

Drosophila Vps35 function is necessary for normal endocytic trafficking and actin cytoskeleton organisation

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Summary

To identify novel proteins required for receptor-mediated endocytosis, we have developed an RNAi-based screening method in *Drosophila* S2 cells, based on uptake of a scavenger receptor ligand. Some known endocytic proteins are essential for endocytosis in this assay, including clathrin and α -adaptin; however, other proteins important for synaptic vesicle endocytosis are not required. In a small screen for novel endocytic proteins, we identified the *Drosophila* homologue of Vps35, a component of the retromer complex, involved in endosome-to-Golgi trafficking. Loss of Vps35 inhibits scavenger receptor ligand endocytosis, and causes mislocalisation of a number of receptors and endocytic proteins. Vps35 has tumour suppressor properties because its loss leads to

overproliferation of blood cells in larvae. Its loss also causes signalling defects at the neuromuscular junction, including upregulation of TGF β /BMP signalling and excessive formation of synaptic terminals. Vps35 negatively regulates actin polymerisation, and genetic interactions suggest that some of the endocytic and signalling defects of *vps35* mutants are due to this function.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/120/24/4367/DC1>

Key words: RNAi, Rac1, Haemocytes, Neuromuscular junction, Retromer

Introduction

Endocytic trafficking is a vesicle-mediated flow of membrane components from the plasma membrane into the cell. Endocytic vesicles are formed by invagination of the plasma membrane, and in most cases, the initial destination of endocytic vesicles is the endosome. Here, specific cargo proteins are sorted and selected for trafficking to other intracellular compartments, including the multivesicular body (MVB) and lysosomal pathway. However, not all endocytosed proteins are destined for degradation in the lysosome. Many are recycled back to the cell surface or trafficked to the Golgi by pathways that can be complex, involving a series of sorting events in several organelles.

Genetic and biochemical approaches in different systems have identified many key protein players in these trafficking steps. For example, clathrin-coated vesicles are generated by the joint action of machinery that includes clathrin itself, adaptor complexes, membrane-bending proteins such as epsin, amphiphysin, syndapin and endophilin A, the proposed ‘pinchase’ dynamin, and many ‘accessory’ proteins that bind to other endocytic proteins, or that interfere with endocytosis

in dominant negative assays of function (Kirchhausen, 2000; Conner and Schmid, 2003). The actin cytoskeleton also plays a dynamic role in endocytic trafficking, with both up- and down-regulation of actin polymerisation affecting the kinetics of endocytosis (Lamaze et al., 1996; Lamaze et al., 1997; Kaksonen et al., 2006). However, we still do not have a full understanding of how clathrin-mediated endocytosis works, or of how complete our knowledge is of its machinery. Proteomic analyses of clathrin-coated vesicles have identified at least several tens of proteins as components of these vesicles (Blondeau et al., 2004; Girard et al., 2005b; Borner et al., 2006), and the roles of most of these are not well understood.

Genetic screens have been useful to identify proteins involved in later endocytic trafficking steps. For example, screens in *Saccharomyces cerevisiae* for components of the vacuolar protein sorting (VPS) pathway have identified many proteins involved in passage of cargo through early endosomes, into MVBs and on to lysosomes (Gruenberg and Stenmark, 2004; Raiborg et al., 2003). A subset of VPS proteins comprises the retromer complex, required for recycling of proteins from early and late endosome to the Golgi

(Seaman et al., 1998; Arighi et al., 2004; Seaman, 2004; Seaman, 2005).

RNAi-based screens now offer an additional route to identify new components of endocytic pathways (Pelkmans et al., 2005). For this purpose, *Drosophila* offers the advantages of convenient RNAi transfection (Bettencourt-Dias et al., 2005), assays for receptor-mediated endocytosis (Guha et al., 2003) and less gene redundancy than in mammalian systems. Here we have used an assay for receptor-mediated endocytosis in the *Drosophila* macrophage-like S2 cell line, that is sensitive to RNAi-mediated knockdown of known components of the clathrin-mediated endocytic machinery, to identify previously unrecognised proteins that are involved in endocytic trafficking. We find that reduction or loss of *Drosophila* Vps35, a core component of the retromer complex, severely affects endocytosis and the localisation of a number of endocytic proteins, causes defects in signalling pathways in haemocytes and at the neuromuscular junction, and leads to increased levels of F-actin. Genetic interactions of *vps35* and *Rac1* mutations suggest that the effects of Vps35 on both the actin cytoskeleton and endocytosis are mediated through Rac1.

Results

RNAi knockdown of certain endocytic proteins inhibits endocytosis in *Drosophila* S2 cells

To screen for effects of RNAi on receptor-mediated endocytosis, we needed to establish an assay in a suitable cell type. Maleylated bovine serum albumin (mBSA) is endocytosed by cultured haemocytes via a scavenger-receptor-mediated pathway through a Rab5 compartment and presumably via clathrin-mediated endocytosis (CME) (Abrams et al., 1992; Guha et al., 2003). It can also be efficiently

internalised by S2 cells (Fig. 1). We used this assay in S2 cells to test whether RNAi-mediated knockdown could be used to identify endocytic proteins, and to verify the pathway used for mBSA uptake (Fig. 1A). Consistent with endocytosis via CME, knockdown of clathrin, α -adaptin, dynamin, Hsc70, auxilin, Rme-8, Lap (AP180) or Rab5 significantly inhibited mBSA uptake (Fig. 1B,C). Inhibition varied from over 80% for clathrin, α -adaptin and dynamin, to as little as 20% for auxilin. By contrast, knockdown of a number of proteins that are important for neurotransmission, Eps15 (Koh et al., 2007), endophilin A (Verstreken et al., 2002) and synaptojanin (Verstreken et al., 2003) had no effect on mBSA uptake, suggesting that the endocytic roles of these proteins are specific for synaptic vesicle (SV) uptake.

The efficient knockdown, particularly of Eps15 (to 1% of control levels) and endophilin A (to 2% of control levels), suggested that the apparent lack of a requirement for these proteins on mBSA uptake was not simply due to insufficient reduction of protein levels. RNAi-mediated knockdown of several proteins was quantified by immunofluorescence (Fig. 2A,B). Also in agreement with the RNAi experiments, no inhibition was observed in cultured haemocytes from *eps15^{EP2513}* (a strong hypomorphic allele) (Koh et al., 2007) and *endoA¹* (a strong hypomorphic null allele) (Verstreken et al., 2002) mutant larvae (Fig. 2C).

Screen for novel endocytic proteins and identification of Vps35

We next used the mBSA uptake assay to test for an endocytic role of other candidate endocytic proteins. We selected proteins containing multiple short motifs WVx[FW], Dx[DxF], FxxFxx[LF] and Dx[FW] predicted to bind to the ear domain

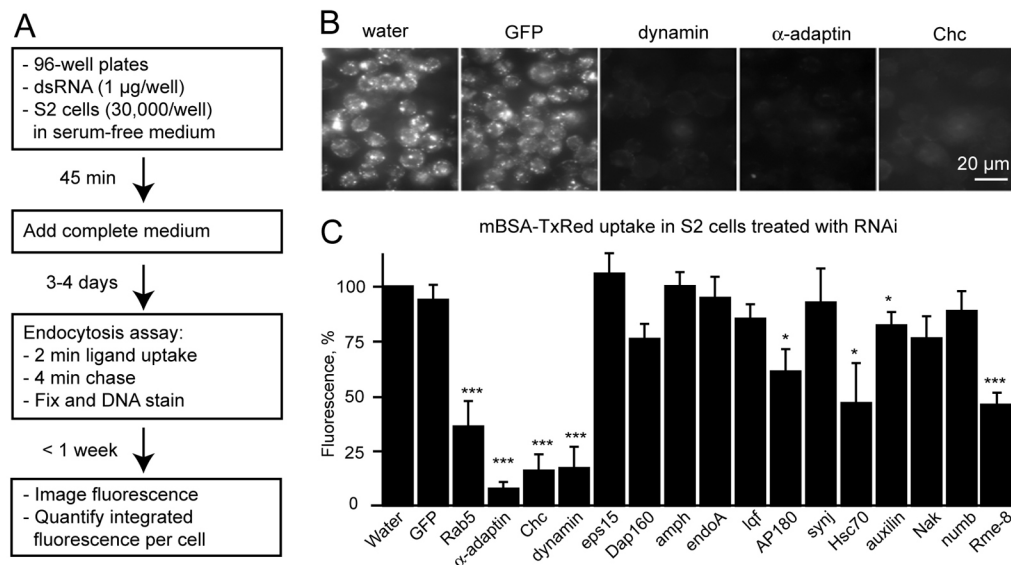


Fig. 1. Uptake of mBSA-Texas-Red by clathrin-mediated endocytosis in *Drosophila* S2 cells. (A) Schematic diagram of the assay.

(B) Representative images of cells treated with RNAi and assayed for uptake of mBSA-Texas-Red. Maximum projection of several Z-sections collected using a wide-field fluorescence microscope is shown. Dynamin, α -adaptin and Chc images have approximately the same number of cells per field as control ones (not shown). (C) Fluorescence levels in cells treated with RNAi against known or proposed endocytic proteins. Quantification was performed using data from 3-4 experiments, each averaged from 10-12 images (average integrated fluorescence per cell), normalised using untreated cells ('Water') in the same experiment, and compared statistically with untreated cells. In this and all subsequent figures, * P <0.05, ** P <0.01, *** P <0.005, Student's t -test; all other comparisons are not significant (NS); error bars indicate s.e.m.

of α -adaptin (Praefcke et al., 2004), or an LLpL[-] motif (where p and [-] denote a polar and a negatively charged residue, respectively) predicted to bind to clathrin heavy chain (Kirchhausen, 2000), or combinations of these. We prioritised proteins that contained either several copies of a single motif, or combinations of multiple motifs, situations that are known to promote high-affinity binding to α -adaptin (Praefcke et al., 2004). In addition, we also selected several candidates that contain predicted membrane-bending ENTH/ANTH or BAR domains.

Using these criteria, 30 candidate proteins were selected (supplementary material Table S1 and Fig. S1) for an RNAi-based screen for defects in endocytosis of mBSA in S2 cells. Knockdown of four proteins showed a significant reduction of endocytosis (Fig. 3A): CG5625 (Vps35), CG2093 (Vps13), CG30268 (a predicted serine protease with some homology to

elastase and kallikrein) and CG17184 (arfaptin-2, partner of Rac1). Since the most severe phenotype was seen with knockdown of CG5625, we chose it for further characterisation.

CG5625 is the only identifiable *Drosophila* orthologue of Vps35 (61% amino-acid identity to human Vps35). This is a core component of the retromer complex that mediates traffic

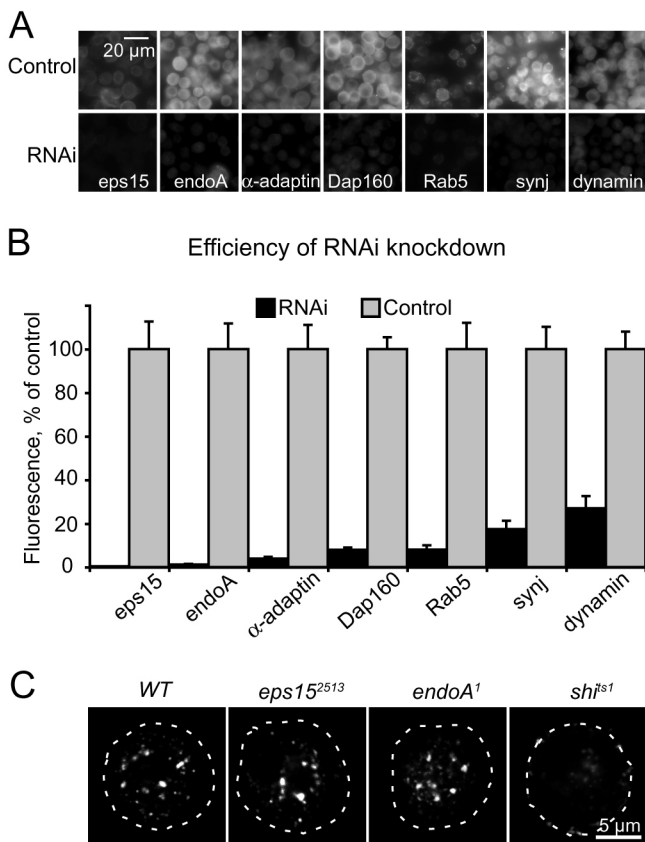


Fig. 2. Efficiency of protein knockdown. (A) Representative images of cells immunostained with antibodies against endocytic proteins 3–4 days after RNAi (RNAi, bottom row) or cells left untreated (Control, top row). Maximum-intensity projections of several Z-sections, collected using fluorescent microscopy, are shown. (B) Protein levels in S2 cells after RNAi knockdown. Images similar to those in A were used to quantify average fluorescence per cell, data represent mean \pm s.e.m. of three independent experiments. (C) Primary haemocytes (inside dotted circles) from *eps15* and *endoA* mutant animals internalise mBSA normally. Haemocytes harvested from wild-type (WT), *eps15* (*EP(2)2513*), *endoA* (*endoA*¹) and dynamin (*shi*^{ts1}) mutant third instar larvae (Guha et al., 2003) were incubated with mBSA-Texas-Red for 2 minutes, followed by a 4-minute chase. Assays were performed at 35°C with WT and *shi*^{ts1} cells, and at room temperature with *eps15* and *endoA* mutant cells.

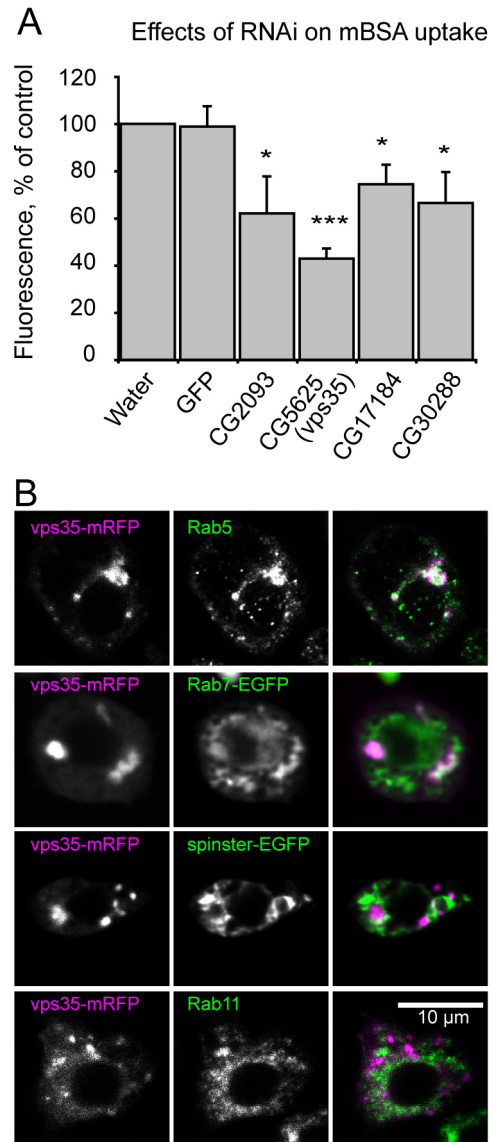


Fig. 3. Vps35 knockdown affects endocytosis and tagged Vps35 localises to endosomes in S2 cells. (A) Uptake of fluorescent mBSA by cells after RNAi knockdown of novel candidate endocytic proteins. Only knockdowns that significantly inhibited endocytosis and relevant control data are shown; the complete data set is presented in supplementary material Fig. S1. Comparisons are to water control. (B) Single confocal sections showing localisation of Vps35-mRFP (magenta) in S2 cells. Colocalisation with markers of early endosome (anti-Rab5), late endosome (Rab7-EGFP) late endosome and lysosome (spinster-EGFP) and recycling endosome (Rab11 antibody) compartments (green) is shown. Cells were co-transfected with each combination of plasmids for 24 hours and expression was induced with 1 mM CuSO₄ for 4 hours before imaging.

of receptors from the endosome to the Golgi (Seaman et al., 1998; Arighi et al., 2004; Seaman, 2004; Seaman, 2005). *Drosophila* Vps35 tagged with monomeric red fluorescent protein (Vps35-mRFP) showed a mainly punctate cytoplasmic distribution (Fig. 3B, supplementary material Fig. S2), that largely overlapped with endosomal compartments that were labelled using anti-Rab5 and Rab7-EGFP, and to a lesser extent with the late endosome-lysosome marker spinster-EGFP. There was no colocalisation with the recycling endosome marker Rab11, nor was Vps35-mRFP detected at the plasma membrane. Therefore, the localisation of *Drosophila* Vps35-mRFP is consistent with its function as a retromer component in other phyla, rather than with direct involvement with endocytosis at the plasma membrane.

Mutation of *vps35* inhibits endocytosis in haemocytes but not SV endocytosis

A *P* element insertion (*P{EPgy2}EY14200*) in the 5' untranslated region of the *vps35* gene (Fig. 4A) is a severe hypomorphic allele, reducing *vps35* mRNA levels to less than 2% of wild-type larvae (Fig. 4B). A deficiency that deletes *vps35* was also identified in the DrosDel collection (Ryder et al., 2004). We designate homozygous *P{EPgy2}EY14200* and *P{EPgy2}EY14200/Df(2R)ED3952* genotypes as *vps35* and *vps35/Df*, respectively. Both genotypes die at late larval or pupal stages, with very rare *vps35* (but not *vps35/Df*) adults eclosing; these are unable to move and die shortly after. Since Vps35 is expected to have an important role in all cells, and appears to be ubiquitously expressed with some enrichment in ovary (<http://flyatlas.org/atlas.cgi?name=FBgn0034708>), the late lethality may be due to a maternal contribution in early development, or to a low level of residual function in *vps35* mutants.

In agreement with the effect of Vps35 knockdown in S2 cells, cultured haemocytes from *vps35* and *vps35/Df* larvae showed significantly reduced mBSA uptake compared with the wild type (Fig. 4C,D). The *P{EPgy2}EY14200* insertion carries a *UAS* element that can drive expression of the *vps35* coding region in the presence of GAL4; in this way we could use a haemocyte-specific driver, *Hml-Gal4*, to rescue the mBSA endocytic defect of *vps35/Df* haemocytes (Fig. 4D), showing that the defect was due to loss of Vps35.

To identify the cellular nature of the endocytic defect in *vps35* mutant haemocytes, we tested the localisation of several proteins with known roles in endocytosis and membrane trafficking. Mutant haemocytes showed intense accumulation of clathrin, α -adaptin and dynamin at the plasma membrane compared with wild-type cells. An increase in plasma membrane localisation was also observed for several receptors, including a *Drosophila* scavenger receptor Croquemort (Crq) (Fig. 4E, supplementary material Fig. S3). This suggests that the endocytic defect is not due to a lack of scavenger receptor at the plasma membrane but rather to a block of receptor internalisation.

However, we found no requirement for Vps35 in SV endocytosis at the neuromuscular junction (NMJ). Mutant *vps35* boutons showed no significant defect in uptake of the styryl dye FM1-43FX (Kuromi and Kidokoro, 2002) during

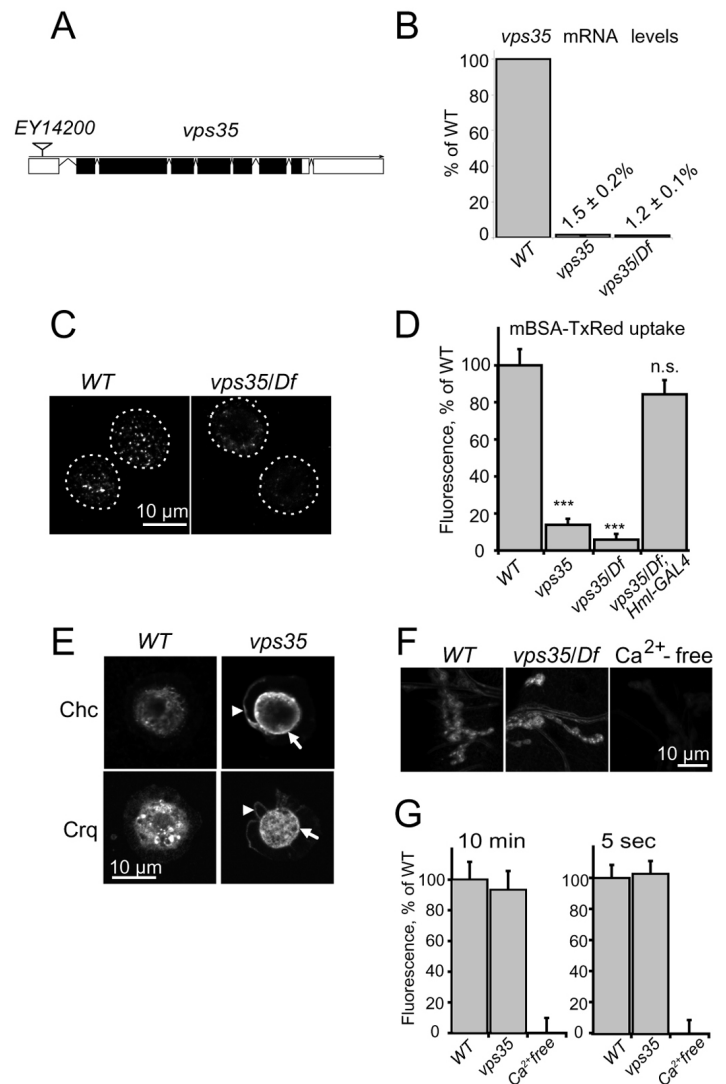


Fig. 4. Vps35 is required for efficient endocytosis in non-neuronal cells but not for SV endocytosis. (A) Map of *Drosophila vps35*, showing exons with coding region (black boxes) and UTRs (open boxes), and insertion *P{EPgy2}EY14200* in the 5' UTR. The sequence of cDNA clone RE65032, and alignment with the genomic sequence suggests that *Drosophila vps35* has nine exons, with the coding sequence stretching from nucleotide 121 of RE65032, at the start of exon 2, to a stop codon at nucleotide 2524 in exon 8. (B) Levels of *vps35* mRNA determined using real-time PCR, normalised to the wild type (WT). $n=2$. (C) Representative images of WT and *vps35/Df* mutant haemocytes (inside dotted circles) after mBSA-Texas-Red uptake (maximum-intensity projections of confocal stacks). (D) mBSA-Texas-Red uptake in haemocytes from animals with genotypes as shown. All comparisons are with WT; $n=12-24$ cells from 6-9 larvae; mean \pm s.e.m. (E) Labelling of clathrin heavy chain (Chc) and the scavenger receptor Crq in WT and *vps35* primary haemocytes, visualised using single confocal sections taken at equal distances (1 μ m) from the base of each cell. Arrows indicate plasma membrane surrounding the body of the cell and arrowheads the leading edge of lamellopodia. (F) Representative images of NMJ boutons labelled with FM1-43FX in a 10-minute loading protocol. (G) FM1-43FX dye internalisation in WT and *vps35* NMJs after 10 minutes or 5 seconds of loading. Non-specific staining of WT boutons in Ca^{2+} -free medium was used as a background control. $n=5-7$ NMJs; mean \pm s.e.m.

stimulation by 90 mM K⁺ (Fig. 4F,G). Similarly, no defect was seen when uptake was allowed only during a brief pulse (5 seconds), a protocol previously used to detect mild impairment of SV recycling (González-Gaitán and Jäckle, 1997).

Since fly stocks carrying insertions in CG17184 (*arfaptin2*, or *partner of Rac1*) were also available, we tested these for an endocytic defect. Haemocytes from larvae homozygous for three such insertions (P{RS3}UM-8176-3, inserted in the second and largest coding exon of CG17184, and thus likely to be a null allele); P{XP}d04253 (inserted in the 3' untranslated region of CG17184); P{Epgy2}EY11874 (inserted in the 5' untranslated region of the longest CG17184 transcript) all showed no significant defect in uptake of maleylated BSA ($P > 0.15$ in all cases; $n = 12-21$ cells). We therefore did not investigate CG17184 further.

Vps35 acts as a tumour suppressor and affects signalling in haemocytes and the NMJ

Many *vps35* mutant larvae develop melanotic masses in the body cavity (Fig. 5A). In normal larvae, the haemocyte

population consists almost entirely of plasmatocytes, which differentiate into lamellocytes after pupation; melanotic tumours result from overproliferation of haemocytes and excessive formation of lamellocytes (Evans et al., 2003). *Vps35* mutant haemolymph contains a large excess of plasmatocytes compared with the wild type (Fig. 5B,C) and significant numbers of lamellocytes (Fig. 5B) similar to other melanotic tumour mutants (Qiu et al., 1998; Minakhina and Steward, 2006).

Formation of melanotic capsules and overproliferation of haemocytes suggests upregulation of one or more signalling pathways that control haemocyte differentiation, such as Toll, Ras-MAP kinase (MAPK), Jun kinase, or JAK/STAT (Qiu et al., 1998; Evans et al., 2003; Zettervall et al., 2004). Indeed, *vps35* cells exhibit elevated levels of Toll signalling as judged by elevated cellular levels of Toll receptor (including increased localisation at the plasma membrane) and by nuclear localisation of the downstream transcription factor Dorsal (Fig. 5D-F). In addition, levels of EGF receptor (EGFR) and PDGF- and VEGF-receptor-related receptor (PVR) were increased at the plasma membrane, and levels of a downstream target of receptor tyrosine kinase signalling, diphospho-MAPK, were elevated (supplementary material Fig. S3).

Although *vps35* NMJs show apparently normal SV recycling, they have many satellite boutons that protrude from larger boutons; these are rare in wild-type synapses but found in some endocytic mutant synapses (Dickman et al., 2006). This causes a twofold increase in total bouton number per NMJ compared with wild-type animals (Fig. 6A,B). Complete rescue of this phenotype requires simultaneous expression of *vps35* using both neuronal (*elav-GAL4*) and muscle (*BG57-GAL4*) drivers, whereas only partial rescue is observed using each driver individually (Fig. 6B). Therefore Vps35 is required both pre- and post-synaptically for normal NMJ development.

Several signalling pathways regulate NMJ development in *Drosophila*, including anterograde Wnt signalling and anterograde and retrograde TGF β /BMP signalling (Packard et al., 2002; McCabe et al., 2003; Marqués, 2005; Dudu et al., 2006; Wang et al., 2007). We therefore looked for changes in some key components of these pathways at *vps35* NMJs. Localisation and levels of the Wnt receptor Frizzled-2 were not significantly affected in *vps35* NMJs (not shown), and heterozygous loss of *Dfz2* had no effect on the *vps35* mutant bouton phenotype (Fig. 6E). However, levels of phosphorylated Mad (pMad) in *vps35* NMJs were elevated compared with the wild type, indicating upregulation of BMP signalling (Fig. 6C,D). Removal of one copy of the *wit* (encoding the type II BMP receptor wishful thinking) or *Mad* genes suppressed the supernumerary bouton phenotype of *vps35* mutants (Fig. 6E). Complete removal of *Mad* suppressed the supernumerary bouton phenotype of *vps35* NMJs even more strongly (Fig. 6E), resulting in bouton numbers similar to those found in homozygous *Mad* NMJs (McCabe et al., 2004). Therefore, synaptic overgrowth in *vps35* mutant animals requires BMP signalling, and is accompanied by elevated levels of BMP signalling.

Role of the actin cytoskeleton in *vps35* phenotypes

Haemocytes isolated from *vps35* mutant larvae have increased numbers of lamellopodia compared with the wild type, and these often appear detached from the coverslip beneath them

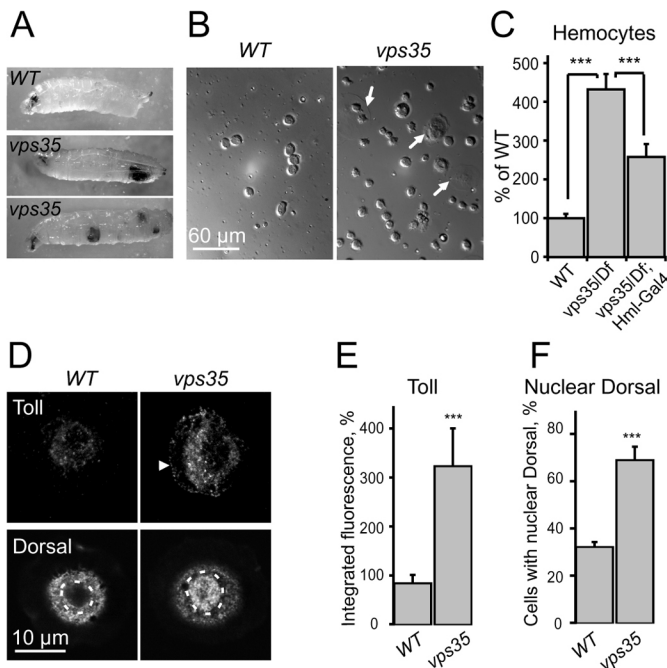


Fig. 5. Proliferation and signalling defects in *vps35* mutant haemocytes. (A) Mutant *vps35* third instar larvae develop melanotic masses. One WT and two *vps35* animals are shown. (B) Representative DIC images of haemocytes isolated from WT or *vps35* third instar larvae. Note increased numbers of small round cells (plasmatocytes) in *vps35* larvae, and flat lamellocytes (arrows) that are absent in WT haemolymph. (C) Increased numbers of haemocytes extracted from *vps35/Df* larvae, relative to WT, and rescue using *Hml-Gal4*. Numbers are normalised relative to WT; $n = 12-15$ images from six larvae; mean \pm s.e.m. (D) Toll signalling is upregulated in *vps35* mutant haemocytes, judged by increased levels of Toll (arrowhead indicates leading edge of lamellopodia that is labelled with Toll in *vps35* but not in WT cell) and nuclear localisation of Dorsal. Dashed circles demarcate nuclei (as defined by bright field image). (E) Toll levels per cell; $n = 14$ cells from five larvae. (F) Percentage of cells in which Dorsal is enriched in nuclei. $n \geq 300$ cells isolated from six larvae; mean \pm s.e.m.

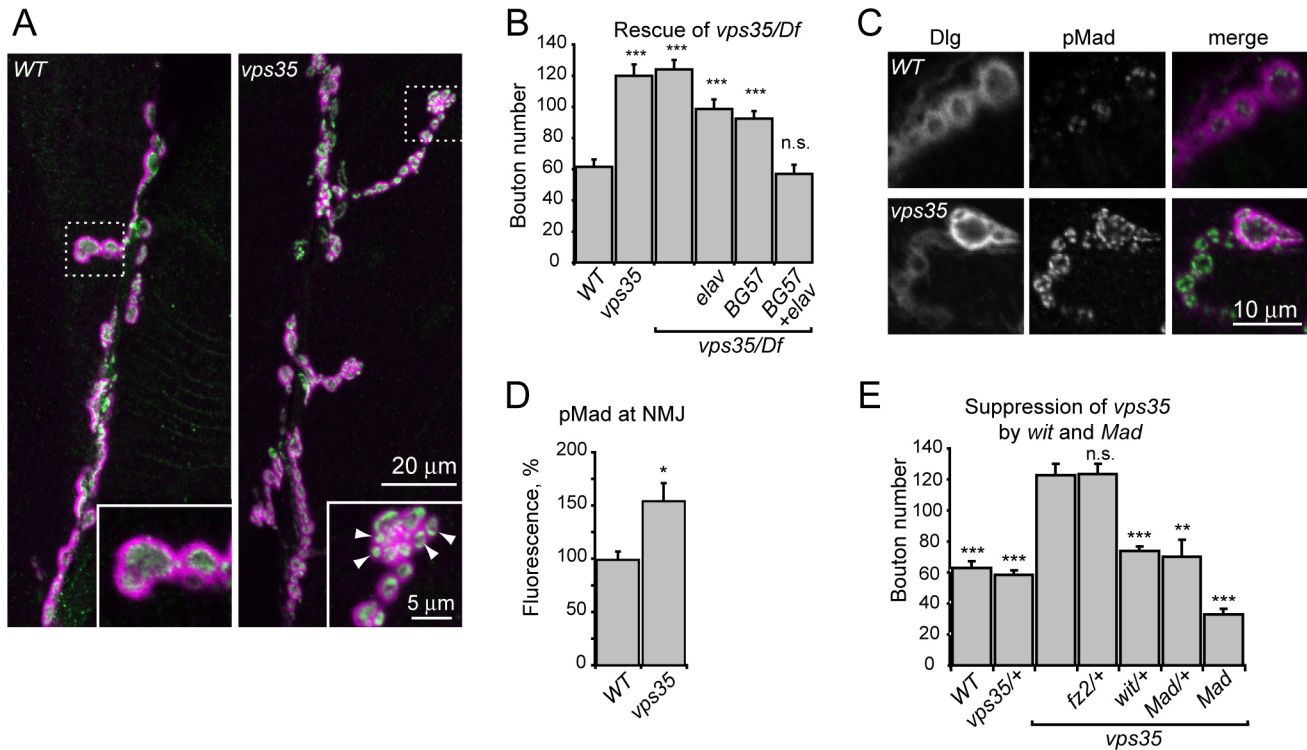


Fig. 6. NMJ bouton phenotype of *vps35*. (A) Projections of confocal sections of wild-type (WT) and *vps35* NMJs stained for the presynaptic marker Eps15 (green) and the largely postsynaptic marker Dlg (magenta). Insets show higher magnification of boxed areas. Arrowheads indicate examples of satellite boutons in *vps35* NMJs, that are rare in WT; note that Eps15 labelling in these is completely surrounded by Dlg. (B) *vps35* mutant larvae have approximately twice as many boutons as WT. The defect is partially rescued when Vps35 expression is driven by *elav-Gal4* or *BG57-Gal4* and completely rescued using a combination of these drivers. $n=5-12$ NMJs from 5-10 larvae; mean \pm s.e.m. (C) Single confocal sections of NMJ boutons showing upregulation of pMad in *vps35* mutant NMJs. Note the pMad puncta (green) predominantly at the inner surface of the mainly postsynaptic Dlg staining (magenta). (D) Quantification of pMad levels at the NMJ ($n=10$ boutons from five animals; mean \pm s.e.m.). (E) The supernumerary bouton phenotype of *vps35* larvae is suppressed by removal of a single copy of the BMP signalling components *wit* or *Mad* (*wit*+/+, *Mad*+/+ respectively) and more strongly by complete loss of *Mad* (*Mad*). $n=5-11$ NMJs from 5-9 larvae; statistical comparisons are with *vps35*. NMJs from muscles 6 and 7 in abdominal segment A3 were used for all analyses; mean \pm s.e.m.

(Supplementary material Movies 1 and 2). This phenotype is similar to that observed when actin polymerisation is upregulated because of increased levels of Rac1, a small Rho family GTPase that stimulates actin polymerisation and has effects on membrane trafficking including inhibition of receptor-mediated endocytosis (Williams et al., 2006; Lamaze et al., 1996; Burridge and Wennerberg, 2004). We therefore hypothesised that knockdown of *vps35* might result in upregulation of Rac1, thus leading to increased formation of filamentous actin and to inhibition of CME.

Consistent with this model, *vps35* mutant haemocytes showed a large increase in levels of filamentous actin (F-actin) relative to the wild type, and this phenotype was partially rescued by driving expression of Vps35 with *Hml-GAL4*, or by removal of a single copy of *Rac1* (Fig. 7A,B). Notably, removal of a single copy of *Rac1* also restored mBSA uptake in haemocytes that lacked Vps35, to about 50% of wild-type levels (Fig. 7C), suggesting that Vps35-dependent inhibition of Rac1 activity was the basis for the involvement of Vps35 in CME. Downregulation of F-actin levels by treatment of haemocytes by cytochalasin D also restored mBSA uptake in *vps35* mutant haemocytes to a level statistically indistinguishable from the wild type (Fig. 7C), suggesting that the inhibition of

endocytosis in mutant haemocytes was in fact due to increased levels of F-actin. Similarly, the increased bouton number phenotype of *vps35* NMJs was suppressed by removal of a single copy of *Rac1* or by expression of dominant-negative Rac1 (*UAS-Rac1.N17*) in either neurons or muscle (Fig. 7D). Neither *rac1*/+ nor dominant-negative Rac1 (in either neurons or muscles) affected bouton numbers in a *vps35*⁺ background (Fig. 7D and data not shown). Therefore, the effects of Vps35 on the actin cytoskeleton, CME and signalling at the NMJ appear to be mediated at least partly through Rac1.

Discussion

Several known endocytic proteins are not essential for endocytosis in *Drosophila* S2 cells

We have established and validated an assay that allows identification of proteins involved in endocytic uptake of mBSA in *Drosophila* S2 cells. As carried out here, the assay is only moderate throughput, but with greater automation of microscopy and image processing, could potentially be a high-throughput assay that could allow screens of a large fraction of the genome for endocytic phenotypes.

Clathrin-mediated endocytosis is the main pathway of mBSA internalisation, since knockdown of clathrin, α -adaptin

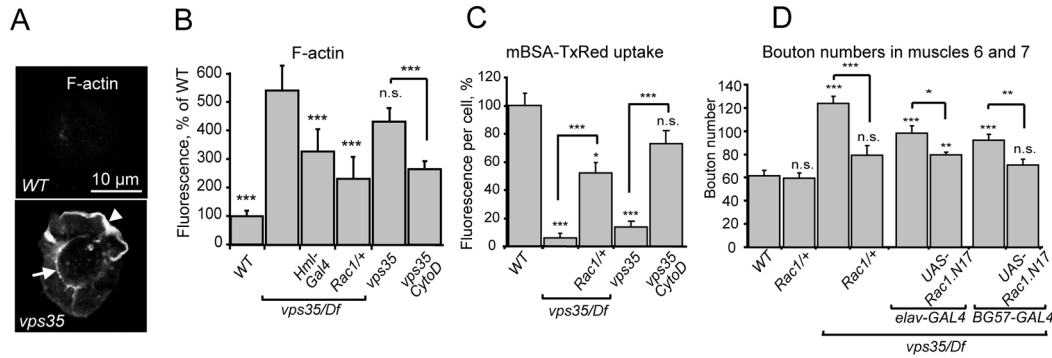


Fig. 7. Involvement of actin cytoskeleton in *vps35* mutant phenotypes. (A) Labelling of F-actin in WT and *vps35* haemocytes, visualised using single confocal sections as in Fig. 4E. Arrow indicates plasma membrane surrounding the body of the cell. Arrowhead indicates leading edge of lamellopodia. (B) F-actin levels in WT, *vps35*, *vps35/Df*, *vps35/Df;Hml-Gal4* and *vps35/Df;Rac1/+* haemocytes and in *vps35* haemocytes treated with 0.1 mM cytochalasin D (cytoD) (average fluorescence per cell). $n=25-30$ cells from four larvae; statistical comparisons are with *vps35/Df*. (C) Removal of a single copy of *Rac1*, or treatment with 0.1 mM cytochalasin D, partially rescues the loss of mBSA-Texas-Red uptake in haemocytes that lack Vps35. $n=25-30$ cells from four larvae. (D) The *vps35/Df* supernumerary bouton phenotype is suppressed by removal of a single copy of *Rac1*, or by presynaptic or postsynaptic expression of dominant-negative Rac1 (*UAS-Rac1.N17*). Note that *elav-GAL4* and *BG57-GAL4* also drive Vps35 expression and partially rescue the *vps35* mutant phenotype (see Fig. 6B). NMJs from muscles 6 and 7 in abdominal segment 3 were used for all analyses; $n=3-8$ larvae. All statistical comparisons are with WT except where indicated. All data are mean \pm s.e.m.

or dynamin inhibits uptake by 80% or more. In addition, knockdown of several other known endocytic proteins including Rab5, Lap (AP180), Hsc70, auxilin and Rme8 (Wucherpfennig et al., 2003; Zhang et al., 1998; Chang et al., 2002; Chang et al., 2004) significantly reduces mBSA uptake. By contrast, Eps15 and endophilin A, and its binding partner synaptojanin (Verstreken et al., 2002; Verstreken et al., 2003; Koh et al., 2004; Majumdar et al., 2006), are not apparently required for mBSA endocytosis, despite their importance in SV endocytosis. Endophilin A (Verstreken et al., 2002) and Dap160 (intersectin) (Koh et al., 2004) have previously been shown to be required for viability only in neurons. Therefore, our work and that of others point to a set of proteins including endophilin A and its binding partner synaptojanin, and Eps15 and its binding partner Dap160/intersectin, are specific for neuronal endocytosis in *Drosophila* and do not belong to the core machinery of CME.

Drosophila Vps35 is involved in endocytic trafficking

Knockdown of Vps35 either by RNA interference or by mutation significantly inhibits scavenger receptor endocytosis. Vps35 is a core component of the retromer complex, involved in selection and trafficking of cargo from the endosome to the Golgi (Seaman, 2005). Physiological roles of retromer include production of a functional long-range Wnt signal, transcytosis of polymeric immunoglobulin A (IgA) and possibly even cell migration and adhesion (Verges et al., 2004; Kerr et al., 2005; Coudreuse et al., 2006; Prasad and Clark, 2006), although it is not known whether these roles depend on the function of retromer in endosome-Golgi recycling.

How might Vps35 play a role in endocytosis? Vps35 is clearly not a core component of the clathrin-mediated endocytic machinery: SV endocytosis is not affected, and in agreement with the known localisation and function of retromer in other organisms (Seaman, 2005), a Vps35-mRFP fusion protein has a predominantly endosomal localisation in *Drosophila* cells. However, Vps35 is not the only endosomal

protein required for endocytosis. For example, Rme-8 is required for retrieval of the mannose phosphate receptor from endosomes to the trans-Golgi network (TGN), endocytosis of the Boss receptor in *Drosophila*, and EGF receptor endocytosis in mammalian cells (Chang et al., 2004; Girard et al., 2005a). Furthermore, *Caenorhabditis* Vps35 is required for secretion of active Wnt, also suggesting an unexpected requirement for endosome-TGN trafficking to allow some proteins to reach the plasma membrane (Coudreuse et al., 2006; Prasad and Clark, 2006). There are a number of ways in which Vps35 could affect endocytic uptake.

First, knockdown of Vps35 as a part of the retromer complex should block trafficking of many cargoes from the endosome to the TGN. Perhaps one or more proteins essential for endocytosis of mBSA are normally recycled via the TGN to the plasma membrane, and in the absence of Vps35 is either trapped in the endosome or shunted towards the lysosomal pathway and to degradation. Notably, intracellular localisation of Crq, EGFR and PVR on large endosomal structures, which is striking in wild-type cells, is significantly reduced in the mutant, and this could be explained if receptors were subject to increased degradation via the lysosomal pathway in *vps35* mutants. However, this could not account for all *vps35* phenotypes, as we observe upregulation of several signalling pathways consistent with accumulation of receptors at the plasma membrane.

Second, a formal possibility is that Vps35 has an as yet unknown role in endocytosis at the plasma membrane. Although we did not detect Vps35 at the plasma membrane, components of the retromer complex (including Vps35) expressed in mammalian cells can be found at the cell surface and lamellopodia (Kerr et al., 2005). However, even an apparent plasma membrane localisation using light microscopy could be explained by association of retromer components with subcortical endosomal structures, as appears to be the case for Rme-8 (Chang et al., 2004).

A third possibility, which we favour, is that loss of Vps35 inhibits endocytosis by causing increased Rac1-dependent

actin polymerisation. This model is supported by the fact that downregulating Rac1 levels and pharmacologically reducing F-actin levels both suppress the endocytic defects caused by loss of Vps35. Increased levels of F-actin resulting from upregulation of small GTPase Rac1 have previously been associated with inhibition of CME, transcytosis of IgA, biosynthetic traffic to the apical membrane and excessive proliferation and differentiation of *Drosophila* blood cells (Lamaze et al., 1996; Jou et al., 2000; Williams et al., 2006). These phenotypes are also caused by loss of Vps35 (Verges et al., 2004; Coudreuse et al., 2006; Prasad and Clark, 2006) (this work). Accumulation of endocytic proteins and receptors at the plasma membrane might therefore be explained by increased levels of F-actin, caused in turn by elevated levels or activity of Rac1, which has been reported as an interactor of *Drosophila* Vps35 (<http://flight.liecr.org>). We do not yet know how Vps35 can define activity or levels of Rac1, but the model is supported by the suppression of several *vps35* phenotypes when activity of Rac1 is reduced. It would be interesting to investigate if physiological consequences of loss of Vps35 – such as its role in Wnt gradient formation in *Caenorhabditis* reported by others (Coudreuse et al., 2006; Prasad and Clark, 2006) – also result from a similar mechanism.

Another question is whether upregulation of actin polymerisation is a result or a consequence of the endocytic defect. Indeed, although increased formation of filamentous actin can lead to accumulation of signalling receptors at the plasma membrane, some receptors including EGFR, PVR and TGF β /BMP receptors, can stimulate polymerisation of actin (Marcoux and Vuori, 2005; Rosin et al., 2004; Eaton and Davis, 2005).

Vps35 is essential for normal physiology of *Drosophila*

Here we identified and characterised for the first time *Drosophila* that are mutant for a component of retromer complex. Mutation of *vps35* causes overproliferation and excessive differentiation of blood cells, thus indicating tumour suppressor properties of retromer. In addition, mutant animals develop melanotic masses that might be an indication of tumour growth. These phenotypes can be a result of upregulation of several signalling pathways, known to cause similar pathologies. For example, upregulation of the Toll-Cactus pathway is sufficient to cause similar defects of blood cell development (Qiu et al., 1998; Evans et al., 2003; Zettervall et al., 2004). *Drosophila* EGFR is also known to promote cell proliferation, differentiation and cell survival, through the Ras-Raf-MAPK pathway (Yoshida et al., 2004; Kurada and White, 1999). Another process that requires normal levels of Vps35 in *Drosophila* is NMJ development, because mutation of *vps35* leads to uncontrolled formation of boutons. Overall, we suggest that Vps35, alone or as a part of retromer complex, is essential for signalling processes that depend on intracellular membrane traffic in multicellular organisms.

Materials and Methods

Cell culture

Adherent *Drosophila* S2 cells (gift from G. Ihrke, Cambridge Institute for Medical Research, Cambridge, UK) were maintained at 25°C in complete medium: *Drosophila* serum-free medium supplemented with 10% heat-inactivated FBS, 2.5 μ g/ml fungizone, 100 U/ml penicillin, 100 μ g/ml streptomycin and 0.292 mg/ml L-Glutamine (all reagents were from Invitrogen, Paisley, UK). Cells were

transfected using Cellfectin (Invitrogen, Paisley, UK) and expression was induced with 1 mM CuSO $_4$ for 4 hours prior to fixation when required. Haemocytes from third instar larvae were prepared in complete medium as previously described (Guha et al., 2003).

RNA synthesis

Motifs were identified using the Sequence Motif Search Program (<http://www.mrc-lmb.cam.ac.uk/genomes/madanm/harvey>; H. McMahon, personal communication). DNA templates were PCR-amplified from genomic DNA, using primers (MWG, Ebersberg, Germany) specified by the Genome RNAi *Drosophila* Resources website (www.dkfz.de/signaling2/e-rnai; supplementary material Table S2), and used for synthesis of double-stranded RNA using MEGAscript[®] High Yield Transcription Kit (Ambion, Huntingdon, UK). RNA was annealed using a PCR cyclor: 95°C for 5 minutes; 85°C for 1 minute; 5°C per minute cooling to 50°C; 0.5°C/second cooling to 25°C; 2°C/second cooling to 4°C. RNA concentrations were estimated by comparison to DNA mass standards in gel electrophoresis, taking into account the 1.2-fold lower fluorescence of ethidium bromide bound to dsRNA compared with dsDNA (<http://www.sigmaaldrich.com/catalog/search/ProductDetail/FLUKA/46047>), and RNA was diluted to a final concentration of 0.6 μ g/ μ l.

RNAi-induced gene silencing and endocytosis assay

1.2 μ g dsRNA was added in duplicate per well of a 96-well plate. 30,000 cells in 50 μ l serum-free medium were added to each well and incubated for 45 minutes. After addition of 150 μ l complete medium, cells were grown to confluency for 3–4 days and used for an endocytosis assay essentially as described for haemocytes (Guha et al., 2003). Briefly, 4 μ g/ μ l mBSA-Texas-Red in complete medium was incubated with cells for 2 minutes at room temperature, washed three times with complete medium within 4 minutes ('chase'), followed by 15 minutes of fixation with 2.5% formaldehyde in PBS (0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl, pH 7.4). After washing with PBS, nuclear DNA was stained with 1 μ g/ml Hoechst 33342 (Invitrogen, Paisley, UK) in PBS for 10 minutes, and cells were washed as before. The cells were kept at 4°C in PBS supplemented with 0.05% NaN $_3$ (Sigma, Dorset, UK) for up to a week before imaging. Cells were imaged using a Zeiss fluorescence microscope equipped with a CoolSnap HQ camera (Photometrics, Marlow, UK) and a 100 \times /1.4 NA oil-immersion objective. Image quantification was performed on a maximum-intensity projection using Metamorph (Molecular Devices Corporation, Sunnyvale, CA), with a purpose-written algorithm allowing the measurement of integrated intensity of fluorescence per average cell. The quantified data were processed and statistically analysed in Excel (Microsoft Corporation, Seattle, WA). Endocytosis assays with larval haemocytes were performed as described (Guha et al., 2003). Treatment with 0.1 mM cytochalasin D (Sigma, Dorset, UK) was performed for 10 minutes in complete medium immediately before the endocytosis assay.

S2 cell expression

Two alternative predicted coding regions of *vps35* were amplified from cDNA clone RE65032 (Geneservice, Cambridge, UK): codons 1–841 (Vp35-1) and 41–841 (Vps35-2). Codon 1 starts at the 5' end of RE65032, but alignment with vertebrate Vps35 sequences suggests that the protein product may only initiate at codon Met41 (nucleotide 121 of RE65032; translation might also potentially initiate at Met39 (nucleotide 115); however, Met41 has a better match to a Kozak consensus sequence [gcgcc(A/G)ccAUGg] G $_3$ (Kozak, 1984) and only sequences after codon 41 appear phylogenetically conserved (data not shown). Vps35-1 was amplified using primers 5'-ggggacaagtgtgtacaaaaagcaggctctatgAATCGATATCAGGACGTCC-3' and 5'-ggggaccacttgtacaaagaagctggctcATTGAGAGTTATGCCCGCAA-3'. Vps35-2 was amplified using primers 5'-ggggacaagtgtgtacaaaaagcaggctc-ATGCCHAATGGTTTGGATGA-3' and 5'-ggggaccacttgtacaaagaagctggctcATTGAGAGTTATGCCCGCAA-3' (Vps35 sequences in uppercase). Primers included att recombination sites (in lowercase) for Gateway cloning. Both PCR products were recombined into pDonor221 (Invitrogen, Carlsbad, CA), and the resulting entry clones were recombined into pMTDest-cRFP; this is a metallothionein-dependent expression vector harbouring a 2.5 kb *XbaI-KpnI* Gateway cassette from pUAST-Dest15 (gift of F. Wirtz-Peitz, Institute of Molecular Biotechnology, Vienna, Austria; identical to pTWR, described at <http://dgrc.cgb.indiana.edu>) subcloned into pMT/V5-His vector (Invitrogen, Carlsbad, CA). The resulting clones express fusions of mRFP to the C-terminus of Vps35, and the entire amplified sequences and in-frame fusion were verified by sequencing before use. pUAST-Rab7-EGFP (Entchev et al., 2000) and pUAST-Spinster-EGFP (Sweeney and Davis, 2002) were expressed by cotransfecting cells with pAcA-Gal4 (McCabe et al., 2003).

Quantification of gene expression

Total RNA was isolated from ten third-instar larvae of each genotype using TRizol Reagent (Sigma, Dorset, UK). Traces of DNA were removed using a DNA-free[™] kit (Ambion, Huntingdon, UK). 1 μ g total RNA was transcribed into single-stranded cDNA using Omniscript Reverse Transcription kit (Qiagen, Crawley, UK) and random primers (Biolone, London, UK). RNA quality was verified by visual

examination in an agarose gel and quantified using a Nanodrop Spectrophotometer (Nanodrop, Wilmington, USA).

Gene-specific oligonucleotide PCR primers (supplementary material Table S2) were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Quantitative PCR was performed using iQ5 real-Time PCR detection system with iQ SYBR Green supermix (Bio-Rad). Quantitative PCR reactions were carried out in quadruplicate in 25 μ l reaction volume containing 100 nM forward and reverse primers and 10 ng template. Quantification of the gene expression was performed using levels of GAPDH for normalisation (Pfaffl, 2001).

Immunostaining

Anti-Eps15 antibody was generated in rabbit using a fragment encoding amino acid residues 586-816 cloned into the pGEX4T1 expression vector in-frame with the GST coding region. Primary antibodies were used at the following dilutions: Eps15, 1:500; dynamin, 1:500 (Roos and Kelly, 1999); α -adaptin, 1:50 (González-Gaitán and Jäckle, 1997); Rab5, 1:50 (Wuchterpfennig et al., 2003); Rab11 clone 47, 1:50 (BD Biosciences Pharmingen, Oxford, UK); Dlg 4F3, 1:500 (Parnas et al., 2001); synaptotagmin, 1:200 (Verstreken et al., 2003); endophilinA, 1:200 (Verstreken et al., 2002); Dap160, 1:500 (Roos and Kelly, 1998); Croquemort, 1:1000 (Franc et al., 1999); Dorsal, 1:100 (Kubota et al., 1993); Toll, 1:500 (Hashimoto et al., 1991); pMad, 1:200 (Liang et al., 2003); synaptotagmin I, 1:500 (Littleton et al., 1993); Ubiquitin, 1:200 (Assay Designs, Ann Arbor, Michigan, USA); EGFR, 1:200 (Jékely and Rørth, 2003); PVR, 1:200 (Duchek et al., 2001); dpMAPK, 1:200 (Sigma, Dorset, UK). Secondary antibodies were labelled with Alexa Fluor 488 and Alexa Fluor 594 (1:200; Molecular Probes, Eugene, OR). Preparations were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and viewed in an MRC BioRad 1024 confocal microscope mounted on a Nikon Eclipse E800 microscope. Images were captured using a 40 \times /1.3 NA or 60 \times /1.4 NA objective, and exported using LaserSharp software (Bio-Rad). Brightness, contrast, levels and colour channels were adjusted using Photoshop (Adobe Systems, San Jose, CA). F-actin in primary haemocytes was stained as described (Williams et al., 2006). Images of unstained primary haemocytes were obtained using differential interference contrast on an Axioskop 2 microscope (Carl Zeiss, Welwyn Garden City, UK), equipped with a Hamamatsu Orca C4742-95 digital camera with Openlab acquisition software (Improvision, Coventry, UK). Protein levels were quantified using ImageJ (<http://rsb.info.nih.gov/ij/>).

FM-43FX-uptake experiments

Larvae were dissected on Sylgard dishes in calcium-free saline and then allowed to internalise a fixable analogue of FM1-43 (Molecular Probes) (4 μ M in saline containing 90 mM KCl) for 5 seconds or 10 minutes. Preparations were washed three times immediately with an excess of zero-calcium saline, and extensively for a further 15 minutes, and fixed for 15 minutes with 4% formaldehyde. Boutons from muscles 6 and 7 in segment A3 were imaged using a 60 \times /1.4 NA oil-immersion objective and an MRC-BioRad 1024 confocal microscope mounted on a Nikon Eclipse E800 microscope (Bio-Rad). All saline solutions were as described (Kuromi and Kidokoro, 2002), and all incubations were carried out at 20–22°C. To quantify uptake, the threshold function of ImageJ was used to define the edges of Type I boutons as closely as possible, and the average pixel intensity within the thresholded area was measured.

Drosophila stocks

Drosophila stocks used were Oregon R as wild-type control, w^{1118} , $eps15^{EP2513}$, $P\{EPgy2\}EY14200$, $Df(2R)ED3952$, $endoA^1$, wir^{A12} and wir^{B11} , Mad^{10} , sh^{s1} , $elav^{3A4-Gal4}$, $BG57-Gal4$, $P\{Hml-GAL4.G\}5-6$, $\{UAS-Rac1.N17\}1$, $Rac1^{J11}$, $Dfz2^{c1}$, $P\{RS3\}UM-8176-3$, $P\{XP\}d04253$, $P\{Epgy2\}EY11874$. Insertions and mutant alleles are described in FlyBase (<http://www.flybase.org>).

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