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Tatsuhiko Nogushi
RIKEN Kobe

Deborah J. Frank
Washington University in St Louis

Mamiko Isaji
Washington University in St Louis

Kathryn G. Miller
Washington University in St Louis

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Coiled-Coil–Mediated Dimerization Is Not Required for Myosin VI to Stabilize Actin during Spermatid Individualization in *Drosophila melanogaster*

Tatsuhiko Noguchi,^{*†} Deborah J. Frank,^{+‡} Mamiko Isaji,[‡] and Kathryn G. Miller[‡]

^{*}Center for Developmental Biology, Riken, Kobe 650-0047, Japan; and [‡]Department of Biology, Washington University, St. Louis, MO 63130

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Myosin VI is a pointed-end–directed actin motor that is thought to function as both a transporter of cargoes and an anchor, capable of binding cellular components to actin for long periods. Dimerization via a predicted coiled coil was hypothesized to regulate activity and motor properties. However, the importance of the coiled-coil sequence has not been tested in vivo. We used myosin VI's well-defined role in actin stabilization during *Drosophila* spermatid individualization to test the importance in vivo of the predicted coiled coil. If myosin VI functions as a dimer, a forced dimer should fully rescue myosin VI loss of function defects, including actin stabilization, actin cone movement, and cytoplasmic exclusion by the cones. Conversely, a molecule lacking the coiled coil should not rescue at all. Surprisingly, neither prediction was correct, because each rescued partially and the molecule lacking the coiled coil functioned better than the forced dimer. In extracts, no cross-linking into higher molecular weight forms indicative of dimerization was observed. In addition, a sequence required for altering nucleotide kinetics to make myosin VI dimers processive is not required for myosin VI's actin stabilization function. We conclude that myosin VI does not need to dimerize via the predicted coiled coil to stabilize actin in vivo.

INTRODUCTION

Due to the facts that myosin VI is unique in its ability to move toward the pointed or slow growing end of an actin filament (Wells *et al.*, 1999) and that it is implicated in human disease, it has been the subject of intense study both in vivo and in vitro. Although mutations in myosin VI cause deafness and are associated with hypertrophic cardiomyopathy (Melchionda *et al.*, 2001; Ahmed *et al.*, 2003; Mohiddin *et al.*, 2004), myosin VI expression is up-regulated in ovarian and prostate cancers, with its level correlating with metastatic potential (Yoshida *et al.*, 2004; Dunn *et al.*, 2006). Studies in *Drosophila* and vertebrates have implicated myosin VI in a number of cellular processes, including endocytosis, basolateral targeting and sorting, cell adhesion and epithelial integrity, cell migration, and actin structure stabilization (Kellerman and Miller, 1992; Mermall *et al.*, 1994; Hicks *et al.*, 1999; Buss *et al.*, 2001; Geisbrecht and Montell, 2002; Aschenbrenner *et al.*, 2003; Petritsch *et al.*, 2003; Millo *et al.*, 2004; Au *et al.*, 2007; Maddugoda *et al.*, 2007). In some processes, myosin VI is proposed to mediate translocation along actin, whereas in others it likely serves as a stable actin anchor. These dual roles are thought to be possible because of myosin VI's ability to move processively along an actin filament and stall in a tightly bound state when placed under load (Altman *et al.*, 2004). How these properties are achieved is

still under investigation, but require significant adaptations of the motor, compared with barbed/plus-end–directed motors (Menetrey *et al.*, 2005, 2007).

Because of a predicted coiled-coil sequence in the tail, myosin VI was thought to work as a dimer. Therefore, myosin VI's biochemical and biophysical properties have been defined in vitro by using altered versions that have been induced to dimerize by adding the well-characterized GCN4 leucine zipper sequence adjacent to the predicted coiled coil (De La Cruz *et al.*, 2001; Rock *et al.*, 2001; Altman *et al.*, 2004; Yildiz *et al.*, 2004; Balci *et al.*, 2005). However, when purified from a heterologous expression system or from native tissue sources, myosin VI is monomeric (Lister *et al.*, 2004; Sakata *et al.*, 2007).

Dimerization has been hypothesized to regulate activity, but the mechanism of dimer formation is controversial. Dimers can be induced to form in vitro by loading onto actin at high density. This mechanism of dimer formation requires the predicted coiled-coil sequence and is inhibited by the presence of the globular tail (Park *et al.*, 2006). In contrast, a recent study suggested that the predicted coiled coil alone cannot dimerize, but a molecule containing the predicted globular region of the tail (also called the cargo-binding domain) and the predicted coiled coil (medial/proximal tail) can form dimers. Whether the globular region is sufficient for dimerization was not examined (Spink *et al.*, 2008). In this work, truncated versions of myosin VI that lacked the head were used, because when the head was present, dimer formation was not observed.

Whether myosin VI functions as a dimer in vivo has not been tested. Determining whether dimer formation is required for function and understanding the mechanism of myosin VI action in vivo requires testing of altered versions of myosin VI for their ability to rescue a robust and well-

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[†] These authors contributed equally to this work.

Address correspondence to: Kathryn G. Miller (miller@biology.wustl.edu).

understood loss of function phenotype in add-back experiments. In most processes in which myosin VI functions, its role is not clearly defined. However, *Drosophila* spermatid individualization provides an ideal system in which to test models of myosin VI action in vivo.

During individualization, the function of mutant forms of myosin VI can be quantitatively assayed by measuring the extent of rescue in several assays. The *jar¹* (*jaguar*) mutant substantially reduces myosin VI in the male germ line and spermatogenesis fails during the final stage, individualization (Hicks *et al.*, 1999). During spermatid individualization, the 64 syncytial spermatids are separated into mature sperm by extensive membrane remodeling and removal of the bulk of the cytoplasm and organelles. This process requires movement of a cone-shaped actin-dense structure along the 2-mm length of each axoneme (Tokuyasu *et al.*, 1972; Fabrizio *et al.*, 1998). As the cones travel, excess cytoplasm and organelles are pushed out of the individualizing sperm tails by the dense actin cones (see schematic diagram, Figure 1A), and the membrane is tightly juxtaposed to the axoneme and mitochondrial derivative. Myosin VI localizes in a tight band at the fronts of the cones. In the absence of myosin VI, the cones fail to accumulate sufficient actin to exclude cytoplasm and organelles, their movement becomes unsynchronized, and they stop moving before completing individualization (Hicks *et al.*, 1999). Actin accumulation during movement correlates with the amount of myosin VI: more actin accumulates when myosin VI is overexpressed, whereas actin is lost when no myosin VI is present (Noguchi *et al.*, 2006). This suggests that myosin VI stabilizes actin. We hypothesized that myosin VI dimers might cross-link filaments to mediate stabilization. Therefore, we have tested the requirement for the predicted coiled-coil sequence and other features that depend on dimer formation. Unexpectedly, our results suggest that the predicted coiled coil, which is the best characterized mediator of dimer formation, is not required for myosin VI to stabilize actin.

MATERIALS AND METHODS

Oregon R was used as the wild type strain throughout this work. Flies were raised on standard cornmeal agar medium at 25°C.

Transgene Construction and Expression Level Determination

Constructs GFP-M6-GCN4 and GFP-Del-Ins 1 were made using QuikChange (Stratagene, La Jolla, CA) and the appropriate primers to modify myosin VI in plasmid pCS2+GFP-Myosin VI (described as an "intermediate vector" in Noguchi *et al.*, 2006). After sequencing to confirm the sequence changes, the GFP-myosin VI sequences were cloned into plasmid CsprHS83 at the Not I site and orientation was confirmed. The HS83 heat-shock promoter is active without heat shock at a low level ubiquitously and at a moderate level in the male germ line. GFP-M6-GCN4 was made by amplifying the 32-amino acid C terminus of GCN4 containing 4.5 heptad repeats. The resulting amplification product was inserted between amino acids 1018 and 1019 (between ANE and SNG). GFP-Del-Ins 1 was created by deleting amino acids 277-302 (GCT.. through... QQK). GFP-H-GT was made by assembly of fragments HT1 and HT2 (both cloned into TA vector; Invitrogen, Carlsbad, CA) as follows: HT1 was made by using primers to amplify the N terminus of myosin VI from the initiating Met to amino acid 832 (PRY). The primers introduced an N-terminal EcoRI site and a C-terminal SpeI site. HT2 was made by using primers to amplify the C terminus of myosin VI from amino acid 1009 (HELA) to the end of the 3' untranslated region contained in plasmid pNB15 (Kellerman and Miller, 1992). The primers introduced an N-terminal SpeI site and C-terminal NotI and XbaI sites. The HT1 and HT2 fragments were then assembled with N-terminal green fluorescent protein (GFP) in vector pUASp and finally transferred to CsprHS83 via NotI digestion and ligation. The junction sites were all confirmed by sequencing. Transformant flies were generated by Genetic Services (Cambridge, MA). Relative expression levels were determined by Western blot. Testes were dissected from young flies (<24 h old) in phosphate-buffered saline (PBS) and kept in culture media on ice until all were collected. After replacing the media with 1× protein sample buffer,

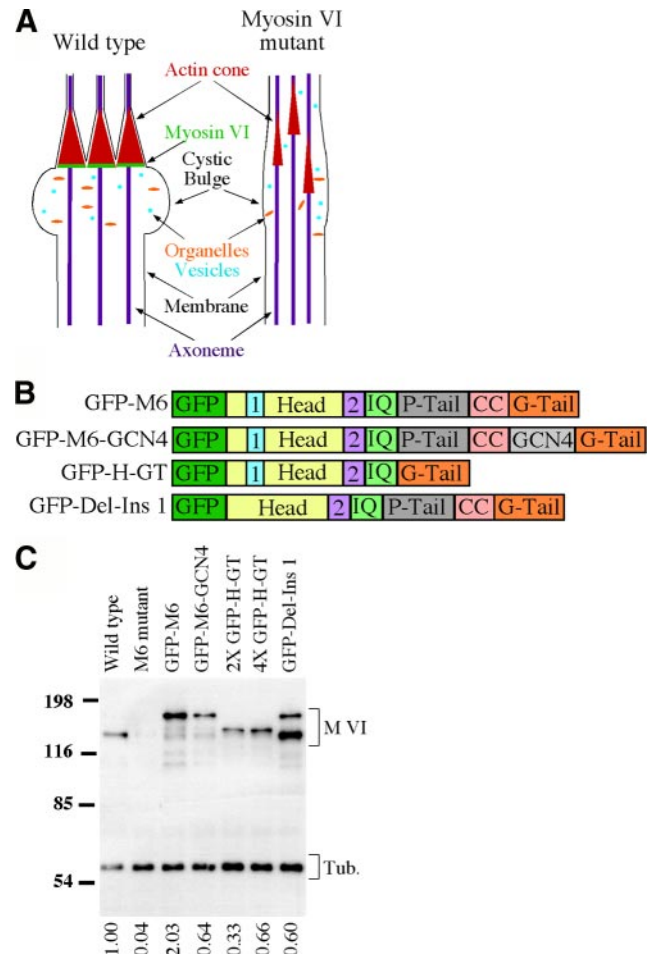


Figure 1. (A) Schematic diagram of spermatid individualization in wild-type and myosin VI mutant. (B) Schematic diagram of constructs used in this study. Head, motor domain containing the ATPase and actin binding domains. 1, 2, inserts found uniquely in class VI myosins. Insert 1 is important for gating and insert 2 binds a light chain. IQ, IQ motif, light chain binding site. P-Tail, proximal tail, not predicted to form a coiled coil. CC, core coiled coil. G-Tail, globular tail. GCN4, leucine zipper dimerization domain from the yeast transcription factor. Drawings are not to scale. (C) Western blot of testis extracts from flies expressing the indicated myosin VI molecules. The top half of the blot was probed with polyclonal anti-myosin VI antibody and the bottom half with anti-tubulin antibody. 2X and 4X indicate the number of copies of the indicated transgene. All others contained one copy of the indicated transgene. GFP-M6-GCN4 and GFP-H-GT samples are in a myosin VI mutant background [*jar¹/Df(3R)S87.5e*], so only the exogenous, GFP-tagged myosin VI is evident in the blot. The GFP-Del-Ins 1 sample is in wild-type background, so both endogenous and the larger, GFP-tagged exogenous myosin VI are evident. The myosin VI band signal intensity was quantitated, standardized to tubulin, and indicated at the bottom of the blot relative to the amount in wild type. Sizes are indicated in kilodaltons.

samples were sonicated for 1 min, boiled for 5 min, and then loaded on a 7.5% polyacrylamide gel. The equivalent of one testis was loaded in each lane. Gel electrophoresis and blotting followed standard procedures. The top halves of the blots were probed with affinity purified rabbit anti-*Drosophila* myosin VI antibody (Kellerman and Miller, 1992) and the bottom halves were probed with anti-tubulin monoclonal antibody DM1A. Detection was performed using Super Signal West Femto (Pierce Chemical, Rockford, IL), and chemiluminescence was captured and quantified using a Fuji Film LAS-1000 imager and ImageGuage software (Fujifilm, Tokyo, Japan). We have observed no difference in transgene expression levels in wild-type versus myosin VI mutant background (data not shown).

Cross-Linking

Testes were dissected from 30 young flies (<24 h after eclosion) in PBS and kept in culture media (Noguchi and Miller, 2003) until ready for processing. Testes were rinsed three times in the buffer described previously (Lister *et al.*, 2004). They were then resuspended in 60 μ l of this buffer and dounced in a 1.5-ml tube with a plastic pestle (Kontes Glass, Vineland, NJ) on ice. 0.5 M N-(3-Dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride (EDC) (Sigma-Aldrich, St. Louis, MO) in dry dimethyl sulfoxide (DMSO) was added to a final concentration of 0.05 M. Cross-linker-containing and mock samples (DMSO only) were incubated for 1 h at room temperature, followed by addition of denaturing sample buffer. Proteins were separated on a 3.5% phosphate SDS gel as described in Sigma technical bulletin MWS-877X and transferred to nitrocellulose in a tank apparatus overnight at 10 V. Nitrocellulose filters were blocked in 5% nonfat dry milk in Tris-buffered saline/Tween 20, and probed with affinity-purified polyclonal anti-myosin VI antibody (Kellerman and Miller, 1992) at 1:100, monoclonal anti-myosin VI antibody 3C7 (Miller *et al.*, 1989) at 1:20, or anti-myosin II (Foe *et al.*, 2000) at 1:1000. Detection was performed using Super Signal West Pico or Femto chemiluminescent systems (Pierce Chemical). For titration experiments, increasing amounts of GFP-M6-GCN4 testis extract was mixed with wild-type testis extract and treated as described above. Cross-linking with BS³ (Pierce Chemical) was performed on extracts prepared as described above following the instructions described in the BS³ product literature.

Rescue Assays

Flies of the following genotypes were generated: w; P[w+ GFP-M6]/+; jar¹/Df(3R)S87.5e, w P[w+ GFP-M6-GCN4]/Y; +; jar¹/Df(3R)S87.5e, and P[w+ GFP-M6-Del-Ins 1]/Y; +; jar¹/Df(3R)S87.5e. These all contained one copy of the indicated transgene. Because one copy of GFP-H-GT resulted in low expression (data not shown), two transgenes were recombined onto the same chromosome. In the text and figures, 2X GFP-H-GT and 4X GFP-H-GT refer to the following genotypes, respectively: w; P[w+ GFP-H-GT] P[w+ GFP-H-GT]/+; jar¹/Df(3R)S87.5e and w; P[w+ GFP-H-GT] P[w+ GFP-H-GT]/P[w+ GFP-H-GT] P[w+ GFP-H-GT]; jar¹/Df(3R)S87.5e.

Fertility Assays

For the data presented in Table 1, where fertility of the mutant lines was extremely low, 10 young (1–3 d after eclosion) males of the test genotype were placed with 25 young (1–3 d after eclosion) wild-type virgin females in a bottle supplemented with moist Instant *Drosophila* media (Carolina Biological Supply, Burlington, NC) and a piece of Kimwipe (Kimberly-Clark, Roswell, GA) to provide the flies with a landing place (day 0). After 5 d at 25°C, adults were transferred to a second bottle (day 0 for the second bottle) and then removed 5 d later. Progeny in each bottle were counted on respective days 13 and 18. The data presented is the sum of both bottles. For GFP-Del-Ins1, where fertility of the mutant lines was identical to full-length GFP-M6, three 1- to 2-d-old males of each test genotype were placed with three 1- to 2-d-old wild-type virgin females in a vial containing standard corn meal media supplemented with moist Instant *Drosophila* media. Adults were removed after 5 d. Water was added if needed to keep the instant food moist and total progeny were counted 13 d later. Ten such vials were counted for each genotype and at least three independent transgenic lines were tested. Data for the same line as is shown in Figure 3A, e–e', is presented.

Immunofluorescence Staining and Microscopy

For the data presented in Figure 3A, a–d', cysts were dissected and processed as described previously (Noguchi and Miller, 2003). For the data presented in Figure 3A, e–e', whole testes were processed as described previously (Rogat and Miller, 2002). In both cases actin was stained with Alexa-568 labeled phalloidin (Invitrogen) and image acquisition was by confocal microscopy (TCS SP2; Leica, Wetzlar, Germany) using 488- and 561-nm lasers.

Table 1. Fertility rescue by myosin VI mutant molecules

Genotype	Avg. progeny	SE	n	p value ^a
M6 mutant	0.6	0.3	10	
GFP-M6-GCN4	2.4	0.7	10	<0.05
4X GFP-H-GT	9.1	2.1	10	<0.005
GFP-M6	1272.3	4.5	3	<10 ⁻⁴

^a p values were determined by a Student's *t* test and indicate the statistical significance of the difference between the myosin VI mutant expressing the indicated transgene and the myosin VI mutant with no transgene.

Cytological Scoring of the Extent of Actin Cone Stabilization and Progression

One author removed testes from 0- to 1-d-old flies and prepared slides by fixing and staining with Alexa-568 phalloidin as described previously (Rogat and Miller, 2002). Coded slides were then scored without knowledge of the genotype by a second author. For each score-able testis, the number of groups of actin cones that were visible at 200 \times magnification (Nikon Eclipse TE2000-S; Nikon, Tokyo, Japan) and had traveled more than one third the length of the testis were counted. Data analysis was performed using Excel (Microsoft, Redmond, WA).

F-Actin Quantitation of Actin Cones

Actin cones in isolated individualizing cysts were stained with Alexa-568-phalloidin as described previously (Noguchi and Miller, 2003). Fluorescent images of actin cone bundles were obtained by laser scanning microscopy (FV-1000; Olympus, Tokyo, Japan) at low magnification (10 \times lens) to include the fluorescent signal from most of the actin cones in a cystic bulge within a single focal plane. The average intensity from three experiments was measured and calculated using ImageJ (National Institutes of Health, Bethesda, MD) and Excel.

Ultrastructure of Mutant Testes

EM cross section of testes was performed as described previously (Noguchi *et al.*, 2006). Testes from mutant flies with myosin VI transgenes were cut at a position one third of the length from the basal end. Because the elongated and individualizing cysts occupy two thirds of the length of the testis from the basal end (the rest of the testis is occupied mostly by primary spermatocytes) (Tokuyasu *et al.*, 1972), this position is approximately the middle of the individualizing cysts. Individualizing cysts were found in the cross sections, and the number of axoneme-mitochondria pairs was counted if multiple pairs were included in a single cell membrane. Data analysis was performed using Excel.

Scoring and Staining of Seminal Vesicles

Seminal vesicles were dissected in PBS and observed on a Nikon dissecting microscope at 10 \times magnification. Scoring categories were as follows: III, very small and completely clear; II, 0–50% larger than III and slightly to almost uniformly white; and I, 2–4 times bigger than III and uniformly white. To facilitate the scoring of many seminal vesicles from each genotype at different time points, we used observations in the dissecting microscope for quantitation. For TOTO-3 (Invitrogen) labeling, seminal vesicles were fixed as described for testes (Noguchi and Miller, 2003), except that the blocking step was omitted. Seminal vesicles were labeled for 1 h with 3.3 μ M TOTO-3 and 0.02 μ M Alexa-488 labeled phalloidin and visualized by confocal microscopy (TCS SP2; Leica) using 633- and 488-nm lasers. Five optical sections 0.13 μ m apart at the Z-axis midpoint of each seminal vesicle (identified by a thin edge of phalloidin staining) were collected and combined in a maximum projection.

RESULTS

The Majority of Myosin VI in the Fly Is Monomeric

Because of the predicted coiled-coil domain present in the tail, it has been assumed that myosin VI works as a dimer. However, when purified from native sources (Lister *et al.*, 2004; Sakata *et al.*, 2007) or expressed in the baculovirus heterologous expression system (Lister *et al.*, 2004), myosin VI is a monomer. To examine whether myosin VI dimers were present in the testis, we performed cross-linking studies on testis extracts using the zero-length cross-linker EDC. When extracts are treated with cross-linker, if myosin VI is a monomer, it should migrate on a denaturing gel at 140 kDa, whereas if it is a dimer, it should migrate at \sim 280 kDa. We observed that all of the myosin VI migrated at the monomer size after EDC treatment (Figure 2A). As a control, we also probed for myosin II and observed that it migrated as a dimer as expected (Figure 2A). To liberate any myosin VI that might be bound to actin and induced to dimerize by clustering on actin or via cargo binding, we treated the extracts with ATP or ATP plus latrunculin A before EDC addition. No dimer was detected under these extraction conditions (data not shown).

In most studies of myosin VI in vitro, an exogenous dimerization sequence, the GCN4 leucine zipper, was added

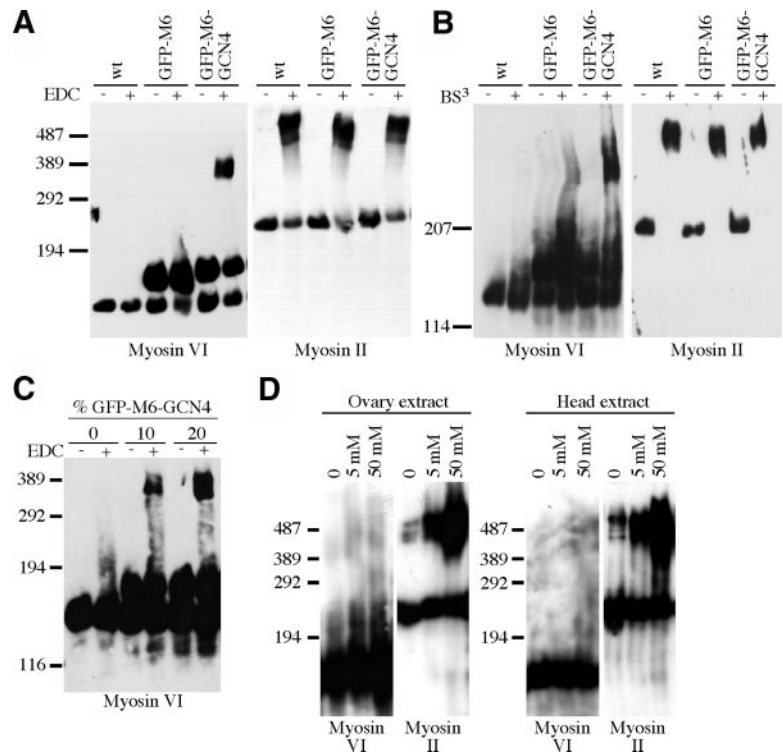


Figure 2. Native myosin VI was not cross-linked by EDC, but an altered version with a dimerization sequence was cross-linked. (A) Extracts of testes from wild-type, GFP-M6-expressing, and GFP-M6-GCN4-expressing flies were treated with DMSO (-) or DMSO + 50 mM EDC (+) for 1 h and then separated on a 3.5% acrylamide gel and probed with a myosin II (right) antibody. The same blot was stripped and reprobed with myosin VI antibody (left). Molecular weight markers (kilodaltons) are on the left. (B) Extracts of testes from wild-type, GFP-M6-expressing, and GFP-M6-GCN4-expressing flies were treated without (-) or with (+) 1 mM BS³ for 30 min and assayed as described in A. (C) Titration of wild-type and GFP-M6-GCN4 testis extracts treated with EDC as described above. (D) Extracts of wild-type heads and ovaries were treated with 0 mM, 5 mM, or 50 mM EDC in DMSO for 1 h and processed as described above.

to force dimer formation. To create a similar molecule, we placed the GCN4 leucine zipper at the end of the predicted coiled coil, in the same position as has been used by others to create dimeric vertebrate myosin VI molecules for *in vitro* motility and single molecule analyses (De La Cruz *et al.*, 2001; Rock *et al.*, 2001; Altman *et al.*, 2004; Yildiz *et al.*, 2004; Balci *et al.*, 2005), yielding the molecule GFP-M6-GCN4 (Figure 1B). Cross-linking of testis extracts from flies expressing GFP-M6-GCN4 showed that myosin VI was a dimer when the GCN4 sequence was included (Figure 2A). A different cross-linker, BS³, which has an 11.4-Å spacer arm length, was similarly able to cross-link GFP-M6-GCN4 but not endogenous wild-type myosin VI (Figure 2B).

We used the GFP-M6-GCN4-expressing flies to determine the percentage of total myosin VI that must be cross-linked to be detectable in this assay. We mixed extracts from wild-type flies with decreasing amounts of extracts from flies expressing GFP-M6-GCN4, treated the mixtures with EDC, and separated the proteins on a gel. When the mixture was as low as 10% GFP-M6-GCN4 extract, we could still detect the cross-linked species (Figure 2C). Because roughly half of the myosin VI in the GFP-M6-GCN4-expressing testes was the endogenous wild-type protein, these results indicate that we should be able to detect dimerized myosin VI if it constitutes 5% of the total. Thus, we conclude that at least 95% of the myosin VI in the *Drosophila* testis is monomeric.

We wondered whether monomeric myosin VI was unique to the testis. Therefore, we performed similar cross-linking experiments using extracts derived from heads or ovaries from adult flies. Consistent with our findings in the testis, we were unable to detect a dimeric form of myosin VI in these different tissues (Figure 2D). Thus, we conclude that myosin VI is likely predominantly monomeric throughout *Drosophila* development.

A Forced Dimer Version of Myosin VI Does Not Fully Rescue Actin Cone Stabilization

If myosin VI works as a dimer, we predicted that the forced dimer molecule, GFP-M6-GCN4, should be able to completely substitute for native myosin VI and behave similarly to GFP-M6, a GFP-tagged version that we previously showed can rescue myosin VI mutants (Noguchi *et al.*, 2006). To test this idea, we compared rescue of myosin VI loss of function phenotypes between flies expressing GFP-M6 and GFP-M6-GCN4.

Actin cones in myosin VI mutants accumulate smaller amounts of actin than cones in wild-type animals and therefore label very weakly with phalloidin. This loss of actin causes the cones to be narrower and to travel asynchronously down the axonemes. Eventually, they arrest movement partway along the cysts (Figure 3A, a-a'); Hicks *et al.*, 1999). These phenotypes can be completely rescued by expression of the full-length molecule GFP-M6 (Figure 3A, b-b'). GFP-M6 localizes to the fronts of actin cones in a manner identical to endogenous myosin VI (Figure 3A, compare b-b' and f-f'; Noguchi *et al.*, 2006). Although the forced dimer, GFP-M6-GCN4, was able to properly localize to the fronts of actin cones (Figure 3A, c-c'), it did not rescue the actin content very well compared with GFP-M6.

To measure the extent of actin stabilization rescue we quantitated the F-actin present in actin cones from each transgene-expressing line by measuring the fluorescence intensity of Alexa568-phalloidin staining of groups of actin cones (Figure 3B). The amount of F-actin in cones recovered significantly in GFP-M6-expressing animals as observed previously (Noguchi *et al.*, 2006). However, GFP-M6-GCN4 rescued actin content only slightly.

In myosin VI mutants, few cone groups are observed that have progressed farther than one third of the length of the axonemes, reflecting the loss of actin, uncoordinated cone

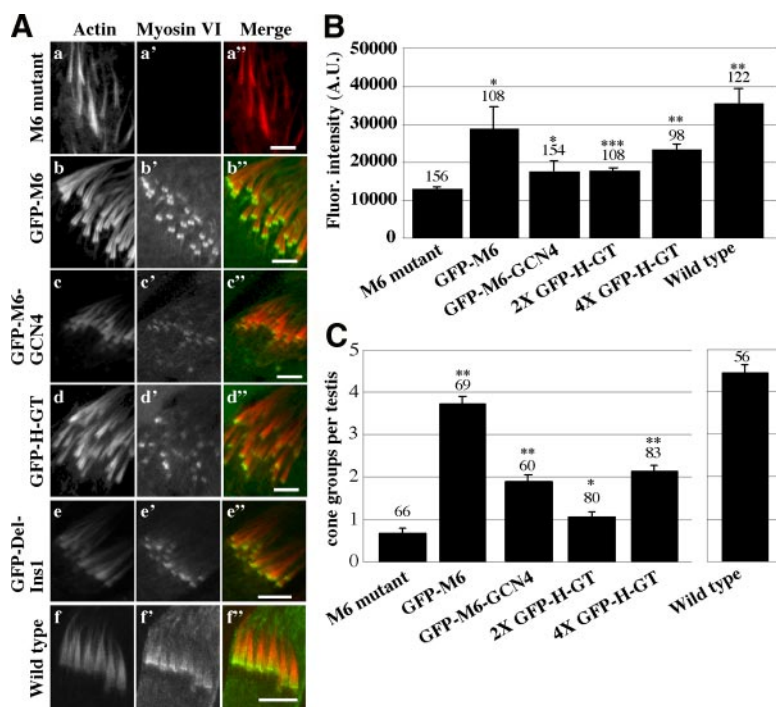


Figure 3. Myosin VI lacking the coiled coil can stabilize actin cones. (A) a–e”, localization of GFP-labeled myosin VI constructs and actin staining in a myosin VI mutant background. f–f”, antibody staining of myosin VI and actin staining in wild-type testes. Bars, 12 μ m. (B) Quantitation of F-actin amount in actin cones in transgenic lines. Average fluorescence intensity (A.U., arbitrary units) of actin cones in each individualizing cyst. Data from three experiments were combined. The number above each bar indicates the number of actin cones that were measured. p values were determined by a Student’s *t* test and indicate the statistical significance of the difference between myosin VI mutant and the indicated transgenes and were as follows: **p* < 0.05, ***p* < 0.005, and ****p* < 0.001. (C) Quantitation of the degree of actin cone stabilization and progression. 2X and 4X indicate two and four copies of the transgene, respectively. The number above each bar indicates the number of testes that were scored. Data from two independent experiments were combined. p values were determined by a Student’s *t* test and indicate the statistical significance of the difference between the indicated transgene and the myosin VI mutant with no transgene (M6 mutant) and were as follows: **p* < 0.05 and ***p* < 10⁻⁷. Actin cone sets in wild-type testes were counted in a separate, nonblinded experiment.

movement and early movement arrest (Noguchi *et al.*, 2006). To quantitate this aspect, we counted (without knowledge of the genotypes) the number of groups of actin cones visible at low magnification that had traveled at least one third of the length of the axonemes (Figure 3C). GFP-M6 rescued well in this assay, whereas GFP-M6-GCN4 rescued to a much smaller extent.

By all assays used, rescue by GFP-M6-GCN4 was greatly reduced relative to that of GFP-M6. Because GFP-M6-GCN4 contains all the sequences present in full-length myosin VI and is expressed at 64% of the level of endogenous myosin VI in wild-type testes, which is comparable to GFP-Del-Ins1 (Figure 1C) that rescues completely (see below), the lack of rescue at a level similar to that seen in GFP-M6-expressing animals is very surprising.

Myosin VI Lacking the Coiled-Coil Dimerization Domain Stabilizes Actin Cones

If coiled-coil mediated dimerization is important for myosin VI function *in vivo*, then a molecule lacking this domain should not rescue myosin VI mutant defects at all. The altered molecule we generated, GFP-H-GT, lacked the proximal tail and core coiled-coil domains, but contained the head, neck (light chain binding sites), and predicted globular tail domain (Figure 1B). The deleted regions included the regions recently more finely described as the proximal, medial, and distal tail domains (Spink *et al.*, 2008). In fly lines with four copies of this transgene, the amount of GFP-H-GT expressed was 66% of the level of endogenous myosin VI expressed in wild-type testes (Figure 1C).

Expression of the GFP-H-GT mutant myosin VI molecules lacking this large region of the tail domain, including the so called “core” coiled-coil domain, was able to somewhat rescue myosin VI loss of function by several criteria. First, this deleted molecule localized on the fronts of the actin cones, in a distribution identical to GFP-M6 (Figure 3A, d–d”). Second, its presence qualitatively improved the structure of the cones: the cones contained more actin (as shown

by phalloidin staining), the cones’ shapes were similar to wild type, and the cones were found in groups, demonstrating synchronous cone movement (Figure 3A, d–d”). The fluorescence intensity of Alexa568-phalloidin staining of actin cone groups was measured and compared with GFP-M6 and GFP-M6-GCN4. The coiled-coil deleted molecule GFP-H-GT was able to rescue actin content slightly better than GFP-M6-GCN4 (Figure 3B). This rescue was dose dependent, because four copies of the GFP-H-GT transgene resulted in more actin in cones than two copies (compare 2X GFP-H-GT to 4X GFP-H-GT in Figure 3B). These results demonstrate that myosin VI lacking the coiled-coil domain is capable of stabilizing F-actin in the cones to a small degree, thus functionally replacing endogenous myosin VI to a greater extent than would be predicted if coiled-coil mediated dimerization was an absolute requirement for function.

Because actin content and cone shape were improved in flies expressing myosin VI without the coiled coil, we expected that they would also travel more synchronously along the axoneme. We found that GFP-H-GT significantly rescued myosin VI function in this assay (Figure 3C). Notably, this rescue was dose dependent, with greater rescue in testes from flies with four copies of the GFP-H-GT transgene than in flies with only two copies of this transgene.

Myosin VI Lacking the Coiled Coil Functions in Spermatid Individualization

Because the actin cones from flies expressing myosin VI without the coiled coil had denser actin and moved more synchronously than those from myosin VI mutants, we expected that they would better function in the membrane remodeling and cytoplasmic and organellar exclusion that occurs during individualization. We performed electron microscopy on cross sections of testes from these flies (Figure 4). The coiled-coil deleted myosin VI molecule GFP-H-GT rescued individualization significantly, showing great improvement in the number of correctly individualized sperm tails and

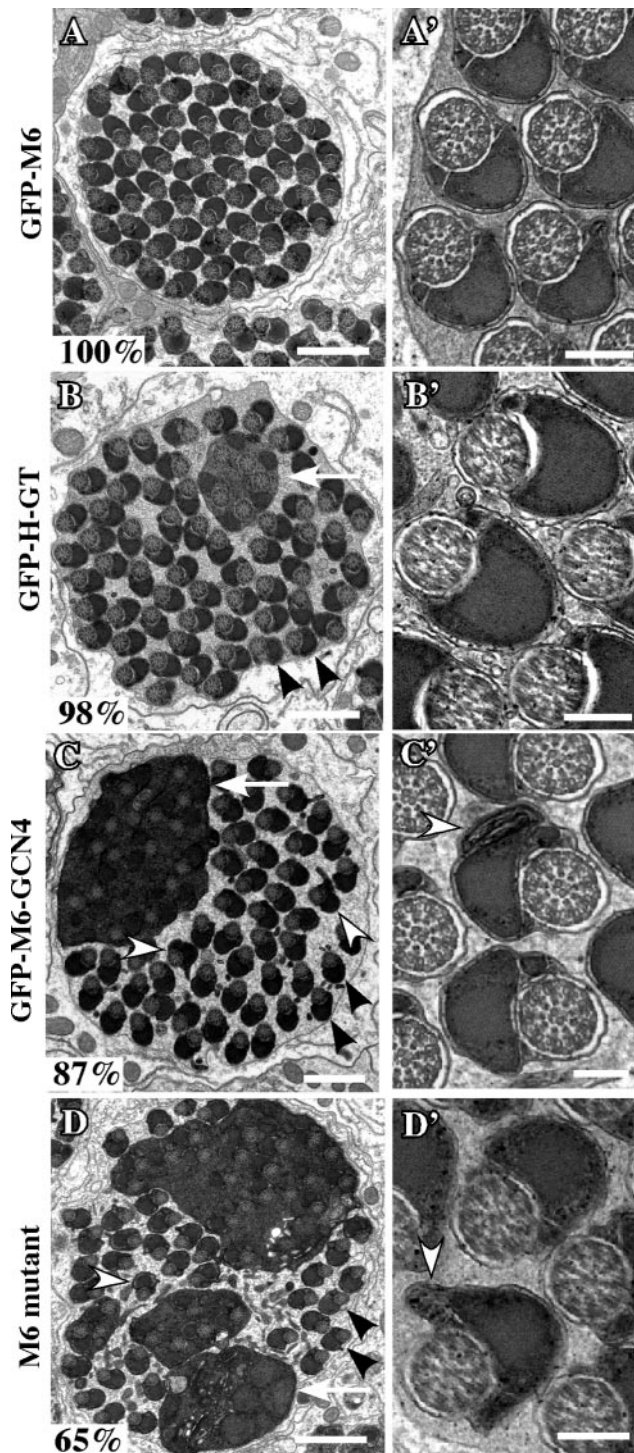


Figure 4. Ultrastructure of individualized sperm tails with three different transgenes. (A–D) Electron microscopy cross sections through the middle of individualizing cysts. In GFP-M6, all the sperm tails were individualized normally, whereas in the 4X GFP-H-GT and GFP-M6-GCN4, the majority of tails were individualized correctly (filled arrowheads), and typically a few sperm tails were seen in a single cell membrane (arrows). In GFP-M6-GCN4 and M6 mutant, some spermatids have individualized but cytoplasmic exclusion is incomplete (open arrowheads in C and D). The number in the box in the lower left corner of each image is the percent of sperm tails per cyst that were correctly individualized. Bars, 1 μm . (A'–D') Cross section of individualized sperm tails in each rescue line at higher magnification. In GFP-M6 and GFP-H-GT, axoneme and

in the removal of cytoplasm and organelles. The number of correctly individualized sperm tails reflects the number of cones that successfully traverse the length of the cysts past the middle. Whereas some small regions in most cysts were not correctly individualized (arrow in Figure 4B), the majority looked normal in the sense that all the cytoplasm was removed and the membrane was tightly juxtaposed to the axoneme and mitochondrial derivatives (arrowheads in Figure 4B; see high magnification images in Figure 4B'). The fact that cytoplasmic exclusion was normal in most sperm tails shows the cones were able to perform their function correctly. In comparison, the GFP-M6-GCN4 spermatids were qualitatively and quantitatively more defective. A higher percentage failed to individualize and in many cases cytoplasmic exclusion was incomplete in those that did individualize (Figure 4C and 4C'). Given that 4X GFP-H-GT and GFP-M6-GCN4 are expressed at similar levels (Figure 1C), we conclude that the molecule lacking the coiled-coil domain functions better than the forced dimer form of myosin VI in spermatid individualization.

Sperm Production Is Rescued by Molecules That Lack the Coiled Coil

Despite the fact that we saw significant rescue of actin stabilization in cones (Figure 3B) and individualization (Figure 4), the fertility rescue by the coiled-coil deleted molecule was minimal (Table 1). A small number of progeny were observed when flies carried the GFP-H-GT transgene. Compared with myosin VI mutant fertility, this increase in progeny was significant (15 times more progeny; compare with GFP-M6-GCN4, which produced 4 times more progeny than the mutant), but it was small in magnitude compared with rescue with the full-length GFP-M6. This lack of fertility was puzzling, because individualization was substantially rescued. Thus, we wanted to determine whether flies expressing mutated myosin VI molecules were able to complete individualization, sperm are transferred to the seminal vesicle, which is connected to the basal end of the testis, where they are stored until they are used in mating. When individualization is defective, sperm are not transferred to the seminal vesicle, resulting in this organ remaining empty. We therefore developed a scoring system to analyze seminal vesicles from flies of the different test genotypes (Figure 5A). This scoring system was based on the observation that large seminal vesicles contained huge numbers of sperm as visualized both by DNA staining (arrows in Figure 5A) and opening of the vesicle. These sperm-filled seminal vesicles were dark when observed by phase contrast microscopy and looked white when observed using a dissecting microscope. Conversely, seminal vesicles that contained very few or no sperm were very small and clear. Based on these characteristics, we were able to assign seminal vesicles from the different genotypes into three categories, I, II, and III (scoring criteria are described in detail in *Materials and Methods*). Examples of these are shown in Figure 5A, where category I seminal vesicles are very large and dark, and when stained with 4,6-diamidino-2-phenylindole, are filled with sperm, and category III are small and clear, and contain very few

mitochondria pairs were nicely packed into cell membranes and there was no evidence of any failure to exclude cytoplasm. In some GFP-M6-GCN4 and M6 mutant spermatids, the axonemes look normal, but cytoplasmic exclusion is incomplete (open arrowheads in C' and D'). Bars, 0.2 μm .

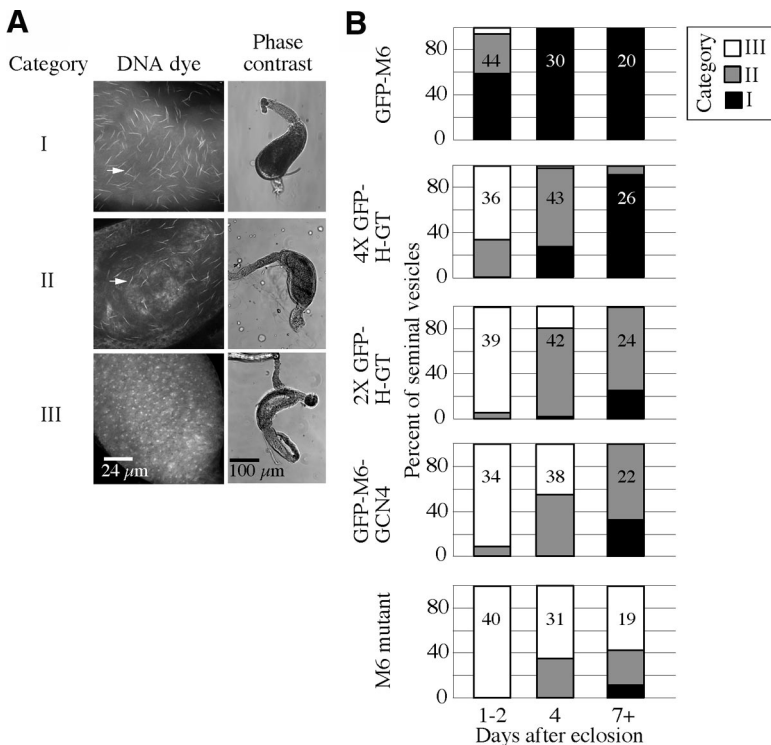


Figure 5. Rescue of sperm production. (A) Representative examples of the classes to which seminal vesicles were assigned in scoring. TOTO-3-labeled seminal vesicles and phase contrast images of seminal vesicles were from flies mutant for myosin VI expressing the following constructs at the indicated time after eclosion: I, GFP-M6 at 3 d; II, four copies of GFP-H-GT at 3 d; and III, myosin VI mutant at 3 d. Arrows indicate sperm nuclei. None are visible in category III. (B) Quantitation of the percentage of seminal vesicles from each genotype and time point scored according to the scale shown in A. The number of seminal vesicles scored is indicated in each bar. Data from two independent experiments were combined.

sperm. The quantity of sperm present in the seminal vesicles also greatly depended on the age of the flies. Seminal vesicles from myosin VI mutant flies (*jar¹*) were all classified as category III at early time points, but a small number were in categories I and II at 1 wk after eclosion (Figure 5B). GFP-M6-GCN4 did not rescue well by this assay, with 68% of seminal vesicles in category II 1 wk after eclosion. Seminal vesicles from flies expressing the GFP-H-GT transgene were substantially rescued in a dose-dependent manner: at 1 wk after eclosion, 75% of 2X GFP-H-GT seminal vesicles were categorized as II, whereas 92% of 4X GFP-H-GT seminal vesicles were categorized as I (Figure 5B). To determine whether the sperm were motile, they were observed by light microscopy. We were unable to discern any difference in sperm motility at a gross level between flies expressing GFP-M6 and two or four copies of GFP-H-GT (Isaji, unpublished observations). We conclude that myosin VI lacking the coiled-coil sequence is able to substantially rescue spermatid individualization, resulting in production of motile sperm that entered the seminal vesicle.

Infertility of GFP-H-GT-expressing Animals Is Due to Defects in Sperm Storage

Because we could not explain the infertility based on problems with steps leading to motile sperm, we examined females to determine whether sperm could be transferred during mating. Sperm were detected in females' uteri within 30 min after mating indicating transfer occurred during mating to males expressing GFP-M6 or GFP-H-GT. If examined 6 h or longer after mating, female sperm storage organs are normally filled with sperm (Bloch Qazi *et al.*, 2003). When storage organs from females who mated with males expressing the GFP-H-GT transgene were examined after 24 h, no sperm were visible, whereas in matings with males expressing GFP-M6, a large number of sperm could be seen. This suggests that the sperm from GFP-H-GT-expressing

flies, although motile and normal looking, were not completely normal and the fertility defect resulted from a problem in sperm entering the female sperm storage organs.

The Gating Activity of Myosin VI Is Not Required for Sperm Individualization

Because the forced dimer could not rescue well, but molecules that lacked the predicted coiled coil were able to substantially rescue, we sought another way to examine the requirement for dimer formation for function *in vivo*. Recent *in vitro* work comparing kinetics of nonprocessive monomeric S1 (head and neck domains) and processive dimeric HMM (the entire coding sequence except with the globular tail replaced by the GCN4 leucine zipper) versions of myosin VI has demonstrated that deletion of the 25 amino acid class-specific Insert 1 in HMM results in loss of processivity of this molecule (Sweeney *et al.*, 2007). Because insert 1 is responsible for the slow rate of ATP binding (Menetrey *et al.*, 2005), the authors concluded that removal of this region results in a loss of the strain-dependent block of ATP binding to the lead head that would allow for gating of the motor. Gating is the mechanism that ensures that the lead head does not release before the trailing head releases and rebinds as the new lead head, thus allowing processivity of a dimeric motor. We thus reasoned that if myosin VI functions as a processive dimer during spermatid individualization, it should require insert 1. To test this, we made the molecule GFP-Del-Ins1 (Figure 1B) in which we deleted all of insert 1, a deletion identical to that used for studies of nucleotide kinetics and movement *in vitro* (Menetrey *et al.*, 2005; Sweeney *et al.*, 2007). We examined whether this deleted molecule could rescue the various defects observed in myosin VI loss of function mutants. When expressed in the myosin VI mutant *jar¹*, in which endogenous myosin VI is reduced to 4% of the level in wild-type testes (Figure 1C), GFP-Del-Ins1 was able to localize properly and restore actin

cone shape and movement (Figure 3A, e–e’). Furthermore, GFP-Del-Ins1 was able to completely rescue fertility of the myosin VI mutant (average number of progeny/vial in controlled matings: myosin VI mutant, 0; GFP-M6, 82; GFP-Del-Ins1, 88). Thus, the alterations in nucleotide binding and release kinetics mediated by insert 1, which are important for coordination of the heads of a dimer, are not required for myosin VI function in spermatogenesis. This result supports the idea that dimerization is likely not required.

DISCUSSION

The Coiled-Coil Sequence Is Not Required for Myosin VI Function

In this report, we tested the requirement for some of the properties and sequence features of myosin VI thought to be important for function *in vivo*, by using the most robust assay system so far characterized for myosin VI functional analysis. We present four lines of evidence that myosin VI does not dimerize via the predicted coiled-coil sequence to stabilize actin during sperm individualization. First, no dimer was detected by cross-linking. Second, molecules forced to dimerize by insertion of the GCN4 leucine zipper were not able to rescue well. Third, a myosin VI molecule that lacks the predicted coiled-coil region was able to somewhat rescue actin cone structure and movement, resulting in improved removal of cytoplasm by the cones, and motile sperm production. Finally, myosin VI did not require gating due to insert 1-dependent blocking of ATP binding, indicating that it is not likely to function as a processive dimer to mediate actin stabilization. Although there has been much speculation about whether myosin VI functions as a monomer or a dimer, and a great deal of biochemical work *in vitro* defining the activities of these different forms, this is the first work to test directly whether myosin VI requires coiled-coil mediated dimerization to be functional *in vivo*.

A key question raised by our observations is how myosin VI lacking this large tail region stabilizes the actin cones. We previously hypothesized that as a dimer, myosin VI could stabilize the actin meshwork at the front of the cones by binding and cross-linking two adjacent actin filaments (Noguchi *et al.*, 2006). As a monomer, actin cross-linking would have to occur via a bridging protein. Alternatively, myosin VI might stabilize actin cones by recruiting the Arp2/3 actin nucleation complex, which is localized at the fronts of the cones (Rogat and Miller, 2002) or by protecting the pointed ends of the filaments at the front of the cones (Noguchi *et al.*, 2006) from depolymerizing factors such as cofilin.

Our data lead us to speculate that myosin VI might function as a monomer during actin cone stabilization. Myosin VI was not cross-linked by two different agents that work with different chemistry (EDC links a carboxy group to an amino group, whereas BS³ links two primary amino groups; Timkovich, 1977; Partis *et al.*, 1983). Although these assays may not be sensitive enough to detect a small pool of dimerized myosin VI (<5% of endogenous protein), the lack of robust rescue by the forced dimer supports the idea that myosin VI does not dimerize to stabilize actin cones. Given that the molecule GFP-M6-GCN4 is a full-length, wild-type version of myosin VI and was expressed at levels comparable with GFP-Del-Ins 1 (Figure 1C) that rescues completely, we expected that it would be capable of fully substituting for endogenous myosin VI, if dimerization were required. The fact that this forced dimer was unable to robustly rescue suggests that dimerization inhibits myosin VI function dur-

ing individualization. This lack of rescue is unlikely to be due to a dominant negative effect, as we observed no effect on fertility when GFP-M6-GCN4 was expressed in a wild-type background (data not shown). The inability of GFP-M6-GCN4 to rescue *in vivo* also calls into question the conclusions that can be drawn from *in vitro* studies using a similarly engineered dimer.

Given the extent of actin cone and individualization rescue by the coiled-coil deleted myosin VI molecule, we expected fertility to be better rescued. Ultrastructural analysis of sections through the middle of the cysts revealed that individualization proceeded fairly normally in flies expressing coiled-coil deleted myosin VI. However, sperm tails are very long (2 mm) and the entire process of individualization takes ~10 h. Thus, we suspect that the lack of fertility might be due to a failure to properly remodel the distal end of the sperm tails. Previous work on effects of loss of *almost* there (*amo*), the polycystin-2 homologue that localizes to the tips of the sperm tails, demonstrated the importance of proper sperm tail formation for fertility. Sperm produced in *amo* males are motile but unable to enter the female sperm storage organs and thus fail to fertilize eggs (Watnick *et al.*, 2003). We have seen a similar effect with GFP-H-GT (see *Results*).

There are several possible explanations for failure to complete individualization properly. First, given that we observed dose-dependent rescue with GFP-H-GT and that overexpression of myosin VI leads to higher actin content in cones (Noguchi *et al.*, 2006), it is possible that further increase in GFP-H-GT expression would result in more complete rescue. The deleted molecule GFP-H-GT seems to be unstable *in vivo*, because all the transgenic lines we isolated had low levels of protein accumulation. GFP-H-GT molecules may thus be depleted by the end of individualization. Second, the sequences deleted from GFP-H-GT may be important for full myosin VI activity. For example, the proximal tail of myosin VI, which is deleted in this molecule, is thought to be important for flexibility of the head domain during movement (Rock *et al.*, 2005). This flexibility may be important for full function. Finally, it is possible that the remodeling that occurs at the distal end of the tails requires different motor properties, which are conferred by the proximal tail and/or coiled-coil domains. Of note is the observation that animals heterozygous for mutations in the *yuri* gene have significantly worse defects in individualization (Texada *et al.*, 2008) compared with the GFP-H-GT rescued animals but are robustly fertile (K. Beckingham, personal communication). This suggests that myosin VI might have functions in other aspects of spermatogenesis that are important for good fertility.

Implications for Myosin VI Function in Other Systems

Our results rule out the idea that myosin VI activity is regulated via coiled-coil mediated dimerization, as has been suggested previously (Sweeney and Houdusse, 2007). We propose several alternate hypotheses for how myosin VI activity might be regulated. First, in a cellular context in which myosin VI stabilizes actin, like in the case of individualization, it may function as a monomer, but in other contexts such as during endocytosis, it may act as a dimer to move vesicles along actin through the cell cortex. If, as has been suggested recently (Buss and Kendrick-Jones, 2008), myosin VI acts as a processive dimer in some processes and as a monomer with different properties in others, it seems likely that regulatory mechanisms would exist to control when it performs these very different functions. Understanding how such regulation is achieved will be important.

Second, it is conceivable that, rather than dimerizing through the coiled coil, myosin VI instead dimerizes through another domain or forms a “pseudodimer” via a binding partner. Several lines of evidence have suggested that such a mechanism might occur. Homo-FRET experiments demonstrated that binding of the globular tail to endocytic vesicles closely positioned the tails in such a conformation that they would be expected to dimerize (Altman *et al.*, 2007), and a C-terminal tail fragment multimerized when bound to lipid vesicles (Spudich *et al.*, 2007). Additionally, tail fragments that include both the predicted coiled coil (also referred to as the medial and distal tail) and the predicted globular domain (GT; also called the cargo binding domain) can dimerize *in vitro*, although the coiled-coil region alone could not (Spink *et al.*, 2008). Whether the globular tail is sufficient for dimerization was not addressed in this work. In addition, myosin VI binds to optineurin, which itself dimerizes (Sahlender *et al.*, 2005). Our cross-linking studies suggest that these mechanisms are unlikely to occur during spermatid individualization, but we cannot completely exclude this possibility. Nonetheless, if this type of dimer does form, it will be important to determine its biochemical and biophysical properties *in vitro*. The increased flexibility and likely different orientation of the dimerization linkage would be expected to alter the inter-head communication that has been previously measured in coiled-coil mediated and forced dimers and is required for motor processivity.

Finally, given that myosin VI is predominantly monomeric in mammalian cells (Lister *et al.*, 2004), sea urchins (Sakata *et al.*, 2007), and during spermatid individualization in *Drosophila* (this work), it is possible that myosin VI always functions as a monomer. Although several reports suggest the involvement of myosin VI in endocytosis as a processive cargo transporter, other mechanisms that do not require processivity, such as stabilizing actin that pushes vesicles or anchoring vesicles to filaments, are possible (Frank *et al.*, 2004). Conversely, it is possible that monomeric myosin VI is processive, as cargo binding promoted processivity for a short distance on actin filaments *in vitro* (Iwaki *et al.*, 2006).

Our results highlight the importance of testing the requirement for the biochemical and biophysical properties of myosin VI, such as dimerization and processivity, which have been defined *in vitro* and predicted from sequence features, in a variety of cellular contexts. Only in doing so, can we understand which pieces of information we learn from studies *in vitro* are relevant to function *in vivo*.

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