# Supporting Information S1 I

## *Supporting Information Methods & References*

### Supporting Information Methods

**Yeast and bacterial methods.** Yeast rich (YPD) and minimal (SD) medium/plates were prepared as described previously [1]; yeast strains and cultures were grown at 30°C.

**Preparation of cell extracts.** Cell extracts were prepared as previously described [2]. Briefly, 2.5 $A_{600}$ eq of logarithmically growing cells were lysed by β-mercaptoethanol/NaOH and proteins were precipitated in 5% trichloracetic acid. The pellet was resuspended in SDS-gel loading buffer.

**Immunoprecipitation.** Cell lysis and immunoprecipitation were performed as previously described [3]. In brief, 25 $A_{600}$ eq of logarithmically growing yeast cells were harvested and resuspended in cold extraction buffer (50 mM Tris, pH 7.5) containing protease inhibitors (625 µM PMSF, 1 µg/ml aprotonin/leupeptin). The cells were disrupted with glass beads by four repeated cycles of vortex mixing for 30 s at maximum speed, interrupted by 30 s incubation on ice. The lysate was diluted with cold extraction buffer and cleared by low-speed centrifugation (2.100 rpm, 5 min). The membrane fraction was collected from the supernatant by centrifugation (13.200 rpm, 10 min) and resuspended in cold resuspension buffer (50 mM Tris, pH 7.5, 200 mM NaOAc, 10% glycerol) containing protease inhibitors (2 µM pepstatin, 1 µg/ml aprotonin/leupeptin and 1 mM Pefabloc). Membranes were solubilized by addition of digitonin (SERVA) to 1% and a 10 min incubation on ice. The digitonin lysate was cleared by centrifugation (13.200 rpm, 10 min) prior to immunoprecipitation. For the immunoprecipitation the digitonin concentration was lowered to 0.5 % by diluting with cold resuspension buffer. An aliquot (5% of total) of the supernatant was removed and served as input for immunoblot. For immunoprecipitation, 10 µl of monoclonal anti-HA agarose beads (Sigma-Aldrich) were added to the digitonin lysate and incubated overnight at 4°C with constant rotation. Beads were collected by low-speed centrifugation, washed four times with cold resuspension buffer, resuspended and heated at 37°C for 15 min in 4x sample buffer (20 mM Tris, pH 6.8, 10% sucrose, 5 mM EDTA, 3% SDS, 10% β-mercaptoethanol, 0.1% bromphenol blue).

**Immunoblotting.** Proteins were separated by SDS-PAGE and electrotransferred onto a PVDF membrane (Immobilon-P, Millipore). Immunodetection was carried out with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Immunoreactive species were visualized using the ECL system (Western Lightning ™ Plus-ECL, PerkinElmer). The following antibodies were used: anti-HA mouse monoclonal antibody HA.11 (Covance); anti-c-MYC rabbit polyclonal antibody sc-789 (Santa Cruz Biotechnology); anti-FLAG mouse monoclonal antibody M2 (Sigma). Quantification was carried out by using Aida image analyzer software (Bio Imaging).

**Northern blot analysis.** Yeast total RNA was prepared by the hot phenol method as described previously [4]. Briefly, cells from a 10 ml YPD culture of 2.5-5.0 $A_{600}$ eq were harvested (3000 rpm, 5 min) and resuspended in 400 µl AE buffer (50 mM NaOAc pH 5.3 , 10 mM EDTA). 40 µl 10% SDS were added and the solution mixed by vortexing. After addition of an equal volume of phenol (equilibrated in AE buffer), samples were vortexed, incubated for 4 min at 65°C and chilled on ice. Following centrifugation (13.200 rpm, 2 min) the aqueous phase was extracted with phenol/chloroform by centrifugation (13.200 rpm, 5 min) at room temperature. RNA was precipitated by addition of 40 µl 3 M NaOAc pH 5.3 and 2.5 volumes of 100% ethanol. The precipitate was washed with 80% ethanol, dried and resuspended in sterile water. For Northern blotting, 25 µg ot total RNA was resuspended to a final concentration of 2 µg/µl in loading buffer (1x MOPS electrophoresis buffer (20 mM MOPS pH 7.0, 2 mM NaOAc, 1 mM EDTA), 50% formamide, 7.4% formaldehyde, 10 µg/ml ethidium bromide). Samples were incubated for 10 min at 65°C, then cooled on ice before loading onto a denaturing 0.9% agarose gel containing 6.6% formaldehyde. Gels were repeatedly washed, first in $H_2O$, then in 50 mM NaOH followed by 100 mM TrisHCl pH7.0. After soaking for 5 min in 20x SSC (3 M NaCl, 0.3 M NaCitrate pH 7.0) the RNA was transferred by standard capillary transfer in 20x SSC onto a positively charged Nylon membrane (Roche). Membranes were baked for 2 h at 80˚C and prehybridization was carried out for 1 h at 45°C in UltraHybOligo hybridization buffer (Ambion), followed by addition of the 5´-[$^{32}$P]-labeled oligonucleotide probe (sequences available upon request) and incubation overnight at 42˚C. Membranes were washed twice in washing buffer (2x SSC, 0.5% SDS) and exposed to a BAS reader PhosphorImager (Raytest) or to film.

**Anisomycin-chase analysis.** *K. lactis* ribosomes are resistent to inhibition by cycloheximide [5]. Therefore, anisomycin was used instead of cycloheximide to inhibit protein synthesis in *K. lactis*.

Anisomycin-chase/Western blot analysis was carried out as described previously [6] except for the use of anisomycin instead of cycloheximide. Briefly, anisomycin (0.15 mg/ml) was added to logarithmically growing *K. lactis* cultures, and cell aliquots were removed at the indicated times after addition. Cells were pelleted, resuspended in cold STOP mix (0.5x SD, 10 mM $NaN_3$), and stored on ice until all time points had been collected. Lysates were generated, and proteins were visualized by immunoblotting.

***K. lactis doa10*Δ strain generation.** The *K. lactis doa10*Δ strain SKY241 was created by replacement of *DOA10* with a *doa10*Δ::KanMX6 allele in strain JA6 [7] by homologous recombination. Briefly, a ~2.4-kb DNA fragment encompassing the region upstream of the Doa10 ORF (-387 to -1), the KanMX6 marker cassette [8] and the first 520 bp of the Doa10 3'-UTR was transformed into strain JA6 by the LiOAc method [9]. Cells were incubated on a YPD+Ade plate for 16 h and were then replica plated on YPD+Ade plus 300 µg/ml G418. The presence of the *doa10*Δ::KanMX6 allele was verified by PCR. The second *K. lactis doa10*Δ strain, SKY281, was created by replacement of *Doa10* with as *doa10*Δ::HphMX4 allele in strain KHO46-12A (OS163; [10]) similar as described for SKY241 (except for the HphMX4 cassette).

**Isolation and sequencing of *K. marxianus* and *K. dobzhanskii DOA10* genes.** The *K. marxianus DOA10* locus was amplified with Phusion DNA Polymerase (Finnzyme) from genomic DNA prepared from *K. marxianus* (DSM 70292). Due to the lack of *KmDOA10* sequences in public databases, we first amplified a ~2.5 kb stretch beginning approx. 300 nts upstream of the *Km*Doa10 start codon and ending within the region encoding the highly conserved RPGVL-motif located within the Doa10 TD-domain. The sequence of primer Kons#215 (5'-CCTTCCACTCGTACTTTAGG-3') which anneals approx. 300 bps upstream of the *Km*Doa10 (Nt-)ORF was deduced from a sequence tag (GenBank: AL422796.1) from an exploratory sequencing project of *K. marxianus* by the Génolevures Consortium [11].The degenerated minus strand primer Kons#218 (5'-GACCTDATRAARAANAGAACNCCNGG-3') "matching" the RPGVL-motif encoding sequence was designed based on a multiple alignment of fungal *DOA10* genes. The second half of the *KmDOA10* locus was amplified as part of an approx. 10.5-kb stretch starting just before the RPGVL-motif encoding sequence and ending in the downstream gene locus with similarity to *S.cerevisiae* YER052c. The ~10.5-kb fragment was amplified using primers Kons#231 (5'-GTATTCTCTGTTATCGGTGG-3') annealing upstream of the RPGVL-motif encoding sequence and primer Kons#305 (5-

GGAATGGCACAATAATCTATCCAAGTG-3') annealing approx. 10.5 kb downstream within the ortholog of *S. cerevisiae* YER052c. The sequence of the ortholog of *S. cerevisiae* YER052c (GenBank: AL423471.1) was retrieved from a sequence tag from the same exploratory *K. marxianus* sequencing project as cited above [11], and the distance from the RPGVL-motif encoding *DOA10* sequence was inferred assuming gene synteny between *K. lactis* and *K. marxianus* over that region. Sequencing of the ~2.5-kb fragment as well as sequencing of part of the ~10.5-kb fragment was performed through primer walking. Primer sequences are available upon request.

An approx. 2.1-kb region starting from the RING-CH encoding sequence (approx. 150 bps downstream of the Nt-Doa10 ORF start codon) to the RPGVL-motif encoding sequence was amplified from genomic DNA prepared from *K. dobzhanskii* (CBS 2104). Semi-nested PCR was carried out with the degenerated primer Kons#324 (5'-CCATCCATGYAAGTGYAAAGG-3') designed to anneal within the region encoding the conserved amino acid stretch HPCKCKG within the RING-CH domain of fungal Doa10 and degenerated primers designed to match the RPGVL-motif encoding region (Kons#218 (5'-GACCTDATRAARAANAGAACNCCNGG-3') and nested primer Kons#219 (5'-ATGAARAANAGAACNCCNGGNCK-3'), respectively). Sequencing of the 2.1-kb fragment was performed through primer walking. Primer sequences are available upon request.

**Supporting Information References**


1. Guthrie C, and Fink, GR (1991) Guide to Yeast Genetics and Molecular Biology; Abelson JN, and Simon, MI, editor. San Diego: Academic Press.
2. Loayza D, Tam A, Schmidt WK, Michaelis S (1998) Ste6p mutants defective in exit from the endoplasmic reticulum (ER) reveal aspects of an ER quality control pathway in Saccharomyces cerevisiae. Mol Biol Cell 9: 2767-2784.
3. Neuber O, Jarosch E, Volkwein C, Walter J, Sommer T (2005) Ubx2 links the Cdc48 complex to ER-associated protein degradation. Nat Cell Biol 7: 993-998.
4. Schmitt ME, Brown TA, Trumpower BL (1990) A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. Nucleic Acids Res 18: 3091-3092.

5. Dehoux P, Davies J, Cannon M (1993) Natural cycloheximide resistance in yeast. The role of ribosomal protein L41. Eur J Biochem 213: 841-848.
6. Kreft SG, Wang L, Hochstrasser M (2006) Membrane topology of the yeast endoplasmic reticulum-localized ubiquitin ligase Doa10 and comparison with its human ortholog TEB4 (MARCH-VI). J Biol Chem 281: 4646-4653.
7. Breunig KD, Kuger P (1987) Functional homology between the yeast regulatory proteins GAL4 and LAC9: LAC9-mediated transcriptional activation in Kluyveromyces lactis involves protein binding to a regulatory sequence homologous to the GAL4 protein-binding site. Mol Cell Biol 7: 4400-4406.
8. Longtine MS, McKenzie A, 3rd, Demarini DJ, Shah NG, Wach A, et al. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast 14: 953-961.
9. Gietz RD, Schiestl RH (2007) Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc 2: 35-37.
10. Heinisch JJ, Buchwald U, Gottschlich A, Heppeler N, Rodicio R (2010) A tool kit for molecular genetics of Kluyveromyces lactis comprising a congenic strain series and a set of versatile vectors. FEMS Yeast Res 10: 333-342.
11. Llorente B, Malpertuy A, Blandin G, Artiguenave F, Wincker P, et al. (2000) Genomic exploration of the hemiascomycetous yeasts: 12. Kluyveromyces marxianus var. marxianus. FEBS Lett 487: 71-75.