

# Requirements of Kettin, a Giant Muscle Protein Highly Conserved in Overall Structure in Evolution, for Normal Muscle Function, Viability, and Flight Activity of *Drosophila*

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**Abstract.** Kettin is a giant muscle protein originally identified in insect flight muscle Z-discs. Here, we determined the entire nucleotide sequence of *Drosophila melanogaster* kettin, deduced the amino acid sequence of its protein product (540 kD) along with that of the *Caenorhabditis elegans* counterpart, and found that the overall primary structure of Kettin has been highly conserved in evolution. The main body of *Drosophila* Kettin consists of 35 immunoglobulin C2 domains separated by spacers. The central two thirds of spacers are constant in length and share in common two conserved

motifs, putative actin binding sites. Neither fibronectin type III nor kinase domains were found. Kettin is present at the Z-disc in several muscle types. Genetic analysis showed that *kettin* is essential for the formation and maintenance of normal sarcomere structure of muscles and muscle tendons. Accordingly, embryos lacking *kettin* activity cannot hatch nor can adult flies heterozygous for the *kettin* mutation fly.

**Key words:** immunoglobulin domain • titin family • *Caenorhabditis elegans* • Z-disc • flight activity

## Introduction

The Z-discs of striated muscles serve as linkers of thin (actin) filaments from adjacent sarcomeres, and may play fundamental roles in the transmission of forces (Vigoreaux, 1994). In *Drosophila* larval somatic muscles, Z-discs appear late in embryogenesis (Bernstein et al., 1993); they are perforated and thick (myosin) filaments frequently penetrate them with muscular contraction. In contrast to Z-discs in larval muscles, the counterparts in *Drosophila* indirect flight muscles (IFMs)<sup>1</sup> are regular in shape and show greater similarity to the Z-discs of vertebrate skeletal muscles, although there is an apparent difference in the lattice structure (Crossley, 1978; Bernstein et al., 1993; Vigoreaux, 1994).

Kettin is one of the Z-disc proteins and was initially identified in muscles of giant waterbug, *Lethocerus* (Lakey et al., 1990, 1993). *Drosophila* Kettin was identified in a cross reaction with antibody raised against *Lethocerus* Kettin (Lakey et al., 1993). IFMs in *Drosophila* exclusively

include a 500-kD major isoform of Kettin; a minor isoform of *Drosophila* Kettin is 700 kD in molecular mass (Lakey et al., 1993). A partial amino acid sequence of *Drosophila* Kettin (~10% of the total) has shown that Kettin possesses repeating units including immunoglobulin C2 (Ig) domains separated by linker sequences (Lakey et al., 1993). Biochemical analysis indicated that an Ig domain flanked by two linkers could bind to actin and  $\alpha$ -actinin but not to myosin (Lakey et al., 1993). Furthermore, plots of the binding data gave a maximum binding of 0.036 mol of Kettin per 1 mol of actin monomer or 1 mol of Kettin per 28 mol of actin monomer (Straaten et al., 1999), leading to the speculation that there are ~30 modules consisting of Ig domain plus a linker sequence and each capable of binding to the actin monomer.

Immunoelectron microscopic observations of IFM showed that Kettin is oriented with the NH<sub>2</sub> terminus in the Z-disc and the COOH terminus outside (Straaten et al., 1999), suggesting possible head-to-head interactions of Kettin molecules at the center of Z-discs. Anti-Kettin antibody signals were restricted to the vicinity of the Z-disc and the length of individual 500-kD Kettin molecules was less than one tenth of the sarcomere length (Straaten et al., 1999), suggesting that it is unlikely that Kettin serves as a molecular ruler to determine thick filament length as proposed for vertebrate titin/connectin (Trinick, 1994). In vertebrates,

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<sup>1</sup>Abbreviations used in this paper: AEL, after egg laying; EST, expressed sequence tag; IFM, indirect flight muscle; MHC, myosin heavy chain; ORF, open reading frame; RT, reverse transcriptase.

titin/connectin molecules are anchored at the Z-disc and M-line through their NH<sub>2</sub> and COOH termini, respectively (Labeit and Kolmerer, 1995).

In developing *Drosophila* muscles, thin filaments appear to grow through the addition of actin molecules to filaments already incorporated into Z-discs (Reedy and Beall, 1993). Kettin and tropomyosin compete for actin, and Kettin appears to prevent tropomyosin from binding to actin filaments in the vicinity of Z-discs in IFM (Straaten et al., 1999). Kettin is susceptible to calpain, a calcium-activated protease (Lakey et al., 1993). Myofibrils treated with calpain lose dense materials of Z-discs and release  $\alpha$ -actinin from myofibrils, possibly suggesting that Kettin is required for  $\alpha$ -actinin localization in Z-discs (Lakey et al., 1993). Thus, as proposed by Straaten et al. (1999), Kettin may reinforce the anchorage of actin filaments through associating with the barbed end of growing actin filaments and promoting the antiparallel assembly of actin filaments, which would be followed by cross-linking with  $\alpha$ -actinin and elongation of filaments by the addition of actin monomers. Muscle proteins similar in property to *Drosophila* Kettin have been found in the crayfish and silkworm (Maki et al., 1995; Suzuki et al., 1999).

Ig domain repeats similar to those of Kettin are present in other giant muscle proteins such as titin/connectin in vertebrate striated muscles and, accordingly, Kettin may belong to the titin family (for review see Benian et al., 1999). Projectin is the first titin family member identified in *Drosophila* and most closely related in sequence to Twitchin, a nematode muscle protein (for review see Benian et al., 1999). A mutation in the Twitchin gene (*unc-22*) causes twitching; that is, in mutant worms, rapid, finely localized movement is frequently observed throughout the body. Muscle structure is disrupted or abnormal in appearance in some mutant worms, particularly those showing abnormal movement (Waterston et al., 1980). Twitchin may be involved in regulating myosin activity (for review see Benian et al., 1999). Projectin appears involved in the connection of myofilaments (Benian et al., 1999). In IFM, Projectin is detected in the I-band, whereas, in other muscles, it is situated solely in the A-band, suggesting that Projectin may have different functions depending on muscle types (Vigoreaux et al., 1991). As with titin, Projectin and Twitchin possess fibronectin type III and protein kinase domains (for reviews see Benian et al., 1999; Kolmerer et al., 1999). Super-repeats consisting of fibronectin type III and Ig domains may be important for the putative ruler function of titin, whereas the kinase domain may be involved in regulatory processes (for reviews see Gautel et al., 1999; Kolmerer et al., 1999).

To further clarify the structure and possible functions of Kettin, we determined the entire amino acid sequences of Kettin of *Drosophila melanogaster* and its counterpart in *Caenorhabditis elegans* in the present study. Both proteins were found to be very similar in overall structure and largely composed of Ig domain repeats separated by spacer sequences. Neither fibronectin type III nor kinase domains were detected, indicating that Kettin is a muscle protein distinct in function and structure from other titin family members. In *Drosophila* embryos lacking *kettin* activity, normal sarcomere structures failed to be formed and, consequently, hatching does not occur. *kettin* is also

required for the maintenance of normal sarcomere structures, and adult flies heterozygous for *kettin* cannot fly.

## Materials and Methods

### Fly Strains and Genetics

Canton-S (CS; wild type [WT]) and *v<sup>36f</sup>;ry<sup>506</sup>* were our laboratory stocks. Gene trap experiments were carried out as follows. A *hedgehog* fragment (nucleotide position 11,206–11,353; Mohler and Vani, 1992), which includes a putative lariat site, splice acceptor, and a 5' portion of the second exon, was connected in-frame to the 5' end of the *Escherichia coli lacZ* coding sequence lacking the initiation codon (a BamHI fragment of pMC1871; Amersham Pharmacia Biotech). The resultant construct was inserted into a P element vector with a *vermillion* marker and pBluescript (Stratagene; Fridell and Searles, 1991) to generate IT1 (see Fig. 1 A). Germ-line transformation was carried out as described previously (Spradling and Rubin, 1982). A transformant line (132) exhibiting muscle-specific *lacZ* expression was selected and analyzed in the present study. When stocked, 132 and derivatives were balanced with TM6. As described in the text, 132 is a hypomorphic *kettin* mutant. Using a P[ry; $\Delta$ 2-3] chromosome (Robertson et al., 1988) the 132 P element was imprecisely excised and two additional *kettin* mutants, *ket<sup>f</sup>* and *ket<sup>l4</sup>*, were obtained; they cannot complement with 132. Note that the *kettin* locus is on the third chromosome and all *kettin* mutant chromosomes examined here are marked with *ry<sup>506</sup>*. *Dp(3;Y)B21/+;ket<sup>l4</sup>* /TM6 flies used in the flight assay were made by crossing *ket<sup>l4</sup>*/TM3 *Sb* flies with *T(Y;3)B21, B, y<sup>+</sup>/TM6;C(1)RM, y/C(1;Y)1, y* flies, obtained from the Bloomington stock center.

### Molecular Cloning, Reverse Transcriptase (RT)-PCR, and Northern Blotting

*Drosophila* genomic DNA fragments at the *kettin* locus were isolated by plasmid rescue and subsequent screening of a *Drosophila* cloned DNA library. cDNA clones, ZB2 and KVB7, were obtained from a randomly primed ZAP cDNA library (8–21-h embryo) and Kauvar's cDNA library (12–24-h embryo; Poole et al., 1985), respectively. CK340 and LD28564 are expressed sequence tag (EST) clones from the Berkeley *Drosophila* Genome Project EST project. RNA was prepared from embryos or larvae using an mRNA purification kit (Amersham Pharmacia Biotech). The nucleotide sequence of genomic DNA was initially determined, and then splicing sites were determined using the nucleotide sequences of relevant cDNAs, which include EST and RT-PCR clones. NB2, identical in sequence to *D-titin* NB (Machado et al., 1998), was prepared by PCR using the total *Drosophila* genomic DNA and suitable primers. Northern blotting was carried out essentially as described by Sambrook et al. (1989). As *kettin*-specific probes, ZB2 or a 5' genomic DNA fragment (a 5-kb EcoRI fragment), which includes exon 1 and 2, were used (see Fig. 1 C). The coding sequence of *C. elegans kettin* was determined based on the genomic DNA sequence recently published (The *C. elegans* Sequencing Consortium, 1998). We presumed that true combinations of splicing acceptor and donor sequences of *C. elegans kettin* give the maximum amino acid sequence homology to *Drosophila* Kettin.

### Antibody Staining and In Situ Hybridization

Antibody staining and in situ hybridization were carried out as described by Shishido et al. (1997). When larvae or adult muscle tissues were subjected to antibody staining, larvae and adults were anesthetized, dissected, and fixed with 4% paraformaldehyde before antibody treatment. Primary antibodies used were as follows: rat monoclonal anti-Kettin antibody (1:20; MAC 155; Lakey et al., 1993), rabbit anti-MHC (myosin heavy chain) antibody (1:500; Kiehart and Feghali, 1986), and rabbit anti-LacZ antibody (1:2,000; anti- $\beta$ -Gal; Cappel). MAC 155 epitopes should be located in the central region of Kettin (see the underlined sequence in Fig. 1 G). FITC-phalloidin and SYTOX (Molecular Probes Inc.) were used to detect actin and DNA, respectively. Both sense and antisense RNA probes for in situ hybridization were prepared using ZB2 (*kettin*) and NB2 (*D-titin*) DNA as templates; *lacZ* RNA probes were similarly prepared.

### Flight Assay

Flight ability was measured essentially as described by Koana and Hotta (1978). A plastic cylinder with a diameter of 7 cm and a height of 32 cm

was used as a flight tester (see Fig. 6). Flies, sorted out soon after eclosion and kept for 2–3 d at 22°C, were subjected to the flight assay.

## Electron Microscopy

Somatic embryonic/larval muscles and IFMs were examined by electron microscopy. Late stage 17 embryos were collected during 20–24 h after egg laying (AEL) at 25°C. First instar wild-type larvae were collected during 24–48 h AEL. *ket<sup>1</sup>* mutant counterparts, collected during the same period, were incapable of hatching but capable of moving inside the egg shell. Second instar larvae homozygous for 132, lacking balancer phenotypes (*Tb* or *Act-GFP*), were collected during 48–72 h AEL. IFMs (adult thoraxes) were prepared 7–10 d after eclosion at 22°C. Pharate adult IFMs were collected from pupae that had developed red eyes. Larvae and thoraxes were dissected, fixed in a mixture of 2.5% glutaraldehyde, 2% formaldehyde, and 0.1 M sodium cacodylate, pH 7.4, for 2 h at room temperature, postfixed with 1% osmium tetroxide in the same buffer for 2 h on ice, stained en bloc with 2% uranyl acetate overnight, and dehydrated in a graded ethanol series. After ethanol was replaced with methyl methacrylate, samples were embedded in Rigolac mixture (Kushida, 1960). Thin sections were stained with uranyl acetate and lead citrate and subjected to electron microscopic observation.

## Results

### Isolation and Entire-Nucleotide-Sequence Determination of *Drosophila kettin* Encoding a Giant Muscle Protein

A trap line of *kettin* (132) was isolated during gene-trap screening of lethal muscle/nervous system genes in *Drosophila*. A *hedgehog* fragment containing a putative lariat signal, 3' splice site and 5' end of exon 2 was connected to the 5' end of the *lacZ* open reading frame (ORF) associated with a polyadenylation signal (Fig. 1 A), and the resultant construct was introduced into *Drosophila* by germline transformation. LacZ protein expression occurs only when *lacZ*, inserted in an intron, is in-frame with an upstream exon. In general, a gene-trap fly line is a hypomorphic or null mutant of the target gene in which *lacZ* is inserted.

In embryos, 132-LacZ was strongly expressed solely in muscle cells and/or precursors (see Fig. 3 I), suggesting that the gene trapped in 132 is a muscle gene. In situ hybridization to polytene chromosome indicated 132 insertion to occur in or near 62C on the left arm of chromosome III (data not shown), where two muscle-related genes, *kettin* and *D-titin*, have been reported to be mapped (Machado et al., 1998; Straaten et al., 1999). Genomic DNA fragments flanking 132-P were isolated by plasmid rescue, followed by screening of a *Drosophila* cloned library. Also isolated were two relevant cDNA clones (ZB2 and KVB7) using as probes genomic DNA fragments showing positive in situ hybridization signals. Nucleotide sequence analysis showed that ZB2 and KVB7 together represented only a portion of the target gene (132 gene; Fig. 1 C) and, thus, the structure of the remaining portion of the 132 gene was first predicted based on the nucleotide sequence of a 20-kb-long genomic DNA region, and then confirmed by those of RT-PCR and EST clones spanning putative splicing junctions (Fig. 1 C). The cDNA information previously published by others (Lakey et al., 1993) was also used.

The presumed RNA start of the 132 gene is 5' TTC-AGTC and not associated with a TATA box (for a consensus RNA start, see Hultmark et al., 1986). Al-

though no cDNA with poly(A)<sup>+</sup> RNA has been isolated to date, two putative polyadenylation signals, AATAAAs, were found ~400 bp downstream of the predicted termination codon, which is situated in the region corresponding to LD28564 cDNA (Fig. 1 C). There is no apparent splicing acceptor signal between the predicted termination codon and polyadenylation signals. In situ hybridization disclosed the presence of a second transcriptional unit unrelated in sequence to *kettin* in the vicinity of the predicted 3' end (Fig. 1 C).

As shown in Fig. 1 C, the 132 gene contains 12 exons with predicted cDNA size of 15 kb. Northern blotting showed that there are two 132-related RNAs in embryos, major 14.5 kb long and minor 18 kb long (Fig. 1 D, lane 1). Thus, the former may correspond to the transcriptional unit analyzed here. The predicted 132 protein is composed of 4,796 amino acids (Fig. 1 G) with molecular weight of  $\sim 5.4 \times 10^5$ . Since a partial amino acid sequence of *Drosophila* Kettin previously determined (Lakey et al., 1993) perfectly matches a central region of the deduced amino acid sequence of the 132 protein (see underlines in Fig. 1 G) and the size of the 132 protein is very close to the major 500-kD component of *Drosophila* Kettin determined by SDS-PAGE (Lakey et al., 1993), we conclude that the 132 gene encodes the major Kettin isoform. Hereafter, we refer to the 132 gene as *kettin*. Since the minor 18-kb RNA has a capacity to encode a 660-kD protein at maximum, it may encode the minor Kettin isoform found in *Drosophila* by SDS-PAGE (Lakey et al., 1993), although we do not know its entire molecular structure yet.

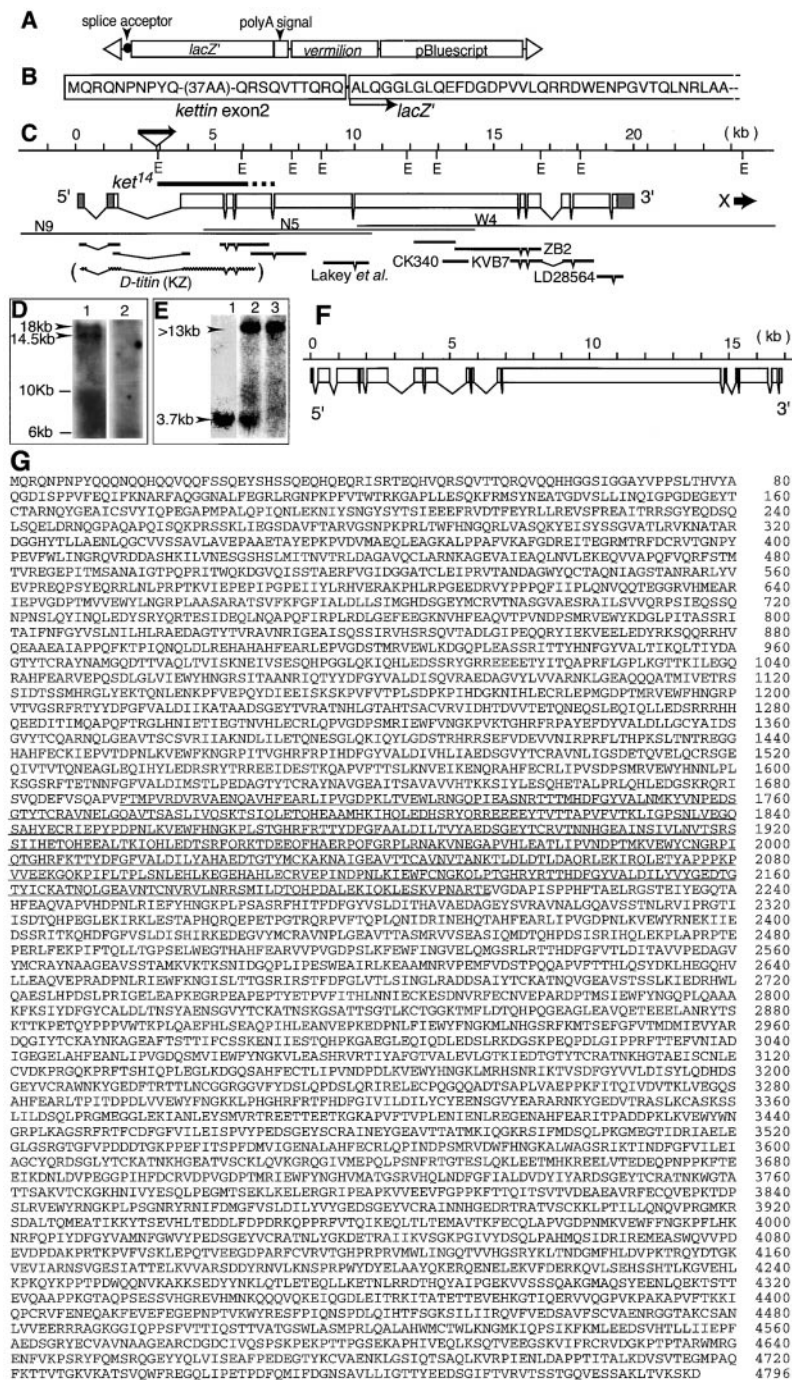
### Production of *kettin-lacZ* Fusion Transcripts in 132

RNA was extracted from 132/TM3,*Sb,ry* larvae and subjected to Northern hybridization. A 5' genomic DNA *kettin* fragment including exons 1 and 2 and a 3-kb *lacZ* fragment were used as probes. As shown in Fig. 1 E, the 132/TM3,*Sb,ry* larvae contained a 3.7-kb component that hybridized with both *kettin* and *lacZ* probes and a component of >13 kb in length and detectable solely with the *kettin* probe; the latter appears to correspond to a mixture of 14.5- and 18-kb wild-type *kettin* RNAs (Fig. 1 D). Nucleotide sequence analysis of RT-PCR products indicated that the 3.7-kb component is a composite of a 0.5-kb exon I/II sequence of *kettin* and the 3.2-kb *lacZ* ORF (Fig. 1 B), confirming the insertion of a P-*lacZ* to occur in the second intron of *kettin* (Fig. 1 C).

### *Kettin* as a Protein Consisting of 35 Repeats with Ig Domain Sequences

A previous partial sequence analysis of Kettin (Lakey et al., 1993) showed that repeats of a 130-amino acid-long sequence, made up of 95 amino acids homologous to Ig domain and a less conserved linker sequence, are included in Kettin. In the following, linker is referred to as spacer since the region appears necessary not only for linking Ig domains, but also possibly for exactly spacing them to fit the size of bound actin molecules (see Discussion).

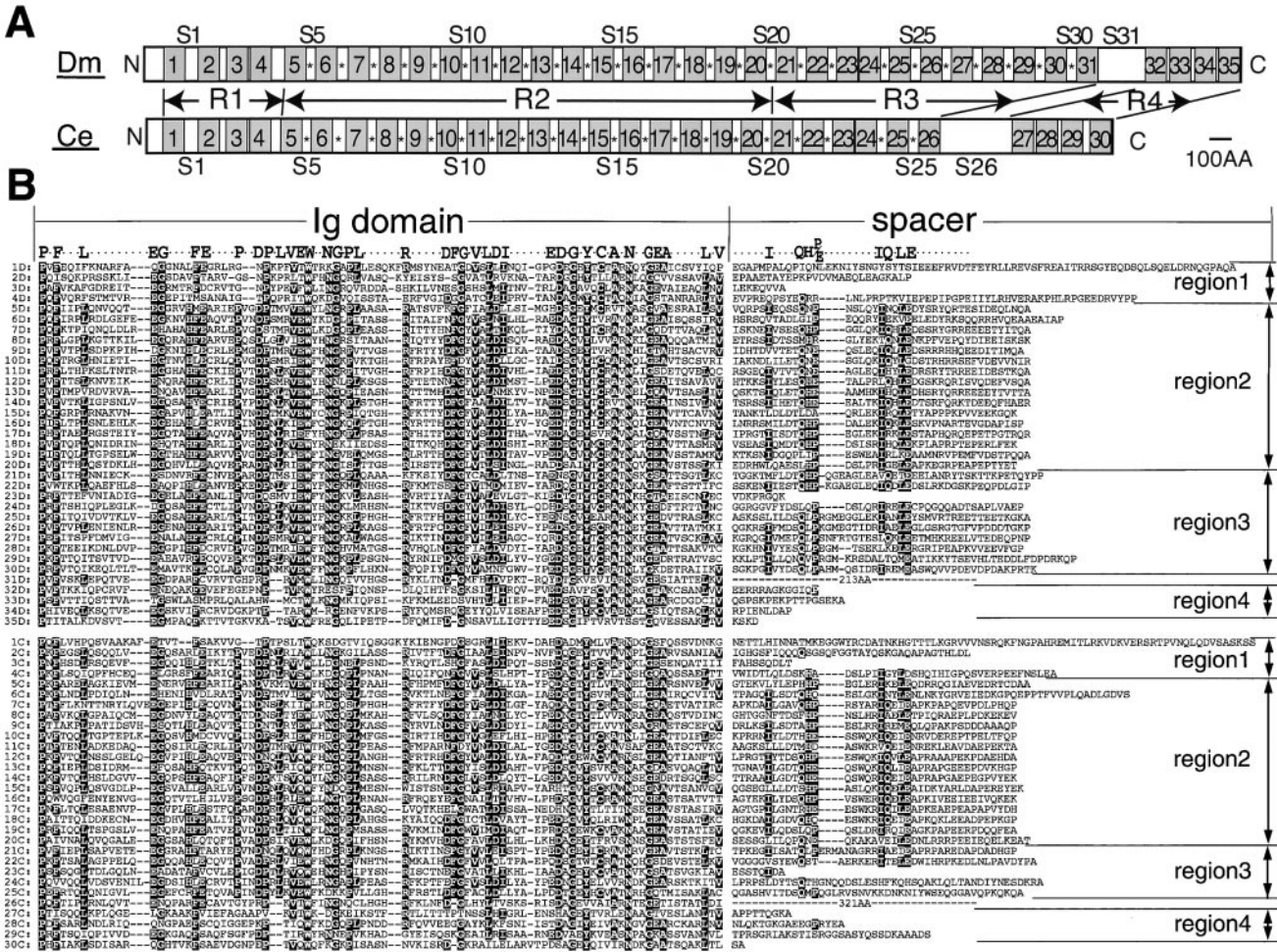
As shown in Fig. 2, A and B, 35 repeating units occupy the entire region of *Drosophila* Kettin, and each includes an Ig domain and a spacer sequence. Except for the NH<sub>2</sub>-terminal region, the protein may be divided into four re-



**Figure 1.** Structures of *Drosophila* and *C. elegans* *kettin* loci and the deduced amino acid sequence of *Drosophila* Kettin. (A) The structure of IT1, a P element vector used for gene trapping in *Drosophila* is schematically shown. Filled circle indicates a *hedgehog* fragment (nucleotide position 11,206–11,341; Mohler and Vani, 1992), including a putative lariar site and splice acceptor within intron 1. *lacZ'* is *E. coli lacZ* in which the initiation codon is replaced with an in-frame 5'-terminal sequence of *hedgehog* exon 2 (nucleotide position 11,342–11,353; Mohler and Vani, 1992). A 255-bp fragment of *hsp70* (nucleotide position 1,918–2,172; Ingolia et al., 1980) was used as a poly(A)<sup>+</sup> signal-containing fragment. Triangles show P element terminal sequences. (B) A part of the deduced amino acid sequence of the polypeptide encoded by the *kettin-lacZ'* chimeric RNA found in 132 mutants (E). A cDNA fragment including the *kettin-lacZ'* junction was recovered as an RT-PCR product of 132/TM3 larval RNA. (C) *Drosophila kettin* gene structure. EcoRI sites on the genomic DNA are shown by Es. The triangle associated with a horizontal arrow shows the location of 132 P insertion and the direction of *lacZ'* transcription. A thick bar labeled *ket*<sup>14</sup> indicates a *kettin* locus region deleted in *ket*<sup>14</sup>. Open boxes show the sizes and locations of coding exons, whereas stippled boxes, noncoding exons. A thick horizontal arrow labeled X shows a putative gene adjacent to *kettin*. Thin lines labeled N9, N5, and W4 show the sizes and locations of genomic DNA clones. ZB2 and KVB7 are cDNA clones isolated from cDNA libraries, whereas CK340 and LD28564 are EST clones obtained from Berkeley *Drosophila* Genome Project. The size and location of cDNA isolated by Lakey et al. (1993) is also shown. Unlabeled cDNA clones are those isolated using RT-PCR. The cDNA enclosed with parentheses and labeled *D-titin* (KZ) is the size and location of fragment KZ, which was reported to represent a part of a hypothetical gene, *D-titin* (Machado et al., 1998). (D) High resolution pictures of Northern blots showing the presence of two high molecular weight *kettin* RNA species (lane 1) and the absence of putative *D-titin* RNA (lane 2). The molecular sizes of two *kettin* RNA were determined using RNA molecular markers and  $\lambda$ /HindIII DNA markers. Arrowheads indicate *kettin* RNA, 14.5 and 18 kb in length. To detect *D-titin* signals in lane 2, fragment NB2 was used as a probe. (E) Northern blotting for the detection of wild-type and mutant *kettin* RNA in 132. (lane 1) 132/TM3 larval RNA hybridized with a *lacZ* cDNA probe. (lane 2) 132/TM3 larval RNA and lane 3, *mutry* larval RNA hybridized with a 5-kb *kettin* genomic DNA probe. The results shown indicate that RNA bands 3.7-kb long and more than 13-kb long, respectively, correspond to a *kettin-lacZ* composite RNA and authentic *kettin* RNA. (F) A putative structure of the *C. elegans kettin* gene. Only the sizes and locations of coding exons are shown. Using published genomic DNA sequences at or near the putative *C. elegans kettin* locus (R05D8; The *C. elegans* Sequencing Consortium, 1998), the entire *kettin* coding sequence was predicted. We assumed that true exon combinations should give the maximum amino acid sequence homology to the *Drosophila* counterpart. The accession number of the resultant *C. elegans kettin* is AB026846. (G) Deduced amino acid sequence of *Drosophila* Kettin, which is 4,796 amino acids in length. A partial amino acid sequence previously reported (Lakey et al., 1993) was underlined. The sequence data of *Drosophila kettin* are available from GenBank/EMBL/DBJ under accession number AB026845.

regions according to spacer length and sequence. Region 1 contains four Ig domains (Igl-4) and spacers (S1-4) of variable length. Ig domains (Igl5-20) in region 2 are separated by spacers almost constant in length; the average

spacer length except for a slightly longer S6 was estimated at  $40.6 \pm 0.7$  amino acids. Spacers in region 3 were slightly variable in length except for a very short one (S23) and contained 40–50 amino acids. Fig. 2 B shows that most, if



**Figure 2.** (A) Highly repetitive structures of *Drosophila melanogaster* (Dm) and *C. elegans* (Ce) Kettin proteins are schematically shown. *Drosophila* and *C. elegans* Kettins contain numerous repeating units consisting of an Ig domain and a spacer sequence. In both *Drosophila* and *C. elegans*, Kettin can be divided into four regions, R1-4, except for the NH<sub>2</sub>-terminal region and a long spacer (S31 and S26, respectively, in *Drosophila* and *C. elegans*). Stippled boxes with numbers show Ig domains. S1–S31 indicate spacer numbers. Spacers with conserved Kettin spacer motifs are marked with asterisks. (B) Comparison of Ig domain amino acid sequences of *Drosophila* and worm Kettins. iD (*i* = 1–35) indicates the Ig domain and spacer sequences of the *i*th repeating unit of *Drosophila* Kettin, while iC (*i* = 1–30), those of the *i*th repeating unit of worm Kettin. Ig domains consist of ~95 amino acids. Highly conserved amino acids are labeled by white letters in black. Note that spacers in regions 2 and 3 share in common two conserved Kettin spacer motifs, I (IXXXQH(P/E)) and II (IQXLE).

not all, spacers in regions 2 and 3 share in common conserved amino acid sequences, IXXXQH(P/E) (Kettin spacer motif I) and IQXLE (Kettin spacer motif II; X, an arbitrary amino acid). Kettin spacer motifs I and II in region 2 are separated by five amino acids, but those in region 3, by five to nine amino acids. Region 4 is separated from other regions by a long spacer (S31) and possesses four Ig domains (Ig32-35) and relatively short spacers.

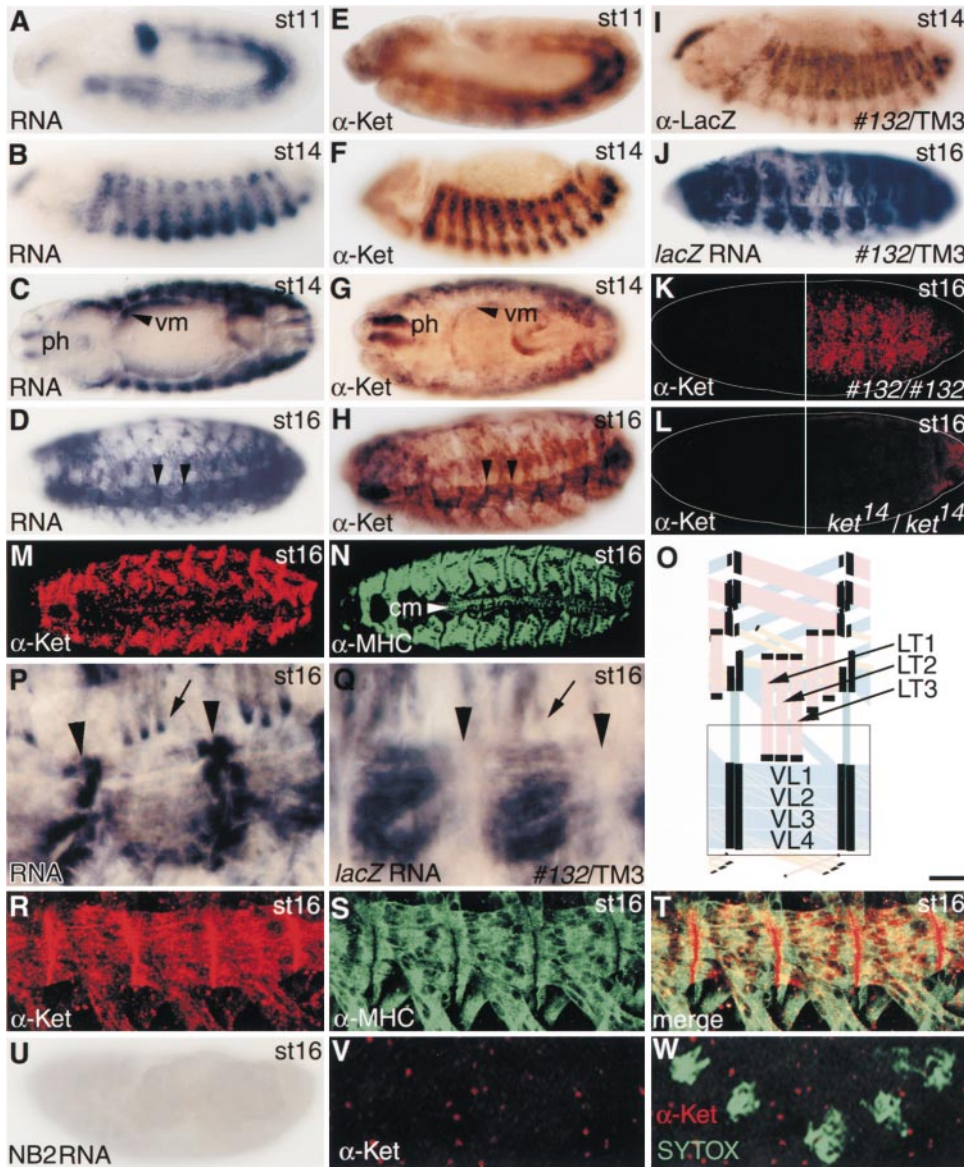
Titin family members such as vertebrate titin, *Drosophila* Projectin, and *C. elegans* Twitchin are characterized by the presence of not only Ig domains, but also a kinase domain and fibronectin type III repeats (Labeit and Kolmerer, 1995; Benian et al., 1999). The PEVK domain, rich in proline, glutamic acid, valine, and lysine, and possibly involved in elasticity, is specific to titin (Kolmerer et al., 1999). Thus, we searched for these sequences in Kettin, but no homology was found. We conclude that, unlike

other titin family members, *Drosophila* Kettin has neither kinase, fibronectin type III, nor PEVK domains.

### Colocalization of RNA and Protein Products of kettin in Muscle Attachments in Developing Embryonic Muscles

Although Kettin was initially found to be present in flight muscle Z-discs (Lakey et al., 1993), 132-LacZ expression indicated that *kettin* is also expressed in embryonic muscle cells (Fig. 3, I and J). To clarify this point, examination was made of temporal and spatial expression of *kettin* RNA and protein in embryos using whole mount in situ hybridization and anti-Kettin antibody staining (Fig. 3, A–H).

*kettin* RNA began to be expressed in the mesoderm at stage 11 several hours before myoblast fusion (Fig. 3 A) and persisted in most, if not all, developing embryonic

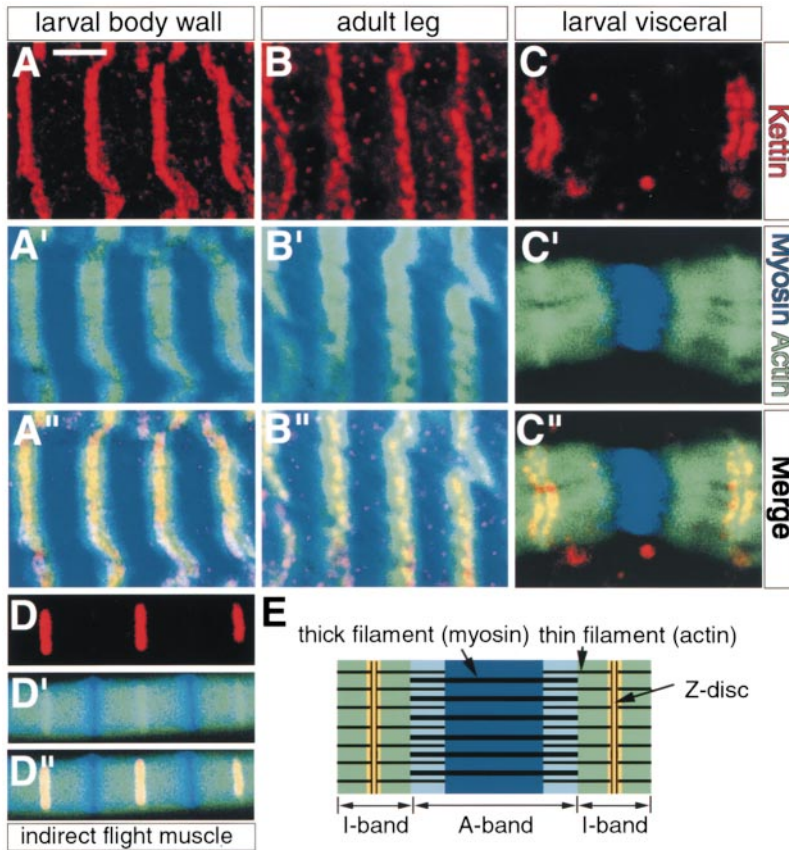


**Figure 3.** *kettin* RNA and protein expression in embryos. (A–D) *kettin* RNA expression occurred in muscle cells or precursors from late stage 11 onwards. As a probe for in situ hybridization, a fragment ZB2 (see Fig. 1 C) was used. At stage 14, not only somatic muscles, but also pharyngeal (ph) and visceral (vm) muscle cells produce *kettin* RNA. Arrowheads indicate the localization of *kettin* RNA at muscle attachment sites (see P for enlarged images). (E–H) Occurrence of Kettin protein expression in embryonic muscle cells or precursors. Arrowheads in H show the accumulation of Kettin at muscle attachment sites. I and J, respectively, show *lacZ* protein and RNA expression in muscle cells heterozygous for 132 insertion. (K) Kettin protein expression in embryos homozygous for 132, a hypomorph of *kettin*. Kettin protein signals in muscles were detected only after signal amplification (right half). (L) Kettin expression in embryos homozygous for *ket*<sup>14</sup>, a strong loss-of-function or null allele of *kettin*. No or little Kettin signals could be detected even after signal amplification (right half). (M and N) Confocal images of wild-type embryos stained for Kettin (M) and myosin heavy chain (MHC) (N). Kettin signals in cardiac muscle cells (cm) are marginal, if any. (O) Embryonic muscles are schematically shown (modified from Bate, 1993). Black bars indicate localization of Kettin sig-

nals at muscle attachment sites. The open box may correspond to regions shown in P and Q. LT1-3, lateral transverse 1-3; VL1-4, ventral longitudinal 1-4. (P) *kettin* RNA localization at muscle attachment sites at stage 16. Arrowheads and arrow, respectively, show *kettin* RNA signals at indirect and direct muscle attachment sites. (Q) *lacZ* RNA expression in stage 16 embryos heterozygous for 132 insertion. Note that there is no or little accumulation of *lacZ* RNA at muscle attachment sites labeled with arrowheads and an arrow. (R–T) Accumulation of Kettin along muscle attachment sites, visualized by confocal microscopy. R, Kettin; S, MHC; and T, a merged picture of R and S. (U) The absence of *D-titin* RNA expression stage in 16 embryos. Whole-mount in situ hybridization was carried out using NB2 (a *D-titin* specific fragment) as a probe. In contrast to strong *kettin* expression (see D), no or little *D-titin* signals were detected. (V and W) The absence of chromosomal Kettin signals in early *Drosophila* embryos. SYTOX was used for the detection of DNA. Kettin and SYTOX signals are colored in red and green, respectively. No or little overlapping between Kettin and SYTOX signals was detected, indicating that Kettin is not chromosomal protein. All embryos are oriented with anterior to the left. For embryos shown in lateral view, dorsal is up. Stages are as described (Campos-Ortega and Hartenstein, 1985). Bar: (A–N and U) 45  $\mu$ m; (P and Q) 10  $\mu$ m; (R–T) 18  $\mu$ m; (V and W) 7  $\mu$ m.

muscle cells until the third instar. At stage 14, strong *kettin* signals were detected in nearly all somatic muscle cells (Fig. 3 B) along with pharyngeal and visceral muscle cells (Fig. 3 C). At stages 14 and 15, myoblast fusion is complete in most somatic muscles (Bate, 1990, 1993). At the end of embryogenesis, somatic muscle tubes become connected to epidermal cells either directly or indirectly (Prokop et al., 1998; Tepass and Hartenstein, 1994). Longitudinal

muscles/epidermis attachments formed at segment borders are of indirect type and muscle tips, and epidermal cells are linked via tendon matrices (see Fig. 5 G). In contrast, transverse muscles/epidermis attachments are formed directly and involve hemiadherens junctions (see Fig. 5 E). Fig. 3, D and P, shows *kettin* RNA to be localized for the most part along edges in nascent transverse and longitudinal muscles at stage 16. In contrast, 132-*lacZ*



**Figure 4.** Z-disc expression of Kettin in several muscle types. (A, A', and A'') Larval body wall muscles. (B, B', and B'') Adult leg muscles. (C, C', and C'') Larval visceral muscles. (D, D', and D'') IFMs. Kettin (red) expression images (A–D) and anti-MHC (blue)/FITC-phalloidin (green) double staining pictures (A'–D') are merged in (A''–D''). FITC-phalloidin staining shows actin expression regions. (E) Schematically shows the relationship between muscle gene expression and sarcomere structures. In all cases examined here, Kettin is expressed exclusively in the region colored in yellow in E, or the center of actin-expressing regions, indicating Kettin in Z-discs and their immediate neighbors. By unknown reasons, the region positive to antimyosin antibody staining was much narrower than expected, in particular, in larval visceral muscles and IFMs. Bar: (A and B) 5  $\mu\text{m}$ ; (C) 4  $\mu\text{m}$ ; (D) 3  $\mu\text{m}$ .

or *kettin-lacZ* chimeric RNA expression was not restricted to muscle tips (Fig. 3, J and Q). Thus, *kettin* RNA is apparently almost uniformly produced from all embryonic muscle cell nuclei, but is transported to and apt to accumulate in the vicinity of muscle tips.

The protein product of *kettin* showed expression quite like that of *kettin* RNA, although the protein appeared somewhat late compared with RNA (Fig. 3, E–H). Although less effective than RNA, Kettin protein was also concentrated at the edges of longitudinal and transverse somatic muscles at stage 16 (Fig. 3 H) as demonstrated by confocal images of double staining with anti-Kettin and anti-MHC antibodies (Fig. 3, R–T). These findings may suggest that, as schematically shown in Fig. 3 O, most Kettin molecules are translated from mRNA localized in muscle tips, and serve as anchors for fixing muscle fibers in the vicinity of direct and indirect muscle attachments, at least in embryonic muscles. No, or only a little, Kettin expression could be detected in cardiac muscle cells (Fig. 3, M and N).

As Z-discs begin to be formed at stage 17, the last stage of embryogenesis (Bernstein et al., 1993), we investigated the spatial distribution of Kettin in mature muscles. Larval body wall, visceral, and adult leg muscles along with IFMs were stained for Kettin, actin, and myosin. As shown in Fig. 4, repetitive Kettin signals were observed in all muscles examined, and Kettin signals were always situated in the center of actin expression regions or putative Z-disc location. Thus, we consider that Kettin is located at or near Z-discs in many types of *Drosophila* muscles and may

have common functions at Z-discs in larval and adult muscles.

### *kettin*, A Gene Essential for Fly Viability

Flies homozygous for 132 were not viable, but this lethality was completely eliminated by removing the 132-P element, indicating that lethality is due to 132-P insertion in *kettin*. By imprecise P excision and subsequent lethality screening, we also obtained two additional *kettin* mutants, *ket<sup>5</sup>* and *ket<sup>14</sup>*.

In *ket<sup>14</sup>*, a genomic sequence ranging from 0 to 4 kb downstream of the P insertion site, which includes the third to fifth exons of *kettin*, is deleted (Fig. 1 C). Embryos homozygous for *ket<sup>14</sup>* produced protein products that could be hardly detected (Fig. 3 L), indicating *ket<sup>14</sup>* to be a strong loss-of-function or null allele of *kettin*. Embryos homozygous for *ket<sup>14</sup>* died as prolonged stage 17 embryos without hatching (Table I).

In contrast to *ket<sup>14</sup>*, *ket<sup>5</sup>* is a weak hypomorphic allele. In *ket<sup>5</sup>*, the structure of the *kettin* gene is identical to that in 132 (Fig. 1 C) except for a deletion within the P element. Almost all *ket<sup>5</sup>* homozygous embryos hatched and grew into pupae and some became pharate adults dying without eclosion (Table I). So far, we have found five escapers, one of which was female and fertile when crossed with 132 heterozygotes.

132 homozygotes died at various times from the very end of embryogenesis to pupal stages. 132 homozygotes appeared to be intermediates in the phenotype between

Table I. Phenotypes of *kettin* Mutants

Genotype	Lethal stage	Mutant rank*	Flight ability	Sarcomere length <sup>‡</sup>	Thin/thick filament ratio <sup>‡</sup>
				$\mu\text{m}$	
<i>ket<sup>14</sup>/ket<sup>14</sup></i>	embryo	6	—	—	—
132/132	larva	5	—	—	—
<i>ket<sup>5</sup>/ket<sup>5</sup></i>	pupa	4	flightless <sup>§</sup>	2.4 $\pm$ 0.2 ( <i>n</i> = 30)	445/1263 (0.35)
<i>ket<sup>14</sup>/+</i>	viable	3	flightless	2.3 $\pm$ 0.2 ( <i>n</i> = 30)	486/1392 (0.35)
132/+	viable	2	normal	ND <sup>  </sup>	ND
+/+	viable	0	normal	2.3 $\pm$ 0.2 ( <i>n</i> = 47)	559/1559 (0.36)

\*The ranks according to phenotype severity and *kettin* expression level are shown. (wild-type rank = 0, null mutant rank = 6).

<sup>‡</sup>Sarcomere length (mean  $\pm$  SD) and thin/thick filament ratios were determined using the electron micrograph of pharate adult IFMs. *n*, number of sarcomeres examined.

<sup>§</sup>Phenotype of five escapers obtained so far.

<sup>||</sup>ND, not determined.

*ket<sup>5</sup>* and *ket<sup>14</sup>*. Although only low level *Kettin* signals were detected in 132 embryos (Fig. 3 K), there was no or little abnormality in anti-MHC staining at stage 16 (data not shown) and most, if not all, 132 embryos normally hatched. Unhatched eggs contained fully formed larvae, capable of head movement but never emerged and eventually died. Hatched larvae showed normal behavior just after hatching, but movement slowly diminished. Many individuals that died at larval stages had double cuticles judging from the number of mouth hooks, suggesting muscular power was too weak to shed old skin. Larvae that

grew to the third instar became incapable of movement by the end of this stage, could not climb the vial wall and died. However, many became pupae when transferred onto wet paper.

Virtually all flies heterozygous for *ket<sup>14</sup>* were viable and became adults (Table I). But, as shown below, they had flight defects. Taken together, these results lead us to the conclusion that *kettin* is an essential muscle gene in *Drosophila*.

### Requirement of *Kettin* for Formation and Maintenance of Normal Sarcomere Structure in Larval Muscles

In wild-type embryos, somatic muscle Z-discs first become detectable at stage 17 (Bernstein et al., 1993). Z-discs could be detected much more clearly in late stage 17 embryos (Fig. 5 A) or in the first instar larvae fixed at 24–36 h AEL (Fig. 5 C). In contrast, sarcomere structure was highly disorganized in any prolonged stage 17 embryos (24–36 h AEL embryos); thick and thin filaments appeared randomly scattered and no regularly arranged Z-disc-like structures could be detected (Fig. 5 D). These findings may indicate that *kettin* is essential for either the formation or maintenance of normal Z-discs or both. To determine which is the case, sarcomere structure of *ket<sup>14</sup>* homozygotes at earlier stages was examined. As shown in Fig. 5 B, in late stage 17 embryos, we occasionally found sarcomere-like bundles of thick and thin filaments associ-

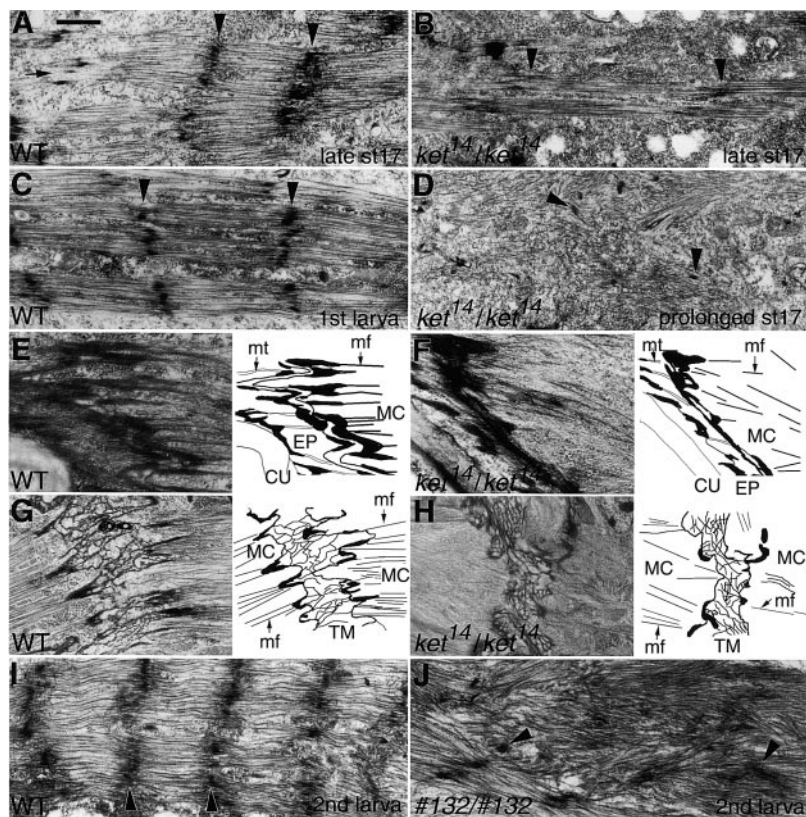


Figure 5. Defects of body wall muscles in *kettin* mutants. Arrowheads indicate electron dense materials of Z-discs or their presumptive derivatives. Only longitudinal muscle sections are shown. (A) Wild type at late stage 17. (B) *ket<sup>14</sup>/ket<sup>14</sup>* homozygotes at late stage 17 or 20–24 h AEL. Z-discs are reported to be formed at stage 17 (Bernstein et al., 1993). Consistent with this, in wild type, Z-disc formation appeared almost completed by late stage 17 (A, arrowheads); immature Z-discs were observed only occasionally (A, arrow). In some mutant muscles (B), the formation of myofilament bundles was apparent but, even in such a case, accumulation of electron dense Z-discs appeared quite incomplete (arrowheads), suggesting that only unstable sarcomere structures are formed in *ket<sup>14</sup>* homozygotes, lacking virtually all *kettin* activity. C and D, respectively, show wild-type first-instar larvae and their counterparts in *ket<sup>14</sup>* homozygotes at prolonged stage 17; *ket<sup>14</sup>* homozygotes cannot hatch. Mutants almost completely lost normal regular sarcomere structures and scattered electron dense materials, putative remnants of Z-discs, were frequently observed (D, arrowheads). E and F and G and H, respectively, show tendon morphologies at direct and indirect muscle attachments. (E and G) Wild type; (F and H) *ket<sup>14</sup>/ket<sup>14</sup>* homozygotes. As schematically shown in the right of each panel, in both direct and indirect muscle attachments, complicated folding of the

muscle cell membrane largely disappeared in mutants. In addition, the size of the tendon matrix (TM), connecting muscle cells (MC) to the epidermis (EP) at indirect muscle attachment sites, reduced in *ket<sup>14</sup>* homozygotes. CU, cuticle; mf, myofilaments; and mt, microtubules. (I and J) Somatic muscles of wild type (I) and 132 homozygotes (J) in second instar. Regularly arranged Z-discs in wild-type muscles appear disorganized in mutants. Bar: (A–D and G–J) 1  $\mu\text{m}$ ; (E and F) 0.5  $\mu\text{m}$ .



ated with incomplete Z-disc-like structures, which is not so dense but almost regularly spaced. Since ~24 h AEL *ket<sup>14</sup>* embryos appeared to possess muscle contraction ability to some extent, we tentatively conclude that *kettin* possesses important roles in both the formation and maintenance of Z-discs and sarcomere structures formed in the absence of Kettin is quite unstable and destroyed immediately with a low level of muscle contraction.

A study was also undertaken to determine the effects of the *ket<sup>14</sup>* mutation on muscle attachments, where *kettin* RNA and protein are localized at embryonic stage 16. As shown in Fig. 5 F, the ends of muscles homozygous for *ket<sup>14</sup>* were abnormally flat, whereas those of wild-type muscles were zigzag (Fig. 5 E; see also Tepass and Hartenstein, 1994). Similar effects were also detected in indirect muscle attachment sites, where muscle tips are connected to epidermal cells via tendon matrices (Fig. 5, compare G with H; Prokop et al., 1998). These results suggest that *kettin* is also essential for the formation and/or maintenance of normal muscle attachment sites.

To determine whether the reduced activity of 132 larvae is also due to possible defects in the contractile apparatus of the musculature, wild-type and mutant larval muscles were examined. With the growth of larvae, the number of sarcomeres per muscle and muscle mass increased for the wild type; in the second instar larvae, all somatic and visceral muscles exhibited well-ordered Z-disc-like structures frequently associated with membrane invagination (Fig. 5 I, and data not shown). In most first instar larvae homozygous for 132, Z-disc-like structures were normal for the most part (data not shown). However, striated muscle patterns of 132 larvae were heavily disrupted in second instar larvae (Fig. 5 J). Thus, it would appear that Kettin must be continuously available for the maintenance of normal Z-disc structures in larvae.

### Flightlessness of *kettin* Mutants

*Drosophila* Kettin was initially identified as a component of Z-discs in flight muscle myofibrils (Lakey et al., 1993),

while our results described above show that continuous Kettin expression may be important for normal muscle development, suggesting the involvement of *kettin* in flying. Thus, examination was made as to whether flies heterozygous for *kettin* are capable of flying, using the cylinder method (Koana and Hotta, 1978). Flies, 2–3 d after eclosion, were dropped through a funnel into a cylinder coated with liquid paraffin so that only those capable of flying would stick to the wall (Fig. 6). As shown in Fig. 6, nearly all wild-type flies stuck to the wall, whereas most, if not all, lacking wings simply dropped to the bottom. Whereas flies heterozygous for 132 or *ket<sup>5</sup>* showed normal flight activity (data not shown), most of those heterozygous for *ket<sup>14</sup>* dropped to the bottom. Five escapers of *ket<sup>5</sup>* homozygotes obtained to date also could not fly at all (data not shown). 132 and *ket<sup>5</sup>* are hypomorphic alleles, whereas *ket<sup>14</sup>* is a strong loss-of-function or null allele. Moreover, flight defects in *ket<sup>14</sup>* heterozygotes were eliminated by introducing *Dp(3;Y)B21* with a wild-type copy of *kettin* (Fig. 6). Thus, the flightless phenotype of *ket<sup>14</sup>/+* flies appears to be due to haploinsufficiency of *kettin*.

*ket<sup>14</sup>/+* IFMs were examined by electron microscopy (Fig. 7) and were found associated with severe myofibrillar aberrations. Transverse sections of IFMs indicated that, for wild type, IFM thick filaments are tightly packed (Fig. 7, A–C), whereas in mutants, they are loosely packed or untied, particularly along the periphery (Fig. 7, F–H). Longitudinal sections showed occasional myofibril branching and branched myofibrils to be connected to and integrated into neighboring ones (Fig. 7, compare D with I). Incomplete and apparently broken Z-discs were also frequently observed (Fig. 7 I, arrows). Since IFMs of late pupae (pharate adults) heterozygous for *ket<sup>14</sup>* were virtually normal in morphology and indistinguishable from those of wild type (Fig. 8, compare A and B with C and D) and there was no apparent difference in sarcomere length and thin/thick filament ratio between wild type and the mutant (Table I), IFM disorganization found in *ket<sup>14</sup>/+* adults may be due to muscle degeneration most probably caused by muscle contraction.

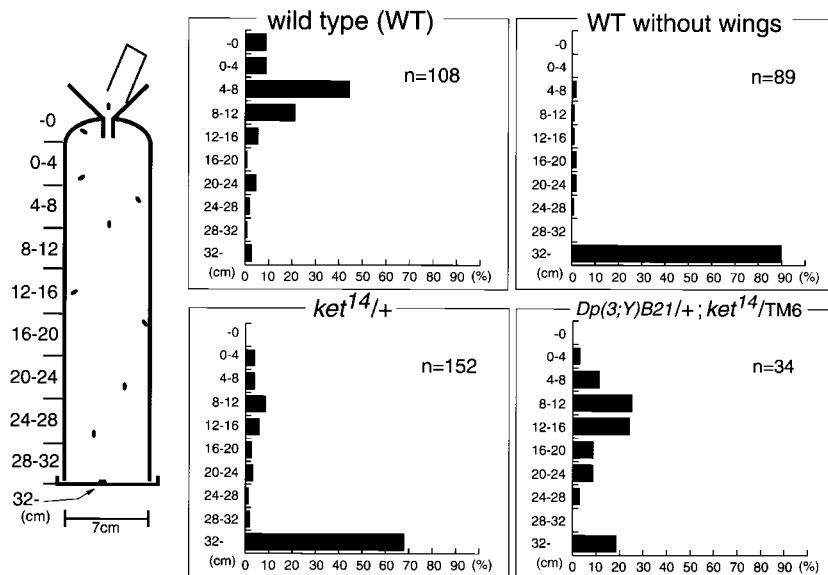
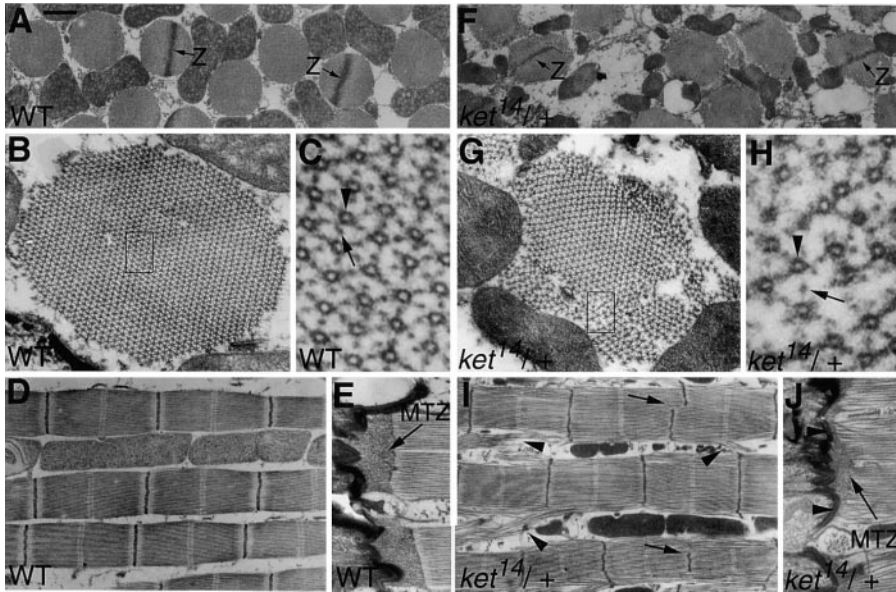


Figure 6. Requirements of *kettin* for acquiring flying capability. Results of flight test of wild-type (Canton S) flies with or without wings, and *ket<sup>14</sup>/+* flies with or without *Dp(3;Y)B21*, which includes the *kettin* locus. An apparatus used for flight test is schematically shown in the left margin. Flies were dropped through a funnel into a column 32 cm in height and coated with liquid paraffin. Virtually all flies capable of flying are expected to stick to the wall. Note that ~70% of *ket<sup>14</sup>/+* flies dropped to the bottom while 80% of those with *Dp(3;Y)B21* stuck to the wall, indicating *kettin* to be essential for acquiring flying capability.



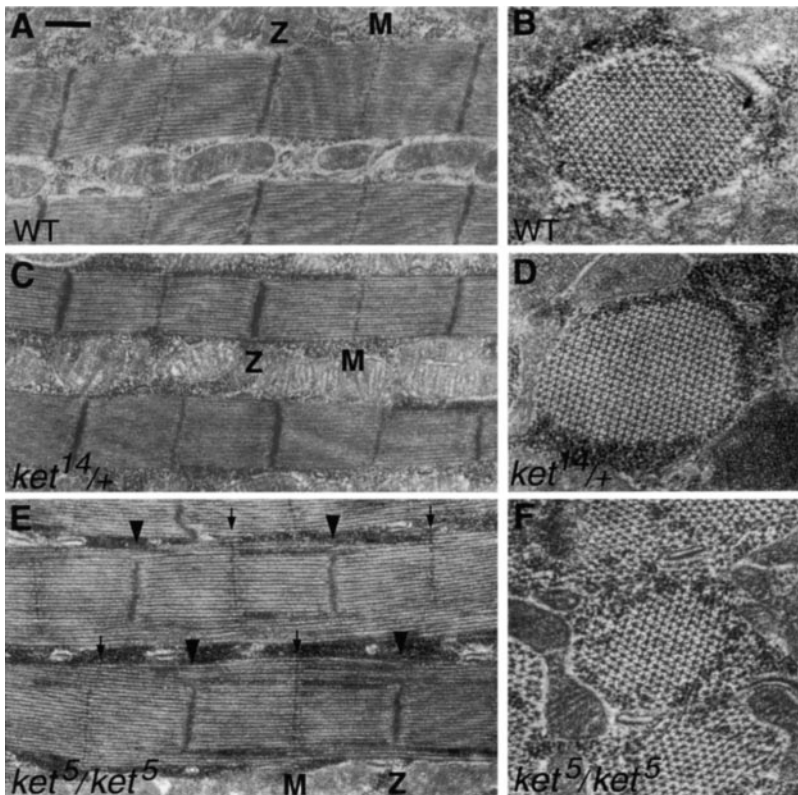
**Figure 7.** Electron micrographs of IFMs of wild-type (A-E) and *ket<sup>14</sup>/+* (F-J) adult flies. (A-C and F-H) Transverse sections of muscles. (D, E, I and J) Longitudinal sections of muscles. (A and F) Arrows labeled Z indicate Z-discs. Note that one of two Z-discs in the mutant (F) does not reach the muscle bundle periphery. (B and G) Enlarged muscle bundles. Peripheral regions of bundles heterozygous for *ket<sup>14</sup>* (G) are considerably disorganized. Regions enclosed by boxes are enlarged in (C and H). Arrowhead, myosin or thick filament. Arrow, actin or thin filament. The hexagonal lattice of normally packed filaments is deformed in the periphery of mutant muscle fiber bundles. (D and I) Z-disc defects and myofibril aberrations such as branching and breakage frequently found in *ket<sup>14</sup>/+* IFMs are shown by arrows and arrowheads, respectively. (E

and J) Myofilaments are connected to muscle cell membrane via modified terminal Z-discs (MTZ). Note that, in *ket<sup>14</sup>/+* heterozygotes, modified terminal Z-discs are diminished in size and some myofilaments appear to contact to muscle cell membrane directly as shown by the arrowhead in J. Bar: (A, D, F, and I) 0.8  $\mu$ m; (B and G) 0.16  $\mu$ m; (E and J) 0.5  $\mu$ m.

However, the above finding may not exclude the possibility that Kettin is involved in IFM sarcomere formation. Indeed, we found morphological defects in late pupal IFMs homozygous for *ket<sup>5</sup>* (Fig. 8, E and F), in which Kettin expression may be lower than that in *ket<sup>14</sup>/+* flies (Table I). As shown in Fig. 8 E, M-lines normally reached the myofibril periphery, whereas most Z-discs did not.

comere length and thin/thick filament ratio appeared normal (Table I). Since IFM contraction is not yet initiated at late pupae, it may follow that Kettin is also required for IFM Z-disc formation.

All myofibrils in IFMs were anchored to the plasma membrane of muscle cells by modified terminal Z-discs (Fig. 7 E; Reedy and Beall, 1993). In *ket<sup>14</sup>/+* flies, the



**Figure 8.** Electron micrographs of IFMs derived from wild-type and *kettin* mutant pharate adults (late pupae). A, C, and E are longitudinal sections, whereas B, D, and F are transverse sections. A and B, wild type; C and D, *ket<sup>14</sup>/+*; and E and F, *ket<sup>5</sup>/ket<sup>5</sup>*. IFM contraction is considered to be initiated after eclosion. *ket<sup>14</sup>/+* IFMs are virtually normal in appearance (compare C and D with A and B), but destroyed after eclosion (Fig. 7 I), indicating that *kettin* is essential for the maintenance of IFMs. On the other hand, *ket<sup>5</sup>/ket<sup>5</sup>* IFMs possessed morphological defects before eclosion; myofilaments were normally packed to generate the typical hexagonal lattice in the center, but the lattice structure appeared loose in the periphery near Z-discs (E and F, arrowheads); as shown by the arrows in E, M-lines are normally formed. These findings may suggest that *kettin* is also involved in normal IFM formation most probably through Z-disc formation. Bar: (A, C, and E) 0.5  $\mu$ m; (B, D, and F) 0.25  $\mu$ m.

terminal Z-discs showed diminished size and departure from normal shape (Fig. 7, compare J with E), suggesting the role of Kettin in terminal Z-disc formation in IFM.

### Identification of *C. elegans kettin*

To determine whether the structure of Kettin is conserved in evolution, the entire genomic DNA of *Caenorhabditis elegans*, with virtually complete nucleotide sequence (The *C. elegans* Sequencing Consortium, 1998), was examined. A genomic clone R05D8 encoding a long polypeptide highly similar in amino acid sequence to Kettin was initially identified, and the study of surrounding genomic DNA sequences confirmed the presence of a putative *C. elegans kettin* gene (Fig. 1 F). The true *C. elegans* Kettin was presumed to have maximum homology with *Drosophila* Kettin. The structure of the putative *C. elegans kettin* gene is outlined in Fig. 1 F. Fig. 2, A and B, shows that *C. elegans* Kettin is a 468-kD protein comprised of 4,219 amino acids, containing 30 Ig domains and highly similar in overall structure to *D. melanogaster* Kettin. As with *Drosophila* Kettin, regions 2 and 3 contain spacer sequences of nearly constant length with two conserved Kettin spacer motifs found in *Drosophila* (Fig. 2 B). The entire Kettin structure is concluded to be highly conserved in evolution.

### *Kettin, as a Protein Distinct from D-Titin*

*D-titin* is a hypothetical gene proposed by Machado et al. (1998) based on circumstantial evidence such as in situ hybridization to polytene chromosome and antibody staining. Although the sequence of *D-titin* was represented by those of four short DNA fragments, KZ, NB, LG, and JT, there is no solid evidence that they are derivatives of an identical gene (Machado et al., 1998). After the nucleotide sequence of the *kettin* coding region had been determined, we found that KZ is completely identical to the 5' terminal sequence of *kettin* cDNA, coding for the NH<sub>2</sub>-terminal region of Kettin (Fig. 1 C). *D-titin* and *kettin* RNA may, thus, be alternative spliced products to each other and if so, Kettin and D-Titin protein may be considered to possess an NH<sub>2</sub>-terminal sequence in common.

To clarify this point further, we carried out Northern blotting and in situ hybridization using an NB probe (NB2). As shown in Fig. 1 D, lane 2, no or little signals were detected when RNA extracted from wild-type embryos or larvae was subjected to northern hybridization. In contrast, apparent hybridization signals were detected when *kettin* probes were used (see lane 1). Fig. 3 U shows a part of the results of whole mount in situ hybridization to embryos. Again, unlike *kettin* probe, *D-titin* probe, NB, gave no or little signals throughout embryonic development. We interpret these results as strongly suggesting that either *D-titin* RNA represents a minor transcriptional unit at the *kettin* locus or *D-titin* is a gene completely different from *kettin*. That there are no or little NB signals in embryos also indicates that, unlike Kettin, putative D-Titin protein may not be involved in embryonic muscle development and/or function.

According to Machado et al. (1998), D-Titin is a chromosomal protein, and anti-KZ antibody can visualize

chromosomes in early *Drosophila* embryos, presumably recognizing the NH<sub>2</sub>-terminal region of Kettin. Thus, we stained *Drosophila* embryonic nuclei by our anti-Kettin antibody. As shown in Fig. 3, V-W, no or little chromosomal signals of Kettin were detected, possibly suggesting that Kettin is not a chromosomal protein.

## Discussion

### *Kettin, a Giant Muscle Protein with Overall Structure Highly Conserved in Evolution and Multiple Actin Binding Sites*

Based on genomic DNA and cDNA sequences, the entire amino acid sequence of the major isoform of *Drosophila* Kettin was deduced (Fig. 2). *Drosophila* Kettin is highly repetitive in sequence, and each repeating unit is comprised of an Ig domain ~95 amino acids long and a spacer sequence. Unlike other titin family members (Benian et al., 1999), Kettin possesses neither protein kinase, fibronectin type III, nor PEVK domains (Fig. 2 A). Kettin structure appears highly conserved in evolution, since the predicted *C. elegans* Kettin is very similar in overall structure to the *Drosophila* counterpart (see Fig. 2).

The central 25 spacers in *Drosophila* Kettin may have unique properties since these spacers share in common two Kettin spacer motifs (I and II; Fig. 2 B). All spacers in region 2 other than S6 show little variation in length. Kettin is thought to bind to actin (Lakey et al., 1993). Plots of binding data showed that 28 mol of actin monomer can bind to 1 mol of the 500-kD *Lethocerus* leg Kettin at maximum (Straaten et al., 1999), suggesting that the 540-kD *Drosophila* Kettin (the major isoform) possesses a similar number of actin binding sites. Ig domain itself has no actin binding activity, whereas an Ig domain flanked with spacers is capable of binding to actin (Lakey et al., 1993), indicating the importance of spacer sequences in actin binding. The number of spacers with conserved motifs I and II is 25, this being essentially identical to that predicted for actin binding sites. Thus, it might be speculated that region 2 and region 3 spacers with conserved Kettin motifs provide actin binding sites, and the major isoform of *Drosophila* Kettin possesses 25 actin binding sites in all. In vertebrates, single nebulin molecules span each thin actin filament and are presumed to serve as protein rulers for thin filament formation (Trinick et al., 1994). However, Kettin may not be involved in such a process since the actin filament distal to the Z-disc is occupied by tropomyosin, a muscle protein competing with Kettin for actin (Straaten et al., 1999). It is also quite feasible that, unlike titin (for review see Gautel et al., 1999), Kettin is not a mechanical integrator of thick and thin filaments during contraction because it lacks myosin binding activity (Lakey et al., 1993).

Kettin may also interact with  $\alpha$ -actinin, since  $\alpha$ -actinin is released from Z-discs of IFMs through the proteolysis of Kettin with calpain (Lakey et al., 1993). Consistent with this, a Kettin Ig domain associated with spacer sequences has been shown to be capable of binding to  $\alpha$ -actinin (Lakey et al., 1993). Immunoelectron microscopy showed, in IFM, Kettin to be oriented with the NH<sub>2</sub> terminus in the

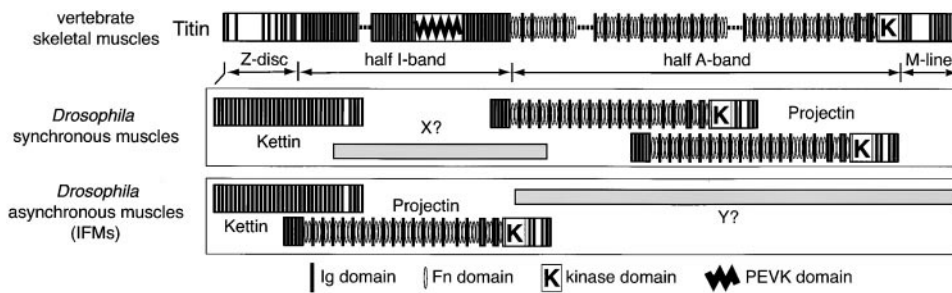


Figure 9. Comparison of structures and intra-sarcomere localizations of *Drosophila* Kettin, *Drosophila* Projectin and vertebrate titin (modified from Benian et al., 1999; Kolmerer et al., 1999). *Drosophila* muscle sarcomeres are boxed. In human muscles, the NH<sub>2</sub>- and COOH-terminal regions of titin are embedded in Z-disc and M-line, respectively, and hence, extend

across the half sarcomere (Labeit and Kolmerer, 1995). As with the A-band region of titin, Projectin possesses not only Ig domains but also fibronectin type III and kinase domains (for review see Gautel et al., 1999). In *Drosophila* synchronous and asynchronous muscles, Projectin is situated in the A-band and I-band regions, respectively (Vigoreaux et al., 1991). In both types of muscles, Kettin is a Z-disc protein. Almost all parts of Kettin consist of repeat units of an Ig domain and a spacer sequence and lack fibronectin type III, kinase, and PVEK domains. X and Y, respectively, are hypothetical *Drosophila* muscle proteins corresponding to the PEVK domain and the A-band region of titin.

Z-disc and the COOH terminus outside (Straaten et al., 1999). Thus, Ig repeats in region 1 and the NH<sub>2</sub>-terminal nonconserved sequences might be involved in possible head-to-head interactions of Kettin molecules and/or interactions between Kettin and other Z-disc proteins including  $\alpha$ -actinin.

In insect IFMs, the size of a half Z-disc is  $\sim 50$  nm (Straaten et al., 1999). A single repeating unit (Ig + Spacer) of Kettin was shown to cover  $\sim 3$  nm of the filament (Straaten et al., 1999) and, thus, the  $\sim 50$  nm may roughly correspond to 16 units, the region spanning repeating units 5–20 (region 2). In region 2, both spacer length and the distance between spacer motifs I and II are almost strictly conserved not only in *Drosophila*, but also in *C. elegans* (Fig. 2 A). Thus, the region 2/region 3 boundary of Kettin might serve as a signal to separate Kettin into two regions: one capable of interacting with Z-disc components, and the other not. Precise spacing of actin binding sites on Kettin may guarantee tight binding of Kettin to thin filament, in which actin monomers may form a helical structure with a constant pitch, to generate stable sarcomere end structures.

### Kettin Functions and Possible Functional Relationships among Kettin, Titin, and Projectin

Our structural analysis showed that *Drosophila* Kettin and vertebrate titin share in common Ig domain repeats, but neither protein kinase nor fibronectin type III domains are included in the former, indicating that Kettin is not a *Drosophila* counterpart of titin.

Projectin is another titin family member found in *Drosophila*, and its nearly complete amino acid sequence has been determined (Daley et al., 1998). Strangely, the location of Projectin within the sarcomere varies depending on muscle types (Vigoreaux et al., 1991). As schematically shown in Fig. 9, Projectin is situated mainly in the A-band region in synchronous muscles, which includes larval somatic muscles, whereas, in asynchronous muscles such as IFM, its location is within the I-band region. The A-band region of titin is constructed in a modular fashion and includes intermingled repeats of Ig and fibronectin type III domains, which together provide binding sites for thick fil-

aments. The A-band region of titin also includes a protein kinase domain. Projectin is quite similar in modular structure to the A-band region of titin, although its size is much smaller than that of titin (Labeit and Kolmerer, 1995a; Daley et al., 1998). A protein kinase domain similar in sequence to that of the A-band region of titin is also included in Projectin (Ayme-Southgate et al., 1995). Thus, at least in synchronous muscles, Projectin might be considered to be a functional substitute for the A-band region of titin (for review see Benian et al., 1999). Consistent with this, as with titin (for review see Kolmerer et al., 1999), Projectin is necessary for the contraction of larval somatic muscles (Benian et al., 1999).

Kettin is located in the vicinity of the Z-disc in all types of *Drosophila* muscles so far examined (Fig. 4), suggesting its general roles at the sarcomere end. The corresponding region of titin consists of Ig domains, specific motifs called Z-repeats, phosphorylation sites, and flanking sequences (Young et al., 1998). The Z-disc region of titin is capable of binding to  $\alpha$ -actinin directly and thin filaments indirectly. Although there is little similarity in the amino acid sequence between Kettin and the Z-disc region of titin except for Ig domains, Kettin is also capable of binding to  $\alpha$ -actinin along with actin, most probably through its repeating units (Lakey et al., 1993). In vertebrates, titin and nebulin may together form a scaffold for Z-disc assembly (Gautel et al., 1999). Our results may indicate that Kettin is also involved in a similar process in *Drosophila*. Indeed, Fig. 5, B and D, shows that Z-disc-like structures formed in synchronous somatic muscles mutant for *kettin* are very unstable (Fig. 5, B and D). Similarly, premature Z-discs were formed in IFM homozygous for *ket<sup>5</sup>* (see Fig. 8, E and F). In IFM heterozygous for *ket<sup>14</sup>*, Z-discs virtually normal in appearance were formed at pupal stages (Fig. 8, C and D) but frequently were destroyed in adults (Fig. 7, F–J), leading to the flightless phenotype (Fig. 6). Consistent with these findings, Kettin promoted antiparallel assembly of actin filaments in vitro in the absence of  $\alpha$ -actinin (Straaten et al., 1999). Our results (Fig. 3 A) indicated that *kettin* expression in muscle cells is initiated at the early stages of muscle cell development when no muscle fiber is yet formed; similar early expression is also known in the case of titin (for review see Fulton, 1999).

The I-band region of titin contains tandem Ig domain modules and the PEVK domain (Labeit and Kolmerer, 1995). Tandem Ig domains elongate with little increase in passive tension, while unfolding of the PEVK domain requires substantial force. These domains may, thus, account for the elasticity of titin (for review see Horowitz, 1999). Projectin, situated in the I-band region of IFM sarcomeres is supposed to be elastic, though there is no PEVK sequence (Benian et al., 1999). D-Titin (the protein product of a hypothetical gene, *D-titin*) was reported to contain a PEVK-like region (Machado et al., 1998), but Fig. 3 U shows that there is no or little expression of *D-titin* at least in embryonic somatic muscles. The function of Ig domains themselves is not yet clear. Since Ig domains are involved in protein-protein interactions (Williams and Barclay, 1988), Kettin Ig domains might interact with themselves or other Ig domain-containing proteins such as Projectin. As with Ig repeats and the PEVK domain of titin (Labeit and Kolmerer, 1995), Kettin Ig repeats might be flexible in structure and involved in regulation of possible breakage/reunion of thin filaments during muscle contraction/relaxation. A recent structural analysis of the Twitchin protein kinase domain indicates Ig domain to be capable of binding to the protein kinase domain (Kobe et al., 1996). Thus, Kettin Ig domains might interact with protein kinases possibly involved in Z-disc assembly to regulate their activity.

### ***kettin* Is a Gene Unrelated to *D-titin***

*D-titin* is a *Drosophila* gene, predicted as the counterpart of vertebrate *titin* by Machado et al. (1998). A short genomic DNA fragment of *D-titin*, LG, was initially isolated from a *Drosophila* genomic expression library using the human autoimmune scleroderma serum. Then, three additional short pieces of cDNA, KZ, NB, and JT, were isolated. In particular, KZ was isolated using P insertion, V(3)ET1, presumed to be located at 62C1-2, the putative *D-titin* locus, but there is no solid evidence that KZ and other *D-titin* fragments are derivatives of an identical gene. Our results show that KZ is identical in sequence to the 5'-terminal region of *kettin* (Fig. 1 C), but *kettin* possesses no homology to other *D-titin* fragments, suggesting that *D-titin* RNA is one of possible alternative splicing products from the *kettin* locus. This hypothesis was first tested by Northern hybridization and whole mount in situ hybridization to embryos using an NB probe, but no signals were detected in both cases (see Fig. 1 D, lane 2, and Fig. 3 U). Thus, *D-titin* might be a very minor transcriptional unit at the *kettin* locus, if any.

KZ is presumed to encode the NH<sub>2</sub>-terminal region of D-Titin (Machado et al., 1998). Machado et al. (1998) showed that anti-KZ antibody is capable of staining the chromosomes of HEP-2 cells (human cells), and maintained that D-Titin serves as a scaffold not only for the sarcomere but for the chromosome as well. However, their experiments using antibodies raising against the NH<sub>2</sub>-terminal region of human titin showed the contrary (Machado et al., 1998). No chromosomal titin signals could be detected in HEP-2 cells when antibodies for the NH<sub>2</sub>-terminal region of human titin were used. To account for this apparent discrepancy, they postulated that there are two forms of titin (or D-Titin), chromosomal and muscle types,

and the NH<sub>2</sub>-terminal sequences of these two types are different from each other (Machado et al., 1998). However, this notion may not be applicable to D-Titin since our results showed that the KZ region, the presumptive NH<sub>2</sub>-terminal region of chromosomal type D-Titin, is essential for muscle function. Indeed, all *kettin* mutants examined in this study have defects in the KZ region, and, in particular, in *ket<sup>14</sup>* mutants, the KZ region is almost completely deleted (Fig. 1 C). As shown in Fig. 5, homozygotes of these *kettin* mutations possess muscle defects at late developmental stages, but are almost normal in early embryonic stages when muscles are not yet formed (Hakeda, S., unpublished data), indicating that KZ is essential for muscle development but not for chromosomal scaffolding. Consistent with this, no chromosomal signals were detected by anti-Kettin antibody raising against the central region of Kettin (see Fig. 3, V and W). Thus, chromosomal KZ signals detected in HEP-2 cells and nuclei in early *Drosophila* embryos by Machado et al. (1998) are quite likely to be due to possible cross-reaction of their anti-KZ antibody to a completely different protein.

An additional problem of *D-titin* is that LG, isolated originally as a fragment encoding the putative PEVK domain of D-Titin is a fortuitous composite of fragments derived from different chromosomal loci. Indeed, by examining the *Drosophila* genomic sequence, we found that a genomic DNA sequence at 62A10-62B5 (accession number, AC005557) is identical to the sequence of a 3' half of LG, while no homology to the remaining half, which encodes the PEVK domain, could be found not only in the sequence of 62A10-62B5, but also in other *Drosophila* genomic regions whose sequences are available. Taken together, these considerations may lead us to the conclusion that *D-titin* is a putative gene unrelated to *kettin* at all.

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