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REVIEW

A-to-I mRNA editing in fungi: occurrence, function, and evolution

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Abstract

A-to-I RNA editing is an important post-transcriptional modification that converts adenosine (A) to inosine (I) in RNA molecules via hydrolytic deamination. Although editing of mRNAs catalyzed by adenosine deaminases acting on RNA (ADARs) is an evolutionarily conserved mechanism in metazoans, organisms outside the animal kingdom lacking ADAR orthologs were thought to lack A-to-I mRNA editing. However, recent discoveries of genome-wide A-to-I mRNA editing during the sexual stage of the wheat scab fungus *Fusarium graminearum*, model filamentous fungus *Neurospora crassa*, *Sordaria macrospora*, and an early diverging filamentous ascomycete *Pyronema confluens* indicated that A-to-I mRNA editing is likely an evolutionarily conserved feature in filamentous ascomycetes. More importantly, A-to-I mRNA editing has been demonstrated to play crucial roles in different sexual developmental processes and display distinct tissue- or development-specific regulation. Contrary to that in animals, the majority of fungal RNA editing events are non-synonymous editing, which were shown to be generally advantageous and favored by positive selection. Many non-synonymous editing sites are conserved among different fungi and have potential functional and evolutionary importance. Here, we review the recent findings about the occurrence, regulation, function, and evolution of A-to-I mRNA editing in fungi.

Keywords RNA modification · Deamination · Adenosine · Inosine · Sexual reproduction · *Fusarium graminearum* · *Neurospora crassa* · Epigenetic · Adaptation · ADAR · ADAT · Non-synonymous editing

Introduction

RNA editing is a post-transcriptional process that alters the RNA sequences relative to their genomic templates. It was first discovered in the mitochondrial mRNA of kinetoplastid protozoa in 1986 [1], in which many uridine nucleotides are inserted into or deleted from the mitochondrial mRNA precursors to restore their open reading frames (ORFs) [2]. Since then, many other types of RNA editing, including nucleotide insertion, deletion, and substitution, have been identified in eukaryotes and their viruses, bacteria, and archaea [3, 4]. The editing occurs in almost all types of cellular RNAs, including but not limited to messenger RNAs (mRNAs), transfer RNAs (tRNAs), and ribosomal RNAs (rRNAs).

RNA editing of mRNAs is of special interest because it can alter the flow of genetic information. Whereas most of the RNA editing types have been documented in eukaryotic organelle-encoded mRNAs, only two types of RNA editing are known in nuclear-encoded mRNAs: one is the adenosine-to-inosine (A-to-I) editing and the other is the cytidine-to-uridine (C-to-U) editing [3, 4]. C-to-U editing is reported only in nuclear mRNAs of mammals and is catalyzed by members of the APOBEC (apolipoprotein B mRNA editing enzyme, catalytic) family of cytidine deaminases [5, 6]. C-to-U editing also occurs commonly in the plastid and mitochondrial RNAs of flowering plants, despite the essentially different underlying deamination mechanisms [7]. A-to-I editing mediated by the ADAR (adenosine deaminase acting on RNA) family of enzymes is the most common form of RNA editing in the animal kingdom [8]. It converts adenosine (A) to inosine (I) in double-stranded RNA (dsRNA) substrates by hydrolytic deamination of the adenine base [9] (Fig. 1a). The newly formed inosine (I) is recognized as guanosine (G) by various cellular machinery,

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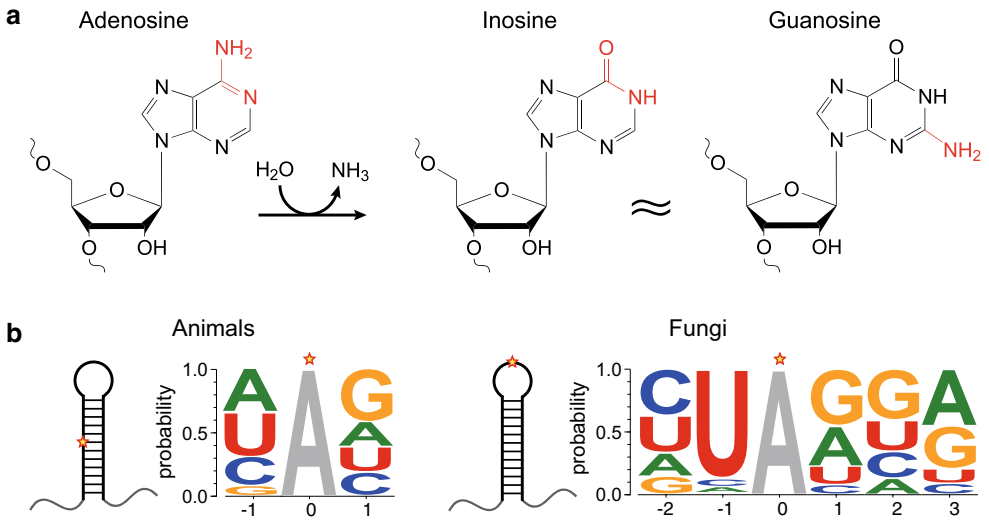

Fig. 1 Deamination of adenosine to inosine. **a** A hydrolytic deamination reaction converts adenosine (A) to inosine (I). Inosine is a guanosine analogue and it pairs with cytidine. Red color marks the difference. **b** Distinct sequence and structure preferences of A-to-I mRNA editing in animals and fungi. In fungi, A-to-I RNA editing occurs mainly in the hairpin loop of folded mRNAs, which differs from the selective targeting of the stem (dsRNA) in animals. Sequence motifs of editing targets in fungi also differ from that in animals [21, 41]. Stars mark the editing site

and the A-to-I editing therefore has a similar cellular function as A-to-G substitution.

A-to-I editing occurs not only in mRNAs, but also in tRNAs in both eukaryotes and prokaryotes [10, 11]. A-to-I editing is found at the wobble anticodon position (position 34) in eight cytoplasmic tRNAs from higher eukaryotes (seven in yeast) and single tRNA ($tRNA^{Arg}$) in bacteria and plant chloroplasts. The position 37 in the anticodon loop of eukaryotic $tRNA^{Ala}$ is also subject to A-to-I editing. The enzymes catalyzing A-to-I deamination in tRNAs are named as adenosine deaminases acting on tRNAs (ADATs). Deamination of A_{37} is catalyzed by ADAT1 (Tad2 in yeast) [10, 12]. Deamination of A_{34} is catalyzed by the TadA (ADATa) homodimer in bacteria and chloroplasts and, in eukaryotes, by the heterodimeric complex composed of two subunits, ADAT2 and ADAT3 (Tad2 and Tad3 in yeast, respectively) [10, 13]. ADAT2 is the catalytic subunit while ADAT3 may serve only a structural role in tRNA substrate recognition [13].

ADAR family proteins exist in all metazoans and appear to be a metazoan innovation [14]. A common feature of ADAR family proteins is a conserved C-terminal deaminase domain and a variable number of N-terminal dsRNA binding domains (dsRBDs) [8, 15]. ADATs contain only a single deaminase domain. The metazoan ADARs were thought to have evolved from an ADAT ancestor by the acquisition of dsRNA-binding domains [16]. Vertebrate genomes encode three members of ADAR family: ADAR1, ADAR2 and ADAR3. ADAR1 and ADAR2 are primarily responsible for the editing activity in repetitive sites and non-repetitive coding sites, respectively, whereas ADAR3 is catalytically inactive and predominantly plays a role to inhibit editing at specific sites [17]. ADAR1 and ADAR2 arose by gene duplication in early metazoan evolution, whereas ADAR3 may have evolved from ADAR2 by gene duplication more recently in vertebrates [14, 18]. Furthermore, ADAR1 or ADAR2 was lost in some lineages during subsequent evolution, such as the loss of ADAR1 in insects [14, 19].

Although A-to-I editing of mRNAs is prevalent in animals, until recently organisms outside the animal kingdom, which do not encode ADAR orthologs, are thought to lack A-to-I mRNA editing. In a recent work, however, Liu and colleagues [20] have identified for the first-time tens of thousands of A-to-I mRNA editing events in *Fusarium graminearum*, a filamentous ascomycetous fungus. Subsequently, A-to-I mRNA editing was also discovered in bacteria, although only 15 A-to-I events were identified [20]. These findings suggest that different mechanisms for A-to-I editing exist in these organisms. Genome-wide A-to-I mRNA editing was also reported in several other different fungal lineages [21, 22], and the dynamic regulation, functional importance and adaptive evolution of A-to-I mRNA editing in fungi were also revealed [21, 23–25]. Collectively, the current studies clearly exhibited the important roles of A-to-I mRNA editing in fungi.

Below we review the most recent and important findings regarding the occurrence, regulation, function, and evolution of A-to-I mRNA editing in fungi. We also discuss differences and similarities of editing characteristics between the fungi and animals. Future research that may shed light onto

the function and mechanism of fungal A-to-I mRNA editing will be proposed.

Discovery of A-to-I mRNA editing in the wheat scab fungus *F. graminearum*

The fungal A-to-I mRNA editing was first observed in the mRNA of a protein kinase named Perithecium unique kinase 1 (Puk1) in *F. graminearum* [24]. *PUK1* is expressed mainly in the later stage of sexual development and plays a specific role in ascospore formation and release [24]. The third intron in the predicted gene model of *PUK1* was found to be inaccurate because it was not spliced in all the transcripts. However, without the splicing of this incorrectly predicted intron, *PUK1* has two premature stop codons $UA^{1831}G$ $UA^{1834}G$ in the kinase domain based on its genomic sequence and encodes a truncated, nonfunctional protein [24]. Surprisingly, in the cDNA of *PUK1* synthesized with mRNA isolated from perithecia, the sequence of these two stop codons was changed to $UG^{1831}G$ $UG^{1834}G$ [24]. Because A-to-I RNA editing events within an mRNA will result in A-to-G changes in its cDNA after reverse transcription, the conversion of $UA^{1831}G$ $UA^{1834}G$ to $UG^{1831}G$ $UG^{1834}G$ indicated the editing of A^{1831} and A^{1834} to Gs in *PUK1* transcripts. This incorrectly predicted intron was artificially introduced into the *PUK1* gene model during automated annotation to cope with these two stop codons.

Genome-wide A-to-I mRNA editing during sexual reproduction in filamentous fungi

Genome-scale analysis of strand-specific RNA-seq data of *F. graminearum* identified more than 26,000 A-to-I editing sites in mature perithecia [24]. In contrast, only around 100 A-to-G changes were detected in conidia (asexual spores) and vegetative hyphae, but there is no enrichment for them relative to other types of nucleotide changes [24]. Manual examination of these A-to-G changes detected in conidia and hyphae revealed that most, if not all, of them were derived from technical artifacts caused by sequencing or read-mapping errors. Consistent with these results, RNA-seq data showed that over 99% of the *PUK1* transcripts had G^{1831} and G^{1834} in perithecia but none in conidia and hyphae [24]. A more in-depth analysis with strand-specific RNA-seq data of different developmental stages of perithecia identified over 41,000 bona fide A-to-I RNA editing sites in *N. crassa* [21]. Likewise, no enrichment of A-to-G changes was observed in conidia and vegetative hyphae of *N. crassa* cultured under different conditions [21]. The average editing level defined as the percentage of edited transcripts over total transcripts at a given site is less than 15% in both *F. graminearum and N. crassa*, which is similar to that of animals [17, 26].

Genome-wide A-to-I mRNA editing was also identified in the perithecia of *F. verticillioides* and *N. tetrasperma*, closely related species of *F. graminearum* and *N. crassa*, respectively [21, 24]. *F. graminearum* is homothallic while *N. crassa* and *F. verticillioides* are heterothallic. They all produce eight ascospores in each ascus. *N. tetrasperma* is a pseudohomothallic fungus that produces four binucleate heterokaryotic ascospores in each ascus. The large number of A-to-I editing sites identified in the perithecia of these species suggests that the prevalence of A-to-I RNA editing is sexual stage-specific but independent of fungal mating systems. The stage-specific occurrence during sexual reproduction is a unique feature of fungal A-to-I RNA editing. In animals, although the abundance varies, A-to-I RNA editing occurs in different tissues and developmental stages [17, 26].

Preferred editing site sequences and secondary structures differ between fungi and animals

Although tens of thousands of editing sites have been identified, only a small portion of As in transcripts are edited in *F. graminearum, N. crassa*, and other fungi, which is similar to RNA editing in animals. However, editing of RNA by animal ADARs has a weak sequence motif flanking the edited adenosines that is depleted of G at the − 1 position and enriched for G or A at the + 1 position [27, 28] (Fig. 1b). This motif preference plays a role in determining the editing specificity but not efficiency [29]. In fungi, a more conserved sequence motif for editing has been identified from the − 2 to + 3 positions [21, 24] (Fig. 1b). The occurrence of U at the − 1 position is 96.7% in *N. crassa* and 85.7% in *F. graminearum* [21, 24], indicating a strong preference. The + 1 and + 3 positions exhibit the enrichment of both A and G. Interestingly, the editing level of edited As with the preferred motif sequences tends to be higher than that of non-preferred ones. These observations suggest that nucleotides surrounding the editing site affect both the editing specificity and efficiency in fungi.

Besides the primary sequence, the secondary structure of RNA around the editing sites also affects the editing specificity and efficiency by ADARs [29] (Fig. 1b). Generally, long, nearly perfect dsRNA structures formed by repetitive elements are edited non-selectively at many sites (hyper-editing), whereas short dsRNAs or long imperfect dsRNAs with mismatches, bulges, and interior loops are edited selectively at one or a fewer specific sites (site-selective editing) [30, 31]. Different from selective editing of adenosines within stems of dsRNA by ADARs, A-to-I RNA editing in fungi preferentially targets adenosines in the hairpin loops

of folded mRNAs [21, 24] (Fig. 1b). The difference in the preferred RNA secondary structure of edited sites in fungi versus animals is consistent with the fact that there are no ADARs in fungi. The preferential editing of adenosines in hairpin loops imply that ADATs might be involved in A-to-I editing of mRNAs in fungi as these might prefer hairpin loops [24]. Furthermore, the sites in hairpin loops have higher editing levels than in other types of elements in the RNA secondary structures. The stability of hairpin loops also affects the editing efficiency [21, 24]. Therefore, the secondary structure features of RNA surrounding the editing sites also play important roles in the specificity and efficiency of fungal A-to-I editing.

Dynamic regulation of A-to-I mRNA editing during sexual development

Perithecium development involves a complex of differentiation processes, including the formation of different sterile tissues and a fertile layer of ascogenous hyphae, asci, and ascospores [32] (Fig. 2). The temporal dynamics of A-to-I mRNA editing in different stages of perithecium development were investigated in *N. crassa* [21]. Overall, the number of A-to-I editing sites increased during perithecium development. A-to-I editing apparently occurs prior to ascus differentiation because approximately 3000 editing sites were detected in developing perithecia at 3-days post-fertilization (dpf) when asci have not been formed. Nevertheless, the vast majority of A-to-I editing events occurred in ascogenous tissues in perithecia. Over 33,000 editing sites were detected in 5-dpf perithecia in the wild type but fewer than 2500 were found in sterile but normal-sized perithecia produced by the Δ*stc*-1 mutant [21]. The number of editing sites in the Δ*sad*-1 mutant that is arrested in the meiotic prophase was 2.5-fold lower than that of the wild type [21]. The editing levels of the A-to-I sites also tend to increase during perithecium development [21]. Therefore, A-to-I RNA editing displays spatiotemporal variations during sexual reproduction, and many editing sites are tissue- or development-specific in *N. crassa*, which may be related to the stage-specific functions of corresponding genes. Nevertheless, the underlying mechanism that regulates the editing activity during sexual development is still unknown. In animals, ADAR enzyme activities are responsible for RNA editing, although additional regulators and modifiers of RNA editing may exist [33]. In fungi, it is also possible that the tissue- or development-specific RNA editing is regulated by the activities of the deaminases responsible for A-to-I editing and their co-factors in perithecia.

High fraction of non-synonymous editing in fungi

The number of editing sites varies considerably in different animal species. For examples, millions of A-to-I editing sites have been identified in human and octopus [17, 27, 34–36], but only tens of thousands of editing sites have been found in mouse, *Drosophila*, and a nematode [17, 27, 37] (Fig. 3a). However, when normalized by the genome size, the abundance of A-to-I RNA editing in humans and octopus is comparable to that of fungi and other animals (Fig. 3b).

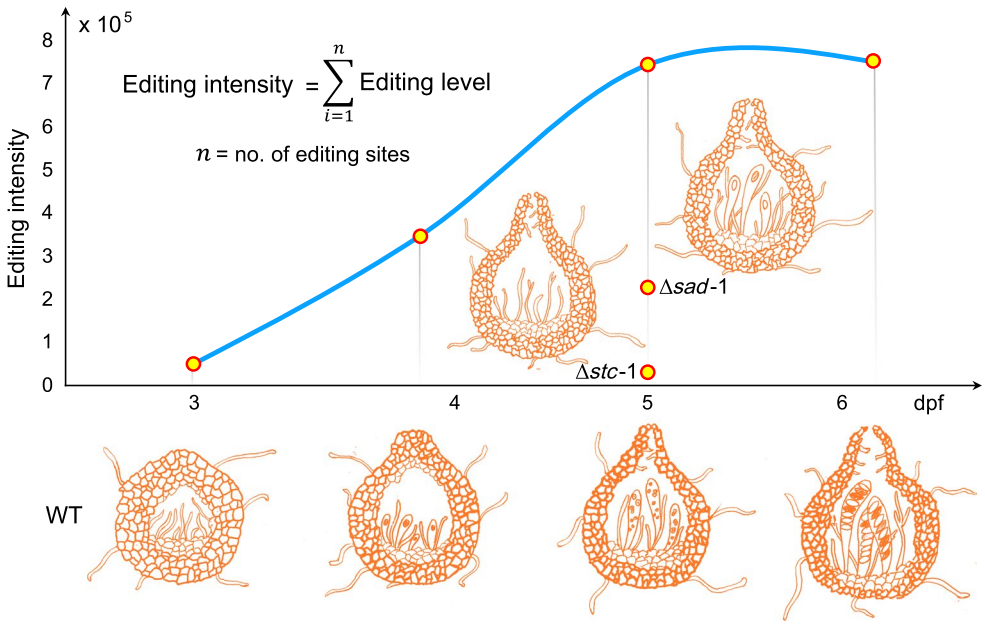

Fig. 2 Spatiotemporal dynamics of A-to-I mRNA editing during sexual development. Illustrations depict perithecia of *N. crassa* at sequential developmental stages from 3 to 6 days post-fertilization (dpf) in the wild type (WT) and 5 dpf perithecia of Δ*stc*-1 and Δ*sad*-1 mutants. The editing intensity for each stage or strain was calculated by summing the editing levels of all A-to-I sites based on published RNA-seq data [21]

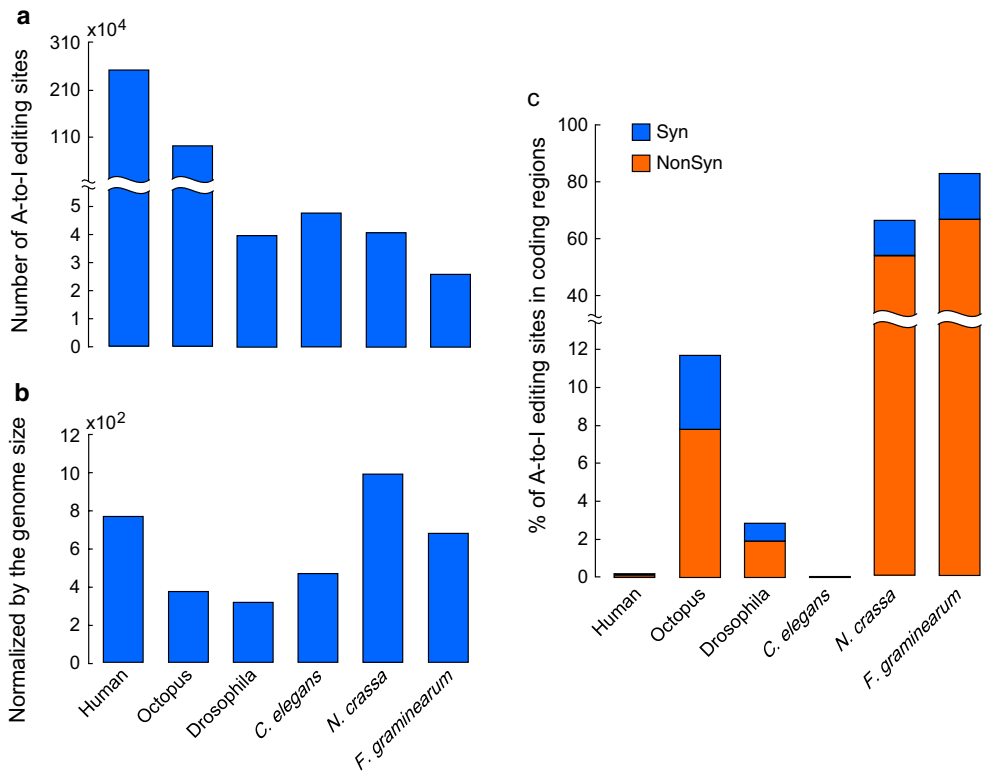

Fig. 3 Comparing number of editing sites and proportion of coding editing sites across different species of animals and fungi. **a** Number of A-to-I RNA editing sites identified in humans [36], *Octopus bimaculoides* [35], *Drosophila* [36], *C. elegans* [37], *N. crassa* [21], and *F. graminearum* [24]. **b** Number of A-to-I RNA editing sites per Mb of the genome sequence of the marked species. **c** Percentages of A-to-I editing sites in coding regions, divided into synonymous (Syn)/non-synonymous (NonSyn) editing sites

Because the readout of inosine is guanosine during translation, A-to-I editing in the coding region of a transcript can result in amino acid substitutions (recoding or non-synonymous editing) that may affect protein function. Despite the capacity for protein recoding, A-to-I editing in coding regions is relatively rare in animals [27, 34, 37, 38], such as 3% in *Drosophila* and less than 0.2% in humans and *C. elegans* (Fig. 3c). Even in coleoid cephalopods that have tens of thousands of recoding sites, the percentage of editing sites in coding regions is still low, less than 12% in octopus [27, 35] (Fig. 3c). However, the majority of the A-to-I editing sites in fungi are in the coding regions [21, 22, 24]. Moreover, about 80% of these coding editing events are non-synonymous (Fig. 3c) and result in variations in protein sequences of about half of the genes expressed during sexual reproduction (4655 out of the 10,652 and 5846 out of the 9302 in *F. graminearum* and *N. crassa*, respectively). A large fraction of proteins is recoded at multiple sites [21, 24].

The vast majority of editing sites in animals occur in non-coding regions associated with repetitive elements [27, 34, 37, 38]. Highly similar repeats can readily hybridize to form long, nearly perfect dsRNA structures that are the preferred targets of the ADAR enzymes for hyper-editing, resulting in the editing of an excessive proportion of adenosines. In humans, ADAR1 is primarily responsible for editing of the repetitive sites, but ADAR2 is primarily responsible for editing of non-repetitive coding sites [17, 27]. *Drosophila* has only the ADAR2 ortholog [14, 19]. Coleoid cephalopods (e.g. squid) have an extra ADAR2 variant with increased editing activities [39]. These observations could explain why the larger fraction of coding editing sites occur in *Drosophila* and coleoid cephalopods relative to other animal lineages. The high fraction of non-synonymous editing in fungi likely results from their distinct editing mechanism.

Non-synonymous editing events are overall adaptive in fungi

It is imperative to ask whether the large fraction of non-synonymous editing in fungi is shaped by natural selection or simply by chance. Analysis in both *N. crassa* and *F. graminearum* showed that the fraction of non-synonymous editing sites is higher than expected under neutral conditions [21, 25], supporting the conclusion that the non-synonymous editing events in fungi are overall beneficial and retained by natural selection through evolution. In addition, the fraction of non-synonymous editing sites generally increases as

editing levels increase [21, 25], suggesting that non-synonymous editing events with higher editing levels are more likely to be advantageous.

Despite a clear benefit of a few editing sites [8], the small fraction of recoding editing sites found in humans is overall non-adaptive, presumably resulting from tolerable promiscuous targeting by the ADARs [40]. However, the non-synonymous editing events occurring in brains or evolutionarily conserved events in *Drosophila* were shown to be generally adaptive even though all non-synonymous editing events were not shown to have an adaptive role [29, 41, 42]. In coleoid cephalopods, although the fraction of all non-synonymous editing sites is expected under neutrality, the recoding sites edited at higher levels and conserved editing sites are commonly adaptive [35, 43]. Together, these results indicate that A-to-I RNA editing plays a larger role in adaptation in the taxa enriched for editing sites in coding regions.

Adaptive advantage of RNA editing events

One intriguing question is what adaptive advantage can be gained from non-synonymous editing rather than directly from non-synonymous DNA substitution. One possible advantage is that non-synonymous editing can generate more proteomic diversity than DNA changes. At a given site, a mutation in DNA generates protein variants only in an all-or-nothing fashion, while RNA editing is almost binary with both edited and unedited versions coexisting in a cell [44] (Fig. 4a). Combinatorial editing of multiple sites can generate numerous protein variants. For instance, two recoding sites will theoretically result in $2^2 = 4$ different protein variants (Fig. 4a). If the proteomic diversity enabled by non-synonymous editing has an overall adaptive advantage, one should expect that recoding adenosine sites are less likely to be replaced with other nucleotides through evolution because such replacements would reduce the protein diversity and fitness. Although no such advantage was found in human [40], the proteomic diversity conferred by non-synonymous editing in fungi was confirmed to provide an adaptive advantage because a lower frequency of replacements was found at the non-synonymous editing sites but not at the synonymous editing sites compared with unedited A sites [21].

Furthermore, genes under stronger functional constraints (more conserved) or functionally critical sites are generally inaccessible through DNA mutations because any mutation would reduce the fitness [40]. Since RNA editing affects only a fraction of transcripts, the non-synonymous editing can generate a low fraction of edited protein variants with an altered function, which are not necessarily beneficial under normal conditions but may be in a changed environment [44] (Fig. 4b). Therefore, another possible advantage is that non-synonymous editing can increase the protein

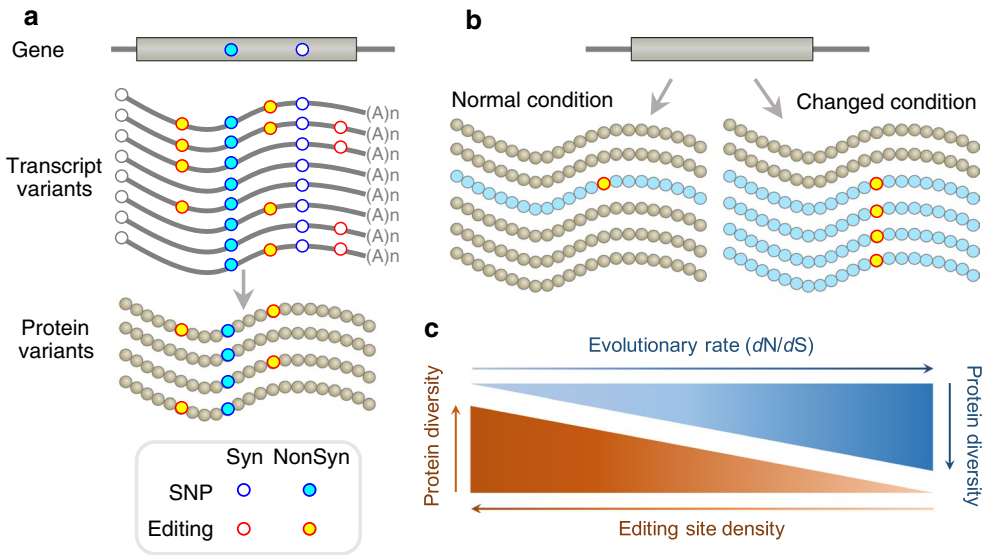

Fig. 4 Advantages of A-to-I mRNA editing for adaptation. A-to-I mRNA editing may be advantageous for adaptation. **a** DNA mutation contributes to genetic diversity for haploid fungi only at the population level, while A-to-I mRNA editing increases transcriptome and proteome diversity for a single cell. **b** RNA editing can generate a low fraction of protein variants with an altered function for functionally constrained genes or at functionally critical sites without reducing the fitness. The edited versions are not necessarily beneficial under normal conditions but may be in a changed environment. The fraction of edited versions can also be regulated for acclimation. **c** RNA editing tends to increase the protein diversity of stronger functionally constrained (more conserved) genes as the density of non-synonymous editing sites were negatively correlated with the rate of protein sequence evolution (d*N*/d*S*). *SNP* single-nucleotide polymorphism, *Syn* synonymous, *NonSyn* non-synonymous

diversity of functionally constrained genes. Indeed, in fungi both the editing density and editing intensity of non-synonymous editing were negatively correlated with the rate of protein sequence evolution (d*N*/d*S*) [21, 25], suggesting that the non-synonymous editing is selected to increase the proteomic diversity of genes under stronger functional constraints (Fig. 4c). Similar results were also observed in *Drosophila* and coleoid cephalopods [29, 35, 41]. Interestingly, in fungi the non-synonymous editing events resulting in somewhat different amino acid changes were found to be favored by positive selection, whereas those resulting in similar or extremely different amino acid changes were not [21, 25]. These results suggest that RNA editing evolved to fine-tune protein functions but avoid destroying protein functions or structures.

In addition, DNA mutations are permanent and hard-wired, whereas RNA editing is dynamic and can be regulated temporally and spatially for function and acclimation. This advantage of RNA editing is reflected in the dynamic regulation of editing in different tissues and developmental stage during sexual reproduction in fungi. Editing level variation of a single non-synonymous editing site in the potassium (K) + channel was demonstrated to be used for temperature adaptation in octopus [45]. In *Drosophila*, several studies have shown that A-to-I RNA editing levels are responsive to temperature alterations and that ADAR plays an important role in temperature adaptation [41, 46, 47].

Functional importance of fungal A-to-I mRNA editing

In animals, the role of A-to-I RNA editing is mainly for fine-tuning neurological functions [8]. The editing events are particularly prevalent in the brain and enriched in genes with neurological functions [17, 29, 35, 42]. Disruption of ADAR function often causes neurological phenotypes [33, 48]. Considering the specificity that occurs during sexual reproduction, the role of A-to-I mRNA editing for fine-tuning sexual functions in fungi is expected. Indeed, massive non-synonymous editing sites are enriched in genes functionally related to sexual reproduction and meiotic cell cycle [21]. Genes with highly edited sites tend to be up-regulated or specifically expressed in perithecia [24]. Therefore, the A-to-I RNA editing may have an important influence on the genes important for sexual development. Beyond the genes related to sexual development, the A-to-I RNA editing may have a global effect on gene expression during sexual development. Genes involved in a variety of biological processes, including chromatin organization and modification, RNA transcription and processing, and protein transport and localization are also enriched for extensive non-synonymous editing sites [21].

Premature stop-codon correction (PSC) editing

Among the numerus nonsynonymous editing events, PSC editing is noticeable. It changes the in-frame stop codons of 'pseudogenes' to amino acid codons in mRNA and enables the expression of full-length proteins during sexual development. The editing of the two tandem stop codons UA^{1831}G UA^{1834}G in *PUK1* ORF is the best example of PSC editing [24]. Tens of PSC editing events were identified in both *F. graminearum* and *N. crassa* [21, 24]. The function of PSC editing is similar to the organelle editing that generally serves as a repair mechanism to correct organelle genome mutations at the RNA level [4].

The importance of individual editing sites for sexual development is well demonstrated by the functional analysis of the 'pseudogenes' with PSC editing events. Because the PSC editing is essential for 'pseudogenes' to express full-length proteins during sexual reproduction, abolishing RNA editing may generate an effect similar to gene deletion. In *F. graminearum*, deletion of the *PUK1* resulted in defects in ascospore formation and release [24]. Expression of the edited version of *PUK1* but not un-edited version rescued the defects in the Δ*puk*1 mutant [24]. Similar results were also reported for *AMD1*, a 'pseudogene' important for ascus maturation and ascospore discharge [23]. In *N. crassa*, three 'pseudogenes' with PSC editing events were found to be important for sexual development [21]. One is the NCU07992, which encodes the ortholog of yeast Spt3, a subunit of the SAGA and SAGA-like transcriptional regulatory complexes [49]. NCU07992 plays an essential role in the early stage of perithecium development; a deletion mutant produced protoperithecia but failed to form mature perithecia [21]. The other two are *stk-21*, the ortholog of *PUK1*, and NCU10184. Both genes are important for ascospore formation [21]. The *stk-21* is also important for ascospore germination [21]. As expected, expression of the un-edited version could not complement the defects in the Δ*stk*-21 and ΔNCU10184 mutants [21]. Therefore, the functional studies carried out in *F. graminearum* and *N. crassa* clearly showed that PSC editing is essential for the function of these pseudogenes and plays an important role in different stages of sexual development. Interestingly, PSC editing was also found to be coordinated with alternative splicing [50] to regulate the expression of genes during sexual reproduction [21].

Stop-loss editing

Different from PSC editing, stop-loss editing changes the canonical stop codons of mRNA to amino acid codons, leading to a C-terminal extension. Although they are rare in animals [51], hundreds of stop-loss edited genes were found in both *Fusarium* and *Neurospora* [21, 24]. When stop-loss

editing occurs, the translation continues to the next in-frame stop codon or to the poly(A) tail at the 3′ UTR of the edited mRNA. Translation of the poly(A) tail can trigger a nonstop mRNA decay mechanism that destabilizes both the mRNA and nascent protein [52, 53]. However, most mRNAs with stop-loss editing events contain downstream in-frame stop codons in their 3′ UTR (unpublished observation), which are outside the scope of the nonstop mRNA surveillance pathway [52, 53]. In animals, the C-terminal-extended proteins that terminated at a downstream stop codon from translational read through were also reported to be destabilized [54, 55]. However, it is well documented in diverse organisms that the translational read through events are functional and regulated to defined levels [56–61]. Remarkably, both the frequency and editing levels of stop-loss editing were significantly higher than those of stop-retaining editing that changed one stop codon to another stop codon, even higher than those of missense editing [21, 25], suggesting that the stop-loss editing is under stronger positive selection and therefore more likely to be functionally important. Therefore, the stop-loss editing may also have an important role in sexual reproduction.

Conserved missense editing

The vast majority of non-synonymous editing events are missense editing that changes one amino acid to a different amino acid. Finding the potentially functional missense editing sites is an important task. RNA editing at the same position shared between different species (conserved) during evolution is more likely to be beneficial and functionally important [62]. As expected, the fraction of conserved editing sites is larger for species that are evolutionarily more closely related [21]. The majority of the editing sites in coding regions are conserved between two *Neurospora* species, and 454 are conserved and shared by all three species—*F. graminearum*, *N. crassa*, and *N. tetrasperma* [21]. Divergence of *Fusarium* from *Neurospora* is estimated to have occurred ~ 364 million years ago (mya), which is similar to that of human from frog (~ 352 mya) (http://www.timetree.org/). In animals, only 59 conserved editing sites are shared between human and mouse [63], and only about 65 editing sites are conserved across the *Drosophila* lineage [42]. A total of 2751 editing sites are conserved and shared between *Octopous bimaculoides* and squid [35], which diverged roughly 200–350 mya [64]. Considering the divergence time, the fraction of conserved editing sites in these fungal species is comparable to that in the coleoid cephalopod species but significantly higher than that in mammals and *Drosophila*.

Similar to that in animals [26, 29, 35, 41, 42, 63], the higher conserved A-to-I editing sites tend to have a higher editing level [21]. The fraction of missense editing sites also increases with the increasing conservation level of A-to-I editing [21]. These results thus suggest potential functional importance and adaptive advantage of highly evolutionarily conserved and highly edited missense sites. This pool of conserved and highly edited sites may serve as the best candidates for future functional validation of their biological roles.

Roles of RNA editing on other epigenetic processes

Besides RNA editing, several epigenetic phenomena are well known in filamentous fungi, including repeat induced point mutation (RIP), DNA methylation, chromatin modification, and two RNA interference (RNAi)-based silencing processes known as quelling and meiotic silencing or meiotic silencing by unpaired DNA (MSUD) [65, 66]. Nevertheless, previously no interactions were known to occur between RNA-based phenomena and DNA- or chromatin-based phenomena [66]. Interestingly, non-synonymous editing sites were found to be significantly enriched in the genes involved in chromatin (histone) modification and gene silencing by RNA in *N. crassa* [21]. Most of the genes known to be important for DNA methylation, histone modifications, quelling and MSUD had multiple recoding sites with high editing levels [21, 67]. These findings indicate that A-to-I mRNA editing may play an important role for the function of DNA methylation, histone modification, and/or RNA silencing during sexual reproduction in fungi.

SAGA is an evolutionarily conserved, multifunctional chromatin-modifying complex that mediates histone acetylation and deubiquitination [68]. The orthologs of two subunits of the SAGA complex are subjected to PSC editing in *N. crassa* [21]. Particularly, the *SPT3* ortholog (NCU07992) was shown to be essential for perithecium development [21]. These results suggest that the components of the SAGA complex may be different between sexual and vegetative stages, and that A-to-I mRNA editing may contribute to the sexual stage-specific function of the SAGA complex. Additionally, in many *Fusarium* and other Nectriaceae species, orthologs of *rid*-1 gene essential for RIP in *N. crassa* [69, 70], were found to contain a premature stop codon that requires A-to-I editing to encode the full-length protein [24, 67]. The finding that the premature stop codon was edited to a sense codon in mRNA during sexual reproduction in *F. graminearum* and *F. verticillioides* [24] suggests that RNA editing may be essential for RIP in these species.

Phylogenetic distribution of A-to-I mRNA editing in fungi

Both *Fusarium* and *Neurospora* belong to Sordariomycetes that produce perithecia. A-to-I mRNA editing also has been reported in another Sordariomycete, *Sordaria macrospora*

[22]. Interestingly, about 2700 A-to-I mRNA editing sites were identified in *Pyronema confluens* [22], an early-diverging filamentous ascomycete belonging to Pezizomycetes, suggesting that the A-to-I mRNA editing occurred in the last common ancestor of filamentous ascomycetes. As in *Fusarium* and *Neurospora*, A-to-I mRNA editing in *S. macrospora* and *P. confluens* also occur specifically in the sexual stage [22].

Considering the importance of *PUK1* and its PSC editing events during sexual development in both *F. graminearum* and *N. crassa*, A-to-I RNA editing is also likely to occur in many other fungal species with the corresponding premature stop codons in the *PUK1* orthologs for encoding full-length functional proteins. By a preliminary analysis, we found at least one of the two premature stop codons in the *PUK1* ORF was commonly detected in members of Sordariomycetes (unpublished results), indicating that A-to-I mRNA editing is most likely to occur commonly in Sordariomycetes. Interestingly, we found several members of Dothideomycetes and Chaetothyriomycetidae that produce pseudothecia [71] also harbor one of the two premature stop codons in *PUK1* orthologs. These results indicate that A-to-I mRNA editing may also exist in these fungal lineages. Although additional data and further analyses are necessary to confirm their occurrence, current data clearly suggest that A-to-I mRNA editing is an evolutionarily conserved mechanism in filamentous ascomycetes. However, we did not detect obvious A-to-I RNA editing events in sexual stage-specific RNA-seq data of *Botrytis cinerea* [72], a member of Leotiomycetes (unpublished results), suggesting that A-to-I mRNA editing was lost in this group of fungi during evolution.

No A-to-I mRNA editing was identified in the budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe* [22, 73]. In basidiomycetes, although over 8000 putative RNA-editing sites were identified in RNA-seq data of mushroom species *Ganoderma lucidum* and *Fomitopsis pinicola* [74, 75], no enrichment of A-to-G mismatches was observed. Therefore, the A-to-I RNA editing is likely a derived trait of filamentous ascomycetes. It will be important to explore the evolutionary origin and current phylogenetic distribution of A-to-I RNA editing in fungi.

Stage-specificity of A-to-I mRNA editing during sexual reproduction

Given the specific occurrence in the sexual stage, it is reasonable to ask why extensive A-to-I mRNA editing occurs only during sexual reproduction. The most straightforward explanation could be that the genes responsible for editing are only active during sexual reproduction. But this cannot answer why the RNA editing activity evolved only in sexual stage. Generation of RNA editing activity in sexual reproductive stages may be neutral, but once it appeared it must have created an immediate adaptive advantage for ancient fungi, and thus was maintained by natural selection [76, 77]. It is possible that RNA editing activity has not arisen in asexual reproductive stages or arose but did not provide an adaptive advantage and was purified by selection.

Fungi often switch to the sexual cycle in response to adverse environmental conditions, such as nutrient starvation and temperature stress [78, 79]. In general, ascocarps of filamentous ascomycetes are complicated structures that are formed under harsh conditions. The potential advantage of sexual reproduction is that sexual recombination during meiosis generates genetic variation into the offspring, some of which may help the progenies to adapt to the changed conditions and survive [80–82]. Consistent with this, the spontaneous mutation rate during meiosis is also elevated in fungi [83, 84]. Therefore, as a mechanism to drive protein diversity, the occurrence of A-to-I RNA editing during the sexual stage is not surprising. However, different from the meiotic recombination and spontaneous mutation that drives adaptation for offsprings, A-to-I mRNA editing provides substantial flexibility of protein diversity for the sexual process itself, possibly to ensure normal sexual development under adverse conditions. Given the abundance of recoding events and important roles of A-to-I editing in sexual development, it is plausible that A-to-I mRNA editing may have driven the evolution of sexual reproduction in filamentous ascomycetes.

Perspectives

The discovery of genome-wide A-to-I mRNA editing during sexual reproduction brings a new perspective to the study of gene expression and sexual development in filamentous fungi. Future research should shed light onto this new and previously unappreciated aspect of fungal biology. The common features of A-to-I mRNA editing in different fungi indicate that editing mechanisms are conserved in these fungi. Because fungi lack orthologs of animal ADARs and the editing features are also distinct from animals, a different enzyme must exist to catalyze RNA editing in filamentous fungi. Because editing preferentially targets adenosine in the hairpin loop of folded mRNAs, a structure similar to the anticodon loop of tRNA targeted by ADATs, we previously speculated that the ADAT2 and ADAT3 may be involved in mRNA editing in fungi [24]. Recent identification of tadA as the enzyme responsible for bacterial mRNA editing activity [20] reinforces our speculation. Therefore, verifying the function of ADAT2 and ADAT3 on fungal A-to-I mRNA editing will be forthcoming in future research. The observation

that ADAT2 and ADAT3 are not specifically expressed in sexual reproduction raises several questions. For example: Why does mRNA editing activity of ADAT2 and ADAT3 occur only during the sexual stage? Are they subjected to stage-specific regulation for activation? Are stage-specific co-factors or accessory proteins required to form editing complexes together with ADAT2 and ADAT3 for mRNA editing? These questions should be addressed in future research. Because ADARs were suggested to evolve from ADATs [16], identification and functional characterization of mRNA editing machinery in fungi will be critical for understanding the evolution of ADARs and their editing activity in animals.

Fungi can be used to study biological functions of RNA editing events in eukaryotic organisms. The studies of several 'pseudogenes' and their PSC editing events clearly showed that these 'pseudogenes' play an important role in different stages of sexual development and that PSC editing is essential for gene function. Therefore, A-to-I mRNA editing is a pivotal regulatory mechanism of fungal sexual development. Although the non-synonymous editing sites were shown to be overall beneficial, more bench work needs to be done to validate the functional importance of individual editing sites for sexual development. It is not surprising that many non-synonymous editing sites may have no obvious functional effects, but the stop-loss and conserved non-synonymous editing sites with higher editing levels are more likely to be functionally important. Therefore, characterizing these editing sites and determining their roles in sexual development should receive priority. It is also important to determine the regulatory roles of RNA editing on other genetic or epigenetic phenomena during sexual reproduction in filamentous ascomycetes.

A-to-I mRNA editing was found to occur before ascus differentiation and primarily in ascus tissues [21]. There still remains the questions of when it first occurs during early sexual development and how it occurs in different tissue types of the ascocarp. The complicated structure of ascocarps present challenges in examining the spatiotemporal aspects of editing. If editing is tissue-specific, the editing levels estimated from present-day RNA-seq data may be underestimated. Fluorescence based real time monitoring or single cell RNA-seq may be useful to determine the spatiotemporal distribution of A-to-I mRNA editing in the future. Whether RNA editing occurs in other developmental stages or conditions at an ultra-low frequency that is difficult to detect remains to be determined.

With respect to acclimation conferred by RNA editing, since sexual reproduction of fungi often occurs in adverse environmental conditions [78, 79], it will be important to investigate how RNA editing assists fungi in adapting to different environments, possibly by examining the editing alteration in response to temperature change and editing divergence of fungal strains from distinct geographical environments.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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