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A GENETIC DEFICIENCY THAT SPANS THE FLIGHTIN GENE OF *DROSOPHILA MELANOGASTER* AFFECTS THE ULTRASTRUCTURE AND FUNCTION OF THE FLIGHT MUSCLES

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Summary

We have developed a reverse-genetic approach to study the function of flightin, a unique protein of the flight muscle myofibril of *Drosophila melanogaster*. We describe the generation and characterization of *Df(3L)fln1*, a lethal genetic deficiency in the 76BE region of the third chromosome which deletes several genes, including the gene for flightin. We show that heterozygous flies harboring the *Df(3L)fln1* mutation exhibit both impaired flight and ultrastructural defects in their flight muscle myofibrils. We found that the mutation does not interfere with assembly of the myofibril but leads to disorganization of peripheral myofilaments in adult myofibrils. Most myofibrils, nevertheless, retain an intact core that represents approximately 80 % of the normal lattice diameter. Mechanical analysis of single skinned flight muscle fibers demonstrates that the mutation has no significant effect on net power output but increases the frequency at which maximum power is delivered to the wings, potentially reducing the overall performance of the flight system. The results suggest that flightin is an indispensable part of the flight muscle contractile mechanism.

Key words: *Drosophila melanogaster*, flightin, flight muscle, muscle mutant, stretch activation, mutation.

Introduction

Striated muscle cells contain a highly ordered, three-dimensional cytoskeletal network specialized for the generation and transmission of contractile forces. Myosin-containing thick filaments and actin-containing thin filaments are organized as interdigitating arrays that allow the cyclical interaction of myosin with actin, thus producing the force for work. The spatial arrangement of contractile myofilaments is dictated in part by two cross-linking structures, the M line and the Z band, whose variation in structure in part reflects tissue and/or fiber-specific differences in muscle properties (Squire, 1986; Schachat *et al.* 1985; Vigoreaux, 1994). The assembly and stability of the sarcomeric lattice results from diverse but poorly defined molecular interactions among structural proteins. Temporal scaffolding structures (e.g. sleeves of microtubules, Reedy and Beall, 1993) have been shown to participate in muscle fiber assembly but play no role in defining the structural stability of the actively contracting fiber.

Contracting muscle cells undergo continuous changes in cell shape. A highly dynamic cytoskeleton well-suited to respond and adjust to internally derived tension and externally applied stress underlies these changes. Although the mechanism for generating contractile force has received a lot of attention, the ability of the muscle cell to contract and return to its original shape unscathed is often taken for granted. As a result, we know very little about the mechanism for preserving cellular integrity and of the molecular interactions that define the dynamic nature of the cytoskeleton.

Genetic approaches have proved exceedingly useful for the analysis of muscle fiber assembly and muscle function (for reviews, see Epstein and Fischman, 1991; Bernstein *et al.* 1993). The indirect flight muscles (IFMs) of *Drosophila melanogaster* are well suited for genetic studies because the effects of a mutation can be studied *in vivo* as well as *in situ* using a combination of well-established functional, ultrastructural, mechanical and biochemical assays (Tohtong *et al.* 1995; Kreuz *et al.* 1996; Dickinson *et al.* 1997). Several mutations in contractile protein genes have been identified that appear to have little or no effect on myofibril assembly but which lead to rapid degeneration of the adult muscle. For example, Kronert *et al.* (1995) reported the characterization of three missense alleles of the myosin heavy chain gene that cause single amino acid changes in the light meromyosin region of the rod. None of the mutations appears to interfere with the normal assembly of myofibrils, but all three lead to sarcomeric degeneration as the adult ages (Kronert *et al.* 1995). An age-dependent degeneration of flight muscle has also been

observed in some troponin T and troponin I mutants, supporting the view that contractile activity is responsible for this process (Fyrberg *et al.* 1990*a*; Beall and Fyrberg, 1991). Further analyses of these mutants will be needed in order to understand the underlying molecular mechanism that leads to structural collapse. Nevertheless, it seems clear that the molecular interactions necessary for muscle fiber assembly are not sufficient to maintain the integrity of the actively contracting muscle.

Here, we report the generation and characterization of a new *Drosophila melanogaster* mutation that affects the ultrastructure and performance of the IFMs. The mutation is a lethal chromosomal deficiency that removes approximately 650 kb of the 76BE region of the polytene chromosome. This region encompasses the single-copy gene for flightin, a 20 kDa myofibrillar protein that is associated with the thick filaments (Vigoreaux *et al.* 1993). We show that flies heterozygous for the deficiency show reduced flightin accumulation in the IFM, which nevertheless assembles normally but degenerates in adult flies. Myofibril fragmentation is accompanied by a decrease in flight performance and slightly altered mechanical properties of skinned IFM fibers. The mutation has a dominant effect on flight muscle structure but a much weaker effect on muscle function *in vivo*. We propose that flightin fulfills an essential role in *Drosophila melanogaster* IFM.

Materials and methods

Genetics

TE4 is a line carrying a single TE transposable element inserted in 76EF (Ising and Block, 1984). This transposable element carries the w^a gene which, when expressed in a *white* background, can be used to score for putative deletions. Homozygous *TE($w^a rst^+$)* flies were first mated to *w/w; e/e* flies, and *TE($w^a rst^+$)e* recombinants were selected from the progeny. Homozygous *w; TE($w^a rst^+$)e* males were irradiated with 4000 rad (40 Gy) and mated to virgin *w; +* females. Progeny with *w* or non-w^a eyes were mated singly to *w/w; TM6e/TM3eSb* to establish balanced stocks (see Fig. 1). Nine non-w^a lethal mutations were recovered from a screen of approximately 25 000 chromosomes. One mutation, *Df(3L)fln1*, is a deficiency that uncovers the flightin gene. Genetic characterization of this mutation will be published elsewhere. *Ax27, Ax7* and *Ax18* are homozygous lethal *w* lines obtained from the same screen as *Df(3L)fln1*. All three complement *Df(3L)fln1* and do not affect the flightin gene. *Df(3L)kto2* was obtained from Jim Kennison.

The original flightin deficiency was recovered in a chromosome that carried a second deletion in the right arm of chromosome 3 (region 91–92). We separated the two mutations by crossing over using the markers *ri* and *e*. Flies carrying only the deletion in region 91–92 had normal flight characteristics, wingbeat frequency and fiber mechanics. Flies that carried both the deletion in region 91–92 and the flightin deficiency behaved similarly to flies carrying only the flightin deficiency.

In situ *hybridization and DNA blots*

In situ hybridizations to third-instar larva polytene chromosomes followed standard procedures (de Frutos *et al.* 1990). The probe used is a 20 kb lambda genomic clone selected from a library screen on the basis of its hybridization to a flightin cDNA probe. This clone contains the entire flightin coding region together with approximately 18 kb of DNA distal to the flightin gene and approximately 2 kb proximal to it. The DNA was labeled with digoxigenin dUTP by random priming (Boehringer Mannheim). Some chromosome preparations were stained with orcein (Ising and Block, 1981).

High-molecular-mass DNA for genomic Southern blots was extracted from 50 flies using the ‘single fly’ preparation method described by Ashburner (1989). The DNA was digested with *Eco*RI or *Sal*I, separated by gel electrophoresis and blotted following standard procedures (Sambrook *et al.* 1989). DNA probes were labeled with ^{32}P[dCTP] using the BioPrime DNA labeling system (Gibco BRL). All DNA fragments used as probes were obtained from a chromosome walk of the flightin genomic region.

Autoradiograms of genomic Southern blots (Kodak X-omat AR film) were scanned with a Molecular Dynamics laser scanning densitometer and analyzed using Quantity One software (PDI, Huntington Station, NY, USA). Background subtraction and image-processing was carried out according to the manufacturer’s instructions using default parameters.

Dot blots of embryonic DNA were performed essentially as described by Kreuz *et al.* (1996) with the following modifications. Embryos from *Df(3L)fln1*/*TM6cSb* were collected on agar plates, dechorionated in 50 % bleach, and transferred individually to nitrocellulose filters. The filter was first hybridized to a radioactive probe derived from a flightin genomic clone and then exposed to X-ray film for approximately 72 h. After viewing, the probe was stripped by boiling, and the filter was re-exposed, to verify that the probe had been removed, and then re-hybridized to a control probe derived from the 98F genomic region.

Flight test and wingbeat frequency analysis

Individual 2- to 5-day-old adult flies were tested for flight ability in an acrylic box (Drummond *et al.* 1991). A flight index was calculated by averaging the weighted scores from six trials following the scoring criteria described previously (Tohtong *et al.* 1995).

Following flight tests, a subset of flies was selected randomly for wingbeat frequency analysis (Hyatt and Maughan, 1994).

Skinned fiber mechanics

Dorsal longitudinal flight muscle fibers were isolated from flies tested for flight ability and wingbeat frequency. Fibers were dissected and skinned in a relaxing solution [5 mmol l^{-1} ATP, 15 mmol l^{-1} creatine phosphate, 240 units ml^{-1} creatine phosphokinase (Sigma Chemicals), 1 mmol l^{-1} free Mg^{2+}, 5 mmol l^{-1} EGTA, 20 mmol l^{-1} Bes (pH 7.0), at an ionic

strength of 175 mmol l^{-1} adjusted using sodium methane sulfonate] containing 0.5 % Triton X-100. After skinning for 1 h at 4 °C, aluminum T clips were attached to the fiber ends, and the fiber was transferred to an oil-filled temperature-controlled chamber. The T clips were then attached to an Akers force transducer and a piezoelectric length driver. Fibers were activated by increasing the Ca^{2+} concentration to a pCa of 5.0. Sinusoidal length changes were applied (0.25 % peak to peak) over 47 frequencies from 0.5 to 1000 Hz using an IBM PC (486DX-100 MHz; ZEOS International, Minneapolis, MN, USA) and a 16-bit data-acquisition board (DT2838; Data Translation, Inc., Malboro, MA, USA). At each frequency, the amplitude ratio of the force response to the length response was calculated and the phase difference between the force response and the length response was measured in order to obtain the complex stiffness of the fiber. The complex stiffness was normalized to the fiber dimensions (cross-sectional area and length) to obtain the complex modulus, which consists of the elastic modulus (stiffness in phase with the length change) and the viscous modulus (stiffness 90 ° out-of-phase with the length change). Oscillatory power output (P, W m^{-3}) (normalized to fiber volume) was calculated from the viscous modulus, the relative amplitude and the frequency of the length change:

$$P = \pi f E_{\mathrm{v}} [(\Delta L/L)_{\mathrm{rms}}]^2 , \quad (1)$$

where f is frequency (Hz), E_{v} is viscous modulus (kN m^{-2}), ΔL is half the peak-to-peak amplitude of the applied sinusoidal length change, and L is the length of the muscle. Additional details of this technique are provided elsewhere (Kreuz *et al.* 1996; Dickinson *et al.* 1997).

At the end of each experiment, the fiber was fixed for electron microscopy by exchanging half the rigor solution volume with rigor solution containing 1 % (w/v) glutaraldehyde.

Electron microscopy

The effect of the mutation on IFM structure was examined by electron microscopy. The structure of mutant IFM was compared with that of the wild type (Oregon R strain) before (pupal stage P15; Bainbridge and Bownes, 1981) and after (2- to 3-day-old adult) the first flight episode. For adults, IFM was examined both in the native state (i.e. fixed in the thorax) and after dissection and skinning (see previous section).

Adult and pupal half-thoraces were prepared for electron microscopy essentially as described by Warmke *et al.* (1992) except that dissection and fixation were carried out in a buffer containing 2 % paraformaldehyde, 1 % glutaraldehyde, 0.1 mol l^{-1} sucrose, 0.001 mol l^{-1} EDTA and 0.2 mol l^{-1} sodium phosphate buffer, pH 7.1. The head, abdomen and legs were removed during dissection, and the complete half-thorax, after fixation and washes (Warmke *et al.* 1992), was embedded in Spurr's low-viscosity embedding media, transferred to gelatin capsules and polymerized overnight at 60 °C. The thorax musculature (IFM and tubular muscles) was surveyed by light microscopy of sections (1 μm thick) stained with Toluidine Blue to identify tissues that may have been damaged during dissection. Only undamaged half-thoraces were further sectioned for electron microscopy.

Quantitative western blots

Sample preparation

Individual flies, aged 2–5 days, were flight tested and assigned a flight index as described above. After the test, flies were cold-anesthetized and stored overnight at –20 °C in acetone. The flies were then lyophilized, and the thorax was dissected and homogenized in a modified Laemmli sample buffer containing 8 mol l^{-1} urea and a cocktail of protease inhibitors (Vigoreaux *et al.* 1991).

Gel electrophoresis and immunoblotting

Thorax homogenates were incubated at 50 °C for 20 min and loaded on a 12 % SDS–PAGE, Bio-Rad Mini-Protean II gel system. Immunoblotting conditions were as described previously (Vigoreaux *et al.* 1993) except that proteins were transferred to ImmuneLite membrane (Bio-Rad). Each blot was cut in half horizontally. The top portion was incubated with an α-actinin monoclonal antibody (mAb) (Vigoreaux *et al.* 1991) and the bottom half with a flightin mAb (Vigoreaux *et al.* 1993). Proteins were detected using a chemiluminescent substrate from Bio-Rad. Each immunoblot was exposed to Kodak XR5 film for different lengths of time, ranging from less than 1 min to 20 min. Exposures in which the intensity of protein bands was within the linear absorbance range of the film were used for quantitative analyses.

Quantification

We first established the linear range of protein detection by performing a serial dilution experiment. Protein concentration was determined using the Bio-Rad DC protein assay. Autoradiograms containing decreasing amounts of thorax proteins were scanned with a laser densitometer and analyzed using Quantity One software (PDI). Each sample was assayed in duplicate. From this, we plotted a standard curve of thorax protein content *versus* absorbance. For quantification, we selected a protein concentration at which the intensities of the α-actinin band and the flightin band fell within the linear range. To control for loading differences, each flightin signal was normalized to the α-actinin signal.

Results

Identification of a chromosomal deficiency that deletes the flightin gene

Using a combination of genetic, molecular and cytogenetic techniques, we mapped the single-copy flightin gene to polytene region 76E1,2 (Fig. 1A). To generate deletions in this region, we used TE4, a line carrying a single TE transposable element inserted in 76EF (Ising and Block, 1984). This TE element contains the *white-apricot* (w^a) gene which serves as a convenient marker for mutagenesis (Ising and Block, 1984). When expressed in a *white* genetic background, flies carrying the TE transposable element can be easily distinguished from

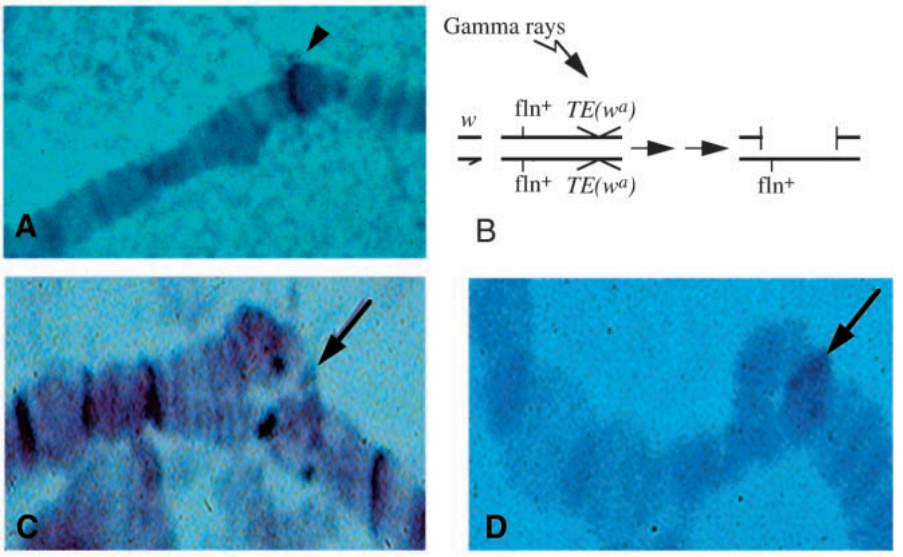

Fig. 1. Generation and cytological characterization of *Df(3L)fln1*. (A) *In situ* hybridization of wild-type polytene chromosomes with a digoxigenin-labeled flightin genomic probe. A single band of hybridization is detected that maps to 76D/E. (B) Schematic diagram of the radiation screen. The w^a gene present in the TE transposable element inserted in 76EF was used as a marker to identify deletions in that region. White-eyed males homozygous for a TE4 third chromosome were exposed to gamma-irradiation and mated to white-eyed females homozygous for a wild-type third chromosome. Progeny with white eyes were examined cytologically for deletions of the 76DE region. (C) Polytene chromosomes from *Df(3L)fln1*/+ stained with orcein. The deleted region extends from approximately 76B to 76EF. (D) *In situ* hybridization of *Df(3L)fln1*/+ polytene chromosomes with the same probe as in A. Note that the hybridization band is found in an asynaptic region. Arrows point to the location of the flightin gene.

those in which the TE has been deleted as a result of exposure to irradiation (Fig. 1B).

We screened approximately 25 000 F_1 progeny and identified nine lines that were *white* and homozygous viable. All these lines expressed flightin and were not studied further. Nine additional lines expressing either *w* or w^a phenotype were also homozygous lethal, suggesting that the mutation could be a deletion because the original TE insertion does not result in lethality. These lines were examined cytologically for visible deletions and by genomic Southern blots for restriction fragment length differences. None of the lines showed qualitative differences on blots using probes encompassing the flightin gene and approximately 26 kb of adjoining DNA (data not shown). One line, herein referred to as *Df(3L)fln1*, contains a cytological deletion that extends from approximately 76B to 76EF (Fig. 1C). *In situ* hybridization with a flightin genomic probe shows that the flightin gene lies within the region deleted by this mutation (Fig. 1D).

Further evidence that the flightin gene is deleted in *Df(3L)fln1* is shown in Figs 2 and 3. The mating scheme outlined in Fig. 2A shows that approximately 25% of the embryos from *Df(3L)fln1*/TM3 parents should be missing both copies of the flightin gene if *Df(3L)fln1* uncovers the flightin gene. DNAs from individual embryos collected from *Df(3L)fln1*/TM3 parents were fixed onto a nitrocellulose membrane and hybridized to a 2.8 kb genomic DNA probe that includes the flightin gene. As seen in Fig. 2B, DNA from 11 of the 40 embryos did not hybridize to this probe; eight of these embryos, however, hybridized to a control probe from the 98F genomic region (Fig. 2C).

Fig. 3 shows a Southern blot of adult genomic DNA extracted from *Df(3L)fln1* and other strains that are known to be diploid for flightin. Probes from genomic regions that are deleted in one of the two chromosomes should, in theory, produce hybridization signals that are approximately 50% less intense than probes from regions that are diploid. Therefore, we hybridized a Southern blot first with a probe from a genomic region not deleted in *Df(3L)fln1* (probe E2, Fig. 3) and then with a probe from the flightin gene region (probe B3). The first probe hybridized to a fragment of 14 kb in *Sal*I-restricted DNA and to two fragments of 12 kb and 3.5 kb in *Eco*RI-restricted DNA (only the 3.5 kb fragment is shown in Fig. 3). After visualization, the probe was stripped and the blot rehybridized to probe B3, from the genomic region just 3′ to the flightin gene. A visual comparison of the intensity of hybridization of the two probes revealed that probe B3 produced a less intense signal than probe E2 in *Df(3L)fln1* DNA but not in DNA from the other strains. To establish that the decreased hybridization is due to a reduction in copy number, we quantified the hybridization signal by scanning densitometry and normalized the optical measurements to that of the control DNA samples. The results showed that the hybridization of probe E2 to *Sal*I-digested DNA produced a signal that is 2.46 times more intense in the *Df(3L)fln1* sample than in the *TE4* sample. Probe B3, in contrast, produced a signal that is only 1.1 times more intense in the *Df(3L)fln1* sample than in the *TE4* sample. This represents a reduction of approximately 54%. Similarly, the intensity of the probe E2 hybridization signal in the *Eco*RI-digested DNA is 2.4 times stronger in the *Df(3L)fln1* sample than in the *TE4* sample, but this is reduced by 67% (0.78 times) when using probe B3. In summary, these results are consistent with the interpretation that the flightin gene is deleted in *Df(3L)fln1*.

Df(3L)fln1 *mutants show impaired flight behavior but normal wingbeat frequency*

Since *Df(3L)fln1* heterozygotes have only one flightin gene, we investigated the effect of this mutation on flight

performance and wingbeat frequency. Flight indices were determined for the mutant line *Df(3L)fln1*, the parental line *TE4*, the mutant line *Ax27* and the mutant line *Df(3L)kto2*. *Ax27* and *Df(3L)fln1* have an identical genetic background since these two lines were obtained from the same screen. In addition, *Ax27* flies have white eyes and are used as a control line for the flight test since this assay relies on the phototrophic response (Drummond *et al.* 1991). *Df(3L)kto2* (76B1-2; 76D5) is a deficiency that removes most of the DNA deleted in *Df(3L)fln1* but whose proximal breakpoint does not extend into the flightin gene region. As seen in Table 1, the flight index of the *Df(3L)fln1* strain is significantly reduced compared with those of all the control strains. In contrast, the flight indices of the control strains are comparable with those reported for wild-type strains (Tohtong *et al.* 1995; Kreuz *et al.* 1996). The wingbeat frequency of *Df(3L)fln1* flies tends to be reduced compared with those of other strains, but the difference is not significant.

One characteristic feature of the *Df(3L)fln1* strain that is not seen in any of the control strains is the high variability in flight performance among individuals. Of 106 individuals scored, 40.6 % were flightless (FI=0–2), 23.6 % had intermediate flight indices (FI=2.1–4.0) and 35.8 % were normal or nearly normal flyers (FI=4.1–6). The variance for *Df(3L)fln1* is more than twice the variance of any of the control strains (Table 1). *Df(3L)fln1* flies also exhibited a wider range of wingbeat frequencies than control strains.

The intrastrain variability in flight performance observed in the *Df(3L)fln1* line could result from different levels of flightin expression in the haploid state given that these flies still retain one functional copy of the flightin gene. To test for this possibility, we measured the amount of flightin in the IFMs of individuals that differed in flight performance. Compared with wild-type flies, *Df(3L)fln1* flies showed reduced levels of flightin accumulation averaging approximately 22 %. However, we did not detect any significant differences in flightin accumulation between extreme flyers and extreme non-flyers. It is possible that small variations in flightin accumulation, too subtle to be detected by our methods, may still account for differences in flight performance. If this were the case, it would suggest that there is a finely defined threshold of flightin accumulation necessary for flight.

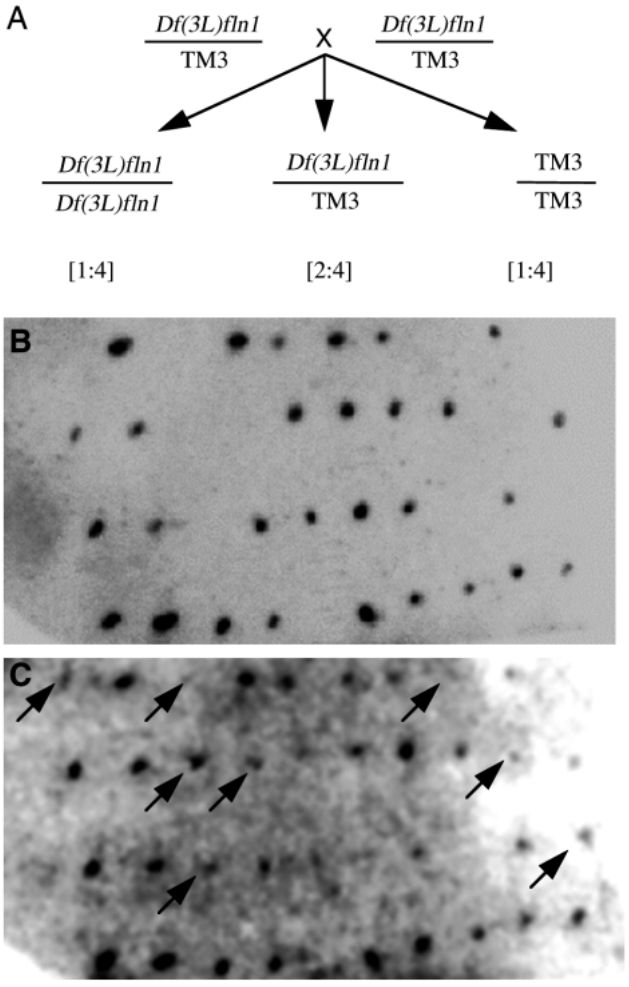

Fig. 2. DNA blots of *Df(3L)fln1/TM3* embryos. (A) Mating between *Df(3L)fln1*/TM3 parents produces embryos of three different genotypes as depicted. If *Df(3L)fln1* uncovers flightin, an expected 25 % of the embryos will not show hybridization to a flightin gene probe. (B) A blot containing 40 embryos was hybridized to a genomic probe that spans the entire flightin gene. (C) The same blot as in B was boiled to remove the hybridized flightin probe and rehybridized with a control probe. Note that an additional eight DNA spots are detected with this probe (arrows). These embryos are homozygous for a deletion that spans the flightin gene.

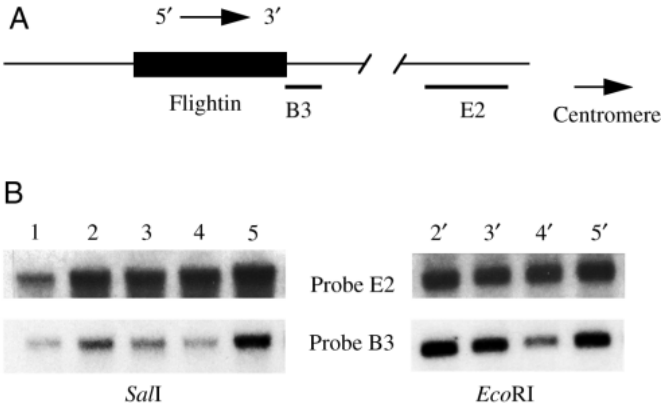

Fig. 3. Southern blot analysis of genomic DNA showing that flightin is deleted in *Df(3L)fln1*. (A) Schematic diagram showing the locations of the probes in relation to the flightin gene. Not drawn to scale. (B) Genomic DNA was extracted and digested with either *Sal*I or *Eco*RI. The same blot was hybridized first to probe E2 and then to probe B3. Only one portion of the blot is shown for each lane, which in some cases showed more than one band of hybridization. Lane 1, *TE4/TE4*; lanes 2 and 2′, *TM3/TM6*; lanes 3 and 3′, *Ax7/TM3*; lanes 4 and 4′, *Df(3L)fln1/TM3*; lanes 5 and 5′, *Ax18/TM3*. Note that the probe B3 hybridization signal is weaker than the probe E2 signal for the *Df(3L)fln1* sample but not for the other samples. The decreased amount of B3 hybridization signal indicates that *Df(3L)fln1* is haploid for the flightin genomic region.

Table 1. *Flight index and wingbeat frequency of fly strains used in this study*

Strain	Flight index†	Variance	*N*	Wingbeat frequency (Hz)	Variance	*N*
TE4‡	4.94±0.09	1.89	44	201±7	565	13
Df(3L)fln1	3.32±0.19*	3.86**	106	193±5	792	37
Ax27	5.41±0.21	1.06	24	204±7	441	9
Df(3L)kto2	6.0±0	0	11	199±10	276	3

All values are means ± S.E.M.
†See Materials and methods.
‡Includes both homozygous and heterozygous flies.
**P*<0.05 for *Df(3L)fln1 versus TE4*.
***From *F* distribution *P*<0.05 for *Df(3L)fln1 versus TE4*; *P*<0.005 for *Df(3L)fln1 versus Ax27* and *Df(3L)fln1 versus Df(3L)kto2*.

Df(3L)fln1 *affects the myofibrillar structure of adult flight muscle*

We examined the ultrastructure of mutant heterozygote IFM by electron microscopy to determine whether the reduced synthesis of flightin has any effect on the assembly and/or integrity of the myofibril. We first looked at the flight muscle of late-stage pupa (stage P15), just prior to eclosion. At this stage, the IFM is almost fully developed (Reedy and Beall, 1993), but has probably not yet been subjected to the mechanical strains of activation and contraction. Fig. 4A shows a transverse section of a wild-type fiber and Fig. 4B shows a similar section from a *Df(3L)fln1* heterozygote. There are no discernible differences between the myofibrillar structure of the mutant pupa and that of the wild-type pupa. In both cases, the myofilaments are arranged in a double-hexagonal array and the myofibrils have a well-defined cylindrical shape of constant diameter (wild type, 23.2±0.29 thick filaments across the fibril diameter, *N*=10; mutant, 26.5±0.22 thick filaments, *N*=6, means ± S.E.M.). Fig. 4C,D shows longitudinal sections of wild-type and mutant IFM, respectively. Both are characterized by well-defined sarcomeres of uniform width and length (wild type, 2.81±0.03 μm long, *N*=16; mutant, 3.29±0.03 μm long, *N*=14) and Z bands that are straight throughout the full width of the

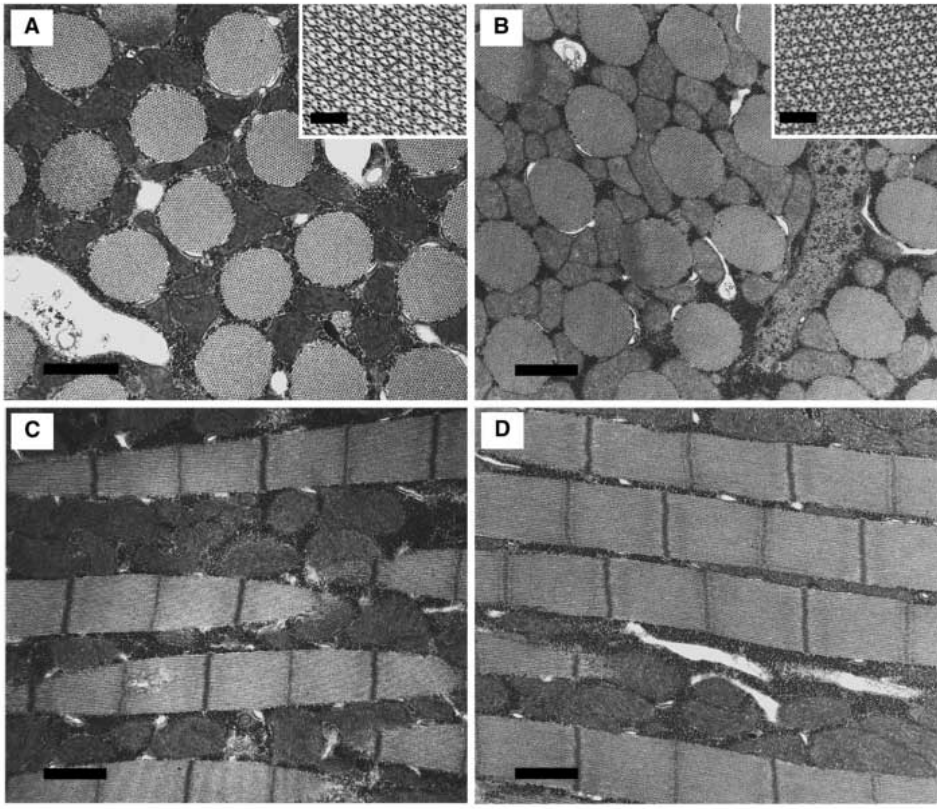

Fig. 4. Flight muscle ultrastructure of wild-type and *Df(3L)fln1/+* late-stage pupa. (A) Transverse section of a wild-type pupal fiber. Indirect flight muscle (IFM) myofibrils are characterized by their cylindrical shape and the regular double-hexagonal array of myofilaments. Other features typical of IFM are the abundant, large mitochondria and pools of glycogen granules. Inset: high-magnification view of a myofibril. Note that each thick filament is surrounded by six thin filaments. This crystalline arrangement is also typical of adult myofibrils. (B) Transverse section of a *Df(3L)fln1* heterozygote pupal fiber. Note the uniformly round myofibrils and overall features that are indistinguishable from those of the wild type. Inset: high-magnification view of a myofibril showing a well-ordered hexagonal array. (C) Longitudinal section of a pupal wild-type fiber. Myofibrils are characterized by sarcomeres of constant length and width that are demarcated by electron-dense Z bands which are straight and of uniform width. (D) Longitudinal section of a pupal *Df(3L)fln1/+* fiber. Myofibrils appear normal by all criteria. The length and width of the sarcomere are similar to those of wild-type sarcomeres. Scale bars, 1 μm; scale bars in insets, 0.25 μm.

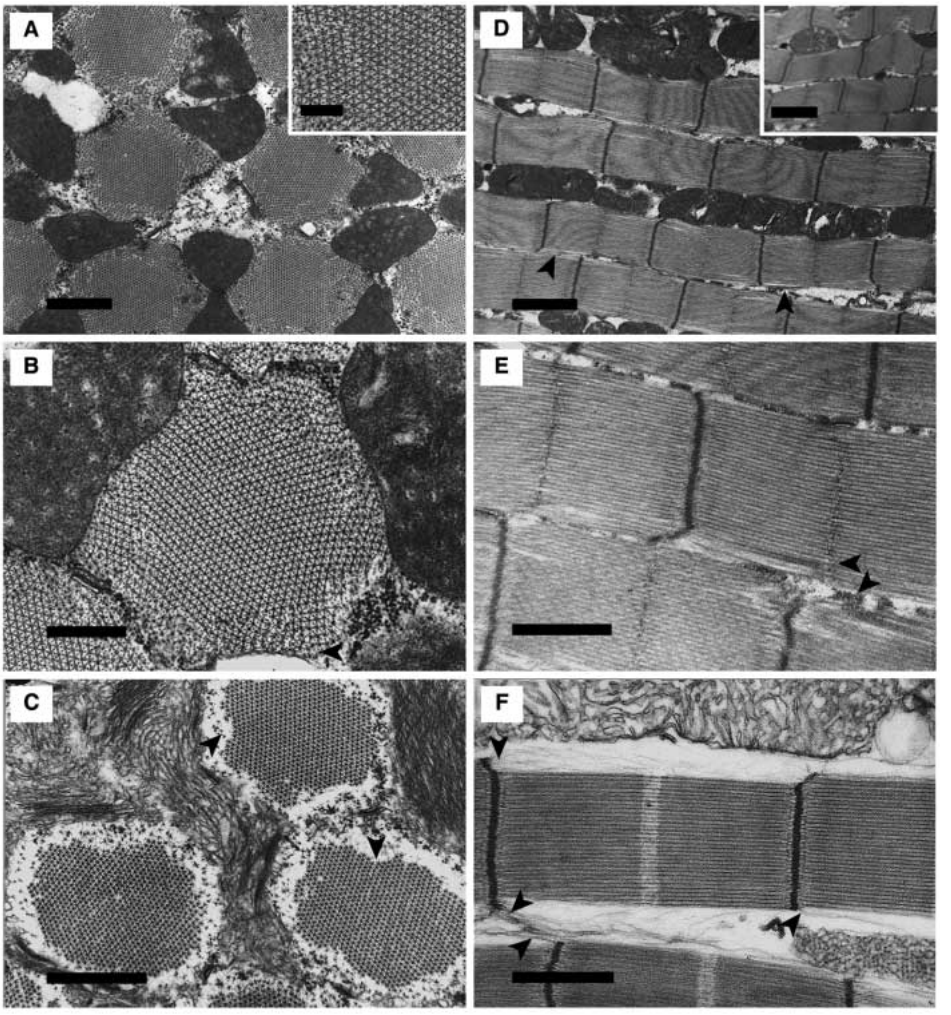

Fig. 5. Myofibrillar defects in adult *Df(3L)fln1* heterozygote indirect flight muscle. (A) As seen in this transverse section, myofibrils from mutant adult flies do not have a well-defined circumference. Note that the central core region of the myofibril retains the regular double-hexagonal arrangement of myofilaments seen in pupal myofibrils but that the arrangement is less regular in the periphery. On some occasions, sections of peripheral myofilaments are separated by large gaps from the rest of the myofibril. (B) Closer view of a myofibril from A. The tight hexagonal packing in the center of the myofibril is obvious but becomes gradually less regular further away from the center; peripheral myofilaments are loosely organized (arrowhead). (C) Transverse section of a fiber taken from a mutant adult and skinned with a detergent solution to solubilize mitochondria and other membrane-bound organelles. Although the integrity of the myofibrillar core is not affected by this treatment, the peripheral myofilaments are washed away, resulting in pronounced surface indentations (lower arrowhead). The majority of the remaining myofilaments are incorporated in hexagonal lattices, but a few peripheral units are seen in which the arrangement is not 6:1. Occasional loose myofilaments are seen (upper arrowhead). (D) Longitudinal section of an adult mutant fiber showing sarcomeres with some structural defects. The majority of sarcomeres are of normal length and nearly normal width, but a few hyperextended or hypercontracted sarcomeres (inset) are also present. Note the large gaps that run along the axis of the myofilament lattice (arrowhead). The edges of the Z band are not straight, as in wild-type fibers, but bent and crooked. (E) Higher magnification of sarcomeres from D showing that the myofilaments are evenly spaced throughout most of the lattice except at the periphery (arrowheads). The distortion of the Z band is limited to the peripheral region. (F) Longitudinal section of a detergent-skinned mutant fiber. Note that the core of the myofibril has been well preserved. As seen in C, the majority of loosely organized peripheral myofilaments have been removed by this treatment, but some remain and are seen to wander off the long axis of the myofibril (arrowheads). Sections of the Z bands near the edge are seen to be devoid of myofilaments. Scale bars: A, C, E, F, 1 μm; A inset, 0.25 μm; B, 0.5 μm; D, 2 μm.

sarcomere. The slightly longer sarcomere length and wider myofibril diameter of mutant fibers suggest that this particular pupa was further ahead in development than the wild-type pupa since sarcomeres are known to continue their growth well into the early hours of adulthood (Reedy and Beall, 1993). More importantly, the lack of any apparent structural defect suggests that an approximately 20 % reduction in flightin synthesis does not affect myofibrillar assembly during the pupal stages of development.

In contrast to pupal IFMs, flight muscles of *Df(3L)fln1* adults show consistent perturbations in the lattice structure that are pronounced in the peripheral region of the myofibril. In transverse sections of adult IFM, the thick filaments and thin filaments form regular hexagonal lattices throughout most of the myofibril, but less so near the outer edges (Fig. 5A,B). Myofilament arrays that deviate from the 6:1 ratio are common, as are loose, randomly scattered thin filaments (arrowhead in Fig. 5B). Occasional gaps in the myofibril appear to divide sections of the lattice from the main core. All the myofibrils have irregular shapes and cross-sectional areas which differ from the regular, cylindrical shape characteristic of pupal IFM (compare Fig. 4A and Fig. 5A).

The myofibrillar defects are also evident in longitudinal sections (Fig. 5D,E). The defects seen most consistently are skewed Z bands and gaps in the myofilament lattice that extend along the long axis of the myofibril. It is also evident, as in cross-sections, that peripheral myofilaments are not well integrated into the lattice (Fig. 5E). While the length and width of most sarcomeres are comparable with those of the wild type, some sarcomeres appear hypercontracted while others appear

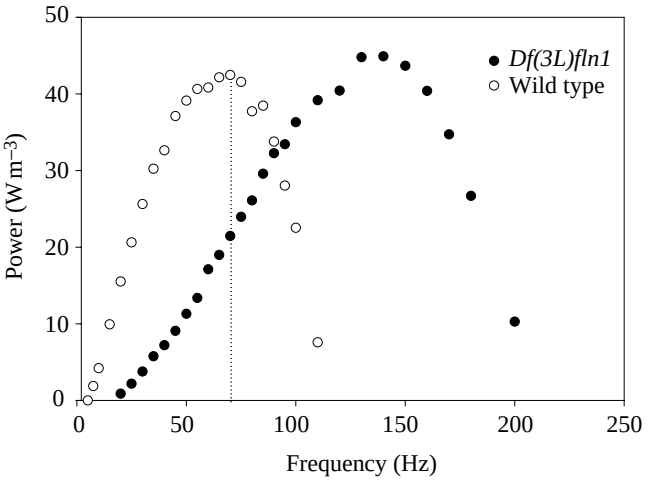

Fig. 6. *Df(3L)fln1* mutant fibers deliver reduced power at normal frequency for maximal power output (f_{max}). Representative results for fibers from wild-type (open circles) and *Df(3L)fln1* (filled circles) flies are shown. Power was calculated from sinusoidal analysis at 12 °C as described in Materials and methods. Both fibers exhibit a similar power profile and a comparable magnitude of power output. However, note that, at f_{max} for the wild-type fiber (dotted line), the mutant fiber is only able to deliver approximately 50 % as much power.

hyperextended (Fig. 5D inset). There are no differences between the numbers of myofibrils per unit area in the mutant and the wild type.

Warmke *et al.* (1992) reported that treating fibers with a detergent skinning solution had a relatively minor effect on the lattice structure of wild-type myofibrils. However, the same treatment resulted in a striking reduction in the number of myofilaments that remained incorporated in the myofibril lattice of the myosin regulatory light chain null heterozygote *Mlc2*^{E38}. The treatment reduced the number of thick filaments per myofibril by approximately 57 %, which is roughly proportional to the reduction in MLC2 synthesis seen in these mutant fibers (Warmke *et al.* 1992). The results suggested to them that peripheral myofilaments slough off the myofibril core as a result of the lack of MLC2, which in turn affects the interaction between thick filaments and thin filaments (Warmke *et al.* 1992).

To establish whether a similar effect is seen in IFM deficient for flightin, we examined by electron microscopy fibers that had been pre-incubated in skinning solution before fixation. As seen in the transverse section in Fig. 5C, skinning the fiber revealed an irregularly shaped myofibril core. Many of the peripheral myofilaments that appeared to be loosely associated with the core were removed by this treatment. In contrast, non-mutant fibers treated in the same solution tended to lose only a few peripheral myofilaments, but not enough to affect the overall cylindrical shape of the myofibril (see Fig. 5 in Warmke *et al.* 1992). In both wild-type and mutant fibers, the myofilaments that remained associated with the myofibril retained, for the most part, their proper hexagonal packing. This is also evident in longitudinal section (Fig. 5F). Note that the bulk of the lattice appears intact except for the myofilaments peeling away from the surface of the myofibril.

The removal of myofilaments that are loosely associated gives us an opportunity to examine what fraction of the myofibrillar lattice has assembled properly. The reduction in myofibril diameter should be proportional to the reduction in flightin stoichiometry assuming that flightin is distributed homogeneously throughout the width of the A band of a normal sarcomere. Skinned fibers from *Df(3L)fln1* have 28.8±0.35 (N=2) thick filaments along their longest diameter, while similarly treated non-mutant fibers (JW60 rescued strain, Warmke *et al.* 1992) have 33.6±0.34 (N=2) (means ± S.E.M.) thick filaments across their diameter. This corresponds to a 26 % reduction in myofibril diameter, which closely parallels the approximately 20 % reduction in flightin accumulation obtained from gel quantification. If the distribution of flightin is non-uniform in the mutant fiber, then the reduction or complete absence of flightin near the myofibril periphery could be the reason why myofilaments become loose. In summary, these results are consistent with the interpretation that *Df(3L)fln1* does not interfere with myofibril assembly during the pupal stages of development but leads to sarcomeric disruptions in the adult IFM.

Df(3L)fln1 *mutant fibers show altered mechanics*

We performed mechanical test on Ca^{2+}-activated skinned IFM fibers to determine whether the mutation had any effect on force production and power output. Specifically, we studied the stretch activation response responsible for powering the oscillatory wing movements in *Drosophila melanogaster* and other insects (Pringle, 1978). The length of individual fibers was sinusoidally oscillated at frequencies ranging from 0.5 to 1000 Hz, and the amplitude and phase of the corresponding force response were used to calculate the negative viscous modulus from which oscillatory work and power were derived (Kawai and Brandt, 1980).

The maximum amplitude of the negative viscous modulus, which is proportional to oscillatory work, tended to be lower in *Df(3L)fln1* fibers (−101±23 kN m^{-2}) than in wild-type fiber controls (−164±24 kN m^{-2}) (means ± S.E.M.), but the difference was not statistically significant (P>0.05). Maximum power, calculated according to equation 1, was also within the normal range (51±12 W m^{-3} *versus* 52±10 W m^{-3} for wild-type fibers), but the frequency required to achieve maximum power (f_{max}) was significantly higher (P<0.005) in *Df(3L)fln1* fibers (127±5 Hz., N=14) than in wild-type fibers (83±11 Hz., N=7). Fig. 6 is a plot of frequency *versus* power for two fibers, one mutant and one wild-type, which showed a similar power profile and a comparable power magnitude. Note that, at the frequency at which power output is highest for the wild-type fiber, the mutant fiber produced approximately 50 % less power.

Crossbridge kinetic parameters (Table 2) can be extracted from the complex stiffness data and analyzed using a simple viscoelastic model of the sarcomere (Dickinson *et al.* 1997).

Table 2. *Mechanical parameters of skinned fibers from* Df(3L)fln1 *and wild-type flies*

	A ($kN\,m^{-2}$)	B ($kN\,m^{-2}$)	C ($kN\,m^{-2}$)	*b* (Hz)	*c* (Hz)	Power ($W\,m^{-3}$)	f_{max} (Hz)
Wild type	586±85	787±69	707±108	69±16	583±51	52±10	83±11
Df(3L)fln1	429±81	587±109	607±104	123±10*	590±21	51±12	127±5**

All values are means ± S.E.M.

For wild-type flies, N=7; for mutants, N=14.

*P<0.05, **P<0.005 compared with the value for wild-type flies.

The viscoelastic properties of the skinned indirect flight muscle fiber are modeled as three composite elements (*A, B* and *C*), each contributing to the total dynamic stiffness. At each frequency (*f*), the combined dynamic stiffness, $y(f)$, is expressed as the sum of the Fourier transforms representing each component: $y(f)=A(i2\pi f/\alpha)^k-Bif/(b+if)+Cif/(c+if)$, where $i=(-1)^{1/2}$, α is a constant (1 Hz, by definition) and k is a unitless exponent proportional to the phase shift angle (Kawai and Brandt, 1980). The coefficients A, B and C ($kN\,m^{-2}$) represent the magnitudes of *A, B*, and *C*, respectively, and *b* and *c* (Hz) the characteristic frequencies of *B* and *C*; 2π times the characteristic frequencies give the respective rate constants of the underlying biochemical transitions. The sum of all three terms reflects the properties of *passive* myofibrillar elements (lumped as the non-exponential component *A*) in parallel with *active* MgATP-dependent crossbridge elements represented by the two exponential components *B* and *C*. Component *B* represents the work-producing step of the crossbridge cycle, whose amplitude B is largest at frequency *b*. A two-step, iterative, least-squares curve-fitting routine was used to fit $y(f)$ to the dynamic stiffness data (Dickinson *et al.* 1997).

The increase in the apparent rate constant of the force-producing step, $2\pi b$, appears to be the basis for the increase in f_{max} (Kawai and Brandt, 1980).

Discussion

A new Drosophila melanogaster *locus that affects flight performance*

The results presented here suggest that *Df(3L)fln1* uncovers a new locus that affects flight muscle structure and function. As discussed below, we propose that this locus is the flightin gene.

Genetic and cytogenetic analyses of *Df(3L)fln1* indicate that the deletion has a distal breakpoint in the 76B region and a proximal breakpoint in the 76EF region. *Df(3L)fln1* complements *Df(3L)VW3* (proximal breakpoint 76B2) and *Df(3L)in61j* (distal breakpoint 76F), but fails to complement *Df(3L)kto2* (76B1,2; 76D5) (C. Hernandez and J. O. Vigoreaux, unpublished result). Unlike *Df(3L)fln1*, *Df(3L)kto2* does not complement *Df(3L)VW3*, suggesting that the distal breakpoint of *Df(3L)fln1* lies within *Df(3L)kto2*.

Despite the substantial overlap of *Df(3L)kto2* with *Df(3L)fln1*, the former does not impair flight. This suggests that the locus responsible for the muscle phenotype is found within the region deleted by *Df(3L)fln1* but not by *Df(3L)kto2*, i.e. the interval between 76D5 and 76EF. Further mutagenesis of this region has failed to reveal genes other than flightin that affect flight muscle function (C. Hernandez and J. O. Vigoreaux, unpublished results). Furthermore, no other gene that is expressed in muscle or that could affect muscle development has been mapped to this interval. This includes results from several flightless screens, muscle cDNA libraries, enhancer trap lines that show muscle staining and expressed sequence tags generated by the *Drosophila* Genome Project.

A large number of flightless mutants have been isolated from genetic screens designed to recover dominant autosomal mutations or mutations on the X chromosome of males, which are hemizygous for the mutant chromosome (for a review, see Bernstein *et al.* 1993). Many of these mutations affect contractile protein genes, although several have been shown to be in genes expressed in myogenic lineage or neuronal tissue. One of the first screens for flightless mutants identified three haploinsufficient genes: myosin heavy chain, actin and tropomyosin (Mogami and Hotta, 1981). It is not surprising that these are haploinsufficient loci given that the sarcomeric lattice is an intricate supramolecular structure that requires strict stoichiometric quantities of the major filamentous components (Homyk and Emerson, 1988; Beall *et al.* 1989). More recent screens have recovered many more alleles of actin and myosin heavy chain and have uncovered one additional haploinsufficient locus, named *laker*, whose function is not yet known (Cripps *et al.* 1994). A large screen to isolate dominant flightless mutants that map to the third chromosome yielded mostly actin alleles (An and Mogami, 1996).

A fourth locus that is haploinsufficient for flight maps to the myosin regulatory light chain gene (Warmke *et al.* 1989). Surprisingly, no mutations in this gene have been recovered from dominant flightless screens despite the pronounced flight impairment of the mutant heterozygotes (Warmke *et al.* 1989) and the fact that some genetically engineered mutants have been shown to affect only the IFM (Tohtong *et al.* 1995; Dickinson *et al.* 1997). Similarly, mutations in many other genes that encode sarcomeric proteins have not been recovered from dominant autosomal flightless screens. Some additional examples include myosin alkali light chain, projectin, troponin H, troponin C, the Z band proteins Z(210) and Z(400/600), paramyosin and flightin. It is possible that haploinsufficiency for flight and dominant flightlessness are phenotypes associated only with the major (i.e. most abundant) structural proteins of the myofibril (Cripps *et al.* 1994). Alternative strategies must be devised to identify mutations in other contractile protein genes. A targeted mutagenesis approach

such as the one described here and by Warmke *et al.* (1989) is extremely valuable when prior cytological mapping of the gene has been carried out.

Df(3L)fln1 *causes IFM degeneration*

Despite the approximately 20 % reduction in flightin accumulation, *Df(3L)fln1* IFM myofibrils assemble normally during pupal development. Myofibrils of uniform shape and diameter are filled entirely with myofilaments arranged in double-hexagonal arrays. During normal IFM development, myosin filaments and actin filaments appear simultaneously in interdigitating arrays approximately 42 h after pupation (Reedy and Beall, 1993). By the time flightin begins to accumulate (approximately 60 h after pupation), striated myofibrils showing hexagonal lattices are abundant. Thus, flightin is not required for the initial alignment of myofilaments. Furthermore, our results suggest that flightin is not required to maintain sarcomeric order during the pupal stages of development. In contrast, a reduction in MLC2 content in the mutant heterozygote $Mlc2^{E38}$ resulted in misregistered myofilaments in pupal IFM (Warmke *et al.* 1992).

Unlike pupal IFM, adult IFM of *Df(3L)fln1* flies is characterized by myofibrils that have an irregular shape and 'rings' of disorganized myofilaments. Peripheral myofilaments pull away from their lateral register and Z bands become distorted at their edges. We have not examined the time-dependent degeneration of the fibers, but an analogous phenomenon is seen in certain point mutants of the troponin I gene (Deak *et al.* 1982; Beall and Fyrberg, 1991), troponin T gene (Fyrberg *et al.* 1990*a*) and myosin heavy chain gene (Kronert *et al.* 1995). As has been proposed for these other mutants, the defect is likely to arise from abnormal interactions between thick filaments and thin filaments upon activation of contraction, shortly after the pupal-to-adult transition (Beall and Fyrberg, 1991). Alternatively, the collapse could result from the failure of the myofibril to respond to externally imposed mechanical strain, which would disrupt the force balance needed to maintain structural integrity (Ingber *et al.* 1994).

Myofibrils with well-preserved hexagonal lattices in the core but with disrupted peripheries are commonly observed in mutant heterozygotes of several myofibrillar proteins. Heterozygotes of the actin null mutation *KM88* were shown to have myofibrils with well-packed lattices except in the periphery, where many unintegrated thick filaments were observed (Beall *et al.* 1989). Flies heterozygous for $Mlc2^{E38}$ also showed disordered peripheral myofilaments (Warmke *et al.* 1992). These results are consistent with the idea that myofibril assembly begins at the core and grows outwards (Warmke *et al.* 1989; Reedy and Beall, 1993). The mutant heterozygote is only able to produce enough gene product to complete assembly at the core, and depletion of the product leads to misaligned peripheral myofilaments (Warmke *et al.* 1992). Our results suggest that myofibrillar incorporation of flightin also begins at the core and radiates outwards, despite the apparent delay in flightin synthesis with respect to initiation of myofibril assembly.

While the structural phenotypes of *Df(3L)fln1* and $Mlc2^{E38}$ adult heterozygotes share similar features (i.e. peripheral disruptions) and both are probably due to a reduction in the amount of gene product, these defects probably arise from different mechanisms. In contrast to *Df(3L)fln1*, the $Mlc2^{E38}$ defect is also evident during the pupal stages (Warmke *et al.* 1992). Thus, the misaligned myofilaments seen in $Mlc2^{E38}$ are the result of improper assembly while the misaligned myofilaments in *Df(3L)fln1* are the result of the instability of properly assembled myofilaments.

We also observed some occasional sarcomeres that had a shorter or a longer length. In particular, the shorter sarcomeres appeared to be hypercontracted, bulging out at the M-line level because of abutting thick filaments from apposing half-sarcomeres. A similar phenotype has been described for three myosin heavy chain alleles with point mutations in the light meromyosin region of the rod (Kronert *et al.* 1995). Interestingly, these three mutations are also characterized by their failure to accumulate flightin (Kronert *et al.* 1995). Thus, the reduction or absence of flightin from the sarcomere could be the cause of the hypercontracted phenotype.

Df(3L)fln1 *has little effect on flight performance and fiber mechanics*

Although *Df(3L)fln1* has a dominant effect on flight muscle structure, its effect on muscle function is less conspicuous. The nearly normal muscle function could be explained by the fact that mutant flies produce approximately 80 % as much flightin as wild-type diploids. The small reduction in flightin accumulation results in myofibrils with disrupted peripheries but whose core is structurally intact and sufficiently wide to produce enough power for some level of flight. We conclude that the flightin gene does not exhibit strict dosage-dependency and that one functional copy of the flightin gene per diploid genome is, in most cases, sufficient for flight muscle function.

The activity of many *Drosophila melanogaster* enzymes has been shown to be dosage-dependent (O'Brien and MacIntyre, 1978). The function of the IFM is in many cases very sensitive to gene dosage. As discussed above, mutant heterozygotes for the major contractile proteins (myosin heavy chain, myosin regulatory light chain, actin and tropomyosin, among others) exhibit dominant flightless behavior. Significant exceptions to the above examples include the null heterozygotes for the myosin light chain kinase gene (Tohtong *et al.* 1997) and for the α-actinin gene (Fyrberg *et al.* 1990*b*), both of which are fully flighted, despite the fact that the latter shows obvious structural defects in terminal sarcomeres. Heterozygotes of the actin null allele *KM88* are not completely flightless despite substantial myofibrillar disruption (Beall *et al.* 1989). In contrast, mutant heterozygotes of the TnH gene in which the amount of protein is reduced by 50 % show only mildly impaired flight and have nearly normal IFM structure (Kreuz *et al.* 1996).

It is possible that the impact of flightin haploidy on IFM function is more significant than our tests were able to measure. For example, the flight test measures the ability of the insect

to fly upwards a short distance after being released in a closed (30 cm×30 cm×40 cm) container. It is not possible to determine from this test whether the fly can sustain flight for a prolonged period or fly long horizontal distances. An apparatus similar to a wind tunnel used for selecting flying speed would be useful in this respect (Weber, 1988). Similarly, our experimental apparatus allows us to measure wingbeat frequencies during brief bouts of flight. However, modulation in flight force (through changes in wing stroke frequency and amplitude) that occur during prolonged flight are not quantifiable in our system (Lehmann and Dickinson, 1997).

The ability of some *Df(3L)fln1* individuals to fly normally suggests that the IFM is able to tolerate some degree of structural disruption. Oscillatory power production (work × frequency) was not compromised in *Df(3L)fln1* fibers despite the reduced diameter of their myofibrils. Although oscillatory work, which is proportional to the viscous modulus, was also reduced in the mutant fibers (consistent with the observed loss of contractile material in the skinned myofibril), this reduction is of marginal statistical significance (P=0.09). However, the optimal frequency at which power is delivered to the wings by these fibers is increased, suggesting that it is this increase, rather than changes in the magnitude of work production or power output, that is responsible for the impairment of flight.

We do not know why alterations in flightin stoichiometry result in increased frequency of power output. One possibility is that myofibrils with a reduced diameter, and hence a reduced number of myosin motors available to do work, somehow compensate by increasing the rate at which the remaining myosin motors produce force. The increase in the model parameter $2\pi b$, which reflects the crossbridge transition from a non-force-producing state to a force-producing state (Zhao and Kawai, 1993), suggests that the kinetics of crossbridge cycling are altered in this mutant. The changes in fiber mechanics cannot be explained solely by the disturbances in myofibrillar structure since the actin mutant *G368E* shows similar myofibrillar disruption yet exhibits a rate constant for the delayed tension response that is decreased by approximately 30 % (Drummond *et al.* 1990). This interpretation raises the possibility that flightin itself has an effect on crossbridge kinetics.

In conclusion, our results strongly suggest that flightin fulfills an essential role in the IFM. The peripheral disruptions in adult myofibrils described here, together with the sarcomeric degeneration that characterizes myosin rod mutants that lack flightin (Kronert *et al.* 1995), indicate that flightin may be an indispensable structural component of the active contractile apparatus. The availability of a genetic deficiency that uncovers the flightin gene should make it feasible to obtain flightin null mutations. Further analyses of new flightin mutants will contribute to our understanding of the functional properties of this intriguing protein.

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