Cbl-associated protein regulates assembly and function of two tension-sensing structures in *Drosophila*

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**SUMMARY**

Cbl-associated protein (CAP) localizes to focal adhesions and associates with numerous cytoskeletal proteins; however, its physiological roles remain unknown. Here, we demonstrate that *Drosophila* CAP regulates the organization of two actin-rich structures in *Drosophila*: muscle attachment sites (MASs), which connect somatic muscles to the body wall; and scolopale cells, which form an integral component of the fly chordotonal organs and mediate mechanosensation. *Drosophila* CAP mutants exhibit aberrant junctional invaginations and perturbation of the cytoskeletal organization at the MAS. CAP depletion also results in collapse of scolopale cells within chordotonal organs, leading to deficits in larval vibration sensation and adult hearing. We investigate the roles of different CAP protein domains in its recruitment to, and function at, various muscle subcellular compartments. Depletion of the CAP-interacting protein Vinculin results in a marked reduction in CAP levels at MASs, and *vinculin* mutants partially phenocopy *Drosophila* CAP mutants. These results show that CAP regulates junctional membrane and cytoskeletal organization at the membrane-cytoskeletal interface of stretch-sensitive structures, and they implicate integrin signaling through a CAP/Vinculin protein complex in stretch-sensitive organ assembly and function.

**KEY WORDS:** CAP, Integrin, Vinculin, Muscle attachment site, Chordotonal organ, *Drosophila*

**INTRODUCTION**

Interactions between cells and the extracellular matrix (ECM) are crucial for many biological processes. These include cell migration, directed process outgrowth, basement membrane-mediated support of tissues and maintenance of cell shape (Bökel and Brown, 2002; Hogg et al., 2011; Nakamoto et al., 2004; Watt and Fujihara, 2011). Communication between cells and ECM proteins often occurs through the action of α/β-integrin heterodimers, a receptor complex that forms adhesive contacts, including focal adhesions, hemiadhersen junctions, costameres and myotendinous junctions (Bökel and Brown, 2002). In response to extracellular forces, focal adhesions undergo structural changes and initiate signaling events that allow adaptation to tensile stress (Geiger et al., 2009). Vinculin is thought to be the primary force sensor in the integrin complex, mediating homeostatic adaptation to external forces (Carisey and Ballestrem, 2011; Grashoff et al., 2010; Hytönen and Vogel, 2008).

Vinculin-binding partners include proteins belonging to the CAP (Cbl-associated protein) protein family (Kioka et al., 1999). However, the physiological significance of this association is unknown. Mammalian CAP proteins are components of focal adhesions in cell culture (Kioka et al., 1999; Zhang et al., 2006). In myocytes, CAP localizes to integrin-containing complexes called costameres that anchor sarcomeres to muscle cell membranes (Zhang et al., 2007). There are three mammalian CAP protein family members: CAP, Vinexin and ArgBP2 (Kioka et al., 2002). CAP associates *in vitro* with many proteins, including the cytoskeletal regulators Paxillin, Afadin and Filamin, vesicle trafficking regulators such as Dynamin and Cbl, and the lipid raft protein Flotillin (Chiang et al., 2001; Mandai et al., 1999; Zhang et al., 2006; Zhang et al., 2007). In *in vitro* studies demonstrate that CAP regulates the reassembly of focal adhesions following nocodazole dissolution (Zhang et al., 2006). However, despite extensive studies on CAP (Kioka, 2002; Zhang et al., 2006), little is known about its functions *in vivo*. *Cap (Sorbs1)* mutant mice are defective in fat metabolism, and targeted deletion of the vinexin gene results in wound-healing defects (Kioka et al., 2010; Lesniewski et al., 2007). *Drosophila* CAP binds to axin and is implicated in glucose metabolism (Yamazaki and Nusse, 2002; Yamazaki and Yanagawa, 2003). Analysis of CAP function in mammals is complicated by potential functional redundancy of the three related CAP proteins. Therefore, we have examined the function of *Drosophila* CAP, the single CAP family member in *Drosophila*, *in vivo*.

The *Drosophila* muscle attachment site (MAS) is an excellent system for studying integrin signaling. Somatic muscles in each segment of the fly embryo and larva are connected to the body wall through integrin-mediated hemiadhersen junctions (Brown, 2000). Somatic muscles in flies lacking integrins lose their connection to the body wall (Brown et al., 2000; Brown et al., 2002; Clark et al., 2003; Zervas et al., 2001). Surprisingly, flies lacking Vinculin, a major component of cytosolic integrin signaling complexes, are viable and show no muscle defects (Alatorvse et al., 1997). Thus, unlike its mammalian counterpart, *Drosophila* Vinculin is apparently dispensable for the initial assembly of integrin-mediated adhesion complexes at somatic MASs.

The fly MAS is structurally analogous to the fly chordotonal organ. These organs transduce sensations from various stimuli, including vibration, sound, gravity, airflow and body wall movements (Caldwell and Eberl, 2002; Kamikouchi et al., 2009;
Kernan, 2007; Yack, 2004; Yorozu et al., 2009). The chordotonal organ is composed of individual subunits called scolopidia, each containing six cell types: neuron, scolopale, cap, ligament, cap attachment and ligament attachment cells (Todi et al., 2004). Chordotonal neurons are monodendritic, and their dendrites are located in the scolopale space, a lymph-filled extracellular space completely enveloped by the scolopale cell (Todi et al., 2004). Within the scolopale cell, a cage composed of actin bars, called scolopale rods, facilitates scolopale cell envelopment of the scolopale space (Carlson et al., 1997; Todi et al., 2004). Thus, like the MAS, the actin cytoskeleton plays a specialized role in defining chordotonal organ morphology. Similarities between MASs and chordotonal organs include the requirement during development in both tendon and cap cells for the transcription factor Stripe (Inbal et al., 2004). Furthermore, both of these cell types maintain structural integrity under force and so are likely to share common molecular components dedicated to this function.

Here, we show that the Drosophila CAP protein is selectively localized to both muscle attachment sites and chordotonal organs. In Drosophila CAP mutants we observe morphological defects that are indicative of actin disorganization in both larval MASs and the scolopale cells of Johnston’s organ in the adult. The morphological defects in scolopale cells result in vibration sensation defects in larvae and hearing deficits in adults. We also find that, like its mammalian homologues, Drosophila CAP interacts with Vinculin both in vitro and in vivo. These results reveal novel CAP functions required for actin-mediated organization of cellular morphology, lending insight into how CAP mediates muscle and sensory organ development and function.

MATERIALS AND METHODS

Drosophila genetics

Drosophila CAP deletion mutants were generated by imprecise excision of the P-element CAP10A06924 inserted in the intron proximal to the SH3 domain-coding exons. We generated multiple excisions, deleting the first two SH3 domain-coding exons. These deletions are dCAP10A and dCAP20A, and delete 2.9 kb (genomic region 2R:6,190,378-6,193,350) and 2.7 kb (genomic region 2R:6,190,378-6,193,141), respectively, downstream of the CAP10A06924 P-element. A precise excision we generated called CAP49a was used as wild-type control. CAP10A06924 and rhea1, flotillin2KC00210 and tensin mutants by1 were from the Bloomington Stock Center; zasp9 and zasp56 mutants were a gift from Frieder Schock (McGill University, Montreal, Canada). Cheerio and paxillin RNAs were obtained from the Vienna Drosophila Research Center.

Drosophila CAP mRNA constructs target the sequences CAAGCTAATGAGAGTGCAGT and TTCAATACGCTGAC-GCAAAATG in the CAP-E transcripts encoding the C-terminal CAP SH3 domains. These constructs were generated as described previously (Chen et al., 2007). The stem-loop backbone and surrounding sequences were unaltered, and the Drosophila Mira-1 gene-targeting miRNA sequence was replaced with the 22 bp complementary to the CAP transcript. Two miRNA constructs targeting distinct CAP sequences were inserted in tandem into the pUAST vector and used to generate transgenic flies.

Drosophila CAP antibody

The Drosophila CAP short isoform (CAP-E), which encodes the three SH3 domains, was cloned into the pGE4X1-1 vector to generate a GST fusion protein. Purified GST-CAP-E was injected into rabbits for polyclonal antibody production. GST-CAP-E-bound Affigel beads were used to affinity-purify CAP-specific antibodies.

Immunostaining and microscopy

Drosophila embryos were processed for immunostaining as described previously (Patel, 1994). For most experiments, fixation used 4% paraformaldehyde. For examination of larval muscle attachment sites, fillet preparations of wandering third instar larvae were prepared in PBS, as described previously (Brent et al., 2009). For Johnston’s organ staining, adult heads with the proboscis removed were fixed in 4% PFA for 90 minutes at 4°C. After three washes with PBS, heads were incubated in 12% sucrose overnight and subsequently frozen in OCT. Cross-sections (20 μm) were cut using a cryostat. The following antibodies were used: anti-BP integrin at 1:100 (DSHB), 22C10 at 1:200 (DSHB), 21A6 at 1:200 (DSHB), rabbit anti-85e-tubulin at 1:20 [a gift from T. C. Kaufman (Indiana University, Bloomington, IN, USA) and A. Salzberg (Rappaport Institute, Haifa, Israel)], rabbit anti-CAP at 1:1000 (our antibody), rabbit anti-Zasp at 1:1000 (a gift from F. Schock), rabbit anti-pincher at 1:500 (a gift from M. C. Beckerle, University of Utah, Salt Lake City, UT, USA), anti-thrombospondin at 1:400 (a gift from T. Volk, Weizmann Institute of Science, Rehovot, Israel), mouse anti-tiggrin at 1:200 (a gift from L. Fessler, UCLA, Los Angeles, CA, USA). Images were acquired using a Zeiss LSM 510 confocal microscope.

Electron microscopy

Drosophila third instar larval fillets from dCAP49a mutants and controls were processed as described with minor variations (Ramachandran and Budnik, 2010). Larval fillets were fixed with 1% glutaraldehyde and 4% paraformaldehyde in cacodylate buffer at 4°C overnight, stained first with 2% osmium tetroxide at 4°C for 1 hour and then with 2% uranyl acetate for 30 minutes. Samples were dehydrated by incubating with increasing concentrations of ethanol and equilibrated in varying concentrations of propylene oxide and Epon resin. Larval fillets were pinned to embedding molds using insect pins and embedded in Epon resin. Horizontal ultrathin sections of the larval fillet were cut using an ultramicrotome. Sections were subsequently stained with uranyl acetate and lead citrate; TEM was performed using a Hitachi H-7000 transmission electron microscope. Ultrastructural analysis of Johnston’s organs was performed as described previously (Todi et al., 2005).

Immunopurification

For each immunopurification experiment, 1.5 g of Drosophila embryos and 100 μg of Drosophila CAP antibody were used. Immunopurification was performed as described previously (Bharadwaj et al., 2004). The protein bands specific to wild-type lysates were excised, pooled, subjected to trypsin digestion and used for MALDI-TOF mass spectrometry analysis (Johns Hopkins Medical School Mass Spectrometry facility).

Vibration sensation assay

The vibration sensation assay was performed as described previously (Swierczek et al., 2011; Wu et al., 2011). Approximately 96-hour-old third instar larvae of the specified genotypes were selected using GFP-balancer lines and housed in 4% paraformaldehyde. For examination of larval muscle attachment sites, fillet sections were cut using an ultramicrotome. Sections were post-fixed with 2% osmium tetroxide at 4°C for 1 hour and then with 2% uranyl acetate for 30 minutes. Samples were dehydrated by incubating with increasing concentrations of ethanol and equilibrated in varying concentrations of propylene oxide and Epon resin. Larval fillets were pinned to embedding molds using insect pins and embedded in Epon resin. Horizontal ultrathin sections of the larval fillet were cut using an ultramicrotome. Sections were subsequently stained with uranyl acetate and lead citrate; TEM was performed using a Hitachi H-7000 transmission electron microscope. Ultrastructural analysis of Johnston’s organs was performed as described previously (Todi et al., 2005).

Auditory nerve electrophysiology

Electrophysiological recording of sound-evoked potentials (SEPs) was performed as described previously (Eberl et al., 2000; Eberl and Kernan, 2011).

RESULTS

Drosophila CAP genomic organization and CAP mutation generation

Drosophila CAP is the only member of the CAP gene family in Drosophila. Like its mammalian counterparts, Drosophila CAP possesses three C-terminal SH3 domains and an N-terminal SoHo (sorbin homology) domain (Fig. 1A). There are three mammalian CAP proteins: CAP, vinexin and ArgBP2. Drosophila CAP shows
the highest amino acid sequence similarity to mammalian CAP (Fig. 1B) (Yamazaki and Nusse, 2002). Genomic DNA sequence, northern blot and western blot analyses reveal that there are multiple CAP isoforms in Drosophila, similar to mammalian CAPs (Fig. 1C) (Yamazaki and Nusse, 2002). The only coding region shared by all predicted Drosophila CAP protein isoforms is at the C terminus and encodes the SH3 domains. Through imprecise excision of a P transposable element (CAPCA06924), we generated two deletions, dCAP42b and dCAP49e, that remove the first two of the three conserved SH3 domains, and also a precise excision, dCAP49a, that serves as a control (Fig. 1A,C). Drosophila CAP mutants are viable and fertile. Western blot analysis of adult whole-body lysates from flies ubiquitously expressing these miRNAs probably lack all N-terminal CAP protein.

**CAP localizes to muscle attachment sites and chordotonal organ scolopale cells**

We next analyzed CAP protein expression throughout development using our CAP polyclonal antibody, which most probably recognizes all predicted Drosophila CAP isoforms. In stage 16 embryos, CAP is expressed predominantly in chordotonal sensory organs, somatic muscles, the dorsal vessel (the Drosophila heart) and the gut (Fig. 2A,C). Drosophila CAP mutants are viable and fertile. Western blot analysis of adult whole-body lysates from flies ubiquitously expressing these miRNAs probably lack all N-terminal CAP protein.
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**Fig. 2. Embryonic expression of CAP protein.** (A-D) Wild-type (A,C) and dCAP<sup>96e</sup> (B,D) embryos stained with anti-CAP and 22C10 antibody (which labels all sensory neurons). (A,B) Wild-type embryos show CAP staining in chordotonal organs (arrowheads), dorsal vessels (thin arrows) and gut (thick arrows); this staining is absent in CAP mutants. (C,D) Higher magnification image of CAP localization in chordotonal organ scolopale cells (arrows) in wild type, but not dCAP<sup>96e</sup> embryos. (E,F) Wild-type and dCAP<sup>96e</sup> embryos immunostained with anti-CAP and anti-βPS integrin; CAP colocalizes with βPS integrin at MASs (arrows). Scale bars: 60 μm in A,B; 20 μm in C-F.

**CAP is required for larval chordotonal organ function.**

We next performed a recently described behavioral assay that provides a sensitive readout of chordotonal organ-mediated locomotor response to vibration (Swierczek et al., 2011; Wu et al., 2011). Fly chordotonal organs are mechanosensory organs that mediate proprioception in larvae and transduction of acoustic signals in adults (Caldwell et al., 2003; Eberl, 1999). Wild-type third instar larvae exhibit a characteristic biphasic vibration response. Larvae first display a fast startle response to vibration by hunching their bodies, followed by a second, relatively slower, head-turning response. These responses are largely dependent on the chordotonal sensory neurons and can be precisely quantified (Wu et al., 2011). The ‘hunch’ response to vibration is quantified by measuring the average larval body length reduction. The mean normalized larval body length is measured over the larval midline before and after a stimulus: for control dCAP<sup>96a</sup> (precise excision) larvae, this was 1.01±0.11 (before) and 0.94±0.1 (after); for dCAP<sup>27b</sup> heterozygotes, it was 0.99±0.07 (before) and 0.91±0.07 (after) (n=100 larvae for each genotype) (Fig. 3C). Thus, wild-type larvae show a significant (P<0.01) reduction in mean normalized body length in response to vibration. By contrast, dCAP<sup>96e</sup> and dCAP<sup>27b</sup> mutant larvae do not exhibit a significant hunching response to vibration (Fig. 3C). The mean normalized midline body length before and after stimuli for dCAP<sup>96e</sup> was 0.99±0.08 (before) and 0.98±0.1 (after); and for dCAP<sup>27b</sup> mutants it was 0.99±0.08 (before) and 1±0.09 (after) (n=100 larvae per genotype). This same phenotype was also observed when dCAP<sup>27b</sup> or dCAP<sup>96e</sup> alleles were placed in trans to a large chromosomal deficiency spanning the entire CAP locus (Fig. 3C). Scolopale cell-specific expression of CAP rescues this vibration-sensation defect observed in CAP mutants, and scolopale cell depletion of CAP by driving CAP miRNA using the NompA-Gal4 driver (Chung et al., 2001) phenocopies CAP mutants (Fig. 3C).

CAP is also enriched at the cap cell attachment site to the ectoderm (but not in ligament cells) (supplementary material Fig. S2A-B’). Integrin is also enriched at these sites. We examined βPS integrin localization in cap cells of dCAP<sup>96e</sup> mutants, and in dCAP<sup>96e</sup> mutants integrin localization in cap cells and the overall morphology of chordotonal organs, revealed by anti-β5e-tubulin labeling, is apparently normal (supplementary material Fig. S2C-D’). These results suggest chordotonal defects observed in dCAP<sup>96e</sup> mutants arise primarily from the lack of scolopale cell CAP.

**Structural and functional Johnston’s organ defects in adult CAP mutants.**

We next examined a distinct chordotonal organ class: the adult Johnston’s organ. The Johnston’s organ resides in the second antennal segment and is composed of ~250 scolopidia (Todi et al., 2004). Johnston’s organ scolopidia and larval chordotonal organs show remarkable morphological similarities. Several proteins, including Atonal and Beethoven, serve specialized functions in both of these organs (Caldwell et al., 2003; Todi et al., 2004). CAP is strongly expressed in wild-type Johnston’s organs but not in dCAP<sup>96e</sup> mutants (Fig. 4A). Immunostaining with phalloidin, which illuminates Johnston’s organ scolopale rods (Todi et al., 2004), and with the 22C10 mAb, which labels sensory neurons, reveals CAP in Johnston’s organ scolopale cells, similar to our observations in embryonic chordotonal organs (Fig. 4A).

Electrophysiological analyses to detect auditory responses were performed by recording from the auditory nerve following a mating song stimulus (Eberl et al., 2000; Eberl and Kernan, 2011). The
sharp contrast to wild-type Johnston’s organs, in which scolopale cells appear circular in cross-section, are of equal size and show fairly regular spacing (Fig. 4B). This morphological defect is consistent with CAP maintaining cytoskeletal structural integrity. In addition, sensory neurons in CAP mutant Johnston’s organs often show accumulation of vacuolar structures (supplementary material Fig. S3B). As CAP localizes predominantly to scolopale cells, these defects are probably secondary to scolopale cell morphological defects and may result from partial disruption of the lymph environment surrounding sensory neuron dendrites in the Johnston’s organ. Taken together, these data show that CAP mutants exhibit functional and morphological Johnston’s organ deficits; the highly localized expression of CAP in scolopale cells, both in Johnston’s organ and the larval chordotonal organ, implicates CAP in the regulation and/or maintenance of scolopale cell membrane morphology.

**CAP mutants exhibit morphological defects at somatic body wall muscle attachment sites**

As CAP is robustly localized to embryonic and larval muscle attachment sites (MASs), we next examined the morphology of MASs in CAP mutants. As shown by immunostaining for βPS integrin (an integral component of the MAS) and also for actin, stage 16 embryonic MASs in CAP mutants appear normal (Fig. 2F; data not shown). However, in third instar larvae, we find significant morphological abnormalities in CAP mutants. Filleted preparations of dCAP^{56e} third instar larvae were stained with rhodamine-phalloidin to determine MAS actin localization. In wild-type larvae, the somatic body wall MAS is bounded on either side by a smooth band of actin (Fig. 5B). Phalloidin staining also reveals that adjacent muscles are apposed to one another with a small gap, of uniform width over its entire length, separating adjacent body wall muscles. By contrast, actin organization at the MAS in CAP mutant larvae is severely disrupted, resulting in a jagged boundary at the gap where the MAS is located (Fig. 5B); this is in stark contrast to the smooth boundaries at this gap observed in wild-type larvae. Furthermore, we observed a significantly enlarged gap between actin filaments from adjacent muscles at most MASs in these mutants (Fig. 5B). We examined MAS morphology between lateral-ventral somatic muscles 6 and 7 in abdominal segments (A2-A5). For each genotype, 30 hemisegments from five to seven larvae, the somatic body wall MAS is bounded on either side by a smooth band of actin (Fig. 5B). Phalloidin staining also reveals that adjacent muscles are apposed to one another with a small gap, of uniform width over its entire length, separating adjacent body wall muscles. By contrast, actin organization at the MAS in CAP mutant larvae is severely disrupted, resulting in a jagged boundary at the gap where the MAS is located (Fig. 5B); this is in stark contrast to the smooth boundaries at this gap observed in wild-type larvae. Furthermore, we observed a significantly enlarged gap between actin filaments from adjacent muscles at most MASs in these mutants (Fig. 5B). We examined MAS morphology between lateral-ventral somatic muscles 6 and 7 in abdominal segments (A2-A5). For each genotype, 30 hemisegments from five to seven larvae were scored, revealing that this phenotype is fully penetrant in both dCAP^{56e} and dCAP^{26b} mutants.

At wild-type MASs, integrin subunits are localized to a smooth, compact band lining the narrow gap between muscles of adjacent segments (Fig. 5B). However, in CAP mutants this band of integrin staining is much broader and includes numerous invaginations into adjacent muscles (Fig. 5B). The average length of the integrin-positive invaginations into the muscles adjacent to the MAS in wild-type larvae is 2±0.18 μM; in dCAP^{56e} mutants it is dramatically increased to 13±0.42 μM (n=30 hemisegments per genotype) (Fig. 5D). The penetrance and expressivity of this phenotype were not enhanced when the dCAP^{56e} allele was placed in trans to a deficiency spanning the entire CAP genomic region, indicating that dCAP^{56e} is either a null, or a very strong loss-of-function, allele (Fig. 5D). In addition, MASs in CAP mutants show partial detachments in ~25% of larval hemisegments, a phenotype suggesting partial disruption of integrin function.

The MAS is composed of two different cell types: multinucleated muscle fibers and tendon cells that connect the muscle fiber to the body wall (Brown, 2000). To determine which of these cell types require CAP function, we selectively depleted
CAP by driving expression of the CAP tandem miRNAs transgene in muscle cells using the MeF2-Gal4 driver (Ranganayakulu et al., 1996), or in tendon cells using the stripe-Gal4 driver (Dorfman et al., 2002). Strikingly, CAP MAS localization was almost completely abolished when CAP miRNAs were expressed under control of MeF2-Gal4, whereas CAP localization was unaltered when CAP miRNAs were driven by stripe-Gal4 (Fig. 5C). Therefore most, if not all, MAS CAP protein is derived from muscles in third instar larvae. miRNA-mediated muscle-specific depletion of CAP phenocopies all attributes of the dCAP^{d9e} phenotype, both qualitatively and quantitatively, providing further evidence that CAP function in muscles is required for MAS development and maybe also maintenance later in development.

Altered distribution of integrin signaling components at the MAS in CAP mutants
Mammalian CAP proteins are implicated in integrin signaling (Kioka et al., 2002; Zhang et al., 2006), so we next investigated whether CAP functions to assemble integrin signaling complex components. We examined the distribution of MAS extracellular matrix proteins tiggrin and thrombospondin (Bunch et al., 1998; Geiger and Yamada, 2011; Subramanian et al., 2007), integrin subunits and the integrin matrix proteins tiggrin and thrombospondin (Bunch et al., 1998; Geiger and Yamada, 2011; Subramanian et al., 2007), integrin subunits and the integrin signaling proteins pinch, Ilk and zasp (Geiger and Yamada, 2011; Jun and Schöck, 2007) at the CAP mutant MAS (Fig. 6A,B; supplementary material Fig. S5; and data not shown). We observed no difference in the levels or colocalization of these proteins at MASs in CAP mutants. However, each of these integrin-associated proteins displays the aberrant, broad, MAS distribution accompanied by numerous aberrant invaginations into adjacent muscles that we observe for βPS integrin expression in CAP mutants and CAP miRNA knock-down larvae (Fig. 6A,B; supplementary material Fig. S5; and data not shown).

To better characterize the ectopic CAP mutant MAS junctional invaginations revealed by anti-βPS integrin immunostaining, we
examined MAS ultrastructure in wild-type and CAP mutant third instar larvae using TEM. We found that close to the muscle cell membrane, myofilaments at MASs are disorganized in CAP mutants (Fig. 6C, thick arrow). Furthermore, we observe numerous junctional invaginations in CAP mutant animals that are larger than those seen in wild-type larvae (Fig. 6C). This is commensurate with our light-level observations of MASs in CAP mutants (Fig. 5B). The presence of these significantly enlarged membranous infoldings, and also the cytoskeletal defects observed in our ultrastructural analysis, suggests that CAP regulates cytoskeletal organization and affects muscle membrane morphology at MASs.

**Distinct functions of CAP isoforms at larval MASs**

CAP protein isoforms are observed from insects to mammals (Kioka et al., 2002). Therefore, we next assessed different CAP isoforms for rescue of CAP mutant MAS defects using cell-type-specific expression in vivo. We assessed rescue ability of a CAP isoform called CAP-C, which includes all CAP protein domains, and also the smaller CAP isoform, CAP-E, which includes only the C-terminal SH3 domains (Fig. 7A). In our immunoblotting experiments, the presence of 100 kDa and 30 kDa bands, which are close to the expected sizes of CAP isoforms C and E, respectively, suggests that these isoforms are found in vivo (Fig. 1D). When expressed in muscles using the Mef2-Gal4 driver, myc-tagged CAP-C fully rescues the MAS defects observed in CAP mutants (Fig. 7B,D). However, there are differences in the distribution of the Mef2-Gal4-driven CAP-C and endogenous CAP protein. The CAP-C isoform is localized evenly to both Z-lines and MASs, whereas endogenous CAP protein shows preferential targeting to MASs (compare Fig. 7B and Fig. 5A). The CAP-E isoform possesses only the three CAP SH3 domains and no additional CAP N-terminal domains, and it does not rescue the CAP MAS phenotype (Fig. 7A,B,D). Interestingly, this isoform is localized predominantly to MASs and not to the Z-lines (Fig. 7B). Therefore, regions of N-terminal CAP are required for targeting CAP protein to Z-lines, whereas the CAP C-terminal SH3 domains are sufficient for targeting to the MAS. Furthermore, though the N-terminal CAP protein motifs are dispensable for targeting to the MAS, these motifs are required for CAP function at MAS, given the inability of the CAP-E isoform to rescue CAP mutants. We also observed that a CAP isoform lacking the conserved SoHo domain is able to rescue the CAP MAS phenotype (Fig. 7B,D). Therefore, the SoHo domain is not required for CAP function at the MAS.

Muscle-specific overexpression of full-length CAP-C, or the CAP-C isoform lacking the SoHo domain, did not result in MAS defects, either at embryonic or third instar larval stages (Fig. 7C,D; data not shown). However, overexpression of the short CAP isoform (CAP-E), which includes only the C-terminal SH3 domains, did lead to defects in actin organization and integrin
distribution at MASs that were very similar to those observed in CAP mutants (Fig. 7C,D). Though these CAP-E gain-of-function defects are not enhanced by removal of one copy of CAP (supplementary material Fig. S6), co-expression of full-length CAP-C and also CAP-E suppresses these defects (supplementary material Fig. S6). Therefore, the short CAP isoform acts in vivo in a dominant-negative fashion, probably through sequestration of CAP-associated proteins, thereby inhibiting endogenous CAP function.

Integrin and Talin are required for CAP recruitment to the embryonic MAS

To examine CAP recruitment, or stabilization, at the larval MAS, we examined its localization in embryos lacking integrin-signaling pathway components. Wild-type embryos show robust CAP localization at MASs (Fig. 2E; Fig. 8A–B). βPS integrin mutants (mys1) exhibit the classic myospheroid phenotype (Brown et al., 2000). MAS CAP localization is missing in mys1 mutants (Fig. 8B–B’). This is also consistent with integrin recruitment to MASs prior to CAP recruitment, whereas CAP recruitment to scolopale cells (arrowheads) is independent of integrins (Fig. 8E–G’). In stage 14 embryos, integrin is expressed at the MAS but CAP is not. At stage 15, CAP expression is observed in regions of the MAS, whereas, at stage 16, CAP expression completely colocalizes with βPS integrin (Fig. 8E–G’).

CAP localization in talin mutants (rhea) (Brown et al., 2002) reveals a dramatic decrease in CAP MAS levels, though a low level of CAP expression is still detectable at MASs in these mutants. These mutants often show MAS splitting, observable by CAP localization in two bands, not one (Fig. 8C’, white arrows). This may reflect partial detachment of muscles from the body wall. We also examined CAP localization in embryos lacking zasp, which is implicated in integrin signaling (Jani and Schöck, 2007), and we find that CAP MAS localization is unperturbed, both at stage 16 and 17 (Fig. 8D). Thus, both integrin and Talin are required for proper localization of CAP at the embryonic MASs, suggesting CAP signals downstream of integrins at the MAS.

Depletion of Vinculin results in MAS defects and disruption of CAP MAS localization

There are several known CAP-interacting proteins, including Flotillin, Tensin, Filamin and Paxillin (Kioka et al., 2002; Zhang et al., 2007). We used null mutants or RNAi-mediated knockdown to determine whether any of these proteins phenocopy CAP mutants; however, none did (supplementary material Fig. S7A). We then sought to identify proteins required for CAP function, immunopurifying endogenous CAP from wild-type and CAP Drosophila embryo lysates using our CAP polyclonal antibody. We isolated protein bands present in wild-type, but not the CAP mutant, immunoprecipitates (Fig. 9A). These bands were pooled and sequenced using MALDI TOF mass spectrometry. The most enriched protein in CAP immunoprecipitates (aside from CAP itself), as indicated by the number of peptides corresponding to any particular protein, was Vinculin. We also identified Talin as a CAP-interacting protein. The other proteins identified in CAP immunoprecipitates were 5-oxo-prolinase, Nervous wreck, 14-3-3 ε and histone H2A.

Vinculin interacts with all mammalian CAP proteins in vitro (Kioka, 2002; Kioka et al., 2002; Zhang et al., 2006); however, none did (supplementary material Fig. S7A). We then sought to identify proteins required for CAP function, immunopurifying endogenous CAP from wild-type and CAP Drosophila embryo lysates using our CAP polyclonal antibody. We isolated protein bands present in wild-type, but not the CAP mutant, immunoprecipitates (Fig. 9A). These bands were pooled and sequenced using MALDI TOF mass spectrometry. The most enriched protein in CAP immunoprecipitates (aside from CAP itself), as indicated by the number of peptides corresponding to any particular protein, was Vinculin. We also identified Talin as a CAP-interacting protein. The other proteins identified in CAP immunoprecipitates were 5-oxo-prolinase, Nervous wreck, 14-3-3 ε and histone H2A.
significance of Vinculin-CAP interactions in audition by electrophysiological recordings from auditory nerves of wild-type and vinculin mutant flies, and did not observe any significant difference in sound evoked potentials between wild type and vinculin mutants (supplementary material Fig. S7B). This suggests that CAP functions in chordotonal organs in a Vinculin-independent manner. However, MASs in vinculin mutants show with full penetrance a marked reduction in CAP levels, though CAP Z-band localization remains unaltered (Fig. 9C). We observed similar morphological defects in In(1LR)pn2a homozygous mutants and in animals carrying this mutation over a deficiency that includes the entire vinculin gene, suggesting the phenotypic defects in In(1LR)pn2a homozygous mutants result from complete disruption of vinculin (data not shown). This suggests Vinculin plays a crucial role in the recruitment of CAP to the MAS, reminiscent of Vinculin-dependent recruitment of CAP to mammalian focal adhesions (Takahashi et al., 2005). Examination of actin staining in vinculin mutants reveals that, as in CAP mutants, the actin bands lining the MAS are serrated and flank a gap wider than that observed in wild-type animals (Fig. 9C,D). However, this gap is not as wide as in CAP mutants, and vinculin mutants show fewer βPS integrin-labeled junctional invaginations relative to CAP mutants (Fig. 9C,D). These results show that CAP and its binding partner Vinculin function together as key regulators of cytoskeletal organization and membrane morphology at the MAS.

**DISCUSSION**

Integrin-based adhesion complexes are crucial for cell attachment to the extracellular matrix. These complexes change their composition and architecture in response to extracellular forces, initiating downstream signaling events that regulate cytoskeletal organization (Geiger et al., 2009). Here, we have investigated the role played by the CAP protein in two stretch-sensitive structures in Drosophila: the MAS and the chordotonal organ. CAP mutants exhibit aberrant junctional invaginations at the MAS and collapse of scolopale cells in chordotonal organs. Our study highlights a crucial integrin signaling function during development: the maintenance of membrane morphology in stretch-sensitive structures.

**CAP functions in somatic muscles**

The morphological defects observed in CAP mutants could result from an excessive integrin signaling, or possibly accumulation of additional membranous components related to integrin signaling, in CAP mutants, owing to defects in endocytosis at the MAS. This is consistent with known interactions between CAP family members and vesicle trafficking regulators, including Dynamin and Synaptojanin, which are required for internalization of transmembrane proteins (Cestra et al., 2005; Tosoni and Cestra, 2009) (Fig. 9E). Alternatively, CAP may be required for proper organization of the actin cytoskeleton at MASs, and the aberrant membrane invaginations we observe are a secondary consequence of these cytoskeletal defects. This idea gains support from known...
interactions between CAP and various actin-binding proteins, including Vinculin, Paxillin, Actinin, Filamin and WAVE2 (Cestra et al., 2005; Kioka, 2002; Kioka et al., 2002; Zhang et al., 2006). A third possibility is that CAP and Vinculin are regulators of membrane stiffness at the MAS, and aberrant junctional infoldings observed in CAP and vinculin mutants derive from diminished membrane rigidity in the presence of persistent myofilament contractile forces (Fig. 9E) (Diez et al., 2011; Goldmann et al., 1998). Biophysical studies demonstrate that Vinculin-deficient mammalian cells in vitro show reduced membrane stiffness (Goldmann et al., 1998). Interestingly, the CAP protein ArgBP2 interacts with Spectrin, a protein important for cell membrane rigidity maintenance (Cestra et al., 2005). These models for CAP function at MASs, however, are not mutually exclusive. Interestingly, disruption of the ECM protein Tiggrin leads to MAS phenotypes similar to CAP. Future studies on CAP interaction with Tiggrin and other CAP-interacting proteins will shed light on mechanisms underlying CAP function. Nevertheless, we demonstrate here in vivo the importance of CAP in stretch-sensitive organ morphogenesis, and it will be interesting to determine whether this function is phylogenetically conserved.

**Role of CAP in chordotonal organs**

Apart from the MAS, CAP is also expressed at high levels in chordotonal organ scolopale cells, and we find that CAP mutants are defective in vibration sensation, a hallmark of chordotonal organ dysfunction. However, only the initial fast hunching response to vibration is disrupted in CAP mutant larvae. This may result from a partial loss of chordotonal function in these organs in the absence of CAP. We also observe a functional defect in the adult Johnston’s organ; CAP mutant flies show diminished sound-evoked potentials. Importantly, the scolopale cells in CAP mutants appear partially collapsed. The extracellular space within the scolopale cell is lined by an actin cage, and CAP may influence the proper assembly of this actin cage or its association with the scolopale cell membrane. Ch organs are mechanosensory detectors and are constantly exposed to tensile forces. Thus, CAP apparently influences cytoskeletal integrity in two actin-rich structures: the MAS and the chordotonal organ, both of which are involved in force transduction.

**CAP interaction with Vinculin**

Mammalian and Drosophila CAP bind to Vinculin (Kioka, 2002; Kioka et al., 2002; Zhang et al., 2006). Vinculin is required for the recruitment of the mammalian CAP proteinvinexin to focal adhesions in NIH3T3 cells in vitro (Takahashi et al., 2005). Consistent with this observation, we observe a dramatic decrease in CAP levels at MASs in vinculin mutants, but residual levels of CAP protein remain. Furthermore, CAP localization at the muscle fiber Z-lines is completely unaltered in vinculin mutants. These observations indicate that Vinculin is not the sole upstream regulator of CAP localization (Fig. 9E). vinculin mutants show some of the phenotypic defects we observe in CAP mutants; however, these defects are less pronounced. Therefore, the residual CAP pool that is recruited to MASs in a Vinculin-independent manner is apparently sufficient for partial CAP function. Our assessment of CAP and Vinculin function at the larval MAS shows that these proteins are required for maintaining the integrity of junctional membranes in the face of tensile forces. CAP proteins may serve as scaffolding proteins at
membrane-cytoskeleton interfaces and facilitate the assembly of protein complexes involved in cytoskeletal regulation and membrane turnover (Fig. 9E).

Mutations in the CAP-binding protein filamin cause myofibrillar myopathy (Vorgard et al., 2005). This, in combination with our data showing a crucial role for CAP in regulation of muscle morphology, sets the stage for investigating how loss of CAP protein function might influence the etiology of myopathies.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References


Fig. S1. Embryonic and larval expression of CAP protein. (A) Stage 16 embryos were immunostained with anti-CAP and anti-MHC. CAP is present at scolopale cells (thick arrows) and muscle attachment sites (MASs) of longitudinal muscles (thin arrows) but is missing from lateral transverse muscles (arrowheads). (B) Expression pattern of CAP-GFP in the GFP-CAP trap line CAPC06924, which harbors a GFP trap in a CAP intron. CAP-GFP localizes to scolopale cells (arrowheads) and MASs (arrow) in stage 16-17 embryos. (C) Wild-type and GFP-CAP trap larval fillets were stained with indicated antibodies. CAP colocalizes at Z-disks with Zasp. Scale bars: 5 μm in A; 10 μm in B; 60 μm in C.
Fig. S2. Cap cells are unaffected in dCAP49e mutants. (A-B") Fillet preparations from GFP-CAP trap animals were stained with GFP and 85e-tubulin antibodies. 85e-tubulin antibody labels cap, cap attachment cells (arrows in A’ and A”), ligament cells and ligament attachment cells (arrows in B’ and B”). CAP-GFP is enriched at the junction of cap and cap-attachment cells (arrowheads in A) and in scolopale cells (arrowhead in B). (C-D") Wild-type and dCAP49e larval fillet preparations were stained with βPS integrin (arrowheads) and 85e-tubulin antibody (arrows). βPS integrin localization in cap cells is unaltered in CAP mutants. Scale bars: 40 μm in A-B”; 20 μm in C-D".
Fig. S3. Morphological analysis of Johnston’s organs in dCAP^{49e} mutants. (A) Sections of Johnston’s organ from wild-type and dCAP^{49e} adult flies were stained with phalloidin, MAb 21A6 and HRP (which labels neurons). No defects were observed in the 21A6 staining in CAP mutants. (B) TEM images of wild-type and dCAP^{49e} Johnston’s organ sections, showing neurons with abnormal vacuolar structure (arrows) in dCAP^{49e} mutants. Scale bars: 30 μm in A; 1 μm in B.
Fig. S4. Morphological analysis of MASs in dCAP^{49e} mutants. (A) Lateral transverse muscles of wild-type and dCAP^{49e} mutant larvae were immunostained with phalloidin and βPS integrin antibody. (B) Muscle attachment sites of dCAP^{49e} larvae often show an aberrant morphology (arrows), with myofilaments organized in a perpendicular orientation relative to wild-type animals and disorganization of myofilaments near the sarcolemma (arrowheads). Middle panel shows magnified image of inset. Scale bars: 20 μm in A; 60 μm in B.
Fig. S5. Tiggrin and Thrombospondin show altered distribution in $dCAP^{49e}$ mutants. (A) Wild-type and $dCAP^{49e}$ larvae were stained with anti-Tiggrin and phalloidin. (B) Wild-type and $dCAP^{49e}$ larvae were stained with anti-thrombospondin and phalloidin. Scale bars: 30 μm.
**Fig. S6. Co-expression of dCAP-C suppresses dCAP-E GOF defects.** *Mef2-Gal4* was used to overexpress dCAP isoforms in muscles. (A) dCAP-E and dCAP-C were co-expressed in muscles. (B) Only the dCAP-E isoform was expressed in muscles. (C) dCAP-E was overexpressed in a *dCAP<sup>49e</sup>* heterozygous background. Scale bars: 30 μm in A-C.
Fig. S7. **MAS and audition analysis in animals lacking CAP-binding proteins.** (A) Muscle attachment sites of larval fillets from indicated genotypes were labeled with phalloidin and CAP antibody. No abnormalities were detected in tensin- and flotillin-null larvae, or in larvae expressing cheerio (filamin) or paxillin RNAi under the Mef2-Gal4 driver. Scale bars: 30 μm. (B) Sound-evoked potentials recorded from the auditory nerves of wild type and vinculin-null flies (n=10 animals).