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Supplemental information

Sestrin2 drives ER-phagy in response to protein misfolding

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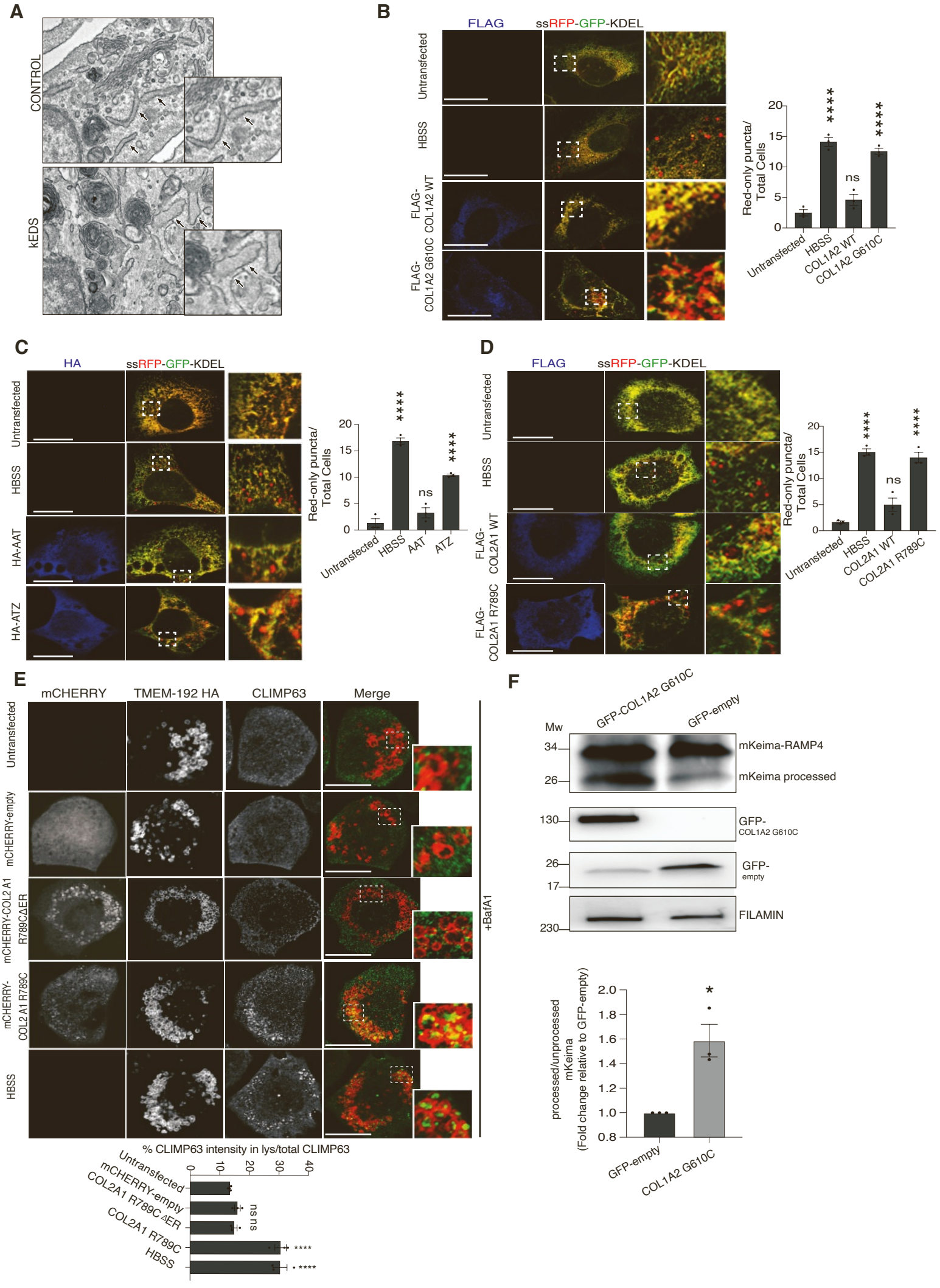
Supplementary Figure 1



Supplementary Figure 1, related to Figure 1. ER storage activates ER-phagy.

A. Representative TEM images of control and kEDS human fibroblasts. Arrows indicate the ER, which is enlarged in kEDS fibroblasts. Scale bar 500 nm.

B. Immunofluorescence staining of U2OS cells expressing FLAG-COL1A2 WT or FLAG-COL1A2 G610C (blue) transfected with the ER-phagy ssRFP-GFP-KDEL reporter. HBSS treatment was used as a positive control. Scale bar 5 μm. Bar graph showing the ratio of red-only puncta/total cells in untransfected, HBSS-treated, and COL1A2 WT or COL1A2 G610C transfected cells. Mean ± standard error of mean (SEM) of $N = 3$ independent experiments. $n = 18$ cells. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: ****$p < 0.0001$; ns ≥ 0.05.

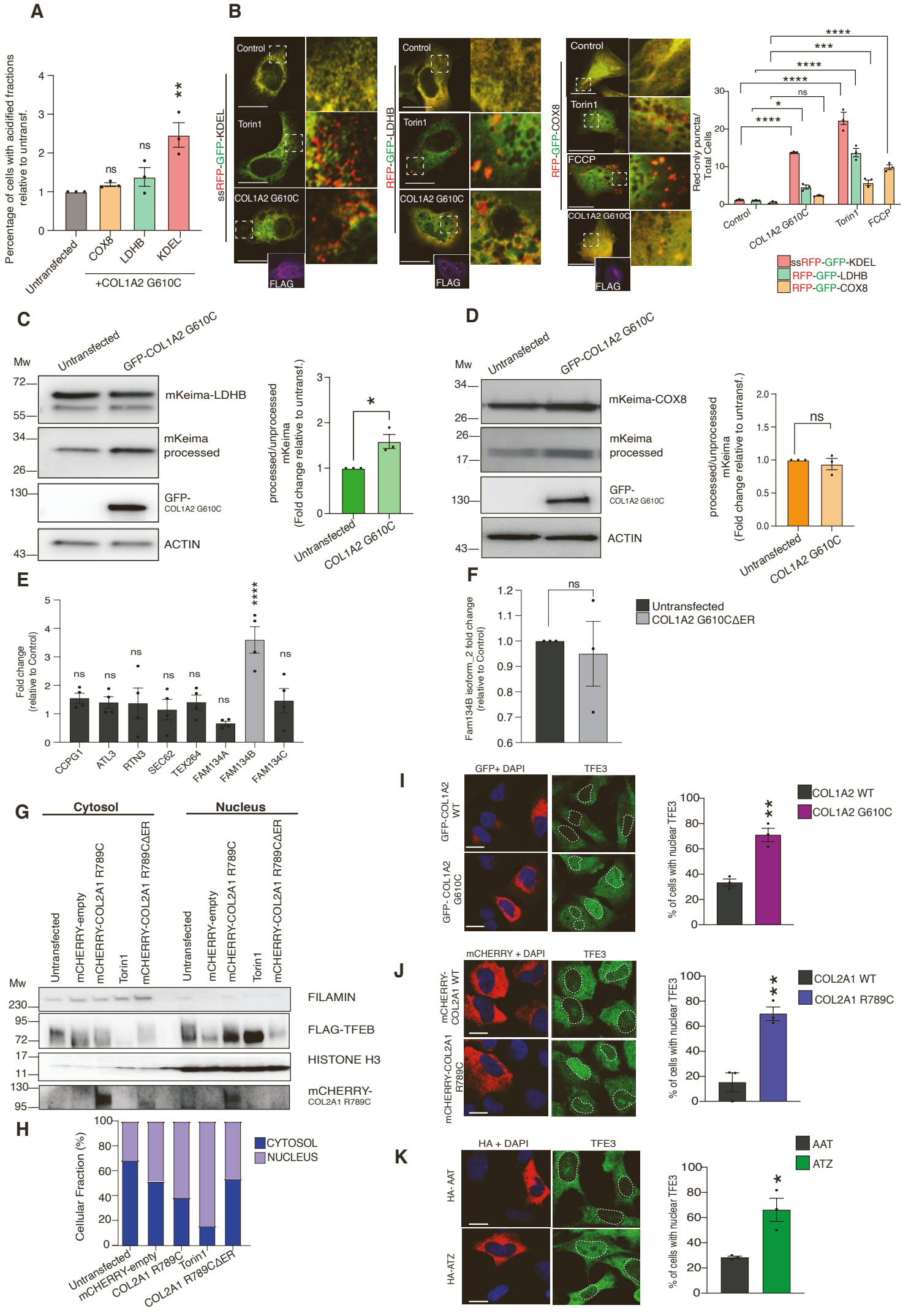
C. Immunofluorescence staining of MEFs expressing HA-Alpha-1 antitrypsin (AAT) and the mutant form (ATZ) (blue) transfected with the ER-phagy ssRFP-GFP-KDEL reporter. HBSS treatment was used as a positive control. Scale bar 5 μm. Bar graph showing the ratio of red-only puncta/total cells in untransfected, HBSS-treated, and AAT- or ATZ-expressing cells. Mean ± standard error of mean (SEM) of $N = 3$ independent experiments. $n = 18$ cells. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: ****$p < 0.0001$; ns ≥ 0.05.

D. Immunofluorescence staining of RCS cells expressing FLAG-COL2A1 WT or FLAG-COL2A1 R789C (blue) transfected with the ER-phagy ssRFP-GFP-KDEL reporter. HBSS treatment was used as a positive control. Scale bar 5 μm. Bar graph showing the ratio of red-only puncta/total cells in untransfected, HBSS-treated, and COL2A1 WT or COL2A1 R789C expressing cells. Mean ± standard error of mean (SEM) of $N = 3$ biological replicates. $n = 18$ cells. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: ****$p < 0.0001$; ns ≥ 0.05.

E. Immunofluorescence staining of CLIMP63 (green) and lysosomes (TMEM192-HA, red) in RCS cells transfected with mCHERRY-empty, mCHERRY-COL2A1 R789CΔER, or mCHERRY-COL2A1 R789C. HBSS was used as positive control for ER-phagy induction. Cells were treated with BafA1 (100 nM, 4 hours). Scale bar 5 μm. Insets show magnification of CLIMP63 accumulation in lysosomes. Bar graph showing quantification (%) of CLIMP63 fluorescence intensity in lysosomes. Mean ± standard error of mean (SEM) of $N = 3$ biological replicates. $n = 30$ cells. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: **** $p < 0.0001$; ns ≥ 0.05.

F. Representative Western blot analysis in RCS cells expressing mKeima-RAMP4 transfected with GFP-COL1A2 G610C or GFP-empty plasmid as control. FILAMIN was used as a loading control. Bar graph showing quantification of mKeima processed/mKeima-RAMP4 ratio in COL1A2 G610C transfected cells relative to GFP-empty. Mean ± standard error of mean (SEM) of $N = 3$ biological replicates. Student's unpaired t test: *$p < 0.05$.

Supplementary Figure 2



Supplementary Figure 2, related to Figure 2. ER storage induces TFE3/TFEB nuclear translocation and transcriptional induction of *FAM134B*.

A. Fluorescence-activated cell sorting (FACS) analysis in U2OS cells co-expressing tandem reporters of ER-phagy (ssRFP-GFP-KDEL), bulk autophagy (RFP-GFP-LDHB) or mitophagy (RFP-GFP-COX8) and FLAG-COL1A2 G610C plasmid. Quantification of red fluorescence shift relative to untransfected cells (control). Mean ± standard error of mean (SEM) of $N = 3$ independent experiments. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: $^{**}p < 0.005$; ns ≥ 0.05.

B. Representative immunofluorescence analysis of U2OS cells co-expressing tandem reporters of ER-phagy (ssRFP-GFP-KDEL), bulk autophagy (RFP-GFP-LDHB) or mitophagy (RFP-GFP-COX8). Cells were left untreated (control) or treated with Torin1 (500nM, 12 hours) or FCCP (1μM, 12 hours), or transfected with FLAG-COL1A2 G610C (insets). Insets show magnification of the boxed areas. Scale bar 5 μm. Bar graph showing the ratio of red-only puncta/total cells. Mean ± standard error of mean (SEM) of $N = 3$ independent experiments. n= 15 cells were counted. One-way analysis of variance (ANOVA) with Sidak's multiple comparisons test: $^{*}p < 0.05$; $^{****}p < 0.0001$; ns ≥ 0.05.

C. – D. Western blot analysis of U2OS cells co-expressing (C) mKeima-LDHB or (D) mKeima-COX8 reporter proteins and GFP-COL1A2 G610C. Cell lysates were probed with the indicated antibodies. Actin was used as a loading control. Bar graphs (right) showing quantification of mKeima processed/mKeima-LDHB and mKeima processed/mKeima-COX8 ratio relative to untransfected cells. Mean ± standard error of mean (SEM) of $N = 3$ biological replicates. Student's unpaired t test: $^{*}p < 0.05$; ns ≥ 0.05

E. q PCR analysis of ER - phagy receptors in RCS cells expressing mutant COL2A1 R789C. Values are normalized to *HPRT* gene expression and are expressed as fold change values relative to untransfected cells (control). Mean ± standard error of mean (SEM) of $N = 4$ biological replicates. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: $^{****}p < 0.0001$; ns ≥ 0.05.

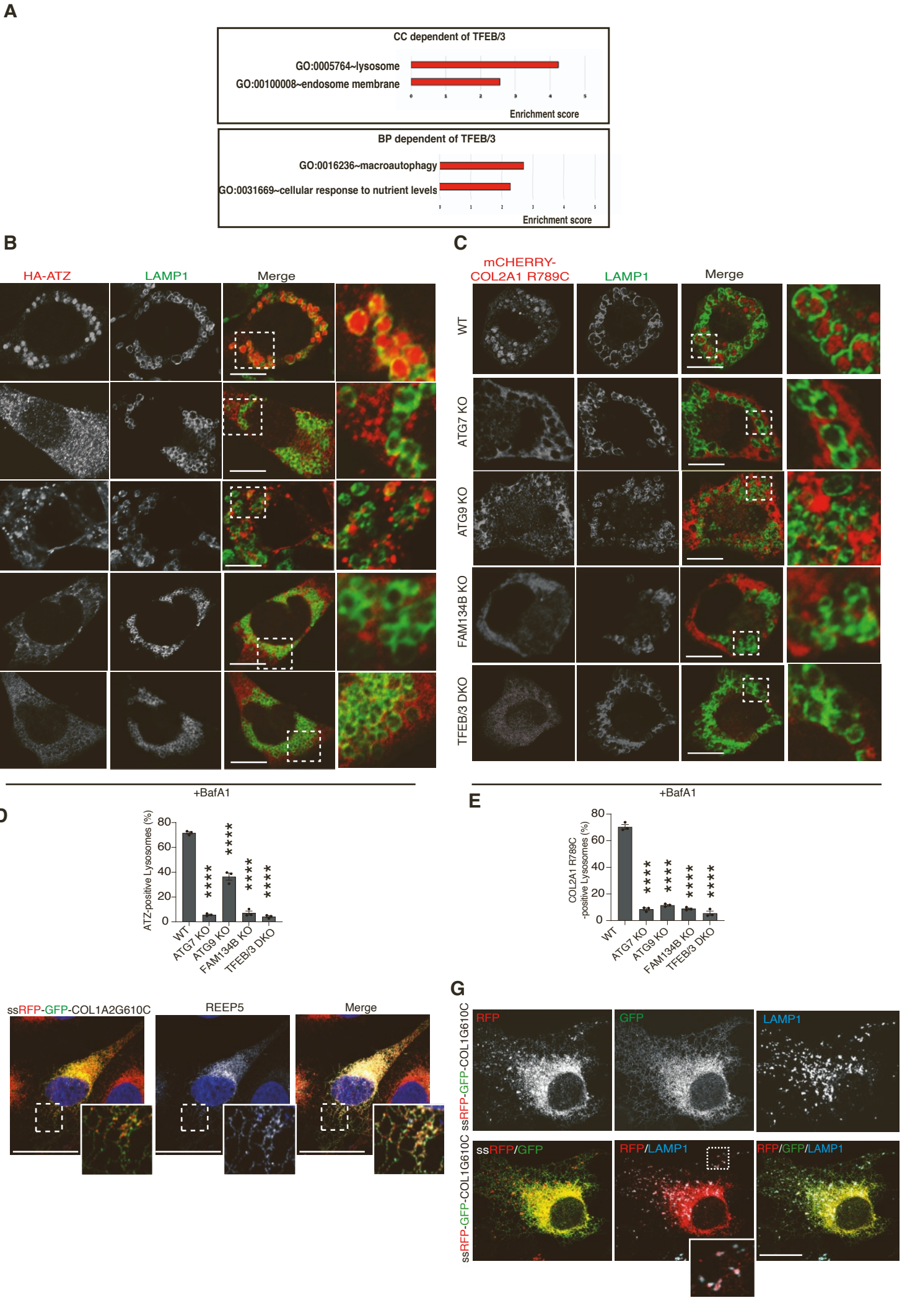
F. qPCR analysis of the ER - phagy receptor *FAM134B isoform 2* in untransfected and U2OS cells expressing mutant COL1A2 G610CΔER. Values are normalized to *HPRT* gene expression and are expressed as fold change values relative to untransfected cells (control). Mean ± standard error of mean (SEM) of $N = 3$ biological replicates. Student's unpaired t test: ns ≥ 0.05

G. Western blot analysis of nuclear and cytosolic fractions from RCS FLAG-TFEB-expressing cells transfected with mCHERRY-empty, mCHERRY-COL2A1 R789C, or mCHERRY-COL2A1 R789CΔER. Torin1 was used as a positive control. FILAMIN and histone H3 were used as a loading control of cytosolic and nuclear fractions, respectively.

H. Quantification of TFEB localization (in G) expressed as % of cytosolic and nuclear fractions relative to the total. Mean of $N = 4$ independent experiments.

I. – K. Immunofluorescence analysis showing the subcellular localization of TFE3 (green) in U2OS cells transfected with (I) GFP-COL1A2 WT or GFP-COL1A2 G610C (red), (J) RCS cells transfected with mCHERRY-COL2A1 WT or mutant mCHERRY-COL2A1 R789C (red), and (K) MEF cells transfected with HA-AAT or HA-ATZ (red). The dotted circle indicates nuclei. Scale bar 8 μm. Bar graphs on right show the quantification (expressed as %) of cells with nuclear TFE3 in I, J, K, respectively. Mean ± standard error of mean (SEM) of $N = 3$ independent experiments. $n = 56$ COL1A2 WT and COL1A2 G610C cells; $n = 55$ COL2A1 WT and COL2A1 R789C; $n = 27$ AAT and ATZ cells. Student's unpaired t test: $^{*}p < 0.05$; $^{**}p < 0.005$.

Supplementary Figure 3



Supplementary Figure 3, related to Figure 3. ER storage activates ER-phagy via TFE3/TFEB mediated transcriptional induction of *FAM134B*.

A. Cellular Compartments (CC) and biological processes (BP) for genes regulated by COL2A1 R789C via TFEB and TFE3 identified by transcriptomic analysis performed in WT and TFEB/3 DKO RCS cells expressing or not the COL2A1 R789C mutant protein (see Supplementary Table 2).

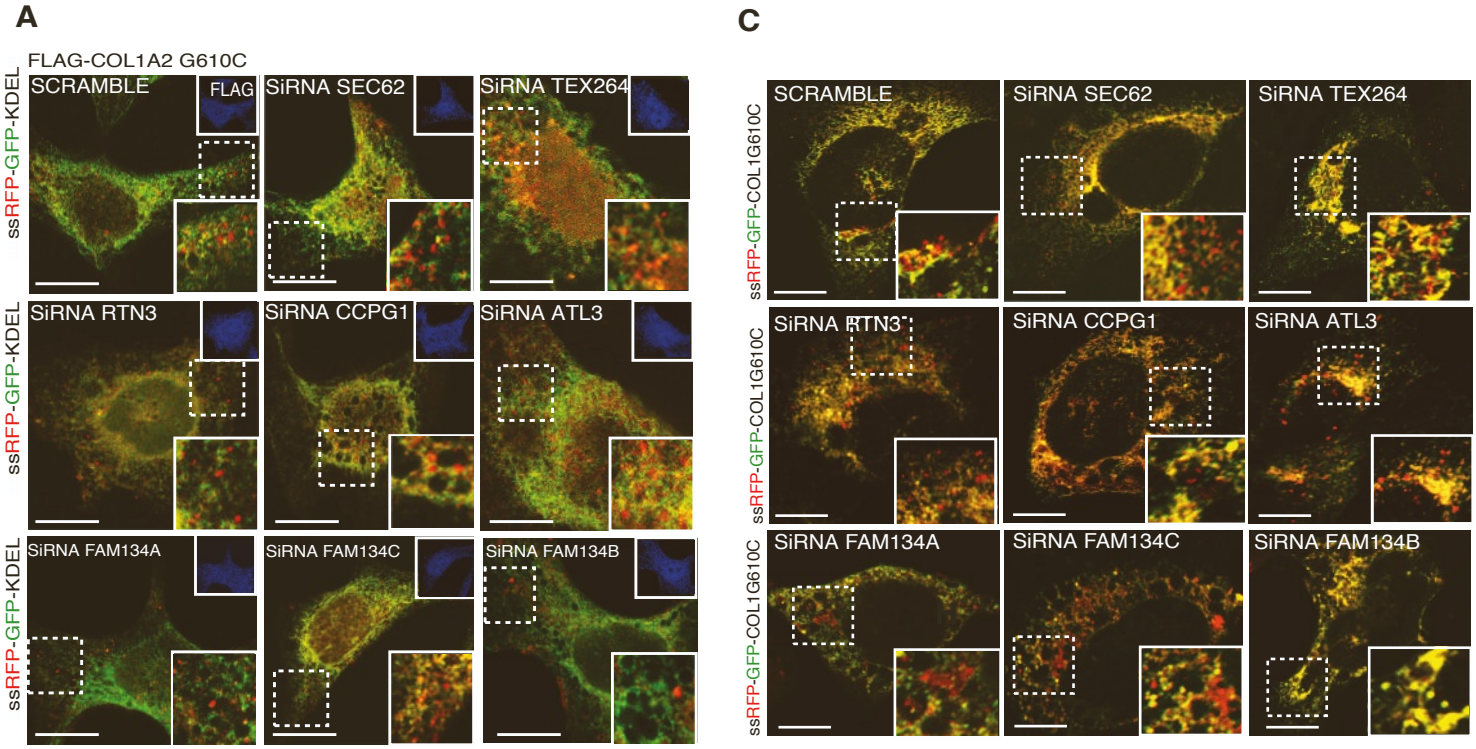
B.- C. Immunofluorescence analysis of intracellular localization of mutant (B) HA-ATZ (red) and (C) mCHERRY-COL2A1 R789C (red) in LAMP1-positive lysosomes (green) in MEFs and RCS cells, respectively, with the indicated genotypes. Cells were treated with BafA1 (50 nM for 12 hours). Scale bar 5 μm. Insets show magnification of boxed area.

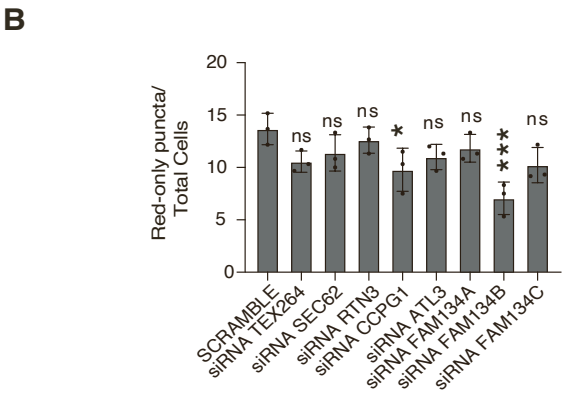
D.-E. Bar graphs showing quantification of (D) HA-ATZ-positive and (E) mCHERRY-COL2A1 R789C-positive lysosomes (expressed in %). Mean ± standard error of mean (SEM) of N = 3 independent experiments. n = 15 cells. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: ****$p < 0.0001$.

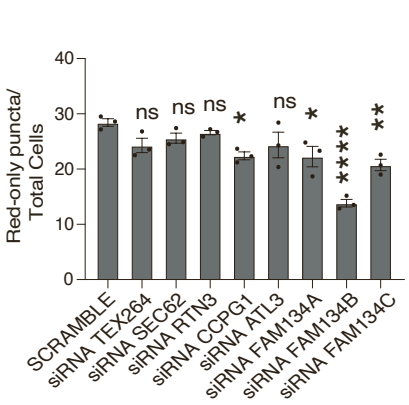
F. Immunofluorescence staining of U2OS cells stably expressing tandem ssRFP-GFP-COL1A2 G610C and the ER protein REEP5 (grey). Scale bar 10 μm.

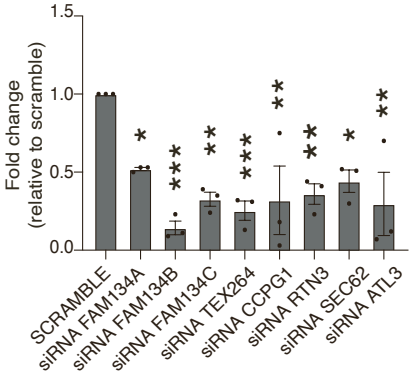
G. Immunofluorescence staining of U2OS cells stably expressing tandem ssRFP-GFP-COL1A2 G610C and the lysosomal protein LAMP1 (grey). Scale bar 10 μm.

Supplementary Figure 4





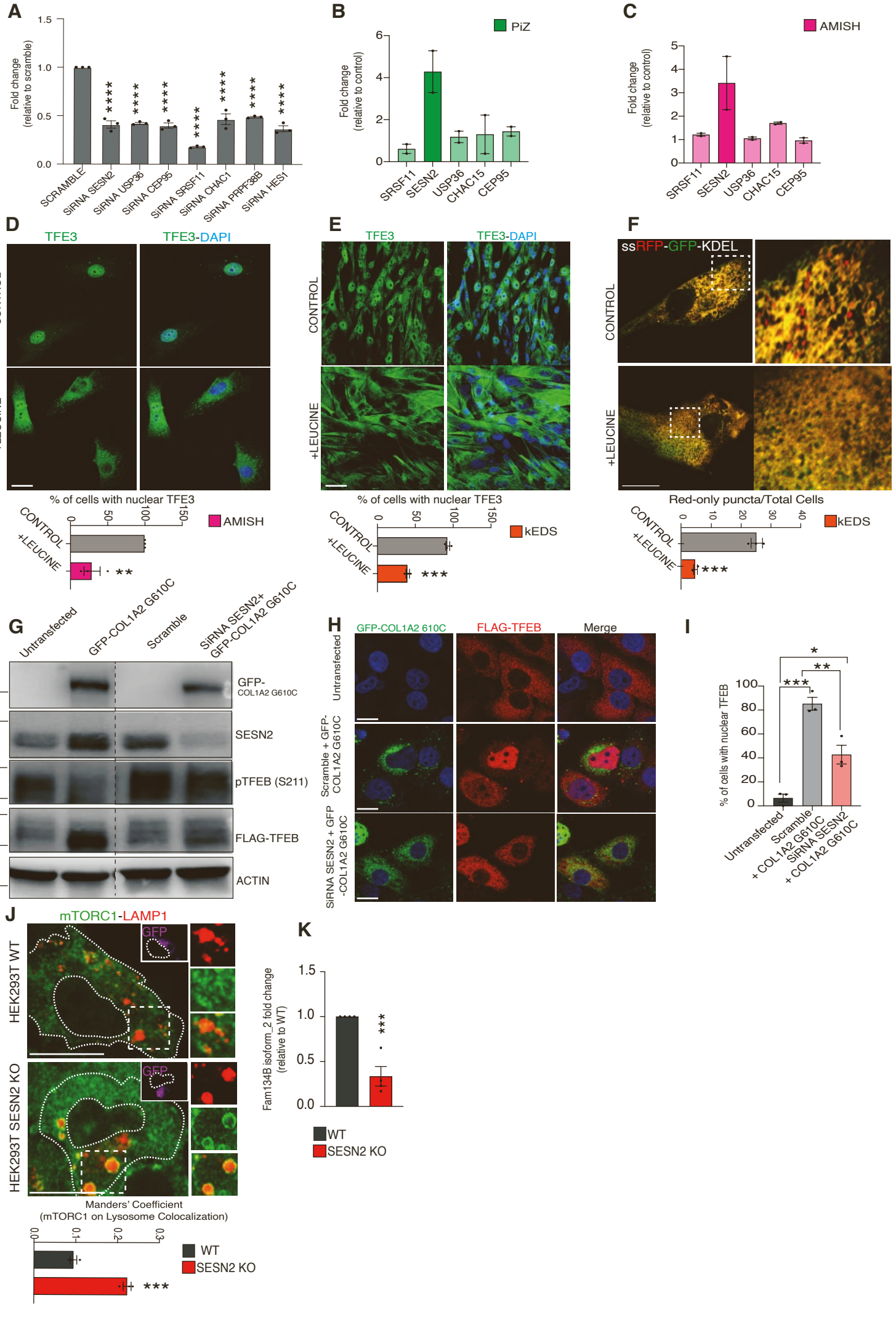




Supplementary Figure 4, related to Figure 4. FAM134B regulates ER-phagy in response to protein misfolding.

A. Representative immunofluorescence of FLAG-COL1A2 G610C plasmid (blue insets) in U2OS cells overexpressing ssRFP-GFP-KDEL reporter transfected with scramble and with the indicated SiRNAs. Scale bar 5 μm. Insets show magnification of boxed area.
B. Bar graph showing the ratio of red-only puncta/total cells in A. Mean ± standard error of mean (SEM) of N = 3 independent experiments. n = 18 cells. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: *$p < 0.05$; ***$p < 0.0005$; ns ≥ 0.05.
C. Fluorescence microscopy analysis of the ssRFP-GFP-COL1A2 G610C reporter in U2OS cells transfected with scramble or with the indicated SiRNAs. Scale bar 5 μm. Insets show magnification of boxed area.
D. Bar graph showing the ratio of red-only puncta/total cells in C. Mean ± standard error of mean (SEM) of N = 3 independent experiments. n = 20 cells. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: *$p < 0.05$; **$p < 0.005$; ****p < 0.0001; ns ≥ 0.05.
E. Silencing efficiency of ER-phagy receptors genes. qPCR analysis of the expression level of the indicated genes in U2OS cells silenced with the corresponding SiRNA. Values are normalized to *HPRT* gene expression and are expressed as fold change values relative to scramble (control). Mean ± standard error of mean (SEM) of $N = 3$ biological replicates. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.

Supplementary Figure 5



Supplementary Figure 5, related to Figure 4 and 5. ER storage downregulates the mTORC1 pathway and induces upregulation of the leucine-sensor Sestrin2.

A. qPCR analysis showing the efficacy of SiRNA knockdown on the expression of genes identified by HCS whose silencing reverts TFEB nuclear localization (as shown in Figure 4B). Values are normalized to *HPRT* and *CYC* gene expression and are expressed as fold change relative to scramble (control). Mean ± standard error of mean (SEM) of $N = 3$ biological replicates. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: $^{****}p < 0.0001$.

B.- C. qPCR analysis of the expression indicated genes in primary PiZ hepatocytes (B) and primary AMISH osteoblasts (C). Values are normalized to *HPRT* and *CYC* gene expression and are expressed as fold change relative to their corresponding controls. Mean ± standard error of mean (SEM) of $N = 2$ biological replicates.

D. Immunofluorescence analysis of TFE3 (green) subcellular localization in Amish osteoblasts untreated (control) or treated with leucine (4 mM, 3 hours). Cells were counterstained with DAPI. Scale bar 20 µm. Bar graph showing % cells with nuclear TFE3 in cells treated with and without leucine. Mean ± standard error of mean (SEM) of $N = 3$ independent experiments. $n = 58$ osteoblasts. Student's unpaired t test: $^{**}p < 0.005$.

E. Immunofluorescence analysis of TFE3 (green) subcellular localization in kEDS fibroblasts untreated (control) or treated with leucine (4 mM, 3 hours). Cells were counterstained with DAPI. Scale bar 20 µm. Bar graph showing % of cells with nuclear TFE3 in cells treated with and without leucine. Mean ± standard error of mean (SEM) of $N = 3$ independent experiments. $n = 216$ fibroblasts. Student's unpaired t test: $^{***}p < 0.0005$.

F. Representative fluorescence microscopy analysis of kEDS fibroblasts transfected with the ssRFP-GFP-KDEL plasmid and treated with leucine (4mM, 3 hours). Scale bar 10 µm. Bar graph showing the ratio of red-only puncta/total cells. Mean ± standard error of mean (SEM) of $N = 3$ biological replicates. $n = 23$ fibroblasts. Student's unpaired t test: $^{***}p < 0.0005$.

G. Western blot analysis of HeLa FLAG-TFEB cells transfected with GFP-COL1A2 G610C and with SiRNA scramble or SiRNA SESN2 as indicated. Cell lysates were probed with the indicated antibodies. Actin was used as a loading control. Representative images of $N = 3$ independent experiments. The dotted line indicates removal of unnecessary lanes.

H. Representative immunofluorescence images of FLAG-TFEB (red) localization in HeLa cells with or without GFP-COL1A2 G610C treated with SiRNA scramble or SiRNA SESN2 as indicated. Cells were counterstained with DAPI. Scale bar 15 µm.

I. Bar graph showing quantification of nuclear TFE3 localization of cells in (H). Mean ± standard error of mean (SEM) of $N = 3$ independent experiments. $n = 45$ cells. One-way analysis of variance (ANOVA) with Šídák's multiple comparisons test: $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$.

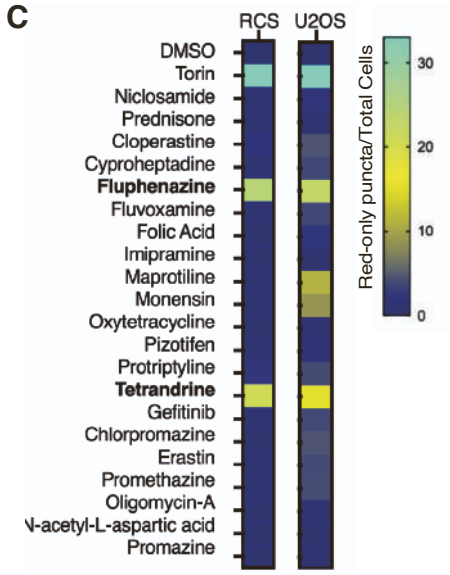
J. Co-immunofluorescence of mTORC1 (green) and lysosomes (LAMP1, red) in HEK293T WT and SESN2 KO cells expressing GFP-COL1A2 G610C (insets violet). The dotted lines delimit cellular and nuclear area. Scale bar 10 µm. Bar graphs showing quantification of the co-localization of mTORC1-Lamp1 (expressed as Manders' Coefficient). Mean ± standard error of mean (SEM) of $N = 3$ independent experiments. $n = 26$ cells. Student's unpaired t test: $^{***}P < 0.0005$.

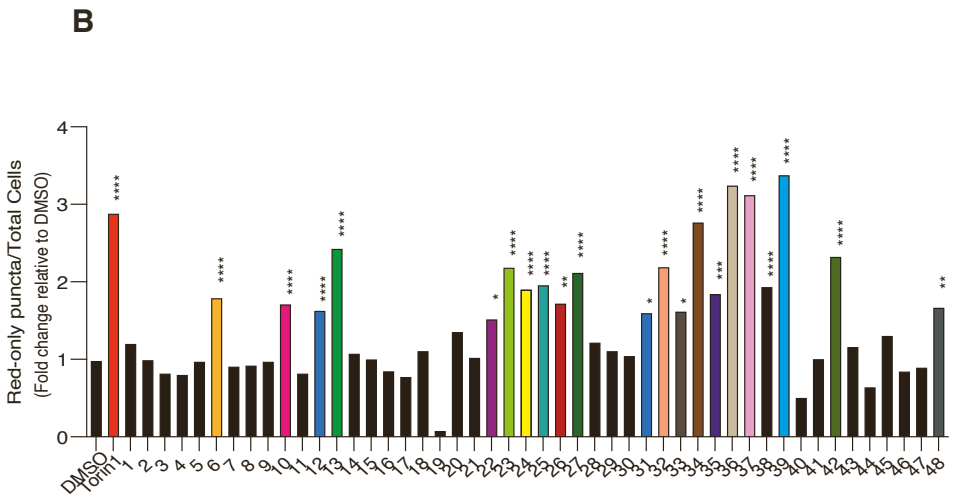
K. qPCR analysis of *FAM134B isoform2* in WT and SESN2 KO HEK283T cells expressing COL1A2 G610C. Values were normalized to *HPRT* gene and are expressed as fold change values relative to WT. Mean ± standard error of mean (SEM) of $N = 4$ biological replicates. Student's unpaired t test: $^{***}p < 0.0005$.

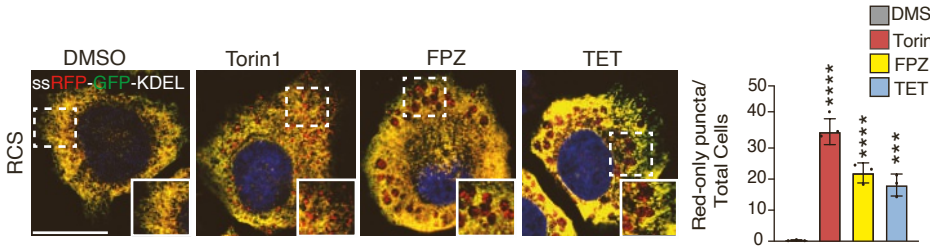
Supplementary Figure 6

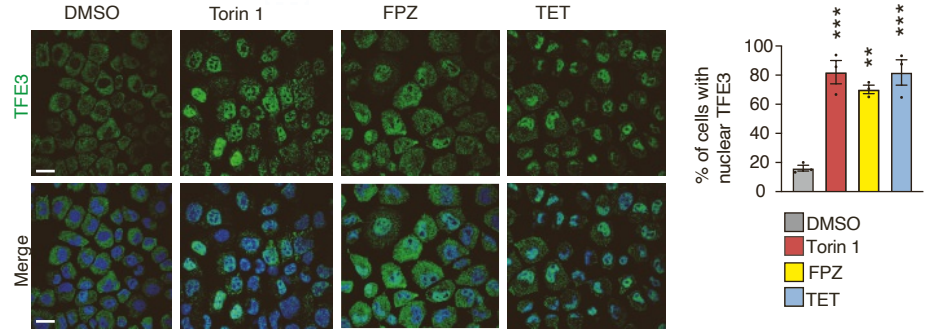
A

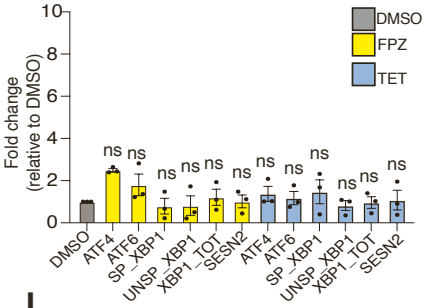
#	Drug name	#	Drug name
1	Azacyclonol	25	Fluvoxamine
2	Carbarsone	26	Folic acid
3	Clofibrate	27	Imipramine
4	Corticosterone	28	Isosorbide
5	Dirithromycin	29	Lactobionic acid
6	Gefitinib	30	Lithocholic acid
7	Imidurea	31	Maprotiline
8	Isoconazole	32	Monensin
9	Mifepristone	33	N-acetyl-L-aspartic acid
10	Niclosamide	34	Oligomycin A
11	Practolol	35	Oxytetracycline
12	Prednisone	36	Pizotifen
13	Rapamycin (Sirolimus)	37	Promazine
14	Ribavirin	38	Protriptyline
15	Rifabutin	39	Tetrandrine
16	Suloctidil	40	Thioridazine
17	Topiramate	41	(-)-MK-801 1
18	Ajmaline	42	Chlorpromazine
19	Alexidine	43	clomifene
20	Beclometasone	44	Colecalciferol
21	Brompheniramine	45	Erastin
22	Cloperastine	46	Latamoxef
23	Cyproheptadine	47	Meptazinol HCL
24	Fluphenazine	48	Promethazine

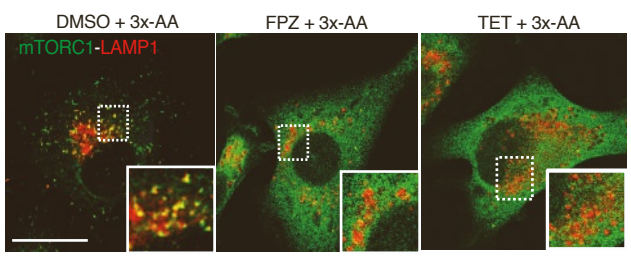


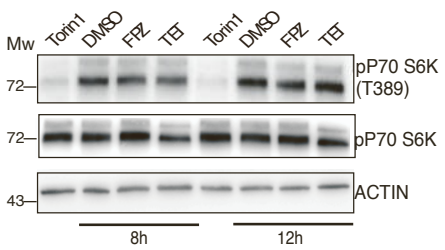


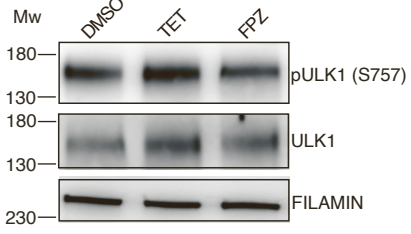


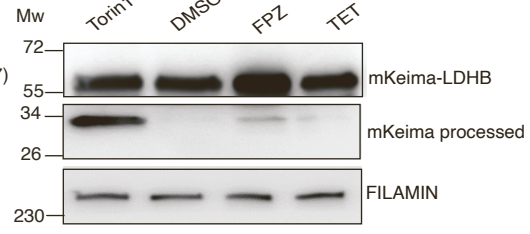


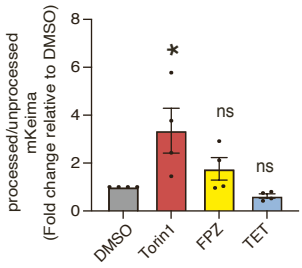


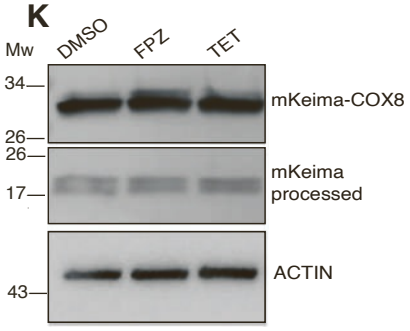


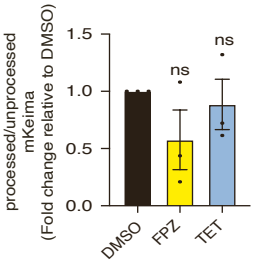


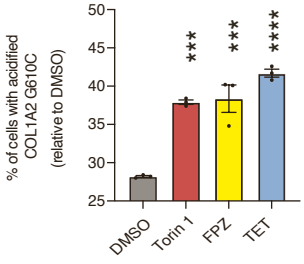












Supplementary Figure 6, related to Figure 7. Pharmacological modulation of ER-phagy as a therapy for ER-storage disorders.

A. List of tested drugs among those identified by the Gene2Drugs tool as *FAM134B* transcriptional inducers.

B. High content screening results of the 48 drugs (shown in A) in A549 cells expressing the ER-phagy reporter ssRFP-GFP-KDEL. The bar graph showing the ratio of red-only puncta/total cells number (expressed as fold increase relative to DMSO). All drugs were tested at 10 μM, 12 hours. Torin 1 (500 nM for 12 hours) was used as a positive control. Mean ± standard error of mean (SEM) of $N = 4$ biological replicates. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; ****$p < 0.0001$.

C. Heat map showing the number of red-only puncta as a measure of ER-phagy induction for the selected drugs, assessed in RCS and U2OS cells stably expressing the tandem ssGFP-RFP-KDEL reporter. Mean ± standard error of mean (SEM) of $N = 18$ and $N = 11$ biological replicates in U2OS and RCS cells, respectively.

D. Representative fluorescence microscopy analysis of RCS cells stably expressing the ssRFP-GFP-KDEL reporter and treated with Fluphenazine (FPZ) and Tetrandrine (TET) (10 μM, 12 hours). Torin1 (500 nM for 12 hours) and DMSO were used as positive control and vehicle, respectively. Scale bar 10 μm. Bar graph showing the ratio of red-only puncta/total cells. Mean ± standard error of mean (SEM) of $N = 3$ independent experiments. $n = 18$ cells. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: ***$p < 0.0005$; ****$p < 0.0001$.

E. TFE3 (green) subcellular localization in RCS cells treated with FPZ and TET (10 μM, 12 hours). Scale bar 30 μm. Quantitative analysis of nuclear TFE3 (expressed as %) in RCS cells treated with FPZ and TET compared to DMSO. Torin 1 (500 nM for 12 hours) was used as a positive control. Mean ± standard error of mean (SEM) of $N = 3$ independent experiments. $n = 40$ cells. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: ** $p < 0.005$; ***$p < 0.0005$.

F. qPCR analysis of the indicated genes in U2OS cells treated with Fluphenazine (FPZ) and Tetrandrine (TET) (10 μM, 12 hours). Values are normalized to *CYC* gene expression and are expressed as fold change values relative to DMSO. Mean ± standard error of mean (SEM) of $N = 3$ biological replicates. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: ns ≥ 0.05.

G. Representative co-immunofluorescence staining of mTORC1 (green) and lysosomes (LAMP1, red) in U2OS cells treated with DMSO, Fluphenazine (FPZ) and Tetrandrine (TET) (10 μM, 12 hours) and 3x-AA for the final 30 minutes. Scale bar 10 μm. Insets show magnification of mTORC1 localization in lysosomes.

H. Representative western blot analysis of phosho-P70S6K (T389) in U2OS cells treated with Fluphenazine (FPZ) and Tetrandrine (TET) (10 μM, 8 hours and 12 hours). Torin1 (500 nM for 12 hours) and DMSO were used as positive control and vehicle, respectively. Actin was used as a loading control.

I. Representative Western blot analysis of phosho-ULK1 (S757) in U2OS cells treated with Fluphenazine (FPZ) and Tetrandrine (TET) (10 μM, 12 hours) compared to DMSO. FILAMIN was used as a loading control. Representative images of $N = 3$ independent experiments.

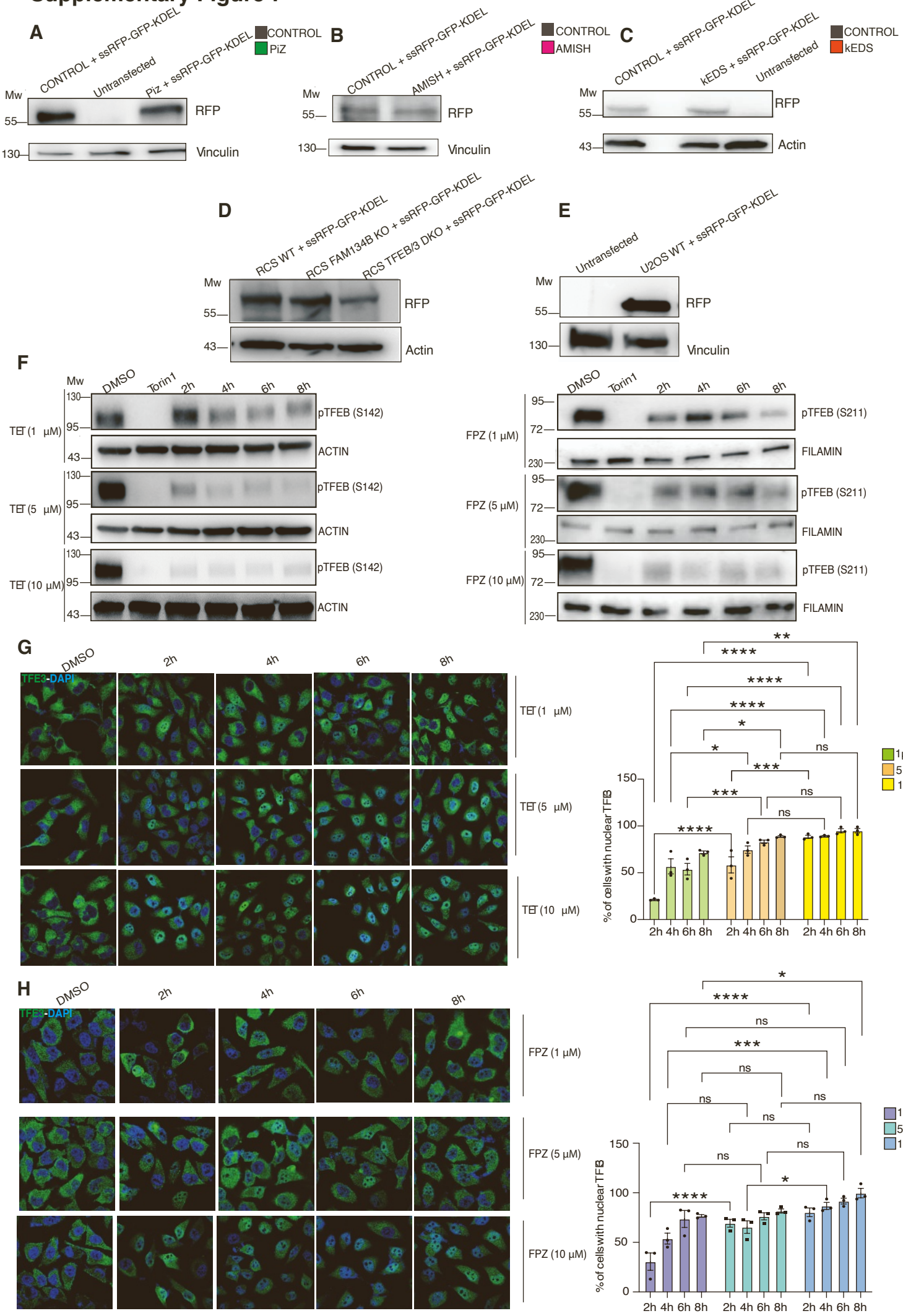
J. Western blot analysis of U2OS cells expressing the mKeima-LDHB reporter protein treated with Fluphenazine (FPZ) and Tetrandrine (TET) (10 μM, 12 hours). Torin1 (500 nM for 12 hours) and DMSO were used as positive control and vehicle, respectively. Cell

lysates were probed with the indicated antibodies. FILAMIN was used as a loading control. The bar graph (left) shows quantification of the mKeima processed/mKeima-LDHB ratio relative to DMSO. Mean ± standard error of mean (SEM) of N = 4 biological replicates. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: *p< 0.05; ns ≥ 0.05.

K. Western blot analysis of U2OS cells expressing the mKeima-COX8 reporter protein treated with Fluphenazine (FPZ) and Tetrandrine (TET) (10 μM, 12 hours). DMSO was used as vehicle. Cell lysates were probed with the indicated antibodies. Actin was used as a loading control. The bar graph (left) shows quantification of the mKeima processed/mKeima-COX8 ratio relative to DMSO. Mean ± standard error of mean (SEM) of *N = 3* biological replicates. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: ns ≥ 0.05.

L. Fluorescence-activated cell sorting (FACS) analysis in U2OS cells stably expressing tandem ssRFP-GFP-COL1A2 G610C upon Torin1, FPZ and TET treatments. Quantification of red fluorescence shift relative to DMSO. Mean ± standard error of mean (SEM) of *N* = 3 independent experiments. One-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test: ***$p < 0.0005$; ****$p < 0.0001$.

Supplementary Figure 7



Supplementary Figure 7, related to the STAR Methods. Validation of ssRFP-GFP-KDEL expressing cells and dose-time dependency of FPZ and TET treatments.

A. – C. Representative Western blot analysis illustrating the expression levels of RFP proteins in primary cells lines (A, PiZ hepatocytes; B, AMISH osteoblasts; C, kEDS fibroblasts) transiently transfected with the ssRFP-GFP-KDEL.

D. – E. Representative Western blot analysis showing the RFP protein in cell lines stably expressing the ssRFP-GFP-KDEL; (D) WT, FAM134B KO and TFEB/3 DKO cells RCS cells; (E) U2OS WT cells.

F. Western blot analysis of phospho-TFEB (S211 and S142) in U2OS treated with Tetrandrine (TET) (left) and Fluphenazine (FPZ) (right) for the indicated time points and concentrations. Torin1 (500 nM for 12 hours) and DMSO were used as positive control and vehicle, respectively. Representative images of $N = 3$ independent experiments. FILAMIN and Actin were used as a loading control.

G.-H. TFE3 (green) subcellular localization in U2OS cells treated with Tetrandrine (TET) (G) and Fluphenazine (FPZ) (H) at the indicated time points and concentrations. Scale bar 30 µm. Bar graph showing quantitative analysis of nuclear TFE3 in cells treated with TET and FPZ expressed as % relative to DMSO. Cells were counterstained with DAPI. Mean ± standard error of mean (SEM) of $N = 3$ independent experiments. $n = 150$ cells. Two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test: $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$; $****p < 0.0001$; ns ≥ 0.05.