The conserved Wobble uridine tRNA thiolase Ctu1–Ctu2 is required to maintain genome integrity

Monique Dewez*, Fanélie Bauer*, Marc Dieu†, Martine Raes†, Jean Vandenhaute*, and Damien Hermand*‡§

*Laboratoire de Génétique Moléculaire and †Unité de Recherche en Biologie Cellulaire, Service de Spectrométrie de Masse, University of Namur, Rue de Bruxelles 61, 5000 Namur, Belgium; and ‡The Hubrecht Institute, 3584 CT, Utrecht, The Netherlands

Edited by Dieter Söll, Yale University, New Haven, CT, and approved January 31, 2008 (received for review October 3, 2007)

Modified nucleosides close to the anticodon are important for the proper decoding of mRNA by the ribosome. Particularly, the uridine at the first anticodon position (U34) of glutamate, lysine, and glutamine tRNAs is universally thiolated (S^2U34), which is proposed to be crucial for both restriction of wobble in the corresponding split codon box and efficient codon–anticodon interaction. Here we show that the highly conserved complex Ctu1–Ctu2 (cytosolic thiouridylase) is responsible for the 2-thiolation of cytosolic tRNAs in the nematode and fission yeast. In both species, inactivation of the complex leads to loss of thiolation on tRNAs and to a thermosensitive decrease of viability associated with marked ploidy abnormalities and aberrant development. Increased level of the corresponding tRNAs suppresses the fission yeast defects, and our data suggest that these defects could result from both misreading and frame shifting during translation. Thus, a translation defect due to unmodified tRNAs results in severe genome instability.

thiolation | yeast

Most sporadic human cancer cells display enhanced levels of mutagenesis including point mutations, frame shifts, rearrangements, or aneuploidy. Mutator phenotypes are therefore thought to be pivotal in tumorigenesis because they allow the coincident occurrence of multiple mutations, which is unlikely in cells with normal mutation rate (1, 2). Such random mutations could contribute to the morphologic heterogeneity of cancer. A genome-wide screen performed to detect genes that contribute to genome stability in the nematode *Caenorhabditis elegans* identified 61 mutator genes (3) based on a *lacZ* construct put out of frame by an A_{17} mononucleotide repeat allowing detection of frameshift mutations. This identified mutators known to be altered in some human cancers including DNA mismatch repair genes required to alleviate DNA replication errors (4). Beside these, a large number of uncharacterized or poorly characterized genes were identified that might be equally relevant in cancer development. We focused on the F29C4.6 gene (hereafter named *CTU1* for reasons that will become clear below) because it encodes a remarkably conserved protein among eukaryotes [supporting information (SI) Fig. S1]. All of the orthologs contain a phosphate-binding loop motif referred to as "PP-loop" (SGGKDS), a specific ATP binding motif found in the ATP pyrophosphate (pyrophosphate synthase) family (5).

tRNAs from all organisms contain modified nucleosides, and, intriguingly, several enzymes implicated in tRNA thiolation harbor a PP-loop motif. TtcA, an enzyme responsible for the synthesis of 2-thiocytidine (S^2C) (Fig. S2) at position 32 of tRNA (6), is the closest *Escherichia coli* sequence to the Ctu1 protein family, but the S^2C modification is not found in eukaryotic tRNAs. In contrast, the thiolation of the wobble uridine (S^2U) at position 34 in $tRNA^{LYS}_{UUU}$, $tRNA^{GLU}_{UUC}$, and $tRNA^{GLN}_{UUG}$ is conserved in nearly all species. Glutamate, lysine, and glutamine are encoded by two degenerate codons ending in purine in the two-codon boxes that specify two amino acids by difference in the third bases in the genetic code. The corresponding tRNAs decode codons of the type NAA and wobble onto NAG. The thiolation of the wobble base on position 2, together with the addition of methoxycarbonylmethyl on position 5 (mcm^5S^2U), was proposed to facilitate and restrict base pairing with purines and to prevent incorrect decoding (7–12).

In *E. coli*, the PP-loop containing protein MnmA is responsible for the S^2U modification, and eukaryotes contain a sequence closely related to MnmA named Mtu1/Slm3 and shown to be the mitochondrial S^2U wobble base thiolase (Fig. S2), whereas the cytosolic thiolase was not identified (13). Recently, a new complex of two proteins from *Thermus thermophilus* (TtuA–TtuB for tRNA-two-thiouridine) was identified as the thiolase of 2-thioribothymidine (S^2U, also known as S^2T) at position 54 of tRNA. Strikingly, the perfect alignment of the PP-loop of TtuA with Ctu1 orthologs extended to five conserved CXXC motifs required for the thiolase activity (Fig. S3) (6).

Interestingly, the deletion of the budding yeast ortholog of *ctu1* (named *NCS6/TUC1*), when combined with a deletion of *ELP3* that results in the lack of the mcm^5 side chain, removes all modifications from the wobble uridine derivatives and is lethal to the cell (14).

In line with this, we show that Ctu1 (cytosolic tRNA thiouridilase) associates with the conserved Ctu2 protein and that this complex is the wobble uridine-34 thiolase of cytosolic tRNAs in eukaryotes. The analysis of deletion mutants in both *C. elegans* and the fission yeast *Schizosaccharomyces pombe* reveals that the complex is essential to maintaining genome stability.

Results

The *C. elegans* F29C4.6 (here named *CTU-1*, cytosolic tRNA thiouridilase; see below) ORF was isolated in a genome-wide screen for mutator genes (3). To investigate its function, we studied a potential null mutation (isolated by S. Mitani, National Bioresource Project for the Nematode, Japan). This *tm1297* allele has an 826-bp deletion that largely removes exon 2. Homozygous mutant animals were viable at 20°C, and synchronous populations of the wild type and mutant displayed similar growth (Fig. 1*A*). At a higher temperature (25°C), the hatched eggs progressed through the L1–L4 larval stages with kinetics similar to those of wild type but presented an important delay in germ-line maturation. Whereas gravid wild-type worms were present 53 h after hatching, germ-line maturation was still ongoing in the mutant after 66 h, and the first adults containing eggs were observed 75 h after hatching, when a large number of new L1 were already present in the wild type (Fig. 1*B*). Strikingly, the eggs that finally appeared in the mutant displayed aberrant morphology and size resulting in both low progeny and high mortality (Fig. 1*C*). This indicated that at 25°C the *CTU-1* gene is required for normal germ-line maturation and for viability.

To test whether the Ctu1 protein was physically associated with other proteins in an evolutionary conserved complex, we first

Author contributions: D.H. designed research; M. Dewez, F.B., M. Dieu, and D.H. performed research; M. Dieu and M.R. contributed new reagents/analytic tools; F.B., M. Dieu, J.V., and D.H. analyzed data; and D.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

§To whom correspondence should be addressed. E-mail: damien.hermand@fundp.ac.be.

This article contains supporting information online at www.pnas.org/cgi/content/full/0709404105/DCSupplemental.

GENETICS

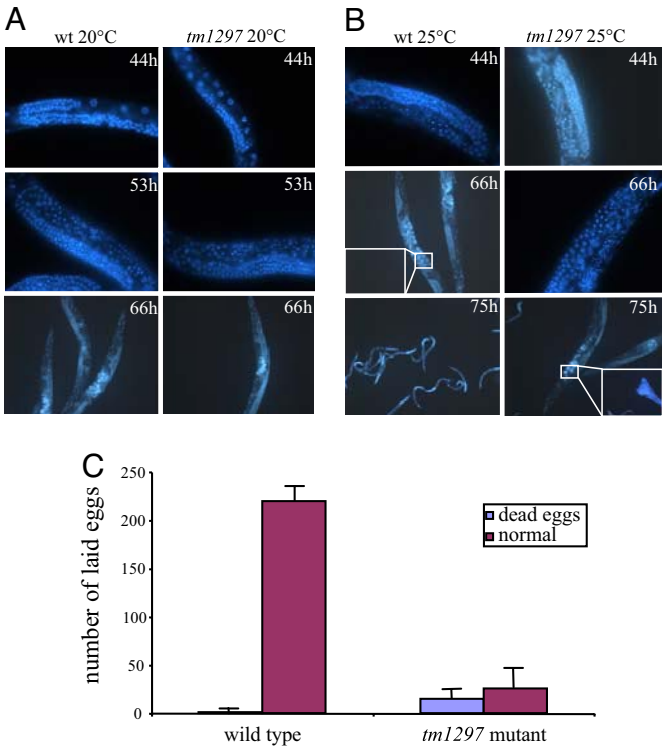

Fig. 1. The conserved F29C4.6 (Ctu1) protein is required for embryogenesis at high temperatures. (*A*) Synchronized L1 worms from wild type or the *tm1297* mutant were grown at 20°C, and samples were collected and stained with DAPI at the indicated time points. Developing eggs are magnified. (*B*) Same as in *A*, except worms were grown at 25°C. (*C*) The number of eggs laid and their viability at 25°C were determined for 10 individuals for both the wild type and the mutant.

performed a tandem affinity purification (TAP) of its fission yeast ortholog (termed SPBC2G5.03). Preliminary experiments revealed that this gene encodes a soluble protein expressed at all stages of the fission yeast cell cycle (data not shown). One copurifying protein was identified by MS as the product of the conserved SPBC19c2.13c gene (Fig. 2*A* and Fig. S4), and this interaction was

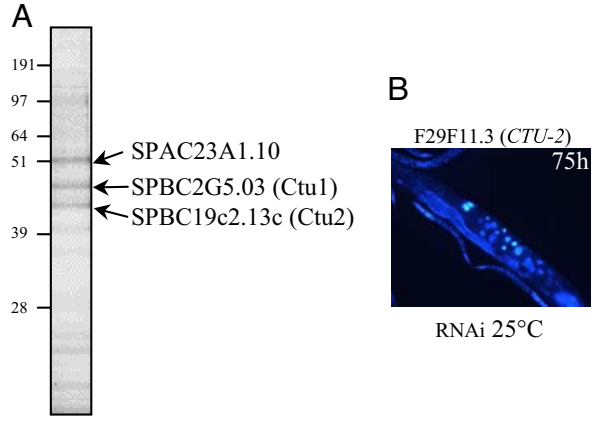

Fig. 2. Ctu1 forms a functional complex with Ctu2. (*A*) TAP purification and MS analysis of polypeptides associated with SPBC2G5.03 (Ctu1). The SPAC23A1.10 is a translation elongation factor often found in TAP purifications and was therefore not further investigated in this study. (*B*) RNAi knockdown of F29F11.3 (*CTU-2*) at 25°C. A representative adult worm 75 h after hatching is shown.

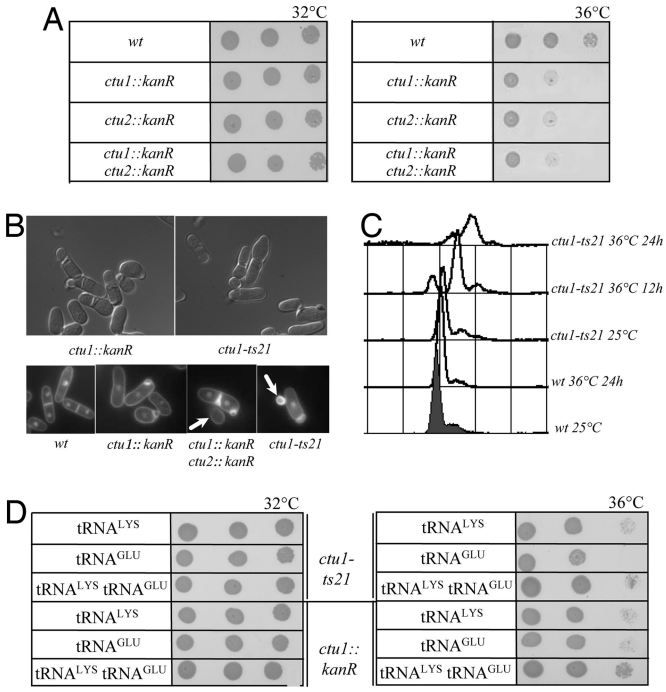

Fig. 3. Deletion of the fission yeast *ctu1* and *ctu2* leads to genome instability. (*A*) Serial dilutions of indicated single or double deleted strains at 32°C (*Left*) and 36°C (*Right*). (*B*) Nomarski pictures of live indicated strains and DAPI/Calcofluor pictures of fixed cells from indicated strains grown at 36°C. Arrows point to aneuploid cells. (*C*) FACS analysis of indicated strains grown at 25°C or 36°C for 12 or 24 h. (*D*) Suppression of the growth defect of strains mutated for *ctu1* by multicopy expression of $tRNA^{LYS}$, $tRNA^{GLU}$, or a combination of both. Serial dilutions at 32°C or 36°C are presented.

confirmed by independent coimmunoprecipitation (data not shown). Based on these data, the new gene was named *ctu2*. The existence of the new complex Ctu1–Ctu2 is likely to be conserved in *C. elegans* because the inactivation of the ortholog of *CTU-2* (termed F29F11.3) by RNAi gave a thermosensitive phenotype very similar to that of the *CTU-1* mutant (Fig. 2*B* and data not shown). In budding yeast, the orthologs of *ctu1* and *ctu2* (named *NCS6* and *NCS2*) were both recovered among 10 other *NCS* (needs Cla4 to survive) as colethal with the PAK (P-21-activated kinase) Cla4 (15, 16). However, we were unable to reconstitute these genetic interactions in fission yeast (data not shown).

Single or double deletion mutants of the fission yeast *ctu1* and *ctu2* genes resulted in a thermosensitive lethal phenotype reminiscent of the inactivation of their worm counterparts (Fig. 3*A*). Double deletions did not aggravate the phenotype of the single mutants, in line with the notion that the encoded proteins belong to the same functional complex (Fig. 2). At the restrictive temperature (36°C) these mutants displayed altered morphology with a high percentage of misplaced septum resulting in aberrant ploidy as shown by DAPI/Calcofluor staining (Fig. 3*B*). FACS analysis confirmed the presence of a high percentage of cells with more than 2C DNA content (Fig. 3*C*). Finally, we generated seven thermosensitive alleles of the *ctu1* gene by *in vitro* mutagenesis and integration at the locus. They displayed a phenotype similar to that of the deletion mutant but with higher penetrance. The representative *ctu1-ts21* strain was further characterized (Fig. 3*B* and data not shown).

We introduced a fission yeast genomic library in the *ctu1-ts21* mutant and the *ctu1* deletion and screened for clones growing at 36°C. In addition to plasmids harboring the wild-type gene, we identified plasmids that partially or completely suppressed the phenotype and allowed growth at 36°C in both strains. They all

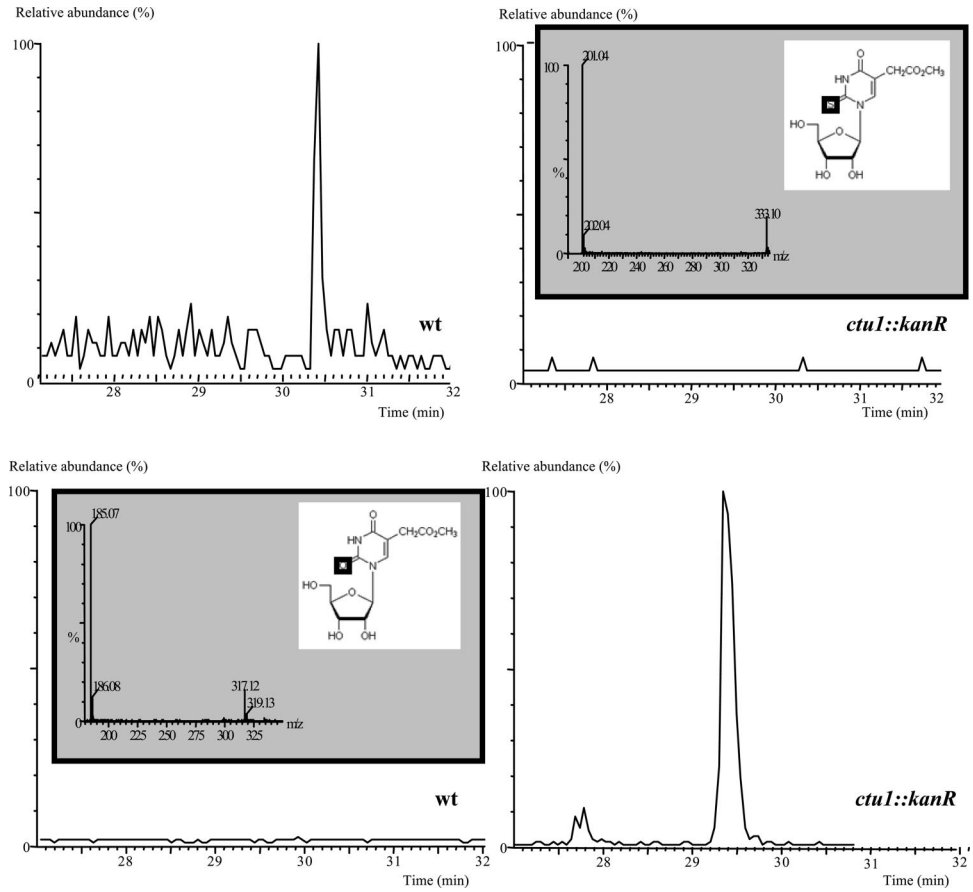

Fig. 4. The thiolated uridine-34 is absent in a strain deleted for *ctu1*. Mass chromatograms for mcm^5S^2U (*Upper*) and mcm^5 (*Lower*) of purified tRNAs from wild type (*Left*) and a strain deleted for *ctu1* (*Right*) with relative abundance (*y* axis) and time in minutes (*x* axis). In each *Inset*, the mass spectrum of the protonated molecule and the protonated free base corresponding to the observed peak is presented with the chemical structure of the nucleoside detected.

contained fragments of the mitochondrial genome, which was surprising because mitochondrial DNA transcription depends on a T3/T7 phage-like polymerase unrelated to the nuclear RNA polymerase II (17). Moreover, the suppressing fragments overlapped only at a severely truncated version of subunit 6 of the ATPase (Fig. S5). We reasoned that the tRNA genes spread all over the mitochondrial genome might act as suppressors because it is likely that their internal promoters (18, 19) are recognized by the nuclear RNA polymerase III, as also suggested by a previously reported case of a mitochondrial tRNA suppressing a nuclear mutation (20).

Moreover, Ctu1 is related to tRNA thiolases (see Introduction), and all of the suppressing fragments of mitochondrial DNA we isolated contained either one or a combination of the thiolated tRNAs (Fig. S5). We thus expressed the corresponding nuclear encoded tRNAs alone or in combination on a multicopy vector (Fig. 3*D* and data not shown) and found that expressing $tRNA^{LYS}$ or $tRNA^{GLU}$ greatly improved growth whereas combination of these two was sufficient to restore wild-type growth.

We next used liquid chromatography/ion trap MS (LC/MS-MS) (21) to analyze the nucleosides obtained from tRNAs purified from wild-type and *ctu1*-deleted *S. pombe* strains. In the wild-type strain mcm^5S^2U was detected as the protonated molecule (MH^+) and the protonated free base (BH_2^+) with the expected *m/z* values of 333 and 201 (21) (Fig. 4). This mcm^5S^2U nucleoside was absent from the mutant strain that rather contained a nucleoside undetected in the wild type and corresponding to mcm^5U based on *m/z* values of 317 and 185 for MH^+ and BH_2^+, respectively (Fig. 4). These data established that *ctu1* is specifically required for the 2-thiouridilation.

To see whether the 2-thiolation occurred specifically on $tRNA^{LYS}$, we used the APM/PAGE band shift method, which relies on the strong retardation of thiolated tRNA in electrophoresis because of the affinity of the thio group with the mercuric compound (APM) in the gel (22). These experiments showed that the $tRNA^{LYS}$, but not the $tRNA^{MET}$, which has a C at position 34, was thiolated *in vivo* in *S. pombe* and *C. elegans* and that the thiolation was absent in a *ctu1* mutant from either species (Fig. 5*A*). They also confirmed that the *ctu1-ts* mutant from *S. pombe* was thermosensitive for the thiolation.

It was reported that the Trm2 tRNA(m^5U_{54}) methyltransferase has an alternate role in tRNA maturation not linked to its catalytic activity (23). To test that the phenotype resulting from *ctu1* deletion is due to the absence of thiolation rather than the absence of Ctu1, we generated mutants in the PP-loop (K62A D63A; Fig. S3) and in a CXXC motif (C142A C145A; Fig. S3), both being expected to be required for the catalytic activity (6). In contrast to wild-type *ctu1*, neither the K62A D63A mutant nor the C142A C145A mutant could rescue the growth defect of the *ctu1*-deleted strain at 36°C or restore thiolation when introduced on a plasmid, although their expression levels were similar to that of the wild-type control (Fig. 5*B*). Taken together with the fact that the level of a $tRNA^{LYS}$ was similar between a wild-type or *ctu1*-deleted strain (Fig. 5*A*), we conclude that the absence of thiolase activity is the main cause of the observed phenotype.

To address the direct role of Ctu1 in the thiolation of some tRNAs, we first tested whether Ctu1 binds tRNAs. Extracts from a Ctu1-TAP or control strains were incubated with ^{32}P-labeled T7-transcribed $tRNA^{LYS}_{UUU}$ or $tRNA^{MET}_{CAT}$. After UV cross-linking, proteins were purified on IgG beads and separated by PAGE. Autoradiography revealed that the $tRNA^{LYS}_{UUU}$ specifically binds to and is coprecipitated by Ctu1 (Fig. 6*A*).

We next attempted to assay the sulfurase activity of Ctu1 *in vitro*. Using an elegant ribonucleome analysis in bacteria, Ikeuchi *et al.* (7) identified the proteins required to reconstitute efficient 2-thiouridine formation from cysteine *in vitro*. Beside the already mentioned

GENETICS

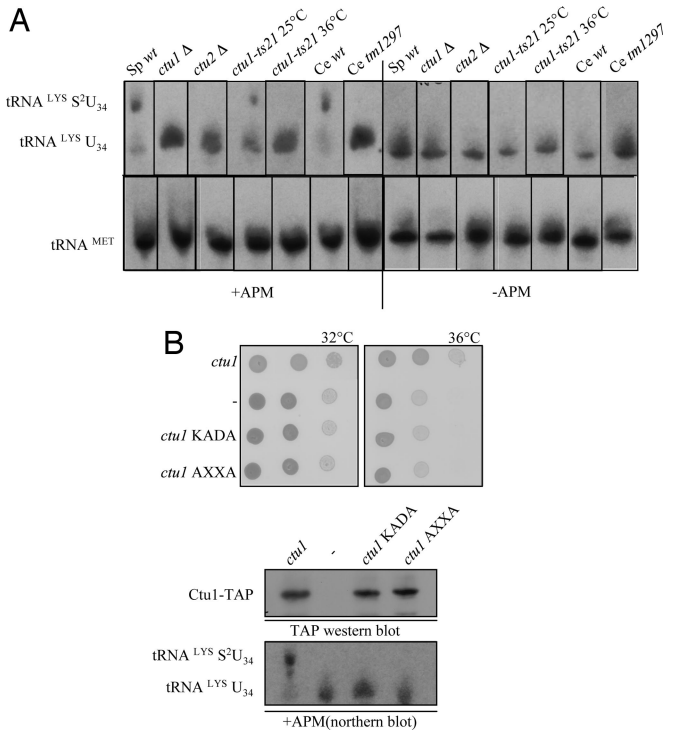

Fig. 5. The Ctu1–Ctu2 complex is required for thiolation of uridine-34 in tRNAs from yeast and nematode. (*A*) Purified tRNA from indicated strains was separated on 8 M urea containing polyacrylamide gels in the presence (*Left*) or absence (*Right*) of APM. Northern hybridization was performed with multiprimed probes corresponding to either $tRNA^{LYS}$ or $tRNA^{MET}$ as indicated. (*B*) The KADA (K62A D63A) and the AXXA (C142A C145A) mutations were introduced in Ctu1-TAP expressed from pREP-4, and the resulting plasmids were transformed in a *ctu1*-deleted strain together with an empty vector as indicated. Growth of corresponding strains was tested at 32°C and 36°C (*Top*), and the expression level of Ctu1-TAP was tested by Western blot (*Middle*). (*Bottom*) Purified tRNA from indicated strains was analyzed by APM gel as in *A*.

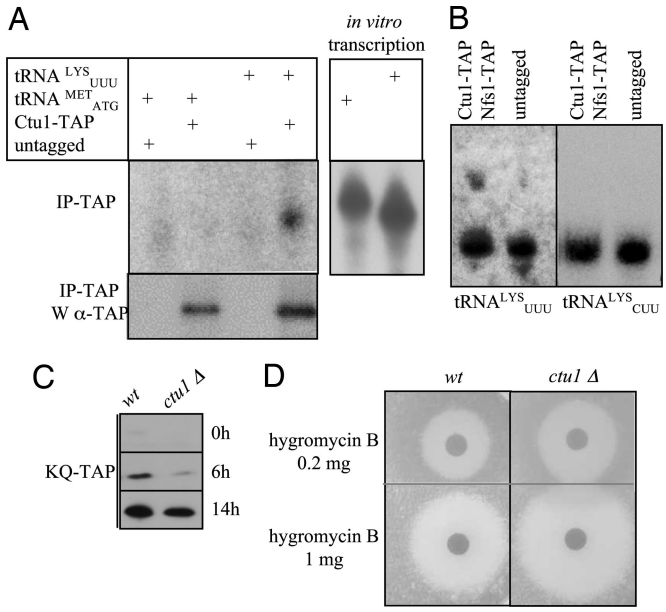

Fig. 6. Ctu1 binds tRNA and is required for efficient translation. (*A*) Extracts from Ctu1-TAP or untagged control were incubated with *in vitro* synthesized, radiolabeled $tRNA^{LYS}$ or $tRNA^{MET}$. After cross-linking, precipitation on IgG beads was performed and the elution product was analyzed on a denaturing gel. Western blot was performed on 10% of the precipitated product to check for the presence of Ctu1-TAP. The untreated transcribed tRNAs are shown in *Right*. (*B*) *In vitro* thiolation assay using purified Ctu1-TAP and Nfs1-TAP or untagged control. Precipitated proteins on beads were incubated with cysteine, ATP, and *in vitro* synthesized $tRNA^{LYS}_{UUU}$ or$tRNA^{LYS}_{CUU}$. Resulting supernatants were separated on 8 M urea-containing polyacrylamide gels in the presence of APM. Northern hybridization was performed with a multiprimed probe corresponding to either $tRNA^{LYS}$. (*C*) A plasmid expressing a KQ-TAP protein (see *Materials and Methods*) under the control of the thiamine-repressed *nmt* promoter was transformed in the indicated strains. Induction was performed in medium lacking thiamine for the indicated time, and total protein extracts were analyzed by Western blot. (*D*) Indicated strains were plated on rich medium, on which a filter containing 0.2 mg or 1 mg of hygromycin B was placed. The size of the halo reflects the sensitivity of the strain toward the drug.

MnmA thiolase, six other proteins were required, and among those only the cysteine desulfurase is clearly conserved in eukaryotes. From this study and others (12, 24) it appeared that the thiolase is the final acceptor of a sulfur flow and recognizes tRNAs while the PP-loop activates the C2 position of the uracil ring at position 34 by forming an adenylate intermediate. We reasoned that combining TAP purifications of both Ctu1-TAP and Nfs1-TAP (the fission yeast cysteine desulfurase) might allow *in vitro* $tRNA^{LYS}$ thiolation detectable by APM/PAGE. As presented in Fig. 6*B*, a retarded band specific to $tRNA^{LYS}_{UUU}$ appeared when the *in vitro* synthesized $tRNA^{LYS}_{UUU}$ was incubated in the presence of cysteine with Nfs1 and Ctu1–Ctu2 purified from *S. pombe*. The band shift was not observed when a $tRNA^{LYS}_{CUU}$ that does not contain an uridine at the wobble position was used in the assay (Fig. 6*B*). As expected, the efficiency was very low, confirming that important components were missing in this assay and that additional work is needed to identify them all.

What are the biological consequences of the absence of thiolation in tRNAs from split codon boxes? The first conclusion we can draw from the mutated worm and yeast is that the modification functions as a thermostabilizer because the phenotype is most obvious at elevated temperature. Beside this, the 2-thio modification has long been known to play a critical role in the decoding mechanism at several stages: it restricts the base-pairing capability and prevents misreading of other near-cognate codons, but it is also needed for efficient reading of the A and G ending codons (7, 9–11, 25). It was also shown to play a role in recognition by the synthetase (26), and it strongly reduces the frequency of frame shifting (27). The identification of the eukaryotic thiolase we describe here allowed us to test some of these possibilities *in vivo* by comparing a wild-type strain and a *ctu1* mutant. We first generated an artificial protein corresponding to the TAP tag where 10 triplets consisting of Lys^{AAA} and Gln^{CAA} codons were inserted downstream of the ATG (KQ^{10}-TAP) and expressed it in a wild-type or the *ctu1-ts* strain under the control of the *nmt41* promoter, which is induced in the absence of thiamine. The three codons CAA (Gln), AAA (Lys), and GAA (Glu) represent 28% of the codon used in this protein. To test whether the presence of the 10 triplets would affect the expression of this protein, the resulting strains were grown in the absence of thiamine at 32°C (semipermissive temperature of the *ctu1-ts*) to induce transcription. Equal protein amounts were separated by PAGE and analyzed by Western blot. Fig. 6*C* shows that the KQ^{10}-TAP protein was much less abundant in the *ctu1* mutant and that a transcriptional effect was excluded (data not shown). Second, the inactivation of *ctu1* in *S. pombe* rendered cells significantly more sensitive to hygromycin B (Fig. 6*D*). This aminoglycoside antibiotic decreases the translational accuracy by stimulating the stable association of near-cognate tRNA to the ribosome (28–30). Our data show that its effect is additive to the presence of unmodified tRNA, and they provide indirect evidence supporting the role of the modification in preventing misreading. Taken together, these data are in line with the known roles of the thiolation

and with the fact that a higher dosage of tRNA can compensate for the absence of the modification.

Discussion

When an uridine is present in the wobble position of tRNAs, this residue is almost universally modified. Particularly, the corresponding tRNAs for lysine, glutamine, and glutamate, whose codons all belong to split codon boxes, are thiolated at the 2-carbon and contain a mcm^5 modification at the 5-carbon on the uridine. Here we have identified and characterized the Ctu1–Ctu2 complex as the cytosolic tRNA thiolase in two model eukaryotes, namely the fission yeast *S. pombe* and the nematode *C. elegans*. Interestingly, early genetic analysis of antisuppressor mutants in fission yeast identified several *sin* mutants where the mcm^5S^2U nucleoside was absent (31, 32). Recently, *sin3* was shown to encode Elp3, a subunit of the elongator complex implicated in the mcm^5 side chain addition (8, 33). Intriguingly, S^2U was also lost in that strain although Ctu1 was present. MS analysis confirmed that both modifications were absent in a *elp3*deleted strain (our unpublished data) whereas a *ctu1* deletion lacks only the thiolation, suggesting that the presence of the mcm^5 modification might be a prerequisite for the thiolation *in vivo*.

The ubiquitous presence of the 2-thiolation in the wobble uridine allows us to analyze the conservation of the enzyme responsible for this modification along evolution. In eukaryotes, the mitochondrial thiolase Mtu1 is clearly related to the bacterial thiolase MnmA (13, 24), but, strikingly, it appears that its cytosolic counterpart, Ctu1, which we have identified in this study, is much more closely related to another bacterial thiolase, TtcA (6), which is involved in synthesis of 2-thiocytidine at position 32 in tRNAs, a modification absent in eukaryotes (see also the Introduction and ref. 14). Therefore, the phylogeny of the wobble uridine thiolase is more complex than what could have been expected in view of the universal presence of wobble uridine thiolation.

The 2-thio modification was suggested for long to play a critical role at several stages in the decoding mechanism by restricting the base-pairing capability to avoid misreading of other near-cognate codons and by allowing efficient reading of the A- and G-ended codons (7, 9–11, 25). Our data showing that overexpression of unmodified tRNA rescues the deletion phenotype and the fact that a decreased level of a synthetic KQ-TAP protein is observed when Ctu1 function is abolished both point to a critical role in the improvement of cognate codon recognition. This requirement of the modification for efficiency might be related to the fact that the corresponding thiolated tRNAs ($tRNA^{LYS}_{\underline{UU}U}$, $tRNA^{GLU}_{\underline{UC}}$, and $tRNA^{GLN}_{\underline{UG}}$) all harbor two uridines in positions 34 and 35 in the anticodon, creating an instable codon–anticodon interaction due to the low stacking potential of uridine (25, 34–36).

In line with a role of the thiolation in the tRNAs binding to their cognate codons, the absence of S^2U in mitochondria results in impaired mitochondrial protein synthesis, which leads to reduced respiratory activity and human pathologies including MERFF (myoclonus epilepsy associated with ragged-red fibers), a subgroup of the mitochondrial encephalomyopathies where the mitochondrial $tRNA^{LYS}$ lacking the wobble modification cannot translate either of its codons (AAA and AAG) (13, 37–39).

What is the impact of a decrease in the efficiency of the cytosolic $tRNA^{LYS}_{\underline{UU}U}$, $tRNA^{GLU}_{\underline{UC}}$, and $tRNA^{GLN}_{\underline{UG}}$ at the proteome scale? Analysis of the codon usage in the lysine, glutamine, and glutamate codon boxes reveals a striking feature: in *S. pombe*, *Saccharomyces cerevisiae*, and *C. elegans*, whose genomes have low G-C content, there is a bias toward the use of the A-ended codon, and this tendency is reversed for organisms with high G-C content, typically higher eukaryotes (Fig. S7 *Upper*). Moreover, GAA (Glu) and AAA (Lys) are the two most abundant codons used in *S. pombe*, *S. cerevisiae*, and *C. elegans* (Fig. S7 *Lower*). Based on this, it is possible that *ctu1* deletion is affecting the entire proteome or that only proteins with a distinctive codon usage enriched for the AAA, GAA, and CAA codons are affected. The specificity of the observed phenotypes both in fission yeast and in worm is in favor of the second possibility and is reminiscent of the specific phenotype (cell cycle arrest) resulting from the absence of wobble inosine in fission yeast (40). This is also supported by a very recent study showing that deletion of the Trm9 $tRNA^{ARG}$ and $tRNA^{GLU}$ methyltransferase affects the translation of transcripts overrepresented with specific arginine and glutamate codons while the translation of average transcripts that contain a normal codon usage pattern is unaffected (41).

In higher eukaryotes, the GAA (Glu) and AAA (Lys) codons are not the most abundant, yet the human *ctu1* gene was identified independently and named *cag-1* (cancer-associated gene) because it is located in a region nonrandomly rearranged in many solid tumors and it is differentially expressed in some cancers (42). Future work is needed to clarify whether the molecular function we assigned to Ctu1 is relevant for cancer etiology.

Materials and Methods

General Methods. The original *tm1297* mutant was outcrossed three times and contains an 826-bp deletion, a +1-bp insertion, and the structure 10824-G-11651. Worms were synchronized by bleaching of adults and hatching in M9 medium. For DAPI staining, worms were fixed in ethanol and DAPI was added at 150 ng/ml. Worms were rehydrated and washed in M9. To assess viability, 10 L4 hermaphrodites were plated. Both eggs and surviving hatched animals were then counted. RNAi was performed by feeding worms with bacteria expressing a double-stranded RNA corresponding to the F29F11.3 sequence. Sequence alignments were performed with the online ClustalW server (www.ebi.ac.uk/clustalw/). Mutations in the *ctu1* sequence were introduced with the QuikChange mutagenesis kit (Stratagene). Primer sequences and details are available upon request.

Analysis of tRNA Hydrolysates by MS (21, 43, 44). An LCQ ion-trap mass spectrometer equipped with an electrospray ionization was used to analyze RNase-P1 (US-Biological)/bacterial alkaline phosphatase (Fermentas)-treated tRNAs (2 mg). The hydrolysates were analyzed by LC/MS-MS (CapLC and QTOF-2; Waters). A Symmetry c18 5-mm, 0.32- × 150-mm column (Waters) with a flow rate of 2 ml/min was used with a solvent system consisting of solvent A (5 mM NH_4OAc, pH 5.3) and solvent B (60% acetonitrile) and a multistep gradient: 0–10 min (0% A to 1% B), 10–45 min (1% B to 35% B), 45–50 min (35% B to 99% B), and 50–60 min (99% B). The effluent was conducted directly into the ion source, and positive ions were scanned over an *m/z* range of 100–500 for MS-MS scan without sequencing of the protonated molecule (MH^+) and the corresponding protonated free base (BH_2^+) with their expected *m/z* values. Purified nucleosides were used to set up the assay. Fig. S6 shows the example of adenosine (21).

TAP. TAP purification was performed essentially as described (45) from 12 liters of the Ctu1-TAP strain grown to an OD of 0.5. The elution products were separated and silver-stained. The bands were cut from the gel; after trypsination peptides were analyzed by MS-MS, and resulting data were compared with the fission yeast proteome.

Generation of Thermosensitive Alleles of *ctu1*. The *natR* selection marker (46) was integrated downstream of the *ctu1* locus. A fragment containing both *ctu1* and *natR* was then reamplified in mutagenic conditions (5.6 mM dATP, 9.0 mM dCTP, 2.0 mM dGTP, and 1.4 mM dTTP with a final Mg^{2+} concentration of 3.26 mM) using *Taq* (Invitrogen) and integrated. Clones showing a thermospecific strong phloxin B staining (47) were selected and sequenced.

Multicopy Suppression of the *ctu1*::*kanR* and *ctu1-ts21* Phenotype. A *S. pombe* genomic library (48) was transformed in both strains, and 10,000 clones were screened by incubating cells for 2 days at permissive temperature and replica plating at 36°C. A total of 87 growing clones were restreaked, and plasmids were extracted and sequenced. Clones containing the wildtype *ctu1* gene were prescreened by PCR.

Cloning and Expression of Cytosolic tRNA. Sequences corresponding to the tRNA SPBTRNALYS.06 and SPBTRNAGLN.02 flanked by ≈500 bp were cloned in a *S. pombe* vector (pJK148 containing ARS1).

Purification of Total tRNA from Yeast and Worm. Total RNA was prepared with the Qiagen RNA/DNA maxi kit and the glass beads method and FastPrep. Low-molecular-weight RNAs were specifically eluted with buffer QRW2 according to the manufacturer.

GENETICS

APM/PAGE (22) and Northern Blot Analysis. Denaturing and 40% acrylamide urea/PAGE (9.6%) were performed in the presence of 1 mg/ml APM [a kind gift of G. Igloi (University of Freiburg, Freiburg, Germany)]. Gels were run at 225 V for 2–3 h and transferred on membranes by using semidry electrotransfer. Probes corresponding to different tRNAs were synthesized by using multiprime labeling (Invitrogen) and long DNA oligos corresponding to the tRNA sequence as template.

tRNA Coimmunoprecipitation. Cells (100 ml) were grown to OD 0.5 and harvested. After washing, cold breaking buffer (10 mM HEPES, pH 7.3/50 mM KCl/10 mM MgAc/5 mM DTT/5% glycerol) was added with protease inhibitors (Boehringer). Cells were disrupted with glass beads, the extracts was centrifuged, and protein concentration was determined. Radiolabeled tRNAs were synthesized *in vitro* by using T7 polymerase and the riboprobe transcription system (Promega). Templates were dsDNA made of long oligos containing the tRNA sequence and the T7 promoter (TAATACGACTCACTATAG). The tRNAs were added to 300 ml of extracts with 20 units of Rnasin (Promega), and 20-ml fractions were transferred to a 96-well plate, incubated for 10 min at room temperature, and exposed five times to 120 mJ/cm^2 UV by using a Stratalinker. Fractions were pooled and used for TAP purification. Beads were washed three times with 10 mM HEPES (pH 7.3), 50 mM KCl, 10 mM MgAc, 5 mM DTT, and 0.1% Triton X-100 and incubated for 15 min at 65°C in 10 ml. This elution product was separated on a denaturing polyacrylamide gel and exposed to film.

***In Vitro* tRNA Thiolation Assay.** A total of 200 ml of Ctu1-TAP and Nfs1-TAP cultures and 400 ml of untagged control were grown to $OD_{0.5}$ and washed with buffer T1 [10 mM Tris, pH 7.5/50 mM KCl/12 mM Mg(OAc)2/protease inhibitors]. Cells were lysed with zirconium beads and FastPrep, and the protein concentration of the extracts was determined. One milligram of Ctu1-TAP and Nfs1-TAP lysates was mixed and incubated with IgG beads (Sigma) for 2 h. An equivalent amount of the untagged control was treated in the same way. Beads were washed four times with buffer T1 and incubated with buffer T2 [10 mM Tris, pH 7.5/50 mM KCl/12 mM Mg(OAc)2/5 mM ATP/1 mM cysteine/3 mg of *in vitro* synthesized tRNA^{LYS} (see above)/20 units of RNAsin/20 mM pyridoxal 5′ phosphate] for 30 min at 30°C. The supernatants were separated on APM/PAGE.

Expression of the KQ-TAP Protein. The TAP tag was amplified with a forward primer containing the CGCGGATCC-ATG-AAA-CAA-AAA-CAA-AAA-CAA-AAA-CAA-AAA-CAA sequence and cloned in BamHI-SmaI in the pREP-41 under the control of the *nmt* promoter, which is repressed by thiamine (49–51).

ACKNOWLEDGMENTS. We are grateful to Ronald Plasterk and the Hubrecht Institute, where this work was initiated. We thank Edouard Delaive for technical assistance; Stéphanie Genevrois, Damien Coudreuse, Pierre Thuriaux, and Marc Vidal for critical reading of the manuscript; Gabor Igloi for providing the APM compound; and the Mitani laboratory for isolating the *tm1297* mutant. D.H. was supported by the Human Frontier Science Program (LT00665/2003).

1. Loeb LA, Loeb KR, Anderson JP (2003) Multiple mutations and cancer. *Proc Natl Acad Sci USA* 100:776–781.
2. Bielas JH, Loeb KR, Rubin BP, True LD, Loeb LA (2006) Human cancers express a mutator phenotype. *Proc Natl Acad Sci USA* 103:18238–18242.
3. Pothof J, *et al.* (2003) Identification of genes that protect the *C. elegans* genome against mutations by genome-wide RNAi. *Genes Dev* 17:443–448.
4. Leach FS, *et al.* (1993) Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell* 75:1215–1225.
5. Bork P, Koonin EV (1994) A P-loop-like motif in a widespread ATP pyrophosphatase domain: Implications for the evolution of sequence motifs and enzyme activity. *Proteins* 20:347–355.
6. Jager G, Leipuviene R, Pollard MG, Qian Q, Bjork GR (2004) The conserved Cys-X1–X2-Cys motif present in the TtcA protein is required for the thiolation of cytidine in position 32 of tRNA from Salmonella enterica serovar Typhimurium. *J Bacteriol* 186:750–757.
7. Ikeuchi Y, Shigi N, Kato J, Nishimura A, Suzuki T (2006) Mechanistic insights into sulfur relay by multiple sulfur mediators involved in thiouridine biosynthesis at tRNA wobble positions. *Mol Cell* 21:97–108.
8. Huang B, Johansson MJ, Bystrom AS (2005) An early step in wobble uridine tRNA modification requires the Elongator complex. *RNA* 11:424–436.
9. Kruger MK, Pedersen S, Hagervall TG, Sorensen MA (1998) The modification of the wobble base of tRNAGlu modulates the translation rate of glutamic acid codons in vivo. *J Mol Biol* 284:621–631.
10. Yarian C, *et al.* (2002) Accurate translation of the genetic code depends on tRNA modified nucleosides. *J Biol Chem* 277:16391–16395.
11. Suzuki T (2005) in *Fine-Tuning of RNA Functions by Modification and Editing*, ed Grosjean H (Springer, Heidelberg, Germany), pp 23–69.
12. Numata T, Ikeuchi Y, Fukai S, Suzuki T, Nureki O (2006) Snapshots of tRNA sulphuration via an adenylated intermediate. *Nature* 442:419–424.
13. Umeda N, *et al.* (2005) Mitochondria-specific RNA-modifying enzymes responsible for the biosynthesis of the wobble base in mitochondrial tRNAs. Implications for the molecular pathogenesis of human mitochondrial diseases. *J Biol Chem* 280:1613–1624.
14. Bjork GR, Huang B, Persson OP, Bystrom AS (2007) A conserved modified wobble nucleoside (mcm5s2U) in lysyl-tRNA is required for viability in yeast. *RNA* 13:1245–1255.
15. Goehring AS, Rivers DM, Sprague GF, Jr (2003) Urmylation: A ubiquitin-like pathway that functions during invasive growth and budding in yeast. *Mol Biol Cell* 14:4329–4341.
16. Goehring AS, *et al.* (2003) Synthetic lethal analysis implicates Ste20p, a p21-activated potein kinase, in polarisome activation. *Mol Biol Cell* 14:1501–1516.
17. Schafer B, Hansen M, Lang BF (2005) Transcription and RNA-processing in fission yeast mitochondria. *RNA* 11:785–795.
18. Marck C, *et al.* (2006) The RNA polymerase III-dependent family of genes in hemiascomycetes: Comparative RNomics, decoding strategies, transcription and evolutionary implications. *Nucleic Acids Res* 34:1816–1835.
19. Sprague KU (1995) in *tRNA: Structure, Biosynthesis and Function*, eds Soll D, RajBhandary U (Am Soc Microbiol, Washington, DC).
20. Fiori A, Francisci S, Falcone C (2002) Suppression of a nuclear frameshift mutation by a mitochondrial tRNA in the yeast Kluyveromyces lactis. *Mol Microbiol* 46:169–177.
21. Pomerantz S, McCloskey J (1990) in *Methods in Enzymology*, ed McCloskey JA (Academic, New York), pp 796–824.
22. Igloi GL (1988) Interaction of tRNAs and of phosphorothioate-substituted nucleic acids with an organomercurial. Probing the chemical environment of thiolated residues by affinity electrophoresis. *Biochemistry* 27:3842–3849.
23. Johansson MJ, Bystrom AS (2002) Dual function of the tRNA(m(5)U54)methyltransferase in tRNA maturation. *RNA* 8:324–335.
24. Kambampati R, Lauhon CT (2003) MnmA and IscS are required for in vitro 2-thiouridine biosynthesis in Escherichia coli. *Biochemistry* 42:1109–1117.
25. Murphy FVt, Ramakrishnan V, Malkiewicz A, Agris PF (2004) The role of modifications in codon discrimination by tRNA(Lys)UUU. *Nat Struct Mol Biol* 11:1186–1191.
26. Kruger MK, Sorensen MA (1998) Aminoacylation of hypomodified tRNAGlu in vivo. *J Mol Biol* 284:609–620.
27. Urbonavicius J, Qian Q, Durand JM, Hagervall TG, Bjork GR (2001) Improvement of reading frame maintenance is a common function for several tRNA modifications. *EMBO J* 20:4863–4873.
28. Singh A, Ursic D, Davies J (1979) Phenotypic suppression and misreading *Saccharomyces cerevisiae*. *Nature* 277:146–148.
29. Palmer E, Wilhelm JM, Sherman F (1979) Phenotypic suppression of nonsense mutants in yeast by aminoglycoside antibiotics. *Nature* 277:148–150.
30. Moazed D, Noller HF (1987) Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature* 327:389–394.
31. Heyer WD, *et al.* (1984) An antisuppressor mutation of *Schizosaccharomyces pombe* affects the post-transcriptional modification of the ''wobble'' base in the anticodon of tRNAs. *J Biol Chem* 259:2856–2862.
32. Grossenbacher AM, *et al.* (1986) Antisuppressor mutations and sulfur-carrying nucleosides in transfer RNAs of *Schizosaccharomyces pombe*. *J Biol Chem* 261:16351–16355.
33. Esberg A, Huang B, Johansson MJ, Bystrom AS (2006) Elevated levels of two tRNA species bypass the requirement for elongator complex in transcription and exocytosis. *Mol Cell* 24:139–148.
34. Durant PC, Bajji AC, Sundaram M, Kumar RK, Davis DR (2005) Structural effects of hypermodified nucleosides in the *Escherichia coli* and human tRNALys anticodon loop: The effect of nucleosides s2U, mcm5U, mcm5s2U, mnm5s2U, t6A, and ms2t6A. *Biochemistry* 44:8078–8089.
35. Ashraf SS, *et al.* (1999) Single atom modification (O–>S) of tRNA confers ribosome binding. *RNA* 5:188–194.
36. Agris PF, Vendeix FA, Graham WD (2007) tRNA's wobble decoding of the genome: 40 years of modification. *J Mol Biol* 366:1–13.
37. Yasukawa T, Suzuki T, Ishii N, Ohta S, Watanabe K (2001) Wobble modification defect in tRNA disturbs codon-anticodon interaction in a mitochondrial disease. *EMBO J* 20:4794–4802.
38. Yasukawa T, *et al.* (2005) Wobble modification deficiency in mutant tRNAs in patients with mitochondrial diseases. *FEBS Lett* 579:2948–2952.
39. Kirino Y, Suzuki T (2005) Human mitochondrial diseases associated with tRNA wobble modification deficiency. *RNA Biol* 2:41–44.
40. Tsutsumi S, *et al.* (2007) Wobble inosine tRNA modification is essential to cell cycle progression in G1/S and G2/M transitions in fission yeast. *J Biol Chem* 282:33459–33465.
41. Begley U, *et al.* (2007) Trm9-catalysed tRNA modifications link translation to the DNA damage response. *Mol Cell* 28:860–870.
42. Yousef GM, *et al.* (2004) Molecular cloning of a new gene which is differentially expressed in breast and prostate cancers. *Tumour Biol* 25:122–133.
43. Kaneko T, *et al.* (2003) Wobble modification differences and subcellular localization of tRNAs in *Leishmania tarentolae*: Implication for tRNA sorting mechanism. *EMBO J* 22:657–667.
44. Nakai Y, *et al.* (2004) Yeast Nfs1p is involved in thio-modification of both mitochondrial and cytoplasmic tRNAs. *J Biol Chem* 279:12363–12368.
45. Tasto JJ, Carnahan RH, McDonald WH, Gould KL (2001) Vectors and gene targeting modules for tandem affinity purification in *Schizosaccharomyces pombe*. *Yeast* 18:657–662.
46. Van Driessche B, Tafforeau L, Hentges P, Carr AM, Vandenhaute J (2005) Additional vectors for PCR-based gene tagging in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* using nourseothricin resistance. *Yeast* 22:1061–1068.
47. Moreno S, Klar A, Nurse P (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol* 194:795–823.
48. Barbet N, Muriel WJ, Carr AM (1992) Versatile shuttle vectors and genomic libraries for use with *Schizosaccharomyces pombe*. *Gene* 114:59–66.
49. Basi G, Schmid E, Maundrell K (1993) TATA box mutations in the Schizosaccharomyces pombe nmt1 promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. *Gene* 123:131–136.
50. Maundrell K (1990) nmt1 of fission yeast. A highly transcribed gene completely repressed by thiamine. *J Biol Chem* 265:10857–10864.
51. Maundrell K (1993) Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene* 123:127–130.