Molecular Cell, Volume *32*

Supplemental Data

Extraproteasomal Rpn10 Restricts Access of the Polyubiquitin-Binding Protein Dsk2 to Proteasome

Yulia Matiuhin, Donald S. Kirkpatrick, Inbal Ziv, Woong Kim, Arun Dakshinamurthy, Oded Kleifeld, Steven P. Gygi, Noa Reis, and Michael H. Glickman

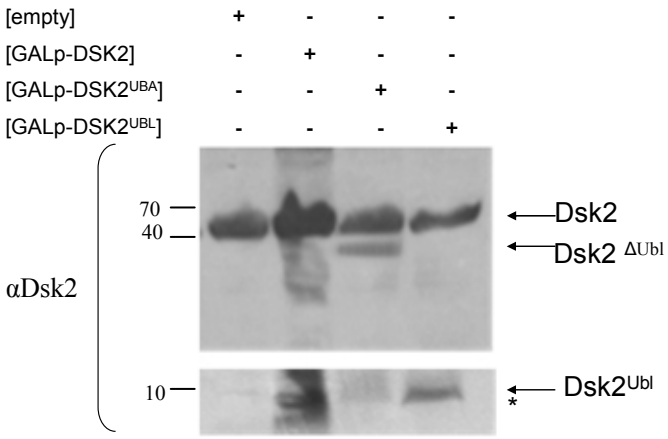

Figure S1: **Cellular levels of Dsk2 variants upon induction.** Extracts from wt cells induced for overexpression of full-length Dsk2 or of either of its truncated versions under *GAL1* promotor, were immunoblotted with anti-Dsk2 antibody. All three genes are expressed producing the corresponding protein or truncated proteins. Similar results were obtained with His_6-tagged Dsk2 constructs expressed in a *ΔDSK2* background (Fig 1 of main text).

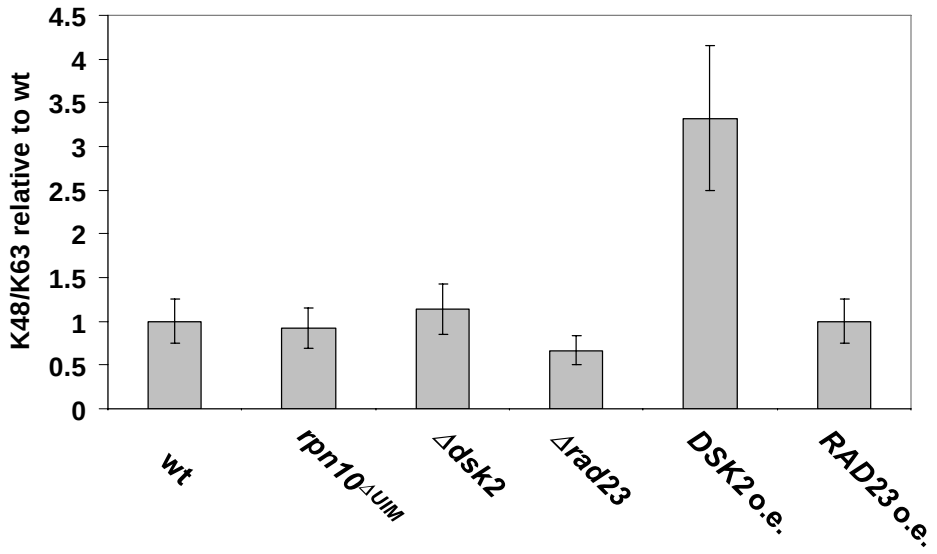

Figure S2: **Summary of effects of the presence or absence of Ub-binding proteins on Lys48 to Lys63 ratio in total conjugated Ub.** The ratio of Lys48 to Lys63 in high MW Ub conjugates was calculated by Ub-AQUA in each of the listed strains and displayed as a deviation from the ratio in the corresponding wt (carried out simultaneously and run in parallel): *rpn10*$^{\Delta UIM}$, *Δdsk2*, *Δrad23*, *DSK2* overexpression, *RAD23* overexpression.

Supplemental Experimental Procedures

Antibodies

Mark Rose (αDsk2), Dan Finley (αUbp6), Akio Toh-e (α20S), Rick Vierstra (αRpn10), Rafi Bahlul (αDsk2, αRad23, αRpt1), Allen Taylor (αUb).

Construction of Plasmids and Strains

Yeast two-hybrid plasmids

RPN10 ORF was cloned into pGBKT7 (Clontech) using EcoR1 and Sal1. *RAD23*, *DSK2* cut with EcoR1/Sal1 inserted into pGADT7 (Clontech) cut EcoR1/Xho1, and *DDI1* was cloned into pGADT7 (Clontech) using Nde1/Sal1.

E. coli expression plasmids

The ORFs of *RAD23*, *DSK2* and *DDI1* were amplified by PCR from wt genomic DNA, cut with BamH1 and Sal1 and cloned into the *E.coli* expression vector - pQE30 (QIAGEN). The truncated versions of Dsk2 were cloned into pQE30 as follows: Ubl domain (2-76aa) was subcloned using BamH1 and Xho1 sites (m483); UBA domain (77-373aa) was subcloned using BamH1 site immediately before codon77 and Sal1 at 3'UTR (m484). 6His-Ub_5 was obtained in a multi-step cloning procedure. Initially, the ubiquitin ORF was amplified using 5' primer containing His_6 encoding sequence flanked by Nco1 at 5' and by BamH1 at 3', which eliminates the native ubiquitin start codon and 3' containing Bgl2 and Xho1 restriction sites immediately after Gly-76 codon. The PCR product then was cloned into pGEM-T (Promega) vector by TA-cloning procedure (m243). Subsequently, two restrictions fragments from m243 were generated separately: 1) Bgl2-Xho1 vector and 2) BamH1-Xho1 insert, which then were ligated, yielding two linearly fused ubiquitin moieties, 6His-tagged at the N-terminus (m244). The procedure was repeated with m244, producing 6His-Ub_4 construct (m245). The fifth ubiquitin moiety was amplified using primers containing BamH1 site

eliminating the native start codon and Xho1 immediately after the native stop codon and cloned into m245 yielding m354. The large 6His-Ub_5 fragment was transferred to pET19b (Novagen) expression vector using Nco1 and Xho1 (m381).

DSK2 expression plasmids were a gift from Mark Rose, and Ub expression plasmids were offered by Dan Finley.

Table S1. Plasmids used in this study.

Plasmid	Genotype	Source
m83	*RPN10* in pET28	(Fu et al., 2001)
m201	ADHp in Yeplac181	this study
m177	pGBKT7	Clontech
m180	pGADT7	Clontech
m243	*6HIS-UBI* in pGEM-T	this study
m244	*6HIS-UBI_2* in pGEM-T	this study
m245	*6HIS-UBI_4* in pGEM-T	this study
m354	*6HIS-UBI_5* in pGEM-T	this study
m381	*6HIS-UBI_5* in pET19b	this study
m451	*$RPN10^{UIM}$* in m201	this study
m453	*RAD23* in pQE30	this study
m454	*DSK2* in pQE30	this study
m455	*DDI1* in pQE30	this study
m461	*RPN10* in pGBKT7	this study
m462	*RAD23* in pGADT7	this study
m463	*DSK2* in pGADT7	this study
m466	*RPN10* in m201	this study
m469	*DDI1* in pGADT7	this study
m480	*$RPN10^{UIM}$* in pET28	(Fu et al., 2001)
m481	*$RPN10^{vWA}$* in pET28	(Fu et al., 2001)
m483	*$DSK2^{Ubl}$* in pQE30	this study
m484	*$DSK2^{UBA}$* in pQE30	this study
m560	*RAD23* in pYes2	this study
m562	*DSK2* in pYes2	this study
m788	*UBI* in pUb70	(Finley et al., 1994)
m891	*CPY*-HA-KAN* in pBG15	(Rabinovich et al., 2002)
m943	*$DSK2^{Ubl}$* in pYes2	this study
m944	*$DSK2^{UBA}$* in pYes2	this study
m411	*Rpn10 ΔUIM*	this study

Yeast strains

All single deletion strains were purchased from Euroscarf (Table II). Double mutants were constructed by mating corresponding single deletion strains and subjecting the resulted diploids to random spore analysis (*Methods in yeast genetics, A cold spring harbor laboratory course manual, 2000 edition*). YGL011c-TAP::K.I URA3 cassette (from position -433 5' UTR to position +1078 3' UTR) was amplified from genomic DNA extracted from TAP-tagged strain (SC0973, Euroscarf) and introduced into the relevant strains by homologous recombination.

YPD and selective yeast media were prepared using standard methods (*Methods in yeast genetics, A cold spring harbor laboratory course manual, 2000 edition*). For canavanine-sensitivity assay, SD plates (arginine dropped out) were supplemented with 3 μg/ml canavanine (Sigma). For cycloheximide media, YPD plated were supplemented with 0.5 μg/ml cycloheximide (Sigma). Cells were grown at 30˚C in all experiments shown unless otherwise is stated. Protein over-expression under *GAL1* promoter is induced upon addition of galactose (Sigma) to media lacking glucose. Random spore analysis, mating assay and lithium acetate transformation assay were performed as described in *Methods in yeast genetics, A cold spring harbor laboratory course manual, 2000 edition*.

Yeast over-expression plasmids

RPN10 and *RPN10*^{UIM} (encompassing residues 191-268) ORFs were cloned into Yeplac181 (Gietz RD, Sugino A, Gene, 1988) bearing ADH promoter (m201) using Sma1 and EcoR1. *RAD23* and *DSK2* ORFs were cloned into GAL1

promotor-bearing vector - pYes2 (Invitrogene) using Xho1/EcoR1 and BamH1/Sal1 respectively. Ubl and UBA segments of *DSK2* were obtained from m483 and m484 using EcoR1/Xba1 and ligated into pYes2 in a similar manner.

Table S2. Yeast strains used in this study.

Strain	Genotype	Background	Source
MY1	*MATa ura3-1, can1-100,GAL+, leu2-3,112, trp1-1,ade2-1,his3-11,15*	W303	(Rinaldi et al., 2004; Rinaldi et al., 2002)
MY9	*MATa his3-Δ200 lys2-801 leu2-3,112 trp1-1 ura3-52 rpn10::LEU2*	Sub62	(Fu et al., 1998; van Nocker et al., 1996)
MY22	*MATa ura3-1 can1-100 GAL+ leu2-3,112 trp1-1 ade2-1 his3-11,15 mpr1-1*	W303	(Rinaldi et al., 2004; Rinaldi et al., 2002)
MY46	*MATa his3-Δ200 lys2-801 leu2-3,112 trp1-1 ura3-52*	Sub62	(Fu et al., 1998; van Nocker et al., 1996)
MY59	*MATα his3ko1 leu2ko0 lys2ko0 ura3ko0*	BY4742	EUROSCARF
MY134	*MATa trp1-901 leu2-3 112 ura3-52 his3-200 gal4Δ gal80ΔLYS2::GAL1UAS-GAL1TATA-HIS3 GAL2UAS-GAL2TATA-ADE2 URA3::MEL1UAS-MEL1TATA-lacZ*	AH109	Clontech
MY163	*MATα his3ko1 leu2ko0 lys2Δ0 ura3ko0 rpn10::kanMX4*	BY4742	EUROSCARF
MY164	*MATa his3ko1 leu2ko0 met15ko0 ura3ko0 rad23::kanMX4*	BY4741	EUROSCARF
MY169	*MATα his3ko1 leu2ko0 lys2Δ0 ura3ko0 dsk2::kanMX4*	BY4742	EUROSCARF
MY208	*MATα his3ko1 leu2ko0 ura3ko0 lys2ko0 rpn10::HIS3 rad23::kanMX4*	BY4742	this study
MY286	*MATα his3ko1 leu2ko0 ura3ko0 lys2ko0 rpn10::HIS3 dsk2::kanMX4*	BY4742	this study
MY325	*MATa ade2 arg4 leu2-3,112 trp1-289 ura3-52 alpha1-TAP::K.I.URA3*	SC0973	EUROSCARF
MY876	*MATa his3ko1 leu2ko0 met15ko0 ura3ko0 alpha1-TAP::K.I.URA3*	BY4741	this study
MY877	*MATα his3ko1 leu2ko0 met15ko0 ura3ko0 dsk2::kanMX4 alpha1-TAP::K.I.URA3*	BY4742	this study
MY884	*MATα his3ko1 leu2ko0 lys2Δ0 ura3ko0 rpn10::kanMX4 alpha1-TAP::K.I.URA3*	BY4742	this study

Supplemental Experimental Procedures

Canavanine, heat-shock or cycloheximide phenotypic analysis.

Yeast strains were grown in liquid YPD to a same optical density (0.75, 600nm) and 10-fold serial dilutions in sterile DDW were spotted (5μL) onto canavanine-, cycloheximide-containing or YPD agar plates. Canavanine and cycloheximide-containing plates were incubated at 30°C and YPD plates were incubated as indicated.

Expression and purification of His_6-tagged protein

For expression of 6His-tagged proteins in bacteria, the relevant strains were grown under appropriate selection to OD_{600} 06.-0.8 at 37ºC. The expression of the proteins was induced in the presence of 0.1-0.4mM IPTG for 2hr at 37ºC. The cells were harvested using TBS (50mM NaCl, 150mM Tris pH 7.4) buffer and deep-frozen. Next, the pellets were thawed on ice and resuspended in a seven-fold volume of buffer lysis (50mM Tris pH 8.0, 150mM NaCl, 10mM Imidazole), passed twice through a French Press, and the resulted extracts were clarified by centrifugation at 30,000g for 20min. The clarified lysates were loaded onto 1ml Ni-NTA Superflow resin (Qiagen) packed in a Poly-Prep Chromatography column (Bio-Rad) and operated by gravity feed or onto 2.5ml Ni-NTA Superflow resin packed in C10/10 column (Amersham Pharmacia) and operated by ÄKTA FPLC (Amersham Pharmacia). Proteins were either eluted at 250mM Imidazole when purified manually or resolved on a 15 ml linear gradient of 250 to 400 mM Imidazole when FPLC was used. 1 ml-fraction were collected and analyzed by SDS-PAGE.

Fractionation of extracts by glycerol gradients

11-ml glycerol gradients were prepared manually using 10% and 40% (v/v) glycerol stock solutions supplemented with 10mM $MgCl_2$, 25mM Tris pH 7.4, 2mM ATP, 1mM DTT. Yeast extracts (1ml) prepared using indicated strains were applied on top of the gradients and resolved by centrifuging at 28,000g for 18hrs in SW-41 rotor in Beckman Ultracentrifuge. Twelve 1-ml fractions were collected and assayed for peptidase activity using suc-LLVY-AMC (Boston Biochem) as described in *Peptidase assay for proteasome activity*. Protein samples were further resolved by SDS-PAGE and analyzed by Western using polyclonal anti-Rpn10, anti-Ddi1 (a gift from Jeffrey Gerst), anti-Ubp6 (a gift from Daniel Finley), anti-Rad23 (this study), anti-Dsk2 (a gift from Mark Rose) antisera.

Purification of the 26S proteasome and Peptidase assay for proteasome activity

Proteasomes were purified from yeast lysates as described previously (Glickman and Coux, 2001; Glickman et al., 1998; Leggett et al., 2005).

To measure peptidase activity, a sample (10μl) was added to 40μl of 0.1mM suc-LLVY-AMC in buffer A. After a 10-min incubation at 30˚C, the reaction was stopped by the addition of 1ml of 1% SDS. The fluorescence of released AMC was measured (excitation, 380nm; emission, 440nm) using an Aminco-Bowman series 2 luminescence spectrometer.

Ub-AQUA analysis

Yeast cell extract preparation for AQUA analysis

5-ml cell culture was grown to stationary phase; OD was measured at 595nm and cell density normalized. The cells were harvested using 20% (v/v) trichloroacetic acid (TCA), resuspended in 100μl, glass beads were added and the mixture was vortexed vigorously for 4 min. The supernatant was collected, and the beads were washed with 5% TCA to retrieve the remains. The supernatants from the two steps were pooled and centrifuged. The pellet was resuspended in Laemmli loading buffer 2X supplemented with 0.4M Tris pH 11.0 and boiled. The pellets were separated by 4-12% Tris-Bis SDS PAGE (Bio-Rad).

Sample preparation for AQUA analysis

Gel regions were excised as indicated, and prepared for Ub-AQUA by liquid-chromatography selected reaction monitoring (LC-SRM) in a TSQuantum Ultra (ThermoElectron, San Jose, CA) similar to published procedures (Kirkpatrick et al., 2005a; Kirkpatrick et al., 2005b; Kirkpatrick et al., 2006; Kirkpatrick et al., 2005c). In brief, excised lanes were excised as indicated, washed in MilliQ water and destained using sequential washes of 50% methanol/50 mM ammonium bicarbonate and 50% acetonitrile/50mM ammonium bicarbonate. Gel pieces were dehydrated with 100% acetonitrile and dried for 45 min under vacuum. Dry gel pieces were rehydrated with 20 ng/μl trypsin (50mM ammonium bicarbonate/5% acetonitrile pH 8) on ice for a minimum of 2 hr and then digested 12-16 hr at 37°C. AQUA peptides were added to digests immediately prior to extraction of peptides from a freshly prepared stock solution containing 0.5 pmol/μl of each peptide. Completed digests containing AQUA standards were extracted twice using a solution of 50% acetonitrile/5% formic acid and samples were dried completely under vacuum. Prior to analysis, samples were resuspended in an appropriate solution containing 10% acetonitrile/5% formic acid/0.005% H_2O_2 and transferred into deactivated glass inserts.

LC-SRM analysis of Ubiquitin-AQUA peptides

The yeast versions of Ubiquitin-AQUA peptides were monitored by liquid-chromatography selected reaction monitoring (LC-SRM) in a TSQuantum Ultra (ThermoElectron, San Jose, CA) according to the transitions presented in Table III. For each SRM, monoisotopic m/z values for parent and fragment ions were derived from GPMAW (Lighthouse Data, Hanstholm, Denmark). Parent ion charge state, optimal fragment ion, collision energy and instrument resolution were all empirically determined. Digested samples containing peptide standards were injected using a Famos autosampler (LCPackings, San Francisco, CA) and loaded onto a reversed phase column (Denali C18, 1mm x 15 mm, Vydac, Hesperia, California) at a flow rate of 60 μl per minute Buffer A (5% ACN, 0.4% acetic acid, 0.005% heptafluorobutyric acid). Peptides were eluted using an Agilent 1100 HPLC across a 19 min linear gradient from 15% to 25 % Buffer B (95% ACN, 0.4% acetic acid, 0.005% heptafluorobutyric acid. Individual SRM transitions were monitored across a 1.2 m/z window for 0.1 sec. Analytically, the run was divided into segments such that 8 SRM transitions (4 peptide pairs) were monitored during a given duty cycle.

Data Analysis

For each peptide, the areas under the curve were determined for the native (trypsin digested) and synthetic (isotope labeled) SRM transitions. The product of this ratio with the known abundance of each synthetic peptide was calculated to determine the abundance of each peptide standard. For the K63 polyUb peptide, two distinct SRM transitions were monitored, with the reported values reflecting the average of these two measurements. To determine the total amount of ubiquitin in a sample the law of conservation of mass was applied to the peptides surrounding both the lysine-63 and lysine 48 locus. The total amount of Ub reported represents the average of the K48 and K63 measurements.

Table S3. Yeast Ubiquitin-AQUA Peptides.

Name	Peptide Sequence	Parent (*z*)	Fragment (ion, z)	Heavy SRM (m/z)	Light SRM (m/z)
K6	$_{ox}$MQIFVK^{GG}T*L*TGK	2	y8, 1	701.89 → 1014.6	698.39 → 1007.6
K11	TLTGK^{GG}TITLEVESSDTIDN*V*K	3	y9, 1	795.41 → 984.5	793.41 → 978.5
K27	TITLEVESSDTIDN*V*K^{GG}AK	3	y16, 2	700.36 → 943.0	698.35 → 940.0
K29	SK^{GG}IQDK	2	y4, 1	419.74 → 503.3	416.73 → 503.3
K33	IQDK^{GG}EGI*PP*DQQR	3	y6, 1	548.61 → 746.4	546.61 → 740.4
K48	LIFAGK^{GG}Q*L*EDGR	3	y10, 2	489.94 → 621.3	487.60 → 617.8
K63_3	TLSDYNIQK^{GG}ESTLHLV*L*R	3	y16, 2	751.07 → 1019.0	748.73 → 1015.5
K63_4		4	y16, 3	563.55 → 679.7	561.80 → 677.4
TLS	T*L*SDYNIQK	2	y7, 1	544.78 → 867.4	541.28 → 867.4
EST	ESTLHLV*L*R	2	y5, 1	537.82 → 644.4	534.31 → 637.4
LIF	LIF*AG*K	2	y4, 1	328.21 → 429.3	324.71 → 422.2
QLE	Q*L*EDGR	2	y2, 1	362.68 → 232.1	359.18 → 232.1
TITLE	TITLEVESSDTIDN*V*K	2	y11, 1	885.45 → 1212.6	882.45 → 1206.6

Supplemental References

Finley, D., Sadis, S., Monia, B. P., Boucher, P., Ecker, D. J., Crooke, S. T., and Chau, V. (1994). Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. Molecular And Cellular Biology *14*, 5501-5509.

Fu, H., Sadis, S., Rubin, D. M., Glickman, M. H., van Nocker, S., Finley, D., and Vierstra, R. D. (1998). Multiubiquitin chain binding and protein degradation are mediated by distinct domains within the 26S proteasome subunit Mcb1. J Biol Chem *273*, 1970-1989.

Fu, H. Y., Reis, N., Lee, Y., Glickman, M. H., and Vierstra, R. (2001). Subunit interaction maps for the regulatory particle of the 26s proteasome and the cop9 signalosome reveal a conserved core structure. EMBO J *20*, 7096-7107.

Glickman, M. H., and Coux, O. (2001). Purification and characterization of proteasomes from Saccharomyces cerevisiae. In Current Protocols in Protein Science (New York, John Wiley & Sons), pp. 21.25.21-21.25.17.

Glickman, M. H., Rubin, D. M., Fried, V. A., and Finley, D. (1998). The regulatory particle of the *S. cerevisiae* proteasome. Mol Cell Biol *18*, 3149-3162.

Kirkpatrick, D. S., Denison, C., and Gygi, S. P. (2005a). Weighing in on ubiquitin: the expanding role of mass-spectrometry-based proteomics. Nat Cell Biol *7*, 750-757.

Kirkpatrick, D. S., Gerber, S. A., and Gygi, S. P. (2005b). The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. Methods *35*, 265-273.

Kirkpatrick, D. S., Hathaway, N. A., Hanna, J., Elsasser, S., Rush, J., Finley, D., King, R. W., and Gygi, S. P. (2006). Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. Nat Cell Biol *8*, 700-710.

Kirkpatrick, D. S., Weldon, S. F., Tsaprailis, G., Liebler, D. C., and Gandolfi, A. J. (2005c). Proteomic identification of ubiquitinated proteins from human cells expressing His-tagged ubiquitin. Proteomics *5*, 2104-2111.

Leggett, D. S., Glickman, M. H., and Finley, D. (2005). Purification of proteasomes, proteasome subcomplexes, and proteasome-associated proteins from budding yeast. Methods Mol Biol *301*, 57-70.

Rabinovich, E., Kerem, A., Frohlich, K. U., Diamant, N., and Bar-Nun, S. (2002). AAA-ATPase p97/Cdc48p, a Cytosolic Chaperone Required for Endoplasmic Reticulum-Associated Protein Degradation. Mol Cell Biol *22*, 626-634.

Rinaldi, T., Pick, E., Gambadoro, A., Zilli, S., Maytal-Kivity, V., Frontali, L., and Glickman, M. H. (2004). Participation of the proteasomal lid subunit Rpn11 in mitochondrial morphology and function is mapped to a distinct C-terminal domain Biochem J *381*, 275-285.

Rinaldi, T., Ricordy, R., Bolotin-Fukuhara, M., and Frontali, L. (2002). Mitochondrial effects of the pleiotropic proteasomal mutation mpr1/rpn11: uncoupling from cell cycle defects in extragenic revertants. Gene *286*, 43-51.

van Nocker, S., Sadis, S., Rubin, D. M., Glickman, M. H., Fu, H., Coux, O., Wefes, I., Finley, D., and Vierstra, R. D. (1996). The multiubiquitin chain binding protein Mcb1 is a component of the 26S proteasome in *S. cerevisiae* and plays a nonessential, substrate-specific role in protein turnover. Mol Cell Biol *11*, 6020-6028.