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ADVANCED REVIEW

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Roles of minor spliceosome in intron recognition and the convergence with the better understood major spliceosome

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Abstract

Catalyzed by spliceosomes in the nucleus, RNA splicing removes intronic sequences from precursor RNAs in eukaryotes to generate mature RNA, which also significantly increases proteome complexity and fine-tunes gene expression. Most metazoans have two coexisting spliceosomes; the major spliceosome, which removes >99.5% of introns, and the minor spliceosome, which removes far fewer introns (only 770 at present have been predicted in the human genome). Both spliceosomes are large and dynamic machineries, each consisting of five small nuclear RNAs (snRNAs) and more than 100 proteins. However, the dynamic assembly, catalysis, and protein composition of the minor spliceosome are still poorly understood. With different splicing signals, minor introns are rare and usually distributed alone and flanked by major introns in genes, raising questions of how they are recognized by the minor spliceosome and how their processing deals with the splicing of neighboring major introns. Due to large numbers of introns and close similarities between the two machinery, cooperative, and competitive recognition by the two spliceosomes has been investigated. Functionally, many minor-intron-containing genes are evolutionarily conserved and essential. Mutations in the minor spliceosome exhibit a variety of developmental defects in plants and animals and are linked to numerous human diseases. Here, we review recent progress in the understanding of minor splicing, compare currently known components of the two spliceosomes, survey minor introns in a wide range of organisms, discuss cooperation and competition of the two spliceosomes in splicing of minor-intron-containing genes, and contributions of minor splicing mutations in development and diseases.

This article is categorized under:

RNA Processing > Processing of Small RNAs

RNA Processing > Splicing Mechanisms

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KEYWORDS

major spliceosome, minor intron, minor spliceosome, splicing, snRNA

1 | INTRODUCTION

Removal of intronic sequences and ligation of flanking exonic sequences from nascent transcripts are carried out by the process of precursor messenger RNA (pre-mRNA) splicing, which is an essential step for RNA maturation and gene expression in all eukaryotes (Reviewed in Maniatis & Reed, 2002; Sharp, 2005). In addition to the pre-mRNAs, RNA splicing is also required for removing introns from long noncoding RNAs (lncRNAs), back ligation of exons to create circular RNAs (circRNAs), and biogenesis of small nucleolar RNAs (snoRNAs) and some micro-RNAs (miRNAs; Hirose et al., 2003; Iyer et al., 2015; Li et al., 2018; Ruby et al., 2007). RNA splicing is catalyzed by the spliceosome, a large and dynamic RNA–protein machinery, which consists of five snRNAs and more than 100 proteins and undergoes multiple dynamic rearrangements during the process of assembly, catalysis and recycling (Reviewed in Hoskins & Moore, 2012; Kastner et al., 2019; Wan et al., 2020). Two separate machineries, the major and minor spliceosomes, have been found to coexist in most metazoans and a few higher species of fungi (Burge et al., 1998; Hall & Padgett, 1996; Larue et al., 2021; Lin et al., 2010; Montzka & Steitz, 1988). The first-known differences were their snRNA components: the major spliceosome has U1, U2, U4, U5, and U6 snRNAs, whereas the minor has U11, U12, U4atac, and U6actac besides the shared U5 snRNA (Montzka & Steitz, 1988; Sharp & Burge, 1997; Shukla & Padgett, 1999; Tarn & Steitz, 1996a, 1996b). Limited by scarcity and instability (Younis et al., 2013), protein components of the minor spliceosome have not been fully addressed, except for the early-identified seven unique proteins in the U11/U12 di-snRNPs (Benecke et al., 2005; Turunen et al., 2008; Will et al., 1999, 2004). Intriguingly, recent investigations allow us to see more differences in protein components between the two spliceosomes (Bai et al., 2021; de Wolf et al., 2021; Suzuki et al., 2021).

It is believed that more than 99.5% of introns are recognized and removed by the major spliceosome in metazoans, while far fewer introns are removed by the minor spliceosome (Chen & Moore, 2015; Lin et al., 2010). Originally, only the AT-AC intronic sequences were identified as minor introns (U12-type introns), in which the first di-nucleotides are AT and the last di-nucleotides are AC, unlike the major introns (U2-type introns), most of which have GT-AG sequences (Hall & Padgett, 1994; Jackson, 1991; Tarn & Steitz, 1996b). Later investigations have found that this AT-AC signature is not stringent; a large subset of minor introns also have GT-AG sequences, and minor introns have more divergent terminal sequences at the 5′ and 3′ splice sites (5′SS and 3′SS) but longer conserved sequences at the 5′SS and the branch site region (BS) than the major introns have (Dietrich et al., 1997). Consensus sequences of minor introns are RUAUCCUUU at the 5′SS, UUCCUURAY at the BS, and YAS (S for G or C) at the 3′SS (Dietrich et al., 1997; Jackson, 1991; Sharp & Burge, 1997). Notably, the minor intronic BSs could form more stable RNA duplexes with U12 snRNA through multiple base-pairings than the major intronic BS-U2 snRNA duplexes could. Based on these characteristics, early bioinformatic tools, such U12DB, ASIP, and ERISdb, were developed to predict minor introns in metazoans (Alioto, 2007; Szczesniak et al., 2013; Wang et al., 2008); more systematic tools have been established in the past 3 years for a wider range of organisms (Gao et al., 2021; Moyer et al., 2020; Olthof et al., 2019).

However, due to a large number of introns, such as the 319,894 introns in human (Olthof et al., 2019), and similarities between the two splicing machineries, competitive recognition of introns by the two spliceosomes have been found and investigated (reviewed in Akinyi & Frilander, 2021; Jacquier et al., 2021; Li et al., 2020). Usually, there is only one minor intron in a gene (reviewed in Baumgartner et al., 2019), it remains unclear how they are recognized by the minor spliceosome in dealing with splicing of neighboring major introns. In addition, many mutations in the two spliceosomes and intronic splicing signals have been identified as key factors causing developmental defects and human diseases (reviewed in Cieply & Carstens, 2015; El Marabti et al., 2021; Padgett, 2012; Xiong et al., 2015). In this review, we summarize recent progress in minor splicing, compare the composition of the two spliceosomes, survey minor introns in broader organisms, discuss cooperation and competition between the two spliceosomes, and discuss minor splicing mutations that cause developmental defects and human diseases.

2 | COMPARISON BETWEEN THE MAJOR AND MINOR SPLICEOSOMES

To fulfill their catalytic activities in the nucleus, spliceosomes are dynamically assembled on each intron and undergo many steps of conformational rearrangement to build intricate interaction networks of RNA–RNA, RNA–protein, and protein–protein contacts (reviewed in Staley & Guthrie, 1998). The dynamics of the major spliceosome have been well studied during the past four decades (reviewed in Wan et al., 2020; Will & Luhrmann, 2011). First, the U1 snRNP recognizes and binds to the 5′SS, largely through multiple base-pairings between the 5′-end of U1 snRNA and the 5′SS (Seraphin et al., 1988; Siliciano & Guthrie, 1988); meanwhile, two U2 snRNP auxiliary factors, U2AF1 and U2AF2, bind to the 3′SS and the polypyrimidine tract

(PPyT) regions, respectively (Shao et al., 2014; Singh et al., 1995; Valcarcel et al., 1996). Then the U2 snRNP stably binds to the BS through base-pairing, which leads to the formation of Complex A (Xu et al., 2004; Xu & Query, 2007). Second, the U4•U6/U5 tri-snRNP joins in, and thereafter release of the U1 and U4 snRNPs forms Complex B, in which U1 is replaced by U5 and U6 at the 5′SS, and the U6 snRNA also base-pairs with U2 snRNA (Ast & Weiner, 1997; Kandels-Lewis & Seraphin, 1993; Madhani & Guthrie, 1994). Several conformational rearrangements subsequently occur and result in the formation of the catalytic spliceosomal C complex to initiate the two transesterification reactions, which join the two flanking exons and release a lariat intron (Moore & Sharp, 1993; Schwer & Gross, 1998; Staley & Guthrie, 1998). The dynamics of minor spliceosome assembly and disassembly have not been fully investigated; however, it presumably would be comparable to the dynamics of the major spliceosome (Frilander & Steitz, 2001). In most eukaryotes, the minor-specific snRNAs have been well identified and documented as their counterparts in the major spliceosome (Davila Lopez et al., 2008), whereas the protein components, except for the seven unique proteins in the minor U11/U12 di-snRNP, have not been well addressed due to their low cellular abundance until the recent identification of several new minor-specific proteins.

2.1 | snRNA components

Although four out of five snRNAs in the two spliceosomes are different, overall secondary structures and locations of their key functional motifs between the snRNA counterparts are highly conserved (Figure 1). For example, both the U1

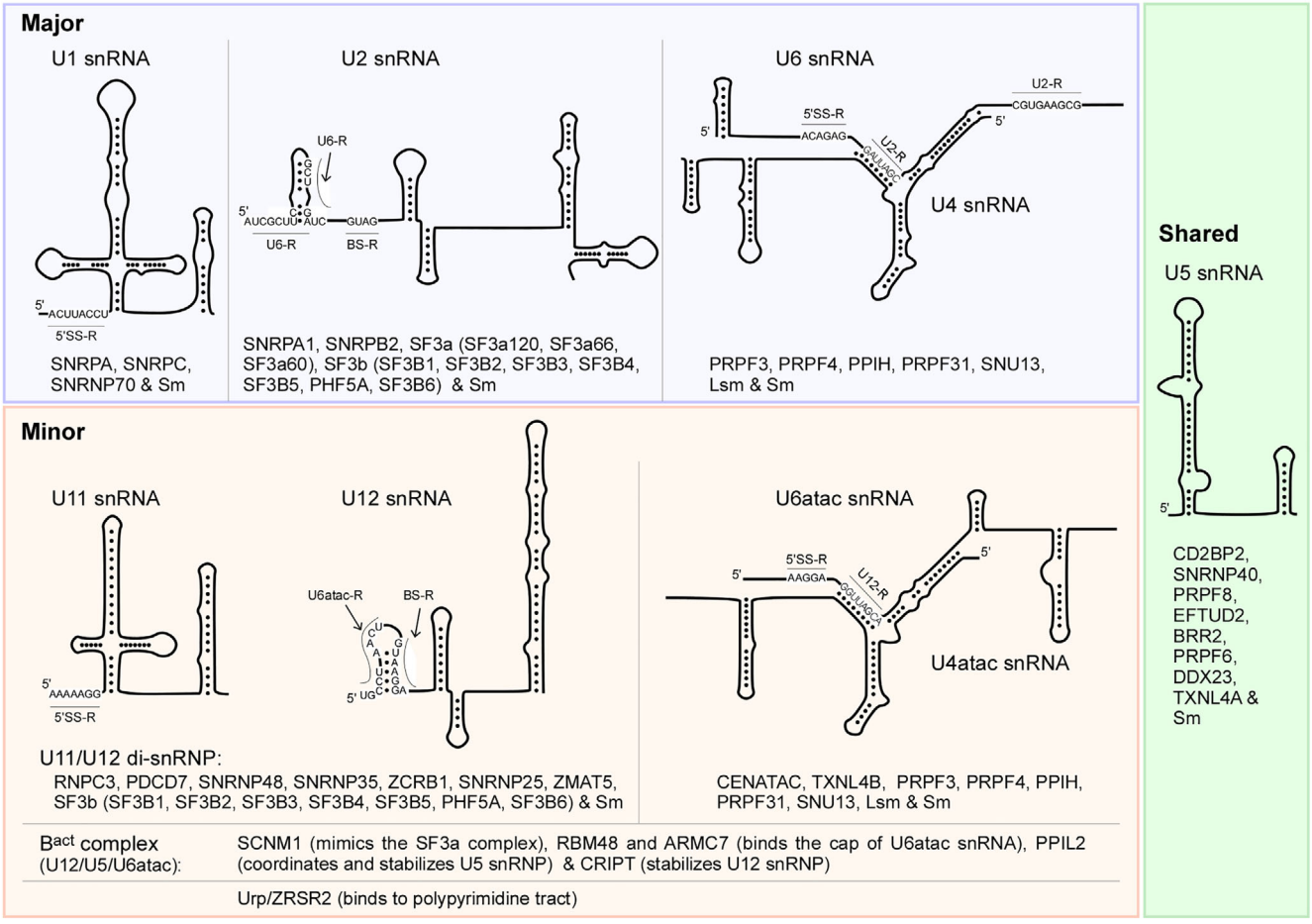

FIGURE 1 Comparison of the human major and minor spliceosomal components. Spliceosomal snRNAs are schematically shown with key functional motifs and secondary structures. 5′SS-R: The 5′SS recognition motif in U1 or U11; BS-R: The BS recognition motif in U2 or U12; U2 (U6, U12, U6atac)-R: Recognition motif that pairs with U2 (U6, U12, U6atac). Protein components from snRNP or complexes are listed below the associated snRNAs. Secondary structures were predicted using RNAstructure v6.2 software, functional motifs are from Turunen et al. (2013). Protein information was obtained from the literature (Bai et al., 2021; Benecke et al., 2005; de Wolf et al., 2021) and the Rfam database (http://rfam.xfam.org).

and U11 snRNAs have the 5′SS recognition motif (5′SS-R) at their 5′-ends, both the U2 and U12 snRNAs have the BS recognition motif (BS-R) in or near their Stem I structures, and both the U4 and U6, and U4atac and U6atac snRNAs are extensively base-paired with each other, exhibiting similar secondary structures (Montzka & Steitz, 1988; Tarn & Steitz, 1996a). In the recently solved cryo-EM structures, the minor and the major spliceosomal B^{act} complexes exhibit close similarities in the overall structures of the U12/U5/U6atac and U2/U5/U6 scaffolds, and RNA elements in the active sites adopt nearly identical conformations (Bai et al., 2021). These characteristics suggest that the two spliceosomes most likely have the same evolutionary origin and share many mechanisms in their intron recognition, assembly, catalysis, and disassembly. However, the primary sequences of key functional motifs are slightly different from those snRNA counterparts. For example, in human, the 5′SS-R is ACUUACCU in U1 but AAAAAGG in U11, the BS-R is GUAG in U2 but GUAAGGA in U12, and the 5′SS-R is ACAGAG in U6 but AAGGA in U6atac (Figure 1). Those differences are consistent with variations of consensus sequences between the major and minor introns, details of which will be discussed below.

Since high-resolution cryo-EM structures of nearly all known major spliceosomal complexes have been successfully achieved in the past decade (reviewed in Kastner et al., 2019; Wan et al., 2020; Wilkinson et al., 2020), the tertiary structures of the five major snRNAs and details of interaction networks in those complexes are now clear, at least for their core parts. However, the small amount of structural information on minor spliceosomal complexes limits our understanding of the tertiary structures of minor snRNAs, except for the U12/U5/U6atac snRNAs in the human minor B^{act} complex (Bai et al., 2021)

2.2 | Protein components

The two spliceosomes share many common proteins and also have their own specific proteins. The first set of minor-specific proteins was discovered in the human U11/U12 di-snRNP (Figure 1, left part below snRNA structures), which lacks all the U1 snRNP specific proteins, U2-A′ and U2-B′ in the U2 snRNP, but has the complete U2-SF3b complex and seven minor-unique proteins, including the 65K/RNPC3, 59K/PDCD7, 48K/SNRNP48, 35K/SNRNP35, 31K/ZCRB1, 25K/SNRNP25, and 20K/ZMAT5 (Will et al., 1999, 2004). In comparison to the major U1 and U2 snRNPs, the U11/U12 di-snRNP is a relatively more stable complex that contributes to 5′SS recognition and intron/exon bridging and definition (Benecke et al., 2005; Olthof et al., 2021; Tidow et al., 2009; Turunen et al., 2008), suggesting that interaction networks differ significantly in the minor versus major spliceosomal Complex A. Later studies demonstrated that this di-snRNP and its protein components are conserved in many minor-intron-containing organisms, such as in mice (Doggett et al., 2018), zebrafish (Markmiller et al., 2014), plants (Jung & Kang, 2014; Kim et al., 2010; Xu et al., 2016), fruit fly (Li et al., 2020; Schneider et al., 2004), and Rhizopus (Russell et al., 2006). Mutations in those protein components result in deficient splicing of minor introns and changed alternative splicing (Doggett et al., 2018; Jung & Kang, 2014; Kim et al., 2010; Markmiller et al., 2014; Xu et al., 2016).

The second set of minor-specific proteins, including SCNM1, RBM48, ARMC7, PPIL2, and CRIPT, were discovered in a recently solved cryo-EM structure of the human minor spliceosomal B^{act} complex, which was captured by a mutated pre-mRNA substrate containing a minor intron (Bai et al., 2021; Figure 1, bottom). The N-terminus of SCNM1 has a C_2H_2-type ZnF domain and shares high homology with that of SF3a66, one of the three subunits of SF3a complex in the U2 snRNP. Interestingly, the whole SCNM1 molecule spans across two opposing sides of the U12 snRNP in the minor B^{act} complex, structurally and functionally mimicking the SF3a complex in the major spliceosome. The RBM48-ARMC7 complex binds the 5′ γ-monomethyl phosphate cap of U6atac snRNA, the U-box protein PPIL2 coordinates loop I of U5 snRNA and stabilizes U5 snRNP, and the cysteine-rich PDZ-binding protein CRIPT stabilizes U12 snRNP. Mutations in RBM48 have been found to result in deficient splicing of minor introns and changed alternative splicing (Bai et al., 2019; Siebert et al., 2020).

In addition, several proteins have been individually identified as minor specific or critical components, including Urp/ZRSR2, CENATAC, and DROL1. The U2AF35-related protein Urp/ZRSR2 is required for splicing of minor introns and is believed to replace two U2AF subunits for 3′SS recognition since minor introns usually do not have an obvious polypyrimidine tract; it is recruited in an ATP-dependent fashion to the 3′SS of minor introns, facilitating the formation of spliceosomal complexes (Horiuchi et al., 2018; Shen et al., 2010). CENATAC is a distinct U4atac/U6atac di-snRNP-associated protein (de Wolf et al., 2021) and DROL1 is a subunit of U5 snRNP and specifically required for splicing of AT–AC-type minor introns in *Arabidopsis* (Suzuki et al., 2021).

TABLE 1 Features of minor intron databases

Database	Species	Algorithm for U12-type intron prediction	References
SpliceRack	4 eukaryotes	PWMs	(Sheth et al., 2006)
U12DB	18 eukaryotes	PWMs	(Alioto, 2007)
ERISdb	7 plants	Random forest classifier	(Szczesniak et al., 2013)
MIDB	Human and mouse	PWMs	(Olthof et al., 2019)
IAOD	24 eukaryotes	*intronIC*	(Moyer et al., 2020)
MMIAD	n.a.	PWMs and probabilistic grammars prediction	(ElKharboutly et al., 2020)

Note: PWMs, positional weight matrices; *intronIC*, intron interrogator and classifier.

3 | MINOR INTRONS AND EVOLUTIONARY DIVERSITY

With one exception (discussed later), the total number of minor introns is less than half a percent of the major introns in each genome. Such a low frequency combined with noncanonical splicing signals have made minor introns often ignored by genome annotation pipelines. Dozens of minor introns that were identified early from mammals, plants, and fruit flies have shown their consensus sequences of RUAUCCUUU at the 5′SS, UUCCUURAY at the BS, and YAS (G or C) at the 3′SS (Dietrich et al., 1997; Jackson, 1991). Using these conserved splicing signals together with the existence of minor-specific (U11, U12, U4atac, and U6atac) snRNA genes, more minor introns in different organisms have been identified, and possible evolutionary models have been proposed.

3.1 | Bioinformatic tools

To find more minor introns, two systematic tools, SpliceRack and U12DB using the above-conserved splicing signals, had surveyed 5 and 20 eukaryotic genomes respectively in 2006 (Table 1) (Alioto, 2007; Sheth et al., 2006). U12DB found 695, 555, 306, 16, 0, and 0 minor introns for *Homo sapiens*, *Mus musculus*, *Arabidopsis thaliana*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae*, respectively. With the improved sequencing techniques of genomes and transcriptomes, more databases for minor introns have been published in recent years, including the ERISdb for seven plants (Szczesniak et al., 2013), the MIDB for humans and mice (Olthof et al., 2019), and IAOD, PID, and MMIAD for more eukaryotes (ElKharboutly et al., 2020; Gao et al., 2021; Moyer et al., 2020).

In the beginning, a prediction method of position weight matrices (PWMs) was used to search the above-conserved splicing signals to define minor introns, such as the representative databases SpliceRack, U12DB, and MIDB. After machine learning approaches appeared in bioinformatics, most intron databases have begun to use the sequences of known major SSs and BSs as the negative data set and the sequences of known minor SSs and BSs as the positive data set to train data in order to obtain a model with suitable parameters, which is then applied to predict classes of new introns. For example, to classify major and minor introns, ERISdb used the random forest classification model which has characteristics of high stability and fast calculation when handling of large amounts of intron data, IAOD used the support vector machine classification model (*intronIC*) which is the latest algorithm to classify introns with greater accuracy, and MMIAD used PWMs together with the probabilistic grammar inference model that improves accuracy. Besides the algorithm, other factors also affect output of each database, such as the length of input sequences, and the number of intron annotations. So far, the *intronIC* is the best algorithm for the prediction of minor introns, while the MIDB reports the largest numbers of minor introns for the human and mouse genomes (770 and 722, respectively), since it added novel introns to genome annotations based on analyses of many recently deep-sequenced human and mouse transcriptomes (Olthof et al., 2019).

Therefore, we updated the consensus sequences of human minor introns according to information from the MIDB that predicts 770 minor introns (Figure 2). In comparison to typical major introns, minor introns usually have a longer and more conserved 5′SS region that pairs with the U11 snRNA, although the last two nucleotides in the upstream exon and the first intronic nucleotide are more flexible (Figure 2, left). Minor introns also have a longer and much more conserved BS that base-pairs with U12 snRNA, which features the sequence UUCCUURAC (Figure 2, middle). The 3′SSs of minor introns are more divergent, usually lacking PPyT, and are recognized by Urp/ZRSR2 (Shen et al., 2010) instead

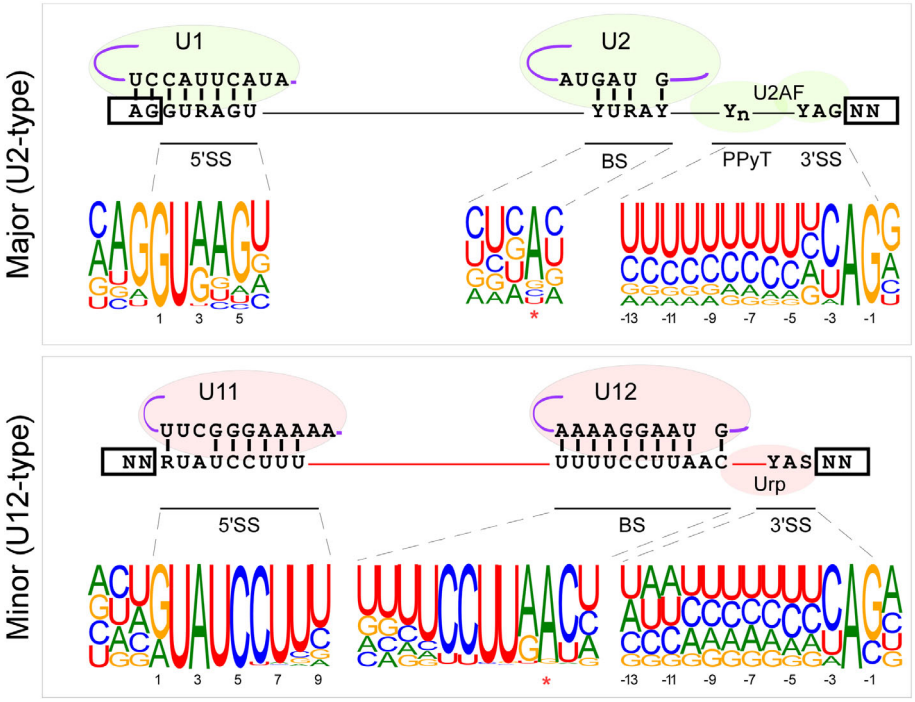

FIGURE 2 Consensus sequences of the human major and minor introns. 5′SS, 5′ splice site region; BS, branch site region; 3′SS, 3′ splice site region; red stars, branch points; R, A, or G; Y, U, or C; S, G, or C. Consensus sequences of the 319,124 major introns and 770 minor introns are projected based on information from MIDB (Olthof et al., 2019).

of the two U2AF subunits that recognized the 3′SSs of major introns (Figure 2, right). In addition, 78 human introns that have AT-AC sequences are classified as major introns due to differences at other positions of minor introns, and this is consistent with previous findings in various species (Chen et al., 2014; Dietrich et al., 1997).

3.2 | Evolutionary diversity of minor splicing

Considering the diversities of genomes and the qualities of sequencing data, it is assumed here that species annotated with one minor-specific snRNA gene would have a minor spliceosome system. So far, 343, 336, 190, and 375 species are listed on the Rfam database (http://rfam.xfam.org) with U11, U12, U4atac, and U6atac snRNA genes, respectively. In the total annotated 14,772 species, 377 species have at least one minor-specific snRNA gene. Analyses of those species suggest that most metazoans and a few evolutionarily higher species of fungi have minor spliceosome (Figure 3). For a better comparison, we here list the numbers of minor and major introns in 31 representative species, ranging from fungi, mold, invertebrates, and plants to vertebrates (Figure 3, outer two circles).

From the view of evolution, several lines of evidence are notable. First, it is notable that no minor snRNAs or minor introns have been found in all the Rfam listed *Chromadorea* nematodes, including *Caenorhabditis elegans* and *Caenorhabditis briggssae*, but have been found in all the *Enoplea* nematodes and other metazoans, suggesting an evolutionary diversity of the minor spliceosome in *Chromadorea* nematode organisms. Second, the length of minor snRNAs in *Drosophila* is particularly variable compared to their counterparts in other organisms (Figure S1). The length of human minor-specific snRNAs are 135 nt for U11, 150 nt for U12, 130 nt for U4atac, and 125 nt for U6atac, whereas those in *D. melanogaster* are 273 nt for U11, 238 nt for U12, 160 nt for U4atac, and 98 nt for U6atac (Li et al., 2020; Otake et al., 2002; Schneider et al., 2004). The obvious differences are multiple extra-large loops in the *Drosophila* U11 and U12 snRNAs, implying that the minor-specific snRNPs in *Drosophila* would have additional or different protein components, as is the case in yeast *S. cerevisiae*, which has large additional sequences in U1 and U2 snRNAs, compared to metazoans (Ares, 1986; Kretzner et al., 1990). Consistent with this, at present, only two homologs of the human seven unique U11/U12 di-snRNP proteins, 65 K and 20 K, have been found in *D. melanogaster* (Schneider et al., 2004).

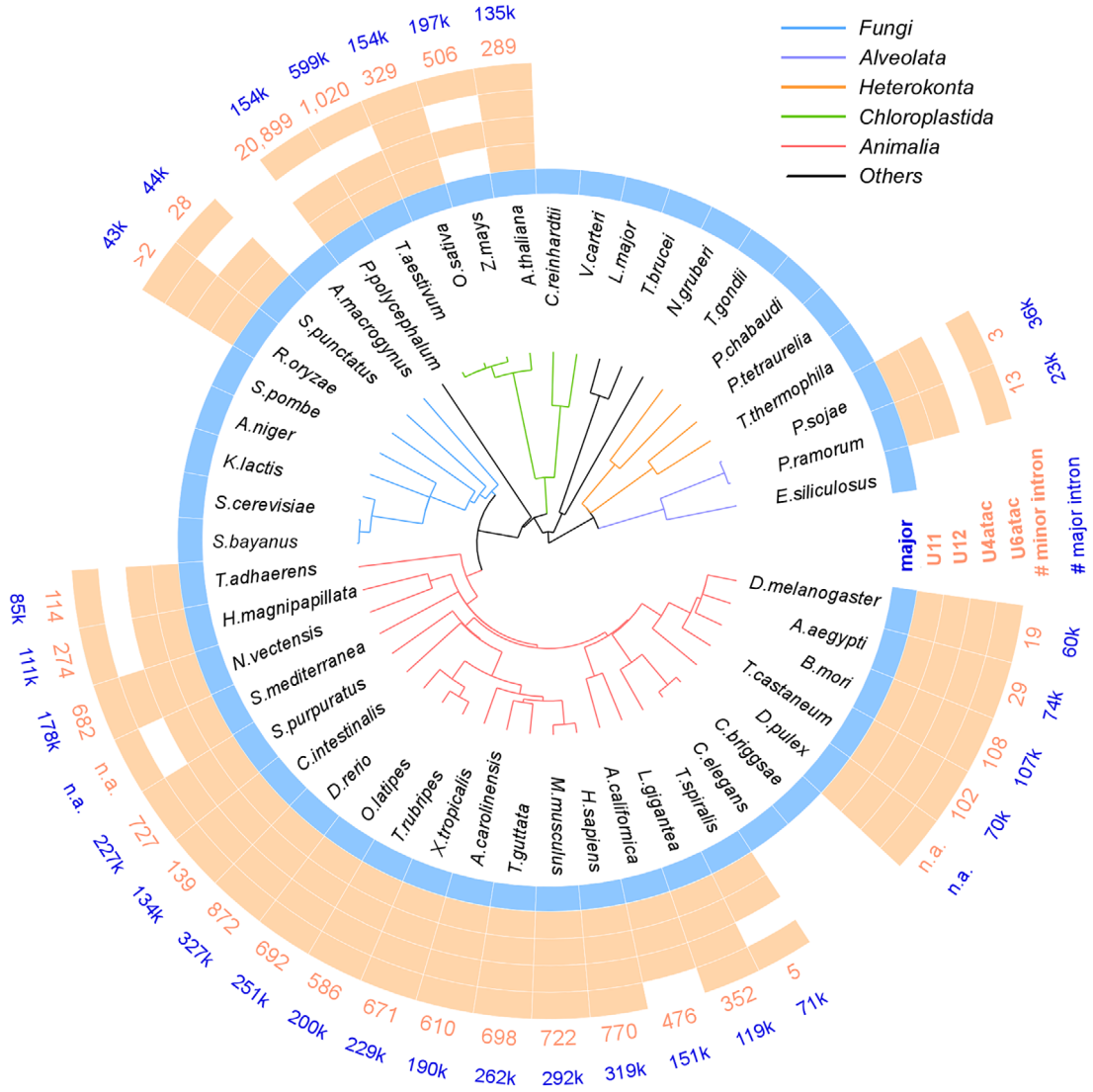

FIGURE 3 Numbers of minor and major introns in the representative species that have minor spliceosomes. Representative species with minor-specific snRNAs are listed according to information on the Rfam database. Numbers of minor introns are obtained from the MIDB for *H. sapiens* and *M. musculus* (Alioto, 2007), from literature for *R. oryzae* (Russell et al., 2006) and *P. polycephalum* (Larue et al., 2021), from ERISdb for *A. thaliana*, *O. sativa*, and *Z. mays* (Szczesniak et al., 2013), and from our calculations using the *intronIC* software (Moyer et al., 2020) for the rest of other species. Numbers of major introns are obtained from literature or *intronIC* calculation.

Third, no known organism has more than 1000 minor introns except a slime mold, *Physarum polycephalum*, which was recently reported to have 20,899 minor introns (Larue et al., 2021). For a unicellular organism, this diversity is huge and interesting. Sequence analysis reveals that the core BS-R motif of U12 snRNA in *P. polycephalum* has two altered nucleotides, from other species' GUAAGGAA sequence to G**C**AA**A**GAA; this allows its perfect base-pairings with the BS consensus sequence UUUUUURAY of minor introns in *P. polycephalum*, maintaining a BS-U12 snRNA duplex with a similar stability as that of other organisms, where the BS consensus sequence is UUCCUURAY (Figure 4). Stability of the BS-U12 snRNA duplex is critical for splicing of minor introns (Brock et al., 2008). This finding in *P. polycephalum* provides a perfect co-evolutionary example of complementary base changes in a core intronic splicing signal and the core part of a spliceosomal snRNA.

To explain the scarcity of minor introns in modern genomes and the existence of some eukaryotic species that lack the minor spliceosome, the origin of spliceosomal introns has been proposed in gain-and-loss and minor-to-major class conversion models (Baumgartner et al., 2019; Burge et al., 1998; Irimia & Roy, 2014; Moyer et al., 2020; Russell et al., 2006). It has been hypothesized that both major and minor introns were gained in eukaryotic common ancestors at similar stages (Baumgartner et al., 2019; Moyer et al., 2020), or the minor introns were gained even earlier (Russell

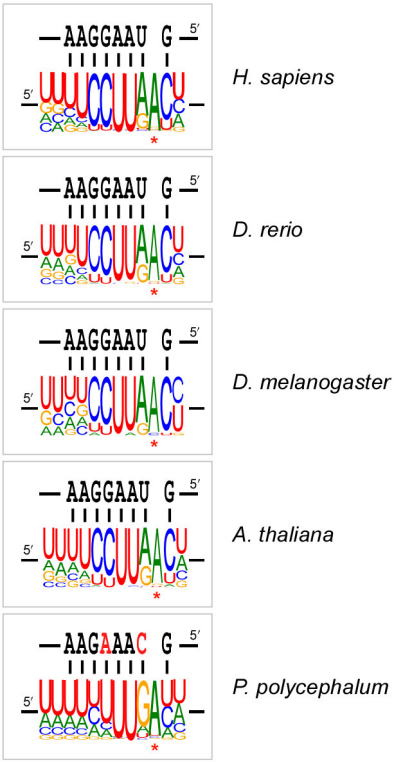

FIGURE 4 Co-evolution of complementary sequence changes of the minor intronic BS and the U12-BS recognition motif. BS consensus sequences are at the bottom in color of each duplex and calculated from minor introns of 770 in *H. sapiens*, 872 in *D. rerio*, 19 in *D. melanogaster*, 289 in *A. thaliana*, and 20,899 in *P. polycephalum*; and the U12-BS recognition motif sequences are at the top in black. The asterisk represents the branch adenosine.

et al., 2006). Introns can be classified into three phases: Phase 0 introns fall directly between two codons of the flanking exons, Phase 1 introns fall between the first and second nts of a single codon, and Phase 2 introns fall between the second and third nts of a single codon. Statistics show that Phase 0 introns are over-represented among major introns, but under-represented among minor introns (Burge et al., 1998; Long et al., 1995; Nguyen et al., 2006; Sheth et al., 2006). Based on this, one hypothesis is that at an early stage in eukaryotic evolution, there were many more minor introns than are currently observed, and the phase bias arose as minor introns were preferentially converted into major introns, resulting in both an over-representation of Phase 0 major introns and an under-representation of Phase 0 minor introns (Moyer et al., 2020). Regarding the identified >20 k minor introns in *P. polycephalum*, the authors found downstream of those minor intron 3′SSs have a more conserved ATAT sequence and proposed a gain model that is driven by DNA transposons, in which the new minor 5′SSs and 3′SSs could be created through the insertion of transposon-elements at a TA motif position with inverted sequences |RTA**TCTTT**... **AAAG**|**A**TAT (|, splice sites; underlines, inverted sequences; Larue et al., 2021).

4 | COOPERATION AND COMPETITION BETWEEN THE TWO SPLICEOSOMES

It has been proposed that exons and introns are recognized and defined by the major spliceosome in two modes, exon definition and intron definition (Berget, 1995; Talerico & Berget, 1994). The exon definition mode is usually for short exons flanked by long introns in higher eukaryotes, whereas the intron definition mode is for short introns that are often flanked by relatively long exons in lower eukaryotes (De Conti et al., 2013; Hertel, 2008; Li et al., 2019; Zlotorynski, 2019). The exon definition mode eventually has to be converted into the intron definition mode for assembly of later-stage active spliceosomal complexes (De Conti et al., 2013; Ellis et al., 2008); one protein, PTB, has been proposed for controlling this conversion (Sharma et al., 2008).

Typically, minor intron is distributed along with multiple major introns in a gene; there are few cases of two minor introns together in one gene. For example, the 770 human minor introns are found in 714 genes, and the 722 mouse minor introns are found in 666 genes (Olthof et al., 2019). In addition, different choices and combinations of splice sites result in alternative splicing that generates multiple mRNA isoforms from one gene, which frequently occurs in higher eukaryotes. More than 95% of human multiple-intron-containing genes are alternatively spliced and produce hundreds of thousands of mRNA products in total (Pan et al., 2008), demonstrating that the proteome complexity is significantly increased and fine-tuned by RNA splicing. Therefore, recognition and splicing of minor introns are inevitably connected with neighboring major introns. Under the exon definition mode, the minor U11 snRNP should cooperate with the upstream binding of major U2 snRNP, meanwhile, the minor U12 snRNP should cooperate with the downstream binding of major U1 snRNP. On the other hand, the existence of nested and overlapped minor and major introns lays a platform for the cell to potentially splice and generate multiple isoforms through competition between the minor and the major spliceosomes. Recently, both cooperation and competition models between the two spliceosomes have been proposed for the splicing of minor introns in multiple-intron-containing genes.

4.1 | Cooperation

In vertebrates, exon definition complexes are usually formed on short exons prior to the assembly of later spliceosomal complexes across the introns. If this applied to exons flanked by two introns of a minor and a major class, the complexes would be composed of mixed snRNPs and splicing factors from both the major and minor spliceosomes, such as U2 snRNP, U2AFs, and U11 snRNPs for definition of the upstream exon, U12 snRNP, Urp/ZRSR2, and U1 snRNPs for definition of the downstream exon. However, these scenarios have been poorly investigated and understood. It was reported that binding of U1 snRNP to the downstream major 5′SS facilitates splicing of the upstream minor intron, and this facilitation would be likely through the definition of the downstream exon (Wu & Krainer, 1996). An in vitro study found that the arginine/serine-rich (RS) domains of SR proteins contact the branch site and 5′SS of a classical human *P120* minor intron, and also contacts the sites of the U6-5′SS interaction and U5-exon 1 interaction (Shen & Green, 2007), suggesting that SR proteins could play an important role in the cooperative cross-talk between the two spliceosomes (Figure 5a). In addition, cooperation of two spliceosomes also occurs for the splicing of mutually exclusive exons (MXEs) in *MAPK8/9* and *TMEM87a/b* genes (Chang et al., 2007; Olthof et al., 2019). Recently, it has been revealed that the minor-specific protein U11-59K directly interacts with the major spliceosomal U2AF complex and other components of the U2 snRNP (Olthof et al., 2021), providing a cooperative cross-talk model in which the minor spliceosome interacts with the major spliceosome across the upstream exon to regulate splicing of minor introns (Figure 5a). Inhibition of U11-59K disrupts this exon-bridging interaction, leading to skipping of the upstream exon, indicating that cross-talk between the two spliceosomal components is critical for efficient splicing of minor introns. However, the definition of the downstream exon of a minor intron remains unclear, and how the upstream and downstream exon definition complexes with chimeric spliceosomal factors would be further converted into across intron definition complexes with sole spliceosomal factors are also unclear.

4.2 | Competition

Competition between the major and minor spliceosomes has been found and/or proposed in many cases, including in vitro and in vivo, under physiological or stress conditions, in mutated organisms or human patients, generating alternatively spliced mRNA products or resulting in aberrant splicing of minor-intron-containing genes, thereby changing the function of genes (reviewed in Akinyi & Frilander, 2021; Chang et al., 2007; Hafez & Hausner, 2015).

According to relative locations of splice sites from the involved major and minor introns, competition models between the two spliceosomes could be roughly divided into three categories: (1) nested introns—a major intron is located inside of a minor intron, or vice versa; (2) overlapped introns—a major intron and a minor intron are overlapped and their splice sites are crossed and interlocked; (3) introns with common SS—a major intron and a minor intron share the 5′SS or the 3′SS, or both SSs (Figure 5b).

Splice site selection of nested introns by two spliceosomes would result in alternative splicing and generate two mRNA isoforms, a short one and a longer one (Figure 5b-I). The presence of *cis*-elements and the corresponding *trans*-factors regulate this competition between the two spliceosomes and determine the cellular ratio of two mRNA isoforms.

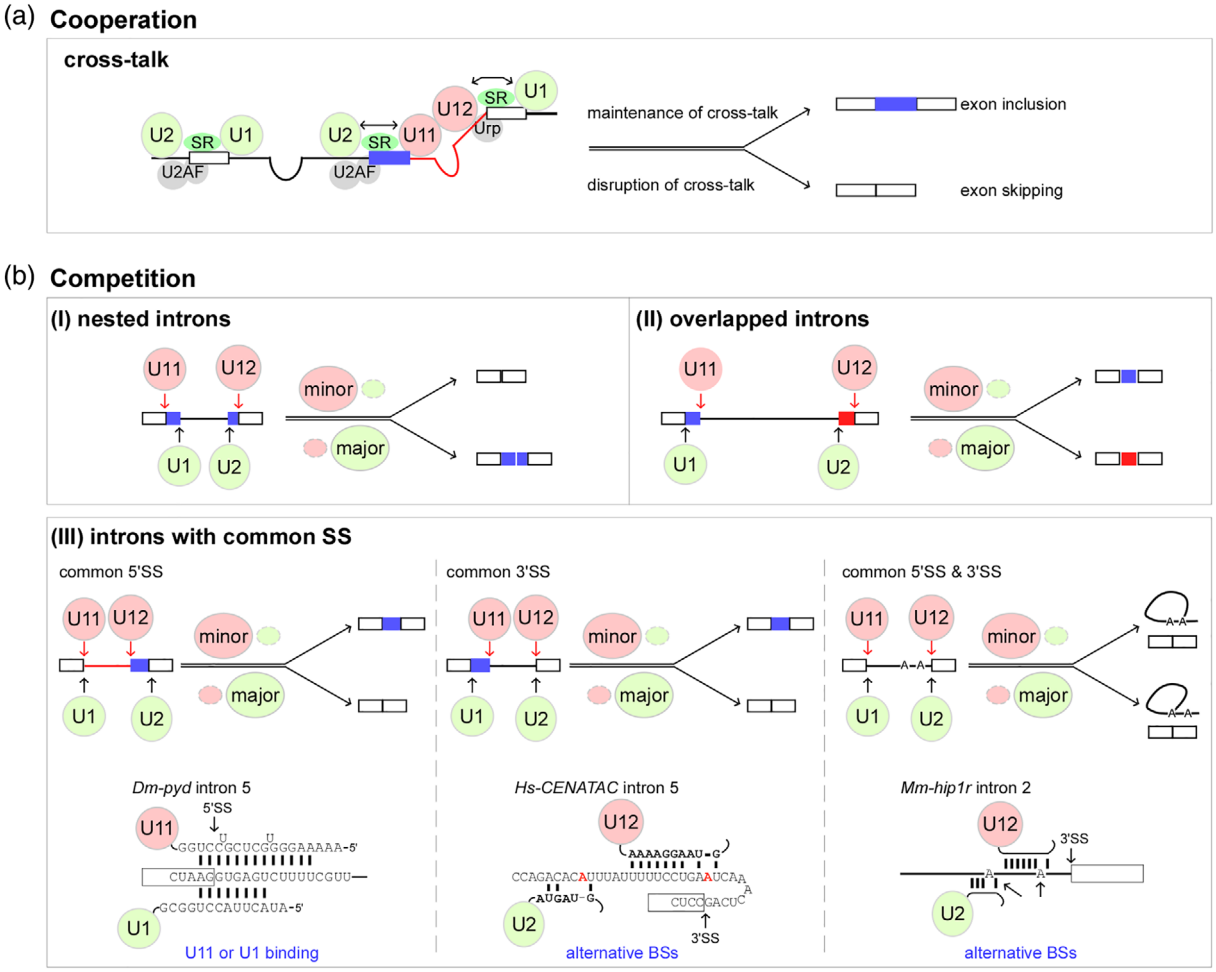

FIGURE 5 Schematics of cooperative and competitive splicing of introns between the major and minor spliceosomes. (a) In the cooperative cross-talk model, factors from the two spliceosomes, such as U11-59K and U2AFs, provide cooperative interactions for the formation of the exon definition complexes to facilitate the splicing of flanking exons of a minor intron. (b) Competitions between the two spliceosomes are divided into three categories, including (1) nested introns, (2) overlapped introns, and (3) common splice sites based on published literature (for references, see the text). In category III, introns or individual splice sites could be recognized by both spliceosomes, each case has one example below the model.

For example, there is a major intron inside of a minor intron in the *Drosophila prospero* gene (Borah et al., 2009; Otake et al., 2002; Scamborova et al., 2004) and in the human *SRSF10* gene (Meinke et al., 2020). Splicing of the minor intron in *prospero* is decreased in the U12-deletion fly, while splicing of the inside major intron is significantly increased (Li et al., 2020). Similarly, splicing of the minor intron in human SRSF10 gene was decreased when the minor protein was knocked down, while splicing of the inside major intron was increased (Meinke et al., 2020). Regulated by hnRNP and SR proteins respectively, alternative splicing of those two genes through two-spliceosome competition generates functionally different mRNA isoforms during development. Many other nested introns have also been reported, such as the *NCBP2*, *PRMT1*, and *CUL4A* genes in humans (Janice et al., 2013).

For the overlapped two introns, their four splice sites from a minor and a major intron are crossed-over in sequence (Figure 5b-II). Splicing of the minor intron in the human RCD8 gene was decreased in the 48K/SNRNP48 knock-down Hela cells, while splicing of the overlapped major intron was increased (Turunen et al., 2008). In addition, noncanonical cryptic splice sites were found to be used in the *LKB1* AT-AC intron mutation that causes Peutz-Jeghers syndrome (Hastings et al., 2005).

Competition of the two spliceosomes on introns with common splice sites has been well investigated recently, including the common 5′SS in the *Drosophila pyd* gene (Li et al., 2020), the common 3′SS in human *CENATAC* and *Drosophila Taf4* genes (de Wolf et al., 2021; Li et al., 2020), and the common 5′ and 3′SSs in mouse *Hip1r* genes (Jacquier et al., 2021). In the *Drosophila* U12 and U6atac knockout strains, analyses of minor-sensitive-SS involved

hundreds of alternative splicing changed events revealing that their common SSs could be recognized by both the major and minor spliceosomes, suggesting a competition mechanism for splicing regulation of minor sensitive introns, through either the common 5′SSs or the common 3′SSs (Figure 5b-III). In the WT strain, the common SSs favor recognition by the minor spliceosome and result in productive splicing of minor introns. However, in the minor spliceosome disrupted strains, the common SSs are recognized by the major spliceosome and result in the productive splicing of introns with alternative SSs (Li et al., 2020). The common 5′SSs are recognized by both U1 and U11 snRNAs; for example, the 5′SS of *pyd*-intron 5 could form stable RNA duplexes with either U1 or U11 snRNAs and the 5′SS-U11 duplex is even more stable than the 5′SS-U1 duplex (Figure 5b-III, left). The common 3′SSs, together with BS, could be recognized by both U2AFs-U2 snRNP and Urp/ZRSR2-U12 snRNP; for example, the 3′SS of human *CENATAC*-intron 5 and *Drosophila Taf4*-intron 3 could be recognized by the major spliceosomal components (Figure 5b-III, middle). Furthermore, the *Drosophila pyd*-intron 5 and *Taf4*-intron 3 are enriched in the co-purification by minor-specific U11/U12-65K protein, providing evidence that these introns are recognized by the minor spliceosome in the WT fly (Li et al., 2020). In addition, several minor introns, such as those in *Ca-α1D*, *Phb2*, *BuGZ*, and *Kcmf1-a* genes, are still well spliced in the *U12 KO* and *U6atac KO* strains, indicating that their two SSs could be recognized by the major spliceosome (Li et al., 2020). This hypothesis has been recently proved in an spinal muscular atrophy (SMA) mouse model, in which the splicing efficiency of minor introns predominantly depends on their alternative BS sequences (Jacquier et al., 2021) and can recruit major spliceosomal components to compensate for the loss of minor splicing activity, such as the mouse *Hip1r*-intron 3 (Figure 5b-III, right).

Importantly, studies from the above competition models reveal that the minor-spliceosome-recognized introns are not limited in the present defined classical minor introns (U12-type introns). Many major introns with close similarities to the minor introns, such as their 5′SSs potentially forming stable duplex with the 5′SS-R motif in U11 and alternative BSs potentially forming stable duplex with the BS-R motif in U12, could be competitively recognized by the minor spliceosome, and vice versa.

5 | DYSFUNCTION OF MINOR SPLICEOSOME COMPONENTS AND MINOR INTRONS

Although the minor spliceosome and minor-intron-containing genes are at very low levels in organisms, it has been demonstrated that mutations in the components of minor spliceosome or key splicing signals in pre-mRNAs cause significant defects in development in a variety of organisms and human diseases.

5.1 | Developmental defects in animals and plants

5.1.1 | By mutations of minor snRNAs

The minor spliceosomal snRNAs are essential for animal and plant development, knock-out or knock-down of individual minor snRNA results in various developmental defects. For example, early studies revealed that P element-mediated disruptions of the *Drosophila* U12 or U6atac snRNA genes result in lethality during the embryonic and the third instar larval stages, respectively (Otake et al., 2002). These are confirmed to be slightly different by recent CRISPR/Cas9-mediated knockout of U12 or U6atac genes that result in lethality at their pupa stages (Li et al., 2020). Loss of U11 snRNA in the developing mouse limb results in micromelia, in which the limb progenitor cells exhibit delayed prometaphase-to-metaphase transition and prolonged S-phase (Drake et al., 2020). Knocking down U6atac snRNA in the neonatal rat ventricular myocytes leads to robust retention of minor introns within the *Scn5a* and *Cacna1c* genes, resulting in reduced protein levels of $Na_v1.5$ and $Ca_v1.2$ channels and Na^+ and L-type Ca^{2+} currents (Montañés-Agudo et al., 2022).

5.1.2 | By mutations of minor proteins

It has also been found that mutations of individual minor spliceosomal protein components cause developmental defects in both animals and plants. For example, a zebrafish mutant, *caliban*, with a point mutation in U11/U12-65K

has shown impaired splicing efficiency of minor introns and an arrested development of the digestive organ, suggesting that efficient minor splicing is a critical process for the growth and proliferation of cells during the development in zebrafish (Markmiller et al., 2014). A *Zrsr1* mutant mice exhibits altered sex-dimorphic behavior and neurogenesis, suggesting that Zrsr1 expression and function are relevant to the organization of the hypothalamic cell network controlling behavior (Alen et al., 2019), and both the Zrsr1 and Zrsr2 in mice are indispensable for early embryo development and 2-cell-like conversion (Gomez-Redondo et al., 2020). In plants, RBP48, the newly identified component of the minor B^{act} complex, is required for maize endosperm differentiation (Bai et al., 2019). The *Arabidopsis* DROL1 is a homolog of yeast U5 snRNP subunit DIB1, seedlings of the *drol1* mutant show retarded growth, and the mutant has specific defects in splicing of many AT–AC minor introns (Suzuki et al., 2021). In addition, chromosomal stability is decreased by mutations in the novel minor spliceosomal component CENATAC (de Wolf et al., 2021).

5.2 | Human diseases

In the past three decades, many human diseases, including neurological disorders and cancers, have been linked to mutations in the minor spliceosome and minor introns (reviewed in El Marabti et al., 2021; Jutzi et al., 2018; Niemela & Frilander, 2014; Verma et al., 2018; Yang et al., 2021). For example, mutations in the U11/U12-65K protein, U12 and U4atac snRNAs, have been identified in growth hormone deficiency, early-onset cerebellar ataxia, and microcephalic osteodysplastic primordial dwarfism type I, Roifman syndrome, and Lowry–Wood syndrome, respectively (Argente et al., 2014; Elsaid et al., 2017; He et al., 2011); mutations in minor introns, the 5′SSs of *STK11* and *TRAPPC2* genes, have been connected with Peutz-Jegher's syndrome and spondyloepiphyseal dysplasia tarda, respectively (Hastings et al., 2005; Shaw et al., 2003). SMA and amyotrophic lateral sclerosis (ALS) are also proposed to be minor splicing-related diseases, in which mutations of *SMN1* in SMA patients and of *FUS* in ALS patients significantly change the splicing of minor-intron-containing genes encoding proteins required for motor neuron survival (Boulisfane et al., 2011; Lotti et al., 2012; Reber et al., 2016; Zhang et al., 2008).

It has been reported that ablation of the *Rnu11* (U11 snRNA) gene in mice results in microcephaly at birth, showing simultaneous cell cycle defects and cell death of radial glial cells in the developing cortex (Baumgartner et al., 2018). Recently, mutations in the *RNU12* (U12 snRNA) gene have also been found in patients with CDAGS Syndrome, which is a rare congenital disorder characterized by craniosynostosis, delayed closure of the fontanelles, cranial defects, clavicular hypoplasia, anal and genitourinary malformations, and skin manifestations (Xing et al., 2021). In those patients, mutations in biallelic rare variants of RNU12 genes alter a highly conserved nucleotide within the 3′ extension of the U12 snRNA precursor in one allele, and disrupt either the secondary structure or the Sm binding site in another allele, providing evidence of the involvement of U12 snRNA in CDAGS syndrome.

A patient with severe primordial microcephalic dwarfism and intellectual disability was recently identified as carrying compound heterozygous variants in 65K/RNPC3, resulting in mutation of the highly conserved Phe410 residue to Val (Yamada et al., 2021). Mutations of four splicing factors have been frequently found in leukemia, including the minor spliceosomal protein ZRSR2 (Wang et al., 2011; Yoshida et al., 2011). Recently, observations of enhanced proliferation in the hematopoietic-specific *Zrsr2*-deletion mice and enhanced clonogenic capacity in the *Zrsr2-null* HSCs were reported, where impaired splicing of minor introns increases self-renewal of hematopoietic stem cells (Inoue et al., 2021).

6 | CONCLUSION

The low abundance of minor spliceosomes and the few numbers of minor introns raise an important question: why do most eukaryotic organisms evolutionarily keep the minor spliceosome system and minor introns? Recent studies discussed in this review partially answer this question and demonstrate that minor splicing is not “small” or “unimportant”. However, findings of novel protein components in the human minor spliceosome, huge numbers of minor introns in *Physarum polycephalum*, competitive recognition of introns by the two spliceosomes, and minor splicing mutations causing developmental defects and diseases, suggest that there are still many more unknowns of minor splicing for us to understand.

The dynamics of the minor spliceosome at present are limited in the U11/U12 di-snRNP and the U12/U5/U6atac-containing B^{act} complex. Searching for new protein components in other minor complexes will reveal novel

mechanisms for the recognition of minor introns and the assembly/disassembly and catalysis of the minor spliceosome. It has been demonstrated that eight RNA-dependent ATPases or ATP-dependent RNA helicases in yeast are critical for facilitating conformational changes of the major spliceosomal complexes by coupling physical movement with a cycle of ATP binding, hydrolysis, and release of ADP (reviewed in Rocak & Linder, 2004; Smith et al., 2008; Staley & Guthrie, 1998). Functional studies of these proteins on the minor spliceosome will reveal mechanisms behind the more stable RNA duplexes that are formed by intronic splicing signals and the minor-specific snRNAs. Investigation of the co-evolution of minor-specific snRNAs and the intronic splicing signals in a wider range of organisms will help to identify more minor introns, to understand functionary regulation of their host genes, and of course to answer why minor splicing is evolutionary important in higher eukaryotes. The competition models are based on a reality of the close similarities between two spliceosomes, although they have distinct characteristics. Which spliceosome mediates excision of a given intron mostly depends on the intronic signals, sequences of the 5′ and the 3′ splice sites, and the branch sites, which are competitively recognized by the major and minor spliceosomal components. Therefore, the affinity and local concentration of the minor spliceosome are important for the recognition and splicing of an intron, which is not limited to the presently defined minor introns. Further studies will reveal more minor-spliceosome-catalyzed introns and their competition mechanisms with the splicing of major introns. Finally, it is important to note that the functional impact of minor splicing and evolutionarily conserved minor-intron-containing genes will be helpful in the understanding of differentiation, development, diseases, and pathologies.

AUTHOR CONTRIBUTIONS

Zhan Ding: Conceptualization (equal); data curation (lead); formal analysis (lead); visualization (lead); writing – original draft (supporting). **Yan-Ran Meng:** Data curation (supporting); visualization (supporting); writing – review and editing (supporting). **Yu-Jie Fan:** Funding acquisition (supporting); project administration (lead); supervision (supporting); visualization (supporting); writing – review and editing (supporting). **Yong-Zhen Xu:** Conceptualization (equal); data curation (supporting); funding acquisition (lead); investigation (lead); methodology (equal); supervision (lead); visualization (equal); writing – original draft (lead).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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