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Trends in
Biochemical Sciences

Review

ADAR RNA Modifications, the Epitranscriptome and Innate Immunity

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Modified bases act as marks on cellular RNAs so that they can be distinguished from foreign RNAs, reducing innate immune responses to endogenous RNA. In humans, mutations giving reduced levels of one base modification, adenosine-to-inosine deamination, cause a viral infection mimic syndrome, a congenital encephalitis with aberrant interferon induction. These Aicardi-Goutières syndrome 6 mutations affect adenosine deaminase acting on RNA 1 (ADAR1), which generates inosines in endogenous double-stranded (ds)RNA. The inosine base alters dsRNA structure to prevent aberrant activation of antiviral cytosolic helicase RIG-I–like receptors. We review how effects of inosines, ADARs, and other modified bases have been shown to be important in innate immunity and cancer.

Highlights

Epitranscriptomic RNA modifications act as marks of innate immune self in RNA; they are critical in current mRNA vaccines.

Studies on adenosine-to-inosine deamination by ADAR enzymes show how inosine acts as a mark of self-RNA by altering the structure of double-stranded (ds)RNA to prevent aberrant activation of antiviral RIG-I–like receptors (RLRs).

Self-marks in RNA threshold and balance innate immune defences against infections; *ADAR* mutations cause inflammatory diseases.

Helicase domains of RLRs scan along perfect dsRNA, and RLRs form signalling complexes cooperatively; base mismatches or I-U base pairs cause RLR disassociation from RNA, and the ancestral helicase domains of DICER proteins probably originated this mechanism.

Knockdown of ADAR1, and potentially also small molecule inhibitors of ADAR1, kill some cancer cells directly and strongly enhance killing of others by immune checkpoint blockade.

The Epitranscriptome, ADARs, and Inosines

Modified bases act as marks of self-RNA to reduce innate immune responses to endogenous cellular RNA. Over 170 different types of enzymatic RNA modifications are known to occur in stable RNAs, such as tRNAs and rRNAs, where many contribute to their high stabilities and optimise the activities of these RNAs [1]. Different types of base modifications have also been found in eukaryotic mRNAs, where they are called **epitranscriptomic** (see Glossary) modifications [2]. Base modifications that have been detected in mRNA include adenosine to inosine (A-to-I) deamination and cytosine to uracil (C-to-U) deamination, N^6-methyladenosine (m^6A), N^1-methyladenosine (m^1A), 5-methylcytosine (5mC), and pseudouridine (ψ), as well as ribose 2′*O*-methyl [1]. Many of these modifications affect innate immune responses to RNA [3–6], and understanding these effects has been critical for development of mRNA therapeutics and vaccines [7].

We focus here on A-to-I deamination, which was one of the first RNA base modifications identified; it is abundant in tRNAs and plays a key role in tRNA wobble decoding. It was also one of the first epitranscriptomic mRNA modifications identified, because it is readily detected by RNA sequencing (RNA-seq). A-to-I **RNA modification/editing** found in mRNAs is catalysed by adenosine deaminases acting on RNAs (ADARs). In mammals, there are three ADAR family members: ADAR1 and ADAR2 deaminate double-stranded (ds)RNA, whereas ADAR3 is enzymatically inactive (for review, see [8]). This review focuses primarily on ADAR1, summarising the evidence for its role in innate immunity (Figure 1) and how this role of ADARs is evolutionarily conserved. We also aim to clarify how and when A-to-I conversion acts as an epitranscriptomic RNA modification versus the rarer cases where it acts as an RNA editing event. This RNA modification role of the ADARs has been, and will continue to be, a trailblazer for understanding epitranscriptomic roles of many types of RNA modifications. Finally, we also summarise human diseases and SNPs associated with *ADAR1* and the roles of ADAR1 in tumour immunity.

ADAR1, the Inosinome, and Effects on Three Levels

ADAR1

The three ADARs are composed of two or more dsRNA-binding domains (dsRBDs) followed by a larger catalytic adenosine deaminase domain. Via their dsRBDs, ADARs recognise and bind to

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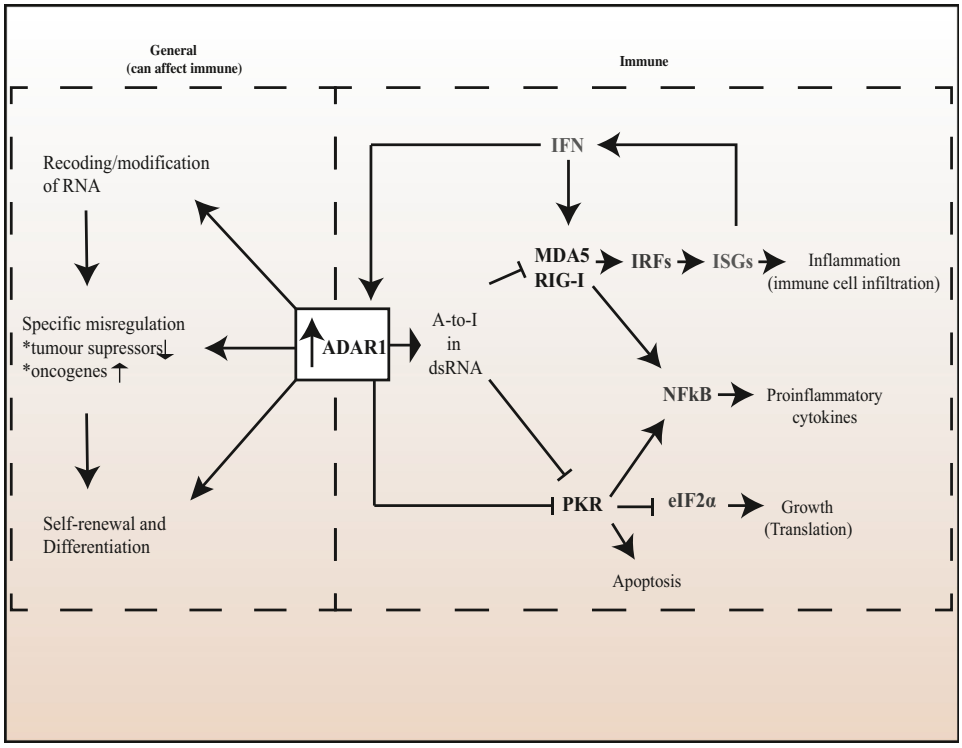

Figure 1. The Biological Roles of Adenosine Deaminase Acting on RNA 1 (ADAR1). When there is increased expression of ADAR1, it can modify mRNA, resulting in recoding of specific mRNA (left side of figure). For example, it can result in downregulation of tumour suppressors and upregulation of oncogenes. Increased expression of ADAR1 can also affect self-renewal and differentiation of cells. Increased expression of ADAR1 in innate immunity also prevents aberrant activation of retinoic acid–inducible gene I (RIG-I)-like antiviral cytoplasmic double-stranded (ds)RNA sensors (RLRs) (right side of figure). It also prevents activation of protein kinase R (PKR). If there is an increase in interferon (IFN), then ADAR1p150 is expressed, which will edit dsRNA in the cytoplasm and inhibit these dsRNA sensors. Abbreviations: eIF2α, eukaryotic initiation factor 2α; IRFs, IFN-regulatory factors; ISGs, IFN-stimulated genes; MDA5, melanoma differentiation-associated gene 5; NFkB, nuclear factor-κB.

the A-form helical structure of dsRNA and flip out the target adenosine [9]. Though they do not have a binding consensus sequence, ADARs prefer a pyrimidine before the edited A and guanine (G) after it (U/CAG). Regarding ADAR1 specifically, there are two isoforms of ADAR1: the constitutively expressed short isoform ADAR1p110 and the **interferon (IFN)**-inducible long isoform ADAR1p150 [10]. Both isoforms of ADAR1 shuttle between the nucleus and the cytoplasm; however, ADAR1p110 is predominantly nuclear, while ADAR1p150 is cytoplasmic [11] (Figure 2). It is possible for ADAR1p110 to be present in the absence of ADAR1p150, but when ADAR1p150 is induced by IFN, ADAR1p110 is also present and its expression is also somewhat increased; this is important to remember when attributing different functions to these isoforms.

The Inosinome

A-to-I edited sites are widespread in pre-mRNAs, mRNAs, and in noncoding RNAs, including miRNAs, with millions of sites identified in the human transcriptome [12], which we call the inosinome. A-to-I deamination changes the base-pairing properties of the edited base; when inosine forms a Watson-Crick base pair during cDNA synthesis, it prefers to pair with C, generating a cDNA with an easily detected A-to-G change [6]. Thus, editing by ADARs is unambiguously identified by RNA-seq, and, unlike other RNA modifications, detection does not rely on recognition by

Glossary

Aicardi-Goutières syndrome type 6 (*AGS6*): a rare childhood autoimmune disorder characterised by high levels of IFN and caused by mutations in seven known genes. *ADAR* was the sixth gene identified; hence the name *AGS6*.

Alu elements: primate-specific short interspersed elements. They are mobile elements comprising 11% of the human genome. When two closely similar Alu elements are inserted in inverted orientations less than approximately 2 kb apart in a transcript, they can base pair and form a stable Alu inverted copy dsRNA hairpin that is promiscuously edited by ADARs.

DICER proteins: in invertebrates, DICERs are antiviral proteins that cleave viral dsRNA or endogenous transposon dsRNA to generate siRNAs of 21 base pairs; one siRNA strand is loaded into an Argonaute protein in the RNA-induced silencing complex to target it to degrade the virus or transposon RNA. DICERs also process miRNA precursors in both invertebrates and invertebrates.

Epitranscriptomic: analogous to epigenetic DNA methylation in that it involves functional changes to RNA that do not include changes in the sequence encoding the RNA.

Interferon (IFN): IFNs are cytokines that communicate between cells to trigger an innate immune response by activating downstream genes. There are three main types of IFNs that signal through different receptors.

Oligoadenylate synthetase (OAS)–ribonuclease (RNase) L pathway: OAS senses dsRNA and produces 2′,5′-oligoadenylate, which bind to and activate RNase L. RNase L can then dimerise and cleave RNA, resulting in inhibition of translation and apoptosis.

Protein kinase R (PKR): a dsRNA-activated protein kinase. Binding to dsRNA induces dimerization, and subsequent autophosphorylation of PKR activates the protein.

RIG-I–like receptors (RLRs): cytoplasmic innate immune pattern recognition receptors that recognise viral dsRNA. The three members are RIG-I, MDA5, and LGP2. Upon binding to dsRNA, RIG-I or MDA5 undergoes conformational changes, enabling it to interact with MAVS, thus initiating an IFN-mediated antiviral innate immune response.

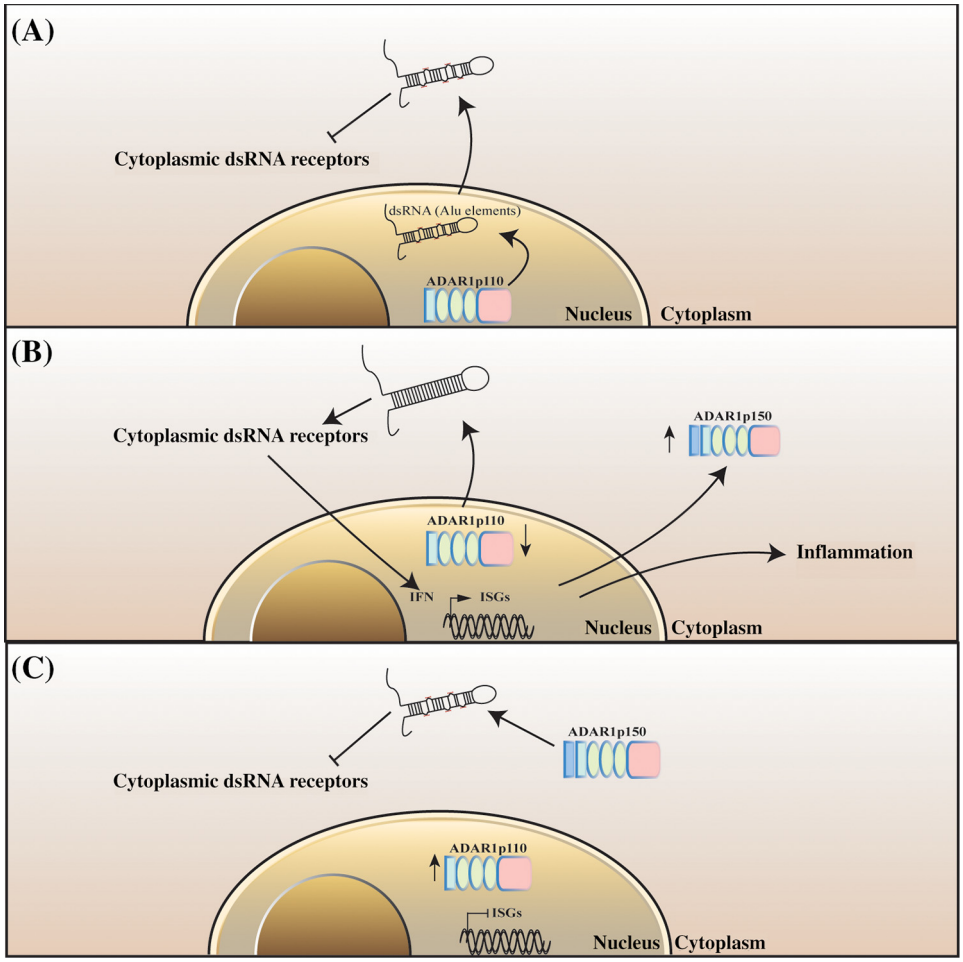

Figure 2. The Adenosine Deaminase Acting on RNA 1 (ADAR1) Expression Cycle During Viral Infection. (A) Under uninfected conditions, ADAR1p110 is mainly located in the nucleus and edits double-stranded RNA (dsRNA). This edited dsRNA can be exported to the cytoplasm and prevents activation of cytoplasmic retinoic acid–inducible gene I (RIG-I)-like receptors (RLRs). (B) Early during a viral infection, there is a temporary decrease in ADAR1p110 expression; thus, unedited dsRNA can be exported to the cytoplasm and can help initiate an innate immune response. This turns on transcription of the interferon (IFN)-stimulated genes (ISGs) and causes inflammation. ADAR1p150 is a late immune response protein, so its expression rises late in infection, and it localises to the cytoplasm. (C) Later in the viral infection, ADAR1p110 levels return to normal. ADAR1p150 is cytoplasmic and edits endogenous dsRNA and may also edit viral dsRNA. This edited dsRNA helps to turns off the innate immune response by binding to the RLRs.

RNA modification/editing: originally, 'RNA editing' was a term used for the very different processes of RNA insertion/deletion in protozoan kinetoplasts (mitochondria) and for A-to-I and C-to-U deamination that resulted in recoding of the RNA to generate a protein or a different protein. However, in the case of ADAR RNA editing, the term 'RNA editing' became arbitrary when it was shown that most vertebrate A-to-I editing events do not result in recoding.

RNA interference (RNAi): siRNAs target virus, transposon, or certain endogenous RNAs for degradation; siRNAs can also target transcriptional silencing to the genes expressing the transposon or 'non–self-RNA.' Transcriptional silencing is a mechanism of genome defence against deleterious effects of parasites; these deleterious effects include endogenous dsRNA formation.

antibodies, which can be problematic. Though this is the primary detection method for inosine, we argue that perhaps many inosines across the transcriptome act as RNA modifications more than as RNA editing events *in vivo*.

Three Effects of A-to-I Modifications

We can distinguish between three levels of ADAR effects *in vivo*, outlined in the subsections below.

Inosine acts as an RNA editing event during a subsequent copying or decoding step

The first and best-known effect of the A-to-I modification is RNA editing. This occurs when inosine acts as an RNA editing event during translation, pairing with a C, which can lead to protein recoding [13]. RNA editing also can also impact protein expression via altering splicing consensus sequences,

or altering base pairing with miRNA, regulating translation or mRNA stability (for review, see [8]). In humans, A-to-I editing only rarely results in recoding of RNA to alter protein sequence.

The majority of ADAR-edited sites are located within dsRNA formed from adjacent, inverted copies of **Alu elements** (short interspersed nuclear elements) [12] embedded in pre-mRNAs and noncoding RNAs. As Alu elements are very abundant in the human genome, there are over 100 million edited sites in human transcriptomes; however, the level of RNA editing at any Alu adenosine is low, <1%. The majority of these editing events are performed by ADAR1, which is ubiquitously expressed [14].

Inosine affects the structure and function of the original RNA molecule

Second, because there are low levels of RNA editing, we suggest that the A-to-I modification acts as other RNA modifications do through effects on the RNA structure and function. For example, when A-to-I editing occurs at an A-U base pair in dsRNA, the resulting I-U base pair adopts a wobble pairing instead of Watson-Crick pairing, which strains the double helix and destabilises dsRNA [15]. The inosine-modified endogenous dsRNA is then recognised as self-dsRNA by innate immune sensors or other proteins, as distinct from newly copied and perfectly paired viral dsRNA [4,16,17]. Additionally, the structural effects of I-U base pairs on dsRNA could occur with other RNA modifications such as m^6A, which is also on the exocyclic N^6 amino group on adenine and strongly impairs dsRNA helical structure [18].

ADARs have editing-independent effects through protein–RNA and protein–protein interactions

A third level of effect occurs when ADAR proteins function in an editing-independent manner by binding to their dsRNA-binding sites, followed by interference with binding by other proteins or interactions with other proteins [19,20]. Binding to dsRNA by dsRBDs is dominated by phosphate backbone interactions more than by interactions with the edited base. One study showed that ADAR2 binds similarly to either the unedited *GRIA2 R/G* RNA substrate or to the inosine-containing edited *GRIA2 R/G* RNA product [21]. Indeed, ADAR binding and editing are separable events; some dsRNAs are bound but not efficiently edited [22], and binding to the most favoured RNA substrates/products may be persistent, even after editing.

Effects of Inosines in Self-dsRNA on Cytosolic Antiviral Innate Immune RLR Activation

ADAR1-mediated RNA modifications can affect the activity of antiviral pattern recognition receptors (PRRs) that sense dsRNA within the cell, in particular the **RIG-I–like receptors (RLRs)** (Figure 3). During infection, viral dsRNA is recognised and bound by RLRs, which include the retinoic acid–inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (for reviews, see [4,23,24]). These signal via mitochondrial antiviral-signalling protein (MAVS) in the affected cell to activate the downstream antiviral immune responses through IFN response factor (IRF) activation of type I and type III IFNs. The resulting secreted inteferon causes autocrine induction of IFN-stimulated genes (ISGs) in the original cell and in other cells through Janus kinase/signal transducer and activator of transduction (JAK/STAT) signalling, and through nuclear factor-κB, activation of proinflammatory cytokines and/or apoptosis (Figure 1).

An example of how ADAR1-mediated A-to-I modifications affect RLR activation is seen in a mouse study. An *Adar* (gene name for Adar1)-null mutant mouse was shown to be embryonic lethal by embryonic day 12.5 (E12.5) and displayed activation of IFN and ISGs, failure of haematopoiesis, and foetal liver disintegration [25,26]. This embryonic lethality was rescued to live birth in *Adar;Mavs* double-mutants [4,17]. An $Adar^{E861A}$ mutant encoding the full-length inactive protein with a mutation in the deaminase active site survives to E14.5, which is longer than the *Adar*-null mutant. Also, $Adar^{E861A}$;*Ifih1* (encoding Mda5) double-mutant mice were

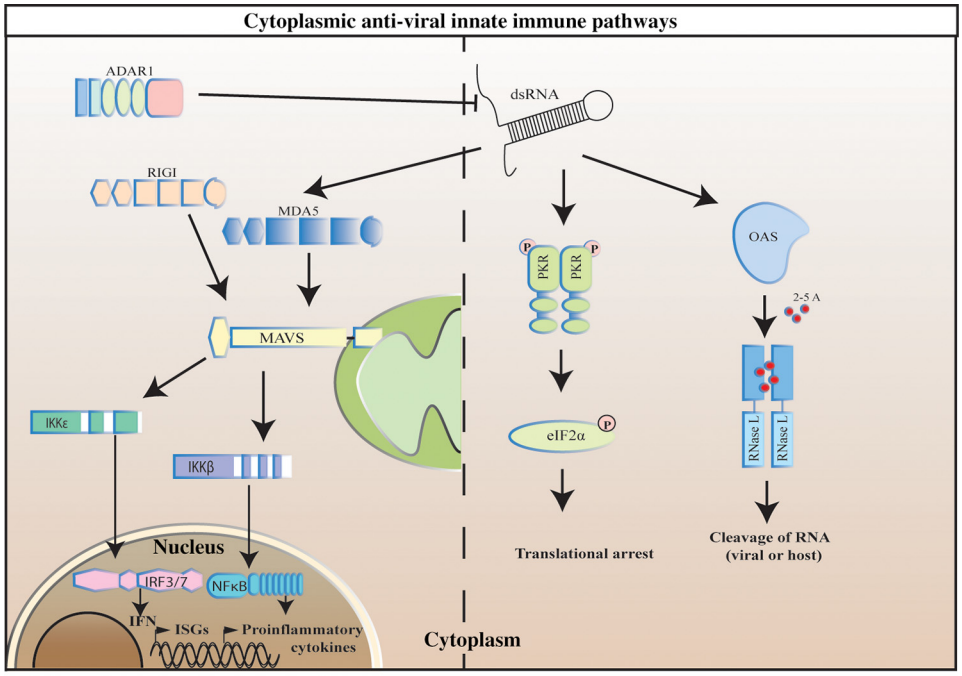

Figure 3. Key Roles of Adenosine Deaminase Acting on RNA 1 (ADAR1) in Innate Immunity. Double-stranded RNA (dsRNA) in the cytoplasm can bind to the retinoic acid–inducible gene I (RIG-I)-like receptors (RLRs), RIG-I and melanoma differentiation-associated protein 5 (MDA5), which have two N-terminal CARD domains (hexagonal boxes), followed by a helicase domain in three parts (three rectangular boxes) and a C-terminal regulatory domain (elliptical box). RIG-I and MDA5 are activated on perfect dsRNA and in turn interact with mitochondrial antiviral-signalling *protein* (MAVS), to form an RNA-protein complex which turns on the downstream innate immune pathway. This results in transcription of interferon (IFN)-stimulated genes (ISGs) and cytokines. However, modification of the dsRNA by ADAR1 prevents the activation of the RLR pathway. ADAR1 can also interfere with activation of protein kinase R (PKR), which is activated by autophosphorylation upon binding to dsRNA. Activated PKR then phosphorylates the translation factor eukaryotic initiation factor 2α (eIF2α), leading to translational arrest. Oligoadenylate synthetase (OAS) is also activated by dsRNA and produces 2′,5′-oligoadenylate (2-5A), which in turn activates ribonuclease (RNase L). It can then dimerise and cleave RNA, either viral or host. Abbreviations: IKK, inhibitor of nuclear factor-κB kinase; NFKB, nuclear factor-κB.

completely rescued with a normal lifespan [16], indicating that Adar1 protein also exerts some unknown, editing-independent function. As described in the previous text, Adar1 is a dsRNA binding protein, so even catalytically inactive Adar1 may be able to shield some of the dsRNA from the RLR sensors that are triggering the aberrant innate immune response, or it may reduce the aberrant immune response by interacting with another protein.

Mechanistically, A-to-I editing by ADAR1 has been shown to alter dsRNA structure so that it prevents aberrant MDA5 activation (Figure 3) [27]. The RLR helicases belong to a particular subset of the SFII helicase homologs that use the ATP hydrolysis between the two moving RecA-like domains to act as dsRNA translocases, binding to dsRNA and scanning along

dsRNA without unwinding it [28]. The helicases are sensitive to dsRNA structure, and, when they reach a bubble or mismatch in the dsRNA, they disassociate [29]. This prevents the RLRs from exposing enough of the CARD domains that recruit the RLR–dsRNA complex to MAVS and activate signalling [23]. Also, many copies of the RLRs bind the same dsRNA and must activate cooperatively to expose the CARD domains [24]. The translocase activities and cooperative activation both contribute to a kinetically based mechanism of discrimination between imperfect self-dsRNA hairpins and perfect foreign, viral dsRNA [23]. Transfecting *Adar* mutant mouse embryonic fibroblasts (MEFs), which have a heightened innate immune response, with dsRNA oligonucleotides containing four inosines located in the middle of their sequence reduces the aberrant innate immune response [4]. We have proposed that RLRs translocating along dsRNA detect I:U wobble base pairs through their perturbation of the structure of the minor groove, deviating it from a perfect A-helix conformation [4,30].

Once ADAR1p150 is induced by IFN and is present in the cytoplasm, it will edit any dsRNA that is present, including viral RNA (Figure 2). Thus, many viruses bear the hallmark of RNA editing in their genomes or replication intermediates, including the recently characterised severe acute respiratory syndrome coronavirus 2 [31]. Editing viral RNA may allow it to evade detection by the innate immune sensors, so editing by ADAR1 can appear to be proviral in cell culture experiments [32]. However, in the whole animal, this editing of all dsRNA in the cytoplasm may be essential to turn off the immune response and to prevent chronic inflammation. Amongst the ADAR proteins, only ADAR1p150 is cytoplasmic and has this role in innate immunity; however, a recent publication demonstrated ADAR2 can edit viral RNA that is nuclear [33]. When a mutant ADAR2 is mislocalised to the cytoplasm, it too can decrease a heightened innate immune response in *Adar1* MEFs [4], demonstrating that it is the generation of inosine in the cytoplasm that is important.

By controlling ADAR1 activity or inosine levels in the cytoplasm, the innate immune response can be modulated, either increased or decreased. In fact, IFN signalling induced by viral infections results initially in a temporary reduction in ADAR1 protein levels (Figure 2) [34]. This temporary reduction in ADAR1 would increase unedited dsRNA, thus stimulating the early innate immune response. Furthermore, high-throughput analyses show that individuals with chronic viral infections have globally reduced levels of A-I editing of endogenous 'self'-RNA [35].

Other ADAR1 Effects on Innate Immunity

The dsRNA-activated **protein kinase R (PKR)** binds to viral dsRNAs, resulting in its activation via dimerisation and autophosphorylation. Activated PKR phosphorylates eukaryotic initiation factor 2α (eIF2α) to inhibit 5′-cap dependent translation, blocking protein synthesis, and therefore preventing viral replication (for review, see [36]). Studies in cell cultures reveal that, during the IFN response, ADAR1 prevents translational shutdown and cell growth arrest by inhibiting hyperactivation of PKR [37] (Figure 3). ADAR1 also plays a role in the **oligoadenylate synthetase (OAS)–ribonuclease (RNase) L pathway**. In a human lung cancer cell line, deletion of RNase L is sufficient to rescue cell death caused by deletion of ADAR1; thus, this pathway may be a mediator of antiviral responses in some specific contexts [38]. However, the studies on PKR and RNase L need to be validated in an animal model.

ADAR1 may regulate IFN pathway components by recoding editing [39–41]; in cancer models, site-specific RNA editing at some sites increased following stimulation with IFN [40]. The idea that ISG transcripts are particularly targeted by ADARs is notable in the context of the proposed central role of ADARs in meditating IFN response. Solomon *et al.* [42] reported that transcripts with more perfect dsRNA structures, which are destabilised when edited, are enriched in ISGs; however, there is no evidence that these particular RNAs can stimulate dsRNA sensors. It is

also well established that miRNAs, including those regulated by ADAR1, such as let7 and mir-21, play central roles in the regulation of gene families in inflammation, immune response, and immune cell development (reviewed in [43]).

Conserved Role of Inosine in dsRNA in *Drosophila* Dicer-2-Mediated Innate Immunity and Genome Defence

ADARs are restricted to the multicellular animals, the metazoans. Both ADAR1 and ADAR2 are present in a simple metazoan, the cnidarian starlet sea anemone *Nematostella vectensis* (Figure 4A) [44]. Later, ADAR1 was lost in some metazoans, such as arthropods, including *Drosophila* [45]; *Drosophila* Adar site-specifically edits many central nervous system (CNS) transcripts and is an orthologue of mammalian ADAR2, which has no known effect on innate immunity and instead site-specifically edits transcripts encoding glutamate receptor subunits to recode them [45]. *Adar*^{5G1}-null mutant flies have severe locomotion defects and aberrant neurotransmitter synaptic vesicle accumulation and develop age-dependent neurodegeneration [46,47]. Unexpectedly,

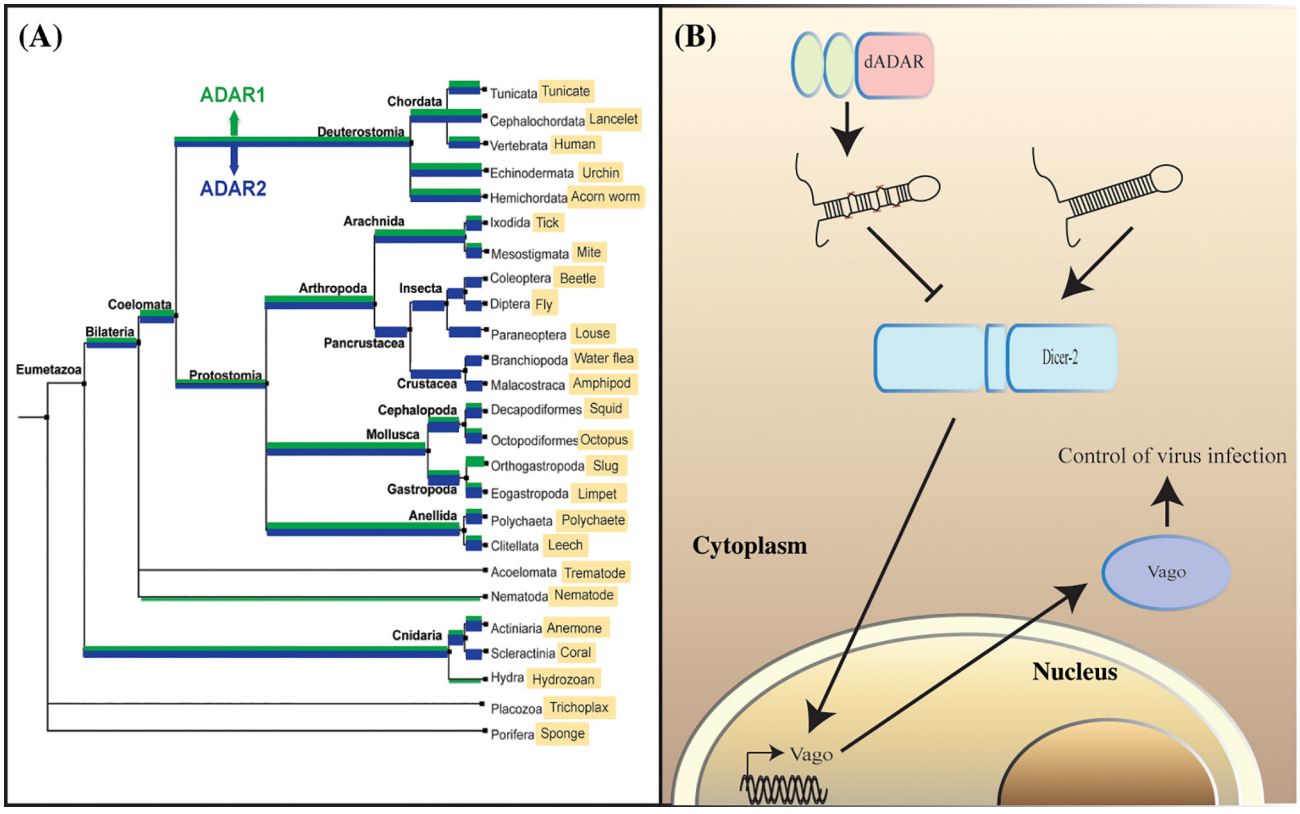

Figure 4. Evolutionary Conservation of Adenosine Deaminase Acting on RNA (ADAR) Innate Immune Effects in *Drosophila*. (A) Phylogenetic tree of ADAR1 and ADAR2 proteins in metazoans. Arthropods have lost ADAR1, and *Drosophila* has one Adar, which is a homolog of human ADAR2, known to be involved mainly in site-specific editing of central nervous system transcripts and not in innate immunity. (B) Like vertebrate *ADAR* mutants, *Drosophila Adar* mutants show an aberrant innate immune induction. *Drosophila* does not have retinoic acid–inducible gene I (RIG-I)-like receptors (RLRs), but the helicase domain of Dicer-2 is evolutionarily related to the helicase domains of vertebrate RLRs. The sensor for double-stranded RNA (dsRNA) in *Drosophila* is Dicer-2; transcriptional signalling downstream of Dicer-2 activates expression of *Vago* transcripts, which encode a secreted protein believed to function as an interferon-like molecule activating the Janus kinase/signal transducer and activator of transduction pathway in invertebrates. The mechanism of helicase domain discrimination between edited and unedited dsRNA to control transcriptional activation of humoral immune defence has been conserved in evolution from DICERs to vertebrate RLRs; in *Drosophila* Dicer-2, it also controls the antiviral RNAi response and, presumably, the related transcriptional silencing and genome defence responses.

the *Drosophila Adar*^{5G1} mutant also has an aberrant induction of innate immune transcripts caused by loss of RNA editing.

The helicase domains of metazoan RLR proteins evolved from helicase domains of **DICER proteins** [48], which have additional RNase III domains that cleave dsRNA to produce siRNAs and miRNAs. Though DICER proteins are present even in unicellular eukaryotes, RLRs evolved later and are restricted to metazoans. RLRs have subsequently been lost in some metazoans; *Drosophila* has no homolog of vertebrate RLRs, and *Adar*^{5G1} mutant aberrant innate immune induction is mediated by Dicer-2 [49]. In *Drosophila*, Dicer-2 mediates the major type of antiviral defence, cleaving long viral dsRNA to give siRNAs. However, Dicer-2 is also an antiviral sensor that detects virus dsRNA and signals to turn on transcription of *Vago* [48], which encodes a secreted protein believed to function as an IFN-like molecule activating the JAK/STAT pathway in invertebrates (Figure 4B) [50]. In the *Adar*^{5G1} mutant, blocking this innate immune Dicer-2 signalling by *Dcr-2* RNAi knockdown suppresses the aberrant immune induction [49].

Editing dsRNA with purified ADAR proteins *in vitro* inhibits cleavage of the edited dsRNA by DICER [51]. For example, in *Caenorhabditis elegans* [52] and in *Drosophila* [19,53], ADARs antagonise Dicer function and inhibit **RNA interference (RNAi)** and also heterochromatin silencing through Dicer-2 in *Drosophila* [54]. The antagonism between ADAR editing and DICER cleavage of dsRNA could have been due to inosine inhibition of DICER at its RNase III active site, but the *Drosophila* findings [49] now indicate that the helicase domain in Dicer-2 is the conserved sensor of inosine in dsRNA. It is likely that many different RNA modifications affecting RLR innate immune sensing similarly affect activation of antiviral helicase DICERs. Viruses have responded by recruiting certain types of RNA base modification to reduce innate immune activation [55].

Disease-Causing *ADAR* Mutations and Clinically Significant Polymorphisms

Mutations in the human *ADAR* gene encoding ADAR1 are associated with disorders of the innate immune system [56]. No homozygous null mutations of *ADAR* have been detected in humans; *ADAR*-null mutations are expected to be embryonic lethal on the basis of mouse studies (described previously). However, genetic mutations and polymorphisms in *ADAR* have given insights into additional roles for ADAR1 in immunity.

Heterozygous *ADAR* mutations cause a skin condition called 'dyschromatosis symmetrica hereditaria' (DSH) [57]. DSH is the least severe *ADAR*-associated disorder, characterised by mixed hyper- and hypopigmented areas of skin discoloration. More than 200 DSH-associated *ADAR* gene mutations have been reported. The deaminase domain appears to be a hotspot for mutations [58,59], while other protein regions usually have frameshift or nonsense mutations [58]. Several DSH patients have heterozygous mutations that exclusively affect the p150 isoform [60,61], indicating that it is haploinsufficiency for the ADAR1p150 isoform that underlies this disorder. It is unclear how ADAR1p150 affects skin pigmentation; however, inappropriate IFN activation can affect the neural crest, leading both to reduced survival of melanocytes, which are important for melanin production, and to reduced Schwann cell differentiation [62]. Factors such as stress, infections, or sun exposure may contribute to the phenotypic variability or incomplete penetrance of the ADAR1-associated disorders [63,64].

Heterozygous dominant-negative (p.Gly1007Arg) *ADAR* mutations or other biallelic *ADAR* mutant combinations lead to the development of neurological disorders ranging in severity from spastic paraparesis [65] and bilateral striatal necrosis (BSN) [66] to the severe early-onset encephalopathy **Aicardi-Goutières syndrome type 6 (*AGS6*)** [56]. A common feature of these

disorders is that the patients present with elevated ISG levels (Table 1). Additional non-neurological features of AGS6 include predisposition to autoimmune phenomena, and patients with biallelic *ADAR* mutations can display cardiac valve calcification [67]. Intriguingly, two siblings with *AGS6* carried biallelic mutations affecting only the ADAR1p150 isoform (start codon mutation and p.Pro193Ala mutation) [68]. This suggests the greater involvement of the ADAR1p150 isoform also in the pathogenesis of *AGS6*. The dominant-negative p.Gly1007Arg mutation has variation in expressivity, and even healthy carriers have been identified [66,69]. The severity of the disorder caused by the loss-of-function *ADAR* mutations in each patient may depend on the overall severity of their effects on editing activity; patients often have compound heterozygous *ADAR* mutations with different mutations in each *ADAR* allele; severity may be further impacted by unknown genetic factors. A decrease in editing activity below a certain threshold probably triggers the development of a neurological disorder [70,71].

Associations have been found between *ADAR* gene polymorphisms and vaccination or therapy outcomes, particularly for IFN-based therapies. It is possible that *ADAR* SNPs may modify the levels of ISGs in healthy or diseased individuals (Table 2). There is only one study where whole-genome SNP analysis was performed [72], though the functional role of the majority of these SNPs was not validated. Probably not all the SNPs in Table 2 are functional; instead, some may be in linkage disequilibrium with functional *ADAR* SNPs. Of particular interest is the SNP rs2229857, which affects a lysine residue that may be ubiquitinated or sumoylated [73]. Degradation of ubiquitinated ADAR1 was previously shown to be important for efficient

Table 1. Summary of Diseases Caused by *ADAR* Gene Mutations Clinically[a]

Disease	Mode of inheritance	Phenotype	IFN signature	Refs
Aicardi-Goutières syndrome 6 (AGS6)	Autosomal recessive, autosomal dominant (p.Gly1007Arg)	Type I interferonopathy; the skin and brain are most severely affected; clinical features include progressive microcephaly, spasticity, dystonia, and psychomotor retardation; neuroradiological evidence of basal ganglia calcification and sometimes white matter abnormalities	Yes	[56,82,83]
Bilateral striatal necrosis (BSN)	Autosomal recessive, autosomal dominant (p.Gly1007Arg)	A neurodegenerative disorder characterised by dystonic/rigid movement; neuroradiological evidence of symmetrical lesions in the corpus striatum and sometimes globus pallidus	Yes	[66,83]
Dyschromatosis symmetrica hereditaria (DSH)	Autosomal dominant (haploinsufficiency of ADAR1 p150)	Hypo- and hyperpigmented macules mainly localised on dorsal aspects of extremities; an occasional occurrence of freckle-like facial macules; otherwise, benign disease	Yes[b]	[57,58,64,84]
Spastic paraparesis	Autosomal dominant (p.Gly1007Arg)	A neurodegenerative disorder characterised by progressive lower limb spasticity; normal imaging of brain and spine	Yes	[65,83]

[a]Abbreviation: IFN, interferon.
[b]Limited evidence; not all the IFN-specific genes measured had elevated levels [56].

Table 2. Clinically Significant *ADAR* Gene Polymorphisms[a]

SNP	Location[b]	Nucleotide change (protein change)	Associated feature or outcome	Notes	Refs
rs1552902	Promoter[c]	Chromosome 1: 154636347C>G	TNF-α secretion upon rubella virus stimulation of PBMCs from MMR-vaccinated individuals	–	[85]
rs4845384	Promoter/ intron 1[d]	Chromosome 1: 154615193A>G	Response to PEG-IFNα-2a/nucleoside analog therapy of chronic hepatitis B (HBeAg seroconversion)	–	[86]
			Spontaneous or IFN-induced HBsAg seroclearance	The A allele results in lower ADAR1 mRNA levels	[87]
rs3738029	Intron 1	Chromosome 1: 154602845G>A	TNF-α secretion upon rubella virus stimulation of PBMCs from MMR-vaccinated individuals	Only in the Caucasian ethnic group	[88]
rs2229857	Exon 2	c.1151A>G (p.Lys384Arg)	Rubella-specific antibody levels in MMR-vaccinated individuals	Only when grouped with other SNPs	[85]
			IL-6 secretion upon rubella virus stimulation of PBMCs from MMR-vaccinated individuals	–	[85,89,90]
			IFN-γ secretion upon rubella virus stimulation of PBMCs from MMR-vaccinated individuals	–	[85,90]
			Severity of liver fibrosis in HIV/HCV-coinfected patients	–	[91]
			IFN-γ ELISpot response upon measles virus stimulation of PBMCs from MMR-vaccinated individuals	Only in the Caucasian ethnic group	[88]
			Response to IFN-β therapy in patients with relapsing-remitting multiple sclerosis	Genome-wide SNP study	[72]
rs903323	Intron 2	Chromosome 1: 154599752C>T	Response to IFN-α/ribavirin therapy of chronic HCV	SNP associated with ADAR gene expression [92]	[93]
rs1127309	Exon 9	c.2682G>A (p.Val894Val)	Response to PEG-IFNα-2a/ribavirin therapy of chronic HCV	–	[94,95]
rs1127313	3′ UTR	Chromosome 1: 154583949G>A	Severity of liver fibrosis in HIV/HCV-coinfected patients	SNP associated with ADAR gene expression [92]	[91]
rs1127314	3′ UTR	Chromosome 1: 154583790G>A	Severity of liver fibrosis in HIV/HCV-coinfected patients	–	[91]
rs1127317	3′ UTR	Chromosome 1: 154583564T>G	IL-6 secretion upon rubella virus stimulation of PBMCs from MMR-vaccinated individuals	–	[89]
			IFN-γ secretion upon rubella virus stimulation of PBMCs from MMR-vaccinated individuals	–	[85]
			Severity of liver fibrosis in HIV/HCV-coinfected patients	–	[91]
			IFN-γ ELISpot response upon measles virus stimulation of PBMCs from MMR-vaccinated individuals	Only in the Caucasian ethnic group	[88]
rs9616	3′ UTR	Chromosome 1: 154583257A>T	IL-6 secretion upon rubella virus stimulation of PBMCs from MMR-vaccinated individuals	–	[85,89]
rs1127326	3′ UTR	Chromosome 1: 154583016T>C	Severity of liver fibrosis in HIV/HCV-coinfected patients	–	[91]
			TNF-α secretion upon rubella virus stimulation of PBMCs from MMR-vaccinated individuals	Only in the Caucasian ethnic group	[88]
rs7515339	Intergenic[e]	Chromosome 1: 154581743T>C	Response to IFN-α/ribavirin therapy of chronic HCV	–	[93]
rs7546383	Intergenic[e]	Chromosome 1: 154581729C>T	Response to IFN-α/ribavirin therapy of chronic HCV	–	[93]
rs9427092	Intergenic[e]	Chromosome 1: 154581246T>C	Rubella-specific antibody levels in MMR-vaccinated individuals	Only when grouped with other SNPs	[96]
			TNF-α secretion upon rubella virus stimulation of PBMCs from MMR-vaccinated individuals	–	[85]

(See Table legend at the bottom of the next page.)

type I IFN signalling [34], hinting at a potential functional role of rs2229857 in the regulation of ADAR1.

The Effects of ADAR1 in Cancer Also Involve Innate Immunity

RNA editing is increased in most tumour types due to higher expression of ADAR1, either by IFN induction or by *ADAR* gene amplification at chromosome 1q21 [74–76]. Experimental knockdown or knockout of ADAR1 can reactivate immune pathways and can result in cancer cell lethality. Cancer types with increased ISG signatures are particularly sensitive to knockdown to ADAR1; an shRNA viability screen across 398 cancer cell lines identified ADAR1 as the top hit to kill cancer cells that have ISG signatures [77]. Some cancer types that are not sensitive to ADAR1 targeting are made sensitive by activation of IFN signalling [78]. Further, therapies that induce IFN signalling, such as radiation treatment, were able to slow growth of transplanted B16 melanoma tumour cells in mice when the tumours were *Adar*-null but not when they were *Adar* wild type [40].

Different mechanisms may explain these requirements for ADAR1 in cancer cells. It has been proposed that transcripts induced by IFN signalling contribute to increasing dsRNA levels and are edited by ADAR1. In support of this, ADAR1 depletion in human cancer cell lines resulted in increased expression of ISGs in an RIG-I–, MDA5-, and STING-dependent manner [77]. In particular, Mda5 was required for the loss of Adar1 to induce inflammation and immune cell infiltration upon IFN stimulation in tumours in mice [40]. Another proposed mechanism is that ADAR1 prevents the activation of PKR. Depletion of ADAR1 in cancer cell lines increased PKR activation, and PKR loss prevented the expression of pro-inflammatory cytokines as well as cancer cell death upon depletion of ADAR1 [77]. In human lung cancer lines, inactivation of PKR also results in partial prevention of cancer cell death caused by ADAR1 inhibition [78]. In this model, PKR is inactivated by ADAR1p150 independently of editing [78], and this, in addition to loss of dsRNA editing leading to aberrant RLR activation, mediates cancer cell death when ADAR1 is knocked down [40,77,78]. Aberrantly increased ADAR1 levels in tumours have also been shown to alter levels of site-specific recoding editing in many mRNAs encoding proteins involved in cancer (reviewed in [81]).

Importantly, ADAR1 can also enhance the therapeutic effect of ‘immune checkpoint blockade’ cancer therapy. Briefly, tumour cells are able to escape antitumour immunity by activating immune checkpoints. For example, they can express programmed cell death ligand 1 (PD-L1) on their cell surface, which interacts with PD-1 on T cells, resulting in negative regulation of antitumour T-cell responses. When this interaction is inhibited with anti–PD-L1/PD-1 antibodies, it restores the antitumour responses (reviewed in [79]). An intact IFN signalling pathway appears to be required for the success of immune checkpoint blockade therapy. Knockdown of ADAR1 was identified as a way to restore the tumour cell response to immune checkpoint blockade therapy [80]. In transplantable tumour models in immunocompetent mice, *Adar*-null tumours were sensitised to anti–PD-1 immune therapy [40]; an intact IFN signalling pathway was required for this effect of the *Adar* knockout [40]. The model proposed is that high levels of A-to-I editing inhibit activation of RLRs and PKR, thus preventing IFN signalling responses [40]. Inhibiting

Notes to Table 2:
[a]Abbreviations: ELISpot, enzyme-linked immunospot; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cell; PEG, polyethylene glycol; MMR, measles, mumps, and rubella; TNF, tumour necrosis factor; UTR, untranslated region.
[b]Genome build GRCh38.p13; ADAR1 p150 transcript ENST00000368474.9; protein isoform ADAR1 p150 (UniProt identifier P55265-1).
[c]Lies closer to ADAR1 p110 transcript ENST00000368471.8.
[d]Promoter region of ADAR1 p150 transcript ENST00000368474.9; intron 1 of ADAR1 p110 transcript ENST00000368471.8.
[e]Lies in proximity to both *ADAR* gene and *CHRNB2* gene.

ADAR1 can therefore convert a tumour from an 'immunologically cold' state that is hidden from T cells to an 'immunologically hot' state, where tumour-specific T cells are present.

Concluding Remarks

The initial research on ADARs focused on their role in recoding of transcripts expressed in the CNS. While this is still an important function of the ADAR2 protein, the research on ADARs, in particular ADAR1, has expanded greatly in the past few years. While its role in immunity was apparent for many years, it took a major shift away from focusing primarily on recoding to its role in editing transcripts encoding Alu elements to elucidate and appreciate ADAR1's role in innate immunity. Over the years, we have also seen the increasingly important role of ADAR1 in cancer progression, and now we are at an exciting stage where the role of ADAR1 in cancer may be harnessed as a possible point of intervention and used for the development of new therapies for cancer treatment. However, ADARs have not yet yielded all their secrets to us, and many outstanding questions remain (see Outstanding Questions).

Acknowledgments

This work was supported by EU Horizon 2020 MSCA 867470 (J.Q.), GAČR 20-11101S, GAČR 21-27329X (M.A.O'C.), MEYS Inter-excellence Programme LTC18052 (M.A.O'C.), and GAČR 19-16963S (L.P.K.).

Declaration of Interests

There are no interests to declare.

Outstanding Questions

Which other RNA modifications are important for innate immunity and help the cell distinguish self- from non–self-RNA? Do m^6A and other modifications also help control the formation of dsRNA?

What is the nature (sequence, structure, common features) of the dsRNAs that trigger the innate immune response in the *Adar*-null mice and in patients with AGS6? Are they Alu inverted repeat hairpins in humans or some other more conserved dsRNAs?

Why does removing *Mavs* only partially rescue the *Adar*-null mutant, whereas it fully rescues the inactive $Adar1^{E861A}$ mutant? What are the molecular mechanisms underlying the editing-independent functions of ADAR1?

How do ADAR1 variants that retain high editing activity on standard editing substrates nonetheless cause AGS? Do these AGS mutations instead interfere with an editing-independent function of ADAR1?

How does ADAR1 help maintain low activity of the innate immune pathways under normal (uninfected) conditions? Upon viral infection, how is ADAR1 regulated to de-repress the innate immune pathways? How is ADAR1 protein regulated at the post-translational level?

What is the molecular mechanism by which ADAR1 and inosine in dsRNA prevent aberrant activation of RLR helicases in vertebrates and Dicers in invertebrates? Do the inhibition mechanisms act dominantly inside the cell, as suggested by experiments using transfection of inosine-containing dsRNA oligonucleotides? That is, could having inosine in some of the dsRNA be enough to lower the overall activation of RLRs in a cell?

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