Chapter 7
Endosarcomeric and Exosarcomeric Cytoskeleton: Emergence of Cell and Molecular Biology in the Muscle Field

Titin and/or nebulin apparently provide axial continuity for the production of resting tension on stretch. (Horowits et al. 1986. With permission Nature Publishing Group)

Horowits et al. (1986)

This four-filament sarcomere model raises the possibility that nebulin and titin might act as organizing templates and length-determining factors for actin and myosin respectively. (Wang and Wright 1988. With permission Rockefeller University Press)

Wang and Wright (1988)

The realization that the M-band is elastic and serves signaling functions opens the perspective that M-band strain might also translate into modulation of metabolic activity, in addition to protein turnover and transcription regulation, and thereby regulate short-term adaptation of muscle to strain. (Gautel 2011. With permission Elsevier)

Gautel (2011)

7.1 Introduction

In the early 1970s the sliding filament, attached cross-bridge, model of muscle contraction had become dogma and attention was directed to elucidating the mechanisms of muscle activation and cross-bridge function. Of course this was a two filament model of contraction with the thin filaments anchored at the Z disc and the thick filaments held in place at the M band. But there were lingering questions. In the classic experiments of Huxley and Hanson (1954), extraction of actin and myosin did not cause the collapse of the myofibril but rather "ghost" myofibrils resulted that still exhibited elasticity. They explained this observation by suggesting that an unseen elastic structure existed that held the Z discs together. With the exciting advances in understanding of activation and cross-bridge function, this observation seemed to have been forgotten. But there was more, striated muscles are striated because of the precise lateral alignment of the myofibrils across the fiber.

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The classic experiments of Huxley and Niedergerke (1954) showed that this alignment did not change when a living fiber shortened actively or was stretched while at rest. What determines this alignment? What is the molecular nature of the Z disc and M band that hold the filaments together transversely and what structure holds the sarcomeres together longitudinally even in the absence of actin and myosin (endosarcomeric structures)? What structures bind the myofibrils together transversely across a muscle fiber (exosarcomeric structures). Electron microscopic evidence provided some early clues but the results were inconsistent and there were concerns about artifacts associated with fixation.

The rapid emergence of cell biology and molecular biology techniques in the 1980s played and continues to play a major role in the advancement of our understanding of the endosarcomeric and exosarcomeric cytoskeleton. Of preeminent importance was the development of immunological approaches to molecular histology with fluorescent antibody and immunoelectron microscopy techniques. These techniques coupled with the amino acid sequence determination of proteins by complementary DNA (cDNA) techniques have led to spectacular advances in the understanding of the muscle cytoskeleton. As a result, new fields of investigation have evolved characterizing intracellular signaling pathways in regulation of muscle cell development and response to mechanical stress and disease.

7.2 Passive Force and the Third Filament of the Sarcomere: Titin First Discovered as Connectin

7.2.1 Introduction

In the early 1970s generally it was thought that the resting elasticity or passive force production observed when a resting muscle was stretched was due simply to the connective tissue that surrounded the muscle fibers and collagen fibrils in the sarcolemma (Fig. 7.1). But there were early observations that suggested that at least some of this elasticity must reside inside the fiber and within the myofibril itself. In the early 1950s, Reiji Natori at the Jikeikai Medical School in Tokyo Japan developed a single skeletal muscle fiber preparation in which the sarcolemma was mechanically removed from a frog fiber. The muscle fiber contained no extracellular collagen but yet when stretched at rest still exhibited the passive force attributed to the intact fiber (Natori 1954). Thus he concluded that much of the resting elasticity of a fiber must exist within the fiber itself. At about the same time, Hugh Huxley and Jean Hanson were describing their evidence for the sliding filament mechanism of muscle contraction. In a remarkable experiment they showed that isolated myofibrils that had their myosin and actin extracted still exhibited Z discs and a "backbone" of virtually zero optical density (see Fig. 2.8) (Huxley and Hanson 1954; Hanson and Huxley 1955). These "ghost" myofibrils were also elastic and could be stretched and released. Because of this observation Hanson and Huxley (1955) speculated that there must be a "third filament" along with the thick and thin

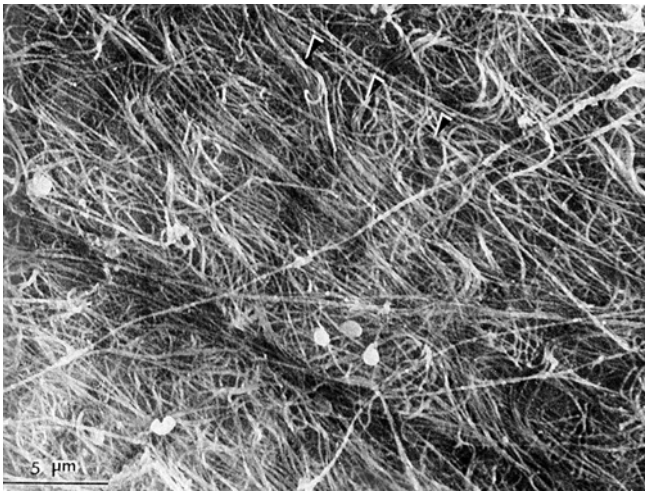

Fig. 7.1 Scanning electron micrograph showing the fibrous layer of connective tissue on the surface of a frog sartorius muscle fiber. Collagenous fibrils densely cover the muscle fiber and take a predominantly longitudinal course. Cross striations can be seen through fibrous layer (Ishikawa et al. 1983. With permission The American Physiological Society)

filaments that composed the sarcomere. They named this hypothetical third filament the "S filament", S for stretch, and envisioned that it connected the ends of thin filaments in each half of the sarcomere (see Fig. 2.9). This third filament vanished from the models of the sliding filament mechanism after 1956 most likely because in Hugh Huxley's classic electron microscopic observations of 1957 there was no visual evidence for an S filament (see Figs. 3.3, 3.4, 3.5).

7.2.2 Early Electron Microscopic Evidence for a Third Filament and Its Skeptical Reception

Despite Huxley's results, there were those scientists who believed that the two filament model of contraction was inherently unstable and that there had to be a third filament to explain the stability of the sarcomere and the elasticity of the myofibril. Electron microscopic evidence started to appear in the early 1960s that suggested a third filament in the sarcomere. For example, Fritiof S. Sjostrand (1962) at the University of California at Los Angeles (UCLA) stretched isolated frog skeletal muscle to the point where there was no overlap of thick and thin filaments. At these lengths a "gap" developed between the thick and thin filaments as seen in the electron microscope. Filaments thinner than the thin filaments could be seen bridging the gap. These filaments were of variable thickness and appeared to be continuous with thick filaments of the A band. Thus he named them "gap filaments". Graham Hoyle at the University of Oregon working with Patricia A. McNeill (McNeill and

Hoyle 1967) also saw very thin filaments in the gap when muscles were stretched to long lengths. These "superthin filaments" or T filaments as Hoyle called them also could be seen in the H zone and McNeill and Hoyle concluded that they ran the whole length of the sarcomere and connected adjacent Z discs together. In 1975 Ronald Locker and N. G. Leet at the Meat Industry Institute of New Zealand published the first of a series of papers examining the structure of highly stretched beef muscle fibers. They found that the thick filaments were staggered and that gap filaments were observed. They concluded that the filaments that "always" span the gap must be responsible for transmitting the force that dislocates and stretches the A-band. There were also other reports of third filaments, particularly in insect muscle (see Wang 1985 for a review).

These results were greeted with skepticism by Hugh Huxley and Jean Hanson (Huxley 1968; Hanson 1968). The issue was discussed extensively at a symposium on muscle in Budapest. There seemed to be multiple reasons for the skepticism. First, Hugh Huxley didn't see them in his classical 1957 study. Second, the third filament proponents could not agree on the exact location of the filaments in the sarcomere. Third, while not denying the observations, there was always the possibility of fixation artifacts. With regard to her own study of the ghost myofibrils, Hanson (1968) stated in the general discussion at the Budapest symposium that she had observed a sparse network of very thin structureless filaments connecting successive Z line but did not know if this material was present in the intact fibril or if it represented an insoluble residue of the actin and myosin filaments or was derived from interfibillar material. In short, in the view of Huxley and Hanson, the electron microscopic evidence was not convincing.

There was a lot of frustration on the part of the third filament proponents. For example, Trombitas (2000) of Washington State University who had seen a third filament in insect muscle provided in 2000 some perspective on those days (Trombitas 2000. With permission Springer):

> Although there was a general consensus about the appearance of the gap filaments in overstretched vertebrate skeletal muscle, the evidence was not convincing enough to firmly establish a third type of filament…Since the sliding filament theory explained muscle contraction so well, the muscle field became conservative and, in my experience, this hindered progress in elastic filament research. It became very difficult to publish new results from this field in high-ranking journals. Referees were not sufficiently open-minded…

Graham Hoyle was more outspoken about the lack of acceptance of the evidence of a third filament by the establishment (Hoyle 1983. With permission John Wiley & Sons Inc):

> The most glaring example of a very real attempt on the part of a majority of muscle scientists to willfully lose a vital piece of information in the last 25 years has been in regard to ultrathin (T or S) filaments…The evidence for their existence, 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. There have been occasional howls from the frustrated few believers, but these believers have been treated by the majority as nasty pests needing to be destroyed. Why? Without them, T filaments would certainly be getting 'lost.' With such obstinacy the majority deserve to be proved wrong!

Reading the arguments on both sides, one comes away with the impression that the majority didn't deny the possible existence of a third filament but rather that the evidence for a third filament didn't reach the level of expectation for wide acceptance. After all, the sliding filament model was greeted with much skepticism when it was proposed in 1954 but Hugh Huxley produced the structural evidence in 1957 that led to general acceptance of the model. It was up to the third filament proponents to do the same.

7.2.3 A Breakthrough: Protein Nature of a Putative Third Filament

It turned out that the required evidence of the existence of a third filament in the sarcomere did not come first from electron microscopy but rather from biochemistry. The breakthrough started with the pioneering studies on the biochemistry of muscle elasticity by Koscak Maruyama (Fig. 7.2a) in Japan. Maruyama was an expert in the isolation and characterization of muscle proteins. He had collaborated with Setsuro Ebashi on the characterization of α-actinin, a protein discovered by Ebashi 1963 (Maruyama and Ebashi 1965). Soon thereafter he discovered another sarcomeric protein, β-actinin (Maruyama 1965) and later showed that it was equivalent to the actin capping protein now known as Cap Z (Maruyama et al. 1990). Maruyama was intrigued by Natori's results on the elasticity of skinned muscle fibers and he set out to determine the protein nature of this elasticity. Along with Natori and Yoshiaki Nonomura, they first showed that skinned muscle fibers from the frog from which actin and myosin were extracted exhibited elasticity when stretched. They then isolated a protein from rabbit ghost myofibrils that was clearly different from elastin and collagen based on its amino acid composition (Maruyama et al. 1976). Furthermore a thread of this protein was elastic as it could be stretched and bear force. They entitled their Nature paper: "New elastic protein from muscle" but did not give it a name. At least in part this may have been because one of the reviewers of the paper apparently suggested that the new elastic protein could be reticulin. The month that the Nature paper was accepted, Maruyama (1976) sent a preliminary communication to the Japanese journal, Journal of Biochemistry, entitled: "Connectin, an elastic protein from myofibrils". In this communication he showed that the new elastic protein had a different amino acid composition than reticulin. Thus he felt confident that he had discovered a genuine new protein and thus gave it a name. He proposed to call the new intracellular elastic protein "connectin". These results were published in full (Maruyama et al. 1977[1]). They knew

[1] Sumiko Kimura, department of biology of Chiba University, collaborated with Koscak Maruyama on virtually all of his publications on connectin over a period of nearly three decades. Her publications span more than 40 years.

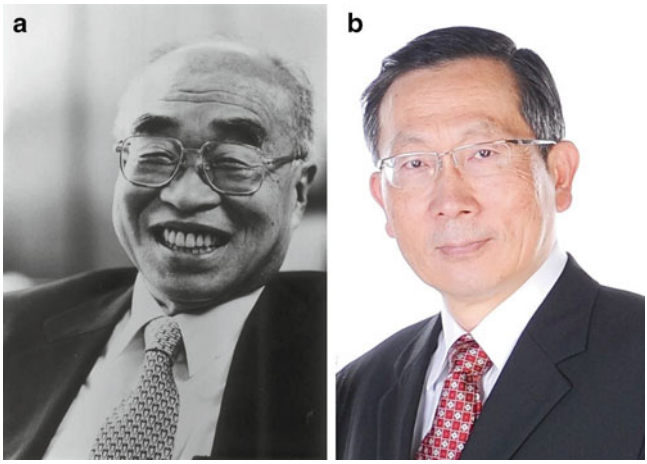

Fig. 7.2 (**a**) Koscak Maruyama (1930–2003) received a Ph.D. from Tokyo University in 1958 and was a postdoctoral fellow in John Gergely's laboratory in Boston from 1959 to 1961. He held faculty positions at the University of Tokyo, Kyoto University and was the head of the biology department at Chiba University. He discovered the protein connectin (now more generally known as titin) and was the first to recognize the elasticity of this protein. He also discovered β-actinin (now called CapZ) and contributed to the characterization of α-actinin that was discovered by Setsuro Ebashi. He had an avid interest in the history of biochemistry and wrote numerous articles on the topic. An able administrator, Maruyama served as the president of Chiba University from 1994 to 1998. For further details, see Gergely and Kimura (2005). Photo: Gergely and Kimura (2005). With permission Springer. (**b**) Kuan Wang received his Ph.D. from Yale University in 1974 and did postdoctoral training with Seymour J. Singer at the University of California at San Diego in 1975. Along with J. Frederick Ash and Singer, Wang isolated and named the protein filamin from smooth muscle. He isolated and named the proteins titin (also called connectin), nebulin and nebulette. Wang was a professor of chemistry and biochemistry at the University of Texas from 1977 to 1997 before becoming the laboratory chief of muscle biology at NIAMS, NIH until 2010. He then became the Director of the Nanomedicine Program and Distinguished Research Fellow of the Institute of Biological Chemistry and Institute of Physics, Academica Sinica, Taiwan. Photo: courtesy K. Wang

that connectin was a large protein but they thought that its size was due to covalent connections among smaller subunits. Looking back, Maruyama (Maruyama and Kimura 2000) stated that they wasted more than one year because they could not believe the presence of a single giant peptide of a few million Daltons. Therefore, it was expected that there were some crosslinks such as in collagen or elastin. It turned out that the crosslink was an artifact resulting from the alkaline treatment of muscle and in fact they were dealing with a giant protein.

Kuan Wang (Fig. 7.2b) at the University of Texas at Austin was also interested in the filamentous proteins of muscle. He had established that filamin existed in smooth muscle cells (Wang et al. 1975) and wondered whether it might also be present in skeletal muscle. It turned out that filamin was not present in skeletal muscle but in the process Wang, Janela McClure and Ann Tu (1979) made a major discovery. When purified chicken and rabbit skeletal myofibrils were analyzed with high-porosity

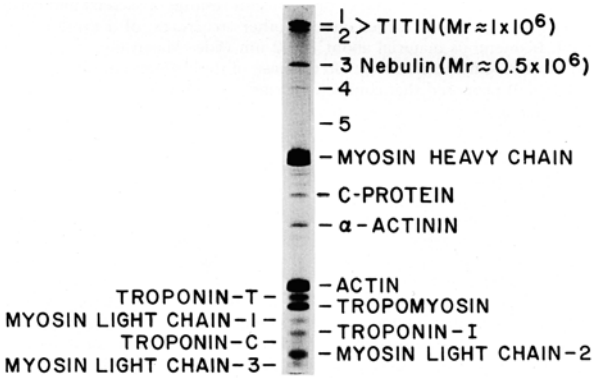

Fig. 7.3 Major myofibrillar proteins of rabbit skeletal muscle. SDS-polyacrylamide gel pattern of thiol-reduced, denatured rabbit myofibrillar proteins. A titin doublet (T1 and T2) and nebulin (originally called band 3) are major components of very low electrophoretic mobility (Wang 1985. With permission Springer)

polyacrylamide gel electrophoresis, all solubilized proteins entered the gels and there were three major bands above the myosin heavy chain band at 200 kDa: a closely spaced doublet and a single band with faster mobility (Fig. 7.3). They estimated the molecular weight of the slow moving doublet to be about 1,000 kDa[2] and the faster moving band at about 500 kDa. They tentatively named the doublet "titin" derived from Titan (Greek, a giant deity, anything of great size.). They called the 500 kDa band "band 3" but later Wang would name it "nebulin" (see below). They purified titin and determined its immunological properties. Immunofluorescent staining of glycerinated chicken breast myofibrils indicated that titin was present in M bands, Z discs, the junctions of A and I bands, and perhaps throughout the entire A bands. Thus they concluded that titin appears to be a "bona fide" structural component of myofibrils. Apparently they were unaware of Maruyama's results since they didn't cite them. Furthermore, despite Maruyama's study, they stated that previously there was no supporting biochemical evidence for a third filament. Also they did not speculate on the function of titin beyond suggesting that titin might be a candidate for the "elusive" third filament of the sarcomere. Nonetheless they discovered the largest protein known in nature. In fact it would continue to become larger as techniques for measuring molecular weights became more precise.

Maruyama naturally wondered whether connectin and titin were the same protein. He and his colleagues compared the two proteins (Maruyama et al. 1981). They found that the amino acid compositions of titin and high molecular weight connectin were almost the same. Furthermore the band 3 protein of Wang, the molecular weight of which is certainly smaller than that of connectin but much larger than that of myosin, was different in amino acid composition from connectin. They therefore concluded that titin and connectin were the same protein and that the band 3 protein (nebulin) should be regarded as an entity distinct from connectin and titin.

[2] As predicted by Wang et al. (1979), the doublet nature of titin was due to limited proteolysis of titin.

7.2.4 What's in a Name: Connectin or Titin?

To Koscak Maruyama, connectin and titin were the same protein and since his work occurred earlier it was natural that he felt that the name connectin should take precedence over the name titin. Kuan Wang did not see it that way. He felt that the original crude extract isolated by Maruyama contained titin, nebulin and other protein contaminants. He summarized his view in a comprehensive review of the subject in the following way (Wang 1985. With permission Springer):

> In 1981 Maruyama et al (1981a) purified titin from KI-extracted muscle residues and proceeded to identify connectin as titin…This redesignation has created much confusion in the literature, because titin is only one of the many components of stroma proteins. Indeed, KI residues contain titin, nebulin, myosin, actin, Z-line proteins, intermediate filament proteins, and contaminating connective tissue proteins (Wang and Ramirez-Mitchell 1983). It is unfortunate that these workers have now attributed most of the properties and structural organization of the poorly defined connectin residue to titin without undertaking a careful reevaluation of the various claims. In the following discussions, we shall adapt the original definitions and use *titin* to designate the large polypeptides near 10^6, *connectin residue* to refer to the insoluble residue, and *connectin* (*titin*) to designate titin-equivalent connectin.

This disagreement in nomenclature has not been completely resolved. Certainly in Japan the elastic protein will likely always be known as connectin in deference to Maruyama's pioneering investigations. Throughout the rest of the world, the usual designation is "titin" or sometimes "titin (also known as connectin)". Sumiko Kimura (2005), Maruyama's long-time research collaborator, avoided the controversy about names in the memorial symposium dedicated to Maruyama when she said: "This symposium commemorated the pioneering work of the late Professor Koscak Maruyama, who discovered elastic proteins in muscle." (Kimura 2005. With permission Springer) Possibly Linke and Kruger (2010) have struck the fairest compromise when in a recent review they stated: "titin, first described as connectin" and referenced Maruyama's 1977 paper and Wang's 1979 paper. Koscak Maruyama, who had an avid interest in the history of biochemistry, wrote a brief paper about a year before his death describing some "extinct names" of discovered proteins, especially the names of his first discovered proteins, β-actinin and connectin (Maruyama 2002). He stated what is, no doubt, a truism (Maruyama 2002. With permission Elsevier): "Only the creators of the extinct names really care about these losses as most scientists are not interested in the history of their fields."

7.2.5 First Comprehensive Model of Titin and Nebulin Location and Function

Based on the ultrastructural studies of Locker and Leet (1975) and his own immunocytological studies, Wang (1985) provided the first comprehensive model of titin and nebulin location and elastic function in the muscle sarcomere (Fig. 7.4). In constructing this model, he made the following predictions:

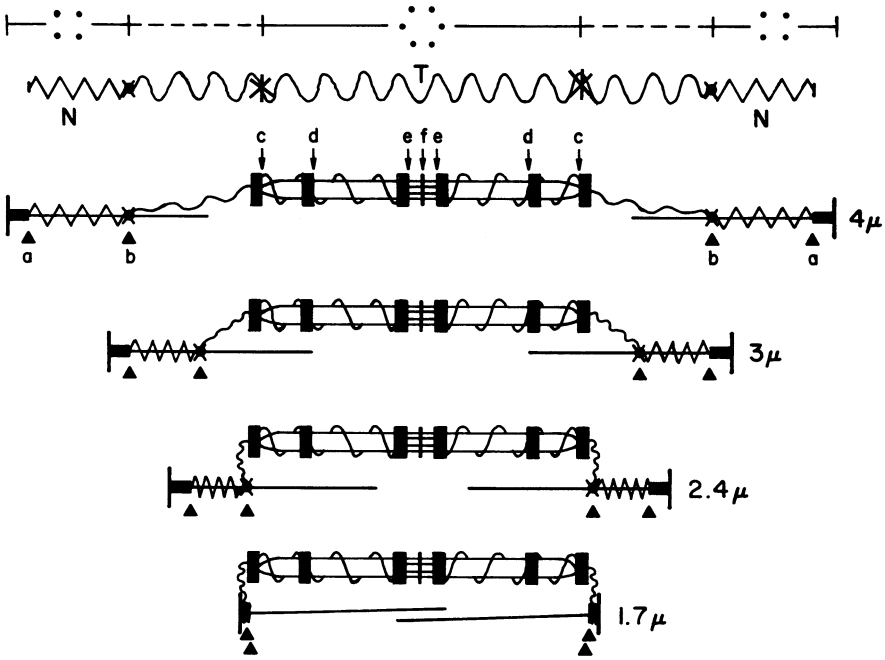

Fig. 7.4 A hypothetical three-filament sarcomere. According to this working hypothesis the third filament constitutes a three-dimensional lattice containing both longitudinal and transverse elements. The longitudinal filament is continuous from Z line to Z line and consists of two alternating filament domains, each containing either titin (T) or nebulin (N). The longitudinal filament need not surround the thick helically as depicted here. One (of many) possible mechanisms by which the third filament may respond to sarcomere length changes is shown; only the I-band domain (**a**–**c**) changes length. See text for further details (Wang 1985. With permission Springer)

1. The longitudinal filaments run parallel and external to thick and thin filaments.
2. The filaments are intrinsically elastic and/or extensible except when and where they interact with other inextensible sarcomeric structures.
3. The filaments are continuous from Z line to Z line.
4. The filaments are anchored at the Z line and M line.
5. Both titin and nebulin are components of the same filament strand.
6. Titin and nebulin are serially connected.
7. The filament is intrinsically elastic along its entire length from Z line to Z line.
8. Weaker or a different mode of interaction exists between the third filament and the thin filament since thin filaments are not displaced with stretch.
9. The filaments are linked to each other at a few spots in the I band to stabilize the sarcomeric lattices.

Wang emphasized that the important point about this model was that it was detailed and testable. Some aspects of the model did not stand the test of time but it was a very valuable guide for future investigation.

7.2.6 *Physiological Role of Titin in Muscle Fibers*

Despite the pioneering work of Maruyama and Wang and their observations that suggested that titin was the protein in the third highest concentration in myofibrils, representing about 5–8 % of the total myofibrillar protein (Maruyama et al. 1977; Wang et al. 1979), there was still great doubt about the observations. The reported gigantic molecular weight of the titin molecule was especially difficult to accept.

An important breakthrough came with the study of the function of this putative giant elastic protein in muscle fibers. Richard J. Podolsky's laboratory at the NIH had a long history of performing creative experiments on muscle. His experiments with postdoctoral fellow Robert Horowits in 1986 are some of the most striking and imaginative in the titin field. They reasoned that one way to examine titin function would be to destroy the molecule and then determine to what extent the mechanical and structural properties of a muscle fiber changed. They established a collaboration with Ellis S. Kempner, also at the NIH, who was utilizing radiation inactivation to estimate the molecular weight of various enzymes (Kempner and Haigler 1982). Since the radiation sensitivity of macromolecules was directly related to their mass, the idea was that the largest molecules would be damaged first by ionizing radiation (destruction of covalent bonds). By controlling the intensity of the irradiation it might be possible to damage titin and nebulin without appreciably destroying the other molecules in the sarcomere. This was not a trivial experiment as radiation inactivation was only effective on dried or frozen samples. They utilized chemically skinned psoas muscle fibers from rabbits (Horowits et al. 1986). They measured the maximum active force production at rest length and the passive force with stretch, then rapidly froze the fibers, irradiated them, thawed the fibers and measured the mechanical properties again. As a control some frozen fibers were not irradiated before thawing. In parallel experiments they examined the effects of irradiation of the proteins with SDS polyacrylamide gel electrophoresis. They established an irradiation dose that resulted in almost complete destruction of the titin bands with only an approximately 15 % reduction in the intensity of the myosin heavy chain, actin, and α-actinin bands. They observed an approximate 20 % decline in active force production with no change in passive force in the control fibers that were frozen and thawed. In contrast in the irradiated fibers, the active force declined but the passive force with stretched declined to a greater extent (Fig. 7.5). Thus they concluded that "Titin and/or nebulin apparently provide axial continuity for the production of resting tension on stretch…" (Horowits et al. 1986. With permission Nature Publishing Group) But what was remarkable was that with prolonged activation, the irradiated fibers exhibited misalignment of the thick filaments in the A bands as seen in the electron microscope. They stated that in some sarcomeres this led to an almost complete disappearance of the I band as individual thick filaments were actively driven toward the nearest Z disk. They envisioned that this effect was due to destruction of some of the titin filaments as shown schematically in Fig. 7.6. Thus they concluded that the findings indicated that irradiation destroyed a structure responsible for centering thick filaments within the sarcomere during both passive and active force generation. Now there was physiological evidence consistent with a role for titin in producing passive force with stretch and in centering the A band in the sarcomere at rest and during contraction.

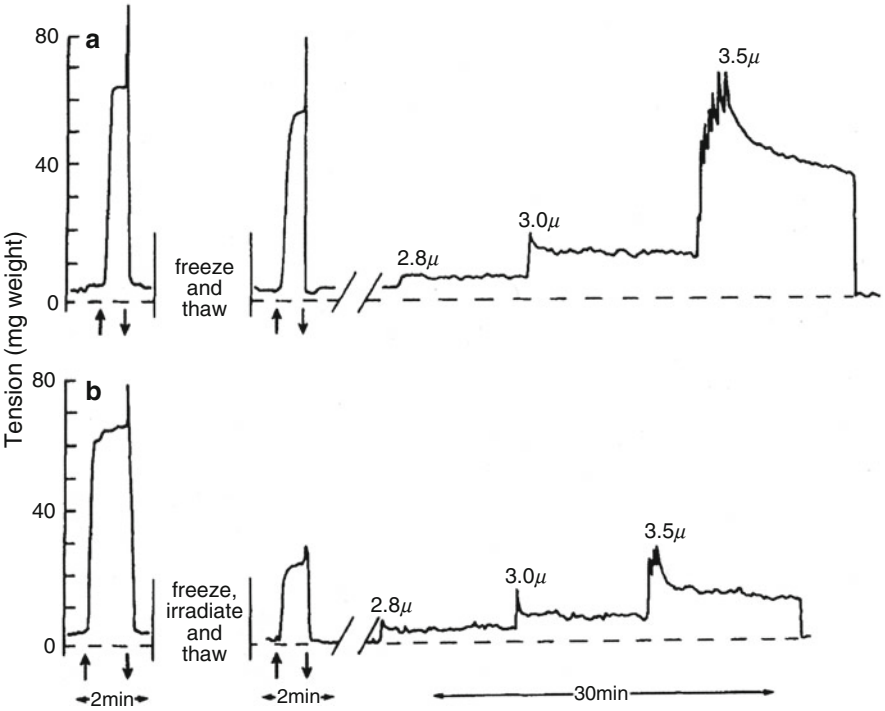

Fig. 7.5 Effects of destruction of titin on active and passive force production of single skinned muscle fibers. (**a**) Dose = 0 Mrad, (**b**) dose = 0.5 Mrad. After maximum isometric force was measured at a sarcomere length of 2.6 μm, the fiber was removed from the force measuring apparatus and frozen, irradiated and thawed. After remounting the fiber in the apparatus, maximum force was again measured at a sarcomere length of 2.6 μm. Passive force was measured by stretching the fibers to various sarcomere lengths as indicated above the force records. Freezing and thawing alone decreased active force but did not significantly affect passive force. Irradiation led to a further decrease in active force but a larger decrease in passive force with stretch (Horowits et al. 1986. With permission Nature Publishing Group)

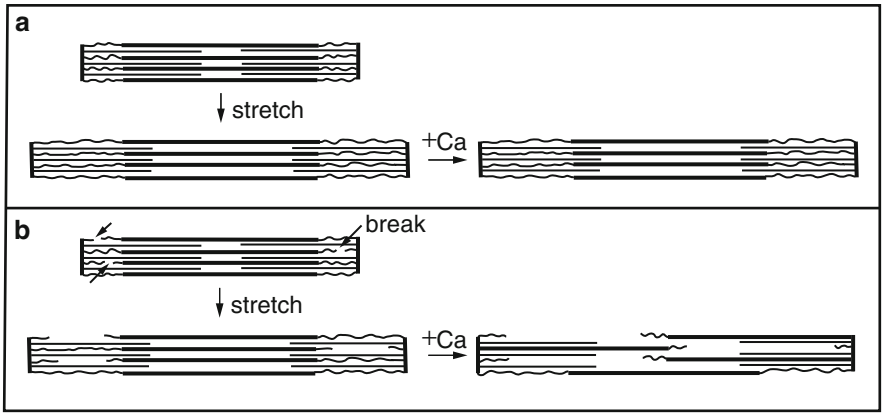

Fig. 7.6 Model of a sarcomere in which each end of the thick filament is linked to the nearest Z disc by elastic filaments made of titin and/or nebulin. (**a**) Control, (**b**) after radiation. Breakage of the elastic filaments by radiation leads to thick filament misalignment on stretch or activation (Horowits et al. 1986. With permission Nature Publishing Group)

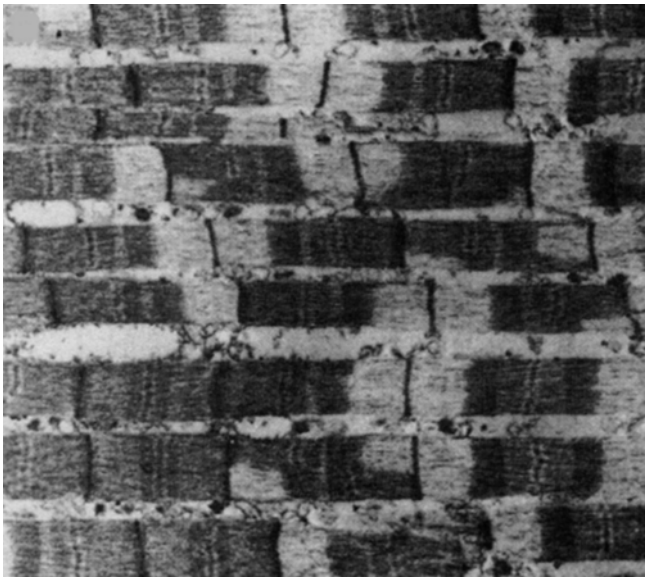

Fig. 7.7 Electron micrograph of a rabbit skinned muscle fiber that was fixed 7.5 min after the start of activation. The fiber was initially at a resting sarcomere length of 2.6 μm and then activated to produce an isometric contraction. Note the dramatic displacements of the A bands from the center of many of the sarcomeres (Horowits and Podolsky 1987. With permission Rockefeller University Press)

Horowits and Podolsky (1987) followed up this study with a more detailed electron microscopic investigation. They reasoned that if it was true that titin helped center the A band in the sarcomere that this centering function might be overcome with very long maximum activation of skinned muscle fibers at rest length. Under these conditions, skinned fibers developed a sarcomere length inhomogeneity. It was possible that a small imbalance of force production in each half of an A band might develop which would be amplified over time and would tend to pull the A band toward one end of the sarcomere. Rabbit skinned psoas fibers were activated to produce an isometric contraction for 5–7.5 min at a resting sarcomere length which would produce maximum force production. These fibers then were examined in the electron microscope. Sarcomeres were of variable length and the A bands were displaced from the center of the sarcomere even though the length of the thick and thin filaments remained constant (Fig. 7.7). This was a most dramatic result. When fibers were fixed after relaxation, the A bands had returned to the center of the sarcomeres as was observed before contraction. In contrast, if a fiber was activated for 5 min at a long sarcomere length where passive force was significant, the A bands remained in the center of the sarcomere. Horowits and Podolsky explained these results quantitatively. At longer sarcomere lengths where active force production is decreased, any imbalance in each half of the A band would not be great enough to

overcome the high resting stiffness and the A band would remain centered in the sarcomere[3]. This resting stiffness was attributed to the elasticity of the titin molecule. Thus the results of Horowits and Podolskly further supported the proposal that titin was responsible for the intracellular resting elasticity of a muscle fiber and for maintaining the A band in the center of the sarcomere upon muscle activation.

7.2.7 Sarcomeric Organization of Titin Molecules

By the mid-1980s much had been learned about the titin molecule (for a review see Ohtsuki et al. 1986). Maruyama determined that the molecular of weight of titin, initially estimated to be about 1,000 kDa by Wang, was actually closer to 3,000 kDa. As expected, the lower band in the titin doublet was a proteolytic breakdown product of the upper band. The titin molecule was isolated in its native form by the Maruyama, Wang and John Trinick laboratories in 1983 and 1984. There was general agreement that titin was the third most abundant protein in the sarcomere and constituted about 10 % of the protein content of myofibrils. In the electron microscope, individual titin molecules appeared as string-like structures up to 1 μm in length with a width of 4–5 nm. Trinick (1981) had earlier identified filaments extruding from both ends of native thick filaments and called them "end filaments" and now speculated that they were titin filaments. Trinick et al. (1984) summarized the status of the third filament models and their relationship to titin. They felt that there was not sufficient data to decide whether any of the various models of elastic filaments was correct or what the exact relationship of titin to such a system might be. What was needed was a precise location of titin in the muscle based on electron micrographs of muscle labeled with titin antibodies. They also emphasized that it would important to demonstrate elasticity in the native molecule.

Thus what clearly was needed was an agreed upon location of the titin molecule in the sarcomere. The definitive results came in a beautiful study from the laboratory of Klaus Weber at the Max Planck Institute for Biophysical Chemistry in Gottingen, Germany. Furst et al. (1988) generated a battery of ten monoclonal antibodies against the native titin molecule. The titin antibodies did not cross react with nebulin. These antibodies were distributed from the amino to the carboxyl end of the titin molecule. They then employed immunofluorescence and immunoelectron microscopy on myofibrils to determine the location of the antibodies in the sarcomere. The result was a linear epitope map for titin in the sarcomere. One antibody bound very near to the Z line, five antibodies bound to titin in the I band, one at the A/I junction and three in the A band. Each of the titin antibodies provided a pair of symmetric decoration lines per sarcomere. The position of these pairs was centrally symmetric to the M band which indicated that the titin molecules were

[3] It should be mentioned that the activation of muscle fibers continuously for 5–7.5 min would likely never occur in vivo. Furthermore it is well known that continuous activation of skinned muscle fibers will cause sarcomere length inhomogeneity.

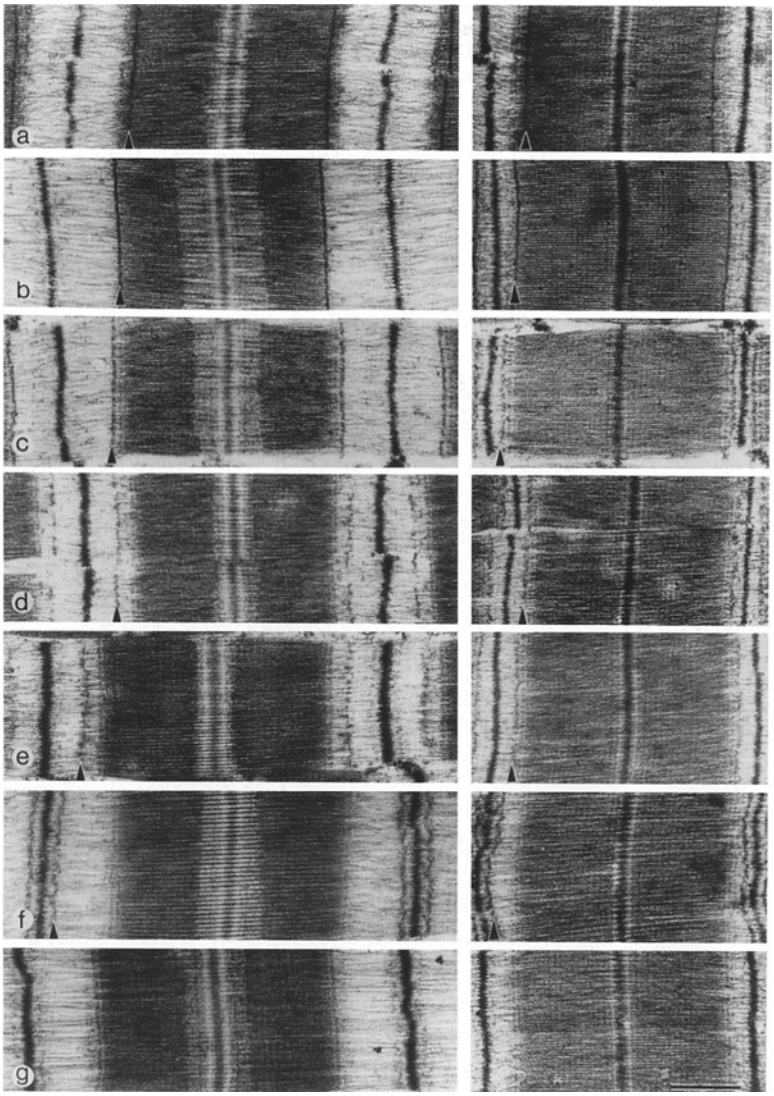

Fig. 7.8 Immunoelectron microscopical localization of titin in chicken pectoralis muscle. Results are presented in pairs. The micrographs on the left show the result on relaxed sarcomeres whereas the micrographs on the right show the result in contracted sarcomeres. Positions of antibody label are indicated by *arrowheads*. Note that each antibody gives only one specific decoration line per half-sarcomere. Six antibody locations are shown (**a**–**f**) and g shows control muscle. Only the three antibodies in the I band (**c**–**e**) closest to the A/I junction change their position upon contraction. Bar: 500 nm (Furst et al. 1988. With permission Rockefeller University Press)

polar structures reaching from the carboxyl end of the molecule at the M band to the amino end at the Z disc (Fig. 7.8). One antibody was located within 0.2 μm of the M band and at the other end of the molecule one antibody decorated the Z disc. Thus the titin molecule ran from the M band to the Z disc in the sarcomere. Since the resting sarcomere length in these experiments was about 2.7 μm, the titin molecule must be about 1.3 μm long under these conditions. They also examined how the antibody location changed when the myofibrils contracted. Only the three antibodies in the I band closest to the A/I junction changed their position upon contraction (Fig. 7.8c–e). Thus the issue of titin location was settled. Certain models of the third filament could now be rejected. A single molecule of titin did not run from Z disc to Z disc. Titin was not connected serially to nebulin but rather it connected the M band to the Z disc.

7.2.8 *Elasticity of the Titin Molecule*

The process of understanding the elasticity of titin at the myofibrillar and molecular level was greatly advanced when Labeit and Kolmerer (1995a) of the European Molecular Biology Laboratory in Heidelberg, Germany, determined the primary sequence of human cardiac titin using complementary DNA (cDNA) analysis. The results predicted a protein of nearly 27,000 residues with a molecular weight of almost 3,000 kDa. Ninety percent of the protein mass is contained in a repetitive structure composed of 100-residue repeats. These repeats encode 112 immunoglobulin (Ig)-like and 132 fibronectin-type III (FN3)-like domains. The I band region of

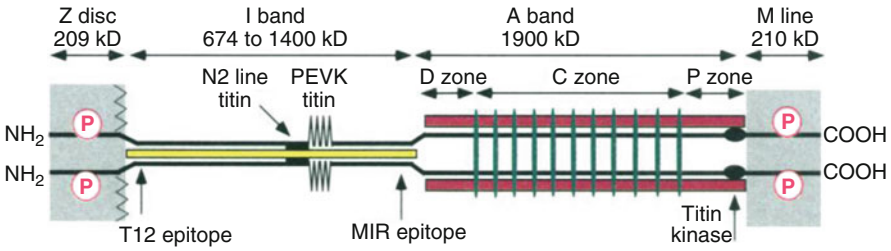

Fig. 7.9 A Model of the titin filament in the sarcomere. The titin filaments (two *thin lines*), the thin filament (actin) (extending from A band to Z disc), and the thick filament (myosin) (*thick lines*). The epitopes of the titin antibodies (T12 and MIR) have been mapped in the sarcomere by immunoelectron microscopy. Antibodies to the titin kinase domain react with the periphery of the M band. For the I band, the range of variation as predicted by the splice variants is indicated. The presumed extensible element of the I band, the PEVK element (*zig-zag* pattern) is shown. Within the thick filament, titin binds to both the C protein (*stripes*) and myosin and is likely to specify the presence of 11 copies of the 430 Å thick filament repeat in vertebrate striated muscles. Phosphorylation of the repeats in the Z disc and the M band titin (P) may control integration of the titin filament into Z discs and M bands during myogenesis (Labeit and Kolmerer 1995a. With permission American Association for the Advancement of Science)

titin is composed of three different segments: tandem Ig-like domains, nonrepetitive sequence insertions in the central region of the I band, and a complex domain architecture near the junction of the A band and the I band. In the central part of the I band, cardiac and skeletal titins branch into distinct isoforms. Alternative splicing of a single titin gene accounts for the tissue-specific variation of the I band titin structure. The four amino acids P (proline), E (glutamic acid), V (valine), and K (lysine) constitute 70 % of this element, and therefore it is referred to as the PEVK domain of titin. This domain comprises 163 or more residues in the cardiac titin sequence and 2,174 residues in the skeletal titin sequence (Fig. 7.9). They concluded that the range of isoforms (see titin isoforms below) of I band titin generated by differential splicing affects the length of the tandem Ig and the PEVK regions of titin. Furthermore the highly conserved A band region of titin could explain the location of C protein along the thick filament (see Chap. 6 for information about C protein [Myosin Binding Protein C, MyBP-C]).

They went on to speculate about the shape of the passive force-extension curve in muscle and how it might relate to the structure of the titin molecule. Labeit and Kolmerer (1995a) suggested the PEVK region might be more easily stretched than the Ig domains because the reduced complexity of the sequence together with the clusters of negative charges could prevent the formation of stable tertiary structure folds. Therefore, the PEVK domain may account for the extensibility of the titin filament at low forces. At longer lengths the stable folds of the Ig domains of I band titin would resist further extension. This would account for the rapid rise in force toward the end of the slack length of sarcomeres. The moderate levels of further extension that occur in vivo in skeletal muscles may be explained by conformational changes in the tandem Ig segments, such as bending and stretching of interdomain linkers. Thus they proposed that titin molecule acts as two springs in series. Also they suggested that the differential expression of the springs provided a molecular explanation for the diversity of sarcomere length and resting tension in vertebrate striated muscle. With this work it was now possible to begin to think about the elasticity of the titin molecule in molecular terms.

Wolfgang A. Linke, then at the University of Heidelberg, and his colleagues Marc Ivemeyer and J. Caspar Ruegg collaborated with Siegfried Labeit and Bernhard Kolmerer and their colleague Nicoletta Olivieri in an effort to locate the elastic region of the titin molecule in myofibrils (Linke et al. 1996). They characterized the elastic properties of both the tandem Ig domain and the PEVK region by a combination of immunofluorescence microscopy and single myofibril mechanics. They measured changes in passive force with stretch of single isolated myofibrils from cardiac and skeletal muscle and the stretched-induced movements of I band titin antibody epitopes that were located adjacent to the PEVK and Ig domains of titin. Since titin is expressed in different isoforms in various muscle tissues, they investigated three types of muscle expressing a long (soleus), intermediate (psoas), and short (cardiac) isoform of titin of known tandem Ig and PEVK content. They found that with myofibril stretch, I-band titin does not extend homogeneously. As an example, the results for soleus myofibrils are shown in Fig. 7.10. An antibody near the Z line (Fig. 7.10a labeled T12 and Fig. 7.10b open circles) hardly moved at

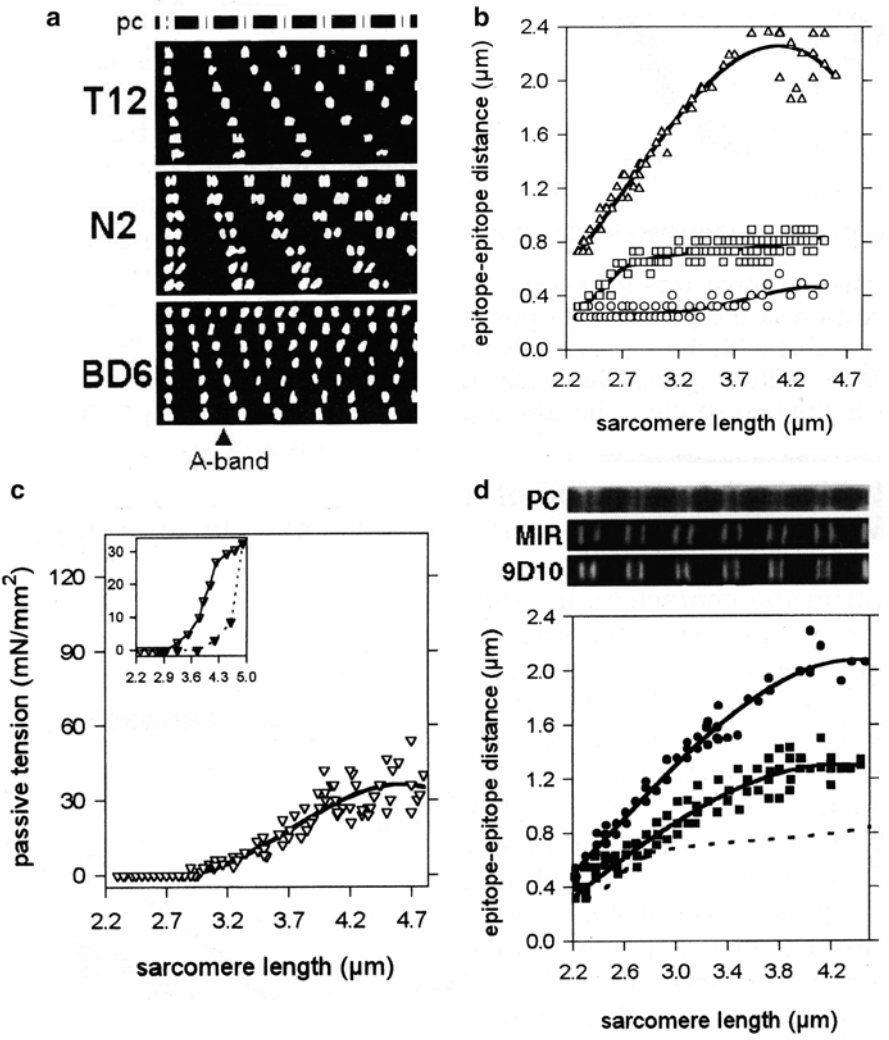

Fig. 7.10 Results of immunofluorescence measurements of titin antibody movement and passive force with stretch of soleus myofibrils. (**a**) Fluorescent antibodies labeled myofibrils near the Z disc (antibody T12), in the Ig region of titin adjacent to the PEVK region (N2 antibody), or in the A band (BD6 antibody). Myofibrils were stretched from sarcomere lengths of 2.4 to ~4.4 μm from *top* to *bottom* and the separation distance of antibody pairs measured across the Z disc. For reference a phase contrast (PC) image is shown at the *top*. (**b**) Summary of antibody movement with stretch. Note that T12 (*open squares*) hardly moves with stretch (no separation of antibodies), N2 (*open squares*) moves only at moderate sarcomere lengths and BD6 (*open triangles*) moves linearly with sarcomere length to the strain limit of titin. (**c**) Passive force versus sarcomere length. (**d**) Summary of antibody movement with stretch for 9D10 in the PEVK region (*filled squares*) and MIR near the A/I junction (*filled circles*) (*dashed line* represents N2 movement). At the *top* of (**d**), phase contrast image and fluorescence images of MIR and 9D10. See text for further explanation (Linke et al. 1996. With permission Elsevier)

all with stretch. Another antibody in the A band (Fig. 7.10a labeled BD6 and Fig. 7.10b open triangles) moved in parallel with the increasing sarcomere length. An antibody that labeled a region adjacent to the Ig domain in the I band (Fig. 7.10a labeled N2 and Fig. 7.10b open squares) initially moved linearly with stretch and then did not move at all with further stretch. This lengthening did not result in measurable passive tension (Fig. 7.10c) and was attributed to straightening, rather than unfolding, of the Ig repeats. An antibody in the PEVK domain region (Fig. 7.10d, 9D10, filled squares) moved linearly with stretch. An antibody near the A/I junction (MIR) also moved linearly with stretch but remained stationary relative to the M band (Fig. 7.10d filled circles). At moderate to extreme stretch, the main extensible region of titin was the PEVK segment whose unraveling was correlated with a steady increase in passive tension (Fig. 7.10c). Thus they concluded that the PEVK domain transition from a linearly extended to a folded state appears to be principally responsible for the elasticity of muscle fibers. Also, the length of the PEVK sequence may determine the tissue-specificity of muscle stiffness, whereas the expression of different Ig domain motif lengths may set the characteristic slack sarcomere length of a muscle type. (See more about titin isoforms below.)

A further step toward understanding the molecular nature of the elasticity of titin was taken in spectacular experiments by three groups who measured the length-force relationship of individual titin molecules or titin segments. The papers all appeared in May of 1997. Larissa Tskhovrebova and John Trinick at Bristol University collaborated with John A. Sleep and Robert M. Simmons at King's College London to measure the elasticity of and unfolding of single titin molecules using the optical trap or optical tweezer technique (see Chap. 9 for more information about optical tweezers) (Tskhovrebova et al. 1997). In this technique a titin molecule is tethered between a glass surface and a polystyrene bead (1.1 μm), using antibodies (covalently coupled to the beads) directed against epitopes located near the ends of the molecule. A bead was trapped using optical tweezers and the molecule was stretched by moving a microscope stage horizontally (Fig. 7.11). They measured the force from the displacement of the bead from the trap center. Miklos S. Z. Kellermayer, Henk L. Granzier at Washington State University collaborated with Steven B. Smith and Carlos Bustamante at the University of Oregon to also use the optical tweezer technique to measure the elastic properties of single titin molecules (Kellermayer et al. 1997). In the final study Mathias Rief, Filipp Oesterhelt, Hermann E. Gaub at the Lehrstuhl fur Angewandte Physik, Muchen, Germany collaborated with Mathias Gautel at the European Molecular Biology Laboratory, Heidelberg, Germany and with Julio M. Fernandez at the Mayo Clinic in Rochester, Minnesota (Rief et al. 1997). They utilized an atomic force microscope[4] (AFM) to

[4] The atomic force microscope (AFM) originated from the principles of scanning tunneling microscopy that were developed by Gerd Binning and Heinrich Rohrer at the IBM Zurich research laboratory. The scanning tunneling microscope (STM) was developed to reveal the structure of surfaces at the atomic level. Binning and Rohrer along with Ernst Ruska, the inventor of the electron microscope, shared the Nobel Prize in physics in 1986. Calvin F. Quate, Christoph Gerber and Binning (Binning et al. 1986) reported the invention of the atomic force microscope in 1986. The AFM can be operated in numerous modes. In the force measuring mode, a sample is attached to the cantilever tip of the microscope and the measured deflections result from the extension of the sample. The

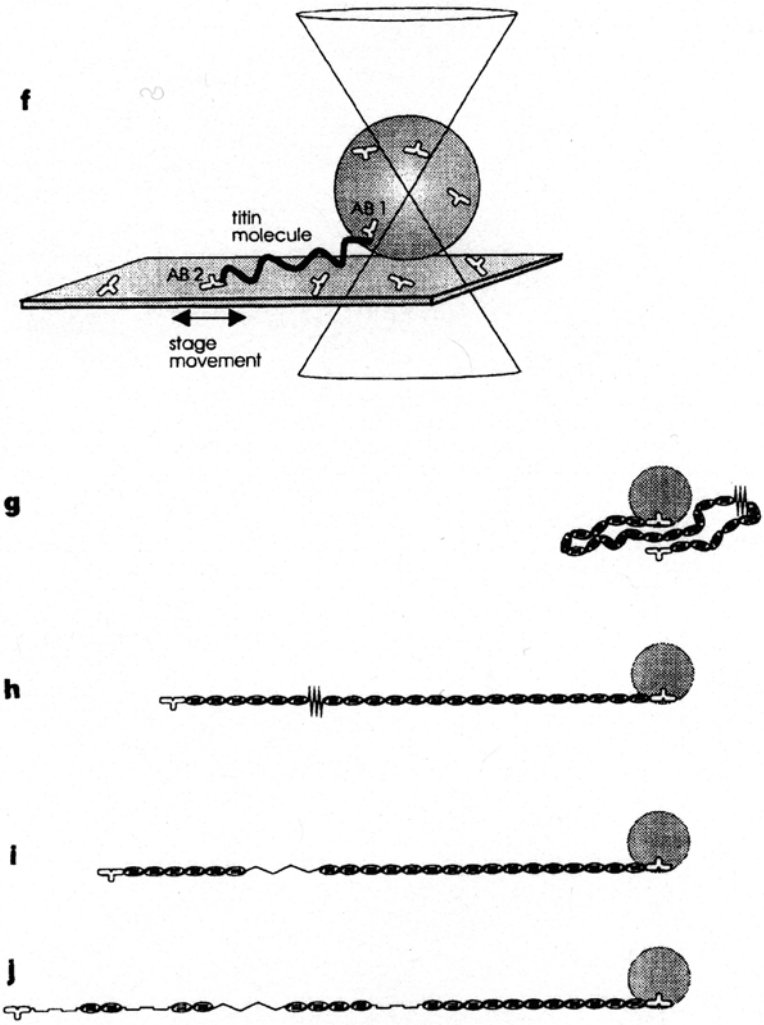

Fig. 7.11 Measurement of the force-extension relationship for a single titin molecule using an optical trap technique. Experimental procedure and mechanisms of elasticity as a single titin molecule is stretched. Extension of the molecule was obtained from the stage movement; force was obtained from the bead movement (Tskhovrebova et al. 1997. With permission Nature Publishing Group)

measure the elasticity of recombinant titin immunoglobulin segments. There was general agreement among these studies that striking elasticity of the titin molecule

AFM is capable of effecting displacements on an Angstrom scale and measuring forces of a few piconewtons. It was first utilized to measure elasticity at the molecular level with the extension of single dextran molecules in 1997. See the review by Fisher et al. (1999) for a discussion of various applications of the AFM for the measurement of the micro-mechanics of single molecules.

was due to two sources: one deriving from the straightening of the molecule and the other from extension of the PEVK region of titin (see Fig. 7.11). Kellermayer et al. (1997) furthermore concluded that scaling the molecular data up to sarcomeric dimensions reproduced many features of the passive force versus extension curve of muscle fibers. Thus by the turn of the century there was a reasonable molecular understanding of the source of titin elasticity and its role in the passive elasticity of the myofibril.

7.2.9 Titin and the Molecular Ruler Hypothesis

Whiting et al. (1989) addressed the question: "Does titin regulate the length of muscle thick filaments?" It had been a puzzle for a long time as to how the thick filaments were precisely regulated to a length of 1.6 μm containing 294 myosin molecules. They suggested that titin molecules provided a mechanism by which the length of thick filaments could be regulated accurately. They described their reasoning in the following way (Whiting et al. 1989. With permission Elsevier):

> If titin molecules are 1 μm or more long and are bound to the outside of the thick filaments, it is not difficult to envisage how, in principle, they could act to determine the length of the filament. In general terms, the simplest way accurately to regulate the length of an extended structure consisting of many similar subunits is by some form of template spanning the entire structure.

Thus among titin's other properties, they envisioned titin as a template or ruler to determine thick filament length. Tskhovrebova and Trinick (2012) have revisited the molecular ruler hypothesis for titin in a brief commentary. They emphasize the intrinsic attractiveness of the hypothesis and also state that titin is one of the first proteins expressed in myofibrillogenesis and thus is likely to form a scaffold before thick filament assembly. But they also point out the nagging problem that insect flight muscle which exhibits a precise thick filament length does not contain titin. Also some gene targeting experiments seem to argue against the molecular ruler hypothesis (see Kontrogianni-Konstantopoulos et al. 2009). Thus the hypothesis is attractive but the evidence is at best inconclusive.

7.2.10 Number of Titin Molecules per Half Thick Filament and the Sarcomere Symmetry Paradox

By the beginning of the twenty-first century, it was agreed that titin molecules constituted the third filament in the sarcomere and that titin was the third most abundant protein in the myofibril after myosin and actin. Therefore it was natural to ask: how many titin molecules were there in a titin filament? Or put another way: how many titin molecules were there per half thick filament? John Trinick and his colleagues summarized the published data on this point in 2001 (Liversage et al. 2001).

The number of titin molecules per half thick filament was very variable and ranged from 3 to 13. The variability seemed most likely to be due to variation in titin extraction and quantification from gel analysis of the amount of titin. They applied an entirely independent technique. Previously Trinick (1981) had described what he called "end filaments" protruding from each end of isolated native thick filaments. These end filaments were shown to be made of titin molecules. Liversage et al. (2001) utilized scanning transmission electron microscopy (STEM) to measure the mass per unit length of the end filaments and compared this value to the expected value for a single titin molecule. Their data was consistent with six titin molecules in an end filament and thus six titin molecules per half thick filament.

It turned out that this number led to a conceptual problem with regard to thick and thin filament symmetry, a problem that they called the "sarcomere symmetry paradox". The thick filaments exhibit three-fold symmetry (see Fig. 6.5). In order to maintain that symmetry, three titin molecules or a multiple of three would have to bind to each thick filament. Six titin molecules thus maintain the thick filament symmetry. At the Z disc titin binds to α-actinin but also at the edge of the Z disc to the thin filament (see description of the Z disc structure below). The thin filament has an approximate twofold symmetry (see Fig. 6.10) and there are twice as many thin filaments as thick filaments in the sarcomere. In order to maintain the thin filament symmetry, two titin molecules or a multiple of two would need to bind to the thin filament at the Z disc. In order to satisfy the thin filament symmetry, of the six titin molecules emanating from a half thick filament, two could bind to each of two thin filaments. This result would leave two extra titin molecules and thus a symmetry mismatch or a paradox seemed to exist. They suggested a model where the remaining two titin molecules went through the Z disc and bound to a thin filament in the adjacent sarcomere. This is an issue that remains unresolved. In conclusion, more than one titin molecule constitutes a titin filament, the likely number of titin molecules in a titin filament is six. The arrangement of these molecules in relationship to the thick and thin filaments is speculative and still a matter for further research.

7.2.11 Titin Isoforms and the Passive Mechanical Properties of Muscle

By the early 1990s it was recognized that there were multiple titin isoforms (Wang et al. 1991). The isoform nature of the titin molecule was firmly established by the cDNA analysis by Labeit and Kolmerer (1995a). Through alternative splicing of a single titin gene, isoforms of titin are generated[5]. These isoforms vary in the length

[5] The complete sequence of the human titin gene could encode a total of 38,138 residues (molecular weight of about 4.2 MDa) and differential expression gives rise to a spectrum of titin isoforms. About a third of titin's 363 exons are differentially spliced. Isoforms currently known differ in size from ~600 kDa to 3.7 MDa corresponding to molecular lengths of ~0.2 to 1.4 μm (Bang et al. 2001).

	Slack length	First Detectable Passive Force	Strain Limit
Soleus myofibrils:	**~2.25 μm**	**~2.9 μm**	**~4.2 μm**
Psoas myofibrils:	**~2.1 μm**	**~2.6 μm**	**~3.6 μm**
Cardiac myofibrils:	**~1.85 μm**	**~2.1 μm**	**~3.0 μm**

Fig. 7.12 Correlation of titin molecule length in soleus, psoas and cardiac muscle with muscle slack length, first detectable passive force and strain limit. Titin isoforms vary in molecular weight and thus length in the order of soleus > psoas > cardiac. The longer the titin molecule, the greater the muscle slack length, first detectable passive force length and strain limit length (Data from Linke et al. 1996)

and amino acid composition of their I band regions. The titin isoforms vary from the shortest (lowest molecular weight) to the longest (highest molecular weight) in the order: cardiac < psoas < soleus muscle. Three predictions arise from these facts: (1) the equilibrium resting sarcomere length of a myofibril, (2) the sarcomere length where passive force is first detectable and (3) the sarcomere length at which the strain limit is reached when a resting myofibril is stretched should all be in the order: cardiac < psoas < soleus muscle. Results describing the passive force versus extension relationships in isolated myofibrils follow this predicted pattern (Fig. 7.12) (Linke et al. 1996). These data are in general agreement with the earlier results of Kuan Wang, Roger McCarter and their colleagues (Wang et al. 1991) with skinned muscle fibers. This analysis of isolated myofibrils, of course, ignores the elasticity of the sarcolemma and extracellular connective tissue which can vary considerably among muscle types and contribute significantly to passive force with stretch. Nonetheless the isoforms of titin are an important component of the resting elasticity of a muscle.

7.2.12 Beyond the "Classical" Functions of the Titin molecule: Titin as a Mechanosensor and Integrator of Myocyte Signaling Pathways

It was generally agreed that the titin molecule connecting the thick filament to the Z disc functions as a molecular spring responsible for much of the passive elasticity of the sarcomere and also functions to maintain the A band in center of the sarcomere. Furthermore the portion of the titin molecule in the A band might act as a scaffold for controlling thick filament length. But there were indications in the 1990s that titin might exhibit more than just these "classical" properties. Titin contains a kinase domain near the carboxyl terminus, the M band end of the molecule, and both ends of the molecule also contain potential phosphorylation sites. These results suggested involvement in signaling mechanisms for both the

M band and Z disc portions of the titin molecule. The possible roles of titin as a mechanosensor and regulator of various signaling pathways has become a rapidly growing field of study. Kontrogianni-Konstantopoulos et al. (2009) have concluded that current evidence suggests that there is a complex of signaling molecules associated with titin that links its kinase activity with sarcomere assembly and maintenance, stress-sensing mechanisms, transcriptional regulation, protein turnover, and hypertrophic responses. For another contemporary review, see Linke and Kruger (2010).

What started out as a search for the source of passive sarcomeric elasticity has evolved, and continues to evolve rapidly, into the study of major regulatory mechanisms for cell growth and maintenance in health and disease. Much more will no doubt be learned about the multi-faceted roles of titin in the near future.

7.3 A Fourth Sarcomeric Filament: Nebulin, Structure and Function

In their classic study, Wang et al. (1979) not only identified the titin doublet on a gel but also observed another high molecular weight band with an approximate molecular weight of 500 kDa which they designated simply as "band 3". Band 3 was an abundant protein which constituted about 3–5 % of the total myofibrillar protein. This protein quickly was shown to be a protein different than titin (Wang and Williamson 1980). The protein seemed to be associated with the N_2 line in the I band which was sometimes seen in electron micrographs of striated muscle (Wang and Williamson 1980). Since the N_2 line seemed to be a "nebulous structure" and since band 3 was associated with it, Wang (1981, 1985) renamed band 3 as nebulin.

Originally Wang (1985) proposed (Fig. 7.4) that nebulin existed in the I band and connected the end of the titin filament to the Z disc. Furthermore he suggested that it was an elastic protein based on the observation by Sally Page (1968) that the N_2 line moved away from the Z disc when a resting muscle was stretched and their similar observations of movement of an antibody bound to nebulin. But later Wang and Wright (1988) showed that multiple antibodies bound to nebulin were located in the I band and did not change their position with respect to the Z disc when rabbit muscle fibers that were split to allow antibody access were stretched[6]. Thus nebulin was not involved in the passive elasticity of a muscle. They concluded that the nebulin molecule appeared to extend the entire length of a thin filament. Thus Wang revised his thinking and now suggested (Wang and Wright 1988) that "nebulin and titin might act as organizing templates and length-determining factors for actin and myosin filaments respectively".

[6] The movement of the N_2 line with stretch of a resting muscle as observed by Page (1968) suggests that the N_2 line is likely associated with titin and not nebulin as Wang originally suggested. Thus the name nebulin was derived from the incorrect assumption that the protein was associated with the N_2 line.

7.3.1 Nebulin as a Molecular Ruler

Siegfried Labeit and John Trinick and their colleagues (Labeit et al. 1991) were thinking along the same lines as Wang and Wright and provided evidence that was consistent with nebulin acting as a "protein-ruler". Based on the partial amino acid sequence of nebulin, derived from human cDNA clones, they found repeating motifs of about 35 residues and super repeats of 7 × 35 residues of a length of 38.5 nm. The repeat motifs were consistent with the suggestion that nebulin is associated with the actin filament since there is one molecule each of tropomyosin and troponin for every seven actin subunits, or 38.5 nm, in the filament. They also speculated that nebulin is likely to interact with tropomyosin and troponin. They went on to suggest that the amount of nebulin in a muscle is consistent with two or four nebulin molecules per thin filament with two being the most likely number. Also they pointed out that the predicted α-helical nebulin strands with a width of about 1 nm would be below the resolution of the electron microscope and thus would not be observed in three dimensional reconstruction of native thin filaments. Finally they showed data that indicated that the molecular weight of nebulin was positively correlated with the variable length of the thin filaments observed in muscles from different animals. The data was consistent with the molecular ruler hypothesis but some of the information was preliminary and discussion speculative.

During the 1990s data was accumulating that was consistent with this attractive hypothesis. Biochemical and structural characterization of the nebulin molecule was not possible because it could not be purified in the native state. Despite this limitation, short partial sequences of nebulin containing the 35 amino acid repeat motifs, derived from human cDNA clones, were examined. Jin and Wang (1991) showed that these nebulin fragments bound to actin with a high affinity under physiological conditions. Nebulin fragments were shown to be α-helical structures and a model was proposed for the structure of the thin filament nebulin complex (Pfuhl et al. 1994). In this model two nebulin molecules occupied symmetrical positions along the central cleft of the actin filament bridging the two strands of the actin helix. Kruger et al. (1991) also found a positive correlation between the molecular weight of nebulin as determined from gels (which varied from 10 to 30 %) and the thin filament lengths in different muscles from chickens, rabbits, snakes and humans (where the thin filament lengths ranged from 1.04 to 1.3 μm). Wright et al. (1993), using monoclonal antibodies, provided further evidence that nebulin extended along the entire length of the thin filament with its C terminal anchored within the Z disc and its N terminal extending out toward the center of the sarcomere. Labeit and Kolmerer (1995b) succeeded in isolating the full-length cDNA encoding human nebulin and determined its sequence. The cDNA predicted a peptide of molecular weight of 773 kDa (a 6,669 residue peptide) with 97 % of its mass consisting of 185 copies of a ~35-residue module. These results confirmed and extended earlier observations from nebulin fragments to the whole molecule. The central 154 copies (with a total length of ~ 0.8 μm) are grouped into 22 seven-module super repeats corresponding to 38.5 nm thin filament repeats. In the thin filament "ruler region", multiple isoforms are generated by alternative exon usage. They concluded that different

types of nebulin molecular rulers were expressed in different types of skeletal muscles by differential splicing. This evidence combined with later data that showed that nebulin bound to the actin filament capping proteins tropomodulin (McElhinny et al. 2001) and CapZ (Pappas et al. 2008) made the molecular ruler hypothesis even more compelling. Capping proteins effectively inhibit actin polymerization and depolymerization at the filament ends and are essential for proper thin filament assembly and length regulation. Thus, nebulin could specify the length of the thin filament by binding a specific number of actin and tropomyosin/troponin molecules and then recruiting capping proteins, which would restrict assembly to that defined length. The hypothesis has great appeal. Nonetheless the evidence, though consistent with the molecular ruler hypothesis, was not conclusive.

Pappas et al. (2010) at the University of Arizona put the molecular ruler hypothesis to a critical test in living cells, i.e., chick skeletal myocytes in culture. They designed and constructed a shorter version of human nebulin, mini-nebulin (missing 18 of the 22 super repeats and about 25 % the length of nebulin) and introduced it into the myocytes where it replaced the endogenous nebulin. The cells then were analyzed by immunofluorescence microscopy. The idea was simple. If the "strict" molecular ruler hypothesis held, then a shorter nebulin should result in shorter and possibly more variable thin filament lengths. What they found was inconsistent with the strict molecular ruler hypothesis. The thin filaments extended beyond the end of the mini-nebulin, an observation which is inconsistent with a strict ruler function. However, under conditions that promote actin filament depolymerization, filaments associated with mini-nebulin were remarkably maintained at lengths either matching or longer than mini-nebulin. This result indicates that mini-nebulin is able to stabilize portions of the filament that it does not contact. They concluded that nebulin regulated thin filament architecture by a mechanism that included stabilizing the filaments and preventing actin depolymerization. These results suggest that the action of nebulin is more complex than acting as a simple ruler. It may best be thought of as a thin filament stabilizer. The work in this area is ongoing. For a recent review see Pappas et al. (2011).

Another reason to question the molecular ruler hypothesis is that cardiac muscle does not seem to express full length nebulin. Rather a shorter version called "nebulette" by Moneman and Wang (1995) is found in cardiac muscle. Nebulette (molecular weight 107 kDa) is about one sixth the size of nebulin and it is difficult to imagine how it could function to determine the length of cardiac thin filaments that are much longer than itself.

7.3.2 Beyond the Molecular Ruler and/or Actin Stabilizer: Possible Roles of Nebulin in Muscle Contraction

One of the intriguing issues relating to nebulin function involves the expected close proximity of nebulin to tropomyosin and troponin in the thin filament and thus the possibility that nebulin may be involved in regulating muscle contraction. With the introduction of knockout mouse models deficient in nebulin, it became possible to

examine the contractile properties of muscle fibers with and without nebulin. It was found that muscle fibers deficient in nebulin developed less force due to reduced myofilament Ca^{2+} sensitivity and altered cross-bridge kinetics. Also the fibers exhibited a higher tension cost, i.e., utilized more ATP to maintain a given force for a given period of time. These novel functions suggested that nebulin might have evolved to enable vertebrate skeletal muscles to develop high levels of force efficiently. Clearly more interesting results will be forthcoming in this rapidly evolving area. See a recent review by Labeit et al. (2011) for further details.

7.3.3 Nebulin and Human Disease

Mutations in nebulin are the leading cause of the human muscle disorder nemaline myopathy. Individuals with nemaline myopathy exhibit muscle weakness, which can be severe, leading to neonatal lethality. Rod-like "nemaline" bodies, composed of Z-disc and thin filament proteins, are prevalent in the muscle fibers of nemaline myopathy patients. To date, more than 60 separate mutations have been identified in nebulin that result in nemaline myopathy. Granzier and his colleagues at the University of Arizona recently have directly linked a nebulin mutation that results in nemaline myopathy to improper assembly of the thin filament and impaired contractility. This mutation results in decreased nebulin levels, which in turn leads to decreased force production because of shorter thin filaments, less thick and thin filament overlap and thus reduced cross-bridge attachment (see Pappas et al. 2011, for a review).

7.4 Sarcomeric Organization: M Band and Z Disc Structure and Function

The regular arrangement of thin and thick filaments within the sarcomere is not simply the result of the self-assembly properties of actin and myosin alone but rather involves specific interactions with a sarcomeric cytoskeletal lattice. The most obvious structures in this context are the M bands and Z disks which are involved in packing thick and thin filaments, respectively. The component that integrates both compartments is the giant protein titin (see above).

7.4.1 M Band Structure

In the middle of the A band in the sarcomere is a structure called the M band or M line. It was observed using light microscopy at least 100 years ago. In a review in 1933, H. E. Jordan called it the "the M membrane or mesophragma", suggesting that it was a septum or partial diaphragm. Today the usual understanding is that the "M" derives from the German "Mittelscheibe". Jordan noted that the M band was

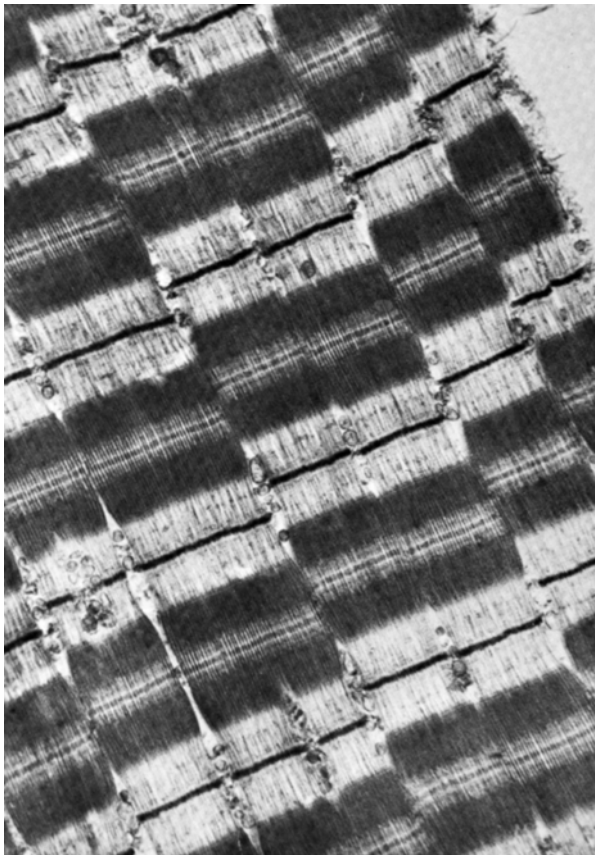

Fig. 7.13 Electron micrograph of a longitudinal section from rabbit psoas muscle. The M band is flanked on either side by a narrow zone of lower density than the rest of the H zone called the pseudo-H zone or M region. It is the area where there are no cross-bridges. Magnification: ×13,700 (Huxley 1972. With permission Elsevier)

located in the middle of a lighter staining region in the A band called the "median disc of Hensen" or H disc[7]. Today the usual designation is H zone. The H zone is that region of the A band where there are no thin filaments (Huxley and Hanson 1954). The M band was clearly seen as a dense band in the H zone in early electron microscopic studies of muscle (Hall et al. 1946; see Figs. 1.5 and 1.6). They noted that in frayed myofibrils the filaments separated laterally but adhered to one another in the region of Z and also M. Hugh Huxley (1972) showed that the M band was flanked on either side by a narrow zone of lower density than the rest of the H zone (Fig. 7.13). These lighter staining bands together with the M band have been referred

[7] With regard to the designation of the H zone after the nineteenth century microscopist V. Hensen, Andrew F. Huxley, who did a thorough investigation of nineteenth century microscopy related to muscle, has implied that Hensen in 1869 was likely looking not at the M band when he described lighter regions on either side but rather at the Z disc. Engelmann (1873) correctly described that lighter region in the middle of the A band and according to Huxley "generously referred to Hensen in this connexion". Thus apparently Hansen's name is associated with a banding region of the sarcomere that he did not discover at all (Huxley 1977, footnote page 35).

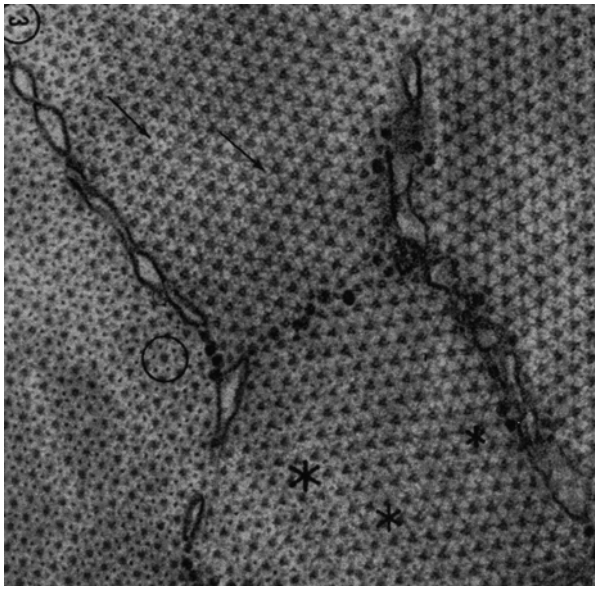

Fig. 7.14 Electron micrograph of transversely oriented bridges connecting thick filaments in the M band of fish skeletal muscle. The bridges are clearly seen in the right and upper portions of the micrograph. Magnification: ×90,000 (Franzini-Armstrong and Porter 1964. With permission Rockefeller University Press)

to as the pseudo-H zone or more recently the M region (Sjostrom and Squire 1977). The pseudo-H zone or M region is that area of the A band where there are no cross-bridges (Huxley 1965).

Details of the M band structure began to emerge after the introduction of glutaraldehyde fixation into electron microscopy which led to much better preservation of structural detail (Sabatini et al. 1963). In transverse sections through the M band, Franzini-Armstrong and Porter (1964) (Fig. 7.14), Page (1965) and Huxley (1965) all noted transversely oriented structures that connected adjacent thick filaments together. At a muscle meeting in 1964, Hugh Huxley was asked about the cross-connections between thick filaments that he observed in the electron micrographs of the muscle sarcomere. Huxley (1965) responded that they only existed in the H zone and that there were three sets of them about 200 Å apart. Knappeis and Carlsen (1968) at the University of Copenhagen performed an influential electron microscopic study of the M band in frog skeletal muscle. In longitudinal sections of the muscle, they found in the M band three, sometimes four or five, dense lines about 200 Å apart that ran transversely across the myofibril. In cross sections through the M band, they observed structures that connected each thick filament with its six neighboring thick filaments. These structures were called M bridges. The M bridges were longer than the cross-bridges observed by Hugh Huxley in 1957 (see Fig. 3.5). They concluded that the dense lines observed in the longitudinal sections of the M band were due to the M bridges. Also they observed filaments oriented parallel to the thick filaments that existed only in the M band and linked each set of M bridges together. They called these structures M filaments. Their observations were summarized in a model that formed the basis for future study

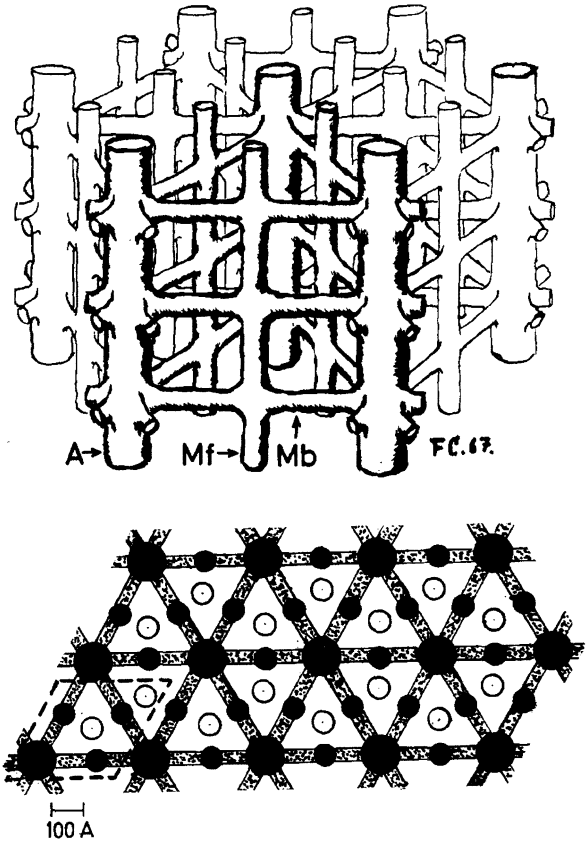

Fig. 7.15 A model of the structure of the M band in vertebrate skeletal muscle. *Top* Arrangement of M bridges (Mb) and M filaments (Mf) between the thick filaments (labeled A). *Bottom* Schematic cross-section through the M band. *Large solid circles* equal thick filaments; small solid circles equal M filaments; *open circles* equal projections of thin filaments to the level of the M region; *dotted bars* equal M bridges (Knappeis and Carlsen 1968. With permission Rockefeller University Press)

(Fig. 7.15). Knappeis and Carlsen speculated that the function of the M band was to keep thick filaments in position in the longitudinal as well as in the transverse direction. They also made the intriguing observation that an increase in the cross-sectional area of the M band must lead to a stretch of the M bridges. This suggestion was consistent with the early X-ray diffraction data that showed that the lateral spacing of the thick filaments increased with muscle shortening (Elliott et al. 1963) (see Chap. 3).

With regard to the variation from three to five in the number of transverse dense lines in the M band of frog muscle, Sjostrom and Squire (1977) observed the same variation in human muscle biopsies. The differences were related to different muscle fiber types. Densitometry scans across the M band exhibited numerous transversely oriented lines of varying intensity. They developed a nomenclature to describe these lines. Starting from the center line, labeled M1, and working outward in one direction, lines were labeled M2–M6 or outward in the other direction M2′–M6′. It was concluded that the central strongest three lines (M1, M4, M4′) represented the M bridges whereas the outer two lines (M6, M6′), in a five line fiber, were

probably non-bridging protein. In the fibers with the four line pattern (M4, M4′, M6, M6′), the central M1 bridge was missing. Since the two M bridges (M4, M4′) on either side of the central M bridge were always present, it was suggested that M4 and M4′ represented the principal cross-links that determined A band ordering. On the basis of this conclusion, Squire (1986) suggested that the central M1 bridge and M6 and M6′ non-bridging protein may not have a structural role but rather may be enzymes located to modify the physiological behavior of the fibers. In general slow twitch fibers exhibited the four line pattern, fast twitch fibers the three line pattern and intermediate fibers the five line pattern (Edman et al. 1988). (The other numbered lines in the M band were weak staining and of unknown origin.)

7.4.2 Protein Content of the M Band

Masaki and Takaiti (1974) in Setsuro Ebashi's laboratory at the University of Tokyo reported the isolation and characterization of a protein that localized to the M band of vertebrate skeletal muscle. This protein, called M-protein, had a molecular weight of 165 kDa and bound to myosin. In 1984 in Hans M. Eppenberger's laboratory at the Swiss Federal Institute of Technology in Zurich, Barbara Kay Grove and colleagues discovered a contaminant in the M-protein preparation. This contaminant was a separate protein of molecular weight 185 kDa and it too located to the M band of skeletal muscle (Grove et al. 1984). They named the protein myomesin. The titin molecule was also shown to enter the M band (see above). Earlier David C. Turner and Theo Wallimann along with Eppenberger discovered another protein that localized to the M band (Turner et al. 1973). This was not a structural protein but rather the muscle specific form of the enzyme creatine kinase (creatine + ATP ↔ creatine phosphate + ADP). This discovery meant that besides a structural role, the M band had a physiological function with a strong link to the energy supply for muscle contraction.

7.4.3 Integrating M Band Proteins with M Band Structure: A Molecular Model

Attention now turned to attempting to integrate the M band proteins into a molecular model that was consistent with M band structure. Dieter O. Furst and his colleagues at the Max-Planck-Institute for Biophysical Chemistry in Gottingen, Germany made an important contribution in this regard (Obermann et al. 1996)[8].

[8] In a tribute to Klaus Weber of the Max-Planck Institute for Biophysical Chemistry, Dieter Furst has commented that Weber's support and interest in the sarcomeric cytoskeleton gave an invaluable impact to the field. (Furst et al. 1999) Weber and his wife Mary Osborn pioneered the development of immunofluorescence microscopy for the localization of molecules in cells. Also see Footnote 11 below and Chap. 6, Footnote 1.

Since the primary sequences of M-protein, myomesin and titin were known, they approached the problem of M band structure by immunoelectron microscopy using a panel of antibodies directed against defined epitopes of each protein. There were four antibodies against the portion of titin that entered the M band, six antibodies against myomesin and six antibodies against M-protein. From experiments with fast and slow twitch fibers from the rabbit psoas, they determined that there was an overlap of titin molecules in the M band where the titin molecules interacted with each other in an antiparallel fashion. Myomesin molecules were oriented parallel to the long axis of the myofibril and neighboring molecules were oriented in an antiparallel fashion. Since the antibodies directed against the amino-terminal domains of myomesin could not be distinguished from one another, this region of the molecule was thought to bend transversely toward the surface of the thick filament and bind to myosin. Also the titin and myomesin molecules exhibited extensive interaction with each other. In contrast M-protein primarily was restricted to the M1 line in the M band. Titin and myomesin were found in both slow and fast twitch fibers but M-protein was found only in the fast twitch fibers. They integrated this information with the structural information and produced the first comprehensive molecular model of the M band shown in Fig. 7.16. In this model the M-protein constitutes the M1 line. They speculated that the M filaments running parallel to thick filaments between M4 and M4′ could be myomesin molecules forming a tight complex with two titin molecules. The M4 and M4′ positions are occupied by the transversely oriented ends of the myomesin molecule.

Irina Agarkova and Jean-Claude Perriard of the Swiss Federal Institute of Technology in Zurich proposed that myomesin was of primary importance in determining the structural and functional properties of the M band (see a review by Agarkova and Perriard 2005, and the references therein). Their proposal is based on the following facts. Myomesin is present in all fiber types at the time of formation of myofibrils during myofibrillogenesis. Myomesin forms antiparallel dimmers that bind to the thick filaments by their N-terminal domains. Myomesin also binds to M band titin and together they might form the M filaments. Furthermore myomesin binds to creatine kinase at a position near to the M4 and M′ lines of the M band. Single molecule studies indicate that the myomesin molecule exhibits an elasticity that is similar to that exhibited by titin. Thus Agarkova and Perriard viewed the M band not as a rigid construction made of transverse and longitudinal rods but rather as an elastic web of fibrillar proteins that were built in the same fashion as titin but on a much smaller scale. Recent evidence is accumulating that suggests that the M band could be a component of a mechanosensitive signaling complex (for a review, see Gautel 2011). Mathias Gautel (2011) of King's College London has speculated that:

> The realization that the M-band is elastic and serves signaling functions opens the perspective that M-band strain might also translate into modulation of metabolic activity, in addition to protein turnover and transcription regulation, and thereby regulate short-term adaptation of muscle to strain.

Even though great progress is being made in elucidating the structure and function of the M band, many questions remain unanswered in this exciting area of research.

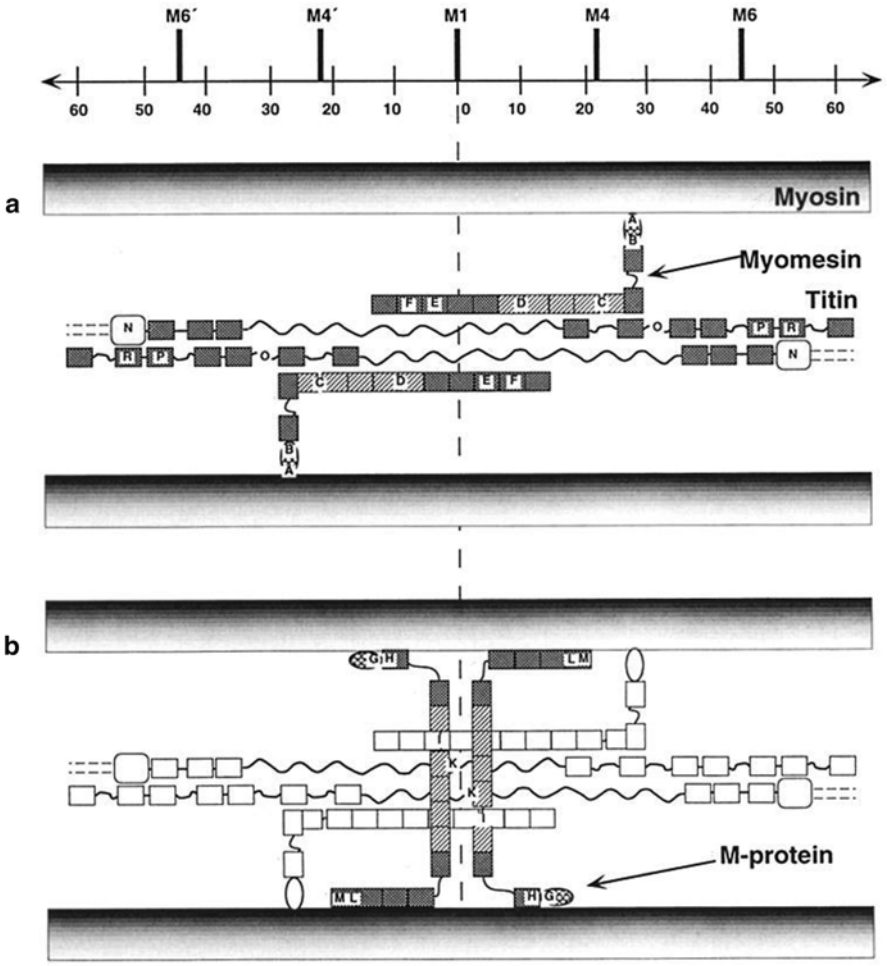

Fig. 7.16 Arrangement of titin, myomesin, and M-protein in the M band compatible with the immunoelectron microscopical results. At the *top* of the diagram, the positions of the prominent M band striations and a scale bar are indicated. Numbers give the distances in nm from the center of the A band, the M1 line. (**a**) The layout of titin and myomesin deduced from labeling data. Only two molecules of each kind are shown in order not to over burden the figure. (**b**) In addition to the structures shown in (**a**), the proposed location of M-protein around the M1 line is shown. Titin, myomesin and M-protein antibody decoration positions are indicated by *letters* (Obermann et al. 1996. With permission Rockefeller University Press)

7.4.4 Z Disc Structure

Another important component of the sarcomeric cytoskeletal lattice is the Z disc which is involved in packing of the thin filaments in the sarcomere. The Z disc or Z line (Zwischenscheibe) or Z band which bisects the I band and delineates the ends

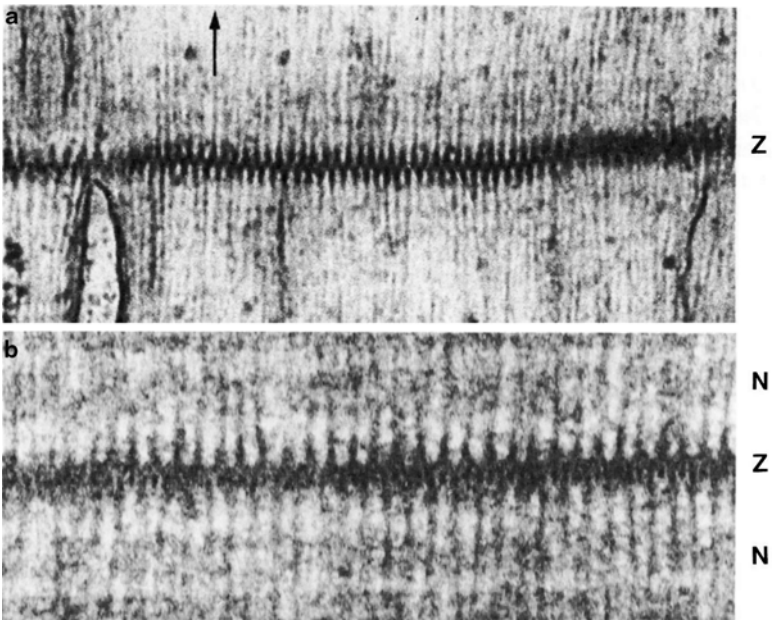

Fig. 7.17 The *zigzag* structure of the Z disc in longitudinal section. Roach white muscle fibers. Magnification: (**a**) ×133,000; (**b**) ×200,000 (Squire 1981. With permission Springer)

of a sarcomere was well known to the nineteenth century light microscopists (Huxley 1977). It also has been called Krause's membrane, Z membrane or telophragma because it was thought to be a membrane barrier between sarcomeres. It was clearly observed in the early electron microscopic studies of Hall et al. (1946) (see Fig. 1.6).

The first three dimensional model of the Z disc was developed by Knappeis and Carlsen (1962). They performed an electron microscopic study of the Z disc of frog skeletal muscle. In longitudinal sections the Z disc exhibited a zigzag line appearance (for examples from a later study, see Fig. 7.17). A thin filament from one sarcomere met the Z disc midway between two thin filaments from the adjacent sarcomere which met the same Z disc from the opposite side. The thin filaments were connected in the Z disc by structures that they called Z filaments (Fig. 7.18a, c). From cross sections through the Z disc, Knappeis and Carlsen found a tetragonal array of thin filaments from one sarcomere interdigitated with a tetragonal array of oppositely oriented thin filaments from the adjacent sarcomere (Fig. 7.18b). The arrays were displaced transversely by half a period from each other. There were extensive cross-links between the two arrays in which one thin filament was attached to four Z filaments and in turn the four Z filaments were attached to four thin filaments from the adjacent sarcomere. The pattern formed at the center of the Z disc was a smaller square lattice positioned at 45° with respect to the original thin filament lattice. Thus the thin filaments were not continuous through the Z disc from

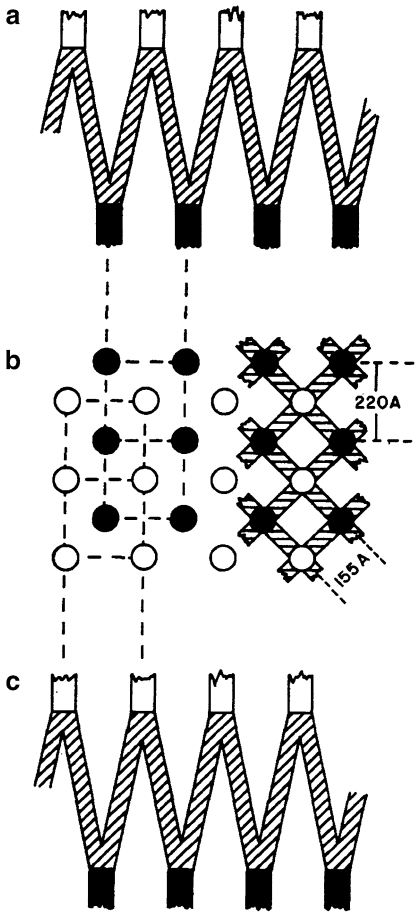

Fig. 7.18 Diagrammatic representation of Z disc structure and I filaments on either side of the Z disc. (**a**, **c**) Longitudinal sections, displaced with respect to each other so that each I filament lies between two opposite I filaments on either side of the Z disc (the all *black* and all *white bars*). The *shadowed bars* represent the Z filaments. (**b**) Projection of the square pattern formed by the cross-cut I filaments on one side of Z disc (*open circles*) onto the square pattern formed by the I filaments on the opposite side of Z disc (*black circles*) such that each I filament on one side faces the *center* of a *square* formed by four I filaments on the opposite side of Z disc (Knappeis and Carlsen 1962. With permission Rockefeller University Press)

one sarcomere to the next. This data fit well with the myosin decorated I-Z-I segments which exhibited reversed polarity of thin filaments at the Z disc as shown by the appearance of arrowheads pointing away from the Z disc (Huxley 1963). The tetragonal arrangement of thin filaments at the Z disc, of course, was in contrast to the hexagonal lattice of the thin and thick filaments in the overlap zone of the sarcomere (Fig. 7.19). Knappeis and Carlsen suggested that the thin filament transition from a hexagonal to tetragonal lattice could be accommodated by a small displacement of the thin filaments as they traverse from the overlap zone to the Z disc.

The so-called small square pattern of thin and Z filaments observed in the Z disc was not the only type of pattern that was observed. Michael Reedy (1964) described what he called a "woven" appearance, as if one were viewing a mat woven of white ribbons having black edges (Fig. 7.20). This remarkable pattern would later become known as a basketweave pattern in contrast to the small square pattern of Fig. 7.18b. Mamoru Yamaguchi and Masatoshi Izumimoto at Ohio State University and Richard

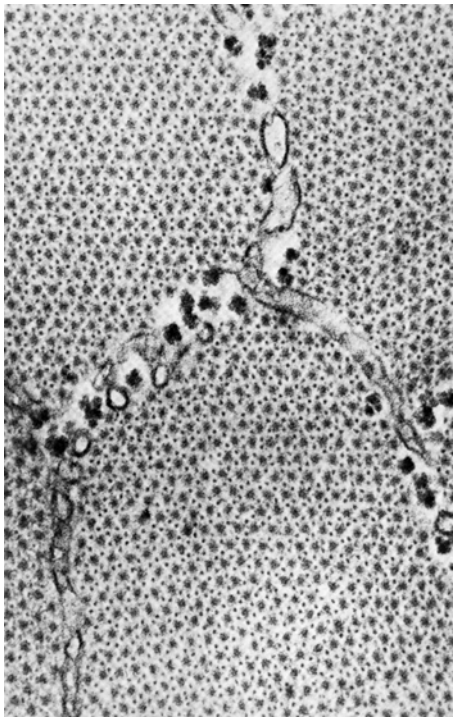

Fig. 7.19 Electron micrograph of a cross section of frog sartorius muscle showing the hexagonal lattice of thin and thick filaments. The thin filaments can be seen at the trigonal points of the hexagonal lattice of thick filaments. Magnification: ×112,000 (Huxley 1972. With permission Elsevier)

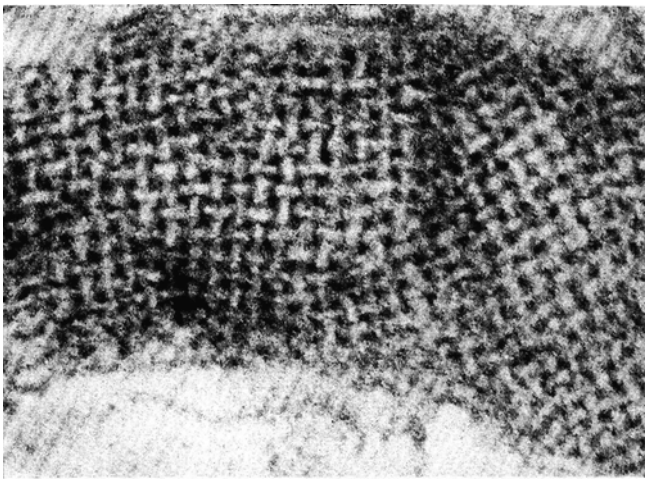

Fig. 7.20 Electron micrograph of cross-section through the Z disc of rat diaphragm muscle showing a basketweave pattern. Magnification: ×170,000 (Reedy 1964. With permission The Royal Society)

M. Robson and Marvin H. Stromer at Iowa State University developed a model of the Z disc that indicated that a small movement of the thin filaments of less than 10 nm could cause a change of appearance of the Z disc from the small square to the basketweave pattern (Yamaguchi et al. 1985). Margaret Goldstein and her colleagues at Baylor University have observed a transition from the small square pattern in resting skeletal muscle to the basketweave appearance during tetanic contraction (Goldstein et al. 1986). After relaxation, the small square pattern returned. They concluded that the Z disc lattice pattern correlated with the physiological state of the muscle. This correspondence with physiological state suggested that the small square to basketweave transition could occur dynamically during contraction. There appeared to be a connection between the molecular events of contraction and structural changes within the Z disc. Yamaguchi and Toshiharu Oba and colleagues also showed that there is a transition in the structure of the Z disc when a resting muscle fiber is stretched (Yamaguchi et al. 1999). Thus both active force production and resting force appear to be "sensed" by changes in Z disc structure.

The interest in characterizing mammalian skeletal muscle fibers into different fiber types began in earnest in the early 1960s. Physiological, metabolic and structural properties were compared in different muscles and muscle fibers in order to establish criteria for fiber type classification. With regard to structural properties, Geraldine F. Gauthier (1971) at Wellesley College, Massachusetts, noted that the width of the Z disc varied considerably with fiber type with fast twitch (white) fibers exhibiting a narrower Z disc than slow twitch (red) fibers. In fact the width of the Z disc was found to be one of the most reliable structural parameters for discriminating fiber types with a range of widths from about 60 nm (fast twitch fiber) to 140 nm for the slow twitch fibers (Eisenberg 1983). Rowe (1973) at the Meat Research Laboratory in Queensland, Australia observed that the width of the Z disc was related to a repeating number of zigzag bands or chevrons seen in longitudinal sections. In rat plantaris muscle, the white, intermediate, and red fibers have Z-bands comprised respectively two, three and four chevrons with a periodicity parallel to the fiber axis of ~40 nm. Pradeep K. Luther at Imperial College, London, has undertaken an extensive study of the three dimensional structure of Z discs of varying widths (for a review see Luther 2009). Figure 7.21 shows the three dimensional models for Z discs of varying width from different muscles. The adjacent thin filaments of opposite polarity overlap to a greater extent as the Z discs become wider. These models differ from that originally proposed by Knappeis and Carlsen in that the thin filaments enter the Z disc to varying extents depending on fiber type and the thin filament end does not attach to four Z filaments. Luther has called the structures linking the thin filaments Z links.

7.4.5 Protein Content of the Z Disc

During the time of the classic investigations that led to the discovery of troponin, Ebashi et al. (1964) described another new protein factor. This protein had an amino acid composition similar to actin but did not function like actin. The protein did not

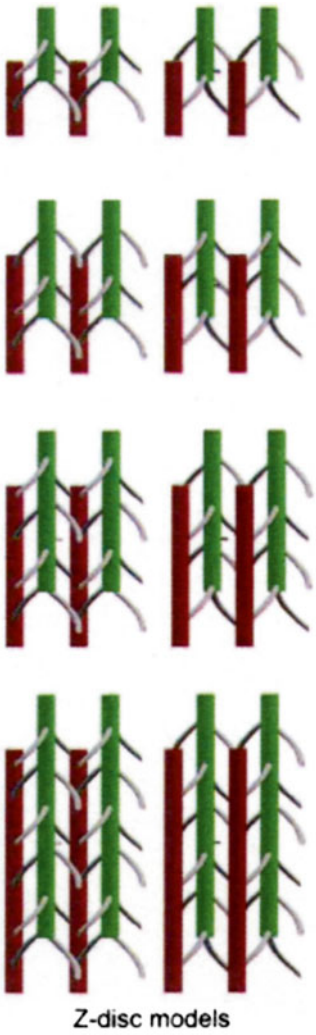

Fig. 7.21 Three dimensional models of the structure of vertebrate muscle Z discs of various widths. The figure shows (from *top* to *bottom*), 2-, 3-, 4- and 6-layer Z discs. *Top* shows the Z disc in fish body muscle which comprises single *zig-zag* links between oppositely oriented actin filaments. This Z disc is composed of two layers of α-actinin and is the minimum width of a Z disc. By adding additional layers of α-actinin, 3-, 4- and 6-layer Z discs are formed (Luther 2009. With permission Springer)

interact with myosin but did promote gelation of F-actin (Maruyama and Ebashi 1965). Ebashi and Ebashi (1965) named this protein α-actinin. α-actinin was localized to the Z disc (Masaki et al. 1967). The Z filaments were presumed to be constructed of α-actinin.

After the discovery of α-actinin and the suggestion that it presented the Z filaments in the model of Knappeis and Carlsen, the investigation of the Z disc was relatively dormant until the 1980s when the development of cell and molecular biology techniques led to an explosion of knowledge with the discovery of new proteins and the identification of their localization in the Z disc and speculation about their function. Alpha-actinin, molecular weight 97 kDa, was shown to be a rod shaped protein of length about 35 nm that formed anti-parallel homodimers

which cross linked actin filaments. The N-terminus of the titin molecule is located in the Z disc and the length of the titin molecule in the Z disc is proportional to the width of the Z disc. This portion of titin interacts with the actin filaments and also contains multiple α-actinin binding sites. Adjacent titin molecules are linked in an antiparallel fashion at their extreme N-terminal ends by the protein telethonin. The Z disc end of the actin filament is capped by a protein called CapZ (originally β-actinin) which binds to α-actinin and the C-terminus of nebulin. The Z disc also contains the actin binding protein nebulin. Numerous other proteins have been localized to the Z disc. It has been suggested that along with its structural role, the Z disc functions as a mechanosensor. Several of these other proteins have been implicated in mechanical strain signaling in skeletal and cardiac muscle (for reviews see Gautel 2011; Pyle and Solaro 2004).

Thus the Z disc and M band which were originally thought to play important but relatively mundane structural roles are now considered important players in strain sensing and signaling in health and disease. Despite the excitement in these rapidly evolving areas of research, there is still no generally agreed upon molecular models of the Z disc and the M band and their proteins.

7.5 Intermediate Filaments: Desmin and the Exosarcomeric Cytoskeleton

The structures considered above have been limited to the endosarcomeric cytoskeleton. Yet striated muscles are striated because of the precise lateral alignment of the myofibrils as can be seen clearly in light and electron micrographs (Fig. 7.22). The striated appearance is maintained when a muscle fiber shortens actively or is stretched while at rest (Huxley and Niedergerke 1954; see Figs. 2.4 and 2.5). This striking appearance implies that there exists an exosarcomeric cytoskeleton that consists of transversely oriented structures that keep the myofibrils in line laterally. The strongest early evidence that intermyofibrillar transverse structures might exist resulted from the observations of N. N. Garamvolgyi (1965) at the Institute of Biophysics, Pecs, Hungary. His electron micrographs of indirect flight muscle of the honey bee clearly showed transverse bridges connecting myofibrillar Z discs laterally. He called these structures "inter-Z bridges". Nonetheless such transverse structures were rarely seen in electron micrographs of vertebrate skeletal muscle.

The breakthrough did not come from observations of adult striated muscle but rather from investigations of structures in developing skeletal muscle and in smooth muscle. Ishikawa et al. (1968) (1935–2008)[9] at the University of Pennsylvania identified a new class of filaments with a diameter between that of the actin filaments

[9] At the time of the publication of this seminal paper, Harunori Ishikawa was a postdoctoral fellow in Lee Peachey's laboratory at the University of Pennsylvania. In a gesture that would not likely happen today, Peachey did not place his name on this paper despite his guidance of Ishikawa. Ishikawa went on to a distinguished career in cell biology (Sanger 2009).

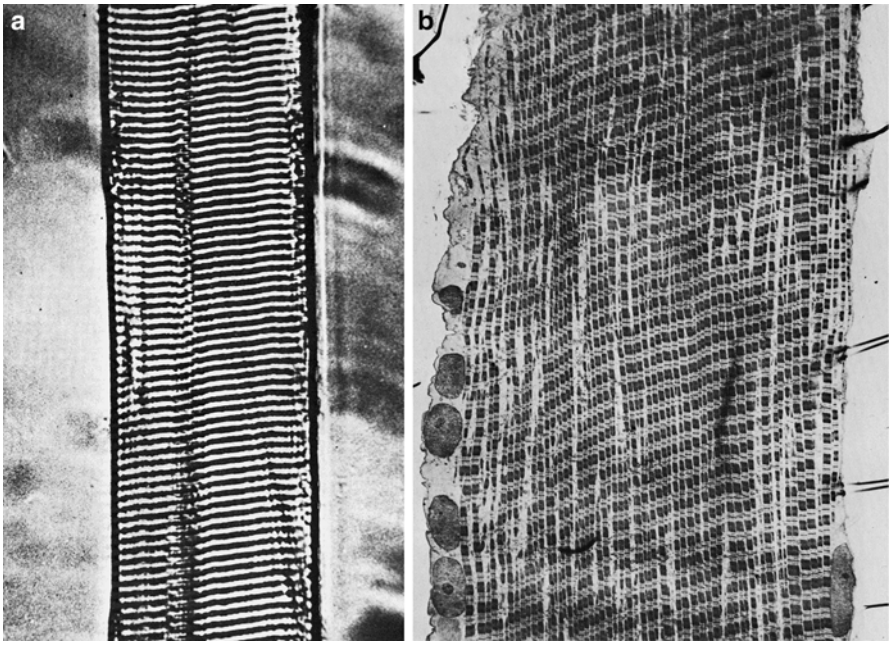

Fig. 7.22 The striated appearance of muscle as seen in the light and electron microscope. (**a**) Single muscle fiber from a rabbit psoas muscle, photographed in the phase contrast light microscopy. A bands are dark and I bands are light. The sarcomere repeat is about 2.6 μm, and the fiber diameter is about 50 μm. Magnification: ×980. (**b**) Single fiber from a human muscle sectioned longitudinally and viewed at low magnification in the electron microscope. The striations arise from the characteristic band pattern of the myofibrils which are laterally aligned. Myofibrillar diameter is about 1 μm and fiber diameter is about 55 μm. Magnification: ×1,480 (Huxley 1972. With permission Elsevier)

and myosin filaments in skeletal muscle cells cultured from chick embryos. These "intermediate filaments" had a diameter, on average, of 10 nm as compared to the 6 nm diameter of actin filaments and 15 nm diameter of myosin filaments. Unlike actin filaments, the intermediate filaments did not form arrowhead complexes in the presence of heavy meromyosin (Ishikawa et al. 1969)[10]. The intermediate filaments were most abundant in smooth muscle. Peter Cooke (1976), then at the University of Kansas, found a filamentous cytoskeleton in vertebrate smooth muscle cells which was made of intermediate filaments. The isolated intermediate filaments contained a new, unnamed, protein of apparent molecular weight of 55 kDa. This protein could form filamentous segments that closely resembled the structure of the native isolated intermediate filaments. Later that same year, Lazarides and Hubbard

[10] This paper was the first to show the utility of formation of arrowhead complexes with myosin decoration to identify actin filaments in non muscle cells.

(1976)[11], then at the University of Colorado, described the isolation of a 50 kDa protein from smooth muscle intermediate filaments and its location in chicken skeletal muscle fibers. They formed an antibody against the protein and employed indirect immunofluorescense to localize the protein in the muscle fiber. The protein was found in close association with the Z discs of the sarcomeres and extended between the Z discs of adjacent myofibrils. It also was associated with filamentous structures that ran along the length of a muscle fiber both in close association with the plasma membrane and between myofibrils. Lazarides and Hubbard (1976) concluded that this protein functioned in muscle as a three dimensional matrix which interconnected individual myofibrils to one another and to the plasma membrane at the level of the Z lines. The molecule thus provided a framework that mechanically integrated the contractile myofilaments during the contraction and relaxation of muscle. To emphasize its linking role in muscle, they named the protein desmin (from the Greek δεσμός = link, bond). Thus whereas intermediate filaments were rarely seen with electron microscopy in adult skeletal muscle fibers, the protein desmin[12] was prevalent and appeared to connect adjacent myofibrils transversely and be responsible for the striated appearance of skeletal muscle fibers.

Lazarides followed up this study with a further investigation of the location of desmin in skeletal muscle fibers. He and Bruce L. Granger isolated, from chicken skeletal muscle, planes of in-register Z discs which appeared as honeycomb-like arrays (Granger and Lazarides 1978). This unique preparation provided a "face-on-view" of Z discs in the same plane. Once again they employed indirect immunofluorescence to localize desmin and also α-actinin in the Z discs. Desmin was present at the periphery of each Z disc, forming a network of collars within the Z plane. α-Actinin was localized within each disc, resulting in a face-on fluorescence pattern that was complementary to that of desmin (Fig. 7.23). Thus the evidence was strong that desmin was a primary component of the cytoskeletal structures responsible for the striated appearance of skeletal muscle fibers. Lazarides (1980) illustrated his vision of the role of desmin in Fig. 7.24.

Wang and Ramirez-Mitchell (1983) described a network of not only transverse but also longitudinally oriented filaments in vertebrate skeletal muscle fibers. They extracted the majority of actin and myosin from rabbit skeletal muscle myofibrils with a potassium iodide solution. The "ghost" myofibrils were examined with transmission and scanning electron microscopy. They observed filaments with the

[11] In 1974 Elias Lazarides, a graduate student at Harvard University, working with Klaus Weber at the Cold Spring Harbor Laboratory, published a classic paper utilizing actin antibodies, indirect immunofluorescence and fluorescence light microscopy to visualize actin filaments in a variety of non muscle cells (Lazarides and Weber 1974). This paper had a major impact on the study of the cytoskeleton and showed the power of indirect immunofluorescence and fluorescence light microscopy for localization of a particular cellular component. The paper became a Citation Classic and is considered to be a landmark paper in cell biology. Also see Lazarides and Revel (1979). The origin of immunofluorescence microscopy can be traced back to the early 1940s and the work of Albert H. Coons at Harvard University (Coons 1961).

[12] Small and Sobieszek (1977) at Aarhus University isolated and characterized an intermediate filament protein from vertebrate smooth muscle and named it skeletin. Skeletin turned out to be equivalent to desmin and desmin is the usual designation today.

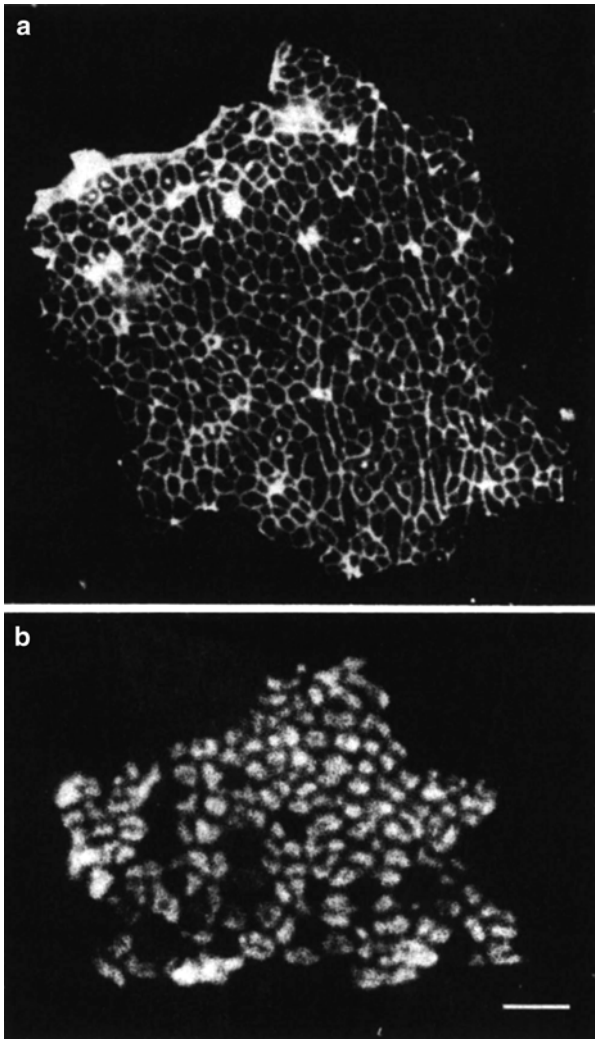

Fig. 7.23 Immunofluorescence localization of desmin and α-actinin at the Z disc of isolated Z disc sheets from chicken skeletal muscle. (**a**) Desmin is distributed at the periphery of Z discs. (**b**) α-actinin is located in the interior of the Z discs. Note the complementarity of the desmin and α-actinin fluorescence patterns. Scale bar: 2.5 μm (Lazarides 1980. With permission Nature Publishing Group)

characteristics of intermediate filaments that were oriented: (1) transversely and connected adjacent myofibrils at the level of the Z discs and M bands and (2) longitudinally and connected the peripheries of successive Z discs and seemed to ensheath the sarcomere (Fig. 7.25).

Besides connecting adjacent myofibrils at the Z discs, desmin is now known to connect myofibrils to mitochondria, the nucleus, the transverse tubular system and the cell membrane through various binding partners (Bar et al. 2004). Desmin also runs longitudinally to surround individual myofibrils. The desmin intermediate filament is only one of many different kinds of intermediate filaments. The various intermediate filaments are heterogeneous in structure and vary somewhat in diameter. The desmin containing intermediate filaments are classified as class III intermediate

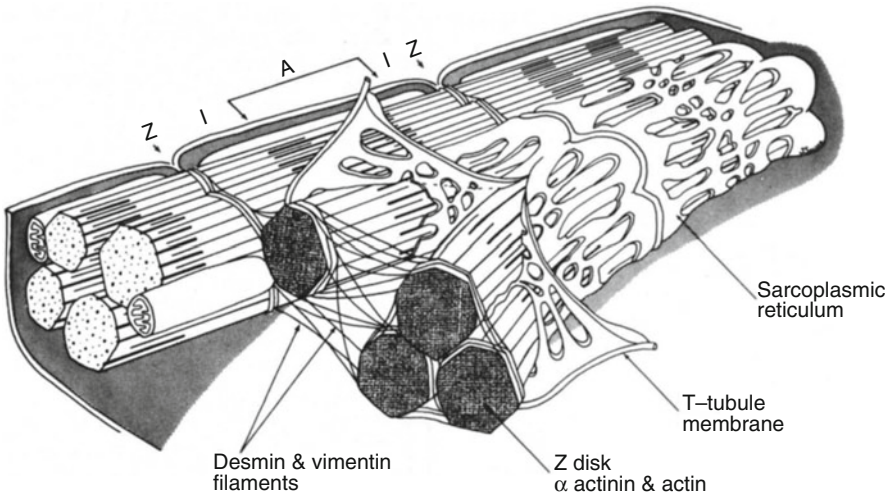

Fig. 7.24 Schematic representation of distribution of desmin, α-actinin, actin and membranous organelles in relationship to skeletal myofibril Z discs. Vimentin is seen early in muscle development and later is replaced by desmin in adult muscle (Lazarides 1980. With permission Nature Publishing Group)

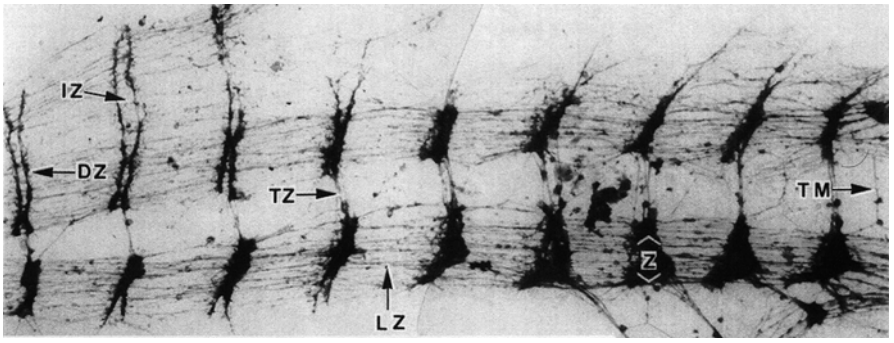

Fig. 7.25 An exosarcomeric network of transverse and longitudinal intermediate filaments. A bundle of rabbit myofibrils is shown from which actin and myosin have been extracted with a potassium iodide solution. The bundle exhibits decreasing degrees of structural integrity from *right* to *left*. Filaments connect myofibrils transversely at the Z disc (TZ) and M band (TM) and filaments connect Z discs longitudinally (LZ). Magnification: ×6,000 (Wang and Ramirez-Mitchell 1983. With permission Rockefeller University Press)

filaments. There are at least five different classes of intermediate filaments based on sequence homology. The desmin molecules form dimeric coiled coils which further associate laterally and end-to-end to form an intermediate filament about 10–12 nm in diameter (Herrmann et al. 2007). Localized at the sarcolemma is a complex lattice network that connects the outer myofibrils to the sarcolemma. The lattice network has been called a costamere (latin costa, rib; Greek, meros, part) (Pardo et al. 1983). Desmin filaments connect to the costameres which in turn connect the outer

myofibrils to the sarcolemma (for a recent review see Ervasti 2003). Investigation is ongoing to establish the molecular nature and function of the exosarcomeric cytoskeleton in greater detail. Of course all cells have a cytoskeleton and the milestones in this area of research was chronicled beautifully in Nature Milestones in 2008 (available at http://www.nature.com/milestones/cytoskeleton).

As impressive as the advancements in understanding the muscle cytoskeleton have been, it is somewhat sobering to realize that there is not yet a complete three dimensional, molecular, description of the architecture of any part of the sarcomere, be it the M band, Z disc, thin or thick filaments. Furthermore the transverse intermyofibrillar connections to other cell organelles are understood in some detail but not in detailed molecular terms. There is still much to learn about the endosarcomeric and exosarcomeric cytoskeleton of muscle.

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