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Activity Regulation of Adenosine Deaminases Acting on RNA (ADARs)

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Abstract Adenosine deaminases acting on RNA (ADARs) are the enzymes that are responsible for the A to I RNA editing process in mammals, which is an important mechanism that increases molecular diversity. A to I RNA editing consists of an enzymatic conversion of specific adenosine in pre-mRNA, leading to alteration of the properties of both the RNA itself and the translated protein. Currently, the importance of this phenomenon is increasingly recognized as it affects a diverse set of cellular pathways. ADAR function within the cell, especially in the neurons, is to diversify the features of a limited set of unique transcripts, mostly neurotransmitter receptors; however, a growing set of target is going to be discovered, increasing the importance of the RNA editing event in the proper physiology of the cell. Despite the functional relevance of these enzymes, there is a gap of knowledge in the mechanisms that regulate ADAR activity and consequently about the modulation of RNA editing process. This review summarizes ongoing investigations of ADAR regulation at the transcriptional, post-transcriptional and post-translational level and addresses new hypothetical mechanisms that are capable of modulating ADAR activity, including subcellular localization, dimerization and interaction with trans-acting factors.

Keywords ADAR1 · ADAR2 · ADAR3 · RNA editing · Post-transcriptional regulation · Post-translational regulation

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Introduction

In mammals, A to I RNA editing is a post-transcriptional process that consists of an enzymatic deamination of either adenosines (A) or cytidines (C) that transforms these nucleosides to either inosines (I) or uridines (U), respectively [1]. Currently, the conversion of A to I is thought to be the most common RNA editing process in higher eukaryotic cells, especially in neurons [2, 3]. This RNA editing process is mediated by adenosine deaminase acting on RNA (ADAR) enzymes [4, 5] that specifically recognize partially double-stranded RNA structures and modify individual adenosines depending on the local structure and sequence environment [5–7]. How ADARs select specific adenosine moieties for deamination is a current matter of research. An NMR-based model of the protein/RNA complex has recently been reported for ADAR2 and the R/G site of GluA2 where the first double-stranded RNA binding motif (dsRBM) recognizes a conserved pentaloop, whereas dsRBM2 recognizes two bulged bases adjacent to the editing site, demonstrating RNA structure-dependent recognition by the ADAR2 dsRBMs [8].

The RNA editing process aids in the diversification of the information that is encoded in the genome of an organism and thereby allows for greater complexity [2]. Because inosine is read as guanosine by the translation machinery [9], editing often leads to codon changes that alter the amino acid sequence of the encoded protein; these changes in codon composition then lead to specific changes in the amino acids that were originally predicted by the genomic DNA sequence. The majority of RNA editing sites are located within intragenic non-coding sequences: the 5′ UTR, 3′UTR and intronic retrotransposon elements, such as Alu and long interspersed elements [6, 10, 11]. Moreover, RNA editing seems to play a role in the control of non-

coding RNA, such us endogenous short interfering RNA (esiRNA) [12, 13], and in the biogenesis and function of certain microRNAs (miRNAs) by editing their precursors [14–18]. A to I RNA editing in mammalian cells has been described for a limited number of protein-coding RNA sequences, including ionotropic glutamate α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (GluA2-4) [5, 7] and kainate receptors (GluK1-2) [5, 19–21], the serotonin receptor 2c (5HT-2cR) [22, 23], the Kv1.1 potassium channel [24], the GABAA-α3 receptor subunit [25, 26] and ADAR2 itself [11]; in all cases, this editing results in dramatic alterations in protein function. In mammalian cells, three highly conserved ADAR genes, ADAR1 [27–29], ADAR2 [30–32] and ADAR3 [33, 34], with common functional domains have been identified (Fig. 1). Improper ADAR functioning has been correlated with several important human diseases [35] such as amyotrophic lateral sclerosis [36–38], depression [39–43] and epilepsy [44, 45]. In addition, the development of a genetic linkage map of the human pigmentation disease called dyschromatosis symmetrica hereditaria has led to the identification of ADAR1 as the responsible gene [46, 47].

Because these enzymes are important for proper cellular function, this review summarizes ongoing investigations of the transcriptional, post-transcriptional and post-translational regulation of ADAR and explores the newly hypothesized mechanisms that are thought to be capable of modulating ADAR activity.

Functional ADAR Protein Domains

The three ADAR enzymes share common structural features, such as their C-terminal deaminase domain and their two or three N-terminal dsRBMs, and contain several subcellular localization signals that allow them to shuttle between different compartments, such as the nucleoli, nucleoplasm and cytoplasm (Fig. 1). At the same time, these enzymes have also specific structural features that are not shared. ADAR1 is characterized by the following properties: (1) It has an N-terminus domain that is longer than that of ADAR2 and ADAR3, (2) it has a Z-DNA/RNA binding domain (Zβ) [48], and (3) it exists in an interferon-inducible form that also contains an arginine–glycine repeat region, another Z-DNA/RNA binding domain (Zα) [49] and a tandem-repeated sequence that is adjacent to the Zβ domain [50]. The ability of ADAR1 enzymes to bind to DNA may serve to localize it to sites of active transcription, which are places where the DNA is in a Z-conformation behind the active polymerase. In this way, ADAR1 can edit its substrates before splicing occurs [51]. ADAR3 and a specific isoform of ADAR2 (ADAR2R) both contain an arginine-rich sequence at the N-terminus that acts as a single-stranded RNA binding motif (ssRBM). This specific domain might operate in combination with the two dsRBMs to target a very selected set of RNA substrates that are yet unknown [33].

Genomic Organization and Post-transcriptional Regulation of ADARs

Transcriptional and post-transcriptional processes are the first steps of ADAR regulation. Therefore, these processes must be explored in great detail when investigating changes in the editing levels of ADAR targets either during development [52–54], under pathological conditions [55, 56] and in response to specific pharmacological treatments [57–60].

ADAR1 Has Different Promoters and Undergoes Alternative Splicing

The human ADAR1 gene contains 17 exons [61, 62]. Three different promoters drive transcription of the ADAR1 gene; this diversity leads to the production of multiple protein variants (Fig. 2) [63, 64]. Two of these promoters (1B and 1C) are constitutive promoters that drive the expression of transcripts that encode a short version (931 aa) of the ADAR1 protein (ADAR1S, cADAR1 or p110), while the third (1A) is an interferon-induced promoter that drives the expression of transcripts that encode an N-terminally extended form (1226 aa) of ADAR1 (ADAR1L, iADAR1 or p150) [48, 61, 63, 65]. Only the interferon-inducible ADAR1 transcript contains a translation initiation codon in exon 1, [63], while the two constitutively expressed forms of ADAR1 start at the downstream AUG296 codon that is present in exon 2 [48, 61]. The relative expression of the three promoter-specific ADAR1 transcripts is tissue specific [65]. In the human brain, a constitutively high expression of ADAR1 is driven by promoter 1B, and a negligible expression results from promoter 1C. In contrast, the inducible-promoter 1A is preferentially expressed in other tissues, including the liver, lungs, cecum, heart, spleen, Peyer's patches and kidney [66, 67].

Two additional ADAR1 splicing variants that involve exons 6 and 7 have also been identified [61, 66]. In contrast to the full length ADAR1a transcript, the ADAR1b variant has a 78 nt in-frame deletion that occurs because the last portion of exon 7 encoding 26 aa in the linker region between the third dsRBM and the catalytic domain is spliced. The ADAR1c variant is characterized by an in-frame deletion of 57 nt (19 aa) that is located between the second and third dsRBMs and derives from the loss of the first part of exon 6. It has been hypothesized that shortening the distance between the functional domains of ADAR1 could consequently change the way in which ADAR1

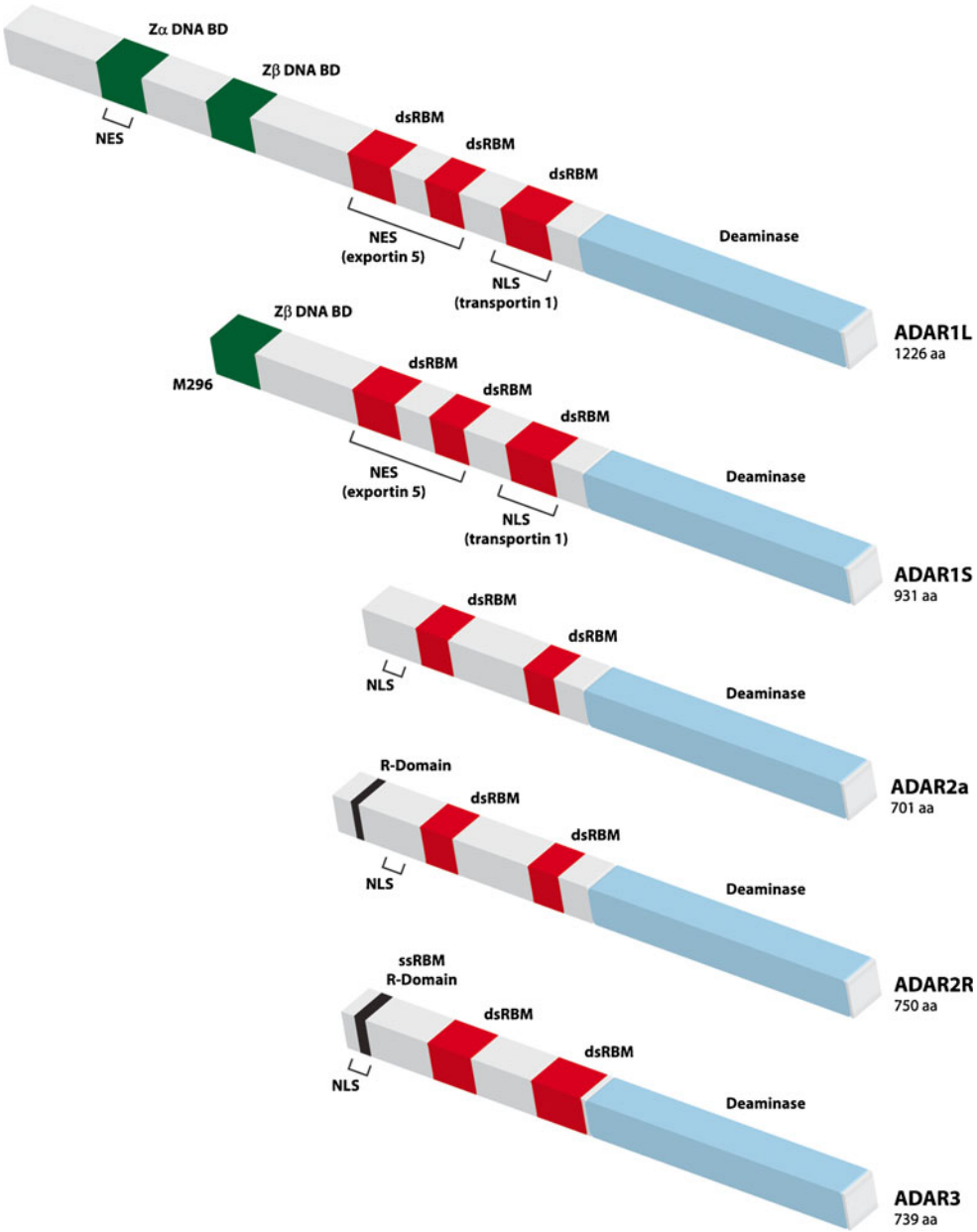

Fig. 1 Functional ADAR protein domains. Schematic representation of ADAR functional domains. *Zα/β DNA BD* Zα/β DNA binding domain, *NES* nuclear exporting signal, *NLS* nuclear localization signal, *dsRBM* double-stranded RNA binding motif, *M296* methionine 296, *R-domain* arginine rich domain

interacts with dsRNA substrates and their position at the catalytic deamination domain [61]. Therefore, ADAR1 splice variants could contribute to mediating the required site selectivity in the editing process of natural substrates. In addition, ADAR1 splice variants differ in their editing efficiency in vitro because ADAR1b and ADAR1c display a greater activity than ADAR1a on the R/G editing site of GluA2 [68]. All three variants have also exhibited a low level of editing activity at the Q/R editing site of this gene [68]. Additionally, these results have been observed with in vitro tests of ADAR1b and ADAR1c enzymatic activity on the 5HT-2cR pre-mRNA. While these two variants have a higher editing efficiency at the A- and C-sites, ADAR1a has higher activity at the B-site [69]. The relative expression of the alternatively spliced ADAR1a and ADAR1b mRNA isoforms correlates with the observed changes at the level of 5HT-2cR RNA editing [70].

Finally, an additional ADAR1 splice variant is generated by the selective exclusion of exon 2 [71]. This leads to the translation of a shorter version of ADAR1 protein (p80) which starts at a methionine 519 (M519) and lacks the putative NLS, the Z-DNA binding domain and the first dsRBM.

ADAR2 Alternative Splicing and Self-editing

The mammalian ADAR2 gene is composed of 15 exons [72, 73]. The main splice variants are defined as ADAR2a,

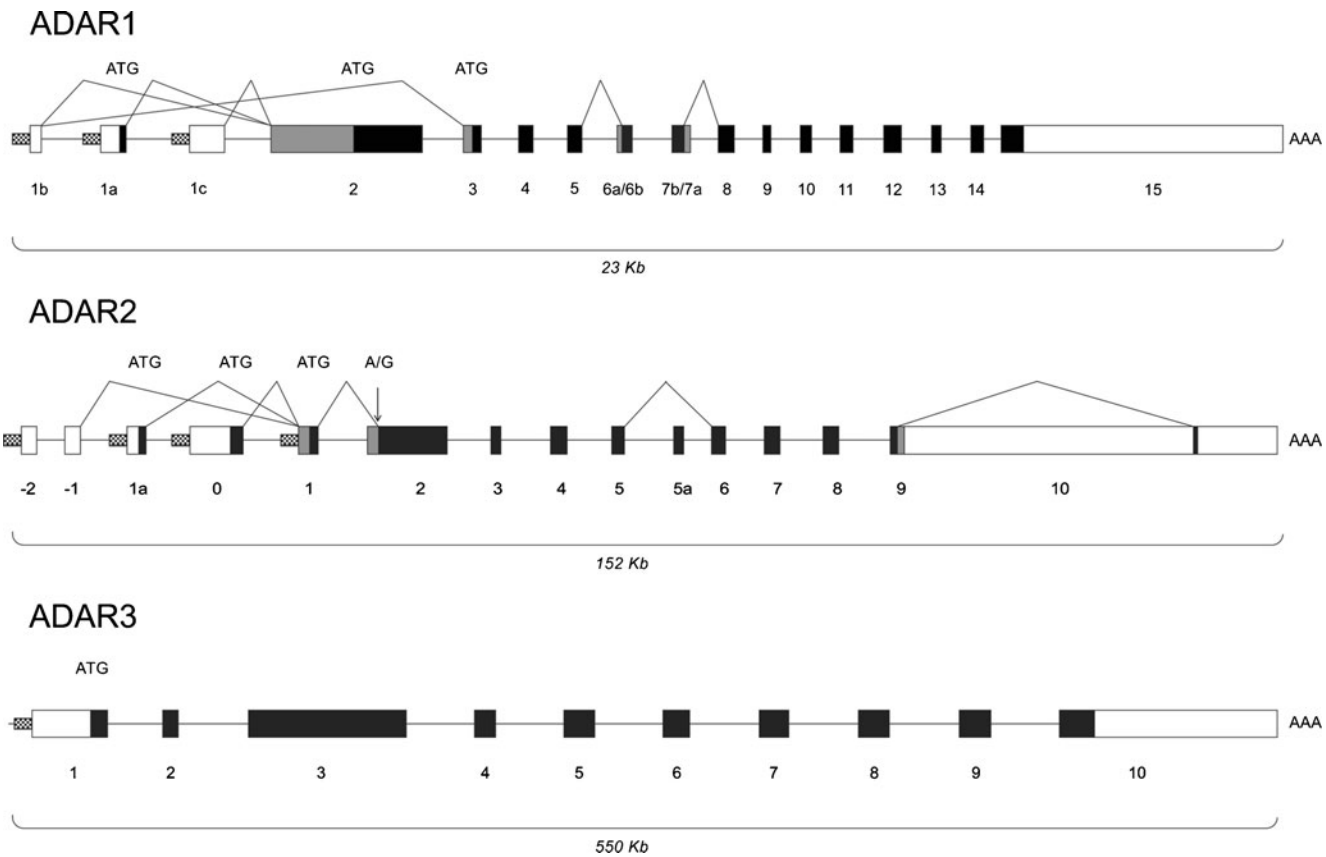

Fig. 2 Genomic organization of human ADAR genes. Exons are represented to scale by *boxes*; introns, not to scale, by *lines*. *Filled boxes* are coding, and *open boxes* are non-coding; alternative promoters are depicted as *striped boxes* in front of their corresponding first exons. *Gray boxes* can be expressed alternately as exons or introns. Alternatively, spliced exons are indicated. Genomic sizes spanning 5′UTRs, coding regions and 3′UTRs are indicated in kilobases. *ADAR1* exon 1a is under control of an interferon-inducible promoter and generates the longest isoform of ADAR1, while exon 1b and exon 1c are under control of constitutive promoters generating shorter versions of the protein. *ADAR2* exons and introns are numbered according to Slavov and Gardiner [73], with the addition of the latest identified exon 0 [72]. The *gray box* before exon 2 indicates the 47 nucleotide cassette, and *A/G* denotes the site of self-editing that produces the AG splice site. *ADAR3* is the largest of the three genes, spanning approximately 550 kb; no alternative splicing has been identified

ADAR2b, ADAR2e and ADAR2f and are produced as a result of two distinct alternative splicing events [11]. A shorter C-terminal sequence has also been characterized, which gives rise to isoforms ADAR2c and ADAR2d that are specific to humans [31].

The first splicing event involves the 5′ end of the coding sequence and generates the ADAR2e and ADAR2f isoforms due to a specific RNA editing event. A self-editing process of ADAR2 that acts on its own pre-mRNA introduces an alternative proximal 3′ acceptor site and converts an intronic AA dinucleotide to an AI dinucleotide, which mimics the highly conserved AG sequence that is normally found at 3′ splice junctions. This new acceptor site adds 47 nt to the ADAR2 coding region [11, 74], which gives rise to a frameshift that codes for a truncated form of ADAR2 protein consisting of only 82 aa in rats and mice and 31 aa in humans [73]. Unexpectedly, ADAR2e and ADAR2f transcripts in rats might encode functionally active proteins using an alternative translation initiation site at methionine 25, which would produce a protein that lacks the 24 aa that are present at the N-terminus of the rat ADAR2a and ADAR2b isoforms [11]. However, this internal translation initiation site is inefficient in the rat brain. Previous studies have found that only 5% of the ADAR2 protein corresponded to the functional N-terminal-truncated isoform, although 80% of the total ADAR2 transcripts contained the 47 nt insertion (ADAR2e and ADAR2f) [11]. Interestingly, the mRNA expression levels of ADAR2e and ADAR2f in human brain are significantly lower and make up only 11% of the total ADAR2 mRNA [11, 73].

Transgenic mice lacking ADAR2 self-editing activity express a significantly higher ADAR2 protein level; this correlates with an increased editing level of ADAR2

substrates [75]. In summary, ADAR2 self-editing regulates the alternative splicing of the 47 nt cassette and thereby reduces the expression levels of functional ADAR2 protein by decreasing its translation efficiency [76]. This negative feedback mechanism could be responsible for a down-regulation of ADAR2 at the protein level that does not correspond to a change in its total mRNA levels [11, 75, 77].

The second alternative splicing site is located within the region near the 3′ end of the coding sequence that encodes the deaminase catalytic domain. Splicing at this site results in the inclusion of 30 nt in the rat and mouse (or 120 nt in the human isoform) between the second and third putative zinc-coordination motifs that generate the ADAR2b isoform [30, 31]. Both ADAR2a and ADAR2b show an almost identical activity level in the rat when measured in vitro [11, 31], although human ADAR2b has been found to be 50% less active than ADAR2a [30].

The last alternative splicing involves the C-terminus and occurs only in humans to create the hADAR2c and hADAR2d isoforms [30, 31, 78]. This event leads to the insertion of a shorter C-terminus and generates a protein that displays essentially no RNA editing activity [31].

Two additional ADAR2 splice variants have been identified in the human brain. The first variant skips all of exon 2, which encodes two dsRBMs; this deletion results in a frameshift that introduces a premature stop codon in the next exon. This variant represents 13–20% of total hADAR2 mRNA in the brain, but it has not been found at the protein level. The second splicing event generates two alternative 3′UTRs that might regulate the translational efficiency and mRNA stability of ADAR2 in vivo [79].

Finally, a novel exon (exon 0) of ADAR2 has been discovered; it is located upstream of exon 1, and it extends the open reading frame at the N-terminal by 49 aa (Fig. 2). Interestingly, this exon contains an R-rich domain that is closely related to the ssRBM of ADAR3; therefore, it has been designated as ADAR2R [72]; however, this isoform seems not to be highly expressed.

Concerning ADAR2 gene expression, it has been reported that it can be induced by activated cAMP response element binding [80].

ADAR3

The editing activity of ADAR3 has not yet been demonstrated [33, 34]. The expression profile of ADAR3 in rat brain has been characterized by northern blot and oligonucleotide-mediated in situ hybridization [34]. The ADAR3 sequence is closely related to ADAR2, but ADAR3 appears to be expressed exclusively in the brain [33] and has a coding region of 2.2 Kb, a 5′UTR of 0.3 Kb and a 3′UTR that is 7 Kb. Among the functional domains encoded by ADAR3 gene is an N-terminal arginine-rich motif that seems to mediate the recognition of specific stem-loop structures that are present in single-stranded RNA substrates; this domain might also be involved in DNA binding [33]. No transcripts arising from alternative splicing have been identified.

Temporal and Spatial Regulation of ADAR Expression

ADAR enzymatic activity is crucial for embryonic development. The deletion of ADAR1 in mice is lethal before E12; the mice develop severe liver defects, impaired hematopoiesis and widespread apoptosis [81, 82]. In addition, *ADAR2*$^{-/-}$ mice develop normally but are prone to early onset of epilepsy and die within 3 weeks of birth [83, 84]. Interestingly, the lethal phenotype of mice that lack ADAR2 could be rescued by introducing a point mutation in the GluA2 Q/R editing site (*ADAR2*$^{-/-}$/*Gria2*$^{R/R}$) that inserts an arginine that encodes directly in the genome [83] showing that this site is the main editing target of ADAR2. However, a very recent analysis of the *ADAR2*$^{-/-}$/*Gria2*$^{R/R}$ mice revealed several specific mutant phenotypes providing a rationale for the identification of new functional ADAR2 targets [85].

The developmental and regional expression of ADARs and the editing levels of their targets have been previously analyzed [30, 32, 52–54, 76, 86, 87]. ADAR1 and ADAR2 are expressed in many tissues [28–30, 52], and their enzymatic substrates have been identified mainly, but not exclusively, in the nervous system; in contrast, ADAR3 expression is entirely brain specific [33, 34].

Northern blot analyses of human ADAR1 [28, 29, 33] and ADAR2 [30, 33] mRNAs have shown that both are highly expressed in the brain, heart, placenta, lung, liver, pancreas and skeletal muscle; these data have also been confirmed at the protein level [30, 32, 86]. Because most previously identified ADAR mRNA substrates are primarily expressed in the brain, detailed studies have been performed to analyze its distribution among various brain regions.

Concerning ADAR2, Paupard and collaborator [53] reported that its mRNA expression was first detectable in the thalamic nuclei formation at embryonic day (E) 19; during the first week of post-natal life, ADAR2 mRNA expression increased gradually in several brain regions, with the higher expression in the thalamus until post-natal day 7 [53]; high levels of RNA editing for several ADAR2-specific substrates have been observed in this brain area [26, 88, 89]. By the third post-natal week, ADAR2 transcripts are more widely distributed throughout the olfactory bulb, the CA3 region and the dentate gyrus of the hippocampus, the thalamus, the inferior colliculus and the molecular cell layer of the cerebellum [53]. Surprisingly, in this work, ADAR2 mRNA has not been detected prior to E19, while AMPA receptor GluA2 pre-mRNA has been 99%

edited by E14 [22, 90]; these data need to be further investigated.

The highest expression of ADAR1 transcripts occur in the hypothalamus and the thalamus [52]. A northern blot analysis of human ADAR3 revealed that the highest levels of expression are in the thalamus, the amygdala and the cerebral cortex [33]. In situ hybridization in the rat brain revealed a differential expression of ADAR3 mRNA with a distribution similar to that of ADAR2, in addition to high levels of expression in the globus pallidus and the thalamus [34]. Another study performed in three post-mortem human brain regions (the cerebellum, the cerebral cortex and the cerebral white matter), at three developmental stages (fetal, neonatal and adult), revealed an increase in the expression of ADAR1-3 mRNA between the neonatal and adult stages in the gray matter areas. In contrast, both ADAR1 and ADAR2 are expressed at a lower level in the white matter, but ADAR3 expression is increased in this region [87]. These discrepancies probably reveal a different subcellular distribution of the ADAR transcripts between tissues where either neurons or oligodendrocytes are the predominant cell type [87]. Finally, two studies failed to establish a close correlation between ADAR mRNA expression levels and the editing frequency of two ADAR substrates, namely, the 5HT-2c and GluA2 receptors [53, 86].

Several studies on brain development found changes in the RNA editing levels in the mRNAs of known ADAR substrates such as GluA subunits [91], the 5HT-2cR [86] and GABAA receptors [25, 26], and ADAR2 itself [11]. For example, the editing of ADAR2 pre-mRNA at six positions does not occur during the embryonic period, but it increases during post-natal development. In addition, the extent of editing in cultured cortical neurons tends to increase with cell maturation from 3 days in vitro (DIV3) to DIV15 [86]. Therefore, although ADAR levels may play a critical physiological role in the brain and other peripheral tissues, changes in their expression alone could not explain the developmental regulation of RNA editing that has been observed in the brain. Therefore, it is clear that looking only at changes in ADAR mRNA expression levels is not an adequate parameter for evaluating the cause of modification in editing frequency [76], and an additional regulatory step may exist at the post-transcriptional or translational level.

Subcellular Distribution of ADARs

The subcellular localization of ADAR proteins is crucial to their regulation of editing efficiency. Most of the RNA editing events occur in the nucleus, which is where the RNA substrates of ADARs are primarily localized. However, the large form of ADAR1 (ADAR1L) is found both in the cytoplasm and in the nucleus [48], while the small nuclear form of ADAR1 (ADAR1S) moves to the cytoplasm in the absence of transcription and displays the hallmarks of a shuttling protein [92, 93]. Furthermore, ADAR2 and ADAR3 are always retained in the nucleus due to the presence of a nuclear localization signal (NLS) [94]. ADAR1 and ADAR2 also have a nucleolar localization that is dependent on RNA binding [95, 96]. There are currently no studies regarding the subnuclear distribution of ADAR3.

The subcellular distribution of ADAR proteins is influenced by the presence of specific functional domains (Fig. 1). A nuclear localization signal (NLS), which overlaps with the third dsRBM, has been described for ADAR1 [92, 97], whereas a nuclear export signal (NES) has been identified only in the N-terminal Zα-domain of ADAR1L, which causes it to act as a nucleocytoplasmic shuttling protein [95, 98]. Transportin-1 is able to bind the third dsRBM-NLS of ADAR1, which interferes with dsRNA binding and acts as its import receptor [99]. An alternative model of ADAR1 nuclear export involves the binding of the first and second dsRBMs with exportin-5, which would allow it to be transported to the cytoplasm even without an NES such as in ADAR1S [93, 99]. In addition, evidence exists for the involvement of the deaminase domain in the regulation of ADAR1 nuclear localization [93]. Generally, the editing process takes place in the nucleus next to sites of active transcription; however, rapid and efficient RNA editing has also been observed in the cytoplasm [100]. The potential cytoplasmic substrates of RNA editing could be viral RNA, which could induce both proviral and antiviral effects [101], or mRNAs in which the editing site is retained after processing.

In contrast, the nuclear localization of ADAR2 and ADAR3 appears to be controlled by members of NLS receptor α importin family. Deletion studies showed that ADAR2 is devoid of any NES, contains a non-canonical NLS within the first 64 amino acid residues and harbors a region between residues 75 and 132 that is necessary but not sufficient for nucleolar targeting [95]. Two-hybrid interaction analysis suggests that ADAR2 has a single NLS in its N-terminal domain and is transported by importin α4 and α5, while an arginine-rich domain (R-domain) in the N-terminus of the ADAR3 protein acts as a NLS and mediates a specific interaction with importin α1 [94].

It has been hypothesized that ADARs may accumulate transiently in the nucleolus, but once a specific dsRNA substrate is present for editing, they relocate to the nucleoplasm [96, 98]. The nucleolar association of ADAR2 is dependent on the dsRBMs and is considered to be related to an interaction with the ribosomal RNA that is present in the nucleolus [96]. ADARs shuttle constantly and rapidly in and out of the nucleolus [95, 96], which suggests the

presence of a dynamic regulation process for RNA editing. The nucleolus might represent a site of enzyme storage in a less active environment of the cell where only ADAR2-mediated—but not ADAR1-mediated—RNA editing occurs [102]. An increasing body of evidence suggests that the nucleolus might operate as a "molecular safe" by sequestering the ADAR2 enzyme, thereby reducing its activity [103], which would prevent any accidental editing activity in the nucleoplasm [104].

The level of accessibility of the RNAs for binding and modification by ADARs is a major issue in RNA editing studies [3]. An intriguing option is the possible physical separation of the enzyme from its substrate, which involves the hypothesis that a concomitant modulation of the distribution of ADARs and their substrates may modulate RNA editing levels.

Dimerization

The dimerization of ADARs is required for their enzymatic activity [105–107]. Both ADAR1 and ADAR2 can form stable, enzymatically active, homodimer complexes [106]; in contrast, ADAR3 does not dimerize in vitro, although dimerization of ADAR3 has been observed in the mouse brain [106]. In Drosophila, the minimal region of ADAR required to form a stable dimer consists of the N-terminus and the first dsRBM (aa 1–133) [108].

The possibility of heterodimerization among ADAR family members has long been debated. A FRET analysis of in vivo dimerization that was performed in HeLa cells has demonstrated for the first time that ADAR1 and ADAR2 heterodimerize [105]. Moreover, the heterodimerization of ADAR1 and ADAR2 has been observed in astrocytes, and the overexpression of ADAR1 in pediatric astrocytomas inhibits the editing activity of ADAR2. These results suggest a possible mechanism for ADAR2 inhibition in which it is sequestered by ADAR1 [109]. However, the association of the heterodimers is likely weaker than the association of the homodimers, and post-translational modifications might be required [106].

Another controversial issue concerns the dependence of ADAR dimerization on RNA binding. RNase treatments do not affect ADAR homo- and heterodimerization, which supports the hypothesis of an RNA-independent dimerization process [105, 106]. In contrast, other studies have suggested that ADAR dimerization could be RNA dependent [107, 108, 110, 111]. Nonetheless, specific amino acid substitutions within the dsRBMs of ADAR1 and ADAR2 result in the total loss of RNA binding properties, whereas the dimerization capability of these enzymes is retained [112]. In summary, ADAR homo- and heterodimerization both occur at an amino acid interface that is formed through protein–protein interactions of the two monomers, and this process appears to be closely related to the enzyme's RNA editing efficiency.

The formation of heterodimers between different ADAR family members has been shown to affect the overall editing efficiency of ADARs. Both in vitro [33] and in vivo analyses [74, 75, 109] have indicated that different combinations of ADARs can attenuate or modulate their editing activity. These observations suggest that ADAR heterodimerization could be a key mechanism in generating a larger spectrum of enzymatic variability, which could, in turn, regulate the efficiency and specificity of RNA editing in the cell [111].

A new idea about the functional regulation of ADAR activity comes from the suggestion that ADAR1 and ADAR2 could heterodimerize with ADAR3, which is a non-functional enzyme. ADAR3 is brain specific and is mainly restricted to the amygdala and the thalamus. It is catalytically inactive on both synthetic dsRNAs and known pre-mRNA substrates in vitro, although it can bind to either double-stranded or single-stranded RNA [33, 34]. Thus, it has been suggested that ADAR3 could either act on unknown RNA targets or could interfere with the activity of ADAR1 and ADAR2 through competitive inhibition [33]. To date, a conceivable hypothesis, based on the results summarized here, is that the ADAR catalytic site would require two deaminase domains of the same ADAR member to be completely active. A combination of different family members at the active site might be responsible for the reduction and/or modulation of ADAR efficiency [30, 108].

ADAR1 SUMOylation

SUMOylation is a highly dynamic post-translational modification where a small ubiquitin-like modifier (SUMO) is covalently linked to a protein; this modification is reversible and is modulated by SUMO-specific proteases. SUMOylation is involved in various cellular processes such as nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, stress response and progression through the cell cycle [113]. SUMOylation appears to alter the long-term fate of the modified protein even though the SUMO modification may be rapidly removed. Thus, a protein that has a history of SUMO modification might have different properties than an identical but unmodified protein [114].

Human ADAR1, but not ADAR2, is modified with SUMO-1 on Lys418 (Fig. 3), which reduces the RNA editing activity of the ADAR1 enzyme without interfering with its proper localization [104]. Both ADAR2 and ADAR3 lack the N-terminal region that contains the consensus sequence for SUMO-1 conjugation. ADAR1

colocalizes with SUMO-1 in subnucleolar regions that are not involved in ribosomal biogenesis [115]. Because the lysine residue in ADAR1 that acts as a SUMO-1 acceptor lies between the Z-DNA binding domain and the first dsRBM, it has been hypothesized that SUMO-1 acts as a stereochemical obstacle for both the binding to dsRNA and the dimerization of ADAR1 [104]. In summary, SUMOylation of ADAR1 may be part of the process that sequesters ADAR1 in the nucleolus in an inactive form [104].

Inositol Hexakisphosphate in the Catalytic Site of ADAR2

The crystal structure of the catalytic domain of human ADAR2 revealed an unusual feature. A zinc ion was found in the active site, and one molecule of inositol hexakisphosphate (IP6) was buried within the enzyme core; both characteristics are thought to contribute to protein folding. The crystal structure suggests that IP6 is required for proper ADAR2 activity because it binds and fills an extremely basic hole located in a deep pocket under the protein surface, which stabilizes the folding of ADAR2 [116]. IP6 is one of the most abundant inositol phosphates within cells and is implicated in many cellular functions [117], including RNA export [118], endocytosis [119], chromatin remodeling [120], vesicle trafficking and receptor compartmentalization [121]. Interestingly, IP6 has been reported to affect glutamatergic AMPA receptors [122] whose mRNAs are well-known substrates of ADAR2 [32].

Several recent studies have emphasized the importance of IP6 in cellular ADAR activity. In one study, negative feedback on RNA editing was shown to occur through the activation of phospholipase C (PLC) in response to serotonin stimulation [70]. PLC activation leads to the cleavage of phosphatidyl inositol 4,5-bisphosphate to form the second messengers diacylglycerol and inositol 1,4,5-triphosphate, whose successive phosphorylation ultimately leads to the formation of IP6. 5HT-2c receptor mRNA is edited at five distinct sites by ADAR1, ADAR2 or both. The edited isoforms exhibit a reduction in G-protein coupling and a reduced affinity for serotonin [22, 89, 123, 124]. Therefore, the serotonin-induced production of IP6 would cause increased production of active ADAR2, which, in turn, edits 5HT-2cR RNA to attenuate serotonin signaling [70]. An increase in IP6 levels may be relevant only for newly synthesized ADAR protein because it cannot move inside and outside of the protein [70]. Further investigation is needed to directly link PLC activation to the regulation of ADAR2 activity. However, if this were demonstrated, it would imply that cellular levels of IP6 are a rate-limiting step in the expression of properly folded and catalytically active ADAR2 [70].

Proteolytic Cleavage of ADAR2

One very recent study has revealed a new mechanism of ADAR2 regulation [125]. Excitotoxic levels of glutamate have been shown to induce the specific proteolytic cleavage of ADAR2. This cleavage is triggered via the activation of the NMDA receptor and through a cascade of events that lead to the activation of calpain protease. It has been demonstrated that cleaved ADAR2 leads to a decrease in GluA2 Q/R editing, which results in high Ca^{2+} influx and excitotoxic neuronal death. Interestingly, the site of ADAR2 cleavage is between the two dsRBMs, thereby leaving the catalytic domain intact; however, the inability to bind the RNA target with both of the dsRBMs prevents the RNA editing activity of ADAR2 [125].

Phosphorylation

Protein phosphorylation is one of the most well-studied post-translational modifications that is used by the cell to dynamically modulate the activity of a single enzyme [126]. However, a detailed characterization of the ADAR phosphorylation pattern and its influence on RNA editing activity has not yet been accomplished.

By using bioinformatic tools such as PhosphoSitePlus® [127] and Phospho.ELM [128], we have explored the phosphorylation pattern of human ADAR1 and ADAR2 obtained by high-throughput phosphoproteomic analyses [129–141]. Data related to human ADAR1 have revealed a clear pattern of ten phosphorylation sites (P-sites) that are concentrated in two specific regions (Table 1). These two regions encompass amino acids 599–614 (6 P-sites) and amino acids 808–825 (4 P-sites). It is conceivable that these two regions represent a crucial regulatory domain of the enzyme. The first region is positioned between the first and second dsRBMs, while the second region is located between the third dsRBM and the catalytic ADAR1 domain (Fig. 3). Two additional phosphorylation sites have been found near the first region at Thr567 and Ser 629; the latter was indentified during the unique mouse phosphoproteomic analysis [135], and the sequence in this region has 100% homology with the human protein.

Another bioinformatic tool that is used to explore phosphorylation patterns is the phosphorylation site database (PHOSIDA) [142, 143] that has been constructed from high-resolution mass spectrometry data [133, 137, 138, 144]. Phosphorylation site analysis of human ADAR1 with PHOSIDA distinguishes between splicing variants and predicts which kinase might be responsible for each single phosphorylation event; data about the accessibility of the phosphorylation sites are also given. After aligning the sequences of the five ADAR1 isoforms that are reported by

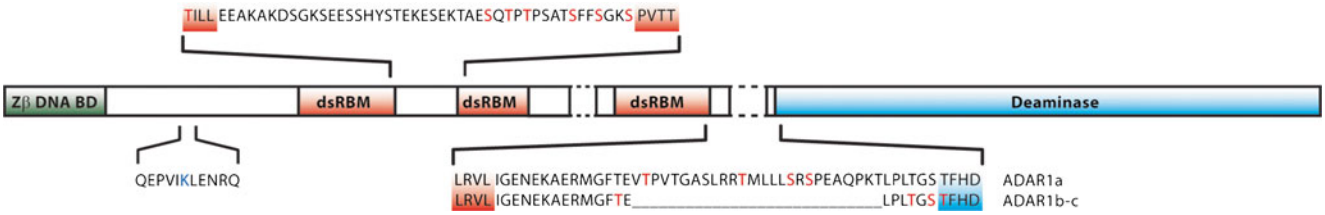

Fig. 3 ADAR1 post-translational modifications. To-scale representation of constitutive ADAR1 (ADAR1S) functional domains with spliced regions and post-translational modifications. *Green* Zβ DNA binding domain, *red* double-stranded RNA binding motifs, *blue* deaminase catalytic domain; *dashed lines* represent alternative spliced exons. Amino acid sequences highlight the SUMO-1 conjugation site (K418) (*blue character*) and the phosphorylation sites obtained from PhosphoSitePlus® (*red characters*) of specific ADAR1 splicing variants. The second phosphorylated sequence coincides with a spliced region that originates ADAR1 variants b and c bearing four new phosphorylation sites (PHOSIDA)

PHOSIDA, we found that the second region containing four phosphorylated amino acids (aa 808–825) is deleted in ADAR1 isoforms ADAR1b and ADAR1c because of the alternative splicing of exon 7. This process gives rise to the

Table 1 Phosphorylation sites and kinase recognition matching motifs

	Amino acid sequence	Kinase recognition matching motifs	References
hADAR1			
T567	DAAMKAM**T**ILLEEAK	FHA2 Rad53p	[138]
S599	ESEKTAE**S**QtPtPSA	DNA damage response kinase (S/T-Q)	[141]
T601	EKTAEsQTPtPSATs	No matching	[130, 132, 134, 136, 138–141]
T603	TAEsQtP**T**PSATsFF	No matching	[136]
S608	tPtPSAT**S**FFsGKsP	CK1 (S-X-X-S/T)	[134, 136]
S611	PSATsFF**S**GKsPVTT	CK1 (S-X-X-S/T; S/T-X-X-X-S)	[132, 134]
S614	TsFFsGK**S**PVTTLLE	CK1 (S-X-X-S/T)	[132, 134, 138]
S629[a]	CMHKLGN**S**CEFRLLS	NEK6 (L-X-X-S/T)	[135]
T786[b]	KAERMGF**T**ELPLtGs	No matching	[138]
T791[b]	GFtELPL**T**GstFHDQ	NEK6 (L-X-X-S/T)	[138]
T793[b]	tELPLtG**S**tFHDQIA	NEK6 (L-X-X-S/T)	[138]
T794[b]	ELPLtGs**T**FHDQIAM	No matching	[138]
T808	RMGFTEV**T**PVTGASL	No matching	[130, 131, 138, 141]
T818	TGASLRR**T**MLLLsRs	NEK6 (L-X-X-S/T); PKA (R-X-S/T); NIMA (F/L/M-R/K-R/K-S/T)	[134, 137, 138]
S823	RRtMLLL**S**RSPEAQP	NEK6 (L-X-X-S/T)	[134, 138]
S825	TMLLLSR**S**PEAQPKT	NEK6 (L-X-X-S/T)	[132–134, 138]
hADAR2			
S26	NRNLDNV**S**PKDGSTP	CDK1 (S/T-P-K/R)	[129, 134, 138]
S31	NVsPKDG**S**TPGPGEG	No matching	[147]
S59	KRPLEEG**S**NGHSKyR	GSK3 (S-X-X-X-S)	[138]
Y65	GsNGHSK**Y**RLKKRRK	No matching	[138]
T304	FNLHLDQ**T**PSRQPIP	NEK6 (L-X-X-S/T); CDK1 (S/T-P-X-K/R); CDK2 (S/T-P-X-K/R)	[138]
T740	EQDQFSL**T**P_____	No matching	[136]
hADAR3			
S63	TEDDDTL**S**TSsAEVK	No matching	[138]
S66	DDTLsTS**S**AEVKENR	CK1 (S-X-X-S/T)	[138]

[a] Phosphosite identified in mouse, Ser582 (100% sequence homology with human)

[b] Phosphosites identified in ADAR1 isoforms b and c (see Fig. 3)

formation of four new phosphorylation sites that replace the skipped ones (Fig. 3) and is likely related to the exposure of a new region that is accessible to kinase activity. This observation strengthens the idea that these phosphorylation sites could be crucial for proper ADAR1 enzymatic activity. The PHOSIDA database also identifies an additional phosphorylation site at Ser112 that should be present only in the interferon-inducible full-length ADAR1 isoform. The main kinase involved in the phosphorylation of ADAR1 is likely never in mitosis gene A (NIMA)-related kinase 6 (NEK6), which is a serine/threonine protein kinase that is implicated in cell cycle regulation [145, 146].

When human ADAR2 was analyzed with PhosphoSitePlus® [129, 134, 136], only two phosphorylation sites, Ser26 and Thr740, were found; Ser26 is located in the N-terminal, while Thr740 is located in the C-terminal domain, and both are not present in the ADAR2 isoforms ADAR2c and ADAR2d (Table 1). PHOSIDA predicts that Ser26 may be phosphorylated by cyclin-dependent kinase 1 (CDK1), which is a key player in cell cycle regulation, and reports three additional phosphorylation sites, namely, Ser59, Tyr65 and Thr304. Again, the functional significance of phosphorylation at these sites remains to be elucidated.

A recent paper revealed for the first time the effect of ADAR2 phosphorylation at Ser26 and at the novel phosphorylation site Ser31 [147]. Peptidyl-prolyl isomerase NIMA interacting protein 1 (Pin1) interacts with ADAR2 when the deaminase is phosphorylated at Ser26 or Ser31. Pin1 acts as a positive regulator of RNA editing efficiency influencing nuclear localization and stability of ADAR2. In the absence of interaction between Pin1 and ADAR2 in the nucleus, ADAR2 mislocalizes to the cytoplasm, where it interacts with NEDD4-like E3 ubiquitin ligase (WWP2). WWP2 plays a negative role by binding ADAR2 at conserved motifs located both at the N-terminal and C-terminal and catalyzing its poly-ubiquitination and subsequent degradation.

Finally, the ADAR3 phosphorylation pattern has been derived only by the PHOSIDA database and includes two serine residues at positions 63 and 66. The latter is predicted to be phosphorylated by casein kinase 1 (CK1) (Table 1).

In summary, phosphorylation may play a central role in the regulation of ADAR enzymatic activity, and the localization of phosphorylation sites near the dsRBMs may suggest that these sites might also regulate substrate selectivity. The data summarized here should encourage a systematic study of ADAR phosphorylation sites and their role in the enzymatic activity of ADARs.

Inhibitors and Trans-acting Factors

The access of ADAR enzymes to RNA editing sites may be negatively controlled by competitive inhibitors. This is the case for the brain-specific C/D small nucleolar RNA MBII-52, which contains a sequence that is complementary to the 5HT-2cR mRNA. As a result, it induces a snoRNA-directed 2′-O-methylation at the C editing site, which seems to be physiologically involved in the regulation of editing at this site [148, 149]. The snoRNA MBII-52 specifically inhibits nucleolar editing [102], and modulations of its expression may shift the balance of RNA editing by trafficking or sequestering transcripts to specific compartments [150].

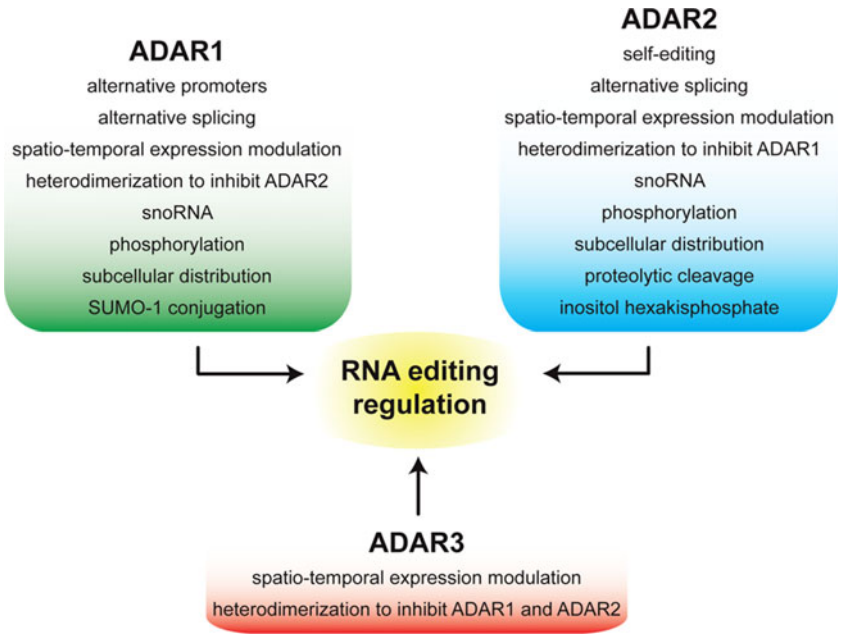

Fig. 4 Mechanisms that can modulate ADAR activity and RNA editing

Moreover, virus-associated RNA I (VAI), a highly structured RNA inhibitor from adenoviruses, may act as an inhibitor and demonstrates a high affinity for the dsRBMs of ADARs [151]. Finally, it has been suggested that ADAR2 exists in a self-inhibited conformation until it binds to a dsRNA that is capable of covering both of its dsRBMs [152], but a detailed analysis of this process has not yet been undertaken.

Conclusion

RNA editing is a process that can alter a critical amino acid residue in proteins and therefore must be tightly regulated. Previous studies have provided data about changes in the editing level of ADAR substrates, but the mechanism that leads to these changes remains unclear. In this review, we have summarized the current state of knowledge about the regulation of ADAR activity and have identified areas where further investigation is needed. Because of the wide variety and physiological significance of ADAR substrates, especially in the brain, a deeper understanding of these mechanisms would further our insight into the molecular basis of neurotransmission.

It is clear that the extent of RNA editing does not always correlate with the expression levels of ADAR1 and ADAR2, which suggest the presence of post-transcriptional or post-translational regulatory mechanisms [31, 54, 56]. Moreover, the subcellular distribution of ADARs and their interaction with inhibitors and activator molecules have also been shown to influence ADAR activity (Fig. 4). Nevertheless, the mechanisms by which ADAR activity is regulated are still largely unclear. It has been demonstrated that phosphorylation events have a role in the regulation of ADAR enzymatic activity modulating their interaction with different intracellular partners, thus determining subcellular localization and stability of ADAR enzymes [147]. Because phosphorylation sites have been found next to dsRBMs, it is possible that phosphorylation might also intervene in the process of ADAR-substrate recognition. Furthermore, an analysis of protein kinases that are responsible for the phosphorylation of ADARs could provide new information about unknown links that correlate ADAR function with a diverse set of cellular pathways.

Another issue to consider is the role of ADAR3 in RNA editing because it is an RNA deaminase with unique features that warrant further investigation. In contrast to the ubiquitous expression of ADAR1 and ADAR2 [28–30], ADAR3 is brain specific [33, 34]. This specific localization suggests two hypotheses: (1) ADAR3 could interact with ADAR1 or ADAR2 to become a negative regulator of RNA editing (these inhibitory properties have already been observed in vitro) [33], and the heterodimerization could prevent the formation of a complete and active catalytic site, or 2) ADAR3 could edit RNA substrates that are different from those edited by ADAR1 and ADAR2. This second hypothesis is supported by the unique properties of ADAR3, such as its capability to bind both ssRNA and dsRNA; nevertheless, ADAR3 might lack enzymatic activity altogether since zinc coordinating amino acids in the catalytic domain are not entirely conserved. All of these events should be further investigated because they might be key points in understanding the regulation of ADAR enzymes.

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Conflict of interest The authors declare that they have no conflict of interest.

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