Mini Review: Novel roles for RNA editing in the course of RNA metabolism

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ABSTRACT

Accurate transcription of genetic information into RNA molecules is vital for proper cellular function. Sequences of RNA serve as templates for the ribosomal translation of proteomes, which execute basic biological tasks. Following RNA synthesis, mature RNA molecules are generated through various RNA processing events. A critical component of the collection of processes involving RNA species, broadly defined as RNA metabolism, is the RNA editing pathway. Acting specifically on double-stranded RNA substrates, RNA editing has the capacity to regulate a plethora of transcriptional outputs, including genomic recoding, RNA splicing, biogenesis and targeting actions of micro RNAs and small interfering RNAs, and global gene expression. Recent evidence suggests that RNA modifications mediated via RNA editing influence the biogenesis of circular RNAs and safeguard from aberrant innate immune responses induced by foreign RNA sources. These novel roles have the potential to contribute new insights into molecular mechanisms underlying RNA editing-mediated pathogenesis. Here, we provide a mini review on the recent advances in the field, which highlight novel roles associated with the RNA editing process and emphasize their importance during cellular RNA metabolism. In addition, we evaluate the relevance of these newly discovered roles in the context of neurological disorders and autoimmune diseases.

KEYWORDS: RNA editing, RNA metabolism, RNA splicing, RNA silencing, circular RNAs, immune responses, neurological disorders, autoimmune disease

INTRODUCTION

The most prevalent mode of RNA editing in metazoans is the adenosine to inosine (A-to-I), mediated by a highly conserved protein family known as adenosine deaminases acting on RNA (ADAR) (Savva et al., 2012b). Exclusively acting on double-stranded RNAs (dsRNAs), ADAR enzymes have the capacity to modify specific adenosines to inosines in short and imperfect dsRNA substrates (Nishikura, 2010). Conversely, in long and perfectly base-paired dsRNA molecules, ADARs exhibit a promiscuous catalytic activity that modifies several adenosines, a phenomenon referred to as hyper-editing (Bass, 2002). Inosine nucleosides in RNA are interpreted as guanosines by the cellular machineries involved in RNA metabolism, including the spliceosome and ribosome (Basilio et al., 1962). Thus, ADAR-mediated modifications in RNA molecules inherently fine-tune RNA metabolism at multiple levels, generating a vast variety of transcriptional outputs which spawn eukaryotic complexity. Characterization of RNA editing landscapes from a broad range of phyla, using next generation sequencing (NGS) technologies, suggests that ADAR modifications are more widespread than previously thought and are distributed throughout genomes. Despite variations between RNA editing landscapes across model organisms, editing sites are observed in both coding and noncoding regions of the genome, with the latter being the most prevalent (St Laurent et al., 2013; Zhao et al., 2015). Similarly, RNA editing sites in humans are over-represented in noncoding *Alu* elements (Ramaswami et al., 2012;Bazak et al., 2014), the most abundant transposable element existing in the genome, corresponding to approximately 10% of its content (Lander et al., 2001).

RNA editing enzymes are enriched in the nuclear compartment and expressed predominantly within the nervous system (Savva et al., 2012b). This specific localization pattern suggests a pivotal role of these enzymes for proper nervous system function. Indeed, the primary function of ADARs is to provide nervous system integrity as exemplified by RNA editing deficiencies in various genetic models. Specifically, invertebrates lacking all ADAR activity exhibit severe neurological defects and behavioral abnormalities. For example, loss of function of the single *Adar* in *Drosophila* leads to frequent seizures, acute uncoordination, and age-dependent neurodegeneration (Palladino et al., 2000). Furthermore, *Caenorhabditis elegans* (*C. elegans*) lacking RNA editing activity through the deletion of both encoded *adar* genes exhibit defects in chemotaxis (Tonkin et al., 2002). Vertebrate genomes encode three RNA editing enzymes (ADAR1-3). While ADAR1 and ADAR2 are catalytically active, ADAR3 lacks any known editing activity. In contrast to ADAR deficiencies in invertebrates, mice lacking either ADAR1 or ADAR2 editing enzymes result in lethal phenotypes. Deletion of ADAR1 leads to embryonic lethality accompanied by elevated cellular apoptosis (Wang et al., 2004), while mice lacking ADAR2 exhibit severe seizure episodes that eventually cause lethality (Higuchi et al., 2000). Two observations suggest that RNA editing systems are functionally pleiotropic in regulating distinct pathways in RNA metabolism that are physiologically critical. First, the chemotaxis defect exhibited by *adar* null *C. elegans* is rescued in double mutants that lack any RNA editing activity as well as the ability to elicit a potent RNA intereference (RNAi) response (Tonkin and Bass, 2003). This observation suggests that without RNA editing improper dsRNAs enter the RNAi pathway and trigger spurious silencing responses. Second, the lethality

observed in ADAR2 null mice can be rescued by the edited version of the glutamate receptor GluR2 (Higuchi et al., 2000). Therefore, depending upon genetic context, some fates of edited RNA molecules are more physiologically relevant compared to others that operate within the same transcriptome.

GENERAL FATES OF EDITED RNAs

Genomic Recoding

Since ribosomes interpret inosines as guanosines, the capacity to generate protein products that are not literally encoded by genomes is enabled by RNA editing in exonic regions. Also known as genomic recoding, this phenomenon extends the genetic information potential through diversification of the protein repertoire, analogous to alternative splicing (Nilsen and Graveley, 2010). In the nucleus and during the synthesis of nascent transcripts, ADAR enzymes bind to dsRNA structures generally formed by highly conserved intronic sequences, which are complementary with the exon to be edited. Occurring co-transcriptionally (Rodriguez et al., 2012), the short and imperfectly base-paired nature of the dsRNA substrates allows ADARs to edit specific adenosines within exonic sequences (Bass, 2002). Edited RNA templates are subsequently exported out to the cytoplasm to be translated by the ribosomal machineries. For instance, a specific RNA editing event within a glutamic acid codon (CAG(Q) $\rightarrow$ C**I**G) is interpreted by the ribosome as CGG(R) (**Figure 1A**), and results in the insertion of an arginine in the polypeptide chain rather than glutamic acid. Genomic recoding is extensively utilized in *Drosophila* as a means of neuronal proteome diversification (St Laurent et al., 2013), while in vertebrates this kind of RNA editing is reported to be limited (Lagarrigue et al., 2013). Intriguingly, proteins involved in neurotransmission are the major targets of specific editing (Hoopengardner et al., 2003). However, recent studies suggest that RNA editing can additionally target transcripts encoding proteins involved in a variety of cellular functions including transcription, RNA splicing, protein metabolism, and DNA replication (Graveley et al., 2011;St Laurent et al., 2013). Generally, genomic recoding events can influence protein function, and in some cases this fine-tuning effect can have broad cellular consequences. For example, a specific RNA editing event that leads to a non-synonymous amino acid substitution regulates the rate of inactivation in human potassium (K^+) channels (Bhalla et al., 2004), while a single RNA editing site within the *Adar* transcript in *Drosophila* reshapes the global landscape of editing events in a manner that impacts complex adult behaviors (Savva et al., 2012a).

RNA splicing

Since RNA editing occurs cotranscriptionally, specific ADAR modifications can influence downstream RNA processing events. Given that the majority of editing sites are found within intronic sequences, editing has the capacity to influence RNA splicing. More specifically, due to the canonical nature of 5' splice donor sites (GU) and 3' splice acceptor sites (AG), specific RNA editing in introns can generate novel splicing signals (AA-to-A**I** $\rightarrow$ AG, AU-to-**I**U $\rightarrow$ GU) (Nishikura, 2010). For example, RNA editing in an intronic *Alu* element within the human nuclear prelamin A recognition factor (NARF) generates a novel 3' splicing acceptor site regulating its exonization in a tissue specific pattern (Lev-Maor et al., 2007). Similarly, the mammalian ADAR2 editing enzyme

generates a new acceptor site in its own transcript that results in a 47 nucleotide inclusion (**Figure 1B**) and subsequent generation of a hypomorphic allele (Rueter et al., 1999), which leads to reduction in RNA editing levels at multiple adenosine targets (Feng et al., 2006). Correspondingly, RNA editing in a 3' acceptor splice site (AG-to-**I**G $\rightarrow$ GG) can prevent its recognition by the spliceosome machinery. The observation that ADAR1 knockdown leads to aberrant exonization of an *Alu* element in the seryl-tRNA synthetase transcript, suggests that RNA editing destroys acceptor sites to ensure proper splicing (Sakurai et al., 2010).

RNA silencing

Cellular defense against endogenous and exogenous parasitic nucleic acids (such as transposons and viruses respectively) is achieved by dsRNA molecules, which trigger a highly conserved biological response known as RNAi (Hannon, 2002). In addition to its safeguarding roles, RNAi also regulates gene expression through target transcript cleavage or by translation repression. Central to RNAi-mediated gene regulation are two kinds of small RNA species, small interfering RNAs (siRNAs) and micro RNAs (miRNAs) (Ghildiyal and Zamore, 2009). These small RNA species are generated via Dicer processing of dsRNA triggers (Bernstein et al., 2001), whose formation is mediated by endogenous genomic sequences. For the biogenesis of siRNAs, Dicer enzymes process long and perfectly base-paired dsRNA molecules usually formed by transposable element sequences (Kawamura et al., 2008). Such dsRNA sources can also serve as ADAR substrates. Typically, RNA editing enzymes are capable of hyper-editing dsRNA molecules and destroying their near perfect duplex nature, leading to inefficient Dicer processing (**Figure 1C**) (Nishikura, 2006). Thus, hyper-editing has the capacity to antagonize cellular RNAi responses through inhibition of siRNA biogenesis (Scadden and Smith, 2001). Intriguingly, ADAR activity can regulate gene expression established through heterochromatin formation by associating with a dsRNA source mediated by transposable elements (Savva et al., 2013). Contrary to the siRNA pathway, the dsRNA triggers for miRNA biogenesis are shorter and usually contain bulges and loops, which result in specific editing of select adenosines (Luciano et al., 2004;Blow et al., 2006). Moreover, specific editing regulates the miRNA pathway at numerous levels (**Figure 1D**). First, as miRNA precursors are edited, pre-miRNA cleavage, in addition to other processing steps of the pathway, is inhibited (Yang et al., 2006). Second, the targeting step, which requires complementarity between the miRNA and its target sequence, may potentially antagonize target recognition due to specific editing within the "seed" region of the small RNA species. Indeed, a single RNA editing event is sufficient to redirect a miRNA to a new complementary target (Kawahara et al., 2007). Finally, the observation that ADAR modifications are abundant at miRNA target sites suggests that RNA editing can regulate gene expression by destruction of miRNA/target complementarity (Gu et al., 2012).

Nuclear retention and degradation

Originally thought of as noise generated by the spurious actions of the transcriptional machinery, antisense transcription occupies a central role in the regulation of gene expression amongst all kingdoms of life (Pelechano and Steinmetz, 2013).

Antisense transcription has the potential to generate a plethora of regulatory dsRNA molecules within the nucleus. However, cells use multiple strategies to control the amount of dsRNA since accumulation can lead to aberrant RNA metabolism and cause human pathology (Kaneko et al., 2011). A cellular strategy that controls the RNA metabolism of dsRNA molecules within cells is through RNA editing (Kumar and Carmichael, 1998). Hyper-edited dsRNAs have at least two fates. First, extensive ADAR modification leads to nuclear retention (Kumar and Carmichael, 1997) through a nuclear matrix protein complex (Zhang and Carmichael, 2001). Second, extensive ADAR modifications are specifically recognized by Tudor SN, a component of the RNA induced silencing complex (RISC), whose actions promote the cleavage and degradation of dsRNAs (Scadden, 2005). These general fates of edited RNAs suggest that ADAR enzymes participate in the optimization of RNA metabolism. Recent results from the field uncover two additional roles for ADAR enzymes during the metabolism of circular RNAs and RNA molecules that trigger innate immune responses, highlighting a more fundamental role for this process within the course of RNA metabolism.

RNA EDITING AND CIRCULAR RNAs

Transcriptional profiling in metazoans revealed mysterious new RNA species that maintain a circular shape (Memczak et al., 2013;Jeck and Sharpless, 2014;Lasda and Parker, 2014). Termed circular RNAs (circRNAs), these RNA species are generated through a non-linear splicing processing mechanism in which a canonical 3’ acceptor site of an exon is connected to a 5’ donor splice site of an upstream exon (Ashwal-Fluss et al., 2014;Starke et al., 2015). While in humans circRNAs can be detected in diverse cell types (Salzman et al., 2012), recent evidence suggests that they are highly enriched in the nervous system, specifically at synapses (Westholm et al., 2014;Rybak-Wolf et al., 2015). A hallmark for circRNA biogenesis in mammals is the presence of reverse complementary intronic sequences that flank the exon destined for circularization. These sequences can base pair extensively forming dsRNA molecules required for the biogenesis of circRNAs. Strikingly, *Alu* repetitive sequences are highly associated with exon circularization (Jeck et al., 2013;Zhang et al., 2014). Since *Alu* element sequences are major targets for ADAR-mediated modifications, RNA editing can potentially regulate the metabolism of circRNAs. Indeed, ADAR knockdown leads to accumulation of circRNAs in human cells (Ivanov et al., 2015). Similarly, several mouse circRNAs are upregulated upon decreasing ADAR expression (Rybak-Wolf et al., 2015). This antagonistic effect between RNA editing enzymes and circRNA biogenesis is a conserved feature in invertebrates. First, intronic sequences flanking circRNAs are enriched for hyper-editing events in *C. elegans* (Ivanov et al., 2015). Second, *Drosophila* raised at 29°C exhibit elevated levels of circRNAs when compared to flies maintained at 18°C (Rybak-Wolf et al., 2015). The accumulation of circRNAs at higher temperatures is thought to occur because of a decrease in ADAR expression at these temperatures, as a recent study suggests that external alterations in temperature dynamically regulate the RNA editing process (Rieder et al., 2015). Clearly, circRNA biogenesis is highly regulated through ADAR activity, a phenomenon that is conserved amongst animals. While the biological roles of circRNAs are currently enigmatic, evidence suggest that

circRNAs can act as sponges for the assembly of miRNAs (Hansen et al., 2013;Westholm et al., 2014). Nevertheless, whatever the functions of these circRNAs might be, RNA editing has the capacity to fine-tune downstream biological phenomena by antagonizing the biogenesis of circRNAs (**Figure 2A**).

RNA EDITING AND INNATE IMMUNE RESPONSES TO dsRNAs

Cellular infection by viruses activates a protective mechanism that involves an inflammatory response (Medzhitov, 2008). The physiological role of this inflamatory response is to provide necessary stimuli required by the host to establish a potent defense mechanism. Referred to as the innate immune response, it is triggered by the recognition of foreign dsRNAs generated during the initial cycles of viral replication (Akira et al., 2006). In vertebrates, the retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation-associated gene 5 (MDA5) operate as sensors for the recognition of foreign dsRNA molecules (Takeuchi and Akira, 2010). In the cytoplasm, RIG-I recognizes dsRNAs that are up to 1kb in legth, while MAD5 senses longer RNA duplexes. Despite this discriminatory recognition, both sensors trigger the actions of a mitochondrial antiviral-signaling protein, MAVS, which signals for the activation of a cascade of events. This cascade involves several factors that orchestrate the expression of cytokines and type I interferons (IFNs) genes required for multiple defense responses (Takeuchi and Akira, 2010). It has been recently realized that genomes are transcribed to their entireties through pervasive transcription (Carninci et al., 2005;Djebali et al., 2012), generating a myriad of RNA species that participate in diverse cellular functions. Moreover, the identification of endogenous RNA-based silencing pathways suggests that a variety of genomic sources generate dsRNA molecules (Nilsen, 2008). Due to their nature, endogenously generated dsRNA duplexes are highly structurally similar to those generated through viral replication after infection. This raises the question of how cells are able to discriminate endogenous dsRNA molecules from exogenous and therefore avoid aberrant immune responces. Previous observations suggest that endogenous RNAs marked with specific nucleoside modifications avoid detection by sensor proteins of the immune response (Kariko et al., 2005). Intriguingly, synthetic dsRNAs containing multiple IU pairs, which mimic the hyper-editing activity of RNA editing enzymes, fail to induce innate immune responses (Vitali and Scadden, 2010). Thus, its was proposed that the presence of IU pairs in dsRNA duplexes interfere with the detection process mediated by the RIG-I and MAD5 sensors, suggesting that RNA editing in princible could regulate innate immune responses. Indeed, two recent studies have uncovered that RNA editing regulates the cascade of events that lead to the activation of innate immune responses. Moreover, this pathway acts upstream to hyper-edit naturally-occuring (self) dsRNA duplexes in order to avoid detection as foreign nucleic acid (nonself) that otherwise would elicit abberant immune responses.

It was reasoned that the elevated cellular apoptosis exhibited by ADAR1 null mice is due to abnormal immune responses triggered by non-edited, endogenous dsRNA sources. In agreement with this notion, Mannion *et al*. examined the transcriptional profiling in ADAR1 null embryos and observed that transcripts of interferon-stimulated genes (ISGs) were elevated significantly (Mannion et al., 2014). Moreover, the embryonic lethal phenotype was rescued, at birth at least, in $Adar1^{(-/-)}/Mavs^{(-/-)}$ double

mutant mice. Finally, transfection of dsRNA species containing multiple IU pairs was unable to elicit an immune response in fibroblast cells of ADAR1 deficient mice, which indicates that editing activity is necessary for blocking the response. In a similar study, Liddicoat *et al*. generated an ADAR1 editing activity-deficient mouse allele (*Adar1*$^{E861A/E861A}$). Through transcriptional profiling of homozygous mutant mice, they observed an atypical upregulation of ISGs, highly similar to the one observed in the ADAR1 null mice (Liddicoat et al., 2015). Furthermore, through analysis of the ADAR1 editing landscape they reavealed that the major substrates of this RNA editing ezyme are dsRNA duplexes, formed by 3' UTR sequences. They reasoned that the absence of IU pairs from these dsRNAs can elicit aberrant immune responces through MDA5 sensing. Indeed, generating *Adar1*$^{E861A/E861A}$/MDA5$^{(-/-)}$ double mutant mice rescued the embryonic lethality phenotype suggesting that MDA5 is the principal sensor of non-edited endogenous dsRNAs. Clearly, these observations suggest a pivotal role for RNA editing in the regulation of innate immune responses towards dsRNA molecules. More importantly, ADAR1 acts hierarchically upstream in the immune cascade and through RNA editing it fingerprints dsRNAs as endogenous, thus preventing undesired cellular immune responses (**Figure 2B**).

CONCLUSION

A universal trait of transcriptomes is the generation of dsRNA entities that orchestrate diverse cellular functions. Despite variation in length, most endogenous dsRNA molecules intersect with the A-to-I RNA editing system, since no sequence specificity is required for ADAR binding and editing (Nishikura et al., 1991). Thus, RNA editing enzymes have the inherent capacity to regulate many dinstinct pathways that are involved in RNA metabolism and serve to fine-tune and optimize transcriptional outputs (Bahn et al., 2015). Although, anomalies in RNA editing are associated with multiple neurological disorders such as epilepsy, schizophrenia, and amyotrophic lateral sclerosis (ALS) (Slotkin and Nishikura, 2013), the molecular determinants of ADAR-mediated pathogenesis still remain elusive. In principle, mutations that affect editing activity (Crow et al., 2015) or lead to abnormal ADAR sequestration (Donnelly et al., 2013) can both lead to disease phenotypes due to detrimental impacts imposed on the known general fates of edited RNAs. In addition, as these recent studies suggest, a severe reduction in RNA editing may lead to the abnormal accumulation of circular RNAs and elicit aberrant innate immune responses. Future research should aim to understand how these novel RNA editing roles may contribute to the etiology of neurological disorders.

Previously, multiple missense mutations in the *Adar1* gene were identified in individuals diagnosed with Aicardi-Goutières syndrome (AGS), an autoimmune disorder (Rice et al., 2012). In addition, individuals with AGS-associated *Adar1* mutations exhibited high levels of expression for a number of interferon-stimulated genes, a phenotype observed previously in ADAR1 deficient mice (Hartner et al., 2009). Due to these observations, it was proposed that ADAR1 theoretically could suppress immunoreactive dsRNA molecules through hyper-editing and control aberrant immune reponses. Certainly, the new role for the A-to-I RNA editing system provided by Mannion *et al*. and Liddicoat *et al*. contributed significantly to a better understanding of how *Adar1* mutations can result in autoimmune diseases. As the search continues for

currently unidentified RNA editing roles, it is becoming increasingly clear that ADAR-mediated modifications occupy a central role in cellular physiology. Therefore, a more comprehensive understanding on how RNA editing systems regulate global cellular RNA metabolism can shed light in the development of therapeutic strategies of ADAR-mediated human diseases by providing insights into their pathogenesis.

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Figure 1. General fates of edited RNAs. (A) Specific RNA editing in coding region of a pre-mRNA. ADAR-mediated hydrolytic deamination of the adenine base within the glutamic acid codon (Q) is interpreted by the ribosomal machinery as an arginine codon (R), leading to an amino acid substitution. **(B)** Specific RNA editing in non-coding regions can generate novel 3' splice acceptor sites (AA $\rightarrow$ AG). In mammals, the ADAR2 RNA editing enzyme modifies its own transcript to generate a novel splicing signal that results in the inclusion of 47 nucleotides in the mature RNA. **(C)** Hyper-editing of dsRNA molecules leads to inefficient Dicer processing. Hyper-editing antagonizes RNA-mediated silencing responses through the generation of fewer siRNAs. **(D)** Specific RNA editing near the base of miRNA precursors leads in the inhibition of Drosha cleavage, which prevents the processing of mature miRNAs. In addition, specific RNA editing within the "seed" region of the mature miRNA may result in redirection to a new target.

Figure 2. Novel Roles for RNA editing. (A) The biogenesis of circular RNAs involves the formation of dsRNA duplexes mediated by intronic sequences flanking the exon(s) destined for circularization. These dsRNA molecules can serve as ADAR substrates. Hyper-editing destroys the dsRNA nature of such substrates, which promotes linear splicing. **(B)** In the cytoplasm, RIG-I and MDA5 act as sensors for dsRNA molecules. Upon recognition, these sensors activate MAVS, which triggers the innate immune response. MAVS-mediated signals result in the translocation of interferon-regulatory factors (IRFs) and NF-κB into the nucleus, which initiates the transcription of ISG and cytokine genes. Hyper-editing of immunoreactive dsRNAs prevents detection by RIG-I and MDA5, inhibiting aberrant innate immune responses towards endogenous dsRNA sources.

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