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Review

The Many Roles of A-to-I RNA Editing in Animals: Functional or Adaptive?

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Abstract

Metazoan adenosine-to-inosine (A-to-I) RNA editing is a highly conserved mechanism that diversifies the transcriptome by post-transcriptionally converting adenosine to inosine. Millions of editing sites have been identified in different species and, based on abnormal editing observed in various disorders, it is intuitive to conclude that RNA editing is both functional and adaptive. In this review, we propose the following major points: (1) "Function/functional" only represents a molecular/phenotypic consequence and is not necessarily connected to "adaptation/adaptive"; (2) Adaptive editing should be judged in the light of evolution and emphasize advantages of temporal-spatial flexibility; (3) Adaptive editing could, in theory, be extended from nonsynonymous sites to all potentially functional sites. This review seeks to conceptually bridge the gap between molecular biology and evolutionary biology and provide a more objective understanding on the biological functions and evolutionary significance of RNA editing.

Keywords: adaptive; animal; A-to-I RNA editing; evolution; functional

1. Introduction

Adenosine-to-inosine (A-to-I) RNA editing is a highly conserved mechanism in metazoans that converts adenosines to inosines within RNA transcripts [1–3]. This A-to-I conversion, mediated by double-stranded RNA-specific adenosine deaminase (ADAR) proteins, is found in all animal clades ranging from coelenterate (coral) [4], pseudocoelomate (worm) [5], arthropods [6–9], mollusks [10,11], and vertebrates [12–15]. A total of 10^3 to 10^7 of editing sites have been identified in the transcriptomes of different species, making RNA editing the most abundant type of RNA modification in metazoans. RNA editing typically takes place in double-stranded RNAs (dsRNA) and since inosines are recognized as guanosines, A-to-I RNA editing leads to similar functional consequences to A-to-G transition mutations within DNA (Fig. 1A).

The functional importance of a mechanism is commonly deduced from the phenotype that arises when the mechanism is knocked-out or knocked-down. As observed in humans and other animals, abnormal RNA editing has led to multiple disorders and human diseases [16–19]. This prompts us to draw an intuitive conclusion that RNA editing in animals is generally functional and thus adaptive. Unfortunately, although this logic seems plausible, in many cases the two independent terms "functional" and "adaptive" are misused, almost promiscuously. In this review, we will base our conclusions on observations of RNA-editing-related animal disorders, together with our current knowledge on the functional essentiality and adaptive evolution of RNA editing, to clarify the following main points:

(1) The term "function/functional" only represents a molecular consequence and is not necessarily connected to "adaptation/adaptive". Conversely, "non-adaptive" [20–23] does not necessarily mean "non-functional". The phenotypic changes arising from the dysregulation of RNA editing events support the notion of functional editing [19], but are not evidence for adaptive editing.

(2) Adaptation of a biological mechanism should be inferred from its conservation and forces of natural selection, which is, to be judged in the light of evolution [6,24–26]. Experimental verification of the adaptiveness of individual editing sites should compare the fitness of "editable" status *versus* "uneditable" status, as well as emphasizing the advantage of temporally/spatially flexible editing [27,28].

(3) Current theories regarding adaptation of RNA editing primarily focus on nonsynonymous variants [11,23,25]. However, it is highly necessary, and feasible, to apply this notion to all functional RNA editing sites such as editing sites in untranslated regions (UTR) or other non-coding regions of RNA, and test their extent of adaptation.

Using the adaptation of animal RNA editing as an example, this review seeks to conceptually bridge the gap between molecular and evolutionary biology. Function is verified case by case, while adaptation is tested at high-throughput level. This review promises to provide a more objective understanding of the biological function and evolutionary significance of RNA editing.

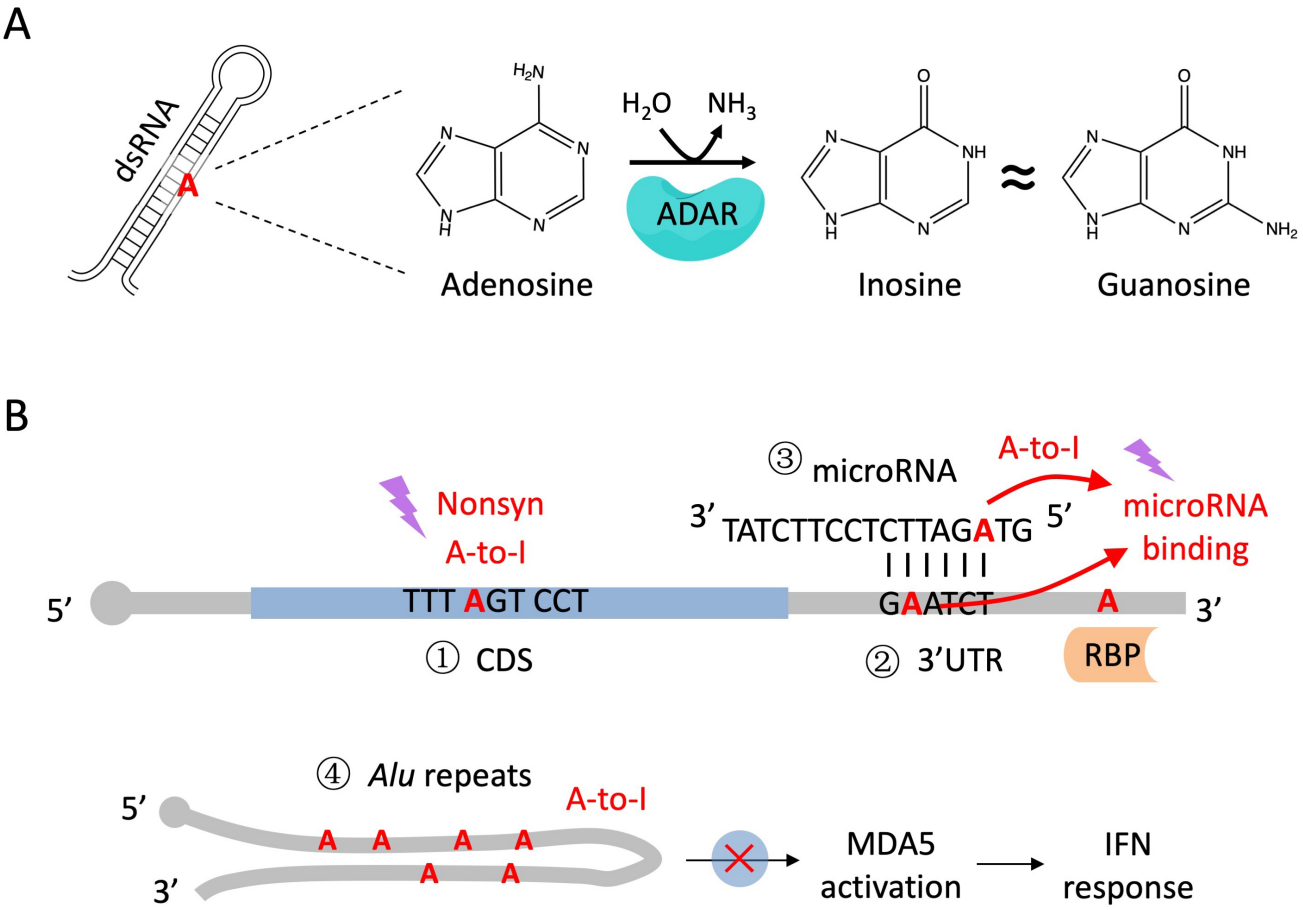

Fig. 1. The occurrence and consequences of Adenosine-to-inosine (A-to-I) RNA editing. (A) A-to-I RNA editing is initiated in dsRNAs and is mediated by adenosine deaminase acting on RNA (ADAR) proteins. (B) A-to-I editing in different RNA regions leads to potentially different biological consequences. Coding sequence (CDS) editing leads to nonsynonymous or synonymous mutations. 3′UTR editing might affect microRNA binding or RNA binding protein (RBP) binding. Editing in a microRNA seed region would change target selection and silencing efficiency of microRNA. Extensive editing in the dsRNA formed by inverted repeats will prevent MDA5 activation in mammals.

2. Molecular Consequence of A-to-I RNA Editing in Different RNA Regions

Prior to discussing how abnormal RNA editing is associated with disorders arising in animals, we should first clearly understand the mechanism and functional consequences of A-to-I RNA editing. RNAs are classified into major categories including mRNA, tRNA, rRNA, microRNA, siRNA, and other non-coding RNAs. The most well-studied classes of RNAs, and those that undergo A-to-I RNA editing, are mRNA, tRNA, microRNA, and a typical RNA transcribed from transposable elements (TE). We note that the editing of tRNA is catalyzed by a specific enzyme family termed adenosine deaminases acting on tRNA (ADAT) [1,29], and ADAT will not be discussed in this review. A-to-I RNA editing events on other RNA classes, such as mRNA, microRNA, and TE, are all mediated by ADARs. Editing of different RNA regions will lead to distinct changes in the molecular function of host RNAs (Fig. 1B).

The most readily conceivable effect of equating A-to-I RNA editing to A-to-G DNA mutation is that A-to-I events in RNA coding sequence (CDS) will likely lead to nonsynonymous mutations (Fig. 1B). As nonsynonymous editing events recode genetic information and may alter the encoded protein sequence, these editing events are also termed "recoding" events. Not surprisingly, recoding events are subjected to strong natural selection [6,7]. For RNA editing events in 3′UTR [18], such editing events have the potential to affect base-pairing between the 3′UTR and microRNAs (Fig. 1B). MicroRNAs are a type of small RNA transcript that generally base-pair with the 3′UTR of target mRNAs using an encoded seed region [30]. microRNA/mRNA recognition depends on sequence complementarity and this interaction will trigger the degradation or silencing of the mRNA transcript. In theory, editing within the target region in 3′UTR could abolish the binding of a microRNA and, conversely, editing could conceivably generate a novel microRNA-binding site within the 3′UTR [18]. Therefore, A-to-I RNA editing within the 3′UTR is capable of generat-

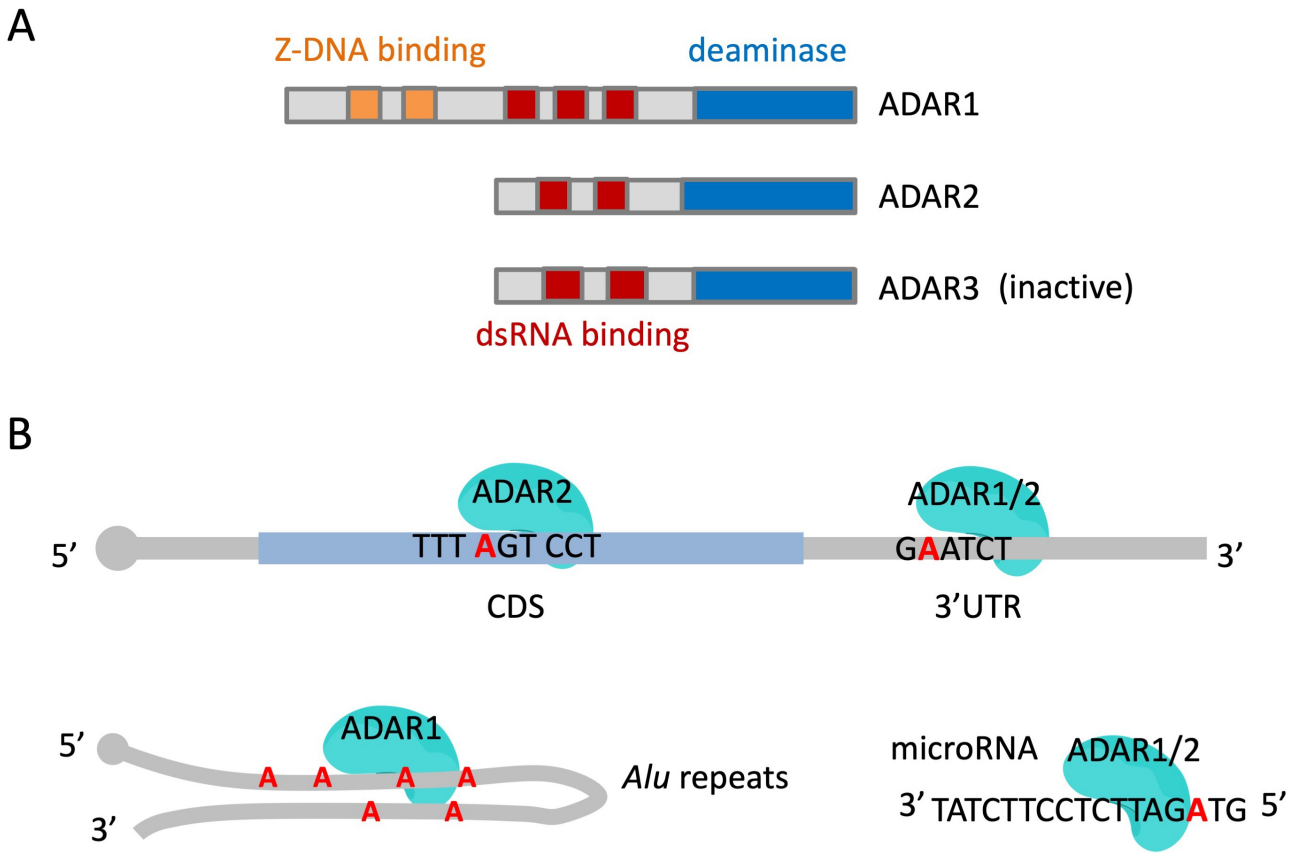

Fig. 2. The ADAR protein family and the preference on different types of RNA editing sites. (A) Illustration of the protein domains of mammalian ADAR1, ADAR2, and ADAR3. (B) Preference of each adenosine deaminase acting on RNA (ADAR) protein on different RNA regions.

ing both gain and loss of microRNA-binding sites and this will, perhaps, alter regulation of host genes. Similarly, A-to-I editing in microRNA seed regions will also affect target selection and gene silencing efficiency (Fig. 1B). Similar to the disruption of microRNA/3′UTR binding, RNA editing in UTRs could also affect the binding of RBPs (RNA binding proteins) due to altered RNA sequence or structure (Fig. 1B) [31].

The RNA editing events outlined above may result in either quantitative or qualitative changes in host/target gene expression. In contrast, there is another special class of A-to-I editing events that takes place on transcribed TEs. A typical example of such a TE are *Alu* elements within the human genome. The editing sites in such repeated elements are highly abundant within the transcriptome and could prevent mammalian MDA5 from sensing endogenous dsRNA as "non-self" [32] and dysregulated MDA5 may lead to a series of immune responses [33] (Fig. 1B). In this scenario, RNA editing acts in an immune-protective role [34]. Notably, while each of the CDS or 3′UTR/microRNA editing sites may have a particular function related to either the host gene or target gene, the RNA editing sites in TEs have the common purpose of avoiding immune response, and this point will be recalled in subsequent sections.

3. Abnormal A-to-I RNA Editing in Human Diseases

As many human and mammalian diseases are associated with abnormal RNA editing, it is tempting to develop the notion that RNA editing is adaptive in mammals. However, this idea appears to be false [22], and a detailed rationale for this view will be discussed later in this review. Overall, mammalian RNA editing events occur in various RNA regions and, interestingly in humans, different editing sites may be targeted by different ADAR family members. Humans, and other mammals, encode three distinct *ADAR* genes in the genome [1,29]. ADAR2 possesses both a dsRNA-binding domain and deaminase domain, ADAR1 additionally has a zDNA-binding domain, and ADAR3 is catalytically inactive (Fig. 2A) [35]. Mammalian ADAR1 is mainly responsible for the millions of A-to-I editing sites in repeats (like human *Alu* elements) while ADAR2 mainly targets exonic regions especially CDS in mRNAs [36]. Some RNA species like microRNAs are likely to be catalyzed by both ADAR1 and ADAR2 [37] (Fig. 2B). Despite that the two active ADAR enzymes in mammals have preference on distinct genomic regions, recent studies in mice have revealed significant overlap between editing capabilities of ADAR1 and ADAR2, and this case especially occurred in brains where both enzymes were

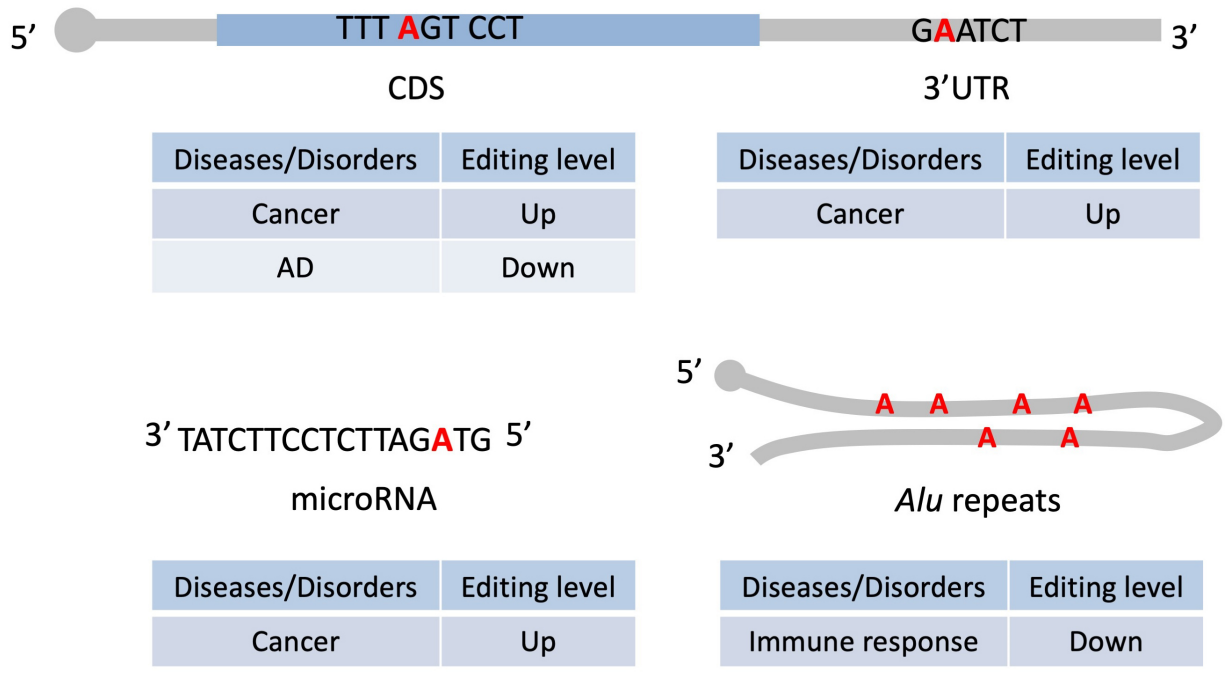

Fig. 3. Examples of diseases or disorders related to RNA editing in different classes and regions of RNAs. Up or down-regulation of RNA editing is indicated.

highly expressed [38,39]. It suggests that although ADAR1 and ADAR2 were non-redundant and could not compensate each other's unique functions, there were indeed many dispensable editing sites that could be targeted by both enzymes.

To clarify the central issue of functional *versus* adaptive RNA editing, we will first list several cases of abnormal RNA editing in human diseases and discuss what conclusion we can base on these observations. Our selected examples will encompass all major classes and regions of RNA transcripts (Fig. 3). Numerous studies revealed that RNA editing participates in various types of cancer. By integrating the thousands of RNA-Seq data covering 17 cancer types currently residing within the TCGA database, Han *et al*. [40] systematically identified RNA editing events in cancer transcriptomes and narrowed this list down to eight clinically-relevant nonsynonymous editing sites, specifically *AZIN1*^{S367G}, *GRIA2*^{R764G}, *COG3*^{I635V}, *COPA*^{I164V}, *ACBD4*^{T262A}, *PPIL3*^{S59G}, *BLCAP*^{Q5R}, and *PODXL*^{H241R}. These editing sites showed clinical relevance to either tumor subtype, tumor stage, or patient survival in more than one cancer type. Moreover, three of the eight nonsynonymous sites, *AZIN1*^{S367G}, *GRIA2*^{R764G}, and *COG3*^{I635V}, were experimentally verified to increase cell viability of normal or cancer cell lines, suggesting an oncogenic role for nonsynonymous RNA editing. A similar study has subsequently verified the correlation between the nonsynonymous editing site *COPA*^{I164V} and the severity of cancer subtype or patient survival time [17]. *In vitro* experiments showed that *COPA*^{I164V} increases proliferation, migration, and invasion of cancer cells; however, the expression of *ADAR1* and *ADAR2* does not correlate with patient survival. This suggests that the prognostic value of nonsynonymous editing sites is independent from global editing regulation catalyzed by ADARs [17]. Notably, the discovery of clinically relevant editing sites, coupled with experimental verification of their tumor promoting activity, implies that such editing sites are up-regulated in cancers.

Apart from the cases that particular nonsynonymous editing sites lead to cancer, another worth-noticing pathway is that ADAR1 frequently plays an immune-suppressing role in tumors [41–43]. Tumor cells might hide themselves from immune detection by the body (e.g., T cells), but the loss of ADAR1 in tumors would trigger the inner-cell immune activity and make the tumor cells sensitive to immunotherapy [42]. This pathway might be convoluted to non-immunologists, but the key message is that ADAR1 is required in some tumor cells and that it becomes a promising therapeutic target in a subset of cancers [41]. The pure observation of elevated editing levels in cancers (Fig. 3) is correlative, but the ADAR1-deficiency datasets coupled with RNA editing analysis would confirm a causative relationship between ADAR1 and cancer. However, we should distinguish the role of ADAR *versus* the role(s) of individual editing sites. Although ADAR1 is functional in tumor cells, the up-regulation of numerous editing sites (by over-expressing *ADAR1*) did not initiate or accelerate the *in vivo* formation of cancer [44], suggesting that a large fraction of the editing sites might be inconsequential and non-functional.

In recent reports that examined abnormal nonsynonymous RNA editing in human diseases, editing levels were

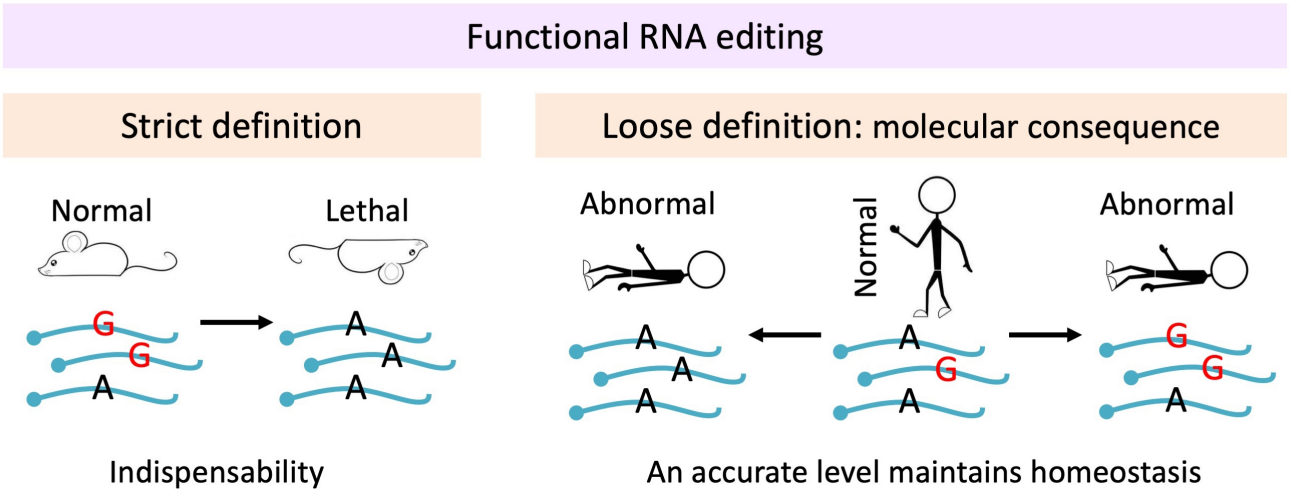

Fig. 4. Definition and criteria for judging functional RNA editing. The strict definition stresses the indispensability of RNA editing, usually inferred from the knock-out or knock-down experiments. Loose definition simply requires an observable consequence of RNA editing which is highly expected from the molecular property conferred by A-to-I editing.

found to be down-regulated (Fig. 3). For example, a well-known recoding site is located in the glutamate receptor gene *GluA2*, is highly conserved across mammals, and is edited 100% of the time in normal cells [45,46]. An A-to-I editing event changes Gln to Arg, and therefore this site has been termed the Q > R site [1]. Strikingly, in different sections of human brain, editing the of Q > R site could not be tolerated with even a 5% reduction in editing rate. Moreover, a roughly ~95% editing level in the hippocampus is likely linked to Alzheimer's disease (AD) [47]. Similarly, a later study reported the reduction of four recoding sites within the *HTR2C* gene in the brain of AD patients [48]. Apart from these recoding sites, editing of transcript 3′UTR as well as microRNAs is also associated with cancer (Fig. 3). Pinto *et al.* [18] retrieved TCGA RNA sequencing data for nine cancer types that have matched tumor and normal tissue controls. Among these samples, the authors identified 63,308 A-to-I editing sites in the 3′UTR of 2687 unique genes. These sites include an editing event that creates a novel microRNAs seed sequence or an editing event that destroys existing microRNA binding sites. The overall editing level of those sites was found to be higher in tumors than in normal samples [18]. Regarding editing within microRNA seed regions, investigators found higher levels of editing in miR-200b and this is associated with worse patient survival and increased cancer cell migration and invasion [37].

A last form of RNA editing is the most abundant type and occurs at repetitive elements within the genome (Fig. 3). It is estimated that, in humans, the number of such editing sites is over 10^8 in abundance [49] although the currently recorded number is roughly ~10^7 [50,51]. Inverted repeats form long and stable dsRNAs that are prone to targeting by ADAR1. Loss of editing in repetitive dsRNAs, usually due to a deficiency in ADAR1, will activate MDA5 and subsequently trigger a series of immune responses or diseases [32]. These diseases include Aicardi-Goutières syndrome (AGS) [52] and potentially Parkinson disease, atopic dermatitis, Crohn's disease, low-density lipoprotein, primary biliary cirrhosis, and ulcerative colitis, all of which were revealed by genome-wide association studies (GWAS) [33].

4. Inference of Functional RNA Editing Sites in Animals

In addition to human diseases, the absence of RNA editing caused by ADAR deficiency, deletion, or mutation is connected to multiple phenotypic forms of disorders in model organisms such as *Drosophila* [53,54] and mice [55]. Given that abnormal RNA editing results in a wide variety of disorders, it is intuitive to posit that RNA editing is functional. In this section of the review, we will demonstrate a dilemma in the logic that gives rise to this conclusion.

The functional importance of a gene, a site, or a biological mechanism is generally inferred from phenotypes observed in the absence of this gene/site/mechanism. For individual RNA editing sites (level >0 under normal conditions), editing function should be seen from the phenotype following editing site is abolishment, such as the requirement for mammalian Q > R site editing [19] (Fig. 4). However, from the standpoint of cancer (Fig. 3), editing levels are either elevated or show a negligible reduction in the tumor. Thus, such cases do not meet the strict definition of functional RNA editing (Fig. 4) because we observe elevated editing levels leading to abnormality rather than the absence of editing leading to abnormality, and virtually no evidence indicates the indispensability of RNA editing events to cancer. Moreover, given recent observations that many RNA editing sites in mice were indeed non-functional and not required for maintaining homeostasis, as inferred

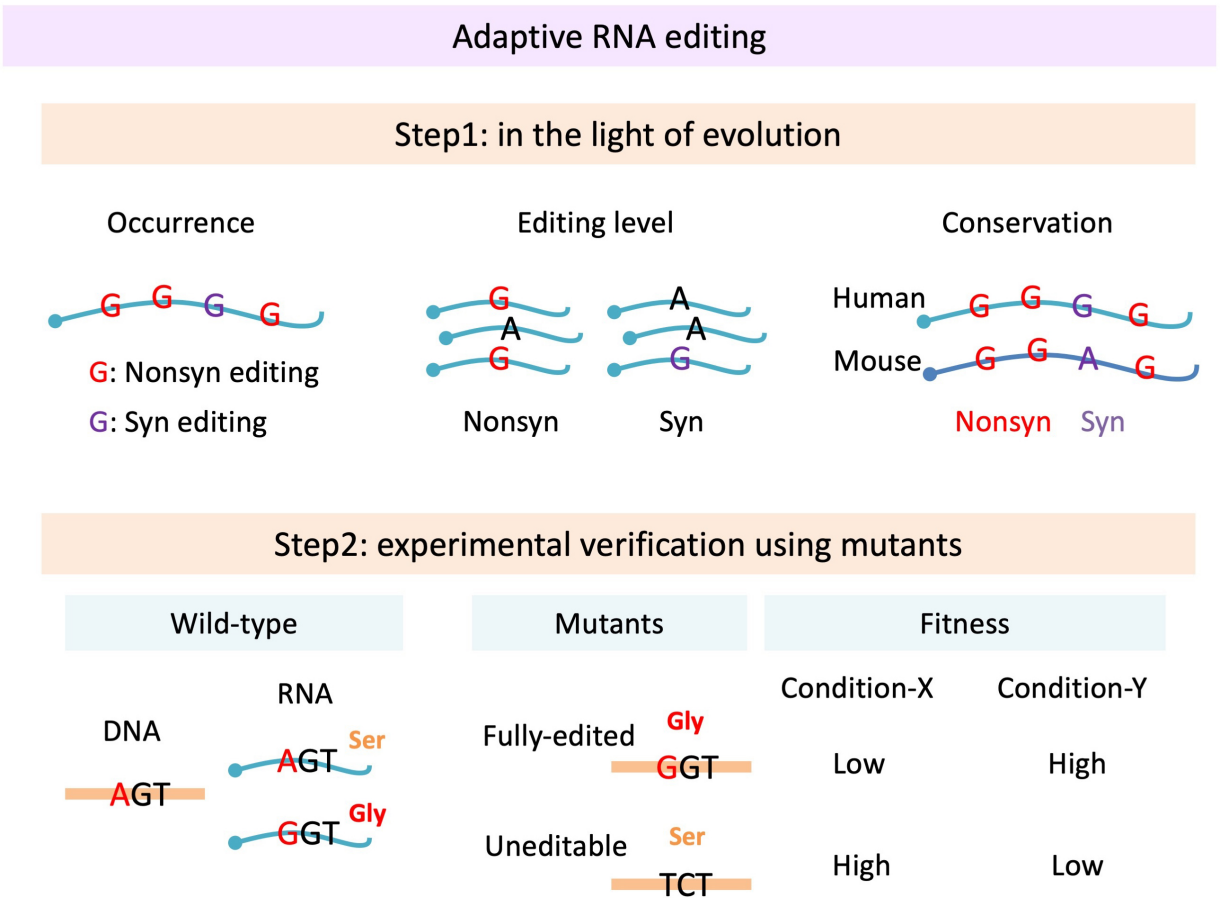

Fig. 5. The definition and verification of adaptive RNA editing. The two progressive steps are needed. The first step is the genome-wide evolutionary analysis. The second step is experimental verification based on the outcome of the first step. The fitness of the edited or unedited allele is judged from the performance of mutant organisms.

from the fitness of *ADAR1*, *ADAR2*, and *ADAR1*/*ADAR2* double mutants [38], further enforcing the notion that some (or many) editing events are non-functional. In fact, the elevated editing level observed in some diseases is more akin to gene over-expression rather than gene knock-out. The observation that altered editing levels results in abnormal phenotype suggests that RNA editing has an observable "molecular consequence" (Fig. 4), which is highly expected given that A-to-I editing molecularly resembles A-to-G mutation. Nevertheless, if one uses molecular consequence to argue for a less stringent, and less rational, definition of "functional", one could also argue that an accurate editing level is functional in the maintenance of cellular homeostasis because too high or too low levels of editing will result in abnormalities (Fig. 4).

Following the establishment of basic criteria for judging whether an editing site(s) is functional, with both stringent or loose definitions, we should also be aware that the detailed function of each editing site often relies on the function of host gene. This evokes an interesting aspect of functional RNA editing, specifically, 98% of the ~10^7 human editing sites are located in repeat elements, and this fraction is 85% in mouse [1]. While the CDS, UTR, or microRNA editing sites could change the activity of various genes with differing functions, the abundant repetitive editing sites all serve the same task, that is to modify dsRNA and prevent the activation of MDA5 and downstream immune response [32,56]. A simple calculation indicates that 100 nonsynonymous sites will produce 2^{100} combinations of primary protein sequence, but 10^7 *Alu* editing sites all have similar functions. With technical improvements, jumping from 10^7 to 10^8 identified *Alu* editing sites does not deepen our understanding regarding the biological function/significance of human RNA editing. The same dilemma applies to the identification of repetitive RNA editing sites in other species. It remains unclear how this will add to our knowledge of function and adaptation of RNA editing and this open question is left to the broad RNA editing community to answer.

5. Adaptive RNA Editing Defined by Conservation and Signals of Natural Selection

Adaptation of RNA editing could be defined in two progressive layers. The first layer is the prerequisite of adaptation which is judged from evolutionary conserva-

tion and selection force, and, moreover, the criteria related to this point have been proposed by Xu and Zhang many years ago [22,57]. The null-hypothesis is straightforward, specifically, assuming synonymous editing as an evolutionarily neutral event, if nonsynonymous editing is adaptive then nonsynonymous editing should exhibit higher occurrence, higher editing levels, and higher conservation levels than synonymous editing sites (Fig. 5). These criteria are well exemplified by investigation of human editing sites, where nonsynonymous editing is less conserved, less frequent, and exhibits lower levels of editing than synonymous editing sites [22]. This suggests that nonsynonymous editing did not confer an advantage to human hosts and thus were eliminated during evolution. Under this definition, functional editing sites are not necessarily adaptive. Some species-specific nonsynonymous editing sites are newly acquired and will naturally have a molecular function (recoding), but no evidence supports the adaptation and selective advantage of these nonsynonymous editing sites. An equivalent statement to "Functional does not mean adaptive" is "non-adaptive does not mean non-functional", and this notion is also well reflected in Xu and Zhang's work [22]. Although the overall trend is that nonsynonymous editing is non-conserved and thus non-adaptive [22], there is definitely a small fraction of recoding sites that are highly conserved across mammals [57] such as the Q $>$ R site controlling the calcium permeability of the glutamate receptor [46]. The global non-adaptive pattern thus does not preclude the existence of functional RNA editing sites.

The second layer is the experimental verification of the advantage of a handful of editing sites selected from the conserved recoding sites. Importantly, this kind of case study on adaptive RNA editing should be inferred from the fitness of the "editable" *versus* the "uneditable" status (Fig. 5). For example, a recent paper has constructed different mutants in fungi to prove that "editable" is superior to "uneditable" [27] commented in [28,58]. Fungi were chosen due to the convenience in measuring the fitness of a particular genotype. In brief, the unedited A-allele is fitter under condition-X, while the edited G-allele is fitter under condition-Y, so the averaged outcome is that being editable is better than the uneditable status (Fig. 5). A comparison is not made between the G-allele and A-allele, instead, the advantage of editing relies on its flexibility in controlling proteomic diversity in a temporal-spatial manner, and avoiding the pleiotropic effects caused by DNA mutation [6,59].

Note a that the "uneditable" allele described in the original Xin *et al*. [27] paper is conceptually different from the "nonedited" alleles in Lewis's commentary [28]. In this example, the adenosine-allele is exactly the "nonedited" allele but it has the potential to be edited in the cell. Nothing can ensure a nonedited allele is not edited, unless the allele itself is strictly "uneditable", specifically, a codon encoding the same amino acid but not containing adenosines. Very few cases could be found within the 64 codon table, for example, in serine codons AGT and TCT the AGT-to-GGT editing could be cancelled by replacing the AGT codon with the uneditable TCT codon. However, not all editable codons have an uneditable counterpart. Therefore, even with technical advances in genetic mutant construction techniques, not every recoding site could prove adaptive by mutant organisms. There remains a lack in convenient and genome-wide methodologies to verify the adaptation of recoding sites. Fortunately, in the microbes that have been studied to date, the editability of an adenosine strictly relies on the UAG motif in fungi [60] or UACG motif in bacteria [61]. This finding suggests that one only needs to mutate the U at the minus-1 position to abolish editing [27]. This natural convenience avoids the need to look for an uneditable codon which is not applicable for most amino acids.

6. Adaptive RNA Editing in Cephalopods is Nearly but not Completely Verified

However, not all organisms can undergo genetical modification to assess the fitness of fully edited individuals compared to uneditable individuals. Thus, this technical limitation hampers the demonstration of the adaptive nature of RNA editing. Nevertheless, a recent paper on Octopus attempted to prove adaptive editing through a "second-best" approach [62]. The authors introduced that although temperature-sensitive RNA editing has been recognized for a long time, it remains unclear whether the changes in editing levels are a result of temperature fluctuations or a deliberate strategy employed by organisms for environmental adaptation? A logical course of action is to investigate whether any functional divergence between the edited and unedited proteins align with the adaptive requirements of the animals under different conditions. The researchers subjected *Octopus bimaculoides* to acute temperature change and subsequently detected RNA editing. The results revealed a substantial increase in editing levels at numerous recoding sites in response to low temperature. The consequences of two recoding sites in kinesin-1 and synaptotagmin were experimentally tested: (1) The I248V edited version of synaptotagmin had lower Ca^{2+} affinity compared to the unedited synaptotagmin; (2) The K282R edited version of kinesin-1 had lower motility compared to the unedited counterpart [62].

In the case of recoding kinesin-1, one might naturally consider that higher editing levels resulting from lower temperatures would lead to increased motility, thus compensating for the cold. However, the authors of the study made an unexpected observation: the edited version of kinesin exhibited lower motility [62]. Although counterintuitive, the authors suggested that this finding could be potentially explained by the "supply matches demand" assumption [62]. Intriguingly, the dilemma arises from our limited understanding of how the fitness of an individual animal is linked to the motility of kinesin under different temperature conditions. We all take it for granted to presume that the animal needs a more active kinesin version under lower tempera-

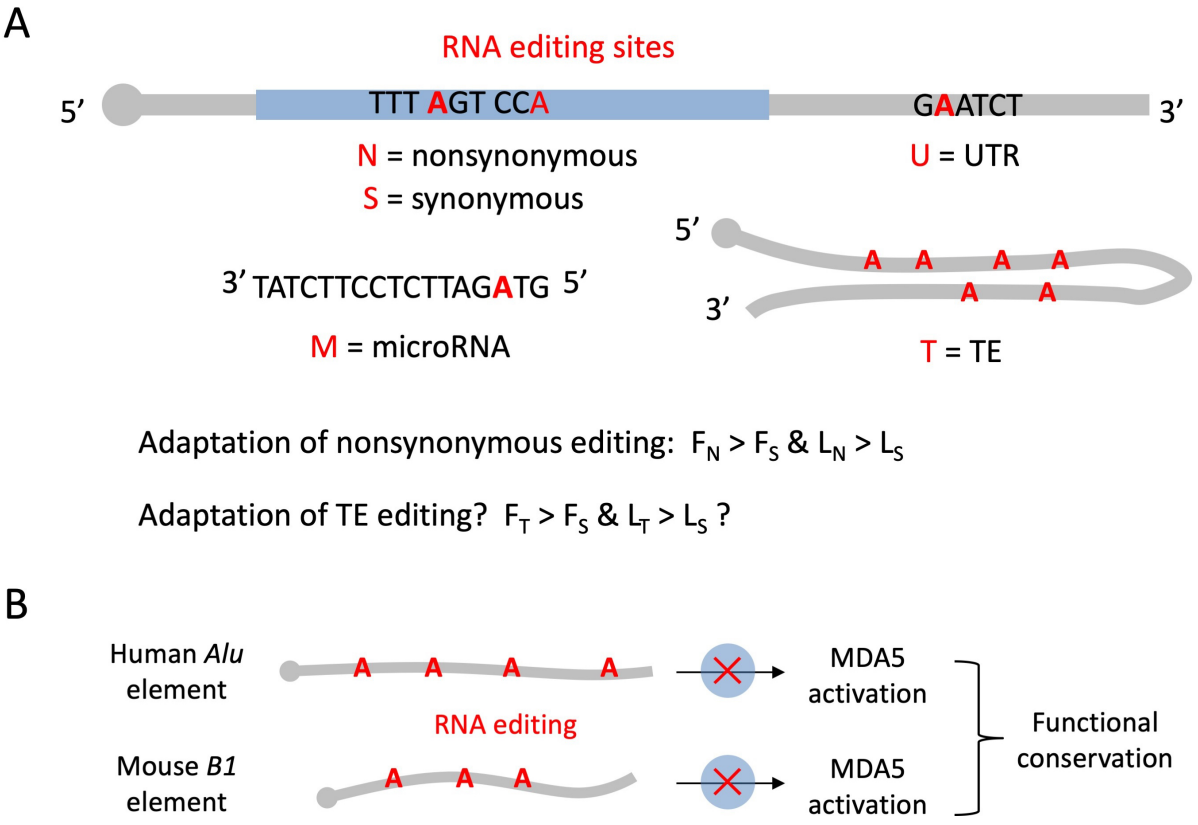

Fig. 6. Extending the concept of adaptive RNA editing. (A) Traditional comparison of the fraction (denoted as F) and editing level (denoted as L) of editing sites. Synonymous sites are used as neutral control. (B) We propose functional conservation as a measurement of adaptation. UTR: untranslated region; TE: transposable element.

ture, but what if the fitness is mainly determined by whether "the supply matches the demand"? This question could only be answered by measuring the fitness of mutant animals where the fully edited animal is fitter under condition-Y while the uneditable animal is fitter under condition-X (Fig. 5). This future plan, outlined in the "Limitations of the study" section of the original literature [62], further emphasizes the importance of utilizing mutant animals as the gold standard for evaluating adaptive editing.

Interestingly, a similar study was conducted on the squid *Doryteuthis pealeii* [63]. The researchers also investigated the motility of the cold-specific kinesin isoform compared to the unedited kinesin. There was a slight difference between the isoform designs in octopuses [62] and squids [63]. In the case of squids, the authors observed a higher occurrence of "multiply recoded transcripts" than expected by chance, which indicated a significant linkage disequilibrium (LD) between editing sites as previously reported by Duan *et al.* in 2018 [3]. Thus, the authors selected three multiply recoded kinesin transcripts as the cold-specific isoforms. Interestingly, under cold condition, those cold-specific kinesin isoforms indeed exhibited higher run distances and landing rates (although slightly lower velocity) than the unedited isoforms [63]. This finding aligns with our intuitive understanding that an active kinesin version might help compensate for the cold. Notably, in the landing rate comparison, the edited versions under cold condition had similar (or even higher) landing rates than the unedited version under normal temperature, which enabled a full compensation [63]. Notably, when comparing landing rates, the edited versions under cold conditions displayed similar (or even higher) rates compared to the unedited version under normal temperatures, indicating complete compensation.

Nevertheless, it is important to emphasize that the observation of a more active kinesin under cold conditions does not inherently establish higher fitness for the organism in cold environments. To definitively conclude adaptive editing, it is still necessary to conduct genetic manipulation along with phenotypic experiments. These experiments should demonstrate that the cold-specific kinesin isoforms confer higher fitness to the animal under cold temperatures (compared to unedited kinesin under cold conditions), while the unedited kinesin offers higher fitness to the animal under normal temperatures.

7. Limitations in the Definition of Adaptive RNA Editing

Current theories examining adaptive RNA editing all focus on nonsynonymous editing sites. Synonymous sites are used as a neutral control to measure the prevalence and selective patterns of nonsynonymous editing (Fig. 6A)

[22,23,25]. This idea is, more or less, affected by traditional and classic theories of evolution that compare the *dN*/*dS* of genes to measure evolutionary rates [64]. Similar cases appear in the estimation of positive selection using the McDonald-Kreitman test (MK-test) where the original paper from McDonald and Kreitman [65] only compared the fixation rate of nonsynonymous to that of synonymous sites [65]. However, the MK-test has been improved to fit any region of interest (RI) [66] and by comparing the fixation rate of the mutations in RI to that of neutral mutations, one would obtain an alpha value representing the strength of positive selection on mutations in RI [66].

Similarly, regarding adaptation of RNA editing, we could also extend the focus from nonsynonymous sites to all "potentially" functional sites (Fig. 6A). Here, potential is inferred from the molecular consequence. Specifically, editing events in CDS, UTR, microRNA, and TEs are all potentially functional. In the example given, let N = nonsynonymous, S = synonymous, U = UTR, M = microRNA, T = TE, F = fraction, L = editing level. The traditional judgement of adaptive nonsynonymous editing is limited to the comparison of F_N *versus* F_S and L_N *versus* L_S [22,23,25]. Here, we propose that the selection force acting on UTR editing sites could be identified by F_U *versus* F_S and L_U *versus* L_S. Similar arguments apply to microRNA and TE, for example, if abundant *Alu* editing in human transcriptome is adaptive, then we would expect $F_T > F_S$ and $L_T > L_S$ (Fig. 6A). Further, the editable *Alu* sites comprise >10% of the total adenosines in *Alu* [49–51], but this fraction is obviously lower for synonymous editing sites [22].

Even with the observation that *Alu* editing is much more prevalent than synonymous editing in the human transcriptome, another traditional criterion largely conceptually excludes the possibility of adaptive *Alu* editing. This criterion is the requirement of conservation level [22]. For example, to show adaptation of nonsynonymous editing, the nonsynonymous editing sites must be more conserved than the synonymous editing sites between two species [6]. *Alu* only exists in primates so if human is compared to mouse, then *Alu* editing sites would receive a conservation level of zero, suggesting the non-adaptive nature of this editing event. This scenario is, of course, absurd as *Alu* editing is indispensable in humans. One solution to this paradox is to examine the conservation of *Alu* editing sites between two closely related primates, and the measured conservation level is hopefully higher than that of synonymous editing sites. Another potential solution that reflects on adaptive *Alu* editing is to focus on "functional conservation" instead of "sequential conservation" between human and mouse (Fig. 6B). The sequence evolution of TEs is commonly beyond the description of evolutionary formula, but the purposes of RNA editing in TEs all converge on the prevention of MDA5 activation and activation of immune response. This functional conservation, although difficult to quantify, could serve as indirect evidence supporting the adaptive editing of TEs.

Although current theories regarding adaptive RNA editing only focus on nonsynonymous editing, it is highly necessary and feasible to apply this idea to all the potentially functional RNA editing sites and test their extent of adaptation compared to neutral (synonymous) sites.

8. Conclusions

In this article, we have clarified some basic concepts in the RNA editing community. We first stressed that the term "function/functional" might have different definitions. Under the strict definition, only when a lower fitness (of host) is observed in the absence of a gene/site could we claim that this gene/site is functional. Even under this stringent definition, functional RNA editing only represents a molecular/phenotypic consequence caused by the editing event and is still insufficient to prove adaptive editing. In fact, adaptive editing should be judged in the light of evolution and emphasize the advantage of temporal-spatial flexibility. Ideally, adaptive editing should be experimentally verified by the fitness of fully edited versus uneditable animals under different conditions. However, due to the theoretical limitations that an uneditable synonymous codon is not available for most recoding sites, and the technical difficulty in genetic manipulation in non-model organisms, the golden standard for adaptive editing is usually unreachable. Thus, many studies tried to measure the functional divergence between the protein isoforms produced by the edited versus unedited RNAs. However, here comes a potentially serious flaw (not an expert here) that how to produce the wildtype protein version translated from the unedited RNAs? As long as the system contains ADAR, the RNAs could be potentially edited, at least partially. Under this situation, the best way to produce the wildtype protein isoform is to design an uneditable codon at the site of interest. However, as we have frequently stressed, the uneditable codon is not applicable for most recoding sites except serine codon AGT. Next, we proposed that the adaptive editing theory could be extended from nonsynonymous sites to all potentially functional sites. The term "adaptation" should represent an evolutionary feature (property) but should not be restricted to nonsynonymous mutations. Taken together, our review might conceptually bridge the gap between molecular biology and evolutionary biology. We tried to broaden our ideas on the nature of the adaptation of a biological feature. Specifically, we have provided a more objective understanding on the biological function and evolutionary significance of RNA editing.

Abbreviations

AD, Alzheimer's disease; ADAR, adenosine deaminase acting on RNA; ADAT, adenosine deaminases acting on tRNA; A-to-I, adenosine-to-inosine; CDS, coding sequence; GWAS, genome-wide association analysis; MK-test, McDonald-Kreitman test; RBP, RNA binding protein; TCGA, The Cancer Genome Atlas; TE, transposable element; UTR, untranslated region.

Author Contributions

YD, WC, and HL: Conceptualization and supervision; YD: Writing original draft; YD, DZ and CZ: Design of work, acquisition of literatures and related data, writing, review, and editing; All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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