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Expanding Role of the Jumonji C Domain as an RNA Hydroxylase*[S]

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JmjC (Jumonji C) domain-containing proteins are known to be an extensive family of Fe(II)/2-oxoglutarate-dependent oxygenases involved in epigenetic regulation of gene expression by catalyzing oxidative demethylation of methylated histones. We report here that a human JmjC protein named Tyw5p (*TYW5*) unexpectedly acts in the biosynthesis of a hypermodified nucleoside, hydroxywybutosine, in tRNA^{Phe} by catalyzing hydroxylation. The finding provides an insight into the expanding role of JmjC protein as an RNA hydroxylase.

The ferrous iron (Fe^{2+})- and 2-oxoglutarate (2-OG)[2]-dependent oxygenases are a superfamily of enzymes that catalyze a wide range of reactions, including hydroxylation, demethylation, and oxidative ring closures for a variety of substrates, including metabolites, nucleic acids, and proteins (1). Recent studies have revealed emerging roles of this family of oxygenases involved in histone demethylation, DNA demethylation, protein stabilization, hypoxia sensing, and fatty acid metabolism. Jumonji C (JmjC) domain-containing proteins, an extensive family of Fe(II)/2-OG-dependent oxygenases, play a key role in epigenetic control of gene expression by catalyzing oxidative demethylation of a series of methylated histones, including histone H3 at Arg-2, Lys-4, Lys-9, Lys-27, and Lys-36, and histone H4 at Arg-3 (2–4). In addition, the JmjC protein FIH-1 hydroxylates an Asp residue in HIF-1α to regulate hypoxic responses (5). JmjC is an evolutionarily conserved domain widely found in proteins from bacteria, fungi, plants, and animals. There are still many JmjC proteins whose functions remain elusive.

Many post-transcriptional modifications required for accurately deciphering the genetic code are found in tRNAs (6). Wybutosine (yW) and hydroxywybutosine (OHyW) (Fig. 1*A*) are hypermodified guanosines found at position 37 of the phenylalanine tRNA (tRNA^{Phe}) in yeast and mammals, respectively (Fig. 1*B*) (7, 8). yW plays a critical role in maintaining the reading frame by stabilizing codon-anticodon pairing (9). The yW synthesis is initiated from N^1-methylation of G37 (m^1G) formation catalyzed by Trm5p (Fig. 1*C*). Further steps of yW synthesis are catalyzed by four enzymes Tyw1p, Tyw2p, Tyw3p, and Tyw4p, which we and other groups identified in yeast (Fig. 1*C*) (10–12). The multistep enzymatic formation of yW from yW-187 can be reconstituted *in vitro* using recombinant Tyw2p, Tyw3p, and Tyw4p (10). In the last step of yW formation, Tyw4p catalyzes both methylation and methoxycarbonylation of the bulky side chain of yW at a single catalytic site, and in the latter reaction, the methoxycarbonyl group is formed through the fixation of carbon dioxide (13). In mammalian tRNA^{Phe}, the β-carbon of the side chain in yW is further hydroxylated to form OhyW (Fig. 1*A*). The functional role and biogenesis of this additional modification have never been studied.

EXPERIMENTAL PROCEDURES

Strains, Media and Plasmid—The following *Saccharomyces cerevisiae* wild-type strain and deletion strains were obtained from EUROSCARF: the BY4742 (*Matα;his3Δ1;leu2Δ0;lys2Δ0; ura3Δ0*) series and strains Δ*TYW3* and Δ*TYW4* (BY4742, *YGL050w::kanmx4* and *YOL141w::kanmx4*). *Aspergillus oryzae* RIB40 and mouse Ehrlich ascites cells were obtained from RIKEN BRC. Yeast strains and *A. oryzae* were grown in rich medium YPD (2% peptone, 1% yeast extract, and 2% glucose). DMEM, 10% FBS medium was used to culture mouse NIH3T3, Ehrlich ascites cells, and HeLa cells. A full-length cDNA of human *TYW5* was amplified by RT-PCR from human total RNA using a primer (5′-gaatccacacaaacacccc-3′) for the first cDNA synthesis and a set of primers for PCR, 5′-ccccgcggatccatggccgggcagcacct-3′ and 5′-atagtttagcggccgcttactcagagttcttgctgt-3′. The amplified cDNA was then cloned into the BamHI/NotI site of the pYES2/CT plasmid (Invitrogen) to generate phTYW5. The phTYW5 plasmid was then introduced into the wild-type and Δ*TYW4* strains of yeast. For protein expression, the h*TYW5* gene was cloned into the NdeI/BamHI site of a modified pET28a vector (Novagen) containing the PreScission protease site (GE Healthcare) instead of the thrombin site.

RNA Mass Spectrometry—To analyze RNA nucleosides, total RNA (20 μg) from the yeast strains, *A. oryzae*, or human tissue culture cells was digested to nucleosides and analyzed by LC/MS using ion trap mass spectrometry as described previously (14, 15). To analyze RNA fragments of tRNA^{Phe} digested

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

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[2] The abbreviations used are: 2-OG, 2-oxoglutarate; JmjC, Jumonji C; OhyW, hydroxywybutosine; yW, wybutosine.

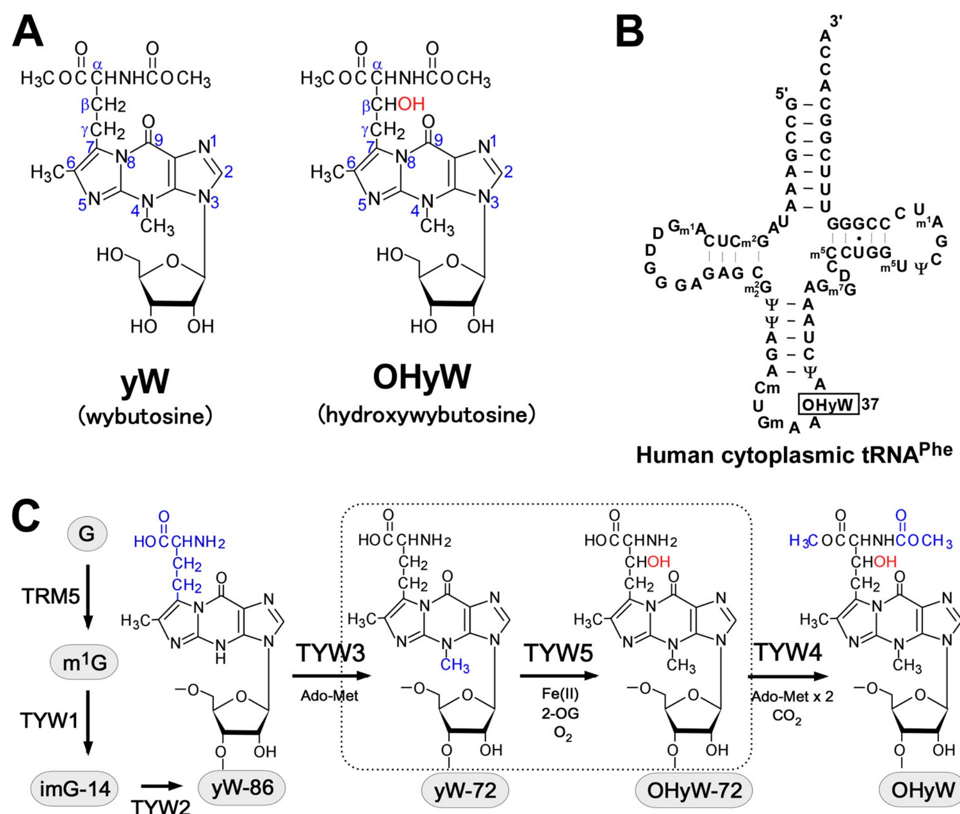

FIGURE 1. **Chemical structure of OHyW in tRNA^{Phe} and its biosynthetic pathway.** *A*, chemical structures of yW (*left*) and OHyW (*right*). The exact name of OHyW is α-(carboxyamino)-β-hydroxy-4,9-dihydro-4,6-dimethyl-9-oxo-1*H*-imidazo [1,2-a]purine-7-butyric acid dimethyl ester. Atoms in the tricyclic ring and side chain are numbered in *blue*. *B*, secondary structure of human cytoplasmic tRNA^{Phe} with modified nucleosides as follows: hydroxywybutosine (*OHyW*), 2′-*O*-methylguanosine (*Gm*), 2′-*O*-methylcytidine (*Cm*), pseudouridine (Ψ), 5-methylcytidine (m^5C), 7-methylguanosine (m^7G), 2-methylguanosine (m^2G), N^2,N^2-dimethylguanosine ($m^2{}_2G$), dihydrouridine (*D*), 1-methyladenosine (m^1A), and 5-methyluridine (m^5U). Peroxywybutosine (*o2yW*), which has been identified in mammalian tRNA^{Phe} (22, 23), was not present in our preparation, similar to a previous report (8). *C*, biosynthetic pathway of OHyW. Chemical structures of intermediates from yW-86 to OHyW are illustrated. Additional groups at each step are shown in *blue*. The hydroxyl group at β-carbon is shown in *red*. *TYW5* hydroxylates yW-72 to produce OHyW-72 utilizing 2-OG, O_2 and Fe(II) as substrates.

by RNase A, a capillary LC coupled to nanoESI-MS was used as described previously (14, 16, 17).

RNA Interference—The short hairpin RNAs (shRNAs) targeting h*TYW5* and GFP, as a control, were designed and prepared as described previously (18). Sense strands of the shRNAs were 5′-gcagcugaagagaaacaua-3′ for h*TYW5* and 5′-ggcacaagcuggaguacaa-3′ for GFP. The shRNAs were introduced into HeLa cells using Lipofectamine RNAiMAX (Invitrogen) as described previously (19). Five days after transfection, the cells were harvested, and RNAs were extracted using TRIzol (Invitrogen). The steady-state level of h*TYW5* mRNA was then measured by real time RT-PCR using a set of primers, 5′-gaggaacagttcttttccagtg-3′ and 5′-actgggcatctcgaggactg-3′, showing that the level of h*TYW5* mRNA was decreased to 33% that of the control cell.

Isolation and Purification of Individual tRNAs from Yeast and HeLa Cells—To isolate individual tRNA^{Phe}, we used reciprocal circulating chromatography (20). The 3′-biotinylated DNA probe complementary to yeast and human cytoplasmic tRNA^{Phe} used in this study was 5′-tgcgaattctgtggatcgaacacaggacct-3′ for yeast tRNA^{Phe} and 5′-gccgaaatagctcagttgggagagcgttagactga-3′ for human tRNA^{Phe}.

Expression and Purification of Human TYW5 Proteins—The recombinant h*TYW5* protein was overexpressed in *E. coli* C41(DE3) Rosetta cells (OverExpress). *E. coli* cells were grown to an A_{600} = 0.8, and hTyw5p expression was then induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside, followed by culture for 18 h at 20 °C. Cells were harvested by centrifugation, resuspended in buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM imidazole, 1 mM PMSF, and 5 mM 2-mercaptoethanol), sonicated, and then centrifuged. The recombinant protein was purified by column chromatography on a nickel-nitrilotriacetic acid Superflow column (Qiagen) and dialyzed against 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM DTT.

In Vitro Reconstitution of OHyW with hTyw5p and Tyw4p—For *in vitro* reconstitution of OHyW-72 from yW-72, reaction mixture (10 μl) contained 40 mM Tris-HCl (pH 7.6), 10 mM KCl, 1 mM DTT, 3 mM $MgCl_2$, 4 mM ascorbic acid, 1.5 mM Fe(II)SO_4, 30 pmol of tRNA^{Phe} with yW-72 isolated from Δ*TYW4* or tRNA^{Phe} with yW isolated from wild-type strain, and 2.8 μM recombinant hTyw5p in the presence or absence of 4 mM 2-oxoglutarate. The reactions were incubated for 1 h at 37 °C. The tRNA^{Phe} was recovered using ISOGEN® (Wako Pure Chemical Industries, Ltd.), precipitated with ethanol, and then subjected to RNase A digestion. RNA fragment analysis by capillary LC nanoESI/MS was conducted. For *in vitro* reconstitution of OHyW from OHyW-72, the reaction mixture (50 μl) contained 50 mM Tris-HCl (pH 8.0), 0.5 mM DTT, 10 mM $MgCl_2$, 1 mM spermidine, 0.5 mM *S*-adenosylmethionine, 30 pmol of tRNA^{Phe} having OHyW-72, and 1.3 μM recombinant Tyw4p (10) was incubated 1 h at 30 °C. The tRNA^{Phe} was recovered as described above and was then digested into nucleosides with nuclease P1, bacterial alkaline phosphatase, and PDase1 (Worthington) for 3 h at 37 °C.

RESULTS

Identification of TYW5 Responsible for OHyW Formation—By searching for *TYW4* homologs among eukaryotes, we found that *TYW4* homologs from several species of fungus, including *A. oryzae*, are fused to a JmjC domain (named *TYW5*) at its C terminus (Fig. 2, *A* and *B*). Because the JmjC domain demethylates the methylated histones by a hydroxylation reaction, we hypothesized that this additional domain catalyzes hydroxylation of yW to form OHyW. In fact, OHyW was detected in *A.*

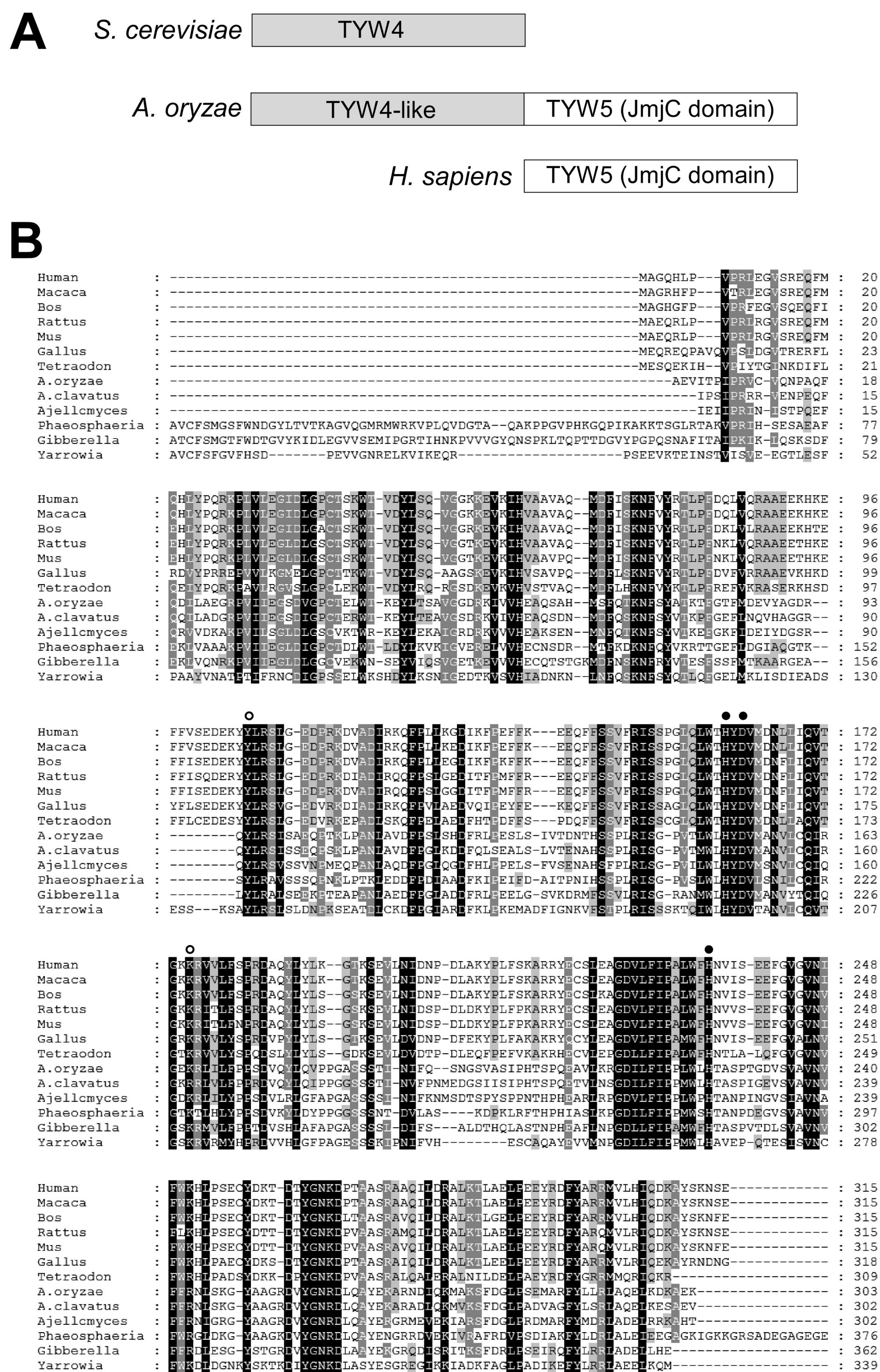

FIGURE 2. **Domain structure and sequence alignment of *TYW5* proteins.** *A,* domain structures of the *S. cerevisiae TYW4*, *A. oryzae TYW4-5*, and *Homo sapiens TYW5*(h*TYW5*) proteins. The *A. oryzae* TYW4-5 protein is a fusion protein composed of TYW4 and TYW5. *B,* sequence alignment of *TYW5* proteins from vertebrates and the C-terminal JmjC domain of TYW4-5 from fungi. In human TYW5, Tyr-106 and Lys-175 (*open circles*) are required for 2-OG binding. His-160, Asp-162, and His-235 (*closed circles*) are residues in the H*X*(D/E)X_nH motif that are required for Fe(II) binding. Accession numbers are NP_001034782 (human), XP_001090415 (*Macaca mulatta*), XP_587720 (*Bos taurus*), XP_217397 (*Rattus norvegicus*), NP_001032831 (*Mus musculus*), XP_421921 (*Gallus gallus*), CAG11227 (*Tetraodon nigroviridis*), XP_001824014 (*A. oryzae*), XP_001274463 (*Aspergillus clavatus*), XP_001543786 (*Ajellomyces capsulatus*), XP_001804235 (*Phaeosphaeria nodorum*), XP_384474 (*Gibberella zeae*), and XP_504718 (*Yarrowia lipolytica*).

oryzae tRNAs (supplemental Fig. 1). Intriguingly, in vertebrates, including humans, this JmjC domain is found as an independent protein (named h*TYW5*) and is not fused to a human homolog of *TYW4* (h*TYW4*) (Fig. 2, *A* and *B*). In human *TYW5*, Tyr-106 and Lys-175 are required for 2-OG binding. His-160, Asp-162, and His-235 are residues in the H*X*(D/E)X_nH motif that are required for Fe(II) binding (Fig. 2*B*). A similar approach to predict an enzyme for hydroxylation of OHyW *in silico* has been reported independently (21).

To demonstrate that h*TYW5* encodes the hydroxylase that specifically generates OHyW, we knocked down h*TYW5* in HeLa cells by RNAi. Mass spectrometric (LC/MS) analysis revealed that the OHyW peak decreased, and the yW peak appeared in the cell treated with an shRNA targeting h*TYW5* (Fig. 3). These data clearly showed h*TYW5* to be a hydroxylase mediating formation of OHyW.

Formation of OHyW in Yeast Cells by Introduction of hTYW5—We then introduced a plasmid encoding h*TYW5* into a wild-type (WT) strain of *S. cerevisiae* and found that a small amount (~10%) of OHyW was formed in the cells (Fig. 4*A* and supplemental Fig. 2*A*). Next, h*TYW5* was expressed in a yeast deletion strain of *TYW4* (Δ*TYW4*) in which yW-72 (hypomodified yW) is formed (Fig. 4*B*). Approximately 60% of yW-72 was converted to OHyW-72 (Fig. 4*B* and supplemental Fig. 2*B*). These data suggested that h*TYW5* preferentially hydroxylates yW-72 rather than the fully modified yW. Introduction of h*TYW5* in Δ*TYW3* cells did not result in the hydroxylation of yW-86 (Fig. 4*C*), suggesting that h*TYW5* needs a methyl group at the N^4 position of the tricyclic base (Fig. 1*A*) for hydroxylation.

In Vitro Reconstitution of OHyW—Using recombinant hTyw5p, we performed an *in vitro* hydroxylation of tRNA. The $tRNA^{Phe}$ isolated from WT, Δ*TYW4*, and Δ*TYW3* strains was used as substrate. In the

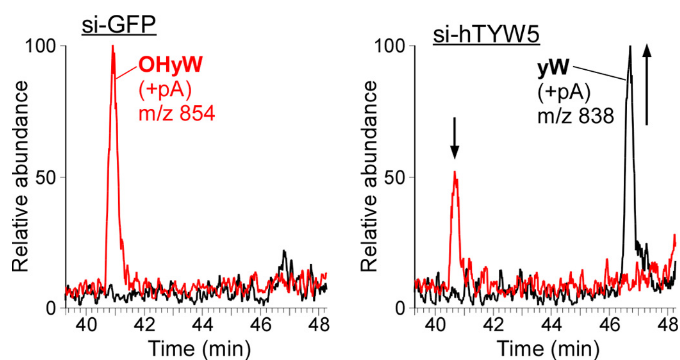

FIGURE 3. **Knockdown of human *TYW5* decreased OHyW.** LC/MS nucleoside analysis of RNA from cells treated with shRNAs targeting for GFP (*si-GFP*, *left*) or h*TYW5* (si-hTYW5, *right*). Mass chromatograms traced with singly charged positive ions for yW(+pA) (*m/z* 838) and OHyW(+pA) (*m/z* 854) are shown by *black* and *red lines*, respectively.

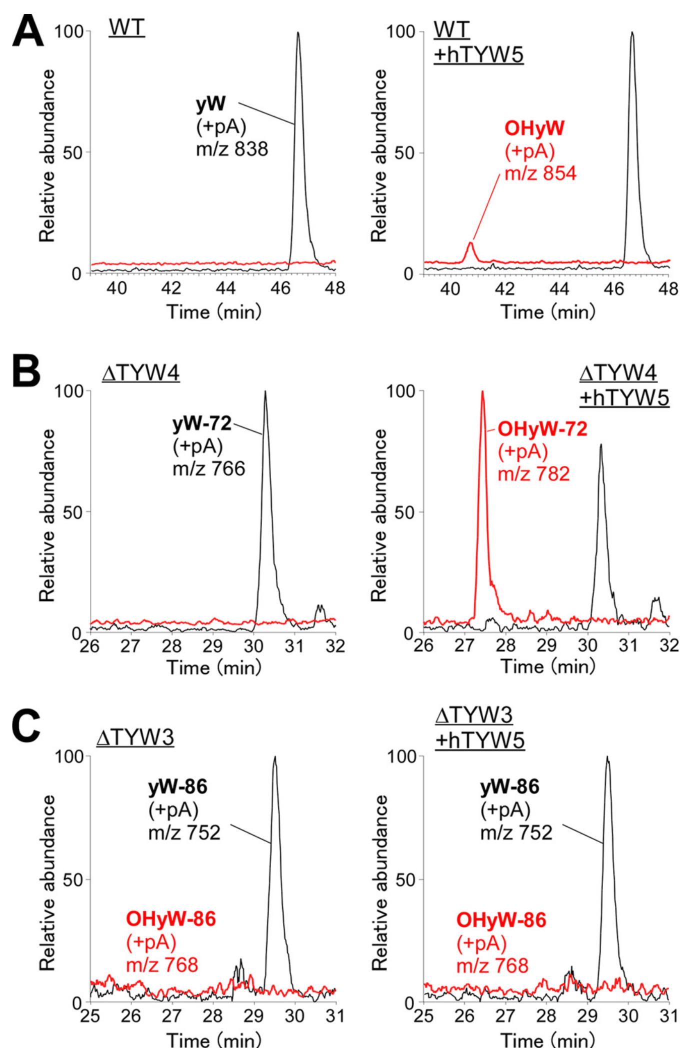

FIGURE 4. **Formation of OHyW in yeast cells by introduction of h*TYW5*.** LC/MS nucleoside analysis is shown of total RNA of yeast strains (*left panels*) and h*TYW5*-introduced strains (*right panels*). The plasmid phTYW5 was introduced into the yeast wild-type strain (*A*), Δ*TYW4* strain (*B*), and Δ*TYW3* strain (*C*).

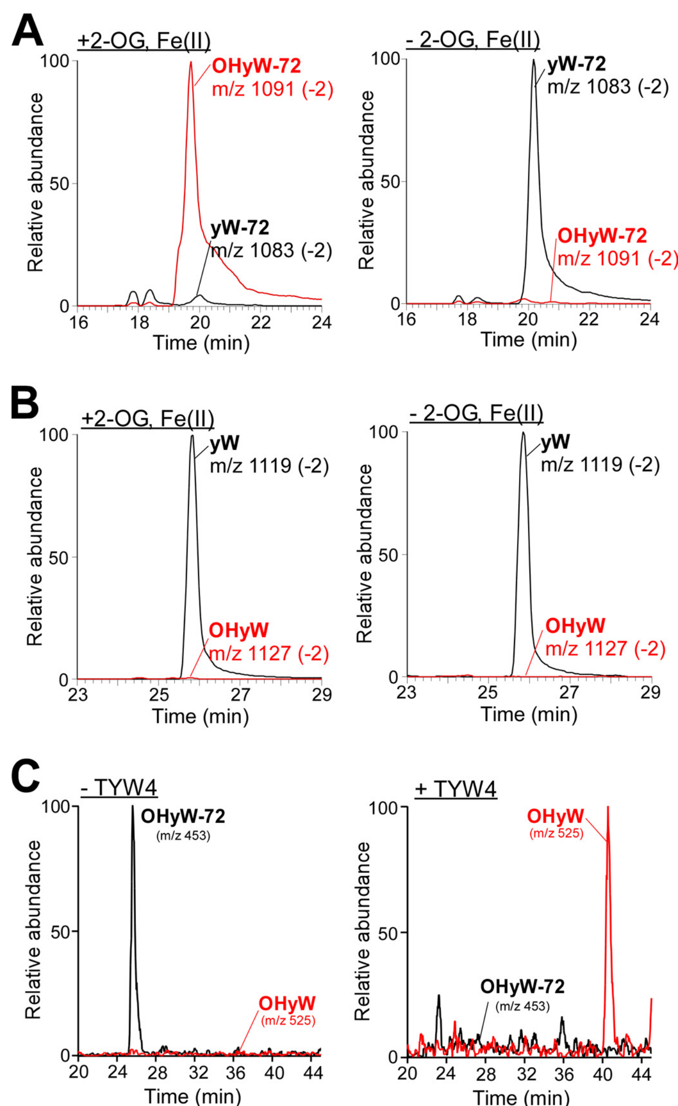

FIGURE 5. ***In vitro* reconstitution of OHyW-72 by the recombinant hTyw5p.** LC/MS analysis of the anticodon-containing fragments (GmAA*OHyW-72*AΨp) of tRNA^{Phe} from Δ*TYW4* (*A*) and WT (*B*) after incubation with the recombinant hTyw5p in the presence (*left panels*) or absence (*right panels*) of 2-OG and Fe(II). Mass chromatograms traced with doubly charged negative ions for yW-72 (*m/z* 1,083), OHyW-72(*m/z* 1,091), yW(*m/z* 1,119), and OHyW(*m/z* 1,127) are shown in *black* or *red lines*. *C, in vitro* reconstitution of OHyW. LC/MS nucleoside analysis of tRNA^{Phe} from Δ*TYW4* treated with the hTyw5p in the presence 2-OG and Fe(II) and then treated with (*right panel*) or without (*left panel*) the recombinant Tyw4p in the presence of *S*-adenosylmethionine and carbonate. Mass chromatograms traced with singly charged positive ions for OHyW-72 (*m/z* 453) and OHyW (*m/z* 525) are shown by *black* and *red lines*, respectively.

presence of Fe(II) and 2-OG, yW-72 in tRNA^{Phe} was efficiently hydroxylated to form OHyW-72 by hTyw5p (Fig. 5*A*), and yW in tRNA^{Phe} was not hydroxylated (Fig. 5*B*). The data are consistent with the observation of OHyW formation in yeast cells by introduction of h*TYW5* (Fig. 4*A*). After hydroxylation, tRNA^{Phe} bearing OHyW-72 was further incubated with yeast Tyw4p in the presence of *S*-adenosylmethionine and carbonate (10, 13). As shown in Fig. 5*C*, OHyW was efficiently reconstituted. To confirm whether OHyW reconstituted *in vitro* was identical to that isolated from HeLa cells, total nucleosides of the reconstituted tRNA^{Phe} were subjected to LC/MS/MS analysis to observe product ions of the base-related ion (BH_2^+) of OHyW produced by collision-induced dissociation. We observed similar profiles of product ions from both collision-induced dissociation spectra (supplemental Fig. 3), demonstrating that OHyW was reconstituted from yW-72 through

two consecutive reactions catalyzed by hTyw5p and Tyw4p *in vitro*. Loss of the OH group, presumably derived from OH of β-carbon, is a characteristic dissociation of OHyW (supplemental Fig. 3). Collectively, in the biogenesis of OHyW, hTyw5p employs yW-72 in $tRNA^{Phe}$ as a substrate by recognizing the N^4-methyl group to hydroxylate the β-carbon of the propyl group, followed by methylation and methoxycarbonylation of the side chain catalyzed by Tyw4p for completion.

DISCUSSION

Here, we showed evidence that hTyw5p is a *bona fide* hydroxylase for OHyW formation in $tRNA^{Phe}$. This is the first study of JmjC domain-containing protein being involved in an RNA modification. This finding revised our knowledge of the JmjC domain as a protein hydroxylase. JmjC domain seems to be a versatile module for hydroxylation of various molecules in the cell. Other instances of hydroxylation mediated by the JmjC domain might be discovered in the future.

It was an intriguing observation that *TYW5* is fused to *TYW4* in fungi. Considering that the last two reactions of OHyW formation are coupled, fusion of enzymes that are involved in the same pathway would bring certain advantages to facilitate the biogenesis. We previously reported a similar case of fusion protein in plants, which consists of *TYW2*, *TYW3,* and C-terminal domain of *TYW4* (10).

In vitro reconstitution of OHyW revealed a strict substrate specificity of Tyw5p that employs yW-72 as a substrate but does not recognize yW-86 nor yW. Structural studies of Tyw5p complexed with $tRNA^{Phe}$ bearing yW-72 will be necessary to clarify the molecular basis of the substrate specificity.

Biological function of OHyW remains elusive. When we knocked down h*TYW5* by siRNA, no significant phenotype was observed (data not shown). In addition, OHyW is a partial modification of yW (Fig. 3 and supplemental Fig. 1). It is difficult for us to consider the functional importance of this modification, although there still might be its modulatory function to maintain the reading frame during decoding. Because Tyw5p requires dissolved oxygen as a substrate for hydroxylation of tRNA, Tyw5p might sense concentration of dissolved oxygen so that the relative fraction of OHyW might be variable due to partial pressure of oxygen in the cell. Otherwise, the hydroxyl group of OHyW confers water solubility of yW, facilitating excretion of the modified base as a metabolic product of tRNAs from our bodies. To investigate the functional importance of OHyW, it will be worth trying to construct *TYW5*-null animal or plant, and its behavioral analysis would be the most informative experiment.

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