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Drosophila D-Titin is required for myoblast fusion and skeletal muscle striation

Yong Zhang, David Featherstone, Warren Davis, Emma Rushton and Kendal Broadie*

Department of Biology, University of Utah, Salt Lake City, Utah 84112-0840, USA
*Author for correspondence (e-mail: broadie@biology.utah.edu)

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SUMMARY

An ethylmethane sulfonate (EMS) mutagenesis of *Drosophila melanogaster* aimed at discovering novel genes essential for neuromuscular development identified six embryonic lethal alleles of one genetic locus on the third chromosome at 62C. Two additional lethal *P* element insertion lines, *l(3)S02001* and *l(3)j1D7*, failed to complement each other and each of the six EMS alleles. Analysis of genomic sequence bracketing the two insertion sites predicted a protein of 16,215 amino acid residues, encoded by a 70 kb genomic region. This sequence includes the recently characterized *kettin*, and includes all known partial *D-Titin* sequences. We call the genetic locus, which encodes both D-Titin and kettin, *D-Titin*. D-Titin has 53 repeats of the immunoglobulin C2 domain, 6 repeats of the fibronectin type III domain and two large PEVK domains. Kettin appears to be the NH2-terminal one third of D-Titin, presumably expressed via alternative splicing. Phenotype assays on the allelic series of *D-Titin* mutants demonstrated that D-Titin plays an essential role in muscle development. First, D-Titin has an unsuspected function in myoblast fusion during myogenesis and, second, D-Titin later serves to organize myofilaments into the highly ordered arrays underlying skeletal muscle striation. We propose that D-Titin is instrumental in the development of the two defining features of striated muscle: the formation of multi-nucleate syncitia and the organization of actin-myosin filaments into striated arrays.

Key words: D-Titin, Kettin, Muscle, Myogenesis, *Drosophila*

INTRODUCTION

Titin (from Greek ‘titan’: giant deity; of great size) is the largest known protein and the third most abundant component of striated muscle (Maruyama, 1976; Wang et al., 1979). Vertebrate titin has a molecular mass of 3,000 kDa, and is composed of 27,000 amino acid (aa) residues, of which 90% are repeats of immunoglobulin C2 (Ig) domains and fibronectin type III (FN3) domains; 10% is unique titin sequence, including a PEVK domain (containing high percentages of P, proline; E, glutamic acid; V, valine and K, lysine) and a titin kinase domain (Labeit and Kolmerer, 1995). During myogenesis, titin is one of the earliest molecules present, localized prior to actin, myosin and other components of the contractile apparatus (Furst et al., 1989; van der Ven and Furst, 1997). In addition to binding actin (Linke et al., 1997; Astier et al., 1998) and myosin (Isaacs et al., 1992), titin also binds several other sarcomeric proteins, including actinin (Sorimachi et al., 1997), T-cap (Gregorio et al., 1998), M-protein and myomesin (Obermann et al., 1996, 1997).

In mature muscle, the filamentous >1 μm titin molecule spans half a sarcomere, with the NH2 terminus localized at the Z disc and the COOH terminus at the M line. Titin is thought to be intertwined with actin thin filaments and myosin thick filaments to provide muscle with elasticity via its tandem Ig repeats and titin specific PEVK domain (Labeit and Kolmerer, 1995; Funatsu, 1996). Titin is specifically expressed in skeletal and cardiac muscle, but absent in smooth muscle (Wang et al., 1979), suggesting that the protein has unique function(s) in striated muscle. These observations have led to the hypotheses that: (1) titin acts as the template or scaffold upon which the sarcomere is assembled during development, and/or (2) titin serves as the elastic element in mature muscle (Fulton and Isaacs, 1991; Trinick, 1994; Gregorio et al., 1998, 1999; Linke et al., 1998; Machado et al., 1998; Trombitas et al., 1998). While a great deal of data support the second hypothesis (for example, Tskhovrebova et al., 1997; for comments see Keller III, 1997), the template/scaffold hypothesis has not been experimentally tested and the in vivo function of titin in muscle development remains unclear.

Members of the titin gene family are present in both vertebrates and invertebrates. In *Drosophila*, three members of the titin family have been identified: projectin, kettin and D-Titin. Like vertebrate titin, projectin has a functional kinase domain, in addition to many repeats of Ig domain and FN3 domain (Ayme-Southgate et al., 1991). However, no titin specific PEVK domain has been found in projectin. Kettin was identified as a large muscle Z-disc protein, consisting of a long chain of Ig domains interspersed with less conserved short amino acid linkers (Lakey et al., 1993). Kettin binds actin and actinin, but not myosin (Lakey et al., 1993; van Straaten et al., 1999). Immunoelectron microcopy showed that the NH2

terminus of kettin localizes within the Z disc, whereas the COOH terminus lies some distance outside. Very recently, the full-length sequence of the *Drosophila* kettin gene has been elucidated (Hakeda et al., 2000; Kolmerer et al., 2000). The vast majority (98% of 4796 aa) of kettin consists of 35 Ig domains separated by spacers; the only unique, non-repetitive sequence is the NH2 terminus 86 aa. Genetic analysis of three kettin mutant alleles demonstrated that kettin is essential for the formation and maintenance of muscle sarcomere structure and muscle tendons (Hakeda et al., 2000).

Using a scleroderma (systemic sclerosis) patient serum, Machado et al. (1998) identified a *Drosophila* homologue of vertebrate titin: D-Titin. On polytene chromosomes, *D-Titin* mapped at 62C1-2, at which only one genetic locus, *l(3)62Ca*, with an exceptionally large number of alleles, was identified during an intensive cytogenetic study in the region (Sliter et al., 1989). However, with very limited sequence information on *D-Titin* (4 partial sequences encoding 1606 aa in total (Machado et al., 1998), compared with 26,926 aa human cardiac titin (Labeit and Kolmerer, 1995)), it has remained controversial whether *Drosophila* indeed has an orthologue of vertebrate titin. More remarkably, recent work (Hakeda et al., 2000; Kolmerer et al., 2000) showed that the D-Titin NH2-terminal is identical to that of kettin, leaving the identity and/or the presence of D-Titin in *Drosophila* elusive.

From a mutagenesis screen for novel genes essential for neuromuscular development in *Drosophila*, we have identified a genetic locus on the third chromosome at 62C with eight alleles, including two lethal *P*-element insertion lines. Computational analysis of the genomic region, containing the two *P*-element insertion sites, predicted a large gene of 70 kb encoding a peptide of 16,215 aa residues, including kettin sequence (Hakeda et al., 2000; Kolmerer et al., 2000) and all four partial D-Titin sequences (Machado et al., 1998). The estimated molecular weight of the protein is approx. 1.8 mega-dalton, consistent with the mega-dalton size shown on western blots (Machado et al., 1998). Kettin appears to be the NH2-terminal one third of D-Titin. We call the genetic locus *D-Titin*, consistent with Machado et al. (1998). Using the eight alleles, we have examined the developmental and structural phenotypes of *D-Titin* mutants, focusing on its muscle functions. We have demonstrated that D-Titin plays an unexpected role in myoblast fusion. Furthermore, our results show that D-Titin is required for the parallel, regular arrangements of myofilaments characteristic of mature myofibrils. This regular array of contractile machinery (visible under the light microscope as striations) is the most fundamental morphological marker for skeletal and cardiac muscle (‘striated’), compared to ‘smooth’ muscle. This work has clarified the molecular relationship between D-Titin and kettin, and demonstrated that D-Titin is not only required for the assembly of sarcomere structure, but also involved in early muscle development.

MATERIALS AND METHODS

Genetic manipulations of *Drosophila*

Ethylmethane sulfonate (EMS) mutagenesis on the 3rd chromosome (approx. 40% of the *Drosophila* genome) was performed using standard techniques. Embryonic lethals with abnormal locomotory behavior (severe uncoordination, paralysis) but grossly normal neuromusculature were selected for characterization. The neuromusculature was visualized by staining embryos with an anti-horseradish-peroxidase antibody (specific to the nervous system) (Desai et al., 1994) and a muscle specific anti-myosin antibody (Kiehart and Feghali, 1986). Selected mutants were mapped on the 3rd chromosome by recombination with a multiple marker chromosome carrying *roughoid*, *hairy*, *thread*, *scarlet*, *curled*, *stripe*, *ebony* and *claret*, followed by fine deficiency mapping.

For the *D-Titin* genetic analyses, deficiency lines *Df(3L)Aprt3*, *Df(3L)Aprt14*, *Df(3L)Aprt71*, *Df(3L)Aprt96*, *Df(3L)Aprt123*, *Df(3L)Aprt198*, *Df(3L)Aprt199* and $Df(3L)R^{G5}$ were from J. Mason (Wang et al., 1994). *P*-element insertion lines: *l(3)S02001* (abbreviated as *20/01*), *l(3)j1D7* (abbreviated as *1D7*), *l(3)04680*, *l(3)rL182*, *l(3)S02385* and *l(3)S61002*, were from public *Drosophila* stock centers, either from the Bloomington *Drosophila* Stock Center or from the Szeged Stock Center in Hungary. Three kettin mutant alleles, ket^{132}, ket^{5} and ket^{14} were from Saigo (Hakeda et al., 2000). For mutant phenotype analyses, all lines were balanced over TM6B, P{ubiquitin-GFP}, so that homozygous or heterozygous mutants could be easily identified based on green fluorescent protein (GFP) expression. Homozygous mutant embryos lacking GFP were manually selected for all phenotypic assays. Embryos were staged from timed egg lays and by the use of precise morphological markers of developmental stages. Isolation of genetic revertants of two lethal *P* element insertion lines, *20/01* and *1D7*, was done as described (Salzberg et al., 1997).

Plasmid rescue of *P*-element insertion lines and polytene chromosome in situ hybridization

Cloning of flanking chromosomal sequences of the *P*-element insertion lines, *20/01*, *1D7* and *l(3)04680*, was done as described (Deak et al., 1997). One end of the rescued genomic fragment was sequenced with primers as described (Deak et al., 1997); the other end of the fragment was sequenced with a newly synthesized primer: primer 3.Y: AGTGCCACCTGACGTCTAAG or primer 5.Y: CACCTCTGACTTGAGCGTCG. Internal primers were then designed to sequence the entire genomic insert of rescued plasmids. For polytene chromosome in situ hybridization (following standard protocols), *D-Titin* probe was prepared from the rescued plasmid of *20/01*; *kettin* probe was prepared from a PCR product, amplified from wild-type *Drosophila* genomic DNA with a pair of primers, primer 1: CACCATGCCGGTAAGGGATG (base 3-22 of database entry X72709) and primer 2: CCTCGGTACGTGCATTCGGC (in opposite direction of base 1545-1564 of database entry X72709). For RT-PCR (**r**everse **t**ranscription-PCR), mRNA was isolated from embryos and first instar larvae of wild-type fly Oregon R using TRIzol^{R} reagent (Jowett, 1998). RT-PCR primers were designed based on the predicted exons.

Database computing

Rescued sequences from *20/01* and *1D7* were used to perform a BLASTN (**b**asic **l**ocal **a**lignment **s**earch **t**ool for a **n**ucleotide query) search against the *Drosophila* genome database, including the htgs (**h**igh **t**hroughput **g**enomic **s**equences) database. Genomic sequences containing the rescued sequences were again used to find overlapping clones. Five sequences: AC019822, AC019792, AC020480, AC020485 and AC017325, produced by Celera genome project (http://www.celera.com/home.asp), plus the two rescued sequences from *20/01* and *1D7*, were used to build a contig. To look for potential genes, the contig sequence was then analyzed with multiple gene finder programs. However the gene structure predicted by FGENESH (*Drosophila* version) at http://genomic.sanger.ac.uk/gf/gfb.html was used in the final analyses presented here, since FGENESH predicted the characterized full-length kettin cDNA (Hakeda et al., 2000; Kolmerer et al., 2000) and the partial D-Titin cDNA sequences (clones KZ, NB and JT; Machado et al., 1989) as exons correctly, except the

3′ end of the last exon of *kettin* was predicted as an intron. A BLASTP (a peptide query against protein database) search was then performed to look for homologies between the predicted peptide and the database entries.

Quantification of muscle nuclei

Mature (22-24 hours after egg laying (AEL) at 25°C) embryos were dechorionated in bleach, manually devitellinized, glued to Sylgard-coated cover slips and minimally dissected along the dorsal midline as previously reported (Fergestad et al., 1999). Immediately after dissection, preparations were incubated in diamidino-phenylindole (DAPI, Sigma) solution (33 ng DAPI /ml of H_2O) at room temperature for 5 minutes. Following incubation in DAPI, the preparations were rinsed in standard *Drosophila* saline and moved for nuclei quantification to a compound microscope equipped with standard UV epifluorescence, a DAPI fluorescence-selective (blue, 461 nm) emission filter, DIC, and a water-immersion objective. Individual muscle fibers were identified based on position and orientation with a ×40 objective using DIC, then fluorescence was used to visualize individual nuclei. For quantification, nuclei in each muscle (6 and 7) for abdominal segments A3-A5 on both sides of each dissected embryo were counted for 6-10 embryos from each genotype (WT, *C872*, *B68* and *B173*).

Immunohistochemical methods and electron microscopy

Antibody staining on whole-mount embryos and on dissected embryos for brightfield and confocal microscopy was done as described (Rushton et al., 1995; Fergestad et al., 1999). Anti-horseradish-peroxidase was from Cappel/ICN (Costa Mesa, CA) and was used at 1/500. Texas red-phalloidin for F-actin staining was from Molecular Probes (Eugene, OR) and used at 1:1,000 dilution; anti-myosin from Kiehart (Kiehart and Feghali, 1986) and used at 1:1,000; anti-actinin from Saide (Saide et al., 1989) and used at 1:40; α-KZ antibody from Andrew (Machado et al., 1998) and used at 1:5,000 for whole mount staining, 1:500 for the dissected embryo staining; kettin antibody (MAC155) from Bullard (Lakey et al., 1993) and used at 1:100.

Electron microscopy of embryos was done following previous procedures (Prokop et al., 1996). Briefly, mature embryos were injected with 5% glutaraldehyde in 0.05 M phosphate buffer, followed by excision of extremities in 2.5% glutaraldehyde in the same buffer. Specimens were post-fixed in 1% osmium tetroxide, stained en bloc in 1% uranyl acetate in dH_2O, dehydrated, and embedded in epoxy resin. Ribbons of thin (50 nm) sections were collected onto slot grids and examined.

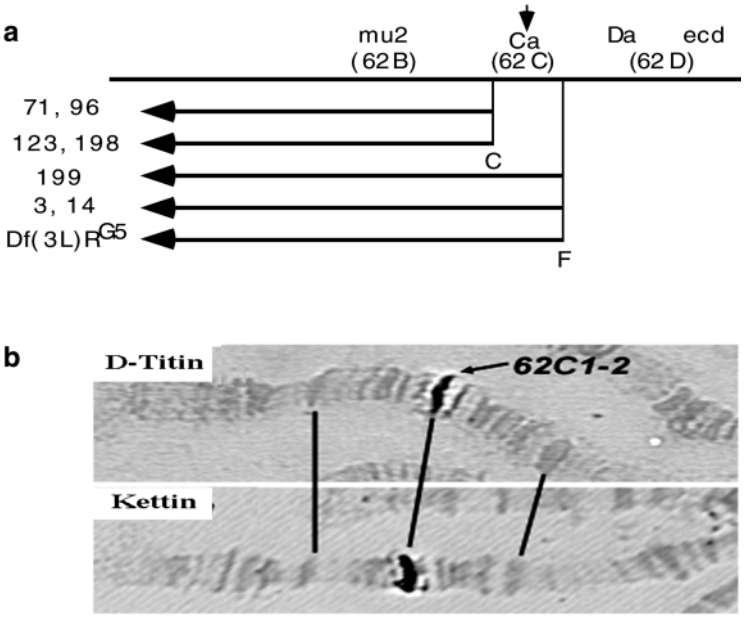

Fig. 1. Deficiency mapping and cytological mapping of the mutated gene. (a) Deficiency mapping of the mutated gene. *mu2*, *Ca*, *Da* and *ecd* are genes in the region with chromosomal location in parenthesis underneath. The mutated gene recovered from our mutagenesis is mapped to the *Ca* region. Five groups of deficiencies are depicted as horizontal lines, with arrow indicating the deficiency limit goes beyond. C indicates complementation; F, failure to complement. The deficiency prefix '*Df(3L)Aprt*' in front of numbers was omitted. (b) Cytological mapping of *D-Titin* and *kettin*. *D-Titin* and *kettin* are co-localized at 62C1-2, shown by an arrow. The equivalent bands in the two panels are indicated by straight lines.

RESULTS

Generation and identification of mutants

We are interested in identifying novel genes essential for neuromuscular functional development. To further this goal, we have screened approx. 3600 *Drosophila* third chromosomes mutagenized by EMS to select embryonic lethal, paralyzed mutants with grossly normal neuromusculature. We have identified 51 independent lethal lines representing 40 genetic loci from this screen. One exceptional locus included six independent EMS alleles: *B68*, *B170*, *B173*, *B247*, *C872* and *C995*. All six lines were embryonic lethal and failed to complement each other in pair-wise genetic crosses. Classical recombination mapping put *B173*, one of the six alleles, at the interval between *roughoid* (61F) and *hairy* (66D15) on the third chromosome. Subsequent extensive deficiency mapping showed that *B173* failed to complement *Df(3L)Aprt3*, *Df(3L)Aprt14*, *Df(3L)R^{G5}* and *Df(3L)Aprt199*; but complemented *Df(3L)Aprt71*, *Df(3L)Aprt96*, *Df(3L)Aprt123* and *Df(3L)Aprt198* (Fig. 1a, for deficiencies see Wang et al., 1994). These mapping data placed the complementation group at cytological position at 62C1-2 (Fig. 1a), at which a single genetic locus *l(3)62Ca* (also known as *l(3)dre8*, Sliter et al., 1989; Wang et al., 1994) was identified. Later on, two genes, *kettin* (van Straaten et al., 1999; Hakeda et al., 2000) and *D-Titin* (Machado et al., 1998), were identified from a reverse genetics approach independently and mapped at 62C by polytene chromosome in situ hybridization. We confirmed by polytene chromosome in situ hybridization that *kettin* and *D-Titin* colocalize at the same chromosomal bands 62C1-2 (Fig. 1b).

To identify the mutated gene, we collected all available *P*-element insertion lines in the 62C region: *20/01*, *1D7*, *l(3)04680*, *l(3)rL182*, *l(3)S02385* and *l(3)S061002*. Two lethal *P*-element lines: *20/01* and *1D7*, failed to complement each other and each of the six EMS-induced alleles isolated from our screen. To confirm that the *P*-element insertions in the two lethal lines, *20/01* and *1D7*, are the only cause of lethality, we tested for reversion of the lethal phenotype after precise excision of the *P*-element. Viable genetic revertants were obtained for both lines, demonstrating that the lethality of the

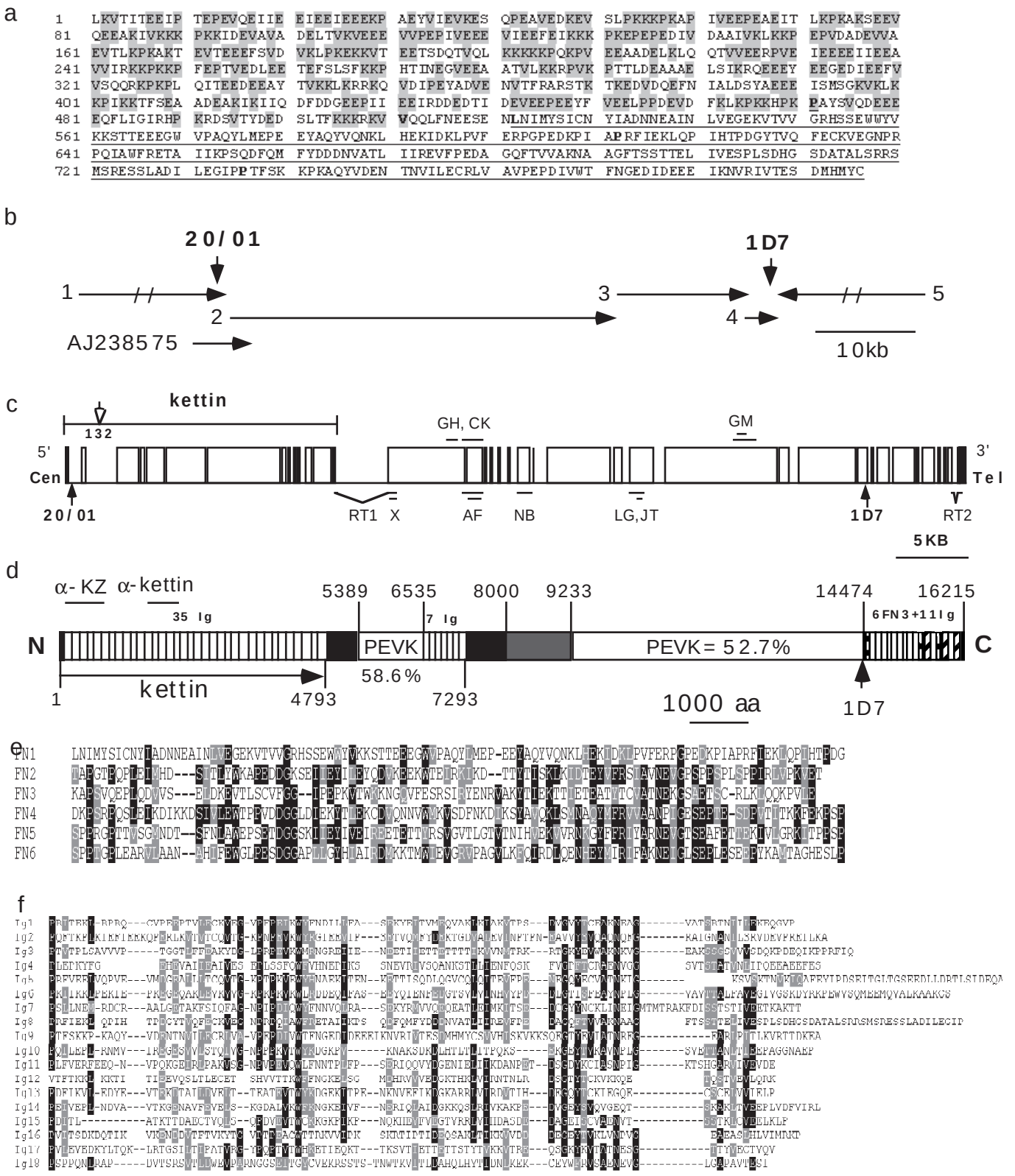

Fig. 2

two *P*-insertion lines was caused by *P*-element insertion at 62C (data not shown). In total, 8 alleles were identified for a gene mapped at 62C. All 8 mutant alleles, 6 EMS alleles and 2 *P*-element insertion alleles, are embryonic lethal except *20/01*, which dies at the pupal/pharate adult stage. No hetero-allelic complementation has been observed between the eight alleles.

Fig. 2. Predicted D-Titin gene structure. (a) Predicted peptide of the rescued *1D7* sequence. The PEVK domain with 55% P, E, V and K is shaded. The FN3 and Ig repeats are underlined. P471 in bold and underlined represents the site for *1D7* insertion. V511 in bold defines the last aa residue of the PEVK domain. L522 in bold indicates the start of FN1 (see panel e); P612 and P736 in bold the start of Ig8 and Ig9, respectively (see panel f). (b) Overlapping genomic sequences encompassing the mutated gene. Five Celera sequences (no. 1, AC019822; 2, AC019792; 3, AC020480; 4, AC020485; 5, AC017325) are in the indicated orientations. The total contig size is approx. 220 kb. The insertion sites of *20/01* and *1D7* are indicated by vertical arrows. (c) Predicted gene structure of *D-Titin*. *Kettin*, located at about 5′ one third of *D-Titin*, is delineated by a horizontal line. *20/01* and *1D7* are shown by solid vertical arrows; *P* element insertion line 132 of *kettin* shown by an open vertical arrow. Five partial *D-Titin* sequences, AF clones (accession number AF135167 and AF241649), NB, LG and JT (Machado at al., 1998), and transcript X (Hakeda et al., 2000) were previously identified. Two RT-PCR products were identified in this study (RT1 with accession number AJ400900 and RT2 AJ400901). Four EST clones: GH25930 (GH), CK00556 (CK), GM05521 and GM05288 (GM) were from the BDGP database. Open box indicates coding regions; empty space between boxes introns; black box at termini untranslated regions, in the middle small introns, small exons or both. 5′ end of *D-Titin* is toward the centromere (Cen); 3′ end toward the telomere (Tel). (d) Domain structure of D-Titin protein. Sequences recognized by α-KZ antibody and α-kettin antibody are indicated. Black boxes, no homology; gray box, low homology (blast score between 40-80) to known proteins; vertical empty strips indicate Ig repeats; 6 hatched strips at COOH-terminal denote FN3 repeats. (e) Alignment of 6 repeats of FN3 domain. FN2 and FN3 are tandemly linked repeats, and so are FN4 and FN5. Identical aa in at least 3 repeats are indicated in black; similar aa in gray. (f) Alignment of 18 repeats of Ig domain (not including the 35 identified kettin Ig repeats). The Ig1-Ig7 and Ig8-Ig16 are continuous from aa 6535-7293 and from aa 14575-15446, respectively. Identical aa in at least 9 repeats are indicated in black; similar aa in gray.

All eight lethal mutations in trans over deficiency *Df(3)Aprt14* showed unchanged lethal stages comparable to homozygous mutants, demonstrating the specificity of the lethal phenotype to the mutations.

The mutants disrupt the genetic locus D-Titin

To identify the mutated gene, we isolated the flanking genomic sequences of *P* insertions from both *20/01* and *1D7* by plasmid rescue. The rescued sequence from *20/01* showed that the *P* element is inserted in the first intron upstream of the start codon ATG of previously described *D-Titin* (Machado et al., 1998; also see Fig. 2b-c and the annotation on the rescued sequence AJ238575 in database). Polytene chromosome in situ hybridization confirmed that the *P* element of *20/01* is inserted at 62C (Fig. 1b). Three other viable *P*-element lines from BDGP (**B**erkeley ***D****rosophila* **G**enome **P**roject at http://www.fruitfly.org/), *l(3)04860*, *l(3)rL182* and *EP(3)3119*, mapped to the first intron downstream of the *D-Titin* ATG, as does the hypomorph *kettin* allele 132 (Fig. 2c). The rescued flanking sequence from *1D7* contains a partial open reading frame encoding a peptide of 796 amino acids, whose COOH terminal 300 aa residues contains one FN3 domain and two Ig domains in tandem (Fig. 2a,d-f). The sum of PEVK in the NH2-terminal is extremely high (55% of the first 511 amino acid residues, Fig. 2a), which is comparable to that (approx. 70%) of the vertebrate titin PEVK domain (Labeit and Kolmerer 1995), and to that (56-63%) of two previously identified PEVK domains in D-Titin (LG and JT clones; Machado et al., 1998). Residue V511 may define the last amino acid residue of the PEVK domain, since the percentage of PEVK in the last 285 aa residues (from aa 512 to 796) drops dramatically from 55% to 32%. The *P*-insertion of *1D7* disrupts the coding region at aa P471 of the peptide (Fig. 2a).

Since *20/01 P* inserts at the first intron upstream of *D-Titin* start ATG, and the *1D7 P* element disrupts a region encoding a titin homologous peptide (a FN3 domain, two Ig repeats and a putative titin PEVK domain; Fig. 2a-d), it would be reasonable to conclude that the two lethal *P* insertion lines are *D-Titin* mutants. However, recent work showed that the NH2-terminal of D-Titin, represented by clone KZ, is also the NH2-terminal of kettin (Hakeda et al., 2000; Kolmerer et al., 2000). *kettin* mutants have been generated and characterized (Hakeda et al., 2000) and the three *kettin* alleles, ket^{132}, ket^{5} and ket^{14}, failed to complement the mutant series we identified, including *1D7*. Based on these results, it could also be concluded that the mutants we identified have mutations in the *kettin* gene. This hypothesis, however, does not explain the lethality of *1D7*, which has a *P* element inserted 35 kb downstream of the kettin coding sequence (Fig. 2c).

To address whether *D-Titin* or *kettin* are one or two genes, we took advantage of the availability of the complete genome sequence of *Drosophila*. BLASTN search of *Drosophila* htgs database with *20/01* and *1D7* rescued sequences showed that *20/01* sequence (AJ238575) was contained in two clones, AC019822 and AC019792, whereas *1D7* sequence overlaps AC020485 and AC017352 (Fig. 2b). These four 'AC' clones, plus *20/01* rescued sequence, were joined together by AC020480, making a contig of 220 kb (see Fig. 2b). The insertion sites of *20/01* and *1D7* were mapped on the contig 57.5 kb apart (Fig. 2c). Within this 57.5 kb region, all four partial *D-Titin* sequences are arranged as reported (Fig. 2c; also see Machado et al., 1998. As pointed out by Hakeda et al., LG is a chimera clone, but its 5′ 856 bp, encoding the PEVK domain, mapped in this region). The full-length *kettin* sequence (AB026845) is localized near the *20/01* insertion site, approx. 35 kb 5′ to the *1D7* insertion site (Fig. 2c).

To study the gene organization in the region, the 57.5 kb fragment defined by *20/01* and *1D7 P* insertions and beyond was analyzed with multiple gene finding programs (see methods). Only one gene was predicted from the region bracketing the *20/01* and *1D7* insertion sites and no second transcription/translation start site was found within the region. In other words, *20/01* and *1D7* represent one gene, instead of two genes. The flanking genes predicted at each side are small and have no sequence homology to vertebrate titin (data not shown). The predicted full-length protein, encoded by 37 exons, has 16,215 aa residues (Fig. 2d), containing 53 Ig domains (for 18 non-kettin Ig domains alignment, see Fig. 2f), 6 FN3 domains (Fig. 2e), and two large putative PEVK domains: one has 1146 aa residues with the sum of P, E, V, and K 58.6%; one has 5241 aa residues with the sum of P, E, V, and K 52.7%. Sequences with no homology to the database entries (1446 aa in total, black boxes in Fig. 2d) comprise 9% of the full protein, similar to that (10%) of human titin (Labeit and Kolmerer, 1995). Unlike human titin, the predicted protein has no previously defined titin kinase domain (Mayans et al.,

1998). Since a titin kinase domain in D-Titin might be mis-predicted as introns in the case of a frameshift, all introns within the D-Titin gene and 20 kb downstream of the gene were translated in three frames and examined specifically for a titin kinase domain, but none was found.

The one-gene prediction was validated by the fact that previously characterized kettin and partial D-Titin sequences were correctly predicted. To further experimentally test the one-gene prediction, RT-PCR was performed. Two RT-PCR products, RT1 and RT2 (Fig. 2c), were obtained. The identification of RT1 demonstrates that the *kettin* stop codon can be spliced out to continue the translation; the identification of RT2, with a stop codon in the exon, demonstrates that we have identified the full length of *D-Titin* gene, at least for one transcript. The single gene prediction is consistent with our genetic data that mutations in a genomic region of 57.5 kb failed to complement each other. The calculated molecular mass (1.8 mega-dalton) of the predicted protein is consistent with the mega-dalton size of D-Titin detected on an immunoblot (Machado et al., 1998). Moreover, the prediction is supported by other lines of molecular evidence (Fig. 2c), i.e., the presence of transcript X (Hakeda et al., 2000), the identification of EST (**e**xpressed **s**equence **t**ag) clones (GH25930, CK00556, GM05288 and GM05521) by BDGP. The sequences in different regions of the gene were originally identified as kettin (Lakey et al., 1993) and D-Titin (Machado et al., 1998) independently. We refer to the genetic locus as *D-Titin*, the predicted product as D-Titin, consistent with Machado et al. (1998). Based on this prediction, kettin is the NH2-terminal one third of D-Titin, probably produced by alternative splicing. Thus the previously characterized *kettin* mutants (Hakeda et al., 2000) are unlikely to be *kettin* specific, as *kettin* mutants failed to complement *1D7*, which has a *P* insertion 35 kb downstream of the kettin gene.

The enormous size of the coding region for D-Titin (approx. 49 kb ORF) provides an easy target for non-selective mutagens, such as EMS. This is consistent with the large number of lethal alleles recovered from our mutagenesis. It has been recently demonstrated that the previously identified *l(3)62Ca* (*dre8*) failed to complement *1D7* (Kolmerer at al., 2000), suggesting that *l(3)62Ca* is *D-Titin*. The enormous size of the gene also explains the single locus recovered at 62C with exceptionally large number of alleles (19 alleles), compared with the two flanking genetic loci (one at each side) with only 1 allele (Sliter et al., 1989).

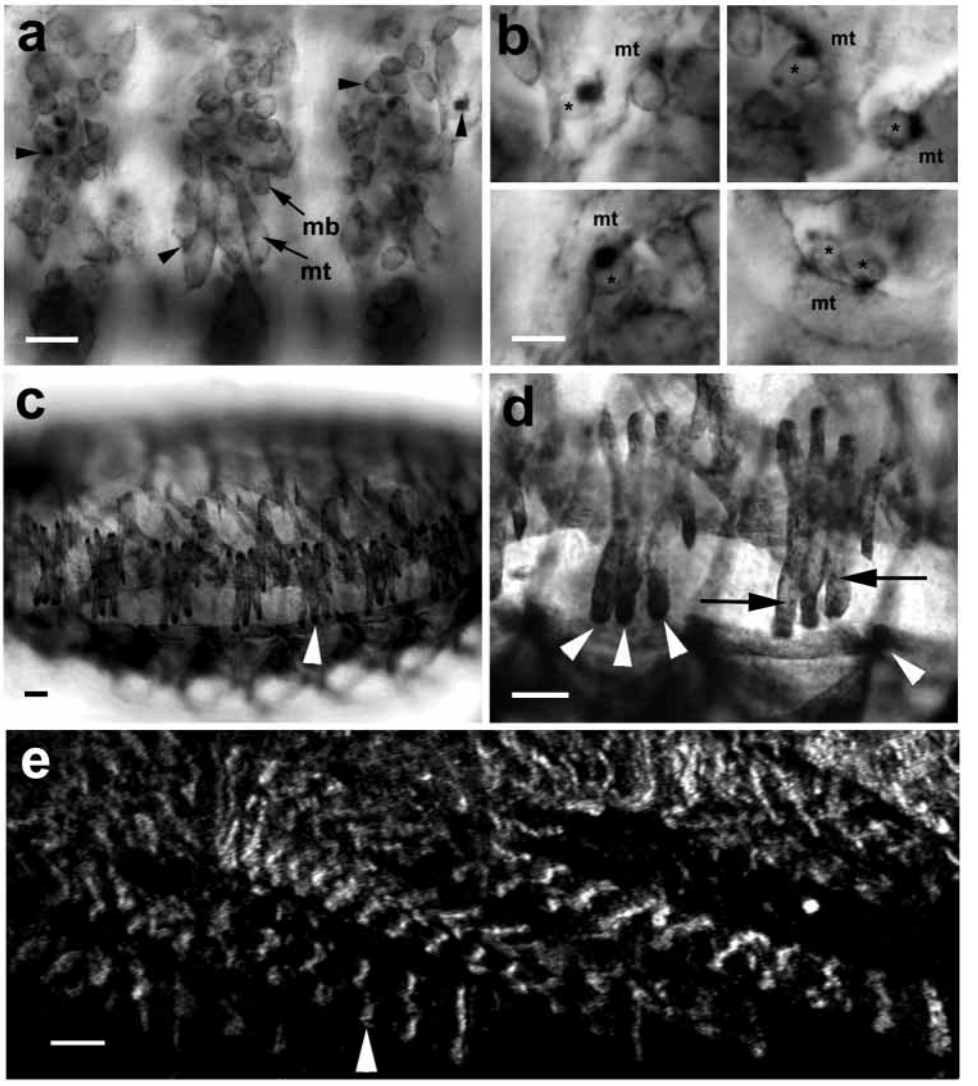

Fig. 3. D-Titin localization in wild-type embryos. (a) 11 hour AEL embryo, showing D-Titin localization in fusing myoblasts (mb) and nascent myotubes (mt). Arrowheads point to peripheral D-Titin expression. (b) Enlarged views of fusing myoblasts in the same embryo. Myoblasts are marked with *. D-Titin is observed to accumulate where myoblasts contact a myotube during fusion. (c) D-Titin localization in the musculature of a 16 hour old embryo. All body wall muscles express D-Titin, and the protein accumulates at the muscle attachment sites with the epidermis (arrowhead). (d) Enlarged view of same embryo. Arrowheads point to muscle attachment sites, arrows point to punctate spots of D-Titin stain. (e) Confocal image of ventral muscles of a mature embryo. D-Titin is restricted to Z-bands (arrowhead). Bars: 10 μm (a,c,d,e); 5 μm (b).

D-Titin expression in wild-type and mutant musculature

The D-Titin antibody (α-KZ) used in this study was raised against the NH2-terminal 636 aa of D-Titin which contains Ig domains (Machado et al., 1998, also see Fig. 2d). It has since been shown that the NH2-terminal of D-Titin is also the NH2-terminal of kettin (Hakeda et al., 2000). The monoclonal kettin antibody recognizes the kettin chain of Ig domains separated by linkers (MAC155, Lakey et al., 1993; also see Fig. 2d). The two antibodies detect different sized proteins on westerns. α-KZ recognized the mega-dalton D-Titin in embryos (Machado et al., 1998), whereas kettin antibody recognized the 500 kDa kettin in adult thoraces (Lakey et al., 1993). The specificity of the two antibodies to the *D-Titin* locus have been genetically reconfirmed in our study since all *D-Titin* mutants have reduced/absent staining compared to that of wild type.

Whole-mount embryo staining with α–KZ on wild type embryos showed the protein localized in muscle lineages (Fig. 3a-e, also see Machado et al., 1998). During skeletal muscle myogenesis, D-Titin expression begins at stage 11 (7 hours AEL at 25°C), and the protein is strongly expressed in pre-fusion myoblasts from 9 hours after fertilization (Fig. 3a,b). D-Titin expression precedes by 2-3 hours the appearance of other structural muscle proteins, such as myosin, which appear at approx. 10 hours after fertilization. This early expression of D-Titin during *Drosophila* myogenesis is consistent with the pattern described for vertebrate titin (Furst et al., 1989; Fulton and Isaacs, 1991; but see also Lin et al., 1994).

A striking observation is that during myoblast fusion, D-Titin is observed on the periphery of myoblasts and myotubes in distinct punctate domains (Fig. 3a,b). These protein localizations are often seen where myoblasts are in direct contact with nascent myotubes during the fusion process (Fig. 3b), consistent with a role for D-Titin in myoblast fusion (see below). In early maturing myotubes, D-Titin is punctate in the cytoplasm and highly concentrated at muscle attachment sites (Fig. 3c,d). This dispersed D-Titin staining is progressively restricted to the Z disc as the muscles mature (Fig. 3e). D-Titin has also been reported as a chromosomal protein (Machado et al., 1998). However we did not observe any nuclear staining of D-Titin in our preparations.

D-Titin protein is mislocalized, reduced or completely absent in muscles in the allelic series of *D-Titin* mutants (Fig. 4a). Staining in mature muscles is normally tightly localized to the Z-disc in wild-type muscles, but dispersed and progressively reduced in quantity in mutants *B173*, *C995*, *20/01* and *1D7* (Fig. 4a). Staining is very weak in *B68*, and the protein is undetectable in the somatic musculature of *C872* (Fig. 4a). Based on the lethal stages and antibody staining on dissected embryos, the allelic series of mutants at the *D-Titin*

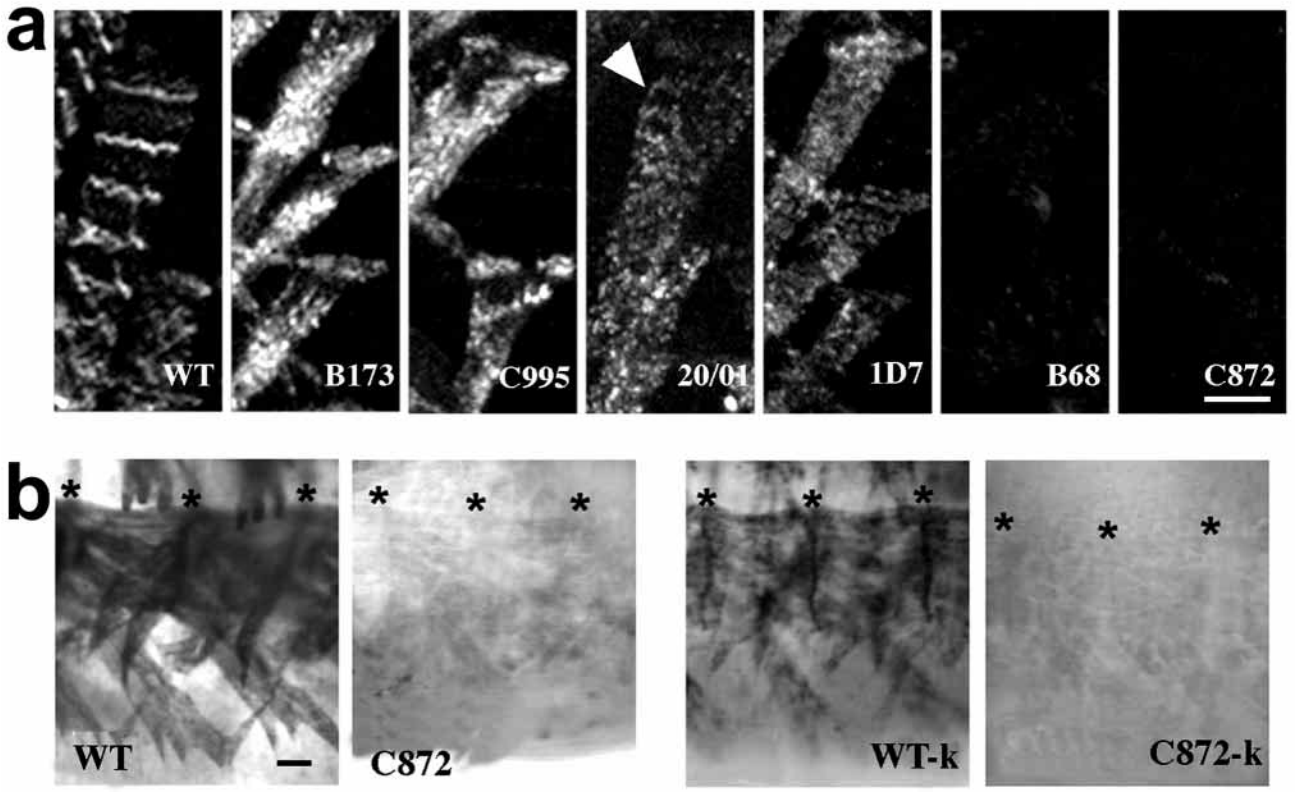

Fig. 4. D-Titin and kettin expression in mutant embryos. (a) Confocal fluorescence microscopy images of ventral oblique muscles stained with D-Titin antibody in dissected, mature embryos. In wild-type muscles (WT), D-Titin is restricted to Z bands, whereas D-Titin is mislocalized, reduced or absent in the shown allelic series of D-Titin mutants. The arrowhead in *20/01* indicates Z-discs in part of a muscle cell. Bars: 10 μm. (b) D-Titin and kettin expression in 16 hour wild-type and mutant embryos. The left two panels represent D-Titin antibody staining in wild-type (WT) and mutant (*C872*) embryos. The right two panels represent kettin antibody staining in wild-type (WT-k) and mutant (C872-k) embryos. Each panel shows ventral muscles in 2 segments. Asterisks mark the muscle attachment sites at equivalent positions in each embryo. Ventral is down, anterior is to the right. Bar, 10 μm.

locus from weakest to strongest is *20/01*, *B173*, *C995*, *1D7*, *B68* and *C872*. The antibody staining on the mutants confirmed our conclusion that the mutants we identified are *D-Titin*, as originally identified by Machado et al. (1998).

Kettin antibody staining on whole mount embryos showed that kettin has a similar expression pattern to that of D-Titin during embryogenesis, though subcellularly kettin antibody staining is spotty and somewhat more concentrated at muscle attachments (compare WT and WT-k in Fig. 4b, also see Machado et al., 1989; Hakeda et al., 2000). Both D-Titin and kettin expression begins at 7 hours of development in cells of the somatic and visceral mesoderm, and become stronger in striated muscle as it develops. In *D-Titin* mutants, kettin expression is reduced/absent in parallel to D-Titin in the allelic series of mutants. The strong allele *C872* had a complete loss of both D-Titin and kettin staining from the somatic muscles (compare C872 and C872-k in Fig. 4b). Both antibodies have a similar staining pattern in both wild-type and mutant embryos. This is not surprising, as they may recognize either the same protein or cross-react with homologous sequences (Ig domains).

D-Titin mutants have myoblast fusion defects and aberrant muscle morphology

All eight *D-Titin* mutant alleles display grossly normal embryo morphology, normal segmentation, normal epidermal denticle belts, and normal patterning of neuromusculature. All alleles, except *20/01* (pupal/pharate lethal), are embryonic lethal. Compared to wild type, mutant embryos showed greatly reduced movement, suggesting possible muscle defects. Staining with an anti-myosin antibody confirmed that the somatic muscle pattern is largely correct (Fig. 5a-c). Occasionally, a few missing muscles are observed in the most severe alleles such as *C872* (Fig. 5c). However, all the mutants showed striking defects in individual muscle fiber morphology in mature embryos. Compared to the wild-type muscles (Fig. 5d), the mutant muscles are smaller, thinner, and spindly with irregular edges and bulges at the sites of muscle nuclei (Fig. 5e for *B173*, 5f for *B68)*. The aberrant muscle shapes are unlikely to be due to muscle attachment defects, as the location and width of the muscle attachments were normal in the mutants, even in cases where the morphology was otherwise severely altered (compare Fig. 5d, wild type, with 5e, *B173* and 5f, *B68*). Although not readily comparable, the muscle morphology defects are more prominent in mature embryos than in developing embryos (compare Fig. 5e-f and Fig. 5b-c). In addition, the mutant embryos contain unfused myoblasts (Fig. 5c) and increased numbers of phagocytes (data not shown), which are not normally seen in wild type.

Nuclear counts in muscle from mature embryos confirmed a significant reduction in nuclei numbers in *D-Titin* mutants (Fig. 5g). All muscles showed a similar quantitative reduction

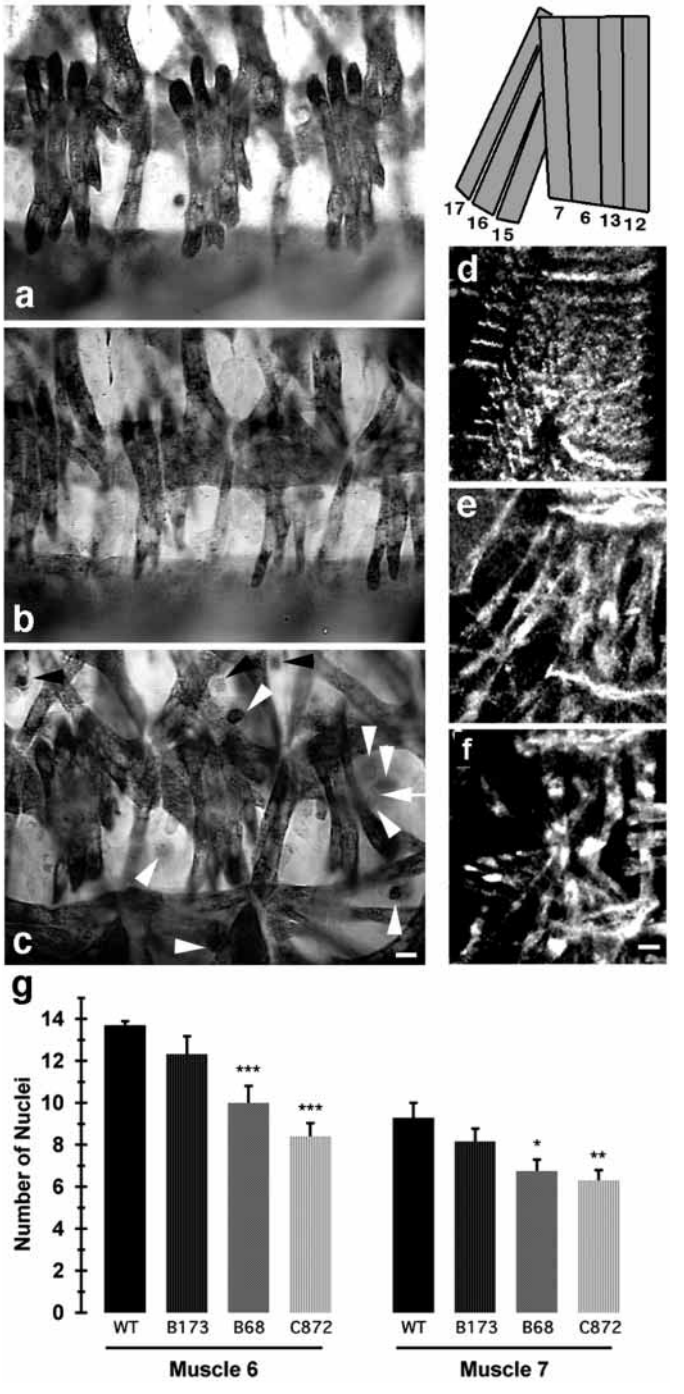

Fig. 5. D-Titin mutants have abnormal muscle morphology and decreased muscle nuclei. (a-c) Whole-mount embryo (12-13 hours, 25°C) stained with anti-myosin antibody. (a) Wild-type, (b) *1D7*, (c) *C872*, showing a lateral view of abdominal hemisegments with a number of transverse and longitudinal muscle fibers. The muscle pattern appears largely unaltered in *D-Titin* mutants, although a few muscles are missing occasionally as shown by an arrow (c). Arrowheads (black and white; c) indicate unfused myoblasts. (d-f) Confocal fluorescence microscopy images of the midline portion of a single ventral abdominal hemisegment in dissected, mature embryos. Preparations were stained with Texas red-phalloidin (a F-actin probe) to reveal muscle morphology (see schematic of WT muscles, upper-right corner). Wild-type muscles (d) have rigid rectangular shapes and display clear striations, whereas *D-Titin* mutants *B173* (e) and *B68* (f) have thin, irregularly shaped muscle fibers. These muscle fibers have uneven F-actin staining and no detectable striations. Bar in c and f, 5 μm. (g) Number of nuclei in mature embryonic muscles 6 and 7. The stronger *D-Titin* alleles (*B68* and *C872*) have significantly reduced numbers of nuclei in both muscles 6 and 7, compared to the wild-type (WT) (n=6-10 embryos for each allele; * indicates P<0.05, ** indicates P<0.01, *** indicates P<0.001).

in nuclei numbers. There was no obvious difference in nuclei numbers between the sides of embryos, and no obvious anterior-posterior gradient for nuclei count within the A3-A5 range. Careful examination of 24 mutant embryos revealed no cases where muscle 6 or 7 were missing in segment A3-A5. Thus, no 'absent muscles' contributed to the average nuclei counts graphed in Fig. 5g. The decreased number of nuclei is most severe in the strongest allele *C872*, with approx. 40% fewer nuclei than wild-type muscles, and progressively less severe in *C872>B68>B173*. This range of phenotype severity agrees well with the severity of the mutants based on α-KZ antibody staining (compare Fig. 5g with Fig. 4a).

The smaller number of cells fused to contribute to each muscle syncitium most likely explains the mutant muscle morphology and persistence of unfused myoblasts. This fusion defect is also consistent with the marked localization of D-Titin at sites of cellular contact between fusing myoblasts and nascent myotubes (Fig. 3b). We conclude that D-Titin is involved in, but not absolutely required for, myoblast fusion and is therefore important for the formation of multi-nucleate myotubes and the development of muscle fiber morphology.

The striation pattern is abolished in D-Titin mutant muscles

To assay the sub-cellular architecture of somatic muscles, we stained dissected mature embryos with antibodies against myosin, actin, and α-actinin, and examined the staining patterns using confocal fluorescence microscopy. Wild-type muscle showed clear striated muscle banding patterns for all three antibodies (Fig. 6, WT). Myosin is present as thick bands, actin as thin bands, and α-actinin sharply defines the muscle Z-disc (Fig. 6, WT). The striking defect observed in the *D-Titin* mutants is that the beautifully ordered sarcomere structure is totally disrupted (Fig. 6, three right columns). Specifically, the highly ordered banding of myosin, actin and α-actinin is completely absent in all mutants. As shown in Fig. 4a, the D-Titin banding pattern stained with α-KZ is also disrupted and/or absent. One exception is that *20/01* displays a weak Z disc staining in parts of muscle cells as shown by α-KZ staining (Fig. 4a) and other sarcomeric markers (not shown). The milder sarcomere phenotype in *20/01* may explain the observation that *20/01* survives to the pupal stage, whereas all other mutants die as mature embryos. This disruption of banding pattern in the mutants is consistent with previous studies showing that over-expression of titin Z-disc region in transfected cells results in disruption of myofibrils (Turnacioglu et al., 1997; Gregorio et al., 1998). Thus, as assayed at the light microscope level, D-Titin is required for the establishment of the highly ordered sarcomere organization characteristic of striated muscle.

D-Titin is required for muscle myofibril organization

We next compared the ultrastructure of muscles in wild-type and *D-Titin* mutant embryos (Fig. 7). Wild-type muscle cells display perfectly parallel arrays of myofilaments in both longitudinal (Fig. 7a, arrow) and transverse (Fig. 7a, arrowhead and inset a2) sections. In contrast, the patterned, parallel arrays of myofibrils are absent in *D-Titin* mutant muscles (Fig. 7b-d). Sections through mutant muscle cells show filaments that are oriented irregularly, both longitudinally (Fig. 7b-d; arrows) and transversely (Fig. 7b-d; arrowheads). This EM phenotype is consistent with the loss of striations observed at the light microscope level (Fig. 6). All three examined *D-Titin* alleles, *B173*, *1D7* and *C872* (Fig. 7b-d), as well as a *D-Titin* deficiency *Df(3L)Aprt14* (data not shown), showed very similar ultrastructural defects. This phenotype is in striking contrast to what has been observed in other sarcomeric protein mutants in *Drosophila*, such as *actin*, *α-actinin* and *myosin* null mutants (Beall et al., 1989; Fyrberg et al., 1990; Roulier et al., 1992; Cripps et al., 1999) which maintain the filaments in parallel array. The neuromuscular junctions (insets a3-d3) appear normal in the D-Titin mutants. Thus, the disruption of D-Titin results in a specific disruption of myofibril structure in striated muscle cells.

A careful examination of micrographs from *D-Titin* mutant muscle reveals that thick myosin and thin actin filaments form normal-looking structures (compare Fig. 7, inset a1 with b1-d1), wherein a thick filament is surrounded by thin filaments to form the basic 'contractile unit' (Hayashi et al., 1977), even when D-Titin is absent from the muscle, i.e., in the most severe allele *C872* (inset d1). However, this 'correct' assembly of actin-myosin units appears almost exclusively in regions where the units are part of a large group of such structures. Thick myosin filaments that are completely or partially isolated (such as at the edge of a group of parallel myosin-actin filaments) rarely show a complete complement of surrounding actin thin filaments, suggesting that this basic 'actin-myosin contractile unit' does not invariably form in vivo without D-Titin, even though these structures can form spontaneously in vitro (Hayashi et al., 1977).

The electron microscopy thus supports the conclusion drawn from our antibody staining: that D-Titin is required in vivo for the correct assembly of cytoskeletal components into functional contractile lattices in regular array.

DISCUSSION

Genomic analysis of the *Drosophila* D-Titin locus

Taking advantage of the newly released *Drosophila* genome

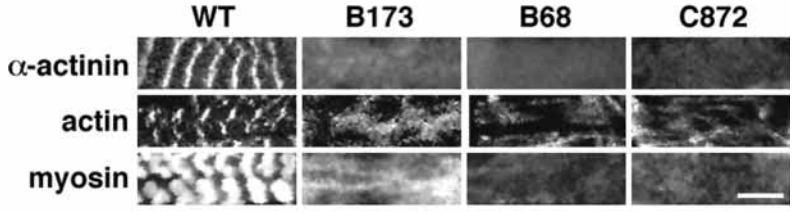

Fig. 6. Muscle striation is disrupted in *D-Titin* mutants. Body wall muscles from mature embryos stained with different markers. Top row stained with anti-α-actinin antibody; middle row stained with Texas Red-phalloidin to visualize actin; lower row stained with anti-myosin antibody. The left column depicts wild-type muscle, which shows clear striated staining patterns for all three markers. The three right columns show muscle staining in three *D-Titin* mutant alleles, *B173*, *B68* and *C872*, which show diffuse staining and lack of detectable striation. Bar, 10 μm.

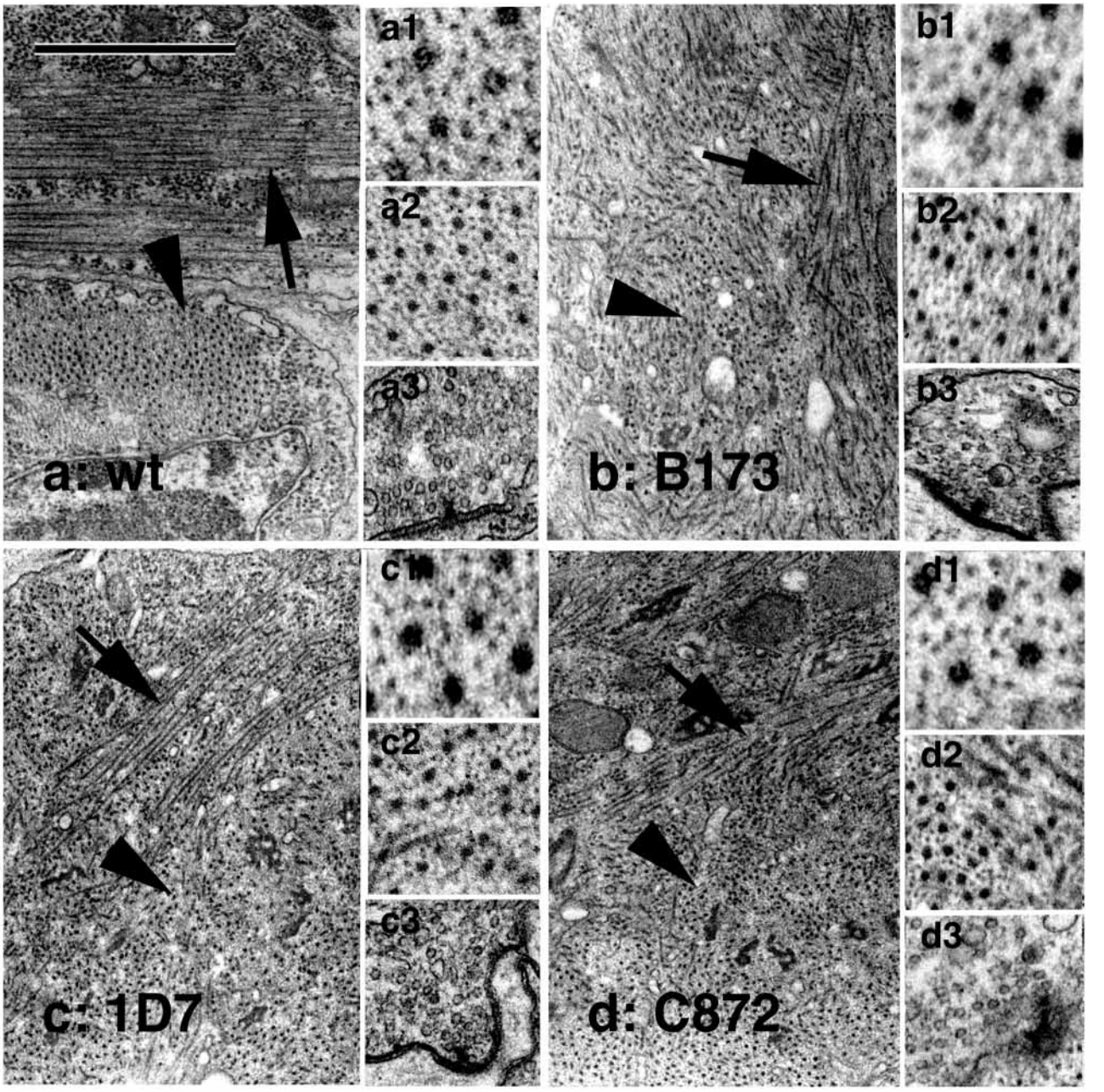

Fig. 7. *D-Titin* mutant muscles lack correctly formed myofibril arrays. Electron micrographs of embryo muscles in wild-type (a) and three *D-Titin* mutant alleles: (b) *B173*,(c) *1D7* and (d) *C872*. Wild-type muscle cells display parallel, highly ordered arrays of myofilaments either longitudinally (a, arrow) or transversely (a, arrowhead). This parallel myofilament array is disrupted and myofilaments are arranged irregularly in all *D-Titin* mutants (see arrow/arrowhead markers in b-d). The cross section at higher magnification showed that the unit of a myosin surrounded by an array of actin molecules (large dots indicate myosin filament; small dots actin filament) can form correctly (compare inset a1 with b1-d1), whereas the overall arrangements of myofilaments in *D-Titin* mutants are largely disrupted (compare inset a2 with b2-d2). No obvious defects were observed in neuromuscular junctions (insets a3-d3) of *D-Titin* mutants; the distribution and size of synaptic vesicles (seen as circles in a3-d3) are comparable between wild type (inset a3) and mutants (insets b3-d3). Bar, 0.5 μm.

sequence, we have confirmed the presence of D-Titin in the *Drosophila* genome and analyzed the structure of the D-Titin gene. D-Titin has a 48 kb coding region and encodes a protein of 16,215 amino acid residues, consistent with the mega-dalton D-Titin detected on immunoblots (Machado et al., 1998). This single gene prediction is also consistent with our genetic analyses showing that two lethal P element insertion alleles, which are located 57.5 Kb apart in the locus, fail to complement each other. Comparison of the predicted D-Titin and kettin showed that kettin (4796 aa) is likely to be a smaller splice variant of D-Titin, i.e. kettin is the NH2-terminal one third of D-Titin (16,215 aa). Thus, the originally identified two genes, kettin (Lakey et al., 1993; Hakeda et al., 2000) and D-Titin (Machado et al., 1998), are most likely two splice variants

of the same genetic locus. Phenotypic assays on the D-Titin mutants showed that D-Titin is involved in myoblast fusion and is required for sarcomere integrity. These results complement and extend the recent phenotype study on kettin (Hakeda et al., 2000). An interesting topic for the future will be to distinguish the individual roles of D-Titin and kettin. The 8 alleles of D-Titin mutants identified from this work, plus 3 identified kettin alleles (Hakeda et al., 2000), as well as classically identified 19 alleles (Sliter et al., 1989), will facilitate functional studies on the gene and its products.

The predicted D-Titin protein has unique features when compared to vertebrate titin. Vertebrate titin has very distinctive sequences, including a titin kinase domain, a PEVK domain and over two hundred copies of the Ig domain and FN3 domain (Labeit and Kolmerer, 1995). It appears that D-Titin has all the vertebrate titin domains, namely, PEVK domain, FN3 repeats and Ig repeats, with the exception of a titin kinase domain. It is possible that the kinase function is provided by projectin, a member of the *Drosophila* titin family, which has FN3 repeats, Ig repeats and a functional kinase domain, but no PEVK domain (Ayme-Southgate et al., 1995). D-Titin and projectin may cooperate/coordinate in *Drosophila* to perform the functions of vertebrate titin in muscles. It has been established that the NH2 terminal of D-Titin localizes at the Z disc (Fig. 3e and Machado et al., 1998). Since D-Titin is about half the size of vertebrate titin and the COOH-terminal of vertebrate titin localizes at the M line, it will be important to know if the COOH-terminal of D-Titin also maps at the M line.

D-Titin plays essential functions in striated muscle development

Using a combination of genetics and cell biology, we have shown two specific roles for D-Titin in striated muscle development: (1) D-Titin plays an important, but non-essential, role in myoblast fusion to form syncitial muscle fibers, presumably by organizing the cytoskeletal elements required for this process; (2) D-Titin organizes actin and myosin into the parallel arrays (visible as 'striations' at the light microscope level) characteristic of mature myofibrils. Multinucleate fibers derived from myoblast fusion and striations due to high-level organization of the contractile cytoskeleton have long been the defining morphological characteristics for so-called 'striated' skeletal and cardiac muscle, as opposed to 'smooth' muscle, which lacks visible striations and is made up of mononucleate cells. It is worth noting that vertebrate titin is specifically expressed in skeletal and cardiac muscles, but absent in smooth muscle (Wang et al., 1979). Thus, although striated and smooth muscles differ in many other molecular components, titin may be instrumental for the morphological differences by which muscle is traditionally classified.

It is not clear exactly how D-Titin might mediate either myofibril organization or myoblast fusion. One possible model, consistent with known data, is that filamentous cytoplasmic D-Titin simultaneously interacts with several other smaller cytoskeletal proteins as they are expressed. Immunohistochemistry in early muscle development does not support the idea that D-Titin is first alone organized into a template or scaffold consistent with the parallel arrays that will exist in the mature myofibril (Furst et al., 1989; Isaacs et al., 1992). Rather, we propose that during early muscle development, D-Titin acts as a flexible sticky rope with specific binding sites for numerous binding partners such as actin, with which kettin (a smaller splice variant of D-Titin) is known to interact in vitro (Lakey et al., 1993; van Straaten et al., 1999). As myogenesis proceeds, D-Titin adheres to the forming Z-disc, stretching the attached contractile cytoskeleton into the final, organized arrangement. Without D-Titin, certain sarcomeric elements, such as actin and myosin, are not organized into any higher-level structure. Consistent with this hypothesis, *α-actinin*, *actin*, and *myosin* mutants in *Drosophila* still have organized, parallel filaments (Beall et al., 1989; Fyrberg et al., 1990; Roulier et al., 1992; Cripps et al., 1999). Thus, D-Titin appears uniquely required for the supramolecular organization characteristic of striated muscle.

Myoblast fusion is a multiple-step process, from cell recognition/adhesion, pre-fusion complex formation, plaque formation, cell alignment/plasma membrane apposition, plasma membrane breakdown to myotube formation (Doberstein et al., 1997). Based on D-Titin's strong peripheral expression in myoblasts and at myoblast contact sites (Fig. 3b), we propose that D-Titin may be involved in organizing cytoskeletal elements required for formation of membrane plaques between apposed, prefused cells, or widening the initial fusion pore to facilitate cytoplasmic continuity, perhaps by directly or indirectly organizing actin-based cytoskeleton, which has been shown to be involved in myoblast fusion in culture (Constantin et al., 1995).

Many important questions remain unanswered. Machado et al. (1998) demonstrated that D-Titin is not only a muscle protein, but also a chromosomal protein. We have studied D-Titin functions in muscle without touching on the chromosomal aspects of D-Titin. Since sarcomeres and chromosomes have completely different structures and functions, it will be of interest to examine how D-Titin is involved in the structure and function of chromosomes. Our mutant phenotype studies suggest that D-Titin is probably not essential for chromosome replication and division during embryogenesis, since all the mutant embryos complete embryogenesis through many rounds of cell divisions. The *D-Titin* mutants described in the present study, will be of use to the study of titin's chromosomal functions.

In conclusion, we have confirmed the presence of D-Titin in the *Drosophila* genome, and clarified the relationship between D-Titin and kettin. We also show that *D-Titin* is a central player in the two key steps of striated muscle differentiation: myoblast fusion and myofibril assembly. These findings predict that the restricted expression of titin in striated muscle lineages is instrumental in initiating the developmental pathways differentiating the two primary muscle types: smooth and striated muscle.

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Celera for the genomic sequences and BDGP for the EST clones. This work was supported by a MDA grant to K.B. and a NIH postdoctoral NRSA fellowship to D.F. Requests for materials should be addressed to K.B. We deposited the following seven sequences in GenBank/EMBL/DDBJ database. Rescued sequence from *l(3)S002001* has accession number AJ238575, *l(3)04680* no. AJ238576, *l(3)j1D7* no. AJ238577. RT-PCR product RT1 has accession no. AJ400900, RT2 no. AJ400901, EST clone GM05288 no. AJ400902, and the predicted D-Titin gene AJ271740.

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