A conserved intronic U1 snRNP-binding sequence promotes *trans*-splicing in *Drosophila*

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Unlike typical *cis*-splicing, *trans*-splicing joins exons from two separate transcripts to produce chimeric mRNA and has been detected in most eukaryotes. *Trans*-splicing in trypanosomes and nematodes has been characterized as a spliced leader RNA-facilitated reaction; in contrast, its mechanism in higher eukaryotes remains unclear. Here we investigate *mod(mdg4)*, a classic *trans*-spliced gene in *Drosophila*, and report that two critical RNA sequences in the middle of the last 5′ intron, TSA and TSB, promote *trans*-splicing of *mod(mdg4)*. In TSA, a 13-nucleotide (nt) core motif is conserved across *Drosophila* species and is essential and sufficient for *trans*-splicing, which binds U1 small nuclear RNP (snRNP) through strong base-pairing with U1 snRNA. In TSB, a conserved secondary structure acts as an enhancer. Deletions of TSA and TSB using the CRISPR/Cas9 system result in developmental defects in flies. Although it is not clear how the 5′ intron finds the 3′ introns, compensatory changes in U1 snRNA rescue *trans*-splicing of TSA mutants, demonstrating that U1 recruitment is critical to promote *trans*-splicing in vivo. Furthermore, TSA core-like motifs are found in many other *trans*-spliced *Drosophila* genes, including *lola*. These findings represent a novel mechanism of *trans*-splicing, in which RNA motifs in the 5′ intron are sufficient to bring separate transcripts into close proximity to promote *trans*-splicing.

[*Keywords*: *trans*-splicing; *mod*(*mdg4*); U1 snRNP; pseudo-5′ splice site; RNA motif; *Drosophila*]

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Removal of introns by pre-mRNA splicing is one of the essential steps of RNA processing during eukaryotic gene expression and regulation (Hoskins and Moore 2012). Additionally, alternative splicing generates multiple mRNA isoforms that play key roles in development, differentiation, and diseases (Kim et al. 2008). Pre-mRNA splicing is catalyzed by a large and dynamic RNA–protein complex, the spliceosome, which contains five small nuclear RNAs (snRNAs; U1, U2, U4, U5, and U6) and >150 proteins (Will and Luhrmann 2011). Exon–exon ligation typically occurs as an intramolecular reaction (*cis*-splicing); however, it can occur less frequently in an intermolecular manner (*trans*-splicing) to generate chimeric mRNA from two different pre-mRNA molecules (Sharp 1987; Lasda and Blumenthal 2011).

Trans-splicing is widespread in lower eukaryotic trypanosomes and nematodes (Sutton and Boothroyd 1986; Zorio et al. 1994). Nearly all transcripts in *Trypanosoma brucei* and ~70% of transcripts in *Caenorhabditis elegans* are *trans*-spliced with a highly conserved short spliced leader (SL), which is 39 nucleotides (nt) in trypanosomes and 22 nt in nematodes (Lasda and Blumenthal 2011). SL is *trans*-spliced to the 5′ end of transcripts from an ~100-nt SL RNA by reactions similar to that of *cis*-splicing except that a Y-structured intermediate is formed instead of a lariat intermediate (Murphy et al. 1986; Hannon et al. 1990). SL RNA was considered to be a chimeric molecule with an exon domain (the SL) and an snRNA-like domain (Sharp 1987) in which the snRNA-like domain contains an Sm core-binding site and a 5′ splice site (5′SS) (Bruzik et al. 1988; Hannon et al. 1992). SL RNA-facilitated *trans*-splicing requires U2, U4, U5, and U6 snRNAs (Tschudi and Ullu 1990) but not U1 snRNA (Hannon et al. 1991). Therefore, SL RNA has been hypothesized to be assembled into SL RNP, an analog of U1

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snRNP (Bruzik and Steitz 1990). However, in vitro purification using cell extracts from the parasitic nematode *Ascaris lumbricoides* revealed that the protein components of SL RNP are specific except for core Sm proteins (Denker et al. 2002). It has been speculated that U5 snRNA and its associated proteins may identify and align the 5′SSs in the SL RNA for the spliceosomal chemical reactions (Denker et al. 1996; Maroney et al. 1996).

Trans-splicing is found less commonly in many higher eukaryotes, including in several chloroplast and mitochondria genes in plants (Koller et al. 1987; Pereira de Souza et al. 1991), in *mod(mdg4)* and *lola* in flies (Horiuchi et al. 2003; Gabler et al. 2005), and in numerous genes in mammals (Fujieda et al. 1996; Hu et al. 2013). Recently, deep sequencing has enhanced the identification of *trans*-splicing events throughout the transcriptome (McManus et al. 2010; Zhang et al. 2010; Shao et al. 2012). In *Drosophila*, ~80 *trans*-spliced genes have been identified and confirmed using interspecies hybrids, including two classic *trans*-spliced genes, *mod(mdg4)* and *lola* (McManus et al. 2010). The *mod(mdg4)* gene functions in establishing and maintaining an open chromatin conformation and therefore is being considered as an enhancer of position effect variegation (Dorn et al. 1993; Gerasimova et al. 1995; Dorn and Krauss 2003). In *mod(mdg4)*, alternative 3′ exons from multiple loci on both DNA strands are *trans*-spliced to a 5′ transcript containing four common exons (Fig. 1A) and result in at least 31 isoforms (Labrador et al. 2001; Dorn and Krauss 2003; Gabler et al. 2005; McManus et al. 2010). *Trans*-splicing of *mod(mdg4)* is conserved in insects, including silkworms (Shao et al. 2012), mosquitos (Krauss and Dorn 2004), and cotton bollworms (Cai et al. 2012). The *lola* gene encodes a transcription factor that is involved in neuron and stem cell differentiation (Neumuller et al. 2011; Southall et al. 2014). The 5′ transcripts of *lola*, containing four alternative exons and four common exons, are *trans*-spliced to multiple 3′ exons to generate at least 80 mRNAs and 20 protein isoforms (Horiuchi et al. 2003; McManus et al. 2010).

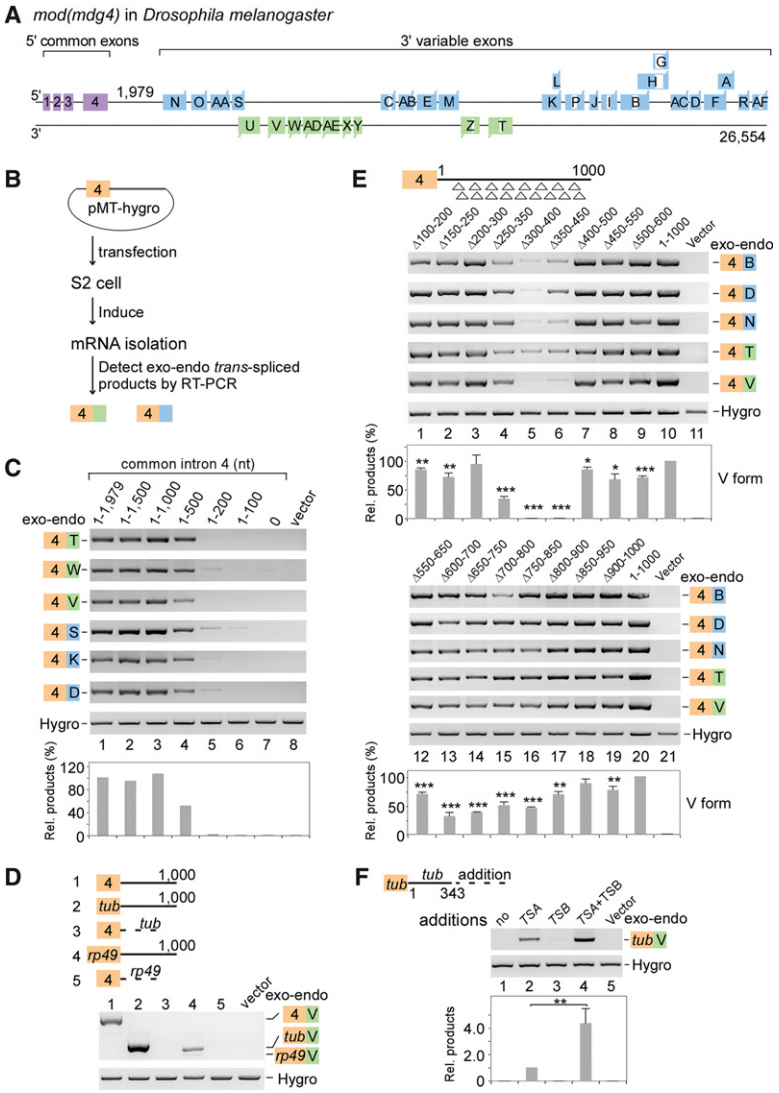


Figure 1. Two intronic RNA sequences are critical for *trans*-splicing of *mod(mdg4)*. (*A*) Schematic of the *mod(mdg4)* gene locus in *Drosophila melanogaster*. Exons and introns from 3′ transcripts are designated according to FlyBase, and the sequence between exon 4 and the first 3′ exon N is defined as the last 5′ intron (intron 4). (*B*) Schematic of a *trans*-splicing system in *Drosophila* S2 cells. (*C*) Truncation assays of intron 4 reveal that two regions are important for *trans*-splicing activity. Relative *trans*-spliced products are averages from six isoforms that were normalized to loading controls and the full-length intron 4. (*D*) Replacing intron 4 of *mod(mdg4)* with other introns abolishes *trans*-splicing activity. Sequences of other genes include exon 1–intron 1 (without the 3′SS) from *tubulin* (*tub*) and *rp49*. (*E*) *Trans*-splicing activity of tiled 101-nt deletions in intron 4. Relative *trans*-spliced products of *mod(mdg4)* V forms were quantitated and normalized to the intact intron 4 (mean ± SEM; $n = 3$). For shorter tiled deletions, see Supplemental Figure S1C. (*F*) TSA RNA is sufficient to promote *trans*-splicing, while TSB RNA enhances the activity. TSA and TSB RNAs are fragments of the 330–500 nt and 650–800 nt in *mod(mdg4)* intron 4, respectively. Relative enhancement by TSB was normalized to TSA alone (mean ± SEM; $n = 3$). (Purple boxes) Chromosomal 5′ exons; (blue boxes) 3′ exons from the same DNA strand; (green boxes) 3′ exons from the opposite strand; (brown boxes) exon 4 on plasmids; (white boxes) internal *cis*-spliced intron in 3′ exons; (Hygro) hygromycin B. Boxes and labels are used similarly in other places unless otherwise indicated. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

Trans-splicing in higher eukaryotes and a few events in nematodes are not promoted by SL RNAs; we refer to these events as non-SL *trans*-splicing. Such non-SL *trans*-splicing was first observed in in vitro assays using HeLa nuclear extract, where exons from two transcripts with mutually complementary intron sequences were spliced together (Konarska et al. 1985; Solnick 1985). In the absence of complementary sequences, *trans*-splicing occurred at lower efficiency, which was further extensively characterized using a 5′SS-containing RNA oligo and a second RNA with a 3′SS (Konforti and Konarska 1995). Therefore, current models favor long complementary sequences bringing two separate transcripts together to promote in vivo non-SL *trans*-splicing. This mechanism was proposed to facilitate *trans*-splicing of RNAi regulatory gene ERI-6/7 in *C. elegans* through base-pairing in the UTRs (Fischer et al. 2008) and facilitate *trans*-splicing of identified genes in *Giardia intestinalis* and *Bombyx mori* through long base-pairing between introns (Kamikawa et al. 2011; Shao et al. 2012). Furthermore, this model of *trans*-splicing has been successfully applied to develop gene therapy strategies to replace mutated exons in several cancer cell lines (Puttaraju et al. 1999; Gruber et al. 2013).

However, it remains unclear whether all *trans*-splicing events in higher eukaryotes are promoted by complementary intronic sequences and how *trans*-splicing is promoted between transcripts that do not have obvious complementary sequences. To address these questions, we investigated *trans*-splicing of *mod(mdg4)* in *Drosophila* S2 cells and identified two critical RNA sequences (TSA and TSB) in the last 5′ intron of *mod(mdg4)*. Mutagenesis analyses reveal that TSA is required and sufficient for *trans*-splicing and that TSB functions as an enhancer. Flies with deletions of TSA and TSB, prepared using the CRISPR/Cas9 system, exhibited defects in viability and embryonic development. Furthermore, two highly conserved core motifs across *Drosophila* species were found in TSA and TSB, respectively. The TSA core motif contains a pseudo-5′SS and recruits U1 snRNP through strong base-pairing with U1 snRNA to promote *trans*-splicing. A similar motif is also found in *lola*, allowing for mutual *trans*-splicing between *lola* and *mod(mdg4)*.

Results

Identification of RNA elements required for trans-*splicing of* mod(mdg4)

Based on the current model that long complementary intronic sequences promote *trans*-splicing between two transcripts (Konarska et al. 1985; Fischer et al. 2008; Kamikawa et al. 2011), we searched the *mod(mdg4)* gene locus in *Drosophila melanogaster* (*Dm*) for regions of complementarity between the last 5′ intron and introns in the 3′ transcripts by BLASTN (*E*-value < 10); however, no obvious long base-paired regions were found. The *trans*-splicing of *mod(mdg4)*, which results from one common 5′ transcript spliced to multiple 3′ transcripts, led us to hypothesize that critical RNA sequences would be located in the last common exon and intron (exon and intron 4) (Fig. 1A). To address this, plasmid-borne constructs that carry exon 4 with various lengths of intron 4 were transfected into *Drosophila* S2 cells; we then tested for *trans*-spliced products between the exogenous exon 4 and the endogenous 3′ exons of *mod(mdg4)* (Fig. 1B). In the presence of the full-length intron 4 (1979 nt), all tested 3′ exons were efficiently *trans*-spliced to the exogenous exon 4 (Fig. 1C, lane 1). When intron 4 was shortened from its 3′ end to 1500 or 1000 nt, similar levels of *trans*-splicing products were still detected; however, it was reduced ~50% when intron 4 was shortened to 500 nt and was undetectable when shortened to ≤200 nt or when the 5′SS was mutated (Fig. 1C; Supplemental Fig. S1A). In addition, replacing the exogenous sequence with other exons and introns from the 5′ transcript of *mod(mdg4)* resulted in no detectable *trans*-splicing (Supplemental Fig. S1B). Similarly, intronic sequences from either *Drosophila tubulin* or *rp49* did not generate detectable *trans*-spliced products (Fig. 1D). However, the presence of intron 4 was sufficient for *trans*-splicing of truncated exon 4 and *trans*-splicing of exons from *tubulin* or *rp49* (Fig. 1D; Supplemental Fig. S1B). Taken together, these results demonstrate that the last common intron, but not other sequences, is required and sufficient for *trans*-splicing of *mod(mdg4)*.

TSA RNA is sufficient for trans-*splicing, whereas TSB enhances*

To further define RNA sequences that facilitate *trans*-splicing, we performed two rounds of tiled deletions of intron 4. In the first round, deletions of nucleotides 250–350, 300–400, and 350–450 nearly abolished the *trans*-spliced products, and deletions of nucleotides 600–700, 650–750, 700–800, and 750–850 significantly decreased *trans*-splicing activity (Fig. 1E), whereas deletions of other regions did not, suggesting that two regions in intron 4 (300–500 nt and 650–800 nt) are important to the *trans*-splicing reaction, consistent with the above truncation assay. In the second round, the 300- to 500-nt region of intron 4 was further examined by shorter tiled deletions. Deletions of the 345- to 375-nt and 420- to 450-nt regions dramatically decreased *trans*-splicing activity (Supplemental Fig. S1C). Taken together, we designated the 330- to 500-nt region as "TSA RNA" and the 650- to 800-nt region as "TSB RNA."

We next asked whether TSA or TSB RNA is sufficient to promote *trans*-splicing in S2 cells. The exogenous *tubulin* sequence (exon1–intron1) did not promote *trans*-splicing with 3′ exons of *mod(mdg4)*; however, the addition of TSA generated a *trans*-spliced mRNA product between the exogenous *tubulin* exon and the endogenous 3′ exon of *mod(mdg4)* (Fig. 1F). In contrast, TSB alone did not promote *trans*-splicing but enhanced *trans*-splicing fourfold when present together with TSA (Fig. 1F). We conclude that TSA is sufficient to promote *trans*-splicing and functions independently of other 5′ common sequences in *mod(mdg4)* and that TSB functions as an enhancer element to the TSA-dependent *trans*-splicing reaction.

Conserved motifs in TSA and TSB across Drosophila *species are critical*

To identify functional motifs in TSA and TSB, we aligned intron 4 sequences from homologous *mod(mdg4)* genes in other available *Drosophila* genomes and found two highly conserved regions (Supplemental Fig. S2). The first conserved region is a nearly invariant 13-nt RNA motif (GGU$^{A}/_{G}$AGUAGAGUG) located in the previously described TSA; the second forms highly conserved secondary structures in TSB and varies from 79 to 95 nt long across *Drosophila* species (Fig. 2A; Supplemental Fig. S3). Therefore, we designate these two conserved regions as the "TSA core" and "TSB core."

We generated a series of mutations to investigate the function of the TSA and TSB cores. In the TSA core, six single G-to-C mutations exhibited various reductions of *trans*-splicing activity in which the G369C mutant provided the strongest inhibition (Fig. 2B). Importantly, when all six guanosines were mutated to cytosines (*6c*), *trans*-splicing activity was almost totally abolished (Fig. 2B). Moreover, the TSA core alone or with additional short sequences was sufficient to promote *trans*-splicing between the exogenous *tubulin* exon and endogenous 3′ exons of *mod(mdg4)* (Supplemental Fig. S1D). These results demonstrate that the highly conserved TSA core is a critical element for *trans*-splicing and suggest that a new mechanism promotes *trans*-splicing in *Drosophila*.

The 79-nt-long *Dm*-TSB core contains 48 nt that are invariant in other *Drosophila* species. Using the mfold server (Zuker 2003), TSB core sequences were predicted

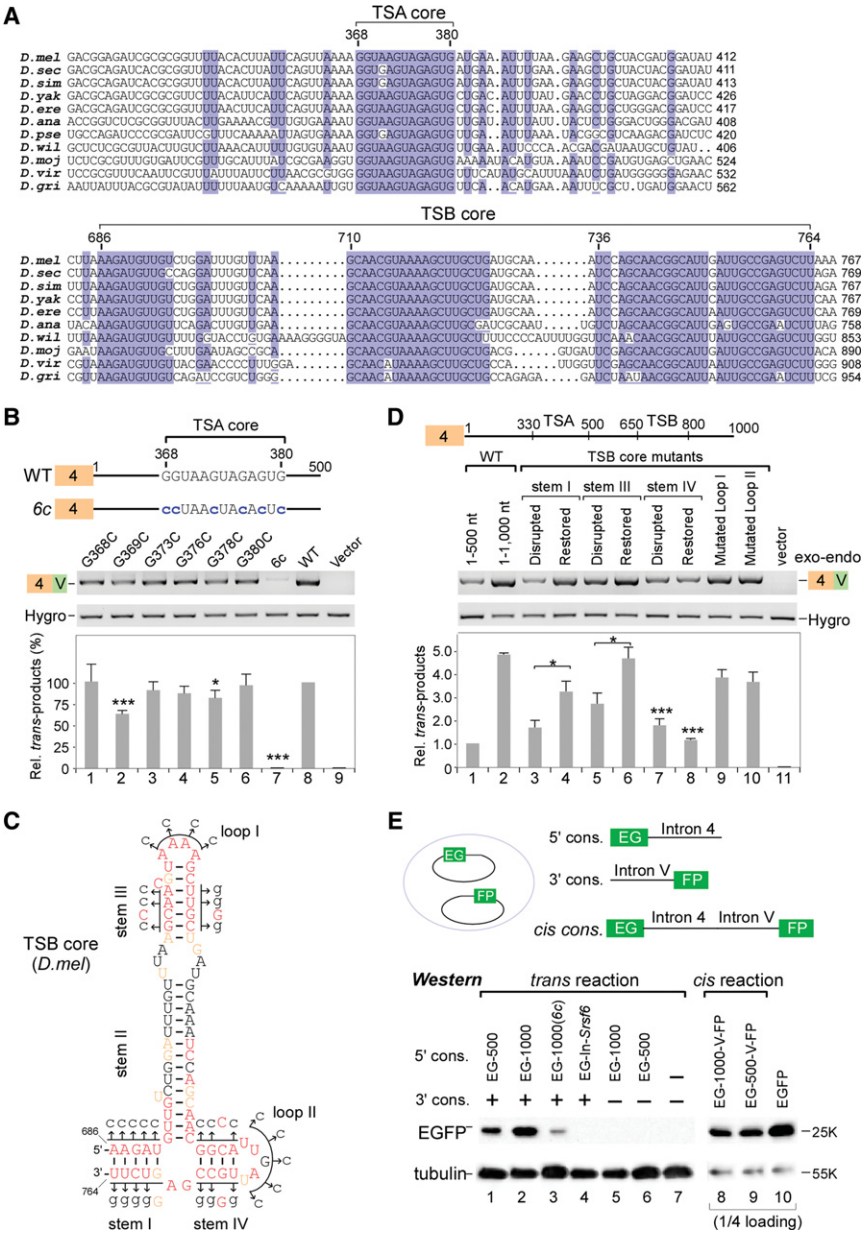


Figure 2. Mutations in the highly conserved RNA motifs of intron 4 significantly decrease *trans*-splicing activity. (*A*) TSA and TSB contain highly conserved core motifs across *Drosophila* species. For sequence alignment of the full-length intron 4, see Supplemental Figure S2. (*B*) Mutations in the TSA core motif significantly decrease *trans*-splicing activity. Relative *trans*-spliced products were normalized to loading controls and the wild-type (WT) intron 4. Mean ± SEM; $n = 3$; (*) $P < 0.05$; (***) $P < 0.001$. (*C*) Highly conserved secondary structure of TSB core RNA from *D. melanogaster*. (Red) 100% conservation; (brown) >75% conservation; (black) <75% conservation. Mutated sites used in *D* are indicated by arrows here. For structures of TSB cores in other *Drosophila* species, see Supplemental Figure S3. (*D*) Secondary structures of stems I and III in the TSB core are required for enhancing *trans*-splicing activity. Mutated sites are indicated in *C*. Relative activities were normalized to loading controls and the wild-type intron 4 (1–500 nt). Mean ± SEM; $n = 3$. (*E*) Validation of *trans*-splicing at the protein level. The coding sequence (CDS) of EGFP is split into two halves (EG and FP) followed by intronic sequences; *trans*-spliced products were detected by Western blot using anti-EGFP antibody. (EG-500) Exon EG with 1–500 nt of intron 4; (EG-1000) exon EG with 1–1000 nt of intron 4; (*6c*) all six Gs are mutated to Cs in the TSA core; (In-*Srsf6*) 1–548 nt of *Srsf6* intron 4. *Cis*-splicing constructs were used as controls.

to form stable conserved secondary structures containing four stems and two loops (Fig. 2C; Supplemental Fig. S3). The most notable difference between TSB core secondary structures across *Drosophila* species is the length of stem II. To address the contribution of these structures in the TSB core, we generated mutations at conserved nucleotides to disrupt base-pairing in stems, restore base-pairing by compensatory mutations, and change sequences in loops (Fig. 2C). When base-pairing in stem I or III was disrupted (nucleotides in one chain were mutated to cytosines), the enhancement by TSB RNA was significantly reduced from 4.9-fold by the wild-type sequence to 1.7-fold and 2.7-fold by stem I and III mutants, respectively (Fig. 2D). Importantly, the reduced enhancement was partially rescued by compensatory mutations that restore base-pairing in stem I or III (Fig. 2D, lanes 3–6). This indicates that the structures of stems I and III are critical for TSB's enhancement to *trans*-splicing activity. Disruption of stem IV base-pairing significantly reduced the enhancement, but compensatory mutations of stem IV could not rescue the impairment (Fig. 2D, lanes 7,8), implying the importance of the primary sequence rather than its structure; alternatively, the restoration construct did not fold as predicted. Last, *trans*-splicing activity was not significantly altered when the loop I or II sequence was mutated (Fig. 2D, lanes 9,10). These results demonstrated that the secondary structures of the TSB core are critical to enhance the *trans*-splicing activity of *mod(mdg4)*.

To verify the effect of these findings on protein expression and confirm that the chimeric mRNAs detected above were not artificially generated by homology-driven template switching during RT–PCR, we split the EGFP coding sequence (CDS) into two halves and separately fused them with intronic sequences from *mod(mdg4)* on two plasmids (Fig. 2E, top). The upstream exon (EG) was followed by various intron 4 sequences, while the downstream exon (FP) was fused with 3′ intron V. Thus, EGFP can be expressed only when the two exons are *trans*-spliced. Consistent with the above mRNA analyses, EGFP was detected when either 1–1000 nt (TSA+TSB) or 1–500 nt (TSA only) of intron 4 were included. The intron 4 sequence containing both TSA and TSB provided higher expression of EGFP, and the TSA core mutant EG-1000 (*6c*) significantly decreased EGFP expression, whereas replacement of intron 4 with an intron from the *Drosophila Srsf6* gene did not produce EGFP (Fig. 2E, lanes 1–4). These results again argue that the TSA core motif is the critical element in *trans*-splicing. In addition, *cis*-splicing constructs were tested and showed more efficient EGFP expression (Fig. 2E, lanes 8,9).

Lack of a 5′SS in 3′ transcripts of mod(mdg4) *determines alternative* trans*-splicing*

The *mod(mdg4)* gene has at least 31 identified 3′ exons, which are located in several regions transcribed from both DNA strands (Yu et al. 2014). Using the splice site-finding software Human Splicing Finder (HSF) (Desmet et al. 2009), we found no strong 5′SS sequences in introns between the alternatively *trans*-spliced exons except the identified internal *cis*-spliced introns (Supplemental Fig. S4). One possible explanation is that the presence of a 5′SS would promote *cis*-splicing between 3′ exons and inhibit its *trans*-splicing to the 5′ exon. To test this, we cloned 3′ intron–exon sequences of *mod(mdg4)* and analyzed their *trans*-spliced products with endogenous exon 4 in S2 cells (Fig. 3A). Two 3′ exons, U and V with their own introns or the introns of *tubulin* or *rp49*, were *trans*-spliced to the endogenous exon 4 in the absence of a 5′SS (Fig. 3A, top); however, *trans*-splicing was abolished and resulted in high levels of *cis*-splicing when a 5′SS (CAAG/GUAAGU) was added (Fig. 3A, bottom). This demonstrates that the presence of a 5′SS in the 3′ intron inhibits *trans*-splicing. Furthermore, in the absence of a strong 5′SS, *trans*-splicing still occurred at high levels when 3′ introns and exons from *mod(mdg4)* were replaced by control genes (Fig. 3B), suggesting that the primary sequence of the 3′ introns and exons is not an important factor for *trans*-splicing of *mod(mdg4)*.

TSA and TSB are required for fly development

Benefiting from the recently developed CRISPR/Cas9 system (Ren et al. 2013), we performed two fly experiments to investigate in vivo functions of TSA and TSB.

We generated flies with deletions of TSA ($TSA^{-/-}$, Δ340–487 nt) or TSB ($TSB^{-/-}$, Δ646–799 nt) (Fig. 4A). Neither $TSA^{-/-}$ nor $TSB^{-/-}$ adults showed visible phenotypes; however, only 44.6% of $TSA^{-/-}$ and 33.5% of $TSB^{-/-}$ embryos hatched, showing a semilethal phenotype (Fig. 4B, lanes 2,3). Since the embryonic development of $TSB^{-/-}$ flies unexpectedly exhibited more defects than $TSA^{-/-}$ flies, we further crossed deletion flies with wild-type flies in both directions and investigated the heterozygous embryos. The hatching rate of $TSB^{+/-}$ was similar to that of the wild type when the paternal fly was $TSB^{-/-}$ but was significantly reduced when the maternal fly was $TSB^{-/-}$. In

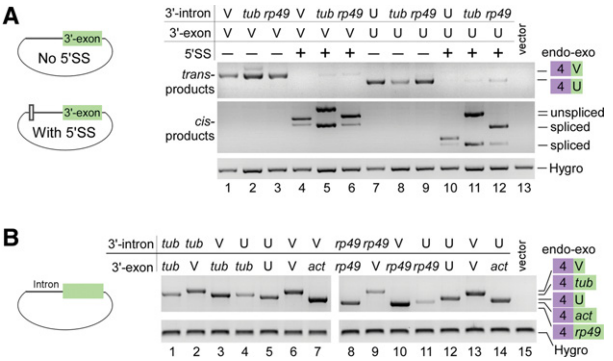


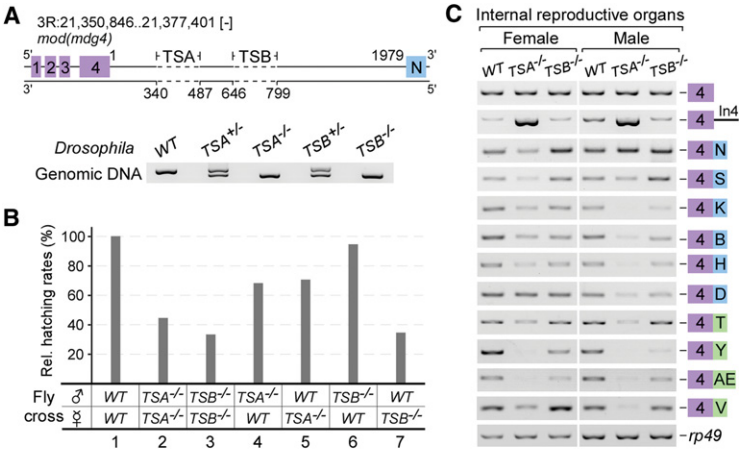
Figure 3. Lack of a 5′SS in the 3′ transcripts is required for *trans*-splicing of *mod(mdg4)*. (*A*) An additional 5′SS abolishes *trans*-splicing and results in self-*cis*-splicing. (*B*) Replacing sequences of the 3′ intron and exons of *mod(mdg4)* did not obviously affect *trans*-splicing activity. (*tub*) Exon 1 and/or intron 1 (no 5′SS) of *tubulin*; (*act*) exon 1 and/or intron 1 (no 5′SS) of *actin*; (endo–exo) *trans*-spliced products between the endogenous 5′ exon 4 and exogenous 3′ exons.

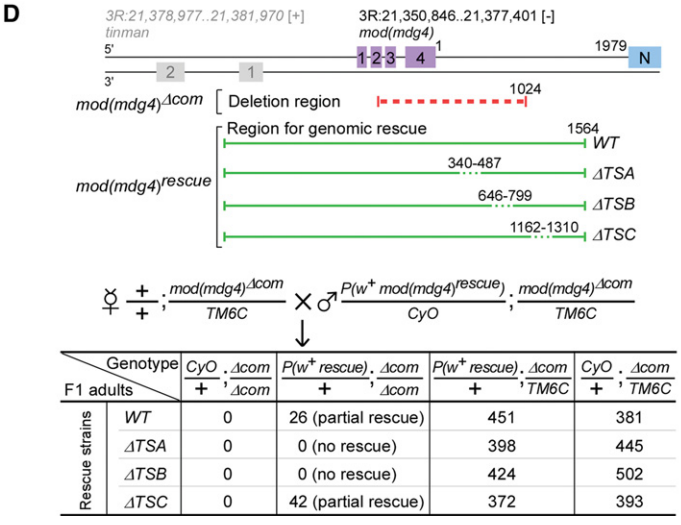
contrast, hatching rates of $TSA^{+/-}$ heterozygotes were significantly affected in crosses of both directions (Fig. 4B, lanes 4–7). Moreover, RT–PCR analyses revealed that all tested *mod(mdg4)* isoforms were obviously decreased in the adult internal reproductive organs from $TSA^{-/-}$ flies and exhibited strong accumulation of unspliced pre-mRNA, whereas *trans*-spliced isoforms in $TSB^{-/-}$ fly samples exhibited subtle and inconsistent changes depending on the isoforms (Fig. 4C). These results reveal that deletion of TSA significantly reduces *trans*-spliced isoforms of *mod(mdg4)* in vivo and affects survival of the progeny of both male and female flies, whereas deletion of TSB does not obviously affect the survival of the progeny of male flies, but a serious reduction in the survival of female flies was observed.

It has been reported that a transgenic fragment containing the entire 5′ transcribed region of *mod(mdg4)* could partially rescue fly lethality caused by transposon insertion at the common region of *mod(mdg4)* (Buchner et al. 2000). To address whether the presence of TSA or TSB is required for the rescue, we first generated a deletion allele, $mod(mdg4)^{\Delta com}$, in which the 5′ common region of the locus was deleted using the CRISPR/Cas9 system, causing recessive lethality; we then generated transgenic flies with various 5′ common regions of *mod(mdg4)* inserted at the attP40 site on chromosome II for genomic rescue crosses (Fig. 4D, top). The wild-type and *ΔTSC* (deletion of a control region in intron 4) transgenic alleles could partially rescue the lethality of $mod(mdg4)^{\Delta com}$; however, the transgenic allele without the TSA or TSB sequence (*ΔTSA* or *ΔTSB* allele) could not (Fig. 4D, bottom), demonstrating that TSA and TSB are critical elements for *trans*-splicing of *mod(mdg4)* in vivo.

TSA RNA associates with U1 snRNP components

The TSA core motif contains a notable 5′SS-like sequence (GGU^{A}/$_{G}$AGUAGAGUG), and *Dm*-TSA RNA could form a secondary structure similar to SL RNAs from trypanosome and nematode (Supplemental Fig. S5A), raising the possibility that TSA could function like SL RNA to facilitate *trans*-splicing in *Drosophila*. However, constructs in which the TSA sequence was replaced with SL RNAs (SL from *T. brucei* or SL1 and SL2 from *C. elegans*) promoted little *trans*-splicing, which was not further decreased by mutations in either the 5′SSs or Sm-binding sites (Supplemental Fig. S5B), indicating that TSA RNA in *Drosophila* is not an analog of SL RNAs in lower eukaryotes.





F1 adults \ Genotype		CyO / Δcom, Δcom	P(w+ rescue), Δcom / Δcom	P(w+ rescue), Δcom / +	CyO / Δcom, TM6C
Rescue strains	WT	0	26 (partial rescue)	451	381
	ΔTSA	0	0 (no rescue)	398	445
	ΔTSB	0	0 (no rescue)	424	502
	ΔTSC	0	42 (partial rescue)	372	393

Figure 4. In vivo deletions of TSA and TSB result in development and viability defects in flies. (*A*) Schematic of in vivo deletions of TSA and TSB induced using the CRISPR/Cas9 system. Homozygous and heterozygous flies were confirmed by genomic PCR and sequencing. (*B*) Deletions of TSA and TSB result in semilethal phenotypes. Hatching rates were determined based on 300 embryos from each strain and normalized to the wild-type (WT) flies. (*C*) Deletion of TSA significantly decreases in vivo *trans*-spliced *mod(mdg4)* isoforms. (*D*) TSA and TSB are both required for the genomic rescue of lethality caused by deletion of the 5′ common region of *mod(mdg4)*. F1 adults were counted based on their genotypes. (Red dashed lines) Deleted common region; (green lines) regions for genomic rescue (in which deleted elements are indicated by green dashed lines).

To gain insights into how TSA and TSB facilitate *trans*-splicing, we identified associated factors by affinity purification of in vitro transcribed RNA with MS2 RNA-binding sequences. After incubation with S2 cell lysate, RNA-associated proteins were purified and identified by mass spectrometry (Fig. 5A). In comparison with TSB and the control (MS2 RNA-binding sequence only) RNAs, TSA RNA specifically purified components of U1 snRNP, including U1-70K, U1A, SmB, D1, D2, D3, E, and G, in which peptides from U1-70K were the most abundant (Fig. 5B; Supplemental Table S1). In contrast, TSB RNA specifically associated with several identified proteins but no U1 proteins (Supplemental Table S1). To confirm the TSA RNA and U1 snRNP interaction, we analyzed associated proteins by Western blot. Consistent with the mass spectrometry data, wild-type TSA RNA purified both U1-70K and SmD2, but the TSA-*6c* mutant, the control, and TSB RNA did not (Fig. 5C). Similarly, TSA RNA specifically pulled down U1 snRNA, whereas the TSA-*6c* mutant and TSB RNA did not (Fig. 5D). These results demonstrate that both RNA and protein components of U1 snRNP are specifically associated with TSA in vitro.

The TSA core contains a pseudo-5′SS and forms 9 base pairs (bp) with the 5′ end of U1 snRNA (Fig. 5E, left), and mutations in the TSA core significantly reduced *trans*-splicing activity. Therefore, we hypothesized that base-pairing between the TSA core and U1 snRNA is a critical interaction for *trans*-splicing of *mod(mdg4)*. To test this, we constructed U1 snRNA mutants that restore (*U1-3g*) or enhance (*hyper-U1*) base-pairing with TSA core mutants and predicted that such compensatory mutations of U1 snRNA would rescue the decreased *trans*-splicing activity of TSA core mutants in S2 cells. Consistent with the previous assays, both the TSA core mutants *3c* and *6c* decreased *trans*-splicing of *mod(mdg4)* (Fig. 5E, lanes 4,6). However, additional expression of U1 snRNA with compensatory mutations, *U1-3g* and *hyper-U1*, significantly rescued the decreased *trans*-splicing of the *3c* and *6c* mutants (Fig. 5E, lanes 6–8), demonstrating that the TSA core motif recruits U1 snRNP through base-pairing with the 5′ end of U1 snRNA to promote *trans*-splicing.

lola *has a TSA core-like motif and is mutually* trans*-spliced with* mod(mdg4)

Trans-splicing of *lola* gene transcripts occurs between the last 5′ exon (exon 8) and alternative 3′ exons (Fig. 6A; Horiuchi et al. 2003; McManus et al. 2010). Aligning available intron 8 sequences of *lola* from five *Drosophila* species, we found nine conserved regions (Supplemental Fig. S6) in which one region ($_{2191}$CAGGU$^{A}/_{G}$AGUAUGCU) was

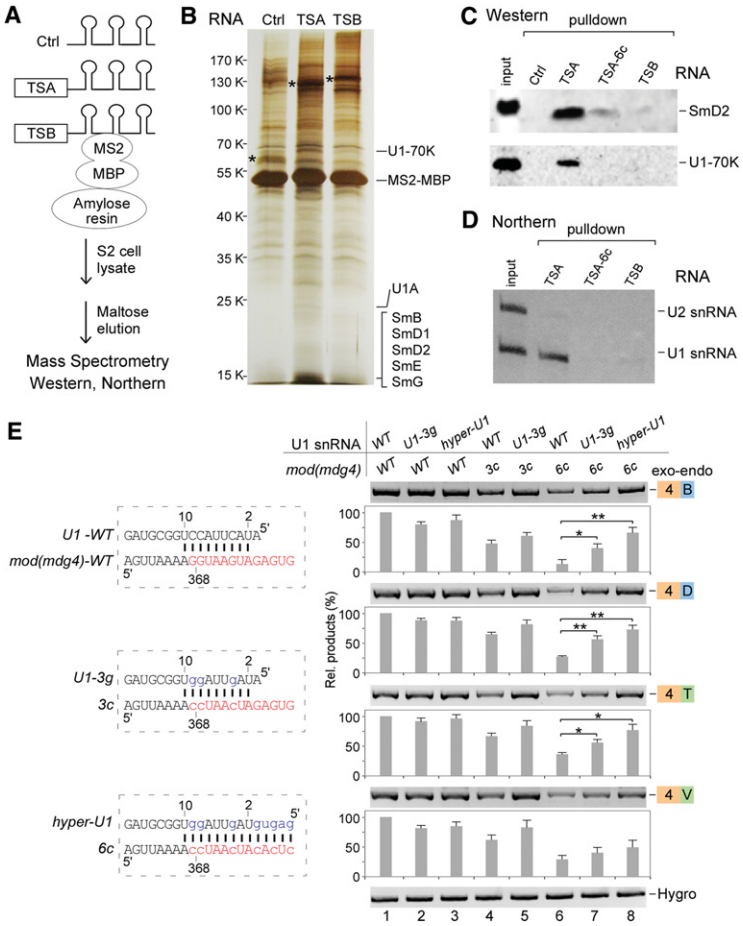


Figure 5. The TSA core motif binds with U1 snRNP to facilitate *trans*-splicing of *mod(mdg4)*. (*A*) Schematic of RNA affinity purification. (Ctrl) 6xMS2-RNA-binding sites only. (*B*) Silver staining of affinity-purified proteins. Analyzed by mass spectrometry, TSA and TSB specifically associated proteins are listed in Supplemental Table S1. Proteins that specifically associated with TSA are indicated. (*) Visualized RNAs. (*C*) Western blot analyses of TSA RNA-associated U1 snRNP proteins. (TSA-*6c*) 6Gs-to-6Cs mutations in the TSA core motif. (*D*) Northern blot analysis revealed that U1 snRNA is specifically associated with TSA. (*E*) Compensatory changes in U1 snRNA partially rescue the decreased *trans*-splicing activities of the TSA core motif mutations. The wild-type (WT) TSA core motif forms 9 base pairs (bp) with the 5′ end of U1 snRNA. *mod(mdg4)* mutants *3c* and *6c* disrupt this base-pairing, but *U1-3g* and *hyper-U1* mutants will restore or enhance the base-pairing. Relative *trans*-spliced products were normalized to loading controls and the wild-type constructs. Mean ± SEM; $n = 3$; (*) $P < 0.05$; (**) $P < 0.01$.

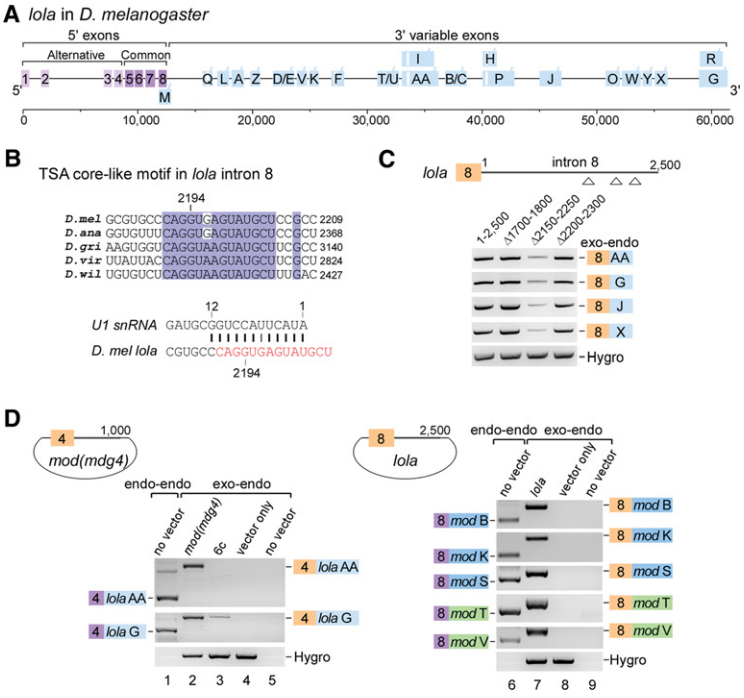


Figure 6. *lola* is mutually *trans*-spliced with *mod (mdg4)*. (*A*) Schematic of the *lola* gene locus in *D. melanogaster*. All 3′ exons are designated according to isoforms in FlyBase. (Light purple boxes) 5′ alternative exons; (dark purple boxes) common exons; (blue boxes) 3′ alternative exons; (white boxes) internal *cis*-introns. (*B*) The conserved TSA core-like motif in the last 5′ intron of *lola*, which forms 12 bp with U1 snRNA. For sequence alignment of the full-length intron 8 from available *Drosophila* species, see Supplemental Figure S6. (*C*) Deletion of a region containing the TSA core-like motif in *lola* significantly decreases *trans*-splicing activity. (*D*) The *lola* and *mod(mdg4)* genes are mutually *trans*-spliced. Both chromosomal and plasmid-borne exon 4s of *mod(mdg4)* are *trans*-spliced to the chromosomal 3′ exons of *lola* (*left*) and vice versa (*right*). *Trans*-spliced products formed between chromosomal exons (endo–endo) and between plasmid-borne and chromosomal exons (exo–endo) are indicated. Boxes in colors are used similarly to in other figures.

similar to the TSA core in *mod(mdg4)* containing a 5′SS with strong base-pairing (12 bp) to the 5′ end of U1 snRNA (Fig. 6B). Deletion of a fragment containing this conserved region (Δ2150–2250 nt) dramatically decreased *trans*-spliced products of *lola* in S2 cells (Fig. 6C), implying the existence of a TSA core-like motif in *lola*. Considering similar gene structures (Figs. 1A, 6A), we hypothesized that *trans*-splicing may occur between *lola* and *mod (mdg4)*, that the 5′ exons of *lola* could be *trans*-spliced to the 3′ exons of *mod(mdg4)*, and vice versa. Indeed, we detected multiple *lola-mod(mdg4)* or *mod(mdg4)-lola* chimeric mRNAs that were generated by *trans*-splicing between endogenous and exogenous transcripts [i.e., chromosomal exon 4 of *mod(mdg4)* efficiently *trans*-spliced to the chromosomal or plasmid-borne 3′ exons AA and G of *lola*] (Fig. 6D). Meanwhile, *mod(mdg4)-lola* chimeric *trans*-splicing activity was significantly reduced when using the *mod(mdg4) 6c* mutant (Fig. 6D, lane 3), demonstrating that *trans*-splicing of *lola* and *mod(mdg4)* requires the TSA core motif and shares a mechanism similar to *trans*-splicing within the *mod(mdg4)* gene locus.

Discussion

Trans-splicing increases genome complexity by generating chimeric mRNAs through intermolecular splicing reactions between two distinct pre-mRNA transcripts, which can be transcribed from distant genomic loci (Lasda and Blumenthal 2011). It has been reported that *trans*-splicing undergoes two transesterification steps similar to those the *cis*-splicing does (Yu et al. 1993; MacMorris et al. 2007) except leaving a Y-structured intron rather than a lariat intron (Murphy et al. 1986; Kamikawa et al. 2011). One of the key questions in *trans*-splicing is how those two nascent transcripts are brought into close proximity to allow subsequent catalysis of splicing. Several mechanisms have been proposed for *trans*-splicing events in higher eukaryotes, such as close cellular transcription sites, SR protein interactions, and complementary intronic sequences (Konarska et al. 1985; Bruzik 1996; Furuyama and Bruzik 2002; Gruber et al. 2013). In this study, through an effective *trans*-splicing detection system in *Drosophila* S2 cells and fly strains with precise deletions generated using the CRISPR/Cas9 system, two critical RNA motifs were successfully identified in the last common intron of the 5′ transcript of *mod(mdg4)*. We demonstrate that one of the conserved motifs, the TSA core, strongly binds to U1 snRNP, providing a novel mechanism to bring separate transcripts together and promote *trans*-splicing in higher eukaryotes.

We obtained multiple lines of evidence to support that the intronic TSA core motif plays a fundamental role in *trans*-splicing of *mod(mdg4)*. First, this 13-nt RNA motif is one of the few highly conserved regions in the nearly 2-kb length of *mod(mdg4)* intron 4s in all *Drosophila* species (Supplemental Fig. S2), and similar sequences are also observed in *mod(mdg4)* homologous genes in other insects (data not shown), showing a significant evolutionary importance of this motif. Second, TSA RNA is required and sufficient to promote *trans*-splicing in our *trans*-splicing detection system. Containing a pseudo-5′SS sequence, the TSA core motif binds U1 snRNP through strong base-pairing with the 5′ end of U1 snRNA. This association can be disrupted by mutations within this sequence, which significantly decreased *trans*-splicing activity, and *trans*-splicing activity was restored by compensatory mutations in U1 snRNA. Last, in vivo

deletion of a region containing the TSA core results in developmental defects of flies. The second conserved motif found in intron 4 of *mod(mdg4)*, the TSB core, has a conserved secondary structure and associates with chromatin proteins and splicing factors to enhance *trans*-splicing of *mod(mdg4)*.

Therefore, we propose a model in which two intronic RNA sequences, TSA and TSB, are critical to promote *trans*-splicing of *mod(mdg4)* (Fig. 7). First, U1 snRNP binds to TSA RNA in the last 5′ intron of *mod(mdg4)* through strong base-pairing between the conserved TSA core and U1 snRNA. Second, TSB RNA forms a highly conserved secondary structure that is required for enhancing the *trans*-splicing activity of *mod(mdg4)*. Recruitment of spliceosomal factors by TSA and TSB RNAs might introduce a bridging interaction between the 5′ and 3′ *trans*-spliced introns; however, it remains unclear how this happens. TSB association with chromatin proteins implies that TSB-specific enhancement of *trans*-splicing could be regulated by transcription and could explain the observed regulation of *trans*-spliced *mod(mdg4)* isoform abundance by *Brahma*, the ATPase subunit of the SWI/SNF chromatin remodeling complex, and the local density of RNA polymerase II (Yu et al. 2014).

Introns often contain many pseudo-5′SSs (Zhang et al. 2005); however, spliceosomal A complex formation on these sites is inefficient due to unproductive U2 snRNP recruitment (Dhir et al. 2010). There are 10 and 16 pseudo-5′SSs predicted with high scores in the last 5′ introns of *mod(mdg4)* and *lola*, respectively (Supplemental Fig. S7). Why is the TSA core motif so important for *trans*-splicing of these two genes? First, we found that among those pseudo-5′SS-containing sequences, the TSA core motif in each intron forms the strongest base-pairing with U1 snRNA: 9 bp in *mod(mdg4)* and 12 bp in *lola* (Supplemental Fig. S7). Second, TSA and TSB core motifs are highly conserved across *Drosophila* species, whereas other pseudo-5′SS sequences are not (Supplemental Figs. S2, S6). In addition, deletion of the TSA core significantly, but not totally, decreased *trans*-splicing activity in both S2 cells and flies, suggesting that other pseudo-5′SS sequences might function as backup and inefficiently facilitate *trans*-splicing in the absence of the optimal sequence.

Additionally, as another determinant of *trans*-splicing, the absence of a strong 5′SS in the *trans*-spliced 3′ introns is necessary to avoid competition from *cis*-splicing. We found that adding a 5′SS to the alternative 3′ transcripts of *mod(mdg4)* resulted in internal *cis*-splicing and abolished *trans*-splicing (Fig. 3A). Consistent with this, *trans*-spliced genes in *Drosophila* have been classified into three categories: genes with multiple 3′-terminal exons, genes with multiple first exons, or genes with very large introns (McManus et al. 2010), where the lack of strong splice sites is common.

After recruitment of spliceosomal factors by TSA and TSB, there are several possible models to explain the follow-up *trans*-splicing of *mod(mdg4)*. Binding of U1 snRNP to nascent transcripts prevents shortening and the use of cryptic polyadenylation sites (telescripting) (Kaida et al. 2010). The presence of a strong pseudo-5′SS in intron 4 of *mod(mdg4)* could increase the abundance of *mod(mdg4)* transcripts containing intron 4 available for *trans*-splicing. However, this is unlikely to be the mechanism, as there are no significant changes in transcript levels when introns either contain a mutated TSA core motif or completely lack the sequence (Fig. 4C). In addition, recursive *cis*-splicing has been well characterized in *Drosophila* and is often used for excising long introns in which the key sequence is a ratcheting point $(Y)_n$-NCAG/GUAAGU, similar to the TSA core motif (Burnette et al. 2005; Venables et al. 2012). However, our data do not support a recursive splicing model for *mod(mdg4)*. First, if the *trans*-splicing reaction occurs between the TSA core motif and downstream 3′SS, the newly generated 3′SS of *D. melanogaster* is a suboptimal 3′SS (AAG/) and could be an even worse in other *Drosophila* species, such as in *Drosophila virilis* (GGG/) (Fig. 2A). Second, compensatory mutations in U1 snRNA rescue the decreased *trans*-splicing activity of TSA core motif mutants in which the 5′SS is totally mutated (Fig. 5E), and recursive splicing cannot occur.

Trans-splicing of *mod(mdg4)* and *trans*-splicing of *lola* share a similar mechanism, both using a conserved intronic TSA core motif to promote the reaction (Fig. 6). In order to verify whether this is a common mechanism of *trans*-splicing in *Drosophila*, we searched the last 5′

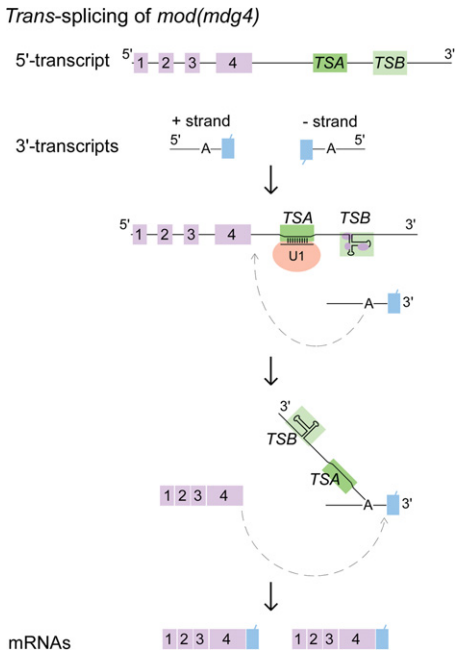


Figure 7. *Trans*-splicing of *mod(mdg4)* is facilitated by two intronic RNA motifs. Highly conserved TSA and TSB RNAs located in the last 5′ intron are critical for *trans*-splicing of *mod(mdg4)*. TSA RNA is sufficient to promote *trans*-splicing in *Drosophila*, which binds U1 snRNP through strong base-pairing between its highly conserved 13-nt TSA core motif and the 5′ end of U1 snRNA. TSB RNA forms a highly conserved secondary structure and enhances the *trans*-splicing activity of *mod(mdg4)*. Including the formation of a Y-structured intermediate, the two-step chemical reactions of *trans*-splicing are indicated.

introns of other *trans*-splicing events in *Drosophila* (McManus et al. 2010) and found that 16 of the 126 introns have pseudo-5′SS-containing sequences, which form ≥9 bp with the 5′ end of U1 snRNA (i.e., TSA core-like motifs) (Supplemental Table S2). Under such strict searching criteria, this result implies that the TSA core-mediated mechanism of *trans*-splicing could represent a general mechanism for a class of *trans*-splicing events in *Drosophila*.

Materials and methods

Strains and plasmids

For plasmids used in *Drosophila* S2 cells, sequences from *mod(mdg4)*, *lola*, or other genes in *D. melanogaster* were inserted into modified pMT/V5-His B vector (Invitrogen) with hygromycin B and P copia promoter as described (Yang et al. 2013). Plasmids were transfected into S2 cells by Effectene transfection reagent (Qiagen) and induced by 0.5 mM $CuSO_4$ at 12 h after transfection; cells were collected 24 h later for isolation of RNA and proteins. The sequence of U1 snRNA, including a ±1-kb region, was amplified from genomic DNA using primers listed in Supplemental Table S3. The CDS of EGFP was amplified from vector pEGFP-N1 (Invitrogen) and split after nucleotide G489.

RT–PCR and sequence analyses

Total RNA was extracted using Trizol (Invitrogen), and reverse transcription was performed by a first strand cDNA synthesis kit (RevertAid, Thermo) using either oligo(dT) or specific primers. *Trans*-spliced products were amplified by Ex Taq DNA polymerase (TaKaRa), confirmed by Sanger sequencing, and quantified using a Tanon-2500 gel analysis system. All sequences from the *Drosophila* species were downloaded from FlyBase and aligned using DNAman version 4.0 (Lynnon BioSoft). Potential splice sites were identified according to scores obtained by the online software HSF (Desmet et al. 2009).

RNA affinity purification and mass spectrometry

Along with 6xMS2 RNA-binding sites (Zhou et al. 2002), studied RNAs were in vitro transcribed by T7 RNA polymerase (Promega) and gel-purified. MS2-MBP protein was expressed in *Escherichia coli* and purified by Superdex 200 10/300 GL column (GE) (Tange et al. 2005). Ninety picomoles of RNA was mixed with 270 pmol of MS2-MBP protein for 20 min on ice in 50 µL of buffer 1 (20 mM Tris-Cl at pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5 mM DTT, 50 µg/mL yeast tRNA, 5 mM creatine phosphate, 0.5 mM ATP, 200 U/mL RNase inhibitor, protease inhibitors). The mixture was then applied to amylose resin (New England Biolabs) and tumbled with 0.1% BSA to reduce background before adding 1.2 mL of lysate from 2×10^7 S2 cells (Spain et al. 2010) in buffer 1. After six washes with buffer 1, bead-bound proteins were eluted with buffer 2 (12 mM maltose, 20 mM Tris-Cl at pH 7.9, 100 mM KCl) and separated on SDS-PAGE. Obtained proteins were then analyzed using a Q-Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Scientific) with a Dionex UltiMate 3000 RSLCnano system. Peptide identification and protein assembly were performed on a Thermo Proteome Discoverer 1.4.1 platform. For each tandem mass spectrometry data set, a single search was performed against the corresponding UniProtKB/Swiss-Prot database using the SEQUEST and percolator algorithms.

Western and Northern analyses

Antiserum against *Drosophila* U1-70K was generated by immunizing rabbits with the peptide DGKKIDSKRVLVDV (amino acids 164–177). Anti-SNRPD2 (SmD2) antibody was purchased from Abcam (ab155030). Western blots of anti-EGFP and anti-tubulin were probed using mAb 7G9 (Abmart) and mAb DM1A (Sigma), respectively. Northern analysis was performed by transferring RNAs from 8 M urea-PAGE gel to Hybond N membrane (Amersham). Membranes were then probed by DIG-labeled (Roche) antisense DNAs complementary to the full-length U snRNAs in *D. melanogaster*.

Fly strains and mutagenesis

Culture and crosses of *D. melanogaster* were carried out on standard medium and at standard temperature. Mutagenesis of the *Drosophila mod(mdg4)* gene were performed using the recently developed CRISPR/Cas9 system (Ren et al. 2013). Briefly, target sequences of two guide RNAs (sgRNA) (listed in Supplemental Table S3) were selected for each genomic deletion. Each pair of sgRNA plasmids was coinjected into embryos of transgenic line *nanos-Cas9*(*attP40*) by Core Facility of *Drosophila* Resource and Technique, Shanghai Institute for Biological Sciences. G0 flies were then crossed with the *TM3*/*TM6* balancer strain (Bloomington no. 5906); subsequently, G1 male flies were crossed with the same balancer strain followed by genomic DNA screening for deletion strains. Obtained flies were then backcrossed at least five generations into a controlled uniform homogeneous genetic background (Bloomington no. 5905) to eliminate potential off-target events.

To generate constructs for genomic rescue of lethality caused by the deletion of *mod(mdg4)* common region, a 6690-bp wild-type fragment containing a sequence from part of the upstream gene *tinman* to nucleotide 1564 of *mod(mdg4)* intron 4 was amplified and cloned into the pUAST(attB) vector. Three parallel deletion constructs—*ΔTSA* (340–487 nt), *ΔTSB* (646–799 nt), and a control, *ΔTSC* (1162–1310 nt)—were constructed by overlapping PCR based on the above wild-type plasmid. Genomic rescue constructs were microinjected and incorporated into the *attP40* site on chromosome II. Obtained flies were then applied in crosses to test their ability to rescue the lethality of the homozygous $mod(mdg4)^{\Delta com}$ strain. For each cross, total offspring from six parallel vials were counted based on their genotypes.

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