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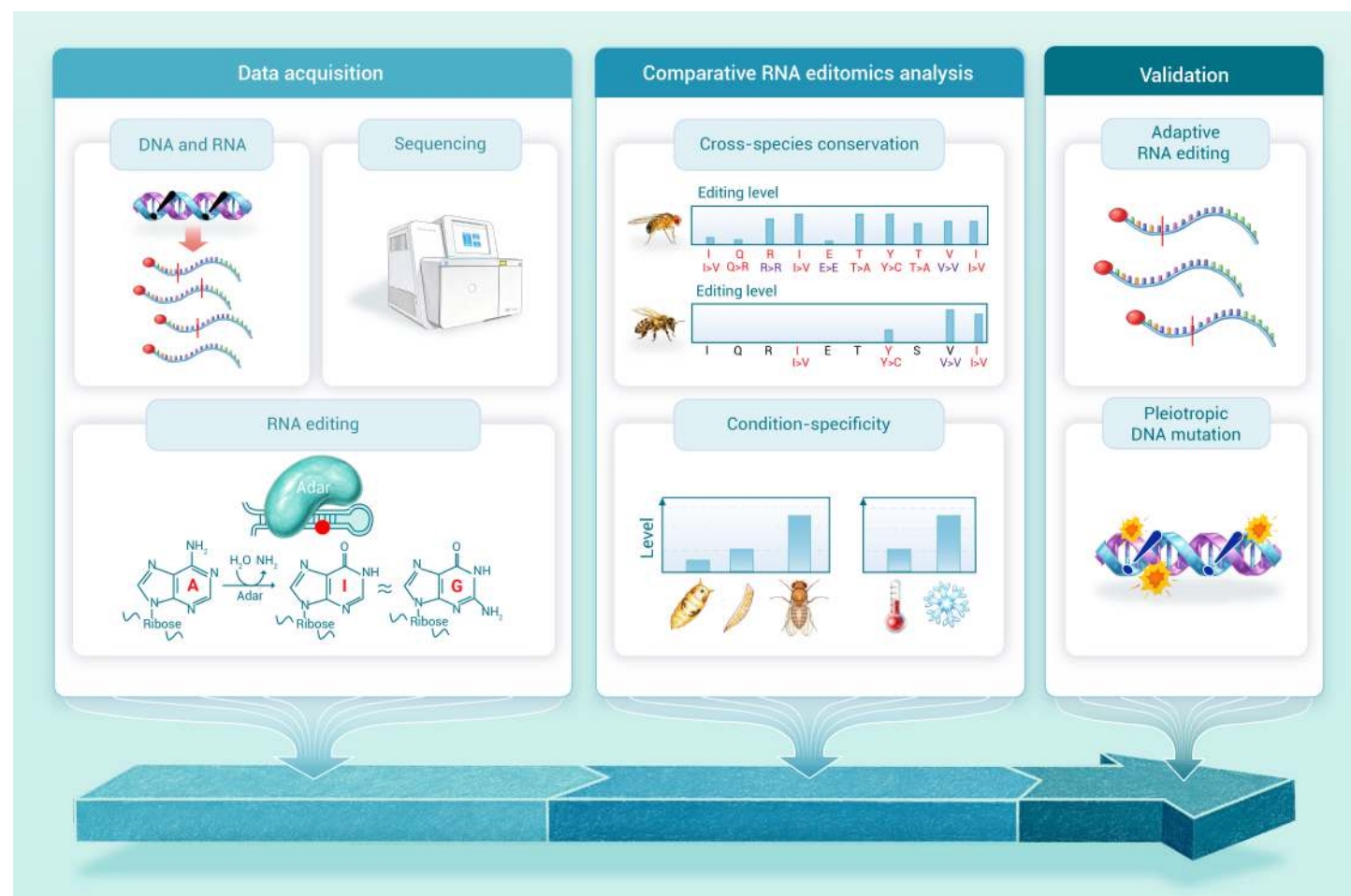
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- The neutral theory and molecular error hypothesis propose most molecular complexity may be non-adaptive.
- Certain RNA editing sites represent a plastic anti-pleiotropic mechanism resolving evolutionary trade-offs.
- Comparative RNA editomics is a pipeline encompassing editing identification and evolutionary analysis.
- Validating adaptive RNA editing relies on the comparisons between fully edited and uneditable mutants.

Comparative RNA editomics prioritizes candidate adaptive RNA editing sites

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A-to-I mRNA editing is a widespread RNA modification that introduces A-to-G variations at mRNA level, enhancing transcriptomic and proteomic diversity without altering the genome. Recently, the term adaptation has gained significant attention but lacks a mechanistic classification. This article aims to discuss conceptual frameworks and research methods related to adaptive and non-adaptive RNA editing. We begin by discussing neutral evolution and the molecular error theory, highlighting that while identifying adaptive signals is desirable, most molecular complexity may be non-adaptive. Conceptually, adaptation could be understood as a continuum between two poles: (1) the permanent mutations leading to fixed adaptation, which is pleiotropic, and (2) the acquisition of plasticity to cope with changing environments, which is anti-pleiotropic. Adaptive RNA editing actually belongs to the latter class due to its regulatable nature. We introduce the term “comparative RNA editomics” to encompass the analyses related to RNA editing, including identification, selection analysis, linkage analysis, condition-specificity, and evolutionary dynamics based on multi-omics data. Such pipelines allow us to prioritize potential adaptive editing sites (e.g., *Adar* auto-recoding in insects) from non-adaptive editing (e.g., *Drosophila* lncRNA *roX1* editing). The adaptive nature of several recoding and stop-codon read-through editing sites in *Fusarium graminearum* have been experimentally validated due to the high feasibility of genetic manipulation and phenotypical assays in fungi. Our deep insights complement traditional views on adaptation and facilitate future studies in functional genomics.

INTRODUCTION

Adenosine-to-inosine (A-to-I) mRNA editing is a wide-spread RNA modification found across animals,^{1,2} fungi,³ and bacteria,⁴ which introduces A-to-G mutations at the mRNA level,⁵ thereby increasing transcriptome and proteome diversity without altering the genome.^{5,7} In recent years, with the rapid advancements in multi-omics approaches, the concept of adaptation has been widely discussed, and the adaptation of RNA editing has been studied across various lineages.^{8–10} In this article, we comprehensively discuss the research methods and underlying conceptual frameworks for studying adaptive RNA editing, drawing on both the current state of the RNA editing field and our own insights. We formally introduce the term “comparative RNA editomics” (abbreviated as comparative editomics) to encompass the analytical processes associated with RNA editing and the identification of adaptive RNA editing sites. Adaptive RNA editing is intuitively defined as editing events that enhance the fitness of organisms, such that possessing editability (the capacity to toggle between edited and unedited states) confers a selective advantage over both the absence of editing and the fully edited state. Notably, the term “editomics” has been rarely mentioned in the literature, appearing only incidentally in the text, without being systematically proposed as a concept.¹¹

We will first introduce the concepts of neutral evolution and the molecular error theory. We emphasize that, while identifying adaptive RNA editing sites is tempting for researchers, the majority of RNA editing sites in the transcriptome are likely non-adaptive.¹² Immune protection is the ancestral role of RNA editing and recoding seems to be a derived benefit.^{1,13} Subsequently, we propose that the commonly discussed concept of adaptation in the scientific community could be understood as a continuum between two conceptual poles. One pole refers to permanent mutation-mediated adaptations, such as the convergent mutations in mammals that

enable adaptation to aquatic environments.¹⁴ Another pole of adaptation involves acquiring a plastic and flexible capability to respond to changing environments. Association studies of A-to-I RNA editing often appear as an example of the first type of adaptation due to its mutation-like characteristics. However, the true elegance of some adaptive RNA editing sites lies in its flexibility,¹⁵ which aligns with the second type of adaptation. Next, we will summarize the research methodologies of comparative RNA editomics, including site identification, selection analysis, linkage analysis, condition-specificity analysis, genome evolution of editing sites, and the evolutionary dynamics of editing events across multiple species. Finally, we will outline experimental validation techniques for adaptive RNA editing sites.

Neutral evolution theory and the molecular error hypothesis

The discovery of adaptive RNA editing sites is exciting, but such perfect sites represent only the tip of the iceberg, and the majority of RNA editing sites are likely non-adaptive.^{16,17} This fact is neither surprising nor disappointing, as the neutral and nearly neutral theory had already been proposed by Kimura and Ohta decades ago.^{18,19} This theory originally stated that the vast majority of fixed divergence sites observed in genomes are close to neutral, with only a small fraction potentially having adaptive significance. While neutral theory was originally formulated for DNA mutations, it can be readily extended to other molecular and biological phenomena.²⁰

In recent years, George Zhang's molecular error hypothesis has further urged the scientific community to reconsider the overall proportion of adaptive traits.¹² Zhang posits that molecular processes generating complexity, such as alternative splicing, RNA editing, RNA methylation, and alternative polyadenylation (APA), are primarily random molecular errors rather than intentional adaptations by organisms to gain a specific benefit.^{17,21–23} Take splicing as an example, the spliceosome machinery may have evolved primarily to ensure the accurate processing of a limited subset of functionally critical transcripts, such as the sex-determination genes in *Drosophila*,²⁴ while the majority of alternative splicing events across the genome may represent nonessential byproducts.¹² The observation that most RNA modifications likely originated as broad immune safeguards rather than as targeted regulatory mechanisms,²⁵ and that their derived benefits including recoding,^{26,27} mutation correction,²⁸ and translational regulation²⁹ were subsequently co-opted during evolution, suggests that precisely regulated molecular variation constitutes only a small fraction of the total transcriptomic diversity.

Multiple lines of evidence at the omics level further support the molecular error hypothesis, such as the negative correlation between gene expression levels and molecular diversity, indicating that this molecular diversity is suppressed in essential genes.¹² Both the neutral theory and the molecular error hypothesis emphasize the importance of genetic drift, with the randomness driven by this drift being negatively correlated with the species' effective population size (N_e). For instance, in humans, where N_e is relatively small,³⁰ non-adaptive RNA editing sites are not effectively purged, resulting in the dilution of truly adaptive sites.¹⁷ In contrast, *Drosophila*, with a larger N_e , exhibits a slightly higher proportion of nonsynonymous editing sites than neutral expectations.³¹ Microbial species, with even larger N_e , such as in fungus *Neurospora crassa*³² and in dozens of Gammaproteobacteria species,³³ exhibit a significantly higher nonsynonymous/synonymous ratio than expected without any filters on editing sites. Regardless of the different global scenarios above, pinpointing particular adaptive RNA editing sites amidst the vast number of total RNA editing sites is a challenging task.

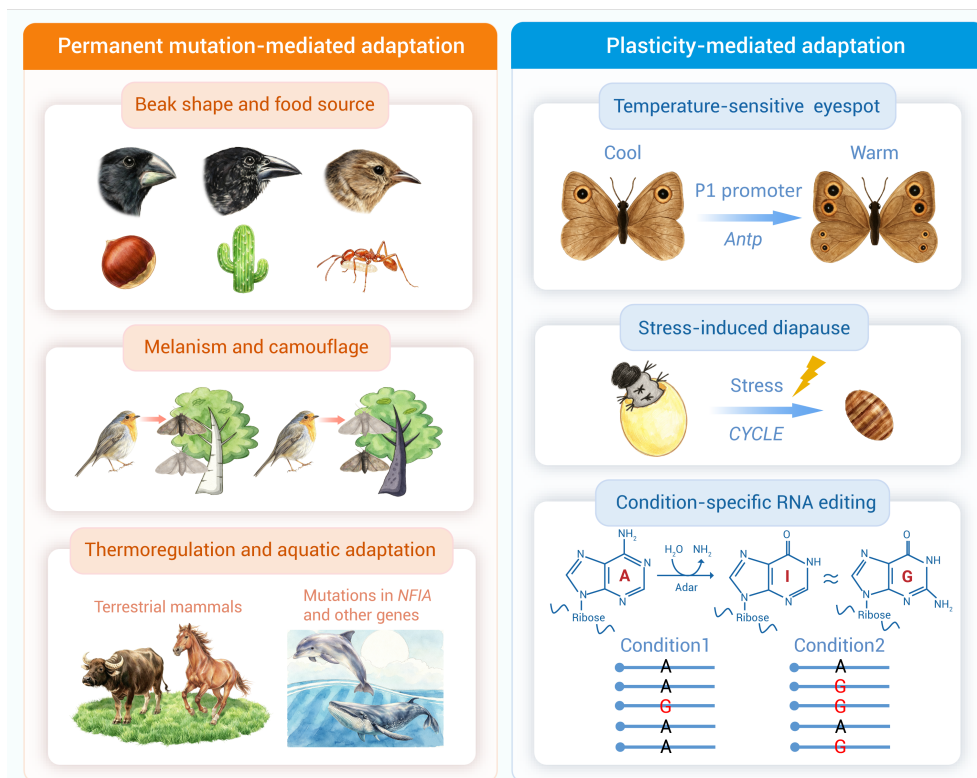


Figure 1. Illustration of two conceptual poles of adaptation The left panel shows the permanent mutation-mediated adaptation, which is pleiotropic. The right panel shows the plasticity-mediated adaptation, which is flexible (anti-pleiotropic). Typical examples of each type of adaptation were displayed. Note that the two conceptual categories are viewed as two poles of a continuum rather than mutually exclusive classes. RNA editing is shown as a potential example of plasticity-mediated adaptation when the editable state provides context-dependent advantages over either hardwired allele.

Before we go deep into the adaptation of RNA editing, we first need to understand the fundamental question of what we are discussing when we talk about “adaptation”.

Two conceptual poles of adaptation: permanent mutation-mediated (pleiotropic) and plasticity-mediated (anti-pleiotropic)

The term “adaptation” has become increasingly prevalent in the fields of evolutionary biology and functional genomics.³⁴ We argue that the concept of adaptation, as traditionally defined, can be viewed as a continuum defined by two conceptual poles, although the distinction between the two poles has not received adequate attention.

One pole of adaptation, the extreme case, can be characterized as “all or none” which is commonly discussed in genomic literatures (Figure 1). A classic example of this type is the beak morphology of Darwin’s finches and its corresponding functional adaptation.³⁵ This paradigm typically assumes (although not explicitly stated) that the presence of a certain mutation (or gene) facilitates adaptation to a specific environment, whereas the absence of that mutation (or gene) hardly adapts to that environment. This logic has become the default setting of some comparative genomics analyses (such as gene family expansions and contractions)³⁶ or population genomics GWAS studies.³⁷ Such idea emphasizes permanent mutations: to be or not to be, that is a binary question.

Intriguingly, understanding adaptation in this way inherently assumes that both the new and old environments are relatively stable. For example, different lineages of aquatic mammals share convergent point mutations in the highly conserved domain of Nuclear Factor I A (*NFIA*), independently adapting to aquatic environments.¹⁴ This case exemplifies the idea of “permanent mutations leading to permanent adaptation” (Figure 1). Once adapted to an aquatic lifestyle, these species do not need to worry about the potential loss of water, and therefore they do not require a flexible mechanism prepared for environmental switching. The color adaptation of the peppered moth is similarly permanent, where dark-colored moths are more advantageous on dark tree bark.³⁸ The tree bark does not change color daily, so the mutation leading to the dark coloration of the moth does not need to evolve a mechanism for flexible regulation between dark and light states (Figure 1).³⁹

The same concept applies to the beak shape changes in Darwin’s finches, which permanently adapted to a new food source without needing to

In sharp contrast, another conceptual pole of adaptation arises from the acquisition of plasticity (Figure 1). Plasticity encompasses various aspects, including the regulation under different life stages, tissues, cell types, ecotypes, and environmental conditions. A prime example of plasticity-mediated adaptation is recent research on satyrid butterflies, where the acquisition of the P1 promoter enables the butterfly to respond to temperature fluctuations and flexibly regulate the expression of the *Hox* gene, *Antp*, ultimately leading to the formation of color patterns (Figure 1).⁴³ Unlike traditional gene family expansion or contraction analyses, in this case, the expansion does not involve the target gene (the *Hox* gene) itself, but rather the promoter, which is specifically regulated by temperature. Such flexible regulation is distinct from the previous water adaptation mediated by hardwired DNA mutation in coding sequence. Moreover, a recent discovery on temperature-responsive spatial-temporal regulation of Hedgehog pathway in honeybee again reinforced the connection between regulatory elements and temperature adaptation.⁴⁴ Another typical example of plasticity is diapause, a strategy used by insects to cope with unfavorable environments. It is reported that moth *CYCLE* gene has three different splicing isoforms, where A/B isoforms functions in rhythms and C isoform is regulated to determine diapause (Figure 1).⁴⁵ Since diapause is temporary, it must not be triggered by a permanent mechanism but should have a plastic regulation.

Our daily experiences tell us that permanent adaptation to fixed environments should be rare. More commonly, environments are variable so that “no turning back” can be dangerous. For instance, under diurnal temperature variations, a mutation that leads to high-temperature adaptation would be incompatible with the cold temperatures at night, and *vice versa*.¹⁵ “All or none” mutations result in contradictions between genotype and environment. In contrast, we anticipate that plasticity-mediated adaptations should be more common, as exemplified by the satyrid butterfly⁴³ and other insects.^{44,45} However, it is surprising that the logic of plasticity-mediated adaptation seems underrepresented in the literatures.

Permanent mutation-mediated adaptation is pleiotropic, while plasticity-mediated adaptation is anti-pleiotropic that resolves evolutionary trade-offs.⁴⁶ These two poles define the conceptual boundaries of adaptation we refer to in our daily research. Virtually all expressional regulation for essential gene should be regarded as adaptation because it resolves gene pleiotropy. However, traditional evolutionary genomic studies paid more attention to the

Box 1 | Two conceptual poles of adaptation: pleiotropic and anti-pleiotropic

Here, we summarize a conceptual distinction: adaptation can be viewed as a continuum between two poles, and propose that adaptive RNA editing belongs to the anti-pleiotropic class.

Definition of two poles

Pleiotropic (mutation-mediated) adaptation: fixed, irreversible changes in DNA sequences that confer stable phenotypic traits (e.g., beak morphology in finches,³⁵ pigmentation in moths).³⁹

Anti-pleiotropic (plasticity-mediated) adaptation: flexible mechanisms that modulate gene expression or molecular states to match environmental changes (e.g., temperature-dependent expression⁴³ and diapause regulation in insects).⁴⁵

Key distinction

Pleiotropic adaptation might lock traits into a single optimum, while plasticity-mediated adaptation enables dynamic balance across fluctuating conditions.

Classification of RNA editing

A-to-I RNA editing looks like the “mutation-like” category due to its equivalence to A-to-G variation. But certain adaptive RNA editing sites actually exemplify anti-pleiotropic plasticity because they are flexible, regulatable, and represent a plastic adaptation mechanism mitigating trade-offs between different conditions.⁴⁶

first class of adaptation such as the default logic underlying GWAS analysis⁴⁷ or convergent substitution,¹⁴ whereas the second class of adaptation attracted less attention or was even considered as falling into the scope of developmental biology. Nevertheless, this biased focus was partially resolved with the advent of Evo-Devo⁴⁸ and Eco-Evo-Devo.⁴⁹

Notably, some seemingly dynamic and flexible concepts, such as balancing selection, negative frequency-dependent selection, and positive frequency-dependent selection, essentially belong to the first category of adaptation.^{47,50} Although multiple alleles may exist at a particular locus within a population, which might appear as flexibility, each individual still harbors only one of these alleles. In essence, different alleles adapt to different environments, and the evolution of the population (e.g., changes in allele frequencies) occurs at the cost of individual sacrifices, not through individuals using flexible regulatory mechanisms to adapt to variable environments. Therefore, these types of adaptation are fundamentally different from plasticity-mediated adaptation. Without distinguishing between different types of adaptation, we might be unable to understand the ways in which organisms adapt to their environments, and we may even risk oversimplifying the scientific stories.

We clarified that our concept of flexible (anti-pleiotropic) adaptation does not fundamentally differ from established concepts such as adaptive plasticity and evolutionary developmental biology.⁴⁸ Although this may resemble a rebranding of established concepts, this does not necessarily mean that it lacks genuine conceptual value. We believe it is useful to introduce our idea of two poles of a continuum when discussing adaptation. To help understand, we raise an analogy. At the early stage of learning mathematics, geometry and algebra may appear to be two separate branches. However, after learning analytic geometry, one realizes that geometry and algebra are complementary and that there is no absolute boundary between them. The study of adaptation is similar. In essence, an adaptive process may occupy a certain position along a continuum between two conceptual poles. However, in some classical examples (like those introduced above), each evolutionary story seems to automatically guide readers toward one of the two poles, while giving little attention to the other. This might create the impression of a dichotomy. In reality, organisms usually adapt to their environments through multiple strategies simultaneously: some rely more on hardwired mutations, whereas others rely more on plasticity. We therefore encourage the community to describe adaptive stories from a broader concept before positioning each case within this continuum.

Adaptive RNA editing is mutation-like but actually involves flexible regulation

A-to-I RNA editing, due to its biochemical similarity to A-to-G mutations,⁵ is often discussed in the context of mutation-driven adaptation, aligning with the first type of adaptation. For instance, *in vitro* experiments might investigate whether protein versions resulting from A-to-G mutations enhance specific molecular functions.²⁶ Moreover, considering the ancient and essential immune protector role of RNA editing in preventing MDA5 from sensing endogenous dsRNA as non-self,⁵¹⁻⁵³ it is speculated that genomic A-to-G mutations may achieve effects similar to those of A-to-I RNA editing,⁵⁴ implying that people are thinking RNA editing in a mutation way.

Nevertheless, here, we stress that the true elegance of particular adaptive

RNA editing sites lies in its flexible regulation (Figure 1). First, RNA editing exhibits a variety of condition-specificities, as will be discussed later. Second, RNA editing it is not an “all or none” mutation; rather, it has an editing level ranging from 0-100%, with editome-wide median level around 20%.^{55,56} This means that the same site may be edited in only 20% of the transcripts.

While we do not deny the feasibility of studying the broad RNA editing sites through the lens of mutation, particularly in molecular experiments *in vitro*,⁵⁷ we emphasize that our plasticity-based ideas should be considered when discussing the adaptation of RNA editing. To demonstrate the adaptive significance of a specific RNA editing site, we should show that RNA editing offers a functional advantage (usually flexibility) over DNA mutations. Otherwise, we risk reverting to the paradigm of studying DNA mutation-driven adaptation, prompting the ultimate question: why would RNA editing occur instead of a DNA mutation?⁵⁸ Nevertheless, as mentioned by the molecular error theory, adaptive RNA editing sites might only represent a corner of an iceberg, and therefore the anti-pleiotropic nature should not be casually extrapolated to all RNA editing sites.

It is worth noting that the non-100% nature of RNA editing levels can sometimes lead to confusion among experimental biologists. For instance, in plants, C-to-U RNA editing, which serves a mutation correction function, may exhibit editing levels of only 80%.^{28,59} One may ask that shouldn't 100% correction be more ideal? However, if we consider that biological processes are fundamentally chemical reactions, we would not expect every process to operate with 100% efficiency. Under the framework of such basic physical law, it is actually those biological phenomena that consistently achieve 100% completion that are surprising. A typical example of 100% editing is the indispensable *GRIA2* Q>R editing event in mammals,^{50,61} where the cellular system can ensure that no mRNA molecule is left unedited, which is truly astonishing.

Discriminating functionality and adaptation under an RNA editing context

Functionality and adaptation can be discussed at the level of entire mechanism (e.g. ADAR-mediated RNA editing) or at the level of individual case (e.g. particular editing site).⁶² Here, we only discuss their definitions at individual editing site level. In general, functional editing sites are not necessarily adaptive, but adaptive editing sites must be functional. If an A-to-G mutation leads to any molecular or phenotypic changes, then this editing site is functional, such as the several recoding sites demonstrated in the later sections. Functional studies do not concern whether the mutation occurs at DNA or RNA levels because they focus on the difference between post-mutation and pre-mutation states. However, adaptive A-to-I editing sites require that the editable status is fitter than hardwired G in the genome or an uneditable state.⁶³ This statement usually requires phenotypic evidence from fully edited and uneditable mutants (see the later section on experimental validation). If an editing site is not functional, then we will not expect any phenotypic differences between mutants and wild-type.

Notably, one may argue that our definition of adaptive RNA editing is overly stringent. Under such a definition, many classical examples of functional RNA editing in animals would not qualify as adaptive. We therefore clarify that our definition used here corresponds to a strict evolutionary definition of adaptive RNA editing. By contrast, readers outside the RNA editing field may

adopt a broader definition of adaptation, in which functionality is implicitly or directly equated with adaptiveness. As discussed above, we do not recommend applying such a broad definition to RNA editing studies. Instead, functionality and adaptation should be explicitly distinguished.⁶²

Retrospection of the development of RNA editome studies reveals that earlier literature (before ~2018) often defined adaptation in a manner analogous to positive selection. For example, if the ratio of nonsynonymous to synonymous editing sites exceeded the neutral expectation, this was often interpreted as an adaptive signal.^{10,64} In more recent studies, however, the definition of adaptive RNA editing has become stringent. For instance, George Zhang argued that even if nonsynonymous RNA editing in cephalopods is, at the global level, subject to positive selection, RNA editing should still be regarded as non-adaptive if the editable state is not more advantageous than a genomic G.⁶⁵ This logic is consistent with the strict definition adopted in the present article. Although this conclusion in cephalopods was later debated,⁶⁶ the debate arose mainly because additional data altered the results, rather than because the underlying logic of the argument was overturned. Such a conceptual definition of adaptive RNA editing can be later experimentally validated by constructing mutants.

Notably, in theory, condition-specific editing (discussed in the following section) is not a prerequisite for either adaptive or functional editing sites. However, there might be a tendency that condition-specific editing sites are more likely to be adaptive, while constantly edited sites simply resemble DNA mutations. Such DNA-like editing pattern can be functional but is not necessarily adaptive. Chloroplast and mitochondrial RNA editing in plants has high level and relatively less condition specificity compared to animal RNA editing, and accordingly, it is believed by botanists that plant RNA editing generally exerts a restorative role to reverse deleterious DNA mutations.^{31,67,68} These plant editing sites are of essential function, but they cannot be regarded as adaptive since they are not fitter than a fully edited version encoded in the genome. This case also demonstrates that non-adaptive sites are not necessarily non-functional.

Together, the definitions of functionality, adaptation, and condition specificity are not mutually exclusive. They are partially overlapped but can be discriminated by *in silico* and experimental evidence.

A general pipeline for comparative RNA editomics analysis

Ideally, adaptive RNA editing sites can be prioritized through a comparative RNA editomics analysis, then followed by experimental validation to demonstrate how a particular RNA editing site is more advantageous than either genomic allele alone. The upstream analysis can be designed in such a way that it allows us to preliminarily distinguish (potentially) adaptive from non-adaptive RNA editing sites, thereby narrowing down the scope for downstream experimental validation. Below, we outline some general or personalized analytical workflows as a reference for the field.

(1) *De novo* identification of RNA editing sites in specific species. In RNA editomics analysis, *de novo* identification of sites serves as the foundation for all downstream works, especially for non-model organisms that have not yet been studied in terms of RNA editing. *De novo* identification has become a relatively mature process, widely used by different research groups, and discussed in several reviews.⁶⁹⁻⁷¹ In brief, it requires RNA-seq and DNA-seq data from a single individual, accurately aligned to a reference genome to identify true RNA-DNA differences (RDDs) (Figure 2A). For small-bodied insects, where it may be challenging to obtain both RNA-seq and DNA-seq from a single individual, a pooling strategy can be employed, wherein samples from multiple individuals are combined.⁵⁵ This ensures that all individuals used for RNA-seq are also included in the DNA-seq analysis, effectively eliminating interference from SNPs in the RNA-seq data.^{72,73} Moreover, for model organisms with a highly purified genomic background, pooled samples and single-individual sequencing yield similar results. In ideal situations, such as with the ISO-1 strain of *Drosophila melanogaster*, where the FlyBase reference genome is exactly aligned with this strain's genome, there is no need to consider SNPs, and DNA resequencing can be omitted.^{31,74} The only exception to this pipeline is RNA virus which does not have a real reference "genome",^{75,76} then the identification of RNA editing on these viruses is challenging and requires additional caution or techniques.⁷⁰

Interestingly, a common question arises regarding why some small insects

are impractical to perform matched RNA-seq and DNA-seq on a single individual. With the feasibility of RNA sequencing at single-cell level, why is it not possible to achieve this at the individual level? The limitations of single-cell transcriptomics have been discussed in a previous review.⁷¹ Droplet-based sequencing like the 10× technique, which targets only the 3' or 5' end of the RNA,⁷⁷ misses a significant portion of the editing sites and thus has limited value for RNA editing studies. In contrast, full-length sequencing techniques like Smart-seq,⁷⁸⁻⁸⁰ although more comprehensive, come at a high cost and introduce sequencing biases, where only one allele can be sequenced at heterozygous sites, making it difficult to accurately capture RNA editing levels (unpublished). Therefore, matched RNA-seq and DNA-seq from a single individual remains the most effective method for *de novo* identification of RNA editing sites. Note that if one tries to quantify the editing level at known editing sites, software REDtools⁸¹ is helpful and the details will not be discussed in this article.

(2) Overall adaptiveness of RNA editome. Before identifying individual adaptive RNA editing sites, it is useful to first examine the selective constraint on nonsynonymous RNA editing sites at the global level. Compared to synonymous sites, if the nonsynonymous RNA editing levels are significantly higher, this indicates that these recoding events are under positive selection, and *vice versa*.¹⁷ Additionally, calculating the nonsynonymous/synonymous ratio based on the absolute number of sites is another way to assess the overall positive or purifying selection (Figure 2B).^{10,17} The control for this analysis is the expected nonsynonymous/synonymous ratio under neutral conditions, which is calculated using unedited adenosines in the genome considering the editing motif.⁶⁴ Other indirect evidence for the adaptive editome includes whether genes that undergo editing are more conserved than unedited genes (lower dN/dS), whether the edited adenosines are more conserved than unedited ones (higher phyloP score), and whether genes that undergo editing exhibit higher expression levels than unedited genes. In certain insect, mollusk, and fungal species, these positive selection signals hold at least partially.^{9,32} However, such phenomena are absent in mammals,¹⁷ which may be related to the smaller effective population size *N_e*, as mentioned earlier. All of the above analyses can be performed with a single-species and single-condition RNA editome.

The approach of comparing edited *versus* unedited adenosines described above is a typical binary method. This method can also be applied to other within-species genomic analyses related to RNA editing. For example, by dividing the CDS regions into those encoding signal peptides and the remaining parts, it was found that RNA editing tends to avoid occurring in signal peptide regions.⁸² This may be because the sequence of signal peptides is crucial for protein localization,⁸³ and thus not only is it constrained from DNA mutations, but it is also constrained at the post-transcriptional level. This example refreshes the traditional theory in RNA editing community, that conserved regions in the genome are compensated for by post-transcriptional modifications.⁶⁴

(3) Linkage analysis to identify driver editing sites. The bioinformatic identification of RNA editing sites essentially treats each site as an independent variation. However, from a biological perspective, different RNA editing sites do not occur independently. Clustered editing sites are likely to undergo collective editing, with some of these sites acting as drivers and others as passengers.⁸⁴ Drivers are the primary editing targets and are more likely to have significant biological roles, whereas passengers are mostly the result of hitchhiking and might be non-functional. Several studies have systematically conducted linkage analysis of RNA editing sites, revealing that driver sites indeed overlap with sites that have been previously studied for their functional roles, while multiple passenger sites are scattered around a driver site.^{84,85}

In the gene lethal-1 G0196 (bifunctional inositol kinase), there is a highly conserved Ser>Gly recoding site in both *Drosophila* and bees, with an editing level exceeding 50%. Within a 27 bp region upstream and downstream of this site, three weakly edited nonsynonymous sites were identified, which are also conserved in *Drosophila* and bees.⁸⁵ This indicates that the Ser>Gly site might be a driver that leads to hitchhiking of the nearby sites.

The definition of driver sites can be established through a relatively quantitative standard (Figure 2A). For instance, we begin by selecting a focal editing site of interest (S1) and require that at least one nearby editing site

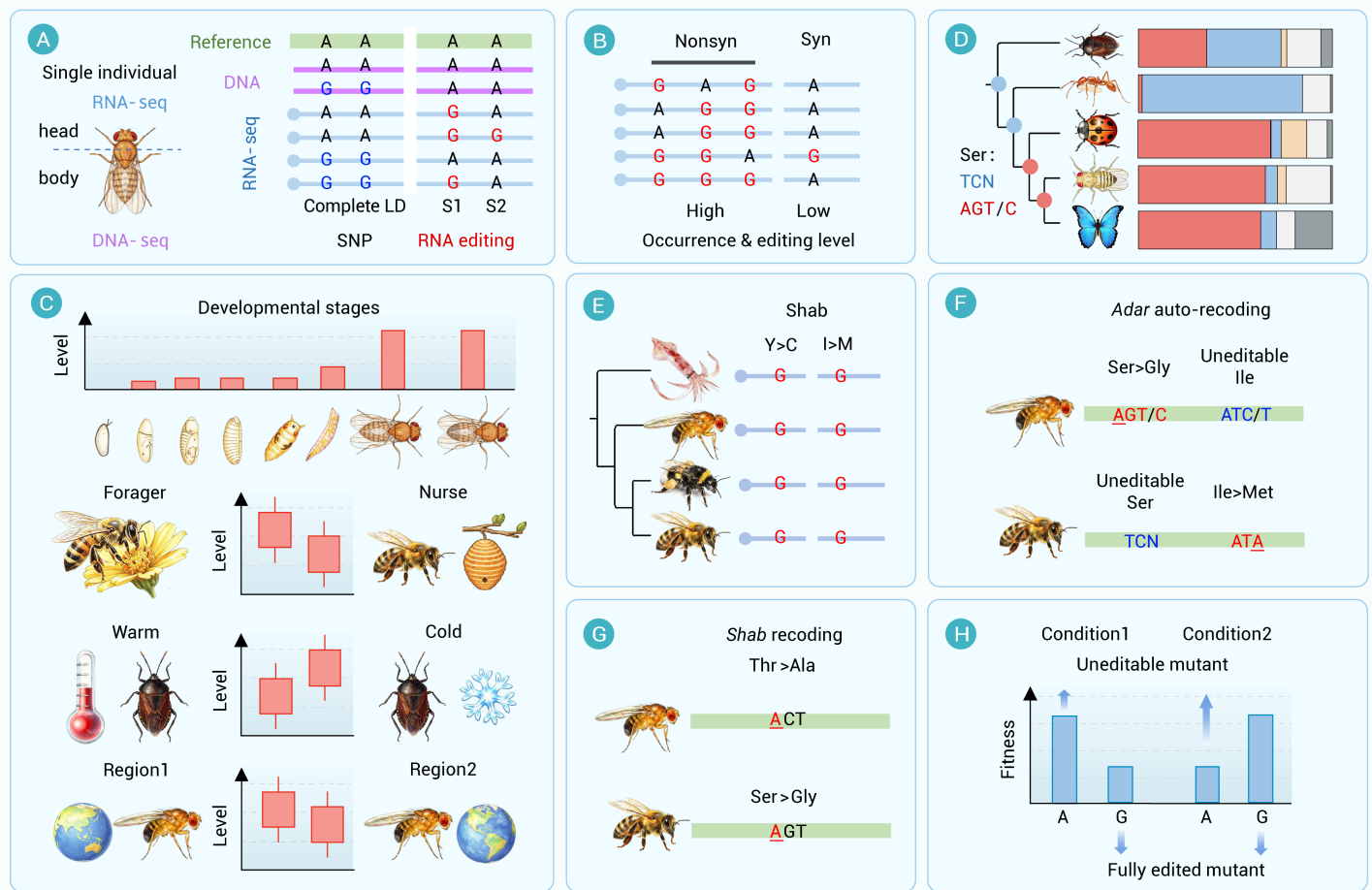


Figure 2. Comparative RNA editomics analysis followed by experimental validation to identify adaptive RNA editing sites (A) Identification of RNA editing sites using RNA-seq and matched DNA-seq from the same individual. Usually RNA-seq from nervous systems like heads/brains was used. Linkage analysis on RNA editing sites can be used to distinguish driver (S1) and passenger (S2) sites. (B) Comparison of the occurrences and editing levels of nonsynonymous and synonymous RNA editing sites. This comparison aims to judge the overall selection force acting on recoding sites. (C) Differential RNA editing across different developmental stages, social castes, environments, and populations. (D) Evolutionary trajectory from uneditable Ser codon (blue) to editable Ser codon (red). The phylogeny of five major insect orders is shown. (E) Deeply conserved recoding sites in potassium channel *Shab* gene. Representative species squid, fruitfly, bumblebee, and honeybee are shown. (F) Convergent evolution of the independent gain of auto-recoding sites in insect *Adar* gene. (G) Conserved editing with non-conserved recoding in *Shab* gene. (H) A schematic diagram showing the expected experimental outcome for adaptive RNA editing sites using fully edited mutant and uneditable mutant. Such results were observed in fungus *Fusarium graminearum* where RNA editing resolves the survival-reproduction trade-off. The approaches illustrated in this figure can serve as a scheme for prioritizing candidate adaptive editing sites. The preceding steps represent *in silico* analyses that help narrow down candidate sites, whereas the final experimental validation provides the decisive evidence.

(S2) be significantly linked to it (FDR < 0.05 in linkage analysis). The significance of linkage disequilibrium (LD) is calculated based on transcriptomic reads following the original LD definition.^{84,86} Next, we require that there are reads where S1 is edited but S2 is not, and that there are no reads where S2 is edited but S1 is not (or allow for a certain margin of error). This standard ensures the importance of the S1 site, indicating that the organism likely aims to edit S1, with S2 editing as a byproduct. In this case, S1 is the driver, and S2 is the passenger. For example, in *Apis mellifera*, the *Adar* Ile>Met auto-recoding site has at least five nearby lowly edited passenger sites that meet the above criteria, which further supports the necessity of editing at the Ile>Met driver site.⁸⁵

Moreover, the combination of LD analysis and manual inspection of reads alignment files offers the advantage of re-confirming and excluding SNP sites. SNPs tend to have strong linkage, exhibiting two clear alleles (haplotypes) in the reads alignment. In contrast, clustered RNA editing sites will show incomplete linkage in reads alignment, exhibiting a rich variety of haplotypes.

(4) Condition specificity analyses narrow down functional sites. The flexibility of RNA editing is manifested in condition specificity, which corresponds to the second type of adaptation defined earlier. There is substantial evidence indicating that particular RNA editing sites can vary across different conditions, including different developmental stages (temporal),⁸⁷ various tissues and cell types (spatial),⁷⁹ different environmental factors,²⁶ different pathological states,⁸⁸ different social castes in eusocial

insects,⁸⁹ different sexes, and different geographical populations (Figure 2C).⁹⁰ It is important to note that condition specific RNA editing is commonly observed as the same site can exhibit differential editing levels across conditions (e.g., 60% editing level in the head, 10% in the body), but occasionally there can be the case of "all or none" difference (e.g., highly edited in the head but unedited in the body, an extreme case similar to the logic of studying DNA mutation). The extent of condition specificity can be quantified by statistical tests such as Fisher's exact test on editing levels under two conditions.⁵⁵

To illustrate the success of specificity analysis in comparative RNA editomics, we first present an opposite example. There are 16 editing sites on the lncRNA *roX1* in *D. melanogaster* which serve as a typical case of non-adaptive RNA editing.⁹¹ The expression of this lncRNA is significantly higher in males than in females; however, no sex-specific differences in RNA editing levels are observed. It is known that the sole function of *roX1* is to participate in male X chromosomal dosage compensation.^{92,93} If the RNA editing events on *roX1* had a functional role, they would likely affect dosage compensation. However, in *Adar* mutants where RNA editing is absent, the dosage compensation of X chromosomal genes in males is unaffected, suggesting that the RNA editing on *roX1* is non-adaptive.⁹¹

Additionally, the analysis of developmental stage specificity, tissue specificity, caste specificity, and environmental sensitivity of editing sites also provides a useful preliminary screening for identifying adaptive sites. This approach has been widely applied in species such as *Drosophila*,^{87,90}

hymenopteran eusocial insects,^{89,94} and hemipteran insects with incomplete metamorphosis.⁵⁵ For example, Hymenoptera *Adar* Ile>Met auto-recoding site has significantly different editing levels between foragers and nurses.⁸⁵ Moreover, the relative level-differences between the two castes are opposite in bumblebees (nurse > forager) and honeybees (forager > nurse), suggesting evolutionary exaptation on an anciently acquired RNA editing site, which might contribute to the flexible genomic regulation of caste differentiation in eusocial insects.

Then, due to the dsRNA preference of ADAR and the temperature sensitivity of dsRNA, it is often observed that temperature is anti-correlated with editing level, serving as a key environmental factor to demonstrate the flexible nature of RNA editing.⁹⁰ Several functional sites that have been recently identified in animals are related to temperature, such as the multiple temperature-dependent recoding of kinesin proteins in cephalopods.^{26,95} Similarly, in *Drosophila*, two local populations living in two sides of the “evolution canyon” exhibit differential editing levels.⁹⁰ The same concept goes for potassium channel *Shab* Ser>Gly recoding in Hemiptera insect *Coridius chinensis* with significantly increased editing level under cold stress,⁵⁵ and the temperature-regulated recoding level in sodium channel subunit TipE of invasive lace bug.⁷³ Of note, these findings have not yet proven adaptation because it has not been clarified why RNA editing instead of DNA mutations were favored in these cases.

In contrast, RNA editing analysis across different populations may easily fall into the GWAS mode in population genomics, which treats RNA editing as DNA mutation (e.g., correlating phenotypes with the presence/absence of an editing event). In fact, a more informative approach would incorporate RNA editing levels into the association analysis and statistical tests, rather than merely considering the presence or absence of RNA editing.⁹⁶ RNA editing sites that exhibit “all or none” differences at the population level are theoretically rare, which is different from the pattern observed in genomic analysis where population-specific variations are common.⁹⁷

(5) Evolutionary analysis on genomic sequences corresponding to editing sites. The above analyses involve the RNA editomes across multiple conditions, but all based on single species. Next, we explore the analysis of RNA editing sites from a macroevolutionary perspective. This type of analysis can be further divided into two levels. First, this part discusses the evolutionary analysis of the genomic sequences at the editing sites.

For instance, the *Drosophila Adar* Ser>Gly auto-recoding site (S>G site) represents a case where the ancestral Ser codon (TCN) was uneditable, but in different insect lineages, it independently evolved into an editable Ser codon (AGC/T).^{98,99} The TCN codon cannot undergo recoding and will only encode the pre-edited Ser version, and the editable Ser codon in *Drosophila* (with editing level ~30% under normal condition) may provide the organism with greater flexibility (Figure 2D).⁷⁴ Interestingly, *Adar* has independently evolved another Ile>Met auto-recoding site (ATA>ATG) in Hymenoptera, with the ancestral sequence being an uneditable Ile codon at the 3rd position (ATC/T), encoding only the pre-edited Ile protein version.¹⁰⁰ Another amino acid that can also undergo an “uneditable-to-editable” evolutionary analysis is Arg, which has six codons.¹⁰¹ We hypothesize that the *Adar* auto-recoding site might be functionally essential. Encouragingly, Savva *et al* has independently validated that S>G recoding reduces the catalytic function of *Adar* enzyme, forming a negative feed-back loop to control the global editing activity.¹⁰² This is the direct evidence justifying the feasibility of our analytical pipeline to preliminarily narrow down functional or adaptive editing sites.

A more general analysis on sequence conservation can be performed to all RNA editing sites. In humans, the overall nonsynonymous editing sites are more likely to be genomically replaced with G in other mammals compared to unedited adenosines in the CDS.¹⁷ This implies the overall non-adaptive nature of recoding. In contrast, the mammalian *GRIA2* Q>R recoding site has a strikingly high conservation level of the genome sequence encoding the pre-edited version Q and is rarely replaced with post-edited R. This demonstrates that the plasticity (editability) at this site is selectively maintained, while the direct genomic substitution might cause unknown trade-offs.¹⁰³

In conclusion, even without cross-species RNA editome, we can still infer the evolutionary trajectory of known editing sites in a particular species. Such analysis can inform us of when did an editing site possibly emerge, and how

an editing site was maintained, providing valuable information about the evolutionary constraint on RNA editing sites.

(6) Multi-species evolutionary analysis on RNA editing events. When RNA editomes from multiple species were obtained, the orthology-based methodology can be employed to align editing sites across different species,⁸⁵ allowing for cross-species comparison of RNA editing levels from the same stage (e.g., adult) or similar tissues (e.g., head or brain). Researchers have found that even highly conserved RNA editing events exhibit species-specific variations in their editing levels.^{64,89,104} This variation may have a neutral explanation, but their highly conserved occurrence indicates functional importance, especially for those recoding sites.¹⁰⁵

Two recoding sites in potassium channel *Shab*, the Tyr>Cys and Ile>Val recoding, are located in the 6th transmembrane domain and are deeply conserved between insects and mollusks.⁹⁴ Both sites have > 50% editing levels in all tested species (> 90% in *Drosophila*), indicating indispensability and functionality (Figure 2E). These sites are promising candidates for adaptive editing, and future studies can investigate whether this strong editing signal has tissue- or stage-specificity, and what trade-off might incur if we directly mutate the genomic adenosine to guanosine.

As a recent literature summarized, high editing level coupled with high conservation level indicates strong candidate for functional RNA editing.⁵⁷ We expand this concept and propose that conservation can be defined in a more flexible way to include the case of convergent evolution, namely the independent acquisition of different RNA editing sites in the same gene.⁹⁸ The two *Adar* auto-recoding sites are the best example, where S>G recoding exists in flies but not bees, and I>M recoding exists in bees but not flies (Figure 2F).

(7) Other evolutionary analyses on recoding events. We propose several additional valuable perspectives that may provide novel insights for the evolution of RNA editing sites. For instance, “convergent recoding” refers to cases where different amino acids are edited in different species or populations, but the resulting amino acid after editing is the same.¹⁰³ Such examples are rare, but the trajectory indicates the demand on the post-edited protein version, resembling that of convergent amino acid substitution in genomic studies.¹⁴ Another example is “conserved editing with non-conserved recoding”, where the edited adenosine in different species is located in non-identical codons, exhibiting conserved editing events but leading to different amino acid changes.¹⁰⁶ A typical example of this can be found in the potassium channel *Shab* gene: in *Drosophila*, the editing site is ACT>GCT (Thr>Ala), while in bees, it is AGT>GGT (Ser>Gly) (Figure 2G). This difference arises because the genomes of *Drosophila* and bees differ by a single nucleotide.^{94,106}

We clarify that comparative RNA editomics is intended to represent a conceptual and analytical framework, rather than a highly standardized analytical pipeline. The specific analyses included in comparative RNA editomics should depend on the available data and the biological question being addressed.

For example, when only a small number of species are available but *de novo* RNA editing identification reveals a large number of editing sites within a species, then global analyses such as the nonsynonymous/synonymous editing ratio may be appropriate for evaluating the overall selective pattern of the editome. However, such datasets may not support extensive cross-species comparisons of RNA editing events. Conversely, when matched DNA-seq/RNA-seq data are unavailable and *de novo* identification of editome is not feasible, researchers may instead use publicly available transcriptomic datasets from multiple species. In this situation, orthology-based approaches are more suitable for investigating the evolutionary trajectories of single or a few conserved editing sites. Similarly, transcriptomic datasets across tissues, developmental stages, physiological states, or environmental conditions can be used to examine the plasticity of known editing sites.

Although comparative RNA editomics integrates existing analytical approaches, we believe that previous RNA editing studies might be constrained by the types of data available, which in turn limits the analytical strategies that can be applied. As datasets become more comprehensive, multiple lines of evidence can be combined, cross-validated, and intersected to narrow down the list of candidate sites. Therefore, giving this integrated

Box 2 | Comparative RNA editomics: a conceptual and analytical framework

Here, we summarize the proposed workflow and its contribution to the field, trying to turn RNA editing analysis into a testable framework for adaptive inference.

Core concept

We propose comparative RNA editomics as a conceptual and analytical framework that integrates RNA editing identification, selection analysis, linkage analysis, condition-specificity analysis, genomic evolutionary analysis, and cross-species comparisons. The output of this framework is not a final adaptive or non-adaptive classification, but a prioritized set of candidate RNA editing sites for downstream functional and evolutionary validation. Adaptive RNA editing is supposed to have an advantage compared to DNA mutations. This is a strict evolutionary definition of adaptation.

Pipeline overview

De novo identification of RNA editing sites using RNA-seq/DNA-seq pairs.

Overall selection analysis on nonsynonymous *versus* synonymous editing sites.

Linkage analysis to distinguish driver and passenger sites.

Condition-specificity analysis across temporospatial or environmental contexts.

Evolutionary trajectory of genomic sites corresponding to RNA editing sites.

Cross-species conservation analysis to detect conserved or lineage-specific editing.

Experimental validation

Using point mutants to test how RNA editing is more advantageous over both fully edited state and uneditable state.

strategy an explicit name (term) is useful for future reference.

Importantly, comparative RNA editomics provides an evidence hierarchy rather than a simple accumulation of existing approaches. It helps distinguish which data can be used for descriptive editome profiling, functional prioritization, candidate adaptive inference, and experimentally validated adaptation. It also shifts the focus from the presence or absence of editing events (the “all or none” logic) to quantitative editability, including editing level, condition specificity, and evolutionary maintenance. Moreover, it can generate both positive and negative prioritization by highlighting candidate adaptive sites while also identifying likely passenger or non-adaptive sites. Then, this framework also encourages researchers to distinguish the origin of an editing event from its evolutionary maintenance. A site may arise through nearly neutral processes, but its long-term maintenance as an editable state requires separate evaluation. Practically, comparative RNA editomics reduces the experimental search space and avoids looking for a needle in a haystack. Overall, comparative RNA editomics is specifically organized around the question of adaptive significance, and its definition of adaptation is formulated in the specific context of RNA editing: namely, whether editability provides an advantage over hardwired genomic states. Functionality is a necessary but not sufficient condition for adaptation, which is one of the central points emphasized in our manuscript. In this sense, comparative RNA editomics has a field-specific conceptual focus that goes beyond the simple integration of existing approaches.

Experimental validation of adaptive RNA editing

When candidate adaptive RNA editing sites have been identified through comparative editomics analysis, functional validation is required. *In vitro* experiments treat RNA editing as a mutation, allowing the demonstration and comparison of the functionality of pre-editing and post-editing RNAs.¹⁰⁷ However, this does not explain why organisms would employ RNA editing instead of DNA mutations. Therefore, further *in vivo* experiments are crucial to demonstrate that the flexible regulation of RNA editing is key to its adaptation.

In *Fusarium graminearum*, several recoding and stop-codon read-through editing sites with adaptive nature have already been validated.^{46,108,109} It is revealed that mutants that exclusively express the edited version (fully edited mutant) show higher fitness during the sexual reproduction stage compared to mutants that only express the unedited version (uneditable mutant), whereas the fitness is reversed during the asexual reproduction stage (Figure 2H). Wild-type fungi specifically edit RNA during sexual reproduction, resolving the survival-reproduction trade-off caused by a hardwired DNA mutation.^{108,109} However, such adaptive RNA editing sites have yet to be discovered in animals. The few functional RNA editing sites verified in *Drosophila* (I>V recoding in glutamate-gated chloride channel GluClα enhances olfactory responses to odors) (Figure 3A),⁵⁷ mice (Q>R recoding in *GRIA2* controls Ca²⁺ permeability) (Figure 3B),⁶⁰ and cephalopods (K>R recoding in kinesin enhances movement) (Figure 3C)²⁶ are insufficient to demonstrate the advantages of RNA editing over DNA mutations, because

the post-editing version seems constantly better than the pre-editing version and thus a genomic mutation would be more straightforward.

However, this does not mean that animals lack adaptive editing sites. It is more likely that their adaptive significance has not been discovered yet. As discussed in the definition of functionality and adaptation, functional studies tend to focus on “whether an A-to-G change causes a functional alteration” instead of from an evolutionary perspective that “why organisms use RNA editing rather than a direct DNA mutation”. Therefore, some studies in animals have constructed either A-to-G mutants or editing-abolished mutants, and compared them with the wild type in terms of fitness.⁹ Once a difference is observed, the RNA editing event is considered functional. However, unlike studies in fungi, animal studies have not yet used two extreme mutant types to demonstrate the adaptive significance of RNA editing. We hypothesize that although the edited version of GluClα in *Drosophila* enhances olfactory responses to odors,⁵⁷ if a broader range of phenotypes were measured in a comprehensive “whole-body checkup” manner, certain conditions might reveal that the edited GluClα version is actually disadvantageous. This would suggest that a DNA mutation could generate a pleiotropic trade-off, whereas RNA editing provides a flexible way to resolve this trade-off. Thus, the issue is not that adaptive editing is absent in animals, but rather that experimental designs seem to focus more on functionality than on adaptation. Moreover, measuring phenotypes and fitness in animals might be more difficult than in fungi, which may explain why adaptive RNA editing has not yet been reported in animals.

Why has research on adaptive RNA editing sites in fungi advanced faster than in animals? (1) First, constructing point mutations in fungi is easier than in animals. Creating point mutants is a direct way to compare the outcomes of editable *versus* hardwired states. (2) Fungi exhibit strong RNA editing sequence preference, enabling the prevention of RNA editing at focal sites by altering a single upstream nucleotide.³ By designing upstream mutations as synonymous mutations, a perfect uneditable mutant expressing only the pre-edited version can be obtained.^{108,109} In animals, RNA editing sequence preference is weaker than in fungi, making such modifications unfeasible. Mutants can only be constructed by designing specific codons, such as the above-mentioned editable Ser codons *versus* uneditable Ser codons, limiting the number of available sites.^{98,99} (3) Fungi are unicellular organisms, and thus lack tissue or cell type specificity. In contrast, animals have numerous cell types in the brain and nervous system, with different editing levels in different cells, potentially diluting signals and hindering the preliminary identification of candidate adaptive editing sites. Moreover, single-cell sequencing has technical limitations.⁷¹ (4) The stage-specificity of mRNA editing in fungi is strong, occurring only during sexual reproduction, whereas during asexual reproduction, the key Ame1 factor is not expressed, and thus RNA editing does not occur.¹¹⁰ This significantly facilitates the discovery of trade-off-related phenotypes. In animals, identifying whether a particular editing site resolves any trade-offs is akin to finding a needle in a haystack, as it requires considering multiple possibilities such as trade-offs at different developmental stages, in different tissues, and between sexes.

Notably, the convenience of adaptive RNA editing study in fungi can be

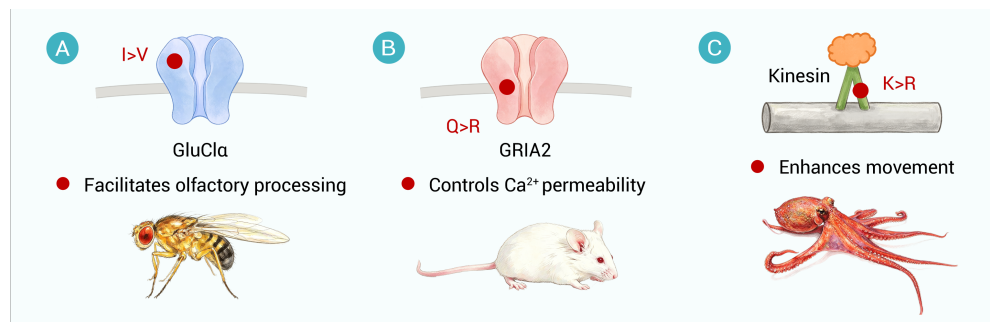


Figure 3. In search of functional and potentially adaptive RNA editing sites in animals (A) The I>V recoding site in *Drosophila* GluClα enhances olfactory responses to odors. (B) The Q>R recoding site in mammalian *GRIA2* controls Ca²⁺ permeability. (C) The K>R recoding in cephalopod kinesin enhances its movement on microtubules. How RNA editing is selectively superior to DNA mutations remains to be validated in these cases.

theoretically applied to bacteria. However, A-to-I mRNA editing in bacteria was considered rare and was mainly associated with a limited number of bacterial species, where the tRNA adenosine deaminase TadA was reported to sporadically modify a small set of adenosines in mRNAs.^{4,111} This view has only been revised by recent studies, which showed that bacterial A-to-I mRNA editing is broadly distributed across bacterial lineages, guided by sequence motifs, and capable of producing functional consequences.^{33,112} The identification of a conserved local motif, YTACGAA, around edited sites suggests that bacterial mRNA editing can be directed by *cis*-regulatory sequence context rather than occurring merely as stochastic noise.³³ Editing events in transcripts such as *hokB* and *fliC* can recode protein sequences and affect toxin-antitoxin regulation, *in vivo* disulfide bond formation, flagellar motility, and adaptation to thermal or oxidative stress.^{33,112} Overall, although bacterial RNA editome research did not begin late, large-scale identification of editing sites and comparative genomic analyses have only emerged recently, delaying the development of the concept of adaptive RNA editing in bacteria.⁹

In plant organelles, C-to-U RNA editing is widely interpreted as a restorative mechanism that compensates for DNA mutations. Early evolutionary discussions proposed that many plant organellar editing events convert genomically encoded residues into amino acids that are more conserved across homologous proteins, suggesting a repair-like function of editing.¹¹³ This interpretation was later examined in an explicit evolutionary framework, in which plant organellar RNA editing was discussed in relation to natural selection *versus* genetic drift, with many sites being consistent with the restoration of conserved or ancestral protein states.¹¹⁴ Consistently, subsequent reviews have summarized chloroplast and mitochondrial C-to-U editing as a process that often repairs point mutations at the RNA level.^{115,116} Together, these studies support the view that a substantial fraction of plant organellar C-to-U editing sites are restorative. According to George Zhang's theory, these editing sites are not fitter than a genomic mutation and therefore should be defined as non-adaptive.⁶⁵ As a consequence, we do not often hear from plant biologist regarding the adaptation of C-to-U RNA editing, unless the definition has not yet been separated from positive selection or functionality.^{28,59}

Differences between comparative RNA editomics, epitranscriptomics, and comparative genomics

Comparative RNA editomics is not meant to replace existing concepts such as epitranscriptomics or comparative genomics. Epitranscriptomics broadly studies RNA modifications, whereas comparative genomics focuses on DNA-level evolutionary changes. Comparative RNA editomics specifically centers on RNA editing events and their editing levels, integrating transcriptomic, genomic, and evolutionary information to evaluate whether particular editing sites are likely to be functional, condition-regulated, or candidate adaptive.

The distinction between RNA editing and other RNA modifications, such as methylation, lies in the recoding nature of RNA editing. A-to-I or C-to-U RNA editing changes the base itself, whereas methylation does not.¹¹⁷ This directly raises the question why organisms use A-to-I RNA editing rather than an A-to-G DNA mutation. By contrast, m⁶A methylation does not raise a comparable question, because m⁶A does not mimic another non-adenosine base and therefore lacks recoding capacity. In this sense, while other RNA modifications are primarily studied through molecular biology and biochemistry, RNA editing is more fundamentally related to evolutionary

biology. This is also why the research approaches discussed here are mainly framed from an evolutionary perspective rather than based on wet-lab experiments.

In addition, editing level is an essential parameter in RNA editing researches. For other modifications such as methylation, although it is theoretically possible to measure and calculate the methylation level at a particular m⁶A site, researchers more often focus on the presence or absence of the modification. In some cases, the scope of analysis even shifts from individual sites to entire genes, asking only whether a gene contains m⁶A sites and comparing modified *versus* unmodified genes, without focusing on the sites themselves.^{118,119} These differences in research logic reflect the fundamental distinction between comparative RNA editomics and epitranscriptomics.

Limitations and future perspectives

Comparative RNA editomics should be viewed as a hypothesis-generating framework. It integrates RNA editing identification, editing level comparison, linkage analysis, conservation analysis, and evolutionary interpretation to narrow down candidate sites. However, this term is conceptual and exploratory in nature, and it cannot by itself prove adaptive significance. Differences in sequencing depth, read length, tissue composition, genome quality, mapping strategy, and statistical power can affect editing site discovery and editing level estimation. Therefore, candidates prioritized by comparative editomics require careful validation using matched genomic data, controlled experimental designs, and functional assays. Nevertheless, while low sequencing depth reduces the accuracy of editing level, we stress that the quantitative differential editing analysis across datasets relies on Fisher's exact tests which take sequencing depth into account,^{72,73} and therefore the differential editing sites (DESSs) are usually of sufficient depth and accurate editing levels. For non-DESSs, their editing levels need to be interpreted with caution.

The advances in sequencing technologies, bioinformatic tools, and optimized pipeline might facilitate the application of editomics analysis. For example, the base accuracy of both next generation sequencing and third generation sequencing has increased through the past decades,^{120,121} which largely benefits the variant calling in RNA editing analysis. In transcriptomic analysis, short reads mapping can be facilitated by using multiple aligners to increase accuracy, and this strategy has been proven successful in reducing misalignment rates in the identification of RNA editing sites.⁷¹⁻⁷³ Moreover, although a sufficiently high sequencing depth is required for *de novo* identification of RNA editing sites, the orthology-based methodology partially alleviates this stringency by looking at the orthologous positions of known editing sites in other species.⁸⁵ This strategy usually requires relatively loose criteria for editing signal detection.

In the future, with reduced RNA input requirements for library construction and sequencing, we would expect more refined tissue- or cell-type-specific RNA editing profiles to be revealed. Meanwhile, more small-sized organisms can have heads or brains sequenced to enrich RNA editing events. With improvements in RNA preservation methods during field sampling, RNA editomes from those rare species living in specialized habitats can be obtained. Such RNA editing atlases will further help us decipher flexible and potentially adaptive RNA editing sites.

Together, comparative RNA editomics may contribute to functional genomics by prioritizing a manageable set of RNA editing sites from thousands of candidates. Downstream studies may combine genome editing,

ADAR perturbation, ribosome profiling, proteomics, electrophysiology, behavioral assays, and tissue- or cell-type-resolved transcriptomics to determine whether candidate sites alter protein function, organismal phenotypes, or fitness. In this sense, comparative RNA editomics serves as a bridge between descriptive editome profiling and experimentally testable functional genomics.

Conclusions

In summary, when we discuss the term adaptation, we could understand it as a continuum between two conceptual poles: one pole involves adaptation driven by permanent mutations (pleiotropic), while the other pertains to the acquisition of plasticity to adapt to a changing environment (anti-pleiotropic). Although A-to-I RNA editing might appear to belong to the first category, the true elegance of adaptive editing sites lies in its flexible regulation, placing it firmly within the second category of adaptation. The methods of comparative RNA editomics are diverse, including site identification, linkage analysis, condition-specificity analysis, genomic evolutionary analysis of sites, and the evolutionary dynamics of editing events across multiple species. Such pipeline enables us to preliminarily focus on potentially adaptive sites like the *Adar* auto-recoding in insects, and preclude some non-adaptive sites like those in *Drosophila* lncRNA *roX1*. Following data analysis, experimental validation is crucial to prove the adaptiveness of particular sites, like several recoding or stop-codon read-through editing in fungus *F. graminearum*. Due to various biological characteristics, fungi are more amenable to validating adaptive RNA editing sites than animals, placing them in a pioneering position in this research field. Nevertheless, although adaptive RNA editing sites are intuitively favored by researchers and are readily testable with various approaches, the majority of editing sites might be non-adaptive according to the neutral theory and molecular error hypothesis. Our insights complement traditional views on adaptation and can be applied to any study subjects in evolutionary biology, facilitating future studies in functional genomics.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

None to disclose.

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AUTHOR CONTRIBUTIONS

Y.D, W.C, and H.L designed the paper. Y.D, W.C, and H.L wrote and reviewed the paper. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

ETHICAL STATEMENT AND PATIENT CONSENT

Not Applicable.

DATA AND CODE AVAILABILITY

No new data were created or analyzed in this study.

SUPPLEMENTAL INFORMATION

Not applicable.

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