The Strip-Hippo Pathway Regulates Synaptic Terminal Formation by Modulating Actin Organization at the *Drosophila* Neuromuscular Synapses

**Highlights**

- A STRIPAK complex component, Strip, localizes presynaptically at neuromuscular junctions
- Strip is required to dephosphorylate Hippo kinase
- Strip inhibits Hippo-mediated synaptic terminal development
- The Strip-Hippo pathway affects F-actin organization through Enabled

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**In Brief**

Sakuma et al. describe how *Drosophila* Strip, a component of the striatin-interacting phosphatase and kinase complex, negatively regulates the formation of synaptic terminal structure. Strip negatively regulates the activity of the core Hippo kinase cassette, whose synaptic function was previously unknown, which in turn locally affects the organization of F-actin through Enabled.
The Strip-Hippo Pathway Regulates Synaptic Terminal Formation by Modulating Actin Organization at the Drosophila Neuromuscular Synapses

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SUMMARY

Synapse formation requires the precise coordination of axon elongation, cytoskeletal stability, and diverse modes of cell signaling. The underlying mechanisms of this interplay, however, remain unclear. Here, we demonstrate that Strip, a component of the striatin-interacting phosphatase and kinase (STRIPAK) complex that regulates these processes, is required to ensure the proper development of synaptic boutons at the Drosophila neuromuscular junction. In doing so, Strip negatively regulates the activity of the Hippo (Hpo) pathway, an evolutionarily conserved regulator of organ size whose role in synapse formation is currently unappreciated. Strip functions genetically with Enabled, an actin assembly/elongation factor and the presumptive downstream target of Hpo signaling, to modulate local actin organization at synaptic termini. This regulation occurs independently of the transcriptional co-activator Yorkie, the canonical downstream target of the Hpo pathway. Our study identifies a previously unanticipated role of the Strip-Hippo pathway in synaptic development, linking cell signaling to actin organization.

INTRODUCTION

Since the Hippo (Hpo) pathway was discovered as the key regulator ensuring appropriate final tissue size by coordinating cell proliferation and cell death (Pan, 2010), large-scale genetic studies have identified numerous regulators of the Hpo pathway (Halder and Johnson, 2011; Staley and Irvine, 2012). While most pathway components identified thus far are positive regulators of Hpo, some negative regulators were recently reported (Yu and Guan, 2013). One such negative regulator is the STRIPAK (striatin-interacting phosphatase and kinase) complex, which is evolutionarily conserved and regulates various cellular processes, including cell-cycle control and cell polarity (Hwang and Pallas, 2014). The core component of the STRIPAK complex is the striatin family of proteins: striatins serve as B000 subunits (a subfamily of regulatory B subunits) of the protein phosphatase 2A (PP2A) complex (Goudreault et al., 2009; Hwang and Pallas, 2014). Beyond this, the A and C subunits of PP2A, Mob3, Mst3, Mst4, Ysk1, Ccm3, Strip1, and Strip2 form the core mammalian STRIPAK complex. We previously reported that Strip, the Drosophila homolog of mammalian Strip1 and 2, is involved in early endosome formation, which is essential for axon elongation (Sakuma et al., 2014). Building on these findings, we hypothesized that the Strip-Hpo pathway may also be involved in neuronal synaptic development.

The Drosophila larval neuromuscular junction (NMJ) is an ideal model for studying synaptic development because of its identifiable, stereotyped morphology, accessibility, broad complement of available reagents, and suitability for a wide range of experimental approaches (Harris and Littleton, 2015). Furthermore, the Drosophila NMJ, like vertebrate central synapses, is glutamatergic, suggesting that the molecular mechanisms that regulate synaptic development in Drosophila NMJ might be applicable to vertebrates (Collins and DiAntonio, 2007). Motor neuron axons are genetically hardwired to target specific muscles by the end of the embryonic stage (Keshishian et al., 1996). There, axonal growth cones subsequently differentiate into presynaptic termini called boutons, each of which contains multiple active zones (Menon et al., 2013). During the larval
stage, muscle size increases nearly 100-fold and boutons are continuously and proportionately added to maintain constant innervation strength (Menon et al., 2013). Various molecules can negatively or positively regulate the growth of synaptic termini (Menon et al., 2013). Among the many factors, elements of the actin cytoskeleton are key effectors of morphological change, functioning downstream of several cell-surface receptors and signaling pathways (Long and Van Vactor, 2013). Of the two types of actin filaments (branched and linear), the activity of the Arp2/3 complex, responsible for nucleation of branched F-actin, the first step of actin polymerization (Insall and McMahon, 2009), should be strictly regulated (Koch et al., 2014). Arp2/3 hyperactivation results in synaptic terminal overgrowth characterized by excess small boutons emanating from the main branch (Ball et al., 2010; Qurashi et al., 2007; Schenck et al., 2004; Zhao et al., 2013) that are termed satellite boutons (Dickman et al., 2006).

Here, we show that Strip negatively regulates synapse terminal development by tuning the activity of the core Hpo kinase cassette. Loss or reduction of strip function in motor neurons increased the number of satellite boutons, which could be suppressed by reducing the genetic dosage of hpo. Similarly, activation of the core Hpo kinase cassette also increased the number of satellite boutons. In this context, the presumptive downstream target of the core Hpo kinase cassette is Enabled (Ena), a regulator of F-actin assembly and elongation that was reported to antagonize the activity of Arp2/3 (Bear and Gertler, 2009). The canonical downstream transcriptional co-activator Yorkie (Yki) appears dispensable for Hpo-mediated synaptic terminal development. Here, we propose that the evolutionarily conserved Strip-Hpo pathway regulates local actin organization during synaptic development.

RESULTS

Strip Is Localized at Presynapses

We first examined the localization of endogenous Strip protein at the NMJ. Using an anti-Strip antibody (Sakuma et al., 2014), we found that Strip is localized at presynaptic sites adjacent to the neuronal membrane marker horseradish peroxidase (HRP). It is excluded from the muscle subsynaptic reticulum (SSR), an in-folded membranous structure that contains many components including neurotransmitter receptors and the postsynaptic signaling complex, and was identified by Spectrin labeling (Figures 1A and 1B). Unfortunately, we could not obtain the negative control for this antibody at the NMJ, probably because strip knockdown using short hairpin RNA against strip was not strong enough. In addition, mutants homozygous for strip<sup>drop</sup>, a strong loss-of-function allele, die at the first-instar stage. Thus, we further confirmed Strip protein localization using a Cas9-triggered homologous recombination strip-myc knockin line, in which the c-Myc tag sequence was inserted at the 3’ end of the strip coding sequence (Figures S1A–S1D). As this strip-myc line is homozygous viable, the insertion is unlikely to impair large (Dlg; D) are shown in magenta. Dlg is the marker of postsynaptic membrane. Scale bars, 2.5 μm. See also Figure S1.
We confirmed that the Strip-myc protein is localized at presynapses, using an antibody against the c-Myc tag (Figures 1C and 1D), and thus hypothesized a role for Strip in synaptic development.

Strip Is Required for Synaptic Terminal Formation and Function

As we reported in our previous studies (Sakuma et al., 2014, 2015), one of the major roles of Strip is to regulate axon elongation. To investigate the role of Strip in synaptic development that occurs after axon elongation, we weakly knocked down strip specifically in motor neurons using short hairpin RNA (The locations of the short hairpin RNA sequences on the strip molecular map are shown in Figure S1A.). We observed a significant increase in the number of satellite boutons, small boutons surrounded by postsynaptic membranes (Figures 2A and 2B; quantified in Figure 3G). A similar phenotype was observed using another short hairpin RNA targeting a different region of strip (Figures S2A and S2B). Furthermore, the satellite bouton phenotype caused by strip knockdown was rescued by expressing a strip transgene resistant for short hairpin RNAs of strip (Figure S2C), demonstrating that strip is the causal gene for the observed phenotype. The total number of boutons and the muscle area were not significantly different between control and strip knockdown samples (Figures S2D and S2E).

We also knocked down other components of the STRIPAK complex in motor neurons. Knockdown of microtubule star (mts, the C subunit of Drosophila PP2A complex) or Connector of kinase to AP-1 (Cka, ortholog of striatins) phenocopied the strip knockdown (Figure S2F), suggesting that Strip regulates synaptic development as a member of the STRIPAK complex. To determine whether the increase in satellite boutons had any functional consequences at the NMJ, we next analyzed the electrophysiological properties of strip knockdown larvae (Figures 2C–2F). Though the amplitude of evoked (Figure 2G) and spontaneous miniature (Figure 2H) excitatory junctional potentials (eEJPs and mEJPs, respectively) were both unchanged (Figures 2C–2H), neuronal strip knockdown...
caused an increase in mEJP frequency (Figures 2E, 2F, and 2I). Therefore, Strip is required for both normal synaptic morphology and function.

**Strip Negatively Regulates Hpo Activity during Satellite Bouton Formation**

Since Strip is a negative regulator of Hpo in the context of growth control in mitotic cells (Ribeiro et al., 2010), we next examined whether Strip and Hippo could function similarly in regulating synaptic morphology. When the genetic dosage of hpo was decreased using a heterozygous hpo background, the satellite bouton phenotype associated with presynaptic strip knockdown was significantly suppressed (Figures 3A and 3G), while the transmission defect of strip knockdown was not suppressed (Figure 2I). hpo heterozygosity alone, however, had no effect on synaptic morphology (Figures 3B and 3G). These results suggest that Strip-mediated inhibition of Hpo activity suppresses satellite bouton formation. Consistent with this idea, Hpo overexpression in motor neurons significantly increased the number of satellite boutons (Figures 3C and 3G). This effect was indistinguishable from that associated with strip knockdown (Figure 3G). Overexpression of the kinase-dead form of Hpo (Jin et al., 2012; Wu et al., 2003), which does not possess catalytic activity to phosphorylate downstream kinases such as Warts (Wts) (Udan et al., 2003), did not alter the number of satellite boutons (Figures 3D and 3G), suggesting that Hpo kinase activity is required for satellite bouton formation. Furthermore, we found that Strip forms a protein complex with FLAG-tagged Hpo (Figure 3H) and that the level of phosphorylated Hpo was drastically increased when strip was knocked down in Drosophila S2 cells (Figure 3I). Taken together, these genetic and biochemical data indicate that Strip negatively regulates satellite bouton formation by inhibiting Hpo activity.

The core of the Hpo pathway (the core Hpo kinase cassette) consists of four proteins: Hpo, Salvador (Sav), Wts, and Mob-as-tumor-suppressor (Mats) (Staley and Irvine, 2012). Hpo and Wts are Ser/Thr kinases that are activated by phosphorylation and act sequentially in the Hpo pathway (Harvey et al., 2003; Wu et al., 2003). Sav, which is phosphorylated by Hpo, acts as a scaffolding protein by binding to both Hpo and Wts (Harvey et al., 2003; Wu et al., 2003), while Mats serves as an essential Wts cofactor (Lai et al., 2005). We confirmed that Wts activity, like Hpo, is also required for satellite bouton formation. First, the strip knockdown phenotype was significantly suppressed in a wts heterozygous background (Figures 3E and 3G). Second, overexpression of Wts resulted in an increase in the number of satellite boutons (Figures 3F and 3G). Third, wts knockdown in motor neurons suppressed the satellite bouton phenotype observed in strip-knockdown motor neurons (Figure S3A), suggesting that Strip negatively regulates the activity of the core Hpo kinase cassette in a cell-autonomous manner.

**The Strip-Hpo Pathway Regulates Satellite Bouton Formation Independent of Yki Activity**

In the context of growth control, the main target of the core Hpo kinase cassette is the transcriptional co-activator Yki, which is...
excluded from the nucleus when phosphorylated (Huang et al., 2005). Upon inactivation of the Hpo pathway, Yki becomes dephosphorylated and translocates to the nucleus, where it binds Scalloped and other DNA binding partners, leading to the upregulation of genes promoting cell proliferation and apoptosis inhibition (Alican et al., 2009; Goulev et al., 2008; Peng et al., 2009; Wu et al., 2008; Zhang et al., 2008). Furthermore, several regulators of the Hpo pathway are also downstream targets of Yki, providing regulation by negative feedback (Cho et al., 2006; Genevet et al., 2010; Hamaratoglu et al., 2006).

In order to investigate the involvement of Yki in Strip-Hpo-mediated regulation of satellite bouton formation, we examined the genetic interactions between strip and yki. The satellite bouton phenotype of strip knockdown was not suppressed by overexpression of Yki, which was expected to increase the level of nuclear Yki (Figure S3B). Furthermore, expression of mutant form of Yki (Yki-3SA) did not affect the number of satellite boutons (Figure S3C). To examine Yki-dependent transcriptional activation in motor neurons, we utilized two types of reporters: a GFP-bantam (ban) sensor and death-associated inhibitor of apoptosis 1 (diap1)-GFP4.3. Both microRNA ban and diap1 are downstream targets of Yki (Harvey et al., 2003; Jia et al., 2003; Nolo et al., 2006; Thompson and Cohen, 2006). The GFP-ban sensor inversely reports ban levels by virtue of a ban recognition sequence in the 3’ UTR of a GFP-expressing transgene (Bennecet et al., 2003). The diap1-GFP4.3 gene, containing a 4.3-kb diap1 genomic fragment from +1.37 kb to +5.68 kb, drove GFP expression in patterns similar to those of the endogenous diap1 gene (Zhang et al., 2009). We investigated the activity of Yki in the ventral nerve cord, where cell bodies of motor neurons reside. Most of the ELAV-positive cells around the midline of the ventral nerve cords were also OK6-Gal4 positive (Sanjal, 2009). Using both methods, we could not detect a change in ban-sensor or diap1-GFP expression between controls and strip knockdown motor neurons (dotted circles in Figures S3D–S3G). These data, along with the failed suppression of the satellite bouton phenotype using Yki overexpression, suggest that Yki is unlikely to be involved in Strip-Hpo-mediated regulation of satellite bouton formation.

**The Strip-Hpo Pathway Regulates Satellite Bouton Formation by Modulating F-Actin Organization through Ena**

Since we could not observe any involvement of Yki in satellite bouton formation, we considered other downstream targets independent of Yki. We examined Ena, an actin assembly and elongation regulator that is the homolog of VASP, the vasodilator stimulated phosphoprotein, Mena, and EVL as potential targets for the following reasons. First, Ena is localized at presynaptic sites in Drosophila motor neurons (Loya et al., 2014). Second, misregulation of actin organization, especially enhanced assembly of branched actin, results in satellite bouton formation at the Drosophila NMJ (Ball et al., 2010; Ourashi et al., 2007; Schenck et al., 2004; Zhao et al., 2013). Third, Ena seems to utilize anti-branched activity to modulate actin dynamics (Bear and Gertler, 2009); Ena antagonizes assembly of branched actin mediated by Arp2/3 and it is also reported that knockdown of the human homolog of Ena (hMena) causes Rac1 activation at the lamellipodia consisting of branched F-actin meshwork (Higashi et al., 2009). Lastly, a recent report demonstrated that Ena is phosphorylated at serine 187 by Wts and is negatively regulated in Drosophila border cell migration (Lucas et al., 2013). Thus, we hypothesized that Ena is phosphorylated at serine 187 by Wts and is negatively regulated at the downstream of the Strip-Hpo pathway in synaptic development at NMJ. If we follow this hypothesis, Ena is hyper-phosphorylated at serine 187 when strip is knocked down. To confirm the involvement of Ena in the Strip-mediated regulation of satellite bouton number, we expressed a phospho mutant or phosphomimetic mutant in Ena strip-knockdown motor neurons. Expression of the phospho mutant EnaS187A (which itself cannot be phosphorylated by Wts) suppressed the satellite bouton phenotype of strip knockdown, while expression of the phosphomimetic mutant EnaS187D did not (Figures 4A–4D). These results indicate that the Wts-dependent phosphorylation state of Ena at serine 187 is crucial for satellite bouton formation and also suggest that an activated core Hpo kinase cassette in strip-knockdown motor neurons inactivates Ena by Ena phosphorylation. Consistent with this idea, ena knockdown in motor neurons increased the number of satellite boutons (Figures 4E and 4F), probably by reducing the levels of active Ena. Taken together, we conclude that the core Hpo kinase cassette regulates synaptic morphogenesis by inactivating Ena, one of the key actin regulators.

**DISCUSSION**

In the present study, we identified Strip and components of the Hpo pathway as regulators of synaptic morphology. In addition to the intensely investigated function of Hpo in growth control in mitotic cells, a few postmitotic roles of the Hpo pathway have recently been uncovered, such as dendrite tiling in Drosophila sensory neurons (Emoto et al., 2006) and cell fate specification of photoreceptor cells in Drosophila retina (Jukam et al., 2013). We now find an additional postmitotic role for Hpo in synaptic terminal development. The results indicate that Strip and the core Hpo kinase cassette regulate satellite bouton formation by regulating the activity of Ena, an actin regulator that is involved in the initiation, extension, and maintenance of linear actin filaments at the barbed end (Winkelman et al., 2014). Although we cannot exclude the possibility that there might be targets of Yki in motor neurons other than diap1 or bantam, whose transcriptional activations were not observed in this study, Yki, a well-known downstream target of the core Hpo kinase cassette, was dispensable for proper synaptic morphology. Ena phosphorylation causes its inactivation (Lucas et al., 2013); therefore, we reason that Strip can act as a positive regulator of Ena by inactivating the Hpo pathway. We propose a model for the regulation of satellite bouton formation by Strip and Hpo pathway components (Figures 4G and 4H). As the presynaptic localization of endogenous Strip was punctate and non-uniform (Figure 1), we expect that Strip localization could be critical for regulating the phosphorylation status of Hpo, Wts, and Ena, which locally alters actin organization and eventually specifies the position of satellite bouton formation that could be a marker for new bouton outgrowth (Torroja et al., 1999) (Figure 4G). When Strip is present, the core Hpo kinase cassette is inactivated,
which in turn locally increases the expression of the active (unphosphorylated) form of Ena (Figure 4H, left). However, the core Hpo kinase cassette can be activated in the absence of Strip, which subsequently phosphorylates and inactivates Ena (Figure 4H, right). Ena prevents Arp2/3-induced branching (Bear and Gertler, 2009), suggesting that Ena inactivation activates Arp2/3 and results in satellite bouton formation, similar to Rac activation (Ball et al., 2010) or to loss of SCAR/WAVE components (Ball et al., 2010; Qurashi et al., 2007; Schenck et al., 2004; Zhao et al., 2013). It is reported that Arp2/3 is involved in bouton formation and axon terminal branching downstream of WAVE/SCAR complex in the NMJ (Koch et al., 2014). Indeed, our findings support this hypothesis. F-actin organization was altered by strip knockdown in motor neurons (Figure S4). We expressed the GFP-moe reporter in motor neurons, the GFP fused to the C-terminal actin-binding domain of Moesin, which is widely used as an F-actin reporter (Chihara et al., 2003; Edwards et al., 1997). The intensity of actin puncta became higher and puncta were unevenly distributed when strip was knocked down (Figure S4B). These data suggest that Strip function is important for the proper organization of F-actin.

There are many indications that Strip and other STRIPAK components (Mst3, Mst4, and Ccm3) regulate the actin network (Bai et al., 2011; Hwang and Pallas, 2014). For example, Strip1, Strip2, Mst3, and Mst4 regulate the actomyosin contractions, which regulate cell migration in cancer cells (Madsen et al., 2015). In addition to regulating the actin network, STRIPAK has been suggested to function in microtubule organization. Mutants of Drosophila Mob4, a member of the core STRIPAK complex and homolog of mammalian Mob3, show abnormal microtubule morphology at NMJs and muscles (Schulte et al., 2010). Furthermore, we previously reported that Strip forms a complex with Glued, the homolog of mammalian p150Glued (Sakuma et al., 2014), a component of the dynactin complex required for dynein motor-mediated retrograde transport along microtubules. Strip also affects microtubule stability (Sakuma et al., 2015). As previously mentioned, microtubules are also key effectors of synaptic development downstream of several receptors and signaling pathways (Long and Van Vactor, 2013). Taken together, the STRIPAK complex can act...
as a regulatory hub for multiple cellular signals, including Hpo-pathway-mediated actin organization, endocytic-pathway-dependent BMP signals, and microtubule stability, for proper synaptic development.

The Hpo pathway has been reported to act as a sensor of the local cellular microenvironment, such as mechanical cues, apicobasal polarity, and actin architecture (Gaspar and Tapon, 2014; Yu and Guan, 2013), to balance cell proliferation and cell death. Although synaptic morphogenesis is a postmitotic process, it is very plastic and depends on a dynamically changing extracellular environment, as exemplified by the nearly 100-fold expansion of muscle size (Menon et al., 2013) during larval development. Thus, our study demonstrates an intriguing function for the Strip-Hpo pathway in the homeostatic control of neuronal synaptic morphology and function.

EXPERIMENTAL PROCEDURES

Fly Strains

The genotypes of D. melanogaster used in each experiment are shown in Table S1. Detailed information regarding the flies used can be found in Supplemental Experimental Procedures.

Immunostaining

Wandering third-instar larvae were dissected in magnetic chambers as described (Budnik et al., 2006). See Supplemental Experimental Procedures for details on immunostaining and antibodies.

Image Acquisition and Analysis

For Figures 1 and S1, a single section was obtained. For other figures, images of NMJs at muscle 6/7 of the A2 segment were acquired every 0.29 μm using a TCS-SP8 confocal laser scanning confocal microscopy (Leica) with an HCX PL APO 63X/1.40-0.60 Oil CS lens (Leica, 506188). See Supplemental Experimental Procedures for a detailed description of data analysis.

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting were performed using Drosophila S2 cells. More information can be found in Supplemental Experimental Procedures.

dsRNA Generation and Treatment

Double-stranded RNA (dsRNA) was designed, produced, and treated as previously described (Rogers and Rogers, 2008). See Supplemental Experimental Procedures for details.

Electrophysiology

Electrophysiology was performed on late third-instar wild-type and motorneuron-specific strip knockdown larvae. See Supplemental Experimental Procedures for details.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software. An unpaired t-test with Welch correction was performed to compare control and strip-knockdown phenotypes. The Kruskal-Wallis test with Dunn’s multiple comparison test was performed to compare multiple genotypes.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.07.066.

AUTHOR CONTRIBUTIONS

C.S., Y.S., and T.C. designed the study with the help of M.M. C.S. and Y.S. conducted most experiments. K.K. and N.M. conducted electrophysiological experiments. T.J.M. contributed intellectually and provided technical advice. C.S., Y.S., T.U., and T.C. analyzed the data. C.S. and T.C. wrote the manuscript with contributions from all authors.

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