SUPPLEMENTARY INFORMATION

FIGURE S1**. Time course of M3 receptor internalization using a radioligand binding assay**. Cells expressing M3R were incubated at 37°C with carbachol (1mM) at different times (See Experimental procedure for details). After drug treatment, the cells were cooled on ice and the loss of cell surface M3 receptor in presence of carbachol was assessed by incubating the cells with the cell-impermeant muscarinic ligand [$_3$H]-NMS (2nM) for 2h at 4°C. Receptor internalization was defined as the loss of the binding with the cell-impermeant [$_3$H]-NMS after carbachol treatment compared to the non-treated cells.
Curve shows average ± SD of a representative experiment of three independent experiments.

FIGURE S2. Constitutive internalization of ®2 and M3 receptors in the presence of antagonists. HeLa cells expressing ®2 (A) or M3 (B) receptors were pretreated for 30 min in the absence or presence of their respective antagonists, 10 ⌈M propranolol (A) or 1 ⌈M atropine (B). The cells were then incubated at 4°C with HA antibody to label the receptors at the surface and then incubated at 37°C for 30 min to allow antibody internalization in the absence or presence of antagonists. After fixation and blocking of surface antibody with unlabeled GAM, internalized antibody-bound receptors were revealed by secondary antibodies in the presence of saponin as described.

FIGURE S3. **Internalization of M3 receptor compared to MHCI and transferrin**. HeLa cells, that were preincubated with anti-HA antibody (IgG1), were incubated at 37°C for 5 min without (A) or with ligand (B) in the presence of mouse anti-MHCI antibody (IgG2a) or Alexa 594-conjugated transferrin (Tf 594). After fixation, unlabeled GAM antibody without saponin was used to block remaining surface mouse antibodies, followed by 488-GAM-IgG1 and 594-GAM-IgG2a in the presence of saponin to detect the internalized ⊂3 receptor and MHCI, respectively. When Alexa 594-transferrin was present, we utilized only the secondary antibody 488-GAM-IgG1. Paired insets show magnified views and indicate M3R, in absence of ligand, on tubular recycling endosomes. Images shown are representative of experiments that were repeated 3 times.

FIGURE S4. **Cellular distribution of ®2-GFP receptor.** Distribution of ®2-GFP receptor in living HeLa cells. Note receptor presence on elongated tubular recycling structures (arrows).
FIGURE S5**. Clathrin depletion using siRNA.** HeLa cells were depleted of clathrin as described in Experiment procedures. Extracts from mock-depleted (Control) and clathrin depleted (Clathrin siRNA) cells were separated by SDS-PAGE and immunoblotted with antibodies to clathrin heavy chain and actin (A). Clathrin was localized by immunofluorescence in control and clathrin depleted cells (B). Images shown are representative of experiments that were repeated 3 times.

FIGURE S6**. ®2 receptor reaches late endosomal compartments.** After preincubation at 4°C for 1h with the rabbit anti-HA antibody to label the ®2 receptor on the plasma membrane, HeLa cells were incubated at 37°C for 8 h in the absence (A) or in presence of Isoproterenol 1mM (B) in media containing 15 mM of NH_4Cl to inhibit degradation and permit the accumulation of antibody into lysosomes. After fixation, the lysosomes were visualized using mouse anti-Lamp1 antibody, followed by the appropriate secondary antibody. Images shown are representative of experiments that were repeated 3 times.

Movie 1. ®**2-GFP receptor and transferrin internalization in the absence of ligand in living cells.** HeLa cells plated onto coverglass chambers expressing ®2-GFP receptor were

incubated on a 37°C stage in CO_2-independent media. After 3-5 min, Alexa 594-transferrin was added to the medium and images were acquired every 10 s for 15 min in total. The left panel shows ®2-GFP receptor alone, while the right one shows the receptor with Tf 594. To reduce the memory space of the file, only selected frames are shown. The experiment was repeated twice.

Movie 2. ®**2-GFP receptor and transferrin internalization in the presence of ligand in living cells.** HeLa cells plated onto coverglass chambers expressing ®2-GFP receptor were incubated on a 37°C stage in CO_2-independent media. After 3-5 min, Alexa 594-transferrin was added to the medium with Isoproterenol (1mM) and images were acquired every 10 s for 15 min in total. The left panel shows ®2- GFP receptor alone, while the right one shows the receptor with Tf 594. To reduce the memory space of the file, only selected frames are shown. The experiment was repeated twice.

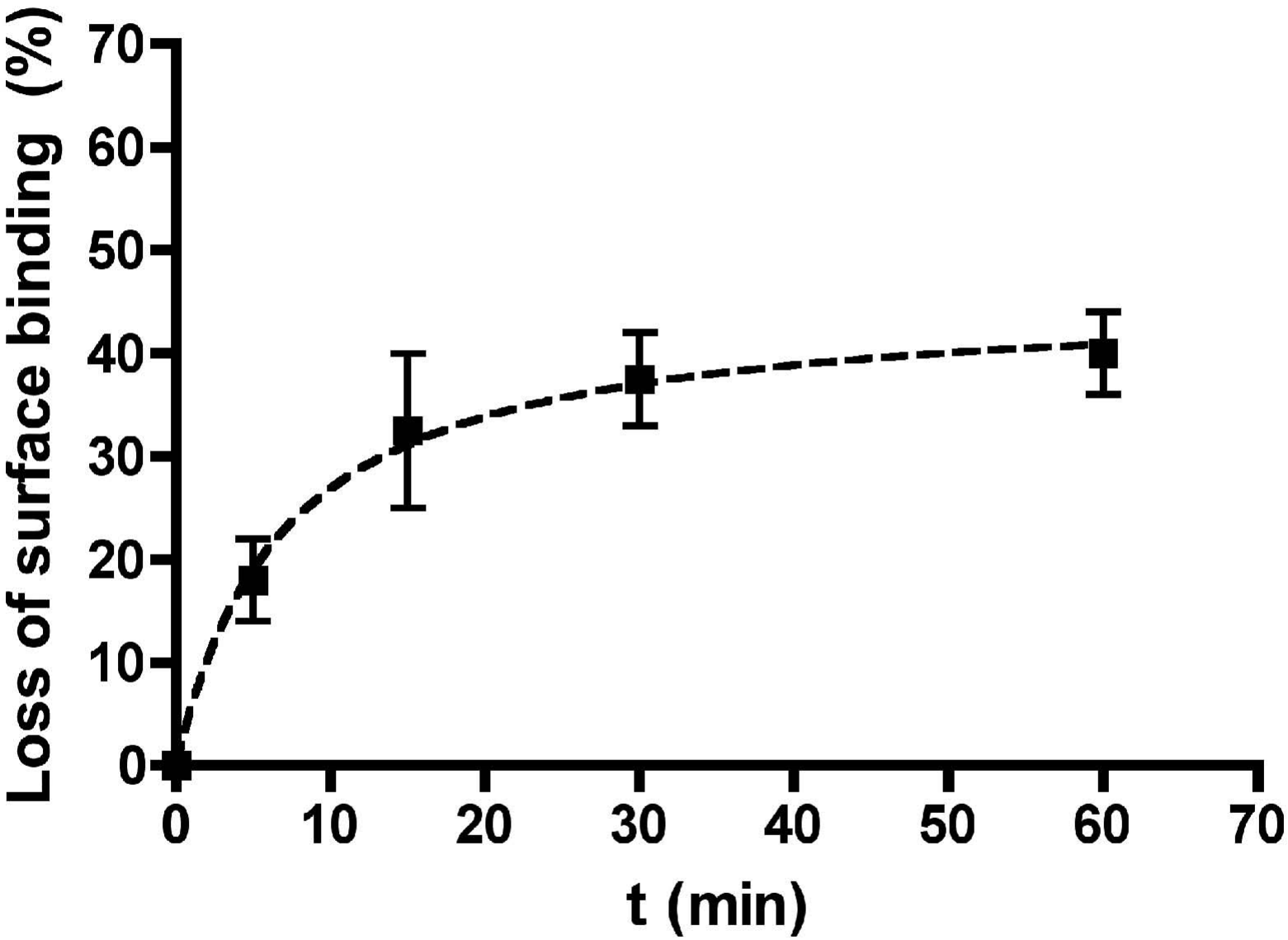
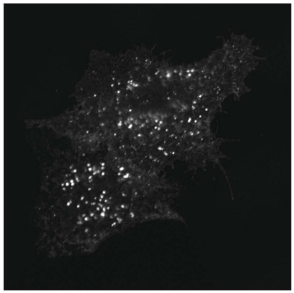
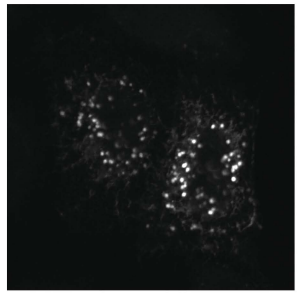

Loss of surface binding (%)
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
t (min)

Fig. S1

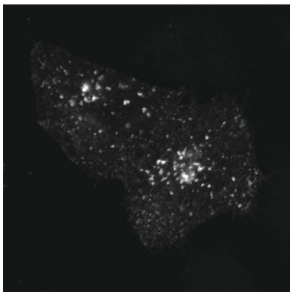
A β2R Control



+ Propranolol



B M3R Control



+ Atropine

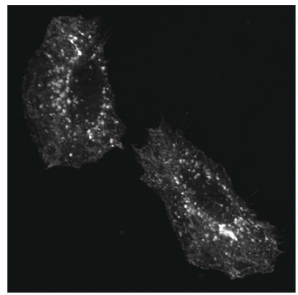


Fig.S2

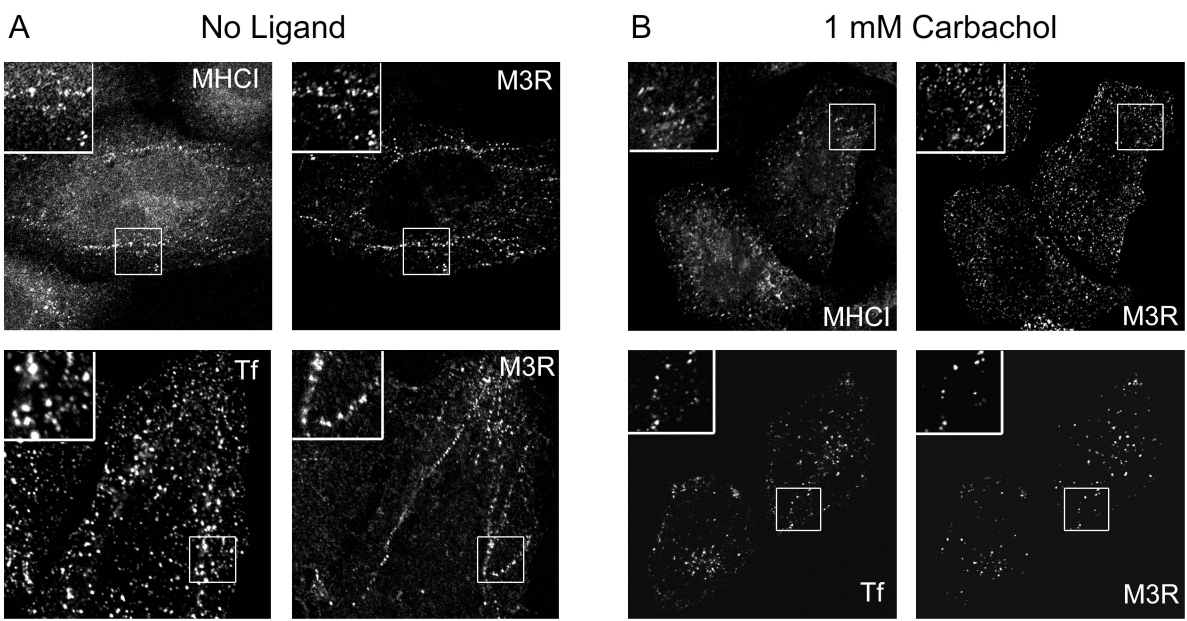


Fig.S3

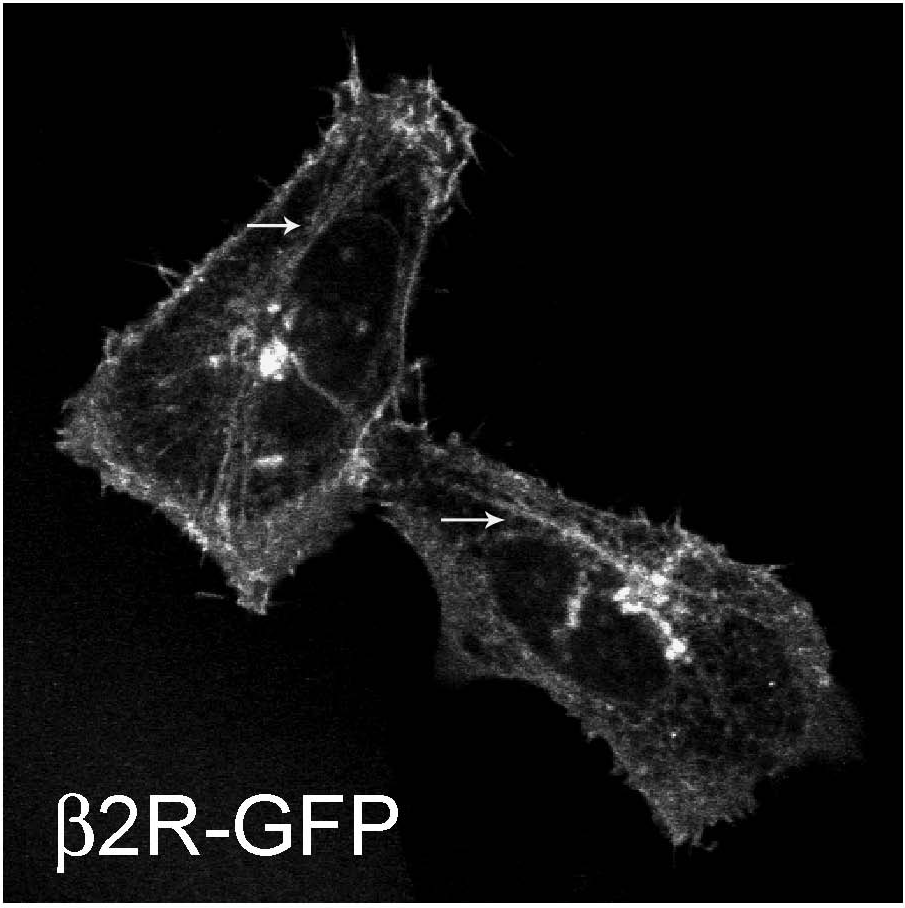
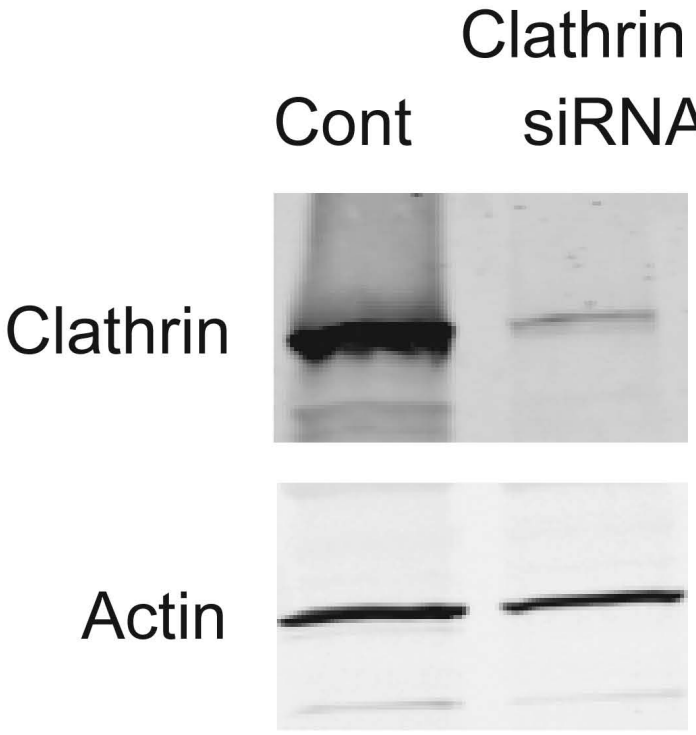


Fig.S4

A


Clathrin
Cont
siRNA
Clathrin
Actin

B

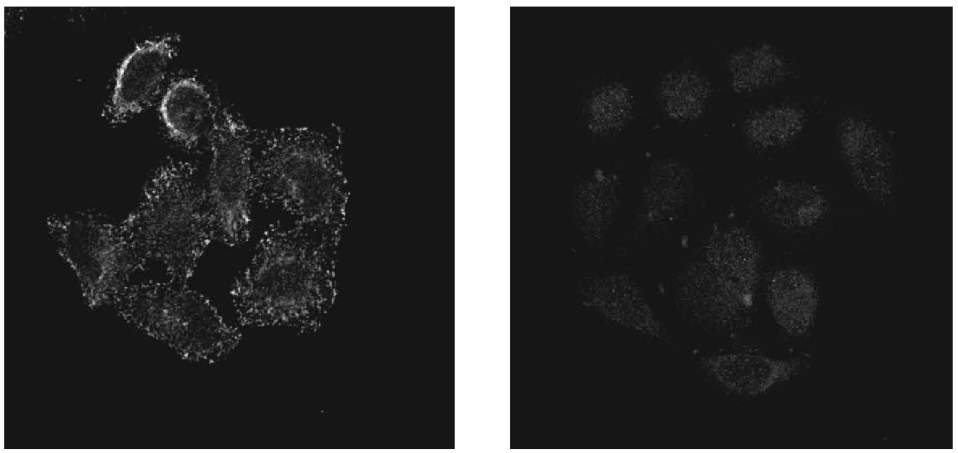

Control
Clathrin siRNA

Fig. S5

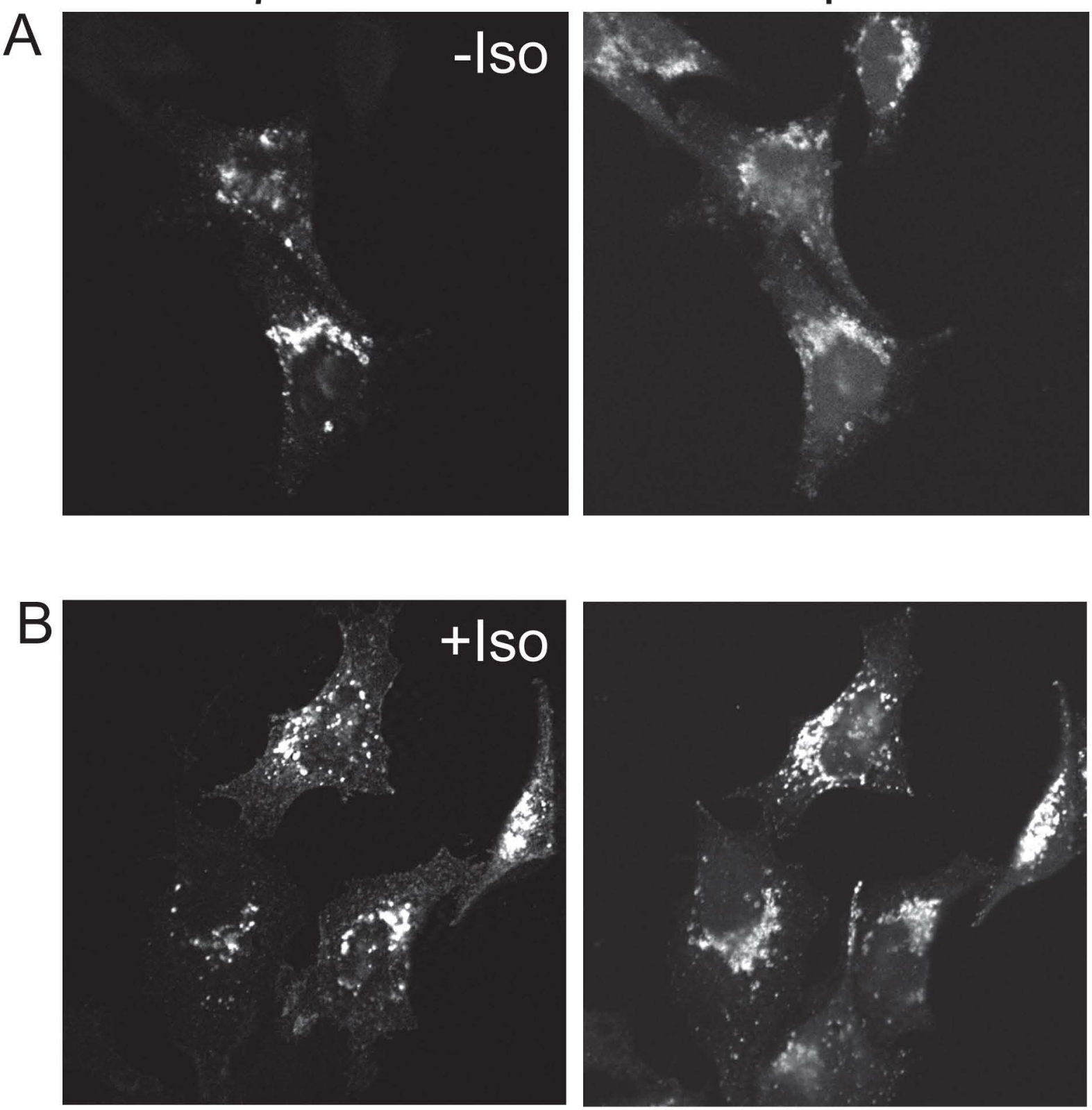


Fig. S6