Prevalent and distinct spliceosomal 3′-end processing mechanisms for fungal telomerase RNA

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Telomerase RNA (TER) is an essential component of the telomerase ribonucleoprotein complex. The mechanism for TER 3′-end processing is highly divergent among different organisms. Here we report a unique spliceosome-mediated TER 3′-end cleavage mechanism in Neurospora crassa that is distinct from that found specifically in the fission yeast Schizosaccharomyces pombe. While the S. pombe TER intron contains the canonical 5′-splice site GUAUGU, the N. crassa TER intron contains a non-canonical 5′-splice site AUAGU that alone prevents the second step of splicing and promotes spliceosomal cleavage. The unique N. crassa TER 5′-splice site sequence is evolutionarily conserved in TERs from Pezizomycotina and early branching Taphrinomycotina species. This suggests that the widespread and basal N. crassa-type spliceosomal cleavage mechanism is more ancestral than the S. pombe-type. The discovery of a prevalent, yet distinct, spliceosomal cleavage mechanism throughout diverse fungal clades furthers our understanding of TER evolution and non-coding RNA processing.

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he telomerase enzyme is responsible for the addition of telomeric DNA repeats onto the ends of eukaryotic chromosomes to maintain genome stability and cellular replicative capacity. The core telomerase ribonucleoprotein (RNP) enzyme is composed of the catalytic telomerase reverse transcriptase (TERT) and telomerase RNA (TER) that contains a short region as the template for telomeric DNA repeat synthesis. TERs are highly divergent across eukaryotic clades, differing immensely in nucleotide sequence, length and biogenesis pathway. The diversity in TER biogenesis is evidenced by the different RNA polymerase (pol) machineries employed for transcription. While TER is transcribed by RNA pol II in most species, it is transcribed by RNA pol III in ciliates. Furthermore, TERs from evolutionary separate groups of species associate with entirely unique groups of accessory proteins—the dyskerin complex and TCAB1 in vertebrates, Est1, Est3 and Sm-ring in budding yeasts, the dyskerin complex and POT1 in the plant Arabidopsis and p50 and p65 in ciliates. These separate sets of TER accessory proteins are essential for localization, RNP assembly and TER cleavage.

The identification of the Schizosaccharomyces pombe TER (SpoTER1) revealed a spliceosome-mediated cleavage mechanism for RNA 3′-end processing. The nascent SpoTER1 transcript harbours a downstream intron followed by a second exon and a 3′-poly(A) tail. The mature SpoTER 3′-end is generated by spliceosomal cleavage at the 5′-splice site (5′-SS), removing the intron, second exon and poly(A) tail. This is in contrast to splicing—whereby the intervening intron sequence is excised and the neighbouring exons are joined. The SpoTER1 intron itself uncouples these two tightly associated splicing steps by inhibiting the second transesterification step at the 3′-splice site (3′-SS). The second transesterification step is abated by a strong branch point sequence (BPS), an extended distance from the BPS to the 3′-SS and a weak poly(A)pyrimidine (Py) in the SpoTER1 intron.

Here we report in N. crassa a spliceosome-mediated mechanism for TER 3′-end processing that is distinct from the S. pombe-type. The N. crassa–type TER spliceosome-mediated 3′-end cleavage relies exclusively on a unique 5′-SS AUAGU sequence, which alone is necessary and sufficient for inhibiting the second transesterification step in splicing. This is the first biologically functional instance of spliceosome-mediated cleavage relying exclusively on the 5′-SS. In addition, the N. crassa-type TER intron is pervasive across Ascomycota, found universally in TERs from filamentous fungi and select early branching fission yeasts. This preponderance and conservation among distinct, evolutionary distant and basal fungal species suggest that this mechanism is more ancestral than the S. pombe-type. The discovery of a unique spliceosome-mediated cleavage mechanism provides new insights into TER biogenesis and evolution.

Results

*N. crassa* TER precursor harbours an intron.

*N. crassa* TER (NcrTER) was recently identified as a 2,049-nt RNA. The 3′-end of the mature NcrTER was determined by rapid amplification of cDNA ends (RACE) from total RNA treated with poly(A) polymerase (Fig. 1a, lane 1). However, 3′-RACE with total RNA untreated with poly(A) polymerase generated two larger products (Fig. 1a, lane 2). While the smaller of these two products was not readily visible by ethidium bromide staining, it was discernable for gel extraction. Cloning and subsequent sequencing of the two PCR products revealed distinct NcrTER variants of ~400 and 470 nt in length. The 470-nt product harboured a 74-nt intron sequence, while the 400-nt product lacked this intron with the two exons joined (Fig. 1b). This intervening sequence contained the conserved features of a canonical intron: 5′-SS, BPS and 3′-SS (Fig. 1c). The presence of an intron in NcrTER transcripts suggested that NcrTER undergoes spliceosome-mediated cleavage for 3′-end processing, similar to that discovered in the fission yeast Spoter1 (ref. 18). Interestingly, the NcrTER intron 5′-SS AUAGU is non-canonical, a stark contrast to the canonical 5′-SS GUAGU sequence found in the Spoter1 and mRNA introns. We then determined the relative abundance of the three NcrTER variants by performing northern blot analysis of total RNA with probes targeting either the first or second exon. The 2,049-nt NcrTER was readily detected by northern blot, while the precursor and spliced variants were below the threshold for detection (Fig. 1d).

This result suggests that the mature 2,049-nt NcrTER variant has far greater abundance, which is consistent with our initial identification of only the 2,049-nt NcrTER species in the purified telomerase holoenzyme.

*NcrTER*-type intron is prevalent in Ascomycota fungal TERs.

Analysis of 72 additional Pezizomycotina filamentous fungal TERs that we previously identified revealed the absolute conservation of the NcrTER-type intron in all species examined (Fig. 2a). All known Pezizomycotina TER introns contain the unique non-canonical 5′-SS AUAGU sequence, the BPS sequence RCURAY that includes an invariant adenine residue as a putative branch point residue and the putative 3′-SS YAG (Supplementary Fig. 1). The 3′-SS was experimentally verified by 3′-RACE for *N. crassa*, Aspergillus nidulans and Mycosphaerella graminicola (Supplementary Fig. 1). The NcrTER 5′-SS AUAGU is extremely conserved in filamentous fungal TERs and distinct from the canonical, yet less conserved 5′-SS GURNGU found in yeast TERs from the fission yeast *S. pombe* as well as species from the budding yeast *Candida* and *Hansenula*.

We then investigated whether the *S. pombe*-type or the *N. crassa*-type TER intron is more ancestral. *S. pombe* belongs to the subphylum Taphrinomycotina that together with the other two subphyla Pezizomycotina and Saccharomycotina constitute the Ascomycota phylum. In addition to TER that we previously cloned from the basal Taphrinomycotina species *Saurochaeta complicata*, we identified two additional Taphrinomycotina TERs from Schizosaccharomyces octosporus and Schizosaccharomyces japonicus by a bioinformatics approach (see Methods). The 3′-ends and intron sequences of these newly identified TERs were experimentally determined by RACE. Surprisingly, there was a clear divide in the intron 5′-SS sequence among Taphrinomycotina TERs. The basal species *S. complicata* and *S. japonicus* harboured the *N. crassa*-type 5′-SS AUAGU, while the later evolving species *S. octosporus* contained the *S. pombe*-type 5′-SS GURNGU (Fig. 2b). Thus, the more prevalent *N. crassa*-type intron appears to be more basal and thus ancestral than the *S. pombe*-type intron (Fig. 2c).

*NcrTER*-type 5′-SS promotes spliceosomal cleavage.

The 5′-SS of the NcrTER and canonical mRNA introns differ with respect to their first and forth residues (Fig. 3a). Newman et al. demonstrated in yeast that an introduced G-to-A substitution at the first residue in an mRNA intron suppresses the second step, yet permits the first step of splicing. We thus hypothesized that the 5′-SS is sufficient for NcrTER 3′-end processing. To test this, we constructed a minimal exogenous expression cassette of the NcrTER gene. This minimal NcrTER expression cassette spanned a 3′-portion (residues 1,888–2,049) of exon 1, the intron (residues 2,050–2,123), exon 2 (residues 2,124–2,200) and the 500 bp downstream flanking genomic sequence that contained a polyadenylation signal (Fig. 3b,c). This expression cassette also
contained an eGFP gene as an internal control for RNA expression and normalization. NcrTER and eGFP mRNA were transcribed independently under separate cgg-1 (clock-controlled gene-1) promoters and were fused to the cgg-1 5′-UTR sequence, generating cgg-1::NcrTER and cgg-1:eGFP, respectively. The cgg-1 promoter is a light induced, strong RNA pol II promoter (Fig. 3d, lane 3). This RNA product had the expected 3′-end, verified by sequencing of PCR products confirmed efficient splicing of the 5′-SS₁ mutant RNA (Fig. 3e, lane 4; Supplementary Fig. 2). In contrast, the 5′-SS₂ mutant with the fourth residue mutated from A to U only slightly reduced the level of mature NcrTER exon-1 RNA (Fig. 3d, lane 7) and did not significantly affect splicing efficiency (Fig. 3e, lane 7; Supplementary Fig. 2). Thus, the first adenosine residue in the NcrTER 5′-SS appears to be the key determinant for NcrTER 3′-end processing.

We then examined if the 5′-SS₁ mutant—which has complete splicing restored—could be converted for S. pombe-type spliceosomal cleavage by extending the distance between the BPS and the 3′-SS (Supplementary Fig. 3). We generated a double mutant 5′-SS₁a that, in addition to the 5′-SS₁ mutation, disrupted the 3′-SS (Fig. 3c). A triple mutant 5′-SS₁b further disrupted a downstream cryptic 3′-SS (Fig. 3c). Both 5′-SS₁a and 5′-SS₁b mutants effectively restored spliceosomal cleavage and accumulated mature RNA at levels comparable to the wild-type NcrTER intron (Fig. 3d, lanes 5 and 6), while reducing the splicing efficiency (Fig. 3e, lanes 5 and 6). RT-PCR and sequencing of the spliced RNA products from 5′-SS₁a and 5′-SS₁b mutants showed that, when the preferred 3′-SS was mutated, two different cryptic 3′-SSs were used instead with low efficiencies (Supplementary Fig. 2). Thus, disruption of the N. crassa-type spliceosomal cleavage in the 5′-SS₁ mutant was...
rescued by extending the distance between the BPS and the 3′-SS to employ S. pombe-type spliceosomal cleavage.

To verify that the NcrTER 3′-end processing is indeed spliceosome-mediated and not through non-specific endo- or exo-nucleolytic RNA degradation, we generated two mutations, BPS_1 and No_intron. Mutant BPS_1 abolished splicing by mutating the essential adenosine residue of the BPS and creating a greater increase in splicing efficiency (Fig. 3d, lanes 3 and 4). The residual spliced products from the mutants 5′-SS and 3′-SS were detected using RT-PCR and sequencing of the spliced products (Supplementary Fig. 2). Mutant 3′-SS_1a further decreased spliceosomal cleavage to 60% of the wild type (Fig. 3f, lane 3) and a greater increase in splicing efficiency (Fig. 3g, lane 3). Thus, the NcrTER intron can be readily converted to an AU/AC splicing intron by mutating the proximal and distal 3′-SS UAG to UAC.

5′-SS AUAGU converts an intron for spliceosomal cleavage. The NcrTER intron can be converted to favour splicing by a simple A-to-G substitution at the first adenine residue of the 5′-SS (Fig. 3). To further confirm that the NcrTER 5′-SS AUAGU is the key determinant for spliceosomal cleavage, we performed a reciprocal experiment to convert an mRNA intron to the NcrTER-type intron for spliceosomal cleavage. We modified our ccg1::NcrTER expression cassette by replacing the NcrTER intron with an mRNA intron from the ccg-1 gene (Fig. 4a).

Mutations were introduced to the ccg-1 mRNA at 5′-SS, BPS and 3′-SS to identify the element(s) necessary for conversion to NcrTER-type spliceosomal cleavage (Fig. 4b). The replacement of the NcrTER intron in our minimal expression cassette with the ccg-1 mRNA intron resulted in severely reduced spliceosomal cleavage (Fig. 4c, lane 1) and markedly increased splicing efficiency (Fig. 4d, lane 1). In mutant 5′-SS_3, replacing the ccg-1 5′-SS with the NcrTER 5′-SS, by two G-to-A substitutions at the first and fourth positions of the 5′-SS, significantly increased the accumulation of mature NcrTER exon-1 fragment (Fig. 4c, lane 2) and reduced splicing efficiency (Fig. 4d, lane 2). Thus, the NcrTER 5′-SS AUAGU alone appears to be sufficient to convert an mRNA intron for spliceosomal cleavage. Interestingly, sequencing the residual spliced products of the 5′-SS_3 mutant showed that a new 3′-SS CAC was utilized, similar to the AU/AC mRNA splicing introns (Supplementary Fig. 4a). In the presence of the 5′-SS AUAGU, there is a shift from the 3′-SS YAG employed by GU/A GU mRNA introns to YAC employed by AU/AC mRNA introns. To minimize the 3′-SS YAC, we generated two double mutants, 5′-SS_3a and _3b, that changed the 3′-SS CAC employed by mutant 5′-SS_3 to CGC or CAU, respectively (Fig. 4b). As expected, preventing AU/AC mRNA splicing for each of these mutants resulted in far greater accumulation of mature NcrTER than the mutant 5′-SS_3 (Fig. 4c, lanes 3 and 4) and similarly reduced splicing efficiency (Fig. 4d, lanes 3 and 4).

The residual spliced products from the mutants 5′-SS_3a and 5′-SS_3b arose from utilizing the 3′-SS CAG or UAG, respectively (Supplementary Fig. 4a). Thus, the 5′-SS_3a and 5′-SS_3b mutants employed the same 3′-SS YAG as the wild-type NcrTER intron.

We next investigated whether a single substitution at the first residue of the ccg-1 5′-SS would convert the ccg-1 mRNA intron to the NcrTER intron. In contrast to mutant 5′-SS_3 that had both the first and fourth residues substituted and accumulated mature NcrTER, mutant 5′-SS_4 with only the first residue substituted failed to accumulate mature NcrTER above the level of the wild-type ccg-1 intron (Fig. 4c, lane 5). The double mutations 5′-SS_4a and 5′-SS_4b eliminated the 3′-SS CAC to prevent AU/AC splicing. The northern blot showed that mutant 5′-SS_4a did not increase mature RNA level (Fig. 4c, lane 6). However, mutant 5′-SS_4b had only a slight increase in mature NcrTER accumulation, which is relatively insignificant compared with the 5′-SS_3 mutant (Fig. 4c, lane 7). Thus, the ccg-1 mRNA intron requires a 5′-SS identical to the NcrTER-type intron for spliceosomal cleavage. The requirement of G-to-A substitutions for the first and fourth residues in the ccg-1 mRNA intron may rely on the first adenosine substitution disrupting snRNA U1 interaction and the fourth position substitution compensating for this disruption.

Figure 2 | Conservation and diversity of fungal TER intron sequence. (a) Sequence conservation of filamentous fungal TER introns. Consensus sequences of 5′-SS, BPS and 3′-SS are derived from a multiple sequence alignment of 73 Pezizomycotina TER introns and presented using Sequence Logo. (b) Sequence conservation of four Taphrinomycotina TER introns. The aligned sequences of 5′-SS, BPS and 3′-SS of TER introns from Schizosaccharomyces pombe (Spa), S. octosporus (Soc), S. japonicus (Sja) and Saitoella complicata (Sco) are shown. (c) Evolutionary relationship of fungal TER introns. The phylogenetic tree of select species from Pezizomycotina (P), Saccharomycotina (S) and Taphrinomycotina (T) is based on a recent fungal six-gene phylogeny study (James et al. (2006); Liu et al. (2009)). Respective intron 5′-SS sequences from select species with known TER introns are shown. The length of the branch line does not reflect evolutionary distance. The evolutionary position of Hansenula polymorpha is tentative (dashed line).
As the ccg-1 mRNA intron can be easily converted into the NcrTER-type intron, we examined whether the ccg-1 mRNA intron can be converted for the S. pombe-type spliceosomal cleavage by extending the distance between the BPS and 3′-SS CAG. Mutant 3′-SS_2 had the proximal 3′-SS eliminated, while mutant Linker_1 introduced a 34-nt insertion between the BPS and the 3′-SS CAG (Fig. 4b). The northern blot and RT-PCR results showed that both mutants had a significantly increased level of mature NcrTER exon-1 RNA (Fig. 4c, lanes 8 and 9) and decreased splicing efficiency (Fig. 4d, lanes 8 and 9). Thus, the ccg-1 mRNA splicing intron can be readily converted to either the NcrTER-type or SpoTER1-type intron for spliceosomal cleavage. We also examined the importance of the BPS in promoting spliceosomal cleavage. Mutant BPS_2 converted the ccg-1 mRNA intron BPS to NcrTER-type intron BPS, while mutant BPS_3 converted it to the strong BPS of spoTER1 intron (Supplementary Figs 3 and 4b). These BPS mutants did not significantly increase the accumulation of mature NcrTER (Fig. 4e) or decrease the splicing efficiency (Fig. 4f) compared with the wild-type ccg-1 mRNA intron.

### Discussion

Telomerase biogenesis requires a 3′-end maturation step for TER, which is remarkably divergent across eukaryotic taxa and utilizes a wide array of distinct mechanisms. Within the phylum Ascomycota, certain budding yeast TERs employ specific transcription termination pathways and nucleolytic cleavage for 3′-end maturation\(^3\)\(^{,}\)\(^{,}\)\(^{,}\)\(^{,}\)\(^{,}\)\(^{,}\), while the fission yeast S. pombe and a few select budding yeast species employ spliceosomal cleavage\(^1\)\(^,\)\(^{,}\)\(^{,}\)\(^{,}\)\(^{,}\)\(^{,}\). Herein we have identified a second, yet vastly more widespread, spliceosome-mediated TER 3′-end processing mechanism initially in N. crassa and virtually ubiquitous throughout Ascomycete fungi. The N. crassa-type spliceosomal cleavage is mechanistically distinct from the S. pombe-type and is dependent on a unique 5′-SS AUAGGU sequence (Fig. 2a and Supplementary Fig. 1). While critical for the S. pombe-type mechanism, the BPS and the distance to the 3′-SS are seemingly unimportant for the N. crassa-type. The prevalence of the N. crassa-type spliceosomal cleavage mechanism is ancestral and the S. pombe-type is later evolved.

We propose that the highly prevalent and basal N. crassa-type TER intron is ancestral and the S. pombe-type intron is later evolved by an A-to-G substitution of the 5′-SS together with strengthening the BPS, extending distance to the 3′-SS and weakening the Py tract. It would seem less likely that multiple TER-intron fusion events occurred during the short evolutionary time within each of the three Ascomycete phyla. Instead, it is more plausible that two transition events converted the N. crassa-type to the S. pombe-type intron independently along the fission and budding yeast lineages. The fission yeast transition event seems to have occurred adjacent to the emergence of the basal S. japonicas species that employs the ancestral N. crassa-type intron (Fig. 5). Similarly, select budding yeast species from the genera Candida and Hansenula harbour the S. pombe-type TER 3′-end maturation (Figs 2c and 5), while later-branching budding yeast species appear to have lost the spliceosomal cleavage intron entirely. These Saccharomyces species with intron-less TERs instead employ the Nrd1/Nab3-dependent transcription termination pathway for generating non-polyadenylated...

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**Figure 3 | Determinants in the NcrTER intron for spliceosomal cleavage.** (a) Schematic of U1 and U2 snRNAs base-paired with NcrTER (top, blue) or ccg-1 (bottom, orange) intron 1. (b) Schematic of the dual gene cassette for expressing the NcrTER 3′-end fragment of exon 1, intron, exon 2 and 500 bp downstream sequence (blue) along with internal control eGFP (green) fused to the 5′-UTR of ccg-1 under the ccg-1 promoter (P_{ccg-1}, orange) with the transcription initiation sites shown (black arrows). Annealing positions for riboprobes (Probe) as well as forward (F) and reverse (R) primers for RT-PCR are denoted (purple). (c) Alignment of NcrTER intron mutations (red), mutations to convert the NcrTER intron into the ccg-1 mRNA intron (orange) and a downstream cryptic 3′-SS (‘; purple) denoted. The accumulation and splicing variants produced from these intron mutations were evaluated by northern blot analysis (d.f) and RT-PCR (e.g). Relative intensity of spliceosomal cleaved ccg-1::NcrTER (Exon 1), determined by the ratio of Exon 1 over eGFP (Exon1/eGFP) with the background of each lane subtracted and relative to the wild-type NcrTER intron, is shown below the blot and the markers (M) were in vitro transcribed ccg-1::NcrTER Exon 1 and ccg-1::eGFP RNA.

**As the ccg-1 mRNA intron can be easily converted into the NcrTER-type intron, we examined whether the ccg-1 mRNA intron can be converted for the S. pombe-type spliceosomal cleavage by extending the distance between the BPS and 3′-SS CAG. Mutant 3′-SS_2 had the proximal 3′-SS eliminated, while mutant Linker_1 introduced a 34-nt insertion between the BPS and the 3′-SS CAG (Fig. 4b). The northern blot and RT-PCR results showed that both mutants had a significantly increased level of mature NcrTER exon-1 RNA (Fig. 4c, lanes 8 and 9) and decreased splicing efficiency (Fig. 4d, lanes 8 and 9). Thus, the ccg-1 mRNA splicing intron can be readily converted to either the NcrTER-type or SpoTER1-type intron for spliceosomal cleavage. We also examined the importance of the BPS in promoting spliceosomal cleavage. Mutant BPS_2 converted the ccg-1 mRNA intron BPS to NcrTER-type intron BPS, while mutant BPS_3 converted it to the strong BPS of spoTER1 intron (Supplementary Figs 3 and 4b). These BPS mutants did not significantly increase the accumulation of mature NcrTER (Fig. 4e) or decrease the splicing efficiency (Fig. 4f) compared with the wild-type ccg-1 mRNA intron.**
TERs. Two independent transition events from a more ancestral N. crassa-type TER intron to the S. pombe-type or the loss of TER intron explain the scattered presence of the S. pombe-type TER within Ascomycete fungal TERs and the uniform presence of the N. crassa-type intron.

While there is strong support for the ancestry of the N. crassa-type TER intron, there are two conceivable possibilities for the origin of the progenitor fungal TER intron. The progenitor fungal TER intron probably arose from a single gene fusion event between an intron-less fungal TER gene and either a common GU/AG or a rare AU/AC mRNA intron (Fig. 5). This newly intron-containing TER gene then evolved into the N. crassa-type and later twice into the S. pombe-type TER intron. Deriving the N. crassa-type TER intron from a GU/AG intron requires alterations in the 5′-SS, while adoption from an AU/AC intron necessitates a C-to-G substitution of the last residue in the 3′-SS. Our mutagenesis studies demonstrate that the NcrTER spliceosomal cleaving intron can be readily converted to either a canonical GU/AG or a rare AU/AC intron—permitting efficient splicing—with only single or double mutations necessary (Figs 3 and 4). Previous studies in yeast have shown that an introduced G-to-A substitution of the first residue in the 5′-SS necessitates a C-to-G substitution of the last residue in the 3′-SS. Arising from an AU/AC intron would negate a G-to-A transition substitution and instead require a 3′-SS mutation for efficient spliceosomal cleavage. Given the abundance of GU/AG mRNA intron in fungal genomes, we favour the possibility that the N. crassa-type intron evolved from
a GU/AG mRNA intron. While all three NcrTER variants reconstituted equal telomerase activity in vitro (Supplementary Fig. 5), it is evident that a spliceosomal cleaving TER intron is evolutionarily selected for as it removes the poly(A) tail from TER, which presumably prevents undesired cytoplasmic localization or poly(A) tail-mediated degradation\(^{33}\). Therefore, spliceosomal cleavage may promote nuclear retention and accumulation of functional TER. In \textit{S. pombe}, disruption of TER spliceosome-mediated 3′-end processing results in reduced accumulation of \textit{SpoTER1} and telomere shortening\(^{33}\). The identification of additional fungal TERs from more basal species is necessary to determine the evolutionary origins of fungal TER introns.

The identification of the widespread \textit{N. crassa}-type spliceosomal cleavage mechanism within fungal TERs provides insights into the evolution of TER 3′-end processing. Spliceosomal cleavage was initially viewed as a molecular idiosyncrasy, unique to a single RNA from a single species. Our findings demonstrate that spliceosomal cleavage is the dominant mechanism for \textit{Ascomycota} fungal TER 3′-end processing, found in the vast majority of species with known TERs. It has yet to be determined whether this mechanism is conserved outside of the \textit{Ascomycota} phylum. Moreover, it is unlikely that this RNA processing mechanism is exclusive to TER and is potentially employed by other non-coding RNAs for 3′-end maturation. This work expands our understanding of the diversity of spliceosomal cleavage mechanisms, the origins and evolution of TER maturation, as well as our knowledge of non-coding RNA processing and biogenesis.

**Methods**

**Fungal strains.** \textit{N. crassa} (FGSC 2489 wild type and FGSC 9720 NC1 his-3 mutant) and \textit{A. nidulans} (FGSC A4 wild type) were obtained from the Fungal Genetics Stock Center (FGSC). \textit{N. crassa} cells were grown at 30°C with continuous light exposure in Vogel's liquid minimal media supplemented with 2% sucrose\(^{34}\). \textit{A. nidulans} cells were grown at 37°C in YG liquid medium (0.5% yeast extract, 2% dextrose, 0.1% \textit{v/v} C\textit{ove}'s trace elements and 0.02% MgSO\(_4\)\(_7\).\textit{H}_2O). \textit{M. graminicola} cells (strain IPO323) were obtained from Dr Gert Kema and grown at 18°C in yeast glucose broth (1% yeast extract and 3% glucose). \textit{S. complicata} (strain Y-17804), \textit{S. japonicus} (strain Y-1361) and \textit{S. octosporus} (strain Y-855) were obtained from ARS Culture Collection (NRRL) and grown at room temperature in YM broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 0.1% sucrose).

**Identification of Schizosaccharomyces TER sequences.** Two protein-coding genes (\textit{sub1}, \textit{SPAC16A10.03c} and \textit{thd4}, \textit{da1}) flanking the \textit{SpoTER1} gene served as queries searching against \textit{S. japonicus} (strain YF5275) and \textit{S. octosporus} (strain YF5286) genomic data sequencing downloaded from the Broad Institute Database\(^{35}\). The intervening sequences between these putative protein coding genes were searched using the program Infernal\(^{36}\) with a covariance model derived from the \textit{S. pombe} TER 3′-end (e-value of 1e-010). The inferred \textit{S. japonicus} and \textit{S. octosporus} were determined by RACE using the FirstChoice RLM-RACE kit (Life Technologies) at room temperature using 300 mg 0.5-mm zirconia/silica beads (BioSpec Products) and a MiniBeadBeater-16 (BioSpec Products) at 3,450 rpm for 1 min. Homogenate was supplemented with 0.1 ml 1-bromo-3-chloropropane, vigorously mixed and centrifuged at 4°C for phase separation.

**Northern blot analysis.** Total RNA was isolated from \textit{N. crassa} mycelia grown in Vogel's minimum medium at 30°C with continuous light exposure. \textit{N. crassa} cells were digested with 10 U BspEI (New England Biolabs) at 100°C in a 1 ml reaction at 37°C overnight. Genomic DNA was phenol/chloroform extracted, ethanol precipitated and resolved on a 8% agarose gel. The agarose gel was denatured by immersion in a solution of 1.5 M NaCl and 0.5 M NaOH for 20 min twice, followed by neutralization in a solution of 1.5 M NaCl and 0.5 M Tris-HCl pH 7.5 for 15 min twice. The gel was dried in a gel dryer and pre-hybridized in 20 ml hybridization solution (50% SSC, 5× Denhardt's solution, 0.1% SDS, 1 mM EDTA pH 7.0) at 58°C for 1 h. In gel hybridization was performed at 58°C overnight in 15 ml fresh hybridization solution with a 32P end-labelled oligonucleotide probe targeting the \textit{his} 3 locus. The gel was then washed at 58°C for 20 min three times with a solution of 3× SSC and 0.1% SDS and three times with a solution of 2× SSC and 0.1% SDS. The gel was exposed to a phosphorstorage screen and imaged on a FX-Pro phosphorimager (Bio-Rad).

**RT-PCR.** Total RNA was treated with RNase-free DNase I (Zymo Research Corporation) to remove any genomic DNA contamination, followed by phenol/ chloroform extraction and ethanol precipitation. For cDNA synthesis, 2 μl total RNA was incubated with 1 μl of 5× First Strand Buffer (Invitrogen), 0.2 μM dNTPs (Roche), 0.5 μg random primers (Invitrogen) and 200 units RNase-free M-MLV Reverse Transcriptase (Promega) at 42°C for 15 min and quickly cooled on ice. A 20 μl reverse-transcription reaction was performed in 1× Transcriptor II Reverse Transcriptase Reaction buffer (50 mM Tris-HCl pH 8.3, 7.5 mM KCl and 3 mM MgCl\(_2\)), 10 mM DTT and 200 U Transcriptase II Reverse Transcriptase (New England Biolabs) at 48°C for 30 min. The reaction was terminated by incubation at 80°C for 5 min. PCR was performed in a 25 μl reaction with 1 μl of 1× diluted reverse-transcription reaction in 1× Q5 Reaction Buffer (25 mM TAPS-HCl pH 9.3, 50 mM KCl, 2 mM MgCl\(_2\), 0.1 mM dNTP, 0.5 U of Q5 DNA Polymerase, 0.2 μM forward primer (5′-CAACACATTCCAAACATTCC-3′) and 0.2 μM reverse primer (5′-TGTCGAAAAACACGAC-3′)) and 100 ng of cDNA. The PCR products were resolved on a 1.5% agarose gel. The DNA bands corresponding to the spliced products were gel extracted using a Wizard SV gel and PCR clean-up system (Promega) and sequenced.

\textit{N. crassa} was transformed with 1 μg of \textit{Stu I}-linearized wild-type or mutant plasmid DNA by electroporation using a Gene Pulser Xcell system (Bio-Rad) set to 1,500 volts and 25 μF. The selection of \textit{his} \textsuperscript{+} transformants was performed in Vogel’s minimum medium supplemented with 2% sorbose in place of sucrose. Following growth at 30°C for 3 days, the colonies were inoculated onto Vogel’s slants. Screening of homokaryon strains was performed by Southern blot analysis.
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