SUPPLEMENTAL DATA

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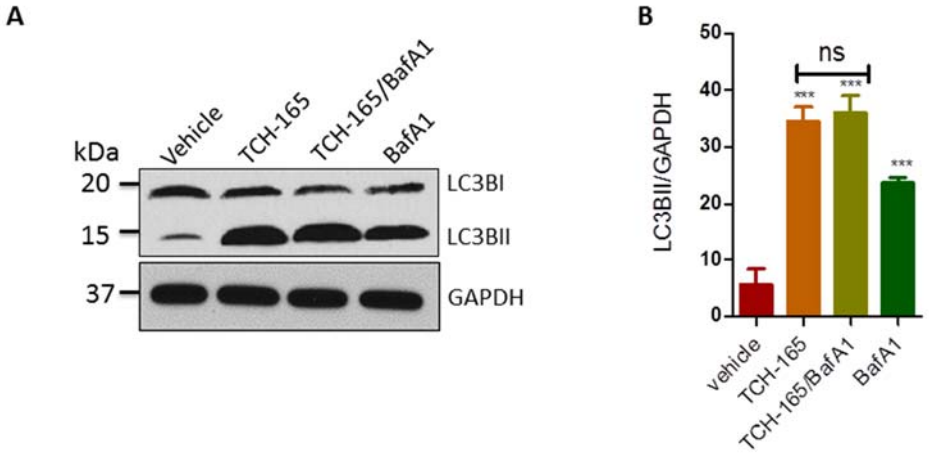

Figure S1: TCH-165 Inhibits autophagy flux in U-87 MG. Related to Figure 2 (A) U-87MG cells were treated with either vehicle (DMSO) or TCH-165 (10 µM) for 16h, followed by treatment with bafilomycin A1 (100 nM) for 4h. Conversion of LC3BI to LC3BII was detected by immunoblot with LC3B-specific antibody. All statistical analyses were performed on densitometry data (imageJ) of five individual experiments. **(B)** Data are graphed as mean ± SD (n=3) and were analyzed by One-Way ANOVA with Bonferroni's multiple comparison test (ns=not significant, *p<0.05, **p<0.01, ***p<0.001).

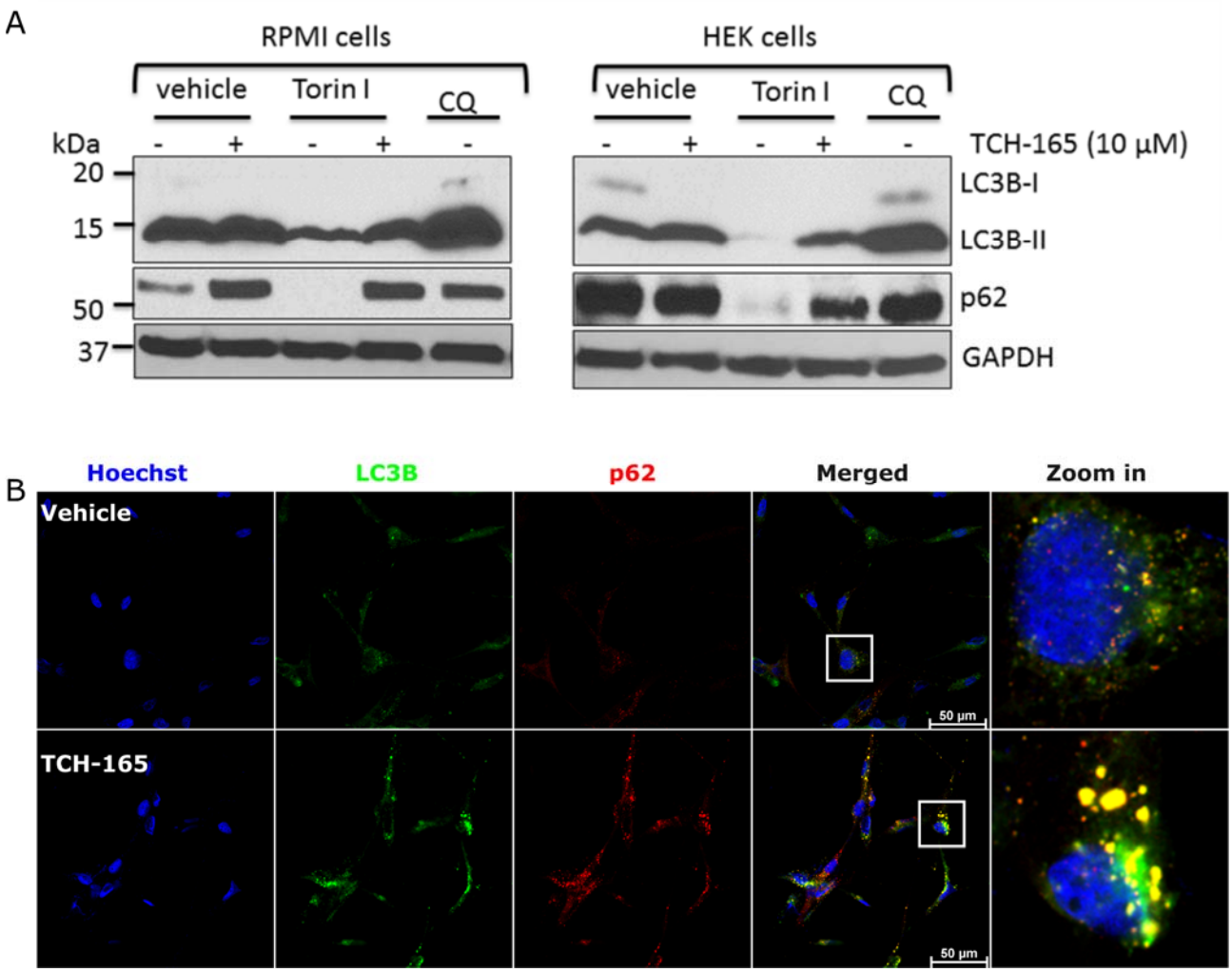

Figure S2: TCH-165 inhibits torin1-induced autophagy in RPMI and HEK 293T cells. Related to Figure 2. (A) RPMI (left) or HEK 293T (right) cells were treated with either vehicle, torin1 (200 nM), TCH-165 (10 µM) chloroquine (CQ; 50 µM) or combinations for 16h. Cell lysates were immunoblotted for LC3B, p62 and GAPDH. **(B)**U-87MG cells were treated with either vehicle or TCH-165 (10 µM) for 24h. Cells were fixed with methanol and immunostained with LC3B specific/Alexa Fluor 488 (green) antibody, p62 specific/Alexa Fluor 594 (red) antibody and Hoechst DNA dye (blue). Images were taken with an upright Nikon A1 confocal microscope using a 60x Plan Apo oil objective, with standard filter for DAPI, GFP and RFP. White square boxes indicate zoomed in areas showing accumulation/co-localization of p62 and LC3B in autophagic vacuoles, for TCH-165 treated cells compared to vehicle control.

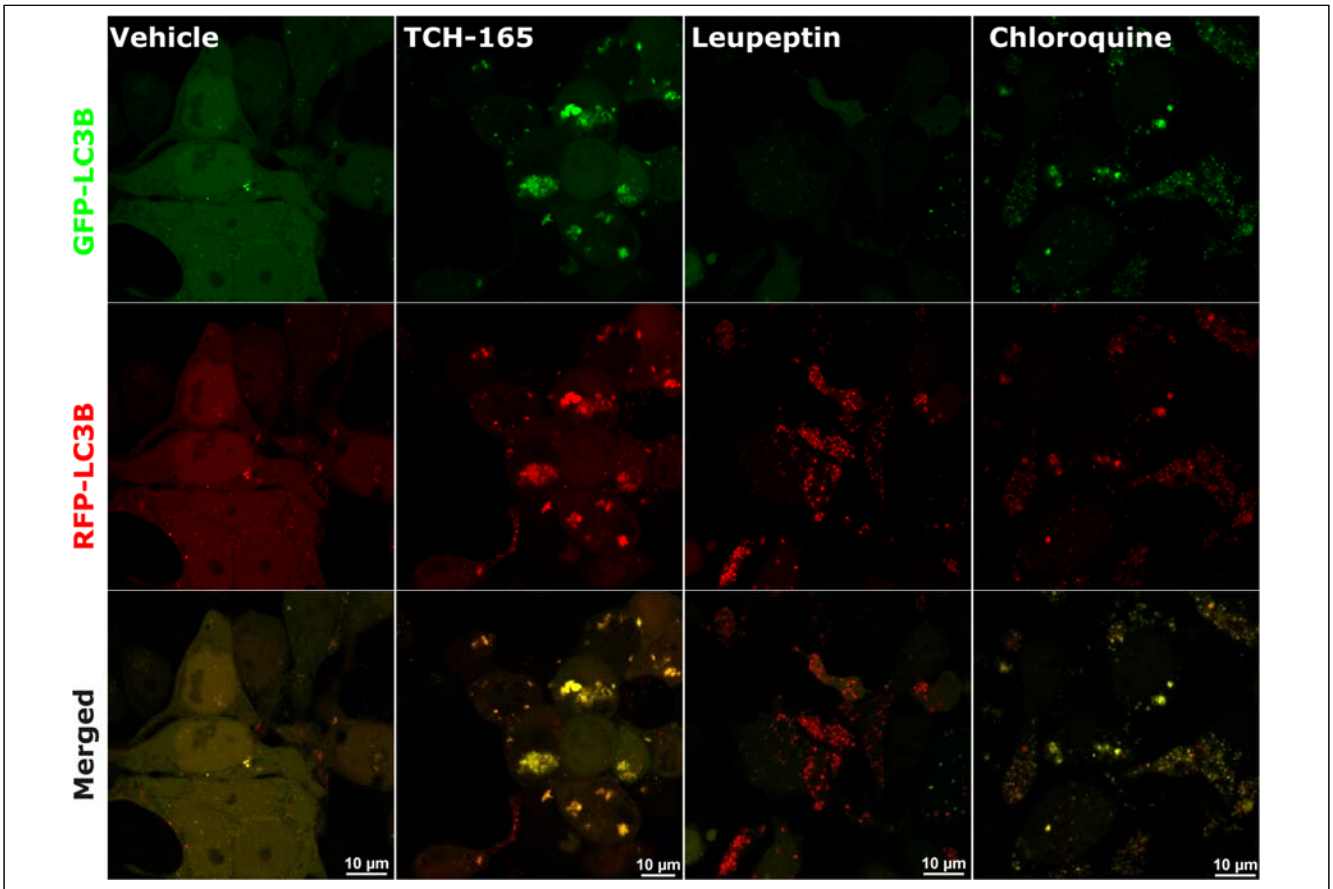

Figure S3: TCH-165 inhibits autophagosome-lysosome fusion in HEK cells. Related to Figure 3. HEK293T cells were transduced with 30 particles per cell of tandem-RFP-GFP-LC3B and cultured for 24h. Cells were then incubated with either vehicle, TCH-165 (10 μM), Chloroquine (100 μM) or Leupeptin A (200 μM) for an additional 24h. Cells were imaged on an upright Nikon A1 confocal microscope using standard filter sets for GFP

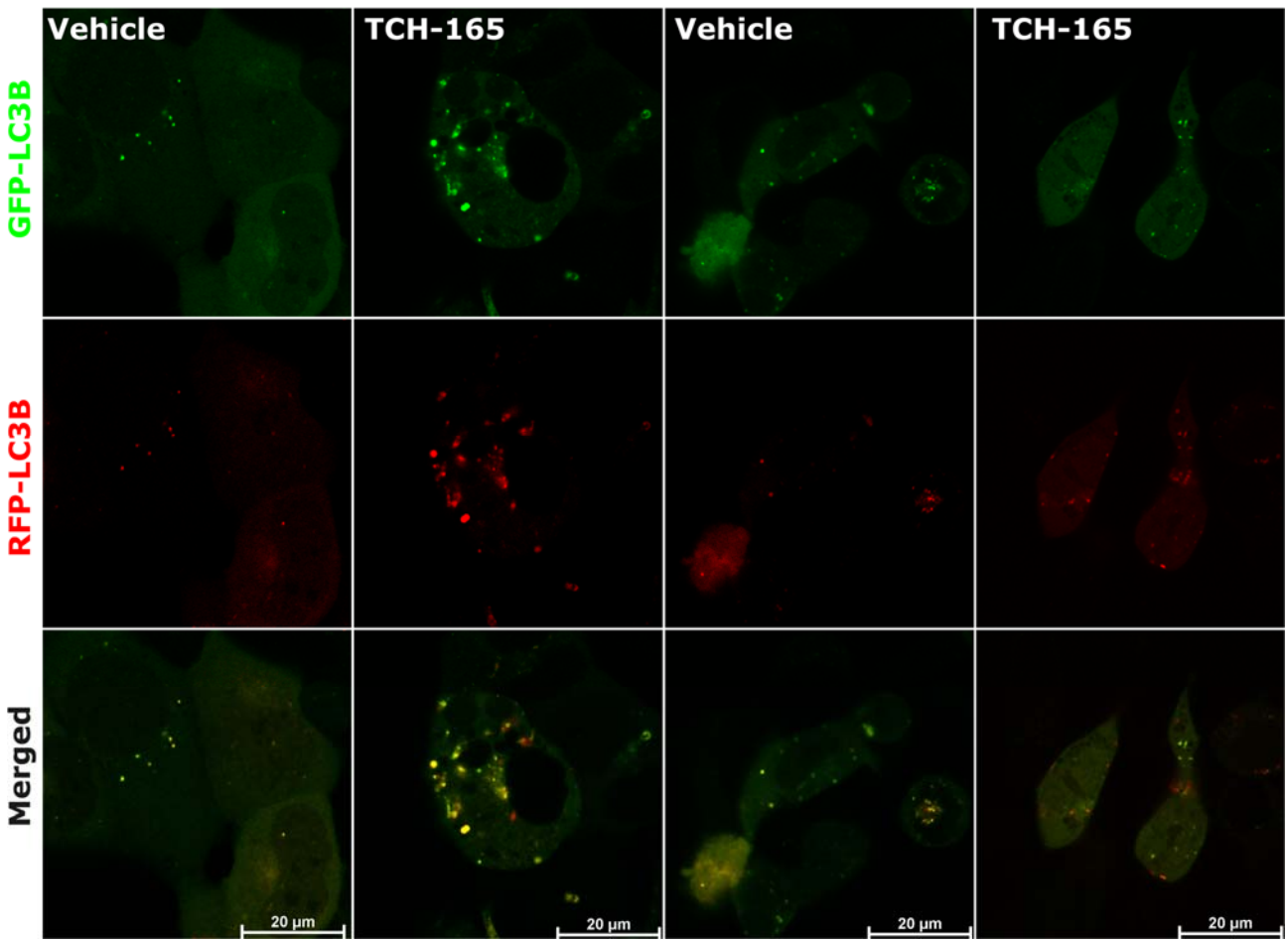

Figure S4: Co-overexpression of FLAG-SNAP29 and FLAG-STX17 ameliorates TCH-165 mediated accumulation of autophagic vacuoles. Related to Figure 5. HEK 293T cells were co-transfected with FLAG-SNAP29 and FLAG-STX17 for 8h. Both wild type (WT) and FLAG SNAP29/FLAG STX17 cells were further transduced with 30 particles per cell of tandem-RFP-GFP-LC3B and cultured for 24h. Cells were then incubated with either vehicle or TCH-165 (10 µM) for an additional 16h. Live cells were imaged on a Nikon A1 confocal microscope using a 60x Plan Apo oil objective with standard filter sets for GFP and RFP. In the merged images, red puncta represent autolysosomes while yellow puncta represent autophagosome.

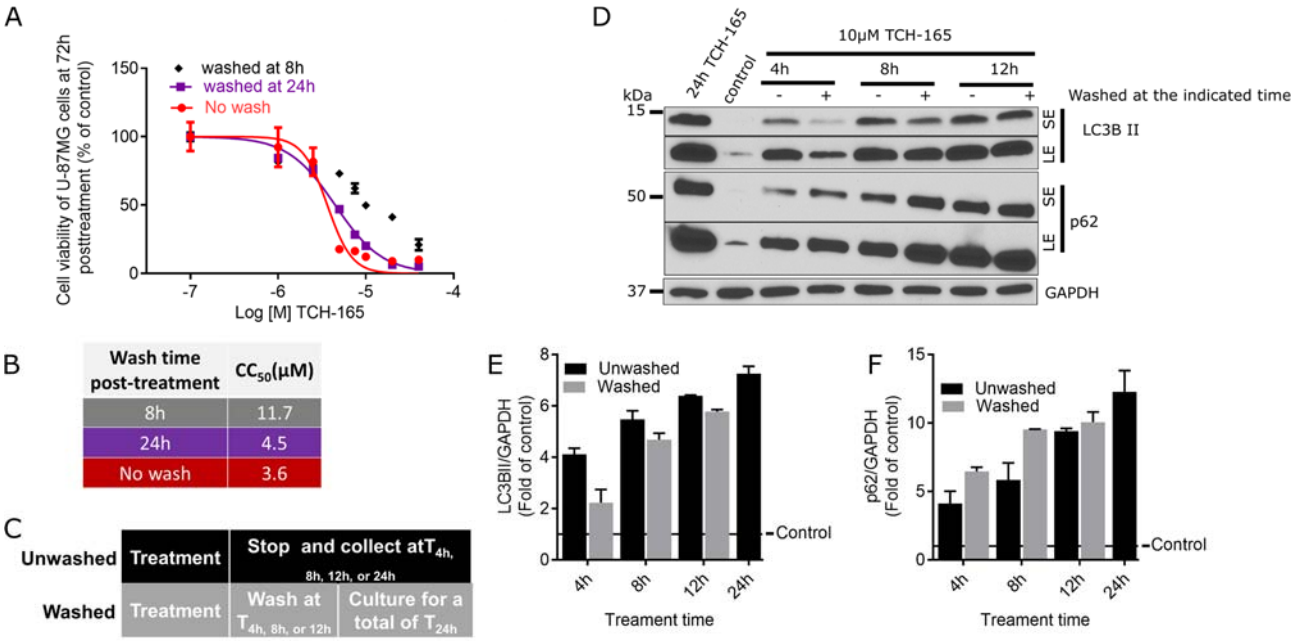

Figure S5: Reversibility of the effect of TCH-165. Related to STARS method Cell viability assay (CellTiter-Glo). (A) U-87 MG cells were treated with either vehicle or different concentrations of TCH-165. Cells were either not washed or washed at 8h, 24h and then cultured for a total of 72h. Cell viability was measured using CellTiter Glo. **(B)** Concentrations of TCH-165 required to reduce cell viability by 50% under the conditions in A. **(C)** Schematic of experiment for data D-F. **(D)** U-87 MG cells were treated with TCH-165 and cultured for 4h, 8h, 12h and 24h (unwashed) OR washed at 4h, 8h or 12h posttreatment with TCH-165 and cultured further, for a total of 24h (washed). Whole cell extracts were immunoblotted for LC3B, p62 and GAPDH. **SE**: Short exposure, **LE**: Long exposure. **(E)** Fold change in the ratio of LC3B-II/GAPDH as a function of 24h vehicle control (lane 2) **(F)** Fold change in the ration of p62GAPDH as a function of vehicle control (lane 2).

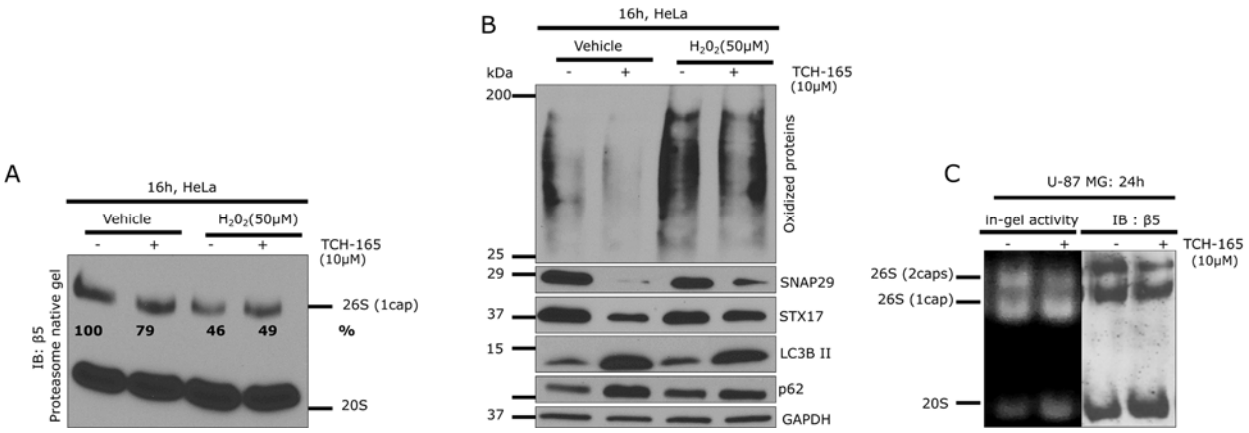

Figure S6: TCH-165 does not act via induction of oxidative stress. Related to STARS method OxyBlot (protein carbonyl quantification). HeLa cells were treated with either vehicle, TCH-165 (10 μM), hydrogen peroxide (H_2O_2; 50 μM) or combinations for 16h. Samples were either immunoblotted under native conditions for proteasome subcomplexes **(A),** derivatized with DNPH for oxidized proteins (**B**, top panel) or immunoblotted for the indicated proteins (**B**, bottom panels)**. (C)** Proteasome native gel for U-87 MG cells treated with vehicle or TCH-165 (10μM) for 24h and exposed to 20S CT-L substrate (Suc-LLVY-AMC) to check for activity (left) and immunoblotted (right) to check for changes in proteasome subcomplexes.

Table S1. Primers for SNAP29, STX17, VAMP8, LC3B, p62 and GAPDH, Related to Figure 4

SNAP 29 NM_004782.3	GTGCTGCAACAGTGCATTAG	CCTATGGAGGCTGTGGATATTT
Syntaxin 17 NM_017919.2	GGGTCATCCATTCTCCTTTACC	GCTGGACACTCAGTAAGGAATC
VAMP8 NM_003761.4	CATCTCCGCAACAAGACAGA	CTTCACGTTCTTCCACCAGAA
LC3B NM_022818.4	CATCACAGTTGGCACAAACG	GACTTTGGGTGTGGTTCTCTTA
P62 NM_003900.4	CTCTGGACACCATCCAGTATTC	TGCAATTCTACGCAAGCTTAAC
GAPDH NM_002046.6	CAGCCTCAAGATCATCAGCA	GTCATGAGTCCTTCCACGATAC