Supplemental Figures

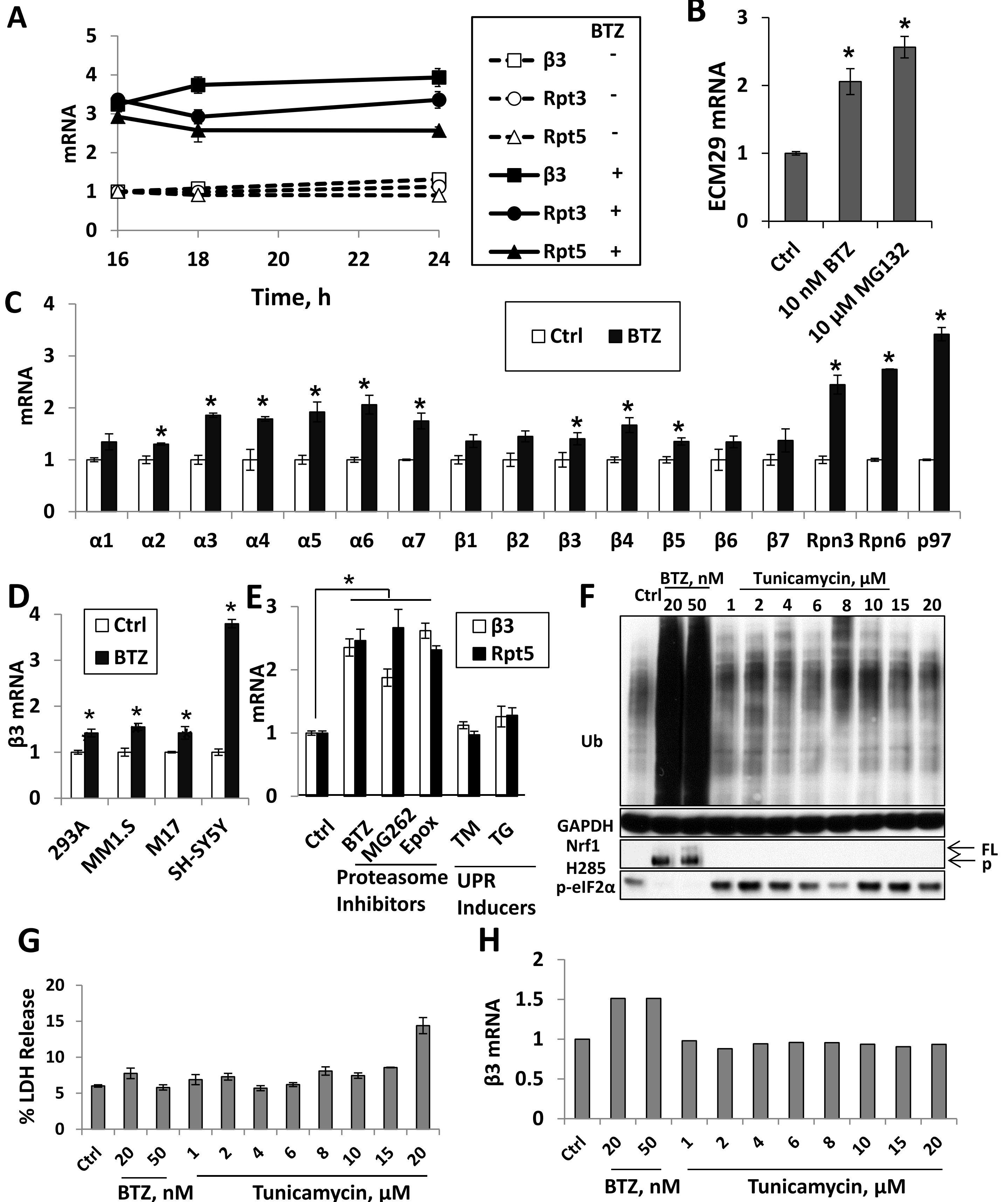


Figure S1

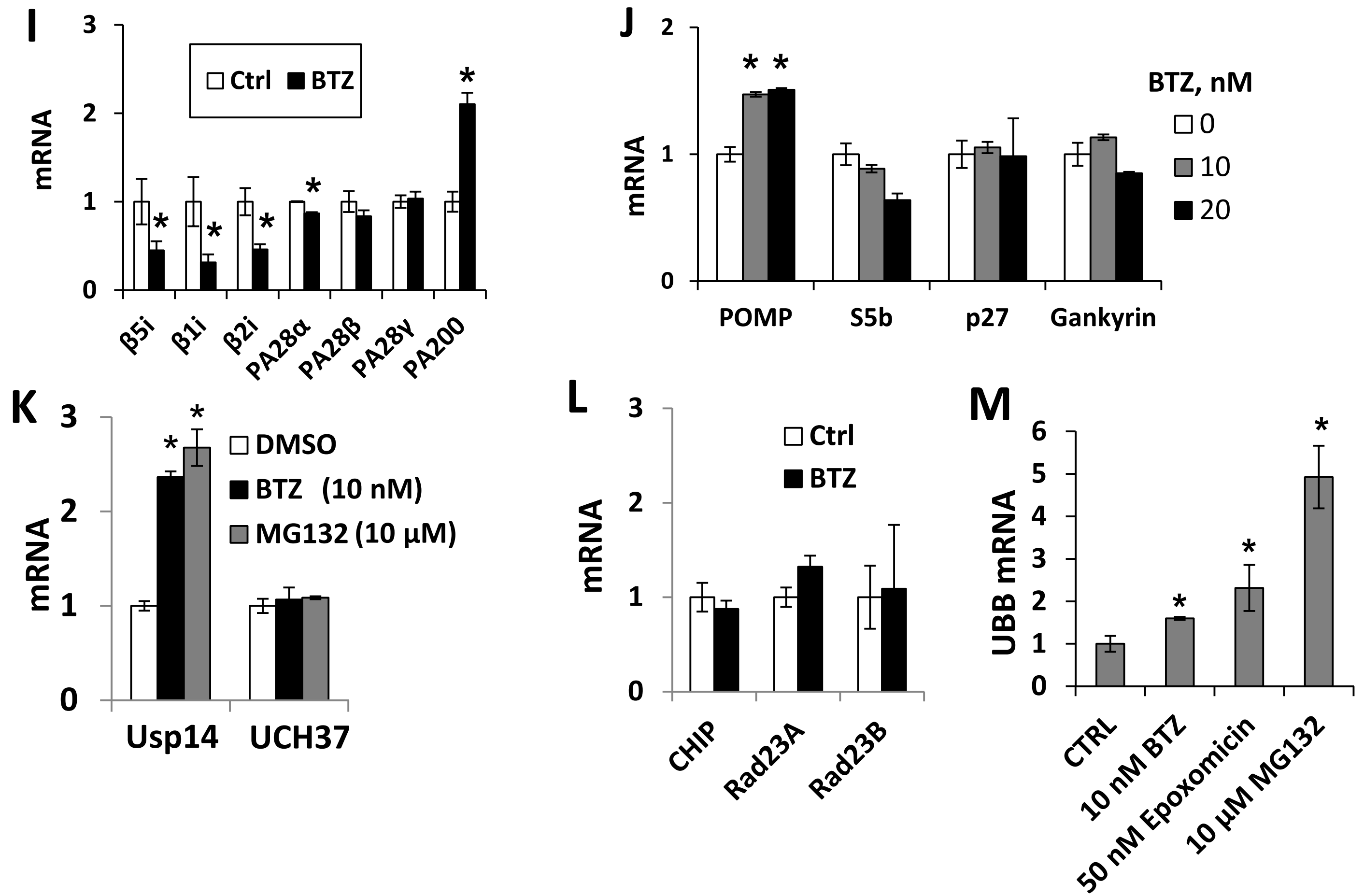


Figure S1 Continued

Figure S1 (related to Fig 1). Induction of proteasome subunits and proteasome-associated proteins by BTZ in SH-SY5Y cells.

(A) The induction of proteasome subunits by BTZ treatment (10 nM) reaches its maximal level by 16hr. (B) Treatment with BTZ or MG132 induced the expression of ECM29. (C) Treatment of SH-SY5Y cells with BTZ (1 μM) for 4h can induce mRNA for proteasome subunits and p97, *: $p<0.05$. (D) A smaller but reproducible induction of β3 mRNA also occurred in HEK293A, MM1.S, and M17 cells upon 10 nM BTZ treatment for 16h. (E) In SH-SY5Y cells, 16 hr treatment with multiple proteasome inhibitors (10 nM BTZ, 1 μM MG262 or 50 nM Epoxomicin (Epox)), but not ER stress inducers tunicamycin (TM, 12 μM) or thapsigargin (TG, 300 nM), induced the mRNA of β3 and Rpt5. (F-H) Tunicamycin does not induce Nrf1 processing or the expression of proteasome subunits in HEK293A cells. HEK293A cells were treated for 16h with indicated concentrations of BTZ or Tunicamycin. The induction of UPR by tunicamycin is validated by measuring the phosphorylation of eIF2α (F). Tunicamycin treatment did not cause significant cell death (measured by LDH release assay) except when used at 20 μM (G). Under these conditions, tunicamycin did not cause the accumulation of ubiquitin conjugates or the processing of Nrf1 (measured by WB, F), and did not induce the mRNA expression of β3 (H). (I-O) Induction of non-26S proteasome subunits, other proteasome-associated proteins, or components of the UPS, or molecular chaperones, by BTZ. (I) Treatment of SH-SY5Y cells were with BTZ (10 nM) for 16h suppressed the expression of mRNAs for immuno-proteasome subunits β1i, β2i, and β5i, and induced the mRNA for proteasome activator PA200, but not PA28 α, β, γ. (J) Treatment of SH-SY5Y cells were with BTZ (10 nM) for 16h also induced the mRNAs for the 20S proteasome assembly chaperone, POMP, but not the 19S chaperones S5b, p27, and gankyrin, (K) Treatment of SH-SY5Y cells with BTZ (10 nM) or MG132 (10 μM) for

16h induced the mRNA of Usp14, but not Uch37. (L) Treatment of SH-SY5Y cells with BTZ (10 nM) for 16h did not induce the mRNA expression of ubiquitin shuttling factors Rad23A and Rad23B, and the E3 CHIP. (M) Treatment with BTZ, Epox, or MG132 induced the expression of the polyubiquitin gene UBB. * indicates significant difference after BTZ treatment $p<0.05$. Error bar = SD.

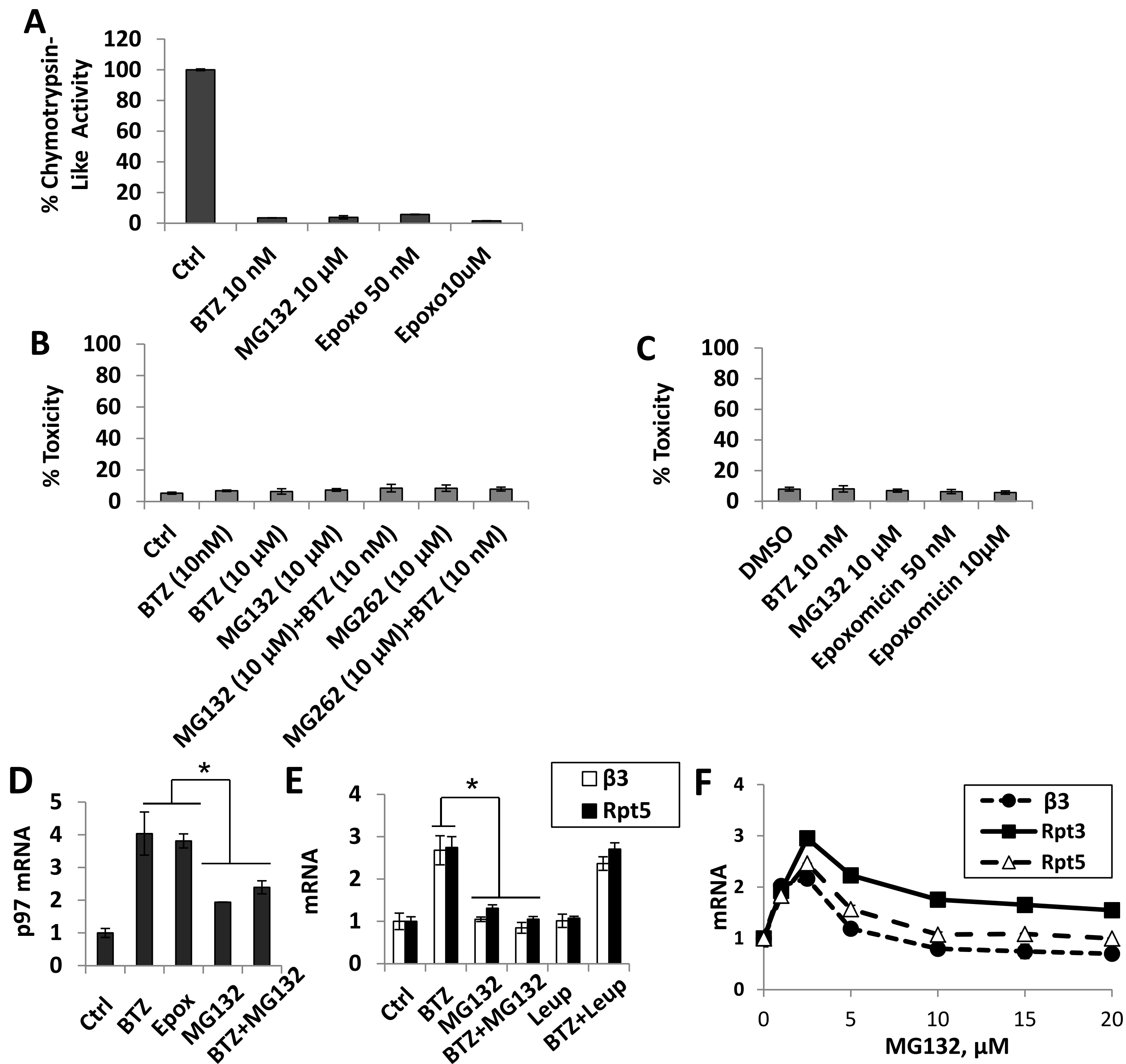


Figure S2

Figure S2 (related to Fig 2). Cell viability upon proteasome inhibitor treatment

(A) Treatment of SH-SY5Y cells for 16h with BTZ (10 nM), MG132 (10 μM), and epoxomicin (50 nM or 10 μM) all completely inhibited the chymotrypsin-like proteasomal peptidase activity assayed in whole cell lysates. (B,C) Treatment with BTZ, MG132, MG262, and Epoxomicin up to 10 μM for 16h did not cause much cytotoxicity to SH-SY5Y cells (LDH release assay). (D) Unlike 10 nM BTZ or 50 nM Epox, 10 μM MG132 alone was unable to induce p97 mRNA and blocked its induction by BTZ (when added together) in SH-SY5Y cells. (E) Treatment of SH-SY5Y cells with 10 μM MG132, but not leupeptin (100 μM), suppressed the induction of β3 and Rpt5 mRNAs by BTZ treatment (10 nM, 16 hr). (F) At concentrations below 2.5 μM, MG132 induced the mRNAs for β3, Rpt3, and Rpt5, but this induction was suppressed at concentrations above 5 μM. Error bar = SD.

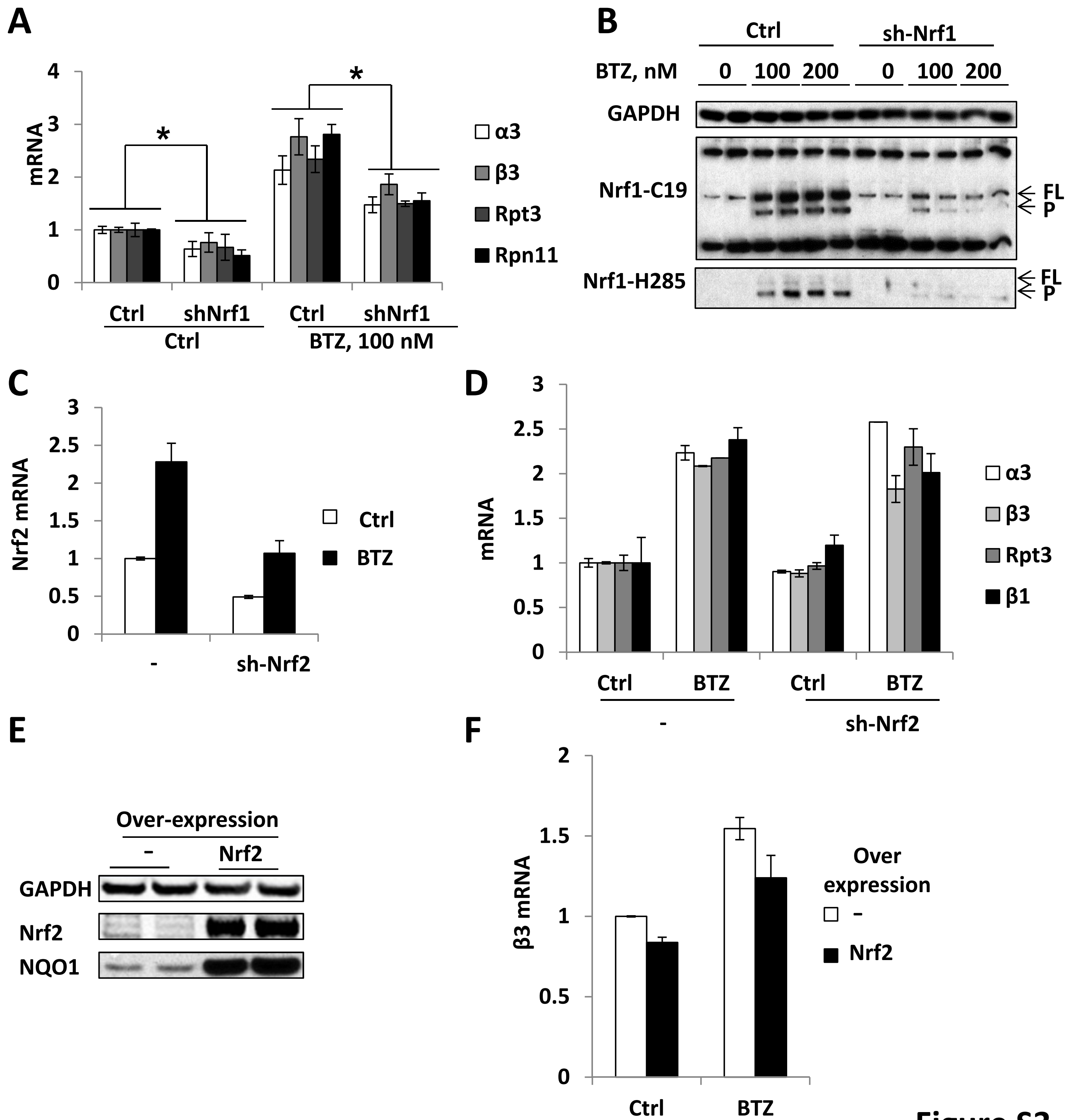


Figure S3

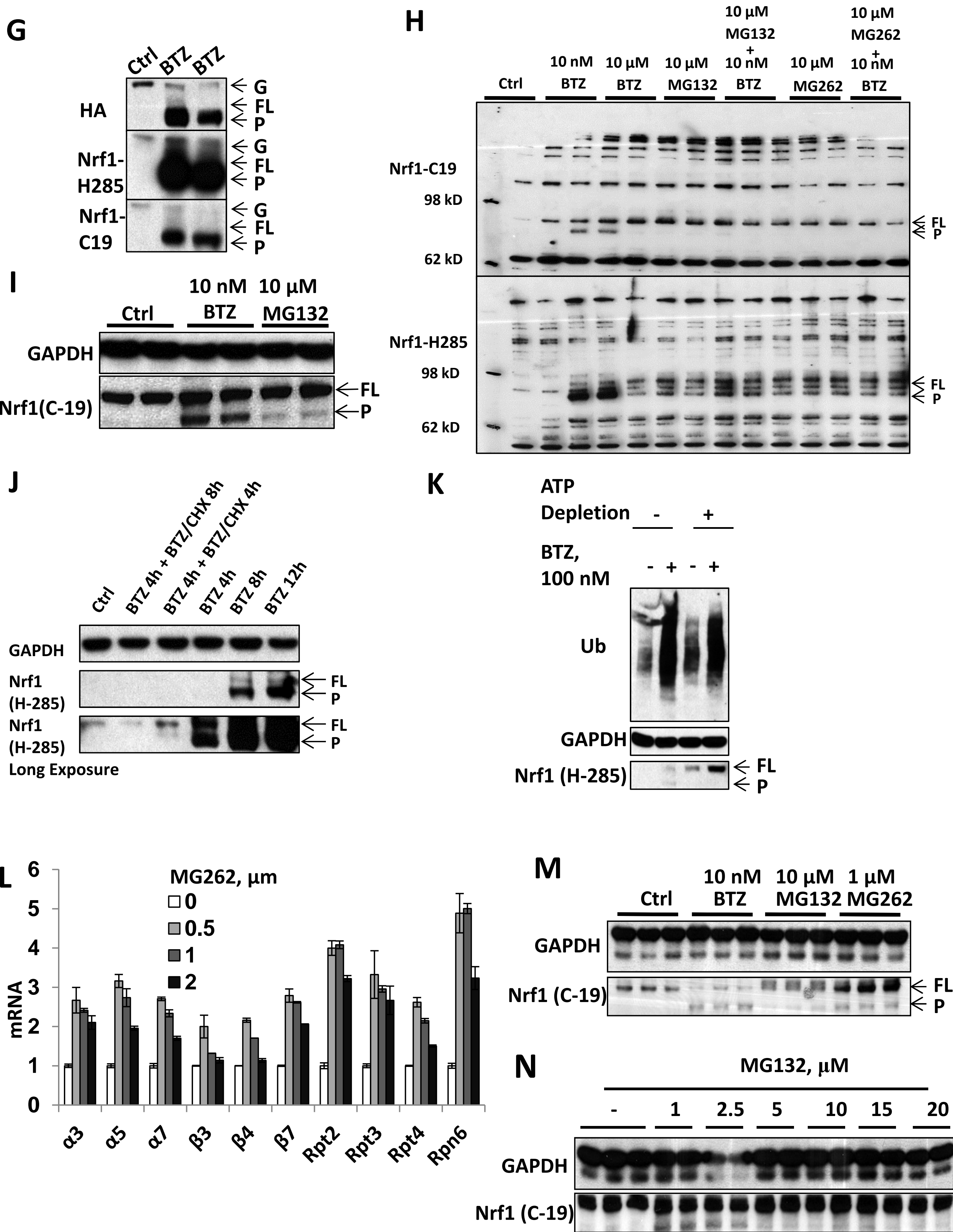
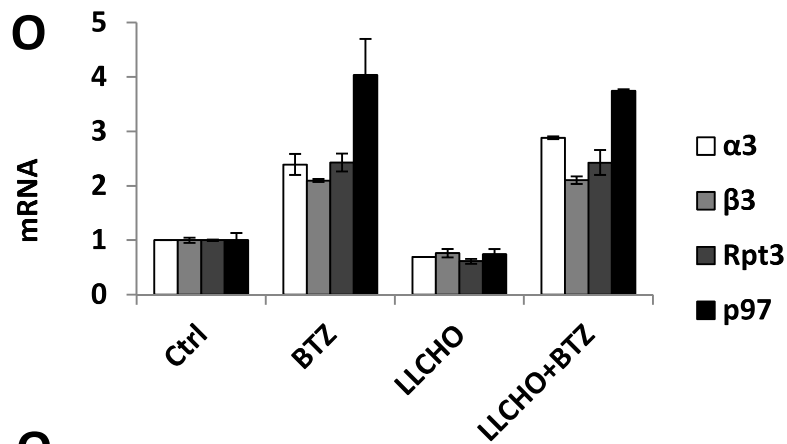
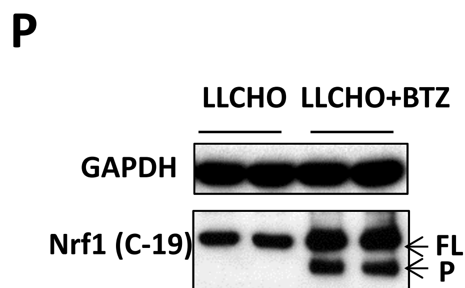
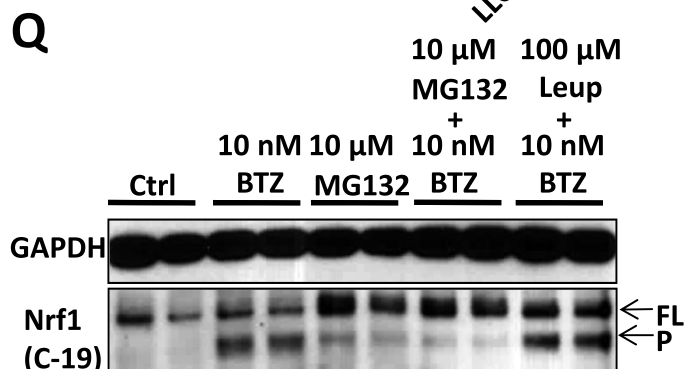
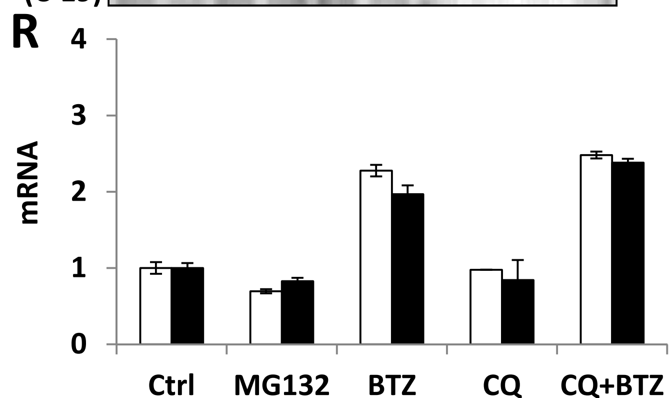


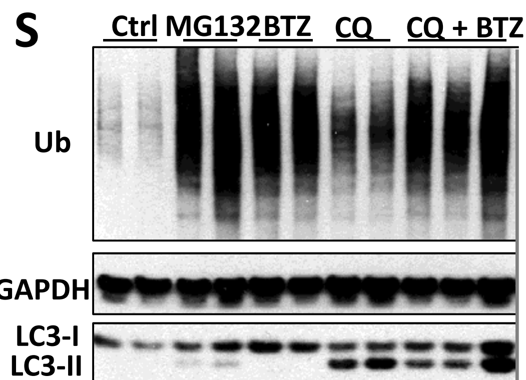
Figure S3 Continued

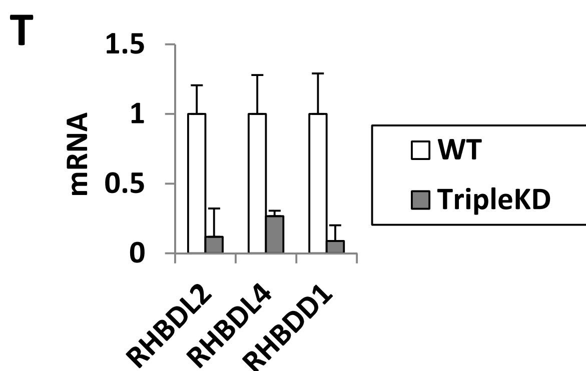


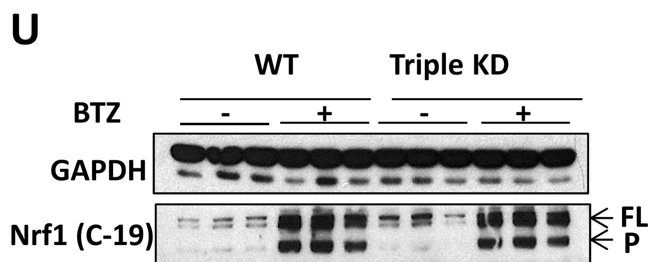


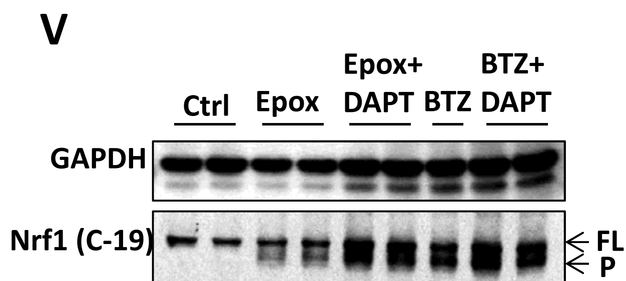












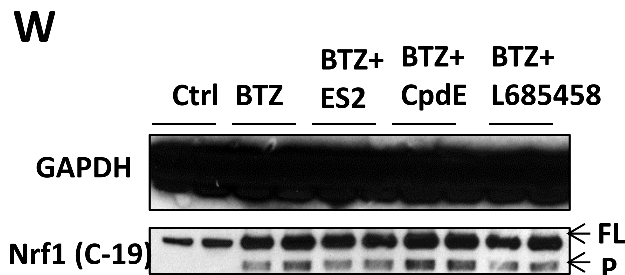


Figure S3 Continued 2

Figure S3 (related to Fig 3). Characterization of Nrf1 processing

(A-F) Nrf1, but not Nrf2, mediates the induction of proteasome subunits upon proteasome inhibition. (A,B) Knock down of Nrf1 suppresses BTZ-induced 26S subunit expression. Nrf1 was knocked down by stable expression of shRNA in SH-SY5Y cells. (A) Knock down of Nrf1 decreased the mRNA expression of α3, β3, Rpt3, Rpn11 in untreated cells or cells treated with 100 nM BTZ (16h). (B) Successful Nrf1 knockdown was confirmed by western blotting using two Nrf1 antibodies (C19 and H285) in untreated cells or cells treated with 100 or 200 nM BTZ (16h). (A) The induction of 26S subunit mRNAs is suppressed by knock down of Nrf1. *: $p<0.05$ (C,D) Knockdown of Nrf2 does not affect BTZ-induced 26S subunit expression. Nrf2 was knocked down by stable expression of shRNA in SH-SY5Y cells, and (C) successful knock down was confirmed by RT-PCR. (D) The induction of 26S subunit mRNAs is unaffected by knock down of Nrf2. (E,F) Overexpression of Nrf2 does not affect BTZ-induced expression of 26S subunits. (E) Nrf2 was overexpressed by transfection of pCI-Nrf2 [S1] (or GFP as a control, -) into HEK293A cells. Successful overexpression of Nrf2 and induction of its target gene NQO1 was validated by western blotting. (F) Overexpression of Nrf2 does not affect the mRNA of β3 in untreated or BTZ (10 nM, 16h) treated cells.

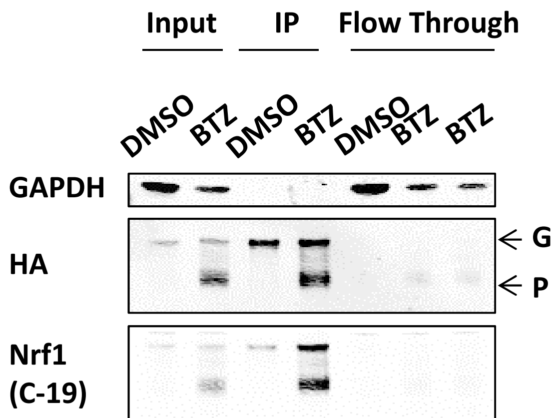
(G-H) Detection of full-length and processed Nrf1 by two Nrf1 antibodies. (G) Validation of the anti-Nrf1 H285 and C19 antibodies. Nrf1-HA was ectopically expressed in HEK293A cells. After BTZ (10 nM) treatment for 16h, the processed Nrf1 was detected by HA and two Nrf1 antibodies (C-19 and H285). Compared to unprocessed form, processed Nrf1 is much more easily detected by western blotting, probably due to the glycosylation of the unprocessed form (see text). (H) The whole gel of the western blot in Fig 3A was shown to demonstrate the molecular weight of full-length and processed Nrf1. The full-length Nrf1 migrates at

approximately 85 kD, which is original molecular weight, while the processed Nrf1 is slightly shorter. The processed Nrf1 is the most prominent form that is recognized by Nrf1 (H285) antibody. (I) Nrf1 processing is blocked by 10 μM MG132 in MM1.S cells**.** MM1.S myeloma cells were treated with 10 nM BTZ or 10 μM MG132 for 12h. Only 10nM BTZ, but not 10 μM MG132, was able to cause Nrf1 processing.(J-K) Nrf1 processing is an ATP-dependent process. (J) HEK293A cells were first treated with BTZ (20 nM) for 4h, then continued to be treated with BTZ in the presence or absence of cycloheximide (CHX, 20 μg/ml) for up to 8h. Without CHX, processed Nrf1 gradually accumulates over the course of 12hr BTZ treatment. However, in the presence of CHX, both full-length and processed Nrf1 rapidly disappeared, indicating that both forms are short-lived even with 20nM BTZ treatment. (K) ATP depletion blocks BTZ-induced Nrf1 processing. To deplete ATP, SH-SY5Y cells were treated for 3h with 0.5 mM dinitrophenol (to inhibit oxidative phosphorylation) and 12.5 mM deoxyglucose (to inhibit glycolysis) [S2] together with 100 nM BTZ. ATP depletion abolishes BTZ-induced Nrf1 processing. (L-N) Effect of MG262 and MG132 on proteasome subunit expression and Nrf1 processing at different concentrations. (L) Treatment of SH-SY5Y cells with 1 μM MG262 (16h) can still induce proteasome subunit expression, but further increasing MG262 to 2 μM reduces their expression. (M) Treatment of SH-SY5Y cells with 1 μM MG262 is still capable of stimulating Nrf1 processing, although to a much less extent than 10 nM BTZ treatment. (N) Treatment with concentrations of MG132 below 2.5 μM for 16h caused processing of Nrf1 in SH-SY5Y cells, but concentrations above 5 μM blocked processing.

(O-W) Nrf1 processing does not require lysosomal proteases, calpain, and several intramembrane proteases. (O,P) Although MG132 can also inhibit two calpains [S3], these cysteine proteases are not inhibited by peptide boronates. Furthermore, co-treatment of SH-

SY5Y cells with the calpain inhibitor LL-CHO (10 μM) for 16h failed to suppress the induction of proteasome subunits and p97 by BTZ (10nM) (O), or the processing of Nrf1 (P), by BTZ treatment. (Q-S) MG132 can also inhibit several lysosomal cathepsins [S3, S4]. However, inhibiting lysosomal proteolysis with leupeptin, a peptide aldehyde inhibitor of several lysosomal thiol proteases (e.g. cathepsin B and L), as well as calpains and trypsin-like serine proteases, did not block Nrf1 processing (Q) nor the induction of 26S subunits (Fig S2E) by BTZ. 100 μM Leupeptin, unlike 10 μM MG132, was unable to suppress the processing of Nrf1 caused by BTZ treatment (10 nM, 16h) in SH-SY5Y cells. Similarly, chloroquine (CQ), which raises intralysosomal pH, did not block BTZ-induced expression of 26S subunits. (R) Treatment of SH-SY5Y cells with CQ (50 μM) failed to suppress the induction of β3 and Rpt5 by BTZ (10 nM, 16h). (S) That CQ treatment inhibited lysosomal proteases is verified by the defective degradation of LC3-II in CQ-treated cells (Two samples loaded). (T-U) Because the cleavage of Nrf1 occurs in the ER, we also tested the possible involvement of three ER-associated intramembrane serine proteases, which are members of the rhomboid family (RHBDL2, RHBDL4, and RHBDD1). Rhomboid family proteases RHBDL2, RHBDL4, and RHBDD1 were all knocked down in the same HEK293A cells, as was confirmed by RT-PCR (T), but the triple knock-down did not suppress the processing of Nrf1 caused by BTZ (10 nM) treatment for 16h (U). (V,W) We tested the involvement of the two major ER-associated intramembrane aspartyl proteases, γ-secretase and signal peptidase (SPP) in the processing of Nrf1 upon proteasome inhibition. Treatment of SH-SY5Y cells with γ-secretase inhibitors DAPT (5 μM, V, [S5]), CpdE(2.5 μM,W, [S6]), L685458 (1 μM, W, [S6]), or the SPP inhibitor ES2 (5 μM, W) failed to suppress Nrf1 processing caused by treatment with BTZ (10 nM) or Epoxomicin (50 nM) treatment for 16h. Error bar = SD.

A



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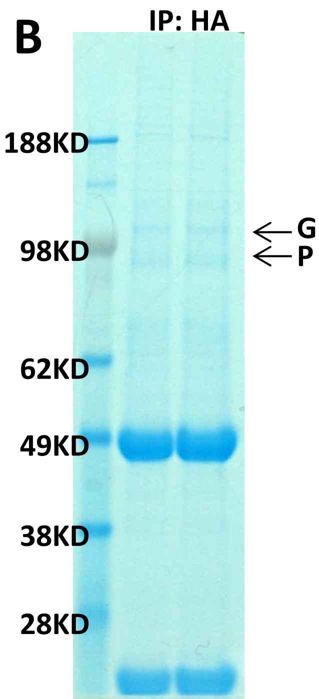


C

Sample: Nrf-HA cleaved (tracking number 37191)

Database search results: Data link

Sample: Nrf-HA full (tracking number 37192)

Database search results: Data link

From-To	Peptide	Peak height cleaved (37191)	Peak height Full (37192)	Δ	Δ
26 to 40	VDVDTYLTSQLPPLR	7.51E+03	5.23E+05		69.6
41 to 59	EIILGPSSAYTQTQFHNLR	9.53E+04	3.73E+06		39.1
60 to 70	NTLDGYGIHPK	5.92E+05	6.75E+06		11.4
71 to 80	SIDLDNYFTAR	6.59E+05	5.67E+06		8.6
108 to 141	DPEGSVSGSQPNSGLALESSSGLQDVTGPDNGVR	1.46E+05	1.53E+06		10.5
142 to 169	ESETEQGFGEDLEDLGAVAPPVSGDLTK	5.02E+04	3.16E+05		6.3
170 to 180	EDIDLIDILWR	7.24E+04	4.35E+04	1.7	
181 to 189	QDIDLGAGR	1.50E+07	1.62E+07		1.1
190 to 197	EVFDYSHR	1.68E+07	4.05E+06	4.1	
714 to 724	VQSLYQEVFGR	5.71E+06	6.72E+06		1.2
779 to 785	NPAFLYK	1.07E+07	9.16E+06	1.2	

Figure S4

Figure S4 (related to Fig 4). Proof of the identity of the glycosylated full-length Nrf1-HA (G form) and the processed Nrf1-HA (P form) by mass-spectrometry

(A-B) HEK293A cells overexpressing Nrf1-HA were treated with BTZ (10 nM) for 16h. Under this condition full-length deglycosylated Nrf1-HA is almost completely processed. Thus, only the original glycosylated full-length form and the processed form of Nrf1-HA were detectable and immunoprecipitated with anti-HA antibody. Successful immunoprecipitation was confirmed by western blotting (A), and purified Nrf1-HA sample was run on SDS PAGE to allow bands for the full-length glycosylated and processed Nrf1-HA to be revealed by coomassie blue staining and excision (B). (C) Abundance (Peak height) of different tryptic peptides from full-length glycosylated (full) and cleaved Nrf1 measured by LC/MS/MS. The substantially lower abundance of N-terminal peptides (26-40 and 41-59) indicates that the cleavage occurs at the N-terminal region of Nrf1. Mass-spectrometry failed to determine the exact cleavage site.

A

HEK293A

Ctrl BTZ BTZ + E1-In

GAPDH

Nrf1 (H-285)

FL

P

Ub

B

mRNA

2

1

0

*

β3 p97 β3 p97

HEK293A HEK293F

Ctrl

BTZ

BTZ + E1-In (0.5 μM)

C

MM1.S

E1-In Ctrl 0.2 μM 0.5 μM

BTZ - - + + - - + + - - + +

Nrf1 (H-285)

P

GAPDH

Ub

D

BTZ, nM 0 20

37°C 43°C 37°C 43°C

Ub

GAPDH

Nrf1 (C-19)

FL

P

E

BTZ, nM - 10 100 100

E1-In - - - +

37°C 42°C 37°C 42°C 37°C 42°C 37°C 42°C

Ub

GAPDH

HSP70

Nrf1 (H-285)

FL

P

G

Ctrl BTZ, 100 nM

$NaAsO_2$ - - + + - - + +

Ub

GAPDH

Nrf1 (C-19)

FL

P

F

mRNA

6

5

4

3

2

1

0

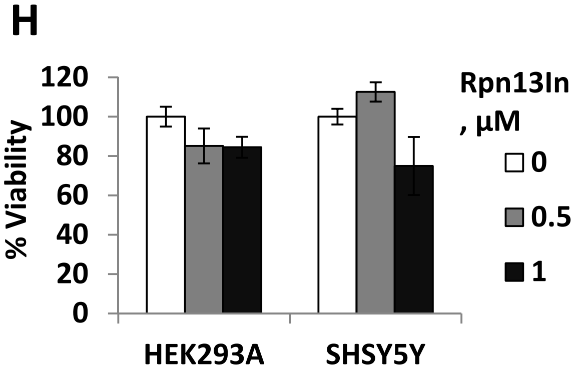
*

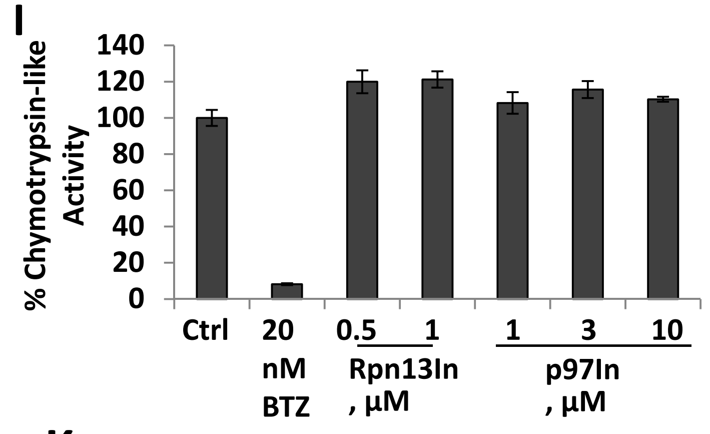
37°C 42°C 37°C 42°C

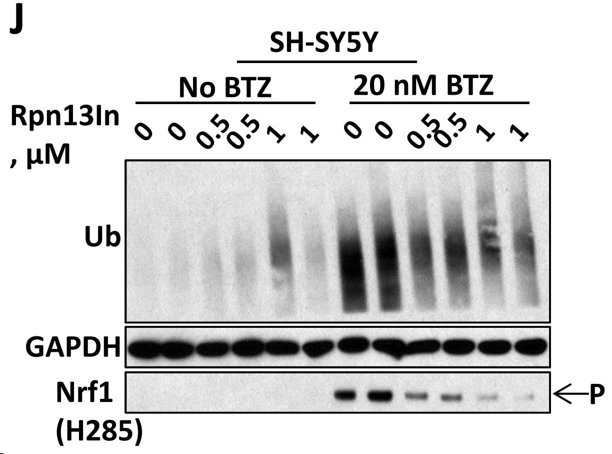
β3 p97

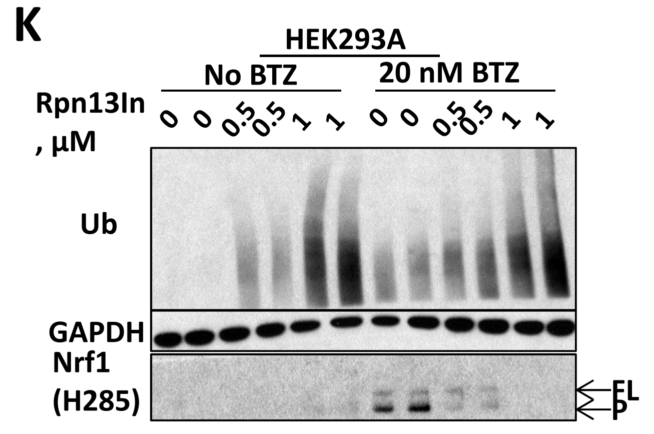
BTZ	E1-In
Ctrl	-
10 nM	-
100 nM	-
100 nM	+

Figure S5









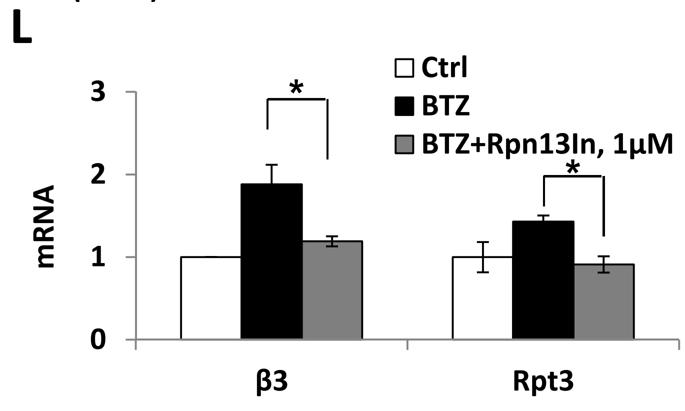


Figure S5 Continued

Figure S5 (related to Fig 6). Blocking ubiquitination blocks Nrf1 processing and the induction of p97 and the proteasome subunit β3 in several cell lines, but merely causing the accumulation of poly-ubiquitinated proteins by proteotoxic stress (heat shock or arsenite) do not promote and actually inhibit Nrf1 processing.

Treatment with ML00603997 (E1-In) for 16h suppressed BTZ (10 nM)-induced Nrf1 processing in HEK293A (A) and MM1.S (C) cells, and (B) the BTZ-induced expression of p97 and proteasome subunit β3 in HEK293A cells and HEK293F cells. Nrf1 is detected with H-285 antibody. *: $p<0.05$ (D) SH-SY5Y cells were incubated for 3h at 37°C or 43°C in the presence or absence of BTZ (20 nM). Heat shock did not cause the processing of Nrf1, and blocked BTZ-induced Nrf1 processing. (E) SH-SY5Y cells were treated with 10 nM or 100 nM BTZ, or 100 nM BTZ together with 0.5 μM ML00603997 for 16h at 37°C or 42°C. The heat shock response was monitored by the induction of Hsp70. At 42°C, Nrf1 processing was completely blocked, and (F) the expression of β3 and p97 was prevented. (G) Treatment of SH-SY5Y cells with 500 μM $NaAsO_2$ for 3h did not cause Nrf1 processing, and further suppressed Nrf1 processing caused by BTZ treatment (100 nM for 3h). *: $p<0.05$. (H) Treatment of HEK293A cells or SH-SY5Y cells with indicated concentrations of Rpn13 inhibitor RA-190 for 16h caused no significant loss of cell viability (MTS assay). (I) Treatment of HEK293A cells with indicated concentrations of Rpn13 inhibitor or p97 inhibitor did not cause any inhibition of the chymotrypsin-like peptidase activity of the proteasome. (J) Treatment of SH-SY5Y cells for 16h with Rpn13 inhibitor inhibited the BTZ-induced processing of Nrf1 to its active P form. (K-L) Treatment of HEK293A cells with Rpn13 inhibitor RA190 (Rpn13In) for 16h blocked BTZ (20nM)-induced Nrf1 processing (K) and also suppressed BTZ-induced expression of 26S subunits (L). Error bar = SD.

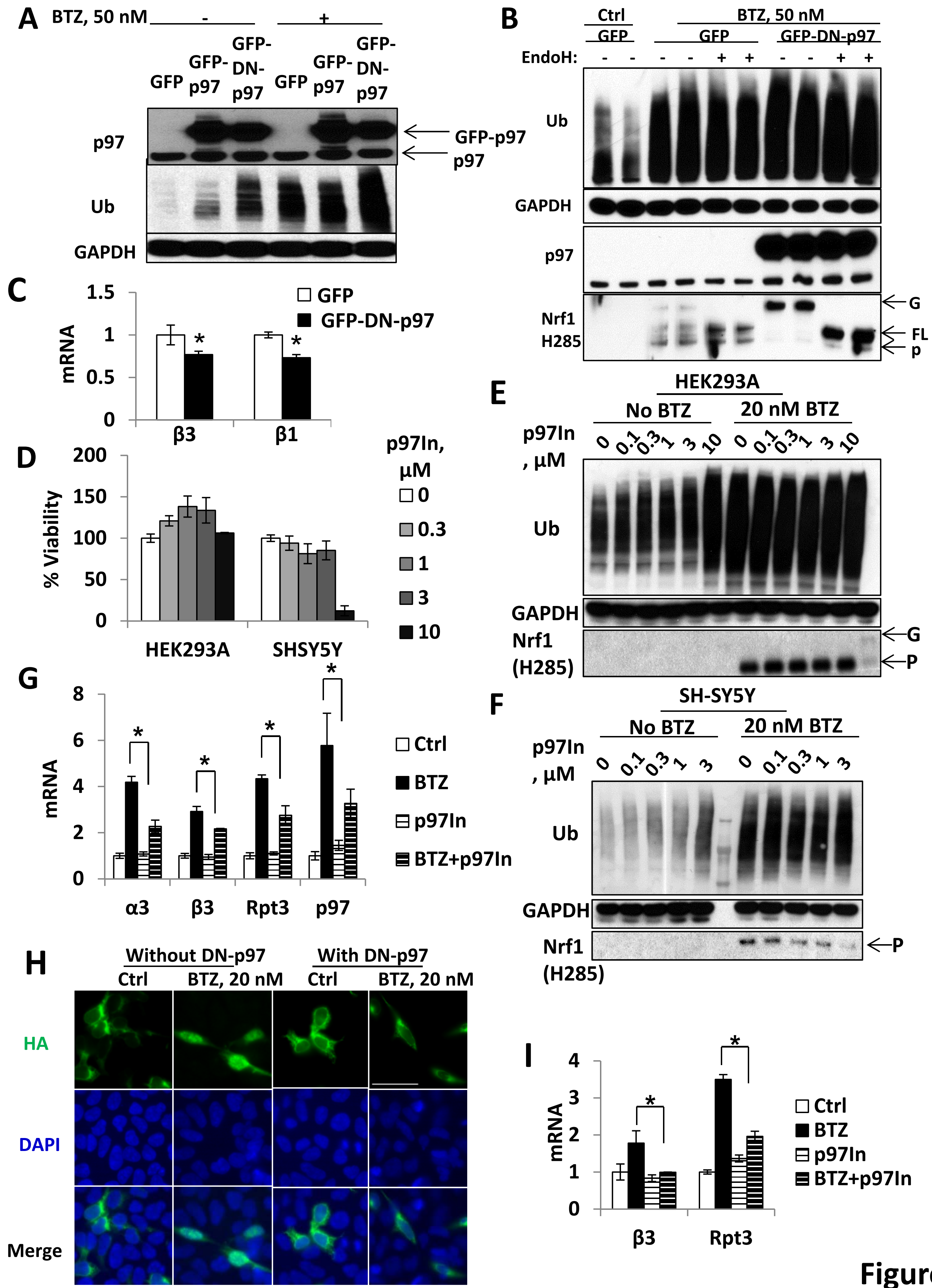


Figure S6

Figure S6 (related to Fig 7). Inhibition of p97 blocks Nrf1 processing and the induction of 26S subunits and p97.

(A) HEK293A cells overexpressing GFP-p97, GFP-DN-p97KA, or GFP (control) were incubated with or without 50 nM BTZ for 4h. GFP-DNp97 caused the accumulation of poly-ubiquitinated proteins. (B) To prove that over-expression of GFP-DN-p97 indeed blocks the deglycosylation of Nrf1, lysates from cells treated as in Fig 7A was treated with endoglycosidase H (Endo H) to remove high mannose N-glycans from glycoproteins. Endo H treatment efficiently caused deglycosylation of the G form of Nrf1 in BTZ-treated cells (Control cells or cells overexpressing GFP-DN-p97) and the deglycosylated Nrf1 migrated at the size of the FL form. (C) Overexpression of GFP-DN-p97 for 24h reduces the expression of β3 and β1. *: $p<0.05$. (D) HEK293A cells or SH-SY5Y cells were treated with indicated concentrations of p97 inhibitor for 16h. Viability of cells were measured by MTS assay. (E) Treatment of HEK293A cells with p97 inhibitor NMS859 (p97In) for 16h blocked Nrf1 processing induced by BTZ (20nM), and caused accumulation of glycosylated. (F-G) p97 inhibitor treatment (3 μM, 16h) of SH-SY5Y cells also blocked Nrf1 processing (F) and the induction of proteasome subunits and p97 (G). (H) DN-p97 also blocks the nuclear translocation of Nrf1-HA upon BTZ (20nM, 12h) treatment. Scale bar is 50 μm. (I) Treatment of HEK293A cells with p97 inhibitor NMS859 (p97In) for 16h suppressed BTZ-induced expression of 26S subunits. Error bar = SD.

Supplemental Experimental Procedures

Cell lines and growth conditions

Neuroblastoma cells M17 (ATCC, CRL-2267) and SH-SY5Y (CRL-2266) cells were cultured in DMEM-F12 (1:1) media (Mediatech, 10-092-CV). HEK293A cells and HEK293F cells were cultured in DMEM media (Mediatech, 10-013-CV). Myeloma cells MM1.S (provided to us by Dr. Teru Hideshima at Dana Farber Institute) were cultured in RPMI1640 media (Mediatech, 10-040-CV). All media contains 10% FBS (SIGMA, F6178-100ml) and 1% Penicillin-Streptomycin solution (Life Technologies, 15070-063). All cells were maintained in a humidified incubator at 37°C and 5% CO_2.

Overexpression of HA-Nrf2, Nrf1-HA, HA-Nrf1, GFP-p97, and GFP-p97K524A in HEK293A cells

To express Nrf1-HA and HA-Nrf1 in HEK293A cells, plasmids were purchased from Genecopoeia Inc. to express Nrf1-HA (EX-F0807-M07) or HA-Nrf1 (EX-F0807-M06). The plasmid (pCI-HA-Nrf2) was kindly provided by Dr. Michael Hannink's group at University of Missouri-Columbia [S1]. Plasmids for GFP, WTp97GFP and GFPp97K524A were described in [S7]. HEK293A cells were seeded in 6-well plates until 80% confluency. Transfection mixture containing 10 μg DNA, 5 μl Lipofectamine 2000 (Life Technologies, 11668-019) was prepared in 500 μl Opti-MEM® I Reduced-Serum Medium (Life Technologies, 51985-034) and allowed to mix at room temperature for 20 min, and then added to each well containing 2.5 ml complete medium. After 8h, the transfection mixture was replaced with fresh medium. Cells were normally incubated for 48-72h before assays, but experiments involving expression of

GFPp97K524 were terminated within 24h of transfection due to the toxicity of p97K524 to cells after prolonged expression.

Transient Knock-down of Rhomboid-like proteases by siRNA

To knock down Rhomboid-like proteases in HEK293F cells, siRNAs were purchased from Thermo Scientific to target RHBDL2 (L-006028-00-0005), RHBDL4 (L-006029-00-0005), and RHBDD1 (L-019378-00-0005). Transfection mixture containing 20 pmol siRNA and 1 μ Lipofectamine 2000 (Life Technologies, 11668-019) was prepared in 100 μl Opti-MEM® I Reduced-Serum Medium (Life Technologies, 51985-034) and allowed to mix under room temperature for 20 minutes before addition to the cells (cultured with 500 μl Pen/Strep-free DMEM in a 24-well plate until 30-40% confluency). 48h after transfection, the cells were treated with BTZ (10 nM) for 16h. As a control, a mixture was used that contained only the transfection reagent but no siRNA.

Construction of stable Nrf1 or Nrf2 knock-down cell lines

Stable knock-downs of Nrf1 or Nrf2 in SH-SY5Y cells were performed using lentiviral particles expressing shRNA for Nrf1 (Santa Cruz, sc-43575-V), Nrf2 (Santa Cruz, sc-156128-V), or control Lentiviral particles expressing cop-GFP (Santa Cruz, sc-108084). Lentivirus infection was set up in 24-well format with 2.5×10^4 cells seeded in each well. 5×10^4 lentiviral particles were mixed with the complete medium to a total volume of 250 μl. Polybrene (Santa Cruz, sc-134220) was added at a final concentration of 8μg/ml. The mixture was applied to each well for 6h, and 350 μl fresh complete medium were added to each well. 24h after infection, stable clones were selected in the presence of 1 μg/ml puromycin.

Treatment with proteasome inhibitors or other compounds

Stock solutions were prepared for the following proteasome inhibitors MG132 (Boston Biochem, I-130, 10 mM, DMSO), BTZ (Millenium Pharmaceuticals, 1 μM, H_2O), MG262 (Enzo Life Sciences, BML-PI109-0100, 1 mM, DMSO), and Epoxomicin (Calbiochem , 324800-100UG , 200 μM, DMSO); UPR inducers: Tunicamycin (SIGMA, T7765-10MG, 10mg/ml, DMSO) and Thapsigargin (SIGMA, T9033-0.5MG, 100 μM, DMSO); Signal peptide peptidase inhibitor ES2 (1 mM, DMSO); γ-secretase inhibitors Compound E(((2S)-2-{[(3,5-Difluorophenyl) acetyl]amino}-N-[(3S)-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl]propanamide, 500 μM, DMSO), L685458(tert-butyl ((2S,3R,5R)-6-(((S)-1-(((S)-1-amino-1-oxo-3-phenylpropan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-5-benzyl-3-hydroxy-6-oxo-1-phenylhexan-2-yl)carbamate, 200 μM, DMSO), DAPT(N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, 5 mM, DMSO); Cycloheximide (10 mg/ml, DMSO); Chloroquin (SIGMA, C6628-25G, 50 mM DMSO); Calpain inhibitors LL-CHO (Bachem, N-1895, 10 mM, DMSO) and PD150606 (3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid, SIGMA, D5946-5MG, 100mM, DMSO); Leupeptin (Boehringer Mannheim, 1017128, 100 mM DMSO), 2-deoxyglucose (SIGMA, D-6134, 1 M, H_2O), 2,4-Dinitrophenol (SIGMA, D198501-100G, 50 mM, H_2O), $NaAsO_2$ (Fisher, S-225, 10 mM, H_2O), NMS-859 (Xcess Biosciences Inc. M60148, 10 mM, DMSO) . ES2, Compound E, L685458, and DAPT were kindly provided by Professor Michael S.Wolfe's group at Harvard Medical School. Treatment time was 16h unless specified. The E1 inhibitor ML00603997 (20 mM, DMSO) was kindly provided by Dr. Laurence R. Dick at Millenium Pharmaceuticals.The Rpn13 inhibitor RA190 (10 mM, DMSO) was kindly provided by Professor Richard Roden's group at the Johns Hopkins University.

Immunostaining

To detect the localization of Nrf1-HA or HA-Nrf1 in HEK293A cells, cells were seeded on coverslips pre-placed in 6-well plates 24h after transfection and allowed to grow for another 24h. After the cells were treated with BTZ (10 nM), Epoxomicin (50 nM), or MG132 (10 μM) for 16h, these cells were fixed with 100% methanol pre-stored at -20°C for 10 minutes, then re-hydrated by washing 3 × 5 min in PBS. Nrf1-HA or HA-Nrf1 was detected with anti-HA antibody (SIGMA, H9658-.2ML, 1:400) or anti-Nrf1(H-285) antibody (Santa Cruz, sc-13031, 1:100), which was further bound with Alexa Fluor 488-conjugated anti-Mouse secondary antibody (Life Technologies, A-11001, 1:500), Alexa Fluor 555-conjugated anti-Rabbit secondary antibody (Life Technologies, A-21428, 1:500), or Tetramethylrhodamine-conjugated anti-mouse secondary antibody (Life Technologies, A-2762, 1:500). Nuclei were counter-stained with DAPI contained in the mounting media (Vector Laboratories, H-1200). Images were taken at a Nikon Ti Inverted Fluorescence Microscope equipped with a Hamamatsu ORCA-R2 cooled CCD camera remotely controlled with Metamorph acquisition software.

Immunoprecipitation

To immunoprecipitate Nrf1-HA, lysate from HEK293A cells overexpressing Nrf1-HA was precleared by normal mouse IgG (Santa Cruz, sc-2025) followed by immunoprecipitation with HA antibody (SIGMA, H9658-.2ML) or normal mouse IgG as a control. Immunoprecipitated proteins were absorbed by Protein A Agarose (SIGMA, P3476-1ML) and eluted by boiling in SDS sample buffer. The amount of antibody and Protein A Agarose was pre-optimized for every preparation of lysate.

Determination of protein sequence by mass-spectrometry

To prepare samples for mass-spectrometry determination of protein sequence, purified Nrf1-HA was ran on SDS page and stained with Coomassie Blue G-250 (SimplyBlue Safe Stain, Life Technologies, LC6065) to allow visualization of the bands for unprocessed (full-length glycosylated) and processed Nrf1. These bands were excised and submitted to the Taplin Mass-spectrometry Facility at Harvard Medical School for in-gel trypsin digestion, and micro-capillary LC/MS/MS analysis.

Western Blotting

Cells were lysed for 30 min in ice-cold TX-100 lysis buffer (50mM NaCl, 30mM $Na_4P_2O_7$, 50 mM NaF, 10 mM Tris-Cl pH 7.6, 5 mM EDTA, 1 mM Na_3VO_4, 1% TX-100, Roche Protease Inhibitor Cocktail Tablet, 100 µl per well (24-well)), and cell debris was cleared by centrifugation (10,000 ×g, 10 min). Antibodies against Rpt5(Enzo Lifesciences BML-PW8770-0100, 1:10,000), α123567 (Enzo Lifesciences, BML-PW8195-0100, 1:10,000), p97 (Cell Signaling 2648 1:1,000), Nrf1(H-285)(Santa Cruz, sc-13031, 1:1000), Nrf1(C-19)(Santa Cruz, s-721, 1:1000), HA(SIGMA, H9658-.2ML, 1:10,000), GAPDH(SIGMA, G8795-200µl, 1:10,000), Nrf2 (Santa Cruz, sc-722, 1:1,000), NQO1 (Abcam, ab2346, 1:1,000), phospho-eIF2α (Cell Signaling Technology, 3597S, 1:1,1000), Ubiquitin (FK2, Enzo Lifesciences BML-PW8810, 1:5,000), HSP70 (Stressgene, SPA-810, 1:1,000) were used to detect corresponding proteins. Unless specified in the figure legend, Nrf1 was always detected with the C-19 antibody.

Real-Time RT-PCR

Primers were designed for individual genes to allow their quantification by real time RT-PCR. mRNA was extracted from cultured MM1.S or SH-SY5Y cells via TRIzol Reagent (Life Technologies 15596-018) and precipitated with isopropanol, cDNA was synthesized using

Multiscribe Reverse Transcriptase (Applied Biosystems, 4311235) according to product instructions. Real-time RT-PCR was performed using ABsolute Blue QPCR ROX Mix (Thermo Scientific AB4162B) on a Bio-Rad C-1000 thermocycler using the following parameters: (Pre-heat 95°C, 5 min, 40×95°C/28sec-60°C/28sec-72°C/28sec, Elongation 72°C, 10 min). Two biological duplicates (separate wells of cells independently treated with identical conditions) were measured to determine the average and standard deviation of each measurement, and the value of each biological duplicates was determined from three replicates in RT-PCR.

Proteasomal Peptidase Activity

Cell lysate was prepared as for western blotting. Peptidase activity assay was set up in a 100 µl reaction (96 well plate format) containing 1 µg lysate protein, 50 mM Tris-HCl pH8.0, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 50 µM Suc-LLVY-AMC (Bachem, I-1395.0100, 20 mM in DMSO, to measure chymotrypsin-like activity) or 50 µM Z-LLE-AMC (Bachem, I-1495.005, 10 mM in DMSO, to measure caspase-like activity). Fluorescence of AMC was measured in a SpectraMax M5 microplate reader (Molecular Devices. now VWR 0310-5625) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Fluorescence was read every 35 sec for a total duration of 1 hr, and the velocity of peptide substrate hydrolysis was determined by the RFU over time slope.

Native Gel Electrophoresis

Native gels and overlay assay with Suc-LLVY-AMC were conducted as describe before (see main text). Native 3–8% Tris-acetate gels (Life Technologies, EA03752BOX) were used as instructed by the manufacturer. To detect the amount of 26S proteasomes separated by native gel

electrophoresis, proteins were transferred to nitrocellulose membrane and 26S proteasomes were detected by the anti-α123567 antibody as in western blotting.

Deglycosylation of glycoproteins by Endoglycosidase H (Endo H)

To prove that the G form of Nrf1 is indeed a glycosylated form, endoglycosidase H (Endo H) was purchased from New England Biolabs (P0702S) and the deglycosylation reaction was set up according to the manufacturer's instructions. Proteins were extracted by TX-100 buffer as in western blotting and the protein concentration of all samples was brought to 2 μg/μl. 10 μg proteins were denatured and incubated with 3 μl Endo H at 37°C for 1 hr to allow complete deglycosylation of glycoproteins. Control reaction was set up with no Endo H.

Measurement of cellular protein degradation

To measure the degradation rate of long-lived proteins, SH-SY5Y cells were first labelled for 24h with Tritium-labelled Phenylalanine (Phe L-[3,4,5 ^{3}H], American Radiolabeled Chemicals, Inc. ART0614, stock 1 mCi/ml in 0.01M HCl, final 5 μCi/ml), then chased for 1 hr with complete media containing 2 mM cold Phenylalanine (Sigma, P5482-25G) to allow turn-over of short-lived proteins. Cells were then allowed to be incubated in media containing both 2mM cold Phe and various concentrations of BTZ. Protein degradation would cause ^{3}H-Phe to be released into the media which could not be precipitated by Trichloroacetic acid (TCA, VWR, BDH3372-2, working concentration 0.625M). Media was collected at 0, 1, 2, 3, 4h during the incubation. 200 μl supernatant after TCA precipitation was mixed with 3 ml Ultima Gold Scintillation Fluid (PerkinElmer, 6013327) and the TCA-soluble count was measured with a PerkinElmer Tri-Carb 2910TR Liquid Scintillation Analyzer equipped with QuantaSmart™ software. To calculate percent protein degradation, cells were lysed after the last time point with 0.1M NaOH and 100

μl lysate was mixed with 3 ml scintillation fluid in order to measure total radioactivity incorporated. Protein degradation rate was determined by plotting percent protein degradation over time. 4 independent samples were used to determine the protein degradation rate under the treatment of various concentrations of BTZ, and the degradation rate without BTZ treatment was set as 100%.

Cell Viability Assay

Cell viability was measured by either CellTiter 96® AQueous Non-Radioactive Cell Proliferation (MTS) Assay (Promega, G5421) or CytoTox 96® Non-Radioactive Cytotoxicity Assay (LDH Release) Assay (Promega, G1780) according to manufacturer instructions.

Statistics

Unpaired student t test was used for statistical analysis throughout the entire manuscript. p value less than 0.05 is the criteria for significant difference.

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