Supplemental Information

Purification of tRNA from yeast

Yeast strains were grown in 2 L of YPD medium and harvested during log phase growth (OD_{660}≈1.5-2.0). Total RNA was extracted as described (Holley, 1963). About 700 A_{260} units of total RNA was obtained from each culture. To isolate individual tRNAs with the highest efficiency, we devised and successfully improved an original solid-phase DNA probe method, which was named 'chaplet' column chromatography (Kaneko et al., 2003; Suzuki, 2005). A 3'-biotinylated DNA probe, 5'-tgcgaattctgtggatcgaacacaggacct-3', complementary to the yeast $tRNA^{Phe}$ was immobilized on avidin Sepharose (Amersham-Pharmacia) packed in a small column with a 200 μL bed volume. The crude total tRNAs dissolved in a binding buffer (1.2 M NaCl, 30 mM HEPES-KOH (pH 7.5), 15 mM EDTA) were circulated through the column by a peristaltic pump at a temperature of 70 °C to entrap the target tRNA. After washing out non-specific tRNAs with a wash buffer [0.6 M NaCl, 15 mM HEPES-KOH (pH 7.5), 7.5 mM EDTA], $tRNA^{Phe}$ was eluted from the column with a low-salt buffer [20 mM NaCl, 0.5 mM HEPES-KOH (pH 7.5), 0.25 mM EDTA] at 70 °C.

Expression and purification of the recombinant proteins from *S. cerevisiae* cells

The hexahistidine-tagged YML005w (TYW2), YGL050w (TYW3) and YOL141w (TYW4) proteins were expressed in *S. cerevisiae* strain YSC3869-9514898, YSC3869-9515336 and YSC3869-9514409 (Open Biosystems), respectively. Single colonies were picked from an SD-uracil selection plate and cells were grown at 30 °C in 300 mL of YPD. The recombinant protein was induced by adding galactose for 20 hours. Cells were harvested and washed with ddH_2O once, and then resuspended in 4 mL of lysis buffer [20mM HEPES-KOH (pH 7.6), 10 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail (Roche)] and disrupted with glass beads (SIGMA). The cell lysate was cleared by centrifugation at 4 °C, 100,000 g, for 45 min and the hexahistidine-tagged protein was purified by AKTA purifier Chromatography system using Hi-Trap chelating HP column (Amersham Biosciences). The column was washed with ddH_2O once and then 10ml of 0.1M $NiSO_4$ was loaded on followed by washing with ddH_2O. The column was equilibrated with the wash buffer [50 mM HEPES-KOH (pH 7.6), 1 M NH_4Cl, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol and protease inhibitor cocktail], and then 25ml of cell lysate was loaded on the column and washed with 25ml of wash buffer. Elution was performed with a -50ml linear-gradient of imidazole(50-450mM) in wash buffer. Eluted fractions were analyzed

by SDS-PAGE. The fractions containing the recombinant protein were pooled and dialyzed against buffer containing 20 mM Tris-HCl (pH 7.6), 4 mM $MgCl_2$, 2 mM EDTA, 55 mM NaCl and 1 mM DTT. The YML005w (TYW2) protein and YGL050w (TYW3) protein were stored in 30% glycerol at -80 °C. The YOL141w (TYW4) protein was further purified by AKTA purifier Chromatography system using Mono Q 5/50 column (Amersham Biosciences). The column was washed with ddH2O once and equilibrated with buffer A [50mM Tris-HCl(pH 7.6), 5mM MgCl2, 50mM KCl, 1mM EDTA, 1mM DTT, 5% glycerol]. Dialyzed elution fraction includes TYW4 protein was loaded on the column and washed 25ml of buffer A(wash fraction). Elution was performed with a -25ml linear-gradient of KCl (0-400mM) in buffer A. Eluted fractions were analyzed by SDS-PAGE. The wash fraction contained the recombinant protein. So the fraction were pooled and concentrated with Amicon Ultra-15 (30,000MWCO) (Millipore). YOL141w (TYW4) protein was stored in 30% glycerol at -80 °C.

Site-directed mutagenesis of TYW1 and complementation test

Site-directed mutagenesis of TYW1 was carried out on plasmid pTYW1 by QuikChange™ Site-Directed Mutagenesis (Stratagene) according to the manufacturer's instruction. Introduced mutations were confirmed by DNA sequencing. The pairs of oligonucleotides were used to create each mutantion; 5'gactccttctttggcagcttcatccaaatgcgttt3' and 5'aaacgcatttggatgaagctgccaaagaaggagtc3' for C479A, 5'ggcatgttcatccaaagccgttttctgttggaggc3' and 5'gcctccaacagaaaacggctttggatgaacatgcc3' for C483A, 5'atccaaatgcgttttcgcttggaggcatggtacaa3' and 5'ttgtaccatgcctccaagcgaaaacgcatttggat3' for C486A, 5'gcccggtgttattgcagcaagatttgcgaaagcgt3' and 5'acgctttcgcaaatcttgctgcaataacaccgggc3' for E532A and 5'cttgtctcttgtcggcgcgcctattctttatcctc3' and 5'gaggataaagaataggcgcgccgacaagagacaag3' for E550A. The strain Y11085 (BY4742, *YPL207w::kanmx4*) was transformed by pTYW1 or its mutants. The transformants were cultivated in YPG media for 20 hours. Then total RNA from each strain was extracted, and their modified nucleosides were analyzed by LC/MS as described above.

Figure S1. Mass spectrometric analysis of the nucleosides of purified $tRNA^{Phe}$ from *S. cerevisiae* wild type and mutant cells.

LC/MS analysis of the nucleosides of the purified $tRNAs^{Phe}$ from wild type (WT), Δ*YPL207w*(*TYW1*), Δ*YML005w*(*TYW2*), Δ*YGL050w*(*TYW3*) and Δ*YOL141w*(*TYW4*). The upper panels are the UV traces at 254 nm. The middle and

lower panels are mass chromatograms detecting yWpA (m/z 838) and yW-base (m/z 377), respectively. Arrows indicate the retention time for yWpA.

Figure S2. Mass spectrometric detection of the modification intermediate of yW in the purified tRNA^{Phe} from *S. cerevisiae* wild type and mutant cells.

The graphs show the UV and mass chromatograms expanded in the range of retention time corresponding to m^{1}G in tRNA^{Phe} from Δ*YPL207w*(*TYW1*) (A), yW-187(yWpA-187) in tRNA^{Phe} from Δ*YML005w*(*TYW2*) (B), yW-86 and yW-14(yWpA-14) in tRNA^{Phe} from Δ*YGL050w*(*TYW3*) (C) and yW-72(yWpA-72) in tRNA^{Phe} from Δ*YOL141w*(*TYW4*) (D). Graphs on the left and right represent chromatograms of WT and mutant samples, respectively. The top panel of each chromatograph is the UV trace at 254 nm. Mass chromatograms are shown by singly charged ions of m^{1}G (m/z 298), yWpA-187 (m/z 651), yW-187 (m/z 322), BH_2^+ of yW-187 (m/z 190), yW-86 (m/z 423), BH_2^+ of yW-86 (m/z 291), yWpA-14 (m/z 824), yW-14 (m/z 495) and BH_2^+ of yW-14 (m/z 363), yWpA-72 (m/z 766), yW-72 (m/z 437) and BH_2^+ of yW-72 (m/z 305), from the top to the bottom.

Figure S3. Chemical structures of the intermediates of yW synthesis and their CID spectra.

The base structures and CID spectra by MS/MS experiments are shown for yW (A), imG-14(yW-187) (B), yW-86 (C), yW-72 (D) and yW-14 (E). Assignment of the dissociation pattern is shown in each chemical structure. Loss of the functional groups is indicated in the CID spectra. Loss of CO originating from C-9 in the center ring of the tricyclic base which is boxed by a dotted line.

As shown in the MS/MS spectrum of the yW base from the wild type tRNA^{Phe} (A), the major dissociation process is the elimination of the CH_3OH of the C-7 side chain ($377^+ \rightarrow 345^+$). The product ion m/z 216 is produced by cleavage of the side chain between β and γ carbons. The m/z 204 product corresponds to the net loss of the entire side chain at C-7, which occurs concomitantly with the transfer of hydrogen from the neutral side chain. The m/z 317 fragment is produced by loss of CO from C-9 in the center ring of the tricyclic nucleus.

The spectrum of yW-187 (B) corresponds well with the product ion pattern of imG-14 (Zhou et al., 2004; Zhou et al., 2004) with the expulsion of CO from C-9 in the center ring ($190^+ \rightarrow 162^+$), followed by dissociation of HCN ($162^+ \rightarrow 135^+$), which is characteristic of purines (Zhou et al., 2004).

In the spectrum of yW-86 (C), the ions m/z 202 and m/z 190 could be detected.

This finding showed that yW-86 has a tricyclic base without N-4 methyl group and the loss of 72 Da corresponds to the atoms distal to the β carbon in the C-7 side chain. Methionine or Ado-Met is thought to be the precursor of this side chain in yeast (Munch and Thiebe, 1975), and the chemical structure of yW-86 should be a compound having an α-amino-α-carboxypropyl group at C-7 of the tricyclic base.

In the spectrum of yW-72 (D), the ions m/z 216 and m/z 204, which are the same product ions produced by cleavage of the C-7 side chain of yW, could be detected. OHyW-72 (OHyW minus 72) has been detected in Ehrlich ascites tumor cells (Kuchino et al., 1982) and is an under-modified derivative of OHyW (hydroxywybutosine) which is found in mouse and rat $tRNA^{Phe}$. OHyW-72 has the same side chain structure as proposed for yW-72, with the exception of the OH group at the β carbon. These compounds lack two methyl esters and one carboxyl group of the C-7 side chain in yW. Dissociation of an OH group ($305^{+}\rightarrow288^{+}$) and loss of an amino group ($-NH_2$, $261^{+}\rightarrow244^{+}$), as found in the MS/MS spectrum of yW-72, corresponds well with this structure. Loss of CO from C-9 in the center ring of tricyclic nucleus was also observed ($288^{+}\rightarrow261^{+}$).

The LC/MS/MS analysis of yW-14 (E) produced the ions m/z 202 and m/z 190 which originate from cleavage of the C-7 side chain of yW. This finding showed that yW-14 has a tricyclic base without N-4 methyl group. Dissociation of CH_3OH from the C-7 side chain ($363^{+}\rightarrow331^{+}$) and the loss of CO from C-9 ($331^{+}\rightarrow303^{+}$) were observed.

Figure S4. Growth properties of strains lacking the enzymes responsible for yW synthesis.

Each deletion strain for yW synthesis and wild type strain were serially diluted (1:10 dilutions) starting with 10^5 cells and then spotted onto YPD and SD plates and incubated at 30 °C for 48 h. There are no visible growth differences between WT and mutants.

Table S1. Subcellular localization of TYW proteins and their protein-protein network.

We have tried to find additional proteins involved in yW synthesis by analyzing proteins that interact with TYW1-4. The database for protein complexes in *S. cerevisiae*, available from the SGD (http://www.yeastgenome.org/) or the CYGD (http://mips.gsf.de/genre/proj/yeast) (Gavin et al., 2002; Guldener et al., 2005) include yeast two-hybrid screening and tandem affinity purification of protein complexes

(Drewes and Bouwmeester, 2003; Gavin et al., 2002; Ito et al., 2001; Marcotte et al., 1999). By searching this database, 38 and 13 ORFs were retrieved as interacting proteins for TYW1 and TYW2, respectively. Only one protein (YPL022w) has been reported as a partner of TYW3. No proteins have been reported to complex with TYW4. Of the 52 proteins that interact one of the four TYW proteins, 31 are non-essential proteins and 21 are essential proteins. We have checked the absence of yWpA in total nucleosides from the deletion strains for the 30 non-essential genes (the deletion strain for *YHR090w* was not available), but yWpA was not absent in any of the strains (data not shown). There is still the possibility that some of the 21 essential genes encode proteins involved in yW synthesis and this will be tested in future studies.

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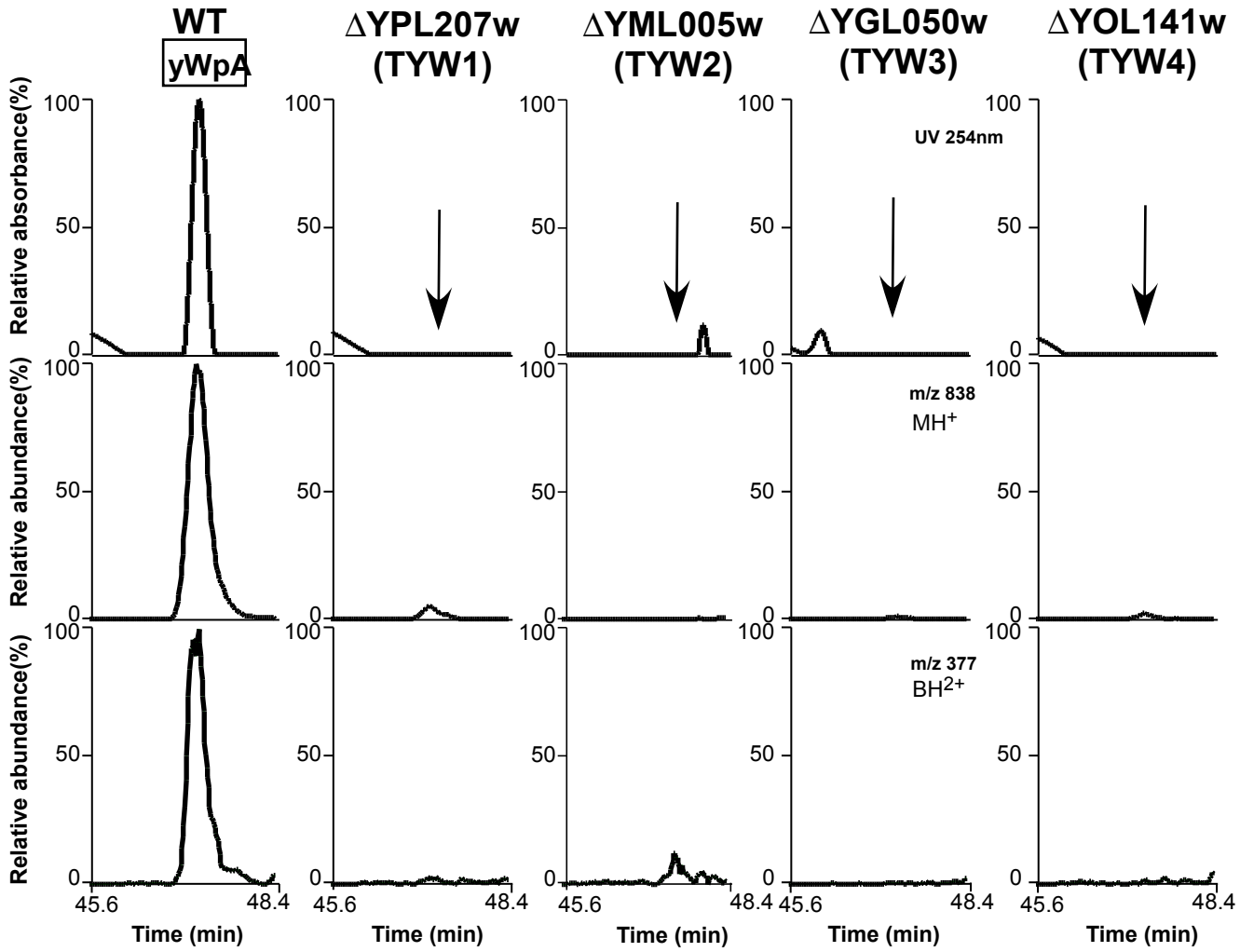
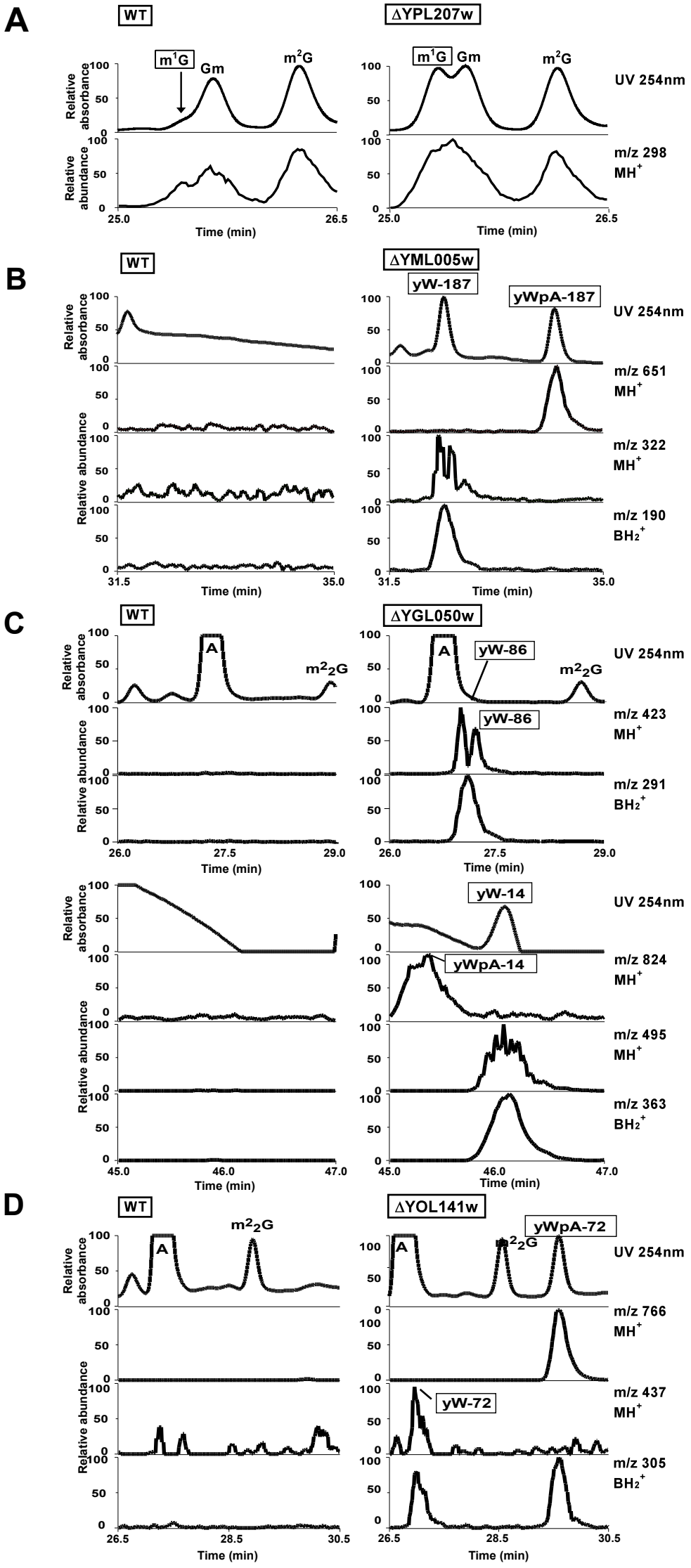
Supplemental Figure S1

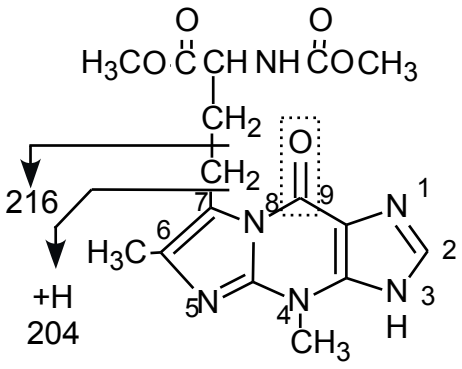


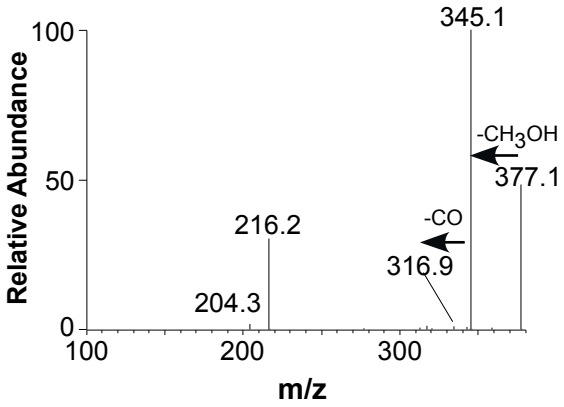
Supplemental Figure S2



Supplemental Figure S3

A yW





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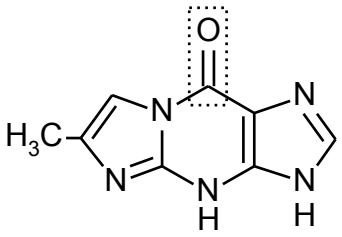

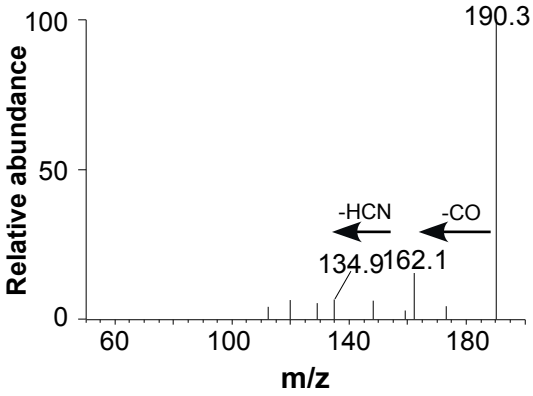

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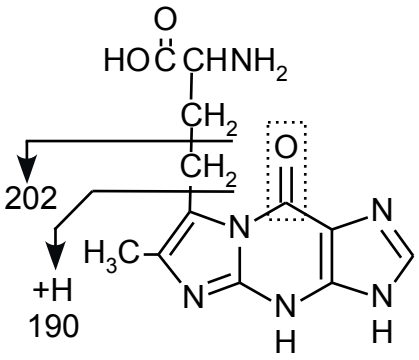

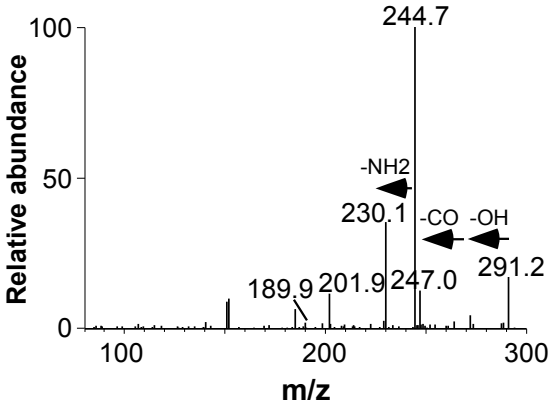

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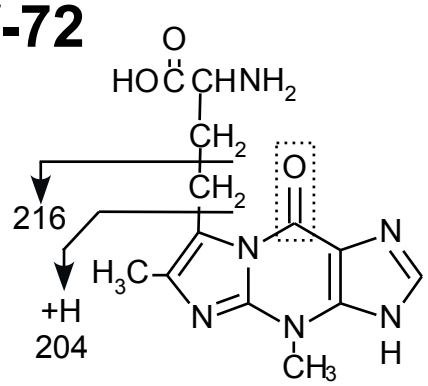
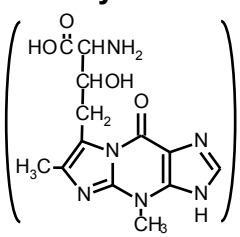
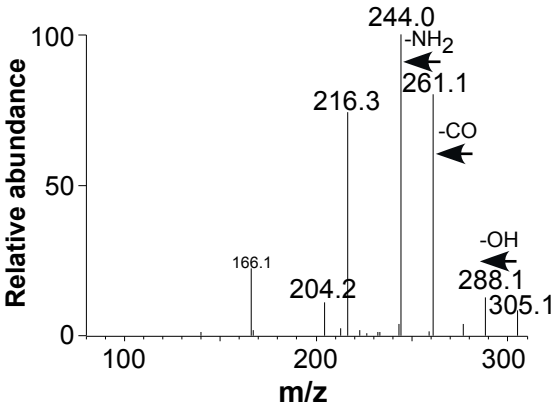


OHyW-72





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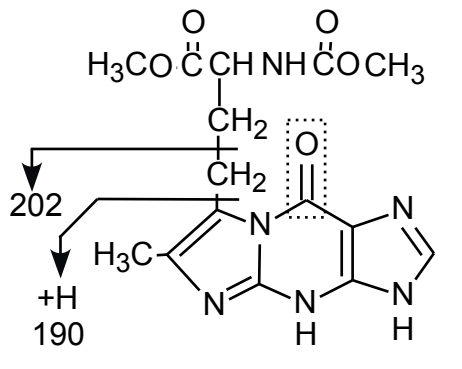

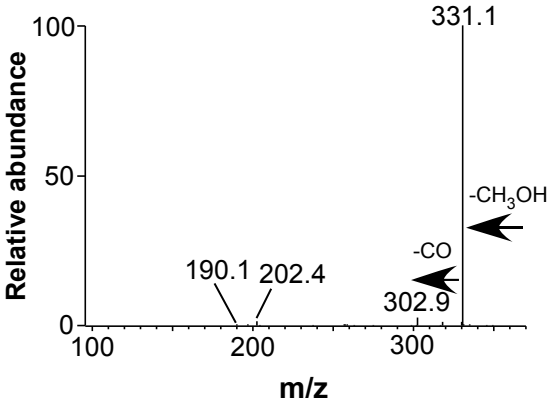

Supplemental Figure S4

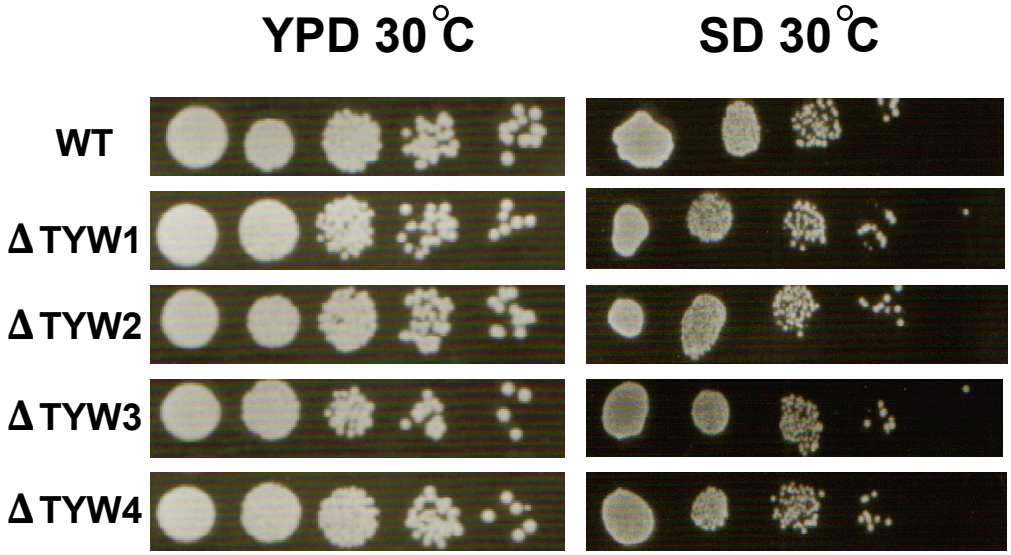

Table S1

Gene name	ORF	intermediate	Localisation	Interacting proteins
TYW1	YPL207w	m^1G	ER	YBL003c,YBR247c,YDL213c,YDR299w,YEL055c,YFL008w,YFL039c,YGL166w, YHR090c,YHR148w,YHR169w,YHR174w,YIL085c,YJL033w,YJL044c,YJL079c, YLR018c,YLR129w,YLR175w,YLR186w,YLR196w,YLR277c,YLR449w,YML006c ,YML015c,YML130w,YMR128w,YMR300c,YNL030w,YNL075w,YNR051c, YNR067c,YOL057w,YOR078w,YOR231w,YPL126w,YPL205c,YPL217c
TYW2	YML005w	imG-14	cytoplasm, ER, nuclear envelope	YHR052w,YJL186w,YKR045c,YNL191w,YNL273w,YNR024w,YOL054w,YOL006c, YOL128c, YOR308c, YPL096w, YPL104w, YPL274w
TYW3	YGL050w	yyW-86 (yW-14)	ambiguous	YPL022w
TYW4	YOL141w	yW-72	cytoplasm, mitochondria	none

*Essential proteins are underlined