FRMD4A regulates epithelial polarity by connecting Arf6 activation with the PAR complex

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The Par-3/Par-6/aPKC/Cdc42 complex regulates the conversion of primordial adherens junctions (AJs) into belt-like AJs and the formation of linear actin cables during epithelial polarization. However, the mechanisms by which this complex functions are not well elucidated. In the present study, we found that activation of Arf6 is spatiotemporally regulated as a downstream signaling pathway of the Par protein complex. When primordial AJs are formed, Par-3 recruits a scaffolding protein, termed the FERM domain containing 4A (FRMD4A). FRMD4A connects Par-3 and the Arf6 guanine-nucleotide exchange factor (GEF), cytohesin-1. We propose that the Par-3/FRMD4A/cytohesin-1 complex ensures accurate activation of Arf6, a central player in actin cytoskeleton dynamics and membrane trafficking, during junctional remodeling and epithelial polarization.

adherens junction | tight junction | cell polarity | epithelial cells | cytohesin

Epithelial polarization and the formation of cell–cell junctions are coupled processes that are essential to the tissue morphogenesis of multicellular organisms. During epithelial polarization, initial cell–cell contacts induce the formation of primordial "spot-like" adherens junction (AJ) complexes (1). Primordial AJs are gradually fused and finally become "belt-like" AJs (2). In parallel with this event, tight junctions (TJs) are formed at the apical side of AJs, and the apical membrane and the basolateral membrane become segregated.

Primordial AJs are also formed in fibroblasts; however, the primordial AJs in epithelial cells are unique because they subsequently become reorganized into continuous belt-like AJs (2). The developmental process of belt-like AJs is coupled with dramatic reorganization of F-actin (3–5). Furthermore, the remodeling of AJs during epithelial polarization is highly dynamic, and cell-adhesion molecules are internalized and recycled back to the plasma membrane (6). The molecular mechanisms underlying these epithelium-specific events during epithelial polarization are little understood.

The Par-3/Par-6/aPKC/Cdc42 complex is known to function in various cell polarization events (7, 8). In epithelial cells, knockdown of Par-3 has been shown to impair the maturation of belt-like AJs from primordial AJs (9). Overexpression of the dominant-negative form of aPKC was also reported to prevent maturation of primordial AJs (10). These data indicate that Par-3/Par-6/aPKC/Cdc42 signaling is essential for the switching of AJs and establishment of membrane domains. Recently, several groups reported that Par3 recruits a guanine-nucleotide exchange factor (GEF) for Rac1, Tiam-1, to primordial AJs, thereby preventing inappropriate Rac activation (9, 11). However, the events downstream of Par-3/Par-6/aPKC/Cdc42 signaling at primordial AJs are only partially understood.

In the present study, we report that Arf6, a central player in actin cytoskeleton dynamics and membrane trafficking, is activated upon primordial AJ formation and is required for the establishment of epithelial polarity. Furthermore, we identified a regulator of epithelial polarity, FERM domain containing 4A (FRMD4A). As reported here, FRMD4A controls the activation of Arf6 by connecting cytohesin family Arf6-specific GEFs and Par-3 at primordial AJs during epithelial polarization.

Results

Considering that primordial AJs are formed between mesenchymal cells but formation of belt-like AJs occurs only in epithelial cells, it may be that unidentified components involved in this process are enriched in epithelial cells. To find previously unidentified components involved in this process, we compared the expression profiles of cultured epithelial cells (EpH4 cells) and EpH4 cells transformed to fibroblastic cells by exogenous expression of Snail (12). Among the genes up-regulated in epithelial cells, we searched for genes encoding proteins with a pleckstrin homology (PH) domain, because phosphatidyl inositol signaling has been implicated in cellular polarization and the determination of membrane identity (13, 14). In the clones that fulfilled both of the above criteria, we identified cytohesin-1 as a PH domain-containing protein (Fig. 1*A*). Cytohesin-1 was expressed at a fivefold or greater rate in EpH4 cells compared to EpH4-Snail cells (Fig. S1 *A* and *B*).

The expression profile of cytohesin-1 suggested by analysis of EST counts confirmed that cytohesin-1 was ubiquitously expressed in epithelial tissues (http://www.ncbi.nlm.nih.gov/UniGene/EST-ProfileViewer.cgi?uglist=Mm.86413). We raised a polyclonal antibody against cytohesin-1. Immunoblotting of EpH4 cell lysate with the cytohesin-1 pAb revealed a strong signal at 48 kDa, the expected molecular weight of cytohesin-1 (Fig. 1*B*). Then, we examined the subcellular localization of cytohesin-1. In mouse intestinal epithelial cells, cytohesin-1 was colocalized with ZO-1 at TJs (Fig. 1*C*). Next, we examined the behavior of cytohesin-1 during epithelial polarization, using the Ca^{2+} switch assay. At 0.15 h after Ca^{2+} repletion, cytohesin-1 was colocalized with ZO-1 at primordial AJs (Fig. 1 *D* and *E*). These observations indicate that cytohesin-1 is a component of both primordial AJs and TJs in epithelial cells.

Cytohesin-1 was originally characterized as a binding protein for the cytoplasmic domain of the integrin beta2 chain in Jurkat T cells (15); however, its function in epithelial cells remains totally unknown. Cytohesin-1 contains the amino (NH2)-terminal coiled-coil domain, the central Sec7 domain, and the carboxy (COOH)-terminal PH domain (Fig. 1*A*). Cytohesin-1 is a GEF for Arf6, and the Sec7 domain is the catalytic domain that stimulates guanine-nucleotide exchange of Arf6, a member of the Arf family small GTPases (16). Therefore, we examined whether cytohesin-1 and Arf6 are involved in epithelial polarization.

We first examined whether Arf6 was actually activated during epithelial polarization using affinity precipitation with GST-GGA3 (amino acids 1–316), which is known to bind to the

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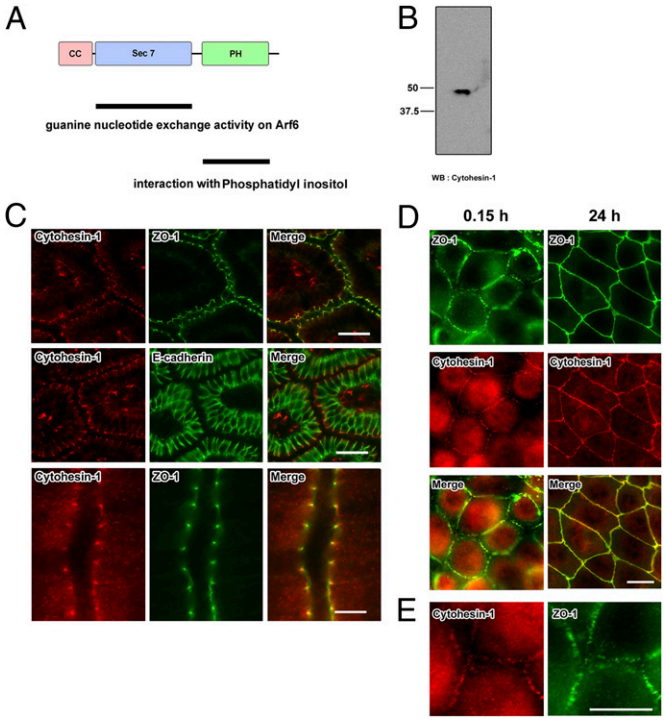

Fig. 1. Cytohesin-1 is a component of primordial AJs and TJs in epithelial cells. (*A*) The domain structure of mouse cytohesin-1. (*B*) Immunoblotting of whole-cell lysate of EpH4 cells with anti-cytohesin-1 pAb. (*C*) (*Top* and *Middle*) Immunofluorescence staining of frozen sections of mouse small intestine with anti-cytohesin-1 pAb (red) and anti-ZO-1 mAb (green) or anti-cytohesin-1 pAb (red) and anti-E-cadherin mAb (green). (Scale bars, 40 μm.) (*Bottom*) Highly magnified image of the *Top*. Cytohesin-1 was concentrated at TJs together with ZO-1 in intestinal epithelial cells in vivo. (Scale bar, 20 μm.) (*D*) Colocalization of cytohesin-1 and ZO-1 during epithelial polarization. EpH4 cells were cultured overnight in low Ca^{2+} medium, and their polarization was initiated by transferring to normal Ca^{2+} medium. After a 0.15- or 24-h incubation, cells were fixed and stained with anti-cytohesin-1 pAb (red) and anti-ZO-1 mAb (green). (Scale bar, 10 μm.) (*E*) Colocalization of cytohesin-1 and ZO-1 at primordial AJs. Highly magnified images of *D* are shown. (Scale bar, 10 μm.)

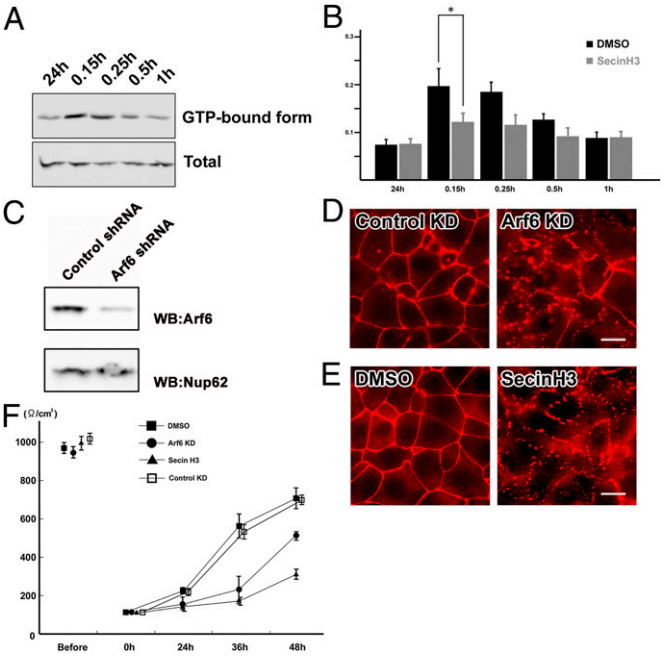

Fig. 2. Activation of Arf6 is essential for epithelial polarization. (*A*) EpH4 cells stably expressing Myc-tagged Arf6 were cultured overnight in low Ca^{2+} medium, and their polarization was initiated by transferring to normal Ca^{2+} medium. GTP-bound Arf6 was affinity precipitated with GST-GGA3 (amino acids 1–316) and immunoblotted with an anti-Myc antibody. (*B*) The ratio of the GTP-bound form to total Arf6 was quantified in EpH4 cells stably expressing Myc-tagged Arf6 after a Ca^{2+} switch in the presence of 20 μM of the cytohesin inhibitor SecinH3 or DMSO (control). Three independent experiments were performed. Data are mean ± SD, $n = 3$, $^*P < 0.05$ (Student's *t* test). (*C*) The results of immunoblotting of whole-cell lysates of EpH4 cells stably transfected with pSuper-control or pSuper-Arf6 with anti-Arf6 mAb and anti-Nup62 mAb are shown. (*D*) After Ca^{2+} switch and a 1-h incubation, control KD cells and Arf6 KD cells were fixed and stained with anti-ZO1 mAb. (Scale bars, 15 μm.) (*E*) After Ca^{2+} switch and a 1-h incubation, DMSO-treated cells (negative control) and SecinH3-treated cells were fixed and stained with anti-ZO1 mAb. (Scale bars, 15 μm.) (*F*) Control KD cells, Arf6 KD cells, and parental EpH4 cells were cultured in Transwell chambers in a low Ca^{2+} medium overnight under confluent conditions, and then their polarization was initiated by transfer to a normal Ca^{2+} medium. In the case of EpH4 cells, Ca^{2+} medium containing 20 μM SecinH3 or DMSO was used.

GTP-bound form of Arf6 (17). Using the Ca^{2+} switch assay, we found that the GTP-bound Arf6 transiently increased during 0.15–0.25 h after Ca^{2+} repletion (Fig. 2 *A* and *B*). As the conversion of primordial AJs to belt-like AJs coincided with the activation of Arf6 (shown in Fig. S2), we next tested whether the activation of Arf6 is essential for epithelial polarization. We established EpH4 cells stably expressing shRNA against Arf6. Arf6 protein expression was suppressed by >75% as determined by Western blot analysis (Fig. 2*C*). Arf6-knockdown EpH4 cells showed retardation of belt-like AJ formation during epithelial cell polarization, as compared to the control-knockdown EpH4 cells (Fig. 2*D*). Furthermore, EpH4 cells transiently expressing a GFP-tagged dominant-negative, GTP binding-defective Arf6 T27N mutant also showed retardation of belt-like AJ formation during epithelial cell polarization, as compared to EpH4 cells transfected with GFP-tagged Arf6 (Fig. S3 *A* and *B*). Collectively, these data suggested that activation of Arf6 is involved in the epithelial polarization.

Cytohesin-1 is a member of the cytohesin family, which also includes ARNO/cytohesin-2, Grp-1/cytohesin-3, and cytohesin-4, and these proteins share the same domain organization and show remarkably high sequence similarities (~70–82% amino acid identity) (18). All four cytohesins were expressed in EpH4 cells (Fig. S1*A*). In addition to cytohesin-1, ARNO and Grp-1 were also concentrated at spot-like AJs and TJs in EpH4 cells (Figs. S1*C* and S3). To test the possibility that the GEF activities of these cytohesin family proteins are responsible for the activation of Arf6 during epithelial polarization, we took advantage of SecinH3, the newly identified chemical inhibitor of all cytohesin family proteins (17). We treated EpH4 cells with 20 μM SecinH3 during polarization after a Ca^{2+} switch. Treatment with SecinH3 blocked the transient activation of Arf6 in the initial stage of epithelial polarization (Fig. 2*B*). The formation of belt-like AJs was delayed in EpH4 cells treated with SecinH3 as compared to the control EpH4 cells (Fig. 2*E*).

Next, we attempted to determine which cytohesin(s) is responsible for this process. Previously, several groups reported that substitution of a Lys residue for a conserved Glu residue in the Sec7 domains of cytohesin family proteins abrogates GEF activity of cytohesin family proteins in vitro, and these catalytically inactive mutants of cytohesin family proteins act in a dominant-negative manner (19, 20). Overexpression of dominant-negative forms of cytohesin-1 and Grp-1, but not those of ARNO and cytohesin-4, blocked the formation of belt-like AJs after a Ca^{2+} switch, implying that cytohesin-1 and Grp-1 play essential roles in the epithelial polarization among cytohesin family proteins (Fig. S3 *A* and *B*).

We assessed the effect of inactivation of Arf6 and cytohesin family proteins on epithelial polarization by measurement of transepithelial electrical resistance (TER). Arf6-knockdown EpH4 cells and control knockdown EpH4 cells were cultured in Transwell chambers under confluent conditions and submitted to a Ca^{2+} switch. After readdition of Ca^{2+}, the TER developed slowly in Arf6-knockdown EpH4 and EpH4 cells treated with SecinH3, as compared to its development in the control EpH4 cells (Fig. 2*F*).

Thus, inactivation of both Arf6 and cytohesin family proteins affected maturation of functional TJs, suggesting that activation of Arf6 is restrictively controlled via cytohesin family proteins in the process of epithelial polarization.

How, then, is the activation of Arf6 by cytohesin family proteins regulated spatially and temporally during epithelial polarization? To identify the binding partner of cytohesin-1 at spot-like AJs and TJs, we employed a yeast two-hybrid system. Using full-length mouse cytohesin-1 as bait, we identified FRMD4A as a binding partner of cytohesin-1 (Fig. 3*A*).

Because the coiled-coil domain of cytohesin-1 is necessary and sufficient for the localization at primordial AJs and TJs (Fig. S5), we examined whether the FRMD4A protein binds directly to the coiled-coil domain of cytohesin-1. The results obtained from the yeast two-hybrid assay indicated that amino acids 343–405 of FRMD4A are responsible for the interaction of cytohesin-1 (Fig. 3*A*). We confirmed the direct interaction between GST fusion proteins encoding the coiled-coil domain of cytohesin-1 (amino acids 1–100) and GFP-tagged FRMD4A (Fig. 3*B*). GFP-tagged FRMD4A was colocalized with cytohesin-1 at primordial AJs and TJs in EpH4 cells (Fig. 3 *C* and *D*). In addition to cytohesin-1, ARNO and Grp-1 also bound to FRMD4A (Fig. S6*E*). Collectively, these results demonstrated that FRMD4A binds cytohesin-1/ARNO/Grp-1 at primordial AJs and TJs in epithelial cells.

We next attempted to identify the binding partners of FRMD4A at primordial AJs and TJs. We examined the direct interaction between FRMD4A and proteins previously known as components of both primordial AJs and TJs, including ZO-1/ZO-2/ZO-3, Tiam-1, Par-3, aPKC, and Par6. Among them, only Par-3 showed binding to FRMD4A. Anti-FRMD4A antibodies coprecipitated Myc-tagged Par-3 in EpH4 cells that stably expressed a low level of Myc-tagged Par-3 (Fig. 3*F*). During the process of epithelial polarization, GFP-tagged FRMD4A and Par-3 were colocalized at primordial AJs and TJs (Fig. 3 *G* and *H*).

To determine the domains of FRMD4A responsible for the localization at primordial AJs and TJs, we examined the localization of a series of deletion mutants of FRMD4A. Among these deletion mutants, the amino acids 565–920 region of FRMD4A was necessary and sufficient for the localization at primordial AJs and TJs (Fig. 3*E* and Fig. S7). Therefore, it was expected that the amino acids 565–920 region of FRMD4A would directly interact with Par-3. An in vitro binding assay showed that the amino acids 931–1,334 region of Par3 bound to the amino acids 565–920 region of FRMD4A (hereafter, FRMD4A Par3BD) (Fig. 4 *A* and *B*). The fact that FRMD4A binds to both Par-3 and cytohesin-1 led us to postulate that FRMD4A mediates the interaction between Par-3 and cytohesin-1.

To examine whether cytohesin-1, FRMD4A, and Par-3 form a ternary complex, HEK293 cells were transfected with the Myc-tagged amino acids 931–1,334 region of Par3, HA-tagged cytohesin-1, and GFP-tagged FRMD4A deletion constructs. HA-tagged cytohesin-1 was coimmunoprecipitated with the Myc-tagged amino acids 931–1,334 region of Par3 in the presence of full-length FRMD4A (Fig. 4*C*).

We then examined whether exogenous expression of FRMD4A can recruit exogenously expressed cytohesin-1 to Par-3-containing AJs in L fibroblasts stably expressing E-cadherin (EL cells) (21). Endogenous Par-3 was recruited to the ectopic AJs (Fig. 4*D*). FRMD4A lacking a Par-3 binding domain was localized in the cytoplasm together with cytohesin-1. Cytohesin-1 was recruited to Par-3-containing AJs in an FRMD4A-dependent manner (Fig. 4*E*).

Finally, we used siRNA against FRMD4A to evaluate the significance of the ternary complex composed of Par3, FRMD4A, and cytohesin-1 in epithelial polarization. Depletion of FRMD4A only slightly affected the formation of belt-like AJs and TJs (Fig. 5 *B* and *D*). To explore the possibility that there are other proteins that compensate for the lack of the FRMD4A protein, we performed a BLAST homology search and found that the FERM domain of FRMD4A shows high sequence similarity to the Grp-1 signaling partner (GRSP-1) (Fig. 5*A*).

GRSP-1 has been reported to bind to Grp-1 and colocalize with F-actin in membrane ruffles upon stimulation with insulin in

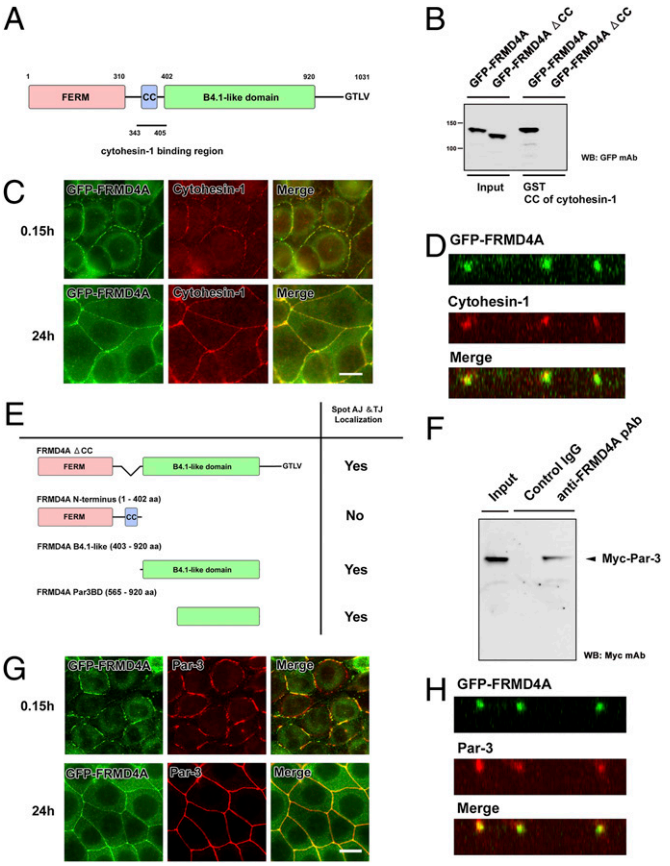

Fig. 3. Identification of FRMD4A as a binding partner of cytohesin-1 and Par-3. (*A*) The domain structure of mouse FRMD4A. (*B*) Interaction of FRMD4A and cytohesin-1. An in vitro binding assay was performed between GST fusion proteins encoding the coiled-coil domain of cytohesin-1 (amino acids 1–100) and total cell lysate of HEK 293 cells expressing GFP-FRMD4A (GFP-FRMD4A full) or GFP-FRMD4A lacking amino acids 343–405 (GFP-FRMD4A lacking CC). (*C*) Colocalization of FRMD4A and cytohesin-1 during epithelial polarization. After Ca^{2+} switch and a 0.15- or 24-h incubation, stable transfectants expressing GFP-FRMD4A were fixed and stained with anti-GFP mAb (green) and anti-cytohesin-1 pAb (red). (Scale bar, 10 μm.) (*D*) Xz section of a confocal image depicting colocalization of cytohesin-1 and FRMD4A. (*E*) Schematic drawings of deletion constructs of FRMD4A. The subcellular localization of these mutants is shown in Fig. S7. FRMD4A (amino acids 565–920) was sufficient and necessary for the localization at spot-like AJs and TJs. (*F*) Cell lysates derived from EpH4 cells stably expressing Myc-tagged Par-3 at a low expression level were subjected to immunoprecipitation with preimmune sera (control IgG) and anti-FRMD4A pAb. The immunoprecipitates were then analyzed with anti-Myc mAb. (*G*) After Ca^{2+} switch and a 0.15- or 24-h incubation, stable transfectants expressing GFP-FRMD4A were fixed and stained with anti-GFP mAb (green) and anti-Par-3 pAb (red). (Scale bar, 10 μm.) (*H*) Xz section of a confocal image depicting colocalization of Par-3 and FRMD4A.

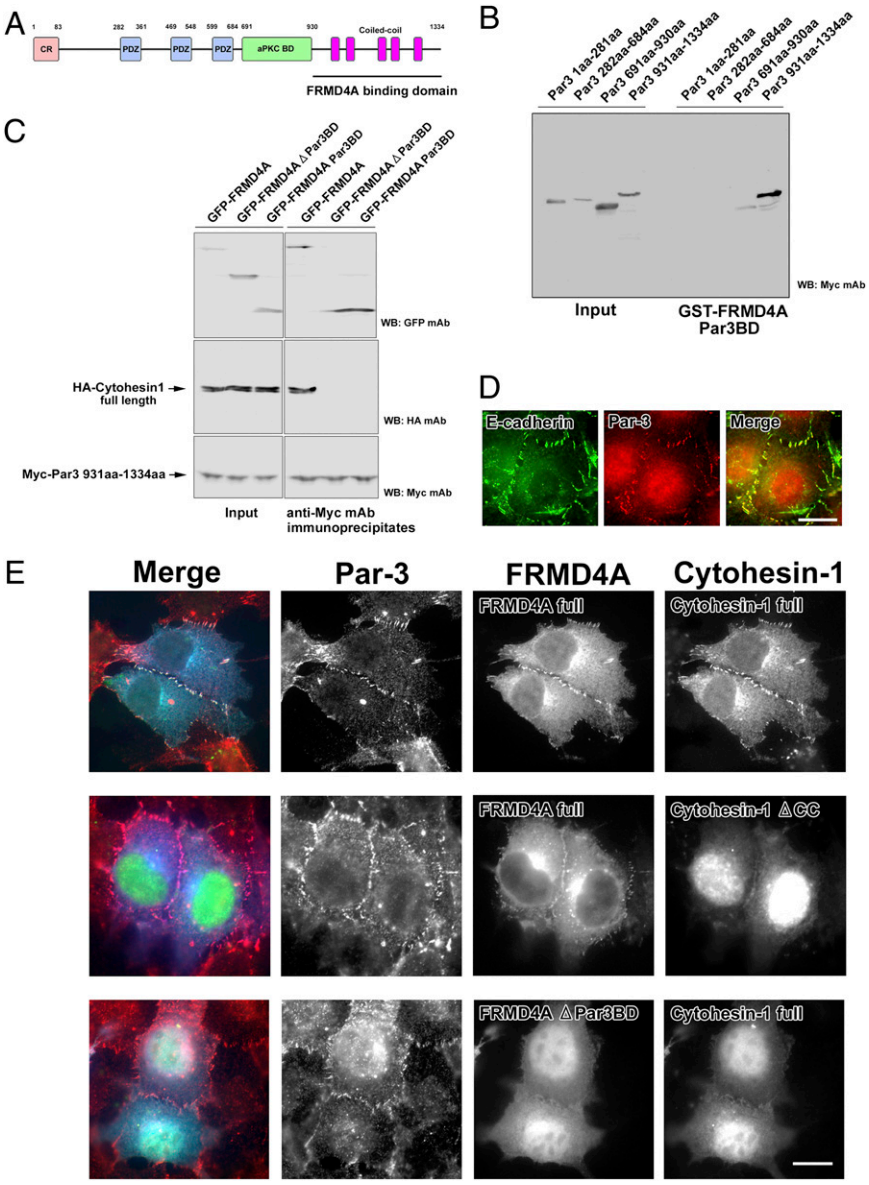

Fig. 4. Cytohesin-1-FRMD4A-Par-3 forms a ternary complex. (*A*) The domain structure of mouse Par-3. (*B*) The coiled-coil region of Par-3 (amino acids 931–1,334) bound to FRMD4A. An in vitro binding assay was performed between GST fusion proteins encoding the Par-3 binding domain of FRMD4A (amino acids 565–920) and total cell lysates derived from HEK293 cells transiently expressing each deletion construct of Par-3. (*C*) HEK293 cells were cotransfected with Myc-tagged Par3 (amino acids 931–1,334), HA-tagged cytohesin-1, and GFP-tagged FRMD4A deletion constructs including full-length FRMD4A, FRMD4AΔPar3BD (amino acids 1–565), and FRMD4A Par3BD (amino acids 565–920). Immunoprecipitates of Myc-tagged Par3 (amino acids 931–1,334) were immunoblotted for HA-cytohesin-1 and GFP-tagged FRMD4A deletion constructs. (*D*) EL cells were fixed and stained with anti-E-cadherin mAb and anti-Par-3 pAb. (Scale bar, 10 μm.) (*E*) Recruitment of cytohesin-1 to Par-3-containing AJs in an FRMD4A-dependent manner. EL cells were transiently cotransfected with Myc-tagged FRMD4A deletion constructs and GFP-tagged cytohesin-1 deletion constructs. Cells were fixed 48 h after transfection and stained with anti-Myc mAb and anti-Par3 pAb. Full-length FRMD4A was recruited to Par-3 containing AJs regardless of cytohesin-1 (*Top* and *Middle*). On the other hand, the deletion mutant of cytohesin-1 lacking its coiled-coil domain (amino acids 61–399), which is responsible for the binding to FRMD4A, does not colocalize with Par-3 and FRMD4A at AJs (*Middle*). The deletion mutant of FRMD4A lacking Par3BD (amino acids 1–565) cannot recruit full-length cytohesin-1 to Par-3-containing spot-like AJs (*Bottom*). (Scale bar, 10 μm.)

CHO-T cells (22). The GFP-tagged GRSP-1 localized together with Par-3 at spot-like AJs and TJs in EpH4 cells and at AJs in NIH 3T3 cells (Fig. S6*A*).

We tested the subcellular localization of a series of deletion mutants of GRSP-1 and found that the amino acids 542–972 region of GRSP-1 was necessary and sufficient for the localization at primordial AJs and TJs (Fig. S6*D*). Furthermore, the GST-tagged amino acids 542–972 region of GRSP-1 bound to Myc-tagged Par-3. GRSP-1 also bound directly to cytohesin-1 and ARNO in addition to Grp-1, suggesting that GRSP-1 can compensate for the loss of FRMD4A in FRMD4A-knockdown cells (Fig. S6*D*).

Therefore, we examined the effects of double knockdown of FRMD4A and GRSP-1 during epithelial polarization. We performed a Ca^{2+} switch assay in cells treated with FRMD4A siRNA, GRSP-1 siRNA, or both. We found that double knockdown of FRMD and GRSP-1 affected the formation of belt-like AJs and TJs (Fig. 5*B*). As expected, the localization of cytohesin-1 at primordial AJs disappeared from the cell–cell boundaries between double knockdown cells, supporting the idea that FRMD4A and GRSP-1 cooperatively recruit cytohesin-1 to primordial AJs (Fig. 5*C*).

We assessed the effect of depletion of FRMD4A and GRSP-1 on epithelial polarization by measuring the TER. The TER experiment clearly showed that double knockdown of FRMD4A and GRSP-1 in EpH4 cells led to the delayed formation of functional TJs (Fig. 5*D*).

Next, we quantified the percentage of cells that showed delayed epithelial polarization in siRNA-treated cells (Fig. 5*E*). EpH4 cells, EpH4 cells stably expressing GFP-tagged human FRMD4A, and EpH4 cells stably expressing GFP-tagged human GRSP-1 were treated with siRNA against FRMD4A and siRNA against GRSP-1. These cells were subjected to a Ca^{2+} switch assay. After Ca^{2+} switch and a 1-h incubation, the percentage of cells arrested at the "spot-like AJs" state was significantly increased in double-knockdown cells when compared with control knockdown cells (from 4.1% ± 0.83%–17.4% ± 4.2%) (P = 0.021) (Fig. 5*E*). Exogenous expression of GFP-tagged human FRMD4A or GFP-tagged human GRSP-1 totally rescued the phenotype caused by depletion of both FRMD4A and GRSP-1 (Fig. 5*E*).

These data clearly showed that the cooperative recruitment of cytohesin-1 to primordial AJs by FRMD4A and GRSP-1 and

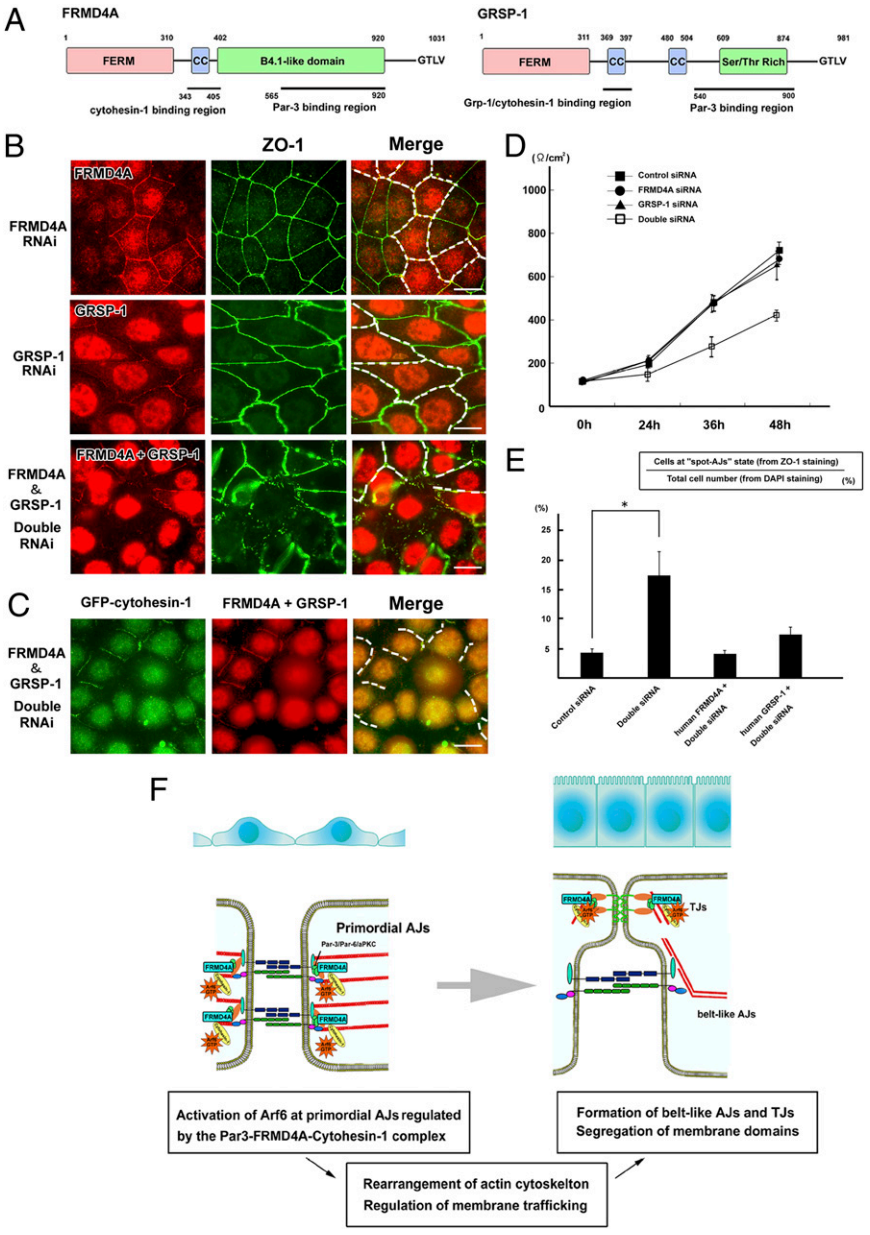

Fig. 5. Cooperative recruitment of cytohesin family proteins to primordial AJs by FRMD4A and GRSP-1 is essential for the epithelial polarization. (*A*) Comparison of domain structures between mouse FRMD4A and mouse GRSP-1. (*B*) EpH4 cells were treated with single siRNA against FRMD4A (*Top*), single siRNA against GRSP-1 (*Middle*), or both siRNA against FRMD4A and siRNA against GRSP-1 (*Bottom*). After Ca^{2+} switch and a 1-h incubation, cells were fixed and stained with anti-FRMD4A pAb (red) (*Top*), anti-GRSP-1 pAb (red) (*Middle*), anti-FRMD4A pAb and anti-GRSP-1 antibody (red) (Bottom), and anti-ZO-1 mAb (green). Dashed white lines indicate FRMD4A- or GRSP-1-positive cells. Strong nonspecific staining induced by anti-GRSP-1 pAb is present in the nucleus. Representative data from three independent experiments are shown. (Scale bar, 15 μm.) (*C*) Recruitment of cytohesin-1 to primordial AJs during epithelial polarization was abolished in both FRMD4A- and GRSP-1-knockdown EpH4 cells. EpH4 transfectants stably expressing GFP-cytohesin-1 were treated with both siRNA against FRMD4A and siRNA against GRSP-1. After Ca^{2+} switch and a 1-h incubation, cells were fixed and stained with anti-FRMD4A pAb and anti-GRSP-1 pAb (red) and anti-GFP mAb (green). Dotted white lines indicate FRMD4A- or GRSP-1-positive cells. (Scale bar, 10 μm.) (*D*) EpH4 cells were treated with control siRNA, siRNA against FRMD4A, siRNA against GRSP-1, or both siRNA against FRMD4A and siRNA against GRSP-1. At the indicated times after Ca^{2+} repletion, cells were analyzed for their TER. (*E*) EpH4 cells, EpH4 cells stably expressing human FRMD4A, and EpH4 cell stably expressing human GRSP-1 were treated with both siRNA against FRMD4A and siRNA against GRSP-1. After Ca^{2+} switch and a 1-h incubation, cells were fixed and stained with anti-FRMD4A pAb (red), anti-GRSP-1 pAb (red), and anti-ZO-1 mAb (green). The percentages were calculated as (the number of cells at the "spot-like AJs" state based on staining with ZO-1)/(total cell number based on staining with DAPI) in the same field. Three independent experiments were performed. Data are mean ± SD, $n = 3$, $^*P < 0.05$ (Student's *t* test). (*F*) In the initial phase of epithelial polarization, cell-adhesion molecules (such as E-cadherin) that mediated cell–cell contacts induce the formation of primordial AJs. FRMD4A and GRSP-1 are recruited to primordial AJs through direct binding to Par-3 and in turn recruit cytohesin family proteins, which are the Arf6 guanine-nucleotide exchange factors. For details of this model, see text.

subsequent activation of Arf6 at primordial AJs are essential for the epithelial polarization.

Discussion

In the present study, we showed that Arf6 activation is spatially and temporally regulated during epithelial polarization by cytohesin family proteins, FRMD4A/GRSP-1, and the Par-3 complex (Fig. 5*F*).

Recently, several lines of evidence have suggested that Arf6 and its GEFs regulate the cell polarity in various systems, such as the cell polarization of neurons (23). Arf6 is reported to regulate dendritic branching in hippocampal neurons and neurite outgrowth in PC12 cells (19, 24). Moreover, in yeast, the Arf6 homolog, Arf3, localizes to the growing bud and plays an important role in polarized growth and bud site selection (25). In regard to the involvement of Arf6 in epithelial polarity, hyperactivation of Arf6 or overexpression of ARNO/cytohesin-2 has been reported to cause disruption of AJs and lead to loss of cell polarity (26–28). Luton et al. previously reported that EFA6, another GEF for Arf6, was recruited to developing junctions upon induction of cell–cell contacts and plays a role in the formation of TJs in MDCK cells (29). However, in EpH4 cells, EFA6 was localized throughout the entire plasma membrane and did not colocalize with ZO-1 at primordial AJs and TJs. On the other hand, cytohesin-1/Grp-1 colocalized precisely with ZO-1 and Par-3 at primordial AJs and TJs, suggesting that cytohesin-1/Grp-1 is responsible for the activation of Arf6 at spot-like AJs and TJs (Figs. S1*D* and S4). One of the causes of this discrepancy may be the difference in cell lines between the two studies, but the precise reason is unclear. We cannot exclude the possibility that other GEFs for Arf6 cooperate in the formation of epithelial polarity together with cytohesin family proteins.

Finally, we briefly discuss the possible functions ascribed to Arf6 in epithelial polarization. First, Arf6 has been shown to activate PIP5-kinase and phospholipase D (30, 31). Phosphatidic acid, the product of phospholipase D, has been shown to function

as a cofactor in the activation of PIP5-kinase (32). PIP5-kinase is responsible for generating PI(4,5)P2, which recruits numerous actin-binding proteins and induces drastic changes in the cortical actin network (33). Furthermore, a previous study reported that Arf6 changes the actin structure at the plasma membrane through activation of Rac1 (34). We previously found that Rac1 activation occurred upon the formation of primordial AJs (35). The ternary complex described in this study may be involved in the formation of linear actin cables and belt-like AJs through spatially restricted activation of PIP5-kinase and/or Rac1. Another important function of Arf6 is its role in endosome recycling (36). Recently, several lines of evidence indicated that Arf6 is involved in the membrane trafficking of E-cadherin (37, 38). Elucidation of the roles of Arf6 at primordial AJs is needed to understand the molecular mechanisms underlying epithelial polarization.

Materials and Methods

Cell Culture, Ca^{2+} Switch Assay, and Transfection. Mouse EpH4 epithelial cells (generously provided by E. Reichmann, Institute Suisse de Recherches, Lausanne, Switzerland), EL cells (generously provided by A. Nagafuchi, Kumamoto University, Kumamoto, Japan), and NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. The low Ca^{2+} medium was the DMEM containing 5-μM calcium. In the Ca^{2+} switch assay, the cells were cultured in a low Ca^{2+} medium containing 5 μM Ca^{2+} overnight under confluent conditions, and their polarization was initiated by transferring to a normal Ca^{2+} medium.

For TER measurement and immunostaining of polarized EpH4 cells, 1×10^6 cells were plated on Transwell polycarbonate filter supports with a pore size of 0.4 μm (Costar). Transfection was performed using Lipofectamine-Plus Reagent (Invitrogen) and Nucleofactor II (V-kit, G-016 program; Amaxa Biosystems) according to the manufacturer's instructions.

Details of expression vectors, antibodies, reagents, immunofluorescence microscopy, yeast two-hybrid screening, TER measurement, in vitro binding assay, gel electrophoresis, and immunoblotting are provided in the *SI Text*.

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