

An association between type I γ PI4P 5-kinase and Exo70 directs E-cadherin clustering and epithelial polarization

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ABSTRACT E-Cadherin–mediated formation of adherens junctions (AJs) is essential for the morphogenesis of epithelial cells. However, the mechanisms underlying E-cadherin clustering and AJ maturation are not fully understood. Here we report that type I γ phosphatidylinositol-4-phosphate 5-kinase (PIPKI γ) associates with the exocyst via a direct interaction with Exo70, the exocyst subunit that guides the polarized targeting of exocyst to the plasma membrane. By means of this interaction, PIPKI γ mediates the association between E-cadherin and Exo70 and determines the targeting of Exo70 to AJs. Further investigation revealed that Exo70 is necessary for clustering of E-cadherin on the plasma membrane and extension of nascent E-cadherin adhesions, which are critical for the maturation of cohesive AJs. In addition, we observed phosphatidylinositol-4,5-bisphosphate (PI4,5P₂) accumulation at E-cadherin clusters during the assembly of E-cadherin adhesions. PIPKI γ -generated PI4,5P₂ is required for recruiting Exo70 to newly formed E-cadherin junctions and facilitates the assembly and maturation of AJs. These results support a model in which PIPKI γ and PIPKI γ -generated PI4,5P₂ pools at nascent E-cadherin contacts cue Exo70 targeting and orient the tethering of exocyst-associated E-cadherin. This could be an important mechanism that regulates E-cadherin clustering and AJ maturation, which is essential for the establishment of solid, polarized epithelial structures.

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INTRODUCTION

The establishment and maintenance of polarized epithelial morphology depend on the organization of adherens junctions (AJs) (Gumbiner, 1996, 2005), protein complexes assembled around E-cadherin and connected to cytoskeletal filaments. AJ assembly is dynamic and stringently regulated during tissue morphogenesis and homeostasis (Gumbiner, 1996, 2005). Abnormal regulation of AJs correlates with loss of epithelial polarity and increased migra-

tory potential, which can lead to abnormal embryogenesis or the development of various diseases such as organ fibrosis (Thiery *et al.*, 2009), inflammatory bowel disease (Bruewer *et al.*, 2006), and epithelial-derived cancer (Bex and Van Roy, 2001; Van Aken *et al.*, 2001; Conacci-Sorrell *et al.*, 2002; Cowin *et al.*, 2005). In addition to transcription factors that control the expression of AJ components, posttranslational elements regulate AJ assembly, maturation, and dynamics (Harris and Tepass, 2010). For example, exocytic and endocytic events that bring E-cadherin on and off the plasma membrane (PM) control the availability of E-cadherin for AJ assembly and dynamics.

Compared to the current understanding of E-cadherin endocytosis, how distribution of E-cadherin on the PM is regulated to favor AJ organization is much less understood. One of the few clues is that PM targeting of DE-cadherin in *Drosophila* requires the exocyst (Langevin *et al.*, 2005), an octameric protein complex that assembles via side-by-side packing of the rod-shaped subunits Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (Hsu *et al.*, 1996;

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Abbreviations used: AJ, adherens junction; ECD, E-cadherin; PI4,5P₂, phosphatidylinositol-4,5-bisphosphate; PIPKI, type I phosphatidylinositol-4-phosphate 5-kinase; PM, plasma membrane.

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TerBush *et al.*, 1996). The exocyst mediates the tethering of post-Golgi secretory vesicles to the PM and plays an important role in the establishment of surface polarity (He and Guo, 2009; Nejsum and Nelson, 2009). It has been indicated that the exocyst associates with E-cadherin and may regulate its polarized distribution on the PM (Yeaman *et al.*, 2004). However, how this association occurs is unknown. The direct evidence showing that the exocyst regulates lateral targeting of E-cadherin also remains to be reported. Recent studies indicate that Sec3 and Exo70 bind to the PM and direct tethering of the arriving exocyst-bound vesicles in yeast (Finger *et al.*, 1998; Boyd *et al.*, 2004; Zajac *et al.*, 2005; He *et al.*, 2007a). This makes it very interesting to investigate whether the association between E-cadherin and the exocyst specifies the destination for lateral cargo delivery and/or facilitates apical-lateral surface differentiation in mammalian epithelial cells.

The PM targeting of yeast Sec3 and Exo70 is mediated by direct binding to phosphatidylinositol-4,5-bisphosphate (PI4,5P₂) (He *et al.*, 2007b; Zhang *et al.*, 2008). Recent studies further demonstrate that mammalian Exo70 is recruited in a similar manner (Liu *et al.*, 2007). PI4,5P₂ is a potent lipid second messenger that regulates multiple fundamental cellular events, such as actin reorganization (Janmey and Lindberg, 2004; Niggli, 2005), vesicular trafficking (Martin, 2001; Di Paolo and De Camilli, 2006), and focal adhesion assembly (DeMali *et al.*, 2002; Bakolitsa *et al.*, 2004; Millard *et al.*, 2004; Ginsberg *et al.*, 2005). In mammalian cells, PI4,5P₂ is mainly synthesized by type I phosphatidylinositol-4-phosphate 5-kinases (PIP1Ks). Among the three isoforms of PIP1K (α , β , and γ), PIP1K γ localizes at AJs via a direct interaction with E-cadherin (Ling *et al.*, 2007), suggesting that regional PI4,5P₂ pools at E-cadherin assembly sites could be created. These PI4,5P₂ pools may cue the targeting of Exo70, the exocyst, and then the exocyst-associated lateral cargoes, including E-cadherin. The clustered E-cadherin targeting to nascent AJs could subsequently promote the maturation of AJs and epithelial polarization. We show here that Exo70 plays an important role in orienting E-cadherin clustering on the PM and in AJ maturation via a direct interaction with PIP1K γ , which recruits Exo70 to the newly formed E-cadherin adhesions on the PM by generating PI4,5P₂. This facilitates the maturation of AJs, the differentiation of apical and lateral membrane domains, and epithelial morphogenesis.

RESULTS

PIP1K γ mediates the association between E-cadherin and the exocyst via a direct interaction with Exo70

Although the exocyst was observed to associate with E-cadherin (Yeaman *et al.*, 2004), it was not known whether this is a direct or indirect interaction. PIP1K γ directly interacts with E-cadherin, and earlier studies indicated that PIP1K γ binding partners are often PI4,5P₂ effectors (Ling *et al.*, 2002; Bairstow *et al.*, 2005, 2006; Ling *et al.*, 2007). Because Exo70 binds and is regulated by PI4,5P₂ (He *et al.*, 2007b; Liu *et al.*, 2007), we examined whether Exo70 associates with PIP1K γ . As shown in Figure 1A, PIP1K γ and Exo70 partially colocalized at AJs in MDCK cells (colocalization coefficient, 0.67 ± 0.02). Immunoprecipitation of endogenous Exo70 pulled down endogenous PIP1K γ (Figure 1B) and vice versa (Figure 1C), strongly suggesting a physical association between Exo70 and PIP1K γ . Endogenous Sec8, one of the other subunits in the exocyst, was also pulled down by anti-PIP1K γ antibody (Figure 1C), indicating that PIP1K γ associates with the exocyst complex. Because E-cadherin was coprecipitated with the exocyst subunits by anti-PIP1K γ antibody, we subsequently determined the association between E-cadherin and the exocyst in polarized epithelial cells. Indeed, Exo70 can pull down E-cadherin in human mammary gland epithelial MCF-10A

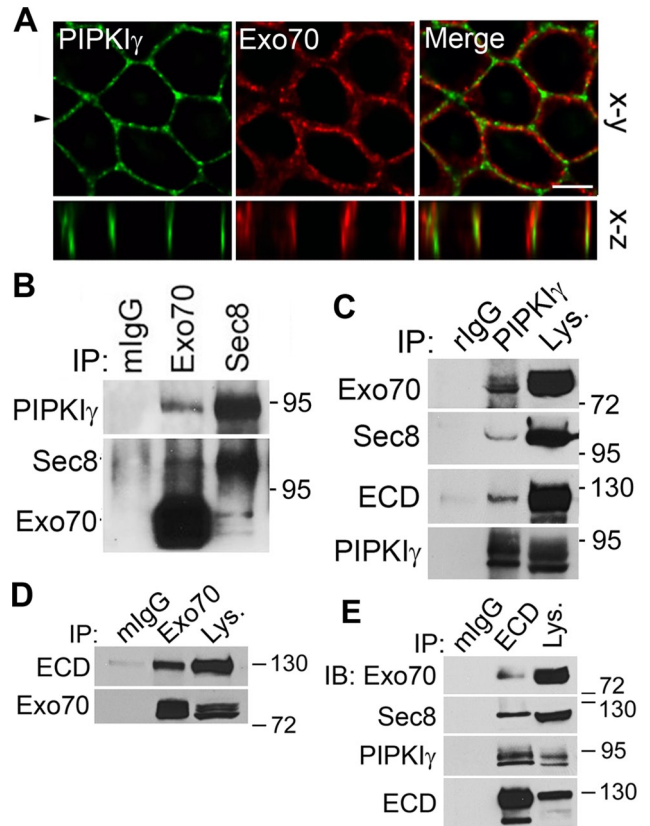


FIGURE 1: PIP1K γ , the exocyst, and E-cadherin form a protein complex. (A) Both PIP1K γ and Exo70 target to the lateral membrane in MDCK cells. Colocalization coefficient, 0.67 ± 0.02 . Horizontal (x-y) and vertical (x-z) sections show colocalization of PIP1K γ and Exo70 along the lateral membrane. Black arrowhead indicates the position where the x-z section was reconstructed. Scale bar, 10 μ m. (B) When immunoprecipitated from MCF-10A cells, endogenous Exo70 pulled down endogenous PIP1K γ and Sec8. mIgG, normal mouse immunoglobulin G. (C) Endogenous E-cadherin (ECD), Exo70, and Sec8 coimmunoprecipitated with endogenous PIP1K γ from MCF-10A cells. rIgG, normal rabbit IgG. (D, E) Endogenous E-cadherin associates with Exo70 and the exocyst in MCF-10A cells. Immunoprecipitation (IP) and immunoblotting (IB) were performed using MCF-10A cells with the indicated antibodies.

cells (Figure 1D) and MDCK cells (data not shown), reinforcing a physical connection between E-cadherin and the exocyst (Yeaman *et al.*, 2004). The result that E-cadherin coprecipitated with Exo70, Sec8, and PIP1K γ (Figure 1E) further reinforced the association between E-cadherin and the exocyst and confirmed that E-cadherin, the exocyst, and PIP1K γ form a protein complex.

Using a glutathione S-transferase (GST) pull-down assay, we observed that GST-tagged Exo70 pulled down PIP1K γ (Figure 2A), indicating that PIP1K γ directly interacts with Exo70. As shown in Figure 2A, PIP1K α did not bind Exo70, which further supported the view that Exo70 interacts specifically with PIP1K γ . Of greater interest, we found that Exo70 does not interact with the cytoplasmic domain of E-cadherin *in vitro* (Figure 2B, lane 3). The addition of PIP1K γ resulted in an association between Exo70 and the cytoplasmic domain of E-cadherin (Figure 2B, lane 6), suggesting that PIP1K γ could be a scaffold between E-cadherin and Exo70. Catenins are important AJ components that bind to E-cadherin and mediate the association of E-cadherin with many structural and signaling proteins (Baum and Georgiou, 2011). To understand how the whole AJ

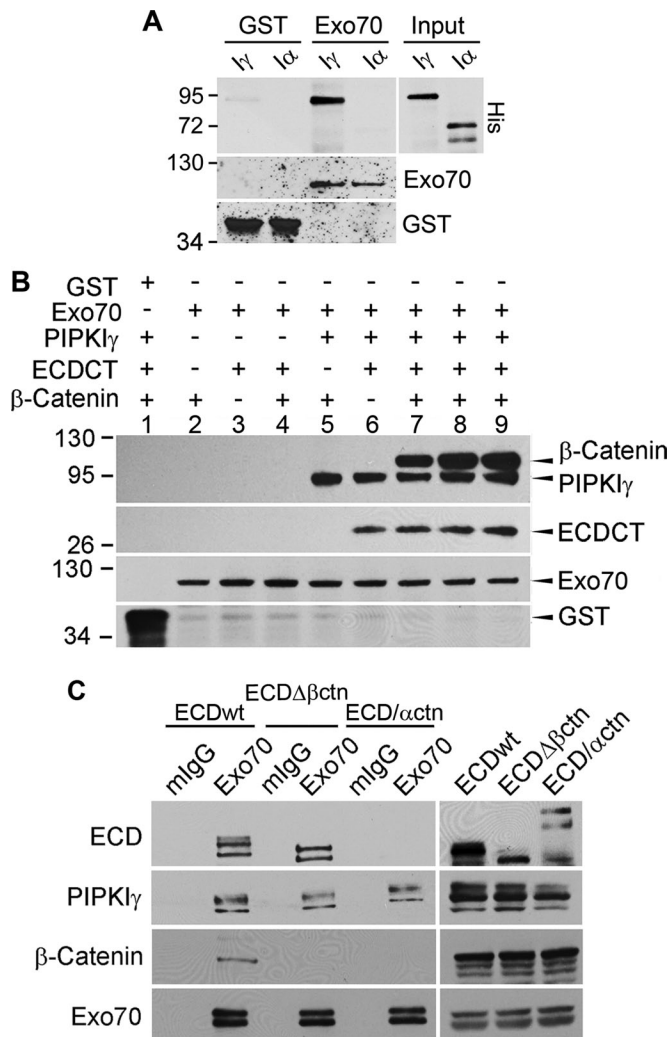


FIGURE 2: PIPK1 γ mediates the association between Exo70 and E-cadherin via a direct interaction with Exo70. (A) GST pull-down assays were performed by mixing glutathione beads with His-tagged PIPK1 γ _{i2} or PIPK1 α . GST- or GST-Exo70-associated proteins were analyzed by IB using the indicated antibody. GST-Exo70, but not GST alone, pulled down His-PIPK1 γ but not His-PIPK1 α . (B) PIPK1 γ , but not β -catenin, can scaffold Exo70 and E-cadherin. GST pull-down assays were performed by incubating 1 μ g of the indicated proteins: GST or GST-Exo70 and His-E-cadherin cytoplasmic domain (ECDCT), with or without His-PIPK1 γ and/or His- β -catenin. GST- and GST-Exo70-associated proteins were analyzed by IB using antibodies recognizing T7 tag (fused to His- β -catenin and His-PIPK1 γ), GST (GST and GST-Exo70), or E-cadherin (ECDCT). Mole of His- β -catenin: same as ECDCT in lane 7; 3-fold of ECDCT in lane 8; 10-fold of ECDCT in lanes 2, 4, and 9. (C) Wild-type (ECDwt), E-cadherin lacking β -catenin-binding site (ECD Δ β ctn), and chimera of E-cadherin and α -catenin were expressed in HEK293 cells. Endogenous Exo70 was immunoprecipitated from these cells, and associated exogenous E-cadherin, endogenous PIPK1 γ , and endogenous β -catenin were determined by IB.

complex associates with Exo70, we examined whether catenins intercede between E-cadherin and Exo70. As shown in Figure 2B, β -catenin at saturating concentration (10-fold of E-cadherin cytoplasmic domain; Supplemental Figure S1) did not show binding to Exo70 (lane 2), mediate the association between Exo70 and E-cadherin (Figure 2B, lane 4), or interrupt the PIPK1 γ -mediated association between E-cadherin and Exo70 (Figure 2B, lane 9), indicating

that E-cadherin associates with Exo70 independent of β -catenin. Although PIPK1 γ alone could not help GST-Exo70 pull down histidine (His)-tagged β -catenin (Figure 2B, lane 5), presence of both E-cadherin C-terminus and PIPK1 γ resulted in the association of β -catenin with Exo70 (Figure 2B, lanes 7, 8, and 9). These results indicate that in our GST pull-down system, β -catenin does not directly interact with Exo70 but can associate with Exo70 via E-cadherin and PIPK1 γ .

To confirm this conclusion, we expressed wild-type or mutated E-cadherin in HEK293 cells (no endogenous E-cadherin) to test the association among Exo70, PIPK1 γ , catenins, and E-cadherin via coimmunoprecipitation. We observed that endogenous Exo70 pulled down wild-type E-cadherin along with endogenous PIPK1 γ and β -catenin (Figure 2C). Truncated E-cadherin that still binds PIPK1 γ but not β -catenin (Ling *et al.*, 2007) can be coprecipitated by Exo70 at a similar level as wild-type E-cadherin; however, the association between β -catenin and Exo70 was abolished (Figure 2C). In addition, a chimera of truncated E-cadherin (deletion of the last 70 amino acids) fusing to a truncated α -catenin (β -catenin-binding site deleted), which lacks both β -catenin binding (Imamura *et al.*, 1999) and PIPK1 γ binding (Ling *et al.*, 2007) but maintains the intact p120-catenin binding site, failed to be pulled down by anti-Exo70 antibody (Figure 2C). Thus our results suggest that β -catenin, α -catenin, or p120-catenin does not likely mediate the association between Exo70 and E-cadherin.

To further understand the interaction between PIPK1 γ and Exo70, a series of GST pull-down analyses was performed using truncated Exo70 (Hamburger *et al.*, 2006; Moore *et al.*, 2007) and PIPK1 γ proteins as shown in Supplemental Figure S2. PIPK1 γ _{i1} (also named PIPK1 γ ₈₇ or PIPK1 γ ₆₃₅) and PIPK1 γ _{i2} (also named PIPK1 γ ₉₀ or PIPK1 γ ₆₆₁) bound Exo70 equally well (Supplemental Figure S2A), indicating that the Exo70-binding site is outside of the binding sites for talin (Di Paolo *et al.*, 2002; Ling *et al.*, 2002) and AP complexes (Bairstow *et al.*, 2005; Ling *et al.*, 2007). Although the PI4,5P₂ binding site is located at the C-terminus of Exo70 (He *et al.*, 2007b), the N-terminus of Exo70 showed the strongest binding to PIPK1 γ , although the input of Exo70 ND is the lowest (Supplemental Figure S2A). This result suggests that the PIPK1 γ -binding site is at the N-terminus of Exo70 and does not overlap with the binding site for the small G-protein TC10, which binds to the first 99 amino acids of Exo70 (Inoue *et al.*, 2003; Supplemental Figure S2A). By testing variant PIPK1 γ truncations (Supplemental Figure S2B), we concluded that Exo70 preferentially binds to the C-terminus of PIPK1 γ _{i1} (Supplemental Figure S2B). We tried to further narrow the interacting regions at Exo70 and PIPK1 γ . However, some of the truncated mutations (GST-tagged Exo70 middle domain; His-tagged, C-terminus-deleted PIPK1 γ ; and His-tagged PIPK1 γ kinase domain) were not soluble or not stable, which limited the possibility to refine the interaction region to amino acids and create Exo70 or PIPK1 γ mutants that specifically interrupt the interaction between them.

PIPK1 γ is required for Exo70 targeting to E-cadherin junctions

Our results strongly argue that PIPK1 γ can scaffold E-cadherin and Exo70 via a direct interaction with Exo70 (Figures 1 and 2), which makes it very interesting to investigate the functional role of this interaction. Because PI4,5P₂ is essential for Exo70 targeting to the PM (He *et al.*, 2007b; Liu *et al.*, 2007), we tested whether PIPK1 γ mediates the targeting of Exo70 to E-cadherin adhesions on the PM. For this purpose, *Lentivirus* was used to deliver PIPK1 γ -specific short hairpin RNA (shRNA) to deplete PIPK1 γ from MDCK cells (~90% depletion; Figure 3B). Although no effect was observed on the protein

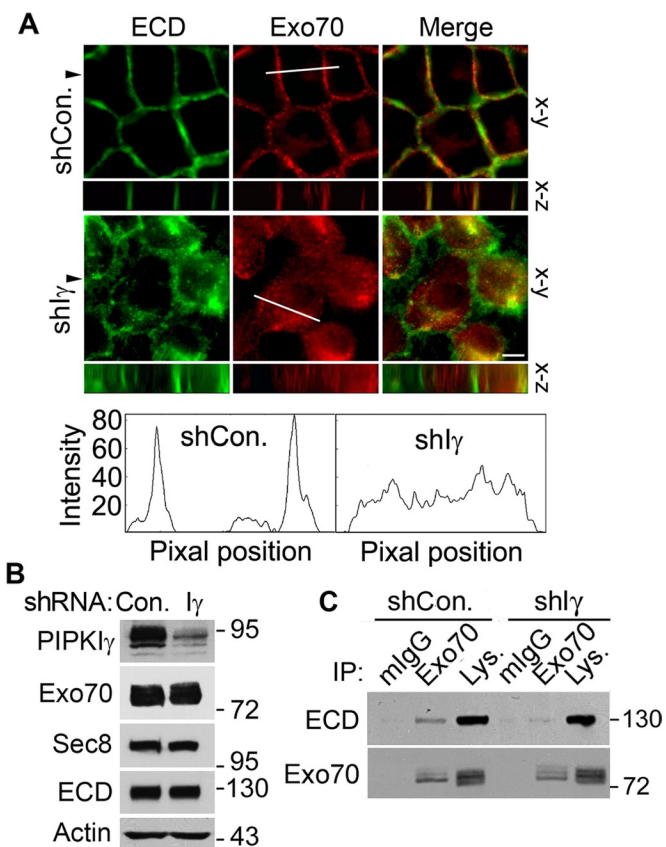


FIGURE 3: PIPKI γ is necessary for Exo70 to target to the PM and to associate with AJ complexes. (A) Loss of PIPKI γ resulted in the redistribution of Exo70 from the PM to the cytoplasm. MDCK cells treated with control (shCon.) or PIPKI γ -specific (shI γ) shRNAs were subjected to immunofluorescence to visualize E-cadherin (ECD, green) and Exo70 (red). Black arrowheads indicate the positions where the x-z sections were reconstructed. Horizontal (x-y) and vertical (x-z) sections show colocalization of Exo70 and E-cadherin at the lateral membrane in control cells. Overlap coefficient, 0.62 ± 0.04 . Accumulation of Exo70 in the cytoplasm was shown in PIPKI γ -depleted cells in both x-y and x-z sections. Scale bars, 10 μ m. Exo70 intensity profiles in representative cells (along the white lines) were determined and plotted using ImageJ. The results (bottom) indicate the targeting of Exo70 on the PM or in the cytoplasm in control or PIPKI γ -depleted cells, respectively. (B) MDCK cells treated with control (Con.) or PIPKI γ -specific (I γ) shRNAs were subjected to immunoblotting. PIPKI γ depletion had no effect on protein levels of E-cadherin (ECD), Exo70, and Sec8. (C) The association between Exo70 and E-cadherin was determined in control or PIPKI γ -depleted MDCK cells using a coimmunoprecipitation assay, followed by immunoblotting. Anti-Exo70 antibody pulled down much less E-cadherin in PIPKI γ -depleted cells (shI γ) than in control cells (shCon.), indicating that PIPKI γ is required for the association between Exo70 and E-cadherin.

levels of E-cadherin or of the exocyst components Exo70 and Sec8 (Figure 3B), knocking down PIPKI γ modified the subcellular localization of Exo70. As shown in Figure 3A, Exo70 localized on the lateral membrane (x-z sections) in control cells and exhibited substantial overlap with E-cadherin staining on the PM (overlap coefficient, 0.62 ± 0.04). However, in PIPKI γ -depleted cells Exo70 accumulated in the cytoplasm and showed little signal on the PM (Figure 3A). Intensity profiles of Exo70 throughout the control or PIPKI γ -depleted cell were determined and plotted using ImageJ (Figure 3A, bottom), which also supports the PM or cytoplasm distribution of Exo70 in

control or PIPKI γ -depleted cells, respectively. In addition, loss of PIPKI γ significantly decreased the association between E-cadherin and Exo70 (Figure 3C), supporting a role for PIPKI γ in scaffolding E-cadherin to Exo70. In the context that Exo70 mediates the polarized PM targeting of the exocyst (He *et al.*, 2007a), our results indicate that PIPKI γ could be the key molecule that orientates Exo70, the exocyst, and the exocyst-associated vesicles that carry E-cadherin to specific PM domains, that is, the forming E-cadherin adhesions. This recruitment could be an important mechanism that promotes E-cadherin clustering, AJ extension, and epithelial surface differentiation.

Exo70 is necessary for AJ assembly and epithelial polarization

To test this hypothesis, we first determined the function of Exo70 in the distribution and assembly of E-cadherin on the PM by depleting endogenous Exo70 from MCF-10A cells using a specific small interfering RNA (siRNA) (Liu *et al.*, 2009). Compared to control siRNA, Exo70-specific siRNA depleted more than 95% of endogenous Exo70 (Supplemental Figure S3A), without affecting the protein levels of other AJ components (E-cadherin and PIPKI γ) or of the exocyst (Sec8; Supplemental Figure S3A). However, E-cadherin staining in Exo70-depleted cells revealed punctate, filopodium-like intercellular adhesive structures (Figure 4B, right, arrows) instead of the linear, cohesive junctions observed in control cells (Figure 4B, left). When the vertical views of these images were obtained via image processing, we found that the E-cadherin signal was diffuse throughout the PM in Exo70-depleted cells (Figure 4B, middle, right) instead of being specifically clustered at the basolateral membrane as in control cells (Figure 4B, middle, left), indicating the loss of epithelial surface polarization. To obtain a better view of AJ structure and cell membrane cohesiveness, we processed these images using the Surface Renderer program. This provided a three-dimensional view in which different colors represent independent objectives without connection with each other (Figure 4B, bottom), which in Exo70-depleted cells revealed immature AJs that failed to expand and fuse into linear, cohesive adhesions. By this means, the occurrence of intermittent AJs and disconnected PMs between Exo70-depleted cells was reinforced (Figure 4B, bottom, right) when compared with the fused AJs and PMs in adjacent control cells (Figure 4B, bottom, left). These discontinued, filopodium adhesions between adjacent cells represent early stage of AJ assembly (Baum and Georgiou, 2011; Niessen *et al.*, 2011), indicating that lack of Exo70 impaired the maturation of AJs.

To determine the dynamics of E-cadherin assembly on the PM, MCF-10A cells treated with control or Exo70-specific siRNA were lifted by enzyme-free disassociation reagent and replated on type I collagen-coated coverslips. At variant time points, cells were fixed, stained for E-cadherin using indirect immunofluorescence labeling, and subjected to epifluorescence microscopy. Vertical views were reconstructed from Z-stack images to show the sides of the cells. As expected, cohesive, linear AJs were not observed in Exo70-depleted cells as in control cells in the time frame that we recorded (Figure 4C). Of greater interest, E-cadherin in control cells preferentially targeted to the sides of the PM as early as 15 min after replating when in Exo70-depleted cells E-cadherin showed even distribution along the PM. At later time points, E-cadherin signals in control cells were only observed on the sides of the cells and were increased in both size and intensity, indicating polarized distribution/targeting of E-cadherin on the PM, maturation of AJs, and formation of lateral membrane. In contrast, E-cadherin remained dispersed in Exo70-depleted cells until 60 min after replating (Figure 4C, right). These

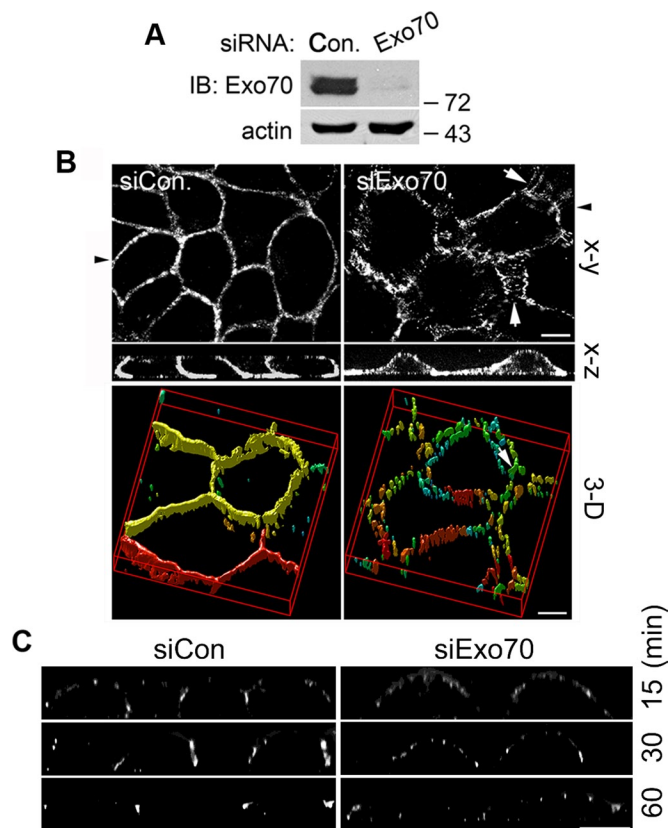


FIGURE 4: Exo70 is required for AJ assembly. (A, B) MCF-10A cells were transiently transfected with Exo70-specific (siExo70) or control siRNA (siCon.). Cells were then subjected to IB to determine Exo70 levels (A) or to indirect immunofluorescence microscopy to visualize E-cadherin in both horizontal (x - y) and vertical (x - z) directions (B). Three-dimensional views of E-cadherin staining in control or Exo70-depleted cells (bottom) were obtained from Z-stack images using the Surface Renderer function of Huygens 3.6. Single color represents individual objectives, which show large, cohesive E-cadherin adhesions between control cells but small, disconnected E-cadherin adhesions between Exo70-depleted cells. Arrows in B highlight filopodial adhesions between Exo70-depleted cells. Scale bars, 10 μ m. (C) Exo70 is required for the polarized distribution of E-cadherin on the PM. MCF-10A cells were lifted by enzyme-free cell disassociation reagent and replated on type I collagen-coated coverslips. At indicated time, cells were fixed with paraformaldehyde, and E-cadherin was visualized in these cells by indirect immunofluorescence microscopy. Vertical views of E-cadherin staining were reconstructed from Z-stack images. E-cadherin localized at the lateral membrane domain in control cells but diffused on the PM in Exo70-depleted cells at the indicated time points.

phenomena indicate that Exo70 is necessary for E-cadherin distribution on the PM, AJ assembly, and formation of lateral membrane domain. These events are very likely occurred simultaneously by promoting each other, as E-cadherin-mediated AJ assembly plays a key role in the initiation and development of epithelial polarity (Nejsum and Nelson, 2009; Niessen *et al.*, 2011).

Therefore we determined whether impaired AJ assembly caused by Exo70 depletion affects epithelial morphology. As shown in Supplemental Figure S3B, tight junctions represented by ZO-1 staining were also broken into discontinued, filopodium-like extensions in Exo70-depleted cells compared with control cells (Supplemental Figure S3B, left). Bright-field images showed a flattened, mesenchymal-like morphology for Exo70-depleted cells compared with polar-

ized control cells, supporting the loss of apical-lateral polarity (Supplemental Figure S3B, middle). In addition, vinculin targeted to focal adhesions in Exo70-depleted cells compared with its diffuse cytoplasmic localization in polarized control cells (Supplemental Figure S3B, right), confirming the morphological transition. Together, our results strongly supported an important role for Exo70 in the organization of cohesive, mature E-cadherin adhesions, which subsequently influences epithelial surface polarization (Nejsum and Nelson, 2009).

Exo70 is required for the polarized targeting and clustering of E-cadherin on the PM

As shown in Figure 4B, the dispersed E-cadherin molecules on the surface of Exo70-depleted cells were able to mediate intercellular contacts, but these junctions failed to extend and fuse into cohesive adhesions. This could be the result of ineffective E-cadherin clustering caused by random E-cadherin insertion in the PM. Because Exo70 is known to direct the polarized membrane tethering of cargoes, we proposed that Exo70 regulates the clustering of E-cadherin on the PM, which is critical for assembly and maturation of AJs. To test this possibility, we expressed green fluorescent protein (GFP)-fused E-cadherin in MCF-10A cells and then performed an E-cadherin adhesion assay by plating cells on glass coverslips coated with purified E-cadherin ectodomain (hE/Fc). Cells were subjected to indirect immunofluorescence 1 h after plating and then analyzed by total internal reflection fluorescence (TIRF) microscopy, which eliminates the cytoplasm signals and allows precise observation in 100-nm depth above and on the basal membrane. By this means, the clustering of E-cadherin and AJ assembly occur at the basal membrane of the cell and can be recorded in live cells. As shown in Figure 5A, GFP-E-cadherin in normal cells formed strong focal adhesion-like structures on the hE/Fc substrata 1 h after being plated on hE/Fc, indicating rapid E-cadherin clustering that promoted the growth of E-cadherin junctions. However, in Exo70-depleted cells, GFP-E-cadherin staining on the basal membrane was diffuse (Figure 5A), suggesting unsystematic insertion of E-cadherin, impaired E-cadherin clustering, and accidental AJ assembly on the membrane-substrata interface. To further explore this possibility, we tracked the assembly of E-cadherin adhesions on the hE/Fc substrata over time with TIRF microscopy. As shown in Figure 5B, GFP-E-cadherin signal rapidly accumulated and assemble into large adhesion patches at the basal surface of control cells (top, arrows), indicating efficient E-cadherin clustering and AJ expansion. However, little expansion was observed with the small, punctate E-cadherin adhesions in Exo70-depleted cells (Figure 5B, bottom). These data support an essential role for Exo70 in AJ maturation by organizing the targeted delivery and clustering of E-cadherin at the adhesion interface.

In addition, we determined the PM targeting of E-cadherin. To do this, we applied calcium depletion followed by calcium restoration in MCF-10A cells, which creates a low E-cadherin signal background on the PM and allows us to observe the targeting of E-cadherin to the cell surface. Calcium depletion was performed at 19°C to ensure the retention of internalized E-cadherin in recycling endosomes instead of its transport to late endosomes for degradation (Le *et al.*, 1999). E-cadherin was then visualized at various time points after calcium restoration by indirect immunofluorescence using an antibody that recognizes the extracellular domain of E-cadherin. As shown in Supplemental Figure S4A, loss of Exo70 delayed the targeting of E-cadherin to the PM. The intensity profiles of E-cadherin signals in control and Exo70-depleted cells were determined as shown in Supplemental Figure S4B. At each time point, E-cadherin intensities on the PM were quantified in more than

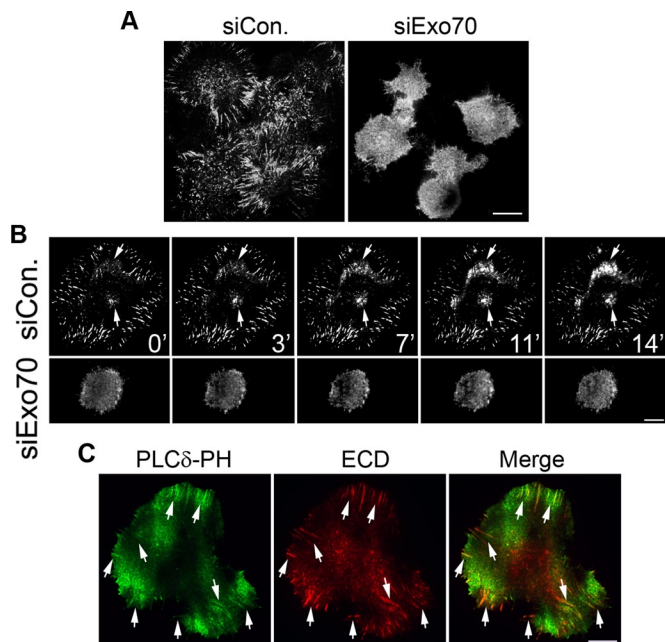


FIGURE 5: Exo70 regulates the clustering of E-cadherin on the PM and AJ assembly. (A) Control (siCon.) or Exo70-depleted (siExo70) cells expressing GFP-tagged E-cadherin were plated on coverslips coated with purified recombinant E-cadherin ectodomain (hE/Fc) for 1 h. These cells were then fixed and subjected to indirect immunofluorescence microscopy. Control cells spread rapidly on the hE/Fc substrata and formed strong E-cadherin cluster adhesions. However, Exo70-depleted cells failed to spread, and no E-cadherin clustering was observed. (B) Control (siCon.) or Exo70-depleted (siExo70) cells expressing GFP-tagged E-cadherin were plated on hE/Fc-coated, glass-bottom Petri dishes for 30 min. Live cells were then subjected to real-time imaging with TIRF microscope for 15 min with 1-min interval. Formation and dynamics of GFP-E-cadherin-mediated adhesions on the hE/Fc substrata were recorded. Arrows show the rapid accumulation of GFP signal and formation of large E-cadherin adhesion patches at the basal surface of control cells. However, in Exo70-depleted cells, E-cadherin adhesions are small, and no growth or extension of GFP signal was observed during the recording, indicating random, inefficient formation of E-cadherin adhesions. (C) PI4,5P₂ enriches at assembly sites of E-cadherin adhesions. Cells were transfected with GFP-fused PLCδ-PH domain for 16 h and then were plated on hE/Fc-coated coverslips. At 1 h postplating, cells were subjected to indirect immunofluorescence using anti-E-cadherin antibody and observed under TIRF microscope. PI4,5P₂ distribution on the basal membrane was represented by GFP signals (PLCδ-PH, green channel), and E-cadherin (ECD) was visualized at the red channel. Arrowheads show the colocalization of PI4,5P₂ pools and E-cadherin adhesions.

200 cells and plotted (Supplemental Figure S4A, bottom), which confirmed that the PM targeting of E-cadherin was impaired in the absence of Exo70. Because Exo70 regulates the transport of other lateral cargoes (Grindstaff *et al.*, 1998), we examined the membrane targeting of GFP-fused vesicular stomatitis virus G (VSVG; Liu *et al.*, 2007). As shown in Supplemental Figure S4C, GFP-VSVG trafficking to the PM was also delayed when Exo70 was missing, which further supports the conclusion that Exo70 is necessary for the transport of lateral cargoes.

Because Exo70 directs the PM targeting of the exocyst to guide vesicle tethering and polarized exocytosis (He *et al.*, 2007a, 2007b), we propose that the impaired PM targeting and distribution of E-cadherin observed in Exo70-depleted cells results from interrupted

PM targeting of the exocyst. Indeed, levels of the PM-associated Sec6 and Sec8 were significantly lower in Exo70-depleted cells than with control cells (Supplemental Figure S5A). We observed much less PM-associated E-cadherin when Exo70 was knocked down (Supplemental Figure S5A), consistent with our observations described in Supplemental Figure S3A. In addition, loss of Exo70 resulted in a significant decrease of PM-associated epidermal growth factor receptor, which is transported by the exocyst, but had little effect on PM-associated Na/K ATPase, which is independent of the exocyst (Supplemental Figure S5A). Levels of both proteins in total cell lysate were not affected by depleting Exo70.

These results suggest that by recruiting exocyst-associated vesicles, Exo70 mediates intentional E-cadherin targeting to existing E-cadherin on the PM, which facilitates E-cadherin clustering and AJ extension and maturation. In addition, Exo70 at AJs orients the arrival of other lateral cargoes carried by the exocyst, which promotes the differentiation of the basolateral membrane from the apical domain. Hence, our results provide direct evidence that supports an important role of Exo70 in epithelial polarization by orchestrating AJ assembly and lateral membrane transport.

PIPK1 γ -generated PI4,5P₂ is necessary for Exo70 to target to the PM and regulate AJ assembly

We showed that PIPK1 γ is necessary for Exo70 to associate with E-cadherin and target to AJs via a direct interaction with Exo70 (Figures 1–3). In the context that PI4,5P₂ is critical for Exo70 targeting to the PM (Liu *et al.*, 2007), we propose that PIPK1 γ -generated PI4,5P₂ pools at E-cadherin assembly sites may function as cues on the PM to guide the targeting of Exo70 and, subsequently, the exocyst and associated lateral cargoes like E-cadherin. Of interest, we indeed observe accumulation of PI4,5P₂ (represented by GFP-fused PH domain of PLCδ, which specifically binds to PI4,5P₂ and is widely used as a PI4,5P₂ marker) at E-cadherin clusters in hE/Fc adhesion assay, which strongly argues for the existence of regional PI4,5P₂ pools at E-cadherin clustering and AJ assembly sites, which likely function as landmarks for Exo70 targeting, since depletion of PIPK1 γ eliminates this PI4,5P₂ accumulation (data not shown).

To test this possibility, we first examined whether Exo70 requires PI4,5P₂ to regulate AJ assembly. For this purpose, RNA interference (RNAi)-resistant wild-type or mutated (PI4,5P₂-binding deficient) rat Exo70 (rExo70 and *exo70-1*, respectively) was introduced to MCF-10A cells in which endogenous Exo70 had been knocked down by siRNA. As shown in Supplemental Figure S5B, wild-type and mutated rExo70 were expressed and pulled down equally well by an anti-PIP1 γ antibody, indicating that PI4,5P₂ binding did not affect Exo70's association with PIP1 γ . However, *exo70-1* did not target to the PM and failed to rescue the filopodium-like junctions caused by depleting endogenous Exo70 (Figure 6A, bottom, arrow). In contrast, wild-type rExo70 targeted to the PM, and transfected cells formed cohesive E-cadherin adhesions (Figure 6A, top, arrowhead) compared with nontransfected cells in which abnormal E-cadherin adhesions were observed (Figure 6A, top, green channel, arrow). To analyze the effect of exogenous Exo70 on AJ assembly, we quantified the fluorescence intensity of E-cadherin along a line crossing neighboring cells that expressed exogenous wild-type or mutated rExo70 (Figure 6A, merge). As shown in Figure 6B, E-cadherin intensity showed a single peak at the contacting PM of cells expressing wild-type rExo70, indicating efficient membrane transport of E-cadherin and formation of cohesive junctions. However, cells expressing *exo70-1* formed filopodium-like adhesions, and the nearby PMs failed to fuse (represented by two peaks). Cells that form cohesive junctions (E-cadherin intensity profile showed a single peak at the

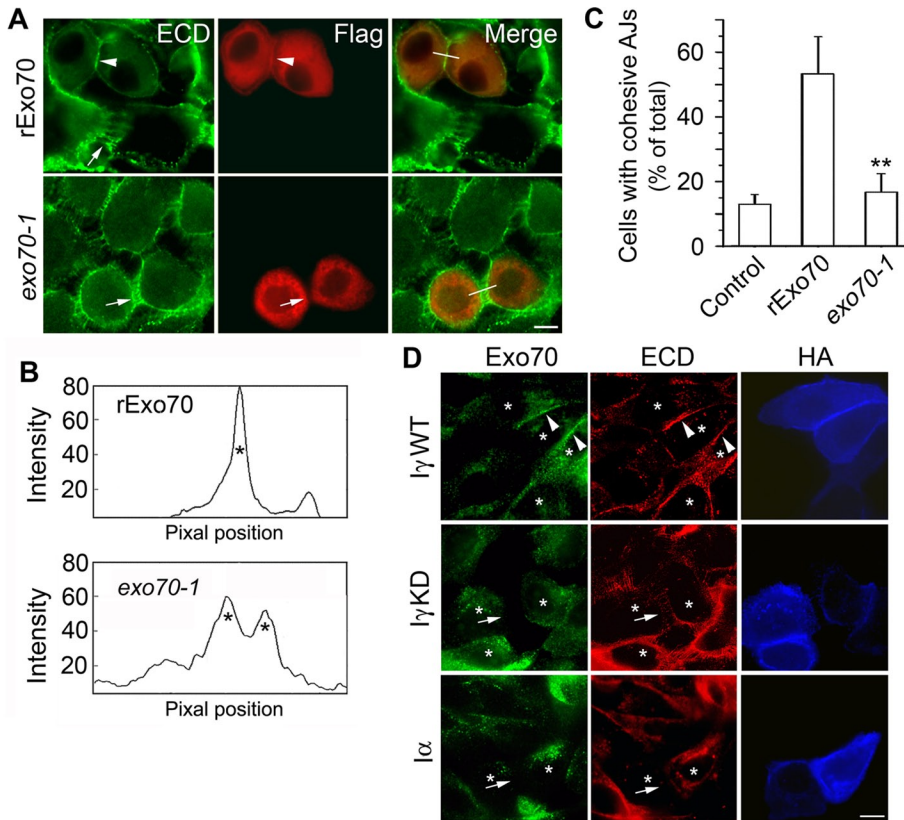


FIGURE 6: PIPKI γ -generated PI(4,5)P₂ regulates the PM targeting and function of Exo70. (A) RNAi-resistant, FLAG-tagged rat Exo70 (rExo70) or *exo70-1* was introduced into Exo70-depleted MCF-10A cells by transient transfection. Then cells were subjected to indirect immunofluorescence microscopy to visualize endogenous E-cadherin (ECD, green) and FLAG-Exo70 (Flag, red). Wild-type rExo70 targeted to the PM (arrowhead, red channel) and rescued AJ assembly (arrowhead, green channel) are compared with nontransfected cells (arrows, green channel). However, overexpressed *exo70-1* did not target to the PM (arrow, red channel) and could not rescue the assembly of AJs (arrow, green channel). Scale bar, 10 μ m. (B) E-cadherin intensity profiles along the straight line in A (merge) were quantified and plotted by ImageJ 1.43. The peak (asterisk) represents E-cadherin accumulation on the PM. (C) Wild-type rExo70, but not *exo70-1*, rescued the assembly of cohesive AJs. Exo70-depleted cells in A were divided into three groups: nontransfected, expressing wild-type rExo70, or expressing *exo70-1*. Ninety pairs of cells were picked randomly from each group and analyzed as described in B. Cells that formed cohesive AJs (single peak in B) were quantified and plotted using SigmaPlot 8.0. Results reflect the mean \pm SD from three independent experiments. ***p* < 0.01. (D) PIPKI γ -depleted MDCK cells were transfected with hemagglutinin-tagged, RNAi-resistant, wild-type mouse PIPKI γ (I γ WT), kinase-dead mouse PIPKI γ (I γ KD), or wild-type human PIPKI α . Exo70 (green), E-cadherin (ECD, red), and HA-tagged PIPKIs (HA, blue) were visualized by immunofluorescence microscopy 24 h later. Adjacent PIPKI γ -depleted cells expressing exogenous PIPKI γ or PIPKI α are marked by an asterisk. PM recruitment of Exo70 and cohesive AJs were observed in cells expressing wild-type PIPKI γ (arrowheads) but not in cells expressing kinase-dead PIPKI γ or wild-type PIPKI α (arrows). Scale bars, 10 μ m.

contacting PM) were quantified in 90 pairs (adjacent to each other) of nontransfected cells or cells expressing rExo70 or *exo70-1*, respectively. Then the data were analyzed and plotted in Figure 6C, which clearly shows that rExo70, but not *exo70-1*, significantly improves the maturation of AJs. Therefore, although the association of Exo70 and PIPKI γ is independent of PI4,5P₂ binding (Supplemental Figure S5B), PI4,5P₂ is essential for Exo70 to facilitate E-cadherin clustering and AJ assembly (Figure 6, A–C). Because Exo70 binds to PI4,5P₂, we examined whether alteration of Exo70 changes the levels of PI4,5P₂. As shown in Supplemental Figure S6, neither depletion of endogenous nor overexpression of exogenous Exo70 altered the global PI4,5P₂ levels.

Next we determined whether PIPKI γ is the kinase that supplies PI4,5P₂ to Exo70. For this purpose, we constructed wild-type mouse PIPKI γ and a kinase-dead mutant that is resistant to the PIPKI γ -specific shRNA. These constructs were transiently expressed in MDCK cells in which endogenous PIPKI γ had been knocked down by Lentivirus-based shRNA. As shown in Figure 6D, Exo70 in cells expressing wild-type PIPKI γ was efficiently recruited to the PM. Consequently, E-cadherin in these cells was transported to the PM, and AJs assembled properly (Figure 6D, top). However, expression of the kinase-dead mutant of PIPKI γ (Figure 6D, middle) or of PIPKI α (Figure 6D, bottom) did not improve the PM targeting of Exo70 and E-cadherin or AJ assembly compared with nontransfected cells, indicating that PIPKI γ is necessary for the targeting of Exo70 to the PM at forming E-cadherin junctions. Thus PIPKI γ -generated PI4,5P₂ pools at nascent E-cadherin adhesions and the physical interaction between PIPKI γ and Exo70 likely complement each other to facilitate recruitment of Exo70 to E-cadherin assembly sites. These events could be critical to ensure E-cadherin transport to the correct PM locales to facilitate E-cadherin clustering and extension of transient AJs.

Our observations are summarized in Figure 7. In this model, we propose that PIPKI γ creates regional PI4,5P₂ pools at nascent intercellular contacts via a direct interaction with E-cadherin. These PI4,5P₂ pools orient the targeting of Exo70 to nascent E-cadherin adhesions, which are likely assembled randomly upon adjacent cell surfaces contacting with one another. Then, PIPKI γ , PIPKI γ -generated PI4,5P₂, and Exo70 together form a cue to guide the directed targeting and clustering of E-cadherin at these newly formed E-cadherin adhesions, which promotes AJ expansion and maturation. In addition, this cue can be important for the targeted delivery of other exocyst-dependent lateral cargoes and therefore defines the E-cadherin assembly sites as nascent lateral membrane patches. By this means, PIPKI γ and Exo70 cooperate for AJ assembly and apical–lateral polarization.

DISCUSSION

E-Cadherin-mediated assembly of AJs not only is essential for cell-to-cell cohesion in solid tissue, but also initiates and promotes the apical–lateral polarization of epithelial cells (Langevin *et al.*, 2005; Blankenship *et al.*, 2007; Georgiou *et al.*, 2008; Leibfried *et al.*, 2008; Wirtz-Peitz and Zallen, 2009). After bringing native cell surfaces to contact via a calcium-dependent homophilic *trans*-binding, E-cadherin molecules undergo a lateral organization on the cell surface that promotes the formation of stable, cohesive AJs (Niessen

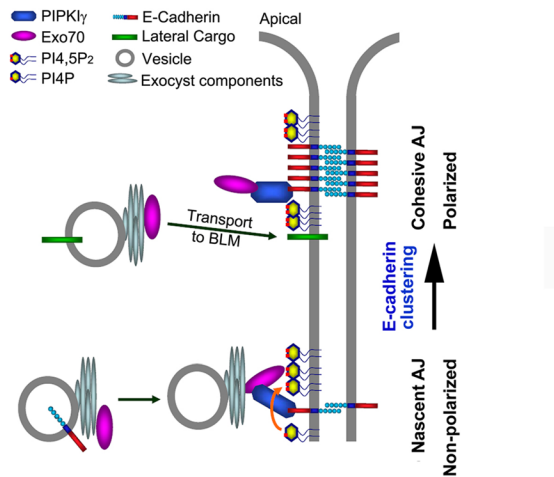


FIGURE 7: PIPK1 γ facilitates AJ maturation and epithelial polarization by recruiting Exo70 to newly formed E-cadherin adhesions. PIPK1 γ , by interacting with E-cadherin, generates regional PI4,5P₂ pools on the PM that serve as landmarks to direct the targeting of Exo70 to nascent AJs. The direct interaction between Exo70 and PIPK1 γ further strengthens the association of Exo70 with E-cadherin adhesions. Exo70 then directs the targeting of the exocyst and exocyst-bound lateral cargoes, such as E-cadherin, to these assembling AJs. In summary, PIPK1 γ and Exo70 function together to identify lateral membrane patches at newly formed E-cadherin adhesions. This mechanism not only contributes to E-cadherin clustering and AJ maturation, but also helps to orchestrate E-cadherin signaling, AJ assembly, and epithelial surface polarization.

et al., 2011). This dynamic process includes the formation of *cis*-oligomer and the organization of larger-scale lateral E-cadherin clusters (Niessen et al., 2011). Compared to our understanding of E-cadherin dimerization (Patel et al., 2003; Harris and Tepass, 2010), it remains vague whether and how exocytosis of cadherins can be oriented to specific regions on the cell surface, thereby supporting the regional accumulation of E-cadherin to promote E-cadherin clustering. It was reported that in *Drosophila* the exocyst recruits E-cadherin to the PM (Langevin et al., 2005), and the Sec6/8 complex was observed to associate with E-cadherin complexes (Yeaman et al., 2004). Because the exocyst defines the docking site on the PM for secretory vesicles carrying lateral cargoes (He and Guo, 2009), it was proposed that the exocyst might mediate the targeted delivery of E-cadherin to the PM. However, whether the exocyst exclusively defines cortical targeting has yet to be directly tested for E-cadherin, in contrast to other basolateral membrane markers (Grindstaff et al., 1998). Moreover, how the exocyst associates with E-cadherin and AJs is not known.

The PM targeting of Exo70 has been shown to be critical for exocyst-mediated polarized exocytosis (He et al., 2007a, 2007b; Liu et al., 2007). Several lines of evidence support that the membrane targeting of Exo70 depends on the binding of PI4,5P₂ in various organisms, including yeast (He et al., 2007b; He and Guo, 2009), fruit flies (Fabian et al., 2010), and mammalian cells (Liu et al., 2007). However, it was proposed that PI4,5P₂ preferentially accumulated on the apical membrane of polarized epithelial cells to direct the exocytosis of apical cargoes (Martin-Belmonte et al., 2007). In addition, PI4,5P₂ distribution on the lateral membrane has also been observed (Martin-Belmonte et al., 2007; our unpublished results). In the context that Exo70 targets to the lateral membrane and helps lateral transport in polarized epithelial cells, how PI4,5P₂ guides the lateral targeting of Exo70, the exocyst, and lateral cargoes like

E-cadherin is not known. Here we showed that PIPK1 γ , which binds to the cytoplasmic domain of E-cadherin (Ling et al., 2007), directly interacts with Exo70, and its lipid kinase activity is necessary for the PM targeting of Exo70. PIPK1 α , which also increases PI4,5P₂ levels on the PM, does not interact with E-cadherin (Ling et al., 2007) or Exo70 and has no effect on the targeting of Exo70 to the PM when overexpressed in cells. This indicates that the physical interaction with PIPK1 γ is important for Exo70 targeting, plausibly by strengthening the association between Exo70 and E-cadherin. These results suggest that PIPK1 γ , by interacting with Exo70 and creating regional PI4,5P₂ pools at E-cadherin assembly sites, can orient the allocation of cytoplasmic Exo70. This working model may help us to understand how the stable PI4,5P₂ pools on the PM serve the PI4,5P₂-dependent events that need to be regulated spatiotemporally such as actin reorganization, focal adhesion assembly, and subcellular vesicle transportation. Indeed, depletion of PIPK1 γ does not cause obvious change in global PI4,5P₂ levels (Bairstow et al., 2006). However, it inhibits specific subcellular events like transferrin uptake (Bairstow et al., 2006), focal adhesion assembly (Di Paolo et al., 2002; Ling et al., 2002), cell migration and invasion (Sun et al., 2007, 2010), and epithelial polarization (Ling et al., 2007) by interacting with and regulating regional binding partners and/or PI4,5P₂ effectors. These results suggest that the specific subcellular targeting of a PI4,5P₂ generator (i.e., PIPK1 γ) is critical for the regional modulation of PI4,5P₂ pools, which may be subtle but efficient enough to serve the local PI4,5P₂-dependent events and therefore ensure spatiotemporal regulation.

Because Exo70 is one of the two membrane-targeting subunits of the exocyst (He et al., 2007a; Zhang et al., 2008), our present result that Exo70 directly interacts with PIPK1 γ and E-cadherin supports the previous hypothesis that the exocyst regulates the targeted delivery of E-cadherin on the PM (Yeaman et al., 2004) and suggests that Exo70 is the key to direct this event. Furthermore, in cells lacking Exo70, we observed decreased PM association of the exocyst, impaired PM targeting of E-cadherin, and loss of E-cadherin clustering at the adhering interface. This is direct evidence that supports the following points: first, the exocyst regulates E-cadherin regional accumulation via targeted delivery of E-cadherin to the cell surface; second, Exo70 directs the orientation of the exocyst in this process; third, this targeted delivery is critical for E-cadherin clustering on the PM, which drives the extension of forming AJs (Niessen et al., 2011). Exo70-depleted cells can still deliver E-cadherin to the cell surface but likely in a random manner, and the newly formed E-cadherin contacts in these cells cannot be expanded efficiently. These immature E-cadherin adhesions could be unstable and lead to increased internalization of surface E-cadherin, which may contribute to the decreased PM-associated E-cadherin or slower surface targeting of E-cadherin as observed in Exo70-depleted cells. Indeed, β -catenin was also proposed to mediate the targeting of the exocyst and directed PM delivery of E-cadherin by interacting with Sec10 (Yeaman et al., 2004). Although this hypothesis has not been tested directly, it is quite plausible that PIPK1 γ –Exo70 interaction and β -catenin–Sec10 interaction may function redundantly to ensure the normal assembly of AJs. In addition, the E-cadherin/ α -catenin chimera has been shown to be functional in many aspects, including mediating intercellular adhesion (Qin et al., 2005), likely because this chimera still can bind to actin cytoskeleton via the C-terminus of α -catenin although the β -catenin-binding site has been removed. However, we observed that this mutant could neither interact with PIPK1 γ (Ling et al., 2007) nor associate with Exo70 (Figure 2C). One possibility is that the reorganization of actin cytoskeleton stimulated by nascent E-cadherin adhesion (mediated by the

E-cadherin/ α -catenin chimera) could have a positive feedback on E-cadherin clustering on the PM. This could be another reason that cells lacking Exo70 can eventually form E-cadherin adhesions and polarize, although much more slowly than with control cells (4–5 d vs. 18–20 h). E-Cadherin plays an essential role in the formation and function of tissues and organs and disease development such as cancers. It is not surprising that the transport and assembly of E-cadherin are regulated at multiple levels so that compensations could be available when one of these regulations is interrupted. It will be interesting to follow up these possibilities in future studies.

It has been widely accepted that E-cadherin, by mediating AJ assembly, plays a key role in the two aspects of epithelial morphogenesis: intercellular cohesion and apical–lateral polarization. Indeed, Exo70-depleted cells could not form mature, cohesive AJs, and the cohesion between adjacent cells was substantially interrupted. These cells also showed loss of the apical–lateral epithelial polarity. In addition, membrane targeting of lateral cargo VSVG was interrupted in Exo70-depleted cells. In the context that the exocyst is necessary for the lateral targeting of multiple lateral cargoes in epithelial cells (Grindstaff *et al.*, 1998; He and Guo, 2009), our present results provide a solid foundation for the hypothesis that targeting of Exo70 to forming AJs defines these surface sites as nascent lateral membrane patches and guides the arrival of other exocyst-dependent lateral cargoes. In this way, Exo70 mediates the communication between AJ assembly and epithelial surface polarization. Furthermore, Sec3 is recruited to the PM in an E-cadherin–dependent manner, and Sec3-containing exocyst complexes specifically regulate the assembly and maintenance of desmosomes (Andersen and Yeaman, 2010). In the present study we showed that Exo70 plays a similar role in AJ maturation via an association with E-cadherin. These results support the importance of the exocyst in apical–lateral surface polarization and epithelial morphogenesis. Exo70 and Sec3, the two membrane-targeting subunits of the exocyst, likely play distinct roles in this process by recognizing sites of two types of intercellular adhesions. These proteins could cooperate to establish and maintain epithelial morphology. Although the PM targeting of yeast Sec3 also requires PI4,5P₂ (Zhang *et al.*, 2008; Yamashita *et al.*, 2010), it is not known whether its mammalian counterpart behaves in the same way. If it does, the E-cadherin dependence of Sec3 targeting (Andersen and Yeaman, 2010) suggests the interesting possibility that PIPK1 γ may also contribute to Sec3 targeting and desmosome assembly by generating PI4,5P₂ at E-cadherin adhesions. However, it is not known whether Sec3 and other exocyst subunits can directly bind to PIPK1 γ or E-cadherin. This should be explored in the future to help understand the whole picture of the association between E-cadherin and the exocyst.

The *trans*-interaction between E-cadherin molecules on the native surface of neighboring cells not only initiates AJ assembly, but also triggers intracellular signaling that activates the remodeling of actin cytoskeleton, which stabilizes nascent adhesions and promotes E-cadherin clustering and AJ maturation (le Duc *et al.*, 2010; Liu *et al.*, 2010; Smutny *et al.*, 2010; Niessen *et al.*, 2011). The cooperation between E-cadherin assembly and actin remodeling is critical for epithelial morphogenesis (Zhang *et al.*, 2005; Mege *et al.*, 2006). PI4,5P₂ is an important regulator of actin reorganization (Janmey and Lindberg, 2004) and AJ formation (Watabe-Uchida *et al.*, 1998; Janmey and Lindberg, 2004; Niggli, 2005; Maddugoda *et al.*, 2007; Spudich *et al.*, 2007; Zemljic-Harpe *et al.*, 2007). It is plausible that PIPK1 γ -generated local PI4,5P₂ pools not only serve Exo70 targeting and E-cadherin transport and assembly, but also are necessary for regional reconstruction of the actin cytoskeleton to facilitate the expansion of nascent AJs. Current literature sup-

ports a model in which Rac1-dependent lamellae formation is necessary for the initiation of E-cadherin adhesion (Ehrlich *et al.*, 2002) and RhoA facilitates the extension of AJs by promoting the configuration of cortical actin bundles (Yamada and Nelson, 2007). In this context, the fact that PIPK1 γ functions upstream of Rho (Mao *et al.*, 2009) further supports the possibility for multiple roles of PIPK1 γ in AJ assembly and epithelial polarization. In addition, we observed a decrease of RhoA activity accompanied with Exo70 depletion, whereas Rac1 and Cdc42 were not affected (data not shown), indicating the cooperation between polarized cargo transport and actin remodeling during AJ assembly. In addition, Exo70 was revealed to bind Arp2/3 and facilitate the formation of actin-based membrane protrusions (Zuo *et al.*, 2006), suggesting another possible connection between the PIPK1 γ –Exo70 complex, regional actin assembly, and AJ maturation. We reported previously that PIPK1 γ , by interacting with clathrin adaptor complex AP1B, mediates the sorting of E-cadherin from *trans*-Golgi network to recycling endosome (Ling *et al.*, 2007). Our present results suggest that PIPK1 γ has multiple roles during the transport and assembly of E-cadherin. Epithelial morphogenesis is a tightly controlled orchestration of E-cadherin signaling, lateral vesicle transport, and actin reorganization. As observed *in vitro* and *in vivo*, engagement of E-cadherin on the adjacent cell surface initiates the assembly of nascent adhesions on contacting membrane protrusions. Although it is possible that E-cadherin may directly interact with other subunits of the exocyst or there is another adapting protein between E-cadherin and the exocyst other than PIPK1 γ , we propose that PIPK1 γ , by generating PI4,5P₂ at E-cadherin assembly sites and mediating the recruitment of Exo70, engages targeted E-cadherin delivery and actin remodeling and finely tunes their cooperation to achieve mature, cohesive AJs and apical–lateral polarization.

MATERIAL AND METHODS

Constructs and antibodies

The coding regions of human Exo70 and its truncations were amplified from total cDNA of the MCF-10A cell line and cloned into pET42a (Novagen, Gibbstown, NJ). shRNA-expressing vectors were constructed by cloning shRNA oligonucleotides (Invitrogen, Carlsbad, CA) into the pLKO.1 vector (AddGene, Cambridge, MA). The shRNA sequence targeting pan-PIPK1 γ was 5'-GGACCUGGACUUC AUGCAG-3'. The siRNA sequence targeting human Exo70 was 5'-GGUUAAAGGUGACUGAUUA-3'. An interfering RNA sequence targeting luciferase (5'-GUACCUGUACUUCAUGCAG-3') was used as negative control. All constructs were confirmed by sequencing.

The following antibodies were used for Western blotting and immunofluorescence: anti-FLAG (Millipore, Billerica, MA); anti-GST (Novagen); anti-E-cadherin, anti-Sec8, and anti-vinculin (BD Biosciences, San Diego, CA); and anti-E-cadherin (ectodomain), anti-Sec6, and anti-actin (Sigma-Aldrich, St. Louis, MO). The mouse monoclonal anti-Exo70 antibody was a gift from S.-C. Hsu (Rutgers University, Piscataway, NJ), and the polyclonal PIPK1 γ antibody was generated as described previously (Ling *et al.*, 2002). Secondary antibodies were from Invitrogen and Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell culture and transfection

MDCK (Clontech Laboratories, Mountain View, CA), HEK293, and 293FT cells were cultured in DMEM (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Invitrogen). The nontumorigenic mammary epithelial cell line MCF10A was cultured in DMEM/F12 (Invitrogen) medium supplemented with 5% horse serum (Invitrogen), 10 μ g/ml insulin (Invitrogen), 0.5 μ g/ml

hydrocortisone (Sigma-Aldrich), 20 ng/ml recombinant epidermal growth factor (PeproTech, Rocky Hill, NJ), and 100 ng/ml cholera toxin (Sigma-Aldrich). MDCK cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For Exo70 siRNA knockdown, MCF-10A cells were transfected twice at 48-h intervals with 75 nM siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Cells were analyzed 48 h after the second transfection. In rescue experiments, these Exo70-depleted MCF-10A cells were infected with *Lentivirus* carrying rExo70 or exo70-1, and analysis was performed 24 h after.

Indirect immunofluorescence and total internal reflection fluorescence microscopy

Indirect immunofluorescence microscopy was performed as described previously (Ling *et al.*, 2002). Briefly, cells were grown on coverslips, washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde at room temperature for 10 min, washed, permeabilized for 10 min with 0.2% Triton X-100, and blocked with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature. The coverslips were incubated sequentially with appropriate primary and secondary antibodies for fluorescence observation using a Plan ApoChromat 60x, 1.49 numerical aperture oil objective (Nikon, Melville, NY) on an imaging microscope (TE 2000-U; Nikon). Z-Series were created by scanning sequentially at 0.2- μ m steps. Single sections were exported to Photoshop CS2 (Adobe, San Jose, CA) for final image processing. Some images were deconvolved and surface rendered by Huygens, version 3.6 (Scientific Volume Imaging, Hilversum, Netherlands), as indicated. Fluorescence intensity was quantified using ImageJ 1.43 (National Institutes of Health, Bethesda, MD). TIRF microscopy was carried out using an Olympus attachment for the IX70 microscope as reported (Singh *et al.*, 2010).

Immunoprecipitation, GST pull-down assay, and immunoblotting

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, and 10% glycerol) and then used for immunoprecipitation (Ling *et al.*, 2002). The immunocomplexes were separated by SDS-PAGE and analyzed. Unless otherwise stated, immunoprecipitation was performed using 1 ml of cell lysate from one confluent 100-mm dish. pET28 and pET42 constructs were transformed into BL21(DE3) (Novagen). Proteins were expressed and purified using His resin following the manufacturer's instructions (Novagen). For GST pull-down assays, 1 μ g of GST or GST-Exo70 or Exo70 truncations was incubated with 1 μ g of appropriate His-tagged PIPK1 proteins, together with Glutathione Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ) in 500 μ l of buffer A (PBS, 1% Triton X-100) for 3 h at 4°C. The beads were washed with 1 ml buffer of A three times and analyzed by Western blot. One microgram of each purified protein was used for GST pull-down assay. Half of the GST beads for each pull-down were loaded on the gel, and 25 μ l of each purified protein was loaded as an input control. Images were scanned and exported to Photoshop CS2 for final processing. Band intensities were quantified using ImageJ 1.43.

Lentivirus

293FT cells were cotransfected with pLKO.1-puro carrying control or PIPK1 γ -specific shRNA or pLOVE carrying rExo70 or exo70-1, pCMV-VSVG, and pCMV-dR8.91 dvpr (gifts from Gaoxiang Ge, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) at a weight ratio of 5:2:3 using

the calcium phosphate–DNA coprecipitation method. Seven hours after transfection, the medium was changed to 10% FBS/DMEM. Supernatants were collected 48–72 h after transfection and concentrated using PEG-it Virus Precipitation Solution (System Biosciences, Mountain View, CA). MCF-10A or MDCK cells were transduced in the presence of 8 μ g/ml polybrene (Sigma-Aldrich). To get better depletion of PIPK1 γ from MCF-10A cells, stable multi-MCF-10A cell clones were selected by 2.5 μ g/ml puromycin 48 h postinfection. MDCK cells were infected twice at a 48-h interval to achieve PIPK1 γ depletion 48 h after the second infection.

Statistical analysis

Data were tested for significance using Student's *t* test by OriginPro 7.0 software (OriginLab, Northampton, MA).

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Supplementary Information

An Association between Type I γ PI4,5P₂ Kinase and Exo70 Directs E-cadherin Clustering and Formation of the Lateral Membrane Domain

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SI Method

Calcium Depletion and Recycling of E-cadherin. These analyses were performed as described previously (Ling *et al.*, 2007). In brief, MCF10A cells were transfected twice with Exo70 siRNA and grown on cover slips. Luciferase siRNA was used as negative control. Forty-eight hours after the second transfection, cells were incubated with 5 mM EGTA at 37 °C for 45 min, then changed to complete medium containing 5 mM CaCl₂ and cultured for 5 min, 15 min, 30 min, or 60 min, respectively. To perform indirect immunofluorescence, cover slips were immediately washed twice in ice-cold PBS, then incubated with rat anti-ecto-E-cadherin antibody at 4 °C for 6 h. Cells were then fixed and stained with anti-rat Alexa555 and FITC-conjugated mouse E-cadherin antibody.

VSV-G Trafficking Assay. The VSV-G trafficking assay was performed as described in (Liu *et al.*, 2007). Briefly, MCF-10A cells were transfected twice with control or Exo70-specific siRNA. Twenty-four hours after the second siRNA treatment, MCF-10A cells were transfected with the VSV-G-45ts-GFP mutant and immediately placed at 40 °C. Twelve hours later, the cells were shifted to 32 °C in the presence of 100 μ g/ml cycloheximide (Sigma-Aldrich). After 0, 30, 60, and 120 min of growth, cells were fixed immediately for GFP observation.

Plasma Membrane Fractionation. After being treated with control or Exo70 siRNA, MCF-10A cells were collected in buffer A (0.225 M sucrose, 20 mM HEPES-OH, pH 7.4, 1 mM EDTA) and homogenized in a 5-ml microtissue grinder with 20 strokes by hand. Plasma membrane was isolated as described previously (Liu *et al.*, 2007). Briefly, the homogenate was centrifuged at 19,000 g for 20 min. The pellet was resuspended in 3 ml buffer A and layered onto 10 ml buffer B (1.12 M sucrose, 20 mM HEPES-OH, pH7.4, 1 mM EDTA) in a 13-ml polyallomer tube and centrifuged at 100,000g for 60 min. About 1 ml interface solution was collected and mixed with 4 ml buffer A. The PM fraction was obtained from the pellet after another centrifugation at 41,000g for 20 min. All steps were performed at 4 °C in the presence of a protease inhibitor cocktail (Research Products International Corp.).

Cellular PI(4,5)P₂ Level Determination. The PI4,5P₂ was extracted and determined using the PI4,5P₂ Mass ELISA Assay Kit (Echelon Biosciences, Inc) according to the manufacturer's protocol.

Ling, K., Bairstow, S.F., Carbonara, C., Turbin, D.A., Huntsman, D.G., and Anderson, R.A. (2007). Type I γ phosphatidylinositol phosphate kinase modulates adherens junction and E-cadherin trafficking via a direct interaction with mu 1B adaptin. *The Journal of cell biology* 176, 343-353.

Liu, J., Zuo, X., Yue, P., and Guo, W. (2007). Phosphatidylinositol 4,5-bisphosphate mediates the targeting of the exocyst to the plasma membrane for exocytosis in mammalian cells. *Molecular biology of the cell* 18, 4483-4492.

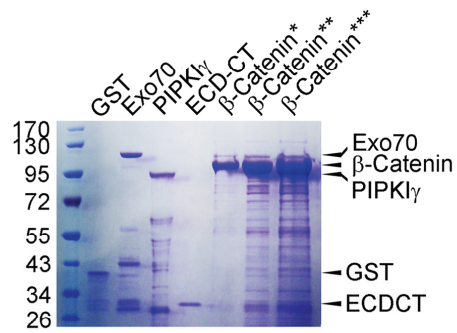


FIGURE S1: Loading control for Figure 2B. Purified recombinant GST, GST-Exo70, His-PIPKI γ , His-E-cadherin C-terminus (ECD-CT), and His- β -catenin were loaded and separated on SDS-PAGE, and then stained using Commassie Blue. The amount of proteins is the same as what was used in GST pull-down assay as described in Figure 2B. Amount of His- β -catenin: *, same as ECD-CT; **, 3-fold of ECD-CT; 10-fold of ECD-CT.

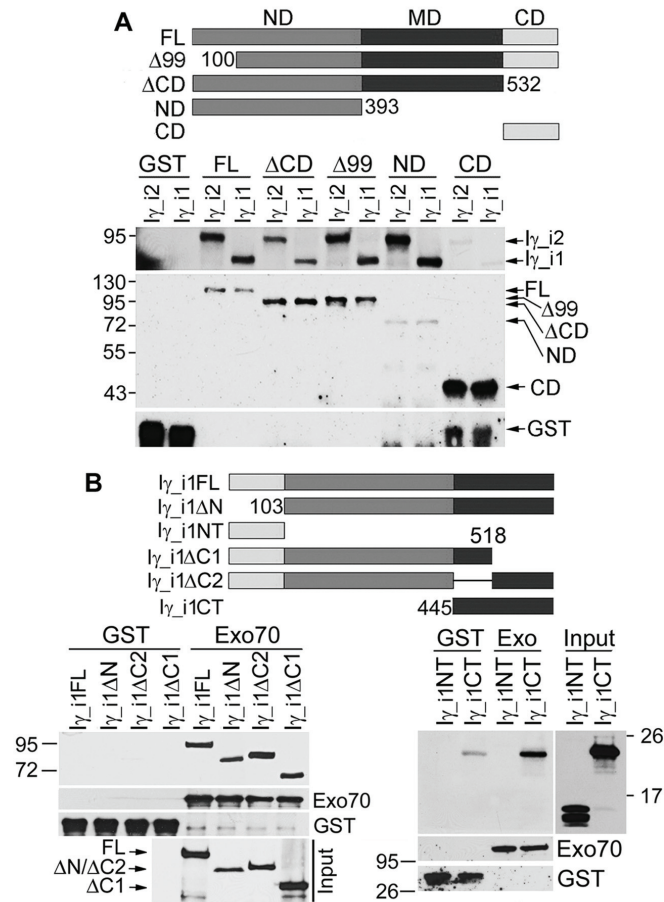


FIGURE S2: Characterizing the direct interaction between PIPKI γ and Exo70. (A) PIPKI γ binds to the N-terminus of Exo70. Top, schematic map of Exo70 truncations. FL, full-length Exo70; Δ 99, Exo70 with N-terminal 99-aa deleted; Δ CD, Exo70 with C-terminal domain deleted; ND, N-terminal domain of Exo70; CD, C-terminal domain of Exo70. (B) Top, schematic map of PIPKI γ truncations. PIPKI γ _i1 is PIPKI γ 87, and PIPKI γ _i2 is PIPKI γ 90. I γ _i1FL, full-length PIPKI γ _i1; I γ _i1 Δ N, PIPKI γ _i1 with N-terminus deleted; I γ _i1NT, N-terminus of PIPKI γ _i1; I γ _i1 Δ C1, PIPKI γ _i1 with C-terminal 122-aa deleted; I γ _i1 Δ C2, PIPKI γ _i1 with the N-terminal 74-aa in the C-terminal domain truncated; I γ _i1CT, C terminus of PIPKI γ _i1. Bottom panels of A and B, GST pull-down assay using 1 μ g of each indicated purified protein and analyzed by IB with indicated antibodies.

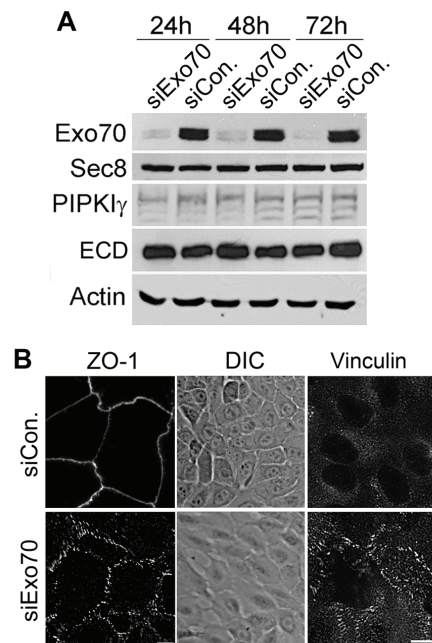


FIGURE S3: Depletion of Exo70 leads to loss of epithelial polarity. (A) Depletion of Exo70 did not affect protein levels of E-cadherin, Sec8, or PIPKI γ . MCF10A cells were transiently transfected twice at a 48-h interval with 75 nM Exo70-specific or control siRNA. Twenty-four, 48, and 72 h after the second transfection, total cell lysates were analyzed by IB using the indicated antibody. (B) MCF-10A cells were double-treated with siRNAs as in (A) to deplete endogenous Exo70. Left panel, tight junction marker ZO-1 was visualized by indirect immunofluorescence. Middle panel, cell morphology under phase contrast microscope (40 \times). Right panel, vinculin visualized by indirect immunofluorescence. Scale bar, 10 μ m.

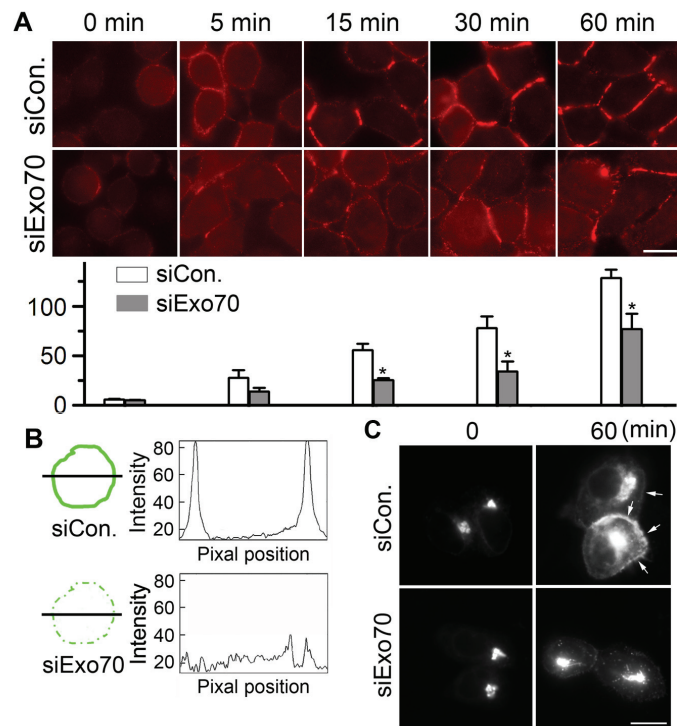


FIGURE S4: Exo70 is required for the polarized PM targeting of E-cadherin and VSV-G. (A) Control or Exo70-depleted MCF-10A cells were subjected to an E-cadherin recycling assay. At the indicated time post-calcium chase, E-cadherin (ECD) in these cells was visualized by immunofluorescence microscopy using an antibody specific to the ectodomain. E-cadherin intensity on the PM was determined as described in panel B by ImageJ and plotted using SigmaPlot 8.0. *, $p < 0.05$. (B) Quantification of the membrane E-cadherin intensity in A. One line was drawn across the control or Exo70-depleted cell, and a density profile plot was generated using NIH Image 1.43 by determining the pixel intensity along the line. (C) Exo70 is necessary for the membrane transport of VSV-G. MCF-10A cells were transfected twice with siRNA to deplete Exo70. 24 h after the second treatment, cells were transfected with temperature-sensitive VSV-G-45ts-GFP and immediately placed at 40 °C overnight. Then, cells were shifted to 32 °C for 0 or 60 min in the presence of cycloheximide (100 $\mu\text{g/ml}$). These cells were then subjected to fluorescence microscopy to visualize GFP signals. GFP-VSVG accumulated in the Golgi apparatus in both control (siCon.) and Exo70-depleted (siExo70) cells. After a 60-min incubation at 32 °C, GFP-VSVG signal was observed on the PM in control cells (arrows) but was still localized to the Golgi in Exo70-depleted cells. Scale bar, 10 μm .

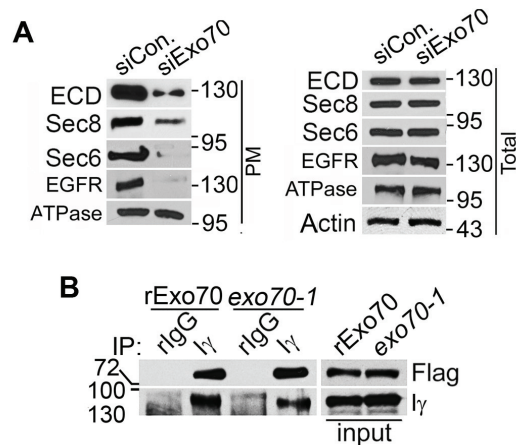


FIGURE S5: (A) Loss of Exo70 led to decreased membrane-associated E-cadherin and exocyst. The PM was fractionated from control (siCon.) or Exo70-depleted (siExo70) MCF-10A cells. Then, PM-associated E-cadherin, Sec6, Sec8, EGFR, and Na/K ATPase (ATPase) were analyzed by immunoblotting. **(B)** Levels of these proteins in total cell lysate were also determined by immunoblotting. The *exo70-1* mutant associates normally with PIPKI γ . HEK293 cells were transfected with Flag-tagged rExo70 or *exo70-1* and then subjected to Immunoprecipitation (IP) using an anti-PIPKI γ antibody. Immunoprecipitates and whole cell lysates were analyzed by immunoblotting (IB) with the indicated antibodies.

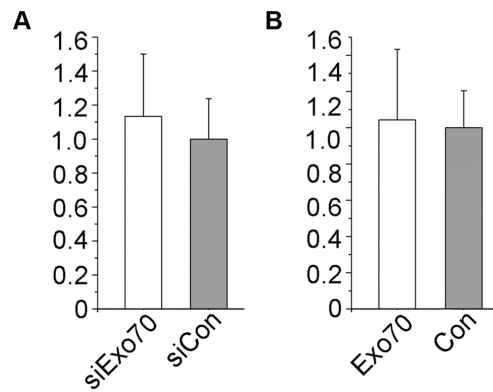


FIGURE S6: Cellular PI4,5P₂ levels are not affected by depletion of endogenous or expression of exogenous Exo70. MCF-10A cells were (A) treated with control (siCon) or Exo70-specific (siExo70) siRNA or (B) transiently transfected with monk (Con) or rat Exo70 (Exo70). Total cellular PI4,5P₂ was extracted from these cells and the levels of PI4,5P₂ were determined using the PI4,5P₂ Mass ELISA Assay Kit (Echelon Biosciences, Inc) according to the manufacturer's protocol.