The J-domain protein Rme-8 interacts with Hsc70 to control clathrin-dependent endocytosis in Drosophila

Henry C. Chang, Michael Hull, and Ira Mellman
Department of Cell Biology, Ludwig Institute for Cancer Research, Yale University School of Medicine, New Haven, CT 06520

Introduction

Endocytosis, a process common to all eukaryotic cells, plays a critical role not only in the internalization of extracellular macromolecules, but also in the down-regulation of signaling receptors from the cell surface. One major route of endocytosis is the clathrin-mediated pathway, characterized by the selective internalization of receptors and bound ligands via clathrin-coated vesicles (CCVs; Schmid, 1997; Kirchhausen, 2000). To form CCVs at the plasma membrane, AP-2 adaptors, coat proteins, and clathrin are recruited to coated pits, where the assembly of clathrin into lattice-like structures induces the inward curvature of the membrane. The pits then grow with successive additions of coat proteins, and eventually detach from plasma membrane as CCVs in a dynamin-dependent process. After detachment, the clathrin coats are dissociated from CCVs and are reused for subsequent rounds of endocytosis.

Hsc70, a constitutively expressed member of the Hsp70 chaperone family, has been implicated in many stages of this CCV cycle. In vitro, Hsc70 can promote the release of clathrin triskelions and other coat proteins from CCVs by binding to clathrin and thus disrupting the clathrin cage concomitant with ATP hydrolysis (Schlossman et al., 1984; Chappell et al., 1986; Ungewickell et al., 1995). In addition, Hsc70 can bind to PP2A which, in turn, can modulate the affinity of adaptor complexes for cargo proteins by dephosphorylation (Ghosh and Kornfeld, 2003). Hsc70 can also bind to PP2A which, in turn, can modulate the affinity of adaptor complexes for cargo proteins by dephosphorylation (Ghosh and Kornfeld, 2003). Thus, Hsc70 may participate in the release of adaptor proteins, as well as clathrin, from CCVs (Hannan et al., 1998; Ghosh and Kornfeld, 2003). After uncoating, Hsc70 remains associated with clathrin triskelions and may have a role in priming clathrin for future rounds of CCV formation (Schlossman et al., 1984; Jiang et al., 2000). Recent evidence has further suggested that Hsc70, along with its cochaperone auxilin (see next paragraph), binds to dynamin in vitro and may have a role in the early steps of CCV formation (Newmyer et al., 2003).

Despite these advances in understanding the biochemical details of Hsc70 in clathrin-mediated endocytosis, its precise function in vivo remains difficult to demonstrate. That Hsc70 is important in intact cells has been substantiated by experiments perturbing Hsc70 function, either by the expression of dominant-negative mutant alleles (Newmyer and Schmid, 2001) or by selection of Hsc70 mutants in Drosophila (Chang et al., 2002). In these examples, inhibition of Hsc70 function under physiological conditions caused defects in clathrin-dependent internalization and in receptor recycling.
(Honing et al., 1994; Morgan et al., 2001; Newmyer and Schmid, 2001; Chang et al., 2002).

Because of the myriad cellular roles of Hsc70, it may be easier to analyze a particular Hsc70 function in vivo by characterizing specific J-domain–containing cochaperones with which it is likely to interact. The J-domain, a conserved motif shared by members of the DnaJ protein family, can bind to Hsp70 family proteins and stimulate their low intrinsic ATPase activity (Ungewickell et al., 1995; Bukau and Horwich, 1998; Kelley, 1999). The relevant cochaperone in the clathrin uncoating reaction is thought to be auxilin, which contains clathrin-binding domains as well as the J-domain (Ungewickell et al., 1995; Umeda et al., 2000). Thus, the proposed mechanism is that auxilin first binds to CCVs, and recruits ATP-bound Hsc70 proteins via its J-domain (Ungewickell et al., 1995; Holstein et al., 1996). The J-domain interaction then stimulates Hsc70 ATPase activity, thereby stabilizing the binding of Hsc70 to clathrin and driving triskelion dissociation (Holstein et al., 1996). Consistent with this, inhibition of auxilin function in vivo causes an accumulation of CCVs, indicative of defects in clathrin uncoating (Gall et al., 2000; Pishvaee et al., 2000; Greener et al., 2001). In addition to auxilin, receptor-mediated endocytosis (Rme) 8, another J-domain–containing protein, was implicated in receptor-mediated internalization and fluid-phase tracer uptake in Caenorhabditis elegans (Zhang et al., 2001), suggesting that distinct cochaperones may mediate diverse functions of Hsc70 in endocytosis. However, a link between Rme-8 and Hsc70 has not been established, and the exact role or site of action of Rme-8 remains poorly understood.

To identify new factors regulating endocytosis, we have screened for mutants altering the rough eye phenotype caused by the overexpression of a dominant-negative shibire (shi), the Drosophila homologue of dynamin (van der Bliek and Meyrowitz, 1991; Chang et al., 2002). Mutations in dynamin, a GTPase required for the detachment of CCVs from the plasma membrane, were first isolated as animals exhibiting temperature-sensitive paralysis, caused by a disruption in neurotransmitter recycling (Poodry et al., 1973; Poodry and Edgar, 1979). Although the thermal sensitivity of shi has been used to identify other endocytic factors (Narayanan and Ramaswami, 2003), we chose the developing eye for conducting a screen because the rough eye phenotype can detect modifying effects in gradation and is, therefore, more sensitive.

Expression of shiK39A, a GTP hydrolysis–defective dynamin, under the control of eye-specific GMR expression cassette has been shown to cause a rough eye phenotype (Chang et al., 2002). Taking advantage of this, we have screened 30,000 progeny and identified several mutations in the Drosophila Rme-8 gene. Here, we present evidence suggesting that Rme-8 cooperates with Hsc70-4 and plays a role in regulating clathrin-mediated endocytosis.

Results

E(shi)2-1 is a dominant modifier of a GTP hydrolysis–defective dynamin

Expression of a GTP hydrolysis–defective dynamin, dynK44A, was shown to block transferrin uptake in HeLa cells (Damke et al., 2001). To test whether this putative dominant-negative dynamin mutant can block endocytosis in Drosophila cells, we expressed the fly dynamin homologue shi carrying analogous mutation (shiK39A) in eye imaginal discs using the GMR expression cassette (Hay et al., 1994; Chang et al., 2002). The mutant eye discs were then stained with an mAb against Bridge of sevenless (Boss; Cagan et al., 1992), and the number of clusters with internalized Boss proteins was counted. Boss encodes the membrane ligand for the Sevenless receptor tyrosine kinase, and is specifically expressed on the apical surface of R8 and internalized into neighboring Sevenless-expressing cells (Cagan et al., 1992). In wild-type discs, 29.6% of the clusters (n = 1,674) exhibited detectable levels of internalized Boss. In the presence of one copy of GMR-shiK39A, only 18.7% of the clusters (n = 2,000) contained internalized Boss, and this number was further reduced to 16.7% (n = 1,439) when two copies of GMR-shiK39A were expressed. These data suggested that, in fly cells, expression of this GTP hydrolysis–defective dynamin, as in mammalian cells, can dominantly inhibit Rme.

In addition to decreasing the level of Boss internalization, GMR-shiK39A caused a rough eye phenotype (Fig. 1 B), presumably reflecting an alteration of one or more signaling processes due to the inhibition of endocytosis during eye development (Chang et al., 2002). In contrast, expression of wild-type shi using the GMR promoter had no effect on eye morphology (Fig. 1 A). As for the Boss internalization defect, the rough eye phenotype became more severe as flies carried more copies of the GMR-shiK39A transgene (unpublished data). This sensitivity to gene dose suggested that we could use GMR-shiK39A to identify genes functioning in the dynamin-mediated pathway by isolating second-site mutations that modify (either suppress or enhance) the rough eye phenotype.

Accordingly, we screened ~30,000 x-ray mutagenized progeny for mutants that altered the rough eye phenotype of GMR-shiK39A (see Materials and methods for details). We isolated 24 enhancers (i.e., those that made the eye phenotype worse) and 1 suppressor (those that made the eye phenotype better), which were assigned into 3 multi-hit and 4 single-hit complementation groups. Here, we describe our characterization of E(shi)2-1 (17 alleles), which exhibited the strongest enhancer interaction with GMR-shiK39A among all our mutants (Fig. 1 C).

E(shi)2-1 affects Rme during eye development

The genetic interaction with GMR-shiK39A suggested that E(shi)2-1 functions in the endocytic pathway. To test if this...
was indeed the case, we examined the trafficking of Boss protein in homozygous E(shi)2-1 mutant tissues during eye development. The localization of Boss proteins can be easily monitored using a functional HRP-Boss chimera in which the cytochemically detectable enzyme HRP was fused to the extracellular domain of Boss (Sunio et al., 1999). In wild-type eye disc (Fig. 2 A), the staining pattern of HRP-Boss fusion was seen as "patches," representing Boss proteins on the apical surface of R8 cells, and "dots," representing those accumulated in structures in Sevenless-expressing cells (Sunio et al., 1999). As shown in Fig. 2 B, the neighboring small dots of HRP-Boss were absent in homozygous E(shi)2-1 mutant disc, indicating that the receptor-mediated internalization of Boss was inhibited.

In addition to Boss internalization, E(shi)2-1 was also required for proper ommatidial development. A tangential section of mosaic adult retina showed that mutant clusters often lack complete complement of photoreceptor cells (Fig. 2 C). In mosaic clusters with a normal complement of photoreceptors (Fig. 2 C, circles), the size of rhabdomeres (the light-sensing organelles) in mutant cells was consistently smaller than those of the wild-type cells. Furthermore, the organization of regular ommatidial array in these mutant tissues was disrupted.

**E(shi)2-1 inhibits tracer uptake at early steps of the endocytic pathway**

To further understand the role of E(shi)2-1 in the endocytic pathway, we examined the defects in the uptake of an endocytic tracer, Texas red–conjugated avidin (TR-avidin), by homozygous E(shi)2-1 mutant larval Garland cells. Garland cells are thought to function as nephrocytes and have a rapid rate of fluid-phase endocytosis (Kosaka and Ikeda, 1983). This internalization of TR-avidin by Garland cells appears to be clathrin dependent, as mutations in α-adapin, a subunit of the AP-2 adaptor complex required for forming CCVs at the plasma membrane (González-Gaitán and Jackle, 1997), inhibit this process (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200311084/DC1). To identify endocytic compartments, the cells also carried an Act5C-GAL4/+; UAS-GFP-rab7/+ transgene, which labels late endosomal and lysosomal structures (Entchev et al., 2000). After a 1-min incubation, in wild-type cells most of the TR-avidin was peripherally localized in Rab7-negative structures just beneath the plasma membrane (Fig. 3 A, red). After a chase of 20 min at 25°C, the TR-avidin was transferred to more central regions of the cytoplasm where it was found associated with Rab7-positive structures, suggesting that the tracer had reached late endosomes or lysosomes (Fig. 3 B). In contrast, in E(shi)2-1/E(shi)2-1 Garland cells, relatively little TR-avidin appeared to be internalized after a 1-min incubation (Fig. 3 D). Moreover, little if any of the tracer was transferred to Rab7-positive structures after a 20-min chase (Fig. 3 E).

To determine whether the small amount of staining seen in E(shi)2-1/E(shi)2-1 cells represented TR-avidin adsorbed to the plasma membrane or trapped in peripheral intracellular vesicles, the uptake assay was modified so Garland cells without the GFP-rab7 transgene were incubated in TR-avidin for 1 min at 25°C, chased for 20 min at 25°C, and then incubated with FITC-avidin for 1 min at 4°C. The incubation of second tracer at 4°C allowed us to assess whether TR-avidin had been internalized into compartments inaccessible to outside because further endocytosis would have been inhibited by cold. In wild-type cells (Fig. 3 C), although TR-avidin was seen as vesicular staining near the center, FITC-avidin staining was mostly peripheral near the cell membrane, indicating that TR-avidin had transited to late endocytic compartments after 20 min. In contrast, the staining of TR- and FITC-avidin showed extensive overlap in mutant cells (Fig. 3 F, arrows), indicating that most of the TR-avidin associated with mutant cells was trapped at the plasma membrane or in structures accessible to outside. Although these results could not definitively distinguish whether the tracers were never internalized, or were once internalized but recycled back to the plasma membrane due to a block in transit through endosomal

---

**Figure 2. Receptor-mediated internalization of Boss is defective in E(shi)2-1 mutant.** Eye discs carrying HRP-Boss dissected from (A) wild-type and (B) E(shi)2-1/E(shi)2-1 third instar larvae. The Boss proteins on the apical surface of R8 cells are labeled by asterisks, and the Boss internalized by the neighboring cells are indicated by arrows. Note that the accumulations of Boss in neighboring cells are absent in B. (C) A tangential section of an E(shi)2-1/+ clone in adult retina. Mutant photoreceptor cells are represented by those lacking white pigment granules at the base of their rhabdomeres, and delineated by the dash line. White pigment granules are the two dark structures (arrows) situated at the bases of rhabdomeres, the light-sensing organelles. Mosaic clusters with a mixture of mutant and wild-type photoreceptors are indicated by circles. In these clusters, the size of rhabdomeres in mutant cells was consistently smaller than those of the wild-type cells. Bars, 5 μm.
compartments, they clearly suggest that E(shi)2-1 affects endocytosis at some early steps.

Endosomal organization and clathrin distribution are disrupted in E(shi)2-1 mutant cells

Given that the uptake of the endocytic tracers into Rab7-positive late endosomes was strongly inhibited, we tested whether E(shi)2-1 exhibited defects in endosomal organization by comparing the staining pattern of Hook (Hk), a cytosolic protein associated with early endosomes (Kramer and Phistry, 1996), and GFP-rab7, in both wild-type and homozygous E(shi)2-1 mutant tissues. In wild-type Garland cells, Hk was localized to the cell periphery and associated with structures that were distinct from the bulk of the Rab7 staining (Fig. 4 A). In contrast, Hk staining was patchy and greatly reduced in homozygous E(shi)2-1 Garland cells (Fig. 4 B). In addition, Rab7 appeared more diffuse and centrally located in E(shi)2-1 cells, their morphology and density of Rab7 labeling varied considerably.

A defect in Hk localization was also detected in mosaic eye imaginal discs generated by FLP/FRT recombination. To identify the z-axis focal plane visualized for each cell cluster, the eye discs were also stained with an anti-Elav antibody, which labels the cell nuclei. Note that these cells are dinucleate. Bar, 5 μm.

Figure 4. Clathrin distribution and endosomal organization are disrupted in E(shi)2-1 mutant Garland cells. Confocal images of (A) wild-type and (B) E(shi)2-1/+E(shi)2-1/+ third instar larval Garland cells stained with a rabbit α-Hk antibody (blue). These cells also carried one copy of the GFP-rab7 transgene (green). (C) A confocal image of homozygous E(shi)2-1/+ clones in a mosaic eye disc. The cells were stained with a rabbit α-Hk antibody (blue) and a rat α-Elav antibody (red) to label early endocytic structures and nuclei of neuronal cells, respectively. Wild-type cells are indicated by the presence of a membrane-associated GFP expression, whereas mutant cells are indicated by the absence of GFP expression and arrows. Because the disc is slanted, the confocal section of the left side is apical (above the nuclei) and the right side more basal. (D–F) Confocal images of Clc-GFP fusion in Garland cells isolated from (D) wild-type, (E) E(shi)2-1/+ E(shi)2-1/+ and (F) Hsc70-4R447H/Hsc70-4R447H animals. N, nucleus. Bars: (A, B, and D–F) 5 μm; (C) 15 μm.
the nuclei of neuronal cells (Robinow and White, 1988). In wild-type cells (indicated by the presence of GFP expression), Hk was concentrated near the apical cortex of photoreceptor cells. In homozygous E(shi)2-1 mutant cells (indicated by the absence of GFP expression), the staining of Hk proteins appeared more vesicular, less restricted to the apical surface, and could be easily detected at lower focal planes of cells (Fig. 4 C). Together, these data suggested that the organization of endosomal compartments was affected by E(shi)2-1 mutation.

The genetic interaction with dynamin and the requirement for Boss internalization during eye development suggested that E(shi)2-1 may play a role in a clathrin-mediated pathway. To determine whether E(shi)2-1 has an effect on clathrin distribution, we compared the localization of a clathrin light chain-EGFP (Clc-EGFP) in wild-type and mutant Garland cells (Chang et al., 2002). This Clc-EGFP was placed under the control of UAS regulatory elements, and was expressed in Garland cells using the Act5C-GAL4 driver line. In wild-type cells, Clc-EGFP was seen as punctate staining around the cell periphery, presumably representing vesicular clathrin-coated structures (Fig. 4 D). However, in homozygous E(shi)2-1 cells, the Clc-EGFP staining was weaker and more diffuse, although clustering near the limiting membrane of some large internal structures was occasionally observed (Fig. 4 E, arrows). This abnormal distribution of Clc-EGFP in mutant Garland cells suggested that clathrin function may be compromised by E(shi)2-1. Interestingly, the phenotype of clathrin mislocalization, as well as those defects in endosomal organization, were remarkably similar to those seen in cells deficient in Hsc70-4, the Drosophila homologue of clathrin uncoating ATPase (Fig. 4 F; Chang et al., 2002), raising the possibility that E(shi)2-1 and Hsc70-4 function in a common pathway.

E(shi)2-1 encodes fly homologue of Rme-8 gene
To identify the gene responsible for E(shi)2-1, the enhancement of the GMR-shi

\[ \text{CG8014} \]

rough eye phenotype was mapped to 2-61 (n = 62) by meiotic recombination. The lethality associated with E(shi)2-1 was included in the deletion Df(2R)G63-73 (45A13-B1:45D5-8) but excluded from Df(2R)w73-3 (45B5-7:45D5-8), placing the gene in the cytological interval of 45A13-B1 to B5-7. The position of gene was further narrowed down to a region between two P-element lines, l(2)k04512 and l(2)k06021 (Spradling et al., 1995, 1999), using P-element-mediated male recombination (Chen et al., 1998).

Although there are ~13 transcriptional units between l(2)k04512 and l(2)k06021, we decided to focus on large transcripts first because 17 alleles of E(shi)2-1 were isolated from a relatively small screen, suggesting that the E(shi)2-1 coding region is large. Indeed, sequencing analysis of E(shi)2-1 revealed that CG8014 (Berkeley Drosophila Genome Project; Rubin et al., 2000), the largest transcript in the region (ORF = 7,162 bp), contained a nonsense mutation at tryptophan1287 (Fig. 5). Consistent with this interpretation, ubiquitous expression of CG8014 under the Act5C-GAL4 driver could revert the enhancement of the GMR-shi

\[ \text{CG8014} \]

rough eye phenotype by E(shi)2-1 alleles, and could rescue the recessive lethality associated with E(shi)2-1 (unpublished data). Together, these observations indicate that CG8014 is the gene for E(shi)2-1. Expression profile analysis from the Berkeley Drosophila Genome Project indicated that CG8014 transcripts were detected ubiquitously throughout all stages of embryonic development, but enriched in Malpighian tubule and Garland cells (Tomancak et al., 2002).

CG8014 encodes a protein of 2,387 amino acids, most homologous to Rme-8, a gene previously identified in C. elegans for defects in Rme (Zhang et al., 2001). Putative homologues were also found in Arabidopsis (GenBank/EMBL/DDBJ accession no. NP_180257), rice (AAP55138), rat (XP_236584), and human (BK001645), suggesting that the function of Rme-8 is conserved (Zhang et al., 2001). Although no obvious motif except an internal J-domain was present in the C. elegans ORF, a J-domain and four IWN domains were conserved in CG8014. The J-domain is a segment (delineated by the dashed line) including the J- and IWN2 domains, which are conserved in many proteins that contain J-domain (delineated by the dashed line) including the J- and IWN2 domains, and Hsp90-4, which plays an important role in a clathrin-mediated pathway. Of the 25 excisions recovered, three were lethal and all failed to complement all alleles of E(shi)2-1, suggesting that E(shi)2-1 is CG8014. This was further confirmed by the sequence analysis of E(shi)2-1, which revealed a premature stop codon before the J-domain in the Rme-8 ORF. To test the importance of its J-domain, a segment (delineated by the dashed line) including the J- and IWN2 domains, was deleted from Rme-8 to generate mRFP-Rme-8.

E(shi)2-1 encodes Drosophila homologue of Rme-8. A schematic representation of the Drosophila Rme-8/CG8014 locus. The exons of Rme8, flanked by Rab-RP1 and rad201 loci, are drawn to scale and shown by white boxes, and the directions of transcription units are indicated by arrows. To test if E(shi)2-1 corresponds to CG8014, we first generated lethal excision lines from p[w]CG02191 (#12659; Bloomington Drosophila stock center), a viable transposon line with a P-element inserted only 92 bp upstream of the CG8014 transcription initiation site. Of the 25 excisions recovered, three were lethal and all failed to complement all alleles of E(shi)2-1, suggesting that E(shi)2-1 is CG8014. This was further confirmed by the sequence analysis of E(shi)2-1, which revealed a premature stop codon before the J-domain in the Rme-8 ORF. Below, a scaled-up diagram of Rme-8 ORF to illustrate the locations of the J-domain (blue box), the four IWN domains (numbered green boxes), and several highly conserved stretches (yellow boxes). To facilitate subsequent analysis of Rme-8 localization, an mRFP (red box) was inserted immediately after the start codon to create a functional mRFP–Rme-8 fusion. To test the importance of its J-domain, a segment (delineated by the dashed line) including the J- and IWN2 domains, was deleted from Rme-8 to generate mRFP–Rme-8N.
detected in Rme-8 protein sequence, comparison of Rme-8 sequences from divergent species revealed four previously defined IWN domains (Zhang et al., 2001) and several conserved regions (Fig. 5). The functions of these domains are not known; however, the high degree of conservation does suggest that they are critical for Rme-8.

Rme-8 proteins are associated with multiple endocytic structures

Although the *C. elegans* Rme-8 proteins appeared to be associated with some multivesicular compartments, the identities of these structures were not established (Zhang et al., 2001). To determine the subcellular localization of Rme-8 proteins, we constructed *UAS-mRFP-Rme-8FL*, where a full-length *Rme-8* genomic/cDNA fusion is tagged at the NH₂ terminus with a monomeric RFP (Campbell et al., 2002; Fig. 5). Ubiquitous expression of this construct could counter the enhancement of the *GMR-shiK39A* rough eye phenotype by loss-of-function *Rme-8* mutations (unpublished data), indicating that this *mRFP-Rme-8FL* was functional.

To help in identifying subcellular structures, *Act5C-GAL4/UAS-mRFP-Rme-8FL*; *UAS-Clc-EGFP-C1/+*; *Act5C-GAL4/UAS-mRFP-Rme-8FL*; *UAS-GFP-Rab5/+*, and *Act5C-GAL4/UAS-mRFP-Rme-8FL*; *UAS-GFP-Rab7/+*. The boxed regions are shown in high magnification. The regions where Rme-8 shows spatial overlaps with various markers are indicated by arrows. Bar, 5 μm.

**Figure 6. Rme-8 proteins are associated with multiple endosomal structures.** Confocal micrographs of Garland cells expressing mRFP-tagged Rme-8 (red) and various subcellular protein markers (green). The genotypes of these cells are (A) *Act5C-GAL4/UAS-mRFP-Rme-8FL*; *UAS-Clc-EGFP-C1/+*, (B) *Act5C-GAL4/UAS-mRFP-Rme-8FL*; *UAS-GFP-Rab5/+*, and (C) *Act5C-GAL4/UAS-mRFP-Rme-8FL*; *UAS-GFP-Rab7/+*. The boxed regions are shown in high magnification. The regions where Rme-8 shows spatial overlaps with various markers are indicated by arrows. Bar, 5 μm.

Rme-8 exhibits genetic interaction with Hsc70

Although the presence of a J-domain suggests that Rme-8 might act as an accessory factor for Hsc70, this possibility has not been addressed experimentally. To determine if the two proteins interact functionally in vivo, an ATP hydrolysis–defective Hsc70-4 (Hsc70-4K71S) was expressed in the eye discs using the *GMR-GAL4* driver (Elefant and Palter, 1999). Expression of a mammalian Hsc70 carrying the analogous mutation in HeLa cells was shown to inhibit transferrin internalization and recycling, suggesting the mutant can interfere with the functions of endogenous wild-type Hsc70 (Newmyer and Schmid, 2001). Similarly, expression of Hsc70-4K71S during eye development caused a disruption of the regular arrays of ommatidia and a
BiP, an ER-associated Hsc70 required for translocation of specific to GAL4 4K71S (Fig. 7 D). This contrast in has little or no effect on the rough eye phenotype of Hsc70-in Hsc70-mediated uncoating of CCVs in vitro, its expression J-domain–containing auxilin has been shown to cooperate Rme-8 enhancement by full-length (D).

Figure 7. Rme-8 exhibits specific genetic interactions with Hsc70-4. Scanning electron micrographs of the adult eyes of (A) +/-; GMR-GAL4/UAS-Hsc70-4K71S, (B) UAS-Rme-8FL/+; GMR-GAL4/UAS-Hsc70-4K71S, (C) UAS-Rme-8J/D; GMR-GAL4/UAS-Hsc70-4K71S, (D) UAS-Aux/+; GMR-GAL4/UAS-Hsc70-4K71S, (E) +/-; GMR-GAL4/UAS-Hsc70-3K97S, and (F) UAS-Rme-8FL/+; GMR-GAL4/UAS-Hsc70-3K97S. Anterior is to the right. Bar, 25 μm.

roughening of the eye (Fig. 7 A). Coexpression of mRFP-Rme-8FL using the GMR-GAL4 driver caused further ommatidial disorganization and a decrease in eye size (Fig. 7 B), indicating that the rough eye phenotype of Hsc70-4K71S was enhanced by Rme-8 expression. Because overexpression of this functional mRFP-Rme-8FL alone has no obvious phenotype, this enhancement of Hsc70-4K71S suggests that Rme-8 genetically interacts with Hsc70-4. Moreover, the directionality of this interaction suggests that Rme-8 acts antagonistically with Hsc70.

To confirm that this genetic interaction is mediated by the J-domain, Rme-8 with its internal J-domain deleted (mRFP-Rme-8J; Fig. 5) was coexpressed with Hsc70-4 K71S using a GMR-GAL4 driver (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200311084/DC1). In contrast to full-length Rme-8, expression of Rme-8 with its internal J-domain deleted (mRFP-Rme-8J) exhibited little or no effect on the Hsc70-4K71S phenotype (Fig. 7 F), suggesting that Rme-8 cooperates with Hsc70-4, but not other Hsc70 family proteins.

The J-domain of Rme-8 binds to Hsc70 in the presence of ADP in vitro

To determine whether the J-domain of Rme-8 can bind to Hsc70 directly, a peptide (aa 1301–1368) containing the J-domain of the Drosophila Rme-8 was fused to GST, and the resulting fusion, GST-JRme-8, was subjected to a pull-down assay with bovine Hsc70. It is generally thought that J-domains recruit ATP-bound Hsc70 (Kelley, 1999), thus the pull-down was performed in either the presence of ATP or ADP to see if the binding was nucleotide dependent. Surprisingly, although GST-JRme-8 exhibited the same level of binding as GST alone in the presence of ATP, GST-JRme-8 showed an elevated level of binding to bovine Hsc70 in the presence of ADP (Fig. 8). Thus, unlike the J-domain from auxilin, the J-domain of Rme-8 appears to interact with ADP-bound Hsc70. These data, along with the enhancement by Rme-8 overexpression of the mutant rough eye phenotype due to dominant-negative Hsc70, suggest that the interaction of Rme-8 with Hsc70 may be different from that of other J-domain–containing proteins.

Discussion

We have isolated mutations in the Drosophila Rme-8 gene from a screen for mutants exhibiting interactions with a GTP hydrolysis–defective dynamin. Consistent with this genetic interaction, phenotypic analysis suggests that Rme-8 is required for the internalization of membrane ligands and endocytic tracers. Our data, along with the previous analysis of newly synthesized membrane proteins (Elefant and Palter, 1999). Expression of this dominant-negative Hsc70-3K97S during eye development caused a slight deformation and roughening of the eye (Fig. 7 E). Coexpression of a full-length Rme-8 using the GMR-GAL4 driver exhibited little or no effect on the Hsc70-3K97S phenotype (Fig. 7 F), suggesting that Rme-8 cooperates with Hsc70-4, but not other Hsc70 family proteins.
C. elegans Rme-8 (Zhang et al., 2001) and the high degree of sequence conservation among Rme-8 homologues from divergent species, strongly support the notion that Rme-8 is an evolutionarily conserved factor in endocytosis.

In C. elegans coelomocytes, Rme-8 proteins appeared to be associated with the membrane of some large multivesicular endosomal structures, although the identity of these structures was unclear (Zhang et al., 2001). In Drosophila Garland cells, the functional mRFP-tagged Rme-8 proteins appeared to be associated with multiple endocytic organelles including clathrin-, Rab5-, and Rab7-positive structures. This, along with the abnormal Clc, Hk, and Rab7 staining exhibited by Rme-8 mutants, might suggest that Rme-8 has a role in organizing and/or maintaining endosomal compartments. Disrupting the integrity of these endosomal compartments by Rme-8 mutations may cause a depletion of the cytosolic clathrin pool, which leads to the apparent block in the uptake of endocytic tracers. Alternatively, Rme-8 may have a more direct role at some early steps of endocytosis. The strong inhibition of tracer uptake and the genetic interaction with dynamin exhibited by Rme-8 mutants are certainly consistent with this possibility. Still, the understanding of the mechanisms of Rme-8 function would require the identification and characterization of its binding partners.

In any case, it seems clear that Rme-8 acts as an unexpected cofactor specific for Hsc70-4 in clathrin-mediated endocytosis. First, the mutant phenotypes of Rme-8, such as defects in Boss internalization, uptake of endocytic tracers, endosomal organization, and clathrin distribution, are remarkably similar to those of Hsc70-4 (Chang et al., 2002). Actually, the phenotypic resemblance appears to extend beyond defects in endocytosis as homoygous Rme-8 animals, like Hsc70-4 mutants, are developmentally delayed and often contain melanotic masses (unpublished data). However, the most compelling evidence is that Rme-8 exhibited a J-domain-dependent genetic interaction with Hsc70-4, but not with other members of Hsc70 family proteins. The in vitro pull-down assay further showed that the J-domain of Rme-8 could bind to Hsc70, suggesting that the Hsc70–Rme-8 interaction is likely to be direct. These data, along with the very similar genetic phenotypes exhibited by Rme-8 and Hsc70-4 mutants, strongly argue that the internal J-domain is critical for Rme-8 to regulate Hsc70 function.

The observation that overexpression of a functional Rme-8 has an inhibitory effect on a dominant-negative Hsc70 suggests that Rme-8 acts antagonistically to Hsc70 during endocytosis. This, along with the binding of the Rme-8 J-domain to ADP-bound Hsc70, suggests that Rme-8 may act as an inhibitor of the Hsc70 ATPase cycle. One proposed mechanism for this dominant-negative behavior of Hsc70K71S is that the mutant protein fails to release substrates upon ATP binding (Elefant and Palter, 1999). Thus, the GMR-Hsc70K71S rough eye phenotype could be caused by the sequestration of limited substrates or effectors. Although the J-domain of Rme-8 does not appear to bind to ATP-bound Hsc70, it is conceivable that overexpression of full-length Rme-8 may interact with the endogenous wild-type ADP-bound Hsc70 and cause a further sequestration of relevant substrates, thereby enhancing the Hsc70K71S eye phenotype.

In any event, although Rme-8 interacts with Hsc70 to regulate clathrin-dependent endocytosis, several evidences suggest that Rme-8 is not itself involved in clathrin uncoating. First, the strong endocytic phenotypes of Hsc70K71S, a point mutation with a moderate reduction in uncoating activity, were recapitulated by Rme-8 mutations, suggesting Rme-8 mutations affected some Hsc70-dependent processes distinct from uncoating. Consistent with this, inspection of the Rme-8 sequence does not reveal any clathrin-binding sites (Morgan et al., 2000). Furthermore, overexpression of auxilin and Rme-8 exhibit different genetic interactions with Hsc70. Together, these data suggest that Rme-8 most likely participates in some novel processes in Hsc70-mediated endocytosis. Thus, by establishing a link between Rme-8 and Hsc70, our data strongly suggest that the function of Hsc70 in endocytosis in vivo is more complex and probably not limited to clathrin uncoating. Although a more extensive role for Hsc70 has been implied by the observation that a decrease in Hsc70 function caused multiple defects in the endocytic pathway, such as the recycling of transferrin (Newmyer and Schmid, 2001; Chang et al., 2002), it has remained unclear whether the associated defects reflected distinct functions of Hsc70 as opposed to indirect consequences of a block in clathrin uncoating. Our identification of a second J-domain co-chaperone that is critical for endocytosis is certainly consistent with the pleiotropic effects of Hsc70 on the endocytic pathway. Conceivably, the interaction of the clathrin-binding protein auxilin with Hsc70 is selectively important for coated vesicle uncoating, and the Rme-8–dependent function of Hsc70 might control a downstream step. In either event, the identification of Rme-8 as a second cofactor for Hsc70 in endocytosis should provide a framework for further understanding the diverse functions of Hsc70 in this process.

Materials and methods
Fly genetics
All fly crosses were performed at 25°C in standard laboratory conditions. For the GMR-shhG69D modifier screen, w; iso 2; 3 males were mutagenized with 4,000 rad of x-ray irradiation, and were mass mated with w/w; TM3, Sb, P[w; GMR-shhG69D]3e, ftz. ry virgins. Progeny exhibiting altered GMR-shhG69D eye phenotype were individually backcrossed to w/w; TM3, Sb, P[w; GMR-shhG69D]3e, ftz. ry flies to ensure the phenotype bred true. Based on the segregation of GMR-shhG69D modifying phenotype, the putative mutants were then maintained over appropriate balances, and were grouped by complementation tests.

All of our Rme-8 alleles appear to be recessive embryonic lethal. However, mutant animals trans-heterozygous for certain allelic combinations, such as Rme-8<sup>−9</sup>Rme-8<sup>−2</sup>, do reach larval stages as rare escapers, allowing isolation of mutant eye discs and larval Garland cells for the HRP-Boss and tracer uptake analysis.

For the tangential section of adult retina, mitotic mutant clones were generated by heat-shocking progeny from crosses between w; FRT<sup>42D</sup>, Rme-8<sup>−</sup> males and hs-FRT<sup>1</sup>; FRT<sup>42D</sup>, P[w]<sup>47A</sup> females (Xu and Rubin, 1993). Mitotic clones in larval eye discs were generated using ey-FLP; FRT<sup>42D</sup>); GMR-myrtGP-2R (Chang et al., 2002).

To facilitate exogenous protein expression in larval Garland cells, UAS-derived transgenes (UAS-mRFP-Rme-8, UAS-GFP-rab5, UAS-GFP-rab7, and UAS-GFP-CIc) were driven with Act5C-GAL4 (#4414; Bloomington Drosophila stock center). For the Rme-8–Hsc70 interaction tests, UAS-Hsc70<sup>−</sup>F751 and UAS-Hsc70<sup>−</sup>3<sup>K71S</sup> (Elefant and Palter, 1999) were expressed during eye development by GMR-GAL4, UAS-GFP-rab5 and UAS-GFP-rab7 flies were obtained from M.A. Gonzalez-Gaitan (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany), UAS-Hsc70-<sup>−</sup>F751, UAS-Hsc70-<sup>−</sup>3<sup>K71S</sup>, and GMR-GAL4 were obtained from the Bloomington Drosophila stock center.
Genetic mapping of Rme-8

To clone E(shi)2-1, the mutation was first placed in the cytological interval of 45B1 to B7 between two P-element lines, f(2)K04512 (#10544; Bloomington Drosophila stock center) and f(2)K06021 (Bloomington Drosophila stock center; see text for details). This region has been subjected to an extensive F2 genetic analysis (Dockendorff et al., 2000), and complementation tests showed that E(shi)2-1 is allelic with f(2)K045a. Consistent with this, f(2)K045a also exhibited a strong interaction with GMC-shb+.

Of all the transcription units in the region between f(2)K04512 and f(2)K06021, CG8014 is the largest one. To test if E(shi)2-1 corresponds to CG8014, we first generated lethal excision lines from p(w+)BG2191 (#12659; Bloomington Drosophila stock center), a viable transposon line with a P-element inserted only 92 bp upstream of CG8014 transcription initiation site. Of the 25 excisions recovered, three were lethal, suggesting that the CG8014 locus might have been disrupted. All three lethal excision lines exhibited strong interaction with GMC-shb+/+. Failed to complement all alleles of E(shi)2-1, suggesting that E(shi)2-1 is CG8014. To confirm this was indeed the case, exons of CG8014 locus were amplified by PCR from the genomic DNA of E(shi)2(+/-) heterozygous animals, and were subjected to sequence analysis.

Histology and immunohistochemistry

For the visualization of HRP-Boss, eye discs dissected from third instar larve were stained in PBS containing 0.5 mg/ml DAB and 0.003% H₂O₂ for 30 min at RT (Sunio et al., 1999). The discs were then washed twice with PBS and fixed in 2% glutaraldehyde/PBS for 40 min at 4°C. After two washes with PBS, the discs were mounted in 80% glycerol/PBS.

For the endocytic tracer uptake assay, dissected Garland cells were incubated in PBS containing 0.2 mg/ml TR-avidin (A-821; Molecular Probes, Inc.) for 1 min at 25°C. The cells were then washed with PBS, chased for 20 min, and chilled on ice. Finally, they were incubated in PBS containing 0.2 mg/ml FITC-conjugated avidin (A-820; Molecular Probes, Inc.) for 1 min at 4°C, washed with ice-cold PBS, and fixed with 4% PFA/PBS for 20 min at 4°C.

Immunostaining of eye discs and Garland cells was performed according to Wolff (2000). Rabbit polyclonal anti-Hk antibody (a gift from H. Henrikson and J. Chappell, T.G., W.J. Welch, D.M. Schlossman, K.B. Palter, M.J. Schlesinger, and M.J. Schlesinger). Mouse monoclonal antibody to BSA (B-270; Sigma, St. Louis, MO) was used as a negative control.

Molecular biology

Because of its relatively large size, no complete Drosophila Rme-8 CDNA was available from the Berkeley Drosophila Genome Project EST collection, as LD15941, the longest EST clone of Rme-8, covered only the COOH-terminal 3.2 kb. To construct a full-length Rme-8 transcription unit, three fragments corresponding to the NH-terminal, central, and COOH-terminal regions of Rme-8 were individually amplified by PCR and sequentially subcloned into pBlueScript® SK (pBSSK; Stratagene). First, an mRFP peptide without the stop codon was PCR amplified from pRSETB-mRFP1 (Campbell et al., 2002) using 5’-GACAAATTCCAGCCGCTTTCCGCGAGGAGCTG-3’ and 5’-GACATGACGATCGCGCGATGTCGCCCAGG-3’ and subcloned as an EcoRI–ClaI fragment into pHFK-mRFP-RC. This mRFP-Rme-8 NH₂-terminal fusion was then excised as a NotI–XbaI fragment and subcloned into pBSSK-Rme-8 NH₂-FL. Finally, the COOH-terminal portion of Rme-8 was amplified from LD15941 using 5’-CCCGCTGCTAGTTAAATGTT-3’ and 5’-GCCGGTCGACGGCGGCCGCTACCGTTTGGCGAGACC-3’ to engineer a NotI site after the stop codon, but before the XhoI site. The resulting 1.9-kb PCR products were subcloned as an XhoI fragment into pBSSK-mRFP-Rme-8 NH₂-FL to complete the construction of pBSSK-mRFP-Rme-8 FL.

The entire transcription unit was then verified by direct sequencing, and subcloned as a NotI fragment into pLUST expression vector (Brand and Perrimon, 1993). To make the J-domain deletion derivative, a BspEI fragment (corresponding to aa 1114–1385) was excised from the central Xbal–Xho fragment before being subcloned into pBSSK-mRFP-Rme-8 FL.

To construct pGMR-auxilin, a DNA fragment containing the entire auxilin (CG1107) ORF was excised from GH26573 (Research Genetics) as an EcoRI–XhoI fragment with the XhoI end blunted and subcloned into the EcoRI–Hal plasmid of pGMR (Hay et al., 1994). Transgenic flies carrying these constructs were generated by P-element–mediated transformation as described previously (Rubin and Spradling, 1982). To construct GST-Jαm, a DNA fragment containing the J-domain was generated by PCR using primers 5’-GAATTCCTACAGCAGCTAGG-3’ and 5’-CTCGAGGGGATCTGCGCCACCACCCGG-3’. The resulting product was subcloned as an EcoRI–Xhol fragment into pGEX-6P.

Rme-8 J-domain pull-downs

In a total volume of 20 μl, 4 μM GST-Jαm was incubated for 15 min at 25°C with 4 μM Hsc70 protein and 2 mM nucleotide in uncoating buffer containing 0.1% ovalbumin and 125 mM KCl. The binding reaction was transferred to 4°C and incubated with 20 μl GSH-Sepharose (50% slurry) with shaking for 30 min. The beads were collected by centrifugation at 16,000 g and washed twice with ice-cold buffer containing the appropriate nucleotide (0.1 mM). The bound material was then analyzed by SDS-PAGE.

Online supplemental material

Fig. S1 showed the spatial overlaps between the localization of Rme-8 and Hsc70 proteins (Wolff et al., 2002) in larval Garland cells. Fig. S2 showed the in vivo expression of various mRFP-tagged Rme-8 constructs in larval eye imaginal discs. Fig. S3 showed the uptake of TR-avidin (A-821; Molecular Probes, Inc.) by α-adaptin mutant Garland cells (González-Gaitán and Jackle, 1997). Videos 1 and 2 showed the uptake of TR-avidin by wild-type and Rme-8 mutant Garland cells, respectively, using time-lapse confocal microscopy. Live Garland cells carrying GFP-rab7 transgenes were dissected and mounted in PBS containing 0.2 mg/ml TR-avidin. Confocal images of these cells were captured at a 10-s interval for 10 min, and were then processed by GraphicConverter software (4 frames/s; Lemke Software). Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200311084/DC1.

We would like to thank Marcos A. González-Gaitán for providing fly strains, Sherri Newmyer (University of California, San Francisco, San Francisco, CA) for providing bovine Hsc70 proteins, and Helmut Kramer for providing anti-Hk antibody. We also thank members of the Mellman/Warren laboratory for their interest and advice.

This work was supported by National Institutes of Health grant GM29765 and by the Ludwig Institute for Cancer Research. H. Chang was a fellow of the Damon Runyon-Walter Winchell Cancer Research Foundation.

Submitted: 17 November 2003
Accepted: 17 February 2004

References

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

Bukau, B., and A.L. Horwich. 1998. The Hsp70 and Hsp60 chaperone machines. Cell. 92:351–366.

Cagan, R.L., H. Kramer, A.C. Hart, and S.L. Zipursky. 1992. The bride of sevenless and sevenless interaction: internalization of a transmembrane ligand. Cell. 69:393–399.

Campbell, R.E., O. Tour, A.E. Palmer, P.A. Steinbach, G.S. Baird, D.A. Zacharas, and R.Y. Tien. 2002. A monomeric red fluorescent protein. Proc. Natl. Acad. Sci. USA. 99:7877–7882.

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

Chappell, T.G., W.J. Welch, D.M. Schlossman, K.B. Palter, M.J. Schlesinger, and J.E. Rothman. 1986. Uncoating ATPase is a member of the 70 kilodalton

Downloaded from jcb.rupress.org on August 19, 2017
family of stress proteins. *Cell* 453:13–17.

Chen, B., T. Chiu, E. Harms, J.P. Gegen, and S. Strickland. 1998. Mapping of *Drosophila* mutations using site-specific male recombination. *Genetics* 149:157–163.

Damke, H., D.D. Binns, H. Ueda, S.L. Schmid, and T. Baba. 2001. Dynamin GTPase domain mutants block endocytic vesicle formation at morphologically distinct stages. *Mol. Biol. Cell.* 12:2578–2589.

Dockendorff, T.C., S.E. Robertson, D.L. Faulkner, and T.A. Jongens. 2000. Genetic characterization of the 44D-45B region of the *Drosophila melanogaster* genome based on an F2 lethal screen. *Mol. Gen. Genet.* 263:137–143.

Elefant, F., and K.B. Pulcher. 1999. Tissue-specific expression of dominant negative mutant *Drosophila* Hsc70 causes developmental defects and lethality. *Mol. Biol. Cell.* 10:2101–2117.

Entchev, E.V., A. Schwabedissen, and M. González-Gaitán. 2000. Gradient formation of the TGF-β homolog Dpp. *Cell* 103:981–991.

Gall, W.E., M.A. Higginbotham, C. Chen, M.F. Ingrain, D.M. Cyr, and T.R. Graham. 2000. The auxilin-like phosphoprotein Swa2p is required for clathrin function in yeast. *Curr. Biol.* 10:1349–1358.

Ghosh, P., and S. Kornfeld. 2003. AP-1 binding to sorting signals and release from clathrin-coated vesicles is regulated by phosphorylation. *J. Cell Biol.* 160:699–708.

González-Gaitán, M., and H. Jackle. 1997. Role of *Drosophila* α-adaptin in presynaptic vesicle recycling. *Cell* 88:767–776.

Greener, T., B. Grant, Y. Zhang, X. Wu, L.E. Greene, D. Hirsh, and E. Eisenberg. 2001. *Caenorhabditis elegans* auxilin: a J-domain protein essential for clathrin-mediated endocytosis in vivo. *Nat. Cell Biol.* 3:215–219.

Hannan, L.A., S.L. Newmeyer, and S.L. Schmid. 1998. ATP- and cytosol-dependent release of adaptor proteins from clathrin-coated vesicles: A dual role for Hsc70. *Mol. Biol. Cell.* 9:2217–2229.

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

Holstein, S.E., H. Ungewickell, and E. Ungewickell. 1996. Mechanism of clathrin basket dissociation: separate functions of protein domains of the DnaJ homologue auxilin. *J. Cell Biol.* 135:925–937.

Honing, S., G. Kreimer, H. Robenek, and B.M. Jockusch. 1994. Receptor-mediated endocytosis and priming clathrin and primes it to interact with vesicle membranes. *J. Biol. Chem.* 275:8439–8447.

Kelley, W.L. 1999. Molecular chaperones: How J domains turn on Hsp70s. *Curr. Biol.* 9:R305–R308.

Kirchhausen, T. 2000. Three ways to make a vesicle. *Nat. Rev. Mol. Cell Biol.* 1:187–195.

Kosaka, T., and K. Ikeda. 1983. Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of *Drosophila melanogaster*, shibire1. *J. Cell Biol.* 97:499–507.

Kramer, H., and M. Pihyster. 1996. Mutations in the *Drosophila* hook gene inhibit endocytosis of the boss transmembrane ligand into multivesicular bodies. *J. Cell Biol.* 135:1205–1215.

Lloyd, T.E., R. Atkinson, M.N. Wu, Y. Zhou, G. Pennetta, and H.J. Bellen. 2002. Hrs regulates endosome membrane invagination and tyrosine kinase receptor signaling in *Drosophila*. *Cell* 108:261–269.

Morgan, J.R., K. Prasad, W. Hao, G.J. Augustine, and E.M. Lafer. 2000. A conserved clathrin assembly motif essential for synaptic vesicle endocytosis. *J. Neurosci.* 20:8667–8676.

Morgan, J.R., K. Prasad, S. Jin, G.J. Augustine, and E.M. Lafer. 2001. Uncoating of clathrin-coated vesicles in presynaptic terminals: roles for Hsc70 and auxilin. *Neuron.* 32:289–300.

Narayanam, R., and M. Ramaswamy. 2003. Regulation of dynamin by nucleoside diphosphate kinase. *J. Bioenerg. Biomembr.* 35:49–55.

Newmeyer, S.L., A. Christensen, and S. Sever. 2003. Auxilin-dynamin interactions link the uncoating ATPase chaperone machinery with vesicle formation. *Dev. Cell.* 4:929–940.

Newmeyer, S.L., and S.L. Schmid. 2001. Dominant-interfering Hsc70 mutants disrupt multiple stages of the clathrin-coated vesicle cycle in vivo. *J. Cell Biol.* 152:607–620.

Pishvaee, B., G. Costaguta, B.G. Yeung, S. Ryzanets, T. Greener, L.E. Greene, E. Eisenberg, J.M. McCaffery, and G.S. Payne. 2000. A yeast DNA J protein required for uncoating of clathrin-coated vesicles in vivo. *Nat. Cell Biol.* 2:958–963.

Poodry, C.A., and L. Edgar. 1979. Reversable alteration in the neuromuscular junctions of *Drosophila melanogaster* bearing a temperature-sensitive mutation, shibire. *J. Cell Biol.* 81:520–527.

Poodry, C.A., L. Hall, and D.T. Suzuki. 1975. Developmental properties of *Shibire*: a pleiotropic mutation affecting larval and adult locomotion and development. *Dev. Biol.* 32:373–386.

Robinson, S., and K. White. 1988. The locus clav of *Drosophila melanogaster* is expressed in neurons at all developmental stages. *Dev. Biol.* 126:294–303.

Rubin, G.M., and A.C. Spradling. 1982. Genetic transformation of *Drosophila* with transposable elements. *Science.* 218:348–353.

Rubin, G.M., L. Hong, P. Brokstein, M. Evans-Holm, E. Frise, M. Stapleton, and D.A. Harvey. 2000. *Drosophila* complementary DNA resource. *Science.* 287:2222–2224.

Schlossman, D.M., S.L. Schmid, W.A. Braell, and J.E. Rothman. 1984. An enzyme that removes clathrin coats: purification of an uncoating ATPase. *J. Cell Biol.* 99:723–733.

Schmid, S.L. 1997. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.* 66:511–548.

Spradling, A.C., D.M. Stern, I. Kiss, J. Roote, T. Laverty, and G.M. Rubin. 1995. Gene disruptions using P transposable elements: an integral component of the *Drosophila* genome project. *Proc. Natl. Acad. Sci. USA.* 92:10824–10830.

Spradling, A.C., D. Stern, A. Beaton, E.J. Rihm, T. Laverty, N. Mozden, S. Misra, and G.M. Rubin. 1999. The Berkeley *Drosophila* Genome Project gene disruption project: single P-element insertions mutating 25% of vital *Drosophila* genes. *Genetics.* 153:35–177.

Sunio, A., A.B. Mercall, and H. Kramer. 1999. Genetic dissection of endocytic trafficking in *Drosophila* using a hereditary peroxidase-bide of sevenless chimera: hook is required for normal maturation of multivesicular endosomes. *Mol. Biol. Cell.* 10:847–859.

Tomanack, P., A. Beaton, R. Weizmann, E. Kwan, S. Shu, S.E. Lewis, S. Richards, M. Ashburner, V. Hartenstein, S.E. Celniker, and G.M. Rubin. 2002. Systematic determination of patterns of gene expression during *Drosophila* embryogenesis. *Genome Biol.* 3: researched0088.1–0088.14.

Umeda, A., A. Meyerholz, and E. Ungewickell. 2000. Identification of the universal cofactor (auxillin 2) in clathrin coat dissociation. *Eur. J. Cell Biol.* 79:336–342.

Ungewickell, E., H. Ungewickell, S.E. Holstein, R. Lindner, K. Prasad, W. Brouch, B. Martin, L.E. Greene, and E. Eisenberg. 1995. Role of auxilin in uncoating clathrin-coated vesicles. *Nature.* 378:632–635.

van der Bleik, A.M., and E.M. Meyerowitz. 1991. Dynamin-like protein encoded by the *Drosophila* shibire gene associated with vesicular traffic. *Nature.* 351:411–414.

Wolff, T. 2000. Histological techniques for the *Drosophila* eye. Parts I and II. In *Drosophila* Protocols. W. Sullivan, M. Ashburner, and R.S. Hawley, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 201–244.