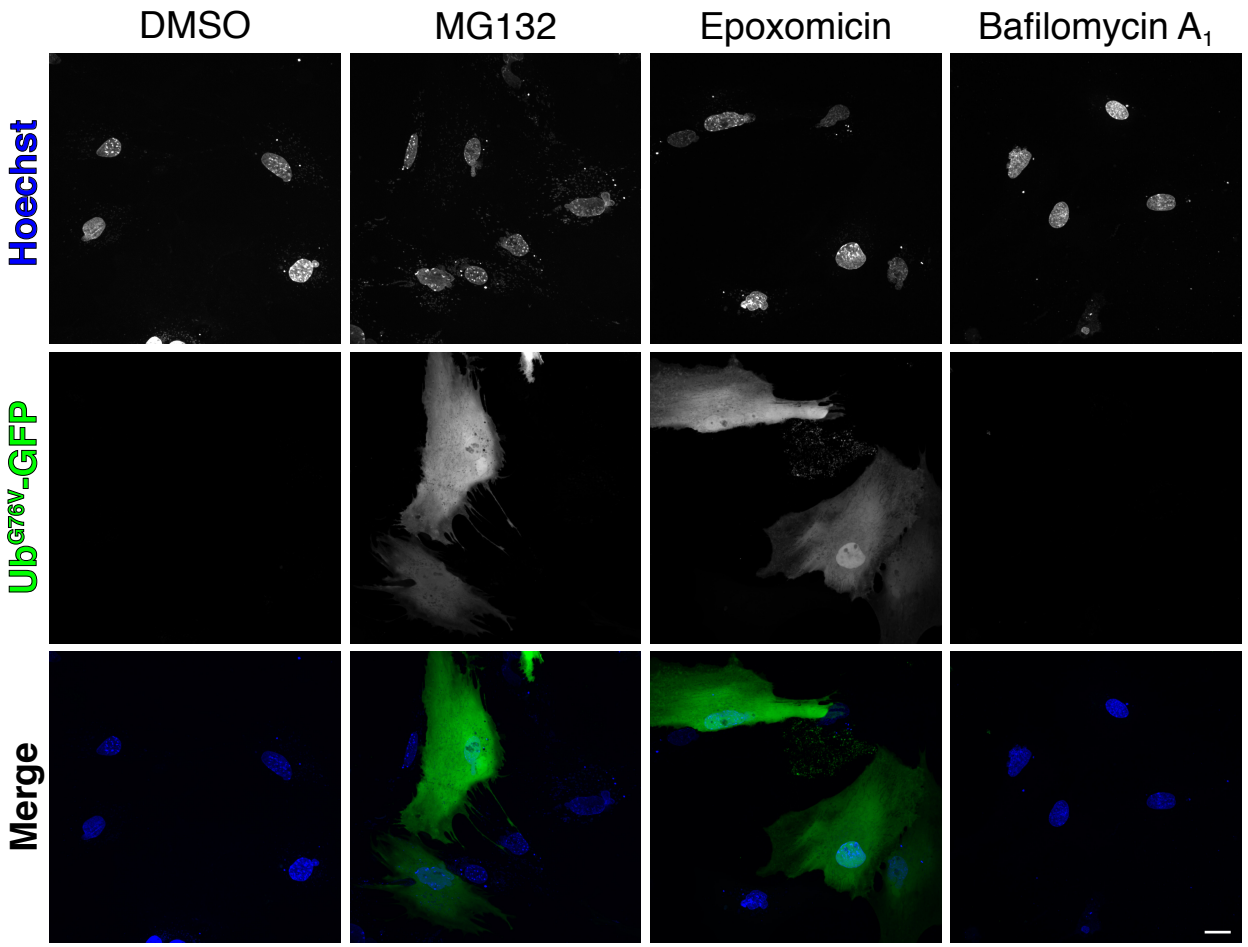
DMSO
MG132
Epoxomicin
Bafilomycin A1
Hoechst
UbG76V-GFP
Merge

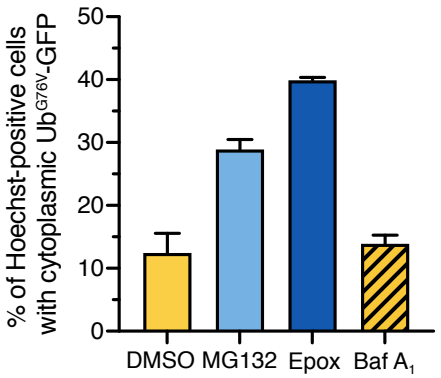
% of Hoechst-positive cells with cytoplasmic UbG76V-GFP
50
40
30
20
10
0
DMSO
MG132
Epox
Baf A1

Fig. S1

Figure S1. UPS-targeted GFP (Ub^{G76V}-GFP) validates UPS inhibition with MG132 and epoxomicin treatments. Live-cell imaging analysis of astrocytes expressing Ub^{G76V}-GFP treated with DMSO, 0.5 µM MG132 or 10 nM epoxomicin for 24 h, or 100 nM Baf A_1 for 4 h. Images in the Ub^{G76V}-GFP channel are grayscale matched to facilitate direct comparisons. Bar: 20 µm. Corresponding quantification of the percentage of Hoechst-positive cells with cytoplasmic Ub^{G76V}-GFP (means ± SEM; n=2 independent experiments; 44-86 cells were analyzed per treatment to generate a percentage for each independent experiment; 6 DIV).

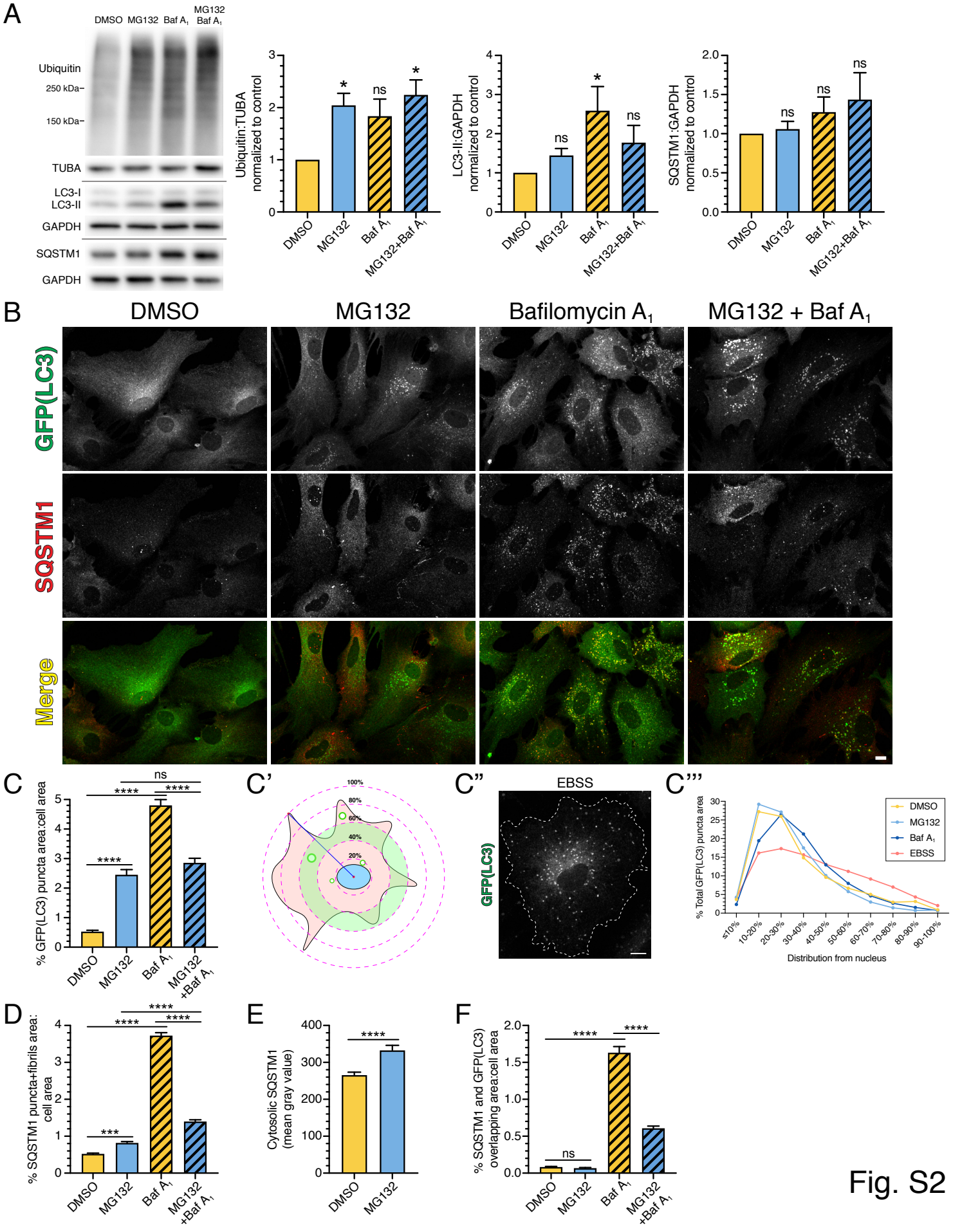
A
DMSO
MG132
Baf A1
MG132 Baf A1
Ubiquitin
250 kDa
150 kDa
TUBA
LC3-I
LC3-II
GAPDH
SQSTM1
GAPDH
Ubiquitin:TUBA normalized to control
LC3-II:GAPDH normalized to control
SQSTM1:GAPDH normalized to control
ns
MG132+Baf A1
B
DMSO
MG132
Bafilomycin A1
MG132 + Baf A1
GFP(LC3)
SQSTM1
Merge
C
% GFP(LC3) puncta area:cell area
C'
100%
80%
60%
40%
20%
C''
EBSS
GFP(LC3)
C'''
% Total GFP(LC3) puncta area
DMSO
MG132
Baf A1
EBSS
≤10%
10-20%
20-30%
30-40%
40-50%
50-60%
60-70%
70-80%
80-90%
90-100%
Distribution from nucleus
D
% SQSTM1 puncta+fibrils area: cell area
E
Cytosolic SQSTM1 (mean gray value)
F
% SQSTM1 and GFP(LC3) overlapping area:cell area

Fig. S2

Figure S2. Short-term UPS inhibition with MG132 leads to SQSTM1 fibril formation, but does not robustly upregulate autophagic flux in primary astrocytes. (**A-F**) Analysis of primary astrocytes treated with 50 μM MG132 supplemented with 100 nM Baf A_1 (or equivalent volume of DMSO as a solvent control) for 4 h. (**A**) Immunoblot analysis and corresponding quantification of glial lysates. GAPDH and TUBA/α-tubulin serve as loading controls; horizontal lines designate individual blots. Ubiquitin levels were normalized to TUBA/α-tubulin, and LC3-II and SQSTM1 levels were normalized to GAPDH (means ± SEM; one-way ANOVA with Dunnett's post hoc test; n=3-4 independent experiments; 7-8 DIV). (**B**) Maximum projections of z-stacks of GFP-LC3 transgenic astrocytes immunostained for GFP and SQSTM1. SQSTM1 images are grayscale matched to show an increase in cytosolic SQSTM1 upon UPS inhibition. Bar: 10 μm. (**C-F**) Corresponding quantification of immunostain analysis shown in B. (**C**) Quantification of total GFP(LC3) puncta area normalized to cell area (means ± SEM; one-way ANOVA with Tukey's post hoc test; n=89-152 cells from 4 independent experiments; 4-5 DIV). (**C', C", C'"**) Quantitation of the distribution of autophagosomes within astrocytes. (**C'**) Schematic of the method used to quantify the total GFP(LC3) puncta distributed within astrocytes from the centroid of the nucleus to the furthest point of the cell, at 10% intervals (only 20% intervals are shown in the schematic). (**C"**) Representative image of (GFP)LC3 in an astrocyte starved in EBSS for 4 h as a positive control for the induction of autophagic flux. Bar: 10 μm. (**C'"**) Quantification using the method in G. Each point represents the pooled distribution of GFP(LC3) puncta measured from 61-103 cells from 3 independent experiments (4-5 DIV). Distribution is measured as the percentage of total GFP(LC3) puncta area localized within each cell region. (**D**) Quantification of total area occupied by SQSTM1 puncta and fibrils normalized to cell area (means ± SEM; one-way ANOVA with Tukey's post hoc test; n=90-114 cells from 3 independent experiments; 3-5 DIV). (**E**) Quantification of cytosolic SQSTM1 signal intensity (means ± SEM; unpaired t-test; n=115-118 cells from 3 independent experiments; 4-7 DIV). (**F**) Quantification of the percentage of overlapping area between GFP(LC3)-positive and SQSTM1-positive structures normalized to cell area (means ± SEM; one-way ANOVA with Tukey's post hoc test; n=83-105 cells from 3 independent experiments; 4-7 DIV).

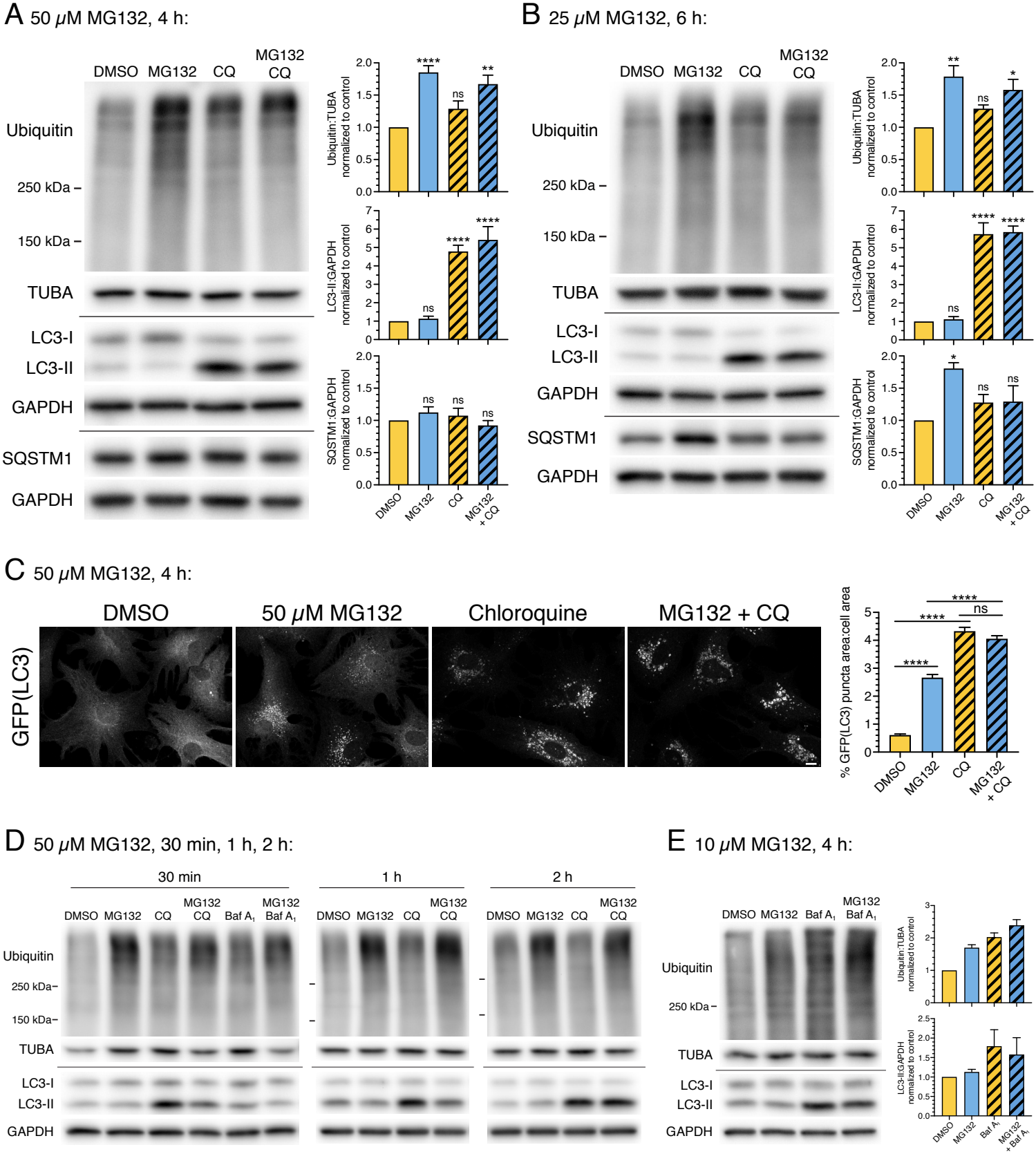
A 50 μM MG132, 4 h:
DMSO
MG132
CQ
MG132 CQ
Ubiquitin
250 kDa –
150 kDa –
TUBA
LC3-I
LC3-II
GAPDH
SQSTM1
GAPDH
Ubiquitin:TUBA normalized to control
LC3-II:GAPDH normalized to control
SQSTM1:GAPDH normalized to control
****
**
ns
DMSO
MG132
CQ
MG132 + CQ
B 25 μM MG132, 6 h:
DMSO
MG132
CQ
MG132 CQ
Ubiquitin
250 kDa –
150 kDa –
TUBA
LC3-I
LC3-II
GAPDH
SQSTM1
GAPDH
Ubiquitin:TUBA normalized to control
LC3-II:GAPDH normalized to control
SQSTM1:GAPDH normalized to control
**
*
****
ns
DMSO
MG132
CQ
MG132 + CQ
C 50 μM MG132, 4 h:
DMSO
50 μM MG132
Chloroquine
MG132 + CQ
GFP(LC3)
% GFP(LC3) puncta area:cell area
****
ns
DMSO
MG132
CQ
MG132 + CQ
D 50 μM MG132, 30 min, 1 h, 2 h:
30 min
1 h
2 h
DMSO
MG132
CQ
MG132 CQ
Baf A1
MG132 Baf A1
Ubiquitin
250 kDa–
150 kDa–
TUBA
LC3-I
LC3-II
GAPDH
E 10 μM MG132, 4 h:
DMSO
MG132
Baf A1
MG132 Baf A1
Ubiquitin
250 kDa –
TUBA
LC3-I
LC3-II
GAPDH
Ubiquitin:TUBA normalized to control
LC3-II:GAPDH normalized to control
DMSO
MG132
Baf A1
MG132 + Baf A1

Fig. S3

Figure S3. Acute inhibition of the UPS with MG132 does not robustly upregulate autophagic flux in astrocytes. Immunoblot analysis and corresponding quantification of primary astrocytes treated with (**A**) 50 µM MG132 ± 50 µM CQ for 4 h or (**B**) 25 µM MG132 ± 50 µM CQ for 6 h. GAPDH and TUBA/α-tubulin serve as loading controls; horizontal lines designate individual blots. Ubiquitin levels were normalized to TUBA/α-tubulin, and LC3-II and SQSTM1 levels were normalized to GAPDH. (**A**) Means ± SEM; one-way ANOVA with Dunnett's post hoc test; 5-7 independent experiments; 3-7 DIV. (**B**) Means ± SEM; one-way ANOVA with Dunnett's post hoc test; 3 independent experiments; 3-8 DIV. (**C**) Maximum projections of z-stacks of GFP-LC3 transgenic astrocytes treated with 50 µM MG132 ± 50 µM CQ for 4 h and immunostained for GFP. Bar: 10 µm. Corresponding quantification of total GFP(LC3) puncta area normalized to cell area (means ± SEM; one-way ANOVA with Tukey's post hoc test; n=69-149 cells from 3 independent experiments; 6-8 DIV). (**D**) Immunoblot analysis of primary astrocytes (5-6 DIV) treated with 50 µM MG132 for 30 min, 1 h, and 2 h. To measure autophagic flux, samples were co-treated with either 50 µM CQ, 100 nM Baf A_1, or an equivalent volume of solvent. GAPDH and TUBA/α-tubulin serve as loading controls; horizontal lines designate individual blots. (**E**) Immunoblot analysis of primary astrocytes treated with 10 µM MG132 ± 100 nM Baf A_1 for 4 h. GAPDH and TUBA/α-tubulin serve as loading controls; horizontal lines designate individual blots. Ubiquitin levels were normalized to TUBA/α-tubulin, and LC3-II levels were normalized to GAPDH (means ± SEM; n=2 independent experiments; 4 DIV).

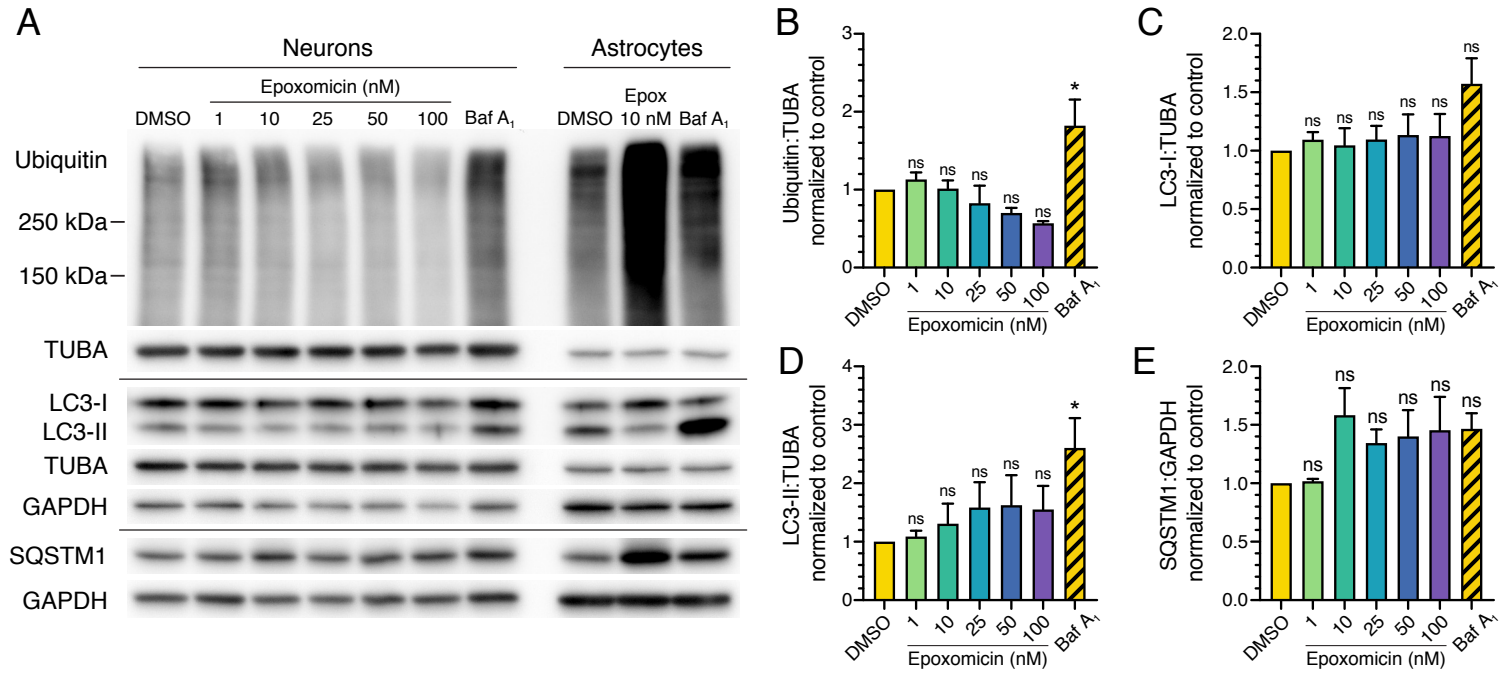
A
Neurons
Astrocytes
Epoxomicin (nM)
DMSO
1
10
25
50
100
Baf A1
Epox
DMSO
10 nM
Baf A1
Ubiquitin
250 kDa
150 kDa
TUBA
LC3-I
LC3-II
TUBA
GAPDH
SQSTM1
GAPDH
B
Ubiquitin:TUBA normalized to control
C
LC3-I:TUBA normalized to control
D
LC3-II:TUBA normalized to control
E
SQSTM1:GAPDH normalized to control
ns
*
Epoxomicin (nM)

Fig. S4

Figure S4. Broader ranges of epoxomicin concentration reveal that UPS inhibition has a reduced effect on SQSTM1 aggresome formation in neurons as compared with astrocytes. (**A-E**) Immunoblot analysis and corresponding quantification of increasing concentrations of epoxomicin (24-h treatment) as compared with 100 nM Baf A_1 for 4 h in primary cortical neurons. GAPDH and TUBA/α-tubulin serve as loading controls; horizontal lines designate individual blots. Ubiquitin, LC3-I, and LC3-II levels were normalized to TUBA/α-tubulin, and SQSTM1 levels were normalized to GAPDH (means ± SEM; one-way ANOVA with Dunnett's post hoc test; n=3 independent experiments; 8 DIV). Astrocytes were treated with 10 nM epoxomicin for 24 h or 100 nM Baf A_1 for 4 h; 3-6 DIV.

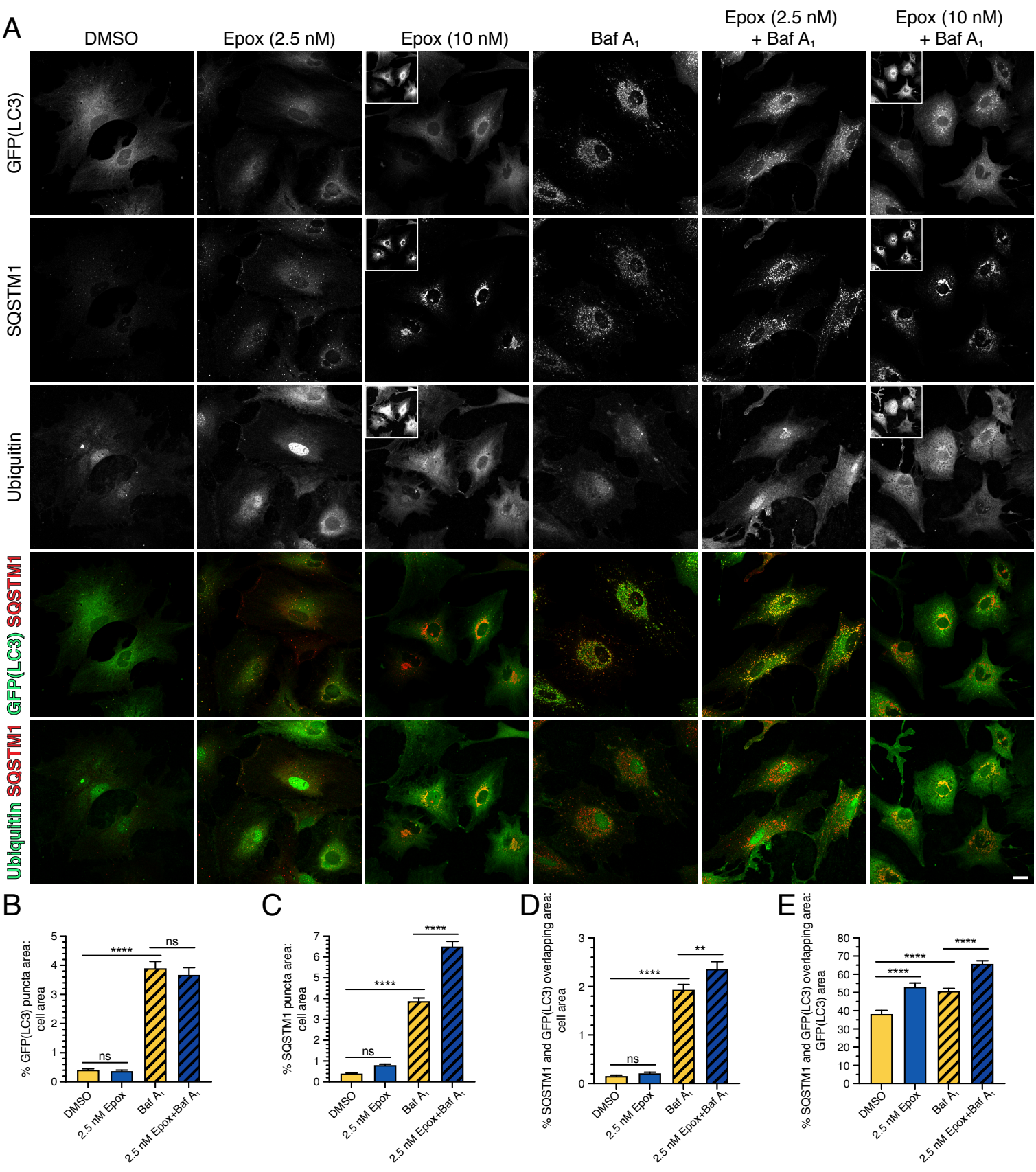
A
DMSO
Epox (2.5 nM)
Epox (10 nM)
Baf A1
Epox (2.5 nM) + Baf A1
Epox (10 nM) + Baf A1
GFP(LC3)
SQSTM1
Ubiquitin
Ubiquitin SQSTM1 GFP(LC3) SQSTM1
Ubiquitin SQSTM1
B
% GFP(LC3) puncta area: cell area
ns
****
ns
DMSO
2.5 nM Epox
Baf A1
2.5 nM Epox+Baf A1
C
% SQSTM1 puncta area: cell area
ns
****
****
D
% SQSTM1 and GFP(LC3) overlapping area: cell area
ns
****
**
E
% SQSTM1 and GFP(LC3) overlapping area: GFP(LC3) area
****
****
****

Fig. S5

Figure S5. Intermediate paradigms of UPS inhibition result in a modest increase in SQSTM1 targeted to autophagosomes in astrocytes. Primary astrocytes treated with DMSO, 2.5 nM or 10 nM epoxomicin for 24 h; 100 nM Baf A_1 (or an equivalent volume of DMSO as a solvent control) was included in the last 4 h. (**A**) Maximum projections of z-stacks of GFP-LC3 transgenic astrocytes immunostained for GFP, SQSTM1, and ubiquitin. Images within the same marker for LC3, SQSTM1, and Ub are grayscale matched to facilitate direct comparisons; grayscale matched 10 nM epoxomicin images are shown as an inset because they reach saturation. Bar: 20 μm. (**B-E**) Corresponding quantification of immunostain analysis shown in A. (**B**) Quantification of total GFP(LC3) puncta area normalized to cell area (means ± SEM; one-way ANOVA with Tukey's post hoc test; n=70-90 cells from 3 independent experiments; 6 DIV). (**C**) Quantification of total area occupied by SQSTM1 puncta normalized to cell area (means ± SEM; one-way ANOVA with Tukey's post hoc test; n=74-94 cells from 3 independent experiments; 6 DIV). (**D-E**) Quantification of the percentage of overlapping area between GFP(LC3)-positive puncta and SQSTM1-positive structures normalized to (**D**) cell area or (**E**) GFP(LC3) puncta area (means ± SEM; one-way ANOVA with Tukey's post hoc test; n=67-85 cells from 3 independent experiments; 6 DIV).

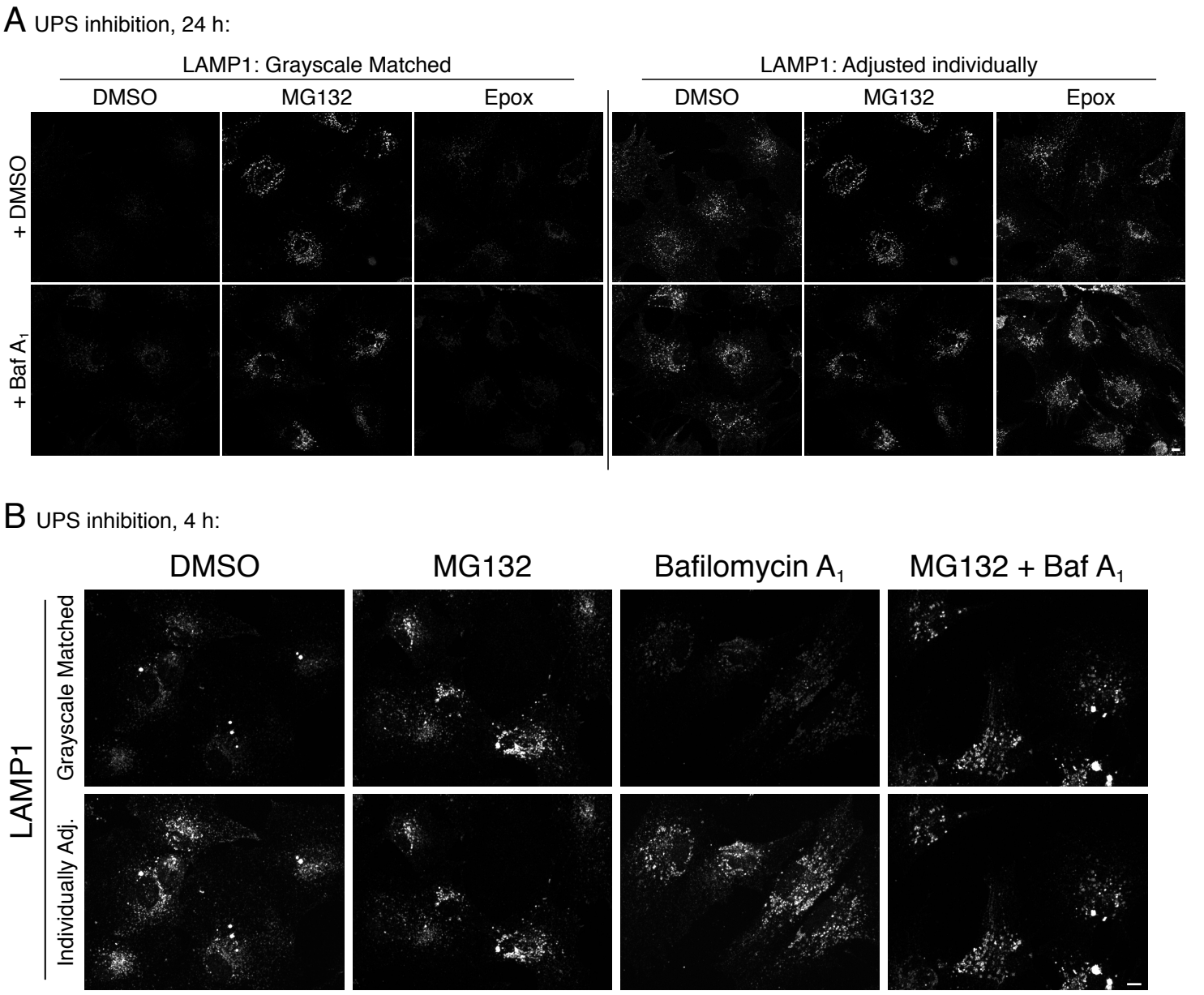
A UPS inhibition, 24 h:
LAMP1: Grayscale Matched
LAMP1: Adjusted individually
DMSO
MG132
Epox
DMSO
MG132
Epox
+ DMSO
+ Baf A1
B UPS inhibition, 4 h:
DMSO
MG132
Bafilomycin A1
MG132 + Baf A1
LAMP1
Grayscale Matched
Individually Adj.

Fig. S6

Figure S6. MG132 alters lysosomal characteristics in primary astrocytes. (**A**) Immunostain analysis of primary astrocytes (5-7 DIV) treated with DMSO, 0.5 µM MG132 or 10 nM epoxomicin for overnight; 100 nM Baf A_1 (or equivalent volume of DMSO as a solvent control) was included in the last 4 h. Maximum projections of z-stacks of astrocytes immunostained for LAMP1 adjusted either identically, or individually, as indicated. Bar: 10 µm. (**B**) Maximum projections of z-stacks of astrocytes (4 DIV) treated with 50 µM MG132 ± 100 nM Baf A_1 for 4 h and immunostained for LAMP1. Images were adjusted either identically, or individually, as indicated. Bar: 10 µm.

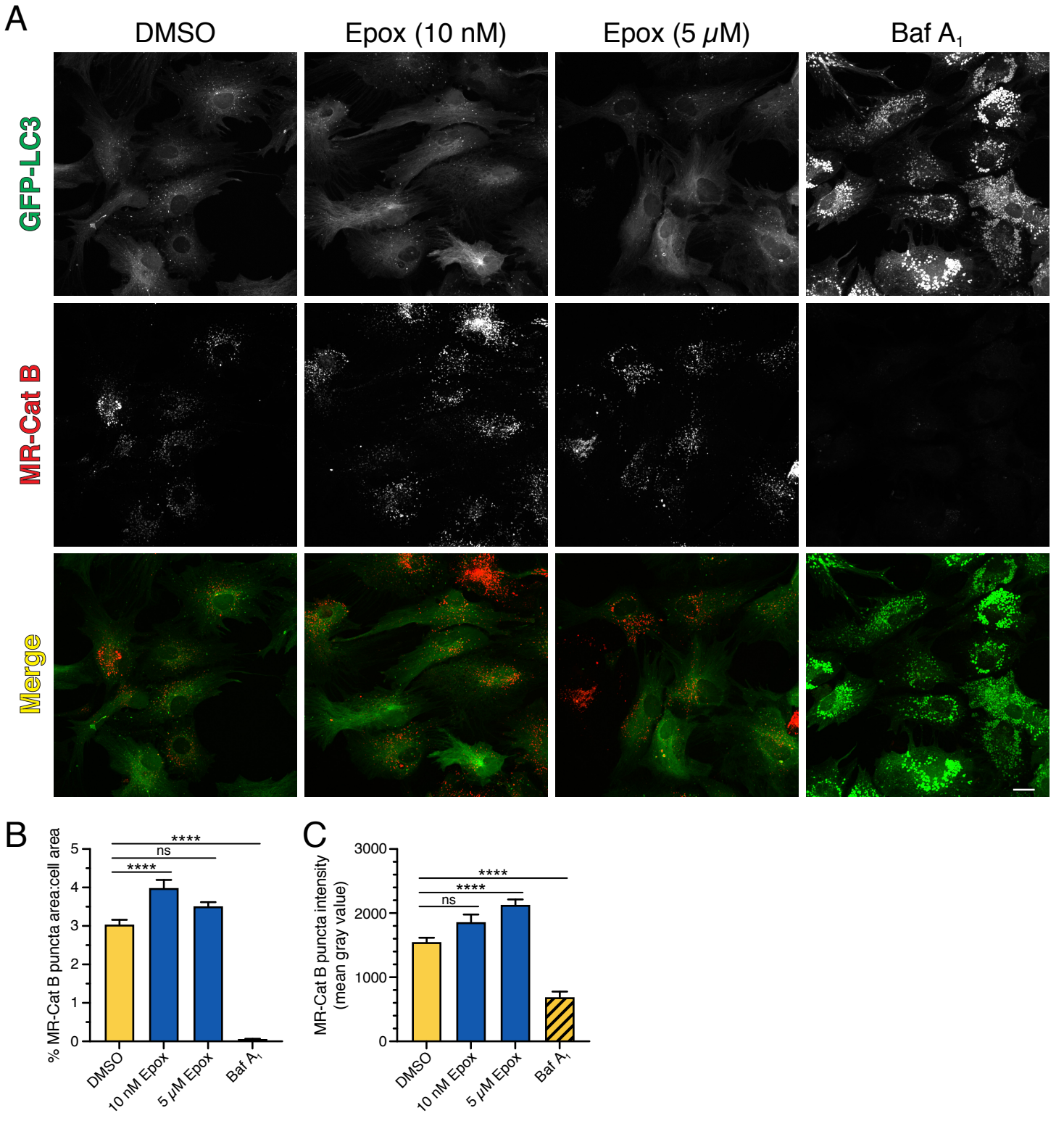
A
DMSO
Epox (10 nM)
Epox (5 μM)
Baf A1
GFP-LC3
MR-Cat B
Merge
B
% MR-Cat B puncta area:cell area
0
1
2
3
4
5
****
ns
****
DMSO
10 nM Epox
5 μM Epox
Baf A1
C
MR-Cat B puncta intensity (mean gray value)
0
1000
2000
3000
****
****
ns
DMSO
10 nM Epox
5 μM Epox
Baf A1

Fig. S7

Figure S7. Short-term inhibition of the UPS results in a modest increase in lysosomal function. (**A**) Live-cell imaging analysis of primary astrocytes treated with 10 nM epoxomicin, 5 µM epoxomicin, 100 nM Baf A_1, or equivalent volume of DMSO solvent control for 4 h. Astrocytes are labeled with Magic-Red Cathepsin B substrate (MR-Cat B) for the final 30 min of treatment. Shown are maximum projections of z-stacks. All images within the same marker are grayscale matched to facilitate direct comparisons. Bar: 20 µm. (**B**) Corresponding quantitation of total area occupied by MR-Cat B-positive puncta normalized to astrocyte area (means ± SEM; one-way ANOVA with Tukey's post hoc test; n=92-96 cells from 3 independent experiments; 4-7 DIV). (**C**) Corresponding quantitation of MR-Cat B puncta intensity (means ± SEM; one-way ANOVA with Tukey's post hoc test; n=72-96 cells from 3 independent experiments; 4-7 DIV).