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Retromer regulates apical–basal polarity through recycling crumbs

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ABSTRACT

Epithelial cells are characterized by an "apical–basal" polarization. The transmembrane protein Crumbs (Crb) is an essential apical determinant which confers apical membrane identity. Previous studies indicated that Crb did not constantly reside on the apical membrane, but was actively recycled. However, the cellular mechanism(s) underlying this process was unclear. Here we showed that in *Drosophila*, retromer, which was a retrograde complex recycling certain transmembrane proteins from endosomes to *t*rans-Golgi *n*etwork (TGN), regulated Crb in epithelial cells. In the absence of retromer, Crb was mis-targeted into lysosomes and degraded, causing a disruption of the apical–basal polarity. We further showed that Crb co-localized and interacted with retromer, suggesting that retromer regulated the retrograde recycling of Crb. Our data presented here uncover the role of retromer in regulating apical–basal polarity in epithelial cells and identify retromer as a novel regulator of Crb recycling.

Introduction

In epithelial cells, apical and basolateral membranes are compositionally and functionally distinct membrane domains, isolated by adhesion junctions (AJs) (Nelson, 2003). The identity and relative size of the apical and basolateral membranes are controlled by several protein complexes (Nelson, 2003). The Lethal giant larval complex (Bilder et al., 2000) and the Yurt/Coracle complex (Laprise et al., 2009) localize on the basolateral side and define the basolateral identity. On the other hand, the Bazooka (Baz) complex (Muller and Wieschaus, 1996; Petronczki and Knoblich, 2001; Wodarz et al., 2000) and the Crumbs (Crb) complex (Bachmann et al., 2001; Hong et al., 2001; Tepass et al., 1990; Wodarz et al., 1995) are located on the apical membrane and regulate the apical identity. The Crb complex contains several components including Stardust, *P*rotein *a*ssociated to *t*ight *j*unctions (Patj) and Lin-7. Crb is a transmembrane protein (Tepass et al., 1990) while Stardust (Bachmann et al., 2001; Hong et al., 2001), Patj (Bhat et al., 1999) and Lin-7 (Bachmann et al., 2008) are adaptors associated with the intracellular domain of Crb.

During development, epithelial cells often undergo various cell movements including invagination, convergent extension and ingression (Wilt and Hake, 2004). How Crb is regulated and maintained during these dynamic processes is of interest. Recent studies suggested that Crb was under dynamic intracellular trafficking control (Blankenship et al., 2007; Lu and Bilder, 2005; Roeth et al., 2009). Blockage of either endocytosis (Lu and Bilder, 2005) or exocytosis (Blankenship et al., 2007) led to an alteration of the Crb protein level on the cell membrane. Moreover, it was recently found that disrupting recycling endosomes could reduce the Crb protein level (Roeth et al., 2009). Collectively, these findings strongly indicate that Crb undergoes active recycling. However, details of how the recycling of Crb is executed and regulated have remained elusive.

Retromer is an evolutionarily conserved intracellular protein complex (Collins, 2008). Retromer interacts with the intracellular domain of its specific transmembrane cargos and regulates their retrograde transportation from endosomes to *t*rans-Golgi *n*etwork (TGN) (Arighi et al., 2004; Seaman, 2004). The retromer complex consists of two sub-complexes (Collins, 2008). *V*acuolar *p*rotein *s*orting 35 (Vps35), Vps26 and Vps29 form one sub-complex for target-recognition (Collins, 2008). Homo- or hetero-dimer of *S*orting *N*exin 1 (SNX1)/SNX2 or SNX5/6 or SNX3 form the other sub-complex for membrane association, curvature sensing and endosome tubulation (Carlton et al., 2004; Collins, 2008; Harterink et al., 2011; Wassmer et al., 2007). Through governing the retrograde trafficking of its multiple distinct cargoes, retromer regulates several biological processes, including lysosome maturation (Arighi et al., 2004; Seaman, 2004), polymeric IgA transcytosis (Verges et al., 2004), Wnt secretion (Belenkaya et al., 2008; Franch-Marro et al., 2008; Harterink et al., 2011; Port et al., 2008), apoptotic cell clearance (Chen et al., 2010) and the efflux of the phytohormone auxin (Jaillais et al., 2007). However, it is currently unknown whether retromer plays a role in regulating apical–basal polarity in epithelial cells.

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Materials and methods

Drosophila strains

Wild type controls: In the follicle mutant clone experiment, cells containing a *minute* (*M(2)58F*) served as the wild type control; in all other experiments, *w1118* was used as the wild type control. *minute* (*M(2)58F*) was marked by GFP expression and was used to generate large *dvps35* follicle mutant clones. *act>y^{+}>Gal4-UAS-GFP* was used to generate flip-out clones and to express *dvps35* or *dvps26* RNAi in follicle cells. Mutant lines: *dvps35^{1}* (Belenkaya et al., 2008) and *crb^{1}* were amorphic alleles. Transgenic lines: *tub-Gal4*, *prd-Gal4*, *UAS-Crb* (wt2e, gift from U. Tepass; (Wodarz et al., 1995)), *UAS-MycVps35* (Belenkaya et al., 2008), *UAS-dvps35RNAi* (Belenkaya et al., 2008), and *UAS-dvps26RNAi* (GD18396 and VDRC (Dietzl et al., 2007)).

Generation of clones

Germ-line clones were generated using the FLP-DFS (FLP-recombinase-dominant female sterile) technique (Chou and Perrimon, 1996). Wandering third instar larvae were heat-shocked at 37 °C for 2 h on 2 consecutive days. Follicle mutant clones were generated using FLP-mediated mitotic recombination (Xu and Rubin, 1993). One-day-old female flies were heat-shocked at 37 °C for 1 h and put in freshly yeasted vials with *w1118* males for another 2–4 days before dissection. Follicle flip-out clones were generated using the FLP-OUT technique (Ito et al., 1997). The heat-shock strategy is the same as for follicle mutant clones.

Drosophila immunostaining and cuticle preparation

Drosophila embryos were fixed as described (Tepass et al., 1990) except for Arm, where embryos were heat-fixed (Muller and Wieschaus, 1996). *Drosophila* ovaries were fixed as described (Tanentzapf et al., 2000) except for Crb, where ovaries were additionally treated with methanol for 5 min after normal fixation (Tanentzapf et al., 2000). The following primary antibodies were used: mouse anti-Crb (Cq4, 1:40, DSHB), rat anti-Crb (1:200) (Bhat et al., 1999), rat anti-Crb (F4, 1:100) (Pellikka et al., 2002), rabbit anti-Patj (1:50) (Tanentzapf et al., 2000), rabbit anti-aPKC (C-20, 1:1000, Santa Cruz), rabbit anti-Baz (1:1000) (Wodarz et al., 1999), mouse anti-Arm (N2-7A1, 1:5, DSHB), rat anti-DE-cad (DCAD2, 1:40, DSHB), mouse anti-Nrt (BP106, 1:5, DSHB), mouse anti-Dlg (4F3, 1:5, DSHB), guinea pig anti-Cad87A (1:50) (Harris and Tepass, 2008), mouse anti-α-Spec (3A9, 1:5, DSHB), rabbit anti-Lva (1:200) (Sisson et al., 2000), guinea pig anti-dRab5 (1:100) (generated according to (Wucherpfennig et al., 2003)), guinea pig anti-Hrs (full-length, 1:100) (Lloyd et al., 2002), rabbit anti-Arl8 (1:100) (Hofmann and Munro, 2006), mouse anti-GFP (1:200, Qbiogene), rabbit anti-GFP Alexa Fluor 488 (1:400, Molecular Probes), mouse anti-Myc (1:50, Invitrogen), and rabbit anti-Myc (1:300, Cell Signaling). Cy3, Cy5 (Jackson Immuno) or Alexa Fluor 488 (Molecular Probes)-conjugated secondary antibodies were used at a dilution of 1:400. Confocal images were collected on a Zeiss LSM 510 confocal microscope with 40× or 63× oil objectives. For cuticle preparations, embryos were dechorionated in 50% bleach for 3 min and mounted in Hoyer's medium, mixed 1:1 with lactic acid. Images were collected on a Zeiss Axioskop 2 plus microscope with a 20× objective. Images were processed and arranged in Adobe Photoshop.

Immunoblotting and co-immunoprecipitation

Drosophila embryos (0–8 h at 25 °C) were homogenized in Laemmli sample buffer (Bio-Rad) plus 5% 2-Mercaptoethanol (FisherBiotech). Embryo lysates were cleared by centrifugation and subjected to immunoblot (IB) analysis. For co-immunoprecipitation (co-IP) experiments, HeLa cells were transfected in 60 mm dishes with 3 μg total DNA, using Polyfect transfection reagent (QIAGEN). 60 h after transfection, co-IP and immunoblot analysis were performed as described (Belenkaya et al., 2008). Cell lysis was performed in the following condition (1% Triton X-100, 50 mM NaCl, 20 mM Tris at pH 7.5, and 1 mM EDTA). Protein G-Sepharose beads (GE Healthcare) were used for co-IP. Washing was performed 5 times using the washing buffer (0.2% Triton X-100, 50 mM NaCl, 20 mM Tris at pH 7.5, and 1 mM EDTA). The following primary antibodies were used: mouse anti-Crb (Cq4, 1:100 for IB, DSHB), mouse anti-V5 (1 μg for IP, 1:1000 for IB, Invitrogen), mouse anti-Myc (1 μg for IP, Invitrogen), rabbit anti-Myc (1:1000 for IB, Cell Signaling), mouse anti-FLAG (1:1000 for IB, Abmart), mouse anti-β-actin (1:5000 for IB, Abcam). Horseradish peroxidase (HRP, Jackson Immuno), or IRDye800CW (LI-COR)-conjugated secondary antibodies were used at a dilution of 1:3000 or 1:10,000. Proteins on the blot were detected using the ECL detection system (Thermo Scientific) or the Odyssey IR imaging system (LI-COR).

Molecular cloning

pUAST-V5Crb: Part of the extracellular domain and the entire transmembrane and intracellular domain of *Drosophila* Crb (the last 406 aa) were fused to the C-terminus of the V5 tag. The resultant V5-dCrb was subcloned into the pUAST construct using EcoRI and XbaI sites. pUAST-MycVps35 was described previously (Belenkaya et al., 2008). pUAST-FLAGVps26-attB: Full length of *Drosophila* Vps26 was subcloned into the pUAST-FLAG-attB construct (gift from B. Gebelein) using NotI and XbaI sites. DVps26 was fused to the C-terminus of the FLAG tag. Illustration of constructs is shown in Fig. 7D.

Results

Drosophila Vps35, DVps35, is required for the apical polarity of epithelial cells

Vps35 is a major component of retromer (Collins, 2008). We previously generated a null mutant of the *Drosophila vps35* gene (*dvps35^{1}*) (Belenkaya et al., 2008). We and others have characterized an important role of *Drosophila* Vps35 in Wnt secretion (Belenkaya et al., 2008; Franch-Marro et al., 2008; Port et al., 2008). To further explore the role of retromer during development, we generated embryos lacking both maternal and zygotic dVps35 activity (referred to as *dvps35* embryos hereafter) using the germ-line clone (GLC) technique (Chou and Perrimon, 1996). Interestingly, *dvps35* embryos displayed a disruption of apical–basal polarity (Fig. 1). When compared with wild type (Fig. 1A), *dvps35* embryos presented a loss-of-cuticle phenotype (Fig. 1C). The phenotype ranged from mild cuticle defects to a massive loss of cuticle (Fig. S1). In the most severe case, only small patches of cuticle were produced (Fig. 1C), which was characteristic of mutants of apical determinants (*crb* zygotic mutants, Fig. 1E). The cuticle defect indicated a disruption of apical–basal polarity in *dvps35* embryos. To examine the disruption of apical–basal polarity at the cellular level, we stained for apical (dPatj) and basolateral markers (Neurotactin, Nrt) in epithelial cells of *dvps35* embryos. In *dvps35* embryos at the germband extension stage, the dPatj staining was greatly reduced and epithelial cells displayed an irregular, non-columnar shape (Figs. 1D–D″) when compared with wild type embryos (Figs. 1B–B″). However, Nrt was still located on the cell membrane, and the protein level was unaltered (Fig. 1D′).

To confirm the apical-specific defect in *dvps35* embryos, we examined more apical and basolateral markers. In wild type embryos at the germband extension stage, apical markers Crb, dPatj, *a*typical *P*rotein *K*inase *C* (aPKC, a component in the Baz complex), Baz, AJs component Armadillo (Arm) as well as basolateral markers Nrt and Discs Large (Dlg) were localized on the cell membrane (Figs. 2A–G). In *dvps35* embryos of the same stage, apical markers were lost from the cell membrane and their remnants were distributed in dot-like structures