Partitioning of the Nuclear and Mitochondrial tRNA 3′-End Processing Activities between Two different Proteins in Schizosaccharomyces pombe*

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Background: Most eukaryotes contain only one tRNase Z gene involved in both nuclear and organellar tRNA 3′-end maturation.

Results: Schizosaccharomyces pombe has two tRNase Z genes required for nuclear and mitochondrial tRNA 3′-end processing, respectively.

Conclusion: The evolution of two tRNase Z genes and their differential expression in fission yeast may avoid toxic off-target effects.

Significance: The results advance our understanding of tRNA 3′-end maturation.

Almost all RNA molecules are synthesized as precursors that must undergo processing and modifications to form functional RNAs (1). tRNA processing and modification steps include removal of 5′- and 3′-extensions, splicing of introns (for only a subset of nuclear pre-tRNAs), base modifications, and addition of 3′-CCA sequence (2–4). For most nuclear pre-tRNAs, 5′-end processing occurs prior to 3′-end processing, and binding by the conserved La protein to pre-tRNAs is required for the orderly processing of tRNA and for the endonucleolytic processing of pre-tRNA 3′-ends (5, 6). In the absence of the yeast La protein, the 3′-end of nuclear tRNA is matured by exonuclease Rexlp (7, 8). In contrast, the La protein is not involved in mitochondrial tRNA (mt-tRNA)2 processing, and the order of mitochondrial pre-tRNA end processing can vary (9). Additionally, unlike nuclear tRNAs, mt-tRNAs are polycistronic. Moreover, mt-tRNAs normally deviate from the classic tRNA cloverleaf secondary structure and L-shaped tertiary structure due to high sequence and size variation in the D- and T-loops.

The metazoan mitochondrial genome (mtDNA) is typically a compact circular molecule (~16 kb). Transcription of metazoan mtDNA is initiated at a single unidirectional or bidirectional promoter on each strand, and mt-tRNAs are produced from large polycistronic RNAs (for reviews, see Refs. 10 and 11). In contrast, the mtDNA of the budding yeast Saccharomyces cerevisiae is large (~75–85 kb in size) and has numerous promoters (20) that direct different levels of transcription (12–14). In the fission yeast Schizosaccharomyces pombe, the mtDNA is compact (~19 kb in size) and contains three promoters (15, 16).

A simple model known as the tRNA punctuation model has been proposed for animal mtRNA processing (17, 18). According to this model, the secondary structures of tRNAs serve as mtRNA-processing signals. Processing of mt-tRNA liberates not only tRNA but also flanking RNAs from the precursor RNA. Thus, tRNA plays an important role in animal mitochondrial mRNA and mtRNA processing. In contrast, mtRNA processing in S. cerevisiae is a complex process (19). After tRNA processing, which liberates the 5′- and 3′-ends of pre-mtRNAs and pre-

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tRNA 3′-End Processing

rRNAs, additional processing steps are required for the generation of mature mRNAs and tRNAs in *S. cerevisiae* (20).

It has been suggested that mtRNA processing is more similar between *S. pombe* and humans than between either and *S. cerevisiae* (19). In the mitochondrial genome of *S. pombe*, the 3′-ends of tRNAs are matched to the 5′-ends of most other RNA species (16, 19). This suggests that the mt-tRNA 3′-end-processing enzyme not only is responsible for tRNA 3′-end maturation but also plays a critical role in the generation of the 5′-ends of other mtRNA species in *S. pombe*. Unlike 5′-end maturation, the generation of the 3′-ends of mRNAs involves additional endonucleolytic cleavage (19).

The endonuclease tRNase Z (also called RNase Z or 3′-tRNase) is near-ubiquitous in all three domains of life and is believed to be responsible for the removal of a 3′-extension of CCA-less tRNA precursors, which is an essential step for the generation of mature CCA termini at the tRNA 3′-end (for reviews, see Refs. 21–24). tRNase Zs belong to the β-CAPS (metallo-β-lactamase-associated CISF/Artemis/SMN1/PSO2) subfamily of the metallo-β-lactamase superfamily with diverse functions (25). Members of the β-CAPS subfamily are various nucleic acid-processing and -degrading enzymes, including cleavage and polyadenylation specificity factors CPSF73 and CPSF100, which are required for the 3′-end formation of mRNA, and RNase J, which is involved in the 5′-end maturation of tRNAs and mRNA stability in bacteria.

tRNase Z can be classified into a short form (tRNase ZS) and a long form (tRNase ZL). Sequence analysis suggests that tRNase ZL, which is about twice the size of the short form, arises from gene duplication of tRNase ZS (26, 27). tRNase ZL is present only in eukaryotes, whereas tRNase ZS exists primarily in prokaryotes, plants, and vertebrates (27–29). Most eukaryotes examined thus far, including *S. cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster*, contain only one tRNase ZL, which is either demonstrated or predicted to be localized in both the nucleus and mitochondria. In contrast, humans contain one tRNase ZL gene (also called ELAC2) and one tRNase ZS gene (also called ELAC1). The human tRNase ZL gene was originally identified as a candidate prostate cancer susceptibility gene by linkage analysis and positional cloning. However, it remains unknown how variations in the human tRNase ZL gene contribute to increased risk of prostate cancer. Human tRNase ZL is localized in both the nucleus and mitochondria (30–32), whereas human tRNase ZS of unknown function is found primarily in the cytosol (30, 31, 33). In plants, the situation is more complex. For example, *Arabidopsis thaliana* has two tRNase ZLs and two tRNase ZSs. The two long forms of tRNase Z are found in both the nucleus and mitochondria and in the mitochondria, respectively, whereas the two short forms are present in the cytoplasm and chloroplasts, respectively (34).

Although different eukaryotic organisms can have different numbers and forms of tRNase Z with different subcellular localizations, it has been suggested that a single tRNase ZL is responsible for the 3′-end processing of both nuclear and mitochondrial pre-tRNAs (27, 28, 35). In support of this hypothesis, it has recently been found that *Drosophila* tRNase ZL is involved in both nuclear and mitochondrial pre-tRNA 3′-end processing (36). In addition, a role for ELAC2 in mitochondrial pre-tRNA 3′-end processing has recently been demonstrated in vivo (30, 32). Although tRNase ZL’s from some species, including *S. cerevisiae* (37), *S. pombe* (38), and *A. thaliana* (34), have been shown to possess tRNA 3′-end processing activity in vitro, their nuclear and mitochondrial tRNA 3′-end processing activity in vivo remains to be experimentally demonstrated.

Unlike most eukaryotes, each of all four sequenced *Schizosaccharomyces* species (*S. pombe*, *Schizosaccharomyces octosporus*, *Schizosaccharomyces japonicus*, and *Schizosaccharomyces cryophilus*) contains two candidate tRNase ZLs encoded by two different essential genes (27). The two proteins from each species share weak sequence similarity. We have previously demonstrated that the two *S. pombe* tRNase ZLs (Trz1 and Trz2) are localized to the nucleus and mitochondria, respectively, and can endonucleolytically remove the 3′-extension from a human nuclear pre-tRNA in vitro (38). However, compelling *in vivo* evidence that the nuclear and mitochondrial tRNase Z activities are separated into two distinct proteins in *S. pombe* is still lacking.

In this work, we analyzed the processing of tRNAs in two strains carrying the temperature-sensitive mutant allele of *trz1* (*trz1-1* or *trz2* (*trz2-1*). We demonstrate that Trz1 and Trz2 are responsible for 3′-end processing of nuclear and mitochondrial pre-tRNAs, respectively. We also show that Trz2 plays a crucial role in 5′-end maturation of other types of RNAs in mitochondria.

**EXPERIMENTAL PROCEDURES**

*S. pombe* Strains, Media, and Genetic Procedures—The *S. pombe* strains used in this study were YHL6381 (wild-type; *h+* leu1-32 his3-D1 ura4-D18 ade6-210), YAS56 (wild-type; *h+* leu1-32 ura4-D18), and YZZ1 (*h+* trz1-1 leu1-32 his3-D1 ura4-D18 ade6-210) carrying the temperature-sensitive *trz1-1* allele (39) and YHD1 (*h+* trz2-1 leu1-32 his3-D1 ura4-D18 ade6-210) carrying the temperature-sensitive *trz2-1* allele. YHD1 was derived originally from D113 (40), which displays a temperature-sensitive phenotype resulting from mutation of Ala to Val at position 623 of *trz2* (which was called *trz1* in that work). However, compared with the wild-type strain, D113 grows extremely slowly in Edinburgh minimal medium even under permissive conditions, making subsequent analysis very hard. We thus constructed the same temperature-sensitive allele of *trz2* (which we termed *trz2-1*) in the YAS56 strain background as follows. A targeting vector containing the 1.3-kb C-terminal coding sequence of *trz2*, its 293-bp terminator sequence, 1.5 kb of *KanMX6*, and the 730-bp 3′-flanking sequence of *trz2* was linearized with Sall and used to transform YAS56. Transformants selected on yeast extract supplement (YES) medium (0.5% yeast extract, 3% (w/v) glucose, and 225 mg/liter each adenine, histidine, leucine, and uracil) containing 100 mg/liter G418 were patched onto YES medium and grown at 26 °C. Patches of cells were then replica-plated onto the same plates grown at 26 °C but not at 37 °C, and the *trz2-1* allele was sequenced to check for the A623V mutation. The *trz2-1* cells grew faster than D113 but still grew much more slowly than wild-type cells. Standard media and protocols for
genetic manipulation of fission yeast were used as described previously (41).

**Northern Analysis**—The trz1-1 and trz2-1 mutants and their isogenic wild-type strains were grown overnight in YES medium at 26 °C. Subsequently, the cells were diluted with fresh YES medium to an A600 of 0.2. When the cells reached an A600 of 0.6–0.8, they were shifted to 37 °C. At the indicated times, a sample was withdrawn, and total RNA was isolated. RNA purification and Northern blot analysis were performed as described (39). Briefly, total RNA was isolated using the acid guanidinium thiocyanate/phenol/chloroform method, separated on a 6% polyacrylamide-urea gel, and transferred to a nylon membrane. The blots were probed with 32P-labeled oligonucleotide probes complementary to precursors or mature forms of mt-tRNAs. The sequences of oligonucleotide probes used for Northern blots are listed in Table 1.

**Quantitative Real-time PCR (qRT-PCR) Measurement**—Total RNA from *S. pombe* cells was isolated using an E.Z.N.A.™ yeast RNA kit (Omega) according to the manufacturer’s instructions, followed by on-column DNase I treatment as described in the TransGen Biotech manual. The absence of genomic DNA was verified by PCR using primers specific for *ain1* (α-actinin). RNA quality was verified on a 1.2% agarose gel and spectrophotometric analysis using a NanoDrop 1000 instrument (Thermo Scientific). 1–2 μg of total RNA was reverse-transcribed using random hexamers and the Transcript First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech) in a 20-μl total volume. qRT-PCR was performed in triplicates on a StepOne real-time PCR system (Applied Biosystems). The primers listed in Table 2 were designed at the adjacent sides of the junctions of mitochondrial tRNA-mRNA and tRNA-rRNA. The PCRs were carried out in a total volume of 20 μl containing 200 nM primers, 2.5–5 ng of product from reverse transcription, 1× TransStart™ Eco Green qPCR SuperMix, and 1× Passive Reference Dye I. The mean threshold cycle number (Ct) of triplicate reactions was determined using StepOne v2.2.2 software. Relative-fold changes in the levels of precursor RNAs in temperature-sensitive trz1 or trz2 mutant cells relative to wild-type cells were calculated using the 2−ΔΔCt method, with normalization to act1 mRNA levels. All graphs were also plotted using Origin version 8.0 (OriginLab).

## Results

**Inactivation of trz1 Affects 3’-End Processing of Nuclear tRNAs**—We first wanted to examine whether trz1 is directly involved in 3’-end processing of nuclear tRNAs. Because trz1 is essential, we used a strain carrying a temperature-sensitive allele of trz1 (trz1-1) (39) to evaluate the role of trz1 in nuclear tRNA 3’-end processing. We examined the processing of two well-characterized nuclear tRNAs (tRNAVal<sup>BAC</sup> and tRNA<sup>Lys</sup>) in the trz1-1 and wild-type strains by Northern blotting using the intron-specific and mature oligonucleotide probes as described previously (5, 39, 42–45). These two tRNAs are encoded by the most abundant intron-containing tRNA genes in *S. pombe*, and thus, their processing intermediates can be easily detected. The intron-containing processing intermediates comprise a precursor tRNA with 5’- and 3’-extensions, a precursor with a mature 5’-end and 3’-extension, and a precursor with mature 5’- and 3’-ends. Because wild-type cells grew much more rapidly than trz1-1 cells even at permissive temperature, wild-type and mutant cells were cultured at the nonpermissive temperature of 37 °C for different periods of time. The A600 of wild-type cells at the 6-h time point was comparable to that of trz1-1 cells at the 10–12-h time point. trz1-1 samples from the later time points (after 10 h) could not be analyzed because trz1-1 cells showed a very substantial loss of viability after a longer incubation at nonpermissive temperature (39).

Comparison of the processing of tRNA<sup>Val</sup> and tRNA<sup>Lys</sup> in the wild-type and trz1-1 cells grown at 37 °C revealed an increased accumulation of a pre-tRNA species containing only a 3’-extension in the trz1-1 cells (Fig. 1), indicating that trz1 inactivation compromises nuclear tRNA 3’-end processing. These results parallel our previous findings (39) that overexpression of trz1 results in a reduced amount of a pre-tRNA species containing a 3’-extension. In contrast, inactivation of trz2 did not cause an increased accumulation of pre-tRNA species containing a mature 5’-end and a 3’-extension (data not shown). To determine whether the levels of mature tRNA<sup>Val</sup> and tRNA<sup>Lys</sup> were affected in the trz1-1 mutant, we reprobed the blots with oligonucleotides that detect the mature tRNA species. It appears that the levels of the mature species of these two tRNAs did not change appreciably in the trz1-1 mutant. We also examined the processing of intron-containing tRNA<sup>Lys</sup> and obtained similar results (data not shown).

**Inactivation of trz2 Affects 3’-End Processing of mt-tRNAs**—We next investigated whether trz2 is involved in mt-tRNA 3’-end processing. For this purpose, we used a temperature-sensitive mutant of trz2 (trz2-1) that permits conditional inactivation of trz2. This mutant strain harbors an Ala-to-Val substitution at position 623 of trz2 and was constructed as described under “Experimental Procedures.” trz2-1 cells grew very poorly in Edinburgh minimal medium at permissive temperature relative to the isogenic wild-type cells and did not grow at nonpermissive temperature. As shown in Fig. 2, the growth defect of trz2-1 cells could be complemented by wild-type trz2 expressed from plasmid pREP81X under the control of a weaker version of the thiamine-repressible nmt1 promoter (46). Full suppression of the temperature sensitivity could be achieved only when trz2 was expressed under repressed conditions. In contrast, trz2 expressed from the pREP4X vector under the control of a strong thiamine-repressible nmt1 promoter could not rescue the temperature sensitivity of trz2-1 (data not shown). These results are consistent with our previous findings that high level expression of trz2 is toxic to the cells.
These results also suggest that a low level of Trz2 activity may be sufficient to function in mt-tRNA maturation.

To examine whether conditional inactivation of \textit{trz2} affects \textit{3}/\textit{H11032}-end processing of mt-tRNAs, we performed Northern analysis of total RNA isolated from the \textit{trz2-1} mutant and its isogenic wild-type strain before and after a temperature shift from the permissive to nonpermissive temperature. The mtDNA of \textit{S. pombe} encodes 25 tRNA species, most of which are organized in six clusters interspersed between the protein-coding and rRNA genes. The intergenic spacers between mt-tRNA genes often vary in size and nucleotide sequence. We chose to examine the processing of mt-tRNA\textsubscript{UUU}\text{Lys}, mt-tRNA\textsubscript{UCG}\text{Arg}, and mt-tRNA\textsubscript{GUG}\text{His} because their processing intermediates significantly differ in length and thus could be easily distinguished by PAGE. We tested multiple probe sequences for each of these mt-tRNA precursors and mature tRNAs and found that the majority of oligonucleotide probes failed to give a detectable hybridization signal. The success of hybridization was highly dependent upon the position of the probe in the tRNA molecule.

Wild-type and \textit{trz2-1} mutant cells were cultured at the nonpermissive temperature of 37 °C for different periods of time because the mutant strain had a much lower growth rate than the wild-type strain even at permissive temperatures (Fig. 2).

We first examined the processing of the mt-tRNA\textsubscript{UUU}\text{Lys} precursor containing a long 3′-spacer (38 nucleotides (nt)). Unlike the
The probe PLys, which was designed to detect the tRNALys gene, but not a tRNA gene, is located near the 5'-end sequence (Fig. 3). The absence of the 5'-end sequence (data not shown). In addition to the monomeric mt-tRNALys and the 3'-end of the monomeric mt-tRNALys precursor containing the 3'-spacer sequence removed from the monomeric mt-tRNALys, we detected a 73-nt band that corresponded to the expected size of the mature form of mt-tRNALys. This band was also probed for U1 snRNA as a loading control (D). The identity of each tRNA species is shown on the right. M, single-stranded DNA markers in nucleotides. Black boxes indicate mt-tRNAs, and thick lines denote intergenic spacers. The positions of the probes are indicated by horizontal bars. Asterisks denote bands whose identities could not be determined unambiguously. These bands may be identified by, for example, RNA deep sequencing.

We next examined whether inactivation of trz2 also affect 3'-end processing of the mt-tRNAArg precursor with a relatively short 3'-spacer (9 nt). The lengths of the 5'- and 3'-spacer sequences of mt-tRNAArg are 111 and 9 nt, respectively. This size difference allows us to distinguish between processing intermediates with the 5'- or 3'-spacer sequence using gel electrophoresis. Using the PArg probe, which is specific for the mt-tRNAArg precursor with the 3'-spacer sequence, we found that accumulation of the monomeric mt-tRNAArg precursor containing the 3'-spacer sequence was increased in the wild-type strain but decreased in the trz2 mutant strain (Fig. 4). In contrast, trz2 inactivation resulted in a marked accumulation of two major bands, which potentially correspond in size to the 175-nt dimeric precursor of tRNAArg-tRNAIle containing the 3'-spacer sequence and the 285-nt dimeric precursor of tRNAArg containing the 3'-spacer sequence, respectively (Fig. 4). It is also notable that the length of the processing intermediates accumulated in the trz2 mutant tended to become progressively longer over time. As expected, the level of mature mt-tRNALys was not significantly affected by trz2-1 inactivation.

We next analyzed the processing of the mt-tRNAHis precursor, which is flanked by spacers of 20 and 38 nt at the 5'- and 3'-ends, respectively (Fig. 5). The PHis probe was designed to detect the precursor of mt-tRNAHis containing the 3'-spacer sequence. Using this probe, we found that, at the nonpermissive temperature, wild-type cells accumulated a weak band corre-
The trz2-1 Mutation Causes Increased Accumulation of Other Unprocessed mtRNAs beyond tRNAs—To examine the effect of the trz2-1 mutation on the processing of other mtRNAs, we measured the abundance of dicistronic precursors containing the 3′- spacer sequence after shifting to the nonpermissive temperature. With the \( M^{\text{His}} \) probe, which detects mature mt-tRNA\(^{\text{His}} \), we found that the level of mature mt-tRNA\(^{\text{His}} \) was dramatically reduced in the trz2-1 mutant strain after a shift to the nonpermissive temperature (Fig. 5). In contrast, inactivation of \( \text{trz1} \) had no effect on the processing of mt-tRNA\(^{\text{His}} \), mt-tRNA\(^{\text{Arg}} \), and mt-tRNA\(^{\text{Lys}} \) (data not shown).

DISCUSSION

Previous studies from our laboratory have shown that \( S. \text{pombe} \) contains two tRNase Z’s, one located in the nucleus and the other in the mitochondria (38, 39). These two proteins can remove the 3′-extension from a T7 RNA polymerase-synthesized nuclear pre-tRNA \( \text{in vitro} \) (38). However, it is unclear that Trz2 can process mitochondrial pre-tRNAs because mt-tRNAs are very different from nuclear tRNAs in sequence and structure. Furthermore, there is no direct \textit{in vivo} evidence that Trz1 and Trz2 are involved in nuclear and mitochondrial tRNA 3′- end processing, respectively.

In this study, we examined the roles of Trz1 and Trz2 in the processing of nuclear and mitochondrial pre-tRNAs \( \text{in vivo} \). We have demonstrated that inactivation of \( \text{trz1} \) and \( \text{trz2} \) impairs nuclear and mitochondrial tRNA 3′-end processing \( \text{in vivo} \), respectively. In addition, we have shown that inactivation of \( \text{trz2} \), but not \( \text{trz1} \), affects the maturation of the 5′-end of mitochondrial mRNAs and rRNAs. Thus, in fission yeast, the nuclear and mitochondrial tRNase Z functions are partitioned between two different proteins.

It is noteworthy that the processing intermediates for different mt-tRNAs accumulated to different levels in the wild-type and tRNase Z\(^{\text{lt}} \) mutant strains. This presumably reflects a complex interplay among transcription, processing, and degradation, which varies for different mt-tRNAs.

Inactivation of tRNase Z\(^{\text{lt}} \) would be expected to cause an increase in the level of precursor species containing a 3′- spacer sequence and a concomitant reduction in the level of mature species. Indeed, the levels of mature mt-tRNA\(^{\text{Arg}} \) and mt-tRNA\(^{\text{Lys}} \) were dramatically reduced upon inactivation of trz2.
However, although the two nuclear tRNAs examined and mitochondrial tRNA exhibited defects in 3'-end processing in response to tRNase Z inactivation, the production of their mature species was not detectably affected upon tRNase Z inactivation. There are two possible explanations, not mutually exclusive, for why the levels of these mature tRNAs were unchanged. The first explanation is that tRNase Z activity is not completely abolished at the nonpermissive temperature, and low levels of residual tRNase Z activity may be sufficient for tRNA 3'-end processing. The second explanation is that these tRNAs may have slower decay rates. It should be noted that the S. pombe genome encodes 171 nuclear tRNAs. Although the levels of the mature nuclear tRNAs we examined seemed to be unchanged, it is likely that the levels of other mature nuclear tRNAs not examined here could have been reduced. Although these results are unexpected, similar results have been observed in tRNase Z-deleted Drosophila. When tRNase Z was knocked down in Drosophila, the levels of all five mature nuclear tRNAs examined and one mature mt-tRNA were not significantly altered even though the levels of the corresponding pre-tRNAs with a 3'-extension increased considerably (36).

Eukaryotic organisms have developed many mechanisms for targeting tRNase Z to different cellular compartments. Metazoans appear to use alternative translation initiation sites. For example, in humans, the mitochondrial and nuclear forms of tRNase Z can be generated from a single gene by the alternative use of two translation start codons (31, 35). Because the first start codon of the human tRNase Z gene is in a suboptimal context, some ribosomes would bypass it to initiate translation at a downstream AUG codon, thus generating essentially two identical tRNase Z proteins, one mitochondrial and one nuclear. In contrast, in Caenorhabditis nematodes, the nuclear and mitochondrial forms of tRNase Z are likely generated by alternative splicing of the tRNase Z gene (35, 47).

Our results provide the first example that S. pombe (and most likely, other fission yeast species) achieves dual localization of tRNase Z to the nucleus and mitochondria by the use of two genes, which is the simplest and basic mechanism of dual targeting. This mechanism also allows for greater flexibility in the control of differential expression of similar genes. Indeed, unlike Trz1, Trz2 seems to be present in very low abundance in cells (it ranks in the bottom 5% of 3521 proteins) and is ~19-fold less abundant than Trz1 (48). These data indicate that trz1 and trz2 are differentially expressed and that different amounts of tRNase Z proteins are required for nuclear and mitochondrial tRNA 3'-end processing in S. pombe. It seems likely that the differential expression of the nuclear and mitochondrial tRNase Z genes may be required to avoid the off-target effects of trz2 in S. pombe because the increased expression of trz2 leads to lethality in a dose-dependent manner (38). Efforts are under way to elucidate the mechanisms involved in differential regulation of these two tRNase Z genes. Because the nuclear and mitochondrial tRNase Z activities reside in two different genes in fission yeast (unlike in the majority of organisms examined to date), fission yeast may provide opportunities for focused studies that separate the nuclear and mitochondrial functions of tRNase Z.

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