Identification of Genes Required for Apical Protein Trafficking in *Drosophila* Photoreceptor Cells

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**ABSTRACT** *Drosophila melanogaster* photoreceptor cells are highly polarized epithelial cells. Their apical membrane is further subdivided into the stalk membrane and the light-sensing rhabdomere. The photopigment Rhodopsin 1 (Rh1) localizes to the rhabdomere, whereas the apical determinant Crumbs (Crb) is enriched at the stalk membrane. The proteoglycan Eyes shut (Eys) is secreted through the apical membrane into an inter-rhabdomeral space. Rh1, Crb, and Eys are essential for the development of photoreceptor cells, normal vision, and photoreceptor cell survival. Human orthologs of all three proteins have been linked to retinal degenerative diseases. Here, we describe an RNAi-based screen examining the importance of 237 trafficking-related genes in apical trafficking of Eys, Rh1, and Crb. We found 28 genes that have an effect on the localization and/or levels of these apical proteins and analyzed several factors in more detail. We show that the Arf GEF protein Sec71 is required for biosynthetic trafficking of both apical and basolateral proteins, that the exocyst complex and the microtubule-based motor proteins dynein and kinesin promote the secretion of Eys and Rh1, and that Syntaxin 7/Avalanche controls the endocytosis of Rh1, Eys, and Crb.

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(Eys) is essential in the formation of the IRS (Husain et al., 2006; Zelhof et al., 2006). Eys is thought to be secreted through the stalk membrane into the IRS (Husain et al., 2006). Similar to mutations in rhodopsin and CRB1, also mutations in human EYS have been linked to retinal degenerative diseases such as retinitis pigmentosa (RP25) (Abd El-Aziz et al., 2008; Collin et al., 2008).

The fact that several apical transmembrane or secreted proteins play key roles in PRC development and disease motivates a careful assessment of the mechanism that transport and target these proteins. Several factors have been identified that are involved in apical trafficking in Drosophila PRCs (see Figure 1). Examples include Rab1 and Syntaxin 5 (Syx5) that are essential in ER to Golgi trafficking (Satoh et al., 1997; Satoh et al., 2016). Rab6 is important in the exit of apical proteins from the Golgi (Iwanami et al., 2016) and Rab11, Rip11, Myosin V and the exocyst complex (e.g., Sec6) are important in the secretion of Rh1 and other rhabdomeral proteins (Satoh et al., 2005; Li et al., 2007; Beronja et al., 2005). Secretory vesicle carrying Rh1 and other rhabdomere-directed proteins are moved along actin fibers of the rhabdomere terminal web driven by the Myosin V motor (Li et al., 2007). Myosin V interacts with Crb to facilitate normal Rh1 transport to the rhabdomere (Pocha et al., 2011). Following the path of Rh1, once at the rhabdomeres, Rab5 and Shibire/Dynamin (Shi) are required for its endocytosis (Satoh et al., 2005; Alloway et al., 2000; Kiselev et al., 2000). Some of this endocytosed Rh1 is recycled back to the rhabdomere through a retromer-dependent pathway that involves the retromer protein Vps26, whereas the rest is sent to the lysosome for degradation (Wang et al., 2014; Chinchore et al., 2009).

It appears that the apical and basolateral trafficking routes diverge somewhere along the Golgi prior to the action of Rab6, whereas the rhabdomeral vs. stalk membrane route diverges downstream of Rab6 following the exit from the Golgi. Crb and Eys are thought to be targeted to the stalk through a pathway distinct from the secretory pathway used by rhabdomeral proteins (Beronja et al., 2005, Husain et al., 2006). The components involved in Crb and Eys exocytosis are currently unknown, except for evidence suggesting that microtubules and microtubule motor proteins are involved in Crb localization during pupal stages (Mukhopadhyay et al., 2010; Chen et al., 2010; League and Nam 2011; Mui et al., 2011; Nam 2016).

To develop a better understanding of the apical trafficking mechanisms that control the distribution of Eys, Crb, and Rh1 in the fly retina we analyzed 237 candidate genes, known or predicted to encode proteins involved in vesicle trafficking. We identified 28 genes that are important for the localization or concentration of apical proteins, and provide a more detailed analysis of four factors: The Arf guanine nucleotide exchange factor (GEF) Sec71, the exocyst complex, the microtubule motor dynein, and the endocytotic regulator Syntaxin 7 (Syx7)/Avalanche (Avl).

MATERIALS AND METHODS

Drosophila stocks and crosses

UAS-RNAi lines were obtained from the Bloomington Drosophila Stock Center (BDSC) and Vienna Drosophila Resource Center (VDRC). See Table S1 for a list of lines used. UAS-RNAi constructs were expressed in the developing eye with UAS-Dicer-2 GMR-Gal4. GMR-Gal4 activity starts in the developing retina posterior to the morphogenetic furrow at third larval instar and continues to adulthood (Freeman 1996). UAS-Dicer-2 was used to amplify the effects of RNAi (Ketting et al., 2002); mouse anti-Rh1 (4C5, 1:50; Developmental Studies Hybridoma Bank), guinea pig anti-Eys (G5, 1:50; Husain et al., 2006), mouse anti-Nervana (nrv57, 1:50, Developmental Studies Hybridoma Bank), rabbit anti-GM130 (ab30637, 1:300, Abcam). Secondary antibodies anti-guinea pig alexa fluor 647 (A21450), anti-rat alexa fluor 555 (A21434), and anti-mouse alexa fluor 488 (A11029) were used at 1:400 (Molecular Probes/Thermo Fisher Scientific). Acti-stain555 (PHDG1-A, 1:75, Cytoskeleton Inc) was used to visualize rhabdomeres. A Leica TCS SP8 confocal microscope with 100x oil objective (NA 1.4) was used to capture images. Image J (Fiji) and Adobe Photoshop and Adobe Illustrator were used to edit and compile figures.

Electron Microscopy

Transmission electron microscopy (TEM) was performed on 0-24 hr old adult flies. Detached heads were bisected in ice-cold fixative solution (2% para-formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4). The dissected tissue was kept in the above-mentioned fixative solution (2% para-formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4). The dissected tissue was kept in the above-mentioned fixative solution for 24 hr or longer. The antibody staining was done according to a standard protocol. The following antibodies were used: rat anti-Crb (F3, 1:500; Pellikka et al., 2002); mouse anti-Rh1 (4C5, 1:50; Developmental Studies Hybridoma Bank), guinea pig anti-Eys (G5, 1:50; Husain et al., 2006), mouse anti-Nervana (nrv57, 1:50, Developmental Studies Hybridoma Bank), rabbit anti-GM130 (ab30637, 1:300, Abcam). Secondary antibodies anti-guinea pig alexa fluor 647 (A21450), anti-rat alexa fluor 555 (A21434), and anti-mouse alexa fluor 488 (A11029) were used at 1:400 (Molecular Probes/Thermo Fisher Scientific). Acti-stain555 (PHDG1-A, 1:75, Cytoskeleton Inc) was used to visualize rhabdomeres. A Leica TCS SP8 confocal microscope with 100x oil objective (NA 1.4) was used to capture images. Image J (Fiji) and Adobe Photoshop and Adobe Illustrator were used to edit and compile figures.
fixative for 3 days at 4°C on a nutator. Next, the tissue was washed with 0.1 M sodium cacodylate and treated with a solution of 1% osmium tetroxide and 0.1 M sorbitol in 0.1 M sodium cacodylate for 1 hr in the dark. After this treatment, the eyes were washed with 0.1 M sodium cacodylate and dehydrated in an ethanol series (50%, 70%, 80%, 100%) and embedded in Spurr’s resin. Ultra-thin sections were stained with uranyl acetate and lead citrate. A Hitachi HT7000 transmission electron microscope was used to view the tissue at 700x magnification and an AMT XR-111 digital camera with AMT capture engine software was used to capture images.

Interrhabdomeral space (IRS) quantification
Retinas were immuno-stained with anti-Eys antibody. Images were taken using a Leica TCS SP8 confocal microscope, with a 100x oil objective (NA 1.4). IRS size was then measured using Imaris software. Mean and standard deviation were calculated. An unpaired non-parametric Mann-Whitney test was performed to establish p values.

Stalk membrane quantification
Image J (Fiji) was used to measure stalk membranes from TEM images taken at 700x magnification. Individual stalk membranes were traced from the base of the rhabdomere to the zonula adherens and the length was measured. Mean and standard deviation were calculated. An unpaired non-parametric Mann-Whitney test was performed to establish p values.

Data availability
All data are included in the paper or the associated supplemental materials. All Drosophila stocks are available from public repositories.

All other reagents are commercially available or can be sent upon request. Supplemental material available at figshare: 10.25387/g3.10010408.

RESULTS AND DISCUSSION
Identification of genes involved in PRC vesicle trafficking
Drosophila PRCs are organized as elongated cylinders surrounding a lumen, the IRS, bound by PRC apical membranes (Figure 2A). PRCs deficient in Rh1, Eys, or Crb have prominent developmental defects. Rh1 is essential for rhabdomeral maintenance (Kumar and Ready 1995) and disruption of factors, such as the exocyt component Sec6 that affects the exocytosis of Rh1 and other rhabdomeral proteins show rhabdomeral deterioration (e.g., Beronja et al., 2005) (Figure 2B). The rhodobemes of crb deficient PRCs appear enlarged and rectangular in cross-sections as a consequence of a distal to proximal extension defect (Pelilikka et al., 2002) (Figure 2B), and eys mutant ommatidia lack an IRS (Husain et al., 2006; Zelhof et al., 2006) (Figure 2B).

To identify factors associated with apical trafficking we screened RNAi lines targeting genes known or predicted to encode vesicle trafficking proteins for defects similar to Rh1, Crb, or Eys reduction. The eye-specific driver GMR-Gal4 (Freeman 1996) was used to express UAS-Dicer2 (to amplify the effects of RNAi; Ketting et al., 2001) and a total of 309 RNAi lines corresponding to 237 genes (Table S1).

The first step in this screen was conducted using transmitted light illumination (TLI). TLI takes advantage of the fact that rhabdomeres act similar to optical fiber cables. When a beam of light is transmitted

Figure 2 RNAi-based screen of known and predicted trafficking factors in Drosophila PRCs. GMR-GAL4 was used to drive the expression of UAS-dicer-2 and UAS-RNAi constructs. UAS-dicer-2/+; pGMR-Gal4/+ was used as control. Scale bars, 5 μm. (A) Schematic of cross-section of PRCs in a Drosophila ommatidium. Apical membranes of PRCs are subdivided into the rhabdomere (RB) and stalk membrane (SM). The stalk membrane connects the rhabdomere to the zonula adherens (ZA). PRCs R1-R8 (R8 is found below R7) surround the interrhabdomeral space (IRS). (B) TEM images of a wild-type, Sec6-RNAi, crb-RNAi, and eys734 mutant ommatidium. (C) TLI images of control, Sec6-RNAi, crb-RNAi, and eys734 mutant ommatidium. (D) Summary of the TLI screen. (E) Distribution of Rh1, Crb, and Eys in ommatidia of control flies. (F) Summary of data obtained from Rh1, Crb, and Eys retinal immunostaining of 66 Class I and Class II candidates identified with TLI (see also Figures S1-S4). Knockdown of 3 genes changed the distribution of Rh1, knockdown of 2 genes changed the distribution of both Crb and Eys, knockdown of 9 genes changed the distribution of both Rh1 and Eys, and knockdown of 14 genes changed the distribution of Rh1, Crb, and Eys.
through the eye, individual rhabdomeres become visible, after the cornea has been optically neutralized in an appropriate medium (Franceschini and Kirschfeld, 1971a). Using TLI, 7 distinct rhabdomeres were visible in control flies (Figure 2C). Deviations from the control were categorized into 3 classes based on the severity and type of the defect. Mild to moderate rhabdomeral defects were categorized as class I. Here, one or more, but not all 7 rhabdomeres were distinguishable as individual entities. For example, the defect caused by Sec6 knockdown would be categorized as class I (Figure 2C). Ommatidia where rhabdomeres appeared as a single diffuse patch were categorized as class II, as seen for example with crb knockdown (Figure 2C). Rhabdomeres do exist in Crb deficient PRCs, but they transmit light poorly, likely as a result of their extension defect (Pellikka et al., 2002). Finally, a class III phenotype showed an eyes-like defect, where a single bright spot of light was visible per ommatidium (Figure 2C), as a result of a loss of the IRS (Husain et al., 2006; Zelhof et al., 2006).

Figure 2D summarizes the results of the TLI screen. Out of the 237 genes screened, we found that the knockdown of 68 genes produced a class I or II defect. We did not observe a class III defect. This suggested that among the trafficking genes tested, no factor exclusively

| Table 1 Trafficking genes affecting Rh1, Crb, and Eys distribution in PRCs |
| Cargo | Gene | CG # | RNAi line tested | Phenotype |
|-------|------|------|------------------|-----------|
| Rh1   | Bet3 | CG3911 | BDSC: 38302, VDRC: 21738 | Cytoplasmic Rh1 |
|       | CdgAPr | CG10538 | BDSC: 6437, 38279 | Cytoplasmic Rh1 |
|       | twf | CG3172 | BDSC: 35365, 57375, VDRC: 25817 | Cytoplasmic Rh1 |
| Crb & Eys | sqh | CG3595 | BDSC: 33892 & 32439 | Larger IRS, longer Crb stained membrane, rectangular rhabdomeres for some ommatidia |
| Eys & Rh1 | Rph | CG11556 | BDSC: 25950, VDRC: 52438 | Larger IRS, longer Crb stained membrane, rectangular rhabdomeres |
| Eys & Rh1 | Dhc64c | CG7507 | BDSC: 36698, 28749, 36583 | Increase in cytoplasmic Rh1, cytoplasmic Eys and decrease in IRS size |
| Khc | CG7765 | BDSC: 25898, 35409 | Cytoplasmic Rh1 and Eys |
| Rab2 | CG3269 | BDSC: 28701, 34922, VDRC: 34767 | Missing/smaller rhabdomeres and reduced IRS size |
| Exocyst | Exo70 | CG7127 | BDSC: 55234 | Increase in cytoplasmic Rh1, cytoplasmic Eys and decrease in IRS size |
|       | Exo84 | CG6095 | BDSC: 28712 |
|       | Sec10 | CG6159 | BDSC: 27483 |
|       | Sec15 | CG7034 | BDSC: 27499 |
|       | Sec5 | CG5341 | BDSC: 27526 VDRC: 105836, 22079 |
| Eys & Rh1 & Crb | Chc | CG9012 | BDSC: 27530, VDRC: 23666 | Reduced Crb, cytoplasmic Eys and Rh1 |
|       | aux | CG1107 | BDSC: 35310, 39017 | Reduced Crb, cytoplasmic Eys and Rh1 |
|       | shi | CG18102 | BDSC: 36921, 28513 | Cytoplasmic Eys, Crb, and Rh1 and retinal degeneration |
|       | TSG101 | CG9712 | BDSC: 35710, VDRC: 23944 | Cytoplasmic Rh1, smaller/missing rhabdomeres, smaller IRS and less Crb |
| Silh | CG3539 | BDSC: 34335, 50940 | Reduction of Rh1, Eys and Crb |
|       | Shrb | CG8055 | BDSC: 38305, VDRC: 106823 | Cytoplasmic Eys, Crb, and Rh1 and retinal degeneration |
| V-ATPase | Vha26 | CG1088 | BDSC: 38996 BDSC: 26290 | Cytoplasmic accumulation of Rh1, Crb and Eys |
|       | Vha100-1 | CG1709 | BDSC: 36795, 39334, 42597 | Cytoplasmic Rh1 and Eys and reduction in Crb levels |
|       | Klc | CG5433 | BDSC: 27299, VDRC: 330620 | Reduction in Crb, Eys and Rh1 |
|       | Rab1 | CG3320 | BDSC: 30518, 34832 | Many ommatidia disintegrated; less affected ommatidia had larger IRS, longer Crb stained stalks and degenerated rhabdomeres; reduced Rh1 at the rhabdomeres |
|       | Rab5 | CG3664 | BDSC: 32366, VDRC: 100300 | Ectopic rhabdomeres at the basolateral membrane. Basolateral Eys and Crb. Cytoplasmic Rh1 and Eys. |
|       | MyosinV | CG2146 | VDRC: 16902 BDSC: 55740 | Significant reduction in Crb, Eys and Rh1, rhabdomeres absent |
|       | Avl/Syx7 | CG5081 | BDSC: 29546, VDRC: 107264 | Larger IRS, basolateral Rh1, longer Crb stained stalks |
|       | Sec71 | CG7578 | BDSC: 32366, VDRC: 100300 | Significance in Crb, Eys and Rh1, rhabdomeres absent |
compromises Eys trafficking without affecting also other apical proteins. Additionally, 13 genotypes were associated with lethality, indicating a leaky expression of the RNAi construct in essential tissues.

RNAi knockdown causing a class I or II defect were further analyzed through immunostaining of retinas for Rh1, Crb, and Eys (Figure 2E). Two phenotypes that were not further analyzed were caused by Rub11 and Rbab21 knockdown as depletion of these genes led to fragile eye tissue that fell apart during dissection. Out of the 66 genes examined, we found that the knockdown of 28 genes changed the amount and/or localization of one or more apical proteins (Figure 2F and Table 1). 21 of our hits were confirmed with a second independent RNAi line. Seven genes were not tested with a second RNAi line as they encoded multiple components of two protein complexes (the exocyst and the V-ATPase) that were associated with similar defects in protein distribution (Tables 1 and S1). We did not find genes that have an exclusive effect on Eys or Crb. We also did not find a case where Crb and Rh1 trafficking was affected in the absence of changes to Eys.

We performed a more in-depth analysis of the defects caused by loss of Sec71 (Figure 3), exocyst (Figure 4), dynein (Figure 5) and Syx7/Avl (Figure 6). We selected these factors as their knockdown was associated with robust defects, and as they represented a component of the Golgi (Sec71), secretory (exocyst and dynein), and endocytic (Sxy7/Avl) pathways. Defects caused by the knockdown of other factors can be found in Figure S1 (for RNAi constructs affecting Rh1), Figure S2 (for RNAi constructs affecting Rh1 and Eys), Figure S3 (for RNAi constructs affecting Crb and Eys), and Figure S4 (for RNAi constructs affecting all three apical proteins).

Sec71 is required for biosynthetic traffic of both apical and basolateral proteins

The Arf GEF Sec71 is essential for the integrity of Golgi compartments in Drosophila daC sensory neurons (Wang et al., 2017). The function of Sec71 in PRCs was unknown. The expression of two different Sec71 RNAi lines led to a class II defect as seen with TLI (Figure 3A). We observed a robust reduction but normal distribution of Eys, Crb, and Rh1 (Figure 3B). Sec71 knockdown affected the integrity and survival of cells in the retina as ultrastructural analysis showed retinal holes, dying and/or absent PRCs, and small or missing rhabdomeres (Figure 3C).

A similar reduction in Eys, Crb, and Rh1 amounts were also observed in Rab1 deficient PRCs (Figure S4). Rab1 has been associated with the ER to Golgi trafficking of Rh1 (Satoh et al., 1997). Proper processing at the Golgi is thought to be important for protein stability, and disruption of factors required for ER to Golgi processing such as Sxy5 and Rab1 are known to cause an overall reduction in cargo levels (Satoh et al., 2016, Satoh et al., 1997). Therefore, the reduction of Eys, Crb, and Rh1 seen in Sec71 compromised PRCs may be due to a lack of Golgi processing, suggesting a role for Sec71 at or prior to the Golgi in the biosynthetic pathway.

This conclusion is further supported by our observations that also the basolateral protein Nerva (Nrv), a subunit of the Na+/K+ ATPase was reduced in Sec71 knockdown PRCs (Figure 3D). This suggests that Sec71 acts prior to the separation of apical and basolateral trafficking routes, which is thought to occur at or prior to the trans-Golgi network (TGN). Similar defects in apical and basolateral protein transport have been described for the Golgi-associated SNARE protein Sxy5 (Satoh et al. 2016). Taken together, our observations indicate that Sec71 contributes to the secretion of both apical and basolateral proteins in PRCs, suggesting a defect in Golgi processing (Figure 3E).

Sec71 is related to the human Arf GEF BIG1/BIG2 family (Christis and Munro 2012). BIG1 and BIG2 are associated with the TGN (Mansour et al., 1999; Yamaji et al., 2000; Shinotsuka et al., 2002; Zhao et al., 2002; Ishizaki et al., 2008), and the recycling endosomes (Shin et al., 2004; Shen et al., 2006; Ishizaki et al., et al., 2017). The function of Sec71 in Drosophila sensory neurons was investigated using RNAi knockdown (Wang et al., 2017). The role of Sec71 in Golgi function was elucidated through biochemical and ultrastructural analyses, leading to the conclusion that Sec71 acts prior to the Golgi in the biosynthetic pathway.
In *Drosophila* sensory neurons, Sec71 is mainly found at the TGN, and the Golgi is severely disrupted in Sec71-deficient cells (Wang et al., 2017). As an Arf GEF, Sec71 is likely to activate one or more resident Golgi Arfs in PRCs. Candidates to consider are Arf1, Arf4, and Arl1. Arf1 (Arf79F in *Drosophila*), is important in regulating trafficking at the Golgi apparatus (Rodrigues and Harris 2017). In sensory neurons, Sec71 activates Arf1, and the Golgi apparatus is disrupted in Arf1 mutant cells (Wang et al., 2017). Arf4 (Arf102F in *Drosophila*), is associated with the TGN and is important in targeting rhodopsin to the cilium of frog PRCs (Mazelova et al., 2009; Wang et al., 2012). Finally, Arl1 (Arf72A in *Drosophila*), localizes to the Golgi in *Drosophila* PRCs and is important in quality assurance of cargo proteins (Lee et al., 2011). Loss of Arf1 results in an increase of Golgi compartments and an acceleration of Rh1 secretion (Lee et al., 2011). In our TLI screen, Arf4 RNAi caused lethality, whereas the expression of Arl1 and Arf1 RNAi lines did not show an effect. This may be due to the inactivity/low activity of the RNAi lines used, or functional redundancies between Arfs in PRCs.

The exocyst complex contributes to both Rh1 and Eys secretion

Depletion of exocyst components Exo70, Exo84, Sec10, Sec15, Sec5 and Sec6 led to class I defects (see Figure 4A for Sec6 and Sec10 data). Rhabdomeral defects were expected as the exocyst is known to be important for the exocytosis of rhabdomeral proteins (Beronja et al., 2005). As previously described for Sec6 mutant PRCs (Beronja et al., 2005), we observed cytoplasmic accumulation of Rh1 in Sec6 and Sec10 deficient retinas. A total of 69 individual IRS were measured for control and 118 for Sec10 RNAi ommatidia, using three different animals per genotype. Values were normalized to the control. Error bars represent standard deviation. Unpaired non-parametric Mann-Whitney test. (F) Summary model indicating the secretion of Rh1 and Eys containing secretory vesicles depends on the exocyst. See Figure 1A for annotation and text for discussion.
reduced by ~20% (Figure 4E). As IRS size is dependent on Eys levels (Husain et al., 2006; Zelhof et al., 2006), it is likely that the IRS is reduced due to ineffective Eys trafficking. We did not observe a noticeable change in Crb (Figure 4C) or basolateral trafficking of Nrv (Figure 4D).

It was previously thought that Eys was secreted through the stalk membrane and would not be affected by defects in rhabdomeral trafficking (Husain et al., 2006). The different impact on Eys trafficking observed here compared to our previous analysis may be the result of the different strategies used to compromise exocyst function (RNAi used here vs. generation of Sec6 mutant clones combined with the expression of a partial rescue construct to avoid cell lethality caused by the loss of exocyst function; Beronja et al., 2005; Husain et al., 2006). We conclude that the exocyst complex, which is crucial for trafficking to the rhabdomere, is also important for Eys trafficking to the IRS (Figure 4F).

**Microtubule motor protein dynein is required for Rh1 and Eys secretion**

The function of dynein, a minus-end directed microtubule motor protein, has not been previously described in fly PRCs. We observed class I defects with three distinct RNAi lines targeting Dynein heavy chain 64C (Dhc64C) (Figure 5A). We observed abnormal cytoplasmic accumulation of Rh1 and Eys for all three RNAi lines (Figure 5C and D). The presence of cytoplasmic Eys partially overlapped with Rh1, suggesting that Eys and Rh1 are trapped in the same compartments. Cytoplasmic Eys was coupled with a reduction in IRS size (Figure 5F). We did not observe an effect on Crb (Figure 5D) or basolateral Nrv (Figure 5E), suggesting that trafficking to the stalk and the basolateral membrane was not affected.

Expanded cell bodies and an accumulation of cytoplasmic vesicles of various sizes are apparent in Dhc64C depleted PRCs (Figure 5B). These vesicles looked similar to the secretory vesicles described for PRCs...
compromised for the exocyst, Rab11, Rip11, or Myosin V (Satoh et al., 2005; Beronja et al., 2005; Li et al., 2007). Notably, we also observed similar defects with the microtubule plus-end directed kinesin motor. Depletion of Kinesin heavy chain (Khc) and Kinesin light chain (Klc) led to a Class I defect in TLI. Rh1 and Eys accumulated in the cytoplasm and IRS size was decreased (Figure S2 and S4). It is curious that the knockdown of both plus-end and minus-end directed motors caused similar Eys and Rh1 secretion defects.

Klc-deficient PRCs (but not Khc-deficient cells) also showed a reduction in Crb (Figure S4). Microtubules and microtubule-related proteins are known to be important for Crb trafficking in pupal PRCs (Mukhopadhyay et al., 2010; Chen et al., 2010; League and Nam 2011; Mui et al., 2011; Nam 2016). It is not clear whether this Crb defect is specific to Klc knockdown or if a stronger knockdown of Khc or Dhc64C would affect Crb in a similar manner.

Previous studies in the developing eye discs showed that the plus-end of microtubules are found at the apical cell membrane whereas the minus-ends are located in the vicinity of the cell nucleus (Mosley-Bishop et al. 1999). Microtubule orientation in adult PRCs is not known and would be important to address in future studies to help in the interpretation of defects resulting from knockdown of motor proteins. Vertebrate PRCs possess microtubules with opposing orientations (for review see Nemet et al., 2015) Rhodopsin is made in the inner segment (IS) of vertebrate PRCs and transported to the vertebrate equivalent of the rhabdomere, the outer segment (OS). Within the IS, microtubules are orientated with their plus-ends at the Golgi and minus-ends at the base of the OS. Microtubule orientation is reversed in the OS, where the minus-end is found at the base of the OS, while the plus-end is found at the distal end of the OS. It has been proposed that dynein is important in the trafficking of rhodopsin from Golgi to the OS, whereas kinesin is important in trafficking from the base of the OS to its tip.

Figure 6 Syx7/Avl deficient PRCs show enhanced surface levels of Rh1, Eys and Crb. RNAi line BDSC 29546 (Syx7-RNAi) was used to deplete Syx7/Avl. Syx7-RNAi was crossed with UAS-dicer-2; pGMR-Gal4. UAS-dicer-2/+; pGMR-Gal4/+ was used as control. Scale bars, 5 μm. (A) Individual rhabdomeres were partially visible in Syx7/Avl deficient retinas. We categorized this defect as Class I. (B) Knockdown of Syx7/Avl led to rhabdomeral fragmentation and degeneration. (C) Syx7/Avl deficient PRCs show increased apical and basolateral accumulation of Rh1. (D) IRS area stained with Eys and stalk membranes labeled with Crb are larger in Syx7/Avl deficient PRCs compared to the control. (E) Levels/localization of the basolateral protein Nrv (K+Na+ ATPase subunit) is normal in Syx7/Avl deficient PRCs. Acti-stain555 (F-actin) was used to visualize rhabdomeres which appear disorganized. (F) IRS size was significantly larger in Syx7/Avl deficient retinas compared to controls. A total of 69 individual IRS were measured for the control and 138 for Syx7/Avl knockdown PRCs using 3 different animals per genotype. Values were normalized to the control. Error bars represent standard deviation. Unpaired non-parametric Mann-Whitney test. (G) Stalk membranes were significantly larger in Syx7/Avl deficient PRCs compared to controls. A total of 182 individual stalk membranes were measured for the control and 126 for Syx7/Avl deficient knockdown PRCs. Error bars represent standard deviation. Unpaired non-parametric Mann-Whitney test. (H) Summary model indicating that the endocytosis of Rh1, Crb, and Eys depends on Syx7/Avl. See Figure 1A for annotation and text for discussion.
(Nemet et al., 2015). Loss of dynein or kinesin function may therefore lead to defects in rhodopsin trafficking.

Taken together, we found that microtubule motors, kinesin and dynein are essential in the secretion of Eys and Rh1. One important conclusion from our findings is that microtubule and actin-based transport mechanisms cooperate in the delivery of rhabdomer-targeted proteins. Previous studies had revealed the importance of actin filament-based routes and the actin motor protein Myosin V in rhabdomeral vesicle trafficking (Li, et al., 2007). It would be interesting to examine how microtubule and actin-based transport mechanisms interface to facilitate apical trafficking in Drosophila PRCs.

**Syntxin 7/Avalanche is required for Rh1, Eys, and Crb endocytosis**

Syx7/Avl is required for apical endocytosis in imaginal disc epithelia. Syx7/Avl deficient cells displayed an excessive accumulation of Crb at the apical membrane (Lu and Bilder 2005). Syx7/Avl co-localizes with Rab5 and is required for the entry of cargo proteins, such as Crb into early endosomes (Lu and Bilder 2005). Previous results suggested that endocytosis is essential for rhabdomer maintenance in Drosophila PRCs. Endocytosis is thought to be important in the proper formation of the interface between the rhabdome and the sub-rhabdomeric space where the rhabdome terminal web is located (Pinal and Pichaud 2011). Here, we found that knockdown of Syx7/Avl using two distinct RNAi lines led to a class I defect with TLI (Figure 6A), and consistent with earlier results indicating that endocytosis promotes rhabdome integrity, we observed structural defects in rhabdomeres of Syx7/Avl knockdown PRCs (Figure 6B and E).

Rh1, Crb, and Eys levels were increased in Syx7/Avl compromised PRCs whereas Nrv levels remained normal (Figure 6C, D and E). Corresponding to the increase in Eys (Zelhof et al., 2006) we found an enlarged IRS in Syx7/Avl knockdown PRCs (Figure 6F). Similarly, the overabundance of Crb in Syx7/Avl knockdown PRCs was associated with an enlarged stalk membrane (Figure 6G) as previously reported for PRCs that overexpress Crb (Pellikka et al., 2002). The apical accumulation of Eys and Crb as a result of compromised apical endocytosis suggests that both proteins similar as Rh1 undergo active turn-over in Drosophila PRCs.

Interestingly, Rh1 was increased not only at the apical rhabdomere but also at the basolateral membrane in Syx7/Avl knockdown PRCs, where in control PRCs only small amounts of Rh1 are found (Figure 6C). It is possible that an over-accumulation of Rh1 at the rhabdemes led to a leakage of Rh1 into the basolateral membrane. Alternatively, it is possible that at least some Rh1 may normally be transcytosed from the apical to the basolateral membrane. It is possible that an over-accumulation of Rh1 at the rhabdomeres led to an excessive accumulation of Crb at the rhabdomeres (Figure 6G) as previously reported for PRCs that overexpress Crb (Pellikka et al., 2002). The apical accumulation of Eys and Crb as a result of compromised apical endocytosis suggests that both proteins similar as Rh1 undergo active turn-over in Drosophila PRCs.

**Concluding remarks**

By exploring apical vesicle trafficking in the fly PRCs through an RNAi-based screen we have uncovered 28 genes involved in apical localization of the key PRC proteins Rh1, Crb, and Eys. We have shown that the Arf GEF Sec71 is essential for proper apical and basolateral protein trafficking, the exocyst complex and microtubule motors dynein and kinesin are important for Eys and Rh1 secretion, and the syntxin Syx7/Avl is involved in Crb, Eys, and Rh1 endocytosis. Our results have implications for the understanding of human eye diseases as mutations in human orthologs of Eys, Crb, and Rh1 cause eye degenerative diseases (Abd El-Aziz et al., 2008; Collin et al., 2008; Richard et al., 2006; den Hollander et al., 2008; Hollingsworth and Gross 2012). The rhabdomere and the stalk membrane in Drosophila correspond topologically and functionally to the vertebrate rod and cone outer segment and inner segment, respectively. It is likely that similar factors are involved in apical trafficking in Drosophila and vertebrate PRCs. One example is Rab1, which was shown to be important in post-Golgi Rh1 exocytosis in both Drosophila and vertebrates (Sato et al., 2005; Mazelova et al., 2009). It will therefore be of interest to further explore the conservation of vesicle trafficking mechanisms in Drosophila and mammalian PRCs.

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**LITERATURE CITED**

Abd El-Aziz, M. M., I. Barragan, C. A. O’Driscoll, L. Goodstadt, E. Pigrimge et al., 2008 EYS, encoding an ortholog of Drosophila spacemark, is mutated in autosomal recessive retinitis pigmentosa. Nat. Genet. 40: 1285–1287. https://doi.org/10.1038/ng.241

Alloway, P. G., L. Howard, and P. J. Dolph, 2000 The formation of stable rhodopsin-arrestin complexes induces apoptosis and photoreceptor cell degeneration. Neuron 28: 129–138. https://doi.org/10.1016/S0896-6273(00)00091-X

Beronja, S., P. Laprise, O. Papoulas, M. Pellikka, J. Sisson et al., 2005 Essential function of Drosophila Sertac in apical exocytosis of epithelial photoreceptor cells. J. Cell Biol. 169: 635–646. https://doi.org/10.1083/jcb.200410081

Bujakowska, K., I. Audo, S. Mohand-Saïd, M. E. Lancelot, A. Antonio et al., 2012 CRB1 mutations in inherited retinal dystrophies. Hum. Mutat. 33: 306–315. https://doi.org/10.1002/humu.21653

Chen, G., G. P. League, and S. C. Nam, 2010 Role of spastin in apical domain control along the rhabdomere elongation in Drosophila photoreceptor. PLoS One 5: e9480. https://doi.org/10.1371/journal.pone.0009480

Chinchore, Y., A. Mitra, and P. J. Dolph, 2009 Accumulation of rhodopsin in late endosomes triggers photoreceptor cell degeneration. PLoS Genet. 5: e1000377. https://doi.org/10.1371/journal.pgen.1000377

Christis, C., and S. Munro, 2012 The small G protein Arl1 directs the trans-Golgi-specific targeting of the Arf1 exchange factors BIG1 and BIG2. J. Cell Biol. 196: 327–335. https://doi.org/10.1083/jcb.201107115

Colley, N. J., J. A. Cassill, E. K. Baker, and C. S. Zuker, 1995 Defective intracellular transport is the molecular basis of rhodopsin-dependent dominant retinal degeneration. Proc. Natl. Acad. Sci. USA 92: 3070–3074. https://doi.org/10.1073/pnas.92.7.3070

Collin, R. W., K. W. Littink, B. J. Klevering, L. I. van den Born, R. K. Koenenkoop et al., 2008 Identification of a 2 Mb human ortholog of Drosophila eyes shut/spacemaker that is mutated in patients with retinitis pigmentosa. Am. J. Hum. Genet. 83: 594–603. https://doi.org/10.1016/j.ajhg.2008.10.014

den Hollander, A. I., J. B. ten Brink, Y. J. de Kok, S. van Soest, L. I. van den Born et al., 1999 Mutations in a human homologue of Drosophila crumbs cause retinitis pigmentosa (RP12). Nat. Genet. 23: 217–221. https://doi.org/10.1038/13848

den Hollander, A. I., R. Roepman, R. K. Koenenkoop, and F. P. Cremers, 2008 Leber congenital amaurosis: genes, proteins and disease mechanisms. Prog. Retin. Eye Res. 27: 391–419. https://doi.org/10.1016/j.preteyeres.2008.05.003

Franceschini, N., and K. Kirschfeld, 1971a In vivo optical study of photoreceptor elements in the compound eye of Drosophila. Kybernetik 8: 1–13. https://doi.org/10.1007/BF00270828
Wang, S., K. L. Tan, M. A. Agosto, B. Xiong, S. Yamamoto et al., 2014 The retromer complex is required for rhodopsin recycling and its loss leads to photoreceptor degeneration. PLoS Biol. 12: e1001847. https://doi.org/10.1371/journal.pbio.1001847

Wang, Y., H. Zhang, M. Shi, Y. C. Liou, L. Lu et al., 2017 Sec71 functions as a GEF for the small GTPase Arf1 to govern dendrite pruning of Drosophila sensory neurons. Development 144: 1851–1862. https://doi.org/10.1242/dev.146175

Xiong, B., and H. J. Bellen, 2013 Rhodopsin homeostasis and retinal degeneration: lessons from the fly. Trends Neurosci. 36: 652–660. https://doi.org/10.1016/j.tins.2013.08.003

Yamaji, R., R. Adamik, K. Takeda, A. Togawa, G. Pacheco-Rodriguez et al., 2000 Identification and localization of two brefeldin A-inhibited guanine nucleotide-exchange proteins for ADP-riboseylation factors in a macromolecular complex. Proc. Natl. Acad. Sci. USA 97: 2567–2572. https://doi.org/10.1073/pnas.97.6.2567

Zelhof, A., R. W. Hardy, A. Becker, and C. S. Zuker, 2006 Transforming the architecture of compound eyes. Nature 443: 696–699. https://doi.org/10.1038/nature05128

Zhao, X., T. K. Lasell, and P. Melançon, 2002 Localization of large ADP-riboseylation factor-guanine nucleotide exchange factors to different Golgi compartments: Evidence for distinct functions in protein traf c. Mol. Biol. Cell 13: 119–133. https://doi.org/10.1091/mbc.01-08-0420

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