Myosin VI Stabilizes an Actin Network during *Drosophila* Spermatid Individualization[□] [▽]

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Here, we demonstrate a new function of myosin VI using observations of *Drosophila* spermatid individualization in vivo. We find that myosin VI stabilizes a branched actin network in actin structures (cones) that mediate the separation of the syncytial spermatids. In a myosin VI mutant, the cones do not accumulate F-actin during cone movement, whereas overexpression of myosin VI leads to bigger cones with more F-actin. Myosin subfragment 1-fragment decoration demonstrated that the actin cone is made up of two regions: a dense meshwork at the front and parallel bundles at the rear. The majority of the actin filaments were oriented with their pointed ends facing in the direction of cone movement. Our data also demonstrate that myosin VI binds to the cone front using its motor domain. Fluorescence recovery after photobleach experiments using green fluorescent protein-myosin VI revealed that myosin VI remains bound to F-actin for minutes, suggesting its role is tethering, rather than transporting cargo. We hypothesize that myosin VI protects the actin cone structure either by cross-linking actin filaments or anchoring regulatory molecules at the cone front. These observations uncover a novel mechanism mediated by myosin VI for stabilizing long-lived actin structures in cells.

INTRODUCTION

Myosin VI is unique among the 18 classes of the myosin superfamily of molecular motors, because it moves along actin in vitro in a direction opposite all other myosins (Cheney and Mooseker, 1992; Mermall *et al.*, 1998; Berg *et al.*, 2001). Genes encoding myosin VI are present in many animals, and myosin VI mutants have phenotypes that suggest a variety of different roles in diverse tissues (Kellerman and Miller, 1992; Hasson and Mooseker, 1994; Buss *et al.*, 1998; Kelleher *et al.*, 2000; Avraham *et al.*, 1995; Seiler *et al.*, 2004). Myosin VI has an ATP-dependent motor domain, a coiled-coil domain that may mediate dimerization, and a predicted globular tail domain that mediates association with membrane vesicles in vivo. Motility properties revealed using in vitro motility assays, the identity of interacting proteins, and phenotypic analysis of myosin VI mutants in various species suggest two speculative molecular functions of myosin VI.

The first role suggested for myosin VI is a motor for cargo transport in the endocytosis pathway. This idea is supported by myosin VI's ability to bind to clathrin-coated pits and proteins associated with compartments of the endosome pathway (SAP97 and DAB2) and localization on endosome vesicles in cultured mammalian cells and neurons in mouse brain (Buss *et al.*, 2001; Aschenbrenner *et al.*, 2003; Dance *et al.*, 2004; Osterweil

et al., 2005). Immunofluorescence localization and time-lapse observation of green fluorescent protein (GFP)-myosin VI-labeled endosome vesicles in cultured mammalian cell shows this association is transient, dynamic, and required for efficient endocytosis (Buss *et al.*, 2001; Aschenbrenner *et al.*, 2003). In addition, when nonnative coiled-coil sequences from yeast GCN4 are inserted into the tail region of myosin VI to induce dimerization, motility assays have demonstrated that myosin VI moves processively with a large step size (up to ~30 nm). Recently, it has been shown that by binding to actin filaments, full-length myosin VI can form dimers and move with processive steps in vitro (Park *et al.*, 2006). These findings support the hypothesis that myosin VI's primary function is as a cargo transporter (Inoue *et al.*, 2002; Morris *et al.*, 2002; Wu *et al.*, 2002).

Another suggested function for myosin VI is a molecular cross-linker for tethering cytoplasmic organelles or components of the actin cytoskeleton. This idea is supported by a number of in vitro observations. First, the majority of myosin VI takes a monomeric form when it is expressed in a baculovirus expression system and in cultured mammalian cells (Lister *et al.*, 2004). In motility assays in vitro, monomeric myosin VI is a nonprocessive motor, making it less likely to play a cargo transport role. It has also been suggested that the predicted coiled-coil sequence of myosin VI could be an α -helix incapable of mediating dimerization (Knight *et al.*, 2005). It remains unclear whether myosin VI works as monomer or dimer in any specific process. Second, even when myosin VI is forced to be a dimer and becomes a processive motor in vitro, some of myosin VI's properties do not suggest a role in cargo transport. Myosin VI takes only a few processive steps (Wells *et al.*, 1999; Rock *et al.*, 2001; Nishikawa *et al.*, 2002) with a transport distance of <200 nm (only a tiny portion of a cell). In addition, myosin VI's stepping in vitro is stalled by backward force. In this situation, myosin

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Abbreviations used: Arp, actin-related protein; *Jar*, *jaguar*.

VI's heads remain tightly bound to actin for long intervals (minutes) (Altman *et al.*, 2004). Thus, by binding to another structure, myosin VI could serve as a structural cross-linker or anchor for particles/vesicles on an actin network.

Analysis of myosin VI mutant phenotypes supports a structural role for myosin VI. In cultured fibroblasts from myosin VI mutant mice (Snell's waltzer mice), the Golgi apparatus is retracted to the periphery of the nuclei (Warner *et al.*, 2003). It seems that the role of myosin VI is to tether the Golgi and resist the inward force exerted by dynein. Moreover, in human, mice and zebrafish, mutations in myosin VI lead to defects in stereocilia formation in the inner ear mechanosensory organ (Self *et al.*, 1999; Kappler *et al.*, 2004; Seiler *et al.*, 2004). Myosin VI localizes to a dense actin network at the base of cilia called the cuticular plate, which is thought to structurally support the stereocilia. In the absence of myosin VI, the cilia fuse together, suggesting that the structural integrity of the cuticular plate is affected.

Although we know a great deal about the mechanisms by which myosin VI is targeted to membrane trafficking pathways, we know little about the motor kinetics of myosin VI *in vivo*. To understand the *in vivo* function of myosin VI and to determine which properties measured *in vitro* are important *in vivo*, direct observation of the motor function of myosin VI in cells is important. Such observations could distinguish between different models for function. An ideal system for performing such studies would be one in which 1) myosin VI functions on an actin structure with clearly defined actin filament orientation and organization, and 2) its localization is solely dependent on its motor domain. Here, we report the development of such a system that is perfectly suited for examining these questions.

Myosin VI is required for the final step of *Drosophila* spermatogenesis, individualization (Hicks *et al.*, 1999). During individualization 64 syncytial spermatids are segregated into individual cells (Supplemental Data 1) (Tokuyasu *et al.*, 1972; Fabrizio *et al.*, 1998; Noguchi and Miller, 2003). To accomplish this, 64 actin cones move synchronously from the sperm nuclei to the ends of the tails. As the actin cones move, the cytoplasm and most organelles are pushed out of the flagella, and the cell membrane is reorganized and attached to the axoneme. The cytoplasm and organelles pushed out by the actin cones accumulate in the cystic bulge and finally are discarded as the waste bag when the cones reach the end of the cyst. In *Drosophila* myosin VI mutants in which the expression of myosin VI is greatly reduced in the testis, actin cone organization is disrupted and individualization stops prematurely (Hicks *et al.*, 1999). In wild-type testes, myosin VI localizes at the fronts of actin cones. Actin-related protein (Arp)2/3 complex and its activator, cortactin also localize to the front of actin cones and their localizations are disrupted in myosin VI mutants (Rogat and Miller, 2002). These findings led us to propose a structural role for myosin VI in *Drosophila* spermatogenesis. Previously, we demonstrated that the whole process of individualization can be observed in culture (Noguchi and Miller, 2003). Here, we have carried out a detailed analysis of myosin VI function using this *in vitro* culture system. In this report, three issues were addressed: 1) Definitive determination of the stage at which myosin VI mutant defect is manifest and the precise nature of those defects using *in vitro* culture and electron microscopy (EM). We show that the defect is specifically in actin cone growth during individualization. 2) Actin cone structure was examined using EM methods that revealed the organization and orientations of actin filaments. 3) Myosin VI motor kinetics on actin *in vivo* were examined using fluorescence recovery after photobleach

(FRAP) of GFP-myosin VI, showing that myosin VI remains bound to actin for long periods.

MATERIALS AND METHODS

Fly Husbandry

Stocks were grown in ordinary cornmeal food and kept at room temperature. A *Drosophila* deficiency stock that lacks the chromosomal region that includes the myosin VI gene (*Df s-87.5*) was obtained from the Bloomington Stock Center (Bloomington, IN). Myosin VI overexpression line was described previously (Hicks *et al.*, 1999).

Primary Culture of Isolated Spermatogenic Cysts

Primary culture of spermatogenic cysts during individualization was described previously (Cross and Shellenbarger, 1979; Noguchi and Miller, 2003). Time-lapse movies were recorded using an inverted microscope (Diaphot; Nikon, Tokyo, Japan) with 10 or 20× ePlan lens with differential interference contrast (numerical aperture [N.A.] = 0.25; Nikon) equipped with a SPOT charge coupled device camera RT Slider (Diagnostic Instruments, Sterling Heights, MI) driven by SPOT software, version 3.5.9. Movies were made using ImageJ software (<http://rsb.info.nih.gov/ij/>).

Electron Microscopy

For cross-sections of spermatogenic cysts, testes were dissected from adult male flies and fixed with 1.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.0, for 2 h on ice. The specimens were then washed twice with 0.1 M PBS, pH 7.0, and postfixed in 1% OsO₄ for 2 h at 4°C. Samples were dehydrated in graded concentrations of ethanol up to 100%. After washing twice in propylene oxide, the specimens were embedded in Poly/Bed 812 resin (Polysciences, Warrington, PA). Sections (60–70 nm) were cut using a SuperNova Reichert-Jung ultramicrotome and stained with 2.5% uranyl acetate and 0.4% lead citrate solutions. Sections were examined using a Hitachi H-600 transmission electron microscope.

For longitudinal sections of actin cones in cystic bulges, individualizing cysts were firmly attached to a piece of glass slide coated with poly-L-lysine for 5 min. This process is important for keeping cystic bulges flattened in one plane for later sectioning. The cysts were fixed with 2% glutaraldehyde in PBS for 2–3 h on ice and then fixed with 1% OsO₄ for 90 min and processed as described above. After polymerization of resin, the glass fragment was ripped off, leaving the sample on the top surface of the resin block. Then, longitudinal sections were cut as described above.

Quantitation of F-Actin in Actin Cones

Actin cones of isolated cysts were stained with Alexa488-phalloidin (Invitrogen, Carlsbad, CA) using the method described previously (Noguchi and Miller, 2003), and examined using a 40× HCX Plan-Apo lens (N.A. = 1.25; Leica, Jena, Germany) on a laser confocal microscope (TCS SP2 attached to DM IRB inverted microscope; Leica). In each experiment, all samples were processed under exactly the same conditions, and all images were recorded with the same exposure settings. To obtain longitudinal optical sections through the middle of the actin cone, focus was adjusted to the position of the axoneme, which runs through the center of the cone. Because the actin cone grows as individualization progresses, the individualizing cysts were divided into four categories: before movement (colocalizing with sperm nuclei), early (0–1/3 of cyst length), middle (1/3–2/3), and late (2/3–3/3), based on the position of actin cones along the cyst. Fluorescence intensity was measured according to each category. However, because the myosin VI mutant cones do not often move more than half the length of the cyst, actin cones located in first half of the cyst were chosen for data acquisition in comparison between wild-type and myosin VI mutants. Fluorescence intensities were analyzed using NIH Image 1.62 (<http://rsb.info.nih.gov/ni-image/>), and data were processed using Microsoft Excel (Microsoft, Redmond, WA). See the legend for Figure 3 for data analysis.

Myosin Subfragment 1 (S1) Fragment Decoration

Purification of rabbit skeletal myosin II and preparation of S1 subfragment were carried out using conventional methods (Margossian and Lowey, 1982). For myosin II S1 fragment decoration, isolated individualizing cysts were permeabilized with 0.1% saponin and 20 μM phalloidin in extraction buffer (50 mM KCl, 50 mM HEPES, pH 7.0, 5 mM EGTA, and 5 mM MgCl₂) for 20 min. Under these conditions, actin cone structure was not disrupted, as judged by Alexa568-phalloidin staining at the light microscope level. After three washes with extraction buffer containing 2.5 μM phalloidin, cysts were treated with 4 mg/ml S1 fragment in extraction buffer for 45 min at room temperature. Then, cysts were washed several times in extraction buffer. S1 decorated cysts were fixed with 1% glutaraldehyde and 0.2% tannic acid in 0.1 M sodium phosphate buffer, pH 6.8, for 30 min at room temperature, followed by several washes in the same buffer. The fixed cysts were stuck on a small piece of plastic sheet (Thermanox; Electron Microscopy Science, Hatfield, PA)

by pushing at both sides of the cystic bulge with a thin glass needle. Then, the cysts were covered with a drop of 0.5% agarose and fixed with 2% OsO₄ in 0.1 M sodium phosphate buffer, pH 6.0, for 2 h at room temperature. After rinsing with Milli-Q-filtered water, samples were dehydrated in a graded series of ethanol and embedded in Poly/Bed 812 resin. Ultrathin sections (40–60 nm) were cut, stained, and examined as described above.

Expression of Myosin VI in Testis

Overexpression of myosin VI was accomplished using a heat shock protein 83 (hsp83)-promoter-driven transgene that expresses myosin VI (Hicks *et al.*, 1999). Myosin VI is highly and constitutively expressed during individualization from this transgene.

For experiments using GFP-myosin VI, GFP was inserted in frame at the N terminus of full-length myosin VI. The GFP-myosin VI transgene was constructed as follows. GFP sequences were amplified from pRSETB (gift from M. Chalfie, Columbia University, New York City, NY) using the primers 5'-cgggatc-catgttctaaggagaaga-3' (forward) and 5'-gaattctgcagcggctttagttatc-3' (reverse), adding BamHI and NotI sites at the 5' end, changing the GFP stop codon, and adding an EcoRI site at the 3' end. pNB15 is a previously isolated full-length myosin VI cDNA (Kellerman and Miller, 1992). The 5' end of myosin VI was amplified from a full-length cDNA, pNB-B15, using the primers 5'-cggaattcattgtggaggacac-3' (forward) and 5'-gcgatcgaatagtcgactgtagat-3' (reverse). The product was digested with EcoRI and SalI. This generated the 5' fragment of myosin VI. The remainder of the open reading frame of myosin VI was obtained by digestion of pNB-B15 with SalI and NotI. The GFP (BamHI-EcoRI), 5' myosin VI (EcoRI-SalI) and 3' myosin VI (SalI-NotI) fragments were assembled in an intermediate vector. The GFP-myosin VI fusion protein was cloned into Casper hsp83 promoter vector (provided by Paul Shedl, Princeton University, Princeton, NJ) using the NotI sites 5' to GFP and 3' to myosin VI open reading frame. Transformed fly lines were established by conventional methods. The truncated version of myosin VI (GFP-myosin VI-Globular-tail) was constructed by PCR amplifying the sequences encoding the predicted C-terminal globular tail domain (fragment containing amino acids 1045–1253) in pNB15 using the primer 5'-cggaattctgtatcagatccgaa (forward) and a reverse primer in the pNB40 vector outside the cDNA. This strategy added an EcoRI site at the 5' end of myosin VI fragment in frame to GFP (see above). The introduced EcoRI site and a NotI site in the pNB40 vector were used to isolate the myosin VI Globular-tail fragment. The GFP and myosin VI mini-tail fragment were assembled in an intermediate vector, cloned into Casper-hsp83, and transformed fly lines isolated as described above.

Protein expression level was examined by Western blot analysis using anti-*Drosophila* myosin VI monoclonal antibody (mAb) (3c7) (Mermall and Miller, 1995) or anti-GFP antibody (Clontech, Mountain View, CA). For loading control, Western blots using anti- α -tubulin mAb (DM1-A) were performed. Forty testes were dissected for each sample. Testes were washed in PBS and homogenized in 100 μ l of homogenization buffer (2 mM Tris-HCl, pH 7.0, 10 μ M leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotted using conventional methods.

ATP Extraction of GFP-Myosin VI

GFP-myosin VI was expressed in male germ line cells of myosin VI mutant (*jar¹*). Adult testes from [*w*; *hsp83-GFP-myosin VI*+/+; *jar¹*/Df-S87.5] were dissected, and cysts were isolated as described above. Cysts were washed with PBS and then incubated for 20 min in permeabilization buffer (50 mM KCl, 10 mM imidazole, pH 7.0, 2.5 mM EGTA, and 4 mM MgCl₂) containing 0.1% Triton X-100 and Alexa568-phalloidin (Invitrogen). After 5-min incubation in permeabilization buffer containing 5 mM ATP or 5 mM ADP, cysts were fixed in 4% paraformaldehyde in PBS, pH 7.0, for 10 min and then washed in PBS. Samples were examined by laser confocal microscopy as described above (Leica).

Fluorescence Recovery after Photobleach

GFP-myosin VI was expressed in male germ line cells as described above. FRAP was carried out as described previously (Noguchi and Miller, 2003) using a laser confocal microscope with a 40 $\times$ lens. Relative fluorescence intensity = (fluorescence intensity of GFP-myosin VI localized on an actin cone – background in cytosol)/fluorescence intensity of GFP-myosin VI on the cone before bleach. Intensity was measured in an area defined to include the region of high-intensity GFP-myosin VI fluorescence (Figure 7). The relative fluorescence intensity of GFP-myosin VI localizing on a cone was plotted against time after bleaching, and the period of linear recovery was determined. Photobleaching due to serial scanning was detected by measuring the decay of fluorescence of GFP-myosin VI on unbleached cones and this amount of fluorescence was added at each time point. The images were analyzed using NIH Image software. The average turnover rate (relative fluorescence intensity recovered/min) was calculated using Microsoft Excel.

RESULTS

Analysis of the Individualization Defect in Myosin VI Mutants

In previous reports, we showed that the actin cone complex is disorganized in myosin VI mutants and individualization stops in the middle of the cyst. The arrested actin cones also have less F-actin in the mutant (Hicks *et al.*, 1999; Rogat and Miller 2002). However, from this work, it was unclear how the defects arose and the exact role of myosin VI in individualization that led to the terminal phenotype we previously observed in fixed preparations. To precisely define the step at which myosin VI acts and to determine whether and how different aspects of individualization were affected, we isolated and cultured individualizing cysts from myosin VI mutants and compared them with wild-type cysts (Figure 1). Fully elongated cysts before individualization from the mutant and wild type looked identical in length and morphology. Individualization seemed to start normally in both genotypes (see also below), but differences between the mutant and wild type were soon apparent. In wild type, the cystic bulge had a round shape. However, in myosin VI mutants (*jar¹/jar¹*), the cystic bulge looked thinner and elongated, suggesting that cytoplasm was not effectively pushed out in front of the actin cones. In cases where the somatic cyst cells that normally enclose the cyst were removed by mechanical shear force during isolation, the individualized part of the sperm tails became apparent. In wild type, individualization occurred simultaneously for all 64 spermatids at the basal side of the cystic bulge. In myosin VI mutant cysts, some sperm tails were individualized, but others were grouped within the same membrane (Figure 1A).

We traced cystic bulge movement to determine if cystic bulge movement was altered in myosin VI mutants. Cystic bulge movement was quite constant along the whole length of the cyst in wild type. In the myosin VI mutant, the bulge moved with normal speed in the initial stages, but movement became irregular and slowed down after a short period of time (Figure 1B and Supplemental Movies 1 and 2). The distance that bulges traveled varied among cysts, ranging from a few hundred micrometers to nearly the full length of the cyst in rare cases (1.8 mm). The bulges became less distinct as they slowed down. Therefore, the exact stopping point is highly variable and difficult to precisely measure. However, cystic bulges typically begin to move abnormally after they reach approximately one-quarter of the way along the cyst. Thus, to assess the defect somewhat quantitatively, we measured the speed of cystic bulge movement during the interval when the cystic bulge was between one-quarter and one-half of the distance along the cyst during a time window of 40–50 min. The average speed was significantly less in the mutant (wild type: 2.7 ± 0.5 μ m/min, $n = 14$; *jar¹/jar¹*: 1.1 ± 0.6 μ m/min, $n = 11$). Because the bulge starts moving normally and sometimes proceeds a long distance before stopping, it is not likely that myosin VI is generating the force of movement directly. Rather, myosin VI is likely to play an indirect role in cone movement. It is also likely that defects arise only after cone movement begins (also see below).

One possible cause of problems with cystic bulge movement could be improperly formed axonemes or other defects before individualization, which would then impede cone movement or affect individualization indirectly. To look for defects in axoneme formation and mitochondria differentiation, which were not apparent at the light microscope level, EM cross-sections of cysts before and in the process of individualization were examined in both wild-type and myosin VI mutants. After elongation but before individualization began, a significant quantity of cytoplasm surrounded the 64 axoneme

Figure 1. Individualization defects of myosin VI mutant cyst. (A) Cystic bulges (small arrows) of individualizing cysts from wild type and myosin VI mutant (*jar¹/jar¹*) male. In a cyst with somatic cyst cells intact, a wild-type cystic bulge had an egg-like shape, whereas the mutant bulge was stretched out and thinner. The individualized sperm tails (small arrowheads) were apparent protruding from the cystic bulge of a cyst lacking somatic cyst cells. In a mutant cyst lacking cyst cells, both large fused sperm tails (large arrowhead) and individualized thin tails (small arrowheads) were seen. Large arrow indicates direction of movement. Bar, 50 μm . (B) Representative plots of cystic bulge movement in wild type (●) and myosin VI mutant (○). The distance of the cystic bulge from the basal end of the cyst was plotted as a function of time during the whole individualization process. Measurements were made at 12 min intervals. (C–F) EM analyses of cross-sections of cysts after individualization. (C) Each wild-type cyst contained 64 pairs of axonemes (arrows) and mitochondria derivatives (arrowheads) enclosed by plasma membrane. (D) Myosin VI mutant cyst contained some individualized (arrows) regions, but some groups of sperm tails are not separated by plasma membrane. (E and F) Sperm tails of wild-type (E) and myosin VI mutant (F) at higher magnification. Both axoneme and major mitochondrial derivative structure look normal, but some cytoplasm remained surrounding the axoneme even in cases where individualization had occurred in myosin VI mutant (white arrow) cysts. Black arrow, axoneme; large white arrowhead, major mitochondria; small white arrowhead, minor mitochondria derivative. Bar, 1 μm (D) and 0.2 μm (F).

and mitochondria derivative pairs (Tokuyasu *et al.*, 1972), and both genotypes looked identical (our unpublished data). Differences were apparent between wild-type and mutants after individualization. In wild type, very little cytoplasm was left, and each axoneme/mitochondria pair was tightly surrounded by plasma membrane (Figure 1, C and E). In myosin VI mutants, individualization had not occurred in some of the axoneme/mitochondria pairs. In some places, several sperm tails were enclosed within the same membrane and surrounded by a large amount of cytoplasm (dark gray area in right side of Figure 1D). Even in individualized sperm tails, a larger than normal amount of cytoplasm remained (Figure 1F, top right). However, axoneme structure and mitochondrial derivative morphology was normal in the mutants (Figure 1, E and F), suggesting that the defects are likely to be restricted to individualization.

We did observe defects in the actin cones themselves in longitudinal sections. In wild type, the region of dense actin that comprises the cones was wide at the front and tapered

off at the rear (Figure 2A). In myosin VI mutants, the severity of the defect was variable, but all cones were smaller and narrower with a decreased diameter at the front (Figure 2, B–D). Fibrous staining is more visible in wild type than myosin VI mutant, suggesting that the mutant cones contained fewer filaments and the filaments were less densely packed. At higher magnification, unlike wild type, cytoplasmic organelles were present in the cone region (Figure 2, E and F). This supports the idea that the mutant actin cones are not effective in pushing out the cytoplasm and organelles. Even when they progress down the cyst and remodel the membrane properly, components that are normally excluded from mature sperm are still present.

F-Actin Amount in the Actin Cone Is Affected by the Amount of Myosin VI

To examine whether myosin VI directly affects the assembly of F-actin in actin cones, we created conditions of significant loss or increase of myosin VI in individualizing cysts. We

Figure 2. EM micrographs of actin cones. Longitudinal sections of actin cones in wild type (A and E) and myosin VI mutant (B–D and F). ax, axoneme; mi, mitochondrial derivative. The front of the membrane surrounding the actin cones is indicated by small arrows. Actin cones were surrounded by a smooth membrane with no obvious vesicles or invaginations. Actin cones of the myosin VI mutant had defects of variable severity (B–D). In all cases, the cones were narrower than wild type. (E and F) Actin cone sections at higher magnification. In a wild-type actin cone, cytoplasmic organelles were excluded from the cone area. In mutant actin cones, there were a number of organelles in the cone area (small arrows). The membrane surrounding actin cones are indicated by large arrowheads. Bar, 0.5 μ m (A) and 0.2 μ m (E).

began by comparing the morphology and quantitated the F-actin content of cones in wild-type and a myosin VI mutant. Before the onset of individualization, the appearance of actin cones in wild-type and myosin VI mutant was identical at the light microscopic level. After individualization began, each cone grew wider as movement progressed, and all cones progressed in register in wild type. In the mutant after movement began, however, F-actin staining was fainter, and the actin cone complex was disorganized (Hicks *et al.*, 1999) (Figure 3A). Width and length were variable and uncoordinated among cones in the same cystic bulge (Figure 3B). The total amount of F-actin in each actin cone and the density of F-actin in the cone were quantitated by measuring fluorescence intensity of Alexa488-phalloidin. Before the onset of individualization, the quantity of F-actin in cones was not significantly different between wild type and the mutant. After individualization began, the density remained constant and the total amount of F-actin increased about twofold in wild type, suggesting that actin cones assemble more F-actin as they move. In the mutant, both total amount and the density of actin declined to about half of their pre-movement level once movement began (Figure 3, C and D).

In the myosin VI overexpression line (Figure 3E), actin cones were similarly shaped as wild-type, and they initially had similar actin content. However, after cone movement began, the cones grew wider and fluorescence intensity increased, compared with wild type (Figure 3, F–H). In wild type, the average amount of actin in cones increased twofold over the course of individualization. However, it increased fourfold in the myosin VI overexpression line. Therefore, the accumulation of F-actin in the cone is correlated with the amount of myosin VI present during individualization process.

Myosin VI might affect the amount of F-actin in cones by altering actin polymerization/depolymerization dynamics. To measure actin dynamics (turnover rate), we performed FRAP experiments on GFP-actin expressed in wild type and myosin VI mutant testes (Supplemental Data 2). Absence of myosin VI did not affect the turnover rate significantly, despite the major disruption of the morphology of actin cones. Thus, we instead favor the hypothesis that myosin VI affects F-actin level by allowing more filaments to accumulate, rather than slowing turn over rate of each filament.

There Are Two Structural Domains in the Actin Cone

To better understand how myosin VI can allow more actin filaments to accumulate in the cone, we asked two questions, comparing mutant and wild-type cones. 1) What is the general organization of actin in the cone? 2) How are the actin filaments oriented in the cone structure? F-actin was visualized at the EM level by decorating with rabbit skeletal muscle myosin II S1. This technique permitted us to better resolve the filament organization and to determine the orientation of the barbed ends of the filaments relative to the overall structure (Figure 4, A–E). Two distinct structural domains, a front meshwork and a rear area of parallel bundles were observed. In wild type, the front meshwork was densely packed with actin filaments oriented at random angles. This structure is very likely built by Arp2/3 actin branching activity (Rogat and Miller, 2002). The rear region was composed of long bundles of filaments that lie parallel to the longitudinal axis of the cone (Figure 4, A and B). In the myosin VI mutant, the most actin cones were narrower and were composed of significantly smaller numbers of filaments, consistent with measurements of actin amount and density by fluorescence. However, the cones had the same basic organization as was seen in wild type (Figure 4, C–E). These data suggest that myosin VI is not essential for forming either the front meshwork or rear bundles, but it has a supportive role in actin cone formation.

The polarities of individual actin filaments were classified relative to cone movement direction (Figure 4A, large white arrow) according to the criteria diagrammed in Figure 5A, as backward (barbed end directed toward area α), perpendicular (toward area β), or forward (toward area γ). The filaments that were classified as perpendicular were further subdivided into those with barbed ends facing the inside (axoneme) or the outside (membrane) of the cone. In the front meshwork, the majority of fast-growing (barbed) ends faced backward (Figures 4F and 5). The actin filaments comprising parallel rear bundles had a very clear polarity, with their barbed ends facing backward (Figure 4, G and H). Based on other motile actin structures such as the *Listeria* actin comet tail, we would have expected that the barbed ends would face in the direction of movement. However,

Figure 3. Protein level of myosin VI affects amount of F-actin in the cone. (A) F-actin staining of actin cones in wild type and myosin VI (*jar¹/jar¹*) mutant. Before actin cone movement began, there was no difference in the appearance of the actin cones between the two genotypes. After the onset of movement, actin cones in wild-type cysts grew larger, whereas in myosin VI mutant cysts, F-actin staining was weaker, and cones were not aligned in the middle of the cystic bulge. (B) Representative actin cone morphology in wild type and myosin VI mutant at higher magnification. Cone shapes were variable in the mutant (fluorescence intensity is raised in these images to show morphology). (C) Relative amount of F-actin in actin cones = (total fluorescence intensity in one actin cone – background intensity in an area of the same size in the cytoplasm)/total fluorescence intensity of one wild-type actin cone before movement. In total, 28–40 actin cones from different cystic bulges from three independent experiments were measured. (D) Relative density of F-actin = (average fluorescence intensity in an actin cone – average intensity of the background in cytoplasm of cystic bulge)/average fluorescence intensity in a wild-type actin cone before movement. Twenty-five to 30 actin cones from different cystic bulges were examined in three independent experiments. (E–H) Effect of overexpression (O/X) of myosin VI on actin cone formation. (E) Western blot analysis of protein expression level in O/X line, using anti-myosin VI antibody and anti- α -tubulin antibody for the control. Signal density, O/X line:wild type = 16:1. (F) F-actin staining of actin cone both in O/P line and wild type at middle stage. (G) Relative amount of F-actin in actin cone (see above). Numbers indicated above the bars are the number of cone examined in the test. (H) Relative density of F-actin in the cone in different stages of individualization (see above). Bar, 5 μ m (A), 2 μ m (B), and 5 μ m (F).

Figure 4. Ultrastructure of S1 decorated actin cones. (A and B) Wild-type actin cones had two structural domains: the front domain was composed of a dense actin filament meshwork and the rear domain was composed of actin bundles parallel to the longitudinal axis of the cone; ax, axoneme; mi, mitochondria; Large arrow in A indicates the direction of movement. (C–E) Disrupted actin cones in myosin VI mutant. In all cases, there were significantly less F-actin in mutant cones. In each mutant cone, both the front and the rear domains were present. (F–J) High-magnification images of the rear and front domain of wild type (F–H) and myosin VI mutant (I and J). Small arrows indicate direction of the pointed ends of each actin filament. (F and I) The front domains were composed of F-actin oriented at random angles. (G, H, and J) The rear domain was composed of parallel-bundled F-actin with barbed ends facing backward. Bar, 0.5 μm (A–E) and 0.1 μm (F–J).

quantitation revealed that only 10% of actin filaments had barbed ends directed forward, whereas the majority of the filaments had barbed ends directed backward (Figure 5B). This orientation also means barbed ends primarily face the membrane that surrounds the rear of the actin cone. In addition, among the perpendicular filaments, the majority of the barbed ends faced outward, toward the membrane surrounding the actin cone. Overall, ~85% of the total filaments had their barbed ends oriented toward the membrane. Interestingly, the front edge of actin cone is dominated by pointed ends, which is consistent with the fact that myosin VI, a pointed end-directed motor, concentrates to the front edge of the cone. We saw no difference in actin filament orientation between wild type and myosin VI mutant (Figures 4, I and J, and 5C), indicating that myosin VI does not play a role in actin filament orientation.

Localization of Myosin VI Depends on Its Motor (Head) Domain

It has been demonstrated that the globular tail is sufficient for the localization of myosin VI to endosomes in mammalian cells (Buss *et al.*, 2001; Aschenbrenner *et al.*, 2003; Dance *et al.*, 2004). We wondered whether the same would hold true in this situation, where myosin VI is not associated with membrane. To address this question, we asked whether myosin VI binding to the actin cone required its head or tail. First, we expressed GFP-myosin VI full-length (GFP-FL) and GFP-myosin VI globular-tail (GFP-G-tail) in testis. G-tail fragment (a.a. 1045–1253) starts at the beginning of predicted globular tail region right after coiled-coil region and ends at the C terminus of the protein. GFP-FL was expressed at a level comparable with endogenous myosin VI in testis,

Figure 5. Quantitation of the orientation of actin filaments in wild type and myosin VI mutant cones. (A) Schematic diagrams show actin cone structure and the criteria used to count the number of actin filaments with different orientations. The right circle is a blow up of a part of the actin cone. Large black arrow indicates longitudinal axis of the actin cone pointing in the direction of cone movement. Along this axis, the area was divided into three sections. In cases where the barbed end was in area α , the filament would be counted as facing backward (opposite the direction of movement) (example: black filament in the circle). Filaments with their barbed ends in area γ were counted as facing forward (in the direction of movement). If the barbed end was in area β , the filament was classified as lying perpendicular to the direction of movement (example: white filament in the circle). The orientation was classified as either barbed end facing the outside edge of the cone where it is surrounded by membrane or the center where the axoneme lies. (B and C) Graphs show orientations of barbed ends categorized according to criteria described above and counted in three different parts of the cone. The number of filaments was counted in wild type (12 cones from 4 cysts) and the mutant (8 cones from 3 cysts). n is number of filaments counted.

whereas the expression level of GFP-G-tail was much lower (Figure 6A; Supplemental Data 3). Quantitation of a Western blot using anti-GFP antibody shows the signal intensity for GFP-G-tail is 54% that of the GFP-FL. However, we suspect this overestimates the amount of GFP-G-tail, because our fluorescence microscopic observations indicate a much lower total amount of fluorescence. We measured the amount of GFP-G-tail as $9.7 \pm 2.9\%$ ($n = 10$) of GFP-FL based on fluorescence intensity. We suspect these measurements differ because the smaller GFP-G-tail (55–60 kDa) is more efficiently transferred to the polyvinylidene difluoride membrane than GFP-FL (180 kDa). However, we think this expression level is high enough to detect GFP localization, if the G-tail does localize specifically. Even at a level of 10% of the GFP-FL, we were able to see GFP-signal in the cysts (see Supplemental Data 3). By comparison, we were easily able to see actin cones in GFP-actin-expressing testes, where the fluorescence was only $7.8 \pm 1.3\%$ ($n = 10$) of the intensity of

GFP-FL (Noguchi and Miller 2003; Supplemental Data 2 and 3). To be sure that the diffuse cytoplasmic signal from unincorporated GFP-myosin VI did not obscure localization to the cones, we fixed the cysts and washed to extract protein that was present diffusely in the cytoplasm. GFP-FL localized properly (Figure 6B), and its expression in the mutant background restored fertility and cystic bulge movement (*hsp83-GFP-myosin VI/+; jar1/Df-S87.5*, speed = $3.1 \pm 0.6 \mu\text{m}/\text{min}$ [$n = 7$], *jar1/Df-S87.5*, speed = $0.7 \pm 1.0 \mu\text{m}/\text{min}$ [$n = 6$]). Therefore, GFP-myosin VI functions properly during individualization. However, GFP-G-tail expressed in wild-type background did not localize to the cone (Figure 6B) and failed to rescue fertility of the mutant (our unpublished data). Even with increased laser power and detection gain settings in GFP-G-tail samples compared with the GFP-FL samples, no enrichment of GFP-G-tail on the cones was observed. At this low level of expression, the GFP-G-tail did not interfere with endogenous myosin VI function, be-

Figure 6. Myosin VI localization is motor dependent. (A) Western blot analysis using anti-myosin VI antibody for GFP-FL (a), and anti-GFP antibody for GFP-G-tail (b). Extract containing the equivalent of two testes was applied to each lane. Genotypes are indicated at the top of each lane. Numbers on the left side indicate the positions of size markers (kilodaltons). As a loading control for each genotype, a Western blot probed with anti- α -tubulin antibody on the same samples is shown. (B) Localization of GFP-FL in myosin VI mutant background and GFP-G-tail in wild type background. GFP-fusion proteins (green), Alexa568-phalloidin staining (red), and merged image are shown. (C) Extraction of GFP-myosin VI from actin cones with ATP. GFP-FL (green) remaining after incubation of permeabilized cysts in the presence of 5 mM ADP. In the presence of 5 mM ATP, GFP-FL was extracted from the actin cone. (D) The fluorescence intensity of remaining GFP on the actin cones was measured. The relative intensity = fluorescence intensity of GFP-FL on an actin cone after extraction with nucleotides/fluorescence intensity before extraction. Bars, 10 μ m (B) and 5 μ m (C).

cause males expressing GFP-G-tail were fertile, and no defects in individualization were observed.

In a second approach to determine which domain of myosin VI was important for localization, we performed an ATP extraction experiment using GFP-FL expressed in the male germ line cells of the myosin VI mutant. If myosin VI's localization is due to the actin binding activity of the motor head, excess ATP should cause GFP-FL to fall off the cone. After permeabilization, GFP-FL disappeared from the actin cone within a few minutes in the presence of excess ATP, whereas adding the same amount of ADP did not affect its localization (Figure 6C). Measurement of fluorescence intensities of GFP-FL on actin cones demonstrated that significantly lower amounts of GFP-FL remained after incubation with ATP ($13 \pm 6\%$ of fluorescence intensity of preincubation, $n = 40$), but not with

ADP ($86 \pm 13\%$ of preincubation, $n = 40$) (Figure 6D). These results suggest that unlike the case of its association with endocytic vesicles, myosin VI localizes to the front of the actin cone using its motor (head) domain.

Dynamics of Myosin VI on Actin Cones

Because myosin VI binds to the actin cones using its motor domain, the dynamics of its binding should directly reflect its motor activity *in vivo* and allow us to determine whether observations of its kinetics *in vitro* are relevant *in vivo*. In motility assays *in vitro*, dimeric myosin VI makes several processive steps before detaching from an actin filament. This behavior would lead to myosin VI remaining bound to actin for on the order of a second (Rock *et al.*, 2001). However, when a backward force is applied to myosin VI walk-

of GFP-myosin VI during recovery. For GFP-myosin VI, turnover rates during the first 80 s after photobleach in moving actin cones were measured. Bar, 10 μ m.

ing on actin *in vitro*, myosin VI stalls and remains bound for several minutes (Altman *et al.*, 2004). If myosin VI takes monomeric form, this nonprocessive motor would fall off of an actin filament in milliseconds unless it is tethered or anchored on actin filaments with its motor domain. We measured the myosin VI turnover rate in moving actin cones using FRAP. The average turnover rate of GFP-myosin VI was $0.25 \pm 0.07/\text{min}$ ($n = 10$). The time required for recovery of half the fluorescence intensity was ~ 2 min (Figure 7). Furthermore, before cone movement, myosin VI turned over even more slowly (Supplemental Data 4). These data demonstrate that myosin VI molecules remain bound to actin for minutes, consistent with the possibility that myosin VI stalls while attached to the filaments. Myosin VI molecule's relatively long dwell time is consistent with a role in structurally stabilizing the actin cone or tethering something to the cone.

DISCUSSION

Myosin VI's Role as an Actin Structure Stabilizer

The data presented here suggest myosin VI's role in *Drosophila* spermatid individualization is to stabilize the branched actin network at the front of the actin cones. Our conclusion is supported by three lines of evidence: 1) The amount of F-actin that accumulates in moving actin cones is correlated to the amount of myosin VI. 2) Initial formation of the cones, before the onset of cone movement, is normal in the mutant. Myosin VI is only required once the branched meshwork begins to grow. 3) Myosin VI remains bound at the cone front for minutes.

The actin cones are composed of two domains, a front meshwork and a rear region of parallel bundles. Even in the most strongly affected cones in the mutant, both meshwork and parallel bundles were present in each cone. Therefore, myosin VI is not absolutely required for generating either of the two structural domains. However, the density of filaments and total area of the cones were both significantly decreased in myosin VI mutant cones. Therefore, we conclude that myosin VI has a supportive role in actin structure formation.

Stabilization of actin structure might explain myosin VI's role in other situations, such as stereocilia formation in inner ear hair cells in vertebrates. In this case, myosin VI localizes to a dense actin network at the bottom of the stereocilia, a structure supporting the cilia (Self *et al.*, 1999; Seiler *et al.*, 2004). Without myosin VI, the stereocilia fuse together, perhaps as a result of the lack of a structural support from this actin meshwork.

Myosin VI Is a Cross-linker or Anchor *In Vivo* not a Cargo Transporter

GFP-G-tail expression and ATP extraction demonstrated that myosin VI binds with its actin binding head domain, rather than its globular tail. In contrast, myosin VI in vertebrate cells associates with compartments of the endocytic pathway and the Golgi using its tail domain (Buss *et al.*, 2001; Aschenbrenner *et al.*, 2003). Because localization of myosin VI to actin cones depends on its actin binding activity, we used FRAP to monitor myosin VI association with actin. These measurements demonstrated that myosin VI remains bound for a long time. This is the first direct observation of myosin VI's motor kinetics *in vivo*. Myosin VI's long dwell time is consistent with the possibility that the myosin VI head stalls in a tightly bound state *in vivo*, as observed *in vitro* (Altman *et al.*, 2004). Myosin VI's slow turnover is also consistent with our hypothesis that it works as a structural cross-linker or anchor *in vivo*.

None of our data support the idea that myosin VI is a cargo transporter during individualization. First, myosin VI at the front edge of actin cone does not translocate along the actin filaments any significant distance, because we see no "flow" of myosin VI in the recovery of fluorescence during FRAP. Second, EM sections of actin cones show no evidence of invaginations or vesicles in the region around the cones (Figure 2). Third, our previous observations of membrane dynamics using membrane dye FM1-43 in live cysts, revealed no endocytosis or exocytosis sites around actin cones (Noguchi and Miller, 2003). Finally, studies of the *shibire* mutant (dynamin) and use of inhibitors showed no requirement for endo- and exocytosis during individualization (Noguchi and Miller, 2003).

Myosin VI also is unlikely to provide force for cone movement. In myosin VI mutants, the cones move with normal speed during the first part of individualization, suggesting that something else is responsible for force production. Actin turnover is required for cone movement, because treatment with inhibitors of both polymerization and depolymerization interfere with movement (Noguchi and Miller, 2003). Whether actin polymerization itself provides the force for movement remains an open question.

Actin Cone Structure Compared with Other Motile Actin Structures

The organization of the actin cone is in some ways reminiscent of other motile actin structures. The cone's triangular shape, the dense meshwork of branched filaments, and the localization of Arp2/3 complex near the front are similar to the actin comet tail of *Listeria monocytogenes* (Gouin *et al.*, 1999; Cameron *et al.*, 2001). The leading edge of the lamelli-

Figure 8. Models for myosin VI's role in cone structure. (A) Schematic drawing of actin cone structure. There are two distinctive structural domains in the cone: the rear domain is composed of long parallel actin bundles that lie along the cone's longitudinal axis, whereas the front domain is composed of meshwork of short actin filaments oriented at random angles. The bulk of the actin filaments have their barbed ends oriented toward the rear or toward the membrane. Therefore, the pointed ends are exposed to cytoplasm at the front edge of the actin cone. Myosin VI localizes at the very front of the actin cone (region indicated by green color), whereas Arp2/3 complex and cortactin localize in a broader region in the front half of the actin cone (region indicated by pink arrows). (B) Models of myosin VI function. Model 1, myosin VI is binding and structurally cross-linking actin filaments in the cone directly. Model 2, myosin VI is binding to some signaling molecule at the G-tail and recruits and tethering it to the front of the cone to stimulate Arp2/3 complex.

podia also is composed of a branched actin meshwork with Arp2/3 complex enriched at the front (Svitkina and Borisy, 1999). However, cones are built using a very different organization (Figure 8A). The filaments in the cone are primarily oriented with their barbed ends away from the direction of movement. In contrast, in *Listeria* comet tails and at the leading edge of motile cells, barbed ends face in the direction of movement. In addition, unlike *Listeria* comet tails and lamellipodia, Arp2/3 complex is not activated by localization of an activator to a membrane or solid surface, because there is no such membrane or structure at the cone front.

Two different actin polymerization mechanisms seem to be important for actin cone formation. Arp2/3-based branching occurs at the front, but the front meshwork is apparently not reorganized into bundles to form the rear region. We see no "flow" of actin from the front meshwork to the rear bundles (Noguchi and Miller, 2003) as would be expected if this were the case. The front meshwork is initially formed just as the cones begin to move on bundles that exist before cone movement (our unpublished data). Formins are more likely candidates to nucleate the formation of the bundles.

Model of Myosin VI Function In Vivo

What is the mechanism by which myosin VI stabilizes the actin meshwork at the front of the cone? Because the actin meshwork grows bigger in cones that contain myosin VI, actin might be predicted to turn over at a slower rate in

cones with myosin VI. However, the filaments turn over with similar rates whether myosin VI is present or not (see Supplemental Data 2). This result indicates that myosin VI does not directly regulate actin dynamics by changing rates of subunit addition or loss at filament ends.

The most direct mechanism for myosin VI to mediate cone growth is by stabilizing actin filaments at the front edge of the cone to prevent their loss through debranching (Figure 8B, model 1). If debranching happens too quickly, the actin cone would lose many filaments from front edge before they were used by the Arp2/3 complex to nucleate new filaments, and the meshwork would not grow. We hypothesize that the growth of the cone is achieved by increasing the number of filaments (which turn over at the same rate). Because normal actin cones approximately triple in actin content by the end of individualization, and filament length does not seem to change, we would expect that the number of filaments approximately triples. At the cone front, this may be particularly important because a filament that debranches may be quickly lost from the meshwork. Filaments turn over with a half-time of ~6 min, whereas cones move for ~600 min. For a cone to remain a constant size with this turnover rate, 50 debranching and branch generation cycles must occur for each initial filament. A threefold increase in number of actin filaments could be achieved by a decrease in debranching rate of only ~2.5% in each cycle. We hypothesize that the supportive role of myosin VI in stabilizing the

branched network at the front edge of the cone, which causes a small change in the rate of actin assembly, has a large impact on over all growth and maintenance of this long-lasting actin structures *in vivo*.

To mediate this branch stabilization, myosin VI might work either as a dimer or monomer. Binding to an actin filament can facilitate dimerization of full-length myosin VI *in vitro* (Park *et al.*, 2006). Myosin VI is highly concentrated at the actin cone front. Myosin VI may form a dimer using the mechanism suggested by Park *et al.* (2006) in this case. A dimer would be able to bridge the branch by holding onto each filament with one head. Myosin VI has a large step size (~36 nm) and a very flexible neck region due to unfolding of the proximal region of the coiled-coil domain (Yildiz *et al.*, 2004; Rock *et al.*, 2005). Such flexibility might allow myosin VI to reach across the Arp2/3 branch (Arp2/3 complex diameter 10~15 nm). If myosin VI works as a monomer (Lister *et al.*, 2004), it might bind to other molecules on nearby actin filaments or dimerize through target protein binding. Other proteins, such as optinuerin (Sahlender *et al.*, 2005), dimerize using such a mechanism.

A second model (Figure 8B, model 2) is that myosin VI serves as an anchor for a molecule that stimulates Arp2/3 complex activity, increasing the rate of branch generation. In other motility systems, Arp2/3 complex activators are thought to bind to and be activated at the leading membrane. However, there is no leading membrane at the cone front. Myosin VI might serve this role instead. Because we still see a branched network without myosin VI, anchoring of the activator cannot be essential for branched network formation, however.

Overall, our data support the idea that myosin VI plays a structural and not a transport role and that it can stall in a tightly bound state, as suggested by motility assays *in vitro*. These studies confirm that myosin VI is indeed an unusual motor.

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