Degradation of several hypomodified mature tRNA species in *Saccharomyces cerevisiae* is mediated by Met22 and the 5′–3′ exonucleases Rat1 and Xrn1

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Mature tRNA is normally extensively modified and extremely stable. Recent evidence suggests that hypomodified mature tRNA in yeast can undergo a quality control check by a rapid tRNA decay (RTD) pathway, since mature tRNA$^{\text{Val}(\text{AAC})}$ lacking 7-methylguanosine and 5-methylcytidine is rapidly degraded and deacylated at 37°C in a *trm8*-Δ *trm4*-Δ strain, resulting in temperature-sensitive growth. We show here that components of this RTD pathway include the 5′–3′ exonucleases Rat1 and Xrn1, and Met22, which likely acts indirectly through Rat1 and Xrn1. Since deletion of *MET22* or mutation of *RAT1* and *XRN1* prevent both degradation and deacylation of mature tRNA$^{\text{Val}(\text{AAC})}$ in a *trm8*-Δ *trm4*-Δ strain and result in healthy growth at 37°C, hypomodified tRNA$^{\text{Val}(\text{AAC})}$ is at least partially functional and structurally intact under these conditions. The integrity of multiple mature tRNA species is subject to surveillance by the RTD pathway, since mutations in this pathway also prevent degradation of at least three other mature tRNAs lacking other combinations of modifications. The RTD pathway is the first to be implicated in the turnover of mature RNA species from the class of stable RNAs. These results and the results of others demonstrate that tRNA, like mRNA, is subject to multiple quality control steps.

*Keywords*: RNA turnover; mature tRNA; *TRM8*; *TRM4*; yeast; tRNA$^{\text{Val}(\text{AAC})}$

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tRNA molecules are stable RNAs that are exquisitely adapted for their central role in translation. In all organisms, tRNAs are constructed to be similar enough for rapid and efficient use in the translation cycle, yet different enough for accurate discrimination by the translation machinery, to ultimately deliver the correct amino acid to the growing peptide chain. tRNA bodies appear to have evolved together with their corresponding amino acids to ensure similar binding to components of the translation apparatus [LaRiviere et al. 2001]. tRNA bodies have also evolved for highly specific decoding through interactions at the anti-codon and at other residues [Cochella and Green 2005; Olejniczak et al. 2005], and for highly specific recognition by aminoacyl tRNA synthetases through multiple determinants to ensure correct aminoacylation [Giege et al. 1998]. In addition, tRNA bodies have evolved to be extremely stable, with half-lives measured in hours or days, during which time each tRNA goes through the translation cycle ~40 times per minute [Waldron and Lacroute 1975].

Many of the properties of tRNAs stem from their ubiquitous modifications. About 100 tRNA modifications have been described, many of which are highly conserved among different organisms. In the yeast *Saccharomyces cerevisiae*, 25 distinct modifications have been identified at 34 different positions on cytoplasmic tRNAs, with an average of 13 modifications per tRNA species [Sprinzl et al. 1999]. Most of the modifications located in and around the anti-codon in yeast are crucial for codon–anti-codon interactions or reading frame maintenance [Agris et al. 2007], based on a range of growth and translation defects of the corresponding yeast mutants [Huang et al. 2005; Waas et al. 2007; for review, see Hopper and Phizicky 2003], and detailed analysis of bacterial mutants [Urbonavicius et al. 2001] and of ribosome–tRNA interactions [Weixlbaumer et al. 2007]. Modifica-
tions are also important for correct charging by aminoacyl tRNA synthetases, as described in yeast for m\(^{12}\)G\(_{37}\) of tRNA\(^{A^{sp}}\) [Putz et al. 1994] and in Escherichia coli for s\(^{5}\)U\(_{34}\) of tRNA\(^{Glu}\) and lysidines\(_{36}\) of tRNA\(^{Ile}\) [Muramatsu et al. 1988; Sylvers et al. 1993].

Recent evidence suggests that tRNA, like mRNA [Doma and Parker 2007] and rRNA [LaRiviere et al. 2006], is subject to quality control steps leading to turnover in vivo. At least two such pathways are known to exist, and these appear to act at different stages of tRNA maturation. First, pre-tRNA\(^{i}i\) and these appear to act at different stages of tRNA maturation. First, pre-tRNA\(^{i}i\) (Doma and Parker 2007) and rRNA (LaRiviere et al. 1988; Sylvers et al. 1993).

Second, mature tRNA\(^{Val}(AAC)\) lacking m\(^{7}\)G\(_{46}\) and which also includes Air1/Air2 and Mtr4 (LaCava et al. 1988; Sylvers et al. 1993).

In this study, we investigate the components of the RTD pathway that is independent of TRF4/RRP6, leading to a temperature-sensitive growth defect of the \(trm8\)-\(\Delta\) \(trm4\)-\(\Delta\) strain (Alexandrov et al. 2006). In addition, there are several other cases where reduced levels of tRNA species are observed, but in each case the mechanism of tRNA loss is largely unknown: Certain mutations of tRNA\(^{Arg(CCG)}\) result in Trf4-independent reduction in tRNA levels [Copela et al. 2006]; reduced levels of tRNA\(^{Ser(CGA)}\) are observed in strains with a tRNA\(^{Ser}\) mutation that also lack m\(^{12}\)U\(_{44}\) or \(\Psi\)\(_{59}\) due to deletion of \(TRM8\) and \(TRM4\) [Fig. 1] is rapidly degraded and deacylated at 37°C by a rapid tRNA decay (RTD) pathway that is independent of TRF4/RRP6, leading to a temperature-sensitive growth defect of the \(trm8\)-\(\Delta\) \(trm4\)-\(\Delta\) strain (Alexandrov et al. 2006).

In this study, we investigate the components of the RTD pathway by which tRNA\(^{Val(AAC)}\) lacking m\(^{12}\)G\(_{46}\) and m\(^{12}\)C\(_{49}\) is rapidly degraded and deacylated in a \(trm8\)-\(\Delta\) \(trm4\)-\(\Delta\) strain at high temperature. We show that loss of functional mature tRNA\(^{Val(AAC)}\) in this strain is mediated by Met22 and the 5′-3′ exonucleases Rat1 and Xrn1, since deletion of \(MET22\), or mutation of \(RAT1\) in combination with deletion of \(XRN1\) prevents degradation of mature tRNA\(^{Val(AAC)}\). The involvement of Met22 in tRNA degradation is likely indirect, since loss of Met22 function has previously been proposed to inhibit Rat1 and Xrn1 through accumulation of its metabolite substrate adenosine 5′,3′-biphosphate [Dichtl et al. 1997]. The involvement of Rat1 and Xrn1 in degradation of mature tRNA\(^{Val(AAC)}\) is the first case in which these proteins have been implicated in degradation of a mature RNA species from the class of stable noncoding RNA. Surprisingly, mutation of components of the RTD pathway also prevents the loss of aminoacylation of tRNA\(^{Val(AAC)}\) that is observed in a \(trm8\)-\(\Delta\) \(trm4\)-\(\Delta\) strain at high temperature, suggesting that the tRNA is at least partially functional and structurally intact under these conditions and that degradation of the tRNA is more complicated than simply the nonspecific removal of waste RNA. Finally, we provide evidence that the RTD pathway is a general tRNA quality control pathway that acts on multiple hypomodified mature tRNA species.

**Results**

**Mutation of MET22 suppresses the temperature sensitivity of a \(trm8\)-\(\Delta\) \(trm4\)-\(\Delta\) strain**

We have shown previously that degradation of hypomodified tRNA\(^{Val(AAC)}\) in \(trm8\)-\(\Delta\) \(trm4\)-\(\Delta\) mutants does not occur via the nuclear pre-tRNA surveillance pathway, since deletion of RRP6 or TRF4 does not prevent degradation of tRNA\(^{Val(AAC)}\) or rescue growth of the \(trm8\)-\(\Delta\) \(trm4\)-\(\Delta\) strain at 37°C [Alexandrov et al. 2006]. To identify components of the RTD pathway by which tRNA\(^{Val(AAC)}\) is degraded and deacylated, we isolated and analyzed 26 spontaneous suppressors of the temperature-sensitive phenotype of the \(trm8\)-\(\Delta\) \(trm4\)-\(\Delta\) strain. All of these suppressors belong to a single complementation group, are cold-sensitive, and are methionine auxotrophs [data not shown].

We cloned the wild-type allele of the suppressor gene by complementation of the methionine auxotrophy. We transformed a suppressor strain (revertant 13) with the genomic movable ORF (MORF) collection of yeast ORF-containing plasmids, each of which expresses an individual ORF under \(P_{GAL}\) control [Gelperin et al. 2005]. Each of three plasmids that conferred methionine prototrophy in media containing galactose encoded the MET22 gene, and expression of this gene also suppressed both the temperature resistance and cold sensitivity of the suppressor strain [Supplemental Fig. S1A]. Furthermore, a \(trm8\)-\(\Delta\) \(trm4\)-\(\Delta\) \(met22\)-\(\Delta\) strain grows as well on plates at 37°C as the original revertant 13 strain [now named \(trm8\)-\(\Delta\) \(trm4\)-\(\Delta\) \(met22\)-13], is cold-sensitive [Fig. 2A], and is a methionine auxotroph, and introduction of a single-copy \(CEN\) plasmid expressing MET22 from its own promoter complements all three phenotypes of the strain [Supplemental Fig. S1B; data not shown]. Thus, we

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Schematic of the secondary structure of tRNA\(^{Val(AAC)}\) and tRNA\(^{Ser(CGA)}\). tRNA sequences are as described [Sprinzl et al. 1999]. Modifications described in the text are highlighted, and the proteins that catalyze their formation are indicated.
Deletion of MET22 suppresses the temperature sensitivity of the trm8-Δ trm4-Δ strain.

Deletion of MET22 in a trm8-Δ trm4-Δ strain prevents degradation and loss of aminoacylation of tRNAVal(AAC)

To determine if met22-mediated suppression of the trm8-Δ trm4-Δ growth defect is due to an effect on the amount of functional tRNAVal(AAC), we analyzed the levels and the aminoacylation status of tRNAVal(AAC) in a trm8-Δ trm4-Δ met22-Δ mutant after growth at 37°C. Consistent with previous results (Alexandrov et al. 2006), 2 h after temperature shift of the trm8-Δ trm4-Δ parent strain, tRNAVal(AAC) is present at only 20% of wild-type levels and is only 25% aminoacylated [Fig. 2B (lane j), C,D]. By contrast, tRNAVal(AAC) levels in the trm8-Δ trm4-Δ met22-Δ strain are maintained at ~80% of wild-type levels throughout the time course [Fig. 2B (lanes q-t), C]. Strikingly, aminoacylation of tRNAVal(AAC) is also almost completely stabilized in the trm8-Δ trm4-Δ met22-Δ strain, remaining at ~60%–65% over the time course, compared with ~75% for wild-type cells [Fig. 2B [cf. lanes q-t and b-e], D]. Since no other change is observed in the levels or aminoacylation of each of three control tRNA species in the met22-Δ or the trm8-Δ trm4-Δ met22-Δ strain [Fig. 2B], we conclude that the met22-Δ mutation suppresses the temperature sensitivity of the trm8-Δ trm4-Δ strain by preventing the degradation and the loss of aminoacylation of tRNAVal(AAC), rather than by global changes in tRNA levels or aminoacylation.

Mutation of both RAT1 and XRN1 also suppresses the temperature-sensitive phenotype of a trm8-Δ trm4-Δ strain and prevents loss of functional tRNAVal(AAC)

The known biochemical function of Met22 suggests that it is not directly responsible for degradation and loss of aminoacylation of tRNAVal(AAC) in the trm8-Δ trm4-Δ strain. Met22 is a phosphatase in the sulfate assimilation pathway leading to methionine biosynthesis [Fig. 3A], in which it removes the 3′-phosphate from the pyridoxal adenosine 3′,5′-bisphosphate (pAp), as well as from the pathway intermediate 3′-phosphoadenosine 5′-phosphosulfate, pApS [Murguia et al. 1995]. One possible role of Met22 in tRNA degradation derives from the observation that mutation of Met22, or inhibition of Met22 by Li+ treatment, leads to inhibition of 5.8S rRNA processing, snoRNA processing, and rRNA spacer fragment degradation, which is attributed to inhibition of the 5′–3′ exonucleases Rat1 and Xrn1 by pAp [Murguia et al. 1996, Dichtl et al. 1997]. Consistent with this, we find that the temperature-sensitive phenotype of a trm8-Δ trm4-Δ strain is suppressed on minimal media containing 0.2 M LiCl, but not on media containing 1 M KCl [Fig. 3B].

To address the possibility that Rat1 and Xrn1 are involved in tRNAVal(AAC) degradation in trm8-Δ trm4-Δ mutants and to identify other possible mechanisms by which tRNAVal(AAC) degradation is effected, we isolated 33 temperature-resistant suppressors of trm8-Δ trm4-Δ mutants that were not met22 alleles, by starting with a trm8-Δ trm4-Δ strain containing a second copy of the wild-type MET22 gene. These suppressors belong to at least three complementation groups, the largest of which are rat1 mutants, based on three lines of evidence. First, a CEN RAT1 plasmid complements each of four mutants tested in this complementation group, restoring the growth defect of these strains to that of a trm8-Δ trm4-Δ

**Figure 2.** met22 mutations suppress the temperature-sensitive growth defect of trm8-Δ trm4-Δ mutants and prevent degradation and loss of aminoacylation of tRNAVal(AAC). [A] Mutation of MET22 in the trm8-Δ trm4-Δ strain allows growth at 37°C. Strains were grown overnight in YPD at 28°C, adjusted to OD600 ~ 1, serially 10-fold-diluted, spotted on YPD plates, and incubated at 18°C, 30°C, and 37°C, as indicated. [B] tRNAVal(AAC) levels and aminoacylation are stable in the trm8-Δ met22-Δ strain. Strains were grown in YPD at 28°C, adjusted to OD600 ~ 2 and shifted to 37°C, and cells were harvested at the indicated times. Ten micrograms of RNA isolated under acidic conditions were analyzed by Northern blotting as described in the Materials and Methods. For each strain, one sample was deacylated prior to gel electrophoresis. Dashed and solid arrows indicate aminoacylated and deacylated tRNA species, respectively. Note that tRNAVal(AAC) from strains lacking met22-Δ and met22-Δ trm8-Δ strains allows growth at 37°C. Deletion of MET22 of tRNAVal(AAC), rather than by global changes in tRNA degradation, which is attributed to inhibition of the 5′–3′ exonucleases Rat1 and Xrn1 by pAp [Murguia et al. 1996, Dichtl et al. 1997]. Consistent with this, we find that the temperature-sensitive phenotype of a trm8-Δ trm4-Δ strain is suppressed on minimal media containing 0.2 M LiCl, but not on media containing 1 M KCl [Fig. 3B].
mutant at 33°C and above (Fig. 3C, Supplemental Fig. S2). Second, sequence analysis of the RAT1 gene from two of the suppressors (rat1-101 and rat1-107) shows that each has the same A661E mutation in the RAT1 gene. Third, a CEN rat1-A661E plasmid does not complement the temperature-resistant phenotype of trm8Δ trm4Δ suppressor strains and controls, transformed with a URA3 CEN RAT1 plasmid or a vector control, were grown at 28°C in SD-Uracil media, plated on SD-Uracil media, and incubated at temperatures indicated. (D) Mutation of both RAT1 and XRN1 improves suppression of the trm8Δ trm4Δ temperature-sensitive phenotype. Strains were grown in YPD at 28°C and plated on YPD media at indicated temperatures.

Although the rat1-A661E allele in the rat1-101 and rat1-107 mutants is the strongest allele isolated and allows the trm8Δ trm4Δ strain to grow at temperatures up to 36.5°C (Fig. 3C,D), growth is not observed at higher temperatures and is not as robust as for a trm8Δ trm4Δ met22Δ strain (Fig. 3D). Since RAT1 and XRN1 have been shown previously to have redundant roles in a number of rRNA and sn[or]RNA processing events, as well as in mRNA degradation (Geyer et al. 1994; Pet- falski et al. 1998; Gerlens et al. 2000; Danin-Kreiselman et al. 2003; Lee et al. 2005), we introduced an xrn1Δ deletion into both the trm8Δ trm4Δ strain and the trm8Δ trm4Δ rat1-107 strain to test the combined effects of Rat1 and Xrn1 on this tRNA degradation pathway. We find that the trm8Δ trm4Δ rat1-107 strain grows significantly better than the corresponding trm8Δ trm4Δ rat1-107 strain at 37.5°C, whereas the trm8Δ trm4Δ xrn1Δ mutant only grows modestly better than its trm8Δ trm4Δ parent strain at 33°C (Fig. 3D). Since each of these growth phenotypes is complemented by introduction of the appropriate XRN1 or RAT1 CEN plasmid (Supplemental Fig. S3), these results suggest that mutation of both RAT1 and XRN1 is required to fully suppress the growth phenotype of a trm8Δ trm4Δ strain.

Rat1 and Xrn1 both appear to mediate the degradation and the loss of aminoacylation of tRNAVal(AAC) in a trm8Δ trm4Δ strain. In a trm8Δ trm4Δ rat1-107 xrn1Δ mutant strain, there is no observed degradation of tRNAVal(AAC) [Fig. 4A (lanes h–k), B], and the aminoacylation levels...
remain constant at 65% after temperature shift, compared with 80% for wild-type cells [Fig. 4A, C]. By contrast, mutation of *RAT1* or *XRN1* alone results in partial prevention of both degradation and loss of aminoacylation of tRNA<sub>Val</sub>(AAC). Thus, in a *trm8-Δ trm4-Δ rat1-107* strain or a *trm8-Δ trm4-Δ xrn1-1* strain, the levels of tRNA<sub>Val</sub>(AAC) decrease to ~40% of wild type after 2 h at 37°C, compared with 20% for the *trm8-Δ trm4-Δ* strain (Fig. 4A [lanes d-g, l-q], B), and ~40% of the tRNA remains aminoacylated at this point, compared with 20% for the *trm8-Δ trm4-Δ* strain [Fig. 4A, C]. Since Rat1 and Xrn1 are known 5’-3’ exonucleases, it is likely that *RAT1* and *XRN1* are directly responsible for degradation of tRNA<sub>Val</sub>(AAC) in the *trm8-Δ trm4-Δ* strain, although indirect effects cannot be excluded.

The *rat1-107* allele acts primarily by preventing degradation of mature tRNA<sub>Val</sub>(AAC) and not by altering transcription or affecting pre-tRNA

Because a *RAT1* allele was previously implicated as an activator of tRNA transcription [Di Segni et al. 1993], it was possible that stabilization of tRNA<sub>Val</sub>(AAC) in the *trm8-Δ trm4-Δ rat1-107* strain was due to a transcription effect. To test this, we examined tRNA<sub>Val</sub>(AAC) levels after temperature shift in the presence of thiolutin, which inhibits RNA polymerases including pol III [Jimenez et al. 1973]. As expected, thiolutin treatment results in rapid disappearance of intron-containing pre-tRNA<sub>Val</sub>(GAA) and pre-tRNA<sub>Val</sub>(GUA) species [Fig. 5A]. However, since levels of tRNA<sub>Val</sub>(AAC) and control tRNAs are similar in the presence or absence of thiolutin in wild-type and *trm8-Δ trm4-Δ rat1-107* cells [Fig. 5A [cf. lanes a–h and q–x], B], we conclude that the *rat1-107* allele does not act as an activator of tRNA<sub>Val</sub>(AAC) transcription but instead stabilizes tRNA<sub>Val</sub>(AAC) levels in the *trm8-Δ trm4-Δ rat1-107* strain by preventing its degradation.

This experiment also underscores two points about the tRNA<sub>Val</sub>(AAC) degradation in *trm8-Δ trm4-Δ* mutants at 37°C. First, since tRNA<sub>Val</sub>(AAC) degradation in *trm8-Δ trm4-Δ* mutants is at least as fast in thiolutin-treated cells as in untreated cells [Fig. 5A [cf. lanes i–l and m–p], B], this demonstrates that the vast majority of loss of tRNA<sub>Val</sub>(AAC) in *trm8-Δ trm4-Δ* mutants is due to degradation of the mature tRNA, rather than pre-tRNA. This finding is consistent with our previous argument that the observed tRNA<sub>Val</sub>(AAC) degradation is too fast to be accounted for by degradation of pre-tRNA [Alexandrov et al. 2006], as occurs for pre-tRNA<sub>Met</sub> in the Trf4/Rrp6 pathway [Kadaba et al. 2004, 2006]. Second, since tRNA<sub>Val</sub>(AAC) degradation occurs to a much larger extent in thiolutin-treated *trm8-Δ trm4-Δ* mutants than in untreated cells, resulting in just 6% of wild-type tRNA<sub>Val</sub>(AAC) levels remaining 2 h after thiolutin treatment compared with 25% in untreated cells [Fig. 5A [cf. lanes i and p], B], we conclude that the full extent of tRNA<sub>Val</sub>(AAC) degradation is masked by transcription of new tRNA<sub>Val</sub>(AAC). We had previously speculated that the slow phase of tRNA<sub>Val</sub>(AAC) degradation that begins ~30 min after temperature shift of *trm8-Δ trm4-Δ* mutants might be due to the presence of a resistant subpool of tRNA<sub>Val</sub>(AAC) or to compensatory synthesis of new tRNA [Alexandrov et al. 2006]. The disappearance of almost all of the tRNA<sub>Val</sub>(AAC) in thiolutin-treated *trm8-Δ trm4-Δ* cells implies that there is no pool of resistant tRNA in the pre-existing tRNA population.

**Mutation of the RTD pathway suppresses the growth defect of strains lacking different combinations of modifications**

The results described above demonstrate that the RTD pathway mediates degradation of mature tRNA<sub>Val</sub>(AAC) lacking m<sup>7</sup>G<sub>46</sub> and m<sup>5</sup>C<sub>49</sub> in *trm8-Δ trm4-Δ* mutants through Met22, Rat1, and Xrn1, a pathway that is distinct from the Trf4/Rrp6-dependent pathway responsible for degradation of pre-tRNA<sub>Met</sub> in mutants lacking m<sup>5</sup>A<sub>58</sub> [Kadaba et al. 2004, 2006; Alexandrov et al. 2006]. To further explore the scope of these tRNA degradation pathways, we investigated the effect of *met22-Δ, trf4-Δ, and rrp6-Δ* mutations on the temperature-sensitive phenotypes of several other strains bearing different combinations of mutations affecting tRNA modifications. As shown below, we find that the growth defect of each of

![Figure 5](https://example.com/figure5.png)  
**Figure 5.** Transcription inhibition does not affect tRNA<sub>Val</sub>(AAC) levels in a *trm8-Δ trm4-Δ rat1-107* xrn1-1 strain. (A) Northern blot analysis of tRNA after treatment with thiolutin. Strains were grown at 28°C to OD<sub>600</sub> = 1.5, treated with 5 µg/mL thiolutin for 10 min, and then shifted to 37°C for indicated times before harvest, RNA preparation, and analysis of 5 µg of RNA by Northern blotting. (B) Quantification of the levels of tRNA<sub>Val</sub>(AAC). Thiolutin-treated samples are indicated by dashed lines and open symbols, and untreated samples are indicated by solid lines and closed symbols. Wild type is indicated by squares, *trm8-Δ trm4-Δ* is indicated by circles, and *trm8-Δ trm4-Δ rat1-107 xrn1-1* is indicated by triangles.
three such strains is strongly suppressed by mutation of components of the RTD pathway but is not suppressed nearly as well by mutation of the Trf4/Rrp6 pathway.

First, the temperature sensitivity of a *trm8*-Δ *pus7*-Δ strain, which lacks m^7^G_46 and ψ_13,35 in its tRNA (Fig. 1), is suppressed efficiently by a *met22*-Δ mutation at 37°C but is suppressed poorly by a *trf4*-Δ mutation or an *rrp6*-Δ mutation at 33°C (Fig. 6A; Supplemental Fig. S4A), although a *trf4*-Δ strain grows well at 38°C and an *rrp6*-Δ strain grows reasonably well at 35°C (Fig. 6B). Since tRNA\(^{Val(AAC)}\) levels are reduced in the *trm8*-Δ *pus7*-Δ strain at high temperature (Alexandrov et al. 2006) and since expression of tRNA\(^{Val(AAC)}\) from a multicopy plasmid suppresses the temperature sensitivity of the strain (Supplemental Fig. S5), this result suggests that tRNA\(^{Val(AAC)}\) degradation in this strain is effected by the RTD pathway.

Second, the temperature sensitivity of a *trm8*-Δ *dus3*-Δ strain, which lacks m^7^G_46 and D_17 (Fig. 1), is suppressed efficiently by a *met22*-Δ mutation at temperatures up to 37°C but is suppressed only at 33°C by a *trf4*-Δ or an *rrp6*-Δ mutation (Fig. 6A; Supplemental Fig. S4A). Although tRNA\(^{Val(AAC)}\) levels are reduced in this strain at 37°C (Alexandrov et al. 2006), the very weak suppression observed in cells expressing tRNA\(^{Val(AAC)}\) from a multicopy plasmid (Supplemental Fig. S5) suggests that one or more other species of tRNA are affected in this strain. Since Trm8 and Dus3 are only known to act on tRNA substrates, it is unlikely that these mutations affect RNA species other than tRNA. The strong suppression of the temperature sensitivity of a *trm8*-Δ *dus3*-Δ strain by a *met22*-Δ mutation suggests that the RTD pathway mediates degradation of the corresponding tRNA species. However, the weak, but distinct, suppression by a *trf4*-Δ mutation or an *rrp6*-Δ mutation suggests that the Trf4/Rrp6 pathway may also be involved at lower temperatures. Mild suppression by *trf4*-Δ or *rrp6*-Δ mutations was also previously observed in *trm8*-Δ *trm4*-Δ mutants at 33°C, but not at 37°C (Alexandrov et al. 2006).

Third, a *met22*-Δ mutation suppresses the growth phenotype of a *trm44*-Δ *tan1*-Δ strain, which lacks U\(_{m44}\) and ac^3^C_12 in its tRNA, and is temperature-sensitive for growth at 33°C due to loss of tRNA\(^{Ser(CGA)}\) and tRNA\(^{Ser(UGA)}\) (Kotelawala et al. 2008). As shown in Figure 6B, a *met22*-Δ mutation suppresses the growth defect of the *trm44*-Δ *tan1*-Δ strain at temperatures up to 38°C, whereas a *trf4*-Δ mutation suppresses only weakly at 35°C, and an *rrp6*-Δ mutation suppresses very weakly at this temperature (see also Supplemental Fig. S4B).

To determine if the *met22*-Δ mutation suppresses the temperature sensitivity of a *trm44*-Δ *tan1*-Δ strain through inactivation of the RTD pathway, we introduced a *rat1-107* mutation to the *trm44*-Δ *tan1*-Δ strain and tested its phenotype. As shown in Figure 6B, a *rat1-107* mutation efficiently suppresses the temperature sensitivity of the *trm44*-Δ *tan1*-Δ strain at 35°C, but not at 38°C (Fig. 6B). This partial suppression by the *rat1-107* mutation is similar to the observed partial suppression of the temperature sensitivity of *trm8*-Δ *trm4*-Δ mutants by this allele (Fig. 3D) and presumably, like for *trm8*-Δ *trm4*-Δ mutants, would be further enhanced by introduction of an additional *xrn1*-Δ mutation.

Since mutation of the RTD pathway suppresses the growth phenotypes of *trm8*-Δ *trm4*-Δ mutants, *trm8*-Δ *dus3*-Δ mutants, *trm8*-Δ *pus7*-Δ mutants, and *trm44*-Δ *tan1*-Δ mutants and since all of these mutants are associated with reduced tRNA levels, this finding suggests that mutation of the RTD pathway suppresses these growth defects by preventing loss of functional tRNA, as is true for *trm8*-Δ *trm4*-Δ mutants. This point is addressed below for *trm44*-Δ *tan1*-Δ mutants.

Figure 6. Deletion of MET22 suppresses the temperature-sensitive phenotypes of multiple strains lacking different sets of tRNA modifications. (A) A *met22*-Δ mutation rescues growth of *trm8*-Δ *pus7*-Δ and *trm8*-Δ *dus3*-Δ mutants at high temperature. Strains were grown at 28°C and plated on YPD media at indicated temperatures. (B) A *met22*-Δ mutation or a *rat1-107* mutation rescues growth of *trm44*-Δ *tan1*-Δ mutants at high temperature. Strains were grown and plated as in A.

Deletion of MET22 in a *trm44*-Δ *tan1*-Δ strain prevents degradation of mature tRNA\(^{Ser(CGA)}\) and tRNA\(^{Ser(UGA)}\)

To quantitatively examine the role of the *met22*-Δ mutation and of the Trf4/Rrp6 pathway in suppression of a *trm44*-Δ *tan1*-Δ mutant, we evaluated the loss of tRNA\(^{Ser(CGA)}\) and tRNA\(^{Ser(UGA)}\) in a *trm44*-Δ *tan1*-Δ strain carrying mutations in the corresponding genes. As observed previously in the *trm44*-Δ *tan1*-Δ strain in medium containing glycerol (Kotelawala et al. 2008), levels of tRNA\(^{Ser(CGA)}\) are reduced at 30°C to 38% of wild-type levels, and after shift to 36.5°C, they decrease further to 15% of wild type after 3 h and to 10% after 8 h (Fig. 7A [lanes d-i], B). Consistent with degradation by the RTD
pathway, levels of tRNA\textsubscript{Ser\textit{CGA}}} in the \textit{trm44-Δ tan1-Δ} met22\textit{-Δ} strain are 89\% of wild-type levels before the temperature shift, and are reduced only mildly after temperature shift to 73\% of wild-type levels after 3 h and 58\% after 8 h [Fig. 7A (lanes g–i), B]. In contrast, levels of tRNA\textsubscript{Ser\textit{CGA}}} are reduced as quickly and completely in the \textit{trm44-Δ tan1-Δ} trf4-Δ strain and the \textit{trm44-Δ tan1-Δ} rrp6-Δ strain as in the parent \textit{trm44-Δ tan1-Δ} strain [Fig. 7A,B]. Similar results are observed for tRNA\textsubscript{Ser\textit{UGA}}} levels [Fig. 7A,B]. We note also that levels of tRNA\textsubscript{Leu\textit{GAG}}} which are modestly reduced in the \textit{trm44-Δ tan1-Δ} parent strain (Kotelawala et al. 2008), are restored in the \textit{trm44-Δ tan1-Δ met22-Δ} strain but not in the \textit{trm44-Δ tan1-Δ trf4-Δ} strain or the \textit{trm44-Δ tan1-Δ rrp6-Δ} strain, whereas levels of the control tRNA\textsubscript{Ser\textit{GAG}}} remain unchanged in all five strains examined [Fig. 7A,B]. These results indicate that the RTD pathway plays a major role in the degradation of tRNA\textsubscript{Ser\textit{CGA}}} and tRNA\textsubscript{Ser\textit{UGA}}} in a \textit{trm44-Δ tan1-Δ} strain and that the Trf4/Rtp6 pathway plays at most a minor role in this process.

The slow loss of tRNA\textsubscript{Ser\textit{CGA}}} and tRNA\textsubscript{Ser\textit{UGA}}} observed in the \textit{trm44-Δ tan1-Δ} strain [Fig. 7, Kotelawala et al. 2008] could be due either to degradation of mature tRNA, partially compensated by new tRNA synthesis, or to degradation of pre-tRNA, manifested as net loss of the mature tRNA species. To distinguish between these possibilities, we examined tRNA levels in cells treated with thiolutin. Consistent with our observation for tRNA\textsubscript{Val\textit{AAC}}} in the \textit{trm8-Δ trm4-Δ} mutant, loss of tRNA species in the \textit{trm44-Δ tan1-Δ} strain is primarily due to degradation of the mature tRNA, rather than pre-tRNA. Thus, thiolutin treatment results in more rapid and complete loss of tRNA\textsubscript{Ser\textit{CGA}}}, tRNA\textsubscript{Ser\textit{UGA}}}, and tRNA\textsubscript{Leu\textit{GAG}}} species at high temperature in the \textit{trm44-Δ tan1-Δ} strain [Fig. 8A (cf. lanes p–t and k–o], B,C], presumably because of the lack of synthesis of new tRNAs, whereas the levels of each of the two control tRNAs are unaffected in the \textit{trm44-Δ tan1-Δ} strain, and thiolutin has no effect on levels of any of the tRNAs in wild-type cells [Fig. 8A (cf. lanes a–e and i–j], B,C]. Since mature tRNA\textsubscript{Ser\textit{CGA}}} \textsubscript{Ser\textit{UGA}}}, and tRNA\textsubscript{Leu\textit{GAG}}} rather than the corresponding pre-tRNA species, are degraded in the \textit{trm44-Δ tan1-Δ} mutant strain and since deletion of \textit{MET22} prevents degradation of these tRNAs, we conclude that the RTD pathway is responsible for degradation of these mature tRNAs in the same manner as it is responsible for degradation and deacylation of mature tRNA\textsubscript{Val\textit{AAC}}} in \textit{trm8-Δ trm4-Δ} mutant strains.

**Discussion**

We identified Met22, Rat1, and Xrn1 as components of the RTD pathway by which mature tRNA\textsubscript{Val\textit{AAC}}} lacking m\textsubscript{7G46} and m\textsubscript{1C26} is rapidly degraded and decyalted at 37°C in a \textit{trm8-Δ trm4-Δ} strain. Introduction of a \textit{met22-Δ} mutation to a \textit{trm8-Δ trm4-Δ} strain leads to robust suppression of the temperature-sensitive phenotype of the strain and to near quantitative prevention of both degradation and loss of aminoacylation of hypothymidine tRNA\textsubscript{Val\textit{AAC}}} mutant alleles of \textit{RAT1}, encoding the essential 5′–3′ exonuclease Rat1, are efficient, but less robust, suppressors of the \textit{trm8-Δ trm4-Δ} temperature-sensitive phenotype and partially prevent degradation and decylation of tRNA\textsubscript{Val\textit{AAC}}} at 37°C. Additional deletion of \textit{XRN1}, encoding the other known 5′–3′ exonuclease, in a \textit{trm8-Δ trm4-Δ rat1-107} strain leads to robust suppression of the growth defect and complete stabilization of valyl-tRNA\textsubscript{Val\textit{AAC}}} at 37°C, suggesting that both Rat1 and Xrn1 participate in degradation and decylation of this tRNA.

We also provided substantial evidence that the RTD pathway is responsible for degradation of several different mature tRNA species lacking different combinations of modifications. Thus, a \textit{met22-Δ} mutation suppresses the temperature-sensitive growth defect of several mutants: a \textit{trm8-Δ pus7-Δ} strain, which lacks m\textsubscript{7G46} and \textit{Ψ} \textsubscript{13,35} and is temperature-sensitive due to loss of tRNA\textsubscript{Val\textit{AAC}}} \textsubscript{Val\textit{AAC}}} \textsubscript{Val\textit{AAC}}} by which mature tRNA\textsubscript{Val\textit{AAC}}} lacking m\textsubscript{7G46} and m\textsubscript{1C26} is rapidly degraded and decyalted at 37°C in a \textit{trm8-Δ trm4-Δ} strain. Introduction of a \textit{met22-Δ} mutation to a \textit{trm8-Δ trm4-Δ} strain leads to robust suppression of the temperature-sensitive phenotype of the strain and to near quantitative prevention of both degradation and loss of aminoacylation of hypothymidine tRNA\textsubscript{Val\textit{AAC}}} mutant alleles of \textit{RAT1}, encoding the essential 5′–3′ exonuclease Rat1, are efficient, but less robust, suppressors of the \textit{trm8-Δ trm4-Δ} temperature-sensitive phenotype and partially prevent degradation and decylation of tRNA\textsubscript{Val\textit{AAC}}} at 37°C. Additional deletion of \textit{XRN1}, encoding the other known 5′–3′ exonuclease, in a \textit{trm8-Δ trm4-Δ rat1-107} strain leads to robust suppression of the growth defect and complete stabilization of valyl-tRNA\textsubscript{Val\textit{AAC}}} at 37°C, suggesting that both Rat1 and Xrn1 participate in degradation and decylation of this tRNA.
Figure 8. Mature tRNA\textsuperscript{Ser(CGA)}, tRNA\textsuperscript{Ser(UGA)}, and tRNA\textsuperscript{Leu(GAG)} are degraded in the \textit{trm44-\Delta tan1-\Delta} strain. (A) Northern blot analysis of tRNA in wild-type and \textit{trm44-\Delta tan1-\Delta} strains after thiolutin treatment. Strains were grown in YP glycerol at 30 \textdegree C to OD\textsubscript{600} = 0.16, treated with 5 \mu g/mL thiolutin (TL) for 5 min at 30 \textdegree C, and shifted to 36.5 \textdegree C for indicated times. Two micrograms of RNA were analyzed by Northern blotting. (B-C) Quantification of the levels of tRNA\textsuperscript{Ser(CGA)} and tRNA\textsuperscript{Ser(UGA)}, tRNA levels quantified as described in Figure 2.

The degradation of multiple mature tRNA species, based on analysis of tRNA levels in a \textit{trm8-\Delta dus3-\Delta} strain, and degradation occurs at the level of mature tRNA. We presume that the tRNAs affected in a \textit{trm8-\Delta dus3-\Delta} strain and a \textit{trm8-\Delta pus7-\Delta} strain are also degraded as mature species by the RTD pathway, and speculate that the RTD pathway may act widely on mature tRNA species that have become unstable or non-functional.

The RTD pathway described here is the only defined pathway by which mature RNA species within the class of stable RNAs are degraded. Moreover, in the case of degradation of tRNA\textsuperscript{Val(\textit{AAC})} lacking m\textsuperscript{C}\textsubscript{44} and m\textsuperscript{C}\textsubscript{3,69}, no other pathway acts at a detectable rate to degrade the mature tRNA, based on analysis of tRNA levels in a \textit{trm8-\Delta trm4-\Delta rat1-107 xrn1-\Delta} strain treated with thiolutin for 2 h at high temperature [Fig. 5]. Recently, Moore and colleagues [LaRiviere et al. 2006] have shown that ribosomes assembled with tRNA containing mutations that adversely affect translation are subject to a quality control pathway leading to degradation of their tRNA, but the components of this pathway are not yet known.

The degradation of multiple mature tRNA species lacking different combinations of modifications could be interpreted as the loss of structural stability of the tRNA, based on a large amount of evidence that modifications contribute to the thermal stability of tRNA. This includes several comparisons between fully modified purified tRNAs and completely unmodified transcripts (Sampson and Uhlenbeck 1988; Perret et al. 1990; Derrick and Horowitz 1993) and some studies of the effect of one or two modifications on tRNA folding [Davanloo et al. 1979; Nobles et al. 2002]. As summarized elsewhere (Shelton et al. 2001), the order of thermal unfolding events in vitro often begins with the tertiary structure of tRNA and progresses to adjacent helices based on their GC content. Thus, one possible interpretation of our data is that lack of the combinations of modifications described here weakens the tertiary structure of the corresponding tRNAs, and leads to increased local unfolding at the neighboring acceptor helix, to trigger degradation from the 5' end by the RTD pathway.

However, two lines of evidence demonstrate that these tRNA species are at least partially functional and structurally intact under the growth conditions that lead to degradation. First, introduction of a \textit{met22-\Delta} mutation leads to near wild-type growth at high temperature of a \textit{trm8-\Delta trm4-\Delta} strain, a \textit{trm44-\Delta tan1-\Delta} strain, and a \textit{trm8-\Delta dus3-\Delta} strain, which implies that the otherwise degraded tRNAs are functional at this temperature. Second, in the case of a \textit{trm8-\Delta trm4-\Delta} strain, suppression by either \textit{a met22-\Delta} mutation or by \textit{rat1-107 xrn1-\Delta} mutations leads to nearly complete aminocacylation of tRNA\textsuperscript{Val(\textit{AAC})} at high temperature, which implies that the tRNA is sufficiently structurally intact and stable to be recognized and charged by its tRNA synthetase, Vas1. Thus, it is not the case that at high temperature the tRNA simply unfolds irreversibly to be discarded; rather, it is clearly functional to a significant extent. Furthermore, there is strong evidence that the observed tRNA degradation is not strictly associated with high temperature, as might be expected of thermal inactivation, since the levels of each of the affected tRNAs are reduced even at 30 \textdegree C in the \textit{trm8-\Delta trm4-\Delta} and \textit{trm44-\Delta tan1-\Delta} mutants, and the tRNAs are restored to wild-type levels at this temperature in the corresponding \textit{met22-\Delta} derivative strains. Degradation of hypomodified tRNA could occur because its presence is damaging to the cell in some manner, perhaps by causing decreased fidelity of translation or by otherwise impairing one of the steps during translation. Alternatively, it is possible that the hypomodified tRNA is in an equilibrium between functional and non-functional states, allowing for degradation or charging depending on the state of the tRNA.
Deletion of MET22 likely prevents tRNA degradation at least in part because of the proposed inhibitory effect of the Met22 substrate pAp on Rat1 and Xrn1 (Dichtl et al. 1997), consistent with our observation that met22-A mutants, and rat1-107 xrn1-Δ mutants, are equally effective in preventing the degradation and the loss of aminoacylation of tRNAVal[Val][AAC]. However, while a met22-Δ mutation efficiently suppresses the temperature-sensitive phenotype of a trm8-Δ trm4-Δ strain at 37°C, the met22-Δ mutation is not lethal like a rat1-Δ mutation and does not cause slow growth like an xrn1-Δ mutation. Thus, if the inhibition of tRNA degradation caused by deletion of MET22 results from pAp accumulation and its effect on Rat1 and Xrn1, we presume that pAp inhibits tRNA degradation more efficiently than it inhibits the essential function[s] of Rat1 or the processes that cause slow growth of xrn1-Δ mutants.

The participation of both Rat1 and Xrn1 in the degradation of tRNAVal[Val][AAC] has two important implications. First, this is the only case in which Rat1 and Xrn1 act to degrade a stable mature noncoding RNA species. This is in contrast to the numerous examples in which Rat1 and Xrn1 both act in tRNA and sn(o)RNA processing, tRNA spacer degradation, degradation of certain unspliced prerNA and corresponding lariat introns, and degradation of aberrant extended mRNAs (Henry et al. 1994; Pettfalski et al. 1998; Geerlings et al. 2000; Danin-Kreiselman et al. 2003; Lee et al. 2005), and to the numerous other individual functions of Rat1 or Xrn1 in RNA quality control [Doma and Parker 2007] and pre-mRNA processing [Kim et al. 2004; Luo et al. 2006]. Second, the participation of Rat1 and Xrn1 in degradation of tRNAVal[Val][AAC] implies that tRNA is degraded in both the nucleus and the cytoplasm, since Rat1 is nuclear and Xrn1 is cytoplasmic (Johnson 1997). Since the substrate for degradation is mature, rather than precursor, tRNAVal[Val][AAC], it seems likely that the mature tRNAVal[Val][AAC] degraded by Rat1 is reimported into the nucleus by retrograde flow (Shaheen and Hopper 2005; Takano et al. 2005), which occurs in response to several environmental signals (Hurto et al. 2007; Whitney et al. 2007). Alternatively, it is possible that mature tRNAVal[Val][AAC] is aberrantly distributed in both the nucleus and cytoplasm of trm8-Δ trm4-Δ cells as a consequence of its hypomodified status, or that some Rat1 is present in the cytoplasm.

Although our results show that this RTD pathway acts on a number of hypomodified mature tRNAs, we do not know the exact aminoacylation state of the substrate tRNAs or the manner in which they are recognized. Since mutations that prevent degradation of hypomodified tRNAVal[Val][AAC] also prevent loss of aminoacylation, these phenomena appear to be linked. Moreover, it appears that during tRNAVal[Val][AAC] degradation, the amount of aminoacylated tRNA decreases, whereas the amount of deacylated tRNA remains constant (Figs. 2B, 4A). This observation suggests that the substrate for degradation is aminoacylated tRNA, although it is also consistent with degradation of newly deacylated tRNA. Degradation from the 5’ end would allow aminoacylated tRNA, as well as deacylated tRNA, to be the substrate.

Recognition of the substrate hypomodified tRNAs described here might be effected in a number of ways. First, substrate tRNAs may be recognized because the acceptor helix is thermally less stable as a consequence of the absence of the modifications in the body of the tRNA, allowing increased access to the 5’ end of the tRNA. Second, recognition could occur because the absence of the modifications affects tertiary structure in such a way that a component such as a helicase can bind the tRNA and unwind the 5’ end so that Rat1 and Xrn1 can gain access to the tRNA. A helicase is an essential component of the TRAMP complex that participates in degradation from the 3’ end of pre-tRNA[^1][[^2]][[^3]][[^4]](LaCava et al. 2005; Vanacova et al. 2005; Wang et al. 2008), and it is known that Rat1 and Xrn1 are less active on substrates with helical structure near the 5’ end [Poole and Stevens 1997]. Third, tRNAs lacking the modifications may also be recognized as a consequence of translation. The hypomodified tRNA might be destabilized as it exits the ribosome after translation, exposing the 5’ end for degradation, or it might be targeted for degradation as a consequence of rejection of the tRNA prior to or during translation in the ribosome. We note that degradation of tRNAVal[Val][AAC] in a trm8-Δ trm4-Δ strain can occur in cells treated with cycloheximide to stop translation (Alexandrov et al. 2006), although the rate of degradation is decreased (our unpublished data).

The RTD pathway described here is one of three surveillance pathways that act at different steps of tRNA maturation to maintain the quality of tRNA in the cell. An initial round of surveillance occurs at the pre-tRNA stage, through the action of the TRAMP complex, Rrp6, and the nuclear exosome [Kadaba et al. 2004, 2006; LaCava et al. 2005; Vanacova et al. 2005]. A second level of surveillance may occur at the export stage, by tRNA synthetases acting in the nucleus [Lund and Dahlberg 1998; Sarkar et al. 1999; Grosshans et al. 2000]. The third level of surveillance occurs at the level of mature tRNA by the RTD pathway, as documented here for the degradation of mature hypomodified tRNAVal[Val][AAC], tRNACys[CGA], tRNAPro[UGA], and tRNAVal[^1][[^2]][[^3]][[^4]][[^5]][[^6]][[^7]][[^8]][[^9]][^10]. It is possible that the Trf4/Rrp6 pathway may also act on mature tRNA that lacks modifications or is unstable, based on in vitro analysis (Vanacova et al. 2005), although our data suggest that the Trf4/Rrp6 pathway has a minimal role with the tRNA substrates examined in this work. Thus, it seems that tRNA is under constant surveillance during and after its maturation and subject to multiple quality control pathways. Degradation of mature tRNA by this and other pathways might be used in wild-type cells when tRNA becomes nonfunctional due to destabilization, loss of modifications [Ougland et al. 2004], or chemical damage, to prevent havoc in the cell.

**Materials and methods**

**Yeast strains**

Strains are shown in Supplemental Table S1. The MET22 gene was deleted in the MATα tram8Δ::natMX trm4Δ::URA3MX
(AA0977) and wild-type [BY4742] strains by transformation of the met22-Δ::kanMX DNA and flanking sequences, obtained by PCR amplification from the corresponding knockout strain [OpenBiosystems], using primers MET22_own_F1 and MET22_own_R1 (Supplemental Table S2), followed by selection on YPD media containing 300 µg/mL geneticin (G418, Gibco).

To delete MET22 in the MATa trm44-Δ::natMX tan1-Δ::kanMX [LY1172] and parent strain [Kotelawala et al. 2008], the met22-Δ::kanMX cassette was converted to a met22-Δ::hphMX cassette (Goldstein and McCusker 1999), which was amplified with primers MET22_own_F1 and MET22_own_R1 and transformed into recipient strains.

The XRN1 gene was deleted in two steps. First, the bleA cassette of pUG66 [Guelden et al. 2002] was transformed into the XRN1 locus of BY4742, by PCR amplification using primers containing sequences directly 5' and 3' of XRN1 (Xrn1 - 43 + Phleo and Xrn1 + 43 + Phleo), followed by selection on YPD media containing 8 µg/mL phleomycin. The resulting xrn1-Δ::bleA cassette and flanking region were then amplified with primers XRN1_del_F and XRN1_del_R and transformed into BY4742, AA0582, as well as into the MATa trm8-Δ::natMX trm4-Δ::kanMX rat1-107 strain [ISC610] carrying a URA3 CEN RAT1 plasmid, followed by growth on 5-fluoroorotic acid (FOA) to remove the plasmid. For experiments in Figures 2–5, strains are derived from AA0582, except that the trm8-Δ trm4-Δ met22-Δ strain is derived from AA0977. Other deletions were constructed in essentially the same manner with appropriate primers (Supplemental Table S2), and all deletions were confirmed by PCR.

The rat1-107 allele (rat1-A661E) was introduced as rat1-107::URA3 into LY1172 by transformation of a construct containing rat1-107 and downstream sequence followed by URA3 and the same downstream sequence.

Plasmids

Plasmids containing MET22 [IC 082 and IC 095], RAT1 [JW 009], and XRN1 [JW 007] were constructed by PCR amplification of the corresponding gene and flanking regions using appropriate primers [Supplemental Table S3], followed by restriction digestion and ligation into a URA3 CEN or a LEU2 CEN vector. Plasmids containing RRP6 [IC 256] and TRF4 [IC 269] were described previously [Kadaba et al. 2004]. Multicopy plasmids containing tRNA^Cys[CGC] [IC 022] and tRNA^Met[ACC] [IC 026] were constructed by PCR amplification of the tCGCAIP2 and tR[ACC]D loci and flanking regions with appropriate primers (Supplemental Table S3), followed by digestion and ligation into a 2 µm URA3 vector. A multicopy plasmid containing tRNA^Val[ACC] was described previously [Alexandrov et al. 2006].

Selection for suppressors

To generate spontaneous suppressors of trm8-Δ trm4-Δ mutants, individual colonies of MATa trm8-Δ trm4-Δ [AA0527] and MATa trm8-Δ trm4-Δ [AA0582] haploids were grown overnight at 30°C, and 200 µL were plated on YPD media and incubated for 3 d at 37°C. Complementation groups were analyzed by mating, and the mutant gene was identified using the MORF plasmid library [Gelpérin et al. 2005]. To generate suppressors of the trm8-Δ trm4-Δ mutants that were not met22 mutations, we first introduced an additional copy of the MET22 gene on a URA3 CEN plasmid, which was removed by growth on FOA media before subsequent analysis.

Temperature shift experiments

trm8-Δ trm4-Δ strains were grown at 28°C and shifted to 37°C by rapid mixing with 2 vol of media at 42°C, and samples were collected using vacuum filter units, followed by freezing on dry ice, as described [Alexandrov et al. 2006]. trm44-Δ tan1-Δ strains were grown in YP media containing 3% glycerol at 30°C, diluted to OD 0.15, and shifted to a water shaker at 36.5°C, and samples were harvested by centrifugation.

RNA preparation

Unless stated otherwise, RNA was prepared using hot phenol [Alexandrov et al. 2006]. Aminoacylated tRNA was isolated under acidic conditions [pH 4.5] at 4°C, and samples were decaying as described [Varshney et al. 1991; Alexandrov et al. 2006].

Northern blot analysis and probes

RNA was separated by 10% PAGE in 8 M urea and TBE buffer, or by 6.5% PAGE for 18 h at 4°C in 8 M urea and 0.1 M sodium acetate [pH 4.5], followed by transfer to Hybond N+ membrane, UV cross-linking, and hybridization with 5′-32P-labeled DNA probes [Supplemental Table S4], as described [Alexandrov et al. 2006].

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