Supplementary material

Materials and Methods

Immunofluorescence (IF) and immunogold (IG) labelling on thin sections. U-87 MG cells were plated in 10 cm petri dishes at $1.5x10^6$ cells per dish and grown overnight before the siramesine treatment. Cells were fixed with 4% PFA in 200 mM HEPES, pH 7.4, overnight. The cell pellet was embedded in 10% bovine gelatine, cut into small cubes and infiltrated with 2.3 M sucrose overnight. The samples were vitrified in liquid $N_{2,}$ and 100 nm Tokuyasu sections were cut using a Leica EM UC7 cryo ultramicrotome. For IF labelling , sections were transferred on L-lysine-coated cover slips, whereas for IG labelling, they were transferred on carbon-coated formvar copper EM grids. One percent cold water fish skin gelatin (Sigma-Aldrich; St. Louis, MO, USA) in PBS was used to block non-specific binding and dilution of antibodies. Mouse mAb against LAMP-1, clone H4A3 (DSHB, Iowa City, IA, USA) was used as undiluted supernatant (for IG) or at 1:50 dilution (for IF) for 30 min. For IF, secondary Cy2-coupled goat-anti-mouse antibody (Jackson ImmunoResearch Europe, Suffolk, UK) was used at 1:500 for 30 min, and then the cells were stained with 1 µg/ml DAPI (Sigma-Aldrich; St. Louis, MO, USA) for 5 min and mounted with ProLong Gold antifade reagent (Invitrogen, Molecular Probes; Eugene, OR, USA). The samples were analysed using an Olympus IX81 microscope. For IG, a rabbit-anti-mouse bridging antibody (Rockland Immunochemicals, Gilbertsville, PA, USA) was used at 1:300 for 20 min, followed by protein A-gold 10 nm at 1:50 for 30 min (Cell Microscopy Center, UMC, the Netherlands). Sections were then embedded in 2% methyl cellulose with 0.2% uranyl acetate (EMS, PA, USA) and analysed with a Philips CM100 TEM microscope. The images were recorded digitally with a Quemsa TEM CCD camera and iTEM software.

NAG activity measurements. HaCaT cells were plated onto a 96-well plate at $0.8x10^4$ cells per well and grown overnight before the siramesine treatment. After incubation with siramesine for the indicated time points, the cells were washed with PBS (PAA; Pasching, Austria), and 15 or 200 µg/ml digitonin (Sigma-Aldrich; St. Louis, MO, USA) in acetate buffer [50 mM Na-acetate, 150 mM NaCl, 0.5 mM EDTA, pH 5.6] was added directly to the wells to lyse only the plasma membrane (cytosolic activity) or all cell membranes (total activity), respectively. After 12-minute incubation with digitonin on ice, the N-acetyl-β-D-glucosaminidase (NAG) activity was measured fluorimetrically on a Tecan Saphire microplate reader using 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Calbiochem; San Diego, CA, USA) at a final concentration of 300 µg/ml in citrate buffer [0.2 M citrate acid, pH=4.5] and incubated for 30 minutes at 37 °C. After incubation, glycine buffer [0.1 M glycine, pH=10] was added. Substrate hydrolysis was measured at excitation and at emission wavelengths of 380 nm and 440 nm, respectively, in one cycle using a Tecan Saphire microplate reader. Three independent experiments were performed, and each experiment was performed in triplicate.

Detection of free cellular calcium. HaCaT cells were plated onto a 96-well plate at $0.8x10^4$ cells per well and grown overnight before the siramesine treatment. Prior to the treatment, cells were stained with 2 µl/ml Fluo-4 (Invitrogen, Molecular Probes; Eugene, OR, USA) in DMEM supplemented with 1% FBS. After 30 minutes at 37 °C, cells were washed twice with Ca^{2+} mobilisation wash/assay buffer [20 mM HEPES, 2.5 mM probenecid, 0.1% BSA, in Hanks' Balanced Salt Solution (HBSS)]. Cells were then treated with 40 µM siramesin, 100 mM EGTA (Sigma-Aldrich, Fluka; St. Louis, USA) or 100 µM ionomycin (Sigma-Aldrich;

St. Louis, MO, USA). Immediately after the treatment, the release of free calcium was measured at excitation and at emission wavelengths of 380 nm and 460 nm, respectively, for 30 min using a Tecan Saphire microplate reader.

Supplementary Figures:

Supplementary Figure 1:
Effect of repetitive α-tocopherol treatment on viability of HaCaT cells treated with siramesine. Cells were treated with 15, 25 and 40 μM siramesine. Lipophilic antioxidant α-tocopherol (0.3 mM) was applied once (2 h before siramesine treatment), twice (2 h before and 1 h after siramesine treatment) and three times (2 h before, 1 h and 4 h after siramesine treatment). After 48-hour incubation with siramesine, the cells were stained with annexin V and propidium iodide (PI), followed by flow cytometer analysis. Live cells were defined as annexin V/PI double negative cells. The experiments were performed in duplicate; the bars represent mean ± SD. S – siramesine, T - α-tocopherol.

Supplementary Figure 2:
Ability of mitochondria to accumulate NAO after siramesine treatment. HaCaT and U-87 MG cells were treated with 25 and 40 μM siramesine and incubated for the indicated time points. Lipophilic antioxidant α-tocopherol (0.3 mM) was applied 2 hours before siramesine treatment. After incubation with siramesine, all the cells were stained at once with NAO at a final concentration of 50 nM and analysed with a flow cytometer. Solid lines represent siramesine-treated cells; dashed lines represent control cells. The bar indicates the position of cells with reduced incorporation of NAO, and their percentage is given. One representative experiment out of two performed is shown. S – siramesine, T – α-tocopherol.

Supplementary Figure 3:
Ultrastructure and immunolabelling for LAMP-1 in siramesine-treated U-87 MG cells.
(a) For the analysis of ultrastructure, cells were fixed with 1% glutaraldehyde and embedded in epon, and thin sections were analysed with TEM.
(b-f) For immunolabelling, cells were fixed with 4% PFA, infiltrated with sucrose and vitrified with liquid nitrogen to make 100 nm thin Tokuyasu sections, which were labelled with LAMP-1 antibodies and analysed with a fluorescence microscope (LAMP-1: green, DAPI: blue) (b) or TEM (LAMP-1: gold 10 nm) (c-f).
Bar size 1 μm. G - Golgi cisterna, E/L - endosome/lysosome.

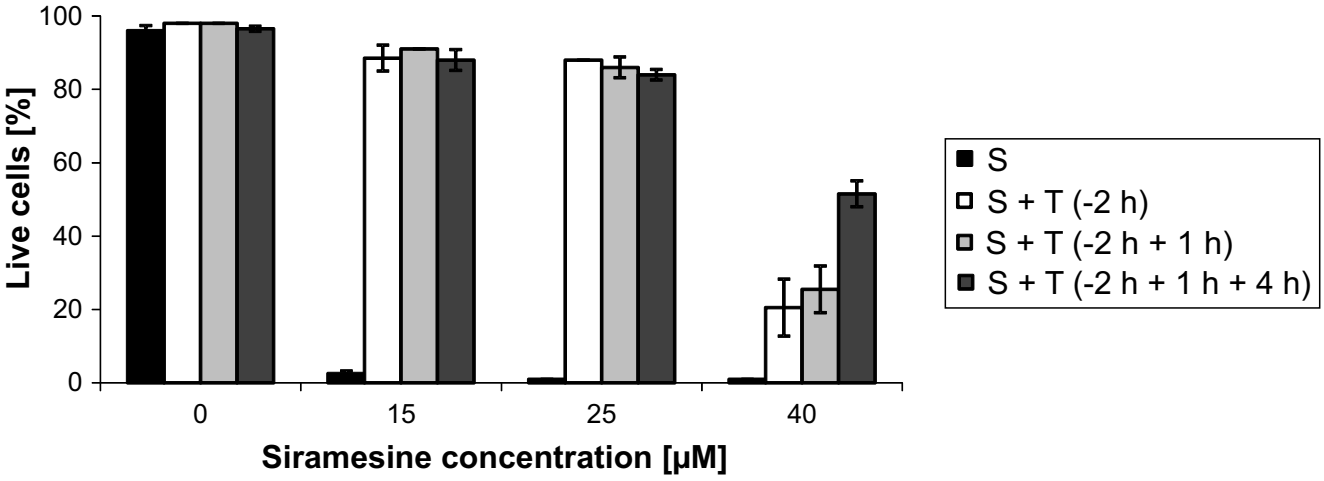
Supplementary Figure 4:
(a) **Optimisation of digitonin concentrations for fluorimetric detection of cytosolic cysteine cathepsin activity after siramesine treatment.** HaCaT cells grown in a 96-well plate were treated with different siramesine concentrations. After 1-hour incubation with siramesine, different concentrations of digitonin (15, 30, 45, 60, 70, 200 μg/ml) were added directly into the wells to lyse only the plasma membrane (cytosolic activity) or all cell membranes (total activity). After 12-minute incubation with digitonin on ice, the cysteine cathepsin activity was measured fluorimetrically using Z-FR-AMC, and the initial velocities of the reactions were calculated. The experiments were performed in triplicate; the bars represent mean ± SD.
(b) **Fluorimetric detection of cytosolic and total cysteine cathepsin activity after siramesine treatment and pretreatment with α-tocopherol.**

HaCaT and U-87 MG cells growing in a 96-well plate were treated with different siramesine concentrations. Alpha-tocopherol was added 2 hours before siramesine. After incubation with siramesine, 25 µg/ml or 200 µg/ml digitonin was added directly to the wells to lyse only the plasma membrane (cytosolic activity) or all cell membranes (total activity), respectively. After 12-minute incubation with digitonin on ice, the cysteine cathepsin activity was measured fluorimetrically using Z-FR-AMC, and the initial velocities of the reactions were calculated. The experiments were performed in triplicate; the bars represent mean ± 2SD.
(c) **Fluorimetric detection of cytosolic and total N-acetyl-β-D-glucosaminidase (NAG) activity after siramesine treatment.** HaCaT cells grown in a 96-well plate were treated with different siramesine concentrations. After incubation with siramesine, 15 or 200 µg/ml digitonin was added directly to the wells to lyse only the plasma membrane (cytosolic activity) or all cell membranes (total activity), respectively. After 12-minute incubation with digitonin on ice, 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide was added in citrate buffer and incubated for 30 minutes at 37 °C. After incubation, a glycine buffer was added, and substrate hydrolysis was measured. The experiments were performed in triplicate; the bars represent mean ± SD. D – digitonin, T – α-tocopherol, TOT – total, CYT – cytosolic.

Supplementary Figure 5:
Effect of siramesine on protein levels of cathepsins B and C. HaCaT cells were treated with different siramesine concentrations, and total cell extracts were prepared in RIPA buffer at the indicated time points. The proteins were resolved in 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. Cathepsin B and C were labelled with specific antibodies. S – siramesine.

Supplementary Figure 6:
Effect of siramesine on ER and glycogen consumption.
(a) Effect of siramesine on free cellular calcium. HaCaT cells growing in a 96-well plate were first stained with Fluo-4 at a final concentration of 2 µl/ml for 30 minutes at 37 °C. After washing, cells were treated with siramesine. Immediately after treatment, the fluorescence was monitored for 30 minutes. One representative experiment out of three performed is displayed.
(b and c) TEM micrographs. U-87 MG cells were untreated or treated with 40 µM siramesine for 6 hours with or without pre-treatment with 0.3 mM α-tocopherol. Cells were fixed with 1% glutaraldehyde, embedded in epon and thin sections were analysed with TEM. (b) TEM micrographs showing ER. ER cisternae are labelled with asterisk. After 6-hour treatment with 40 µM siramesin, in some cells ER cisternae were enlarged, whereas in cells pre-treated with α-tocopherol ER appeared normal. (c) TEM micrographs showing glycogen grains. Abundant glycogen grains (roughly twice the ribosome size) are seen in control cells and cells treated with α-tocopherol and siramesine, but not in cells treated only with siramesine. Bar size 1 µm. S – siramesine, T – α-tocopherol, G – glycogen.

Suppl. Fig. 1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80
60
40
20
0
Live cells [%]
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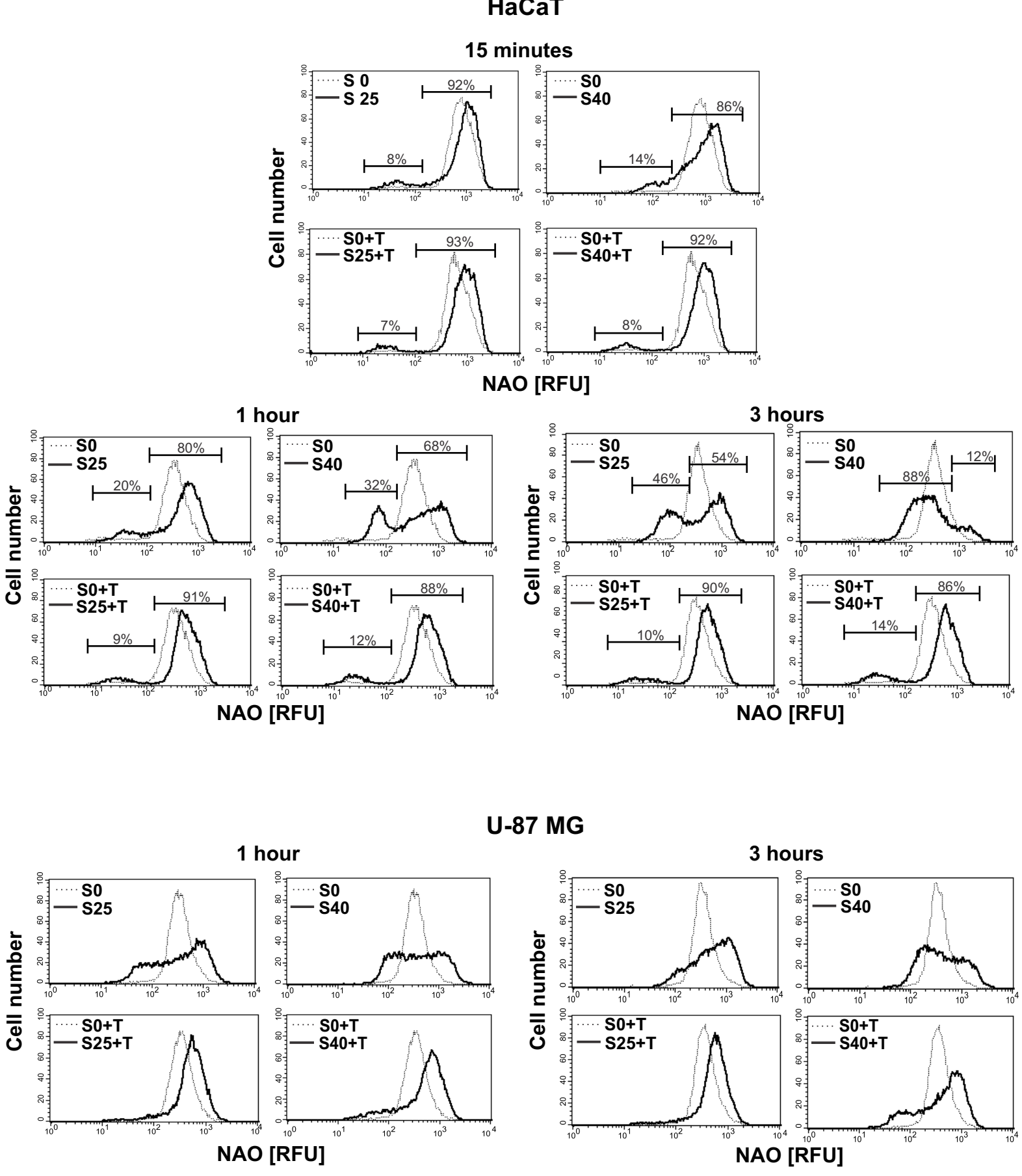
15
25
40
Siramesine concentration [µM]
S
S + T (-2 h)
S + T (-2 h + 1 h)
S + T (-2 h + 1 h + 4 h)

Suppl. Fig. 2


HaCaT
15 minutes
S 0
S 25
92%
8%
S0
S40
86%
14%
S0+T
S25+T
93%
7%
S0+T
S40+T
92%
8%
Cell number
NAO [RFU]
1 hour
3 hours
S0
S25
80%
20%
S0
S40
68%
32%
S0
S25
54%
46%
S0
S40
12%
88%
S0+T
S25+T
91%
9%
S0+T
S40+T
88%
12%
S0+T
S25+T
90%
10%
S0+T
S40+T
86%
14%
Cell number
NAO [RFU]
U-87 MG
1 hour
3 hours
S0
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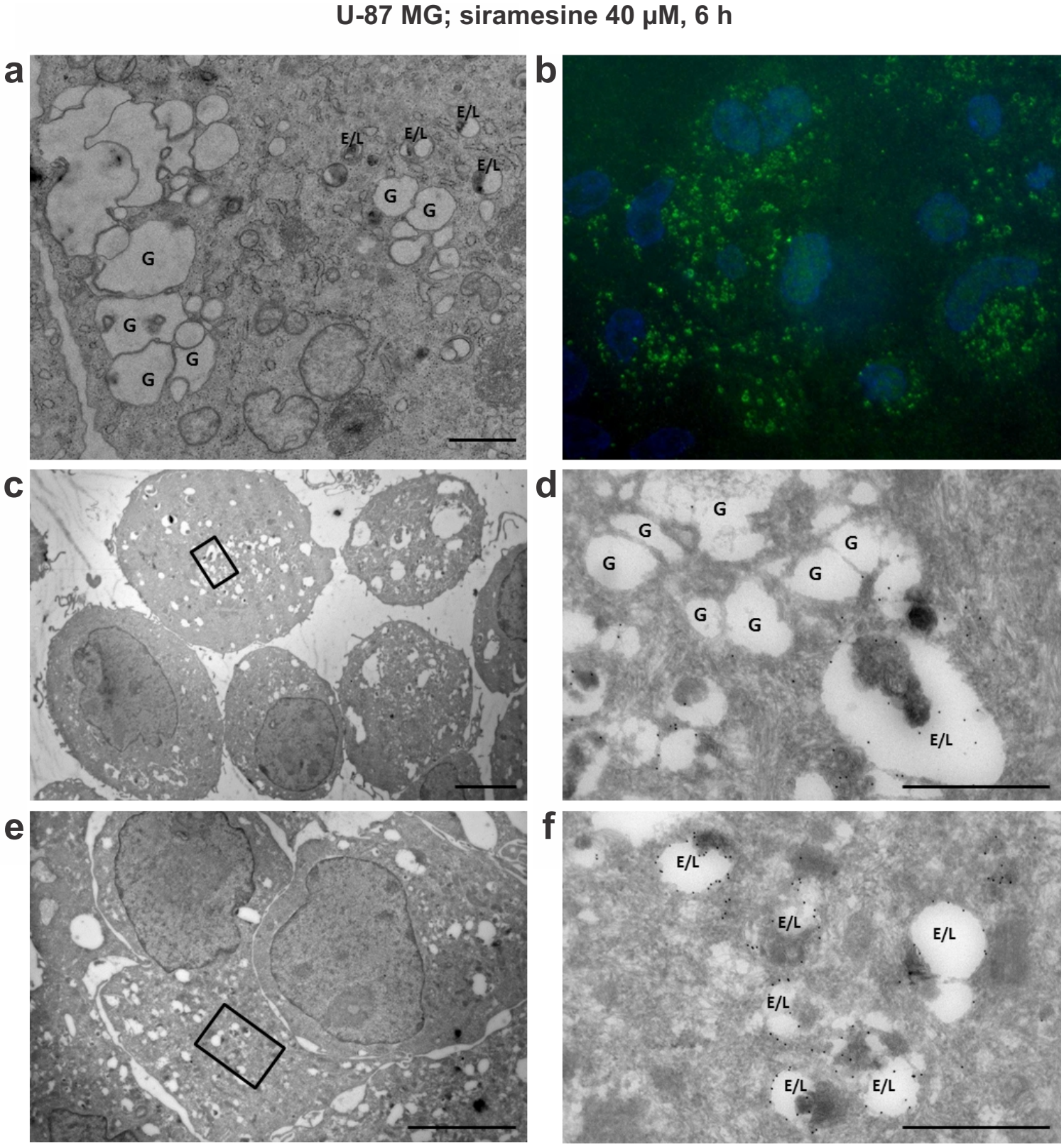
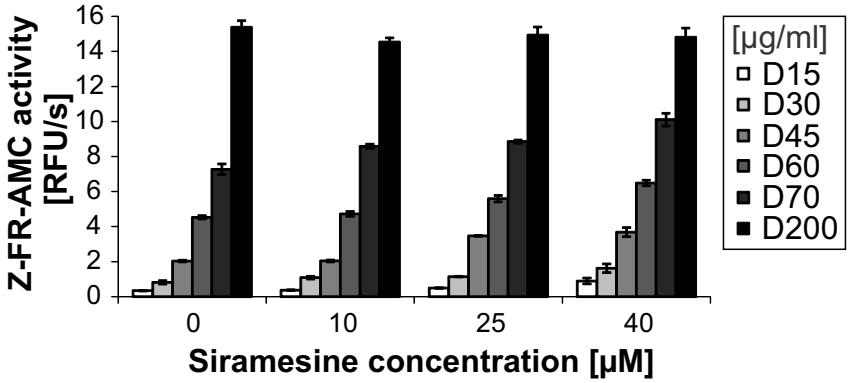
Suppl. Fig. 3



Suppl. Fig. 4

a



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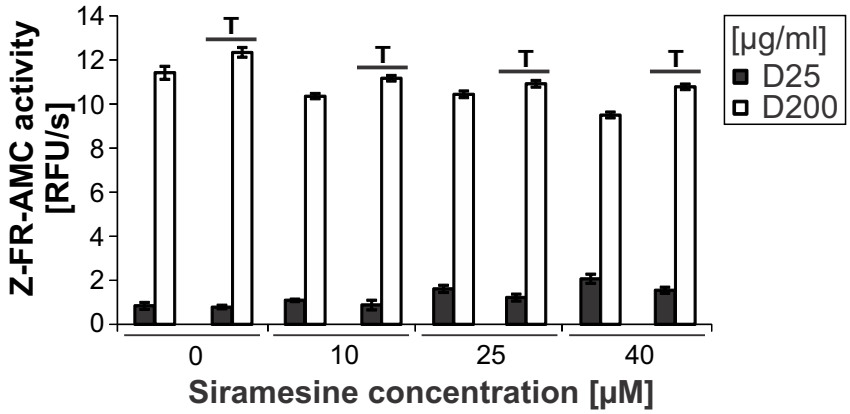

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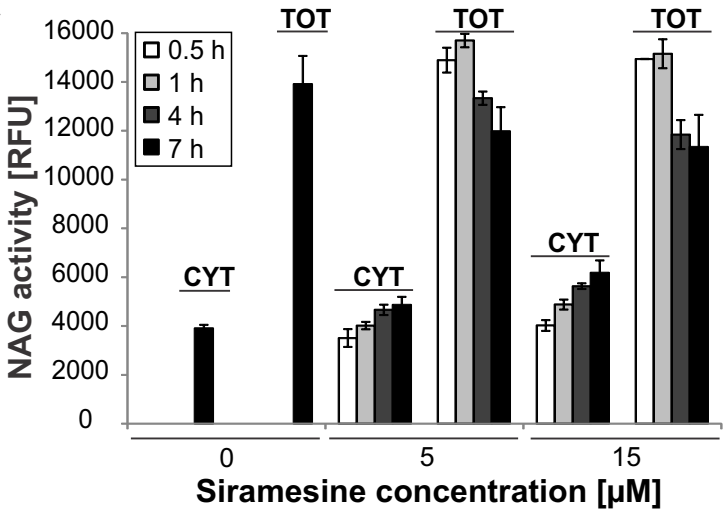
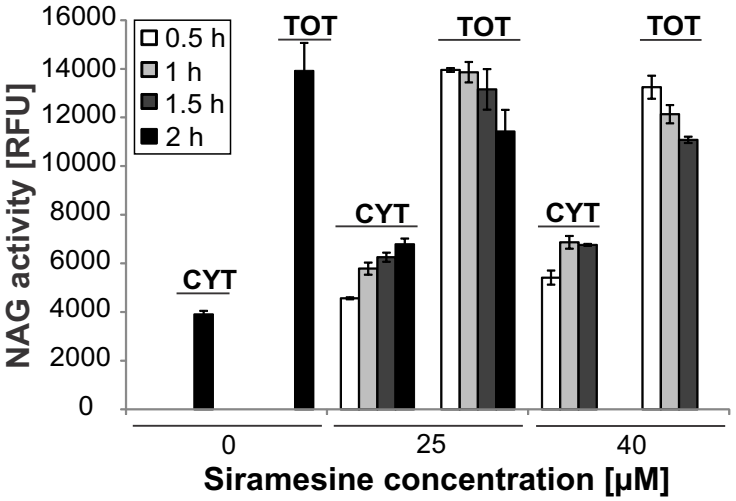
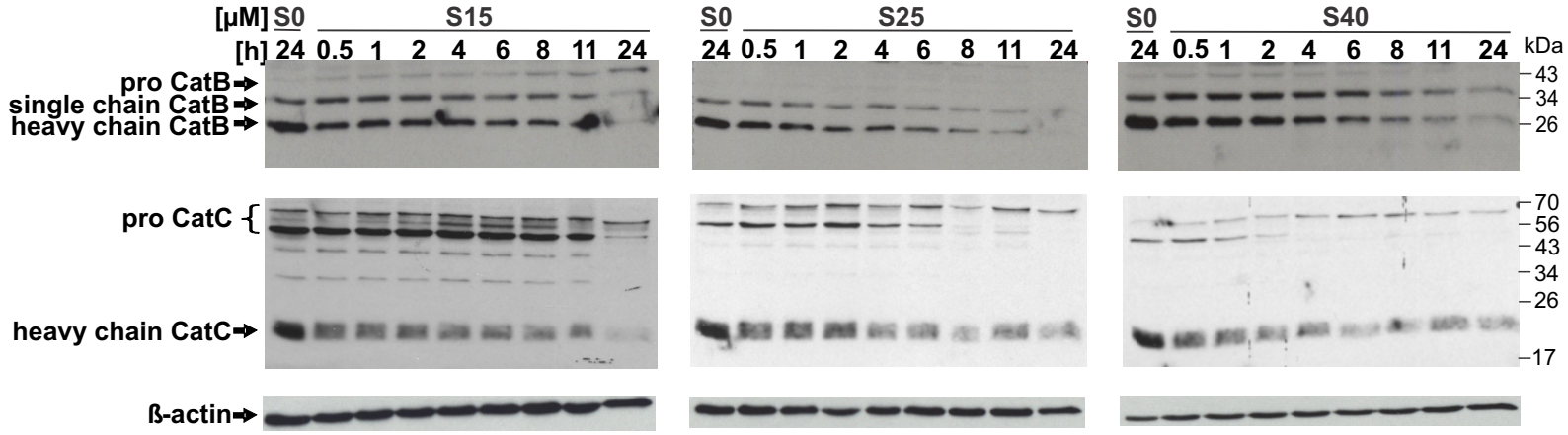




Suppl. Fig. 5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S0 S40
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kDa
pro CatB
single chain CatB
heavy chain CatB
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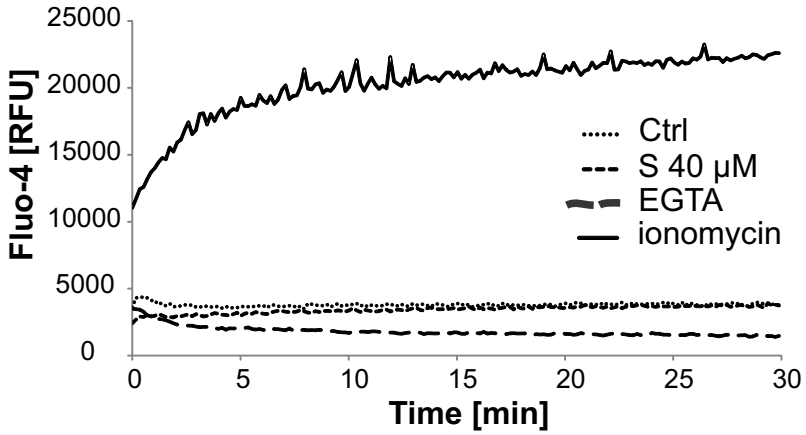
34
26
pro CatC
heavy chain CatC
70
56
43
34
26
17
ß-actin

Suppl. Fig. 6

a



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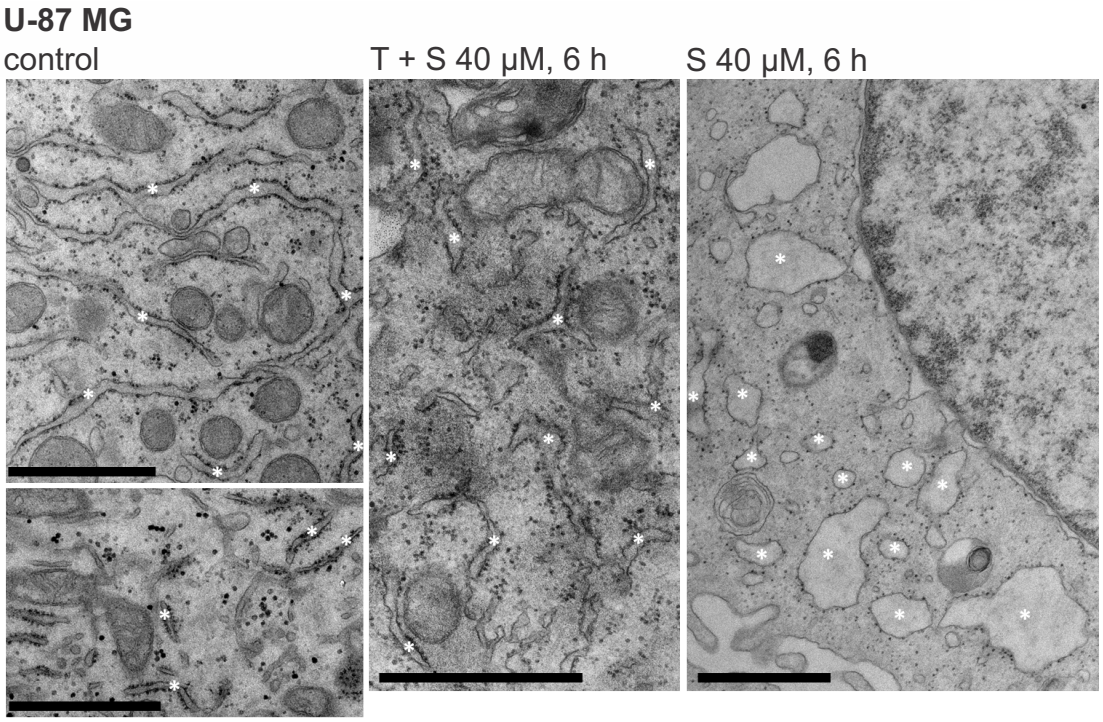

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