RESOURCE AVAILABILTY

Lead contact

Requests for resources or further information can be directed to the Lead Contact Eric J. Bennett (e1bennett@ucsd.edu).

Materials availability

All reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability

Original western blot images have been deposited at Mendeley and are publicly available as of the date of publication. The DOI is listed in the key resources table. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All HEK293, HEK293T, HCT116 and 293Flp-In cells were grown in DMEM (high glucose, pyruvate and L-Glutamine) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and maintained in a 5% CO2 humidified incubator.

METHOD DETAILS

Plasmids

Using Gateway cloning (Invitrogen) all protein coding regions were cloned into Myc- of GFP-tagged CMV expression vectors. Mutations were introduced using QuickChange site-directed mutagenesis utilizing PCR-based approaches (primers 5' to 3': RNF10-C225S, CATGAAGTGCCATCTTCCCCAATATGCCTCTATC). Template DNA was digested by Dpn1 followed by transformation of the mutated plasmids into TOP10 E. coli cells. Plasmids were confirmed by sequencing and screened for expression by immunoblotting.

Treatments, transfections and siRNA

Prior to harvesting cells were treated with either 1uM Tg, 5mM DTT, 2ug/ml HTN, 100ug/ml CHX, 500uM NaAsO2, 150nM Torin1, 50-100nM Bafilomycin A, 1uM SAR405, 10uM MG132 or were exposed to 0.02J/cm2 UV radiation using a SpectorlinkerTM XL-1000 (Spectronics).

Lentiviral transduction was used to generate stable cells lines expressing Flag-HA tagged USP10. Using Mirus TransIT 293 transfection reagent cells were transfected with five helper plasmids pHAGE-GAG-POL; pHAGE-VSVG; pHAGE-tat1b; pHAGE-rev and pHAGE-Flag-HA-USP10 (wild type or catalytic mutant), followed by the addition of fresh media after 24 hours. The supernatant was filtered using a 0.45 mm sterile syringe filter and mixed with 2ul of 6mg/ml polybrene. The viral mixture was then added to cells

seeded at 50% confluency and infected for 24hours. Stable expression clones were selected with 1ug/ml Puromycin.

The Flp-In™ system (Thermo Fisher) through single locus integration and hygromycin selection was used to generate stable doxycycline inducible cell lines expressing Flag-HA-tagged proteins. Flp-In 293 cells were transfected with Flp-In expression vectors for RNF10 using TransIT 293 transfection reagent (Mirus) according to manufacturer guidelines. Cells were seeded at 60% confluency, transfected for 24 hours followed by selection of stable expression clones with 100ug/mL Hygromycin. Treatment with 2ug/mL doxycycline for 16 hours prior to harvesting was used to induce protein expression.

All transient transfections were carried out using Lipofectamine 2000 (Thermo Fisher) and all siRNA knockdown transfections were performed using Lipofectamine RNAiMAX (Thermo Fisher) according to manufacturer instructions. A list of all RNAi oligonucleotides used in this study can be found in table below.

Immunoblotting

For all immunoblot analysis, cell pellets were resuspended in urea denaturing lysis buffer (8M urea, 50mM Tris-Cl, pH 8.0, 75mM NaCl, 1mM NaV, 1mM NaF, 1mM β-glycerophosphate, 40mM NEM in the presence of EDTA-free protease inhibitor cocktail) and kept on ice during preparation. Cell lysates were sonicated for 10 s (output of 3W on a membrane dismembrator model 100 (Fisher Scientific) with a microtip probe then centrifuged for 10 min at 15,000rpm at 4°C. Lysate protein concentrations were measured by BCA Protein Assay (23225, Thermo Scientific Pierce). Laemmli sample buffer with β-mercaptoethanol was then added to cell lysates and heated at 95°C for 10 min. Samples were then cooled to room temperature and centrifuged briefly. Lysates were resolved on 12% Tris-glycine SDS -PAGE gels, followed by transfer to PVDF membranes (1620177, BioRad) using Bjerrum semi-dry transfer buffer (48mMTris Base, 39mM Glycine-free acid, 0.0375% SDS, 20% MeOH, pH 9.2) and a semi-dry transfer apparatus (Bio-Rad Turbo Transfer) for 30 min at 25V. Immunoblots were blocked with 5% blotting grade nonfat dry milk (APEX Bioresearch) in TBST for 1 hour. Primary antibodies were diluted in 5% BSA and rocked overnight. Immunoblots were developed using Clarity Western ECL Substrate (1705061, BioRad) and imaged on a Bio-Rad Chemi-Doc XRS+ system. All blots were processed using Imagelab (BioRad) software, with final images prepared in Adobe Illustrator. All plots were prepared using GraphPad Prism 9.0.

Phos-Tag SDS-PAGE

For Phos-tag analysis, cell pellets were resuspended in 500ul of lysis buffer (8M urea, 50mM Tris-Cl, pH 8.0, 75mM NaCl, 1mM NaV, 1mM NaF, 1mM β-glycerophosphate in the presence of EDTA-free protease inhibitor cocktail). Lysates were sonicated for 10s (as described above) followed by centrifugation for 10 min at 15,000rpm at 4°C. 125ul of

TCA was added to each sample, then incubated on ice for 2h at 4°C. Protein was collected by spinning tube in microcentrifuge at 15,000 rpm for 30min at 4°C. The TCA protein pellet was washed with 200ul cold acetone, followed by centrifugation at 15,000 rpm for 10min at 4°C. The acetone wash step was repeated two more times. Pellets were left to dry for 30min at room temperature to evaporate any remaining acetone, then resuspended in 50ul 8M urea/20mM DTT. Protein concentrations were measured by Bradford Assay (protein assay dye reagent concentrate, 500-0006, BioRad). Laemmli sample buffer with β-mercaptoethanol was then added to protein samples and heated at 95°C for 10 min. Samples were resolved on 12.5% SuperSepTM Phos-tagTM gels (198-17981, Fujifilm), followed by Zn2+ ion elimination. Gel was soaked in 1X transfer buffer (25mM Tris, 192mM Glycine, 10% v/v methanol) with 10mM EDTA for 20min with gentle agitation. This step was repeated three times with buffer exchanges, followed by 10min without EDTA. Wet-tank transfer to PVDF membranes using Towbin transfer buffer (25mM Tris, 192mM Glycine, 20% v/v methanol) was done overnight (16h) at 30V. Immunoblots were blocked, developed, and imaged as described above.

Sucrose density gradient fractionation

Cell pellets were lysed in 500 ul of lysis buffer (20mM Tris-Cl, pH 8.0, 150mM NaCl, 15mM MgCl2, 1% Triton-X 100, 40U Turbo DNase I, 40mM NEM, 1mM DTT, EDTA-free protease inhibitor cocktail in DEPC treated water) followed by vigorous pipetting and incubated on ice for 15min. The cell lysates were centrifuged at 15,000 rpm for 10min at 4°C and the supernatant was transferred to a new microcentrifuge tube. Total RNA concentration of each lysate was determined using a nanodrop (Thermo Scientific). 500ug of total RNA was digested with 3.5ug/ml of RNaseA for 15min at 25°C on a thermomixer (Eppendorf) at 500rpm. The digestion was stopped with 166.5U of SUPERaseIn RNase Inhibitor. Samples were fractionated over a 10–30% sucrose gradient containing 150ug/ml cycloheximide (prepared on Gradient Master 108 (Biocomp): 1min 54s, 81.5 degrees, 16rpm). Samples were centrifuged at 41,000rpm for 2 hr at 4°C in an SW41i rotor. 1ml fractions were collected using a PGFip piston gradient fractionator (Biocomp). Protein fractions were precipitated overnight with 10% TCA at 4°C, followed by three ice-cold acetone washes. Pellets were dried in Vacufuge plus (Eppendorf) at room temperature for 5 min. Pellets were then resuspended in Laemmli sample buffer with β-mercaptoethanol, heated at 95°C for 10 min.

SILAC LC-MS-MS analysis

Cells were grown in a media containing dialyzed FBS (FB03, Omega Scientific) and either light (K0) lysine and arginine (R0) or 13C615N2-labeled (K8) lysine and (R10) arginine (Cambridge Isotopes). Cells were harvested and mixed 1:1 by cell count and were processed for mass spectrometry as described previously (Markmiller et al., 2019). Briefly, cells were lysed using 8M urea lysis buffer with 40mM fresh NEM and lysates were quantified for protein content using the BCA assay. 20µg of total cell extract was diluted to a final urea concentration of 1M and then digested overnight with trypsin (V5111, Promega) at a 1:100 (enzyme:protein) ratio. The digests were reduced with 1mM DTT for 30 min and then alkylated with 10mM NEM in a dark for 30min. The

digests were desalted using Stage-Tip method and analyzed by LC-MS/MS as described below. Mixed SILAC lysates were fractionated over sucrose gradients as described. Fractions were TCA precipitated, followed by resuspension in 50mM ammonium bicarbonate and digested overnight with 500ng/ul of trypsin (V5111, Promega) at 37°C. Digests were reduced, alkylated and desalted as described above.

All the samples (1ug digested peptides) were analyzed in triplicate by LC-MS/MS using a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, CA) with the following conditions. A fused silica microcapillary column (100 mmID, 20 cm) packed with C18 reverse-phase resin (XSELECT CSH 130 C18 2.5 mm, Waters Co., Wilford, MA) using an in-line nano-flow EASY-nLC 1000 UHPLC (Thermo Scientific) was used to resolve the peptides. Peptides were eluted over a 45 min 2%–30% ACN gradient, a 5 min 30%–60% ACN gradient, a 2 min 60%–95% gradient, with a final 8 min isocratic step at 0% ACN for a total run time of 60 min at a flow rate of 250 nl/min. All gradient mobile phases contained 0.1% formic acid. MS/MS data were collected in a data dependent fashion using a top 10 method with a full MS mass range from 300–1750 m/z, 70,000 resolution, and an AGC target of 3e6. MS2 scans were triggered when an ion intensity threshold of 1e5 was reached with a maximum injection time of 60 ms. Peptides were fragmented using a normalized collision energy setting of 25. A dynamic exclusion time of 20 s 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 analyzed using Andromeda/MaxQuant (version 1.6.12.0) using the combined UniProt reviewed only database for Homo sapiens (Dec 2020). The default parameters were used and ‘match between the runs’ and ‘requantify’ options were enabled in the MaxQuant settings. The proteingroups output table was imported into Microsoft Excel for subsequent data analysis. Normalized SILAC ratios and LFQ intensities were used for data analysis.

Purification of RNF10

Cells were seeded at 50% confluency in ten 10cm plates one day prior to transfection of a N-Flag-TEV-RNF10 expression plasmid using the calcium phosphate method. 20ug of total DNA was mixed with 2M CaCl2 in distilled water. The mixture was added in a dropwise manner to equal volumes 2XHBS (280mM NaCl, 10mM KCl, 1.5mM Na2HPO4, 12mM glucose and 50mM HEPES pH 7.05) solution at room temperature with continuous mixing, followed by incubation at room temperature for 30 minutes. Transfection mixture was added to each plate and incubated overnight at 37°C. 48 hours post transfection cells were collected by scrapping into cold 1X PBS and pelleted at 1,000 rpm for 5min at 4°C. Cells were lysed in 2mL of lysis buffer (50mM HEPES, pH 7.4, 100mM KAc, 5mM MgAc2, 0.5% NP40, 1 mM DTT (made fresh) and 1X EDTA-free Complete protease inhibitor cocktail) and incubated on ice for 20min. Lysates were clarified by centrifugation at 15,000 rpm for 10min at 4°C. 200ul of clarified lysate was added to a 1:1 slurry of pre-equilibrated (in lysis buffer with 0.1% NP40) anti-Flag M2 resin (A2220, Sigma) and incubated with rotation for 2 hours at 4°C. Resin was collected by centrifugation at 3,000 rpm for 1min at 4°C, while flow through was saved

in a new tube. Resin was washed three times in 1ml of IP buffer (50mM HEPES, pH 7.4, 100mM KAc, 5mM MgAc2, 0.1% NP40, 1mM DTT (made fresh) and 1X EDTA-free Complete protease inhibitor cocktail) for 2min with rotation, followed by centrifugation. Resin was then washed three times with 1ml of high salt buffer (50mM HEPES, pH 7.4, 400mM KAc, 5mM MgAc2, 0.1% NP40, 1mM DTT), followed by three washes with 1ml of elution buffer (50mM HEPES, pH 7.4, 100mM KAc, 5mM MgAc2, 1mM DTT). Following elution, 100U of His-TEV protease (Z03030-1K, GenScript) was added to the 1:1 slurry of resin in elution buffer and incubated at room temperature for 30min. Resin was washed with an additional 100ul of elution buffer and then pooled with the first elution. 50ul of pre-equilibrated NiNTA agarose resin (30210, Qiagen) was added to the pooled elution fractions and incubated with rotation for 1h at 4°C. Cleared elution was collected by centrifugation, followed by silver stain and immunoblotting for confirmation of protein purification.

In vitro ubiquitylation assay

All in vitro ubiquitylation reactions were carried out for 60min at 37°C. Single reactions consisted of 400nM recombinant human His6-Ubiquitin E1 enzyme Ube1 (E-304, BostonBiochem), 2uM recombinant human UbcH5c/UBE2D3 protein (E2-627, BostonBiochem), 200uM recombinant human ubiquitin no K (UM-NOK, BostonBiochem), 125nM 40S ribosomes (Purified from Hap1 cells, gift from Jody Puglisi and Alex Johnson, Stanford University), 50mM Tris-Cl pH 7.5, 50mM MgCl2, 20mM ATP, 6U/ml pyrophosphatase, 35U/ml creatine kinase and 100mM creatine phosphate, and 8uM RNF10. Reactions were inactivated with Laemmli buffer, then incubated for 10min at 95°C. Proteins were resolved by 12% SDS-PAGE and visualized by immunoblotting.

Generation of knockout and knockin cell lines

Using CRISPR/Cas9 genome engineering USP10 and RNF10 knockout was done in 293Flp-In and 293T cells. Three individual guide RNAs were designed for each gene using CHOPCHOP website (https://chopchop.cbu.uib.no). RNF10: 5'-GCCGGCGAGTCTAAACCCAA-3', 5'- GCCACGTTAGACTCGGGAAG-3', 5'-CCGTTGATGCCGCTGAGCTC-3', USP10: 5'- GACTCCTCGATCTTCAGTTG-3', 5'-CTTACCTCAACTGAAGATCG-3' and 5'- GCCTGGGTACTGGCAGTCGA-3. Cells were transfected with the pSpCas9(BB)-2a-GFP plasmid containing individual guide RNAs using lipofectamine 2000. 48 hours post transfection, GFP positive cells were either single cell sorted on a BD FACSAria Fusion (BD BioSciences) cell sorter, or pooled cell sorts were clonally isolated by limiting dilution method. Cells were validated for loss of USP10 and RNF10 by immunoblotting and sequencing. For HaloTag7 knock-in, guide RNA (gRNA) targeting the C-terminal region of human RPL26 gene was designed using the CHOPCHOP website (http://chopchop.cbu.uib.no/). The guide sequence for RPL26 gene (5′- GAAACCATTGAGAAGATGC-3′) was assembled into a pX459 plasmid. Donor vector was constructed by assembling a HaloTag7 transgene with upstream and downstream homology arms (650 nucleotide each) into a digested pSMART plasmid by Gibson assembly. Wild type HCT116 cells were transfected with donor and gRNA

vectors (1 to 1 ratio) by Lipofectamine 3000 (Invitrogen). Five days after the transfection, the pool of transfected cells was treated with 100 nM Halo-TMR ligand for 1h, followed by washing three times. Fluorescence-positive cells were sorted into 96-well plates by flow cytometry (MoFlo Astrios EQ, Beckman Coulter). Three weeks later, the expanded single-cell colonies were screened for the integration of the HaloTag7 transgene by immunoblotting with α-RPL26.

Ribo-Halo microscopy

HCT116 Ribo-HaloTag7 cells were transfected with either GFP-RNF10 WT or CS expression plasmid (2 ug/dish) using lipofectamine 3000 (Invitrogen). 24 hours post transfection, the cells were plated onto 35 mm-glass bottom dishes (No. 1.5, 14 mm glass diameter, MatTek) pre-treated with poly-L-lysine. 48 hours later, Halo-TMR containing medium (50 nM) was added to the cells and incubated for 1 hour. The medium was removed, and the cells were washed with warm DMEM for two times. DMEM was replaced by FluoroBrite™DMEM (Thermo Fisher) before the live cell imaging. The cells were imaged using a Yokogawa CSU-X1 spinning disk confocal with Spectral Applied Research Aurora Borealis modification on a Nikon Ti motorized microscope equipped with a Nikon Plan Apo 60×/1.40 N.A objective lens. Pairs of images for TMR and GFP fluorescence were collected sequentially using 100 mW 488 nm and 100 mW 561 solid state lasers attenuated and controlled with an AOTF (Spectral Applied Research LMM-5), and emission collected with a 525/50 nm or 620/60 nm filter (Chroma Technologies), respectively. Confocal images were acquired with the Hamamatsu ORCA-ER cooled CCD camera and MetaMorph software. The images were analyzed using FiJi software.

Flowcytometry analysis for Ribo-Halo labeling

Ribo-Halo cells were seeded at 40% confluency in 12-well plates one day prior to transient transfections. 36 hours post transfection cells were treated with 100nM TMR-ligand (G8251, Promega) for 1-2 hours. After TMR-labeling, cells were washed with fresh warmed DMEM without the Halo-ligand three times with 10min incubations in between washes. Fresh warm DMEM was added to cells and cells were collected at various time points post washout. Cells were trypsinized then collected in fresh media. Following a short 3min centrifugation at 3,500rpm, cell pellets were resuspended in 800ul of FACS buffer (2% FBS in 1x DPBS) and passed through a nitex nylon mesh (Genesee Scientific). Samples were analyzed by flow-cytometry on a BD LSRFortessaTM X-20 cell analyzer (BD Biosciences). FACS data was analyzed using FlowJo (v10.6.2).

qPCR analysis

For qPCR analysis, cells were plated at 50-60% confluency prior to lipofectamine based transfection, as described previously. 48 hours post transfection cells were collected in TRIzol and RNA was isolated using Direct-zol RNA miniprep kit (11-331, Zymo Research). Using 2ug RNA template, cDNA was synthesized is SuperScript III First

Strand Synthesis system (18080-051, Invitrogen). Five standards were prepared by making four-fold dilutions of a sample pool. cDNA samples were each diluted 1:5 in water prior to plating. 8ul of each standard or sample was plated into a 96-well thermocycler plate, followed by 12ul of master mix containing SYBR green super mix (1725121, BioRad) and primers for gene of interest. The following primers were used in this study: RPS3: 5'-CAGAACAGAAGGGTGGGAAG-3', 5'-GCAACATCCAGACTCCAGAATA-3', RPS6: 5'-GAGCGTTCTCAACTTGGTTATTG-3', 5'-GCGGATTCTGCTAGCTCTTT-3', RPL7: 5'-GGCGAGGATGGCAAGAAA-3', 5'-CTTTGGGCTCACTCCATTGATA-3', GAPDH: 5'-AACGGGAAGCTTGTCATCAATGGAAA-3', 5'-GCAGGAGGCAGCTGATGATCTT-3'. The following PCR conditions were run on a C1000 Thermo Cycler (BioRad): 50°C for 10min, 95°C for 15min, 95°C for 10s, 60°C for 30s (repeat for 40 cycles). All relative quantifications were calculated using the delta delta Ct method.

QUANTIFICATION AND STATISTICAL ANALYSIS

All FACS-based assays were performed in triplicate (n = 3) as biologically distinct samples. The median 561nm/488nm ratio and SD were calculated. Transient overexpression experiments were compared to a transfection control. Immunoblot quantification of the relative % ubiquitylation and % phosphorylation was calculated by normalization of the individual intensities for each concentration to that of the no treatment control. Significance (p value) was calculated using an unpaired two-tailed Student's t test using GraphPad Prism 9.0.