Supporting information materials and methods

Antibodies and reagents

Antibodies against β-actin, ubiquitin, p53, PARP-1, RPS27, JNK, USP10, and RNF123 were obtained from Santa Cruz Biotechnology (USA). Antibodies against RPL26, pJNK, FLAG, and α-TUBULIN were purchased from Cell Signaling Technology (USA). Anti-RPS3 antibodies and siRNAs were from HAEL (Korea). Ara-C, camptothecin, DON, and pactamycin were purchased from Sigma-Aldrich (USA). NSC119889 and puromycin were purchased from Merck (Germany).

Cell Culture

HT1080, HeLa, and 293T cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10 % fetal bovine serum (FBS) (Hyclone, Korea). MDA-MB-231 (human breast adenocarcinoma) was cultured in RPMI supplemented with 10% FBS.

Transfections of DNA and siRNA

Wild-type and K214R RPS3 were subcloned into pcDNA3-Flag (Invitrogen, Germany) using EcoRI and XhoI restriction sites to generate Flag-RPS3 and Flag-RPS3^{K214R}, respectively. To establish stable transfectants, Lipofectamine 2000 reagent (Invitrogen) was used followed by selection with G418. Specific siRNAs for RNF123 (sc-398340) and USP10 (sc-90255) were purchased from Santa-Cruz. Scrambled control (SN-1013) was purchased from Bioneer (Korea). Anti-RPS3 siRNA was from HAEL and the sequence targeting RPS3 was 5’-GGATGTTGCTCTCTAAAGA-3’. All siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s recommendations.

Ribosomal Fractionation and pelleting

Cells for ribosomal fractionation were incubated with cycloheximide (100 μg/ml) for 15 min. They were then harvested and resuspended in HMK lysis buffer (25 mM HEPES-OH pH 7.5, 5 mM $MgCl_2$, 100 mM KCl, 1 % Triton X-100, 2 mM DTT, 50 units/ml RNasin and protease inhibitors) at 4°C. The suspension was then passed twelve times through a 25-gauge needle with syringe. Cell lysates were centrifuged at 15,000 × *g* at 4 °C for 10 min. Supernatants were collected and loaded on 10 ~ 50 % (w/v) linear sucrose gradient (20 mM Tris-Cl pH 7.5, 150 mM NaCl, and 2.5 mM $MgCl_2$). Each ribosomal profile was monitored and fractionated by measuring absorbance at 254 nm using a Duo-Flow system (Bio-Red). For ribosomal pelleting, the supernatant was layered over a sucrose cushion (20 % w/v) followed by centrifugation at 32,000 rpm for 3h at 4°C in a Beckman SW41Ti rotor. Next, the ribosome pellet was solubilized with resuspendend buffer (10 mM Tris-HCl pH 7.5, 2 % SDS and 150 mM NaCl) containing 2 mM sodium orthovanadate and 50 mM sodium fluoride.

Western Blotting and immunoprecipitation

Cells were lysed in lysis buffer (50 mM Tris-Cl, pH 7.4, 150mM NaCl, 0.5 % NP-40, 0.25 % sodium deoxycholate and protease inhibitors) and cleared by centrifugation. Proteins from total cell lysates or immunoprecipitates were separated by SDS-PAGE and transferred onto PVDF membrane (Bio-Rad). For immunoprecipitation of ribosomal pellets with anti-ubiquitin antibody, the resuspensions of ribosomal pellets were boiled and sonicated followed by dilution with ten volumes of dilution buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA and 1 % Triton X-100). Western blotting was performed using chemiluminescence blotting substrate (Boehringer Mannheim). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (Boehringer Mannheim) were used as secondary antibodies.

Nuclei Fractionation

For subcellular fractionation, cells were harvested, washed with PBS, and resuspended in buffer I (10 mM Hepes pH 7.9, 10 mM NaCl, 0.1 mM EDTA, 1 mM DTT and protease inhibitors). After incubation on ice for 15 min, plasma membranes were disrupted by the addition of 0.3 % Nonidet P-40 followed by mixing for 10 secs. The nuclei were pelleted by centrifugation at 13,000 rpm for 1 min and the cytoplasmic fraction (supernatant) was discarded. After washing three times, the pellets were resuspended in buffer II (20 mM Hepes pH 7.9, 0.5 mM EDTA, 0.5 mM EGTA, 0.4 M NaCl, 1 mM DTT, and protease inhibitors), rocked for 30 mins on ice, and centrifuged at 3000 × *g* for 15 mins at 4 °C to recover the nuclear fraction.

Proteomics of RPS3

RPS3 was purified from solubilized ribosome pellet by immunoprecipitation using anti-RPS3 antibody. Gel bands were excised and destained with 25 mM NH_4HCO_3 in 50 % acetonitrile. Proteins in bands were reduced with 10 mM DTT in 25 mM NH_4HCO_3 at 56 °C for 1 h and alkylated with 55 mM iodoacetamide at 25 °C for 1 h in the dark. Gel slices were washed with 25 mM NH_4HCO_3 in 50% acetonitrile followed by soaking in a chilled 25 mM NH_4HCO_3 solution containing 12 ng/μl of sequencing grade trypsin (Promega, USA) for 45 min. After removing residual trypsin, gel slices were incubated at 37 °C for 16 h. Digested peptides were extracted sequentially with 25 mM NH_4HCO_3 in 50 % acetonitrile, 0.25 % TFA in 50 % acetonitrile, and 0.25 % TFA in 70 % acetonitrile. These three extracts were pooled and dried using a vacuum evaporator.

Mass spectrometric analyses were performed using a linear ion trap mass spectrometer (LTQ, Thermo Electron, USA) connected with an Agilent nanoflow-1200 HPLC system. The dried

trypsinized peptides were reconstituted with 5 μl of 0.4 % acetic acid and injected into a reverse-phase Magic C18aq column (13 cm × 75 μm) equilibrated with 95 % buffer A (0.1 % formic acid in H_2O) + 5 % buffer B (0.1 % formic acid in acetonitrile). These peptides were eluted in a linear gradient containing 10 ~ 40 % acetonitrile over 75 mins. MS survey was scanned from 300 to 2000 *m/z* followed by three data-dependent MS/MS scans with the following options: isolation width, 1.5 *m/z*; normalized collision energy, 25 %; dynamic exclusion duration, 180 secs. The acquired MS/MS spectra were automatically compared against the human International Protein Index database including known contaminants (IPI, versions 3.24, European Bioinformatics Institute, www.ebi.ac.uk/IPI) using SEQUEST allowing 2 missed cleavage sites (trypsin) with ± 0.5 and ± 3 Da mass tolerance for MS/MS and MS, respectively. Modifications were: alkylation of cysteine (+ 57.02 Da), oxidation of methionine (+ 15.99 Da), and ubiquitination of lysine (+ 114.04 Da, mono-ubiquitinated trypsinized peptide has an additional glycine residue on lysine). Peptide assignment was performed using the Trans-Proteomic Pipeline (TPP, version 4.0, http://www.proteomecenter.org). Proteins matched with over 90 % confidence were manually inspected.

Supplemental figure legends

Fig S1. Modification of ribosomal proteins under UV-irradiation.

HT1080 cells were irradiated with UV (150 J/m^2) and harvested after 2 h. Ribosomes were purified from control or UV irradiated cells and immunoprecipitated with anti-RPS3 antibody. (A) MS/MS spectrum of the ubiquitinated peptide, KPLPDHVSIVEPKDEILPTTPISEQK, from 40S ribosomal protein S3 (rpS3). K* denotes ubiquitinated lysine. (B) Immunoprecipitants were analyzed by immunoblotting with indicated antibodies.

Fig S2. Stability of Rps3p and Rps3p^{K212R} mutant cells.

Each strain grown to early log phage was treated with or without 200 μg/ml of CHX for the indicated times. Cell extracts were analyzed by immunoblot with indicated antibodies. “L” and “S” indicates long exposure and short exposure, respectively.

Fig S3. Screening for E3 ligase responsible for Rps3p mono-ubiquitination.

(A-H) Various gene deletion strains were treated with rapamycin. Cell lysates were then analyzed by immunoblot with anti-Rps3p and anti-Pgk1p antibodies.

Fig S4. Mono-ubiquitination of RPS3 is not induced by inhibitors of translation initiation.

(A) HT1080 cells treated with rapamycin or emetine were incubated for 2 h. Cell lysates were subjected to immunoblot analysis using the indicated antibodies. (B) HT1080 cells were treated with 10 μM NSC119889 for the indicated times and cell lysates were subjected to immunoblot analysis using the indicated antibodies.

Fig S5. Mono-ubiquitination of Rps3p induced by rapamycin disappears in *ufd3*Δ strain.

Wild-type (wt) and *ufd3*Δ strains were grown to early log phase in YPD media, treated with

rapamycin (100 ng/ml), and incubated for 1 h. Whole cell lysates were analyzed by immunoblot with anti-Rps3p, eIF2a~p, and Pgk1p antibodies.

Fig S6. Mono-ubiquitination of RPS3 is not induced by DNA damaging agents.

HT1080 cells were treated with 10 μM ara-C or 10 μM camptothecin for the indicated times and cell lysates were subjected to immunoblot analysis using the indicated antibodies.

Fig S7. Mono-ubiquitination of RPS3 is predominant in 80S monosome and independent of p53.

(A) Time course of RPS3 ubiquitination. HT1080 cell extracts were prepared at the indicated times after UV-irradiation. (B) HeLa cells were irradiated with UV (200 J/m^2) and fractionated into cytosolic or nuclear fractions after incubation in fresh media for 2 h. RPS3 mono-ubiquitination was determined by western blotting. PARP serves as a fraction control. (C) HeLa cells were irradiated with UV (200 J/m^2). After 2 h, ribosomal fractions were performed as described in "Materials and Methods" and then 20 μl of each fraction was used for immunoblotting. (D) p53-negative 293T or MDA-MB231 cells were irradiated with the indicated dose of UV. Cell lysates were subjected to immunoblot analysis using the indicated antibodies.

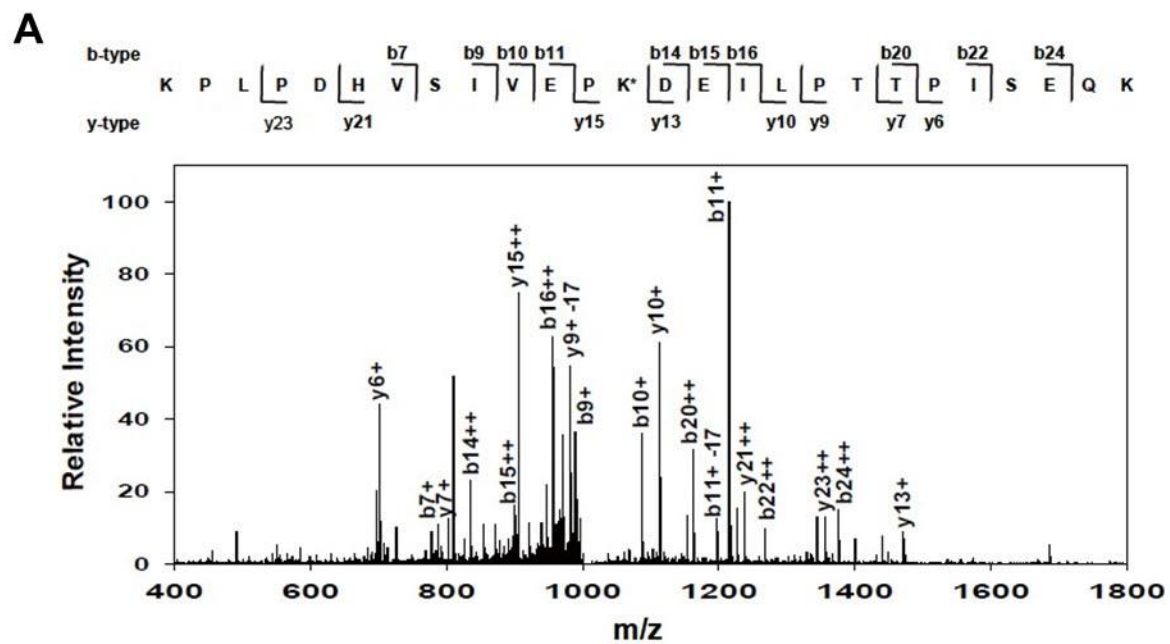
Fig S8. The residue of K214 of RPS3 is a major mono-ubiquitination site.

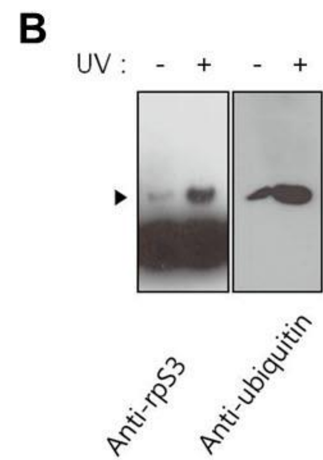
HT1080 cells stably expressing Flag-RPS3 wild-type, K90R, K214R, K227R, or K230R mutants were transfected with RPS3 siRNA. After 2 days, cells were irradiated with 200 J/m^2 of UV, harvested after 2 h, and subjected to immunoblotting with anti-RPS3 or anti-Flag antibody.

Fig S9. Asc1p/RACK1 is associated with rapamycin induced Rps3p mono-ubiquitination.

(A) Wild-type (wt) and *asc1*Δ strains were grown to early log phase in YPD media, treated with rapamycin (100 ng/ml), and incubated for 1 h. Whole cell lysates were analyzed by immunoblot with anti-Rps3p, eIF2a~p, and Pgk1p antibodies. (B) HT1080 cells were transfected with siRNAs against SC or RACK1, treated with 150 J/m^2 of UV irradiation, and harvested after incubation in fresh media for 2 h. Lysates were subjected to immunoblotting using the indicated antibodies.

Figure S1





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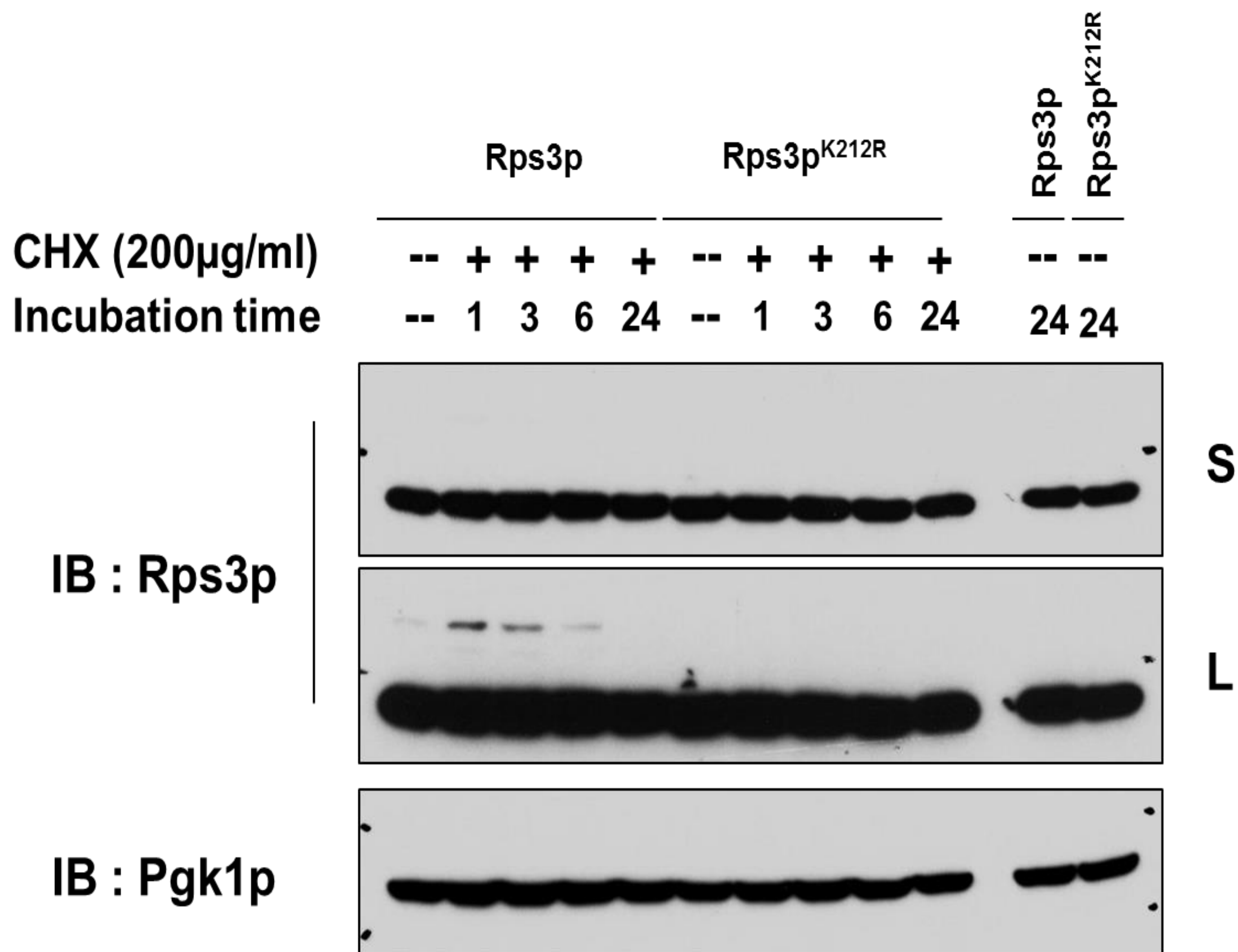

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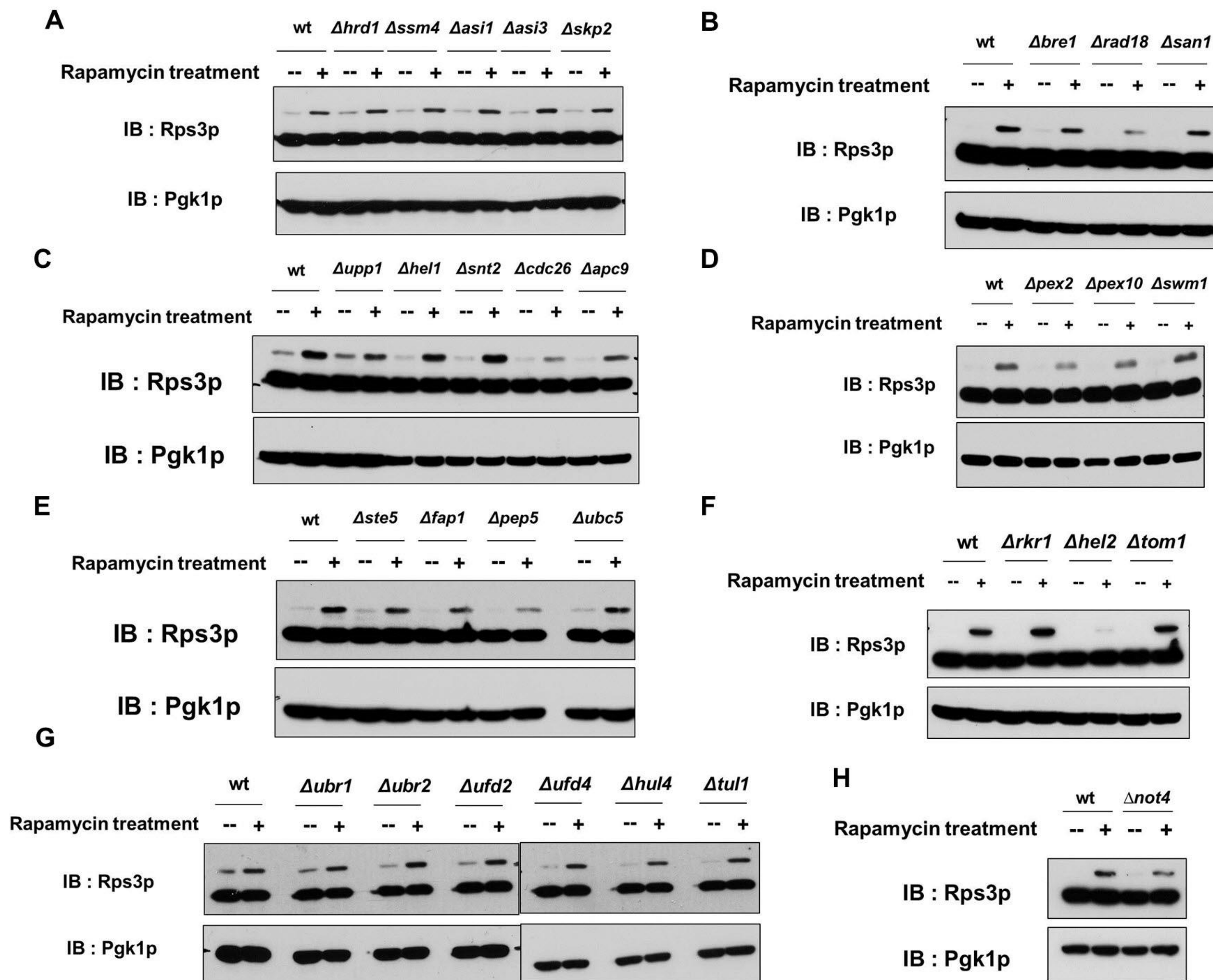

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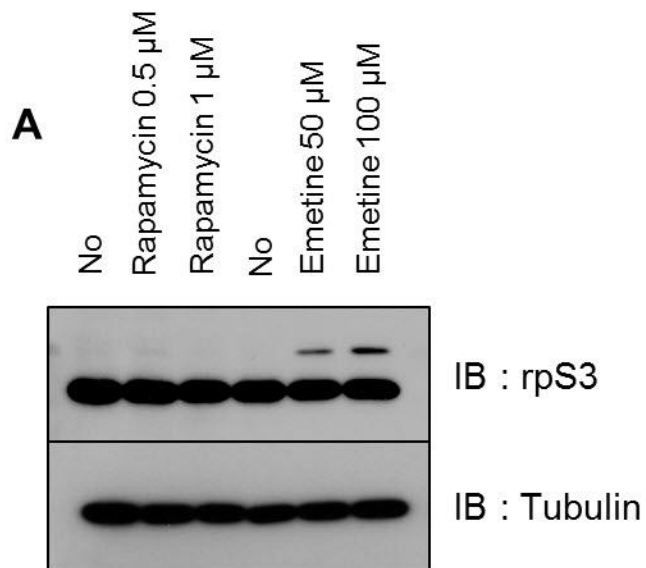

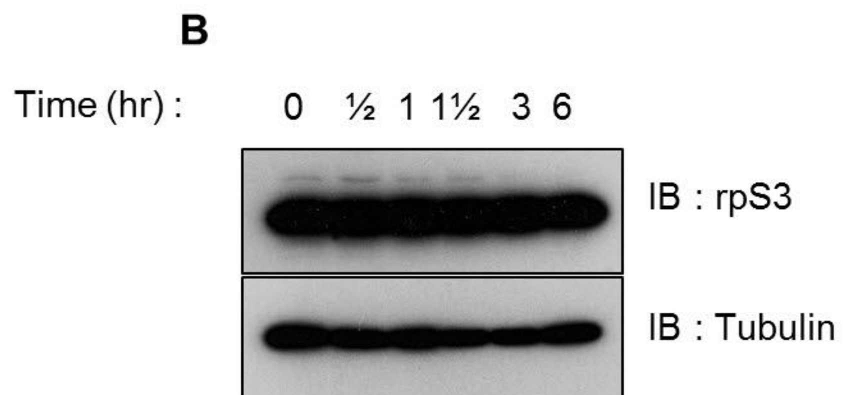

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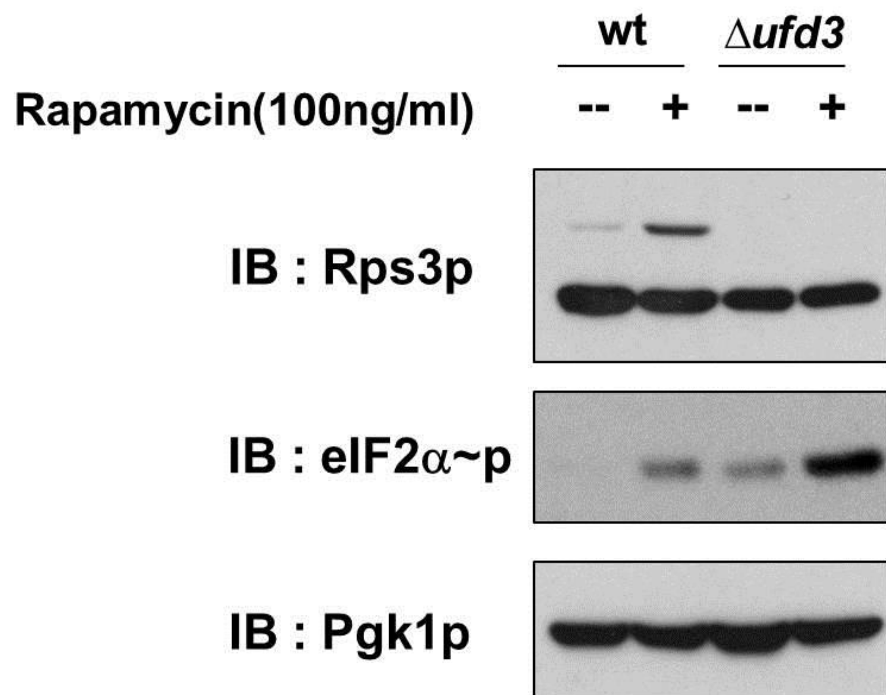

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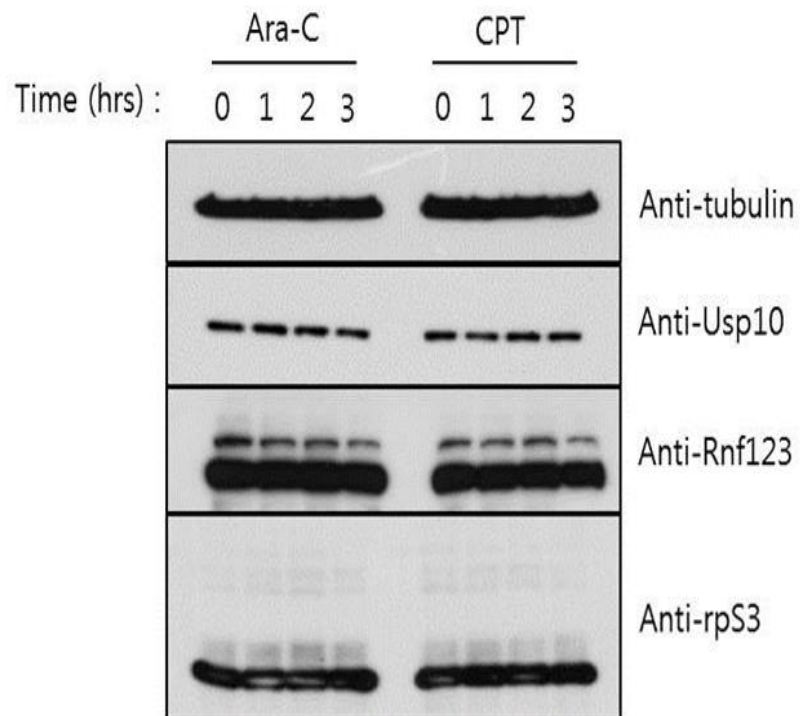
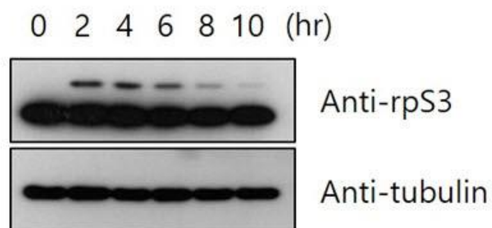


Figure S7

A



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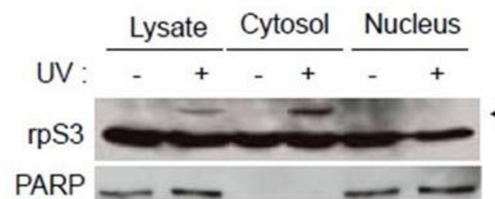

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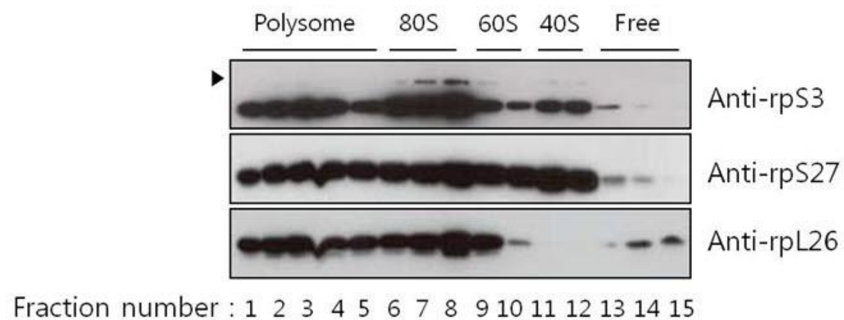

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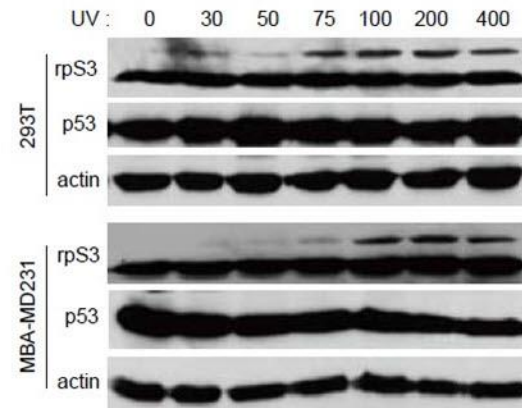

Figure S8

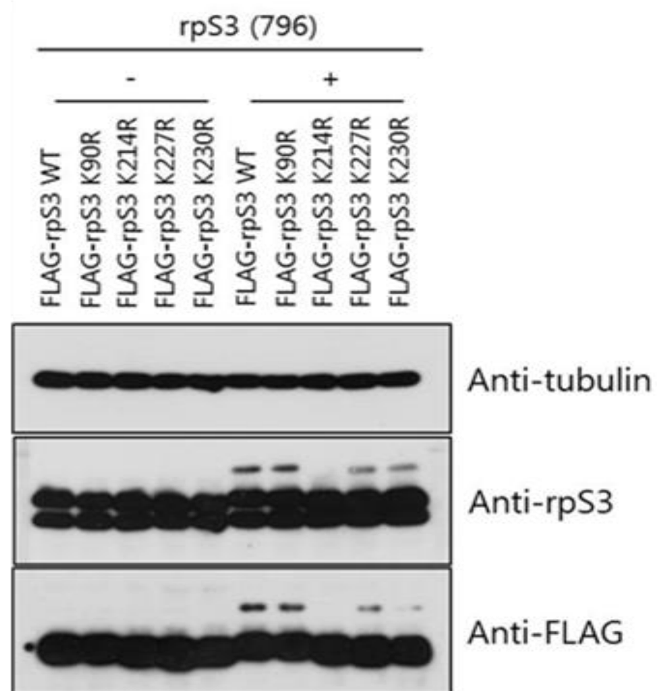

Figure S9