Drosophila Vps16A is required for trafficking to lysosomes and biogenesis of pigment granules

Suprabha Pulipparacharuvil[1], Mohammed Ali Akbar[1], Sanchali Ray[1], Evgueny A. Sevrioukov[1], Adam S. Haberman[1], Jack Rohrer[2] and Helmut Krämer[1,*]

[1]Center for Basic Neuroscience, UT Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390-9111, USA
[2]University of Zürich, Institute of Physiology, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland
*Author for correspondence (e-mail: hkrame@mednet.swmed.edu)

Accepted 17 May 2005
J. Cell Sci. 118, 3663-3673 Published by The Company of Biologists 2005
doi:10.1242/jcs.02502

Summary

Mutations that disrupt trafficking to lysosomes and lysosome-related organelles cause multiple diseases, including Hermansky-Pudlak syndrome. The *Drosophila* eye is a model system for analyzing such mutations. The eye-color genes *carnation* and *deep orange* encode two subunits of the Vps-C protein complex required for endosomal trafficking and pigment-granule biogenesis. Here we demonstrate that *dVps16A* (CG8454) encodes another Vps-C subunit. Biochemical experiments revealed a specific interaction between the dVps16A C-terminus and the Sec1/Munc18 homolog Carnation but not its closest homolog, dVps33B. Instead, dVps33B interacted with a related protein, dVps16B (CG18112). Deep orange bound both Vps16 homologs. Like a *deep orange* null mutation, eye-specific RNAi-induced knockdown of dVps16A inhibited lysosomal delivery of internalized ligands and interfered with biogenesis of pigment granules. Ubiquitous knockdown of dVps16A was lethal. Together, these findings demonstrate that *Drosophila* Vps16A is essential for lysosomal trafficking. Furthermore, metazoans have two types of Vps-C complexes with non-redundant functions.

Key words: Endocytic trafficking, Vps-C genes, Pigment granules, Lysosome-related organelles, Autophagosomes

Introduction

Hermansky-Pudlak syndrome (HPS) is a genetic disorder caused by mutations that interfere with the biogenesis of lysosome-related organelles (Gahl et al., 1998). Defects in the generation of melanosomes are responsible for oculo-cutanous albinism in such patients (Nguyen et al., 2002; Nguyen and Wei, 2004). Prolonged bleeding times are due to defects in the biogenesis of platelet secretory granules (White, 1982). Furthermore, distinct steps in the biogenesis and secretion of granules in cytotoxic T-lymphocytes are altered by several HPS-related mutations (Stinchcombe et al., 2004).

At least 16 HPS-related genes have now been identified based on mutations in humans and mice (Huizing and Gahl, 2002; Li et al., 2004; Spritz and Oh, 1999). Most of these genes encode proteins that assemble into one of the three biogenesis of lysosome-related organelles complexes, BLOC-I, -II, and -III (Ciciotte et al., 2003; Gwynn et al., 2004; Li et al., 2003a; Martina et al., 2003; Nazarian et al., 2003; Starcevic and Dell'Angelica, 2004). In addition, the *HPS-2* gene encodes a subunit of the AP-3 adaptor complex (Dell'Angelica et al., 1999).

An additional set of proteins has been implicated in HPS-related functions by a point mutation in the murine *vps33A* gene that causes the HPS-like phenotypes observed in *buff* mutants (Suzuki et al., 2003). The yeast homolog of Buff is encoded by the vacuolar protein sorting (VPS) gene *Vps33/Pep14* (Banta et al., 1990; Subramanian et al., 2004). Vps33 proteins are members of the family of Sec1/Munc18 (SM) proteins, which are essential regulators of membrane fusion events (Jahn et al., 2003; Toonen and Verhage, 2003).

SM proteins have been shown to interact with SNARE proteins in three different ways. First, Munc18 binds directly to the closed form of Syntaxin I, which cannot participate in SNARE complex formation (Dulubova et al., 1999; Misura et al., 2000). Second, yeast Sec1p binds to the syntaxin homolog Sso1p only when Sso1p is complexed with other SNARE proteins (Carr et al., 1999). Third, Vps33p, as part of the Class C Vps protein complex, may only indirectly interact with the syntaxin homolog Vam3p (Dulubova et al., 2001; Sato et al., 2000).

The Class C Vps protein complex comprises four core subunits that originally were defined by mutations that severely disrupt vacuolar morphology (Raymond et al., 1992). In addition to Vps33p/Pep14p, the VPS-C complex comprises Vps18p/Pep3p, Vps11p/Pep5p and Vps16p (Rieder and Emr, 1997). This complex has been implicated in multiple membrane fusion events including fusion of endosomes with vacuoles (Rieder and Emr, 1997; Sato et al., 2000), early endosome fusions (Peterson and Emr, 2001; Richardson et al., 2004; Srivastava et al., 2000; Subramanian et al., 2004) and homotypic fusion between vacuoles (Seals et al., 2000). The Vps-C protein complex binds to Vps41p/Vam2p and Vps39/Vam6p, thus constituting the HOPS complex (for homotypic vacuole fusion and protein sorting), which acts as effector and regulator of the Rab7 homolog Ypt7 (Price et al., 2000; Wurmser et al., 2000).

Vps-C complexes have also been described in mammalian and *Drosophila* cells (Kim et al., 2001; Richardson et al., 2004;

Sevrioukov et al., 1999; Sriram et al., 2003). A fly homolog of Vps33p is encoded by the *carnation* (*car*) gene, and a homolog of *vps18* is encoded by *deep orange* (*dor*) (Sevrioukov et al., 1999; Shestopal et al., 1997). Furthermore, *light* encodes a homolog of Vps41p (Warner et al., 1998). Because mild loss-of-function alleles of these genes impair the biogenesis of pigment granules, these genes were originally discovered as eye-color mutants (Beadle and Ephrussi, 1936). Stronger phenotypes, including lethality, result either from genetic interactions of mild alleles, e.g. car^1 and $light^1$ (Nickla, 1977) or null alleles such as dor^8 (Shestopal et al., 1997). Such strong alleles revealed the additional roles of *dor* in membrane trafficking. For example, cells homozygous for dor^8 are impaired in the delivery of internalized ligands to lysosomes (Sevrioukov et al., 1999).

Here, we identify dVps16A as an additional subunit of the Dor/Car complex. RNAi-induced knockdown of dVps16A reveals phenotypes in lysosomal delivery and pigment granule biogenesis similar to those of a *dor* null allele. Furthermore, we demonstrate that a second Vps16 homolog, dVps16B, specifically interacts with a second Vps33p homolog, dVps33B. These results suggest the presence of two distinct Vps-C-like protein complexes in *Drosophila*.

Materials and Methods

Molecular biology

The cDNAs encoding dVps16A (GM04828), dVps16B (SD23896), dVps33B (LD23683) and ROP (Wu et al., 1998) were identified in the collection of *Drosophila* EST sequences (Rubin et al., 2000). Their sequences were identical to those predicted (Adams et al., 2000) with the exception of a 60 base pair (bp) exon [encoding a 20 amino acid (aa) insertion after aa 472] that was missing in the GM04828 cDNA. In the various dVps16A constructs listed below, amino acid numbers refer to the GM04828-encoded protein sequence. Expression vectors encoding epitope-tagged versions of full-length Car, Dor, dVps33B, Rop, dVps16B and dVps16A, or the truncations dVps16N1 (aa 2-224), dVps16ΔN (aa 260-813), dVps16M (aa 260-520), dVps16C (aa 489-813), and dVps16C2 (aa 643-813) dVps16C3 (aa 694-813), dVps16ΔC1 (aa 1-555), dVps16ΔC2 (aa 1-633), dVps16ΔC3 (aa 1-717) were generated using PCR and placed under control of the metallothionein promoter (Bunch et al., 1988) using standard molecular biology techniques (Ausubel et al., 1994).

Fly work

For expression in transgenic flies, cDNAs encoding the relevant proteins were cloned into the p{UASt} vector (Brand and Perrimon, 1993). The GFP-LAMP fusion protein is designed to encode a cleavable preprolactin signal sequence followed by eGFP and a transmembrane domain and cytoplasmic tail derived from human LAMP1 (accession number CAI13797). The LAMP1 cytoplasmic tail is sufficient to direct proteins to lysosomes (data not shown) (Rohrer et al., 1996). Flies carrying the P{UAS-Dor} construct have been described (Sevrioukov et al., 1999). RNAi constructs containing inverted repeats of about 300 bp of dVps16A cDNA were generated in pWIZ (Lee and Carthew, 2003). The chosen region did not contain any stretch of more than 12 bp of identity to the dVps16B cDNA. Transgenic flies were established using standard procedures (Rubin and Spradling, 1982). Expression was driven by P{GMR-Gal4} (Hay et al., 1994) P{ey-Gal4} (Hazelett et al., 1998) or P{da-gal4} (Wodarz et al., 1995), which were obtained from the Bloomington Stock center. To assess effects on pigment granules, expression in the eye was induced in a $white^+$ background.

Antibodies

To raise anti-dVps16A antibodies, a GST-fusion protein containing aa 663-833 of dVps16A, GST-dVps16C2, was expressed in bacteria, purified and injected into rabbits. Specific antibodies were affinity-purified from sera using the GST-dVps16C2 fusion proteins coupled to agarose beads (Affigel 10, Bio-Rad) as described (Harlow and Lane, 1988). An antibody recognizing the C-terminus of Dor (anti-DorY81) was raised in guinea pigs using a peptide encompassing aa 987-1002. For detection of endogenous proteins by western blots we used anti-dVps16A (1:3000), anti-Car (1:2000) (Sevrioukov et al., 1999), anti-DorY81 (1:1000), anti-HA 12CA5 (BABCO, 1:500), anti-Myc 9E10 (Sigma, 1:2000) anti-tubulin DM1A (Sigma, 1:3000), dSyn7-45 (1:5000) and anti-Hook (1:2000) (Krämer and Phistry, 1996).

Tissue culture and biochemistry

cDNAs encoding full-length or truncated tagged forms of Dor and Car (Sevrioukov et al., 1999), Rop (Wu et al., 1998) dVps16A, dVps16B, or dVps33B were transfected into S2 cells and expression was induced using 0.7 μM $CuSO_4$ (Krämer and Phistry, 1996). After 16 hours, S2-cell extracts were used for co-immunoprecipitations as described (Sevrioukov et al., 1999).

For analysis of endogenous proteins, *Drosophila* cytosol (~12 μg protein μl^{-1}) was prepared from S2 cells (Walenta et al., 2001). For sucrose density gradient centrifugation (10-20%), 50 μl cytosol was fractionated and sedimentation coefficients (S_{20w}) determined as described (Sevrioukov et al., 1999). Individual fractions were separated by SDS-PAGE and probed with the indicated antibodies. Extracts for developmental western blots were prepared from different developmental stages as described (Sevrioukov et al., 1999).

For western blots of dVps16A-RNAi larvae, the homozygous da-Gal4 driver was crossed to a line homozygous for the dVps16A-RNAi transgene. Larvae hemizygous for dor^8 were recognized by the absence of the GFP-tagged FM7 balancer (Casso et al., 2000).

Histology

Eye imaginal discs were stained as described using anti-Boss (Krämer et al., 1991) or anti-Sca1 antibodies (Lee et al., 1996). Alexa-488- or Alexa-568-conjugated secondary antibodies were detected with a Leica TCS SP2 confocal microscope with a 63× NA 1.32 lens. All digital images were imported into Photoshop and adjusted for gain and contrast.

For the quantification of LAMP1-GFP levels, third instar eye discs were stained with antibodies against Boss using Alexa-568-conjugated secondary antibodies and imaged on a Leica TCS SP2 confocal microscope as described above. A Z-stack of images 120 μm × 120 μm in size were obtained to a depth of about 10 μm, with images taken every 0.5 μm. Boss staining on the apical surface of R8 cells was used as a reference to ensure that images were collected at the same depth and distance from the morphogenetic furrow. Image stacks were imported into Volocity software for thresholding and quantification of GFP levels. The intensity of each LAMP1-GFP pixel was determined and the sum of pixel intensities in the Z-stack was determined to be the total GFP fluorescence for each sample. For electron microscopy, adult heads were fixed, embedded in plastic and sectioned as described (Van Vactor et al., 1991).

Results

Identification of two *Drosophila* Vps16 homologs

The *Drosophila* Vps-C proteins Dor and Car are subunits of a cytosolic complex of about 370 kDa (Fig. 1) (Sevrioukov et al., 1999). In yeast, Vps16p is a necessary subunit of the

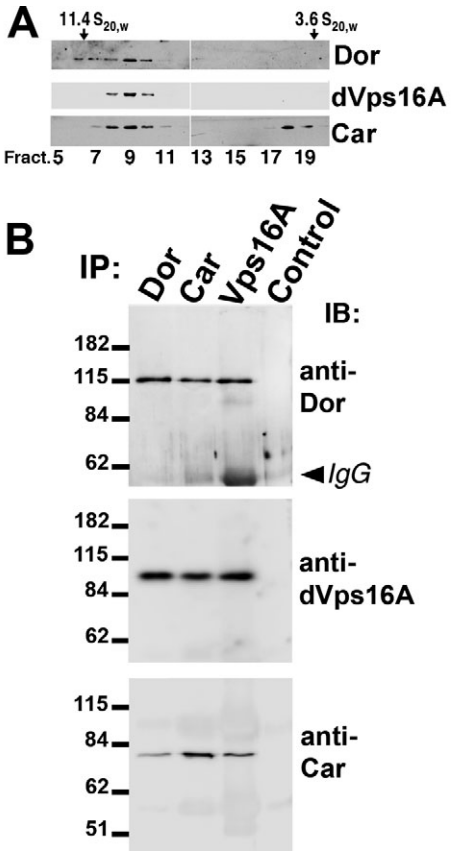

Fig. 1. dVps16A physically interacts with Dor and Car. (A) Cytosol from S2 cells was fractionated by sucrose density gradient centrifugation (10-20%). Proteins in each fraction were detected by western blotting with the indicated antibodies. Sedimentation coefficients (S_{20w}) are indicated for two markers. (B) Proteins were immunoprecipitated from S2-cell cytosol with anti-Dor, anti-Car, anti-dVps16A, or anti-Boss antibodies as controls. Immunoprecipitates were detected on western blots with the indicated antibodies to Dor, dVps16 or Car.

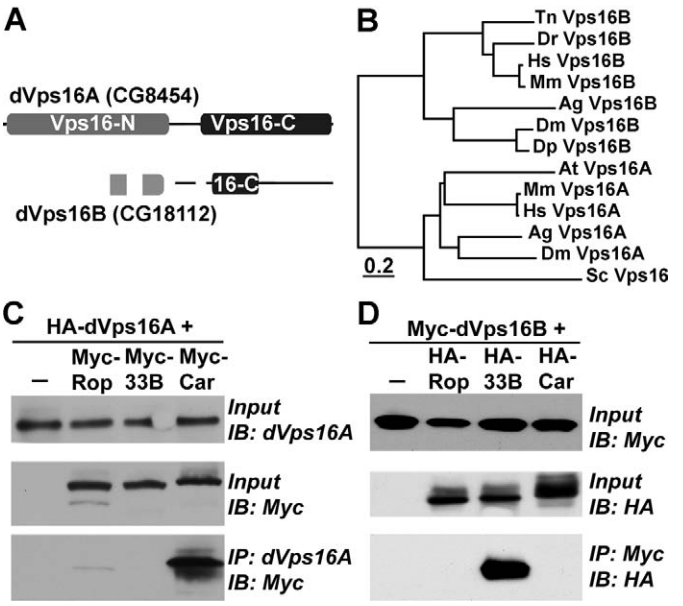

Fig. 2. dVps16A and dVps16B bind different Vps33 homologs. (A) The domain of highest similarity between the two Vps16 homologs in the *Drosophila* genome is the Vps16-C domain [Pfam04840 in the Conserved Domain Database (Marchler-Bauer et al., 2005)]. (B) Sequences of Vps16 proteins were aligned using ClustalW and a phylogenetic tree was constructed using 1000 bootstraps. Accession numbers: *Tetraodon nigroviridis* Vps16B (TnVps16: CAF92974), zebrafish Vps16B (DrVps16B: AAQ94572.1), human Vps16s (Hsvps16A: AAG34678.1 and HsVps16B: AAD09624.1) and mouse Vps16s (MmVps16A: AAH25626.1 and MmVps16B: BAC32680.1), *Anopheles gambia* (AgVps16A: XP_310557.1 and AgVps16B: XP_321923.1), *Drosophila melanogaster* (DmVps16A: NP_649877.1 and DmVps16B: AAF56946.1) *Drosophila pseudoobscura* (DpVps16B: EAL28124.1), *Arabidopsis thaliana* VACUOLELESS1 (AtVps16: AAM98105.1) and *Saccharomyces cervisiae* Vps16p (ScVps16p: NP_015280.1). (C) Myc-tagged Rop, dVps33B or Car proteins were co-expressed in S2 cells with HA-tagged dVsp16A and detected in input samples by immunoblots (IB) with anti-dVps16A or anti-Myc antibodies. Proteins were immunoprecipitated with anti-dVps16A antibodies. Immunoprecipitated proteins (IP) were detected with anti-Myc antibodies. (D) HA-tagged Rop, dVps33B or Car proteins were co-expressed in S2 cells with a Myc-tagged dVsp16A and detected in input samples by immunoblots (IB) with anti-HA or anti-Myc antibodies. Proteins were immunoprecipitated with anti-Myc antibodies. Immunoprecipitated proteins (IP) were detected with anti-HA antibodies.

corresponding Vps-C complex (Horazdovsky and Emr, 1993; Sato et al., 2000). Two *Drosophila* transcription units, CG8454 and CG18112 (Fig. 2A,B), exhibit significant similarity to the yeast and mammalian Vps16 proteins (Horazdovsky and Emr, 1993; Huizing et al., 2001; Kim et al., 2001). The 813 amino acid (aa) protein encoded by CG8454 shares 35% identity with the human Vps16 protein and 21% with *Saccharomyces cerevisiae* Vps16p. A more distant relationship characterizes CG18112, which encodes a 447 aa protein that displayed 24% identity with human Vps16 and 20% with yeast Vps16p. The domain best conserved between these proteins is the C-terminal Vps16-C domain (Fig. 2A). Based on this similarity and the functional characterization described below, we named these genes *dVps16A* and *dVps16B*.

dVps16A is part of the Vps-C complex and binds to Car and Dor

When S2 cytosol was separated in a 10-20% sucrose density gradient, the majority of dVps16A was found in the same fractions in which the level of Dor and Car proteins peaked (Fig. 1A). To test for a physical interaction between dVps16A and the Dor/Car complex we used co-immunoprecipitation of endogenous proteins. Antibodies against Dor, Car and dVps16A, or control antibodies, were used to immunoprecipitate proteins from *Drosophila* S2 cell cytosol. Immunoprecipitates were separated by SDS-PAGE and probed for the presence of the three proteins for which antibodies are available: Dor, Car and dVps16A. Antibodies against each of these proteins, but not control antibodies, precipitated each of the three proteins (Fig. 1B) consistent with the model that the *Drosophila* homologs of the C-class Vps proteins form a stable complex.

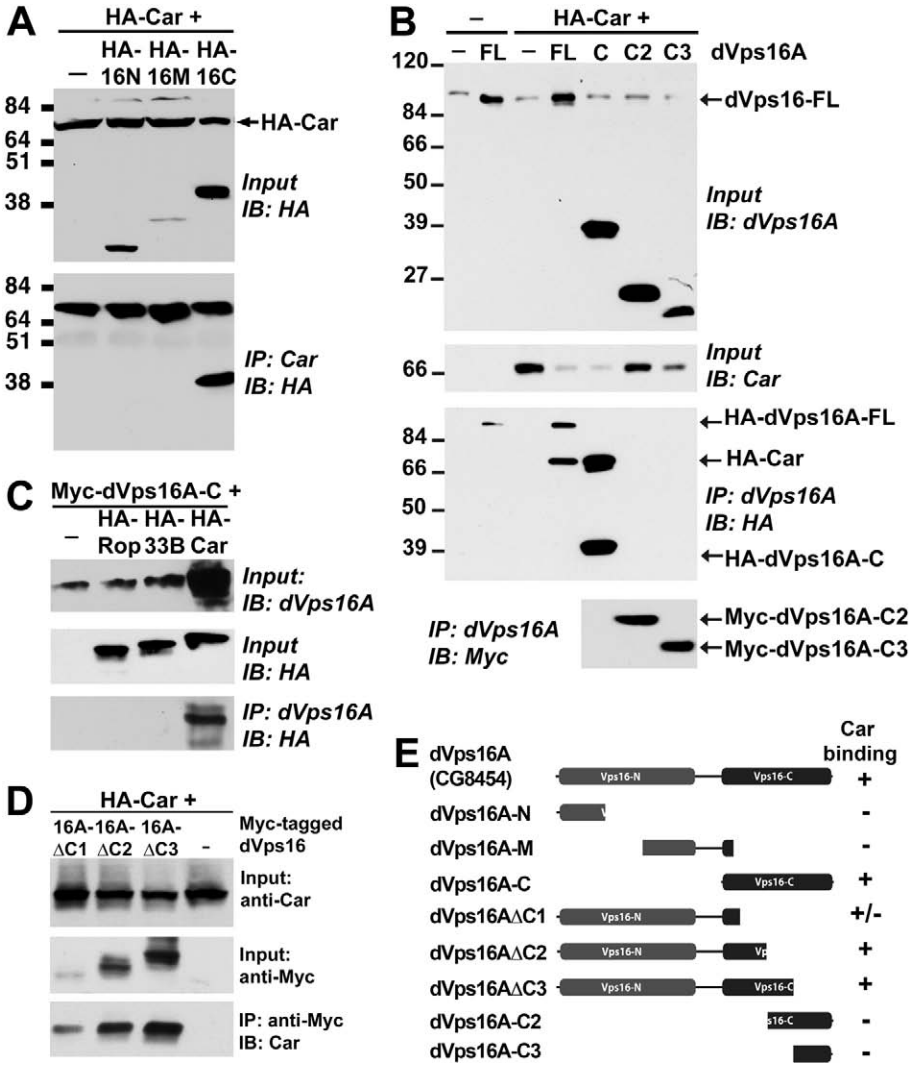

Fig. 3. A small domain in dVps16A is required for binding to Car. The binding of different dVps16A truncations (outlined in panel E) to Car was evaluated by co-immunoprecipitation experiments. (A) HA-tagged truncations of dVps16A were co-expressed with HA-Car and in whole cell extracts (input) detected with HA antibodies. After IP with anti-Car antibodies only dVps16A-C was co-immunoprecipitated. (B) HA- or Myc-tagged truncations of dVps16A were co-expressed with HA-Car protein and detected in input samples with anti-Vps16 antibodies. After IP with anti-dVps16A antibodies, HA-Car was co-immunoprecipitated only with full-length dVps16A or dVps16A-C. (C) HA-tagged Rop, dVps33B or Car were co-expressed in S2 cells with the Myc-tagged dVsp16A-C domain and detected in input samples with anti-dVps16A or anti-HA antibodies. After IP with anti-dVsp16A antibodies, proteins were detected with anti-HA antibodies. (D) Myc-tagged truncations of dVps16 were co-expressed with HA-Car. After IP with anti-Myc antibodies co-precipitated Car was detected with anti-Car antibodies. (E) The summary of the co-immunoprecipitation results indicates that only a small domain of dVps16A from aa 489-555 is required for binding to Car.

dVps16A and dVps16B bind distinct Vps33 homologs

We compared the biochemical interactions of the two Vps16 homologs with subunits of the Vps-C complex using co-immunoprecipitation experiments after co-expression with epitope-tagged subunits of the Dor/Car complex. First, dVps16A was co-expressed with epitope-tagged Car or the SM proteins dVps33B and Rop, the *Drosophila* ortholog of Munc18 (Wu et al., 1998). Upon pull-down of the Myc-tagged SM proteins, we found that only Car exhibited significant binding to dVps16A (Fig. 2C). By contrast, in corresponding experiments dVps16B specifically co-immunoprecipitated with dVps33B but not Car or Rop (Fig. 2D). An interaction between dVps16B and dVps33B has also been observed in a genome-wide two-hybrid analysis (Giot et al., 2003).

Co-immunoprecipitation experiments between Car and different dVps16A truncations were used to define the domain within dVps16A that is responsible for its binding to Car (Fig. 3). After immunoprecipitation of Car from co-transfected cells, the C-terminal domain of dVps16A was pulled down, but not the N-terminus or the well-conserved central part (Fig. 3A). We noted that in these experiments that the C-terminal domain was consistently higher expressed than other truncations; however, even 10-fold longer exposure did not detect Vps16A-N or -M in the immunoprecipitates (data not shown). Because in these experiments Car and the respective dVps16A truncations were both HA-tagged, the levels of co-immunoprecipitated proteins could be directly compared. Full-length dVps16A as well as the dVps16A-C domain bound Car in a ratio close to 1:1, indicating this is a direct interaction between the over-expressed proteins (Fig. 3A,B). The specificity of the interaction with SM proteins is encoded within the C-terminal domain of dVps16A, which only pulled down Car but not dVps33B or Rop (Fig. 3C). In these co-expression experiments, we consistently noticed that the level of dVps16A-C in the cytosol was increased in response to the co-expression of Car but not other SM proteins (Fig. 3C). This adds further weight to the notion that the Vps16-C domain determines the specific interaction of Vps16 proteins with SM protein family members. Further truncation analysis indicated that aa 489-555 of dVps16A are necessary for its binding to Car (Fig. 3B,D,E).

We also probed the interaction between Dor and the Vps16 homologs using co-immunoprecipitation of epitope-tagged Vps-C subunits. Western blots of immunoprecipitates revealed that Myc-tagged Dor pulled down full-length dVps16A or its C-terminal domain, dVps16A-C (Fig. 4A). C-terminal truncations narrowed the region of dVps16A that is required for binding to Dor at aa 489-633 (Fig. 4B,C). This region is partially overlapping with the Car-binding domain, but the presence of all three endogenous proteins in a single

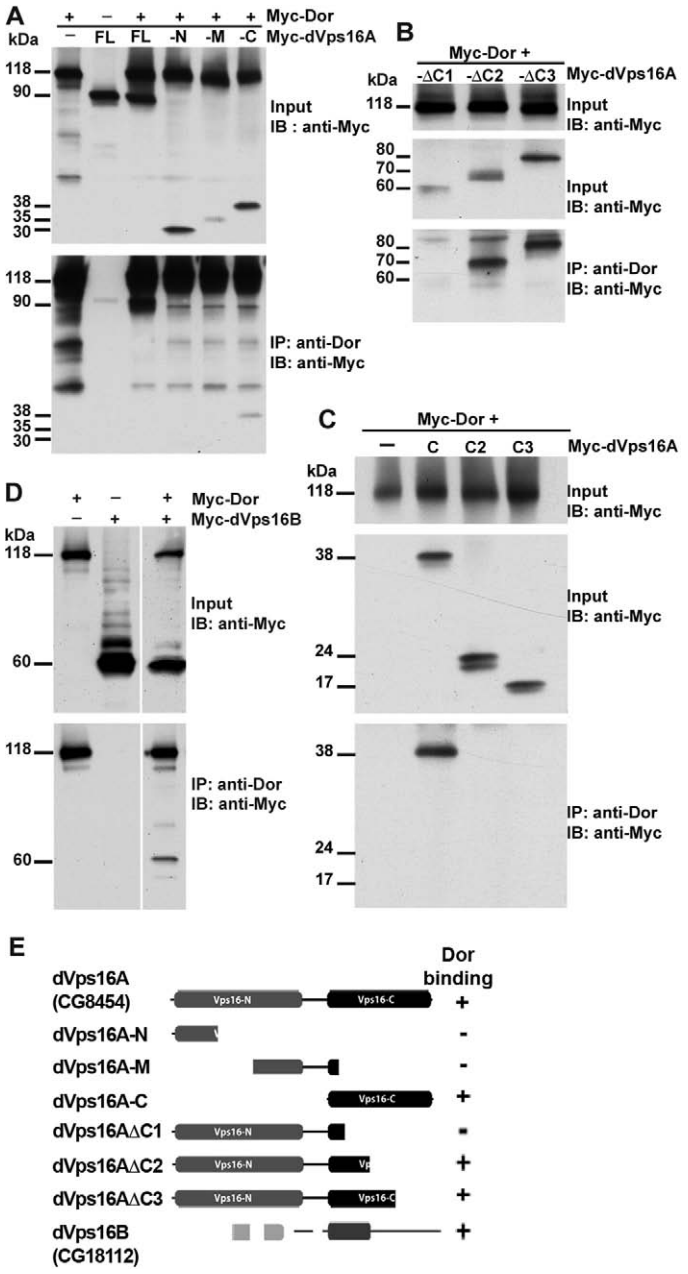

Fig. 4. dVps16 proteins bind to Dor. The binding of different dVps16A truncations (outlined in panel E) to Dor was evaluated by co-immunoprecipitation experiments. (A) Myc-tagged full-length or truncated dVps16A proteins were co-expressed with Myc-Dor and detected with Myc antibodies in whole cell extracts (Input). After IP with anti-Dor antibodies, only the co-immunoprecipitated full-length dVps16A protein (FL) or the C-terminal domain (-C) were detected by anti-Myc antibodies. (B,C) Myc-tagged truncated dVps16A proteins were co-expressed with Myc-Dor and in input samples detected with Myc antibodies. (B) After IP with anti-Dor antibodies, only dVps16A-ΔC2 (-ΔC2) and dVps16A-ΔC3 (-ΔC3) were co-immunoprecipitated as detected by anti-Myc antibodies, but not the shorter dVps16A-ΔC1 (-ΔC1). (C) After IP with anti-Dor antibodies, only the dVps16A-C (C) truncation co-immunoprecipitated, but not the shorter dVps16A-C2 or -C3 peptides. (D) Myc-tagged Dor and dVps16B were expressed alone or together in S2 cells. After IP with anti-Dor antibodies, dVps16B was only pulled down when co-expressed with Dor protein. (E) The summary of the co-immunoprecipitation results indicates that a small domain of dVps16A from amino acid 489 to 633 is required for binding to Dor.

complex (Fig. 1) suggests that these interactions are not competitive.

Interestingly, the Dor-binding domain of dVps16A is the most similar to dVps16B. We therefore tested its interaction with Dor and found that after co-expression in S2 cells Dor pulled down dVps16B (Fig. 4D). Together, these experiments suggested that the two distinct Vps-C complexes in *Drosophila* use Dor as a subunit.

dVps16A knockdown causes loss of Dor

Because no mutation has yet been identified in the *dVps16A* gene, we used RNAi to analyze its loss-of-function phenotypes. Two snap-back dVps16A-RNAi constructs were placed under UAS control. One of these constructs was efficient in knocking-down dVps16A after expression in transgenic flies and is further characterized below. In wild-type *Drosophila* tissue, affinity-purified anti-dVpsd16A antibodies recognized a major band of about 100 kDa (Fig. 5A), close to the predicted mass of dVps16A. This band was not detected in extracts from larvae in which the dVps16A-RNAi transgene was ubiquitously expressed using the da-Gal4 driver (Fig. 5B). These larvae developed into pupae but no flies emerged, indicating that *dVps16A* is an essential gene. No changes in the levels of Car, dSyntaxin-7 or tubulin proteins were detected in larvae and pupae after dVps16A knockdown, but the level of Dor protein was dramatically reduced (Fig. 5B).

We therefore wondered whether the reciprocal is true, and dVps16A is destabilized in *dor* mutants. We found no appreciable decrease in dVps16A in adult flies homozygous for the mild dor^1 and dor^4 alleles, despite the noticeable reduction in their Dor protein levels (Fig. 5C). However, in 4-day-old larvae that were hemizygous for the lethal dor^8 allele we observed a significant reduction of dVps16A. This effect was specific, as no changes were seen for several other proteins tested (Fig. 5D). Thus, Dor and dVps16A stability depend on each other's presence, whereas Car remains stable in the absence of the Vps-C complex. This is consistent with our observations that the majority of cytosolic Dor and dVps16A protein is found in high molecular mass complexes, whereas a significant amount of Car is detected in fractions that may represent unbound protein (Fig. 1A) (Sevrioukov et al., 1999).

dVps16A is required for the formation of the compound eye

To analyze the cellular consequences of loss of dVps16A function, we focused on the compound eye. Eye-specific expression of the dVps16A-RNAi construct was induced with the combined ey-Gal4 (Hazelett et al., 1998) and GMR-Gal4 drivers (Hay et al., 1994), which initiate expression early in eye development and maintain high levels in the adult eye. Externally, the most obvious change in response to dVps16A knockdown was a dramatic reduction of eye pigmentation compared with wild-type flies (Fig. 6A-C). The resulting eye

color exhibited a gradient with the most anterior ommatidia having lost most of their pigmentation (Fig. 6B). This gradient reflects the expression pattern of the ey-Gal4 driver, as we consistently see such gradients with this driver using different RNAi transgenes (T. Endicott and H.K., unpublished). The eye-color phenotype of dVps16A knockdown flies was not rescued by over-expressing Dor or Car protein using the uas/Gal4 systems with the combined ey-Gal4 and GMR-Gal4 drivers (data not shown).

Thin sections revealed strong retinal degeneration after eye-specific dVps16A knockdown. All cell types analyzed showed accumulation of autophagosomes (Fig. 7), which are characterized by the limiting double membrane and the presence of internal cytosol and organelles such as mitochondria and rough endoplasmic reticulum (e.g. inset in Fig. 7D). Because autophagosomes fuse with lysosomes to initiate the digestion of their content (Klionsky, 2005), the massive accumulation that we observe upon dVps16A knockdown is consistent with a requirement for dVps16A in lysosome-autophagosome fusion.

Different cell types exhibited additional specific defects. Lenses overlaying each ommatidium ('L' in Fig. 6D and Fig. 7A,B) appeared correctly formed, but the pseudocones just below them ('P' in Fig. 6D and Fig. 7A,B) were essentially absent after dVps16A knockdown (Fig. 6E and Fig. 7B). Pseudocones contain a transparent fibrous material that is secreted by cone cells (Cagan and Ready, 1989). After dVps16A knockdown, the remnants of the pseudocones were surrounded by a dense structure (arrow in Fig. 6E) that EM revealed to be composed of vesicles and vacuolar structures containing internal vesicles (Fig. 7B). Furthermore, the smaller type I pigment granules normally found in primary pigment cells (PPC in Fig. 7A) as well as the larger type II pigment granules characteristic for secondary pigment cells (SPC) were absent (Fig. 7B,G). The accumulation of autophagosomes in pigment cells paralleled their expansion from the typical width of 1-2 μm to often more than 5 μm (Fig. 7G).

In plastic sections, photoreceptor cells are easily identified based on their darkly stained rhabdomeres (arrowheads in Fig.

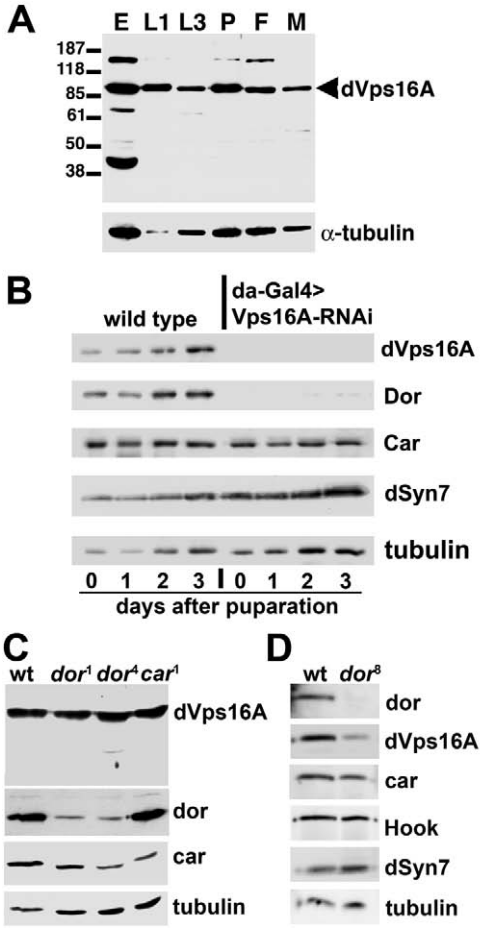

Fig. 5. RNAi-induced knockdown of dVps16A. (A) dVps16A is expressed throughout development. Extracts for western blotting were prepared from embryos (E), first and third instar larvae (L1 and L3), pupae (P) and adult females (F) and males (M). (B) Western blots of extracts from wild-type or dVps16A RNAi knockdown larvae (0) or 1-, 2- and 3-day-old pupae (1, 2 and 3). In addition to dVps16A, Dor was drastically reduced after dVps16A knockdown, but Car, dSyntaxin7, and tubulin levels were unchanged. (C) Western blots of extracts from adult wild-type, *dor*^{1}, *dor*^{4} or *car*^{1} flies did not show a reduction in dVps16A, although Dor protein levels were reduced in the *dor* alleles. (D) Western blots from extracts of day-four wild-type or *dor*^{8} third instar larvae show the loss of Dor and the reduced expression of dVps16A whereas Car, Hook, dSyn7 or tubulin were unchanged.

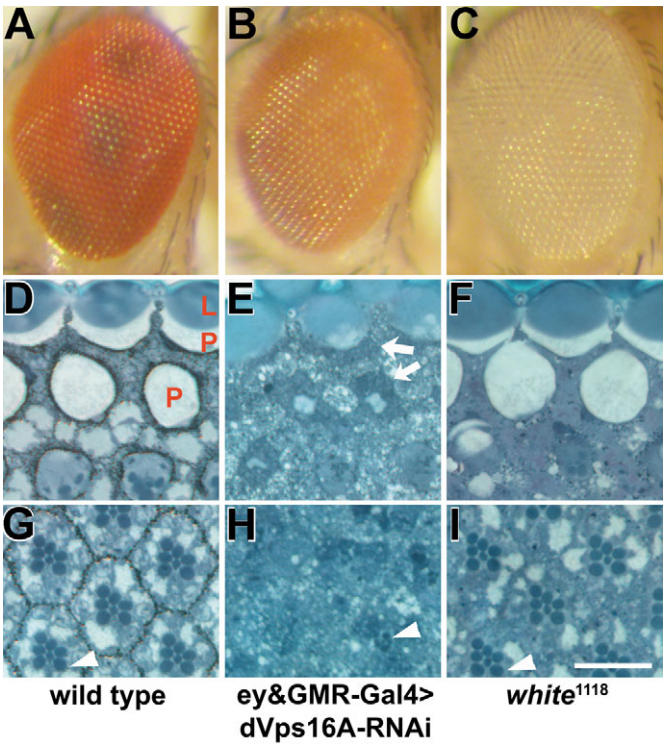

Fig. 6. dVps16C is required for eye development. (A-C) Micrographs of compound eyes from wild type (A), ey-Gal4&GMR-Gal4>dVps16A-RNAi (B), or *white*^{1118} flies (C). (D-I) Micrograph of sections of plastic embedded wild type (D,G), or ey-Gal4&GMR-Gal4>dVps16A-RNAi (E,H) or *white*^{1118} eyes (F,I). Lenses (L) and pseudocones (P) are indicated in (D). Arrows in E point to the dense material next to a malformed pseudocone. Arrowheads in panels G-I point to wild-type (G,I) or degenerate (H) rhabdomeres. Scale bar in I represents 15 μm in panels D-I.

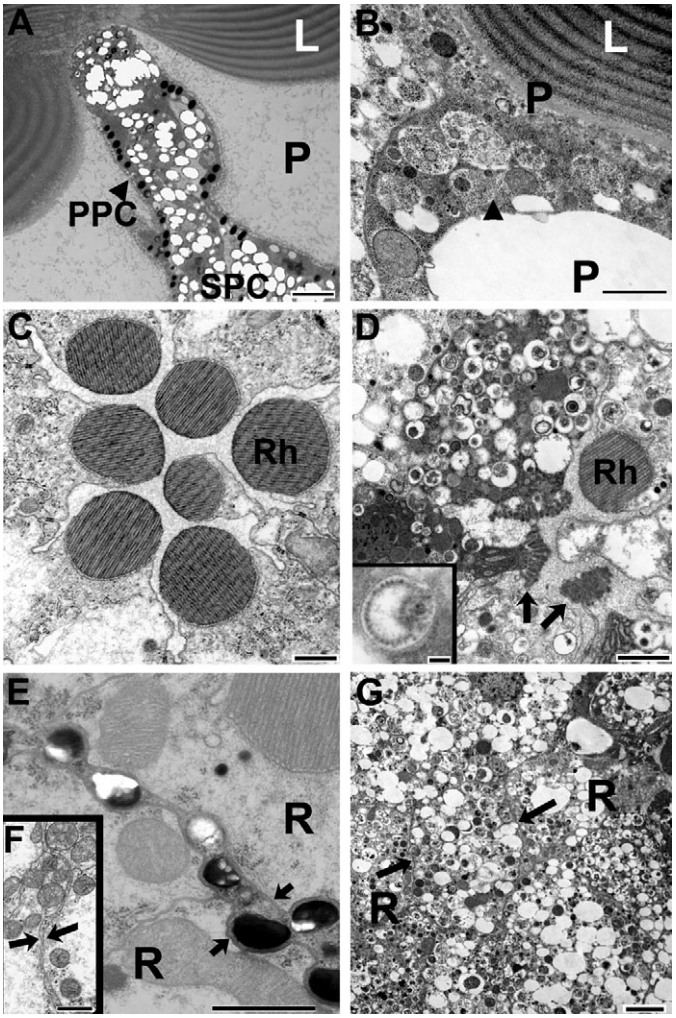

Fig. 7. Ultrastructural defects in compound eyes lacking dVps16A. Electron micrographs show details of compound eyes from wild type (A,E), *white*^{1118} (C,F) or ey-Gal4 and GMR-Gal4>dVps16A-RNAi flies (B,D,G). Lenses (L) and pseudocones (P) are indicated in A and B. Primary pigment cells (PPC) face the pseudocones and contain type I granules, which appear electron dense. The larger type II granules are predominant in secondary pigment cells (SPC); they appear translucent because their content of pigments were washed out during the embedding process (A). Both types of pigment granules are absent after dVps16A knockdown (B). Rhabdomeres (Rh) contain the phototransduction cascade and are composed of tightly packed microvilli (C). After dVps16A knockdown (D), most rhabdomeres are only detected as remnants (arrows in D) in degenerating photoreceptors cells full of vacuoles and autophagosomes. The inset in D shows an example of an autophagosome with an internal piece of rough endoplasmic reticulum. (E-G) Photoreceptor cells (R) of neighboring ommatidia are separated by a layer of pigment cells. In wild-type eyes (E), the width of pigment cells (arrows) between photoreceptor cells (R) is dominated by type II pigment granules. These granules are essentially absent in *white*^{1118} mutant eyes (F) resulting in a thin layer of pigment cells (between the arrows in F). After dVps16A knockdown (G), pigment cells are full of vacuoles and autophagosomes resulting in expanded width of the pigment cell layer (indicated between the arrows in G). Photoreceptors (R) in panel G were recognized by the remnants of rhabdomeres outside the field shown. Scale bar: 2 μm in A and G, 1 μm in B-F, and 100 nm for the inset in D.

6G-I). Upon dVps16A knockdown, however, most photoreceptor cells contained only remnants of rhabdomeres (e.g. arrowhead in Fig. 6H and arrows in Fig. 7D). The loss of rhabdomeres was accompanied by the accumulation of autophagosomes and vacuoles. Together the different ultrastructural phenotypes support a requirement for dVps16A in the trafficking to lysosomes and pigment granules.

dVps16A is required for lysosomal delivery

Because the retinal degeneration detected in the adult compound eyes might be an indirect consequence of defects in endocytosis or secretion we looked earlier in development for defects in either pathway. For this purpose, we analyzed the trafficking of two ligands, Boss and Scabrous, in the developing eye disc. Boss is targeted to the apical surface of R8 cells and, upon binding to the sevenless receptor, internalized into R7 cells (Fig. 7A) (Krämer et al., 1991). The pattern of Boss expression on the R8 cell surface is diagnostic for several developmental decisions in eye development (e.g. Van Vactor et al., 1991). Vps16A knockdown did not change this pattern indicating that R8 cell specification was not affected.

The level of Boss protein detectable in R7 cells is sensitive to mutations that perturb endocytic trafficking (Chang et al., 2002; Sevrioukov et al., 1999; Sunio et al., 1999). After dVps16A knockdown using the da-Gal4 driver, the level of Boss expressed on the R8 cell surface was not significantly altered, indicating no change in secretion. By contrast, the level of Boss protein in R7 cells was increased (Fig. 8A,B), consistent with an inhibition of lysosomal delivery.

This effect was even more pronounced with the Scabrous ligand. Scabrous is expressed in pro-neural clusters in the morphogenetic furrow and accumulates in endosomes before being degraded (Li et al., 2003b). As a consequence, very little Scabrous protein can be detected in wild-type ommatidia two or three rows posterior to the furrow, which corresponds to 4-6 hours after the initial burst of Scabrous expression (Fig. 8C). After dVps16A knockdown, the level of Scabrous protein remained high far posterior to the morphogenetic furrow (Fig. 8D). These data are consistent with a requirement for dVps16A in lysosomal delivery, but not protein targeting to the apical surface of cells in the developing eye.

We also tested the effect of dVps16A knockdown on lysosomal trafficking using a GFP-LAMP fusion protein. In *Drosophila* cells, the LAMP1-derived cytoplasmic tail is sufficient to target this fusion protein from the Golgi to late endosomes and lysosomes, where hydrolases degrade GFP (data not shown) (Rohrer et al., 1996). Thus, GFP-LAMP did not accumulate to high levels in wild-type eye discs (Fig. 8E). By contrast, massive accumulation of GFP-LAMP in dVps16A knockdown flies was observed. Quantification revealed a more than 12-fold increase in average GFP-LAMP fluorescence after dVps16A knockdown ($12.4\times10^{7}\pm5.5$; n=5) compared with wild-type controls ($1.0\times10^{7}\pm0.4$; n=3). Because the number of GFP-positive vesicles was at least threefold increased this is likely to reflect the accumulation of endosomal intermediates due to the inhibited fusion with lysosomes and earlier intermediates in the pathway. This demonstrated that delivery to lysosomes is inhibited for Golgi-derived biosynthetic cargo, along with the internalized ligands described above.

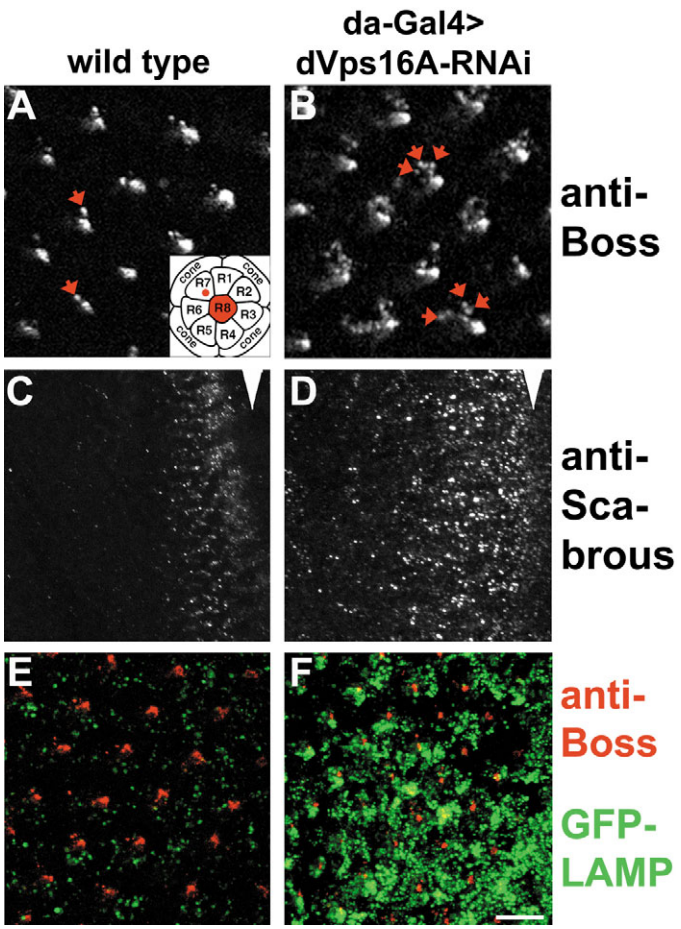

Fig. 8. Cells lacking dVps16A are deficient in lysosomal delivery. Eye discs from wild-type (A,C) or dVps16A knockdown (B,D) third instar larvae were stained for Boss (A,B) or Scabrous (C,D). As indicated in the inset in panel A, the large dots of Boss staining represent its expression on the apical surface of R8 cells in each ommatidium whereas the small dots next to it (arrows in A and B) show the ligand after its internalization into the neighboring R7 cells (Cagan et al., 1992). Note that, after dVps16A knockdown, R8 surface levels are unchanged whereas the level of Boss staining in R7 cells is increased. Similarly, posterior to the furrow (arrowhead in panel C and D), Scabrous protein is rapidly degraded in wild-type cells (C) but after dVps16A knockdown Scabrous levels remain high (D). (E) In wild-type eye discs, a GFP-LAMP fusion protein is targeted to lysosomes and efficiently degraded. (F) After dVps16A knockdown, this degradation is inhibited and GFP-LAMP accumulates in vesicles. All images are projections of confocal z-series. Posterior is to the left. Scale bar: in F is 7 μm in A and B, 20 μm in C and D, and 10 μm in E and F.

Discussion

Vps16 was originally discovered in yeast as one of the four class C *vps* genes whose disruption results in the absence of a morphologically recognizable vacuole (Horazdovsky and Emr, 1993; Robinson et al., 1988). The four class C vps proteins form a complex that is necessary for the membrane fusion in early endosomes (Peterson and Emr, 2001; Srivastava et al., 2000; Subramanian et al., 2004), as well as fusion of endosomal intermediates and autophagosomes with the vacuole (Rieder and Emr, 1997; Seals et al., 2000; Wurmser et al., 2000). Within this complex, Vps16p interacts directly with Vps18p/Pep3p and the SM protein Vps33p/Pep14p (Sato et al., 2000). Here, we demonstrate that the *Drosophila* genome encodes two Vps16 homologs: dVps16B (CG18112) binds specifically to dVps33b, but not to Car. By contrast, dVps16A (CG8454) binds specifically to Car, but not dVps33B.

Most phenotypes after dVps16A knockdown were similar to those observed for *dor* mutations, which is consistent with the loss of Dor protein after dVps16A knockdown (Fig. 5). Both cause deficits in lysosomal delivery as well as eye-color defects due the loss of pigment granules. Additionally, we observed a loss of pseudocones, whose gelatinous content is secreted by cone cells starting ~110 hours after pupariation (Cagan and Ready, 1989). Although it is possible that the loss of pseudocones indicates a primary role of dVps16A in secretion, it is likely that this secretory defect is a secondary consequence of the massive changes we notice in cells after dVps16A knockdown (Fig. 7). For example, the lenses overlaying each ommatidium are secreted by cone cells and primary pigment cells about 1 day before the pseudocones (~70 hours after pupariation) (Cagan and Ready, 1989). At this stage, secretion is normal as indicated by the fully formed lenses in dVps16A knockdown flies. Furthermore, 3 days earlier in development, delivery of endocytosed ligands and Golgi-derived biosynthetic cargo to lysosomes is inhibited in eye discs, yet secretion of Boss to the R8 cell surface is unchanged (Fig. 8), indicating that the primary defect is in endosomal trafficking. Finally, indirect effects on secretion have also been observed in loss-of-function mutations in VACUOLELESS1, which encodes an *Arabidopsis* homolog of Vps16 (Rojo et al., 2001).

Another phenotype of VACUOLELESS1 mutant cells that we also observed in dVps16A knockdown flies is the accumulation of autophagosomes. Autophagy is a process in which cytosol and organelles are engulfed by double-membrane bounded vacuoles. These vacuoles fuse with lysosomes, which exposes their content to lysosomal hydrolases for degradation (Klionsky, 2005). In *Drosophila*, as in other organisms, autophagy is induced by starvation (Scott et al., 2004) and is observed during developmentally induced cell death (Baehrecke, 2003; Rusten et al., 2004). The accumulation of autophagosomes after dVps16A knockdown indicates that the dVps16A containing complex is required for the fusion of autophagosomes to lysosomes, similar to the function of the Vps-C complex in yeast (Klionsky, 2005; Rieder and Emr, 1997).

Because dVps16A binds tightly and specifically to Car (Figs 2 and 3), it is interesting to compare the dVps16A phenotypes with those of *car* and its mammalian ortholog *buff*, which causes some HPS-like phenotypes in mice (Suzuki et al., 2003). Similar to the eye-color defect observed in Vps16A knockdown and *car* mutant flies, the *buff* mutation causes strong pigmentation defects in the skin and the eye. Knockdown of dVps16A also causes defects in lysosomal delivery. Similar defects in HPS-related mutations are indicated by the deficiency to secrete lysosomal enzymes into urine (Li et al., 2004). However, little or no secretion deficiency was observed in *buff* mice (Suzuki et al., 2003). In *Drosophila*, car^1 exhibits only a slightly reduced rate of degradation for internalized cargo (Sriram et al., 2003). Some of the mild phenotypes observed for *car* and *buff* are likely due to the fact that both mutations are single amino acid changes. Complete

loss-of-function mutations will be necessary to define their functions more precisely.

In addition to dVps16A, we have described a second Vps16 homolog, encoded by CG18112. We have called this protein dVps16B based on its similarity to Vps16 proteins and its binding to the second Vps33 homolog in flies, dVps33B. Homologs of dVps16B are encoded by other insect and vertebrate genomes (Fig. 2). The presence of two Vps16 homologs appears to parallel the presence of two Vps33 homologs in metazoans consistent with the formation of two distinct Vps-C complexes. No mutation has yet been identified in *dVps16B* or *dVps33B*, but an indication for their function comes from the phenotypes observed in humans with mutations in *VPS33B*. These patients suffer from arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome (Gissen et al., 2004). The hallmark of this syndrome is occurrence of muscle atrophy that limits the motion of joints, in conjunction with renal dysfunction and liver defects that prevent bile secretion (Eastham et al., 2001). Cellular phenotypes in the liver and kidney of ARC patients suggest an underlying deficiency in the targeting of proteins to the apical cell surface: proteins normally restricted to the apical domain are distributed over the entire cell surface of hepatocytes and kidney cells (Gissen et al., 2004). Because endosomes play a key role in the maintenance of apical-basal polarity (Nelson and Rodriguez-Boulan, 2004) these phenotypes are consistent with a role of Vps33B proteins in endosomes.

Although the distinct phenotypes suggest non-overlapping function between the two Vps33 homologs in mammals, an interaction with other VpsC proteins was suggested by overexpression of human Vps33B, which resulted in the clustering of lysosomes (Gissen et al., 2004). This phenotype mimics closely the consequences of overexpression of mammalian homologs of Vps18p (Poupon et al., 2003). Similar lysosome clustering has also been observed upon expression of human Vam6p (Caplan et al., 2001). In yeast, Vam6/Vps39 is part of the HOPS complex (Seals et al., 2000; Wurmser et al., 2000). A connection between the Vps33B/Vps16B complex and other subunits of the Vps-C complex is also supported by our detection of a physical interaction between dVps16B and Dor (Fig. 4D). To define to which extend the two Vps-C complexes have distinct and overlapping functions in different membrane fusion events the consequences of complete loss-of-function mutations need to be analyzed in a single organism.

We thank Ellen Lumpkin for the critical reading of the manuscript, Dean Smith for help with the design of the RNAi constructs and Rick Carthew for the pWIZ vector. We are grateful to Mary Kuhn for technical assistance. We like to thank the Bloomington Stock Center and the Developmental Studies Hybridoma Bank at the University of Iowa for fly stocks and reagents. This work was supported by grants to H.K. from The Welch Foundation (I-1300), and the NIH (EY10199 and NS43406).

References

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al. (2000). The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185-2195.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1994). *Current Protocols in Molecular Biology*. New York, NY, USA: John Wiley & Sons.

Baehrecke, E. H. (2003). Autophagic programmed cell death in *Drosophila*. *Cell Death Differ.* **10**, 940-945.

Banta, L. M., Vida, T. A., Herman, P. K. and Emr, S. D. (1990). Characterization of yeast Vps33p, a protein required for vacuolar protein sorting and vacuole biogenesis. *Mol. Cell. Biol.* **10**, 4638-4649.

Beadle, G. and Ephrussi, B. (1936). The differentiation of eye pigments in *Drosophila* as studied by transplantation. *Genetics* **21**, 225-247.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Bunch, T. A., Grinblat, Y. and Goldstein, L. S. (1988). Characterization and use of the *Drosophila* metallothionein promoter in cultured *Drosophila melanogaster* cells. *Nucleic Acids Res.* **16**, 1043-1061.

Cagan, R. L. and Ready, D. F. (1989). The emergence of order in the *Drosophila* pupal retina. *Dev. Biol.* **136**, 346-362.

Caplan, S., Hartnell, L. M., Aguilar, R. C., Naslavsky, N. and Bonifacino, J. S. (2001). Human Vam6p promotes lysosome clustering and fusion in vivo. *J. Cell Biol.* **154**, 109-122.

Carr, C. M., Grote, E., Munson, M., Hughson, F. M. and Novick, P. J. (1999). Sec1p binds to SNARE complexes and concentrates at sites of secretion. *J. Cell Biol.* **146**, 333-344.

Casso, D., Ramirez-Weber, F. and Kornberg, T. B. (2000). GFP-tagged balancer chromosomes for *Drosophila melanogaster*. *Mech. Dev.* **91**, 451-454.

Chang, H. C., Newmyer, S. L., Hull, M. J., Ebersold, M., Schmid, S. L. and Mellman, I. (2002). Hsc70 is required for endocytosis and clathrin function in *Drosophila*. *J. Cell Biol.* **159**, 477-487.

Ciciotte, S. L., Gwynn, B., Moriyama, K., Huizing, M., Gahl, W. A., Bonifacino, J. S. and Peters, L. L. (2003). Cappuccino, a mouse model of Hermansky-Pudlak syndrome, encodes a novel protein that is part of the pallidin-muted complex (BLOC-1). *Blood* **101**, 4402-4407.

Dell'Angelica, E. C., Shotelersuk, V., Aguilar, R. C., Gahl, W. A. and Bonifacino, J. S. (1999). Altered trafficking of lysosomal proteins in Hermansky-Pudlak syndrome due to mutations in the beta 3A subunit of the AP-3 adaptor. *Mol. Cell* **3**, 11-21.

Dulubova, I., Sugita, S., Hill, S., Hosaka, M., Fernandez, I., Sudhof, T. C. and Rizo, J. (1999). A conformational switch in syntaxin during exocytosis: role of munc18. *EMBO J.* **18**, 4372-4382.

Dulubova, I., Yamaguchi, T., Wang, Y., Sudhof, T. C. and Rizo, J. (2001). Vam3p structure reveals conserved and divergent properties of syntaxins. *Nat. Struct. Biol.* **8**, 258-264.

Eastham, K. M., McKiernan, P. J., Milford, D. V., Ramani, P., Wyllie, J., van't Hoff, W., Lynch, S. A. and Morris, A. A. (2001). ARC syndrome: an expanding range of phenotypes. *Arch. Dis. Child.* **85**, 415-420.

Gahl, W. A., Brantly, M., Kaiser-Kupfer, M. I., Iwata, F., Hazelwood, S., Shotelersuk, V., Duffy, L. F., Kuehl, E. M., Troendle, J. and Bernardini, I. (1998). Genetic defects and clinical characteristics of patients with a form of oculocutaneous albinism (Hermansky-Pudlak syndrome). *New Engl. J. Med.* **338**, 1258-1264.

Giot, L., Bader, J. S., Brouwer, C., Chaudhuri, A., Kuang, B., Li, Y., Hao, Y. L., Ooi, C. E., Godwin, B., Vitols, E. et al. (2003). A protein interaction map of *Drosophila melanogaster*. *Science* **302**, 1727-1736.

Gissen, P., Johnson, C. A., Morgan, N. V., Stapelbroek, J. M., Forshew, T., Cooper, W. N., McKiernan, P. J., Klomp, L. W., Morris, A. A., Wraith, J. E. et al. (2004). Mutations in VPS33B, encoding a regulator of SNARE-dependent membrane fusion, cause arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome. *Nat. Genet.* **36**, 400-404.

Gwynn, B., Martina, J. A., Bonifacino, J. S., Sviderskaya, E. V., Lamoreux, M. L., Bennett, D. C., Moriyama, K., Huizing, M., Helip-Wooley, A., Gahl, W. A. et al. (2004). Reduced pigmentation (rp), a mouse model of Hermansky-Pudlak syndrome, encodes a novel component of the BLOC-1 complex. *Blood* **104**, 3181-3189.

Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* **120**, 2121-2129.

Hazelett, D. J., Bourouis, M., Walldorf, U. and Treisman, J. E. (1998). decapentaplegic and wingless are regulated by eyes absent and eyegone and interact to direct the pattern of retinal differentiation in the eye disc. *Development* **125**, 3741-3751.

Horazdovsky, B. F. and Emr, S. D. (1993). The VPS16 gene product associates with a sedimentable protein complex and is essential for vacuolar protein sorting in yeast. *J. Biol. Chem.* **268**, 4953-4962.

Huizing, M. and Gahl, W. A. (2002). Disorders of vesicles of lysosomal lineage: the Hermansky-Pudlak syndromes. *Curr. Mol. Med.* **2**, 451-467.
Huizing, M., Didier, A., Walenta, J., Anikster, Y., Gahl, W. A. and Krämer, H. (2001). Molecular cloning and characterization of human VPS18, VPS **11**, VPS16, and VPS33. *Gene* **264**, 241-247.
Jahn, R., Lang, T. and Sudhof, T. C. (2003). Membrane fusion. *Cell* **112**, 519-533.
Kim, B. Y., Krämer, H., Yamamoto, A., Kominami, E., Kohsaka, S. and Akazawa, C. (2001). Molecular characterization of mammalian homologues of class C Vps proteins that interact with syntaxin-7. *J. Biol. Chem.* **276**, 29393-29402.
Klionsky, D. J. (2005). The molecular machinery of autophagy: unanswered questions. *J. Cell Sci.* **118**, 7-18.
Krämer, H. and Phistry, M. (1996). Mutations in the *Drosophila* hook gene inhibit endocytosis of the boss transmembrane ligand into multivesicular bodies. *J. Cell Biol.* **133**, 1205-1215.
Krämer, H., Cagan, R. L. and Zipursky, S. L. (1991). Interaction of bride of sevenless membrane-bound ligand and the sevenless tyrosine-kinase receptor. *Nature* **352**, 207-212.
Lee, E. C., Hu, X., Yu, S. Y. and Baker, N. E. (1996). The scabrous gene encodes a secreted glycoprotein dimer and regulates proneural development in *Drosophila* eyes. *Mol. Cell. Biol.* **16**, 1179-1188.
Lee, Y. S. and Carthew, R. W. (2003). Making a better RNAi vector for *Drosophila*: use of intron spacers. *Methods* **30**, 322-329.
Li, W., Zhang, Q., Oiso, N., Novak, E. K., Gautam, R., O'Brien, E. P., Tinsley, C. L., Blake, D. J., Spritz, R. A., Copeland, N. G. et al. (2003a). Hermansky-Pudlak syndrome type 7 (HPS-7) results from mutant dysbindin, a member of the biogenesis of lysosome-related organelles complex 1 (BLOC-1). *Nat. Genet.* **35**, 84-89.
Li, W., Rusiniak, M. E., Chintala, S., Gautam, R., Novak, E. K. and Swank, R. T. (2004). Murine Hermansky-Pudlak syndrome genes: regulators of lysosome-related organelles. *BioEssays* **26**, 616-628.
Li, Y., Fetchko, M., Lai, Z. C. and Baker, N. E. (2003b). Scabrous and Gp150 are endosomal proteins that regulate Notch activity. *Development* **130**, 2819-2827.
Marchler-Bauer, A., Anderson, J. B., Cherukuri, P. F., DeWeese-Scott, C., Geer, L. Y., Gwadz, M., He, S., Hurwitz, D. I., Jackson, J. D., Ke, Z. et al. (2005). CDD: a conserved domain database for protein classification. *Nucleic Acids Res.* **30**, D192-D196.
Martina, J. A., Moriyama, K. and Bonifacino, J. S. (2003). BLOC-3, a protein complex containing the Hermansky-Pudlak syndrome gene products HPS1 and HPS4. *J. Biol. Chem.* **278**, 29376-29384.
Misura, K. M., Scheller, R. H. and Weis, W. I. (2000). Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. *Nature* **404**, 355-362.
Nazarian, R., Falcon-Perez, J. M. and Dell'Angelica, E. C. (2003). Biogenesis of lysosome-related organelles complex 3 (BLOC-3): a complex containing the Hermansky-Pudlak syndrome (HPS) proteins HPS1 and HPS4. *Proc. Natl. Acad. Sci. USA* **100**, 8770-8775.
Nelson, W. J. and Rodriguez-Boulan, E. (2004). Unravelling protein sorting. *Nat. Cell Biol.* **6**, 282-284.
Nguyen, T. and Wei, M. L. (2004). Characterization of melanosomes in murine Hermansky-Pudlak syndrome: mechanisms of hypopigmentation. *J. Invest. Dermatol.* **122**, 452-460.
Nguyen, T., Novak, E. K., Kermani, M., Fluhr, J., Peters, L. L., Swank, R. T. and Wei, M. L. (2002). Melanosome morphologies in murine models of hermansky-pudlak syndrome reflect blocks in organelle development. *J. Invest. Dermatol.* **119**, 1156-1164.
Nickla, H. (1977). Maternal effects determine effective lethal phase of carnation-light synthetic lethal in *Drosophila melanogaster*. *Nature* **268**, 638-639.
Peterson, M. R. and Emr, S. D. (2001). The class C Vps complex functions at multiple stages of the vacuolar transport pathway. *Traffic* **2**, 476-486.
Poupon, V., Stewart, A., Gray, S. R., Piper, R. C. and Luzio, J. P. (2003). The role of HmVps18p in clustering, fusion, and intracellular localization of late endocytic organelles. *Mol. Biol. Cell* **14**, 4015-4027.
Price, A., Seals, D., Wickner, W. and Ungermann, C. P. (2000). The docking stage of yeast vacuole fusion requires the transfer of proteins from a cis-SNARE complex to a Rab/Ypt protein. *J. Cell Biol.* **148**, 1231-1238.
Raymond, C. K., Howald-Stevenson, I., Vater, C. A. and Stevens, T. H. (1992). Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E *VPS* mutants. *Mol. Biol. Cell* **3**, 1389-1402.
Richardson, S. C., Winistorfer, S. C., Poupon, V., Luzio, J. P. and Piper, R. C. (2004). Mammalian late vacuole protein sorting orthologues participate in early endosomal fusion and interact with the cytoskeleton. *Mol. Biol. Cell* **15**, 1197-1210.
Rieder, S. E. and Emr, S. D. (1997). A novel RING finger protein complex essential for a late step in protein transport to the yeast vacuole. *Mol. Biol. Cell* **8**, 2307-2327.
Robinson, J. S., Klionsky, D. J., Banta, L. M. and Emr, S. D. (1988). Protein sorting in Saccharomyces cerevisiae: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* **8**, 4936-4948.
Rohrer, J., Schweizer, A., Russell, D. and Kornfeld, S. (1996). The targeting of Lamp1 to lysosomes is dependent on the spacing of its cytoplasmic tail tyrosine sorting motif relative to the membrane. *J. Cell Biol.* **132**, 565-576.
Rojo, E., Gillmor, C. S., Kovaleva, V., Somerville, C. R. and Raikhel, N. V. (2001). VACUOLELESS1 is an essential gene required for vacuole formation and morphogenesis in Arabidopsis. *Dev. Cell* **1**, 303-310.
Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.
Rubin, G. M., Hong, L., Brokstein, P., Evans-Holm, M., Frise, E., Stapleton, M. and Harvey, D. A. (2000). A *Drosophila* complementary DNA resource. *Science* **287**, 2222-2224.
Rusten, T. E., Lindmo, K., Juhasz, G., Sass, M., Seglen, P. O., Brech, A. and Stenmark, H. (2004). Programmed autophagy in the *Drosophila* fat body is induced by ecdysone through regulation of the PI3K pathway. *Dev. Cell* **7**, 179-192.
Sato, T. K., Rehling, P., Peterson, M. R. and Emr, S. D. (2000). Class C Vps protein complex regulates vacuolar SNARE pairing and is required for vesicle docking/fusion. *Mol. Cell* **6**, 661-671.
Scott, R. C., Schuldiner, O. and Neufeld, T. P. (2004). Role and regulation of starvation-induced autophagy in the *Drosophila* fat body. *Dev. Cell* **7**, 167-178.
Seals, D. F., Eitzen, G., Margolis, N., Wickner, W. T. and Price, A. (2000). A Ypt/Rab effector complex containing the Sec1 homolog Vps33p is required for homotypic vacuole fusion. *Proc. Natl. Acad. Sci. USA* **97**, 9402-9407.
Sevrioukov, E. A., He, J. P., Moghrabi, N., Sunio, A. and Krämer, H. (1999). A role for the deep orange and carnation eye color genes in lysosomal delivery in *Drosophila*. *Mol. Cell* **4**, 479-486.
Shestopal, S. A., Makunin, I. V., Belyaeva, E. S., Ashburner, M. and Zhimulev, I. F. (1997). Molecular characterization of the *deep orange* (*dor*) gene of *Drosophila melanogaster*. *Mol. Gen. Genet.* **253**, 642-648.
Spritz, R. A. and Oh, J. (1999). HPS gene mutations in Hermansky-Pudlak syndrome. *Am. J. Hum. Genet.* **64**, 658.
Sriram, V., Krishnan, K. S. and Mayor, S. (2003). deep-orange and carnation define distinct stages in late endosomal biogenesis in *Drosophila melanogaster*. *J. Cell Biol.* **161**, 593-607.
Srivastava, A., Woolford, C. A. and Jones, E. W. (2000). Pep3p/Pep5p complex: a putative docking factor at multiple steps of vesicular transport to the vacuole of Saccharomyces cerevisiae. *Genetics* **156**, 105-122.
Starcevic, M. and Dell'Angelica, E. C. (2004). Identification of snapin and three novel proteins (BLOS1, BLOS2 and BLOS3/reduced pigmentation) as subunits of biogenesis of lysosome-related organelles complex-1 (BLOC-1). *J. Biol. Chem.* **279**, 28393-28401.
Stinchcombe, J., Bossi, G. and Griffiths, G. M. (2004). Linking albinism and immunity: the secrets of secretory lysosomes. *Science* **305**, 55-59.
Subramanian, S., Woolford, C. A. and Jones, E. W. (2004). The Sec1/Munc18 protein, Vps33p, functions at the endosome and the vacuole of Saccharomyces cerevisiae. *Mol. Biol. Cell* **15**, 2593-2605.
Sunio, A., Metcalf, A. B. and Krämer, H. (1999). Genetic dissection of endocytic trafficking in *Drosophila* using a horseradish peroxidase-bride of sevenless chimera: *hook* is required for normal maturation of multivesicular endosomes. *Mol. Biol. Cell* **10**, 847-859.
Suzuki, T., Oiso, N., Gautam, R., Novak, E. K., Panthier, J. J., Suprabha, P. G., Vida, T., Swank, R. T. and Spritz, R. A. (2003). The mouse organellar biogenesis mutant buff results from a mutation in Vps33a, a homologue of yeast vps33 and *Drosophila* carnation. *Proc. Natl. Acad. Sci. USA* **100**, 1146-1150.
Toonen, R. F. and Verhage, M. (2003). Vesicle trafficking: pleasure and pain from SM genes. *Trends Cell Biol.* **13**, 177-186.
Van Vactor, D. L., Jr, Cagan, R. L., Krämer, H. and Zipursky, S. L. (1991). Induction in the developing compound eye of *Drosophila*: multiple mechanisms restrict R7 induction to a single retinal precursor cell. *Cell* **67**, 1145-1155.

Walenta, J. H., Didier, A. J., Liu, X. and Krämer, H. (2001). The Golgi-associated hook3 protein is a member of a novel family of microtubule-binding proteins. *J. Cell Biol.* **152**, 923-934.

Warner, T. S., Sinclair, D. A., Fitzpatrick, K. A., Singh, M., Devlin, R. H. and Honda, B. M. (1998). The light gene of *Drosophila melanogaster* encodes a homologue of VPS41, a yeast gene involved in cellular-protein trafficking. *Genome* **41**, 236-243.

White, J. G. (1982). Membrane defects in inherited disorders of platelet function. *Am. J. Pediatr. Hematol. Oncol.* **4**, 83-94.

Wodarz, A., Hinz, U., Engelbert, M. and Knust, E. (1995). Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*. *Cell* **82**, 67-76.

Wu, M. N., Littleton, J. T., Bhat, M. A., Prokop, A. and Bellen, H. J. (1998). ROP, the *Drosophila* Sec1 homolog, interacts with syntaxin and regulates neurotransmitter release in a dosage-dependent manner. *EMBO J.* **17**, 127-139.

Wurmser, A. E., Sato, T. K. and Emr, S. D. (2000). New component of the vacuolar class C-Vps complex couples nucleotide exchange on the Ypt7 GTPase to SNARE-dependent docking and fusion. *J. Cell Biol.* **151**, 551-562.