In Vivo X-Ray Diffraction of Indirect Flight Muscle from *Drosophila melanogaster*

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ABSTRACT Small-angle x-ray diffraction from isolated muscle preparations is commonly used to obtain time-resolved structural information during contraction. We extended this technique to the thoracic flight muscles of living fruit flies, at rest and during tethered flight. Precise measurements at 1-ms time resolution indicate that the myofilament lattice spacing does not change significantly during oscillatory contraction. This result is consistent with the notion that a net radial force maintains the thick filaments at an equilibrium interfilament spacing of ~56 nm throughout the contractile cycle. Transgenic flies with amino-acid substitutions in the conserved phosphorylation site of the myosin regulatory light chain (RLC) exhibit structural abnormalities that can explain their flight impairment. The I_{20}/I_{10} equatorial intensity ratio of the mutant fly is 35% less than that of wild type, supporting the hypothesis that myosin heads that lack phosphorylated RLC remain close to the thick filament backbone. This new experimental system facilitates investigation of the relation between molecular structure and muscle function in living organisms.

INTRODUCTION

Over 35 years ago, Worthington (1961) compared x-ray diffraction patterns from thoracic muscles of living blow flies with isolated, glycerinated thoracic muscle fibers as an elegant control for muscle structure studies. Intrigued by his use of a living fly, we used improved x-ray technology to obtain high-quality, in vivo patterns from the flight muscles of the fruit fly *Drosophila melanogaster*. We compared and contrasted the patterns from living *Drosophila* with those reported from isolated, glycerinated flight muscle fibers of the giant waterbug, *Lethocerus.* We chose *D. melanogaster* because, among insects, it is readily amenable to genetic manipulation and therefore of greatest interest for studies of mutants designed to probe specific aspects of muscle function.

Using high-speed video microscopy of intact *Drosophila virilis*, Chan and Dickinson (1996) demonstrated that the dorsal longitudinal muscle (DLM) undergoes a ~3.5% change in length during oscillatory contraction. This change implies that the spacing of the myofilament lattice varies by ~1.7% assuming a constant lattice volume. Long believed to be a feature of intact muscle during active contraction, constant volume behavior has only been convincingly demonstrated in relaxed muscle (Elliott et al., 1963). Recent mechanical and x-ray diffraction studies of isolated, intact amphibian muscle fibers, however, demonstrate a radial component of cross-bridge force that develops during contraction (Cecchi and Bagni, 1994, and references therein). This compressive radial force alters the equilibrium of forces that, in resting muscle, keeps the lattice volume constant. During tension generation, constant volume behavior of the myofilament lattice is no longer obeyed (Cecchi and Bagni, 1994), at least in isolated amphibian muscle. In this report, we show, by measuring lattice spacings at two points in the wing beat cycle of *Drosophila*, that the volume of the lattice during muscular activity in vivo is not constant, but that the lattice spacing is constant over the wingbeat cycle in the indirect flight muscle of this insect.

MATERIALS AND METHODS

The experiments were carried out at the Biophysics Collaborative Access Team (BioCAT) undulator-based beam line (Irving et al., 2000) at the Advanced Photon Source at Argonne National Laboratory. Adult flies (2–3-day-old females) were held at right angles to the x-ray beam while allowing unimpeded movement of the wings and legs (Fig. 1). The collimated beam intercepted all 12 DLM fibers located in two muscle sets on each side of the median plane of the thorax. Time-resolved experiments used a rotating shutter to isolate two phases of the wing beat cycle.

Before mounting, flies were anesthetized using CO_2 gas. The top of the head, the neck and thoracic cuticle adjacent to the neck were glued to a stainless steel pin. After recovery from anesthesia, the fly was mounted with the long axis of its thorax perpendicular to the x-ray beam (head up) such that the x-ray beam intersected the two layers of DLM fibers, with little or no contamination of signal from other muscles. "Rest" diffraction patterns from the DLM were obtained when the fly's wings were folded back (legs moving); "active" patterns were obtained when the fly's wings were beating (legs quiescent and extended backward). We triggered flight activity by blowing gently on the fly, using a long flexible tube. X-ray diffraction patterns were recorded by a CCD-based detector constructed by Dr. W. Phillips's group at Brandeis University (active area, 60 × 60 mm; 60-μm pixels). Images were corrected for detector distortions and analyzed using the program FIT2D (Hammersley, 1998) on a UNIX workstation. Values for the spacing and intensity of the equatorial reflections were obtained from nonlinear fits to one-dimensional (1D) projections (Irving and Millman, 1989). Intensities of the 14.5-nm near-meridional reflections

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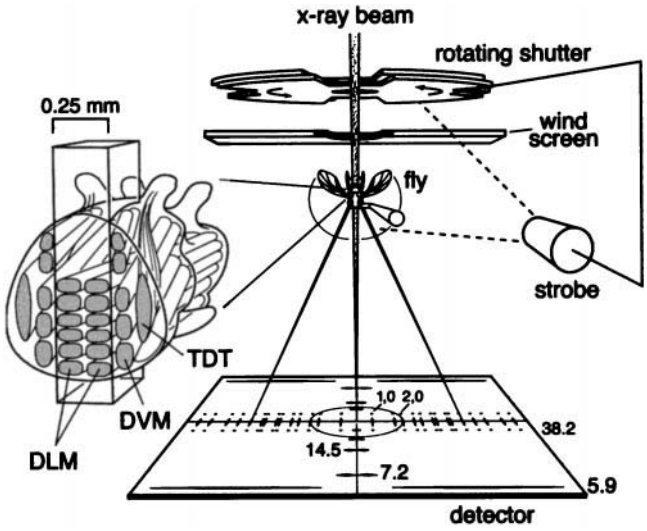

FIGURE 1 Experimental set-up and preparation. The incident-beam intensity of the synchrotron x-ray source was modulated by aluminum filters of varying thickness (maximum flux, $\sim 2.0 \times 10^{13}$ photons/s; wavelength, 0.103 nm). The camera length was 1.5 m (fly-to-detector distance); the spot size at the CCD-based detector was $\sim 30 \times 200$ μm (full width, half maximum); the spot size at the sample was $\sim 250 \times 250$ μm. A primary shutter controlled the x-ray exposure. During the wing beat, the x-ray aperture was modulated by a rotating shutter, coupled to a stroboscope (see text). Shutter opening was synchronized to wing-beat frequency by manually adjusting the strobe frequency and shutter disc rotation until the wing image froze at a desired position. The fly's image was recorded on videotape using a video camera equipped with telescopic lens. The video image field included the rotating shutter and a light-emitting diode that signaled when the primary shutter was open (at the point the wings were in the desired position). A wind-shield blocked the fly from shutter-generated air currents. The support wire glued to the fly could be manipulated by a motorized stage to orient the DLM fibers in the x-ray beam path. The inset shows details of the thoracic musculature (DLM, DVM, and TDT), with the orientation of the x-ray beam indicated.

were obtained from 1D projections along the meridian using the multiple 1D routines in FIT2D.

For time resolved experiments, a chopper-type shutter mounted on a DC motor was used to isolate two broad phases of the wing-beat cycle, using ⅙ of a complete cycle (unless otherwise noted). The shutter consisted of a sandwich of two ⅛-inch-thick aluminum disks with four equally spaced slots, the width of which could be adjusted by rotating the disks with respect to one another (Fig. 1). The center of the temporal aperture was set such that the wings were either at the top of the up-stroke or at the bottom of the down-stroke, i.e., at times that included the maximum and minimum muscle length, respectively. Wing position was monitored using a video camera and stroboscope, which illuminated the fly every fourth wing beat. Shutter frequency was coupled electronically to the strobe frequency, which was adjusted by means of a potentiometer to find a frequency at which the wings appeared to "freeze" in either the up or the down position. Although wing beat frequency fluctuated continuously, there were brief, occasional periods of 1–2 s during which the rotating shutter and wing-beat frequencies matched and the primary shutter was opened. The average wing-beat frequency was ~200 Hz, so the effective time resolution of the exposure was ~0.8 ms [i.e., $(⅙ \times 200\ s^{-1})^{-1}$].

The variation in wing-beat frequency precluded attempts to systematically vary the phase of aperture opening through a full contraction–extension cycle (see, e.g., Tregear and Miller, 1969). We considered using an opto-electronic tracking device to gate the beam at a constant phase point of the wing-beat cycle, but the response time of the primary shutter was too slow to gate the beam reliably on a beat-to-beat basis. Thus, for these preliminary studies, we chose the simpler two-point approach using the rotating shutter.

We assessed the extent to which the phases fluctuated during an x-ray exposure by videotaping flies at 30 frames/s and evaluating the tapes one frame at a time over the exposure period. The x-ray patterns were analyzed if >70% of the frames showed wings in either extreme position (up or down); otherwise, the patterns were rejected. For the four experiments listed in Table 1, for example, the fraction of frames showing wings in the up position was 0.83 ± 0.06 and the fraction showing wings down was 0.88 ± 0.05. For both fractions, wing position was assumed to be in strict phase with muscle length. Thus, signal contamination due to phase fluctuations was estimated to be ~15%. Because the temporal aperture was a significant portion of the wing-beat cycle (⅙, or 27% by area), muscle lengths other than the extremes were sampled, thereby adding to the signal contamination. Thus, for an apparent reduction in lattice spacing of 1.7%, for example, the combined effects of these two sources of signal contamination could lead, in the worst case, to an estimated reduction in lattice spacing of ~0.95% rather than 1.7% over one cycle.

RESULTS AND DISCUSSION

Figure 2 shows a resting pattern from the DLM of a wild-type fly. This pattern shows many similarities to those obtained from glycerinated fibers isolated from *Lethocerus* (Tregear et al., 1998). Both patterns show better lattice ordering than those from vertebrate muscle, with numerous diffraction peaks on the equator, and layer lines that index as various orders of a 232-nm repeat (Reedy et al., 1992). All of these diffraction features are candidates for time-resolved experiments that aim to elucidate various aspects of acto–myosin interaction (see e.g., Tregear et al., 1998).

The pattern in Fig. 2 shows a split 14.5-nm meridional reflection with reduced intensity on the meridian itself (unlike *Lethocerus*, which shows a 14.5-nm layer strong on the

TABLE 1 DLM diffraction pattern data

Genotype*	State†	Position of Wings	I_{20}/I_{10}	d_{10} (nm)	n
Canton S	Rest	Folded	0.866 ± 0.040	48.87 ± 0.08	8
	Active	Average‡	0.928 ± 0.050	48.48 ± 0.10	8
	Active	Up§	0.865 ± 0.045	48.68 ± 0.05	4¶
	Active	Down§	0.900 ± 0.040	48.64 ± 0.15	4¶
Mlc2^{S66,67A}	Rest	Folded	0.562 ± 0.040	47.44 ± 0.40	7
Mlc2^{+}	Rest	Folded	0.841 ± 0.026	48.71 ± 0.24	6

Mean ± standard errors of the mean; n = number of flies.
*Canton S is a wild type strain. *Mlc2*^{S66,67A} (line RT6) is a homozygous mutant strain in which the two conserved serines of the myosin RLC are replaced by alanines (Tohtong et al., 1995). *Mlc2*$^{+}$ (line JW63.1) is a rescued strain created by transforming *Mlc*^{E38}/+, a heterozgyous MLC2 null host, with a wild type *Mlc2* gene (Tohtong et al., 1995).
†Rest versus active intensity ratios were significantly different ($p < 0.05$).
‡Average over all wing positions.
§Up = wings raised (top of stroke), Down = wings lowered (bottom of stroke). The variation in repeated spacing measurements of a given diffraction pattern was <0.05% (<0.025 nm). In pair-wise comparisons, up versus down intensity ratios and lattice spacings were not significantly different ($p > 0.05$).
¶Three cases, temporal aperture ⅙ wing beat cycle; 1 case, ⅓.

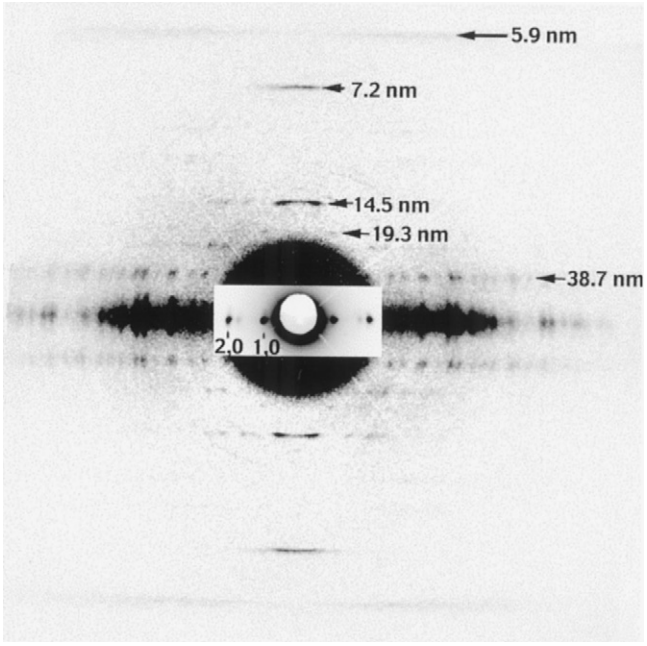

FIGURE 2 Two-dimensional x-ray pattern from DLM of resting (wings folded) *D. melanogaster*. Layer lines (the 6th, 12th, 16th, 32nd, and 39th orders of the 232-nm-long spacing repeat) are indicated by their lattice spacing (in nm). A radially symmetric background has been subtracted from the outer parts of the pattern for clarity. The inset shows the 1, 0 and 2, 0 equatorial reflections on a reduced intensity scale. Exposure time was 0.36 s with an incident flux of $\sim 2.5 \times 10^{12}$ photons/s (main beam attenuated fourfold).

meridian). The ratio of the integrated intensity of the 14.5-nm reflection in the active state to that of the resting state is 0.97 ± 0.04 ($n = 7$), consistent with no change in intensity upon activation. This result is in marked contrast to that observed in glycerinated *Lethocerus* DLM, where the intensity of the 14.5-nm reflection decreases by ~50% upon activation by stretch (Tregear et al., 1998). In *Lethocerus,* the loss of intensity is assumed to be due to myosin heads losing their thick-filament based symmetry when they bind to target sites on the thin filaments. In relaxed *Drosophila* DLM, however, the heads do not appear as well ordered in electron micrographs as they do in *Lethocerus* (Menetret et al., 1990), suggesting that, in *Drosophila*, fewer myosin heads are held close (docked) to the thick-filament backbone. The loosely-docked heads will therefore undergo a proportionately smaller loss of order upon binding to the thin filaments. This view is consistent with the relatively small change in the I_{20}/I_{10} intensity ratio observed upon activation (see below).

Table 1 lists ratios of the intensities of the reflections from the 2, 0 and 1, 0 lattice planes (I_{20}/I_{10}, analogous to I_{11}/I_{10} in vertebrate muscle: e.g., Elliott et al., 1963, 1967; Huxley, 1968). Measurements were taken in flies at rest and during tethered flight. Paired comparisons of the I_{20}/I_{10} ratios show an increase of 4.8% ($p < 0.005$) in active flies (averaged over all wing positions) compared to flies at rest (wings folded). An increase in I_{20}/I_{10} is consistent with a movement of a myosin-related mass from the thick filament to the thin filament (Rapp et al., 1991), in this case due to calcium- or stretch-activation.

The changes in I_{20}/I_{10} ratios upon activation are in the same direction but less than those reported for stretch-activated skinned fibers from *Lethocerus* (Rapp et al., 1991; Tregear et al., 1998). The results from *Lethocerus* showed an increase in I_{20}/I_{10} of ~5% upon raising the calcium concentration, and another 5% increase upon stretch-activation. These intensity changes were accompanied by a decrease in d_{10} of ~1.5% relative to the relaxed state (Tregear et al., 1998). As proposed above, the center of mass of the numerous loosely-docked myosin heads in relaxed fibers of *Drosophila* will be nearer the thin filament than in the case of *Lethocerus* with its more numerous tightly-docked heads. This initial condition could account for the relatively small equatorial intensity shift seen upon activation in *Drosophila* compared to *Lethocerus*. Alternatively, the differences between *Drosophila* and *Lethocerus* may be due simply to the fact that the latter is a skinned preparation possessing a greater lattice spacing and therefore producing a different sampling of the filament transform.

Thick filament spacing in vivo

The numerous, well-resolved reflections on the equator allowed changes of myofilament lattice spacing to be measured very accurately (±0.1%, or 0.05 nm). Table 1 lists spacings between the 1, 0 planes (d_{10}), alongside the values of I_{20}/I_{10}, at rest and during tethered flight. Paired comparisons show that the lattice shrinks slightly upon activation, by 0.8% ($p < 0.001$), to a d_{10} of 48.5 nm (center-to-center thick-filament spacing, 56.0 nm $= 2 \times 3^{-1/2} \times 48.5$ nm). Chan and Dickinson (1996) observed that the mean oscillating length of both DLM and dorsal-ventral muscle (DVM) is not significantly different from the measured resting lengths. Thus, the decrease in mean lattice spacing in vivo indicates that cross-bridge forces have radial components that compress the lattice, as in vitro studies of frog muscle have also indicated (Cecchi and Bagni, 1994, and references therein).

The time-resolved results of Table 1 indicate that, near the bottom of the down-stroke (i.e., DLM shortened), d_{10} was virtually identical (within 1 Å) to the spacing near the top of the upstroke (DLM lengthened). Paired comparison also showed no difference between the spacings at the extremes and the average spacing in a particular fly during tethered flight (0.00 ± 0.07 nm; $p < 0.05$). (Note that the average active spacing given in Table 1 includes data from flies from which the average "up" and average "down" values were obtained as well as a number of additional flies in which only active and relaxed spacings were compared. The result is that the mean average active spacing of the

larger population is less than either extremes reported in the smaller population. This seemingly anomalous finding is due to small (~1%) variations in mean lattice spacing between flies, emphasizing the importance of making paired comparisons). The observed changes of less than 0.25%, are ~4–7 times less than predicted with constant volume (1.7%, or ~0.95% with contamination: see Methods). This surprising constancy of lattice spacing in a given fly during the wing-beat cycle, despite significant changes in muscle length, suggests that living *Drosophila* do not maintain constant lattice volume during oscillatory contraction. Rather, a net radial force generated as cross-bridges attach appears to bring the thick filaments to an equilibrium inter-thick-filament spacing (of ~56 nm) that is maintained throughout the contractile cycle and for many thousand such cycles (Millman, 1998; Xu et al., 1993; Cecchi and Bagni, 1994). The constancy of lattice spacing at the two endpoints of the wing-beat cycle implies that the average radial component of the cross-bridge force is the same under these conditions. It would be informative to know the variation of total force in skinned fibers under conditions that closely mimic the normal wing-beat cycle (i.e., oscillations that allow for a 3.5% length change) to estimate the expected magnitude of changes in radial forces. Such data, however, is not yet available.

Nonconstant volume behavior may produce interesting consequences with regard to bulk movement of cytosolic constituents across the myofibrillar interface. As the DLM shorten during the down-stroke, the volume of each muscle cell remains constant, resulting in an increase in cell width. During the upstroke, a reciprocal decrease in cell width occurs. Assuming the cell volume remains constant, a 3.5% change in cell length (Chan and Dickinson, 1996) with no associated change in lattice spacing implies that at least 3.5% of the myofibrillar fluid (by volume) must leave and re-enter the lattice with each wing beat. At 200 or more beats/second, this rapid fluid exchange may provide an effective means of assisting the diffusion of MgATP from mitochondria outside the myofibril to working cross-bridges within. The rapid fluid exchange may also assist removal of the products of MgATP hydrolysis (MgADP, free phosphate and H^+).

Other musculature

In some flies, the thorax was slightly off-axis with the x-ray beam, a fortuitous misalignment that produced distinct equatorial patterns (Fig. 3) from the DVM and tergal depressor of the trochanter (TDT: see Fig. 1, *inset*). The DVM and TDT patterns were at increasing angles to the DLM pattern, a separation that allowed additional tests of constant volume. Because the DLM and DVM are antagonistic muscles, one set will lengthen while the other shortens, and vice versa. Going from rest to activation, the lattice spacings of the DVM moved in the same direction and by about the same amount as those of the DLM. This similarity of movement suggests that the reduced lattice spacing in working DLM is most likely related to an inwardly directed radial component of the cross-bridge force upon activation rather than to changes in cell length. Any changes in the radial component of cross-bridge forces are likely to be much larger than any changes in electrostatic repulsive forces upon going from passive to active muscle (see Millman, 1998 and references therein). Changes in the lattice spacing of the TDT, when measurable, showed no consistent relationship to changes in the DLM or DVM spacings.

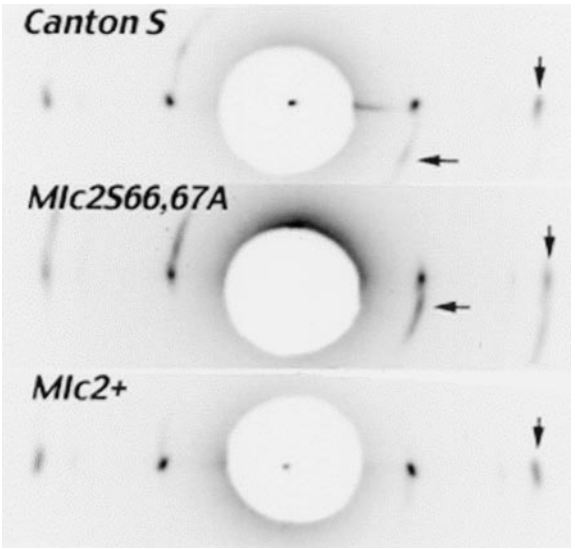

FIGURE 3 Equatorial x-ray diffraction patterns from DLM of *D. melanogaster* at rest. Canton S, wild-type; $Mlc2^{S66,67A}$, mutant with unphosphorylatable alanines at the conserved phosphorylation site; and $Mlc2^+$, rescued transformant. The 2, 0 reflections are indicated by vertical arrows. Note the weak 2, 0 reflection in $Mlc2^{S66,67A}$. The patterns have been scaled so that the 1, 0 reflection has approximately equal intensity in each of the three patterns. Horizontal arrows indicate reflections from the DVM muscles that were visible in some patterns.

Experiments were also conducted on two lines of transformed flies (Table 1): $Mlc2^{S66,67A}$, in which two phosphorylated serine residues (66 and 67) of the myosin RLC are replaced with unphosphorylatable alanines, and a control line $Mlc2^+$, in which wild-type myosin RLC protein is expressed in an MLC2 null background (Tohtong et al., 1995; Dickinson et al., 1997). Both $Mlc2^{S66,67A}$ and $Mlc2^+$ display wild-type myofibrillar and sarcomeric structures. $Mlc2^{S66,67A}$, however, is flightless (most transformants are unable to beat their wings), in contrast to $Mlc2^+$, whose flight ability is similar to wild type (*Canton S*).

Isolated, skinned DLM from $Mlc2^{S66,67A}$ exhibit marked reductions in the amplitude of stretch-activated force, oscillatory work, and power output compared to $Mlc2^+$ and wild type (Tohtong et al., 1995), with little or no affect on cross-bridge kinetics (Dickinson et al., 1997). It was hypothesized that removal of the phosphorylated residues

somehow reduces the number of cross-bridges available for stretch-activation without affecting the kinetics of the remaining power-producing cross-bridges. The present results show that the I_{20}/I_{10} equatorial intensity ratio of the mutant fly is 35% less ($p < 0.001$) than that of wild type (Table 1; Fig. 3, compare *B* to *A*). The reduced mass associated with the thin filament (implied by the reduced I_{20}/I_{10} ratio) supports the hypothesis that myosin heads that lack phosphorylated RLC remain close to the thick-filament backbone as suggested by Levine et al. (1996) in their study of rabbit muscle. We speculate that these flies are flightless because too few myosin heads are positioned for productive actomyosin interactions. The I_{20}/I_{10} equatorial intensity ratio and interfilament spacing of $Mlc2^{+}$ were not significantly different than those of wild type (Table 1; Fig. 3, compare *C* to *A*). Thus, the changes observed in $Mlc2^{S66,67A}$ mutant flies cannot be attributed to secondary effects of in vivo transformation of the regulatory light chain gene per se.

In summary, we have used *Drosophila* to obtain detailed, two-dimensional x-ray diffraction patterns of working muscle in a living organism. We demonstrated a new application of coupling the molecular genetic toolkit of *Drosophila* to high-resolution biophysical measurements. The in vivo technique complements experiments on excised, skinned preparations, and advances *Drosophila* as a model system for studying integrative biology (Maughan and Vigoreaux, 1999). The demonstrated ability to carry out x-ray studies in transgenic flies with defined alterations of muscle proteins will increase our understanding of how they function in vivo.

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