Supplementary Materials

Reconstitution of pseudouridylation

About 1000 cpm (<0.001 fmol) of single $^{32}$P-radiolabeled yeast U2 snRNA (Ma et al, 2003) was mixed with 2 µl of each micrococcal nuclease–treated TAP-tagging preparation and 10% of the total RNA (isolated by PCA extraction and ethanol precipitation) or gel-fractionated RNA from the 20-µl original TAP-tagging preparation that had not been treated with micrococcal nuclease. To screen the Box H/ACA RNA library (see below) for Ψ42 guide activity, 25 ng of each in vitro transcribed Box H/ACA RNA (instead of total RNA or fractionated RNA) was added to the reconstitution mixture. As a control, we used the same volume of water in place of the RNA. The mixture was then brought to a final volume of 20 µl containing 100 mM Tris-HCl, pH 8.0, 100 mM ammonium acetate, 5 mM MgCl$_2$, 2 mM DTT and 0.1 mM EDTA. The pseudouridylation reaction was performed at 30°C for 1 h. The singly radiolabeled U2 was then recovered via PCA extraction and ethanol precipitation followed by gel purification, and was subsequently subjected to P1 nuclease digestion and TLC analysis, exactly as described (Ma et al, 2003).

To reconstitute rRNA pseudouridylation, a yeast 25S rRNA fragment (corresponding to nucleotides 908-1093) containing a single $^{32}$P label at the 5' side of U989, a naturally-occurring pseudouridylate site, was synthesized according to previously
published methods (Ma et al, 2003). This singly radiolabeled rRNA fragment was used as a substrate in place of the singly radiolabeled U2 snRNA in the pseudouridylation assay.

**Construction of a Box H/ACA RNA library**

For the construction of a Box H/ACA RNA library, RNAs eluted from fraction E were polyadenylated in a 25 µl reaction containing 0.5 µg of fraction E RNAs, 0.5 mM ATP, 600 U of poly(A) polymerase (USB), and 1× reaction buffer provided by the supplier (USB). Polyadenylated RNAs were recovered by PCA extraction and ethanol extraction, and were reversely transcribed into cDNAs using 25 U of AMV reverse transcriptase (Promega) and 10 pmol poly(dT) primer. The resulting cDNAs were further 3’ poly(dG) tailed using 20 U of terminal deoxynucleotidyl transferase (TDT, Promega) and 0.25 mM dGTP. After 3’ poly(dG)-tailing, the cDNAs were then used as templates for PCR amplification. The 5’ primer was a poly(dT) oligodeoxynucleotide carrying an EcoRI site (5’ CTGCAGTTTTTTTTTTTTTTT 3’) and the 3’ primer was a poly(dC) oligodeoxynucleotide carrying a XhoI site (5’GAATTCCCCCCCCCCCCCCCCC3’). The PCR products were digested with EcoRI and XhoI, and inserted between the EcoRI and XhoI sites of pBluescript II KS vector. The resultant plasmids were transformed into the E. coli DH5α strain, which was then plated on a solid LB-Amp medium to generate single colonies.

In order to generate Box H/ACA RNAs with limited 5’ and 3’ flanking sequences, each plasmid isolated from single colonies was amplified again by PCR using a 5’ primer
containing a T7 promoter sequence followed by a poly(dC) sequence
(5’TATAATAGGACTCTATAGGGCCCCCCCCCCCCCCCCCCC3’) and a 3’ poly(dT)
primer (TTTTTTTTTTTTTTT). The PCR products were directly used for in vitro
transcription to generate individual Box H/ACA RNAs, which were subsequently used
for pseudouridylation reconstitution assay (see above).

Construction of Nhp2p- and Cbf5p-conditional depletion (promoter shut-off) strains

The yeast strains used in this study, including strain 23906 (MATa/α, his3-
Δ1/his3-Δ1, leu2-Δ0/leu2-Δ0, met15-Δ0/met15-Δ0, ura3-Δ0/ura3-Δ0, NHP2/nhp2-
Δ0::Kan°), strain 24124 (MATa/α, his3-Δ1/his3-Δ1, leu2-Δ0/leu2-Δ0, met15-Δ0/met15-
Δ0, ura3-Δ0/ura3-Δ0, CBF5/cbf5-Δ0::Kan°), and the haploid strain BY4741 (MATα,
his3-Δ1, leu2-Δ0, met15-Δ0, ura3-Δ0), were all purchased from Invitrogen.

Plasmid pAVA0040-NHP2 (CEN URA3 Pgal1-NHP2) was constructed by
inserting the NHP2 gene into the pAVA0040 vector (Alexandrov et al., 2002) between the
BamHI and PstI sites. Using the same strategy, pAVA0040-CBF5 (CEN URA3 Pgal1-
CBF5) was constructed by inserting the CBF5 gene into pAVA0040 between SalI and
HindIII sites. To construct a conditional Nhp2p-depletion strain, the haploid yeast strain
BY4741 was transformed with pAVA0040-NHP2. Subsequently, a PCR-amplified DNA
fragment, derived from genomic DNA of strain 23906, containing the nhp2-Δ0::Kan°
cassette and flanking sequences (~305 bp to +310 bp, relative to the 5’ and 3’ ends of
ORF, respectively), was transformed into the strain. Selection on a galactose-containing
rich medium (YPGal) containing 0.2 mg/ml geneticin generated the conditional Nhp2p-depletion strain. The same procedure was used to generate the conditional Cbf5p-depletion strain. The deletion strains were confirmed by PCR analysis.

For depletion of Nhp2p or Cbf5p, cells (the conditional Nhp2p-depletion strain or conditional Cbf5p-depletion strain, respectively) growing exponentially at 30°C under permissive conditions (galactose-containing rich medium, YPGal) were harvested by brief centrifugation and were resuspended in glucose-containing medium (YPD). During growth, cells were diluted with YPD and maintained in the exponential phase. Cell growth was monitored by measuring OD$_{600}$ of the cell culture. Yeast cells were collected at different time points before and after YPGal was switched to YPD, and total RNA was extracted and analyzed for pseudouridylation as described (Ma et al., 2003).

**Construction of yeast snR81-deletion strain**

To delete the snR81 gene, PCR was used to generate a DNA fragment containing the snR81-Δ::Kan$^r$ cassette and snR81-flanking sequences (−45 bp to +45 bp, relative to the 5’ and 3’ ends of snR81 gene, respectively). Specifically, the template for the PCR reaction was the genomic DNA isolated from strain 24124. The 5’ primer was a 60-mer oligodeoxynucleotide (snR81-F1, 5’

ACATGATGGAGTACCCTCATGCTTTGGCGCGTGCGCATCTATATACGGATC

CCCGGGTTA 3’) corresponding to the 45 nucleotides immediately upstream of snR81 gene and the first 15 nucleotides of a KanMX4 cassette, and the 3’ primer was a 60-mer
oligodeoxynucleotide (snR81-R1 5’
GACTGAATCATATTACTTAGTTACTACTACTCTTTCTATCATGCAAGAGAAAAACT
CATCGAG 3’) complementary to the 45 nucleotide sequence immediately downstream
of the snR81 gene and the last 15 nucleotides of KanMX4 gene. The PCR product was
transformed into strain BY4741, and the snR81-deletion strain was selected on a YPD
medium containing 0.2 mg/ml geneticin. The deletion of snR81 was verified by PCR
analysis using the 5’ primer (snR81-C1, 5’ CATCAAAACATGACGTTAGCG 3’)
corresponding to a 20 nucleotide sequence upstream of snR81 gene, and either of the two
3’ primers (KanB, 5’ GCCGCCTGCAGTACTCTTTATCATGCAAGAAG 3’, and snR81-
R2, 5’ GCCGCCTGCAGTACTCTTTATCATGCAAGAAG 3’) complementary to a
KanMX4 sequence or a snR81 sequence, respectively. A PCR product with predicted size
was generated when the 5’ snR81-C1 primer and the 3’ KanB primer were used; no
product was observed when the 3’ KanB primer was replaced by snR81-R2 (data not
shown).

Construction of an snR81-containing plasmid

To carry out functional complementation analysis, we constructed a plasmid
containing a wild-type snR81 gene under the control of P_{GAL1} promoter. The snR81
double-stranded DNA was generated by PCR using the wild-type genomic DNA as a
template and the following pair of oligodeoxynucleotides as primers: 5’ primer,
Gal-snR81-F corresponding to the BamHI sequence and the first 20 nucleotides of
snR81; 3’ primer, Gal-snR81-R complementary to PstI sequence and the last 20
nucleotides of snR81. The PCR product was first digested with BamHI and PstI, and subsequently inserted into the AVA0040 vector between the BamH1 and Pst1 sites. The plasmid was transformed into the snR81-deletion strain, which was then grown in either glucose- or galactose-containing medium. Total RNA was isolated and pseudouridylation of U2 snRNA and 25S rRNA was assayed.