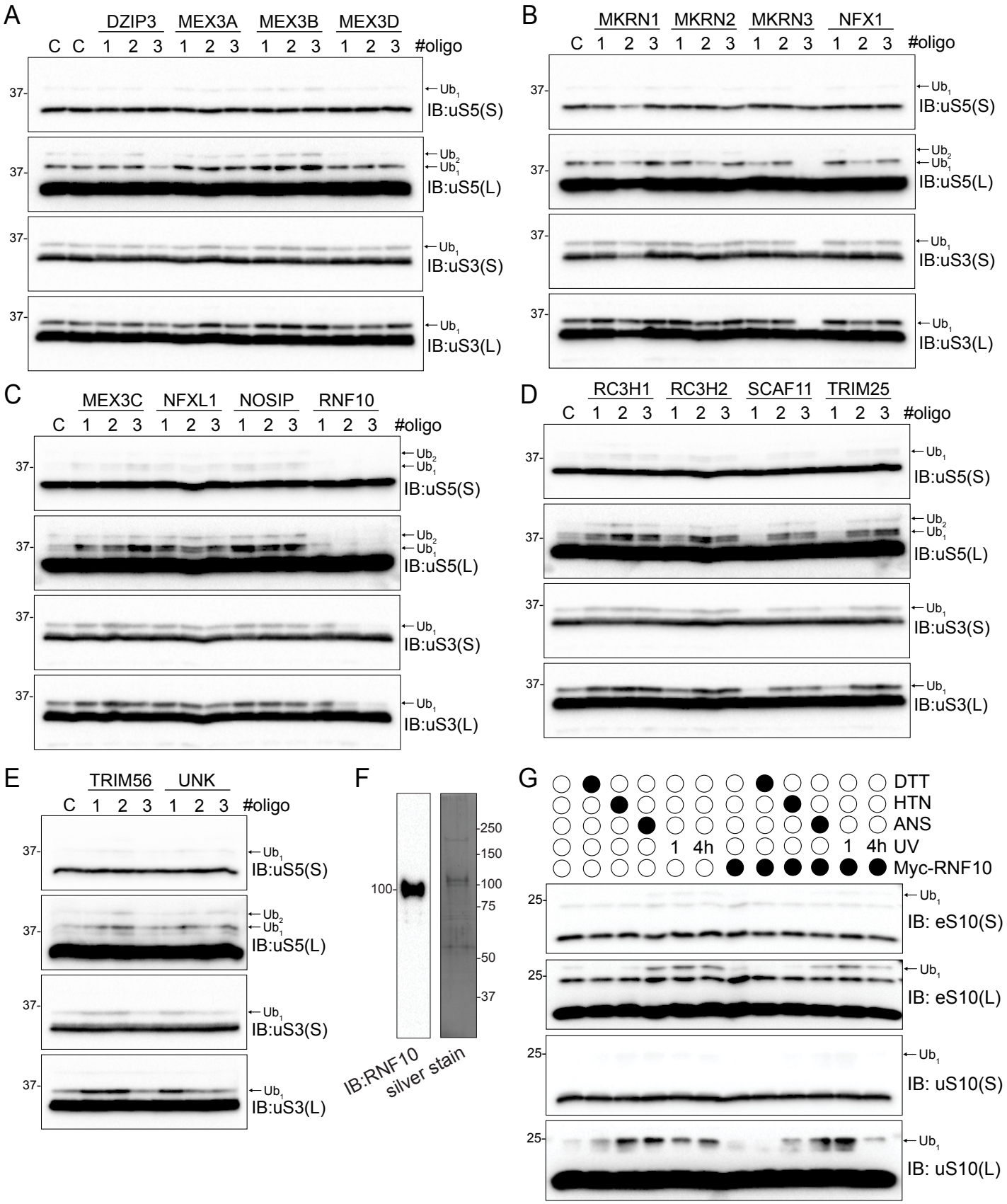

Figure S1. Screening RNA-associated ubiquitin ligases identifies RNF10 as the uS3/uS5 ubiquitin ligase, Related to Figure 1.

(A-E) 293T cells were transfected with either a control siRNA oligo or three separate siRNA oligos targeting the indicated E3 ligase, followed by treatment with DTT (5mM) for two hours. Cell lysates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. For all blots, the ubiquitin-modified ribosomal protein is indicated by the arrow. S and L denote short and long exposures, respectively.

(F) RNF10 immunoblot and silver stain of affinity purified wild type RNF10 expressed in 293T cells.

(G) 293T cells with and without Myc-tagged wild type RNF10 expression were drug treated as indicated. UV indicates that cells were exposed to UV (0.02J/cm2) and were allowed to recover for 1 or 4 hours. Cell extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

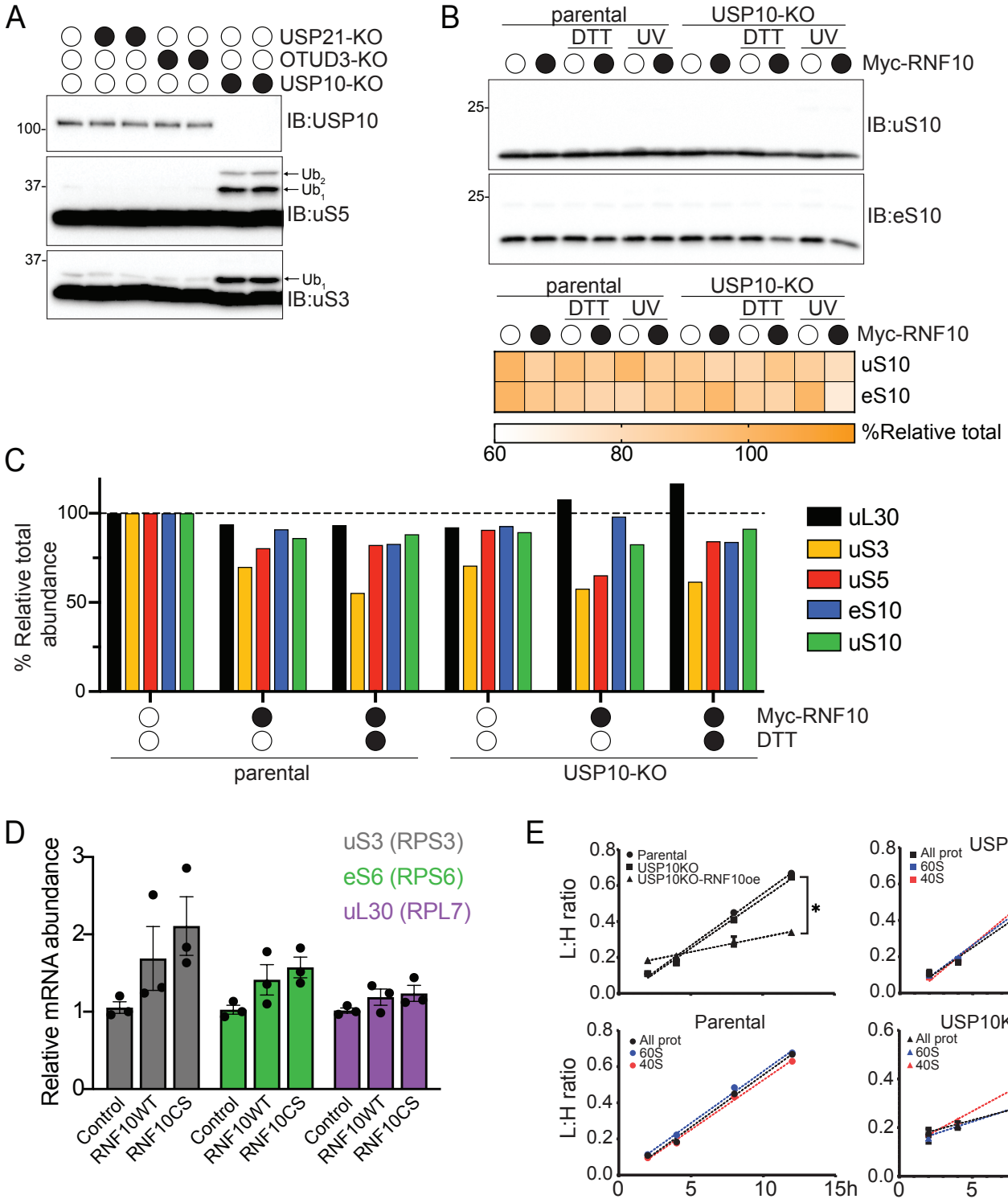

Figure S2. uS3 and uS5 ubiquitylation trigger 40S subunit turnover, Related to Figure 2.

(A) Cell extracts from parental 293T cells or USP21-KO, OTUD3-KO or USP10-KO cells were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. For all blots, the ubiquitin-modified ribosomal protein is indicated by the arrow. S and L denote short and long exposures, respectively.

(B) Top: parental 293T or USP10-KO cells expressing Myc-tagged wild type RNF10 were either untreated, treated with DTT or exposed to UV. Cell lysates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. Bottom: quantitative representation of percent relative total abundance for eS10 and uS10.

(C) Quantitative representation of percent relative total protein abundance for uL30, uS3, uS5, eS10 and uS10 from immunoblots in Figures 2C and S2B.

(D) Relative mRNA abundance measured by qPCR for uS3 (RPS3), eS6 (RPS6), and uL30 (RPL7) in parental cells, or parental cells expressing wild type (WT) or inactive mutant (CS) RNF10. n=3, error bars denote SEM.

(E) Total protein (black line), 40S (red line), or 60S (blue line) ribosomal protein synthesis rates from parental 293T, USP10-KO, and USP10-KO cells overexpressing wild type RNF10 were determined using SILAC label swap from cells collected at the indicated time point post label swap. Light to heavy ratios were determined by mass spectrometry. n=3, error bars denote SEM of triplicate experiments. *$p<0.05$ using unpaired, two-tailed Student's t-test comparing slope of best-fit line for replicate experiments.

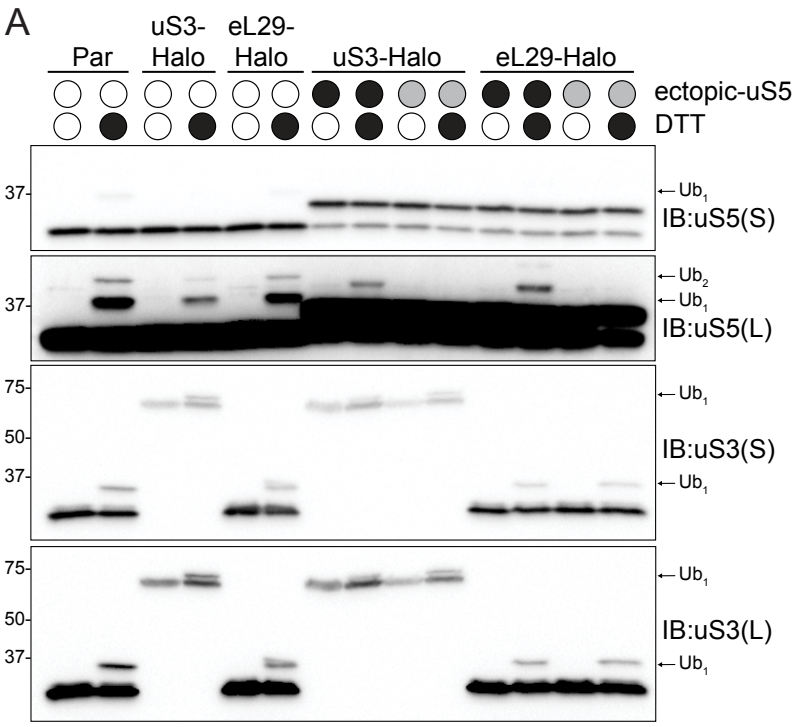

Figure S3. Loss of uS5 ubiquitylation blocks RNF10-mediated 40S protein turnover, Related to Figure 4.
(A) 293T parental cells (Par) or 293T-uS3-Halo or eL29-Halo cells alone or with constitutive wild type (black circles) or K54R/K58R mutant (grey circles) uS5 expression were either untreated or treated with DTT (5mM) for 2 hours. Cell lysates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

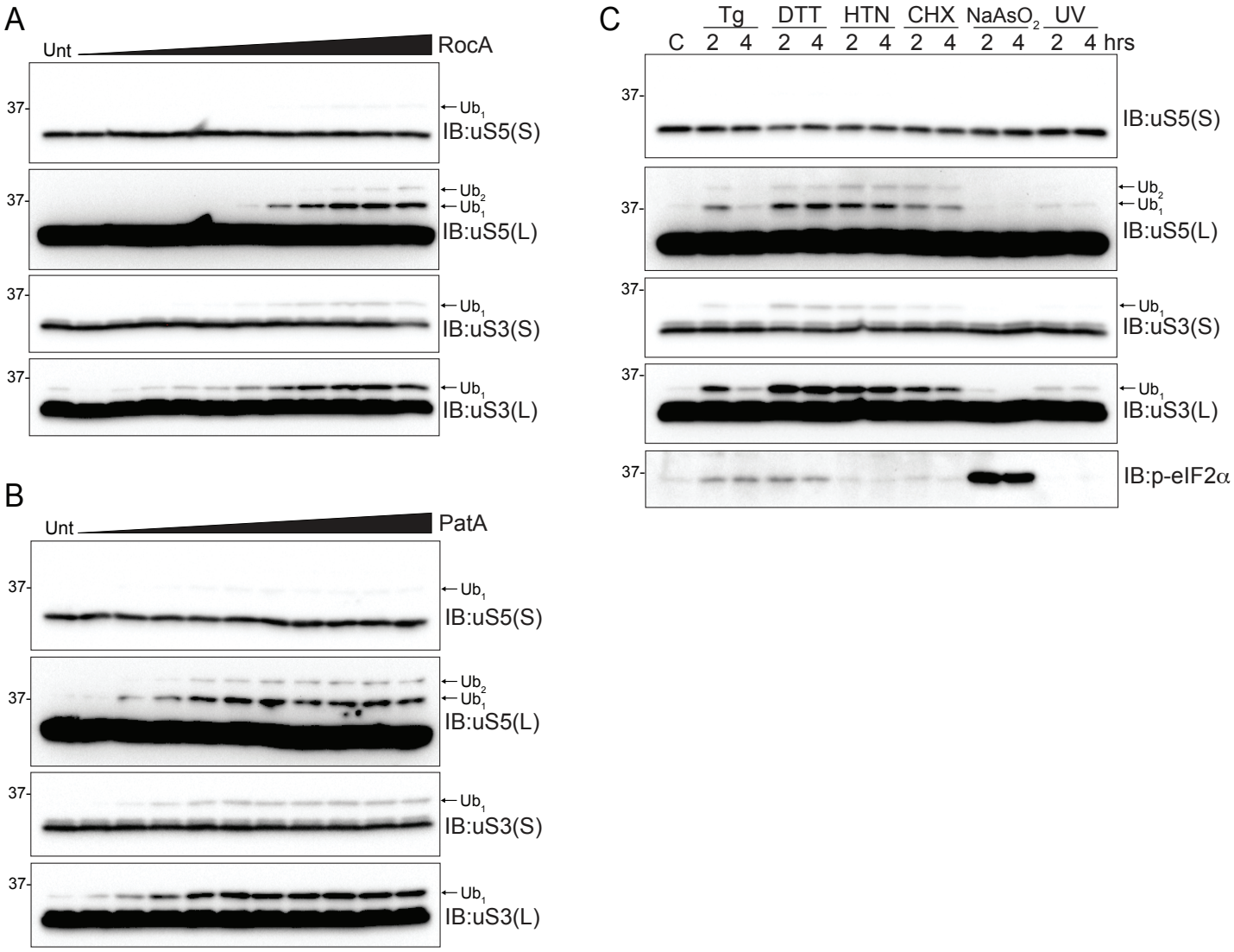

Figure S4. Translation initiation inhibition triggers ribosome ubiquitylation, Related to Figures 5 and 6.
(A,B) 293T cells were treated with increasing concentrations of either RocA (0.031-3.2uM) (A) or PatA (0.31-100nM) (B) for two hours. Whole-cell lysates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. For all blots, the ubiquitin-modified ribosomal protein is indicated by the arrow. S and L denote short and long exposures, respectively.
(C) Cell extracts from cells treated with Tg (1uM), DTT (5mM), HTN (2ug/ml), CHX (100ug/ml), NaAsO2 (500uM) or UV (0.02J/cm2) (washout) for two or four hours were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

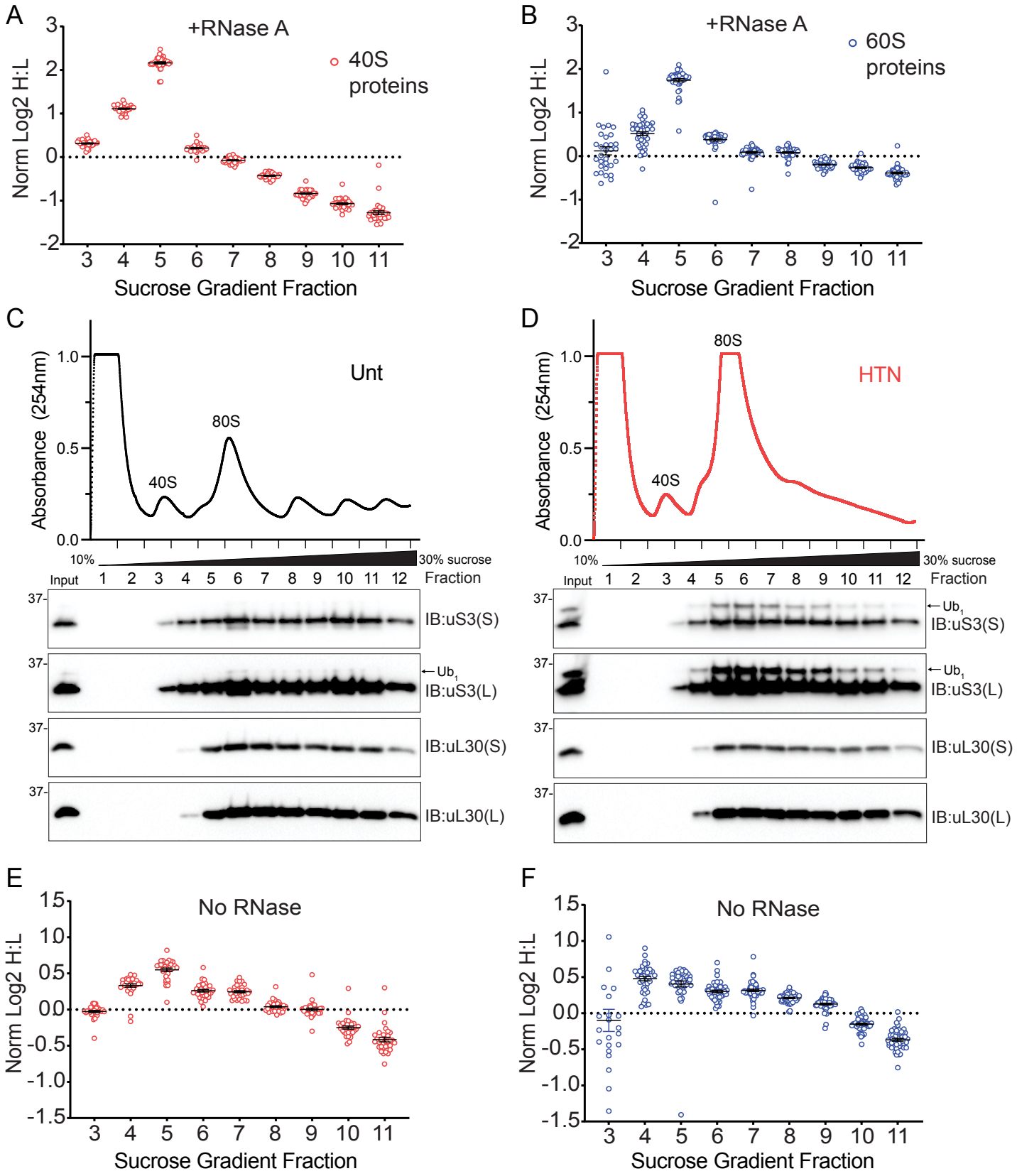

Figure S5. Ribosomal subunit imbalance is present in gradient fractions containing ubiquitylated ribosomes, Related to Figure 7.

(A-B) The normalized log2 SILAC ratio (H-HTN:L-untreated) of all quantified 40S (A, red circles) and 60S (B, blue circles) proteins within indicated sucrose gradient fractions from lysates treated with RNaseA prior to density gradient centrifugation. Each data point is an individual ribosomal protein, and the black bar denotes the median value.

(C-D) Cell extracts from untreated (C) or HTN (2ug/ml) treated (D) 293T cells were fractionated on 10-30% sucrose gradients. The 254-nm absorbance trace is depicted. Fractions were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. The ubiquitin-modified ribosomal protein is indicated by the arrow. S and L denote short and long exposures respectively.

(E-F) The normalized log2 SILAC ratio (H-HTN:L-untreated) of all quantified 40S (E, red circles) and 60S (F, blue circles) proteins within indicated sucrose gradient fractions from untreated lysates prior to density gradient centrifugation. Each data point is an individual ribosomal protein, and the black bar denotes the median value.

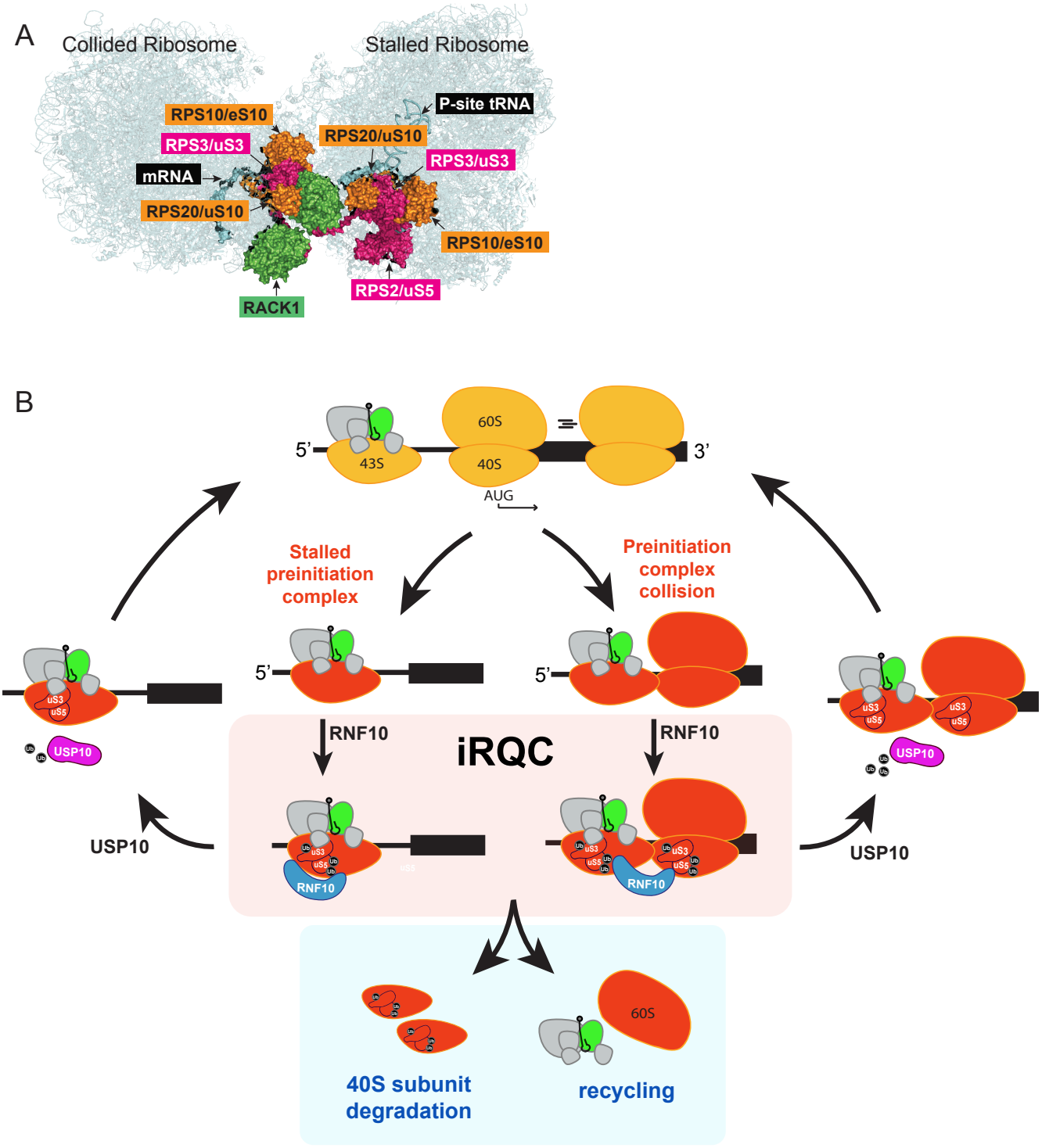

Figure S6. Model of iRQC activation, Related to Figure 7.
(A) Collided disome structure with ubiquitylated 40S proteins that function either within (uS10/RPS20, eS10/RP10 shown in orange) or outside (uS3/RPS3, uS5/RPS2 shown in magenta) the characterized RQC pathway. RACK1 (green) mRNA (blue), and P-site tRNA (blue) are indicated. Structure from PDB:6HCQ and 6HCM.
(B) Under normal homeostatic conditions, cap-dependent translation proceeds with 43S scanning of the 5' untranslated region, followed by start codon recognition, and 60S subunit joining to form an elongation competent 80S ribosome (indicated by yellow ribosomes). Stalled preinitiation complexes or collisions between 43S and 80S ribosomes (red ribosomal subunits) activate the iRQC pathway in which RNF10 is recruited to ubiquitylate uS5 and uS3 on specific lysine residues. Persistent ribosome ubiquitylation triggers 40S subunit destruction and recycling of initiation factors and the 60S subunit. Stalled and/or collided preintiation complexes can alternatively undergo USP10-dependent ribosome deubiquitylation to allow for translation initiation progression.