Supplemental data

MONOUBIQUITINATION OF RPN10 REGULATES SUBSTRATE RECRUITMENT TO THE PROTEASOME

Marta Isasa, Elijah J. Katz, Woong Kim, Verónica Yugo, Sheyla González, Donald S. Kirkpatrick, Timothy M. Thomson, Daniel Finley, Steven P. Gygi and Bernat Crosas

Supplemental Experimental Procedures

Yeast Methods and Media

Tetrad dissection and strain transformation were performed following standard techniques (Rose *et al*., 1990). YPD medium consisted of 1% yeast extract, 2% Bacto-Peptone, and 2% dextrose. Synthetic media consisted of 0.7% Yeast Nitrogen Base supplemented with amino acids, adenine and uracil as described (Rose *et al*., 1990), 2% dextrose (SDC) or, if necessary, 1% galactose (SGRC). For plasmid selection, synthetic media lacking leucine or uracil, or both were prepared. To remove plasmid carrying URA3 marker, 5-fluoroorotic acid (5-FOA) 0.1% w/v final was used. For cadmium sensitivity screenings, cadmium sulfate to the concentrations indicated was added to media. Samples taken from growing cultures were normalized by optical density at 600 nm using an Ultrospec 2000 spectrophotometer (GE Healthcare). To rescue growth of *RSP5* gene deletion or thermosensitive mutants at restrictive temperatures sorbitol 1M was added to media (Figure S3C; Kee *et al*., 2005). Images of colony spot assays were taken in the Image Service of CRAG (CID-CSIC) by A. Sànchez. Detailed description of genotypes of strains is provided in Table S1.

Analysis of endogenous Rpn10

Yeast wild-type strain was grown under normal conditions and, starting at an OD_{600} of 5, an equivalent number of cells were taken at the indicated time points (Figure 1A). For Figures S1A and S3D, *rpn10*Δ and *rsp5*Δ strains were respectively used. Cells were harvested and resuspended with buffer composed of 50 mM Tris-HCl (pH 7.8), 1 mM EDTA and 1x concentration of protease complete inhibitor cocktail (-EDTA, Roche). Cells were lysated by means of ultrasounds and by vortexing with glass beads. Supernatant was resolved by SDS-PAGE and analyzed by immunoblot against Rpn10.

UR Fractions

Fractions UR8 and UR10 were obtained following the method described in Crosas *et al* ., 2006. bulk ubiquitin conjugating and deconjugating activities of UR8 and UR10 fractions are shown in Figure

S2A.

Expression and purification of GST-fusion proteins in *E.coli*

In *E.coli*, glutathione S-transferase fusion vectors (pGEX-4T-3) were used to express and purify the following proteins: Rpn10, Rpn10^{K84only}, Rpn10-Ub, Rpn10-Ub^{I44A}, Rsp5, Ubc4, Ubp2, Ubp6, S5a, Ub and Ub^{I44A}. Ubp6^{C118A} was kindly provided by John Hanna. Bacterial cultures (2L) were grown to an OD_{600} of 0.7, induced with 500 µM isopropylthiogalactoside (IPTG) for 15 h at room temperature, resuspended with 2 volumes of 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1x concentration of protease complete inhibitor cocktail EDTA free (GE Healthcare) buffer and lysed using a cell disrupter (Constant Cell Disruptor Systems). The supernatant was mixed with Glutathione (GSH) Sepharose 4B beads at a ratio of 1 mL of 50% beads slurry to 1 L of initial culture size. The mixture was incubated at 4ºC rolling for 1 h. Beads were washed with 50 bed volumes of the previous lysis buffer supplemented with 150 mM NaCl. Proteins were eluted either with SDS loading buffer or with a reduced glutathione buffer of 50 mM Tris-HCl pH 7.4, 1 mM EDTA and 30 mM reduced glutathione. When necessary (Figures 6D and 6E), GST-fused proteins were digested with biotinylated thrombin (Novagen) (1U enzyme for 10 µg target). Reactions were incubated at room temperature for 2, 4 or 8 hours. The efficiency of cleavage was determined by SDS-PAGE analysis. Thrombin was removed by benzamidine beads (Amersham Biosciences) according to the manufacturer's instructions.

Expression and purification of GST-fusion proteins in *Saccharomyces cerevisiae*

To analyze the involvement of Rsp5 and Ubp2 in Rpn10 monoubiquitination, we used Rsp5 thermosensitive mutant (Rsp5-1), *ubp2*Δ and *rpn10*Δ*ubp2*Δ strains, all of them carrying GST-Rpn10 expressing plasmid (pEGH, Mitchell *et al*., 1992). Rsp5-1 was inactivated by a temperature shift to 35ºC for 5 hours; identical cultures were grown at 28ºC to provide a control at permissive temperature (Figure 3A). To characterize the lysines of Rpn10 modified by ubiquitin, GST-Rpn10 and mutants were expressed by means of a pEGH plasmid (Mitchell *et al*., 1992) using *ubp2*Δ*rpn10*Δ (Figures 5B-D, Lu *et al*., 2008) or *rpn10*Δ strains (Figure S3B). In both cases, cells were cultured overnight in SDC-URA to an OD_{600} of 4-5, harvested, washed twice in sterile water, resupended in SGRC-URA and grown. Cells were collected, resuspended with 2 volumes of buffer composed of 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM ATP, 5 µM MG132, 1x concentration of protease complete inhibitor cocktail EDTA free (GE Healthcare) and lysated by means of ultrasounds and by vortexing with glass beads. The lysate was mixed with Glutathione (GSH) Sepharose 4B beads at a ratio of 1 mL of 50% beads slurry to 1 L of initial culture size and incubated at 4ºC rolling for 1 h. Beads were washed with 50 bed volumes of the previous lysis buffer supplemented with 150 mM NaCl. Proteins were eluted with SDS loading buffer and analyzed by immunoblot against Rpn10.

Size exclusion chromatography

WT, *ubp2*Δ and *ubp6Δ* strains were cultured (2L) and cells were harvested, resuspended in a 2-fold volume of 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl buffer and lysed using a cell disrupter (Constant Cell Disruptor Systems). Lysate was centrifuged at 12,000xg for 30 min. The supernatant was recovered and centrifugation was repeated three times. Final lysate was filtered through a 0.45 µm filter before applying it to a Superose 6 gel filtration chromatography (GE Healthcare). The eluted fractions were 10-fold concentrated by means of Microcon YM-30 (Millipore), resolved by SDS-PAGE and analyzed by immunoblot against specific antibodies.

Binding experiments in GSH Sepharose

Rpn10, Rpn10-Ub and Rpn10-Ub^{I44A} were purified as GST-fusions and used as immobilized phase in the binding assays. Equal amounts of input were incubated with 30 µL of a fraction of cellular ubiquitin conjugates (Figure 6B) or 0.5 µg purified proteasomes *rpn10*Δ (Figure 6F) for 1 h at 4ºC in the presence of binding buffer containing 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 µg mL^{-1} BSA, 1x concentration of protease complete inhibitor cocktail EDTA free (GE Healthcare). Beads were washed with 10 volumes of the previous buffer supplemented with 150 mM NaCl. Bound proteins were eluted by boiling 5 min in 2x Laemmli buffer and separated by SDS-PAGE gel for immunoblot against specific antibodies.

Binding experiments in Ni-NTA agarose

The fractions of ubiquitin conjugates were obtained from strain SJR125 or SBC9 (See table S1). Elution was performed by an increasing concentration of imidazole (from 25 mM to 1M). Total protein concentration (mg/mL) and LLVY-AMC activity were assessed for all fractions (Figure S5A). For the binding assay shown in Figure 6A, the last fraction was used. Previous to binding assay, conjugate sample was incubated with 5 mM ATP and 5 µM of MG132 for 1 h at 37ºC. Then, conjugates were bound to Ni-NTA agarose beads and equilibrated with buffer containing 50 mM Tris-HCl pH 7.4, 1 mM EDTA and 1x concentration of protease complete inhibitor cocktail EDTA free (GE Healthcare), 100 µg mL^{-1} BSA, 1 µg of ubiquitin aldehyde and 1 µM of phenanthroline. Ubiquitin conjugates were then incubated at 4 ºC with equal amounts of unmodified Rpn10 and mUb-Rpn10 (20 µg). To analyze the status of mUb-Rpn10 during the binding assay, samples were taken every 30 minutes and analyzed by SDS-PAGE and immunoblot against Rpn10 (Figure S5B). After 90 minutes, beads were washed with 50 bed volumes of the previous buffer with 200 mM NaCl and then eluted with SDS-PAGE buffer. Equivalent volumes of eluate were analyzed by SDS-PAGE followed by immunoblot against anti-HIS and anti-Rpn10 antibodies. For Figure 6C, to amplify polyubiquitin, 5 µg of commercial pure ubiquitin chains (Enzo Life Sciences) were incubated 2 hours at 37ºC with 1 µg human activating E1 (Enzo Life Sciences), 2 µg Ubc4, 2.5 µg 6HISxUb (Enzo Life Sciences) and buffer consisting of 100

mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM ATP, 10 mM $MgCl_2$ and 1 mM DTT. Polyubiquitin chains were bound to Ni-NTA agarose beads and equilibrated with 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 μg mL^{-1} BSA, 1 μg of ubiquitin aldehyde and 1 μM of phenanthroline. *In vitro* monoubiquitination reactions of Rpn10 and $Rpn10^{k84only}$ were performed (see below). 10 μg of unmodified Rpn10, mUb-Rpn10, mUb $Rpn10^{K84only}$, Rpn10-Ub and Rpn10-Ub^{I44A} were used to test the affinity to ubiquitin chains. The binding assay was carried out for 90 minutes at 4ºC. Beads were washed, eluted and analyzed as described for the previous binding assay.

Assays of Rpn10 ubiquitination *in vitro*

Reactions involving ubiquitin conjugation of GST-Rpn10 and GST-$Rpn10^{K84only}$ contained, per 100 μL of reaction: 0.5-1 μg of recombinant protein, 1 μg of human activating E1 (Enzo Life Sciences), 2 μg GST-Ubc4, 5 μg GST-Rsp5 and 1 μg ubiquitin. If required, 0.5 μg of purified proteasomes (WT and $Rpn10^{UIM}$) were added. Reactions were carried out in 100 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM ATP, 10 mM $MgCl_2$, 1 mM DTT buffer at 37ºC for the indicated times. Different types of commercial ubiquitins were used: wild-type, 6His-tagged, biotinylated and methylated ubiquitin (Enzo Life Sciences). When necessary, reactions using recombinant Ub^{I44A} were also performed (Figure 6D). Reactions were stopped by the addition of 5x SDS-PAGE loading buffer and separated by SDS-PAGE for western blot analysis against Rpn10.

Assays of deubiquitinating activities

For the deubiquitinating assays, approximately 22.5 μg of fractions rich in proteasomes and mUb-Rpn10 from a *ubp6Δ* strain (Figure S3E) were incubated separately with equimolar amounts of GST-Ubp2 (5.5 μg), GST-Ubp6 (1.8 μg) or GST-$Ubp6^{C118A}$ (1.8 μg), in buffer containing 100 mM Tris-HCl pH 7.4, 200 mM NaCl and 5 μM MG132. Reactions were performed at 37ºC and stopped by the addition of 5x Laemmli buffer at the indicated times (Figure 3B). Samples were analyzed by SDS-PAGE and immunoblot against Rpn10. To obtain proteasome fractions rich in mUb-Rpn10, strains SJR125 and SBC10 (carrying the deletion of *UBP6* gene, see table S1) were used in a standard Ni-NTA agarose chromatography. The fraction was isloated from the peak of elution of LLVY-AMC activity in an imidazole gradient elution. Fractions were analyzed as shown in Figure S3E.

Purification of Proteasomes

Proteasomes were purified from yeast strains carrying Rpn11-TEV-ProA tag (Leggett *et al*., 2002). Cells were harvested, resuspended in a 2-fold volume of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA buffer, and lysed in a cell disrupter (Constant Cell Disruptor Systems). Lysate was clarified at 15,000x*g* for 25 min, incubated with IgG resin (GE Healthcare) for 1 hr at 4°C, and the resin washed with 100 bed volumes 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl buffer. Proteasomes

were eluted by equilibrating the IgG resin with 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT buffer (TEB), then incubating with 1.5 volumes of TEB containing 100 U/ml of 6His-TEV protease at 30°C for 1 hr. TEV protease was subsequently removed from the eluate by incubation with Ni-NTA resin (Qiagen) at 4°C for 15 min.

Assays of S5a ubiquitination *in vitro*

HeLa cells were lysed in buffer containing 20 mM Tris-HCl pH 7.4, 0,5% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1x concentration of protease complete inhibitor cocktail EDTA free (GE Healthcare) and lysates were then cleared by centrifugation. The ubiquitination reaction of S5a contained per 50 µL of final volume: 10 µL HeLa cells extract, 2 µg S5a GST-fused and 1 µg ubiquitin. To optimize reaction efficiency, 1 µg of human activating E1 (Enzo Life Sciences) and 5 µg UbcH5a (Enzo Life Sciences) were added. Reactions were incubated at 37ºC with buffer containing 100 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM ATP, 10 mM $MgCl_2$, 1 mM DTT and 1x concentration of protease complete inhibitor cocktail EDTA free (GE Healthcare). Reactions were stopped at the indicated times by adding 5x SDS-PAGE loading buffer. Proteins were resolved by SDS-PAGE and visualized by immunoblot using Rpn10 antibody.

Degradation assays of cyclin B1

Purified proteasomes lacking Rpn10 subunit (25 nM) were incubated 15 min at 30ºC with *E. coli* expressed Rpn10 forms (50 nM each) and buffer containing 50 mM TrisHCl pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$ and 1 mM DTT. Then the degradation assay was initiated by adding 5 µL of polyubiquitinated cyclin B1 (stock of 50 µg/mL) and 5 mM ATP. At the indicated times, samples were taken and the reaction was terminated by adding SDS sample buffer and analyzed by western blot with specific antibodies.

Stress sensitivity assays

Wild-type and *ubp2*Δ cells were cultured under normal growth conditions to an OD_{600} of 0.8-1, then cells were exposed to different stresses: cold (14ºC) and heat (37ºC) shock, oxidative stress ($CdSO_4$, final concentration of 200 µM), osmotic stress (NaCl, final concentration of 500 mM) and DNA damage stress (EMS, final concentration of 0.08%). Cultures were grown for additional 21 hours and an equivalent number of cells were taken at the indicated time points (Figures 7A to 7G). Cells were harvested, resuspended, lysated and analyzed as described for Figure 1A (Section of *Analysis of endogenous Rpn10*).

Electrophoresis, isoelectric focusing and immunoblot analysis

For two-dimensional electrophoresis, the sample was applied in 8 M urea, 2% CHAPS, 0.5% ampholytes, 20 mM DTT, and 0.002% bromophenol blue**.** Isoelectric focusing extended over a pH range of 3-10 using appropriate ampholytes and strips (Invitrogen). Applied voltage was increased stepwise to 2000 V. The second dimension, 4-12% SDS-PAGE (Invitrogen), was performed according to manufacturer's instructions. For one-dimension electrophoresis, 4-12% gradients PAGE system with 1xMES or 1xMOPS buffers were used (Invitrogen). For immunoblots, proteins were transferred to polyvinylidene difluoride membranes, which were then blocked, and incubated with antibodies using TBST buffer (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) with 5% w/v nonfat powdered milk, and washed with TBST and with H_2O. Detection was performed by chemiluminescence, using horseradish peroxidase-conjugated donkey anti-rabbit antibodies (GE Healthcare).

Antibodies

Polyclonal antibodies to Rpn10 were raised in rabbits against full length protein and second bleed antisera was used. Anti-cyclin B antibody (Ab-2) was obtained from Neomarkers. Anti-ubiquitin and anti-HIS antibodies were obtained from Enzo Life Science. Antibody was from α7, rpn12 were kindly provided by D. Finley.

Protein detection in gels by silver staining

The gel was soaked for 10 min in fixing solution containing 40% methanol and 13.5% formaldehyde and washed twice in MilliQ water for 5 min each. Gel was incubated for 1 min in 0.02% sodium thiosulfate and washed twice, 30 seconds each, in MilliQ water. Silver solution (0.1% silver nitrate) was added, shaken for 10 min and rinsed twice with deionized water. Then the gel was rinsed shortly with the developing solution (3% sodium carbonate and 0.05% formaldehyde). The solution was discarded and a new portion of the developing solution was added. The protein image was developed by incubating the gel with the developing solution for 5 min. When the desired intensity of the bands was reached, reaction was stopped by adding 6 grams of solid citric acid and gel was shaken for 10 min. Finally, the gel was washed in MilliQ water for 30 min.

Plasmid constructions

Plasmid and oligonucleotid primer detailed information is included in Tables S2 and S3, respectively. All single and multiple nucleotide mutations were performed using Quick Change Site Directed Mutagenesis kit (Stratagene). Plasmid pMIC24 was made by removing the STOP codon of pMIC26 by means of PCR amplification using *Rpn10_No TAG Fw* and *Rpn10_No TAG Rev* primers. UBI4 was derived from genomic DNA by PCR using primers *Ubi4 Fw* and *Ubi4 Rev* and cloned into *SalI* and *NotI* sites of pMIC24, generating pMIC27. To mutate isoleucine 44 of the ubiquitin group fused at the C-terminus of Rpn10 we used pMIC27 as a template. The primers used for the PCR reaction were

UbI44A Fw and its reverse complement. Rpn10 was derived from a cloning vector and then cloned into *HindIII* and *XbaI* sites of pYES2 (Invitrogen), generating plasmid pMIC33. For the ubiquitin fusion at Ct of Rpn10 and the subsequent mutation of isoleucine 44 to alanine (pMIC15 and pMIC39, respectively), we followed the same strategy described for plasmids pMIC27 and pMIC28. pMIC71 was derived from pMIC70 by PCR amplification (primers *R84K-EcoRI Fw* and *R84K-SalI Rev*) and cloned into *EcoRI* and *SalI* cleavage sites. pMIC72 was kindly provided by Dr. I. Dikic and was used as a template to generate pMIC73. Alanine 44 of ubiquitin was mutated to isoleucine by means of a mutagenesis reaction using primers *Ub pGEX Fw* and its reverse complement.

Rpn10 WT and its lysine to arginine mutants are all cloned into pEG-KT vector described in Mitchell *et al.*, 1992. Plasmid pMIC18 was kindly provided by Dr. Heng Zhu and was used as a template for constructing pMIC20, pMIC21, pMIC48, pMIC50, pMIC52, pMIC55 and pMIC57. The primers used were *K104R Fw*, *K130-134R Fw*, *K40R Fw*, *K71R Fw*, *K268R Fw*, *K84R Fw*, *K99R Fw* and its reverse complement, respectively. pMIC30 was derived from pMIC21 using *K1045R Fw* and its reverse complement. pMIC54 was made using pMIC30 as a template and *K99R Fw* and its reverse complement. From pMIC54 we generated pMIC60 using *K268R Fw* and its reverse complement. pMIC66 was derived from pMIC60 using *K84R Fw* and its reverse complement. pMIC67 was built from pMIC66 using *K71R Fw* primer and its reverse complement. pMIC69 was made from pMIC67 using *K40R Fw* and its reverse complement. From pMIC55 we generated pMIC59 and pMIC61 using *K268R Fw*, *K71R Fw*, and its reverse complement, respectively. pMIC63 was derived from pMIC59 using *K99R Fw* and its reverse complement. pMIC65 was derived from pMIC63 using *K71R Fw* and its reverse complement. Finally, pMIC70 was derived from pMIC69 using *R84K Fw* primer and its reverse complement. Both DNA strands of cloned and mutated fragments in all plasmids described in this study were sequenced twice.

Supplemental References

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P. and Boeke, J.D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast *14*, 115-132.

Chen, L. and Madura, K. (2002). Rad23 promotes the targeting of proteolytic substrates to the proteasome. Mol. Cell. Biol. 22, 4902-4913.

Finley, D., Ozkaynak, E. and Varshavsky, A. (1987). The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation and other stresses. Cell *48*, 1035-1046.

Huibregtse, J.M., Yang, J.C. and Beaudenon, S.L. (1997) The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase. Proc Natl Acad Sci USA 94, 3656-3661.

Kee, Y., Lyon, N. and Huibregtse, J.M. (2005). The Rsp5 ubiquitin ligase is coupled to and antagonized by the Ubp2 deubiquitinating enzyme. EMBO J. 24, 2414-2424.

Leggett, D.S., Hanna, J., Borodovsky, A., Crosas, B., Schmidt, M., Baker, R.T., Walz, T., Ploegh, H. and Finley, D. (2002). Multiple associated proteins regulate proteasome structure and function. Mol. Cell 10, 495-507.

Lu, J.Y., Lin, Y.Y., Qian, J., Tao, S.C., Zhu, J., Pickart, C. and Zhu, H. (2008) Functional dissection of a HECT ubiquitin E3 ligase. Mol. Cell. Proteomics 7, 35-45.

Mitchell, D.A., Marshall, T.K. and Deschenes, R.J. (1992) Vectors for the inducible overexpression of glutathione S-transferase fusion proteins in yeast. Yeast 9, 715-722.

Rose, M.D., Winston, F. and Hieter, P. (1990). Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Siepe, D. and Jentsch, S. (2009) Prolyl isomerase Pin1 acts as a switch to control the degree of substrate ubiquitylation. Nature Cell Biology, 11, 967-972.

Table S1: Strains used in the present study

Strain	Genotype	Reference
SUB62	***MATa lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1***	**Finley *et al*., 1987**
Sub62 based strains (relevant genotype):		
SDL133	*rpn11::RPN11-TEVProA (HIS3)*	Leggett *et al*., 2002
SDL145	*rpn11::RPN11-TEVProA (HIS3) ubp6::URA3*	Leggett *et al*., 2002
SBC6	*rpn11::RPN11-TEVProA (HIS3) hul5::kanMX4*	Crosas *et al*., 2006
SBC50	*rpn11::RPN11-TEVProA (HIS3) Ubr1::kanMX4*	This study
SBC51	*rpn11::RPN11-TEVProA (HIS3) ufd4::kanMX4*	This study
SJR125	*[am] ubi1-Δ1::TRP1 ubi2-Δ2::ura3 ubi3-Δub-2 ubi4-Δ2::LEU2 arg4::HYG pdr5::ura3 [pUB221] [pUB100]*	Crosas *et al*., 2006
SBC9	*[am] ubi1-Δ1::TRP1 ubi2-Δ2::ura3 ubi3-Δub-2 ubi4-Δ2::LEU2 arg4::HYG pdr5::ura3 hul5::kanMX4 [pUB221] [pUB100]*	Crosas *et al*., 2006
SBC10	*[am] ubi1-Δ1::TRP1 ubi2-Δ2::ura3 ubi3-Δub-2 ubi4-Δ2::LEU2 arg4::HYG pdr5::ura3 Ubp6::kanMX4 [pUB221] [pUB100]*	This study
SY74	*rpt1::HIS3 rpn10::rpn10-uim-natMX pEL36 (TRP1)*	S. Elsasser
sJH177	*ubp6::URA3 rpn11::RPN11-TEV-ProA (HIS3) [pJH60]*	J. Hanna
SDL73	*MATα rpn11::RPN11-TEVProA (HIS3)*	Leggett *et al*., 2002
SY36	*rpt1::HIS3 pEL36 (TRP1)*	S. Elsasser
SBC52	*Rpn10::natMX*	This study
SBC53	*Rad23::KanMX4*	This study
SBC54	*Rpn10::natMX Rad23::KanMX4*	This study
YDS124	*Rsp5Δ::HIS3MX [Ycplac33pRSP5 URA3]*	Siepe and Jentsch, 2009
BY4741	***MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0***	**Brachmann *et al*., 1998**
BY4741 based strains (relevant genotype):		
SBC55	*ubp2::kanMX4*	Euroscarf
SBC56	*rpn10::RPN10-TAP (HIS3)*	Euroscarf
FY56	***MATa ura3-52 his4-912σR5 lys2Δ 128***	**Huibregtse *et al*., 1997**
FY56 based strains:		
FW1808	*MATa rsp5-1 ura3-52 his4-912σR5 lys2Δ 128*	Huibregtse *et al*., 1997
JYL01	*MATa ura3-52 his4-912σR5 lys2Δ 128 RPN10::URA3*	Lu *et al*., 2008
JYL02	*MATa rsp5-1 ura3-52 his4-912σR5 lys2Δ 128 RPN10::URA3*	Lu *et al*., 2008
JYL19	*MATa ura3-52 his4-912σR5 lys2Δ 128 RPN10::URA3 ubp2Δ::KanMX*	Lu *et al*., 2008
JYL20	*MATa rsp5-1 ura3-52 his4-912σR5 lys2Δ 128 RPN10::URA3 ubp2Δ::KanMX*	Lu *et al*., 2008

Table S2: Plasmid vectors used in the present study

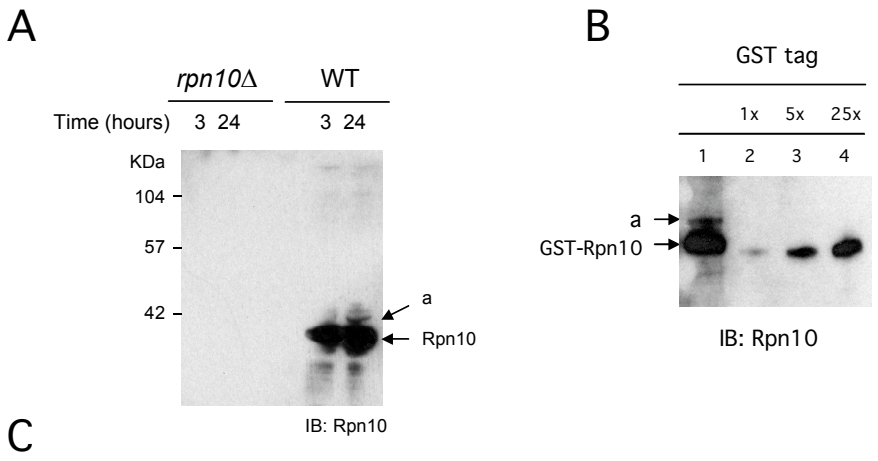
Plasmid	Details	Reference
pMIC15	Ubiquitin fused at Ct of Rpn10 (pYES2)	This study
pMIC18	Rpn10 WT (pEG-KT)	Provided by Dr. H. Zhu
pMIC20	GST-Rpn10 K104R (pEG-KT)	This study
pMIC21	GST-Rpn10 K130, 133, 134R (pEG-KT)	This study
pMIC24	GST-Rpn10 No STOP (pGEX-4T-3)	This study
pMIC26	GST-Rpn10 STOP (pGEX-4T-3)	Crosas, *et al*., 2006
pMIC27	Ubiquitin fused at Ct of GST-Rpn10 (pGEX-4T-3)	This study
pMIC28	Ubiquitin I44A fused at Ct of GST-Rpn10 (pGEX-4T-3)	This study
pMIC30	GST-Rpn10 K104, 130, 133, 134R (pEG-KT)	This study
pMIC33	Rpn10 WT (pYES2)	This study
pMIC39	Ubiquitin I44A fused at Ct of Rpn10 (pYES2)	This study
pMIC48	GST-Rpn10 K40R (pEG-KT)	This study
pMIC50	GST-Rpn1'0 K71R (pEG-KT)	This study
pMIC52	GST-Rpn10 K268R (pEG-KT)	This study
pMIC54	GST-Rpn10 K99, 104, 130, 133, 134R (pEG-KT)	This study
pMIC55	GST-Rpn10 K84R (pEG-KT)	This study
pMIC57	GST-Rpn10 K99R (pEG-KT)	This study
pMIC59	GST-Rpn10 K84, 268R (pEG-KT)	This study
pMIC60	GST-Rpn10 K99, 104, 130, 133, 134, 268R (pEG-KT)	This study
pMIC61	GST-Rpn10 K71, 84R (pEG-KT)	This study
pMIC63	GST-Rpn10 K84, 99, 268R (pEG-KT)	This study
pMIC65	GST-Rpn10 K71, 84, 99, 268R (pEG-KT)	This study
pMIC66	GST-Rpn10 K84, 99, 104, 130, 133, 134, 268R (pEG-KT)	This study
pMIC67	GST-Rpn10 K71, 84, 99, 104, 130, 133, 134, 268R (pEG-KT)	This study
pMIC69	GST-Rpn10 K40, 71, 84, 99, 104, 130, 133, 134, 268R (pEG-KT)	This study
pMIC70	GST-Rpn10 K40, 71, 99, 104, 130, 133, 134, 268R (pEG-KT)	This study
pMIC71	GST-Rpn10 K40, 71, 99, 104, 130, 133, 134, 268R (pGEX-4T-3)	This study
pMIC72	GST-Ubiquitn I44A (pGEX)	Provided by Dr. I. Dikic
pMIC73	GST-Ubiquitin (pGEX)	This study

Table S3: Oligonucleotid primers used in the present study

Only forward sequences of mutagenic primers are shown

Name	Sequence
Rpn10_NoTAG Fw	CGTAGGTACCGAATTCCATGGTATTGGAAGCTACAGTG
Rpn10_NoTAG Rev	GCATGTC GACTTTGTCTTGGTGTTGTTCAGGCTG
Ubi4 Fw	CTGAGTCGACATGC AGATTTTCGTCAAGACT
Ubi4 Rev	CGATGCGGCCGCCTAACCACCTCTTAGCC TTAGCACAAG
UbI44A Fw	TCCAGATCAACAAAGATTGGCCTTTGCCGGTAAGCAGCT
K40R Fw	TCATATTTCAAGCCAGGAGAAACAGCAATCC
K71R Fw	CCGCCGAGTTTGGGAGGATTCTTGCTGGACT
K84R Fw	CGCAGATCGAGGGTAGGCTGCATATGGCCAC
K99R Fw	CTCAGCTGACTTTGAGGCATCGCCAGAATAA
K104R Fw	TTGAAGCATCGCCAGAATAGGGTCCAACATCAAAGGATT
K130-134R Fw	TTGATCAGATTGGCAAGAACACTGAGAAGGAATAATGTTGCCGTG
K268R Fw	AGCCTGAACAACACCAAGACAGATAGCATGGCAATTCCCGGG
R84K Fw	GACACGCAGATCGAGGGTAAGCTGCATATGGCCACTGCG
R84K-EcoRI Fw	CGTACCGAATTCCATGGTATTGGAAGCTACAGTG
R84K-SalI Rev	CGCTAGTCGACCTATCTGTCTTGGTGTTGTTCA
Ub PGEX Fw	CTGATCAACAAAGATTGATCTTTGCCGGTAAGCAGC

Supplementary Figures



C

Rpn10

MVLEATVLVIDNSEYSRNGDFPRTRFEAQIDSVEFIFQAKRNSNPENTVGLISGAGANPRVLSTFTAEFGKILAGLHDTQ
IEGKLHMATALQIAQLTLKHRQNKVQHQRIVAFVCSPISDSRDELIRLAKTLKKNNVAVDIINFGEIEQNTELLDEFIAA
VNNPQEETSHLLTVTPGPRLLYENIASSPIILEEGSSGMGAFGGSGGDSDANGTFMDFGVDPSMDPELAMALRLSMEEEQ
QRQERLRQQQQQQDQPEQSEQPEQHQDK

Ubiquitin

MQIFVKTLTGKTITLEVESSDTIDNVKSKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG

Figure S1. (A) Time points from exponentially growing cultures (t=3 h and t=24 h correspond to OD_{600} of 5 and 11.5, respectively) were taken and analyzed by western blotting with Rpn10 antibody. **(B)** GST-Rpn10 expressed in yeast by means of a pEGH vector, pulled down and analyzed by western blotting using anti-Rpn10 (lane 1). Different amounts of *E. coli* expressed GST-Rpn10 (lanes 2-4). **(C)** Band '*a*' shown in Figure 1B was analyzed by mass spectrometry and two abundant proteins were found: Rpn10 and ubiquitin. Sequences of Rpn10 and ubiquitin are represented, showing in green regions covered by MS analysis.

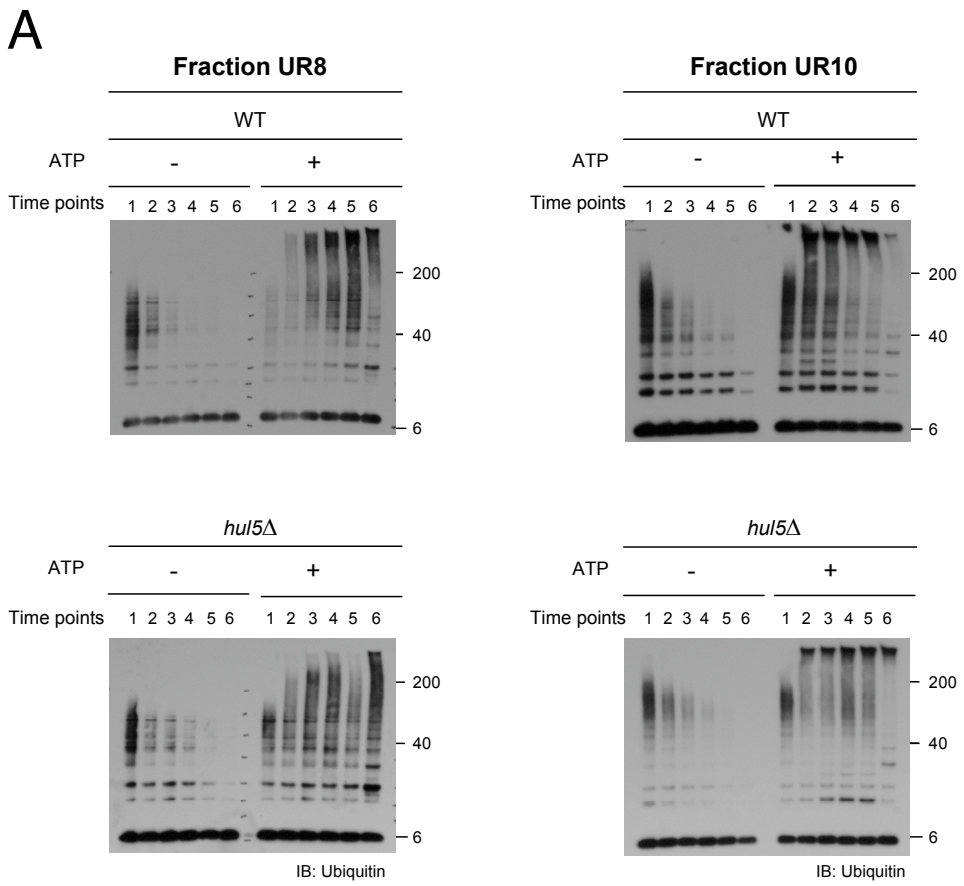


Figure S2. (A) Bulk ubiquitinating and deubiquitinating activities of fractions UR8 and UR10. Fractions, containing 5 μM MG132, were incubated at 28°C and time points were taken each 30 min, except for point 6, which was taken at 10 hours. ATP was used at 5 mM. **(B)** Monoubiquitination reactions using proteasomes and fraction UR8. Purified proteasomes (4.5 μg), incubated in the presence of 5 mM ATP and 5 μM MG132 (Lanes 1 to 3). Reactions containing wild-type proteasomes and UR8 extracts (0.45 μg) (Lanes 4 to 6). Reactions containing *hul5*Δ proteasomes and *hul5*Δ UR8 extracts (0.45 μg) (Lanes 7 to 9).

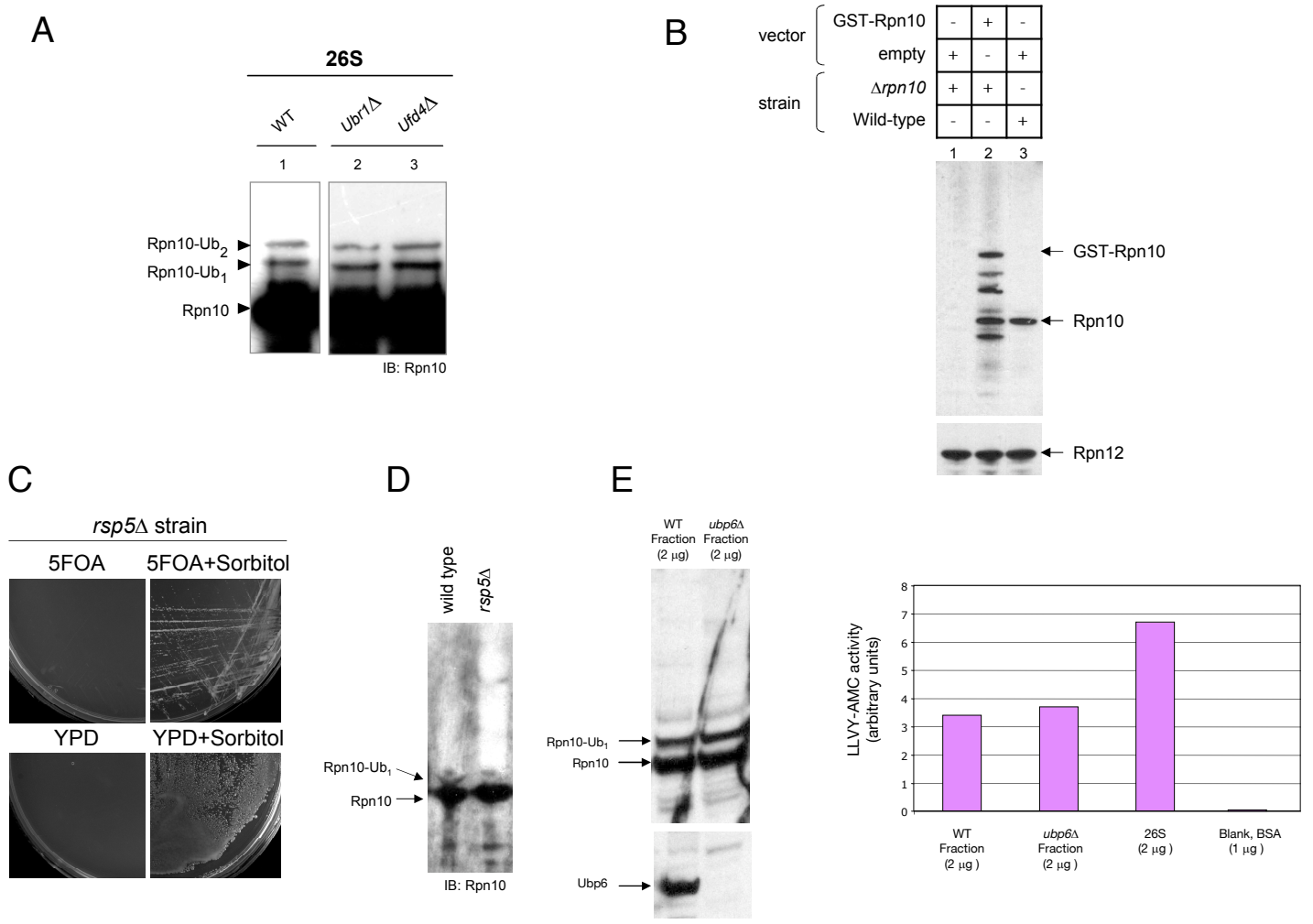
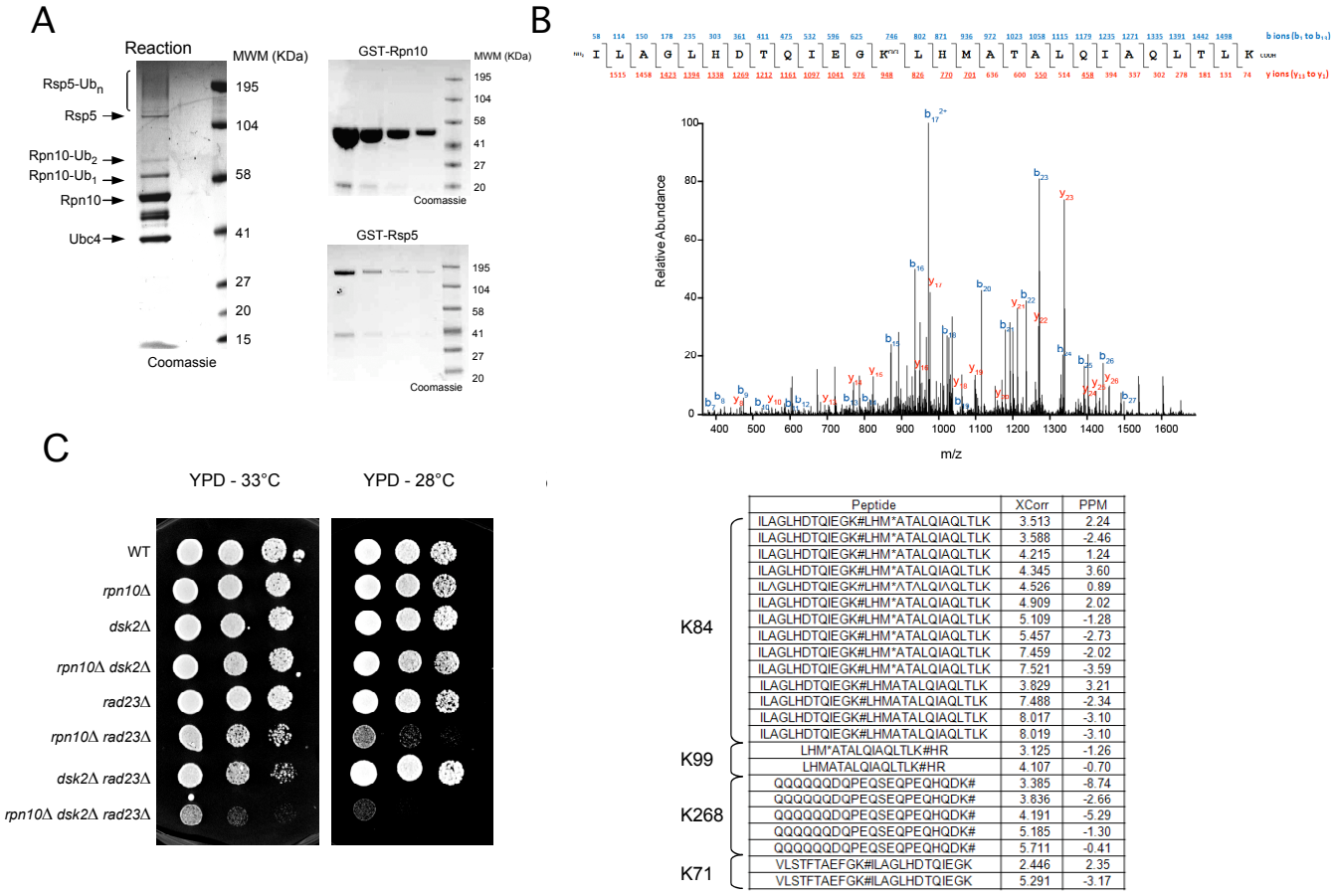


Figure S3. (A) Ubr1 and Ufd4 do not regulate levels of Rpn10 monoubiquitination. Ufd4 interacts with Rpt6 ATPase and Ubr1 binds to non-ATPase subunit Rpn2 and to Rpt6 (Xie and Varshavsky, 2000), components of the base of the proteasome regulatory particle (Glickman *et al.*, 1998). Purified proteasomes from WT, *ubr1*Δ and *ufd4*Δ strains, in the presence of 5 mM ATP and 5 μM MG132, were resolved by SDS-PAGE and analyzed by immunoblotting against Rpn10. **(B)** Levels of Rpn10 induction using galactose. Sample show levels after 5 hours of induction. Western blotting against Rpn12 is included as a loading control. **(C)** Strain YDS124 (Siepe and Jentsch, 2009) was consecutively streaked in 5FOA plates containing 1M sorbitol (3 times, third streak is shown above). Strain lacking RSP5 plasmid was viable in the presence of sorbitol (below panel). Plates were incubated 4 days at 28 °C. **(D)** Analysis of Rpn10 in wild-type and *rsp5*Δ growing cultures, as in Figure S1A. **(E)** Fractions containing proteasomes and monoubiquitinanted Rpn10, isolated from strains SJR125 and SBC10. Status of Rpn10 in fractions, analyzed by anti Rpn10 and Ubp6 western blotting (left) and LLVY-AMC hydrolyzing activities (right). Activity of fractions was compared to the activity of affinity purified proteasomes (Rpn11-Protein A tagged, third column).



	Peptide	XCorr	PPM
K84	ILAGLHDTQIEGK#LHM*ATALQIAQLTLK	3.513	2.24
	ILAGLHDTQIEGK#LHM*ATALQIAQLTLK	3.588	-2.46
	ILAGLHDTQIEGK#LHM*ATALQIAQLTLK	4.215	1.24
	ILAGLHDTQIEGK#LHM*ATALQIAQLTLK	4.345	3.60
	ILAGLHDTQIEGK#LHM*ATALQIAQLTLK	4.526	0.89
	ILAGLHDTQIEGK#LHM*ATALQIAQLTLK	4.909	2.02
	ILAGLHDTQIEGK#LHM*ATALQIAQLTLK	5.109	-1.28
	ILAGLHDTQIEGK#LHM*ATALQIAQLTLK	5.457	-2.73
	ILAGLHDTQIEGK#LHM*ATALQIAQLTLK	7.459	-2.02
	ILAGLHDTQIEGK#LHM*ATALQIAQLTLK	7.521	-3.59
	ILAGLHDTQIEGK#LHMATALQIAQLTLK	3.829	3.21
	ILAGLHDTQIEGK#LHMATALQIAQLTLK	7.488	-2.34
	ILAGLHDTQIEGK#LHMATALQIAQLTLK	8.017	-3.10
	ILAGLHDTQIEGK#LHMATALQIAQLTLK	8.019	-3.10
K99	LHM*ATALQIAQLTLK#HR	3.125	-1.26
	LHMATALQIAQLTLK#HR	4.107	-0.70
K268	QQQQQQDQPEQSEQPEQHQDK#	3.385	-8.74
	QQQQQQDQPEQSEQPEQHQDK#	3.836	-2.66
	QQQQQQDQPEQSEQPEQHQDK#	4.191	-5.29
	QQQQQQDQPEQSEQPEQHQDK#	5.185	-1.30
	QQQQQQDQPEQSEQPEQHQDK#	5.711	-0.41
K71	VLSTFTAEFGK#ILAGLHDTQIEGK	2.446	2.35
	VLSTFTAEFGK#ILAGLHDTQIEGK	5.291	-3.17

Figure S4. (A) The product of Rpn10 *in vitro* monoubiquitination was analysed by SDS-PAGE and Coomassie staining. Samples of Rsp5 and Rpn10 purified from E. coli are shown (right panels). Bands corresponding to Rpn10-Ub1 and Rpn10-Ub2 were excised and analyzed by MS. **(B)** Identification of ubiquitinated lysines by MS. Above, representative MS/MS spectrum of lysines with di-glycine motif (remaining residues after tryptic digest of ubiquitinated lysines) at lysine 84 (K^{GG}). The precursor peptide ion (m/z 1047.9167) was isolated and fragmented in mass spectrometer. Fragment ions from both termini are shown (*b*- and *y*- ions respectively) where the detected ions are underlined. Below, Lysines 71, 84, 99, and 268 showed ubiquitination on Rpn10. The peptide sequence, Sequest score (XCorr) and mass deviation are shown. Ubiquitinated lysines are indicated by K#. **(C)** . Selection of spores obtained by tetrad dissection of a diploid strain that contains *RPN10*/*rpn10*Δ, *RAD23*/*rad23*Δ and *DSK2*/*dsk*Δ heterozygotic background, kindly provided by S. Elsasser. Spores were genotyped and their growth at different temperatures was tested. *rpn10*Δ*rad23*Δ strain, used in Figure 5F, shows slow growth, as reported by Chen and Madura, 2002.

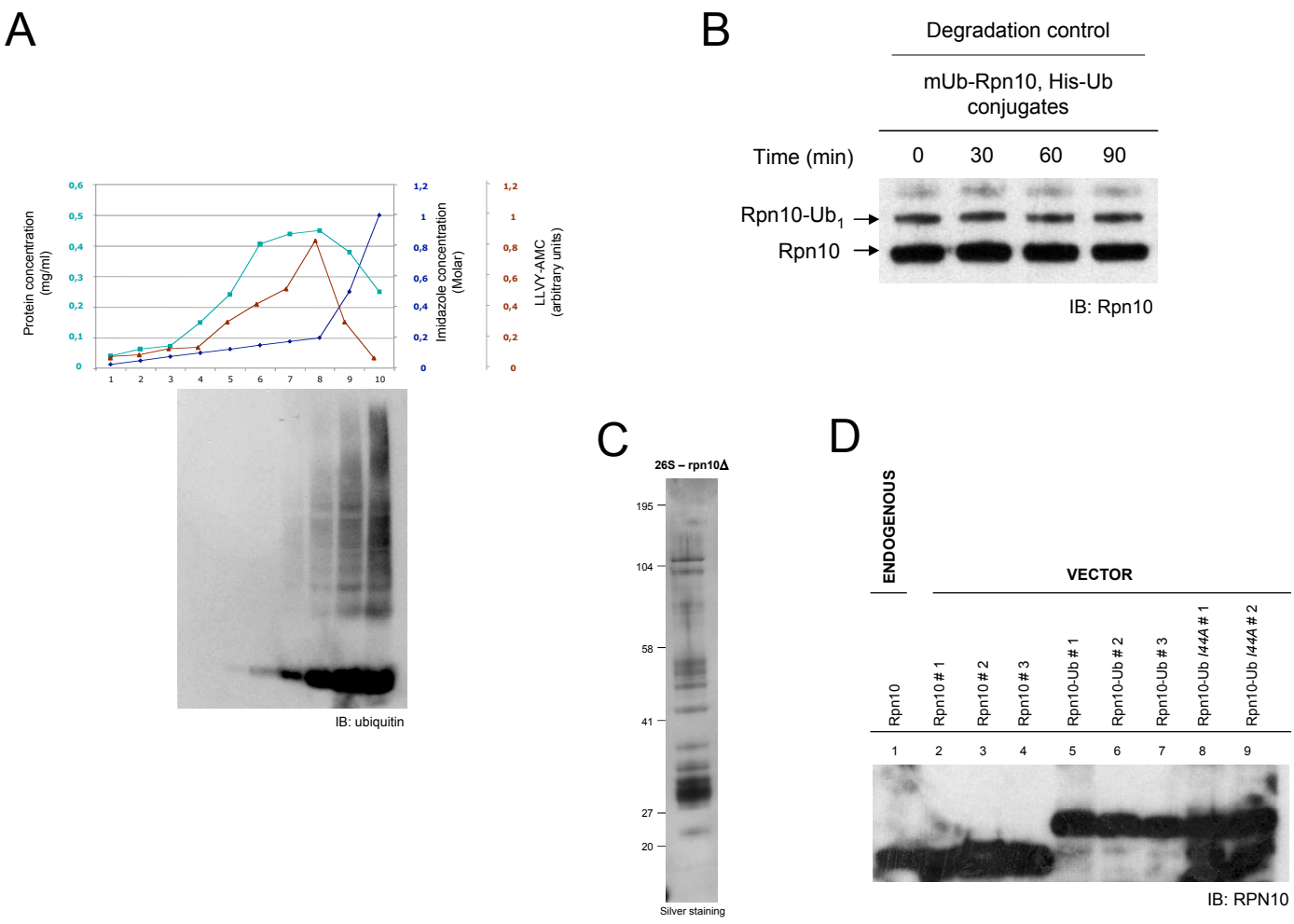


Figure S5. **(A)** Fractionation of HIS-Ubiquitin cell conjugates from strain SJR125 performed as described in Crosas *et al*., 2006. Fraction from lane 10 was used in assay shown in Figure 6A. **(B)** Deubiquitination control. A sample of monoubiquitinated Rpn10 *in vitro* was incubated with beads containing a fraction of cellular ubiquitin conjugates (panel A, lane 10) using the conditions used in the binding assay shown in Figure 6A. Samples were taken as indicated and analyzed by anti Rpn10 western blotting. See section 'Binding experiments with Ni-NTA agarose' **(C)** Proteasomes were purified from a *rpn10*Δ strain, resolved by SDS-PAGE and visualized by silver staining to check composition. **(D)** Expression levels of plasmid-borne RPN10. Strain SBC52, carrying plasmids (pYES2) expressing Rpn10 (lanes 2-4), Rpn10-Ub (lanes 5-7) or Rpn10-Ub^{I44A} (lanes 8-9), was grown in SGRC medium. Cells were harvested, resuspended and lysed with buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 2.5 mM ATP, 5 µM MG132, and 1x protease complete inhibitor cocktail EDTA free (GE Healthcare). A WT strain transformed with an empty vector was grown in parallel to evaluate endogenous levels of Rpn10 (lane 1). Supernatant was analyzed by SDS-PAGE and immunobloted against Rpn10.