Supplementary Material

Pathogenic missense variants in *SLC2A1* (GLUT1) misroute to lysosomes in endothelial cells: insights into molecular pathology of GLUT1 deficiency

Sara Lazzari[1,2], Agnese Giovannetti[3], Francesco Petrizzelli[3], Veronica De Paolis[2], Francesca Matarazzo[2], Tommaso Biagini[3], Tommaso Mazza[3], Chiara Parisi[2*], Viviana Caputo[4*]

1. Clinical Genomics Laboratory Fondazione IRCCS Casa Sollievo Della Sofferenza, S. Giovanni Rotondo (FG), Italy
2. Institute of Biochemistry and Cell Biology, CNR-National Research Council, Monterotondo Scalo (RM), Italy
3. UOS Computational Biology and Bioinformatics, Fondazione Policlinico Universitario A. Gemelli, IRCCS, Rome, Italy
4. Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy

Correspondence to:
Chiara Parisi - chiara.parisi@cnr.it
Viviana Caputo - viviana.caputo@uniroma1.it

*co-last and co-corresponding authors

Contents

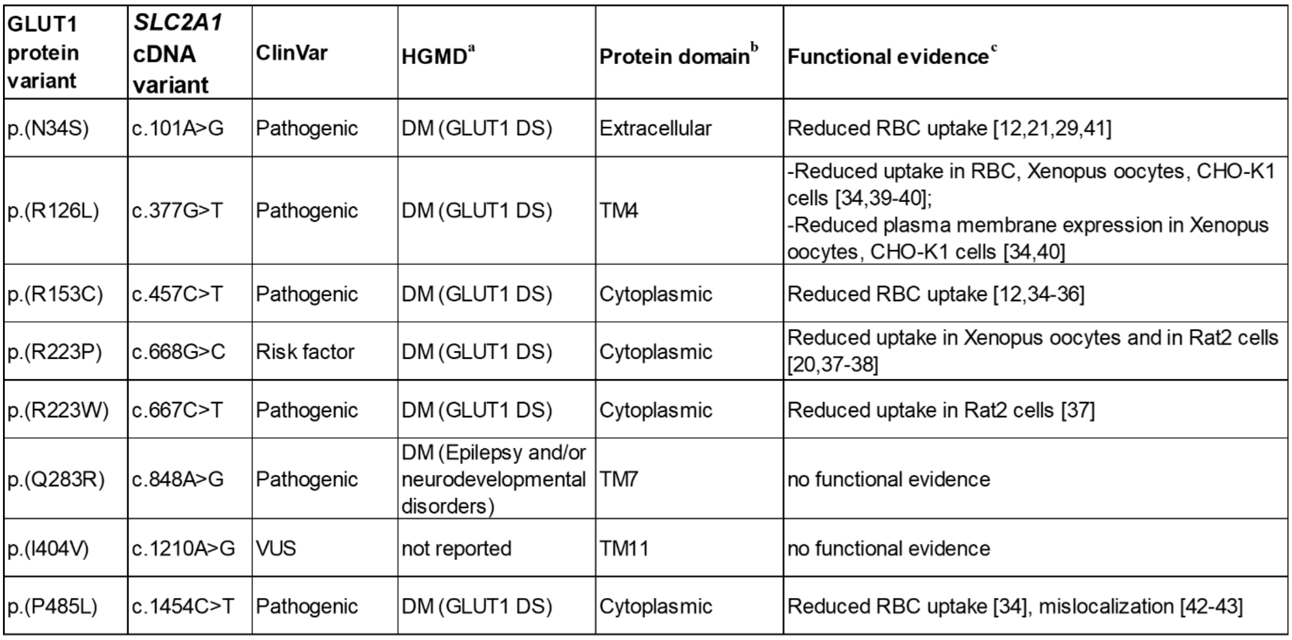
Supplementary Table S1.

List of the selected GLUT1 missense variants, specifying the amino acid substitution on protein sequence (NP_006507.2), the nucleotide substitution found in the coding sequence (NM_006516.4), and the relative primer pairs used for mutagenesis experiments shown in the 5' to 3' direction.

GLUT1 protein variant	**SLC2A1 cDNA variant**	**Forward Primer**	**Reverse Primer**
p.(N34S)	c.101A>G	TGGAGTCATCAGTGCCCCCCAGAAG	GTGTTGTAGCCAAACTGCAGGGAGC
p.(R126L)	c.377G>T	CTGATCCTGGGCCTCTTCATCATCGGTG	CATCTCAAAGGACTTGCCCAGTTTCGAG
p.(R153C)	c.457C>T	CCACAGCCCTTTGTGGGGCCCTG	GTGACACTTCACCCACATACATGGG
p.(R223P)	c.668G>C	AACGAGGAGAACCCGGCCAAGAGTG	GCGGTTGATGAGCAGGAAGCGGGGAC
p.(R223W)	c.667C>T	AACGAGGAGAACTGGGCCAAGAGTG	GCGGTTGATGAGCAGGAAGCGGGGAC
p.(Q283R)	c.848A>G	GCTGTCCCAGCGGCTGTCTGGCATC	TGCAGCACCACAGCGATGAGGATG
p.(I404V)	c.1210A>G	CGTCCAGCTGCCGTTGCCGTTGC	TGGACCCTGGCTGAAGAGTTCAG
p.(P485L)	c.1454C>T	AGCTGTTCCATCTCCTGGGGGCTG	CCTCGGGTGTCTTGTCACTTTGGC

Supplementary Table S2.

Clinical and experimental features of the GLUT1 missense variants reported in this work. We reported classification of variants from clinical databases, namely ClinVar and HGMD, including associated phenotypes, localization in GLUT1 protein domains, and functional evidence relevant to this work.

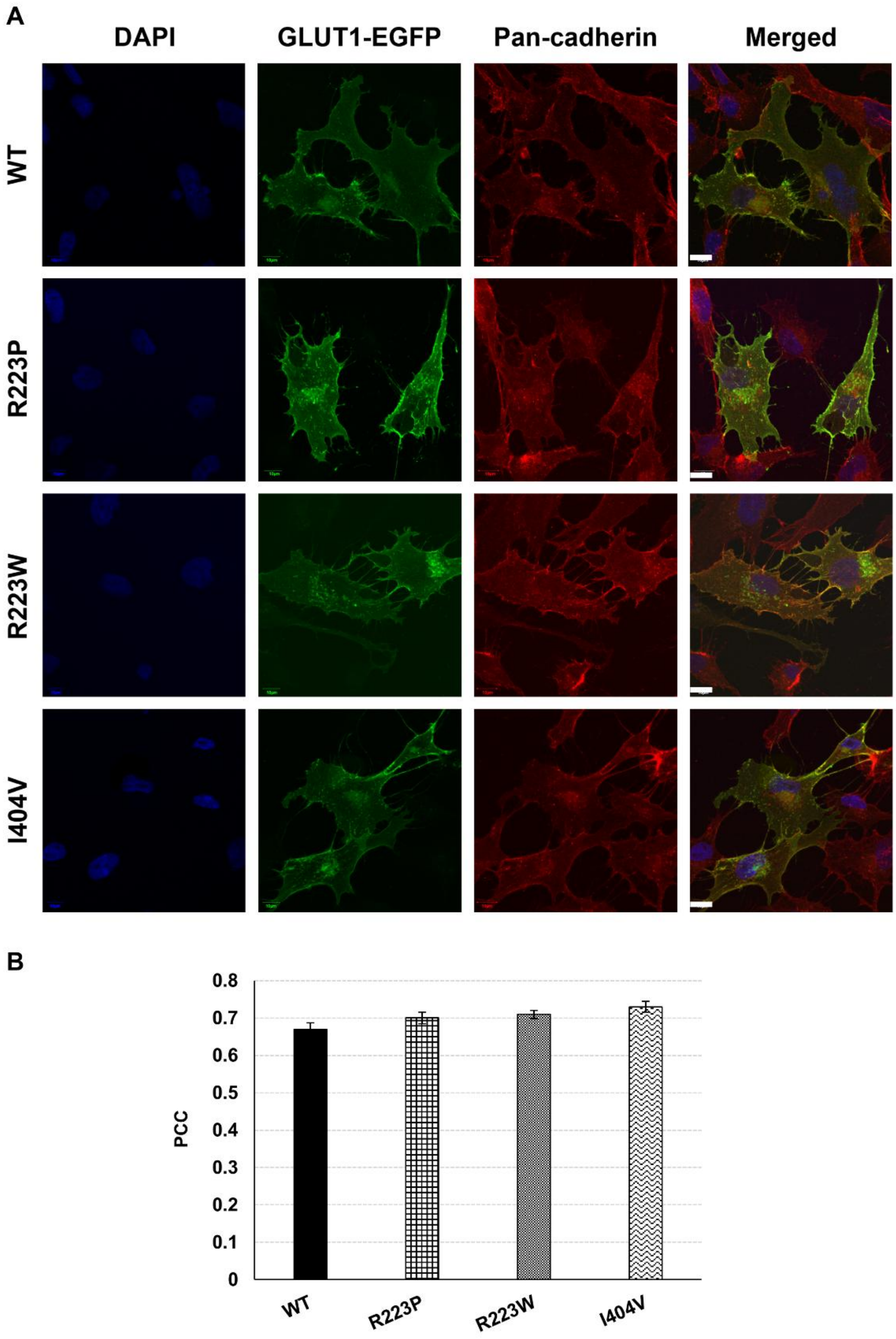


GLUT1 protein variant	*SLC2A1* cDNA variant	ClinVar	HGMD[a]	Protein domain[b]	Functional evidence[c]
p.(N34S)	c.101A>G	Pathogenic	DM (GLUT1 DS)	Extracellular	Reduced RBC uptake [12,21,29,41]
p.(R126L)	c.377G>T	Pathogenic	DM (GLUT1 DS)	TM4	-Reduced uptake in RBC, Xenopus oocytes, CHO-K1 cells [34,39-40]; -Reduced plasma membrane expression in Xenopus oocytes, CHO-K1 cells [34,40]
p.(R153C)	c.457C>T	Pathogenic	DM (GLUT1 DS)	Cytoplasmic	Reduced RBC uptake [12,34-36]
p.(R223P)	c.668G>C	Risk factor	DM (GLUT1 DS)	Cytoplasmic	Reduced uptake in Xenopus oocytes and in Rat2 cells [20,37-38]
p.(R223W)	c.667C>T	Pathogenic	DM (GLUT1 DS)	Cytoplasmic	Reduced uptake in Rat2 cells [37]
p.(Q283R)	c.848A>G	Pathogenic	DM (Epilepsy and/or neurodevelopmental disorders)	TM7	no functional evidence
p.(I404V)	c.1210A>G	VUS	not reported	TM11	no functional evidence
p.(P485L)	c.1454C>T	Pathogenic	DM (GLUT1 DS)	Cytoplasmic	Reduced RBC uptake [34], mislocalization [42-43]

[a] DM indicate variants classified as Disease-Mutation in HGMD

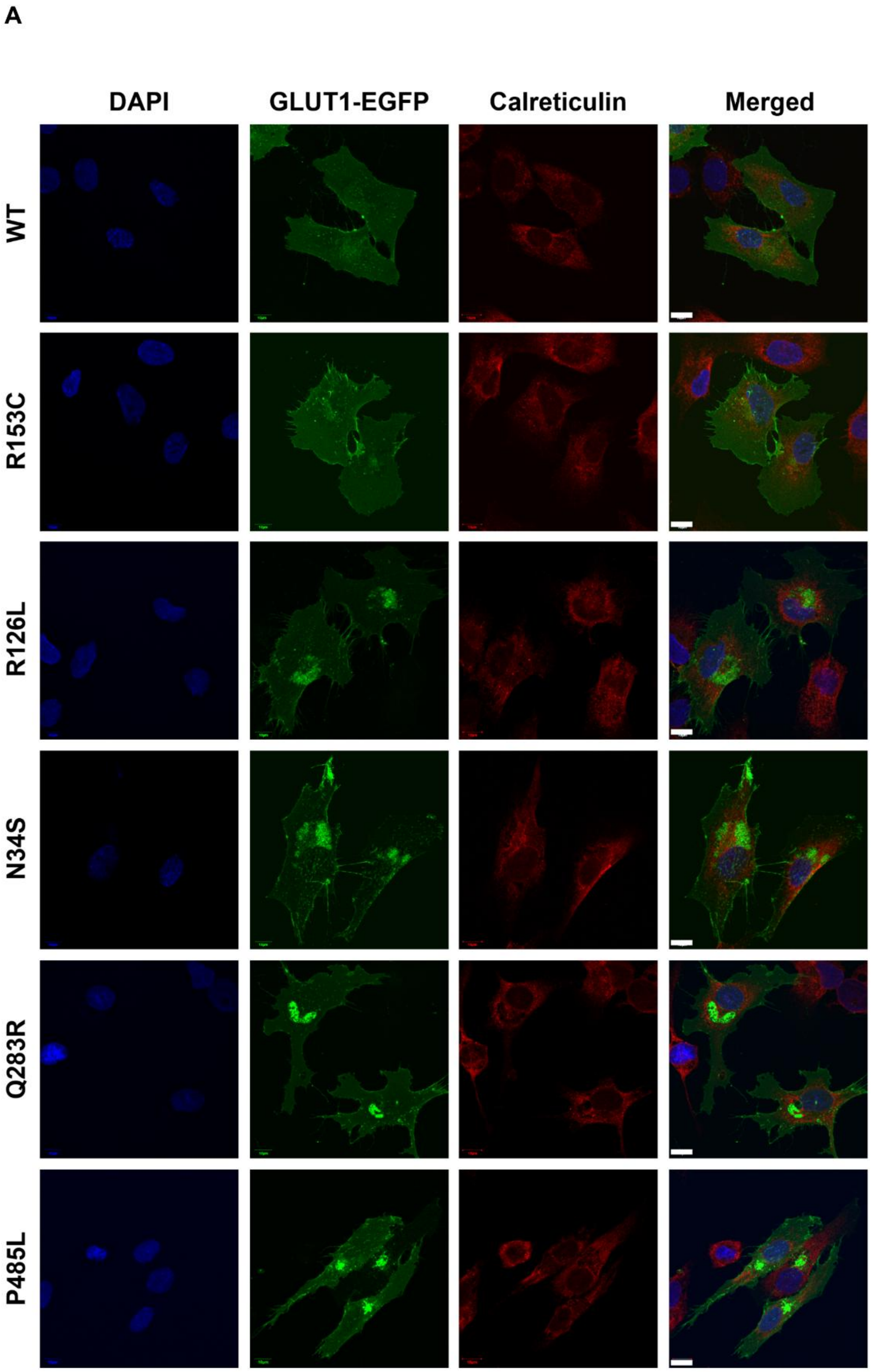
[b] TM indicate variant localization in a TransMembrane alpha-helix, followed by the domain number

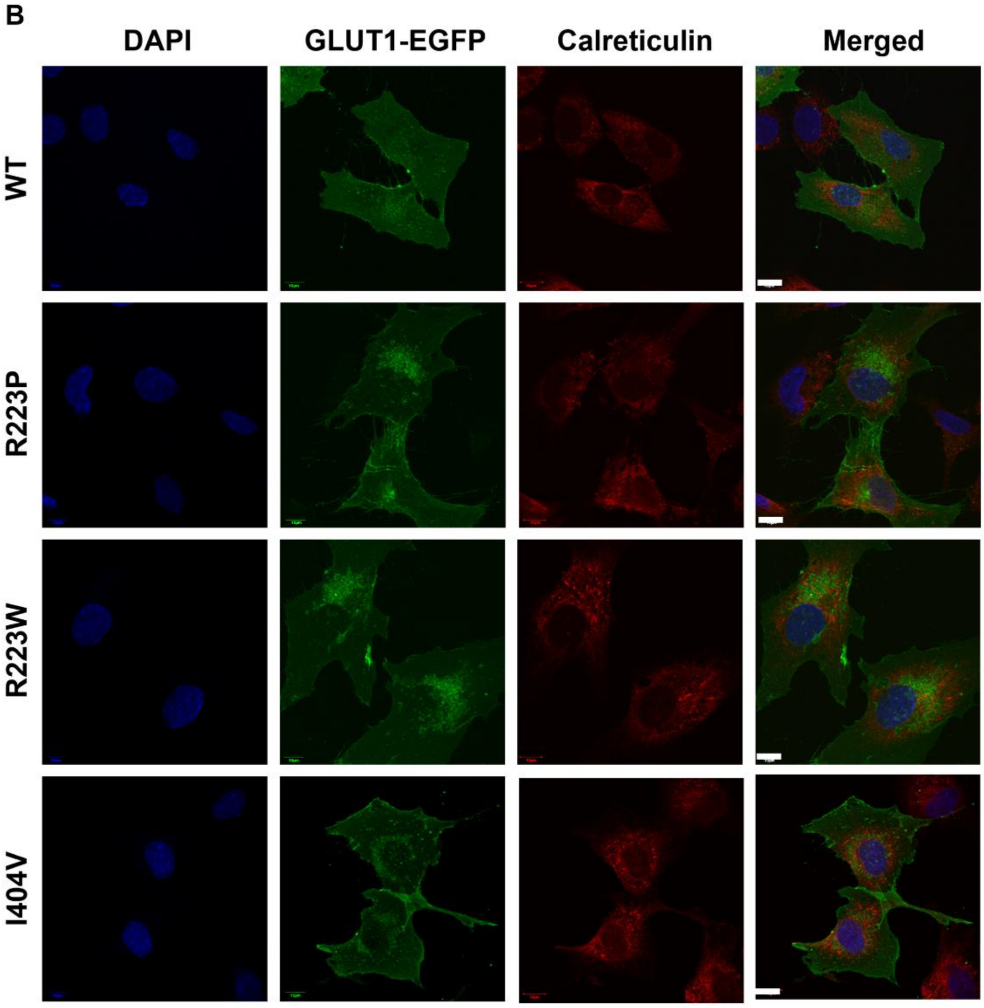
[c] RBC, Red Blood Cells, refers to the cellular type in which reduced glucose uptake was observed



Supplementary Fig. S1.

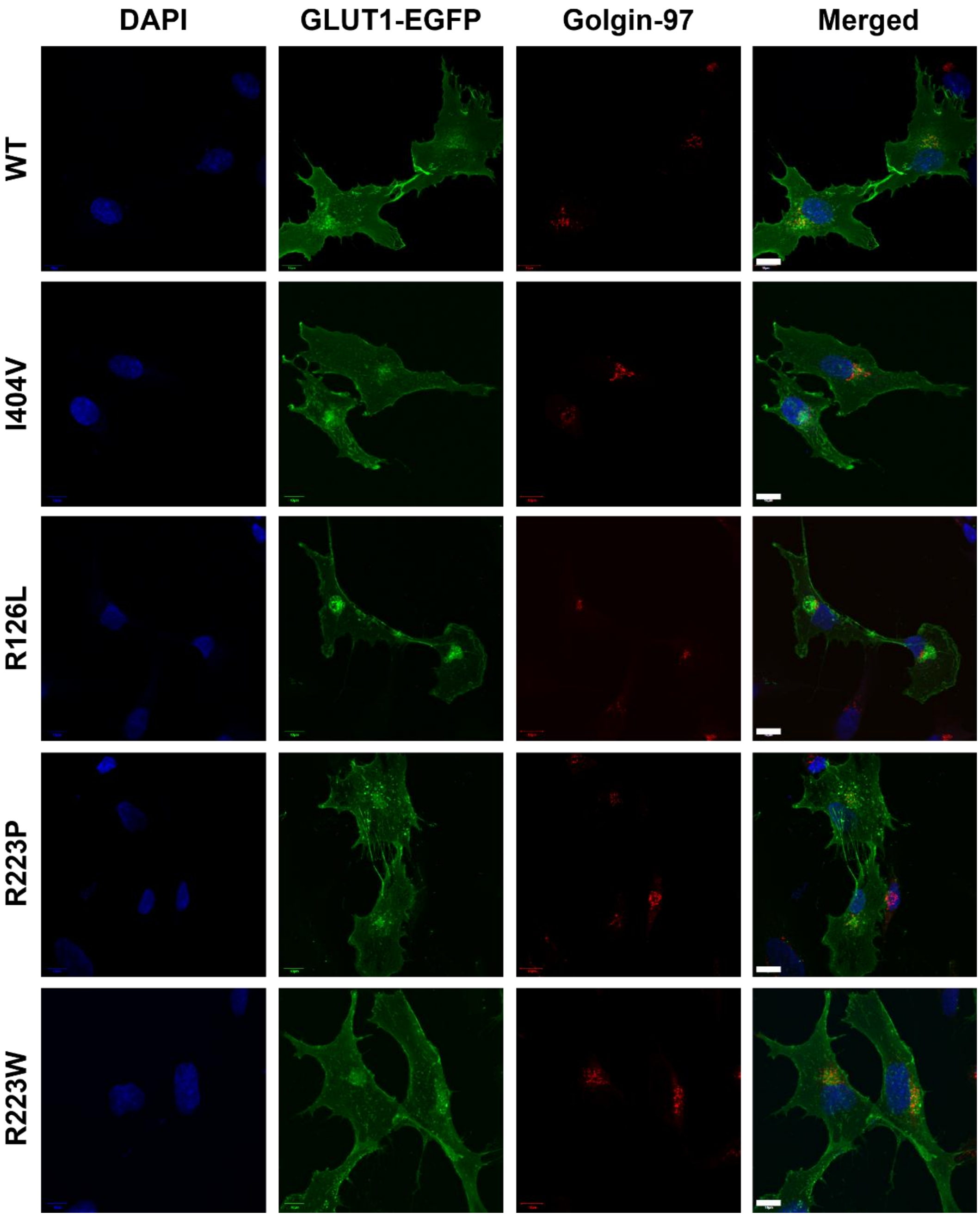
(**A**) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in hCMEC/D3 cells transfected for 48 h with the following constructs: pEGFPN1-GLUT1-WT, pEGFPN1-GLUT1-R223P, pEGFPN1-GLUT1-R223W, and pEGFPN1-GLUT1-I404V. DAPI (blue) was used to visualize nuclei, cadherins staining (red) detected plasma membranes. Images are maximum intensity projections of z-stacks. Scale bars 10 μm. (**B**) Quantification of colocalization between GLUT1-EGFP and cadherins signals. For each experimental condition, Pearson's correlation coefficient (PCC) was calculated within the areas of colocalization using Fiji. PCC was recorded for each cell and reported as mean ± sem on 30 cells. Differences between WT and mutants were analyzed by unpaired Student's t-test. *p<0.01


A
DAPI
GLUT1-EGFP
Calreticulin
Merged
WT
R153C
R126L
N34S
Q283R
P485L



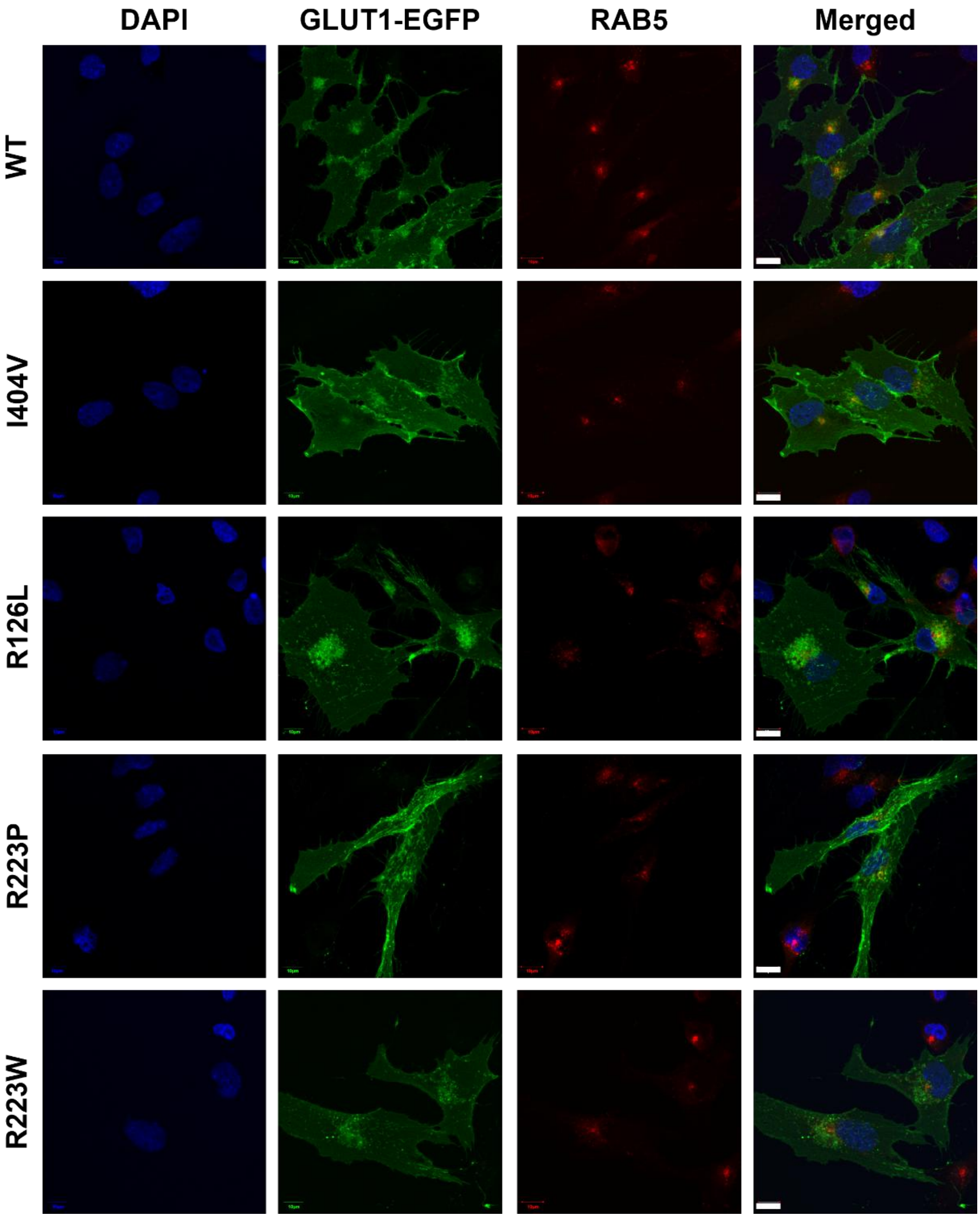
Supplementary Fig. S2.

(A) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in hCMEC/D3 cells transfected for 48 h with the following constructs: pEGFPN1-GLUT1-WT, pEGFPN1-GLUT1-R153C, pEGFPN1-GLUT1-R126L, pEGFPN1-GLUT1-N34S, pEGFPN1-GLUT1-Q283R, and pEGFPN1-GLUT1-P485L. DAPI (blue) was used to visualize nuclei, Calreticulin staining (red) detected endoplasmic reticulum. Images are maximum intensity projections of z-stacks. Scale bars 10 µm. (**B**) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in hCMEC/D3 cells transfected for 48 h with the following constructs: pEGFPN1-GLUT1-WT, pEGFPN1-GLUT1-R223P, pEGFPN1-GLUT1-R223W, and pEGFPN1-GLUT1-I404V. DAPI (blue) was used to visualize nuclei, Calreticulin staining (red) detected endoplasmic reticulum. Images are maximum intensity projections of z-stacks. Scale bars 10 µm.



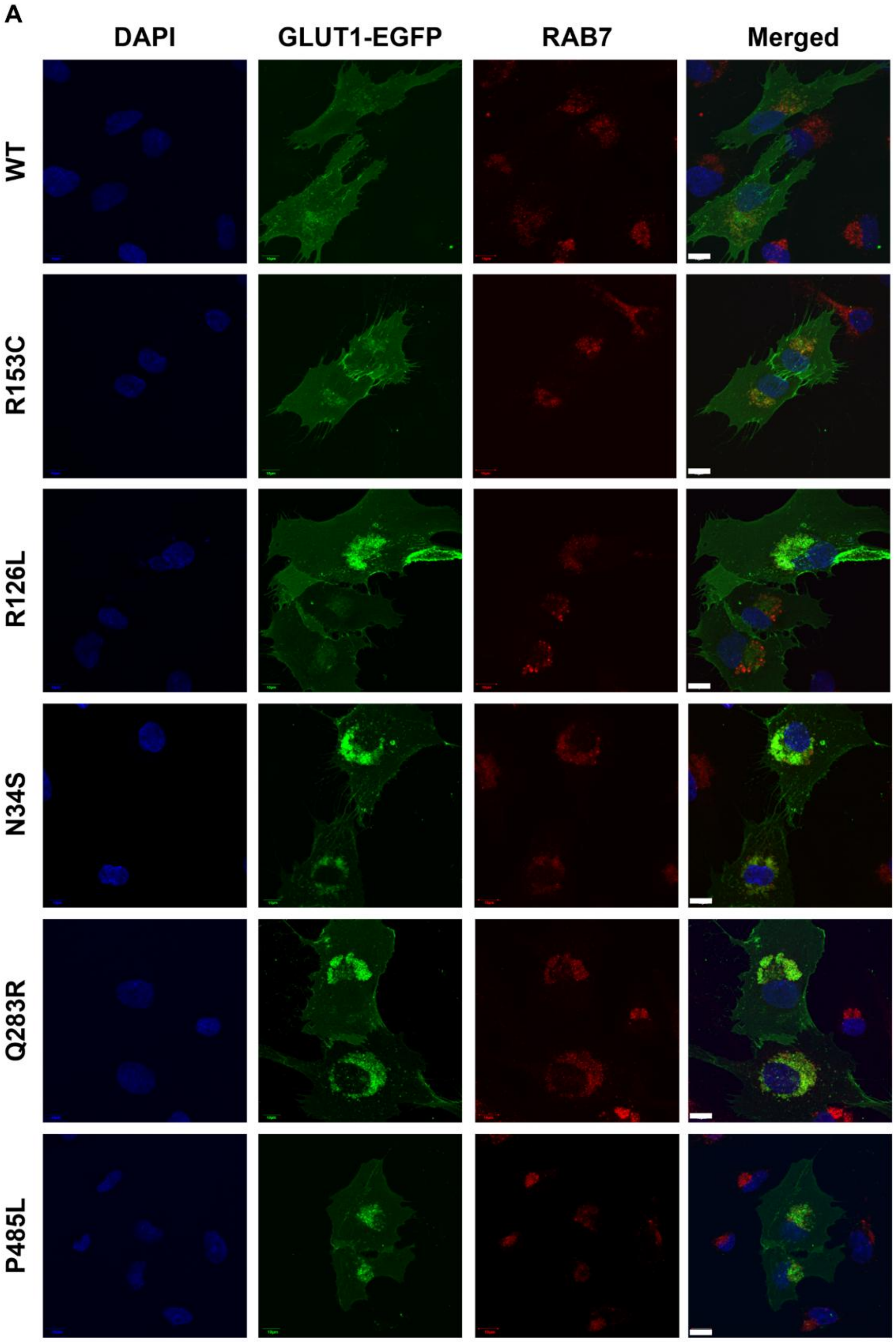
Supplementary Fig. S3.

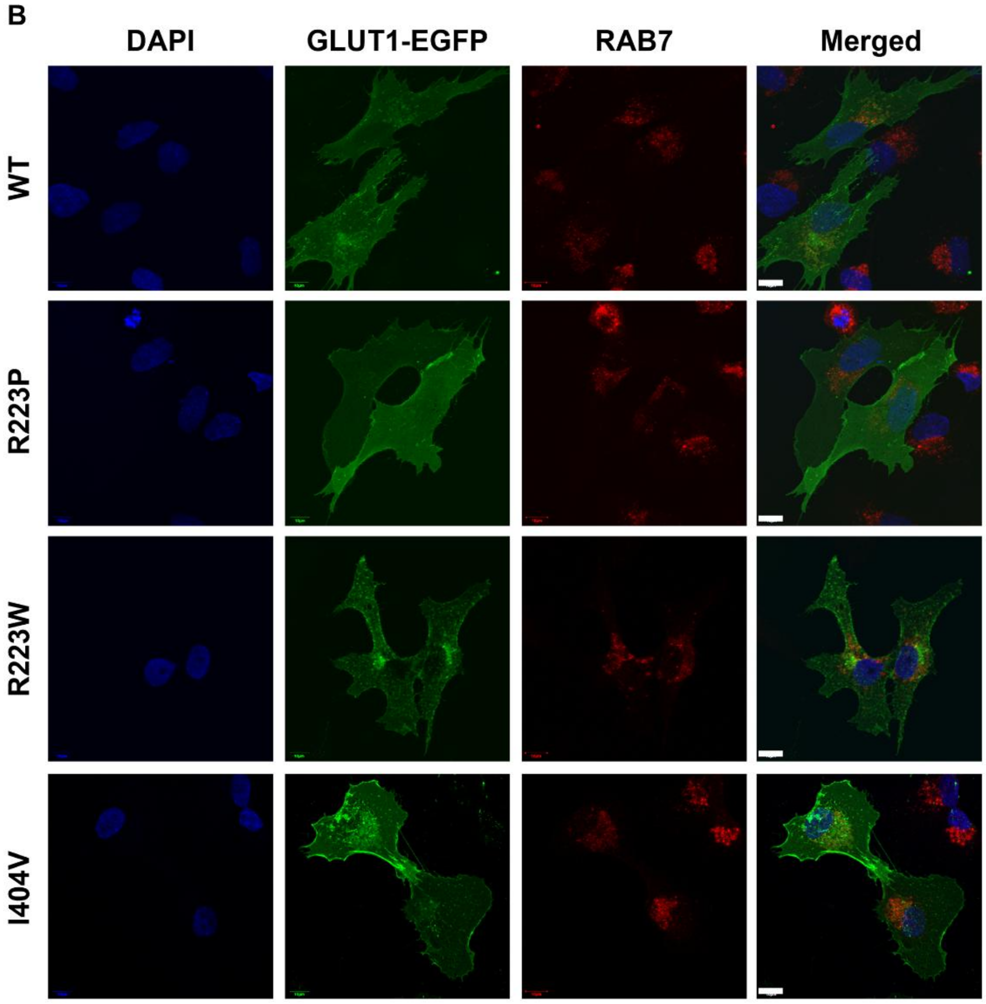
Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in hCMEC/D3 cells transfected for 48 h with the following constructs: pEGFPN1-GLUT1-WT, pEGFPN1-GLUT1-I404V, pEGFPN1-GLUT1-R126L, pEGFPN1-GLUT1-R223P, and pEGFPN1-GLUT1-R223W. DAPI (blue) was used to visualize nuclei, Golgin-97 staining (red) detected Golgi apparatus. Images are maximum intensity projections of z-stacks. Scale bars 10 µm.



Supplementary Fig. S4.

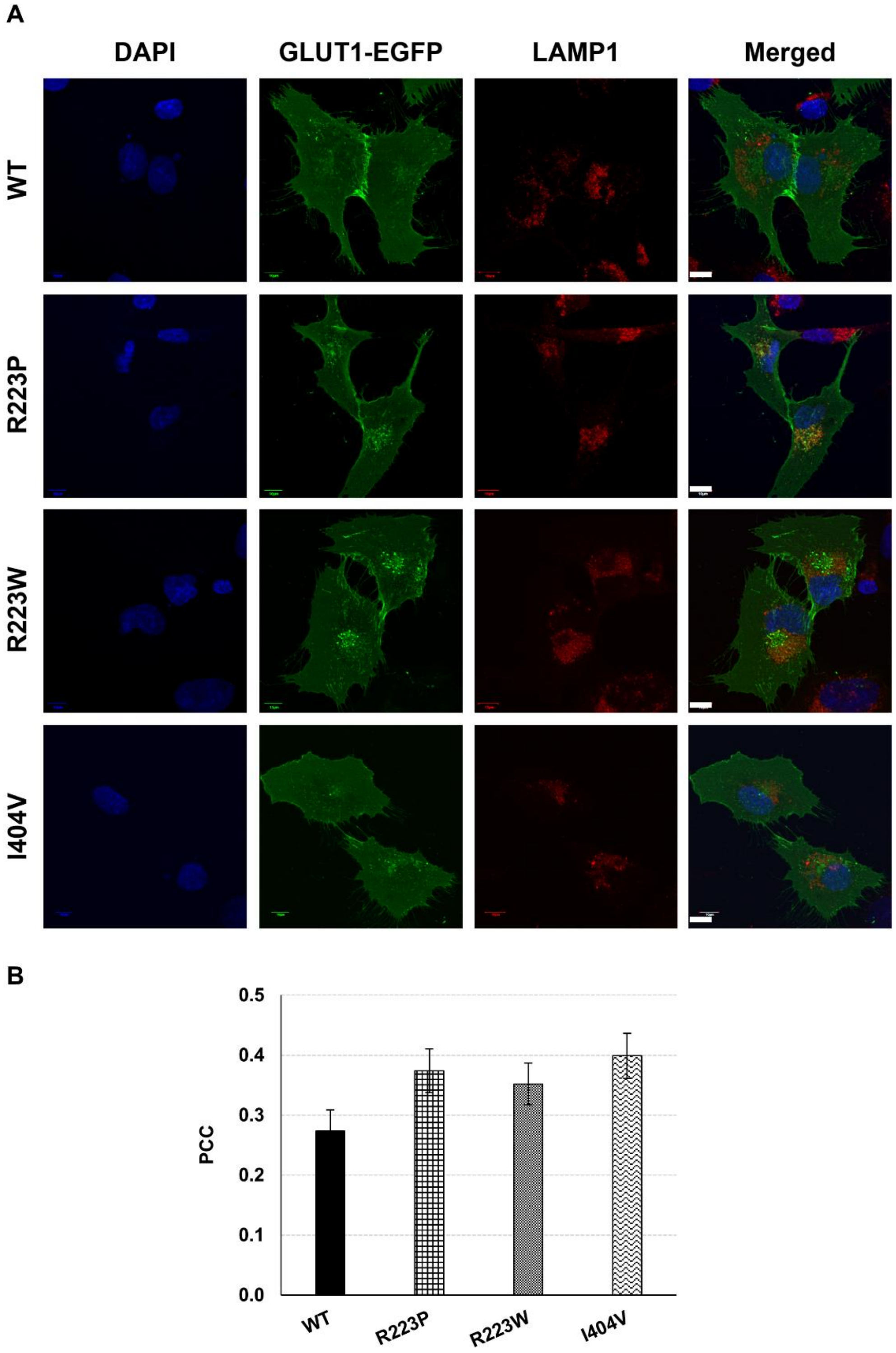
Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in hCMEC/D3 cells transfected for 48 h with the following constructs: pEGFPN1-GLUT1-WT, pEGFPN1-GLUT1-I404V, pEGFPN1-GLUT1-R126L, pEGFPN1-GLUT1-R223P, and pEGFPN1-GLUT1-R223W. DAPI (blue) was used to visualize nuclei, RAB5 staining (red) detected early endosomes. Images are maximum intensity projections of z-stacks. Scale bars 10 µm.


A
DAPI
GLUT1-EGFP
RAB7
Merged
WT
R153C
R126L
N34S
Q283R
P485L



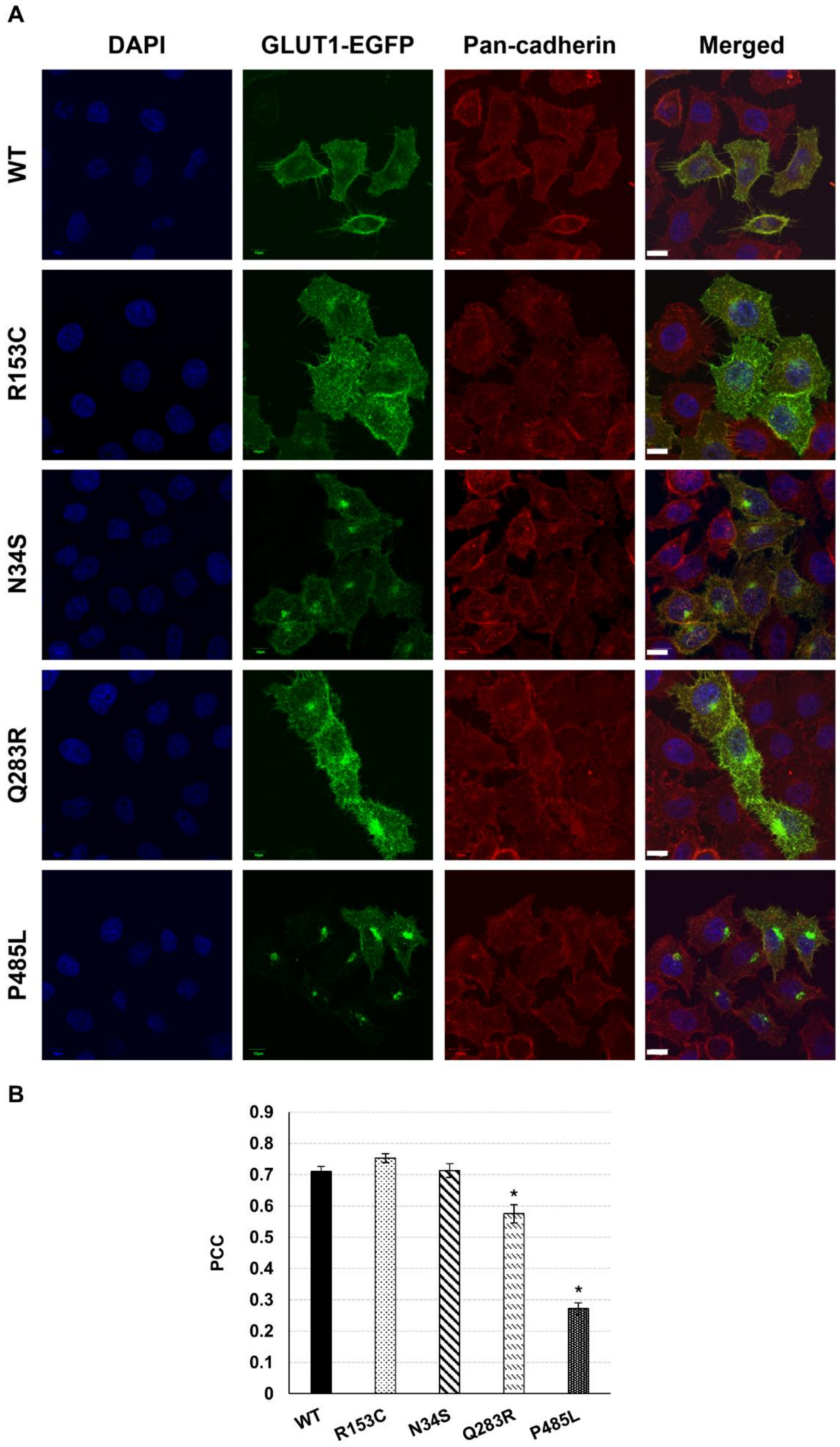
Supplementary Fig. S5.

(A) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in hCMEC/D3 cells transfected for 48 h with the following constructs: pEGFPN1-GLUT1-WT, pEGFPN1-GLUT1-R153C, pEGFPN1-GLUT1-R126L, pEGFPN1-GLUT1-N34S, pEGFPN1-GLUT1-Q283R, and pEGFPN1-GLUT1-P485L. DAPI (blue) was used to visualize nuclei, RAB7 staining (red) detected late endosomes. Images are maximum intensity projections of z-stacks. Scale bars 10 µm. (**B**) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in hCMEC/D3 cells transfected for 48 h with the following constructs: pEGFPN1-GLUT1-WT, pEGFPN1-GLUT1-R223P, pEGFPN1-GLUT1-R223W, and pEGFPN1-GLUT1-I404V. DAPI (blue) was used to visualize nuclei, RAB7 staining (red) detected late endosomes. Images are maximum intensity projections of z-stacks. Scale bars 10 µm.



Supplementary Fig. S6.

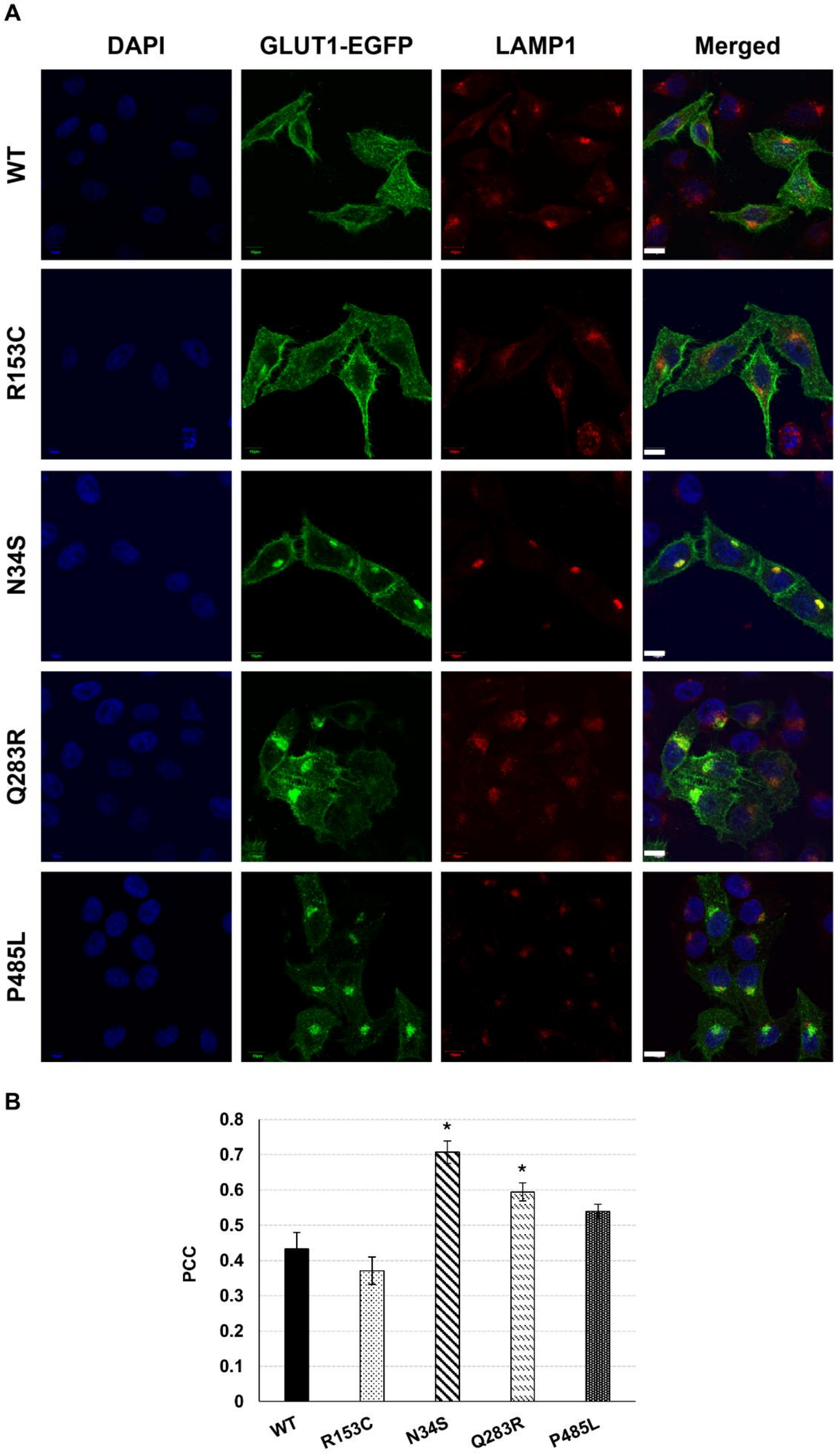
(**A**) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in hCMEC/D3 cells transfected for 48 h with the following constructs: pEGFPN1-GLUT1-WT, pEGFPN1-GLUT1-R223P, pEGFPN1-GLUT1-R223W, and pEGFPN1-GLUT1-I404V. DAPI (blue) was used to visualize nuclei, LAMP1 staining (red) detected lysosomes. Images are maximum intensity projections of z-stacks. Scale bars 10 µm. (**B**) Quantification of colocalization between GLUT1-EGFP and LAMP1 signals. For each experimental condition, Pearson's correlation coefficient (PCC) was calculated within the areas of colocalization using Fiji. PCC was recorded for each cell and reported as mean ± sem on 30 cells. Differences between WT and mutants were analyzed by unpaired Student's t-test. $^*p<0.01$



Supplementary Fig. S7.

(**A**) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in HeLa cells transfected for 48 h with the following constructs: pEGFPN1-GLUT1-WT, pEGFPN1-GLUT1-R153C, pEGFPN1-

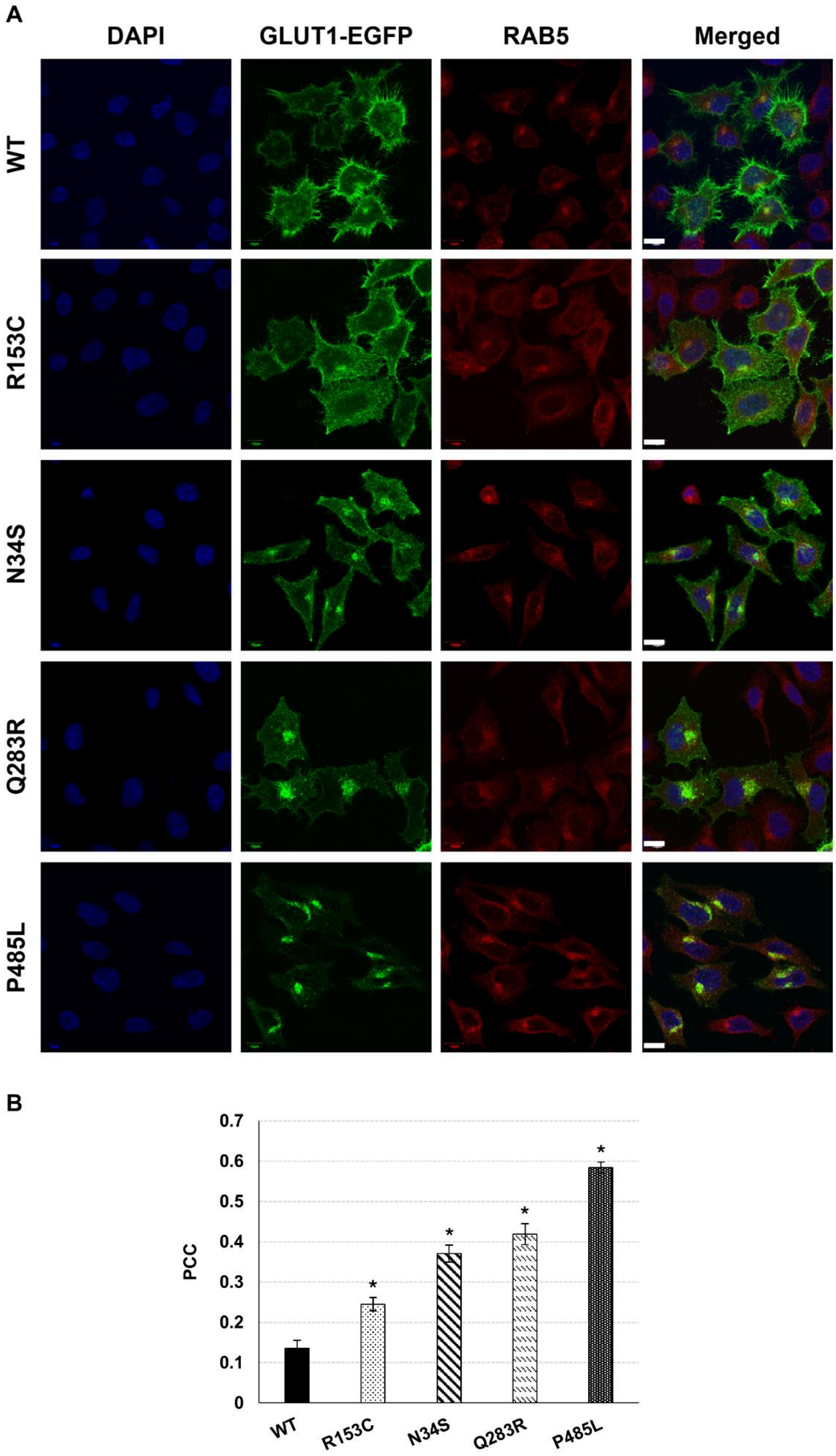
GLUT1-N34S, pEGFPN1-GLUT1-Q283R, and pEGFPN1-GLUT1-P485L. DAPI (blue) was used to visualize nuclei, cadherins staining (red) detected plasma membranes. Images are maximum intensity projections of z-stacks. Scale bars 10 µm. (**B**) Quantification of colocalization between GLUT1-EGFP and cadherins signals. For each experimental condition, Pearson's correlation coefficient (PCC) was calculated within the areas of colocalization using Fiji. PCC was recorded for each cell and reported as mean ± sem on 30 cells. Differences between WT and mutants (R153C, N34S, Q283R, P485L) were analyzed by unpaired Student's t-test. *p<0.01



Supplementary Fig. S8.

(**A**). Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in HeLa cells transfected for 48 h with the following constructs: pEGFPN1-GLUT1-WT, pEGFPN1-GLUT1-R153C, pEGFPN1-

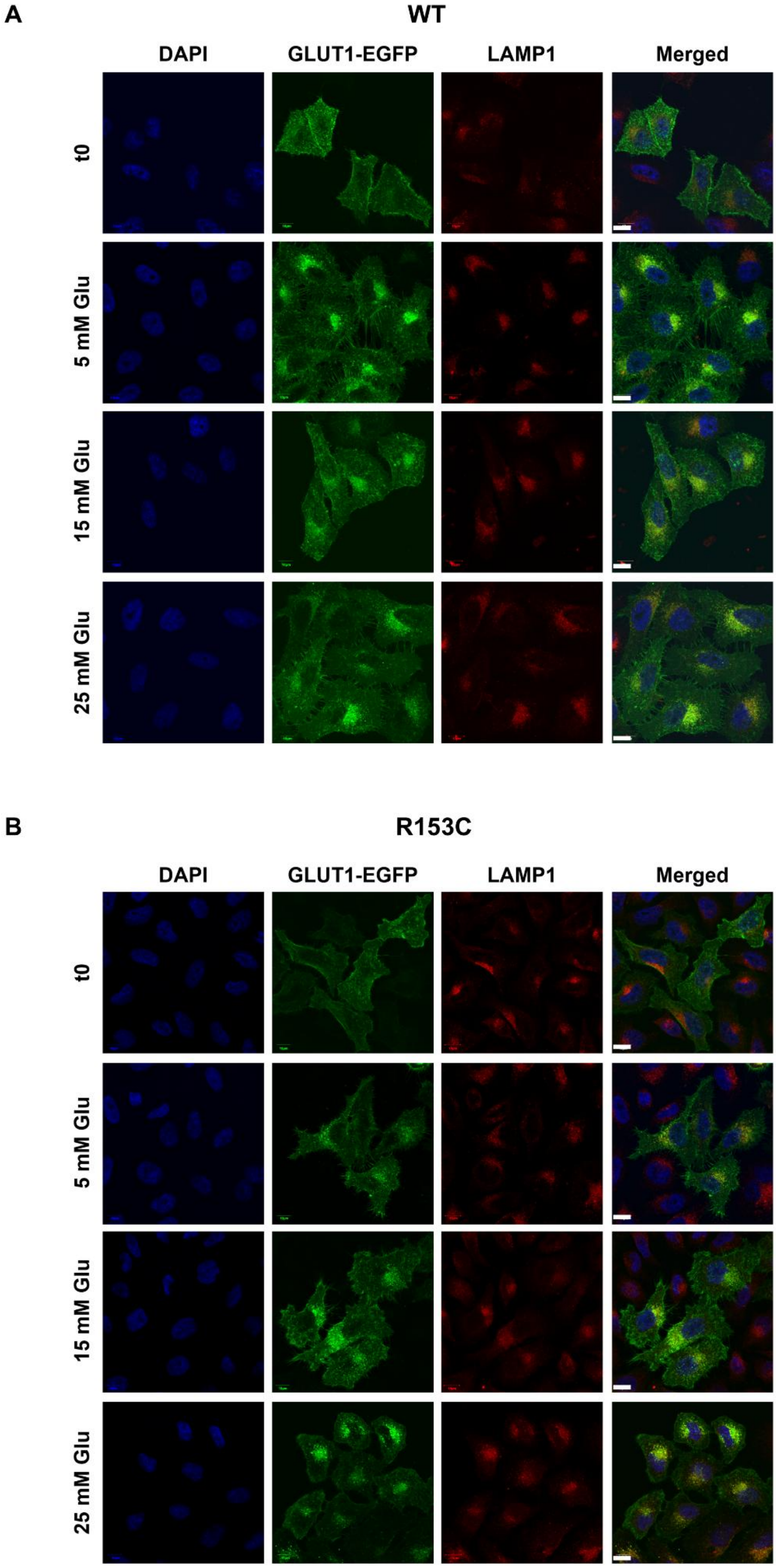
GLUT1-N34S, pEGFPN1-GLUT1-Q283R, and pEGFPN1-GLUT1-P485L. DAPI (blue) was used to visualize nuclei, LAMP1 staining (red) detected lysosomes. Images are maximum intensity projections of z-stacks. Scale bars 10 µm. (**B**) Quantification of colocalization between GLUT1-EGFP and LAMP1 signals. For each experimental condition, Pearson's correlation coefficient (PCC) was calculated within the areas of colocalization using Fiji. PCC was recorded for each cell and reported as mean ± sem on 30 cells. Differences between WT and mutants (R153C, N34S, Q283R, P485L) were analyzed by unpaired Student's t-test. $^*p<0.01$

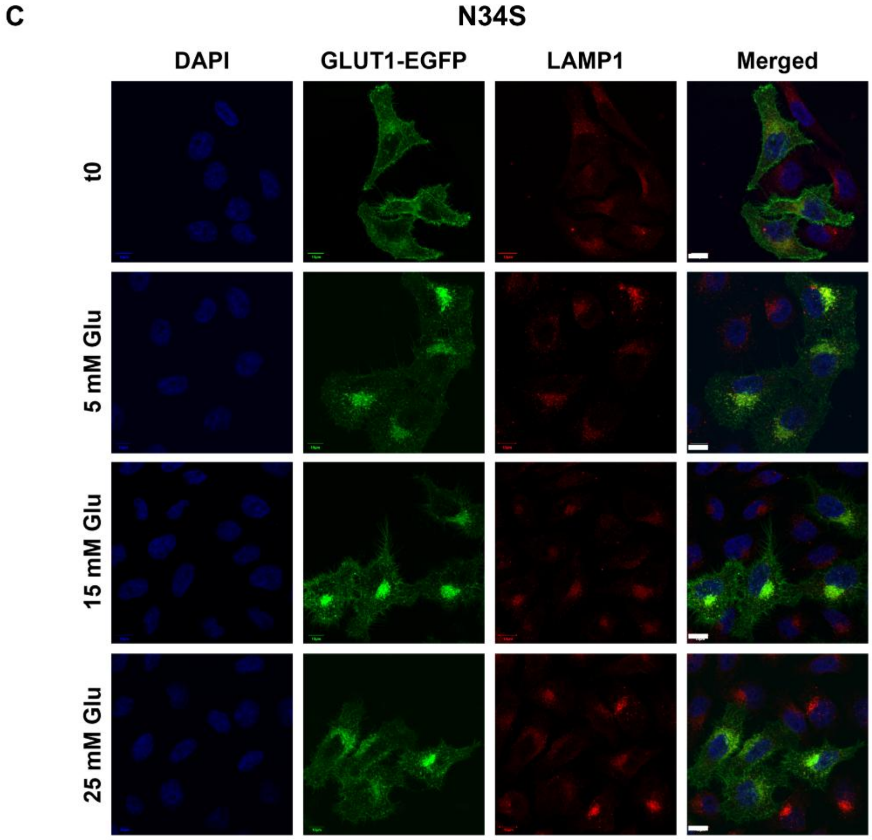


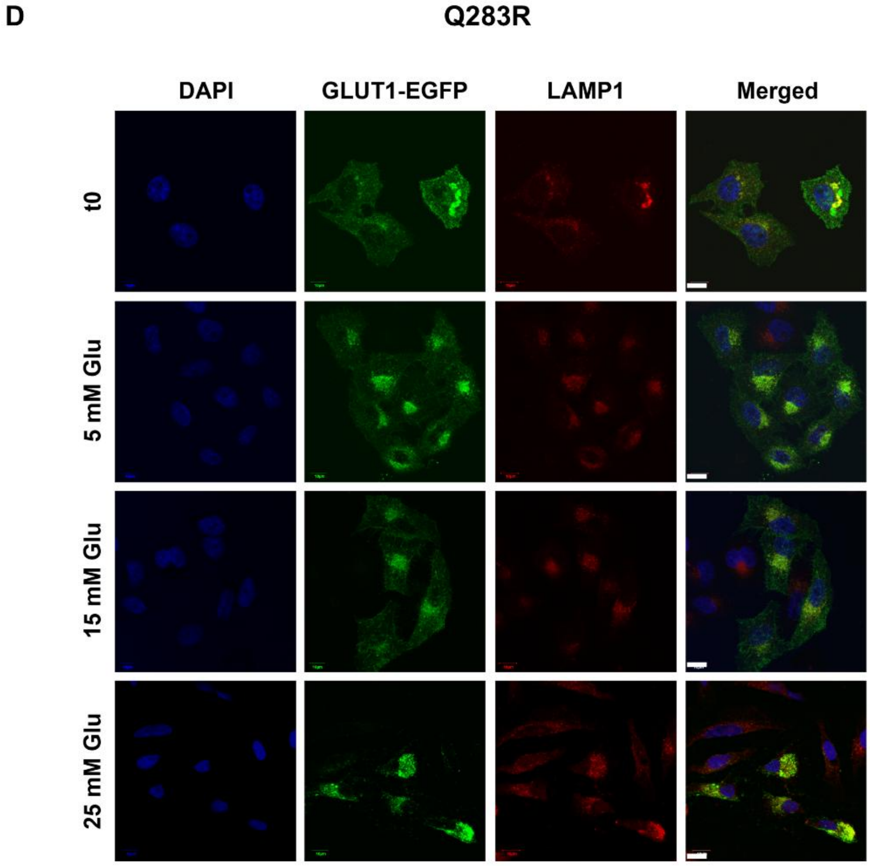
Supplementary Fig. S9.

(**A**) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in HeLa cells transfected for 48 h with the following constructs: pEGFPN1-GLUT1-WT, pEGFPN1-GLUT1-R153C, pEGFPN1-

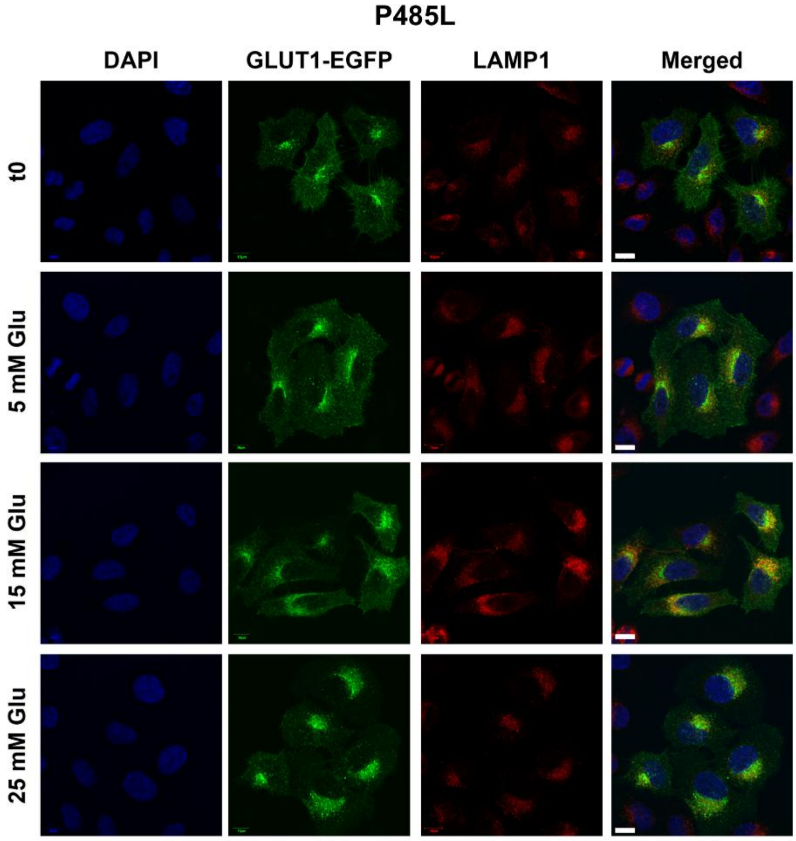
GLUT1-N34S, pEGFPN1-GLUT1-Q283R, and pEGFPN1-GLUT1-P485L. DAPI (blue) was used to visualize nuclei, RAB5 staining (red) detected early endosomes. Images are maximum intensity projections of z-stacks. Scale bars 10 µm. (**B**) Quantification of colocalization between GLUT1-EGFP and RAB5 signals. For each experimental condition, Pearson's correlation coefficient (PCC) was calculated within the areas of colocalization using Fiji. PCC was recorded for each cell and reported as mean ± sem on 30 cells. Differences between WT and mutants (R153C, N34S, Q283R, P485L) were analyzed by unpaired Student's t-test. $^*p<0.01$


A
WT
DAPI
GLUT1-EGFP
LAMP1
Merged
t0
5 mM Glu
15 mM Glu
25 mM Glu
B
R153C
DAPI
GLUT1-EGFP
LAMP1
Merged
t0
5 mM Glu
15 mM Glu
25 mM Glu


C
N34S
DAPI
GLUT1-EGFP
LAMP1
Merged
t0
5 mM Glu
15 mM Glu
25 mM Glu

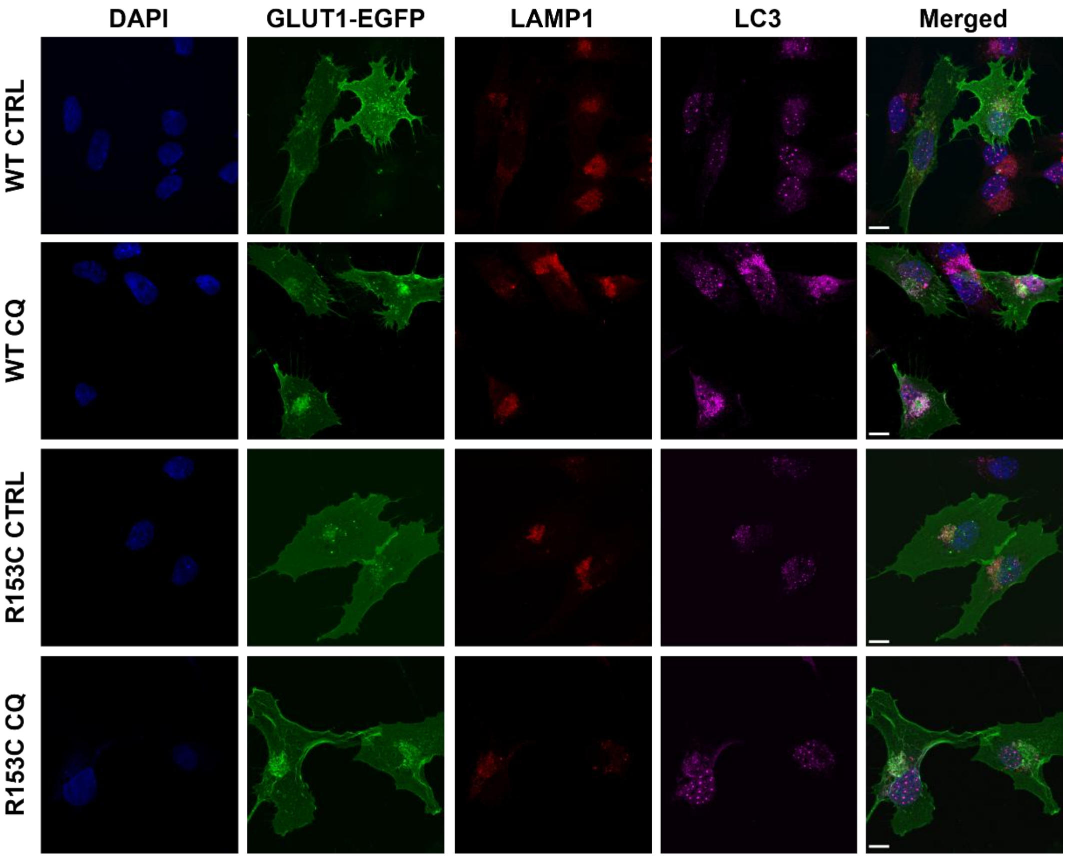

D
Q283R
DAPI
GLUT1-EGFP
LAMP1
Merged
t0
5 mM Glu
15 mM Glu
25 mM Glu

E



Supplementary Fig. S10.

(**A**) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in HeLa cells transfected with pEGFPN1-GLUT1-WT, subjected to glucose deprivation for 24 h and then stopped (t0) or shifted towards different glucose concentrations (5 mM, 15 mM, 25 mM) for 24 h. DAPI (blue) was used to visualize nuclei, LAMP1 staining (red) detected lysosomes. Images are maximum intensity projections of z-stacks. Scale bars 10 µm. (**B**) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in HeLa cells transfected with pEGFPN1-GLUT1-R153C, subjected to glucose deprivation for 24 h and then stopped (t0) or shifted towards different glucose concentrations (5 mM, 15 mM, 25 mM) for 24 h. DAPI (blue) was used to visualize nuclei, LAMP1 staining (red) detected lysosomes. Images are maximum intensity projections of z-stacks. Scale bars 10 µm. (**C**) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in HeLa cells transfected with pEGFPN1-GLUT1-N34S, subjected to glucose deprivation for 24 h and then stopped (t0) or shifted towards different glucose concentrations (5 mM, 15 mM, 25 mM) for 24 h. DAPI (blue) was used to visualize nuclei, LAMP1 staining (red) detected lysosomes. Images are maximum intensity projections of z-stacks. Scale bars 10 µm. (**D**) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in HeLa cells transfected with pEGFPN1-GLUT1-Q283R, subjected to glucose deprivation for 24 h and then stopped (t0) or shifted towards different glucose concentrations (5 mM, 15 mM, 25 mM) for 24 h. DAPI (blue) was used to visualize nuclei, LAMP1 staining (red) detected lysosomes. Images are maximum intensity projections of z-stacks. Scale bars 10 µm. (**E**) Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in HeLa cells transfected with pEGFPN1-GLUT1-P485L, subjected to glucose deprivation for 24 h and then stopped (t0) or shifted towards different glucose concentrations (5 mM, 15 mM, 25 mM) for 24 h. DAPI (blue) was used to visualize nuclei, LAMP1 staining (red) detected lysosomes. Images are maximum intensity projections of z-stacks. Scale bars 10 µm.



Supplementary Fig. S11.

Representative images of immunofluorescence (IF) staining detecting GLUT1-EGFP localization (green) in hCMEC/D3 cells transfected for 24 h with pEGFPN1-GLUT1-WT and pEGFPN1-GLUT1-R153C constructs after chloroquine (CQ) treatment for 2 h or under control condition (CTRL). DAPI (blue) was used to visualize nuclei, LAMP1 (red) and LC3 (magenta) stainings detected lysosomes and autophagosomes, respectively. Images are maximum intensity projections of z-stacks. Scale bars 10 µm.