

## TITIN: PROPERTIES AND FAMILY RELATIONSHIPS

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In striated muscles, the rapid production of macroscopic levels of force and displacement stems directly from highly ordered and hierarchical protein organization, with the sarcomere as the elemental contractile unit. There is now a wealth of evidence indicating that the giant elastic protein titin has important roles in controlling the structure and extensibility of vertebrate muscle sarcomeres.

### RESTING LENGTH

The length to which the relaxed sarcomeres freely shorten in the absence of external load.

### OPERATING RANGE

The length range over which sarcomeres shorten and extend in muscle *in vivo*.

The giant protein **titin** (which is also known as **connectin**) is the third most abundant protein of vertebrate striated muscle, after **myosin** and **actin**. The titin molecule is a flexible filament that is more than 1  $\mu\text{m}$  long. This spans half the sarcomere, the repeating contractile unit that gives striated muscle its characteristic striped appearance (BOX 1). The molecule is formed by a single polypeptide with a molecular weight of up to  $\sim 4$  MDa, which is the largest polypeptide found so far in nature.

Studies during the past decade have uncovered the enormous complexity and multiple functions of titin. Different regions of the molecule mirror the different parts of the sarcomere and can have mechanical and catalytic functions, as well as the ability to bind to many other sarcomere proteins. During muscle development, titin probably controls the assembly of the contractile proteins actin and myosin — especially the structure and size of thick filaments — and the RESTING LENGTH of the sarcomere. In the mature muscle, titin contributes to mechanisms that control elasticity, and, consequently, the OPERATING RANGE of sarcomere lengths and tension-related biochemical processes.

Titin therefore seems to be a key component in the assembly and functioning of vertebrate striated muscles. But is it an essential component of sarcomere-based actomyosin contractile systems in general? It is striking that exact homologues of titin that span half the sarcomere have not been found in invertebrate muscles, and this could be argued to bring into question the importance of titin.

The reality is likely, however, to be more complex. There is an intimate relationship between the particular

type of vertebrate muscle and the titin isoform that it contains. This indicates that related molecules in other muscle types and cells must be very diverse. From this point of view, such proteins, even those that differ in most properties but possess one or more of the primary functions of titin — in particular, elastic stabilization of the relative positions of myosin and actin filaments — are functional homologues of titin. Such proteins are indeed found in invertebrate striated muscles, and might also be present in non-striated muscles and non-muscle cells.

Here, we review current ideas concerning the structure and numerous functions of titins. We then give an overview of related proteins in invertebrate muscles. Finally, we discuss the general relationship between the structure and elastic properties of titins and other cytoskeletal muscle proteins, and their roles in elastic deformability of sarcomeres and muscles.

### The structure of titin

There were early reports of elastic proteins in muscle<sup>1</sup>, but it was not until the advent of large-pore polyacrylamide gels that the presence of polypeptides in the megadalton range could be shown<sup>2</sup>. This allowed the newly identified components to be monitored throughout purification and then further characterized. The largest of these new components was named connectin/titin, and under the electron microscope it appeared as a flexible filament of about 1  $\mu\text{m}$  long and 3–4 nm wide<sup>3–5</sup> (FIG. 1). The filament had a beaded substructure with 4-nm periodicity, which pointed to the presence of multiple domains in the molecule. This was

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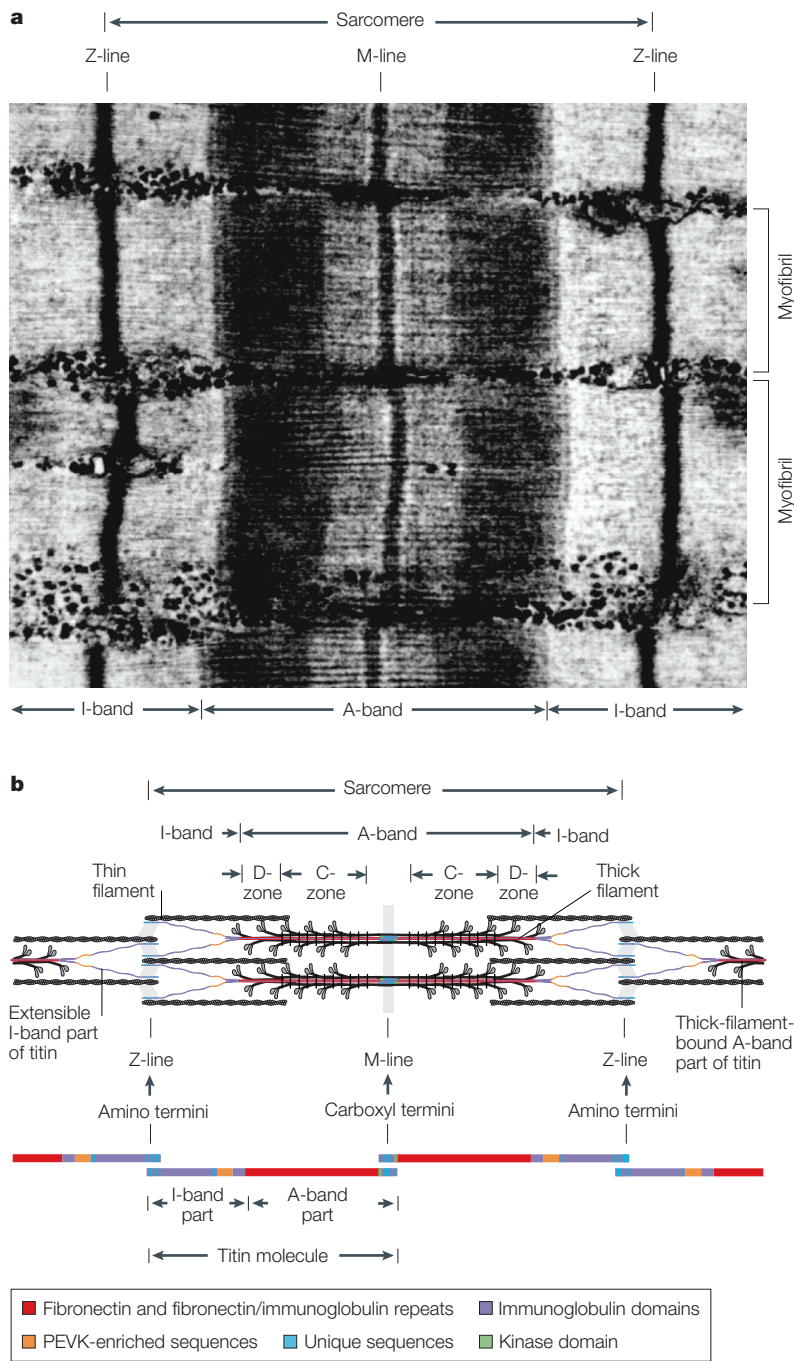
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Box 1 | The structure of the vertebrate striated muscle



The beautiful near-crystalline appearance of striated muscle (a; micrograph courtesy of R. Craig, University of Massachusetts, USA) results directly from the highly ordered and hierarchical organization of the proteins that form the contractile apparatus, principally actin and myosin. These both self-associate into filamentous polymers known as myofilaments — myosin forms bipolar thick filaments, and actin forms polar thin filaments. Remarkably, in most cases, both filament types contain an exact numbers of subunits, so there are probably 294 myosin molecules in most vertebrate thick filaments. The myofilaments assemble into elemental contractile units, sarcomeres, which are concatenated to form MYOFIBRILS. The myofibrils are cross-connected and aligned in register, and fill most of the interior of muscle cells. The resulting highly ordered network provides the basis for rapid generation of macroscopic levels of force and displacement.

Within the sarcomere (b), the myofilaments are assembled into parallel bundles with exactly aligned ends. The thick filament bundle is located in the centre of the sarcomere and appears as a dark band — the anisotropic A-band. On both sides, the thick filaments overlap with thin filaments that extend from the Z-lines at the ends of the sarcomere. The thin-filament region that is not overlapped by thick filaments is termed the light, isotropic I-band. A very dense, narrow Z-line bisects the I-band. A central, less dense region of the A-band is known as the H-band, and this, in turn, is bisected by a dark M-line. The assembly of thick and thin filaments into bundles in register is mediated by a number of crosslinking proteins that are located at the centre of the sarcomere in the M-line and within the Z-lines.

Except in a small central region that is called the bare zone, myosin heads are distributed in helical arrays over the surface of each half of the thick filament, ready to interact with actin. The inner two-thirds of these regions are termed the C-zones, because C-protein and its homologues, H- and X-proteins, all of which are composed of Ig and fibronectin domains, are located here. The functions of these proteins are unknown, but they bind to myosin and are spaced at 43-nm intervals along the filament, which is also the repeat distance of the helix that describes the myosin head spacing. The ends of thick filaments do not have these other proteins and are known as the D-zones. Activation of muscle initiates the interaction of myosin heads with actin filaments. The force produced by this interaction results in relative sliding of the thin and thick filaments and sarcomere contraction (see FIG. 5).

**MYOFIBRIL**  
The structural apparatus of striated muscle, consisting of elemental contractile units — the sarcomeres — concatenated at their boundaries, the Z-lines.

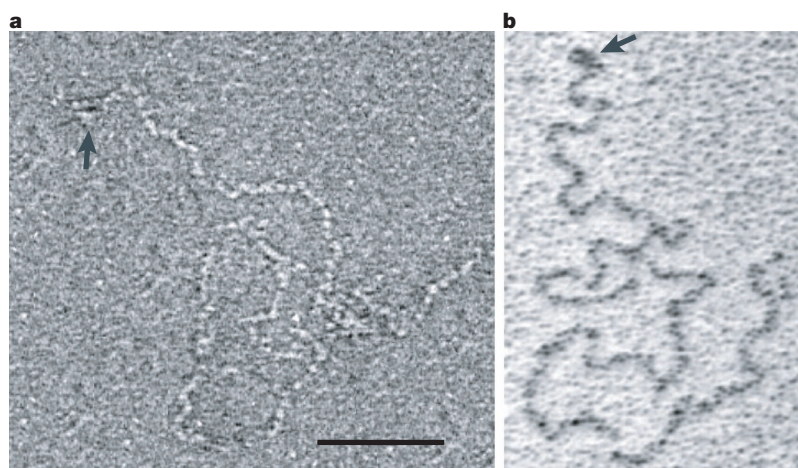
**M-LINE**  
The transverse line at the midpoint of the sarcomere. The M-line consists of proteins that connect the thick filaments at their midpoint.

later confirmed by sequence analysis. There was also a small 'head' at one end that contained two other polypeptides of ~160–190 kDa<sup>6</sup>. Antibodies that recognize these proteins bind to the M-LINE region of the sarcomere, indicating the location and interactions of the head *in situ*.

The complete sequence of the human titin gene could encode a total of 38,138 residues (MW ~4.2 MDa)<sup>7</sup>, and differential expression gives rise to a spectrum of isoforms (BOX 2, parts a–e). In fact, about a third of titin's 363 exons are differentially spliced. The isoforms that are currently known differ in size from ~600 kDa to

3.7 MDa, corresponding to molecular lengths of ~0.2–1.4 μm. Although most sequence information is derived from human cardiac and skeletal muscle isoforms<sup>7–10</sup>, partial sequences have also been obtained from other muscle types and species<sup>8,10–13</sup>.

Sequencing has shown that titin is largely composed of two types of domain that are similar to IMMUNOGLOBULIN (Ig; I-set) and fibronectin (fibronectin type-III) domains (FIG. 2). Both these domain types are β-sandwiches of seven or eight strands that contain about 100 residues. They occur in many other proteins, most of which are



**Figure 1 | Electron micrographs of titin molecules.** Titin molecules isolated from rabbit skeletal muscles were (a) negatively stained with uranyl acetate on a carbon substrate, or (b) dried on a mica substrate and rotary shadowed by platinum. The molecules are more than 1  $\mu\text{m}$  in length, and about 4 nm in diameter. The flexible filamentous shape with a small 'head' at one end (arrows) is evident in both micrographs. The head contains the carboxyl terminus, which *in situ* is located in the M-line region of the sarcomere. The head also has two M-line proteins of ~160 and ~190 kDa that remain tightly bound during purification of titin<sup>6</sup>. Negative staining also shows the bead-like sub-structure with a periodicity of about 4 nm; this correlates with the multi-domain structure that is inferred from primary sequence.

extracellular. The first intracellular protein to be found with these domains was twitchin (~750 kDa), which is present in the striated muscle of *Caenorhabditis elegans*<sup>14</sup>. Since then, titin and a number of other muscle proteins have been shown to have a similar composition.

Several Ig and fibronectin domains that are representative of different regions of titin have been solved to atomic resolution by nuclear magnetic resonance and X-ray diffraction, and the predicted folds of seven or eight  $\beta$ -strands have been confirmed<sup>15–18</sup>. In addition to ~300 Ig and fibronectin domains, titin also contains a kinase domain near the carboxyl terminus, and several 'unique' sequence regions — that is, sequences that are not similar to existing database entries. Both ends of the polypeptide also contain potential phosphorylation sites<sup>19,20</sup>. At the level of whole molecule, experiments<sup>21</sup> and modelling<sup>22,23</sup> indicate that there is torsional stiffness and variations of flexibility along its length.

**Four distinct regions in titin and the sarcomere.** *In situ*, a titin molecule extends half the sarcomere, from the Z-LINE to the M-line (BOX 1), and four distinct regions in the sarcomere — the M-line, A-band, I-band and Z-line — correlate with different parts of the molecule (FIG. 2 and BOX 1, part b). The Z-line contains the overlapped amino-terminal regions of titin molecules from adjacent sarcomeres<sup>24</sup>, and the M-line contains the overlapped carboxy-terminal regions<sup>25</sup>. The Z-line and M-line parts of titin contain Ig domains interspaced with unique sequences of different size. Some of these are differentially expressed in different tissues and species<sup>12,13,26,27</sup>, which is likely to reflect variations in Z-line and M-line organization<sup>28,29</sup>.

Within the Z-line, titin interacts with other Z-line proteins. The two amino-terminal Ig domains of Z-line titin contain a binding site for **telethonin** (also known as T-cap), which is a small protein that is involved in cellular signalling mechanisms<sup>24,30</sup>. Adjacent to the telethonin/T-cap site, there is a differentially spliced unique sequence that has a repeated motif of ~45 residues. These 'Z-repeats' bind to  $\alpha$ -actinin, which crosslinks anti-parallel thin filaments from adjacent sarcomeres<sup>28,29</sup>. The two Ig domains that are carboxy-terminal to this unique sequence contain a binding site for **obscurin**, a giant multimodular protein that probably has signalling functions<sup>31</sup>. The relationship between titin and actin within the Z-line region is not yet clear, but it is known that near the edge of the Z-line, titin attaches to the thin filament<sup>32</sup>.

The carboxyl terminus of titin is in the head region, which can be seen in the isolated molecule by electron microscopy. As mentioned above, the head contains two other polypeptides that are found at the M-line — M-protein (~165 kDa) and **myomesin** (~185 kDa). Myomesin might directly bind to Ig domains in the M-line part of titin, and therefore might interact with both myosin and titin components of thick filaments within the M-line<sup>25</sup>.

The A-band contains the largest part of the molecule (~2.1 MDa) and the sequence here is highly conserved, both between different muscle types and in different species<sup>9,11</sup>. The Ig and fibronectin domains are arranged in long-range patterns or so-called 'super-repeats'<sup>8,9</sup>. A domain at a particular position within a super-repeat is most similar to comparable positions in different super-repeats<sup>23</sup>. There are two types of super-repeat that are composed of seven and eleven domains, respectively. These are about 25–30 nm and 43 nm long, the latter being the repeat distance of the helix that describes the arrangement of myosin heads on the thick filament. Six copies of the smaller super-repeat are located near the end of the A-band. Contiguous with these are 11 copies of the large super-repeat. The sizes of the super-repeat zones correlate with what are known as the D- and C-zones of the thick filament (BOX 1). These structural correlations, together with the highly conserved sequence in this region of the molecule, reflect the intimate association of titin with the thick filament. The main proteins that interact with titin in thick filaments are myosin, C-protein (which is also known as myosin-binding protein C) and M-line proteins. The interaction with myosin might particularly involve the fibronectin domains of titin<sup>8</sup>. Binding sites for C-protein are localized within the C-zone part and are periodically spaced with Ig domains of the 11-domain super-repeat<sup>33</sup>. This explains the attachment of C-protein to the filament at 43-nm intervals and its restriction to the C-zone.

The I-band region contains only Ig domains and unique sequences. The amino-terminal (or 'proximal') and carboxy-terminal (or 'distal') I-band segments contain 15 and 22 Ig domains, respectively, that are arranged in tandem. These segments are conserved between isoforms. The part joining them is isoform

#### IMMUNOGLOBULIN-LIKE DOMAIN

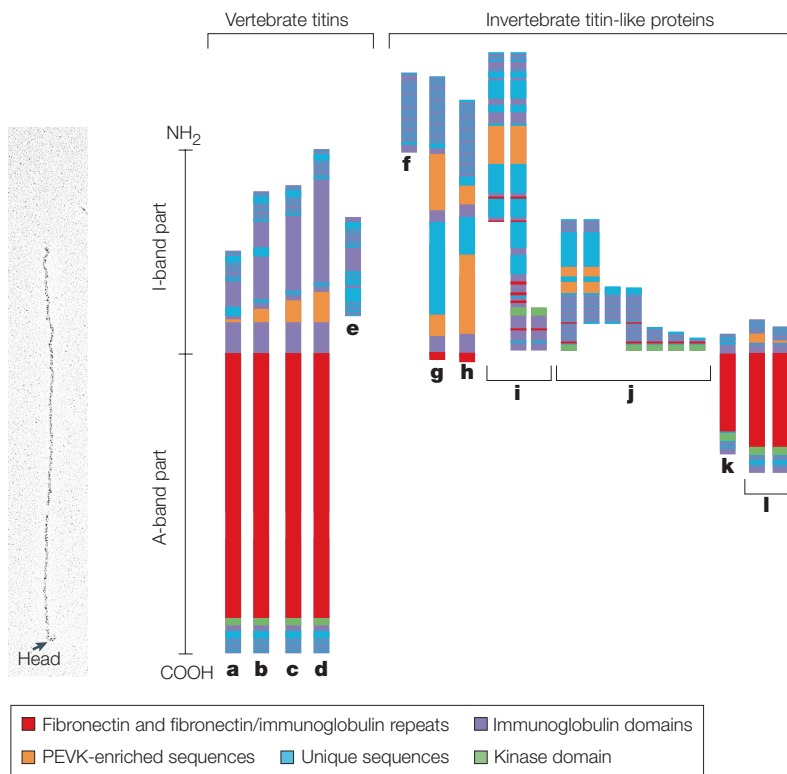
A type of polypeptide fold that was first identified in antibodies and extracellular-matrix proteins. The domain contains ~100 amino acids and is folded into two sandwiched  $\beta$ -pleated sheets of 3 or 4 strands.

#### Z-LINE

A region of muscle sarcomere to which the plus ends of actin filaments are attached. It appears as a dark transverse line in micrographs.



Box 2 | The titin family



The titin family of proteins is characterized by morphological, sequence and functional similarities. In addition to the titins that are present in striated muscles of vertebrates (a–e), the family includes a number of related proteins from invertebrates. Some of these have been studied in purified form, whereas others have only been identified in sequence databases.

Kettins (f) are 0.5–0.7 MDa<sup>85,86</sup> and ~180-nm long<sup>93</sup>. They are composed mainly of immunoglobulin (Ig) domains joined by relatively long linker sequences of 35 residues. Towards their carboxyl terminus they also have a unique sequence insert. Some, if not all, kettin isoforms are likely to form elastic connections between thick filaments and the Z-line<sup>92</sup>.

Kettins are probably expressed as truncated versions of larger molecules by alternative gene splicing. Among such larger molecules are D-titin (~2.0 MDa; g), which is found in *Drosophila melanogaster*<sup>87,88</sup>, and I-connectin (~2.0 MDa; h), which is found in crayfish<sup>89</sup>. Different isoforms are thought to be expressed in different muscle types or during different developmental stages. The large isoforms span the I-band and form elastic connections between the ends of the thick filaments and Z-lines in giant invertebrate sarcomeres (where the resting length is ~8–10 μm). The small-isoform kettins might also have similar function in sarcomeres with relatively narrow I-bands — for example, in INDIRECT FLIGHT MUSCLES of insects.

Two families of titin-like proteins — stretchins<sup>90</sup> (j) and *Caenorhabditis elegans* titins<sup>70</sup> (i) — were identified in the *C. elegans* genome. Members of each family are expressed as alternative products of the same genes and consist mainly of Ig domains and unique sequences. Both groups also have kinase domains near the carboxyl terminus that belong to the same family as the titin and smooth-muscle myosin light-chain kinase.

Twitchins<sup>14</sup> (k) and projectins<sup>91</sup> (l) have polypeptide chain weights of 0.8–1 MDa and are 0.2–0.5-μm long<sup>93,122</sup>. They are composed mainly of tandem Ig and fibronectin domains and also have a carboxy-terminal kinase domain, similar to the A-band part of titin. In projectins, the amino-acid composition of the unique sequence regions is similar to the titin PEVK region. Twitchins are mainly located in the A-band<sup>123</sup>, whereas projectins are found either in the A- or I-band, depending on the muscle type<sup>124</sup>. When they are located in the I-band, projectins are likely to function as connecting filaments between the ends of thick filaments and the Z-line, and therefore influence sarcomere extensibility<sup>92</sup>.

dependent and varies greatly in structure and size. It has three main sub-regions: first, a segment with a variable number of Ig domains joining the proximal Ig segment; second, the N2 region, which consists of alternating Ig domains and unique sequences; and third, the unique PEVK region, which is named on the basis of its preponderance of proline (P), glutamate (E), valine (V) and lysine (K) residues. There are two alternative forms of the N2 region — N2A and N2B. Skeletal titins contain N2A sequences, whereas cardiac titins contain either N2B or both N2A and N2B sequences (N2BA isoforms). Additional stretches of unique sequences with single Ig-domain insertions (novex-1 and novex-2) are present in some of the cardiac isoforms, and are amino-terminal to the N2B region<sup>7</sup>. Both cardiac and skeletal muscles also express small amounts of a truncated titin isoform called novex-3, which spans only the I-band<sup>7</sup>. The amino-terminal part of novex-3 is identical to titin in the Z-line part and the conserved proximal Ig segment, but its carboxy-terminal region is mainly a large unique sequence (~350 kDa) with a few interspersed Ig domains. The folds that are adopted by the unique sequences are not well understood, but the PEVK region contains repeats of a 28-residue motif that forms a POLYPROLINE TYPE II HELIX<sup>34–36</sup>.

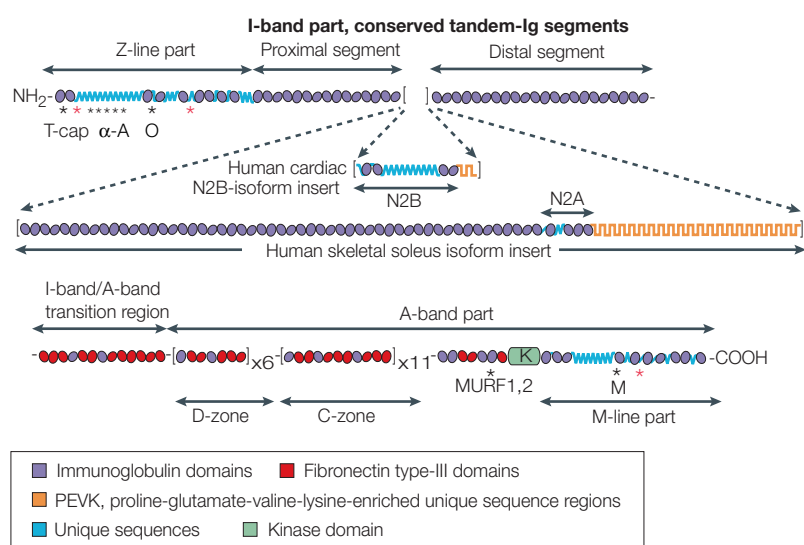
**A-band titin and the thick filament**

The packing of titin within the thick filament is poorly understood, but accessibility to antibodies implies that the molecules are located at the surface of the filament. It is also likely that they are aligned parallel to the long axis of the filament, rather than following helical tracks around the filament<sup>37</sup>. The presence of six molecules in each half of the thick filament<sup>38–39</sup> correlates with the trigonal symmetry of the filament.

Titin forms 25–30% of the mass of the thick-filament backbone and contributes to the diameter of the filament and to its flexibility. Preliminary estimates from rabbit skeletal muscle indicate that native thick filaments have higher bending stiffness than synthetic filaments that are made from purified myosin<sup>40</sup>. The contribution to thick-filament stiffness in striated muscles of vertebrates can be expected to be similar, given the high degree of sequence conservation in the A-band part of titin and the likely invariant number of molecules in vertebrate sarcomeres.

Exogenously expressed fibronectin modules of A-band titin interact *in vitro* with the actin-binding head parts of myosin<sup>41</sup>. If the interaction could be proved to correlate with the functional state of muscle, this would point to titin as being part of the control mechanisms that regulate actin–myosin interactions. Interacting with myosin heads in relaxed muscle, titin could contribute to the helical order of the heads at the surface of thick filaments, which is a characteristic feature of their resting state.

*The molecular ruler hypothesis.* More than a decade ago, it was proposed that titin acts as a template or ruler to determine the length of thick filaments. This hypothesis stemmed from the need to explain how assembly of what is probably an exact number of myosin molecules



**Figure 2 | Scheme for the tertiary structure of titin.** This diagram has been derived from sequence considerations (the two isoforms that are shown — the human cardiac isoform and the human skeletal soleus isoform — correspond to the EMBL data library entries x90568/x90569). The amino-terminal, Z-line, and carboxy-terminal, M-line, parts of the molecule contain immunoglobulin domains (Ig; I-set) interspersed with unique sequences. Black asterisks indicate binding sites for the Z-line proteins, telethonin/T-cap,  $\alpha$ -actinin ( $\alpha$ -A), obscurin (O), the muscle-specific RING finger proteins MURF1 and MURF2, and for the M-line protein, myomesin (M). The I-band part has two conserved regions of tandem Ig domains. The central part between these is isoform dependent and has three sub-regions: an additional segment of tandem Ig domains, the N2 region and the PEVK region. The N2 region differs only between skeletal and cardiac muscles, whereas the size of the Ig segments and PEVK region varies between all muscle types. The sequence in the A-band part of the molecule is highly conserved. Here, the Ig and fibronectin domains are arranged in patterns or 'super-repeats'. The seven-domain small super-repeats are adjacent to the I-band/A-band junction. These are followed by eleven-domain large super-repeats. The kinase domain is at the edge of M-line part. The MURF binding site is in the three domains that are amino-terminally adjacent to the kinase domain. These include one fibronectin and two Ig domains. Potential phosphorylation sites in the Z- and M-lines are indicated by red asterisks. Additional phosphorylation sites are thought to be present elsewhere in the molecule, depending on the isoform<sup>125</sup>.

#### INDIRECT FLIGHT MUSCLES

High-frequency muscles that are also known as asynchronous or fibrillar muscles. In contrast to synchronous muscles, in which there is a direct correspondence between stimulus (action potential) and contractile cycle, indirect muscles can respond to an individual stimulus with a series of contractile cycles in an oscillatory fashion. In insects that use indirect muscles, the wings and thorax form a mechanically resonant system that allows muscles to oscillate with the resonant frequency.

#### POLYPROLINE TYPE II HELIX

A preferred conformation for proline-rich regions of protein sequences, with an axial translation of 3.20 Å and three residues in each turn of a left-handed helix. Other common polypeptide conformations are  $\alpha$ -helix and  $\beta$ -structure.

in the filament (thought to be 294 in vertebrate striated muscle) is terminated. After antibody labelling had shown that individual titin molecules span half the filament and are integral, a role as a ruler was an obvious possibility<sup>42</sup>. Several subsequent discoveries are consistent with this hypothesis. These include the exact match between the titin domain super-repeats and the myosin and C-protein periodicities in the thick filament; the apparent delay of filament assembly relative to titin expression in cells<sup>43</sup>; and a lack of thick filaments in the cells that express defective titin molecules<sup>44</sup>. Although these data are indicative, the molecular ruler mechanism remains unproved.

#### I-band titin and sarcomere extensibility

In mature muscle, one of the main tasks of titin is to maintain thick filaments centrally in the sarcomere during cycles of contraction and extension. This is important, because it ensures development of balanced forces between both halves of the sarcomere by myosin<sup>45</sup>. Within the normal operating range of sarcomere lengths, this function is carried out by the I-band part of the titin molecule, which connects the end of the thick

filament to the Z-line. When muscle changes length, stress is transmitted to this part of titin, which coils up or extends so that its end-to-end distance can approximately halve or double.

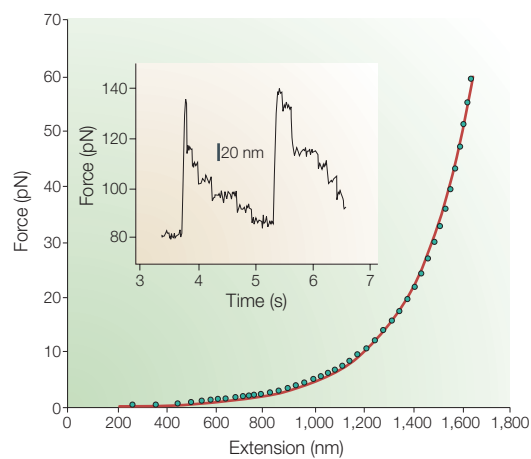
The tension that is developed in titin by sarcomere length changes is the main source of the passive tension that is developed by muscle. Both the contour length of I-band titin and its intrinsic extensible properties are the main determinants of this tension. Relatively extensible muscles, such as the skeletal SOLEUS, contain larger titin isoforms that have longer and more compliant I-band regions. Stiffer muscles, such as cardiac muscles, have smaller isoforms with shorter and stiffer I-band parts<sup>46</sup>.

Remarkably, sarcomeres can be extended reversibly to well beyond the point at which the I-band part of titin becomes straight, and on release there is retraction almost to the original length. To explain this phenomenon, the polypeptide was proposed to reversibly unfold, and the domain-folding energies were shown to be comparable with the mechanical work involved<sup>47</sup>. Erickson<sup>48</sup> extended these ideas to consideration of the critical forces that are necessary to break bonding networks in the domains. Subsequently, studies of the mechanical behaviour of titin *in situ* and of single molecules *in vitro* have confirmed these predictions. They have also shown how non-uniform mechanical properties derive directly from variations in structure along the molecule.

The current view is that sarcomere extension beyond the resting length first results in straightening of I-band titin from an initially coiled conformation. Further extension stresses interdomain links and induces unfolding of the polypeptide. Unfolding occurs first in the unique PEVK sequence and N2B regions that are located in the middle of the I-band, which have lower mechanical stability than the Ig domains<sup>49–52</sup>. The large gains in length that are produced by unfolding — by a factor of 2–4 over the folded N2B and PEVK regions — help ensure sarcomere integrity at large extensions. This is probably important for minimizing the probability of Ig-domain unfolding. However, at present, occasional unfolding of Ig domains cannot be excluded<sup>53</sup>. The folding and mechanical properties of the different regions of titin, as illustrated by the *in vitro* studies (see below), seem to have evolved to meet these needs.

#### Single-molecule experiments on titin

The folding and mechanical properties of the whole titin molecule and its component parts have also been explored in detail *in vitro*. This has been greatly facilitated by the exciting development of new, highly sensitive methods — OPTICAL TWEEZERS, ATOMIC-FORCE SPECTROSCOPY and so on — that allow manipulation and mechanical measurements on individual biomolecules. These new methods have opened the way for new types of information that were previously masked in data from ensemble averages of the molecules. This has allowed measurement of the entropic and enthalpic forces that maintain structure and conformation of biomolecules and their complexes. The many advantages of titin — not least its giant size — have made it a model for single-molecule studies.



**Figure 3 | Force-extension relationship of single titin molecules measured by optical tweezers.** To exert force on a molecule, optical tweezers (also called optical traps) use the radiation pressure of high-intensity light. The molecule is stretched between a surface and a micron-sized plastic bead held at the focus of a laser beam, or between two beads held in two independent optical traps. In this experiment, the ends of the molecule were attached between a surface and a plastic bead by antibodies specific to the amino- and carboxy-terminal regions of titin. The molecule was then extended ~1.5-fold. The main curve shows an exponential rise of tension that accompanied extension. This curve could be fitted using a model that consists of two serially linked worm-like chains with different stiffness. This correlates with the presence of two structurally different parts in the molecule — segments of immunoglobulin (Ig) and fibronectin domains, and the PEVK region. After fast extension, tension relaxes in a characteristic step-like manner (insert) due to sequential unfolding of Ig and fibronectin modules. Reproduced from REF. 55 © (1997) Macmillan Magazines Ltd.

#### SOLEUS MUSCLE

A muscle in the leg that controls postural stability. It contains one of the largest titin isoforms. This results in sarcomeres that have a comparatively long resting length and are more compliant than in other muscles. At the other end of this range is cardiac muscle, which has short, stiff sarcomeres.

#### OPTICAL TWEEZERS

A technique that is used for the manipulation of individual protein molecules and is based on the radiation pressure of light. A micron-sized transparent bead tends to stay at the focus of a laser beam. When the bead is attached to a protein molecule, movement of the laser beam can be used to impart small forces and displacements to the protein.

#### ATOMIC-FORCE SPECTROSCOPY

An imaging and force-measuring (pico/nano-Newton range) technique that is based on a sharp probing tip attached to a flexible cantilever. The probe can scan across a surface in a raster to form an image. Alternatively, the probe can pull upwards to measure forces in a molecule stretched between the probe and a surface.

The picture that has emerged shows how extensibility correlates directly with structure in different regions of the titin molecule. Uncoiling and straightening of the molecule occurs with forces in the picoNewton range and precedes any unfolding events. This is characterized by an exponential increase in tension in the molecule<sup>54,55</sup> (FIG. 3). With further stretching, the polypeptide starts to unravel, and different segments of the molecule extend independently. The PEVK region has the lowest mechanical stability and extends first. The N2B region in cardiac isoforms is only marginally more stable than the PEVK region, and it extends next. Finally,  $\beta$ -sandwich Ig and fibronectin domains unfold; these show wide variation in their ability to resist force, and unfold hierarchically starting from the least stable. Both PEVK and N2B regions unravel as random coils, which is illustrated by an exponential force-extension relationship<sup>56,57</sup>. Unfolding of the domains produces a characteristic sawtooth-like pattern in the force-extension relationship<sup>58</sup>, or a step-like pattern in stress-relaxation curves<sup>55</sup> (FIG. 3, insert), with each peak or step corresponding to the unfolding of a single domain.

**Force extension — muscle fibres versus titin molecules?** As a general outline, the response of muscle to stretching seems to be well explained by extrapolating directly from

the mechanical properties of single titin molecules. This explanation could, however, be too simplistic, because the intracellular arrangement of titin, its environment and its interactions are all likely to modulate its extensibility. Moreover, there are also recent data indicating the presence of more than one full-length isoform<sup>59</sup>, as well as truncated isoforms<sup>7</sup>, within a single sarcomere.

The presence of more than one isoform might be required to accommodate titin to symmetry rearrangement between the A-band and Z-line regions of the sarcomere<sup>7,39</sup>. In the A-band, titin is attached to threefold rotationally symmetric thick filaments that are arranged on a hexagonal lattice, whereas near the Z-line it attaches to the twofold symmetric thin filaments on a tetragonal lattice. How both symmetries can be satisfied by identical titin molecules at the stoichiometry of six titins per half thick filament is unclear. The presence of more than one isoform might be related to this problem. It is equally possible that the presence of more than one isoform in a sarcomere reflects the pattern of expression. The tight relationship between properties of the molecule and sarcomere extensibility indicates that isoform synthesis might constantly be adjusted depending on the immediate history of the muscle. It therefore cannot be excluded, at present, that the apparent similarity in force-extension behaviour of muscle fibres and individual titin molecules might simply result from the limited sensitivity of experiments.

An important question that has arisen from comparisons between *in situ* and *in vitro* studies is whether there is reversible unfolding of I-band Ig domains in muscle under physiological conditions. The wide range of mechanical stabilities of the individual domains, as well as what seem to be systematic differences in stability along the molecule<sup>60,61</sup>, seem to favour this idea. *In situ* studies indicate a probabilistic mechanism of Ig unfolding<sup>60,62</sup>. Sudden local unfolding or ‘popping’ of individual Ig domains, leading to an abrupt drop of tension in the molecule, could provide a mechanism that is similar to PEVK and N2B unfolding and could concentrate strain in a relatively narrow region of the molecule. However, the likelihood of Ig unfolding *in vivo* is relatively low<sup>63</sup>. In the absence of more direct evidence, this question remains open, whereas our understanding of the forced unfolding of the domains is improving<sup>64,65</sup>.

#### Titin's role in muscle signalling mechanisms

The discovery in titin of a kinase domain towards its carboxy-terminal, M-line end, as well as potential phosphorylation sites near both ends, pointed to an involvement in signalling mechanisms. This stimulated a search for phosphorylation substrates, related enzymes and the role(s) of the enzymatic activity in muscle development and function. Later, these ideas were enhanced by evidence that both the Z-line<sup>66</sup> and M-line<sup>67,68</sup> ends of the molecule are components of signalling pathways that control tension- and protein-turnover-related processes. These ideas are also consistent with likely roles of titin in thick-filament and sarcomere assembly, and with its optimal location for sensing strain during muscle length changes.



**The role in sarcomere assembly.** The titin kinase domain is of the serine/threonine type and is similar to the smooth-muscle myosin light-chain kinase<sup>69</sup>. Potential regulators of the activity of the kinase activity include Ca<sup>2+</sup>-dependent binding of calmodulin, a phosphorylation–dephosphorylation cycle, and RING-FINGER PROTEINS, which are the likely components of protein-turnover signalling pathways<sup>67,69</sup>. Early studies had indicated that titin could be autophosphorylated. Later, activation of the kinase and phosphorylation were linked to the early phases in muscle development. Telethonin/T-cap — a Z-line protein that interacts with the amino terminus of titin<sup>24,30</sup> — was found to be phosphorylated by the activated titin kinase in *in vitro* experiments in differentiating muscle cells<sup>69</sup>. The importance of telethonin/T-cap for sarcomere assembly and, therefore, the significance of its phosphorylation by titin kinase is, however, controversial<sup>70</sup>. The existence of other substrates is likely and the role of titin in sarcomere assembly requires clarification. There is, however, general agreement that titin synthesis precedes or occurs simultaneously with sarcomere assembly<sup>43,71–73</sup>, and that titin is vital for normal sarcomere formation<sup>44,68,74</sup>. There is also evidence that transient interactions of titin with signalling proteins might help align the thin- and thick-filament arrays in the sarcomere during the early stages of muscle assembly<sup>75</sup>.

**Linking the contractile system with protein-breakdown/expression machinery.** A site near the titin kinase domain has been shown to interact *in vitro* with two members of the muscle-specific RING-finger (MURF)-family of signalling proteins, MURF1 (REFS 67,68) and MURF2 (REF. 75). *In situ* interactions of titin with MURF2 are likely to be only transient, whereas MURF1 might form long-term complexes in the M- and Z-line regions of the mature sarcomere. Early in muscle development, both proteins undergo reorganization in a way that indicates a potential role in aligning the contractile filaments. In adult muscle, the likely function of MURF proteins as ubiquitin ligases indicates a role in the regulation of contractile protein breakdown. Their M- and Z-line locations and interactions with titin indicate that these regions might be the earliest targets, and that disruption of the ends of titin could be one of the crucial steps in sarcomere disassembly that would facilitate further breakdown. The role of titin as a link between the contractile system and mechanisms controlling the balance between muscle growth and degradation is further supported by the finding that myostatin, a muscle growth factor, is one of the ligands of telethonin/T-cap<sup>76</sup>, and interacts with titin at the Z-line periphery.

**Linking contractile and excitation systems.** The Z-line part of titin is also likely to be physically linked to intracellular membrane systems, T-TUBULES and the sarcoplasmic reticulum (SR). T-tubules are invaginations of the plasma membrane that are involved in spreading of action potentials into the interior of muscle cells, and the SR is the store of calcium that activates contraction. A link between titin and T-tubules might occur through telethonin/T-cap, through interactions of telethonin

with T-tubule potassium channels<sup>66</sup>. This link might exist in cardiac muscles, where T-tubules traverse myofibrils at the level of the Z-line and are therefore near the titin–telethonin/T-cap complex. Links with the SR could involve either direct interactions between titin and ankyrins (integral-membrane proteins)<sup>77</sup>, or indirect interactions through obscurin, which could link titin with ankyrin<sup>78</sup>. Links between titin and the membrane systems would point to a tension-related feedback mechanism between the contractile and excitation systems, which could regulate muscle activity<sup>66</sup>.

**Relationship with muscle pathology.** In parallel with *in vitro* experiments showing that titin mutations seriously disrupt the sarcomere assembly and function<sup>24,44,68,74,79</sup>, several recent reports have described mutations in different regions of the molecule that are associated with muscle pathologies. Dilated cardiomyopathy (DCM) is associated with mutations in the N2B region<sup>80</sup>, or with expression of a truncated molecule that has only the amino-terminal part of titin up to the PEVK region<sup>81</sup>. Both mutations affect the I-band part of the molecule and consequently alter the elastic properties of muscle. Other mutations that are associated with DCM are found near and within the Z-line<sup>81,82</sup>, and these are likely to result in defective tension transmission through muscle. In skeletal muscles, tibial muscular dystrophy was found to be associated with mutations in the M-line part of titin<sup>83</sup>, or in the N2A region<sup>84</sup>. These mutations are also likely to result in altered elasticity or force transmission in the sarcomere. All these findings indicate the importance of intact titin for normal muscle structure and function.

### Invertebrate titin-like proteins

Compared with vertebrates, invertebrate sarcomeres are much more diverse in size and structure. The size and structure of thick filaments and their ratio to thin filaments are also less conserved. Considering the strong relationship between titin isoforms and vertebrate sarcomere properties, it is to be expected that related proteins in invertebrates will also be more diverse. Biochemical and genetic analyses have confirmed and extended this prediction.

No giant molecule that is similar to titin and that spans half the sarcomere has been found in the striated muscles of invertebrates. Instead, titin-like proteins with diverse sizes have been isolated biochemically or identified in databases (BOX 2). Two main classes — those that bear some resemblance in their domain composition to the I-band part of titin and those that resemble the A-band part — can be distinguished on the basis of their abundance of Ig or fibronectin domains. Distinct genes encode the two classes, and within the classes some of the protein groups are expressed as splice isoforms. Members of both classes might be present within a single sarcomere.

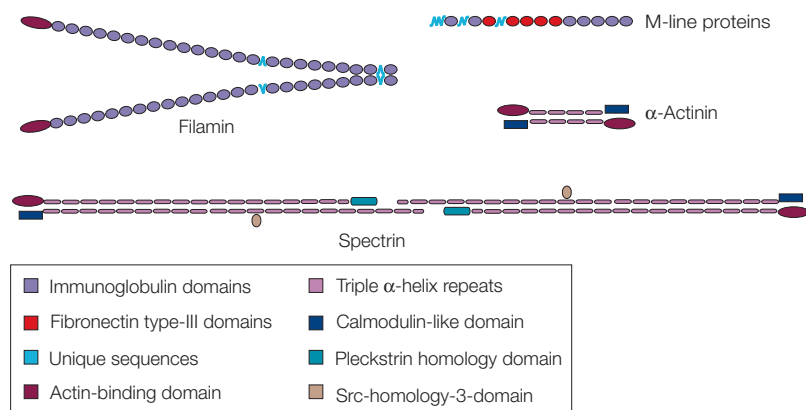
Kettins<sup>85,86</sup> and their larger splice isoforms, D-titins<sup>87,88</sup> and I-connectins<sup>89</sup>, as well as stretchins<sup>90</sup> and *C. elegans* titins<sup>70</sup>, have domain compositions that are reminiscent of the I-band part of titin. Accordingly, some of these

#### RING-FINGER PROTEINS

A family of proteins that are structurally defined by the presence of the zinc-binding RING-finger motif. The RING consensus sequence is: CX2CX(9–39)CX(1–3)HX(2–3)C/HX2CX(4–48)CX2C. The cysteines and histidines represent metal binding sites. The first, second, fifth and sixth of these bind one zinc ion and the third, fourth, seventh and eighth bind the second.

#### TRANSVERSE (T)-TUBULES

A system of surface-connected membranes in muscle that enables a nerve impulse to travel to the interior of the muscle fibre.



**Figure 4 | Scheme for tertiary structure in Z-line and M-line proteins.** The Z-line protein filamin is formed mainly by immunoglobulin (Ig) domains arranged in tandem. M-line proteins (M-protein, myomesin and skelemin) have a similar architecture of Ig and fibronectin domains. Note the occasional inserts of unstructured polypeptide between domains shown in turquoise, which are likely to provide increased local flexibility, hinges or bends. Myomesin is present in all types of striated muscle. It interacts with myosin and titin in the M-line and forms bridges between thick filaments. Skelemin is likely to be part of crosslinks between myofibrils. Similarly, the Z-line proteins filamin and spectrin interact with actin mainly at the surface of myofibrils and are involved in bridging myofibrils.  $\alpha$ -Actinin, another Z-line protein, interacts with actin and titin and forms bridges between thin filaments within the Z-line. In contrast to filamin and M-proteins,  $\alpha$ -actinin and spectrin structures are based on a triple  $\alpha$ -helix module. Differences in the domain fold, the number and length of inter-domain sequence inserts, as well as dimerization (for example, in  $\alpha$ -actinin), will all influence mechanical properties in the molecules. Not all of the known Z- and M-line proteins are shown and some other proteins have similar modular structures.

have been shown to reside in the Z-line and I-band and to contribute to sarcomere elasticity. The large variation in the overall size of these proteins is in agreement with the large range of sarcomere and I-band lengths in invertebrates. The diversity of unique sequences that are present in their structures indicates, in turn, that there might be more complex extensibility and unfolding patterns than in vertebrate titins.

Twitchins<sup>14</sup> and projectins<sup>91</sup>, on the other hand, have domain compositions that resemble the A-band part of titin, and in most muscles are found in the A-bands of sarcomeres. Details of their association with thick filaments remain unknown, but their relatively short molecular lengths indicate that single molecules cannot span half the filament. It is therefore unlikely that they function as rulers to determine exact filament length. In the cases in which they are located in the I-band, projectins are likely to connect the ends of thick filaments to the Z-line, and therefore to influence sarcomere extensibility<sup>92</sup>. Similar to titin, some of the invertebrate proteins from both main classes have kinase domains and are capable of autophosphorylation. They are also expected to have external substrates. These are likely to vary and to be different from substrates of the titin kinase, as can be judged from sequence variations in their catalytic sites.

The invertebrate proteins are also morphologically similar to titins. They have a similar filamentous shape, seem flexible, and vary in length in proportion to their molecular weights<sup>93–99</sup>. Mini-titins — titin-like proteins that can be isolated from molluscan muscle and that are thought to be identical to twitchins and

projectins — are  $\sim 0.25 \mu\text{m}$  long<sup>96</sup>. In electron micrographs, they show a tendency to separate off a part of the tail. This results from hydrodynamic forces that are exerted on a substrate during drying. The gap between two separated parts of the molecule is likely to be spanned by unfolded unique sequence that is invisible at the resolution of the micrographs. A similar phenomenon is seen in titins<sup>100,101</sup>.

The length of the largest purified crayfish titin-like protein is  $0.5\text{--}0.8 \mu\text{m}$ <sup>97</sup>, which is similar to estimates from the sequence of I-connectin<sup>89</sup>. This correspondence has implications for the elusive structure of unstressed PEVK and other unique sequences of the molecule. Estimates from sequences assume that the unique sequences that comprise more than half of the polypeptide are folded with the same packing density as the Ig domains — that is,  $\sim 100$  residues per 4 nm. This indicates that the unique sequences, including the PEVK polypeptide, are probably coiled up relatively tightly in the relaxed molecule.

**A unified function for titins?** This perspective of vertebrate titins and invertebrate titin-like proteins indicates that, despite the divergence in physical properties, most of the group might be unified by the ability to interact with thick filaments and to function as a special class of myosin crosslinkers, connecting thick filaments axially to Z-lines. Such crosslinks elastically stabilize the positions of thick filaments relative to thin filaments, centralizing them in the sarcomere. Providing connections at the level of individual myofilaments, titin and titin-like proteins contribute to the fine balance of forces between the two halves of the sarcomere. The size and extensibility of the crosslinks are the main determinants of sarcomere extensibility.

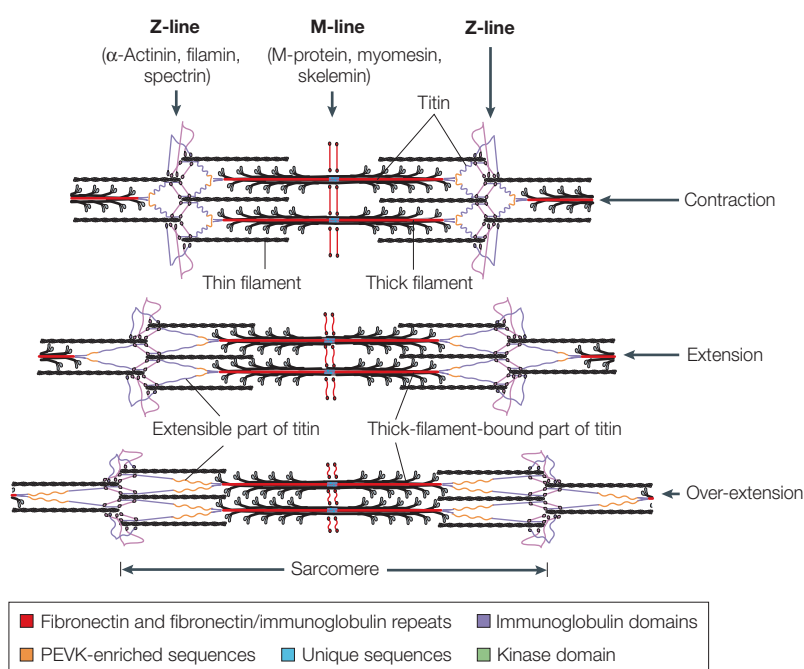
Sarcomeres are unique to striated muscles; however, a related ordering and integration of the actomyosin system is also seen in non-striated muscles and in non-muscle cells<sup>102,103</sup>. This is consistent with claims of titin-like proteins outside striated muscles<sup>86,104–109</sup>. There are not yet sufficient data for the detailed comparisons of these proteins with titins. But if the structural and functional similarities are confirmed, this will indicate that universal mechanisms exist to control assembly of actomyosin contractile systems in cells.

#### Cytoskeletal proteins and sarcomere elasticity

When muscle contracts or extends, there are reversible changes not only in sarcomere length but also in cross-sectional area. This is indicated by changes in the spacings between thick and thin filaments in the A-band and in the Z-line<sup>110</sup>. This, in turn, strongly indicates that the proteins that crosslink myosin and actin filaments transversely at the M- and Z-lines are likely to deform mechanically.

M-protein (165 kDa) and myomesin (185 kDa) — the main proteins that crosslink thick filaments within the M-line — are, like titin, intracellular members of the Ig-fibronectin superfamily<sup>111–112</sup> (FIG. 4). And, like titin, they have flexible filamentous structures. Although details of their arrangement within the M-line region





**Figure 5 | Sarcomere structure at different degrees of extension.** During muscle contraction, sarcomere length decreases, which results in coiling of the I-band part of titin. Simultaneously, inter-filament spacings increase, which is likely to reorientate or stretch Z- and M-line proteins that crosslink thin and thick filaments and myofibrils. During extension, sarcomere length increases and inter-filament spacings decrease. This leads to the extension of titin, while releasing tension on the Z- and M-line proteins. Over-extension of muscle results in the unravelling of titin polypeptide, which starts from the least mechanically stable PEVK region. This is accompanied by compression and reorientation of Z- and M-line proteins. Thin and thick filaments are shown in black,  $\alpha$ -actinin and spectrin in pink, filamin in purple and M-line proteins in red. Titin is coloured in differently along its length according to regional differences in structure.

differ, the bridges they form are oriented nearly perpendicular to the long axes of the thick filament<sup>25</sup>. Therefore, an increase or decrease in the spacing between thick filaments must be accompanied by an extension or coiling up of these proteins to accommodate changes in the bridge lengths (FIG. 5).

The main protein that crosslinks thin filaments within the Z-line region of the sarcomere is  $\alpha$ -actinin. This is an elongated molecule with two antiparallel subunits. The modular structure of the central rod part of the subunit is based on a triple-stranded  $\alpha$ -helix as the repeating unit<sup>113</sup> (FIG. 4). In contrast to the M-line proteins, the bridges that are formed by  $\alpha$ -actinin are oriented obliquely with respect to the axis of the thin filament. Therefore, changes in the interfilament spacing are likely to lead only to the reorientation and/or bending of the bridges, with no significant distortion in their lengths (FIG. 5). These are likely to be accommodated by the flexibility of the molecule, especially near its actin-binding domains<sup>114</sup>.

Many other muscle cytoskeletal proteins, located within and at the periphery of the sarcomere, have multi-modular structures that are based on either Ig and fibronectin, or a triple-stranded  $\alpha$ -helix, as the repeating structural units. These include skelemins (200–220 kDa)<sup>115</sup>, which are thought to bridge myofibrils at the periphery of M-lines; C- and H-proteins

(~150 kDa and 86 kDa)<sup>116</sup>, which are minor components of vertebrate thick filaments with unknown functions; *unc-89* (732 kDa)<sup>117</sup>, which contributes to the M-line structure in muscles of invertebrates; obscurin (~800 kDa), a possible component of Z-lines and M-lines in vertebrate sarcomeres, and a potential bridge between the sarcomere and SR; and *filamin*<sup>118</sup>, spectrin and *dystrophin*<sup>119</sup>, which are actin crosslinking proteins that are involved in inter-myofibrillar bridging and linking with the SARCOLEMMA at the periphery of Z-lines.

At present, many functional aspects of these proteins, especially their responses to changes in muscle length, remain unknown. However, their flexible filamentous shapes and the relatively low mechanical strength of their domains<sup>120,121</sup> indicate that, like titin, they are adapted to bear mechanical stress. A capacity for reversible mechanical deformation is probably a key feature of these proteins. This will stem directly from their molecular designs, which allow entropy and non-bonded polypeptide packing to have significant roles determining their structures and conformations. These two parameters provide the means to manipulate the length of the molecule over a large range and at relatively low energy cost. Integration of the actomyosin system based on elastically deformable crosslinking proteins therefore results in an elastically deformable molecular network that allows reversible reshaping of muscle cells during contractions and extensions.

### Conclusion and future directions

The sarcomere is the elemental contractile unit of striated muscles. A population of modular cytoskeletal proteins is used to organize and support the sarcomere structure and, therefore, the spatial order of the force-producing proteins, actin and myosin. In vertebrates, the structural integrity and elasticity of sarcomeres is largely controlled by the giant cytoskeletal protein titin. Establishing the relationship between the intrinsic properties of single titin molecules and the structure and passive mechanical properties of the sarcomere has been one of the main achievements in muscle studies during recent years. Key questions about the role of titin in sarcomere elasticity now concern the oligomeric state and interactions of the elastic I-band part of the molecule.

Another important outstanding issue concerns the role of titin in defining the structure and size of thick filaments. The 'ruler hypothesis' is attractively simple, but is not proven and more direct tests are clearly needed. One way to test this hypothesis would be to delete the sequence that encodes one or more large super-repeats from the titin gene. If the length of thick filaments were reduced by predicted multiples of 43 nm, this would provide strong support for the hypothesis. The detailed structure of the thick filament is still largely unknown and the exact position and functions of titin molecules within it are important unresolved problems. Solving these problems will also give pointers to the relationship between myosin and titin-like proteins in other contractile systems. Finally,

**SARCOLEMMA**  
The membrane (plasmalemma) of a muscle cell that makes an osmotic barrier around the contents of the cell.

the role of titin in muscle regulation is only just starting to be understood. Defining the molecular pathways that relate titin to signalling mechanisms is one of the most important tasks for the future.

The broader remaining question is whether titin-like molecules are universal components of actomyosin

contractile systems, and, if so, what their general functions are. Establishing the exact nature of the titin-like proteins and their relationships with the architecture of their host contractile systems will be fruitful in deepening our understanding of structural, functional and evolutionary aspects of the actomyosin machinery.

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