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RNase III cleavage signals spread across splice junctions enforce sequential processing of co-hosted snoRNAs.

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Small nucleolar RNAs (snoRNAs) are a class of non-coding RNA molecules whose precursor transcripts are capped and polyadenylated. However, these end modifications are detrimental to snoRNA function and must be removed, a process typically involving excision from introns and/or endonucleolytic cleavage. In the case of polycistronic RNA precursors that host multiple snoRNAs, the sequence of maturation events is not well understood. Here we report a new mode of maturation concerning snoRNA pairs that are co-hosted in the intron and the adjacent exon of a precursor transcript. For such a pair in the model eukaryotic species *Schizosaccharomyces pombe*, we identified a double-stranded RNA hairpin folding across the exon-exon junction. The hairpin recruits the RNase III Pac1 that cleaves and destabilizes the precursor transcript while participating in the maturation of the downstream exonic snoRNA, but only after splicing and release of the intronic snoRNA. We propose that such RNase III degradation signal hidden in an exon-exon junction evolved to enforce sequential snoRNA processing. Sequence analysis suggest that this mechanism is conserved in animals and plants.

Introduction

Small nucleolar RNAs (snoRNAs) constitute a class of eukaryotic non-coding RNA that guide chemical modifications on other RNA molecules, an essential function that also exist in archaea, despite their lack of nucleolus. Their evolutionary origin has been proposed to trace back to the RNA world[1–3]. Most snoRNA can be classified in one of two major snoRNA families based on distinct structural features, functions, and conserved sequence motifs (or boxes). The C/D box snoRNAs fold into a closed loop and associate with methyl-transferase proteins to deposit 2′-O-methylation on targeted RNAs. In contrast, H/ACA box snoRNAs fold into a double hairpin structure and associate with pseudouridine synthase proteins to guide pseudouridination. Members of both snoRNA families target RNA substrates through base-pairing interactions between guide sequences in the snoRNA and complementary sequences in the substrate[4,5]. One of the major targets for snoRNA-directed modification is nascent ribosomal RNA (rRNA), which is consistent with the preferential nucleolar localization of snoRNAs.

SnoRNA maturation and biogenesis in eukaryotes relies on distinct mechanisms – often perceived as unusual – compared to other classes of genes. Being transcribed by RNAPII, snoRNA precursor transcripts (pre-snoRNAs) are, like messenger RNAs (mRNAs), subject to co-transcriptional capping and polyadenylation. The 7-methylguanosine (m^7G) cap and poly(A) tail are essential modifications that protect mRNA from exonucleolytic degradation and favor their export and translation in the cytoplasm[6]. In contrast, mature snoRNAs do not require end modifications as they are intrinsically stable due to their structure and the association of partner proteins. In fact, unprocessed caps or poly(A) tails can even be detrimental to snoRNA function. For instance, capped snoRNAs in yeast have been shown to mis-localize in the cytoplasm where they are targeted for degradation[7]. Therefore, snoRNA maturation involves the removal or alteration of the m^7G cap and poly(A) tail from the pre-snoRNAs, relying on distinct mechanisms depending on snoRNA genomic organization: intronic, (mono)-exonic, or polycistronic, reviewed in[8] and briefly summarized hereafter.

In mammals, snoRNAs are often encoded in the introns of host genes. For instance, 61% of all annotated snoRNAs in human are intronic[9,10]. Following splicing of the intron, intronic snoRNAs can be excised from the lariat either by endonucleolytic cleavage or by the combined action of the debranching enzyme and exonucleases, resulting in cap- and poly(A)-less linear transcripts. Remarkably, intronic snoRNAs are often hosted in genes coding for proteins involved in ribosome synthesis, suggesting that snoRNA and ribosome production are coordinated. However, host genes are not exclusively protein coding: a significant fraction of intronic snoRNA (23% in human)[9,10] are hosted in long non-coding RNAs (lncRNA) genes, for which, most frequently, no apparent function has been described besides their role as snoRNA precursors.

Exonic snoRNAs (*sensu lato*, referring to all snoRNA that are not intronic) are more common in yeast where they account for 90 and 81% of all snoRNA in budding yeast and fission yeast, respectively[9]. Exonic snoRNAs are organized either as polycistrons or as individual genes. For individually encoded snoRNAs, 3' end processing can differ between species. In fission yeast, it occurs through the mRNA

cleavage and polyadenylation machinery[11] with subsequently deadenylation by the nuclear exosome together with a nuclear poly(A)-binding protein Pab2[12]. Mechanisms of 5'-end processing of individually encoded snoRNAs differ between snoRNA families with most individual C/D box pre-snoRNAs being transcribed with a capped 5' leading sequence. This leading sequence is removed through endonucleolytic cleavage and subsequent trimming by exonucleases[13,14]. In contrast, for individually transcribed pre-snoRNA without a leading sequence (including most H/ACA snoRNA in yeast) the m^7G cap of the pre-snoRNA is converted in trimethylated $m^{2,7,7}G$ (TMG) cap[15].

Finally, exonic snoRNA genes can also be organized in operon, where snoRNAs are typically matured from polycistronic transcripts through endonucleolytic cleavage between the cistrons. In budding yeast, this process is mediated by the RNaseIII family protein Rnt[13,16]. In other species, including fission yeast and human, the actors mediating the 5' and 3' maturation of exonic snoRNA co-hosted in the same precursor transcripts remain largely unknown. In these cases, the sequence of maturation events is particularly significant, as precursor cleavage exposes snoRNAs and surrounding sequences to exonucleolytic degradation. Whether and how the order of snoRNA processing is controlled remains an open question.

In this study, we address these questions using the model eukaryote *Schizosaccharomyces pombe*. In *S. pombe*, the RNase III homolog Pac1 is an endoribonuclease involved in RNA polymerase II termination and ncRNA biogenesis, including the 5' maturation of several exonic C/D box snoRNAs[17]. Mechanistically, Pac1 targets double-stranded RNA (dsRNA) hairpins with a minimum stem length of 20 base pairs, tolerating small bulges and varied loop structures[18,19] and cut them in two staggered cuts. This contrasts with the budding yeast RNase III homolog Rnt1 that specifically targets RNA hairpins with apical NGNN tetraloops[20].

Here, we report a new role for Pac1 in the maturation of a snoRNA pair co-hosted within the intron and adjacent exon of a precursor transcript. Remarkably, Pac1 activity at this locus depends on a dsRNA hairpin that folds across the exon-exon junction after splicing of the intron. Our findings suggest that this exon-exon junction serves as a conditional degradation signal, ensuring sequential snoRNA processing, a mechanism that may be conserved across eukaryotes, including animals and plants.

Results

snoU14 and the mamRNA are transcribed from a polycistronic precursor stabilized by Pac1 inactivation.

Based on high-throughput genomic and transcriptomic data, we previously identified Pac1 cleavage signatures for at least five C/D box snoRNA[17]. Accordingly, northern blot experiments on three of these snoRNA (snoU14, snr88 and sno16) revealed that 5'-extended snoRNA precursor transcripts accumulate when Pac1 is inactivated. Based on these data, we have proposed that Pac1 is involved in snoRNA maturation through 5'-end processing[17]. However, the recent discovery by the Rougemaille group of a previously unannotated ncRNA named *mamRNA* (for *Mmi1 and Mei2-associated RNA*) directly upstream of snoU14[21] suggests that the role of Pac1 at the locus could be more complex than previously thought. Moreover, a preprint from the same research group now describes that the mamRNA and snoU14 are expressed from a common transcript precursor[22], a conclusion we also reached independently when re-evaluating the contribution of Pac1 to mamRNA/snoU14 expression.

To assess Pac1 role in modulating mamRNA expression, we first performed northern blot experiments using radiolabeled probes complementary to the mamRNA (*C*), snoU14 (*B*) and the intergenic region between the two genes (*A*) in conditions where the activity of Pac1 is compromised either with the conditional nuclear exclusion of Pac1 in the Pac1-AA (anchor-away) strain or with the Pac1-ts (thermosensitive at 37°C) strain that is hypomorphic for Pac1 function even at growth-permissive temperature[17]. As expected, the *A* and *B* probes recapitulated our previous observation that a long 5'-extended precursor containing the mature snoU14 sequence accumulates in conditions of Pac1 deficiency (Figure 1.A, *A*-*B* probes). More surprisingly, at least four different transcripts were detected with the mamRNA probe (Figure 1.A, *C* probe). The two shortest ones were the most abundant within the control strains (when Pac1 is active) and their length match the annotated mamRNA *short* and *long* isoforms[21,23]. In addition, a longer, previously uncharacterized mamRNA isoform specifically accumulated in the Pac1-deficient strains. As it migrates at the same size as the 5'-extended snoU14 precursor, we tentatively named this isoform *pre-mamRNA/snoU14*. Long read RNA-sequencing validated the existence of such transcript isoforms containing both the mamRNA and snoU14 sequences (Supplementary Figure 1.A), as also recently reported[22]. Further suggesting that they are transcribed together, previously published CAGE data[24] highlighted a single transcription start site for the entire locus, located directly upstream of the mamRNA (Figure 1.B). Together, these results confirm that snoU14 and the mamRNA are transcribed from a single transcript precursor[22], a precursor whose expression appears dependent on Pac1 activity.

We then corroborated our northern blot experiments with short read RNA-sequencing data, confirming that transcripts originating from the locus encompassing both the mamRNA and snoU14 genes were up-regulated in Pac1-deficient strains (Figure 1.B). However, a 227 nucleotides gap in the read density profiles could be observed within the mamRNA. This gap corresponds to a newly described intron in the pre-mamRNA/snoU14 transcript[22] which is confirmed here by spliced reads spanning the exon-exon junction in both our short and long read RNA sequencing data (Figure 1.C, Supplementary Figure 1.A). Of note, this intron is also host to a newly annotated C/D box snoRNA, called snR107[22], whose expression was not tangibly affected by Pac1 inactivation (Supplementary Figure 1.B).

Across the mamRNA intron, we observed that the spliced reads accumulated more than the intronic reads when Pac1 is deficient (Figure 1.B-C), suggesting that the isoform whose expression depends on Pac1 activity is spliced. In comparison, the steady-state level of the unspliced pre-mamRNA/snoU14 isoform (Figure 1.A) remains largely unaffected. Confirming these observations, splice-specific RT-qPCR experiments showed a specific increase in relative expression with the amplicon spanning

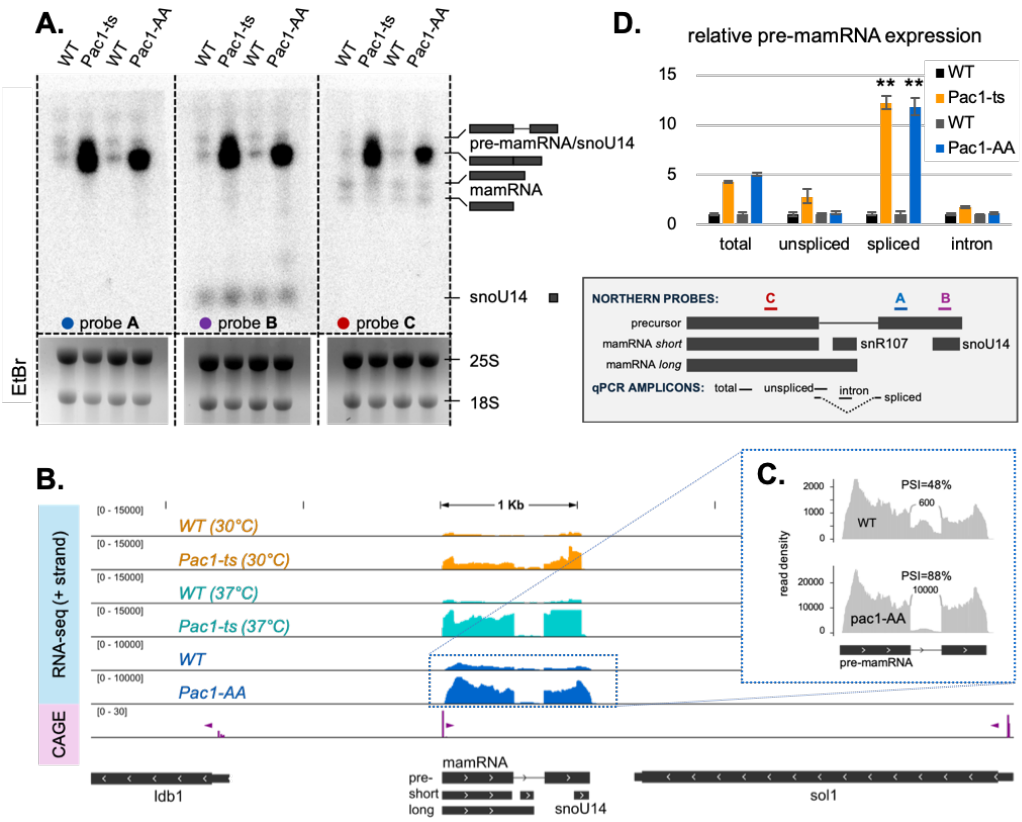

Figure 1: A common precursor for the mamRNA and snoU14 depends on Pac1 activity. A. Representative northern blot analysis of transcripts originating from the mamRNA/snoU14 locus in conditions where the activity of Pac1 is compromised. Probes positions are indicated below panel D. **B.** Genome browser snapshot of the normalized read density over the mamRNA/snoU14 locus for RNA-seq experiments (+ strand only) and CAGE-seq (+ and - strand). For the CAGE-seq experiments, the direction of transcriptional firing is indicated with purple arrowheads. Gene annotation is indicated in dark grey. **C.** Sashimi plot showing the junctions reads plotted as arcs over the overall read density profile for the Pac1-AA and matching WT RNA-seq experiments over the mamRNA locus. The number of junction reads (rounded to the closest 100) and percent spliced-in values (PSI) are indicated. **D.** RT-qPCR analysis (n=3) of transcript isoforms spanning the mamRNA locus. qPCR amplicon position is indicated below the panel. Statistical significance of the differences in spliced isoform level in the Pac1 mutants compared to their control strains is indicated (**: Student's t test pvalue < 0.01). EtBr: Ethidium Bromide; ts: thermosensitive; AA: anchor-away; WT: wild-type.

the exon-exon junction within the mamRNA, but not with the amplicon spanning the exon-intron junction nor with the intronic amplicon (Figure 1.D). Therefore, we conclude that Pac1 activity specifically opposes the expression of the spliced pre-mamRNA/snoU14 isoform.

A stemloop structure from the pre-mamRNA exon-exon junction directs Pac1 cleavage.

We next wanted to identify *cis*-acting elements in the pre-mamRNA/snoU14 transcript responsible for Pac1 recruitment and/or activity. The sequence directly upstream snoU14 has been previously predicted to fold in a stemloop that could constitute a genuine Pac1 substrate, although this has not been experimentally tested[17]. To challenge this prediction, we seamlessly introduced 8 point mutations expected to dramatically disrupt the predicted stemloop structure in a strain we named *snoU14-SD* (*StemDead*) (Figure 2.A). In this strain, we observed that the mature level of snoU14 was significantly decreased by about 34% (Figure 2.B, *B* probe; quantified in supplementary Figure 2.B), suggesting that the mutated sequence might be important for snoU14 expression and/or maturation. However, the snoU14-SD mutant did not recapitulated the effects of Pac1 deficiency as the expression of the spliced pre-mamRNA/snoU14 isoform remained at wild-type level in both Northern blot and splice-specific RT-qPCR experiments (Figure 2.B-C). From these observations, we concluded that the predicted stemloop proximal to snoU14 (Figure 2.A) is not responsible for Pac1 activity on the precursor transcript.

We therefore scanned both the unspliced and spliced pre-mamRNA/snoU14 sequences for other secondary structures that could be potentially recognized by Pac1. Strikingly, a very stable stemloop (ΔG=-35Kcal/mol) with symmetric bulges, was predicted to fold across the exon-exon junction (-15 of exon 1 to +121 of exon 2) of the spliced precursor (Figure 2.A). In contrast, the same segment from the unspliced mamRNA precursor isoform was predicted to form a branched structure due to the disruptive presence of the intron (Supplementary Figure 2.A). Given our knowledge of Pac1 substrate specificity[18], only the stemloop predicted to fold across the splice junction could be expected to recruit Pac1. To test this hypothesis, we scarlessly introduced three sets of point mutations in the endogenous mamRNA gene and named the resulting mutants *mamRNA-SD₁*, *-SD₂*, or *-SD₃* (Figure 2.A). The four and three disruptive mutations in the SD_1 and SD_2 mutant, respectively, are located in the exon 2 of the pre-mamRNA gene, close to the 3' end of the predicted secondary structure. Conversely, the three disruptive mutations in the mamRNA-SD_3 mutant are located in the exon 1 of the mamRNA gene, and are close to the 5' end of the predicted structure. They were designed in such a way that the combination of the SD_2 and SD_3 mutations should complement each other in maintaining the structure, allowing to discriminate between sequence- or structure-dependent effects.

In all of the mamRNA-SD individual mutants (mamRNA-$SD_{1,2,\ or\ 3}$), the steady state level of the spliced pre-mamRNA/snoU14 isoform was dramatically increased (Figure 2.B-C) while the mature mamRNA forms were less abundant (Figure 2.B , *C* probe). Strikingly, these effects were not observed in the mamRNA-SD_{2+3} mutant where the SD_2 and SD_3 complementary mutations reform a stable structure. These results strongly suggest that the RNA secondary structure spanning the exon-exon junction of the mamRNA is responsible for its regulation by Pac1.

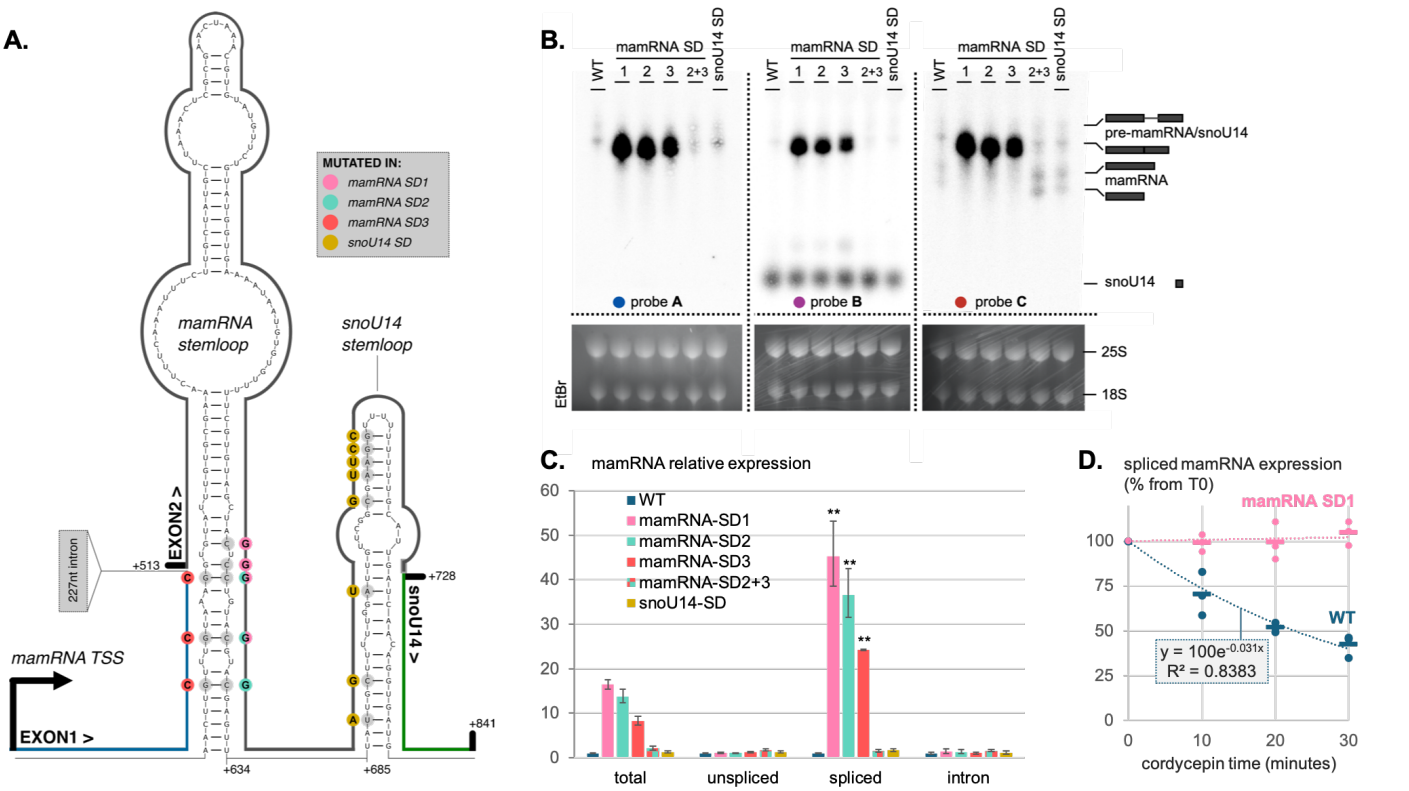

Figure 2: A stemloop spanning the exon-exon junction directs Pac1 cleavage at the mamRNA. A. Secondary structures of the spliced pre-mamRNA/snoU14 transcript. Nucleotide positions are numbered relatively to the mamRNA transcription start site (TSS). Mutated bases are colored according to the stemdead (SD) mutant strains in which they are changed. **B.** Northern blot analysis of transcripts originating from the mamRNA/snoU14 locus. Probes positions are the same as in Figure 1.A. EtBr: ethidium bromide. **C.** RT-qPCR analysis (n=3) of transcript isoforms spanning the mamRNA locus. The qPCR amplicons are the same as in figure 1.D. Statistical significance of the differences in spliced isoform level in the CS mutants compared to their control strains is indicated (**: Student's t test pvalue < 0.01). **D.** Decay rate of the spliced pre-mamRNA/snoU14 transcript measured by RT-qPCR after transcriptional inhibition with 0.6mM cordycepin (n=3).

Confirming this hypothesis, we found that mutations in the secondary structure are epistatic to Pac1 loss of function, with no further increase in the expression of the spliced pre-mamRNA/snoU14 isoform when Pac1 is excluded from the nucleus in the mamRNA-SD1 background (Supplementary Figure 2.C).

Arguably, the most straightforward explanation for how Pac1 represses the *pre-mamRNA/snoU14* isoform would be that its endonucleolytic activity destabilizes the transcript. To test this hypothesis, we measured the decay rate of the mamRNA isoforms in time course experiments following transcription inhibition with the chain-terminating adenosine analog cordycepin (see material and methods section for comments about the choice of transcriptional inhibitor). In wild-type, cordycepin treatment caused a rapid decrease of the relative abundance of the spliced mamRNA/snoU14 precursor (half-life estimation assuming a complete transcriptional inhibition = 20 minutes). However, the mamRNA-SD$_1$ mutation drastically stabilized the isoform, that did not decay over the course of the experiment (Figure 2.D). From these results, we conclude that the stemloop structure over the splice junction destabilizes the spliced mamRNA-snoU14 precursor in a Pac1-dependent manner.

snoU14 can mature independently from Pac1 activity

The degradation signal localized at the mamRNA splice junction raises the question of its role in snoRNA maturation. We initially hypothesized that Pac1 cleavage at this locus was necessary to release snoU14 from the mamRNA precursor. Supporting this idea, Northern blot experiments indicated that snoU14 is sequestered in the longer precursor transcript when Pac1 activity is compromised in Pac1-deficient strains or mamRNA stemdead mutants (Figure 1A and 2B, *B* probe). However, mature snoU14 levels remained unaffected. This suggests that alternative pathways are able to mature snoU14 from its precursor, potentially with slower kinetics.

SnoU14 is a conserved and essential C/D box snoRNA involved in the maturation of the 18S rRNA from the polycistronic 35S rRNA precursor. Accordingly, deficiencies in snoU14 expression have been reported to dramatically affect rRNA processing, 18S levels, and cellular growth in yeast[25,26]. Here, northern blot experiments on rRNA precursors showed that reduced snoU14 expression in the snoU14-SD strain (Figure 2B) correlated with a small (about 20%) but statistically significant increase in 35S and 33/32S rRNA precursor (Supplementary Figures 3.A-B). In contrast, mamRNA-SD mutants did not exhibit perceptible differences in mature or precursor 18S rRNA levels (Supplementary Figure 3.A-B), indicating that snoU14 matured by putative Pac1-independent pathway are fully functional. Moreover, all the mamRNA SD strains grew normally in rich medium (Supplementary Figure 3.C). From these experiments, we conclude that Pac1 cleavage on the pre-mamRNA/snoU14 transcript is not strictly required for snoU14 expression or function.

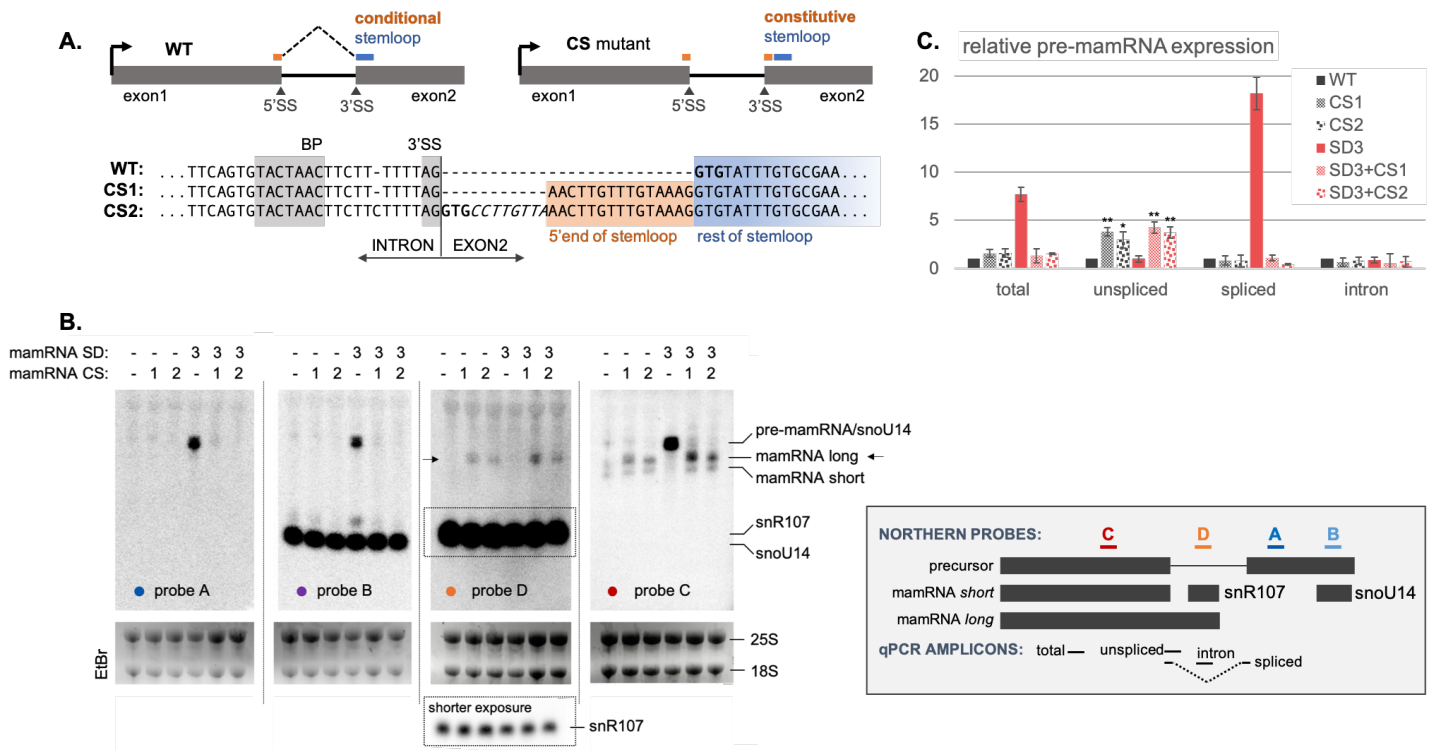

Figure 3: Conditional Pac1 cleavage ensures sequential snoRNA processing. A. Top: schematic representation of the mamRNA Constitutive Stem (CS) mutations. Bottom: mutated sequence of the mamRNA CS mutants. For the mamRNA-CS_2 mutant, the nucleotides in bold and in italic also correspond to the original WT sequence immediately downstream the 3' splice-site and immediately upstream the 5'end of the hairpin, respectively. SS: splice-site; BP: branch point **B.** Northern blot analysis of transcripts originating from the mamRNA/snoU14 locus. Probes positions are indicated on the right of the figure. Arrows indicates the snR107-containing long mamRNA isoform. EtBr: ethidium bromide. **C.** RT-qPCR analysis (n=3) of transcript isoforms spanning the mamRNA locus in the indicated strains. Statistical significance of the differences in unspliced isoform level in the CS mutants compared to their control strains is indicated (* and **: Student's t test pvalue < 0.05 and < 0.01, respectively). The qPCR amplicons are the same as in figure 1.D.

Pac1 cleavage across splice junction coordinates the sequence of snoRNA maturation events

Disjoint secondary RNA structure spanning splice junctions are not unique to *S. pombe*; they can also be predicted upstream of the snoU14 genes of related *Schizosaccharomyces* species, suggesting conservation (Supplementary figure 4.A-B). We reasoned that such a peculiar localization forces RNase III cleavage to occur only after splicing of the intron, a condition that could be required for proper expression of the cohosted intronic snoRNA. To test this hypothesis in *S. pombe*, we re-engineered the mamRNA/snoU14 locus by inserting a copy of the 5'end of the base of the stemloop (the last 15 nucleotides of the exon1) at the beginning of the exon2 (figure 3.A). In the resulting strain called mamRNA-CS_1 (*Constitutive Stemloop 1*), the folding of the hairpin should be uncoupled from the splicing of the intron, potentially leading to faster/unconditional Pac1 cleavage kinetics. An alternative version of the mutant (mamRNA-CS_2), preserves the original sequence immediately downstream of the 3' splice-site and immediately upstream of the 5'end of the hairpin (figure 3.A). Strains with either the mamRNA-CS_1 or -CS_2 mutations grew normally in rich medium (Supplementary figure 3.D). More importantly, they suppressed the increased levels of spliced precursor in the SD_3 mutant (Figure 3.B, *A-B* probes; Figure 3.C), demonstrating that the re-engineered "constitutive" stemloops are functional cleavage substrates for Pac1.

In Northern blot analysis, we observed in the CS mutants that a small fraction of intronic snR107 remained trapped in the long mamRNA isoform (Figure 3.B, *D* probe) whose expression was also increased (Figure 3B, *C* probes), suggesting that unconditional Pac1 cleavage can cause aberrant snR107 processing, but not to an extent sufficient to affect mature snR107 level. To interpret these results, we propose that in the CS mutants, Pac1 cleavage has the potential to occur before splicing on the unspliced precursor transcript. This relatively rare event could lead to 3'->5' exonucleolytic trimming of the 3' splice-site, antagonizing splicing and subsequent maturation of snR107 (Figure 4.A). Consistent with this model, qPCR analysis indicates a significant increase in unspliced isoforms for the mamRNA CS strains (Figure 3.C).

Conservation of secondary structure spanning the splice junction in mixed snoRNA clusters.

Our results suggest that a cleavage signal (hairpin) spread across splice junctions enforces sequential processing of intronic-exonic snoRNA pairs co-hosted in the same precursor transcript (Figure 4.A). We next wondered whether such a mechanism could also exist in other eukaryotes. To address this question, we mined the *Ensembl* genome annotation[27] for similarly organized snoRNA pairs, limiting our search to seven model species reputed for their high quality annotation: the budding yeast *S. cerevisiae*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the mouse *Mus musculus*, the rat *Rattus norvegicus*, the plant *Arabidopsis thaliana*, and *Homo sapiens*.

In total, we found twelve snoRNA clusters co-hosted in the same precursor transcript comprising at least one

RNAse III cleavage signals spread across splice junctions enforce sequential processing of co-hosted snoRNAs.

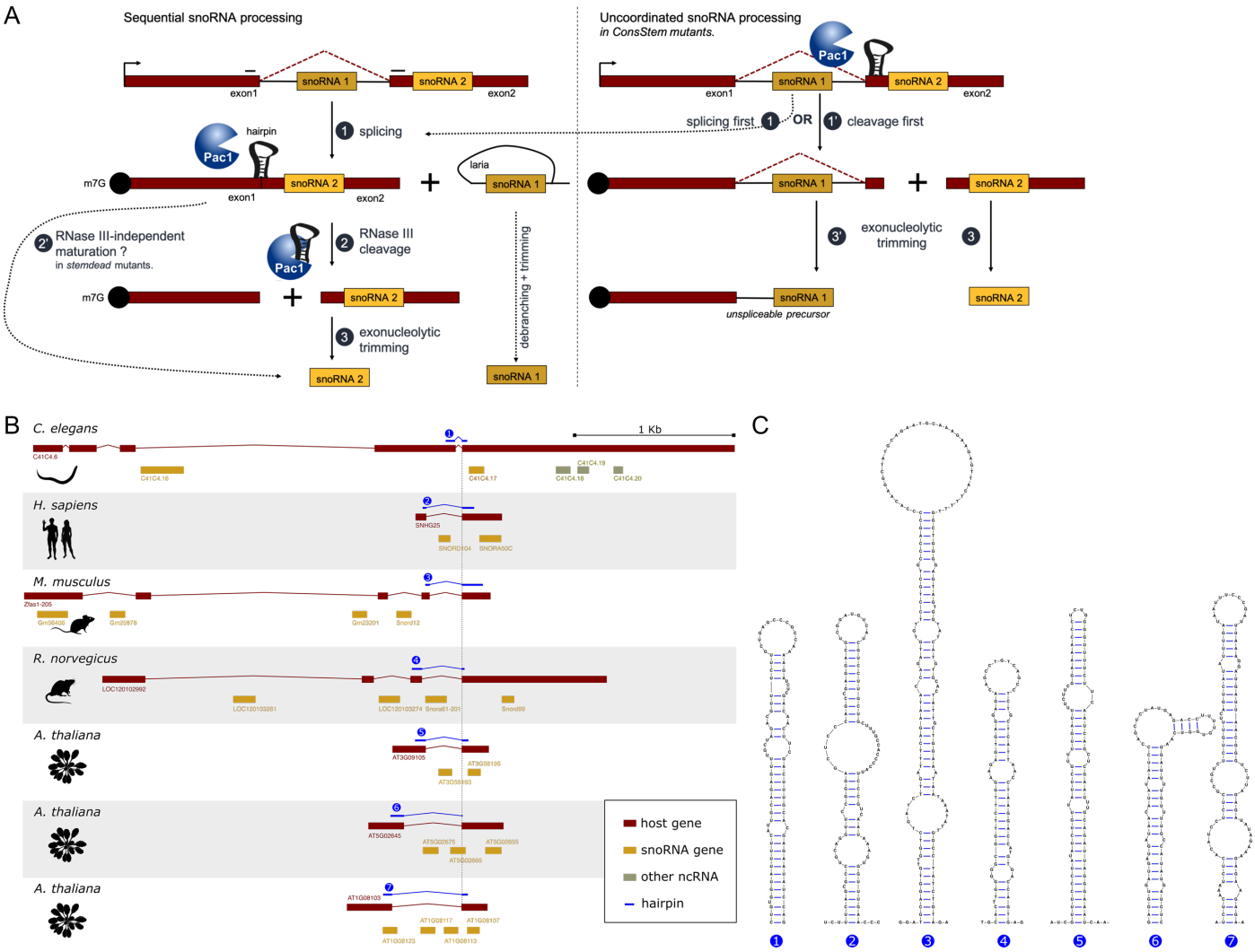

Figure 4: Sequential snoRNA processing of co-hosted intronic-exonic snoRNAs. A. Model for sequential vs uncoordinated processing of co-hosted snoRNAs. In those models, m^{7}G cap (black circles), polyA tails, and snoRNAs are barrier to exonucleolytic trimming. In sequential processing (left panel), splicing occurs first (1) allowing the release and maturation of the intronic snoRNA. By bringing together the two exons, splicing also allows for a secondary structure to fold across the exon-exon junction. This structure recruits the RNase III Pac1 in fission yeast (2), leading to the cleavage of the spliced precursor and subsequent maturation of the exonic snoRNA by exonucleolytic trimming (3). In the case of snoU14 in *S. pombe*, a secondary Pac1-independent maturation pathway exists (2'). In contrast, snoRNA processing would become uncoordinated (right panel) with cleavage potentially occurring before splicing (1') if the RNA secondary structure folded constitutively. This could lead to the exonucleolytic degradation of the 3' splice-site, leaving the intronic snoRNA trapped in the precursor (3'). **B.** Genomic arrangements of selected genes host to both intronic and exonic snoRNAs, aligned to the 5' end of their last exon. **C.** Predicted secondary structure from the sequences spanning the last exon-exon junction of host genes displayed in panel B.

exonic snoRNA and one intronic snoRNA. None of these clusters were found in the intron-poor *S. cerevisiae* genome and between one and three were found in the other species considered (Supplementary table 1). It suggests that, although relatively rare, *mixed* (exonic-intronic) snoRNA host exist in species beyond *S. pombe*. Among the identified clusters, most (ten out of twelve) were organized in such orientation that the last hosted snoRNA was exonic, preceded by one or more intronic snoRNAs (Figure 4.B). From these ten clusters, at least seven harbored predictable RNA hairpin structures folding from the sequence of the last two exons of their spliced host precursor (Figure 4.C). Further work will determine whether the secondary RNA structures spanning the exon-exon junction of cohosted snoRNAs also function as a degradation signal in other eukaryotic species.

Discussion & Conclusions

In this study, we have demonstrated that in *S. pombe*, snoU14 and an upstream non-coding RNA, the mamRNA[21], are expressed from a common polycistronic precursor whose transcription initiates about 1kb upstream of snoU14. These findings independently confirm the conclusion of a recently published preprint[22]. The updated transcript model provides a straightforward explanation for earlier observations that *S. pombe* snoU14 is functional when expressed from a *S. cerevisiae* promoter but cannot be expressed in *S. cerevisiae* in its native context, *i. e.* preceded by 800 bp upstream[25]. To explain this discrepancy, Samarsky and colleagues hypothesized that specific transcription signals from *S. pombe* snoU14 were poorly utilized in *S. cerevisiae*. However, it now appears that promoter elements, located further upstream, were not included in the transgene sequence.

Multiple transcripts arise from the mamRNA-snoU14 precursor, including an exonic snoRNA (snoU14), an intronic snoRNA (snR107)[22], and the long and short mamRNA isoforms[21]. This diversity suggests that multiple RNA maturation processes coexist at the locus. In this study, we demonstrated that the endoribonuclease Pac1 participates in the release of mature snoU14 from the precursor (figure 1) even though this activity is dispensable for snoU14 maturation (figure S3), hinting at a secondary maturation pathway which likely involves another endoribonuclease. In *S. cerevisiae*, snoU14 is released from a dicistronic precursor through cleavage by the RNase III ortholog Rnt1. However, partial functional redundancy between Rnt1 cleavage and another unknown factor has been reported[28]. This raises the possibility that the homolog of this unknown factor also operates in *S. pombe* in a secondary snoU14 maturation pathway.

We have identified that Pac1 cleaves the mamRNA-snoU14 precursor at a stemloop RNA structure encoded disjointly over its two exons (Figure 2). The particular localization of this degradation signal is remarkable as it makes Pac1 activity specific towards the spliced version of the precursor transcript, delaying cleavage until splicing is complete. This unique mechanism explains the dramatic mamRNA-snoU14 precursor accumulation previously reported in splicing-deficient mutants[22]. We propose that splicing as a prerequisite to Pac1 cleavage allows to precisely control the sequence of processing events. Indeed, we found that unconditional Pac1 cleavage of the mamRNA-snoU14 precursor leads to the accumulation of snR107-containing unspliced transcripts (Figure 3), suggesting that premature cleavage stochastically occurring before splicing can antagonize the later, possibly by exposing the 3' splice site to degradation (Figure 4A). It is likely that the core snoRNP proteins bound to snR107 sequence[22] can shield what remains of the precursor from further exonucleolytic trimming, similarly to the lncRNA transcripts with snoRNA ends (sno-lncRNAs) described in mammals[29] and to hybrid messenger RNA–snoRNA (hmsnoRNA) described in budding yeast[30]. Therefore, we propose that enforcing delayed cleavage for exonic snoU14 maturation ensures proper intronic snR107 maturation which is critical to avoid the accumulation of the snR107-containing long mamRNA isoform. Control over the expression of this isoform might be especially important since both the mamRNA and snR107 contribute to the control of the gametogenesis in fission yeast by interacting with the Mmi1 and Mei2 proteins[21,22].

Previous studies have also described close mechanistic relationships between eukaryotic RNase III activity and intron splicing. In *S. cerevisiae*, Rnt1 cleavage sites are located within introns of seven pre-mRNAs coding for RNA binding proteins, promoting the degradation of unspliced pre-mRNA and lariat introns[31,32]. In mammals, Drosha cleavage sites span exon-intron junctions, antagonizing splicing either by direct cleavage or by sterically hindering splice-site recognition by the splicing machinery[33–36]. Our findings introduce a new, distinct configuration, with RNase III cleavage spanning exon-exon junctions and requiring prior splicing (Table 1). A similar case has been briefly reported among the non-canonical Drosha substrates identified by genome-wide fCLIP-seq in human cell lines[37].

Localization	Consequence of cleavage	Shown in
Within intron	quality control vs mis-spliced transcripts, regulation of targeted mRNA expression	S. cerevisiae (Rnt1)
Exon-intron junction	competition with splicing, antagonizes splicing	mammals (Drosha)
Exon-exon junction	splicing as a prerequisite to cleavage, delayed cleavage	*S. pombe* (Pac1) mammals ?

Table 1: Consequences of RNase III cleavage site localization relative to intron boundaries.

The enforced sequential snoRNA processing mechanism described in this study might have evolved to accommodate the very particular genomic organization of the two co-hosted snoRNAs from the mamRNA locus, where one snoRNA (intronic) depends on splicing for its biogenesis[22], whereas the other (exonic) relies on endonucleolytic cleavage. Such an organization can be qualified as *polycistronic-mixed* and can be found in a wide range of eukaryotic species, although it is quite uncommon compared to other types of snoRNA clusters[9]. However, its prevalence might be underestimated as the detection of polycistronic-mixed snoRNA clusters is highly reliant on high-quality snoRNA and host RNA annotation. Ongoing efforts to improve these annotations using machine learning and RNA-sequencing optimized for snoRNA detection (Fafard-Couture et al., manuscript in preparation) may enhance classification accuracy for snoRNA organization. Nevertheless, we found that in the majority of polycistronic-mixed clusters detected in the current annotation, a single exonic snoRNA is located downstream of one or multiple intronic snoRNAs (Figure 4B). This consistent genomic organization combined with the presence of predictable stemloop structures folding across the last splice junction (Figure 4C) suggest that spreading cleavage signal on two exons might be a conserved mechanism to delay cleavage-dependent exonic snoRNA maturation after the last intronic snoRNA of the cluster is spliced. Since the mechanism of 5'end processing of exonic snoRNAs is currently unknown in higher eukaryotes, it remains to be determined which endonuclease(s) are involved (if any) and whether the predicted stemloops in the polycistronic-mixed clusters can indeed function as cleavage signal. Thus, the mechanisms underlying 5'-end processing of exonic snoRNAs in eukaryotes remain a promising area for future investigation.

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Acknowledgements

We thank Marc Hennequart, Mathieu Rougemaille, and François-Xavier Stubbe for helpful discussion and proofreading the manuscript. We further thank Mathieu Rougemaille for openly sharing unpublished data concerning the existence of intronic snR107 in the mamRNA precursor. C.Y-S. is a scientific collaborator of the Fonds de la Recherche Scientifique – FNRS.

Author contributions

C.Y-S designed the study and wrote the manuscript with inputs from M.S and F.B. V.M, Y.M, and C.Y-S performed all experiments, with the exception of the nanopore sequencing experiment performed by P.L. E.F-C and C.Y-S

analyzed transcriptomic and genomic data. C.Y-S prepared the figures. All authors reviewed the first draft of the manuscript.

Competing interest statement

None.

Materials and Methods

Yeast strains, drugs and media.

Unless stated otherwise, yeast cells were grown at 32°C in liquid minimal medium supplemented with adenine, uracil, histidine and leucin (EMM+AS, All Supplements) until an optical density of 0.4-0.6. For the anchor-away experiments, yeast cultures were treated for two hours with 2.5 µg/ml rapamycin (Sigma R8781) or equal volume of DMSO carrier to allow for Pac1 nuclear exclusion. For the stability assays, yeast were grown at 26°C in EMM+AS over the full course of the experiment. Cordycepin (Sigma C3394, 10 mM stock in H_2O, required heating at 55°C to fully dissolve) was added to a final concentration of 0.6 mM at the appropriate time so that all sample from the same experiments could be pelleted together.

Gene disruptions and C-terminal tagging of proteins were performed by PCR-mediated gene targeting using lithium acetate method. Cloning-free CRISPR/Cas9-mediated mutagenesis was used to generate the stem-loop mutants[38]. Yeast strains used in this study are listed in Supplementary Table 2.

Choice of cordycepin as a transcriptional inhibitor.

Chemical inhibition of transcription in yeast is most commonly performed by using divalent cation chelator such as 1-10 phenanthroline or thiolutin[39,40]. However, divalent cation are required for other processes beyond transcriptional elongation by the RNA polymerases; they are also important for the spliceosome to catalyze the second step of splicing[41] as well as for Pac1 cleavage activity[42]. Therefore, these transcriptional inhibitors interfere at different levels with the expression of the pre-mamRNA/snoU14 (data not shown), making them ill-suited to study its decay rate. As an alternative, we used the adenosine analog cordycepin. Compared to adenosine, cordycepin lacks an hydroxyl group required for chain elongation and therefore blocks transcription elongation when its triphosphorylated form is incorporated into nascent RNA[43].

RNA extraction and analysis.

Total RNA was extracted following the classical hot phenol method[44] as previously described[45]. Briefly, the cells were first resuspended in 750 µl of TES solution (10mM Tris-HCl [pH 7.5], 10mM EDTA [pH8.0] and 0.5% SDS) and 750 of acidic phenol-chloroform-isoamyl alcohol 125:24:1 (Sigma P1944) then incubated at 65 °C for 60 minutes with high-speed agitation for 10 seconds every 10 minutes. After centrifugation, the upper, aqueous, phase was transferred to a new tube containing equal volume of acidic phenol-chloroform-isoamyl alcohol 125:24:1. After thorough mixing and centrifugation, the upper phase was transferred to a new tube containing equal volume µl of chloroform-isoamyl alcohol 24:1 (Sigma 25666). After mixing and centrifugation, 500 µL of the upper phase was precipitated with 1.5 mL 100% ethanol and 50 µL of 3M NaOAc pH5.2. The precipitated RNA pellet was washed twice with 70% ethanol, air-dried and resuspended in DEPC-treated (Sigma D5758) water.

For RT-qPCR analysis, 0.5µg of total RNA was retro-transcribed with the High-Capacity cDNA Reverse Transcription Kit (Thermo 4368813) following the manufacturer's instructions. The real-time PCR amplification was performed with SYBR Green 2X Supermix (Bio-Rad 172-5124) in a Bio-Rad CFX96™ Real-Time machine. The PCR program was 3 minutes at 95 °C followed by 40 cycles of (15'' at 95 °C; 30'' at 60 °C) and a melt curve. The PCR reactions were performed in technical duplicates, and all experiments were performed at least in biological triplicates.

Relative RNA quantification relied on the $2^{-\Delta\Delta Cq}$ method using the actin gene as internal reference except for the transcription inhibition assays (with cordycepin) in which we used the stable 18S rRNA instead. For statistical analysis, the normalized relative expression levels were brought back in the Cq scale by log2 transformation and used in one-sample Student's t test. Primer sequences are listed in Supplementary Table 3.

For northern blot analysis, two types of denaturing gel were used: 1.5% agarose gel in MOPS buffer with 1.6M formaldehyde to separate (pre-)snoRNAs, as previously described[21], and 0.8% agarose gel in Tri/Tri buffer with 0.4M formaldehyde[46] to separate pre-rRNAs, as described[47]. After migration, RNAs were transferred onto a positively charged nylon membrane (Amersham Hybond N+, Cytiva), crosslinked with UV in a Stratalinker or with heat (2h in the oven at 80°C), and pre-hybridized in hybridization buffer (Sigma H7033) at 42 °C. DNA probes were 5' radiolabeled with [γ-32P]-ATP using T4 polynucleotide kinase (Thermo Scientific EK0031, 30 minutes at 37°C) and were added to the membrane. After over-night incubation, membranes were washed once with 2×SSC/0.1% SDS and twice with 0.1×SSC/0.1% SDS. A storage phosphor screen was exposed to the membrane for up to 24h and scanned in a Typhoon biomolecular imager (Amersham). When relevant, the samples were run in duplicates or triplicates on the same gel to allow the study of multiple probes without stripping. All northern blot experiments were performed at least in duplicates, with only one representative scan shown. Probe sequences are listed in Supplementary Table 3.

For long read sequencing direct RNA-seq polyA+ libraries (SQK-RNA002, Oxford Nanopore Technologies) were prepared according to manufacturer's instructions starting from 500ng of total RNA. The libraries were sequenced on a MinION apparatus during 48 hours.

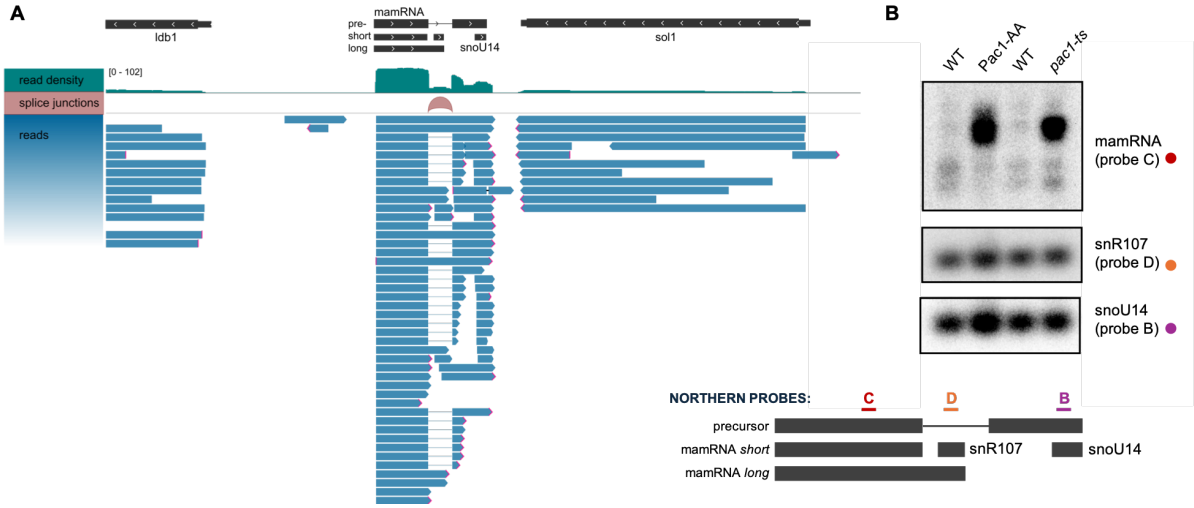
Sequence analysis

RNA secondary structures were predicted using the RNAfold2 webserver[48] and visualized in vaRNA[49]. Short read RNA-seq data were processed as described[17]. For long read RNA-seq, the raw signal was basecalled with the Guppy algorithm provided by ONT (version 6.5.7) with options `--flowcell FLO-MIN106 --kit SQK-RNA002 --device auto --calib_detect --reverse_sequence --u_substitution`. The resulting reads were mapped onto S. pombe genome using minimap2[50], version 2.24, with options `-Y -G 1000 -t 8 -R "@RG\tID:Sample\tSM:hs\tLB:ga\tPL:ONT" --MD -ax splice -uf -k14 --junc-bed` and visualized in the IGV software[51].

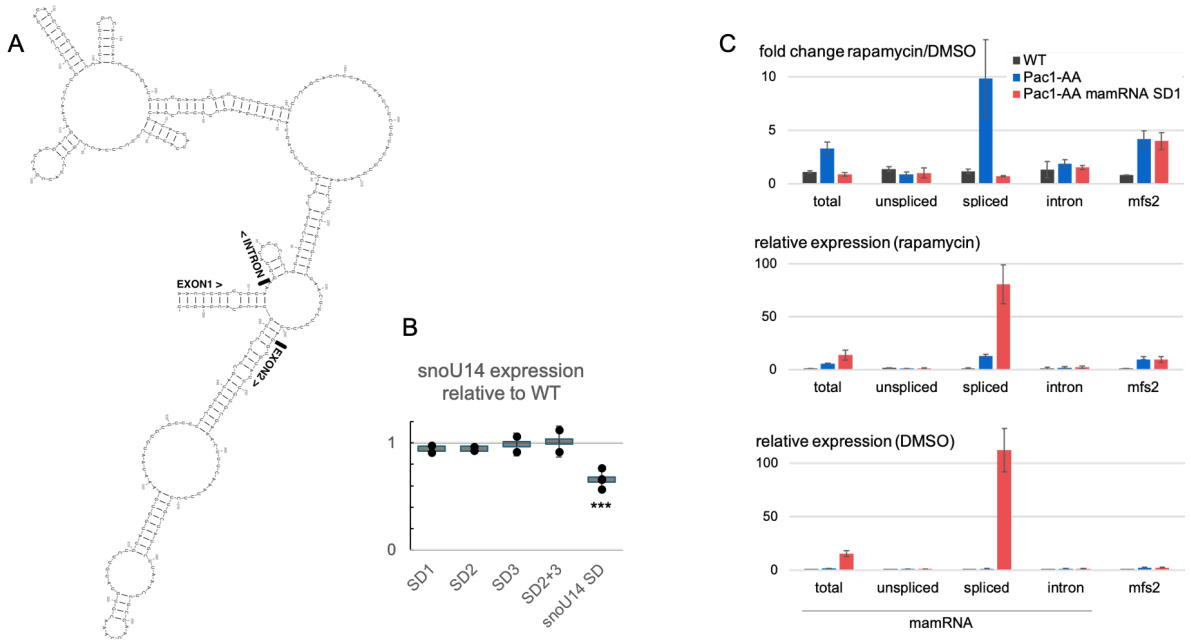
Data availability

Short read RNA-seq data analyzed in this study are available on GEO under the accession numbers GSE167041. Long read RNA-seq data analyzed in this study are available on ENA under the accession number PRJEB83877.

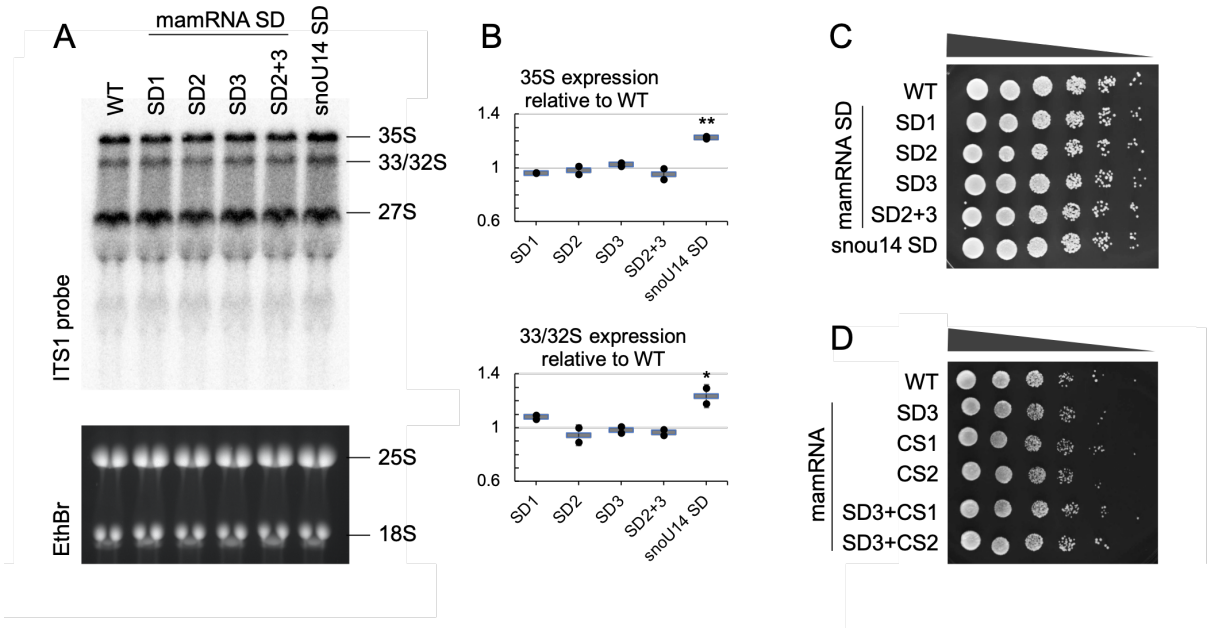
RNAse III cleavage signals spread across splice junctions enforce sequential processing of co-hosted snoRNAs.



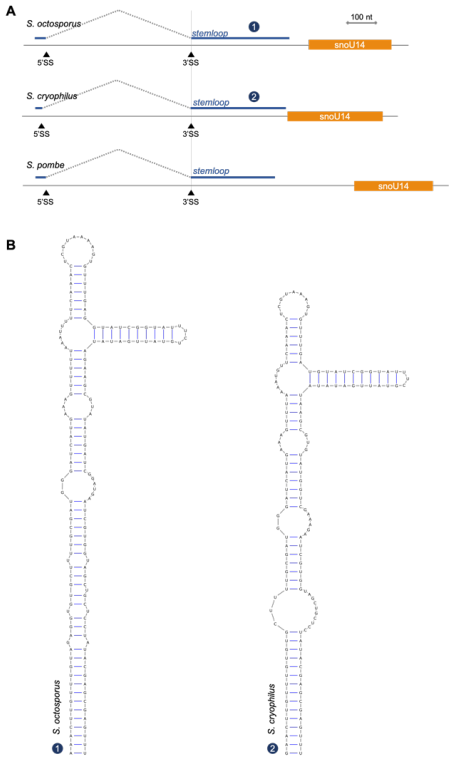
Supplementary Figure 1: A. Read density, splice-junctions and individual reads from a long read sequencing experiment (wild-type strain) over the mamRNA/snoU14 locus. **B.** Representative northern blot analysis of transcripts originating from the mamRNA/snoU14 locus in conditions where the activity of Pac1 is compromised. Probes positions are indicated below panel B.



Supplementary Figure 2: A. Predicted secondary structures of the unspliced pre-mamRNA/snoU14 transcript. **B.** Semi-quantitative determination of mature snoU14 relative expression from pixel measurement of the northern blot experiments presented in Figure 2.B. Individual replicates are represented by filled circles. Means +/- SD are depicted by grey rectangle with error bars. Statistical significance of the differences in mature snoU14 level in the snoU14-SD mutant compared to the WT strains is indicated (***: Student's t test pvalue < 0.001). **C.** RT-qPCR analysis (n=3) of transcript isoforms spanning the mamRNA locus in the indicated strains treated for two hours with rapamycin (middle panel) or with its solvent (DMSO) as control (bottom panel). The qPCR amplicons are the same as in figure 1.D with the addition of mfs2 as a positive control – mfs2 have been previously shown to be upregulated by conditional nuclear exclusion of Pac1 by rapamycin in the Pac1-AA strain (Yague-Sanz et al, 2021). The fold change between the relative expression in rapamycin and the control was calculated (top panel).



Supplementary Figure 3: snoU14 matured independently from Pac1 is functional. A. Northern blot analysis of pre-rRNA transcripts in the indicated strains with internal transcribed spacer (ITS) probe. Mature rRNA forms (25S and 18S) are revealed by ethidium bromide (EthBr) staining. **B.** Semi-quantitative determination of mature 35 and 33/32S rRNA precursor relative expression from pixel measurement of the northern blot experiments presented in panel A. Individual replicates are represented by filled circles (n=2). Their mean +/- their standard deviation are depicted by grey rectangle with error bars. Statistical significance of the differences in snoU14-SD mutant compared to the WT strain is indicated (*: Student's t test pvalue < 0.05; **: Student's t test pvalue < 0.01). **C.** 5-fold dilutions of yeast cultures from the indicated strains spotted on YES-agar plates and incubated for 2 days at 32°C. **D.** 10-fold dilutions of yeast cultures from the indicated strains spotted on YES-agar plates and incubated for 3 days at 32°C.



Supplementary Figure 4: A. Genomic arrangements of snoU14 orthologs in *Schizosaccharomyces* species, aligned to their upstream 3'splice-site (3'SS) **B.** Predicted secondary structure of the sequences spanning the exon-exon junction in (A) except for S. pombe snoU14, already shown in main Figure 2.A.