

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY TABLES

Supplementary Table 1. Reagents used in this paper

Reagent		Source	Identifier
Antibodies	Working dilution		
Mouse monoclonal Anti- β -actin clone AC-15	1:5000	Sigma-Aldrich	Cat# A5441, RRID: AB_476744
Mouse monoclonal Anti-GAPDH	1:10000	Millipore	Cat#CB1001
Mouse monoclonal anti-tubulin	1:5000	Millipore	Cat#CP06
Mouse monoclonal anti-FLAG	1:10000	Sigma-Aldrich	Cat# F3165, RRID: AB_259529)
Rabbit polyclone anti-FLAG	1:5000	Sigma-Aldrich	Cat#F7425, RRID: AB_439687
Anti-FLAG M2 affinity gel		Sigma-Aldrich	Cat#A2220 RRID:AB_10063035
Mouse anti-Myc Tag, clone 4A6, agarose conjugate		Millipore	Cat# 16-219
Rabbit monoclonal Anti-Myc Tag	1:1000	Cell signaling technology	Cat#2278
Mouse monoclonal anti-S Tag,	1:2000	Millipore	Cat#71549-3 RRID: AB_11210600
Mouse monoclonal anti-HA Tag	1:2000	Sigma-Aldrich	Cat# H9658 RRID:AB_260092
S-Protein Agarose		Novagen	Cat#69704
GammaBind Plus Sepharose		Cytiva	Cat#17088601
Rabbit IgG isotype control		Abcam	Cat# ab172730 RRID:AB_2687931
Goat Anti-Rabbit IgG (H+L) Antibody, Alexa Fluor 647 Conjugated	1:1000	Thermo Fisher Scientific	Cat#A21245 RRID: AB_141775
Rabbit polyclonal anti-ZNF598	1:1000	Invitrogen	Cat# PA559777 RRID: AB_2650226
Rabbit polyclonal anti-LTN1	1:1000	ProteinTech	Cat# 28452-1-AP RRID: AB_2881144
Rabbit polyclonal anti-UFL1	1:5000	Bethyl Laboratories	Cat# A303-456A, RRID: AB_10951658
Rabbit polyclonal anti-CDK5RAP3	1:3000	ProteinTech	Cat#11007-1-AP RRID: AB_2076869
Rabbit polyclonal anti-DDRGK1	1:3000	Thermo Fisher Scientific	Cat# PA5-100467 RRID: AB_2849977
Rabbit monoclonal anti-UFC1	1:5000	Abcam	Cat#ab189251
Rabbit Monoclonal anti-UFM1	1:3000	Abcam	Cat# ab109305, RRID: AB_10864675

Rabbit polyclonal anti-Histone H3	1:2000	Millipore	Cat# 07-690
Rabbit monoclonal anti-UFSP2	1:3000	Abcam	Cat# ab185965
Rabbit polyclonal anti-SEC63	1:2000	Bethyl Laboratories	Cat# A305-084A, RRID: AB_2631479
Mouse monoclonal anti-RPN1	1:1000	Santa Cruz Biotechnology	Cat# sc-48367, RRID: AB_628221
Rabbit polyclonal anti-SRPRB	1:2000	Bethyl Laboratories	Cat# A305-440A, RRID: AB_2631831
Rabbit polyclonal anti-SRP68	1:2000	Bethyl Laboratories	Cat# A303-955A, RRID: AB_2620304
Rabbit monoclonal anti-RPS10	1:2000	Abcam	Cat# ab151550 RRID:AB_2714147
Rabbit monoclonal anti-RPS20	1:2000	Abcam	Cat# ab133776 RRID:AB_2714148
Rabbit Polyclonal anti-RPL26	1:2000	Abcam	Cat# ab59567, RRID: AB_945306
Rabbit polyclonal anti-SEC61B	1:10000	Thermo Fisher Scientific	Cat# PA3-015 RRID: AB_2239072
Mouse monoclonal anti-eIF4G1 (Clone 2A9)	1:1000	Abnova	Cat# H00001981-M10, RRID: AB_606171
Rabbit anti-CYB5R3	1:2000	ProteinTech	Cat# 10894-1-AP RRID:AB_2292715
Rabbit anti-LC3B	1:1000	Sigma-Aldrich	Cat#L7543 RRID:AB_796155
Rabbit Polyclonal anti-SUMO2+3	1:1000	Abcam	Cat# ab3742
Mouse monoclonal anti-ubiquitin	1:1000	Santa Cruz	Cat#sc8017
Rat monoclonal anti-EBV-BPLF1	1:1000	van Gent, et al. 2014 ¹	N/A
Chemicals, peptides, and recombinant proteins			
IGEPAL CA-630	Sigma-Aldrich	I3021; CAS: 9002-93-1	
Ciprofloxacin	Sigma-Aldrich	Cat#17850; CAS: 85721-33-1	
MgCl ₂	Merck	Cat# M1028	
Sodium dodecyl sulphate	Sigma-Aldrich	L3771; CAS:151-21-3	
N-Ethylmaleimide	Sigma-Aldrich	E1271; CAS:128-53-0	
Iodoacetamide	Sigma-Aldrich	I1149; CAS:144-48-9	
Sodium deoxycholate monohydrate	Sigma-Aldrich	D5670; CAS:145224-92-6	
Triton X-100	Sigma-Aldrich	T9284; CAS:9002-93-1	
Bovine serum albumin	Sigma-Aldrich	A7906; CAS:9048-46-8	
Tween-20	Sigma-Aldrich	P9416; CAS: 9005-64-5	
PMSF	Sigma-Aldrich	P7626; CAS: 329-98-6	
Trizma base	Sigma-Aldrich	93349; CAS:77-86-1	
Doxycycline cyclate	Sigma-Aldrich	D9891; CAS: 24390-14-5	
Anisomycin	Sigma-Aldrich	A5862; CAS:22862-76-6	
Cycloheximide	Sigma-Aldrich	C4859; CAS:66-81-9	

Carfilzomib	Medchem Express	Cat# 253339
Bafilomycin A1	Sigma-Aldrich	B1793; CAS:88899-55-2
Epoxomicin	Sigma-Aldrich	Cat#324801
Thapsigargin	Sigma-Aldrich	T9033; CAS:67526-95-8
Digitonin	Calbiochem	Cat#300410
TAK-243	MedChem Express	Cat#HY-100487
ML792	MedChem Express	Cat#HY-108702
Imidazole	Sigma-Aldrich	I5513; CAS: 288-32-4
Polybrene	Sigma-Aldrich	TR-1003-G;
Complete protease inhibitors cocktail	Roche Diagnostic	Cat#04693116001
Phosphatase inhibitor cocktail	Roche Diagnostic	Cat#04906837001
DAPI	Sigma-Aldrich	Cat#9542 CAS:28718-90-3
Mowiol	Calbiochem	Cat# 475904 CAS:9002-89-5
Dabco(1,4-Diazabicyclo[2.2.2]octane)	Sigma-Aldrich	Cat#D2522 CAS:280-57-9
HA-Ubiquitin-VS	R&D systems	Cat# U-212-025
Histone H3.1/H4 Tetramer	NEB Biolabs	Cat# M2509S
Glutathione	ThermoFisher Scientific	Cat# 78259
ATP	ThermoFisher Scientific	Cat# R0441
Kits		
Lipofectamine 2000 transfection kit	Invitrogen	Cat#L2000-015
jetOPTIMUS DNA transfection reagent	Polyplus	Cat#101000006
Q5® Site-Directed Mutagenesis Kit	New England Biolabs	Cat#E0554S
Quick-RNA MiniPrep kit	Zymo Research	Cat#R1055
RNA clean and concentrator	Zymo Research	Cat#R1013
SuperScript VILO cDNA Synthesis kit	Invitrogen	Cat#11754050
LC FS DNA Master SYBR Green I	Roche	Cat#12239264001
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Thermo Scientific	Cat# 10777019
DC Protein Assay quantification kit	Bio-Rad	Cat#A500-0116
SuperSignal™ WestPico PLUS Chemiluminescent Substrate	Thermo Scientific	Cat#XE356732
Protein purification columns		
HisPur NiNTA resin	Thermo Scientific	Cat# 88222
Pierce Glutathione Agarose	Thermo Scientific	Cat# 16101
StrepTrap XT	Cytiva	Cat# 29401317
Superose 6HR 10/30	Cytiva	Cat# 17-0537-01
Microcon-10 Centrifugal filter	Merck	Cat# MRCPR010

Mediums and Buffers		
RPMI1640	Sigma-Aldrich	Cat# R8758
DMEM	Sigma-Aldrich	Cat# D6429
EBSS	Gibco	Cat# 1854705
Hepes Buffer solution	Gibco	Cat#15630-080
Experimental models: Cell lines		
HeLa	ATCC	RR-B51S
HEK293T	ATCC	CRL3216
HEK293T znf598 knock out	Liu et al., 2023 ²	N/A
HEK293T ufsp2 knock out	This paper	N/A
U2OS	ATCC	HTB-96
U2OS ufsp2 knock out	This paper	N/A
HCT116	ATCC	CCL-247
HCT116-EATR	This paper	N/A
LCLwt/cm (Tet-on BZLF1)	Li et al. 2021 ³	N/A
Recombinant DNA		
pCMV3-RPL26-Myc	Sino Biological	HG16834-CM
pCMV3-UFSP2-FLAG	Sino Biological	HG17133-CF
pTRIPZ lentiviral vector	ThermoScientific	Cat#RHS4750
pCW57.1	gift from David Root	Addgene #41393
psPAX2	gift from Didier Trono	Addgene # 12260
pMD2.G	gift from Didier Trono	Addgene # 12259
TetOn-mCherry-eGFP-RAMP4	gift from Jacob Corn	Addgene #109014
pLenti-X1-Neo-DDRGK1-WT-HA	gift from Jacob Corn	Addgene # 139845
pLenti-X1-Neo-HA-UFL1	gift from Jacob Corn	Addgene #139860
ER-K20	gift from Yihong Ye	Addgene # 133861
px330-UFSP2 sgRNA1	gift from Yihong Ye	Addgene # 134638
px330-UFSP2 sgRNA2	gift from Yihong Ye	Addgene # 134637
pCMV10-3xFLAG-BPLF1	Gastaldello et al., 2010 ⁴	N/A
pCMV10-3xFLAG-BPLF1 ^{C61A}	Gastaldello et al., 2010 ⁴	N/A
pET28a-His-BPLF1 (1-235)	Gastaldello et al., 2012 ⁵	N/A
pET28a-His-BPLF1 ^{C61A}	This paper	N/A
pET28a-His-UFSP2	This paper	N/A
pACYC-EGFP	Dantuma et al., 2000 ⁶	N/A
pACYC-Ubiquitin-EGFP	Dantuma et al., 2000 ⁶	N/A
pACYC-UFM1-EGFP	This paper	N/A
pCDNA3.1-RPL26-S	gift from Ron R. Kopito	N/A
pCDNA3.1-UFM1-GFP	Gift from Yogesh Kulathu	N/A
pET15b-His-UBA5	Gift from Yogesh Kulathu	N/A
pGX-6P1-GST-UFC1	Gift from Yogesh Kulathu	N/A
pET-15b-His-UFM1	Gift from Yogesh Kulathu	N/A
pETDuet1-His-UFL1-Strep-DDRGK1	Gift from Yogesh Kulathu	N/A

Supplementary Table 2. PCR and qPCR primers

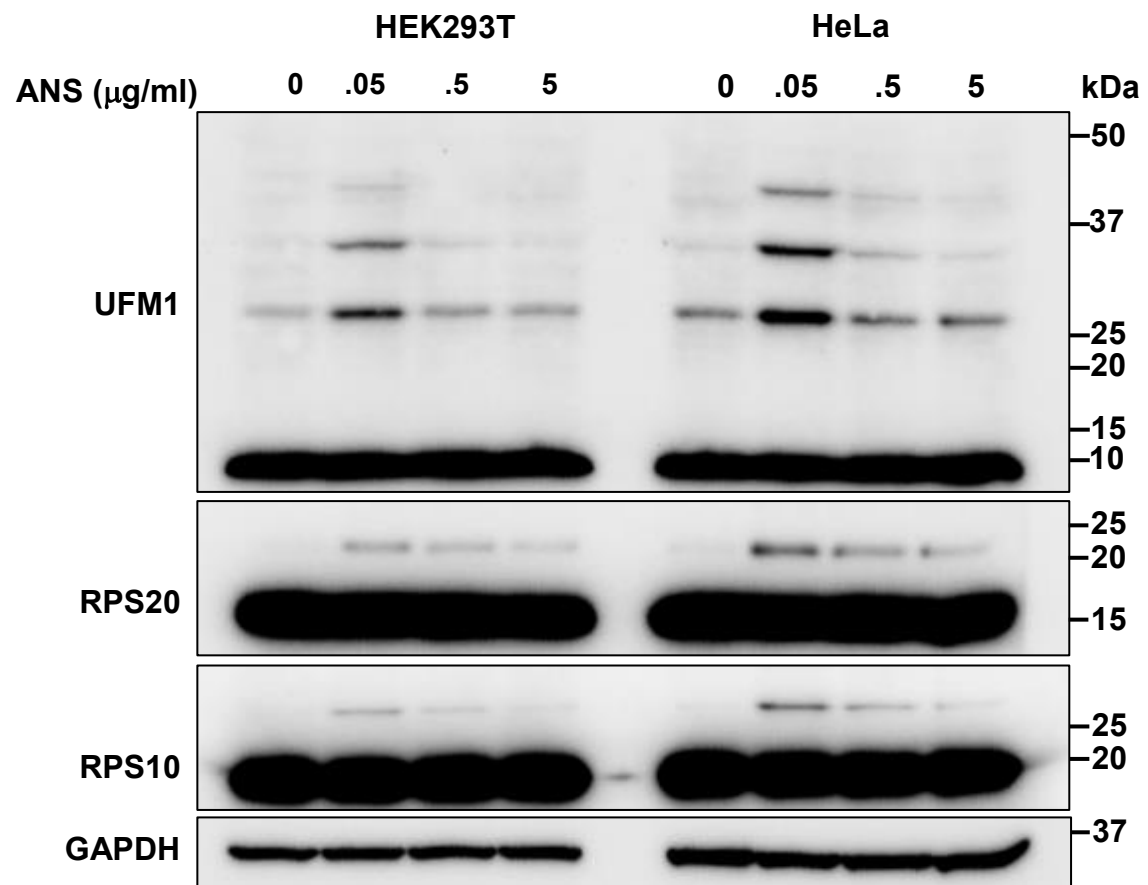
Gene	Forward primer (5'-3')	Reverse primer (5'-3')
pACYC-UFM1-EGFP PCR	GTTCCAAAAAGAATTCATCCCCC ATGTCGAAGGTTTCCTTT	GGTGCCTCACAAGATCTTCCAAC ACGATCTCTAGGAATAATCCGC
pET28a-His-UFSP2 PCR	CTGGTGCCGCGCGGCAGCCATA TGGTGATTTCAGAAAGTATG	GTCGACGGAGCTCGAATTCGTTA AATCATATTTGGTCGCTG
pET28a-His-BPLF1 ^{C61A} PCR	CGGCATCCAGGCAGTCAGCAAC TGC	GCAAAGCGGCCAAACTTG
ER_K20 PCR (no FLAG Tag)	GGATCTCCTGGTCCGACCC	TCCGGACTTGTACAGCTCGTC
Spliced Xbp1 qPCR	TGCTGAGTCCGCAGCAGGTG	GCTGGCAGGCTCTGGGGAAG
BiP qPCR	TGTTCAACCAATTATCAGCAAAC TC	TTCTGCTGTATCCTCTTCACCAGT
GAPDH qPCR	AAGGTCGGAGTCAACGGATT	CTCCTGGAAGATGGTGATGG

REFERENCES

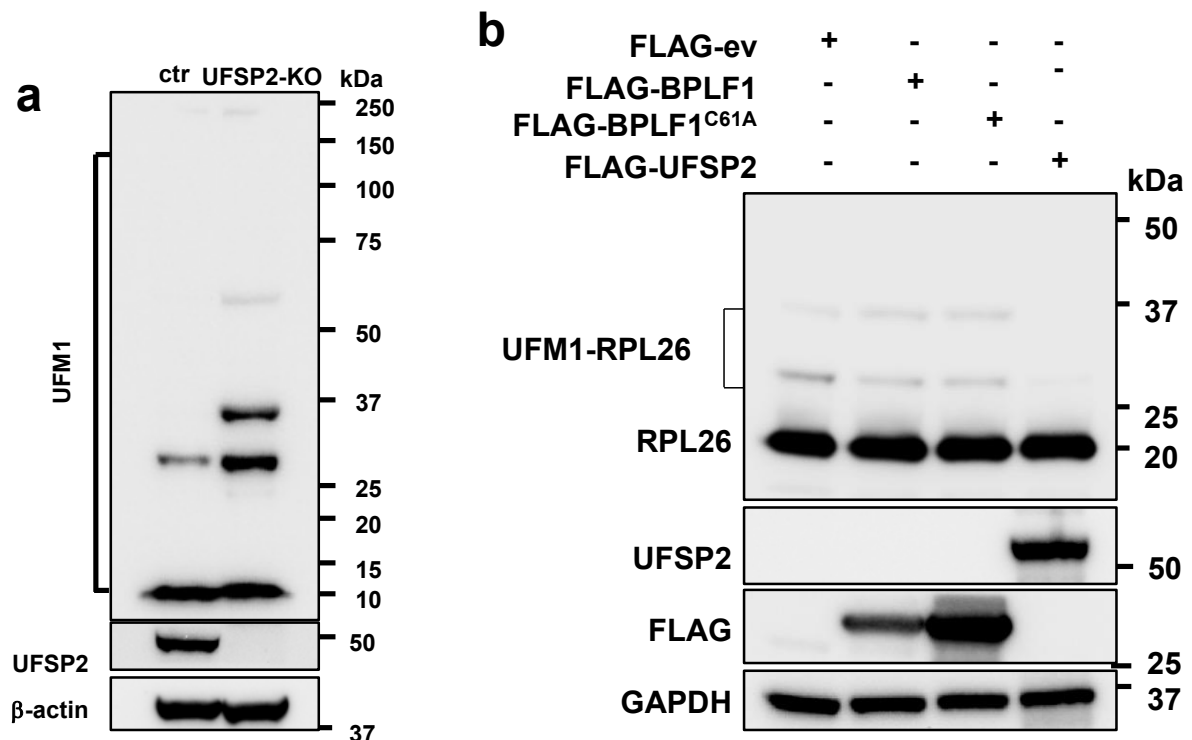
1. van Gent M, *et al.* Epstein-Barr virus large tegument protein BPLF1 contributes to innate immune evasion through interference with toll-like receptor signaling. *PLoS Pathog* **10**, e1003960 (2014).
2. Liu J, *et al.* Remodeling of the ribosomal quality control and integrated stress response by viral ubiquitin deconjugases. *Nat Commun* **14**, 8315 (2023).
3. Li J, *et al.* The Epstein-Barr virus deubiquitinating enzyme BPLF1 regulates the activity of topoisomerase II during productive infection. *PLoS Pathog* **17**, e1009954 (2021).
4. Gastaldello S, *et al.* A deneddylase encoded by Epstein-Barr virus promotes viral DNA replication by regulating the activity of cullin-RING ligases. *Nat Cell Biol* **12**, 351-361 (2010).
5. Gastaldello S, Callegari S, Coppotelli G, Hildebrand S, Song M, Masucci MG. Herpes virus deneddylases interrupt the cullin-RING ligase neddylation cycle by inhibiting the binding of CAND1. *J Mol Cell Biol* **4**, 242-251 (2012).
6. Dantuma NP, Lindsten K, Glas R, Jellne M, Masucci MG. Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. *Nat Biotechnol* **18**, 538-543 (2000).

SUPPLEMENTARY FIGURES

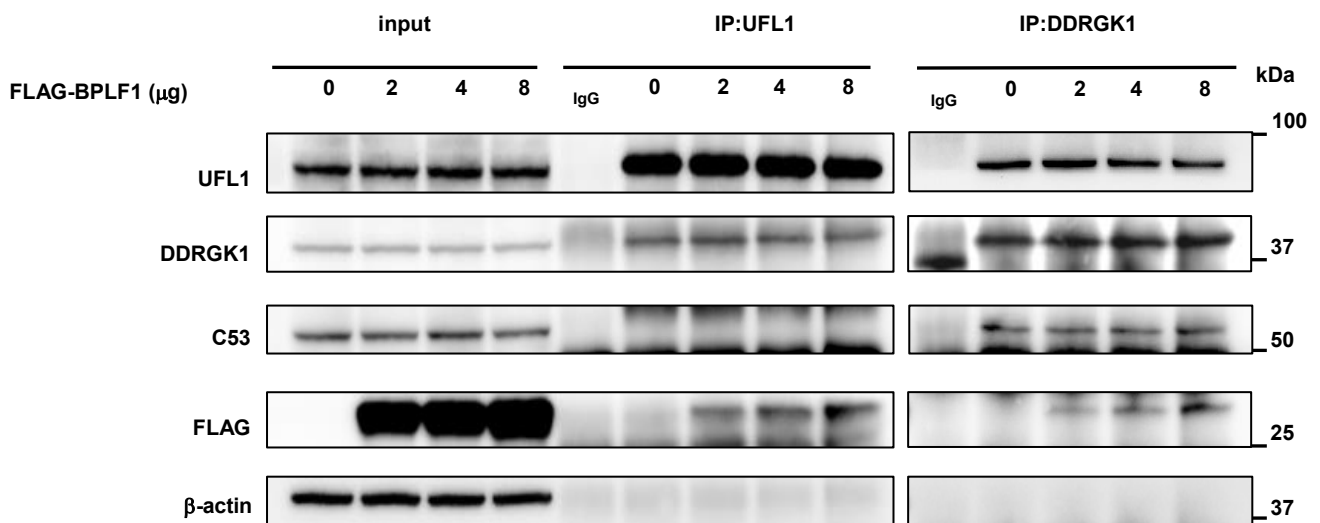
Supplementary Figure 1. Titration of the amount of ANS required for induction of RPL26 UFMylation. The cell lines were treated with the indicated ANS concentrations for 20 min before analysis of RPL26 UFMylation and RPS10 and RPS20 ubiquitination. Immunoblots from one representative experiment out of two are shown in the figure.



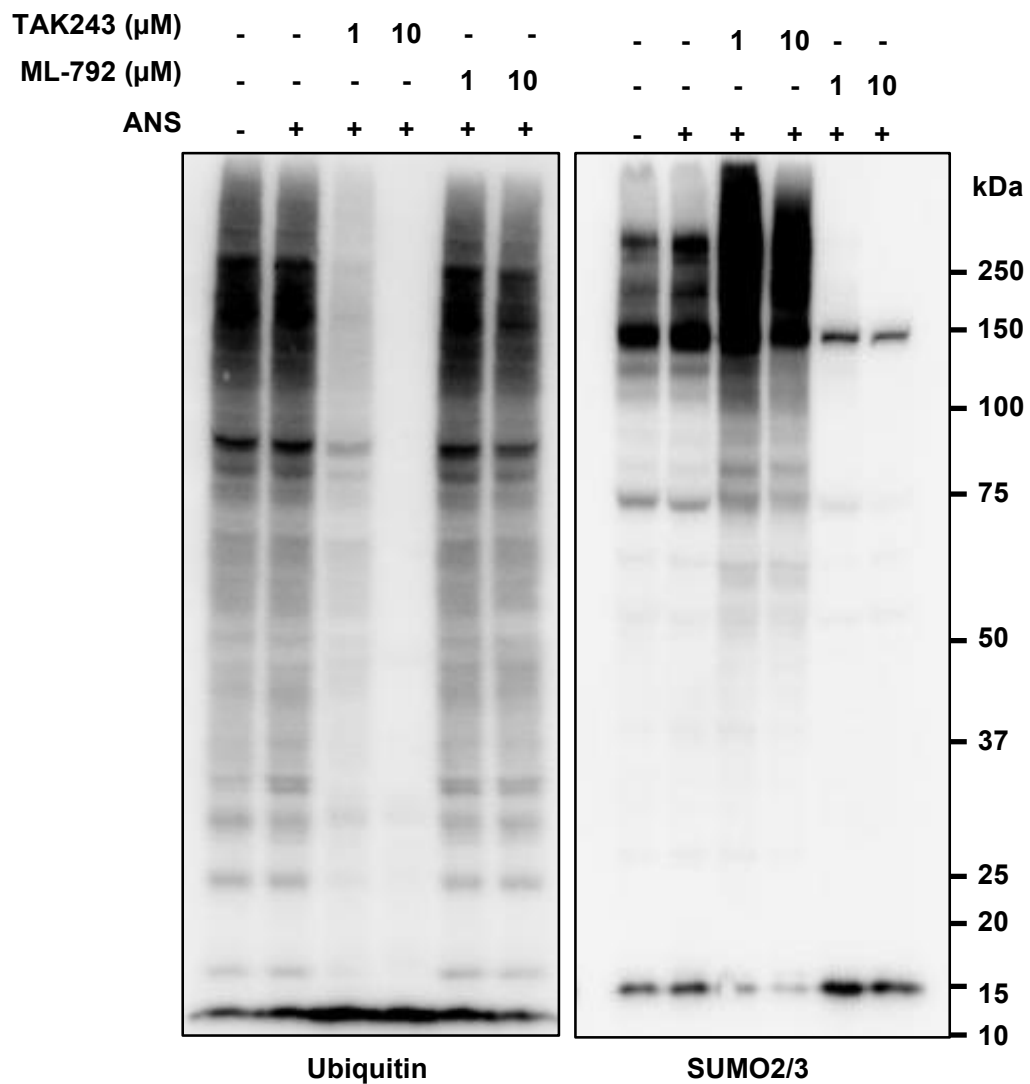
Supplementary Figure 2. Characterization of the U2OS-UFSP2-KO cell line. (a) The main cellular de-UFMyrase UFSP2 was knocked down in U2OS cells by co-transfection with GFP and px330-UFSP2-sgRNAs expressing plasmids. After two rounds of FACS sorting, the expression of UFSP2 was assessed in immunoblots. A representative immunoblot illustrating the loss of UFSP2 and concomitant accumulation of endogenous UFMylated substrates is shown. (b) BPLF1 does not promote the deUFMylation of endogenous RPL26 in UFSP2-KO cells. The UFSP2-KO cells were cotransfected with FLAG-ev/BPLF1/ BPLF1^{C61A} or FLAG-UFSP2, and immunoblots were probed with the RPL26 antibody. Immunoblots from one representative experiment out of two are shown.



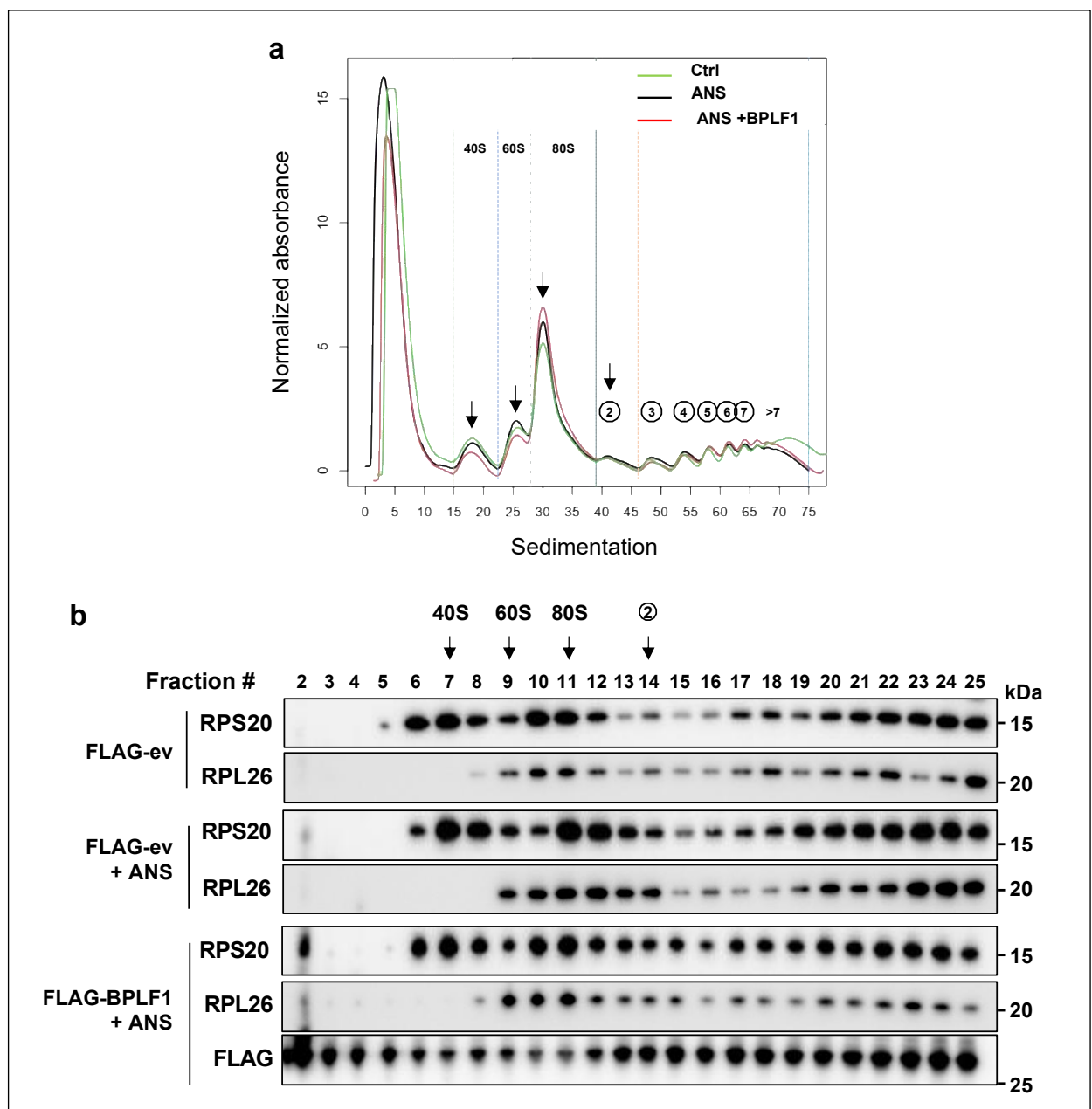
Supplementary Figure 3. Overexpressed BPLF1 does not affect the assembly of the UFM1 ligase complex. HEK293T cells were transfected with increasing amounts of the FLAG-BPLF1 plasmid. Cell lysates were immunoprecipitated with the indicated antibodies. Immunoblots from one out of two independent experiments are shown in the figure.



Supplementary Figure 4. Validation of the ubiquitin and SUMO E1 inhibitors. HEK293T cells were treated with the indicated concentrations of TAK243 or ML-792 for 3 h before treatment with 50 ng/ml ANS for 20 min. Immunoblots of cell lysates from the experiment shown in Figure 6C were probed with the indicated antibodies.



Supplementary Figure 5. BPLF1 cofractionates with ribosomes and is enriched on polysomes. (a) Sucrose gradient sedimentation of untreated HEK293T cells and cells treated with ANS in the absence or presence of catalytically active BPLF1. Polysome tracing from one out of two independent experiments is shown. The peak fractions enriched in 40S, 60S, 80S ribosomes and disomes are indicated by arrows. (b) Collected fractions were analyzed by immunoblotting. The 40S and 60S ribosome proteins were identified by probing the blots with RPS20- and RPL26-specific antibodies. Although a large proportion of the transfected BPLF1 was recovered in the pre-ribosomal fractions, as expected, the vDUB was detected in all ribosome-containing fractions and was enriched in the polysome fractions.



Supplementary Figure 6. Characterization of the HCT116 ER-phagy reporter cell line.

(a) HCT116 cells were stably transfected with an ER-phagy expressing in frame the ER-membrane protein RAMP4, GFP, and ChFP under the control of a tetracycline-regulated promoter. Immunoblots probed with an anti-GFP antibody confirmed the expression of the fusion protein in cells treated with 2 μ g/ml Dox for 24 h. **(b)** Visualization of ER-phagy. Stably transfected HCT116 growing on cover slides were treated with Dox for 24 h followed by starvation overnight in EBSS medium. Red dots corresponding to ER-loaded phagolysosomes were visualized by confocal microscopy.

