Biosynthesis of wybutosine, a hyper-modified nucleoside in eukaryotic phenylalanine tRNA

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Wybutosine (yW) is a tricyclic nucleoside with a large side chain found at the 3'-position adjacent to the anticodon of eukaryotic phenylalanine tRNA. yW supports codon recognition by stabilizing codon-anticodon interactions during decoding on the ribosome. To identify genes responsible for vW synthesis from uncharacterized genes of Saccharomyces cerevisiae, we employed a systematic reverse genetic approach combined with mass spectrometry ('ribonucleome analysis'). Four genes YPL207w, YML005w, YGL050w and YOL141w (named TYW1, TYW2, TYW3 and TYW4, respectively) were essential for vW synthesis. Mass spectrometric analysis of each modification intermediate of yW revealed its sequential biosynthetic pathway. TYW1 is an iron-sulfur (Fe-S) cluster protein responsible for the tricyclic formation. Multistep enzymatic formation of yW from yW-187 could be reconstituted in vitro using recombinant TYW2, TYW3 and TYW4 with S-adenosylmethionine, suggesting that yW synthesis might proceed through sequential reactions in a complex formed by multiple components assembled with the precursor tRNA. This hypothesis is also supported by the fact that plant ortholog is a large fusion protein consisting of TYW2 and TYW3 with the C-terminal domain of TYW4.

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Introduction

A characteristic structural feature of functional RNAs is the presence of post-transcriptional modifications. During RNA processing and maturation, RNA molecules undergo various chemical modifications by RNA-modifying enzymes. Accurate maintenance of RNA modifications is required for various biological functions (Björk, 1995; Suzuki, 2005). Our recent studies revealed that lack of tRNA modification leads to translational defects, which have been associated with human diseases (Kirino and Suzuki, 2004; Kirino *et al*, 2005).

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To date, more than 100 different RNA modifications have been reported in RNA molecules from all domains of life (Rozenski et al, 1999). Most have been identified and characterized in tRNA molecules. Many tRNAs have modifications at the first (wobble) position of the anticodon (position 34) and adjacent to the 3'-position of the anticodon (position 37). The wobble modifications at position 34 are important for precise decoding mediated by the codon-anticodon interaction (Suzuki, 2005). Position 37 of the tRNA usually contains a modified purine nucleoside. This modification is often a hyper-modified nucleoside with large molecular weight, such as N⁶-threonylcarbamoyladenosine (t⁶A), 2-methylthio- N^6 -isopentenyladenosine (ms²i⁶A) or wybutosine (yW). These modifications play a critical role in the stabilization of codon-anticodon pairing through base-stacking interactions and function to maintain the reading frame. yW (whose base is wye, Figure 1A), which contains a tricyclic base, is one of the most complexly modified guanosine residues. yW and its derivatives are found at position 37 of eukaryotic and archaeal phenylalanine tRNA (tRNA^{Phe}) (Figure 1B) (Blobstein et al, 1973), with the exception of Bombyx mori (Keith and Dirheimer, 1980) and Drosophila melanogaster (Altwegg and Kubli, 1979), where 1-methylguanosine (m¹G) is present. yW does not have any major influence on the phenylalanylation of tRNA^{Phe} (Thiebe and Zachau, 1968), but may stabilize the first base pair of the codon-anticodon duplex in the ribosomal A site by base stacking (Konevega et al, 2004). tRNA^{Phe} that lacks the yW modification has been shown to enhance frameshifting (Carlson et al, 1999) and influence the replication of human immunodeficiency virus (HIV) RNA (Hatfield et al, 1989). In addition, mouse tRNA^{Phe} isolated from neuroblastoma cells has m¹G instead of the naturally occurring hydroxywybutosine (OHyW), and tRNA^{Phe} from Ehrlich ascites tumors contains an undermodified OHyW (OHyW-72; OHyW minus 72 Da) (Kuchino et al, 1982). These data imply that disruption of the yW modification may have pathological consequences.

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The biosynthesis of yW is a multienzymatic process. yW originates from a genetically encoded guanine (Li et al, 1973; Thiebe and Poralla, 1973), and the presence of m¹G37 in tRNAs^{Phe} from neuroblastoma cells, as well as *B. mori* and *D. melanogaster*, suggested that m¹G is the first precursor in the synthesis of vW. N^1 -methylation of G37 in tRNA^{Phe} is catalyzed by an S-adenosylmethionine(Ado-Met)-dependent tRNA methylase (TRM5) in the initial step of yW synthesis (Droogmans and Grosjean, 1987). Disruption of the TRM5 gene led to a severe growth defect (Björk et al, 2001), suggesting that m¹G37 has a critical role in maintaining the correct reading frame in addition to initiating yW synthesis in tRNA^{Phe}. The GAA anticodon of tRNA^{Phe} is necessary for yW synthesis (Droogmans and Grosjean, 1987), although the anticodon is not required for m¹G formation by TRM5 (Brule et al, 2004). Thus, subsequent steps in the multienzymatic process from m¹G to yW strictly discriminate tRNA^{Phe} from other m¹G-containing tRNAs. Very little is

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Figure 1 Chemical structure of wybutosine (yW) and secondary structure of tRNA^{Phe}. (A) Chemical structure of yW. Carbon and nitrogen atoms in the tricyclic base are numbered. The α -amino- α -carboxypropyl group at C-7 is boxed by a dotted line. (B) Secondary structure of the *S. cerevisiae* tRNA^{Phe} with modified nucleosides: wybutosine (yW), 2'-O-methylguanosine (Gm), 2'-O-methylguanosine (Cm), pseudouridine (Ψ), 5-methylcytidine (m⁵C), 7-methylguanosine (m²G), 2-methylguanosine (m²G), N²,N²-dimethylguanosine (m²G), dihydrouridine (D), 1-methyladenosine (m¹A) and 5-methyluridine (m⁵U). The anticodon-containing fragment produced by RNase T₁ digestion is shown in gray. Arrows indicate the sites for RNase T₁ cleavage.

known about the biosynthetic steps from m^1G to yW. It has been reported that the α -amino- α -carboxypropyl side chain at the C-7 position of the yW-base (Figure 1A) is transferred from methionine or *S*-adenosylmethionine in yeast cells (Munch and Thiebe, 1975). However, in Vero cells, the lateral chain of the yW-base is derived from lysine (Pergolizzi *et al*, 1979). These data imply that the biosynthesis of yW in tRNA^{Phe} might vary from one species to another.

Although the number of genes involved in biosynthesis of yW is not known, the genes responsible for yW synthesis must be present among the uncharacterized genes in the Saccharomyces cerevisiae genome. To identify genes responsible for RNA modifications, we have employed a reverse genetic approach combined with mass spectrometry ('ribonucleome' analysis) (Soma et al, 2003; Suzuki, 2005; Ikeuchi et al, 2006). This analysis utilizes a series of gene-deletion strains of S. cerevisiae. Total RNA extracted from each strain was analyzed by liquid chromatography-mass spectrometry (LC/MS), to determine whether a particular gene deletion leads to the absence of a specific modified base and, thus, permits us to identify the enzyme or protein responsible for this modification. In the case of essential genes, we analyzed temperature-sensitive mutants cultured at the nonpermissive temperature, or expression-controlled strains (Soma et al, 2003). This analysis enables us to identify the enzymes directly responsible for RNA modification, as well as genes that encode nonenzymatic proteins necessary for the biosynthesis of RNA modifications. These include carriers of the metabolic substrates used in the RNA modification and subunit proteins needed for RNA recognition.

Here, using the ribonucleome analysis, we identified four new genes responsible for biosynthesis of yW in *S. cerevisiae*.

Chemical structures of the modification intermediates of yW in these deletion strains enabled us to model a biosynthetic pathway of yW. In addition, yW synthesis was partially reconstituted *in vitro*. This study will enable us to now develop a complete understanding and *in vitro* reconstitution of yW synthesis.

Results

Identification of four genes responsible for yW synthesis by ribonucleome analysis

Since yW is a nonessential RNA modification, the complete set of S. cerevisiae deletion strains (4829) serves as a parent population to identify genes responsible for yW synthesis. To reduce the size of the starting population, we selected 3482 genes that have orthologs in Shizosaccharomyces pombe, because S. pombe tRNAs possess all the modified nucleosides found in S. cerevisiae tRNAs. Next, we chose 351 genes with ORFs coding for proteins of unknown function in S. cerevisiae (CYGD: http://mips.gsf.de/genre/proj/yeast) (Guldener et al, 2005) and started the ribonucleome analysis with this population. Parallel small-scale cultivation of each deletion strain was performed in deep 24-well plates. We developed a system that enabled us to obtain total RNA from each cultured strain and perform nuclease digestions in a 96-well format. Samples were then automatically analyzed by LC/MS using ion-trap MS to determine which modified nucleosides, if any, were absent. Our LC/MS analysis routinely detects 20 species of modified nucleosides in yeast total RNA, most are from tRNAs and some are from rRNAs or other noncoding RNAs. In the mass chromatogram, yW is detected as a proton adduct form (MH⁺) of yWpA (m/z 838, RT 46.8 min), which is a P1 nuclease-resistant dimer (Figure 2A). The protonated base fragment (BH₂⁺) related to vW-base (m/z 377) is also detected (Figure 2A). To search for genes responsible for yW synthesis, we examined mass chromatograms of the 351 selected deletion mutants for peaks at m/z 838 and m/zand identified four deletion strains, YPL207w, 377 YML005w, YGL050w and YOL141w, in which vW was absent (Figure 2A). Since other modified nucleosides in these strains were detected normally (data not shown), the absence of yW is not due to nonspecific reduction of RNA modifications. Moreover, as normal amounts of tRNA^{Phe} could be isolated from each deletion strain, the absence of yW is not due to a specific defect in tRNA^{Phe} transcription (data not shown).

Cell growth of each deletion strain was measured in rich medium as well as in the minimal medium (Supplementary Figure S4). However, we did not observe any significant differences in growth between wild-type and the deletion strains.

Mass spectrometric analysis of the modification intermediates of yW

To confirm the absence of yW at position 37 of tRNA^{Phe} and to determine the chemical structure of each modification intermediate, we isolated individual tRNA^{Phe} from each deletion strain. As expected, the nucleoside analysis revealed that the purified tRNA^{Phe} from each deletion strain had no yW nucleoside (Supplementary Figure S1), but other modified nucleosides were present (data not shown). These results demonstrated that the four new genes encode proteins involved in biosynthesis of yW at position 37 of tRNA^{Phe}. In addition, biosyntheses of other modifications in tRNA^{Phe} (Figure 1B) were not affected by the absence of yW, indicating that yW is not required for biosynthesis of other modifications. We have renamed *YPL207w*, *YML005w*, *YGL050w* and *YOL141w* as *TYW1*, *TYW2*, *TYW3* and *TYW4* (<u>tRNA-yW</u> synthesizing protein 1–4), respectively. To determine the mass of each modification intermediate of yW, the purified $tRNA^{Phe}$ was subjected to RNase T_1 digestion and analyzed by LC/MS (T_1 mapping), as shown in Figure 2B. In the case of wild-type $tRNA^{Phe}$, the anticodon-



containing RNA fragment (ACmUGmAAvWAΨm⁵CUGp, MW 4167) produced by RNase T₁ digestion (Figure 1B) was clearly observed as a triply charged ion with m/z 1388 (Figure 2B). The mass of the same RNA fragment in tRNA^{Phe} obtained from the $\Delta TYW1$ strain was MW 3956 (*m*/*z* 1317), which is the mass of a fragment having m¹G37 instead of yW37 (Figure 2B). Appearance of $m^{1}G$ was also confirmed by nucleoside analysis of purified tRNA^{Phe} from the $\Delta TYW1$ strain (Supplementary Figure S2A). The data clearly demonstrate that TYW1 encodes a protein responsible for the second step of yW synthesis (see Figure 6). According to T_1 mapping of purified tRNA^{Phe} from the other three deletion strains, $\Delta TYW2$, $\Delta TYW3$ and $\Delta TYW4$ generated the modification intermediates yW-187 (yW minus 187 Da, MW 321), yW-86 or yW-14 (yW minus 86 or 14 Da, MW 422 or 494), and yW-72 (yW minus 72 Da, MW 436), respectively, judging from changes in the mass of each anticodon-containing fragment (Figure 2B). These intermediates were also confirmed by nucleoside analysis of the purified tRNA^{Phe} using LC/MS (Supplementary Figure S2B-D). It is intriguing that tRNA^{Phe} from $\Delta TYW3$ strain has two yW intermediates, yW-86 and yW-14. It is likely that yW-86 is a real intermediate at this step and yW-14 is a product by skipping TYW3-dependent reaction. Judging from the intensity of the mass chromatogram, about 50% of tRNA^{Phe} has yW-14 instead of yW-86.

Chemical structures of the modification intermediates of yW

To further define the chemical structures of the modification intermediates of yW, nucleosides from each purified mutant tRNA^{Phe} were subjected to LC/MS/MS analysis to observe product ions of the bases produced by collision-induced dissociation (CID). The structures of the compounds can then be determined by referring to the reported MS/MS spectra, since characteristic patterns of the product ions are highly correlated to the chemical structure. When yWpA and its intermediates are ionized as proton adducts (MH⁺) by electrospray ionization (ESI), base fragments of yW and its derivatives are spontaneously generated in the ion source. The protonated base fragment (BH_2^+) of the each tRNA was selected as a parent mass and decomposed in collision with helium gas to obtain the spectrum of product ions (Supplementary Figure S3). Most of our data could be interpreted by referring to the CID spectra for chemically synthesized vW derivatives, part of the pioneering work by J McCloskey's group (Zhou et al, 2004a, b). Interpretation of each CID spectrum and determination of chemical structure of each vW intermediate are described in the legend for Supplementary Figure S3. The determined chemical structures for yW-187, yW-86, yW-72 and yW-14 are shown in Figure 6 and Supplementary Figure S3.

TYW genes encode highly conserved proteins in eukaryote

Using each yeast TYW gene as a query, we have retrieved homologs from *S. pombe*, human, mouse and *Arabidopsis thaliana*. The sequence alignments (Figure 3A–C) show that these proteins share many conserved regions.

TYW1 (810 aa in *S. cerevisiae*) and its homologs contain two conserved domains (Figure 3A), a flavodoxin-1 domain in the N-terminus (pfam00258) and a radical-Ado-Met domain in the C-terminus (pfam04055). Flavodoxin is a protein that includes an iron–sulfur (Fe–S) cluster and employs FMN as a cofactor (Wakabayashi *et al*, 1989). Radical Ado-Met proteins contain a conserved CxxxCxxC motif (Figure 3A), which is responsible for binding a [4Fe–4S] cluster, as a catalytic core for diverse reactions (Sofia *et al*, 2001). These structural features of the conserved domains suggest that TYW1 is an Fe–S cluster protein responsible for ring formation of the tricyclic base of yW.

TYW2 (462 aa in *S. cerevisiae*) and its homologs contain a conserved Ado-Met-binding motif found in Met-10 + -like protein family (lower part of Figure 3B) (Niewmierzycka and Clarke, 1999). In addition, TYW2 was found to be a member of COG2520, which also includes TRM5. The data indicate that TYW2 is an Ado-Met-dependent enzyme.

TYW3 (273 aa in S. cerevisiae) was found to be a highly conserved protein (pfam02676/DUF207, COG1590), but its function is unknown. A. thaliana and Oryza sativa homologs of TYW3 have a long extended C-terminal domain (CTD) of about 1000 aa (Figure 3B), and it is interesting to note that the long extended CTD of the plant TYW3 actually contains the CTD of TYW4 as well as the entire sequence of TYW2. Thus, the plant homologs appear to be a large fusion protein including TYW3, the CTD of TYW4 and TYW2, which we have named TYW3-4C-2 (Figure 3B and D). Additionally, the plant TYW4 is a short protein without the CTD (Figure 3C). These data suggest that TYW3-4C-2 acts as a multienzymatic complex for yW synthesis in A. thaliana and O. sativa. Schematic depiction of the topological orientation of TYW2, TYW3, TYW4 and TYW3-4C-2 is shown in Figure 3D.

TYW4 (695 aa in *S. cerevisiae*) has a conserved leucine carboxyl methyltransferase (LCM) domain (pfam04072) in its N-terminal region (Figure 3C). TYW4 has an apparent paralog, PPM1 (protein phosphatase methyltransferase 1), which is a yeast methyltransferase responsible for methylesterification of the C-terminal Leu residue of PP2A (protein phosphatase 2A) (Wu *et al*, 2000; Kalhor *et al*, 2001). Thus, YOL141w (TYW4) had been previously named PPM2 (Kalhor *et al*, 2001; Wei *et al*, 2001), but its biochemical function was never demonstrated directly. The PPM family has three highly conserved Ado-Met-binding motifs (motifs I, II and III). This finding strongly suggests that, using Ado-Met as a

Figure 2 Mass spectrometric analysis of total nucleosides and tRNA^{Phe} from *S. cerevisiae* wild-type and mutant cells. (A) LC/MS analysis of the total nucleosides in the wild-type (WT), $\Delta YPL207w$ (TYW1), $\Delta YML005w$ (TYW2), $\Delta YGL050w$ (TYW3) and $\Delta YOL141w$ (TYW4). The upper panel is the UV trace at 254 nm. The middle and lower panels are mass chromatograms detecting MH⁺ of yWpA (m/z 838) and BH₂⁺ of yW-base (m/z 377), respectively. Arrows indicate the retention time for yWpA. (**B**) LC/MS fragment analyses of RNaseT₁-digested tRNA^{Phe} obtained from wild-type and $\Delta TYW1-4$. The graphs on the left describe the mass chromatograms shown by triply charged ions of anticodon-containing fragments containing yW (m/z 1388) from WT, m¹G (m/z 1317) from $\Delta YPL207w$ (TYW1), yW-187 (m/z 1325) from $\Delta YML005w$ (TYW2), and yW-86 (m/z 1359) or yW-14 (m/z 1383) from $\Delta YGL050w$ (TYW3) and yW-72 (m/z 1364) from $\Delta YOL141w$ (TYW4). RNA sequences, including modifications for each fragment, are indicated. The graphs on the right show the mass spectrum for each anticodon-containing fragment. Charge states are indicated in parentheses.

A ScerettW1: SponbttW1: HUMANTTW1: MOUSETTW1: AthaltTW1:	TYW1NG-PRAGALVVGALTAANL FEGERSEARVETUGVGTYCNEASGGSODSQERLELNKOOKKPCCSDKKIADGGKKTGGCCSSKGGKKGGCCSSKGGKKGGCCSSKGKKGGCGSSKGKKGGCCSSKGKKGGCCSSKGKKGGCCSSKGKKGGCCSSKGKKGGCCSSKGKKGGCCSSKGKKGGCCSSKGKKGGCGSSKGKKGGCCSSKGKKGGCGSKGKGGKGGCGSKGKGGKGGKGGKGGCGSKGCGSKGKGGCGSKGCGSKGCGSKGKGCGSKGCGSKGKGGCGSKGCGSKGCGSKGCGSKGCGSKGKGGCGSKGCGCGSKGCGSKGCGCGSKGCGSKGCGCGSKGCGCGSKGCGSKGCGCGSKGCGSCGSKGCGSKGCGSKGCGKGGCSSKGCGSKGCGSKGCGSGCGS	7 0
ScereTTW1 : SponbTTW1 : HumanTTW1 : NouseTTW1 : AthalTTW1 :	EVFREPTKERSSTPKVFSKNSSSNSRVGKKLSVSKKIGPDGLIKSALTISNETLLSSOIYGLÜSELGEADSKAUKSYTDKIKEN LITHEPKOLNLUDLSDFDUYFDVEVANDYLLGIPENDIDGLDGULOGUANTBODS : 299 	5
ScereTTW1 :		
SponDTTW1 : HumanTTW1 : MouseTTW1 : AthalTTW1 :	TYDER R-VANGLENSATASHENENGENNER LUMLEAN UNSPERED VVESKHÖSBEADEPARKTEFISCUOALOKGERKESCEGHCKEGKCESBOR SEEREEGSBEODELHHRU EEEDPFESSSEEEFGGDHOSLNSIVD : 313 TYDER R-VANGLENSATESHENENSTIM E LUMLEAOUNLTREEGD AVOSKHÖSBEADETAKKTEFISCUOALOKGE-KRACEGNCKREKESADE PGEARPHPOGELHPOHAEEDPCESSSEDELGT DYOSLTSVD : 302	2
	479 483 486	
ScereTTW1 : SpombTTW1 : HumanTTW1 : MouseTTW1 : AthalTTW1 :	VEDICGKOSNON FSGADEI	L
	532 550 [4Fe-4S] motif	
ScereTTW1 : SponbTTW1 : NumanTTW1 : MouseTTW1 : AthalTTW1 :	OVPOY LA DE ANAFEVRICALSLUGEPH VERINKTECHER KETSFLUCNAGED AL DE VEVTOLVUSIOAFTELRKUDEPH VEVENGECH IN KTVORTUKETUKETUKETUKETUKETUKETUKETUKETUKETUKE	9
	Radical-Ado-Met domain	
ScereTTW1 : SpombTTW1 : HumanTTW1 : MouseTTW1 : AthaiTTW1 :	ESA SLTNAHOPPHERVOPVED VOLD-VOLD-VOLD-VOLD-PEVELACHENSIOLLIA-HEITERGEDUNTINVARIOELICEYEDSGESKTFANDYN FITHVALFGLSEFGFDNOITHOS N SKAISGC	

Figure 3 Sequence alignments of the TYW proteins. Each TYW protein is aligned with a set of protein homologs from *S. cerevisiae* (Scere), *S. pombe* (Spomb), *Homo sapiens* (Human), *Mus musculus* (Mouse), *A. thaliana* (Athal) and *O. sativa* (Osati). Multiple alignment of each sequence was carried out by Clustal X (Thompson *et al*, 1997) and displayed by Genedoc multiple sequence alignment editor (Nicholas *et al*, 1997). 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 residues with ~60% homology. (A) Sequence alignment of TYW1 with its homologs. Two conserved domains, flavodoxin-1 domain and Radical-Ado-Met domain, are underlined. The boxed region represents the [4Fe-4S] motif. Positions for site-directed mutagenesis are indicated by arrowheads. (B) Sequence alignment of TYW2 and TYW3 with its homologs. *A. thaliana* and *O. sativa* homologs are large fusion proteins including TYW3 and the C-terminal region of TYW4 and TYW2 (TYW3-4C-2). A set of TYW2 homologs and TYW3 homologs are aligned with plant TYW3-4C-2. The Met-10 + -like protein family domain in TYW2 is indicated by a line. The C-terminal region of TYW4 in plant TYW3-4C-2 is shaded. (C) Sequence alignment of TYW4 with its homologs. The conserved domain, LCM, is indicated by a line. The boxed regions are Ado-Met-binding motifs, including motif I-III and post-I. The residues important for protein carboxyl methyltransferase activity are indicated by arrowheads. The C-terminal region of TYW4 is shaded. (D) Domain structure of the plant TYW3-4C-2 protein. The plant TYW3-4C-2 protein is a large fusion protein composed of TYW3, the CTD of TYW4 and TYW2.

methyl donor, TYW4 catalyzes methylation of the carboxyl group of the methionine moiety in yW-72 to convert it to yW-58 (see Figure 6).

Biogenesis of an Fe–S cluster is required for TYW1 function in yW synthesis

The S. cerevisiae NFS1 gene encodes a cysteine desulfurase involved in Fe-S cluster biogenesis. To test whether yW synthesis requires biosynthesis of an Fe-S cluster, we analyzed total nucleosides from S. cerevisiae YN101 cells in which expression of NFS1 is controlled under the GAL1 promoter (Nakai et al, 2004). We previously demonstrated that inhibition of NFS1 expression resulted in a severe reduction of thio-modifications in both mitochondrial and cytoplasmic tRNAs (Nakai et al, 2004). When cells were cultured in galactose-containing medium, yWpA was clearly observed (Figure 4A). However, when the expression of NFS1 was downregulated by culturing cells in glucose-containing medium, yWpA was severely reduced (Figure 4A). These data demonstrated that biosynthesis of Fe-S clusters by NFS1 is absolutely required for yW synthesis. The Fe-S cluster in TYW1 is likely to be critical for the formation of the tricyclic base of vW.

The radical-Ado-Met domain in the C-terminal region of TYW1 has a conserved $C^{479}xxxC^{483}xxC^{486}$ motif (Figure 3A)

for [4Fe-4S] cluster binding. In addition, E532 and E550 are candidate residues for GGE motif in the radical Ado-Met domain (Pierrel et al, 2004). To clarify functional importance of these conserved residues, site-directed mutagenesis of Cys479, Cys483, Cys486, Glu532 and Glu550 was carried out. The plasmid expressing TYW1 (pTYW1) with each mutation was introduced into $\Delta TYW1$ strain to test complementation activity of each mutant for yW synthesis. As shown in Figure 4B, vW in the $\Delta TYW1$ strain was restored by the introduction of wild-type pTYW1. No activity for yW synthesis could be observed when pTYW1 with C479A, C483A, C486A or E550A mutation was introduced. E532A mutation did not affect TYW1 functionality. These results revealed that three conserved Cys in the CxxxCxxC motif as well as E550 in the GGE motif is critical for yW synthesis. Taken together with the result of NFS1 data, biogenesis of an Fe-S cluster in TYW1 is required for TYW1 function in yW synthesis.

Reconstitution of yW synthesis in vitro using recombinant TYW2, TYW3 and TYW4

Next, we performed *in vitro* reconstitution of yW synthesis using recombinant TYW proteins. A hexahistidine-tagged TYW2, TYW3 and TYW4 were expressed in *S. cerevisiae* and purified using an Ni⁺-chelating column. tRNA^{Phe} having

Novel genes involved in the biosynthesis of wybutosine in tRNA^{Phe} A Noma *et al*

B ScereTTW2	TYW2, TYW3
NouseTTW2 . HumanTTW2 AtbalTTW3=4C=2	
ScereTTW2 SponDTTW2 MouseTTW2 HumanTTW2 AthaLTW3-4C-2 CsaLITW3-4C-2 ScereTTW3 SponDTTW3 MouseTTW3 HumanTTW3	S OUT VALUES ASTRONOM TO CONCERN AND AND AND AND AND AND AND AND AND AN
ScereTTW2 SpombTTW2 MouseTTW2 HumanTTW2 AthalTTW3-4C-2 GearTTW3 SpombTTW3 MouseTTW3 HumanTTW3	KELIPLEKLSIVGEPVEKLHLWGKSACTIDESDEKEVIVTGGFGGFGHARENESLLINPSCOTLKLIAVNESFSARLGHTASHVGDFHFVIGGRADPLNILNDVWRLDISTGEWSSQRCVGSEFPFHRHAAASVGTKVYIFGGLVNDK KELIPLEKLSIVGEPVEKLHLWGGSACALTVGREHHLTTGGFGGFGHARENTSLLVNPGGLUTELKVTGSPSPRHGHTITVVGNDIYVVGGSGDFSEILNDIWVLERSNNRWSNVDCSGDFFFFHRHAAASVGTKVYIFGGLVNDK LCDD
ScereTTW2 SpeabTTW2 MouseTTW2 NumarTW2 AthalTW3-4C-2 OsatiTW3-4C-2 ScereTTW3 SpeabTTW3 MouseTTW3 MouseTTW3	IV358HILLDTKDLQWKEVEQ0QQWPCARHSHARVAY0SQSFNF0GYNOENVLNDLYSFPVQSCSUKLEVISGKVPHARFSHSHFVYKHTIGIIGGCPVSQNCQELTLLDLKHELWPSVRLEFNNKELFVRSTASILGDDLIVIGGAACT 546 LCSCKNINDTASIQUNVISPDDKWPCARHSHSLVSYOSKLFLFGGHDQQALINDFYSFDTTLKUNKENTNOKAPSPBFSHCHFIYKDYLGILGGCPIFESSQEIALLNLKHKIWFVYSIPSLSQCLCVRSSSVIIDDDLIVIGGASCY 548 Homologous to C-terminal of TYW4
SoereTW2 SpeebTW2 MouseTW2 AtbalTW3-4C-2 OsatITW3-4C-2 SoereTW3 SpeebTW3 MouseTW3 MouseTW3	
ScereTW2 SpenbTW2 MouseTW2 AthalTW3-4C-2 OsatITW3-4C-2 ScereTW3 SpenbTW3 MouseTW3 HumanTW3	:
Sceretinz Spenbtinz Humantinz Athalting-4c-2 CsatitN3-4c-2 Sceretin3 Spenbtin3 Housetin3 Humantin3	EITERENTISTIKKULTTFPDICNNDU UUTAGIGVTITSULTKORTUFUSULTKOSTERU BOGKLAOFN
HumanTTW2 AtbalTTW3-4C-2 OsatiTTW3-4C-2 ScereTTW3 SpombTTW3	: MILEONANIDA LEDOSFENNU TULLA INES IAI IENROI KLOFVR

Figure 3 continued.

yW intermediate purified from each deletion strain was employed as a substrate for each step of yW synthesis *in vitro*.

In vitro reconstitution of the tRNA^{Phe} having yW-187 by recombinant TYW2 was carried out in the presence or absence of Ado-Met. As shown in Figure 5A, in the absence of Ado-Met, the substrate tRNA yielded an anticodoncontaining RNA fragment whose mass is unchanged (ACmUGmAA(yW-187)A Ψ m⁵CUGp, MW 3980). In the presence of Ado-Met, yW-187 in tRNA^{Phe} was clearly converted to yW-86 (101 Da increase). No other product, such as yW-173 (14 Da increase), was detected. The data revealed that recombinant TYW2 has an activity to transfer α -amino- α carboxypropyl group from Ado-Met to the side chain at the C-7 position of the yW-base. This result is consistent with a previous observation that α -amino- α -carboxypropyl group of yW originates from methionine or Ado-Met in yeast cells (Munch and Thiebe, 1975).

In $\Delta TYW3$ strain, tRNA^{Phe} has two yW intermediates, yW-86 and yW-14 (Figure 2B). As shown in Figure 5B, recombinant TYW3 clearly methylated yW-86 to yield yW-72 in Ado-Met-dependent manner. Thus, TYW3 is an N-4 methyltransferase for yW synthesis. On the other hand, yW-14 was not methylated by TYW3 (no yW product is ob-

С	TYW4
ScerePPM1	:
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ScerePPM1	1
AthalPPM1-like OsatiPPM1-like	
ScereTIW4 SpombTIW4	: GDVDVAGNDVFYNEESNFYNEEILCHS HYDKIDHKNIEVSSSEVPVARUCHFTTDSRNCLLLGGERABHOCLSINWIFDHKTREBHIKSLSHTRFEHSACSUPDGN
RumanTTW4 MouseTTW4	: ELPSLRLPAVCDLDDSUTVORELSTAGRSKDAYD ISEKDDGSINKITTDSLTSCHGGEVYSIDKK-TCHFIGGRESKKILSSCIYFADGN
AthalTTW3-4C-2	2460 ACTIDESDRKEVIVF GOOG-GRHADENESLINPSCGTIKLIAVNESPSANGGHASKNGD-FHFVIGGALGLAIINNDVWRIDISTGESQCCVGSEFPPRERAAASMGTK
OsatiTTW3-4C-2	I (14 VCFLAAREUUTE LEELAAL-ONUNDMULDEBULADADELEENA LOOLDANGAUTIAN MDELANDORDEBOTINDIAAERSUNK
ScerePPM1	
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	1 LAGVTEGPANLE VN/TEFEIDENDY FPENEFFONSUUSAGLEEDPVSKOGIDE SET NOTTVSDKAL [FKVDAENATEPITVIKKLOHPLKORYGGQIKVITPR-KLEUWGTSPSGLFDRINSIESDPISKITSPPISK 1 LAG
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	1 YCORSVAEFVLEDCRFLHVETHAUVE DVQCASPECREBESACSEQOGALECSLOASEELLSSVLFLKFVSSGFLUESIDIGPSITPRYSHTARUPNG-KLLWOZVUHSSVPOUVICLGTTGLSEYG DTAS : 689 1 YC-LNNEXUSAEHLDIXULGUKS AQQQCUCASPECREBENHAVAIGSGSFUG THGENVINDLIS DVGCSGKLWISTGLASESHHIYTKH-TIG 1 YC-LSDGLCSCNNIDTASI GVV SPDDRUCAS HEBHANVAIGSGSFUG THGENVINDIYSDTTTLKNKKHTMAKASPSFPSBCNIYTKH-TIGLIGCPSGCACGA-LILDUARENURSVNGFFN : 522
OsatiTTW3-4C-2	: For-LSDDGLCSCHNIHDTASICENVESPDDKUPCARHSHSLVSYGSKLFEFEHDGGRAINDFYSHDTTTLKNNKENTHGKAPSPRFSHCNFIYKDY-LGHAGECPIRESQE-IALENEKHKIUFYVSEPSLS : 546
ScerePPM1	
AthalPP81-like OsatiPP81-like	
ScereTTW4 SpombTTW4	RIVEDUS[IN]AOFSI.GTSHOTHAN INGATUYOFGSVINVOJKI.ALAK
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OBALLTIW3-4C-2	: ACECERSEAMTODDRAMARQARSEIVERERSCAREDURESIE (204)
D	A. thaliana PPM1-like
	O. sativa
	S. cerevisiae PPM1
	(S. pombe, M. musculus, H. sapiens)
	S. cerevisiae TYW 4 catalytic region TYW 4 C-terminal
	(S. pombe, M. musculus, H. sapiens)
	A. thaliana
	O. sativa TYW 3-like TYW 4 C-terminal-like 74W/2-like
	S. cerevisiae TYW 3 S. cerevisiae TYW 2
	(S. pombe, M. musculus, H. sapiens) (S. pombe, M. musculus, H. sapiens)

Figure 3 continued.

tained). This suggests that complete synthesis of the side chain at C-7 position inhibits N-4 methylation by TYW3.

The strong homology between TYW4 and PPM1 prompted us to test the evolutionary link between RNA methylase and protein methylase. tRNA^{Phe} having yW-72 purified from $\Delta TYW4$ cells was employed as a substrate for the yW reconstitution. In the presence of Ado-Met (Figure 5C, middle panels), UV trace of the chromatogram showed that about 60% of the anticodon-containing RNA fragment with yW-72 was converted to yield the fragments with yW-58 and yW in which the mass had increased by 14 and 72 Da, respectively. We could detect scanty amount of the RNA fragment with yW-58, which is expected as a direct product of TYW4 methylation, by the deconvolution analysis of the mass spectrum (Figure 5C). The data clearly demonstrated that recombinant TYW4 has an apparent Ado-Met-dependent methylation activity. However, it was unexpected that yW was a major product by TYW4-catalyzed reconstitution of yW-72. As RNA fragments with yW-14 or other possible intermediates were not observed (Figure 5C), these results indicate that multistep enzymatic formation of yW from yW-72 is triggered by Ado-Met-dependent TYW4 methylation. We thought small quantity of other protein involved in methoxy-carbonylation of yW-58 might be copurified with the histagged TYW4 protein, since the recombinant TYW4 was obtained by partial purification by an Ni⁺-chelating column. Then, the recombinant TYW4 protein was subjected to further purification by anion exchange chromatography.



Figure 4 Fe–S cluster in TYW1 involved in yW synthesis. (**A**) LC/MS analysis of the total nucleosides in the YN101 cells in which expression of NFS1 is controlled under the GAL1 promoter. The left- and right-hand graphs describe the LC/MS chromatograms of yWpA from YN101 cultured with galactose and glucose, respectively. The upper panels are the UV trace at 254 nm. The lower panels are mass chromatograms detecting the yW-base (m/z 377). The relative amount of yWpA in both conditions was normalized to the pseudouridine (Ψ) content. (**B**) Complementation test of Δ TYW1 by introducing a series of mutant plasmid pTYW1 in which critical residue for Fe–S cluster is mutated. Total nucleosides from each strain were analyzed by LC/MS to detect yWpA. Merged mass chromatograms of yWpA (m/z 838) and yW-base (m/z 377) are shown. Arrows indicate the retention time for yWpA. yWpA was restored by introducing wild-type pTYW1 or pTYW1-E532A. Other mutant plasmids did not complement yW synthesis in Δ TYW1

In vitro reconstitution of yW-72 was then performed using the purified TYW4. As shown in the right panels in Figure 5C, almost all yW-72 was directly converted to yW without showing any yW-58 peak. The result strongly suggests that TYW4 is a bifunctional enzyme to synthesize yW by catalyzing methylation and methoxycarbonylation of yW-72.

Discussion

Biosynthesis of yW is a multienzymatic process, and to determine the precise order of each gene in the biosynthetic pathway of yW, we analyzed the chemical structures of the yW intermediates produced by each deletion strain. In the $\Delta TYW1$ strain, the modification intermediate was m¹G. Since m¹G is the first intermediate for yW synthesis catalyzed by TRM5, *TYW1* therefore encodes an enzyme responsible for the second step of the reaction employing m¹G37-containing tRNA^{Phe} as a substrate. The chemical structure of the reaction product is unknown. The fact that a *TYW1* homolog is not encoded in the *Drosophila* genome is consistent with the observation that insect tRNA^{Phe} utilizes m¹G37 instead of yW37 (Altwegg and Kubli, 1979; Keith and Dirheimer, 1980). In addition, mouse tRNA^{Phe} from neuroblastoma cells have m¹G37 instead of OHyW37 (Kuchino *et al*, 1982), suggesting that a functional defect in the mouse homolog of TYW1 or its partner proteins might occur in these cells. TYW1 and its

homologs share the flavodoxin-like domain and the radical Ado-Met domain (Figure 3A). The flavodoxin-like domain is found in a number of proteins including flavodoxin and nitric oxide synthase. Flavodoxin is found in electron-transfer proteins that function in various electron transport systems. These domains bind one FMN molecule, which serves as a redox-active prosthetic group and are functionally interchangeable with ferredoxins (Wakabayashi *et al*, 1989).



Radical Ado-Met proteins catalyzes diverse reactions, including methylation, isomerization, sulfur insertion, ring formation, anaerobic oxidation and protein radical formation (Sofia et al, 2001). The conserved CxxxCxxC motif ([4Fe-4S] motif in Figure 3A) in the radical Ado-Met proteins is responsible for binding the [4Fe-4S] cluster, which is involved in the reductive cleavage of Ado-Met to generate the 5' deoxyadenosyl radical (Ado°) (Fontecave et al, 2001; Frey and Booker, 2001). Ado° initiates radical-based chemistry on various substrates. MiaB, which is responsible for biosynthesis of ms²i⁶A, is shown to be a radical Ado-Met enzyme involved in methylthiolation of isopentenyl-adenosine (i⁶A) at position 37 of some tRNAs (Pierrel et al, 2004). In this study, inhibition of NFS1 expression resulted in severe reduction of yW. Three conserved Cys residue and Glu550 in the motifs were essential for TYW1 function. These results clearly demonstrated that the involvement of Fe-S clusters in yW synthesis. In the crystal structure of RumA, which is an Fe-S clustercontaining protein responsible for rRNA methylation, its Fe-S cluster domain is directly involved in RNA recognition (Lee et al, 2005). It can be speculated that Fe-S cluster in TYW1 might have an important role in tRNA recognition and ring formation of tricyclic base of yW. To elucidate the detailed mechanism of this reaction, it is necessary to determine the carbon source of C-6 and its methyl group in the tricyclic base.

yW-187 (imG-14) was the yW intermediate obtained from the $\Delta TYW2$ strain. TYW2 has an Ado-Met-dependent methyltransferase motif found in the Met-10 + -like protein family, implying that TYW2 catalyzes the N-4 methylation of imG-14 to produce imG (wyosine) using Ado-Met as a methyl donor. imG has an isomer named imG2 (isowyosine) (Blobstein *et al*, 1973; Kasai *et al*, 1976), which has a methyl group at C-7 of imG-14 and there was another possibility that TYW2 methylates the C-7 position of imG-14 to produce imG2. However, according to our *in vitro* reconstitution, TYW2 has an activity to transfer α -amino- α -carboxypropyl group from Ado-Met to the side chain at the C-7 position of the yW to synthesize yW-86. Recently, Kalhor *et al* (2005) reported that deletion of YML005w (also called TRM12) generates imG-14, although the enzymatic activity was not directly demonstrated.

yW-86 was identified to be an yW intermediate in the $\Delta TYW3$ strain. Recombinant TYW3 was found to be N-4 methyltransferase that methylates yW-86 to yield yW-72 in Ado-Met-dependent manner. In addition, yW-14, which lacks N-4 methyl group from yW, was also found in this strain, suggesting that N-4 methyl group is not essential for complete synthesis of the C-7 side chain mediated by TYW4. Since yW-14 was never a substrate for TYW3, it is considered that complete C-7 side chain inhibits N-4 methylation by TYW3.

yW-72, found in the Δ*TYW4* strain, is a yW intermediate, which has an α-amino-α-carboxypropyl group at C-7 of the tricyclic base (Figure 6). TYW4 (previously named PPM2) was expected to be an Ado-Met-dependent methyltransferase having a conserved LCM domain also present in the protein methyltransferase PPM1 (Wu *et al*, 2000; Kalhor *et al*, 2001). In this study, yW-72 of tRNA^{Phe} was methylated in an Ado-Met-dependent manner in the presence of TYW4 *in vitro*, demonstrating that TYW4 is a *bona fide* methyltransferase for yW-72. The N-terminal domain of TYW4/PPM2 is homolo-



Figure 6 Biosynthetic pathway of yW in *S. cerevisiae* tRNA^{Phe}. TRM5 methylates G37 of tRNA^{Phe} to produce m¹G37, utilizing Ado-Met as a methyl donor. TYW1, an Fe–S cluster protein, may catalyze tricyclic formation of yW using Ado-Met and FMN as cofactors. TYW2 transfers α -amino- α -carboxypropyl group from Ado-Met to the side chain at the C-7 position of the yW-187 to produce yW-86. TYW3 methylates N-4 position of yW-86 in Ado-Met-dependent manner to yield yW-72. Finally, TYW4, an Ado-Met-dependent carboxymethyltransferase, methylates the α -carboxy group of yW-72 to form yW-58, which triggers methoxycarbonylation of α -amino group of yW-58 to complete yW. TYW4 is likely responsible for the last two steps. Substrate for methoxycarbonylation and requirement of partner protein still remain to be investigated.

Figure 5 *In vitro* reconstitution of yW synthesis using recombinant TYW2, TYW3 and TYW4. LC/MS fragment analysis of RNaseT₁-digested tRNA^{Phe} whose yW is partially reconstituted by recombinant TYW2 (**A**), TYW3 (**B**) and TYW4 (**C**) in the presence or absence of Ado-Met. All mass chromatograms are produced by integration of triply (-3), quadruply (-4) and quintuply (-5) charged ions of anticodon-containing fragments. (A) tRNA^{Phe} having yW-187 treated by recombinant TYW2 in the absence (left panels) or in the presence (right panels) of Ado-Met. The top and bottom graphs show mass chromatographs for anticodon-containing fragments containing yW-187 (m/z 1359 + 1019 + 815), respectively. (B) tRNA^{Phe} having yW-86 (and yW-14) treated by recombinant TYW3 in the absence (left panels) or in the presence (right panels) of Ado-Met. The top, middle and bottom graphs show mass chromatographs for anticodon-containing fragments containing yW-86 (m/z 1359 + 1019 + 815), yW-72 (m/z 1364 + 1023 + 818) and yW-14 (m/z 1383 + 1037 + 829), respectively. (C) tRNA^{Phe} having yW-72 treated by recombinant TYW4 in the absence (left panels) or in the presence (middle and right panels) of Ado-Met. Experiment in the right panels was carried out by highly purified recombinant TYW4. The top, middle and bottom graphs show mass chromatographs for anticodon-containing fragments containing yW-72 (m/z 1364 + 1023 + 818), yW-58 (m/z 1368 + 1026 + 821) and yW (m/z 1388 + 1041 + 832), respectively. Deconvolution spectra of the mass spectra (RT 34–36 min) for the anticodon-containing fragments having yW-72 reconstituted in the absence (left bottom) or presence (right bottom) of Ado-Met. The fragment with yW-58 was clearly detected in the presence of Ado-Met.

gous to PPM1. Considering that PPM1 methylates the Cterminal carboxyl group of two PP2A proteins (PPH21 and PPH22) (Wu et al, 2000; Kalhor et al, 2001), TYM4/PPM2 should methylate the carboxyl group of the Met moiety of yW-72. The observation that PPM2 has no methylation activity for PP2A proteins (Kalhor et al, 2001) is consistent with our findings in this study. The crystal structure of PPM1 complexed with Ado-Met showed a common Ado-Met-dependent methyltransferase fold with a cavity for binding the PP2A C-terminal peptide (Leulliot et al, 2004), where C202, Y206, N250, L251 and R255 of PPM1 were shown to be important for protein methylation (Leulliot et al, 2004). In the case of TYW4, the surface of the cavity is composed of TYW4-specific residues, such as V225, H272 and F273, in the flexible $\alpha 5$ helix (Figure 3C). Since the flexibility of the $\alpha 5$ helix is involved in substrate binding, the differences in this region may recognize yW-72-containing tRNA^{Phe}. TYW4 has a specific extension of the CTD, which is not present in PPM1. The TYW4-specific CTD is likely to be involved in recognition of tRNA^{Phe}. The presence of the TYW4-specific CTD in the large fusion protein TYW3-4C-2 in Arabidopsis also supports the importance of this domain for yW synthesis. The high sequence similarity suggests that TYW4 may have evolved from the PPM1-like carboxyl methyltransferase by acquiring the CTD, which is responsible for recognition of the RNA molecule.

The plant homologs of TYW2 and TYW3 are part of a large fusion protein that we have named TYW3-4C-2, which includes the CTD of TYW4 (Figure 3B and D). It is interesting that the absence of the CTD in plant TYW4 is explained by the presence of a TYW4-CTD in TYW3-4C-2. As there is one TYW4 (or PPM1) homolog in each plant genome, plant TYW4/PPM1 might be a bifunctional protein responsible for methylation of the C-terminal carboxyl group of PP2A protein and methylation of yW intermediates. The topological orientation of TYW3-4C-2 in plant prompted us to speculate that vW synthesis proceeds through consecutive sequential reactions in a multienzyme complex. If TYW4-CTD is responsible for recognition of tRNA^{Phe}, plant TYW4 might coassemble with the tRNA^{Phe}-TYW3-4C-2 complex (Figure 7). In S. cerevisiae or in mammals, TYW2, TYW3 and TYW4 might assemble with tRNA^{Phe} in an analogous manner to carry out the multistep reactions in yW synthesis (Figure 7). Since TYW proteins were not identified as interacting partners of other TYW proteins in the S. cerevisiae protein-protein network database (Supplementary Table S1), it is possible that tRNA^{Phe} serves as a scaffold to form this multienzymatic complex. Other unidentified proteins involved in yW synthesis might be found in the tRNA^{Phe}-dependent complex. In general, assembly of several proteins responsible for multistep biosynthesis with a substrate enables sequential reactions to proceed more smoothly, due to the close proximity of enzymes and substrates within the complex.

The predicted biosynthetic pathway of yW is depicted in Figure 6. First, the N^1 -methyltransferase TRM5 methylates G37 of tRNA^{Phe} to convert it to m¹G37. Second, m¹G37 serves as a substrate for the Fe–S cluster protein TYW1, to form the tricyclic core of yW, using FMN as a cofactor. Next, TYW2 transfers α -amino- α -carboxypropyl group from Ado-Met to the lateral side chain at the C-7 position of the yW-187 to form yW-86. TYW3 catalyzes N-4 methylation of yW-86 to yield yW-72 in Ado-Met-dependent manner. Finally, TYW4,



Figure 7 Schematic depiction of a multienzymatic complex of TYW proteins. In *S. cerevisiae* and in mammals, TYW2, 3 and 4 might coassemble with tRNA^{Phe} to carry out the sequential reactions of yW synthesis. In plants, a large fusion protein of TYW3-4C-2 and TYW4 might coassemble with tRNA^{Phe}.

an Ado-Met-dependent carboxymethyltransferase, methylates the α -carboxy group of yW-72 to form yW-58. Presumably, second function of TYW4 or involvement of unidentified factor(s) should be responsible for methoxycarbonylation of the α -amino group of the side chain of yW-58 to form yW. This multistep enzymatic formation from yW-72 to yW is triggered by TYW4 methylation in a tRNA^{Phe}-dependent protein complex.

Localization of many yeast ORFs has been investigated (Kumar et al, 2002; Huh et al, 2003; Sickmann et al, 2003) and the localization of each TYW protein is listed in Supplementary Table S1. TRM5 localizes to the nucleus and cytoplasm (Huh et al, 2003) and the initial step of yW synthesis could occur in the nucleus. However, other tRNAprocessing enzymes localize to the mitochondrial outer membrane (Yoshihisa et al, 2003). More recently, active shuttling of tRNA between the nucleus and cytosol in yeast has been reported (Takano et al, 2005). Thus, localization of the first step of yW synthesis remains an open question. TYW1 localizes on the endoplasmic reticulum (ER). TYW2 localizes to the cytoplasm, ER and nuclear envelope, and TYW4 localizes to the cytoplasm and mitochondria. In addition, the predicted localization of the A. thaliana TYW3-4C-2 is cytoplasmic (http://www.arabidopsis.org/). These data suggest that the subsequent step of yW synthesis takes place mainly in the cytoplasm. Post-transcriptional modifications serve as signals that control the subcellular localization of eukaryotic RNA molecules (Kaneko et al, 2003; Suzuki, 2005). In future studies, biosynthesis of yW should be investigated in association with subcellular localization of a precursor tRNA^{Phe} having vW intermediates with different chemical structures.

Materials and methods

Strains and media

S. cerevisiae wild-type strain and deletion strains were obtained from EUROSCARF: the BY4742 ($Mat\alpha$; $his3\Delta1$; $leu2\Delta0$; $lys2\Delta0$; $ura3\Delta0$) series and strains Y14418, Y10571, Y16650 and Y11085 (BY4742, YGL050w::kanmx4, YML005w::kanmx4, YOL141w:: kanmx4, YPL207w::kanmx4). For recombinant protein production, the S. cerevisiae strain BY4741 (Mat a; $his3\Delta1$; $leu2\Delta0$; $met15\Delta0$; $ura3\Delta0$) overexpressing hexahistidine-tagged YML005w (YSC38699514898), YGL050w (YSC3869-9514409) and YOL141w (YSC3869-9515336) were obtained from Open Biosystems. The BG1805derived plasmid containing the YPL207w (named pTYW1) was obtained from Open Biosystems. Strains were grown in rich medium (YPD, 2% peptone, 1% yeast nitrogen base without amino acids and 2% glucose) or in synthetic complete medium (SC, 0.67% yeast nitrogen base without amino acids, 0.5% casamino acid and 2% glucose) supplemented by auxotrophic nutrients as specified. YN101 was cultured as described (Nakai *et al*, 2004).

Ribonucleome analysis

Parallel preparation of total RNAs from yeast strains. Yeast strains were grown in 5 ml of YPD in a 24-well format deep well plates at 30°C for 36 h and cells were harvested during log phase growth (OD₆₆₀ \approx 1.5–2.0). Cell pellets were resuspended in 500 µl of lysis buffer (20 mM Tris–HCl (pH 7.5), 10 mM MgCl₂), and extracted with neutral pH phenol/chloroform (500/100 µl), which was added to the lysate and shaken for 3 h at room temperature. RNA was then recovered by ethanol precipitation from the aqueous phase. The total RNA samples were dissolved in 100 µl of ddH₂O and stored at -20° C.

Mass spectrometry. To analyze RNA nucleosides, total RNA (20 µg) obtained from each strain was digested to nucleosides with nuclease P1(Yamasa) and bacterial alkaline phosphatase derived from Escherichia coli strain C75 (BAP.C75, Takara) for 3 h at 37°C, and analyzed by LC/MS using ion-trap MS as described previously (Kaneko et al, 2003) with slight modifications. Nucleosides were separated by an ODS reverse-phase column (Intertsil ODS3 $5\,\mu m$, 2.1×250 mm, GL Science) using an HP1100 liquid chromatography system (Agilent). The solvent consisted of 0.1% acetonitrile in 5 mM NH₄OAc (pH 5.3) (Solvent A) and 60% acetonitrile in H₂O (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, 99-1% B in 50-50.1 min and 1% B in 50.1-60 min. The chromatographic effluent was directly conducted to the ESI source to ionize the separated nucleosides, which were analyzed on an LCQ DUO ion-trap mass spectrometer (ThermoElectron). 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 100-900

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T₁ mapping and CID

RNA fragments produced by RNase T₁ digestion were analyzed by LC/MS using ion-trap MS according to previously described methods (Kaneko et al, 2003; Soma et al, 2003) with slight modifications. Purified $tRNA^{Phe}$ (2.5 µg) was subjected to digestion by RNase T₁ (Seikagaku corporation; 0.6 U) for 30 min at 37°C in a buffer of 10 mM NH₄OAc (pH 5.3). The hydrolysates were analyzed by LC/MS using a ODS column (Xterra MS C18 $2.5\,\mu m,$ 2.1×50 mm, Waters). The solvent consisted of 0.4 M 1,1,1,3,3,3hexafluoro-2-propanol (pH 7.0, adjusted with triethylamine) in H₂O (Solvent A) and 50% methanol (Solvent B) in the following gradients: 5% B in 0-5 min, 5-95% B in 5-35 min, 95% B in 35-40 min, 95–5% B in 40–42 min and 5% B in 42–60 min. Negative ions were scanned over an m/z range of 620–2000 throughout the separation. LC/MS/MS analysis for the yW-base and its derivatives was performed by ion-trap mass spectrometry using the LCQ DUO. The collision energy was 25-30%. Product ions were scanned over an *m*/*z* range of 100–900.

In vitro reconstitution of yW synthesis using recombinant proteins

Reaction mixtures of 10µl containing 50 mM Tris-HCl (pH 8.0), 0.5 mM DTT, 10 mM MgCl₂, 1 mM spermidine, 2µg of tRNA^{Phe} having yW intermediate obtained from each deletion strain, and 1.4µM recombinant TYW2, TYW3 or TYW4 protein, with or without 0.5 mM Ado-Met, were incubated 1 h at 30°C. After incubation, the reaction was stopped by adding 0.5 M Tris-HCl (pH 8.0) and phenol. The tRNA^{Phe} was extracted and precipitated by ethanol and then subjected to RNase T₁ digestion. The RNA fragment analysis by LC/MS was carried out as described above.

Supplementary data

Supplementary data are available at The EMBO Journal Online.

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