Supporting Information

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SI Materials and Methods

Isolation of Proteins Associated with Tandem Affinity Purification-Tagged Mod5. The proteins identified as associating with the tandem affinity purification (TAP) affinity-tagged Mod5 protein are shown in Table S1. Table S1 can also be found in the Yeast Resource Center (http://depts.washington.edu/yeastrc/) public repository at the University of Washington. The isolation protocol was modified from previous work (1). Cells were grown in 600 mL YPD to an OD_{600} between 0.8 and 1.2, and cell pellets were stored at −80 °C. Pellets were resuspended in 1 mL buffer of 10 mM Tris (pH 8.0), 150 mM NaCl, 0.5% Nonidet (Nonidet P-40), and Complete protease inhibitors. The cells were lysed by passing through 200- and 100-μm chambers of a Microfluidizer 110Y (Microfluidics) 10 times each. Lysates were spun for 30 min at 20,000 × *g* and the supernatant was collected. Extracts (3 mL) were added to 100 μL IgG Sepharose beads (GE Healthcare) and incubated with mixing at 4 °C for 3 h. Beads were washed four times with 10 mL IgG wash buffer (10 mM Tris, 150 mM NaCl, 0.1% NonidetP-40) and one time with TEV cleavage buffer (10 mM Tris, 150 mM NaCl, 01% Nonidet P-40, 0.5 mM EDTA, 1 mM DTT). Beads were resuspended with 300 μL TEV cleavage buffer plus 50 μg TEV protease and incubated for 3 h.

Two hundred microliters packed calmodulin beads (Stratagene) were used for the second affinity step. Beads were washed with 10 mL calmodulin binding buffer (10 mM Tris, pH 8.0, 10 mM 2-mercaptoethanol, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% Nonidet P-40); 3 mL calmodulin binding buffer and 3 μL 1 M $CaCl_2$ were added to 1 mL elutate recovered after TEV cleavage. This solution is then added to the column containing the equilibrated calmodulin beads and incubated for 2 h at 4 °C. The beads were then washed with an addition 100 mL calmodulin binding buffer. After washing, the beads of the bound proteins were eluted with 1 mL calmodulin elution buffer (10 mM Tris·Cl, pH 8.0, 10 mM 2-mercaptoethanol, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 0.1% Nonidet P-40, 20 mM EGTA).

The eluted proteins were precipitated using 1 mL ice-cold 20% trichloroacetic acid and incubated on ice for 30 min. The resulting precipitate was centrifuged at 12,000 × *g* for 15 min at 4 C. Supernatant was decanted, and pellets were washed with 300 μL acetone and centrifuged again. Air-dried pellets were suspending in 100 μL 8 M urea/Tris solution with 5 mM tris (2-carboxyethyl) phosphine (TCEP) and incubated for 30 min. Iodoacetamide was added to a concentration of 10 mM in the dark for 30 min. Proteins were diluted to 2 M urea, and tryptic digests were performed in 2 mM $CaCl_2$ and 0.01 μg/mL trypsin ON at 37 C. Reaction was quenched with 5% wt/vol formic acid.

Multidimensional Protein Identification Technology. The protein digest was pressure-loaded onto a fused silica capillary desalting column containing 5 cm 5 μm Polaris C18-A material (Metachem) packed into a 250-μm i.d. capillary with a 2 μm filtered union (UpChurch Scientific). The desalting column was washed with buffer containing 95% water, 5% acetonitrile, and 0.1% formic acid. After desalting, a 100-μm i.d capillary with a 5-μm pulled tip packed with 10 cm 3 μm Aqua C18 material (Phenomenex) followed by a 3 cm 5 μm Partisphere strong cation exchanger (Whatman) was attached to the filter union, and the entire split column (desalting column–filter union–analytical column) was placed in line with an Agilent 1100 quaternary HPLC and analyzed using a modified 12-step separation as described previously (1). The buffer solutions used were 5% acetonitrile/0.1% formic acid (buffer A), 80% acetonitrile/0.1% formic acid (buffer B), and 500 mM ammonium acetate/5% acetonitrile/0.1% formic acid (buffer C). Step 1 consisted of a 100 min gradient from 0% to 100% buffer B. Steps 2–11 had the following profile: 3 min of 100% buffer A, 2 min of X% buffer C, a 10 min gradient from 0% to 15% buffer B, and a 97 min gradient from 15% to 45% buffer B. The 2 min buffer C percentages (X) were 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, and 60% for the 12-step analysis. In the final step, the gradient contained 3 min of 100% buffer A, 20 min of 100% buffer C, a 10 min gradient from 0% to 15% buffer B, and a 107 min gradient from 15% to 70% buffer B.

MS. As peptides eluted from the microcapillary column, they were electrosprayed directly into an LTQ 2D ion trap mass spectrometer (ThermoFinnigan) with the application of a distal 2.4 kV spray voltage. A cycle of one full-scan mass spectrum (400–1,400 *m*/*z*) followed by eight data-dependent MS/MS spectra at a 35% normalized collision energy was repeated continuously throughout each step of the multidimensional separation. Application of mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system.

Analysis of Tandem Mass Spectra. MS/MS spectra were analyzed using the following software analysis protocol. Poor quality spectra were removed from the dataset using an automated spectral quality assessment algorithm (2). MS/MS spectra remaining after filtering were searched with the SEQUEST algorithm (3) against the *Saccharomyces cerevisiae* Uniprot version 34 database, which was concatenated to a decoy database in which the sequence for each entry in the original database was reversed (4). All searches were parallelized and performed on a Beowulf computer cluster consisting of 100 1.2 GHz Athlon central processing units (CPUs) (5). No enzyme specificity was considered for any search. SEQUEST results were assembled and filtered using the DTASelect (version 2.0) program (6, 7). DTASelect 2.0 uses a linear discriminant analysis to dynamically set XCorr and DeltaCN thresholds for the entire dataset to achieve a user-specified false-positive rate (5% in this analysis). The false-positive rates are estimated by the program from the number and quality of spectral matches to the decoy database.

Coimmunoprecipitation of tRNA-Bound Proteins by Mod5-TAP. C-terminal Myc tags were added to Tfc1, Smc2, or Smc4 by standard methods (8) using coinsertion of a kanamycin resistance cassette. The affinity pull-down protocol was modified from previous work (9). Cells were grown in 500 mL YPD to an OD at 600 nm of 0.8–1.2, and cell pellets were stored at −80 °C. Pellets were resuspended in 1 mL buffer [10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Nonidet (Nonidet P-40), complete protease inhibitors]. Zymolyase 20T was added to the cells (20 μL 12 μg/μL stock) and incubated for 1 h, and the cells were lysed by mechanical bead disruption. Lysates were spun for 30 min at 20,000 × *g* and the supernatant was collected. Extracts (3 mL) were added to 100 μL IgG Sepharose beads (GE Healthcare) and incubated with mixing at 4 °C for 3 h. Beads were washed four times with 10 mL lysis buffer and one time with TEV cleavage buffer (10 mM Tris, 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, 1 mM DTT). Beads were resuspended with 300 μL TEV cleavage buffer plus 50 μg TEV protease. Elutions were precipitated by trichloroacetic acid and resolved using an SDS/PAGE gel 10% (Bio-Rad). Proteins were transferred to PVDF membrane and probed with anti-myc

antibody (Santa Cruz) followed by anti-mouse from sheep (GE Healthcare), with signals detected by ELC Plus (GE Healthcare).

Creation of Random and Site-Directed Mutations in *MOD5*. To test if the catalytic ability of Mod5p is necessary for tRNA gene-mediated (tgm) silencing, we created both site-directed mutations in residues thought to be at the Mod5p active site (10–12) and a large number UV-induced random mutations to screen for loss of tgm silencing function. All mutations that released tgm silencing were tested phenotypically for the ability to modify the *SUP7* tRNA as well. In all cases (3 site-directed and 18 randomly generated loss-of-function mutations), loss of tgm silencing function was accompanied by loss of tRNA modification capability, but in most cases, conclusions could not be drawn, because the mutations destabilized the protein so that it did not accumulate. The destabilization of the protein by most of the mutations is perhaps unsurprising in light of recent results showing that Mod5p can behave as a prion (13). Few mutant proteins with tgm-inactivated phenotypes were stably expressed, and only one of those mutants was in the active site, for which data are shown in Fig. 4.

To create the site-directed mutations, plasmid pMPH3 was used to produce two overlapping PCR products that have mutations in the overlapping region. Plasmid pMPH3 was digested with Age I and Kas I. The two PCR products were then transformed into BY4741 + mod5Δ with the digested plasmid. Gap repair of the plasmid was selected for by growth on synthetic dextrose complete-ura, and the sequences of the mutated genes were confirmed by DNA sequencing.

Testing Steady State Levels of Mutant Proteins. Strains were grown in 200 mL synthetic dextrose complete-ura to an OD at 600 nm of 1.5. Cells were harvested and resuspended with 0.5 mL lysis buffer, 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.5% Nonidet (Nonidet P-40). Samples (20 μg protein) were subjected to SDS/PAGE 10% electrophoresis and electroblotted to a PVDF membrane (Millipore). Western analyses were done with anti-TAP (Open Biosystems) and anti-rabbit from donkey (GE Healthcare). After exposure to detect TAP-tagged proteins, blots were probed with antiactin A5060 (Sigma) and anti-rabbit from donkey.

Complementation of tgm Silencing and tRNA Modification Activities. Complementation of the *mod5*Δ strain for tgm silencing and modification of *SUP4* tRNA were performed as in Fig. 4.

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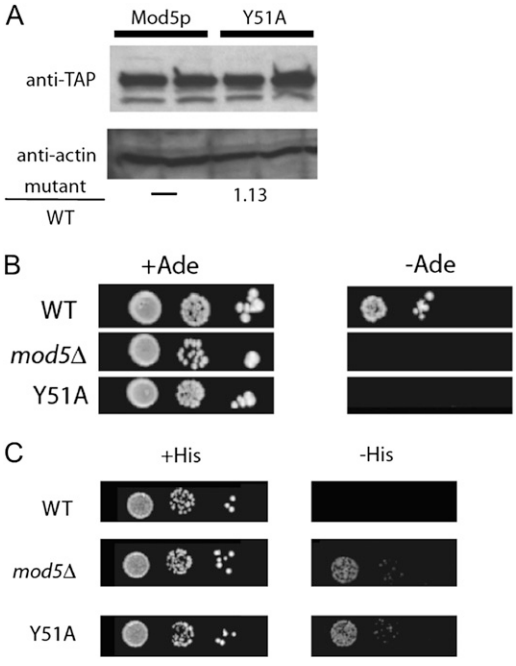

Fig. S1. Mutations that affect tRNA modification also affect tgm silencing. (*A*) A mutation in the putative active site of Mod5 for tRNA modification [conversion of the tyrosine at position 51 to an alanine (Y51A)] was stably expressed but not competent to support either (*B*) tRNA modification or (*C*) tgm silencing.

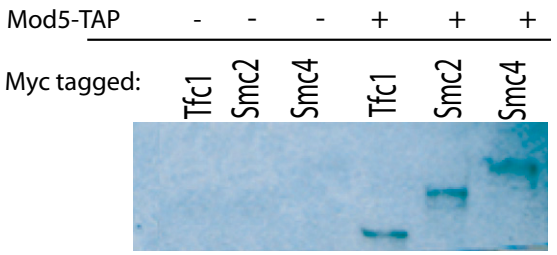

Fig. S2. Mod5-TAP coimmunoprecipitates other proteins bound at tRNA genes. Haploid yeast containing the chromosomal *MOD5* ORF with or without a C-terminal TAP tag was obtained from Open Biosystems. C-terminal 13xmyc tags were added to the indicated gene ORFs in these strains, and TAP-tagged Mod5 was isolated in a single affinity step. Probes of Western blots for coisolation of Myc-tagged proteins. These results indicate that Mod5-TAP but not untagged Mod5 pulls down myc-tagged Tfc1, Smc2, and Smc4. Methods are provided in *SI Materials and Methods*.

Table S1. Table of proteins that coisolate with Mod5-TAP

Table S1