Development

AP2 Regulates Thickveins Trafficking to Attenuate NMJ Growth Signaling in Drosophila

Saumitra Dey Choudhury,1,* Manish Kumar Dwivedi,1,* Srikanth Pippadpally,1 Abhinandan Patnaik,1 Shirish Mishra,2 Raghu Padinjat,2 and Vimlesh Kumar1

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1Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Bhopal, Bhopal, Madhya Pradesh 462 066, India and 2National Centre for Biological Sciences, Bangalore, Karnataka 560065, India

Abstract
Compromised endocytosis in neurons leads to synapse overgrowth and altered organization of synaptic proteins. However, the molecular players and the signaling pathways which regulate the process remain poorly understood. Here, we show that α2-adaptin, one of the subunits of the AP2-complex, genetically interacts with Mad, Medea and Dad (components of BMP signaling) to control neuromuscular junction (NMJ) growth in Drosophila. Ultrastructural analysis of α2-adaptin mutants show an accumulation of large vesicles and membranous structures akin to endosomes at the synapse. We found that mutations in α2-adaptin lead to an accumulation of Tkv receptors at the presynaptic membrane. Interestingly, the level of small GTPase Rab11 was significantly reduced in the α2-adaptin mutant synapses. However, expression of Rab11 does not restore the synaptic defects of α2-adaptin mutations. We propose a model in which AP2 regulates Tkv internalization and endosomal recycling to control synaptic growth.

Key words: α2-adaptin; BMP receptors; Rab11; synaptic growth; Thickveins

Significance Statement
Understanding the regulation of synaptic development and refinement could provide mechanistic insights into the neural basis of fundamental processes such as learning, memory and adaptation. In this study, we describe the role of α2-adaptin in attenuating neuromuscular junction (NMJ) growth signaling and show its involvement in the trafficking of Thickveins receptor at the Drosophila NMJ. α2-Adaptin interacts genetically with the BMP pathway components and NMJ synapses lacking α2-adaptin show reduced levels of the endosomal recycling marker, Rab11. Our study contributes to a broader understanding of AP2 complex-dependent regulation of synaptic growth signaling, which might have implications in neurodevelopment under conditions where endocytosis and trafficking of the BMP receptors are perturbed.

Introduction
Synapse development and refinement is an interplay of multiple signaling pathways, namely, the Bone morphogenetic protein (BMP), Wingless (Wnt), and Ubiquitin-Proteasome-mediated protein degradation (Highwire-Wallenda) pathways (McCabe et al., 2003, 2004; Franco et al., 2004; Collins et al., 2006; Ball et al., 2010; Budnik and Salinas, 2011; Piccioli and Littleton, 2014; Choudhury et al., 2016). Besides, there are also pathways mediated by endocytic, cytoskeletal, and actin regulatory proteins (Franco et al., 2004; Piccioli and Littleton, 2014; Choudhury et al., 2016). Understanding the crosstalk among them is crucial to our understanding of the processes that regulate synapse development, refinement, and plasticity (Deshpande and Rodal, 2016). BMP signaling pathway at the Drosophila neuromuscular junction (NMJ) synapses is a well-studied growth-promoting pathway...
The canonical BMP signaling is dependent on phosphorylated Smad (pMad in *Drosophila*) and its translocation into the motor neuron nuclei, followed by the transcription of BMP target genes (Keshishian and Kim, 2004; O’Connor-Giles et al., 2008; Ball et al., 2010; Kim and Marqués, 2012; Sulkowski et al., 2016; Vuilleumier et al., 2019).

At the *Drosophila* NMJ, the retrograde BMP signaling is initiated by secretion of Glass bottom boat (Gbb) from the muscle and the motor neurons (Goold and Davis, 2007). Gbb binds to Wit (a Type II receptor) as well as Tkv and Sax (Type I receptors) at the presynaptic nerve terminals to control NMJ growth and function (Aberle et al., 2002; Marqués et al., 2002; McCabe et al., 2003). Gbb binding to Wit triggers the tetramerization of BMP receptors that, in turn, phosphorylates the Smad transcription factor. Mothers against decapentaplegic (Mad). Following BMP activation, these receptors are endocytosed for retrograde transport to the motor neuron nuclei in the soma (Rodal et al., 2011; Smith et al., 2012; Vanlandingham et al., 2013).

Multiple studies have shown a tight correlation between defective endocytosis, altered synapse growth, and elevated synaptic pMad levels, implicating increased BMP signaling in synaptic development (O’Connor-Giles et al., 2008; Ball et al., 2010; Nahm et al., 2010; Shi et al., 2013; Piccioli and Littleton, 2014). One such study has shown that Nwk, an F-BAR and SH3 domain-containing protein along with Dap160 and Dynamin (both endocytic molecules in endosomes or intermediate structures of the endosomal pathway (Dubois et al., 2001; Lloyd et al., 2002; Sweeney and Davis, 2002; Rodal et al., 2011). In the neuronal context, the efficacy of intercellular signaling is regulated by the trafficking of activated receptor/ligand complexes following endocytosis from the presynaptic membrane.

Tightly regulated endocytic transport of BMP receptors relies on the spatiotemporal regulation of Rab GTPase function (Kelly et al., 2012). The Rab-family of GTPases regulates the progression of receptor endocytosis and participates in the successive steps of membrane maturation, receptor transport, and turnover (Horgan and McCaffrey, 2011). In particular, Rab5 regulates vesicle formation and is associated with early endosomes, while Rab7 and Rab11 associate with late and recycling endosomes, respectively (Chavrier et al., 1990; Ullrich et al., 1996). Endosomal trafficking of BMP signaling complexes at the nerve terminals is known to fine-tune the intensity and persistence of BMP signaling (Cosker and Segal, 2014; Deshpande and Rodal, 2016). Altered distribution or misregulation of Rab11 has been shown to suppress Tkv trafficking from early endosome to presynaptic membrane resulting in elevated BMP signaling (O’Connor-Giles et al., 2008; Rodal et al., 2008; Liu et al., 2014; Deshpande and Rodal, 2016).

An important, yet enigmatic question, is the correlation between defective Clathrin-mediated endocytosis (CME) and aberrant synaptic growth. For instance, it is not known whether specific modes of endocytosis externalize specific cargos whose trafficking defect perturbs synaptic signaling. CME, the major endocytic pathway, is required not only for basal synaptic transmission at nerve terminals but also for synapse development (Koh et al., 2004; Dickman et al., 2006; Choudhury et al., 2016). For instance, perturbations in CME resulting from mutations in Dynamin, AP2 subunits, Endo, or Synj all exhibit NMJ structural defects resulting in increased number but decreased size of synaptic boutons in *Drosophila* (Dickman et al., 2006; Choudhury et al., 2016). It is unclear whether the NMJ structural defects associated with the endocytic mutants is a consequence of deficient endosomal trafficking leading to aberrant synaptic signaling. It is likely that perturbing CME deregulates signaling modules of BMP pathway that leads to elevated pMad in the endocytic mutants (O’Connor-Giles et al., 2008; Choudhury et al., 2016).

In central synapses, AP2-dependent CME is dispensable for membrane regeneration from the presynaptic plasma membrane following high-frequency nerve stimulation (Kononenko et al., 2014). However, the critical role of CME in generating vesicles from endosome-like structures following bulk membrane endocytosis cannot be ruled out (Watanabe et al., 2014). Previous studies support a model in which compromised CME can lead to defective signalosome trafficking by trapping signaling molecules in endosomes or intermediate structures of the endosomal pathway (Dubois et al., 2001; Lloyd et al., 2002; Sweeney and Davis, 2002; Wang et al., 2007; Papagiannouli et al., 2019; Joseph et al., 2020). Another study has shown clathrin-independent role of the AP2 in synapse development (Di Fiore and De Camilli, 2001; Dubois et al., 2001; Sweeney and Davis, 2002; Rodal et al., 2011).
the endocytic retrieval of select synaptic vesicle (SV) cargos from the presynaptic cell surface (López-Hernández et al., 2021).

Our previous study has shown elevated levels of synaptic as well as motor-nuclei pMad in \(\alpha 2\)-adaptin mutants (Choudhury et al., 2016). In order to investigate the underlying signaling mechanisms leading to elevated pMad levels, we performed epistatic interactions between \(\alpha 2\)-adaptin mutants with the components of BMP signaling. Introducing a mutant copy of \(tkv\) in \(\alpha 2\)-adaptin mutants significantly reduces the NMJ overgrowth. Conversely, introducing a mutant copy of inhibitory Smad, \(Dad\) in a heterozygous \(\alpha 2\)-adaptin background leads to NMJ overgrowth. Ultrastructural analysis of NMJ revealed accumulation of large vesicles and supports a role of \(\alpha 2\)-adaptin in generating signalosomes containing vesicles, possibly from endosomal structures. Further analysis of vesicular trafficking using endosomal markers shows that Rab11 is reduced in \(\alpha 2\)-adaptin mutant NMJ synapses. Thus, our studies reveal a novel function of \(\alpha 2\)-adaptin in attenuating BMP signaling by facilitating trafficking and recycling of the Tkv receptor.

Materials and Methods

Fly stock

Flies were grown and maintained at 25°C temperature in a standard cornmeal medium as described earlier (Choudhury et al., 2016). Both males and females were used for experiments in the current study. \(w^{1118}\) was used as control unless otherwise stated. Two \(\alpha 2\)-adaptin alleles were used: \(AP2_\alpha^{ANG}\), which is a hypomorphic allele obtained through a \(P\)-element mobilization of Synd\(_{EP877}\) located 2416 bp upstream of the Syndapin ORF, and \(AP2_\alpha^{KG02457}\), which is a \(P\)-element insertion in the third exon of the \(\alpha 2\)-adaptin ORF making it a null allele (Choudhury et al., 2016). All the mutants, controls, and rescued larvae of either sex were grown under noncrowded conditions on apple agar plates with yeast paste dollop. The following stocks were obtained from Bloomington Drosophila Stock Center (BDSL): \(tkv^\prime\) (BL3242), \(Med^{A13}\) (BL7340), \(w^{AI2}\) (BL5173), \(Dad^{1E4}\) (BL10305), \(AP2_\alpha^{KG02457}\) (BL13478), UAS-Rab11-GFP (BL8506), \(Mad^{d00237}\) (BL10474), \(\alpha\)-adaptin dsRNA (BL32866), Clc dsRNA (BL27496), \(Syn^{7}\) (BL24883), \(Syn^{2}\) (BL24884). Other lines used in this study are: \(D42-Gal4\) (Yeh et al., 1995; Sanyal, 2009), UAS-tkv-EGFP (BL51653; Ayyaz et al., 2015), UAS-YFP-Rab11\(_{S25N}\) (BL9792; Guan et al., 2020), UAS-Rab11\(_{Q70L}\)-GFP (BL23260; Sorvina et al., 2016), Rab11\(_{ex2}\) and Rab11\(_{93Bi}\) (Khodosh et al., 2006).

Antibodies and immunocytochemistry

Wandering third instar larvae were dissected in cold calcium-free HL3 saline (70 mM NaCl, 5 mM KCl, 20 mM MgCl\(_2\), 10 mM NaHCO\(_3\), 5 mM Trehalose, 115 mM sucrose, and 5 mM HEPES, pH 7.2) to expose the NMJs and fixed in 4% paraformaldehyde in PBS (pH 7.2) for 30 min at room temperature. Fillets were then washed in PBS containing 0.15% Triton X-100, blocked for 1 h with 5% bovine serum albumin (BSA), followed by overnight incubation with primary antibody at 4°C. The monoclonal antibody anti-CSP (1:100) was obtained from the Developmental Studies Hybridoma Bank (DSHB). The polyclonal antibody against Rab5 (1:500; Mottola et al., 2010) was a gift from Marino Zerial, Max Planck Institute, Germany. The polyclonal Rab7 (1:500) and Rab11 (1:500; Tanaka and Nakamura, 2008; West et al., 2015) antibodies were a gift from Tsubasa Tanaka, RIKEN Center for Developmental Biology, Japan. The pMad antibody was a gift from Peter ten Dijke and used at 1:1000 dilution (Persson et al., 1998). The secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen, Thermo Fisher Scientific) were used at 1:800 dilution. The Alexa Fluor 488 or Rhodamine-conjugated anti-HRP (Jackson ImmunoResearch) were used at 1:800 dilution. Stained larval fillets were mounted in VECTASHIELD (Vector Laboratories). All the images were captured with a laser scanning confocal microscope (LSM780, Carl Zeiss or FV3000, Olympus Corporation).

Electrophysiology

All intracellular recordings were performed on wandering third instar larvae as described previously (Choudhury et al., 2016). Briefly, HL3 buffer containing 1.5 mM Ca\(^{2+}\) was used for larval dissection. Recordings from muscle 6 of A2 hemisegment were performed using sharp glass electrodes having a resistance of 20–25 MΩ. Miniature excitatory junction potentials (mEJPs) were recorded for 60 s, followed by recordings of EJPs at 1 Hz stimulation. For High-frequency recording, nerves were stimulated at 10 Hz, and EJPs were recorded for 5 min. For recording EJPs, stimulation pulse was delivered using Grass S88 stimulator (Grass Instruments, Astro-Med, Inc). The signals were amplified using Axoclamp 900A, digitized using Digidata 1440A, and acquired using pClamp10 software (Molecular Devices). Muscles with resting membrane potential between –60 and –75 mV were used for analysis. The data were analyzed with the Mini Analysis program (Synaptosoft, Decatur).

Intensity profile

Confocal images of muscle four NMJ at A2 hemisegment were used to plot the intensity profile. A single bouton section was used, and the intensities of Tkv and HRP were analyzed by drawing a line across the bouton using Fiji/ImageJ software. The graph was plotted in Microsoft Excel using the intensity values obtained from Fiji/ImageJ software. As the intensity of Tkv in control was too low to plot the graph, all the intensity values were multiplied two-fold.

Electron microscopy

TEM was performed as described previously (Deivasigamani et al., 2015). Briefly, third instar larvae were dissected in cold PBS. The larval fillets were then fixed in 0.12 M cacodylate buffer containing 2% glutaraldehyde for 10 min at room temperature, transferred to a fresh fixative, and kept overnight at 4°C. The fillets...
were postfixed for 1 h with 2% osmium tetroxide (OsO₄) solution prepared in 0.12 M cacodylate buffer. The samples were rinsed with 0.12 M cacodylate buffer followed by washes with distilled water to avoid precipitation of cacodylate with Uranyl acetate. Subsequently, the samples were subjected to en bloc staining with 2% uranyl acetate. The stained fillets were again washed with distilled water and dehydrated using graded solutions of ethanol before final infiltration of the samples through propylene oxide for 30 min. Stained and dehydrated fillets were embedded in epoxy resin and hardened overnight at 60°C. Muscles embedded in epoxy resin were sectioned at 60 nm. Ultrathin sections of the muscles stained with 2% uranyl acetate (in 70% ethanol) and 1% aqueous lead citrate were examined at 120 KV on a Tecnai G2 Spirit BioTWIN (FEI) electron microscope. The number of synaptic vesicles per bouton were counted manually using the Multi-point tool in ImageJ/Fiji software and then divided by their respective bouton areas to obtain the vesicle density/m² area of a bouton. For vesicle size, diameters of at least 100 vesicles from 10 bouton sections of each genotype were used for quantification. For calculating the number of synaptic vesicles docked at the active zones, only those vesicles that were touching the T-bar platform were counted. For calculating the SSR thickness, the scale bar in the images was first calibrated to the number of pixels using the Set scale function in ImageJ/Fiji software. This was followed by using the Straight-line tool to draw a line across the SSR and Measure tool to calculate the thickness.

**Western blot analysis**

The western blot analysis was done as previously described previously (Choudhury et al., 2016). Briefly, VNCs from wandering third instar larvae were dissected out in ice-cold HL3 buffer and homogenized in buffer containing 50 mM Tris–HCl, pH 6.8, 25 mM KCl, 2 mM EDTA, 0.3 M sucrose, and 2% SDS in water. The homogenized sample was then mixed with an equal volume of 2× Laemmli buffer. The protein equivalent to 50 μg was separated on 12% SDS-PAGE and transferred to Hybond-LFP PVDF membrane (GE Healthcare, GE Healthcare Life Sciences). The membrane was then blocked with 5% skimmed milk for 1 h, followed by overnight incubation with anti-Rab11 (1:2000) and anti-Tubulin (1:5000) antibody. IRDye 800 (1:10,000) was used as a secondary antibody, and signals were visualized on LI-COR Odyssey platform. The density of Western bands was quantified using ImageJ/Fiji software.

**Quantification and statistical analysis**

For fluorescence quantification, images were captured using a laser scanning confocal microscope (LSM780; Carl Zeiss or FV3000, Olympus). All the control and experimental fillets were processed in the same way, and the fluorescence images were captured under the same settings for every experimental set. For bouton quantification, CSP-labeled structures were counted at muscle 6/7 of A2 hemisegment. The number of boutons from each NMJ was normalized to the respective muscle area. To calculate the bouton number, NMJs from A2 hemisegment were captured using a plan apochromat 40× objective, 1.4 NA and all the CSP positive boutons were counted manually in ImageJ/Fiji software. For muscle area quantification, images from A2 hemisegment were captured using 20× objective, and the area was quantified using ZEN2 software (Carl Zeiss, Germany). For bouton number quantification, the total number of boutons per NMJ was divided by the respective muscle areas. For fluorescence intensity quantification, NMJs from muscle four were captured using a plan apochromat 60×, 1.4 NA objective. For each NMJ, the fluorescence intensity from each bouton was subtracted from the background intensity, and the average intensity was normalized to the control. The fluorescence intensity was calculated using ImageJ/Fiji software. For bouton area quantification, NMJs from muscle 6/7 at A2 hemisegment were captured, and the area was calculated by drawing a free-hand sketch around CSP positive bouton using ImageJ/Fiji software. For multiple comparisons, one-way ANOVA followed by post hoc Tukey’s test, or Student’s t test was used. GraphPad Prism 8 was used to plot the graph. Error bars in all the histograms represent SEM. *p < 0.5, **p < 0.01, ***p < 0.001.

**Results**

α2-adaptin genetically interacts with regulators of BMP signaling

In a previous study, we showed α2-adaptin mutants caused NMJ overgrowth and upregulation of pMad, an effector of the BMP pathway. Two previously characterized α2-adaptin alleles, AP2αang7, a strong hypomorph and AP2αKG02457, a null allele was used in the current study (Choudhury et al., 2016). To explore the role α2-adaptin in regulating BMP signaling at the NMJ, we first assessed the epistatic interaction between α2-adaptin and components of the BMP-signaling pathway. We found that introducing one mutant copy of tkv, Med, or Mad in α2-adaptin mutant background significantly suppressed the synaptic overgrowth phenotype in these animals (Fig. 1A–F). The number of boutons normalized to the muscle area was significantly rescued in tkv+/+; AP2αKG02457/AP2αang7 (1.91 ± 0.12, p < 0.001), Med13, AP2αKG02457/AP2αang7 (1.82 ± 0.07, p < 0.001), and Mad60023+/+; AP2αKG02457/AP2αang7 (2.25 ± 0.11, p < 0.001) when compared with AP2αKG02457/AP2αang7 (2.85 ± 0.08, p < 0.001). However, there was no significant difference between w1118 control, heterozygous AP2αKG02457/+, and tkv+/+ (w1118: 1.26 ± 0.06, AP2αKG02457/+: 1.33 ± 0.03 and tkv/+: 1.34 ± 0.05; Fig. 1G).

Consistent with the above observations, we found that mutating one copy of wit significantly reduced the α2-adaptin induced synaptic overgrowth (witA12, AP2αKG02457/AP2αang7: 2.00 ± 0.08 vs AP2αKG02457/AP2αang7: 2.50 ± 0.11; p = 0.01; Extended Data Fig. 1-1). Since elevated BMP signaling results in the formation of smaller boutons, we quantified the bouton area in these genotypes. We found that introducing one copy of tkv7 (tkv/++; AP2αKG02457/AP2αang7: 2.9 ± 0.05, p < 0.01), Med13
Figure 1. Downregulating BMP signaling components reduced the synaptic overgrowth and clustering in the α2-adaptin mutant. **A–F.** Confocal images of NMJ synapses at muscles 6/7 of A2 hemisegment showing synaptic growth in **A, A’** (Control), **B, B’** tkv/+, **C, C’** AP2αKG02457/AP2αang7, **D, D’** tkv/+; AP2αKG02457/AP2αang7, **E, E’** Med13, **F, F’** Madk00237/+; AP2αKG02457/AP2αang7 double immunolabeled with a presynaptic vesicle marker, CSP (magenta), and a neuronal membrane marker, HRP (green), to mark the bouton outline. Reducing the levels of BMP signaling components in the AP2αKG02457/AP2αang7 background reduces the synaptic overgrowth. Scale bar in **F** (for **A–F**) and **F’** (for **A’–F’**) represents 20 and 5 μm, respectively. 

**G.** Histogram showing the averagebouton number normalized to the muscle area from muscle 6/7 NMJ at A2 hemisegment in control animals (8.57 ± 0.53), AP2αKG02457/AP2αang7 (8.90 ± 0.35), tkv/+ (9.08 ± 0.47), AP2αKG02457/AP2αang7 (1.34 ± 0.05), Med13, AP2αKG02457/AP2αang7 (1.91 ± 0.12), AP2αKG02457/AP2αang7 (1.98 ± 0.03), tkv/+; AP2αKG02457/AP2αang7 (2.79 ± 0.05), and Madk00237/+; AP2αKG02457/AP2αang7 (3.86 ± 0.14). Error bar represents SEM; the statistical analysis was done using one-way ANOVA followed by post hoc Tukey’s test. **H.** Histogram showing the average bouton area from muscle 6/7 NMJ at A2 hemisegment in control animals (2.59 ± 0.05), AP2αKG02457/AP2αang7 (2.59 ± 0.05), tkv/+ (2.59 ± 0.05), AP2αKG02457/AP2αang7 (2.85 ± 0.05), Med13, AP2αKG02457/AP2αang7 (2.79 ± 0.05), and Madk00237/+; AP2αKG02457/AP2αang7 (3.86 ± 0.14). Error bar represents SEM; the statistical analysis was done using one-way ANOVA followed by post hoc Tukey’s test. **I.** Histogram showing the average NMJ length normalized with muscle area from muscle 6/7 NMJ at A2 hemisegment in control animals (3.43 ± 0.18), AP2αKG02457/AP2αang7 (2.56 ± 0.22), tkv/+ (2.56 ± 0.22), AP2αKG02457/AP2αang7 (3.49 ± 0.15), Med13, AP2αKG02457/AP2αang7 (3.91 ± 0.39), Madk00237/+; AP2αKG02457/AP2αang7 (4.08 ± 0.22). Error bar represents SEM; the statistical analysis was done using one-way ANOVA followed by post hoc Tukey’s test. ns, not significant. **J.** Histogram showing the average number of branches from muscle 6/7 NMJ at A2 hemisegment in control animals (8.95 ± 0.71), AP2αKG02457/AP2αang7 (8.77 ± 0.43), tkv/+ (9.11 ± 0.53), AP2αKG02457/AP2αang7 (7.8 ± 0.36), tkv/+; AP2αKG02457/AP2αang7 (8.88 ± 0.55), Med13, AP2αKG02457/AP2αang7 (9.25 ± 0.56), and Madk00237/+;
Thus, our data suggest that is shown in Extended Data Figure 1-1.

In order to further strengthen our observation that α2-adaptin attenuates BMP signaling, we tested the interaction between α2-adaptin and Dad, a negative regulator of BMP signaling (O’Connor-Giles et al., 2008; L Zhao et al., 2013). We first examined the total number of synaptic boutons in transheterozygotes of α2-adaptin and Dad mutants (Fig. 2). While the number of boutons in larvae heterozygous for α2-adaptin [AP2αKG02457/+/] and Dad [Dad1Ed/+] (1.39 ± 0.09) was comparable with w1118 control (1.26 ± 0.06), transheterozygous Dad1Ed/AP2αKG02457 (1.89 ± 0.09, p < 0.001) showed significantly higher bouton number when compared with w1118 controls (Fig. 2H). However, there was no significant difference in the bouton area in transheterozygous of α2-adaptin and Dad mutants Dad1Ed4/AP2αKG02457 (8.51 ± 0.61) when compared with w1118 control (10.91 ± 0.87; Fig. 2).

Next, we examined whether neurally expressing UAS-Dad in α2-adaptin mutant could rescue the synaptic overgrowth phenotype (Fig. 2E,F). Neuronal expression of Dad in an α2-adaptin mutant background UAS-Dad/+; D42-Gal4, Ap2αang7/AP2αKG02457 (1.87 ± 0.15, p < 0.001) significantly rescued the synaptic overgrowth in α2-adaptin mutant AP2αang7/AP2αKG02457 (3.1 ± 0.18, p < 0.001; Fig. 2G). Moreover, we found that neuronal expression of Dad in α2-adaptin mutant background partially rescued the average bouton area (UAS-Dad/+; D42-Gal4, Ap2αang7/AP2αKG02457; 5.94 ± 0.29, p < 0.001; Fig. 2I). Taken together, these data suggest that α2-adaptin regulates BMP signaling to attenuate synaptic growth at the Drosophila NMJ.

The functional and morphologic aspects of α2-adaptin can be genetically discriminated

Since one mutant copy of BMP receptors in the α2-adaptin mutant background significantly restores the morphologic defects, we asked whether the electrophysiological defects associated with the α2-adaptin mutant could also be rescued. To address this, we measured evoked excitatory junction potential (EJP), quantal content (QC), and high-frequency intracellular recording on w1118 control, heterozygous tkv+/+, AP2αKG02457/AP2αang7, and tkv+/+; AP2αKG02457/AP2αang7 larvae (Fig. 3). We found that both mEJP amplitude and quantal content in tkv+/+; AP2αKG02457/AP2αang7 (mEJP amplitude = 1.01 ± 0.08, QC = 38.20 ± 3.09) were not significantly different from α2-adaptin mutant AP2αKG02457/AP2αang7 (mEJP = 1.04 ± 0.09, QC = 39.29 ± 5.81; Fig. 3A–D). The scatter plot for mEJP amplitude distribution showed that both α2-adaptin mutant and tkv+/+; AP2αKG02457/AP2αang7 had more mEJPs with higher amplitude compared with w1118 controls (Fig. 3C). Furthermore, we found that reducing the levels of Tkv receptor did not rescue the activity-dependent decline in EJP amplitude. The line plot showed that both α2-adaptin mutant and tkv+/+; AP2αKG02457/AP2αang7 followed the same trend of EJP amplitude decline during the high frequency (10 Hz) stimulations (Fig. 3E,F). Moreover, there was no significant difference between w1118 and tkv+/+ controls in mEJP amplitude, QC, and high frequency recordings (w1118: mEJP = 0.65 ± 0.03, QC = 61.92 ± 4.02; tkv+/+: mEJP = 0.71 ± 0.05, QC = 69.77 ± 6.98; Fig. 3B,D,F).

Loss of α2-adaptin leads to the accumulation of endosome-like structures at the NMJ

Studies have shown that internalization and recycling of the membrane and BMP receptors depend on CME and endosomal proteins such as Rab5 and Rab11 (Mitchell et al., 2004; Liu et al., 2014). Mutants with defects in endocytosis and endosomal trafficking cause functional and ultrastructural abnormalities at the synapses. To get a deeper understanding of how loss of α2-adaptin affects synapse ultrastructure, we performed transmission electron microscopy on α2-adaptin mutant NMJs. Interestingly, ultrastructural analysis of α2-adaptin deficient synapses showed an accumulation of large endosome-like structures, similar to what has been shown in mutants that affect endocytic and endosomal recycling machinery such as clathrin (Kasprowicz et al., 2008; Kawasaki et al., 2011), AP180 (B Zhang et al., 1998), Rab5 (Shimizu et al., 2003), Rab8 (West et al., 2015), and Rab11 (Inoshita et al., 2017). We found decreased synaptic vesicle (SV) density (w1118: 85.18 ± 12.26 vs AP2αKG02457/AP2αang7: 28.02 ± 14, p < 0.01) and increased size of the SVs in the α2-adaptin mutants (w1118: 43.16 ± 0.94 vs AP2αKG02457/AP2αang7: 71.53 ± 3.70, p < 0.001). While there was no change in the number of SVs docked at the active zones (w1118: 0.91 ± 0.20 vs AP2αKG02457/AP2αang7: 0.78 ± 0.28, p > 0.05), we found significantly decreased thickness of the subsynaptic reticulum (SSR; w1118: 0.68 ± 0.03 vs AP2αKG02457/AP2αang7: 0.45 ± 0.03, p < 0.001) in the α2-adaptin mutants. These ultrastructural...
defects were rescued on ubiquitous expression of a α2-adaptin transgene \([\text{actin5C-Gal4/+; AP2}\alpha_{\text{KG02457}}/\text{AP2}\alpha_{\text{ang7}}]/\text{AP2}\alpha_{\text{ang7}}/\alpha2\text{-adaptin transgene} [\text{actin5C-Gal4/+; AP2}\alpha_{\text{KG02457}}/\text{AP2}\alpha_{\text{ang7}}], \text{SV density} (107.9 \pm 11.32), \text{SV size} (43.1 \pm 1.46), \text{and SSR thickness} (0.61 \pm 0.01); \text{Fig. 4A–G}]. \text{Together, these data indicate that compromised regeneration of vesicles from the presynaptic membrane and defective membrane recycling in α2-adaptin mutants results in the accumulation of large endosome-like structures.}
Figure 3. Structural and functional deficits in α2-adaptin mutant can be genetically discriminated. A, Representative traces of mEJP in control, tkv\( ^{+/+} \), heteroallelic AP2\( \alpha ^{KGO2457}/AP2\alpha ^{ang7} \), and tkv\( ^{+/+} \); AP2\( \alpha ^{KGO2457}/AP2\alpha ^{ang7} \) larvae. B, Quantification of average mEJP amplitude in control (0.65 ± 0.03), tkv\( ^{+/+} \) (0.71 ± 0.05), heteroallelic AP2\( \alpha ^{KGO2457}/AP2\alpha ^{ang7} \) (1.04 ± 0.09) and tkv\( ^{+/+} \); AP2\( \alpha ^{KGO2457}/AP2\alpha ^{ang7} \) (1.01 ± 0.08). At least eight NMJ recordings of each genotype were used for quantification. Error bars represent the SEM; statistical analysis is based on one-way ANOVA followed by post hoc Tukey’s multiple-comparison test. **p < 0.01; ns, not significant. C, Scatter plot shows quantification of mEJP amplitude distribution in control, tkv\( ^{+/+} \), heteroallelic AP2\( \alpha ^{KGO2457}/AP2\alpha ^{ang7} \) and tkv\( ^{+/+} \); AP2\( \alpha ^{KGO2457}/AP2\alpha ^{ang7} \). Note that events with mEJPs greater than 4 mV occurred more frequently in AP2\( \alpha ^{KGO2457}/AP2\alpha ^{ang7} \) and tkv\( ^{+/+} \); AP2\( \alpha ^{KGO2457}/AP2\alpha ^{ang7} \) compared with controls. D, Quantification of quantal content in control (61.92 ± 4.02), tkv\( ^{+/+} \) (69.77 ± 6.98), AP2\( \alpha ^{KGO2457}/AP2\alpha ^{ang7} \) (39.29 ± 5.81) and tkv\( ^{+/+} \); AP2\( \alpha ^{KGO2457}/AP2\alpha ^{ang7} \) (38.2 ± 3.09). At least eight NMJ recordings of each genotype were used for quantification. Error bars represent the SEM; statistical analysis is
a2-adaptin mutants display increased Tkv receptors at the NMJ

AP2 complex has been shown to regulate CME and activity-dependent vesicle regeneration from endosome-like vacuoles (Kononenko et al., 2014; Kadlecova et al., 2017). Since the ultrastructural analysis of α2-adaptin mutants revealed an accumulation of large endosome-like membranous structures similar to mutants with perturbed endocytosis or endosomal trafficking such as Rab5, Rab8, and Rab11 mutants (Shimizu et al., 2003; West et al., 2015; Inoshita et al., 2017), we hypothesized that α2-adaptin could be involved either in endocytosis of BMP receptors from the presynaptic membrane or in the endosome-dependent trafficking of the receptors. To test this possibility, we first assessed the level of Tkv receptors at the larval NMJ. Since specific antibodies against Tkv receptors are not available, we expressed an EGFP-tagged Tkv receptor transgene in the motor neurons of α2-adaptin mutants. Interestingly, we found a significant accumulation of Tkv receptors at the mutant synapses (D42-Gal4, AP2αKG02457/AP2αang7, UAS-tkv-EGFP: 427.5 ± 20.20, p < 0.001) when compared with control (D42-Gal4/UAS-tkv-EGFP: 100 ± 8.49; Fig. 5A–D-i). In order to analyze the subcellular accumulation, we plotted the intensity profiles of Tkv-EGFP and HRP (a presynaptic membrane marker) in single sections of the acquired images. While in control synapses, Tkv localized both at the presynaptic membrane as well as within the bouton; we found a higher intensity peak of Tkv-EGFP at the presynaptic membranes of α2-adaptin mutants (Fig. 5J–O). Consistent with these observations, we found that knocking down α-adaptin (D42-Gal4>α-adaptin dsRNA; 276.1 ± 14.75, p < 0.001) or clathrin light chain (Cic; D42-Gal4>Cic dsRNA: 408.61 ± 21.17, p < 0.001) in motor neurons showed significantly increased synaptic Tkv levels (Fig. 5E–I) with intensity profiles similar to that of α2-adaptin mutants (Fig. 5P–U). In contrast, mutants defective in proteins involved in later stages of endocytosis did not show Tkv accumulation at the presynaptic membrane (Fig. 5i), which is consistent with previous studies (O’Connor-Giles et al., 2008; G Zhao et al., 2015). These data indicate that endocytosis/trafficking of Tkv receptors in α2-adaptin mutants is severely compromised, leading to their accumulation at the synaptic membranes.

Loss of α2-adaptin results in decreased levels of recycling endosomal marker Rab11

Receptors are known to be endocytosed, trafficked to early endosomes, and sorted out for recycling back to the membrane or degraded in the lysosomes (Dunn et al., 1989; Gruenberg et al., 1989; Jovic et al., 2010). This process is mediated by the Rab family of small GTPases (Schwartz et al., 2007). Since the ultrastructural analysis of α2-adaptin mutant synapse showed an accumulation of enlarged synaptic vesicles similar to mutants of the endosomosomal pathway (Shimizu et al., 2003; West et al., 2015; Inoshita et al., 2017), we next asked whether this pathway has any role in the accumulation of Tkv in α2-adaptin mutants. We found that the synaptic levels of the early endosomal marker, Rab5 (w1118: 100 ± 3.0 vs AP2αKG02457/AP2αang7: 97.25 ± 4.81) or the late endosomal marker, Rab7 (w1118: 100 ± 5.71 vs AP2αKG02457/AP2αang7: 115.6 ± 8.17) were not altered in α2-adaptin mutant synapses (Fig. 6A–J). However, we observed reduced synaptic Rab11 levels (w1118: 100 ± 5.73 vs AP2αKG02457/AP2αang7: 61.81 ± 7.10, p < 0.001) in α2-adaptin mutant synapses (Fig. 6K–S). Western blot analysis revealed that total levels of Rab11 protein in α2-adaptin mutants were not altered compared with w1118 controls (Extended Data Fig. 6-1). Synaptic Rab11 levels were restored to w1118 control levels on neuronal expression of a α2-adaptin transgene in the α2-adaptin mutant (D42-Gal4, AP2αKG02457/UAS-AP2α, AP2αang7):112.9 ± 8.28; Figure 60,P,S). Taken together, these data indicate that: (1) compromised endocytosis, and possible defective recycling of the endocytosed Tkv receptors result in their accumulation at the synapse, or (and) (2) AP2 complex may regulate Tkv sorting possibly through the endosomosomal pathway.

Rab11 mutants phenocopy BMP-signaling and NMJ defects of α2-adaptin

Rab11 has been shown to regulate BMP signaling at the Drosophila NMJ (Khodosh et al., 2006; Liu et al., 2014). Because α2-adaptin mutant synapses showed reduced Rab11 levels, we next asked whether Rab11 had a role in regulating Tkv receptor trafficking. We first examined the levels of pMad and Tkv in Rab11 mutants. We found that Rab11 mutants, as well as animals expressing a dominant-negative form of Rab11 in motor neurons, showed accumulation of pMad at the NMJ synapses (w1118: 100 ± 5.87; Rab11Δz/ΔzB; 147.9 ± 8.58, p < 0.01; UAS-YFP-Rab11Δz:125N: 148.1 ± 8.6; D42-Gal4/++; 134.2 ± 4.36, p < 0.05; AP2αKG02457/AP2αang7:218.8 ± 7.5, p < 0.001; Fig. 7A–H,M). We further assessed Tkv receptor levels in these mutant synapses and found that Rab11 mutants also showed increased Tkv levels at the synapse (D42-Gal4, Rab11Δz: tkv-EGFP, Rab11ΔzB; 189.5 ± 10.57) compared with control animals (D42-Gal4/tkv-EGFP: 100 ± 8.71). However, the Tkv enrichment pattern was not the same as that in α2-adaptin mutants (Fig. 7I–L,N). While α2-adaptin mutants showed Tkv enrichment at the synaptic membranes, Rab11 mutants had punctate Tkv localization within the boutons, but showed no enrichment at the presynaptic...
Figure 4. α2-adaptin mutant synapses show accumulation of large endosome-like vesicular structure. A–C, Electron micrographs of third instar Type Ib boutons of control (A), AP2α<sup>ΔKGO2457</sup>/AP2α<sup> Δang7</sup> (B), and actin5C/++; AP2α<sup>ΔKGO2457</sup>, UAS-AP2α/ AP2α<sup> Δang7</sup> (C). Arrows point to the large endosome-like vesicular structures observed in AP2α<sup>ΔKGO2457</sup>/AP2α<sup> Δang7</sup> boutons but are absent in control and rescued boutons. The insets show a magnified area around the active zones. The presynaptic compartment is pseudocolored in cyan, and the subsynaptic reticulum is marked in green. Scale bar represents 500 nm. D, Histogram showing average vesicle diameter in control (43.16 ± 0.94), AP2α<sup>ΔKGO2457</sup>/AP2α<sup> Δang7</sup> (71.53 ± 3.7), and actin5C/++; AP2α<sup>ΔKGO2457</sup>, UAS-AP2α/ AP2α<sup> Δang7</sup> (43.1 ± 1.46). Error bar represents SEM; statistical analysis was done using one-way ANOVA followed by post hoc Tukey’s test. **p < 0.01, ***p < 0.001. E, Histogram showing SV density per unit area in control (85.18 ± 12.26), AP2α<sup>ΔKGO2457</sup>/AP2α<sup> Δang7</sup> (28.02 ± 14), and actin5C/++; AP2α<sup>ΔKGO2457</sup>, UAS-AP2α/ AP2α<sup> Δang7</sup> (107.9 ± 11.32). At least 10 images from three different larvae per genotype were used for quantification. Error bar represents SEM; statistical analysis was done using one-way ANOVA followed by post hoc Tukey’s test. **p < 0.01, ***p < 0.001. F, Histogram showing average number of synaptic vesicles docked at the active zones in control (0.91 ± 0.20), AP2α<sup>ΔKGO2457</sup>/AP2α<sup> Δang7</sup> (0.78 ± 0.28), and actin5C/++; AP2α<sup>ΔKGO2457</sup>, UAS-AP2α/ AP2α<sup> Δang7</sup> (0.86 ± 0.34). At least five images from three different larvae per genotype were used for quantification. Error bar represents SEM; statistical analysis was done using one-way ANOVA followed by post hoc Tukey’s test. ns, not significant. G, Histogram showing SSR thickness in control (0.68 ± 0.03), AP2α<sup>ΔKGO2457</sup>/AP2α<sup> Δang7</sup> (0.45 ± 0.03), and actin5C/++; AP2α<sup>ΔKGO2457</sup>, UAS-AP2α/ AP2α<sup> Δang7</sup> (0.61 ± 0.01). At least five images from three different larvae per genotype were used for quantification. Error bar represents SEM; statistical analysis was done using one-way ANOVA followed by post hoc Tukey’s test. ***p < 0.001.
Figure 5. Neuronal reduction of subunits of AP2 complex or clathrin leads to accumulation of synaptic Tkv. A–H, Confocal images of NMJ synapses at muscle 4 NMJ at A2 hemisegment in control, D42-Gal4/UAS-tkv-EGFP (A, B), D42-Gal4, AP2<sup>x20,KG02457</sup>/AP2<sup>x20,ang7</sup>, UAS-tkv-EGFP (C, D), D42-Gal4, tkv-EGFP/α-adaptin dsRNA (E, F), and D42-Gal4, tkv-EGFP/Clc dsRNA (G, H). The neuronal membrane is marked with HRP (magenta), and EGFP fluorescence is shown in grayscale/green. The bouton area is outlined in the gray channel. Scale bar in H represents 5 μm. I, Histogram showing the relative Tkv level normalized to HRP in D42-Gal4/UAS-tkv-EGFP (100 ± 8.489), D42-Gal4, AP2<sup>x20,KG02457/AP2<sup>x20,ang7</sup>, UAS-tkv-EGFP (427.5 ± 20.2), D42-Gal4, tkv-EGFP/α-adaptin dsRNA (276.1 ± 14.75), D42-Gal4, tkv-EGFP/Clc dsRNA (408.6 ± 21.17), and Synj1/2<sup>x20</sup> (110.1 ± 6.65) synapses. Error bars represent SEM; statistical analysis is based on one-way ANOVA followed by post hoc Tukey’s multiple-comparison test. ***p < 0.001; ns, not significant. J–U, A single confocal section of a bouton labeled for Tkv (represented in grayscale) and presynaptic membrane marker HRP (magenta) in D42-Gal4/UAS-tkv-EGFP (J, K) or D42-Gal4, AP2<sup>x20,KG02457/AP2<sup>x20,ang7</sup>, UAS-tkv-EGFP (M, N), D42-Gal4, tkv-EGFP/α-adaptin dsRNA (O), or D42-Gal4, tkv-EGFP/Clc dsRNA (P, Q, S, T).
membrane (Extended Data Fig. 7-1). This suggests that while Rab11 does not affect the internalization of Tkv, a defective vesicular trafficking pathway in Rab11 mutants probably leads to the enrichment of Tkv receptors in endosome-like compartments.

Since α2-adaptin mutants showed reduced Rab11 levels at the NMJ, we next asked whether Rab11 was responsible for the morphologic defects in these mutants. To assess this, we first examined whether reducing Rab11 levels phenocopied α2-adaptin mutations. Interestingly, we found that Rab11 mutants or neuronal expression of a dominant-negative form of Rab11 (Rab11^S25N) indeed phenocopied the NMJ morphologic defects in α2-adaptin mutants and showed NMJ overgrowth (w^1118: 1.56 ± 0.06; Rab11^S25N/+; D42-Gal4/+; AP2α^KG02457/AP2α^ang77; 2.26 ± 0.08, p ≤ 0.001; and AP2α^KG02457/AP2α^ang77; 2.83 ± 0.12, p ≤ 0.001; Fig. 8A–F). Expressing a wild-type or constitutively active form of Rab11, however, did not alter the synaptic morphology (Extended Data Fig. 8-1). We next examined whether neuronal expression of Rab11 could restore the synaptic overgrowth in α2-adaptin mutants. We found that expressing the wild-type form of Rab11 in α2-adaptin mutant background (UAS-YFP-Rab11^WT/+; D42-Gal4, AP2α^ang77/AP2α^KG02457; 2.83 ± 0.16, ns) did not prevent the synaptic overgrowth (Fig. 8E,F). Moreover, expressing a constitutively active form of Rab11 (Rab11^CA: UAS-YFP-Rab11^CA/+; D42-Gal4, AP2α^ang77/AP2α^KG02457; 2.5 ± 0.14, ns) failed to prevent the synaptic overgrowth in α2-adaptin mutants (Extended Data Fig. 8-2). Consistent with this, expression of Rab11^WT, Rab11^DN, or Rab11^CA in α2-adaptin mutant background did not rescue the functional defects in α2-adaptin mutant (Extended Data Figs. 8-3, 8-4). Taken together, these results suggest that α2-adaptin is crucial for the proper localization of Rab11 at the synapses and possibly required for the formation of the Rab11 positive recycling endosomes. We surmise that these recycling endosomes could be essential for targeting and distributing Tkv receptors at the synapses to regulate BMP signaling.

In conclusion, α2-adaptin and Rab11 increase Tkv accumulation and BMP signaling to generate similar NMJ phenotypes via independent pathways.

Discussion
Compromised endocytosis not only perturbs synaptic transmission but also has been implicated in deregulating synaptic growth as demonstrated in Endo, Synj, nwk, shi, Clc, brat, and α2-adaptin mutant NMJs (Rikhy et al., 2002; Verstreken et al., 2002; Verstreken et al., 2003; Choudhury et al., 2016). The underlying molecular mechanism by which these proteins regulate synaptic growth, however, has only been demonstrated for nwk and brat (O’Connor-Giles et al., 2008; Shi et al., 2013). Our previous study on α2-adaptin mutants showed no change in levels of endocytic proteins like Endo, Synj, and Dyn (Choudhury et al., 2016), prompting us to investigate the role of α2-adaptin in synaptic growth signaling. Mutations that affect endocytosis, in general, show synaptic overgrowth and increased BMP effector, pMad. The signaling output of growth-regulating pathways is often dependent on intracellular traffic that in part is dependent on endocytosis of activated receptors, ultimately impinging on the BMP, JNK, or Wingless pathways (Shi et al., 2013; Deshpande and Rodal, 2016). Here, we show for the first time that α2-adaptin genetically interacts with BMP signaling pathway at the synapse. Loss of α2-adaptin leads to accumulation of Tkv receptor at the NMJ. Additionally, we provide evidence that α2-adaptin regulates the localization of recycling endosomal protein Rab11.

α2-adaptin genetically interacts with BMP pathway to regulate neuronal BMP signaling
Endosomal trafficking of BMP receptors is a crucial regulatory mechanism that controls synaptic growth (Rodal et al., 2011). Various proteins interact with BMP receptors to facilitate or attenuate the BMP-dependent signaling cascade (McCabe et al., 2004; XW Zhang et al., 2017). Endocytic proteins appear to be fascinating candidates as BMP receptor interactors. Drosophila loss-of-function endocytic mutants correlate with elevated BMP signaling and neuronal overgrowth phenotype (Dickman et al., 2006; Rodal et al., 2011; Deshpande and Rodal, 2016). Consistent with this, we show that increased Tkv levels at the NMJ result in elevated BMP signaling in α2-adaptin mutants. If the BMP pathway is responsible for the synaptic overgrowth in α2-adaptin mutants, we reasoned that reducing the levels of BMP signaling components should rescue the NMJ phenotype. In agreement with this, we found that partially reducing BMP receptors Tkv, Wit, and cytosolic co-Smad molecule, Medea, significantly rescues the NMJ defects in α2-adaptin mutants. Our data reveal that α2-adaptin genetically interacts with the negative regulator of BMP signaling, the inhibitory Smad, Dad. Transheterozygotes of Dad and α2-adaptin mutants have increased number of boutons compared with heterozygotes of either mutant alone. Consistent with this inference, neuronal expression of UAS-Dad in α2-adaptin mutant background significantly reduces the synaptic overgrowth phenotype. However, reducing BMP signaling only partially reduces the bouton size in α2-adaptin mutants. There could be at least two plausible explanations for the partial rescue of the bouton size: first, removing only one copy of tkv may not be sufficient for rescuing this defect. Since mutating both copies of tkv results in embryonic lethality, we could not test the epistatic interactions by removing both the copies of Tkv in α2-adaptin mutants. Second, α2-adaptin may regulate NMJ bouton size through a different signaling pathway, which
**Figure 6.** α2-adaptin mutant synapses show a reduction in the recycling endosome marker, Rab11.  
**A–D.** Confocal images of NMJ synapses at muscle 4 of A2 hemisegment in control (**A, B**), AP2αKG02457/AP2αang7 (**C, D**), double immunolabeled with early endosomal marker Rab5 (represented in grayscale/green) and neuronal membrane marker, HRP (magenta). **E,** Histogram showing the Rab5 level in control (100.0 ± 3.0) and AP2αKG02457/AP2αang7 (97.25 ± 4.81) synapse. Error bar represents SEM; statistical analysis was done using Student’s t test. ns, not significant.  
**F–I.** Confocal images of NMJ synapses at muscle 4 of A2 hemisegment in control (**F, G**), AP2αKG02457/AP2αang7 (**H, I**), double immunolabeled with late endosomal marker, Rab7 (represented in grayscale/green), and neuronal membrane marker, HRP (magenta). **J,** Histogram showing the Rab7 level in control (100.0 ± 5.71) and AP2αKG02457/AP2αang7 (115.6 ± 8.17) synapse. Error bar represents SEM; statistical analysis was done using Student’s t test. ns, not significant.  
**K–R,** Confocal images of NMJ synapses at muscle 4 of A2 hemisegment in control (**K, L**), AP2αKG02457/AP2αang7 (**M, N**), D42-Gal4, AP2αKG02457/UAS-AP2α, AP2αang7 (**O, P**), and Rab11^{62-93 BI} (**Q, R**), double immunolabeled with recycling endosomal marker, Rab11 (represented in grayscale/green), and neuronal membrane marker, HRP (magenta). Scale bar in **R** represents 3 μm. **S,** Histogram showing relative Rab11 level normalized to HRP in control (100 ± 5.73), AP2αKG02457/AP2αang7 (61.81 ± 7.11); D42-Gal4, AP2αKG02457/UAS-AP2α, AP2αang7 (142.9 ± 8.29) and Rab11^{62-93 BI} (142.1 ± 1.57) synapses. Error bars represent SEM; statistical analysis is based on one-way ANOVA followed by post hoc Tukey’s multiple-comparison test. ***p < 0.001; ns, not significant. The data supporting that the levels of Rab11 is not altered in α2-adaptin mutants is provided in Extended Data Figure 6-1.
remains to be elucidated. Overall, our data suggest that $\sigma$2-adaptin negatively regulates the BMP growth signaling pathway to attenuate synaptic growth.

$s$2-adaptin regulates trafficking of Thickveins at the NMJ

BMP signaling has been extensively studied in the context of neuronal growth in which the activated Tkv is endocytosed and fuse with the early endosomes, where it activates downstream signaling molecules. The signaling is attenuated when these activated receptor-containing vesicles recycle back to the plasma membrane or fuse with lysosomes for degradation (Rodal et al., 2011; Smith et al., 2012). Trafficking of these receptors into and out of such endosomes provides an additional tier for spatial and temporal modulation of signal transduction. The members of the Rab family of small GTPases regulate various stages of endocytosis (Kelly et al., 2012). Our immunocytochemistry data show elevated Tkv receptor levels at the synapses and motor neuron soma (data not shown) of $\sigma$2-adaptin mutants. Besides, levels of Rab11 (known for its role in the recycling of Tkv receptor) are reduced by half in $\sigma$2-adaptin mutant synapses.

Interestingly, levels of early and late endosomes marked with Rab5 and Rab7, respectively, remain unaffected at the $\sigma$2-adaptin mutant synapses. The intensity profile of Tkv and HRP across the bouton shows that $\sigma$2-adaptin mutant has a higher intensity of Tkv at the membrane, indicating that a significant proportion of the Tkv receptors are accumulated at the presynaptic membrane. The pattern of Tkv enrichment in $\sigma$2-adaptin and Rab11 mutants were distinct. While $\sigma$2-adaptin mutant synapses showed Tkv enriched at synaptic membranes, Rab11 mutants had a rather punctate distribution within the bouton. The differential distribution of these proteins could be because of their distinct roles in the neurons. While $\sigma$2-adaptin plays a critical role in retrieving the SV-membrane from the presynaptic membrane, Rab11 is a component of the endolysosomal machinery. A clear separation of Tkv signals/pixels distinguishing plasma membrane Tkv from endosomal Tkv is challenging at the NMJ, given the resolution limit of the confocal system.
mutants also argues for additional pathways, other than Rab11, through which α2-adaptin may regulate BMP signaling.

Tkv receptors in α2-adaptin mutants could be accumulated either at the plasma membrane because of inefficient CME or at the early endosomes caused by inefficient recycling. If Tkv were accumulated at the early endosomes, we would expect greater colocalization with Rab5, which is not the case. However, based on reduced Rab11 staining at the α2-adaptin mutant synapses, we conclude that the portion of the receptors in Rab5 positive early endosomes fail to recycle back to the plasma membrane. This conclusion also fits with previous observations that defective CME results in the accumulation of endosome-like structures in cultured hippocampal neurons (Kononenko et al., 2014) and substantiated by our electron microscopy data. The link between clathrin-mediated dynamin-dependent endocytosis and BMP signaling is still a contentious topic. A recent study using human umbilical vein endothelial cells (HUVECs) has shown that treating these cells with BMP-9 triggered Caveolin-1 and dynamin-2-mediated...

Figure 8. Rab11 mutants phenocopy NMJ structural defects of α2-adaptin mutation. A–D, Confocal images of NMJ synapses at muscle 6/7 NMJ at A2 hemisegment in control (A), AP2α<sup>CGO2457</sup>/AP2α<sup>ang7</sup> (B), Rab11<sup>ex2/93 Bi</sup> (C), and D42-Gal4>YFP-Rab11<sup>S25N</sup> (D) double immunolabeled with a presynaptic synaptic vesicle marker, CSP (magenta), and a neuronal membrane marker, HRP (green), to mark the bouton outline. Scale bar in D represents 20 μm. E, Histogram showing average bouton number normalized to the muscle area from muscle 6/7 NMJ at A2 hemisegment in control (1.56 ± 0.06), Rab11<sup>ex2/93 Bi</sup> (2.77 ± 0.11), D42-Gal4-driven dominant-negative YFP-Rab11<sup>S25N</sup> (2.26 ± 0.08), AP2α<sup>KG02457</sup>/AP2α<sup>ang7</sup> (2.83 ± 0.12), and UAS-YFP-Rab11<sup>WT</sup>/+. F, Histogram showing average bouton area from muscle 6/7 NMJ at A2 hemisegment in control (9.97 ± 0.62), Rab11<sup>ex2/93 Bi</sup> (5.51 ± 0.33), D42-Gal4-driven dominant-negative YFP-Rab11<sup>S25N</sup> (6.63 ± 0.31), AP2α<sup>KG02457</sup>/AP2α<sup>ang7</sup> (3.71 ± 0.2), and UAS-YFP-Rab11<sup>WT</sup>/+. Error bar represents SEM; statistical analysis was done using one-way ANOVA followed by post hoc Tukey’s test. ***p < 0.001, *p < 0.05; ns, not significant. Data supporting that expression of wild-type or constitutive active Rab11 does not alter NMJ morphology and does not restore NMJ morphologic defects of α2-adaptin mutants is shown in Extended Data Figures 8-1 and 8-2, respectively. Data supporting that expression of wild-type or dominant negative Rab11 and constitutive active Rab11 does not restore the functional defects of α2-adaptin mutants is shown in Extended Data Figures 8-3 and 8-4, respectively.
endocytosis of its receptor, activin-like kinase 1 (ALK-1). Surprisingly, this ALK-1 endocytosis was not mediated by Clathrin heavy chain (Tao et al., 2020). At the Drosophila NMJ, endocytosis attenuates BMP signaling (O’Connor-Giles et al., 2008), whereas in Drosophila wing discs and intestinal stem cells, endocytosis facilitates the signaling cascade by internalizing Tkv (Gui et al., 2016; Tracy Cai et al., 2019), pointing toward a tissue-specific mechanism. Our results suggest a model where bulk membrane endocytosis is insufficient in removing Tkv from the plasma membrane; besides, the synapses in \( \sigma_2 \)-adaptin mutants fail to recycle remaining receptors from early endosomes leading to enhanced signaling and NMJ growth defects.

Functional and morphologic aspects of \( \sigma_2 \)-adaptin-mediated BMP signaling can be discriminated

Morphologic features of synapses often dictate functional outcomes, and physiological analyses of BMP signaling mutants reveal the same. In \( \text{wit} \) mutants, the size of the NMJ is significantly reduced with concomitant reduced evoked excitatory potentials (McCabe et al., 2003). \( \sigma_2 \)-adaptin mutant synapses show a modest reduction in evoked potentials, and the protein is dispensable for maintaining basal synaptic transmission (Choudhury et al., 2016). However, a rundown of EJP amplitudes during high-frequency stimulation in synaptic mutants implicated in CME, such as \( \text{Endo} \), \( \text{Synj} \), and \( \text{Dap160} \), show a rapid stimulus-dependent decline in EJP amplitude that recovers following a period of rest after the high-frequency stimulation paradigm (Verstreken et al., 2003; Koh et al., 2004). In our previous study, we reported that \( \sigma_2 \)-adaptin mutants do not recover from synaptic depression even after the 90s rest after the cessation of high-frequency stimulation (Choudhury et al., 2016). This observation suggested that in addition to its requirement in synaptic membrane retrieval, the \( \sigma_2 \)-adaptin function is also required during the much slower process of SV trafficking, possibly at one of the rate-limiting steps in SV regeneration. This conclusion is supported by our EM data that shows the accumulation of endosome-like structures more frequently at the mutant synapses when compared with \( \text{wit}^{7179} \) controls. EJP and high-frequency recordings from \( \sigma_2 \)-adaptin mutant synapses with one copy of \( \text{tkv}^7 \) did not show any rescue in synaptic function. These data drive the conclusion that partial reduction of BMP pathway components can only rescue morphologic defects in \( \sigma_2 \)-adaptin mutants but not functional aspects and that morphologic and functional deficits can be discriminated in these mutants. Besides, the partial rescue of bouton size and bouton
clustering at the NMJ argues for possible deregulation of multiple signaling pathways in α2-adaptin mutants that remain to be explored.

Our study uncovers and extends the existing knowledge of synaptic growth signaling and endocytosis. We provide four lines of evidence on the critical role of α2-adaptin in modulating BMP-dependent synaptic growth signaling at the Drosophila NMJ. First, we show using genetics that the morphologic defects in α2-adaptin mutant synapses can be partially rescued by introducing a mutant copy of the BMP receptors, tkv, and wit. We also show a direct epistatic interaction between α2-adaptin and the inhibitory Smad, Dad. Second, using immunohistochemistry, we show that α2-adaptin mutant synapses accumulate Tkv at the plasma membrane and some of these receptors that are endocytosed and make it to the early endosomes fail to recycle back to the plasma membrane. Third, our electrophysiology data establish that morphologic and functional defects can be discriminated in α2-adaptin mutants. Finally, our electron micrographs provide evidence for the presence of large endosomes and support our conclusion that α2-adaptin is critically required at a later step of vesicle regeneration following endocytosis from the plasma membrane.

While this study does not report a direct biochemical or epistatic interaction between α2-adaptin and Rab11, we observed a significant reduction in Rab11 immuno-reactivity in α2-adaptin mutant synapses that could be restored by neuronal expression of α2-adaptin transgenes. However, the structural and functional defects of α2-adaptin mutants could not be restored by neuronal expression of Rab11WT or Rab11CA. This rules out the notion that α2-adaptin phenotypes result from the observed reduction of Rab11. The Rab11 mutant used in this study is a hypomorph with substantial Rab11 protein being detected by us and others (Khodosh et al., 2006). This precludes further epistatic analyses of this mutant. The fact that Rab11 protein could still be detected in these Rab11 hypomorphic mutants may explain why pMad levels were not as upregulated when compared with α2-adaptin mutants. Finally, the separation of Tkv immunoreactivity from the synaptic membrane versus that of the endosomal membrane is challenging, given the resolution of the imaging system used here. Our electron micrographs, however, convincingly show an accumulation of endosome-like structures close to the plasma membrane in α2-adaptin mutant boutons. The endosome-like structures observed in the α2-adaptin mutants are strikingly similar to structures previously reported for clathrin (Khodosh et al., 2006), AP180 (B Zhang et al., 1998), and Rab11 (Inoshita et al., 2017), among others. Thus, Tkv receptors could likely be enriched in these endosome-like compartments.

We propose a model in which α2-adaptin/AP2-complex is required for the attenuation of BMP signaling at the Drosophila NMJ. In the absence of AP2, recycling of Tkv is compromised, which results in its enrichment at the presynaptic membrane and/or in the early endosomes leading to elevated BMP signaling and synaptic overgrowth (Fig. 9). This study thus opens new avenues where the role of other CME components and their interaction with various growth signaling pathways can be studied. Since receptor localization and regulation appear to be the central theme in modulating BMP signaling and synapse growth, it will be interesting to perform structure-function analyses of BMP receptors and identify key residues/motifs that interact with AP2 and facilitate its endocytosis. Mutating tyrosine-based signal (YXXϕ) and dileucine-based signal (DE) motifs in Tkv and Wit could lead to further understanding of these intracellular interactions.

References

Aberle H, Hagighi AP, Fetter RD, McCabe BD, Magalhães TR, Goodman CS (2002) wishful thinking encodes a BMP type II receptor that regulates synaptic growth in Drosophila. Neuron 35:545–558.
Ayyaz A, Li H, Jasper H (2015) Haemocytes control stem cell activity in the Drosophila intestine. Nat Cell Biol 17:736–748.
Ball RW, Warren-Paquin M, Tsurudome K, Liao EH, Elazzouzi F, Cavanagh C, An BS, Wang TT, White JH, Haghighi AP (2010) Retrograde BMP signaling controls synaptic growth at the NMJ by regulating trio expression in motor neurons. Neuron 66:536–549.
Budnik V, Salinas PC (2011) Wnt signaling during synaptic development and plasticity. Curr Opin Neurobiol 21:151–159.
Chavrier P, Parton RG, Hauri HP, Zerial M (1990) Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. Cell 62:317–329.
Choudhury SD, Mushtaq Z, Reddy-Alla S, Balakrishnan SS, Thakur RS, Krishnan KS, Raghu P, Ramaswami M, Kumar V (2016) α2-Adaptin facilitates basal synaptic transmission and is required for regenerating endo-exo cycling pool under high-frequency nerve stimulation in Drosophila. Genetics 203:369–385.
Collins CA, Wairkar YP, Johnson SL, DiAntonio A (2006) Highwire restrains synaptic growth by attenuating a MAP kinase signal. Neuron 51:57–69.
Cosker KE, Segal RA (2014) Neuronal signaling through endocytosis. Cold Spring Harb Perspect Biol 6:a020669.
Deivasigamani S, Basargekar A, Shweta K, Sonavane P, Ratnaparkhi GS, Ratnaparkhi A (2015) A presynaptic regulatory system acts transsynaptically via Mon1 to regulate glutamate receptor levels in Drosophila. Genetics 201:651–664.
Deshpande M, Rodal AA (2016) The crossroads of synaptic growth signaling, membrane traffic and neurological disease: insights from Drosophila. Traffic 17:87–101.
Dickman DK, Lu Z, Meierzhagen IA, Schwarz TL (2006) Altered synaptic development and active zone spacing in endocytosis mutants. Curr Biol 16:591–598.
Di Fiore PP, De Camilli P (2001) Endocytosis and signaling, an inseparable partnership. Cell 106:1–4.
Dubois L, Lecourtiois M, Alexandre C, Hirst E, Vincent JP (2006) Regulated endocytic routing modulates wingless signaling in Drosophila embryos. Cell 105:613–624.
Dunn KW, McGraw TE, Maxfield FR (1989) Iterative fractionation of early endosome and putative endocytic carrier vesicles in vivo and the intestine. Nat Cell Biol 17:736–748.
Franco B, Bogdan L, Bobinney C, Debec A, Bockaert J, Parmentier ML, Grau Y (2004) Shaggy, the homolog of glycogen synthase kinase 3, controls neuromuscular junction growth in Drosophila. J Neurosci 24:6573–6577.
Goold CP, Davis GW (2007) The BMP ligand Gbb gates the expression of synaptic homeostasis independent of synaptic growth control. Neuron 56:109–123.
Gruenberg J, Griffiths G, Howell KE (1989) Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle fusion in vitro. J Cell Biol 108:1301–1316.
Guan Z, Quiñones-Frias MC, Akbergenova Y, Littleton JT (2020) *Drosophila* Synaptotagmin 7 negatively regulates synaptic vesicle release and replenishment in a dosage-dependent manner. Elife 9:e55443.

Gui J, Huang Y, Shimmi O (2016) Scribbled optimizes BMP signaling through its receptor internalization to the Rab5 endosome and promote robust epithelial morphogenesis. PLoS Genet 12:e1006424.

Hartung A, Bitton-Worms K, Rechtman MM, Wenzel V, Boergermann JH, Hassel S, Henis YI, Knaus P (2006) Different routes of bone morphogenetic protein (BMP) receptor endocytosis influence BMP signaling. Mol Cell Biol 26:7791–7805.

Horgan CP, McCaffrey MW (2011) Rab GTPases and microtubule motors. Biochem Soc Trans 39:1202–1206.

Inoshita T, Arano T, Hosaka Y, Meng H, Umezaki Y, Kosugi S, Jovic M, Sharma M, Rahajeng J, Caplan S (2010) The early endosomal trafficking of clathrin-mediated endocytosis by NIMA family kinases. PLoS Genet 16:e1006833.

Jovic M, Sharma M, Rahajeng J, Caplan S (2010) The endosome: a busy sorting station for proteins at the crossroads. Histol Histopathol 25:99–112.

Kadlecova Z, Spielman SJ, Loerke D, Mohanakrishnan A, Reed DK, Schmid SL (2017) Regulation of clathrin-mediated endocytosis by hierarchical allosteric activation of AP2. J Cell Biol 216:167–179.

Kasprowicz J, Kuenen S, Miskiewicz K, Habets RL, Smitz L, Verstreken P (2008) Inactivation of clathrin heavy chain inhibits synaptic recycling but allows bulk membrane uptake. J Cell Biol 182:1007–1016.

Kawasaki F, Iyer J, Posey LL, Sun CE, Mammen SE, Yan H, Ordway RW (2011) The DISABLED protein functions in CLATHRIN-mediated synaptic vesicle endocytosis and exocytotoxic coupling at the active zone. Proc Natl Acad Sci U S A 108:E222–E229.

Kelly EE, Horgan CP, Goud B, McCaffrey MW (2012) The Rab family of proteins: 25 years on. Biochem Soc Trans 40:1337–1347.

Keshishian H, Kim YS (2004) Orchestration development and function: retrograde BMP signaling in the *Drosophila* nervous system. Trends Neurosci 27:143–147.

Khodr AS, Augsburger A, Schwarz TL, Garriot PA (2006) Bchs, a BEACH domain protein, antagonizes Rab11 in synapse morphogenesis and other developmental events. Development 133:4655–4665.

Kim NC, Marquès G (2012) The Ly6 neurotoxin-like molecule targets of wit regulates spontaneous neurotransmitter release at the developing neuromuscular junction in *Drosophila*. Dev Neurobiol 72:1541–1558.

Koh TW, Verstreken P, Bellen HJ (2004) Dap160/intersectin acts as a stabilizing scaffold required for synaptic development and vesicle endocytosis. Neuron 43:193–205.

Kononenko NL, Puchkov D, Classen GA, Walter AM, Peichstein A, Sawada L, Kamps P, Trimbuch T, Lorenz D, Rosenmund C, Maritzen T, Haucke V (2014) Clathrin/AP-2 mediate synaptic vesicle reformation from endosome-like vacuoles but are not essential for membrane retrieval at central synapses. Neuron 82:981–988.

Liu Z, Huang Y, Hu W, Huang S, Wang Q, Han J, Zhang YQ (2014) dAcs, the *Drosophila* ortholog of acyl-CoA synthetase long-chain family member 3 and 4, inhibits synapse growth by attenuating bone morphogenetic protein signaling via endocytic recycling. J Neurosci 34:2785–2796.

Lloyd TE, Atkinson R, Wu MN, Zhou Y, Pennetta G, Bellen HJ (2002) Hrs regulates endosome membrane invagination and lysosome kinase receptor signaling in *Drosophila*. Cell 108:261–269.

López-Hernández T, Takenaka K-I, Mori Y, Kongspracha P, Nagamori S, Haucke V, Takamori S (2021) Clathrin-independent endocytic retrieval of SV proteins mediated by the clathrin adaptor AP-2 at mammalian central synapses. bioRxiv. doi: 10.1101/2021.06.24.449713.

Marquès G, Bao H, Haerry TE, Shimell MJ, Duchek P, Zhang B, O’Connor MB (2002) The *Drosophila* BMP type II receptor Wishful Thinking regulates neuromuscular synaptic morphology and function. Neuron 33:529–543.

McCabe BD, Marquès G, Haghighi AP, Fetter RD, Crotty ML, Haerry TE, Goodman CS, O’Connor MB (2003) The BMP homolog Gbb provides a retrograde signal that regulates synaptic growth at the *Drosophila* neuromuscular junction. Neuron 39:241–254.

McCabe BD, Hom S, Abele H, Fetter RD, Marquès G, Haerry TE, Wan H, O’Connor MB, Goodman CS, Haghighi AP (2004) Highwire regulates presynaptic BMP signaling essential for synaptic growth. Neuron 41:891–905.

Mitchell H, Choudhury A, Pagano RE, Leof EB (2004) Ligand-dependent and -independent transforming growth factor-beta receptor recycling regulated by clathrin-mediated endocytosis and Rab11. Mol Biol Cell 15:4166–4178.

Mottola G, Classen AK, González-Gaitán M, Eaton S, Zerial M (2010) A novel function for the Rab5 effector Rabenosyn-5 in planar cell polarity. Development 137:2353–2364.

Nahm M, Long AA, Paik SK, Kim S, Bae YC, Broadie K, Lee S (2010) The Cdc42-selective GAP rich regulates postsynaptic development and retrograde BMP transsynaptic signaling. J Cell Biol 191:661–675.

O’Connor-Giles KM, Ho LL, Ganetzky B (2008) Nervous wreck interacts with thickveins and the endocytic machinery to attenuate retrograde BMP signaling during synaptic growth. Neuron 58:507–518.

Papagianouli F, Berry CW, Fuller MT (2019) The Dlg module and clathrin-mediated endocytosis regulate EGFR signaling and cyst cell-germline coordination in the *Drosophila* testis. Stem Cell Reports 12:1024–1040.

Persson U, Izumi H, Souchelnytskyi S, Itoh S, Grimsby S, Engström U, Heldin CH, Funa K, ten Dijke P (1998) The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. FEBS Lett 434:83–87.

Piccioli ZD, Littleton JT (2014) Retrograde BMP signaling modulates rapid activity-dependent synaptic growth via presynaptic LIM kinase regulation of colfiiin. J Neurosci 34:4371–4381.

Rikhy R, Kumar V, Mittal R, Krishnaswami S (2006) Endophilin is critically required for synapse formation and function in *Drosophila* melanogaster. J Neurosci 22:7478–7484.

Rodal AA, Motola-Barnes RN, Littleton JT (2008) Nervous wreck and Cdc42 cooperate to regulate endocytic actin assembly during synaptic growth. J Neurosci 28:8316–8325.

Rodal AA, Blunk AD, Akbergenova Y, Jorquera RA, Buhl LK, Littleton JT (2011) A presynaptic endosomal trafficking pathway controls synaptic growth signaling. J Cell Biol 193:201–217.

Sanyal S (2009) Genomic mapping and expression patterns of C380, OK6 and D42 enhancer trap lines in the larval nervous system of *Drosophila*. Gene Exp Patterns 9:371–380.

Schwartz SL, Cao C, Pylypenko O, Rak A, Wandler- Ness A (2007) Rab GTases at a glance. J Cell Sci 120:3905–3910.

Shi W, Chen Y, Gan G, Wang D, Ren J, Wang Q, Xu Z, Xie W, Zhang YQ (2013) Brain tumor regulates neuromuscular synaptic growth and endocytosis in *Drosophila* by suppressing mad expression. J Neurosci 33:12352–12363.

Shimizu H, Kawamura S, Ozaki K (2003) An essential role of Rab6 in uniformity of synaptic vesicle size. J Cell Sci 116:3583–3590.

Smith RB, Machamer JB, Kim NC, Hays TS, Marquès G (2012) Relay of retrograde synaptogenic signals through axon terminal of BMP receptors. J Cell Sci 125:3752–3764.

Sorvina A, Shandalia T, Brooks DA (2016) *Drosophila* Pkaap regulates Rab4/Rab11-dependent traﬁc and Rab11 exocytosis of innate immune cargo. Bio Open 5:678–688.

Sulkowski MJ, Han TH, Ott C, Wang Q, Verheyen EM, Lippincott-Schwartz J, Serpe M (2016) A novel, noncanonical BMP pathway modulates synapse maturation at the *Drosophila* neuromuscular junction. PLoS Genet 12:e1005810.
Sweeney ST, Davis GW (2002) Unrestricted synaptic growth in spin- ster-a late endosomal protein implicated in TGF-beta-mediated synaptic growth regulation. Neuron 36:403–416.

Tanaka T, Nakamura A (2008) The endocytic pathway acts downstream of Oskar in Drosophila germ plasm assembly. Development 135:1107–1117.

Tao B, Kraehling JR, Ghaffari S, Ramirez CM, Lee S, Fowler JW, Lee WL, Fernandez-Hernando C, Eichmann A, Sessa WC (2020) BMP-9 and LDL crosstalk regulates ALK-1 endocytosis and LDL transcytosis in endothelial cells. J Biol Chem 295:18179–18188.

Tracy Cai X, Li H, Safyan A, Gawlik J, Pyrowolakis G, Jasper H (2019) AWD regulates timed activation of BMP signaling in intestinal stem cells to maintain tissue homeostasis. Nat Commun 10:2988.

Ullrich O, Reinsch S, Ubré S, Zerial M, Parton RG (1996) Rab11 regulates recycling through the pericentriolar recycling endosome. J Cell Biol 135:913–924.

Vanlandingham PA, Fore TR, Chastain LR, Royer SM, Bao H, Reist NE, Zhang B (2013) Epsin 1 promotes synaptic growth by enhancing BMP signal levels in motoneuron nuclei. PLoS One 8:e65997.

Verstreken P, Kjaerulff O, Lloyd TE, Atkinson R, Zhou Y, Meinertzhagen IA, Bellen HJ (2002) Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release. Cell 109:101–112.

Verstreken P, Koh TW, Schulze KL, Zhai RG, Hiesinger PR, Zhou Y, Mehta SQ, Cao Y, Roos J, Bellen HJ (2003) Synaptojanin is recruited by endophilin to promote synaptic vesicle uncoating. Neuron 40:733–748.

Vuilleumier R, Lian T, Filibotte S, Khan ZN, Fuchs A, Pyrowolakis G, Allan DW (2019) Retrograde BMP signaling activates neuronal gene expression through widespread deployment of a conserved BMP-responsive cis-regulatory activation element. Nucleic Acids Res 47:679–699.

Wang X, Shaw WR, Tsang HT, Reid E, O’Kane CJ (2007) Drosophila spichthyin inhibits BMP signaling and regulates synaptic growth and axonal microtubules. Nat Neurosci 10:177–185.

Watanabe S, Trimbuch T, Camacho-Pérez M, Rost BR, Brokowski B, Söhl-Kielczynski B, Felies A, Davis MW, Rosenmund C, Jorgensen EM (2014) Clathrin regenerates synaptic vesicles from endosomes. Nature 515:228–233.

West RJ, Lu Y, Marie B, Gao FB, Sweeney ST (2015) Rab8, Posh, and TAK1 regulate synaptic growth in a Drosophila model of frontotemporal dementia. J Cell Biol 208:931–947.

Yeh E, Gustafson K, Boulianne GL (1995) Green fluorescent protein as a vital marker and reporter of gene expression in Drosophila. Proc Natl Acad Sci U S A 92:7036–7040.

Zhang B, Koh YH, Beckstead RB, Budnik V, Ganetzky B, Bellen HJ (1998) Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. Neuron 21:1465–1475.

Zhang XW, Rui ML, Gan GM, Huang C, Yi JK, Lv HH, Xie W (2017) Neuroligin 4 regulates synaptic growth via the bone morphogenetic protein (BMP) signaling pathway at the Drosophila neuromuscular junction. J Biol Chem 292:17991–18005.

Zhao L, Wang D, Wang Q, Rodal AA, Zhang YQ (2013) Drosophila cyfip regulates synaptic development and endocytosis by suppressing filamentous actin assembly. PLoS Genet 9:e1003450.