Mechanistic characterization of the sulfur-relay system for eukaryotic 2-thiouridine biogenesis at tRNA wobble positions

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ABSTRACT

The wobble modification in tRNAs, 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U), is required for the proper decoding of NNR codons in eukaryotes. The 2-thio group confers conformational rigidity of mcm^5s^2U by largely fixing the C3′-*endo* ribose puckering, ensuring stable and accurate codon–anticodon pairing. We have identified five genes in *Saccharomyces cerevisiae*, *YIL008w* (*URM1*), *YHR111w* (*UBA4*), *YOR251c* (*TUM1*), *YNL119w* (*NCS2*) and *YGL211w* (*NCS6*), that are required for 2-thiolation of mcm^5s^2U. An *in vitro* sulfur transfer experiment revealed that Tum1p stimulated the cysteine desulfurase of Nfs1p, and accepted persulfide sulfurs from Nfs1p. *URM1* is a ubiquitin-related modifier, and *UBA4* is an E1-like enzyme involved in protein urmylation. The carboxy-terminus of Urm1p was activated as an acyl-adenylate (-COAMP), then thiocarboxylated (-COSH) by Uba4p. The activated thiocarboxylate can be utilized in the subsequent reactions for 2-thiouridine formation, mediated by Ncs2p/Ncs6p. We could successfully reconstitute the 2-thiouridine formation *in vitro* using recombinant proteins. This study revealed that 2-thiouridine formation shares a pathway and chemical reactions with protein urmylation. The sulfur-flow of eukaryotic 2-thiouridine formation is distinct mechanism from the bacterial sulfur-relay system which is based on the persulfide chemistry.

INTRODUCTION

Post-transcriptional RNA modifications are characteristic structural feature of RNA molecules. RNA modifications play critical roles in various aspects of RNA molecules, including biogenesis, metabolism, structural stability and functions. To date, more than 100 species of RNA modifications have been reported (1). The majority of these modifications have been identified and characterized in tRNA molecules (2–4). In the region of the tRNA anticodon in particular, various modified nucleosides with diverse chemical structures have been found at the anticodon first (wobble) position 34, and at position 37 3′-adjacent to the anticodon. The wobble modifications play pivotal roles in deciphering genetic codes that are mediated by the precise codon–anticodon interactions at the ribosomal A-site. The wobble base of the tRNAs for Glu, Gln and Lys are universally modified to 5-methyl-2-thiouridine derivatives (xm^5s^2U), such as 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) and 5-carboxymethyl-2-thiouridine ($cmnm^5s^2U$) in bacterial tRNAs, 5-taurinomethyl-2-thiouridine (τm^5s^2U) in mammalian mitochondrial tRNAs and 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) in cytoplasmic tRNAs in eukaryotes (4) (Figure 1). It is known that the conformation of xm^5s^2U is largely fixed in the C3′-*endo* form of its ribose puckering, since the large van der Waals' radius of the 2-thio group causes a steric repulsion with its 2′ OH group (5). The xm^5s^2U modification base-pairs preferentially with purine and prevents misreading of near cognate codons ending in pyrimidine due to this conformational rigidity (5,6). It is known that the 2-thio group of mnm^5s^2U is required for efficient codon recognition on the ribosome (7). In addition, the 2-thio group of mnm^5s^2U in tRNA^{Glu} acts as the identifying element for specific recognition by glutaminyl-tRNA synthetase (8). Lack of xm^5s^2U modification in the mutant mitochondrial tRNA^{Lys} from myoclonus epilepsy associated with ragged-red fibers (MERRF), results in a marked defect in whole mitochondrial translation (9,10). Thus, the 2-thio modification xm^5s^2U plays a critical role in protein synthesis.

It is known that sulfur is an essential element for all living organisms. In fact, sulfur occurs in all the major classes of biomolecules, such as Fe/S clusters (iron/sulfur clusters or ISCs) in proteins, enzyme cofactors such as

A

B
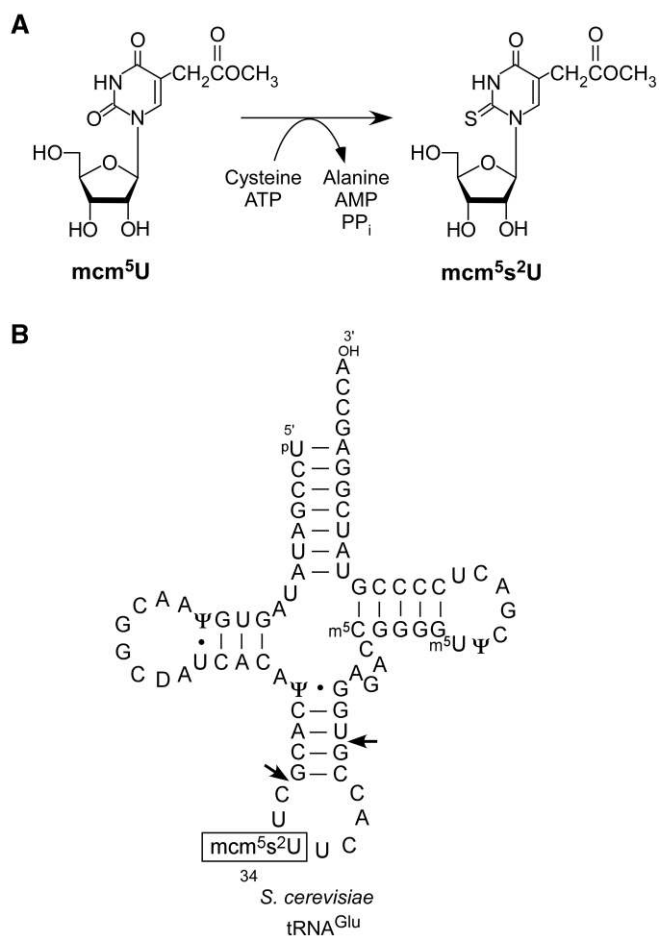
Figure 1. Chemical structure of 5-methoxycarbonylmethyl-2-thiouridene (mcm^{5}s^{2}U) and secondary structure of tRNA^{Glu}. (**A**) Chemical structures of mcm^{5}U and mcm^{5}s^{2}U. 2-thiolation of mcm^{5}s^{2}U utilizes cysteine and ATP as substrates. (**B**) Secondary structure of *S. cerevisiae* tRNA^{Glu} with modified nucleosides: 5-methoxycarbonylmethyl-2-thiouridine (mcm^{5}s^{2}U), pseudouridine (Ψ), dihydrouridine (D), 5-methylcytidine (m^{5}C) and 5-methyluridine (m^{5}U). Arrows indicate the sites for RNase T$_1$ cleavage needed to produce the anticodon-containing fragment.

thiamine, biotine and molybdopterin, and thionucleosides in tRNAs (11–13). The trafficking and delivery of sulfur to these molecules remains to be elucidated. Cysteine desulfurases have been shown to play a key role in the generation of sulfur-containing metabolites. In eubacteria, IscS is a widely distributed cysteine desulfurase that catalyzes the desulfuration of L-cysteine using pyridoxal-5′-phosphate (PLP) as a cofactor to generate cysteine persulfide (IscS-S-SH) by transferring the sulfur atom from the L-cysteine to the active-site cysteine residue of IscS. The resultant persulfide can be utilized by subsequent reactions in various sulfur pathways (14,15). In fact, it is known that IscS is required for the generation of several thionucleosides in bacterial tRNAs (16,17). The biogenesis of 2-thiouridine in tRNAs requires a complicated sulfur-transfer system that involves multiple sulfur mediators (18). We previously identified five sulfur mediators, named as TusA, B, C, D and E, which are involved in a bucket-brigade transfer of the persulfide responsible

for 2-thiolation of mnm^{5}s^{2}U in *Escherichia coli* (18). Efficient 2-thiouridine formation *in vitro* could be reconstituted with recombinant proteins of IscS, TusA, the TusBCD complex, TusE and MnmA. TusA directly interacts with IscS, and stimulates its desulfurase activity. TusA accepts the persulfide sulfur from IscS, and transfers the sulfur to TusD in the TusBCD complex. TusE can interact with TusBCD and accepts the sulfur from TusD. TusE also interacts with MnmA, a thiouridylase responsible for 2-thiouridine formation, and transfers the persulfide to it. MnmA activates the C2-position of the uracil base at the wobble position of the tRNA by forming an acyl-adenylated intermediate (19). Then, nucleophilic attack by the persulfide sulfur on the activated carbon generates a thiocarbonyl group by releasing the AMP. The crystal structure of the MnmA–tRNA complex clearly revealed snapshots of 2-thiouridine formation via the acyl-adenylated intermediate (19). These studies indicated that the biogenesis of 2-thiouridine in *E. coli* utilizes persulfide chemistry and proceeds through a complex sulfur-relay system by multiple sulfur mediators that select and facilitate specific sulfur flow to 2-thiouridine from various cellular sulfur pathways.

In the yeast *Saccharomyces cerevisiae*, the cysteine desulfurase *NFS1* is essential for the biogenesis of the 2-thiouridines in both mitochondrial and cytoplasmic tRNAs (20). Since *NFS1* not only functions as a direct supplier of persulfide to thionucleosides, but also provides a sulfur atom to the Fe/S cluster formation, it was uncertain whether biogenesis of the Fe/S cluster is required for 2-thiouridine formation. Since three components of the cytosolic Fe/S protein assembly (CIA) apparatus (Cfd1, Nbp35 and Cia1), and two scaffold proteins (Isu1 and Isu2), for the mitochondrial ISC machinery, were required for 2-thiolation of mcm^{5}s^{2}U in cytoplasmic tRNAs, but not for 2-thiolation of cmnm^{5}s^{2}U in mitochondrial tRNAs (21), cytoplasmic 2-thiouridine formation essentially requires a protein containing an Fe/S cluster, while mitochondrial 2-thiouridine formation is an independent reaction from the biogenesis of the Fe/S cluster. We previously showed that *MTU1*, which is the eukaryotic homolog of *mnmA*, is a mitochondria-specific 2-thiouridylase responsible for the generation of cmnm^{5}s^{2}U in yeast and τm^{5}s^{2}U in mammals (22). As no homologs of bacterial Tus-proteins have been identified in eukaryotes, unidentified sulfur mediators might be involved in connecting the specific sulfur-flow between *NFS1* and *MTU1* in mitochondria.

In the case of cytoplasmic 2-thiouridine formation, it was reported that deletion of *TUC1/NCS6/YGL211* resulted in the loss of the 2-thio group of mcm^{5}s^{2}U in *S. cerevisiae* tRNAs (23). Recently, in nematode and fission yeast, Ctu1, a homolog of TUC1, was shown to be involved in the 2-thiouridine formation of cytoplasmic tRNAs (24). Ctu1 forms a functional complex with Ctu2. These results indicate that the Ctu1-Ctu2 complex is a putative enzyme for the formation of 2-thiouridine. Although *in vitro* 2-thiouridine formation could be partially reconstituted using the immunoprecipitated Ctu1–Ctu2 complex and Nfs1p, the activity was quite low, indicating that there are still components missing

from the pathway. The proteins mediating the transfer of the activated sulfur from the cysteine residue to the 2-thio group of mcm^5s^2U in cytoplasmic tRNAs, and the mechanism by which this is achieved, remain unclear.

To elucidate a sulfur-flow system for cytoplasmic 2-thiouridine formation, we searched for the genes responsible for 2-thiolation of mcm^5s^2U in cytoplasmic tRNAs using a genome-wide screen of uncharacterized genes in *S. cerevisiae*. The screen employed a reverse genetic approach combined with mass spectrometry, which we called 'ribonucleome' analysis (4,18,25,26). This analysis utilizes a series of gene-deletion strains of *S. cerevisiae* (or *E. coli*). The total RNA extracted from each strain is analyzed by liquid chromatography/mass spectrometry (LC/MS) to determine whether a particular gene deletion leads to the absence of a specific modified nucleoside, thereby permitting us to identify the enzyme or protein responsible for this modification. The ribonucleome analysis enables us to identify not only the enzyme genes directly responsible for RNA modifications, but also genes that encode non-enzymatic proteins necessary for the biosynthesis of RNA modifications. These include carriers of the metabolic substrates used for RNA modifications and partner proteins needed for RNA recognition. In fact, using this approach, we previously identified *tilS* for lysidine formation (27), *tusA-E* for 2-thiouridine formation (18) and *tmcA* for N^4-acetyl-cytidine formation (28) in *E. coli*. In *S. cerevisiae*, *TYW1-4* for wybutosine synthesis were identified and characterized (25). In this study, we have used ribonucleome analysis to identify five genes that are required for 2-thiouridine formation of mcm^5s^2U. Biochemical characterization revealed that eukaryotic 2-thiouridine formation occurs through a chemical reaction related to ubiquitination, the sulfur-flow of which employs a different system from the bacterial sulfur-relay system which is based on the persulfide chemistry.

MATERIALS AND METHODS

Strains, media and plasmids

Saccharomyces cerevisiae wild-type strain and deletion strains were obtained from EUROSCARF: the BY4742 (*Mat α; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*) series of strains ΔURM1 (*YIL008w::kanMX4*), ΔUBA4 (*YHR111w::kanMX4*), ΔTUM1 (*YOR251c::kanMX4*), ΔNCS2 (*YNL119w::kanMX4*), ΔNCS6 (*YGL211w::kanMX4*), ΔELP4 (*YPL101w::kanMX4*), ΔTRM9 (*YML014w::kanMX4*) and ΔTHI4 (*YGR144w::kanMX4*). YSC3869-9518112 (Y258; *Mat a; pep4Δ3; his4Δ580; ura3Δ53; leu2Δ3;/pYOR251c*) for expression of recombinant Tum1p, and YSC1178-7500542 for TAP tagged Ncs6p were obtained from OpenBiosystems.

Strains were grown in rich medium YPD (2% peptone, 1% yeast extract and 2% glucose) or in synthetic complete medium SC [0.67% yeast nitrogen base (YNB) without amino acids, 0.5% casamino acid and 2% glucose] supplemented with auxotrophic nutrients as specified. Thiamine-deficient medium was prepared by mixing YNB without

amino acids, ammonium sulfate and thiamine, 0.5% ammonium sulfate, 2% glucose supplemented by auxotrophic nutrients as specified. For control experiments this medium was supplemented with 0.4 µg/ml thiamine hydrochloride. The plasmid pET15b-YCL017c (pNFS1) was kindly provided by Dr Roland Lill (University of Marburg) (29), and was transformed into Rosetta(DE3)(Invitrogen) for expression of Nfs1p. pURM1 was constructed by inserting His-tag at the N-terminus and a TAA stop codon into the C terminus of the *URM1* ORF of the BG1805-amp plasmid obtained from the yeast ORF collection of the *E. coli* strain YSC3867-9521287 (OpenBiosystems). pURM1 was introduced into the ΔPEP4 strain (BY4741; *Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; pep4::kanMX4*) for expression of Urm1p. The ORF of UBA4 (YHR111w) was PCR-amplified from *S. cerevisiae* genomic DNA with primers bearing BamHI and NotI sites, and was then integrated into the same sites of pETDuet-1 (Invitrogen) to construct pETDuet/UBA4. pETDuet/UBA4 was introduced into Rosetta(DE3) for expression of Uba4p. For site-directed mutagenesis and complementation test, pUBA4 was constructed by inserting a TAG stop codon at the C-terminus of the UBA4 ORF of the BG1805-amp plasmid obtained from the yeast ORF collection of the *E. coli* strain YSC3867-9523618 (OpenBiosystems). pTUM1 was a series of BG1805-amp plasmids obtained from the yeast ORF *E. coli* strain YSC3867-9523911 (OpenBiosystems). pNCS2 and pNCS6 were obtained from the yeast ORF *E. coli* strains YSC3867-9519368 and YSC3867-98812192, respectively (OpenBiosystems).

Ribonucleome analysis

The basic procedure was carried out as described previously (25,26). Yeast strains were grown in 5 ml of YPD in 24-well format deep-well plates at 30°C for 8–10 h and cells were harvested during log phase growth ($OD_{660} = 1.5$–2.0). Cell pellets were resuspended in 500 µl of lysis buffer [20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$]. Then it was added by neutralized phenol/chloroform (500 µl/100 µl), and shaken for 3 h at room temperature, to extract the RNA fraction. The extracted RNA was then precipitated twice with 2-propanol and ethanol. The total RNA samples were dissolved in 100 µl of ddH_2O and stored at –20°C until use. To analyze RNA nucleosides, total RNA (20 µg) obtained from each strain was digested to nucleosides with nuclease P1 (Yamasa) and bacterial alkaline phosphatase (BAP.C75, Takara) for 3 h at 37°C, and analyzed by LC/MS using ion trap mass spectrometry as described previously (30) with slight modifications. Nucleosides were separated by an ODS reverse-phase column (Intersil ODS3 5 µm, 2.1 × 250 mm, GL Science) using an HP1100 liquid chromatography system (Agilent). The solvent consisted of 0.1% acetonitrile in 5 mM NH_4OAc (pH 5.3) (Solvent A) and 60% acetonitrile in H_2O (Solvent B) in the following gradients: 1–35% B in 0–35 min, 35–99% B in 35–40 min, 99% B in 40–50 min, 0.99–1% B in 50–50.1 min and 1% B in 50.1–60 min. The chromatographic effluent was directly conducted to the electrospray ionization (ESI) source to ionize the

separated nucleosides, which were analyzed on a LCQ DUO ion trap mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was operated with a spray voltage of 5 kV and a capillary temperature of 245°C. The sheath gas flow rate was 95 arb, auxiliary gas flow rate was 5 arb. Positive ions were scanned over an m/z range of 103 to 900.

Isolation and purification of individual tRNAs from yeast

Yeast strains were grown in 2 L of YPD medium and harvested during late-log phase growth (OD_{660} = 1.5–2.0). Total RNA was extracted as described above, to obtain about 700 A_{260} units. To isolate individual tRNAs, we employed the chaplet column chlomatography (31) and the reciprocal circulating chromatography (32). The 3′-biotinylated DNA probes complementary to yeast cytoplasmic tRNAs used in this study are 5′-ctccgatacggggagtcgaaccccggtctc-3′ for tRNA^{Glu} and 5′-ctcctcataggggggctcgaacccctgacat-3′ for tRNA^{Lys2}. As appropriate, the isolated tRNAs were further purified by denaturing polyacrylamide gel electrophoresis.

Mass spectrometry of tRNA

A detailed description of tRNA analysis by mass spectrometry was described previously (26). The purified tRNA (24 ng each) was digested at 37°C for 1 h, then heated at 55°C for 10 min in 10 μl of a reaction mixture containing 10 mM ammonium acetate (pH 5.3) and 5 U/μl of RNase T_1 (Epicentre). After digestion, 10 μl of 0.1 M triethyl-amine-acetate (TEAA) (pH 7.0) was added to the reaction mixture. To analyze the limited quantity of RNA fragments, we employed a system of capillary LC nano ESI/ mass spectrometry consisting of a tandem quadrupole time-of-flight (QqTOF) mass spectrometer (QSTAR® XL, Applied Biosystems) equipped with nanoelectrospray ionization sources (NANOSPRAY II), and a splitless nanoflow HPLC system (DiNa, KYA Technologies) equipped with a nano injection valve (Nanovolume Valve,Valco Instruments). Twelve nanograms of the digested tRNA were loaded onto a nano-LC trap column (C18, $\Phi0.5 \times 0.1$ mm) and desalted, by 0.1 M TEAA (pH 7.0). The RNA fragments were eluted from the trap column, directly injected into a C18 capillary column (HiQ Sil; 3 um C18, 100A pore size; $\Phi0.15 \times 50$ mm, KYA Technologies) and chromato-graphed at a flow rate of 500 nl/min in a linear gradient of 0–80% B for 40 min. The solvent system consisted of 0.4 M 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (pH 7.0, adjusted with triethylamine) (Solvent A) and 0.4 M HFIP in 50% methanol (Solvent B). The chromatographic eluent was sprayed from an energized sprayer tip attached to the capillary column. Ions were scanned with a negative polarity mode over an m/z range of 600 to 2000 through-out the separation. The parameters for the QSTAR used in this analysis were: spray voltage, –2.0 kV; curtain gas, 15; accumulation time, 1 s.

APM–polyacrylamide gel electrophoresis/northern blotting

The presence of thiouridine in tRNAs was analyzed by the retardation of electrophoretic mobility on polyacrylamide gels containing 0.02 mg/ml (N-acryloylamino)phenyl]mer-curic chloride (APM) (kindly provided by Dr Naoki Shigi of AIST). The procedure was originally developed by Igloi (33). Total RNA (0.05 A_{260} units) was separated by PAGE containing APM and blotted onto Hybond N$^+$ mem-branes (GE Healthcare). Individual tRNAs were detected by northern blotting with 5′ ^{32}P-labeled oligonucleotide probes as follows: 5′-ctccgatacggggagtcgaacccccg-3′ for tRNA^{Glu}, 5′-ctcctcataggggggctcgaacccct-3′ for tRNA^{Lys2} and 5′-tgagaatagctggagttgaaccaag-3′ for mt tRNA^{Lys}. Following hybridization, membranes were washed, dried and then exposed to a phosphor-imaging plate. The radio-activity was visualized by an FLA-7000 imaging analyzer (Fuji Film).

Expression and purification of the recombinant proteins

The yeast strains expressing hexahistidine-tagged Urm1p and Tum1p were grown at 30°C in 300 ml of YPD. The expression of the recombinant proteins was induced by 2% galactose. After cultivation for 20 h, cells were har-vested and washed with ddH$_2$O, and then resuspended in the lysis buffer [20 mM HEPES-KOH (pH 7.6), 10 mM KOAc, 2 mM Mg(OAc)$_2$, 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail (Roche)] and dis-rupted by four passages through the French press (Thermo Fischer Scientific). The cell lysate was cleared by ultracentrifugation with 100 000 g for 1.5 h at 4°C. The supernatant of the lysate was passed through a His-Trap chelating HP column (GE Healthcare) to purify the His-tagged protein using an AKTA purifier chromatogra-phy system (GE Healthcare) according to the manufac-turer's instruction. Briefly, the column was equilibrated with wash buffer [50 mM HEPES-KOH (pH 7.6), 100 mM KCl, 10 mM MgCl$_2$, 7 mM β-mercaptoethanol and a protease inhibitor cocktail], and then the super-natant was loaded onto the column and washed with 25–50 ml of the wash buffer. The His-tagged proteins were eluted from the column with a linear-gradient of imidazole (50–450 mM) in the wash buffer. The eluted fractions were analyzed by SDS–PAGE. The fractions containing the recombinant proteins were pooled and dialyzed against a buffer containing 20 mM Tris–HCl (pH 7.6), 4 mM MgCl$_2$, 55 mM NaCl and 1 mM DTT. Urm1p was further purified by anion-exchange chroma-tography using a Mono Q (GE healthcare) column. His-tagged Nfs1p and Uba4p were recombinantly expressed in the *E. coli* strain Rosetta(DE3). The expres-sion of these recombinant proteins was induced by 2% lactose. The *E. coli* cells, grown for 20 h at 18°C for Nfs1p (or 25°C for Uba4p), were harvested and resus-pended in lysis buffer, and lyzed by sonication. The lysate was cleared by ultracentrifugation at 4°C and 100 000 g for 1.5 h. The His-tagged proteins were purified by the AKTA purifier chromatography system using a His-Trap chelating HP column (GE Healthcare) as described above.

Site-directed mutagenesis

Site-directed mutagenesis of *URM1*, *UBA4* and *TUM1* was carried out on the plasmids pURM1, pUBA4 and pTUM1 by QuikChange™ Site-Directed Mutagenesis (Stratagene) according to the manufacturer's instructions. Introduced mutations were confirmed by DNA sequencing. The following pairs of oligonucleotides were used to create each mutantion:

5′-ttacttcaacattacattaaaacccagctttctt-3′ and 5′-aagaaagctgggttttaatgtaatgttgaagtaa-3′ for pURM1 ΔGG, 5′-aaatgccgtgacctcttcccaagaaggcggtgtga-3′ and 5′-tcacaccgccttcttggaagaggtcacggcattt-3′ for pUBA4 C225S, 5′-aaatgccgtgacctctgcccaagaaggcggtgtga-3′ and 5′-tcacaccgccttcttgggcagaggtcacggcattt-3′ for pUBA4 C225A, 5′-taatatagtgattctttcccgctacggtaacgact-3′ and 5′-agtcgttaccgtagcgggaaagaatcactatatta-3′ for pUBA4 C397S, 5′-accaactatttgctcttctggaactggcgtttcag-3′ and 5′-ctgaaacgccagttccagaagagcaaatagttggt-3′ for pTUM1 C259S, 5′-accaactatttgctctgctggaactggcgtttcag-3′ and 5′-ctgaaacgccagttccagcagagcaaatagttggt-3′ for pTUM1 C259A.

In vitro sulfur transfer experiments

Sulfur transfer from Nfs1p to Tum1p was performed as follows: a 10 μl reaction mixture consisting of 50 mM Tris–HCl (pH 7.5), 12 mM Mg(OAc)$_2$, 20 μM pyridoxal 5′-phosphate (PLP), 0.4 mM DTT, 0.1 mM [^{35}S] cysteine (370 MBq/mmol), 25 pmol of Nfs1p and 2.5–60.2 pmol of Tum1p was incubated at 30°C for 30 min. In the case of sulfur transfer from Nfs1p to Urm1p, a 10 μl reaction mixture consisting of 50 mM Tris–HCl (pH 7.5), 12 mM Mg(OAc)$_2$, 20 μM PLP, 0.4 mM DTT, 0.1 mM [^{35}S]cysteine (370 MBq/mmol) with or without 2 mM ATP and recombinant proteins (50 pmol of Urm1p and 25 pmol each of Nfs1p, Tum1p and Uba4p) was incubated at 30°C for 30 min. Following the reaction, the reaction mixture was mixed with the sample loading solution for SDS–PAGE to give a final concentration of 4% glycerol, 25 mM Tris–HCl (pH 6.8), 1% SDS, and 0.0015% bromophenol blue. SDS–PAGE was performed on a 9% acrylamide gel. The gel was dried and exposed on a phosphor-imaging plate, and the radioactivity was visualized by an FLA-7000 imager (Fuji Film).

To characterize the thiocarboxylation of Urm1p by mass spectrometry, 0.1% trifluoroacetic acid (TFA) was added to the reaction mixture following sulfur transfer from Nfs1p to Urm1p to adjust the reaction mixture to pH 3. It was then subjected to capillary LC nano ESI/mass spectrometry as described above. The reaction mixture containing 10 pmol of Urm1p was loaded onto a trap-column packed with C4 reverse-phase resin to be desalted with 0.1% TFA, then directly injected into a C4 capillary column (HiQ Sil, 5um C4, 100 Å poresize; Φ0.15 × 50 mm, KYA Technologies) and chromatographed at a flow rate of 500 nl/min in a linear gradient of 0–80% Solvent B for 30 min. The solvent system consisted of 0.1% formic acid and 2% acetonitrile (Solvent A) and 0.1% formic acid and 70% acetonitrile (Solvent B). The applied voltage on the sprayer was set +2.2 kV to generate a nanoelectrospray. TOF MS and product ion spectra were acquired on

positive mode using the information-dependent data acquisition (IDA) of the Analyst QS software (Applied Biosystems). Mass ranges for TOF MS and MS/MS were set to 400–2000 and 200–2000, respectively. The collision energy was set at +40 to decompose the acyl adenylated Urm1p (-COAMP). To reconstruct the zero-charge deconvoluted spectra from the raw data, the Bayesian protein reconstruct tool in the Bioanalyst software (Applied Biosystems) was used.

Pulling down TAP-tagged Ncs6p by IgG beads

The yeast strain expressing TAP-tagged Ncs6p was cultivated in 1 L of YPD at 30°C (until OD$_{660}$ = 0.7) and washed with ddH$_2$O, and then resuspended in lysis buffer. The cells were disrupted by three passages through a homogenizer an EmulsiFlex C-3 (AVESTIN). The lysate was cleared by ultracentrifugation at 100 000 *g* and 4°C for 1.5 h. The lysate was mixed with IgG beads (Sigma), and incubated for 2 h at 4°C. The beads were washed twice with IPP300 buffer [25 mM Tris–HCl (pH 8.0), 300 mM NaCl, 0.1% NP-40, 1% Empigen BB(Fluka), 1 mM DTT and protease inhibitor cocktail (Roche)], washed twice with IPP150 buffer [25 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% NP-40, 1% Empigen BB(Fluka), 1 mM DTT and a protease inhibitor cocktail (Roche)], and washed once with a buffer consisting of 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 12 mM MgCl$_2$. Finally, the beads were washed once with T buffer [50 mM Tris–HCl (pH 7.5), 50 mM KCl, 12 mM MgCl$_2$, 20 μM PLP, 1 mM cysteine and 5 mM ATP] and then suspended in T buffer. As a negative control, we prepared IgG beads which were passed through the cell lysate of the wild-type strain, and treated with the same procedure as used in pulling down Ncs6p.

In vitro 2-thiouridine formation

The isolated tRNA^{Lys2} from the Δ*URM1* strain was dephosphorylated by bacterial alkaline phosphatase (BAP.A19, Takara), and labeled at its 5′ terminus with [γ-^{32}P]ATP (111 TBq/mmol) by T4 polynucleotide kinase (Toyobo). The end-labeled tRNA was then purified by 10% denaturing PAGE. Formation of 2-thiouridine formation was carried out as described (18) with following modifications: the reaction mixture (20 μl) consisting of 50 mM Tris–HCl (pH 7.5), 12 mM Mg(OAc)$_2$, 20 μM PLP, 1 mM cysteine, 2 mM ATP, 0.1 mM DTT and 50 pmol of Urm1p and 25 pmol each of Nfs1p, Tum1p and Uba4p and 5′-^{32}P-labeled tRNA^{Lys2} (50 000 cpm) was incubated at 30°C for 60 min in the presence or absence of the TAP-tagged Ncs6p trapped on the IgG beads. Reaction mixtures without ATP or with IgG beads which were passed through the wild-type cell lysate tRNA were employed as negative controls for 2-thiouridine formation. After the reaction, tRNAs were recovered by ISOGEN (Wako), ethanol-precipitated and dissolved by APM–PAGE as described above. The gels were exposed on a phosphor-imaging plate and analyzed by FLA-7000 imager (Fuji Film).

RESULTS

Genome-wide identification of genes responsible for 2-thiouridine formation of mcm⁵s²U by the ribonucleome analysis

To identify the genes responsible for 2-thiouridine formation of mcm^{5}s^{2}U, we performed ribonucleome analysis, a genome-wide reverse genetic approach combined with mass spectrometry (26). Since 2-thiouridine is a non-essential RNA modification, the complete set of *S. cerevisiae* deletion strains (4829) serves as a parent population for this analysis. To reduce the size of the starting population, we selected 3482 genes that have orthologs in *Schizosaccharomyces pombe*, because *S. pombe* tRNAs possess mcm^{5}s^{2}U. Next, we chose 767 genes which are described as proteins of unknown function, and proteins with weak similarity to known functions in *S. cerevisiae* (CYGD: http://mips.gsf.de/genre/proj/yeast) (34). We then started the ribonucleome analysis using this population. In the mass chromatogram (Figure 2A), mcm^{5}s^{2}U was detected as a proton adduct form (MH$^+$; m/z 333) with its base fragment (BH$_2^+$; m/z 201) in wild-type cells. mcm^{5}U (MH$^+$; m/z 317) with its base fragment (BH$_2^+$; m/z 185), which is a precursor form of mcm^{5}s^{2}U, could also be detected in wild-type cells (Figure 2A). Among the 767 selected deletion mutants, we identified four deletion strains, *YHR111w(UBA4), YNL119w(NCS2), YGL211w(NCS6)* and *YOR251c*, in which mcm^{5}s^{2}U was absent (Figure 2A, upper panels), while levels of its precursor mcm^{5}U were increased (Figure 2A, lower panels), demonstrating that these genes are specifically involved in 2-thiolation of mcm^{5}s^{2}U. We have renamed *YOR251c* as tRNA-thiouridine modification protein 1 (*TUM1*). *NCS2* is an orthologous protein of Ctu2 in *Caenorhabditis elegans* and fission yeast (24). Although it was reported that *NCS6/TUC1* is responsible for 2-thiouridine formation by comparative genomic approach in 2007 (23), we have independently identified the same gene in this analysis. It was reported that *UBA4, NCS2* and *NCS6* are essential in the *ΔCLA4* strain (35), and that their deletion affects the protein urmylation, which is a ubiquitin-like conjugation pathway (36,37). *UBA4* encodes a paralog of a ubiquitin-activating enzyme (E1) (38). In fact, *UBA4* was shown to function as an E1-like enzyme for *URM1*, which is an ubiquitin-related modifier involved in protein urmylation. We therefore analyzed the total nucleosides of *ΔYIL008w(URM1)* and found that *URM1* is also responsible for 2-thiolation of mcm^{5}s^{2}U (Figure 2A).

Analyses of individual tRNAs isolated from a series of the knockout strains

To confirm the absence of the 2-thio group of mcm^{5}s^{2}U at the wobble position of individual tRNAs, we isolated individual tRNA^{Glu} from each of *ΔURM1, ΔUBA4, ΔTUM1, ΔNCS2* and *ΔNCS6* strains. As shown in Figure 2B, the anticodon-containing fragments of the isolated tRNAs^{Glu} were analyzed by LC/MS (RNaseT$_1$ mapping). In the wild-type tRNA^{Glu}, the anticodon-containing RNA fragment with mcm^{5}s^{2}U modification (CUmcm^{5}s^{2}UUCACCGp,

MW 2918.344), produced by RNase T$_1$ digestion was clearly observed as doubly-charged (m/z 1458.277), triply-charged (m/z 971.856) and quadruply-charged (m/z 728.586) ions (Figure 2B, top left panel). The same RNA fragment in tRNA^{Glu} isolated from the *ΔURM1, ΔUBA4, ΔTUM1, ΔNCS2* and *ΔNCS6* strains weighed 2902.367 Da (detected as m/z 1450.183, 966.455 and 724.591 for doubly- to quadruply charged ions), which is the mass of a fragment composed of mcm^{5}U instead of mcm^{5}s^{2}U. Very recently, it has been reported that 5-carboxymethyluridine (cm^{5}U) was detected as a modification intermediate in *ΔNCS2* and *ΔNCS6* strains (39). However, no such intermediate could be observed in the isolated tRNAs in our analysis. In the case of the *ΔTUM1* strain, the mass of a fragment with a fully modified mcm^{5}s^{2}U was also detected as a minor product (Figure 2B, as shown in red).

Next, we also employed APM–PAGE/northern blotting to detect thiolated tRNA^{Glu} (Figure 4A and B) from each of the knockout strains. In the wild-type cells, a large fraction of the tRNA^{Glu} migrates slowly on the PAGE in the presence of APM due to a specific interaction between the thiocarbonyl group of mcm^{5}s^{2}U and the APM in the gel. No retarded band for tRNA^{Glu} from *ΔURM1, ΔUBA4* and *ΔNCS2* was observed, showing that no thiouridine formation occurred in these strains. The thiouridine in these deletion strains was partially or completely restored by the introduction of a plasmid encoding *URM1, UBA4* or *NCS2*. With respect to *NCS6*, we failed to restore 2-thiouridine formation in the *ΔNCS6* strain, by introduction of a plasmid encoding *NCS6*, for unknown reasons (data not shown). In the case of *ΔTUM1*, the large fraction of tRNA^{Glu} lacked 2-thiouridine, but the small fraction of tRNA^{Glu} remained 2-thiolated. This result is consistent with the LC/MS analysis (Figure 2B). Thus, although *TUM1* is not essential for thiolation, it is a major component involved in this pathway.

As it is known that *NFS1* is involved in the biogenesis of the 2-thiouridines in both mitochondrial and cytoplasmic tRNAs (20), we investigated whether these genes affect mitochondrial 2-thiouridine formation. We employed APM–PAGE/northern blotting to detect thiolated mitochondrial tRNA^{Lys} from each of the five knockout strains, and found that none of these genes affected the retarded bands of mitochondrial tRNA^{Lys} on the APM gel (Supplementary Figure 1). The data demonstrated that *URM1, UBA4, TUM1, NCS2* and *NCS6* are specific to cytoplasmic 2-thiouridine formation.

TUM1 is a sulfur mediator stimulating the desulfurase activity of *NFS1*

NFS1 is a cysteine desulfurase that accepts a sulfur atom from a cysteine to form a persulfide group using PLP as a cofactor. This activated sulfur is considered to be a direct substrate for 2-thiouridine formation (18,20). According to the sequence alignment of *TUM1* (Figure 3A), *TUM1* contains two rhodanese-like domains in the N-terminal and C-terminal regions, respectively. Rhodaneses are widespread sulfur carrier proteins that catalyze the transfer of sulfur atoms from thiosulfate to cyanide
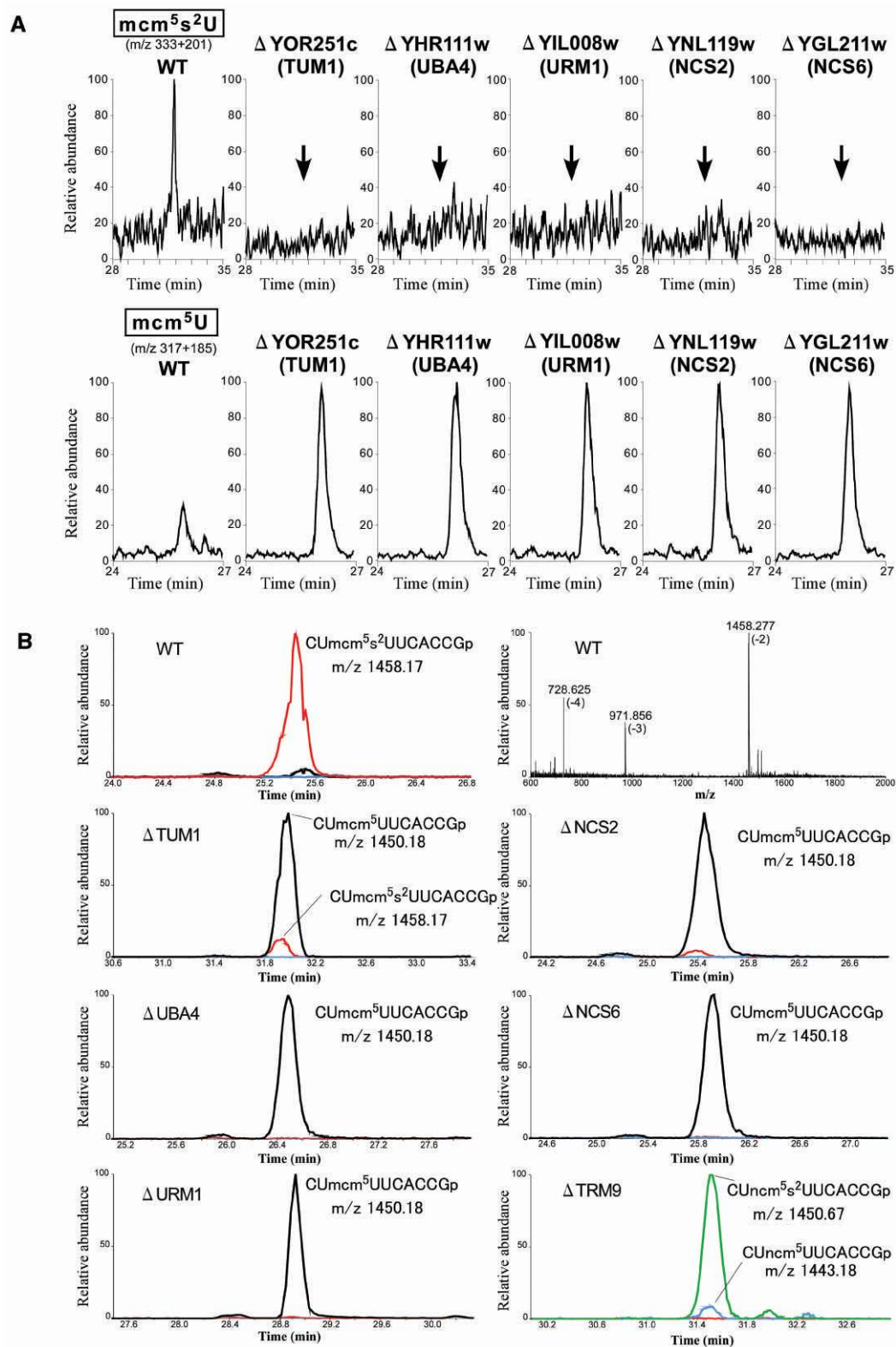
Figure 2. Mass spectrometric analyses of total nucleosides and purified tRNAs^{Glu} from *S. cerevisiae* wild-type and mutant cells. (**A**) LC/MS analyses of total nucleosides from strains of wild-type (WT), $\Delta YOR251c$ (*TUM1*), $\Delta YHR111w$ (*UBA4*), $\Delta YIL008w$ (*URM1*), $\Delta YNL119w$ (*NCS2*) and $\Delta YGL211w$ (*NCS6*). The upper panels show the merged mass chromatograms detecting MH$^+$ (m/z 333) and BH$_2^+$ (m/z 201) of mcm^{5}s^{2}U. The lower panels show the mass chromatograms detecting MH$^+$ (m/z 317) and BH$_2^+$ (m/z 185) of mcm^{5}U. Arrows in the upper panels indicate the retention time for mcm^{5}s^{2}U. (**B**) LC/MS fragment analyses of RNase T$_1$-digested tRNAs^{Glu} obtained from wild-type strains: $\Delta TUM1$, $\Delta UBA4$, $\Delta URM1$, $\Delta NCS2$, $\Delta NCS6$ and $\Delta TRM9$. A graph on the top-right represents the mass spectrum for the anticodon-containing fragment (CUmcm^{5}s^{2}UUCACCGp) (Figure 1B) from the wild-type strain. Charge states of multiply charged ions are indicated in parentheses. Other graphs describe the mass chromatograms shown by triply charged ions of anticodon-containing fragments bearing mcm^{5}s^{2}U (m/z 1458.17, red line), mcm^{5}U (m/z 1450.18, black line), ncm^{5}s^{2}U (m/z 1450.67, green line) and ncm^{5}U (m/z 1443.18, blue line). The RNA sequence including the wobble modification is indicated on each graph.
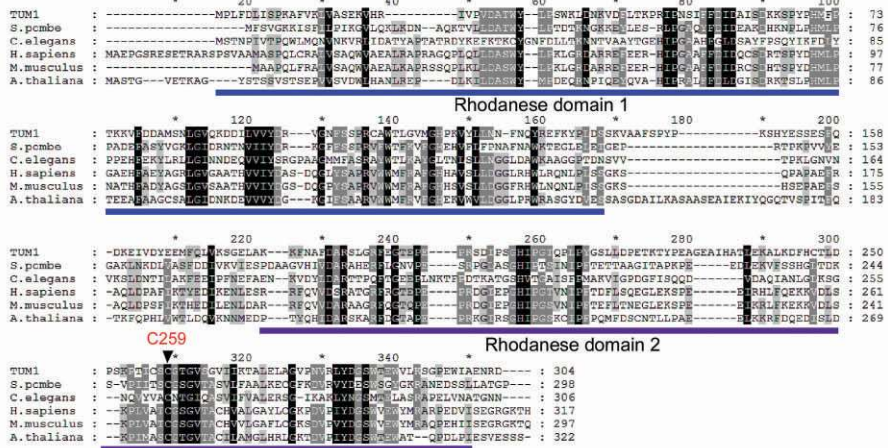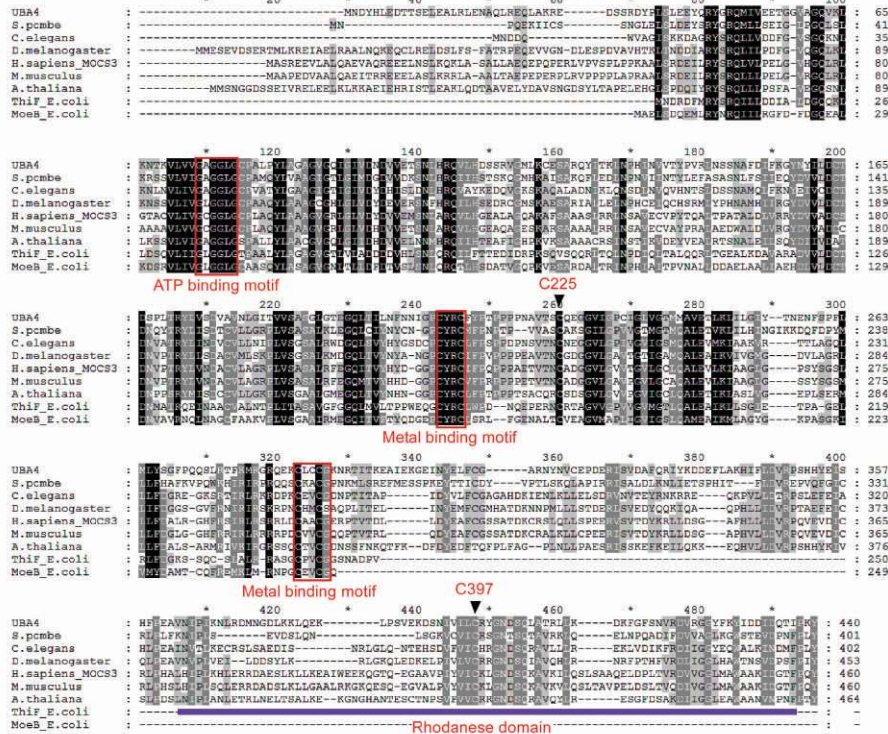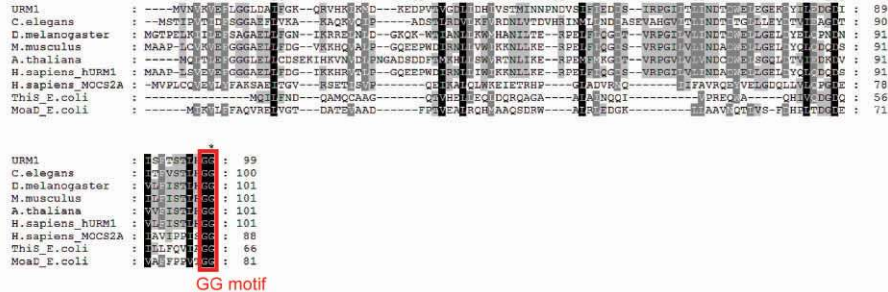
Figure 3. Sequence alignments of *TUM1*, *UBA4* and *URM1*. Each protein is aligned with its homologs. Multiple alignment of each sequence was carried out by Clustal X analysis (82) and displayed using the Genedoc multiple sequence alignment editor. White letters in black boxes represent amino-acid residues identical in all species, while white letters in gray boxes represent residues with ~80% homology. Black letters in gray boxes represent restudies with ~60% homology. (**A**) Sequence alignment of *TUM1* with its homologs. Two conserved rhodanese domains are underlined. The conserved C259 is indicated. (**B**) Sequence alignment of *UBA4* with its homologs and *E. coli ThiF* and *MoeB*. The boxed regions are the ATP-binding motif (GxGxxG) and the metal-binding motif (CxxC, CxxCG). The conserved C225 and C397 are indicated. The rhodanese-like domain in the C-terminal region is underlined. (**C**) Sequence alignment of *URM1* with its homologs and *E. coli ThiS* and *MoaD*. The conserved C-terminal GG motif is boxed.

in vitro (40). It is known that a conserved cysteine residue in the active-site loop of rhodanese plays a key role in substrate recognition and catalytic activity. *TUM1* has one conserved cysteine residue (Cys259) in the C-terminal rhodanese-like domain (RLD) (Figure 3A). To clarify the functional importance of Cys259 in thiouridine formation, plasmids expressing wild type *TUM1* and its mutants, in which Cys259 was replaced with Ser (pTUM1 C259S) or Ala (pTUM1 C259A), were introduced in a Δ*TUM1* strain to test for complementation activity of these mutations. We performed APM–PAGE/northern blotting to estimate 2-thiouridine formation. As shown in Figure 4, the reduced level of 2-thiolated tRNA^{Glu} in the Δ*TUM1* strain was restored by the introduction of wild-type *TUM1*. Compared with the Δ*TUM1* strain, no enhancement for 2-thiouridine formation could be observed when *TUM1* with a C259S or C259A mutation was introduced. This result revealed that Cys259 in the C-terminal RLD of *TUM1* is critical for enhancing 2-thiouridine formation.

Next, we tested whether Tum1p accepts a sulfur atom from Nfs1p using an *in vitro* sulfur transfer experiment. Recombinant Nfs1p and Tum1p were mixed and incubated with [^{35}S] cysteine and immediately electrophoresed without a reducing agent and boiling. As shown in Figure 5A, Nfs1p alone was slightly labeled by [^{35}S] sulfur due to its intrinsic desulfurase activity (lane 1), while Tum1p alone was also labeled by [^{35}S] sulfur due to its rhodanese activity (lane 2). When they were mixed, both Nfs1p and Tum1p were significantly labeled by [^{35}S] sulfur. As the [^{35}S] labeled sulfur was readily dissociated from Nfs1p and Tum1p by DTT treatment (data not shown), Nfs1p and Tum1p carried the sulfur atoms as a persulfide form. The labeling efficiency of Nfs1p was enhanced significantly in the presence of increasing amounts of Tum1p. Concomitantly, the activated sulfur of Nfs1p was efficiently transferred to Tum1p (Figure 5A). This result suggested that the desulfurase activity of Nfs1p was enhanced in the presence of Tum1p, then the activated sulfur of Nfs1p was specifically transferred to Tum1p. Thus, Tum1p is a persulfide mediator as well as an activator for Nfs1p. The catalytic Cys259 in the RLD of Tum1p is likely to be the site for the persulfide formation.

Rhodanese domain of *UBA4* acts as a persulfide carrier

It is known that *UBA4* also contains an RLD in the C-terminal region (41). According to the sequence alignment (Figure 3B), *UBA4* has a highly conserved cysteine residue (Cys397) in its RLD. We constructed a plasmid harboring a *UBA4* mutant in which Cys397 was replaced with Ser (pUBA4 C397S). Approximately half the fraction of tRNA^{Glu} in the Δ*UBA4* strain could be 2-thiolated by the introduction of a plasmid encoding the wild-type *UBA4* (Figure 4A and B). When pUBA4 C397S was introduced, no 2-thiouridine formation of tRNA^{Glu} occurred. This result demonstrated that Cys397 in the RLD of *UBA4* is critical for 2-thiouridine formation.

We then examined whether Uba4p directly accepts a sulfur atom from Nfs1p by means of an *in vitro* sulfur transfer experiment. As shown in Figure 5B, both

recombinant Nfs1p and Uba4p were slightly labeled by [^{35}S] sulfur due to their intrinsic sulfur transfer activities (lanes 1 and 2). When they were mixed together, Uba4p was apparently, but inefficiently, labeled by [^{35}S] sulfur. The labeling efficiency of Uba4p was enhanced in proportion to increasing amounts of Uba4p, indicating that the activated sulfur of Nfs1p was transferred to Uba4p. As the [^{35}S] labeled sulfur was removed from Uba4p by DTT treatment (data not shown), Uba4p accepted the sulfur atom as a persulfide form. However, we did not observe any apparent activation of Nfs1p with the addition of increasing amounts of Uba4p. These results suggested that Uba4p has the potential to act as an acceptor of the persulfide sulfur from Nfs1p, but does not function as an activator for Nfs1p. It is likely that the catalytic Cys397 in the RLD of Uba4p is involved in the sulfur transfer reaction.

In addition to *TUM1*, we also found that the RLD of *UBA4* acts as a sulfur carrier. In the Δ*TUM1* strain, we found that the small fraction of tRNA^{Glu} still remained to be 2-thiolated, indicating a minor pathway for sulfur flow. On the other hand, no thiolation of tRNA^{Glu} could be observed in the Δ*UBA4* strain introduced with pUBA4 C397S. These observations prompted us to speculate that Tum1p first activates Nfs1p and accepts the sulfur, then probably transfers the sulfur to the RLD of Uba4p. Meanwhile, Uba4p also acts as a minor sulfur mediator to accept the sulfur atom directly from Nfs1p, by bypassing Tum1p-mediated sulfur transfer.

Urm1p is thiocarboxylated by Uba4p through an acyl-adenylated intermediate

URM1 has been known as an ubiquitin-related modifier (38). Conjugation of Urm1p (urmylation) to its target proteins depends on the E1-like activating enzyme Uba4p. Similar to ubiquitination, at the first step of urmylation, Urm1p is known to form a thioester link with Uba4p (38). *URM1* has conserved Gly-Gly residues at its C-terminus (Figure 3C) and it was shown that the conjugation reaction requires the C-terminal glycine of Urm1p (38). The conserved C-terminal Gly–Gly sequence of ubiquitin, or ubiquitin-like proteins, is also generally required for protein conjugation (42–44). It is thought that Urm1p attaches to a target protein through an isopeptide bond between the C-terminal Gly of *URM1* and a lysine residue of the target protein (38). To test the functional importance of the C-terminal Gly-Gly of *URM1*, we constructed a plasmid harboring a *URM1* mutant (ΔGG) in which the C-terminal Gly–Gly was removed. As shown in Figure 4, 2-thiouridine formation of tRNA^{Glu} in the Δ*URM1* strain could be restored by the introduction of a plasmid harboring wild-type *URM1* (Figure 4A and B). When the *URM1* with the ΔGG mutation was introduced, no thiouridine formation of tRNA^{Glu} took place, showing that the C-terminal Gly–Gly of *URM1* is a critical motif not only for protein conjugation, but also for 2-thiouridine formation.

UBA4 is an E1-like enzyme which was assumed to activate *URM1* (38). In this study, we found that *UBA4* has an RLD in the C-terminal region, which acts as a carrier
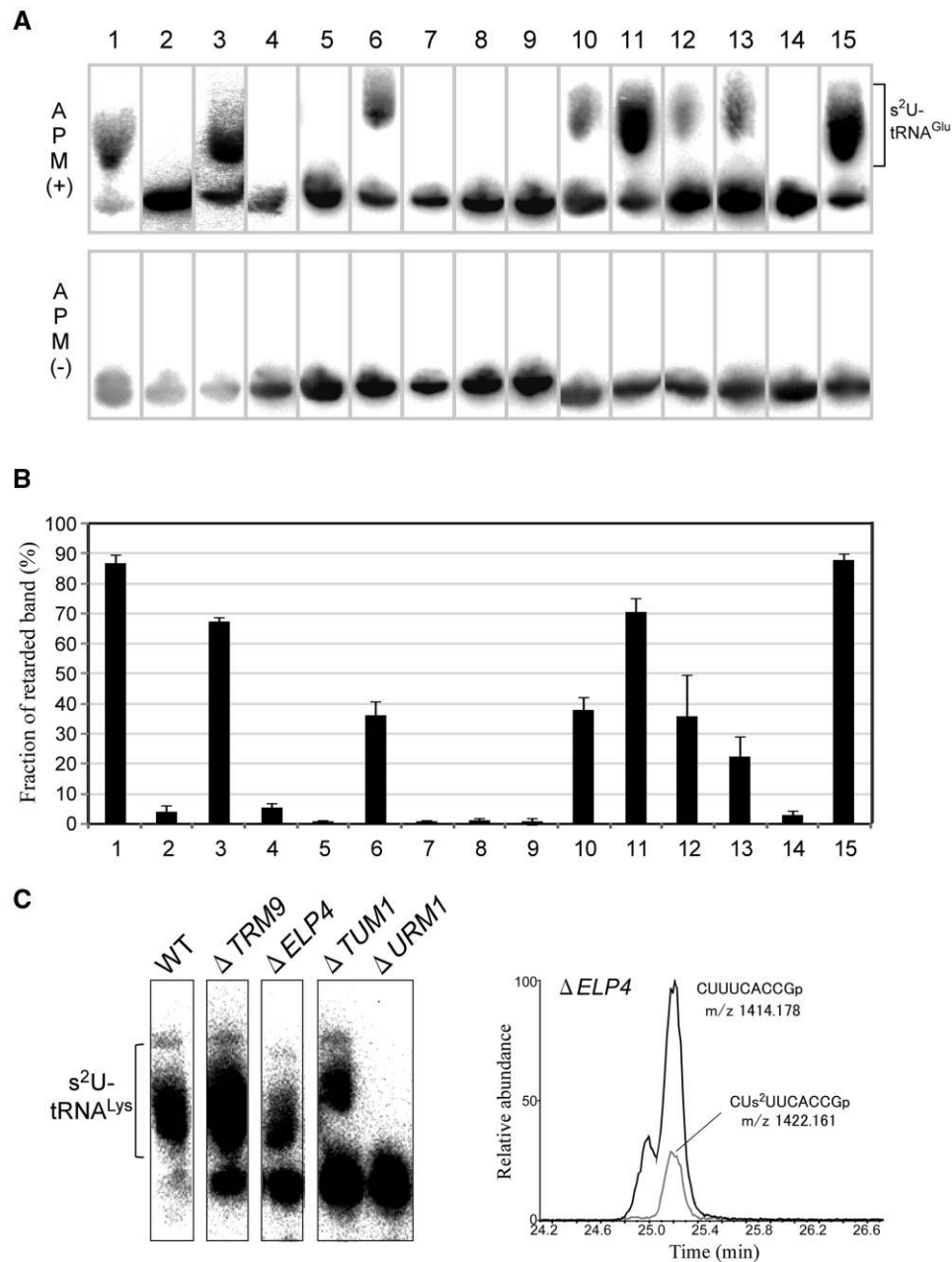
Figure 4. APM Northern analyses of thiolation-status of tRNAs in mutant strains and transformants. (**A**) APM northern analyses of tRNA^{Glu} in the s^{2}U-deficient strains by introducing a series of mutant plasmids. Total RNA from each strain was resolved by denaturing polyacrylamide-gel electrophoresis in the presence (upper panels) or absence (lower panels) of APM, by northern blotting. 2-thiolated tRNA^{Glu} in each strain was detected as a shifted band only in the presence of APM, by northern blotting. Lanes 1 to 12 correspond to wild-tye (1), $\Delta URM1$ (2), $\Delta URM1$ harboring pURM1 (3), $\Delta URM1$ harboring pURM1ΔGG (4), $\Delta UBA4$ (5), $\Delta UBA4$ harboring pUBA4 (6), $\Delta UBA4$ harboring pUBA4 C225S (7), $\Delta UBA4$ harboring pUBA4 C225A (8), $\Delta UBA4$ harboring pUBA4 C397S (9),$\Delta TUM1$ (10), $\Delta TUM1$ harboring pTUM1 (11), $\Delta TUM1$ harboring pTUM1 C259S (12), $\Delta TUM1$ harboring pTUM1 C259A (13), $\Delta NCS2$ (14) and $\Delta NCS2$ harboring pNCS2 (15), respectively. (**B**) Quantification of the thiolation-status of tRNA^{Glu} by APM/northern analyses. The fraction of the retarded band in the total intensity of the bands was calculated. Data are shown as values $\pm$ SD and reflect the average of four independent experiments. (**C**) APM/northern analyses of tRNAs^{Lys} from wild-type, $\Delta TRM9$, $\Delta ELP4$, $\Delta TUM1$ and $\Delta URM1$. The right panel represents a mass chromatogram shown by triply charged ions of the anticodon-containing fragments of tRNA^{Glu} isolated from $\Delta ELP4$. The unmodified fragment (m/z 1414.18, black line) and the s^{2}U-containing fragment (m/z 1422.16, gray line) are specifically detected.

of persulfide sulfur. In the N-terminal region, *UBA4* contains some conserved motifs shared with E1-like enzymes, such as an ATP-binding motif and a metal-binding motif (Figure 3B). Considering the analogy to ubiquitination, the active-site cysteine residue of the E1 enzyme resides

about 10-to-20-amino-acid residues downstream of the metal-binding motif (45–47). Therefore, the conserved Cys225 was predicted as the active-site cysteine residue for the E1-like function. When a plasmid harboring *UBA4* with a C225S mutation was introduced into the
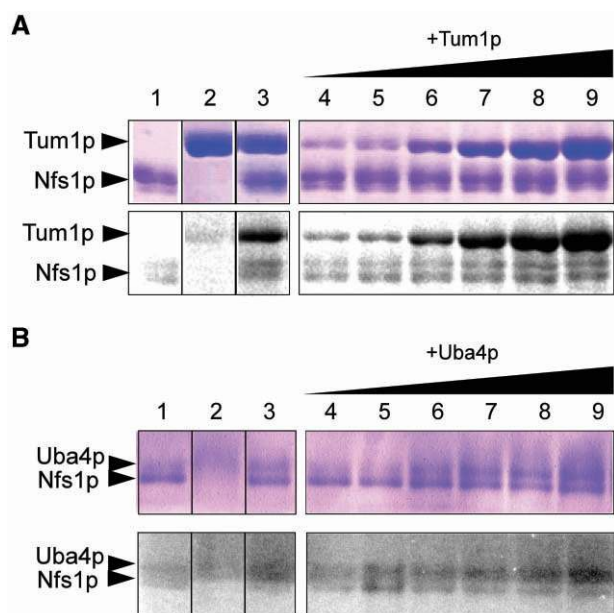
Figure 5. *In vitro* sulfur transfer from Nfs1p to Tum1p and Uba4p. (**A**) Sulfur transfer from Nfs1p to Tum1p. The gel was stained with Coomassie brilliant blue (CBB) (upper panel) and [^{35}S] radioactivity was visualized on a phosphor-imaging plate (lower panel). Lane 1, Nfs1p (25 pmol); lane 2, Tum1p (25 pmol); lane 3, Nfs1p (25 pmol) and Tum1p (25 pmol), lanes 4–9, Nfs1p (25 pmol) and Tum1p (2.5, 5, 12.5, 25, 37.5, 50 pmol). (**B**) Sulfur transfer from Nfs1p to Uba4p. The gel was stained with Coomassie brilliant blue (CBB) (upper panel) and [^{35}S] radioactivity was visualized on a phosphor-imaging plate (lower panel). Lane 1, Nfs1p (25 pmol); lane 2, Uba4p (25 pmol); lane 3, Nfs1p (25 pmol) and Uba4p (25 pmol), lanes 4–9, Nfs1p (25 pmol) and Uba4p (2.5, 5, 12.5, 25, 37.5, 50 pmol).

$\Delta UBA4$ strain, no thiouridine formation of tRNA^{Glu} could be observed (Figure 4A and B), demonstrating that Cys225 is the active-site cysteine residue of *UBA4* required for 2-thiouridine formation.

To test whether Urm1p receives a sulfur atom from Nfs1p as a thiocarboxylated form, we carried out an *in vitro* sulfur transfer assay with recombinant Nfs1p, Tum1p, Urm1p and Uba4p. Recombinant proteins were incubated with [^{35}S] cysteine in the presence, or absence, of ATP. In the presence of ATP, Urm1p was specifically labeled with [^{35}S] sulfur (Figure 6A). In the absence of ATP, no incorporation of [^{35}S] sulfur into Urm1p was observed. Thus, Urm1p accepts a sulfur atom from cysteine in an ATP-dependent manner. Nfs1p receives a sulfur atom from cysteine as a persulfide form by employing PLP as a cofactor. This reaction is stimulated by the RLDs of Tum1p and Uba4p as observed in Figure 5. Both proteins accept the persulfide sulfur from Nfs1p. In fact, recombinant Nfs1p, Tum1p and Uba4p were labeled with [^{35}S] sulfur during the reaction. The labeling efficiency of Urm1p was not altered when the reaction mixture was treated with DTT, whereas [^{35}S]-labeled persulfide sulfurs were readily dissociated from Nfs1p, Tum1p and Uba4p by this treatment (Figure 6A). The data suggested that Urm1p was not labeled by nonspecific sulfur transfer as a persulfide, but that the C-terminal Gly of Urm1p was thiocarboxylated by the E1-like activity of Uba4p.

We further characterized the thiocarboxylation of Urm1p by mass spectrometry. The recombinant Urm1p was directly analyzed by LC/MS using tandem quadrupole mass spectrometry. Proton adducts of Urm1p were detected as a series of multiply charged ions (i.e. +7 to +15). The exact molecular mass of Urm1p was calculated as $12\,083 \pm 1$ Da by deconvoluting these ions (Figure 6B), which was consistent with the theoretical mass of 12 082.37 Da. We also found a small fraction of the Urm1p derivative with a molecular mass of $12\,099 \pm 1$ Da (Figure 6B), which was assumed to be the thiocarboxylated form of Urm1p (Urm1p-COSH) whose theoretical value is 12 098.37 Da. As Urm1p was recombinantly expressed in yeast cells, it is likely that the C-terminal Gly of Urm1p was partially thiocarboxylated in the cell. Urm1p was then incubated with Nfs1p, Tum1p and Uba4p in the presence or absence of ATP (Figure 6B). In the absence of ATP, no change in the molecular mass of Urm1p was observed, while in the presence of ATP, we could clearly observe the acyl-adenylated form of Urm1p (Urm1p-COAMP; 12 413 Da) which is consistent with its theoretical value (12 411.59 Da). To confirm the acyl-adenylated intermediate, the proton adduct ion with +9 charge state of Urm1p-COAMP (Figure 6C) was subjected to collision-induced dissociation to be decomposed in the instrument using an MS/MS experiment. As shown in Figure 6D, AMP from the acyl-adenylate intermediate of Urm1p was clearly observed as a product ion. These results revealed that the C-terminal Gly of Urm1p was first activated by Uba4p to synthesize the acyl-adenylated intermediate, then thiocarboxylated by releasing AMP.

In vitro reconstitution of 2-thiouridine formation

To investigate whether 2-thiouridine formation occurs through a ubiquitin-like pathway, we attempted an *in vitro* reconstitution of 2-thiouridine of tRNAs with recombinant proteins. With respect to Ncs2p and Ncs6p, we failed to obtain recombinant proteins in soluble form (data not shown). The TAP-tagged Ncs6p was pulled down by IgG beads. Since it was reported that Ctu1/Ncs6p in fission yeast is physically associated with Ctu2/Ncs2p (24), it should be possible to isolate a small fraction of endogenous Ncs2p as an Ncs6p/Ncs2p hetero-complex in this preparation. We employed nonthiolated tRNA^{Lys2} which was obtained from a $\Delta URM1$ strain as a substrate for the *in vitro* 2-thiouridine formation. Recombinant Nfs1p, Tum1p, Urm1p and Uba4p were mixed with the IgG beads retaining the Ncs6p and 5′-^{32}P-labeled tRNA^{Lys2} in a reaction mixture containing ATP, PLP and cysteine. After the reaction, the substrate tRNA^{Lys2} was subjected to APM-containing PAGE to detect 2-thiouridine formation. As shown in Figure 7, we partially succeeded in reconstituting the ATP-dependent 2-thiouridine formation of tRNA^{Lys2}, but only in the presence of the recombinant proteins (Nfs1p, Tum1p, Urm1p and Uba4p), and with Ncs6p trapped on the IgG beads. As a negative control for TAP-tagged Ncs6p, we employed the IgG beads treated with wild-type cell lysate for the *in vitro* 2-thiouridine formation, and observed no 2-thiouridine formation (Figure 7). It is
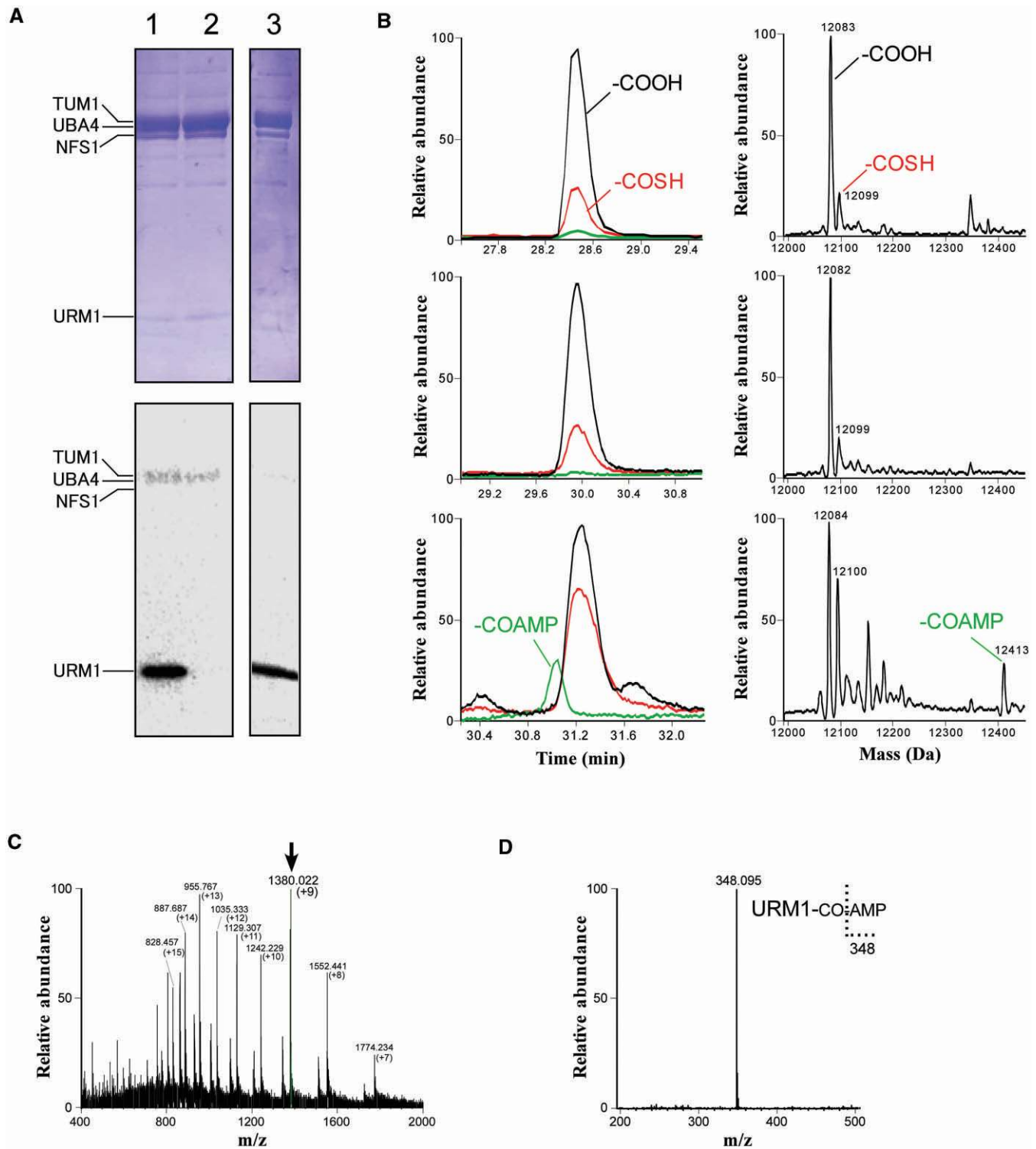
Figure 6. Thiocarboxylation of Urm1p through an acyl-adenylated intermediate. (**A**) *In vitro* sulfur transfer from Nfs1p to Urm1p. Recombinant Nfs1p, Tum1p, Urm1p and Uba4p were mixed and incubated with [^{35}S] cysteine in the presence (lane 1) or absence (lane 2) of ATP. The reaction mixture was further incubated with 50 mM DTT (lane 3). The gel was stained with CBB (upper panels) and [^{35}S] radioactivity was visualized on a phosphor-imaging plate (lower panels). The band for each protein is indicated. (**B**) Whole-mass analysis of the thiocarboxylation of Urm1p. Left panels are mass chromatograms for detecting +9-charged ions of Urm1p with unmodified C-terminus (-COOH, black line), Urm1- with thiocarboxylated C-terminus (-COSH, red line) and Urm1p with acyl-adenylated C-terminus (-COAMP, green line). Right panels are deconvoluted mass spectra for Urm1p detecting unmodified Urm1p (-COOH, 12 083 Da), thiocarboxylated Urm1p (-COSH, 12 099 Da) and acyl-adenylated Urm1p (-COAMP, 12 413 Da). Top panels show whole-mass analysis of purified Urm1p used in this study. *In vitro* thiocarboxylation of Urm1p by Uba4p was carried out in the absence (middle panels) or presence (bottom panels) of ATP. *In vitro* thiocarboxylation of Urm1p by Uba4p was carried out in the absence (middle panels) or presence (bottom panels) of ATP. A series of multiply charged ions are detected. The parent ion (m/z 1380.022, +9) for collision-induced dissociation (CID) analysis is indicated. (**D**) CID spectrum of Urm1p-COAMP. The product ion (m/z 348.095) for AMP originated from Urm1p-COAMP is shown.
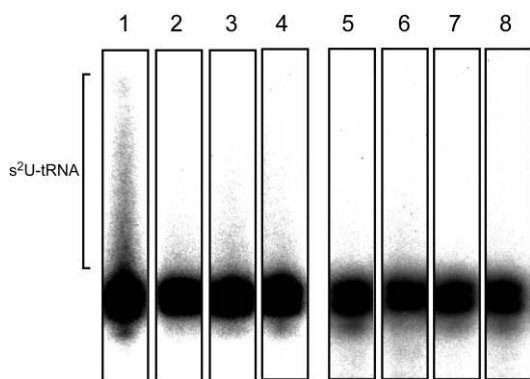
Figure 7. Reconstitution of 2-thiouridine formation *in vitro* using recombinant proteins. Thiouridine formation was detected by retardation of the tRNA band on the gel in the presence (lanes 1–4) or absence (lanes 5–8) of APM. 2-thiouridine formation of [^{32}P]-labeled tRNA^{Lys2} was carried out with recombinant proteins (Nfs1p, Tum1p, Uba4p and Urm1p) in the presence (lanes 1and 5) or absence (lanes 2 and 6) of IgG beads retaining the Ncs6p, or in the presence of IgG beads treated with wild-type cell lysate (lanes 4 and 8) as a negative control. The reaction was also performed only in the presence of IgG beads retaining the Ncs6p without recombinant proteins (lanes 3 and 7).

possible that there are still unidentified proteins, and as the exact conditions and requirements for this reaction, further studies will be necessary for the complete reconstitution of 2-thiouridine in eukaryote.

Biogenesis of 5-methoxycarbonylmethyl-group is required for efficient 2-thiouridine formation

Escherichia coli MnmA and MnmE/GidA are RNA-modifying enzymes responsible for synthesizing 2-thio and 5-methylaminomethyl groups of mnm^{5}s^{2}U, respectively (48,49). It is known that mnm^{5}U and s^{2}U are observed respectively observed in tRNAs from $\Delta mnmA$ and $\Delta mnmE$ (or $\Delta gidA$) strains (50), indicating that 2-thiolation and C5-modification occurred independently. In the case of mitochondrial cmnm^{5}s^{2}U, we previously showed that 2-thio and cmnm^5 groups are modified independently (22). In this study, we have investigated whether, or not, 2-thio and 5-methoxycarbonylmethyl groups of cytoplasmic mcm^{5}s^{2}U are synthesized independently. As shown in Figure 2A and B, mass spectrometry analyses revealed that mcm^{5}U was clearly detected instead of mcm^{5}s^{2}U in a series of knockout strains, suggesting that lack of 2-thiouridine formation does never inhibit mcm^5-modification. We employed APM/northern blotting to analyze the thiolation status of tRNA^{Lys2} and tRNA^{Glu} from a series of strains in which genes (*ELP4* and *TRM9*) responsible for mcm^5-formation (51,52) were knocked out. In tRNA^{Glu} from $\Delta ELP4$, we observed a complete loss of mcm^5-modification by LC/MS analysis (Figure 4C). Thus, *ELP4* is one of the genes responsible for the initial step of mcm^5-formation (51). In the APM/northern analysis, we could detect the small fraction of thiolated tRNA^{Lys2} in the $\Delta ELP4$ strain, but the large fraction remained nonthiolated (Figure 4C). In tRNA^{Glu} from $\Delta TRM9$, we have identified 5-aminocarbonylmethyluridine (ncm^{5}U) as a

modification intermediate of mcm^{5}U by mass spectrometry (Figure 2B). The thiolation status of tRNA^{Glu} was clearly increased as compared to that observed in $\Delta ELP4$, indicating that biosynthesis of the C5-substituent of the uracil ring promotes 2-thiouridine formation. However, the small fraction of non-thiolated tRNA^{Glu} still existed in $\Delta TRM9$ as compared to the wild-type strain (Figure 4C). These results demonstrated that mcm^5-formation is required for efficient 2-thiouridine formation.

DISCUSSION

We here described identification of five genes responsible for 2-thiouridine formation of mcm^{5}s^{2}U, through exploratory search of uncharacterized genes in *S. cerevisiae* by using 'ribonucleome analysis'. Although there may still be, as yet uncharacterized, enzymes or proteins responsible for this modification, we have revealed a large portion of the mechanism involved in cytoplasmic 2-thiouridine formation in *S. cerevisiae*. During the time we were revising this paper, Bystrom and co-workers (53) published identification of the same subset of genes responsible for 2-thio modification by the genetic screen. Ploegh and co-workers (54) published human Urm1 to be required for 2-thio modification. Our present study is completely independent from their studies.

Similar to bacterial 2-thiouridine formation, it was known that the cysteine desulfurase *NFS1* is involved in 2-thiolation of mcm^{5}s^{2}U (20). However, since 2-thiolation in cytoplasmic tRNAs essentially requires a protein component with an Fe/S cluster, it was uncertain whether *NFS1* functions as a direct supplier of active sulfur to 2-thiouridine. We identified *TUM1* which contains tandem rhodanese-like domains (RLDs). Rhodanese is a widespread and versatile sulfur-carrier enzyme catalyzing the sulfur-transfer reaction in distinct metabolic and regulatory pathways (40). An *in vitro* sulfur transfer reaction revealed that Tum1p stimulated Nfs1p and accepted a persulfide sulfur atom from Nfs1p (Figure 5A). In addition, we have shown that Cys259 in RLD2 is responsible for efficient 2-thiouridine formation. RLD1 of Tum1p seems to be a catalytically inactive RLD, often found in various rhodanese-containing proteins, because it has no conserved cysteine residue. The result demonstrated that *NFS1* not only provides a sulfur atom to Fe/S cluster formation, but also directly supplies a sulfur atom to the formation of 2-thiouridine. Tum1p acts as an activator for the desulfurase of Nfs1p as well as a mediator of the persulfide from Nfs1p.

Although the large fraction of tRNA^{Glu} lacked 2-thio modification in $\Delta TUM1$ strain, we found that the small fraction of tRNA^{Glu} still contained mcm^{5}s^{2}U (Figures 2B, and 4A and B). *TUM1* is not an essential protein for the biogenesis of 2-thiouridine, but it is required for efficient 2-thiouridine formation in the cell. This finding implies the presence of a minor sulfur flow pathway, capable of bypassing the Tum1p-mediated major pathway to 2-thiouridine formation. Since *UBA4* also has an RLD in the C-terminal region, we speculated that Uba4p
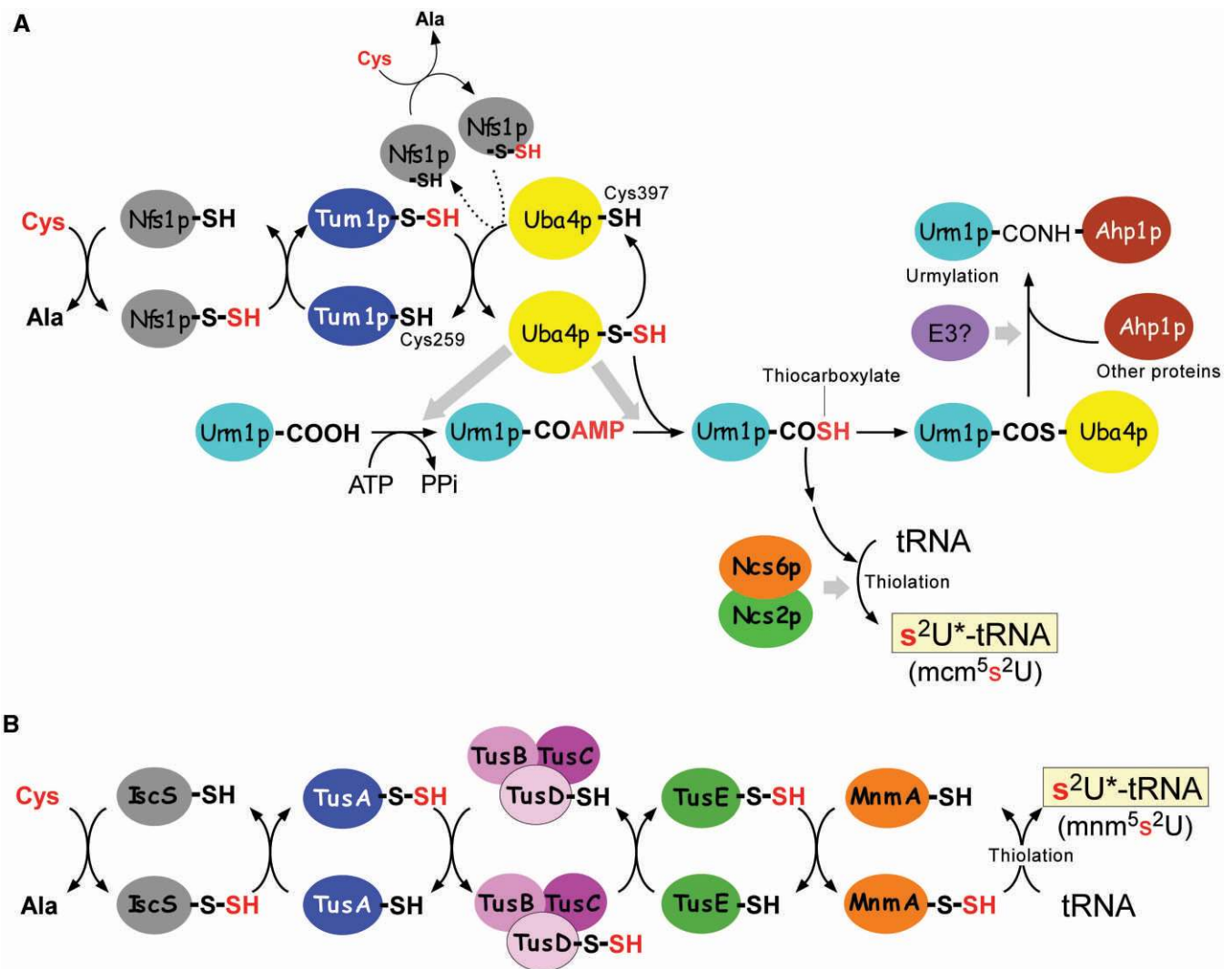
Figure 8. Comparison of cellular sulfur trafficking related to thiouridine formation of tRNA anticodon in *S. cerevisiae* and *E. coli*. (A) Proposed sulfur flow system for 2-thiouridine formation of mcm^{5}s^{2}U. Nfs1p accepts sulfur from Cys to form a persulfide using PLP as a cofactor. The persulfide sulfur is mainly transferred to the RLD2 (Cys259) of Tum1p, and partially to the RLD (Cys397) of Uba4p. Uba4p, as an E1-like enzyme in the ubiquitin-related pathway, activates the C-terminus of Urm1p to form the acyl-adenylated intermediate, then transfers the persulfide sulfur from the RLD to form thiocarboxylated Urm1p (Urm1p-COSH) by releasing AMP. Urm1p-COSH is a substrate of 2-thiouridine formation catalyzed by Ncs2p and Ncs6p. For protein urmylation, Urm1p-COSH is conjugated with Uba4p, then the putative E3 enzyme transfers Urm1p to target proteins such as Ahp1p. (B) Sulfur-relay system by Tus-proteins in *E. coli*. IscS accepts sulfur from Cys to form a persulfide using PLP as a cofactor. TusA interacts with IscS to stimulate its desulfurase activity, and accepts the persulfide sulfur. The sulfur is transferred to TusD in the TusBCD complex. It is then transferred to TusE. TusE interacts with MnmA to transfer the sulfur. MnmA recognizes the tRNA and activates the wobble uridine by forming an adenylated intermediate, then the persulfide sulfur attacks this position to release the AMP resulting in the synthesis of the 2-thiouridine of mnm^{5}s^{2}U.

functions as a minor sulfur mediator and accepts the persulfide sulfur directly from Nfs1p. In an *in vitro* sulfur transfer experiment, Uba4p accepted the sulfur from Nfs1p in the absence of Tum1p but did so inefficiently (Figure 5B). In addition, it seemed that Uba4p did not activate the Nfs1p desulfurase. Thus, the direct transfer of sulfur from Nfs1p to Uba4p is a minor pathway in the biogenesis of 2-thiouridine. Very recently, it has been reported that the C-terminal RLD of Uba4p has an *in vitro* sulfur transfer activity from thiosulfate to cyanide (41). This result is completely consistent with our observations. Although sulfur transfer from Nfs1p to Uba4p is a minor pathway, Cys397 in the RLD of Uba4p was found

to be essential for 2-thiouridine formation. Taken together with the role of Tum1p, we speculate that the persulfide sulfur of Nfs1p is mainly transferred to the RLD2 (Cys259) of Tum1p, and the sulfur is then relayed to the RLD (Cys397) of Uba4p. In addition, direct sulfur transfer from Nfs1p to the RLD (Cys397) of Uba4p also takes place as a minor pathway (Figure 8).

Ubiquitin and ubiquitin-related modifiers (Ubls) are involved in various biological processes through conjugation of target proteins (55,56). As the first step of ubiquitination, the C-terminal glycine of Ubl is activated by the activating enzyme (E1) to form an acyl-adenylate intermediate, which is then transferred to an active-site cysteine

residue of the E1 enzyme via a thioester-linkage. *URM1* is a ubiquitin-related modifier, and *UBA4* is an E1-like enzyme of *URM1* involved in protein urmylation (38). In this study we have demonstrated that Uba4p first activates the C-terminus of Urm1p as an acyl-adenylated form by employing ATP, then converts the C-terminal acyl-adenylate (Urm1p-COAMP) to the thiocarboxylate (Urm1p-COSH) by replacing AMP (Figure 6B). Moreover, the *in vitro* sulfur transfer experiment revealed that the labeled sulfur of [^{35}S] cysteine was actually transferred to the thiocarboxylated C-terminus of Urm1p via the catalytic functions of Nfs1p, Tum1p and Uba4p (Figure 6A and B).

Although there may still be missing proteins needed for the formation of 2-thiouridine, we were able to partially reconstitute 2-thiouridine formation of tRNA^{Lys2}, *in vitro*, with recombinant Nfs1p, Tum1p, Urm1p, Uba4p and TAP-tagged Ncs6p, which was pulled down with IgG beads (Figure 7). It is possible that Ncs2p may be co-precipitated with Ncs6p, as Ctu1/Ncs6p is complexed with Ctu2/Ncs2p in fission yeast (24). The thiocarboxylate at the C-terminus of Urm1p might be utilized in the subsequent reactions mediated by Ncs2p/Ncs6p. *NCS6/CTU-1/TUC1* is a homolog of bacterial *ttcA* (23), which is required for the biogenesis of 2-thiocytidine (s^{2}C) at position 32 of bacterial tRNAs (57). *NCS6* and *ttcA* share a PP-loop motif (SGGxDS) in the N-terminal region (23). The PP-loop is a catalytic motif of N-type ATP pyrophosphatases including TilS involved in lysidine formation (27,58), MnmA for bacterial s^{2}U formation (19,48) and ThiI for s^{4}U formation (59). These RNA-modifying enzymes commonly activate the target positions of pyrimidine bases by forming acyl-adenylate intermediates, which are then converted to their respective modifications. Although there is no evidence that TtcA catalyzes s^{2}C formation, it is likely that s^{2}C can be synthesized through similar chemical reactions with other RNA modifications generated by PP-loop containing enzymes. To characterize the final step of 2-thiouridine formation, it will be necessary to generate a recombinant Ncs6p/Ncs2p complex. We still neither know which protein receives the thiocarboxylate from Urm1p nor how the Ncs6p/Ncs2p complex recognizes the tRNA and catalyzes 2-thiouridine formation. Since we have shown that biogenesis of the mcm^5-group of the wobble base is required for efficient formation of 2-thiouridine (Figure 4C), it is possible that the Ncs6p/Ncs2p complex might recognize the C5 substituent of uracil base.

Where does 2-thiouridine formation take place in the cell? Nfs1p is mainly localized in mitochondria (60–62). According to a high-throughput search on the subcellular localization of yeast proteins (63–65), Tum1p is localized in mitochondria and cytoplasm. Thus, Tum1p might interact with Nfs1p and accept a persulfide sulfur in mitochondria. It is assumed that Tum1p is then exported to the cytoplasm where Uba4p relays the activated sulfur from Tum1p. Given this perspective, Tum1p could be a shuttling protein between the cytoplasm and mitochondria. Urm1p is thiocarboxylated by Uba4p in the cytoplasm. The subsequent 2-thiouridine formation of tRNAs, mediated by Ncs2p/Ncs6p, also takes place in the cytoplasm.

URM1 and *UBA4* show strong similarities to bacterial proteins responsible for the biogenesis of sulfur-containing cofactors. In *E. coli*, MoaD and MoeB, which are homologous to *URM1* and *UBA4* respectively (Figure 3B and C), are involved in molybdopterin biogenesis (66). The C-terminus of MoaD is first activated as an acyl-adenylated form (MoaD-COAMP) by its E1-like enzyme MoeB. The acyl-adenylated C-terminus of MoaD is then converted to thiocarboxylated MoaD (MoaD-COSH) through the action of MoeB and cysteine desulfurases (67,68). In the case of thiamine biogenesis in *E. coli*, ThiS and ThiF, which are homologous to *URM1* and *UBA4*, respectively (Figure 3B and C), undergo the same chemical reactions to generate a thiocarboxylated form of ThiS (ThiS-COSH), which is then used as a substrate for thiamine biogenesis (69). In this reaction, it is known that the RLD-containing protein ThiI accepts a persulfide sulfur from IscS, then transfers the sulfur for thiocarboxylate formation to the C-terminus of ThiS which is catalyzed by ThiF. ThiI has another function as an RNA-modifying enzyme as it is involved in the synthesis of 4-thiouridine at position 8 of tRNAs (70). Human *MOCS2A* is a paralog of human *URM1* (Figure 3C). Human ortholog of *UBA4* is *MOCS3* which is an E1-like enzyme for *MOCS2A* as well as *URM1*. *MOCS2A* and *MOCS3* encode proteins required for molybdopterin biogenesis in humans (71,72). Although a system of molybdopterin biogenesis is well conserved in eukaryotes, *S. cerevisiae* is one of the rare organisms that has lost molybdopterin biogenesis (41). Thus, *URM1* and *UBA4* are only involved in protein urmylation and 2-thiouridine formation in *S. cerevisiae*. With respect to thiamine biogenesis, the yeast functional homolog of ThiF is *THI4* (73) which is a different gene from *UBA4*. To confirm whether *URM1* and *UBA4* are involved in thiamine biogenesis in *S. cerevisiae*, we tested the requirement of thiamine for normal growth of Δ*URM1* and Δ*UBA4* strains. When we inoculated Δ*URM1* and Δ*UBA4* strains, together with a Δ*THI4* strain as a control on thiamine-deficient plates, normal growth of Δ*URM1* and Δ*UBA4* strains was observed, while no growth of Δ*THI4* strain was observed as expected (data not shown). This result is consistent with the previous observation that temperature sensitivity of Δ*URM1* and Δ*UBA4* was not suppressed by thiamine addition (38). These data revealed that thiamine biogenesis in *S. cerevisiae* is a distinct pathway from the *URM1/UBA4*-mediated pathway.

According to sequence alignment (Figure 3B), *UBA4* along with other eukaryotic homologs contain a C-terminal RLD with a conserved cysteine residue, whereas *E. coli* MoeB and ThiF lack a C-terminal RLD. We have shown in this study that Cys397 in the RLD of Uba4p plays an essential role in s^{2}U formation. Moreover, a recent study on molybdopterin biogenesis clearly reported that Nfs1p specifically interacts with the RLD of human *MOCS3* and transfers the persulfide sulfur to this domain (74). These facts reveal a mechanistic difference between cytoplasmic s^{2}U formation and bacterial cofactor biogenesis; namely, Uba4p utilizes its own RLD as a sulfur carrier for

generating Urm1p-COSH, while ThiF uses the rhodanese-containing protein ThiI as a sulfur carrier for generating ThiS-COSH. Even if Tum1p is assumed to be a functional homolog of ThiI, the RLD of Uba4p is essential for cytoplasmic s^2U formation.

It was reported that the loss of *URM1* together with the loss of *CLA4*, a p21-activated kinase required for the budding process resulted in a synthetic lethal phenotype (35,75). The loss of *URM1* resulted in deficient invasive growth and rapamycin sensitivity, indicating that the urmylation pathway has a genetic interaction with the TOR pathway (35). In addition, it has been reported that the urmylation pathway regulates the expression of genes involved in sensing and controlling amino acids levels (37). Thus, the urmylation pathway has been implicated in budding and nutrient sensing. It is known that Urm1p conjugates with a number of proteins in *S. cerevisiae*. Ahp1p was identified as the first *in vivo* target of urmylation (36). Ahp1p is a peroxiredoxin, an antioxidant protein, that eliminates alkyl peroxide or thiol oxidant (76,77). Loss of the urmylation pathway ($\Delta URM1$ and $\Delta UBA4$) results in sensitivity to a thiol-specific oxidant, as does the loss of *AHP1*, indicating that the urmylation pathway has a possible link with an oxidative stress response (36). In this study, we have shown that the urmylation pathway is shared with the biogenesis of s^2U. Lack of 2-thiouridine in the tRNA anticodon modulates the decoding activity of NNR codons (10). tRNAs bearing hypomodified wobble bases may affect the global translation profile (69). Therefore, various cellular responses related to loss of the urmylation pathway can be explained, not only by protein modification, but also by the lack of 2-thiouridine formation of tRNA anticodon. Futher studies will be required to reveal both sides of the urmylation pathway in various cellular functions.

In *E. coli*, we have previously shown that the sulfur-relay system mediated by Tus-proteins is required for 2-thiouridine formation (18) (Figure 8B). IscS initially accepts a persulfide sulfur from cysteine. TusA then interacts with IscS to stimulate its desulfurase activity, and accepts the persulfide sulfur. The sulfur is transferred to TusD in the TusBCD complex, then to TusE. TusE interacts with MnmA to transfer the sulfur. MnmA recognizes the tRNA and facilitates the 2-thiouridine synthesis of mnm^5s^2U using the persulfide sulfur. Thus, the sulfur flow system in *E. coli* is based on persulfide chemistry. In *S. cerevisiae*, Tum1p is a functional homolog of TusA in *E. coli*. However, the subsequent sulfur-relay system in *S. cerevisiae* is completely distinct from the bacterial sulfur-relay system (Figure 8A). The most striking aspect in eukaryotic 2-thiouridine formation is that the activated sulfur is further relayed by the protein conjugation system of a ubiquitin-related modifier. A similar system of sulfur-flow can be found in some thermophilic bacteria. In *Thermus thermophilus*, 2-thiolated ribothymidine (s^2T) can be found at position 54 in the T-loop of tRNAs (78). It is known that s^2T54 confers tRNA stability at higher temperatures (79). Shigi *et al.* (80,81) identified three genes from *T. thermophilus*, named *ttuA*, *ttuB* and *ttuC*, which are responsible for 2-thiouridine formation of s^2T54 (80,81). *ttuA* is a homologous protein of *E. coli ttcA*

and *NCS6*, indicating that s^2T formation in these organisms occurs through a similar mechanism to that found in eubacterial s^2C formation and eukaryotic 2-thiouridine formation. In addition, *ttuB* and *ttuC* are homologous to *URM1* and *UBA4*, respectively. In fact, the C-terminal glycine of TtuB was shown to be acyl-adenylated, then thiocarboxylated by TtuC (81). The activated sulfur atom of the thiocarboxylate is further transferred to tRNA by the action of TtuA. Therefore, sulfur flow systems for 2-thiouridine formation are diversified in various living organisms.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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