Supplementary Material

Methods

Plasmid constructs

cDNAs for *FAB1* and PIKfyve were as described previously (McEwen et al, 1999). GST-Fab1p and GST-PIKfyve were isolated from *S. cerevisiae* as described previously (McEwen et al, 1999). GST-p110α was isolated from baculovirus infected insect cells as described elsewhere (Dhand et al, 1994). The tandem FYVE domain construct was kindly provided by Harald Stenmark.

In vivo phospholipid labelling

6 cm dishes of sub-confluent NIH3T3 cells were labelled with inorganic ^{32}P-orthophosphate ($^{32}P_i$, Amersham). Cells were placed in phosphate free medium, 0.3 mCi/ml $^{32}P_i$ added and cells incubated for 1 hour, followed by treatment -/+ YM201636 for 20 minutes. Cells were lysed by the addition of 1 ml of ice-cold 1 M HCl, harvested by scraping and lipids extracted and analysed by HPLC as described previously (Dove et al, 1997b).

Western Blots

NIH3T3 cells were seeded on to 6-well plates (200,000 per well) and grown in DMEM with 10% donor calf serum (DCS). The cells were then washed with DMEM and placed in DMEM with 0.1% DCS for 18 hours. The cells were pre-treated for 15 minutes with

vehicle (DMSO), 800 nM YM201636, 20 nM rapamycin (10 μM stock dissolved in ethanol; Sigma), or 10 μM LY294002 (Calbiochem). The cells were then either left unstimulated or were stimulated for a further 15 min with 10% DCS. Following these treatments the cells were placed on ice, washed immediately with Phosphate Buffered Saline (4°C) and harvested in 250 ml 1X sample buffer. The extracts were resolved by SDS-PAGE on a 10% polyacrylamide gel, transferred onto a PVDF membrane by semidry blotting and probed with specific antibodies as indicated. Immunoreactions were visualised by ECL (Amersham). The following primary antibodies were used: PW88 a rabbit phospho-specific polyclonal antibody raised against P-ser473 of PKB, a gift from Dr Julian Downward (1:1000); PW56 a rabbit polyclonal anti-PKB, a gift from Dr Julian Downward (used at 1:1000); a rabbit polyclonal anti-P-ser473 from Signal Transduction laboratories (used at 1:1000). Lamp1 antisera were from Santa Cruz (sc-8098) and recognise human, mouse and rat Lamp1.

Live Cell Time Lapse Microscopy

For live cell imaging, cells were grown on 35mm glass bottom dishes (Matek Corporation). Imaging was performed in an environmental chamber at 37°C with 10% CO_2. Cells were grown in phenol red-free DMEM supplemented with 10% DCS prior to imaging. NIH3T3 cells were treated with 800 nM YM201636 and examined using a Nikon Diaphot 200 microscope fitted with a 60X/1.4 NA plan Apo DM ph4 160/0.17 oil immersion objective lens. Images were captured using AQM 2001 Kinetic Acquisition Manager software (Kinetic Imaging Ltd). Treated NIH3T3 cells were then washed 3 times, placed in medium without YM201636 and image acquisition continued.

Transfected Cos7 cells were examined 20-24 hours post-transfection using an Olympus IX70 microscope fitted with a Wallac UltraVIEW Confocal Scanner (Perkin Elmer), an E-662 LYPZT Amplifier Pieza Disk using the UltraVIEW Imaging Suite software package set in Temporal Module (Perkin Elmer). The GFP-Rab5 chimera was excited at 488 nm and detected with split emission spectrum 525/550 nm. Films were constructed using AQM 2001 Kinetic Acquisition Manager software (Kinetic Imaging Ltd).

Confocal and Electron Microscopy

NIH3T3 cells and Cos7 cells were seeded on acid washed glass coverslips. In some experiments transient transfections were performed using Lipofectamine 2000 (Invitrogen) and all subsequent manipulations then performed 48 hours post-transfection. Cells were washed and fixed in 4% paraformaldehyde for 15min. When processing for immunofluorescence, cells were permeabilized with 0.2% (v/v) Triton X-100 for 5min, washed and incubated in 2% bovine serum albumin (BSA) in PBS for 20min. Coverslips for immunofluorescence were then incubated with primary antibody in 1% (w/v) BSA in PBS for 1 hour. The cells were washed and incubated with secondary antibody fluorescent dye-conjugate for 1 hour then washed three times in PBS and once in water. The cells were mounted on glass slides under MOWIOL (100mM TrisHCl pH 8.8, 10% (w/v) MOWIOL 4-88 (Calbiochem) and 25% (v/v) glycerol) containing antiphotobleaching agent (2.5% (w/v) 1,4-diazabicyclo[2.2.2]; Sigma). For all preparations, slides were examined using a confocal laser-scanning microscope (Axioplan2 with LSM 510 Carl Zeiss Inc.) equipped with 63x/1.4 Plan-APOCHROMAT

oil immersion objective. Each image represents a single 0.4 μm optical section. Ultrastructural electron microscopy images were obtained as described previously (Ivaska et al, 2002).

Small Interfering RNA (siRNA)

Small interfering RNAs were designed and synthesised using the "Silencer siRNA construction kit" (Ambion). Four regions were selected based on AA sequence, low GC content and unique sequence. NIH3T3 cells and Cos7 cells were seeded on acid washed glass coverslips in 6-well plates. They were then transfected with approximately 2 μg of siRNA using Lipofectamine transfection reagent (Invitrogen). The cells were monitored over 48 hrs (distinct changes were observed after 24 hrs) then the cells were washed and fixed in 4% paraformaldehyde for 15 min. The cells were mounted on glass slides and imaged as described above.

Protein Degradation Assay

The protein degradation assay was based on the original method described previously (Gronostajski et al, 1984). In brief, NIH3T3 cells were labelled with ^{14}C-leucine (0.2 mCi/ml) in DMEM (without leucine) supplemented with 10% dialysed donor calf serum (DCS) and 2 mM cold leucine for 24 hours. This was followed by a chase in DMEM (without leucine) supplemented with 10% dialysed DCS and 2 mM cold leucine for 24 hours. The cells were washed and then placed in either (i) DMEM (without leucine) supplemented with 10% dialysed DCS, 2 mM cold leucine in the absence of YM201636, (ii) DMEM (without leucine) supplemented with 10% dialysed DCS, 2 mM

cold leucine and 800 nM YM201636, (iii) Earl's balanced saline solution (EBSS), 0.1% BSA (Sigma) 2m M cold leucine in the absence of YM201636 (iv) Earl's balanced saline solution (EBSS), 0.1% BSA (Sigma) 2 mM cold leucine and 800 nM YM201636. Samples of medium (200 ml) were taken at each time point indicated. Trichloroacetic acid (TCA) to 5% was added to each sample and the radioactivity in the TCA-soluble material was measured. After the final sample was taken the cells were harvested and the radioactivity in the total-acid insoluble bulk protein was determined. The extent of protein degradation was calculated as a percentage: 100 x acid soluble cpm in medium/{acid insoluble cpm in bulk protein + acid soluble cpm in medium}.

Viral Production Assays

A TEGH-human fibrosarcoma producer line (TEFLY Mo (Cosset et al, 1995)) was employed to monitor retrovirus production. This producer line had been stably transfected with pBabe-Puro and generated ecotropic MLV retrovirus with puromycin resistance. TEFLY Mo cells were grown in DMEM supplemented with 10% fetal calf serum. TEFLY Mo cultures were pre-treated with 0nM, 400nM or 800 nM YM201636 for 3 hours. The cells were washed 3 times and fresh medium containing YM201636 compound added. The cells were incubated for a further 9 hours before the virus-containing medium was harvested, filtered through a 0.45 μM membrane and diluted 1/10, 1/100, 1/1000, 1/10000. These dilutions were used to infect NIH3T3 cell cultures on 6cm plates in the presence of 8 μg/ml polybrene. After 2 hrs the polybrene was diluted to 2 μg/ml. 48 hours later the medium was changed and selection begun by addition of puromycin at 2 μg/ml. Fresh medium containing puromycin was added every 48-72 hrs

until distinct colonies formed. The cells were then fixed and stained with 0.5% crystal violet 10% methanol and de-stained with distilled water before the numbers of colonies were quantified visually.

Supplementary Figures and Movie Legends

Movie 1. Time-lapse analysis of the induction of the swollen vesicle phenotype and its reversion, following treatment of NIH3T3 cells with 800nM YM201636 and the subsequent withdrawal of the drug. NIH3T3 cells were treated with 800nM YM201636 and imaged for 120min as described in supplementary material and methods. At 120min the 35mm Matek dish was removed from the microscope stage, the cells were washed free of YM201636, placed in fresh medium and the dish return to the microscope. The filming was then continued for a further 130min in the absence of YM201636. This changeover can be seen at 1.11-1.12 minutes of the film. Frames were captured at 10sec. intervals and the film playback is at 10fps.

Movie 2. Rab5 positive compartments contribute to the swollen vesicle phenotype induced by YM201636. Cos7 cells transfected with EGFP-Rab5 plasmid were treated with 800nM YM201636. Image capture was begun 5min after YM201636 treatment (with an Olympus IX70 microscope fitted with a Wallac UltraVIEW Confocal Scanner [Perkin Elmer] as described in supplementary material and methods) and continued for

approximately 83min. 500 frames were captured at 10sec. intervals and the film playback is at 10fps.

Figure S1. Golgi, Endoplasmic Reticulum and Lysosomal membrane markers do not localise to the outer membrane of the swollen vesicles.

A, the localisation is shown for Golgi marker GM130 in NIH3T3 cells untreated (left column panels) or treated with 800nM YM201636 for 2 hours (right column panels). The phase image (Ph) is merged with the fluorescent image (Fl) in the bottom panel (Mer). B, the localisation of Golgi marker p230 is shown in Cos7 cells untreated (left column panels) or treated with 800nM YM201636 for 2 hours (right column panels). C, the localisation is shown for ER marker p62 in NIH3T3 cells untreated (left column panels) or treated with 800nM YM201636 for 2 hours (right column panels). D, the localisation is shown for lysosomal marker Lamp1 in NIH3T3 cells untreated (left column panels) or treated with 800nM YM201636 for 2 hours (right column panels).

Figure S2. Localisation of endosomal marker EEA-1 in untreated control cells.

The localisation of EEA1 is shown in NIH3T3 cells untreated with YM201636. The phase image (Ph) is merged with the fluorescent image (Fl) in the bottom panel (Mer).

Figure S3. YM201636-induced vesicles are actin associated and require microtubules for their formation.

A, NIH3T3 cells treated with 800 nM YM201636 for 2 hours were stained for actin filaments with phalloidin-TxR. B, C, NIH3T3 cells were treated with 800 nM YM201636

for 2 hours in the absence (B) or presence of 2 mM vinblastin (C) and the microtubules were stained with anti α-tubulin and anti-mouse-cy3. NIH3T3 cells were treated with 800 nM YM201636 for 2 hours in the presence (E) or absence (D) of 66 μM Nocodazole and then the microtubules stained with anti α-tubulin and anti-mouse-cy3.

Figure S4. EM analysis of the swollen vesicles induced by YM201636

Representative thin section electron micrographs of NIH3T3 cells (A) untreated with YM201636 or (B) treated with 800nM YM201636 for 60 min or (C) treated with 800nM YM201636 for 120min.

Figure S5. Localisation of CI-MPR and GFP-tandem-FYVE^{Hrs} domain (2XFYVE) in untreated control NIH3T3 cells.

A, the localisation of CI-MPR is shown in NIH3T3 cells untreated with YM201636. The phase image (Ph) is merged with the fluorescent image (Fl) in the bottom panel (Mer). B, the localisation of GFP-tandem-FYVE^{Hrs} domain (2XFYVE) is shown in NIH3T3 cells untreated with YM201636.

Figure S6. Localisation of endosomal and lysosomal tracers in untreated control NIH3T3 cells.

A, NIH3T3 cells were incubated with 2mg/ml Lucifer Yellow for 30min. B, NIH3T3 cells were serum starved (20 h, 0.1% FCS) and then stimulated with 10 nM EGF for 60 min. EGF receptor was then stained with anti-EGFR and anti-mouse-cy3. C, NIH3T3 cells were incubated with 100nM Lysotracker Red DND-99 for 35 min. Lysotracker is

acidotropic and accumulates in acidified organelles. A, B, and C, the phase images (Ph) are merged with their respective fluorescent images (Fl) in the bottom panels (Mer).

Figure S7. YM201636 partially inhibits protein degradation and autophagosomal marker GFP-LC3 accumulates inside the swollen vesicles.

A, GFP-LC3 transfected NIH3T3 cells were untreated (left column panels) or treated for 2 h with 800nM YM201636 (right column panels). The GPF, phase and overlay are shown. B, NIH3T3 cells were pre-labelled with ^{14}C-leucine as described in the Experimental Procedures. Cells were then placed in EBSS in the presence or absence of YM201636 as indicated and the total protein and released TCA-soluble counts monitored over time.

Figure S8. YM201636 blocks retroviral release.

A, B, viral release was assayed in the packaging line TEGH expressing the Moloney leukaemia virus. Released viral particles were detected by infection of NIH3T3 cells with serial dilutions of virus and selection with puromycin followed by colony counting (see Experimental Procedures; the colonies resulting from the infections using virus dilutions of 1/1,000 and 1/10,000 were used to calculate the figures shown). The effect of YM201636 in virus production was tested at 0, 400 and 800 nM as indicated. Representative plates of puromycin selected cells stained with crystal violet, which had been infected with a 1/1000 dilution of virus containing media from retro-viral packaging cells treated with the indicated concentrations of YM201636 are shown in B. The appearance of virus particles within cells was scored from EM images as illustrated in D

and scored as particles/cell panel C. Thin section Electron Micrographs of the retro-virus packaging line TEGH human fibrosarcoma (TEFLY Mo) untreated (left panel) or treated with 800 nM YM201636 for 12 hours (right panel). The small, far right inset panel illustrates a tethered viral particle (see text).

Figure S1

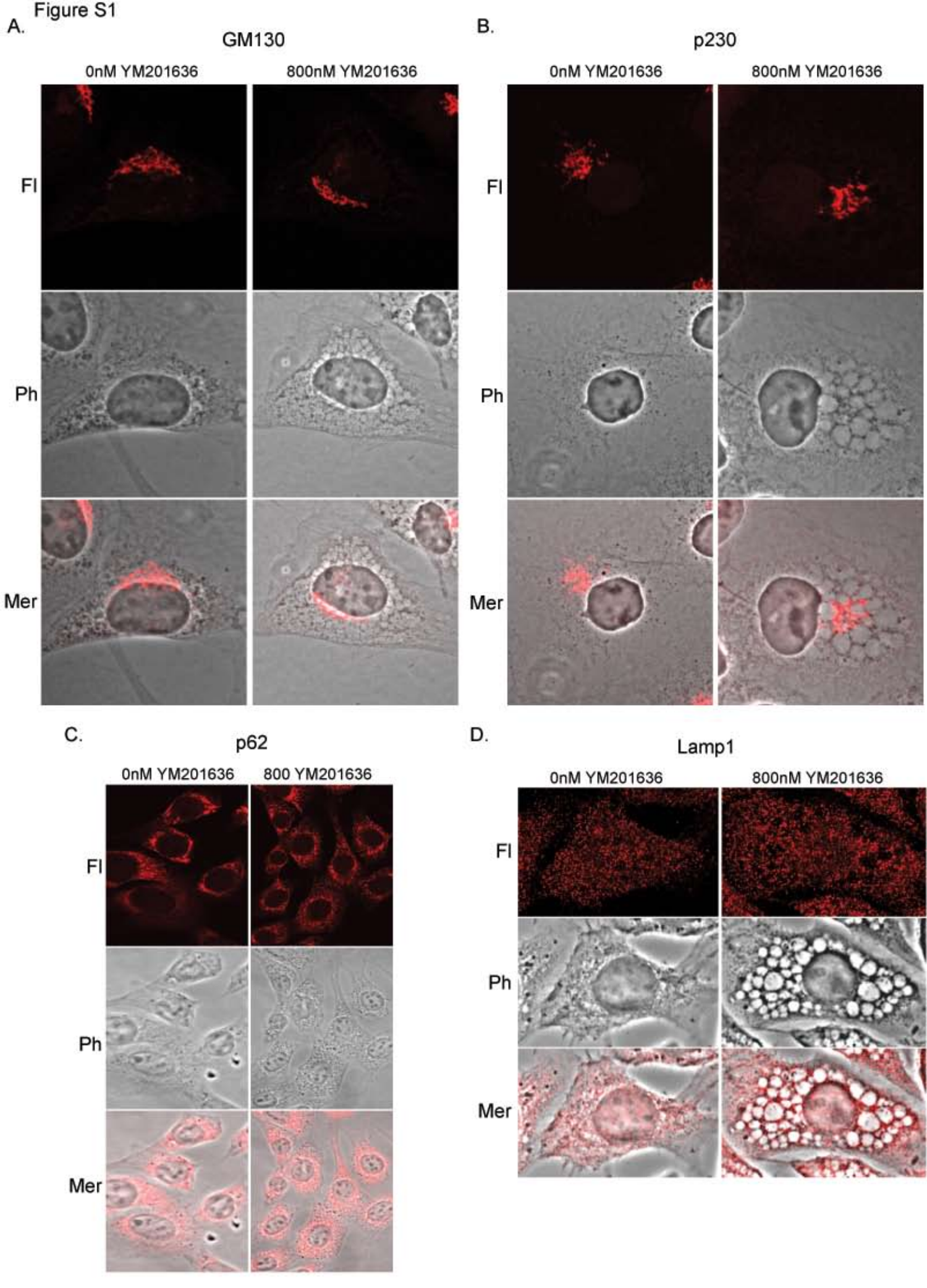

A.
GM130
0nM YM201636
800nM YM201636
Fl
Ph
Mer
B.
p230
0nM YM201636
800nM YM201636
Fl
Ph
Mer
C.
p62
0nM YM201636
800 YM201636
Fl
Ph
Mer
D.
Lamp1
0nM YM201636
800nM YM201636
Fl
Ph
Mer

Figure S2

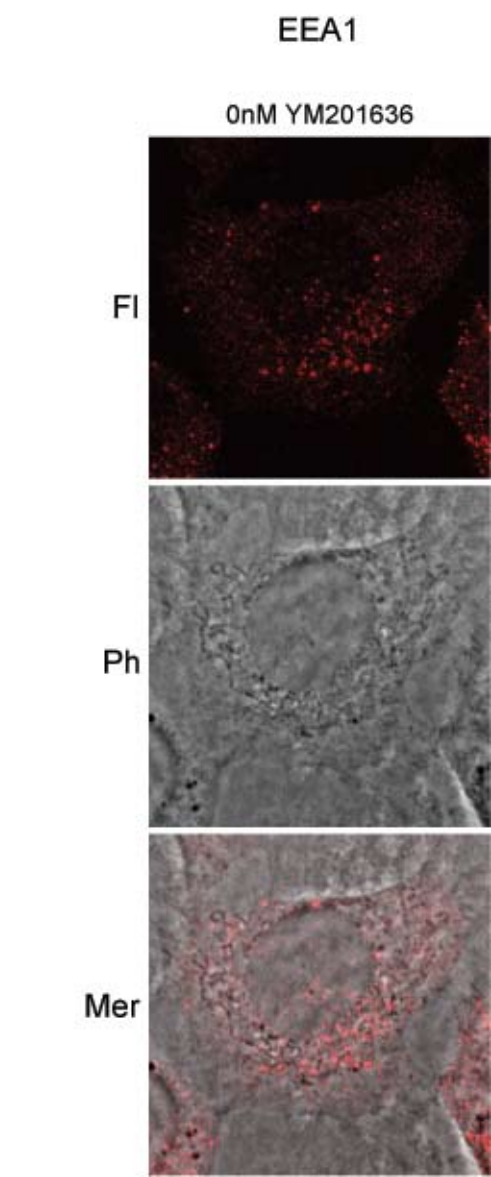


Figure S3

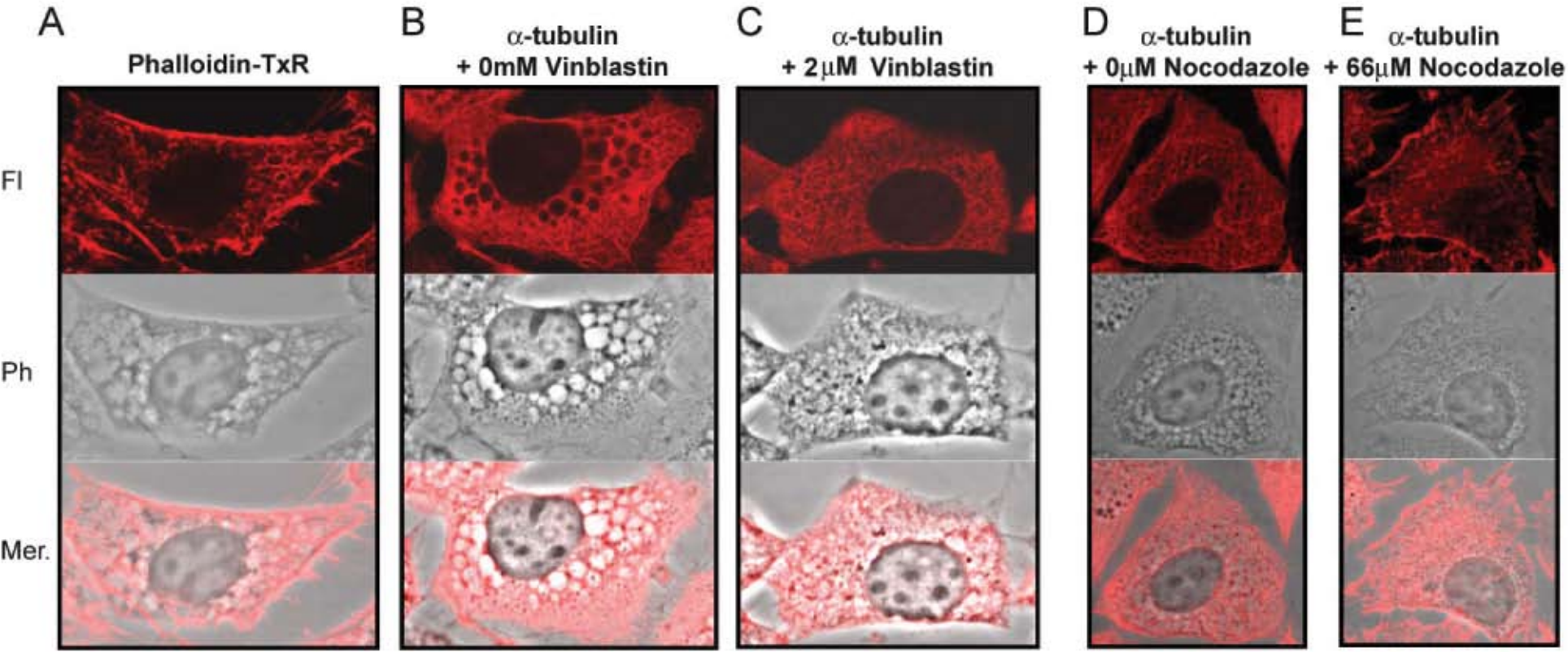

A
Phalloidin-TxR
B
α-tubulin
+ 0mM Vinblastin
C
α-tubulin
+ 2μM Vinblastin
D
α-tubulin
+ 0μM Nocodazole
E
α-tubulin
+ 66μM Nocodazole
Fl
Ph
Mer.

Figure S4

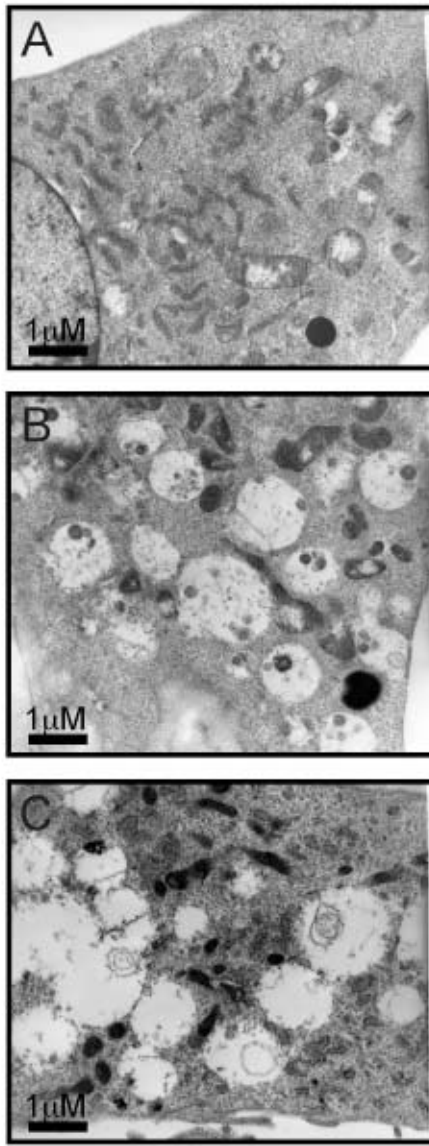


Figure S5

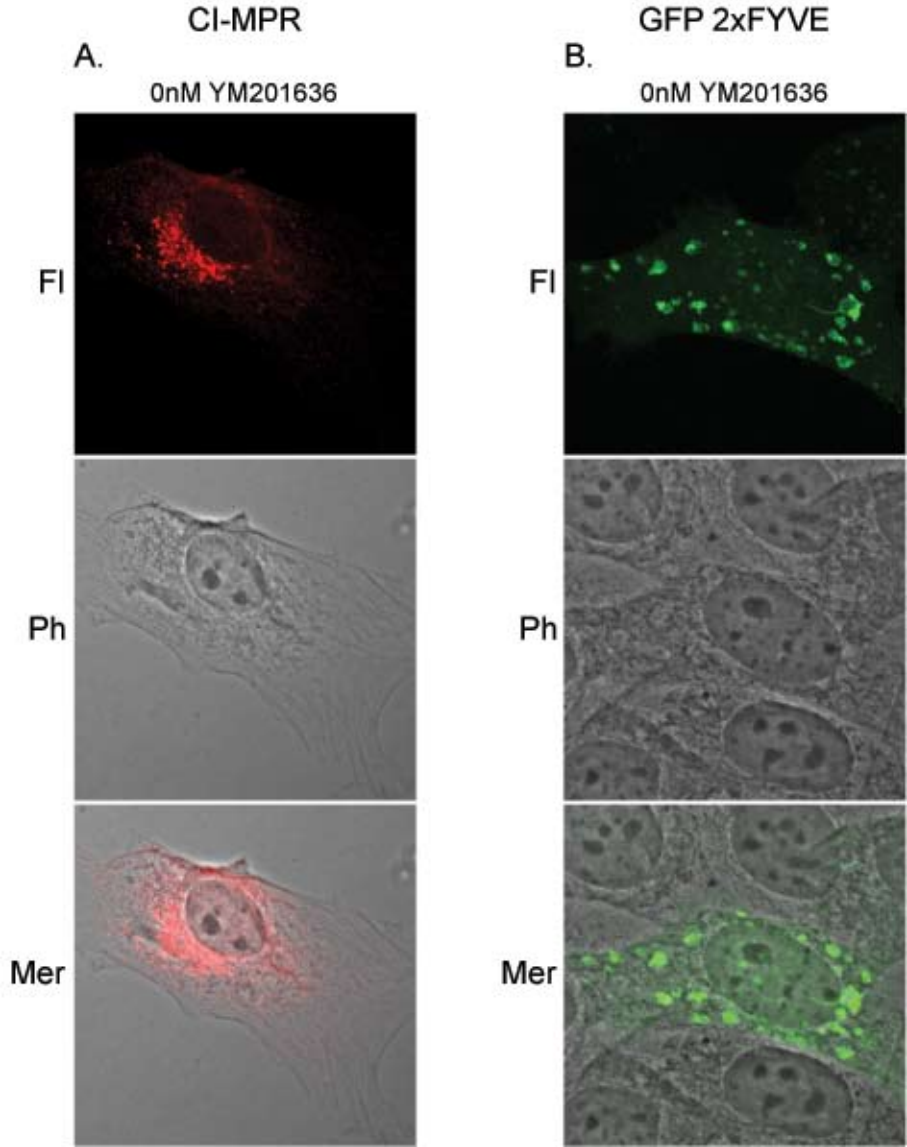

CI-MPR
A.
0nM YM201636
Fl
Ph
Mer
GFP 2xFYVE
B.
0nM YM201636
Fl
Ph
Mer

Figure S6

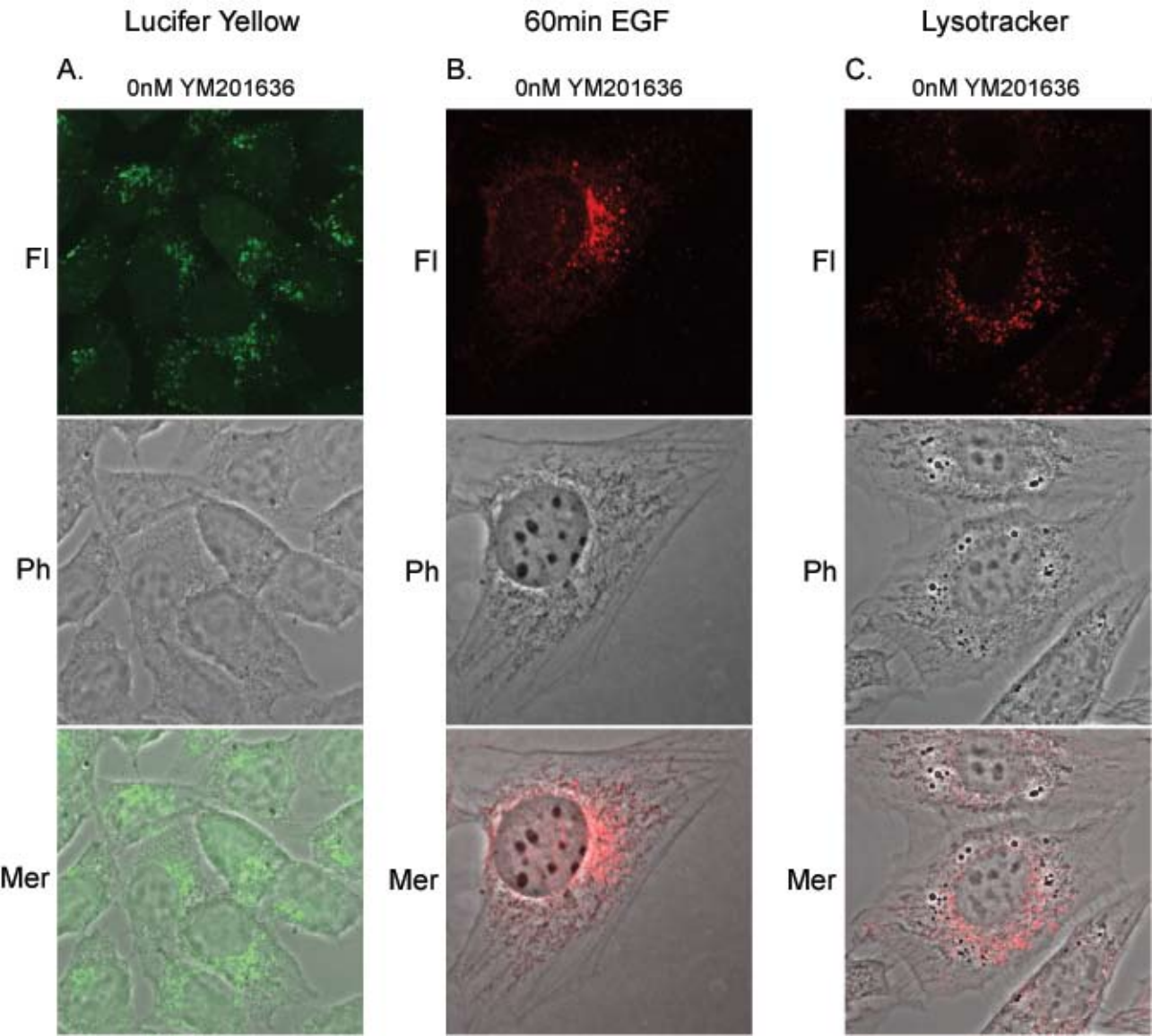
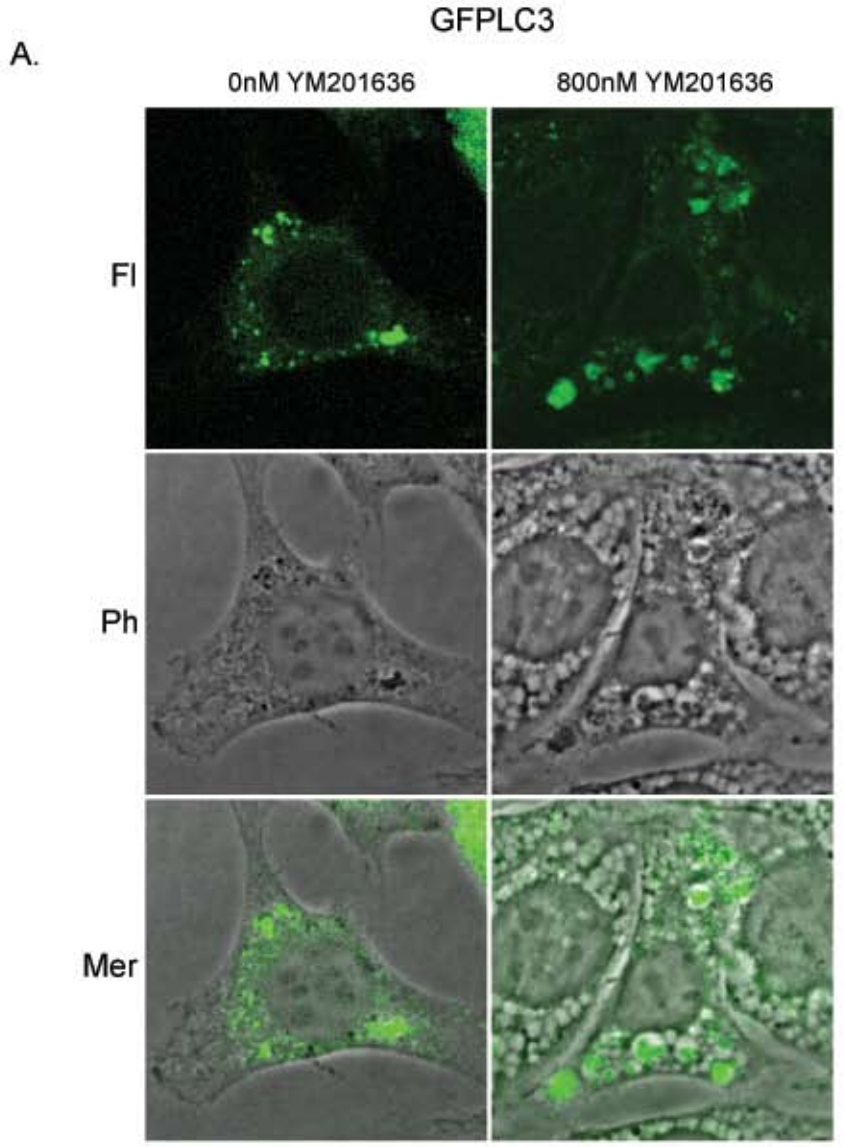
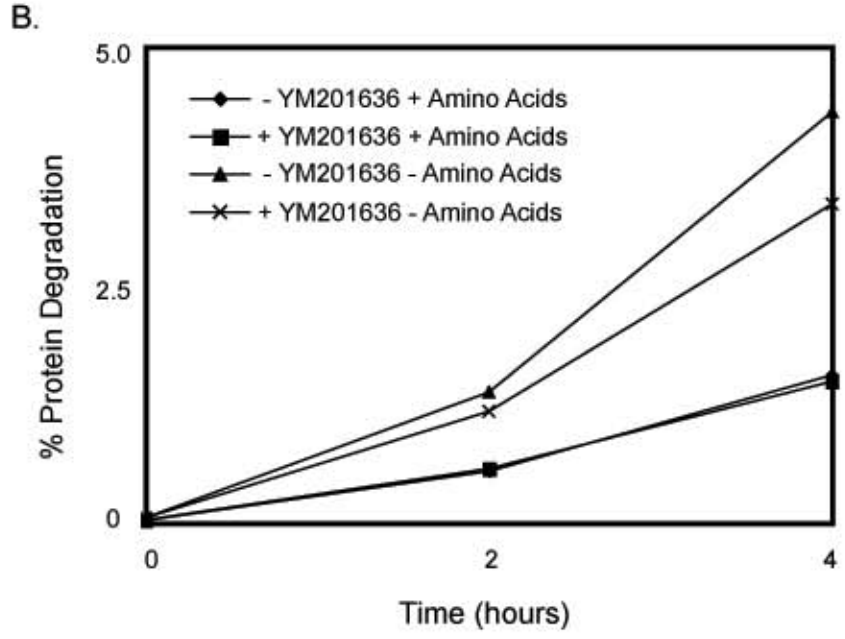
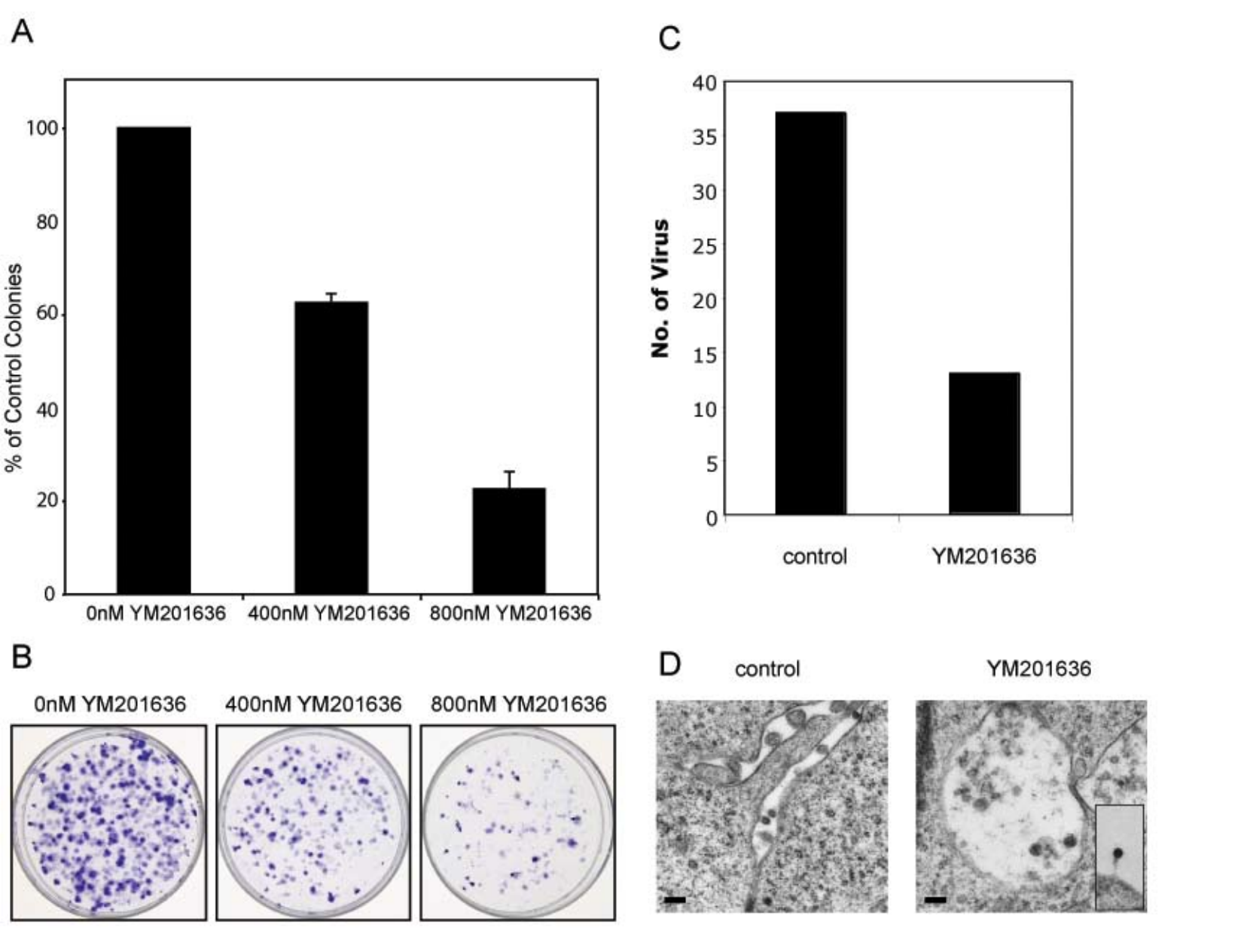

Lucifer Yellow
60min EGF
Lysotracker
A.
0nM YM201636
B.
0nM YM201636
C.
0nM YM201636
Fl
Ph
Mer
Fl
Ph
Mer
Fl
Ph
Mer

Figure S7


GFPLC3
A.
0nM YM201636
800nM YM201636
Fl
Ph
Mer


B.
5.0
2.5
0
% Protein Degradation
- YM201636 + Amino Acids
+ YM201636 + Amino Acids
- YM201636 - Amino Acids
+ YM201636 - Amino Acids
0
2
4
Time (hours)


A
100
80
60
40
20
0
% of Control Colonies
0nM YM201636
400nM YM201636
800nM YM201636
C
40
35
30
25
20
15
10
5
0
No. of Virus
control
YM201636
B
0nM YM201636
400nM YM201636
800nM YM201636
D
control
YM201636