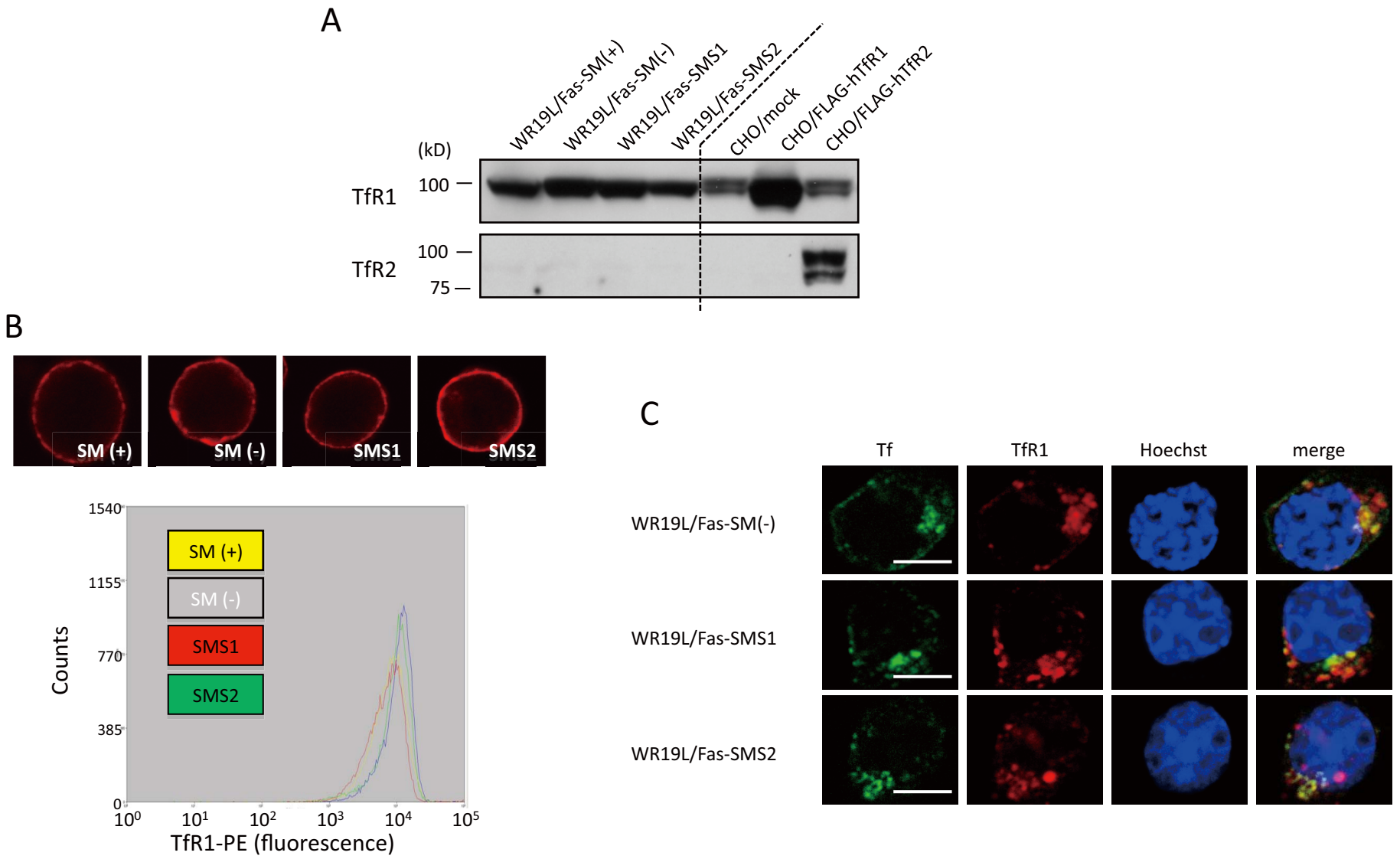
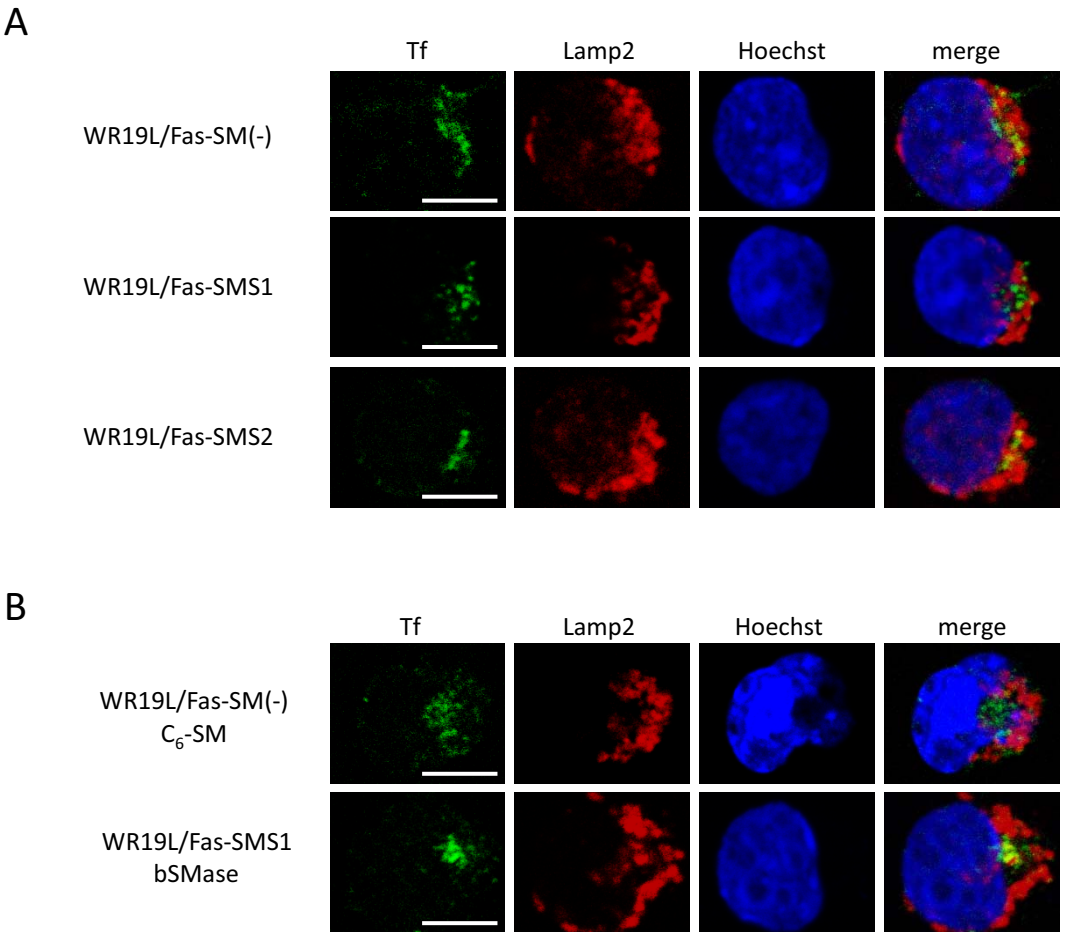
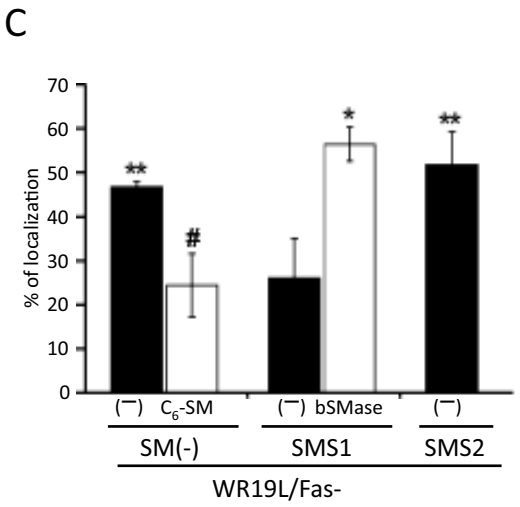
Supplemental Figure S1

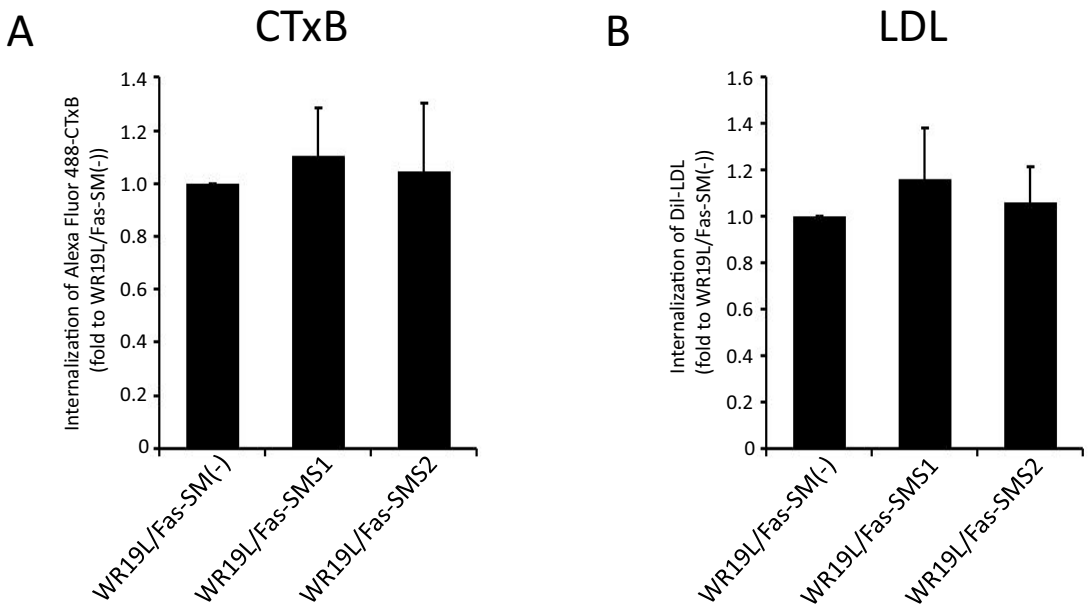


Supplemental figure S2





Supplemental Figure S3



SUPPLEMENTAL INFORMATION

Experimental Procedures

Materials—Alexa fluor 488-conjugated cholera toxin subunit B (CTxB) and dil-LDL were purchased from Molecular Probes (Eugene, OR). Plasmid vectors of TfR1 and TfR2 were kindly gifted by Dr. Kawabata (Osaka University). PE-conjugated anti-TfR1 antibody was from Biolegends (San Diego, CA). Rabbit polyclonal anti-TfR2 antibody was purchased from Abcam. Anti-Lamp2 antibody was from Santa Cruz Biotechnology Inc.

Flow cytometry analysis—WR19L cells were washed with PBS and incubated with anti-TfR1 conjugated with PE for 60 min on ice. After washing with BSA/PBS, the cells were analyzed by flow cytometry using an EPICS ALTRA (Beckman Coulter, Brea, CA).

Internalization assay—The cells were pre-incubated in serum-free RPMI for 60 min and further incubated with 2 μg/ml alexa fluor 488-conjugated CTxB or Dil-LDL for 10 min at 37°C. After washing with ice-cold PBS, cells were treated with acidic buffer for 10 min on ice, washed with ice-cold PBS, and fixed with 1% paraformaldehyde for 30 min at 4°C. Fluorescence was detected by FACS.

Figure Legends

Supplemental Fig. S1. Protein expression of transferrin receptor (TfR) and Tf/TfR colocalization in WR cells. (A) Transferrin receptor 1 (TfR1) and TfR2 levels in WR19L and CHO cells transiently transfected with FLAG-hTfR1 or FLAG-hTfR2, and mock-transfected cells were determined by western blot analysis. (B) Cell surface TfR1 levels were determined with FACS by using anti-TfR1 antibody-conjugated PE. (C) Cells were incubated with Alexa Fluor 488-conjugated Tf (green) for 5 min, fixed, stained with anti-TfR (red), and analyzed by confocal microscopy. Scale bars, 5 μm.

Supplemental Fig. S2. Sphingomyelin is important for Tf to avoid the degradation pathway. (A) Cells were processed for Tf uptake as described previously and then immunocytochemical detection of Lamp2 was done using an anti-Lamp2 antibody, followed by Alexa Fluor 564-conjugated goat anti-mouse IgG. Scale bars, 10 μm. (B) Exogenous C_6-SM addition and bSMase treatment were done as described in Fig. 6 and then cells were processed to take up Tf. Immunostaining for Lamp2 was performed as described. Scale bars, 5 μm. (C) Colocalization analysis of A and B. Error bars, SD. $^{*}P < 0.01$, $^{**}P < 0.05$ vs WR19L/Fas-SMS1 cells; $^{\#}P < 0.01$ vs WR19L/Fas-SM(-) cells.

Supplemental Fig. S3. Uptake of cholera toxin subunit B and LDL in WR19L cells. Cells were pre-incubated for 60 min and treated with Alexa Fluor 488-conjugated cholera toxin subunit B (CTxB) (B) and dil-LDL (C) for 10 min. After treatment, cells were washed with ice-cold PBS and treated with ice-cold acidic buffer (pH 5.0) for 10 min. Intracellular fluorescence was analyzed with FACS. Values are indicated as a fold increase over WR19L/Fas-SM(-) cells. Error bars, SD. $^{*}P < 0.005$, vs WR19L/Fas-SM(-) cells.