FLY
2016, VOL. 10, NO. 3, 123–127
http://dx.doi.org/10.1080/19336934.2016.1182273

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EXTRA VIEW

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Rab6 functions in polarized transport in *Drosophila* photoreceptors

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ABSTRACT

Selective membrane transport pathways are essential for cells *in situ* to construct and maintain a polarized structure comprising multiple plasma membrane domains, which is essential for their specific cellular functions. Genetic screening in *Drosophila* photoreceptors harboring multiple plasma membrane domains enables the identification of genes involved in polarized transport pathways. Our genome-wide high-throughput screening identified a Rab6-null mutant with a rare phenotype characterized by a loss of 2 apical transport pathways with an intact basolateral transport. Although the functions of Rab6 in the Golgi apparatus are well known, its function in polarized transport is unexpected.

The mutant phenotype and localization of Rab6 strongly indicate that Rab6 regulates transport between the *trans*-Golgi network (TGN) and recycling endosomes (REs): basolateral cargos are segregated at the TGN before Rab6 functions, but cargos going to multiple apical domains are sorted at REs. Both the medial-Golgi resident protein Metallophosphoesterase (MPPE) and the TGN marker GalT::CFP exhibit diffused co-localized distributions in Rab6-deficient cells, suggesting they are trapped in the retrograde transport vesicles returning to *trans*-Golgi cisternae. Hence, we propose that Rab6 regulates the fusion of retrograde transport vesicles containing medial, *trans*-Golgi resident proteins to the Golgi cisternae, which causes Golgi maturation to REs.

ARTICLE HISTORY
Received 23 March 2016
Revised 9 April 2016
Accepted 20 April 2016

KEYWORDS
Photoreceptor; Rab6; recycling endosome; retrograde transport; *trans*-Golgi network

Fly photoreceptor cells as a model to investigate the mechanism of polarized transport

Fully differentiated cells are often highly polarized *in vivo*; epithelial cells and neurons are 2 well-known examples. Epithelial cells contain apical and basolateral plasma membrane domains, while neurons contain an axon, dendrites, and a cell body. Polarized vesicle transport is essential for establishing and maintaining these polarized structures. However, the underlying mechanisms are not well elucidated. Highly polarized fly photoreceptors are a good genetics-based model for studying the mechanism of polarized transport. In a single plane of the retina, 3 distinct plasma membrane domains of many photoreceptors can be observed. The first domain is the photoreceptive membrane domain, i.e., the rhabdomere, which is formed at the center of the apical plasma membrane during pupal development. Proteins involved in photo-transduction—such as the photosensitive molecule rhodopsin1 (Rh1) and the Ca^{2+} permeable channel TRP—as well as a protein essential for rhabdomere architecture, chaoptin (Chp), specifically localize in the rhabdomeres. The second domain is the peripheral apical domain surrounding the rhabdomere, i.e., the stalk membrane, which is where the apical determinant Crb is localized. The third domain is the basolateral membrane, which is separated from the apical membrane by the adherens junctions. $Na^{+}K^{+}$ATPase localizes on the basolateral membrane, similar to typical polarized epithelial cells.[1]

Rab6-null mutant fails to accumulate Rh1 in the rhabdomeres in Drosophila photoreceptors

One of the advantages of the *Drosophila melanogaster* model is that forward genetics can be used for genome-wide screening. Thus, to identify the genes essential for polarized membrane transport to the rhabdomeres, we performed retinal mosaic screening of P-element–inserted lines maintained in stock centers[2] or EMS-mutagenized flies using the FLP/FRT method[3] with *in vivo* fluorescent imaging[4] of Arrestin2::GFP.[5] Arrestin2::GFP specifically binds to Rh1, enabling the visualization of Rh1 localization in live animals. Accordingly, we identified a Rab6-null

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allele, $Rab6^{546P}$, as the mutant that failed to accumulate Rh1 in the rhabdomeres. Rab6 is a member of the Rab family of small GTPases, which regulate the specificity of vesicle budding, docking, tethering, and fusion steps during transport.[6-8] Several reports have shown that Rab6 regulates vesicle trafficking around the Golgi complex, including retrograde transport from the Golgi complex to the endoplasmic reticulum;[9,10] the retrograde transport of the B subunit of Shiga toxin from early endosomes or recycling endosomes (REs) to the Golgi complex;[11,12] and the post-Golgi trafficking of herpes simplex virus 1 (HSV1) envelop proteins, tumor necrosis factor (TNF), and vesicular stomatitis virus G-protein (VSV-G) to the plasma membrane.[13-15] However, the function of Rab6 in polarized transport has been studied scarcely. We recently investigated the phenotypes of $Rab6^{546P}$, another Rab6 null allele ($Rab6^{D23D}$), and a null allele ($Rich^{1}$) of the putative guanine nucleotide exchange factor (GEF) Rich that recruits Rab6. The results of these studies showed that Rab6 functions in apical transport pathways but not in basolateral transport pathways.[16]

Defects in the polarized trafficking of membrane proteins in $Rab6^{546P}$, $Rab6^{D23D}$, and $Rich^{1}$ photoreceptors

Flies have an "open rhabdom," in which the rhabdomeres are separated from each other by the inter-rhabdomeric space. Thus, individual rhabdomeres can respond differently to light coming from slightly different angles. In contrast, bees and butterflies have a "closed rhabdom," in which rhabdomeres within an ommatidium are closely bundled and cannot separate light coming from slightly different directions. In other words, an open rhabdom yields better spatio-temporal resolution. "Eye shut" (Eys) is an extracellular matrix protein secreted from the stalk membrane domain of fly photoreceptors in pupal stages,[17,18] and its secretion is necessary and sufficient to form the inter-rhabdomeric space; therefore, *eys*-deficient fly ommatidia exhibit a closed rhabdom. Interestingly, $Rab6^{546P}$, $Rab6^{D23D}$, and $Rich^{1}$ mutant ommatidia exhibit a closed rhabdom, indicating a loss of Eys secretion. Immunostaining of Eys confirmed that Eys was not secreted into the inter-rhabdomeric space, but rather accumulated within multi-vesicular bodies (MVBs) in these mutants. A stalk membrane protein, Crb was also detected within the same MVBs as Eys localizes. The rhabdomere proteins, Rh1, TRP and Chp co-localize with Eys and Crb within MVBs in $Rab6^{546P}$, $Rab6^{D23D}$, and $Rich^{1}$ mutant photoreceptors.

In contrast to the deficient apical transport of secretory and membrane proteins, the basolateral transport of a membrane protein, $Na^{+}K^{+}$ATPase was not affected in $Rab6^{546P}$, $Rab6^{D23D}$, or $Rich^{1}$ mutant photoreceptors. DE-Cadherin, which is presumably transported to the basolateral membrane before accumulating in the adherence junctions, also exhibited normal localization in these mutant photoreceptors. These results indicate that transport pathways to 2 apical domains—the rhabdomeres and stalk—but not the transport pathway to the basolateral membrane, are inhibited in $Rab6^{546P}$, $Rab6^{D23D}$, and $Rich^{1}$ mutant photoreceptors. Concordant with the localizations of plasma membrane proteins, the basolateral membrane in $Rab6^{546P}$, $Rab6^{D23D}$, and $Rich^{1}$ mutant photoreceptors was extended as in the wild type, despite the reduced stalk and rhabdomere membrane. In addition, we determined if Rab6 deficiency affects the transport of another basolateral protein, FasIII, in ovarian follicle cells. FasIII localized normally on the basolateral membrane, whereas apically localized Notch membrane protein was diminished in mutant follicle cells. These results indicate Rab6 can involve generally in apical but not in basolateral transport in polarized cells.

Rab6 localizes at the TGN and REs

We previously showed that Rab11 is essential for the post-Golgi transport of Rh1 and that most Golgi units are accompanied by Rab11-positive puncta adjacent to the *trans* side in fly photoreceptors.[19] As Rab11 is typically considered an RE marker, these Rab11-positive puncta are likely fly photoreceptor REs. Rab6 exhibits widespread localization from CFP::Galactosy-transferase (GalT::CFP)-labeled TGN to Golgi-associated Rab11-positive REs. Consistent with Rab6 localization, the detailed analysis of Rh1 transport in Rab6-deficient cells by blue light-induced chromophore supply (BLICS), a method to induce synchronous ER exit of Rh1[20,21] indicated that Rab6 functions during the stage close to but before Rab11-dependent post-Golgi trafficking. Thus, Rab6 is likely involved in transport between the TGN and REs. Since rhabdomeric transport as well as stalk transport is

inhibited in Rab6-deficient cells, membrane proteins destined for the rhabdomere and stalk are likely transported together to REs by Rab6-regulated processes before sorting.

Clathrin and clathrin adaptor protein complex 1B (AP1B) are involved in basolateral transport.[22,23] In fly photoreceptors, loss of the AP1 gamma or AP1 mu subunit causes mis-transport of Na^+K^+ATPase to the stalk membrane,[5] indicating the involvement of AP1 in basolateral transport. Despite a lack of direct evidence, clathrin likely plays a role in basolateral transport in fly photoreceptors. We found that clathrin heavy chain and Rab6 partially co-localize, but clathrin heavy chain and Rab11 are substantially separated. These results indicate that the basolateral transport pathway likely branches off before the Rab6-dependent transport from the TGN to REs.

Medial, trans-Golgi resident proteins but not cis-Golgi proteins are dispersed in Rab6-deficient photoreceptors

Electron microscopy revealed that Golgi cisternae are dilated and accompanied by multi vesicular bodies (MVBs) in Rab6-deficient cells. To further understand the abnormality of Golgi units in Rab6-deficient cells, we investigated the distribution of Golgi resident proteins in Rab6-deficient photoreceptors (Fig. 1). In Rab6-null photoreceptors, both the medial-Golgi resident protein MPPE and the *trans*-Golgi/TGN marker GalT::CFP exhibited diffuse distributions. These patterns are clearly distinct from that observed in typical staining of Golgi or MVBs, in which apical cargo are accumulated in Rab6-deficient photoreceptors. However, MPPE and GalT::CFP were still largely co-localized in loose cytoplasmic condensation in Rab6-deficient photoreceptors. In contrast, the *cis*-Golgi protein GM130 localized normally. These results indicate that *cis*-Golgi compartments are normal, whereas the medial, *trans*-Golgi compartments can lose their normal functionality. These findings are concordant with the results of the detailed analysis of Rh1 transport in Rab6-deficient cells by BLICS.

Rab6 regulates apical cargo trafficking from the TGN to REs in fly photoreceptors

The results of our recent study indicate that Rab6 is involved in the transport of 2 apically directed cargo proteins from the TGN to REs. However, the direction of Rab6-mediated transport (i.e., anterograde or retrograde) remained unresolved, because anterograde cargo transport can be a result of cisternal progression and retrograde vesicle transport retrieving TGN-resident proteins. The present study showed the diffusion of medial- and *trans*-Golgi/TGN enzymes but normal *cis*-Golgi–localizing proteins in Rab6-deficient photoreceptors. Dispersed but co-localized condensations of MPPE and GalT::CFP in Rab6-deficient cells could be interpreted as trapped retrograde transport vesicles

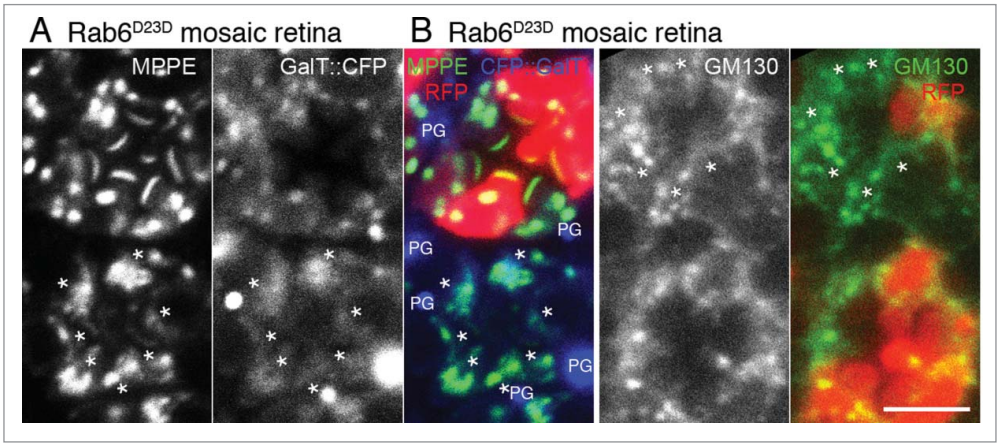

Figure 1. Diffusion of medial, *trans*-Golgi markers in Rab6-null photoreceptors. *Rab6^{D23D}* mosaic retinas were immunostained by the following antibodies. RFP (red) marks wild-type cells. Asterisks show *Rab6^{D23D}* homozygous photoreceptors. In (A), all peripheral photoreceptors in mosaic retinas express GalT::CFP. (A) Antibody staining for MPPE, a medial-Golgi marker (green) and anti-GFP antibody staining visualizing GalT::CFP, a *trans*-Golgi/TGN marker (blue) shows that these late Golgi enzymes are diffused but still largely co-localized in loose cytoplasmic condensation. GalT::CFP (blue) was originally detected by 473-nm laser, indicating contamination of auto-florescence from pigment granules (PGs). (B) Anti-GM130 antibody (green) shows that cis-Golgi is normal in Rab6 null photoreceptors. Scale bar: 5 mm.

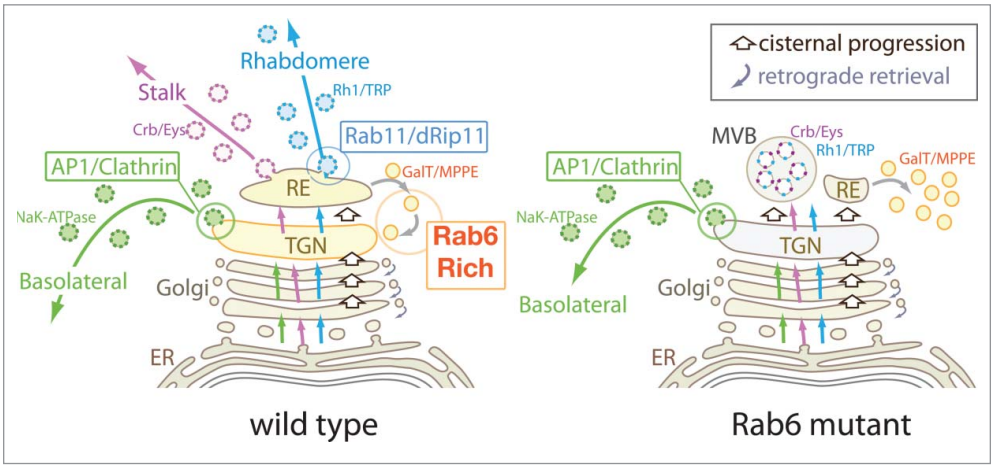

Figure 2. Proposed model of Rab6 function in apical cargo transport. In wild-type fly photoreceptors (left), membrane proteins going to distinct plasma membrane domains are synthesized in the endoplasmic reticulum and transported together into the Golgi units. In the first sorting event at the *trans*-Golgi network (TGN), some cargo proteins are sorted into Chc/AP1 vesicles and exported to the basolateral membrane. Apical cargo proteins bound for the rhabdomere or stalk membrane remain within the TGN. As TGN cisternae mature, Rab6-mediated retrograde transport returns TGN-resident proteins to younger cisternae. As the result of cisternal progression and retrograde retrieval of TGN enzymes, apical cargo proteins are transported into Rab11-positive recycling endosomes. In Rab6-deficient photoreceptors (right), TGN-resident proteins on the retrograde transport vesicles fail to be retrieved to the TGN and accumulate in the cytoplasm,resulting in TGN malfunction and ultimately apical cargo degradation in multi-vesicular bodies. However, basolateral cargo proteins can still leave the TGN via Chc/AP1-dependent, Rab6-independent budding of transport vesicles.

retrieving medial- and *trans*-Golgi resident proteins (Fig. 2). Thus, Rab6 likely mediates the fusion of retrograde transport vesicles required for cisternal maturation to REs, resulting in the loss of trafficking of apical cargos from the TGN to REs and mis-transport of apical cargos to MVBs. This interpretation is concordant with previous reports showing Rab6 involvement in the retrograde transport of the B subunit of Shiga toxin from early endosomes or REs to the Golgi complex.[11,12] Nevertheless, additional direct assays should be performed in future studies to elucidate the function of Rab6 in the fusion of RE-derived retrograde vesicles to the TGN.

Materials and methods

Drosophila stocks and genetics

Flies were grown at 18–25°C on standard cornmeal–glucose–agar–yeast food. Males of w; $Rab6^{D23D}$ FRT40A/SM1 were crossed to females of y w ey-FLP; P3RFP FRT40A/SM1.

Immunohistochemistry

Fixation and staining were performed as described previously.[24] The following primary anti-sera were used: rabbit anti-GM130 (1:300) (Abcam, Cambridge, UK), rabbit anti-MPPE (1:500) (a gift from Dr. Han, Southeast University, Nanjing, China), and chicken anti-GFP (1:1000) (Chemicon International Inc., Billerica, MA, USA). Secondary antibodies were anti-rabbit and anti-chicken labeled with Alexa Fluor 488 or 647 (1:300) (Life Technologies., Carlsbad, CA, USA). Samples were examined and images recorded using an FV1000 confocal microscope (60x, 1.42NA lens; Olympus, Tokyo, Japan).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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