**Introduction**

Endocytosis is crucial for the reformation of functional synaptic vesicles (SVs) after exocytosis of neurotransmitters. Although multiple mechanisms have been observed during SV endocytosis (Gad et al., 2000; Teng and Wilkinson, 2000; Sun et al., 2002; Granseth et al., 2006; He et al., 2006), all of these involve the detachment of the vesicle from the plasma membrane, a step that is known as fission. Current evidence indicates that the GTPase dynamin functions at the fission step. For example, endocytic vesicles are arrested at the fission step in *shibire*<sup>ts1</sup>, a temperature-sensitive mutant of dynamin, and in the presence of the nonhydrolyzable analogue of GTP (Kosaka and Ikeda, 1983; Takei et al., 1998). As inhibition of dynamin leads to complete blockage of SV endocytosis in *shibire* mutants, dynamin activity is absolutely required for SV endocytosis (Koenig and Ikeda, 1989; Shupliakov et al., 1997; Verstreken et al., 2002; Poskanzer et al., 2003).

To further understand dynamin function, we and others have previously characterized a major binding partner of dynamin, Dap160 (dynamin-associated protein 160 kD) and shown that it maintains proper dynamin localization at synapses (Broadie, 2004; Koh et al., 2004; Marie et al., 2004; Evergren et al., 2007). Although there is a complete block in endocytosis during inhibition of dynamin function, residual endocytic activity persists in the absence of Dap160. This prompted us to study other proteins that may function in conjunction with dynamin.

EGF receptor pathway kinase substrate clone 15 (Eps15) has been found in protein complexes with multiple endocytic proteins, including dynamin and the vertebrate homologue of Dap160, intersectin (Santolini et al., 1999; Sengar et al., 1999; Salcini et al., 2001). Originally discovered as an EGF receptor kinase pathway substrate clone 15 (Eps15) is a protein implicated in endocytosis, endosomal protein sorting, and cytoskeletal organization. Its role is, however, still unclear, because of reasons including limitations of dominant-negative experiments and apparent redundancy with other endocytic proteins. We generated *Drosophila eps15*-null mutants and show that Eps15 is required for proper synaptic bouton development and normal levels of synaptic vesicle (SV) endocytosis. Consistent with a role in SV endocytosis, Eps15 moves from the center of synaptic boutons to the periphery in response to synaptic activity. The endocytic protein, Dap160/intersectin, is a major binding partner of Eps15, and *eps15* mutants phenotypically resemble *dap160* mutants. Analyses of *eps15 dap160* double mutants suggest that Eps15 functions in concert with Dap160 during SV endocytosis. Based on these data, we hypothesize that Eps15 and Dap160 promote the efficiency of endocytosis from the plasma membrane by maintaining high concentrations of multiple endocytic proteins, including dynamin, at synapses.
substrate and a putative oncogene, Eps15 was later implicated in endocytosis and endosomal trafficking by in vitro studies (Fazioli et al., 1993; Tang and Cai, 1996; Tebar et al., 1996; Wendland et al., 1996; Carbone et al., 1997; Benmerah et al., 1999; Torrisi et al., 1999; Bache et al., 2003). Intriguingly, RNA interference knockdown of Eps15 in a nonneuronal cell line indicated that Eps15 is redundant with other endocytic proteins during the endocytosis of EGF receptor (Sigismund et al., 2005). This prompted several groups of investigators to examine its role in SV endocytosis. In Caenorhabditis elegans, eps15-null mutants undergo a temperature-sensitive paralysis, and mutant nerve terminals exhibit vesicle depletion at high temperatures, indicating a role for Eps15 in maintaining the presence of SVs at the nerve terminals (Salcini et al., 2001). More recently, a Drosophila mutant with reduced Eps15 levels was shown to undergo impairment in neurotransmission and paralysis only at high temperatures but not at physiological temperatures (Majumdar et al., 2006). In contrast, injection of dominant-negative peptides to inhibit Eps15 function in axonal axonal terminals leads to a very subtle decrease in the number of SVs but a strong inhibition of neurotransmitter release (Morgan et al., 2003). The in vivo data therefore suggest that Eps15 plays either an accessory role or no role in endocytosis. We therefore decided to generate eps15 protein-null alleles and investigate the role of Eps15 in flies in more detail.

Here, we describe the functional characterization of Drosophila Eps15 to address the following questions: (1) Does Eps15 function in endocytosis of SVs from the plasma membrane at the nerve terminal? (2) If so, at which step is Eps15 involved? (3) Is there a functional relationship between Eps15 and Dap160? Our data indicate that Eps15 is required for efficient SV endocytosis and that loss of Eps15 causes a severe reduction in dynamin and Dap160 protein levels at neuromuscular junctions (NMJs). Furthermore, the endocytic defects of eps15- and dap160-null mutations are not additive, suggesting that Eps15 and Dap160 act at the same step during endocytosis.

Results

Drosophila eps15 is an essential gene

The Drosophila genome contains a single homolog of mammalian Eps15 (Lloyd et al., 2000). It has three Eps15 homology (EH) domains, a coiled-coil domain, a region with multiple DPF (aspartate-proline-phenylalanine) motifs, and at least one, possibly two, ubiquitin-interacting motifs (UIMs) at the C terminus, similar to its mammalian homologue (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200701030/DC1; Polo et al., 2002). To determine its spatial and subcellular distribution, we raised two different polyclonal antibodies against epitopes shown in Fig. S1 A: a rabbit antibody and a guinea pig antibody. Both antibodies are specific, as mutants that lack the eps15 gene display no immunoreactivity on Western blots (Fig. 1 D and Fig. S1 B) and immunohistochemically stained tissues (Fig. 2 A and Fig. S1, C and D). Western blots of fly heads show an ∼160-kD and a 90-kD band, whereas the predicted mol wt is ∼132 kD (Fig. 1 D). Immunoprecipitation experiments with rabbit anti-Eps15 and fly head protein extracts show that Eps15 binds to Dap160, as detected by Western blot and mass spectrometry of Coomassie blue–stained bands (Fig. 1 A and Table S1). The Coomassie blue–stained gel indicates that Dap160 is a major binding partner of Eps15, in agreement with the biochemical interaction data for the vertebrate homologue of Dap160, intersectin (Sengar et al., 1999). In addition, rabbit anti-Eps15 also coimmunoprecipitates Eps15 with α-adaptin, epsin/Liquid facin (Sengar et al., 1999). In addition, rabbit anti-Eps15 also coimmunoprecipitates Eps15 with α-adaptin, epsin/Liquid facin (Sengar et al., 1999). In addition, rabbit anti-Eps15 also coimmunoprecipitates Eps15 with α-adaptin, epsin/Liquid facin (Sengar et al., 1999). In addition, rabbit anti-Eps15 also coimmunoprecipitates Eps15 with α-adaptin, epsin/Liquid facin (Sengar et al., 1999). In addition, rabbit anti-Eps15 also coimmunoprecipitates Eps15 with α-adaptin, epsin/Liquid facin (Sengar et al., 1999). In addition, rabbit anti-Eps15 also coimmunoprecipitates Eps15 with α-adaptin, epsin/Liquid facin (Sengar et al., 1999).

To determine the function of Eps15 in vivo, we disrupted the eps15 gene using two different reverse genetic approaches. We imprecisely excised E2513, a semilethal P element insertion in the 5′ untranslated region of the eps15 gene (Fig. 1 B; Lloyd et al., 2000). This allowed isolation of a lethal allele, eps15575.
eps15 effects the same gene, presumably containing transposons flanking the recombination between two FLP recognition target (FRT)–cDNA using a neuronal-specific driver.

To determine whether Eps15 is required in the nervous system, we also deleted the entire eps15 locus by inducing site-specific recombination between two FLP recognition target (FRT)–containing transposons flanking the eps15 locus, generating eps15Δ29 (Fig. 1 B; Golic, 1994; Thibault et al., 2004). Complementation analysis revealed that eps15EP2513, eps15Δ29, and Df(2R)Dll-MP (a large deficiency uncovering the genomic region encompassing the eps15 locus), all fail to complement each other’s lethality, indicating that each of these mutations affects the same gene, presumably eps15 (Fig. 1 C). To determine the severity of these mutations, we examined the lethal phase of the mutants in various allelic combinations. The eps15Δ29 homozygote and eps15Δ75/eps15Δ29 transheterozygote animals die as pharate adults, eps15Δ75 homozygous animals die as first instars, and eps15Δ75/Df(2R)Dll-MP transheterozygotes die as third instars (Fig. 1 C). To ensure that the lethality is due to mutations in the eps15 gene, we introduced a genomic fragment encompassing the eps15 locus into the mutant backgrounds (Fig. 1 B; genomic rescue construct). This rescues eps15Δ29 homozygotes and eps15Δ75/eps15Δ29 transheterozygotes to normal adults but only partially rescues eps15Δ75/Df(2R)Dll-MP transheterozygotes, as the adults are uncoordinated. The lethal phase and the rescue data indicate that the lethality of eps15Δ29 is solely due to deletion of the eps15 locus and that eps15Δ75 carries a second site mutation that is not shared by eps15Δ29. To determine whether Eps15 is required in the nervous system, we expressed the eps15 cDNA using a neuronal-specific driver (elav-Gal4). This rescued eps15Δ75/eps15Δ29 to normal adults with no obvious morphological defects, indicating that Eps15 is predominantly required in the nervous system (Fig. 1 C).

To determine which mutations correspond to null alleles, we performed Western blot analyses. The eps15Δ75/eps15Δ29 and eps15Δ75/eps15Δ29 animals do not express detectable levels of Eps15 protein on Western blots (Fig. 1 D and Fig. S1 B). In summary, we have isolated two independent protein-null mutations, eps15Δ75 and eps15Δ29, which cause lethality, indicating that eps15 is an essential gene in Drosophila, unlike the C. elegans eps15-null mutation, which is viable (Salcini et al., 2001). In addition, the essential role of Eps15 is confined to the nervous system in flies.

Eps15 is enriched at synapses in the SV pool and migrates to the plasma membrane during stimulation

To determine where Eps15 is expressed, we performed immunohistochemistry of third instar larvae (Bellen and Budnik, 2000). Both anti-Eps15 antibodies reveal the same expression profiles, and this immunoreactivity is lost in null mutant animals, indicating that the labeling is specific (Fig. 2 A and Fig. S1 C and D). Eps15 is expressed broadly in the nervous system and is much enriched in the neuropil of the central nervous system and at the NMJs (Fig. 2, A–I). In addition, most, and possibly all, imaginal discs also express Eps15 (unpublished data). To determine the subsynaptic localization of Eps15 at the NMJ, we costained Eps15 with Dlg, which at this resolution is effectively a post synaptic compartment of the NMJ (Fig. 2, B–I). (B) Eps15 (green) is closely associated with Dlg (magenta) in the neuropil of the larval ventral nerve cord. (Bottom) Merge of anti-Eps15 and anti-Dlg staining patterns. (C) Dlg (green), which labels the postsynaptic compartment of the NMJ at this resolution, shows a pattern that envelopes that of Eps15 (magenta). This indicates that Eps15 is enriched in the presynaptic compartment of the NMJ. (D) Eps15 (magenta) is excluded from puncta decorated by Bruchpilot (nc82 antigen; green), which marks T-bars. (E) Eps15 (magenta; left) and Dap160 (green; right in merged panel) largely colocalize in the boutons, resulting in large areas of white in the merged image (right). A and C–E show single confocal slices, and B shows a projection view. (F) Eps15 immunogold labeling in two zones located within 500 nm lateral to T-bars (thick arrows in H and I). The first zone (gray bars) refers to areas 0–200 nm from the plasma membrane, and the second zone (white bars) refers to areas 200–400 nm from the plasma membrane. Relative distribution shows the percentage of total immunogold labeling distributed in either zones. Error bars indicate standard deviation. **, P < 0.01; ***, P < 0.001 [t test]. Bar graphs represent mean values from 15 resting wild-type boutons, 18 wild-type boutons stimulated with 60 mM K+, and 20 shibire214a boutons stimulated with 60 mM K+. (G) Silver-enhanced immunogold labeling (black precipitates; see Materials and methods) of Eps15 using guinea pig anti-Eps15 reveals that Eps15 is associated with vesicle-rich regions of a wild-type resting bouton. (H) A high-magnification view of anti-Eps15 immunogold labeling in a wild-type resting bouton shows that Eps15 is associated with vesicles but largely excluded from the area around the synaptic dense body or T-bar (short wide arrow). (I) Upon stimulation with 60 mM K+, anti-Eps15 immunogold labeling becomes concentrated at plasma membrane regions adjacent to the active zone (long arrows). Bars: (G) 1 μm; (H and I) 200 nm. Guinea pig anti-Eps15 was used in A, B, and E–I, and rabbit anti-Eps15 was used in C and D.
High K+ stimulation of shibirets1 NMJs at the restrictive temperature leads to a dramatic redistribution of Eps15 in boutons independent of SVs. (A) Silver-enhanced immunogold labeling of Eps15 using guinea pig anti-Eps15 reveals that Eps15 is predominantly localized to the plasma membrane (thin arrows) in areas surrounding the T-bars (thick arrow; the 60 mM K+ stimulus did not completely deplete the preexisting pool of vesicles in the shibirets1 bouton). As reported previously in shibirets1 mutants, this stimulus leads to mild depletions of the vesicle pool in a subset of boutons (A) and more severe depletions in other boutons (not depicted), even though endocytosis is blocked at the restrictive temperature (Estes et al., 1996). (Inset) Confocal imaging of guinea pig anti-Eps15 staining reveals that Eps15 is localized in the lumen of a resting bouton (rest) and is redistributed to the periphery in a bouton stimulated with 60 mM K+ (stim). m, mitochondria; a, axoplasm. (B) Boxed area in A is shown at higher magnification. (C) Electron micrograph of the same area from an adjacent ultrathin section. Gold particles were often located close to the rims of invaginating pits at the plasma membrane. Small arrows indicate fuzzy coats on vesicles. Although the areas that are immunolabeled for Eps15 seem to be reduced after stimulation, levels of Eps15 proteins are unchanged in resting vesicles or pits at the plasma membrane surrounding the T-bars (Fig. 2, F and I). This redistribution of Eps15 is particularly evident in shibirets1 mutants stimulated at a restrictive temperature (Fig. 2 F and Fig. 3 A). Interestingly, a subset of Eps15 is localized to the rim of invaginating vesicles or pits at the plasma membrane in stimulated shibirets1 mutant boutons (Fig. 3, B and C). In summary, Eps15 is associated with vesicle in resting boutons and is mobilized to areas adjacent to active zones upon stimulation.

Eps15 regulates neuromuscular synapse development

A larval NMJ typically consists of a series of boutons connected by neurites, like beads on string. These boutons are dynamic structures that sprout and retract during larval development (Eaton et al., 2002). Because Dap160 mutants display numerous extra NMJ boutons and branching (Koh et al., 2004; Marie et al., 2004), and because Eps15 and Dap160 interact, we quantified the number of boutons and branches in eps15 mutant NMJs. Compared with controls, mutant larvae show a pronounced increase in bouton number at three different NMJ synapses (Fig. 4, A–D and F). In addition, control NMJs typically have few branchpoints from which boutons sprout (Fig. 4, C and G, arrows). In contrast, eps15 mutant NMJs show more branchpoints, some of which lead to more than two branches (Fig. 4, D and G). For example, mutant muscle 4 synapses commonly exhibit boutons with three branches or more, but such hyperbranched boutons are infrequent in controls (Fig. 4, A–D). Branch points from which boutons sprout are similar to Dap160 mutants (Koh et al., 2004; Marie et al., 2004).

Eps15 is required to sustain high-frequency neurotransmitter release

Eps15 has been implicated in endocytosis and endosomal trafficking (Tebar et al., 1996; Benmerah et al., 1999; Torrisi et al., 1999; Bache et al., 2003). In addition, loss of Eps15 in C. elegans leads to a defect in vesicle cycling at the restrictive temperature (Salcini et al., 2001). To define the role of Eps15 in the SV cycle more precisely, we performed electrophysiological assays of mutant eps15 NMJs. To determine whether the exocytic machinery is intact, we elicited low-frequency stimuli and recorded evoked excitatory junctional potentials (EJPs) in 0.5, 1, and 5 mM extracellular Ca2+. Under these conditions, the EJPs are similar to wild-type amplitudes (Fig. 5 A). In addition, spontaneous release or miniature EJPs (mEJPs) of eps15 mutants to show a similar honeycomb-like localization pattern (Roos and Kelly, 1999). These data prompted us to investigate Eps15 localization at the ultrastructural level. Silver-enhanced immunogold labeling of resting wild-type NMJ boutons with anti-Eps15 reveals that Eps15 is associated with vesicles in the lumen of boutons but is at neither presynaptic dense bodies (T-bars) nor vesicle-free areas of the boutons (Fig. 2, G–H, black precipitates; see Materials and methods). This immunogold labeling pattern is not observed when a nonrelevant primary antibody was used, indicating that the anti-Eps15 label is specific (unpublished data). Upon a mild stimulation with 60 mM K+, Eps15 becomes more concentrated in regions close to the plasma membrane surrounding the T-bars (Fig. 2, F and I). This redistribution of Eps15 is particularly evident in shibirets1 mutants stimulated at a restrictive temperature (Fig. 2 F and Fig. 3 A). Interestingly, a subset of Eps15 is localized to the rim of invaginating vesicles or pits at the plasma membrane in stimulated shibirets1 mutant boutons (Fig. 3, B and C). In summary, Eps15 is associated with vesicle in resting boutons and is mobilized to areas adjacent to active zones upon stimulation.

Eps15 is required to sustain high-frequency neurotransmitter release

Eps15 has been implicated in endocytosis and endosomal trafficking (Tebar et al., 1996; Benmerah et al., 1999; Torrisi et al., 1999; Bache et al., 2003). In addition, loss of Eps15 in C. elegans leads to a defect in vesicle cycling at the restrictive temperature (SalCini et al., 2001). To define the role of Eps15 in the SV cycle more precisely, we performed electrophysiological assays of mutant eps15 NMJs. To determine whether the exocytic machinery is intact, we elicited low-frequency stimuli and recorded evoked excitatory junctional potentials (EJPs) in 0.5, 1, and 5 mM extracellular Ca2+. Under these conditions, the EJPs are similar to wild-type amplitudes (Fig. 5 A). In addition, spontaneous release or miniature EJPs (mEJPs) of eps15 mutants

marker (Lahey et al., 1994). Eps15 is enveloped by Dil at the NMJ, indicating that Eps15 is enriched in the presynaptic compartment (Fig. 2 C). Collectively, we conclude that Eps15 is widely expressed but is enriched in the neuropil and in the presynaptic compartment of the NMJ. At the NMJ, Eps15 is present in the central region of the boutons in a honeycomb-like pattern, where most Eps15 staining was found surrounding active zones, defined by anti-Bruchpilot/ncc82 (Fig. 2 D; Wucherpfennig et al., 2003; Wagh et al., 2006). In addition, Eps15 shows extensive colocalization with Dap160 (Fig. 2 E). Therefore, at the level of confocal microscopy, Eps15 is enriched in areas surrounding active zones and colocalizes with Dap160, which has been reported
shows similar amplitude but higher frequency than controls (Fig. 5, B and C). Therefore, in the absence of Eps15, exocytosis of neurotransmitters is normal under low-frequency stimulation, but spontaneous release is altered. To determine whether eps15 mutant NMJs are able to efficiently recycle vesicles under repetitive stimulation, we applied 10 Hz of stimulation for 10 min in 5 mM extracellular Ca^{2+}. Control NMJs are able to maintain EJPs at ~90% of the initial amplitudes, but eps15 NMJs show a strong synaptic depression to <40% of initial EJP amplitudes (Fig. 5 D). In this assay, shibire^ts1-encoded a temperature-sensitive dynamin mutant, depresses to zero as a result of a complete block in endocytosis, whereas dap160 mutant NMJs show profiles of synaptic depression similar to those of eps15 mutant NMJs (Fig. 5 D; Koh et al., 2004). Hence, eps15 mutant NMJs show a defect in vesicle recycling that is similar to dap160 mutant NMJs. Compared with our current data on eps15 and dap160 mutants, other endocytic mutants like shibire, synaptojanin, or endophilin show faster synaptic depression under a milder stimulation condition (higher Mg^{2+}/Ca^{2+} ratio) in our previous experiments (Verstreken et al., 2002). In summary, although exocytosis of neurotransmitters is essentially normal at eps15 mutant NMJs, vesicle recycling is impaired but not blocked.

**Eps15 is required for efficient SV endocytosis**

One possible cause for the vesicle recycling defect in eps15 mutants is impaired endocytosis. To assay for endocytosis at

---

**Figure 4.** eps15 mutants show bouton overgrowth at the NMJ. Muscle 12 NMJ of eps15^e75/eps15^e75 L3 larva (B) possess more boutons and branches than a control larva (A) as revealed by mouse anti-Dlg staining. A close-up view of anti-HRP–stained muscle 4 type IB boutons reveals that there are more branchpoints (arrows) in eps15^e75/eps15^e75 (D) than in controls (C). A bouton that was connected to more than one distal neurite or bouton (arrowheads) was counted as a branchpoint (arrow). (E) Neuronal expression of the full-length eps15 cDNA rescues the supernumerary boutons at the muscle 12 in the eps15^e75/eps15^e75 background (see Fig. 9 B for quantification). (F) Comparison of mean bouton numbers per NMJ at muscle 12, 4, and 6/7 in eps15^e75/eps15^e75 (e75/∆29), eps15^e75/DF(2L)Dll-MP (e75/Df), and control larvae reveals that eps15 mutant NMJs possess significantly more boutons per synapse than control NMJs. In addition, bouton numbers at Df/Df NMJs are significantly more than those at e75/∆29 NMJs (m4, P < 0.01; m6/7, P < 0.05). (G) Comparison of branchpoint numbers per NMJ (arrow in inset shows an example of a bouton with three branches) reveals that eps15 mutant muscle 4 NMJs possess more boutons with two branches (gray bar) and three or more branches (white bar) than controls. In addition, Df/Df NMJs exhibit somewhat more branchpoints than e75/∆29, but the difference is not significant (P > 0.05). w^1 larvae were used as controls. Bars: (A, B, and E) 20 μm; (C and D) 10 μm. *, P < 0.05; **, P < 0.01 (controls vs. mutants; Mann-Whitney U-test). Error bars indicate SEM, and the numbers in histograms indicate the number of larvae.

**Figure 5.** eps15 mutants show normal neurotransmitter release when stimulated at low frequency but are unable to sustain release under high-frequency stimulation. (A) Mean EJP measured at the NMJ reveals that neurotransmitter release is normal at eps15^e75/eps15^e75 (e75/∆29) and eps15^e75/DF(2L)Dll-MP (e75/Df) during 0.1 Hz of stimulation (0.5 and 1 mM Ca^{2+}) and single nerve stimulations (5 mM Ca^{2+}). (B) Mean mEJP amplitudes recorded in 0.5 mM Ca^{2+} and 3 μM tetrodotoxin were not different between e75/∆29 and w controls. (C) However, mean mEJP frequency of e75/∆29 is higher than controls. (D) When stimulated at 10 Hz, eps15 mutant NMJs show defects in maintaining release. Specifically, the mean EJPs of both e75/∆29 and e75/Df were significantly different from controls (P < 0.01) after 60 s. Introducing one copy of eps15 genomic fragment in the e75/∆29 background rescued this defect (genomic vs. control, P > 0.1; genomic vs. e75/∆29, P < 0.01). Note that the two different eps15-null transheterozygotes show defects that are similar to that of dap160^ts1/DF(2L)burK1 (dap160 null) but less severe than that of shibire^ts1 (shibire^ts1 data is adapted from Koh et al. [2004] for comparison). w^1 larvae were used as controls. *, P < 0.05 (controls vs. mutants; Mann-Whitney U-test). Error bars indicate SEM, and the numbers in histograms indicate the number of larvae.
In contrast to its role at the synapse, Eps15 is not essential for receptor-mediated endocytosis in hemocytes (Fig. S2, A–C and F; available at http://www.jcb.org/cgi/content/full/jcb.200701030/DC1) and in the Drosophila S2 cell line (RNAi knockdown experiment; unpublished data). Therefore, Eps15 is required for efficient SV endocytosis during nerve stimulation.

Endocytosis from the plasma membrane consists of sequential steps that can be observed with transmission EM (TEM). To further investigate the endocytic defect in eps15 mutants, we quantified vesicle density and size of boutons in the resting state, during a strong stimulation with 90 mM K+, and recovery after stimulation (Fig. 7, A–F). The vesicle densities of eps15 mutant boutons at rest are not significantly different from those of controls (Fig. 7 A, B, and G; P > 0.05), in agreement with a lack of defect in exocytosis. Although the majority of vesicles in both control and eps15 mutant boutons are <45 nm in diameter, we observe an increased proportion of abnormally large vesicles or cisternae in resting mutant boutons (Fig. 7, A, B, and H).

Because intense stimulations reveal reduced FM1-43FX uptake and more severe synaptic depression at eps15 NMJs compared with controls, we applied 90 mM K+ stimulation to control and eps15 mutant boutons before processing for TEM. As shown in Fig. 7 (C, D, and G), we observed a reduction in vesicle densities in eps15 mutant boutons when compared with control boutons. When allowed to recover for 1 min without stimulation, the vesicle densities in wild-type control boutons return to levels that are not significantly different from resting levels, but vesicle densities in eps15 mutant boutons show a poor recovery (Fig. 7, E–G; P > 0.05). Relative to control boutons, the proportion of cisternae >85 nm is increased by stimulation in eps15 mutant boutons but is reduced and returns to wild-type levels after a 1-min recovery (Fig. 7, I–K). In stimulated eps15 mutant boutons, some cisternae appear to be large invaginations, which are contiguous with the plasma membrane (Fig. 7, K and L). In summary, eps15 mutant boutons undergo vesicle depletion, accumulation of cisternae, and large membrane invaginations during stimulation. After 1 min of recovery, the relative size distributions of vesicles in eps15 mutant and control boutons become similar, but the control boutons still have approximately twice the number of vesicles observed in mutant boutons. Thus, the TEM data indicate that Eps15 is required for efficient vesicle retrieval during intense stimulation. In its absence, bulk membrane invagination is not abolished, but budding of small clear vesicles from the plasma membrane and cisternae occur with much lower efficiency than in wild-type synapses.

The presence of large cisternae in eps15 mutant boutons is reminiscent of dapi60 mutant boutons after a 1-min recovery from stimulation (Koh et al., 2004). This prompted us to examine dapi60 mutant bouton morphology during stimulation in more detail. As seen in eps15 mutants, we observe cisternae and large membraneous bodies that appear to be contiguous to the plasma membrane (Fig. 7, M and N). The similarity of the large membrane invaginations in eps15 and dapi60 mutant boutons suggests that both Eps15 and Dap160 act at a similar step in endocytosis.
Eps15 maintains proper levels of dynamin and Dap160 at NMJs

The endocytic defects, in particular, the retrieval defects observed in eps15 mutants resemble those observed in dap160 mutants (Koh et al., 2004; Marie et al., 2004; Fig. 7, K–N) and are quite different from those associated with the loss of endophilin and synaptojanin (Verstreken et al., 2002, 2003). This suggests that Eps15 may be functionally related to Dap160, prompting us to examine the localization of Dap160 and its partner dynamin (Roos and Kelly, 1998), as well as other synaptic proteins in eps15 mutant NMJs. Immunohistochemistry of eps15 mutant NMJs shows that dynamin and Dap160 levels are severely reduced to 10–16% and 25% of control levels, respectively (Fig. 8 A). In addition, we observe reductions of other proteins associated with SV cycling, including Stoned B, synaptotagmin I, α-adaptin, and endophilin (Fig. 8 B; Littleton et al., 1993; Andrews et al., 1996; González-Gaitán and Jäckle, 1997; Verstreken et al., 2002). Cysteine string protein (Csp), a cochaperone protein required for proper neurotransmitter release, and Fas II, a cell-adhesion molecule implicated in synapse formation and function, are not significantly reduced at eps15 mutant NMJs (Fig. 8 C; Zinsmaier et al., 1994; Schuster et al., 1996; P > 0.05). The strong reduction of Dap160 at eps15 mutant NMJs prompted us also to examine the Eps15 levels at dap160 mutant NMJs. Although Eps15 levels are variably affected in different boutons and synapses, there is only a slight reduction, which is not statistically significant (Fig. 8 D; P > 0.05). These data indicate that Eps15 maintains normal levels of multiple synaptic proteins at the synapse.

The severe reductions of dynamin and Dap160 at eps15 mutant NMJs suggest that these two proteins are critical partners of Eps15 in endocytosis. We then asked whether the reductions...
of these two proteins are restricted to the synapse. Western blots of third instar larval brains indicate a subtle reduction of Dap160 levels and normal dynamin levels in the central nervous system (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200701030/DC1), suggesting that the reductions of these two proteins in eps15 mutants occur predominantly at the synapse.

The C-terminal domains of Eps15 are not essential for SV endocytosis and synapse development

Because Eps15 possesses different domains that biochemically interact with various protein partners, it is possible that different domains are involved in distinct processes. To address this possibility, we verified the binding properties of three major protein–protein interaction domains of Eps15 using in vitro binding assays. Consistent with similar studies on the mammalian Eps15, the EH-, DPF- and the UIM-containing regions of Drosophila Eps15 bind Dap160, α-adaptin, and ubiquitin, respectively (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200701030/DC1), suggesting that the reductions of these two proteins in eps15 mutants occur predominantly at the synapse.

To determine the contributions of Eps15 domains to synaptic bouton development, we quantified bouton numbers of the muscle 6/7 synapses (Fig. 9 B). The eps15-null mutants show approximately twice the number of NMJ boutons compared with controls. The supernumerary bouton phenotype is rescued by neuronal expression of Eps15wt and partially rescued by ∆DPF. Thus, the Eps15 C-terminal domains play minor roles in synaptic development.

Figure 8. Synaptic markers are reduced at eps15 mutant NMJs. Anti-Dlg outlines the NMJ boutons (magenta). The synaptic markers stained in green are quantified. (A) Dynamin and Dap160 are strongly reduced at eps15 mutant NMJs. (B) Stoned B, synaptotagmin I, α-adaptin, and endophilin are mildly reduced. (C) Csp and FasII are not significantly reduced at the eps15 mutant NMJs (P > 0.05). (D) Eps15 level is slightly but not significantly reduced at dap160+/Df(2L)bur-K1 NMJs compared with controls (P > 0.05). w larvae were used as controls. At least 10 muscle 12 type I boutons from abdominal segment 3 of each larva were used to obtain a pixel intensity for each larva, and at least three larvae were used to arrive at mean values for statistical comparisons. *, P < 0.05; **, P < 0.01 (controls vs. mutants; t test). Error bars indicate SEM.
expression of ΔDPF in eps15-null mutants show rescue of FM1-43FX uptake to a level significantly less than, but close to, control levels (P < 0.05). Therefore, the DPF-containing region plays a minor role in SV endocytosis. In summary, the roles of Eps15 in synaptic bouton development and SV endocytosis can be partially substituted by an N-terminal fragment containing the EH and coiled coil domains.

**Eps15 and Dap160 function at the same endocytic step**

Several observations suggest a functional relationship between Eps15 and Dap160. First, Eps15 and Dap160 physically bind to each other and colocalize at NMJs (Fig. 1 A and Fig. 2 E and Fig. S4 A; Sengar et al., 1999). Second, eps15 mutant NMJs, like dap160 mutant NMJs show extra boutons and branches (Fig. 4; Koh et al., 2004; Marie et al., 2004). Third, both eps15 and dap160 mutants exhibit endocytic defects but no exocytic defects at the NMJ (Figs. 5 and 6). Fourth, we observe large membranous bodies in stimulated eps15 and dap160 mutant boutons (Koh et al., 2004; Fig. 7 K–N), which suggest a defect in the resolution of these large invaginations into small clear vesicles. Fifth, we observe a reduction of Dap160 levels at eps15 mutant NMJs. These data suggest that the two proteins may function at very similar steps in endocytosis, namely, during vesicle budding from the plasma membrane and from large invaginations during bulk endocytosis. Because Dap160 and Eps15 share N-terminal EH domains, which are more homologous to each other than EH domains of other Drosophila proteins (40–60% similarity), it is possible that the EH domains of these two proteins serve redundant roles and act in parallel during endocytosis; if so, genetic ablation of both eps15 and dap160 should lead to more severe defects than either mutant alone. Alternatively, if Eps15 and Dap160 function at the same step during endocytosis from the plasma membrane, double-null mutants should have a phenotype similar to the single mutants.

To investigate the functional relationship between Eps15 and Dap160, we determined the endocytic defects of eps15 and dap160 mutants in double mutant larvae. First, we examined the lethal phases of double mutants and showed that dap160- and eps15-null mutants live to pharate adult stage, similar to the single eps15- and dap160-null mutants. Second, dap160- and eps15-null mutants show normal evoked EJPs in response to single stimuli and during 10 Hz of stimulation; hence, these double mutants also show the same depression kinetics as the eps15- and dap160-null single mutants (Fig. 10, A and B). Thus, the loss of Dap160 does not enhance the endocytic defects in eps15 mutants. To provide further support for the electrophysiology data, we performed FM1-43FX dye uptake experiments. As shown in Fig. 10 C, we observed a reduction in dye uptake in the double-null mutant that is similar to that of eps15 and dap160 single mutants. Thus, the dap160-null mutation does not affect the residual vesicle recycling in the eps15-null mutant background. In summary, these data argue that Eps15 and Dap160 act at the same step in SV endocytosis.

**Discussion**

Eps15 stabilizes multiple proteins in the presynaptic compartment.

eps15 mutant NMJs exhibit a severe reduction in dynamin and Dap160 protein levels, suggesting a functional relationship between these proteins and Eps15. At the same time, we observe relatively mild reductions of α-adaptin, Stoned B, synaptotagmin I, and endophilin. The reduction of protein levels of dynamin, Dap160/intersectin, α-adaptin, and Stoned B in eps15 mutants may be accountable by the interaction of these proteins.
with Eps15 (Benmerah et al., 1999; Sengar et al., 1999; Martina et al., 2001; Salcini et al., 2001; Fig. 1 A). As our data suggest that Eps15 is not required to regulate global protein levels of dynamin and Dap160 (Fig. S3), Eps15 may facilitate anterograde axonal transport of dynamin and Dap160 through inter-action of the EH domains with vesicular membrane proteins containing NPF motifs (Fernandez-Chacon et al., 2000). Alternatively, because Eps15 has been implicated in the endocytosis of synaptotagmin I (Jarousse et al., 2003), Eps15 may retain high concentrations of synaptic proteins by promoting the local recycling and sorting of vesicle-/membrane-associated proteins at synapses. Interestingly, a similar role has been proposed for Stoned in recycling synaptotagmin I at the synapse (Ferguson and Brodie, 2001; Diril et al., 2006).

Eps15 and Dap160 are required to maintain proper levels of dynamin at the NMJ

The endocytic defect in eps15 can be largely ascribed to impaired vesicle budding from plasma membrane and cisternae. Compared with control boutons, stimulations of eps15 mutant boutons result in more severe vesicle depletions, concurrent with the appearance of cisternae and large invaginations, which appear to be contiguous with the plasma membrane (Fig. 7, K and L). Importantly, 1 min after stimulation, wild-type control boutons recover to near normal vesicle densities, whereas eps15 mutant boutons show little recovery. Note that internalization of cisternae, an early step in bulk endocytosis (Teng and Wilkinson, 2000; Royle and Lagano, 2003), does occur in the mutants, but the cisternae are not efficiently resolved into properly sized SVs.

The endocytic intermediates found in eps15 mutants resemble those observed when dynamin or Dap160 function is perturbed. Cisternae and invaginations contiguous with the plasma membrane have been reported at neuronal synapses during genetic or dominant-negative peptide perturbations of dynamin function in Drosophila and lampreys, respectively (Kosaka and Ikeda, 1983; Koenig and Ikeda, 1989, 1996, 1999; Koenig et al., 1993; Shupliakov et al., 1997). We have previously reported that dap160 mutant NMJs show accumulation of cisternae during recovery from stimulation (Koh et al., 2004). Here, we fixed dap160 mutants during stimulation and observed severe vesicle depletion concurrent with large invaginations from the plasma membrane, similar to those observed in eps15. Because dynamin levels are severely reduced at eps15 and dap160 NMJs, it is likely that both Eps15 and Dap160 act in concert to maintain high concentrations of dynamin at the synapse. Indeed, dap160 eps15 double mutants show the same defects in dye uptake and in maintaining release during prolonged stimulations as the eps15 and dap160 single mutants, suggesting that both Eps15 and Dap160 act at the same step in endocytosis. Furthermore, the notion that Eps15 is localized at the site of dynamin action is consistent with the mobilization of Eps15 to the plasma membrane in the vicinity of endocytic intermediates in stimulated nerve terminals (Figs. 2 and 3). In addition, a functional interaction between Eps15 and dynamin is further bolstered by the recent demonstration of genetic interaction between shibire mutants and the hypomorphic eps15 mutant, eps15<sup>ES221</sup> (Majumdar et al., 2006). Note that the lack of endocytic defect in eps15 mutant hemocytes also correlates with normal dynamin levels in these cells (Fig. S2), further corroborating with a link between SV endocytosis and the Eps15–Dap160–dynamin interaction. In agreement with the correlation of endocytic defects with reduced dynamin levels in eps15 and dap160 mutants, the GTPase activity of dynamin is allosterically dependent on dynamin protein concentration (Stowell et al., 1999). Therefore, these data suggest that the maintenance of high dynamin levels at synapses is one of the key functions of Eps15 and Dap160 in SV endocytosis.

Although we observed severe reduction of dynamin levels in both eps15 and dap160 mutants, there are subtle differences in synaptic protein levels between eps15 and dap160 mutants. For example, there is a reduction of synaptotagmin I in eps15 mutants, which was not observed in dap160 mutants. This may have resulted in minor differences in phenotypes, such as an increase in mEJP amplitudes in dap160 mutants but not in eps15 mutants.

Eps15 has been implicated in clathrin-coated pit assembly in nonneuronal cells, based on its interaction with α-adaptin (Tebar et al., 1996; Carbone et al., 1997; Benmerah et al., 1999; Torrisi et al., 1999). However, our data indicate that a truncated Eps15 protein lacking the α-adaptin–interacting DPF motifs (∆DPF) partially rescues the FM1-43FX uptake defect of the eps15-null mutant. This suggests that a direct interaction between Eps15 and α-adaptin is not essential for SV endocytosis. Consistent with the nonessential role of the DPF domain of Eps15, the Eps15 binding region of α-adaptin is not essential for the rescue of transferrin endocytosis after α-adaptin siRNA knockdown (Motley et al., 2006). Hence, we propose that the dynamin–Dap160–Eps15 interaction is important for SV endocytosis.

A model of Eps15 function during SV endocytosis and synapse development

Based on our data and previous work, we propose a two-tier model for Eps15 function during SV endocytosis and synapse development. During SV endocytosis, Eps15 and Dap160 act together to stabilize several proteins at the synapse. In particular, the Eps15–Dap160 complex maintains high concentrations of dynamin, allowing allosteric activation of its GTPase activity, which is crucial for the resolution of newly internalized membranes into SVs (Stowell et al., 1999). It has been proposed that actin polymerization propels endocytosed vesicles toward the interior of the cell during dynamin-mediated endocytosis (Merrifield et al., 2002; Shupliakov et al., 2002; Itoh et al., 2005; Tsujita et al., 2006). Given that Eps15, Dap160, and related EH domain proteins in yeast have been implicated in regulating the actin cytoskeleton (Tang and Cai, 1996; Duncan et al., 2001; Zamanian and Kelly, 2003; Koh et al., 2004; Marie et al., 2004), it is tempting to speculate that the Eps15–Dap160 complex functions as a molecular scaffold to coordinate dynamin function in SV endocytosis with actin polymerization, a role that has been previously proposed for Dap160 (McPherson, 2002). It is thus interesting that the redistribution of Eps15 and Dap160 within NMJ boutons during stimulation suggests a dynamic role.
of Eps15 and Dap160 during endocytosis (Fig. 2, F–I; and Fig. 3 A; Roos and Kelly, 1998, 1999).

In addition to its role in SV endocytosis, Eps15 may function in concert with Dap160 during synapse development through regulation of signal transduction pathways and/or cytoskeletal organization. Both Eps15 and Dap160 have been implicated in signal transduction pathways (Irie and Yamaguchi, 2002; Polo et al., 2003). In addition, Dap160/intersectin has been proposed to regulate cytoskeletal organization during synapse development (Coyle et al., 2004; Koh et al., 2004). This is consistent with our recent data on lamprey reticulospinal synapses, which show that actin at synaptic endocytic sites is reorganized when intersectin interactions are perturbed (Evergren et al., 2007). Interestingly, an EH domain protein is involved in cytoskeletal reorganization during budding of yeast cells (Tang and Cai, 1996; Duncan et al., 2001), which is thought to be analogous to Drosophila NMJ bouton formation (Penetta et al., 2002). We speculate that Eps15 and Dap160 may function as a protein complex—with Nervous wreck and WASp—that regulates cytoskeletal organization during synapse development (Coyle et al., 2004; Koh et al., 2004; Marie et al., 2004). In this respect, Eps15 and Dap160 may serve to bridge upstream signal transduction pathways and cytoskeletal organization during synapse development.

Materials and methods

Antibody generation

Two fragments encoding amino acid residues 586–816 and 915–1093 from Eps15 cDNA (SD09478 from Research Genetics) were cloned into the pGEX4T1 expression vector in frame with the GST coding region. GST–Eps15586–816 was used to generate the rabbit polyclonal antisera, Gp Ab.

Fly genetics

P element excisions, w; EP[2]2512S (Rath, 1996) was crossed to y w+/CyO, H{w+mcPa2-3}H02P1, 1/6C Efg1; Gerlitz et al., 2002) to generate 810 independent excision lines. Lines that failed to complement Df[2R]Dil-MP (Cohen et al., 1989) were characterized. Excisions were balanced over CyO, P[w+mc+Gal4-4wi.G] P[w+mc=UAS-ZeEGFP] (Halfon et al., 2002), and immunohistochemistry and Western blot were performed to identify allele eps15Δ29. Remnants of the P element are still inserted in its original genomic location, but the exact lesion is not known.

FRT-mediated deletion. Two transposons carrying FRT elements, P[XP=FRT]d00445 and PBac[WH=FRT]j02085, were used for site-specific deletion of the eps15 locus as described previously (Parks et al., 2004). In brief, second chromosomes containing both transposons were placed in trans with each other in a genetic background containing P[yr=y w+;+hsFLP]12 [Bloomington Stock Center]. 1–8 °C heat-shock treatments to the locus over the consecutive days to induce expression of the FLP recombinase. P[yr=y w+;+hsFLP]12, y w+; P[XP=FRT]d00445 and PBac[WH=FRT]j02085 females were then crossed to y w+; L/CyO. In the next generation, dark-eyed males were isolated as putative recombintants and were crossed to eps150/– to test for noncomplementation of lethality, leading to the isolation of the eps15Δ29 allele. About 80% of the dark-eyed males failed to complement eps150/– PCR and sequencing showed that the junctions of P[XP=FRT]d00445 and PBac[WH=FRT]j02085 with their respective flanking DNA in the eps15 locus were retained in homologous eps15Δ29 larvae, confirming recombination.

Genomic rescue construct. 10.8 kb of genomic DNA containing the wild-type eps15 (2R region bp: 19811150–19821904; Berkeley Drosophila Genome Project release 3 [http://www.fruitfly.org/CGI-bin/annot/ ggbrowse]) was recovered from BAC RP88-387 (BACRC, Resources) by gap repair in the vector, 2.28 β (Pacman) [Venken et al., 2006]. In brief, we cloned 500-bp left and right homology arms separated by a BamHI site in Pacman. The vector was linearized using BamHI and transformed into recombination-competent DH10B bacteria containing both BACRP98-387 and mini-t-etr [Court et al., 2003; Liu et al., 2003]. Gap-repaired vectors were selected with Amp, screened by PCR, and verified by restriction enzyme digestion and sequencing. As a third chromosome insertion of this genomic construct, P[eps15-SJ12-2.5b, eps15Δ29; elav–Digo1, ΔDPF] and P[eps15-SJ12-2.5b, eps15Δ29; elav–Digo1, ΔDPF] were inserted into a compound chromosome consisting of chromosomes 2 and 3. We then cross this line to eps15Δ29 and Df[2R]Dil-MP to test for rescue of lethality. The presence of the eps15 lesions was verified by homozygous lethality of the parental eps15Δ29 and Df[2R]Dil-MP stocks used for the final rescue cross.

cDNA rescue constructs. Full-length eps15 (1S5w1r), Ehmut (full length with wild-type W5A4, W17A4A, and W536A substitutions in the EH domains), and ADP (residues 1–639) were subcloned from SD09478 cDNA into pUAST (Brand and Perrimon, 1993). eps15 cDNAs were driven by one of the elav–based neuronal drivers, the elav+55Gal4 enhancer trap in the elav locus (Lin and Goodman, 1994), or an elav promoter:Gal4 fusion transgene (elav–Gal4Δ50) inserted in the third chromosome [Luo et al., 1994]. The elav–Gal4 driver drove higher transgene expression levels and rescued the lethality of eps15Δ29/eps15Δ29 more effectively than the third chromosome elav–Gal4 driver. Rescue of eps15 mutants was only observed when elav+55Gal4 or elav–Gal4Δ50 was introduced in the same fly as the UAS-cDNA constructs. Presence of eps15 lesions in rescue individuals was verified by lethality associated with homozygosity on the second chromosome in the parental lines, eps15Δ29; UAS-cDNA, eps15Δ29; elav–Gal4Δ50, and C153; eps15Δ29.

Larval culture

Mutant larvae were separated from larval bearing balancer chromosomes and cultured on grape juice agar with yeast paste (Loewen et al., 2001). This allows lethal phase of mutants to be determined without complications from overcrowding and competition from larval bearing balancer chromosomes.

Immunohistochemistry and quantification of fluorescent images

Labeling of third instar NMJs, third instar brains, and adult brains was performed as described by Verstreken et al. (2003). Animals were dissected in PBS and fixed in 4% formaldehyde in PBS for 20 min. Tissue was extensively washed in PBS and permeabilized with 0.1% Triton X-100 for anti-endophilin labeling, permeabilization was performed using 0.1% tween 20. Antibodies were used in the following dilutions: affinity-purified anti-Dap160, 1:500 (Roos and Kelly, 1998); mouse anti-Dlg, 1:150 (AF3; Parnas et al., 2001); rabbit anti-Dlg, 1:2,000 [Cho et al., 2000]; guinea pig anti-Dlg, 1:500 [Parmentier et al., 2000]; rabbit anti-HRP, 1:1,500 [Jackson Immunoresearch Laboratories]; rabbit anti-Stoned B, 1:200 [Andrews et al., 1996]; mouse anti-Csp mb49, 1:20 [Zinsmaier et al., 1994]; rat anti-Nck, 1:500 [Coley et al., 2004]; rabbit anti-dynamin, 1:500 [Rinze and Kelly, 1998]; mouse anti-dynamin, 1:50 [Rinze et al., 1994]; mouse anti–Fas II 1D4 guinea pig anti-endophilin, 1:200 [Verstreken et al., 2002]; mouse anti–Bruchpilot ncb2, 1:100 [Waghs et al., 2006]; and anti–synaptotagmin I, 1:500 [Littleton et al., 1993, 1994]. Secondary antibodies conjugated to Cy3 or Cy5 (Jackson Immunoresearch Laboratories) or Alexa 488 or 594 (Invitrogen) were used at 1:250. Samples were mounted in Vectashield mounting medium (Vector Laboratories). For Fig. 2 (A, B, and E), Fig. 4, Fig. 8, and Fig. S1, images were captured with a confocal microscope (LSM 510; Carl Zeiss MicroImaging, Inc.) with a 40×/1.3 differential interference contrast-Neofluor oil immersion lens using the LSM 5 software [Carl Zeiss MicroImaging, Inc.] and were processed using Amira software (Mercury Computer Systems) followed by Photoshop (Adobe). For Fig. 2 (C and D) and Fig. S2, images were viewed using a confocal system (MRC-1024; Bio-Rad Laboratories) mounted on a microscope (Eclipse E800; Nikon) and were captured using a 40×/1.3 NA or 60×/1.4 NA oil-immersion objective and LaserSharp software (Bio-Rad Laboratories). Brightness and contrast levels and color channels were adjusted using ImageJ (http://rsb.info.nih.gov/ij) or Photoshop. A binary mask was used to quantitate synaptic proteins. Anti-Dlg labeling was used to outline type I boutons for quantitation of synaptic protein levels (Leehey et al., 1994). Boutons on muscle 12 were scanned with 2 steps of 0.7 μm. Using Amira software, the Dlg-stained type I boutons in each confocal slice were highlighted, and the mean pixel intensity of all slices of the highlighted boutons of each muscle 12 NMJ were computed. Background fluorescence in muscle areas adjacent to the boutons was quantified similarly, and the background was subtracted from the bouton values to yield the mean intensity.
of labeling in the boutons. The mean value from at least three mutant NMJs was then expressed as a percentage of the corresponding control value. The primary antibodies against presynaptic proteins were used at concentrations that produced strong specific labeling in the NMJ boutons without giving excessive background labeling in other tissues.

Biochemistry

Immunoprecipitation experiments were performed essentially as described previously (Bean et al., 2000; Verstreken et al., 2003). The Dap160 band was detected by in-gel trypsin digest followed by peptide determination using a QSTAR hybrid tandem mass spectrometer (MDS Sciex). Spectra were searched against a NCBI nonredundant database with MACSOT (Matlab software, Matrix Science). The only other two Coomassie blue-stained bands that were successfully identified were keratin (likely a contaminant from the experimenter) and CG5214, a mitochondrial enzyme in the tricarboxylic acid cycle. For Western blots, rabbit anti-Eps15 was used at 1:2,500, rabbit anti-α-adaptin (Dornan et al., 1997) at 1:200, rabbit anti-dynamin (Roos and Kelly, 1998) at 1:300, and rabbit anti-Dap160 (Roos and Kelly, 1998) at 1:500. For pull-down assays, Histidine (His)-tagged and GST fusion proteins were expressed in BL21 (DE3) (Novagen) cells and purified from soluble fraction using Ni-NiTA (Qiagen) and glutathione (GE Healthcare) beads, respectively, before being used for binding assays. Ni-NiTA agarose-coupled His-tagged UIM region of Eps15 (amino acids 1177–1253) was incubated with GST or GST-Ubiquitin (G76A, Lloyd et al., 2002) for 2 h at 4°C, washed extensively in PBS (0.01 M sodium phosphate buffer, 0.15 M NaCl, pH 7.4) supplemented with 0.1% Triton X-100 and Complete protein inhibitor cocktail (Roche) and prepared for electrophoresis. For pull downs from fly head extracts, glutathione Sepharose–coupled GST, GST-3xEH (amino acids 1–403), and the GST-DPF region of Eps15 (amino acids 551–1001) were incubated with extracts (frozen heads homogenized in PBS supplemented with 2 mM MgCl2, 1.5 mM MgCl2, 1% Triton X-100, and Complete protein inhibitor cocktail) cleared soluble fraction adjusted to 0.1% Triton X-100 concentration) for 2 h at 4°C, washed extensively in the same buffer, and prepared for electrophoresis.

Electrophysiology and FM1-43FX uptake assay

Third instar electrophysiology was also performed essentially as described previously (Verstreken et al., 2002, 2003). Larvae were dissected, and recordings were performed in modified HL3: 110 mM NaCl; 5 mM KCl; 10 mM NaHCO3; 5 mM Heps; 30 mM sucrose; 5 mM trehalose; 10 mM MgCl2; and 0, 0.5, or 5 mM CaCl2 (as indicated in the text and figure legends). For 10-Hz stimulations, the above medium was modified to contain 4 mM MgCl2 and 5 mM CaCl2. Recordings of low-frequency stimulations and 10-Hz stimulations were made from muscles with resting potential lower than −60 and −65 mV, respectively.

For the FM1-43FX (Invitrogen) styryl dye uptake experiments, larvae were dissected on Sylgard plates and incubated in modified HL3 with 4 μM FM1-43FX, 5 mM CaCl2, 90 mM KCl, and 25 mM NaCl for 10 min. Excess dye was then washed away with zero Ca2+ solution for 10–15 min, and labeling was imaged using a 40×/0.75w Apochromat water-immersion objective on the LSM 510 confocal microscope. Data acquisition as well as data processing and quantification were performed as described previously (Verstreken et al., 2003). The FM1-43FX dye uptake assay in Fig. 8 was performed as above, but the data were acquired after fixation with 4% formaldehyde in PBS, imaged using a 60×/1.4 NA oil-immersion objective on the MRC-1024 confocal system, and quantified using NIH ImageJ. Data acquired using the two methods were comparable. All electrophysiology and FM1-43FX uptake was performed at room temperature (−21–23°C), except when indicated otherwise.

TEM

Larval fillets were prepared in HL3 without Ca2+ and fixed in 4% paraformaldehyde and 1% glutaraldehyde/0.1 M cacodylic acid, pH 7.2, or 3% paraformaldehyde/0.5% glutaraldehyde/0.1 M cacodylic acid, pH 7.2. Samples were postfixed in 2% OsO4 and stained in 2% uranyl acetate, dehydrated in alcohol, and embedded in Spurr’s resin (Electron Microscopy Sciences) or Durcupan (Fluka). Ultrathin sections were cut with a diamond knife (Diatome) and stained with 2% uranyl acetate and lead citrate on grids. The grids were then visualized with an electron microscope (JEM-1010; JEOL) fitted with a charge-coupled device digital camera (Gatan). To study the morphology of stimulated boutons, larval fillets were incubated in HL3 with 5 mM Ca2+ and 60 or 90 mM K+ for 10 min and fixed immediately. In stimulation-recovery experiments, stimulated NMJs were allowed to recover for 1 min in normal HL3 without Ca2+ before fixation. Images were quantified by NIH ImageJ.

Preembedding immunocytochemistry

Muscles 6 and 7 were dissected from third instar Drosophila larvae were fixed in 4% paraformaldehyde and embedded in agarose (Sigma-Aldrich). Vibratome slices of the agarose blocks were incubated with guinea pig anti-Eps15 antisera followed by secondary antibodies conjugated to 1.4-nm gold particles (Nanoprobes, Inc.). The immunogold labeling was silver enhanced using IntenSE Silver Enhancement kit (GE Healthcare), and samples were embedded in Durcupan ACM (Fluka) for ultrathin sectioning. Serial ultrathin sections were counterstained with uranyl acetate and lead citrate and examined in a microscope (Tecnai 12; FEI Company). Images were quantified using NIH ImageJ, and statistical evaluation was performed using Excel (Microsoft). Note that the silver enhancement technique works by the precipitation of silver particles around the gold particles, resulting in the formation of irregularly shaped black precipitates.

Online supplemental material

Fig. S1 illustrates the domain structure of Drosophila Eps15 and shows that the eps I-5 null alleles do not express detectable amounts of Eps15. Fig. S2 shows that m85A endocytosis and dynamin levels are both normal in eps I-5 null hemocytes. Fig. S3 shows that dynamin and Dap160 levels are not substantially altered in the larval brains of eps I-5 mutants. Fig. S4 shows that the DH, DPF, and UIM domains of Eps15 bind to Dap160, α-adaptin, and ubiquitin, respectively. Table S1 shows mass spectroscopy data of peptide sequences corresponding to Dap160, which immunoprecipitated with Eps15 (Fig. 1 A). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200701030/DC1.

We are grateful to C.-K. Yao for critical assessment of data on bouton morphology, and we thank H. Veltman for mass spectroscopy. Y.-T. L. for quantification of morphological data, N. Tomlin for expert advice in EM, and K. S. Krishnan and A. Majumdar for sharing results and reagents before publication. In addition, we are grateful to K.W. Cho, R. Kelly, L. Benyetez, M. González-Gaítan, E. Buchner, and A. Jackson for antibodies; D. Court for minia Tet; and P. Verstreken, A. Fayyazuddin, and C. ly for critical comments on this manuscript.

We acknowledge stocks and antibodies from the Bloomington and Szeged Drosophila stock centers and the Developmental Biology Hybridoma Center, Iowa. Confocal microscopy was supported by the Mental Retardation and Developmental Disabilities Research Center. T.-W. Koh acknowledges support from the Baylor College of Medicine-Karolinska Institutet Exchange Program. Y.P. Waiikar was supported by a Nehru scholarship from the Cambridge Commonwealth Trust. This work was supported by a grant (B/8/19002) and a Research Development Fellowship from the Biotechnology and Biological Sciences Research Council to C. O’Kane, a Career Development Award (G120/414) from the Medical Research Council to I.M. Robinson, fellowship from Härmofonden to E. Evergren, The Swedish Research Council [13473] and Forsmions Stiftelse to O. Shupilakov, and a Howard Hughes Institutional Grant [76200-549202] to J.M. Robinson. H.J. Bellon is supported by the Howard Hughes Medical Institute.

Submitted: 5 January 2007
Accepted: 18 June 2007

References

Andrews, J., M. Smith, J. Merakovsky, M. Coulson, F. Hannan, and L.E. Kelly. 1996. The stoned locus of Drosophila melanogaster produces a dicistronic transcript and encodes two distinct polypeptides. Genetics. 143:1699–1711.

Bache, K.G., C. Raiborg, A. Mehlum, and H. Stenmark. 2003. STAM and Hrs interact with Eps15. J. Cell Biol. 162:1303–1311.

Bellen, H.J., and V. Budnik. 2000. The neuromuscular junction. In Protocols. W. Sullivan, M. Ashburner, and R.S. Hawley, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 175–199.

Benmerah, A., C. Lamaze, B. Begue, S.L. Schmid, A. Dautry-Varsat, and N. Cerf-Bensussan. 1998. AP-2/Eps15 interaction is required for receptor-mediated endocytosis. J. Cell Biol. 140:1055–1062.

Benmerah, A., M. Bayrou, N. Cerf-Bensussan, and A. Dautry-Varsat. 1999. Inhibition of clathrin-coated pit assembly by an Eps15 mutant. J. Cell Sci. 112:1303–1311.
Koenig, J.H., and K. Ikeda. 1989. Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval. J. Neurosci. 9:3844–3860.

Koenig, J.H., and K. Ikeda. 1996. Synaptic vesicles have two distinct recycling pathways. J. Cell Biol. 135:797–808.

Koenig, J.H., and K. Ikeda. 1999. Contribution of active zone subpopulation of vesicles to evoked and spontaneous release. J. Neurophysiol. 81:1495–1505.

Koenig, J.H., K. Yamazaki, and K. Ikeda. 1993. Calcium-induced translocation of synaptic vesicles to the active site. J. Neurosci. 13:2313–2322.

Koh, T.W., P. Verstreken, and H.J. Bellen. 2004. Dapl610/intersectin acts as a stabilizing scaffold required for synaptic development and vesicle endocytosis. Neuron. 43:193–205.

Kosaka, T., and K. Ikeda. 1983. Possible temperature-dependent blockage of synaptic vesicle recycling induced by a single gene mutation in Drosophila. J. Neurobiol. 14:207–225.

Lahey, T., M. Gorczyca, X.X. Jia, and V. Budnik. 1994. The Drosophila tumor suppressor gene dgl is required for normal synaptic bouton structure. Neuron. 13:823–835.

Lim, D.M., and C.S. Goodman. 1994. Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. Neuron. 13:507–523.

Littleton, J.T., M. Stern, K. Schulze, M. Perin, and H.J. Bellen. 1993. Mutational analysis of Drosophila synaptotagmin demonstrates its essential role in Ca2+-activated neurotransmitter release. Cell. 74:1125–1134.

Littleton, J.T., M. Stern, M. Perin, and H.J. Bellen. 1994. Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in Drosophila synaptotagmin mutants. Proc. Natl. Acad. Sci. USA. 91:10888–10892.

Liu, P., N.A. Jenkins, and N.G. Copeland. 2003. A highly efficient recombinase-based method for generating conditional knockout mutations. Genome Res. 13:476–484.

Lloyd, T.E., P. Verstreken, E.J. Ostrin, A. Phillippot, O. Lichtarge, and H.J. Bellen. 2000. A genome-wide screen for synaptic vesicle cycle proteins in Drosophila. Neuron. 26:45–50.

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

Loewen, C.A., J.M. Mackler, and N.E. Reist. 2001. Fission and uncoating of Drosophila synaptic vesicles. J. Neurosci. 21:1218–1227.

Martina, J.C., C.J. Bonangeli, R.C. Aguilar, and J.S. Bonifacino. 2001. Stonin, a homologue of AP-2. Neuron. 27:379–390.

Martina, J.C., J.S. Bonifacino, and R.C. Aguilar. 2003. Stonin 2 is an AP-2-dependent endocytic sorting adaptor for synaptotagmin internalization and recycling. Dev. Cell. 10:233–244.

Merrifield, C.J., M.E. Feldman, L. Wan, and W. Almers. 2002. Imaging actin and dynein motors in axonal outgrowth and myoblast fusion. Neuron. 35:291–306.

Morgan, J.R., K. Prasad, S. Jin, G.J. Augustine, and E.M. Lafer. 2003. Ep15 homology domain-NPF motif interaction regulates clathrin coat assembly during synaptic vesicle recycling. J. Biol. Chem. 278:33583–33592.

Motley, A.M., N. Berg, M.J. Taylor, D.A. Sahl tender, J. Hirst, D.J. Owen, and M.S. Robinson. 2006. Functional analysis of AP-2 α and β subunits. Mol. Biol. Cell. 17:5298–5308.

Pennetta, G., P. Hiesinger, R. Fabian-Fine, I. Meinertzhagen, and H. Bellen. 2002. Drosophila VAP-33A directs formation at neuromuscular junctions in a dosage-dependent manner. Neuron. 35:291–306.

Parks, A.L., K.R. Cook, M. Belvin, N.A. Dompe, R. Fawcett, K. Huppert, L.R. Farris, W.C. Anderson, and M.S. Robinson. 2006. Functional analysis of AP-2 α and β subunits. Mol. Biol. Cell. 17:5298–5308.

Pennetta, G., P. Hiesinger, R. Fabian-Fine, I. Meinertzhagen, and H. Bellen. 2002. Drosophila VAP-33A directs formation at neuromuscular junctions in a dosage-dependent manner. Neuron. 35:291–306.

Parnet tier, M.L., D. Woods, S. Greig, P.G. Phan, A. Radvic, P. Bryant, and C.J. O’Kane. 2000. Rapsynoid/partner of in scutable controls asymmetric division of larval neuroblasts in Drosophila. J. Neurosci. 20:RC84.

Pennemann, D., G. Fries, E. Kautler, S. Kim, and H.J. Bellen. 2001. Regulation of post synaptic structure and protein localization by the Rho- type guanine nucleotide exchange factor dPlex. Neuro. 32:415–424.

Perry, M., F. Schmorak, and H. Jäckle. 2000. Capacity for dynamic synaptic vesicle distribution is dependent on syntaxin. Genes Dev. 14:233–238.

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

Prentice, N., and K. Ikeda. 1995. The Drosophila tumor suppressor gene dgl is required for normal synaptic bouton structure. Neuron. 13:823–835.

Purves, D., P. Anand, A. Eichler, E. Eichler, L. Goldstein, E. Korngold, J. Lee, S. Lichtenberg, J. Saito, E. Shapira, and J. Stojilковић. 2004. Dapl610/intersectin acts as a stabilizing scaffold required for synaptic development and vesicle endocytosis. Neuron. 43:193–205.

Quinn, C., E. Maccioni, and O. Shupliakov. 2002. A screen for genes that alter motoneuron growth cone guidance. Neuron. 34:135–138.

Radcliffe, M.E., T. Proctor, D. Brem, L. Wiberg, and M.S. Robinson. 2006. Functional analysis of AP-2 α and β subunits. Mol. Biol. Cell. 17:5298–5308.

Rakic, P., D. Edelman, L. Kalil, R. Watanabe, and M. Bellen. 2002. Drosophila VAP-33A directs formation at neuromuscular junctions in a dosage-dependent manner. Neuron. 35:291–306.

Rapaport, D.M., B. Odermatt, S.J. Ro, and N.J. Gay. 1997. Alpha-adaptin, a marker for endocytosis is the dominant mechanism of vesicle retrieval at hippocampal synapses. Neuron. 19:791–804.
ubiquitin recognition and monoubiquitination in endocytic proteins. Nature. 416:451–455.

Polo, S., S. Confalonieri, A.E. Salcini, and P.P. Di Fiore. 2003. EH and UIM: endocytosis and more. Sci. STKE. 2003:re7.

Poskanzer, K.E., K.W. Marek, S.T. Sweeney, and G.W. Davis. 2003. Synaptopagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. Nature. 426:559–563.

Roos, J., and R.B. Kelly. 1998. Dap160, a neural-specific Eps15 homology and multiple SH3 domain-containing protein that interacts with Drosophila dynamin. J. Biol. Chem. 273:19108–19119.

Roos, J., and R.B. Kelly. 1999. The endocytic machinery in nerve terminals surrounds sites of exocytosis. Curr. Biol. 9:1411–1416.

Rorth, P. 1996. A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. Proc. Natl. Acad. Sci. USA. 93:12418–12422.

Royle, S.J., and L. Lagnado. 2003. Endocytosis at the synaptic terminal. J. Physiol. 553:345–355.

Salcini, A.E., M.A. Hilliard, A. Croce, S. Arbusci, P. Luzzi, C. Tacchetti, L. Daniell, P. De Camilli, P.G. Pelicci, P.P. Di Fiore, and P. Bazzicalupo. 2001. The Eps15 C. elegans homologue EHS-1 is implicated in synaptic vesicle recycling. Nat. Cell Biol. 3:755–760.

Santolini, E., A.E. Salcini, B.K. Kay, M. Yamabhai, and P.P. Di Fiore. 1999. The EH network. Exp. Cell Res. 253:186–209.

Schuster, C.M., G.W. Davis, R.D. Fetter, and C.S. Goodman. 1996. Genetic dissection of structural and functional components of synaptic plasticity. I. Fasciclin II controls synaptic stabilization and growth. Neuron. 17:641–654.

Sengar, A.S., W. Wang, J. Bishay, S. Cohen, and S.E. Egan. 1999. The EH and SH3 domain Ese proteins regulate endocytosis by linking to dynamin and Eps15. EMBO J. 18:1119–1171.

Shupliakov, O., P. Low, D. Grabs, H. Gad, H. Chen, C. David, K. Takei, P. De Camilli, and L. Brodin. 1997. Synaptic vesicle endocytosis impaired by disruption of dynamin-SH3 domain interactions. Science. 276:259–263.

Shupliakov, O., O. Bloom, J.S. Gustafsson, O. Kjaerulff, P. Low, N. Tomilin, V.A. Pieribone, P. Greengard, and L. Brodin. 2002. Impaired recycling of synaptic vesicles after acute perturbation of the presynaptic actin cytoskeleton. Proc. Natl. Acad. Sci. USA. 99:14476–14481.

Sigismund, S., T. Woelk, C. Puri, E. Maspero, C. Tacchetti, P. Transidico, P.P. Di Fiore, and S. Polo. 2005. Clathrin-independent endocytosis of ubiquitinated cargos. Proc. Natl. Acad. Sci. USA. 102:2760–2765.

Stowell, M.H., B. Marks, P. Wigge, and H.T. McMahon. 2001. The Eps15 EH network. Exp. Cell Res. 253:186–209.

Sun, J.Y., X.S. Wu, and L.G. Wu. 2002. Single and multiple vesicle fusion induce different rates of endocytosis at a central synapse. Nature. 417:555–559.

Takei, K., V. Hauke, V. Slepev, K. Farsad, M. Salazar, H. Chen, and P. De Camilli. 1998. Generation of coated intermediates of clathrin-mediated endocytosis on protein-free liposomes. Proc. Natl. Acad. Sci. USA. 95:2768–2773.

Tang, H.Y., and M. Cai. 1996. The EH-domain-containing protein Pn1 is required for normal organization of the actin cytoskeleton in C. elegans. Mol. Cell. Biol. 16:4897–4914.

Tebar, F., T. Sorkina, A. Sorkin, M. Ericsson, and T. Kirchhausen. 1996. Eps15 is recruited by endophilin to promote synaptic vesicle uncoating. Neuron. 40:733–748.

Wagh, D.A., T.M. Rasse, E. Asan, A. Hofbauer, I. Schwenkert, H. Durrbeck, S. Buchner, M.C. Dabauvalle, M. Schmidt, G. Qin, et al. 2006. Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila. Neuron. 49:833–844.

Wendland, B., J.M. McCaffery, Q. Xiao, and S.D. Emr. 1996. A novel fluorescence-activated cell sorter-based screen for yeast endocytosis mutants identifies a yeast homologue of mammalian eps15. J. Cell Biol. 135:1485–1500.

Wucherpfennig, T., M. Wilsh-Brauninger, and M. Gonzalez-Gaitán. 2003. Role of Drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. J. Cell Biol. 161:609–624.

Zamanian, J.L., and R.B. Kelly. 2003. Intersectin 1L guanine nucleotide exchange activity is regulated by adjacent src homology 3 domains that are also involved in endocytosis. Mol. Biol. Cell. 14:1624–1637.

Zinsmaier, K.E., K.K. Eberle, E. Buchner, N. Walter, and S. Benzer. 1994. Paralysis and early death in cysteine string protein mutants of Drosophila. Science. 263:977–980.