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Basolateral sorting of the Mg^{2+} transporter CNNM4 requires interaction with AP-1A and AP-1B

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ABSTRACT

Ancient conserved domain protein/cyclin M (CNNM) 4 is an evolutionarily conserved Mg^{2+} transporter that localizes at the basolateral membrane of the intestinal epithelia. Here, we show the complementary importance of clathrin adaptor protein (AP) complexes AP-1A and AP-1B in basolateral sorting of CNNM4. We first confirmed the basolateral localization of both endogenous and ectopically expressed CNNM4 in Madin–Darby Canine Kidney cells, which form highly polarized epithelia in culture. Single knockdown of μ1B, a cargo-recognition subunit of AP-1B, did not affect basolateral localization, but simultaneous knockdown of the μ1A subunit of AP-1A abrogated localization. Mutational analyses showed the importance of three conserved dileucine motifs in CNNM4 for both basolateral sorting and interaction with μ1A and μ1B. These results imply that CNNM4 is sorted to the basolateral membrane by the complementary function of AP-1A and AP-1B.

1. Introduction

Proteins in the ancient conserved domain protein/cyclin M (CNNM) family are evolutionarily conserved integral membrane proteins [1]. Multiple genetic and biochemical studies have suggested that CNNMs function in Mg^{2+} transport [2,3] and magnesium homeostasis in humans [4,5]. In addition, our recent study revealed the crucial role of CNNM4 in basolateral extrusion of Mg^{2+}; CNNM4 localizes at the basolateral membrane of the intestinal epithelia, where it extrudes intracellular Mg^{2+} in exchange for Na^+, thereby mediating transcellular Mg^{2+} transport [6]. Indeed, *CNNM4*-knockout mice showed mild hypomagnesemia due to the malabsorption of magnesium in the intestine [6], indicating the physiological importance of the basolateral localization of CNNM4. However, the molecular mechanism underlying the basolateral localization is completely unknown.

Localization of many basolateral proteins is mediated by clathrin-dependent selective sorting of proteins to the basolateral surface [7]. Previous studies have revealed that the clathrin adaptor protein (AP) complex-1B (AP-1B, a subtype of AP-1, described below) plays a major role in the recruitment and subsequent basolateral sorting of cargo proteins [8]. AP-1 consists of four subunits, γ, β1, σ1, and μ1, the last of which is involved in cargo recognition via direct binding to sorting signal motifs present in cargo proteins [9]. There are two subtypes of AP-1, AP-1A and AP-1B, which contain either μ1A or μ1B (isoforms of the μ1 subunit), respectively, but share the other subunits [10].

The involvement of AP-1B in basolateral sorting was initially revealed by comparative analyses using LLC-PK1 kidney cells and Madin–Darby Canine Kidney (MDCK) cells [11]. Transferrin receptor and low-density lipoprotein receptor (LDLR) localized basolaterally in MDCK cells, which possess endogenous μ1B, but localized apically in LLC-PK1 cells, which lack endogenous μ1B. In addition, ectopic expression of μ1B in LLC-PK1 cells forced both proteins to localize at the basolateral membrane as in MDCK cells, demonstrating the critical role of AP-1B in basolateral sorting [11]. However, it has also been suggested that basolateral sorting requires adaptors in addition to AP-1B; several native epithelia, such as liver [12], retinal pigment epithelium [13], and kidney proximal tubules [14], constitutively lack μ1B, and furthermore, *μ1B*-knockout mice are viable [15]. Recent reports have shown that the basolateral localization of Na,K-ATPase in MDCK cells is not affected by single knockdown of either μ1B or μ1A, but is substantially disrupted by double knockdown of both μ1B and μ1A [16,17], suggesting that AP-1A and AP-1B play complementary

Abbreviations: CNNM, cyclin M; AP, adaptor protein; MDCK, Madin–Darby Canine Kidney; LDLR, low-density lipoprotein receptor; ZO-1, zonula occludens-1; WT, wild-type.

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roles in basolateral sorting of proteins. Therefore, in this study, we examined the contributions of AP-1A and AP-1B to the basolateral localization of CNNM4.

2. Materials and methods

2.1. Constructs

cDNA for human CNNM4 was generated in a previous study [6]. Amino acid-substitution mutants of human CNNM4 (Y351A, Y405A, Y488A, Y581A, Y708A, L323/324A, L550/551/552A, L575/576A, L578/579A, L758/759A, L765/766A, and L575/576/758/759/765/766A) were generated using the QuickChange site-directed mutagenesis kit (Agilent). These cDNA fragments were inserted into pCMV-Tag 4A vector (Agilent). cDNAs for mouse μ1A and human μ1B were kindly provided by Dr. Heike Fölsch [18], and inserted into pEF-BOS.

2.2. Antibodies

Anti-CNNM4 rabbit polyclonal antibody was generated in a previous study [6]. Anti-μ1B rabbit polyclonal antibody was kindly provided by Dr. Heike Fölsch [11]. Anti-zonula occludens-1 (ZO-1) mouse monoclonal antibody was kindly provided by Drs. Masahiko Itoh and Mikio Furuse [19]. We also used the following commercially available antibodies: anti-Na,K-ATPase mouse monoclonal antibody (Upstate/Millipore), anti-μ1A rabbit polyclonal antibody (Proteintech), anti-FLAG mouse monoclonal and rabbit polyclonal antibodies (Sigma–Aldrich), and anti-HA mouse monoclonal antibody (Covance).

2.3. Cell culture and transfection

COS7, MDCK, and Bosc23 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Expression plasmids were transfected using Lipofectamine 2000 (Invitrogen), unless otherwise noted.

2.4. RNAi knockdown

For transient knockdown of μ1A, a duplex siRNA against canine *μ1A* (5′-GTGCTCATCTGCCGGAATT-3′), purchased from Invitrogen, was transfected into MDCK cells by electroporation using a Nucleofector kit T (Lonza), as follows. Trypsinized cells were suspended at 4×10^6 cells per 100 μl of Nucleofector Solution T, supplemented with 375 pmol of siRNA, and electroporated (Program "T-23"). After 4 days, cells were transfected again with the same siRNA, and used for experiments after the additional culture for 3 days. A non-targeting siRNA duplex sequence (Stealth RNAi, Invitrogen) was used as a negative control. Stable knockdown of μ1B in MDCK cells was achieved according to a previously described method using pSUPER-retro vector (OligoEngine) [20]. Oligonucleotides including the following target sequence for canine *μ1B*, 5′-CAAGCTGGTGACTGGCAAA-3′, were used. For control experiments, oligonucleotides including the following irrelevant target sequence, 5′-GGAAAGCTAGGAAGAGAGA-3′, were used.

2.5. Cell-surface biotinylation

MDCK cells were seeded on 12-mm polycarbonate transwell culture filters (pore size 0.4 μm, Costar) at a density of 1×10^5 cells per filter and cultured for 5 days; the medium was refreshed daily. Cell-surface labeling with sulfo-NHS-biotin (Pierce) was performed as previously described [21], except that the reactive biotin remaining after labeling was quenched by incubating with 50 mM NH_4Cl in PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 10 min. After washing, the cells were lysed in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, and 1 mM PMSF. The cell lysates were mixed with avidin-agarose beads (Sigma–Aldrich), and the bound proteins were analyzed by immunoblotting.

2.6. Immunofluorescence microscopy

MDCK (3×10^6) cells were seeded on coverslips in a 3.5-cm dish. In experiments involving RNAi knockdown, the same number of cells were seeded, following the second siRNA transfection by electroporation. 2 days after seeding, cells were transfected twice with each CNNM4-FLAG construct. At 24 h after the first transfection, cells were washed with PBS, and fixed with 1% formaldehyde for 15 min at room temperature. For staining endogenous ZO-1, cells were permeabilized with 0.5% TritonX-100 in PBS for 10 min at room temperature. For staining endogenous Na,K-ATPase, cells were washed with 50 mM NH_4Cl in PBS, incubated with 5% FBS in PBS for 15 min, and permeabilized with 0.1% TritonX-100 for 5 min at room temperature. After blocking with PBS containing 1% BSA for 1 h, cells were incubated overnight with the primary antibody at 4 °C, followed by incubation for 30 min with the appropriate secondary antibodies at room temperature. After washing, coverslips were mounted and observed with a confocal scanning laser microscope (FLUOVIEW FV1000; Olympus). Horizontal section images were captured at intervals of 0.45 μm in the z-direction. Using the software FV10-ASW (Olympus), vertical section images (X-Z) were reconstructed by stacking the horizontal section images. Quantitative analyses of the basolateral localization of CNNM4 were performed as follows. X-Z image of each cell was used for analyses using ImageJ (NIH software). The plasma membrane either above or beneath the tight junction marker ZO-1 (defined as apical or basolateral surface, respectively) was traced with a line of 0.5-μm width. The sum total of the fluorescence signal for CNNM4 on each line was divided by its line length to obtain the signal intensity per length on either apical or basolateral surface (F_{AP} and F_{BL}, respectively). The ratio (F_{AP}/F_{BL}) values will be 0 if CNNM4 exclusively localizes at the basolateral membrane, while it will be 1 if CNNM4 is evenly distributed on both surfaces.

2.7. Co-immunoprecipitation

To reduce the level of background noise, anti-FLAG rabbit polyclonal antibody was cross-linked to Protein-A Sepharose beads (Pierce) before use in immunoprecipitation as previously described [22], with minor modifications. Briefly, the beads and anti-FLAG antibody were incubated overnight, and then mixed with 200 mM sodium borate containing 20 mM dimethyl pimelimidate (Sigma–Aldrich) and incubated for 45 min, followed by incubation with 200 mM glycine (pH 8.0) for 2 h at room temperature. COS7 cells transfected with various expression constructs were lysed with lysis buffer [23], and centrifuged at 16,000×*g* for 15 min. The supernatants were mixed with the FLAG antibody-bound beads for immunoprecipitation.

3. Results and discussion

3.1. Basolateral localization of CNNM4 in MDCK cells

MDCK cells have been widely used as a model for studying epithelial polarity, because they acquire clear apico-basal polarity in monolayer culture [24,25]. Therefore, we used MDCK cells to examine the mechanism of basolateral localization of CNNM4. To

investigate the localization of endogenous CNNM4 proteins, polarized MDCK cells cultured on the transwell filters were examined by cell-surface biotinylation and immunoblotting analyses. CNNM4 was specifically detected in the avidin-precipitated fractions of basolaterally biotinylated samples, but not in those of apically biotinylated samples (Fig. 1A). These results are very similar to those reported for Na,K-ATPase, a basolateral marker protein [26], showing that endogenous CNNM4 is selectively expressed on the basolateral surface of MDCK cells. To further confirm the basolateral localization of CNMM4 in MDCK cells, we next performed immunostaining for CNNM4. However, because immunofluorescence staining with our anti-CNNM4 antibody did not generate clear results (data not shown), polarized MDCK cells were transfected with CNNM4-FLAG and were studied using confocal immunofluorescence microscopy using anti-FLAG antibody. As shown in Fig. 1B, the CNNM4 signal was not observed in the horizontal section positive for the tight junction marker ZO-1 [27], but was clearly observed on the plasma membrane below this section. Furthermore, vertical section images, reconstructed by stacking the horizontal images, showed that CNNM4 was present throughout the membrane immediately beneath ZO-1, but not on the membrane above ZO-1, similar to the basolateral marker Na,K-ATPase. Thus, we confirmed that both endogenous and ectopically expressed CNNM4 localize basolaterally in MDCK cells.

3.2. Both AP-1A and AP-1B contribute to the basolateral localization of CNNM4

It has been shown that the AP-1B plays a key role in the recognition and intracellular transport of many membrane proteins destined for the basolateral surface of epithelial cells [8]. To determine the importance of AP-1B for the basolateral localization of CNNM4,

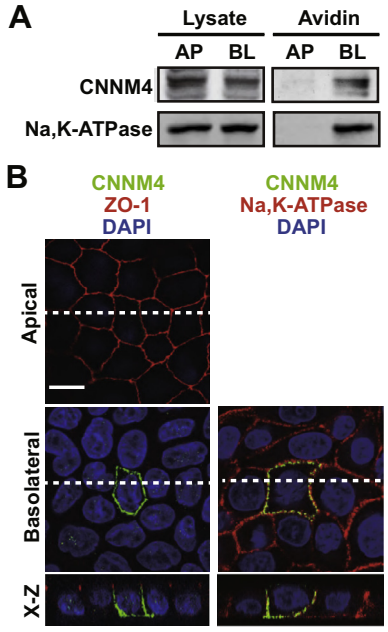

Fig. 1. Basolateral localization of CNNM4 in MDCK cells. (A) Polarized filter-grown MDCK cells were biotinylated from either the apical (AP) or the basolateral (BL) side. Biotinylated proteins were precipitated from cell lysates with avidin-agarose, and were subjected to immunoblotting with antibodies against CNNM4 or Na,K-ATPase. (B) MDCK cells transfected with CNNM4-FLAG were subjected to immunofluorescence staining with antibodies against FLAG (green) and ZO-1 (red) or Na,K-ATPase (red). Additionally, nuclei were stained with DAPI (blue). Horizontal section images, at the level of the apical and basolateral membranes, and vertically reconstituted images (X-Z) are shown. Vertical sections were taken at the white dotted lines indicated in the horizontal section images. Bar, 10 μm.

we performed knockdown experiments for μ1B, which is an AP-1B subunit involved in cargo recognition. We generated stable *μ1B*-knockdown MDCK cell lines by retrovirally introducing a vector designed to express an shRNA that had been confirmed to provide an effective knockdown in a previous study [28] (Fig. 2A). However, immunofluorescence analyses showed that ectopically expressed CNNM4 primarily localized basolaterally, as in control cells (Fig. 2B). To evaluate the localization of CNNM4 quantitatively, we compared signal intensity of CNNM4 expressed on the apical surface with that on the basolateral surface (for details, see Section 2). The mean ratio (F_{AP}/F_{BL}) values were 0.17 and 0.20 in the control and the *μ1B*-knockdown cells, respectively, indicating the major expression of CNNM4 on the basolateral surface in these cells (Fig. 2C). These results suggest the involvement of additional adaptor proteins in the basolateral localization of CNNM4. It was recently reported that the basolateral localization of Na,K-ATPase is mediated by both AP-1A and AP-1B in a complementary manner; its localization was not affected by single knockdown of either μ1A (a subunit of AP-1A) or μ1B, but was substantially disrupted by simultaneous knockdown of μ1A and μ1B [16,17]. Therefore, we performed knockdown of μ1A, using a previously published siRNA sequence [17]. As shown in Fig. 2A, *μ1A*-siRNA treatment significantly decreased the expression of μ1A in both the control and *μ1B*-knockdown cells, compared to cells treated with the respective control siRNAs. These cells were then transfected with CNNM4-FLAG and assessed using immunofluorescence analyses. As shown in Fig. 2B and C, CNNM4 was expressed at both the apical and basolateral surfaces in the majority of double knockdown cells, and the mean ratio (F_{AP}/F_{BL}) value was significantly increased to 0.79. These results indicate that the basolateral localization of CNNM4 was strongly disrupted by simultaneous knockdown of both μ1A and μ1B, but not by single knockdown of either μ1A or μ1B, suggesting that AP-1A and AP-1B contribute to the basolateral localization of CNNM4 in a complementary manner. AP-1A and AP-1B mediate basolateral sorting of proteins through their interactions with cargo proteins, which are largely dependent on the μ1A and μ1B subunits, respectively [16,17,29]. To examine the interactions of CNNM4 with μ1A and μ1B, we performed immunoprecipitation analyses using lysates of COS7 cells co-expressing FLAG-tagged CNNM4 and HA-tagged μ1A or μ1B. Significant co-immunoprecipitation signals for μ1A and μ1B were observed in anti-FLAG immunoprecipitates, showing that CNNM4 interacts with both μ1A and μ1B (Fig. 2D). Collectively, these results suggest that both AP-1A and AP-1B recognize CNNM4 as a cargo, and play complementary roles in the basolateral sorting of CNNM4.

3.3. Three dileucine motifs contribute to the basolateral localization of CNNM4

Both AP-1A and AP-1B interact with cargo proteins by recognizing basolateral sorting signals, such as tyrosine-based YXXΦ (X, any amino acid; Φ, bulky hydrophobic amino acid) and dileucine motifs, located in the cytosolic domains of cargo proteins [10]. Therefore, we searched for these motifs in the C-terminal cytosolic regions of CNNM4, and found several corresponding motifs, which are conserved in mammalian CNNM4 (human, cow, rat, and mouse) (Fig. 3A). To investigate the importance of each motif in the basolateral localization of CNNM4, we constructed amino acid-substituted mutants of CNNM4 by introducing tyrosine-to-alanine substitutions into each YXXΦ motif or leucine-to-alanine substitutions into each dileucine motif. MDCK cells were transfected with wild-type (WT) and mutants of CNNM4-FLAG and analyzed by immunofluorescence analyses. As shown in Fig. 3B and C, basolateral localization of CNNM4 was not affected by the Y351A, Y405A, Y488A, Y581A, L323/324A, or L550/551/552A mutations. In

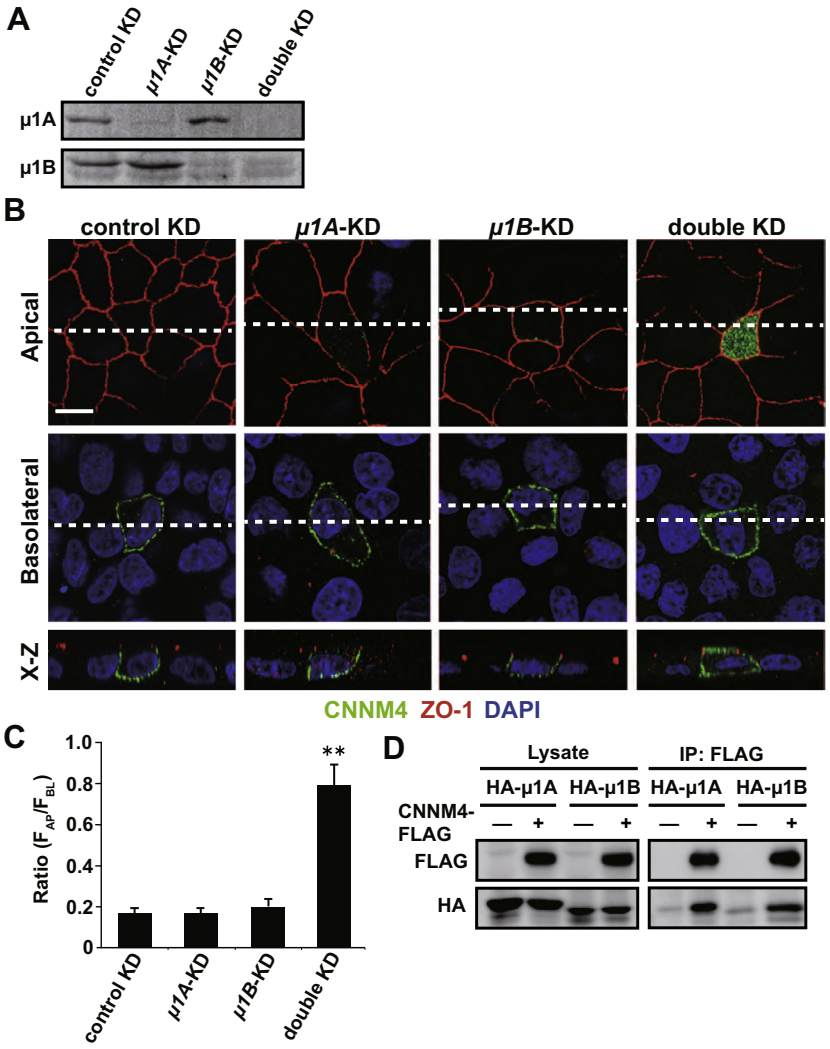

Fig. 2. Complementary roles of AP-1A and AP-1B in the basolateral localization of CNNM4. (A) MDCK cells stably expressing control shRNA transfected with control siRNA (control KD cells) or *μ1A*-siRNA (*μ1A*-KD cells), and MDCK cells stably expressing *μ1B*-shRNA transfected with control siRNA (*μ1B*-KD cells) or *μ1A*-siRNA (double KD cells) were subjected to immunoblotting with antibodies against μ1A or μ1B. (B) The above MDCK cells were transfected with CNNM4-FLAG, and subjected to immunofluorescence staining, as explained in the legend to Fig. 1B. Bar, 10 μm. (C) Quantitative analyses of the basolateral localization of CNNM4 in MDCK cells immunostained in (B). F_{AP}/F_{BL} values of 20 cells were determined for each cell group, and the data are shown as mean ± SEM. $^{**}P < 0.01$ (vs control KD; Kruskall–Wallis test followed by Steel–Dwass test). (D) Lysates of COS7 cells transfected with the indicated constructs were subjected to immunoprecipitation with anti-FLAG antibody. The precipitated proteins were analyzed by immunoblotting with the indicated antibodies.

contrast, L575/576A, L758/759A, and L765/766A mutants were expressed on both apical and basolateral surfaces in approximately half of the observed cells, and the mean ratio (F_{AP}/F_{BL}) values of these cells were 0.49, 0.46 and 0.46, respectively, which are moderately higher than that of WT CNNM4-expressing cells (Fig. 3B and C). In the cases of Y708A and L578/579A mutants, signals were observed throughout the cytoplasm, and we were unable to evaluate the effects of these mutations on the basolateral localization of CNNM4. Collectively, these results show that basolateral localization of CNNM4 is partially disrupted by mutations in any of the three dileucine motifs (L575/576A, L758/759A, and L765/766A), suggesting that these motifs contribute to the basolateral localization of CNNM4 mediated by AP-1A and AP-1B.

3.4. Simultaneous mutations in three dileucine motifs disrupted basolateral localization and interaction with μ1A and μ1B

The localization of several basolaterally localized proteins, such as LDLR [30], is mediated by the cooperative action of multiple basolateral sorting motifs. Therefore, we constructed a triple dileucine mutant by introducing leucine-to-alanine substitutions into all three dileucine motifs (L575/576/758/759/765/766A), in which individual mutations partially disrupted the basolateral localization of CNNM4 (Fig. 3). Subsequent immunofluorescence analyses revealed that the triple dileucine mutant was expressed on both apical and basolateral surfaces in almost all of the observed cells; the mean ratio (F_{AP}/F_{BL}) value was drastically increased to 0.89, while that of WT was only 0.14 (Fig. 4A and B). To investigate the effect of these mutations on the interaction of CNNM4 with μ1A and μ1B, we performed immunoprecipitation analyses using lysates of COS7 cells co-expressing FLAG-tagged CNNM4 (WT or the triple dileucine mutant) and HA-tagged μ1A or μ1B. As shown in Fig. 4C, significant signals for co-immunoprecipitation of μ1A and μ1B were observed in the anti-FLAG immunoprecipitates from WT CNNM4-expressing cells, but not in those from triple dileucine mutant CNNM4-expressing cells, indicating that simultaneous mutations of the three dileucine motifs abrogated the interaction of CNNM4 with μ1A and μ1B. These results suggest that the three

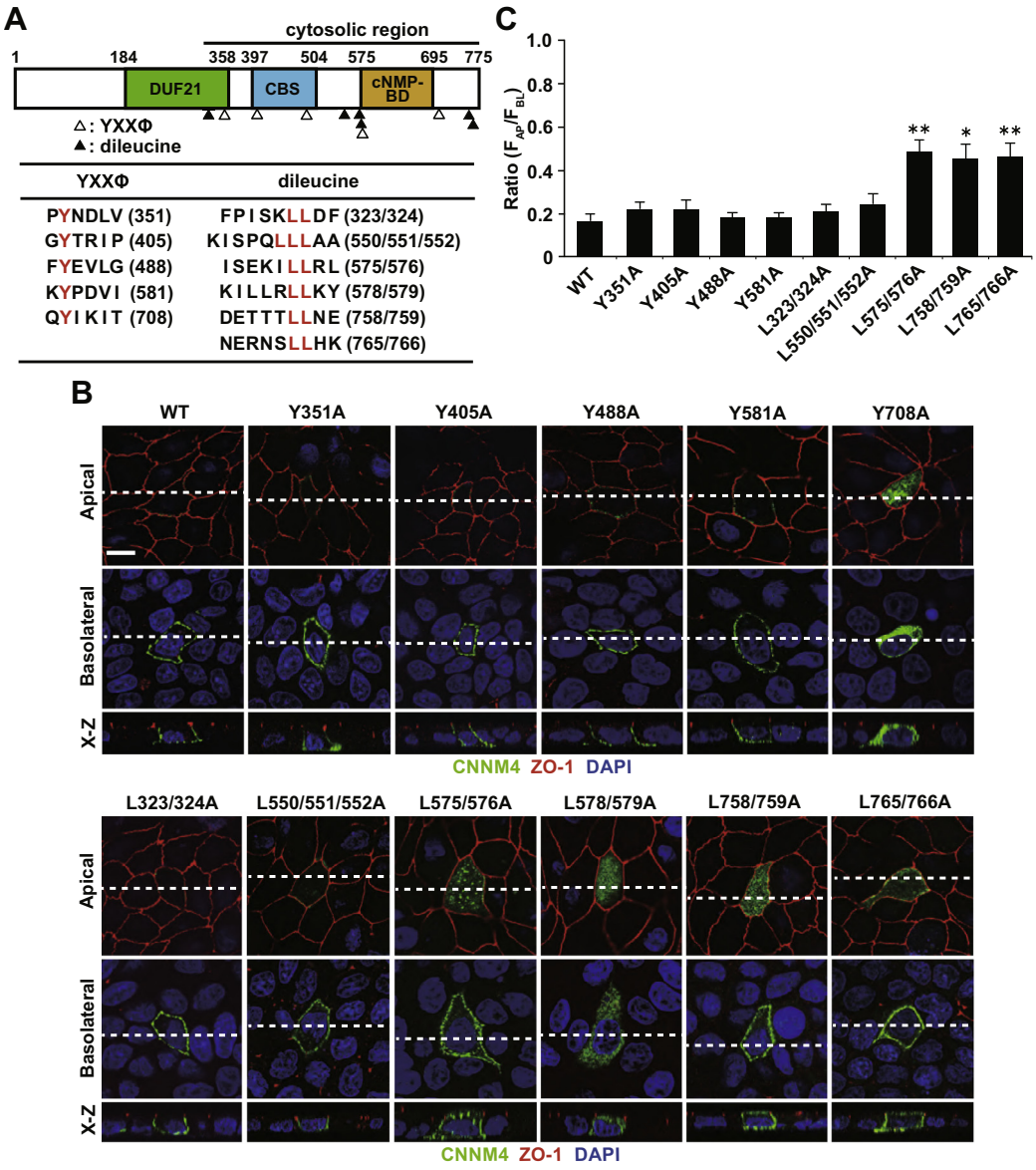

Fig. 3. Mutational analyses of basolateral sorting motifs of CNNM4. (A) Basolateral sorting motifs present in the C-terminal cytosolic regions of human CNNM4. The positions of the tyrosine-based YXXΦ motifs and the dileucine motifs conserved in mammalian CNNM4 (human, cow, rat, and mouse) are shown as open and filled triangles, respectively, in the schematic illustration of human CNNM4. The DUF21 domain, the CBS domains, and the cyclic nucleotide-monophosphate-binding domain (cNMP-BD) are boxed and the amino acid residue numbers are indicated. The amino acid sequences around these motifs are also shown below the illustration of CNNM4. The tyrosines in the YXXΦ motifs and the dileucines are shown in red letters, and their respective amino acid positions in human CNNM4 are shown in parentheses. (B) MDCK cells transfected with the indicated constructs were subjected to immunofluorescence staining, as explained in the legend to Fig. 1B. Bar, 10 μm. (C) Quantitative analyses of the basolateral localization of CNNM4 in the MDCK cells immunostained in (B). The ratio (F_{AP}/F_{BL}) values of 20 cells were determined for each cell group, and the data are shown as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ (vs WT; Kruskall–Wallis test followed by Steel–Dwass test).

dileucine motifs in CNNM4 (L575/576, L758/759, and L765/766) cooperatively mediate the interaction with AP-1A and AP-1B, and thereby contribute to the basolateral localization of CNNM4.

3.5. Conclusion

Our knockdown experiments revealed that μ1A and μ1B play complementary roles in the basolateral localization of CNNM4 in polarized MDCK cells (Fig. 2). The proportional contributions of AP-1A and AP-1B to basolateral sorting have been shown to differ according to the cargo protein being transported [16,17,31,32]. To the best of our knowledge, Na,K-ATPase is the only protein whose basolateral localization has been demonstrated to be mediated by the complementary function of AP-1A and AP-1B [16,17]. Thus, we identified CNNM4 as the second example of a protein whose basolateral localization is mediated by both AP-1A and AP-1B in a complementary fashion.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgments

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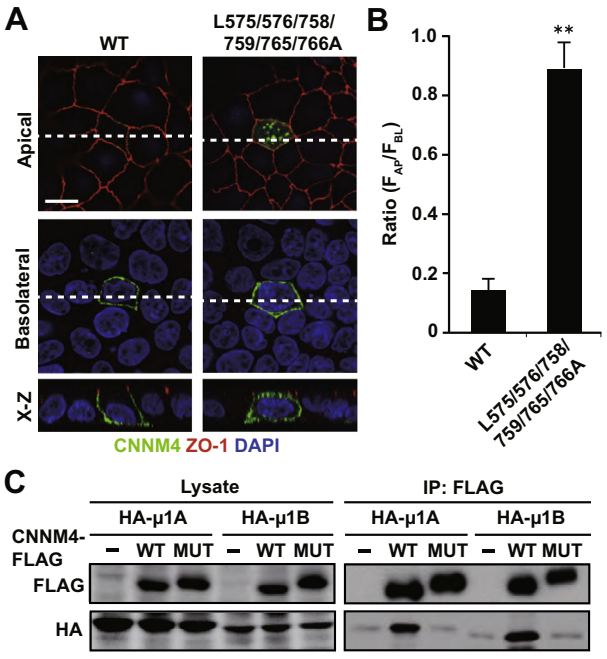

Fig. 4. Disruption of the basolateral localization of CNNM4 by simultaneous mutations of three dileucine motifs. (A) MDCK cells transfected with the indicated constructs were subjected to immunofluorescence staining, as explained in the legend to Fig. 1B. Bar, 10 μm. (B) Quantitative analyses of the basolateral localization of CNNM4 in the MDCK cells immunostained in (A). The ratio (F_{AP}/F_{BL}) values of 20 cells were determined for each cell group, and the data are shown as mean ± SEM. $^{**}P < 0.01$ (vs WT; Kruskall–Wallis test followed by Steel–Dwass test). (C) Lysates of COS7 cells transfected with the indicated constructs were subjected to immunoprecipitation with anti-FLAG antibody. "MUT" indicates the CNNM4 mutant L575/576/758/759/765/766A. The precipitated proteins were analyzed by immunoblotting with the indicated antibodies.

providing the anti-ZO-1 antibody. We are also grateful to T. Kasashima for technical assistance. Y.H. was supported by the Taniguchi Memorial Fellowship project funded by the Research Foundation for Microbial Diseases of Osaka University. This study was supported by Funding Program for Next Generation World-Leading Researchers from Japan Society for the Promotion of Science (JSPS) to H.M. (LS083), Exciting Leading-Edge Research Projects from Osaka University to H.M., and Grants-in-Aid for Scientific Research from JSPS and Ministry of Education, Culture, Sports, Science and Technology-Japan to H.M. (26111007 and 26291042) and Y.F. (22770195 and 23117710). The funding sources had no role in study design, data collection, data analysis, data interpretation, writing of the report, or decision to submit the report for publication.

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