

Note

Genetic Characterization of the *Drosophila jaguar*³²² Mutant Reveals That Complete Myosin VI Loss of Function Is Not Lethal

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ABSTRACT

Myosin VI is an actin-based motor that has been implicated in many cellular processes. Studies in vertebrates have demonstrated that animals lacking this ubiquitously expressed myosin are viable. However in *Drosophila*, myosin VI loss of function has been thought to be lethal. We show here that complete loss of myosin VI is not lethal in flies and that the previously reported lethality of the null mutation (*jar*³²²) is most likely due to deletion of a neighboring gene. Maternally provided myosin VI does not account for the survival of myosin VI null animals. Mutant animals are recovered at a lower than expected Mendelian frequency, suggesting that myosin VI participates in processes which contribute to normal development, but its participation is not essential.

MYOSIN VI is an actin motor that is conserved from flies and worms to humans. This motor is unique among myosins in that it can move along actin filaments toward the pointed or minus end, a direction opposite other characterized myosins. In addition, myosin VI can both move processively and bind tightly to an actin filament, stalling under backward load. These properties may allow it to act as a transporter in some processes and an anchor in other processes (BUSS *et al.* 2004; FRANK *et al.* 2004; SWEENEY and HOUDUSSE 2007).

Myosin VI has been implicated in a variety of cellular functions in *Drosophila* using loss-of-function techniques. Microinjection of myosin VI antibodies into syncytial blastoderm embryos caused defects in actin pseudocleavage furrow formation. This phenotype was hypothesized to be the result of defects in transport of furrow components (MERMALL and MILLER 1995). Promoter mutations (*jar*¹ and *jar*^{mmw14}), which prevent myosin VI expression in the testes, cause male sterility due to a failure of spermatid individualization. Defects in the actin structures that mediate the separation of the syncytial spermatids are observed (HICKS *et al.* 1999; NOGUCHI *et al.* 2006). Using a Gal4-UAS targeted expression system combined with antisense RNA, which allows for disruption of function in specific groups of cells at different developmental stages, myosin VI has been implicated in border cell migration in oogenesis,

tissue integrity during late embryogenesis, cuticle development in third star larvae, and imaginal disc morphogenesis during metamorphosis (DENG *et al.* 1999; GEISBRECHT and MONTELL 2002). Myosin VI promoter mutations (*jar*^{R39} and *jar*^{R235}), which cause a loss of myosin VI expression in late embryogenesis and expression of a myosin VI dominant negative protein that lacks the ATP binding site cause lethality in late embryos due to defects in the integrity of the epithelial cell layers during dorsal closure (MILLO *et al.* 2004). A null allele of myosin VI (*jar*³²²) causes abnormalities in asymmetric localization of determinants and, therefore, neural fate determination (PETRITSCH *et al.* 2003). Animals homozygous for this mutation die as first or second instar larvae.

In contrast to *Drosophila*, studies in vertebrates show that myosin VI loss of function is not lethal. Myosin VI mutations in mice, fish, and humans result in deafness due to degeneration of hair cell stereocilia (AVRAHAM *et al.* 1995; SELF *et al.* 1999; AHMED *et al.* 2003; KAPPLER *et al.* 2004). In addition, vertebrate myosin VI has been implicated in processes similar to those affected in *Drosophila*, such as endocytosis, cell adhesion, and basolateral sorting (DANCE *et al.* 2004; AMEEN and APODACA 2007; AU *et al.* 2007; MADDUGODA *et al.* 2007; MORRISWOOD *et al.* 2007). Since myosin VI is involved in similar processes in vertebrates as *Drosophila*, the lethality of myosin VI in flies but not in vertebrates seems surprising.

Because the *jar*³²² mutation deletes all of the amino acid coding sequences in the myosin VI gene, it offers the best means to understand myosin VI's role *in vivo*. However, this small deletion affects both myosin VI and

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a neighboring gene, CG5706. Thus, it is unclear which gene is responsible for the lethal phenotype. To determine if this lethality is due to loss of myosin VI, we have examined the *jar*³²² allele in transheterozygous combination with two large deletions in this region. Our experiments demonstrate that myosin VI null animals can survive, but do so at less than the expected Mendelian frequency. This phenotype is consistent with myosin VI playing a role in a variety of processes during development but not being essential for them. The ability to examine myosin VI null animals at all stages of the *Drosophila* life cycle provides an opportunity to investigate its contribution to a wide variety of cellular processes and in many developmental contexts.

MATERIALS AND METHODS

Fly stocks and fertility tests: All *Drosophila melanogaster* fly stocks and crosses were maintained on standard *Drosophila* cornmeal food at 25° unless otherwise noted. For crosses and fertility tests, parents were removed after 5 days and progeny were counted after 18 days. For fertility tests, three males or females of the test genotype were placed with three Oregon-R males or females in a vial supplemented with instant *Drosophila* medium (Carolina Biologicals, Burlington, NC). A total of five vials were scored for each test genotype. The previously described *jaguar*³²² (*jar*³²²) mutant (PETRITSCH *et al.* 2003) was obtained from C. Petritsch and Y. N. Jan. Deletion chromosomes *Df(3R)S87-5* and *Df(3R)S87-4* were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN). Zygotic null myosin VI animals were generated by crossing *jar*³²²/*TM3 Sb ubx lacZ e pp* females to *Df(3R)S87-5/TM3 Sb e pp* males. To generate flies lacking maternal myosin, *jar*³²²/*Df(3R)S87-5* females from the above cross were back-crossed to *jar*³²²/*TM3 Sb ubx lacZ e pp* males. For rescue of *jar*³²² in the *Df(3R)S87-5* background, *w; P[C95w+]; Df(3R)S87-5 st e/TM3 Sb e pp* females were crossed to *jar*³²²/*TM3 Sb ubx lacZ e pp* males. *P[C95w+]* is a transgene containing a myosin VI cDNA under the control of a hsp83 promoter, which is ubiquitously expressed without heat shock. Animals were not heat-shocked in rescue experiments, since basal expression resulted in myosin VI amounts similar to endogenous protein levels. *P[C95w+]* flies were generated and first reported by (HICKS *et al.* 1999). Flies lacking both myosin VI and a neighboring gene, CG5706, were generated by crossing *jar*³²²/*TM3 Sb ubx lacZ e pp* males to *Df(3R)S87-4 st e/TM3 Ser e* females. To test the ability of myosin VI expression to rescue *jar*³²² in the *Df(3R)S87-4* background, *w; P[C95w+]; Df(3R)S87-4 st e/TM3 Sb e pp* females were crossed to *jar*³²²/*TM3 Sb ubx lacZ e pp* males. A total of two vials were scored for each test genotype.

Genomic PCR: PCR was performed on DNA extracted from a single fly and products were analyzed on a 1.8% agarose gel. Primers used were as follows: set 1, CAGGCGAGTGAAA GTGGT CGGGCC/CTTGGTCTATGGGACCGGCACTG; set 2, TACATGCTCCTCGCCGGAG CTC/TGCATCACTCGG GATACCAGGG; set 3, GCGAATCACTATCGCCTGGGTC/ACGC GATGGATAACCGTGCTCC; set 4, CTGGGAGCCC TCTGCGTGATCAAGC/GATTTCTT CGCGCTGGCGCTGC TCC; set 5, GGAGCAGCGCCAGCGGAGGAAATC/CACC TGGC CATTGGACTCGTTGGCC; set 6, GGCCAACGAGTC CAATGGCCAGGTG/GGGAGACGC GTCATTGACACCACTG; set 7, GCCCAACAACAGGCCCTGGCAAGC/CCAGCCGTG GTACACCTTCAAGCGG; and set 8, AACCGCAAGCGCAC CACCATGGATG/GTTTCTG CATTGCTGCAGCCGGCCC.

RT-PCR: RNA was extracted from OreR and *jar*³²²/*Df(3R)S87-5* adult flies using RNAeasy mini kit (QIAGEN, Valencia, CA). RT-PCR was performed with the above primers using the One-Step RT-PCR kit (QIAGEN).

Western analysis: Ovaries (20), testes (20), or adults (5) were homogenized in 75 μ l 1 \times testes IP buffer (100 mM NaCl, 20 mM Hepes, 1% NP₄O, pH 7.5), protease inhibitor cocktail (Sigma, St. Louis) and 1mM PMSF supplemented with 4 \times SDS-PAGE sample buffer. Samples with whole adults were homogenized and then briefly centrifuged to remove insoluble debris before 4 \times sample buffer was added. Proteins were separated on a 7.5% SDS polyacrylamide gel and blotted onto nitrocellulose using conventional methods. Detection was performed using Supersignal West Pico chemiluminescence substrate (Pierce, Rockford, IL). Antibodies used were mouse monoclonal anti-myosin VI (3C7) (KELLERMAN and MILLER 1992) at 1:20 dilution and a monoclonal anti- β -tubulin antibody, DMI-A (Sigma) at 1:1000 dilution. Flies were fed yeast for several days before collecting ovaries.

Fluorescence microscopy: *jar*³²²/*TM3 Sb Green* males were crossed to *jar*³²²/*Df(3R)S87-5* females and progeny were collected. Embryos were scored as either expressing GFP [*jar*³²²/*TM3 Sb Green*, *Df(3R)S87-5/TM3 Sb Green*] or not expressing [*jar*³²²/*Df(3R)S87-5*, *jar*³²²/*jar*³²²] at approximately stage 16 using fluorescence of the Bolvic's organ as a marker (CASSO *et al.* 2000). Third instar mutant larvae of the genotype *jar*³²²/*Df(3R)S87-5* and GFP-expressing larvae [*jar*³²²/*TM3 Sb Green*, *Df(3R)S87-5/TM3 Sb Green*] were selected, put into vials with instant food (which increases survival), and scored for the number of adults obtained. GFP expression was visualized using a Nikon Eclipse TE 2000 microscope (Nikon, Japan) equipped with a GFP band pass filter.

RESULTS AND DISCUSSION

***jar*³²²/*Df(3R)S87-5* flies lack myosin VI protein expression:** *jar*³²²/*Df(3R)S87-4* flies are not viable while *jar*³²²/*Df(3R)S87-5* flies are viable, but male sterile (PETRITSCH *et al.* 2003; J. K. MORRISON, unpublished observations). Previous studies showed that *jar*¹/*Df(3R)S87-5* were male sterile due to the loss of myosin VI expression in the testis but not other tissues (HICKS *et al.* 1999). The complementation observed among the myosin VI alleles and deficiencies used in this study is shown in Table 1. To determine if *jar*³²²/*Df(3R)S87-5* animals are sterile due to loss of myosin VI function only in the testis [similar to *jar*¹/*Df(3R)S87-5*], we examined expression in adult testis and other tissues using Western blot analysis with monoclonal anti-myosin VI antibody (3C7). This antibody recognizes a determinant in the predicted core coiled-coil region of the tail (Figure 2A, exon 13), which immediately precedes the predicted globular region at the C terminus of the molecule (exons 14–17). Carcass, ovaries, and testes of *jar*³²²/*Df(3R)S87-5* adults and *jar*³²²/*Df(3R)S87-5* adults expressing a myosin VI cDNA transgene were selected. No myosin VI was detected in the *jar*³²²/*Df(3R)S87-5* adults (Figure 1A). Additionally, myosin VI expression was restored in these flies when the myosin VI cDNA transgene was present (Figure 1C).

***Df(3R)S87-5* endpoint maps in the region of exon 13 of myosin VI:** Since we could not detect intact myosin VI

TABLE 1
Complementation among the myosin VI alleles and deficiencies used in this study

	<i>jar</i> ³²²	<i>jar</i> ¹	<i>jar</i> ^{mmw14}
<i>jar</i> ³²²	Lethal ^a	—	—
<i>jar</i> ¹	Viable, male sterile ^b	Viable, male sterile ^c	—
<i>jar</i> ^{mmw14}	Viable, male sterile ^b	Viable, male sterile ^c	Lethal ^{c,e}
<i>Df(3R)S87-5</i>	Viable, male sterile ^d	Viable, male sterile ^c	Viable, male sterile ^c
<i>Df(3R)S87-4</i>	Lethal ^d	Viable, male sterile ^c	Viable, male sterile ^c

^a PETRITSCH *et al.* (2003).
^b J. K. MORRISON and K. G. MILLER (unpublished results).
^c HICKS *et al.* (1999).
^d This study.
^e The lethality in this genotype is unlinked to myosin VI.

protein in *jar*³²²/*Df(3R)S87-5* adults and they were viable, we wondered whether a low level of expression of myosin VI earlier in development from sequences still present in *Df(3R)S87-5* allowed these animals to bypass a larval lethal phase and account for their survival. We therefore wished to examine the myosin VI genomic region in the *Df(3R)S87-5* chromosome to determine if a functional myosin VI protein was encoded. The myosin VI gene lies on the right arm of the third chromosome at cytological map position 95F6-95F8 (Figure 2A). Downstream (centromere proximal) of myosin VI lies a predicted gene, CG5706, which encodes a phenylalanine tRNA ligase. The *jar*³²² mutation was generated by imprecise excision of the *P* element inserted in myosin VI's first intron in the male sterile allele *jar*¹. In the *jar*³²² mutation, both myosin VI (exons 3–17) and at least the first exon of CG5706 are absent (PETRITSCH *et al.* 2003). There are two deficiencies that uncover this region. *Df(3R)S87-4* removes the region 95D11-96A2, thus uncovering both myosin VI and CG5706. *Df(3R)S87-5* removes the region 95F7-96A18, but its exact endpoint was unknown. Eight primer sets were designed to span sequences across the entire myosin VI amino acid coding region to determine if any part of the myosin VI gene was retained in the *Df(3R)S87-5* chromosome (Figure 2A). PCR amplification of genomic DNA using these primers showed that *jar*³²²/*Df(3R)S87-5* animals

only contain sequences corresponding to the globular tail (exons 14–17) of the myosin VI gene (Figure 2B). Molecularly, the left endpoint of *Df(3R)S87-5* thus lies in the region of exon 13 of myosin VI.

***jar*³²²/*Df(3R)S87-5* flies lack myosin VI transcripts:** It was possible that the globular tail sequences (exons 14–17) retained in *jar*³²²/*Df(3R)S87-5* might encode a fragment that provided some myosin VI function and accounted for our ability to recover these flies (although this seemed unlikely). Neither of the antibodies (monoclonal 3C7 and polyclonal) that recognize myosin VI can detect the C-terminal globular tail fragment (M. ISAJI, unpublished observations). To determine if a globular tail fragment was expressed, RT-PCR was performed using the primers that amplified this region. While we were able to detect transcripts for the CG5706 gene in *jar*³²²/*Df(3R)S87-5* flies (data not shown), we were unable to detect transcripts corresponding to the myosin VI tail (Figure 2C). We conclude that no portion of myosin VI is expressed in *jar*³²²/*Df(3R)S87-5* flies and, therefore, expression of a truncated myosin VI protein does not account for their viability.

Together, these data resolve three important issues. First, *jar*³²²/*Df(3R)S87-5* adults completely lack myosin VI protein and are viable. Second, *Df(3R)S87-5* removes most of the myosin VI coding region, through exon 13, and no myosin VI sequences are transcribed. Third,

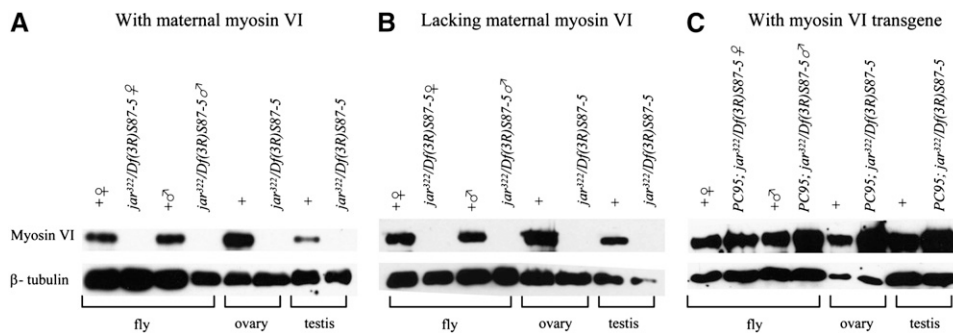


FIGURE 1.—*jar*³²²/*Df(3R)S87-5* adults do not express the myosin VI protein in any tissue. Western blot analysis of carcasses, ovaries, and testes in a (A) zygotic null myosin VI mutant, (B) maternal and zygotic null myosin VI mutant, and (C) zygotic null myosin VI mutant that expresses a myosin VI cDNA transgene. One-half of a whole fly, two ovaries, and two testes were loaded except for the mutant expressing the myosin VI transgene (one-quarter of an ovary).

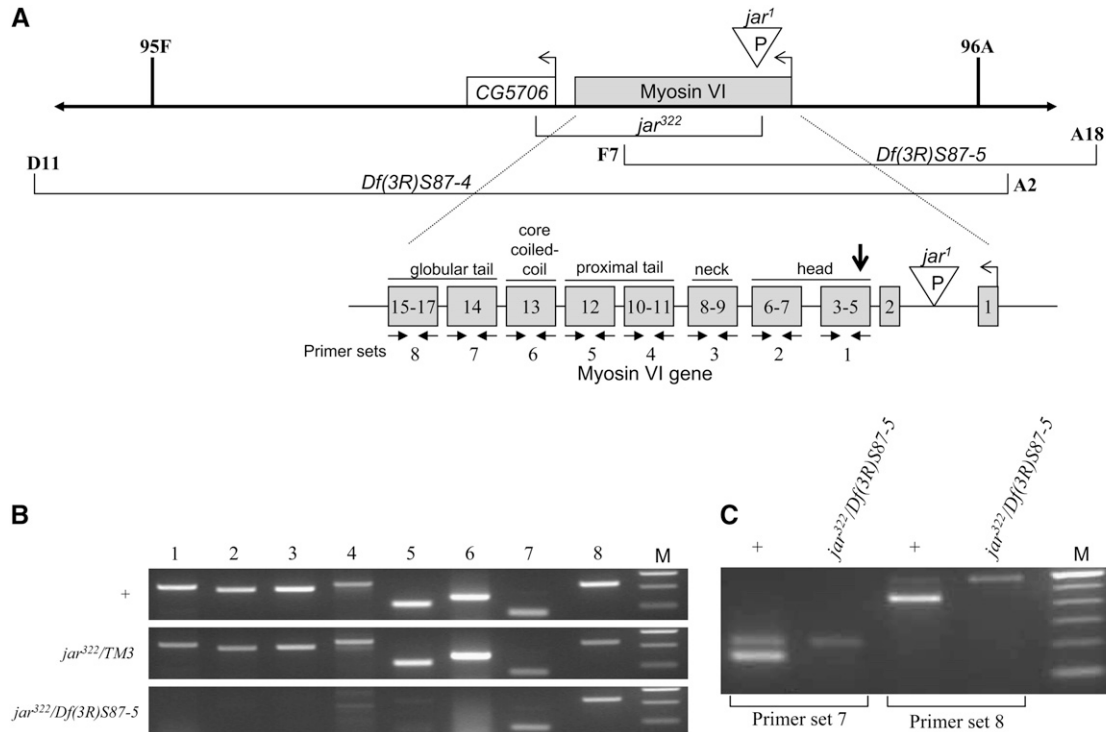


FIGURE 2.—Characterization of the genomic region of myosin VI in the *Df(3R)S87-5* deletion chromosome. (A) Schematic depicts a portion of the right arm of the third chromosome in *Drosophila*, showing the position of the myosin VI gene, the deficiencies that remove portions of this region, and the mutations used. The unfilled arrows indicate the transcription start site and direction. Below, an expanded schematic illustrates the exons encoding different parts of the myosin VI protein with eight primer sets (solid arrows) designed to detect different regions (not drawn to scale). The vertical arrowhead indicates the translation start, which is encoded in exon 3. (B) PCR products generated in amplification reactions of genomic DNA using the primers indicated in A, resolved on a 1.8% gel. Size markers (M) are in increments of 100 bp starting with 600 bp at the top. Note that in *jar*³²²/*Df(3R)S87-5*, no PCR products are obtained from exons 3–13, but products are obtained from exons 14–17. (C) RT-PCR products obtained from amplification of total RNA using the primers indicated, resolved on a 1.8% gel. In wild-type animals the higher band is derived from genomic DNA and the lower band from cDNA generated in the RT reaction. Note the lack of the lower band in the mutant genotype. The bands corresponding to genomic DNA amplification were verified by reactions in which the reverse transcription step was not performed (not shown). Size markers (M) are in increments of 100 bp starting with 600 bp at the top.

Df(3R)S87-5 does not remove the sequences encoding CG5706. These data indicate that a complete myosin VI loss of function is not lethal in *D. melanogaster*.

Maternally contributed myosin VI is not essential for viability: It was possible that maternally contributed myosin VI enabled null animals to survive. To test this idea, we compared viability of animals with and without maternally contributed myosin VI. *jar*³²²/*Df(3R)S87-5* adults with no maternal myosin VI eclosed at the same frequency as *jar*³²²/*Df(3R)S87-5* adults that had maternally contributed myosin VI [18% ($n = 415$) and 16% ($n = 443$) of total progeny, respectively]. In addition, *jar*³²²/*Df(3R)S87-5* females derived from embryos with or without maternal myosin VI were fertile. When compared to control genotypes, both types of mutant females produced progeny in numbers equal to nonmutant animals (data not shown). Males were sterile, as expected based on the *jar*¹ phenotype. These adult flies expressed no intact myosin VI protein (Figure 1B), as expected. Thus, maternally provided myosin VI is not responsible for our ability to obtain *jar*³²²/*Df(3R)S87-5*

adults, further supporting the idea that complete myosin VI loss of function is not lethal.

***jar*³²²/*Df(3R)S87-5* mutants display partially penetrant heterogeneous lethality:** Although we were able to obtain *jar*³²²/*Df(3R)S87-5* flies, they were found at ~40% of the expected Mendelian frequency (Table 2).

TABLE 2

Viability of *jar*³²²/*Df(3R)S87-5* adult flies

Cross	n^a	<i>jar</i> ³²² / <i>Df(3R)S87-5</i> viability (%) ^b
<i>Df(3R)S87-5/TM3</i> × <i>jar</i> ³²² / <i>TM3</i>	443	37
<i>jar</i> ³²² / <i>Df(3R)S87-5</i> × <i>jar</i> ³²² / <i>TM3</i>	415	44
<i>P[C95]; jar</i> ³²² / <i>Df(3R)S87-5</i> × <i>P[C95]; jar</i> ³²² / <i>TM3</i> ^c	398	105

^a Total number of progeny from three crosses.

^b Calculated as observed/expected × 100.

^c Expressing myosin VI cDNA transgene.

No obvious abnormalities were observed in the null mutant adults that would suggest a developmental defect responsible for this partially penetrant lethality. The viability of these myosin VI null animals was completely rescued by basal expression (without heat shock) of the hsp83-myosin VI cDNA transgene (Table 2). To investigate the arrest point of myosin VI deficient animals, we used a GFP balancer to identify genotypes of progeny during development. *jar³²²/Df(3R)S87-5* virgin myosin VI null females were crossed to *jar³²²/TM3 Green* males. If the number of non-GFP-expressing embryos [*jar³²²/jar³²², jar³²²/Df(3R)S87-5*] are equivalent to the number of GFP-expressing embryos [*jar³²²/TM3 Green, Df(3R)S87-5/TM3 Green*], then no defect occurred during oogenesis due to lack of myosin VI in ovaries of adult females. Of 165 embryos, 37% expressed GFP (contained the balancer) and 63% did not express GFP (did not contain the balancer and thus were null for myosin VI). Since we see >50% myosin VI null embryos, developmental defects during oogenesis do not account for the 40% viability we observed. Recovery of only 37% GFP-expressing embryos (*i.e.*, nonmutant), rather than the expected 50%, may be explained by the observation that animals carrying both the TM3 balancer and *Df(3R)S87-5* appear somewhat unhealthy. In this cross, it is impossible to distinguish *jar³²²* homozygotes and *jar³²²/Df(3R)S87-5* transheterozygotes, so examining phenotypes during embryogenesis and early larval development specifically attributable to loss of myosin VI function was not possible. Since *jar³²²* homozygotes die during the first to second instar larval stage (PETRITSCH *et al.* 2003; J. K. MORRISON, unpublished observations), we were able to unambiguously identify non-GFP-expressing third instar larvae as *jar³²²/Df(3R)S87-5* and compare their viability to GFP-expressing control animals (*jar³²²/TM3 Green*). Third instar *jar³²²/Df(3R)S87-5* larvae eclosed in reduced numbers compared with controls [77% ($n = 239$) and 87% ($n = 499$), respectively]. Examination of *jar³²²/Df(3R)S87-5* pupae revealed that some were normal appearing but did not eclose, some died trying to eclose, and some did not complete metamorphosis. Because myosin VI null animals are present at the expected frequency at egg laying and only 10% of the animals die during metamorphosis, null animals must die at other developmental stages to account for our inability to recover 60% of the expected number of progeny.

Conclusion: Previous experiments suggested that in *Drosophila*, myosin VI loss of function was lethal. However, the studies reported here using genetic null animals demonstrate conclusively that myosin VI loss of function is not lethal in flies and that the lethality attributed previously to loss of myosin VI function in *jar³²²* deletion must be caused by loss of the neighboring gene. Interestingly, our results are consistent with results in other species that loss of myosin VI function is not lethal. Despite the fact that animals completely lacking myosin VI can survive, they are not obtained at

the expected frequency. This partially penetrant lethality is attributable to myosin VI loss of function, because we can rescue this defect by ubiquitous expression of a myosin VI cDNA transgene. The same transgene fails to rescue the lethality of the *jar³²²* homozygotes and *jar³²²/Df(3R)S87-4* transheterozygotes. Thus, the lethality observed in these animals is not associated with myosin VI loss of function.

In addition to observations reported here, an ENU mutagenesis screen for new myosin VI mutants in which we screened both for lethal and male sterile alleles lends additional support to the idea that myosin VI loss of function is not lethal. We did not obtain any myosin VI lethal mutations, even though this screen was successful in identifying three new male sterile myosin VI alleles (J. K. MORRISON, unpublished observations).

Because of the phenotypes previously observed using other loss-of-function techniques and other myosin VI alleles, we were surprised that the null mutant animals were viable. The phenotypes attributed to myosin VI loss of function in other mutant alleles, including *jar³²²* homozygous mutants, and defects generated by other loss of function techniques, such as antibody injection and antisense expression, must be reassessed in the *jar³²²/Df(3R)S87-5* background. In neuroblasts, both expression of dominant negative fragments and lack of myosin VI (*jar³²²* homozygotes) resulted in a partially penetrant defect in basal determinant localization (PETRITSCH *et al.* 2003), but perhaps such a defect is not severe enough to cause lethality. During early embryonic development, function-blocking antibody injection prevented metaphase pseudocleavage furrow formation (MERMALL and MILLER 1995), leading to massive defects in nuclear division. This severe phenotype would be expected to cause embryonic lethality. However, loss of myosin VI does not cause embryonic lethality. Perhaps the furrow defects caused by antibody injection can be explained by the sequestration of myosin VI-associated proteins, rather than as a direct effect of inhibiting myosin VI function. When myosin VI is absent (in null embryos), the myosin VI-associated proteins may still be able to function in furrow formation. Transgenic antisense expression caused severe defects in epithelial integrity and morphogenesis at a number of times in development and these animals were not viable (DENG *et al.* 1999; MILLO *et al.* 2004). It is unclear why antisense expression causes more severe defects than the null mutation, but one explanation is off-target effects of the antisense. Possibly, impairment of epithelial integrity and defects in morphogenesis due to lack of myosin VI could contribute to the partially penetrant lethality we observe. However, the processes in question cannot be completely blocked by loss of myosin VI function, since 40% of the null animals survive and these survivors have no obvious defects. The significant differences in phenotype of this null mutation compared to those observed using other functional

manipulations reinforces the importance of careful genetic analysis of loss-of-function alleles in understanding *in vivo* functions.

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