Titin: Function in Life and Changes Postmortem

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This presentation will be divided into two parts. The initial section will describe our present knowledge about the structure and function of titin in living muscle. The second section will summarize what we know regarding changes in titin postmortem and how these may relate to use of muscle as food. This is actually the third presentation I have made at the RMC that include information on this protein; recent progress is particularly striking (compare with Greaser et al. 1981; Greaser, 1991). Further information on titin function can be gathered from several excellent reviews (Granzier and Labeit, 2007; Fukuda et al. 2008; Kontrogianni-Konstantopoulos et al. 2009; Anderson and Granzier, 2012; LeWinter and Granzier, 2013; Meyer and Wright, 2013; Linke and Hamdani, 2014).

TITIN STRUCTURE AND FUNCTION IN LIVING MUSCLE

Titin Discovery and Properties

The giant protein titin was independently discovered by two groups. Wang and co-workers (1979) isolated a high molecular weight protein (> 1,000,000 Daltons) by chromatography of SDS extracts of myofibrils. Two bands appeared at the top of sodium dodecyl sulfate polyacrylamide gels, T1 and T2. The T2 was later shown to be a degradation fragment of the T1, the intact protein. Koscak Maruyama (1976) had earlier found an elastic protein, which he called connectin, after extensive extraction of purified myofibrils. The protein had a rubber-like consistency. The high molecular weight component of the crude connectin was found to be identical to titin (Maruyama et al. 1981). For many years both names were used, but the titin name currently dominates. Fürst and coworkers (1988, 1989) obtained 14 unique monoclonal antibodies that perpendicularly stained different regions of the sarcomere with bands spanning from near the M-line to the Z-line. Work at Wisconsin also described a number of titin monoclonals, including the 9D10 that is still widely used for Western blotting and histochemistry (Wang and Greaser, 1985). These observations all led to the currently accepted model in which there are two sets of parallel and oppositely directed titin molecules, each spanning half a sarcomere (Figure 1). The amino terminal end of titin is anchored at the Z-line. The carboxyl end binds the thick filaments in the A-band, and it extends to the M-line. The model is supported by rotary shadowing studies that showed titin molecules are up to 1.0 μm in length, very thin, and flexible (Wang et al. 1984; Nave et al. 1989; Tskhovrebova and Trinick, 1997).

Our understanding of titin moved forward dramatically with the determination of the full cDNA sequence for titin (Labeit and Kolmerer, 1995). Concerns that a protein this size could occur were removed when it was shown that the cardiac titin cDNA sequence coded for a protein of 3,000,000 Daltons. The sequence for human soleus (a skeletal muscle) yielded a protein of 3,700,000 Daltons. Both the cardiac and skeletal muscle titins were derived from a single gene; this was in contrast to several other muscle proteins with separate isoform specific genes. The protein has many repeating modules of 90-100 amino acids in length that are similar in sequence to those found in immunoglobulins (Ig) and the third domain of fibronectin (FNIII). Both the Ig and FNIII domains are folded into 7 stranded beta sheets. The motif structure is highly complex, but most of the full molecule contains repeating patterns of these Ig and Fn III modules. The A-band has three different super-repeat zones: D zone $(Ig\text{-}Fn\text{-}Fn\text{-}Ig\text{-}Fn\text{-}Fn\text{-}Fn)_6$; C zone $(Ig\text{-}Fn\text{-}Fn\text{-}Ig\text{-}Fn\text{-}Fn\text{-}Fn\text{-}Ig\text{-}Fn\text{-}Fn\text{-}Fn)_{11}$; and P zone (Ig-Ig-Fn-Fn-Ig-Ig-Fn)(Figure 2). The I-band region is composed mostly of repeating Ig domains. In addition there is an unusual region near the center of the I-band part of titin that contains predominantly proline (P), glutamic acid (E), valine (V), and lysine (K) residues (nearly 80%) and has been termed the "PEVK" region. Labeit and Kolmerer (1995) also showed that the length of this region varied between tissue sources with the soleus having a much longer PEVK segment than that from cardiac muscle. Titins from other skeletal muscles have intermediate length PEVKs. Since the PEVK length is negatively

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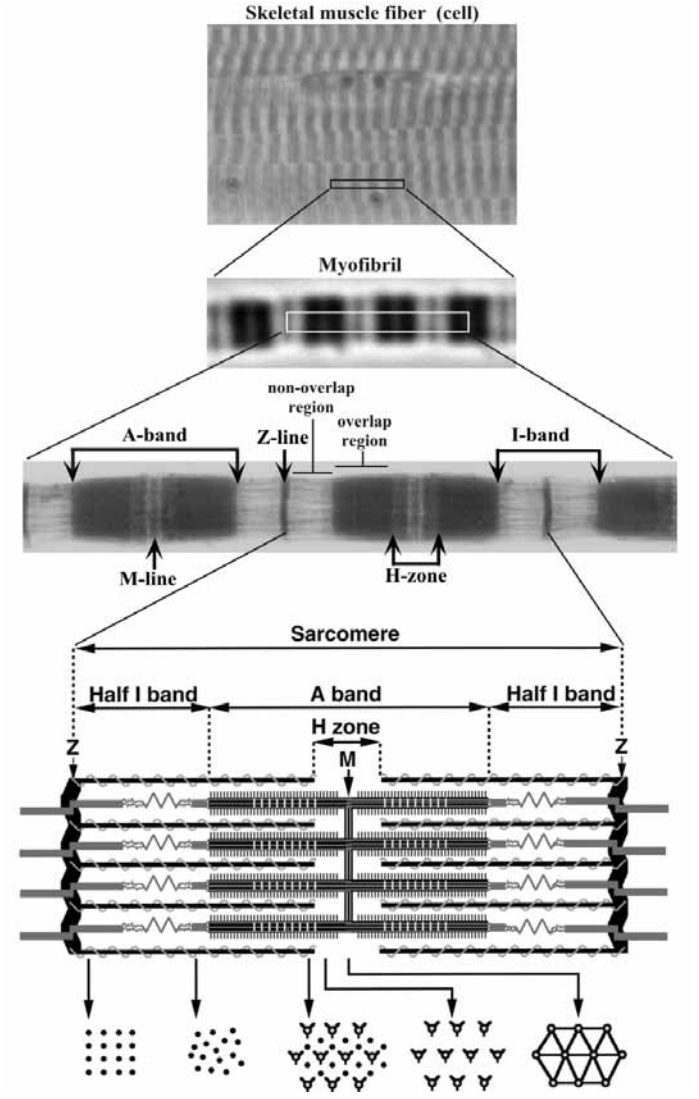

Figure 1.Myofibril structure. Titin extends from the Z-line to the M-line; nebulin is shown wrapped around the thin filament. From Swartz et al. 2009, used by permission.

correlated with resting tension levels of the corresponding cell types, Labeit and Kolmerer concluded that the PEVK region was the principle location of titin extension during stretch. They also showed that there were different numbers of Ig domains in the region amino terminal to the PEVK zone in titins from different tissue sources. Originally two different human titin isoforms were identified, termed N2A and N2B (Labeit and Kolmerer, 1995). The N2B isoform was thought to be cardiac muscle specific and the N2A isoform found only in skeletal muscle. The N2A isoform was larger and contained all the N2B sequence except for an approximately 500-600 amino acid segment found in the latter termed "N2B unique" that was also stretchable (Freiburg et al. 2000).

However, it was subsequently determined that there were two major classes of cardiac isoforms, the original cardiac N2B type as well as a hybrid with elements from both the N2B and N2A forms (Freiberg et al. 2000, Bang et al. 2001). The two major cardiac isoform classes are now termed "N2BA" and "N2B." There are significant species differences in the proportions of the two major classes; rats have approximately 90% N2B while larger mammals (such as dog, pig, bovine, and human) have 50% or greater of the N2BA type (Greaser et al. 2002). Although the N2B exon splicing patterns are fairly uniform, a plethora of pathways exist for the cardiac N2BA isoforms in human, rabbit, dog, and rat. Similarly there appear to be many alternatively spliced skeletal muscle titins since their sizes vary widely (Prado et al. 2005; Li et al. 2012). The size of cardiac and skeletal muscle titins changes during development, moving from larger to smaller. In rats this change occurs during the first month in the heart (Warren et al 2004; Opitz et al. 2004; Lahmers et al. 2004), but may take several months in some skeletal muscles (Li et al 2012).

The unusual PEVK region of titin was also found to contain both repeating sequences and alternating regions varying in charge (Greaser, 2001). "PPAK" repeats of

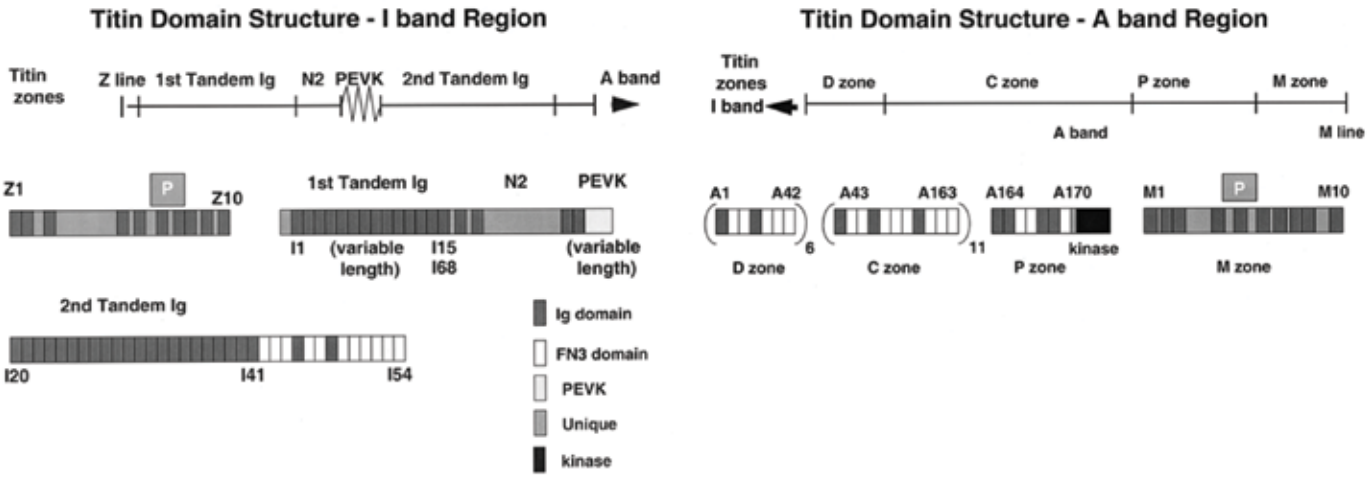

Figure 2. Domain structure of titin. Re-drawn from Labeit and Kolmerer, 1995.

26-28 amino acids (named after the first four residues of each module) are separated by regions rich in glutamic acid residues (about 45%) termed "polyE". The PEVK region is believed to lack a set secondary structure, and thus is well suited for unraveling when the sarcomere is stretched.

Titin Role in Myofibril Assembly

It might be expected that a protein that extends half the length of the sarcomere would play a key role in the assembly of the myofibrils. Indeed it was early demonstrated that overexpression of a segment of the titin gene blocked myofibril formation (Peckham et al. 1997). Later experiments using anti-sense nucleotides reduced titin expression and myosin incorporation into myofibrils (Person et al., 2000). Much of the work on myofibril assembly mechanisms has been conducted with both skeletal and cardiac muscle cells in culture. Even cells that initially contain myofibrils will disassemble them and start over. This allows the assembly process to be observed in synchrony.

A diagram of the steps in assembly is shown in Figure 3. Initially the cells form bundles of alpha-actin filaments adjacent to the cell membranes with a non-striated longitudinal appearance. These structures are also muscle tropomyosin positive and described as "stress-fiber-like" (Dlugosz et al. 1984). Non-muscle myosin is associated with these stress fibers in a structure termed "pre-myofibril" (Rhee et al, 1994). Titin staining becomes visible (9D10 antibody) and perpendicular clearing zones for alpha actin and muscle tropomyosin appear at the positions of developing Z-lines (Figure 3, stage b). Irregular but periodic sarcomeres become visible with muscle myosin antibody staining (Figure 3, stage b). Sometimes a single or groups of 2-3 sarcomeres form on a stress-fiber like structure. Wagon wheel like structures (Figure 4) with a titin staining node eventually collapse into longitudinally oriented sarcomeres. In all cases a titin stained band is associated with each end of the nascent A-band (Figure 5). Each of these A-bands is 1.6 microns in length. Other workers have suggested that the mini-sarcomeres progressively increase in Z-line spacing during development (Sanger et al., 1986; Dabiri et al. 1997; Sanger et al., 2010), but we have not observed this in our studies (Wang et al.1988, Handel et al. 1991). The myofibrils become progressively more ordered (Figure 3, stages c and d). Intense alpha-actin staining is restricted to the non-thick-thin filament overlap regions, and myosin staining also is strong only in the H zone regions (Figure 3, stage e). This implies that antibody penetration is more complete in the immature sarcomere than the mature because the development of the thick-thin filament lattice structure (perpendicular order) lags behind the filament longitudinal alignment. Thus titin staining attains its mature pattern in the sarcomere prior to that of alpha-actin, muscle tropomyosin, or muscle myosin (Wang et al. 1988; Handel et al 1989). The proteins in the myofibril continue to exchange throughout adult life (da Siva Lopes et al. 2011).

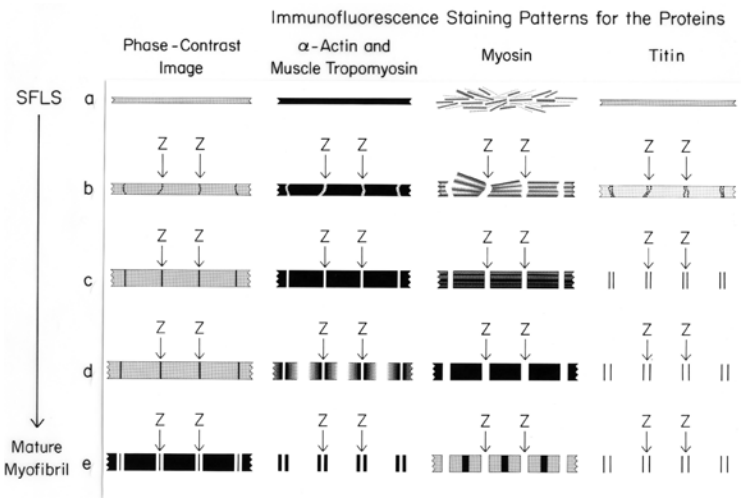

Figure 3. Diagram showing steps in myofibril assembly. From Handel et al. 1991, used by permission.

Electrophoresis Methods for Titin

Although titin is the third most prevalent protein in the myofibril (approximately 8%), its discovery lagged behind that of other proteins because of difficulty in observing it by electrophoresis. A protein with a single chain size of over 3,000,000

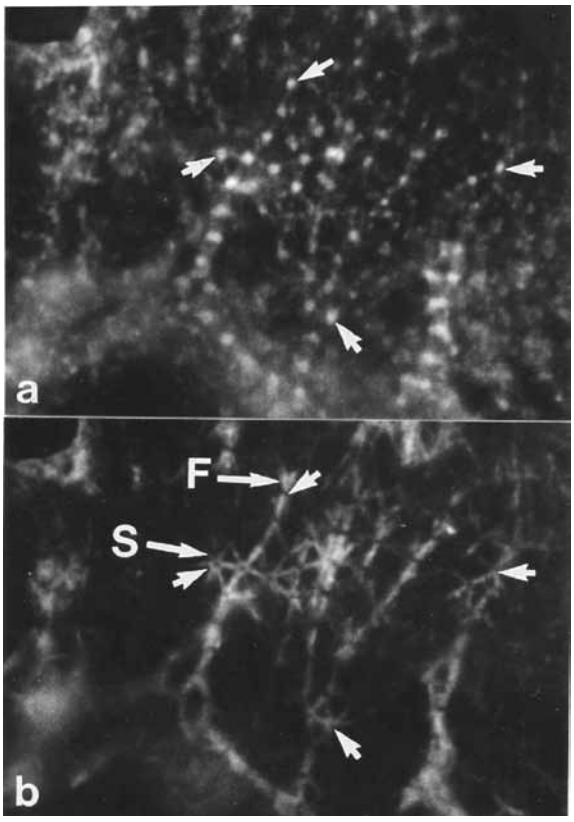

Figure 4. Myofibril assembly in cultured cardiomyocytes. Note the wagon wheel like patterns using anti-titin 9D10 (a) and myosin (b). From Handel et al 1991, used by permission.

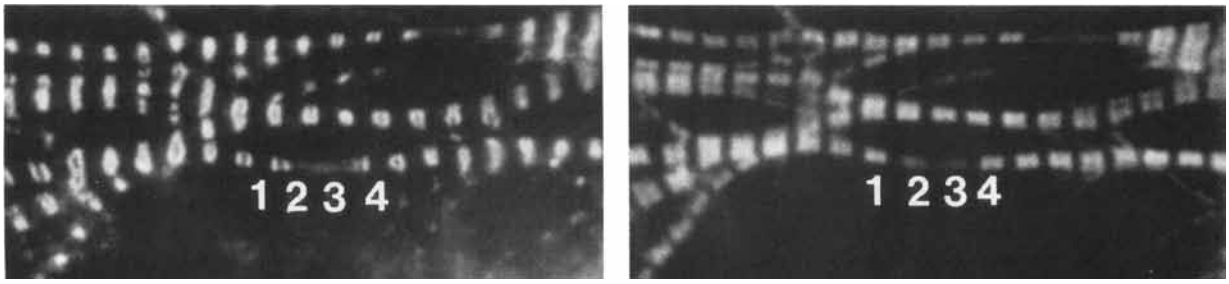

Figure 5. Titin assembly in developing myofibrils (a) in concert with myosin (b). From Wang et al 1988, used by permission.

Daltons will barely move into a polyacrylamide gel. Other groups have used very low acrylamide gel concentrations or gradient gels, but these methods are difficult and not very reproducible. We were able to achieve some minor improvements by replacing the cross-linking bis-acrylamide in the stacking gel with N,N′ diallyltartardiamide and including a sulfhydryl reductant in the upper reservoir buffer (Fritz et al., 1989). This allowed the use of a single concentration polyacrylamide separating gel (10%) that resulted in titin moving a few millimeters and the resolution of the rest of the myofibrillar proteins down to the 15,000 Dalton myosin light chain (Figure 6). Resolution of different sized titin isoforms, however, was not possible. A new system using SDS and agarose provided dramatic separation (Warren et al 2003). The full sized titin protein migrated 10 cm through a 15 cm long gel (Warren et al. 2003)(Figure 7). A study was initiated to determine if there were changes in titin size during development. Rats were used as a model, and samples were obtained at days 1, 5, 10, 15, 20, 25, and 30 after birth. The results showed a gradual progressive reduction in titin size with age with the adult pattern achieved by one month (Figure 8A).

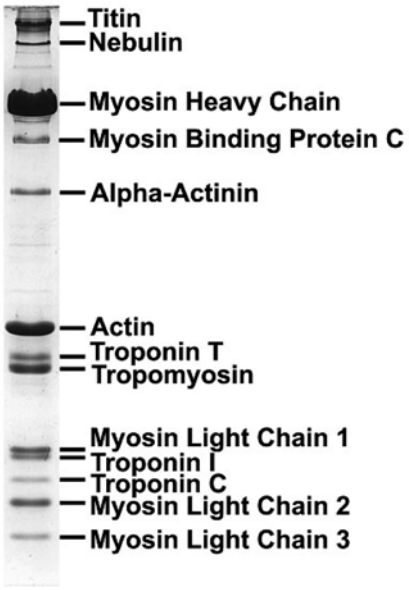

Figure 6. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis pattern for muscle myofibrils using the method of Fritz et al. 1989.

Titin and RBM20

Most of the samples obtained in the developmental study had consistent patterns appropriate for their age, but the titin sizes in a few animals were much larger than expected (Greaser et al. 2005) (Figure 8B). In checking it was determined that all the samples with altered sizes came from the same litter. This suggested that some genetic factor was involved. It was subsequently determined that these rats had an autosomal dominant mutation that resulted in this increased titin size. In collaboration with Michael Gotthardt and coworkers, the mutation was traced to a 95,000 base pair deletion on chromosome 1(Guo et al 2012). This deletion was in the middle of a poorly characterized gene called Rbm20 (Guo et al 2012). The Rbm20

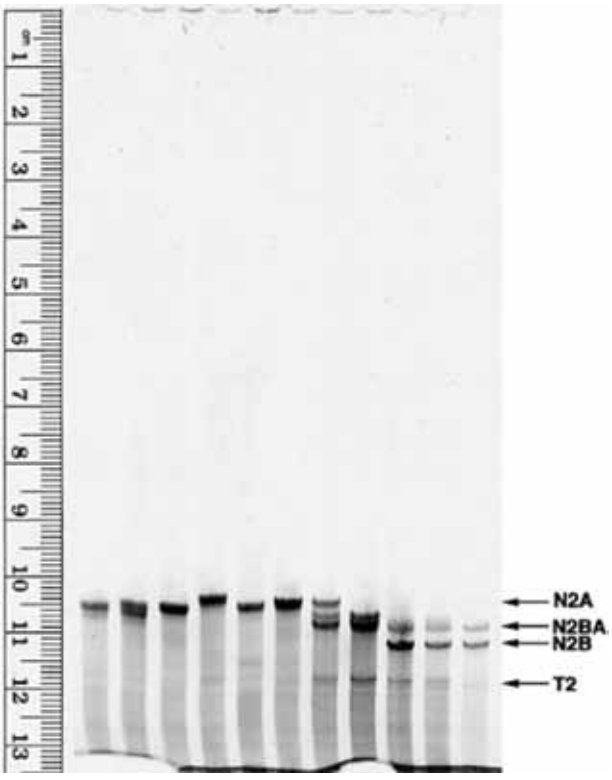

Figure 7. SDS agarose gel showing separation of different titin isoforms. From Warren et al 2003, used by permission.

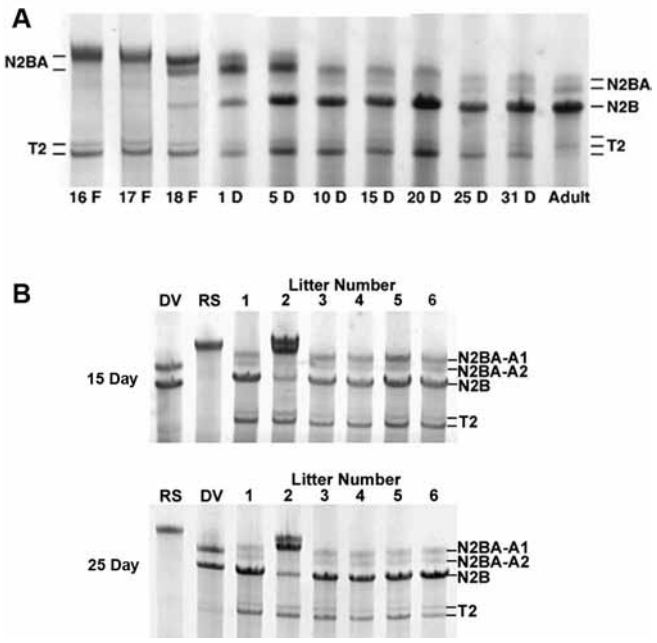

Figure 8. A. Agarose gel changes in cardiac titin size with age in the rat. B. Patterns for 15 day (upper) and 25 day (lower) rats. Note the larger size in animals from litter number 2. From Warren et al. 2004 and Greaser et al. 2005, used by permission.

was found to be involved in a process called alternative splicing that affects the number of exons used to make a messenger RNA (Li et al. 2013). At least 30 genes are affected by this splicing factor, with the most extensive alteration being with titin. Amazingly, homozygous Rbm20 mutant rats have titin sizes of about 3.8 million Daltons as opposed to the major cardiac isoform of 3,000,000 Daltons, a difference of 800,000. Although the animals are visibly normal, they do develop dilated cardiomyopathy and have compromised exercise ability (Guo et al. 2013). Mutations found in RBM20 in humans cause a similar heart problem (Guo et al. 2012). Thus we have discovered a major animal model for a serious human health condition.

TITIN CHANGES POSTMORTEM AND IMPLICATIONS FOR MUSCLE AS FOOD

Titin and Tenderness - Electrophoresis

It was early recognized that titin's size and role in sarcomere structure made it a logical candidate for alterations postmortem. Meat typically improves in tenderness in the process called aging, so it was hypothesized that titin breakdown postmortem might be involved. Early studies showed that the conversion of the intact titin T1 to the degraded T2 progressed with time postmortem (Lusby et al 1983; Anderson and Parrish, 1989), and this paralleled improvement in tenderness. Similarly the appearance of a 1.2 MDa titin fragment postmortem also was correlated with tenderness improvement (Huff Lonergan et al 1995, 1996A; Melody et al 2004). Results from the work of Fritz and Greaser (1991) confirmed that titin and nebulin were progressively degraded with time postmortem using both electrophoresis and Western blotting. The rate of titin postmortem degradation has been shown to be affected by sarcomere length (England et al. 2012).

Although these studies pointed to the potential role of titin breakdown to explain tenderness development during aging, a more critical test would be to show that sample tenderness correlated with degree of titin breakdown at the same time postmortem. Such a study was conducted on beef samples observed at 48 hours and 16 days postmortem (Fritz et al 1993). Even though Warner–Bratzler sample tenderness at 48 hours varied two-fold, neither the total titin content nor the ratio of the T1 to T2 showed any correlation for either raw or cooked samples (Figure 9). Attempts have been made to explain the disparate conclusions from these studies. First, sample heating time has been shown to affect

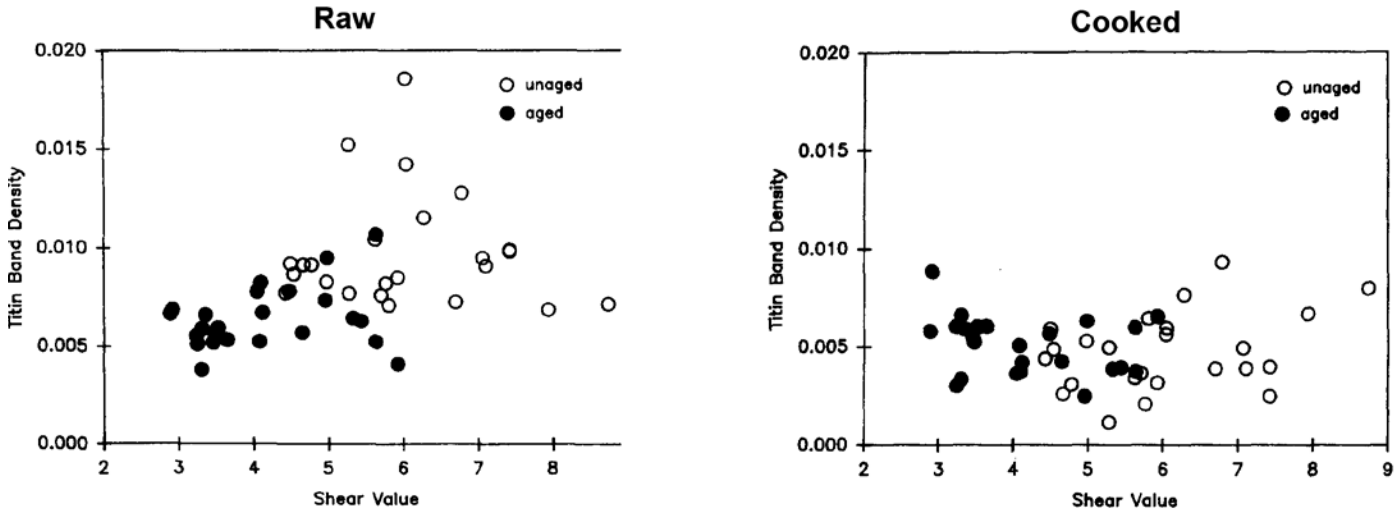

Figure 9. Relationship between titin T2 to T1 ratios and tenderness using raw or cooked samples. No relationship was found. From Fritz et al. 1993, used by permission.

the titin T1 to T2 ratios. The minimum heating induced titin degradation has been shown to occur if samples are heated at 60 degrees C for 10 minutes versus the 100 C – 3 minutes used in the Fritz et al 1993 study. However, Warren and coworkers (2003) demonstrated that the difference between these two heating protocols was minimal (see Figure 2 in Warren et al. 2003). Repeating these studies using the new agarose electrophoresis methodology will be necessary to resolve the tenderness versus titin degradation question.

Titin Structural Changes Postmortem

Evidence that there may be structural changes in titin postmortem was obtained when myofibrils were examined after staining with the 9D10 anti-titin monoclonal antibody. Antibody staining shows two perpendicular bands that are located in the I-band region of myofibrils obtained early postmortem. The position of these bands varies with sarcomere length. Since the titin is anchored at both the Z-line and in the A-band, one might expect that the epitope position would move away from both the Z-line and the edge of the A-band when the muscle is stretched. This was found to be true in most cases, but some longer myofibrils showed the 9D10 band at the immediate edge of the A-band (Figure 10). The most straightforward explanation would be that the titin molecules were broken and the titin epitope retracted back to the A-band edge.

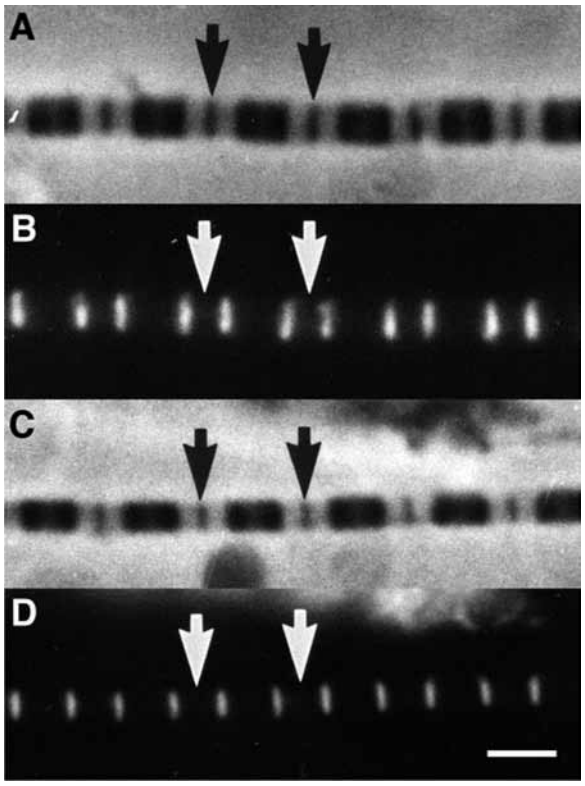

Figure 10. Different patterns of 9D10 staining of myofibrils. A,C- phase contrast; B, D- immunofluorescence. Although the sarcomere lengths of the upper and lower myofibrils were the same, the positions of the 9D10 stained band was different.

A new type of 9D10 staining pattern was observed in bovine myofibrils subjected to stretch. One of the most highly stretched muscles in a beef carcass is the psoas with sarcomere lengths typically greater than 3 microns. Psoas myofibrils consistently showed 2 titin bands per sarcomere at 3 hours postmortem, but most myofibrils had a 4 band per sarcomere pattern at 48 hours postmortem (Ringkob et al 1988)(Figure 11). Although extremely puzzling at the time, new insights about the interpretation of these patterns was gained from several other observations. First, any possible break in the titin would probably be in the PEVK region that is structurally disordered and thus more susceptible to proteolytic cleavage. Second, the size of the titin T2 fragment is consistent with the expected size for the section of titin between the PEVK and the M line end. Third, work in collaboration with Henk Granzier and coworkers demonstrated that the 9D10 monoclonal stained a broad zone in the I-band in human soleus muscle, consistent with its much longer PEVK (Trombitas et al. 1998). Fourth, the PEVK contains multiple repeating sequences (Greaser, 2000) and it was therefore reasonable that the antibody might bind to multiple similar sequences throughout this region. Therefore a break in the PEVK middle might result in staining in two regions at the ends of the retracting titin fragments.

Another kind of titin structural change was observed using an antibody against a different part of the titin (Boyer-Berri and Greaser, 1998). The FE-RE antibody was developed against an expressed titin fragment from the region near the Z-line, just prior to the 1st tandem Ig segment (Sebestyen et al. 1995). This antibody is close enough to the Z-line that one typically sees a single fused fluorescent image at the Z-line when observed by immunofluorescence light microscopy in Day 1 postmortem samples (Fig 12A). Samples taken at increasing times postmortem

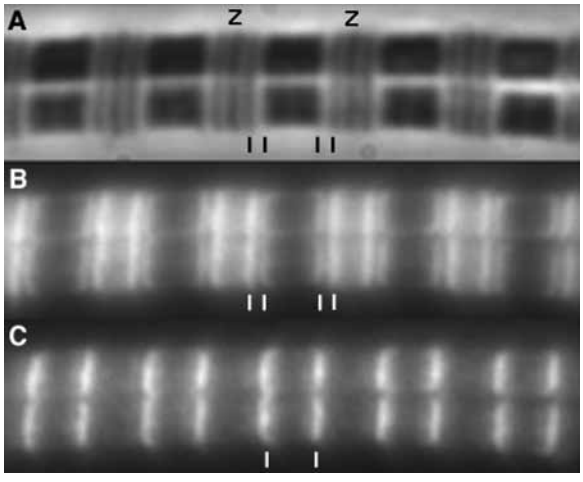

Figure 11. A. Staining of myofibrils from 48 hour bovine psoas. A. Phase contrast. B. Anti-titin 9D10. C. Anti-titin 514. Note that the 514 antibody, directed against an epitope at one end of the PEVK, only stains two bands per sarcomere.

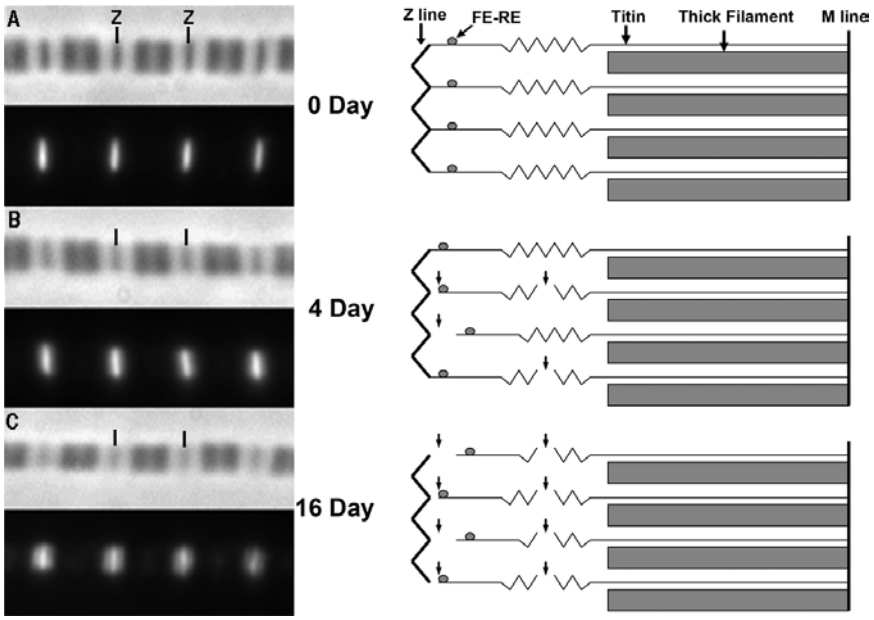

Figure 12. Changing titin staining patterns with the FE-RE antibody. A. Phase contrast (upper) and Immunofluorescence (lower) patterns with FE-RE anti-titin staining. Note that the stained region becomes wider during ageing. B. A model used

showed a gradual widening of the fluorescent band region. This has been interpreted as being due to a proteolytic cleavage occurring near the Z-line and a retraction of the broken titin molecule towards the A-band direction. Such movement would presumably only occur if the Z-line region break occurred before the break in the PEVK since the movement would require tension on the molecules. Figure 12B is a model showing the proposed structural rearrangement. Since such a break would only remove approximately 100,000 Daltons or less of the titin molecule, such a change might not be observed using conventional polyacrylamide electrophoretic methods. A titin break near the Z-line would be consistent with previous electron microscopic observations with postmortem muscle (Davey and Gilbert, 1969; Greaser et al. 1969; Taylor et al. 1995; Ho et al 1997) showing Z-line damage. There is also increased myofibril fragmentation during aging (Davey and Gilbert, 1969; Culler et al. 1977).

Titin and Calpains

The calcium activated proteases first found in muscle by Dayton et al (1976) are now believed to play a major role in postmortem meat tenderization. Three different isoforms constitute the largest amounts in skeletal muscle (m calpain – CAPN1, M calpain – CAPN2, and P94 calpain – CAPN3)(for review see Goll et al 2003, Goll et al. 2006). CAPN1 and CAPN2 are activated by micromolar and millmolar calcium levels. The CAPN1 is believed to be most important since its action on myofibrils mimics the patterns of protein breakdown seen in postmortem muscle (Huff-Lonergan et al. 1996B; 2010). CAPN1 binds to titin at both the Z-line and M-line ends (Raynaud et al 2005) so it would be positioned favorably to initiate titin breakdown when the calcium levels in the cytosol rise during the postmortem time frame. Although such a mechanism seems logical, it remains only as speculation.

The activation of CAPN3 is more complex. It rapidly autolyzes in the free state without calcium (Ono et al. 2006). Later it was shown that sodium ion activates the CAPN3 (Ono et al 2010). Surprisingly the CAPN3 is bound to titin in the N2A region, and this binding protects the CAPN3 from breakdown (Ono et al. 2006). The potential role of CAPN3 in postmortem protein degradation remains unclear.

SUMMARY

The size and number of protein-protein interactions involving titin is daunting. Although much information has been obtained regarding titin structure and function, many details remain to be determined. Titin's role in postmortem meat tenderization during aging also requires further investigation.

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