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Supplemental information

ATG9A regulates proteostasis through reticulophagy receptors FAM134B and SEC62 and folding chaperones CALR and HSPB1

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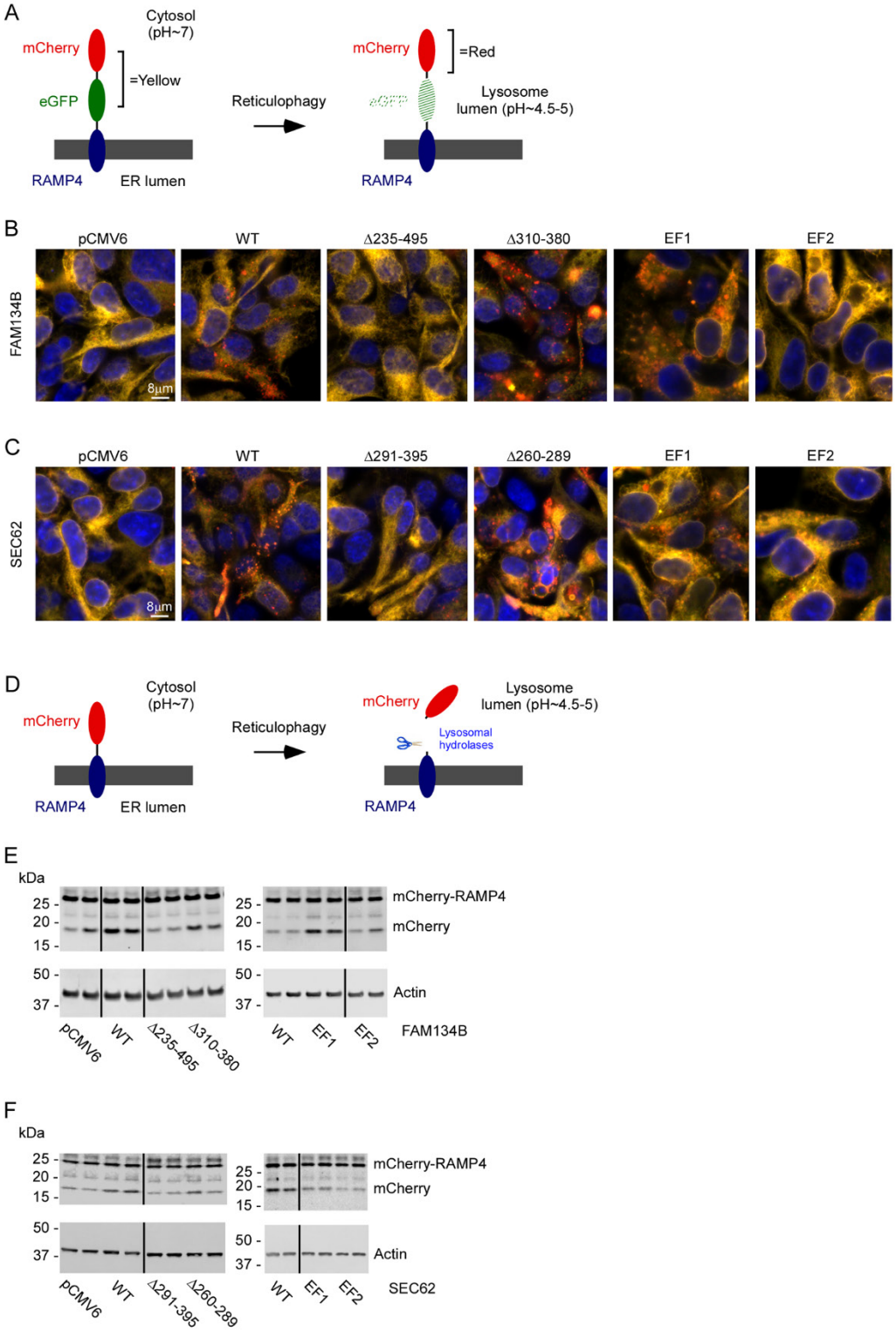
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Cytosol
(pH~7)
mCherry
=Yellow
eGFP
RAMP4
ER lumen
Reticulophagy
mCherry
=Red
Lysosome
lumen (pH~4.5-5)
RAMP4
B
pCMV6
WT
Δ235-495
Δ310-380
EF1
EF2
FAM134B
8μm
C
pCMV6
WT
Δ291-395
Δ260-289
EF1
EF2
SEC62
8μm
D
Cytosol
(pH~7)
mCherry
RAMP4
ER lumen
Reticulophagy
mCherry
Lysosome
lumen (pH~4.5-5)
Lysosomal
hydrolases
RAMP4
E
kDa
25 -
20 -
15 -
50 -
37 -
mCherry-RAMP4
mCherry
Actin
pCMV6
WT
Δ235-495
Δ310-380
WT
EF1
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FAM134B
F
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25 -
20 -
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50 -
37 -
mCherry-RAMP4
mCherry
Actin
pCMV6
WT
Δ291-395
Δ260-289
WT
EF1
EF2
SEC62

Figure S1. FAM134B and SEC62 mutants that engage ATG9A can induce reticulophagy, Related to Figure 4.

(A) Schematic view of the EATR assay design.

(B and C) Live imaging showing induction of reticulophagy as caused by different FAM134B (B) or SEC62 (C) mutants.

(D) Schematic view of the CCER assay design.

(E and F) Western blotting showing cleavage of mCherry as caused by different FAM134B (E) or SEC62 (F) mutants.

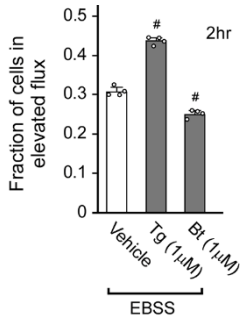
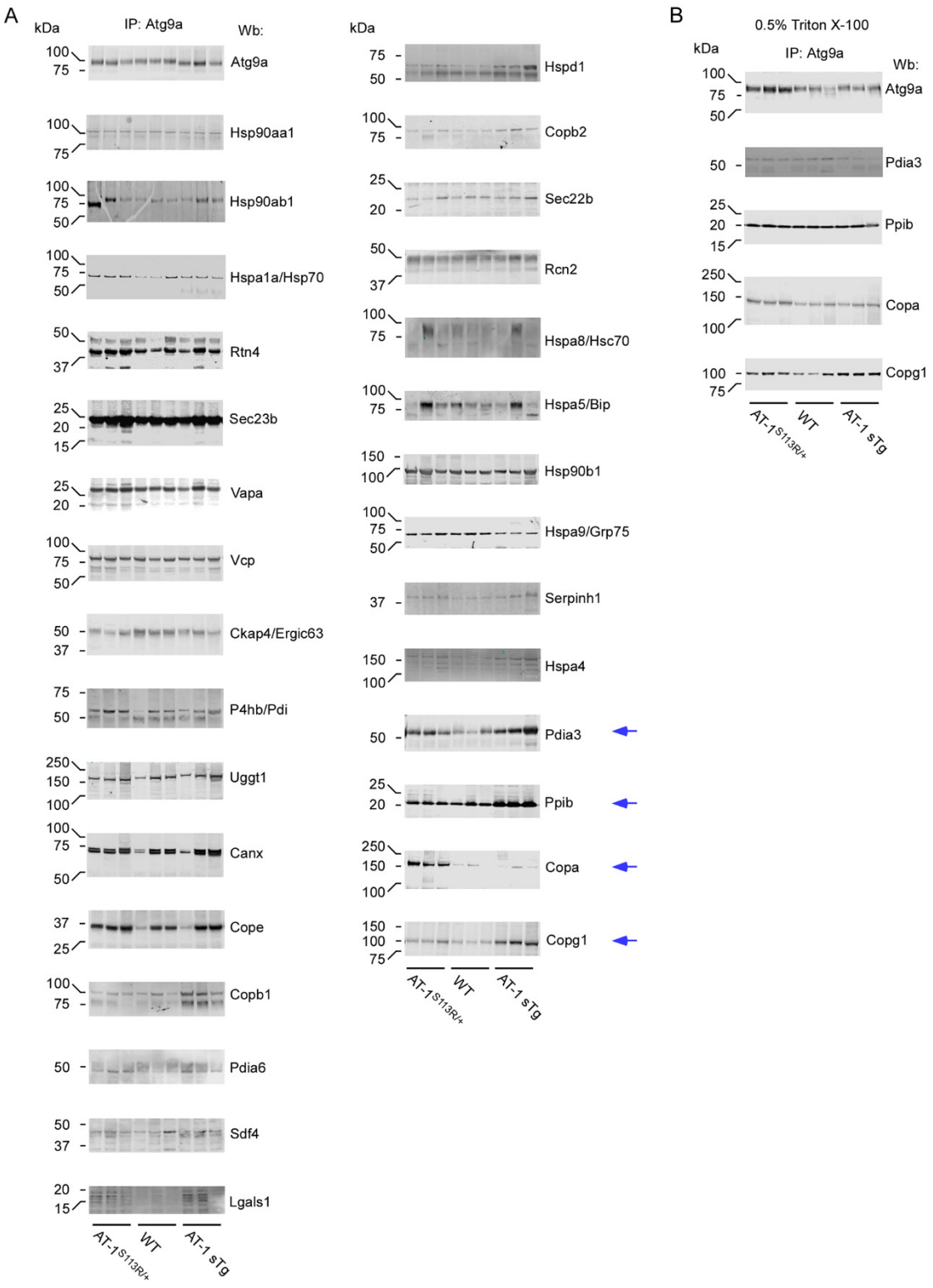


Figure S2. Calcium modifying drugs, Thapsigargin (Tg) and BAPTA (Bt), differentially affected the induction of reticulophagy, Related to Figure 4.

Reticulophagy flux as regulated by Tg and BAPTA. Data are represented as mean $\pm$ SD. $^{\#}p < 0.0005$.


A
kDa
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Wb:
Atg9a
Hsp90aa1
Hsp90ab1
Hspa1a/Hsp70
Rtn4
Sec23b
Vapa
Vcp
Ckap4/Ergic63
P4hb/Pdi
Uggt1
Canx
Cope
Copb1
Pdia6
Sdf4
Lgals1
AT-1S113R/+
WT
AT-1 sTg
kDa
Hspd1
Copb2
Sec22b
Rcn2
Hspa8/Hsc70
Hspa5/Bip
Hsp90b1
Hspa9/Grp75
Serpinh1
Hspa4
Pdia3
Ppib
Copa
Copg1
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WT
AT-1 sTg
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0.5% Triton X-100
kDa
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Wb:
Atg9a
Pdia3
Ppib
Copa
Copg1
AT-1S113R/+
WT
AT-1 sTg

Figure S3. Identification and characterization of the ER-based ATG9A interactome, Related to Figure 5.

(A) Western blotting showing that Atg9a interacting proteins co-immunoprecipitate with ER-based endogenous Atg9a. Co-immunoprecipitation was performed with intact native ER vesicles.

(B) Functional co-immunoprecipitation of selected proteins from (A; see arrows) was also performed with permeabilized native ER vesicles.

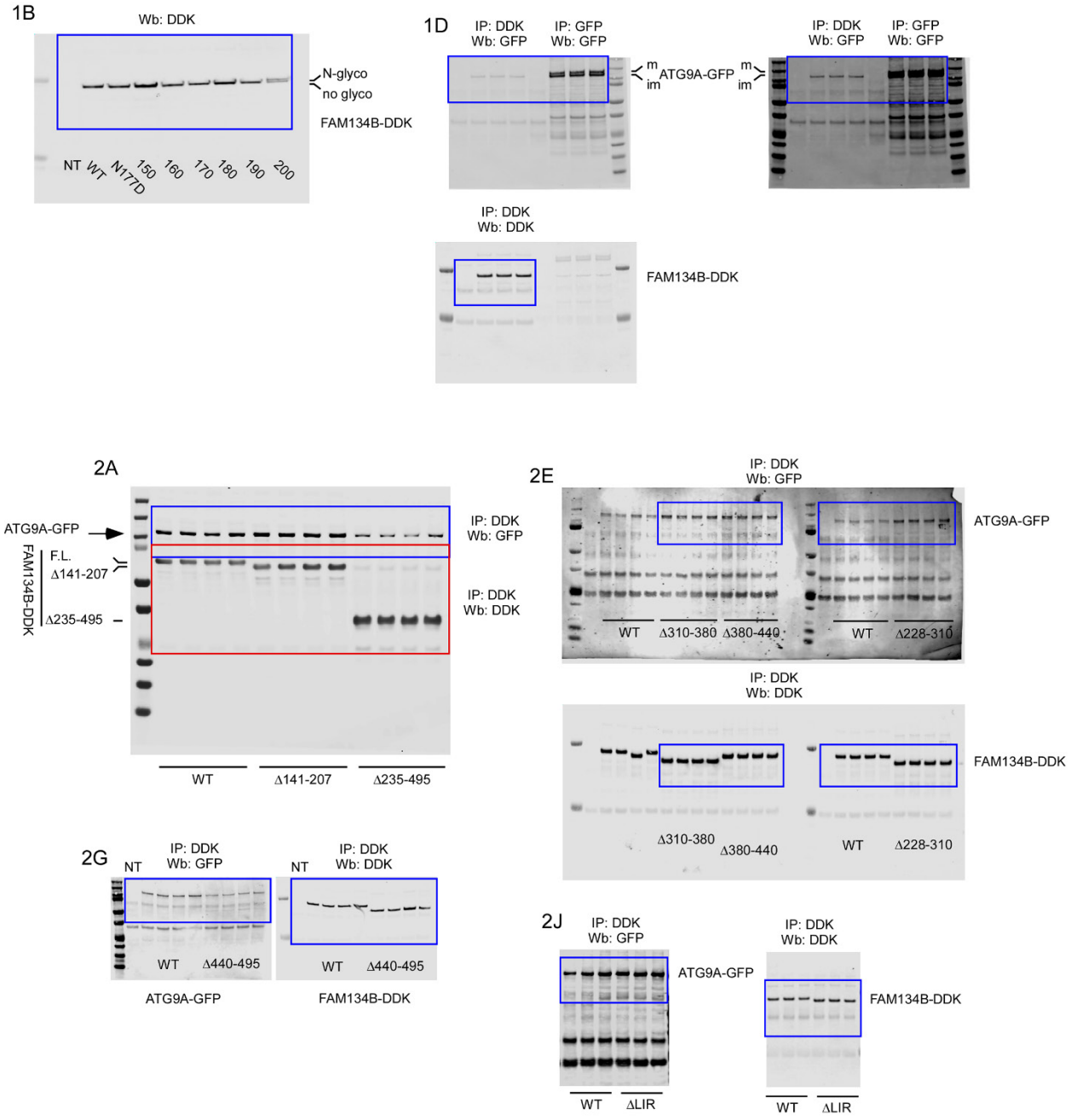

Figure S4. Uncropped blots included in the main manuscript, Related to Figures 1-2.

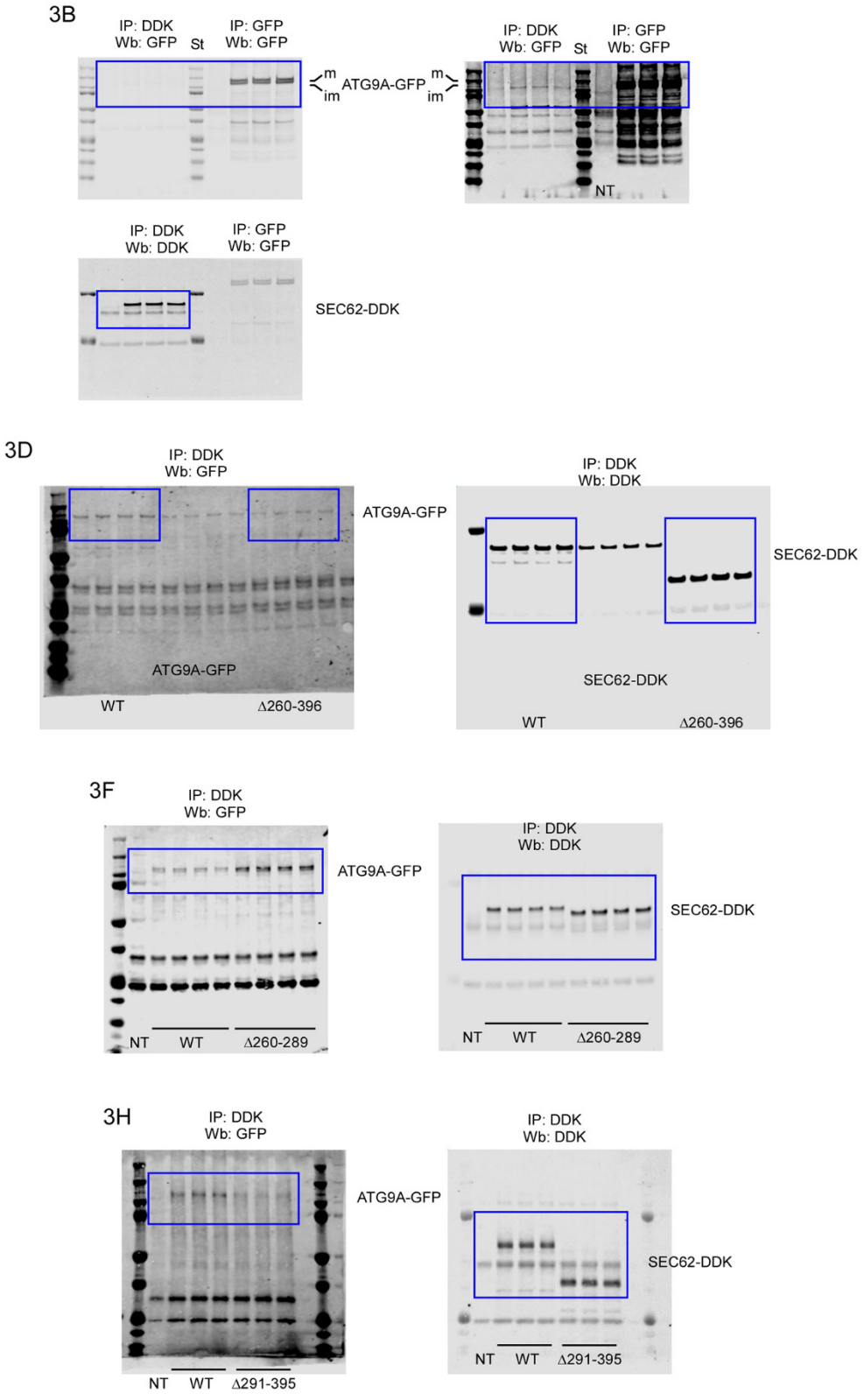

Figure S5. Uncropped blots included in the main manuscript, Related to Figure 3.

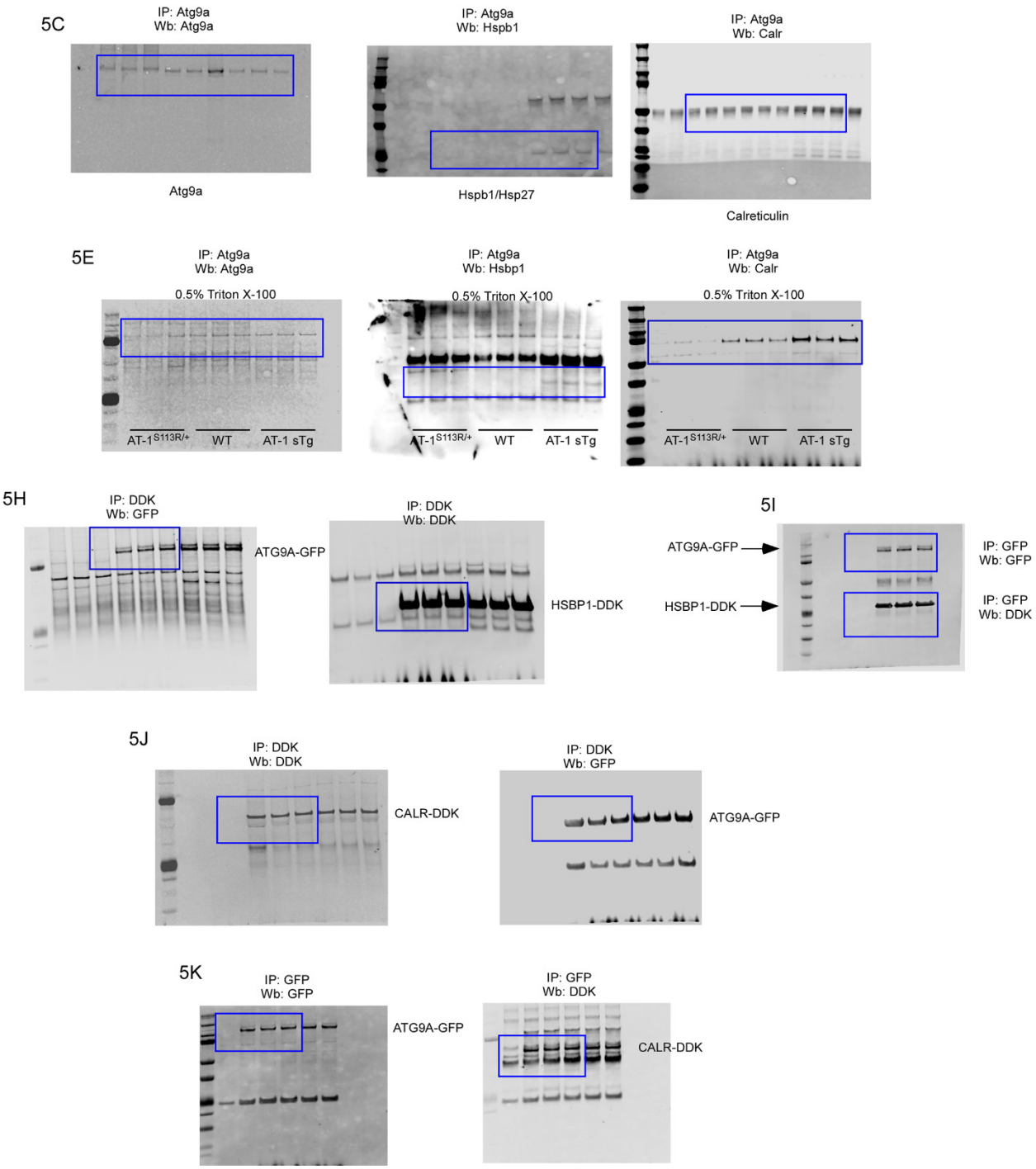

Figure S6. Uncropped blots included in the main manuscript, Related to Figure 5.

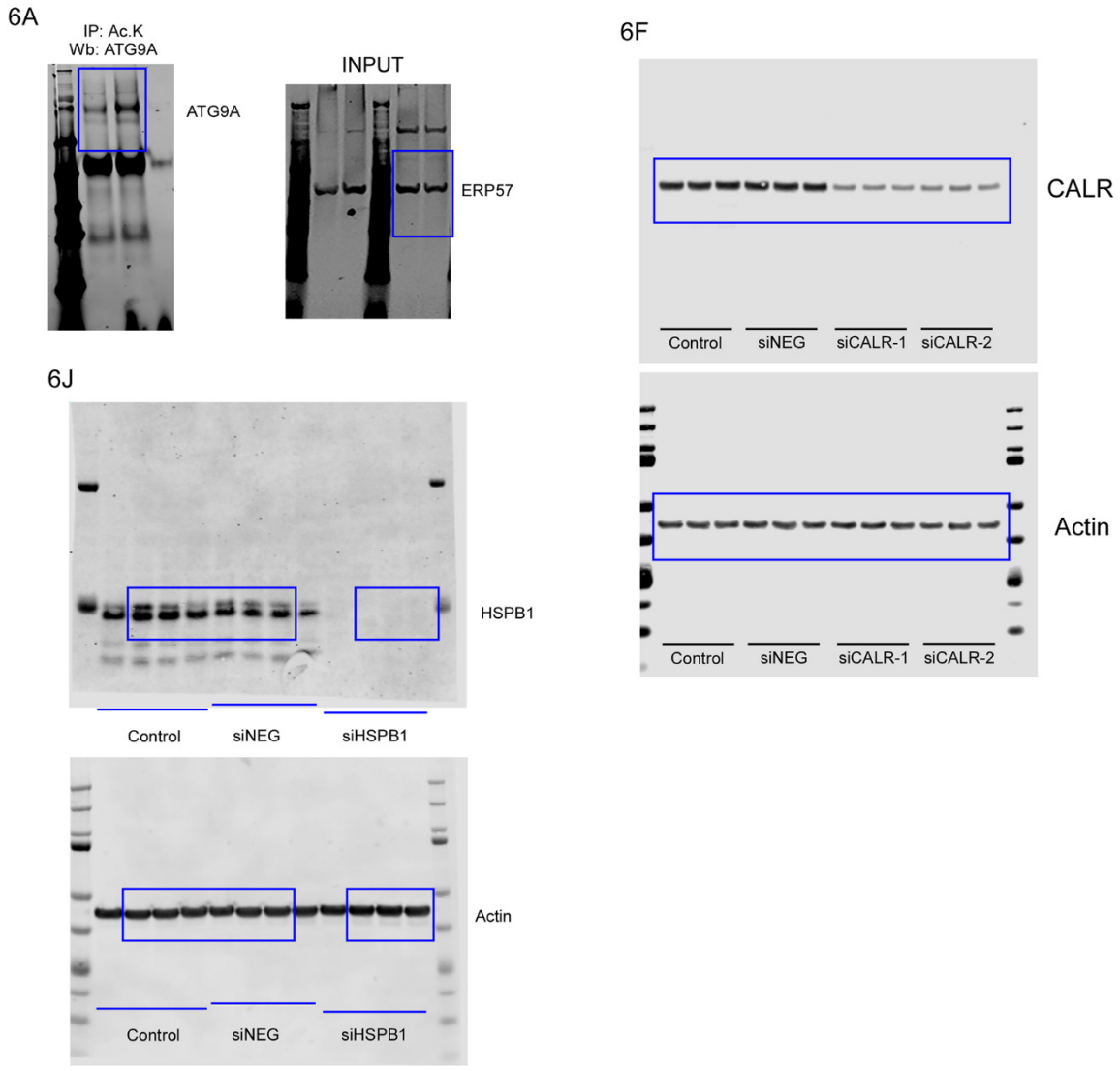

Figure S7. Uncropped blots included in the main manuscript, Related to Figure 6.

TRANSPARENT METHODS

Animals

All mouse studies were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and received ethical approval by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison. Generation of AT-1$^{S113R/+}$ and AT-1 sTg mice is described elsewhere (Peng et al., 2014; Peng et al., 2018). Mice were studied at ~2-3 months of age. Age-matched wild-type (WT) littermates were used as controls. Both males and females were used for the experiments reported in this study.

Cell cultures

H4 (human neuroglioma; RRID:CVCL_1239; ATCC:HTB-148) and HEK293 (human embryonic kidney; RRID: CVCL_0045; ATCC:CRL-1573) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Corning #10–013-CV) supplemented with 10% Fetal Bovine Serum (Corning #35–011-CV) and 1% penicillin/streptomycin (Gibco™ #10378016), thereafter referred to as DMEM-10. Cells were maintained at 37°C in a humidified atmosphere with 5% CO2. Cells were obtained and authenticated by American Type Culture Collection, and are not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee (ICLAC; Version 9). Unless otherwise specified, cells were harvested by scraping in ice-cold PBS and pelleting by centrifugation at 6,000g for 5 minutes at 4°C.

Structural modeling

Structural modeling was performed using the Iterative Threading ASSEmbly Refinement (I-TASSER) platform, which analyzes PDB structural templates to select high-scoring 3D

models (Roy et al., 2010; Yang et al., 2015). PDB analysis employed the following threading platforms: CEthreader, FFAS3D, HHpred, HHsearch, MUSTER, Neff-MUSTER, PPAS, PRC, PROSPECT2, SP3, and SparksX. Models were further validated by combining secondary structure and solvent accessibility analysis. The top five scoring templates were built using MODELLER; they were then assigned a confidence score based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. The structure that gave the highest confidence score was used for our analysis. In the case of FAM134B, the following top structure templates were identified from the PDB library by LOMETS: 6gmhM; 2pff; 6fltX; 2pff; 5gaoE; 5yfpD; 5yz0C; 5ly9A; 4jioA. In the case of HSPB1, the following top structure templates were identified from the PDB library by LOMETS: 6dv5B; 6dv5A; 4ydzA; 6dv5; 2ygd; 6t1rA; 6dv5; 6dv5A; 6dv5B; 2bolA. The dimeric structure of the α-crystallin domain of HSPB1 (PDB:2n3j) was also used to generate our final HSPB1 model (Rajagopal et al., 2015). Secondary structure prediction was performed with the Jpred 4, PredictProtein, and NetSurfP-2.0 platforms. Ligand-protein binding interactions were assessed through the BioLip database. Disorder prediction was performed with DisEMBL, PredictProtein and NetSurfP-2.0 platforms. Images were prepared with Pymol (Schrödinger, Inc).

Cell transfection

H4 and HEK293 cells were transiently transfected with Lipofectamine 3000 (Invitrogen #100022234) according to the manufacturer's instructions. Original plasmids used: human pCMV6-ATG9A-tGFP (OriGene #RG222568); human pCMV6-ATG9A-DDK (OriGene #RC222513); human pCMV6-CALR-DDK (OriGene #RC203222); human pCMV6-HSPB1-

DDK (OriGene #RC201800); human pCMV6-FAM134B-DDK (OriGene # RC208619); human pCMV6-SEC62-DDK (OriGene #RC204452); pCMV6-Entry empty plasmid (OriGene #PS100001). Co-transfections were performed with 300fmol of DDK-tagged protein and 600fmol ATG9A-tGFP, unless otherwise noted. Cells were harvested 48 hours after transfection.

Site-directed mutagenesis

Plasmids were obtained from OriGene and are listed in the "Cell transfection" section. Molecular constructs were prepared by Integrated DNA Technologies. Primers are listed in **Table S2**. Site-directed mutagenesis was performed using the QuikChange Lightning Site-Directed Mutagenesis kit per manufacturer's instructions (Agilent Technologies, 210518 and 210519). All constructs were prorogated from a single colony. Plasmid DNA was extracted using either QIAprep Spin Miniprep Kit or QIAGEN Plasmid Maxi Kit (Qiagen, 27106 and 12165). All constructs were confirmed by DNA sequencing.

Generation of EATR, CCER, and mCherry-ER-3 stable lines

To generate the EATR stable line, HEK293 cells were transfected with SspI digested TetOn-mCherry-eGFP-RAMP4 vector (gift from Jacob Corn; Addgene plasmid #109014) and selected in DMEM-10 with 400ug/mL G418 (Invitrogen #10131027). To generate the CCER stable line, HEK293 cells were transfected with MluI digested pLenti-X1-hygro-mCherry-RAMP4 vector (gift from Jacob Corn; Addgene plasmid #118391) and selected in DMEM-10 + 100ug/mL Hygromyocin B (Invitrogen #10687010). To generate the mCherry-ER-3 stable line, HEK293 cells were transfected with undigested mCherry-ER-3 vector (gift from Michael Davidson; Addgene plasmid #55041) and selected in DMEM-10 with 400ug/mL G418.

Polyclonal resistant cells were expanded and cultured in G418 or Hygromycin B until plating for experiments.

EATR assay

HEK293 cells stably expressing TetOn-mCherry-eGFP-RAMP4 were plated on 12-well plates two days before transfection. On the day of transfection, media was changed to DMEM-10 with 400ug/mL G418 and 16ug/mL Doxycycline to enhance expression of the tandem reporter. Twenty-four hours before flow cytometry analysis, cells were transfected with 200fmol of plasmid using Lipofectamine 3000 according to manufacturer's instructions. To stimulate autophagy, cells were incubated for 2 or 4 hours in 50% DMEM-10 and 50% EBSS then trypsinized, centrifuged, and resuspended in PBS containing 2% FBS, 10mM EDTA, and 1ug/mL DAPI on ice. For time course experiments, autophagy was not stimulated and cells were trypsinized, centrifuged, and resuspended as above, but were placed in a 37^{o}C water bath at time 0 and sampled every 10 minutes. Flow cytometry was performed on Attune NxT flow cytometers (Thermo Fisher Scientific) configured with 405, 488, 561, and 637nm lasers. Single, live, and mCherry-expressing gates were set using the entire experiment population. The "elevated flux" gate was defined as events one standard deviation above the least-squares regression line of the eGFP vs mCherry plot of non-transfected cells incubated in 100% DMEM-10 and 16ug/mL Doxycycline under equivalent experimental conditions. Data analysis was performed using Attune NxT Sofware version 4.2 and in-house Python scripts.

For calcium modulating experiments, cells were prepared equivalently to other EATR assay experiments except media was changed to 100% EBSS 2 hours before analysis instead of a 50% DMEM-10 and 50% EBSS solution to force induction of reticulophagy. Immediately after

media change, cells were treated with a 10x solution containing additives and either calcium modulating agent or DMSO. The final solution contained 1mM Probenecid (Sigma, P8761) and 0.05% Pluronic F-127 (Sigma, P2443) as well as either 1uM Thapsigargin (Sigma, T9033), 1uM BAPTA-AM (Thermo Fisher Scientific, B6769), or 0.1% DMSO (vehicle). Probenecid and Pluronic F-127 were added to improve loading of BAPTA-AM into cells.

EATR Live Imaging

HEK293 cells stably expressing mCherry-eGFP-RAMP4 were transfected as for the EATR assay. Cells were trypsinized and replated 24 hours post-transfection on a poly-D-lysine-coated, glass-bottomed, 96-well plate (Cellvis P96-1.5H-N) and incubated for 48hrs to allow for maximum construct expression. Single 1024x1024, 0.208um/pixel images were acquired on a Nikon A1R-SI microscope equipped with 405, 488, 561, and 640 nm lasers and a CFI Plan Apochromat Lambda 60X Oil 1.4 NA objective at a pinhole size of 219.67um.The acquisition software was NIS-Elements AR 5.11.01.

CCER Assay

HEK293 cells stably expressing mCherry-RAMP4 were plated on 12-well plates two days before transfection. On the day of transfection, media was changed to DMEM-10 and cells were transfected with 200fmol of the indicated plasmids using Lipofectamine 3000 according to manufacturer's instructions. After 24 hours, cells were harvested via trypsin.

ICC

HEK293 cells stably expressing mCherry-ER-3 were plated on poly-D-lysine (Sigma P6407) coated glass coverslips and allowed to adhere overnight. Cells were then starved with EBSS for 6hrs to stimulate reticulophagy and fixed in 4% PFA for 15min. Cells were washed in PBS for 5min and permeabilized in 0.5% Saponin (Sigma 47036) in PBS for 20min. Saponin was used to preserve ER membrane structure. Fixed and permeabilized cells were then washed for 5min in PBS to remove excess detergent. Antibody staining was done sequentially to avoid cross-reaction, starting with ATG9A then FAM134B, SEC62, CALR, or HSPB1. Cells were blocked for 1hr in 5% goat serum and 0.1% Saponin in PBS, then incubated in primary antibody in 5% goat serum and 0.1% Saponin in PBS overnight at 4°C. Cells were washed 3x5min in PBS with 0.1% Tween-20, incubated in secondary antibody in 5% goat serum and 0.1% Saponin in PBS for 1 hour, and again washed 3x5min in PBS with 0.1% Tween-20. After sequential stainings, cells were washed one more time in PBS to remove excess detergent and mounted onto slides using ProLong™ Diamond Antifade Mountant (Invitrogen P36961).

SIM and Image Reconstruction

Z-stack 1024x1024, 0.032um/pixel images were acquired at 0.2um intervals on a Nikon-Structured Illumination Microscope (Eclipse Ti-E) equipped with 405, 488, 561, and 647 nm lasers, a CFI Apochromat TIRF 100XC Oil 1.49 NA objective, and iXon 3 EMCCD (Andor Technologies). The acquisition software was NIS-Elements AR 4.60.00. Reconstructions were generated using Imaris 9.6.0.

ER enrichment

Total ER membranes were isolated from *8*-week-old WT, AT-1$^{S113R/+}$, and AT-1 sTg mice using the ER Enrichment Kit (Novus Biologicals, NBP2-29482) according to the manufacturer's instructions.

LC-MS/MS

ATG9A complex isolation. Enriched ER fractions prepared as described above were extracted in either 1% Triton X-100 or 1% DDM. The ATG9A-myc complex was then purified by affinity chromatography using AminoLink immobilized anti-c-Myc monoclonal antibodies (Pierce ProFound, VWR-PI23620) (Jonas et al., 2010; Ko and Puglielli, 2009). Eluted ATG9A-associated proteins were analyzed by high-resolution high-accuracy MS.

Enzymatic "In Liquid" Digestion. TCA/acetone precipitated protein pellets were re-solubilized and denatured in 7.5µl of 8M Urea / 50mM NH_4HCO_3 (pH8.5) / 1mM TrisHCl. Subsequently diluted to 30µl for reduction step with: 1.25µl of 25mM DTT, 2.5µl MeOH, and 18.75µl 25mM NH_4HCO_3 (pH8.5). Incubated at 52°C for 15 minutes, cooled on ice to room temperature then 1.5µl of 55mM IAA was added for alkylation and incubated in darkness at room temperature for 15 minutes. Reaction was quenched by adding 4µl of 25mM DTT. Subsequently 1µl of Trypsin/LysC solution [100ng/µl of 1:1 Trypsin (Promega) and LysC (FujiFilm) mix in 25mM NH4HCO] and 13.5µl of 25mM NH_4HCO_3 (pH8.5) was added to 50µl final volume. Digestion was conducted for 2 hour at 42°C then additional 0.5µl of Trypsin/LysC solution was added and digestion proceeded for 4hrs at 37°C. Reaction was terminated by acidification with 2.5% TFA [Trifluoroacetic Acid] to 0.3% final.

NanoLC-MS/MS. Digests were cleaned up using OMIX C18 SPE cartridges (Agilent, Palo Alto, CA) per manufacturer protocol and eluted in 20µl of 60/40/0.1% ACN/H_2O/TFA, dried to completion in the speed-vac and finally reconstituted in 12µl of 0.1% formic acid. Peptides were analyzed by nanoLC-MS/MS using the Agilent 1100 nanoflow system (Agilent) connected to hybrid linear ion trap-orbitrap mass spectrometer (LTQ-Orbitrap Elite™, Thermo Fisher Scientific) equipped with an EASY-Spray™ electrospray source. Chromatography of peptides prior to mass spectral analysis was accomplished using capillary emitter column (PepMap® C18, 3µM, 100Å, 150x0.075mm, Thermo Fisher Scientific) onto which 3µl of extracted peptides was automatically loaded. NanoHPLC system delivered solvents A: 0.1% (v/v) formic acid , and B: 99.9% (v/v) acetonitrile, 0.1% (v/v) formic acid at 0.50 µL/min to load the peptides (over a 30 minute period) and 0.3µl/min to elute peptides directly into the nano-electrospray with gradual gradient from 0% (v/v) B to 30% (v/v) B over 78 minutes and concluded with 5 minute fast gradient from 30% (v/v) B to 50% (v/v) B at which time a 5 minute flash-out from 50-95% (v/v) B took place. As peptides eluted from the HPLC-column/electrospray source survey MS scans were acquired in the Orbitrap with a resolution of 120,000 followed by MS2 fragmentation of 20 most intense peptides detected in the MS1 scan from 350 to 1800 m/z; redundancy was limited by dynamic exclusion.

MS data analysis. Raw MS/MS data were converted to mgf file format using MSConvert (ProteoWizard: Open Source Software for Rapid Proteomics Tools Development). Resulting mgf files were used to search against Uniprot *Homo sapiens* proteome database (UP000005640) with a decoy reverse entries and a list of common contaminants (134,183 total entries) using in-house *Mascot* search engine 2.2.07 [Matrix Science] with variable Methionine oxidation, Asparagine or Glutamine deamidation plus Lysine and protein N-terminus acetylation. Peptide mass tolerance

was set at 15 ppm and fragment mass at 0.6 Da. Protein annotations, significance of identification and spectral based quantification was done with Scaffold software (version 4.3.2, Proteome Software Inc., Portland, OR). Protein identifications were accepted if they could be established at greater than 97.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, AI Anal Chem. 2003 Sep 1;75(17):4646-58). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. MS parameters as well as the final output of the MS analysis is reported in **Table S1**. Identified proteins were further analyzed using the KEGG (Kanehisa, 2019; Kanehisa et al., 2020) and STRING (Szklarczyk et al., 2019) platforms.

siRNA Mediated Gene Silencing

Silencer Select siRNAs (siNeg: *Silencer*™ Select Negative Control No. 1, siCALR 1: s115, siCALR 2: s116; siHSPB1: s194537 and s194538) were purchased from Thermo Fisher Scientific. siRNAs were resuspended in RNase-free water to a concentration of 50uM for long term storage at -20°C. siRNAs were diluted to a 2uM working solution for short term use. Lipofectamine 3000 was used for all siRNA transfections per manufacturer's instructions. For a 12-well plate, 15uL of 2uM siRNA was suspended in mixture containing 100uL Opti-MEM (Invitrogen, 31985-070) and 3uL Lipofectamine 3000. In the case of siHSPB1, 7.5uL 2uM siRNA s194537 and siRNA s194538 working solutions were added. HEK293 cells or HEK293 cells stably expressing TetOn-mCherry-eGFP-RAMP4 were plated on 12-well plates and immediately transfected with siRNA. After 24 hours, cells were again transfected with siRNA. After 48 hours, media was changed and cells were transfected a third time with siRNA. The next

day, cells were either harvested via trypsin for Western blot, or were prepared for the EATR assay as described above. DMEM-10 without G418 was used during these experiments in order to minimize toxic effect of gene silencing.

Western blotting

Western blotting was performed on a 4–12% Bis-Tris SDS-PAGE system (NuPAGE, Invitrogen) (Costantini et al., 2006; Jonas et al., 2010; Peng et al., 2014; Peng et al., 2018). The primary antibodies used in this study are listed in **Table S3**). Appropriate secondary antibodies were diluted 1:15000 or 1:20000 in blocking solution at room temperature for 1 hour. Membranes were washed with 1X Tris-Buffered Saline with 0.1% Tween® 20 Detergent (Thermo Fisher Scientific 28360), and protein bands were developed using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences). The original uncropped Western blot images included in the main manuscript can be found in **Figures S4-S7**.

Co-immunoprecipitation

Protein extracts were generated using GTIP buffer (10 mM Tris, pH 7.6, 2 mM EDTA, 0.15 M NaCl) supplemented with 1% Triton X-100 (Roche Applied Science 11332481001), 0.25% Nonidet P-40 (Sigma N-6507), complete protein inhibitor mixture (Roche Applied Science 11836170001), and phosphatase inhibitors (mixture set I and set II; Calbiochem). Extracts were pre-cleared with protein A magnetic beads (Polysciences 84600, Bio-Rad 1614013). For immunoprecipitation, protein A magnetic beads were washed with cold GTIP buffer solution and pre-incubated for 4hr at 4°C with rotation in the presence of the specific antibody. The lysate was then added to washed beads and incubated for 12hr at 4°C.

Elution of protein-coupled beads was carried out in 20-µl LDS sample buffer 4X with heating at 95°C for 5 minutes.

Statistics

Data are expressed as mean ± standard deviation of separate experiments. Data analysis was performed using GraphPad Prism Version 7.0.5. Comparison of the means was performed using Student's *t* test, or one- or two-way ANOVA. Differences were declared statistically significant if $p<0.05$, and the following statistical significance indicators are used: *$p<0.05$; **$p<0.005$; #$p<0.0005$.

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