Supplemental Material and Methods

Recombinant protein production and purification

Plasmid constructs encoding Ubp3-6His, 6His-Cdc48, 6His-Flag-Ufd3 and GST-Bre5 and related deletion mutants were described previously (Cohen *et al.*, 2003a; Mullally *et al.*, 2006). Plasmid encoding 6His-Flag-Cdc48 was constructed by insertion of the Flag epitope into 6His-Cdc48 plasmid. Plasmid encoding 6His-Bre5 was constructed by cloning of Bre5 cDNA into pET28a vector. Recombinant proteins were expressed in *Escherichia coli* strain BL21 (DE3). Cells were grown at 37°C up to OD_{600} = 0.6. Protein expression was then induced for 24 hours at 23°C with 0.5 mM of IPTG and fusion proteins were purified on Ni-agarose beads (Qiagen) or Glutathione-Sepharose (GE Healthcare) according to the manufacturer's instructions.

Pull-down assays

For pull-down experiments, indicated amounts of recombinant proteins with 1 μg of bovine serum albumin were allowed to bind for 30 min at 25 °C prior incubation with anti-Flag-agarose (clone M2; Sigma-Aldrich) for 1h at 4°C in binding buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM Dithiothreitol, 0.1% TX100, 10% glycerol, 2,5 mM ATP, 20 mM $MgCl_2$ and protease inhibitors cocktail). Beads were washed with binding buffer and bound proteins were eluted by heating samples at 95°C for 5 min in Laemmli sample buffer prior WB analysis.

Sample preparation for proteomic analysis of Ubp3 and Bre5 partners

Yeast cells expressing genomically protein A-tagged Bre5 (Bre5-PrA) or Ubp3 (Ubp3-PrA) were grown up to an OD_{600}= 1.5. Cells were harvested and rapidly frozen in liquid nitrogen prior cryolysis (Alber *et al.*, 2007). Grindate (1 g) was resuspended in 9 ml of ice-cold IP buffer (50 mM Hepes pH 7.5, 75 mM NaCl, 1 mM Dithiothreitol, 0.1% Triton-X100, 5% glycerol, protease inhibitors cocktail), homogenized and cleared by centrifugation. This soluble extract was incubated for 1 hour at 4°C with~9 x 10^8 of IgG-conjugated magnetic beads per gram of cells. Beads were then washed with ice-cold IP buffer containing 150 mM NaCl. Bound proteins were

eluted with 0.5 M NH_4OH, 0.5 mM EDTA. The eluate was lyophilized, resuspended in SDS-PAGE sample buffer, separated on a 4-12% Bis-Tris NuPAGE NOVEX gel (Invitrogen), and visualized with Coomassie blue (R-250) staining. Gels were systematically cut into slices, and in gel digestion was performed with an automated protein digestion system, MassPREP Station (Waters, Manchester, UK). The gel slices were washed three times in a mixture containing 25 mM NH_4HCO_3: CH_3CN (1:1, v/v). The cysteine residues were reduced by 50 µL of 10 mM dithiothreitol at 57°C and alkylated by 50 µL of 55 mM iodoacetamide. After dehydration with acetonitrile, the proteins were cleaved in gel with 40 µL of 12.5 ng/µL of modified porcine trypsin (Promega, Madison, WI, USA) in 25 mM NH_4HCO_3 at 37°C for 4 hours. The tryptic peptides were extracted with 60% acetonitrile in 0.5% formic acid, followed by a second extraction with 100% (v/v) acetonitrile.

Mass spectrometry analysis

NanoLC-MS/MS analyses were performed on a nanoACQUITY Ultra-Performance-LC system (UPLC) coupled with a SYNAPT High Definition Mass Spectrometry (HDMS) quadrupole time-of-flight tandem mass spectrometer (Waters, Milford, MA) equipped with a nano-electrospray source.

5µL of each sample were loaded on a Symmetry C18 precolumn (20 x 0.18 mm, 5 µm particle size, Waters). The peptides were separated on an ACQUITY UPLC® BEH130 C18 column (75 µm x 200 mm, 1.7 µm particle size, Waters). The solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Trapping was performed during 3 min at 5 µL/min with 99% of solvent A and 1% of solvent B. Elution was performed at a flow rate of 400 nL/min, using a 1-40% linear gradient of solvent B for 35 min at 45°C followed by a 5 min stage at 65% of solvent B.

The Q-TOF .instrument was operated with the following settings: source temperature was set to 80°C, cone gaz flow was 30l/h, cone voltage was 40V and the nanoelectrospray voltage was 3.5kV.

Mass calibration of the TOF was achieved using phosphoric acid (H3PO4) on the 50 to 2000 m/z range in positive mode. Online correction of this calibration was achieved using lock-mass on product ions derived from the [Glu1]-fibrinopeptide B (GFP). The ion $(M+2H)^{2+}$ at m/z 785.8426 is used to calibrate MS data and the fragment ion (M+H)+ at m/z 684.3469 is used to calibrate MS/MS data during the analysis.

For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes. The MS spectra were collected over 0.5 s in the range 250-1500 m/z and the MS/MS over 0.7 s in the range 50-2000 m/z. The 3 most abundant peptides (intensity threshold 60 counts/s), preferably doubly and triply charged ions, were selected on each MS spectrum for further isolation and CID fragmentation. Fragmentation was performed using argon as the collision gas. The system was fully controlled by MassLynx 4.1 (SCN 566, Waters).

Protein identification

The MS and the MS/MS data were searched using a local Mascot server (MASCOT version 2. 2. 0, MatrixScience, London, UK). The MS/MS data were analyzed against a composite target-decoy database including the protein sequences of the Swiss-Prot database (version 57.2) and reversed versions of these sequences (total 933478 entries).

Searches were performed with a mass tolerance of 50 ppm in MS mode and 0.2 Da in MS/MS mode. One missed cleavage per peptide was allowed and variable modifications were taken into account such as carbamidomethylation of cysteine residues and oxidation of methionine residues. Neither protein molecular weight nor isoelectric point constrains were applied.

The Mascot results were loaded into the Scaffold software (Proteome Software, Portland, USA). To minimize false positive identifications, results were subjected to very stringent filtering criteria as follows. For the identification of proteins with two peptides or more, a Mascot ion score above 30 was required. In the case of single peptide hits, the score of the unique peptide had to be greater (minimal “difference score” of 0) than the 95% Mascot significance threshold. The target-decoy database search allowed us to control and estimate the false positive

identification rate of our study (Elias et al., 2007; Peng et al., 2003). Thus, the final catalogue of proteins presents an estimated false positive rate below 1.1%.

Elias, J.E. and S.P. Gygi, *Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry.* Nat Methods., 2007. **4**(3): p. 207-14.

Peng, J., et al., *Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome.* J Proteome Res., 2003. **2**(1): p. 43-50.

Synthetic lethality assay

To analyze the genetic interaction between UBP3 and CDC48, the *cdc48-6* thermosensitive mutant cells were transformed with p426-UBP3 (URA3) plasmid, prior deletion of the genomic copy of *UBP3* gene. Growth phenotype of resulting cells was assessed on 5-FOA plates at 23°C.

Supplemental Table 1: Peptide sequence identified by tandem MS/MS (see XLS table attached)

Supplementary Table II: Strains and plasmids used in this study

Strains	**Genotype**	**Reference**
DF5-BRE5-Protein A	MAT α, his3-Δ200, leu2-3,2-112, lys2-801, trp1-1(am), ura3-52, BRE5-PrtA-HIS	This study
DF5-UBP3-Protein A	MAT α, his3-Δ200, leu2-3,2-112, lys2-801, trp1-1(am), ura3-52, UBP3-PrtA-HIS	This study
FY1679-08A-BRE5-GFP-UPB3-HA	MAT a, ura3, trp1, his3, leu2, BRE5-GFP-KanMX6, UPB3-HA-TRP1	Cohen et al., 2003a
FY1679-08A-BRE5-GFP-*ubp3Δ*	MAT a, ura3, trp1, his3, leu2, BRE5-GFP-KanMX6, YER151c::TRP1	Cohen et al., 2003a
FY1679-08A-*bre5Δ*-UPB3-HA	MAT a, ura3, trp1, his3, leu2, YNR051c::KanMX6, UPB3-HA-TRP1	Cohen et al., 2003a
DF5	MAT α, his3-Δ200, leu2-3,2-112, lys2-801, trp1-1(am), ura3-52	Gift from S. Jentsch
DF5 *bre5Δ*	MAT α his3-Δ200, leu2-3,2-112, lys2-801, trp1-1(am), ura3-52, YNR051c::KanMX6	This study
DF5 *ubp3Δ*	MAT α, his3-Δ200, leu2-3,2-112, lys2-801, trp1-1(am), ura3-52, YER151c::TRP1	This study
DF5 *ufd3Δ*	MAT α, his3-Δ200, leu2-3,2-112, lys2-801, trp1-1(am), ura3-52, YKL213c::KanMX6	This study
DF5 *ubp3Δ ufd3Δ*	MAT α, his3-Δ200, leu2-3,2-112, lys2-801, trp1-1(am), ura3-52, YER151c::TRP1 YKL213c::HIS	This study
BY4742	MAT α, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0	Euroscarf
BY4742 *ubp3Δ*	MAT α, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, Yer151c::kanMX4	Euroscarf
BY4742 *ufd3Δ*	MAT α, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, YKL213c::His	This study
BY4742 *ubp3Δ ufd3Δ*	MAT α, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, Yer151c::kanMX4 YKL213c::His	This study
BY4741 *erg6Δ*	Mat **a,** his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, YML008c::KanMX6	Euroscarf
DF5 *cdc48-6*	MAT α, his3-Δ200, leu2-3,2-112, lys2-801, trp1-1(am), ura3-52, cdc48-6	Gift from S. Jentsch
DF5 *cdc48-6* UBP3-HA	MAT α, his3-Δ200, leu2-3,2-112, lys2-801, trp1-1(am), ura3-52, UPB3-HA-TRP1	This study
FY1679-08A-BRE5-GFP-UPB3-HA-*ufd3Δ*	MATa, ura3, trp1, his3, leu2, BRE5-GFP-KanMX6, UPB3-HA-TRP1, YKL213c::HIS	This study
Plasmids		
p426-Ubp3	2μ URA3 ADH-Ubp3-6His	Cohen et al., 2003a
YEp96-6HisUb	2μ TPR1 CUP-6His-UB	Gift from S. Dupre
pRS315-RPL25eGFP		Gadal et al., 2001
pCK71	pRS316 HOG1-GFP	Kraft et al., 2008
pCK15	pRS316 GFP-ATG8	Susuki et al., 2001

Supplementary Figures Legends

Suppl. Figure 1. Identification of the Ubp3/Bre5 interactome

(A) Cells expressing Protein A (PrA), Bre5-PrA or Ubp3-PrA were cryolyzed and grindates were affinity purified on IgG-coupled magnetic beads and bound proteins were identified by mass spectrometry analysis. Baits are indicated in black and preys relevent for this study are indicated in grey. (B) Functions of the Ubp3/Bre5 interaction partners.

Suppl. Figure 2 : Lysates from wt and mutant cells expressing Bre5-GFP were immunoprecipitated using anti-Cdc48 antibodies. Immunoprecipitates were resolved by SDS–PAGE and analyzed by WB with anti-Cdc48 or anti-GFP antibodies.

Suppl. Figure 3 : Wild-type and mutant cells were cultured in rich medium in the absence or presence of of Rapamycin (20 ng/ml).

Suppl. Figure 4 : Ufd3-dependent regulation of ubiquitin expression level is not involved in ribophagy. Wild-type and ufd3Δ cells expressing both Rpl25-GFP and a cupper-inducible 6His-tagged version of ubiquitin were starved for the indicated period of time. Degradation of Rpl25-GFP was analyzed by western-blotting in whole-cell extracts using anti-GFP antibodies and quantified as in Figure 4.

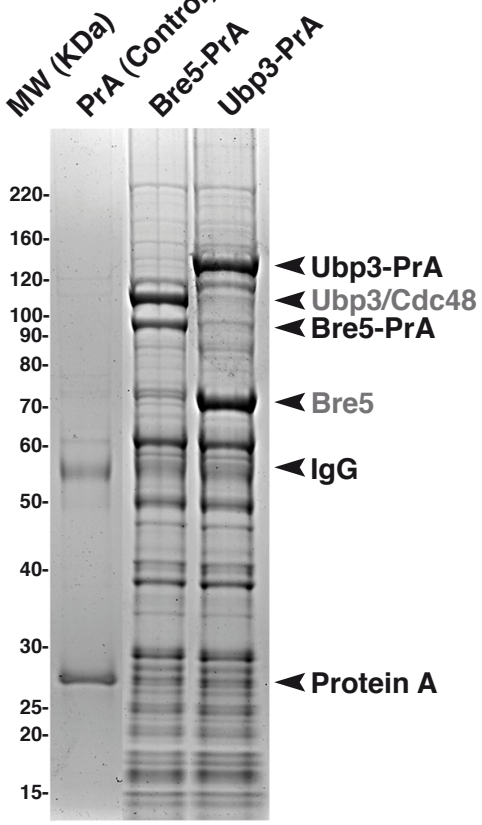
Suppl. Figure 5 : Cells expressing Rpl25-GFP were grown in rich medium (prior starvation), starved in SD-N for 24h at 25°C (starvation) and examined both by fluorescence microscopy (Rpl25-GFP) and interferential contrast (DIC).

Suppl. Figure 6 : Wild-type and mutant cells expressing GFP-Atg8 were starved for the indicated period of time. Degradation of GFP-tagged proteins was then analyzed by western-blotting in whole-cell extracts using anti-GFP antibodies and quantified as in Figure 4.

Suppl. Figure 7 : erg6Δ cells expressing Rpl25-GFP were starved at 25°C in the absence or presence of MG132 (50 μM), clasto-lactacystin β-lactone (20 μM) for the indicated period of time as in Figure 5E. Accumulation of total poly-ubiquitylated proteins upon treatment with proteasome inhibitors was analyzed by western blotting using anti-ubiquitin antibodies

Suppl. Figure 8: Quantification of the ribophagy assay shown in Figure 3C.

A



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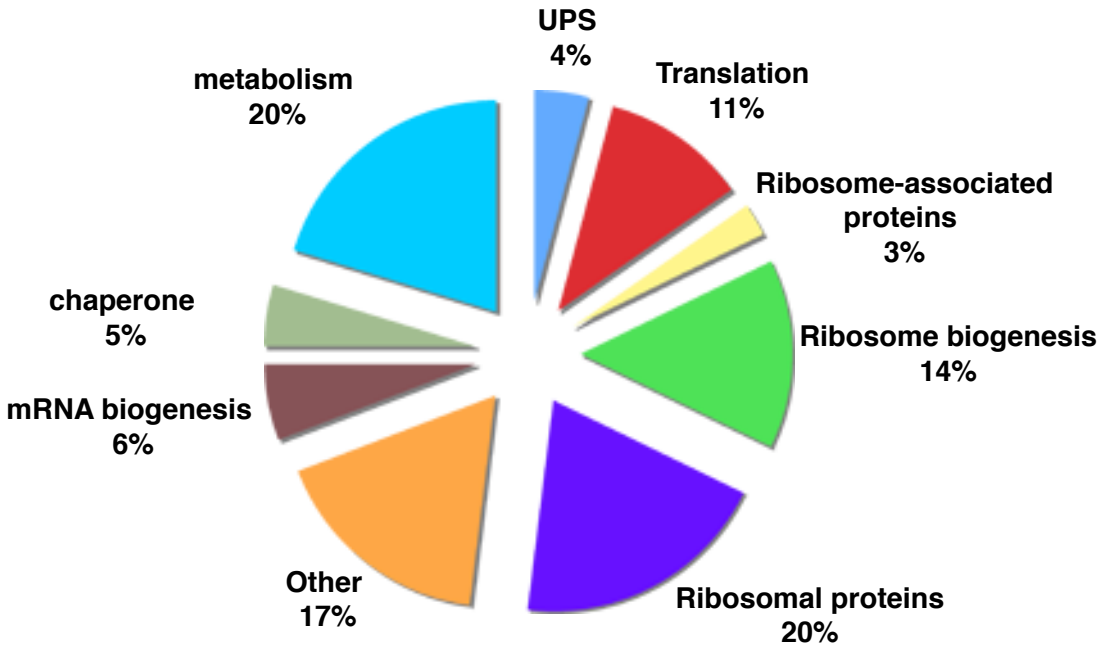
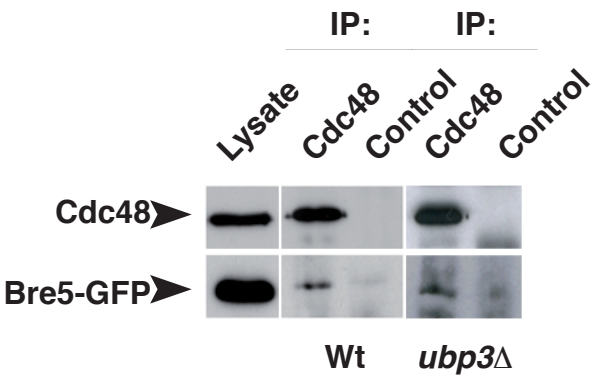
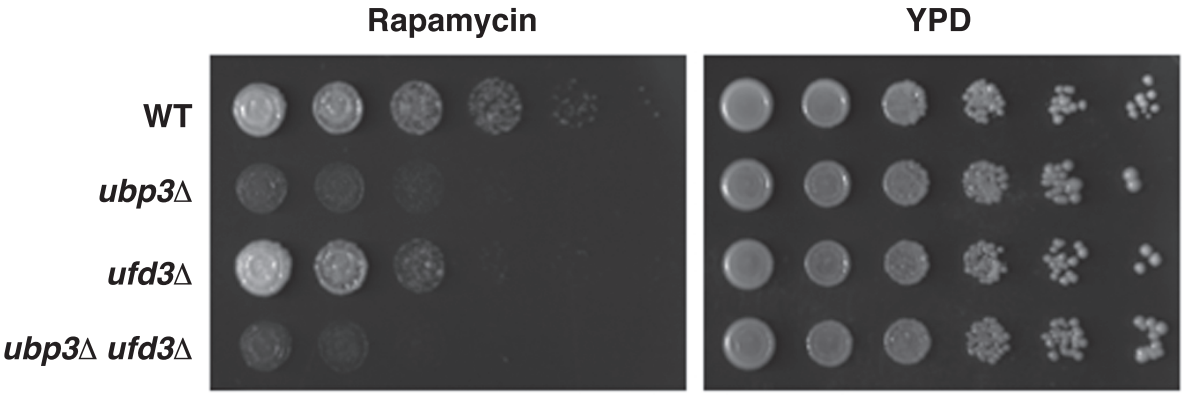


Ossareh-Nazari et al., Supplementary Figure 1

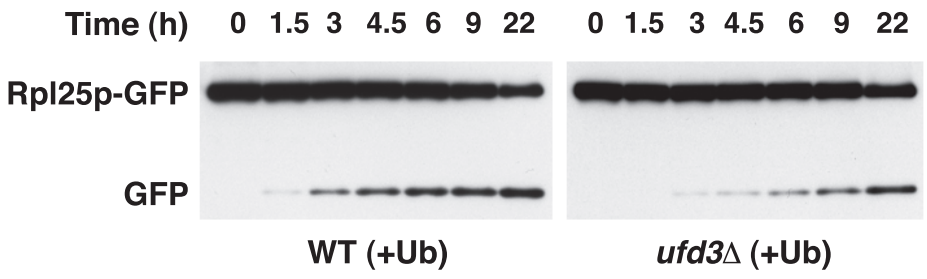
Ossareh-Nazari, Supplementary Figure 2



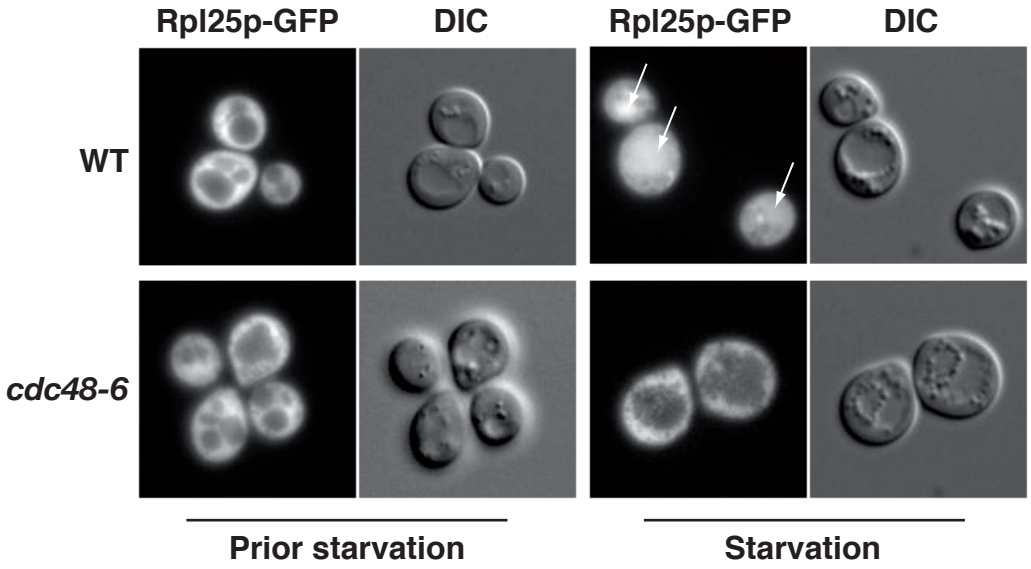
Ossareh-Nazari, Supplementary Figure 3



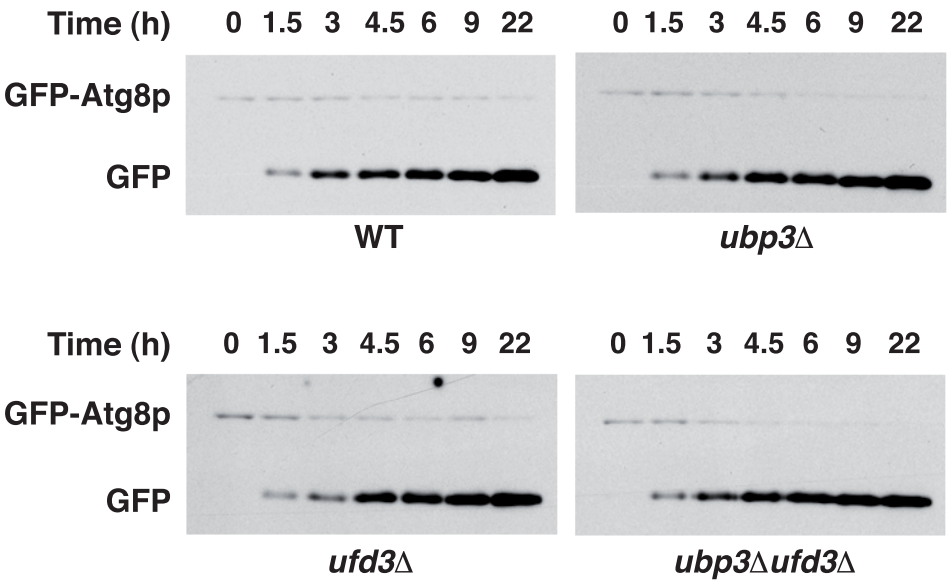
Ossareh-Nazari, Supplementary Figure 4



Ossareh-Nazari, Supplementary Figure 5



Ossareh-Nazari, Supplementary Figure 6



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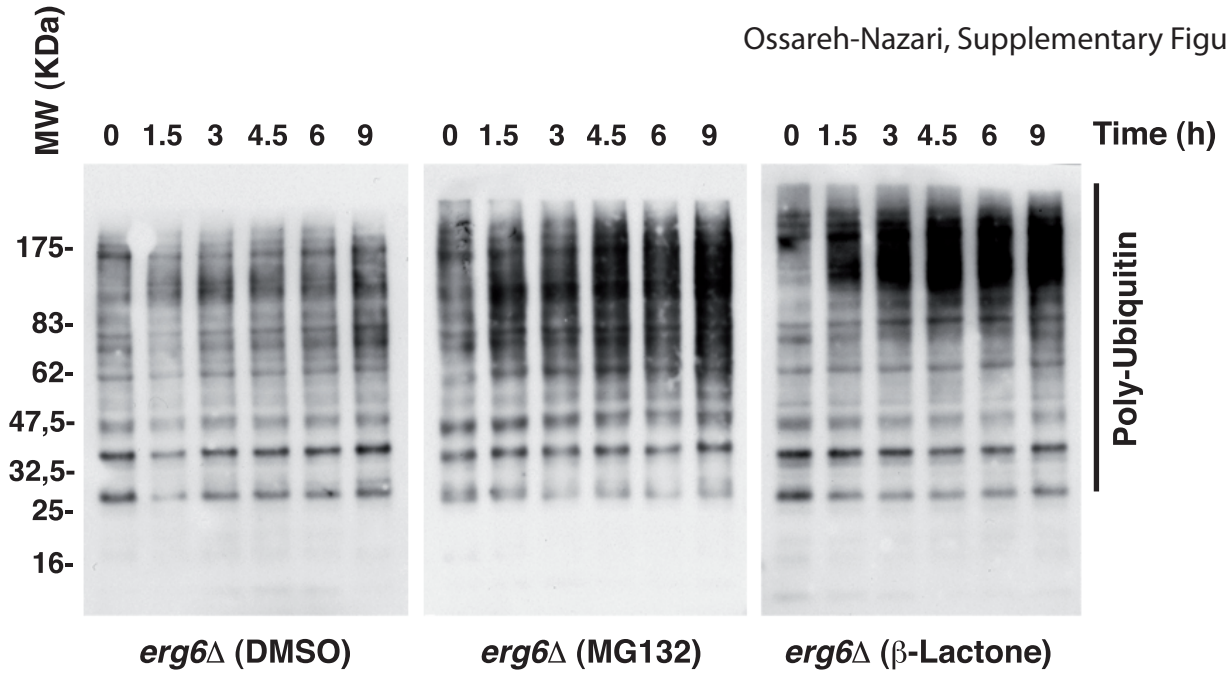

Ossareh-Nazari et al., Supplementary Figure 8