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REVIEW

RNA Editing Adds Flavor to Complexity

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Abstract—A-to-I RNA editing results in the conversion of single adenosines into inosines, which alters coding and non-coding sequences in RNA molecules, increasing the complexity of the transcriptome. This modification is vital in a number of brain-specific coding transcripts, where the introduced alternative amino acids impact protein function substantially. Indeed, deviations from normal editing levels have been detected in tissues from individuals affected by neurological diseases and cancer, underscoring the importance of correct and regulated editing. Since the discovery of A-to-I RNA editing, considerable effort has been made to uncover additional editing targets and analyze the subsequent functional consequences for the recoded substrates. The effects of editing on non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) or long ncRNAs are less well explored. ncRNAs act as regulators of gene expression through chromatin modification, imprinting, alternative splicing, and mRNA translation and stability. Editing has the potential to dynamically alter and diversify ncRNAs, thereby redirecting their functions. How editing intersects, interferes with, and modulates the roles of ncRNAs, possibly in response to external stimuli, therefore warrants a deeper look. This review discusses recent advances and new insights in the field.

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Key words: RNA editing, ADAR, inosine, deamination, ncRNAs, transcriptome complexity

UNEXPLORED RNA WORLD

RNA may well be the most underrated biological molecule of the 20th century. Apart from being extensively studied for its central roles in spliceosomes and ribosomes during transcriptional processing and translation, respectively, it has by and large led a wallflower existence in the wider research community. For a long time, the main function of RNA was thought to be serving as intermediary between DNA and protein, facilitating the portability and translation of genetic information. However, this view has been revised in recent years and the prominence of RNA, particularly within regulatory networks, has started to surface [1-3]. In fact, RNA is extraordinarily well suited to serve as regulatory molecule, since it can provide both exquisite one-dimensional sequence-specificity and versatile tertiary structures that can either be recognized by proteins or have catalytic activity themselves [1, 4]. RNA therefore resembles a converter of digital to analog signals, directly bridging two worlds, that of informational content stored in DNA and the functional output embodied by proteins [5, 6]. Classic examples of RNAs with these properties are tRNAs, whose tertiary structures interact selectively with both aminoacyl tRNA synthetases and ribosomes, while specifically base-pairing to the respective codons of an mRNA. This versatility of functional RNAs, such as ribozymes, enables them to catalyze enzymatic reactions and allow for certain long non-coding RNAs (ncRNAs) to nucleate and propagate epigenetic silencing across chromatin or parts thereof [1, 4, 7].

Recent advances show that well-studied functional RNAs like tRNAs are not, as long presumed, solitary remnants of the primordial RNA world in which self-replicating, catalytic RNA reigned. Instead, RNA has continued to play a central role throughout evolution. In fact, non-coding portions of genomes, the majority of which is pervasively transcribed from yeast to human [8-11], increase with increasing complexity of organisms, while the quantity of sequences coding for proteins remains comparatively similar [12-14]. Although alternative splicing considerably expands proteome diversity from a limited number of genes, it can only partially explain the elaborateness of metabolic, developmental, architectural, and cognitive systems in higher organisms. The true force underlying organismal complexity may be more related to the sophistication of regulatory networks

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encrypted in non-coding sequences. Therefore, it has been proposed that the complexity of higher organisms is directly related to the information stored in the non-protein coding part of a genome [15]. Contrary to the previous belief, RNA has not been replaced by the supposedly more resourceful proteins, but instead has continued to perform critical functions, assuming pertinent responsibilities we are just beginning to fathom. Even though ncRNAs are starting to receive the attention they deserve only of late, their involvement in many regulatory networks is already apparent, such as regulating the epigenetic state of a cell and controlling gene expression on transcriptional and translational levels in both *cis* and *trans* [3, 16, 17]. Consequently, ncRNAs are now subject of intense research efforts, aimed at trying to elucidate their various roles in cellular functions. A critical aspect of how a cell controls the actions of such ncRNAs is its ability to modulate and regulate them both on a transcriptional level and through posttranscriptional regulation and modification. Adenosine-to-inosine conversion in RNA molecules represents a mechanism to alter and adjust the fate of RNAs and thus may significantly impact the regulatory potential of ncRNAs.

ADARs

All types of RNA transcripts can be subject to modification by A-to-I editing, which converts adenosine (A) to inosine (I) through hydrolytic deamination in double-stranded RNA (dsRNA) (Fig. 1a). Inosine preferentially base-pairs with cytidine and is thus interpreted as guanosine in most cases. Consequently, editing provides a means by which cells can manipulate primary sequence readouts as well as higher order RNA structures. Editing is catalyzed by members of the adenosine deaminases acting on RNA (ADAR) protein family, which are found in most metazoans, but have not been detected in plants, yeast, and fungi [18]. There are four ADAR family members in mammals (ADAR1-3 and TENR = testis nuclear RNA binding protein), one in *Drosophila* (dADAR) and two in *Caenorhabditis elegans* (adr-1 and adr-2). ADAR deficiencies lead to a wide range of phenotypes, which underlines their importance for proper development and behavior in different species. The dADAR$^{-/-}$ *Drosophila* show incapacitated coordination of locomotion and abnormal behavior [19]. Homozygous deletion of ADARs in *C. elegans* results in defective chemotaxis [20]. ADAR2$^{-/-}$ mice die within three weeks *post natum*, after repeated episodes of epileptic seizures [21], and ADAR1$^{-/-}$ mice have an embryonically lethal phenotype associated with liver disintegration and defects in hematopoiesis [22-25].

All ADARs share a similar domain structure, with one to three double-stranded RNA binding motifs (DRBMs) and a C-terminal deaminase domain (Fig. 1b). However, of the four mammalian ADAR family members, only ADAR1 and ADAR2 have a catalytically active deaminase, while ADAR3 and TENR (also called ADAD1 = adenosine deaminase domain containing 1) are presumed to be deamination deficient as catalytically critical residues are not conserved and the enzymes are unable to modify any of the known substrates [26-28]. Sometimes ADAR1 and ADAR2 have overlapping target-specificity, but more often one adenosine within a given sequence is primarily edited by one or the other [29, 30]. Preliminary evidence suggested that this target-specificity can mostly be credited to the respective deaminase domains [29]. However, editing specificity is mediated to a considerable extent also by the DRBMs, as shown by hydroxyl radical footprinting [31, 32] and recently confirmed by the crystal structure of DRBMs 1 and 2 of ADAR2 bound to a pre-mRNA target [33]. There are at least two types of ADAR substrates – fully double-stranded RNAs of 50 or more base pairs that are promiscuously edited, and partially double-stranded RNAs with loops and bulges that are edited at specific adenosines only [34, 35]. In the former, nonspecific editing occurs because ADARs bind long dsRNA via their DRBMs without sequence preference. The presence of loops and bulges in the dsRNA, conversely, mediates the defined positioning of the active site on the substrate through sequence-specific interactions of the DRBMs and provides for precise targeting of one or few adenosines within the tertiary RNA structure [33, 36]. Thus, DRBMs and active sites unite to promote exquisitely controlled editing in some contexts, while allowing promiscuous editing in others.

Our understanding of how ADAR activity is regulated is still incomplete, but new findings continue to paint an increasingly complex picture. ADARs are expressed in a large number of tissues but are most abundant in the central nervous system (CNS) [37]. ADAR expression and editing activity are regulated in a tight spatio-temporal manner and generally increase during development [38-40]. On the other hand, a decrease of editing is seen in specific subsets of ADAR targets during differentiation of human embryonic stem cells into the neural lineage [41]. Several lines of evidence thus point toward complex and precise regulation.

In *Drosophila*, which expresses different isoforms through alternative splicing of one ADAR transcript, substrate dsRNA induces cooperative interaction between two dADAR monomers to promote dimerization, and heterodimerization of different dADAR isoforms affects editing efficiency [42]. In human, homodimerization of ADAR1 and ADAR2 is also required for enzymatic activity [43], yet heterodimerization between ADAR1 and ADAR2 was not detected. However, this analysis did not take into account heterodimerization between alternative splice isoforms of ADAR1 and ADAR2, respectively (Fig. 1b), creating a possible regulatory mechanism that remains to be explored.

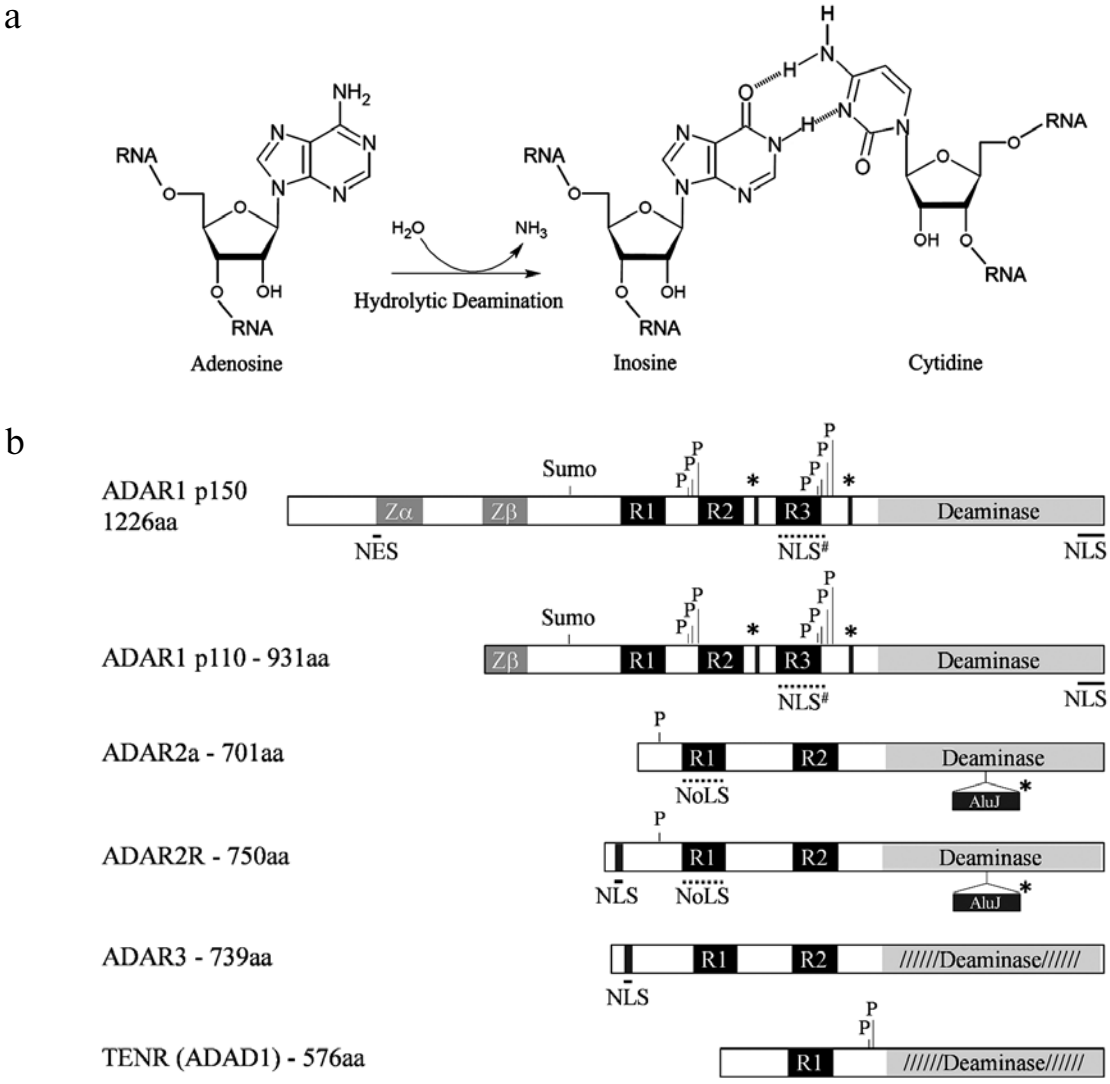

Fig. 1. Hydrolytic deamination of adenosines is catalyzed by the ADAR family of proteins. a) Hydrolytic deamination of adenosine yields inosine. Inosine forms Watson–Crick base-pairing with cytidine, analogous to that of guanosine. b) Simplified schematic representation of the four mammalian ADAR family members. Regulation of ADAR expression and activity occurs on several levels. **ADAR1.** *Alternative promoters*: ADAR1p150 is expressed from an interferon-inducible, ADAR1p110 from a constitutive promoter [45, 157]. *Alternative splicing*: leads to differential spacing between DRBMs and the deaminase domains (marked with an asterisk *), potentially altering target specificity and/or activity of homodimers. *Subcellular localization*: ADAR1 dynamically associates with the nucleolus due to the presence of a NoLS (overlapping with NLS#) [158]. N-Terminal NES renders ADAR1p150 almost exclusively cytoplasmic [47]. ADAR1p150 has both NLS and NES, facilitating nucleocytoplasmic shuttling [44, 48, 49]. Other motifs not indicated further contribute to the complex subcellular localization of ADAR1 [159]. *Posttranslational modifications*: sumoylation of K418 reduces editing activity, possibly due to stereochemical inhibition of dimerization [160]. ADAR1 has several phosphorylation sites, but their functional relevance is unknown. **ADAR2.** *Alternative promoters*: ADAR2a and ADAR2R are expressed from different promoters [161]. *Alternative splicing*: insertion of an AluJ cassette (marked with *) into the deaminase domain reduces activity approximately 2-fold [162]. *Editing*: ADAR2 self-editing creates a 3′-acceptor site, which leads to a frameshift and the creation of a premature termination codon, providing for a negative autoregulatory mechanism [155] (not shown). *Subcellular localization*: ADAR2 is primarily nuclear and also dynamically associates with the nucleolus due to the presence of a NoLS [158]. *Posttranslational modifications*: one phosphorylation site with unknown function is present. **ADAR3 and TENR.** The deaminase domains of ADAR3 and TENR are believed to be non-functional due to mutations at positions important for catalytic activity [26, 163]. Abbreviations: Zα and Zβ, Z-DNA binding domains; R1-R3, DRBMs; NES, nuclear export signal; NLS, nuclear localization signal; NoLS, nucleolar localization signal; Sumo, sumoylation site; P, phosphorylation site; AluJ, AluJ alternative cassette; NLS# overlaps with NoLS.

Transcription from alternative promoters leads to the expression of variant ADAR isoforms, as indicated in Fig. 1b. Aside from allowing regulation of protein levels in response to external stimuli, transcription from alternative promoters can result in different translation start sites and produce proteins with additional domains and/or UTRs. ADAR1, for example, exists in two isoforms. The constitutive ADAR1p110 harbors a nuclear localization signal

(NLS) within the DRBM3 and is thus mostly nuclear [44]. In contrast, ADAR1p150 is transcribed from an interferon-inducible promoter [45, 46] and not only contains an additional Z-DNA binding domain, but also a nuclear export signal (NES), which allows its nucleocytoplasmic shuttling [44, 47-49]. Of course, both alterations in functional domains and cellular localization allow ADARs to access different sets of substrates; and so it is believed that the main targets of ADAR1p150 are dsRNA from viral infections present in the cytoplasm [50]. Considering the controlled spatio-temporal regulation, tissue-specificity, and responsiveness to environmental cues, ADAR activity must undergo tight regulation. Future research will reveal how such regulation is achieved.

PRE-mRNA EDITING IN CODING SEQUENCES

Site-specific editing in coding regions was first discovered in brain-specific mRNAs, where the resulting codon changes lead to the expression of protein isoforms with altered amino acid sequences, as inosine is interpreted as guanosine by the translational machinery [51, 52]. One editing site of the glutamate receptor 2 (GluR2) changes a glutamine to an arginine codon (Q/R site), which renders the channel Ca^{2+}-impermeable [51]. This is also the only site known to date to undergo 100% editing in mammals. In fact, the lethal ADAR2$^{-/-}$ phenotype has been entirely ascribed to the lack of editing at the GluR2 Q/R site [21]. Another thoroughly studied editing target is the G-protein coupled serotonin receptor ($5HT_{2c}R$), where editing occurs at five adenosines that change the coding of three amino acids. These are located in the second intracellular loop, important for receptor activity, and the combinatorial editing of these five adenosines alters G-protein interaction, agonist affinity, and receptor trafficking [52-55]. The profound effects of editing on the functions of these two exemplary frontrunners have inspired the intensive search for additional recoding sites [56-60]. A list of targets for which an alteration in protein function has been determined is given in the table. A comprehensive list of all currently known recoding targets has recently been assembled by Pullirsch and Jantsch [61] and will not be further discussed here.

Deep sequencing of predicted target sites determined that high levels of recoding editing (leading to amino acid

List of editing events that lead to codon changes and where a functional impact has been determined

Gene name	Function	Amino acid substitution	Functional impact of editing	Reference
GluR-2	glutamate-gated ion channel subunit 2	Q606R, R763G	decreased Ca^{2+} permeability (Q606R); faster recovery from desensitization (R763G)	[51, 150]
GluR-3	glutamate-gated ion channel subunit 3	R775G	faster recovery from desensitization	[150]
GluR-4	glutamate-gated ion channel subunit 4	R765G	faster recovery from desensitization	[150]
GluR-5	glutamate-gated ion channel subunit 5	Q621R	variation in ion permeability	[51]
GluR-6	glutamate-gated ion channel subunit 6	I567V, Y571C, Q621R	Q621R: increased Ca^{2+} permeability if I/V and Y/C are edited	[51, 151]
$5HT_{2c}R$	G-protein coupled serotonin receptor	I156V/M, N158S/D/G, I160V	altered G-protein coupling, agonist affinity, receptor trafficking	[52]
KCNA1	voltage-gated potassium channel	I400V	increased recovery rate from inactivation	[152]
Gabra-3	γ-aminobutyric acid gated chloride channel subunit	I342M	altered receptor sensitivity and deactivation rate	[39, 153, 154]
ADAR2	adenosine deaminase acting on RNA	creates 3′ splice acceptor site in intron	frameshift, premature termination codon, negative feedback	[155]
NEIL1	DNA repair enzyme	K242R	changes lesion specificity	[57, 156]

changes) is relatively rare, but can occur at low levels at many sites [56, 57, 60-62]. It has been proposed that this may provide a mechanism for continuous probing of potentially advantageous editing events, without compromising the genomic information content [63]. Such a mechanism would manifest itself in the observed low levels of editing in many transcripts. The fluctuating nature of pre-mRNA secondary structure may allow such continuous probing of potentially beneficial editing sites. Accordingly, an editing event would become engraved if it conferred an adaptive advantage under a given selection pressure, and only then would be edited at relatively high levels [63].

PRE-mRNA EDITING IN NON-CODING SEQUENCES

While the number of high-level, site-selective recoding editing sites appears to be relatively low, bioinformatic screenings of human sequence databases have revealed that most (>85%) pre-mRNAs are edited at least at one position. However, in primates the bulk of editing takes place in Alu repeat elements within introns and untranslated regions (UTRs) [64-67]. Alu elements belong to the short interspersed nuclear elements (SINE) family of transposable elements and comprise about 10% of the human genome [13]. They are approximately 300 nucleotides long and are found in gene rich regions. Due to their high copy number, they are often present multiple times in one transcript, sometimes in opposite orientation to one another. Alu elements have a conserved sequence owing to their relatively recent expansion in the primate genome [68]. Therefore, transcribed oppositely oriented Alu elements form long dsRNA structures and are potent substrates for ADARs [64]. Alu elements appear to be evolutionarily important for primates, especially in conjunction with A-to-I RNA editing [69-73]. This notion has been supported by the finding that editing can lead to the exonization of an Alu element by changing an AA dinucleotide into an AG 3′ acceptor splice site [74, 75]. RNA editing of Alu repeats in introns and UTRs also appears to affect transcripts in other ways, such as retention in the nucleus [76, 77], altered stability [78, 79], and inhibition of transcription and translation [79-81], although conflicting results [82, 83] foretell multi-layered regulatory mechanisms involved in determining the fate of inosine-containing pre-mRNAs. A recent review by Hundley and Bass outlines how some seemingly contradictory findings could be reconciled [81].

Evidently, A-to-I RNA editing has been shown to effectuate a wide range of consequences for pre-mRNAs, and it is expected that additional functions remain to be discovered. Figure 2 summarizes known and potential ways A-to-I RNA editing may influence pre-mRNA fate.

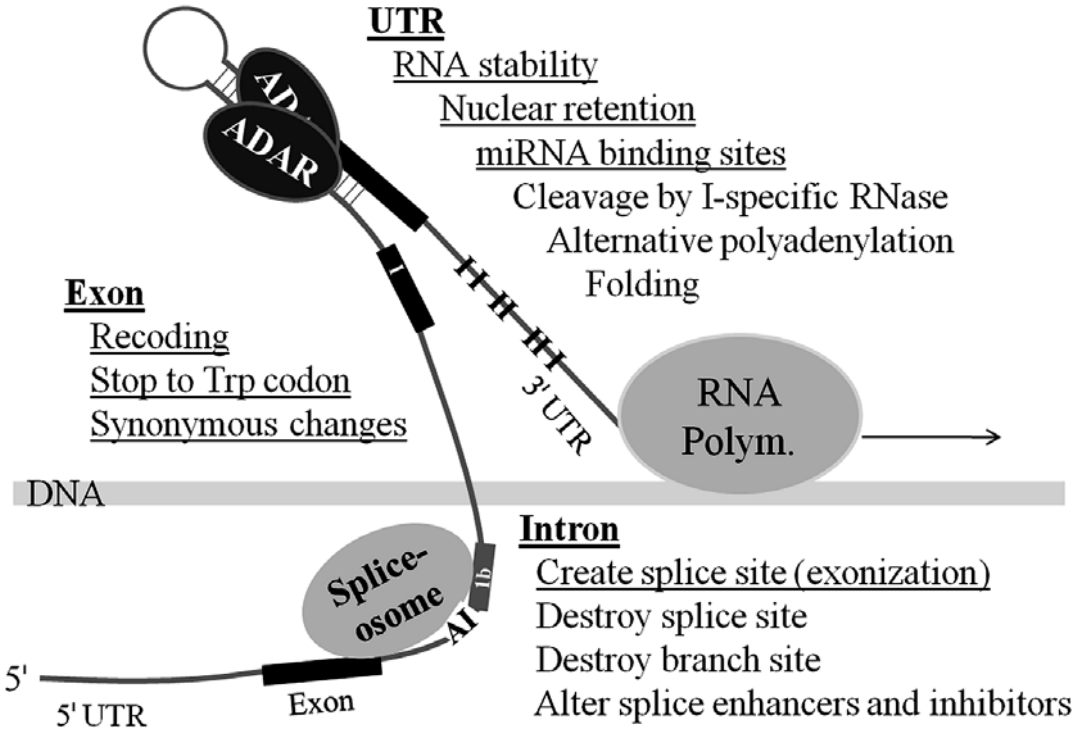

Fig. 2. Known and potential effects of editing on pre-mRNA. Some of the effects of editing on the fate of pre-mRNA targets are shown. Effects, which have been demonstrated *in vivo*, are underlined (for references see text), while the other indicated consequences are hypothetical.

SMALL NON-CODING RNAs

Other classes of RNA molecules targeted by the editing machinery are 18-200 base-pair small non-coding RNAs. They are primarily associated with regulation of expression through sequence-specific RNA interference (RNAi). RNA silencing is thought to have evolved as a form of nucleic acid-based immunity to inactivate viruses and transposable elements [84, 85]. Two classes of small non-coding RNAs, namely microRNAs and siRNAs, and their relationship with RNA editing will be discussed here.

EDITING AND THE microRNA PATHWAY

MicroRNAs (miRNAs) are a class of small non-coding RNAs of approximately 22 nucleotide length. A single miRNA can regulate the expression of dozens or even hundreds of genes by repressing translation or promoting transcript destabilization of mRNAs [86]. They occupy important roles in development, differentiation, proliferation, and apoptosis. At the time of submission of this review, the miRNA database (www.mirbase.org [87]) reported 1048 miRNAs in human, which together regulate thousands of protein-coding genes [88]. They are usually produced from long primary miRNAs (pri-miRNAs) by two processive steps [89]. In the nucleus, a protein complex containing Drosha and DGCR8 recognizes characteristic hairpin structures within the pri-miRNAs and cleaves them at the base, resulting in stem-loop precursor miRNAs (pre-miRNAs) of about 70 nucleotide length. Pre-miRNAs are transported by exportin-5 and RanGTP into the cytoplasm, where they are further processed by the Dicer/TRBP-containing protein complex into 22-bp miRNA/miRNA duplexes consisting of the 5′ (miR-5p) and 3′ strands (miR-3p). The strand of the duplex with lower base-pairing stability at the 5′-end will selectively associate with one of the Argonaute (AGO) proteins, which are part of the multi-protein microRNA-induced silencing complex (miRISC). However, in some instances, both strands of the miRNA/miRNA duplex can give rise to mature miRNAs [90]. The charged miRISC complexes are guided by the seven nucleotide "seed" region of the miRNA located at its 5′-end to partially complementary sequences most commonly located in the 3′-UTRs of target mRNAs, where they direct translational repression and/or mRNA degradation [86]. In specific cases, miRNAs are generated through alternative, non-canonical pathways, which are either Drosha- or Dicer-independent [91]. Transcriptional regulation can only incompletely account for the tight monitoring of miRNA levels, as pri-miRNAs are often transcribed in clusters or as part of introns and UTRs of protein-coding genes. Their expression is therefore governed by the promoter activity of other genes [91, 92]. The multi-step maturation path provides several focal points where regulatory mechanisms find leverage to stop or divert production, and/or adjust the amount of final product [93].

The stem–loop structures of both pri- and pre-miRNAs are potential targets for editing. Indeed, pri-miRNAs have been shown to undergo editing, often at more than one adenosine [94-98]. Pri-miR-22 was the first microRNA precursor identified to be edited, where it occurs at six different positions, including sites within the mature miRNA [94]. To further elucidate how A-to-I RNA editing intersects with the miRNA biogenesis pathways, Yang and coworkers analyzed editing in eight randomly chosen pri-miRNAs, and they found that four were edited [95]. Notably, editing at two adenosines close to the Drosha cleavage site (positions +4 and +5 from the 5′-end of pri-miR-142) inhibits processing of pri- to pre-miR-142. Inhibition also occurs when these positions are mutated to guanosines, showing that Drosha/DGCR8 recognizes A→G and A→I changes alike. Surprisingly, the authors could not detect an increase of edited pri-miR-142 in this cell culture system, but mutated pri-miR-142 accumulated. Therefore, inosine-containing pri-miR-142 RNAs are short-lived, whereas pre-edited pri-miR-142 (with guanosines at positions +4 and +5) is stable and accumulates. Tudor-staphylococcal nuclease (Tudor-SN) has been identified as a ribonuclease, or a critical component thereof, that is specific to inosine-containing dsRNA *in vitro* [99, 100]. Higher levels of editing in pri-miR-142 RNAs render them progressively sensitive to Tudor-SN, and as such, edited pri-miR-142 accumulates in cells treated with a Tudor-SN specific inhibitor. Moreover, endogenous miR-142 levels are increased in ADAR null mice, underscoring that ADAR activity significantly modulates proper miR-142 output.

Drosha cleavage is not the only miRNA biosynthesis step that can be modulated by editing. Editing in pri-miR-151 at a position close to the Dicer cleavage site is accomplished in a tissue-specific manner [96]. Detailed analysis of the editing levels in human pri-, pre-, and mature miR-151 revealed that in this case, processing by Dicer, but not Drosha, is inhibited by editing of miR-151 precursors. Unlike inosine-containing pri-miR-142, high levels of editing are detected in pre-miR-151, indicating that the presence of inosine in pre-miR-151 does not render it unstable, in contrast to what happens in pri-miR-142.

It is clear that editing interferes with miRNA maturation at several steps, but does editing also affect miRNA targeting as had been speculated [101]? Examples where this is the case are the six human miR-376 RNAs (miR-376a1, -376a2, -376b, -378, -B1, -B2), which are encoded in a cluster and transcribed into one long primary transcript. They share highly similar sequences, and undergo tissue-specific editing (nearly 100% in cortex and medul-

la) at one or both of two positions (+4 and +44 from the 5′-end of the pri-miRNAs) [97]. Both sites are located within the 5′-proximal "seed" sequences of the mature miRNAs that, in this case, are derived from both strands of the miRNA/miRNA duplexes. Target predictions of the unedited and edited seed regions of miR-376a1-5p yield 78 and 82 candidate genes, respectively, with only two in common. Endogenous expression levels of one target gene for the edited miR-376a1-5p, phosphoribosyl pyrophosphate synthetase 1 (PRPS1), are reduced twofold in wild-type versus ADAR2$^{-/-}$ mouse cortexes. This effect was tissue-specific, for miR-376a1-5p is not edited in liver tissue and consequently PRPS1 expression levels in liver of wild-type versus ADAR2$^{-/-}$ mouse are comparable. PRPS1 overexpression causes a human disorder characterized by neurodevelopmental impairment with hyperuricemia (2-4-fold above normal). Indeed, cortexes of ADAR2$^{-/-}$ mice, which lack PRPS1 downregulation by edited miR-376a1-5p, have twofold higher uric acid levels.

Yet another point where ADARs intersect the miRNA pathway was identified in viruses. Acute and lytic infection with Epstein–Barr virus (EBV) causes mononucleosis, while persistent latent infection, regulated by specific viral genes, has been associated with diverse human cancers (i.e. Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma [102, 103]). Epstein–Barr virus expresses 23 miRNAs, four of which are edited in virus-transformed cell lines [104]. Pri-miR-BART6 is edited to 50-70% at position +20 (from the 5′-end of the 3p-arm). Both wild-type and pre-edited pri-miR-BART6, wherein the adenosine at +20 is mutated to a guanosine, are efficiently processed by Drosha and Dicer *in vitro*. However, editing of the 3p-arm suppresses loading of miR-BART6-5p into the miRISC complex, probably due to increased stability of the 5′-end of the 5p strand. Interestingly, Dicer mRNA has four miR-BART6-5p target sites in the 3′-UTR. Dicer levels are substantially (70%) reduced by miR-BART6-5p, which results in a global repression of miRNA biogenesis. Such miRNA repression, as is observed after infecting cells with EBV, has been implicated in the transition from lytic to latent infection and the attenuation of antiviral host responses [105, 106]. Thus, in this case A-to-I RNA editing of miR-BART6-5p is an antiviral mechanism to prevent its loading into the miRISC. However, ADAR activity is also often exploited by viruses, which have found ways to direct the editing machinery against host virus defenses, as reviewed recently by Samuel [50].

A systematic interrogation of editing of known pri-miRNA across different tissues was first performed by Blow et al. [107]. Medium to high (10-70%) levels of editing that varied across tissues were observed in six out of 99 analyzed pri-miRNAs. The authors caution that low editing levels escape detection by their analysis, and thus their findings may represent an underestimate. Another study found that 16% (47 of 209) pri-miRNA of human brain are edited to 10-100% at 86 sites [98]. Editing in the seed sequences of mature miRNA was only detected in four cases. It was observed that the fraction of edited miRNA is lower than that of edited pri-miRNA. This reveals that editing almost always inhibits miRNA maturation, which was confirmed by *in vitro* processing analysis of a subset of edited pri-miRNA. Interestingly, in two of the six analyzed pri-miRNAs, editing increases Drosha cleavage rate as much as twofold, thus promoting processing.

Deep sequencing studies corroborate the findings that editing can rarely be detected in mature miRNAs [108]. Rather, ADARs seem to modulate miRNA biogenesis and steady-state levels. Editing of 16% of pri-miRNAs demonstrates the regulatory impact posttranscriptional A-to-I modification has on miRNA-based silencing in human. This impact may be extended even further when considering that binding alone of pri- or pre-miRNAs by ADAR, without the actual editing event taking place, can interfere with Drosha or Dicer processing due to substrate competition. As outlined above, ADAR2 re-directs the edited miR-376a2-5p to a different set of targets. However, at the same time ADAR2 binding to pri-miR-376a2 also specifically competes with Drosha processing, independent of its editing activity [109]. Together, deaminase-independent effects and quick degradation of inosine-containing RNAs probably further increase the percentage of miRNAs affected by ADARs compared to what has been reported so far.

At the other end of miRNA regulation, editing in the 3′-UTRs of target mRNAs could potentially affect mRNA–miRNA binding by creating or destroying seed matches. While editing within known seed match sequences appears to be selected against [110], the opposite seems true for the creation of seed matches [111]. Strikingly, over 3,000 of the 12,723 adenosine deaminations identified by Levanon and coworkers [67] were found to generate 7-mer seed-matches to a subset of human miRNAs. The editing machinery thus substantially intercepts and regulates miRNA gene regulatory pathways at multiple levels, and future research may uncover additional mechanisms. For instance, the structure of the 3′-UTR around the seed match plays a significant role in determining miRNA function [112], and such structures can be significantly altered by editing events taking place even outside of the seed match.

EDITING AND THE siRNA PATHWAY

Double-stranded RNA (dsRNA) in the cytoplasm can be cleaved by Dicer into 21-23-nucleotide-long small interfering RNAs (siRNAs) [113]. The siRNAs associate with the RNA-induced silencing complex (RISC) that recognizes and cleaves the original message, leading to

posttranscriptional gene silencing called RNA interference (RNAi). RNAi is evoked by dsRNA internalized by cells during viral infection, for example, and in recent years has been used as a tool to knock-down the expression of genes of interest [114]. ADARs can potentially interfere with the RNAi pathway as shown in *C. elegans*. As mentioned earlier, knockout worms that do not express ADAR enzymes show defects in chemotaxis [20]. This phenotype is dependent on the RNAi pathway, and is reverted in RNAi-defective worms [115]. Transgene silencing in *C. elegans*, i.e. the silencing of dsRNAs arising from transcription of inverted genomic repeats, is much more effective in ADAR-deficient animals [116]. Again, it is posited that ADAR binding alone, without the editing activity, interferes with suppression of endogenous dsRNA [117]. The authors speculate that normal chemotaxis may depend on the expression of transgenes in somatic cells, which is suppressed in ADAR-null worms due to the unchallenged action of the RNAi system.

In mammals, ADAR1-p150 binds strongly to siRNA. However, binding affinity of ADAR1-p110 to siRNA is 15-times lower [118]. This may indicate that the Zα-DNA binding domain present in ADAR1-p150, but absent in ADAR1-p110, grants higher binding affinity to siRNA. Importantly, RNAi is much more efficient in mouse fibroblasts that lack ADAR1, implicating ADAR1-p150 as an antagonistic factor against RNAi [118]. The ability of human ADAR1 to interfere with the siRNA pathway was also demonstrated in a *Drosophila* reporter system: human ADAR1-p150 quantitatively antagonizes hairpin-mediated RNA interference [109]. This effect is only partially dependent on the editing activity, as even deaminase-deficient ADAR1-p150 achieves substantial relief of reporter gene expression from RNAi suppression. This may be especially relevant for the catalytically inactive but dsRNA-binding-competent ADAR members, ADAR3 and TENR.

It is apparent that ADARs can significantly counteract RNAi efficiency. Consistent with this conclusion is the finding that simultaneous down-regulation of *c-myc* and *ADAR1* by RNAi resulted in effective tumor growth inhibition in mice, whereas high concentrations of siRNAs directed against *c-myc* alone were less effective [119]. Through an unknown mechanism, the presence of siRNA leads to an increase in PU.1 transcription factor, which binds to the *ADAR1* promoter region [120], attracts chromatin remodelers that displace nucleosomes, and renders the sequence accessible for other proteins [121]. PU.1 acts as an enhancer to control lymphoid and myeloid cell proliferation and differentiation [122]. Interestingly, ADAR1 is required for the maintenance of hematopoiesis by opposing a global interferon response [25]. Similar mechanisms thus may take place during hematopoiesis and viral infection that lead to expression of ADAR1, which subsequently buffers the interferon response.

EDITING IN LONG ncRNAs

Long ncRNAs (lncRNAs) are 200 to tens of thousands of nucleotides long. Analysis of the transcriptome in human and mouse revealed the expression of an unforeseen number of lncRNAs [10, 11, 123, 124]. It is estimated that there are at least 30,000 lncRNAs [123], yet the functions of only a handful have been analyzed. Kapranov and coworkers determined that a fraction (2-4%) of long ncRNAs carry sequences of small ncRNAs and can thus be viewed as their precursors [10]. About 36% of all lncRNAs reside exclusively in the nucleus, 17% are exclusively cytoplasmic, and 48% are found in both compartments. They are transcribed from intergenic and intronic regions, as well as in antisense or bidirectional configuration to protein-coding genes. Such overlapping transcription directly regulates expression of these genes (see for example [125]). Furthermore, lncRNAs can be transcribed from imprinted genomic loci where they are understood to direct epigenetic processes that involve chromatin remodeling by DNA methylation and histone modification (reviewed in [126]).

In the central nervous system (CNS), epigenetic mechanisms are pertinent for development, homeostasis, and plasticity in responses to external stimuli ([127] and references therein). Selective targeting of genetic loci by initiators of epigenetic silencing is now thought to be mediated by ncRNAs (see for example [16]). Thus, lncRNAs are proposed to be particularly important for a properly operating CNS, above all by mediating cell identity [128]. They have been implicated in a wide array of neurological disorders and diseases such as Prader–Willi syndrome, Alzheimer disease, multiple sclerosis, glioblastomas, schizophrenia, and depression (reviewed in [127]). Analysis of *in situ* hybridization data revealed that the majority (849 of 1328 examined) of lncRNAs are expressed in the adult mouse brain, often in a cell-type specific manner or in association with defined subcellular compartments [129], indicating functional importance particularly pertaining to cell identity.

It is tempting to speculate that editing of lncRNAs adds another layer of complexity by increasing the diversity of regulatory RNA networks in the CNS. Perhaps not incidentally, editing levels are highest in brain [37], and aberrant editing has been implicated in neurological disorders (reviewed in [130]). The lncRNAs can have ordered secondary structure, which may be important for their function (i.e. to recruit proteins [131]). Additionally, a look at the UCSC human genome browser reveals that they may also harbor Alu elements [132]. As discussed earlier, inverted Alu repeats form long dsRNA structures that can be promiscuously edited [64]. It seems likely that at least some lncRNAs are subject to editing. However, the editing potential and implications thereof remain to be investigated. Editing not only changes the primary sequence of an RNA, which could alter targeting of

lncRNAs to specific sites, but can also impact its secondary and tertiary structure, which may abolish or create protein recognition sites and/or induce changes in subcellular localization as shown for other RNAs. Furthermore, proteins that recognize inosine-containing RNA, such as the ribonuclease Tudor-SN, may find additional substrates in the lncRNA pool. It is possible that the mere binding of ADARs to lncRNAs has biological significance, as is the case for miRNAs and siRNAs. Related to this possibility, the deaminase-deficient ADAR3 is exclusively expressed in brain, where it may interact with dsRNA through its DRBM or with ssRNA through its Arg-domain [26]. In view of the importance of both lncRNAs and RNA editing for the CNS, future efforts should investigate the degree and consequences of their interconnection.

EDITING IN TRANSPOSABLE Alu ELEMENTS

Alu elements not transcribed as part of protein-coding genes or ncRNAs can function as transposable elements in intergenic regions. There are at least 852 active, or transposable, consensus Alu sequences, each present in several copies in the genome [133]. Intrinsic RNA pol III promoters allow low expression, which can be increased during times of stress such as heat shock, viral infection, and treatment with protein synthesis blockers or DNA damaging agents [134, 135]. Alu elements expressed during heat shock act as transacting repressors by binding to RNA polymerase II, thereby globally downregulating transcription [136]. Since Alu elements do not contain open reading frames, retrotransposition, dependent on the reverse transcription followed by insertion of the DNA into different sites in the genome, has to rely on other mobile elements. LINE elements are autonomous retrotransposons still active in the human genome today. They contain two open reading frames, one encoding a reverse transcriptase and the second an endonuclease. Retrotransposition of Alu elements mediated by expressed LINE elements has been shown in cell culture systems [137]. Recently, comparison of individual genomes revealed several thousand Alu insertions not annotated in the human reference database [138], suggesting that LINE-mediated retrotransposition of Alu elements continues to occur and contribute to polymorphism.

The expression of LINE elements is usually suppressed by DNA methylation or the activity of piRNAs (PIWI-interacting short RNAs), as their uncontrolled retrotransposition would wreak havoc on the genome. However, it appears that occasionally their activity is permitted. For instance, transposon silencing is transiently relaxed at the onset of mammalian meiosis due to global DNA methylation reprogramming [139]. Expression does not imply retrotransposition, as the latter is efficiently inhibited by the APOBEC family (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) of the innate antiretroviral defense system [140-142]. However, retrotransposition of a LINE-1 element during early embryogenesis was detected in a case where a new insertion was the underlying cause of the patient's choroideremia, an X-linked eye disease [143]. Somatic and germ-line mosaicism in the mother suggest that retrotransposition of this LINE-1 occurred in early development. Transgenic mouse and rat models expressing traceable elements revealed that LINE-1 RNA is abundant in germ cells and embryos, but integration mainly occurs during embryogenesis [144]. The LINE-1 RNA in germ cells can be carried over into the embryo. Therefore, it appears that the germ line is by and large protected from retrotransposition but this inhibition is lifted, at least in part, in the embryo, allowing some degree of somatic mosaicism as a source of genomic variation among individuals. Human embryonic stem cells (hESCs) express a wide range of Alu as well as LINE-1 elements, possibly due to a relaxation in transcriptional control elicited by the epigenetic remodeling typical of pluripotent cells and reminiscent of that seen at the onset of mammalian meiosis [145]. In hESC, Alu sequences located in introns and UTRs of transcripts display high levels of A-to-I RNA editing [41]. It seems possible, if not probable, that editing also occurs in Alu elements expressed from pol III promoters, especially where two Alu elements occur in opposite orientation on the same transcript. Such edited Alu elements could be retrotransposed into the genome by the activity of the expressed LINE elements as a source of polymorphisms. Conversely, ADAR binding and/or editing of Alu elements could act protectively by thwarting retrotransposition, analogous to the activity of APOBECs [140-142].

In contrast to the previous studies, expression of LINE-1 elements was recently shown to be widespread in somatic tissue as well as adult stem cells, where they are proposed to act as endogenous mutagens, contributing to genetic instability, aging, and cancer [146]. They could also be required to increase the variability of cellular identity through somatic mosaicism in specific organs, which may be particularly important for the complexity of the nervous system. LINE-1 elements were shown to retrotranspose in neuronal precursors *in vitro* and during embryonic and adult neurogenesis in a mouse system [147]. Interestingly, exercise stimulates neuronal proliferation in the hippocampuses of mice and activates a LINE-1 expression reporter gene [148]. This suggests that LINE-1 retrotransposition could be induced to promote neuronal plasticity in response to environmental stimuli. Evidence indicates that LINE-1 retrotransposition also occurs in the human brain [149]. LINE-mediated retrotransposition of other transposable elements such as Alu elements in the CNS is likely. If and how ADARs influence this retrotransposition by binding to and/or editing

Alu transcripts is an unexplored, yet fascinating avenue of research.

Proteins have the capacity to execute structural, enzymatic, and signaling functions that are facilitated by the chemical properties of the amino acids, the polypeptide chain three-dimensional structure, its ability to undergo conformational changes, and to interact with multiple partners. Interwoven with the protein world is a much more ephemeral but no less essential and multifaceted component of life, functional RNA molecules. The combinatorial control of complex networks governed largely by regulatory RNAs seems to be particularly important for the highly complex human nervous system. The sophisticated regulation of neurons mediated by RNAs may in part explain the difference between human and other species. RNA interacting partners in general and RNA modifying enzymes in particular assume the critical responsibility of directing and modulating functional RNAs. The ability of ADARs to bind to dsRNA and alter RNA primary and higher-order structures has been shown to profoundly impact the regulation and function of small non-coding RNAs, but we are just beginning to explore their regulatory power on other RNAs. In order to comprehend how regulatory RNAs exert their functions, it is important to elucidate how these RNAs are governed by their own superimposed regulatory networks – attacking this uncharted territory promises to break new ground in a young research field.

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