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Drosophila Exocyst Components Sec5, Sec6, and Sec15 Regulate DE-Cadherin Trafficking from Recycling Endosomes to the Plasma Membrane

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Summary

The E-Cadherin-catenin complex plays a critical role in epithelial cell-cell adhesion, polarization, and morphogenesis. Here, we have analyzed the mechanism of *Drosophila* E-Cadherin (DE-Cad) localization. Loss of function of the *Drosophila* exocyst components *sec5*, *sec6*, and *sec15* in epithelial cells results in DE-Cad accumulation in an enlarged Rab11 recycling endosomal compartment and inhibits DE-Cad delivery to the membrane. Furthermore, Rab11 and Armadillo interact with the exocyst components Sec15 and Sec10, respectively. Our results support a model whereby the exocyst regulates DE-Cadherin trafficking, from recycling endosomes to sites on the epithelial cell membrane where Armadillo is located.

Introduction

Epithelial cells are characterized by an apical, a basolateral, and a junctional domain. The establishment and maintenance of epithelial cell polarity depends upon transmembrane and cortical protein complexes including the Crumbs/PALS1/PATJ, the Par, and the E-Cadherin/catenin complexes (for review, see Bilder, 2004). Epithelial cell polarity also relies upon the polarized exocytosis of proteins and lipids to the apical, basolateral, and junction domains of the epithelial cell (for review, see Rodriguez-Boulan et al., 2005). The exocytosis of vesicles to specific regions of the plasma membrane requires recognition between the vesicles and the target membranes prior to their fusion. This initial step, known as tethering, is thought to be dependent upon large multisubunit complexes such as the exocyst (Whyte and Munro, 2002).

The exocyst is a conserved complex composed of eight subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (Hsu et al., 1996; Kee et al., 1997; Lloyd et al., 2000; Matern et al., 2001; TerBush et al., 1996; Ting et al., 1995). In yeast, mutations in any member of the exocyst complex block bud growth and cause post-Golgi vesicles to accumulate in the cytosol (Novick et al., 1980; Salminen and Novick, 1989). The exocyst complex, without Sec3p, is localized on the post-Golgi vesicles by an interaction between Sec15p and the Rab family protein Sec4p (Boyd et al., 2004; Guo et al., 1999; Salminen and Novick, 1989). On the bud membrane, Exo70p and Sec3p interact with Rho1 and create a landmark to direct polarized exocytosis at the bud membrane and thereby promote bud growth (Boyd et al., 2004; Finger et al., 1998; Guo et al., 2001; Wiederkehr et al., 2003). Hence, it has been proposed that the exocyst regulates polarized exocytosis by tethering post-Golgi vesicles to the bud membrane compartment, prior to their fusion.

In both mammals and *Drosophila*, components of the exocyst complex regulate vesicle delivery and polarized membrane growth in neuronal and exocrine cells (Andrews et al., 2002; Beronja et al., 2005; Inoue et al., 2003; Kanzaki and Pessin, 2003; Mehta et al., 2005; Murthy et al., 2003, 2005; Murthy and Schwarz, 2004; Riefler et al., 2003; Vega and Hsu, 2001). In the mammalian epithelial MDCK cell line, exocyst components have been implicated in the establishment of epithelial cell polarity (Grindstaff et al., 1998; Lipschutz et al., 2000). In polarizing MDCK cells, Sec8 and Sec6 are recruited from the cytosol to sites of cell-cell contact upon initiation of E-Cadherin-dependent cell-cell adhesion, and Sec8 can be immunoprecipitated with E-Cadherin (Grindstaff et al., 1998; Yeaman et al., 2004). In polarized MDCK cells, Sec6 and Sec8 localize to the trans-Golgi network and tight junctions (Grindstaff et al., 1998; Yeaman et al., 2001, 2004). In these cells, the inhibition of Sec8 function by Sec8-blocking antibodies inhibits the delivery of LDL receptor to the basolateral membrane, but not $p75^{NTR}$ protein to the apical membrane (Grindstaff et al., 1998). Therefore, it has been proposed that the exocyst promotes the formation of the basolateral domain of epithelial cells by polarizing the delivery of vesicles from the Golgi to the sites of E-Cadherin cell-cell contacts and thereby establishing epithelial cell polarity. The function of the exocyst as a regulator in Golgi to plasma membrane trafficking has been recently challenged. In mammalian cells, Exo70, Sec8, Sec15, and Sec10 are also localized on the recycling endosomes (RE), and Sec15 interacts with the RE-associated GTPase Rab11 (Folsch et al., 2003; Prigent et al., 2003; Zhang et al., 2004). In *Drosophila* extracts, Rab11-GFP was shown to be immunoprecipitated in a complex with Sec5, but a role of Sec5 in the RE remains to be established (Beronja et al., 2005). Finally, Sec5 was recently shown to be associated with

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clathrin-coated pits and vesicles at the plasma membrane and proposed to regulate the endocytosis of the Yolkless receptor (Sommer et al., 2005). Hence, what remains unclear during epithelial cell polarization is whether the exocyst contributes to the polarized exocytosis of molecules known to be involved in the establishment or maintenance of epithelial polarity and whether this function would implicate exocytosis from the Golgi or the RE.

The E-Cadherin/β-catenin/α-catenin complex plays a central role in the establishment and the maintenance of apico-basal cell polarity of epithelial tissues both in mammals and *Drosophila*. In *Drosophila*, DE-Cadherin (DE-Cad), β-catenin (known as Armadillo, Arm), and Dα-catenin (Dα-Cat) localized at the apical zonula adherens (ZA) where they form adherens junctions (AJs). In *Drosophila*, AJs have also been described along the basolateral domain of epithelial cells both in the embryos and in the larva (for review, see Tepass and Hartenstein, 1994). Here, by initially analyzing the role of Sec5 in the monolayered epithelium of the dorsal thorax (notum) of the pupae, we have found that the exocyst regulates the trafficking of DE-Cad.

Results

DE-Cad and Catenins Accumulate Intracellularly in *sec5* Mutant Epithelial Cells

The *sec5* null mutant $sec5^{E10}$ is characterized by a nonsense mutation at amino acid 31 and is lethal at the first instar larva stage (Murthy et al., 2003). To analyze its function in the epithelium of the notum of *Drosophila* pupa, somatic *sec5* mutant clones (marked by loss of GFP expression) were generated using the FLP/FRT system (Xu and Rubin, 1993). However, *sec5* mutant clones were not recovered when larvae and pupae were grown at 25°C. Serendipitously, it was found that *sec5* mutant clones survived at 18°C. The survival of these clones may represent the persistence of sufficient Sec5 protein from heterozygous precursor cells at the lower temperature. The function of *sec5* was therefore analyzed by generating mutant clones in larvae harboring $sec5^{E10}$ somatic clones grown at 18°C. Upon puparium formation, pupae were shifted to 25°C and dissected at least 18 hr later.

In order to examine the function of *sec5* in the notum, the localization of epithelial cell polarity markers was compared between *sec5* mutant epithelial cells and the surrounding control epithelial cells. The localization of the Crumbs protein, which forms a complex with Stardust and dPATJ at the apical membrane (for review, see Bilder, 2004), was unaffected in *sec5* mutant epithelial cells (24 out of 25 clones; Figures 1A and 1A′). At confocal resolution, the components of *Drosophila* Par complex, Bazooka (Baz), DmPar6, and DaPKC overlap with the Crumbs-containing region and the ZA formed by the AJs, where the DE-Cad/Arm/Dα-Cat complex is located. Whereas the localization of Baz was unaffected in *sec5* mutant epithelial cell clones (36 out of 39 clones; Figures 1A, 1A′, 1B′, 1H, and 1H′), in more than half of the *sec5* mutant epithelial cell clones compared with the control cells (12 out of 21 clones), DE-Cad was found to have accumulated in the apical cytoplasm and around the nucleus, in numerous punctate structures (Figures 1D, 1D′, 1E′, 1G, 1G′, and 1H). Below the AJs, the septate junctions, marked by the enrichment of cortical Discs-large (Dlg) and Scribble, describe the apical limit of the basolateral membrane domain, along which the transmembrane protein Fas3 is located. The localization of all these basolateral proteins was unaffected in *sec5* mutant epithelial cell clones (Figures 1B, 1B′, 1C, and 1C′ and not shown; Fas3, 36 out of 39 clones; and Dlg or Scrib, 14 out of 14 clones). Thus, in these experimental conditions, the loss of Sec5 function preferentially affected the intracellular distribution of DE-Cad, but not the distribution of other membrane and cortical proteins examined.

A few *sec5* mutant cells were found to have a small apex. Within such cells, DE-Cad, Baz, Fas3, and Crumbs appeared to accumulate in large cytoplasmic puncta (3 out of 39 clones for Baz and Fas3 and 1 out 25 for Crumbs; and data not shown). These cells may represent cells more affected by the 25°C temperature shift, and such cells may have been delaminating due either to the internal DE-Cad accumulation or to a requirement of the *sec5* gene for cell viability.

As the localization of DE-Cad was disrupted, the localization of Arm and Dα-Cat was also examined in *sec5* mutant cells. Where DE-Cad was found to accumulate intracellularly, Arm was also found to accumulate intracellularly (Figures 1E, 1E′, 1F′, and 1G). The DE-Cad puncta often colocalized with or were closely apposed to Arm intracellular puncta. Similarly Dα-Cat was found to accumulate in intracellular puncta in *sec5* mutant epithelial cells where Arm was found to accumulate intracellularly, and again intracellular puncta of Dα-Cat were colocalized with or closely apposed to Arm intracellular puncta (Figures 1F and 1F′). Thus, Sec5 regulates the localization of DE-Cad, Arm, and Dα-Cat.

In *sec5* Mutant Cells, Arm and DE-Cad Accumulate in an Enlarged Recycling Endosome Compartment

In order to determine the nature of the DE-Cad and Arm intracellular structures observed with the loss of Sec5 function, markers of different intracellular trafficking compartments were examined. These included the RE marker Rab11 (Dollar et al., 2002), the Golgi marker Lava Lamp (Lva, Sisson et al., 2000), the early endosome marker Rab5 (Wucherpfennig et al., 2003), and the late endosome marker Hepatocyte growth factor-Regulated tyrosine kinase Substrate (HRS, Lloyd et al., 2002). Whereas the distribution of Lva (Figure 2A), HRS (Figure 2B), and Rab5 (Figure 2C) were similar in control and *sec5* mutant epithelial cells, the distribution of Rab11 was disrupted in 94% of the mutant cells (n = 35, Figure 2D). Rab11 was found in punctate structures, mostly concentrated in the apical part of both the mutant and control epithelial cells. However, the fluorescence staining level of Rab11 was much higher and was associated with larger punctate structures in the *sec5* mutant epithelial cells compared with the control cells, with some of these larger structures being formed by the coalescence of smaller ones, indicating that the RE compartment was expanded. To confirm this result, Nuclear fallout (Nuf) was used as an additional marker

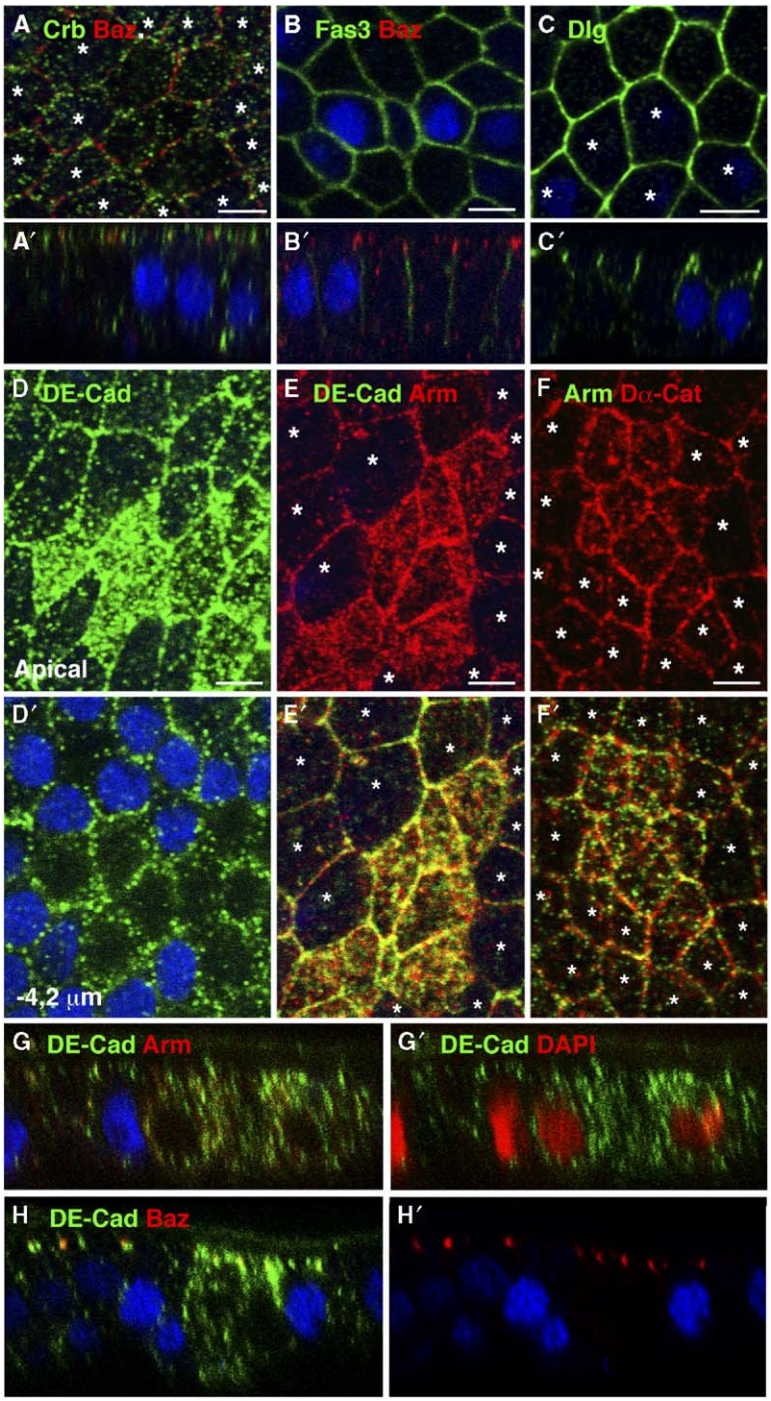

Figure 1. DE-Cad, Arm, and Dα-Cat Accumulate in the Cytoplasm in *sec5* Mutant Epithelial Cells

Confocal immunodetection showing XY (A–C and D–F′) and XZ (A′–C′ and G–H′) sections in the plane of fixed epithelial cells.

(A–C′) Localization of Crumbs (green in [A] and [A′]), Fas3 (green in [B] and [B′]), Dlg (green in [C] and [C′]), and Baz (red in [A], [A′], and [B′]) in control (indicated by asterisks in [A] and [C]) and *sec5* mutant epithelial cells. *sec5* mutant epithelial cells were identified by the absence of nls-GFP (GFP is blue).

(D–H′) Localization of DE-Cad (green in [D], [D′], [E′], [G], [G′], and [H]), Arm (red in [E], [E′], and [G] and green in [F′]), Dα-Cat (red in [F] and [F′]), and Baz (red in [H] and [H′]) in control (indicated by asterisks in [E]–[F′]) and *sec5* mutant epithelial cells. *sec5* mutant epithelial cells were identified by the absence of nls-GFP (GFP is blue in [D], [D′], [G], [H], and [H′] and not shown in [E]–[F′] and [G′]). (D′) is a XY confocal section acquired 4.2 μm below (D). In (G′), control and *sec5* mutant cell nuclei are stained by DAPI (red). Note, DE-Cad, Arm, and Dα-Cat were also found in intracellular puncta in control cells. However, the number of puncta and the staining intensity was lower and variable (compare control and *sec5* mutant cells in [D] and [E′]).

In all sections, scale bar equals 5 μm.

of the REs. Nuf belongs to the Arfophilin family of Rab11 effectors, colocalizes with Rab11, and is required for Rab11 localization in the early *Drosophila* embryo (Hickson et al., 2003; Riggs et al., 2003). In wild-type notum epithelial cells, 43% of the Nuf puncta (n = 583) were found also to be positive for Rab11 (n = 883; see also Experimental Procedures for method of quantification; see Figure S1 and Table S1 in the Supplemental Data available with this article online). Therefore, it was assumed that Rab11 or Nuf were each associated only with subcompartments of the RE. Consistent with the enlargement of Rab11-positive compartment observed in *sec5* mutant cells, Nuf was found at greater levels and in more numerous puncta in *sec5* mutant epithelial cells (Figure 2E, Figure S1 and Table S1), further confirming that the RE compartment is enlarged in *sec5* mutant cells.

The localization of DE-Cad and Arm was then compared with that of Nuf and Rab11 both in wild-type and mutant *sec5* epithelial cells. In wild-type cells, 11% of

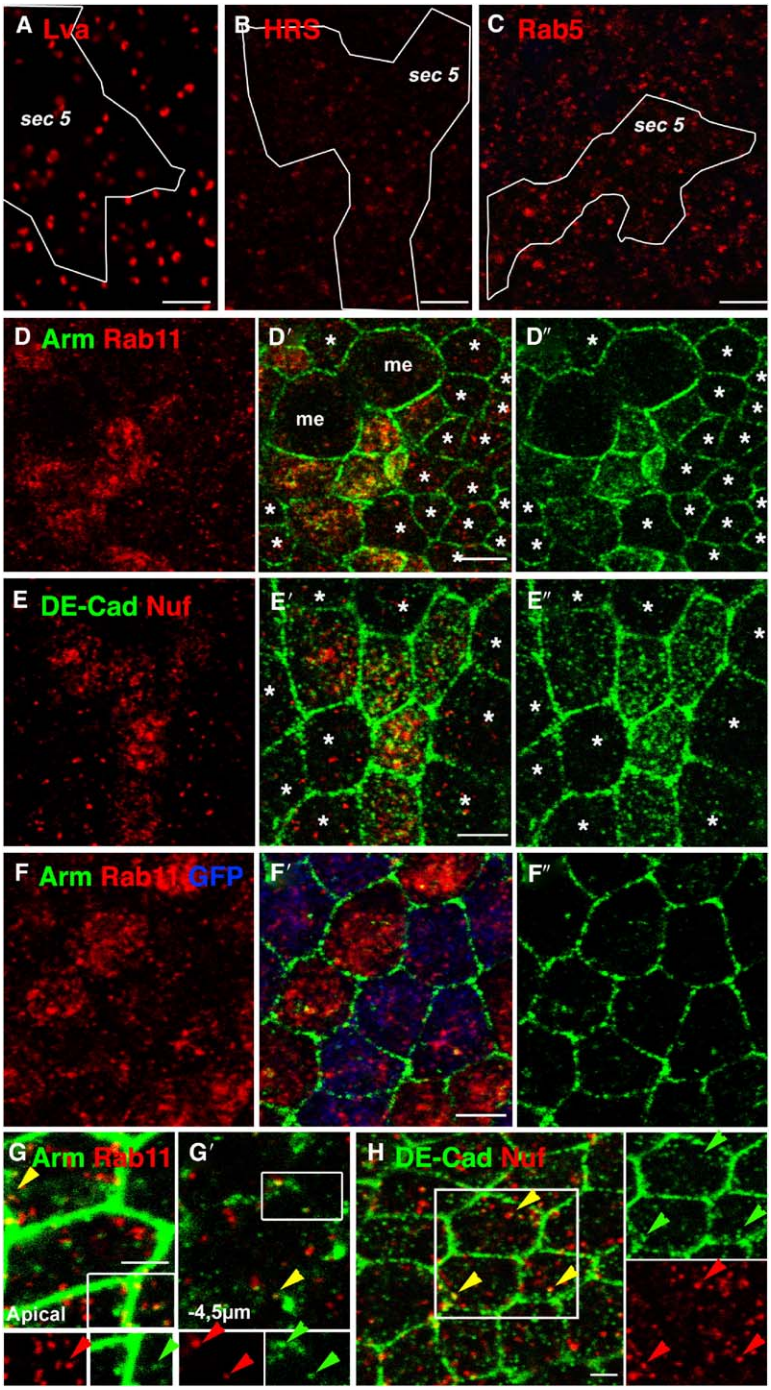

Figure 2. More Numerous, More Intense, and Larger Rab11-Positive Puncta in *sec5* Mutant Epithelial Cells

(A–C) The localization of Lva (red in [A]), HRS (red in [B]), and Rab5 (red in [C]) in *sec5* mutant cells, depicted by a white outline, where DE-Cad had accumulated (not shown).

(D–D″) Localization of Rab11 compartment (red in [D] and [D′]) and Arm (green in [D′] and [D″]) in wild-type and *sec5* mutant cells marked by loss of nls-GFP (not shown). Control cells are indicated by asterisks in (D′) and (D″). In mitotic cells (me), the accumulation of Rab11 and Arm puncta was much less apparent, probably due to the fragmentation of the Rab11 compartment at mitosis.

(E–E″) The localization of Nuf (red in [E] and [E′]) and DE-Cad (green in [E′] and [E″]) in *sec5* mutant clones. Control cells are indicated by asterisks in (E′) and (E″).

(F–F″) Localization of Rab11 (red in [F] and [F′]) and Arm (green in [F′] and [F″]) in a *sec5* mutant clones identified by loss of nls-GFP (blue in [F′]).

(G and G′) The localization of Rab11 (red) and Arm (green) in wild-type cells. (G′) is a confocal section acquired 4.5 μm below (G). The panels below (G) and (G′) detail the inset defined by the rectangular outline. Red arrowheads indicate puncta of Rab11 and green arrowheads indicate puncta of Arm at points of overlap. Yellow arrowheads indicate colocalization of Rab11- and Arm-positive puncta.

(H) Localization of Nuf (red) and DE-Cad (green) in wild-type cells. The panels on the right of (H) detail the inset defined by the rectangular outline. Red arrowheads indicate puncta of Nuf and green arrowheads indicate puncta of DE-Cad at points of overlap. Yellow arrowheads indicate colocalization of Nuf- and DE-Cad-positive puncta.

Scale bar equals 5 μm.

DE-Cad (n = 339, Figure 2H) and 17% of Arm intracellular punctate structures (n = 80, Figures 2G and 2G′) were colocalized or closely apposed with Nuf (n = 412) or Rab11 (n = 93) vesicles, respectively, indicating that both Arm and DE-Cad are associated with the RE in wild-type epithelial cells. Strikingly, in *sec5* mutant clones where Arm or DE-Cad accumulated, Arm or DE-Cad were enriched in REs (Figures 2D′, 2D″, 2E′, and 2E″). In fact, in such *sec5* mutant clones, 40% and 60% of intracellular punctate structures of DE-Cad (n = 542) and Arm (n = 319) were colocalized or closely apposed with Nuf (n = 440) or Rab11 (n = 282) puncta. Importantly, Rab11 and Nuf were found to accumulate in *sec5* mutant clones even when the distribution of Arm or DE-Cad was normal (Figures 2F–2F″ and not shown). Thus, the DE-Cad and Arm intracellular accumulation and their accumulation in the expanded RE compartment is probably a consequence and not a cause of the Rab11 or Nuf intracellular accumulation in *sec5* mutant epithelial cells.

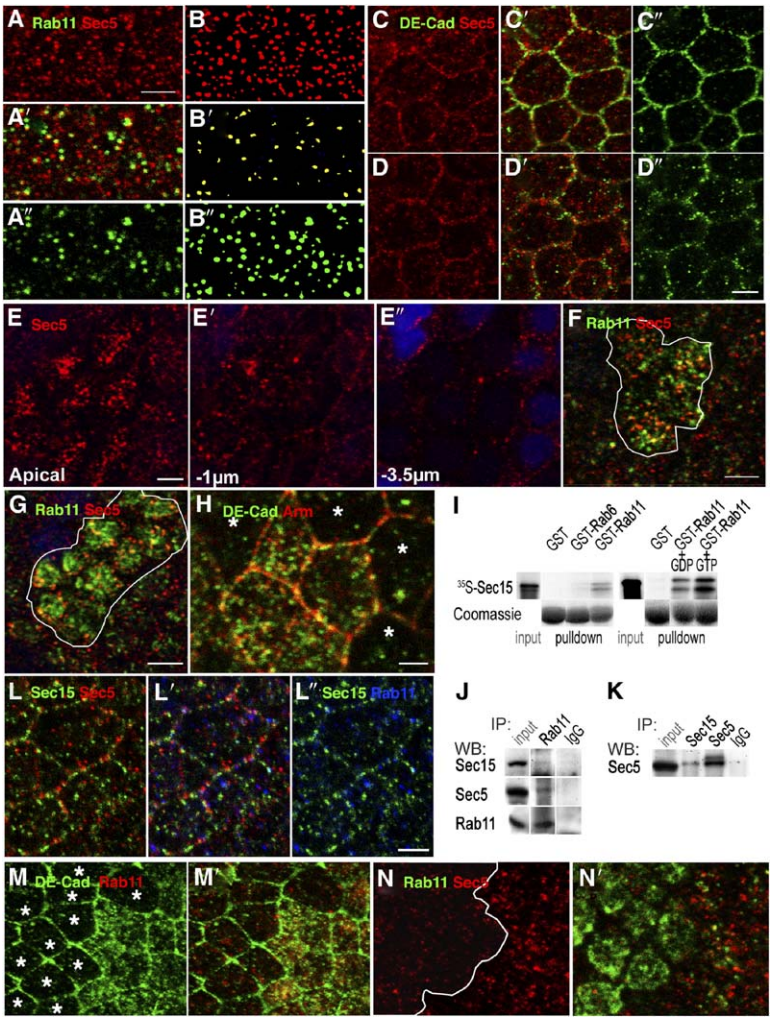

Figure 3. Sec5 Localization and DE-Cad Delivery to the Plasma Membrane

(A–A″) In wild-type cells, apical Sec5-positive puncta (red in [A] and [A′]) were colocalized or in close apposition with Rab11-positive (green in [A′] and [A″]) puncta.

(B–B″) Detection of Sec5 (red in [B]) and Rab11 (green in [B″]) puncta colocalization or close apposition using Spot Detection Software. In (B′), the yellow dots correspond to colocalization or close apposition of Rab11 and Sec5 puncta. Blue regions were not considered.

(C–D″) Apical (C–C″) and lateral (D–D″) Sec5 (red in [C], [C′], [D], and [D′]) localization relative to DE-Cad (green in [C′], [C″], [D′], and [D″]). The plane of acquisition was not parallel to the plane of the epithelium, hence the levels of DE-Cad appear graded in quantity across the sections. Note that the DE-Cad staining levels in (D′) and (D″) were enhanced to better reveal the lateral DE-Cad.

(E–E″) Sec5 (red) was found to accumulate at the apical pole of *sec6* mutant epithelial cells identified by loss of nls-GFP (blue). (E) is at the level of the AJs, (E′) and (E″) are acquired 1 μm and 3.5 μm below (E), respectively. Note that the Sec5 staining levels in (E″) were enhanced to better reveal the lateral Sec5.

(F and G) More numerous, more intense, and larger (especially in [G]) Rab11-positive puncta (green) overlap with more numerous, more intense, and larger Sec5-positive puncta (red) in *sec6* mutant cells compared with control cells. The *sec6* mutant cells are depicted by a white outline in (F) and (G).

(H) The accumulation of DE-Cad (green) and Arm (red) in *sec6* mutant cells relative to adjacent control cells indicated by asterisks.

(I) *Drosophila* Sec15 interacts with *Drosophila* GST-Rab11 preferentially in its GTP bound form but not with GST-human Rab6 or GST.

(J) Embryonic extracts prepared from wild-type *Drosophila* embryos were subjected to immunoprecipitation (IP) with anti-Rab11 and IgG. The immunoprecipitates (IP) were examined by Western blotting using (WB) *anti*-Rab11, *anti*-Sec15, and *anti*-Sec5 antibodies.

(K) Embryonic extracts prepared from wild-type *Drosophila* embryos were subjected to immunoprecipitation (IP) with anti-Sec5 and IgG. The immunoprecipitates (IP) were examined by Western blotting using (WB) *anti*-Sec15 and *anti*-Sec5 antibodies.

(L–L″) Localization of Sec15 (green), Sec5 (red in [L] and [L′]), and Rab11 (blue in [L′] and [L″]) in wild-type cells. 29% and 17% of Sec15 punctate structures (n = 1301) are colocalized or closely apposed with Sec5 (n = 1171) punctate structures and Rab11 (n = 810) puncate structures, respectively. 15% of the punctate structures containing both Sec15 and Sec5 staining (n = 377) are colocalized or closely apposed with Rab11 punctate structures (n = 810).

(M and M′) Localization of DE-Cad (green) and Rab11 (red in [M′]) in *sec15* mutant cells and control cells (indicated by a asterisk).

(N and N′) Localization of Sec5 (red) and Rab11 (green in [N′]) in *sec15* mutant cells and control cells. The *sec15* mutant cells are depicted by a white outline in [N].

Scale bar equals 2.5 μm in (A), (F), (H), and (L) and 5 μm in (C), (D), (E), (G), (M), and (N).

Sec5 Is Associated with the Recycling Endosomes and the Lateral Cortex

The localization of Sec5 was then studied in both wild-type and *sec6* mutant epithelial cells. In wild-type cells, Sec5 was found in punctate structures both in the cytoplasm and at the cell cortex. The cytoplasmic Sec5 distribution was compared with that of Rab11, Rab5, HRS, and Lva. 28% of the Sec5 puncta were colocalized with or in close apposition with Rab11 (Figures 3A–3B″, n_{Sec5} = 503 and n_{Rab11} = 469), whereas fewer Sec5 puncta were colocalized or in close apposition with HRS (11%, n_{Sec5} = 503 and n_{HRS} = 435), Rab5 (12%, n_{Sec5} = 621 and n_{Rab5} = 237), or Lva (3.2%, n_{Sec5} = 1679 and n_{Lva} = 123) puncta (not shown). Along the cortex, Sec5 puncta were colocalized with DE-Cad (Figures 3C–3C″) and hence Arm, at the level of the AJs. Furthermore, Sec5 puncta were also found along the lateral cortex of the cells, and again they were colocalized or closely apposed with punctate structures positive for DE-Cad (Figures 3D–D″). Thus, consistent with a role of Sec5 in regulating the size of the RE compartment and the distribution of DE-Cad, Sec5 localized both along the cortex with DE-Cad and is found associated with the RE.

In *sec6* mutant epithelial cells compared with control cells, Sec5 accumulated at a greater level in intracellular punctate structures in the apical part of the cell and cortical Sec5 was reduced at the AJs and along the

lateral membrane (Figures 3E–3E″). As observed in *sec5* mutant cells, the Rab11-positive compartment was enlarged in *sec6* mutant epithelial cells and strikingly, most of the intracellular punctate Sec5 structures (64%, n = 200) colocalized with Rab11-positive puncta (n = 153, Figures 3F and 3G). Thus, in the absence of Sec6, Sec5 accumulated in an expanded RE compartment and its cortical localization was reduced. Therefore, the partial colocalization observed between Sec5 and Rab11 in wild-type cells may represent an association of Sec5 with the REs prior to the localization of Sec5 at the cell cortex. DE-Cad and Arm also accumulated intracellularly in *sec6* mutant epithelial cells, indicating that cortically localized Sec5 may be necessary to locate DE-Cad and Arm at the cortex (Figure 3H). Hence, the *sec5* and *sec6* mutant phenotypes are similar, suggesting that Sec5 and Sec6 function within the exocyst complex in the regulation of DE-Cad and REs.

The localization of Sec5 on the REs is further supported by the interaction of Sec15 with Rab11. *Drosophila* Sec15 (synthesized and radiolabeled in vitro) was pulled down by GST-Rab11 (Figure 3I). Furthermore, Sec15 interacted more efficiently with GTPγS-loaded GST-Rab11 compared with GDP-loaded GST-Rab11 (Figure 3I), and endogenous Sec15 was detected in a complex with endogenous Rab11 in *Drosophila* embryo extracts (Figure 3J). Thus, in agreement with findings in mammals (Zhang et al., 2004), *Drosophila* Sec15 is an effector of Rab11. In both yeast and mammals, Sec5 indirectly interacts with Sec15 via Sec10 (Guo et al., 1999; Matern et al., 2001). Therefore, the association of Sec5 with the REs could be dependent on its interaction with GTP bound Rab11 via Sec15 and Sec10. The existence of such a complex is supported by the coimmunoprecipitation of Sec5 with Sec15 and of Rab11 with Sec5 (Figures 3J and 3K). Furthermore, the recruitment of Sec5 on the RE appears to depend on Sec15. Sec15 punctate structures colocalized with or were closely apposed to Sec5- and Rab11-positive puncta (Figures 3L–L″). In *sec15* mutant epithelial cells, Sec5 staining was more diffuse in the cytoplasm and was not so strongly associated with large intracellular puncta (Figures 3N and 3N′). Finally, *sec15* mutant epithelial cells were characterized by an accumulation of DE-Cad in an enlarged recycling endosomal compartment (Figures 3M and 3M′). Hence, *sec15* loss-of-function phenotype is similar to that of *sec5* and *sec6* regarding the enlargement of the recycling endosomes and the localization of DE-Cad. But Sec5 accumulated on the enlarged REs in *sec6* mutant cells, while Sec5 appeared not to be recruited on the enlarged REs in *sec15* mutant cells. Altogether this strongly argues that Sec15 links Sec5 on the REs, maybe via Sec10, whereas Sec6 is dispensable for the recruitment of Sec5 on the REs; however, Sec5, Sec6, and Sec15 are all necessary to regulate the distribution of DE-Cad and the size of the RE compartment.

Sec5 Is Required for Delivery of DE-Cad to the Membrane

The intracellular accumulation of DE-Cad in the REs suggested that DE-Cad is not properly delivered to the plasma membrane. To detect DE-Cad present at the membrane and not in the intracellular DE-Cad pool, antibody binding experiments were performed without cell permeabilization and at 4°C to prevent antibody endocytosis. Dissected notum were therefore incubated at 4°C for 30 min in culture media with the DCAD2 antibody, which recognizes an epitope on the extracellular domain of DE-Cad (Oda and Tsukita, 1999; the experiments are schematically described in Figure 4A). In wild-type cells, DCAD2 was detected on the lateral membrane below the AJs (Figures 4B and 4B′). This was expected, as DE-Cad at the AJs is not exposed to DCAD2 without cell permeabilization, because the cuticle of the notum and the septate junctions prevent its access to the AJs. Therefore, DE-Cad is delivered to the lateral membrane of epithelial cells, and DCAD2 detection along the lateral cortex was used as a readout of DE-Cad delivery to the plasma membrane. Importantly, DE-Cad and Arm were also found to colocalize along the lateral cortex of the cell below the level of AJs (Figure 4B, lower panel).

In *sec5* mutant epithelial cells where Arm accumulated intracellularly, DCAD2 staining was detected along the lateral membrane but its level was strongly reduced (Figures 4C and 4C′). As Arm accumulated intracellularly in *sec5* mutant cells only when DE-Cad accumulated intracellularly, then DE-Cad accumulation within *sec5* mutant cells correlated with a reduction of DE-Cad delivery to the lateral membrane (n = 18 clones). Similar results were obtained in *sec6* and *sec15* mutant epithelial cells (data not shown). Finally, the delivery of Fas3 to the lateral membrane was not affected in *sec5* mutant epithelial cells even when DE-Cad delivery was reduced (Figures 4D–4D″). The preferential effect of loss of Sec5 function on DE-Cad delivery may represent a selective requirement of Sec5 for the delivery of DE-Cad or may be due to a higher turnover of DE-Cad relative to Fas3 on the lateral membrane.

Thus, loss of Sec5 or Sec6 or Sec15 function is associated with an enlargement of the REs and an accumulation of DE-Cad in the REs. Furthermore, loss of Sec5 and Sec6 function inhibits DE-Cad delivery to the lateral membrane, suggesting that Sec5, Sec6, and Sec15 are necessary for the delivery of DE-Cad from the REs to the lateral membrane where both Sec5 and Arm are localized.

Lateral DE-Cad Is Recycled to the AJs in a *sec5*-Dependent Manner

The reduction of DE-Cad on the lateral membrane could be due either to a failure to deliver newly synthesized DE-Cad to the lateral domain or to a defect in DE-Cad recycling at the lateral membrane in *sec5* mutant epithelial cells. To analyze DE-Cad recycling, a DE-Cad transcytosis assay was developed. In wild-type cells, DE-Cad was located both at the AJs of the ZA and along the lateral membrane with Arm. AJs have been described along the lateral domain of epithelial cells in *Drosophila* larvae (Tepass, 1997; Tepass and Hartenstein, 1994), and therefore DE-Cad and Arm along the lateral membrane may contribute to epithelial cohesion by forming basolateral AJs. Furthermore, DE-Cad along the lateral membrane was found to recycle to the AJs of the zonula adherens. Following incubation with DCAD2

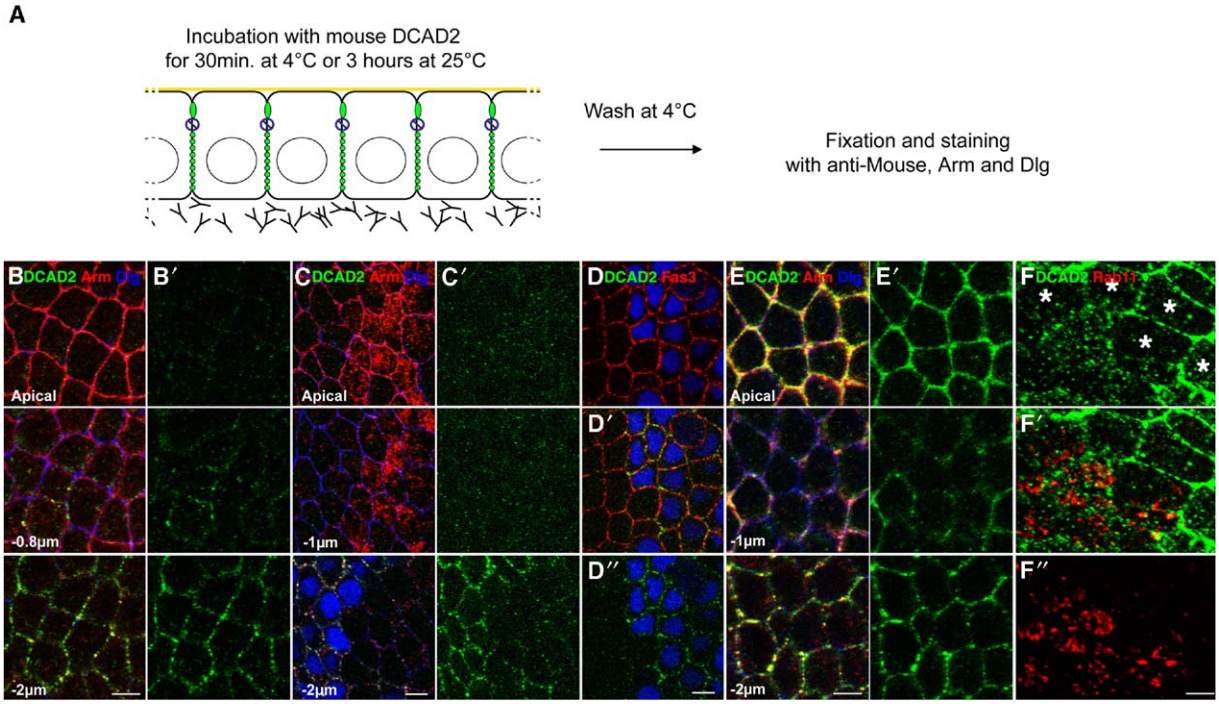

Figure 4. DE-Cad Delivery to the Lateral Membrane and Transcytosis Are Dependent on *sec5* Function

(A) Schematic representation of the endocytosis assay. A longitudinal view of notum epithelial cells in black outline (with circular nuclei) is shown, with the overlying cuticle represented as a thick yellow line. On the apical and lateral membrane, DE-Cad is shown in green and septate junctions are shown as blue circles containing oblique lines.

(B and B′) Vertical columns of three sequential confocal sections in an apical-basal direction are shown, each separated by 0.8 μm and 2 μm, respectively. Wild-type epithelial cells fixed after 30 min of incubation at 4°C with DCAD2 and stained with Alexa488-anti-Rat (green), Arm (red in [B]), and Dlg (blue in [B]).

(C and C′) Vertical columns of three sequential confocal sections in an apical-basal direction are shown, each separated by 1 μm and 2 μm, respectively. Dissected notum harboring *sec5* mutant cells identified by loss of nls-GFP (blue) fixed after 30 min of incubation at 4°C with DCAD2 and stained with Alexa488-anti-Rat (green), Arm (red in [C]), and Dlg (blue in [C]).

(D–D″) Dissected nota were incubated at 4°C for 30 min with both DCAD2 (green in [D′] and [D″]) and Fas3 (red in [D] and [D′]) antibodies prior to fixation. *sec5* mutant epithelial cells were identified by loss of nls-GFP (blue).

(E and E′) Vertical columns of three sequential confocal sections in an apical-basal direction are shown, each separated by 1 μm and 2 μm, respectively. Wild-type dissected notum fixed after 3 hr of incubation at 25°C with DCAD2 and stained with Alexa488-anti-rat (green), Arm (red in [E]), and Dlg (blue in [E]).

(F–F″) Dissected nota were incubated at 25°C for 3 hr with DCAD2 (green in [F] and [F′]) and stained for Rab11 (red in [F′] and [F″]). *sec5* mutant epithelial cells were identified by loss of nls-GFP expression (not shown). Control cells are indicated by a asterisk.

Scale bar equals 2.5 μm in (G″) and 5 μm in (B)–(E).

antibody for 3 hr at 25°C to allow endocytosis and recycling, DCAD2 was detected at the AJs of the ZA above the septate junction marked by Arm and Dlg, respectively (Figures 4E and 4E′). This indicated that basolateral DCAD2 was recycled to the AJs. Therefore, the basolateral membrane appears to be a transcytosis route DE-Cad can follow in order to reach the AJs.

The role of Sec5 in this transcytosis route was then tested by incubating dissected notum harboring *sec5* mutant epithelial cell clones with the DCAD2 antibody for 3 hr at 25°C. As expected, the lower level of DCAD2 antibody binding the lateral membrane of *sec5* mutant epithelial cells led to a lower overall level of DCAD2 staining compared with the control cells. Nevertheless, DCAD2 was not detected at the AJs (n = 13 clones), but was found inside the cells, and 29% of DCAD2 puncta were colocalized with or closely apposed to the enlarged recycling compartment (n_{DCAD2} = 221 and n_{Rab11} = 94, Figures 4F–4F″). Thus, although DCAD2 was internalized, it failed to be recycled to the AJs and accumulated in the REs in the absence of Sec5 function. Therefore, lateral DE-Cad is not recycled to the AJs in absence of the Sec5 and accumulates in the recycling endosomal compartment, indicating that Sec5 is necessary at least for recycling of DE-Cad from the REs to the apical AJs.

Arm Interacts with Sec10

In parallel, potential exocyst binding partners were identified by yeast two-hybrid screens. Thus, it was found that an Exo70 bait interacted with four distinct clones encoding overlapping regions of the *arm* gene. These clones delineated an Exo70-interacting domain within Arm, encompassing Arm repeats 8 to 12 (amino residues 373 to 754, Figure 5A and schematically described in Figure S2). The interaction between Exo70 and Arm could not be confirmed by GST pull-down. Therefore, additional components of the exocyst were

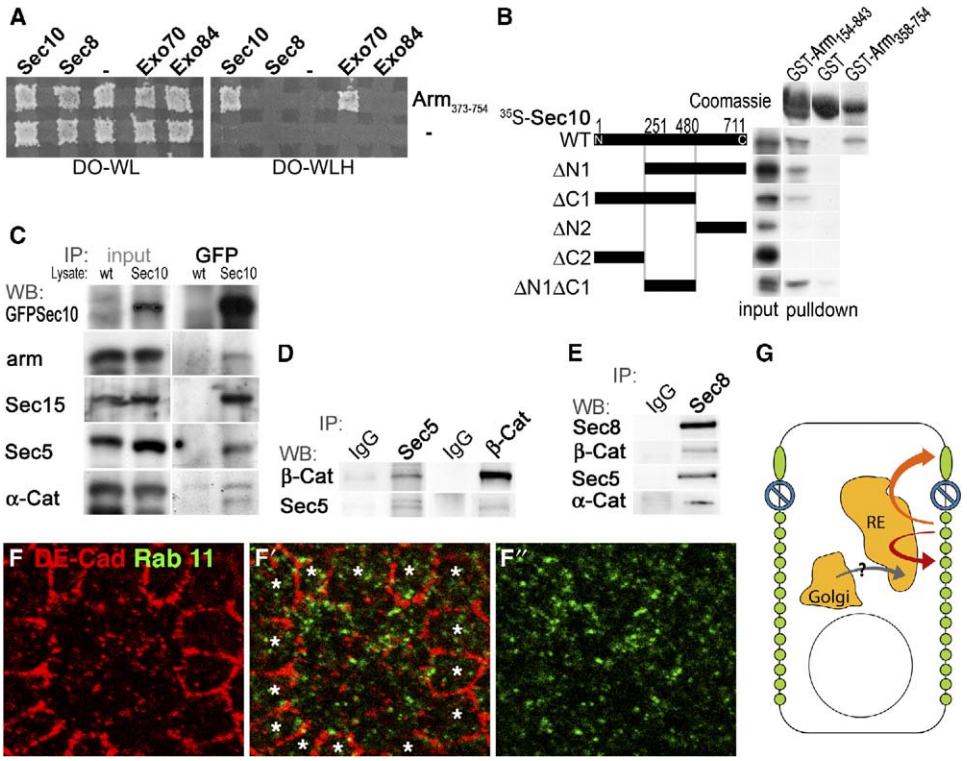

Figure 5. Exocyst Components Interact with Armadillo/β-catenin

(A) The replica plated growth of yeast diploids, from matings between strains expressing Sec8, Sec10, Exo70, and Exo84 fused to LexA or empty plasmid pLex12; and strains expressing GAL4 fused to amino residues 373–754 of Arm; on DO-WL medium (all diploids grow, left grid) and on selection medium (DO-WLH). On selection medium, diploids grow only when bait and prey protein interact.

(B) *Drosophila* Sec10 interacts with Arm. Six different constructs of radiolabeled Sec10 were tested for interaction with GST-Arm154-843.

(C) Embryonic extracts prepared from *Drosophila* wild-type and Sec10-GFP-expressing embryos were subjected to immunoprecipitation (IP) with *anti*-GFP antibodies. The immunoprecipitates (IP) were examined by Western blotting using (WB) *anti*-GFP, *anti*-Sec15, *anti*-Sec5, *anti*-Arm, and *anti*-Dα-Cat antibodies.

(D and E) Rat exocyst components interact with β-catenin in NRK cells. Subconfluent NRK lysates were subjected to immunopecipitation (IP) with (E) IgG or *anti*-Sec5, *anti*-β-catenin (F), or IgG and *anti*-Sec8 antibodies. The immunoprecipitates (IP) were examined by Western blotting using (WB) *anti*-Sec5, *anti*-Sec8, *anti*-β-catenin, and *anti*-α-catenin antibodies.

(F–F″) Localization of DE-Cad (red in [F] and [F′]) and Rab11 (green in [F′] and [F″]) in *arm* mutant clones identified by loss of GFP expression (not shown). Control cells are indicated by asterisks. Note that some *arm* mutant clones are characterized by a strong reduction of Rab11 staining, by loss of nuclei, or by mispositioned or very large nuclei as revealed by DAPI staining.

(G) A schematic model describing exocyst function during DE-Cad trafficking in an epithelial cell. The exocyst contributes to lateral and apical junction delivery of DE-CAD via the REs by regulating DE-Cad (green circle) trancytosis (orange arrow) from the lateral membrane to the apical AJs (green oval) above the septate junctions (blue circles containing oblique lines). The strong reduction of DE-Cad present on the lateral membrane is interpreted as a failure to recycle DE-Cad from the lateral membrane back to the lateral membrane (red arrow). The existence of a pathway of neosynthesized DE-Cad from the Golgi to the membrane via the RE remains to be established (gray arrow). If so, this pathway may also be regulated by Sec5, Sec6, and Sec15.

tested to determine whether they interact with Arm by yeast two-hybrid assays. An interaction between the same internal domain of Arm and Sec10 was identified, but no interaction was found with Sec8, Exo84, or Sec6 (Figure 5A and not shown). The interaction between Sec10 and Arm was further confirmed by GST pull-down, and the amino residues 251 to 480 of Sec10 were shown to be sufficient for interaction with GST-Arm (Figure 5B). In addition, in extracts prepared from *Drosophila* embryos expressing Sec10-GFP, which colocalized with Sec5 and Arm (not shown), anti-GFP antibody was found to immunoprecipitate Arm, Sec5, Sec15, and Dα-Cat (Figure 5C). Furthermore, in cell lysates from the normal rat kidney (NRK) cell line, anti-Sec5 was found to immunoprecipitate β-catenin, and reciprocally, anti-β-catenin was found to immunoprecipitate Sec5 (Figure 5D). Finally, Sec5, β-catenin, and α-catenin were found in anti-Sec8 immunoprecipitates (Figure 5E). Thus, Arm and its mammalian homolog β-catenin can exist in a complex with exocyst components.

In order to test whether Arm regulates the delivery of DE-Cad from REs to the lateral membrane, the localization of DE-Cad and Rab11 were analyzed in mutant arm^{YD35} epithelial cell mutant for the amorphic arm^{YD35} allele (Cox et al., 1996). Similar to what has been found in arm^{XP33} mutant embryos (Cox et al., 1996), in large arm^{YD35} mutant epithelial cell clones, the overall level of DE-Cad was reduced and DE-Cad was almost completely absent from the AJs and the lateral membrane (Figure 5F and not shown). While the level

of DE-Cad was reduced in *arm* mutant epithelial cells, DE-Cad was found in intracellular punctate structures, and DE-Cad was enriched in the REs (Figures 5F–5F″). Indeed, 27% of the intracellular DE-Cad puncta (n = 69) were colocalized with or closely apposed with Rab11-positive vesicles (n = 81, Figure 5F′) versus 11% in wild-type cells and 40% in *sec5* mutant epithelial cells. Even though the *arm* mutant phenotype was distinct from the one observed in *sec5* and *sec6* mutant epithelial cells, DE-Cad was found in the REs and was not localized at the lateral membrane in *arm* mutant epithelial cells as observed in the absence of Sec5 or Sec6. As indicated by the interaction between Arm and the exocyst, our results suggest that Arm together with the exocyst regulates the delivery of DE-Cad from the REs to the lateral membrane and the AJs.

Discussion

Deciphering the Role of Exocyst Components in *Drosophila* Epithelial Cells

Exocyst components have been implicated in different developmental contexts where they appear to function in polarized exocytosis. Sec5 and Sec6 have implicated during neurite growth, synaptic transmission, and polarization of the *Drosophila* oocyte (Beronja et al., 2005; Murthy et al., 2003, 2005; Murthy and Schwarz, 2004). Furthermore, Sec6 was shown to regulate apical exocytosis in photoreceptor cells (Beronja et al., 2005). Recently, Sec15 was implicated in synaptic specificity, as *sec15* loss of function causes a defect in the targeting of specific adhesion and signaling molecules. In this context, Sec15 is proposed to function as part of an exocyst subcomplex, since *sec15* loss of function in contrast to that of *sec5* or *sec6* does not lead to cell death (Mehta et al., 2005). Here we have identified that the loss of *sec5*, *sec6*, or *sec15* function similarly disrupts the localization of DE-Cad, which accumulates in an enlarged RE compartment. Our work demonstrates a role of exocyst components in regulating the localization of DE-Cad in epithelial cells and the delivery of DE-Cad from the REs. Furthermore, using antibody binding uptake experiments, Sec5 was found to regulate the recycling of basolateral DE-Cad to the apical AJs. While a connection between exocyst and the Rab11 recycling endosomal compartment has been proposed both in *Drosophila* and mammalian cells (Beronja et al., 2005; Folsch et al., 2003; Prigent et al., 2003; Zhang et al., 2004), the characterization of *sec15* phenotype demonstrates the functional significance of this interaction. Finally, the exocyst has been viewed as an important regulator of exocytosis, but the mechanisms by which the exocyst complex could regulate the exocytosis of specific cargoes to specific domains within the cell have remained poorly understood. Below we propose a model whereby the interaction between Sec10 and Arm enables the exocyst to regulate DE-Cad trafficking.

Tethering of Vesicles Originating from the RE to the Lateral Membrane

In budding yeast, the exocyst has been proposed to tether post-Golgi vesicles to the membrane of the growing bud prior to fusion. This model is supported by several observations. First, exocyst components localize both on post-Golgi vesicles and on the bud membrane (Boyd et al., 2004). Analogously in *Drosophila*, Sec5 and Sec15 localized along the lateral membrane and on the REs. Second, mutations in genes encoding components of the exocyst complex lead to the accumulation of post-Golgi vesicles (Novick et al., 1980). Analogously, Sec5, Sec6, and Sec15 loss of function leads to an enlargement of the RE compartment that we interpret as an accumulation of RE vesicles. Third, the localization of Sec8p and Exo70p at the growing bud, i.e., the site of polarized exocytosis, depends on the function of the other exocyst components (Finger et al., 1998; Guo et al., 2001). Analogously, Sec5 is localized along the lateral membrane, where DE-Cad delivery is affected, and its localization along the cortex depends on Sec6. We therefore propose that in *Drosophila* epithelial cells, Sec5, Sec6, and Sec15 act by tethering vesicles originating from the recycling endosomal compartment to the lateral membrane of epithelial cells, as a prerequisite for their exocytosis.

In epithelial cells, Arm and DE-Cad colocalize to the AJs of the ZA as well as along the lateral membrane. In the absence of Sec5, Sec6, and Sec15 function, DE-Cad trafficking is affected and DE-Cad accumulates in the RE. Similarly, in the absence of *arm*, DE-Cad failed to localize at the membrane and localized in the RE. The identification of an interaction between Arm and Sec10 is therefore consistent with a model whereby this interaction provide a landmark at the site where Arm is enriched in order to deliver DE-Cad from the recycling endosomes. Nevertheless, Arm may play an additional role in stabilizing DE-Cad at the AJs. A direct demonstration of the function of Arm in regulating the delivery of DE-Cad will therefore require the identification of *arm* mutant alleles that do not perturb its function as a regulator of DE-Cad stabilization and only affects its interaction with Sec10.

The Exocyst Regulates DE-Cad Transcytosis in *Drosophila*

In the absence of Sec5, Sec6, or Sec15 function, DE-Cad delivery to the lateral membrane is inhibited and DE-Cad accumulates in the REs. Furthermore, DE-Cad was found to transcytose in a Sec5-dependent manner from the lateral membrane of epithelial cells to the apical AJs. Therefore, our study reveals at least a role of the exocyst in the recycling of DE-Cad from the lateral membrane to the apical AJs. Furthermore, the strong reduction of DE-Cad present on the lateral membrane is interpreted as a failure to recycle DE-Cad from the lateral membrane back to the lateral membrane, which cannot be compensated for by the delivery of newly synthesized DE-Cad to the lateral membrane (see model, Figure 5G). The loss of DE-Cad on the lateral membrane may also lead to a reduction of DE-Cad delivery at the AJs. This may have also contributed to the loss of epithelial cell polarity observed in some of the *sec5* mutant epithelial cells.

In polarized MDCK cells, the apical REs are well known as a site of sorting during endocytic and transcytotic transport (for review, see Hoekstra et al., 2004). More recently, the REs were also shown to serve as an

intermediate during the transport of newly synthesized proteins from the Golgi to the plasma membrane in nonpolarized MDCK cells (Ang et al., 2004). Similarly, upon overexpression of GFP-E-Cad in HeLa cells, E-Cad was shown to transit from the Golgi to the Rab11 endosomes (Lock and Stow, 2005). Nevertheless, the existence of such a pathway remains to be established in polarized MDCK cells. In fact, the overexpression of a dominant-negative form of Rab11 led to sequestration of E-Cad in the REs, but whether sequestered E-Cad represented newly synthesized or recycled E-Cad was not determined (Lock and Stow, 2005; Miranda et al., 2001). The existence of such a Golgi-to-RE pathway also remains to be established in *Drosophila* epithelial cells. If so, a role of the exocyst in regulating the delivery of newly synthesized DE-Cad from the Golgi to the lateral membrane via the REs remains plausible (see model in Figure 5G).

Delivery of E-Cadherin from the RE to the Membrane

Whether the exocyst regulates E-Cad localization in mammalian cells has not been directly analyzed. However, E-Cad is proposed to act as a regulator of the localization of the exocyst complex in polarizing mammalian cells since E-Cad- and Nectin-2α-dependent cell-cell contacts were proposed to recruit the exocyst complex in order to promote the growth of the lateral epithelial cell domain (Yeaman et al., 2004). Our study suggests that upon the recruitment of the exocyst complex by E-Cad, the exocyst promotes the delivery of more E-Cad to the lateral membrane during the establishment of apico-basal polarity. In fact, several reports can be reconciled with a function of the exocyst in regulating the transport of E-Cad in mammalian cells. Thus, polarized exocytosis of E-Cad to the lateral membrane is dependent upon its interaction with Arm (Chen et al., 1999; Miranda et al., 2001). And, as stated above, REs were shown to serve as an intermediate during the transport of E-Cad from the Golgi to the lateral membrane where E-Cad, β-Catenin, and α-Catenin form the AJs (Lock and Stow, 2005). Furthermore, the overexpression of a dominant-negative form of Rab11 impairs the delivery of E-Cad to the lateral membrane (Lock and Stow, 2005). Consistent with the exocyst regulating trafficking from the REs, exocyst components also localize on the REs, and Sec15 is an effector of Rab11 (Folsch et al., 2003; Prigent et al., 2003; Zhang et al., 2004). Finally, DE-Cad and catenins are associated with exocyst components (Yeaman et al., 2004, and this report).

In conclusion, our work provides evidence for a conserved role of the exocyst in regulating the delivery of E-Cad from REs to sites on the plasma membrane and in thereby contributing to the maintenance of epithelial cell polarity.

Experimental Procedures

Flies

w pupae were used as wild-type controls. Clones were recovered from pupae of the following genotypes: (1) hs-flp; FRT40A, Ubi-nls-GFP/FRT40A,*sec5*^{E10}, (2) hs-flp; FRTG13 *sec6* ^{ex15}/FRTG13 Ubi-nlsGFP, (3) *arm*^{YD35}, FRT101/Ubi-nls-GFP,FRT101; hs-flp, and (4) hs-flp; FRT82B, *sec15*^{1}/FRT82B Ubi-nlsGFP. *arm*^{YD35} (Cox et al., 1996), *sec5*^{E10} (Murthy and Schwarz, 2004), *sec6*^{ex15} (Murthy et al., 2005), and *sec15*^{1} (Mehta et al., 2005) mutant clones were generated by heat-shocking late L2 larvae for 1 hr at 37°C. Sec10-GFP embryonic extracts were prepared from embryos collect from maternal-GAL4 females (flybase: http://fbserver.gen.cam.ac.uk/) crossed with UAS-Sec10-GFP males (Andrews et al., 2002).

Immunocytochemistry

Pupal nota were dissected from staged pupae and fixed and stained as described in Bellaiche et al. (2001). Primary antibodies were mouse *anti*-Sec 5 (1/50, specificity of Sec5 staining was verified by strong reduction of Sec5 staining in *sec5*^{E10} mutant epithelial cells, see Movie S1), rat *anti*-DE-cadherin (DCAD2) (gift from H. Oda; 1/500), mouse *anti*-Arm (from DSHB; 1/500), rat *anti*-Crumbs (gift from U. Tepass; 1/3000), rabbit *anti*-Lava lamp (gift from J.C. Sisson; 1/5000), rat *anti*-Dα-cat (gift from H. Oda; 1/500), rat *anti*-Rab11 (gift from R. Cohen, 1/1000), rabbit *anti*-Rab11 (gift from D. Ready, 1/3000), rabbit *anti*-Rab5 (from G. Gonzales; 1/100), rabbit *anti*-Nuf (gift from W. Sullivan; 1/1000), rabbit *anti*-Baz (gift from A. Wodarz; 1/4000), mouse *anti*-GFP (Roche; 1/300), rabbit *anti*-GFP (Molecular Probes; 1/4000), mouse *anti*-Fas3 (7G10, obtained from DSHB,1/50), guinea pig *anti*-HRS (gift from H. Bellen, 1/600), mouse *anti*-Dlg (from DSHB; 1/1000), rabbit *anti*-Dlg (gift from P. Bryant; 1/2000), and guinea pig *anti*-Sec15 (gift from H. Bellen, 1/500). The Cy3- and Cy5-coupled secondary antibodies were from Jackson Laboratory, and Alexa-488-coupled secondary antibodies were from Molecular Probes. Images were acquired on Leica SP2 confocal microscope.

Antibody Binding and Recycling Assay on Dissected Notum

Pupal nota were dissected in Schneider's *Drosophila* medium (GIBCO-BRL, Europe) supplemented with 1% fetal calf serum and 1 μg/ml of 20-OH ecdysone (Sigma, Europe). The nota were incubated with either the *anti*-DE-Cadherin DCAD2 diluted at 1/100 alone or with DCAD2 and Fas3 7B10 antibodies in the dissection medium for 30 min at 4°C. Following three washes in Schneider's *Drosophila* medium at 4°C, nota were fixed and then stained as described above. DCAD2 and 7B10 antibodies were detected using Cy3- or Cy5-coupled *anti*-rat antibodies or Cy3- or Cy5-coupled *anti*-mouse antibodies, respectively. Similar binding assays using anti-Arm antibody results in not staining along the membrane or intracellularly.

To perform recycling assays, dissected nota were incubated for 3 hr as above but at 25°C. Incubation without antibody followed by incubation at 4°C with DCAD2 revealed staining only below the AJs, indicating that incubation of dissected nota for 3 hr does not affect septate junction functions or cell integrity.

Yeast Two-Hybrid Assay

The open reading frames of fly *exo70*, *sec6*, *sec8*, *sec10*, and *exo84* were amplified by PCR and cloned in the two-hybrid bait vector pLex12. The screening procedure has been described elsewhere (Formstecher et al., 2005). Two-hybrid methods and pairwise mating assays were performed using standard procedures (Vojtek and Hollenberg, 1995) with strains L40Δgal4 and Y187.

Glutathione-S-Transferase Fusion Protein Pull-Down

The *exo70* and *sec10* templates for T7 primed protein synthesis were prepared from two rounds of PCR using appropriate primers. GST-ΔN1Arm was a kind gift of Dr. K. Basler. The plasmid coding for the GST-Armadillo repeats 7–12, termed GST-ΔN2ΔCArm, was constructed by cloning a PCR fragment in-frame and downstream from the GST coding sequence in the vector pGEX4T1. GST pull-downs were performed according to Bellaiche et al. (2004).

Immunoprecipitation

Fly protein extracts were prepared from embryos less than 24 hr old and raised at 18°C, in a lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5% NP40, and Complete Protease inhibitor (Roche Europe). For each immunoprecipitation, lysate containing at least 1 mg of protein was precleared in Protein G sepharose (Amersham, Europe) and incubated with Protein G sepharose and antibody for 16 hr at 4°C (one of the following: 2 μl rabbit

anti-GFP, kind gift from Ahmed Zahraoui; 5 μl mouse *anti*-Sec5, ascites clone 16A; 1 μl rabbit *anti*-Rab11; or 1 μl guinea pig *anti*-Sec15). Cell lysates and immunoprecipitates were examined using a standard Western blotting protocol, with either rabbit *anti*-GFP, mouse *anti*-Sec5, rabbit *anti*-Rab11, guinea pig *anti*-Sec15, mouse *anti*-Arm, or rat *anti*-Dα-cat primary antibodies; appropriate HRP-conjugated secondary antibodies; and Amersham's (Europe) ECL Advance chemiluminescence detection system.

With NRK cells, subconfluent cultures were lysed, and cell extracts corresponding to 1.5 million cells per immunoprecipitation were prepared in Tris-HCl 20 mM (pH 7.4), NaCl 100 mM, $MgCl_2$ 1 mM, DTT 0.1 mM, 1% Triton X100, 10% glycerol. Immunoprecipitation and further analysis were performed according to Prigent et al. (2003) using 5 μg for the immunopreciptation and 1/1000 for immunodetection of *anti*-Sec8, *anti*-α-catenin, and *anti*-β-catenin (all from Transduction Laboratories, USA) and *anti*-Sec5 antibodies.

Supplemental Data

Supplemental Data include two figures, one table, and one movie and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/9/3/365/DC1/.

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