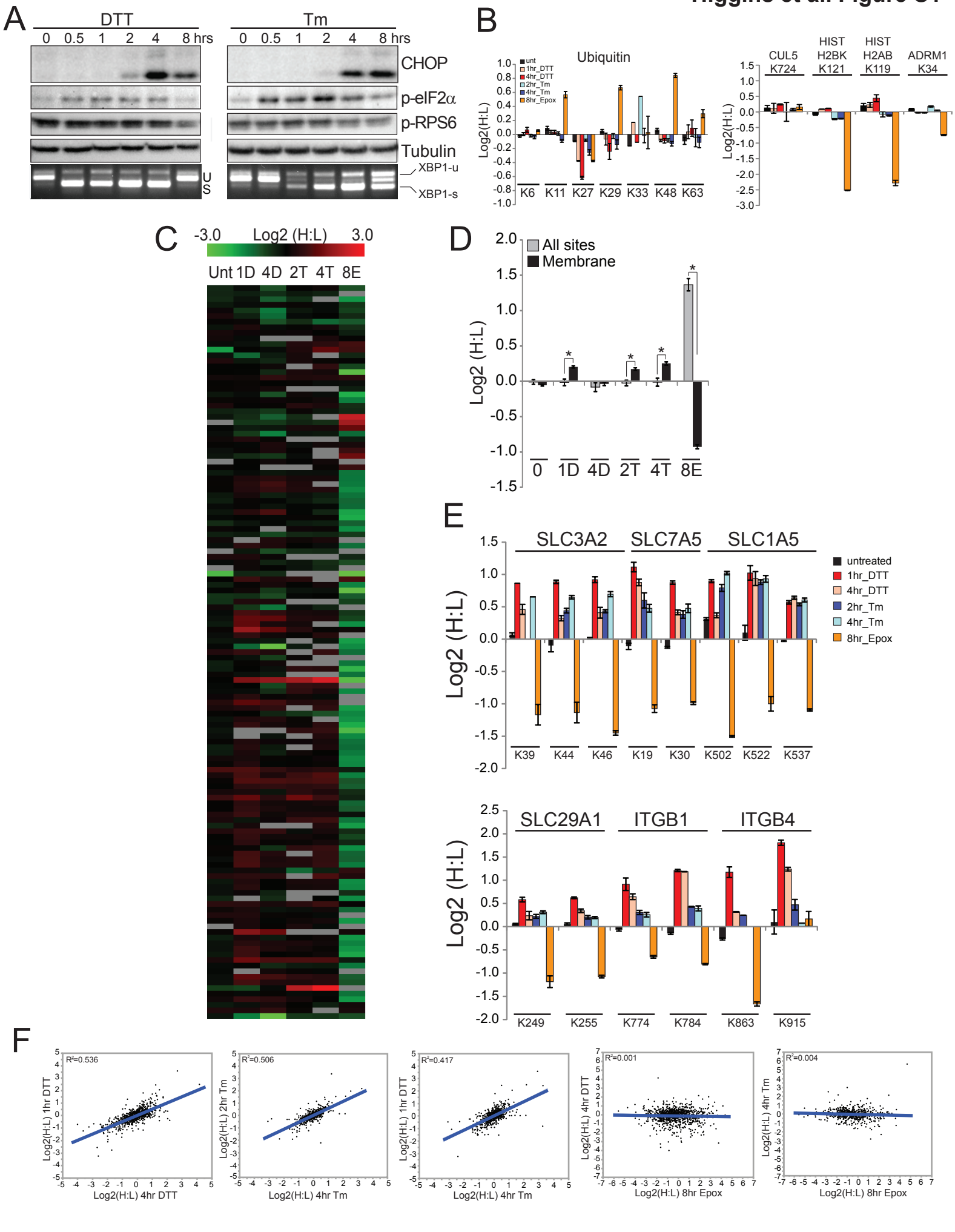
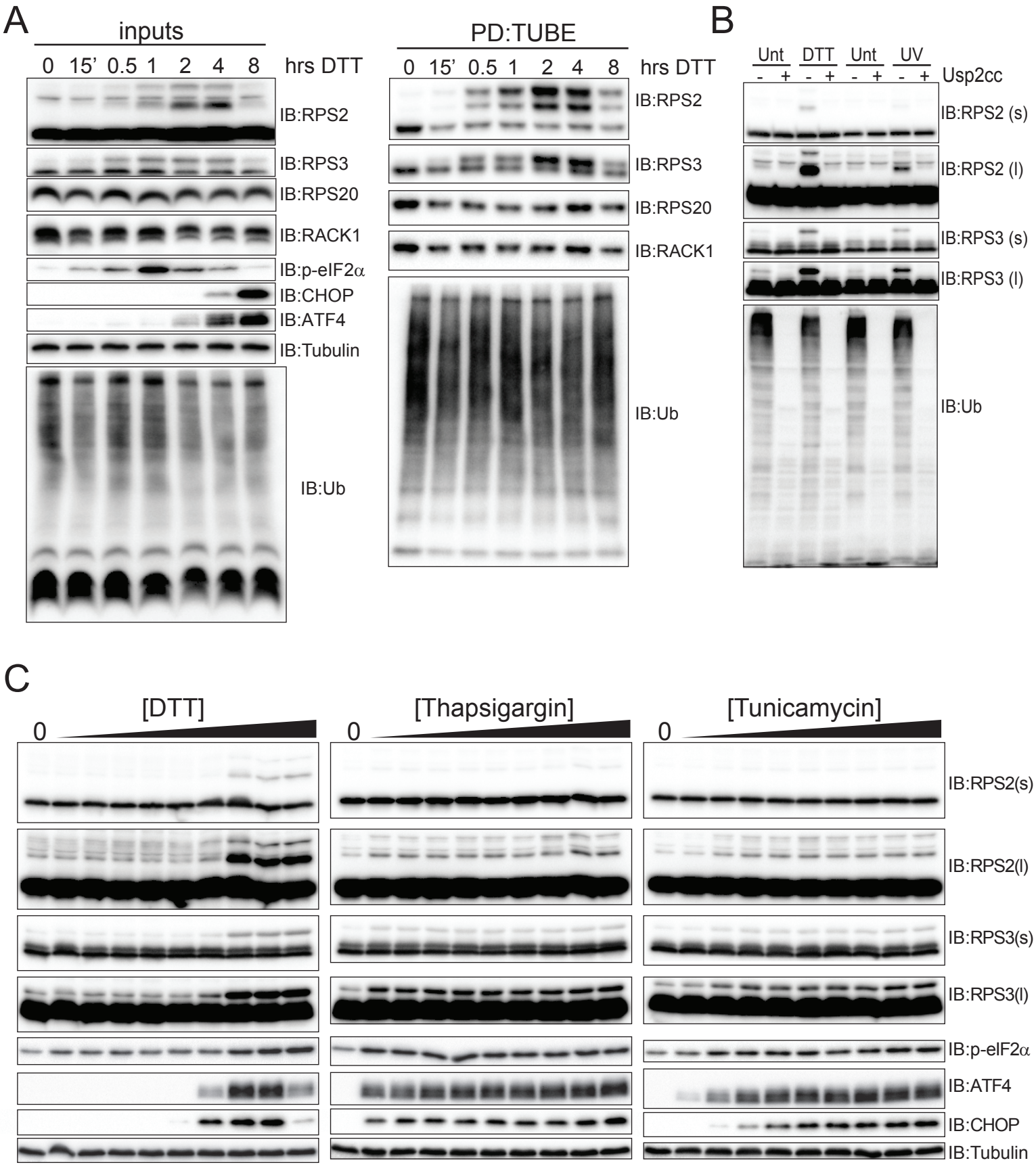
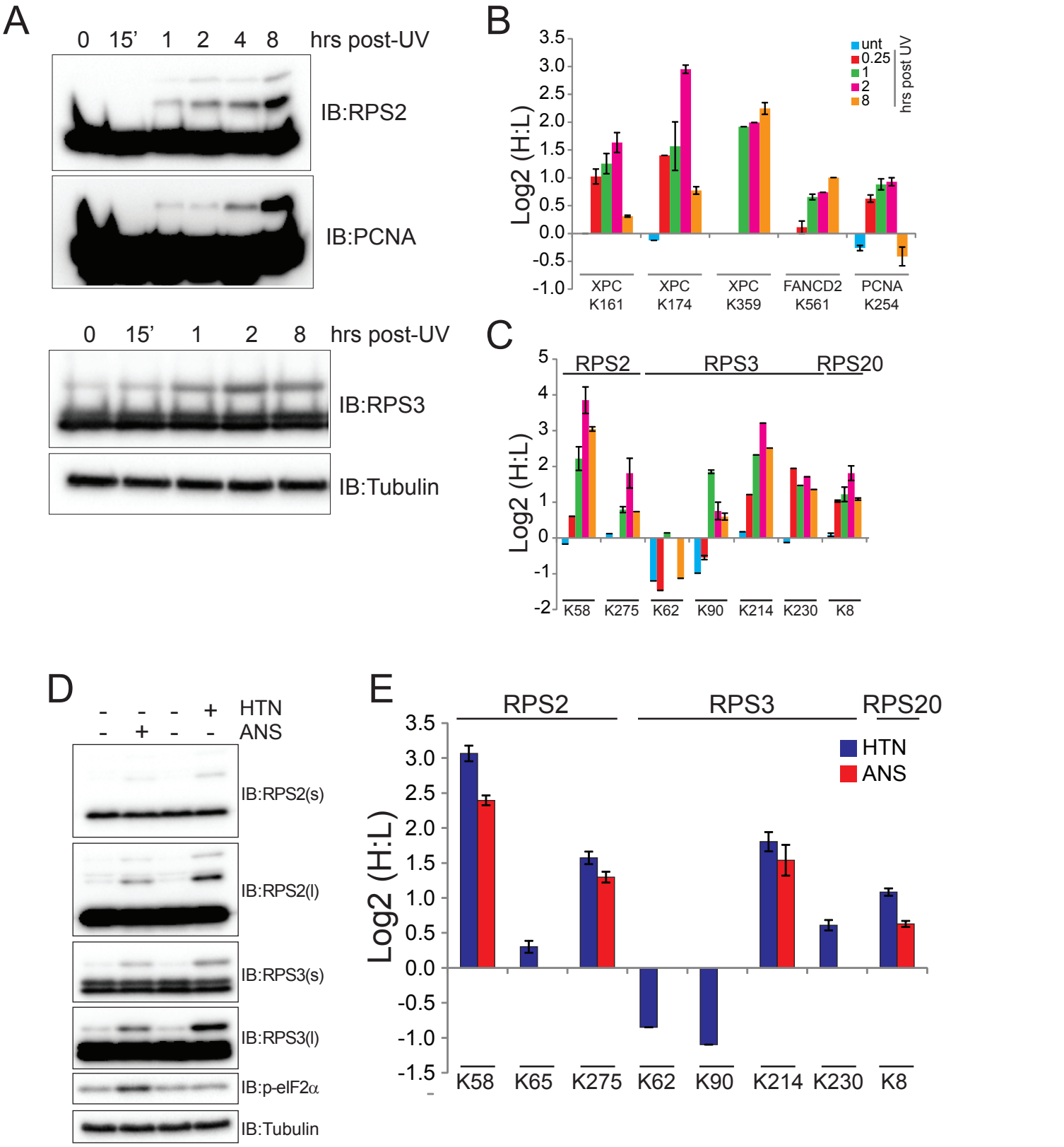
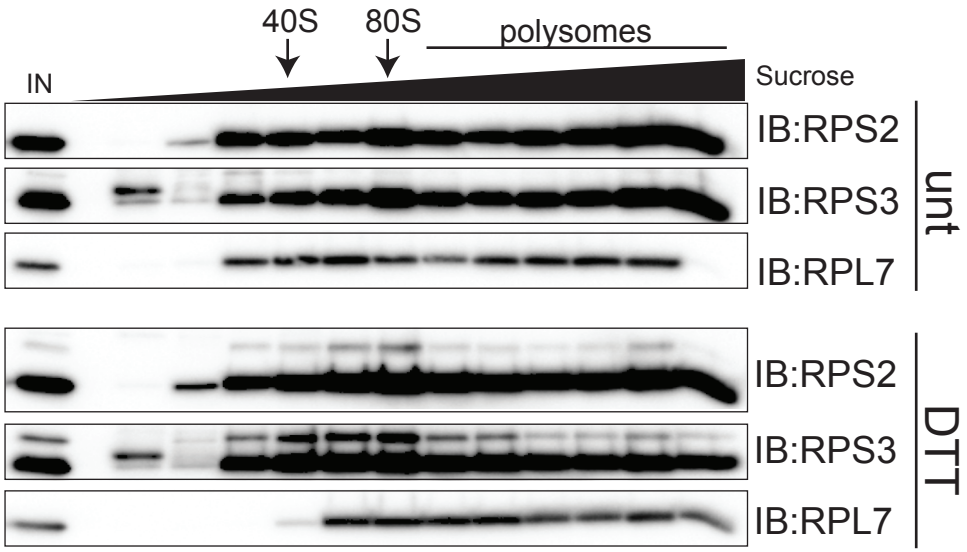

A
DTT
Tm
0 0.5 1 2 4 8 hrs
CHOP
p-eIF2α
p-RPS6
Tubulin
XBP1-u
XBP1-s
U
S
B
Ubiquitin
unt
1hr_DTT
4hr_DTT
2hr_Tm
4hr_Tm
8hr_Epox
Log2(H:L)
K6 K11 K27 K29 K33 K48 K63
CUL5 K724
HIST H2BK K121
HIST H2AB K119
ADRM1 K34
C
-3.0 Log2 (H:L) 3.0
Unt 1D 4D 2T 4T 8E
D
All sites
Membrane
Log2 (H:L)
0 1D 4D 2T 4T 8E
E
SLC3A2
SLC7A5
SLC1A5
untreated
1hr_DTT
4hr_DTT
2hr_Tm
4hr_Tm
8hr_Epox
Log2 (H:L)
K39 K44 K46 K19 K30 K502 K522 K537
SLC29A1
ITGB1
ITGB4
K249 K255 K774 K784 K863 K915
F
R²=0.536
Log2(H:L) 1hr DTT
Log2(H:L) 4hr DTT
R²=0.506
Log2(H:L) 2hr Tm
Log2(H:L) 4hr Tm
R²=0.417
Log2(H:L) 1hr DTT
Log2(H:L) 4hr Tm
R²=0.001
Log2(H:L) 4hr DTT
Log2(H:L) 8hr Epox
R²=0.004
Log2(H:L) 4hr Tm
Log2(H:L) 8hr Epox

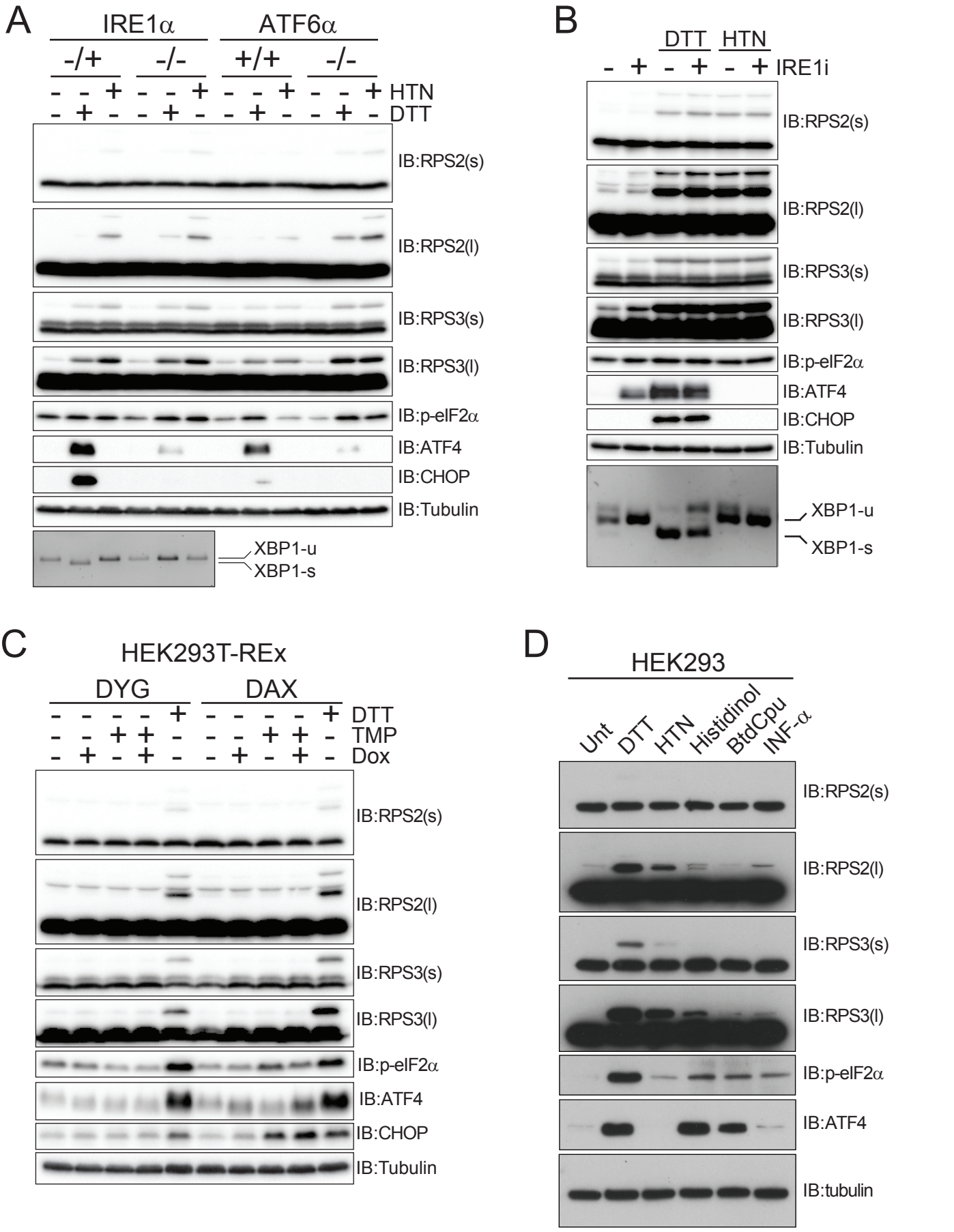
Higgins et al. Figure S2

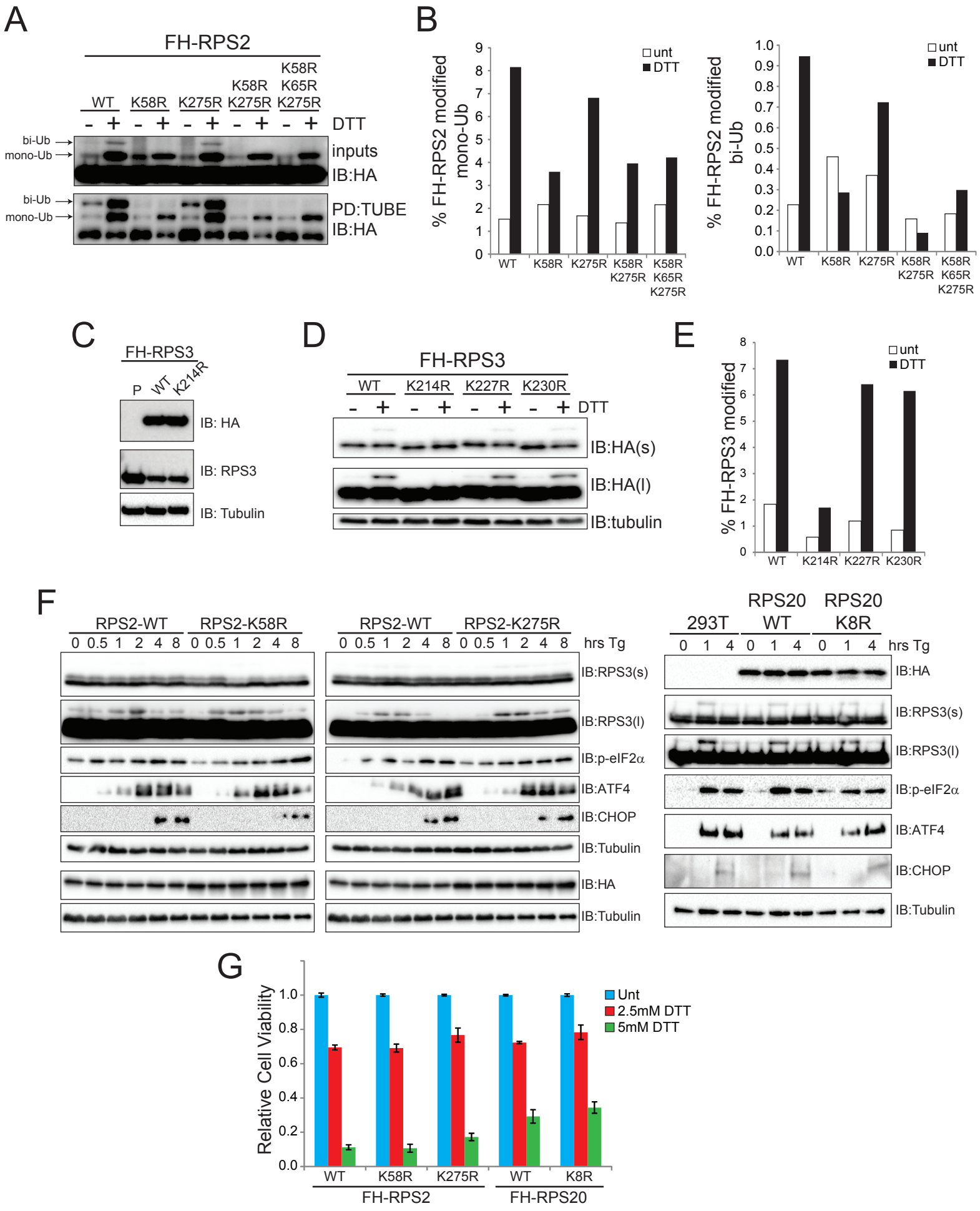


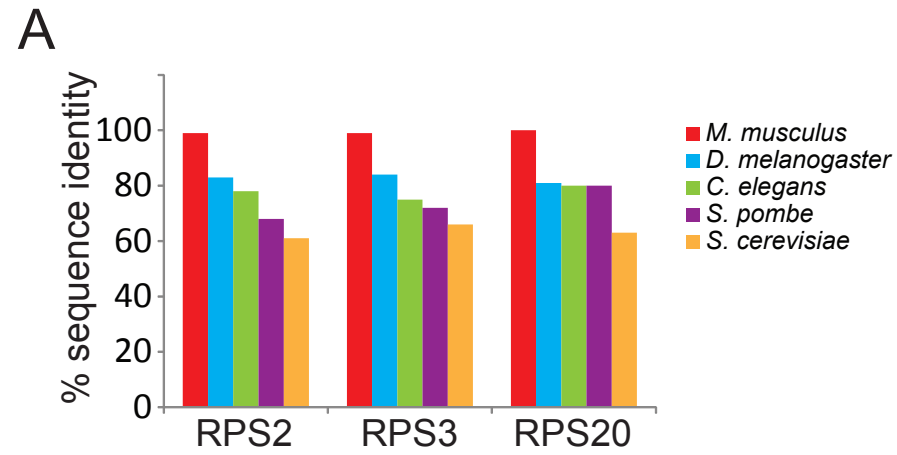

A
0 15' 1 2 4 8 hrs post-UV
IB:RPS2
IB:PCNA
0 15' 1 2 8 hrs post-UV
IB:RPS3
IB:Tubulin
B
Log2 (H:L)
unt
0.25
1
2
8
hrs post UV
XPC K161
XPC K174
XPC K359
FANCD2 K561
PCNA K254
C
RPS2
RPS3
RPS20
Log2 (H:L)
K58
K275
K62
K90
K214
K230
K8
D
HTN
ANS
IB:RPS2(s)
IB:RPS2(l)
IB:RPS3(s)
IB:RPS3(l)
IB:p-eIF2α
IB:Tubulin
E
RPS2
RPS3
RPS20
HTN
ANS
Log2 (H:L)
K58
K65
K275
K62
K90
K214
K230
K8

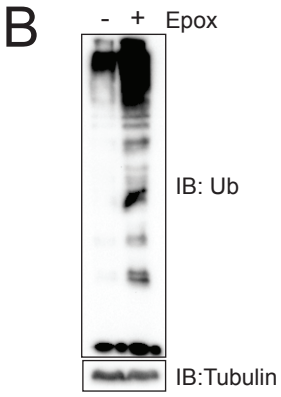
Higgins et al. Figure S4

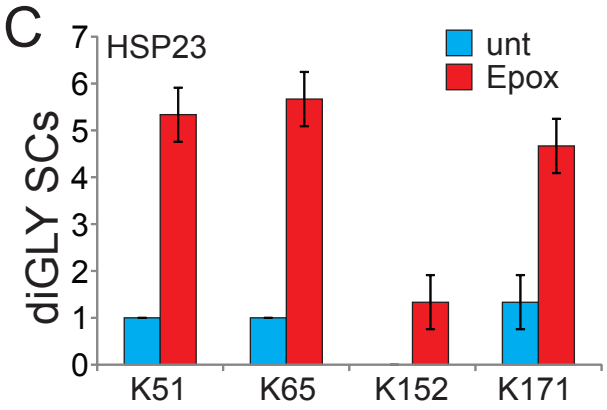


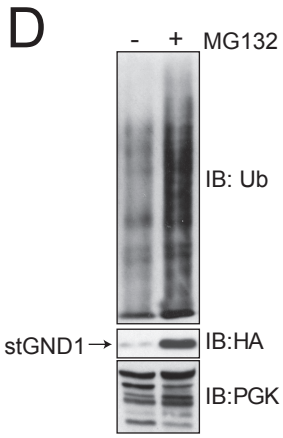

A
IRE1α
ATF6α
-/+
-/-
+/+
-/-
HTN
DTT
IB:RPS2(s)
IB:RPS2(l)
IB:RPS3(s)
IB:RPS3(l)
IB:p-eIF2α
IB:ATF4
IB:CHOP
IB:Tubulin
XBP1-u
XBP1-s
B
DTT
HTN
IRE1i
IB:RPS2(s)
IB:RPS2(l)
IB:RPS3(s)
IB:RPS3(l)
IB:p-eIF2α
IB:ATF4
IB:CHOP
IB:Tubulin
XBP1-u
XBP1-s
C
HEK293T-REx
DYG
DAX
DTT
TMP
Dox
IB:RPS2(s)
IB:RPS2(l)
IB:RPS3(s)
IB:RPS3(l)
IB:p-eIF2α
IB:ATF4
IB:CHOP
IB:Tubulin
D
HEK293
Unt
DTT
HTN
Histidinol
BtdCpu
INF-α
IB:RPS2(s)
IB:RPS2(l)
IB:RPS3(s)
IB:RPS3(l)
IB:p-eIF2α
IB:ATF4
IB:tubulin

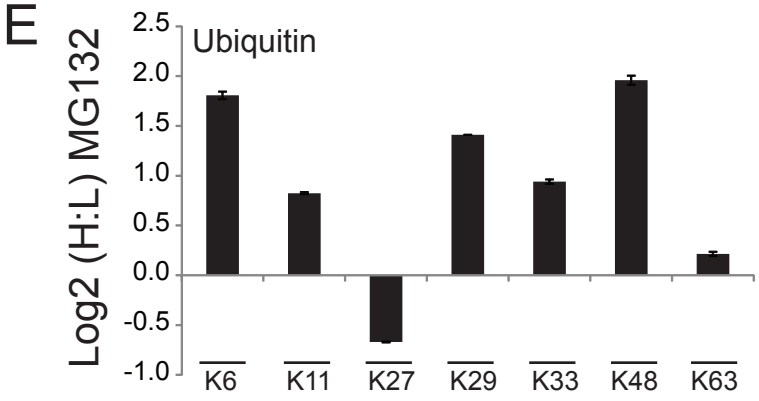

A
FH-RPS2
WT
K58R
K275R
K58R K275R
K58R K65R K275R
- + - + - + - + - + DTT
bi-Ub
mono-Ub
inputs IB:HA
PD:TUBE IB:HA
B
% FH-RPS2 modified mono-Ub
% FH-RPS2 modified bi-Ub
unt
DTT
WT
K58R
K275R
K58R K275R
K58R K65R K275R
C
FH-RPS3
P
WT
K214R
IB: HA
IB: RPS3
IB: Tubulin
D
FH-RPS3
WT
K214R
K227R
K230R
- + - + - + - + DTT
IB:HA(s)
IB:HA(l)
IB:tubulin
E
% FH-RPS3 modified
unt
DTT
WT
K214R
K227R
K230R
F
RPS2-WT
RPS2-K58R
RPS2-K275R
0 0.5 1 2 4 8
hrs Tg
IB:RPS3(s)
IB:RPS3(l)
IB:p-eIF2α
IB:ATF4
IB:CHOP
IB:Tubulin
IB:HA
293T
RPS20 WT
RPS20 K8R
0 1 4
G
Relative Cell Viability
Unt
2.5mM DTT
5mM DTT
WT
K58R
K275R
K8R
FH-RPS2
FH-RPS20


A
% sequence identity
100
80
60
40
20
0
RPS2
RPS3
RPS20
M. musculus
D. melanogaster
C. elegans
S. pombe
S. cerevisiae


B
- + Epox
IB: Ub
IB:Tubulin


C
HSP23
unt
Epox
diGLY SCs
7
6
5
4
3
2
1
0
K51
K65
K152
K171


D
- + MG132
IB: Ub
stGND1→
IB:HA
IB:PGK


E
Ubiquitin
Log2 (H:L) MG132
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
K6
K11
K27
K29
K33
K48
K63

Supplemental Figure Legends

Figure S1 – ER stress and proteasome inhibition induces distinct alterations to the diGly-modified proteome. (Related to Figure 1)

A) HCT116 cells were treated with 5mM DTT, or 5μg/ml tunicamycin (Tm) for the indicated times. Whole cell lysates were blotted with the indicated antibodies. XBP1 splicing was detected by PCR from total cDNA isolation.

B) Left, SILAC Log2 H:L ratios of all quantified ubiquitin linkage peptides for each treatment. The lysine position on ubiquitin is indicated. Error bars represent the standard error of the mean (SEM) for multiple quantifications. Right, SILAC Log2 H:L ratios for specific diGly-modified peptides. The protein and diGly-modified lysine position is indicated.

C) Heat map of the Log2 peptide ratios for all diGly-modified peptides from proteins containing an annotated transmembrane domain. The Log2 ratio from each experiment (Unt=untreated, 1D=1hr DTT, 4D=4hr DTT, 2T=2 hr Tm, 4T=4 hr Tm, 8E=8 hr Epox) is shown.

D) The average Log2 ratio from all quantified diGly-modified peptides (grey bar) is compared to the average Log2 ratio from quantified diGly-modified peptides from transmembrane domain containing proteins (black bar) in each experiment. Error bars represent the SEM for each group. * indicated a p-value < 0.05 using Student's t-test.

E) SILAC Log2 H:L ratios for a selection of diGly-modified peptides from transmembrane-domain containing proteins. The protein and diGly-modified lysine position is indicated. Error bars represent SEM from multiple peptide MS quantifications.

F) Log2 H:L peptide ratios for all diGly-modified peptides that were quantified in both experiments as indicated by the axes labels are depicted. The best-fit line from linear regression analysis is shown as the coefficient of determination.

See also Table S4

Figure S2 – RPS2 and RPS3 ubiquitylation are early events upon UPR induction. (Related to Figure 2)

A) Whole cell lysates from HCT116 cells treated with 5mM DTT for the indicated times were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. Ubiquitylated proteins were captured using a tandem ubiquitin binding entity (TUBE) resin (Halo-4xUBAUBQLN1) and eluates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

B) Native whole cell extracts from HCT116 cells treated as indicated prior to lysis were mixed with or without purified Usp2cc. After Usp2cc treatment lysates were denatured and analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

C) Whole cell lysates from HCT116 cells treated with increasing concentrations of the indicated UPR inducers for 4hrs were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. Each experiment is a two-fold dilution series starting with 20mM DTT, 10μM Thapsigargin, or 20μg/ml Tunicamycin. Short (s) and long (l) exposures are presented.

Figure S3 – Site-specific ubiquitylation of 40S ribosomal proteins is induced by exposure to UV or translation elongation inhibitors. (Related to Figure 3)

A) Whole cell lysates from HCT116 cells exposed to UV (50 J/m^2) and allowed to recover for the indicated times were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

B) Heavy (K8) lysine labeled cells were either untreated or exposed to UV (50 J/m^2) and allowed to recover for the indicated times prior to mixing with untreated light labeled control cells. The SILAC Log2 H:L ratios for each experimental condition for a selection of specific diGly-modified peptides are depicted. The protein and diGly-modified lysine position is indicated. Error bars represent SEM from multiple peptide MS quantifications.

C) SILAC Log2 H:L ratios for each experimental condition for diGly-modified peptides on the 40S ribosomal proteins RPS2, RPS3, and RPS20. The protein and diGly-modified lysine position is indicated. Error bars represent SEM from multiple peptide MS quantifications.

D) Whole cell lysates from HCT116 cells either untreated or treated with anisomycin (ANS) or harringtonine (HTN) were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

E) SILAC Log2 H:L ratios from anisomycin (ANS, red) or harringtonine (HTN, blue) treated cells for diGly-modified peptides on the 40S ribosomal proteins RPS2, RPS3, and RPS20. The protein and diGly-modified lysine position is indicated. Error bars represent SEM from multiple peptide MS quantifications.

Figure S4 –DTT-induced RPS2 and RPS3 ubiquitylation occurs on polysomes. (Related to Figure 4)

HCT116 cells were untreated (top) or treated with DTT (bottom). Cycloheximide was included in the lysis buffer to help maintain the integrity of the polysomes. Whole cell lysates were separated on a 10-50% linear sucrose gradient. Sucrose gradient fractions from cells treated as indicated were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. IN represents the whole cell lysate input. The position of the 40S and 80S particles is depicted by the arrows.

Figure S5 – IRE1 or ATF6 signaling is not necessary or sufficient for UPR-induced RPS2 or RP3 ubiquitylation. (Related to Figure 5)

A) Whole cell lysates from immortalized mouse embryonic fibroblasts derived from IRE1α heterozygous or homozygous knockout mice or wildtype or ATF6α knockout mice treated with DTT or HTN were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. XBP1 splicing was detected by PCR from total cDNA isolation.

B) Whole cell lysates from HCT116 cells treated with DTT or HTN alone or in combination with an IRE1α endonuclease inhibitor (Irestatin, STF-083010) were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. XBP1 splicing was detected by PCR from total cDNA isolation.

C) HEK293T-REx cells expressing tet-inducible GFP and DHFR-YFP (DYG) or tet-inducible spliced XBP1 and DHFR-constitutively active ATF6(1-373) (DAX) were treated with DTT, Doxycycline (Dox), or trimethoprim (TMP) alone or a combination of Dox and TMP. Whole cell lysates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

D) HEK293 cells were treated with a panel of individual eIF2α kinase inducers. Whole cell lysates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

Figure S6 – Mutational analysis of RPS2 and RPS3 ubiquitylation. (Related to Figure 6)

A) Longer exposure of immunoblot depicted in figure 6A indicating the positions of mono and bi-ubiquitin modified RPS2. 293T cells stably expressing Flag-HA (FH) tagged wild type or mutant RPS2 were untreated or treated with DTT. Whole cell lysates (inputs) or TUBE-enriched fractions were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

B) The percent of exogenous RPS2 that is modified with one (mono, left) or two (bi, right) ubiquitin molecules with (filled) or without (open) DTT treatment is quantified from immunoblots in panel A.

C) Whole cell lysates from 293T cells stably expressing Flag-HA (FH) tagged wild type RPS3 (WT) or RPS3^{K214R} were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. P=parental 293T cell line.

D) 293T cells stably expressing Flag-HA (FH) tagged wild type RPS3 (WT) or RPS3^{K214R} were untreated or treated with DTT. Whole cell lysates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

E) The percent of exogenous RPS3 that is ubiquitin modified with (filled) or without (open) DTT treatment is quantified from immunoblots in panel D.

F) 293T cells with stable expression of the indicated wild type or mutant ribosomal proteins were treated with thapsigargin for the indicated times. Whole cell lysates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

G) 293T cells with stable expression of the indicated wild type or mutant ribosomal proteins were wither untreated (Unt) or treated with DTT at the indicated concentrations for 48hrs. Cell viability was measured using Celltiter-Glo. Error bars represent SEM from triplicate measurements for each condition.

Figure S7– Efficacy of proteasome inhibitors in *D. melanogaster* S2 cells and *S. cerevisiae*. (Related to Figure 7)

A) The percent sequence identity compared to the human sequence from multiple sequence alignments of RPS2, RPS3, and RPS20 for the indicated species.

B) *Drosophila* S2 cells were untreated or treated with epoxomicin for 8 hours and whole cell extracts were analyzed by SDS-PAGE and immunoblotted as indicated.

C) Spectral counts (SCs) for observed diGly-modified peptides in Hsp23 from untreated (blue bars) and epoxomicin treated (red bars) *Drosophila* S2 cells. Error bars represent SEM from triplicate measurements.

D) Cultures of heavy labeled (K8) *S. cerevisiae* expressing HA-stGND1 were treated with MG132 for 1.5 hours. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted as indicated.

E) SILAC (H:L) Log2 ratios for diGly-modified peptides from polyubiquitin chains from heavy labeled yeast cultures treated with MG132 prior to mixing with untreated, unlabeled control cells. Error bars represent SEM from multiple peptide MS quantifications.

Table S1 - **List and SILAC quantification values for all diGly-modified peptides observed and quantified in response to ER stress or proteasome inhibition.**

Related to Figure 1.

Table S2 - List and SILAC quantification values for all proteins observed and quantified in response to ER stress.

Related to Figure 1.

Table S3 - List and SILAC quantification values for all proteins observed and quantified in response to proteasome inhibition.

Related to Figure 1.

Table S4 - List and SILAC quantification values for diGly-modified peptides on transmembrane domain-containing proteins.

Related to Figure S1.

Table S5 - List and SILAC quantification values for all diGly-modified peptides on ribosomal proteins.

Related to Figure 2.

Table S6 - List of and quantification values for diGly-modified peptides on ribosomal proteins from human, yeast, and fly.

Related to Figure 7.

Table S7 - List of all MS filenames and corresponding experiments uploaded to proteomexchange.

Supplemental Experimental Procedures

Antibodies, Chemicals, and Plasmids

The following antibodies were utilized in this study. Antibodies for Nrf2 (ab62352) and RPS20 (ab133776) were from Abcam Inc. Antibodies for RPS2 (A303-794A), RPS3 (A303-840A), RPL7 (A300-741A), and RACK1 (A302-545A) were from Bethyl Laboratories Inc. Antibodies for α-tubulin (3873), Ubiquityl-Histone H2B (5546), ATF4 (11815), Phospho-SAPK/JNK (Thr183/Tyr185, 4668), Phospho-p38 MAPK (Thr180/Tyr182, 4511), Phospho-S6 Ribosomal Protein (Ser235/236, 4858), and Phospho-eIF2α (Ser51, 3398) were from Cell Signaling Technology. Ubiquitin antibody (MAB1510) and ubiquityl-histone H2A antibody (05-678) was from EMD Millipore/Chemicon. CHOP antibody (ALX-804-551) and KDEL (ADI-SPA-827) was from Enzo Life Sciences. Antibodies for c-Myc (sc-40), GAPDH (sc-69778), and PCNA (sc-56) were from Santa Cruz Biotechnology Inc. Antibodies for HA (rabbit, H6908) and FLAG M2 (F3165) were from Sigma-Aldrich, Inc. HA antibody (mouse, MMS-101P) was from BioLegend (formerly Covance Research Products, Inc.).

The following chemicals and concentrations were used to treat cells in this study unless otherwise indicated:

Dithiothreitol (DTT) – 5mM (165680050) was from ACROS Organics. Tunicamycin (Tm) – 5μg/ml (3516) was from R&D Systems, Inc. Thapsigargin (Tg) – 100ng/ml (BML-PE180) was from Enzo Life Sciences. Cycloheximide – 100 μg/ml (94271) was from AMRESCO. Anisomycin 10μM (80055-088) was from EMD Millipore Biosciences. Harringtonine 2 μg/ml (H0169) was from LKT Laboratories. 4E1RCat - 50μM (4215), PERK inhibitor (GSK2606414) (Harding et al., 2012) - 1μM, and Torin-1 – 100nM (4247)

were from Tocris Bioscience/R&D Systems, Inc. Sodium Arsenite – 500μM (35000), Pifithrin-μ - 50μM (P0122), Antimycin A – 100μM (A8674), Rotenone – 100nM (R8875), and Canavanine – 500μM (C9758) were from Sigma-Aldrich. Etoposide –20μM (BML-GR307), Mitomycin-C – 5μM (BML-GR311), and Cisplatin – 10μM (ALX-400-040) were from Enzo Life Sciences. Azetidine-2-carboxylic Acid – 5mM (A1043) was from TCI America (Tokyo Chemical Industry). UV – 50 J/m2 (UV Stratalinker 2400, Stratagene). Heat shock was induced by exposure to 42°C. Serum starvation conditions were induced by Serum starvation overnight followed by re-addition of full media (SS Recovery). The IRE1 inhibitor (Irestatin, STF-083010) was used at 60μM and was provided by Maho Niwa-Rosen (UCSD) (Tam et al., 2014). Histidinol (H6647) – 2mM was from Sigma (Zhang et al., 2002). BTdCPU (324892) – 50μM was from EMD Millipore (Chen et al., 2011). Interferon-α (300-02A) – 1000U/ml was from Peprotech.

Cell culture

HCT116 and 293T cell lines were both purchased from American Type Culture Collection (ATCC) and grown in complete DMEM media (Gibco) containing 10%FBS (Omega Scientific), penicillin (50 I.U./ml), and streptomycin (50 μg/ml) (Mediatech). PERK knockout and control MEFs were provided by David Ron (University of Cambridge). ATF6α and IRE1α knockout MEFs as wells as eIF2α (S/S) and (A/A) knock-in MEFS were provided by Randy Kaufman (Sanford-Burnham Medical Research Institute). The DAX and DYG cell lines were provided by Luke Wiseman (Scripps Research Institute) (Shoulders et al., 2013). HEK293 cells with Fv2E-PERK expression were provided by Jonathan Lin (UCSD) (Lin et al., 2009). 293T cells with stable

expression of wild-type or mutant RPS2, RPS3, or RPS20 were created using lenti-viral mediated transduction of Flag-HA-tagged ribosomal proteins and subsequent selection using puromycin (1μg/ml Mediatech). For stable isotope labeling by amino acids in cell culture (SILAC) experiments, cells were cultured in custom DMEM without arginine or lysine (Mediatech) supplemented with 10% dialyzed FBS (Life Technologies), penicillin (50 I.U./ml) streptomycin (50 μg/ml) (Mediatech), L-Arginine hydrochloride (85μg/ml Sigma) and either "light" L-Lysine hydrochloride (50μg/ml Sigma) or heavy $^{13}C_6$,$^{15}N_2$ L-Lysine-hydrochloride (50μg/ml Cambridge Isotopes) and 292 μg/mL L-Glutamine (Mediatech). All cell lines were grown at 37°C in the presence of 5% CO_2.

Drosophila S2 cell culture

Drosophila S2 cells were cultured at 28 °C in Schneider's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 2.5 g/ml fungizone, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded at a density of 106/ml and cultured for three days prior to treatment. Following treatment, cells were harvested by gentle centrifugation and washed three times with PBS.

Plasmid construction

Full length clones for human RSP2, RPS3, and RSP20 were obtained from the human orfeome collection (Version 8.1). Stop codons were added using standard site-directed mutagenesis. Lysine to arginine substitutions were created using standard site-directed mutagenesis. Lentiviral based expression vectors containing N-terminal tandem Flag-HA-tagged plasmids containing wild-type or mutant RPS2, RPS3, or RPS20 were

generated using Gateway-based recombination cloning.

Cell viability assays

Cells were plated (10,000 cells per well) onto 96-well white-walled plates prior to treatment with thapsigargin for 48hrs. Cell viability was measured using the CellTiter-Glo assay system with a Glomax microplate Luminometer (Promega).

Yeast proteomics

S. cerevisiae strain RHY 3968 (MATa ade2-101 met2 lys2-801 ura3-52 trp1::hisG leu2Δ *his3Δ200 padre*Δ*::KanMX)* was derived from S288C background. Yeast cultures were grown at 30°C in minimal medium (-Ura) with 2% (vol/vol) dextrose. For SILAC labeling experiments, cultures were grown in YPD media overnight. A 1:1000 dilution of cells was transferred to SILAC media (light K or heavy K in 10mL of minimal medium, -Ura) and cultured overnight. This overnight culture was diluted in to 200mL of either light or heavy SILAC media and cultures were allowed to grow until an OD 600 of 0.7-0.8 was obtained. Cultures were then treated with DMSO or 50μM MG132 for 1.5hr at 30°C. After treatment, cells were collected by centrifugation and mixed 1:1 (by OD). Cells were lysed with glass bead disruption in denaturing lysis buffer (3:1 v/v). Lysates were then prepared for proteomic analysis as described.

Cell Lysis and Immunoblot Analysis

For Immunoblot analysis, cells were resuspended in denaturing lysis buffer (8 M urea, 50 mM Tris-Cl, pH 8.0, 75 mM NaCl, 1 mM NaF, 1 mM NaV, 1 mM β-glycerophosphate, 5 mM NEM) in the presence of EDTA-free Protease Inhibitor Cocktail (Roche

Diagnostics, 5056489001). Cells/lysates were kept on ice during preparation. Lysis buffer was added to cells, and cells were then sonicated briefly (10 s at output of 3 W on a membrane dismembrator model 100 (Fisher Scientific) with a microtip probe and centrifuged for 10 minutes at 21,000 x *g* at 4°C. Supernatants were transferred to clean 1.5 ml tubes, and protein levels were determined by BCA Protein Assay (Thermo Scientific Pierce, 23225). Laemmli sample buffer with β-Mercaptoethanol was then added to cell lysates. Cell lysates were heated at 95^{o}C for 5 minutes, then cooled to room temperature and centrifuged briefly. Cell lysates were resolved by Tris-glycine SDS-polyacrylamide gel electrophoresis (SDS -PAGE) using hand-cast 12% polyacrylamide gels made with 30% Acrylamide/Bis Solution, 37.5:1 mixture (Bio-Rad, 1610158). Proteins were transferred using Bjerrum semi-dry transfer buffer (48 mM Tris Base, 39 mM Glycine-free acid, 0.0375% SDS, 20% MeOH, pH 9.2) to PVDF membranes (Bio-Rad Immun-Blot, 1620177) using a semi-dry transfer apparatus (Bio-Rad Turbo Transfer) for 30 minutes at 25V. Membranes were blocked with 4% nonfat dry milk in TBST (20mM Tris Base, 137 mM NaCl, 0.1% Tween-20 [v/v]) for either 1 hour at room temperature or overnight at 4^{o}C with gentle rocking. Membranes were washed in TBST and then incubated with primary antibodies diluted in 5% BSA in TBST for either 1 hour at room temperature, or overnight at 4^{o}C, with gentle rocking. Blots were washed with TBST, 5-6X over 30-45 minutes at room temperature, then incubated with secondary antibodies diluted in 4% milk in TBST (Promega, Anti-Rabbit IgG (H+L), HRP Conjugate, W4011 or Anti-Mouse IgG (H+L), HRP Conjugate, W4021) for 1 hour at room temperature, with gentle rocking. Membranes were washed again with TBST, 5-6X over 30-45 minutes at room temperature, then developed using Clarity Western

ECL Substrate (Bio-Rad, 1705061), and imaged on a Bio-Rad Chemi-Doc XRS+ system. The intensity of the protein bands were quantified using Image Lab software (Bio-Rad, version 4.1).

XBP-1 splicing assay

Total RNA was prepared from cell pellets using the RNeasy mini kit (Qiagen). cDNA was generated using the iScript cDNA Synthesis kit (Bio-Rad). The oligonucleotide primer sequences used for PCR of human XBP1 are as follows: hXbp1_F- 5' TTACGAGAGAAAACTCATGGC 3' and hXBP1_R 5' GGGTCCAAGTTGTCCAGAATGC 3'.

DiGly-peptide enrichment and Mass spectrometry

diGly-peptide enrichment was done essentially as described previously (Kim et al., 2011). HCT116 cells were grown in media containing either light (K0) lysine or $^{13}C^{15}N$-labeled (K8) lysine. Cell pellets containing a 1:1 mixture of heavy and light cells were lysed in 4 ml of denaturing lysis buffer (8 M urea, 75 mM NaCl, 50 mM Tris-Cl pH 8.2, Roche complete protease inhibitor, 1 mM NaF, 1 mM β-glycerophosphate, 1mM sodium orthovanadate, 1mM PMSF, 5mM NEM). Lysates were sonicated twice for 5s with 30s rest on ice between cycles. Clarified lysates were digested with Lys-C (Wako) at final concentration of 10 ng/μl for 4 hours at 37°C. Lysates were then diluted to 2 M urea with 50 mM Tris-Cl pH 7.2. Trypsin (Sigma) was added at a ratio of 1:100 enzyme:substrate and allowed to incubate overnight at 37 °C. Trypsin digestion was halted by addition of Trifluoroacetic acid (TFA, Sigma) to a final concentration of 0.4%. Digested samples

were clarified by centrifugation (2,500 xg for 15 min) at room temperature and the supernatant was collected. Digested peptides were desalted with C18 solid-phase extraction cartridges (Waters) as described previously (Villen and Gygi, 2008). Eluted peptides were flash frozen with liquid nitrogen dried down to completion in a lyophilizer. Peptides were resuspended in 1.3 ml of 2 × IAP buffer (50 mM MOPS-NaOH pH 7.5, 10 mM Na_2HPO_4, 50 mM NaCl). Resuspended peptides were incubated with α-diGly antibody (Cell Signaling Technologies) pre-conjugated to Protein-A (Thermo) beads at 4 °C for 2 hours with rotating. Beads were then washed 4× with 1 ml IAP buffer with rotating for 10 minutes between each wash. Peptides were eluted with 5% formic acid.

The resulting peptides were desalted with in-house prepared C18 stage-tips using a previously described protocol and dried in a vacuum centrifuge (Rappsilber et al., 2003). Samples were resuspended in 5% formic acid, 5% ACN and analyzed in triplicate by LC-MS/MS using a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, CA) with the following conditions. As more than 100 separate MS runs were done to generate the proteomic data within this study, the exact nHPLC and instrument methods vary between samples. For the precise instrument methods associated with each run, find the uploaded .RAW files at ProteomeXchange (http://www.proteomexchange.org/concept)

The following is a generalized nHPLC and instrument method that is representative of individual analyses. Peptides were first separated by reverse-phase chromatography using a fused silica microcapillary column (100 μm ID, 20 cm) packed with C18 reverse-phase resin (XSELECT CSH 130 C18 2.5 μm, Waters Co.) using an in-line nano-flow EASY-nLC 1000 UHPLC (Thermo Scientific). Peptides were eluted over a 1 minute 0-

5% ACN gradient, followed by a 125 minute 5-30% ACN gradient, a 3 minute 30-45% ACN gradient, a 1 minute 45%- 98% gradient, with a final 10 minute isocratic step at 98% ACN for a total run time of 130 minutes at a flow rate of 500 nl min-1. MS/MS data were collected in a data-dependent fashion using a top 10 method with a full MS mass range from 400-1800 m/z, 70,000 resolution, and an AGC target of 1 e6. MS2 scans were triggered when an ion intensity threshold of 2.5 e2 was reached. Peptides were fragmented using a normalized collision energy setting of 22. A dynamic exclusion time of 30 seconds was used and the peptide match setting was disabled. Singly charged ions, charge states above 8 and unassigned charge states were excluded. The resultant RAW files were converted into mzXML format using the ReAdW program. The SEQUEST search algorithm (version 28) was used to search MS/MS spectra against a concatenated target-decoy database comprised of forward and reversed sequences from the reviewed UniprotKB/Swiss-Prot FASTA database for the respective species (Human, *Drosophila*, *S. Cerevisiae)*. The search parameters used are as follows: 50 ppm precursor ion tolerance and 0.01 Da fragment ion tolerance; up to three missed cleavages were allowed; dynamic modifications of 15.99491 Da on methionine (oxidation), and 114.04293 Da on lysine. For SILAC labeled samples, each .RAW file was searched separately with a “light-lysine” database containing the medication setting above, and a “heavy-lysine” database containing a static (8.0141988132 Da) modification on lysines. Peptide matches were filtered to a peptide false discovery rate of less than 1% and a protein false discovery rate of less than 2% using the linear discriminant analysis (Huttlin et al., 2010). Peptides were assembled into proteins using maximum parsimony and only unique and razor peptides were retained for subsequent

analysis. Ub-modified lysines were localized on peptides using ModScore (Huttlin et al., 2010) and sites with a localization score above 13 were retained for further analysis. Unique sites were filtered and quantified as described previously (Kim et al., 2011).

Mammalian cell fractionation

Mammalian cell fractionation was performed as described (Ramsby and Makowski, 2011) with the following modifications. 293T cells were grown to 80% confluence and treated with 5 mM DTT or vehicle (water) for 0, 1, 2, or 4 hours. All subsequent steps were carried out on ice or at 4°C. Following treatment, cells were washed twice with PBS and harvested in resuspension buffer (50 mM Tris-Cl pH 7.6, 100 mM KCl, 5 mM $MgCl_2$ • 6H2O, 300 mM sucrose). Cytosolic proteins were extracted by resuspending cells in digitonin buffer (resuspension buffer supplemented with 0.015% digitonin, 1× protease inhibitor cocktail (Roche), 5mM EDTA, 5 mM NEM). The insoluble material was pelleted by centrifugation (10 minutes at 500 × *g*) and washed twice in resuspension buffer. ER proteins were extracted by resuspending the digitonin-permeabilized pellet in Triton X-100 buffer (resuspension buffer supplemented with 0.5% Triton X-100, 1× protease inhibitor cocktail (Roche), 5 mM EDTA, 5 mM NEM). The insoluble material was pelleted by centrifugation (10 minutes at 5,000 × *g*) and washed twice with resuspension buffer. Triton X-100-insoluble pellets were resuspended in denaturing lysis buffer (8 M urea, 50 mM Tris pH 8.0, 75 mM NaCl, 1 mM NaF, 1 mM NaV, 1 mM β-glycerophosphate, 5 mM NEM, 1 × protease inhibitor cocktail (Roche)), and soluble nucleoplasmic proteins were extracted by sonication followed by centrifugation (10 minutes at 21,000 × g).

Sucrose gradient separation

HCT116 cells were grown to 60% confluency and treated with 10μM MG132, 5mM DTT, or 5μg/ml Tunicamycin for 4hrs at 37°C in the presence of 5% CO_2. Following treatment, cells were collected using trypsin-EDTA (Gibco) and lysed in Polysome Extraction Buffer (10mM Tris pH 7.4, 0.5% Triton X-100, 15mM $MgCl_2$, 50μg/mL cycloheximide, 0.3M NaCl, 0.2μg/mL Heparin). 3mg of protein were run through 10-50% linear sucrose gradients using a Beckmann Coulter SW 41 Ti rotor at 41,000 rpm, at 4°C for 2hrs. Gradients were fractionated into 12-1ml fractions using the Biocomp piston gradient fractionator or manually via top-sipping. The absorbance at 254 was measured using a Model EM-1 Econo UV monitor (Bio-Rad) or NanoDrop (Thermo Scientific). Protein was precipitated from the solution using a final concentration of 20% Trichloroacetic acid (TCA). The resulting pellets were washed with 10% TCA, followed by 100% acetone, and dried using a Concentrator Plus (Eppendorf). Samples were resuspended in Laemmli sample buffer containing β-mercaptoethanol and boiled at 95°C for 5min.

Supplemental References

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