The C-terminal region of BLT2 restricts its localization to the lateral membrane in a LIN7C-dependent manner

Title: The C-terminal region of BLT2 restricts its localization to the lateral membrane in a LIN7C-dependent manner

Short running title: BLT2 localizes to the lateral membrane via LIN7C

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Nonstandard Abbreviations:

12-HHT: 12(*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid

ANOVA: analysis of variance

APEX: ascorbate peroxidase

BLT: leukotriene B_4 receptor

CASK: calcium/calmodulin-dependent serine protein kinase

DAPI: 4′,6-diamidino-2-phenylindole

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GPCR: G protein-coupled receptor

ICL: intracellular loop

LIN7: lin-7 homolog

LTB_4: leukotriene B_4

MDCK II: Madin-Darby canine kidney II

MS: mass spectrometry

qPCR: quantitative PCR

RCAS: receptor-binding cancer antigen expressed on SiSo cells

SD: standard deviation

siRNA: small interfering RNA

TER: transepithelial electrical resistance

Abstract

Leukotriene B_4 receptor type 2 (BLT2) is a G protein-coupled receptor (GPCR) mainly expressed in epithelial cells, where it enhances barrier function. A unique characteristic of BLT2 is its restricted localization to the lateral membrane. However, the molecular mechanism underlying the localization of BLT2 to the lateral membrane and the physiological roles of laterally localized BLT2 are unknown. BLT1 is the most homologous GPCR to BLT2 and localizes to both the apical and lateral membranes. In this study, we generated chimeric receptors of BLT2 and BLT1 as well as deletion mutants of BLT2 to determine the region(s) of BLT2 responsible for its localization. Chimeric receptors containing the C-terminal domain of BLT2 localized only to the lateral membrane, and the C-terminal deletion mutant of BLT2 accumulated at the Golgi apparatus. Furthermore, the middle and C-terminal regions of BLT2 were important for maintaining epithelial barrier function. Proteomics analysis using the chimeric BLT-ascorbate peroxidase 2 biotinylation method showed that some proteins involved in intracellular protein transport, cell-cell junctions, and actin filament binding were located very close to the C-terminal domain of BLT2. Knockdown of lin-7 homolog C (LIN7C), a membrane trafficking protein, led to accumulation of BLT2 in the Golgi apparatus, resulting in diminished epithelial barrier function. These results suggest that the C-terminal region of BLT2 plays an important role in the transport of BLT2 from the Golgi apparatus to the plasma membrane in a LIN7C-dependent manner.

Keywords: LIN7C, APEX2, barrier, GPCR, trafficking

Introduction

Leukotriene B_4 (LTB_4) receptor type 2 (BLT2), a G protein-coupled receptor (GPCR) for 12(*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid (12-HHT) and LTB_4,[1, 2] is mainly expressed in keratinocytes and intestinal and corneal epithelial cells. We previously reported that BLT2 plays important roles in cell migration, barrier function, and wound healing processes[3-6] and also interestingly found that BLT2 localizes to the lateral membrane of intestinal epithelial cells and Madin-Darby canine kidney (MDCK) II cells.[5] However, the molecular mechanism underlying localization of BLT2 to the lateral membrane and the physiological roles of laterally localized BLT2 are totally unknown.

Epithelial cells have unique properties that enable sorting of membrane proteins to apical and basolateral regions.[7] To maintain these two distinct regions and to protect the invasion of foreign organisms, epithelial cells are sealed together by a junctional complex comprising four main components, namely, tight junctions, adherens junctions, gap junctions, and desmosomes. Sorting of membrane proteins is a multistep process involving initial sorting in the endoplasmic reticulum, passage through the *trans*-Golgi network, and transport to correct positions in the plasma membrane. Recycling or degradation of membrane proteins via clathrin-dependent or -independent endocytosis regulates membrane retention.[8] Tightly controlled sorting of membrane proteins is pivotal for the functions of epithelial cells. Disturbances in cellular polarization are integral to the development of epithelial cancers.[9, 10] Additionally, mistargeting or defective membrane localization of specific membrane proteins causes many diseases including skin Darier's disease,[11] cystic fibrosis,[12] and polycystic kidney disease.[13]

We have used MDCK II cells as a model to study protein trafficking and barrier function in polarized epithelial cells. Our previous research showed that BLT2 localizes to the lateral membrane and BLT2 expression enhances barrier function.[5] BLT1, a high-affinity LTB_4 receptor, localizes to both the apical and lateral membranes. In the present study, we generated chimeric receptors by domain swapping between BLT2 and BLT1 as well as deletion mutants of BLT2 to determine the region(s) of BLT2 responsible for its lateral localization. Moreover, we searched for BLT2-interacting proteins required for the lateral localization of BLT2

using an ascorbate peroxidase 2 (APEX2) biotinylation method in which APEX2[14, 15] fused to BLT2 was used as a bait.

Materials and Methods

Cell culture

MDCK II cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. MDCK II cells stably expressing chimeric BLT (HA-tagged) or chimeric BLT-APEX2 (FLAG-tagged) were selected in the presence of G418 (1 mg/mL) and then sorted (Aria III, BD Biosciences, San Jose, CA, USA) after staining with an anti-HA (3F10, Roche Diagnostics, Indianapolis, IN, USA) or anti-FLAG (2H8[16]) antibody and a fluorescently-labeled secondary antibody (see below for details).

Plasmids

Constructs for chimeric BLT (HA-tagged), BLT-APEX2 (FLAG-tagged), and BLT2 deletion mutants (HA-tagged) were generated by standard PCR amplification and In-Fusion methods (Takara Bio USA, Mountain View, CA, USA). All constructs were generated using pCXN2.1 expression vector.[17] All constructs were confirmed by DNA sequencing.

Transepithelial electrical resistance (TER) measurement and immunocytochemistry

MDCK II cells (4×10^4 cells/well) were seeded onto membrane transwell inserts (diameter of 12 mm, Millipore, Billerica, MA, USA) and incubated for the indicated number of hours. Thereafter TER was measured using a Millicell-ERS 2 V ohmmeter (EMS-Millipore). For immunocytochemistry, MDCK II cells on the membrane were embedded in OCT compound (Sakura Finetek, Chuo, Tokyo, Japan) after 48 h of culture. Frozen sections (7-μm thick) were fixed in cold acetone, blocked with phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) for 30 min, stained with an anti-HA (0.5 μg/mL, 3F10) or anti-FLAG (2 μg/mL, 2H8)

antibody overnight at 4°C, and then incubated with an Alexa Fluor 555-conjugated anti-rat IgG (2 μg/mL, Thermo Fischer Scientific, Waltham, MA, USA) or Alexa Fluor 488-conjugated anti-mouse IgG (2 μg/mL, Thermo Fischer Scientific) antibody overnight at 4°C. Nuclei were visualized by staining with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/mL). All images were acquired using a fluorescence microscope (BZ-X700, KEYENCE, Higashiyodogawa, Osaka, Japan).

Flow cytometry

MDCK II cells were incubated with an anti-HA antibody (0.5 μg/mL, 3F10) for 1 h at room temperature, and then with an Alexa Fluor 488-conjugated rabbit anti-rat IgG antibody (2 μg/mL) for 1 h at room temperature. Cells were analyzed using a flow cytometer (FACSCalibur, BD Biosciences).

Quantitative PCR (qPCR)

MDCK II cells (1×10^5 cells/well) were seeded onto 24-well plates and cultured for 48 h. Total RNA was purified with TRIzol reagent (Thermo Fisher Scientific). cDNA was generated using a QuatiTect Reverse Transcription Kit (Qiagen, Netherlands, Hilden, Germany). qPCR was performed with FastStart SYBR Green Master (Roche) on an ABI StepOnePlus system (Thermo Fisher Scientific). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control. The primer sequences were as follows: *canis lupus familiaris* claudin-4 (*CLDN4*) upstream, 5’-AGCTGATCCAACCGCCTCTG-3’ and downstream, 5’-CCAAGCGGTGAGGACAGACA-3’; *canis lupus familiaris* lin-7 homolog C (*LIN7C*) upstream, 5’-TCCGGGTGGAATTGCTGATAG-3’ and downstream, 5’-ACCTTTCCCTGAGCTGCTTTC-3’; and *canis lupus familiaris GAPDH* upstream, 5’-ACTGCTTGGCTCCTCTAGCC-3’, and downstream, 5’-GTCCACGGTCTTCTGGGTGG-3’.

Immunostaining

MDCK II cells (1×10^5 cells/well) were seeded onto 24-well plates and incubated for 48 h. Cells were fixed in 0.1 M phosphate buffer containing 4% paraformaldehyde, blocked with 2% BSA prepared in PBS containing 0.5% Triton X-100 for 30 min, stained with anti-HA (0.5 μg/mL, 3F10) and anti- receptor-binding cancer antigen expressed on SiSo cells (RCAS1; 1:100, Cell Signaling Technology, Danvers, MA, USA) antibodies overnight at 4°C, and then incubated with Alexa Fluor 555-conjugated anti-rat IgG (2 μg/mL) and Alexa Fluor 488-conjugated anti-rabbit IgG (2 μg/mL, Thermo Fischer Scientific) antibodies overnight at 4°C. Nuclei were visualized by staining with DAPI (1 μg/mL). All images were acquired using a fluorescence microscope (BZ-X700, KEYENCE).

Pull-down of biotinylated proteins

MDCK II cells (2×10^6 cells/well) expressing BLT-APEX2 were seeded onto 10-cm dishes and cultured for 48 h. Cells were treated with biotin-phenol (1.5 mM) for 6 h and then biotinylation was initiated by adding H_2O_2 (1 mM) and samples were incubated for 1 min. Cells were washed with quenching buffer (PBS containing 10 mM sodium ascorbate, 5 mM Trolox, and 10 mM sodium azide) and lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 10 mM sodium ascorbate, 5 mM Trolox, and 10 mM sodium azide) for 1 h at 4°C. Cell lysates were centrifuged at 13,000 rpm for 20 min and their supernatants were incubated with streptavidin beads (Dynabeads, Invitrogen, Waltham, MA, USA).

Proteomics analysis

Biotinylated proteins bound to streptavidin beads were incubated with 100 mM Tris-HCl (pH 8.0), 5 mM DTT, and 8 M urea for 30 min at 37°C and then with 100 mM Tris-HCl (pH 8.0), 15 mM iodoacetamide, and 8 M urea in the dark for 30 min at room temperature. Proteins were digested with Trypsin/Lys-C Mix (Promega, Madison, WI, USA) in 100 mM Tris-HCl (pH 8.0) and 4 M urea for 2 h at 37°C, diluted with five volumes of 100 mM Tris-HCl (pH 8.0), and incubated overnight at 37°C. Protein digestion was terminated by adding trifluoroacetic acid (final concentration of 1%). Digested proteins were desalted using GL-Tip SDB (GL

Sciences, Shinjuku, Tokyo, Japan) and subjected to proteomics analysis by nano-LC-MS/MS using a TripleTOF 5600 mass spectrometer coupled with Eksigent nano-LC system (SCIEX, Framingham, MA, USA). Data were normalized to the abundance of acetyl-CoA carboxylase, an endogenous biotinylated protein, in all streptavidin pull-down samples.

Small interfering RNA (siRNA) knockdown

Synthetic siRNAs targeting *canis lupus familiaris LIN7C,* 5'- UCAUAUACCUCUCUCACAGCAUUGC -3' (sense) and 5'- GCAAUGCUGUGAGAGAGGUAUAUGA -3' (anti-sense) (Stealth RNAi, Thermo Fisher Scientific) were used. The Medium GC Duplex of Stealth RNAi Negative Control Duplexes 2 (Thermo Fisher Scientific) was used as a negative control. MDCK II cells (6×10^5 cells/well) seeded onto 6 well-plates were transfected with 20 nM siRNA using RNAiMAX (Thermo Fisher Scientific) and cultured in DMEM containing 10% fetal calf serum. After 24 h, cells were detached and seeded onto membrane transwell inserts (1.6×10^5 cells/well) for TER measurement as described elsewhere[18] and onto 24-well plates (2×10^5 cells/well) for qPCR or immunostaining.

Statistical analysis

Results are expressed as mean ± standard deviation (SD) of the mean. Statistical analyses were performed with the Student's *t*-test, the paired *t*-test, a one-way analysis of variance (ANOVA), or a two-way ANOVA followed by Dunnett's or Tukey's multiple comparison test using GraphPad Prism 8 software (GraphPad, San Diego, CA, USA).

Results

The C-terminal region of BLT2 is important for its lateral localization

We previously reported that BLT2 only localizes to the lateral membrane, while BLT1 localizes to both the apical and lateral membranes.[5] To identify the domain(s) of BLT2 essential for its lateral localization, we

assessed the expression and localization of chimeras of BLT1 and BLT2. We generated several HA-tagged chimeric BLT constructs (Fig. 1A, B) and examined the localizations of these chimeric BLTs stably expressed in MDCK II cells. Flow cytometric analyses showed that BLT (2-2-1) and BLT (2-1-1) were not expressed on the plasma membrane (data not shown), while BLT (1-2-2), BLT (2-1-2), BLT (1-2-1), and BLT (1-1-2) were expressed on the cell surface (Fig. 1C). MDCK II cells expressing these chimeric BLTs on the plasma membrane were cultured on membrane inserts to form a monolayer, and then longitudinal sections of the monolayer were stained using an anti-HA antibody. Whereas BLT1 and BLT (1-2-1) localized to both the apical and lateral membranes, BLT (1-1-2), BLT2, BLT (1-2-2), and BLT (2-1-2) localized only to the lateral membrane (Fig. 1D). These results show that the C-terminal region of BLT2 (244-358) is important for its restricted localization to the lateral membrane.

Regions of BLT2 required for barrier function

BLT2 expression increases TER by upregulating claudin-4 in MDCK II cells.[5] To determine the region of BLT2 required for the increase in TER and maintenance of barrier function, we measured the TER of monolayers of MDCK II cells expressing chimeric BLT receptors. Expression of BLT2 and BLT (1-2-2) increased TER and claudin-4 expression, but expression of the other chimeric BLTs with lateral localizations including BLT (1-1-2) and BLT (2-1-2) did not (Fig. 2A, B). These results suggest that a region of BLT2 (107–358) is required for TER elevation and claudin-4 upregulation.

The C-terminal cytosolic region of BLT2 is important for its lateral localization

To precisely determine the region of BLT2 required for its lateral localization, we divided the previously determined C-terminal region of BLT2 (244–358) into three regions and constructed three new chimeric BLTs (Fig. 3A). Flow cytometry confirmed that these chimeric BLTs were expressed at comparable levels on the plasma membrane (Fig. 3B). Immunocytochemical staining showed that BLT (1-1-2-1-1) and BLT (1-1-1-2-1) localized to both the apical and lateral membranes, while BLT (1-1-1-1-2) localized only to the lateral

membrane (Fig. 3C). These results indicate that the C-terminal cytosolic region of BLT2 (295–358) restricts its localization to the lateral membrane.

The C-terminal cytosolic region of BLT2 is necessary for its transport from the Golgi apparatus to the plasma membrane

We also generated two deletion mutants of BLT2 (Fig. 4A) and examined their localizations. BLT2 (334del) was expressed on the plasma membrane, but BLT2 (310del) was not (Fig. 4B). BLT2 (334del) was present only at the lateral membrane in the longitudinal section of the monolayer (Fig. 4C). To clarify the intracellular localization of BLT2 (310del), we performed co-immunostaining of BLT2 (310del) and organelle markers. BLT2 (310del) co-localized with RCAS1, a Golgi apparatus marker (Fig. 4D). These observations indicate that the C-terminal region of BLT2 (311–334) is important for its transport from the Golgi apparatus to the plasma membrane, especially to the lateral membrane.

Neighboring proteins of the C-terminal region of BLT2

To investigate the detailed molecular mechanism that underlies localization BLT2 to the lateral membrane, we attempted to identify BLT2-interacting proteins by an APEX2 biotinylation method, in which chimeric BLT-APEX2 biotinylates neighboring proteins. In this system, APEX2 rapidly produces short-lived (< 1 ms) biotin-phenoxy radicals that covalently biotinylate electron-rich amino acids, such as Tyr, Trp, His, and Cys, of neighboring proteins (within a diameter of 20 nm).[14, 15]

Fusion with APEX2 did not alter the localizations of BLT receptors; BLT1-APEX2 localized to both the apical and lateral membranes, while BLT (1-1-1-1-2)-APEX2 and BLT2-APEX2 localized only to the lateral membrane (Fig. 5A). MDCK II cells expressing BLT-APEX2 were cultured for 48 h and subjected to biotin-phenol-dependent biotinylation (Fig. 5B). Biotinylated proteins were purified using streptavidin beads as described in the Materials and Methods, and analyzed by mass spectrometry (MS) to identify candidate binding partners of BLT2. Proteins detected in the BLT2-APEX2 sample with high ratios of MS scores (BLT

(1-1-1-1-2)-APEX2/BLT1-APEX2 > 1.5) are listed in Figure 5C. These proteins included trafficking proteins, cell-cell junction proteins, and cytoskeletal proteins, and are candidate proteins that interact with the C-terminal cytosolic region of BLT2.

Knockdown of LIN7C impairs BLT2 expression at the plasma membrane

Several proteins involved in membrane trafficking were detected by MS analysis (Fig. 5). Clathrin, AP-2, and dynamin-2 are key proteins for endocytosis. Furthermore, the α2B adrenergic receptor localized to the apical membrane is internalized more rapidly by endocytosis than that localized to the lateral membrane.[19] Therefore, we hypothesized that these trafficking proteins contribute to the lateral localization of BLT2. To verify this, we performed knockdown and inhibition experiments using siRNAs and inhibitors against the candidate proteins. Treatment with methyl-β-cyclodextrin, which inhibits clathrin-dependent endocytosis, dynasore, which inhibits dynamin, FLOT2-targeting siRNA, and SNX8-targeting siRNA did not affect the lateral localization of BLT2 (data not shown). However, LIN7C-targeting siRNA affected BLT2 localization in MDCK II cells (Fig. 6). LIN7C knockdown resulted in accumulation of BLT2 at the perinuclear area, which was co-stained with an anti-RCAS1 antibody (Fig. 6B). By contrast, LIN7C-targeting siRNA did not affect the localization of BLT1, despite a comparable reduction in the level of LIN7C mRNA (Fig. 6A). Furthermore, we examined the effect of LIN7C knockdown on barrier function. LIN7C knockdown decreased TER and claudin-4 mRNA expression in BLT2-expressing MDCK II cells, but not in mock MDCK II cells (Fig. 6C, D). These results indicate that LIN7C is important for transport of BLT2 from the Golgi apparatus to the lateral membrane and BLT2-dependent barrier enhancement via claudin-4 upregulation.

Discussion

Here, we showed that the C-terminal cytosolic region of BLT2 is important for its localization to the lateral membrane and transport from the Golgi apparatus to the plasma membrane via LIN7C-dependent manner in MDCK II cells. Previous studies of membrane trafficking reported that the GPI-anchor[20, 21] and N- and O-linked

glycosylation[22-24] mediate sorting to the apical plasma membrane, while the tyrosine motif,[25] dileucine motif,[26, 27] and AP1 complex[28-30] mediate sorting to the lateral plasma membrane. Our data suggest that the C-terminal cytosolic region of BLT2, especially BLT2 (311–334) with the sequence of "SGEARGGGRSREGTMELRTTPQLK", is a candidate trafficking motif for transport from the Golgi apparatus to the plasma membrane. This sequence does not contain any typical sorting sequences related to membrane trafficking. The APEX2 biotinylation system and siRNA experiments indicated that LIN7C modulates trafficking of BLT2 to the lateral membrane. LIN7C is a LIN7 family member (LIN7A, B, and C) and a small scaffold protein. LIN7 forms membrane-associated protein complexes with calcium/calmodulin-dependent serine protein kinase (CASK), Mint1, and discs large membrane-associated guanylate kinase scaffold protein 1,[31-34] and regulates protein trafficking to the correct membrane domains in epithelial cells and neurons.[35-38] A complex comprising LIN7, CASK, and Mint1 serves as a kinesin-cargo linker that assists trafficking of transport vesicles containing *N*-methyl-D-aspartate receptor via kinesin family member 17.[39, 40] LIN7 contains protein-protein interaction domains, including a PDZ domain and an L27 domain.[41] The PDZ domain of LIN7 is necessary for the lateral localization of epidermal growth factor receptor, the Kir 2.3 channel, and IRSp53.[33, 38, 42] The L27 domain mediates a protein interaction between LIN7 and CASK.[38] CASK is necessary for the lateral localization of LIN7C.[34] CASK at the lateral membrane interacts with syndecan and protein 4.1 bound to the actin cytoskeleton.[43] Junctional proteins such as zonula occludens-1 and affadin directly bind to F-actin and other components of the cytoskeleton in polarized cells.[44, 45] The results of our APEX2 biotinylation experiments suggest that cell-cell junctional proteins and actin-related proteins are responsible for the localization of BLT2 to the lateral membrane. Given that the 12-HHT/BLT2 axis enhances actin polymerization,[46] interactions among BLT2, junctional proteins, and actin-related proteins might stabilize BLT2 at the lateral membrane. We hypothesize that the interaction between LIN7C and the C-terminal region of BLT2 is one mechanism underlying localization and stabilization of BLT2 at the lateral membrane.

On the other hand, the middle and C-terminal domains of BLT2 were required to increase TER and upregulate claudin-4 mRNA expression. The intracellular loop (ICL) 2 and ICL3 domains of GPCRs interact

with G proteins,[47-49] and Gαi, which mediates 12-HHT/BLT2 signaling for barrier enhancement, localizes to the lateral membrane.[50] These studies support the idea that the middle domain of BLT2 is necessary for the pathway downstream of BLT2. However, the importance of the lateral localization of BLT2 for barrier function is unclear. Further experiments are necessary to clarify the physiological roles of BLT2 at the lateral membrane.

In conclusion, the C-terminal region of BLT2 is important for its transport from the Golgi apparatus to the plasma membrane and its localization to the lateral membrane. LIN7C is important for localization of BLT2 to the plasma membrane and BLT2-dependent barrier formation in epithelial cells.

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Conflict of Interest

The authors have no conflicts of interest directly relevant to the content of this article.

Author contributions

T. Hara, K. Saeki, Y. Miura, and T. Yokomizo designed the experiments. T. Hara, H. Jinnouchi, and S. Kazuno performed the experiments. T. Hara, K. Saeki, H. Jinnouchi, S. Kazuno, and Y. Miura analyzed the data. T. Hara, K. Saeki, and T. Yokomizo wrote the manuscript.

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Figure legends

Fig. 1 The C-terminal region of BLT2 restricts its localization to the lateral membrane

(A) Sequence alignment of hBLT1 and hBLT2. Blue and red numbers indicate the number of amino acid residues of hBLT1 and hBLT2, respectively. The transmembrane regions are marked by yellow. (B) Overview of HA-tagged chimeric BLT constructs. (C) Cell surface expression of chimeric BLTs on MDCK II cells was examined by flow cytometry. (D) Chimeric BLT-expressing MDCK II cells cultured on membrane inserts were stained with an anti-HA antibody to determine the localizations of HA-tagged chimeric BLTs. TM: transmembrane domain.

Fig. 2 The middle and C-terminal regions of BLT2 enhance barrier function

(A) TER of chimeric BLT-expressing MDCK II cells was measured. (B) Claudin-4 mRNA expression was assessed in chimeric BLT-expressing cells. Data represent the mean ± SD (n=4 for A; n=3 for B). Statistical significance was assessed by a two-way ANOVA (A) and one-way ANOVA (B) followed by Dunnett's multiple comparisons test. *p<0.05, **p<0.01, and ***p<0.001.

Fig. 3 The C-terminal cytosolic region of BLT2 restricts its localization to the lateral membrane

(A) Overview of HA-tagged chimeric BLT constructs. (B) Cell surface expression of chimeric BLTs on MDCK II cells was examined by flow cytometry. (C) Chimeric BLT-expressing MDCK II cells cultured on membrane inserts were stained with an anti-HA antibody to determine the localizations of HA-tagged chimeric BLTs.

Fig. 4 The C-terminal region of BLT2 is necessary for its export from the Golgi apparatus to the plasma membrane

(A) Overview of HA-tagged BLT2 deletion mutants. (B) Cell surface expression of BLT2 deletion mutants on MDCK II cells was examined by flow cytometry. (C) BLT2 (334del)-expressing MDCK II cells cultured on membrane inserts were stained with an anti-HA antibody to determine the localization of HA-BLT2 (334del). (D) Wild-type BLT2- or BLT2 (310del)-expressing MDCK II cells cultured on a dish were stained with an anti-HA antibody to detect HA-BLT2 or HA-BLT2 (310del) and with an anti-RCAS1 (Golgi marker) antibody.

Fig. 5 Candidates proteins that bind to the C-terminal region of BLT2

(A) FLAG-BLT1-APEX2-, FLAG-BLT (1-1-1-1-2)-APEX2-, and FLAG-BLT2-APEX2-expressing MDCK II cells cultured on membrane inserts were stained with an anti-FLAG antibody to detect FLAG-BLT-APEX2. (B) Schematic presentation of APEX2-mediated protein labeling. (C) Biotinylated proteins detected in the BLT2-APEX2 sample with high ratios of MS scores (FLAG-BLT (1-1-1-1-2)-APEX2/FLAG-BLT1-APEX2 > 1.5) are listed.

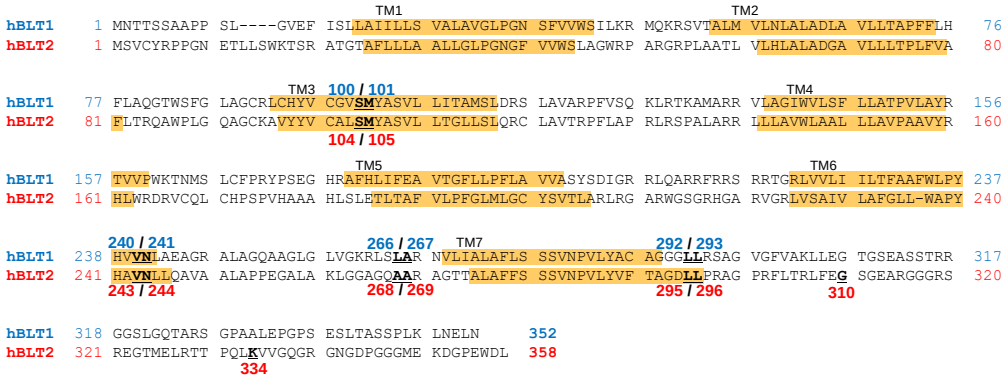
Fig. 6 LIN7C knockdown impairs BLT2 expression at the plasma membrane

(A) LIN7C mRNA levels in MDCK II cells were measured after treatment with LIN7C-targeting siRNA. (B) Wild-type BLT2- or BLT1-expressing MDCK II cells cultured on a dish were stained with an anti-HA antibody to detect HA-BLT2 or HA-BLT1 and with an anti-RCAS1 (Golgi marker) antibody. (C) TER of MDCK II cells was measured after treatment with LIN7C-targeting siRNA. (D) Claudin-4 mRNA levels in MDCK II cells were measured after treatment with LIN7C-targeting siRNA. Data represent the mean ± SD (n=4-5 for A; n=5 for C; n=5 for D). Statistical significance was determined by the paired t-test (A) and a one-way ANOVA followed by Tukey's multiple comparisons test (C, D). *p<0.05, **p<0.01, and ***p<0.001.

Fig. 7 A possible mechanism by which BLT2 is transported from the Golgi apparatus to the plasma membrane and stabilized at the lateral membrane in a LIN7C-dependent manner

Figure 1

A




```
hBLT1    1 MNTTSSAAPP SL----GVEF ISLLAIILLS VALAVGLPGN SFVVWSILKR MQKRSVTALM VLNLALADLA VLLTAPFFLH   76
hBLT2    1 MSVCYRPPGN ETLLSWKTSR ATGTAFLLLA ALLGLPGNGF VVWSLAGWRP ARGRPLAATL VLHLALADGA VLLLTPLFVA   80

hBLT1   77 FLAQGTWSFG LAGCRLCHYV CGVSMYASVL LITAMSLDRS LAVARPFVSQ KLRTKAMARR VLAGIWVLSF LLATPVLAYR  156
hBLT2   81 FLTRQAWPLG QAGCKAVYYV CALSMYASVL LTGLLSLQRC LAVTRPFLAP RLRSPALARR LLLAVWLAAL LLAVPAAVYR  160

hBLT1  157 TVVPWKTNMS LCFPRYPSEG HRAFHLIFEA VTGFLLPFLA VVASYSDIGR RLQARRFRRS RRTGRLVVLI ILTFAAFWLPY  237
hBLT2  161 HLWRDRVCQL CHPSPVHAAA HLSLETLTAF VLPFGLMLGC YSVTLARLRG ARWGSGRHGA RVGRLVSAIV LAFGLL-WAPY  240

hBLT1  238 HVVNLAEAGR ALAGQAAGLG LVGKRLSLAR NVLIALAFLS SSVNPVLYAC AGGGLLRSAG VGFVAKLLEG TGSEASSTRR  317
hBLT2  241 HAVNLLQAVA ALAPPEGALA KLGGAGQAAR AGTTALAFFS SSVNPVLYVF TAGDLLPRAG PRFLTRLFEG SGEARGGGRS  320

hBLT1  318 GGSLGQTARS GPAALEPGPS ESLTASSPLK LNELN      352
hBLT2  321 REGTMELRTT PQLKVVGQGR GNGDPGGGME KDGPEWDL   358
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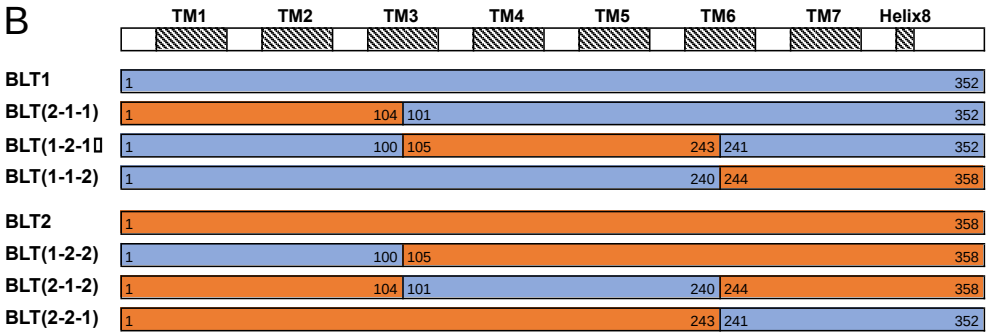
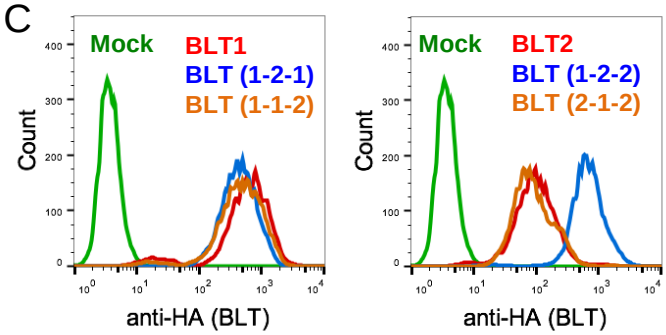
B



C



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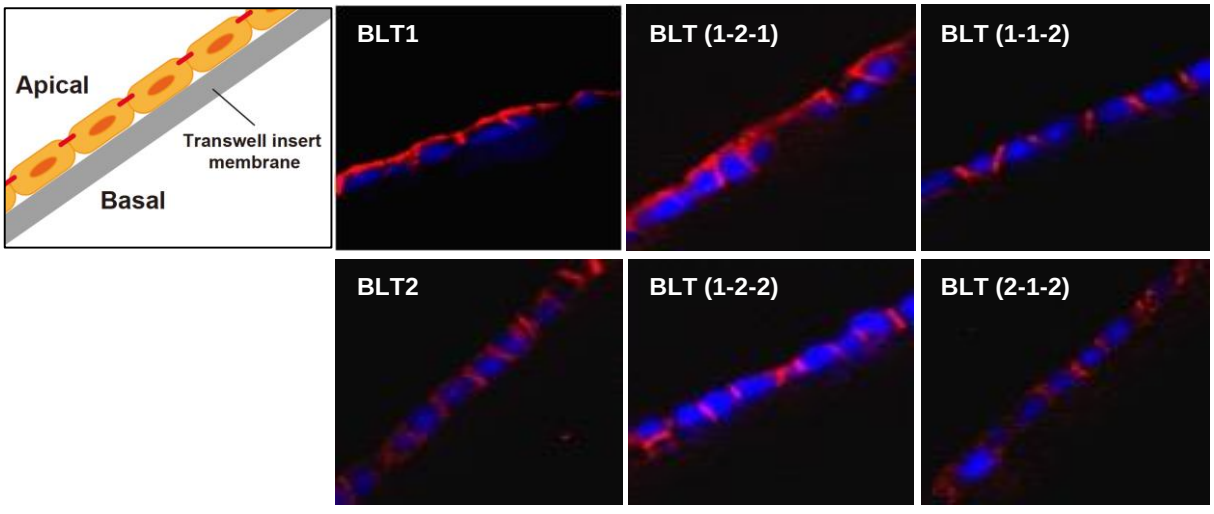


Figure 2

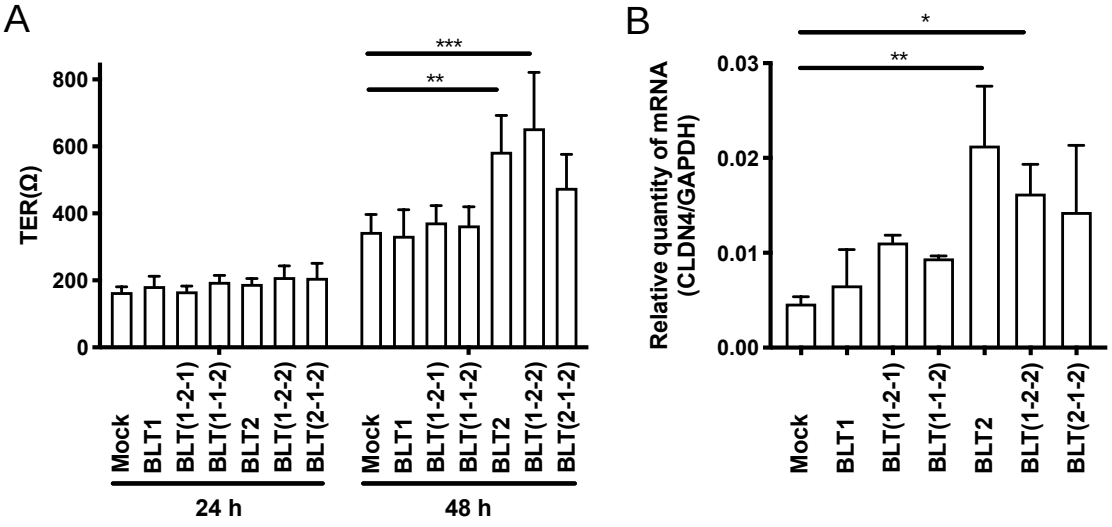
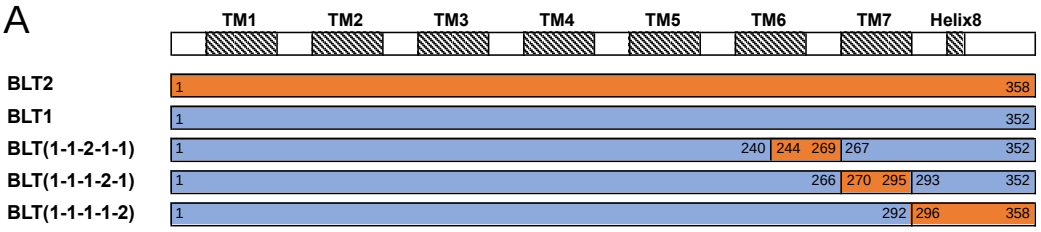
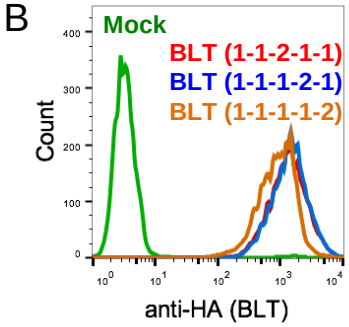

A
B
TER(Ω)
800
600
400
200
0
***
**
Mock
BLT1
BLT(1-2-1)
BLT(1-1-2)
BLT2
BLT(1-2-2)
BLT(2-1-2)
24 h
48 h
Relative quantity of mRNA
(CLDN4/GAPDH)
0.03
0.02
0.01
0.00
*
**

Figure 3


A
TM1
TM2
TM3
TM4
TM5
TM6
TM7
Helix8
BLT2
1
358
BLT1
1
352
BLT(1-1-2-1-1)
1
240
244
269
267
352
BLT(1-1-1-2-1)
1
266
270
295
293
352
BLT(1-1-1-1-2)
1
292
296
358


B
Mock
BLT (1-1-2-1-1)
BLT (1-1-1-2-1)
BLT (1-1-1-1-2)
Count
400
300
200
100
0
10^0
10^1
10^2
10^3
10^4
anti-HA (BLT)

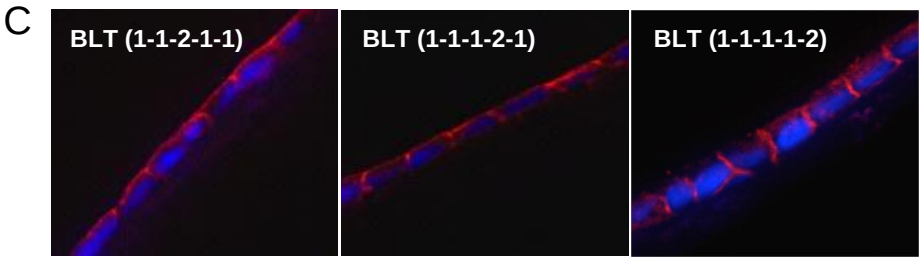
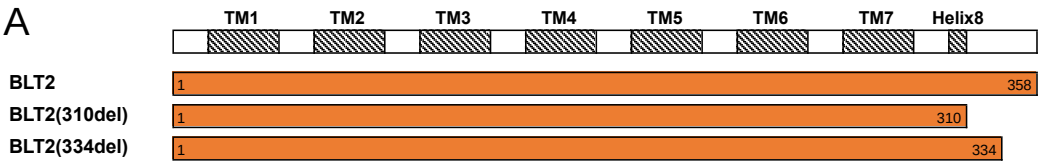
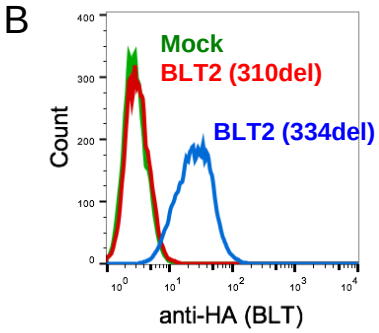
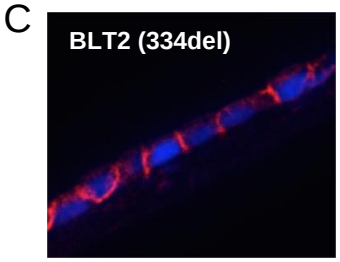

C
BLT (1-1-2-1-1)
BLT (1-1-1-2-1)
BLT (1-1-1-1-2)

Figure 4


A
TM1
TM2
TM3
TM4
TM5
TM6
TM7
Helix8
BLT2
1
358
BLT2(310del)
1
310
BLT2(334del)
1
334


B
Mock
BLT2 (310del)
BLT2 (334del)
Count
anti-HA (BLT)


C
BLT2 (334del)

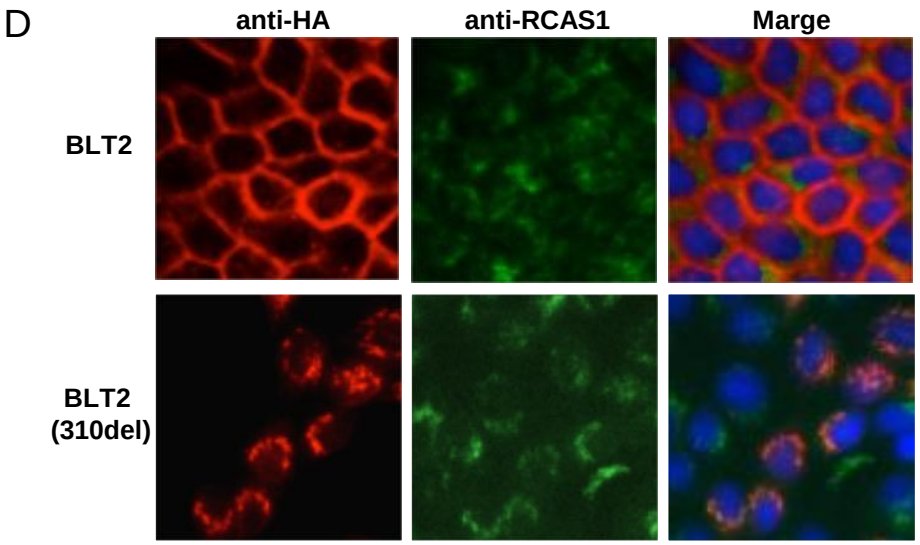
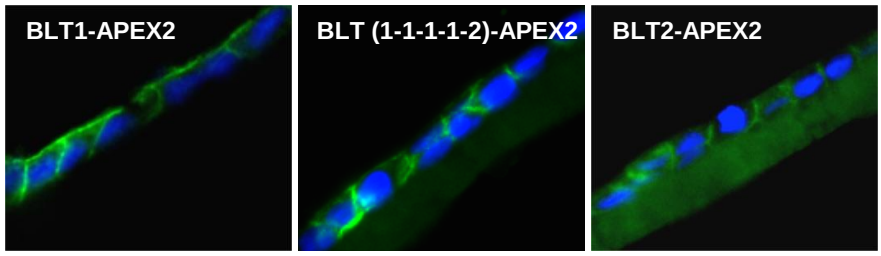
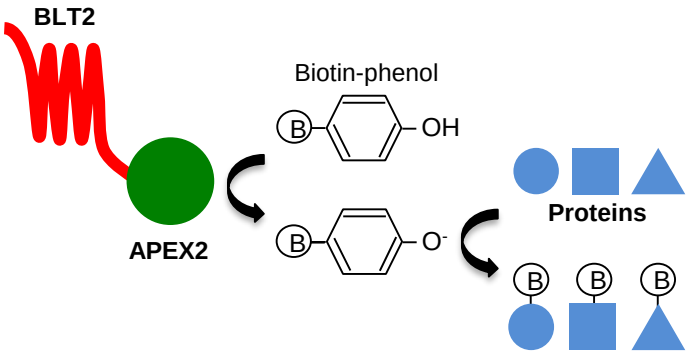

D
anti-HA
anti-RCAS1
Marge
BLT2
BLT2
(310del)

Figure 5

A



B



C

Intracellular protein transport	Cell-cell junction	Actin filament binding
AP-2 complex subunit alpha-1	afadin	actin-related protein 2/3 complex subunit 4
AP-2 complex subunit beta-1	desmocollin-2	actin-related protein 2/3 complex subunit 2
clathrin heavy chain 1	desmoglein-2	annexin A8
dynamin-2	desmoplakin	ezrin
flotillin-2	junctional adhesion molecule A	plastin-3
protein lin-7 homolog C	periplakin	talin-1
sorting nexin-8	tight junction protein ZO-1	tensin-3
sorting nexin-27	tight junction protein ZO-2	utrophin
src substrate cortactin	tight junction protein ZO-3	vinculin
	vasodilator-stimulated phosphoprotein	

Figure 6

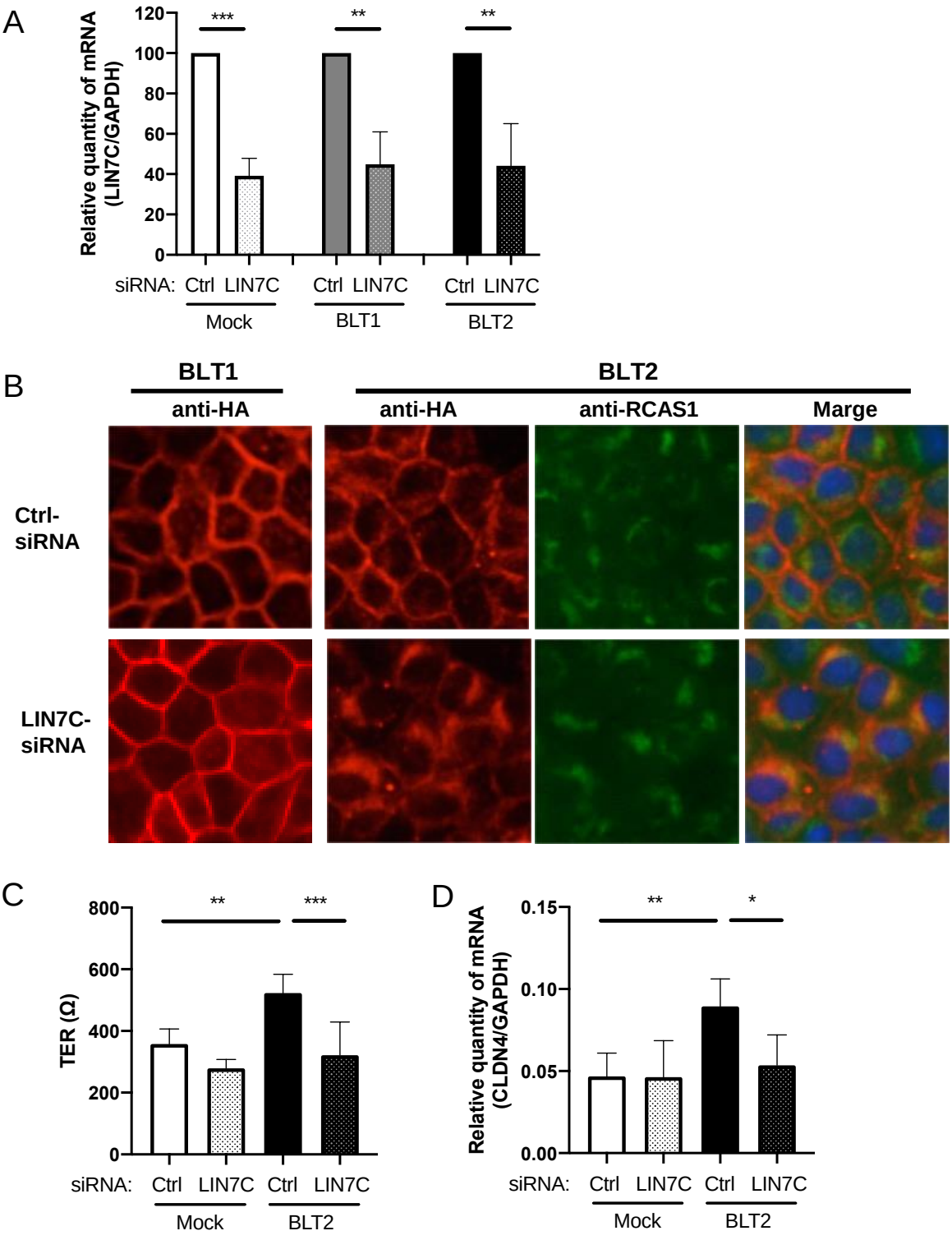
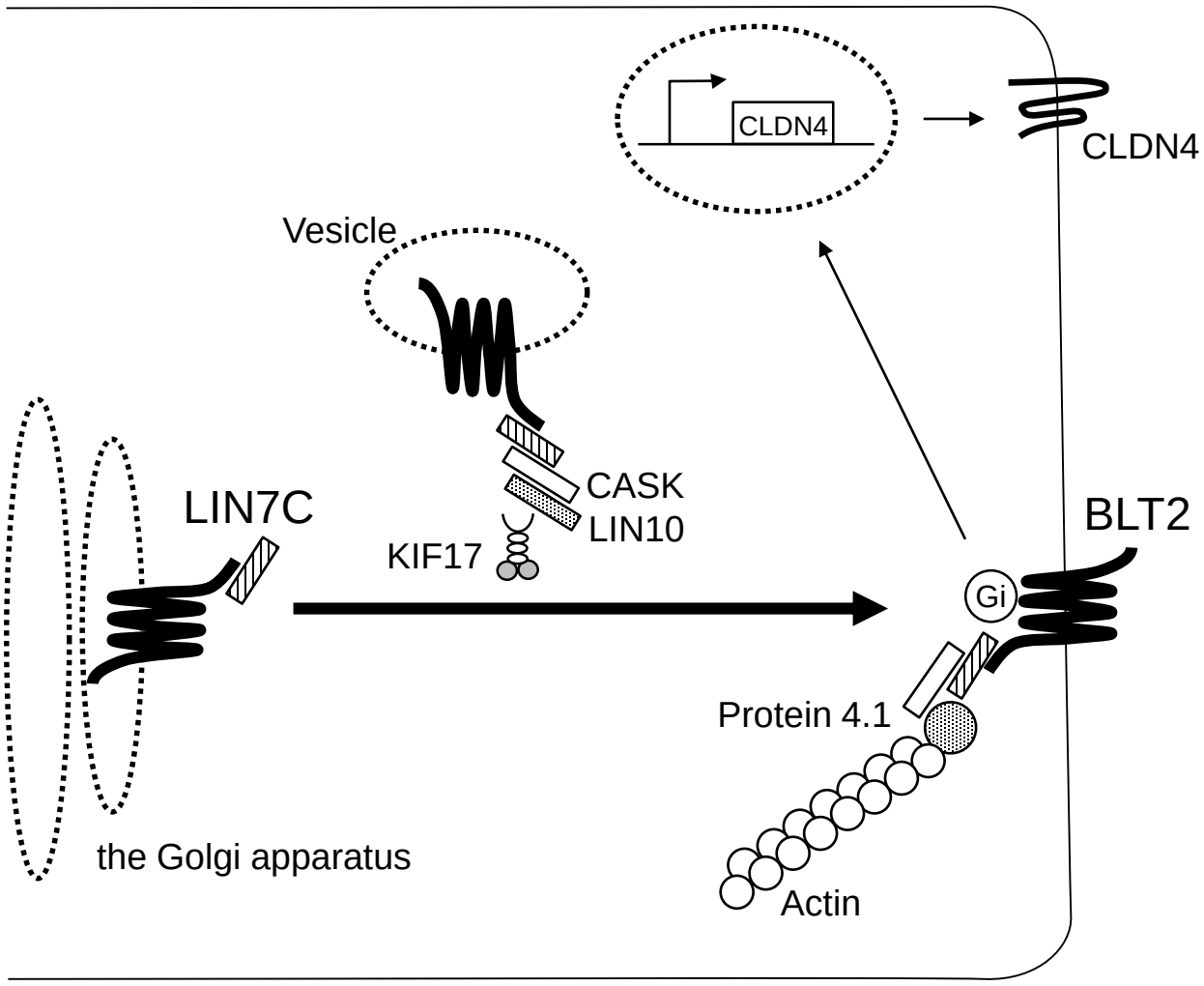

A
Relative quantity of mRNA (LIN7C/GAPDH)
120
100
80
60
40
20
0
***
**
**
siRNA: Ctrl LIN7C Ctrl LIN7C Ctrl LIN7C
Mock
BLT1
BLT2
B
BLT1
BLT2
anti-HA
anti-HA
anti-RCAS1
Marge
Ctrl-siRNA
LIN7C-siRNA
C
TER (Ω)
800
600
400
200
0
**
***
siRNA: Ctrl LIN7C Ctrl LIN7C
Mock
BLT2
D
Relative quantity of mRNA (CLDN4/GAPDH)
0.15
0.10
0.05
0.00
**
*
siRNA: Ctrl LIN7C Ctrl LIN7C
Mock
BLT2

Figure 7


CLDN4
CLDN4
Vesicle
CASK
LIN10
KIF17
LIN7C
BLT2
Gi
Protein 4.1
Actin
the Golgi apparatus