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$^{Val(AAC)}$ 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$^{Val(AAC)}$ in a *trm8*-Δ *trm4*-Δ strain and result in healthy growth at 37°C, hypomodified tRNA$^{Val(AAC)}$ 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$^{Val(AAC)}$]

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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). Many 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-

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tions are also important for correct charging by aminoacyl tRNA synthetases, as described in yeast for m^1G_{37} of $tRNA^{Asp}$ (Putz et al. 1994) and in *Escherichia coli* for s^2U_{34} of $tRNA^{Glu}$ and $lysidine_{34}$ 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-$tRNA_i^{Met}$ lacking m^1A_{58} due to mutation of *GCD10* or *GCD14* is degraded by Rrp6 and the nuclear exosome, after polyadenylation by Trf4 (Kadaba et al. 2004, 2006), a component of the TRAMP complex, which also includes Air1/Air2 and Mtr4 (LaCava et al. 2005; Vanacova et al. 2005; Wyers et al. 2005; Wang et al. 2008). Second, mature $tRNA^{Val(AAC)}$ lacking m^7G_{46} and m^5C_{49} 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*-Δ *trm4*-Δ 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^5U_{54} or Ψ_{55} due to deletion of *TRM2* or *PUS4* (Johansson and Bystrom 2002); and reduced levels of $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ are observed at high temperature in strains lacking Um_{44} and ac^4C_{12} due to deletion of *TRM44* and *TAN1* (Fig. 1; Kotelawala et al. 2008). Thus, although tRNA is among the most stable RNA species in vivo, it appears to undergo turnover both during and after biosynthesis when the sequence or modifications of the tRNA are altered.

In this study, we investigate the components of the RTD pathway by which $tRNA^{Val(AAC)}$ lacking m^7G_{46} and m^5C_{49} is rapidly degraded and deacylated in a *trm8*-Δ *trm4*-Δ 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′ bisphosphate (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*-Δ *trm4*-Δ 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.

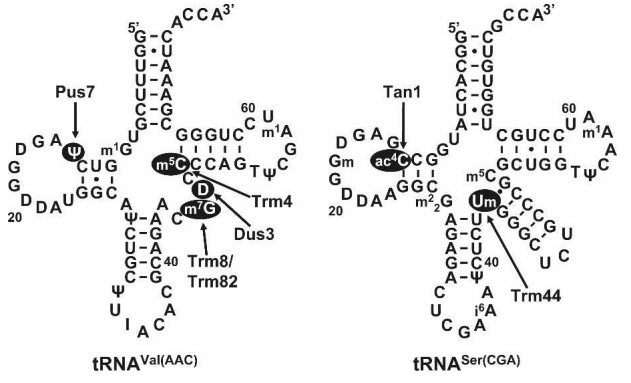

Figure 1. Schematic of the secondary structure of $tRNA^{Val(AAC)}$ and $tRNA^{Ser(CGA)}$. tRNA sequences are as described (Sprinzl et al. 1999). Modifications described in the text are highlighted, and the proteins that catalyze their formation are indicated.

Results

Mutation of MET22 *suppresses the temperature sensitivity of a* trm8-Δ trm4-Δ *strain*

We have shown previously that degradation of hypomodified $tRNA^{Val(AAC)}$ in *trm8*-Δ *trm4*-Δ 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*-Δ *trm4*-Δ 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*-Δ *trm4*-Δ 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*-Δ *trm4*-Δ *met22*-Δ strain grows as well on plates at 37°C as the original revertant 13 strain (now named *trm8*-Δ *trm4*-Δ *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

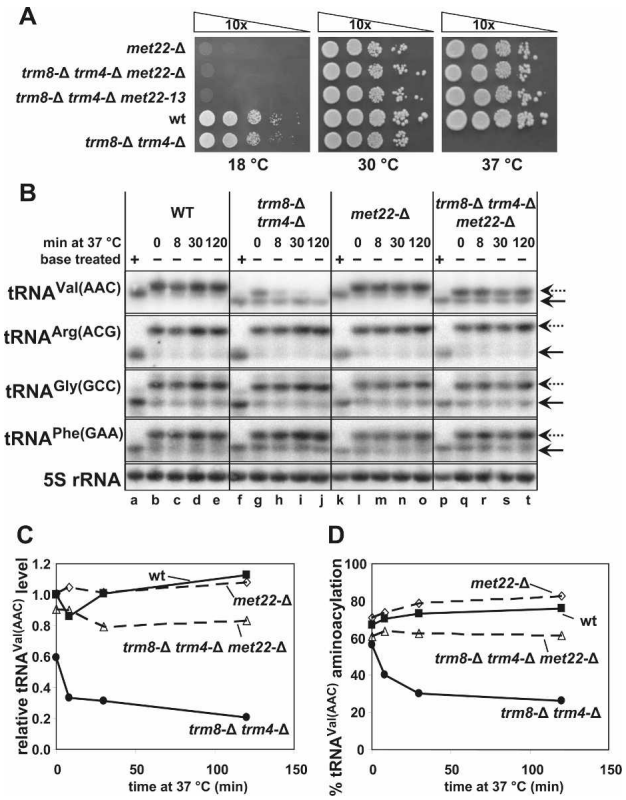

Figure 2. *met22* mutations suppress the temperature-sensitive growth defect of *trm8*-Δ *trm4*-Δ mutants and prevent degradation and loss of aminoacylation of $tRNA^{Val(AAC)}$. (*A*) Mutation of *MET22* in the *trm8*-Δ *trm4*-Δ strain allows growth at 37°C. Strains were grown overnight in YPD at 28°C, adjusted to OD_{600} ~ 1, serially 10-fold-diluted, spotted on YPD plates, and incubated at 18°C, 30°C, and 37°C, as indicated. (*B*) $tRNA^{Val(AAC)}$ levels and aminoacylation are stable in the *trm8*-Δ *trm4*-Δ *met22*-Δ strain. Strains were grown in YPD at 28°C to OD_{600} ~ 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 $tRNA^{Val(AAC)}$ from strains lacking *TRM8* migrates faster than from other strains. (*C*) Quantification of the levels of $tRNA^{Val(AAC)}$. The ordinate shows the ratio of the levels of $tRNA^{Val(AAC)}$ at each time point relative to its level in the wild-type strain immediately before temperature shift (each value itself first normalized to 5S RNA). (*D*) Quantification of the percentage of aminoacylation of $tRNA^{Val(AAC)}$.

conclude that 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* $tRNA^{Val(AAC)}$

To determine if *met22*-mediated suppression of the *trm8*-Δ *trm4*-Δ growth defect is due to an effect on the amount of functional $tRNA^{Val(AAC)}$, we analyzed the levels and the aminoacylation status of $tRNA^{Val(AAC)}$ 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, $tRNA^{Val(AAC)}$ is present at only 20% of wild-type levels and is only 25% aminoacylated (Fig. 2B [lane j], C,D). By contrast, $tRNA^{Val(AAC)}$ 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 $tRNA^{Val(AAC)}$ 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 $tRNA^{Val(AAC)}$, 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* $tRNA^{Val(AAC)}$

The known biochemical function of Met22 suggests that it is not directly responsible for degradation and loss of aminoacylation of $tRNA^{Val(AAC)}$ 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 byproduct 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 $tRNA^{Val(AAC)}$ degradation in *trm8*-Δ *trm4*-Δ mutants and to identify other possible mechanisms by which $tRNA^{Val(AAC)}$ 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

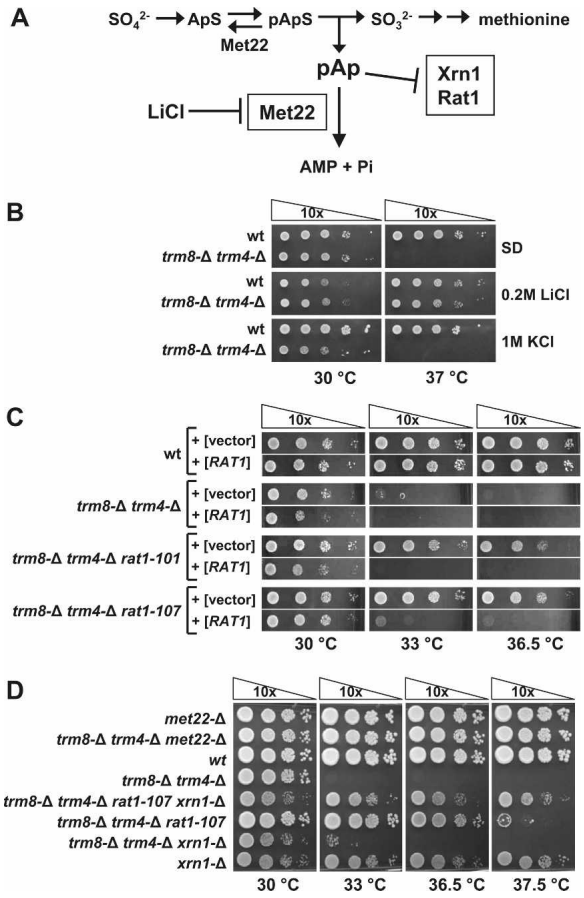

Figure 3. Mutation of *RAT1* and *XRN1* suppresses the temperature-sensitive phenotype of *trm8*-Δ *trm4*-Δ mutants. (*A*) Schematic of the role of Met22 in the sulfate assimilation pathway. (*B*) The temperature-sensitive phenotype of *trm8*-Δ *trm4*-Δ mutants is suppressed on media containing LiCl. Wild-type and *trm8*-Δ *trm4*-Δ strains were grown in YPD at 28°C, plated as in Figure 1 on SD media containing 0.2M LiCl or 1M KCl, and incubated at 30°C or 37°C. (*C*) *RAT1* complements suppressors of *trm8*-Δ *trm4*-Δ strain. *trm8*-Δ *trm4*-Δ 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 *trm8*-Δ *trm4*-Δ temperature-sensitive phenotype. Strains were grown in YPD at 28°C and plated on YPD media at indicated temperatures.

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 *trm8*-Δ *trm4*-Δ *rat1* suppressors (Supplemental Fig. S2). Since *RAT1* is essential (Amberg et al. 1992), it was not possible to analyze a *rat1*-Δ mutant.

Although the *rat1-A661E* allele in the *rat1-101* and *rat1-107* mutants is the strongest allele isolated and allows the *trm8*-Δ *trm4*-Δ 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 *trm8*-Δ *trm4*-Δ *met22*-Δ strain (Fig. 3D). Since *RAT1* and *XRN1* have been shown previously to have redundant roles in a number of rRNA and sn(o)RNA processing events, as well as in mRNA degradation (Henry et al. 1994; Petfalski et al. 1998; Geerlings et al. 2000; Danin-Kreiselman et al. 2003; Lee et al. 2005), we introduced an *xrn1*-Δ deletion into both the *trm8*-Δ *trm4*-Δ strain and the *trm8*-Δ *trm4*-Δ *rat1-107* strain to test the combined effects of Rat1 and Xrn1 on this tRNA degradation pathway. We find that the *trm8*-Δ *trm4*-Δ *rat1-107 xrn1*-Δ strain grows significantly better than the corresponding *trm8*-Δ *trm4*-Δ *rat1-107* strain at 37.5°C, whereas the *trm8*-Δ *trm4*-Δ *xrn1*-Δ mutant only grows modestly better than its *trm8*-Δ *trm4*-Δ 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 *trm8*-Δ *trm4*-Δ strain.

Rat1 and Xrn1 both appear to mediate the degradation and the loss of aminoacylation of $tRNA^{Val(AAC)}$ in a *trm8*-Δ *trm4*-Δ strain. In a *trm8*-Δ *trm4*-Δ *rat1-107 xrn1*-Δ mutant strain, there is no observed degradation of $tRNA^{Val(AAC)}$ (Fig. 4A [lanes h–k], B), and the aminoacylation levels

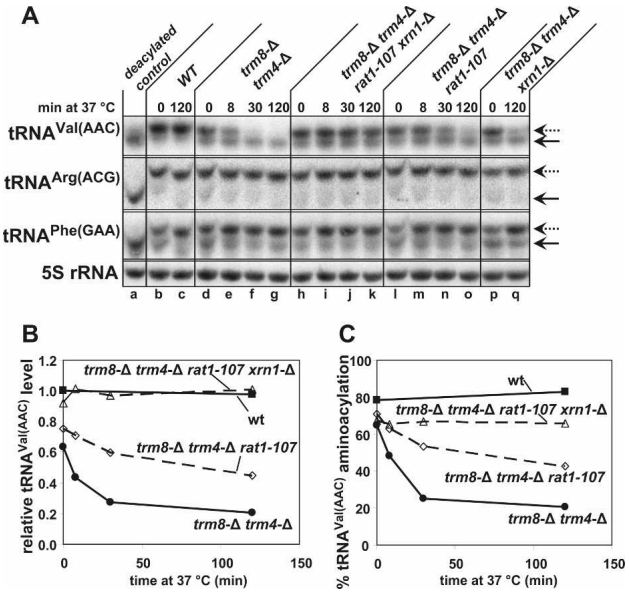

Figure 4. Both Rat1 and Xrn1 contribute to degradation of $tRNA^{Val(AAC)}$ in the *trm8*-Δ *trm4*-Δ strain. (*A*) $tRNA^{Val(AAC)}$ is stable in *trm8*-Δ *trm4*-Δ mutants containing mutations in *RAT1* and *XRN1*. Strains were grown in YPD at 28°C to OD_{600} ~ 1.5 and shifted to 37°C, and cells were harvested at the indicated times. RNA (5 µg) isolated under acidic conditions was analyzed by Northern blotting. One sample was deacylated prior to gel electrophoresis. Dashed and solid arrows indicate aminoacylated and deacylated tRNA species, respectively. (*B*) Quantification of the levels of $tRNA^{Val(AAC)}$. tRNA is quantified as in Figure 2. (*C*) Quantification of the percentage of aminoacylation of $tRNA^{Val(AAC)}$.

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 $tRNA^{Val(AAC)}$. Thus, in a *trm8*-Δ *trm4*-Δ *rat1-107* strain or a *trm8*-Δ *trm4*-Δ *xrn1*-Δ strain, the levels of $tRNA^{Val(AAC)}$ 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 $tRNA^{Val(AAC)}$ in the *trm8*-Δ *trm4*-Δ strain, although indirect effects cannot be excluded.

The rat1-107 *allele acts primarily by preventing degradation of mature $tRNA^{Val(AAC)}$ 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 $tRNA^{Val(AAC)}$ in the *trm8*-Δ *trm4*-Δ *rat1-107 xrn1*-Δ strain was due to a transcription effect. To test this, we examined $tRNA^{Val(AAC)}$ 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-$tRNA^{Phe(GAA)}$ and pre-$tRNA^{Tyr(GUA)}$ species (Fig. 5A). However, since levels of $tRNA^{Val(AAC)}$ and control tRNAs are similar in the presence or absence of thiolutin in wild-type and *trm8*-Δ *trm4*-Δ *rat1-107 xrn1*-Δ 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 $tRNA^{Val(AAC)}$ transcription but instead stabilizes $tRNA^{Val(AAC)}$ levels in the *trm8*-Δ *trm4*-Δ *rat1-107 xrn1*-Δ strain by preventing its degradation.

This experiment also underscores two points about the $tRNA^{Val(AAC)}$ degradation in *trm8*-Δ *trm4*-Δ mutants at 37°C. First, since $tRNA^{Val(AAC)}$ 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 $tRNA^{Val(AAC)}$ 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 $tRNA^{Val(AAC)}$ degradation is too fast to be accounted for by degradation of pre-tRNA (Alexandrov et al. 2006), as occurs for pre-$tRNA_i^{Met}$ in the Trf4/Rrp6 pathway (Kadaba et al. 2004, 2006). Second, since $tRNA^{Val(AAC)}$ 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 $tRNA^{Val(AAC)}$ levels remaining 2 h after thiolutin treatment compared with 25% in untreated cells (Fig. 5A [cf. lanes l and p], B), we conclude that the full extent of $tRNA^{Val(AAC)}$ degradation is masked by transcription of new $tRNA^{Val(AAC)}$. We had previously speculated that the slow phase of $tRNA^{Val(AAC)}$ 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 $tRNA^{Val(AAC)}$ or to compensatory synthesis of new tRNA (Alexandrov et al. 2006). The disappearance of almost all of the $tRNA^{Val(AAC)}$ 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 $tRNA^{Val(AAC)}$ lacking m^7G_{46} and m^5C_{49} 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-$tRNA_i^{Met}$ in mutants lacking m^1A_{58} (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

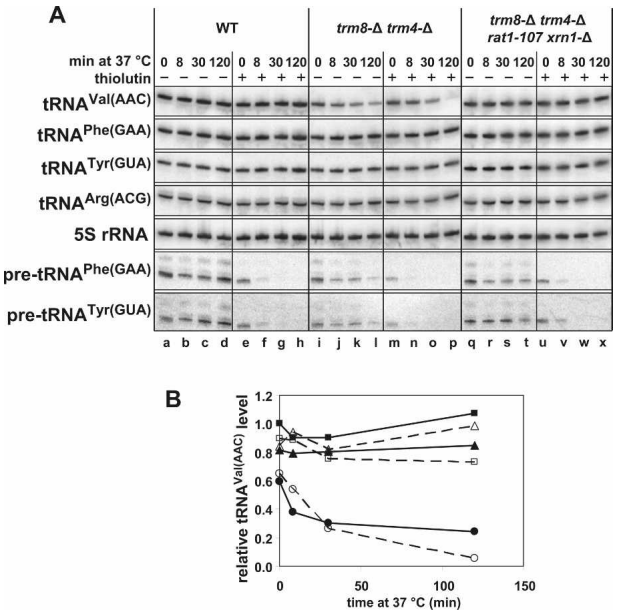

Figure 5. Transcription inhibition does not affect $tRNA^{Val(AAC)}$ levels in a *trm8*-Δ *trm4*-Δ *rat1-107 xrn1*-Δ strain. (*A*) Northern blot analysis of tRNA after treatment with thiolutin. Strains were grown at 28°C to OD_{600} ~ 1.5, treated with 5 µg/mL thiolutin for 10 min, and then shifted to 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 $tRNA^{Val(AAC)}$. 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*-Δ 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^7G_{46} and $\Psi_{13,35}$ 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^7G_{46} and D_{47} (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).

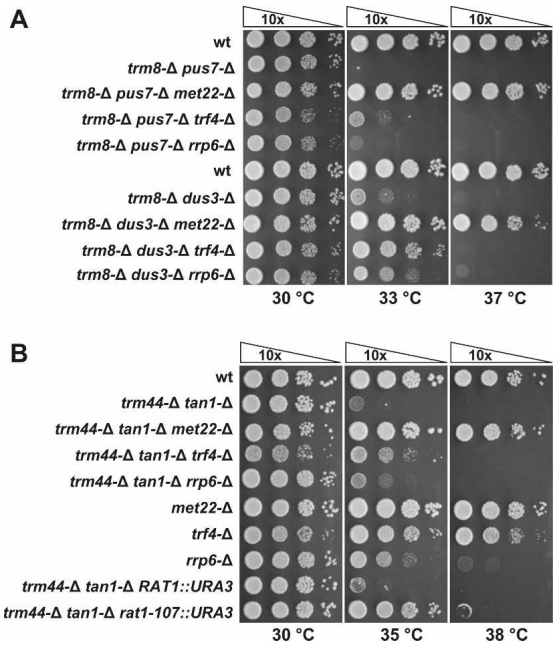

Figure 6. Deletion of *MET22* suppresses the temperature-sensitive phenotypes of multiple strains lacking different sets of tRNA modifications. (*A*) A *met22*-Δ mutation rescues growth of *trm8*-Δ *pus7*-Δ and *trm8*-Δ *dus3*-Δ mutants at high temperature. Strains were grown at 28°C and plated on YPD 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*.

Third, a *met22*-Δ mutation suppresses the growth phenotype of a *trm44*-Δ *tan1*-Δ strain, which lacks Um_{44} and ac^4C_{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.

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–f], B). Consistent with degradation by the RTD

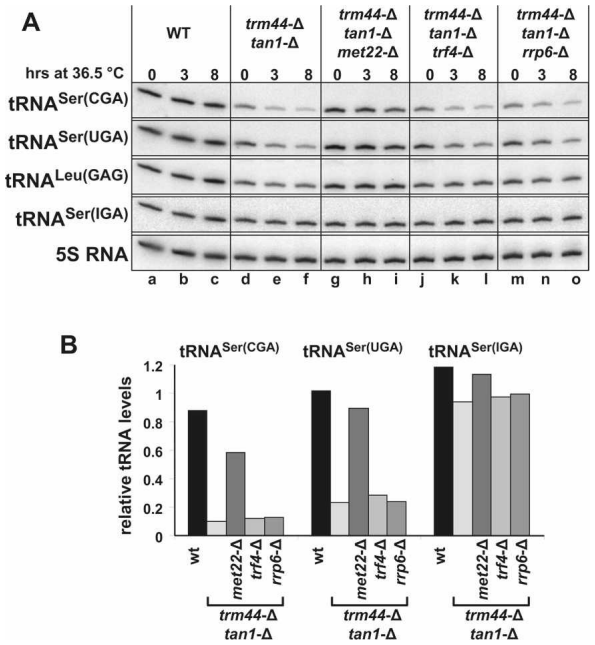

Figure 7. Deletion of *MET22* stabilizes $tRNA^{Ser(CGA)}$, $tRNA^{Ser(UGA)}$, and $tRNA^{Leu(GAG)}$ in *trm44*-Δ *tan1*-Δ mutants. (*A*) Northern blot analysis of the effect of *met22*-Δ, *trf4*-Δ, and *rrp6*-Δ mutations on tRNA levels in *trm44*-Δ *tan1*-Δ mutants. Strains were grown in YP glycerol media at 30°C and shifted to 36.5°C for the indicated times. RNA (1.5 µg) was analyzed by Northern blotting. (*B*) Quantification of the levels of tRNA species 8 h after temperature shift. For $tRNA^{Ser(CGA)}$, $tRNA^{Ser(UGA)}$, and $tRNA^{Ser(IGA)}$, the bars indicate the ratio of tRNA levels after 8 h at 36.5°C, relative to levels in the wild-type strain before temperature shift (each value itself first normalized to 5S RNA).

pathway, levels of $tRNA^{Ser(CGA)}$ in the *trm44*-Δ *tan1*-Δ *met22*-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 $tRNA^{Ser(CGA)}$ are reduced as quickly and completely in the *trm44*-Δ *tan1*-Δ *trf4*-Δ strain and the *trm44*-Δ *tan1*-Δ *rrp6*-Δ strain as in the parent *trm44*-Δ *tan1*-Δ strain (Fig. 7A,B). Similar results are observed for $tRNA^{Ser(UGA)}$ levels (Fig. 7A,B). We note also that levels of $tRNA^{Leu(GAG)}$, which are modestly reduced in the *trm44*-Δ *tan1*-Δ parent strain (Kotelawala et al. 2008), are restored in the *trm44*-Δ *tan1*-Δ *met22*-Δ strain but not in the *trm44*-Δ *tan1*-Δ *trf4*-Δ strain or the *trm44*-Δ *tan1*-Δ *rrp6*-Δ strain, whereas levels of the control $tRNA^{Ser(IGA)}$ 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 $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ in a *trm44*-Δ *tan1*-Δ strain and that the Trf4/Rrp6 pathway plays at most a minor role in this process.

The slow loss of $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ observed in the *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 $tRNA^{Val(AAC)}$ in the *trm8*-Δ *trm4*-Δ mutant, loss of tRNA species in the *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 $tRNA^{Ser(CGA)}$, $tRNA^{Ser(UGA)}$, and $tRNA^{Leu(GAG)}$ species at high temperature in the *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 *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 f–j], B,C). Since mature $tRNA^{Ser(CGA)}$, $tRNA^{Ser(UGA)}$, and $tRNA^{Leu(GAG)}$, rather than the corresponding pre-tRNA species, are degraded in the *trm44*-Δ *tan1*-Δ mutant strain and since deletion of *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 $tRNA^{Val(AAC)}$ in *trm8*-Δ *trm4*-Δ mutant strains.

Discussion

We identified Met22, Rat1, and Xrn1 as components of the RTD pathway by which mature $tRNA^{Val(AAC)}$ lacking m^7G_{46} and m^5C_{49} is rapidly degraded and deacylated at 37°C in a *trm8*-Δ *trm4*-Δ strain. Introduction of a *met22*-Δ mutation to a *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 hypomodified $tRNA^{Val(AAC)}$. Mutant alleles of *RAT1*, encoding the essential 5′–3′ exonuclease Rat1, are efficient, but less robust, suppressors of the *trm8*-Δ *trm4*-Δ temperature-sensitive phenotype and partially prevent degradation and deacylation of $tRNA^{Val(AAC)}$ at 37°C. Additional deletion of *XRN1,* encoding the other known 5′–3′ exonuclease, in a *trm8*-Δ *trm4*-Δ *rat1-107* strain leads to robust suppression of the growth defect and complete stabilization of valyl-$tRNA^{Val(AAC)}$ at 37°C, suggesting that both Rat1 and Xrn1 participate in degradation and deacylation 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 *met22*-Δ mutation suppresses the temperature-sensitive growth defect of several mutants: a *trm8*-Δ *pus7*-Δ strain, which lacks m^7G_{46} and $\Psi_{13,35}$ and is temperature-sensitive due to loss of $tRNA^{Val(AAC)}$; a *trm8*-Δ *dus3*-Δ strain, which lacks m^7G_{46} and D_{47} and has reduced levels of $tRNA^{Val(AAC)}$ and likely one or more other tRNA species; and a *trm44*-Δ *tan1*-Δ strain, which lacks Um_{44} and ac^4C_{12} and is temperature-sensitive due to loss of $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$. Since the *rat1-107* mutation also suppresses the temperature sensitivity of a *trm44*-Δ *tan1*-Δ strain at 35°C, it is likely that suppression by *met22*-Δ mutation is due to inhibition of tRNA degradation by

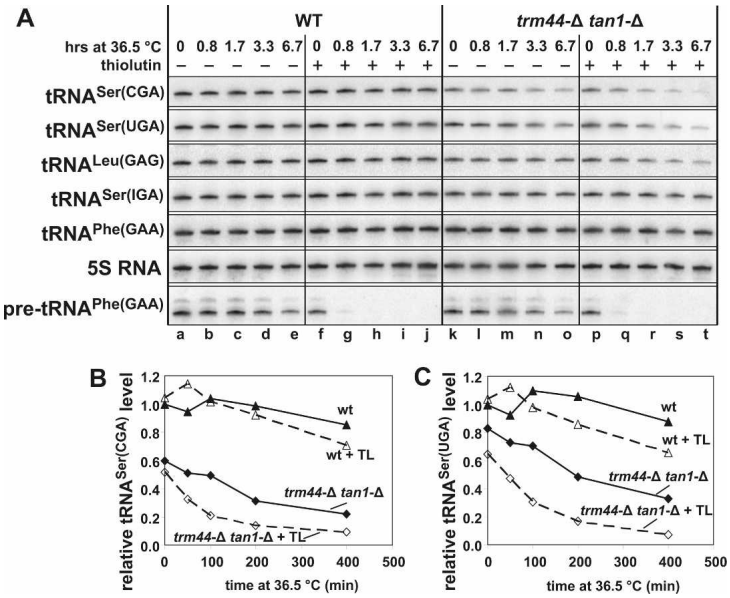

Figure 8. Mature $tRNA^{Ser(CGA)}$, $tRNA^{Ser(UGA)}$, and $tRNA^{Leu(GAG)}$ are degraded in the *trm44*-Δ *tan1*-Δ strain. (*A*) Northern blot analysis of tRNA in wild-type and *trm44*-Δ *tan1*-Δ strains after thiolutin treatment. Strains were grown in YP glycerol at 30°C to OD_{600} ~ 0.16, treated with 5 µg/mL thiolutin (TL) for 5 min at 30°C, and shifted to 36.5°C for indicated times. Two micrograms of RNA were analyzed by Northern blotting. (*B*,*C*) Quantification of the levels of $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$. tRNA levels quantified as described in Figure 2.

the RTD pathway. In support of this, the *met22*-Δ mutation prevents the degradation of mature $tRNA^{Ser(CGA)}$, $tRNA^{Ser(UGA)}$, and $tRNA^{Leu(GAG)}$ in a *trm44*-Δ *tan1*-Δ strain, and degradation occurs at the level of mature tRNA. We presume that the tRNAs affected in a *trm8*-Δ *dus3*-Δ strain and a *trm8*-Δ *pus7*-Δ 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 nonfunctional.

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 $tRNA^{Val(AAC)}$ lacking m^7G_{46} and m^5C_{49}, no other pathway acts at a detectable rate to degrade the mature tRNA, based on analysis of tRNA levels in a *trm8*-Δ *trm4*-Δ *rat1-107 xrn1*-Δ 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 rRNA containing mutations that adversely affect translation are subject to a quality control pathway leading to degradation of their rRNA, 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 *met22*-Δ mutation leads to near wild-type growth at high temperature of a *trm8*-Δ *trm4*-Δ strain, a *trm44*-Δ *tan1*-Δ strain, a *trm8*-Δ *pus7*-Δ strain, and a *trm8*-Δ *dus3*-Δ strain, which implies that the otherwise degraded tRNAs are functional at this temperature. Second, in the case of a *trm8*-Δ *trm4*-Δ strain, suppression by either a *met22*-Δ mutation or by *rat1-107 xrn1*-Δ mutations leads to nearly complete aminoacylation of $tRNA^{Val(AAC)}$ 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°C in the *trm8*-Δ *trm4*-Δ and *trm44*-Δ *tan1*-Δ mutants, and the tRNAs are restored to wild-type levels at this temperature in the corresponding *met22*-Δ 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 nonfunctional 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*-Δ mutants, and *rat1-107 xrn1*-Δ mutants, are equally effective in preventing the degradation and the loss of aminoacylation of $tRNA^{Val(AAC)}$. 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 $tRNA^{Val(AAC)}$ 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 rRNA and sn(o)RNA processing, rRNA spacer degradation, degradation of certain unspliced pre-mRNAs and corresponding lariat introns, and degradation of aberrant extended mRNAs (Henry et al. 1994; Petfalski 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 $tRNA^{Val(AAC)}$ 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, $tRNA^{Val(AAC)}$, it seems likely that the mature $tRNA^{Val(AAC)}$ 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 $tRNA^{Val(AAC)}$ 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 $tRNA^{Val(AAC)}$ also prevent loss of aminoacylation, these phenomena appear to be linked. Moreover, it appears that during $tRNA^{Val(AAC)}$ 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-$tRNA_i^{Met}$ lacking m^1A_{58} (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 $tRNA^{Val(AAC)}$ 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 $tRNA^{Val(AAC)}$, $tRNA^{Ser(CGA)}$, $tRNA^{Ser(UGA)}$, and $tRNA^{Leu(GAG)}$. 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 trm8*-Δ::*natMX trm4*-Δ::*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 *MAT*a *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 ble^R cassette of pUG66 (Gueldener 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::ble^R 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 *MAT*α *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* (B256) and *TRF4* (B269) were described previously (Kadaba et al. 2004). Multicopy plasmids containing $tRNA^{Cys(GCA)}$ (IC 022) and $tRNA^{Arg(ACG)}$ (IC 026) were constructed by PCR amplification of the tC(GCA)P2 and tR(ACG)D loci and flanking regions with appropriate primers (Supplemental Table S3), followed by digestion and ligation into a 2µ *URA3* vector. A multicopy plasmid containing $tRNA^{Val(AAC)}$ 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 *MAT*α *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 (Gelperin 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 deacylated 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′ ^{32}P-labeled DNA probes (Supplemental Table S4), as described (Alexandrov et al. 2006).

Acknowledgments

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