Supporting information for Du *et al.* (2002) *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.172527399

Supporting Methods

Extracts of *Sacchromyces cerevisiae* Expressing UBR1 or its Derivatives. *S. cerevisiae* SC295 (*MATa, GAL4 GAL80 ura3-52, leu2-3,112 reg1-501 gal1 pep4-3*) was a gift from S. A. Johnston (Univ. of Texas, SW Medical Center, Dallas, TX). N-terminally flag-tagged (1), full-length *S. cerevisiae* UBR1 (^{f}UBR1) and its truncated derivatives were expressed in SC295 from the P_{ADH1} promoter in the high-copy pFlagUBR1SBX based on the vector Yeplac181 (1). Construction details are available upon request. ^{f}UBR1 is functionally indistinguishable from WT UBR1 (data not shown). A single colony of yeast transformant was inoculated into 20 ml of yeast SD medium (1), and the cells were grown at 30°C until mid-exponential phase, then re-inoculated into 2 liters of SD medium and grown to A_{600} of ~1.0. The cells were harvested by centrifugation, washed once with cold 1× PBS, and frozen in liquid N_2. The frozen pellet was ground to fine powder in liquid N_2, using a mortar and pestle. The powder was thawed and resuspended (6 ml of buffer per 1 g of pellet) in the lysis buffer [10% glycerol/0.5% Nonidet-P40 (NP-40)/0.2 M KCl/1 mM DTT/50 mM Hepes, pH 7.5] containing protease inhibitors (1 tablet per 10 ml of buffer; Roche Molecular Biochemicals). The suspension was centrifuged at 11,200 × *g* for 30 min at 4°C. Samples of the supernatant were stored at –80°C.

A fragment of mouse *UBR1* cDNA (2, 3) encoding the 1,031 residue N-terminal fragment of mUBR1 N-terminally tagged with the flag epitope ($m^{f}UBR1^{1-1031}$) was expressed in SC295 *S. cerevisiae* from the from the P_{ADH1} promoter in the high-copy plasmid pFlagmUBR1NT1031. To subclone the $m^{f}UBR1^{1-1031}$ cDNA, a PCR-produced fragment of the 3,093-bp *mUBR1* ORF was constructed that also contained a sequence encoding the flag tag and a 3-residue linker at the 5' of the *mUBR1* ORF. This fragment was subcloned into low-copy pBluescript II SK(+) (Stratagene). The *Sal*I/*Xho*I fragment of the resulting insert was then ligated into *Sal*I/*Xho*I-cut (and dephosphorylated)

pUBR1CT870, derived from pFlagUBR1SBX, and the ligation mixture was used to transform SC295 *S. cerevisiae*, as described (3). Construction details are available upon request.

GST Fusions. DNA fragments encoding either WT CUP9, or its mutant derivatives, or RAD6, or the 273-residue C-terminal fragment of *S. cerevisiae* UBR1 ($UBR1^{1678-1950}$), or the 259-residue C-terminal fragment of mouse UBR1 ($mUBR1^{1499-1757}$) were subcloned into pGEX-2TK (Amersham Pharmacia), downstream of, and in frame with, the ORF of GST. The final constructs (except the one containing RAD6) also bore the C-terminal His_6 tag (1). The resulting plasmids were cotransformed into *E. coli* BL21(DE3) together with pRI952 (a gift from S. W. Stevens, Division of Biology, CIT), expressing tRNAs for the codons AGG, AGA, and AUA, which are rare in *Escherichia coli*. Transformants were grown in LB medium (1) containing ampicilin (60 μg/ml) and chloramphenicol (25 μg/ml) at 37°C to A_{600} of 0.8–1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM, followed by a 3-hr incubation. The cells were harvested by centrifugations, washed once with PBS, and frozen in liquid N_2. The cell pellet was resuspended in buffer A (10% glycerol/0.3 M NaCl/10 mM imidazole/10 mM β-mercaptoethanol/25 mM K_2HPO_4-KH_2PO_4, pH 7.8) (6 ml of buffer per 1 g of pellet). Freshly dissolved chicken egg white lysozyme in the same buffer was added to a final concentration of 0.5 mg/ml, followed by incubation at 4°C for 30 min. The cells were further disrupted by sonication, 3 times for 1 min each, at 1-min intervals, followed by the addition of NP40 to a final concentration of 0.5%. The suspension was centrifuged at 11,200 × *g* for 30 min. The supernatant was added to a tube containing 2 ml of Ni-NTA resin (Qiagen). After gentle rotation at 4°C for 10 min, the resin was transferred to a 5-ml column and washed with 10 ml of buffer A. A GST fusion protein was then eluted with buffer A containing 0.3 M imidazole.

GST-Pulldown Assay. Either a GST fusion protein or GST alone (~2 μg) was diluted to 0.5 ml in the loading buffer (10% glycerol/0.5 M NaCl/1 mM EDTA/1% NP-40/50 mM Tris•HCl, pH 8.0), and incubated with 20 μl of glutathione-Sepharose beads (Amersham Pharmacia) for 20 min at 4°C. The beads were washed once with 0.5 ml of the loading

buffer and once with 0.5 ml of the binding buffer (10% glycerol/50 mM NaCl/0.05% NP-40/50 mM Na-Hepes, pH 7.8). The washed beads, in 100 μl of the binding buffer, were incubated at 4°C for 1 hr with a yeast extract (160 μl) containing ^{f}UBR1 or its truncated derivative, in the absence or presence of dipeptides (Sigma) at stated concentrations. Ovalbumin (0.1 mg/ml in the binding buffer) was used to dilute yeast extract. The beads were washed three times with 0.2 ml of the binding buffer containing dipeptides at the same concentrations. The beads then were suspended in 20 μl of SDS/PAGE loading buffer and heated at 100°C for 5 min, followed by SDS/8% PAGE, electrophoretic transfer of proteins onto PVDF membrane (Millipore), and immunoblotting with anti-FLAG M2 antibody (Sigma; ref. 4).

Pulldown Assay with Peptide Beads. 12-mer peptides, XIFSTDTGPGGC (in single-letter abbreviations for amino acids), with the N-terminal residue X being Arg, Phe, Gly, Ser, Thr, Ala, or Asp, were synthesized at the Caltech peptide facility. From residue 2 to residue 9, the (identical) sequences of these peptides were the same as the (identically positioned) sequence of nsP4, the 70-kDa Sindbis virus RNA polymerase, a natural substrate of the N-end rule pathway (5, 6). The nsP4's residue 9 of these peptides was followed by two Gly residues and C-terminal Cys residue; the latter was used to crosslink each peptide to microbeads. Each peptide was purified by HPLC and verified by mass spectroscopy. A peptide (1.5 mg) was crosslinked, via its C-terminal Cys residue, to 1 ml (packed volume) of UltraLink Iodoacetyl beads (Pierce) as described in the manufacturer's protocol. *S. cerevisiae* extract containing the overexpressed, full-length *S. cerevisiae* ^{f}UBR1 was diluted by the lysis buffer to 1.2 mg/ml of total protein, and thereafter incubated with or without an added dipeptide for 15 min on ice. When present during the incubation, the dipeptides Arg-Ala, Ala-Arg (Bachem), Phe-Ala, or Ala-Phe (Sigma) were at 10 mM each. Bestatin (Sigma), an aminopeptidase inhibitor, was added to samples to the final concentration of 50 μM. A sample (0.3 ml) was then transferred to a new tube containing 5 μl (packed volume) of a carrier-linked 12-mer peptide, followed by gentle mixing, through rotation of the tube, for 1 hr at 4°C. The beads were pelleted by a brief centrifugation in a microcentrifuge, then washed 3 times, for 2 min each, with the lysis buffer containing (or lacking) the same dipeptides (at 10 mM each) that were

present during the incubation. The beads were then suspended in 20 μl of SDS/PAGE loading buffer, and heated at 95°C for 5 min, followed by a brief spin in a microcentrifuge, SDS/8% PAGE of the supernatant, and detection of ^{f}UBR1 by immunoblotting with anti-FLAG M2 antibody.

Pulse–Chase Assay. Ub fusion proteins of the UPR (Ub/protein/reference) technique (7, 8) were expressed in *S. cerevisiae* JD52 (*MAT***a** *lys2-801 ura3-52 trp1-*Δ*63 his3-*Δ*200 leu2-3,112*) (9) from low-copy plasmids and the P_{MET25} promoter. Upon cotranslational cleavage by deubiquitylating enzymes (DUBs), these ^{f}DHFR-Ub-CUP9^{f} fusions yielded the long-lived reference protein flag-dihydrofolate reductase-Ub (^{f}DHFR-Ub) and either the C-terminally flag-tagged WT CUP9 (CUP9^{f}) or its mutant derivatives. Cells were labeled with [^{35}S]methionine/cysteine for 5 min at 30°C, followed by chase for 5, 15, 30, and 60 min, extraction of proteins, immunoprecipitation with anti-flag antibody, SDS/PAGE, and quantitation, essentially as described (7, 9, 10).

1. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G. & Struhl, K. (2002) *Current Protocols in Molecular Biology* (Wiley Interscience, New York).

2. Kwon, Y. T., Reiss, Y., Fried, V. A., Hershko, A., Yoon, J. K., Gonda, D. K., Sangan, P., Copeland, N. G., Jenkins, N. A. & Varshavsky, A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 7898–7903.

3. Kwon, Y. T., Xia, Z., Davydov, I. V., Lecker, S. H. & Varshavsky, A. (2001) *Mol. Cell. Biol.* **21,** 8007–8021.

4. Xie, Y. & Varshavsky, A. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 2497–2502.

5. deGroot, R. J., Rümenapf, T., Kuhn, R. J. & Strauss, J. H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8967–8971.

6. Varshavsky, A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 12142–12149.

7. Suzuki, T. & Varshavsky, A. (1999) *EMBO J.* **18,** 6017–6026.

8. Turner, G. C. & Varshavsky, A. (2000) *Science* **289,** 2117–2120.

9. Turner, G. C., Du, F. & Varshavsky, A. (2000) *Nature* **405,** 579–583.

10. Rao, H., Uhlmann, F., Nasmyth, K. & Varshavsky, A. (2001) *Nature* **410,** 955–960.