results in double-stranded regions with single-stranded overhangs. These are available for base-pairing with other molecules, potentially resulting in the formation of more complex, branched RNA structures, which have been proposed to play a role in MDA5 activation [34].

### RNA Sensing by MDA5 during Virus Infection

Early studies in MDA5-deficient mice reported that these animals are highly susceptible to infection with encephalomyocarditis virus (EMCV), failing to induce type I IFNs [18,30]. More recently, MDA5 deficiency in humans has been shown to increase susceptibility to viral infection [36–38]. EMCV is a widely used model belonging to the Picornaviridae family that includes important human pathogens such as hepatitis A virus, coxsackie B virus, enterovirus, and rhinovirus. As summarised in Table 1, subsequent work by many laboratories revealed that MDA5 is involved in type I IFN induction during infection with several other types of viruses. This includes virus families characterised by genomes consisting of single-stranded (ss), positive- or negative-sense RNA, dsRNA, and dsDNA. However, in contrast to EMCV infection, in which MDA5 is essential for type I IFN induction, MDA5 plays a partial role in other infections (Table 1). For example, dsDNA viruses are also detected by the cytosolic DNA sensing pathway, and some RNA viruses trigger both MDA5 and RIG-I.

Immunofluorescence experiments using monoclonal antibodies, called J2 and K1, that recognise dsRNA [39] revealed strong staining in cells infected with many of the viruses that activate MDA5 [34,40–42]. In the case of ssRNA viruses, this dsRNA is likely to be generated during replication of the viral genome, which involves the synthesis of a complementary RNA molecule. For dsDNA viruses, overlapping transcription of both the positive and negative DNA strand may generate dsRNA [42].

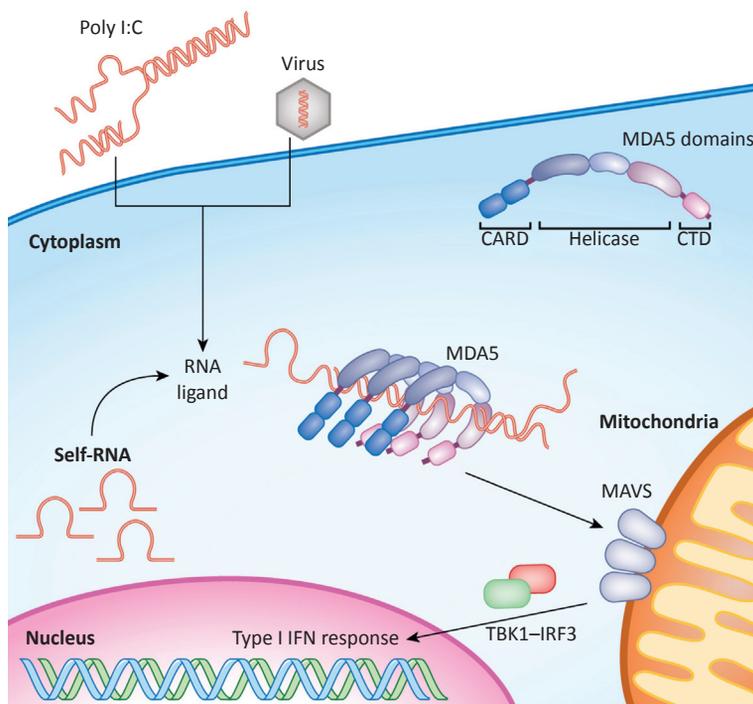
Since many viruses have evolved mechanisms to inhibit nucleic acid sensors, one protocol to study immunostimulatory RNAs is to isolate total RNA from virus-infected cells, followed by retransfection of this sample containing both cellular and viral RNAs into uninfected reporter cells. The transfected RNA then stimulates innate immune receptors in recipient cells without the presence of inhibitory viral mechanisms. For example, total RNA from cells infected with picornaviruses or Zika virus triggers MDA5-dependent IFN responses [34,43,44]. Fractionation of the RNA isolated from infected cells can be used to further characterise MDA5 agonists. Using two picornaviruses, Mengo virus and Coxsackievirus B3, Feng *et al.* showed that RNA from a band containing long viral dsRNA, called the replicative form, triggers MDA5 [43], and similar results were reported by Triantafilou *et al.* [41]. In another study using EMCV, fractionation revealed that the activity stimulating MDA5 is not found in a dsRNA band specifically present in infected cells [34]. Instead, MDA5-stimulatory RNA was recovered from a high-molecular-weight fraction containing both ssRNA and dsRNA, and similar observations were made analysing RNA from vaccinia virus-infected cells [34].

Although a useful approach, transfecting total RNA from infected cells has limitations; for example, the role of RNA-binding proteins may be overlooked. In order to directly study MDA5-stimulatory RNAs generated during virus infection, a number of studies attempted to purify these RNAs from infected cells using different pull-down approaches [45–47]. Indeed, immunoprecipitations (IPs) have been used successfully to identify RIG-I-associated RNAs [8,9,12,16,48]. However, in contrast to RIG-I, we and others found that similar protocols using

used to stimulate type I IFN production and engages both TLR3 as well as RLRs.

**Single-nucleotide polymorphisms (SNPs):** this term describes substitutions of individual nucleotides relative to the reference genome. SNPs can occur within or outside of protein-coding sequences, and some SNPs affect the amino acid sequence of the encoded protein or gene expression, respectively.

**Systemic lupus erythematosus (SLE):** this common autoimmune disease is characterised by a range of symptoms, including rash, photosensitivity, arthritis, fatigue, and nephritis. IFN- $\alpha$  and autoantibodies play crucial roles in the development of SLE.



Trends in Microbiology

**Figure 1. Overview of the MDA5 Pathway.** MDA5 comprises two N terminal CARD domains, a central helicase domain, and a C terminal domain (CTD). Both the helicase domain and the CTD are involved in RNA binding. MDA5 recognises synthetic RNAs, such as poly I:C, viral RNAs, and endogenous RNAs. Length and secondary structure are likely to be key determinants of MDA5 activation, which has been suggested to be mediated by multimerisation of the protein on RNA agonists. This results in the clustering of multiple CARD domains, which, in turn, allows the engagement of MAVS. Activation of this adaptor protein triggers a signalling cascade involving TBK1 and IRF3, leading to transcriptional induction of the genes encoding type I IFNs and other antiviral genes. CARD, caspase activation and recruitment domain; MAVS, mitochondrial antiviral signalling protein.

Table 1. Viral Infections Detected by MDA5

Virus family	Genome	Examples	Role of MDA5 in type I IFN induction	Selected Refs
Picornaviridae	ssRNA (+)	Encephalomyocarditis virus; Rhinovirus; Coxsackie B virus	Essential	[18,30,43,102,103]
Flaviviridae	ssRNA (+)	West Nile virus; Hepatitis C virus; Zika virus	Partial	[44,104,105]
Togaviridae	ssRNA (+)	Sindbis virus	Partial	[34,106,107]
Coronaviridae	ssRNA (+)	SARS coronavirus	Partial	[57,108,109]
Paramyxoviridae	ssRNA (-)	Measles virus; human Metapneumovirus; Sendai virus <sup>a</sup>	Partial	[110-112]
Reoviridae	dsRNA	Rotavirus	Partial	[8,32,113]
Poxviridae	dsDNA	Vaccinia virus	Partial	[34,114]
Herpesviridae	dsDNA	Herpes simplex virus 1	Partial	[115]
Hepadnaviridae	dsDNA	Hepatitis B virus <sup>b</sup>	Partial	[116]

<sup>a</sup>Some Sendai virus stocks, particularly the Cantell strain, activate mostly RIG-I.

<sup>b</sup>Hepatitis D virus, a satellite virus that only infects HBV-infected cells and has a circular, ssRNA(-) genome, is also sensed by MDA5 [117].

native conditions for IP of MDA5 do not copurify IFN-stimulatory RNAs [25,47]. One possible explanation for this observation is that MDA5 forms multimers on its natural RNA agonists, as has been reported for synthetic RNAs, and that such MDA5 filaments are unstable after cell lysis [19,20,25]. To circumvent this technical obstacle, one study used photoactivatable ribonucleoside enhanced crosslinking and immunoprecipitation (PAR-CLIP) [46]. This method involves incubating cells with modified nucleosides, which are incorporated into newly synthesised RNA and allow UV-A-induced cross-linking of the RNA to nearby proteins [49]. Using antibodies against endogenous MDA5, Runge *et al.* isolated IFN-stimulatory RNAs from cells infected with measles virus (MV, a paramyxovirus) [46]. Sequencing of these RNAs revealed an enrichment of AU-rich sequences derived from mRNA of the MV L gene [46]. A later study using a streptavidin-based pull-down approach and native conditions, however, concluded that MDA5 binds to the MV N mRNA [45] and did not confirm the preference of MDA5 for AU-rich sequences observed with PAR-CLIP [45,46]. The reason for these different results is currently unclear and may relate to the different experimental methods used. Another approach to purify MDA5-stimulatory RNAs from virus-infected cells utilised IP of LGP2 [47]. This RLR lacks CARD domains and plays a dual role regulating both RNA interference by association with Dicer [50] as well as RIG-I and MDA5 signalling [51]. Indeed, LGP2 may enhance MDA5-dependent type I IFN responses by facilitating the access of MDA5 to RNA agonists [52,53]. IP of LGP2 from EMCV-infected cells under native conditions captures an RNA that activates MDA5 upon retransfection into reporter cells [47]. Deep sequencing of this RNA revealed an enrichment of negative-sense RNA corresponding to the L region of the EMCV genome [47]. Further experiments showed that *in vitro* transcribed L antisense RNA activates MDA5, and that the L region is required for type I IFN induction following EMCV infection in cells and mice [47].

Despite the differences in methodologies employed and results obtained, all of these studies find that MDA5-stimulatory RNAs are derived from one strand of the investigated viral sequences, namely, positive-sense RNA for MV [45,46] and negative-sense RNA for EMCV [47]. Although a degree of intramolecular base-pairing is conceivable, these findings are consistent with the notion that physiological MDA5 agonists may not always simply correspond to viral dsRNA. Instead, dsRNA – as a product of infection – might be a correlate of MDA5 activation [34,40,42]. We speculate that a more complex RNA motif or structure may activate MDA5 in some settings. Further studies are needed to identify and characterise this viral PAMP detected by MDA5 with precision. The recently described MDA5-RNase-protection assay [25], and advances in CLIP technologies [49], are likely to facilitate research into this question. Although CLIP analysis reveals both activating and nonactivating RNA ligands, it may be particularly informative as it allows interrogation of MDA5-associated RNAs in virus infected cells. This is important because viral RNAs are typically coated by viral proteins, and RLRs have been suggested to compete with viral RNA-binding proteins [54]. As such, the actual RNA agonist recognised by MDA5 in infected cells is likely to be determined by a combination of factors, including (i) the RNA binding preferences of MDA5, (ii) accessory host factors such as LGP2 [53], DHX29 [55], and LncITPRIP-1 [56] that facilitate RNA binding by MDA5, (iii) the abundance and properties of viral RNA-binding proteins [54], and (iv) viral evasion strategies such as RNA methylation [57] and RNA degradation [58]. Another interesting avenue for future research is to study at the subcellular level MDA5 activation, the formation of aggregates or filaments, and interaction with MAVS. For example, the subcellular localisation of MAVS suggests that active MDA5 signalling complexes may be formed on mitochondria and/or peroxisomes [59–61]. Super-resolution and live-cell microscopy methods should be utilised to address these questions.

### Activation of MDA5 in Autoimmune and Autoinflammatory Diseases

In addition to its role in the detection of viral infections, MDA5 has also been implicated in a number of sterile, inflammatory conditions. For example, **single-nucleotide polymorphisms (SNPs)** in *IFIH1*, the gene encoding MDA5, have been associated with T1D, psoriasis, rheumatoid arthritis, vitiligo, multiple sclerosis, and SLE [62–68]. How *IFIH1* risk alleles relate to the development of these diseases is not fully understood but it is likely that this involves chronic induction of type I IFNs, which then initiate or enhance autoinflammation and autoimmune responses. In the context of T1D, the *IFIH1* risk alleles may result in increased MDA5 protein levels or increased responses to RNA derived from cellular sources or viral infection [69–71]. Alternatively, it is possible that some *IFIH1* SNPs change the conformation of MDA5 such that the protein becomes constitutively active, irrespective of RNA binding [72].

*IFIH1* mutations can also cause a number of rare disorders characterised by aberrant type I IFN production, some of which are monogenic diseases and, as such, are good models for molecular studies [22]. This includes Singleton–Merten syndrome [73] and AGS [74,75]. AGS is characterised by severe neurological dysfunction and often results in death in childhood [76]. Type I IFNs are detectable in cerebrospinal fluid and in peripheral blood [77]. The AGS-associated *IFIH1* mutations result in substitutions of single amino acids in the helicase domain of MDA5 [74,75]. How do these missense mutations lead to chronic type I IFN production? In overexpression assays, mutated versions of MDA5 are more potent than wild-type MDA5 in activating the *IFN-β* promoter in the absence of an exogenous stimulus [74,75]. Two explanations have been proposed for this. In one, mutated MDA5 signals constitutively in an RNA-independent manner [75]. Indeed, Oda *et al.* find that type I IFN induction following EMCV infection is not enhanced by expression of mutated MDA5 [75], and similar observations were reported in a mouse model in which an *Ifih1* mutation leads to type I IFN induction [72]. The other explanation suggests that MDA5 mutations alter the interaction between the protein and RNA, resulting in spontaneous signalling triggered by cellular RNAs. In line with the idea of enhanced RNA sensing, Rice *et al.* found that overexpression of mutated MDA5 in cell lines potentiates the type I IFN response to transfected synthetic RNA agonists [74]. Furthermore, coexpression of viral RNA-binding proteins prevents the response triggered by overexpression of MDA5 without additional stimulation [25]. This observation suggests that mutated MDA5 aberrantly detects cellular RNAs.

In order to identify cellular RNAs that stimulate mutant MDA5, Ahmad *et al.* recently described an RNase protection assay where total RNA extracted from cells is mixed in the test tube with recombinant MDA5 protein bearing a mutation in its helicase domain, which had been identified in an AGS patient [25,74]. RNA not bound by MDA5 is then degraded with RNase A and any remaining material is sequenced [25]. This analysis revealed that RNA derived from *Alu* elements arranged as inverted repeats (IRs) is bound by MDA5 [25]. Transcription of IR-*Alu* elements generates single-stranded RNA molecules with extensive self-complementarity, resulting in the formation of a long, base-paired stem. In cell-free systems, mutant MDA5 forms filaments on synthetic IR-*Alu* RNAs, which also stimulate its ATPase activity and MDA5-dependent dimerisation of a key downstream transcription factor, IRF3 [25]. Ahmad *et al.* further propose that the presence of mismatched bases in RNA stem structures formed by IR-*Alu* elements prevents recognition by wild-type MDA5 [25]. These elegant experiments provide a molecular explanation for type I IFN induction in cells from AGS patients: mutant MDA5 is less selective than wild-type MDA5 in terms of RNA binding and associates with base-paired RNA stems formed by IR-*Alu* elements containing bulges and mismatches [25]. Furthermore, these data lend support to the notion that nucleic acids derived from endogenous retroelements play important roles in autoinflammatory responses [78].

In addition to *IFIH1*, mutations in six other genes have been shown to cause AGS: *TREX1*, *RNASEH2A*, *RNASEH2B*, *RNASEH2C*, *SAMHD1* and *ADAR1* [22]. *TREX1*, *RNASEH2A-C* and *SAMHD1* mutations all result in aberrant activation of the dsDNA sensing cGAS-STING pathway, driving chronic type I IFN production [79–84]. In contrast, the molecular consequence of *ADAR1* mutations is the engagement of MDA5 [85–87]. ADAR1 (adenosine deaminase acting on RNA 1) is an RNA-editing enzyme that binds to dsRNA and converts adenosine to inosine (A-to-I). A-to-I edits are predicted to change RNA structure and stability, in addition to introducing mutations into proteins as the translational machinery reads 'I' bases as guanosines instead of adenosines [88]. ADAR1 has two isoforms: ADAR1-p110 is constitutively expressed, and localises to the cell nucleus, while ADAR1-p150 is type I IFN-inducible and additionally found in the cytoplasm. In AGS patients, mutations in the *ADAR1* gene mostly map to regions of ADAR1 involved in A-to-I editing [89], and some mutations diminish editing activity [89,90]. In mice, ADAR1-deficiency triggers a type I IFN response and is embryonically lethal [91]. This phenotype is partially rescued by crossing *Adar1*<sup>-/-</sup> mice with either *Ifnar*<sup>-/-</sup> animals lacking the type I IFN receptor or *Mavs*<sup>-/-</sup> mice lacking the common adaptor protein for RLRs [87,90]. Similarly, IFN induction and embryonic lethality are rescued in *Adar1*<sup>-/-</sup> mice by additional knock-out of MDA5, but not RIG-I [87]. Interestingly, much like the situation in *Adar1*<sup>-/-</sup> mice (lacking both ADAR1-p110 and -p150), specific knock-out of ADAR1-p150 results in embryonic lethality that can be rescued by additional MAVS-deficiency [87]. Further evidence for the role of MDA5 and MAVS in inducing type I IFN downstream of ADAR1-deficiency was obtained in animals expressing an editing-deficient version of ADAR1 (E861A) [86]. *Adar1*<sup>E861A/E861A</sup> animals die during *in utero* development and recapitulate the type I IFN induction seen in *Adar1*<sup>-/-</sup> mice, and these effects are reversed in *Adar1*<sup>E861A/E861A</sup>; *Ifih1*<sup>-/-</sup> animals [86,92].

Sites bound and edited by ADAR1 have been identified and, in human cells, are often found within or close to *Alu* elements [85,93–95]. Moreover, in the RNase protection assay described above, wild-type MDA5 protects IR-*Alu* RNAs amongst total RNA extracted from ADAR1-deficient cells, but not amongst RNA from wild-type cells [25]. Together with the MDA5-dependency of type I IFN induction in ADAR1-deficient mice and cells [85–87], these data suggest an attractive model: ADAR1 edits RNAs derived from IR-*Alu* repeats, leading to destabilization of their base-paired structure, which in turn prevents activation of MDA5 [25,96]. This model is further consistent with the notion that *IFIH1* mutations found in AGS change the RNA specificity of MDA5 such that it binds to IR-*Alu* elements containing mismatches and bulges [25].

Collectively, these observations highlight the importance of long base-paired stretches of RNA formed by IR-*Alu* elements in the activation of MDA5 in autoinflammatory disease. It is interesting, however, to note that Chung *et al.* recently reported that adenosines edited by ADAR1 tend to be found in single-stranded, rather than base-paired, regions of both single *Alu* and IR-*Alu* elements [85]. Moreover, A-to-I editing can in some circumstances stabilise RNA duplexes formed by *Alu* elements [96] and inosine-containing RNAs may in some settings inhibit RLRs [97]. Finally, RNAs derived from IR-*Alu* elements are bound by additional proteins such as DHX9 [98], which may compete with ADAR1 and MDA5. It will therefore be important to study RNAs bound by MDA5 in living cells harbouring *ADAR1* or *IFIH1* mutations.

A recent study found that mitochondrial dsRNA escapes into the cytosol when the mitochondrial enzymes SUV3 and PNPase, which are involved in RNA degradation, are depleted from cells or are dysfunctional due to mutations [17]. At the same time, type I IFN is induced in an MDA5-dependent manner. This observation further highlights the potential of MDA5 to detect

cellular RNAs and parallels the finding that RIG-I can detect nuclear RNAs mislocalised to the cytosol [16].

### The Role of MDA5 in Cancer

In addition to its myriad roles in viral and autoimmune diseases, MDA5 has recently been found to be activated during cancer treatment with DNA-demethylating agents [23,24]. Indeed, much like during viral infection, exposure of colorectal cancer cells to inhibitors of DNA methylation, such as 5-azacytidine-2-deoxycytidine, results in accumulation of RNAs detected by the dsRNA-specific antibody J2 [23]. Furthermore, these and similarly treated ovarian cancer cells contain extractable IFN-stimulatory RNAs, secrete type I and III IFNs, and upregulate ISGs [23,24]. These effects are MDA5- and MAVS-dependent and result in reduced cell growth and self-renewal [23,24]. Mechanistically, demethylation of normally repressed areas of the genome may result in transcription of endogenous retroviruses, and such RNAs have been suggested to activate MDA5 [23,24]. In broad agreement with these data, a recent study, using acute myeloid leukemia cell lines, also shows that loss of epigenetic regulation can result in de-silencing of retroelements, dsRNA accumulation, and MDA5 activation [99]. It would be interesting to systematically investigate expression, subcellular localisation, and MDA5-association of RNAs derived from endogenous retroviruses and other endogenous retroelements such as *Alu* elements in cancer cells. Furthermore, *IFIH1* genotype and MDA5 expression levels may be instructive in predicting the response of patients to drugs targeting epigenetic regulation. In another study, overexpression of MDA5 using adenoviral vectors induced cell death in cancer cells and also facilitated antitumour immunity via type I IFN production [100]. Taken together, these observations further support the notion that self-RNAs can trigger MDA5. In addition, synthetic MDA5-stimulatory RNAs, such as poly I:C, may induce cancer cell apoptosis and immune responses against tumours (reviewed in [101]).

### Concluding Remarks

MDA5 is an important RNA sensor implicated in the detection of viral infections, in a range of autoimmune and autoinflammatory diseases, as well as in cancer. Recent efforts in identifying an RNA molecular pattern detected by MDA5 suggest that length and secondary structure of RNA are important determinants; however, we speculate here that activation of MDA5 in some settings may require more than simply dsRNA and that a yet-to-be identified consensus RNA PAMP detected by MDA5 may exist. We predict that recent technical advances, including an RNase protection assay [25] and, in particular, CLIP protocols [49], will facilitate research in this direction. The description of a defined RNA that selectively activates MDA5 would be of great value (see Outstanding Questions). MDA5 agonists could be used to boost antiviral and antitumoural immunity and may guide the developments of MDA5 antagonistic RNAs for use in autoinflammation.

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### Outstanding Questions

What are the RNAs detected by MDA5? This question should be studied in the contexts of different virus infections, autoinflammation, and cancer. Models using living cells will be important given that other RNA-binding proteins are likely to compete with MDA5 for RNA binding. CLIP technologies may be particularly useful given that RNA-MDA5 complexes can be crosslinked in live cells.

Is there a common RNA structure or motif that is recognised by MDA5 in both virus infection and sterile conditions leading to MDA5 activation?

Is it possible to design synthetic RNAs that either activate or inhibit MDA5? Would such MDA5 agonists and antagonists have therapeutic value?

What is the relationship between endogenous retroelements and MDA5? At present, a systematic study of different retroelements in terms of their ability to generate MDA5-stimulatory RNAs has not been reported. Other interesting questions pertain to the stage in the life cycle of retroelements at which MDA5 is activated and to the role of MDA5 in suppressing these elements.

What are the molecular consequences of *IFIH1* risk or pathologic alleles? Is mutated MDA5 spontaneously active or is RNA binding required? In which types of cells is MDA5 activated, and are there differences in the amount of MDA5-stimulatory RNA between cell types?

Does MDA5 form higher-order structures, such as filaments, in living cells? What is the spatiotemporal behaviour of MDA5 during virus infection? Advanced imaging technologies, such as super-resolution microscopy, are likely to provide insights into these questions.

What are the molecular mechanisms by which accessory proteins, such as LGP2 and DHX9, regulate the activity of MDA5?

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