Huxleys' Missing Filament: Form and Function of Titin in Vertebrate Striated Muscle

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Abstract

Although superthin filaments were inferred from early experiments on muscle, decades passed before their existence was accepted. Phylogenetic analyses suggest that titin, the largest known protein, first appeared in the common ancestor of chordates and nematodes and evolved rapidly via duplication. Twitchin and projectin evolved later by truncation. Sallimus mutants in *Drosophila* exhibit disrupted sarcomere and chromosome structure, suggesting that giant proteins may have evolved as chromosomal scaffolds that were co-opted for a similar purpose in striated muscles. Though encoded by only one gene, titin comprises hundreds of exons and has the potential for enormous diversity. Shorter isoforms typically confer greater passive stiffness associated with smaller in vivo muscle strains. Recent studies demonstrate unequivocally that titin stiffness increases upon muscle activation, but the mechanisms are only now being uncovered. Although some basic principles have been established, a vast opportunity remains to extend our understanding of titin function in striated muscle.

INTRODUCTION

The history of muscle physiology reveals a pattern of contentiousness, as the characterization of muscle force production greatly preceded the acceptance of the responsible mechanisms. Not surprisingly, both the existence and roles of giant proteins in muscle sarcomeres were also only begrudgingly accepted. We briefly examine this history as we discuss the initial discovery, structural characteristics, and evolutionary history of titin, first described as "connectin" by Maruyama (1), and other closely related giant sarcomeric proteins. The structure of titin and its related giants reveals a protein spring with great adaptability, capable of storing elastic potential energy. We discuss these elastic properties of titin molecules and their contribution to passive tension in cardiac muscle. Titin in skeletal muscle is a much more compliant spring, suggesting a modified role. Thus, we conclude this review by addressing current discoveries in muscle physiology related to the roles of titin and other giant sarcomeric proteins in active muscle contraction.

DISCOVERY OF MUSCLE FILAMENTS

The advent of the electron microscope, first developed by the engineer Max Knoll and physicist Ernst Ruska in 1932, ushered in the modern era of muscle physiology research. With this remarkable tool, it became possible to break the optical resolution barrier of the wavelength of light. It was now possible to view objects orders of magnitude smaller than could be resolved with the light microscope. However, it would take an additional two decades before this technology became useful for examining biological samples.

For electron microscopy to probe biological samples, two additional achievements were required. To resolve structures within the cell, the section thickness itself had to be minimized below what was useful for light microscopy. Without ultrathin sections, no image could be brought into clear focus using transmission electron microscopy. In 1950, Latta & Hartman (2) developed a knife-edge microtome that provided this essential technique. The other key necessity was the proper preparation of biological tissues for electron microscopy. George Palade introduced fixation of tissue using osmium tetroxide in 1952 (3). These two innovations ultimately resulted in the Nobel Prize in Physiology or Medicine in 1974, awarded jointly to Albert Claude, Christian de Duve, and George Palade "for their discoveries concerning the structural and functional organization of the cell" (**http://www.nobelprize.org/nobel_prizes/medicine/laureates/1974/**).

Hugh Huxley and Jean Hanson relied on these techniques to present the first description of the organization of striated muscle sarcomeres (4, 5). They described what they called filaments within the myofibril, thick (primary) filaments and thin (secondary) filaments. Huxley (5, p. 388) concluded that the thick filaments were likely composed of myosin and the thin filaments, actin. He even observed that, "In some cases 'bridges' appear to extend between the primary and secondary filament."

These seminal papers provided the underpinnings of myofibril ultrastructure that resulted in the publication the following year of two papers that described the sliding filament theory of muscle contraction. Hugh Huxley, working at MIT in Cambridge, Massachusetts, and Andrew Huxley, working at the University of Cambridge in the United Kingdom, were neither related nor collaborators, though they were certainly influenced by each other's work. They met in Woods Hole, Massachusetts in 1953, where the simultaneous publication of their two papers in *Nature* in 1954 (6, 7) was orchestrated.

The initial reaction to this new sliding filament theory was hardly welcoming, as it did not conform to any existing paradigm in biochemistry. In fact, this and subsequent discoveries in muscle physiology (see below) are excellent examples of scientific inertia. Novel concepts are

initially rejected and finally accepted only with great reluctance; ironically these then became the new paradigm that in turn resists any significant modification. By the early 1960s, the idea of sliding filaments was finally adopted and is now the keystone of our understanding of muscle contraction. Maruyama (8) provides an outstanding history of the development of the sliding filament theory. A comprehensive and engaging history of muscle physiology can be found in Rall's book (9), which serves as a framework for numerous observations noted here.

HUXLEYS' MISSING FILAMENT

In the process of developing the sliding filament theory, Huxley & Hanson (7) observed that myofibrils exhibited elasticity even after removal of the thick and thin filaments. From these observations, they inferred that additional elastic filaments that connect the Z-disks must be present in muscle sarcomeres. They named this hypothetical filament the S filament (10). When evidence for a third filament eventually appeared in electron micrographs (11, 12), both Huxley (13) and Hanson (14) were skeptical, and it would be several decades before the existence of these filaments became widely accepted. Rall (9, p. 288) documents the reluctance to accept the presence of the third filament with two quotes, from Karoly Trombitas (15), "the muscle field became conservative . . . and this hindered progress in elastic filament research. It became very difficult to publish new results . . . in high-ranking journals. Referees were not sufficiently open-minded . . . . " The late physiologist Graham Hoyle (9, p. 288) was more adamant: "The evidence . . . both theoretical, based on dynamic properties, and visible, based on electron microscopy, has been amply provided . . . Yet, for reasons I have not been able to understand, the majority of muscle scientists have ignored them completely." Acceptance of the existence of giant elastic filaments in muscle would eventually require biochemical isolation and identification of giant proteins (1) as well as their localization within the sarcomere using immunohistochemistry (16).

Maruyama (1) called the new elastic protein connectin. Working independently, Wang et al. (17) discovered giant muscle proteins using gel electrophoresis and named the largest titin. Maruyama et al. (18) later demonstrated that titin and connectin were the same protein. For historical reasons, the name titin stuck despite Maruyama's priority, although Japanese authors still refer to a variety of giant sarcomeric proteins as connectin (9, 19). Immunohistochemical studies would soon reveal that titin extends from the Z-disk to M-line in muscle sarcomeres (16).

STRUCTURE OF TITIN AND OTHER GIANT SARCOMERIC PROTEINS

At up to 4.2 MDa, titin is the largest known protein (20). It is also the third most abundant protein in striated muscle (21). Its carboxy terminus is located at the M-line, where a kinase domain is present, and filaments from adjacent half-sarcomeres with opposite polarity overlap (**Figure 1**). In the A-band, titin is composed of repeating fibronectin (FNIII) and immunoglobulin (Ig) domains, arranged into distinct patterns of super-repeats, which are tightly associated with the myosin thick filaments (22, 23). A relatively inelastic distal Ig segment connects A-band to I-band titin at the end of the thick filaments (24). The structure of the elastic I-band region of titin varies depending on the isoform (25). I-band titin is composed of the proximal tandem Ig segment, N2A sequence, and PEVK region, enriched in P (proline), E (glutamate), V (valine), and K (lysine) (25). Finally, Z-disk titin is composed of inelastic residues that bind to actin, alpha-actinin, and titin molecules of opposite polarity from adjacent sarcomeres. Overlap of adjacent titin molecules in the Z-disk and M-line results in the formation of a titin filament system that is contiguous along the entire length of a myofibril (25).

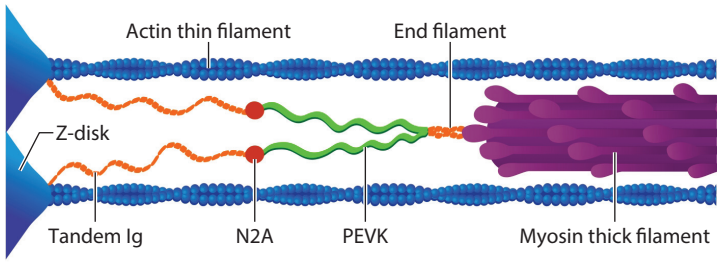

Figure 1

Layout of titin and other muscle proteins in muscle sarcomeres. Each titin molecule is bound to the thin filaments (*blue*) in the I-band and to the thick filaments (*purple*) in the A-band. The N2A segment (*red*) is located between the proximal tandem Ig segments (*orange*) and the PEVK segment (*green*). Reproduced with permission from Nishikawa et al. (99). Copyright 2012, The Company of Biologists Ltd. Abbreviations: Ig, immunoglobulin; PEVK, proline-glutamate-valine-lysine.

Compared to chordate titin, the structure of invertebrate giant proteins, such as sallimus/kettin (all invertebrates), twitchin (nematodes and mollusks), and projectin (arthropods), is simpler (26). None of these elastic proteins span the entire length of a sarcomere. The sallimus protein is located in the I-band and Z-disk. At its N-terminal, sallimus contains Ig-linker modules that bind to actin, similar to the N-terminal Z-repeats of titin (27). In the I-band, sallimus contains two PEVK domains and several unique sequences with tandem Ig and fibronectin-like domains at the C-terminus (26). In *Drosophila* and other arthropods, kettin is a splice variant of sallimus. Sallimus/kettin proteins connect the Z-disk to the ends of the thick filaments in muscle sarcomeres but unlike titin do not extend to the M-line (19). Twitchin and projectin proteins are composed mainly of repeating fibronectin-like and immunoglobulin-like domains similar to A-band titin. Their location within muscle sarcomeres varies among different types of muscle. Twitchin in *Caenorhabditis elegans* and projectin in insect synchronous flight muscles, similar to sallimus, also appear to connect the A-band to the Z-disk, known as the dense bodies in *C. elegans* (19).

EVOLUTION OF GIANT SARCOMERIC PROTEINS

Because titin is the largest known protein (20), the question of how it evolved is of particular interest. The family of giant titin-like proteins includes twitchin, projectin, sallimus, kettin, smooth muscle myosin light chain kinase (smMLCK), myosin binding protein C, and many other giant proteins (28). These proteins share a basic modular structure consisting of repeats of fibronectin class III domains and immunoglobulin c2 domains, and most also contain a protein kinase domain near the C-terminus. Giant titin-like proteins are found in the muscles of all bilaterian metazoans, including the protostome annelids, arthropods and mollusks, and the deuterostome echinoderms, hemichordates, and chordates (29). Genes that encode giant sarcomeric proteins appear to be absent in cnidarians, whose muscles appear to have evolved independently from those of bilaterians (30).

An understanding of the evolution of giant sarcomeric proteins is hindered by both the low sequence homology and enormous isoform diversity of these proteins, as well as by their nomenclature (19). Invertebrates appear to possess at least two different genes for titin-like giant sarcomeric proteins, each with different isoforms. One gene expresses D-titin/sallimus/kettin proteins, and a different gene expresses twitchin/projectin proteins (31). The proteins expressed

by these genes are generally much smaller than vertebrate titin and therefore have a more limited distribution within the sarcomere, with sallimus proteins typically found in the Z-disk and I-band and twitchin/projectin proteins in the A-band. The greater diversity of giant sarcomeric proteins in invertebrate muscle appears to be commensurate with their greater diversity of sarcomere structure, which varies widely among larval and adult muscles, direct and indirect flight muscles, supercontractile gut muscles, and obliquely striated muscles (19, 29). Invertebrate muscles exhibit a large range of sarcomere and filament lengths, with thick filament lengths up to 10 μm and thin filament lengths up to 6 μm (26), in contrast to vertebrates whose sarcomere and filament lengths are shorter and much less variable (32).

Vertebrates possess a single large titin gene and express a diversity of titin isoforms in different muscles (33). Most isoforms span the entire half-sarcomere from Z-disk to M-line (25). The titin gene of *Amphioxus* (34) is highly similar to the vertebrate titin gene in the A-band, but it lacks the I-band PEVK sequence typical of vertebrate titin and instead possesses an I-band Ig-PEVK region similar to that of giant sarcomeric proteins found in obliquely striated muscles of annelids. In contrast to *Amphioxus*, ascidians possess a short PEVK sequence in the I-band (35). The twitchin gene of *C. elegans* and the projectin gene of arthropods appear to be most closely related to the vertebrate titin gene, with loss of a sallimus-like gene in the lineage leading to vertebrates (26).

Relatively few studies have examined the evolution of titin-like proteins using phylogenetic analysis, and these studies include only small numbers of taxa (28, 34–36). The analyses of Higgins et al. (28) and Kenny et al. (36) suggest that duplication of immunoglobulin and fibronectin domains occurred rapidly in the early evolution of these giant proteins with the appearance of striated muscle in the common ancestor of nematodes and vertebrates. Domains at the same position within different super-repeats are more similar to each other than they are to domains within the same super-repeat, revealing the steps involved in the evolution of the 7- and 11-domain super-repeat patterns.

Higgins et al. (28) used the nucleotide sequence of the kinase domain of titin, twitchin projectin, and smMLCK for phylogeny reconstruction with trees rooted to the *Dictyostelium* myosin light chain kinase (MLCK). They found, somewhat unexpectedly, that the other giant proteins were derived from a titin-like ancestor via truncation events, although the confidence levels for the trees were relatively low. Ohtsuka et al. (34) and Hanashima et al. (35) conducted similar analyses with trees rooted to human MLCK. Similar to Higgins et al. (28), they also found that titin was ancestral to twitchin and projectin proteins of arthropods, mollusks, and nematodes. In muscle sarcomeres of early metazoans, it seems likely that giant sarcomeric proteins may have evolved to facilitate movement of worm-like animals through media of differing viscosity. It is curious that the largest and most complicated giant protein would appear first during phylogeny, with subsequent evolution occurring by a series of truncation events (**Figure 2**).

Although most evidence suggests that twitchin and projectin genes evolved from a titin-like ancestor by truncation, no phylogenetic study to date has clarified the relationships among vertebrate titin and sallimus (Ce-titin in *C. elegans*, 37; D-titin/sallimus in *Drosophila*, 38). Kenny et al. (36) tentatively include sallimus (D-titin) in the same family as titin, twitchin, and projectin but do not clarify the relationships within this group. Ohtsuka et al. (34) place sallimus (Ce-titin) at the base of the phylogeny before the appearance of chordate titin, whereas Hanashima et al. (35) place it at the base of the clade leading to twitchin and projectin.

Why did giant proteins evolve in the common ancestor of vertebrates and nematodes? An interesting observation suggests an unexpected answer that makes understanding the phylogenetic relationships between titin and sallimus an important goal for future work. Machado et al. (39) identify D-titin as a chromosomal protein in both human cells (vertebrate titin) and *Drosophila* embryos (sallimus/D-titin), using multiple antibodies developed against D-titin (sallimus protein).

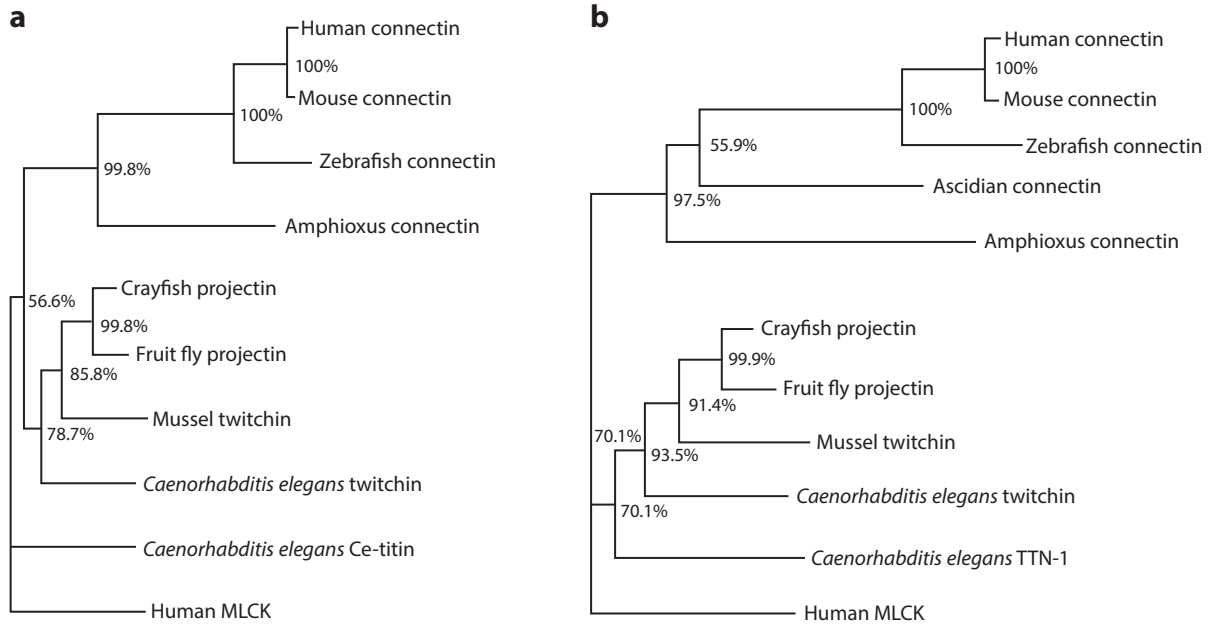

Figure 2

Two recent phylogenetic analyses of the evolution of giant sarcomeric proteins. (*a*) Ohtsuka et al. (34) place *Caenorhabditis elegans* titin at the base of the phylogeny, whereas (*b*) Hanashima et al. (35) place titin at the base of the phylogeny. More data are needed to resolve the early evolution of these proteins in the common ancestor of nematodes and vertebrates. Reproduced with permission from Ohtsuka et al. (34) and Hanashima et al. (35). Copyright 2011, 2012, Elsevier Ltd.

Subsequent studies also reveal the presence of titin in nuclei of human cells using titin-specific antibodies (40) and proteome analysis (41).

Machado & Andrew (38) further demonstrate that mutations in the *Drosophila* D-titin (sallimus) gene resulted in not only muscle but also chromosomal defects. The chromosomal defects observed in mitotic nuclei of fly larvae include polyploidy, chromosome fragmentation, undercondensation, and irregular condensation. The same mutants exhibit defects in myoblast fusion. These results strongly suggest a role for D-titin in providing an elastic scaffold for muscle sarcomeres and chromosomes in *Drosophila*.

Why would giant elastic proteins evolve rapidly in a common ancestor of nematodes and vertebrates and then undergo truncation during subsequent evolution as invertebrate sarcomere scaffolds? These studies (38–43) suggest the possibility that the earliest giant proteins, precursors of Ce-titin, D-titin/sallimus, and vertebrate titin, served as chromosomal scaffolds required for DNA supercoiling and condensation (42, 43), and were subsequently co-opted as scaffold proteins in muscle sarcomeres. A stronger test of this intriguing hypothesis requires more thorough phylogenetic analyses that include more representative genes from the Ce-titin/D-titin/sallimus lineage, as well as inclusion of more taxa. Although it seems unlikely that the role of D-titin in chromosome condensation is a derived characteristic within the phylogeny, additional direct evidence for involvement of titin in chromosomal condensation in other organisms is needed.

GIGANTIC VARIETY: MUSCLE-SPECIFIC ISOFORM EXPRESSION

Vertebrates possess a single titin gene with hundreds of exons, resulting in an enormous number of potential splicing pathways (33). Titin mutations also exhibit altered exon splicing. Removal

of even a few immunoglobulin domains from I-band titin altered splicing of several downstream exons, resulting in a much shorter and stiffer protein than predicted based on the primary mutation alone (44). The mechanisms for controlling exon splicing are only now just beginning to come to light (45, 46).

Numerous studies have documented the differential expression of titin isoforms in different tissues (47, 48). Cardiac muscle expresses the smallest, and soleus the largest, titin isoforms (20). Some exons (N2B) are expressed only in cardiac muscle (22), whereas others are expressed only in soleus (49). At least two different titin isoforms are expressed in cardiac muscle, N2B and N2BA (47). Both isoforms can be expressed in a single cardiac myocyte, and the proportion of the two isoforms varies regionally within the heart as well as across species. In skeletal muscle, Prado et al. (48) find that a sample of 37 muscles exhibits at least 9 different titin isoforms, which vary in their molecular weight from 3,300 kDa in psoas to 3,700 kDa in soleus. Some individual muscle fibers express at least two different isoforms. Titin isoforms change during normal development (50, 51), as well as in the progression of chronic diseases such as heart failure (52). It is speculated that titin isoforms may change as a result of eccentric training (53, 54). However, direct evidence is lacking.

Although the differential expression of different titin isoforms in diverse tissues within an organism is well established, an understanding of why expression differs is more elusive. Biomechanically, it is not obvious why the soleus muscle expresses the longest titin isoform and psoas expresses the shortest skeletal isoform in mammals from mice to rabbits to humans (49). One possibility may be that different titin isoforms are expressed in slow versus fast muscles. If so, why? We have barely scratched the surface of understanding the biophysical and biomechanical implications of differential titin expression in skeletal muscle.

THE ROLE OF TITIN IN SKELETAL MUSCLE PASSIVE TENSION

Although Maruyama (1) first identified connectin (titin) as an intracellular elastic protein in his short preliminary communication, it would be decades before significant effort was devoted to investigating its elastic function within muscle sarcomeres (55). Eventually, investigations of titin's elastic properties shed some light on this function. When single myofibrils are stretched, they exhibit passive stiffness (resistance to stretch). Linke et al. (56) demonstrate that the passive stiffness of rabbit psoas myofibrils is consistent with the passive stiffness of single titin molecules. This observation provides an explanation for how sarcomere integrity is maintained when a myofibril is stretched beyond the overlap of thick and thin filaments.

The currently accepted explanation for the passive force-extension behavior of intact myofibrils is that stretch of muscle sarcomeres first straightens the folded proximal tandem Ig domains with little increase in passive tension (57–60). At longer sarcomere lengths, as the proximal tandem Ig domains straighten toward their contour length, the stiffer PEVK domain elongates, and the passive tension increases steeply (57, 61). Titin is thus functionally two springs in series, one compliant and one stiff. In passive skeletal muscles, the stiffer PEVK region never elongates at physiological lengths (56), which seems puzzling as a design feature. Therefore, in passive skeletal muscle, titin appears to be more of a bungee than a spring. During active contraction of skeletal muscles, it appears that titin is too compliant to play a role.

Numerous studies have now established the relationships among titin isoform size, passive tension of single titin molecules (62, 63), and passive tension of single myofibrils or single muscle fibers (47–49). In general, the shorter the I-band region of titin, the higher the passive tension will be (15, 49). The length of the proximal Ig domain region determines the slack length of muscle sarcomeres, whereas the length of the PEVK sequence determines their stiffness (59, 64).

However, the passive stiffness of titin is highly variable and adaptable. In addition to differential exon splicing of I-band titin, the force-extension behavior and stiffness of both cardiac and skeletal muscles can be adjusted by changing the ratios of different isoforms expressed in the same myocyte or fiber (47, 65). Downhill (eccentric) training in rats results in an increase in passive muscle (and, apparently, titin) stiffness (54), and there is evidence that high frequency muscles in small mammals have stiffer titin isoforms than the same, lower frequency muscles in larger animals (53).

Although less well studied, the enormous diversity of sarcomere structure and function exhibited by invertebrate muscles shows a similar relationship between the length of giant sarcomeric proteins and their stiffness (31, 66, 67). Isoform expression changes during development, and the very stiff indirect flight muscles of insects (similar to cardiac titin) express much smaller isoforms of sallimus protein than synchronous flight muscles or other body muscles.

The available evidence strongly suggests that titin structure is closely linked to myofibrillar passive tension, though the exon splicing mechanisms that produce this association are largely unknown. Understanding the signaling pathways that lead to expression of specific exons in specific tissues in health, aging, and disease is one of the most exciting areas for future titin research.

Whereas much is known about the role of titin in passive tension of skeletal muscles, there are significantly fewer studies that relate titin structure to physiology or biomechanics at the whole-organism level. One such study examined the titin isoforms in red and white anterior and posterior axial muscles of carp (68). The results show that red muscles express larger isoforms of titin than white muscles and also that posterior muscles express larger isoforms than anterior muscles. The isoform size is correlated with higher passive tension and stiffness in anterior muscles than in posterior muscles. In fishes, red muscles experience larger sarcomere strain for a given body curvature than white muscles due to their placement in the periphery farther from the center of the body (69). Likewise, the posterior muscles experience a greater sarcomeric strain than anterior muscles at a given swimming speed due to the greater amplitude of oscillation at the tail. This distribution of titin isoforms in carp suggests that more compliant titin isoforms provide less resistance to stretch in parts of the body where deformations are greatest, thereby reducing energy loss during cyclic movement (68). The results also suggest that sarcomere strain, rather than muscle fiber type, may be an important determinant of titin isoform expression.

THE ROLE OF TITIN IN CARDIAC PASSIVE TENSION

Although there are many different isoforms of skeletal muscle titin, they are all more similar to each other than they are to the titin isoforms found in cardiac muscle (**Figure 3*a***). Regardless of the specific isoform, the passive tension of skeletal muscle titin develops at relatively long sarcomere lengths and is relatively more compliant, so that significant passive forces develop only at very long sarcomere lengths beyond the physiological operating range for most muscles (60). Although the passive tension of skeletal muscle isoforms varies among muscles and species, in all cases the titin in skeletal muscles is functionally a bungee cord, rather than a spring (70). In contrast, cardiac titin is a relatively stiff spring; even a very small stretch at short sarcomere lengths is accompanied by an increase in passive tension (71). The shortest and stiffest titin isoform so far described, N2B, is found exclusively in cardiac muscle, and even the longer more compliant N2BA isoform is much stiffer than any skeletal muscle isoforms (47). The net result is that the passive length-tension curve of cardiac muscle is shifted far to the left relative to that of skeletal muscle (**Figure 3*b***).

The passive stiffness of titin was described as the kingpin in the Frank-Starling law of the heart (i.e., the property of cardiac muscle that increased end-diastolic volume, or preload, results in increased cardiac contractility; see 58, 72). Titin plays several purported roles in the Frank-Starling

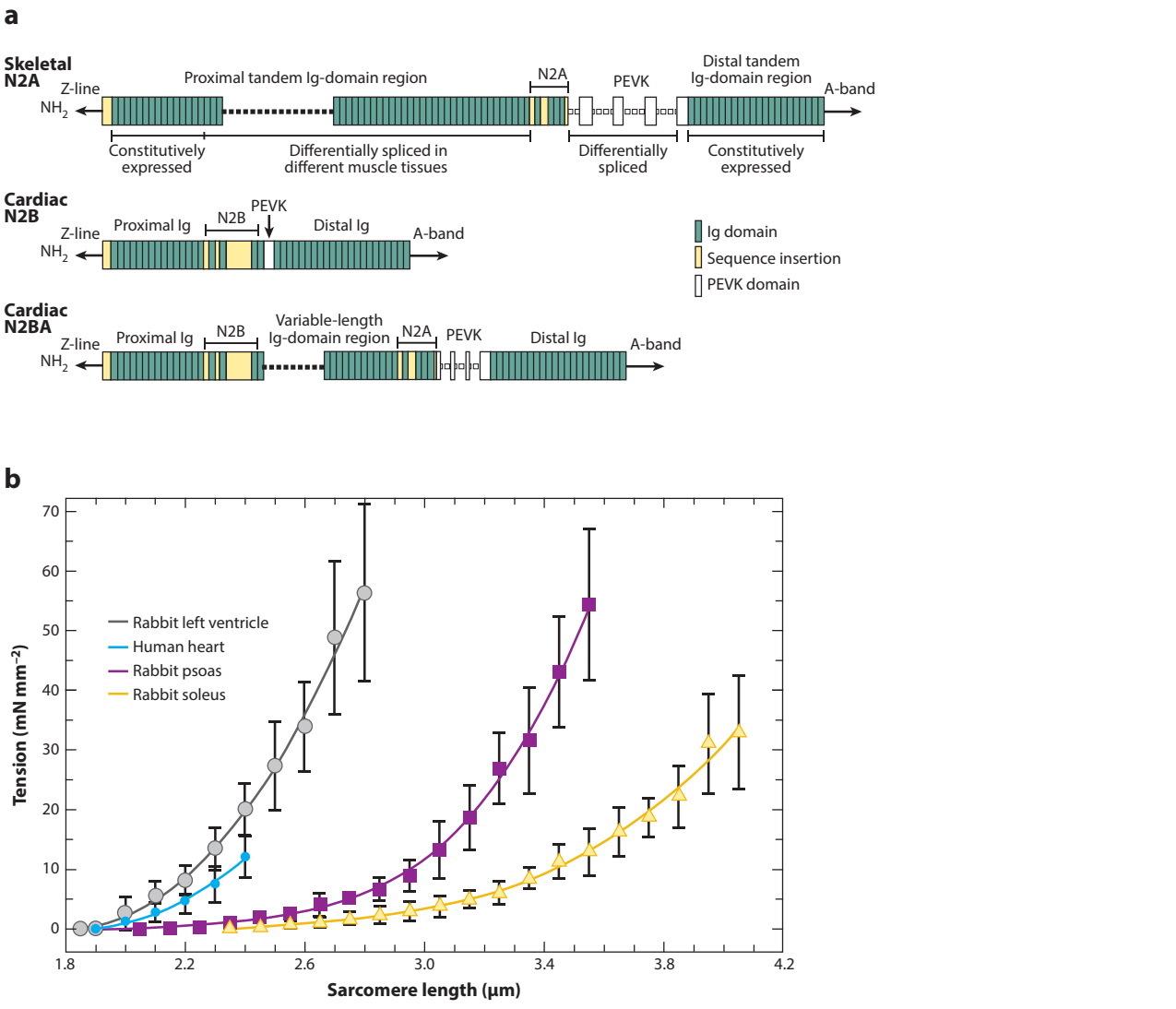

Figure 3

(*a*) The domain architecture of I-band titin isoforms in skeletal (N2A) and cardiac (N2B, N2BA) muscle. N2B is the shortest and stiffest isoform, whereas N2A is the longest and most compliant. (*b*) Titin passive stiffness as a function of sarcomere length. In cardiac titin, passive tension increases at sarcomere lengths well within the functional range during normal ventricular diastole. In contrast, skeletal muscle titin displays passive stiffness only at sarcomere lengths outside the physiological operating range of sarcomere lengths. Reproduced with permission from Neagoe et al. (47). Copyright 2003, Springer. Abbreviations: Ig, immunoglobulin; PEVK, proline-glutamate-valine-lysine.

law, all triggered by the increased passive tension that accompanies stretch during ventricular filling. These roles include: (*a*) increased Ca^{2+} sensitivity, directly causing increased contractility (73), (*b*) stress-sensing and signaling within the myocardium (65), and (*c*) length-dependent thin filament regulation (74). Titin in cardiac muscle has been characterized as a "bidirectional spring

that determines not only ventricular rigidity and diastolic function, but systolic function" (75, p. 332). Thus, cardiac titin was demonstrated to be critically important in cardiac function.

Importantly, the myocardium is only stretched passively during filling (diastole). For that reason, the passive stiffness of cardiac titin must be tuned to the in vivo length changes that occur during normal ventricular diastole. An example of how titin stiffness is tuned is apparent in animals with drastically different cardiac demands during hibernation. Accompanying greatly reduced metabolic rate, there is a dramatic reduction in cardiac output in hibernating animals (76). Whereas stroke volume remains unchanged during hibernation in large animals such as bears, squirrels increase stroke volume during hibernation. The hearts of bears decrease in mass and increase in ventricular stiffness during hibernation, whereas the hearts of ground squirrels increase in mass and decrease in ventricular stiffness. The ventricles in bears become stiffer due to upregulation of the shorter N2B titin isoform and downregulation of the longer N2BA isoform (77). In contrast, the ventricles of hibernating rodents become more compliant by downregulating the shorter N2B titin isoform and upregulating the longer N2BA isoform (78). The results seem to parallel those from fish axial muscles, as the cardiac isoforms become more compliant when sarcomere strain increases due to increasing stroke volume in ground squirrel hearts. A parallel phenomenon is also observed in the hearts of trout (79), which regulate cardiac output by increasing stroke volume. Trout have a higher ratio of N2BA to N2B isoform in their ventricles than rats, which regulate cardiac output by increasing heart rate and have a much lower ratio of N2BA to N2B.

Although these examples document one link between titin isoform expression and organismal physiology, it is clear that much work remains to elucidate the structure–function relationships of titin and other giant sarcomeric proteins in animals. Some intriguing questions include the relationships between titin isoforms and body size, muscle fiber type, concentric versus eccentric training, and aging.

A ROLE FOR TITIN IN ACTIVE SKELETAL MUSCLE

The foregoing discussion has centered upon the role of titin in muscle passive tension. Absent in cardiac muscle, isometric and eccentric (lengthening) contractions are common in skeletal muscle, opening the possibility that titin may play a key role during active force production. Certainly, the distinct differences in titin isoforms in skeletal versus cardiac muscle suggest a different role. The idea that titin might play a role in active muscle gained traction when studies showed that the structure of purified I-band titin is altered by Ca^{2+} in vitro (80). In active muscle fibers, studies had shown that Ca^{2+} influx increases tension and stiffness of a noncrossbridge structure, possibly titin (81–85). In both PEVK fragments and single muscle fibers, Labeit et al. (86) demonstrate not only that Ca^{2+} influx increases titin stiffness but also that increasing Ca^{2+} results in a leftward shift of the force-extension curve.

The effects of Ca^{2+} on titin stiffness observed in these studies are ten times too small to account for the observed increase in stiffness of muscle fibers upon calcium activation (83, 87) or active stretch (88–90). Edman et al. (91) first suggested that enhancement of force during active stretch was due to recruitment of viscoelastic structures in muscle, based on the observation that single fibers shorten faster in the enhanced state, shifting the force-velocity curve to the right. Edman & Tsuchiya (92) made similar observations during load-clamp and unloaded shortening tests.

In an innovative series of experiments, Leonard & Herzog (93) stretched myofibrils, both passive and active, far beyond overlap of the thick and thin filaments (i.e., sarcomere lengths up to 6 μm). These experiments unequivocally demonstrated that titin stiffness is increased by Ca^{2+} influx and force development in active muscle. At sarcomere lengths beyond overlap, it is not possible for crossbridges per se to contribute directly to active force. In addition, depletion

of troponin C had no effect on myofibril force during stretch beyond overlap, suggesting that the mechanism is unrelated to thin filament activation (94). Furthermore, there was no evidence of yielding [a decrease in tension with stretch due to material failure as seen by Wang et al. (64)] during these slow stretches to long sarcomere lengths, suggesting that there was little or no unfolding of Ig domains (90, 95, 96).

Leonard & Herzog (97) speculate that, in addition to relatively small direct effects of Ca^{2+} on titin stiffness (86, 98), titin may bind to actin when Ca^{2+} is present, thereby decreasing its free length and increasing its stiffness. The observation that force increased less steeply with stretch when myofibrils were activated at a sarcomere length of 3.4 μm than when activated at 2.4 μm suggests that an interaction with the crossbridges also affects active titin stiffness. If true, the lower titin stiffness upon activation at 3.4 μm would reflect the decreasing overlap of thick and thin filaments.

THE WINDING FILAMENT HYPOTHESIS

The winding filament hypothesis (99) was proposed to account for Leonard & Herzog's (93) observations. The hypothesis suggests that titin is activated by Ca^{2+} influx, and that activated titin is then wound upon the thin filaments by the crossbridges, which both translate and rotate the thin filaments (99). The N2A region of titin in skeletal muscle (**Figure 4*a***) is in an ideal position for modulation of titin stiffness through Ca^{2+}-dependent binding to thin filaments. Binding of titin to actin at this location would eliminate low-force straightening of proximal tandem Ig domains in the I-band that normally occurs upon passive stretch of myofibrils at slack length (56). Furthermore, when Ca^{2+}-activated sarcomeres are stretched, elongation of the PEVK segment will generate

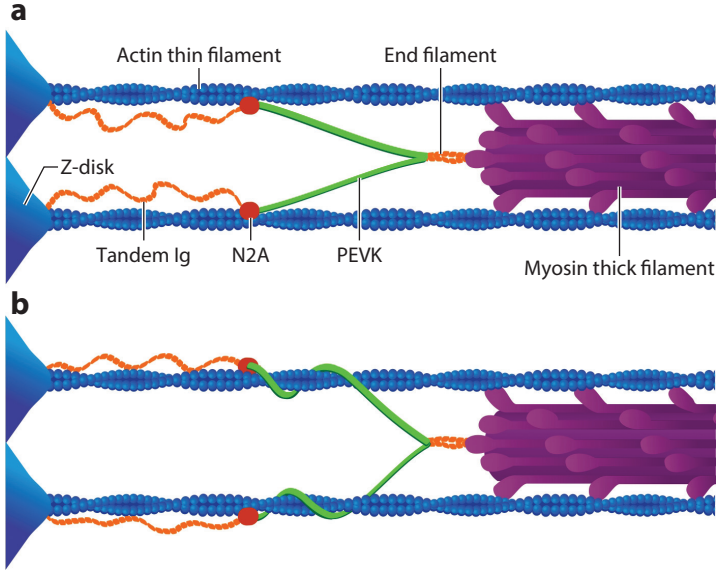

Figure 4

The winding filament hypothesis. (*a*) Upon Ca^{2+} influx, N2A titin (*red*) binds to thin filaments (*blue*). (*b*) Crossbridges (*purple*) wind PEVK titin (*green*) on thin filaments in active muscle. As shown, all titins in the same half-sarcomere must wind in the same direction around actin filaments. Reproduced with permission from Nishikawa et al. (99). Copyright 2012, The Company of Biologists Ltd. Abbreviations: Ig, immunoglobulin; PEVK, proline-glutamate-valine-lysine.

high force. The absence of the N2A segment in the shortest cardiac titin isoforms suggests that they are not activated by Ca^{2+} ions, perhaps because cardiac muscle is never active when stretched.

In active muscle sarcomeres, crossbridges likely rotate and translate the thin filaments (**Figure 4*b***). At least some isoforms of all known motor proteins—dynein (100, 101), kinesin (102), and nonmuscle myosins (103, 104)—follow a spiral path as they translate along helical microtubules or actin filaments. Given the structure of the thick and thin filaments, maintenance of stereospecific binding between an actin monomer and its three neighboring thick filaments requires the thin filaments to rotate by ~28° as the myosin heads translate the length of one actin monomer (105). This would produce one full rotation of the thin filaments for every ~71.5 nm of translation.

Because titin is bound to thick filaments in the A-band and to thin filaments in the Z-disk (106), rotation of thin filaments by the crossbridges must inevitably lead to winding of titin upon them. Rotation of thin filaments by the crossbridges would also produce a torque in alpha-actinin in the Z-disk. Winding of titin on the thin filaments is predicted to change the length and stiffness of PEVK, storing elastic potential energy during isometric force development and active stretch. This energy would be recoverable during active shortening.

Unwinding of titin from the thin filaments could be prevented by electrostatic interactions between thin filaments and PEVK (107). Spontaneous dissociation rates of PEVK bound to actin are low, and the force required to break the bonds is approximately equal to the force required to break an actomyosin crossbridge. Unwinding of PEVK from the thin filaments is hypothesized to occur during active shortening at low loads when the combined PEVK-actin and crossbridge forces are too low to hold the torques in titin and alpha-actinin, as well as during muscle relaxation.

When the sliding filament theory is enhanced by the addition of a dynamic role for titin that includes N2A binding to actin and PEVK winding on thin filaments, it becomes possible to explain several puzzling aspects of muscle physiology (108). The winding filament hypothesis provides an explanation for enhancement of muscle force at low energy cost during active stretch (89, 99, 109, 110). When muscles are stretched while active, the work done in stretching will extend titin, storing elastic energy in PEVK with no additional ATP requirement. The added force will increase with stretch amplitude. Ca^{2+}-dependent binding of titin to thin filaments explains why force increases faster during active stretch (**Figure 5*b***) than during passive stretch (**Figure 5*a***). Stretch of resting sarcomeres would straighten the N-terminal tandem Ig domain and elongate PEVK, whereas stretch of active sarcomeres would only elongate PEVK (108). Winding of PEVK on thin filaments by the crossbridges explains why residual force enhancement recovers in an activated muscle fiber that is shortened prior to stretch (111–113). It also explains why residual force enhancement does not increase monotonically with initial sarcomere length at activation (111).

In addition to the winding filament hypothesis, several alternatives are proposed. These include different hypotheses for titin–actin interactions (114–116), titin–myosin interactions (117), and titin Ig domain unfolding and refolding (118). How can these alternative hypotheses be tested? Granzier (95) suggests labeling single myofibrils with antibodies specific to different regions of titin and comparing the effects of active and passive stretch on epitope movement. For example, the winding filament hypothesis suggests that the N2A antibody should move relative to the Z-line in passively stretched myofibrils but should become anchored to the thin filaments in actively stretched myofibrils. By conducting these tests with different antibodies, it should be possible to test whether any epitopes of titin change their behavior during active versus passive stretch.

A spontaneously occurring mutation in mice may also help to discover mechanisms of titin activation in vertebrate skeletal muscle. A titin mutation that produces muscular dystrophy with myositis (*mdm*) in mice results in a predicted 83-amino-acid deletion in the N2A and PEVK regions of the titin protein (119). Muscles from *mdm* mice are actively more compliant (108, 120) possibly

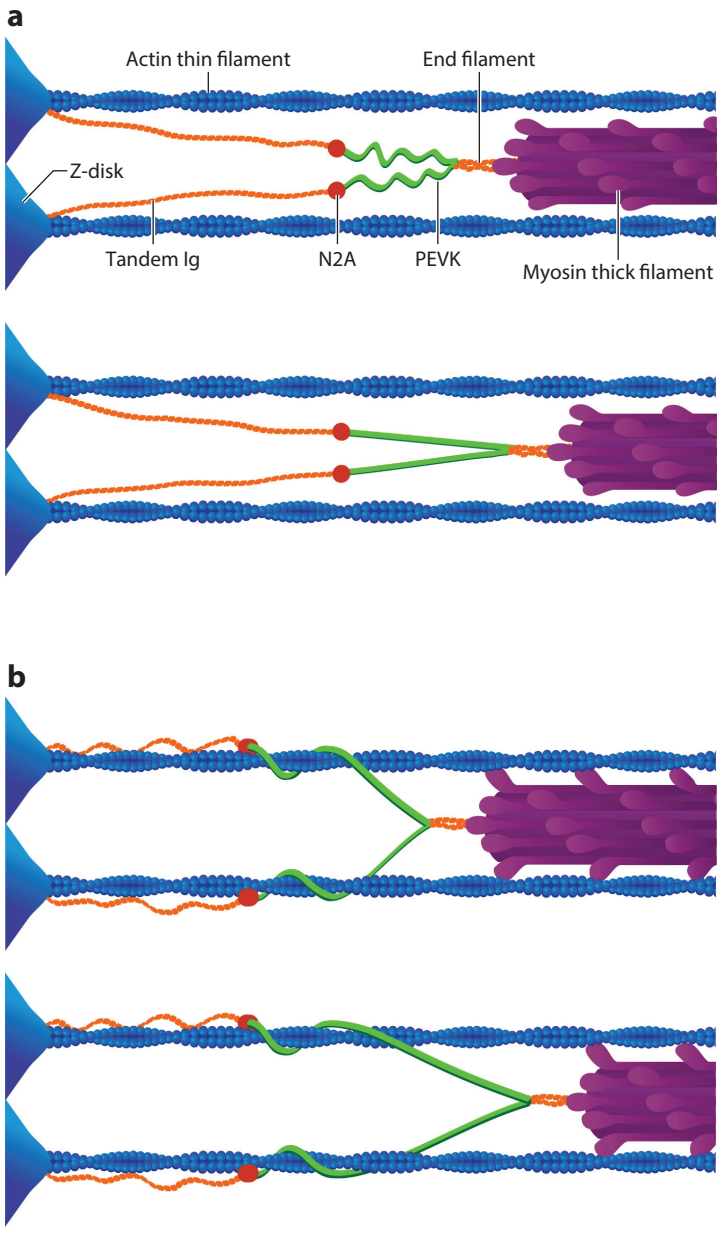

Figure 5

(*a*) Passive stretch of muscle sarcomeres. As a sarcomere is stretched beyond its slack length, the proximal tandem Ig segments unfold approximately to their contour length (*above*). After the proximal tandem Ig segments have reached their contour length, further stretching extends the PEVK segment (*below*). Adapted from Granzier & Labeit (58). (*b*) Active stretch of muscle sarcomeres. Upon activation, N2A titin binds to actin (*above*). Only the PEVK segment (*green*) extends when active muscle is stretched (*below*), due to binding of N2A to thin filaments. Reproduced with permission from Nishikawa et al. (99). Copyright 2012, The Company of Biologists Ltd. Abbreviations: Ig, immunoglobulin; PEVK, proline-glutamate-valine-lysine.

owing to the deletion in titin's I-band region. This suggests that modulation of titin stiffness in active sarcomeres by a titin-thin filament interaction may be affected by the *mdm* mutation.

To test this hypothesis, Powers et al. (121) stretched psoas myofibrils from normal and *mdm* mice from sarcomere lengths of 2.5 to 6.0 μm. Actively stretched myofibrils from normal mice were stiffer and generated more force than passive myofibrils at all sarcomere lengths. In contrast to wildtype myofibrils, no increase in stiffness and just a small increase in force were observed in actively compared to passively stretched *mdm* myofibrils. These results support the idea that titin is activated upon Ca^{2+} influx, stiffening and stabilizing the sarcomere during contraction, possibly by N2A binding to actin, and that this mechanism is lost in the *mdm* mutation. Future studies should focus on determining which amino acids in titin, absent in the *mdm* mutation, are required for titin activation. Identification of these amino acid sequences may suggest hypotheses for the biochemical processes involved in titin activation.

DIVERSITY OF MUSCLE PHYSIOLOGY AMONG ANIMALS

Animals from nematodes to vertebrates exhibit a wide range of muscular specializations, from the smooth cardiac and skeletal muscles of vertebrates to the catch and asynchronous muscles of invertebrates (122). Virtually all of these diverse types of muscles produce more force at a lower energy cost when actively stretched. Yet, no consensus has emerged regarding the mechanisms for how this occurs in any type of muscle (123). Clearly, giant sarcomeric proteins contribute to the passive stiffness of cardiac muscle in vertebrates (47) and asynchronous indirect flight muscles in invertebrates (124). Yet, a role for giant proteins in active muscle contraction is only now—begrudgingly—becoming accepted.

In molluscan catch, known for more than 150 years from bivalve adductor and other muscles, an elastic element develops upon muscle activation, persists for long periods after deactivation, and adjusts its stiffness during shortening to maintain its force at a shorter length (125). This elastic element is twitchin (126). A role for giant proteins in invertebrate catch was unexpected (122). Although no fewer than 26 proteins change their phosphorylation state during catch (122, 127), Yamada and colleagues (127) demonstrated that catch could be observed in an in vitro assay containing only myosin, actin, and twitchin. During catch, Ca^{2+} influx triggers dephosphorylation of twitchin and binding of twitchin to actin (125). Although it was observed originally in molluscan smooth muscles, Wilson & Larimer (128) show that catchiness is a general property of all invertebrate muscles.

The structural similarities (26) and evolutionary relationship between twitchin and titin (see above), as well as the functional similarities between catch and residual force enhancement, suggest that residual force enhancement in vertebrate skeletal muscle and invertebrate catch could be evolutionary homologues. However, the rapid evolution of the giant sarcomeric proteins, along with their low sequence homology, also makes it likely that biochemical mechanisms for calcium-dependent binding to actin may have diverged during evolution. Nevertheless, our understanding of twitchin-based catch force may provide potentially fruitful hypotheses for the regulation of force enhancement in vertebrate muscle.

SUMMARY AND RECOMMENDATIONS FOR FUTURE WORK

New discoveries in muscle physiology have been met with vigorous resistance, from the initial proposal of a sliding mechanism for muscle contraction, to the existence of superthin filaments. However, as a role for these proteins in muscle passive tension is now accepted, we can focus our attention to the roles of giant sarcomeric proteins in active muscle. From nematodes to vertebrates, striated muscles of all animals express one or more giant sarcomeric proteins. These enormous

proteins possess a similar structure consisting of super-repeats of fibronectin, immunoglobulin, PEVK, and kinase domains. It appears that these proteins first appeared in the common ancestor of nematodes and vertebrates via rapid duplication of repeating fibronectin and immunoglobulin motifs. It is unclear whether these proteins may have evolved first as scaffolds for the chromosomes and were subsequently co-opted to serve as scaffolds for muscle sarcomeres.

Because muscles are complex structures composed of hundreds of interacting proteins, and giant sarcomeric proteins are composed of tens to hundreds of different exons, sorting out the roles of titin and other giant sarcomeric proteins in muscle physiology is a difficult enterprise, as amply demonstrated by the historical examples discussed herein. Although biochemical processes are readily identifiable, it is much more difficult to demonstrate that any given process is necessary or sufficient to account for physiologically relevant phenomena.

As discussed previously, phylogenetic studies of giant sarcomeric proteins have included relatively few taxa, and none to date has included both *C. elegans* titin, D-titin/sallimus, and twitchin/projectin from multiple taxa in the same analysis. The relationship between *C. elegans* titin and vertebrate titin thus remains unclear. If *C. elegans* titin is ancestral to vertebrate titin, it would imply that the largest proteins evolved not in the common ancestor of bilaterians but somewhat later, perhaps in the common ancestor of deuterostomes. Alternatively, if vertebrate titin is ancestral to *C. elegans* titin and sallimus, then that would support the hypothesis that the largest known protein appeared very early in animal evolution.

Perversely, there is very strong genetic evidence in *Drosophila* that the sallimus protein is found not only in muscle sarcomeres but also in chromosomes. Analysis of mutants suggests that titin plays a role in chromosome condensation, and it seems highly unlikely that a chromosomal role for sallimus would be unique to insects or even arthropods. Yet, the evidence for localization of titin-like proteins in the chromosomes of other animals and humans is based entirely on immunohistochemistry where cross-reactivity is always a potential artifact. Furthermore, no studies have explored the biophysics of titin function in chromosomes. Although a few studies have demonstrated that titin interacts with histones (129), none have tested whether titin interacts directly with DNA. Much work remains to definitively demonstrate a physiological role for titin-like proteins in chromosomes, to say nothing of the underlying mechanisms.

Another curious question is why the genomes of any animals would contain a single large gene that encodes a giant protein the size of a thousand average-sized proteins, and then use exon splicing to create different isoforms. Why not have multiple genes? Why use post-transcriptional processing to control isoform expression? Some insight into this question may come from understanding why invertebrates have at least two genes, projectin and sallimus, that express giant sarcomeric proteins, whereas vertebrates have only one. It remains to be determined whether their common ancestor possessed one or two genes, and therefore whether a gene was lost in deuterostomes or gained in protostomes. In addition, the invertebrate genes have many fewer exons than vertebrate titin (26).

The processes that control post-transcriptional processing and exon splicing in titin remain largely unknown. It was demonstrated that titin isoform expression changes during development and disease, but it remains unknown whether isoform expression may change in response to training, aging, or other signals. We know that different skeletal muscles express different titin isoforms, but whether or how isoform expression is adaptive physiologically remains unknown. The signaling mechanisms involved in isoform expression in different muscles are likely related to the different types of muscular dystrophy, which can target specific muscles or muscle groups (130).

Recent studies on twitchin and titin demonstrate that at least some giant sarcomeric proteins play a role in active muscle. Whereas the mechanism is at least partly understood for molluscan twitchin, the mechanism of activation of titin is completely unknown. Several alternative

hypotheses were advanced, and it seems likely that immunohistochemical labeling of different epitopes in single myofibrils during active and passive stretching may help to distinguish among the alternatives. We are only now beginning to understand the roles of giant sarcomeric proteins in different physiological processes among the diverse types of muscles found in the animal kingdom.

Lastly, in addition to spontaneously occurring mutants such as the *mdm* mouse, it appears that the ability to selectively manipulate gene expression in model organisms like *Drosophila* (131) and mouse (44) will ultimately be required to link biochemical events, such as dephosphorylation of twitchin, to biophysical events like binding of twitchin to actin and ultimately to physiological processes at the organismal level, such as invertebrate catch, residual force enhancement, or stretch activation of insect asynchronous flight muscle. It seems likely that these organism-level phenomena may result from multiple biochemical and biophysical events that are tuned to produce large changes in muscle function in response to activation and other signals. In this context, titin and other giant sarcomeric proteins appear to play a crucial role in transmitting crossbridge forces outside the sarcomere (132). In this role, variable stiffness that increases with activation and muscle force would be highly advantageous.

It is a very exciting time for research on giant sarcomeric proteins. Recent research strongly suggests that important new discoveries are imminent. This is somewhat ironic given that 15–20 years ago it was widely believed that the field of muscle physiology was largely solved. In fact, numerous questions remain concerning the evolution of giant sarcomeric proteins, the regulation of exon splicing that leads to the production of different isoforms, the role(s) of these proteins in active muscle contraction, and the underlying mechanisms of activation in different types of muscles. These range from skeletal and cardiac muscles of vertebrates to the supercontracting, oblique, synchronous, and asynchronous muscles of invertebrates. Regardless of which hypotheses are supported and which are refuted, it is clear that several new chapters remain to be written in the annals of muscle physiology.

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