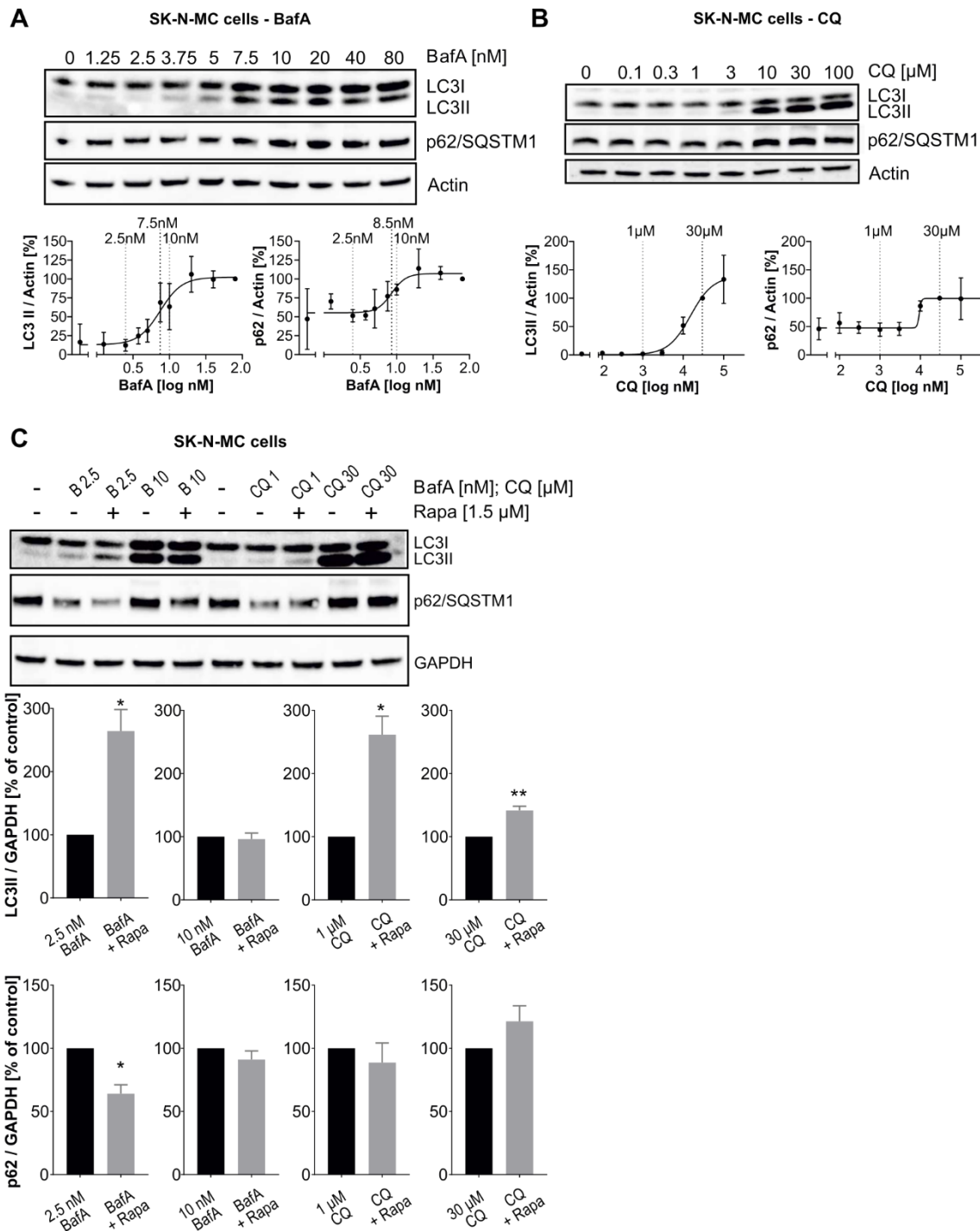


Robust LC3B lipidation analysis by precisely adjusting autophagic flux

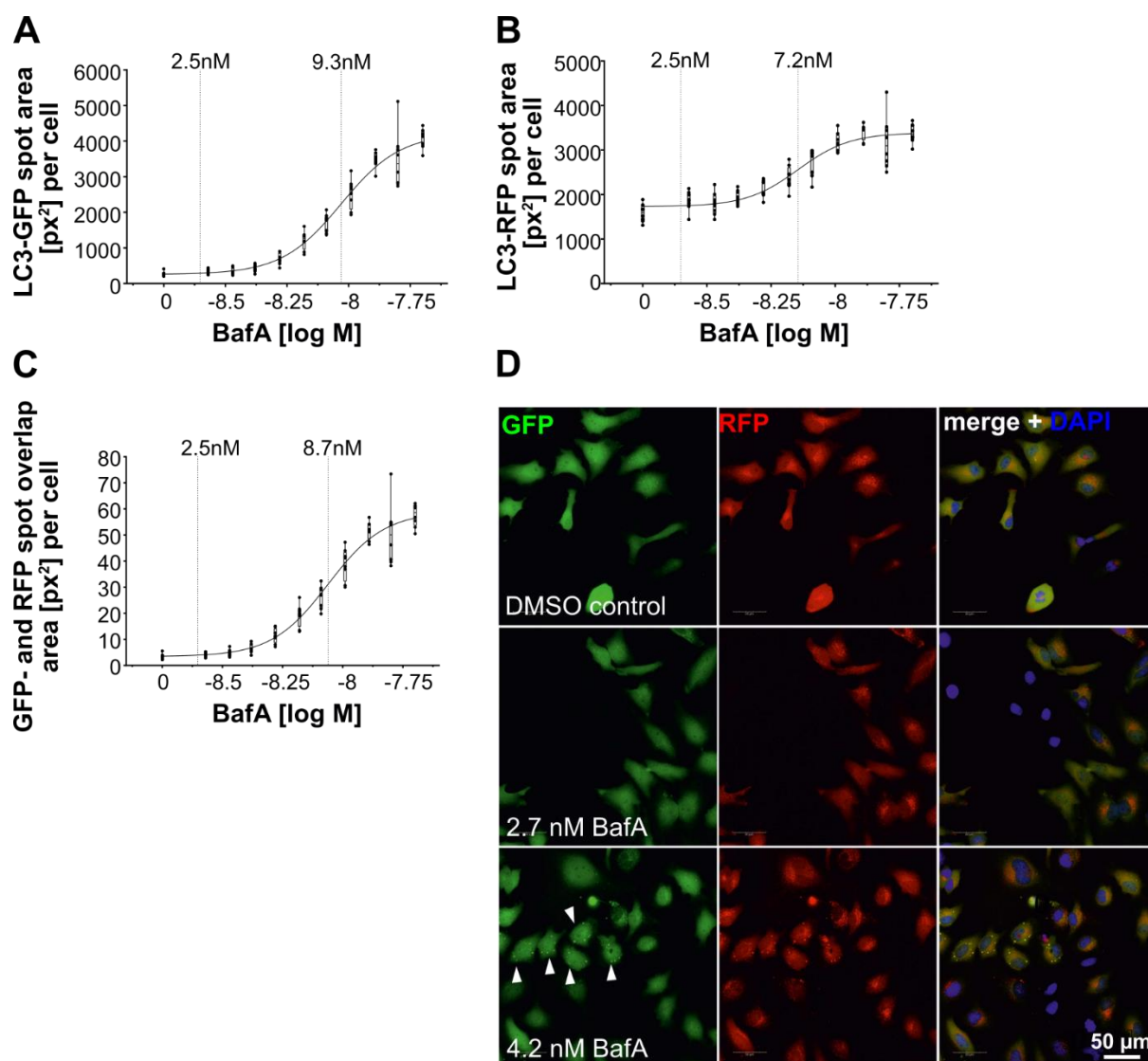
Martina P. Liebl, Sarah C. Meister, Lisa Frey, Kristina Hendrich, Anja Klemmer, Bettina Wohlfart, Christopher Untucht, Judith Nuber, Christian Pohl, Viktor Lakics

Supplementary information

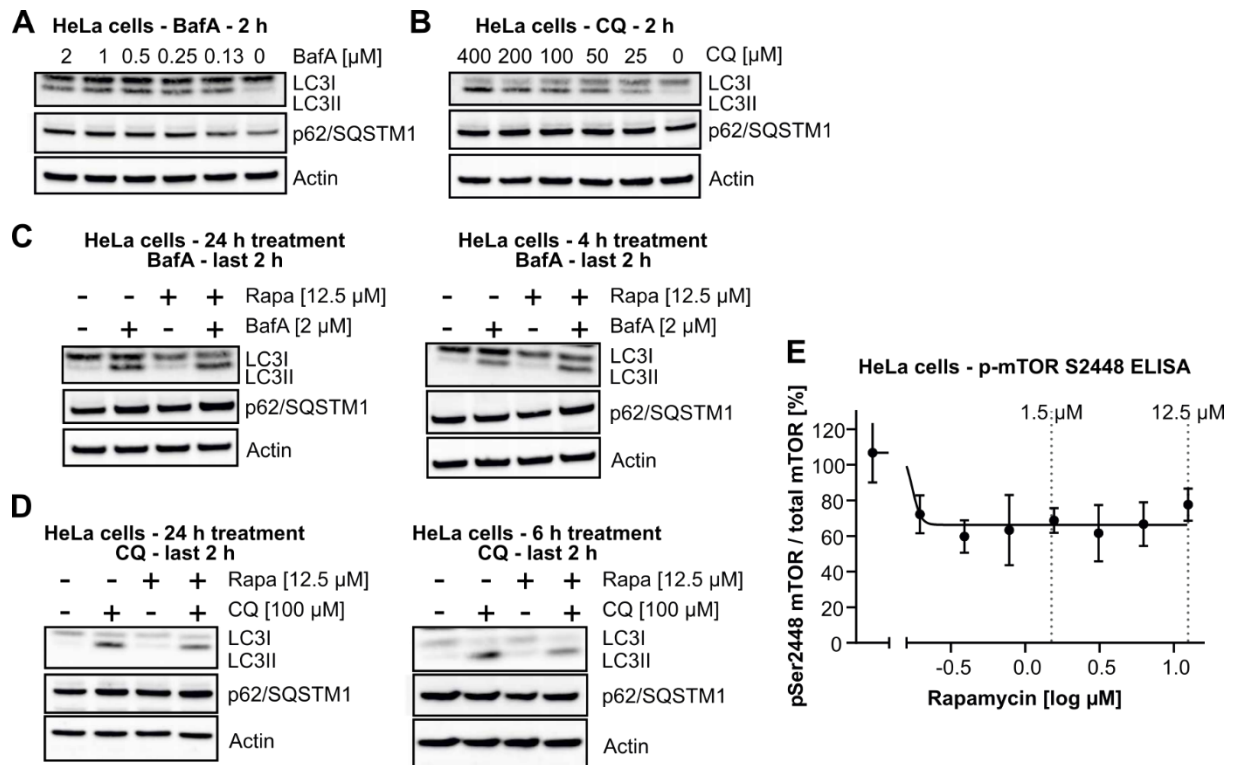


Supplementary Figure 1. Applying non-saturating and saturating concentrations of late-stage autophagy inhibitors to SK-N-MC cells. (A) SK-N-MC cells were treated with a concentration range of BafA (1.25 nM – 80 nM) for 24 h. Shown is a representative Western blot for LC3, p62/SQSTM1 and actin (top) as well as quantification based on Western blot analysis from 3 independent biological replicates ($n = 3$; mean $\pm$ SEM). BafA inhibited the basal autophagic flux with an EC_{50} of 7.5 and 8.5 nM based on the increases of LC3-II and p62/SQSTM1, respectively. For curve display, LC3-II or p62/SQSTM1 levels at 80 nM BafA were set to 100 %. For curve fitting, a non-linear regression curve fit (log(inhibitor) vs. response- variable slope (four parameters)) was applied. (B) SK-N-MC cells were treated with a concentration range (0.1 – 100 μ M) of CQ for 24 h. Shown is a representative Western blot for LC3, p62/SQSTM1 and actin (top) as well as quantification based on Western blot analysis from 3 independent biological replicates ($n = 3$; mean $\pm$ SEM). The calculated EC_{50} s with

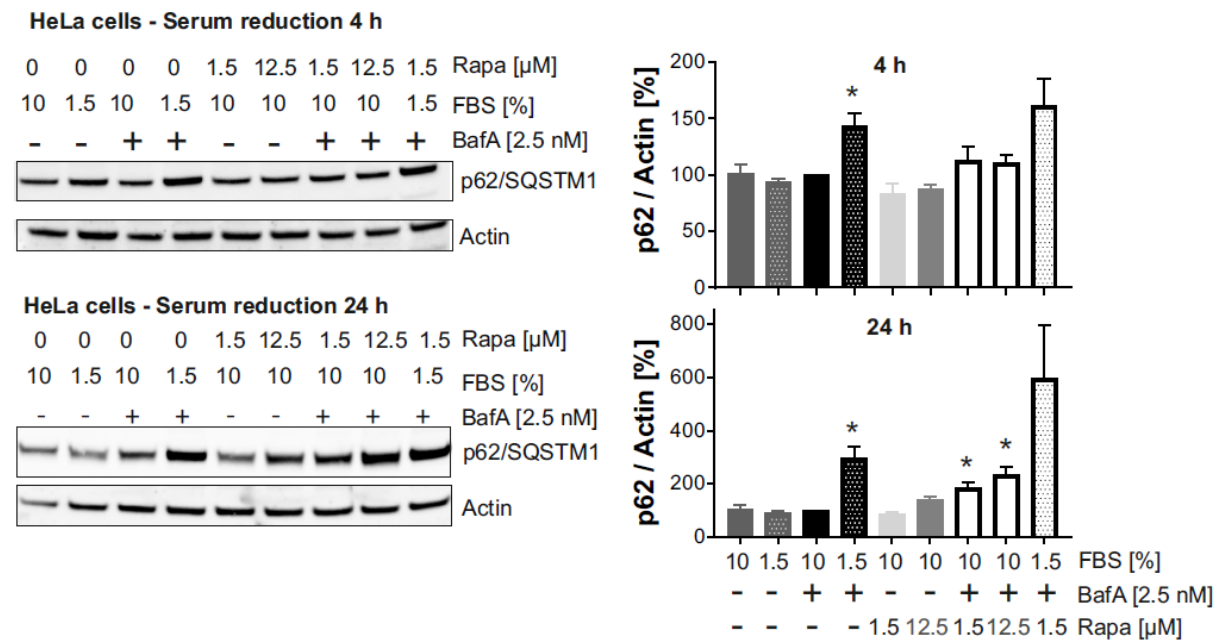
respect to LC3-II increase as well as p62/SQSTM1 accumulation were 15 μ M and 9 μ M, respectively. Curve calculation was done based on 3 independent biological replicates (mean $\pm$ SEM). For curve display, LC3-II or p62/SQSTM1 levels at 30 μ M CQ were set to 100 %. For curve fitting, a non-linear regression curve fit (log(inhibitor) vs. response- variable slope (four parameters)) was applied. **(C)** SK-N-MC cells were cotreated with either 2.5 nM or 10 nM BafA or either 1 μ M or 30 μ M CQ and 1.5 μ M Rapamycin for 24 h. Shown is a representative Western blot for LC3, p62/SQSTM1 and GAPDH (left) as well as the quantification (right) from Western blots from 4 independent biological replicates (n = 4; mean $\pm$ SEM). Statistics: One-sample t-test; compared to 100 % control (late-stage autophagy inhibitor only control, from left to right: 2.5 nM BafA, 10 nM BafA, 1 μ M CQ, 30 μ M CQ).



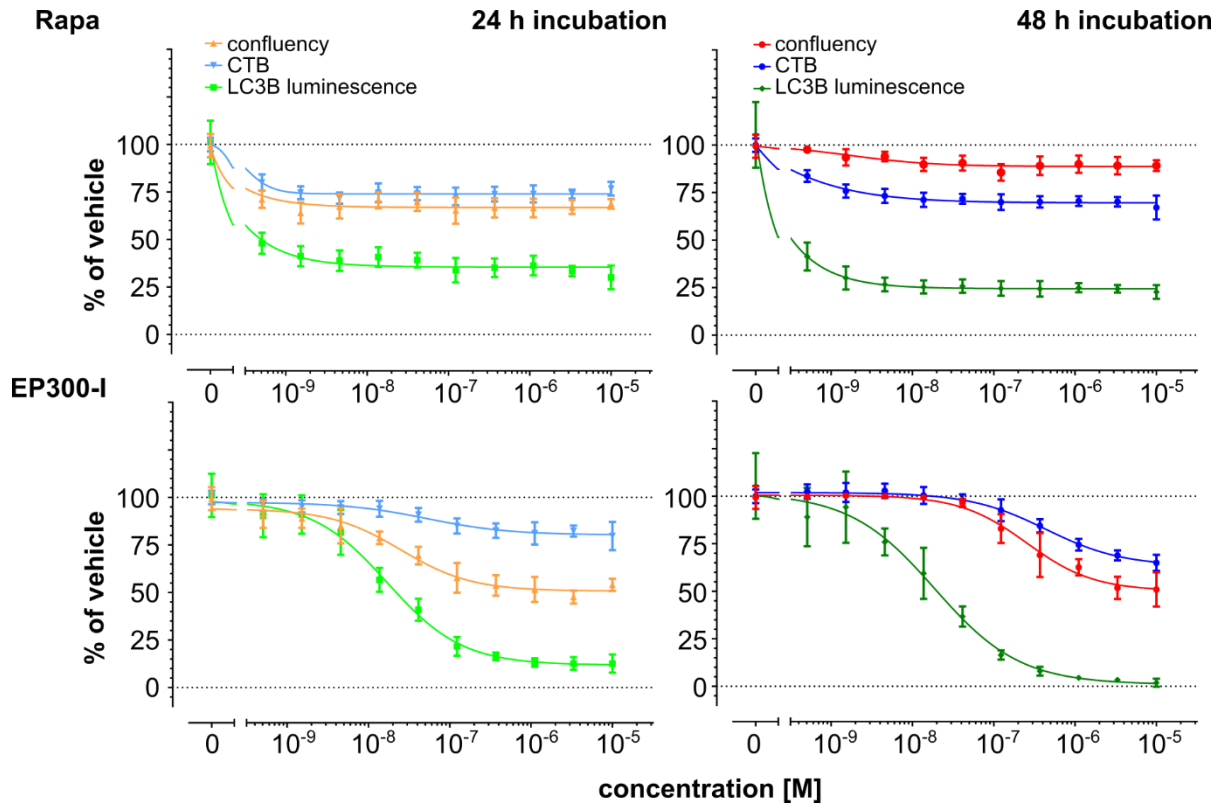
Supplementary Figure 2. Titration of BafA in HeLa GFP-RFP-LC3B Difluo cells and evaluation by fluorescence microscopy. **(A)** Quantification of GFP spot area, **(B)** RFP spot area and **(C)** co-localization of GFP and RFP positive spots for a concentration range of BafA (n = 8). **(D)** Representative fluorescence images from treatments quantified in panels (A) and (B). Only concentrations around the “non-saturating” concentration of BafA are shown. Arrowheads in the micrographs from 4.2 nM BafA treatments point to cells with LC3B puncta. For curve fitting, a non-linear regression curve fit (sigmoidal, 4PL, X is log (concentration)) was applied.



Supplementary Figure 3. Short-time treatments of HeLa cells with late-stage autophagy inhibitors and mTOR ELISA assay. For all experiments, representative Western blots for LC3, p62/SQSTM1 and actin are shown. **(A)** HeLa cells were treated with a concentration range (130 nM – 2 μ M) of BafA for 2 h to determine a saturating concentration based on the LC3-II and p62/SQSTM1 increase. **(B)** HeLa cells were treated with a concentration range (25 μ M – 400 μ M) of CQ for 2 h to determine a saturating concentration based on the LC3-II and p62/SQSTM1 increase. **(C)** HeLa cells were treated with rapamycin for either 24 (left) or 4 h (right). BafA at a saturating concentration (2 μ M) was added during the last 2 h of incubation with rapamycin. **(D)** HeLa cells were treated with rapamycin for either 24 (left) or 6 h (right). A saturating concentration of CQ (100 μ M) was added during the last 2 h of incubation with rapamycin. **(E)** Lysates from HeLa cells treated with a dose range of rapamycin (200 nM – 12.5 μ M) for 24 h were analyzed with a mTOR MSD ELISA assay multiplexing total mTOR and pS2448 mTOR. A maximum reduction of mTOR phosphorylation by 30-40 % was observed. The curve represents one biological replicate with 3 technical replicates ($n=1$; technical mean $\pm$ SEM). For curve fitting, a nonlinear regression curve fit was applied (sigmoidal, four parameter logistic, X is log(concentration)).



Supplementary Figure 4. Capturing autophagic flux increase through serum starvation by applying a non-saturating concentration of BafA. HeLa cells were kept in standard medium (10 % FBS) or in FBS-reduced medium (1.5 %) for 4 or 24 h. Medium change was combined with (co)-treatment of cells with 2.5 nM BafA and / or 1.5 or 12.5 μ M rapamycin. Shown are representative Western blots of p62/SQSTM1 and actin (top) as well as quantifications (n=3, mean $\pm$ SEM; bottom). Statistics: One-sample T-test; compared to 100 % control (DMSO, 10% FBS).



Supplementary Figure 5. LC3B luminescence complementation assay comparing concentration ranges of rapamycin to EP300-I. Measurement of confluency (orange/red), Cell Titer Blue (CTB, light and dark blue) and LC3B fluorescence complementation (light and dark green) for cells treated with different concentrations of rapamycin (top) and the EP300 inhibitor (bottom) for 24 and 48 h, respectively (n = 8).

Uncropped Western Blots

Figure 1A

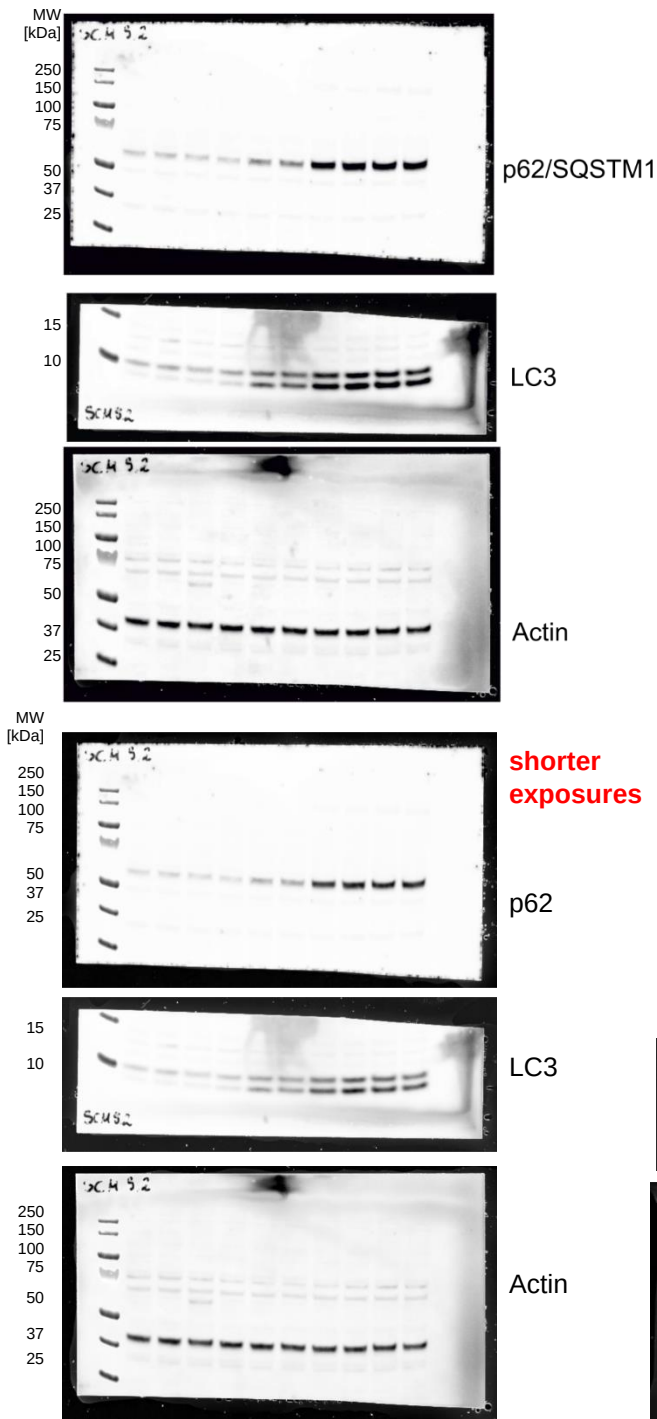


Figure 1B

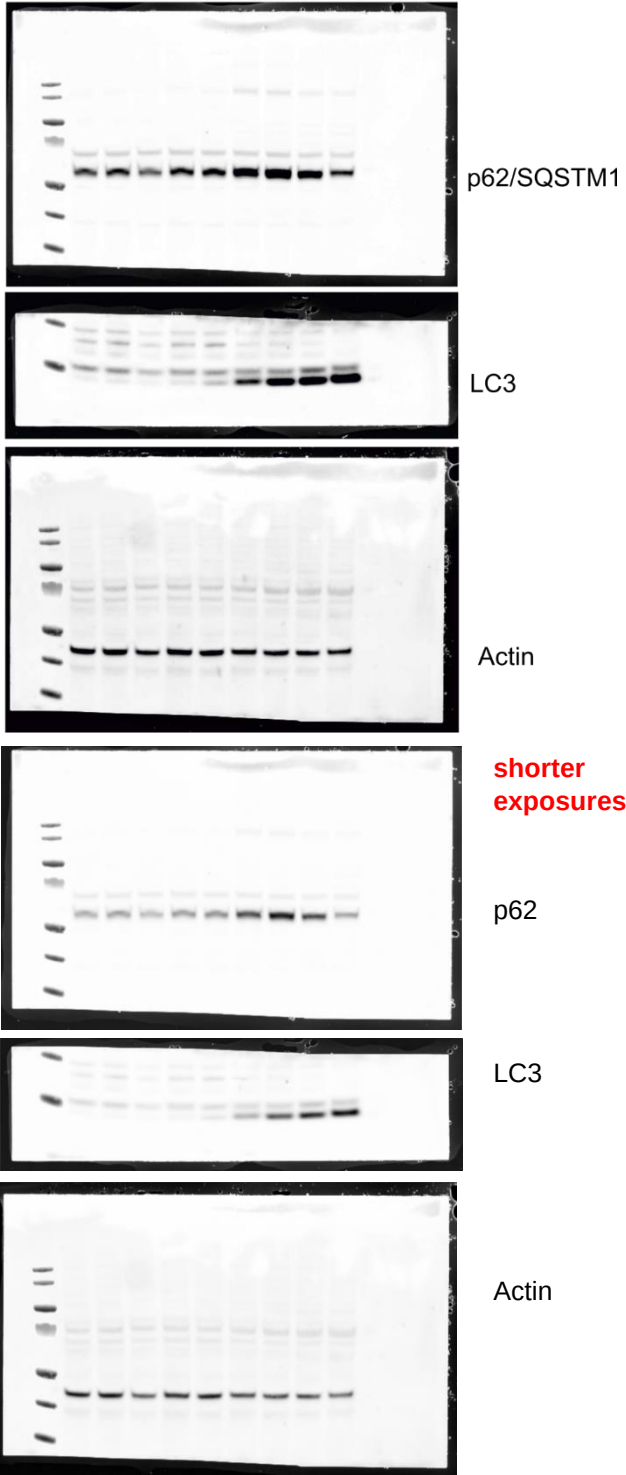


Figure 2A

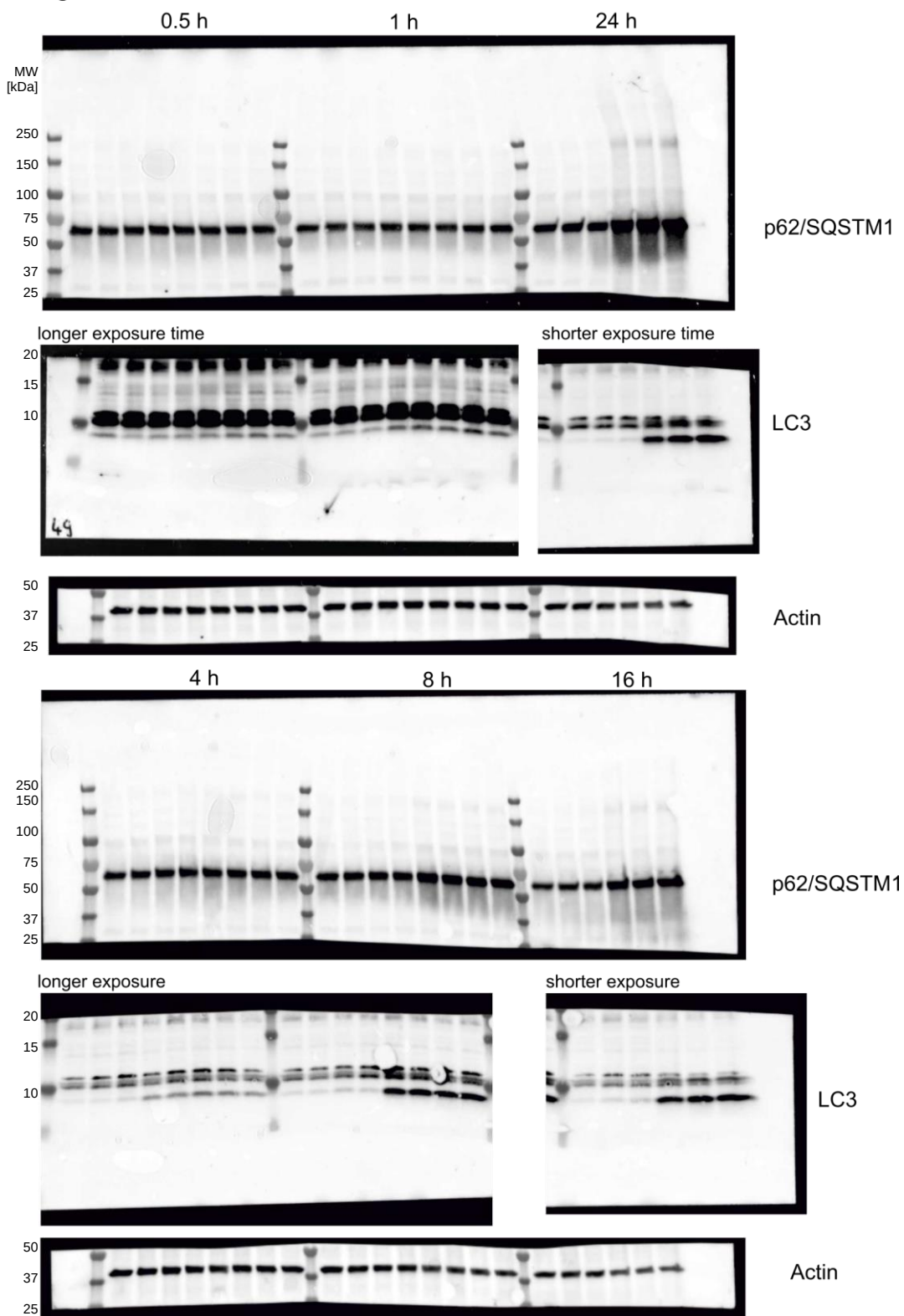


Figure 3A

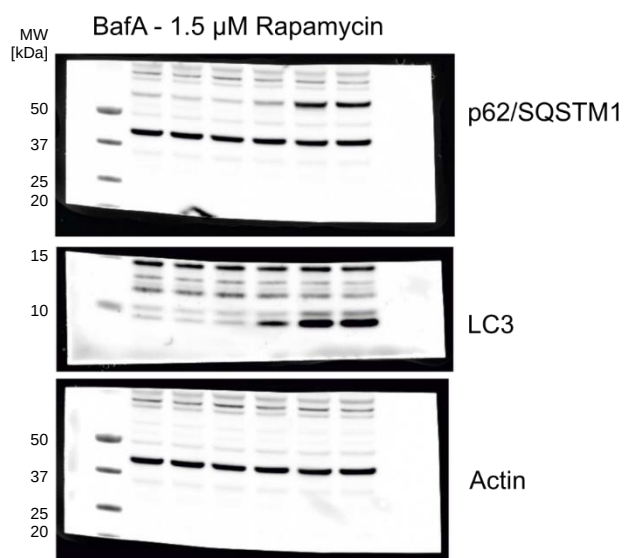


Figure 3C

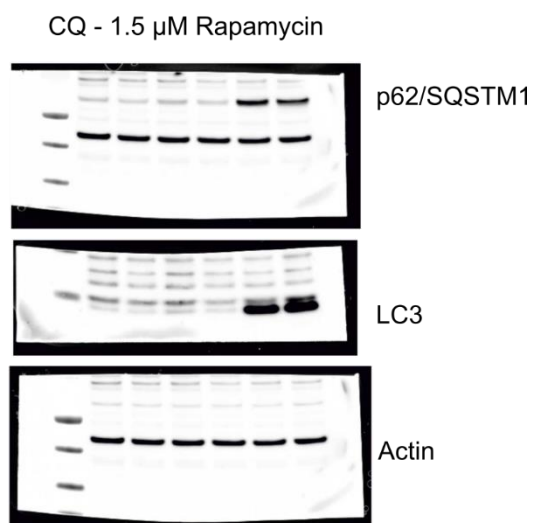


Figure 3B

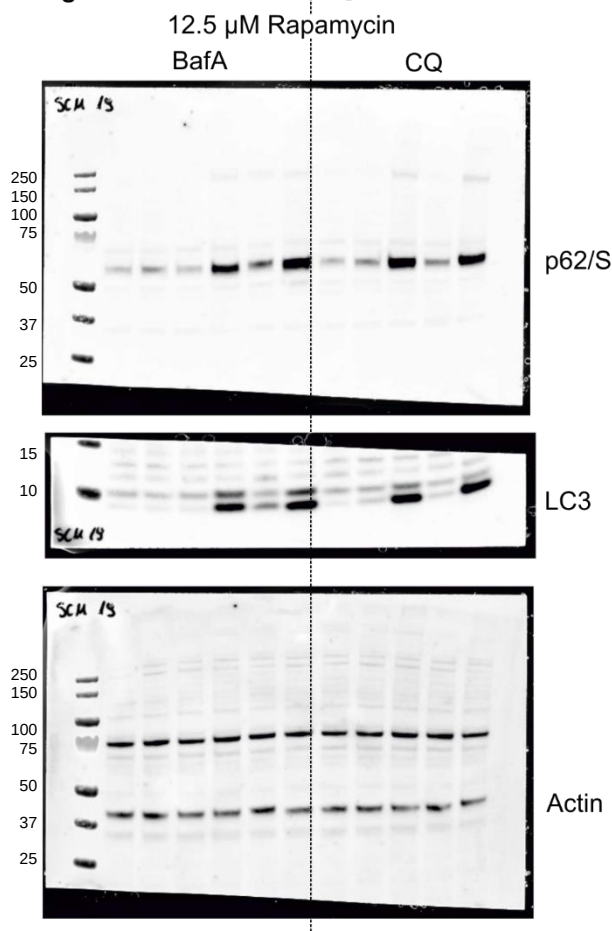


Figure 3A

BafA - 1.5 μ M Rapamycin

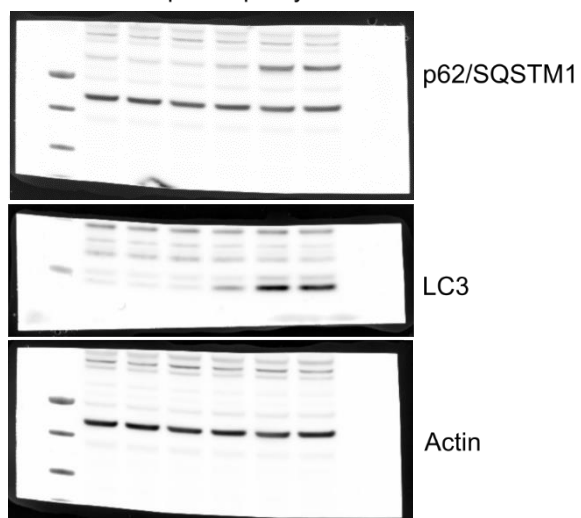


Figure 3C

shorter exposures

CQ - 1.5 μ M Rapamycin

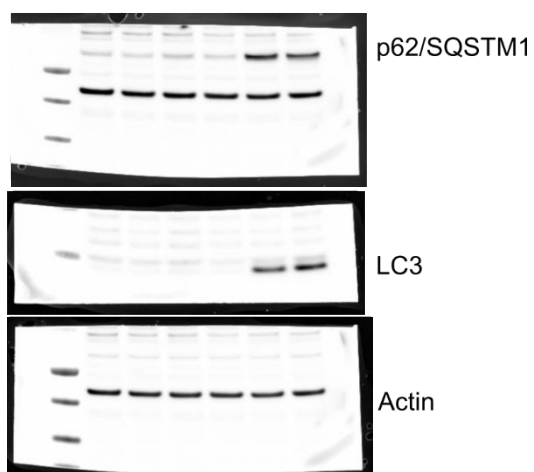


Figure 3B

12.5 μ M Rapamycin

BafA

Figure 3C

CQ

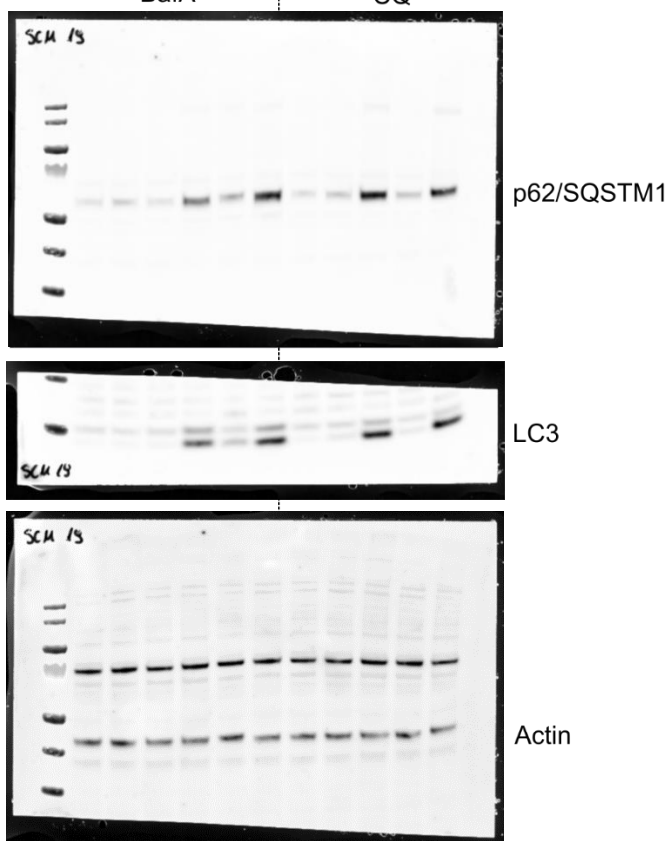


Figure 4A

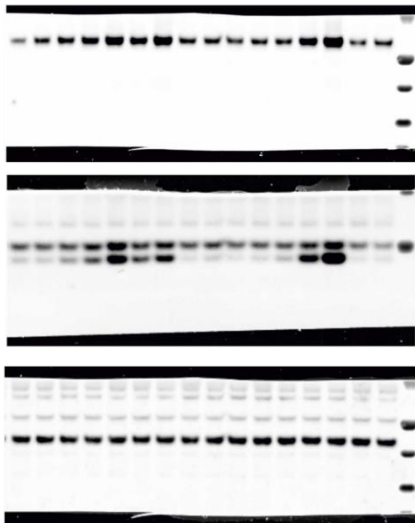
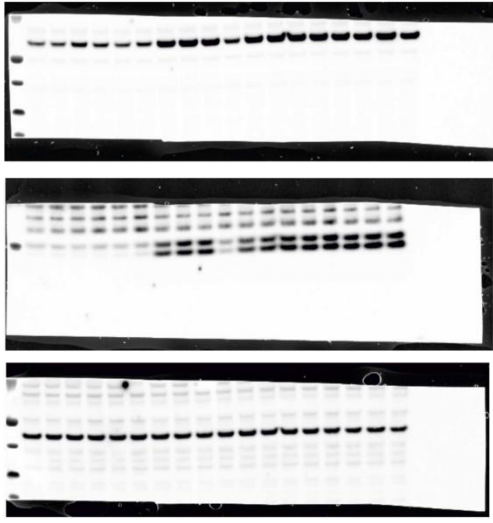


Figure 4B



p62/SQSTM1

LC3

Actin

Figure 4A

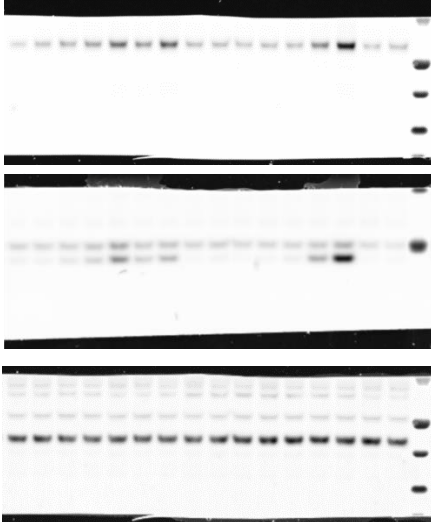
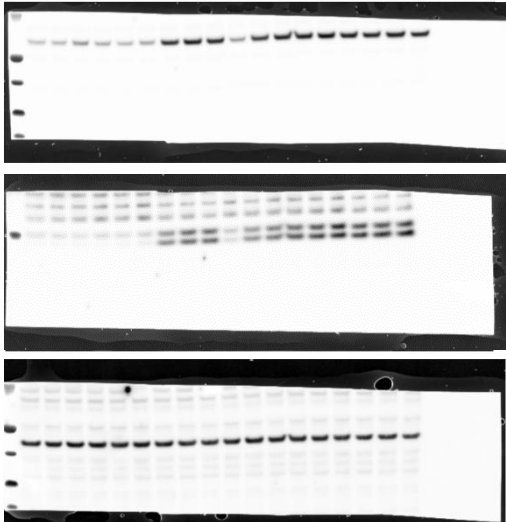


Figure 4B



shorter exposures

p62/SQSTM1

LC3

Actin

Figure 5A and Supplementary Figure 4

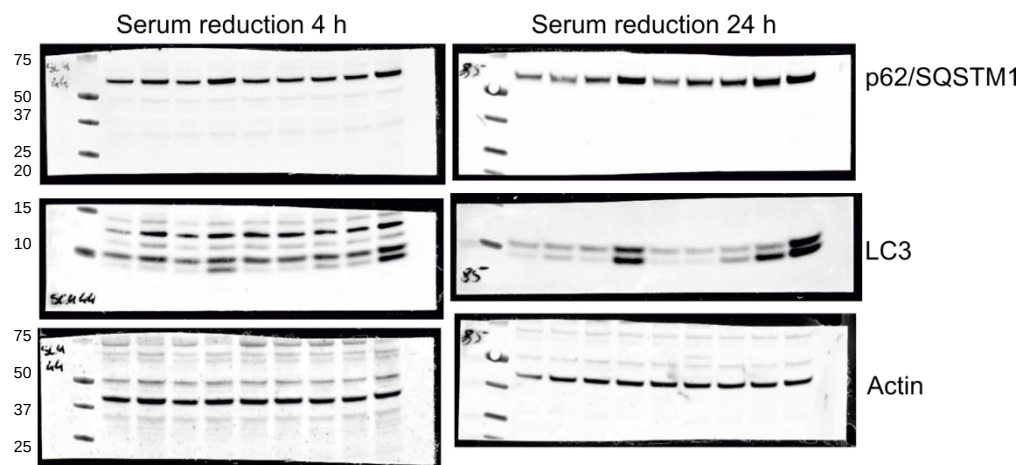


Figure 5B

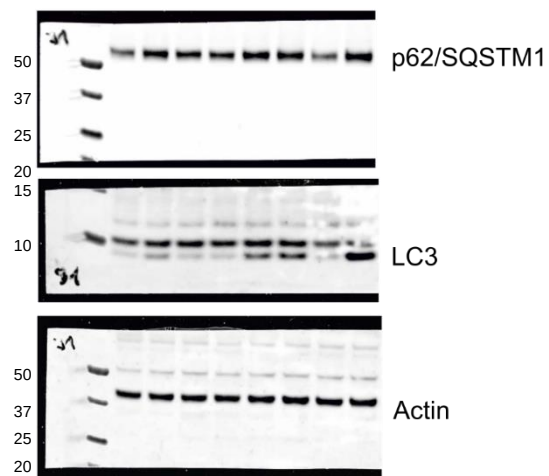


Figure 5A and Supplementary Figure 4

shorter exposures

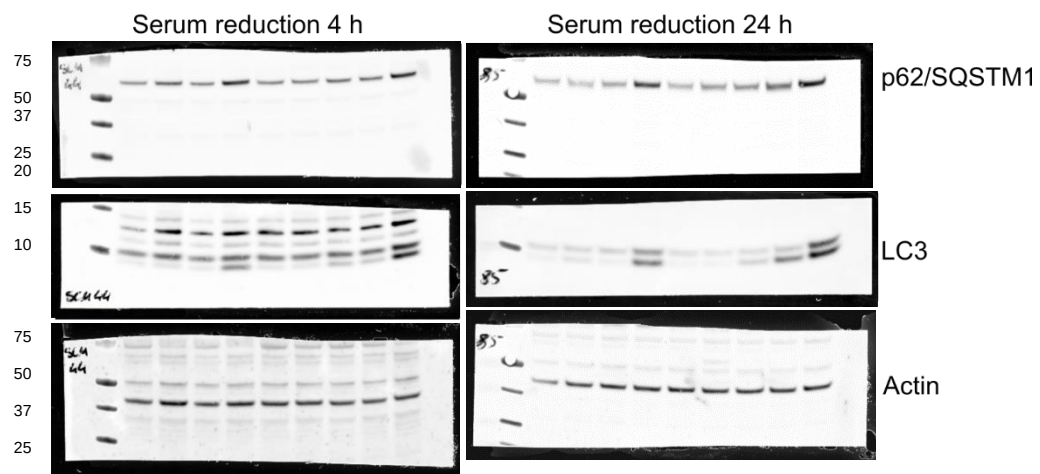


Figure 5B

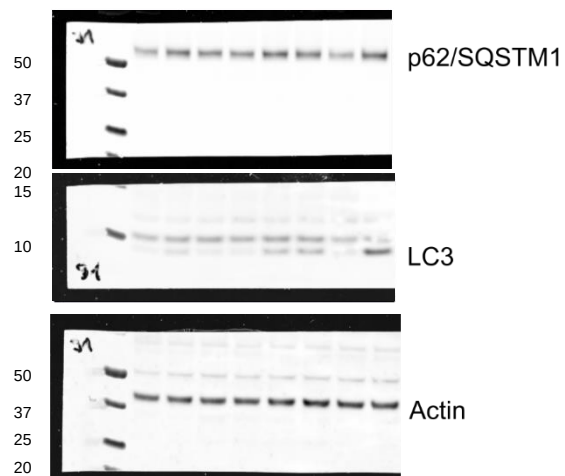


Figure 6 B

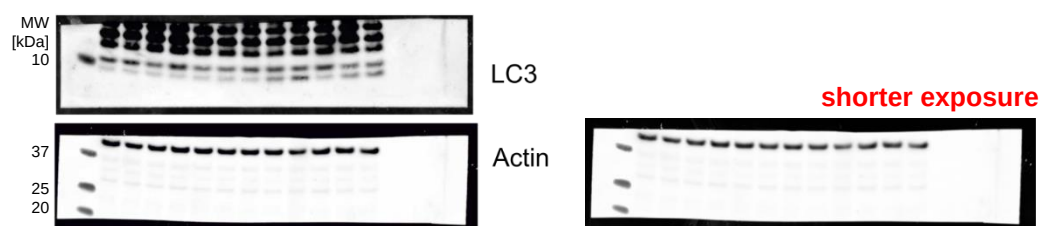


Figure 6 C

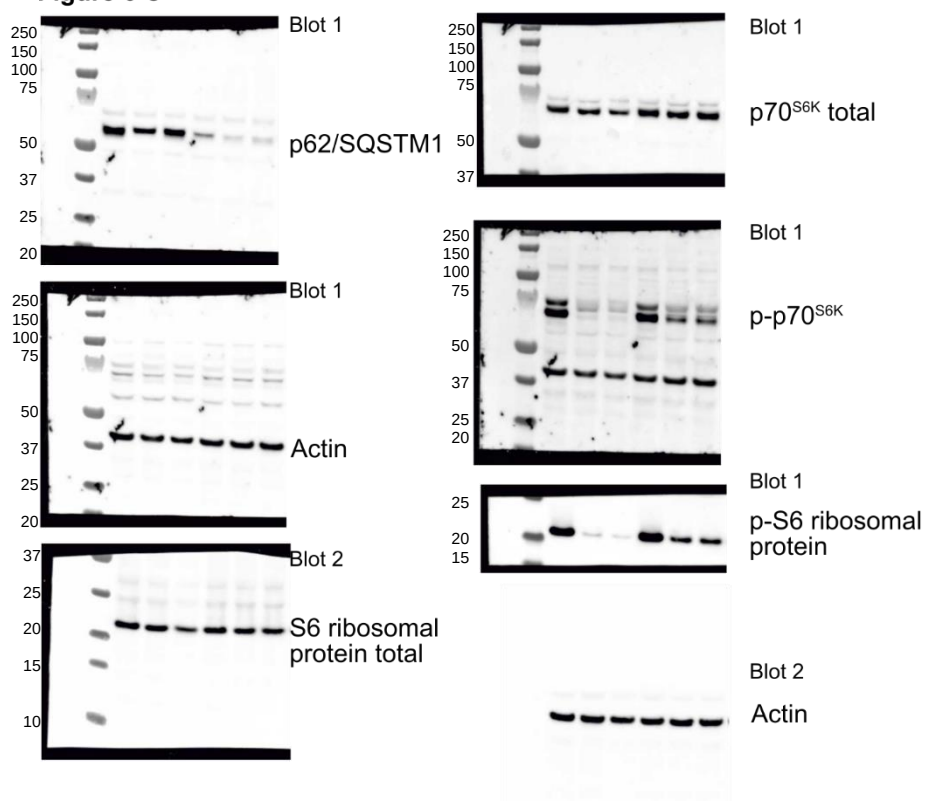
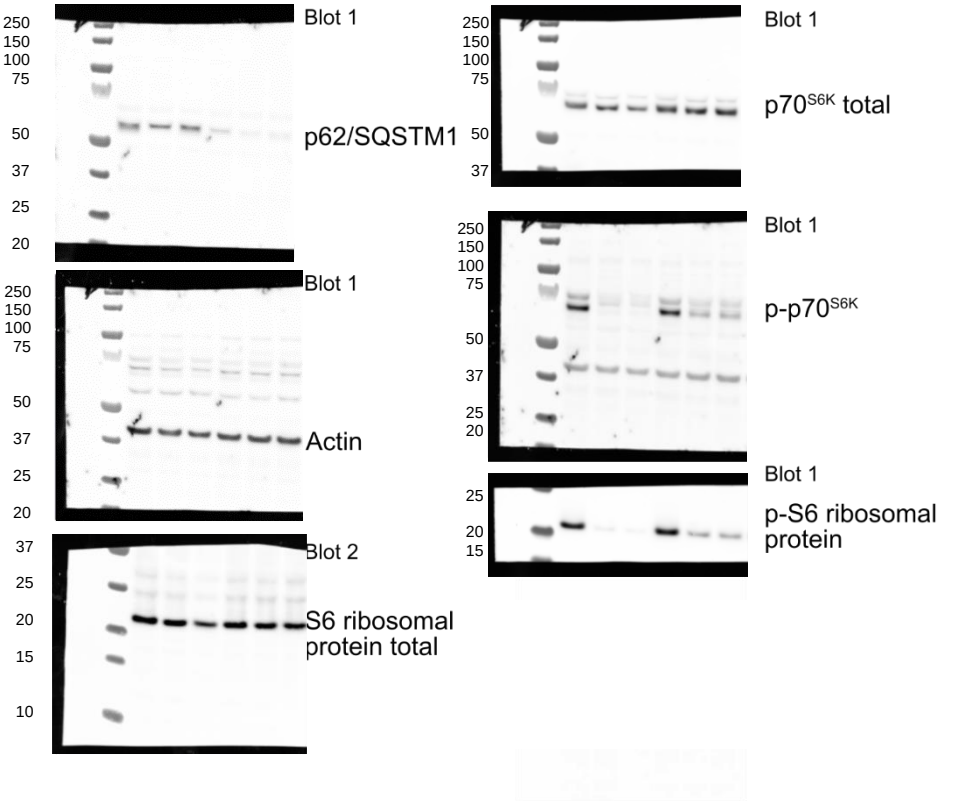
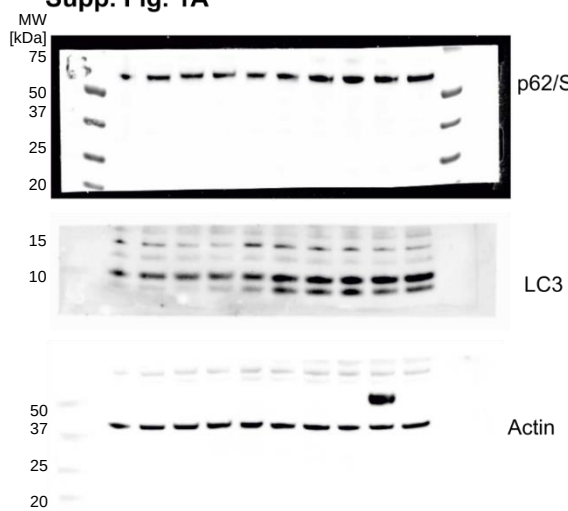


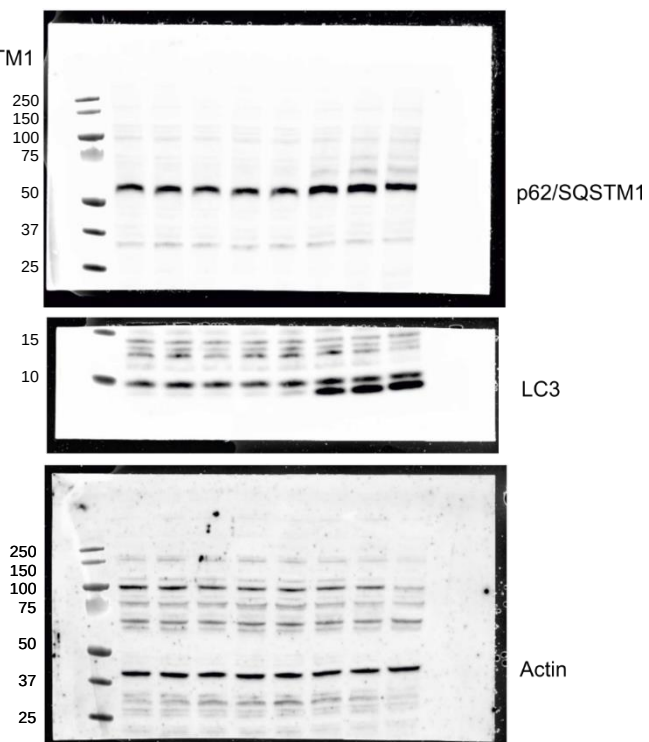
Figure 6 C



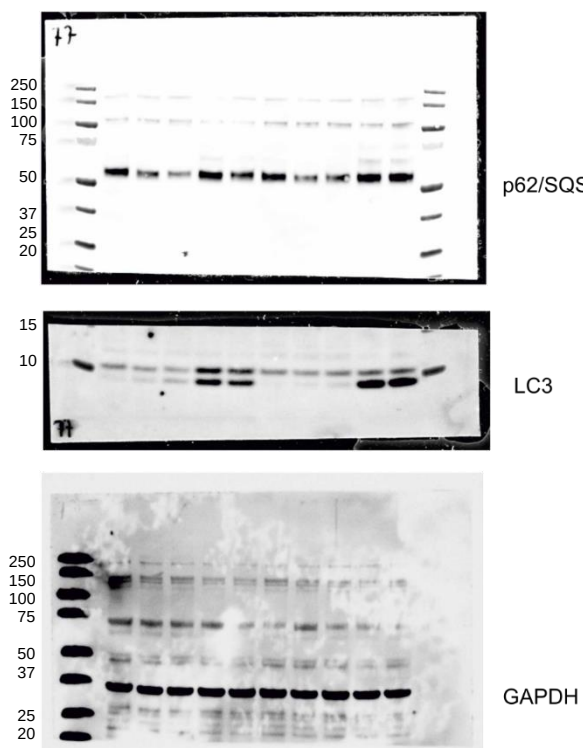
Supp. Fig. 1A



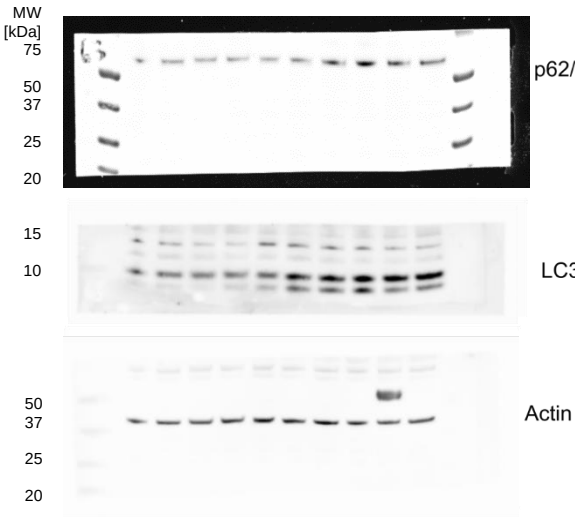
Supp. Fig. 1B



Supp. Fig. 1C

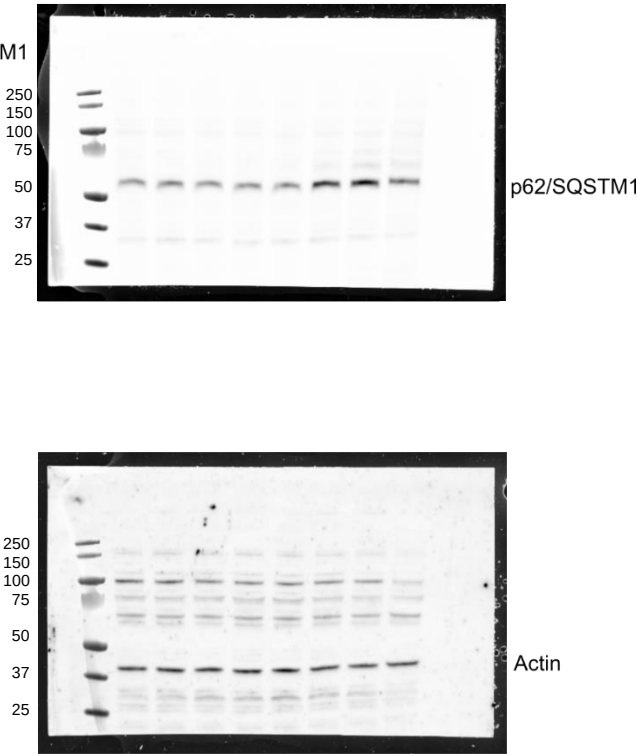


Supp. Fig. 1A

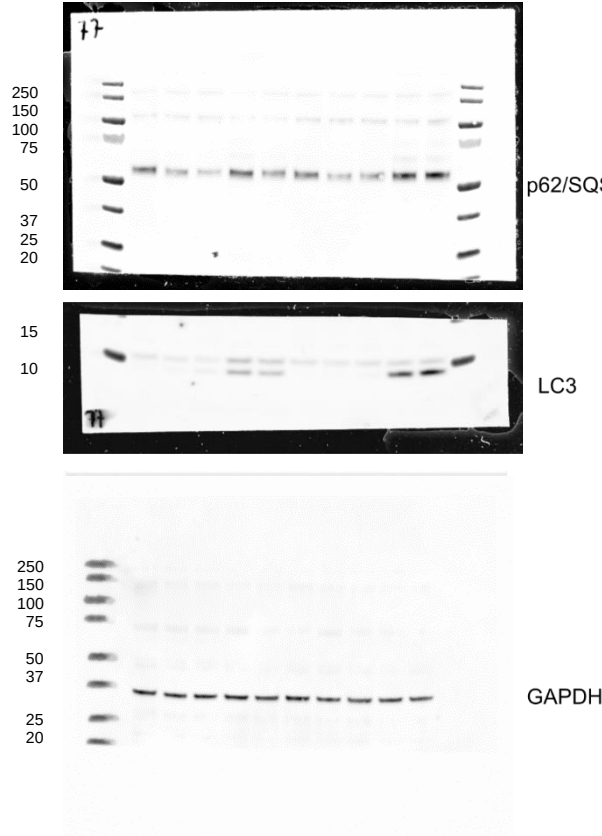


Supp. Fig. 1B

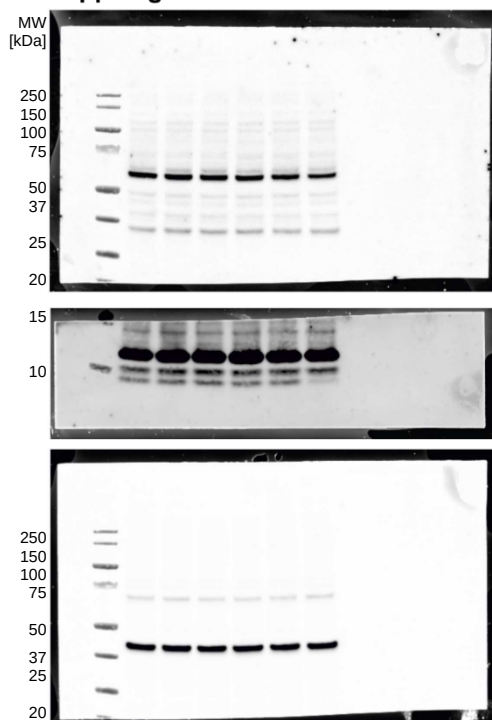
shorter exposures



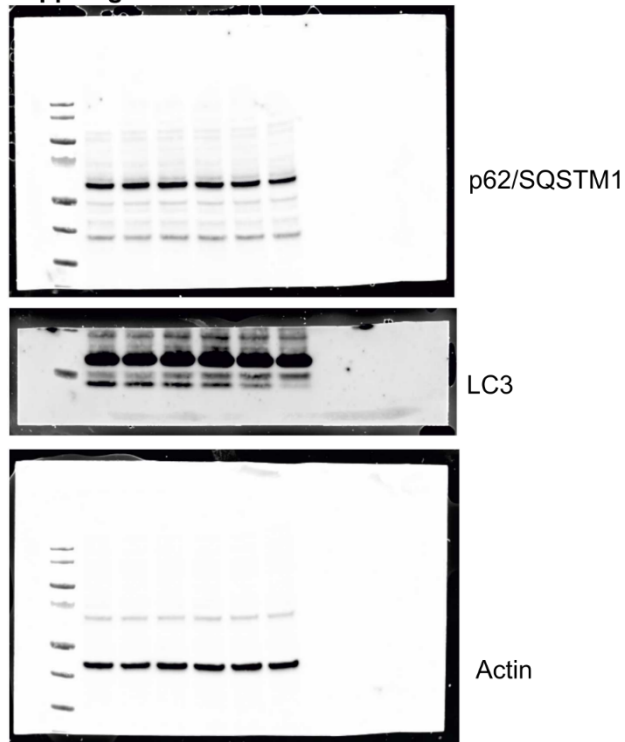
Supp. Fig. 1C



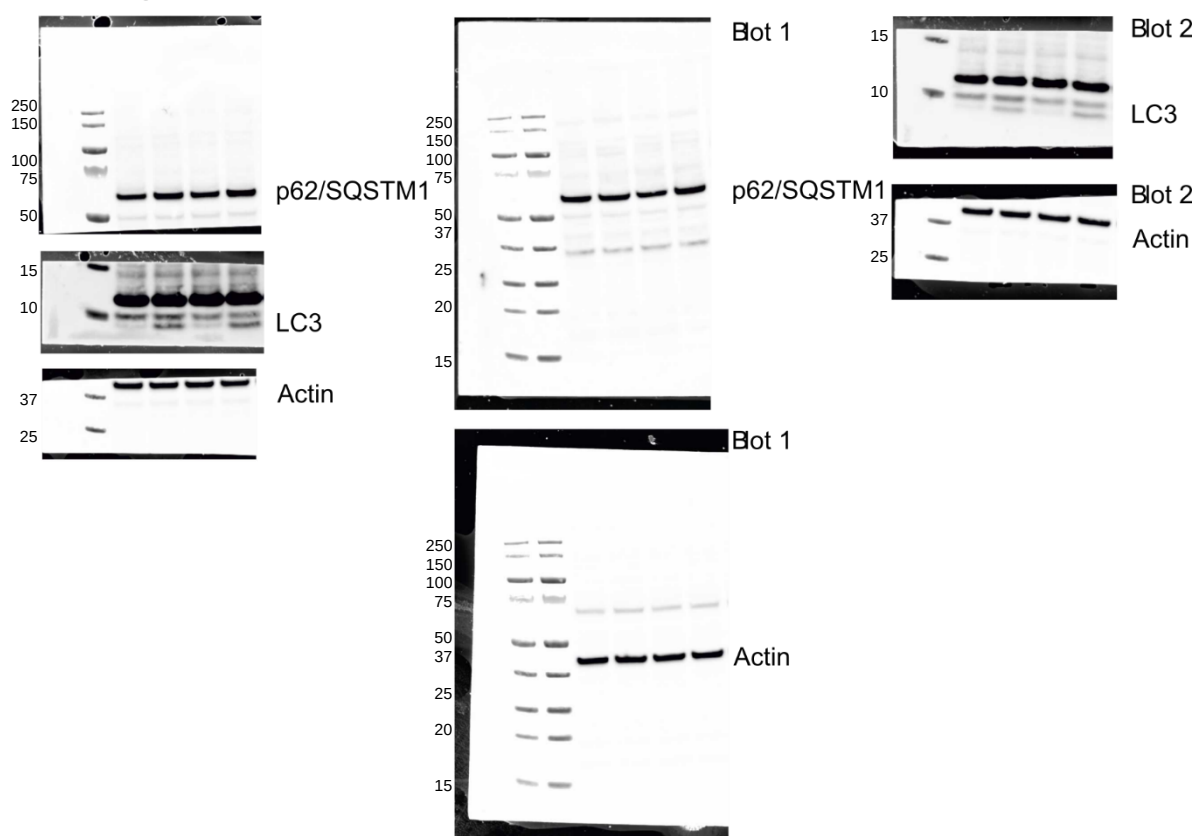
Supp. Fig. 3A



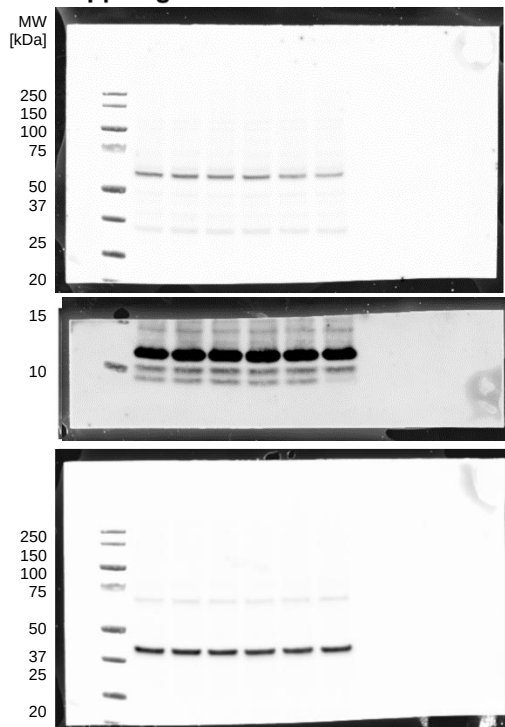
Supp. Fig. 3B



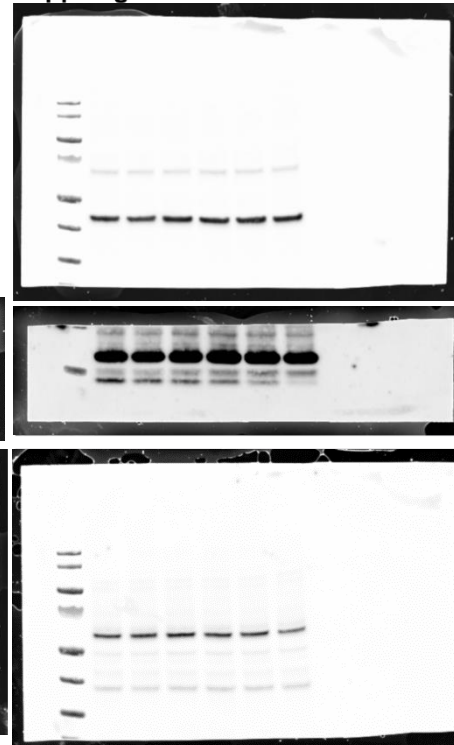
Supp. Fig. 3C



Supp. Fig. 3A



Supp. Fig. 3B



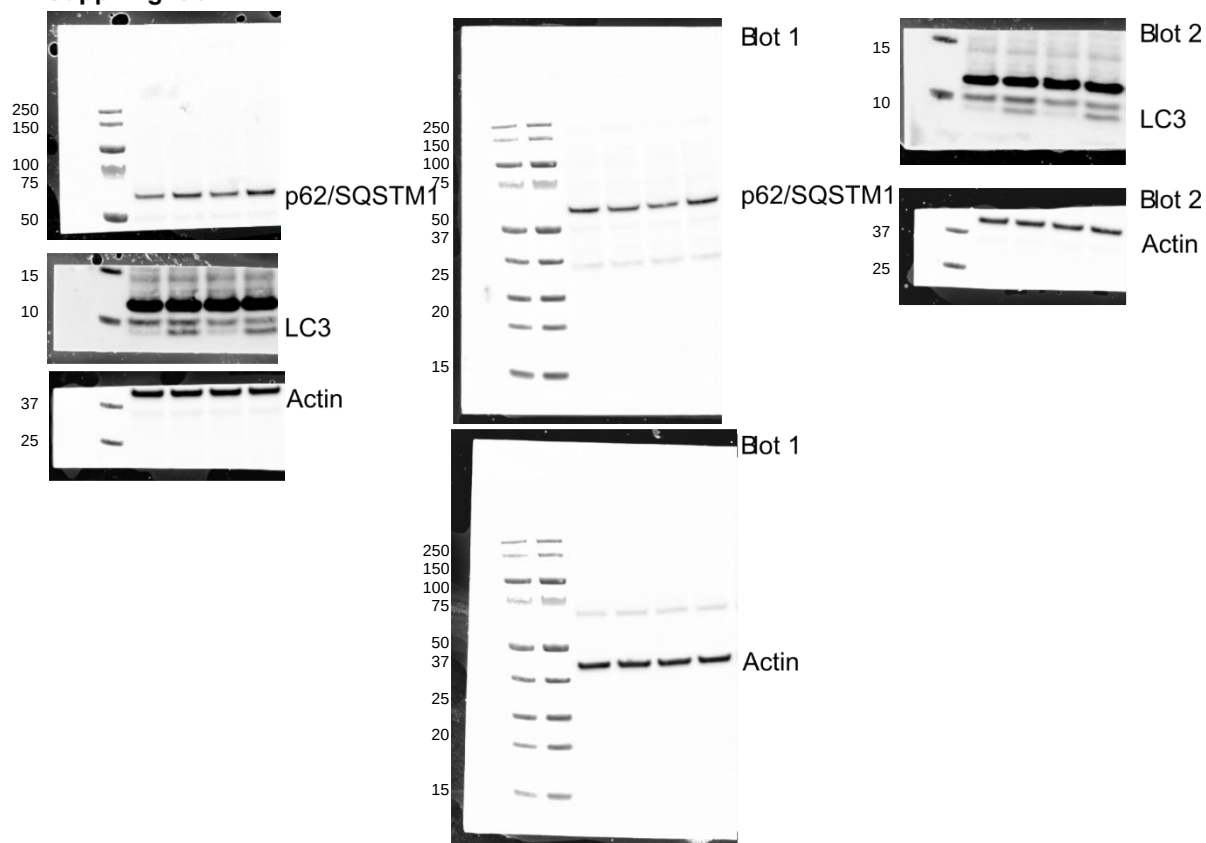
shorter exposures

p62/SQSTM1

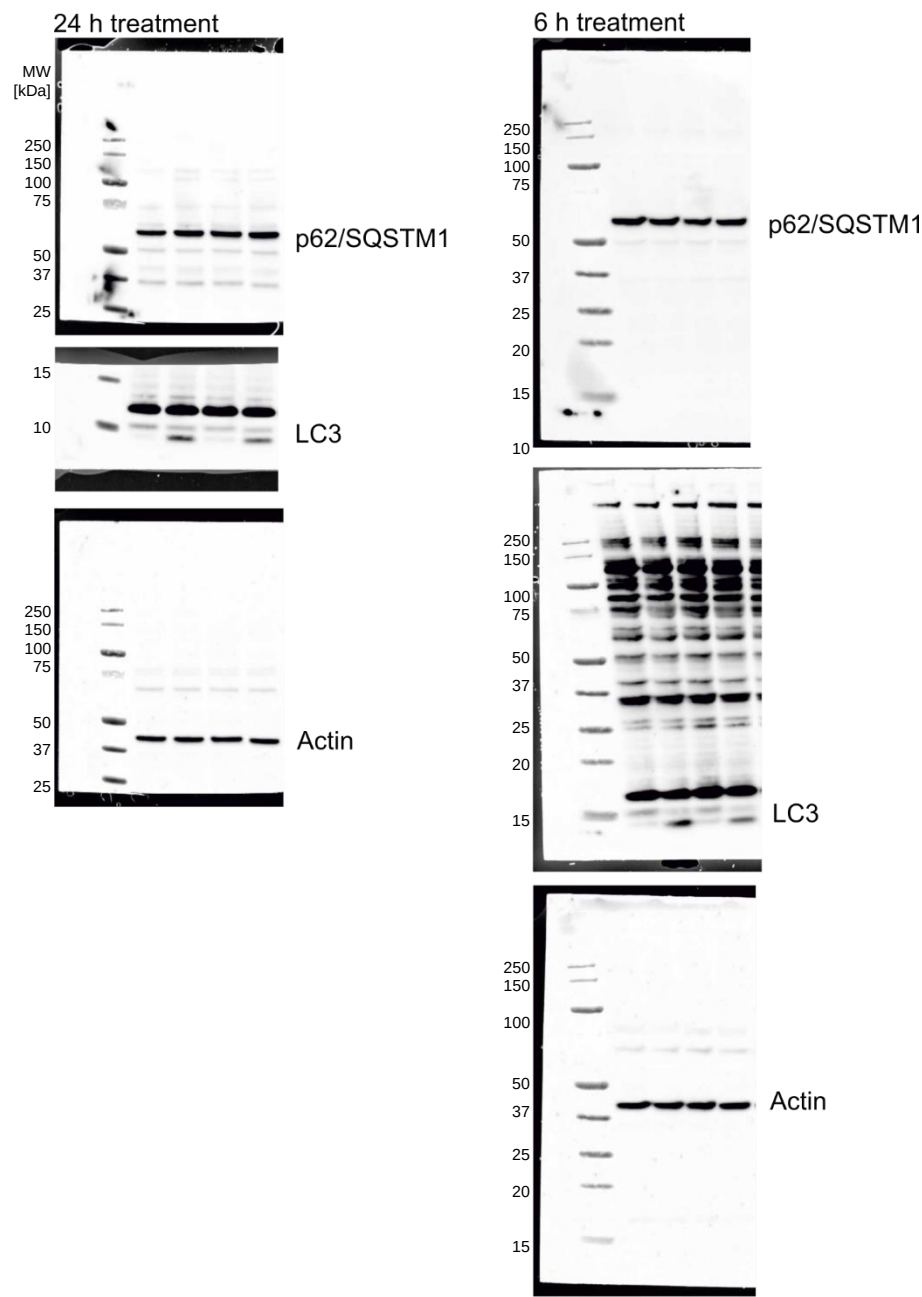
LC3

Actin

Supp. Fig. 3C



Supp. Fig. 3D



Supp. Fig. 3D

shorter exposures

