Supporting Information

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SI Materials and Methods

DNA Constructs. To generate Lys4Gln (K4Q) αβ phosphotransferase, the following primers were used in quick-change site-directed mutagenesis: 5′-ATG CTG TTC CAG CTC CTG CAG AGA CAG ACC-3′ and 5′-GGT CTG TCT CTG CAG GAG CTG GAA CAG CAT-3′; Ser15Tyr (S15Y) αβ phosphotransferase was generated with the primers 5′-CCT ATA CCT GCC TGT ACC ACA GGT ATG GGC-3′ and 5′-GCC CAT ACC TGT GGT ACA GGC AGG TAT AGG-3′ and S399F phosphotransferase αβ subunits with 5′-CGC ATC GAA GGG CTG TTC CAG AAG TTT ATT TAC C-3′ and 5′-GGT AAA TAA ACT TCT GGA ACA GCC CTT CGA TGC G-3′. The last 17 amino acids in the C terminus of αβ phosphotransferase were deleted (ΔCT) by quick-change site-directed mutagenesis with primers 5′-GCA CTT AAG CGG AAG ATA AGA GGG CCC TTC GAA GGT AAG CC-3′ and 5′-GGC TTA CCT TCG AAG GGC CCT CTT ATC TTC CGC TTA AGT GC-3′.

Immunofluorescence Microscopy. HeLa cells were maintained in DMEM containing 10% (vol/vol) FBS, transferred the day before transfection to 12-well plates containing coverslips and transfected at 50% confluency with 700 ng DNA, using Lipofectamine Plus (Life Technologies), according to the manufacturer's protocol. The cells were fixed 16–20 h after transfection with 4% (wt/vol) paraformaldehyde in PBS for 30 min at room temperature and stained for immunofluorescence microscopy as described in ref. 1.

The following antibodies were used: mouse anti-GM130 (BD Transduction Laboratories) at dilution 1:500, rabbit anti-α subunit (kind gift from W. Canfield, Genzyme, Cambridge, MA) at dilution 1:1,000, mouse anti-calnexin (Millipore) at dilution 1:150, mouse anti-Sec31A (BD Transduction Laboratories/BD Biosciences) at dilution 1:100, and mouse anti-LAMP-2 (CD 107b, BD Pharmingen) at dilution 1:100.

1. Qian Y, Flanagan-Steet H, van Meel E, Steet R, Kornfeld SA (2013) The DMAP interaction domain of UDP-GlcNAc:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase is a substrate recognition module. *Proc Natl Acad Sci USA* 110(25): 10246–10251.

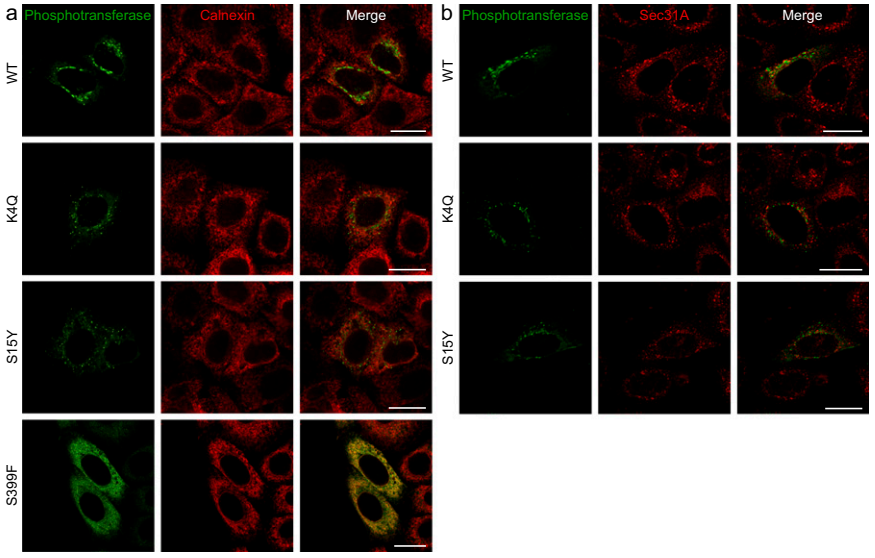

Fig. S1. K4Q and S15Y αβ phosphotransferase do not accumulate in the endoplasmic reticulum (ER) or at ER exit sites. Confocal immunofluorescence microscopy shows no significant colocalization between the K4Q and S15Y phosphotransferase containing punctae (anti-α subunit) and calnexin (*A*) or Sec31A (*B*), markers of the ER and ER exit sites, respectively. For comparison, the mutant Ser399Phe is shown, which is retained in the ER and completely colocalizes with calnexin (*A*). (Scale bars, 20 μm.)

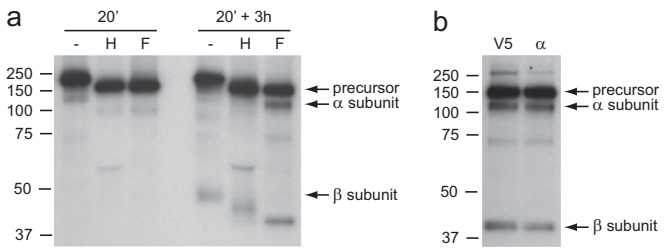

Fig. S2. Pulse-chase labeling and immunoprecipitation of αβ phosphotransferase. (*A* and *B*) Transfected HEK293 cells were labeled with TRAN ^{35}S-LABEL methionine/cysteine for 20 min, and αβ phosphotransferase was immunoprecipitated with anti-V5 (or anti-α subunit in *B*, second lane) either immediately or after a 3-h chase. In *B*, the chase was 3 h. (*A*) Immunoprecipitated wild-type phosphotransferase was treated with endoglycosidase H_f (Endo H_f) (H) or *N*-glycosidase F (PNGase F) (F) for 3 h at 37 °C. –, Untreated samples. After the 3-h chase, note the smear above the 190-kDa precursor after Endo H_f and treatment with PNGase F shows the presence of the α subunit (~110-kDa band). (*B*) All samples were treated with PNGase F (3 h at 37 °C).

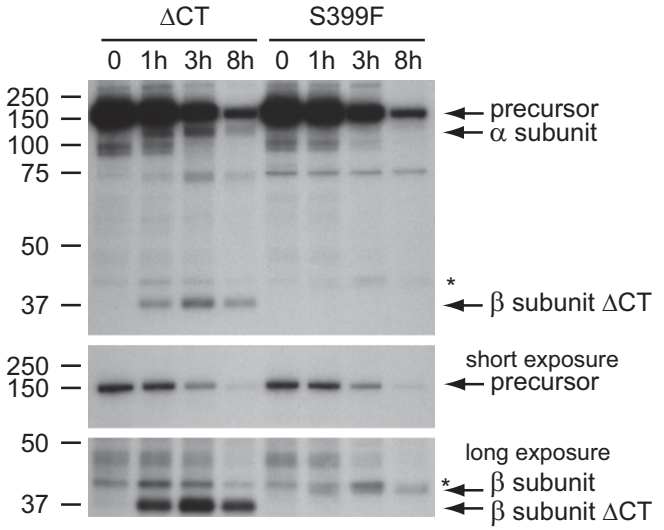

Fig. S3. Pulse-chase labeling and immunoprecipitation of Ser399Phe (S399F) and ΔCT αβ phosphotransferase. The bulk of S399F αβ does not exit the ER, and therefore only trace amounts of β subunit are formed. The ΔCT β subunit migrates faster than the wild-type β subunit because of deletion of the last 17 amino acids, whereas the migration of the background band (*) is unchanged.

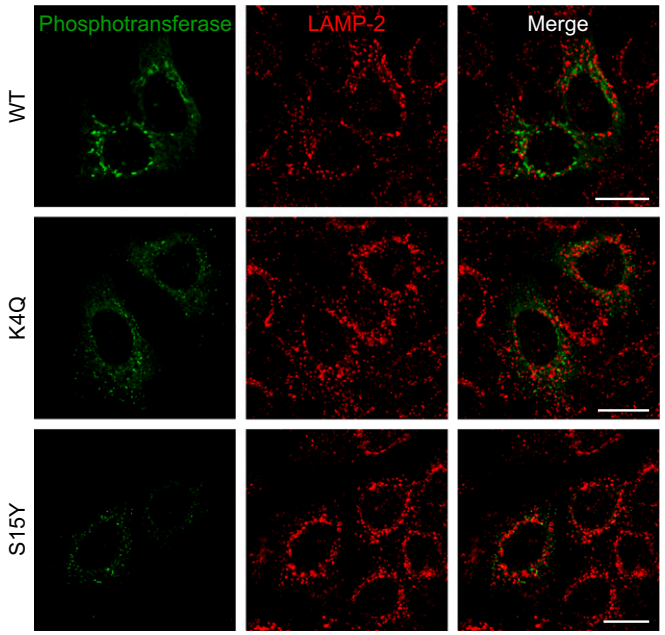

Fig. S4. K4Q and S15Y αβ phosphotransferase do not significantly colocalize with LAMP-2. Transfected HeLa cells were stained for the late endosomal/lysosomal marker lysosome-associated membrane protein-2 (LAMP-2) and phosphotransferase (anti-α subunit). (Scale bars, 20 μm.)

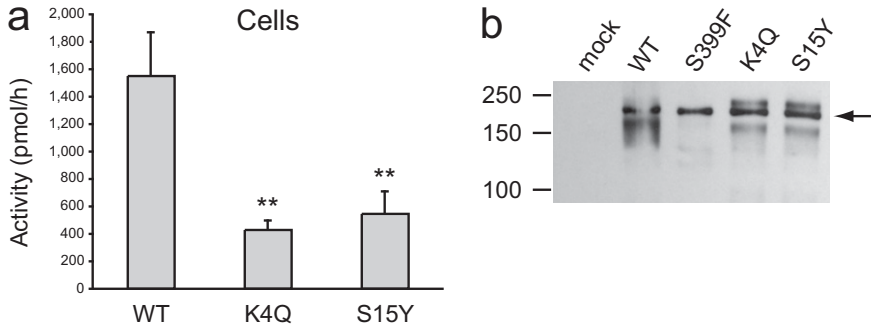

Fig. S5. Activity and expression of αβ phosphotransferase in HEK293 cells. (*A*) The activity of wild-type, K4Q and S15Y αβ phosphotransferase toward α-methyl D-mannoside was measured in HEK293 cell lysates 48 h after transfection. Data are average values of 3 independent experiments, error bars represent SD. $^{**}P < 0.01$ (Student *t* test). (*B*) Western blots (anti-α subunit antibody) of HEK293 cell lysates expressing wild-type, Ser399Phe, K4Q, or S15Y αβ phosphotransferase. Half a microgram total protein was loaded.