Mutations in the anticodon stem of tRNA cause accumulation and Met22-dependent decay of pre-tRNA in yeast

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ABSTRACT

During tRNA maturation in yeast, aberrant pre-tRNAs are targeted for 3'–5' degradation by the nuclear surveillance pathway, and aberrant mature tRNAs are targeted for 5'–3' degradation by the rapid tRNA decay (RTD) pathway. RTD is catalyzed by the 5'–3' exonucleases Xrn1 and Rat1, which act on tRNAs with an exposed 5' end due to the lack of certain body modifications or the presence of destabilizing mutations in the acceptor stem, T-stem, or tRNA fold. RTD is inhibited by mutation of *MET22*, likely due to accumulation of the Met22 substrate adenosine 3',5' bis-phosphate, which inhibits 5'–3' exonucleases. Here we provide evidence for a new tRNA quality control pathway in which intron-containing pre-tRNAs with destabilizing mutations in the anticodon stem are targeted for Met22-dependent pre-tRNA decay (MPD). Multiple *SUP4_{oc}* anticodon stem variants that are subject to MPD each perturb the bulge-helix-bulge structure formed by the anticodon stem–loop and intron, which is important for splicing, resulting in substantial accumulation of end-matured unspliced pre-tRNA as well as pre-tRNA decay. Mutations that restore exon–intron structure commensurately reduce pre-tRNA accumulation and MPD. The MPD pathway can contribute substantially to decay of anticodon stem variants, since pre-tRNA decay is largely suppressed by removal of the intron or by restoration of exon–intron structure, each also resulting in increased tRNA levels. The MPD pathway is general as it extends to variants of tRNA^{Tyr(GUA)} and tRNA^{Ser(CGA)}. These results demonstrate that the integrity of the anticodon stem–loop and the efficiency of tRNA splicing are monitored by a quality control pathway.

Keywords: rapid tRNA decay; pre-tRNA; tRNA splicing; *S. cerevisiae*; MPD

INTRODUCTION

In all organisms, the central role of tRNAs in decoding mRNA during translation places restrictions on the properties of tRNA. tRNAs must be selective in charging by their cognate synthetase (Giege et al. 1998; Fechter et al. 2000; Kermgard et al. 2017), and in their anticodon–codon interactions during decoding (Murphy et al. 2004; Agris et al. 2007; Demeshkina et al. 2012; Rozov et al. 2016; Loveland et al. 2017); they must adopt a conserved structure to uniformly participate in translation, with sufficient flexibility for accommodation and passage through the ribosome (Kim et al. 1974; Marck and Grosjean 2002; Valle et al. 2003; Schmeing et al. 2009; Giege et al. 2012; Ramrath et al. 2013; Zhou et al. 2013, 2014); and they must be stable enough to have long half-lives, enabling participation in multiple rounds of translation (Kadaba

et al. 2004; Alexandrov et al. 2006; Chernyakov et al. 2008; Gudipati et al. 2012).

tRNAs undergo a highly complex maturation process. In the yeast *Saccharomyces cerevisiae* and other eukaryotes, tRNAs are transcribed by Pol III as pre-tRNAs with a 5' leader, a 3' trailer, and in some cases an intron, all of which must be removed before the tRNA can participate in translation (Hopper 2013). Although introns only occur in a subset of tRNAs in different organisms, they are found ubiquitously in eukaryotes, and tRNA splicing is essential in all eukaryotes examined. In vertebrates and higher eukaryotes, the end trimming and splicing steps are completed in the nucleus (Nishikura and De Robertis 1981; Lund and Dahlberg 1998). However, in *S. cerevisiae* and *Schizosaccharomyces pombe*, end-processed intron-containing pre-tRNAs are

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exported from the nucleus after end trimming and then spliced in the cytoplasm, initiated by the splicing endonuclease (SEN) complex on the surface of the mitochondria (Trotta et al. 1997; Yoshihisa et al. 2003, 2007; Wan and Hopper 2018). In addition, an extensive set of modifications are added to the tRNA throughout the course of processing (Etcheverry et al. 1979; Peebles et al. 1979; Nishikura and De Robertis 1981), including a final set of modifications that occur in the anticodon loop after splicing, after the subsequent retrograde import of spliced tRNAs into the nucleus, or after re-export of the spliced tRNAs back to the cytoplasm (Shaheen and Hopper 2005; Takano et al. 2005; Murthi et al. 2010; Ohira and Suzuki 2011).

The necessity for proper tRNA maturation and function is evident from the numerous human diseases associated with incorrectly processed tRNAs. Hypomodification of tRNAs, caused by mutation of either the modifying enzyme or the tRNA itself, can result in numerous mitochondrial and neurological diseases (Yarham et al. 2010, 2014; Suzuki et al. 2011; Gillis et al. 2014; Karlsborn et al. 2014; Torres et al. 2014; Guy et al. 2015; Shaheen et al. 2016; de Brouwer et al. 2018); mutations in the human SEN complex responsible for the cleavage step of tRNA splicing can cause pontocerebellar hypoplasia and neurodegeneration (Budde et al. 2008; Bierhals et al. 2013; Breuss et al. 2016); and mutations in the aminoacyl tRNA synthetases can cause a host of neurological diseases (Lee et al. 2006; Boczonadi et al. 2018).

In *S. cerevisiae*, the fidelity of tRNA maturation is monitored both as pre-tRNA, by the nuclear surveillance pathway, and as mature tRNA, by the rapid tRNA decay (RTD) pathway. The nuclear surveillance pathway acts through the TRAMP complex and the nuclear exosome to degrade pre-tRNA_i^{Met} lacking the 1-methyladenosine (m¹A) modification (Kadaba et al. 2004, 2006; LaCava et al. 2005; Vaňáčová et al. 2005), and may also degrade as much as half of the total transcribed pre-tRNA, likely due to transcription errors or folding defects (Gudipati et al. 2012). After maturation, tRNAs are subject to quality control by the RTD pathway, which degrades certain hypomodified tRNAs and tRNAs with destabilizing mutations, using the 5′–3′ exonucleases Rat1 in the nucleus, and Xrn1 in the cytoplasm (Alexandrov et al. 2006; Chernyakov et al. 2008; Whipple et al. 2011; Dewe et al. 2012; Kramer and Hopper 2013; Guy et al. 2014; Payea et al. 2018). The RTD pathway targets tRNAs due to exposure of the 5′ end arising from reduced stability of the acceptor/T-stem, as shown by both structure probing and Xrn1 assay of hypomodified tRNA^{Ser(CGA)} [tS(CGA)] variants, and by Xrn1 assay of hypomodified tV(AAC) (Whipple et al. 2011). The degradation of all identified substrates of the RTD pathway is inhibited genetically and biochemically by a *met22Δ* mutation (Chernyakov et al. 2008; Dewe et al. 2012; Guy et al. 2014; Payea et al. 2018). The sup-

pression of RTD by a *met22Δ* mutation is likely an indirect effect due to elevated levels of the Met22 substrate adenosine 3′, 5′ bisphosphate (pAp) (Murguía et al. 1996), which inhibits both Xrn1 and Rat1 in vitro and in vivo (Dichtl et al. 1997; Yun et al. 2018). There is also evidence that the RTD pathway may be conserved in humans, since Xrn1 and the human Rat1 ortholog Xrn2 can mediate the decay of tRNA_i^{Met} after heat shock (Watanabe et al. 2013).

As the RTD pathway targets substrates due to 5′ end exposure (Whipple et al. 2011), it was surprising to find from high-throughput analysis of variants of the suppressor *SUP4_{oc}* (tY(GUA) with a UUA anticodon) that *met22Δ* mutants prevented the decay of numerous variants with destabilizing mutations in the anticodon stem (Guy et al. 2014; Payea et al. 2018). Although the Met22-dependent decay of anticodon stem variants implicated the RTD pathway, anticodon stem mutations were not expected to significantly destabilize the 5′ end to trigger RTD, as there is little interaction of nucleotides in this stem with other parts of the tRNA body, other than limited stacking of the anticodon stem with the D-stem (Ladner et al. 1975; Westhof et al. 1985).

Here we describe a new Met22-dependent pre-tRNA decay pathway that, unlike RTD, targets end-matured unspliced pre-tRNA variants with mutations in the anticodon stem–loop that perturb exon–intron structure. We first show that the Met22-dependent decay of anticodon stem *SUP4_{oc}* variants is quantitatively equivalent to that of acceptor stem variants in vivo, despite their reduced sensitivity to 5′–3′ exonucleases in vitro. We explain this discrepancy by showing that these anticodon stem variants have a defect in pre-tRNA maturation, which causes an accumulation of end-matured unspliced pre-tRNA that is targeted for decay. We show that this Met22-dependent pre-tRNA decay (MPD) is due to the perturbed secondary structure in the region comprising the anticodon stem–loop and the intron, and that MPD contributes significantly to overall decay of anticodon stem variants. We further find that MPD is a general pathway of quality control for pre-tRNA since it also occurs in tY(GUA) and tS(CGA) variants. These results document for the first time that the tRNA exon–intron structure is subject to quality control in yeast. As introns are found in many tRNAs throughout eukaryotes, MPD may be conserved in higher eukaryotes as well.

RESULTS

Anticodon stem variants are susceptible to Met22-dependent tRNA decay in vivo, but not to RTD exonucleases in vitro

Because of our finding that *SUP4_{oc}* anticodon stem variants could provoke Met22-dependent tRNA decay in the absence of known mechanisms by which they could significantly destabilize the tRNA 5′ end (Guy et al. 2014), we

quantitatively compared the decay of anticodon stem and acceptor stem *SUP4_{oc}* variant substrates. We examined the tRNA levels of biological triplicates for three acceptor stem variants, seven anticodon stem variants, and one anticodon loop variant (Fig. 1A). We used poison primer extension to directly compare mature tRNA levels of *SUP4_{oc}* variants as a percentage of endogenous tY(GUA) in a *MET22⁺* strain and in a *met22Δ* strain, in which the 5'–3' exonucleases of the RTD pathway are inhibited, and then calculated the ratio of these two values (*met22Δ*/*MET22⁺*) to obtain a tRNA decay ratio, previously called the RTD ratio (Guy et al. 2014; Payea et al. 2018).

Consistent with previous findings, we found that a *met22Δ* mutation resulted in a large quantitative increase in the tRNA levels for all 11 tested variants, and thus a substantial tRNA decay ratio (Fig. 1B,C; Supplemental Fig. S1). Seven variants had a tRNA decay ratio greater than four (C₅U, A₂₉U, G₃₀A, C₄₀U, U₄₁A, U₄₂A, G₆₉C), and four had a ratio between two and four (U₂C, A₂₈U, A₃₁U, C₃₂A), indicating that they were subject to substantial Met22-dependent decay. Moreover, the average tRNA decay ratio of 5.4 for anticodon stem variants was at least as large as the average decay ratio of 4.4 for acceptor stem variants. This finding emphasizes the unusual nature of anticodon stem substrates, in that despite their predicted inability to substantially increase the exposure of the 5' end to exonucleases, they are subject to Met22-dependent decay in vivo as effectively as mutations in the acceptor stem.

To directly determine if the similar in vivo tRNA decay ratios of anticodon stem variants and acceptor stem variants were due to similar 5' end accessibility, we measured 5'–3' exonuclease activity in vitro on a set of *SUP4_{oc}* variants, as we had previously done with tS(CGA) variants and Xrn1 (Whipple et al. 2011). To assay decay, we used a biotinylated oligomer to purify *SUP4_{oc}* variants together with endogenous tY(GUA), incubated the tRNAs with purified preparations of Xrn1 or Rat1/Rai1 complex (Xue et al. 2000; Xiang et al. 2009), and assayed decay by poison primer extension, using the endogenous purified tY(GUA) as a control (Payea et al. 2015).

With Xrn1, we found that the U₂C acceptor stem variant was highly susceptible to decay at 37°C, whereas the A₂₉U and A₃₁U anticodon stem variants were nearly as resistant as the negative control *SUP4_{oc}* (Fig. 1D,E; Supplemental Fig. S2A). This assay was done at 37°C, as many known RTD substrates are more sensitive to decay in vivo at high temperature (Chernyakov et al. 2008; Payea et al. 2018). At 30°C, at which tRNAs are expected to be less prone to decay, we still observed substantial sensitivity of the U₂C variant to Xrn1, whereas the anticodon stem variants were both highly resistant (Supplemental Fig. S2B–D).

We performed similar experiments with the purified Rat1/Rai1 complex at 37°C and again observed that anticodon stem variants were more resistant to decay than ac-

ceptor stem variants. The *SUP4_{oc}* acceptor stem U₂C and C₅U variants were highly susceptible to decay over a range of Rat1/Rai1 concentrations, whereas the A₃₁U anticodon stem variant was highly resistant, and the A₂₉U and C₄₀U anticodon stem variants were moderately resistant (Fig. 1F; Supplemental Figs. S3, S4).

The finding that anticodon stem variants were uniformly more resistant in vitro to 5'–3' exonucleases than acceptor stem variants is consistent with our expectation that a destabilized anticodon stem would have a limited effect on 5' end exposure, but is seemingly contradictory to our in vivo data showing that anticodon stem and acceptor stem variants were similarly sensitive to Met22-dependent tRNA decay. The discrepancy between our in vitro and in vivo data implied that the degradation of anticodon stem variants might have an additional aspect in vivo that we were not able to account for in vitro.

Anticodon stem variants accumulate pre-tRNA that is targeted for decay

We considered that the tRNA decay ratio of anticodon stem variants measured in vivo might actually be the sum of conventional decay of mature tRNA by the RTD pathway and decay of the pre-tRNA by a separate Met22-dependent pathway. As it was previously shown that anticodon stem variants of both tY(GUA) and tS(CGA) could impair tRNA maturation (Nishikura et al. 1982; Yoo and Wolin 1997), it seemed reasonable that *SUP4_{oc}* anticodon stem variants might accumulate pre-tRNA that was subject to decay.

To determine if the pre-tRNAs of anticodon stem variants were also subject to Met22-dependent decay, we used poison primer extension to look for increased pre-tRNA in a *met22Δ* strain relative to a *MET22⁺* strain. The primer for this experiment extended from N₅₁ in the 3' tRNA exon through to the most 5' residue of the intron (In₁), and would detect any of the unspliced precursors in the tRNA maturation pathway (Fig. 2A).

We found evidence for substantial Met22-dependent decay of pre-tRNA in our set of anticodon stem variants. Initial experiments showed that for both the *SUP4_{oc}* A₂₉U and U₄₁A anticodon stem variants in a *MET22⁺* strain, there was a large accumulation of pre-tRNA [107% and 66.7% of the endogenous pre-tY(GUA)] relative to that for wild type (WT) pre-*SUP4_{oc}* (14.7%), an increase of 7.3-fold and 4.5-fold, respectively. Moreover, the pre-tRNA levels of the *SUP4_{oc}* A₂₉U and U₄₁A anticodon stem variants were further increased in a *met22Δ* strain (16.2-fold and 8.6-fold, relative to WT pre-*SUP4_{oc}*), resulting in pre-tRNA decay ratios (pre-tRNA in *met22Δ*/pre-tRNA in *MET22⁺*) of 2.3 and 1.9, respectively (Fig. 2B). In contrast, although there was a modest relative accumulation of pre-tRNA for the *SUP4_{oc}* C₅U acceptor stem variant (2.4-fold),

FIGURE 1. The tRNA decay ratio of anticodon stem *SUP4_{oc}* variants is quantitatively similar to that of acceptor stem variants. (A) Schematic of *SUP4_{oc}* secondary structure and variants examined. The secondary structure of tY(GUA) is shown, along with the G₃₄U ochre mutation of *SUP4_{oc}* (purple); variants examined (red); and the P7 primer used for poison primer extension analysis of mature tRNA (blue line). (B) Poison primer extension analysis of *SUP4_{oc}* anticodon stem and acceptor stem variants. Strains containing an integrated copy of a *SUP4_{oc}* tRNA variant as indicated were grown at 28°C to mid-log phase, and bulk RNA was analyzed by poison primer extension with the P7 primer (complementary to nt 62–43) in the presence of ddCTP, producing a stop at G₃₄ for endogenous tY(GUA) (blue arrow) and at G₃₀ for integrated *SUP4_{oc}* variants (red arrow), visualized after electrophoresis. A sequencing ladder is shown at the left. To confirm that all the measured G₃₀ signal is from *SUP4_{oc}* variants, bulk RNA was also analyzed from a strain containing no *SUP4_{oc}* variant (lane a) and an intronless *SUP4_{oc}* variant (lane b). *SUP4_{oc}* variants were quantified as a percentage of the endogenous tY(GUA) (% tY), and an average value was determined for each *SUP4_{oc}* variant from the triplicate samples in each genetic background (Avg. % tY). As detailed in Materials and Methods, data obtained from variants measured at the same time as their controls are highly consistent. (C) *SUP4_{oc}* anticodon stem and acceptor stem variants have increased tRNA levels in *met22Δ* relative to *MET22⁺*. Bar chart depicting average % tY values and tRNA decay ratios with associated standard deviations for variants analyzed. tRNA levels are indicated by diagonal hatching for *MET22⁺* (green); and for *met22Δ* (blue); tRNA decay ratios are indicated by red diagonal hatching. For *SUP4_{oc}*, *n* = 12 derived from four different measurements of biological triplicates analyzed on different days; for all other variants, *n* = 3 for biological triplicates analyzed in the same experiment on the same day. The depicted error for tRNA decay ratios represents propagated error for the quotient of *met22Δ*/*MET22⁺* average % tY values. The red asterisks above the G₃₀A data for *MET22⁺* levels and tRNA decay ratio indicate that tRNA levels in *MET22⁺* were at or below background, and therefore those values could not be accurately measured. (D) Analysis of in vitro degradation of purified *SUP4_{oc}* variants by purified Xrn1 at 37°C. Purified mixtures of *SUP4_{oc}* variants and tY(GUA) were incubated with purified Xrn1 at the indicated concentrations at 37°C for 20 min. Reaction products were analyzed by measuring the remaining *SUP4_{oc}* variant tRNA relative to tY(GUA) by using poison primer extension analysis with primer P5 (nt 57–37) in the presence of ddCTP, producing a stop at G₃₄ for endogenous tY(GUA) (blue arrow) and at G₃₀ for *SUP4_{oc}* variants (red arrow). (E) Anticodon stem variants are more resistant than an acceptor stem variant to in vitro degradation by Xrn1 at 37°C. The chart shows the quantification of poison primer extensions used in the analysis of in vitro digests of *SUP4_{oc}* variants with purified Xrn1 at 37°C, performed at two different concentrations (*n* = 2, replicates assayed on different days). (F) Anticodon stem variants are more resistant than acceptor stem variants to in vitro degradation by Rat1/Rai1 at 37°C. The bar chart shows the quantification of poison primer extension analysis of in vitro digests of purified *SUP4_{oc}* variants with purified Rat1/Rai1 complex at 37°C, performed at three different concentrations as indicated, with two to four replicates as indicated, conducted on different days.

FIGURE 2. SUP4_{oc} anticodon stem variants accumulate pre-tRNA that is subject to Met22-dependent decay. (A) Schematic of unspliced tRNA precursors during biogenesis of tY(GUA). The initial tY(GUA) transcript is depicted with a 5' leader (orange), 3' trailer (purple), anticodon (red), and intron (green). The primer MP 497 (51-In₁) used for measurement of pre-tRNA is indicated by a blue line, with the black dot denoting the probe start. Maturation of pre-tY(GUA) in cells proceeds from the initial transcript (left), to the 3' extended species (center), and then to the end-matured species (right). Cartoon depictions of each respective pre-tRNA species are above. (B) Poison primer extension analysis of unspliced pre-tRNA levels for SUP4_{oc} variants. Bulk RNA from strains with integrated SUP4_{oc} variants was analyzed for pre-tRNA levels by poison primer extension as in Figure 1B, using primer MP 497 (51-In₁) in the presence of ddCTP, producing a stop at G₃₄ for the endogenous pre-tY(GUA) (blue arrow) and at G₃₀ for pre-SUP4_{oc} variants (red arrow). To confirm that all the measured G₃₀ signal is from SUP4_{oc} pre-tRNA, bulk RNA was also analyzed from a strain containing no SUP4_{oc} variant (lane a) and an intronless SUP4_{oc} variant (lane b). (C) The pre-tRNA of SUP4_{oc} anticodon stem variants is subject to Met22-dependent pre-tRNA decay. Bar chart depicting average % pre-tY(GUA) values and pre-tRNA decay ratios with associated standard deviations for each of the analyzed variants; MET22⁺, green; met22Δ, blue; pre-tRNA decay ratio, red. For SUP4_{oc}, n = 12 derived from four different sets of biological triplicates analyzed at separate times; for all other variants n = 3. (D) The end-matured unspliced pre-tRNA species of SUP4_{oc} anticodon variants is targeted for decay in a MET22⁺ strain and accumulates in a met22Δ strain. Northern blot analysis of bulk RNA resolved on a 10% polyacrylamide 7 M urea gel and hybridized with radiolabeled probes indicated at the left; lanes represent biological triplicates of indicated strains with integrated SUP4_{oc} A29U. The 5S rRNA is used as a loading control.

there was only a minor further increase in a met22Δ strain, resulting in a pre-tRNA decay ratio of 1.1.

These results extended to all SUP4_{oc} variants we examined. Each of the five other tested anticodon stem variants also had significant relative accumulation of pre-tRNA in MET22⁺ strains (2.1-fold or more), and in each case this was further increased in a met22Δ strain, resulting in pre-tRNA decay ratios ranging from 1.6 to 5.9 (Fig. 2C; Supplemental Fig. S5). In contrast, the SUP4_{oc} acceptor stem variants U₂C and G₆₉C showed no substantial accumulation of pre-tRNA and no significant pre-tRNA decay ratio. We note that for the SUP4_{oc} C₄₀U, U₄₁A, and U₄₂A variants, the pre-tRNA levels estimated by poison primer extension may be biased downward since the primer en-

compasses this region and is complementary to WT, and the amount of pre-tRNA is benchmarked to endogenous pre-tY. Thus, the increase in pre-tRNA levels in these variants is, if anything, an underestimate. However, the pre-tRNA decay ratios for these variants are accurate as the pre-tRNA accumulation was examined in the MET22⁺ and met22Δ strains at the same time, mitigating the importance of any differences in hybridization efficiency to these SUP4_{oc} variants and endogenous tY(GUA).

Since our primer extension assay cannot distinguish between the three discrete pre-tRNA species that exist during processing (Fig. 2A), we further analyzed several variants by northern analysis. Our results showed that the end-matured unspliced pre-tRNA species of SUP4_{oc} A29U

had substantially increased levels in the *met22Δ* strain compared to the *MET22⁺* strain, whereas the levels of the other pre-tRNA species were unchanged in the two strains, indicating that the end-matured unspliced pre-tRNA species was the target for the Met22-dependent decay (Fig. 2D). We observed the same results for the *SUP4_{oc}* G₃₀A and A₃₁U anticodon stem variants (Supplemental Fig. S6). Thus, it is a general property that *SUP4_{oc}* anticodon stem variants subject to Met22-dependent tRNA decay have substantially increased levels of end-matured unspliced pre-tRNA in *MET22⁺* and *met22Δ* strains, and have substantial pre-tRNA decay ratios, now referred to as Met22-dependent pre-tRNA decay (MPD). This finding offers a simple explanation for the discrepancy between the similar tRNA decay ratios of anticodon stem and acceptor stem variants in vivo, and the relative resistance of anticodon stem variants to 5′–3′ exonucleases in vitro, since only mature tRNA decay was accounted for in our in vitro assays.

Destabilization of anticodon-intron base-pairing causes pre-tRNA accumulation and decay

The large accumulation of end-matured unspliced pre-tRNA associated with MPD of *SUP4_{oc}* anticodon stem variants suggested that perturbation of the pre-tRNA structure might in some way inhibit maturation to trigger decay. In intron-containing pre-tRNAs, the anticodon forms part of a central helix with residues in the intron, surrounded by two bulges that comprise a bulge-helix-bulge region, which is recognized and cleaved by the SEN complex in the first step of splicing (Ogden et al. 1984; Swerdlow and Guthrie 1984; Lee and Knapp 1985; Fruscoloni et al. 2001; Xue et al. 2006). This characteristic pre-tRNA structure of the anticodon stem–loop and intron is predicted to be relatively unstable in pre-tY(GUA) (Reuter and Mathews 2010), which has only four base pairs in the central helix between the anticodon region and the intron (Fig. 3A); moreover, the G₃₄U *ochre* mutation of *SUP4_{oc}* is predicted to destabilize the anticodon–intron pairing by disrupting the normal G₃₄ pairing with intron residue In₁₁, and to alter the pre-tRNA structure in the region. Thus, it seemed plausible that the G₃₄U *ochre* mutation could be contributing to the accumulation of *SUP4_{oc}* pre-tRNAs observed in some of the anticodon stem RTD variants.

We tested the importance of exon–intron structure in pre-tRNA decay of three *SUP4_{oc}* variants (A₂₉U, G₃₀A, and A₃₁U) by constructing tY(GUA) derivatives, bearing a native GUA anticodon instead of the G₃₄U mutation in *SUP4_{oc}*, and comparing pre-tRNA levels and pre-tRNA decay ratios to the corresponding *SUP4_{oc}* variants by poison primer extension. The pre-tRNA decay ratios were dramatically reduced in the tY(GUA) A₂₉U and A₃₁U anticodon stem variants compared to the corresponding *SUP4_{oc}* variants, from 2.4 to 1.3 for the A₂₉U variant, and from 2.0 to

1.1 for the A₃₁U variant (Fig. 3B,C; Supplemental Fig. S7A). Moreover, pre-tRNA accumulation in the *met22Δ* strain was also dramatically reduced in the tY(GUA) variants compared to the *SUP4_{oc}* variants, 3.3-fold for the A₂₉U variant [from 146% to 44% of pre-tY(GUA)] and 4.1 fold in the A₃₁U variant. Furthermore, for these two variants, pre-tRNA decay comprises a major component of their overall tRNA decay, since the dramatic reduction in pre-tRNA decay ratios for the tY(GUA) A₂₉U and A₃₁U variants (relative to the corresponding *SUP4_{oc}* variants) was matched by a similar reduction in their tRNA decay ratios (Fig. 3D; Supplemental Fig. S8).

To further examine the extent that perturbation of the pre-tRNA structure could provoke MPD, we generated a variant we called *SUP4_{CAC}*, in which the anticodon of tY(GUA) was mutated to prevent any substantial pairing between the intron and exon, but the tRNA was otherwise WT. We found that *SUP4_{CAC}* significantly accumulated pre-tRNA in a *MET22⁺* strain (26.9% of endogenous pre-tY, compared to 16.4% for *SUP4_{oc}*) and further accumulated pre-tRNA in a *met22Δ* strain, leading to a pre-tRNA decay ratio of 1.6 (Supplemental Fig. S9), similar to the ratios obtained for the *SUP4_{oc}* A₂₈U and U₄₂A anticodon stem variants. This result, and the results from the tY(GUA) A₂₉U and A₃₁U variants, are consistent with a model in which pre-tRNA decay is driven by pre-tRNA accumulation, which is in turn due to perturbation of the pre-tRNA structure that is important for splicing.

In contrast to the substantial reduction of pre-tRNA decay in the tY(GUA) A₂₉U and A₃₁U variants relative to the corresponding *SUP4_{oc}* variants, the pre-tRNA decay ratio was only modestly reduced for the tY(GUA) G₃₀A variant, compared to the *SUP4_{oc}* G₃₀A variant (from 3.3 to 2.4), and the pre-tRNA accumulation was only slightly reduced in the *met22Δ* strain for the tY(GUA) G₃₀A variant, relative to the *SUP4_{oc}* G₃₀A variant [1.2-fold, from 148% to 124% tY(GUA)] (Fig. 3C; Supplemental Fig. S7B). The persistence of the G₃₀A mutation in causing substantial pre-tRNA decay and pre-tRNA accumulation with either a native GUA anticodon or an *ochre* anticodon is consistent with the greater predicted disruption in pre-tRNA structure caused by the G₃₀A mutation, compared to either the A₂₉U or A₃₁U mutations (Reuter and Mathews 2010). This result also emphasizes that substantial amounts of pre-tRNA decay can occur in anticodon stem tY(GUA) variants with a natural GUA anticodon, accompanied by comparably large accumulations of pre-tRNA in the *met22Δ* strain, and large tRNA decay ratios (Supplemental Fig. S10).

We further tested the importance of anticodon–intron pairing in tRNA decay by making a compensatory mutation at In₁₁ to restore pairing with residue U₃₄ of *SUP4_{oc}* (making a *SUP4_{oc}* variant denoted as *SUP4_{oc}* In^{oc}), albeit with a U–A pair instead of the native G–C pair (Fig. 3A). We found that the resulting *SUP4_{oc}* In^{oc} A₂₉U and *SUP4_{oc}* In^{oc} A₃₁U variants each had the anticipated reduction in

FIGURE 3. Anticodon-intron base-pairing has a substantial effect on pre-tRNA accumulation and Met22-dependent pre-tRNA decay. (A) Schematic of the structure of the anticodon stem-loop and intron of pre-tY(GUA). Nucleotides N₂₇–N₄₃ of the anticodon stem-loop are shown, as well as the intron (residues In₁ to In₁₄) arranged in the bulge-helix-bulge secondary structure common to substrates of the SEN complex. Anticodon, red; intron, green. The G₃₄U mutation of SUP_{4oc} is indicated in red. (B) Analysis of Met22-dependent pre-tRNA decay of the SUP_{4oc} A₃₁U and tY(GUA) A₃₁U variants. Variants as indicated were analyzed for pre-tRNA levels by poison primer extension as described in Figure 2B. Primer MP 497 (51-In₁) and ddCTP were used for the analysis of SUP_{4oc} variants, and Primer MP 538 (49-[In]-34) and ddTTP were used for analysis of tY(GUA) variants, producing stops at A₃₁ for endogenous pre-tY(GUA) (green dot) and at A₂₉ for SUP_{4oc} A₃₁U pre-tRNA (purple dot). (C) Restoration of anticodon-intron base-pairing suppresses MPD for the SUP_{4oc} A₂₉U and A₃₁U variants, but not the SUP_{4oc} G₃₀A variant. Bar chart of % pre-tY levels and pre-tRNA decay ratios for variants that have either mismatches or pairing between N₃₄ and In₁₁ as indicated in the Anticodon (5'-3') and Intron (3'-5') rows; no pairing, red text and no dash; pairing, green text with dash. (D) The suppression of MPD by the restoration of anticodon-intron pairing also suppresses tRNA decay ratios. Bar graph of % tY levels and tRNA decay ratios for variants as indicated. Symbols and pairings as in C. The red asterisks above the G₃₀A data for MET22⁺ levels and tRNA decay ratio indicate that tRNA levels in MET22⁺ were at or below background, and therefore those values could not be accurately measured.

tRNA decay ratio relative to the corresponding SUP_{4oc} A₂₉U and A₃₁U variants, but not as much of a reduction as in the corresponding tY(GUA) variants (Fig. 3D; Supplemental Fig. S8). Presumably, the reduced tRNA decay ratio reflects reduced pre-tRNA decay; however, we note that we could not accurately compare pre-tRNA accumulation or pre-tRNA decay ratios of these variants to the corresponding SUP_{4oc} and tY(GUA) variants, as the In₁₁ mutation could affect the hybridization of the primer used for these measurements.

Removal of the intron suppresses tRNA decay

To quantitatively assess the importance of the intron in decay of SUP_{4oc} and tY(GUA) anticodon stem variants subject to MPD, we compared tRNA decay with and without the

intron. However, since the intron of tY(GUA) is known to be required for pseudouridine (Ψ) modification of U₃₅ (Urban et al. 2009), we performed these comparisons in a *pus7Δ* strain, in which both the intron-containing and intronless variants would lack Ψ₃₅, enabling a more accurate comparison of the effect of intron removal.

Our results show that most of the decay of the SUP_{4oc} A₂₉U and A₃₁U variant is due to MPD, provoked by the presence of both the intron and the G₃₄U mutation of SUP_{4oc}. Thus, the tRNA decay ratio of SUP_{4oc} A₂₉U is substantially reduced by intron removal, falling from 7.3 to 2.9, and the tRNA decay ratio is further reduced (to 1.7) in an intronless tY(GUA) A₂₉U variant (Fig. 4A,B; Supplemental Fig. S11A). We infer that the increased access of Met22-dependent nucleases to SUP_{4oc} A₂₉U [compared to tY(GUA) A₂₉U] explains its increased tRNA decay ratio in

FIGURE 4. tRNA decay of the *SUP4_{oc}* A₂₉U and A₃₁U anticodon stem variants is suppressed by removal of the intron and restoration of G₃₄. (A) Poison primer extension analysis of *SUP4_{oc}* A₂₉U intron-containing and intronless variants. Variants as indicated were analyzed for tRNA levels after growth in *pus7Δ* strains, by poison primer extension analysis using primer P5 (57–37) in the presence of ddCTP. a and b, strain with no *SUP4_{oc}* variant in *MET22⁺* and *met22Δ*. (B) The decay of *SUP4_{oc}* A₂₉U is suppressed by intron removal and restoration of G₃₄. Bar graph depicting % tY levels and tRNA decay ratios for anticodon stem variants with or without an intron in *SUP4_{oc}* and tY(GUA), as indicated. (C) Poison primer extension analysis of *SUP4_{oc}* A₃₁U intron-containing and intronless variants. (D) The decay of *SUP4_{oc}* A₃₁U is suppressed by intron removal and restoration of G₃₄.

the intronless variants. These data demonstrate that for the *SUP4_{oc}* A₂₉U variant most, but not all, of the tRNA decay is due to MPD, with the remainder presumably due to RTD. Similarly, removal of the intron from *SUP4_{oc}* A₃₁U reduces the tRNA decay ratio from 3.8 to 1.5, and restoration of G₃₄ to generate an intronless tY(GUA) A₃₁U variant eliminates virtually all remaining tRNA decay, resulting in a tRNA decay ratio of 1.1 (Fig. 4C,D; Supplemental Fig. S11B).

Anticodon stem mutations cause both RTD and MPD in tS(CGA)

The finding of Met22-dependent decay of both mature tRNA (RTD) and pre-tRNA (MPD) associated with *SUP4_{oc}* and tY(GUA) anticodon stem variants led us to speculate that anticodon stem variants of other intron-containing tRNA species would also be subject to some combination of RTD and MPD. We therefore attempted to design tS(CGA) anticodon stem variants that would be subject to

RTD (Fig. 5A), since RTD of tS(CGA) variants is well studied biochemically, and since tS(CGA) is a single copy essential tRNA gene that is amenable to a genetic test for Met22-dependent tRNA decay (Whipple et al. 2011). We integrated a tS(CGA) anticodon stem variant into a tS(CGA)Δ [*URA3* tS(CGA)] strain, so that cell growth dependent on the integrated variant could be tested after FOA selection to remove the *URA3⁺* plasmid bearing the WT tS(CGA) gene. We found that strains bearing each of the three tested anticodon stem tS(CGA) variants (A₂₉U, C₄₀U, and C₄₂U) behaved like substrates of RTD or MPD, because they exhibited temperature sensitivity that could be suppressed by a *met22Δ* mutation (Fig. 5B), as was observed for the control *tan1Δ trm44Δ* mutant, previously shown to be due to RTD (Chernyakov et al. 2008; Kotelawala et al. 2008).

To assess tRNA decay of these variants, we first introduced a plasmid containing a tS(GCU/CGA) hybrid tRNA, bearing the body of tS(GCU) and a CGA anticodon, into

FIGURE 5. Anticodon stem variants of tS(CGA) provoke RTD and MPD. (A) Schematic of mature tS(CGA) and variants analyzed. Variants are indicated in red. (B) Genetic analysis demonstrates that several tS(CGA) anticodon stem variants provoke Met22-dependent decay. *MET22*⁺ and *met22Δ* strains with a tS(CGA)Δ mutation and a single integrated tS(CGA) variant as indicated were tested for growth by plating serial dilutions on YPD media and incubating plates at the temperatures indicated. (C) Northern analysis of tS(CGA) and tS(CGA) C₄₂U tRNA levels in *MET22*⁺ and *met22Δ* strains grown at 34°C. Strains with a tS(CGA)Δ mutation, an integrated tS(CGA) variant, and a [tS(GCU/CGA) *LEU2 CEN*] plasmid (to allow for growth at a nonpermissive temperature), were grown at 34°C, and bulk RNA was resolved by gel electrophoresis and hybridized using labeled probes as indicated at the left. To confirm selective hybridization relative to endogenous tS(UGA), bulk RNA from a strain with no integrated tS(CGA) variant (lane a) was probed at the same time. (D) The tS(CGA) C₄₂U variant is subject to RTD and MPD. Pre-tS(CGA) and mature tS(CGA) were quantified relative to hybridization of tY(GUA) (×100) to control for loading, to obtain relative tRNA and pre-tRNA levels, and associated tRNA decay ratios and pre-tRNA decay ratios. The statistical significance of pre-tRNA accumulation was evaluated using a one-tailed Student's *t*-test assuming equal variance. Analysis of mature tRNA, diagonal stripes; pre-tRNA, solid colors; *MET22*⁺, green; *met22Δ*, blue; decay ratios, red.

the tS(CGA)Δ strain with an integrated tS(CGA) variant, thus allowing growth of strains at nonpermissive temperatures and facile measurement of tS(CGA) variant levels by northern analysis. At 34°C, the tS(CGA) C₄₂U variant had a substantial tRNA decay ratio of 3.8, accompanied by a large accumulation of unspliced end-matured pre-tRNA in the *met22Δ* strain [compared to that of WT tS(CGA)], and a substantial pre-tRNA decay ratio of 2.8 (Fig. 5C,D). At 32°C, the tS(CGA) C₄₀U variant also had a substantial tRNA decay ratio of 3.9 and a distinct pre-tRNA decay ratio of 1.8 (Supplemental Fig. S12), albeit accompanied by a modest accumulation of pre-tRNA in the *met22Δ* background (*P*-value of 0.065). It was also clear from our northern analysis of the tS(CGA) C₄₂U and C₄₀U variants that the end-matured intron-containing pre-tRNA species was the target

of MPD. In contrast, although the tS(CGA) A₂₉U variant had a substantial tRNA decay ratio of 2.0, there was no obvious accumulation or decay of pre-tRNA (Supplemental Fig. S13).

DISCUSSION

The results described here show that decay of anticodon stem tRNA variants occurs in large part through a previously undescribed Met22-dependent decay pathway that targets pre-tRNAs that accumulate due to altered structure of the anticodon stem-loop and intron. Pre-tRNA decay by the MPD pathway can contribute substantially to the overall decay of tRNA variants as mutations that suppress MPD or remove the intron result in a substantial reduction of the

tRNA decay ratio. Furthermore, MPD is a general phenomenon, as it can occur in both tY(GUA) and tS(CGA) variants bearing native anticodons and thus participating in normal translation. In all previously described cases, Met22 has only been shown to regulate mature tRNA decay by the RTD pathway (Alexandrov et al. 2006; Chernyakov et al. 2008; Dewe et al. 2012), and the decay of pre-tRNA has only been attributed to 3'–5' exonucleolytic decay by the nuclear surveillance pathway (Kadaba et al. 2004, 2006; Gudipati et al. 2012).

The accumulation of pre-tRNA that drives MPD is likely directly related to the structure of the pre-tRNA anticodon stem-loop and intron, which is important for splicing. Pairing between intron residues and residues of the anticodon and neighboring nucleotides is highly conserved in archaeal and yeast intron-containing pre-tRNAs, comprising the central helix of the bulge-helix-bulge motif important for splicing (Ogden et al. 1984; Swerdlow and Guthrie 1984; Lee and Knapp 1985; Kjems et al. 1989; Thompson and Daniels 1990; Marck and Grosjean 2003; Xue et al. 2006). The importance of the central helix is underscored by our results that disruption of this helix by a G₃₄U mutation tended to increase MPD, whereas restoration of the helix tended to reduce MPD. Remarkably, we found that a complete disruption of this interaction in the SUP4_{CAC} variant is sufficient to provoke MPD without any other accessory mutations. The importance of the 3' bulge structure is underscored by prominent pre-tRNA decay in variants predicted to have an intact central helix, but with mutations affecting the bulge, including the tY(GUA) G₃₀A, tS(CGA) C₄₀U, and tS(CGA) C₄₂U variants.

The Met22-dependent decay of end-matured unspliced pre-tRNA documented here might extend to other organisms. Introns are found in at least some tRNA genes throughout eukaryotes, including humans, and the metazoan tRNA splicing pathway uses a similar endonuclease, likely recognizing pre-tRNA in a similar fashion (Fruscoloni et al. 2001). Presumably, the integrity of pre-tRNA structure is under similar surveillance in other eukaryotes.

Our data shows that the target of pre-tRNA decay by the MPD pathway is the end-matured unspliced species, for both tY(GUA) and tS(CGA) variants. This pre-tRNA species is the last end processing intermediate before nuclear export of the pre-tRNA for splicing on the surface of the mitochondrion (Yoshihisa et al. 2003, 2007; Wan and Hopper 2018). Thus, MPD could occur in the nucleus just after end processing, or in the cytoplasm after export. Since perturbed exon–intron structure is a strong determinant of MPD and is linked to impaired splicing, we anticipate that MPD is more likely to occur in the cytoplasm, where splicing occurs. Although we do not yet unequivocally know the nucleases responsible for MPD, the Met22-dependence of pre-tRNA decay suggests strongly that MPD is due to a 5'–3' exonuclease, as this class of nucleas-

es is known to be inhibited in a *met22Δ* strain (Dichtl et al. 1997; Yun et al. 2018). Indeed Xrn1 and Rat1 are the known 5'–3' exonucleases of RTD, which are inhibited in *met22Δ* strains (Chernyakov et al. 2008; Whipple et al. 2011). However, we note that other nucleases could also be involved as there is at least one report of a 3'–5' exonuclease that is inhibited by pAp (Mechold et al. 2006), and in principle an endonuclease could also be inhibited by pAp.

We envision two mechanisms by which MPD might occur. First, the accumulation of pre-tRNAs due to inefficient endonucleolytic cleavage during splicing may provide the substrate for degradation by 5'–3' exonucleases, due to the increased availability of the pre-tRNAs. We note that a pre-tRNA that accumulates before the onset of splicing might be uniquely vulnerable to attack by 5'–3' exonucleases, as decay of the pre-tRNA, unlike decay of mature tRNA during RTD, would not be blocked by binding to EF-1A or the aminoacyl tRNA synthetases, both of which prevent RTD (Dewe et al. 2012; Turowski et al. 2012), and would also not be expected to be protected by the ribosome. Second, MPD may be mediated through a more complex decay pathway involving the SEN complex itself. In this model, pre-tRNA with a compromised structure might be inefficiently spliced by the SEN complex at either the 5' or 3' splice junction, generating tRNA fragments with exposed 5' and 3' ends at the acceptor stem. At this step, the tRNA fragments would be in competition among three different fates: completion of endonucleolytic cleavage by the SEN complex; healing and religation by the tRNA ligase (Trl1); or decay by exonucleases. This type of model was recently proposed as a mechanism regulating decay of *HAC1* mRNA, encoding a major regulator of the unfolded protein response; this mRNA is spliced by a similar mechanism to tRNA, using Ire1 endonuclease and tRNA ligase (Sidrauski et al. 1996; Sidrauski and Walter 1997), and ligation of its cleavage products is in competition with decay mediated by Xrn1 (Cherry et al. 2019; Peschek and Walter 2019). Future experiments should cast light on the mechanism by which the MPD pathway monitors pre-tRNA quality and mediates decay.

The identification of MPD also has implications for the tRNAs of higher eukaryotes, which are often made up of several isodecoders, tRNAs with identical anticodons, but variations in the remaining sequence and the intron (Goodenbour and Pan 2006). Of particular note to the work described here, the four human tRNA isodecoder families with introns have substantial intron variations in both length and sequence content (Chan and Lowe 2016). It has been previously reported that tR(UCU) isodecoders are subject to tissue specific regulation of expression (Ishimura et al. 2014), and based on the work described here, intronic variations could in principle participate in regulating expression through an MPD-like pathway.

TABLE 1. Strains used in this study

Strain name	Genotype	Origin
BCY 123	<i>MATa pep4 Δ::HIS3 prbΔ::LEU2 bar1Δ::HISG lys2Δ::GAL1/10-GALΔ4 can1 ade2 ura3 leu2-3, 112</i>	(Macbeth et al. 2005)
BY 4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Open biosystems
ISC 566	BY 4741 <i>met22Δ::kan^R</i>	This study
JMW 223	BY 4741 <i>tS(CGA)Δ::ble^R [tS(CGA) URA3 CEN]</i>	(Whipple et al. 2011)
JMW 451	BY 4741 <i>tS(CGA)Δ::ble^R ade2Δ::MET15::tS(CGA) tan1Δ::kan^R trm44Δ::nat^R</i>	(Whipple et al. 2011)
JMW 549	BY 4741 <i>tS(CGA)Δ::ble^R ade2Δ::MET15::tS(CGA) tan1Δ::kan^R trm44Δ::nat^R met22Δ::hyg^R</i>	(Whipple et al. 2011)
QB 1112AD	BCY 123 [2μ <i>P_{GAL}-XRN1-PT</i>]	(Quartley et al. 2009)
QB 1222AD	BCY 123 [2μ <i>P_{GAL}-RAT1-PT/His10-RAI1</i>]	(Quartley et al. 2009)
YK 380-1	BY4741 <i>can1Δ::RNA-ID GFP_{oc} MET15</i>	(Guy et al. 2014)
YK 391-1	BY4741 <i>can1Δ::RNA-ID GFP_{oc} MET15 met22Δ::kan^R</i>	(Guy et al. 2014)
YAH592-1	YK380-1 <i>pus7Δ::ble^R</i>	This study
YAH593-1	YK391-1 <i>pus7Δ::ble^R</i>	This study

MATERIALS AND METHODS

Yeast strains

The yeast strains used are shown in Table 1. Deletions were introduced by standard PCR amplification of the appropriate strain from the YKO collection (Giaever et al. 2002) (or of a derivative strain with a different drug marker), followed by a linear transformation, and PCR of transformants to confirm the deletion.

Construction and integration of variants of *SUP4_{oc}* and *tS(CGA)*

SUP4_{oc} variants were constructed by annealing of overlapping oligomers (IDT) followed by ligation into the Bgl II Xho I site of the tRNA gene cassette of plasmid AB230-1, as previously described (Guy et al. 2014). *tS(CGA)* variants were constructed in a similar manner. tRNA variants were integrated at the *ADE2* site of strains by a linear transformation of a Stu I fragment containing the tRNA cassette, the adjacent *HIS3* marker, and flanking *ADE2* sequences, deleting part of *ADE2* (Whipple et al. 2011). Three biological isolates of each strain were isolated and used for all of the experiments reported, unless otherwise noted.

Growth of cells for isolation of bulk RNA

Three independent isolates of strains with integrated tRNA variants were grown overnight at 28°C in 5 mL YPD media (1% yeast extract, 2% peptone, 2% dextrose, supplemented with 80 mg/L adenine hemisulfate), inoculated into 5 mL fresh media at OD₆₀₀ ~ 0.1, and grown to an OD ~ 1, and then 2-OD samples were harvested with a microcentrifuge, washed with 1 mL water, frozen on dry ice, and stored at -80°C. Then bulk RNA was extracted using hot phenol as described previously (Jackman et al. 2003), followed by addition of 20 μg glycogen, phenol-chloroform-isoamyl alcohol (PCA) extraction, two ethanol precipitations, and resuspension in ddH₂O.

Purification of Xrn1 and the Rat1/Rai1 complex

Xrn1 was purified from yeast strain QB1122AD after galactose-induced expression of a *P_{GAL1}-XRN1* construct in which *XRN1* was fused at its 3' terminus to the PT tag, bearing a protease 3C site- HA epitope- His6- ZZ domain of protein A, as described previously (Whipple et al. 2011). Rat1/Rai1 was purified analogously from strain QB1222AD bearing a dual *P_{GAL1,10}* expression plasmid expressing *RAT1-PT* and *His10-RAI1* (Quartley et al. 2009).

TABLE 2. Primers used in poison primer extension

Name	Target RNA	Probe (5'-3')	Sequence
P5	tY(GUA)	57-37	CGAACGCCCCGATCTCAAGATT
P7	tY(GUA)	62-43	CGAGTCGAACGCCCCGATCTC
P9	tY(GUA)	57-33	CGAACGCCCCGATCTCAAGATTTACA
MP 539	tY(GUA)	55-31	AACGCCCCGATCTCAAGATTACAGT
MP 497	pre-tY(GUA)	51-In1	CCCGATCTCAAGATTTCGTAGTGATAAA
MP 538	pre-tY(GUA)	49-[In]-34	CGATCTCAAGATTTCGTAGTGATAAATTAC
MP 540	pre-tY(GUA)	46-[In]-31	TCTCAAGATTTCGTAGTGATAAATTACTGT

TABLE 3. Oligomers used for northern analysis

Name	Target RNA	Probe (5'–3')	Sequence
TDZ 231	<i>SUP4_{oc}</i> A29U	48–27	GATCTCAAGATTTAAAGTCATG
TDZ 271	<i>SUP4_{oc}</i> G30A	48–27	GATCTCAAGATTTAAAGTTTGG
TDZ 249	<i>SUP4_{oc}</i> A31U	48–27	GATCTCAAGATTTAAAGACTTG
tRNAPheLoop	tF(GAA)	64–45	TGTGGATCGAACACAGGACC
MP 586	tS(CGA)	37–17	TTCGAGTCTCTCGCCTTAACCA
MP 197	tY(GUA)	76–53	TGGTCTCCCGGGGGCGAGTCGAACG
Phe GAA intron	pre-tF(GAA)	In16–35	TTGACCGAAGTTTTTCTT
MP 564	pre-tY(GUA)	43–In1	CAAGATTTCTAGTGATAAA
MP 567	pre- <i>SUP4_{oc}</i> A29U	In14–27	TTCGTAGTGATAAATTAAGTCATG
TDZ 238	pre- <i>SUP4_{oc}</i> G30A	In14–27	TTCGTAGTGATAAATTAAGTTTGG
TDZ 239	pre- <i>SUP4_{oc}</i> A31U	In14–27	TTCGTAGTGATAAATTAAGACTTG
5S	5S	20–1	TGGTAGATATGGCCGCAACC

Purification of tRNAs

SUP4_{oc} variants were purified together with tRNA^{Tyr} using 1.0 mg bulk RNA from the corresponding *met22Δ* strain and the biotinylated oligomer MP 129 (nt 76–53), as previously described (Gehrke and Kuo 1989; Whipple et al. 2011).

In vitro digestion with exonucleases

Purified tRNAs were analyzed for exonuclease sensitivity essentially as previously described (Whipple et al. 2011). A purified mixture of tY(GUA) and *SUP4_{oc}* (7.5 ng) was melted at 95°C for 10 min in buffer containing 25 mM Tris pH 8.0 and 150 mM NaCl, immediately placed on ice, refolded at 37°C for 20 min in buffer containing 13.9 mM Tris pH 8.0, 83.3 mM NaCl, 2.2 mM MgCl₂, 0.55 mM DTT, 0.11 mg/mL BSA, and then incubated as indicated with purified Xrn1 or Rat1/Rai1 in buffer containing 4.5 mM Tris pH 8.0, 90 mM NaCl, 2.1 mM MgCl₂, 0.6 mM DTT, 0.1 mg/mL BSA, 5% glycerol. Reactions were stopped on dry ice, extracted with PCA, ethanol precipitated with 20 μg of glycogen, and then resuspended in ddH₂O.

Poison primer extension analysis of *SUP4_{oc}* variants

To analyze relative amounts of *SUP4_{oc}* and tRNA^{Tyr} in bulk RNA or purified mixtures, we used a poison primer extension assay with an appropriate primer (Table 2) and ddCTP or ddTTP, essentially as previously described (Guy et al. 2014). Briefly, ~0.5 pmol of 5' radiolabeled primer and ~1.0 μg of bulk RNA was denatured by heating at 95°C and then annealed in water, followed by primer extension with 2U AMV reverse transcriptase (Promega) in supplied buffer containing 1 mM ddNTP, and 1 mM remaining dNTPs, incubation for 1 h at 50°C, and transfer to –20°C. Aliquots of reactions were diluted twofold in formamide dye, heated at 95°C for 3 min, and resolved on a 15% polyacrylamide gel (29:1) containing 7 M urea in 1× TBE. The gel was dried on a Model 583 Biorad gel dryer at 78°C for 40 min, exposed and an-

alyzed using an Amersham Typhoon phosphorimager and Image Quant v5.2.

The relative amount of *SUP4_{oc}* variant in each sample is expressed as % tY(GUA) (% tY), calculated as the quotient of the signal for the *SUP4_{oc}* variant (typically the G₃₀ stop) divided by the endogenous tY(GUA) signal (typically the G₃₄ stop), each first corrected for background. The % tY was averaged over three biologically independent strains grown and analyzed in parallel, and the error calculated as a standard deviation. The RTD ratios were calculated as the quotient of the average % tY value in a *met22Δ* strain divided by the corresponding average % tY value in a *MET22⁺* strain (analyzed in the same gel from primer extensions done at the same time), with the error propagated appropriately for a division operation.

We note that despite some variability in % tY from experiment to experiment, the RTD ratios obtained from *MET22⁺* and *met22Δ* strains grown in triplicate and analyzed at the same time are largely consistent between experiments. The variability in % tY likely arises from variations in the hybridization efficiencies of the primer extension probe to *SUP4_{oc}* variants in different sets of assays. This results in variable % tY measurements for the same bulk RNA preparations assayed at different times, but the same % tY from biological triplicates assayed at the same time.

Northern analysis of *SUP4_{oc}* variants

For northern analysis, independently made strains were grown in parallel, bulk RNA was isolated, and 1 μg of bulk RNA was mixed with an equal volume formamide dye, heated at 60°C for 5 min, and resolved on a 10% polyacrylamide (19:1), 7 M urea 1× TBE gel, which was transferred onto an Amersham Hybond-N⁺ membrane (GE Healthcare), crosslinked on a UV Stratalinker 2400, and hybridized to probes (~12 pmol 5' ³²P-labeled DNA oligomer) as indicated. Oligomers are described in Table 3.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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