Splicing of branchpoint-distant exons is promoted by Cactin, Tls1 and the ubiquitin-fold-activated Sde2

Anupa T. Anil1,†, Karan Choudhary1,†, Rakesh Pandian1,†, Praver Gupta1, Poonam Thakran1, Arashdeep Singh1, Monika Sharma2 and Shravan Kumar Mishra1,*

1Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali, Sector 81, 140306 Punjab, India and 2Department of Chemical Sciences, Indian Institute of Science Education and Research (IISER) Mohali, Sector 81, 140306 Punjab, India

Received July 21, 2022; Revised August 22, 2022; Editorial Decision August 23, 2022; Accepted August 27, 2022

ABSTRACT

Intron diversity facilitates regulated gene expression and alternative splicing. Spliceosomes excise introns after recognizing their splicing signals: the 5′-splice site (5′ss), branchpoint (BP) and 3′-splice site (3′ss). The latter two signals are recognized by U2 small nuclear ribonucleoprotein (snRNP) and its accessory factors (U2AFs), but longer spacings between them result in weaker splicing. Here, we show that excision of introns with a BP-distant 3′ss (e.g. rap1 intron 2) requires the ubiquitin-fold-activated splicing regulator Sde2 in Schizosaccharomyces pombe. By monitoring splicing-specific ura4 reporters in a collection of S. pombe mutants, Cay1 and Tls1 were identified as additional regulators of this process. The role of Sde2, Cay1 and Tls1 was further confirmed by increasing BP–3′ss spacinss in a canonical th05 intron. We also examined BP-distant exons spliced independently of these factors and observed that RNA secondary structures possibly bridged the gap between the two signals. These proteins may guide the 3′ss towards the spliceosome’s catalytic centre by folding the RNA between the BP and 3′ss. Orthologues of Sde2, Cay1 and Tls1, although missing in the intron-poor Saccharomyces cerevisiae, are present in intron-rich eukaryotes, including humans. This type of intron-specific pre-mRNA splicing appears to have evolved for regulated gene expression and alternative splicing of key heterochromatin factors.

INTRODUCTION

Splicing of precursor mRNAs into protein-coding mRNAs is an essential step in gene expression. It also promotes regulated gene expression and alternative splicing. The process is completed by the spliceosome assembled from five small nuclear RNA-protein (snRNP) complexes. Spliceosomes excise pre-mRNA introns detected by their splicing signals. The 5′-splice site (5′ss) is detected by U1 snRNP, while the branchpoint (BP), the polypyrimidine tract and the 3′-splice site (3′ss) are detected by U2 snRNP and its accessory factors (U2AFs) (1,2). Further, the U4/U6-U5 tri-snRNPs are recruited to form the B complex spliceosome (3,4). The unwinding of U4/U6 snRNAs by Brr2 helicase mediates the formation of the U2/U6 snRNP complex. The active site embedded within the U2/U6-U5 snRNP complex is formed with two catalytic metal-binding sites in U6 snRNA, which catalyses both transesterification reactions needed to excise the intron and ligate the two exons (5–8).

Introns differ in positions and sequences of splicing signals, lengths and location in the pre-mRNA. Furthermore, the presence of sequence motifs that act as splicing enhancers or silencers, and the propensity to form secondary structures, also differ (9–13). This diversity is critical for regulated gene expression and alternative splicing; therefore, intron-rich eukaryotes experience an enormous impact of RNA splicing on their physiology. To tackle the processing of diverse introns, intron-specific splicing regulators that...
include RNP assembly factors, RNA-binding proteins or RNA- and protein-modifying proteins and enzymes are recruited to the spliceosome (14).

Ubiquitin and ubiquitin-like proteins (e.g. SUMO, Hub1 and Sde2; collectively referred to as UBLs) are post-translational modifiers of proteins controlling diverse cellular activities. They also regulate pre-mRNA splicing by modifying spliceosomes, thereby potentiating the machinery to act on specific introns and pre-mRNAs (15). UBLs appear dispensable for constitutive pre-mRNA splicing involving introns with canonical splicing signals, but become critical for excising introns with non-canonical sites. For example, the UBL Hub1/UBL5 is conserved from *Saccharomyces cerevisiae* to humans. It promotes alternative splicing and facilitates spliceosomal recognition of suboptimal 5′ss. It modifies spliceosomes by binding to HIND-containing splicing factors (16–19). Hub1 from *S. cerevisiae* also activates the spliceosomal RNA helicase Prp5, allowing Hub1-modified spliceosomes to use a non-canonical 5′ss and allow alternative splicing (19).

Spliceosomes modified by the ubiquitin-fold-activated Sde2 promote the excision of selected introns in a subset of pre-mRNAs in *Schizosaccharomyces pombe*. This splicing regulator is conserved among intron-rich eukaryotes up to humans, but is absent in intron-poor organisms such as *S. cerevisiae*. Sde2 was recently shown to be important in pre-mRNA splicing in mammalian cells (20). Sde2 is translated as an inactive precursor harbouring an insert at the N-terminus. The removal of Sde2UBL and the free lysine of K3Sde2-C are critical for its intron-specific splicing function. However, the question of why only selected pre-mRNAs required Sde2 for efficient splicing remained unanswered.

Here, we report that Sde2 facilitates excision of introns with longer spacing between the BP and 3′ss (referred to as BP-distant 3′ss). This activity requires the cleavage of Sde2UBL by DUB and the free lysine of processed K3Sde2-C. Using splicing reporters made with the *ura4* gene split by introns of varying distances between the BP and 3′ss, we searched for *S. pombe* deletion mutants of putative splicing factors. We identified Cactin/Cay1 and Tsl1 as additional regulators of BP-distant splicing. These three splicing regulators are absent in budding yeast but are conserved from the fission yeast *S. pombe* to metazoans. Furthermore, by altering the spacing between the BP and 3′ss in selected targets, we show that introns with longer spacing between the BP and 3′ss require Sde2, Cay1 and Tsl1 for efficient splicing. They regulate gene expression and alternative splicing of various heterochromatin factors, including the telomeric factor Rap1.

**MATERIALS AND METHODS**

**Plasmids, strains, *S. pombe* transformation and growth assays**

Plasmids and strains used in this study are listed in Supplementary Tables S2 and S3, respectively. The *S. pombe* deletion strains of splicing factors were obtained from the Bioneer haploid deletion library. Competent cell preparation and transformation were performed following previously published protocols (23,24). Double knockout strains for genetic interactions were made by tetrad dissection of spores. The plasmid clones indicated in Supplementary Figure S1A were used to tag genes at their chromosomal loci. The NotI inserts were transformed into wild-type (wt) *S. pombe* strains, and tagging was confirmed by western blot analysis. Polymerase chain reaction (PCR) and western blotting confirmed gene deletions and tagging, respectively (23,24). The reporter strain for telomeric silencing in Δsde2 (25) was obtained from NBRP-yeast, Japan. Plasmids expressing chromatin factors had the *nmt81* promoter inducible in the absence of thiamine and the *S. cerevisiae* tef2 terminator. Overexpression was carried out by expressing clones under a strong *eno101* promoter and a tef2 terminator. For growth/spot assays, 5-fold serial dilutions of cells were spotted on the indicated agar plates, and plates were incubated at temperatures indicated in the figure legends.

**Splicing reporters**

The reporters used in this study are listed in Supplementary Table S4. Splicing reporter plasmids have the *S. pombe* ars1, leu2+ selection marker and *ura4* cDNA with introns inserted between nucleotides 426 and 427. Insertions at this position in *ura4* were previously used to study exon skipping (26). The reporters have a 400 bp segment of the *S. pombe* eno101 promoter, an N-terminal 3MYC tag and the tef2 terminator. Intronic variants were inserted into the *ura4* reporter using specific primers in SOE (splicing by overlap extension) PCR. Point mutations in the BP and 3′ss were made by QuickChange site-directed mutagenesis (Agilent). For reporter assays and screens, competent cells from *S. pombe* strains were transformed with the reporter and transformants were selected in media lacking uracil, and counter-selection was performed on media containing 0.1% 5-fluoroorotic acid (5-FOA). The plates were incubated for 3–5 days at 30°C.

**Bioinformatics of *S. pombe* introns**

The Sde2-dependent introns were obtained from the splicing-sensitive microarray of the Δsde2 strain (GEO accession number GSE97097) (22). Nucleotide sequences were obtained from the PomBase server. RNA structures were predicted on the RNAfold WebServer (27). For intron analysis, the complete dataset of introns was downloaded from PomBase and then mapped with the microarray data. A cut-off of 0.5 log₂Δsde2/wt ratio (~1.4-fold difference) of intron-retention values obtained with the strains grown at 30°C was applied. Introns with ratios >0.5 were considered Sde2 dependent, and the remaining introns were considered Sde2 independent. The database was then matched with the following list of BP sequences: ‘C‘CAC’, ‘C‘AAC’, ‘U‘AAC’, ‘U‘AAAC’, ‘UC‘AAC’, ‘CC‘GAC’, ‘C‘UGAC’, ‘U‘UGAC’, ‘UC‘GAC’, ‘CCA‘AU’, ‘C‘UA‘AU’, ‘U‘UA‘AU’, ‘U‘CA‘AU’, ‘C‘CG‘AU’, ‘C‘UG‘AU’, ‘U‘UG‘AU’, ‘UC‘GA‘U’, ‘GU‘AC’.
RNA isolation and cDNA synthesis

For RNA isolation, logarithmically growing cells equivalent to 2 OD_{600} were harvested (at OD_{600} ~0.5–0.6). The hot acid–phenol method was used for RNA isolation, and 2 ml phase-lock tubes (Qiagen) were used for phase separation (29). Multiscribe reverse transcriptase with random hexamer primers (Thermofischer Scientific) was used to synthesize cDNAs from 1 μg of total RNA incubated at 42°C for 16 h. Splicing defects were monitored by detecting intron-containing transcripts or transcripts matured post-splicing. Reporter-specific intron-containing transcripts were detected using a cDNA template by PCR with a 3MYC tag-specific primer and a ura4 exon 2-specific primer. The reporter-specific transcripts matured post-splicing were detected using the 3MYC tag-specific primer and the exon–exon junction-specific primer (where the first half of the primer annealed to ura4 exon 1 and the second half of the primer annealed to exon 2). The PCR products were run on a 2% agarose gel. Real-time quantitative PCR (qRT-PCR) was performed using SYBR green (Roche). The primers used for the splicing assay and qRT-PCR were listed in Supplementary Table S5. Lariat PCR was described in (30). Briefly, 2 μg of DNase I-treated RNA was heat denatured at 65°C for 5 min, and cDNA synthesis was performed in the presence of 3 mM MgCl₂ and 3 mM MnCl₂ for 2 h at 37°C with a reverse primer specific for the target introns.

Western blots

For protein western blots (immunoblotting), logarithmically growing cells equivalent to 2 OD_{600} were harvested. Whole protein was extracted using the trichloroacetic acid (TCA) method (24). Total proteins used for western blots in Supplementary Figure S12B were isolated by heating cells in high urea buffer at 65°C for 15 min. The protein samples were run on sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to a polyvinylidene difluoride (PVDF) membrane and probed with specific primary and secondary antibodies.

Antibodies

Anti-MYC antibodies raised in rabbits (MYC polyclonal), anti-haemagglutinin (HA) raised in mice (HA, monoclonal), anti-mouse horseradish peroxidase (HRP; goat) and anti-rabbit HRP (HRP; goat) were procured from Sigma-Aldrich.

RESULTS

Intronic features define the role of Sde2 in pre-mRNA splicing

We have previously reported that _S. pombe_ Sde2 is a ubiquitin-fold-activated splicing regulator that facilitates pre-mRNA splicing in an intron-specific manner (22). Splicing-sensitive microarrays were performed with the wt and the Δsde2 strain to understand the role of Sde2 in pre-mRNA splicing. The microarray data, further verified by RT–PCR, showed that the lack of Sde2 (Δsde2) leads to the retention of only selected introns (22). A key splicing target of Sde2 is _rap1_ pre-mRNA encoding the telomere-binding shelterin complex subunit Rap1 (Figure 1A; Supplementary Figure S1A, B). However, it is unknown why only specific introns, such as intron 2 but not intron 1 of _rap1_, required Sde2 for splicing.

To understand the intron specificity of Sde2, we made two splicing reporters with the _S. pombe_ _ura4_ gene split individually by the two _rap1_ introns (Figure 1B). We tested the reporters’ ability to complement a _ura4_ auxotrophic _S. pombe_ strain. Functional _ura4_ mRNA and protein were expected only after accurate splicing of the pre-mRNAs that would allow the cells to grow on plates lacking uracil (–URA) but not on uracil counter-selection plates containing 5′-FOA (+FOA) (Figure 1C). With the reporter split by the Sde2-independent _rap1_ intron 1 (_rap1-i1_ reporter), both the wt and Δsde2 strains grew on –URA plates but not on +FOA. In contrast, with the reporter split by the Sde2-dependent _rap1_ intron 2 (_rap1-i2_ reporter), wt cells grew on –URA but not on the counter-selection plate containing FOA, while Δsde2 cells did not grow well on –URA but grew on the +FOA plate (Figure 1D).

The above results suggest that _rap1_ intron 2 (but not intron 1) requires Sde2 for splicing. The intron specificity was further confirmed by detecting reporter-specific proteins in western blots and transcripts in reverse transcription–PCR (RT–PCR) assays. For these assays, a DNA sequence encoding the 3MYC epitope tag was inserted at the N-terminus of the reporters (Figure 1B). In RT–PCR, one primer was annealed to 3MYC and another to ura4 exon 2 or the junction of the two exons. The _rap1-i1_ reporter was spliced in both _wt_ and Δsde2 strains. In contrast, the _rap1-i2_ reporter was spliced only in the _wt_ but not in Δsde2 (Supplementary Figure S1C, D). The binding specificity of the junction primers for the ura4 cDNA was verified by performing multiplex PCR on plasmid DNA with and without the intron as templates (Supplementary Figure S1E). Anti-MYC western blots were used to detect reporter-specific proteins. The _rap1-i1_ reporter formed the full-length Ura4 protein in both _wt_ and Δsde2 strains, whereas the _rap1-i2_ reporter formed the full-length Ura4 protein only in the _wt_ but not in Δsde2. In general, both reporters were partially spliced. They showed truncated Ura4-N proteins resulting from intron retention in all strains, indicating that both _rap1_ introns were weak once kept out of their native location (Supplementary Figure S1F). However, _rap1_ intron 2 needed Sde2 for splicing. Thus, the splicing role of Sde2 was attributed...
to specific features present in the intron. These assays also ruled out the possible involvement of transcription, untranslated regions (UTRs) or the protein-coding parts of the rap1 gene for its Sde2 dependency (see the next section).

Sde2 precursors in S. pombe and humans are processed into Sde2UBL and K-Sde2-C (21,22). After the DUBs Ubp5 and Ubp15 cleave the S. pombe precursor at the conserved GG–K motif, the processed K-Sde2-C enters the spliceosome (Figure 1A). K-Sde2-C is short-lived due to its pro-tesosomal degradation by the N-end rule pathway (22,31). A ubiquitin–K-Sde2-C chimaera complemented growth defects in the Δsde2 strain, suggesting that ubiquitin could replace the Sde2UBL activity of producing functional K-Sde2-C. We tested the importance of ubiquitin-like processing for reporter splicing in three strains: (i) a DUB deletion strain Δubp5 Δubp15; (ii) a processing-defective sde2 (AAK) mutant (amino acid changes underlined); and (iii) a ubiquitin–K-Sde2-C chimaera that replaces chromosomal sde2 (Figure 1E; Supplementary Figure S1G, H). Like the Δsde2 strain, the sde2 processing-defective strains Δubp5 Δubp15 and sde2 (AAK) spliced only the rap1-i1 reporter and became uracil positive. However, neither of the strains could splice the rap1-i2 reporter and remain uracil negative. In contrast, the Sde2-replacing ubiquitin–Sde2-C chimeric strain spliced both reporters and became uracil positive (Figure 1E). We also tested the need for N-terminal lysine in K-Sde2-C in a lysine to methionine mutant strain sde2 (GGM). This mutant, which forms 35Sde2-C, could splice only the rap1-i1 reporter but not rap1-i2 (Figure 1F). Thus, both ubiquitin-like processing and the N-terminal lysine of K-Sde2-C are essential for the rap1 intron 2-specific splicing activity of Sde2.

Introns with longer spacing between BP and 3′ss require Sde2 for splicing

Next, we ask which features of the intron targets detected in the microarrays made them dependent on Sde2. The target introns (defined as log2 Δsde2/wt signal ratio at 30℃ ≥ 0.5) lacked a common sequence motif and did not match with respect to the strengths of their splicing signals, lengths or position in resident pre-mRNAs. The 5′ss in the Sde2 target introns showed a reduced enrichment of the nucleotide ‘A’ at positions +3 and +4 compared with Sde2 non-targets. Although most targets did not have a weak BP or 3′ss, a few had a non-canonical BP and 3′ss or even their combinations (Supplementary Table S1). The Sde2-dependent introns had an average of 3.32 poly(pyrimidine) tracts between the 5′ss and BP (compared with 2.40 in non-targets) and 0.38 be-
between the BP and 3'ss (0.25 in non-targets) (Supplementary Figure S2A, B). For testing whether Sde2 dependency was due to a non-canonical 5'ss, the 5'ss of rap1 intron 2 was changed to a canonical site (GTATGA to GAAAGT) in the genomic construct of rap1. RT–PCR was used to study splicing efficiency in wt and Δsde2 strains (Supplementary Figure S3A). We did not observe an improvement in its splicing in the Δsde2 strain. We also made reporters with non-canonical variants of the BP and 3'ss in an Sde2-independent tho5 intron 1 (tho5-ii) [Tho5 is a THO complex subunit (32)] and tested their splicing in wt and Δsde2 strains. For example, the BP and 3'ss subtle splicing defects of BP and 3'ss showed most striking difference in splicing efficiency between the wt and Δsde2. This result indicated that Sde2-C becomes critical for using the 3'ss at ≥21 nt from the BP (Figure 3C). Interestingly, the intron with a 6 nt gap between the BP and 3'ss showed reduced levels of Ura4 protein on immunoblot. The defect might be due to steric clashes in the spliceosome when the BP and 3'ss are too close. The results were further validated by RT–PCR and qRT-PCR assays for selected reporters (Figure 3D, E; Supplementary Figure S4D). Furthermore, there is a limit to the spacing between the BP and 3'ss for efficient splicing. Splicing efficiency decreased drastically when we further increased BP–3'ss spacing, and introns with ≥30 nt spacing between the BP and 3'ss were poorly excised even in wt cells (Figure 3B, C). The correct usage of the newly introduced BP was verified by sequencing PCR products from lariats in the rap1-i2 distance-reduced reporter (BP to 3'ss = 12 nt) and a tho5-ii reporter variant (BP to 3'ss = 30 nt) (Supplementary Figure S5A, B).

We next tested whether Sde2 could enhance splicing through a BP-distant 3'ss in the presence of a BP-near 3'ss. For this, we made a reporter with two competing 3'ss, BP-near and BP-distant, and monitored its splicing in wt cells (Figure 4A–C). A protein corresponding to the mRNA formed after the usage of only the BP-near 3'ss could be detected, suggesting that BP-distant splicing is avoided in the presence of a more favourable BP-near 3'ss.

**RNA between BP-distant 3'ss could be structured**

Among ~5250 introns in S. pombe, the spacing of BP–3'ss in ~40 introns is ≥30 nt. The spacing is ≥20 nt in ~90 introns. Despite a longer spacing between the BP and 3'ss, several introns did not appear to require Sde2 (22). Thus, the question arose of how those introns were excised in Δsde2. One possibility was that the sequence between the BP and 3'ss of these introns might form secondary structures, thus bringing their 3'ss closer to the BP. BP–3'ss intervening segments in several introns were predicted to fold into secondary structures with shorter effective distances between the BP and 3'ss. Such secondary structures were detected in, for example, intron 1 of cam1 [encoding calmodulin (35)], intron 1 of ste4 [encoding MAPK cascade adaptor protein (36)], intron 2 of fmd1 [encoding formaldehyde dehydrogenase (37)], intron 1 of whi5 [encoding a transcription repressor (38)], intron 2 of atm20 [encoding an autophagy protein (39)] and intron 2 of mug65 [encoding a dyserlin-like membrane trafficking factor (40)] (Supplementary Figures S6 and S7A). In such cases, the disruption of secondary structures by exposure to elevated temperatures could open the fold and increase the distance between the BP and 3'ss. We examined this possibility by RT–PCR assays. Indeed, excision of introns with secondary structures such as rap1 intron 2, whi5 intron 1, atm20 intron 2 and mug65 intron 2 was sensitive to 15 min treatment at 42°C. After lowering the temperature to 25°C, splicing defects of these introns recovered in wt cells, but the recovery with whi5 and atm20 introns was slower in Δsde2. The heat-sensitive splicing defects and the slower recovery of splicing in Δsde2 seem to correlate with the presence of secondary structures between the BP and 3'ss. This defect could be attributed to the presumed opening of secondary structures resulting in
Figure 2. Sde2 target introns have longer spacing between the BP and 3′ss. (A) Distribution of distances (number of nucleotides) between the BP adenine and the 5′- and 3′-splice sites (ss) of the Sde2-dependent and -independent introns (Sde2-dependent ≥ 0.5 log₂ Δsde2/wt ratio at 30°C; Sde2-independent ≤ log₂ Δsde2/wt ratio at 30°C). Red peaks and histograms show 122 Sde2-dependent introns, and blue peaks and histograms show 4418 Sde2-independent introns. The numbers on the peaks show their maxima. (B) Nucleotide sequence of rap1-i2 with different spacing between the BP and 3′ss. (C and D) Growth assay and RT–PCR showing that rap1-i2 with 39 nt between the BP and 3′ss was Sde2 dependent (incubation time: –LEU, –URA 4 days; +FOA 5 days). The reduction of this spacing made its splicing Sde2 independent. RT–PCR was performed by two sets of primers as indicated in the schematic. ex1-ex2 indicates PCR performed with primers that bind to exon 1 and exon 2, thereby monitoring both spliced and unspliced ura4 transcripts, and ex1-Jn indicates PCR performed with primers that anneal to exon 1 and the exon 1–exon 2 junction, thus monitoring only the spliced ura4 transcripts. The arrows in the schematics depict the binding of primers in the reporter. (E) Nucleotide sequence of tho5-i1 with different spacing between the BP and 3′ss. (F and G) Growth assay and RT–PCR showing that an Sde2-independent intron, tho5-i1, required Sde2 for splicing after increasing the distance between the BP and 3′ss. Growth assay and RT–PCR were performed similarly to (C) and (D) (incubation time: –LEU, –URA 4 days; +FOA, 5 days). Arrows in the schematics depict primer binding to the reporter.
increased effective distances between the BP and 3′ss. Excision of introns lacking secondary structures [e.g. *tho5* intron 1; introns in *vps55* encoding a vacuolar sorting protein (41); and introns in *hse1* encoding the ESCRT-0 complex subunit (42)] did not appear heat sensitive, suggesting optimal spliceosomal activity (Supplementary Figure S7B). Structured introns such as *cam1* intron 1, *ste4* intron 1 and *fmd1* intron 2 did not show temperature-sensitive splicing, suggesting that the heat shock regimes tested above might not have opened their structures. Also, structures in introns are likely to have specific regulatory elements and roles beyond RNA splicing.

Specific Sde2 target introns, including 39 nt RNA between the BP and 3′ss of *rap1* intron 2, were also predicted to fold into weak secondary structures (Supplementary Figure S8). To test whether the secondary structures could affect pre-mRNA splicing, *rap1* intron 2 was mutated to weaken the structure. The effective distance between the BP and 3′ss was predicted to increase by the change. Weakening of the structure caused splicing defects even in wt *S. pombe*. Defects due to altered bases could be rescued by regaining an RNA fold through complementary mutations (Figure 5A–C). Changes were also introduced to stabilize the structure in *rap1* intron 2 to increase the abundance and lifetime of folded transcripts. (Figure 5D–F). RT–PCR and qRT-PCR analyses showed that excision of structurally stabilized *rap1*-i2 improved in the Δsde2 strain (Supplementary Figure S9A, B). These results suggested that Sde2 activities and potential RNA structures synergize splicing of introns with BP-distant 3′ss. To independently verify the above data in another target, mutations were introduced to abolish the predicted structure in *atg20* intron 2. Excision of this intron variant became Sde2 dependent. Furthermore, complementary mutations that regain an RNA fold in the variant intron also partially rescued the splicing defect in Δsde2 (Supplementary Figure S9C–E).

---

**Figure 3.** Threshold distance between the BP and 3′ss for the Sde2 target introns. (A) Schematics of *tho5-i1* reporters with increasing spacing between the BP and 3′ss. (B, C) Growth assay and immunoblot analysis show that an increase in the distance between the BP and 3′ss makes the intron Sde2 dependent (incubation time: –LEU, –URA, 3 days; +FOA, 5 days). * marks the stop codon that arises during the translation of intron-retained mRNA. * marks proteins possibly arising from aberrant splicing or proteolytic cleavage. (D) Semi-quantitative RT–PCR showing splicing of *tho5-i1* reporters with different spacing between the BP and 3′ss. RT–PCR was performed in the same way as in Figure 2D. Small amounts of Ura4 protein appeared enough for the cells to grow on –URA, but the cDNA and protein’s detection by RT–PCR and immunoblots seems to require more efficient splicing. Thus, growth assays discriminate the reporters’ activities better with lower splicing efficiency, whereas RT–PCR and immunoblots discriminate the reporters’ activities better with higher splicing efficiency. (E) qRT-PCR analysis to quantify spliced transcripts from the *tho5-i1* reporter with different spacings between the BP and 3′ss in wt (grey bars) and Δsde2 (black bars). The quantitation was against *leu2* transcripts arising from the same plasmid. The forward primer used in qRT-PCRs binds to exon 1, and the reverse primer binds to the exon 1–exon 2 junction, thus specifically amplifying the spliced transcripts. The y-axis denotes log2 spliced ura4 transcripts against leucine transcripts, and the x-axis depicts different spacings between the BP and 3′ss in the *tho5-i1* reporter.
Sde2 functions with Cactin/Cay1 and Tls1

Besides Sde2, additional splicing factors might facilitate the usage of a BP-distant 3′ss. Thus, we searched for more factors needed for splicing the BP-distant 3′ss rap1 intron 2. Splicing of rap1-i1 and rap1-i2 reporters was tested in a collection of 48 viable deletion mutants of putative splicing factors and 10 conditional mutants of splicing factors essential for viability (Supplementary Figure S10A, B). Assuming the activities of both reporters in the above mutants allowed us to exclude constitutive splicing factors, including the ones involved in splicing rap1 intron 1 (mutants defective in constitutive splicing would not splice either reporter). Reporter-specific growth assays could not be used under restrictive conditions of mutants in essential factors (when their splicing defects become visible). Therefore, to identify Δsde2-like defects in these mutants, the splicing of Sde2 target introns, rap1 intron 2 and mcs2 intron 2, was assayed by RT–PCR (Supplementary Figure S10B) [Mcs2 is a TFIIH complex cyclin (43)]. Among the mutants studied, Δcay1 and Δtls1 strains also showed splicing defects specific for rap1 intron 2 (Supplementary Figure S10A). The defects in Δcay1 and Δtls1 were also specific to introns with a BP-distant 3′ss. Shortening the distance to 12 nt made the splicing independent of all three factors (Figure 6A, B). In contrast, the tho5-i1 reporter with the BP and 3′ss 12 nt apart did not require these factors but became dependent on all three when the BP was shifted 39 nt away from the 3′ss (Figure 6C, D). These results suggested that Sde2, Cay1 and Tls1 play a common role in facilitating the usage of a BP-distant 3′ss.

We assessed genetic interactions between sde2, cay1 and tls1 by combining their deletions into double mutants. These factors showed negative genetic interactions; co-deletion of any two genes in the cell was synthetically sick or probably lethal (Figure 6E). These phenotypes suggested that the three factors acted separately. Overexpression of one protein did not complement defects from the lack of another, although overexpression of Tls1 enhanced growth in the Δsde2 strain (Supplementary Figure S11A). Thus, the splicing activities of these proteins were not redundant. Although Sde2 helps recruit Cay1 to the spliceosome (22), yeast two-hybrid assays did not detect interactions between Sde2, Cay1 and Tls1 (Supplementary Figure S11B), suggesting that these proteins may not bind to one another directly. The genetic relationships between these factors were further verified by monitoring the splicing of the reporters in the respective double mutants. Like single mutants, the viable double mutants could efficiently splice the rap1-i1 reporter, but not rap1-i2 (Supplementary Figure S11C). Concerning the BP–3′ss distance threshold required for splicing, Δcay1 Δtls1 and Δsde2 Δtls1 double mutants showed splicing defects similar to their single mutants (Supplementary Figure S11D).

Sde2, Cay1 and Tls1 control the expression of RNA interference (RNAi) and heterochromatin factors via splicing

Spectosomes use Sde2, Cay1 and Tls1 to splice pre-mRNAs containing introns with BP-distant 3′ss, whereas introns with BP-near 3′ss in the same pre-mRNA do not require these factors. These proteins have also been involved in heterochromatin silencing (25,44,45). Tls1 was reported to regulate the expression of genes of the shelterin complex via pre-mRNA splicing. In contrast, splicing factors have...
Figure 5. RNA structures may bring the 3′ss closer to the BP. (A) Predicted secondary structures of the RNA between the BP and 3′ss in rap1-i2 variants. Arrows indicate nucleotides where mutations were made in the rap1-i2 construct. The bold letters in the table indicate the mutations, and the underlined letters indicate the BP and 3′ss. (B) S. pombe growth on the indicated plates with rap1-i2 reporter variants (incubation time: –LEU, –URA, 3 days; +FOA, 4 days). (C) Western blot analysis to check for splicing defects with different rap1-i2 reporter variants. (D–F) The assays with the atg20-i2 reporter variant are similar to (A–C). –LEU and –URA plates were scanned after 3 days, and +FOA plates after 4 days.
also been shown to act in heterochromatin silencing independently of their splicing functions (46–48). These factors could regulate heterochromatin formation via splicing across BP-distant 3′ss introns. Thus, we monitored protein levels of selected Sde2 targets functioning at the chromatin level. A 6HA epitope tag was inserted at the C-termini of the genes at their chromosomal loci in S. pombe. The genes rap1, bqt3, dsh1, hil2, psf2 and rxt2 were tagged in the wt, Δsde2, Δcay1 and Δtls1 strains (Supplementary Figure S12A). Anti-HA western blots showed diminished protein levels of key heterochromatin factors [Rap1, RNAi factor Dsh1 (49); Set3 histone deacetylase complex protein Hif2 (50); and histone deacetylase complex subunits Rxt2 (51) and Psf2 (52); and the telomere bouquet-forming protein Bqt3 (53)] in all three mutants (Figure 7A; Supplementary Figure S12B). RT–PCR showed retention of specific introns in the above heterochromatin and RNAi factors in Δsde2, Δcay1 and Δtls1 strains (Figure 7B).

Protein levels of Mcs2, Hif2, Psf3, Rxt2 and Rap1 were also monitored by expressing N-terminal 3MYC epitope-tagged genomic constructs in wt and Δsde2 strains. These proteins were lower in the Δsde2 strain, further confirming the role of Sde2 in gene expression. As expected, the full-length Rap1 protein diminished in Δsde2, but an alternative Rap1 protein of ~19 kDa (Rap1-N) was detected in this strain. Similarly, the full-length Rxt2 protein decreased in Δsde2, but an alternative form of ~13 kDa accumulated (Supplementary Figure S13A). These results indicated that controlled BP-distant 3′ss usage could promote alternative splicing through intron retention.

Since an alternative Rap1-N protein accumulated in Δsde2, Δcay1 and Δtls1 mutants, we used Rap1-N as a
proteins are lower in deletion mutants of intron-specific splicing factors. Immunoblotting was performed for the chromosomally C-terminal 6HA-tagged strains using an anti-HA antibody. (B) Semi-quantitative RT–PCR was performed to assay the splicing of heterochromatin factors in the indicated strains. Arrows in the schematic indicate where the primer binds.

Figure 7. Sde2, Cay1 and Tls1 control the expression of heterochromatin factors through intron-specific pre-mRNA splicing. (A) Rap1, Hif2 and Dsh1 proteins are lower in deletion mutants of intron-specific splicing factors. Immunoblotting was performed for the chromosomally C-terminal 6HA-tagged strains using an anti-HA antibody. (B) Semi-quantitative RT–PCR was performed to assay the splicing of heterochromatin factors in the indicated strains. Arrows in the schematic indicate where the primer binds.

The loss of Rap1 in S. pombe is reported to produce defective silencing of telomeric reporters and aberrant expression of telomeric transcripts (44). However, ectopic expression of Rap1-N in a telomeric reporter strain did not reveal silencing defects (Supplementary Figure S14B). Therefore, the physiological significance of Rap1-N remains unknown. We also tested whether Rap1-N would accumulate under certain conditions by expressing the 3MYC epitope-tagged rap1 genomic clone in wt S. pombe. Protein accumulated at high temperature and after treatments with hydroxyurea (HU), cadmium and sorbitol (Supplementary Figure S14C). This accumulation could result from silencing defects arising from the sensitivity of structured RNA between the BP and 3′ss of rap1 intron 2. Thus, intronic diversity and dedicated splicing regulators of the kind discussed in this study allow the cell to control gene expression and promote alternative splicing to create protein variants that help the cell under specific conditions.

Thus, we propose a model based on our findings and structural studies (6,43) explaining the spliceosomal processing of BP-distant 3′ss. The two signals could be brought into proximity by two potentially related mechanisms. With the help of Sde2, Cay1 and Tls1, and RNA secondary structures, the spliceosome could position extra RNA between the BP and 3′ss into the complex (Supplementary Figure S15; also see the Discussion). The model has also proposed an alternative possibility that these factors function as post-mRNA release factors.

**DISCUSSION**

The regulators of BP-distant 3′ss usage in *S. pombe*

This study shows that Sde2, Cay1 (Cactin in humans) and Tls1 (C9ORF78/hepatocellular carcinoma-associated antigen 59 in humans) form a specific group of spliceosomal regulators that facilitate the use of BP-distant 3′ss. These proteins were previously reported to function in pre-mRNA splicing (22,45,55–58), but their specificity for target pre-mRNAs or introns was unknown. Their intron-specific splicing function was revealed in two independent approaches in this study. The first approach monitored the activities of *S. pombe* urad4 splicing reporters harbouring introns with a BP-near and BP-distant 3′ss in a collection of *S. pombe* mutants. A similar reporter with nda3 introns was used to study exon skipping in *S. pombe* (26). The reporters were assayed by three techniques: growth, RT–PCR and western blot, which collectively made the outcome more sensitive and specific. The second approach detected an alternatively spliced form of the telomeric factor Rap1 resulting from retention of its second intron due to poor usage of a BP-distant 3′ss.

The splicing with a BP-distant 3′ss became independent of these factors once the spacing between the BP and 3′ss was reduced. Intrinsic choice of the spliceosome for a BP-near 3′ss made by essential RNA-binding proteins (59–65) dominated over activities of these regulators; a BP-near 3′ss was preferred even in the presence of Sde2, Cay1 and Tls1. Despite obvious growth defects, none of these factors was essential for the viability of *S. pombe* under standard growth conditions, and none was needed for constitutive splicing. Therefore, the activities of these regulators improve splicing efficiency, specifically of pre-mRNAs containing introns with a BP-distant 3′ss. Genetic interactions among Sde2, Cay1 and Tls1 confirmed the specificity of these factors. Like single deletion mutants, splicing defects in double mutants were also specific to such introns. However, these molecules do not appear to have fully overlapping functions since the growth phenotypes of individual deletion mutants...
were distinct. Also, they could function beyond pre-mRNA splicing. Sde2, for example, is reported to regulate the replication stress response (21,31,66,67) and ribosome biogenesis in humans (20) and anthocyanin biosynthesis in Arabidopsis (68).

**An added layer of controls for BP-distant 3′ss usage**

Sde2, Cay1 and Tls1 appear to act beyond the known mechanisms of BP-distant 3′ss usage in *S. cerevisiae*. Although conserved from *S. pombe* to humans, these molecules are absent in *S. cerevisiae*. The BP and 3′ss are separated by an average spacing of 36 nt in *S. cerevisiae* and 29.3 ± 11.9 nt in humans. (9,69–71). The optimal distance between the BP and 3′ss in humans is 19–23 nt (65). The conserved and essential splicing factors Slu7 and Prp18 are known to promote the usage of a BP-distant 3′ss, not only in *S. cerevisiae* (72–75) but also in *S. pombe* (60). Distant 3′ss in *S. cerevisiae* are also selected by reducing the effective distance between the BP and 3′ss through RNA folding (76–78). However, despite highly optimized introns, uniformity in splicing and the presence of the above mechanisms of 3′ss selection, *S. cerevisiae* spliceosomes used all available 3′ss within a distance of 10–45 nt (46) [though BP-proximal 3′ss were preferred (79)]. This mode of BP-distant 3′ss selection could result in error-prone splicing. The activities of Sde2, Cay1 and Tls1 were possibly retained in intron-rich organisms for added layers of control to avoid errors caused by competing branch points or 3′ss. Therefore, intron-rich eukaryotes use (at least) two mechanisms to excise introns with a BP-distant 3′ss through RNA structures and specific regulators of the spliceosome, and the two mechanisms are likely to be complementary.

**Potential mechanism of Sde2, Tls1 and Cay1**

The activities of Sde2, Cay1 and Tls1 that make spliceosomes competent for BP-distant 3′ss need further investigation. We propose that these factors could guide the incoming 3′ss towards the spliceosome’s catalytic centre. Loss of these factors led to the retention of selected introns. The sde2 deletion also resulted in mild splicing defects for introns with a non-canonical BP and 3′ss, suggesting that Sde2 may act with U2 snRNP and U2AF that recognize the BP and 3′ss. This possibility is also suggested by the homology between mammalian Sde2 and the U2 snRNP assembly factor SF3A3 (80–82). However, as discussed below, these regulators are more likely to act once splicing signals have been recognized. Schizosaccharomyces pombe Sde2-C facilitates the recruitment of Cay1/Cactin to the spliceosome (22), a result supported by the cryo-electron microscopy structure of the human post-catalytic spliceosome (6). The structure shows that Sde2-C and Cactin function as exon ligation factors by stabilizing the branch helix in a suitable conformation for the ligation.

Sde2, Cay1 and Tls1 may facilitate the positioning of the RNA intervening the BP and 3′ss. The RNA between the BP and 3′ss must loop during exon ligation to juxtapose the two exons in the catalytic centre. This RNA structure in the spliceosome is yet to be seen (5,6,83), but its flexible or longer trajectories could hinder the spliceosome by slowing down the docking of the incoming 3′ss in the catalytic centre. Indeed, *tho5* intron 1 variants with longer gaps between the BP and 3′ss were poorly excised even in wt cells. A positively charged amino acid patch in human Cactin was proposed to follow a path predicted for the RNA between the BP and 3′ss (6). The role of this surface might become more critical with longer RNAs between the two sites.

Furthermore, these factors could also act by modulating the RNA structures between the BP and 3′ss. Tls1 has been reported to bind Brr2 (a U5-snRNP-specific RNA helicase that unwinds U4/U6 snRNA duplexes during spliceosomal activation) in *S. pombe* and humans (45,84). Tls1 has been shown to regulate Brr2’s activity in humans (84). The helicase has been proposed to play an additional role in the second step of splicing via substrate repositioning. A Brr2 mutant showed second step splicing defects for BP-distant 3′ss introns and introns with potential secondary structures between the BP and 3′ss (85). Thus, the Tls1–Brr2 interaction and the roles of the two proteins in the BP-distant splicing suggest that Tls1 could regulate Brr2 activity on RNA structures between the BP and 3′ss. Increasing the effective distance between the BP and 3′ss by weakening the RNA structure through temperature stress aggravated the splicing defect of *rap1* intron 2, whereas decreasing the effective gap by strengthening the structure alleviated the defect.

Notwithstanding the above evidence, definitive proof for Sde2, Cay1 and Tls1 function can come from structures where these factors and the RNA intervening the BP and 3′ss are resolved. However, we cannot rule out the possibility that these factors play a role in spliceosomal disassembly post-catalysis. Similar activity has been ascribed to the RNA helicase Prp22 in *S. cerevisiae*; the enzyme promotes the disassembly of the spliceosome in *S. cerevisiae*. Extracts lacking Prp22 also showed defects in the splicing of BP-distant 3′ss introns (86,87).

**The physiological relevance of splicing through BP-distant 3′ss**

We report that Sde2, Cay1 and Tls1 control gene expression and alternative splicing of selected chromatin and RNAi factors in *S. pombe*. We and others have shown that their mutants are defective in heterochromatin silencing and genomic instability (22,37,42,45,47,78). RNAi defects in the *sde2* mutant have also been reported (47). These phenotypes could result from defects in the expression of chromatin factors, e.g. the shelterin complex subunit Rap1, the histone deacetylases Hif2 and Rxt2, or the RNAi machinery assembly factor, Dsh1. The Sde2 mutant also accumulated alternative forms of Rap1 and Rxt2. The function of the alternative Rap1 and Rxt2 has not yet been elucidated. Although their aberrant origin cannot be ruled out, these proteins might function only under specific conditions. Indeed, the alternative Rap1 accumulated in wt cells under chemical and temperature stress. Sde2, Cay1 and Tls1 could also make gene expression and alternative splicing conditional or tissue specific in multicellular eukaryotes rich in diverse introns. Indeed, mammalian introns with BP-distant 3′ss have a higher tendency to undergo alternative splicing (88,89). Further regulation of splicing by these factors is plausible, considering a stringent control over Sde2 protein,
involving its activation by DUB and degradation by the proteasome (21,22,31).

Heat-sensitive splicing of the structured S. cerevisiae APE2 intron is reported to work like a thermosensor (78). Notably, splicing defects upon heat shock could arise from multiple causes; among them may be the potential opening of RNA structures between the BP and 3′ss. Splicing of selected pre-mRNAs with possible structures between the BP and 3′ss appeared sensitive to elevated temperatures, suggesting that BP-distant 3′ss may play regulatory roles in gene expression. Similar sensitivities could be expected with cellular metabolites that modulate RNA structures. Mutants of several splicing factors, including Δsde2 and Δcay1, show sensitivities to low and high temperatures, metabolites or chemicals such as formamide, possibly for this reason (25,44,90). It would be interesting to check if some of the structured introns studied here could work as sensors for ions, chemicals or heat. Also, chemical probing should be done to confirm the presumed folding in the introns. Another interesting example of gene regulation by intronic RNA is the intron in S. pombe telomerase RNA terl (91). This RNA controls its own maturation. The sequence between the BP and 3′ss impedes exon ligation but activates a discard pathway essential for terl maturation. Another class of introns with RNA structures prevalent in zebrafish genes has been reported. Splicing of these structured pre-mRNAs was independent of the essential U2AF2 protein (92).

In conclusion, Sde2, Cactin/Cay1 and Tls1 bring more advanced controls over pre-mRNA splicing involving introns with a BP-distant 3′ss. Activities of these regulators appear in sync with the RNA structures to bring the BP and 3′ss closer to each other in the spliceosome. These processes become more relevant to intron-rich organisms because they add an extra layer of specificity and control over gene expression. Such regulations would become critical when the cell is faced with suboptimal conditions. Furthermore, given the vast diversity of introns available in eukaryotic genomes, these controls would also allow spliceosomes to receive more messages from the existing gene pools through alternative splicing.

DATA AVAILABILITY
All data are available in the main text or the supplementary materials. The microarray data analysed in this study is available in Gene Expression Omnibus (GEO) database under the accession number GSE97097.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We thank S. Datta and P.A. Pandit for reagents and early contributions to the project; M. Raghavan (University of California, San Francisco) for helping us with lariat PCR; R. Allshire (The University of Edinburgh) and NBRP-Yeast for yeast strains. We also thank A.K. Bachhawat (IISER Mohali) and K. Babu (IISc Bangalore) for critically reading the manuscript. K.C. was supported by CPSDE and a scholarship from UGC/CSIR; A.T.A., R.P. A.S. and P.G. by scholarships from IISER Mohali; P.T. received an INSPIRE scholarship from the Department of Science and Technology (DST), Government of India; and M.S. is supported a DST INSPIRE award and research grant.

Author contributions: S.K.M. conceived the project; all authors designed the experiments, analysed the data and prepared the manuscript; S.K.M., A.T.A. and R.P. wrote the paper with input from all authors; A.T.A., K.C., R.P., P.G., P.T., A.S., and M.S. produced the figures.

FUNDING
This work was supported by IISER Mohali and the Centre for Protein Science Design and Engineering (CPSDE) of the Ministry of Human Resource and Development (MHRD), Government of India; the Max Planck Society, Germany; and the Wellcome Trust/DBT India Alliance Fellowship/Grant [IA/1/18/2/504020 to S.K.M.]. Funding for open access charge: IISER Mohali.

Conflict of interest statement. None declared.

REFERENCES
1. Plaschka,C., Lin,P.C., Charenton,C. and Nagai,K. (2018) Pre-spoolosome structure provides insights into spliceosome assembly and regulation. Nature, 559, 419–422.
2. Reich,C.I., VanHoy,R.W., Porter,G.L and Wise,J.A. (1992) Mutations at the 5′ splice site can be suppressed by compensatory base changes in U1 snRNA in fission yeast. Cell, 69, 1159–1169.
3. Wahl,M.C., Will,C.L. and Lührmann,R. (2009) The spliceosome: design principles of a dynamic RNP machine. Cell, 136, 701–718.
4. Will,C.L. and Lührmann,R. (2011) Spliceosome structure and function. Cold Spring Harb. Perspect. Biol., 3, a003707.
5. Fica,S.M., Oubridge,C., Galej,W.P., Wilkinson,M.E., Bai,X.C., Newman,A.J and Nagai,K. (2017) Structure of a spliceosome remodelled for exon ligation. Nature, 542, 377.
6. Fica,S.M., Oubridge,C., Wilkinson,M.E., Newman,A.J and Nagai,K. (2019) A human postcatalytic spliceosome structure reveals essential roles of metazoan factors for exon ligation. Science, 363, 710–714.
7. Yan,C., Wan,R., Bai,R., Huang,G. and Shi,Z. (2016) Structure of a yeast activated spliceosome at 3.5 Å resolution. Science, 353, 904–911.
8. Bertram,K., Agafonov,D.E., Dybkov,O., Haselbach,D., Leelaram,M.N., Will,C.L., Urlaub,H., Kastner,B., Lührmann,R. and Stark,H. (2017) Cryo-EM structure of a pre-catalytic human spliceosome primed for activation. Cell, 170, 701–713.
9. Kapfer,D.M., Drabenstot,S.D., Buchanan,K.L., Lai,H., Zhu,H., Dyer,D.W., Roe,B.A. and Murphy,J.W. (2004) Introns and splicing elements of five diverse fungi. Eukaryot. Cell., 3, 1088–1100.
10. William Roy,S. and Gilbert,W. (2006) The evolution of spliceosomal introns: patterns, puzzles and progress. Nat. Rev. Genet., 7, 211.
11. Hooks,K.B., Delneri,D. and Griffiths-Jones,S. (2014) Intron evolution in Saccharomycetaceae. Genome Biol. Evol., 6, 2543–2556.
12. Taggart,A.J., Lin,C.L., Sreshtha,B., Heintzelman,C., Kim,S. and Fairbrother,W.G. (2017) Large-scale analysis of branchpoint usage across species and cell lines. Genome Res., 27, 639–649.
13. Mishra,S.K. and Thakran,P. (2018) Intron specificity in pre-mRNA splicing. Curr. Genet., 64, 777–784.
14. Lee,Y. and Rin,D.C. (2015) Mechanisms and regulation of alternative pre-mRNA splicing. Annu. Rev. Biochem., 84, 291–323.
15. Chanarat,S. and Mishra,S.K. (2018) Emerging roles of ubiquitin-like proteins in pre-mRNA splicing. Trends Biochem. Sci., 43, 896–907.
16. Wilkinson,C.R.M., Dittmar,G.A.G., Ohl,M.D., Uetz,P., Jones,N. and Finley,D. (2004) Ubiquitin-like protein Hub1 is required for pre-mRNA splicing and localization of an essential splicing factor in fission yeast. Curr. Biol., 14, 2283–2288.
17. Mishra,S.K., Ammon,T., Popowicz,G.M., Krajewski,M., Nagel,R.J., Ares,M., Holak,T.A. and Jentsch,S. (2011) Role of the ubiquitin-like
protein Hub1 in splice-site usage and alternative splicing. Nature, 474, 173–178.

18. Ammon,T., Mishra,S.K., Kowalska,K., Popowicz,G.M., Holak,T.A. and Jentsch,S. (2014) The conserved ubiquitin-like protein Hub1 plays a critical role in splicing in human cells. J. Mol. Cell Biol., 6, 312–323.

19. Karaduman,R., Chanarat,S., Pfander,B. and Jentsch,S. (2017) Error-prone splicing controlled by the ubiquitin relative Hub1. Mol. Cell., 67, 423–432.

20. Floro,J., Dai,A., Metzger.A., Mora-Martin,A., Ganem,N.J., Cifuentes,D., Wu,C.S., Dalal,J., Lyons,S.M., Labadorf,A. et al. (2021) SDE2 is an essential gene required for ribosome biogenesis and the regulation of alternative splicing. Nucleic Acids Res., 49, 9424–9443.

21. Jo,U., Cai,W., Wang,J., Kwon,Y., D’Andrea,A. and Kim,H. (2016) PCNA-dependent cleavage and degradation of SDE2 regulates response to replication stress. PLoS Genet., 12, e1006465.

22. Thakran,P., Pandit,P.A., Datta,S., Kolathu,K.K., Pleiss,J.A. and Mishra,S.K. (2018) Sde2 is an intron-specific pre-mRNA splicing regulator activated by ubiquitin-like processing. EMBO J., 37, 89–101.

23. Janke,C., Magiera,M.M., Rothfelder,N., Taxis,C., Reber,S., Maeß,A., Tascioli,D., Diepen,B., Doerschug,G., Schwob,E., Schiebel,E. et al. (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast, 21, 947–962.

24. Knop,M., Siegers,K., Pereira,G., Zachariae,W., Winsor,B., Wang,J., Kwon,Y., D’Andrea,A. and Kim,H. (2016) Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast, 15, 963–972.

25. Sugioka-Sugiyama,R. and Sugiyama,T. (2011) Sde2: a novel nuclear protein essential for telomeric silencing and genomic stability in Schizosaccharomyces pombe. Biochem. Biophys. Res. Commun., 406, 444–448.

26. Haraguchi,N., Andoh,T., Frendewey,D. and Tani,T. (2007) Mutations in the SF1–U2AF59–U2AF23 complex cause exon skipping in Schizosaccharomyces pombe. Mol. Biol. Chem., 282, 2221–2228.

27. Gruber,A.R., Lorenz,R., Bernhart,S.H., Neuböck,R. and Schiebel,E. (2008) Structural toggle in the RNaseH domain of the yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. FEBS J., 275, 77–87.

28. Haraguchi,N., Andoh,T., Frendewey,D. and Tani,T. (2007) Mutations in the SF1–U2AF59–U2AF23 complex cause exon skipping in Schizosaccharomyces pombe. Mol. Biol. Chem., 282, 2221–2228.

29. Inada,M. and Pleiss,J.A. (2010) Structural toggle in the RNaseH domain of the yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. FEBS J., 275, 77–87.

30. Mayerle,M., Raghavan,M., Ledoux,S., Price,A., Stepankiw,N., Rentas,S., Saberianfar,R., Grewal,C., Kanippayoor,R., Mishra,M., McCollum,D. and Karagiannis,J. (2012) The SSe7p domain protein, Set3p, promotes the reliable execution of cytokinesis in fission yeast centromeres. J. Biol. Chem., 285, 5630–5638.

31. Kawakami,K., Hayashi,A., Nakayama,J. and Murakami,Y. (2012) A novel nuclear protein, Dsh1, assembles RNAi machinery on chromatin to amplify heterochromatic siRNA. Nucleic Acids Res., 40, 11419–11432.

32. Carrozza,M., Florens,L., Swanson,S., Shia,W.J., Anderson,S., Guthrie,C. et al. (2017) Structural toggle in the RNaseH domain of the yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. FEBS J., 275, 77–87.

33. Carrozza,M., Florens,L., Swanson,S., Shia,W.J., Anderson,S., Guthrie,C. et al. (2017) Structural toggle in the RNaseH domain of the yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. FEBS J., 275, 77–87.

34. Carrozza,M., Florens,L., Swanson,S., Shia,W.J., Anderson,S., Guthrie,C. et al. (2017) Structural toggle in the RNaseH domain of the yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. FEBS J., 275, 77–87.

35. Carrozza,M., Florens,L., Swanson,S., Shia,W.J., Anderson,S., Guthrie,C. et al. (2017) Structural toggle in the RNaseH domain of the yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. FEBS J., 275, 77–87.

36. Carrozza,M., Florens,L., Swanson,S., Shia,W.J., Anderson,S., Guthrie,C. et al. (2017) Structural toggle in the RNaseH domain of the yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. FEBS J., 275, 77–87.

37. Carrozza,M., Florens,L., Swanson,S., Shia,W.J., Anderson,S., Guthrie,C. et al. (2017) Structural toggle in the RNaseH domain of the yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. FEBS J., 275, 77–87.

38. Carrozza,M., Florens,L., Swanson,S., Shia,W.J., Anderson,S., Guthrie,C. et al. (2017) Structural toggle in the RNaseH domain of the yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. FEBS J., 275, 77–87.

39. Carrozza,M., Florens,L., Swanson,S., Shia,W.J., Anderson,S., Guthrie,C. et al. (2017) Structural toggle in the RNaseH domain of the yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. FEBS J., 275, 77–87.
