Stratum is required for both apical and basolateral transport through stable expression of Rab10 and Rab35 in Drosophila photoreceptors

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ABSTRACT Post-Golgi transport for specific membrane domains, also termed polarized transport, is essential for the construction and maintenance of polarized cells. Highly polarized Drosophila photoreceptors serve as a good model system for studying the mechanisms underlying polarized transport. The Mss4 Drosophila ortholog, Stratrum (Strat), controls basal restriction of basement membrane proteins in follicle cells, and Rab8 acts downstream of Strat. We investigated the function of Strat in fly photoreceptors and found that polarized transport in both the basolateral and the rhabdomere membrane domains was inhibited in Strat-deficient photoreceptors. We also observed 79 and 55% reductions in Rab10 and Rab35 levels, respectively, but no reduction in Rab11 levels in whole-eye homozygous clones Strat. Moreover, Rab35 was localized in the rhabdomere, and loss of Rab35 resulted in impaired Rh1 transport to the rhabdomere. These results indicate that Strat is essential for the stable expression of Rab10 and Rab35, which regulate basolateral and rhabdomere transport, respectively, in fly photoreceptors.

INTRODUCTION
Post-Golgi transport for specific membrane domains, also called polarized transport, is essential for the development and maintenance of polarized cells (Román-Fernández and Bryant, 2016). The Drosophila retina is an excellent model system for studying the mechanism of polarized transport. In a single cross-section of the Drosophila retina, three types of plasma membrane domains (a rhabdomere, stalk, and basolateral membrane) of many photoreceptors are simultaneously observed. Rh1, the rhodopsin expressed in R1–6 outer retinal photoreceptor cells, is specifically localized to an apical membrane domain, the rhabdomere. In contrast, the sodium-potassium ATPase (Na+/K+-ATPase) is localized to the basolateral membrane domain.

The Rab family of small GTP-binding proteins is an important regulator of membrane trafficking. More than 60 mammalian and 31 Drosophila Rab proteins regulate specific transport steps and pathways (Stenmark, 2009; Pfeffer, 2013). In Drosophila photoreceptors, Rab11 regulates the post-Golgi transport of Rh1 to the rhabdomere, and Rab10 is required for the post-Golgi transport of Na+/K+-ATPase to the basolateral membrane domain (Satoh et al., 2005; Nakamura et al., 2020). However, other Rab proteins might be involved in post-Golgi transport in fly photoreceptors. The function of Mss4 has been controversial for a long time. Mss4 and its Saccharomyces cerevisiae ortholog Dss4 were originally isolated as the first putative Rab guanine nucleotide exchange factor (GEF) (Burton et al., 1993; Moya et al., 1993). Several studies have demonstrated the GEF activity of Mss4 in vitro (Burton et al., 1993; Moya et al., 1993; Burton et al., 1994; Miyazaki et al., 1994; Coppola et al., 2002); however, some studies have indicated that the GEF activity of Mss4 is rather
weak compared with that of other typical GEFs (Itzen et al., 2006; Itzen et al., 2007). Given its tight binding to nucleotide-free Rabs, Mss4 has been proposed to function as a nucleotide-free chaperone (Nuooffer et al., 1997; Strick et al., 2002; Wu et al., 2014). In agreement with this notion, Mss4 was shown to promote the stability of Rab10 against proteasome degradation and to play crucial roles in glucose transporter type 4 (GLUT4) exocytosis in vivo (Gulbranson et al., 2017). In Drosophila, the Mss4 ortholog Stratum (Strat) controls the basal restriction of basement membrane proteins in follicle cells, and Rab8 acts downstream of Strat (Devergne et al., 2017). Strat is also known to localize Rab8 in the trans-Golgi network (TGN) and regulate the exit of Notch, Delta, and Splotch from the TGN. Furthermore, it is involved in the stable expression of Rab8 and Rab10 but not Rab1, Rab11, and Rab3 (Belloc et al., 2018; Belloc et al., 2020). Strat was also shown to interact with Rab3, Rab10, and Rab35 in a genomewide interaction map (Guruharsha et al., 2011). In this study, we investigated the impact of Strat deficiency on polarized transport in fly photoreceptors and found that Strat is required for both rhabdomere and basolateral transport through stable expression of Rab10 and Rab35.

RESULTS

Strat is required for both rhabdomere and basolateral transport

R1–6 outer retinal photoreceptor cells contain three different plasma membrane domains: rhabdomere, stalk, and basolateral membranes (Supplemental Figure S1). The rhabdomere is a tight bundle of photoreceptive microvilli protruding from the photoreceptor into the central lumen of the ommatidium. The rhabdomeres appeared oval in the cross-sections of the photoreceptors. Rh1 is an excellent marker of rhabdomere membranes. The basolateral membrane is a Na+/K+-ATPase-positive membrane that contacts pigment cells and neighboring photoreceptors. The stalk membrane, positive for Crb, is located between the rhabdomere and the basolateral membrane and faces the interrhabdomeric space (IRS) (Tepass and Harris, 2007; Xiong and Bellen, 2013; Schopp and Huber, 2017; Laffanian and Tepass, 2019).

To understand whether Strat is necessary for post-Golgi transport in these three plasma membranes, we investigated the homozgyous photoreceptor phenotypes of the Stratnull and Strattwt alleles (Belloc et al., 2018) using a mosaic retina formed by the FLP/FRT method (Xu and Rubin, 1993). The P3RFP markers in the original Stratnull and Strattwt alleles were removed using Cre recombinase to visualize heterozygous and wild-type RFP-positive and homzygous Strat alleles as RFP-negative in FLP/FRT mosaic retinas. In Stratnull homozygous photoreceptors, Na+/K+-ATPase was mislocalized in the stalk membrane (Figure 1A, top panel). Together with the shrinkage of the basolateral membrane and the expansion of the stalk membrane, these phenotypes were similar to those of Rab10-deficient photoreceptors (Figure 1B). At the same time, Stratnull homozygous photoreceptors accumulated Rh1 in the cytoplasm (Figure 1A, top panel) which resembles that in Rab11-reduction by Rab11RNAi expression (Figure 1C). In contrast, in Strattwt homzygous photoreceptors (Figure 1A, bottom panel), Na+/K+-ATPase and Rh1 localized normally in the basolateral and rhabdomere domains. We also investigated the phenotypes of the Strat RNAi-expressing photoreceptors (Figure 1D). Similar to Stratnull homozygous photoreceptors, Strat RNAi(GD10605)-expressing photoreceptors accumulated Rh1 in the cytoplasm, Na+/K+-ATPase was mislocalized to the stalk membrane, and shrinkage of the basolateral membrane and expansion of the stalk membrane were observed in these cells. The stalk and basolateral membranes in Stratnull homozygous photoreceptors were longer (1.44x) and shorter (0.56x) than those of the wild-type photoreceptors, respectively, in the same cross-section of the mosaic retina (Figure 1E). The stalk and basolateral membranes in Strat RNAi(GD10605)-expressing photoreceptors were also longer (1.42x) and shorter (0.70x), respectively, than those of the wild-type photoreceptors in the same cross-section of mosaic retinas (Figure 1E).

Quantification of the ratio of Rh1 staining in the cytoplasm against that in the whole photoreceptor that includes both rhabdomeres and cytoplasm confirmed 74 and 81% Rh1 staining in the cytoplasm of Stratnull homozygous and Strat RNAi(GD10605)-expressing photoreceptors, respectively (Figure 1F).

To perform a detailed phenotypic comparison of the wild-type, Rab10null, Rab11RNAi, and Strat-deficient photoreceptors, we observed thin sections of pupal photoreceptors using electron microscopy (Figure 2, A–J, and Supplemental Figure S2, A–J). In agreement with the data obtained by confocal microscopy, we found that the basolateral and stalk membranes of Stratnull homzygous photoreceptors appeared shorter and longer than those of the wild-type photoreceptors, respectively (Figure 2, A and D, and Supplemental Figure S2, A and D), similar to those of Rab10null homzygous photoreceptors (Figure 2B and Supplemental Figure S2B). The basolateral and stalk membrane lengths of the wild-type photoreceptors were 12.49 ± 0.40 μm and 5.28 ± 0.08 μm, respectively (Figure 2K). In contrast, the basolateral and stalk membrane lengths of Stratnull homzygous photoreceptors were 8.63 ± 0.76 μm and 6.29 ± 0.50 μm, and those of Strat RNAi(GD10605)-expressing photoreceptors were 8.47 ± 0.55 μm and 7.15 ± 1.76 μm, respectively (Figure 2K). Thus in Stratnull homzygous photoreceptors and Strat RNAi(GD10605)-expressing photoreceptors, the cross-sectional lengths of the basolateral membranes were 0.69x shorter and those of stalk membranes were 1.19x and 1.35x longer than those of the wild-type photoreceptors, respectively; however, the difference between the stalk membranes lengths of the wild-type and Strat RNAi(GD10605)-expressing photoreceptors was not significant. In the case of Rab10null homzygous photoreceptors, the basolateral and stalk membrane lengths were 8.08 ± 0.25 μm and 7.39 ± 0.39 μm and were 0.65x shorter and 1.40x longer than those of the wild-type photoreceptors, respectively. Adherance junctions (AJs) were localized on the circumference of Stratnull homzygous, Strat RNAi(GD10605)-expressing, and Rab10null homzygous ommatidia, but in the central region of the wild-type ommatidia (Figure 2, A–J, and Supplemental Figure S2, A–J), likely because of the shorter basolateral and longer stalk membranes in the Stratnull and Rab10null homzygous photoreceptors. These results indicate that the phenotypes of Strat-deficient photoreceptors were similar to those of Rab10null homzygous photoreceptors. However, unlike Rab10null homzygous photoreceptors, Stratnull homzygous photoreceptors accumulated many vesicles in the cytoplasm and resembled Rab11RNAi(gsk2)-expressing photoreceptors (Figure 2, C, D, I, and J, and Supplemental Figure S2, C, D, I, and J). Strat RNAi(GD10605)-expressing photoreceptors phenocopied the Stratnull homzygous photoreceptors, but the same was not observed in Stratwt homzygous photoreceptors (Figure 2, E and F, and Supplemental Figure S2, E and F). These observations strongly indicate that both basolateral and rhabdomere transport are inhibited in Strat-deficient photoreceptors.

Strat is required for stable expression of Rab10 but not Rab11 in Drosophila photoreceptors

As Strat-deficient photoreceptors exhibited phenotypic characteristics of Rab10 and Rab11 deficiency, we next investigated the levels of Rab10 and Rab11 proteins in Stratnull photoreceptors. In
FIGURE 1: Impaired transport of both rhabdomeres and basolateral membrane in Strat-deficient photoreceptors. (A) Immunostaining of mosaic retinas from Stratnull (top panel) and StratWT (bottom panel) mosaic retinas with anti-Na+/K+ -ATPase-α (green) and anti-Rh1 (blue) antibodies (left) or anti-Crb (green) and Na+/K+ -ATPase-α (blue) antibodies (right). RFP (red) shows the wild-type cells. Asterisks show Stratnull (top panel) and StratWT (bottom panel) cells. (B, C) Immunostaining of late pupal retinas from Rab10null hemizygous flies (B) and longGMR-Gal4/UAS-Rab11RNAi pWIZ (C) with anti-Na+/K+ -ATPase-α (green) and anti-Rh1 (blue) antibodies (left), or anti-Crb (green) and Na+/K+ -ATPase-α (blue) antibodies (right). F-actin was stained with phalloidin (red). (D) Immunostaining of late pupal retinas from coinFLP-Gal4/UAS-StratRNAi GD10605 UAS-GFP flies with anti-Na+/K+ -ATPase-α (green) and anti-Rh1 (blue) antibodies (left), or anti-Crb (green) and Na+/K+ -ATPase-α (blue) antibodies (right). GFP (red) and asterisks indicate cells expressing StratRNAi GD10605. (E) Lengths of the basolateral (white and gray) and stalk (light green and dark green) membranes of photoreceptors. The lengths of the membranes in the cross-sections of retinas stained with anti-Crb and Na+/K+ -ATPase-α antibodies were measured using Fiji. A Crb-positive membrane was defined as a stalk membrane, and a Na+/K+ -ATPase-α-positive but Crb-negative membrane was defined as a basolateral membrane. White and light green bars indicate the length of the basolateral and stalk membranes, respectively, of wild-type cells. Gray and dark green bars indicate the length of the basolateral and stalk membranes, respectively, of mutant cells in Stratnull, StratWT, Rab10null, longGMR-Gal4/UAS-Rab11RNAi pWIZ, and coinFLP-Gal4/UAS-StratRNAi GD10605. (F) Plot of Rh1 cytoplasmic accumulation. In retinas stained with anti-Rh1 and Na+/K+ -ATPase-α antibodies, the ratio of fluorescence intensity for Rh1 staining in the cytoplasm to that in whole cells was measured using Fiji. The details of the methodology for this measurement are described in Materials and Methods. White bars indicate wild-type cells, and gray bars indicate mutant cells in Stratnull, StratWT, Rab10null, longGMR-Gal4/UAS-Rab11RNAi pWIZ, and coinFLP-Gal4/UAS-StratRNAi GD10605. Scale bar: 5 μm (A–D). Error bars indicate SD of three retinas. Significance according to two-tailed unpaired Student’s t test: ***p < 0.001, **p < 0.01, and *p < 0.02.
FIGURE 2: Ultrastructure of Strat-deficient photoreceptors. Green and pink lines indicate the basolateral and stalk membranes, respectively. AJs are indicated by the red boxes. (A–C) Electron micrographs of the wild-type fly, w¹¹¹⁸ (A), Rab10null (B), and longGMR-Gal4/UAS-Rab11RNAi⁴⁴⁴⁷ (C) ommatidia from late-pupal flies. (D, E) Electron micrographs of Stratnull (D) and StratWT (E) ommatidia obtained from a whole-eye homozygous clone of Stratnull (D) and StratWT (E) late pupal flies. (F) Electron micrographs of StratRNAi⁴⁴⁴⁷ expressing ommatidium obtained from coinFLP-Gal4/UAS-StratRNAi⁴⁴⁴⁷ late pupal flies. (G–I) Electron micrographs of wild-type flies and w¹¹¹⁸ (G), Rab10null (H), and longGMR-Gal4/UAS-Rab11RNAi⁴⁴⁴⁷ (I) photoreceptors from late pupal flies. (J) Electron micrographs of StratRNAi⁴⁴⁴⁷ expressing photoreceptors obtained from coinFLP-Gal4/UAS-StratRNAi⁴⁴⁴⁷ in late-pupal flies. (K) Lengths of the basolateral (white) and stalk (gray) membranes of photoreceptors in wild-type, Rab10null, longGMR-Gal4/UAS-Rab11RNAi⁴⁴⁴⁷, Stratnull, and coinFLP-Gal4/UAS-StratRNAi⁴⁴⁴⁷. The lengths of the membranes in the retinas were measured using the Fiji software. Scale bars: 2 μm (A–F) and 1 μm (G–J). Error bars indicate SD of three retinas. Significance according to two-tailed unpaired Student’s t test: ***p < 0.001, **p < 0.01, *p < 0.05.
Stratnull mosaic retinas expressing myc::Rab10, the fluorescence signal of the anti-myc antibody was greatly reduced in Stratnull homozygous photoreceptors (52% reduction) as compared with that in the wild-type photoreceptors (Figure 3A). In line with this observation, immunoblotting of Stratnull whole-eye clones formed by the GMR-hid method (Stowers and Schwarz, 1999) and the wild-type eyes used anti-Rab10 antibody showed a 79% reduction in endogenous Rab10 levels in Stratnull whole-eye clones (Figure 3B and Supplemental Figure S3A). In contrast, endogenous Rab10 levels were not reduced in Stratnull homozygous photoreceptors or whole-eye clones both by immunostaining and immunoblotting (Figure 3, C and D, and Supplemental Figure S3B). These results indicate that Strat is required for stable expression of Rab10 but not Rab11 in Drosophila photoreceptors.

Rab10 but not Rab11 levels are reduced in Strat-deficient photoreceptors. (A) Immunostaining of Stratnull mosaic retinas expressing Myc::Rab10 with anti-Myc antibody (blue). F-actin was stained by phalloidin (green). RFP (red) shows the wild-type cells. Asterisks show Stratnull homozygous cells. Plot of the relative amounts of Myc::Rab10 in Stratnull photoreceptors as compared with those in wild-type photoreceptors. Error bars indicate SD of four retinas. Significance according to two-tailed unpaired Student’s t test: *p < 0.02. (B) Immunoblotting of retinas from wild-type and a whole-eye homozygous clone of Stratnull flies with anti-α-tubulin and anti-Rab10 antibodies. Relative amounts of Rab10 in Stratnull retinas compared with those in wild-type retinas normalized by the amount of α-tubulin. Error bars indicate SD of 10 independent experiments. Significance according to two-tailed unpaired Student’s t test: ***p < 0.001. (C) Immunostaining of Stratnull mosaic retinas with anti-Rab11 (green) and anti-Rh1 (blue) antibodies. RFP (red) indicates the wild-type cells. Asterisks indicate the Stratnull homozygous cells. Relative amounts of Rab11 in Stratnull photoreceptors compared with those in wild-type photoreceptors. Error bars indicate SD of four retinas. Significance was determined using a two-tailed unpaired Student’s t test. (D) Immunoblotting of retinas from the wild-type and a whole-eye homozygous clone of Stratnull flies with anti-α-tubulin and anti-Rab11 antibodies. Relative amounts of Rab11 in Stratnull retinas compared with wild-type retinas normalized by the amount of α-tubulin. Error bars indicate SD of 10 independent experiments. Significance was determined using a two-tailed unpaired Student’s t test. Scale bar: 5 μm (A, C).

FIGURE 3: Rab35 is a novel Rab protein involved in rhabdomere transport
Stratnull homozygous photoreceptors showed Rh1 accumulation in the cytoplasm; however, Strat deficiency did not reduce Rab11 levels. We assumed that there is an unidentified Rab protein that is necessary for the post-Golgi transport of Rh1 and that its stable expression depends on Strat, Rab1, Rab3, Rab8, Rab10, Rab13, Rab15, and Rab35 were previously reported to bind to the mammalian ortholog Mss4 (Burton et al., 1994; Strick et al., 2002; Guruharsha et al., 2011; Gulbranson et al., 2017; Bellec et al., 2018; Moissoglu et al., 2020). Among these, Rab13 and Rab15 do not exist in the fly genome, and we have already shown that photoreceptors lacking Rab1 and Rab10 do not accumulate Rh1 in the cytoplasm (Satoh et al., 1997; Nakamura et al., 2020). Thus Rab3, Rab8, and Rab35 are strong candidates for Strat-dependent Rab proteins.
that are essential for the post-Golgi transport of Rh1. We investigated whether the loss of Rab3, Rab8, or Rab35 affected the localization of Rh1 and Na+/K+ ATPase. The photoreceptors with a viable Rab3 null allele, Rab3+/-, over the deficient Df(2)E02076 showed normal localization of both Rh1 and Na+/K+ ATPase in the rhabdomere and basolateral membranes, respectively (Figure 4, A and F). As the hypomorphic allele Rab8- (Giajットzgliou et al., 2012) and Rab8 null allele Rab8null (produced in this work) are lethal, we generated mosaic retinas containing Rab8- or Rab8null homozygous photoreceptors using the FLP/FRT method (Xu and Rubin, 1993). In both Rab8-deficient photoreceptors, Rh1 and Na+/K+ ATPase showed normal localization in the rhabdomere and basolateral membranes (Figure 4, B and F). In contrast, we observed severe cytoplasmic accumulation of Rh1 in Rab35RNAiJF02978- and Rab35RNAiK108640-expressing photoreceptors (Figure 4, C and D, F). In the electron micrograph, the cytoplasm of Rab35 knockdown photoreceptors was filled with vesicles and resembled those that accumulated in Rab11- or Strat-deficient photoreceptors (Figure 4, G and H). Na+/K+ ATPase was normally localized in the basolateral membrane of Rab35 RNAiJF02978- and Rab35RNAiK108640-expressing photoreceptors (Figure 4, C and D). We also investigated Rab35null hemizygous flies (Kohrs et al., 2021) and found some accumulation of Rh1 in the cytoplasm (Figure 4E). The ratio of Rh1 in the Rab35null hemizygous photoreceptor cytoplasm tended to be higher than that in the Rab35null+/+ heterozygous photoreceptor cytoplasm, but the difference was not statistically significant (Figure 4, E and F). The electron micrograph revealed the accumulation of some vesicles in the cytoplasm of Rab35null hemizygous photoreceptors (Figure 4I), although the degree of accumulation was milder than that in Rab35RNAiJF02978- and Rab35RNAiK108640-expressing photoreceptors. Our results indicated that neither Rab3 nor Rab8 but Rab35 is a candidate responsible for the post-Golgi transport of Rh1.

Strat is required for stable expression of Rab35

Next, we investigated Rab35 localization in fly photoreceptors. As three kinds of anti-Rab35 antibodies did not work for immunostaining of retinas (Zhang et al., 2009), we generated transgenic flies with UAS-tagBFP2:FLAG::Rab35. In photoreceptors expressing tagBFP2:FLAG::Rab35, anti-FLAG antibody staining was observed exclusively in the rhabdomeres (Figure 5A). Rhabdomeres are tight bundles of microvilli that protrude from the apical plasma membrane. This result is consistent with many studies indicating Rab35 localization on the apical plasma membrane in mammalian cells (Klinkert et al., 2016; Mrozowska and Fukuda, 2016).

We expressed tagBFP2:FLAG::Rab35 in Stratnull photoreceptors and found some anti-FLAG antibody staining in the cytoplasm but not in rhabdomeres (Figure 5B, left). The fluorescence intensity of anti-FLAG antibody staining in Stratnull photoreceptor rhabdomeres was 49% lower than that in wild-type photoreceptor rhabdomeres (Figure 5B, right). These results indicate that Strat is not functional without Strat. In agreement with the immunostaining results, immunoblotting of Stratnull whole-eye clones generated using the GMR-hid method (Stowers and Schwarz, 1999) and the wild-type eyes, performed using an anti-Rab35 antibody (Zhang et al., 2009), showed a 55% reduction in endogenous Rab35 in Stratnull whole-eye clones (Figure 5C and Supplemental Figure 5SC). Thus Strat is required for stable and functional Rab35 expression.

As both Rab11 and Rab35 are required for the post-Golgi Rh1 transport, we next investigated Rab11 localization in Rab35RNAiJF02978-expressing photoreceptors. We found there is no Rab11 staining at the base of the rhabdomeres and Rab11 is colocalized with Rh1 in the cytoplasm (Figure 5D). This result indicates the possibility that Rab35 is required to tether Rh1 bearing post-Golgi vesicles to the base of the rhabdomeres.

DISCUSSION

In the present study, we found that Strat was required for both basolateral and rhabdomere transport through the stable expression of Rab10 and Rab35 (Figure 5E). We previously indicated that the reduction of Rab10 activity causes mislocalization of Na+/K+ ATPase to the stalk membrane, shrinkage of the basolateral membrane, and expansion of the stalk membrane, suggesting Rab10 is required for basolateral transport (Charron et al., 2000). In our previous work, we failed to obtain Rab10null flies; however, in this study, we successfully showed that Rab10null photoreceptors indeed display these phenotypes. We found that in Strat-deficient photoreceptors, the amount of Rab10 protein was reduced, the basolateral membrane had shrunk, and Na+/K+ ATPase was mislocalized to the expanded stalk membrane. Rab10 reduction by the loss of Strat is consistent with the chaperone hypothesis and likely to be the reason of these phenotypes (Figure 5E).

We previously showed that Rab11 is localized at the trans-side of Golgi stacks and post-Golgi vesicles near the base of the rhabdomeres. Rab11, together with MyoV and dRip11, promotes the migration of post-Golgi vesicles toward the rhabdomere base (Satoh et al., 2005; Li et al., 2007). Here we found that in addition to basolateral transport, rhabdomere transport was inhibited in Strat-deficient photoreceptors. We failed to detect Rab11 reduction in Strat-deficient photoreceptors; however, we found that the protein levels of Rab35 were reduced. In the wild-type photoreceptors, Rab35 localized exclusively at the rhabdomere membrane, which is the downstream of Rab11-mediated Rh1 transport. Moreover, Rab11 is lost at the base of the rhabdomeres and colocalized with Rh1 accumulated in cytoplasm of Rab35-deficient photoreceptors. Thus Rab35 is likely involved in tethering or fusion of Rh1-containing post-Golgi vesicles to the rhabdomere at the downstream of Rab11 (Figure 5E).

The function of Mss4 is under debate of whether it is a RabGEF or a chaperone for Rab proteins (Burton et al., 1993; Moya et al., 1993; Burton et al., 1994; Miyazaki et al., 1994; Nuoffer et al., 1997; Coppola et al., 2002; Strick et al., 2002; Itzen et al., 2006; Itzen et al., 2007; Wu et al., 2014; Guiblinson et al., 2017). Recent studies support the role of Mss4 as a chaperone rather than RabGEF (Nuoffer et al., 1997; Strick et al., 2002; Itzen et al., 2006; Itzen et al., 2007; Wu et al., 2014; Guiblinson et al., 2017). In agreement with these studies, our data suggest that Strat functions as a chaperone for Rab10 and Rab35 in Drosophila photoreceptors, as Rab10 and Rab35 protein levels are greatly reduced in Strat-deficient retinas.

This is the first report to highlight the essential role of Rab35 in the post-Golgi transport of Rh1 to apical membrane rhabdomeres in Drosophila photoreceptors. Consistent with our results, several studies have indicated that Rab35 plays an important role in polarized transport to the apical membrane. Rab35 physically couples cytokinesis with the initiation of apico-basal polarity by tethering intracellular vesicles containing key apical determinants at the cleavage site (Klinkert et al., 2016). Rab35 also regulates podocalyxin trafficking in two- and three-dimensional epithelial cell cultures, which are differentially activated by Rab35GEFs, DENND1A, and folliculin (Mrozowska and Fukuda, 2016; Kinoshita et al., 2020). The I-Bar protein IRSp53 and the actin-capping protein EP58 form a complex with Rab35 that is necessary for controlling the trafficking of apical determinants and maintaining the integrity of the luminal plasma membrane (Bisi et al., 2020). Recent work has also indicated that CD13 establishes Rab35 at the apical membrane initiation site and
is necessary for capturing vesicles containing apical determinates for apical membrane initiation (Wang et al., 2021).

A recent report revealed an interesting interaction between Rab11FIP1 and Rab35 and the critical role of Rab11FIP1 for Rab35 function in actin removal prior to cytokinesis. Interestingly, the Drosophila homolog of Rab11FIP1, dRip11, is essential for Rh1 transport in photoreceptors as well as cytokinesis and is localized to the intracellular bridge between daughter cells (Li et al., 2007; Iannantuono and Emery, 2021). Future studies should explore the relationship between Rab11 and Rab35 in the final stage of Rh1 transport into the rhabdomeres.

**MATERIALS AND METHODS**

Request a protocol through Bio-protocol.

**Drosophila stocks and genetic background**

Drosophila were grown at 20–25°C on standard cornmeal–glucose–agar–yeast medium either in the laboratory with room light or in an

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**FIGURE 4:** Impaired post-Golgi transport of rhabdomeres in Rab35-reduced photoreceptors. (A) Immunostaining of late pupal retinas from Rab3<sup>35<sup>+/Df<sup>ED2076 flies with anti-Na<sup>+/K<sup>+-ATPase-α (green) and anti-Rh1 (blue) antibodies (left). F-actin was stained by phalloidin (red). (B) Immunostaining of mosaic retinas from Rab8<sup>del11 (top panel) or Rab8<sup>1 (bottom panel) mosaic retina with anti-Na<sup>+/K<sup>+-ATPase-α (green) and anti-Rh1 (blue) antibodies (left). RFP (red) shows the wild-type cells. Asterisks show Rab8<sup>del11 or Rab8<sup>1 homozygous cells. (C) Immunostaining of late pupal retinas from coinFLP-Gal4/UAS-Rab35RNAi<sup>JF02978 flies with anti-Na<sup>+/K<sup>+-ATPase-α (green) and anti-Rh1 (blue) antibodies. GFP (red) and asterisks show cells with Rab35RNAi<sup>JF02978. (D) Immunostaining of late pupal retinas from longGMR-Gal4/UAS-Rab35RNAi<sup>KK108660 flies with anti- Na<sup>+/K<sup>+-ATPase-α (green) (E) Immunostaining of late pupal retinas from Rab35<sup>null hemizygous flies with anti-Na<sup>+/K<sup>+-ATPase-α (green) and anti-Rh1 (blue) antibodies and phalloidin (red). (F) The ratio of signal strength of Rh1 staining in the cytoplasm against that of the whole cells was plotted. White bars indicate the wild-type or Rab35 heterozygous cells, and the gray bars indicate mutant cells in Rab3<sup>35<sup>/Df<sup>ED2076, Rab8<sup>del11, coinFLP-Gal4/UAS-Rab35RNAi<sup>JF02978, longGMR-Gal4/UAS-Rab35RNAi<sup>KK108660, and Rab35<sup>null hemizygous flies. Error bars indicate SD of three retinas. Significance according to two-tailed unpaired Student’s t test: *p < 0.02. (G) Electron micrographs of Rab35RNAi<sup>JF02978 ommatidium and the photoreceptor obtained from coinFLP-Gal4/UAS-Rab35RNAi<sup>JF02978. (H) Electron micrographs of Rab35RNAi<sup>KK108660 expressing photoreceptor by longGMR-Gal4. (I) Electron micrographs of Rab35<sup>null hemizygous ommatidium and photoreceptors. Scale bar: 5 μm (A–E), 2 μm (G, I left panel) and 1 μm (G, I right panel and H).
incubator without light. The following fly stocks were used: Rh1-Gal4 (Chihiro Hama, Kyoto Sangyo University, Japan), longGMR-Gal4 (Bloomington Drosophila Stock Center No. 8605, Bloomington, IN; indicated as BL8605 in the following stocks), coinFLP-Gal4 (BL58751), y w; GMR-hid FRT40A/CyO; ey-Gal4 UAS-FLP/TM6B flies (BL5250), UAS-Rab11RNAi pWIZ, (Satoh et al., 2005), UAS-Strat RNAi GD10605 (Vienna Drosophila Resource Center No. 45715, Vienna, Austria; indicated as v45715 in the following stocks), Rab35RNAi JF02978 (BL28342), Rab35RNAi KK108660 (v101361), Rab10null /FM7, Rab35null /FM7 (gifts from Hiesinger), and UAS-tagBFP2::FLAG::Rab35 (produced in the present study).

Stratnull with RFP FRT40A/CyO and StratWT with RFP FRT40A/CyO were kindly provided by Le Borgne (Bellec et al., 2018) and RFP was removed using Cre recombinase (BL106201). Males of Stratnull FRT40A/CyO GFP and StratWT FRT40A/CyO GFP were crossed with y w eyFLP; RFP FRT40A/CyO to generate mosaic eyes. Rab81

FIGURE 5: Rab35 levels are reduced in Strat-deficient photoreceptors. (A) Immunostaining of wild-type cells expressing tagBFP2::FLAG::Rab35 with anti-FLAG (green) and anti-GM130 (blue) antibodies. F-actin was stained by phalloidin (red). (B) Immunostaining of Stratnull mosaic retinas expressing tagBFP2::FLAG::Rab35 by Rh1-Gal4 with anti-FLAG antibody (blue). F-actin was stained by phalloidin (green). RFP (red) shows the wild-type cells. Asterisks indicate the Stratnull homozygous cells. Plot of the relative amounts of tagBFP2::FLAG::Rab35 in Stratnull photoreceptors as compared with those in wild-type photoreceptors. Error bars indicate SD of five retinas. Significance according to two-tailed unpaired Student’s t-test: ***p < 0.001. (C) Immunoblotting of retinas from wild-type and a whole-eye homozygous clone of Stratnull with anti-α-tubulin and anti-Rab35 antibodies. Plot of the relative amounts of Rab35 in Stratnull retinas as compared with those in the wild-type retinas normalized by the amount of α-tubulin. Error bars indicate SD of 10 independent experiments. Significance according to two-tailed unpaired Student’s t-test: **p < 0.01. (D) Immunostaining of late pupal retinas from coinFLP-Gal4/UAS-Rab35RNAiJF02978 flies with anti-Rab11 (green) and anti-Rh1 (red) antibodies. GFP (blue) and asterisks show cells with Rab35RNAi JF02978. (E) The proposed model of functions of Strat and Rab proteins in polarized transport in Drosophila photoreceptors. Strat ensures the stable expression of Rab10 and Rab35, which regulate basolateral and rhabdomere transport, respectively. Rab11 is localized on trans-side of Golgi stacks and post-Golgi vesicles, and Rab35 is localized on the apical plasma membrane, rhabdomere. These localizations suggest that Rab35 works after Rab11 for Rh1 transport. Scale bar: 5 μm (A, B).
(BL26173) was combined with FRT80B. Males of Rab8/ FRT80B/ TM6B were crossed with y w eyFLP; RFP FRT80B to generate mosaic eyes.

**Construction of a Rab8 null allele, Rab8/Δnull, and formation of Rab8/Δnull mosaic retinas**

A null allele of Rab8 was generated by FLP/FRT-based recombination between two P-element insertions, P(RS3)CB-5247-3 and P(RS3)CB-0752-3, located upstream and downstream of Rab8, respectively. Briefly, male flies carrying P(y+7.2) = hsFPL2)Δ2 on X chromosome, P(RS3)CB-5247-3 and P(RS3)CB-0752-3 on the third chromosome were heat-treated at 37°C for 1 h daily for 3 d in larval stages. Isogenic lines balanced with TM6C were established and analyzed by PCR using genomic DNA as the template. The Rab8 null allele, Rab8/Δnull, was identified by the presence of upstream of P(RS3)CB-5247-3 (amplified with primers Rab8-GF2 and 5′-out) and downstream of P(RS3)CB-0752-3 (Rab8-GR1 and 3′-out) but the absence of downstream of P(RS3)CB-5247-3 (Rab8-GR2 and 3′-out) or upstream of P(RS3)CB-0752-3 (Rab8-GF1 and 5′-out). To obtain Rab8/Δnull mosaic retinas, Rab8/Δnull was crossed with the original P-element insertion P(RS3)CB-5247-3, located on the proximal side of the Rab8 gene. Primer information; 5′-out: 5′-CAAGCAAAACGTGCACT-GAAAT-3′, 3′-out: 5′-TCGCTGTCTCAGTACAGAC-3′.

**Transgenic flies for UAS-tagBFP2::FLAG::Rab35**

The entire coding region of Rab35 was amplified from cDNA reverse-transcribed from total RNA extracted from the third instar larval of the wild-type w1118 strain. The DNA fragment corresponding to A2 to the stop codon was integrated into a common P-element transformation vector pUAST together with N-terminal tagBFP2 connected with a FLAG tag linker encoding “TSGGGYKDDDDKGSSGGGAAAGGRSGGAPGGGGSGGGSSG” resulting in the plasmid vector pUAST-tBFP2-FLAG-Rab35. The plasmid was injected into embryos to generate transgenic lines.

**Immunohistochemistry**

Fixation and staining were performed as previously described, except for the fixative (Sato et al., 2005). PLP (10 mM periodate, 75 mM lysine, 30 mM phosphate buffer, and 4% paraformaldehyde) was used as a fixative (Otsuka et al., 2019). The primary antiserum used were as follows: rabbit anti-Rh1 (1:1000) (Satoh et al., 2005), mouse monoclonal anti-Na+/K+-ATPase alpha subunit (1:500 asciites; Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA), rat anti-Crb (1:300) (Ulich Tepass, University of Toronto, Toronto, Ontario, Canada), rat anti-Rab11 (1:250) (Otsuka et al., 2019), mouse monoclonal anti-γ-tubulin (1:20; DSHB), and mouse anti-FLAG M2 (1:1000) (Sigma-Aldrich Japan, Tokyo, Japan). The secondary antibodies were anti-mouse, anti-rabbit, and/or anti-rat antibodies labeled with Alexa Fluor 488 and 647 (1:300) (Life Technologies, Carlsbad, CA) or Cy2 (1:300) (GE Healthcare Life Sciences, Pittsburgh, PA). F-actin was stained with phalloidin conjugated with Alexa Fluor 568 (Life Technologies, Carlsbad, CA). Sample images were recorded using a Model FV1000 confocal microscope (60× 1.42 NA objective lens; Olympus, Tokyo, Japan). To minimize bleed-through, each signal in the double- or triple-stained samples was sequentially imaged. Images were processed following the Guidelines for Proper Digital Image Handling using ImageJ and affinity photos. To quantify the intensity of Rh1 staining in the photoreceptor cytoplasm, we used more than three mosaic retinas with more than eight wild-type and eight mutant photoreceptors in each retina. The area of the cytoplasm or whole cells and their staining intensities were measured using Fiji (Schindelin et al., 2012). Na+/K+-ATPase-α and Rh1 staining were used to define the outline of the cell and rhodobioem. Rhabdomeres were defined as Rh1-positive oval structures protruding from the photoreceptors to the center of the ommatidium. The area of the cells, except for the rhodobioem, was regarded as the cytoplasm. The integrated densities of Rh1 staining in cytoplasm and whole cells were measured. The ratio of the density of cytoplasm to that of whole cell was calculated for each cell. The sectional lengths of the stalk (defined as the membrane stained by anti-Crb antibody and used as the sum of both sides of the rhodobioem) and basolateral membrane (defined as the membrane stained with anti-Na+/K+-ATPase-α antibody but not by anti-Crb antibody) were measured using Fiji. More than five photoreceptors in three flies for more than three independent samples for each genotype were used for these measurements.

**Electron microscopy**

Electron microscopy was performed as previously described (Sato et al., 1997). The samples were observed under a JEM1400 electron microscope (JEOL, Tokyo, Japan), and montages were prepared using a charge-coupled device camera system (JEOL). The phenotypes were investigated using a section at the depth at which a couple of photoreceptor nuclei within the ommatidia was observed.

**Immunoblotting**

For quantitative isolation of the retina, we used freeze-dried flies stored in acetone (Fujita et al., 1987; Nakamura et al., 2020). W; GMR-hid 40A/ Stratnull FRT40A; ey-Gal4 UAS-FLP/+ flies and w; GMR-hid FRT40A/+; ey-Gal4 UAS-FLP/+ flies at 0–7 d were collected and frozen in liquid nitrogen. The heads were collected using two sieves with different mesh sizes. The heads were then immersed in acetone, cooled to −80°C, and maintained for >1 wk. The acetone was replaced once or twice during this time to remove water. The retinas were dissected with forceps, and the proteins were extracted in SDS sample buffer (0.05 M Tris, 10% vol/vol glycerol, 5% vol/vol β-mercaptoethanol, and 2.3% wt/vol SDS) in phosphate buffer solution at 80°C for 2 min. Ten independent samples, each containing 40 eyes in 40 μL SDS sample buffer, were prepared for both the wild-type and a whole-eye homozygous clone of Stratnull.

Immunoblotting was performed as previously described (Satoh et al., 1997). The blotted membranes were cut, the upper halves were used to detect α-tubulin as loading controls, and the lower halves were used to detect Rab proteins. Mouse anti-α-tubulin (1:200 supernatant) (DSHB), guinea pig anti-Rab10 (1:2500) (Nakamura et al., 2020), rat anti-Rab11 (1:2500) (Otsuka et al., 2019), and rabbit anti-Rab35 (1:1000) (Zhang et al., 2009) were used as the primary antibodies. HRP-conjugated anti-mouse, anti-rabbit, anti-ant, and anti-guinea pig IgG antibodies (1:20 000; Life Technologies) were used as secondary antibodies. The signals were visualized by enhanced chemiluminescence (Clarity Western ECL Substrate; Bio-Rad, Hercules, CA) and imaged using ChemiDoc XRS+ (Bio-Rad). The intensities of the 60 bands were measured using Fiji software, the intensity of the α-tubulin band was used to standardize the loading amount for each lane, and the amounts of Rab proteins between the wild-type and Stratnull mutant retinas were compared.
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