Supplementary Materials for

Control of lysosomal-mediated cell death by the pH-dependent calcium channel RECS1

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Published 12 November 2021, Sci. Adv. 7, eabe5469 (2021)
DOI: 10.1126/sciadv.abe5469

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  Movie S1
SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Reagents. Tunicamycin was purchased from Calbiochem EMB Bioscience. Cell culture media, fetal calf serum and antibiotics were obtained from Life Technologies. Fluorescent probes and secondary antibodies coupled to fluorescent markers were purchased from Molecular Probes, Invitrogen. All other reagents used were from Sigma or of the highest grade available.

Cell culture, cell lines and DNA constructs. All MEF, HeLa and HEK cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, non-essential amino acids and grown at 37 °C and 5% CO₂. Full length wild-type (clone 3899632, Dharmacon) or D295Q mutant human RECS1 constructs were cloned into the Dox-inducible vector pRetrox-TRE3G (631188, Clonetech) in frame with an N-terminal Flag-StrepII fusion tag. Full length wild-type or C-terminal deletion mutants (∆868 and ∆910) human RECS1 constructs were cloned into pMSCV (24828, addgene) in frame with an N-terminal MYC fusion tag. The production of amphotropic retroviruses using the HEK293 cell line and transduction of target MEF and HeLa cells were performed according to standard protocols (61). Cells with stable expression of shRNA directed against the endogenous mouse Recs1 (shRECS1) or a Luciferase gene (Broad Institute) as control (shLuc) were generated as previously described (62).

RNA isolation, RT-PCR and real time PCR. Total RNA was extracted using the TRizol reagent (ThermoFischer Scientific) following the manufacturer’s instructions. The analysis of transcription targets was performed by real-time PCR using the following primers: Recs1 (mouse): 5’-GGCTTTGTGACAGGCACTATT-3’ and 5’-CTATGCTTTGTGACAATTCCGGT-3’; RECS1 (human): 5’-CCTACCTGATCCTTGCCTGC-3’ and 5’-TACTGGAAATGGTGCCCGTC-3’; Bi-1: 5’-GAAGCTCCGGAGAGGTGGGCT-3’ and 5’-ATTGCAGGCTGTTGAGGCT-3’; Bcl-2: 5’-GCTGAGCAGGGTCTTCAGAG-3’
and 5'- AGTACCTGAACGGCATCTG-3'; *Puma*: 5'-ATGGCGGACGACCTCAAC-3' and 5'- AGTCCCCATGAAGAGATTGTACATGAC-3'; *Bip/Grp78*: 5'- TCATCGGACGCACTTGGAA-3' and 5'-CAACCACCTTGAATGGCAAGA-3'; *Chop*: '5'- TGGAGAGCGAGGGCTTTG-3' and 5'-GTCCCTAGCTTGGCTGACACA-3'; *β-ACTIN* (human): 5'- GCGAGAAGATGACCCAGATC-3' and 5'- CCAGTGGTACGGCCAGAGG-3'.

**Western blot.** Cells were collected and homogenized in RIPA buffer (20 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% Triton X-100) containing a protease inhibitor cocktail (Roche) in presence of 50 mM NaF and 1 mM Na3VO4. Protein concentrations were determined in all experiments by micro-BCA assay (Pierce), and 20-40 μg of total protein was loaded onto 8–12 % SDS–PAGE minigels (Bio-Rad Laboratories) before transfer onto polyvinylidene fluoride membranes. Membranes were blocked using PBS, 0.1% Tween20 (PBST) containing 5% milk for 60 min at room temperature then probed overnight with primary antibodies. The following antibodies diluted in blocking solution were used: anti-HSP90 (1:5000, sc13119, Santa-Cruz); anti-Flag (1:3000, F7425, Sigma Aldrich); anti-TMBIM1 (1:1000, MBS1499661, MyoBioSource); anti-BAX (1:1000, 06-499, EMD Millipore); anti-MYC (1:3000, ab9106, Abcam); anti-PERK (1:1000, 3192, Cell Signaling); anti-ATF4 (C-20) (1:1000, sc-200, Santa Cruz); anti-Bip (1:1000, ab21685, Abcam); anti-CHOP (1:1000, ab11419, Abcam); and anti-actin (1:10000, sc-8432, Santa-Cruz). Bound antibodies were detected using peroxidase-coupled secondary antibodies and the enhanced chemiluminescence (ECL) system.

**Cell death assays.** Cell death was monitored by propidium iodide (PI) and Annexin V co-staining followed by flow cytometry using FACScan and FACSCalibur systems (BD Biosciences). Cell death kinetics were determined by either staining the cells with the cell-impermeable DNA-binding dye SYTOX™ green (S7020, ThermoFischer) or co-staining them with propidium iodide and SYTO™ 16 (S7578, ThermoFischer) followed by imaging using a one color FLR IncuCyte® or a two color ZOOM™ IncuCyte (Essen Biosciences) respectively. Clonogenic survival assay was performed as previously reported (63).

**Cell death screening.** For cell screening, MEF 3G wild-type (WT) cells carrying a doxycycline-inducible Flag-tagged version of the human RECS1 (Flag-RECS1) were
seeded into µclear imaging plates (Greiner Bioone, Kremsmünster, Austria) and let to adapt for 24 h. RECS1 expression was induced by treatment with doxycycline at 1 g/mL for 16h. The cells were treated in supplemented DMEM with the following compounds: mitoxantrone dihydrochloride (MTX; #M6545, Merck-Millipore, 1 µM and 3 µM), doxycycline hydrochloride (#D3447, Merck-Millipore, 50 µM), chloroquine (#C6628, Merck-Millipore, 50 µM), hydroxychloroquine sulfate (#1327000-200MG, Merck-Millipore, final 50 µM), Leu-Leu-Ome (#CH6371378738, Merck-Millipore, 1 mM), cisplatin (CDDP, #C2210000, Merck-Millipore, 150 µM), oxaliplatin (OXA, Accord Healthcare, 500 µM) and the agents from a custom arrayed anti-cancer drug library composed by 71 drugs diluted in dimethyl sulfoxide (DMSO; #D8418; Merck-Millipore) (64), at 10 µM and 3 µM for 24 h. Upon treatment, Hoechst 33342 (#H3570; ThermoFisher Scientific, 1/5000 v/v) and propidium iodide solution (PI, #P4864, Merck-Millipore 1/1000 v/v) were added for 30 min before imaging acquisition using an ImageXpress Micro XL automated microscope (Molecular Devices, Sunnyvale, CA, USA) equipped with environmental control (maintaining a humidified atmosphere containing 5% CO₂ and a temperature of 37 °C). Four view fields per well were imaged at 20X magnification and images were then analyzed and processed using the MetaXpress analysis software. Data was then further processed and statistically evaluated using the R software (https://www.r-project.org/).

**Indirect immunofluorescence analysis.** Flag-RECS1, MYC-RECS1, BAX, ERp57, LAMP1/2, LGALS1/galectin-1 and LGALS3/galectin-3 proteins were visualized by immunofluorescence. Cells were fixed for 30 min with 4% paraformaldehyde and permeabilized with 0.5% NP-40 in PBS containing 0.5% bovine serum albumin (BSA) for 10 min. After blocking for 1 h with 10% FBS in PBS containing 0.5% BSA, cells were incubated with specific antibodies (see below) overnight at 4 °C. Cells were washed three times in PBS containing 0.5% BSA, and incubated with Alexa-conjugated secondary antibodies (Molecular Probes) for 1 h at 37 °C. Nuclei were stained with Hoechst dye. Coverslips were mounted with Fluoromount G onto slides and visualized by confocal microscopy (Fluoview FV1000). The following antibodies were used: anti-Flag (F7425, Sigma Aldrich); anti-Flag M2 (F1804, Sigma Aldrich); anti-TBMIM1 (MBS1499661, MyoBioSource), anti-ERp57 (ab13506, Abcam): anti-LAMP-1 (an24170, Abcam); LAMP-2 (ab18528, Abcam), BAX (ab5714, Abcam); anti-LGAL1/galectin-1 (ab25138, Abcam) and LGAL3/galectin-3 (556904, BD Biosciences). All antibodies were diluted 1:1000. We used a sensitive method based on a confined displacement analysis algorithm to calculate
colocalization coefficients between Flag-RECS1 and ERp57 (ER), GM130 (Golgi apparatus) and LAMP1/2 (endosomes/lysosomes) (65). The colocalization of images was performed as previously described (65). Briefly, images obtained by confocal microscopy using a × 60 oil objective lens (NA: 1.35) were subjected to Huygens deconvolution software. Each channel used for Flag-RECS1 and an organelle-localized protein was then segmented using a series of filters using the IDL software to obtain masked images. These images were used to determine Manders colocalization coefficients and to quantify true and random colocalization between channels.

**Transmission electron microscopy (TEM) and immunogold staining.** For TEM analysis, cells were fixed in 2% glutaraldehyde (EM-grade) in 0.1 M sodium cacodylate buffer, pH 7.4 for 30 minutes at room temperature. Samples were washed and post-fixed with reduced 1% osmium tetroxide for 1 h, dehydrated through increasing concentrations of ethanol and acetone prior to infiltration into Epon (TAAB, cat# 812). After Epon polymerization, a pyramid was trimmed, and 60-nm-thick sections were cut, picked up on copper grids and post-stained with uranyl acetate and lead citrate. The images were acquired using a Hitachi HT7800 microscope (Hitachi High-Technologies, Tokyo, Japan) operated at 100 kV, and Rio9 CMOS-camera (AMETEK Gatan Inc., Pleasanton, CA). Random systematic sampling was applied for the collection of TEM micrographs: from randomly selected cells, one image was acquired above the nucleus and one below, at nominal magnification of 4000x. Segmentation and quantification of cytosolic and lysosomal areas were performed using the MIB software.

For immuno-EM studies, MEF Flag-RECS1 cells (non-induced) were fixed with periodate-lysine-paraformaldehyde fixative for 2 h at RT. Samples were washed, permeabilized with 0.01% saponin and labeled with anti-TMBIM1 antibody (MyoBioSource, MBS149966, diluted 1:20) for 1 h and nano-gold-conjugated anti-rabbit FAB fragments (Nanoprobes, cat# 2004; diluted 1:60) for 1 h prior post-fixation with 1% glutaraldehyde and quenching with 50 mM glycine in a 0.1 M sodium phosphate buffer, pH 7.4. Nano-gold particles were then intensified for 5 min using the HQ SILVER Enhancement Kit (Nanoprobes, catalog #2012) according to the manufacturer’s instructions followed by gold toning in 2% sodium acetate, 0.05% HAuCl4, and 0.3% sodium thiosulfate. Samples were then osmicated, dehydrated and flat embedded into Epon as described above.
BH3 profiling and competition assays. The profiling of BH3 peptides derived from RECS1 C-terminal domain was performed as previously described (66). In brief: wild-type (WT) or BAX/BAK double knockout (BB DKO) lymphocytes were resuspended in DTEB buffer (135 mM trehalose, 50 mM KCl, 20 mM EDTA, 20 mM EGTA, 0.1% BSA, 5 mM succinate, 10 mM HEPES-KOH pH 7.5). Cells were stained and permeabilized with 1 mM JC-1, 10 µg/mL oligomycin, 5 mM 2-mercaptoethanol, and 0.002% digitonin (w/v) for 10 min in DTEB. 10000 cells were mixed with 100-1000 nM of peptides and the JC-1 fluorescence was measured using Tecan Safire 2 fluorescent plate reader at 30°C every 5 min for 180 min using the following excitation and emission wavelengths: 545/590 nm. 1 mM of FCCP in DMSO was used as a positive control.

The ability of RECS1280-299 (SPEDYITGALQYRDIIYI) and RECS1296-311 (IYIFTFVLQLMGDRN) peptides to displace the interaction of the BH3-only protein BIM with BCL-2, BCL-XL or MCL-1 was assessed by fluorescent polarization, as previously performed (67). Fluorescent BIM (3 nM) was incubated with purified human BCL-2, BCL-XL or MCL-1 protein (6 nM) in the absence or presence of increasing concentrations (i.e. 30-3000 nM) of RECS1280-299 or RECS1296-311 peptides in the binding affinity buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% BSA and 5 mM DTT) in a 96-well assay plate. The plate was mixed on a shaker for 1 min and incubated at room temperature for an additional 15 min. Polarization, defined as millipolarization units (mP), was measured at room temperature with a fluorescence microplate reader at 485/530 nm (Gemini XPS, Molecular Devices). BIM protein (3 nM) and a BH3-defective PUMA mutant (PUMA2A, 3 nM) were used as positive and negative controls, respectively.

Measurement of lysosomal pH and calcium. To measure lysosomal intraluminal calcium, lysosomal pH and calcium were measured simultaneously using two ratiometric dyes (see detailed protocol in (68)) according to a modified protocol based on the methods described by Christensen and colleagues (40). HeLa, MEF and MEF BAX/BAK double knockout (BB DKO) Flag-RECS1 cell lines were seeded in glass-bottom 4-well (35 mm) dishes. A mixture of 150 µg/ml 10000 MW Fura-2-dextran (F3029, ThermoFischer) and 50 µg/ml 10000 MW Oregon-Green-488-dextran (D7117, ThermoFischer) was sonicated in a 37°C water bath sonicator in full cell culture medium containing selection antibiotics for 15 min. The mixture was split into two volumes and either 1µg/ml of doxycycline or the equivalent volume of DMSO was added to each mixture. Cells were incubated with the dextran mixture overnight. Prior to imaging, the dextran mixture was chased into
lysosomes for 90 min by washing 5 times in warm full medium containing 20 mM HEPES and DMSO or doxycycline without dextran. Cells were then washed 3 times in a warm, modified Ringer’s solution (140 mM NaCl, 5 mM KCl, 1mM MgCl₂, 2 mM CaCl₂, 20 mM HEPES, 10 mM glucose, adjusted to pH 7.4 with NaOH), and imaged immediately in 4 channels, using the following excitation/emission wavelengths (nm): 340/510, 380/510, 490/535 and 440/535. At least 5 different fields of cells, representing ~5-30 cells per field were analyzed per well. 2-4 wells were analyzed per experimental day and at least 4 independent experiments per condition were performed.

One pH and one calcium calibration was performed per experimental day. Calibration solutions from pH 3.0-7.0, containing nigericin/monensin (Sigma) were prepared as described in (69) except that for pH 3.0 and 3.5 a 10 mM EGTA was used to buffer pH instead. For calcium calibrations, 2 solutions were prepared: calcium maximum and calcium minimum solutions where the 7.0 pH calibration solution was adjusted to pH 7.2 using KOH, either 10 mM CaCl₂ or 10 mM EGTA was added respectively. 10 µM ionomycin was also added just prior to measurements. Calibration was performed at 37°C, and calcium calibration solutions were added to cells and allowed to equilibrate for at least 5 min prior to imaging, whereas pH calibration solutions were equilibrated for at least 3 min each.

Images were analyzed using ImageJ software (NIH). Images were background subtracted using the BG Subtraction from ROI plugin from manually drawn background ROIs. To obtain individual lysosomal ROIs, the Analyze Particles plugin was used along with a custom macro to apply a threshold ramp that isolated particles in the 440 nm pH-insensitive channel of 4-25 pixel units (~0.6-1.5 μm²), the size of typical lysosome clusters. A small percentage of extreme ratio values were present in the population, particularly where dextran clumps coincided with cellular zones. Since both pH (Oregon Green, 490/440) and calcium (Fura-2, 340/380) ratio values showed a unimodal, near Gaussian distribution, the median rather than average lysosomal ratio value of each field was used to minimize the effects of extreme values. For each field, the median Oregon Green lysosomal ratio value was first converted to pH using the daily calibration curve values, which were fitted to a Boltzmann sigmoidal curve using Prism software (Graphpad). Then, pH values were used in combination with the corresponding median lysosomal Fura-2 ratio value and calcium calibration values to convert the Fura-2 ratio values to calcium concentration, taking into account the effect of pH on the Fura-2 Kd. This conversion was calculated using the published Maxchelator Excel files from Patton and colleagues (70)
and https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/downloads.htm as described in (40). Finally, calcium concentration values were expressed as pCa values (log[Ca]) for comparability with pH values. The average of lysosomal median pH and pCa were aggregated per experimental day.

**Molecular modeling.** Molecular models of human RECS1 in its open and closed conformations were generated by molecular similarity with Modeller (71), using the crystal structure of the homolog protein YetJ from *Bacillus subtilis* (BsYetJ) as template (11). We used the chain A from 4PGS and 4PGW and from 4PGR and 4PGV PDB structures as templates for the models of the open and closed conformations, respectively. The FASTA sequence for human RECS1 was retrieved from Uniprot (Q969X1). The corresponding sequences were aligned with MAFFT using the linsi protocol (72, 73). 100 models were generated and the one with better discrete optimized protein energy (DOPE) was selected as the best model per conformation. The resulting models cover the region between R92 and N311. Given the lack of corresponding residues in the templates, the following regions were poorly modeled: R130 – V139 in the open conformation model and F129 – S140, S238 – L241 in the closed conformation model.

**RECS1 expression in Xenopus laevis oocytes.** Full-length wild-type (WT) human RECS1 and RECS1 D295Q were subcloned into the pBSTA vector, which is optimized for expression in *X. laevis* oocytes. DNA was amplified and cleaned using QIAprep kit (QIAGEN). 10 µg of plasmid DNA were linearized with NotI and 1 µg was used for *in vitro* transcription with mMESSAGE mMACHINE kit (Ambion). *Xenopus* oocytes were injected with a 50 nL of ~5 ng of RECS1 WT or mutant cRNA, per oocyte. Recordings were performed 1–3 d after injection.

**Electrophysiology.** *Patch clamp:* Using the patch-clamp technique, ionic single channel currents were recorded in the cell-attached mode by a constant voltage or a ramp potential. The pipette and bath solutions contained: 140 mM KCl, 5 mM EGTA, 10 mM HEPES, 2 mM MgCl2, pH 7.4. MES (2-(N-morpholino)ethanesulfonic acid) buffer was used instead of HEPES to adjust and maintain the pH at 6.5. The pH was adjusted using KOH. Currents were recorded at -100 mV for constant voltage during 90 s, and the ramp was elicited between -250 mV to 150 mV during 40 s. Experiments were performed at room temperature (20°C). Pipette resistance was 1.5–3 MΩ after being fire-polished with a microforge (MF-900, Narishige). Data were acquired with an Axopatch 200B amplifier.
Both the voltage command and current output were filtered at 20 kHz with an eight-pole Bessel low-pass filter (Frequency Devices). Current signals were sampled with a 16-bit A/D converter Digidata 1550B (Molecular Devices), using a sampling rate of 100 kHz. Experiments were performed using Clampex 10.6 acquisition software (Molecular Devices).

_Cut open:_ Macroscopic currents were recorded using the Cut-open technique (Cut-open Oocyte Vaseline Gap (COVG) voltage clamp), recording a wide fraction of the total oocyte membrane (20 to 25% of total) (74). The membrane fraction clamped was physically and electrically isolated by three superposed chambers and connected by holes of 0.5 to 0.8 mm in diameter. The recordings were performed using a CA-1 amplifier (Dagan Corp., Minneapolis, MN-USA) and an acquisition board (National Instrument BNC-2090, USA). The acquisition program was developed in IGORpro platform (WaveMetrics, version 6.3.4.1, OR-USA). To obtain a low resistance path to the intracellular side, the oocytes were permeabilized with 0.1% saponin during 15-30 s. The internal solution contained: 120 mM L-Gluconic acid potassium salt, 10 mM HEPES, pH 7.0, adjusted with KOH. The external solutions contained: 96 mM NMDG, 10 mM HEPES and 10 mM XOH where X was replaced by: Na⁺, K⁺, Ca²⁺, Cs⁺ or Ba²⁺, with pH 7.0 adjusted with methanesulfonic acid. Oocytes were dialyzed in internal solution during 30 min before individual recordings with different extracellular cations were performed. K⁺ was used as the internal reference ion. Pipettes were filled with: 3000 mM Sodium methanesulfonate, 10 mM NaCl and 10 mM HEPES, pH 7.0. Currents were elicited by one pulse of 200 mV during 350 ms from a holding potential of -90 mV by 20 ms. Tail current versus voltage relationships were measured from 150 mV to -350 mV, every -20 mV, during 120 ms. Currents recordings were sampled at 30 KHz and filtered at 5 KHz. The linear components were subtracted by P/-4 prepulse protocol (75).

All data recorded were analyzed using Clampfit 10.6 (Molecular Devices), IGORPro and Excel 2013 (Microsoft). In microscopic current analysis, as the index for channel steady-state functioning, NPo was obtained after traces idealization for different conditions at -100 mV, from 90 s of continuous recording. Current amplitude distribution were collected and plotted to obtain channel conductance (γ) from the slope of current amplitudes versus membrane potential. Unitary current amplitudes were obtained from all data points of the amplitude histograms from ramp recordings and conductance calculated from linear regressions of the curves obtained. For macroscopic current analysis, current versus potential plots were generated for each Cation tested. The linear part of each curve
was adjusted to a linear regression and the reversal potential \( (V_r) \) was extrapolated. With these data, the permeability ratio \( (P_X/P_K) \) was calculated for each external Cation, as follows:

\[
\frac{P_X}{P_K} = \frac{K}{X_0} \times e^{\frac{V_rF}{RT}} \quad \text{for monovalent cations}
\]

\[
\frac{P_X}{P_K} = K \times e^{\frac{V_rF}{RT}} \times \left( e^{\frac{V_rF}{RT}} + 1 \right) / 4 \times X_0 \quad \text{for divalent cations}
\]

where \([K^+] = 120 \text{ mM} \) (concentration of the reference ion); \([X]_0 = \text{external Cation concentration} \); \(V_r = \text{reversal potential for the cation} \ X \); \(T = \text{temperature in Kelvin} \) (293,15); \(R \) is the universal gas constant 8,314 J/mol*K; and \(F \) is the Faraday’s constant 96.5 kJ/mol.

**Generation of D. melanogaster strains and cell death assays.** *Fly strains:* All experiments were performed with *Drosophila melanogaster* grown at 25°C unless otherwise indicated. We generated transgenic animals allowing the UAS/Gal4-guided \( (76) \) expression of the full-length (dRECS1) or truncated version (dRECS1\(^{\Delta C}\)) of the CG9722/dRECS1 gene, or the human RECS1 coding sequence (hRECS1), all of them tagged with a 3xFlag sequence at the amino terminal region. The coding sequence of CG9722 was amplified with the primers 5’-CACCATGCTGGATATTTTACAAGAA-3’ and 5’-ATCGGATCCTCCGATTAATTG-3’, as well as a truncated version missing 63 nucleotides at its 3’ end with the primers CACCATGCTGGATATTTTACAAGAA-3’ and 5’-TAGGGCAGCGAATATGTACTC-3’. The human RECS1 gene was amplified with the primers 5’-CACCATGTCCAACCCAGCGCCCCA-3’ and 5’-ATTGCGATCCCACCCTACAGCTG-3’. PCR products were directionally inserted using the pENTR™/D-TOPO cloning kit (K240020, ThermoFischer). The constructs were subcloned in a pTFW vector from the Drosophila Genomics Resource Center (NIH Grant 2P40OD010949) using the Gateway cloning system (Gateway™ LR Clonase™ Enzyme mix, Invitrogen, 11791019). The pTFW vector contains a UAS promoter as well as an N-terminal 3xFlag tag sequence. DNA constructs were prepared and injected by BestGene, using the P-element based transposition (https://www.thebestgene.com/). All additional *Drosophila* strains were obtained from Bloomington *Drosophila* Stock Center: \( w^{118} \) (BDSC:3605), \( y^{1}, w^{+} \) (BDSC:1495), CG9722-KO \( (y^{1}, w^{+}) \); Mi(76)CG9722\(^{M108483}\).
Immuno-fluorescence: Third instar larval wing discs were dissected from animals expressing dRECS1-Flag at the wing disc pouch, guided by the nub-Gal4 driver. The tissue was fixed for 20 min in 4% paraformaldehyde and washed three times with a PBS solution containing 0.3% Triton X-100. Permeabilization and blocking were performed during 1 h with a solution containing 0.3% Triton X-100 and 1 % BSA in PBS. The same solution was used for the incubation of both primary and secondary antibodies. Primary antibodies (anti-cleaved caspase 3, Cell Signaling #9664, dilution 1:100; and anti-Flag epitope, Sigma F1804, dilution 1:500) were incubated overnight at 4°C, and after washing 4 times, Alexa-conjugated secondary antibodies (anti-IgG rabbit-488 and mouse-633, Invitrogen A27034 and A21052, both diluted 1:200) were incubated during 2 hours at room temperature with mild shaking. The tissue was washed three times and mounted in VectaShield (Vector Laboratories). Images were acquired in a Zeiss LSM510 Meta confocal microscope.

Wing phenotypes: Flies (1-2 days) were immersed in 70% ethanol and right sided wings were dissected and transferred to a microscope slide for imaging. Animal with vestigial wings were mounted in glycerol for imaging. For the initial characterization of dRECS1 function in Drosophila, animals were grown at 29°C to increase the expression levels of the transgene and male phenotypes were analyzed. To determine the effect of lysosomal or ER stress, animals were grown in media supplemented with 100 µg/ml chloroquine or 10 µg/ml tunicamycin throughout development until hatching, where wing phenotypes were analyzed (N ≥ 3 independent experiments, n ≥ 80 wings/condition).

Survival assays: Depending on the case, animals were grown in vials containing normal food (Instant Drosophila Medium, Carolina, #173200) or starvation media (1% agarose in PBS), either alone or supplemented with chloroquine or tunicamycin. To evaluate eclosion rate, embryos were collected in vials containing rich food alone or with 10 µg/ml tunicamycin for 6 h, after which adults were discarded and eggs developed at 25°C until hatching. Starting from the first hatch, we quantified eclosion daily (N ≥ 2 independent experiments, n ≥ 40 animals/condition). For the determination of adult viability, animals grew in rich food and hatched flies (1 day after eclosion) were isolated in a clean new vial containing rich food alone, rich food supplemented with 10 µg/ml tunicamycin or starvation media, after which animal viability was determined every 12 or
24 hours during 11 consecutive days (N ≥ 4 independent experiments, n ≥ 15 adults/condition).

**Autophagy in the fat body:** All fly crosses and larvae were maintained in vials containing 'standard' food. For starvation induced-autophagy, 2nd instar larvae were individually selected and no more than 20 larvae per experiment were transferred to vials containing 1% agarose in PBS for 0, 3 and 6 h, after which fat tissues were dissected and directly imaged under a confocal microscope (Zeiss LSM510 Meta confocal). For each genotype, at least 6 larvae from three independent vials were analyzed.

**Zebrafish studies.** Zebrafish embryos were raised in E3 medium, kept at 28 °C and staged according to age (hours post fertilization; hpf). Wild-type AB/Tübingen zebrafish were microinjected at one-cell stage with 39 pg of full-length human RECS1, C-terminal deletion mutants, RECS1 D295Q, mouse GRINA or left uninjected as a control. Embryos’ viability and morphology was quantified 24 hpf by visual inspection. Additionally, at 24 hpf, apoptotic cells were visualized by acridine orange (Sigma Aldrich) staining followed by epifluorescence microscopy in full-mounted embryos, as previously reported (77). The effect of RECS1 overexpression in ER stress-induced cell death was evaluated by microinjecting one-cell stage embryos with 50–100 pg RNA of full-length RECS1 or mouse GRINA. At 24 hpf, embryos were incubated with 1-5 mM of Thapsigargin (Sigma) for 4 h and apoptosis was evaluated by acridine orange staining. For immunofluorescence staining, wild-type Tübingen embryos were fixed at 24 hpf in PFA 4% overnight and washed three times in PBS for 10 min. After 6 min of proteinase K treatment, larvae were incubated with 1:100 dilution of rabbit anti-TBMIM1 (MBS1499661, MyoBioSource), followed by incubation with 1:200 dilution of anti-rabbit Alexa 488-conjugated antibody (Invitrogen). Fluorescence micrographs of fixed embryos were taken using a Leica LSI confocal microscope and Velocity ViewVox spinning disc (Perkin Elmer) coupled to a Zeiss Axiovert 200 inverted microscope. Images were processed with ImageJ (http://rsbweb.nih.gov/ij/) and Adobe Photoshop CS3 software (Adobe).

**Statistics and reproducibility.** Results were statistically compared using one-way analysis of variance (ANOVA) for unpaired groups, followed by Holm-Sidak’s multiple comparison tests. When there were two independent variables, a two-way ANOVA followed by Holm-Sidak’s multiple comparison test was performed. For cell death kinetics experiments, the goodness-of-fit of the different curves was compared using the extra-sum-of-squares F test. When pertinent, two-tailed Student’s t-test was performed for
unpaired or paired groups. In all plots, p values are shown as indicated: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ and ****: $p < 0.0001$ and were considered significant. All results are presented as the mean ± SD or mean ± s.e.m. as indicated in the figure legends. Analyses were performed using PRISM software.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1.

(A) Phylogenetic tree depicting the evolutionary relationship between the human members of the TMBIM superfamily of proteins. (B) Multiple alignments of the C-terminal domain of the human TMBIM proteins. (C) Multiple sequence alignment of the C-terminal domain of RECS1 from indicated species. (D) Phylogenetic tree constructed using the sequence of RECS1/TMBIM1. Species where RECS1 C-terminal BH3-like motif is conserved are shown in red. (E) Wild-type MEFs were transiently transfected with MYC-RECS1 wild-type (WT) or C-terminal deletion mutants (RECS1Δ910 and RECS1Δ868) for 48 h and protein levels monitored by western blot. (F) MEF cells were transiently transfected with RECS1 wild-type (WT) or C-terminal deletion mutants for 48 h. Cells were fixed and immunofluorescence with an anti-MYC antibody was performed. (G) MEFs were transiently transfected with RECS1 WT, C-terminal deletion mutants, BIM or empty vector (MOCK) for 48 h and cell death was determined by PI staining followed by FACS ($n = 3$). (H) Three-dimensional structure and primary aminoacid sequence of peptides derived from RECS1 C-terminal domain. (I) WT DHL4 and BAX/BAK double knockout (DKO) DHL10 lymphoblast cells were permeabilized with digitonin, loaded with the mitochondrial potential sensitive dye JC-1 and treated with increasing concentrations of RECS1\textsubscript{280-299} or RECS1\textsubscript{296-311} peptides or fixed concentrations of BIM, BAD or PUMA2A (BH3-only mutant). Mitochondrial membrane potential was measured using a fluorescence plate reader. FCCP was used as a positive control. Data shown is a representative experiment of at least 2 independent experiments. (J) BCL-2, BCL-X\textsubscript{L} or MCL-1 GST-fused recombinant proteins were incubated with BIM-FITC \emph{in vitro} to form stable dimers. The dimers were treated with increasing concentrations of RECS1\textsubscript{280-299}, RECS1\textsubscript{296-311}, BIM, BAD or PUMA2A for 24 h and fluorescence light polarization was measured ($n = 3$ independent experiments). (K) Flag-RECS1 MEFs were fixed, permeabilized and labeled with anti-TMBIM1 and nano-gold-conjugated anti-rabbit FAB fragments and prepared for transmission electron microscopy (TEM) imaging. Image magnification: 4000x. (L) Left
Panel: HE LA Flag-RECS1 cells were treated with 1 µg/ml doxycycline for 24 h, washed, fixed, permeabilized and stained with primary antibodies against Flag and indicated organelle proteins. Co-localization was assessed by immunofluorescence (IF) followed by confocal microscopy. Right panel: Quantification of co-localization signals between RECS1 (Flag) and indicated organelle markers was determined using the confined displacement algorithm (CDA) (n = 3-15 cells per condition). Data represents mean ± SD (G) or mean ± s.e.m. (G and L). Statistically significant differences were determined by One-Way ANOVA followed by Holm-Sidak’s multiple comparisons test (**: p < 0.01).

Supplementary Figure 2.

(A) MEF Flag-RECS1 cells were cultured in the presence or absence of 1 µg/ml of doxycycline (DOX) for 16 h and treated with an arrayed anti-cancer drug library composed by 77 drugs at for 24 h. *CQ and HCQ were used at 50 µM. **Cisplatin (CDDP) was used at 150 µM. Normalized cell death percentages correspond to doxycycline-treated PI values after the subtraction of cell death induced by doxycycline alone (only DOX) and by the compound in the absence of doxycycline. Positive and negative values represent compounds sensitized and inhibited by RECS1 respectively. (B) MEF Flag-RECS1 cells were cultured as in (A) and treated with indicated concentrations of CQ or HCQ for 24 h. Cell death was determined by PI staining followed by automatic microscopy (n = 3 independent experiments, 1000-10000 cells per condition per experiment). (C) Examples of microtubule-targeting compounds that were sensitized by RECS1 overexpression. (D) Heat map showing the top 10 compounds that were inhibited by RECS1 overexpression from the screening shown in (A). Data was normalized as in (B). (E) Examples of compounds inhibited by RECS1 overexpression. (F) Frequency and function of compounds sensitized (red) or inhibited (blue) by RECS1 overexpression. HDAC: histone deacetylase. Data are shown as mean ± SD.

Supplementary Figure 3.

(A) MEF Flag-RECS1 cells were cultured in the presence of 1 µg/ml doxycycline for 16 h and then treated with indicated concentrations of Chloroquine (CQ) for 24 h. Cell death was determined by A5 and PI co-staining followed by FACS. Left panel: Representative A5 vs. PI density plot of cells treated with 50 µM CQ for 24 h (n = 3 independent
experiments). (B) Cell death kinetic analyses of MEF Flag-RECS1 cells cultured in the presence of 1 µg/ml doxycycline for 16 h and then treated with 25 µM CQ (n = 2 independent experiments). (C) MEFs were cultured in normal media (NT), EBSS or in 1 µg/ml of doxycycline for 16 h, treated with 50 µM of Chloroquine for 6 h, fixed, permeabilized and stained with primary antibodies against RECS1 (red) in combination with LAMP-1 (green). Co-localization was assessed by immunofluorescence (IF) followed by confocal microscopy. Right panel: Quantification of co-localization signals between RECS1 and LAMP-1 was determined using the confined displacement algorithm (CDA) (n = 3 independent experiments, 9-12 cells per condition). (D) MEFs were treated as in (C) and stained with primary antibodies against RECS1 (red) and LAMP-2 (green) (n = 3 independent experiments, 10-12 cells per condition). (E) Cells were cultured and treated as in (C) and stained with primary antibodies against RECS1 and the late endosome protein RAB7A (green) (n = 2 independent experiments, 19-23 cells per condition). (F) MEFs were cultured in EBSS for indicated time points and the mRNA levels of the endogenous mouse Recs1 were detected by qPCR using specific primers (n = 4 independent experiments). (G) Flag-RECS1 MEF cells expressing LC3-GFP were cultured with 1 µg/ml of doxycycline for 16 h, treated with 50 µM of Chloroquine for 8 h and the number of LC3-GFP puncta per area was quantified (n = 3 independent experiments). Data are shown as mean ± SD (B) or mean ± s.e.m. (A, D, E, F and G). Statistically significant differences were determined by Extra-sum-of-squares F test (B), Two-Way (A and G) or One-Way (C, D, E and I) ANOVA followed by Holm-Sidak’s multiple comparisons test (*: p < 0.05; ***: p < 0.001; ****: p < 0.0001).

Supplementary Figure 4.

(A) HeLa Flag-RECS1 cells were cultured in the presence of 1 µg/ml doxycycline for 24 h and then treated with 100 µM of CQ, 40 µM of QvD or both for 16 h. Cells were fixed and immunofluorescence against galectin-1 was performed. (B) Quantification of galectin-1 puncta per field (n = 2 independent experiments; 10-12 fields in total). (C) Quantification of galectin-1 puncta per field and per cell (n = 120-190 cells per condition). (D) HeLa Flag-RECS1 cells were cultured as in (A) and treated with 50 µM of CQ for 8 and 16 h. Cells were fixed and immunofluorescence against galectin-3 was performed. (E) Number of galectin-3 puncta per field (6-13 fields in total). (F) Quantification of galectin-3 puncta per
field and per cell (n = >100 cells per condition). (G) MEF BAX/BAK double knockout (DKO) Flag-RECS1 cells were cultured with 1 µg of doxycycline for 16 h and then treated with 50 µM of CQ in the presence of indicated concentrations of necrostatin-1 (Nec1) and cyclosporine A (CsA). Cell death was determined by PI staining followed by FACS (n = 3 independent experiments). (H) MEF Flag-RECS1 cells were treated with 50 µM of CQ for 16 h and fixed. Immunofluorescence against LAMP-2 (green), active BAX (red) and Flag (blue) was performed. Lower panels correspond to zoomed white boxes in the upper panel. In all panels, data are shown as mean ± s.e.m. For panels (B, C, E, F and G) a Two-Way ANOVA followed by Holm-Sidak’s multiple comparisons test was used (*: p < 0.05; **: p < 0.01; ***: p < 0.001; ****: p < 0.0001).

Supplementary Figure 5.

(A) MEF cells were treated with 100 ng/ml of tunicamycin for indicated time points and the mRNA levels of the endogenous mouse *Recs1* and *Bi-1* were determined by qPCR using specific primers. Actin was used as a housekeeping gene (n = 5 (*Bi-1*) and 6 (*Recs1*) independent experiments). (B) Cells were treated as in (A) and the endogenous levels of *Bcl-2* and *Puma* were determined by qPCR (n = 2 independent experiments). (C) MEFs were treated with 50 nM (left panel) or 100 nM (right panel) of thapsigargin for indicated time points and the mRNA levels of *Recs1* were determined by qPCR. Actin mRNA levels were used as a housekeeping gene (n = 4 independent experiments). (D) Cells were treated as in (A) and the levels of *Chop/Gadd153* and *Bip/Grp78* were determined by qPCR (n = 2 (*Bip/Grp78*) and 3 (*Chop*) independent experiments). (E) MEF Flag-RECS1 cells were cultured with 1 µg/ml of doxycycline for indicated time points and the levels of Flag-RECS1 and RECS1 were determined by western blot. HSP90 protein levels were monitored as a loading control. (F) MEF Flag-RECS1 cells were treated with 1 µg/ml of doxycycline for indicated time points or with 100 ng/ml of Tunicamycin for 8 h, and the levels of *Xbp1* mRNA splicing were determined by RT-PCR. Spliced and un-spliced PCR fragments are indicated. In addition, the levels of spliced *Xbp1* and *Actin* were determined by qPCR. (G) The levels of *Xbp1* mRNA splicing were determined from MEF Flag-RECS1 cells treated as in (F) by qPCR (n = 3 independent experiments). (H) Flag-RECS1 MEF cells were treated as in (F) and the levels of *Bip/Grp78* and *Chop/Gadd153* were determined by qPCR (n = 3 independent experiments). (I) Flag-RECS1 MEFs were treated with 1 µg/ml of doxycycline for indicated time points or with 250 ng/ml of Tunicamycin for 6
h and the levels of Sel1L and Pdia4 mRNA levels were determined by qPCR (n = 3 independent experiments). (J) MEF Flag-RECS1 cells were treated with 1 µg/ml doxycycline of 500 ng/ml of Tunicamycin for indicated time points and the levels of the proteins PERK, BIP, CHOP and ATF4 were detected with specific antibodies by western blot. The protein levels of HSP90 were monitored as a loading control. (K) Flag-RECS1 MEF cells were cultured in the presence of 1 µg/ml of doxycycline for 16 h, treated with 50 µM of chloroquine for indicated time points and the levels of Xbp1 mRNA splicing were determined by RT-PCR. Spliced and un-spliced PCR fragments are indicated. Cells treated with 500 ng/ml of tunicamycin and 500 nM of thapsigargin were used as positive controls. In addition, the levels of actin were determined by RT-PCR. (L) The levels of Xbp1 mRNA splicing, BiP/Grp78 and Chop/Gadd154 were determined from MEF Flag-RECS1 cells treated as in (C) by qPCR (n = 3 independent experiments). (M) HeLa Flag-RECS1 cells were cultured with 1 µg/ml doxycycline for indicated time points and the levels of Flag-RECS1 were determined by western blot. HSP90 protein levels were monitored as a loading control. (N) HeLa Flag-RECS1 cells were cultured in 1 µg/ml of doxycycline for 24 h and treated with indicated concentrations of Tg for 24 h. Cell death was measured by PI staining followed by FACS (n = 3 independent experiments). (O) MEFs were transfected with two siRNAs directed against the endogenous mouse Recs1 or a non-targeting scrambled control (siSCR) for 24 h and the percentage of cell death was determined by PI staining followed by FACS (n = 3 independent experiments). (P) MEF cells were treated as in (O) and the levels of cell death were determined by SYTOX green staining and live imaging microscopy (representative kinetic from 4 independent experiments). (Q) Knockdown of the endogenous human RECS1 using a siRNA pool was confirmed by qPCR from total mRNA extracts after 48 h of transient transfection. (R) HeLa cells transfected with RECS1 siRNA were treated with Tm or Tg and cell death was determined by SYTOX® green staining using live microscopy. A representative experiment from 2 independent experiments. (S) Left panel: MEF wild-type cells were stably transduced with lentiviral vectors expressing two shRNAs directed against mouse Recs1 (shRecs1) or luciferase (shLuc) mRNAs. Total RNA was extracted and the levels of endogenous Recs1 were determined by qPCR. Actin mRNA levels were monitored as a housekeeping control gene. Right panel: shLuc and shRecs1 cell lines were treated with indicated doses of Tm or Tg for 24 and cell death was determined by PI staining followed by FACS (for cells treated with Tm: n = 10 independent experiments; for cells treated with Tg: n = 3 independent experiments). Data are shown as mean ± SD (P and R) or mean ±
s.e.m. Statistically significant differences were determined by One-Way (A, C, G, H, I and O) or Two-Way (L, N and S) followed by Holm-Sidak’s multiple comparisons test (*: \( p < 0.05 \); **: \( p < 0.01 \); ***: \( p < 0.001 \); ****: \( p < 0.0001 \)).

**Supplementary Figure 6.**

**(A)** Representative immunofluorescence images of MEF, HeLa and BAX and BAK double knock-out (DKO) Flag-RECS1 cell lines loaded with ratiometric probes for lysosomal pH (OGDx) and calcium (Fura2Dx). **(B)** Left panel: HeLa Flag-RECS1 cells were loaded with 150 \( \mu \text{g/ml} \) 10000 MW Fura-2-dextran and 50 \( \mu \text{g/ml} \) 10000 MW Oregon-Green-488-dextran for 15 min, incubated with calibration solutions pH 3.0-7.0 for 3 min and imaged. Calibration curves were performed for each experimental day. The ratio 490/440 was calculated for each individual cell (n = 3-7 cells/per experiment). Right panel: HeLa Flag-RECS1 cells loaded as previously described were incubated with 0 or 10 mM calcium solution for 5 min and imaged (n = 13-14 cells/experiment). **(C)** Representative TEM images of MEF Flag-RECS1 cells cultured with doxycycline for 16 h and treated with 50 \( \mu \text{M} \) of Chloroquine for 8 h. For each experimental condition, four representative images were taken. Image magnification: 4000x. **(D)** MEF Flag-RECS1 cells were cultured and treated as in (C), stained with Lysotracker™ red for 1 h and visualized by confocal microscopy. A representative image for each experimental condition is shown. **(E)** Quantification of the total area per cell of lysotracker particles (n = 46-63 cells per condition). **(F)** Number of lysotracker positive particles per cell (n = 46-63 cells per condition). **(G)** Area frequency distribution of lysotracker positive particles in MEF Flag-RECS1 cells cultured in 1 \( \mu \text{g/ml} \) of doxycycline for 24 h and treated with 50 \( \mu \text{M} \) CQ for 8 h (n = 46-63 cells per condition). **(H)** Number of lysotracker positive particles with areas higher (left panel) or lower (right panel) than 1.5 \( \mu \text{m}^2 \). Data are shown as mean ± s.e.m. (E and F) or boxes and whiskers, where the horizontal line represents the median; the boxes the 1\textsuperscript{st} and 3\textsuperscript{rd} quartiles, and whisker the minimum and maximum observation values. For all panels, Two-Way ANOVA followed by Holm-Sidak’s multiple comparisons test was used (*: \( p < 0.05 \); **: \( p < 0.01 \); ***: \( p < 0.001 \); ****: \( p < 0.0001 \)).

**Supplementary Figure 7.**

Comparison of protein primary aminoacid identity between the Bacillus subtilis protein BsYetJ and the human proteins of the TMBIM family.

**Supplementary Figure 8.**
(A) Comparison of protein primary aminoacid identity between the drosophila protein CG9722/dRECS1 and the human proteins of the TMBIM family. (B) Phylogenetic tree comparing the D. melanogaster RECS1 ortholog GC9722 (dRECS1) with the human members of the TMBIM family. (C) Three-dimensional model of the human RECS1 and dRECS1 from top view. (D) Representative images of fat bodies expressing the mCherry-GFP-ATG8a autophagy reporter from yw (control) and dRECS1 mutant (dRECS1KO) animals. In all cases, 2nd instar larvae were starved during 0, 3 and 6 h (n = 3 independent experiments, 6 animals per experiment). (E) Quantification of autophagosomes (left panel, yellow puncta) and autophagolysosomes (right panel, red puncta) per cell from images shown in (D) (n = 7-15 cells per condition). (F) Autophagy flux, as determined by the ratio of autophagolysosomes to total vesicles per cell. Data are shown as mean ± s.e.m. For panel (E) Two-Way ANOVA followed by Holm-Sidak’s multiple comparisons test was used (*: p < 0.05; **: p > 0.001; ****: p < 0.0001).

**Supplementary Figure 9.**

(A) Endogenous distribution of zebrafish RECS1 in different cell types of zebrafish embryos 24 h after fertilization. Left panels: low magnification Hoechst staining (white), showing the regions analyzed by immunofluorescence with the anti-RECS1 antibody (yellow). A. Dorsal view of the brain at the level of the forebrain (Fb) and midbrain (Mb). A1, A2 and A3 represent different consecutive planes of confocal stacks, with A1 being the most ventral, showing RECS1 positive spots into the neuroepithelial cells. The A2 is a more dorsal plane and A3 is the most dorsal plane where the distribution of RECS1 can be observed in the skin cells above the head. B. Lateral view, showing the stained somites in B1, where the distribution of RECS1 in the muscle cells can be appreciated. (B) Real-time qPCR analyses from total RNA extracts of zebrafish embryos at 24 h post fertilization after they were microinjected with wild-type or the mutant D295Q RECS1 mRNA at one-cell stage. Primers are specific for human RECS1. In panel (B) data represents mean ± s.e.m.

**Supplementary video 1.**

MEF Flag-RECS1 cells were cultured in the absence (NT) or presence of 1 µg/ml doxycycline (DOX) for 16 h, stained with propidium iodide (PI, red) and then treated with 50 µM of Chloroquine (CQ). Phase contrast and fluorescence images were obtained each hour for 30 h using a ZOOM Incucyte live imaging system.
Supplementary Figure 2.

A

Screening of cytotoxic compounds (10 μM)
MEF Flag-RECS1

Lysosomotropic compounds

B

2μM Dose-Response (μM)

CQ & HCQ

C

Lysosomotropic

Docetaxel
Vinorelbine
Vinblastine
Vincristine
Paclitaxel

C

Microtubule disruptive

Docetaxel
Vinorelbine
Vinblastine
Vincristine
Paclitaxel

D

Top inhibited compounds

Concentration (μM): 3
Methotrexate
Flouxuridine
Cladribine
Gemcitabine HCl
Cytarabine HCl
Dactinomycin
Oxaliplatin*

Concentration (μM): 10

E

Antifolate

Methotrexate

Cladribine
Gemcitabine

DNA damage

Flouxuridine

Cladribine
Gemcitabine

F

# of compounds

Antifolate
DNA Damage
HDAC inhibition
DNA damage
MTB disruption
Lysosome
Supplementary Figure 3.
Supplementary Figure 7.

| BeYetU vs. | Per. Ident | E-value   | Total score |
|------------|------------|-----------|-------------|
| GAAP       | 27.38%     | 1.00E-09  | 45.1        |
| RECS1      | 35.59%     | 2.00E-05  | 32.7        |
| GHITM      | 26.09%     | 5.00E-04  | 28.5        |
| BI-1       | 23.47%     | 0.001     | 27.3        |
| LFG        | 31.58%     | 0.001     | 45.4        |
| GRINA      | 25.00%     | 0.003     | 26.2        |
Supplementary Figure 8.

A

| Protein | Per. Ident | E-value | Total score |
|---------|------------|---------|-------------|
| GRINA   | 39.00%     | 5.00E-58| 179         |
| LFG     | 39.17%     | 8.00E-45| 143         |
| RECS1   | 34.55%     | 2.00E-42| 136         |
| GAAP    | 33.33%     | 2.00E-26| 94.0        |
| GHITM   | 27.97%     | 6.00E-07| 38.1        |
| Bi-1    | 21.71%     | 0.005   | 25.8        |

B

LFG family

C

hRECS1

dRECS1

D

Starvation (h)

E

Autophagosomes/cell

F

Autophagy flux

| Starvation (h) | 0  | 3  | 6  |
|----------------|----|----|----|
| YW RECS1  | 0  | 3  | 6  |
| RECS1 KO  | 0  | 3  | 6  |
Western blots

Raw Figures
Pihán et al., 2021
Science Advances
Figure 1A

Membrane 1, blot 1: αFlag (1:1000)  
Exp.: 31.9s

Membrane 1, blot 2: αHSP90 (1:3000)  
Exp.: 16.0s

Membrane 2, blot 1: αTMBIM1 (1:1000)  
Exp.: 31.9s

Membrane 2, blot 2: αHSP90 (1:3000)  
Exp.: 14.0s

MEF  
Flag-RECS1

Dox (h): 0 16 24

Mw (kDa)

1  αFlag
2  αHSP90
3  αTMBIM1
4  αHSP90
Membrane 1, blot 1:
Upper: αFlag (1:1000)
Lower: αTMBIM1 (1:1000)
Exp.: 109.9s
Same samples for both gels

Membrane 1, blot 2:
Lower: αHSP990 (1:3000)
Exp.: 19.4s
Figure 1F (2)

Membrane 1, blot 1: αBAK (1:1000)
Exp.: 231.0s

Membrane 1, blot 2: αBAX (1:1000)
Exp.: 300.0s

Membrane 1, blot 2: αBAX (1:1000)
Exp.: 177.3s

Membrane 1, blot 3: αHSP90 (1:1000)
Exp.: 60.0.3s
Figure 7H

Membrane 1, blot 1: αFlag (1:1000)
Exp.: 71.7s

Membrane 1, blot 2: αHSP90 (1:3000)
Exp.: 55.8s

No-DOX controls
Additional mutants

1 2 3
1 2 3

MOCK WT D295Q
αFlag
αHSP90

M_w (kDa)
40
90
1 2 3
Supplementary Figure 5E

Blot 1:
αTMBIM1 (1:1000)
Exp.: 31.9s

Blot 2:
αHSP90 (1:3000)
Exp.: 34.6s

Blot 1:
αFlag (1:1000)
Exp.: 31.9s

Blot 2:
αHSP90 (1:3000)
Exp.: 34.6s

MEF Flag-RECS1

| Dox (h): | 0 | 2 | 4 | 8 | 16 | 24 |
|---------|---|---|---|---|----|----|
| αFlag   |   |   |   |   |    |    |
| αHSP90  |   |   |   |   |    |    |
| αTMBIM1 |   |   |   |   |    |    |
| αHSP90  |   |   |   |   |    |    |
Supplementary Figure 5M

Membrane 1, blot 2: 
αHSP90 (1:3000)  
Exp.: 16.0s

Membrane 2, blot 1: 
αTMBIM1 (1:1000)  
Exp.: 176.3s

Membrane 2, blot 2: 
αHSP90 (1:3000)  
Exp.: 16.0s

Membrane 1, blot 1: 
αFlag (1:1000)  
Exposition: 52.5s in both images
Supplementary Figure 5J

Membrane 1, blot 1: αPERK (1:1000)
Membrane 1, blot 2: αBiP (1:1000)
Membrane 1, blot 3: αHSP90 (1:3000)

Membrane 2, blot 1: αATF4 (1:1000)
Membrane 3, blot 1: αCHOP (1:1000)
Membrane 4, blot 1: αATF5 (1:1000)
Membrane 5, blot 1: αHSP90 (1:3000)

| Time (h) | DOX | Tm | Mₚ (kDa) |
|---------|-----|----|----------|
| 0       |     |    | 170      |
| 2       |     |    | 70       |
| 4       |     |    | 40       |
| 8       |     |    | 35       |
| 16      |     |    | 90       |
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