Online Supplementary Material

Supplementary Methods:

Cell Culture and Transfection. BHK cells were cultured and proteins were expressed using a recombinant vaccinia virus system for 6 h as described (Mackett *et al.,* 1992; Feng *et al.,* 1995). A431 cells were cultured in DMEM with 10% FBS. SCC-12F cells were grown in a 1:1 mixture of DMEM and nutrient mixture F-12 Ham's medium supplemented with 10% FBS. Cells were transfected at 30% confluence with 100 nM siRNA duplex with or without plasmids using Lipofectamine 2000 (Invitrogen) and cultured 48 to 96 h prior to analysis.

EGFR Analyses. For flow cytometry based EGFR cell surface and internalization assays, A431 cells or SCC-12F cells were mock transfected or transfected with MTM1 or MTMR2 siRNA duplexes for 48 h, serum starved for 2 h (SCC-12F cells) or overnight (A431 cells) and ligand stimulated with EGF, 20 nM EGF for 10 min (SCC-12F cells) or 100ng/ml EGF for 3 h (A431 cells). Basal EGFR levels were measured in serum starved unstimulated cells. Flow cytometry measurements were performed after cells were released with 1 mM EDTA, blocked with 3% bovine serum albumin in PBS, labeled with a fluorescein-conjugated mAb directed against EGFR at 4^0C. A FACScan flow cytometer (Becton Dickinson) was used to analyze 10^6 cells/ml and a minimum of 20,000 cells were counted.

Statistical evaluation of changes in EGFR levels employed multiple analysis tools. Based on Student's t-test analyses, EGFR levels were statistically different in MTM1 or MTMR2 depleted samples relative to control samples where designated by an asterisk. In addition to Student's t-test, the data was further evaluated using repeated measures analysis, one-way ANOVA and the Tukey method. Repeated measures confirmed significant differences ($p=0.003$) among groups (Mock, GAPDH, MTM1 and MTMR2) and across time ($p<0.000$) in the two cell lines. Based on repeated measures analysis, the group-by-time interactions in SCC cell samples nearly achieved statistical significance ($p=0.056$), suggestive that the rates of

change across time for the GAPDH, MTM1 and MTMR2 samples may differ. Follow-up one-way ANOVA analysis of relative change over time or absolute change over time and Tukey pairwise comparisons were also conducted, but did not achieve statistical significance, suggesting that the nature of the interaction may be more complicated than these simple measures. In the A431 cell trials, repeated measures did not identify a significant group-by-time interaction ($p=0.341$). However, one-way ANOVA confirmed that the differences in mean response among the four groups (Mock, GAPDH, MTM1 and MTMR2) in A431 cells was statistically significant at each point in time (0 h, $p=0.007$; 1 h, $p=0.017$; 2 h, $p=0.042$; 3 h, $p=0.001$). Tukey multiple comparisons of all possible pairs of A431 samples at each time point (using 5% family error rate) revealed that EGFR levels in the presence of MTM1 siRNA were significantly different from controls at 2 and 3 h. Similarly, EGFR levels in the presence MTMR2 siRNA were significantly different from controls at all time points. In all cases where significant differences are noted p-values were 0.01 or less. The observed statistical differences between SCC and A431 cells is likely due to the fact that in SCC cells where EGFR levels are low, myotubularins cause a delay in degradation at the 15 min and 30 min timepoints, but not an absolute block in degradation over the entire experimental timecourse, while in A431 cells where receptor levels are 10-fold higher degradation is decreased over the entire timecourse.

Reagents and Antibodies. Cell lines and bacteria were from American Type Culture Collection (Rockville, MD). SCC-12F, a human squamous cell carcinoma cell line was from Dr. Laurie Hudson (University of New Mexico, NM). Cell culture media was from Invitrogen. DNA primers were synthesized by Integrated DNA Technologies (Coralville, IA). Restriction enzymes were from New England Biolabs, and reagents for the purification of DNA purchased from QIAGEN. Reagents were from Sigma-Aldrich. Primary antibodies used from commercial sources directed against: FLAG epitope tag (mAb, Sigma-Aldrich); V5 epitope tag (mAb, Invitrogen); EGFR (unconjugated: mAb, Sigma-Aldrich; rabbit pAb, Santa Cruz Biotechnology;

mAb, NeoMarkers, Fremont, CA) or EGFR (mAb conjugated to fluorescein, Santa Cruz Biotechnology); actin (mAb, ICN Biomedicals); GST (mAb, Santa Cruz Biotechnology); $PI(3,5)P_2$ (mAb, Echelon Research Labs); human transferrin receptor (goat pAb, Santa Cruz Biotechnology). Secondary antibodies used from commercial sources: biotinylated rabbit pAb directed against goat Ig (Vector Laboratories, Burlingame, CA), fluorescent secondary antibodies raised in donkey (Jackson ImmunoResearch Laboratories, West Grove, PA) and horseradish peroxidase conjugated antibodies against mouse or rabbit Ig (Amersham Biosciences). Noncommercial antibodies were as follows: chicken IgY (Stein *et al.,* 2005) or rabbit pAb (Press *et al.,* 1998) directed against Rab7; rabbit pAb directed against Rab5 (Chavrier *et al.,* 1990); rabbit pAb directed against hVps34 (Stein *et al.,* 2005); rabbit pAb directed against MTMR2 (Previtali *et al.,* 2003); mAb or rabbit pAb directed against human MTM1 (Blondeau *et al.,* 2000); human pAb directed against EEA1 (Callaghan *et al.,* 1999).

Plasmids and siRNA Construction. Plasmids used were: pCMVTag2B human MTMR2wt, pCMVTag2B MTMR2 D320A (Laporte *et al.,* 2002); pGEM Rab5 (Chavrier *et al.,* 1990); pGEM Rab7; GST tagged Rab7; pcDNA3.1 human Vps34 (hVps34) (Stein *et al.,* 2005); human cDNAs encoding full-length hVps15, hVps15 PKDΔ, hVps15 WD40Δ, hVps15 HEATΔ, and the WD40 or HEAT domains of hVps15 in pcDNA3.1V5/His (Murray *et al.,* 2002). Flag tagged MTMR2wt, MTMR2 D320A or MTMR2 coil• (GeneBank NM_016156, 1-1764 nt) and the contiguous WD40/HEAT domain of hVps15 (GeneBank NM_014602, 1299-4077 nt) were PCR amplified and subcloned into pcDNA3.1 Hygro (Invitrogen) using standard methods. GST fusion proteins of full-length human MTMR2 and the PH (1-546 nt), PTP (547-1764 nt), and coiled-coil (1765-1932 nt) domains were constructed using pCMVTag2B MTMR2wt as a template and subcloned into pGEX-5x-2 (Amersham Biosciences). pEGFP-2xFYVE^{Hrs} was prepared by subloning FYVE^{Hrs} from pGEX-5x-3-2xFYVE^{Hrs} (Gillooly *et al.,* 2000) into pEGFP-C2 (Clontech Laboratories. Mountain View, CA). All constructs were confirmed by DNA

sequencing.

Optimal regions in MTM1 (GeneBank NM_000252) or MTMR2 (GeneBank NM_016156) mRNA for targeted siRNA were identified using the Ambion siRNA design tool and following Reynold's rational design criteria (Reynolds *et al.*, 2004). Only targets scoring higher than 6 and meeting all eight criteria were selected. Sense and antisense siRNA oligonucleotide templates for each target sequence included a T7 promoter homologous sequence (CCTGTCTC) at the 3' ends to allow enzymatic preparation and purification of siRNA duplex using the Ambion construction kit (Ambion, Austin, TX). GAPDH siRNA and scrambled negative control siRNA template sets were purchased from Ambion. Chemically synthesized siRNA products were quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE). For immunofluorescence staining, siRNAs were labeled with Cy3 using the Ambion labeling kit.

Quantitative Real-Time PCR. A431 cells were mock transfected or transfected with MTMR2, GAPDH or scrambled negative control siRNA duplex. Total RNA was purified using RNeasy mini kit (QIAGEN). The human MTMR2 gene specific primer (GSP) set forward (5'-TGCTTATTCGGAACATTCCTCTG-3') and reverse (5'- AGGTTCCTGTGGTTTCATCCG-3') and a human GAPDH GSP were from Invitrogen. Quantitative real-time PCR was performed in a 96 well optical PCR plate with DyNAmo HS SYBR green qPCR kit (Bio-Rad) with the DNA Engine Opticon 2 instrument (Bio-Rad).

PI(3,5)P2 Analyses by HPLC and Immunofluorescence.

For immunofluorescence, control and siRNA transfected cells were fixed with paraformaldehyde without pre-permeabilization and stained for PI(3,5)P_2 using a commercial antibody directed against PI(3,5)P_2. Quantification of fluorescence intensity represents the average pixel intensity above background in individual cells (n=20-30) and was determined using Slidebook 4.1 software (Intelligent Imaging Innovations, Inc., Denver, CO). For HPLC, metabolic labeling of

A431 cells with myo-[2-^{3}H]inositol and HPLC separation of glycerophosphoinositol phosphates was performed as detailed in the manuscript Methods section.

Endogenous MTMR2 Staining.

Endogenous MTMR2 was detected by Tyramide signal amplification fluorescence system purchased from Perkin Elmer (Boston, MA) according to the manufacturer's protocols and colocalized with early and late endosomes using a human pAb directed against EEA1 (Callaghan *et al.,* 1999) and a chicken IgY (Stein *et al.,* 2005) directed against Rab7, respectively.

MTMR2 Overexpression and EEA1 Staining.

Flag tagged MTMR2wt was constructed as detailed in the manuscript methods section and used to transfect A431 cells by lipofection with Lipofectamine 2000 (Invitrogen). Cells were fixed, permeabilized and stained 48 h post-transfection as described (Stein *et al.,* 2005). MTMR2 was detected using a mAb directed against the Flag epitope (Sigma-Aldrich, St. Louis, MO) and EEA1 was detected using a human pAb directed against EEA1 (Callaghan *et al.,* 1999).

Confocal Imaging.

Coverslips were viewed on a Zeiss LSM 510 confocal microscope. All supplementary images are single 0.5 μm optical sections collected using plan-Neofluor 40x 1.30 oil objective from one of three independent experiments.

Statistical Analyses.

Supplementary data are representative of multiple independent trials with the n values given in the legend. All bar graphs include error bars that represent standard errors of the mean. Student's t-test was used to calculate p-values with individual p-values included in figure legends.

Supplementary Figure 1. Silencing Endogenous MTM1 or MTMR2 in A431 Cells by siRNA. (A) Optimal regions in MTM1 or MTMR2 mRNA for targeted siRNA-mediated silencing were identified using Ambion's design tool. (B) A431 cells transfected with 100 nM Cy3-labeled MTMR2 #19 siRNA demonstrated high (95%) transfection efficiencies detected by confocal microscope (left panel, 40x objective and scan zoom 1.0, bar represents 20 μm) and flow cytometry (right panel). (C) A431 cells were transfected with MTM1 or MTMR2 siRNA. Mock transfected samples served as negative controls. At 48 to 96 h after transfection, equal amounts of total protein were resolved by SDS PAGE. Western blot shows MTM1 or MTMR2 along with actin in indicated samples (top panel). The ratio of MTM to actin in each sample was quantified with chemiluminescent signal on immunoblots by densitometry and shown in a bar graph. (D) A431 cells were transfected with MTMR2, GAPDH or scrambled control siRNA. At 48 h after transfection, total RNA was purified and processed for quantitative real-time PCR using GSP for MTMR2 and GAPDH, respectively. Each bar represents the fold mRNA decrease relative to a scrambled control and is plotted with mean $\pm$ s.e.m. (n=2). (E) Western blot shows sample treated with MTM1 or MTMR2 siRNA depletes the individual siRNA targeted proteins but does not result in the non-specific depletion of a different myotubularin. Actin served as an equal protein loading control.

Supplementary Figure 2. MTM1 and MTMR2 Regulate Cellular PI(3,5)P_2 Levels. A431 cells were transfected with MTM1 or MTMR2 siRNA. GAPDH siRNA or mock transfected samples served as controls. (A) Cells were metabolically labeled with myo-[2-^{3}H]inositol. Glycerophosphoinositol phosphates (GroP) were resolved by HPLC. Chromatogram shows percentage of the summed radioactivity from all GroP species (total [^{3}H] radioactivity) as a function of elution time for indicated samples. PI(3,5)P_2 are quantified by area integration and normalized to control for each sample (bar graph, mean $\pm$ s.e.m., n=3, unpaired t test, two-tailed $p>0.05$). (B) Cells were fixed without pre-permeabilization and stained for PI(3,5)P_2. All

samples were scanned at identical settings on a confocal microscope, 40x objective and scan zoom 1.0, bar represents 10μm (left top panels). Fluorescence intensity is the average pixel intensity in the selected cell that is above the threshold (left bottom panels). Bar graph shows increase in PI(3,5)P_2 of 80% in MTM1 or MTMR2 siRNA transfected cells versus mock control (mean $\pm$ s.e.m., n= 20-30, unpaired t test, *** two-tailed $p<0.001$).

Supplementary Figure 3. MTM1 and MTMR2 Differentially Regulate Endosomal PI(3)P Levels - Supporting Micrographs. (A-B) Effects of MTM1 siRNA or (C-D) MTMR2 siRNA on total and endosomal PI(3)P levels. A431 cells were co-transfected with Cy3-labeled (A-B) MTM1 #209 or (C-D) MTMR2 #19 siRNA and GFP-2xFYVE^{Hrs}. Cy3-labeled GAPDH siRNA or mock transfected samples served as controls. At 48 h post transfection, cells were fixed and processed for immunofluorescence. GFP-2xFYVE^{Hrs} (green channel) and Cy3-labeled siRNA (red channel) were detected directly and endogenous EEA1 and Rab7 were first stained with specific primary antibodies and detected with Cy5-labeled secondary antibodies (blue channel). All samples were scanned at identical settings on a Zeiss LSM510 confocal microscope, 40x objective and scan zoom 1.0, bar represents 10μm. Total PI(3)P levels (total fixed, membrane bound GFP-2xFYVE^{Hrs}), early endosomal PI(3)P levels (GFP-2xFYVE^{Hrs} and EEA1 colabel) and late endosomal PI(3)P levels (GFP-2xFYVE^{Hrs} and Rab7 colabel) were quantified using Slidebook 4.1 image analysis software. Quantification was performed on 10-20 cells from three independent experiments and data are summarized in Figure 1C of main text.

Supplementary Figure 4. Endogenous MTMR2 Localizes to Rab7-positive Late Endosomes and MTMR2 Overexpression does not Affect EEA1 Membrane Association. (A) A431 cells were permeabilized with saponin, fixed and stained for endogenous MTMR2 (green, with tyramide signal amplification) and EEA1 (red) or Rab7 (red) by immunofluorescence staining. Insets of areas marked with arrows demonstrate colocalization with Rab7. 40x objective and scan zoom 3.0, bar represents 10 μm. (B) A431 cells overexpressing Flag-MTMR2wt (green) were

detected together with endogenous EEA1 (red) by immunofluorescence staining. 40x objective and scan zoom 1.7, bar represents 10 μm. (C) A431 cells were left untreated or siRNA depleted for MTMR2, permeabilized with saponin, fixed and stained for endogenous MTMR2 with tyramide amplification.

Supplementary Figure 5. Alterations in EGFR Degradation Kinetics Following siRNA-mediated Silencing of MTM1 or MTMR2. A431 cells were transfected with MTM1 or MTMR2 siRNA. Mock transfected samples served as negative controls. At 48 h after transfection, cells were prepared for immunofluorescence staining with 100 ng/ml EGF stimulation for 1.5 h. EGFR (green) was co-stained with EEA1 (red) or Rab7 (red) in indicated samples. (A and B) Mock transfected cells, (C and D) MTM1 siRNA transfected cells, (E and F) MTMR2 siRNA transfected cells. 40x objective and scan zoom 1.0, bar represents 10 μm.

Supplementary Figure 6. In vitro Translated hVps15 WD40 deletion mutant Exhibits Reduced Binding to GST-MTMR2. Equimolar amounts of GST-MTMR2 or GST were immobilized on glutathione-Sepharose beads and incubated with equal amounts of in vitro synthesized, [^{35}S]-L-methionine labeled full-length hVps15, domain deletion mutants or individual hVps15 domains. Shown are composite data from multiple experiments. The binding to GST versus GST-MTMR2 is directly compared for each hVps15 derivative, but different hVps15 derivatives can not be compared without normalization to the GST control. Normalized binding of each hVps15 derivative to GST-MTMR2 from multiple trials are shown in Figure 6E of main text.

Supplementary Figure 7. hVps15 WD40 Deletion Mutant Exhibits Reduced MTMR2 Phosphatase Inhibitory Effect. GST-MTMR2 (60 ng) immobilized on glutathione-Sepharose beads were incubated with equivalent amounts of V5-hVps15-containing or hVps15 WD40Δ-containing cell lysates. Phosphatase reaction products were resolved by TLC with the migration

of PI and PI(3)P as indicated (top panel). Western blot shows the amounts of GST-MTMR2 and bound V5-hVps15 protein present in the phosphatase assay (bottom panel).

Supplementary References

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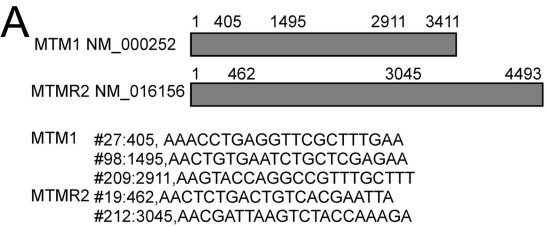
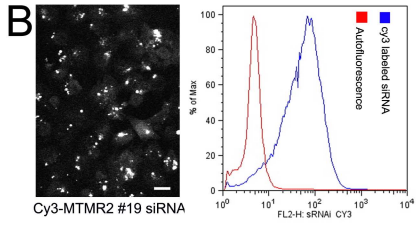
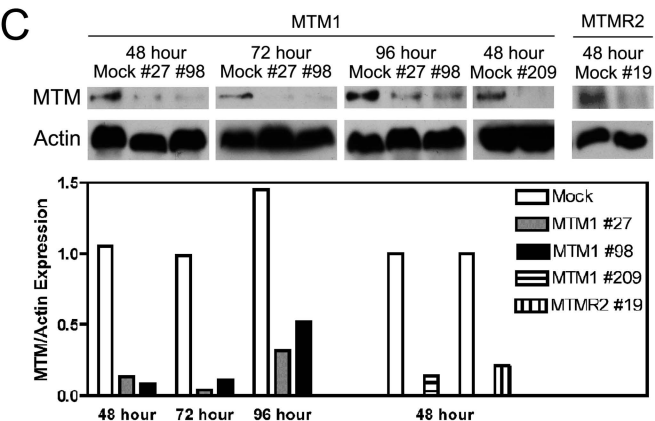
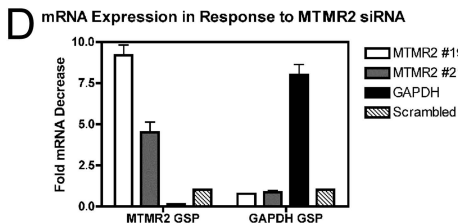
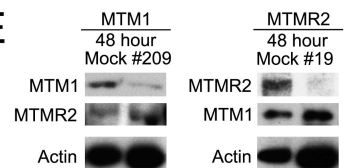
Supplementary Figure 1











Supplementary Figure 2

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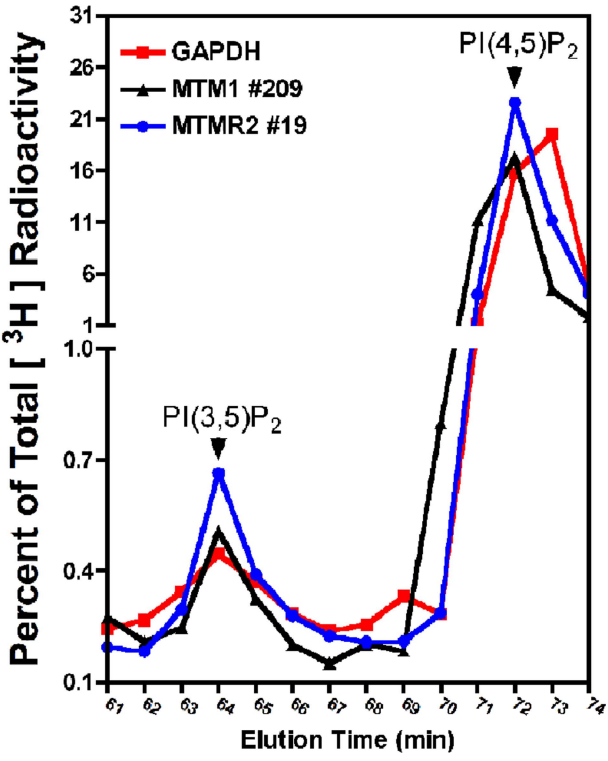

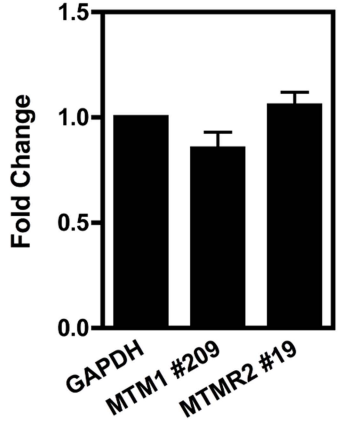

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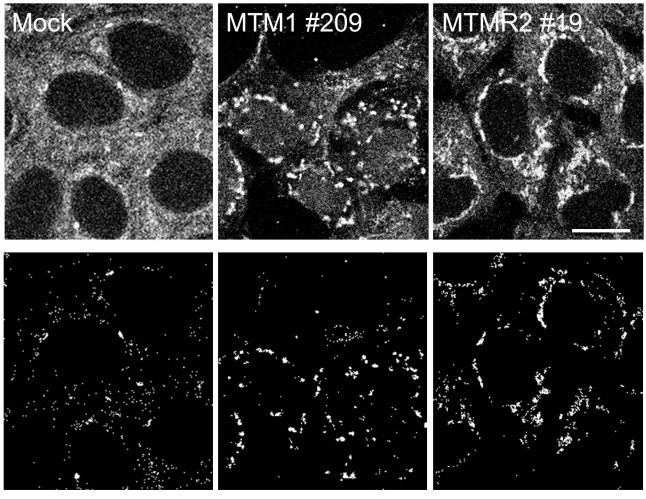
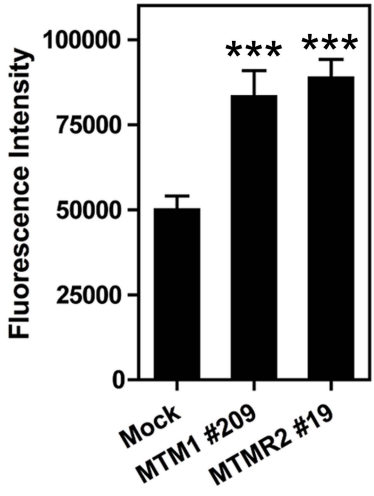
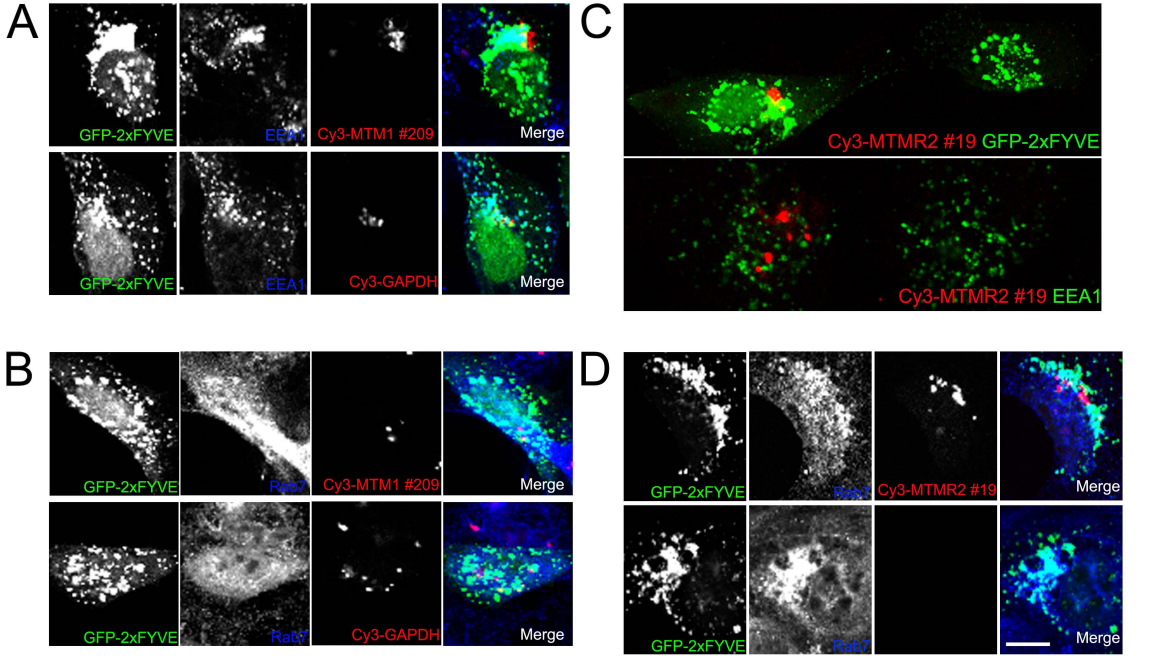
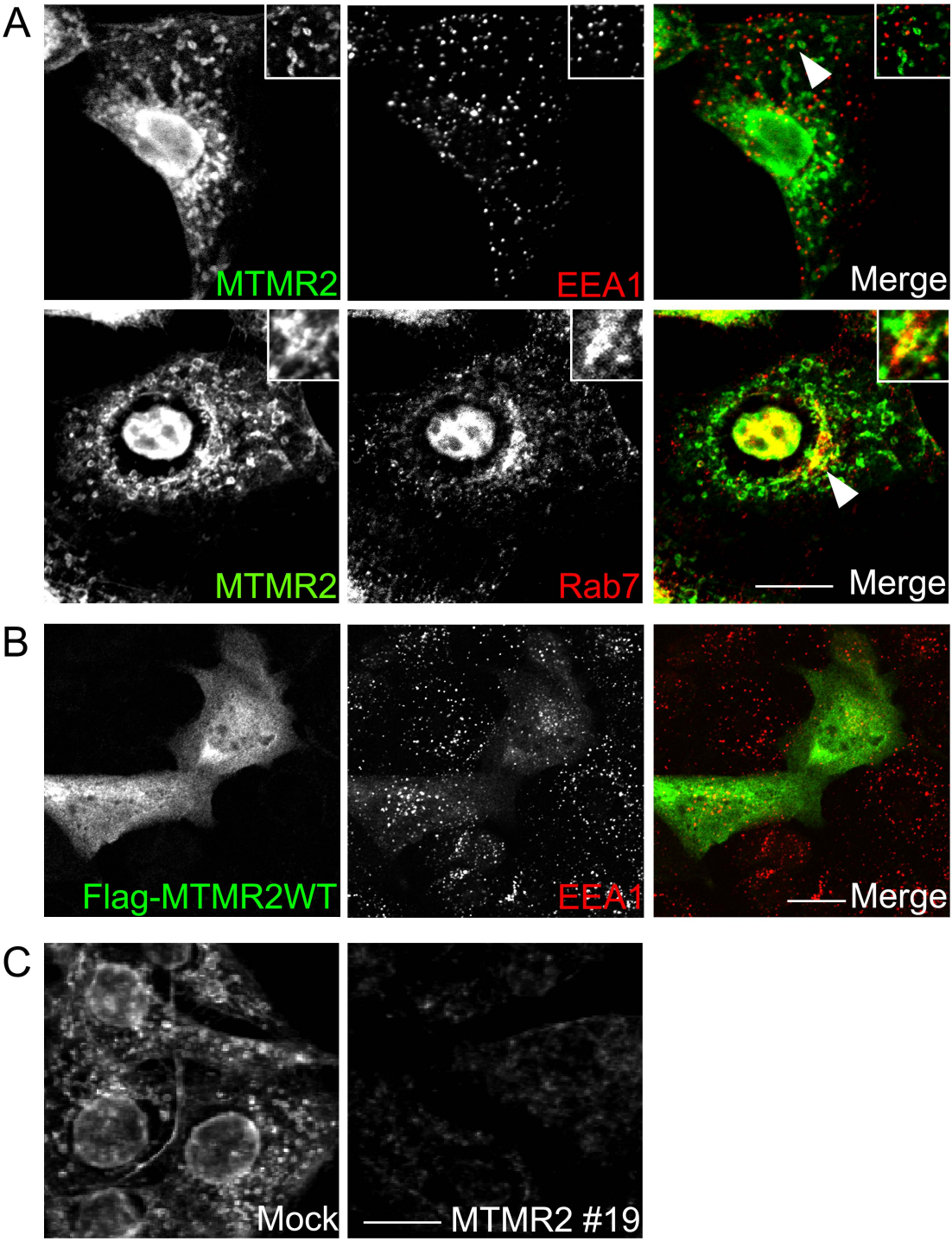




Supplementary Figure 3



Supplementary Figure 4



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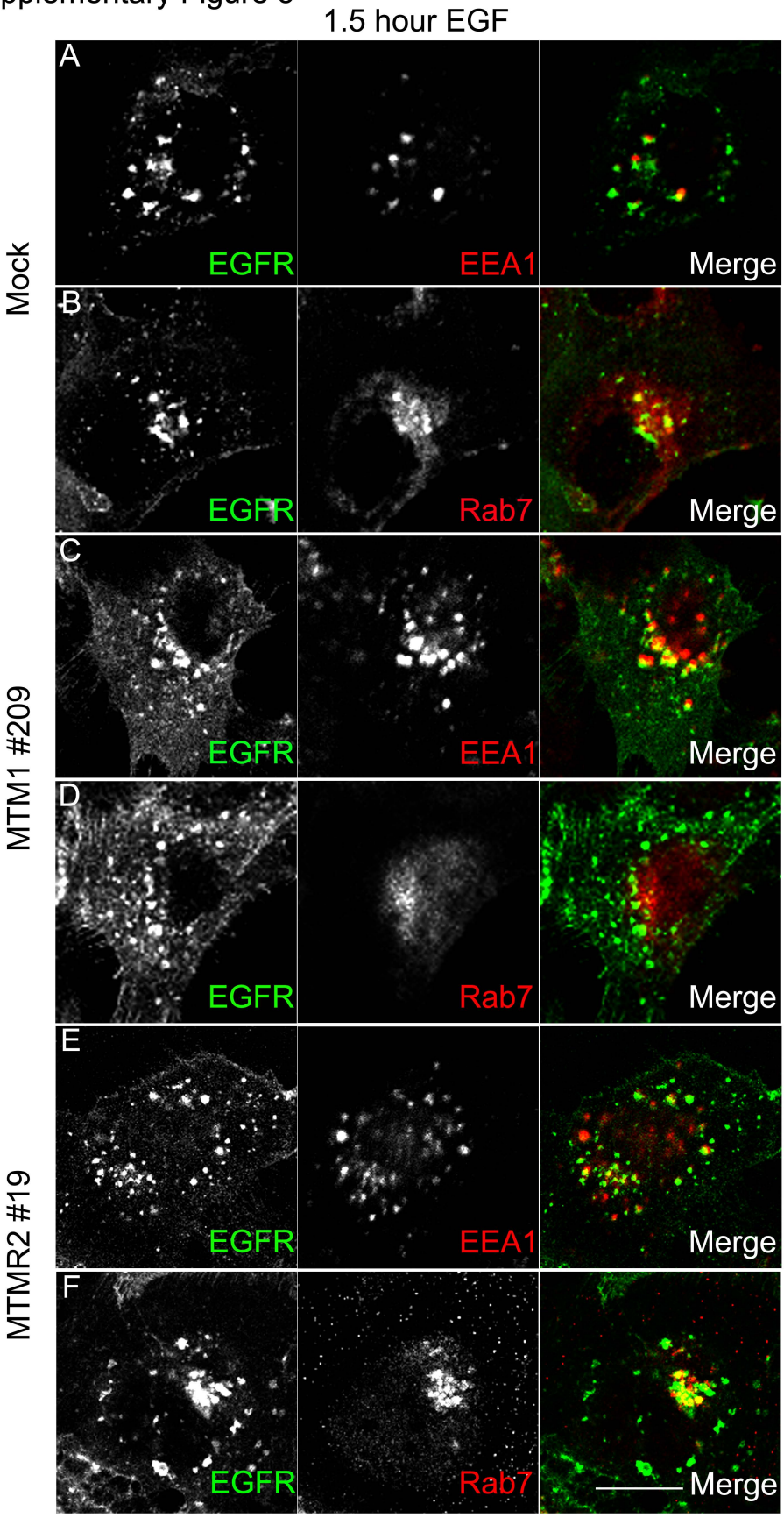
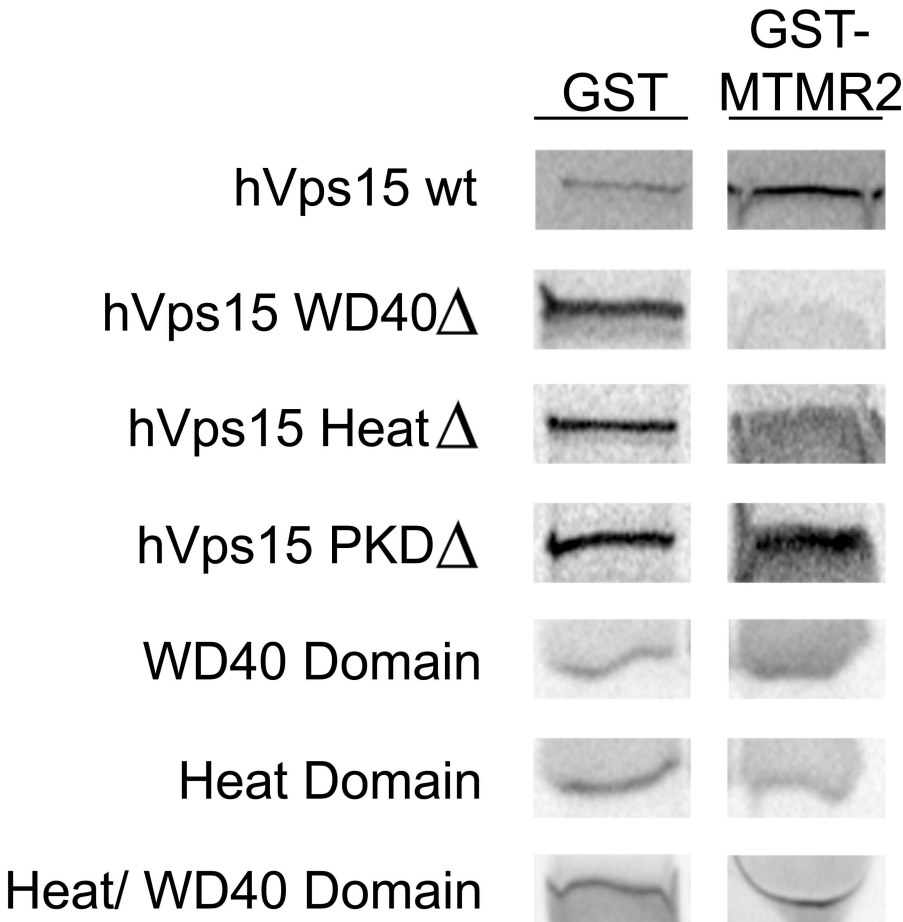


Supplementary Figure 6



Supplementary Figure 7