The actin-microtubule cross-linking activity of Drosophila Short stop is regulated by intramolecular inhibition

Derek A. Applewhite,a Kyle D. Grode,a Mara C. Duncanab, and Stephen L. Rogersa,b,c,d

aDepartment of Biology, bCurriculum in Genetics and Molecular Biology, cCarolina Center for Genome Sciences, and dLineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280

ABSTRACT  Actin and microtubule dynamics must be precisely coordinated during cell migration, mitosis, and morphogenesis—much of this coordination is mediated by proteins that physically bridge the two cytoskeletal networks. We have investigated the regulation of the Drosophila actin-microtubule cross-linker Short stop (Shot), a member of the spectraplakin family. Our data suggest that Shot’s cytoskeletal cross-linking activity is regulated by an intramolecular inhibitory mechanism. In its inactive conformation, Shot adopts a “closed” conformation through interactions between its NH₂-terminal actin-binding domain and COOH-terminal EF-hand-GAS2 domain. This inactive conformation is targeted to the growing microtubule plus end by EB1. On activation, Shot binds along the microtubule through its COOH-terminal GAS2 domain and binds to actin with its NH₂-terminal tandem CH domains. We propose that this mechanism allows Shot to rapidly cross-link dynamic microtubules in response to localized activating signals at the cell cortex.

INTRODUCTION

Although often studied as isolated networks, actin filaments and microtubules are functionally intertwined during cellular processes such as polarization, directed migration, and asymmetric cell division (Waterman-Storer et al., 2000; Schaefer et al., 2002; Rodriguez et al., 2003; Wittmann et al., 2003; Drabek et al., 2006; Gupta et al., 2010). This type of cross-talk often requires mechanical coupling between the two cytoskeletal networks by proteins or protein complexes that act as physical cross-bridges (Salmon et al., 2002; Rothenberg et al., 2003; Wittmann et al., 2003; Miller et al., 2004; Rosales-Nieves et al., 2006; Lee and Suter, 2008; Applewhite et al., 2010). The spectraplakins are one such family of giant cytoskeletal cross-linking proteins that have been highly conserved across most animal lineages (Lee et al., 2000; Röper et al., 2002; Röper and Brown, 2003; Jefferson et al., 2004; Sonnenberg and Liem, 2007; Kim et al., 2011; Suzzo et al., 2012). Wherever studied genetically, spectraplakins are found to be essential genes that play multiple roles in development. For example, Drosophila has a single spectraplakin, Short stop (Shot), that is required for cell–cell and cell–matrix adhesion. Shot mutants exhibit defects in axon outgrowth, trachea development, and muscle attachment to the cuticle (Strumpf and Volk, 1998; Lee et al., 2000; Lee and Kolodziej, 2002a,b; Röper and Brown, 2003; Subramanian et al., 2003; Bottenberg et al., 2009). Mammals possess two spectraplakin genes, best studied in mouse, in which the orthologues are called dystonin/BPAG1 and ACF7/MACF1 (Bernier et al., 1996; Gong et al., 2001; Jefferson et al., 2007). BPAG1 acts as a cytoskeletal cross-linker in epidermal cells and neurons; mutants with tissue-specific loss of the protein exhibit skin-blistering phenotypes and neurodegeneration (Jefferson et al., 2004; Sonnenberg and Liem, 2007; Edwardson et al., 2012). Studies conducted with cells derived from ACF7 knockout mice revealed that ACF7 regulates microtubule dynamics and plays important roles in cell migration (Kodama et al., 2003; Wu et al., 2008, 2011). Collectively these studies have revealed essential roles for spectraplakin-mediated cytoskeletal cross-linking in tissue morphogenesis and cell migration.

Spectraplakins are characterized by a complex, multidomain architecture that is alternatively spliced to generate a diversity of...
isoforms (Röper et al., 2002; Sonnenberg and Liem, 2007; Suozzi et al., 2012). Spectraplakins typically possess an NH$_2$-terminal actin-binding domain (ABD) composed of two calponin homology repeats, a central rod-like domain comprising plakin- and spectrin-like repeats, two EF-hand motifs, a GAS2-like domain that binds to microtubules, and a 300-residue COOH-terminal domain (CTD) that binds to the microtubule plus end–binding protein EB1 (Lee et al., 2000; Wu et al., 2008; Honnappa et al., 2009; Appelwhite et al., 2010; Alves-Silva et al., 2012). Thus spectraplakins are thought to be elongated, bivalent, rod-like proteins with actin- and microtubule-binding sites at each end.

Given the many important roles that spectraplakins play throughout development, investigating the spatiotemporal regulation of these proteins is key to understanding their function at the molecular level. We previously published a structure–function study of Drosophila Shot that led us to propose a model in which its actin-microtubule cross-linking activity is regulated by an intramolecular inhibition mechanism (Appelwhite et al., 2010). In the current report, we used a combination of biochemical interaction studies and advanced imaging techniques to test our hypothesis. Our data indicate that Shot is able to adopt a conformation in which the ABD contacts the EF-hand-GAS2 domains. While in this autoinhibited conformation, Shot does not interact with actin or the microtubule lattice, but does interact with EB1 to localize to the plus ends of growing microtubules. On reaching the actin-rich zones located at the cell periphery, the ABD and EF-hand-GAS2 domains dissociate and are able to bridge nearby actin filaments and microtubules, respectively. These data provide the first mechanistic detail of how spectraplakin-mediated cytoskeletal cross-linking is regulated at the molecular level.

RESULTS

The NH$_2$-terminal ABD of Shot directly interacts with the EF-hand/GAS2 domain

Our previous work showed that Shot localizes either to the microtubule plus end or to the microtubule lattice and that the lattice-associated pool was actively engaged as a cross-linker to actin (Appelwhite et al., 2010). Our hypothesis is that actin-microtubule cross-linking is inhibited by a direct intramolecular contact between the actin- and microtubule-binding domains of the protein. To test this idea, we purified 6xHis- and glutathione S-transferase (GST)-tagged fusions of the ABD and EF-hand-GAS2-CTD from Shot and tested their ability to interact by Ni-NTA resin pull-down assays (Figure 1A). We observed that 6xHis-ABD bound to GST-EF-hand-GAS2-CTD, but not to GST alone. As a positive control, we also tested their ability to interact by Ni-NTA resin pull-down assays and found that 6xHis-EB1 also bound to GST-EF-hand-GAS2-CTD. We noted that recombinant GST-EF-hand-GAS2-CTD always exhibited some degradation products; 6xHis-EB1 selectively bound to the higher-molecular-weight fragments, consistent with our previous observation that the EB1-binding sites are present in the CTD of Shot (Slep et al., 2005; Appelwhite et al., 2010). 6xHis-ABD, however, interacted with all the observed truncated fragments, consistent with an interaction with the EF-hand-GAS2 domains (Figure 1A, black boxes). As a negative control for specificity of the interaction, we found that the first two tumor overexpressed gene (TOG) domains of the microtubule tip-tracking protein Mini spindles (6xHis-TOG1-2) did not interact with GST-EF-hand-GAS2-CTD. To test whether the ABD and EF-hand-GAS2 domains could also interact in vivo, we cotransfected S2 cells with epitope-tagged versions of ABD-myc and a more refined Flag-EF-hand-GAS2 construct and immunoprecipitated with antibodies to each epitope (Figure 1B). Immunoblotting revealed that the ABD and EF-hand-GAS2 fusion coimmunoprecipitated, while the ABD did not coimmunoprecipitate with the CTD alone. These data indicate that the ABD and EF-hand-GAS2 domains are able to directly interact.

Shot exhibits an intramolecular head-to-tail interaction when localized to microtubule plus ends

To examine the conformation of Shot within living cells, we designed a series of bimolecular fluorescent complementation (BiFC) probes to serve as a readout for interactions between the ABD and EF-hand-GAS2 domains (Figure 2). The basis of this technique is that two nonfluorescent fragments of the yellow fluorescent protein Venus are fused to candidate proteins; if the chimeric proteins directly interact, they reconstitute full-length Venus and become fluorescent (Kerppola, 2008, 2009). Coexpressed complementary halves of split Venus probe alone (VN and VC) did not fluoresce in S2 cells; however, coexpression of two EB1 constructs, EB1-VN and EB1-VC, heterodimerized and reconstituted fluorescence at growing microtubule plus ends (Figure 2, A and B). To further validate the specificity of this approach, we differentially tagged EB1 and Shot with VC and VN, as both proteins interact via their COOH-termini (Figure 2C). Expression of EB1-VN and Shot-VC reconstituted fluorescence and the proteins localized to microtubules; however, as expected, expression of EB1-VC and VN-Shot failed to produce fluorescence above background levels (unpublished data). We next fused VN and VC to the Shot coding sequence to determine whether its NH$_2$- and COOH-termini could interact in cis (Figure 2D and Supplemental Movie S1). Both VN-Shot-VC and VC-Shot-VN reconstituted fluorescence in S2 cells and localized exclusively to microtubule plus ends, indicating that both ends of the protein were in close proximity. To determine whether Venus reconstitution occurred as the result of inter- or intramolecular interaction, we coexpressed VN-Shot and Shot-VC (Figure 2F) but failed to observe fluorescence, indicating that Shot does not form an intermolecular interaction between NH$_2$- and COOH-terminal domains in trans. These data suggest that Shot is able to adopt a head-to-tail, folded conformation when localized to the microtubule plus end.

The central rod domain of shot is required for interactions between the ABD and EF-hand-GAS2 domains

Given Shot’s size, we reasoned that its rod domain must be flexible enough to allow the long-distance interactions between the NH$_2$- and COOH-termini we observed using the BiFC assay. The triple-helical, coiled-coil motifs of spectrin endow it with a high degree of flexibility; thus we hypothesized that the spectrin-like rod domain of Shot confers a similar degree of flexibility and that deletion of this domain would eliminate intramolecular inhibition (Kusunoki et al., 2004; Määttä et al., 2004; Bhasin et al., 2005). To test this, we used the BiFC assay to determine whether the NH$_2$- and COOH-termini of Shot could interact if the rod domain were deleted (Figure 2H and Movie S2). When transfected into S2 cells, VN-ShotΔRod-VC (lacking residues 1205–4599) was unable to reconstitute Venus fluorescence above background levels. However, when we coexpressed VN-ShotΔRod-VC with EB1-VN (Figure 2I), we did observe reconstitution of fluorescence along the lattice of microtubules, indicating that the lack of fluorescence from VN-ShotΔRod-VC alone was not due to misfolding of the protein, as it was still competent to bind EB1. These results indicate that the spectrin-like domain is necessary for interactions between the NH$_2$- and COOH-termini and for normal dynamics of the molecule. They also suggest that, upon binding to the microtubule lattice, ShotΔRod acts to mislocalize EB1 when bound via reconstituted split Venus.
Next we measured the degree of colocalization between Shot-green fluorescent protein (Shot-GFP) and mCherry-tubulin in living cells, using Mander’s coefficient (M; a scale of zero to one, where zero indicates no colocalization; Bolte and Cordelières, 2006; Figure 3). In our experiments, Shot-GFP exhibited an intermediate colocalization with microtubules (M = 0.34; Figure 3, A and H) that reflected its dual localization to the microtubule lattice and plus end. As a reference, ShotΔGAS2-EGFP, a construct that localizes exclusively to microtubule plus ends, exhibited a significantly lower value (M = 0.23; Figure 3, D and H), and ShotGAS2-GFP, a construct that binds exclusively to the lattice, produced a significantly higher value (M = 0.66; Figure 3, G and H). To test the contribution of the central rod domain to Shot localization, we expressed ShotΔRod-EGFP in S2 cells and examined its interactions with microtubules (Figure 3E). We found that ShotΔRod-EGFP predominantly associated with the microtubule lattice in the central cytoplasm, as well as in the cell periphery. Consistent with this localization pattern, ShotΔRod-EGFP exhibited a significantly higher degree of colocalization with microtubules (M = 0.70; Figure 3H) than full-length Shot A-GFP and was statistically indistinguishable from that of ShotΔGAS2-EGFP. These data suggested that deletion of the central rod domain disrupted regulation of ShotΔRod-EGFP and shifted it to a lattice-bound conformation.

The EF-hand motifs contribute to regulation of Shot

The two EF-hand motifs that lie immediately upstream of the GAS2 domain are essential for axon extension, a process that requires actin-microtubule cross-linking by Shot (Lee and Kolodziej, 2002b). To test the hypothesis that the EF-hand motifs contribute to intramolecular inhibition of Shot, we constructed a full-length Shot BiFC probe lacking the EF-hand motifs (VC-ShotΔEF-hand-VN; lacking residues 5072–5135) and tested whether this construct could reconstitute fluorescence (Figure 2, J–L). We did not observe fluorescence above background levels when we expressed this construct in S2 cells (Figure 2K). However, when we coexpressed VC-ShotΔEF-hand-VN with EB1-VC, we did observe fluorescence along the lattice of microtubules in a manner similar to that seen in ShotΔEF-hand-EGFP, which contains a similar deletion (residues 5011–5152; Figure 2L).

Figure 1: Shot’s NH2-terminal ABD interacts with its COOH-terminal EF-hand-GAS2 domain. (A) Coomassie-stained polyacrylamide gel of purified 6xHis-tagged NH2-terminal and COOH-terminal halves of Shot. Left, GST alone failed to interact with 6xHis-tagged ABD (Shot’s tandem calponin homology domains), EB1, or TOG domains 1 and 2 from Mini spindles (control). Right, GST-EF-handGAS2CTD did interact with 6xHis-ABD and 6xHis-EB1 (black boxes), but did not interact with TOG1/2. (B) Western blot analysis of S2 cell lysate collected from cells expressing NH2-terminal Flag-tagged and COOH-terminal Myc-tagged fragments of Shot. NTABD is the actin-binding domain plus an NH2-terminal leader sequence (residues 1–142); ABD-Myc lacks this NH2-terminal leader sequence. CTD is Shot’s COOH-terminal domain lacking both EF-hand motifs and the GAS2 domain. Only Shot’s ABD and EF-hand-GAS2 domain communoprecipitated. Also included are Myc-tagged Drosophila α-actinin, which contains both NH2-terminal CH domains, and a COOH-terminal EF-hand motif (control). (C) Diagram of the Shot fragments used in A and B.
To test the hypothesis that the EF-hand motifs of Shot regulate its activity by binding to calcium, we constructed a full-length Shot BiFC probe carrying mutations (D5080A, D5082A, N5084A) predicted to abolish calcium binding (Figure 2, M and N, and Movie S3). When expressed in S2 cells, this probe did reconstitute fluorescence at the microtubule plus end, which was similar to results seen with the full-length VC-Shot-VN BiFC probe (Figure 2, D and E). The coordinating residues of the second EF-hand motif appear to diverge from other canonical calcium-binding EF-hands, and we did not test their contribution. To further test the potential role of EF-hand motifs, Shot remains in the “open” lattice-bound conformation. EF-hand motifs are most often found in tandem, whereby they function as calcium-dependent molecular switches (Yap et al., 1999; Atkinson et al., 2001; Gifford et al., 2007). The presence of tandem EF-hand motifs in Shot raises the intriguing possibility that intramolecular inhibition could be regulated by calcium. In fact, a recent study of a mammalian homologue of Shot, BPG1n4, demonstrated that increased calcium led to a switch in how the COOH-terminal domain of this protein interacted with microtubules (Kapur et al., 2012). To test the hypothesis that the EF-hand motifs of Shot regulate its activity by binding to calcium, we constructed a full-length Shot BiFC probe carrying mutations (D5080A, D5082A, N5084A) predicted to abolish calcium binding (Figure 2, M and N, and Movie S3). When expressed in S2 cells, this probe did reconstitute fluorescence at the microtubule plus end, which was similar to results seen with the full-length VC-Shot-VN BiFC probe (Figure 2, D and E). The coordinating residues of the second EF-hand motif appear to diverge from other canonical calcium-binding EF-hands, and we did not test their contribution. To further test the potential role of EF-hand motifs, Shot remains in the “open” lattice-bound conformation. EF-hand motifs are most often found in tandem, whereby they function as calcium-dependent molecular switches (Yap et al., 1999; Atkinson et al., 2001; Gifford et al., 2007). The presence of tandem EF-hand motifs in Shot raises the intriguing possibility that intramolecular inhibition could be regulated by calcium. In fact, a recent study of a mammalian homologue of Shot, BPG1n4, demonstrated that increased calcium led to a switch in how the COOH-terminal domain of this protein interacted with microtubules (Kapur et al., 2012). To test the hypothesis that the EF-hand motifs of Shot regulate its activity by binding to calcium, we constructed a full-length Shot BiFC probe carrying mutations (D5080A, D5082A, N5084A) predicted to abolish calcium binding (Figure 2, M and N, and Movie S3). When expressed in S2 cells, this probe did reconstitute fluorescence at the microtubule plus end, which was similar to results seen with the full-length VC-Shot-VN BiFC probe (Figure 2, D and E).

Figure 2, J and L). This result suggests that without the EF-hand motifs, Shot remains in the “open” lattice-bound conformation.
Inhibition by intramolecular interaction

To experimentally control the conformation of Shot, we used the rapamycin-induced FKBP/FRB dimerization system to engineer FKBP-Shot-FRB-EGFP (Banaszynski et al., 2005, 2006; Slep and Vale, 2007; Figure 4). With the coding sequence of Shot flanked by FKBP and FRB, we predicted that addition of rapamycin would force the molecule into a closed and potentially inactive conformation. When FKBP-Shot-FRB-EGFP was transfected into S2 cells, its dynamics were indistinguishable from Shot A-EGFP; it tracked with microtubule plus ends in the central regions of the cells and localized to the microtubule lattice in the cell periphery (Figure 4, A and B). On perfusion with rapamycin, however, FKBP-Shot-FRB-EGFP interaction with microtubules was dramatically altered (Figure 4C and Movies S4 and S5). We observed a reduction in microtubule lattice association of FKBP-Shot-FRB-EGFP association compared with dimethyl sulfoxide (DMSO) controls and documented this change by measuring the lengths of microtubule lattice decorated by FKBP-Shot-FRB-EGFP from the plus end (Supplemental Figure S1). We also measured this change by comparing the distribution of FKBP-Shot-FRB-EGFP with that of EB1, using fluorescence intensity line scans, and observed a decrease in the extent of lattice binding (Figure 4, D–F). In addition we calculated the Mander’s coefficient to quantify the degree of colocalization between FKBP-Shot-FRB-EGFP and mCherry-tubulin in S2 cells treated with DMSO or 500 nM rapamycin (Figure 3, B, C, and H). Consistent with our live-cell imaging results, the Mander’s coefficient for FKBP-Shot-FRB-EGFP and mCherry-tubulin was similar to that of Shot A-EGFP in cells treated with DMSO (M = 0.35); however, upon treatment with rapamycin, the degree to which Shot and mCherry-tubulin colocalized decreased (M = 0.22). The Mander’s coefficient for rapamycin-treated cells was statistically indistinguishable from that of ShotΔGAS2-EGFP (Figure 3, D, C, and H). These data indicate that intramolecular association between Shot’s NH2- and COOH-termini is sufficient to target it to microtubule plus ends.

Our previous data suggested that Shot functions as a cross-linker when it is bound to the microtubule lattice (Appelwhite et al., 2010). Shot depletion causes microtubules to exhibit abnormal lateral, “fishtail” movements in S2 cells that can be measured from time-lapse images of fluorescently-tagged tubulin (Figure 5). In this assay an increase in fluorescence correlates with increased microtubule displacement or “fishtailing.” Microtubule fishtailing was rescued in S2 cells depleted of endogenous Shot (3'depletion by transfection with full-length Shot isoform A, but not with a version of Shot with the GAS2 domain deleted (ΔGAS2) nor with the Shot isoform C, which lacks a functional actin-binding domain (Figure 5A). These domains are also required for axon extension during Drosophila neurogenesis, a process that depends on Shot’s cross-linking activity (Lee and Kolodziej, 2002b). Reconstitution of split Venus is an irreversible reaction and, therefore, is predicted to "trap" the NH2- and COOH-termini of Shot in its folded conformation. Because neither VN-Shot-VC nor VC-Shot-VN exhibited robust localization to the microtubule lattice (Figure 2, D and E), we hypothesized that these constructs would be unable to act as...
cytoskeletal cross-linkers. We tested the ability of VC-Shot-VN to rescue cells depleted of endogenous Shot with RNAi and found that this construct did not suppress microtubule fissioning movements (Figure 5B). Comparison of the amount of microtubule lateral movements revealed that VC-Shot-VN—expressing cells were statistically indistinguishable from cells treated with 3′UTR Shot RNAi alone. Furthermore, we found that expression of FKBP-Shot-FRB-EGFP could rescue fissioning, but that FKBP-Shot-FRB-EGFP treated with rapamycin could not (Figure 5C). These data support our hypothesis that, when Shot adopts a “closed” conformation at microtubule plus ends, it is unable to bind to the microtubule lattice and to function as an actin-microtubule cross-linker.

**DISCUSSION**

A major outstanding question that arises from our work is: What is the regulatory input that causes Shot to undergo this conformational change and actively cross-link actin filaments to microtubules? Two recent studies have addressed this issue and have presented two (although not necessarily exclusive) activation signals. Wu et al. (2011) demonstrated that microtubule association of mouse ACF7/MACF1 is regulated by phosphorylation by GSK3β. In this system, phosphorylation of two GSK3β consensus sites in its COOH-terminus uncoupled ACF7 from microtubules, thus abrogating actin-microtubule cross-linking. Shot possesses putative Zeste-white 3 (Zw3; the fly GSK3β homologue) consensus sequences in the CTD; therefore, we looked for a regulatory role for this pathway by overexpressing constitutively active mCherry-Zw3 in S2 cells or inhibited kinase by treatment with LiCl or Zw3 RNAi. None of these treatments affected the localization or dynamics of endogenous Shot, full-length Shot-GFP, or CTD-GFP (Figures S2 and S3).

In a second study, Kapur et al. (2012) proposed a different intramolecular inhibitory mechanism for human BPAG1n4 in which the EF-hand motif binds to the GAS2 domain to inhibit microtubule lattice association, and this inhibition is relieved by free calcium. The authors also showed that interactions between BPAG1n4 and EB1 were also inhibited in the presence of calcium. To test whether calcium affected Shot dynamics in our system, we manipulated cytosolic calcium levels in S2 cells by treatment with ionophore, thapsigargin, or BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) but did not observe significant changes in Shot-GFP dynamics (unpublished data). We did confirm that the EF-hand motifs contribute to Shot regulation; however, our data do not support a role for calcium binding as the regulatory trigger. Thus the signal that relieves Shot autoinhibition in Drosophila remains an open area of investigation.

In this article, we demonstrate that Shot is able to adopt a folded conformation through an interaction between its NH2- and COOH-termini. We propose a model in which Shot interconverts between an extended conformation that is able to make cross-bridges between microtubules and actin filaments via the ABD and EF-hand-GAS2 domains, and a folded conformation that is targeted to the microtubule plus end by interaction with EB1 (Figure 6). A central component of this model is that the central rod domain of Shot, composed of 17 tandem spectrin repeats, affords the molecule sufficient flexibility for the NH2- and COOH-termini to interact. It will be interesting to examine the contributions of these individual repeats, which represent potentially flexible elements of the protein, and the plakin repeat domain to intramolecular inhibition of Shot. Given the high degree of conservation between Shot and spectraplakins in other species, as well as the published observations that mammalian homologues exhibit similar transitions between microtubule tip- and lattice-associated states, we speculate that this mechanism of regulation is likely conserved among members of this protein superfamily.

While our BiFC data are consistent with our biochemical data in supporting an intramolecular inhibition model, we were unable to directly observe the dynamics of this conformational change due to the irreversibility of this assay (Kerppola, 2008, 2009). Further experiments involving the use of Förster resonance energy transfer (FRET) may be required to capture the spatiotemporal regulation of this process. Additionally, techniques that can examine the structure of purified Shot, such as atomic force microscopy or electron microscopy, will provide a more conclusive test of our model (Lansbergen et al., 2004).

Autoinhibition is a common theme for protein regulation and has been described for a number of cytoskeletal proteins, such as conventional kinesin, CLIP-170, moesin, and the Diaphanos family of proteins (Alberts, 2001; Li and Higgs, 2003; Lansbergen et al., 2004; Wong et al., 2009; Wong and Rice, 2010; Lee et al., 2010; Kaan et al., 2011; Ben-Aissa et al., 2012). We speculate that this regulatory mechanism has evolved in spectraplakins so that they may utilize microtubule dynamic instability to search for localized upstream activation signals by a “search and capture” mechanism.
MATERIALS AND METHODS

Cell culture and RNAi

Drosophila S2 cell culture and RNAi were performed as described by Rogers and Rogers (2008). Briefly, S2 cells (Drosophila Genomics Resource Center, Bloomington, IN) were cultured in SF900II medium supplemented with 100× antibiotic–antimycotic (Invitrogen, Carlsbad, CA). RNAi was administered in six-well plates by treating cells (∼50% confluent) with 10 μg double-stranded RNA (dsRNA) in 1 ml of medium each day for 7 d.

DNA constructs and transfection

The pUAST-ShotA-EGFP, pUAST-ShotC-EGFP, pUAST-ShotΔGAS2-EGFP, and pUAST-ShotΔRod1-EGFP plasmids were first described by Lee and Kolodziej (2002b). For expression in S2 cells, pUAST-plasmids were cotransfected with a pMT-Gal4 plasmid. For BiFC-tagged constructs, the NH2 half (VN, residues 1–173) and COOH half (VC, residues 155–239) of Venus were generated by standard PCR procedures and cloned into pMT vectors (Invitrogen). We included a five–amino acid linker region (RSIAT) on both ends. Full-length Shot PCR products were generated by standard procedures using KOD Xtreme polymerase (EMD Millipore, Billerica, MA). pMT-Shot fragments used in immunoprecipitation experiments were cloned using standard PCR procedures. Expression was achieved with 250–500 μM final concentration of copper sulfate.

Protein purification and in vitro binding assays

Full-length EB1, GST, and fragments of Mini spindles and Shot were expressed and harvested from BL21 Escherichia coli. For production of 6xHis-tagged fusion proteins, the NH2-terminal ABD of Shot A isoform (residues 143–398), full-length EB1, and the first two TOG domains from Mini spindles (residues 1–512) were cloned into pET28a and purified on Ni-NTA resin (Qiagen, Valencia, CA). GST-tagged fusion of the EF-hand-GAS2-CTD fragment of Shot (residues 5001–5409) was cloned into pGEX6P2 and purified using glutathione fast-flow resin (GE Healthcare, Pittsburgh, PA).

In vitro binding assays were performed by incubating Ni-NTA resin with ∼10 mg 6xHis fusion prey proteins in 20 mM Tris (pH 8)
and 150 mM NaCl and mixing with ~10 μg GST fusion protein bait proteins for 30 min. The beads were washed three times and then boiled in SDS–PAGE sample buffer, separated by SDS–PAGE, and visualized with Coomasie Blue stain.

**Immunoprecipitation/Western blotting**
Transfected S2 cells were induced to express the various epitope-tagged constructs with 500 μM CuSO4 for 24 h. Cells were then lysed in cell lysis buffer (CLB; 50 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100, and 2.5 mM phenylmethylsulfonyl fluoride) and precleared, and lysates were diluted twofold in CLB. Lysates were mixed with anti-Myc (Sigma-Aldrich, St. Louis, MO) or anti-FLAG (Sigma-Aldrich) for 2 h at 4°C, and then protein A-Sepharose beads or protein G-Sepharose beads (Sigma-Aldrich) were incubated with the antibody–lysate mixture for 2 h at 4°C. The beads were washed three times with 0.5 ml of CLB and boiled in SDS–PAGE sample buffer, and then proteins were analyzed by SDS–PAGE immunoblotted with anti-FLAG Clone M2 (Sigma-Aldrich) or anti-Myc 9E10 (provided by the Developmental Hybridoma Bank, Department of Biology, University of Iowa, Iowa City, IA). Western blots were developed with horseradish peroxidase–tagged secondary antibodies and ECL reagents (Thermo Fisher Scientific). In addition to the antibodies listed above, Western blots were also probed with anti-D1M1α (Sigma-Aldrich), anti-dsRed (Clontech, Mountain View, CA), and anti-Armadillo (Developmental Hybridoma Bank).

**Immunofluorescence and live-cell imaging**
Immunofluorescence was performed on S2 cells plated on 0.5 mg/ml concanavalin A (Con A)–treated glass coverslips and fixed with cold methanol as described by Rogers et al. (2002). Antibodies used for this study include the anti-Rod (Short-stop; Developmental Hybridoma Bank), diluted 1:300 in phosphate-buffered solution and 0.1% Triton X-100 (PBST), and anti-EB1 (Rogers et al., 2002), diluted 1:400 in PBST. Secondary antibodies (Cy2 and Rhodamine Red; Jackson ImmunoResearch, West Grove, PA) were used at a final dilution of 1:100 in PBST. For live-cell imaging, S2 cells were plated on 0.5 mg/ml Con A–treated coverslips attached to drilled 35-mm tissue culture dishes with UV-curable adhesive (Norland Products, Cranbury, NJ) in Schneider’s Drosophila medium supplemented with 10% fetal bovine serum and 100x antibiotic–antimycotic (Invitrogen). Cells were allowed to attach for at least 1 h before imaging. When treated with rapamycin (EMD Millipore), cells were perfused with 250–500 nM final concentration. Time-lapse imaging was performed on two microscopes. The first was a Hawk-VT multipoint array scanning confocal (VisiTech International, Sunderland, UK) mounted on an inverted microscope (Eclipse TE300; Nikon, Tokyo, Japan) equipped with a 100×/1.4 numerical aperture objective lens driven by VisiTech VxO software. The second was a laser total internal reflection fluorescence (TIRF) system (Nikon) mounted on an inverted microscope (Ti; Nikon) equipped with a 100×/1.49 objective lens driven by Nikon Elements software. Images were captured with an Orca-R2 camera (Hamamatsu, Hamamatsu, Japan) or an Andor-Clara Interline camera (Andor Technology, Belfast, UK). All images were processed for brightness and contrast and prepared for publication using Photoshop (CS version 8.0; Adobe Systems, Mountain View, CA).

**Colocalization analysis**

The mean fraction of overlap between tubulin and EGFP-tagged Shot was calculated using Mander’s coefficient (described in Bolte and Cordelières, 2006) and the JACoP plug-in in ImageJ (National Institutes of Health [NIH], Bethesda, MD). Prior to analysis, images were thresholded. The Mander’s coefficient varies from zero to one, with zero representing nonoverlapping images and one representing complete overlap/colocalization. All images used for analysis were acquired from live cells by TIRF microscopy using identical microscope settings.

To exclude the possibility that overexpression artifacts affected the degree of colocalization between the EGFP-tagged Shot constructs and mCherry-tubulin, we compared the Mander’s coefficient with the fluorescence intensity per cell area (Figure S4). The correlation between the Mander’s coefficient and the fluorescence per cell area in cells expressing Shot A-EGFP, FKBP-Shot-FRB-EGFP (DMSO treated), and ShotGAS2-EGFP was statistically significant, indicating that the extent of colocalization increased with increasing expression levels of these constructs. In contrast, correlation between the Mander’s coefficient and the fluorescence intensity per cell area was not statistically significant for ShotΔEFhand-EGFP, ShotGAS2-EGFP, and ShotARod-EGFP, indicating that microtubule localization of these constructs was independent of expression level and did not reflect an artifact of overexpression.

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