Supplementary Information:

Direct control of lysosomal catabolic activity by mTORC1 through regulation of V-ATPase assembly

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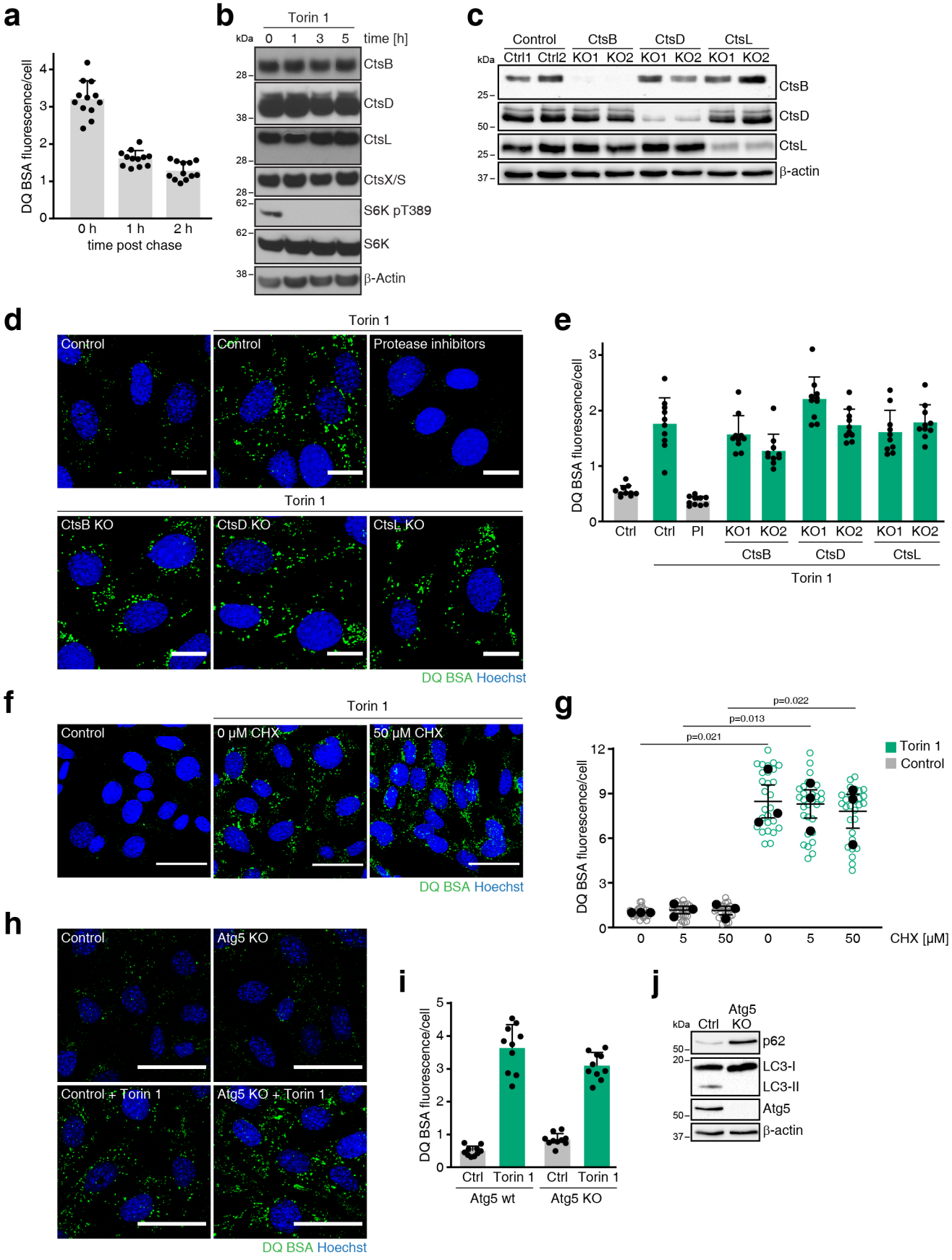
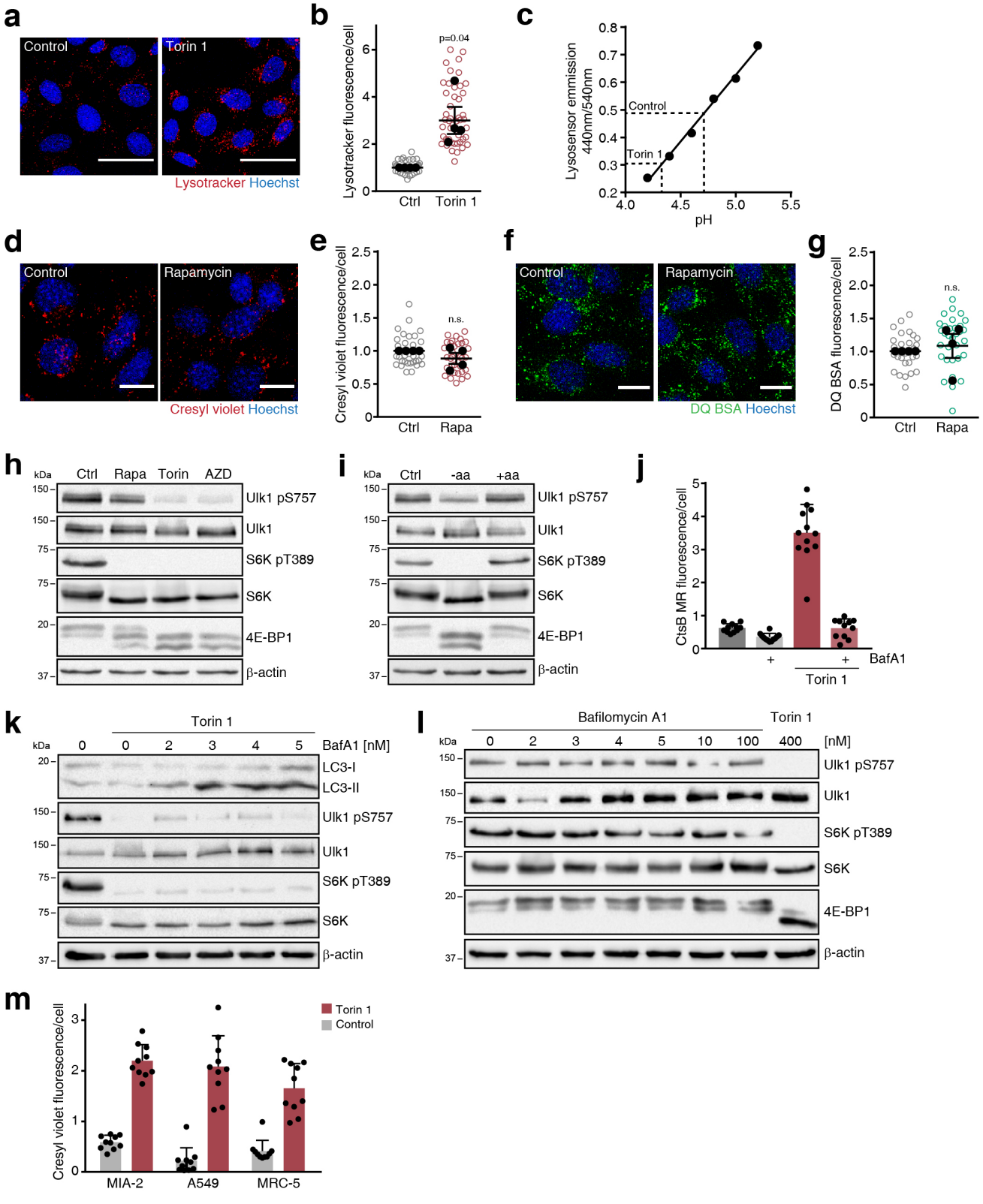
Supplementary figures



Supplementary Figure 1 mTORC1 does not block lysosomal catabolism of extracellular proteins through changes in cathepsin levels, protein synthesis or autophagy

a) Quantification of DQ BSA degradation in MEFs after 4 h pre-loading followed by chase for indicated periods of time. Data are mean ± SD (10 fields of view). **b)** Mature cathepsin levels in MEFs + torin 1 [250 nM] for indicated periods of time, analysed by western blot. **c)** CRISPR/Cas9-mediated deletion of cathepsin B, cathepsin D or cathepsin L, analysed by western blot. **d)** DQ BSA degradation in MEFs deficient for cathepsin B, cathepsin D or cathepsin L, or treated with protease inhibitors (PI; 20 μM pepstatin, leupeptine, E64, AEBSF) after 5 h DQ BSA uptake + torin 1 [400 nM]. Scale bars = 20 μm. **e)** Quantification of DQ BSA fluorescence of cells shown in d). Data are mean ± SD (10 fields of view). **f)** DQ BSA degradation in MEFs after 5 h DQ BSA uptake ± torin 1 [250 nM] and cycloheximide (CHX) at indicated concentrations. Scale bars = 50 μm. **g)** Quantification of DQ BSA fluorescence of cells treated as in f). Data are normalized replicate mean ± SEM (closed circles) and fields of view (open circles; 6-12 per replicate). **h)** DQ BSA degradation in Atg5-deficient MEFs after 5 h DQ BSA uptake ± torin 1 [400 nM]. Scale bars = 20 μm. **i)** Quantification of DQ BSA fluorescence of cells shown in h). Data are mean ± SD (10 fields of view). **j)** CRISPR/Cas9-mediated deletion of Atg5, analysed by western blot. a), e), i) One representative of n=3 biologically independent experiments. b), c), j) One representative of n=2 biologically independent experiments. g) n=3 biologically independent experiments. p-values were calculated using a two-tailed unpaired t-test with Welch correction. Source data are provided as a Source Data file.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Control
Torin 1
pH
d
Control
Rapamycin
Cresyl violet Hoechst
e
Cresyl violet fluorescence/cell
n.s.
Ctrl
Rapa
f
Control
Rapamycin
DQ BSA Hoechst
g
DQ BSA fluorescence/cell
n.s.
Ctrl
Rapa
h
kDa
Ctrl
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Torin
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Ulk1
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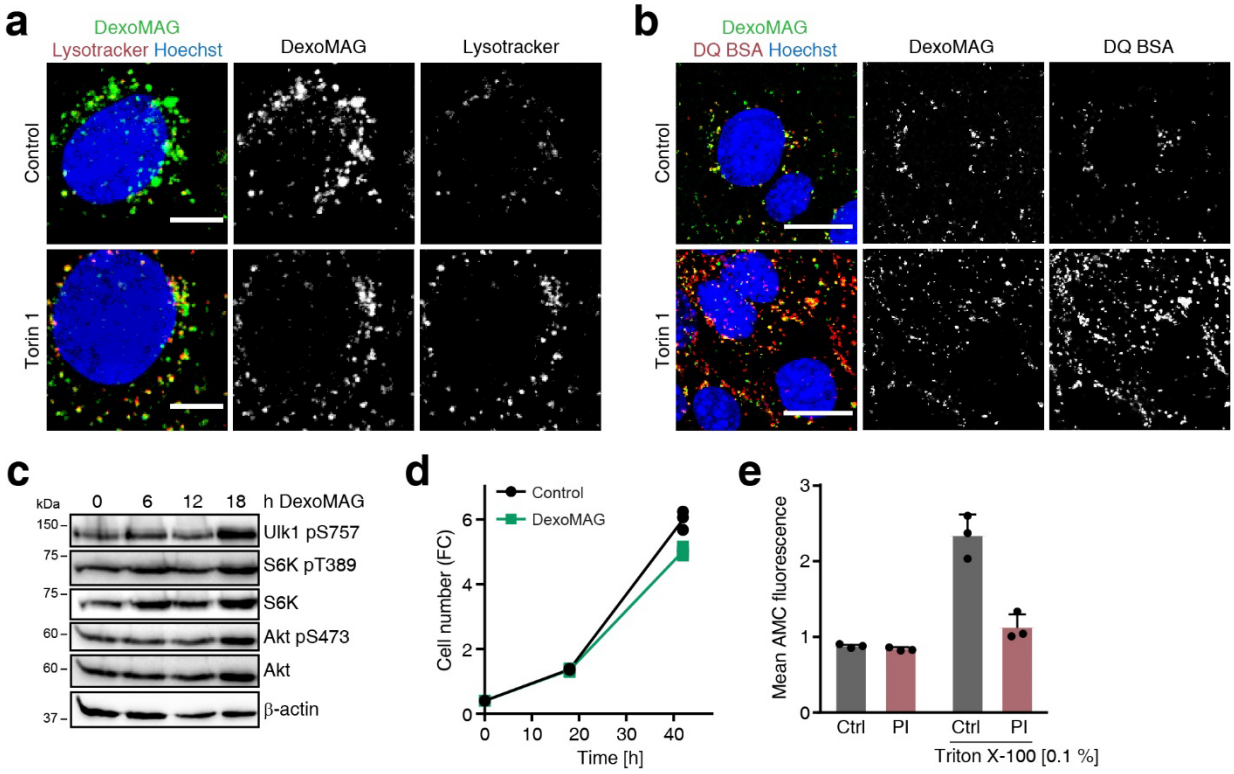
β-actin
m
Cresyl violet fluorescence/cell
Torin 1
Control
MIA-2
A549
MRC-5

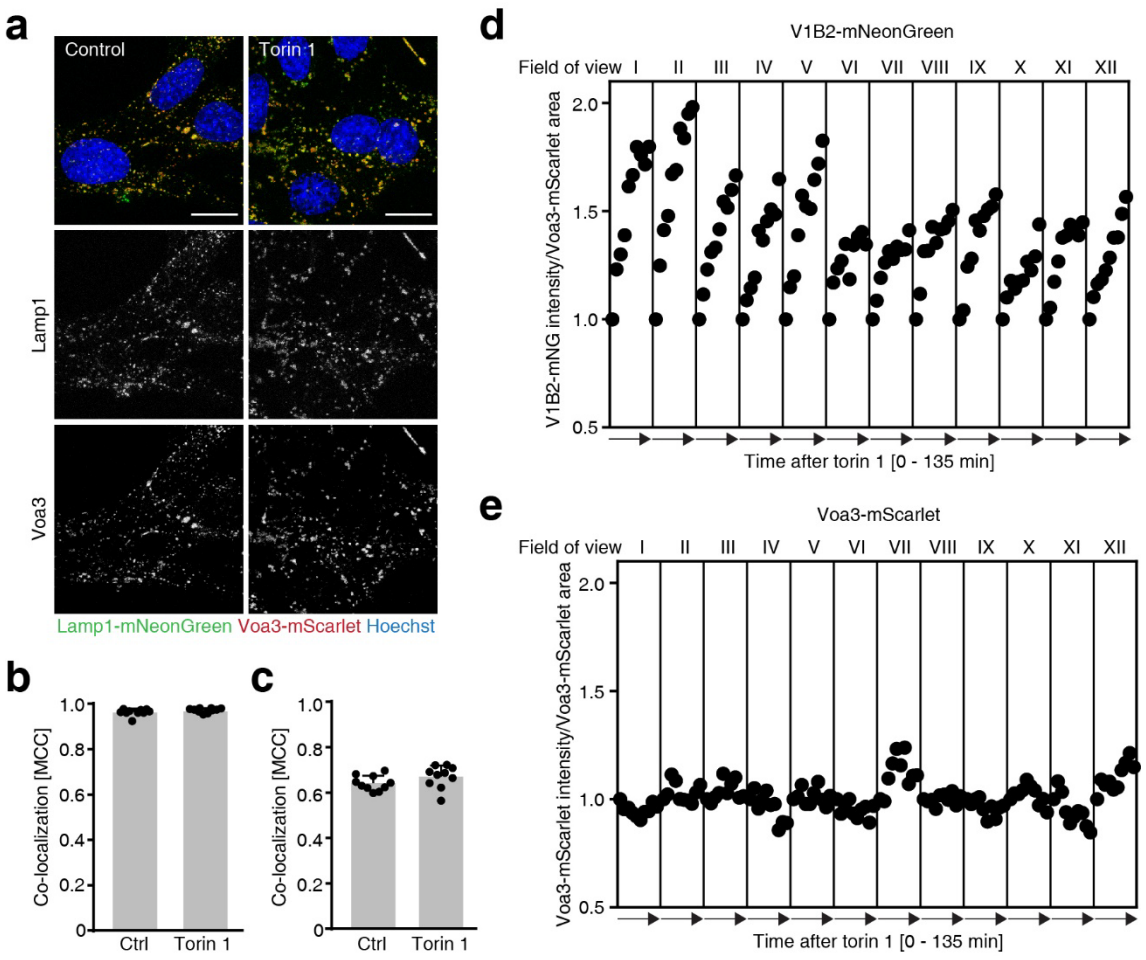
Supplementary Figure 2 mTORC1 regulates lysosomal catabolism

a) Lysotracker accumulation in MEFs after 1 h ± torin 1 [400 nM]. Scale bars = 50 µm. **b)** Quantification of lysotracker fluorescence in cells treated as in a). Data are normalized replicate mean ± SEM (closed circles) and fields of view (open circles; 10-15 per replicate). **c)** Exemplary calibration curve for lysosensor-based pH measurements. Dashed lines indicate dual-emission ratiometric measurements and corresponding pH values in MEFs after 1 h ± torin 1 [400 nM] in the same experiment. **d)** Cresyl violet accumulation in MEFs after 1 h ± rapamycin [200 nM]. Scale bars = 20 µm. **e)** Quantification of cresyl violet fluorescence in cells treated as in d). Data are normalized replicate mean ± SEM (closed circles) and fields of view (open circles; 8-10 per replicate). **f)** DQ-BSA degradation in MEFs after 5 h DQ BSA uptake ± rapamycin [200 nM]. Scale bars = 20 µm. **g)** Quantification of DQ BSA degradation in cells treated as in f). Data are normalized replicate mean ± SEM (closed circles) and fields of view (open circles; 6-10 per replicate). **h)** mTORC1 signalling activity in MEFs after 1 h rapamycin [100 nM], torin 1 [250 nM], or AZD8055 [250 nM] as in Fig. 2c), analysed by western blot. **i)** mTORC1 signalling activity in MEFs after 1 h amino acid starvation (-aa), or 1 h aa starvation + 30 min aa restimulation (+aa) as in Fig. 2d), analysed by western blot. **j)** Quantification of cathepsin B magic red substrate degradation in MEFs pre-treated ± 1 h torin 1 [250 nM], bafilomycin A1 [2.5 nM]. Data are mean ± SD (10 fields of view). **k)** LC3-II levels and mTORC1 signalling activity in MEFs after 2 h + torin 1 [400 nM] and bafilomycin A1 at indicated concentrations, analysed by western blot. **l)** mTORC1 signalling activity in MEFs after 2 h + bafilomycin A1 at indicated concentrations or torin 1 [400 nM], analysed by western blot. **m)** Quantification of cresyl violet accumulation after 3 h ± torin 1 [400 nM] in cell lines shown in Fig. 2h). Data are mean ± SD (10 fields of view). b), e), g) n=4 biologically independent experiments. h) - m) One representative of n=3 biologically independent experiments. p-values were calculate using a two-tailed unpaired t-test with Welch correction. n.s. not significant. Source data are provided as a Source Data file.



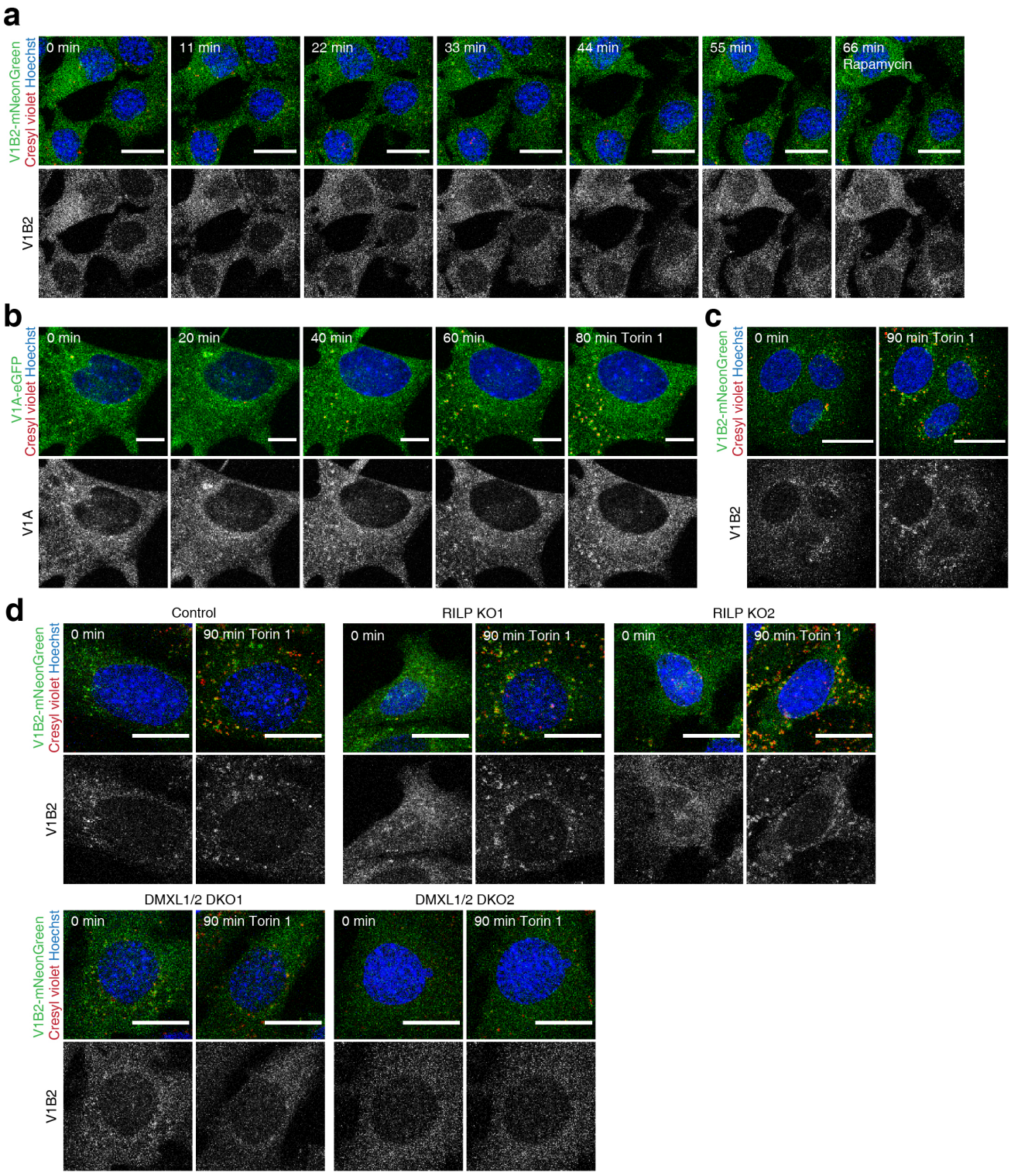
Supplementary Figure 3 Quality controls for magnetic enrichment of lysosomes

a) Co-localization of fluorescent DexoMAG and lysotracker in MEFs ± torin 1 [250 nM]. Scale bars = 10 µm. **b)** Co-localization of fluorescent DexoMAG and DQ BSA in MEFs after 5 h ± torin 1 [250 nM]. Scale bars = 20 µm. **c)** mTORC1 signalling activity in MEFs after incubation with DexoMAG for indicated periods of time, analysed by western blot. **d)** Proliferation of MEFs ± DexoMAG. Data are mean ± SD (3 technical replicates). **e)** Integrity of magnetically enriched lysosomes, as assessed by fluorescence dequenching of the cathepsin B substrate Z-Arg-Arg-AMC. Note that cathepsin activity increases substantially upon permeabilization of lysosomes with 0.1 % Triton X-100. PI: protease inhibitor (10 µM leupeptin). Data are mean ± SD (3 technical replicates). a) - e) One representative of n=2 biologically independent experiments. Source data are provided as a Source Data file.



Supplementary Figure 4 Live cell imaging reveals regulation of V-ATPase assembly at lysosomes by mTORC1

a) Co-localization of Voa3-mScarlet with Lamp1-mNeonGreen in MEFs after 1 h ± torin 1 [400 nM]. Scale bars = 20 µm. **b)**, **c)** Manders correlation coefficient (MCC) for co-localization of b) Voa3-mScarlet with Lamp1-mNeonGreen, c) Lamp1-mNeonGreen with Voa3-mScarlet of cells shown in a). Data are mean ± SD (10 fields of view). **d)**, **e)** Quantification of d) V1B2-mNeonGreen levels in Voa3-mScarlet-containing organelles, e) organellar Voa-3-mScarlet levels after torin 1 treatment [400 nM] over time. Data are individual fields of view in 15 min intervals. See also Fig. 4c). d), e) One representative of n=3 biologically independent experiments. Source data are provided as a Source Data file.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33 min
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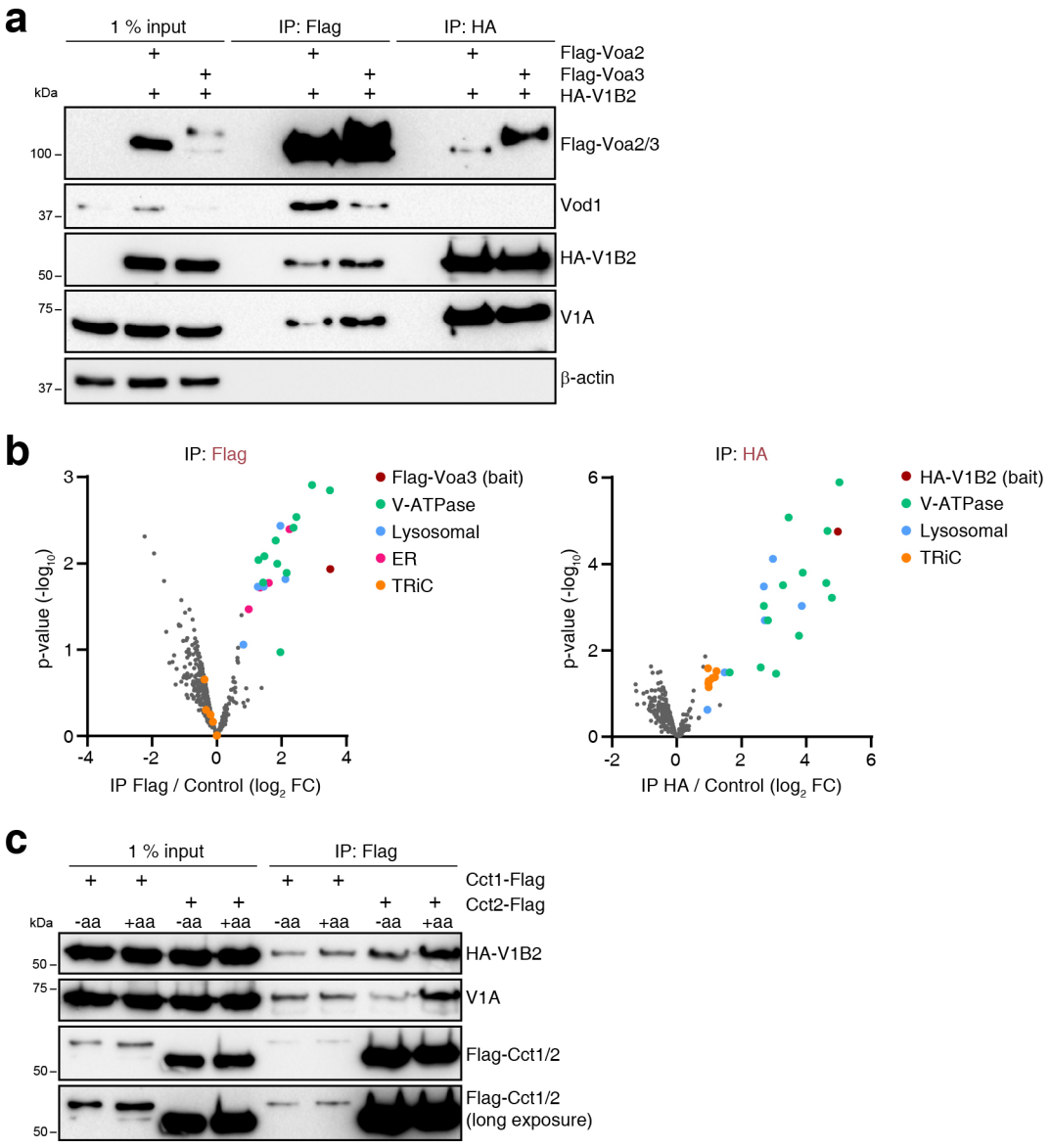
V1A-eGFP
Cresyl violet Hoechst
0 min
20 min
40 min
60 min
80 min Torin 1
V1A
c
V1B2-mNeonGreen
Cresyl violet Hoechst
0 min
90 min Torin 1
V1B2
d
Control
RILP KO1
RILP KO2
V1B2-mNeonGreen
Cresyl violet Hoechst
0 min
90 min Torin 1
V1B2
DMXL1/2 DKO1
DMXL1/2 DKO2

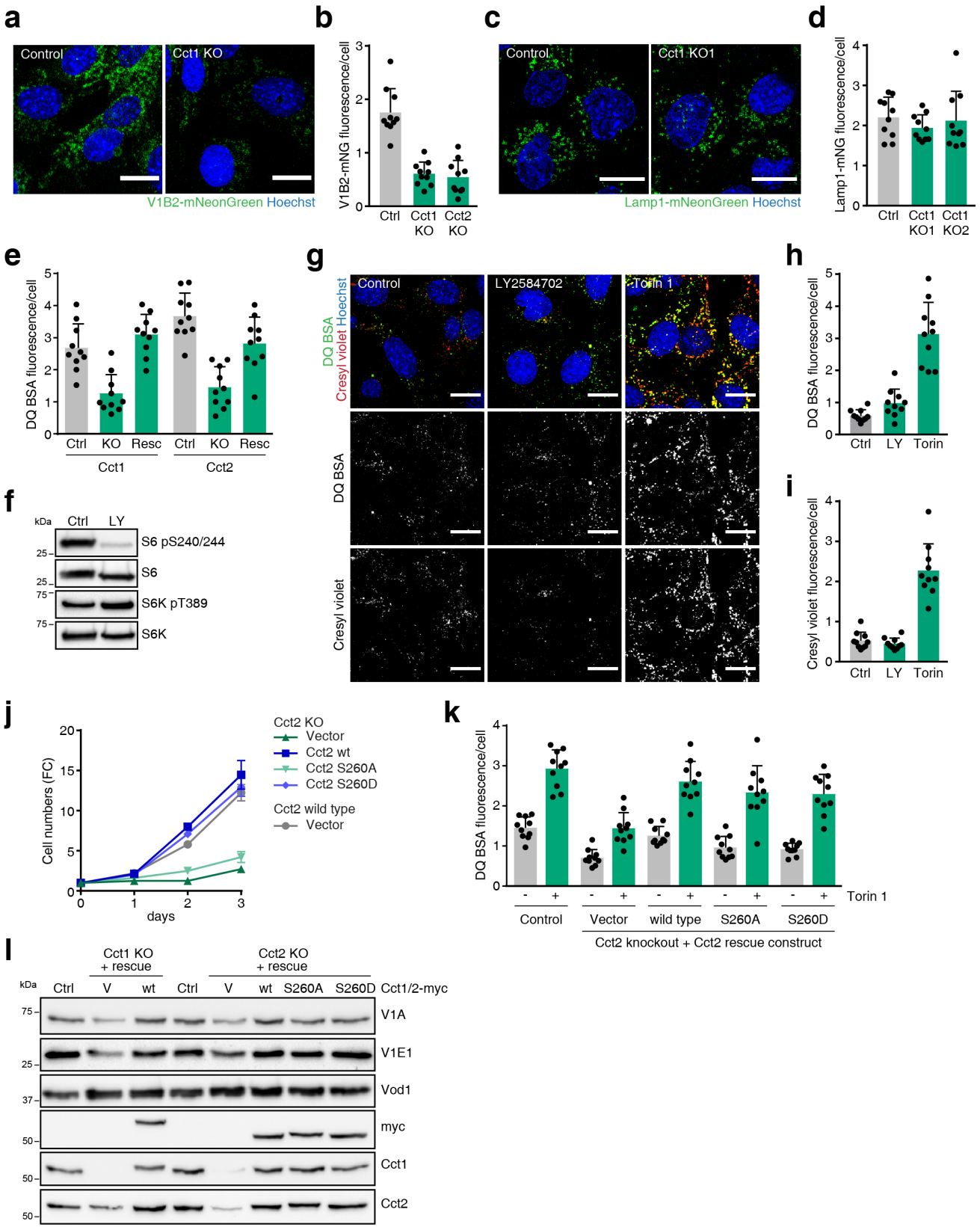
Supplementary Figure 5 Lysosomal movement of V-ATPase V_1 domains and lysosomal acidification in response to mTORC1 inactivation

a) No lysosomal accumulation of V1B2-mNeonGreen and cresyl violet in MEFs over time upon treatment with rapamycin [200 nM]. Scale bars = 20 µm. **b)** Lysosomal accumulation of V1A-eGFP and cresyl violet in MEFs over time upon treatment with torin 1 [400 nM]. Scale bars = 10 µm. **c)** Lysosomal accumulation of V1B2-mNeonGreen and cresyl violet in A549 cells before and after 90 min + torin 1 [400 nM]. Scale bars = 20 µm. **d)** Changes in lysosomal accumulation of V1B2-mNeonGreen and cresyl violet in MEFs deficient for RILP or DMXL1/2 before and after 90 min + torin 1 [400 nM]. Scale bars = 20 µm. a) - d) One representative of n=3 biologically independent experiments.



Supplementary Figure 6 The chaperonin TRiC reversibly associates with the V-ATPase V_1 domain

a) Co-IP of V-ATPase subunits in MEFs by Flag-Voa3, Flag-Voa2 or HA-V1B2, analysed by western blot. **b)** Co-IP – SILAC proteomics of V-ATPase subcomplex-interacting proteins. Shown is the $\log_2$ fold change of proteins from cells expressing the respective bait (Flag-Voa3 or HA-V1B2) over vector control cells. **c)** Increased Co-IP of V-ATPase V_1 domain subunits in MEFs with TRiC subunits Cct1 or Cct2 after 1 h amino acid starvation + 30 min amino acid restimulation (+aa) and after 1 h amino acid starvation (-aa), analysed by western blot. a) One representative of n=3 biologically independent experiments. b) n=4 biologically independent experiments. p-values were calculated using limma moderated t-statistic. c) One representative of n=2 biologically independent experiments. Source data are provided as a Source Data file.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d
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Ctrl
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Ctrl
KO
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l
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Cct2 KO + rescue
kDa
Ctrl
V
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V
wt
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S260D
Cct1/2-myc
V1A
V1E1
Vod1
myc
Cct1
Cct2

Supplementary Figure 7 Stabilization of the V-ATPase V_1 domain by TRiC is not regulated by S6K-mediated phosphorylation of Cct2

a) V1B2-mNeonGreen levels in Cct1-deficient MEFs. Scale bars = 20 µm. **b)** Quantification of V1B2-mNeonGreen levels in Cct1 and Cct2-deficient MEFs. **c)** Lamp1-mNeonGreen levels in Cct1-deficient MEFs. Scale bars = 20 µm. **d)** Quantification of Lamp1-mNeonGreen levels in Cct1-deficient MEFs. **e)** Quantification of DQ BSA degradation in Cct1 and Cct2-deficient MEFs ectopically expressing Cct1 and Cct2 rescue construct, respectively, after 5 h DQ BSA uptake + torin 1 [400 nM]. **f)** Inhibition of S6K activity after 6 h LY2584702 [5 µM], analysed by western blot. **g)** DQ BSA and cresyl violet in MEFs after 5 h + LY2584702 [5 µM] or torin 1 [400 nM]. Scale bars = 20 µm. **h)**, **i)** Quantification of h) DQ BSA, i) cresyl violet in MEFs after 5 h DQ BSA uptake + LY2584702 [5 µM] or torin 1 [400 nM] as shown in g). **j)** Proliferation of Cct2-deficient MEFs ectopically expressing Cct2 wild type, S260A or S260D. **k)** Quantification of DQ BSA degradation in Cct2-deficient MEFs ectopically expressing Cct2 wild type, S260A or S260D after 5 h DQ BSA uptake ± torin 1 [400 nM]. **l)** Changes in V-ATPase subunit abundance in Cct1 and Cct2-deficient MEFs ectopically expressing Cct1 and Cct2 rescue constructs, respectively, analysed by western blot. b), d), e), h), i), k) Data are mean ± SD (10 fields of view). j) Data are mean ± SD (3 technical replicates). a) - i) one representative of n=3 biologically independent experiments. k), l) one representative of n=5 biologically independent experiments. Source data are provided as a Source Data file.

Supplementary Tables

Supplementary Table 1 sgRNA sequences

Gene	Species	Name	sgRNA sequence 1	sgRNA sequence 2
Chr1.1	mouse	Control_sgRNA1	GACAATGAACATAAGCACAT	
Rosa1	mouse	Control_sgRNA2	GAAGATGGGCGGGAGTCTTC	
Cct1	mouse	CCT1_sgRNA1	GGTGGCACCATCGTTAGTAA	
Cct2	mouse	CCT2_sgRNA1	GGTTGGAGAGAAGCCACAA	
Atg5	mouse	ATG5_dsgRNA	ATCAAATAGTAAACCAAT	GAACATCACAGTACATTTCA
Rilp1	mouse	RILP1_dsgRNA1	TCTTGGAAAAGGCCGCCGTG	GCTCGTGTACCATCTAGCGG
Rilp1	mouse	RILP1_dsgRNA2	GGAGGTGACAGACAGACAGC	TCTTGGAAAAGGCCGCCGTG
Dmxl1	mouse	DMXL1_dsgRNA1	AGTTGGGGAAAGTACATGAG	TGATGGTGAGAGATCTAAGG
Dmxl1	mouse	DMXL1_dsgRNA2	AGTAGGGAGCCATGCCACAT	GCATTAAGGACACCAAATGT
Dmxl2	mouse	DMXL2_dsgRNA1	GTGGGACACAGTCAGAACAG	TATATGACAAAGGTCCAATG
Dmxl2	mouse	DMXL2_dsgRNA2	GGTAAGAGATGGAATATTGG	TTGAATGCGAATCTACAGGA
CtsB	mouse	CTSB_dsgRNA1	GGAGTCTACAATTCTCATGT	CTGGAGAAGGAGATACTCCC
CtsB	mouse	CTSB_dsgRNA2	TCGGCCATTGGTGTGAATGC	ATTGGACAGATTAGAGACCA
CtsD	mouse	CTSD_dsgRNA1	GTATCTTGCAATGAATGGAG	CCTGAGCCAGGACACTGTAT
CtsD	mouse	CTSD_dsgRNA2	GGGGCAGTGCCTCTTATCCA	CACTCGAAAGGCCTACTGGC
CtsL	mouse	CTSL_dsgRNA1	GCAAGAGAAAGCCCTCATGA	GGACAGATGTTCCTTAAGAC
CtsL	mouse	CTSL_dsgRNA2	AAGAGTGGAGGAGAGCGATA	CCAAGCAGAGGACAGCCAAA
Cct1	mouse	CCT1_dsgRNA1	AGTTGGCTTGGATAAAATGT	GTTCGGGGACCGCAGCACTG
Cct1	mouse	CCT1_dsgRNA2	GTTCGGGGACCGCAGCACTG	CAGATCATCAATCCGAAGGA
Cct2	mouse	CCT2_dsgRNA1	AGTGGCACCACGAAGCACAA	GATCCTAGAACAGTTTACGG

Supplementary Table 2 PCR primers for endogenous tagging of Lamp1

Primer ID	Sequence (5' to 3')
M1_mLAMP1	G*T*G*G*G*CGGTGCCCTGGCAGGGCTGGTCCTCATCGTCCTCATTGCCTACCTCATTGGC AGGAAGAGGAGTCACGCCGGCTATCAGACCATCTCAGGTGGAGGAGGTAGTG
M2_mLAMP1	G*C*T*T*G*GGGATGTGAGAACAGGCCCCTGTGCATCTCTGGTGCACCTGCCCACCAGGAA AAAACCCACCAGGCTAGATGGTCTATCTACAAGAGTAGAAATTAGCTAGCTGCATCGGTA CC