Rab11 mediates post-Golgi trafficking of rhodopsin to the photosensitive apical membrane of *Drosophila* photoreceptors

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Summary

In developing *Drosophila* photoreceptors, rhodopsin is trafficked to the rhabdomere, a specialized domain within the apical membrane surface. Rab11, a small GTPase implicated in membrane traffic, immunolocalizes to the trans-Golgi network, cytoplasmic vesicles and tubules, and the base of rhabdomeres. One hour after release from the endoplasmic reticulum, rhodopsin colocalizes with Rab11 in vesicles at the base of the rhabdomere. When Rab11 activity is reduced by three different genetic procedures, rhabdomere morphogenesis is inhibited and rhodopsin-bearing vesicles proliferate within the cytosol. Rab11 activity is also essential for development of MVB endosomal compartments; this is probably a secondary consequence of impaired rhabdomere development. Furthermore, Rab11 is required for transport of TRP, another rhabdomeric protein, and for development of specialized membrane structures within Garland cells. These results establish a role for Rab11 in the post-Golgi transport of rhodopsin and of other proteins to the rhabdomeric membranes of photoreceptors, and in analogous transport processes in other cells.

Key words: Rhodopsin, Rab11, Retina, *Drosophila*

Introduction

During photoreceptor terminal differentiation, massive biosynthetic membrane traffic delivers rhodopsin and other phototransduction proteins to an apical plasma membrane subdomain to form photosensory organelles, invertebrate rhabdomeres and vertebrate outer segments. The proper targeting of rhodopsin to this domain is crucial for normal development and, if impaired, leads to retinal degeneration (Colley et al., 1995; Li et al., 1996; Liu et al., 1997; Sung et al., 1994). Rab proteins and their effectors are known to control membrane traffic and maintain distinct organelle identities (Deneka et al., 2003). Indeed, observations in *Xenopus* (Deretic et al., 1995; Moritz et al., 2001) and *Drosophila* (Satoh et al., 1997; Shetty et al., 1998) support a role for particular Rab proteins in rhodopsin transport to the photosensitive organelles.

Surprisingly, these studies on rhodopsin trafficking identified Rab11 as active in exocytosis. Rab11 is thought to regulate endosomal/plasma membrane interactions by controlling membrane traffic through recycling endosomes. These endosomes receive endocytosed plasma membrane and either return it to the cell surface or direct it to degradative pathways (Ullrich et al., 1996). Rab11 localizes to the pericentriolar recycling endosome, the *trans*-Golgi network (TGN), and post-Golgi vesicles (Chen et al., 1998; Deretic, 1997; Ullrich et al., 1996). Dominant-negative Rab11a inhibits apical recycling and basolateral-to-apical transcytosis in polarized MDCK cells (Wang et al., 2000), blocks stimulus-induced recruitment of endosome-sequestered H^+-K^+ ATPase-rich membrane to the apical membrane of acid-secreting parietal cells (Duman et al., 1999), and inhibits exosome release in human leukemic K562 cells (Savina et al., 2002). During cellularization of *Drosophila* embryos, apical membrane recycled to the expanding lateral membranes trafficks through Rab11-dependent recycling endosomes (Pelissier et al., 2003).

In addition to these extensive reports linking Rab11 activity to endocytic pathways, other reports suggest a role for Rab11 in biosynthetic exocytic membrane traffic. In PC12 cells, Rab11 was detected in association with TGN and TGN-derived secretory vesicles (Urbe et al., 1993). In baby hamster kidney cells, overexpression of dominant-negative Rab11S25N decreased delivery of the basolaterally targeted vesicular stomatitis virus (VSV) G protein to the cell surface (Chen et al., 1998), and expression of wild-type Rab11a accelerated delivery of new protease activated receptors to kidney epithelial cell surfaces following trypsin exposure (Roosterman et al., 2003). Of particular interest to this study, the movement of rhodopsin to the apical surface may also be dependent on Rab11. Rhodopsin has been detected in Rab11-positive post-Golgi vesicles of *Xenopus* retina cell-free extracts (Deretic, 1997). Immature rhodopsin, which is indicative of defective rhodopsin transport, accumulated in *Drosophila* photoreceptors that expressed dominant-negative $Rab11^{N124I}$ (Satoh, 1998).

In this study, we characterize the movement of rhodopsin and other rhabdomeric membrane proteins in the developing *Drosophila* photoreceptor. This experimental system allows us to define the role of Rab11 in this process. We find that

vigorous light-dependent endocytosis competes with exocytosis from the outset of rhabdomere morphogenesis. We show that, independent of a requirement in endosomal recycling, Rab11 activity is essential for the initial exocytic rhodopsin delivery to the growing rhabdomere. We also show that loss of Rab11 activity disrupts endocytic pathways, but this is likely to be a secondary consequence of attenuated exocytic delivery. Thus, our results demonstrate Rab11 promotes the *trans*-Golgi to rhabdomere membrane traffic responsible for elaboration of the sensory membranes of these cells.

Materials and methods

Fly stocks

Flies were reared at 20°C. White-eye flies (w^{1118}) were used as wild type. Transgenic flies also carried the w^{1118} allele. *Rh1-Gal4* flies were a kind gift from Dr Chihiro Hama (Kobe Riken), *UAS-GFP-actin* flies from Dr Hiroki Oda (JT Biohistory Research Hall) and *UAS-GFP-Rab7* from Dr Hideyuki Shimizu (Osaka University). Construction of *UAS-Rab11*^{N124I} and *UAS-CFP-Golgi* flies are described below. Genetic mosaics were induced by the FLP-FRT technique with an eyFLP driver (Stowers and Schwarz, 1999) to generate mitotic clones in the eye (Xu and Rubin, 1993). Males of the genotype *y w eyFLP; FRT82B Rab11*^{EP3017}*/TM3* were crossed to females of the genotype *y w eyFLP; FRT82B* w^{+} virgins to generate mitotic clones. We refer to these flies as 'severely reduced' in the text because the *Rab11* gene was removed from the cell lineage during development. Residual Rab11 protein and activity may remain from prior expression within this lineage.

Construction of transgenic flies expressing dominant-negative Rab11^{N124I}, Rab11 dsRNA and CFP-Golgi

Rab11^{N124I} substitutes isoleucine for asparagine at amino acid 124 within the third conserved region required for guanine nucleotide binding. A similar substitution in mammalian Rab11 acts as an inhibitor for Rab11 when expressed in gastric parietal or MDCK cells (Duman et al., 1999; Wang et al., 2000). To create the *Rab11*^{N124I} gene, Quick Change site-directed mutagenesis (Stratagene) was used according to the manufacturer's manual using primers: DRab11 M1-F1, CTGGTGGGCA**T**CAAGTCCGAC; and DRab11 M1-R1, GTCGGACTTGA**A**TGCCCACCAG. The *Rab11*^{N124I} mutant cDNA was inserted into pUAST. For Rab11 RNAi, complementary 403 bp fragments of *Rab11* cDNA were amplified using primers: RAB11-D, GGGCTCGAGGTGAGCCAACGACAAACGC; RAB11-F, GGGC-CTAGGGGCACCGCGGTAGTAGGCAG; RAB11-E, GGGGCTA-GCGTGAGCCAACGACAAACGC; and RAB11-F. The resulting fragments were inserted into the RNAi vector, pWIZ, a kind gift from Dr Richard Carthew (Northwestern University) (Lee and Carthew, 2003). To make Golgi-marked flies, the *Nhe*I/*Not*I fragment containing the entire coding sequence of CFP-Golgi fusion protein was cut from CFP-Golgi vector (Clontech) and inserted to pUAST. *pUAST-Rab11*^{N124I} and *pUAST-CFP-Golgi* were transformed into *Drosophila* and insertion strains containing a single copy of each transgene were generated by standard means (Spradling, 1986). Immunoblot analysis and immunohistochemistry (data not shown) confirmed Rab11N124I protein expression by hs-Gal4. CFP-galactosyl transferase colocalized with a *Drosophila* Golgi-specific antibody (Stanley et al., 1997) (data not shown).

Generation of anti *Drosophila* Rab11 and Rh1 antibodies

6xHis-Rab11 fusion proteins were expressed in *E. coli* cells, purified on polyhistidine affinity resin (Qiagen) and injected into mice. The Rab11 antiserum generated in this way recognizes a single 27 kDa protein in fly head homogenate when assayed on protein blots. This antiserum recognizes the 6xHis-Rab11 fusion protein but does not recognize *E. coli*-expressed 6xHis-Rab1, 6xHis-Rab2, 6xHis-Rab6 or 6xHis-RabRP4. Rabbit anti-Rab11 was also raised against 6xHis-Rab11. This antibody recognizes Rab11 in western blots. Rabbit affinity-purified anti-Rh1 antibody was raised against the Rh1 (21-36) peptide GSVVDKVTPDMAHLIS. The antibody recognizes Rh1 in western blots, and, unlike the previously characterized 4C5 anti-Rh1 monoclonal antibody, its epitope is not masked by arrestin binding to rhodopsin (Orem and Dolph, 2002).

Immunohistochemistry

Fixation and staining methods are described elsewhere (Fan and Ready, 1997). For fixation of dark-reared flies, eyes were dissected using infrared illumination and image intensifier eyepieces. Primary antisera were: mouse anti-Rab11 (1:250) (this report), Rabbit anti-Rab11 (1:1000), mouse anti-Rab1 (1:500) (Satoh et al., 1997), mouse monoclonal anti-Rh1 (4C5) (1:50 supernatant) (DSHB), rabbit anti-Rh1 (1:1000) (this report), rabbit anti-TRP (1:2000) (gift from Dr Craig Montell), guinea pig anti-Hrs (1:2000) (gift from Dr Hugo Bellen), chicken anti-GFP (Chemicon) or rabbit anti-GFP (1:2000) (MBL International Corporation). Secondary antibodies were anti-mouse and/or anti-rabbit labeled with Alexa488, Alexa647 (1:300) (Molecular Probes), Cy2 (1:500) or Cy5 (1:150) (Amersham-Pharmacia). Samples were examined and images recorded using a BioRad MRC1024 confocal microscope (Nikon 60×, 1.4NA lens). To minimize bleed through, each signal in double or triple stained samples was imaged separately using a single line and then merged. Acquired images were processed by image J and/or Photoshop 5.5. Anti-Hook (gift from Dr Helmut Kramer) and anti-LBPA (gift from Dr Jean Gruenberg), antibodies that stain MVBs in other cells, did not show any signal in photoreceptors. CFP-labeled Golgi were triple immunostained using mouse anti-Rab1, rabbit anti-Rab11 and chicken anti-GFP to determine Rab11 cisternal association.

Synchronous release of Rh1 accumulated in the ER

Flies were raised from egg to 2nd or 3rd instar larvae on carotenoid-deprived food, and then crystalline all-trans-retinal (Sigma) was added to the media; cultures were kept in constant dark. These conditions allowed for development of pupae in which the 40 K intermediate of rhodopsin accumulated within the ER. Late stage, dark-winged pupae were then irradiated with blue light (410 nm) using a CFP filter on a 50 W Hg lamp to isomerize the all-trans retinal to the 11-cis-form and initiate rhodopsin maturation.

Electron microscopy

Conventional electron microscopic methods have been described previously (Satoh et al., 1997). For immunoelectron microscopy, heads were dissected and incubated in PLP fixative (2% paraformaldehyde, 0.075 M lysine, 0.01 M $NaIO_4$, PBS pH 7.4) with 0.1% glutaraldehyde for 2 hours. The heads were then postfixed in 0.5% OsO_4, in 0.75% $K_4Fe(CN)_6$ in 0.1 M cacodylate buffer (pH 7.4) for 30 minutes on ice, serial dehydrated in alcohol and embedded in LR-White (Electron Microscopy Sciences). Ultrathin sections (silver or gray) were etched by saturated solution of sodium meta-periodate for 30 minutes. For immunogold labeling of the sections, specimens were reacted overnight at 4°C with mouse anti-Rh1 (4C5) (1:20 ascites), and then reacted overnight at 4°C with anti-mouse IgG-15 nm gold conjugates (1:40, British Bio Cell International). Samples were observed on a Philips 300 electron microscope.

Organelle counts

The number of RLVs, defined as rhodopsin-positive, spherical vesicles >400 nm was counted in 10 flies: wild type, 0.67 (s.d.=0.13); *Rab11*^{N124I} photoreceptor, 0.074 (s.d.=0.054). The number of MVBs, defined as >300nm spherical vesicles with at least four internal vesicles, was counted in five flies: wild type, 0.49 (s.d.=0.06); *Rab11*^{N124I} photoreceptor, 0.0013 (s.d.=0.015).

Endocytic tracer uptake in Garland cells

Garland cells were dissected in the *Drosophila* standard saline, and incubated for 10 minutes with 20 mg/ml Texas Red-conjugated avidin (Molecular Probes). Garland cells were fixed immediately after a brief wash.

Results

Developmental expression of TRP and Rh1

Drosophila rhabdomere differentiation begins in mid-pupal life with the establishment of a morphologically and molecularly distinct apical plasma membrane subdomain, which is then amplified and specialized for phototransduction by targeted membrane delivery (Karagiosis and Ready, 2004; Longley and Ready, 1995). Between ~50% and 60% of pupal development (% pd), nascent rhabdomeres begin to load with TRP (Fig. 1A), a light-activated Ca^{2+} channel that serves phototransduction (Hardie and Raghu, 2001). TRP is first immunodetectable in photoreceptor cytoplasm beginning at about 45% pd and accumulates in large cytoplasmic vesicles by 50% pd. Expression of the major rhodopsin (Rh1), the photosensory protein of photoreceptors R1-6, initiates later, at about 70% pd. Rh1 is first detected as faint diffuse signals and small puncta spread throughout the cytoplasm. In animals raised in standard 12/12 hour light/dark conditions, Rh1 concentrates in large (>200 nm) cytoplasmic vesicles, 'Rh1-containing large vesicles' (RLVs), prior to its appearance in the rhabdomere (Fig. 1B). During the stage when Rh1 and TRP synthesis overlap (Li and Montell, 2000), the proteins colocalize in RLVs (Fig. 1C), suggesting the same vesicle accommodates both rhabdomere proteins. One or more small dots of F-actin decorate each RLV (Fig. 1D), resembling the actin patches associated with vesicles that mediate transport in yeast (Pelham and Chang, 2001) and other systems (Rozelle et al., 2000). In both fixed and living preparations, RLVs appear tethered via actin patches to the rhabdomere terminal web (RTW), a specialization of the cortical actin cytoskeleton (Fig. 1E,F).

RLVs are MVBs

Immunogold electron microscopy using anti-Rh1 identified RLVs as multivesicular bodies (MVBs) (Fig. 2A). The delicate detail of MVBs is poorly preserved by fixation protocols that retain Rh1 antigenicity. However, MVBs are the only organelle observed in the EM with the size, shape and distribution characteristic of RLVs observed in our confocal immunofluorescence studies. We further observed that RLVs label with GFP-tagged Rab7 (Entchev et al., 2000) (Fig. 2B), an endosomal protein that marks the limiting membrane of MVBs in *Drosophila* (Sriram et al., 2003) and other systems (Raiborg et al., 2003). RLVs also immunostain for Hrs (Fig. 2C), the endosome-associated Hepatocyte growth factor-regulated tyrosine kinase substrate associated with MVBs in *Drosophila* (Lloyd et al., 2002; Sriram et al., 2003) and other systems (Bache et al., 2003). These observations identify RLVs as MVBs.

Light dependent endocytosis is responsible for the presence of Rh1 in MVBs

The early detection of rhodopsin and TRP within MVBs was

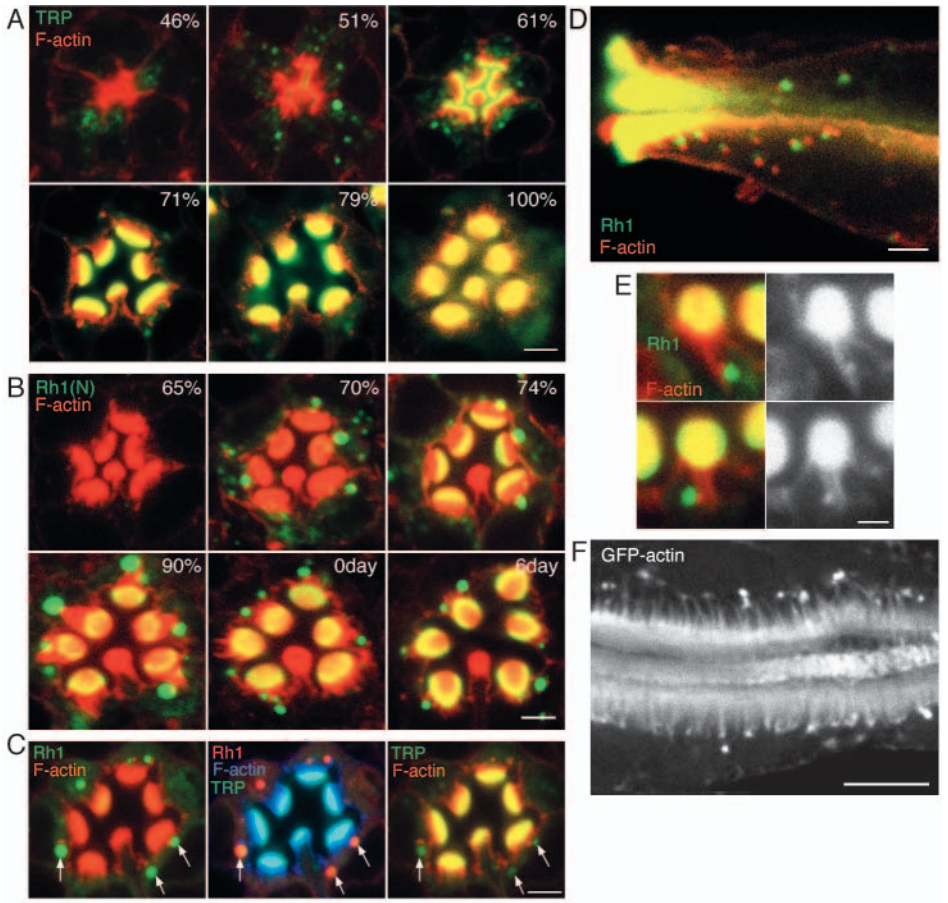

Fig. 1. TRP and Rh1 accumulate in large cytoplasmic vesicles during photoreceptor maturation. Confocal cross-sections of staged pupal eyes (% pd=% pupal development) stained for F-actin using rhodamine-phalloidin (red) and either (A) anti-TRP or (B) anti-Rh1 (green). Actin-rich rhabdomeres stain red at all stages; overlapping green immunostain (Rh1 or TRP) renders rhabdomeres yellow. The yellow-red boundary marks the rhabdomere base, the cytoplasmic ends of microvilli and the origin of the actin-rich rhabdomere terminal web (RTW). (A) TRP concentrates in large cytoplasmic vesicles during its synthetic peak, between 50 and 80% pd. (B) Following the onset of Rh1 expression at about 70% pd, the protein concentrates in large cytoplasmic (Rh1-containing large) vesicles (RLVs), before it is detected in rhabdomeres at 70% pd. (C) TRP and Rh1 colocalize in RLVs of 74% pd photoreceptors. (D) A side view of an ommatidium isolated from a newly eclosed wild-type fly shows RLVs associate with one or more small dots of F-actin. (E) Most RLVs appear tethered to the RTW via actin patches. (F) A side view of a live ommatidium isolated from a 6-day-old fly expressing GFP-actin driven by Rh1Gal4. Actin patches decorate the RTW of living cells. Scale bar: 2 μm in A-D; 1 μm in E; 5 μm in F.

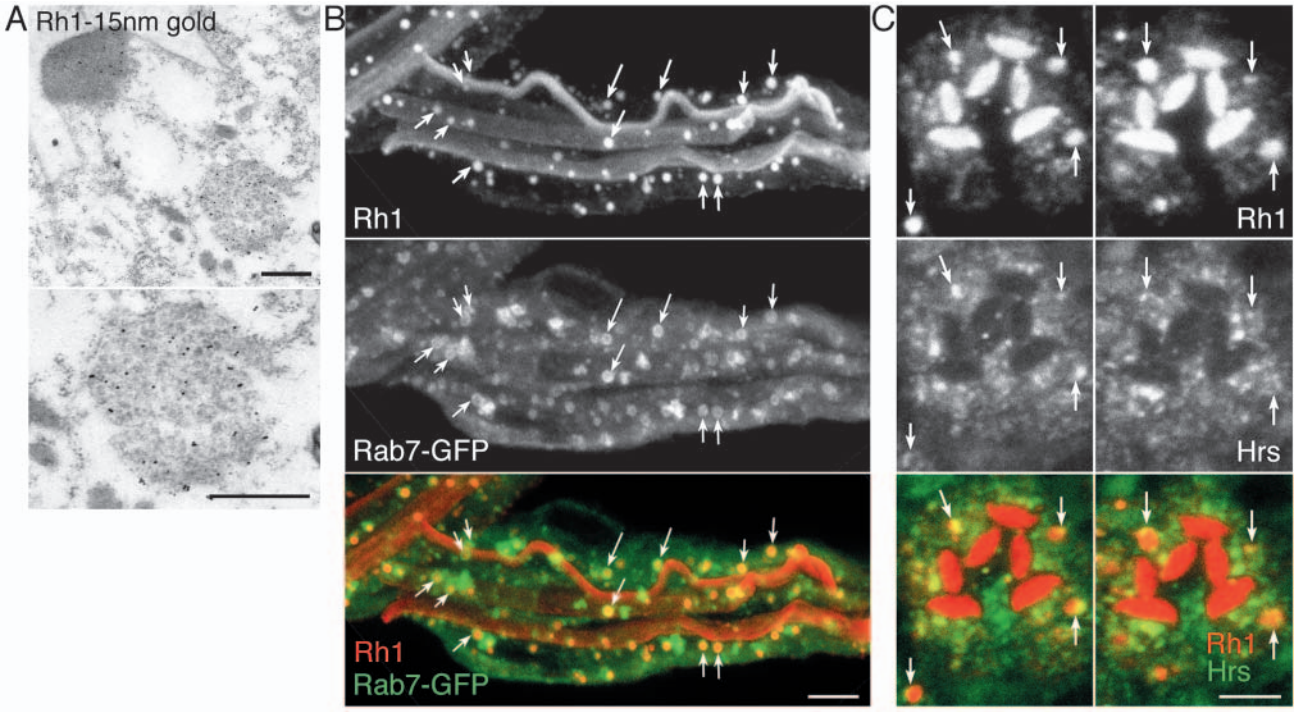

Fig. 2. RLVs are MVBs. (A) Immunoelectron microscopy localizes Rh1 to MVBs in 87% pd photoreceptors. (B) Confocal projections of ommatidia isolated from newly eclosed *Rh1-Gal4, UAS-Rab7-GFP* flies. Rab7-GFP outlines RLVs, whereas Rh1 stains more uniformly. Arrows indicate corresponding RLVs. RLVs, ER and Golgi (not shown) are distributed evenly throughout photoreceptors, unlike cell types in which pericentriolar cytoplasm is a focus for endosomal organization. (C) Hrs localizes to RLVs in 78% pd ommatidia. Arrows indicate corresponding RLVs. Scale bar: 500 nm in A; 4 μm in B; 2 μm in C.

not anticipated because MVBs are generally considered to be late endosomal compartments, delivering cargo retrieved from the plasma membrane to the lysosomes for degradation. Thus, the presence of Rh1 in an endocytic degradative organelle during the time the cell is increasing its sensory membrane is noteworthy. However, light-dependent endocytosis of Rh1 is well documented in adult photoreceptors (Blest, 1980; Xu et al., 2004), and it is possible that the Rh1 found in RLVs has already been retrieved from the developing rhabdomere.

To investigate this possibility, we observed Rh1 transport in dark-reared flies (Fig. 3A). Rh1 begins to accumulate in the rhabdomere just after Rh1 expression starts at 70% pd. There are few RLVs in all stages in dark-reared flies. RLVs form within 30 minutes of light exposure, and disappear within 13 hours of return to dark (Fig. 3B). These observations suggest that in light-reared flies, Rh1 is first transported to the rhabdomere, but light-induced internalization quickly transports Rh1 into RLVs. Thus, even during the developmental period in which the photoreceptor cell is increasing rhabdomeric volume and Rh1 content, vigorous endocytosis can exceed the rate of biosynthetic delivery of Rh1.

Rab11 colocalizes with TGN and newly synthesized Rh1 at the rhabdomere base

To position Rab11 in the sensory membrane transport pathway,

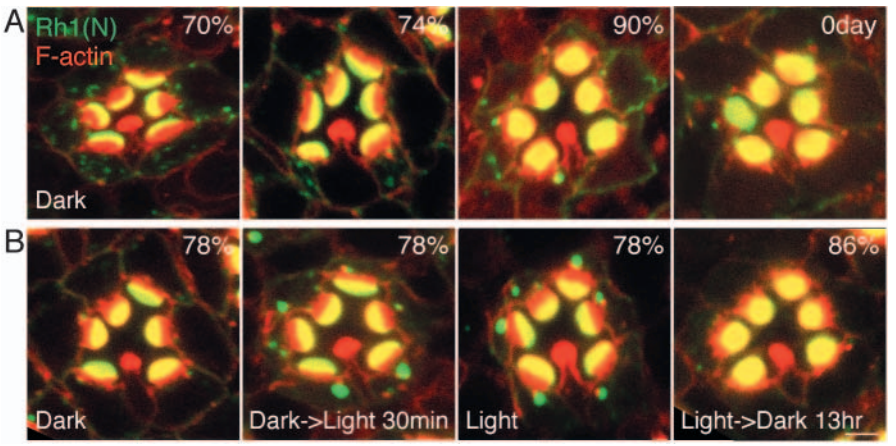

Fig. 3. Rh1 internalization is light dependent. (A) In dark-reared flies, Rh1 accumulates in the rhabdomere shortly after the onset of its expression, about 70% pd. There are few RLVs in developing (74% pd and 90% pd) and newly-eclosed (0 day) flies. (B) Many RLVs appear within 30 minutes of light exposure (Dark→Light 30 minutes), approximating the distribution of RLVs in 12L/12D raised flies fixed 4 hours after light (Light). RLVs are absent 13 hours after flies are returned to the dark following light exposure at 78% pd (Light→Dark 13 hours). Scale bar: 2 μm.

we generated flies expressing the Golgi marker, CFP-galactosyl-transferase and immunolocalized Rab11 in developing photoreceptors. Rab11 is present on small vesicles (<200 nm in diameter) scattered throughout the cytoplasm (Fig. 4A). Many of these appear associated with Golgi structures; others are located at the base of developing rhabdomere (Fig. 4A arrows). Rab11 is not associated with RLVs. To further characterize the Golgi association of Rab11, we immunostained developing photoreceptors for Rab11 and the cis-Golgi marker, Rab1 (Fig. 4B). Rab1 localizes to the convex side of the Golgi, while Rab11 localizes to the opposite, concave, side. Triple staining of CFP-labeled Golgi directly shows Rab1 and Rab11 localize to opposite sides of the Golgi (Fig. 4C). Therefore, Rab11 must localize to the trans-Golgi surface.

To investigate the possibility that Rab11 is associated with maturing Rh1, we used blue light to trigger synchronized release of Rh1 accumulated in the ER because of lack of the correct chromophore isomer (Ozaki et al., 1993; Satoh et al., 1997). Rh1 and Rab11 were then immunolocalized in pupal eyes 0, 40, 60, 90 and 180 minutes after blue-light irradiation (Fig. 4D,F). Prior to blue light (0 minutes), Rh1 was distributed throughout photoreceptor cytoplasm, colocalizing with an ER marker (not shown). At 40 and 60 minutes, most Rh1 colocalized with the Golgi (Fig. 4F). By 40 minutes, some Rh1 was concentrated in Rab11 immunopositive vesicles, some of these at the rhabdomere base (Fig. 4D, arrow). At both 60 and 90 minutes, Rh1 and Rab11 showed extensive colocalization in vesicles at the rhabdomere base (Fig. 4D,F, arrows). By 90 minutes, there are few Rh1 positive cytoplasmic vesicles, and by 180 minutes most Rh1 is found in the rhabdomeres (Fig. 4D).

These immunofluorescence results suggest Rh1, upon exit from the ER, associates first with the Golgi, then within Rab11-

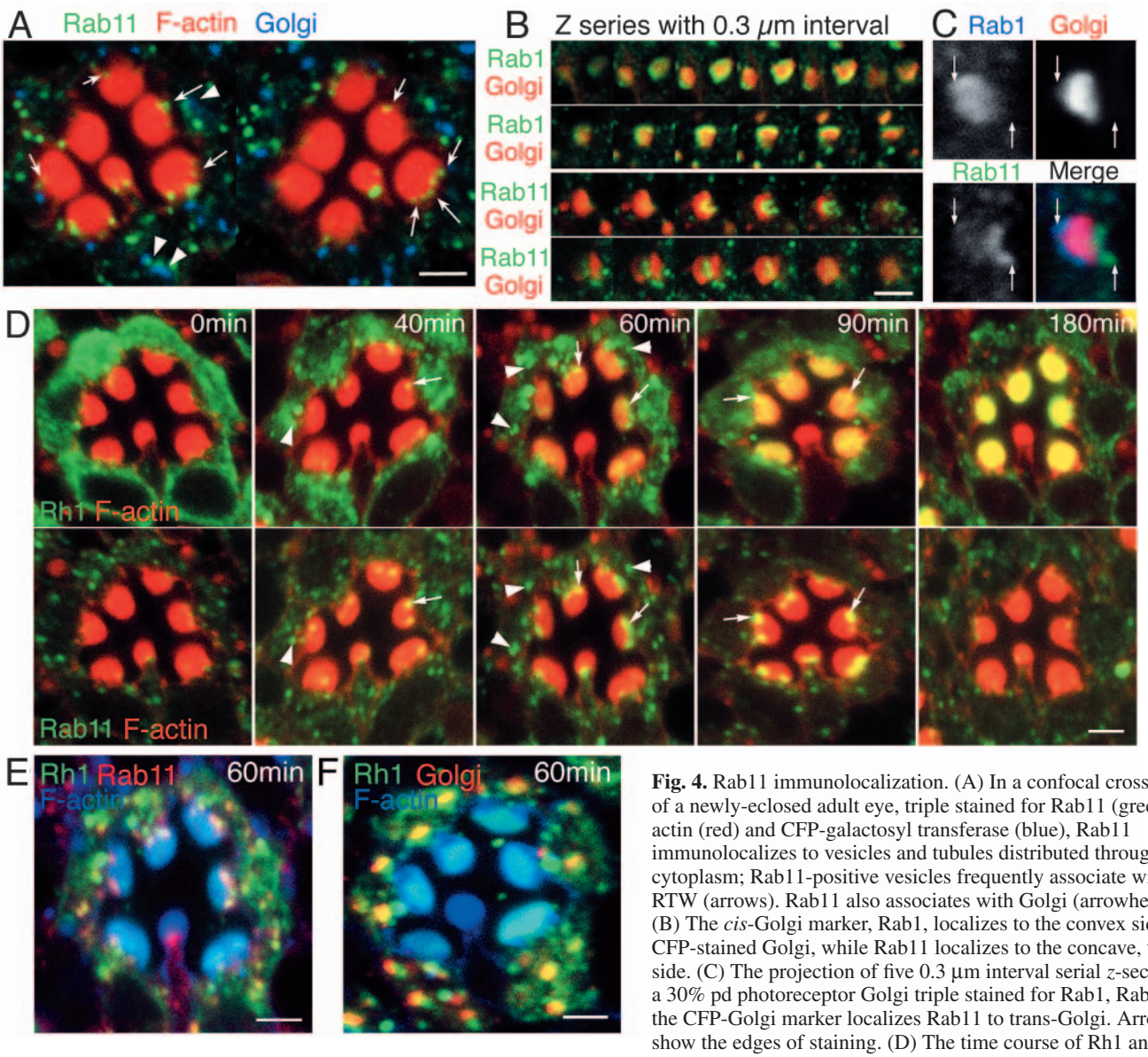

Fig. 4. Rab11 immunolocalization. (A) In a confocal cross-section of a newly-eclosed adult eye, triple stained for Rab11 (green), F-actin (red) and CFP-galactosyl transferase (blue), Rab11 immunolocalizes to vesicles and tubules distributed throughout the cytoplasm; Rab11-positive vesicles frequently associate with the RTW (arrows). Rab11 also associates with Golgi (arrowheads). (B) The *cis*-Golgi marker, Rab1, localizes to the convex side of CFP-stained Golgi, while Rab11 localizes to the concave, trans side. (C) The projection of five 0.3 μm interval serial *z*-sections of a 30% pd photoreceptor Golgi triple stained for Rab1, Rab11 and the CFP-Golgi marker localizes Rab11 to trans-Golgi. Arrows show the edges of staining. (D) The time course of Rh1 and Rab11 localizations after blue light irradiation. Arrows and arrowheads indicate colocalization of Rab11 and Rh1 at the rhabdomere base and in cytoplasmic organelles. (E) Rh1 and Rab11 intensely colocalize at the base of rhabdomere 60 minutes after blue light. (F) Most of Rh1 positive organelles are Golgi bodies. Scale bar: 2 μm in A-E.

positive vesicles, before being deposited in the rhabdomere. Thus, the Rab11 localization is consistent with a role in TGN→rhabdomere transport. Transport visualized in these studies is completed by 180 minutes (Fig. 3D), in good agreement with previous immunoblot data showing intermediate Rh1 is completely processed into mature 35K Rh1 within 180 minutes (Satoh et al., 1997). RLVs are not prominent at any time during ER→rhabdomere transport, consistent with the proposal that RLVs do not participate in biosynthetic traffic.

Rab11 activity is essential for Rh1 transport to rhabdomere and MVB formation

To further evaluate the role of Rab11 in Rh1 transport, we investigated Rh1 transport in Rab11 mutants. As animals lacking Rab11 die as embryos (Dollar et al., 2002; Jankovics et al., 2001), we made mosaic animals with eyes containing a mixture of normal photoreceptors and photoreceptors with severely reduced Rab11. Comparatively few Rab11 mutant photoreceptors were observed in mosaic eyes, probably reflecting a cell-essential role for the protein. Rab11-reduced photoreceptors fail to transport Rh1 to the rhabdomere (Fig. 5A). Mutant rhabdomeres are reduced in size and a profusion of vesicles fills the photoreceptor cytoplasm (Fig. 5B,C). These cells lack normal globular MVBs, but contain infrequent, irregular vesicular organelles resembling defective MVBs (Fig. 5D). Other vesicular compartments, including ER and Golgi, retain normal appearance.

Rab11 reduction via dsRNA expression in developing eyes similarly blocks Rh1 delivery to the rhabdomere and disperses it throughout photoreceptor cytoplasm (Fig. 6A-D). Electron microscopy shows MVBs are lost and irregular vesicles fill the cytoplasm (Fig. 6E). We also reduced Rab11 activity via expression of a dominant-negative *Rab11*, $Rab11^{N124I}$, a GTP-binding mutant (Duman et al., 1999). $Rab11^{N124I}$ expression during the time when TRP is normally delivered to the rhabdomere blocks TRP delivery with a concomitant accumulation of TRP in photoreceptor cytoplasm (Fig. 7A,B). Expression during the Rh1 delivery period recapitulates genetic Rab11 reduction: Rh1 fails to reach the rhabdomere and instead accumulates in vesicles dispersed throughout the cytoplasm (Fig. 7F). Confocal and electron microscope examination of $Rab11^{N124I}$ photoreceptors shows a parallel loss of RLVs and MVBs (Fig. 7K-O).

Rab11, but not MVBs, are required for Rh1 transport to the rhabdomere

The results above show that loss of Rab11 activity results in the accumulation of Rh1-containing vesicles in the cytoplasm and the absence of MVBs. We considered the possibility that the Rh1 vesicles may originate from a defective endocytic pathway, such that the cytoplasm accumulates early endocytic vesicles unable to consolidate into MVBs. To address this possibility, we examined the effect of a dominant-negative form of Rab5, $Rab5^{N142I}$, on the process of rhodopsin transport and MVB accumulation. This mutant has previously been shown to inhibit endocytosis and prevents MVB formation (Shimizu et al., 2003). Indeed, MVBs containing both TRP and Rh1 (Fig. 7A,E) are lost in flies expressing $Rab5^{N142I}$ (Fig. 7C,G). The same images show that $Rab5^{N142I}$ does not inhibit TRP and rhodopsin delivery to the

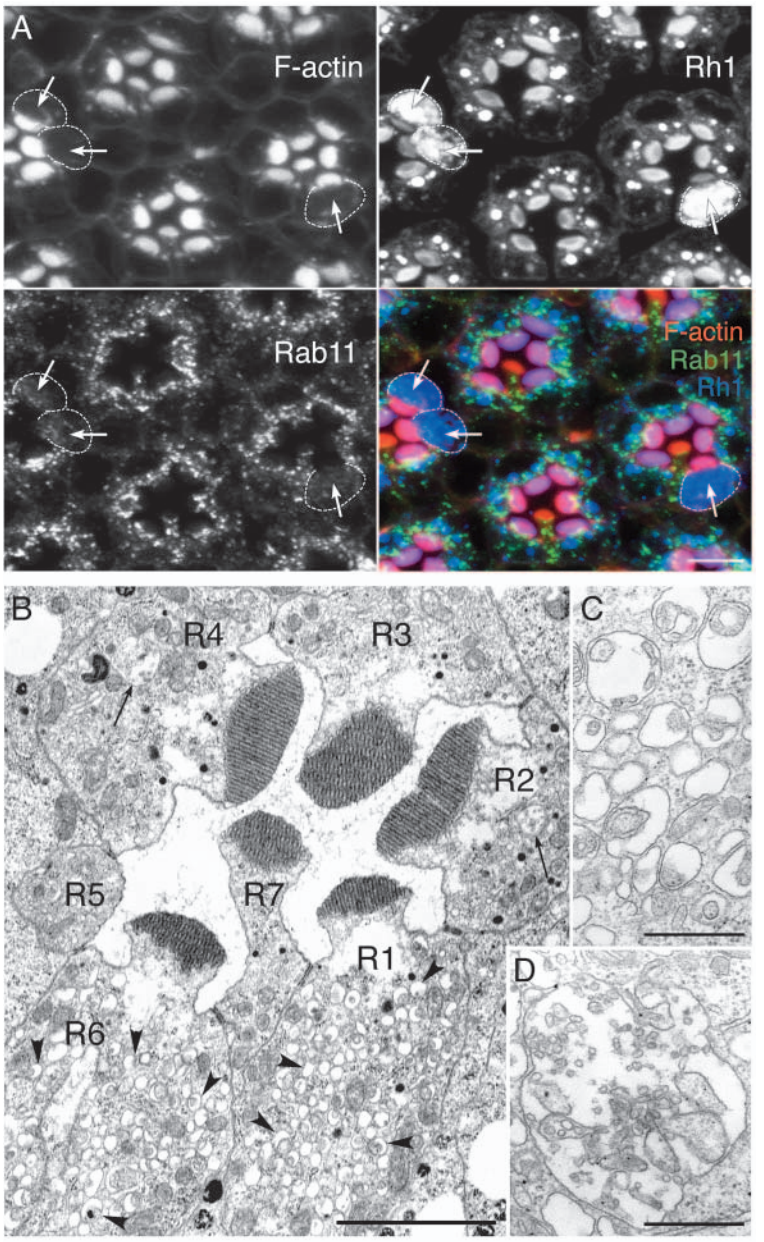

Fig. 5. Rh1 transport requires Rab11. (A) Rab11-reduced photoreceptors (arrows) in a mosaic eye triple stained for Rh1 (blue), Rab11 (green) and F-actin (red) show disrupted RLVs and Rh1 dispersed throughout the photoreceptor cytoplasm. Rh1 accumulation in rhabdomeres and rhabdomere size is decreased in the Rab11-reduced photoreceptors. (B) Electron micrographs of mosaic ommatidia show photoreceptors with normal MVBs (arrows) and vesicle-filled cells lacking MVBs (arrowheads). Other internal membranes appear normal. (C,D) Higher magnification of abnormal cytoplasmic vesicles shows unusual irregular MVB vesiculation. These appear incomplete, leaving openings, or iterated, resulting in vesicles within endovesicles not seen in normal MVBs. Scale bar: 5 μm in A; 2 μm in B; 500 nm in C,D.

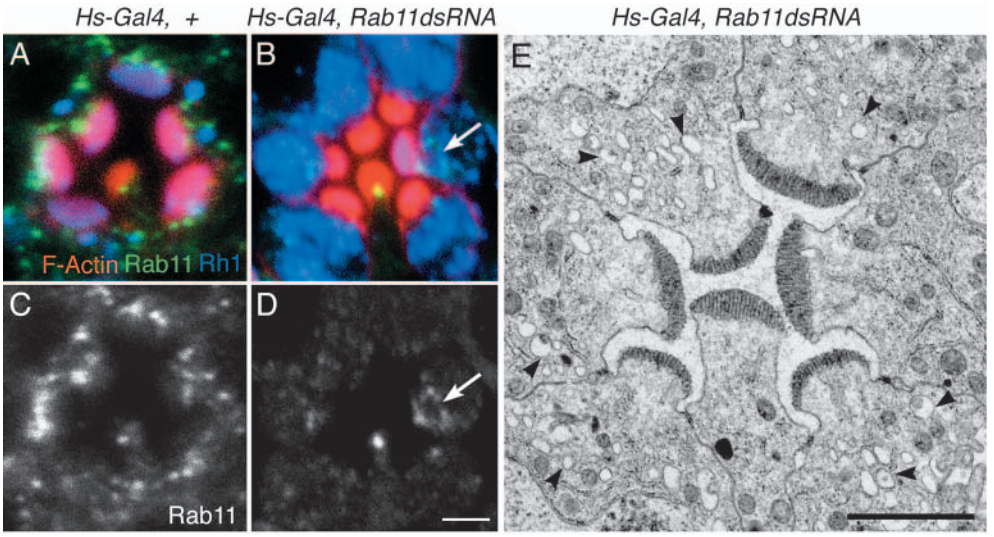

Fig. 6. Rab11 RNAi inhibits Rh1 transport. (A) In control Hs-Gal4 flies, heat shocked at 27% pd, then fixed and stained at 85% pd, Rh1 (blue) transport is normal. Rh1 is present in cytoplasmic RLVs and in rhabdomeres stained red using rhodamine phalloidin. Rab11-positive (green) vesicles are distributed throughout the cytoplasm. (B) In experimental Hs-Gal4, dsRNA flies, Rh1 is dispersed throughout photoreceptor cytoplasm and delivery to the rhabdomere is inhibited. (C) Shown separately, Rab11 immunostaining of control flies is brighter than D, Hs-Gal4, Rab11 dsRNA flies. The R2 photoreceptor (arrow) shows a higher level of Rab11 signal and detectable Rh1 delivery to the rhabdomere. R7, which also shows Rab11 signal here, does not express Rh1; transport of its rhodopsin, either Rh3 or Rh5, cannot be determined. (E) Electron micrographs of *Hs-Gal4, UAS-Rab11 dsRNA* pupae. Rhabdomere size is small. Abnormal cytoplasmic vesicles (arrowheads) proliferate and MVBs are absent. Scale bar: 2 μm in A-E.

rhabdomere, confirming that MVBs are not required for transport to the rhabdomere.

Simultaneous expression of $Rab11^{N124I}$ and $Rab5^{N142I}$ allowed us to determine if the $Rab11^{N124I}$ phenotype is the result of absence of Rab11 activity prior to TRP and Rh1 delivery to the rhabdomere, or after endocytic removal of these two proteins from the rhabdomere. If the TRP and Rh1 vesicles accumulating in cytoplasm upon $Rab11^{N124I}$ expression are endocytosed from the rhabdomere, $Rab5^{N142I}$ expression should inhibit their biogenesis. Fig. 7D,G show that this is not the case; numerous cytoplasmic TRP- or Rh1-bearing vesicles accumulate and rhabdomeres do not stain for TRP and Rh1.

The interpretation of these results could be complicated by consideration that guanine-nucleotide-deficient small GTPase dominant negatives sequester activating GEF proteins (Feig, 1999), and crosstalk may exist among RabGEF signaling pathways. However, the fidelity with which $Rab11^{N124I}$ recapitulates genetic and RNAi Rab11 loss, the observation that $Rab5^{N142I}$ mutant shows the expected endocytic defect, and the marked contrast in the $Rab11^{N124I}$ and $Rab5^{N142I}$ individual phenotypes, suggest these dominant negatives do generate a specific loss of function for each of these genes. From this perspective, the failure of $Rab5^{N142I}$ expression to impact the $Rab11^{N124I}$ phenotype argues Rab11 is required upstream of Rab5 and prior to the initial delivery of TRP and Rh1 to the rhabdomere.

We also observed that Golgi morphology visualized by CFP-galactosyl transferase in confocal microscopy was unaffected in $Rab11^{N124I}$ photoreceptors (Fig. 7I,J). These data, in agreement with the localization studies, suggest Rab11 is required for a post-Golgi step in rhodopsin and TRP movement to the rhabdomere.

Rab11 has an indirect role in MVB formation

The proposed role of Rab11 in delivery of membrane proteins to the rhabdomere does not account for the loss of MVBs in the *Rab11* mutant photoreceptors. It is possible that loss of Rab11 activity depletes the rhabdomere of Rh1 and other membrane proteins, and the lack of protein in these membranes limits the rate of endocytosis and MVB formation. To investigate this possibility more directly, we examined the effect of $Rab11^{N124I}$ on uptake of an endocytic tracer, Texas Red-conjugated avidin (TR-avidin), by larval Garland cells, large and easily accessible endocytic specialists (Chang et al., 2002; Kosaka and Ikeda, 1983). In normal cells, internalized TR-avidin could be seen in peripheral, vesicular structures 10 minutes after exposure to TR-avidin, (Fig. 8A). In Garland cells expressing $Rab11^{N124I}$, however, TR-avidin is not internalized (Fig. 8B).

Electron microscopic observations provided insight into this defect. In wild-type Garland cells, numerous labyrinthine channels invaginate deeply from plasma membrane (Fig. 8C). These channels are the sites of active endocytosis; clathrin-coated buds mark the tips of the channels and the channels elongate when endocytosis is inhibited in the dynamin mutant $shibire^{ts}$ (Kosaka and Ikeda, 1983). The labyrinthine channels are absent in Garland cells expressing $Rab11^{N124I}$ (Fig. 8D). Thus, the labyrinthine channels do not form correctly in the absence of Rab11 activity. These results are consistent with the view that membrane components essential to sustaining vigorous endocytosis are lost when Rab11-dependent apical delivery is compromised.

Discussion

Rab proteins organize and maintain physiologically distinct endosomal compartments (Zerial and McBride, 2001). In the work reported here, we show that Rab11 is essential for trafficking two membrane proteins, TRP and Rh1, to the *Drosophila* photosensory organelle, the rhabdomere. When Rab11 activity is reduced in developing photoreceptors, Golgi-derived TRP- and Rh1-bearing vesicles accumulate in photoreceptor cytoplasm instead of exocytosing to expand the growing rhabdomere. Thus, the results presented here show Rab11 activity supports a distinct plasma membrane

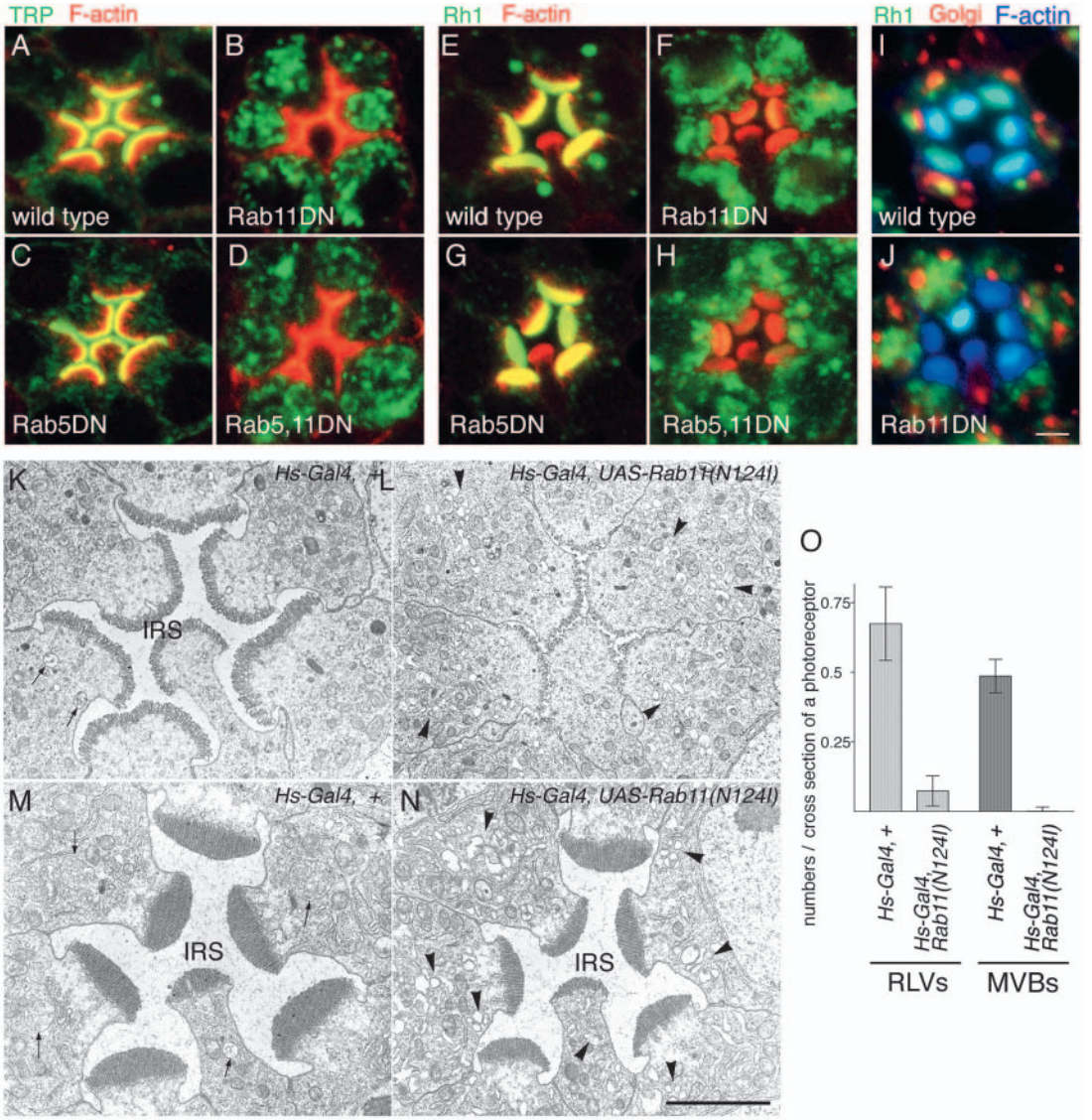

Fig. 7. Dominant-negative Rab11 inhibits TRP and Rh1 transport, and disperses RLVs and MVBs. (A) Wild-type rhabdomeres are TRP immunopositive at 64% pd. (B) Rab11^{N124I} expression beginning at 50% pd prevents TRP transport to the rhabdomere; TRP is dispersed throughout photoreceptor cytoplasm. (C) There is no TRP containing vesicles in *Rab5*^{N142I}-expressing cells, but TRP is normally transported to the rhabdomere. (D) *Rab11*^{N124I} and *Rab5*^{N142I} co-expression beginning at 50% pd prevents TRP transport to the rhabdomere. (E) Wild-type rhabdomeres are Rh1 immunopositive at 83% pd; cytoplasmic Rh1 is present in RLVs. (F) *Rab11*^{N124I} expression beginning at 70% pd prevents Rh1 transport to the rhabdomere. RLVs are lost and Rh1 accumulates diffusely throughout photoreceptors. (G) RLVs are absent in *Rab5*^{N142I}-expressing cells, but Rh1 is normally transported to the rhabdomere. (H) *Rab11*^{N124I} and *Rab5*^{N142I} co-expression beginning at 70% pd prevents Rh1 transport to the rhabdomere. RLVs are lost and Rh1 accumulates diffusely throughout photoreceptors, similar to Rab11^{N124I} single expression. (I,J) Golgi morphologies are not affected by *Rab11*^{N124I} expression. (K-N) Electron micrographs of 65% pd pupae heat shocked from 50% pd (K,L) or 83% pd pupae heat shocked from 70% pd (M,N). *Rab11*^{N124I} expression reduces rhabdomere size and causes proliferation of abnormal cytoplasmic vesicles (arrowheads). MVBs are evident in control photoreceptors (arrows), but are absent in photoreceptors expressing *Rab11*^{N124I}. IRS is reduced by *Rab11*^{N124I} expression. (O) *Rab11*^{N124I} expression decreases RLVs and MVBs in parallel (see Materials and methods). Scale bar: 2 μm in A-N.

compartment, the apical plasma membrane subdomain specialized for phototransduction.

Rab11 has previously been implicated in control of membrane traffic through the pericentriolar recycling endosome. In cultured baby hamster kidney (BHK) cells, return of internalized transferrin receptor to the cell surface is inhibited by dominant-negative Rab11 expression (Ullrich et al., 1996). During cellularization of *Drosophila* embryos, apical membrane redeployed to the growing basolateral surface transits a Rab11-dependent recycling endosome (Pelissier et al., 2003). Rab11 has also been implicated in *trans*-Golgi to plasma membrane transport. In non-polarized BHK cells in culture, expression of dominant-negative Rab11S25N inhibited transport of a basolateral marker protein marker, vesicular stomatitis virus G protein, but had no impact on delivery of an apical marker protein, influenza hemagglutinin (Chen et al.,

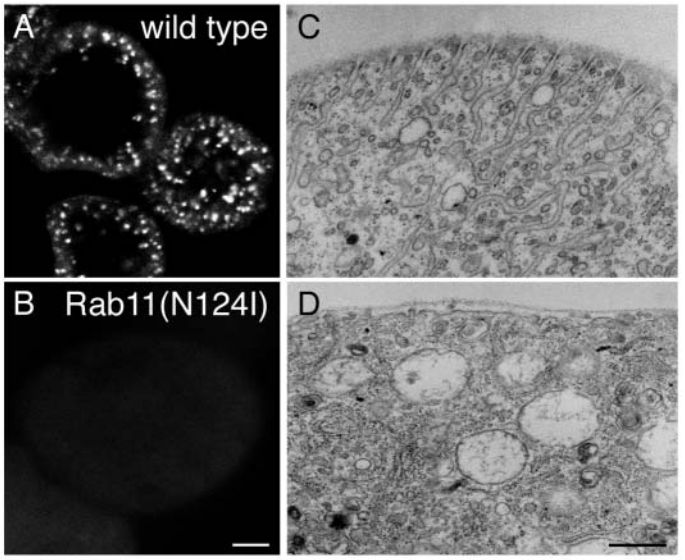

Fig. 8. *Rab11*^{N124I} inhibits endocytosis in Garland cells. (A,B) Confocal cross sections of wild-type or Rab11^{N124I}-expressing Garland cells after 10 minutes TR-avidin uptake. Many vesicles stain in wild-type Garland cells. However, there is no avidin uptake in *Rab11*^{N124I} expressing Garland cells. (C,D) Electron micrographs of Garland cells show labrynthine channels, deep invaginations of the plasma membrane. These are lost in *Rab11*^{N124I}-expressing Garland cells. Scale bar: 5 μm in A,B; 500 nm in C,D.

1998). Recent observation that recycling endosomes can serve as an intermediate during transport from the Golgi to MDCK cell plasma membranes (Ang et al., 2004) raises the possibility that biosynthetic traffic transits a recycling endosome and the site of Rab11 action is at the recycling endosome. However, we observed no pericentriolar endosome in *Drosophila* photoreceptors, and that Rh1 moved directly from the *trans*-Golgi to the rhabdomere when released into the biosynthetic pathway by blue light. Thus, there is no evidence for Rh1 moving through an intermediate compartment when en route to the rhabdomere.

Rh1-bearing post-Golgi vesicles and recycling endosome-derived vesicles may both traffick to the cell surface because they share common Rab11 effectors. Rab11 interacts with unconventional class V myosins and expression of dominant-negative Myo-Vb inhibits delivery from early endosomes to the cell surface (Lapierre et al., 2001). An extensive F-actin terminal web, the RTW, extends from the rhabdomere base into photoreceptor cell cytoplasm (Chang and Ready, 2000) and disruption of the photoreceptor actin cytoskeleton inhibits the vesicular traffic that builds crab rhabdomeres (Matsushita and Arikawa, 1996). Rab11, together with a Myo-V effector, may promote post-Golgi vesicle motility along the actin RTW to focus delivery to the rhabdomere.

Loss of Rab11 activity also disrupts normal photoreceptor MVB morphology. MVBs are often identified as late endosomal compartments, delivering cargo destined for lysosomal degradation (Gruenberg, 2001; Katzmann et al., 2002; Kramer, 2002). However, several recent studies show MVBs can be also exocytic carriers, delivering endosomal contents to the cell surface. Examples include the secretory lysosomes of immune system cells (Griffiths, 2002), melanosomes of pigment cells (Marks and Seabra, 2001), exosomes of maturing red blood cells (Johnstone et al., 1991) and secreted vesicles mediating cell-cell signaling (Denzer et al., 2000; Seto et al., 2002). The accumulation of newly synthesized MHCII receptors within MVBs of unstimulated dendritic cells, and the stimulus-induced reorganization of MVBs and appearance of MHCII receptors at the plasma membrane, led to consideration of MVBs as an exocytic compartment (Kleijmeer and Raposo, 2001). Autoradiography of crayfish eyes following ^{3}H-leucine injection showed newly synthesized protein first in the cytoplasm, then in MVBs and then in rhabdomere rhabdomeres (Hafner and Bok, 1977), prompting the conjecture that MVBs are a post-Golgi organelle of biosynthetic traffic (Piekos, 1987).

The work reported here discounts the possibility that MVBs are exocytic vesicles in *Drosophila* photoreceptors. First, we show that appearance of Rh1 in the MVBs is dependent on light treatment. Previously, light was shown to trigger endocytosis of rhabdomeric membrane (Blest, 1980; Xu et al., 2004), so this light dependency suggests MVBs originate from an endocytic process. Second, depletion of Rab5 activity, which is known to regulate the fusion between endocytic vesicles and early endosomes, also eliminates Rh1 and TRP containing MVBs, without affecting Rh1 and TRP transport to the rhabdomere. Thus, all the results support the view that MVBs are endocytic vesicles. The early and rapid appearance of Rh1 and TRP in these vesicles is remarkable, showing that the machinery of light-dependent receptor internalization is fully operational at the outset of morphogenesis. Vigorous light-dependent endocytosis competes with exocytosis from the outset of rhabdomere morphogenesis, internalizing rhodopsin and TRP from the growing sensory membrane even as exocytosis expands it.

Rab5 loss-of-function analysis also supports the view that Rab11 acts before Rab5. In Rab5, Rab11 double mutants, photoreceptors retain the Rab11 phenotype. These results are consistent with a role of Rab11 in the exocytic process, but not with an exclusive role in endocytic recycling. Yet, we have also shown that Rab11 activity is required for accumulation of MVBs. We propose that this is an indirect effect of the Rab11 requirement in the exocytic pathway. Rab11 inhibition 'starves' the rhabdomere, the target of Rab11-mediated transport, of required proteins, which in turn slows the rate of endocytosis and eliminates endocytosis-dependent MVBs. In support of this view, we have shown that Rab11 activity is required for the presence of labyrinthine channels on Garland cells, membrane specializations that promote vigorous endocytosis. Rab11 loss plausibly depletes membrane components that sustain vigorous endocytosis.

Drosophila and vertebrate photoreceptors share fundamental cellular and molecular mechanisms and Rab family members and their functions are strongly conserved across eukaryotes (Pereira-Leal and Seabra, 2001). Rab11 has been identified in rhodopsin-containing post-Golgi vesicles formed within a vertebrate retina cell-free system (Deretic, 1997), raising the likelihood vertebrate photoreceptors also contain a Rab11-dependent vesicular compartment essential for rhodopsin transport and outer segment development. Failure to traffic Rh1 in *Drosophila* leads to retinal degeneration (Colley et al., 1995; Green et al., 2000; Kurada et al., 1998), and similar

mechanisms are implicated in rhodopsin mutations and other mutations causing the human disease retinitis pigmentosa (Sung and Tai, 2000; Trudeau and Zagotta, 2002). The involvement of Rab11 in the post-Golgi processes provides an entry point to discover the cellular components and pathways responsible for elaborating the specialized photosensitive membranes. These events are likely to be key regulators of normal cellular development and the triggering events of retinal disease.

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