Nuclear surveillance and degradation of hypomodified initiator tRNA^{Met} in *S. cerevisiae*

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The tRNA m^{1}A58 methyltransferase is composed of two subunits encoded by the essential genes *TRM6* and *TRM61* (formerly *GCD10* and *GCD14*). The *trm6-504* mutation results in a defective m^{1}A methyltransferase (Mtase) and a temperature-sensitive growth phenotype that is attributable to the absence of m^{1}A58 and consequential tRNA$_i^{Met}$ instability. We used a genetic approach to identify the genes responsible for tRNA$_i^{Met}$ degradation in *trm6* cells. Three recessive extragenic mutations that suppress *trm6-504* mutant phenotypes and restore hypomodified tRNA$_i^{Met}$ to near normal levels were identified. The wild-type allele of one suppressor, *DIS3/RRP44*, encodes a 3′–5′ exoribonuclease and a member of the multisubunit exosome complex. We provide evidence that a functional nuclear exosome is required for the degradation of tRNA$_i^{Met}$ lacking m^{1}A58. A second suppressor gene encodes Trf4p, a DNA polymerase (pol σ) with poly(A) polymerase activity. Whereas deletion of *TRF4* leads to stabilization of tRNA$_i^{Met}$, overexpression of Trf4p destabilizes the hypomodified tRNA$_i^{Met}$ in *trm6* cells. The hypomodified, but not wild-type, pre-tRNA$_i^{Met}$ accumulates as a polyadenylated species, whose abundance and length distribution both increase upon Trf4p overexpression. These data indicate that a tRNA surveillance pathway exists in yeast that requires Trf4p and the exosome for polyadenylation and degradation of hypomodified pre-tRNA$_i^{Met}$.

[*Keywords*: Transfer RNA; exosome; RNA processing; RNA turnover; tRNA modification]

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The relatively unstable nature of messenger RNAs fueled the discovery of pathways that control the degradation of normal and abnormal mRNAs in the nucleus and cytoplasm (Hilleren and Parker 1999; Mitchell and Tollervey 2001; Wilusz et al. 2001; Maquat 2002; Moore 2002; Long and McNally 2003). Two general pathways of mRNA decay have been characterized in the yeast *Saccharomyces cerevisiae*, and homologs of most of the yeast proteins involved in mRNA turnover have been identified in metazoans. The first pathway initially requires shortening of the mRNA polyadenylate tail, followed by removal of the 5′ cap structure (Wilusz et al. 2001), which leaves the body of the mRNA susceptible to 5′–3′ exonucleolytic degradation by Xrn1p. The second pathway involves deadenylation of mRNAs and the 3′–5′ degradation of the body of the mRNA by the exosome (Jacobs et al. 1998; Burkard and Butler 2000; van Hoof et al. 2000b; van Hoof and Parker 2002; Mitchell and Tollervey 2003).

The exosome is a multisubunit complex of proteins with multiple functions in the processing, degradation, and retention of stable and unstable RNAs in the nucleus and cytoplasm. The cytoplasmic exosome directly interacts with Ski7p (Araki et al. 2001) and recruits the Ski2p, Ski3p, and Ski8p complex to the 3′ end of a deadenylated mRNA (Brown et al. 2000) or an mRNA that is stalled on the ribosome because it lacks a stop codon (Jacobs et al. 1998; van Hoof et al. 2000b), and in turn each is degraded in a 3′-to-5′ direction. In the nucleus, the exosome has been implicated in elimination of by-products of rRNA processing (ETS sequence). The nuclear exosome possesses an exonuclease, Rrp6p, not found in the cytoplasmic form (Allmang et al. 1999b). A specialized function of Rrp6p and the nuclear exosome appears to be in retaining mRNAs incorrectly processed at their 3′ ends at the site of transcription to prevent their release into the cytoplasm (Hilleren et al. 2001; Libri et al. 2002). Thus far, the exosome has not been implicated in the destruction of stable RNAs that are rendered unstable due to mutations or defects in processing.

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In contrast to mRNA, tRNA and rRNA are highly stable RNA species with half-lives measured in days. The longevity of tRNA and rRNA can be disrupted by mutations in the RNA themselves (Eisenberg and Yarus 1980; Colby et al. 1981; Yuo and Weiner 1989; Eschenlauer et al. 1993; Johansson and Bystrom 2002) or by inactivation of a protein required for the processing or modification of a normally stable RNA (Anderson et al. 1998). Recent studies have shown that precursor tRNAs (pre-tRNAs) rendered unstable because of a mutation activate a surveillance mechanism in *Escherichia coli* that results in 3′ adenylation and degradation of the precursor form of the mutant tRNA. This degradation mechanism requires poly(A) polymerase and the exoribonuclease PNpase (Li et al. 2002). In *E. coli*, the degradation of mRNA also involves, but is not limited to, poly(A) polymerase and PNpase (Kushner 2002). The fact that poly(A) polymerase and PNpase play an active role in mRNA decay and tRNA degradation suggests that aberrant pre-tRNA degradation and mRNA turnover share similar mechanisms in *E. coli*.

The processing of eukaryotic pre-tRNA is a multistep pathway consisting of 5′ end cleavage by RNaseP, intron removal, 3′ end-trimming by endo- and/or exonucleases, CCA addition to the 3′ end, and ribose or nucleotide modification (Hopper and Phizicky 2003). One such nucleotide modification is the addition of a methyl group to the N^1 position of adenosine at position 58 of the T Ψ C loop. The yeast 1-methyladenosine 58 methyltransferase (m^1A58 Mtase) is composed of two proteins encoded by genes essential for cell viability (Garcia-Barrio et al. 1995; Cuesta et al. 1998). Mutation of the genes *TRM6* (formerly named *GCD10*) or *TRM61* (formerly named *GCD14*) encoding the m^1A Mtase lead to $tRNA_i^{Met}$ instability, supporting the postulate that m^1A58, along with several other modified nucleosides found in tRNA from all three kingdoms, is important for tRNA structure or function (Bjork 1995). Apart from the requirement for aminoacylation of some tRNAs prior to nucleocytoplasmic transport (Lund and Dahlberg 1998; Sarkar et al. 1999; Grosshans et al. 2000), there are no known surveillance mechanisms to ensure the structural or functional integrity of nuclear tRNA processing events prior to nucleocytoplasmic transport.

We previously showed that the defect in $tRNA_i^{Met}$ expression in *trm6* or *trm61* mutants due to a lack of (m^1A58) is overcome by increasing the copy number of *IMT4*, encoding $tRNA_i^{Met}$, showing that increasing the level of $tRNA_i^{Met}$ transcription is one mechanism of suppression. We also found that increasing the copy number of *LHP1*, encoding the tRNA 3′-end processing factor Lhp1p, augmented the synthesis of $tRNA_i^{Met}$ or stabilized pre-$tRNA_i^{Met}$ during processing (Anderson et al. 1998; Calvo et al. 1999). The instability of pre-$tRNA_i^{Met}$ lacking m^1A58 is unique in that no other tRNAs exhibit instability in strains possessing an inactivated m^1A Mtase (Anderson et al. 1998). The three-dimensional structure of $tRNA_i^{Met}$ from *S. cerevisiae* has been solved by X-ray crystallography (Basavappa and Sigler 1991). Those authors showed that adenosines at positions 20, 54, and 60 (rarely found in noninitiator tRNAs in eukaryotes) along with m^1A58 in $tRNA_i^{Met}$ form a substructure that is predicted to be crucial for the maintenance of D- and T-loop interactions. Based on these data, we postulated that the instability of $tRNA_i^{Met}$ occurs from a weakened tertiary structure through disruption of normal D- and T-loop interactions, which stem from the absence of 1-methyl on adenosine 58 (Anderson et al. 1998). Because the substructure described is unlikely to be represented in elongator tRNAs from *S. cerevisiae*, the initiator $tRNA^{Met}$ is unique in exhibiting instability in the absence of m^1A58. Having determined that the absence of m^1A58 from pre-$tRNA_i^{Met}$ leads to its degradation, we reasoned that impairing the ability of the cell to degrade pre-$tRNA_i^{Met}$ lacking m^1A58 should restore normal levels of mature hypomodified $tRNA_i^{Met}$ in *trm6* cells. In this report, we outline a pathway in yeast for the degradation of $tRNA_i^{Met}$ lacking m^1A58 by identifying mutations in *TRF4* and *DIS3/RRP44* as spontaneous suppressors of the *trm6-504* Ts^- phenotype. Trf4p was first identified as a DNA polymerase, along with its redundant homolog Trf5p, in *S. cerevisiae* (Castano et al. 1996b; Wang et al. 2000). *DIS3/RRP44* was first identified as a homolog of *Schizosaccharomyces pombe DIS3* (Noguchi et al. 1996), and later as a component of a purified exosome fraction from *S. cerevisiae* (Mitchell et al. 1997). Dis3p/Rrp44p is a member of the RNR superfamily of exoribonucleases (Zuo and Deutscher 2001), and the purified protein possesses 3′–5′ riboexonuclease activity in vitro (Mitchell et al. 1997). Our results show that mutation of *TRF4* or *DIS3/RRP44* restores mature hypomodified $tRNA_i^{Met}$ to near normal levels. By blocking degradation of $tRNA_i^{Met}$ lacking m^1A58, we found that the hypomodified molecule accumulates as an adenylated precursor, whose abundance and size distribution increase upon Trf4p overexpression. These findings establish that Trf4p and the exosome are functioning together in the nucleus to degrade the aberrant pre-$tRNA_i^{Met}$ lacking m^1A58.

Results

Extragenic suppressors of trm6-504

We took a genetic approach to identify the molecular pathway responsible for the degradation of pre-$tRNA_i^{Met}$ lacking m^1A58 by isolating second-site mutations that suppress the growth defect associated with the *trm6-504* mutation in the m^1A Mtase. This mutation confers a temperature-sensitive (Ts^-) growth phenotype at 36°C, and resistance to the drug 3-aminotriazole ($3AT^r$; Harashima and Hinnebusch 1986). 3-AT is an inhibitor of the His3p protein; in this context 3-AT simulates amino acid starvation and induces the general control pathway through activation of the Gcn2p kinase (Wolfner et al. 1975), and the attendant induction of *GCN4* mRNA translation (Hinnebusch 1997). The *trm6-504* mutant bypasses the requirement for Gcn2p to induce the general control pathway because of reduced $tRNA_i^{Met}$ levels, and thus a *trm6-504* mutant exhibits a constitutive 3-AT resistant ($3\text{-}AT^r$) phenotype in the absence of

Gcn2p function (Harashima and Hinnebusch 1986). Increasing the level of mature hypomodified $tRNA_i^{Met}$ in a *trm6 gcn2* mutant reverses the 3-AT^r phenotype to yield a 3-AT-sensitive phenotype (3-AT^s; Anderson et al. 1998). We isolated 150 spontaneous revertants of the Ts$^-$ and 3AT^r growth phenotypes of the *trm6-504 gcn2-101* mutant, of which 51 confer a cold-sensitive (Cs$^-$) phenotype at 16°C. Initial genetic characterization revealed that all 51 Cs$^-$ revertants contained recessive suppressor mutations. Upon tetrad dissection of revertants backcrossed to the *trm6-504 gcn2-101* parental strain, the Cs$^-$ phenotype always cosegregated 2:2 with suppression of *trm6-504*, indicating that suppression is linked to the Cs$^-$ phenotype and due to mutation of a single suppressor gene. We assigned the 51 Cs$^-$ suppressors to three complementation groups, designated *sup1* to *sup3*, and the phenotypes of representatives of each complementation group are shown in Figure 1. We ruled out the possibility that suppression was due to intragenic reversion of *TRM6*, by showing that a wild-type copy of *TRM6* on a single-copy plasmid failed to complement the recessive Cs$^-$ phenotype of each complementation group (data not shown).

Identification of suppressor genes

We used the cold-sensitive phenotypes of stable representatives of *sup1* and *sup2* to clone the corresponding wild-type alleles of the suppressor genes by complementation using a yeast genomic DNA library constructed in a single-copy shuttle vector (Rose et al. 1987). The ends of the genomic DNA inserts in the complementing plasmids were sequenced, and the DNA sequences were used to query the Saccharomyces Genome Database to identify all predicted open reading frames (ORFs) within the identified genomic intervals. So far, we have been unable to clone the wild-type allele of *sup3* due to its weak Cs$^-$ phenotype.

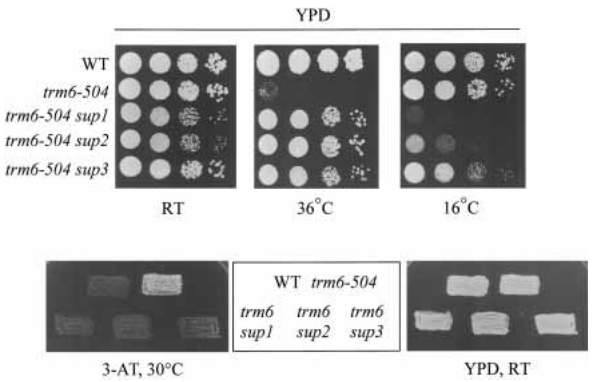

Figure 1. Mutations of *sup1*, *sup2*, or *sup3* suppress the mutant phenotypes of *trm6-504*. *TRM6*, *trm6-504* and representative *trm6-504, sup–* strains all harboring *gcn2-101* were grown to saturation in YPD medium, and serial 10-fold dilutions were spotted onto YPD plates and incubated at 36°C, 16°C, and 26°C. The same strains were grown on YPD at 26°C and replica-plated to synthetic complete (SC) plates lacking histidine and supplemented with 30 mM 3-aminotriazole (3-AT) or YPD and incubated at 30°C and 26°C, respectively.

The sequence of two independent plasmids that complemented *sup1* (p*Sup1.1* and p*Sup1.2*) had identical fragments ~12 kbp in length from Chromosome XV (coordinates 100202–112210 bp) containing nine complete ORFs (Fig. 2A). By constructing subclones and testing them for complementation of *trm6-504*, we identified *TRF4* as the suppressor gene (Fig. 2A,C). Because *TRF4* is a nonessential gene, we were able to confirm its identity as *SUP1*$^+$ by crossing a *trm6-504* mutant with a *trf4*Δ strain and finding that *trf4*Δ suppresses the Ts$^-$ phenotype of *trm6-504* (data not shown; see Materials and Methods). Moreover, we determined that *TRF4* is allelic to *sup1* using marker rescue (Materials and Methods).

We identified three independent plasmids from the single-copy genomic library that complemented the *sup2* Cs$^-$ phenotype. DNA sequence analysis and searches of the *S. cerevisiae* genome database revealed that all three genomic clones were derived from a single chromosomal region (Fig. 2B; Table 1). Only *DIS3/RRP44* and *SUF17*, encoding $tRNA_{GCC}^{Gly}$, were represented as complete ORFs in all three clones, and we found that the *DIS3/RRP44* gene subcloned into a single-copy plasmid lacking *SUF17* (Fig. 2B) complemented the *sup2* Cs$^-$ phenotype and restored the Ts$^-$ and 3AT^r phenotypes of the *trm6-504 gcn2-101* strain (Fig. 2C). We confirmed the identity of *SUP2*$^+$ as *DIS3/RRP44* by conducting linkage analysis after directing integration of *URA3* near the *DIS3/RRP44* locus in *sup2* (see Materials and Methods). From these results, we concluded that mutation of either *TRF4* or *DIS3/RRP44* can suppress the growth phenotypes of a *trm6-504* mutant. Throughout the remainder of this report, *DIS3/RRP44* will be referred to as *RRP44*.

TRF4 was originally discovered in a genetic screen to identify mutants that require topoisomerase I for growth (Sadoff et al. 1995a). It has been shown that Trf4p exhibits DNA polymerase activity with β-polymerase-like characteristics and is required for sister-chromatid cohesion (Wang et al. 2000). Trf4p is an ortholog of *S. pombe* Cid13p, a cytoplasmic poly(A) polymerase that is required for the stability of ribonucleotide reductase mRNA by maintaining its polyadenylated state (Saitoh et al. 2002). It was further demonstrated that both Cid13p and Trf4p exhibit poly(A) polymerase activity in vitro (Saitoh et al. 2002). Rrp44p is a core component of the yeast exosome, a complex of 10 predicted 3′–5′ exohydrolases or phosphorylases involved in numerous RNA processing events in the nucleus and cytoplasm of yeast (van Hoof and Parker 1999). Rrp44p has been classified as a member of the RNR superfamily of exoribonuclease based on its 3′-5 hydrolytic exonuclease activity (Mitchell et al. 1997; Zuo and Deutscher 2001).

$tRNA_i^{Met}$ lacking m^1A58 is stabilized by mutation of TRF4, RRP44, *or* sup3

Previously, we established that *trm6-504* confers selective degradation of pre-$tRNA_i^{Met}$ due to the absence of m^1A58 (Anderson et al. 1998), and that genetic suppression of *trm6-504* can occur by increasing the level of hypomodified $tRNA_i^{Met}$ (Anderson et al. 1998). We

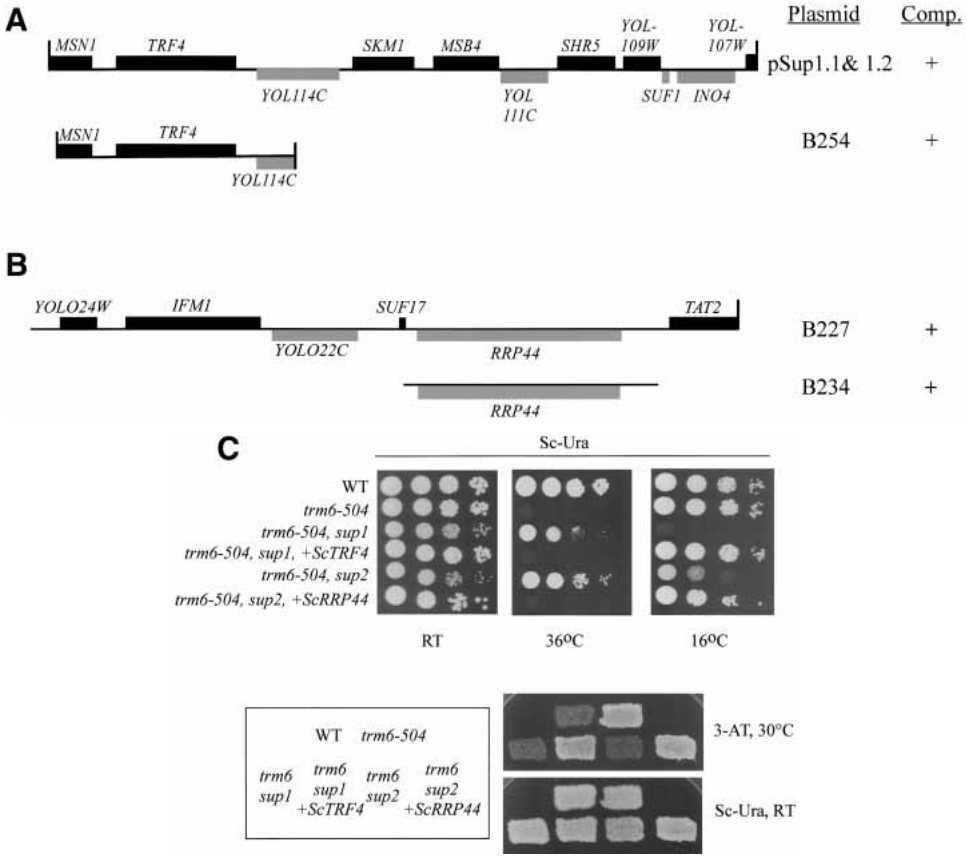

Figure 2. Identification of *SUP1*$^+$ and *SUP2*$^+$ as *TRF4* and *RRP44*, respectively. (*A*) Schematic representation of genomic DNA inserts present in YCp50 (pSUP1.1 and pSUP1.2) and the single-copy plasmid subclone derived from pSUP1.1 (B254). All three plasmids complemented the Cs$^-$ and 3-AT^r phenotypes of the *sup1*, *trm6-504*, *gcn2*-101 strain. (*B*) Schematic representation of the largest genomic DNA insert that complemented *sup2* Cs$^-$ phenotype (B227) and the single-copy plasmid subclone derived from B227 (B234). Both plasmids complemented the Cs$^-$ and 3-AT^r phenotypes of the *sup1*, *trm6-504*, *gcn2-101* strain (see Materials and Methods for details). The chromosomal coordinates for all three genomic clones containing *DIS3/RRP44* (B227, B228, and B229) are given in Table 1. Black boxes, ORFs encoded on the Watson strand; stippled boxes, ORFs encoded on the Crick strand; vertical lines, incomplete ORFs. (*C*) Growth of strains wild type, *trm6-504*, *sup1*, *trm6-504*, and *sup2*, *trm6-504* all harboring *gcn2-101* transformed with YCpLac33 (rows 1–3, 5), with sc*TRF4* (B254, row 4) or sc*RRP44* (B234, row 6). Cultures of each strain grown in SC-Ura and serial 10-fold dilutions were spotted onto SC-Ura and incubated at 36°C, 16°C, and 26°C (RT). The same strains were grown as patches on SC-Ura plates and replica-plated to SC-Ura, His medium supplemented with 30mM 3-AT or SC-Ura and incubated at 30°C and 26°C, respectively.

sought to determine whether mutations in *TRF4* or *RRP44* suppress *trm6-504* by this same mechanism. Total RNA was isolated from the isogenic strains *TRM6*, *trm6-504*, *trm6-504 sup1* (*trf4-20*), *trm6-504 sup2* (*rrp44-20*), and *trm6-504 sup3* grown at 26°C and following a shift to 36°C, the nonpermissive temperature for *trm6-504* (Anderson et al. 1998). Northern blot analysis of the steady-state levels of $tRNA_i^{Met}$ revealed a significant increase in the level of mature $tRNA_i^{Met}$ in all three suppressor-containing strains compared to the parental *trm6-504* mutant (Fig. 3). Quantification of mature $tRNA_i^{Met}$ after normalization to 5S rRNArRNA revealed that the levels of mature $tRNA_i^{Met}$ in the *trm6-504* single mutant were 58% and 48% of the wild-type levels in the *TRM6* strain at 26°C and 36°C, respectively (Fig. 3), in agreement with our previous findings (Anderson et al. 1998). Importantly, the levels of mature $tRNA_i^{Met}$ in the suppressor strains were elevated to 88%–94% of the wild-type level at 26°C and to 68%–78% of wild type at 36°C (Fig. 3). The fact that the level of mature $tRNA_i^{Met}$ in the suppressor strains was significantly lower than that in wild type cells may reflect degradation of $tRNA_i^{Met}$ by a Trf4p- and Rrp44p-independent pathway that is revealed only at elevated temperatures. Alternatively, the suppressor mutations may not completely inactivate the functions of Trf4p and Rrp44p involved in degradation of hypomodified $tRNA_i^{Met}$. The high level of pre-$tRNA_i^{Met}$ in the *trm6-504* mutant compared to the *TRM6* strain at 26°C (Fig. 3 *TRM6* vs. *trm6-504*, 0 h) indicates a reduced efficiency of pre-$tRNA_i^{Met}$ processing in the absence of m^1A58. Consistent with the idea that processing of pre-$tRNA_i^{Met}$ is slowed in a *trm6* mutant (Calvo et al. 1999) and that mutant pre-$tRNA_i^{Met}$ is the target for degradation at elevated temperatures

Table 1. *Plasmids used*

Name	Description	Reference
B176	YCp50: *CEN4, URA3* cloning vector	Rose et al. 1987
B181	YCpLac33: *CEN4, URA3* cloning vector	Gietz and Sugino 1988
B184	YEpLac195: *URA3* high copy vector	Gietz and Sugino 1988
B187	YIpLac211: *URA3* integrative vector	Gietz and Sugino 1988
B234	3.9 kb XhoI/XmaI PCR fragment of *RRP44/DIS3* in SalI/XmaI-digested YCpLac33	This study
B251	3.9 kb XhoI/XmaI product of *RRP44/DIS3* in SalI/XmaI YEpLac195	This study
B254	3.0 kb SspI/PmeI fragment pSup1 containing *TRF4* into SmaI-digested YCpLac33	This study
B256	3.1 kb SacI/PstI fragment of *TRF4* from B254 into SacI/PstI YEpLac195	This study
B269	3.4 kb XbaI/EcoRI fragment of *RRP6* in XbaI/EcoRI YCpLac33	Briggs et al. 1998
CB432	3.8 kb SmaI/HindIII genomic fragment containing *TRF4* into SmaI/HindIII pRS306	Sadoff et al. 1995a
B227	YCp50 containing chromosome XV DNA coordinates 280049 to 286635	This study
B228	YCp50 containing chromosome XV DNA coordinates 280689 to 287099	This study
B229	YCp50 containing chromosome XV DNA coordinates 276767 to 287099	This study
B285	1.1 kb EcoRI fragment of *IFM1* cloned into EcoRI-digested YIpLac211	This study
B145	A 3.0 kb XbaI/XhoI fragment containing *TRM6* into SalI/XbaI-digested YIpLac211	This study
[a]pSup1.1	YCp50 containing chromosome XV DNA coordinates 100202-112210	This study
AK*YOL114C*	2.5 kb *YOL114C* HindIII fragment from CB432 into HindIII-digested YCpLac33	This study
p2704	3.0 kb XbaI/XhoI fragment containing *TRM6* into XbaI/SalI-digested YCplac33	Anderson et al. 1998

[a]Genomic DNA contained in pSup1.1 and pSup1.2 is identical.

(Anderson et al. 1998), the steady-state level of pre-$tRNA_i^{Met}$ in *trm6-504* is reduced at 36°C compared to wild type (Fig. 3). In the *trm6-504* suppressor strains, pre-$tRNA_i^{Met}$ is more stable at 36°C and its accumulation varies between suppressors at 26°C, suggesting that pre-$tRNA_i^{Met}$ is more stable in the suppressors strains at 36°C and that processing rates of pre-$tRNA_i^{Met}$ vary significantly between individual suppressors (Fig. 3). To rule out the remote possibility that increased levels of mature $tRNA_i^{Met}$ in the suppressors was due to an increase in m^1A production, we measured the level of m^1A in total tRNA from *TRM6*, *trm6-504*, and the *trm6-504 trf4-20*, *rrp44-20*, or *sup3* double mutants by HPLC analysis (Table 2). As anticipated, m^1A was nearly undetectable in the parental *trm6-504* strain, and the low-level of m^1A detected in tRNA from *trm6-504* cells was unchanged in the two suppressor strains. We conclude from these results that the mechanism of suppression is most likely due to defects in the machinery that normally degrades hypomodified pre-$tRNA_i^{Met}$.

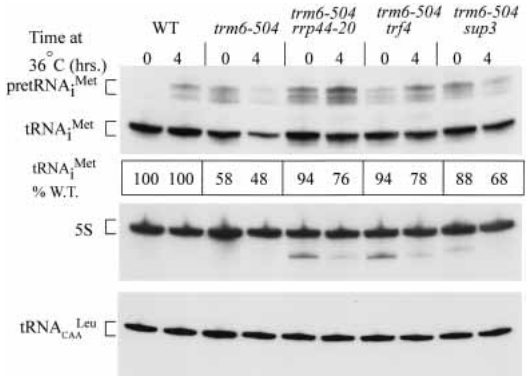

Figure 3. Mutations in *TRF4*, *RRP44*, and *sup3* increase the steady-state level of $tRNA_i^{Met}$ in a *trm6-504* background. Northern blot analysis of total RNA isolated from wild-type, *trm6-504*, or *trm6-504 trf4-20*, *rrp44-20* or *sup3* double mutants grown at a permissive (26°C) or nonpermissive (36°C) temperature. Total RNA (10 µg) was separated by electrophoresis on a 6% denaturing acrylamide (19:1) gel cast in 8 M urea. Hybridization with probes JA11 ($tRNA_i^{Met}$), JA99 (5S rRNA), and JA151 ($tRNA_{CAA}^{Leu}$) were performed as described in Materials and Methods. $tRNA_i^{Met}$ was visualized by autoradiography and quantified by PhosphorImager analysis using Image Quant software; normalized to 5S rRNA or $tRNA_{CAA}^{Leu}$ and then expressed as a percentage of $tRNA_i^{Met}$ in wild type at the same temperature. Normalization of $tRNA_i^{Met}$ to 5S rRNA or $tRNA_{CAA}^{Leu}$ yielded similar results, and the quantity of $tRNA_i^{Met}$ shown is after normalizing to 5S rRNA.

Degradation of $tRNA_i^{Met}$ occurs in the nucleus

Based on results from previous work, we hypothesized that the precursor form of $tRNA_i^{Met}$ lacking m^1A58 is rapidly degraded whereas the mature form of the hypomodified $tRNA_i^{Met}$ is stable upon reaching the cytoplasm (Anderson et al. 1998). The exosome exists in two forms, with the nuclear form containing an additional nonessential 3′–5′ exonuclease, Rrp6p. If degradation of hypomodified pre-$tRNA_i^{Met}$ occurs in the nucleus, then it should be affected by deletion of *RRP6*. To test this

Table 2. *Detection of m^1A in total tRNA by HPLC analysis*

Strains	m^1A as % of WT
WT	100
trm6-504	7.5
trm6-504 rrp44-20	7.2
trm6-504 trf4-20	7.8
trm6-504 sup3	3.0

m^1A levels normalized to m^1G are shown; normalization to m^5C and Ψ gave similar results.

prediction, we created a *trm6-504 rrp6*Δ double mutant by genetic cross (see Materials and Methods) and examined its phenotype. The results in Figure 4A suggest that the *rrp6*Δ mutation suppressed the Ts⁻ phenotype of *trm6-504*. This conclusion was confirmed by showing that a single-copy plasmid bearing *RRP6* (Briggs et al. 1998) complemented the ability of *rrp6*Δ to suppress *trm6-504* by restoring the original Ts⁻ phenotype of *trm6-504* (Fig. 4A). Because *trm6-504* suppression by *trf4-20*, *rrp44-20*, and *sup3* were accompanied by an increase in the steady-state level of mature $tRNA_i^{Met}$, we predicted the same would be true for suppression by *rrp6*Δ. To test this possibility, we determined the steady-state level of $tRNA_i^{Met}$ in the *trm6-504 rrp6*Δ mutant at 26°C and 36°C by Northern blot analysis. As expected, suppression of the *trm6-504* Ts⁻ phenotype was accompanied by an increase in mature $tRNA_i^{Met}$ level compared to the *trm6-504* mutant (Fig. 4B, *trm6-504* vs. *trm6-504 rrp6*Δ). Taken together, these results strongly support the conclusion that degradation of hypomodified pre-$tRNA_i^{Met}$ does occur in the nucleus, although they do not exclude the possibility that mature $tRNA_i^{Met}$ degradation also occurs in the cytoplasm.

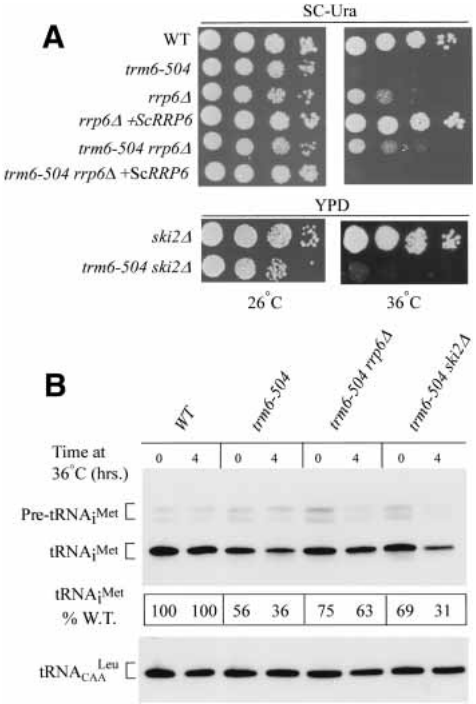

Figure 4. (*A*) Degradation of hypomodified $tRNA_i^{Met}$ occurs in the nucleus. Wild-type (Y200), *trm6-504* (Y190), *rrp6*Δ (F23), and *trm6-50 rrp6*Δ (Y298) strains were transformed with YCpLac33 (B181, rows 1–3, 5) or Sc*RRP6* (B269, rows 4,6), or untransformed strains ski2Δ (F24) and *trm6-504 ski2*Δ (Y299) were tested for growth. Transformants or ski2Δ (F24) and *trm6-504 ski2*Δ (Y299) were grown to saturation in SC-Ura or YPD, respectively, and serial 10-fold dilutions were spotted on SC-Ura or YPD plates and incubated at 26°C and 36°C. (*B*) Northern blot analysis of total RNA (10μg) isolated from wild-type (F27), *trm6-504* (Y190), *trm6-504 rrp6*Δ (Y298), and *trm6-504 ski2*Δ (Y299) strains grown in YPD at permissive (26°C) and after shift to the nonpermissive temperature, 36°C. RNAs separated on a 6% polyacrylamide (19:1) 8 M urea gel and transferred to a membrane were probed with JA11 to detect $tRNA_i^{Met}$ and JA151 to detect $tRNA_{CAA}^{Leu}$. $tRNA_i^{Met}$ and $tRNA_{CAA}^{Leu}$ were visualized by autoradiography and quantified by PhosphorImager analysis using Image Quant software. $tRNA_i^{Met}$ was normalized to the amount of $tRNA_{CAA}^{Leu}$ in the same sample and is expressed as percentage of wild type at the same temperature.

To address whether the cytoplasmic exosome might also play a role in the degradation of aberrant $tRNA_i^{Met}$, we created a *trm6-504 ski2*Δ double mutant by genetic cross (see Materials and Methods). Ski2p is an accessory factor to the exosome that is required for the degradation of mRNA in the cytoplasm (Jacobs et al. 1998; van Hoof et al. 2002), but thus far has not been shown to function in the nuclear exosome (Jacobs et al. 1998; van Hoof et al. 2000a). The *trm6-504 ski2*Δ double mutant showed no significant difference in growth at 36°C compared to *trm6-504*, and the mature $tRNA_i^{Met}$ level at 36°C in the double mutant was not significantly different than that seen in *trm6-504* (Fig. 4A,B). From these results we conclude that it is unlikely that the cytoplasmic exosome is involved in the degradation of hypomodified $tRNA_i^{Met}$; however, we cannot rule out the possibility that the cytoplasmic exosome can degrade the aberrant $tRNA_i^{Met}$ independently of the accessory factor Ski2p.

Overexpression of TRF4 *exacerbates the slow growth phenotype of* trm6-504 *and results in a further reduction of* $tRNA_i^{Met}$ *levels*

Our results demonstrated that the exosome and Trf4p are required for the degradation of pre-$tRNA_i^{Met}$ lacking m^1A58 in *trm6-504* cells. Next, we wished to address whether Trf4p and the exosome function independently or in conjunction to bring about degradation of hypomodified pre-$tRNA_i^{Met}$. Toward this end, we asked whether overexpression of Trf4p would accelerate the degradation of $tRNA_i^{Met}$ lacking m^1A58 in a manner dependent on the exosome. This was tested by introducing a multicopy-number plasmid containing wild-type *TRF4* (hc*TRF4*) into *trm6-504*, *TRM6*, and *trm6-504 rrp44-20* haploid strains. We found that the presence of hc*TRF4* exacerbated the slow growth of the *trm6-504* mutant at 26°C or 30°C while having no effect on the growth of *TRM6* cells (Fig. 5A). This suggested that high levels of Trf4p might further destabilize pre-$tRNA_i^{Met}$ lacking m^1A58 in the *trm6-504* mutant while having no affect on wild-type $tRNA_i^{Met}$. This idea was borne out by the results of Northern blot analysis. The $tRNA_i^{Met}$ level in a *trm6-504* mutant bearing hc*TRF4* was reduced to 43% of the level found in the *trm6-504* containing an empty plasmid (Fig. 5B). Thus, the level of $tRNA_i^{Met}$ in the *trm6-504* mutant was 37% of that seen in *TRM6* cells but only 16% of wild type in the *trm6-504*+hc*TRF4* cells (Fig. 5B). The reduction in the mature $tRNA_i^{Met}$ levels in the presence of excess Trf4p requires that the $tRNA_i^{Met}$ lacks m^1A58, because the *TRM6*+hc*TRF4* and *TRM6* cells had indistinguishable amounts of mature $tRNA_i^{Met}$ (Fig. 5B). As might be expected for a protein residing in a

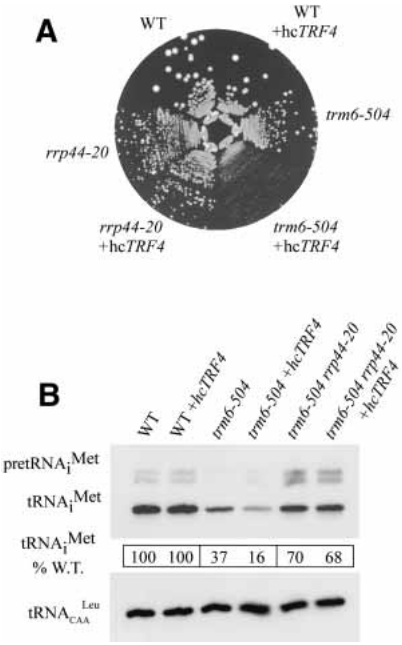

Figure 5. Overexpression of Trf4p enhances degradation of hypomodified tRNA$_i^{Met}$ in an exosome-dependent manner. (*A*) Wild type (Y200), *trm6-504* (Y190), and *trm6-504 rrp44-20* were transformed with a high-copy-number plasmid YEpLac195 (B184) or a high-copy-number plasmid bearing *TRF4* (B256), and single transformants were streaked to SC-Ura and incubated for 4 d at 30°C. (*B*) Northern blot analysis of total RNA (10μg) isolated from the same strains described in *A* grown at 30°C. Detection of precursor and mature tRNA$_i^{Met}$ was done by hybridization with probes JA11 (tRNA$_i^{Met}$) and JA151 (tRNA$_{CAA}^{Leu}$), followed by autoradiography or PhosphorImager analysis and quantification using Image Quant software. tRNA$_i^{Met}$ was normalized to the amount of tRNA$_{CAA}^{Leu}$ in the same sample and is expressed as percentage of wild type bearing the same plasmid.

multisubunit complex such as the exosome, similar experiments overexpressing *RRP44* in *trm6-504* or *TRM6* strains did not result in a measurable change in growth at 26°C or 30°C (data not shown).

Trf4p function is required prior to exosome-mediated degradation of aberrant tRNA$_i^{Met}$

To determine whether a functional exosome is required for Trf4p-mediated degradation of tRNA$_i^{Met}$ lacking m^{1}A58, we asked whether overexpression of Trf4p in a strain bearing *rrp44-20* would affect the growth rate or levels of mature tRNA$_i^{Met}$ in a *trm6-504* background. If exosome function is not required for the accelerated pre-tRNA$_i^{Met}$ degradation mediated by Trf4p, then overexpression of Trf4p should reduce the growth rate of the *trm6-504 rrp44-20* double mutant. At odds with this prediction, we found that hc*TRF4* had no effect on the growth rate (Fig. 5A) or levels of tRNA$_i^{Met}$ (Fig. 5B) in the *trm6-504 rrp44-20* double mutant. Thus, it appears that Trf4p mediates degradation of pre-tRNA$_i^{Met}$ lacking m^{1}A58 via the exosome.

Polyadenylation of hypomodified precursor tRNA$_i^{Met}$

Exosome-mediated degradation of hypomodified pre-tRNA$_i^{Met}$ occurs in the nucleus, and the direct involvement of Trf4p (also a nuclear protein) in the degradative process suggested that this mechanism might require the poly(A) polymerase function of Trf4p to polyadenylate pre-tRNA$_i^{Met}$ prior to degradation. This would be reminiscent of what has been shown for degradation of a mutant tRNA^{Trp} in *E. coli* (Li et al. 2002). Thus, we reasoned that if polyadenylation of hypomodified pre-tRNA$_i^{Met}$ occurs in yeast, it could signify the existence of a tRNA surveillance mechanism similar to that identified in bacterial cells. We predicted that polyadenylated hypomodified tRNA$_i^{Met}$ would migrate in gel electrophoresis as a heterogeneous smear above the pre-tRNA$_i^{Met}$ from *trm6-504* cells. However, after long exposures, Northern blots of total RNA from *trm6-504* cells failed to provide evidence that hypomodified pre-tRNA$_i^{Met}$ was polyadenylated (data not shown).

Because *rrp6*Δ suppresses *trm6-504* and previous reports showed that deletion of *RRP6* led to accumulation of polyadenylated pre-sno and snRNAs (Allmang et al. 1999a; van Hoof et al. 2000a), we reasoned that by combining *rrp6*Δ and *trm6-504* in the same strain we might increase the abundance of polyadenylated pre-tRNA$_i^{Met}$ by inactivating Rrp6p. Similarly, we reasoned that if Trf4p influences the polyadenylation of pre-tRNA$_i^{Met}$, then overexpression of Trf4p may cause a greater accumulation of the polyadenylated pre-tRNA$_i^{Met}$ in the *trm6-504 rrp6*Δ mutant. Northern blot analysis of total RNA from *trm6-504 rrp6*Δ under conditions that favor hybridization of precursor tRNA$_i^{Met}$ (Materials and Methods) and extended autoradiography revealed a small amount of slower migrating, heterogeneously sized tRNA$_i^{Met}$ (data not shown). In order to enrich for the presumptive poly(A)$^+$ pre-tRNA$_i^{Met}$, we selected poly(A)$^+$ RNA from wild-type, *rrp6*Δ, *trm6-504 rrp6*Δ, and *trm6-504 rrp6*Δ +hc*TRF4* total RNA using oligo-d(T) cellulose (Materials and Methods). We conducted a Northern blot analysis of equivalent proportions of the resulting poly(A)$^+$ RNAs after correcting for the different input amounts of total RNA used in the oligo-d(T) selection. As predicted, we detected a smear of tRNA$_i^{Met}$ molecules longer than pre-tRNA$_i^{Met}$ in poly(A)$^+$ RNA from *trm6-504 rrp6*Δ that was not present in the corresponding sample from wild type or *rrp6*Δ, indicating that hypomodified but not wild-type tRNA$_i^{Met}$ was polyadenylated (Fig. 6, lanes 5–9). Significantly, the abundance of polyadenylated tRNA$_i^{Met}$ increased, and it displayed a wider length distribution, in the poly(A)$^+$ RNAs isolated from *trm6-504 rrp6*Δ +hc*TRF4* versus *trm6-504 rrp6*Δ cells (Fig. 6, lanes 8,9), demonstrating that Trf4p stimulates polyadenylation of hypomodified tRNA$_i^{Met}$. Pretreatment of the poly(A)$^+$ RNA from *trm6-504 rrp6*Δ +hc*TRF4* with oligo-d(T) and RNaseH converted the smear of polyadenylated tRNA$_i^{Met}$ to a single species similar in size to pre-tRNA$_i^{Met}$ (Fig. 6, lane 13) These data strongly support our model that hypomodified pre-tRNA$_i^{Met}$ is

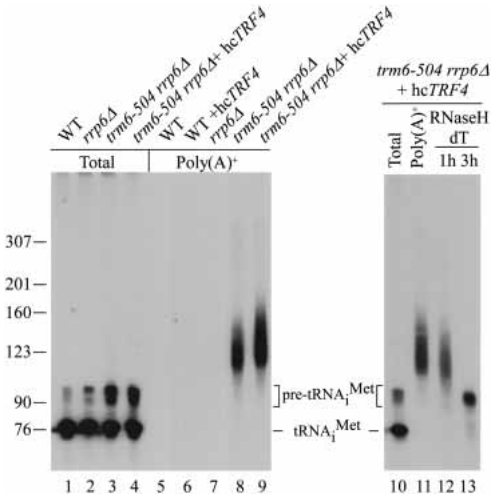

Figure 6. Pre-tRNA$_i^{Met}$ lacking m^{1}A58 is polyadenylated. RNA isolated from the indicated strains cultured at 30°C was subjected to Northern blot analysis using total RNA (lanes *1–4*, 5 µg; lane *10*, 1 µg) or poly(A)$^+$ RNA (lanes *5*,*6*, 1 µg; lanes *7–9*, 2 µg; lanes *11–13*, 1 µg) after separation by denaturing polyacrylamide gel electrophoresis and transfer to a membrane. Hybridization with probe JA11 (tRNA$_i^{Met}$) was done as described in Materials and Methods with the exception that the hybridizing temperature was 60°C, and the tRNA$_i^{Met}$ was visualized by autoradiography. Pretreatment of 1 µg of poly(A)$^+$ RNA from the *trm6-504 rrp6*Δ +hc*TRF4* strain with oligo-d(T$_{18}$) and RNaseH was done as described in Materials and Methods; the reactions were terminated after 1 or 3 h (lanes *12*,*13*, respectively). The standards used in size determination were radiolabeled pBR322 digested with MspI.

polyadenylated prior to degradation by the nuclear exosome.

Discussion

We previously determined that *TRM6* and *TRM61* (formerly *GCD10* and *GCD14*) comprise the essential tRNA m^{1}A58 methyltransferase. Mutations in *TRM6* or *TRM61* lead to the selective instability of tRNA$_i^{Met}$ due to the absence of the m^{1}A58 modification, conferring temperature sensitivity and resistance to 3-AT in a *gcn2* background (Harashima and Hinnebusch 1986; Garcia-Barrio et al. 1995; Anderson et al. 1998). Here we used genetic suppressor analysis to establish that mutation of the exosome subunit Rrp44p, or inactivation of Trf4p, suppresses the Ts$^-$ and 3-AT^r phenotypes of a *trm6-504* mutant. Our molecular analysis showed that the *rrp44* and *trf4* mutations suppress *trm6-504* by increasing the steady-state amounts of hypomodified tRNA$_i^{Met}$ to nearly wild-type levels. Deletion of another exosome subunit gene, *RRP6*, also suppressed the Ts$^-$ phenotype conferred by *trm6-504*, providing strong evidence that impairment of exosome function is the mechanism of suppression by the *rrp44-20* mutation. Because Rrp6p is associated only with the nuclear form of the exosome, we conclude that the bulk of the degradation of hypomodified pre-tRNA$_i^{Met}$ in *trm6-504* cells occurs in the nucleus. Consistent with this, inactivation of Ski2p, an accessory factor for the cytoplasmic form of the exosome, did not suppress the Ts$^-$ *trm6-504* mutant phenotype.

Overexpression of Trf4p exacerbated the reduction in hypomodified tRNA$_i^{Met}$ levels in *trm6-504* cells in a manner that was completely suppressed by the *rrp44-20* mutation. Thus, we conclude that Trf4p is required for efficient degradation of unmodified tRNA$_i^{Met}$ by the nuclear exosome. Trf4p belongs to the family of β-polymerase-like nucleotidyltransferases (Aravind and Koonin 1999) that was also shown to have poly(A) polymerase activity (Saitoh et al. 2002). Below, we propose a working model to account for the function of Trf4p in exosome-mediated degradation of hypomodified tRNA$_i^{Met}$.

The exosome is required for degradation of tRNA$_i^{Met}$ lacking m^{1}A58

The role of the exosome in the degradation of tRNA$_i^{Met}$ lacking m^{1}A58 represents a novel function for this multisubunit RNA processing machinery. The exosome facilitates the 3′-to-5′ degradation of mRNA and the processing of many small stable RNAs, and it possesses other functions related to RNA metabolism in both the nucleus and cytoplasm (Mitchell et al. 1997; Jacobs et al. 1998; van Hoof et al. 2000a). The ability of *rrp6*Δ to suppress *trm6-504* (Fig. 4A,B) clearly demonstrated that the nuclear exosome is heavily involved in the degradation of tRNA$_i^{Met}$ lacking m^{1}A58. This is consistent with the localization of Trf4p to the nucleus (Walowsky et al. 1999) and our previous finding that pre-tRNA$_i^{Met}$ but not mature tRNA$_i^{Met}$ lacking m^{1}A is subject to degradation (Anderson et al. 1998). Thus, we conclude that the degradation of hypomodified tRNA$_i^{Met}$ is carried out in the nucleus.

A proposed pathway for the surveillance and degradation of tRNA$_i^{Met}$ lacking m^{1}A58

TRF4 was originally identified in a genetic screen for mutations that lead to synthetic lethality in combination with a topoisomerase I mutant, *top1-7* (Sadoff et al. 1995a). This finding was followed by several studies that provided strong support for a model where Trf4p functions as a DNA polymerase, termed pol σ, which may couple DNA replication with the formation of sites of sister chromatid cohesion during mitosis (Castano et al. 1996a; Wang et al. 2000, 2002). Additional genetic studies uncovered *TRF5*, encoding a highly related protein Trf5p, which is 55% identical to Trf4p (Castano et al. 1996b). More recently, *TRF4* and *TRF5* were found to physically and functionally interact with DNA polymerase ε (Edwards et al. 2003).

How does the loss of Trf4p function lead to an increased level of tRNA$_i^{Met}$ lacking m^{1}A58? As pol σ, Trf4p might function to repress transcription of the *IMT* genes during mitosis. This possibility is suggested by the

failure of a *trf4 top1* double mutant to condense rDNA repeats during mitosis (Castano et al. 1996a) and to repress *ACT1* transcription after cells reach stationary phase (Sadoff et al. 1995b). If true, then loss of Trf4p function should lead to increased *IMT* gene transcription and higher steady-state levels of pre-$tRNA_i^{Met}$ in the *trm6-504* mutant at all growth temperatures, analogous to the effect of increased *IMT4* dosage (Anderson et al. 1998). However, the *trf4* mutation did not produce a significant increase in the steady-state level of pre-$tRNA_i^{Met}$ in the *trm6-504* mutant at the permissive temperature (Fig. 3), nor did it affect the steady-state levels of other tRNAs (data not shown). Instead, we favor a model whereTrf4p is directly involved in the detection and degradation of aberrant tRNA independently of its function as a DNA polymerase. This hypothesis is supported by our finding that overexpression of Trf4p in *trm6-504* exacerbated its slow growth phenotype and reduced the level of $tRNA_i^{Met}$ lacking m^1A58. Because Trf4p overexpression in the *trm6-504 rrp44-20* mutant did not decrease $tRNA_i^{Met}$ levels or reduce growth (Fig. 5), we conclude that Trf4p promotes the degradation of $tRNA_i^{Met}$ lacking m^1A58 via the exosome. It is important to note that overexpression of Trf4p reduced the level of $tRNA_i^{Met}$ lacking m^1A58, but had no effect on wild-type $tRNA_i^{Met}$ expression (Fig. 5B). We propose that this selective degradation results from an abnormal structure of the mutant $tRNA_i^{Met}$ that is recognized as aberrant and targeted for degradation, rather than being a specific response to the absence of m^1A58. This argument is supported by the unique tertiary structure of $tRNA_i^{Met}$ involving m^1A58 (Basavappa and Sigler 1991) and the fact that $tRNA_i^{Met}$ is the only known tRNA that is degraded in the absence of m^1A.

Mutation of *TRF4* affects rRNA processing as well as $tRNA_i^{Met}$ levels in *trm6-504* cells (Fig. 3; S. Kadaba and J. Anderson, unpubl.), suggesting that the activity of Trf4p may be required for the exosome to efficiently process rRNA precursors. By extending this idea, Trf4p could be required for all functions of the exosome. In this regard, Trf4p could be functioning as a poly(A) polymerase, like the related Cid13p in *S. pombe* (Saitoh et al. 2002) to stabilize one or more mRNAs in the cytoplasm. The predicted target of Trf4p would be one or more mRNAs encoding an exosome subunit. In this view, loss of Trf4p function would result in destabilization of an mRNA encoding an exosome subunit, with attendant reduction in exosome function and subsequent stabilization of $tRNA_i^{Met}$ lacking m^1A58. This hypothesis is inconsistent with the finding that a GFP–Trf4p fusion was found localized to the nucleus of yeast (Walowsky et al. 1999), although a small amount of GFP–Trf4p could be undetectable in the cytoplasm under these conditions and might be enough to carry out polyadenylation of a limited number of mRNAs.

A more direct role for Trf4p in promoting the degradation of unmodified $tRNA_i^{Met}$ is suggested by the mechanism recently established for degradation of a mutant tRNA in bacteria (Saitoh et al. 2002). A mutation in *E. coli* $tRNA^{Trp}$ renders it unstable and results in temperature-sensitive growth. Introduction of mutations in poly(A) polymerase and polynucleotide phosphorylase stabilized the mature mutant $tRNA^{Trp}$ and caused its precursor to accumulate (Li et al. 2002). Subsequently, it was shown that the mutant precursor $tRNA^{Trp}$ is adenylated in a poly(A) polymerase-dependent manner, and the presence of adenosines is required for most of the observed $tRNA^{Trp}$ instability. It was thus concluded that a mechanism requiring adenylation and exonucleolytic cleavage was responsible for eliminating mutant precursor $tRNA^{Trp}$ from *E. coli*. In this report, we provide strong evidence that a pathway similar to the one described in *E. coli* exists in *S. cerevisiae*. Our working model is that Trf4p marks pre-$tRNA_i^{Met}$ lacking m^1A58 by polyadenylation, which targets the tRNA for rapid degradation by the exosome. A tacit assumption of this model is that the polyadenylated pre-$tRNA_i^{Met}$ is too short-lived to be detected by Northern blot analysis of total RNA in *trm6-504* cells.

We gained considerable support for our model by identifying polyadenylated pre-$tRNA_i^{Met}$ in a *trm6-504 rrp6*Δ mutant (Fig. 6). The fact that polyadenylated pre-$tRNA_i^{Met}$ is found in *trm6-504 rrp6*Δ but not in the *rrp6*Δ or wild-type strain indicates that hypomodified, but not wild-type pre-$tRNA_i^{Met}$, is subject to polyadenylation. The increased abundance and shift to longer poly(A) tail lengths we observed in the *trm6-504 rrp6*Δ+hc*TRF4* mutant compared to *trm6-504 rrp6*Δ further supports the proposed role for Trf4p as the poly(A) polymerase responsible for adenylation of hypomodified pre-$tRNA_i^{Met}$. However, we cannot rule out the possibility that Pap1p, the major poly(A) polymerase in yeast, is responsible for some or all of the pre-$tRNA_i^{Met}$ adenylation. In this regard, a Pap1p-dependent polyadenylation of wild-type pre-snoRNAs U14, U18, snR73, and snR72 in a *rrp6*Δ mutant has been described (Allmang et al. 1999a; van Hoof et al. 2000a). Furthermore, Van Hoof et al. (2000a) proposed that polyadenylation represents a normal but nonessential step in the 3′-end processing of wild-type pre-snoRNAs. This is in contrast to the polyadenylation of pre-$tRNA_i^{Met}$ described here, which is limited to the hypomodified molecules in *trm6-504* cells and seems more consistent with an RNA surveillance pathway that eliminates abnormal tRNAs. Moreover, the poly(A) tail lengths on hypomodified pre-$tRNA_i^{Met}$ are ~10–50 nucleotides in length (Fig. 6), in contrast to the 40–100 nucleotide lengths of poly(A) tails found on pre-sno and snRNAs (Allmang et al. 1999a; van Hoof et al. 2000a). This distinction may reflect differences in the biochemical activities of yeast Pap1p and Trf4p, or it could underlie the differences between a pathway of normal pre-snRNA and snoRNA processing versus degradation of hypomodified pre-$tRNA_i^{Met}$ and possibly other abnormally processed stable RNAs. In stark contrast to the polyadenylation of pre-$tRNA_i^{Met}$ in yeast, degradation of mutant pre- $tRNA^{Trp}$ in *E. coli* is preceded by adenylation of the tRNA precursor with only 1–3 adenosines (Li et al. 2002).

We propose that the degradation of mutant tRNAs in yeast and bacteria represent similar mechanisms that ex-

ist to eliminate aberrant tRNAs from cells that might otherwise negatively affect their growth. Elucidating the exact role of Trf4p in the degradation of hypomodified $tRNA_i^{Met}$ and demonstration that adenylation is a prerequisite for degradation will allow us to refine our model and deepen our understanding of the role played by the exosome in nuclear RNA surveillance and degradation.

Materials and methods

Plasmid constructions

Plasmids used in this study are described in detail in Table 1. B269 and CB432 were generous gifts from Dr. S. Butler (University of Rochester, Rochester, NY) and Dr. M. Christman (Boston University, Boston, MA). B227, B228, B229, and pSup1.1 and 1.2 were identified as complementing plasmids from a genomic library during suppressor screening. The entire *RRP44* ORF plus 600 base pairs upstream and 175 base pairs downstream were amplified by PCR using primers JA126 and JA127 and B228 DNA as a template. The PCR product was digested with XhoI and XmaI and cloned into SalI/XmaI-digested YCplac33 (B234) or YEpLac195 (B251). B285 was created by cloning a 1.1-kb EcoRI fragment isolated from EcoRI-digested B227 into EcoRI-digested YIpLac211. B254 was created by cloning an ~3.0 kb SspI/PmeI fragment from pSup1.1 into SmaI-digested YCplac33. Cloned DNAs were propagated through transformation of *E. coli* DH10B cells (Invitrogen) by electroporation. Yeast transformations were done as described (Ito et al. 1983).

Yeast strains and media

Standard genetic techniques and culture media were employed as described (Sherman et al. 1974b). Growth analysis using plates containing 3-AT was done as described (Hinnebusch and Fink 1983). Yeast strains used in this study are described in Table 3. Standard genetic techniques and culture media were employed as described (Sherman et al. 1974a). Y193 was created by self mating of H2457 after transformation with YCp50-*HO* plasmid, containing the *HO* endonuclease gene required for mating type switching (Herskowitz and Jensen 1991). Diploid cells were identified by their inability to mate with mating testers of *Mat a* or α genotype. Tetrad dissection and analysis of Y193 yielded four isogenic haploid spores (Y189, Y190, Y191, and Y192) from a single tetrad. Y190 and Y192 were used to create Y200 and Y201 by introducing ClaI-digested B145 (YIp*TRM6*) into Y190 (*trm6-504*) and selecting for Ura^+ transformants on synthetic complete medium lacking uracil (SC-Ura). The transformants containing both *TRM6* and *trm6-504* genes were identified by growth on Sc-Ura at 36°C. Eviction of the integrated plasmid by homologous recombination was selected by replica plating transformants to SC + Ura containing 1.0 μg/mL 5-FOA (Boeke et al. 1987; Rothstein 1991). 5-FOA-resistant colonies retaining the *TRM6* allele were identified by their growth on YPD at 36°C and failure to grow on SC plates containing (30mM) 3-AT (Hinnebusch and Fink 1983). Y298, Y299, and Y300 were created by mating F23, F24, and F22 to Y190 (*trm6-504*), respectively. The resulting diploids were subjected to sporulation, and tetrad analysis was conducted to identify haploids containing trm6-504 and the corresponding mutant. Y306 was created by transformation of *sup2 trm6-504* with BglII-digested B285 to integrate the *URA3* gene within the *IFM1* locus upstream of *RRP44*. Ura^+ transformants were selected on SC-Ura medium and tested for growth on YP glycerol medium (YPG). Genomic DNA was isolated from one positive respiratory-deficient transformant incapable of growth on YPG medium, and disruption of the *IFM1* locus was verified by PCR analysis of genomic DNA with primers JA239, JA240, and JA241 (Table 4).

Generation and identification of trm6-504 *suppressors*

Selection of suppressors was carried out using strains Y190 and Y192. One hundred individual colonies from each mating type were patched and grown at 26°C overnight on YPD medium. The plates containing the patches were replica-plated to YPD and grown at 36°C for 3–7 d. Individual papillae were picked and streaked for single colonies on YPD plates and grown at 26°C. Two individual colonies from each streak were patched onto YPD plates, grown at 26°C, and replica-plated to YPD and

Table 3. *Genotypes of strains used*

Strain	Genotype	Reference
H2457	*MATα trm6-504, gcn2-101, his1-29, ura3-52, ino1 (HIS4-lacZ, ura3-52)*	Anderson et al. 1998
Y193	*MATa/MATα trm6-504/trm6-504, gcn2-101/gcn2-101, his1-29/his1-29 ura3-52/ura3-52, ino1/ino1 (HIS4-lacZ, ura3-52)*	This study
Y190	*MATa trm6-504, gcn2-101, his1-29, ura3-52, ino1 (HIS4-lacZ, ura3-52)*	This study
Y192	*MATα trm6-504, gcn2-101, his1-29, ura3-52, ino1 (HIS4-lacZ, ura3-52)*	This study
Y200	*MATa TRM6, gcn2-101, his1-29, ura3-52, ino1, (HIS4-lacZ, ura3-52)*	This study
Y201	*MATα TRM6, gcn2-101, his1-29, ura3-52, ino1, (HIS4-lacZ, ura3-52)*	This study
Sup1	*MATa trf4-20, trm6-504, gcn2-101, his1-29, ura3-52, ino1, (HIS4-lacZ, ura3-52)*	This study
Sup2	*MATα rrp44-20, trm6-504, gcn2-101, his1-29, ura3-52, ino1, (HIS4-lacZ, ura3-52)*	This study
Sup3	*MATαsup3, trm6-504, gcn2-101, his1-29, ura3-52, ino1, (HIS4-lacZ, ura3-52)*	This study
Y298	*MATa rrp6Δ:KanMX4 trm6-504, ura3*	This study
Y299	*MATα ski2Δ:KanMX4 trm6-504, ura3*	This study
Y300	*MATα trf4Δ:KanMX4 trm6-504, ura3*	This study
Y306	*MATα rrp44-20, trm6-504, gcn2-101, his1-29, ura3-52, ino1, ifm1::URA3<B285> (HIS4-lacZ, ura3-52)*	This study
F22	*MATα his3Δ, leu2Δ, lys2Δ, ura3Δ, trf4Δ:KanMX4* clone#*11777*	Invitrogen
F23	*MATα his3Δ, leu2Δ, lys2Δ, ura3Δ, rrp6Δ:KanMX4* clone#*16265*	Invitrogen
F24	*MATα his3Δ, leu2Δ, lys2Δ, ura3Δ, ski2Δ:KanMX4* clone#*15307*	Invitrogen
F27	*MATα his3Δ, leu2Δ, lys2Δ, ura3Δ*	Invitrogen

Table 4. *Oligonucleotides used*

Oligo	Target	Sequence
JA126	*RRP44*	TCCCCCCGGGTTTGGGAAATGTCGTGCTCGACGT
JA127	*RRP44*	CCGCTCGAGGTCCACCACCAAAATGTCAA
JA11	$tRNA_i^{Met}$	TCGGTTTCGATCCGAGGACATCAGGGTTATGA
JA99	5S rRNA	TCGCGTATGGTCACCCACTACA
JA239	YIpLac211	GTTCTGCTATGTGGCGCGGTATTATC
JA240	YIpLac211	CTCTCAAGGATCTTACCGCTGTTG
JA241	*IFM1* 5′	GATGCCCCAGACATTAGAAGCC
JA125	5.8S rRNA	GCGTTSTTCATCGATGC
JA151	$tRNA_{CAA}^{Leu}$	TGGTTGCTAAGAGATTCGAAC

grown at 36°C, 16°C, or 30°C, and to SC-His supplemented with 30 mM 3-AT in order to identify Ts^+ and $3\text{-}AT^s$ revertants. We identified 150 such revertants, and 49 exhibited a cold-sensitive phenotype (Cs^-). A revertant's dominance or recessiveness was determined by mating each revertant of one mating type to the corresponding opposite mating type *trm6-504 Sup*$^+$ strain (Y190 or Y192) and testing for complementation of the revertant phenotype. Complementation of the revertant phenotypes Ts^+ $3\text{-}AT^s$ to Ts^-, $3\text{-}AT^r$ indicated that the revertant was recessive. Complementation groups were assigned by mating revertants from Y190 with revertants from Y192 and testing for complementation of Ts^+, $3\text{-}AT^s$ phenotypes. Complementation to Ts^- and $3\text{-}AT^r$ indicated that the revertants were in different complementation groups, and failure to complement placed the revertants in the same complementation group. Of the 49 Cs^- revertants, we assigned three complementation groups, designating them *sup1*–*3*. The wild-type alleles of *sup1* and *sup2* were identified by transforming representative strains of each complementation group with a single-copy yeast genomic plasmid library (Rose et al. 1987) and plating on minimal media lacking uracil at 30°C overnight. After this preincubation period at a permissive temperature, transformation plates were incubated at 16°C for 3 d. Growth at 16°C was taken to indicate complementation of the Cs^- phenotype linked to the suppressor gene. The Cs^+ transformants were tested for growth on YPD plates at 36°C and 16°C, and on plates containing 3-AT as described above. Plasmids were rescued from Ts^-, Cs^+, and $3\text{-}AT^r$ transformants in *E. coli* (Hoffman and Winston 1987), and the rescued plasmids were used to confirm complementation of the cognate mutants. Plasmids that passed this test were analyzed to determine the ends of the genomic DNA inserts by DNA sequencing using primers complementary to sequences flanking the BamHI site of YCp50. These sequences were compared to the yeast genome using the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces). *sup1* was confirmed as *TRF4* by transforming the *sup1* strain with sc*TRF4* (B254) and also by creating a *trm6-504 trf4*Δ double mutant and showing that deletion of *TRF4* suppressed the *trm6-504* mutant phenotypes. To confirm the identity of *sup1* as a mutant allele of *TRF4*, we conducted marker-rescue experiments using two integrative plasmids containing the 5′ or 3′ half of *TRF4* derived from YIpLac211 (Gietz and Sugino 1988). Integration of plasmid trf4mp2 bearing the 5′ half of *TRF4* (~1.5-kb SspI–HindIII fragment) but not plasmid trf4mp3 bearing 3′ half of *TRF4* (~1.5-kb HindIII–PmeI fragment) complemented the *sup1* phenotypes and restored *trm6-504* mutant phenotypes. Eviction of the integrated plasmids was selected by replica-plating transformants to 5-FOA-containing medium, and the $5FOA^r$ colonies were screened for *trm6-504* phenotypes. Of the 20 $5\text{-}FOA^r$ colonies from the transformant containing the 5′ half of *TRF4*, 12 exhibited Sup^+ and eight showed *sup*$^-$ phenotype.

sup2 was confirmed as *RRP44* by linkage analysis. After mating Y190 *ura3-52*, *gnc2-101*, *trm6-504*, Sup^+ to Y306 *ura3-52*, *gcn2-101*, *trm6-504*, *sup2*, *ifm1*::*URA3*, the resulting diploid was subjected to sporulation and tetrad analysis. In 18 out of 18 tetrads we observed cosegregation of the Ts^+, Cs^-, and $3\text{-}AT^s$ phenotype of *sup2*, with the Ura^+ phenotype of the disrupted *ifm1*::*URA3* allele integrated near *RRP44*.

RNA isolation and blotting

Total RNA was isolated as described (Kohrer and Domdey 1991). For RNA blotting, total RNA was separated on a 6% polyacrylamide-bis-acrylamide (19:1) gel cast in 8 M urea and 1× Tris-Borate-EDTA (TBE) buffer (Maniatis et al. 1982) by electrophoresis at 450 V for 1 h unless otherwise stated. The separated RNA was transferred to a Hybond N^+ membrane (Amersham Pharmacia) at 12 V for 5 h in 0.5× TBE. The blot was probed using radiolabeled oligonucleotides (Table 4) for specific RNAs in 0.25 M Na_2HPO_4, 1 mM EDTA, 1% BSA, and 7% SDS buffer. The oligonucleotides were radiolabeled by incubating (γ-^{32}P)-ATP (Amersham Pharmacia) and T4 Polynucleotide Kinase (New England Biolabs) with 10 pmole of oligonucleotide at 37°C for 1 h.

tRNA isolation and modification studies

Total RNA was extracted from yeast grown in YPD (Kohrer and Domdey 1991). tRNA was isolated by DEAE-cellulose chromatography of total RNA (Hatfield et al. 1979). Three hundred µg of purified tRNA was lyophilized and analyzed by HPLC as described (Gehrke et al. 1982; Gehrke and Kuo 1990). The amount of m^1A present in a tRNA sample was determined by taking the area under the m^1A peak and dividing it by the area of the peak for m^1G, m^5C, or Ψ from the same sample. This normalized m^1A content was compared to the same value derived from tRNA isolated from the wild-type strain, Y200.

Oligo d(T) purification of PolyA$^+$ *RNAs*

Yeast strains, wild type, wild-type +hc*TRF4*, *rrp6*Δ, *trm6-504 rrp6*Δ, and *trm6-504 rrp6*Δ +hc*TRF4* were grown to an optical density at A_{600}nm of 0.5–0.6 in 500 mL SC-Ura at 30°C. Cells were harvested at 6000 × g for 5 min at 4°C and washed with 1× phosphate buffered saline (PBS). The pellet was resuspended in 2.0 mL of lysis buffer (20 mM Tris at pH 7.4, 50 mM LiCl, 1% SDS, 1% BME, 1 mg/mL Heparin, 10 mM Vanadyl–ribonucleoside complex [New England Biolabs] 300 µg/mL Proteinase K [New England Biolabs]) and passed through a French pressure cell (20,000 psi) twice to effect cell lysis. The lysate was incubated at 42°C for 15 min, and then EDTA was added to a final concentration of 10 mM, and incubation at 42°C was continued for 15 min. The lysate was incubated at 65°C for 10 min and put on ice until cool. Lithium chloride was added to a final concentration of 0.5 M, and the lysate was centrifuged at 6000 × g for 5 min at 4°C. Fifty microliters of the cleared lysate was removed for total RNA extraction as described (Kohrer and Domdey 1991). The remaining lysate was incubated with 0.1 g of oligo d(T) Cellulose (New England Biolabs) pre-equilibrated with binding buffer (10 mM Tris at pH 7.4, 1 mM EDTA, 0.5% SDS and 0.5 M LiCl) at room temperature for 25 min. with constant mixing. The lysate-oligo-d(T) mixture was applied to an econo-column (Bio-Rad) and washed with 10 mL of binding (20× bed volume). $Poly(A)^+$ RNA was eluted using 5.0 mL of elution buffer (10 mM Tris, 1 mM EDTA and 0.05% SDS). The eluate was heated to 65°C, cooled on ice, and brought to 0.5 M LiCl

before being incubated and chromatographed with oligo-d(T) a second time. The poly(A)$^+$ RNA was eluted from oligo-d(T) with elution buffer by collection 0.5-mL fractions. The O.D.$_{260}$ of each fraction was determined, and fractions having O.D.$_{260}$ of 0.2 or greater were pooled, phenol chloroform-extracted, and precipitated with 0.3 M sodium acetate and three volumes of ethanol.

Oligo d(T) RNaseH treatment of PolyA$^+$ RNAs

First, 1.0 µg of PolyA$^+$ RNA from *trm6-504/rrp6*Δ +hc*TRF4* was dried with 300 ng of oligo d(T_{18}) and resuspended in 10 µL of hybridization buffer (25 mM Tris at pH 7.5, 1 mM EDTA, and 50 mM NaCl) and incubated at 68°C for 20 min, 42°C for 10 min, 30°C for 10 min, and then placed on ice for 5.0 min. The reaction was started by the addition of 10 µL of 2× RNaseH buffer containing 250 units RNaseH (40 mM Tris at pH 7.5, 20 mM $MgCl_2$, 100 mM NaCl, 2 mM DTT, and 60 µg/mL of BSA) and incubated at 30°C for 1 h. A duplicate reaction was incubated at 30°C for 3 h with the addition of 0.5 µL (250 units) RNaseH (NEB) after 1 h. The reaction was stopped by the addition of 0.1 mL of stop buffer (4 µg/mL *E. Coli* $tRNA_{Phe}$, 20 mM EDTA, and 300 mM NaoAc) The samples were phenol chloroform-extracted and precipitated with three volumes of ethanol.

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