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FLORIDA STATE UNIVERSITY
COLLEGE OF ARTS AND SCIENCES

IDENTIFICATION AND CHARACTERIZATION OF CELLULAR TITIN

By
PETER JAMES CAVNAR

A Dissertation submitted to the
Department of Biological Science
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Degree Awarded:
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members.

This work is dedicated to my wife Katie, my daughter Grace, and my son Joseph.
Without their unconditional love and support, this could not have been possible.

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TABLE OF CONTENTS

LIST OF FIGURES	vi
ABSTRACT	viii
CHAPTERS	
1. GENERAL INTRODUCTION.....	1
2. MOLECULAR IDENTIFICATION AND LOCALIZATION OF CELLULAR TITIN, A NOVEL TITIN ISOFORM IN THE FIBROBLAST STRESS FIBER	12
Introduction.....	12
Materials and Methods	15
Results	19
Discussion	22
3. CHARACTERIZATION OF CELLULAR TITIN KINASE AND ITS ROLE IN STRESS FIBER ORGANIZATION THROUGH INTERACTION WITH HAX-1	39
Introduction.....	39
Materials and Methods	41
Results	43
Discussion	46
4. SUMMARY AND CONCLUSION	56
APPENDIX A: APOPTOSIS DETECTION IN CELLS EXPRESSING CTKIN4.....	61
APPENDIX B: CELLULAR TITIN AND MYOSIN LOCALIZATION IN ACTIVATED PLATELETS	62
APPENDIX C: HUMAN SUBJECTS APPROVAL FORM.....	63
REFERENCES.....	65
BIOGRAPHICAL SKETCH.....	73

LIST OF FIGURES

1. Skeletal titin structural layout within the sarcomere according to Labeit and Kolmerer (1995)	1
2. The titin interactome: a summary of current known direct and indirect titin interactions according to Linke (2007)	2
3. Four step unfolding of cardiac N2B titin in the extending sarcomere	5
4. Organization and layout of the nonmuscle stress fiber according to Langanger G and Small JV (1986)	9
5. Model for dorsal stress fiber assembly in human osteosarcoma (U2OS) cells according to Hotulainen and Lappalainen (2006).....	10
6. Molecular determination of cellular titin mRNA exon composition using gene specific RT-PCR of CHRF cell total RNA	32
7. Western blot analysis of total protein isolated from human platelets using antibodies raised against bacterially expressed N-terminal and C-terminal regions of titin.....	33
8. Immunofluorescence localization of the cellular titin Z-repeat-Zq region with respect to actin filaments.....	34
9. Immunofluorescence localization of cellular titin kinase domain region with respect to actin filaments	35
10. Histogram of center-to-center distances between labeled Z-repeat and titin kinase epitopes along the stress fibers.....	36
11. Immunofluorescence localization of the cellular titin PEVK domain with respect to actin filaments	37
12. Immunofluorescence localization of the cellular titin Z-repeat-Zq and kinase regions with respect to alpha-actinin.....	38
13. Cellular titin kinase constructs, ctkin4, expression and localization in HeLa cells	50

14. Quantification of ctKin4 constructs displaying a rounded phenotype	51
15. Time lapse imaging of HeLa cell expressing ctKin4-GFP	52
16. Yeast two-hybrid using cellular titin kinase identifies Hax-1 as a novel interacting partner	53
17. Pull down assay using expressed cellular titin kinase to interact with native HeLa cells Hax-1	54
18. Native Hax-1 localization with respect to actin filaments.....	55
19. Apoptosis detection of cells expressing ctKin4	61
20. Cellular titin and myosin localization in activated platelets.....	62

ABSTRACT

We previously discovered a large titin-like protein - c-titin - in chicken epithelial brush border and human blood platelet extracts that binds alpha-actinin and organizes arrays of myosin II bipolar filaments *in vitro*. RT-PCR analysis of total RNA from human megakaryoblastic (CHRF-288-11) and mouse fibroblast (3T3) nonmuscle cells have revealed sequences identical to known titin gene exon sequences that encode parts of the Z-line, I-band, PEVK domain, A-band, and M-line regions of striated muscle titins. These sequences are differentially spliced in patterns not reported for any striated muscle titin isoform. Rabbit polyclonal antibodies raised against expressed protein fragments encoded by the Z-repeat and kinase domain regions react with the c-titin band in Western blot analysis of platelet extracts and immunoprecipitate c-titin in whole platelet extracts. Immunofluorescent localization reveals the majority of the c-titin colocalizes with alpha-actinin and actin in 3T3 and Indian Muntjac deer skin fibroblast stress fibers. Our results suggest that differential expression of titin gene exons in nonmuscle cells yields multiple novel isoforms of the protein c-titin which are incorporated with the actin stress fiber structures.

Titin contains a dually regulated serine/threonine kinase domain belonging to the myosin light chain kinase family localized in the M-line region of titin. Titin kinase has a direct effect on myofibrillogenesis and protein turnover. Our lab has previously reported the identification of a novel nonmuscle titin isoform, cellular titin. Cellular titin localizes to the stress fibers of spread fibroblast and epithelial cells and contains sequence identical to the skeletal titin kinase domain. Expression of the cellular titin kinase domain in epithelial cells localize in a punctate manner previously reported, followed by a breakdown of stress fiber architecture resulting in cell rounding and eventual loss of adhesion to the substratum. Yeast two-hybrid analysis of the titin kinase domain has identified a novel binding partner HS1 associated protein X-1 (Hax-1). Previously identified as anti-apoptotic; its interaction with cortactin, Rac, and G_α13 illustrates Hax-1 as a mediator in stress fiber regulation and organization. Taken together our results suggest a novel role for cellular titin kinase in stress fiber integrity and regulation.

CHAPTER 1

GENERAL INTRODUCTION

Skeletal Muscle Titin Background

The sarcomere, a highly organized array of filament systems containing actin, myosin, and titin, is the basic contractile unit of skeletal and cardiac muscle. Titin, considered the third major filament system of the muscle sarcomere, is a modular protein ranging in size from 2000 kDa. to 4000 kDa, and is approximately 1 μ m long in resting length. Titin spans one-half sarcomere with the N-terminus anchored in the Z-line extending linearly to the center of the M-line (Figure 1).

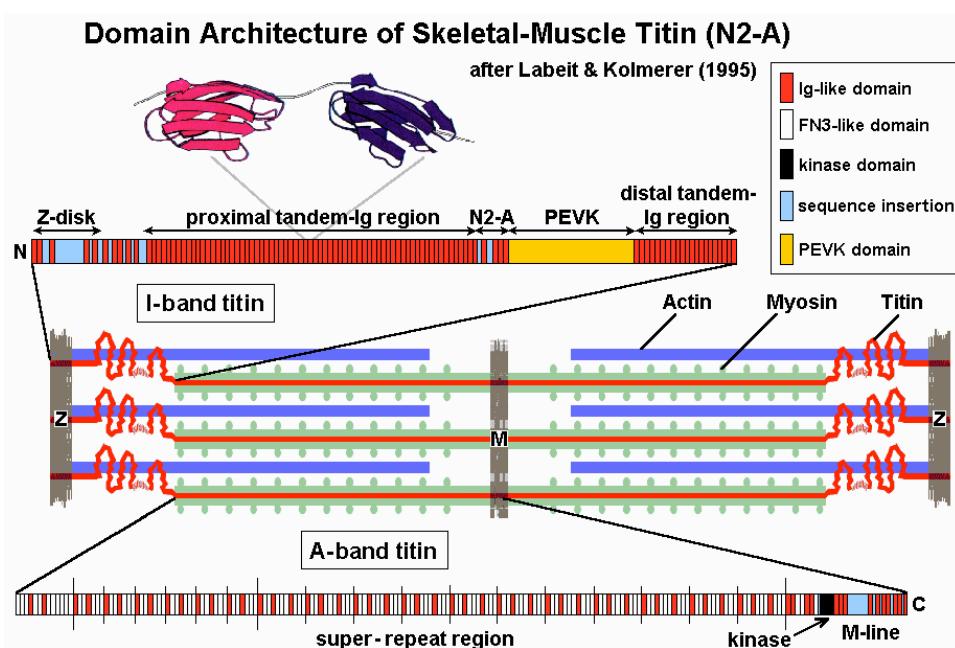


Figure 1. Skeletal titin structural layout within the sarcomere according to Labeit and Kolmerer (1995). The titin N-termini lies in the Z-line anchored to telethonin and alpha-actinin. In the I-band, titin contains tandem arrays of immunoglobulin-like domains and the elastic PEVK region, which can be unfolded and extended. The A-band region of titin is firmly bound to one half of the myosin bipolar filament with the C-termini of titin molecules, containing the kinase domain, localized in the M-line.

Titin is the major “elastic element” within the muscle sarcomere through its unique ability to stretch and retract providing passive tension through intra- and intermolecular interactions. Made up predominantly of immunoglobulin-like (Ig) and fibronectin type-III-like (Fn3) domains attached through short linker segments, titin resembles “beads on a string” when imaged using electron microscopy. Titin contains approximately 240-300 of the 4nm long globular beta-folded Ig and Fn3 domains and the Ig and Fn3 composition predominately governs the slack length of the molecule and thus of the muscle sarcomere (Tskhovrebova and Trinick, 2004).

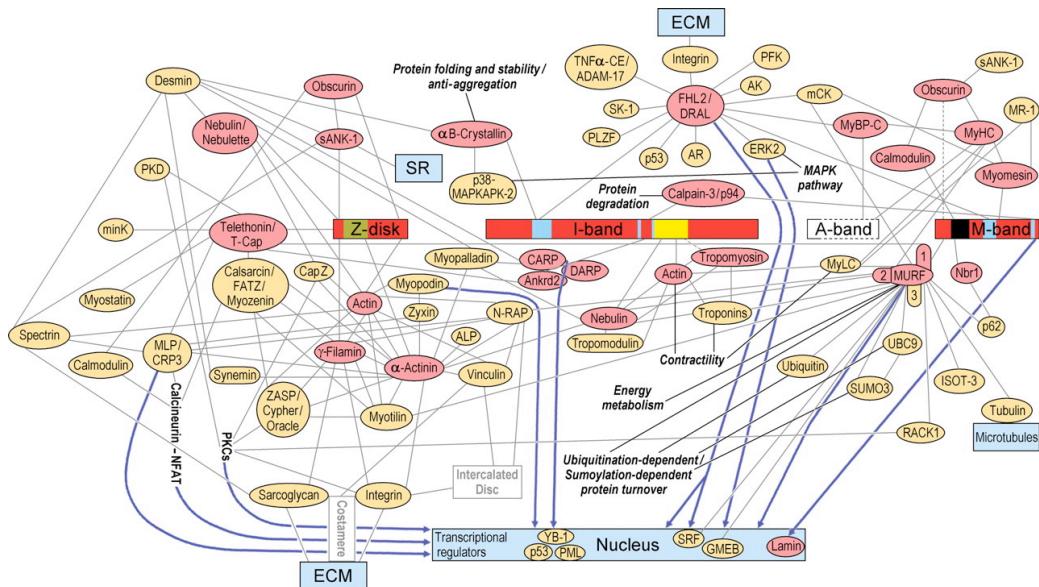


Figure 2. The titin interactome. A summary of the current known direct (red ovals) and indirect (yellow ovals) titin interactions with muscle proteins. (Linke WA 2007).

Through the direct and indirect interactions that have been discovered to date, it is apparent that titin functions as a scaffold that ties the sarcomere together (Figure 2). In the Z-line, titin contains two important Ig domains, Z1 and Z2, which are on the

extreme C-terminal end of the molecule. Two titin molecule Z1Z2 domains interact in a palindromic manner with the Z-line protein telethonin (T-cap) (Zou et al., 2006). This titin-t-cap interaction is hypothesized to be a “spot-weld” anchoring the titin filaments in the Z-disk, conferring an ability to resist strong forces imposed on the Z-line during muscle stretch (Lee et al., 2006). Moreover, just downstream of Z1Z2 are the Z-repeats (Zr), a series of seven differentially spliced unique sequences. The Z-repeat region binds to alpha-actinin, which crosslinks the titin filament with the actin filament. Zr7 appears to be most important in alpha-actinin binding (Sorimachi et al., 1997). The titin-t-cap and titin-alpha-actinin interactions have been shown to be important for proper sarcomere formation and organization, and are what anchors the titin N-terminal region to withstand sarcomere forces.

The I-band region of titin contains serially linked extensible regions that unfold in a stepwise manner (Figure 3). The I-band region of titin is predominantly composed of proximal and distal Ig domains flanking a PEVK region, all of which are differentially spliced (Figure 1). Moreover, the I-band contains two unique regions, the N2B region, also differentially spliced, and the N2A region. The number and composition of the Ig and PEVK domains and the presence or absence of the N2B domain govern the slack length of the titin isoform and the overall stretch length and passive tension of the sarcomere.

The highly extensible and elastic domain adjacent to the N2A region is named PEVK because of the prevalence of the amino acids proline (P), glutamate (E), valine (V), and lysine (K). The PEVK domain contains 110 to 225 exons, which encode domains comprised of 26-28 highly conserved amino acid residues (Greaser, 2001). The PEVK domain, which functions as an entropic spring, is differentially spliced to modulate the elastic nature of the titin molecule. The PEVK is a region of unordered structure and is composed of two distinct motif classes; the PPAK motifs, which are rich in lysine and fold into polyproline type-II left-handed helices in the relaxed sarcomere (Ma et al., 2001), conferring an overall positive charge, and the poly-E-motifs, regions rich in glutamate containing an overall negative charge (Greaser, 2001). PEVK motif charge characteristics are hypothesized to be crucial in determining how the region folds unstretched and how the tension generated within the PEVK region changes in

different ionic strength environments (Ma et al., 2001). The PEVK also may serve as a mechanical and signaling stretch response center. The PEVK region contains many SH3 binding sites, which run in the forward and reverse direction. It is hypothesized that folding and unfolding alters the exposed SH3-containing protein binding sites (Ma et al., 2006). In this regard, the directed inclusion and exclusion of each PEVK exon confer different properties to each titin molecule.

Exclusively expressed in cardiac sarcomeres, N2B is a unique region that can unfold providing an additional level of elasticity (Linke, 2008). In the cardiac sarcomere at slack length, the PEVK and N2B region are folded with the proximal and distal Ig domains resting in an unordered state. As the sarcomere lengthens the Ig domains straighten out, further extension results in PEVK and N2B unfolding to a linear form. The N2B region is hypothesized to be modulated through protein kinase A phosphorylation, which reduces the amount of passive force generated by the titin molecule (Fukuda et al., 2005).

The N2A region, unlike the N2B region, is ubiquitously expressed in all isoforms of titin. N2A is not extensible, but recent reports show it to be a potential signaling center within the sarcomere. The N2A region contains binding sites for p94/calpain 3 and the muscle ankyrin repeat proteins (MARPs), proteins involved in muscle stress-activated pathways which are upregulated in both cardiac and skeletal injury and hypertrophy (Miller et al., 2003). It is hypothesized the N2A-p94/calpain interaction modulates MARP and p94 activity and that p94 binding to N2A titin facilitates proper proteolytic function preventing muscle damage (Hayashi et al., 2008). Interestingly, it has recently been shown that p94/calpain 3 when activated cleaves both MARPs and titin at the N2A region and that loss of binding to N2A results in titin degradation and ultimately muscular dystrophy (Hayashi et al., 2008).

The A-band of the sarcomere contains another filament system, the myosin-II bipolar filament, spanning 1600nm in length. The A-band region of titin spans one-half the length of the bipolar filament, a distance of 800nm and consists of Ig and Fn3 domains in a distinct 7- and 11-domain super-repeat. This super-repeat region binds to the myosin-II tail and is hypothesized to act as a molecular ruler for myosin-II bipolar

filament formation and localization within the sarcomere (Houmeida et al., 1995; Gautel, 1996).

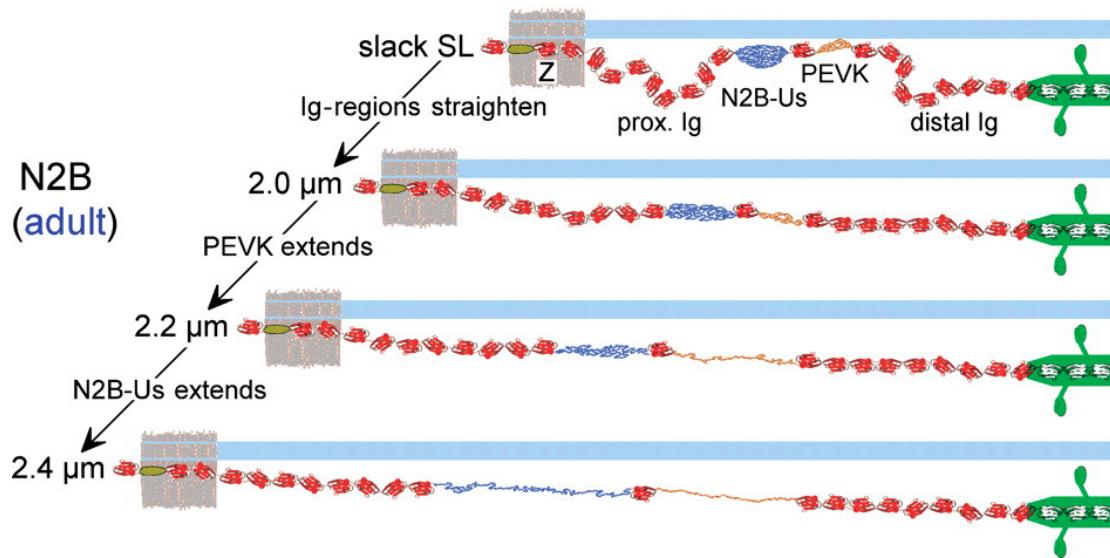


Figure 3. Four step unfolding of cardiac titin N2B isoform in the stretching cardiac sarcomere. At slack length, the extensible N2B unique sequence region and the PEVK domain are both folded and the proximal and distal Ig domains are at rest. Upon sarcomere extension, the Ig domains straighten out, followed by N2B and PEVK unfolding. (Linke WA 2007).

The M-line region of titin contains a serine/threonine kinase domain belonging to the myosin light chain kinase family. It is dually regulated through a mechanism involving Ca^{2+} /calmodulin binding to an inhibitory regulatory tail and an active site tyrosine phosphorylation by an unknown kinase (Mayans et al., 1998). Titin kinase is important in myofibrillogenesis and phosphorylates the Z-line associated protein telethonin (T-cap). The relevance of T-cap being a substrate of titin kinase is yet to be determined, however proper muscle architecture is dependent on titin kinase function (Gregorio et al., 1998; Miller et al., 2003; Musa et al., 2006; Gotthardt et al., 2003). Regions flanking the titin kinase domain also are important in maintaining sarcomere

and muscle stability. Homozygous deletion of the titin M-band region containing titin kinase prevents cardiomyocyte differentiation and myofibrillogenesis (Miller et al., 2003; Musa et al., 2006). Likewise, conditional knockout of the Mex1/Mex2 exons, which encode the kinase domain plus flanking regions, in early mouse development results in embryonic lethality, whereas knockout later in development produces muscle weakness and subsequent death (Gotthardt et al., 2003). Interestingly, these knockout mice display an upregulation of the titin N2A-region binding proteins CARP and ankrd2 proteins which belong to the family of MARPs.

Using kinase-flanking Ig/FN3 domains, which are highly conserved in titin and in the invertebrate titin isoforms projectin and twitchin, for yeast two-hybrid screening revealed the RING finger protein MURF-1 binds to exons A168/A169 just N-terminal to the titin kinase domain (Centner et al., 2001). MURF-1 belongs to a family of RING finger proteins including MURF-2 and MURF-3, which can interact as homodimers and heterodimers, that are involved independent of titin through the ability of MURF-1 to act as a ubiquitin ligase in muscle atrophy through proteosome-dependent degradation of muscle proteins, microtubule stabilization through the ability to form glutamic acid-modified microtubules (Glu-MTs) which exhibit increased stability, and transcriptional regulation through its ability to shuttle between the sarcomere and the myonuclei and interact with serum response factor (Gregorio et al., 2005). MURF-1 interactions with the titin kinase region is important for sarcomeric M-line integrity and through its interaction with glucocorticoid modulatory element binding protein-1 (GMEB-1), a transcriptional regulator, the titin M-line region may be linked to MURF-1 signalling pathways (McElhinny et al., 2002). Together, these data suggest titin kinase and its flanking regions may play crucial roles in sarcomeric integrity and regulation of skeletal and cardiac muscle structure and homeostasis.

It is hypothesized that titin kinase acts as a stress response signalosome through its association with the zinc-finger protein nbr1 which in turn interacts with an nbr1-related zinc-finger protein p62. p62 is found to interact with MURF-2 which has been shown to be able to translocate to the myonuclei and initiate serum response factor-driven transcription of genes important in muscle protein expression and turnover (Lange et al., 2005). Moreover titin kinase *in vitro* can be activated by mechanical

stretch (Grater et al., 2005; Greene et al., 2008) suggesting a possible stretch sensor role. The C-terminal region of titin also contains binding sites for p94/calpain 3 which suggests, like the N2A region, the C-terminus of titin may have a role in muscle protein degradation and turnover (Ojima et al., 2005).

Initially characterized as a filament system and shown to be the important elastic element within the sarcomere, titin is now emerging to be the epicenter of sarcomere organization and regulation. These data implicate titin to be a fundamental protein involved in Z-line architecture, I-band signaling, elastic force generation, A-band myosin organization, and M-line structure and signaling.

Titin Isoforms

The human titin gene is located on the long arm of chromosome 2. The titin gene contains 363 differentially spliced exons that encode a total of 38,138 amino acids. In skeletal muscle, the predominant isoform is the full length N2A-titin with a mass of approximately 4000 MDa and length of 1 μ m. In cardiac muscle, there is a balance between two isoforms of titin: the N2B titin, which is a shorter and stiffer isoform that contains the unique extensible N2B region, a spliced PEVK region encoding 186 amino acids, and is approximately 3 MDa in mass, and the N2BA titin, which is a larger and more compliant (3.2-3.7 MDa) isoform containing both the N2A and N2B domains. The N2BA and N2B isoforms of titin, which govern myocardial stiffness, are expressed in cardiac muscles at varying ratios in different species and during different developmental stages. During cardiac development, the predominant isoform is the more compliant N2BA isoform, however an isoform switch takes place throughout development with a shift to the stiffer N2B titin (Linke, 2008). This molecular switching tunes the cardiac sarcomere function. Titin isoform expression is tissue specific, with a high degree of differential splicing predominantly in the I-band Ig domains and PEVK region.

Titin isoforms have also been found in smooth muscle (Kim and Keller, III, 2002; Labeit et al., 2006) and are part of the smooth muscle contractile apparatus (Kim and Keller, III, 2002). Moreover, titin has been found in the nuclei of drosophila and nonmuscle cells (Zastrow et al., 2006; Machado and Andrew, 2000). It is hypothesized that these nuclear titins are involved in nuclear envelope integrity and chromosome structure. Our lab discovered cellular titin (c-titin) in the chicken intestinal epithelial

brush border and in platelets and demonstrated c-titin is similar to striated muscle titin in structure and binds both nonmuscle myosin-II and alpha-actinin (Eilertsen and Keller, III, 1992; Eilertsen et al., 1994).

The sequencing of the full length human titin gene revealed three novel exons, Novex-1, Novex-2, and Novex-3. Only Novex-3, which contains a cryptic stop codon at the C-terminus of the exon, has been studied in detail. Novex-3 is typically spliced out of the full length titin isoforms, but when expressed it encodes a ~700-kDa isoform that is found in all human muscles. The Novex-3 isoform extends from the Z-disc into the I-band and interacts with the signaling protein obscurin. It is hypothesized that Novex-3 is involved in stretch initiated muscle organization during muscle adaptation and disease (Bang et al., 2001).

Stress Fibers Are Muscle-Like Organizations In Nonmuscle Cells

Stress fibers are highly organized and dynamic structures within the cell that play crucial roles in cell motility, adhesion, and division. Composed of alpha-actinin cross-linked anti-parallel actin filaments interdigitated with myosin-II bipolar filaments (figure 5), stress fibers resemble muscle sarcomeres in both protein composition and organization. Stress fiber organization is regulated through Rho activation in response to activators such as lysophosphatidic acid (LPA) or sphingosine-1 phosphate (S1P). Rho activated kinase (ROCK) and the FH1 domain of the formin protein mDia are downstream Rho effectors which induce stress fiber assembly through myosin-II bipolar filament formation as well as formin induced actin polymerization (Watanabe et al., 1997). It is hypothesized that some forms of stress fibers form from nascent alpha-actinin cross-linked actin filaments polymerized by the formin mDia, after which myosin-II bipolar filaments displace alpha-actinin at distinct spots in a periodic manner (figure 5) (Hotulainen and Lappalainen, 2006).

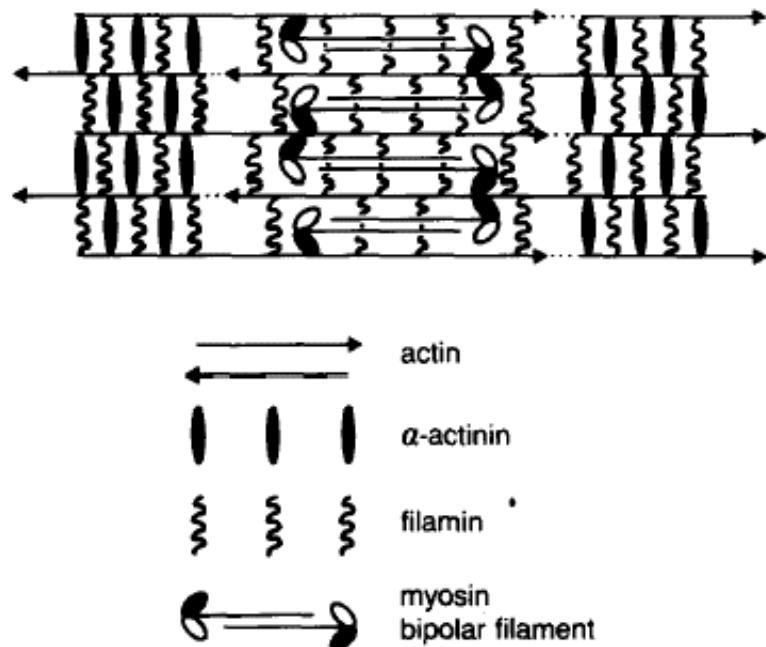


Figure 4. The organization of the nonmuscle stress fiber. Similar in protein composition to the muscle sarcomere, filamin cross-linked anti-parallel actin filaments are bound to alpha-actinin in an alternating periodic fashion with the nonmuscle myosin-II bipolar filament (Langanger G 1986).

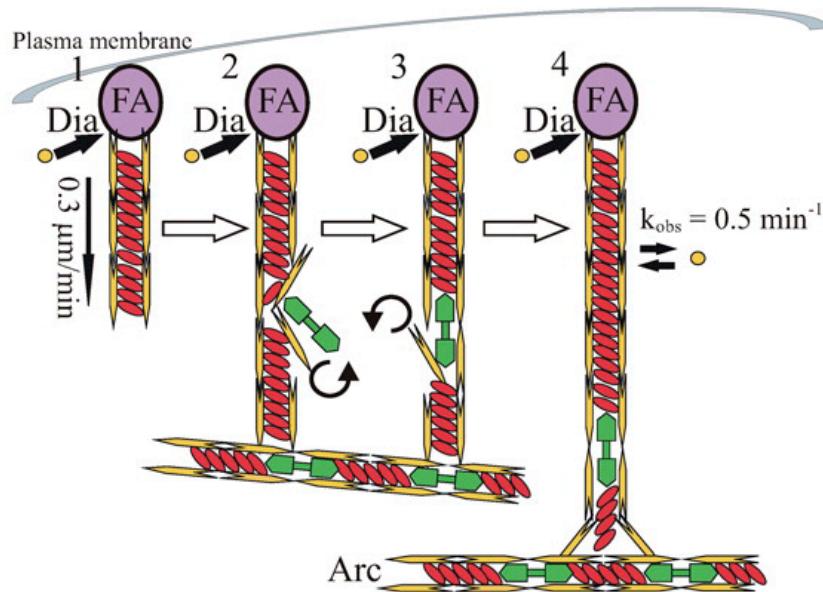


Figure 5. Model for dorsal stress fiber assembly in human osteosarcoma (U2OS) cells. Formin mDia nucleates actin polymerization at the plasma membrane. As actin filaments grow, they are cross-linked through alpha-actinin binding the length of the nascent filament. As the filament grows, myosin incorporates at periodic points along the stress fiber displacing alpha-actinin. Eventually the actin filament fuses with transverse stress fibers (Arc). (Hotulainen and Lappalainen 2006).

Cellular Titin: a Novel Titin Isoform Integral in Stress Fiber Organization and Regulation

We have previously discovered a nonmuscle titin-like protein we named cellular titin, c-titin. This dissertation reports sequence for a novel c-titin isoform encoded by the titin gene on chromosome 2 [Chapter 2 and (Cavnar et al., 2007)] and its involvement in the nonmuscle cell stress fiber apparatus. We have discovered three titin transcripts in total RNA isolated from human megakaryocytes: two full length titin isoforms and the truncated Novex-3 isoform. C-titin exhibits an exon organization in the alpha-actinin binding Z-repeat region, which contains Z-repeats Zr1, Zr2, Zr3, and Zr7. C-titin is also distinctly arranged in the PEVK region, which contains sequence encoded by exons previously reported to be used for only fetal titin isoforms or not previously known to be used at all. These molecular data now confirm our previous hypothesis that nonmuscle cells contain titin. Given the distinct similarity of the stress fiber structure to the muscle

sarcomere this suggests c-titin plays a role in stress fiber organization through the interactions with alpha-actinin and myosin-II.

The two reported full length c-titin isoforms contain the C-terminal titin kinase domain. We have investigated the possible role of cellular titin kinase in regulating the cytoskeleton organization of nonmuscle cells. We show here c-titin kinase is an important element in stress fiber maintenance. Expression of the c-titin kinase domain results in a rapid rounding up phenotype resulting in complete loss of cell shape and adherence. Yeast two-hybrid analysis demonstrated cellular titin kinase interacts with Hax-1, a ubiquitous nonmuscle protein hypothesized to be anti-apoptotic (Cilenti et al., 2004;Lee et al., 2008) and involved in cytoskeletal regulation through its interaction with G α 13, cortactin, and Rac. We report here our findings that cellular titin and its kinase domain are integral to stress fiber organization and maintenance.

CHAPTER 2

MOLECULAR IDENTIFICATION AND LOCALIZATION OF CELLULAR TITIN, A NOVEL TITIN FORM IN THE FIBROBLAST STRESS FIBER.*

Introduction

Cells assemble actin and myosin II filaments into structures such as stress fibers and cleavage furrows to produce contractile force for cell motility (Sandquist et al., 2006), adhesion (Peterson et al., 2004), and division. The organization of stress fibers, in which actin filaments are crosslinked by alpha-actinin and interdigitated with myosin II bipolar filaments, resembles that of muscle sarcomeres (Sanger et al., 1983;Langanger et al., 1986;Hotulainen and Lappalainen, 2006). Like sarcomeres, stress fibers maintain structural integrity while contracting and extending, but stress fibers are more dynamically organized than the stable muscle sarcomeres (Peterson et al., 2004). The long protein titin plays major roles in assembling and maintaining the integrity of the vertebrate muscle sarcomeres, but underlying organizing components of stress fibers remain poorly understood.

Human striated muscles express several muscle type-specific and developmental stage-specific isoforms of titin, all of which are encoded by differential splicing of the 363 exons of the titin gene on chromosome 2 (Warren et al., 2004;Tskhovrebova and Trinick, 2003;Granzier and Labeit, 2002). The longest isoforms span the ~1 μm length across one-half of the sarcomere [for reviews of titin see (Keller, III et al., 2000;Tskhovrebova and Trinick, 2003;Labeit and Kolmerer, 1995)]. The N-terminus of each of these titin molecules is anchored in the sarcomere Z-disk through interaction with telethonin/Tcap (Zou et al., 2006;Gregorio et al., 1998) and with alpha-actinin (Young et al., 1998). The titin C-terminus is anchored in the sarcomere A-band and M-line through interactions with the myosin II thick filament (Houmeida et al.,

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1995;Muhle-Goll et al., 2001), myosin binding protein C (Furst et al., 1992), and myomesin (Obermann et al., 1997).

In the Z-disk, telethonin/Tcap crosslinks titin molecules through interaction with titin Z1 and Z2 Ig domains. Titin Z-repeat domains and the Zq domain interact with alpha-actinin. Differential splicing yields different numbers of the seven possible Z-repeats in different titin isoforms. Other Z-disk domains provide binding sites for a variety of other muscle proteins (Hoshijima, 2006).

In the I-band region of titin, inclusion of the Novex-3 exon upstream of the PEVK region introduces a stop codon that encodes a truncated isoform of titin. The Novex-3 isoform is ubiquitously expressed in skeletal muscle and includes the Z-disk anchorage but lacks most of the I-band region and all of the myosin-filament binding A-band region and M-line kinase domain. Novex-3 interacts with the muscle-specific protein obscurin in the sarcomere I-band (Bang et al., 2001).

In the long titin isoforms, the region of titin that spans the I-band between the Z-disk and the end of the myosin bipolar filament contains serially linked regions that confer passive elasticity in response to physiological stretch. Differential splicing of exons encoding the I-Band region of titins, including the Ig domains and the elastic cardiac titin-specific N2B and PEVK exons, produces isoforms that confer varying degrees of compliance to specific muscle types and are consistent with different sarcomere lengths (Tskhovrebova and Trinick, 2003;Granzier et al., 2000). These elastic regions include a cardiac titin-specific N2B insert, certain Ig domains, and PEVK domains, which are composed predominately of the amino acid residues P, E, V, and K (Bang et al., 2001). There are two distinct types of PEVK domains encoded by individual differentially-spliced exons: Group P exons encode neutral or basic PPAK domains and Group E exons encode acidic polyglutamate-rich domains. The number, specific charge characteristics, and interactions of the domains are important in the overall elasticity of the PEVK segment (Forbes et al., 2005). Additionally, certain PEVK segments contain SH₃-binding motifs that may be important for SH₃-based signaling in the muscle sarcomere (Ma et al., 2006).

The titin C-terminal region contains a kinase domain, which is anchored in the sarcomere M-line. This kinase domain activity is dually regulated by tyrosine

phosphorylation and Ca^{2+} /calmodulin binding (Mayans et al., 1998). In developing muscle, the titin kinase domain is important in myofibrillogenesis (Miller et al., 2003), at least in part because titin kinase can phosphorylate the Z-disk protein telethonin/Tcap (Mayans et al., 1998), but the direct physiological implications of this phosphorylation remain elusive. Titin kinase activity also can be activated by mechanical stretch, supporting the possibility that the titin kinase domain plays a unique signaling role as a force sensor in the M-line region of the muscle sarcomere (Grater et al., 2005). In addition to the kinase domain, the C-terminal region of titin contains binding sites for a variety of proteins that may play roles in muscle protein turnover and gene regulation (McElhinny et al., 2002; Ojima et al., 2005; Lange et al., 2005).

Titin-like proteins and titin isoforms have been found outside striated muscles. Following several reports of a large protein in smooth muscle cells, a protein named smitin because of its similarity to titin was isolated from smooth muscle sources (Kim and Keller, III, 2002). Smitin also interacts with alpha-actinin as well as with both bipolar and side polar smooth muscle myosin II filaments and colocalizes with both proteins in the smooth muscle contractile apparatus (Chi et al., 2005; Kim and Keller, III, 2002). More recently, evidence for expression of bona fide titin isoforms encoded by the titin gene in smooth muscle has been presented (Labeit et al., 2006).

Previously, our lab has identified a titin-like protein (cellular titin, c-titin) in nonmuscle cells, first in the terminal web domain of the intestinal cell brush border cytoskeleton and then in human blood platelets (Keller, III et al., 2000; Eilertsen et al., 1994; Eilertsen and Keller, III, 1992). Characterization of c-titin isolated from brush borders and platelets revealed that it interacts with alpha-actinin and arranges nonmuscle myosin II bipolar filaments in a side-by-side and end-to-end ordered array similar to that of myosin II organization in the stress fibers of nonmuscle cells (Cavnar et al., 2007; Eilertsen et al., 1994; Eilertsen and Keller, III, 1992). An antibody raised against cellular titin demonstrated it colocalizes with nonmuscle myosin II along the stress fibers of fibroblast cells (Eilertsen et al., 1994). Here we report the molecular identification of c-titin as a novel product of the human titin gene on chromosome 2. We demonstrate the presence in human nonmuscle cells of multiple distinct cellular titin isoforms encoded by exon organizations not reported for any known titin. We also

demonstrate that the alpha-actinin-binding Z-repeat region and kinase domain of cellular titin localize in a periodic pattern along the lengths of stress fibers in cultured nonmuscle cells.

Materials and Methods

Cellular Titin Gene Sequence Analysis

The human megakaryoblastic cell line, CHRF-288-11 (Fugman et al., 1990), was used as a source of nonmuscle total RNA. Cells were grown to 1×10^6 cells/ml in Dulbecco's Modified Eagles Medium (Invitrogen Corp.) supplemented with 10% Cosmic Calf Serum (Invitrogen Corp.) in 225 cm² culture flasks (Corning). Cells were grown in suspension and pelleted by centrifugation at 500 x g for 10 minutes. Total RNA was extracted using TRIzol® (Invitrogen Corp.). The total RNA was aliquoted for RT-PCR at a concentration of 1 µg/µl and stored frozen.

RT-PCR primers were designed using the IDT primer quest software (Integrated DNA Technologies, Inc.) and directed against specific exon sequences from the human titin gene using the titin exon map reported in [(Bang et al., 2001) and <http://www.embl-heidelberg.de/ExternalInfo/Titin/genomic/titin-page-genomic1.html>]. RT-PCR reactions were performed with SuperScript™ Reverse Transcriptase III (Invitrogen Corp.) at 55°C for 50 minutes. PCR reactions were run with Platinum® Taq Polymerase (Invitrogen Corp.) at melting temperatures governed by the individual primer pairs used. Reactions typically were carried out for 30 cycles with extension times of 1 minute for every one thousand bases of predicted product. Sizes of the PCR products were determined using agarose gel electrophoresis by comparison to various DNA ladders.

Products of interest were purified either from the reaction solution directly with a PCR Purification kit (Qiagen, Inc.) or if multiple bands were present from excised gel slices with a Gel Extraction Purification kit (Qiagen, Inc.). PCR products were sequenced with the primers used to generate the product. The nucleotide sequences were analyzed for the presence of titin gene exon-exon junctions using the human titin gene map. Exons were judged to be absent only when PCR products contained junctions between flanking exons.

Rabbit Polyclonal Antibody Production

RT-PCR was performed to yield two distinct products of human cellular titin gene sequence to use for antibody production. The cellular titin Z-repeat region product contained the code for amino acids 398-554,694-963 (GenBank NP_596869; N2A isoform amino acid numbering). This product was cloned into the multiple cloning site of the pET41 vector for expression. The cellular titin kinase domain product contained the code for amino acids 31202-31371 (GenBank NP_596869; N2A amino acid numbering). This product was cloned into the multiple cloning site of the pGEX-2T vector for expression. Both fragments were expressed as GST-fusion proteins in *E. coli* strain BL21 (DE3), and the GST-fusion proteins were partially purified using glutathione-agarose (Sigma-Aldrich) column chromatography. Each protein was further purified by SDS-PAGE. Target bands were excised from the SDS gel, macerated in PBS, and injected into rabbits. Two booster injections were given over the course of 1 month. IgG was isolated from rabbit serum by affinity chromatography using protein A conjugated agarose beads (Pierce Chemical Company). The reactivity of each antibody was tested by Western Blot analysis of the expressed GST-fusion proteins.

Western Blot Analysis of Total Platelet Extract

Human platelet rich plasma was obtained from Southeastern Community Blood Center (Tallahassee, FL). Platelets were further purified by centrifuging the plasma at 1400 x g for 15 minutes to pellet any remaining white or red blood cells. The platelet rich plasma supernatant was decanted and the calpain protease inhibitor calpeptin (ICN Biomedicals, Inc.) was added to a final concentration of 0.5 µg/ml. The platelet rich plasma was incubated at room temperature for 10 minutes before pelleting the platelets by centrifugation at 2400 x g for 15 minutes. The platelet pellet was washed once by gentle resuspension in Tyrodes buffer (140 mM NaCl, 3 mM KCl, 12 mM NaHCO₃, 1 mM MgCl₂, 0.4 mM NaH₂PO₄, 0.4 mM Na₂HPO₄, 0.35% BSA, 0.1% D-Glucose, 2 mM CaCl₂, brought to pH 7.33 with 1 M HEPES) and pelleted again at 2400 x g for 15 minutes. The platelet pellet was frozen in liquid nitrogen and stored at -80°C. For protein extraction, the platelet pellet was thawed on ice, refrozen in liquid nitrogen and thawed again on ice before resuspension in 10 volumes of extraction buffer (500 mM KCl, 10 mM EDTA, 10 mM EGTA, 10 mM Imidazole, 0.2 mM DTT, 5 mM ATP, 0.2

mM PMSF, 0.05 mM Leupeptin, pH 7.3) at 4°C. After 15 minutes on ice, the extract was clarified by centrifugation at 35,000 x g for 15 minutes.

The clarified extract was fractionated on a 4%-20% SDS-PAGE and transferred to nitrocellulose overnight at 30 V for 8 hours in 4°C. To assess the transfer of cellular titin, blots containing total platelet extract were stained for protein content using Ponceau S (2% Ponceau S, 30% Trichloroacetic acid, 30% sulfosalicylic acid). The blots were blocked for 2 hours in 5% non-fat dry milk (Nestlé USA) in T-PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 0.1% Tween-20, pH 7.4). Blots were subsequently incubated with the anti-titin kinase rabbit polyclonal antibody (TKK) at a concentration of 1:1000 for 1 hour in 5% non-fat dry milk in T-PBS. Blots were washed rapidly with 3 changes of T-PBS, followed by a 15 minute wash with gentle agitation, and then 3 more washes for 5 minutes with gentle agitation all with T-PBS. Anti-rabbit IgG peroxidase conjugated secondary antibodies (Sigma-Aldrich) then were incubated with the blots at a concentration of 1:75,000 for 1 hour in 5% non-fat dry milk in T-PBS. Blots were washed as described after primary antibody incubations, and chemiluminescent signal was detected using ECL detection substrate (Pierce Chemical Company).

Immunoprecipitation of Cellular Titin

Platelet pellets were washed and lysed as described above. Platelet total protein was extracted (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.2 mM PMSF, and 0.05 mM leupeptin, pH 8.0) and clarified by centrifugation at 35,000 x g for 15 minutes. Proteins that bound nonspecifically to the beads were removed by incubating the extract with Protein G-conjugated agarose beads (Pierce Chemical Company) for 1 hour at 4°C and pelleting the beads at 14,000 x g for 10 minutes. The supernatant was transferred to a fresh tube and incubated at 4°C for 1 hour with the anti-titin antibody TKZ at a concentration of 5-10 µg/ml. Protein G-conjugated agarose beads were added to the solution and incubated for 1 hour at 4°C before pelleting the beads by centrifugation at 10,000 x g for 30 seconds. The supernatant was discarded and the bead pellet was washed 5 times by resuspension in extract buffer and pelleting by centrifugation. The washed bead pellet was suspended in a small volume of 1x SDS sample buffer, heated at 90°C for 10 minutes, and centrifuged at 14,000 x g for 5 minutes. The SDS solution

was then fractionated by 4%-20% SDS-PAGE, blotted onto nitrocellulose membranes overnight, and analyzed using the anti-titin TKK antibody, as described previously.

Indirect Immunofluorescence of Cultured Deer Skin Fibroblasts and Mouse Fibroblasts

Indian Muntjac cells (ATTC #CCL-157), or NIH 3T3 cells were grown on No. 1.5 glass coverslips (Fisher Scientific) in Ham's F10 media (HyClone) supplemented with 10% fetal bovine serum (Invitrogen Corp.) until they reached roughly 70% confluence. The cells were washed twice with cytoskeletal buffer (60 mM PIPES, 27 mM HEPES, 10 mM EGTA, 4 mM MgSO₄, pH 7.0) for 5 minutes at 37°C. The coverslips were gently agitated in this and all subsequent incubation steps. The cells were fixed for 10 minutes at 37°C using freshly prepared 3.7% paraformaldehyde in a 1:1 mixture of PBS (137 mM NaCl, 2.7 mM KCL, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) and Ham's F10 medium (HyClone). The coverslips were then washed with three changes of cytoskeletal buffer at room temperature for 5 minutes. The cell membranes were permeabilized with 0.2% Triton-X-100 in PBS for 15 minutes at room temperature. After permeabilization, coverslips were washed with one change of cytoskeletal buffer for 5 minutes at room temperature and then with 2 additional washes of PBS for 5 minutes each at room temperature. Cells were incubated in blocking buffer (10% normal goat serum (Sigma-Aldrich) and 0.05% Triton-X-100 in PBS) for 60 minutes at room temperature. Primary antibodies were diluted in a PBS solution containing 5% normal goat serum, 0.05% Triton-X-100. The coverslips were incubated with the primary antibody cocktail at 37°C for 1 hour in a humidified chamber. After primary antibody incubations, the cells were washed 3 times in wash buffer (1% normal goat serum, 0.05% Triton-X-100, and PBS) for 5 minutes at room temperature. Coverslips were incubated for 1 hour at 37°C in a humidified chamber with secondary antibodies and Phalloidin diluted in the blocking solution. The coverslips were washed 3 times with wash buffer and 2 times with dH₂O for 5 minutes prior to mounting onto glass microscope slides using ProLong® Gold mounting medium (Invitrogen Corp.).

Microscopy

Immunofluorescence microscopy was performed using a Zeiss 510 LDM confocal microscope or the DeltaVisionRT Restoration Imaging system (Applied

Precision, Inc.) using an Olympus IX71 inverted microscope with a PLAN-APO 60x Oil immersion objective, 1.4NA. Images were captured using the CoolSnap CCD camera (Roper Scientific, Inc.) and colocalization analysis was performed using softWoRx® imaging software. Distance measurements were performed using softWoRx® imaging software and bands of staining were measured from the center of one to the center of the next using the phalloidin stained stress fiber as a track.

Results

RT-PCR Analysis Illustrates Cellular Titin is a Novel Isoform Encoded by the Titin Gene

Total RNA was isolated from the human megakaryoblastic CHRF-288-11 cell line (Fugman et al., 1990), and analyzed for the presence of human chromosome 2 titin gene transcripts by RT-PCR analysis using primers based on human titin gene exon sequences [(Bang et al., 2001) and <http://www.embl-heidelberg.de/ExternalInfo/Titin/genomic/titin-page-genomic1.html>]. Sequences of the RT-PCR products were compared to the known titin gene exon sequences (Figure 6). The sequences of all titin gene products obtained contained exon-exon junctions and no intron sequence, indicating spliced mRNA was the source of the sequence. Of the 363 known titin gene exons, sequence from 218 exons has been confirmed to be present in or absent from the nonmuscle cell mRNA. Key Z-disk, I-band, A-band, and M-line functional regions of striated muscle titins are present in the nonmuscle cellular titin isoforms, but at least two unique patterns of exon usage were found, both of which differ from those of all reported titin gene transcripts, indicating the nonmuscle cell protein products represent novel titin isoforms (Figure 6).

RT-PCR products covering the exons encoding the Z-disk domain of striated muscle titin isoforms contain the sequence of exons 2 through 4, which encode the Z1 and Z2 domains that interact with muscle-specific telethonin/Tcap and enable telethonin/Tcap to crosslink the N-termini of titin molecules in the muscle sarcomere Z-disk (Gregorio et al., 1998;Zou et al., 2006). These products also contain sequence from exons 8-10 spliced directly to exons 14-15, which encode the 45-residue Z-repeat

domains Zr1, 2, 3, and 7 and the Zq domain that bind to alpha-actinin (Sorimachi et al., 1997; Young and Gautel, 2000; Atkinson et al., 2000).

In the region equivalent to that spanning the I-band in striated muscle, cellular titin RT-PCR products contain the exons that encode the N2A domain (Linke et al., 1996; Labeit and Kolmerer, 1995), which in striated muscle titin binds muscle-specific p94/Calpain 3 (Ojima et al., 2005). RT-PCR products revealing a junction between exons 44 and 50 confirm lack of the extensible cardiac titin isoform-specific N2B region (Helmes et al., 1999; Labeit and Kolmerer, 1995). Likewise, no products contained sequence from the Novex-1 domain-encoding exon 45 or the Novex-2 domain-encoding exon 46.

Sequences from a majority of PEVK-encoding exons were present in the nonmuscle cell RT-PCR products. Interestingly, several of these exon sequences never have been reported to be present in any striated muscle titin isoform sequence and several others have been found previously only in fetal muscle titin isoform sequences (Gutierrez-Cruz et al., 2001). Moreover, unique patterns of differential exon splicing were found in two PEVK regions (Figure 6, two color exons), indicating the presence of at least two cellular titin isoforms in cultured CHRF cells. These two regions of differential splicing have yet to be connected in continuous RT-PCR products, leaving open the question of the number of isoforms encoded and the actual PEVK sequence of each isoform. Several PEVK exons, many of which belong to the group P exons, have been confirmed to be missing from the nonmuscle cell RT-PCR products covering this region.

In the equivalent of the A-band region of striated muscle titin, the cellular titin sequence resembles that of the major isoforms of skeletal muscle titin containing the D- and C-zone immunoglobulin and fibronectin III domain super-repeats, which interact with one pole of the myosin II bipolar filament (Labeit and Kolmerer, 1995; Muhle-Goll et al., 2001; Houmeida et al., 1995). Cellular titin also contains the titin kinase domain and surrounding sequence, which is localized in the sarcomere M-Line.

RT-PCR using an exon 48-specific primer revealed the existence of a Novex-3 transcript in CHRF cells. The sequence of the Novex-3 exon 48 contains an in frame stop codon followed by an additional 76 bases of gene sequence before a canonical

Polyadenylation signal. Inclusion of the Novex-3 exon encodes a 700 kDa isoform of titin known as Novex-3 (Bang et al., 2001). PCR products from first strand cDNA transcribed using the Novex-3 primer exhibited the same Z-repeat region splicing pattern of inclusion of Zr1, 2, 3, and 7 found in the longer cellular titin transcripts.

Western Blot Analysis of Human Platelet Cellular Titin

Two RT-PCR products encoding the Z-repeat region and part of the kinase domain were cloned and expressed in bacteria as GST-fusion proteins. The expressed Z-repeat fragment consisted of sequence from exons 7-10 and 14-18 encoding Z-repeats 1, 2, 3, and 7 (N2A amino acids 398-554), the Zq domain, and additional unique sequence out to the Ig domain encoded by exon 18 (N2A amino acids 694-963). The expressed kinase domain fragment contains a 169 residues (N2A amino acid numbering 31,202-31,371) encoded by sequence within exon 358. The expressed proteins were purified and used to raise the rabbit polyclonal antibodies against the Z-repeat region, TKZ, and against the kinase domain region, TKK. Both the TKZ and TKK antibodies reacted specifically in Western blot analysis with the high molecular weight protein c-titin band in blots of human platelet extracts fractionated by 4%-20% SDS-PAGE (Figure 7), confirming that the N- and C-terminal domains found by RT-PCR analysis are present in the native c-titin protein. The relative Western blot reactivity of the TKK antibody (Figure 7, lane 3) was much stronger than that of the TKZ antibody, which was weak and difficult to document directly. Because of this, the reactivity of the TKZ antibody was confirmed by using it to immunoprecipitate cellular titin from platelet extracts and to confirm the cellular titin immunoprecipitation by Western blot analysis with the TKK antibody (Figure 7, Lane 2).

Immunolocalization of Cellular Titin in Cells

The TKZ and TKK rabbit polyclonal antibodies and the commercially available 9D10 mouse monoclonal antibody were used to immunofluorescently localize three regions of cellular titin in deer skin fibroblasts, which assemble robust stress fibers. Double labeling the cells with the TKZ antibody (Figure 8B) and phalloidin for actin filaments (Figure 8A) reveals that most of the Z-repeat region of cellular titin is localized in a periodic pattern along the stress fibers (Figure 8D). Most of the distances between bands of TKZ signal throughout the cell range from 500 nm to 1.2 μ m (Figure 10A).

Double labeling the fibroblasts with the TKK antibody and phalloidin demonstrated that much of the cellular titin kinase domain labeling (Figure 9B) also is associated with the actin-based stress fibers (Figure 9C) in a periodic pattern. The TKK antibody labeling pattern associated with the stress fibers (Figure 9D) is less ordered than that of the TKZ antibody, suggesting that the kinase domain may be tethered to the stress fiber but able to extend out from the core structure of the stress fiber into the cytoplasm. As with the TKZ staining, most of the distances between bands of TKK signal throughout the cell also range from 500 nm to 1.2 μ m (Figure 10B).

The 9D10 monoclonal antibody (Wang and Greaser, 1985) binds to two epitopes in the PEVK region of skeletal titin, which are located near the A/I-junction in striated muscle. In the fibroblasts, some of the 9D10 antibody staining was punctate and aligned along the stress fibers (Figure 11). As with the TKK and TKZ antibodies, some of the punctate 9D10 staining appears to be aligned in arrays that are not coincident with the stress fibers. 3T3 mouse fibroblast cells were used to compare the immunolocalization of the cellular titin domains and alpha-actinin in stress fibers (Figure 12). The anti-titin Z-repeat polyclonal antibody TKZ displays a periodic localization, which in many cases is close but not perfectly coincident with the alpha-actinin-containing dense bodies of the stress fibers (Figure 12A). Moreover, there is a significant amount of staining throughout the cell body not directly associated with the stress fibers. This may result from the polyclonal TKZ binding to multiple epitopes on cellular titin. TKK displays a similar periodic distribution pattern (Figure 12B) as seen previously (Figure 9), and which alternates with the alpha-actinin bands along the stress fibers.

Discussion

Cellular Titin- a Titin Isoform

We previously isolated a very high molecular weight protein from extracts of intestinal epithelial cell brush borders (Eilertsen et al., 1994; Eilertsen and Keller, III, 1992) and human blood platelets (Keller, III et al., 2000). This protein was named cellular titin (c-titin) because of its similarity in size and molecular morphology to striated

muscle titins. C-titin migrates only slightly faster than striated muscle titin on SDS-PAGE, and both c-titin and striated muscle titin molecules are ~1 μ m long linear molecules. In addition, c-titin organizes nonmuscle myosin II bipolar filaments end-to-end and side-by-side in striated arrays in vitro and interacts with alpha-actinin (Eilertsen et al., 1997), as might be expected of a titin-like protein. Despite similarities between the c-titin and striated muscle titin proteins, the molecular identity of c-titin remained a mystery because of difficulty in obtaining and purifying quantities of c-titin sufficient for protein sequence determination. Moreover, several investigations failed to find evidence for titin in nonmuscle cells (Bang et al., 2001; Labeit et al., 2006). To test more directly whether nonmuscle cells contain transcripts from the titin gene on chromosome 2, we used RT-PCR analysis with gene-specific primers to assay for titin transcripts in total RNA isolated from the CHRF-288-11 human megakaryocyte cell line. Products obtained from this RT-PCR analysis confirmed the presence of titin gene-encoded mRNA in the nonmuscle cells. Screening over 60% of the titin gene exon sequence has confirmed the presence and absence of specific exon sequence in CHRF-288-11 cell cDNA lacking intron sequence and containing exon-exon junctions.

To determine whether the cDNA products represent sequence that encodes the c-titin protein, we raised rabbit polyclonal antibodies against bacterially-expressed peptides representing two distinct regions of titin; the Z-repeat region to the first I-band Ig domain (TKZ), and a region within the titin kinase domain (TKK). Both the TKZ and TKK antibodies reacted with the high molecular weight c-titin protein in total platelet extracts. The TKK antibody Western analysis signal was strong and specific for the c-titin protein. In contrast, the TKZ antibody signal was very weak and sometimes undetectable. Although its reactivity with blotted SDS-denatured c-titin was weak, the TKZ antibody immunoprecipitated c-titin from platelet extracts (Figure 7, lane 2) and yielded strong immunofluorescence localization signal. Overall, the Western and immunoprecipitation analyses with the TKZ and TKK antibodies provide compelling evidence that the c-titin protein contains Z-repeat and kinase domains encoded by the titin gene and strongly suggests the RT-PCR products represent sequence encoding c-titin. It has been reported that a ~500 kDa splice isoform comprising the C-terminus of

titin might be present in the nucleus of nonmuscle cells (Zastrow et al., 2006); however a protein of this size has not been found in platelet extracts using our TKK antibody.

Positive reactivity of the TKZ and TKK antibodies with c-titin raises the question of why monoclonal antibodies against striated muscle titin previously have failed to confirm the presence of titin isoforms in nonmuscle cells. Indeed, our Western blot analysis of c-titin from intestinal epithelial cells failed to demonstrate reactivity with the 9D10 antibody (Eilertsen et al., 1994). In this investigation, we confirmed poor reactivity of the 9D10 antibody with platelet c-titin in Western blot analysis (data not shown) but found immunofluorescence signal consistent with localization of c-titin in the nonmuscle cells. The source of this discrepancy may be the relatively low abundance of c-titin in nonmuscle cells compared to that of titin in striated muscle. The low abundance of c-titin makes it difficult to blot sufficient molar equivalents to detect with 9D10 and other monoclonal antibodies. Moreover, titin isoforms in nonmuscle cells may be missing some of the epitopes anti-striated muscle titin antibodies are directed against.

Analysis of the megakaryocyte RT-PCR products presented here, however, reveals that c-titin contains several of the domains known to be important for the function of striated muscle titins, including domains from the Z-disk, I-band, A-band, and M-line regions of titin. From the Z-disk region, the RT-PCR products contain sequence encoding the titin Z1 and Z2 immunoglobulin domains. In the muscle sarcomere, the Z1Z2 domains of adjacent titin molecules interact in a palindromic manner with the 19 kDa. protein telethonin/Tcap (Zou et al., 2006), which plays crucial roles in titin Z-disk anchorage through its association with FATZ (Faulkner et al., 2001) and in a signaling pathway through its interaction with myostatin, a key muscle growth factor (Nicholas et al., 2002). No telethonin/Tcap or telethonin/Tcap-like protein has been found in nonmuscle cells, raising the possibility that in nonmuscle cells a yet to be identified cellular titin binding partner plays roles similar to those of telethonin/Tcap.

Cellular titin also contains N-terminal region Z-repeat sequences Zr1-3+Zr7 and the Zq domain. The Z-repeats are a series of seven 45 residue repeats, which in muscle titins are localized in the Z-disk and differentially spliced in different muscles (Gautel et al., 1996). The Zr1-3+Zr7 pattern of splicing has been reported previously for mouse psoas muscle and human fetal cardiac muscle (Sorimachi et al., 1997), and the

Zr3+Zr7 junction is reported in a database entry for a human skeletal muscle cDNA sequence clone (GenBank BF670351). At least one of the functions of the Z-repeat domains is to interact with the Act-EF34 domain at the C-terminus of each peptide of the alpha-actinin antiparallel dimer. Of the Z-repeats, Zr1 and Zr7 have the highest affinities for alpha-actinin binding (K_{DS} in the 100-300 nm range in vitro). Z-repeat Zr2 has relatively low affinity for alpha-actinin (K_D in the millimolar range) and Zr3 has an intermediate affinity. The Zq domain interacts with the alpha-actinin rod (Young et al., 1998). Inclusion of the Zr1, Zr7, and Zq domains in cellular titin, therefore, is consistent with our previous reports of native c-titin-alpha-actinin interaction in vitro (Keller, III et al., 2000;Eilertsen et al., 1997).

The RT-PCR products reveal that cellular titin also contains sequence matching that of the region of striated muscle titin in the Z/I-junction and N1 line of the sarcomere. This includes the sequence of exon 28, which encodes several unique binding domains. The Z9 and Z10 Ig domains encoded by exon 28, for example, bind obscurin, a large 800 kDa Rho guanine nucleotide exchange factor important in sarcomere assembly and association with the sarcoplasmic reticulum (Kontogianni-Konstantopoulos et al., 2006;Young et al., 2001). Obscurin has not been found outside striated muscle, raising the question of whether this region interacts with any protein in nonmuscle cells. The N1-line region of striated muscle titin also binds actin (Linke et al., 1997) and Ca^{2+} with a high affinity (Coulis et al., 2004). Whether cellular titin interacts directly with actin filaments and Ca^{2+} remains to be determined.

The serially linked tandem Ig domains, the cardiac titin-specific N2B domain, and the PEVK domains comprise elastic spring elements in the I-band region of striated muscle titins (Linke et al., 1996;Linke et al., 1999;Witt et al., 1998). Through alternative splicing of these various spring elements, titin isoforms establish elastic properties of various muscle types (Warren et al., 2004;Granzier and Labeit, 2002). The nonmuscle cell RT-PCR products confirm the absence of the N2B domain and the presence of proximal I-band Ig domains and unique arrays of PEVK domains. Much of the elasticity of skeletal muscle titin derives from the PEVK region, which is encoded by a series of differentially spliced exons each encoding a modular domain of ~28 amino acids. The PEVK exons encode two classes of domains: group P domains contain high levels of

the amino acid residues proline (P), glutamate (E), valine (V), and lysine (K) and have pl_s of ~9; group E domains are rich in glutamate (E) residues and have a pI of ~4 (Forbes et al., 2005). Conformational studies have shown PEVK domains fold as polyproline type II left-handed helices, beta-turns, and unordered coils (Ma and Wang, 2003). The overall PEVK region, therefore, is a flexible chain that can be extended and individual domains can be unfolded when stretched.

Some PEVK exons have been found expressed only in adult striated muscles, where the titin isoform specific pattern of differential splicing and overall length of the PEVK domain determines the extensibility of each titin molecule and thereby directly affects the passive tension of the muscle (Granzier et al., 2000). Other PEVK exons have been found expressed only in fetal muscles, whereas expression of other PEVK exons has yet to be reported. The nonmuscle cell RT-PCR products reported here reveal two unique patterns of PEVK domains including exons expressed in adult striated muscle titins, fetal muscle titins and others not previously found expressed. Cellular titin has two differentially spliced PEVK regions, one near the middle and one near the C-terminal end of the PEVK region. Each of the regions involves differential inclusion or exclusion of three Group P and two Group E exons with SH₃-binding domains. Whether one isoform includes all the exons and the other excludes all the exons making the PEVK region of one isoform significantly longer than the other or whether the differential splicing is reciprocal making the region a similar length but perhaps introducing a subtle difference in certain positions along the region in each isoform remains to be determined. Moreover, there are other distinct single exon exclusions in both isoforms, all of which are of the group P class of exons spread throughout the PEVK regions in the cellular titins, which have yet to be understood.

The physiological significance of these isoforms exhibited within the PEVK remains unclear but suggests that cellular titin contains unique elastic properties in nonmuscle cells. Extensibility of cellular titin likely contributes directly to passive tension of stress fibers, which stretch and contract along their length in a manner similar to that of sarcomeres (Katoh et al., 1998). The SH₃ binding domains present in some PEVK domains, including those found here in cellular titin may confer an important signaling role within this region of titin (Ma et al., 2006). Whether or not these SH₃

binding domains are important *in vivo* and whether cellular titin exhibits binding to nonmuscle SH3 domain-containing proteins has yet to be determined, but presents an interesting possibility for cellular titin-based signaling. Also, remaining to be determined is why there are (at least) two isoforms of cellular titin that differ in the PEVK region. Megakaryocytes produce platelets, which we have shown contain cellular titin. It is conceivable that one of the cellular titin isoforms functions in the megakaryocyte, and the other isoform is produced to function in the platelets. Analysis of cellular titin isoform expression from other sources and development of isoform specific probes will help resolve this issue.

In the A-band region of striated muscle titins, two tandem super-repeating patterns of Ig and Fn3 domains comprise the region of the protein that binds to the myosin II bipolar thick filament in the sarcomere (Houmeida et al., 1995; Muhle-Goll et al., 2001). The RT-PCR products found here reveal that cellular titins resemble the major isoforms of striated muscle titin in this A-band region. This molecular evidence for inclusion of a myosin bipolar filament binding region in cellular titin supports our previous observations that native cellular titin interacts with and organizes nonmuscle myosin bipolar filaments in striated arrays *in vitro* and that cellular titin is localized in stress fibers where myosin bipolar filaments are organized with sarcomere-like periodicity (Eilertsen et al., 1994; Langanger et al., 1986). Lack of a significant difference between cellular titins and striated muscle titins in this region, however, fails to account for our previous observation that cellular and muscle titins and myosins interact in an isoform-specific manner (Eilertsen et al., 1994). Moreover, striated muscle titin has been proposed to provide a ruler for templating the size of the ~1 μm long striated muscle bipolar filaments (Trinick, 1994) and nonmuscle myosin bipolar filaments are considerably smaller and shorter (~300 nm long). Resolution of this discrepancy requires further investigation.

The nonmuscle cell RT-PCR products also reveal inclusion of sequence from exon 358, which encodes a kinase domain located in the M-line of the mature striated muscle sarcomere. The activity of this kinase domain is dually regulated by binding of a Ca^{2+} -regulated protein such as calmodulin and phosphorylation of an active site tyrosine by an unknown kinase. The titin kinase domain activity also can be activated by

physical extension (Grater et al., 2005). One function of the titin kinase domain activity in developing muscle is to regulate the timing of myofibrillogenesis by phosphorylating telethonin/Tcap, a titin binding protein localized in the mature sarcomere Z-disk (Mayans et al., 1998). In the mature sarcomere, where telethonin/Tcap is located in the Z-disk and the kinase domain is located in the M-line, stretch activation of the kinase domain activity may initiate signaling through phosphorylation of other unknown substrates. Inclusion of the kinase domain in cellular titin raises the intriguing possibilities that it plays roles in regulating when and where in cells stress fibers are assembled, and in stretch-dependent signaling from those stress fibers once formed. Cellular titin kinase activity therefore may play a major role in how cells sense stress and strain forces. Understanding such a signaling mechanism will require identifying nonmuscle cell substrates of the cellular titin kinase.

Interestingly, the cellular titin isoforms described here contain many of the sites in striated muscle titins known to bind other proteins. Except for alpha-actinin and myosin II (which have nonmuscle homologues), however, most of the striated muscle titin-binding proteins, including telethonin/Tcap, p94/calpain 3, and obscurin have been found only in muscle. It will be important to determine whether these known binding sites in cellular titin bind nonmuscle proteins with functions similar to those of the muscle proteins or whether undiscovered sites mediate interactions with cellular proteins whose roles are specific to the nonmuscle cell cytoskeleton. The organization of some nonmuscle actin-myosin II contractile structures such as the belt desmosome-associated apical rings in columnar epithelial cells is stable like that of muscle sarcomeres. The organization of others, such as stress fibers and the cleavage furrow contractile ring, is dynamic. Roles for cellular titin in assembly and disassembly of these dynamic structures are likely to be highly regulated directly or indirectly by local cytoplasmic conditions and the Rho-based signaling pathway that promotes stress fiber formation. Possibilities for even greater roles for cellular titin in stretch sensing and gene regulation remain to be explored.

Of the three exons designated Novex-1, -2, and -3 immediately preceding the N2B exon in the titin gene, we found evidence in the nonmuscle cell RT-PCR products for expression of only the Novex-3 exon sequence. The N-terminal region of the Novex-

3 protein resembles that of other titin isoforms, but the Novex-3 exon contains an in frame stop signal and a canonical polyadenylation signal. Inclusion of this exon produces a ~700 kDa titin isoform, which is anchored in the Z-disk and projects into the I-band where it binds to obscurin. The titin/obscurin complex may be important in the organization of myosin in the A-band (Kontogianni-Konstantopoulos et al., 2004), in maintaining the overall integrity of the sarcomere (Kontogianni-Konstantopoulos and Bloch, 2005), as well as in SH₃ and GTPase signaling (Bang et al., 2001). The TKZ antibody failed to reveal a 700 kDa protein in the western blot analysis presented here, suggesting that Novex-3 is a rare protein in nonmuscle cells.

Localization of Cellular Titin in Nonmuscle Cells

Previous immunofluorescence and immunogold localization analysis demonstrated localization of c-titin in fibroblast stress fibers, as well as in the intestinal epithelial cell brush border terminal web domains from which c-titin was originally isolated (Keller, III et al., 2000). Development of the TKZ anti-Z-repeat region antibody and the TKK anti-kinase domain antibody provided probes for investigating the layout of confirmed N- and C-terminal regions of cellular titin in cells. Because CHRF-288-11 megakaryocyte cells spread poorly in culture and assemble stress fibers that are suboptimal for analysis, we investigated the localization of the N- and C-terminal regions of cellular titin in Muntjac deer skin fibroblasts and 3T3 cells, both of which spread well and assemble robust stress fibers in culture.

Immunolocalization with TKZ, TKK, and 9D10 antibodies indicate that cellular titin is periodically distributed along stress fibers and aligned with respect to alpha-actinin. The distance measurements between bands of TKZ and TKK staining (mostly 500 nm to 1.2 μm) supports a model in which stress fibers are sarcomere-like, with cellular titin maintaining a complementary pattern of alpha-actinin and myosin filaments. The periodic distribution of the cellular titin Z-repeat region is similar to and closely associated with that of alpha-actinin, with which it likely interacts in the stress fibers. This interaction could be crucial for maintaining the integrity of the stress fiber structure when under tension. Cellular titin-alpha-actinin interaction also could be important in the assembly and disassembly of the dynamically organized stress fibers.

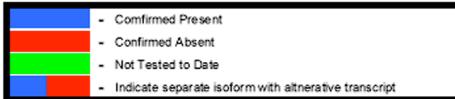
Much of the PEVK domain localization detected with the 9D10 antibody also appears to be periodically distributed along the core of the stress fiber. The presence of this elastic domain could provide passive tension capable of resisting stretch of the stress fiber in the absence of contractile force production. A stress fiber can be differentially contracted along its length (Peterson et al., 2004). Contraction in one region of a stress fiber, therefore, may impart stress on a region in which the myosin is inactive. This stretch could be resisted by the passive tension of the cellular titin PEVK domain while allowing that region of the stress fiber to extend to its uncontracted length. Moreover, SH₃ binding domains in the PEVK could provide sites for yet to be discovered binding partners that play a role in stretch sensing by the cellular titin in the stress fiber.

The localization pattern of the kinase domain along the stress fibers would place it in proximity to the myosin filaments, as is the case for the kinase domain in striated muscle titin, which is localized in the M-line. There is, however, no known structural homologue of the sarcomere M-line in stress fibers. Although periodically distributed along the stress fiber, much of the TKK staining appears to project from the core of the stress fiber, as if the kinase domain is tethered close to the stress fiber by interaction of the A-band-like domain with myosin filaments but itself is not confined to a highly organized M-line-like structure. Projection from the stress fiber may make the kinase domain more available to phosphorylate substrates outside the stress fiber or in regions of the stress fiber distal from the bare zone of the myosin bipolar filaments. If the C-terminus of the cellular titin is anchored to a particular stress fiber structure, kinase domain activation by stretch also could be part of a stretch sensor function for cellular titin, as has been proposed for striated muscle titin.

All three antibodies revealed some cytoplasmic staining distinct from that associated with the fibroblast stress fibers. The additional staining could be due to the presence of a pool of unassembled cellular titin or the presence of cellular titin that is not aligned along stress fibers. There is some degree of linearity of the extra staining, but it fails to perfectly align with microtubules or intermediate filaments in the cells (Cavnar and Keller, unpublished observation). This raises the possibility that cellular titin alone or perhaps interacting in an unknown way with yet to be identified partners or

known cytoskeletal structures can form a superstructure that more fully integrates cell activity than currently realized.

Exon	Functional Region	Exon	Functional Region	Exon	Functional Region
1	5' UTR	143-147	PEVK, Group P, Class 2 SH3 Motif	195-196	PEVK, Group P, Class 1 & 2 SH3 Motif
2-4	Z1 and Z2	148	PEVK, Group E	197	PEVK, Group P, Class 1 SH3 Motif
5-7	Zis	148b	PEVK, Group P, Class 1 & 2 SH3 Motif	198	PEVK, Group P
8-10	Z-repeats 1, 2 and 3	149	PEVK, Group P	199-203	PEVK, Group P, Class 1 & 2 SH3 Motif
11-13	Z-repeats 4, 5, and 6	150	PEVK, Group P	204	PEVK, Group P
14	Z-repeat 7	151	PEVK, Group E, Class 2 SH3 Motif	205	PEVK, Group E
15-23	Z-line	152	PEVK, Group E	206-208	PEVK, Group P
24-28	Zl - Junction	153	PEVK, Group P, Class 1 SH3 Motif	209-211	PEVK, Group P, Class 2 SH3 Motif
29-44	I-band	154	PEVK, Group P	212-214	PEVK, Group P, Class 2 SH3 Motif
45-46	Novex I + II	155	PEVK, Group P, Class 2 SH3 Motif	215-216	PEVK, Group E
47	I band	156	PEVK, Group E	217-218	PEVK, Group P, Class 1 & 2 SH3 Motif
48	Novex III	157	PEVK, Group P	219-220	PEVK, Group P
49	N2B region	158-159	PEVK, Group P, Class 2 SH3 Motif	221-222	PEVK, Group P
50	I band	160	PEVK, Group P, Class 2 SH3 Motif	223	PEVK, Group P, Class 1 SH3 Motif
51-59	I band Ig domains	161	PEVK, Group P	224	PEVK, Group P, Class 2 SH3 Motif
90-101	I band Ig domains	162	PEVK, Group P, Class 2 SH3 Motif	225	PEVK, Group P
102-111	N2A Region	163	PEVK, Group E	226-227	IIA Junction Tandem Ig domains
112-114	PEVK, Group P	164-166	PEVK, Group P	228-236	IIA Junction Tandem Ig domains
115	PEVK, Group E	167-169	PEVK, Group P	237-247	IIA Junction Tandem Ig domains
116	PEVK, Group P, Class 1 SH3 Motif	170	PEVK, Group P	248-252	IIA Junction
117	PEVK, Group P	171	PEVK, Group P, Class 2 SH3 Motif	253-255	IIA Junction
118	PEVK, Group P, Class 1 & 2 SH3 Motif	172-173	PEVK, Group P, Class 1 & 2 SH3 Motif	256-261	IIA Junction
119-120	PEVK, Group P	174	PEVK, Group P	262-269	IIA Junction
121-122	PEVK, Group P, Class 1 & 2 SH3 Motif	175	PEVK, Group P, Class 1 & 2 SH3 Motif	270-276	A-Band, D-Zone, Superrepeat 1
123	PEVK, Group P	176	PEVK, Group P	277-283	A-Band, D-Zone, Superrepeat 2
124	PEVK, Group P, Class 2 SH3 Motif	177-178	PEVK, Group P, Class 1 & 2 SH3 Motif	284-293	A-Band, D-Zone, Superrepeat 3
125	PEVK, Group P	179	PEVK, Group P, Class 1 SH3 Motif	294-300	A-Band, D-Zone, Superrepeat 4
126-127	PEVK, Group P, Class 2 SH3 Motif	180	PEVK, Group P	301-303	A-Band, D-Zone, Superrepeat 5
128	PEVK, Group P	181-182	PEVK, Group P, Class 1 & 2 SH3 Motif	304	A-band, D-Zone, Superrepeat 5, 6
129	PEVK, Group E	183	PEVK, Group P	305	A-band, D-Zone, Superrepeat 6
130	PEVK, Group E	184	PEVK, Group P, Class 1 & 2 SH3 Motif	306-316	A-band, C-Zone, Superrepeat 1
131-133	PEVK, Group P, Class 2 SH3 Motif	185	PEVK, Group P	317-325	A-band, C-Zone, Superrepeat 2
134	PEVK, Group P	186-187	PEVK, Group P, Class 1 & 2 SH3 Motif	326	A-band, C-Zone, Superrepeat 2-8
135	PEVK, Group P, Class 2 SH3 Motif	188	PEVK, Group P, Class 1 SH3 Motif	327-328	A-band, C-Zone, Superrepeat 8
136	PEVK, Group P	189	PEVK, Group P	329-334	A-band, C-Zone, Superrepeat 8
137	PEVK, Group E	190-191	PEVK, Group P, Class 1 & 2 SH3 Motif	335-345	A-band, C-Zone, Superrepeat 9, 10
138-139	PEVK, Group P, Class 1 SH3 Motif	192	PEVK, Group P	346-351	A-band, C-Zone, Superrepeat 11
140-141	PEVK, Group P, Class 2 SH3 Motif	193	PEVK, Group P, Class 1 & 2 SH3 Motif	352-354	A-band, C-Zone, Superrepeat 11
142	PEVK, Group E	194	PEVK, Group P	355-357	A-band
				358	Titin Kinase Domain
				359-362	M-line
				363	3' UTR



- Confirmed Present
- Confirmed Absent
- Not Tested to Date
- Indicate separate isoform with alternative transcript

Figure 6. Molecular determination of cellular titin mRNA exon composition using gene specific RT-PCR of CHRF cell total RNA. Blue exons indicate those which have been confirmed to be included in cellular titin mRNA. Red exons indicate those which have been confirmed to be absent or alternatively spliced. Green exons indicate those which have yet to be investigated with gene specific primers. Half blue and half red boxes indicate exons that have been confirmed present and absent in different products. Exons are grouped and listed with respect to their functional region in the muscle titin paradigm. Exons in the PEVK region are listed as Group P (PPAK) or Group E (poly E) exons based on amino acid composition. Sites of predicted class I and II SH3 motifs are indicated in the PEVK exons. Exons comprising the six copies of D zone seven module super-repeats and the C zone eleven module super-repeats in the A-band region of striated muscle titin are indicated.

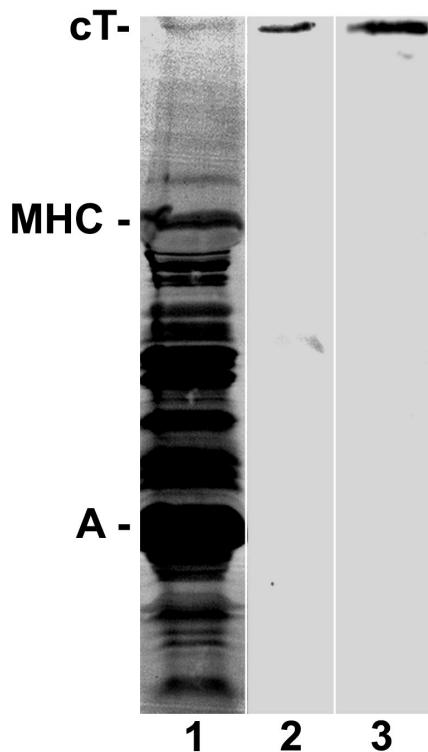


Figure 7. Western blot analysis of total protein isolated from human platelets using antibodies raised against bacterially expressed N-terminal and C-terminal regions titin. The TKZ antibody was raised against the N-terminal Z-repeat-Zq region encoded by exons 7-10 and 14-18. The TKK antibody was raised against the C-terminal kinase domain region encoded by sequence within exon 358. Lane 1 is a ponceau S stain of the human platelet total protein extract run on 4%-20% SDS-PAGE and blotted onto nitrocellulose membrane used for subsequent Western analysis. Lane 2 is a Western blot using TKK antibody of an immunoprecipitation pellet of the total platelet extract with the TKZ antibody. Lane 3 is a Western blot of the human platelet protein extract using the TKK antibody. Both antibodies react with a single high molecular weight band corresponding to platelet cellular titin.

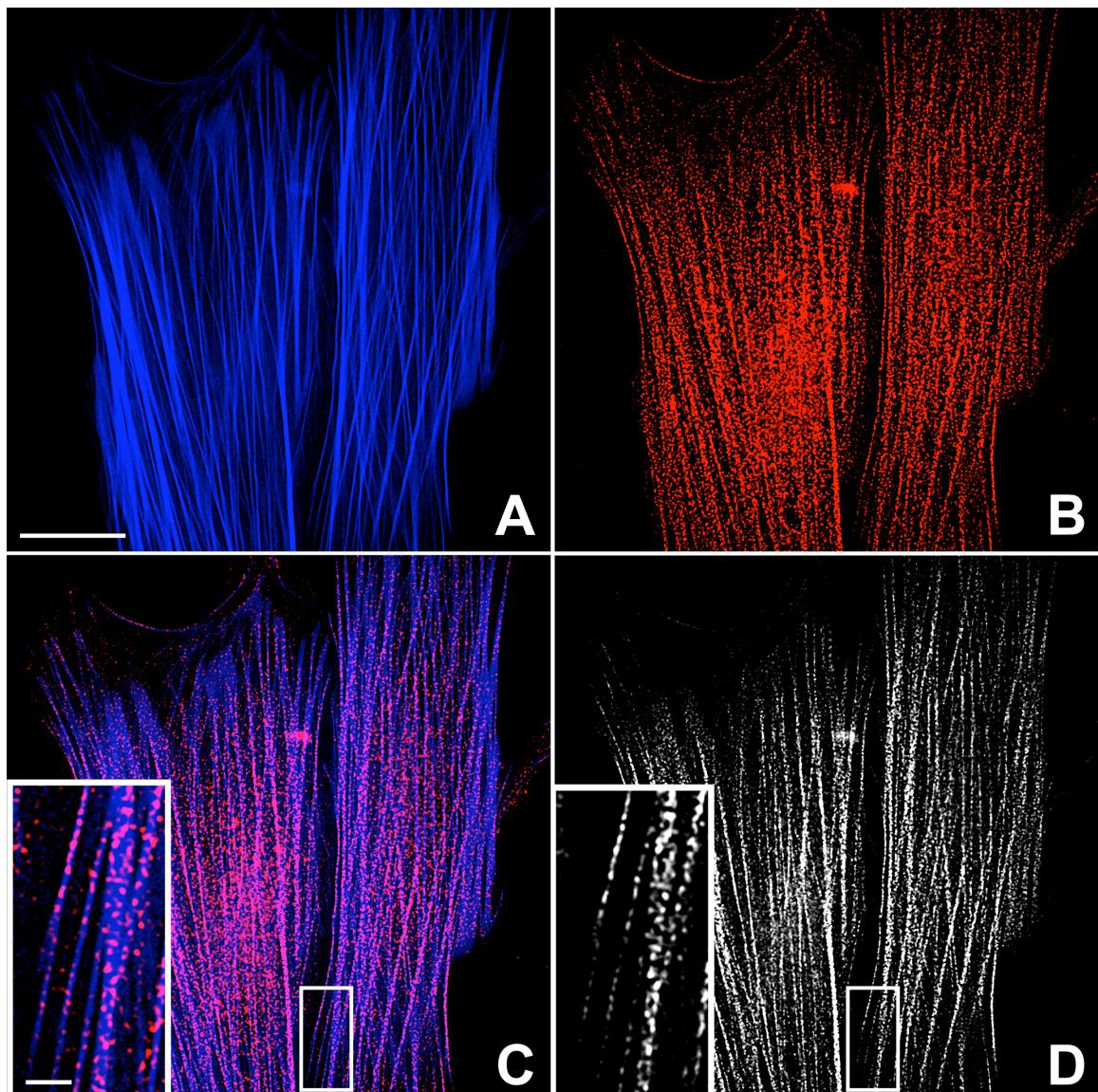


Figure 8. Deconvolution immunofluorescence localization of the cellular titin Z-repeat-Zq region with respect to actin filaments in cultured deer skin fibroblast stress fibers. Cultured Indian Muntjac cells were double labeled with Alexa Fluor 350[®] conjugated to Phalloidin (A) and with the TKZ primary antibody detected with Alexa Fluor 633[®] conjugated to an anti-rabbit IgG secondary antibody (B). (C), Overlay of A and B. (D), Colocalization of phalloidin and TKZ antibody signals. Bars, 20 μ m and 2 μ m (inset box).

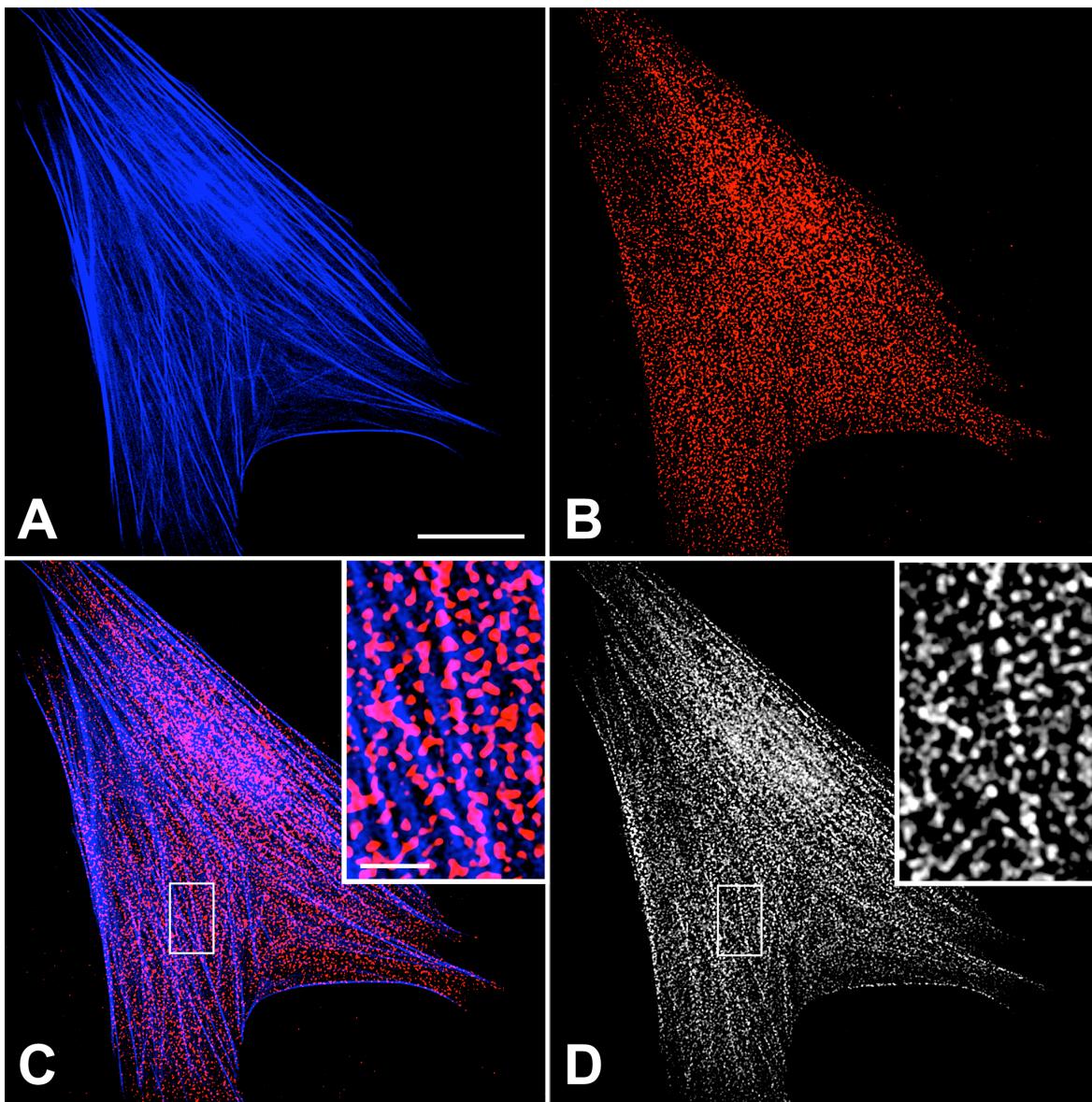


Figure 9. Immunofluorescence localization of the cellular titin kinase domain region with respect to actin filaments in cultured deer skin fibroblast stress fibers using deconvolution microscopy. Cultured Indian Muntjac cells were double labeled with Alexa Fluor 350[®] conjugated to Phalloidin (A) and with the TKZ primary antibody detected with Alexa Fluor 633[®] conjugated to an anti-rabbit IgG secondary antibody (B). (C), Overlay of A and B. (D), Colocalization of phalloidin and TKZ antibody signals. Bars, 20 µm and 2 µm (inset box).

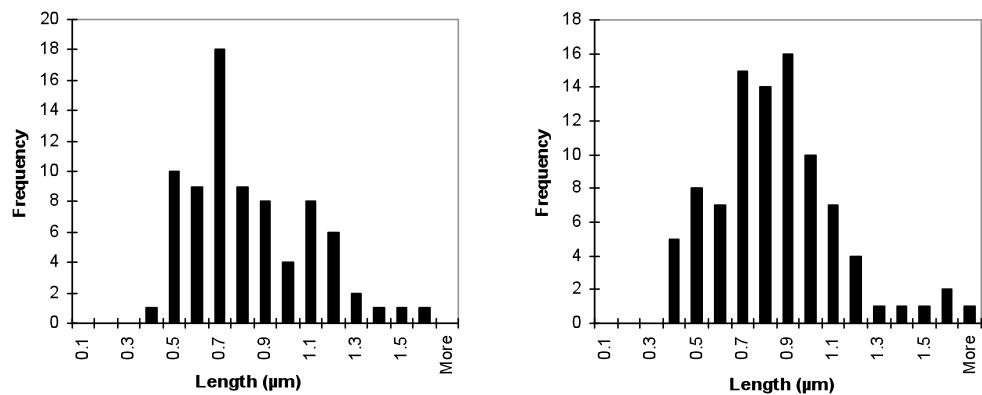


Figure 10. Histogram of center-to-center distances between spots labeled with TKZ (A, n=78) and TKK (B, n=92) along the stress fibers of cultured Indian Muntjac cells. Individual lengths were binned into 100 nm length categories.

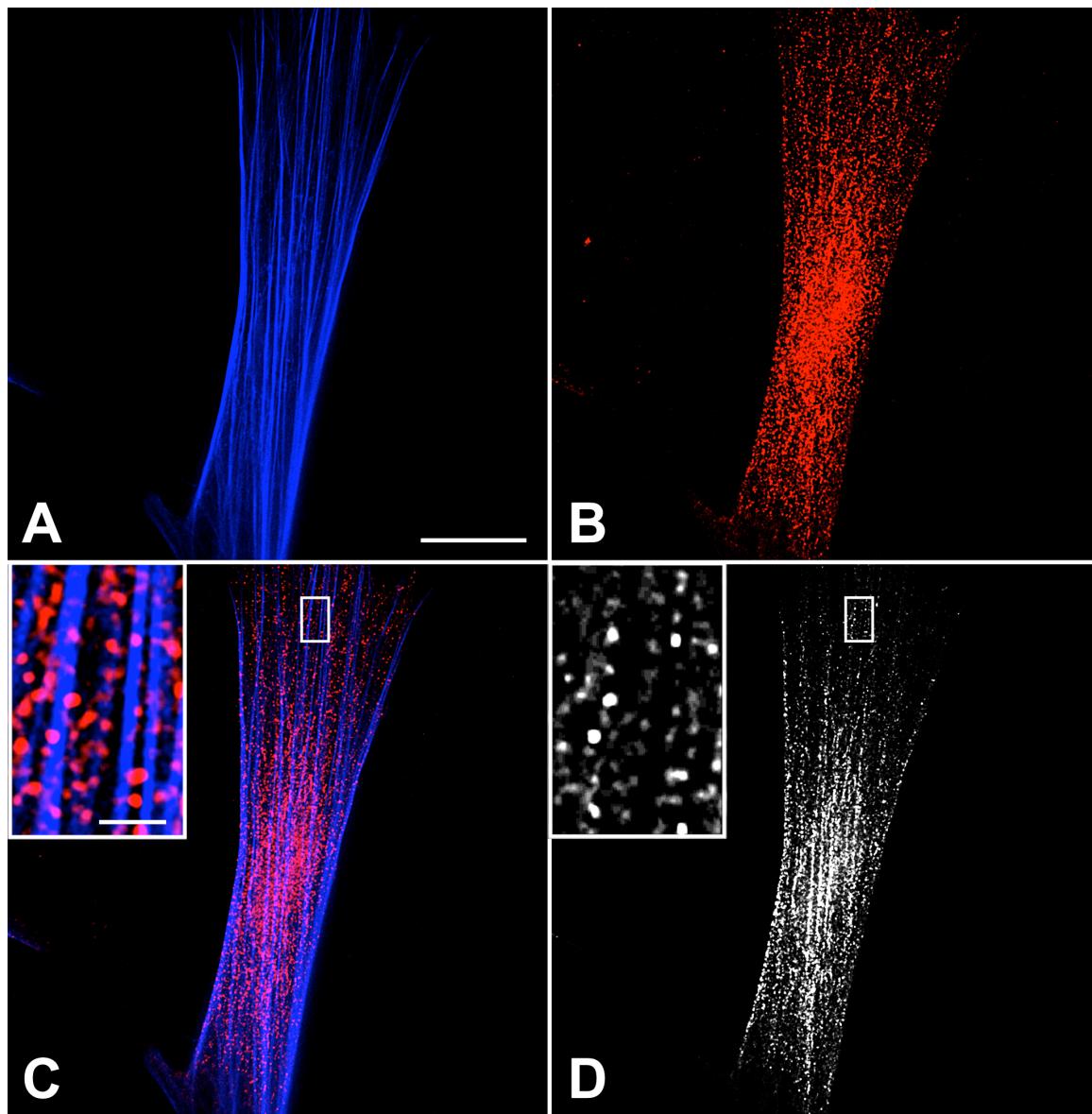


Figure 11. Immunofluorescence localization of the cellular titin PEVK domain with respect to actin filaments in cultured deer skin fibroblast stress fibers using deconvolution microscopy. Indian Muntjac cells were double labeled with Alexa Fluor 350[®] conjugated to Phalloidin (A) and with the 9D10 monoclonal antibody detected with Alexa Fluor 633[®] conjugated to an anti-mouse IgM secondary antibody (B). (C), Overlay of A and B. (D), Colocalization of phalloidin and 9D10 antibody signals. Bars, 20 μ m and 2 μ m (inset box).

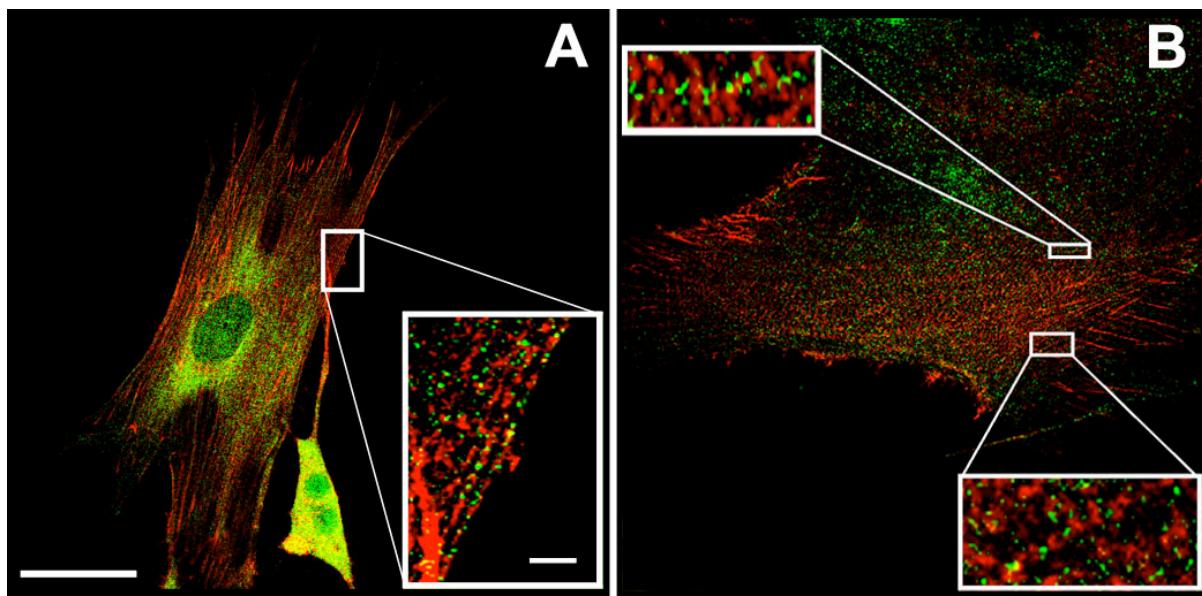


Figure 12. Immunofluorescence localization of the cellular titin Z-repeat-Zq and kinase regions with respect to alpha-actinin in mouse fibroblast 3T3 cell stress fibers using confocal microscopy. Cells were double labeled with the TKZ (A) or TKK (B) primary antibodies detected with an Alexa Fluor 488[®] conjugated anti-rabbit IgG secondary antibody (A and B) and an anti-alpha-actinin mouse IgM monoclonal antibody detected with an Alexa Fluor 568[®] conjugated anti-mouse IgM antibody (A and B). In both images cellular titin exhibits a periodic pattern with the Z-repeat domains overlapping some alpha-actinin dense bodies (A), and the kinase domain falling in an alternating pattern with respect to α -actinin (B). Bars, 20 μ m and 2 μ m (inset box).

CHAPTER 3

CHARACTERIZATION OF CELLULAR TITIN KINASE AND ITS ROLE IN STRESS FIBER ORGANIZATION THROUGH INTERACTION WITH HAX-1

Introduction

Striated muscle titin is a large >3000 kDa protein which spans 1 μ m in length from the Z-line to the M-line of one-half sarcomere and is considered the third major filament system in skeletal muscle [for reviews see (Tskhovrebova and Trinick, 2003)]. Predominately made up of immunoglobulin- (Ig) and fibronectin type-III-like (Fn3) domains (Labeit et al., 1992) titin is modular in structure consisting of four major structural regions in muscle. The N-terminal Z-line region of titin, which comprises the telethonin (Gregorio et al., 1998) and alpha-actinin (Sorimachi et al., 1997) binding sites anchored in the Z-disc, provides potential signaling and structural roles crucial to the stability of the sarcomere. The I-band region, which consists of “proximal” and “distal” Ig-containing domains, is partly responsible for conferring titin slack length and “stretch resistance” (Linke, 2008). The PEVK region, so called due to the prevalence of proline (P), glutamate (E), valine (V), and lysine (E) residues, is an additional “spring-like” region folded in unordered polyproline type-II helices (Ma et al., 2001). The A-band region is made up of alternating bands of Ig and Fn3 domains in two distinct super-repeats, which correspond to the helical repeat nature of the myosin-II bipolar filament and are thought to be responsible for myosin bipolar filament formation and localization. The C-terminal M-line region of titin contains a kinase domain, which phosphorylates telethonin (Mayans et al., 1998), and also several other binding sites for proteins hypothesized to be involved in signaling protein turnover and gene expression changes in myocytes (Lange et al., 2005; Gregorio et al., 2005). The M-line domain also has been shown to interact with A- and B-type lamins in the nuclear envelope of nonmuscle cells (Zastrow et al., 2006).

The titin kinase domain is a serine/threonine kinase belonging to the MLCK-like family of kinases. The kinase activity is dually regulated through Ca²⁺/calmodulin

binding to a C-terminal autoinhibitory regulatory tail, which unblocks the catalytic site, and subsequent phosphorylation of an active site tyrosine by an unknown kinase, which significantly increases the kinase activity (Mayans et al., 1998). To date the Z-disk protein telethonin, which binds to the first two Ig domains of titin, is the only substrate reported for skeletal titin kinase (Mayans et al., 1998). In the sarcomere, the telethonin in the Z-disk is separated from the titin kinase domain in the M-line by about 1 μ m and likely inaccessible to the kinase activity. Nevertheless, titin kinase function affects sarcomere stability. Expression of a constitutively active titin kinase domain results in cytoskeletal breakdown of the sarcomere (Mayans et al., 1998). Moreover, titin kinase might act as a “stretch sensor” that when activated through mechanical stretch activates a “signalosome” that regulates muscle protein transcription and turnover. In support of this it has been shown *in vitro* that titin kinase can be activated by force-induced stretch (Grater et al., 2005; Greene et al., 2008), but whether this is relevant physiologically remains unknown.

We recently reported the discovery of novel isoforms of titin, cellular titins (c-titin), localized in the stress fiber structures of nonmuscle cells (Cavnar et al., 2007). Our RT-PCR mapping of the exon sequences encoding c-titin revealed that c-titin is a unique isoform encoded by the single human titin gene that shares many of the protein domains found in most striated muscle titin isoforms including the kinase domain region near its C-terminus. This domain localizes in a punctate pattern that is loosely associated with the actin based stress fibers in nonmuscle cells.

Here, we report that overexpression of the cellular titin kinase domain results in a dramatic disassembly of stress fiber structures in spread epithelial cells. We also found using yeast two-hybrid screening of a nonmuscle cell cDNA library that the cellular titin kinase domain interacts with the cortactin interacting protein HS1 associated protein X-1 (HAX-1).

HAX-1 is a ubiquitously expressed protein that contains two Bcl-2 homology domains, a PEST domain, and a predicted transmembrane domain. Hax-1 has been found associated with the mitochondrial membrane in B lymphoma cells (Suzuki et al., 1997) and with the endoplasmic reticulum (ER), nuclear envelope, and cytoplasm of COS-7 and HeLa cells (Gallagher et al., 2000; Sharp et al., 2002). Hax-1 reportedly

plays roles in regulating cell motility and apoptosis, and consequently perturbations of Hax-1 are associated with several human disease states. Hax-1 expression, for example, is upregulated in psoriatic skin as well in as a variety of melanoma cell lines (Mirmohammadsadegh et al., 2003), which may be related to its proposed anti-apoptotic activity. Its proposed anti-apoptotic activity is based on evidence that Hax-1 can associate with the mitochondrial membrane and is an early cleavage substrate of the pro-apoptotic protein Omi/HtrA2 (Cilenti et al., 2004) and caspase-3 (Lee et al., 2008). Moreover, overexpression of Hax-1 prevents cardiomyocyte apoptosis through caspase-9 inhibition (Han et al., 2006).

Early evidence that Hax-1 is involved in cell motility came from the observation that patients deficient in Hax-1 exhibit a severe congenital neutropenia, known as Kostmann disease (Rezaei et al., 2007), in which the neutrophils exhibit decreased chemotaxis and impaired F-actin actin polymerization (Elsner et al., 1993). Subsequently Hax-1 was shown to bind to HSC-1, which is a member of the ubiquitously expressed cortactin family of actin polymerization regulatory proteins, (Gallagher et al., 2000). Hax-1 also interacts with G_α13 (Radhika et al., 2004), the α-subunit of the heterotrimeric protein G13. It has been proposed that through these interactions Hax-1 may be a mediator between the stress fiber forming Rho signaling pathway and the cell migration Rac mediated pathway (Radhika et al., 2004). Hax-1 also has been shown to regulate integrin α₅β₆-dependent cell migration by clathrin-mediated endocytosis in oral squamous cell carcinomas by direct interaction with the integrin (Ramsay et al., 2007).

Materials And Methods

Cellular Titin Kinase Cloning

Cellular titin kinase fragments ctKin4 and ctKin1 were cloned from total RNA isolated from the megakaryocyte cell line CHRF-288-11 as described previously (Cavnar et al., 2007) using primers directed against amino acids 24705-25005 and amino acids 24705-25054 of cardiac titin EMBL accession number X90568 respectively. The constitutively active titin kinase fragment ctKin4(Y170E) and inactive kinase

ctKin4(K36A) were created by site-directed mutagenesis (Quick Change Site-Directed Mutagenesis Kit, Stratagene). All titin fragments were cloned into the multi-cloning site of pEGFP-C1 and pmCherry-C1 (Clontech).

Yeast-Two Hybrid

Cellular titin kinase fragment ctKin4 was cloned into the multicloning site of pGBKT7 (Clontech) to create ctKin4 fused to the GAL4-DNA binding domain (Kin4-BD). *Saccharomyces cerevisiae* strain AH109 was transformed with ctKin4-BD and plated on selective media SD/-Trp. One CFU was obtained and grown in liquid SD/-Trp and frozen as a glycerol stock. For two-hybrid screening, the bait was subsequently transformed with a HeLa cell cDNA library in the pGAD-GH GAL4 activation domain vector (Clontech). 1.35×10^7 transformants were screened using quadruple selection plates SD/-Leu/-Trp/-His/-Ade and incubated at 30°C for 5-7 days. Of the 1.35×10^7 transformants screened 400 CFUs were isolated and streaked on SD/-Leu/-Trp and grown at 30°C for 3-5 days to segregate colonies. Colonies were then rescreened on SD/-Leu/-Trp/-His/-Ade for a second time and incubated at 30°C for 5-7 days. Positive clones were grown in 5ml of SD/-Leu/-Trp/-His and cDNA insert containing plasmid DNA was isolated. *Escherichia coli* strain DH5 α and the pGAD-GH vector was selected using Ampicillin resistance. Plasmid DNA was isolated from *E. coli* colonies and submitted for sequencing. Sequencing results were blasted against the human genome. Of the 30 clones screened, 22 of them were 4 different constructs of Hax-1.

Immunoprecipitation and Immunoblot Analysis of Hax-1

HeLa CCL.2 cells were grown in Ham's F10 with 10% fetal bovine serum (Gibco) in 75cm² culture dishes (Corning) to a density of ~85%. Cells were placed on ice and washed once with ice cold PBS. Cells were then extracted for 20 minutes with gentle rocking in lysis buffer (20mM HEPES, pH 7.5, 50mM KCL, 1mM EDTA, 1% NP-40, .2mM PMSF, 1 μ g/ml pepstatin, 2 μ g/ml aprotinin, 1 μ g/ml leupeptin). ctKin4 fused to maltose binding protein (Kin4-MBP) was expressed in *Escherichia coli* strain BL21 and purified through conjugation to amylose resin (New England BioLabs). The HeLa extract was incubated with 100 μ l of Kin4-MBP for 1 hour at 4°C. Beads were gently pelleted for 30sec at 12,000xg and supernatant was saved. Beads were washed five times in lysis buffer and afterwards in 50 μ l of 2x Laemmli sample buffer. Samples were

boiled at 90°C for 10 minutes and pelleted at 13,000xg for 1 minute. Supernatant was run on 15% SDS-PAGE and subsequently blotted onto nitrocellulose. Western blots were performed as described (Cavnar et al., 2007) and assayed for the presence of Hax-1 using anti-Hax-1 antibody (BD Biosciences).

Kin4 Counting Experiments

HeLa cells CCL.2 were grown in triplicate in 65mm culture dishes (corning) to a density of roughly 75%. Cells were subsequently transfected with ctKin4-GFP, ctKin4(Y170E)-GFP, ctKin4(K36A)-GFP, and GFPC1 using Efectene reagent (Qiagen) according to the manufacturers protocol. After 48 hours, five fields from each culture dish were imaged using a 10x objective with phase microscopy and fluorescence microscopy. Images were overlaid and transfected spread and transfected round cells were counted using ImageJ cell counter (NIH).

Immunofluorescence Microscopy

HeLa cell line CCL.2 was grown in Ham's F10 with 10% FBS to a confluence of ~75%. Cells were transfected with ctKin4 fused to GFP and mCherry and transfected into cells using DMRIE-C transfection reagent (Gibco) following manufacturer's protocols. Cells were stained for F-actin using Alexa Fluor conjugated phalloidin as described in (Cavnar et al., 2007). Cells were imaged using an Olympus BX61 fluorescent microscope using a 60x, 1.42 NA oil objective.

Live Cell Microscopy

Live cell microscopy was performed using the DeltaVision® personalDV (Applied Precision). HeLa cells were grown an uncoated glass bottom culture dish (MatTek Corporation) to a density of 75%. Cells were transiently transfected with 2 μ g of ctKin4-GFP using the DMRIE-C reagent (Gibco). Cells were allowed to express for 8 hours at which time 20mM HEPES, pH 7.5 was added to the media and placed on the microscope in a 37°C chamber. Cells were imaged using a PLAN-APO 60x Oil immersion objective with 1.4NA, taking images every 5 minutes for 2 hours. Images were collected using the CoolSnap ES2 camera (PCI) and compiled into mpeg form using the SoftWorx® imaging software.

Results

Expressed Cellular Titin Kinase Catalytic Core Localization

Using RT-PCR of RNA isolated from the human megakaryocyte cell line CHRF-288-11, a construct of the titin kinase domain containing the catalytic domain but missing the Ca^{2+} /CaM-binding regulatory tail was cloned. This construct, hereafter designated ctKin4 because of its c-titin origin, is identical to the Kin4 construct previously characterized as having unregulated kinase activity (Mayans et al., 1998). The maximally active phosphomimetic Y170E [ctKin4(Y170E)] mutation and the putatively inactive K36A [ctKin4(K36A)] mutation of this construct, described by Mayans et al, also were made. In addition, all three constructs were cloned into the pEGFP-C1 vector that when transfected into cells results in expression of GFP-kinase chimeras.

ctKin4 Expression Results In A Rounded Phenotype Through Stress Fiber Disassembly

When expressed in HeLa cells, all three GFP-Kin4 constructs, GFP-ctKin4 (Figure 13), GFP-ctKin4(Y170E), and GFP-ctKin4(K36A), initially localized in a linear punctate pattern loosely associated with the phalloidin stained actin stress fibers similar to the pattern previously found with the TKK antibody for the native titin kinase domain (Cavnar et al., 2007). Over time, however, cells expressing any of the three GFP-ctKin4 constructs but not those expressing GFP alone rounded up and ultimately lost adherence to the substratum. This rounding appeared to correlate with disassembly of the stress fibers. To quantify the phenotype effect, the transfected cultures of HeLa cells were imaged 48 hours after transfection. The number of transfected cells in 15 imaged fields for each construct were classified either as rounded or spread (Figure 14). At 48 hours post-transfection, there was a marked increase in rounded cells expressing each of the three ctKin4 constructs compared to GFP only. Over time, this difference increased to the point where all cells expressing any of the three Kin4 constructs were rounded (data not shown).

Live cell fluorescence microscopy revealed that spread HeLa cells expressing any of the three GFP-ctKin4 constructs undergo a rapid and dramatic cytoskeletal breakdown over the course of approximately 15 minutes once the process becomes evident (Figure 15, supplemental data). Once rounded, most cells eventually lose

adhesion. This effect is reproducible even in the presence of Blebbistatin, a myosin II activity inhibitor.

Identification of a Cellular Titin Kinase Binding Partner - Hax-1

The rounded phenotype resulting from overexpression of all three GFP-Kin4 constructs, including the GFP-ctKin4(K36A) with little to no kinase activity, raised the possibility that the direct interaction of the titin kinase domain rather than phosphorylation of another protein caused the phenotype. To identify such proteins, we used the yeast two-hybrid system to screen a HeLa cell cDNA library for interacting partners to ctKin4 fused to the Gal4 DNA binding domain (Clontech). From the 1.35×10^7 CFUs screened, 400 CFUs were isolated on selection media lacking Tryptophan, Leucine, Histidine, and Adenine. Of those, 30 CFUs were analyzed and 22 CFUs were identified as one of four clones of HS1 associated protein X-1 (Hax-1) (Figure 16). The ctKin4 construct as well as the ctKin4(Y170E), and ctKin4(K36A) constructs were re-screened against the four Hax-1 cDNA clones, and all were found to interact equally.

In order to verify the c-titin kinase domain interaction with Hax-1, bacterially-expressed ctKin4 fused to maltose binding protein (MBP) was added to the soluble fraction from a HeLa extract. Addition of amylose resin to pellet the MBP-ctKin4 also specifically pulled down Hax-1 (Figure 17). Because Hax-1 is a known cortactin interacting partner, the pellet was analyzed for the presence of cortactin copelleted from the HeLa cell extract with the Hax-1 bound to the MBP-ctKin4. The pellet was found to be devoid of cortactin (Figure 17), which is consistent with the possibility that cortactin binding site on Hax-1, amino acids 114-279 (Suzuki et al., 1997), overlaps with the apparent titin kinase domain binding site on Hax-1, amino acids 175-279.

Whether Hax1 interacts with the titin kinase domain when titin is associated with stress fibers was investigated by immunolocalization of Hax-1 in HeLa cells containing stress fibers. Hax-1 was found to be distributed in a punctate pattern throughout the cell cytoplasm, but exhibited little evidence of direct association with the stress fibers (Figure 18).

Discussion

We previously reported the identification of novel nonmuscle isoforms of titin encoded by the titin gene on the long arm of chromosome 2 (Cavnar et al., 2007). Over 60% of the c-titin sequence has been mapped and shown to contain the identical sequence to skeletal titin within the kinase domain, indicating c-titin contains a functional kinase domain. The titin kinase domain belongs to the myosin-light-chain kinase family, and is dually regulated by Ca^{2+} /CaM-binding to an inhibitory regulatory tail and subsequent tyrosine phosphorylation within the catalytic domain (Mayans et al., 1998). The titin kinase has been hypothesized to be important in sarcomeric signaling and integrity. Indeed the kinase domain in striated muscle titin isoforms recently has been shown to interact with a signaling protein nbr1 stimulating a signaling cascade that ultimately leads to activation of serum response factor (SRF). SRF is an important transcription factor which stimulates muscle protein transcription and turnover (Lange et al., 2005). Moreover deletion or overexpression of the titin kinase domain results in impaired sarcomeric integrity (Mayans et al., 1998; Gotthardt et al., 2003).

To investigate titin kinase domain activity in nonmuscle cells, the catalytic domain without the regulatory tail, was cloned using total RNA isolated from a megakaryocyte cell line CHRF-288-11. In addition, both a maximally active kinase domain with a phosphomimetic mutation of the regulatory tyrosine ctKin4(Y170E) and a minimally active kinase domain with a lysine to alanine mutation of an active site residue ctKin4(K36A) were made. Surprisingly, expression of each of the three constructs as GFP-fusion proteins caused a dramatic breakdown of the cytoskeleton and rounding up of HeLa cells (Figure 14).

Live cell fluorescence microscopy shows the action of rounding up due to ctKin4 is a rapid process taking on average 15 minutes to complete (Figure 15). This action can take place even in the presence of the myosin inhibitor Blebbistatin, suggesting this is a result of a complete breakdown in stress fiber structure as opposed to a myosin based contraction.

We had hypothesized the ctKin4(Y170E) and perhaps the ctKin4 would have a detectable affect on the cytoskeleton of cells, if the titin kinase domain activity regulates

nonmuscle cytoskeleton stability in a manner similar to its effects on sarcomere stability in striated muscle cells. It is unclear, however, why the putatively inactive ctKin4(K36A) kinase domain yielded the same result. Based on the observation that an alanine mutation (K72A) of Protein Kinase A (PKA) active site lysine, which also is highly conserved in the active site of myosin light chain kinase family members, significantly decreases PKA activity by inhibiting Mg²⁺/ATP binding (Taylor et al., 1992), the ctKin4(K36A) mutation is predicted to significantly decrease titin kinase activity (Mayans et al., 1998). Our finding suggests activity of the titin kinase domain is unnecessary for causing cell rounding.

If the titin kinase domain activity is not the primary cause of the phenotype, cell rounding may be caused by dominant negative interaction of all three ctKin4 constructs with a binding partner. We used yeast two-hybrid screening with ctKin4 to search for titin kinase domain binding proteins. Of the 30 positive clones analyzed, 22 were one of four independent clones of the cortactin homologue HS1 associated protein X-1 (Hax-1). This interaction was confirmed *in vitro* using bacterially expressed ctKin4 fused to maltose binding protein. This ctKin4-MBP fusion protein bound to amylose resin specifically pulled down native Hax-1 from the soluble fraction of a HeLa cell extract.

Of the remaining 8 positive CFUs from our yeast two-hybrid screen, 3 encoded unique proteins unrelated to the nonmuscle cytoskeleton and 5 contained unidentifiable cDNA. Whether these clones are legitimate interacting partners with ctKin4 or not remains to be determined.

Hax-1 is a small ~26 kDa protein containing two Bcl-2 homology domains, a PEST domain, and a putative transmembrane domain. Initial investigations characterized Hax-1 to be an anti-apoptotic protein due to the Bcl-2 homology domains and its localization to the mitochondria in B lymphoma cells (Suzuki et al., 1997). Evidence for the anti-apoptotic function of Hax-1 was further strengthened by recent studies showing that cells overexpressing Hax-1 have increased viability under conditions that induce cell death (Vafiadaki et al., 2007) and that Hax-1 is a substrate of the pro-apoptotic mitochondrial serine protease Omi/HtrA2 (Cilenti et al., 2004) as well as of caspase-3 (Lee et al., 2008). It is unclear whether overexpression of the cellular titin kinase domain in our experiments modulates Hax-1 anti-apoptotic role; TUNEL

assays on ctKin4 transfected HeLa cells indicated the rounded cells were not apoptotic (Appendix B), therefore we do not believe ctKin4 to be indirectly inducing apoptosis through its interaction with Hax-1.

In addition to its role in apoptosis, evidence is accumulating that Hax-1 plays important roles in regulating actin cytoskeleton activity in lamellipodia and stress fibers. Hax-1 knockdown decreases cell motility through inhibition of clathrin-mediated endocytosis of integrin $\alpha_5\beta_6$, with which the Hax-1 interacts directly (Ramsay et al., 2007). Patients with a homozygous mutation in the Hax-1 gene exhibit Kostmann disease, a rare form of severe congenital neutropenia (Klein et al., 2007). Neutrophils from these patients exhibit impaired chemotaxis and a higher than normal F-actin concentration, showing an inability to disassemble actin filaments (Elsner et al., 1993). In contrast, Hax-1 overexpression in transformed cells is correlated with increased invasiveness, implicating Hax-1 in tumor progression and metastasis (Ramsay et al., 2007).

Hax-1 has been implicated in signaling changes in cytoskeletal organization through its association with the family of Rho GTPases, specifically through its interaction with the α subunit of G protein 13 (Radhika et al., 2004). $G_{\alpha}13$ specifically binds to a complex of Hax-1, cortactin, and Rac when the Rac pathway is stimulated, stimulating formation of the actin network producing protrusive force in lamellipodia. When Rho is stimulated, $G_{\alpha}13$ interacts with RhoGEF, promoting cell spreading and stress fiber formation. It is hypothesized that Hax-1 is a scaffolding protein, which through its interaction with $G_{\alpha}13$ brings cortactin to the cell cortex promoting actin filament assembly at the leading edge when Rac is activated. We found that in well spread HeLa cells Hax-1 localizes in a punctate manner throughout the cell (Figure 18). Because the Hax-1 binding sites for the titin kinase domain and cortactin may overlap, we hypothesize that the titin kinase domain outcompetes cortactin for binding to Hax-1 under conditions that promote stress fiber assembly. Alternatively, when the Rac pathway is activated, the Hax-1/cortactin interaction may be favored over interaction with the c-titin kinase domain, and the Hax-1-cortactin complex shuttles to the cell cortex where it interacts with $G_{\alpha}13$ to promote cell migration. It remains unknown at this time whether Hax-1 is indeed a substrate of cellular titin kinase or purely a binding

partner; however, it is intriguing that cellular titin kinase has a role in mediating cytoskeleton regulation and organization, possibly through the Rho/Rac signaling pathways.

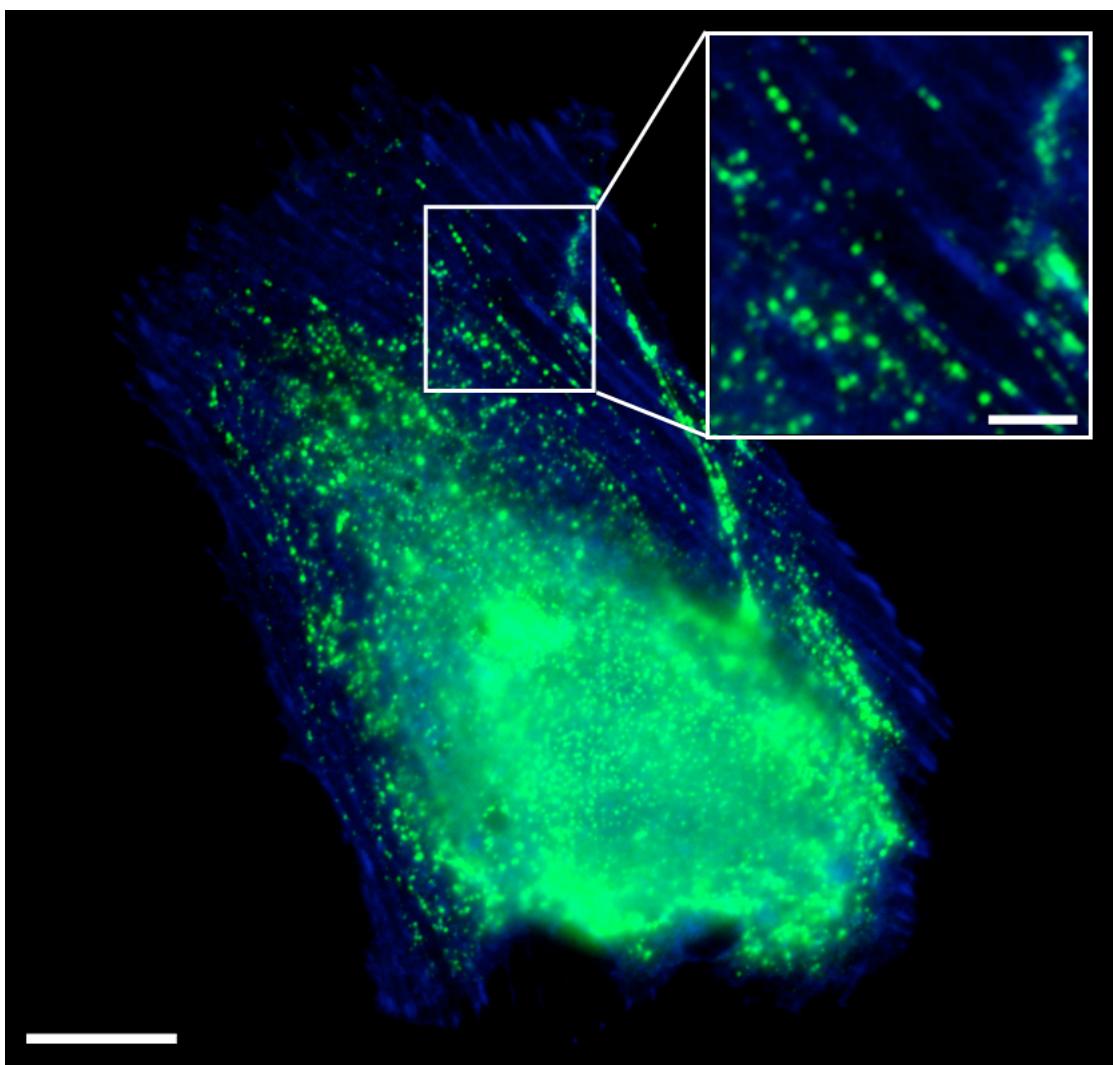


Figure 13. ctKin4-GFP localization in HeLa cells. ctKin4-GFP was transiently transfected into HeLa CCL.2 cells. The cells were fixed and counterstained with Alexa Fluor 350[®] phalloidin for F-actin (Blue). ctKin4-GFP shows a linear punctate distribution in loose association with the actin based stress fibers. Scale bars 20 μ m, 5 μ m (inset).

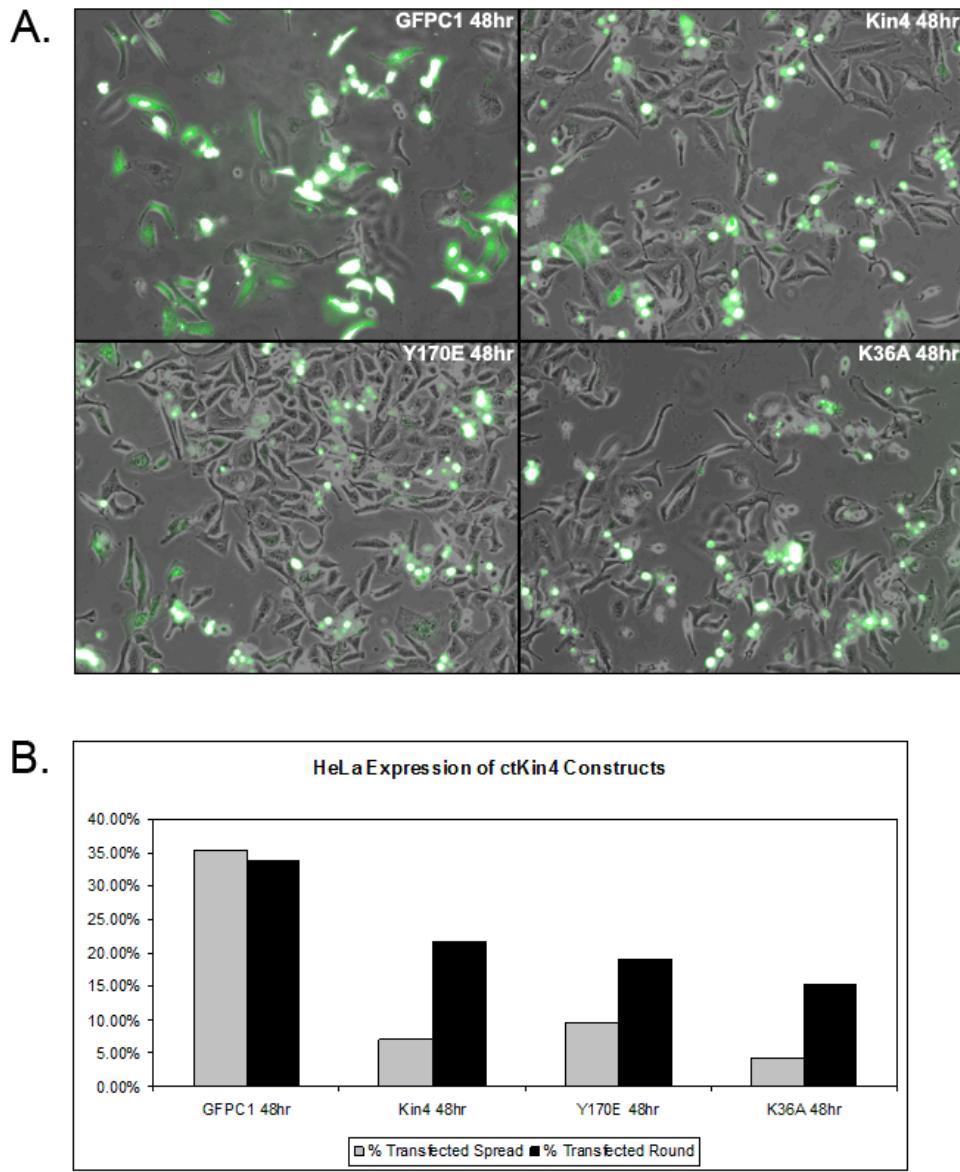


Figure 14. ctKin4 expression results in an overall rounded phenotype. (A) ctKin4, ctKin4(Y170E), and ctKin4(K36A) were expressed as GFP fusion proteins along with GFP alone in HeLa CCL.2 cells for 48 hours. The vast majority of the cells expressing ctKin4 constructs, but not those expressing GFP alone, exhibited a rounded phenotype with eventual loss of adherence. (B) At 48 hours post transfection, five 10x fields from triplicate cultures were imaged with phase and fluorescence microscopy. Total number of cells were counted, pooled, and categorized as a percentage of the total cell population as either transfected spread or transfected round. n=1641 (GFP), n=2263 (ctKin4), n=2935 [ctKin4(Y10E)], n=2907 [ctKin4(K36A)]. Cells expressing ctKin4, ctKin4(Y170E), and ctKin4(K36A) displayed a marked increase in the percentage of round versus spread cells compared to the GFP only control.

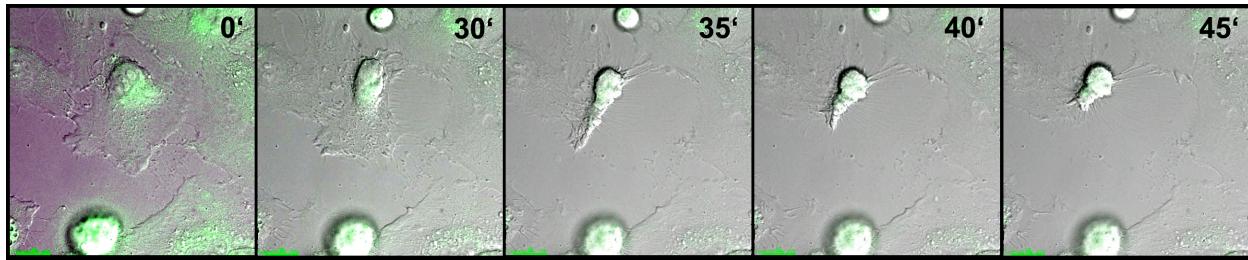


Figure 15. Time lapse imaging of HeLa cell expressing ctKin4-GFP. HeLa CCL.2 cells were transiently transfected with ctKin4-GFP and allowed to express the fusion protein for 8 hours. Twenty candidate cells expressing ctKin4-GFP were chosen and imaged every 5 minutes for 2 hours using fluorescence and DIC. Cells expressing ctKin4 displayed a rapid breakdown in their cytoskeleton from a spread phenotype (0 minutes) to a rounded phenotype (45 minutes). This phenotype was observed even in the presence of Blebbestatin. Many of the ctKin4-GFP expressing cells eventually lost adherence to the substrate.

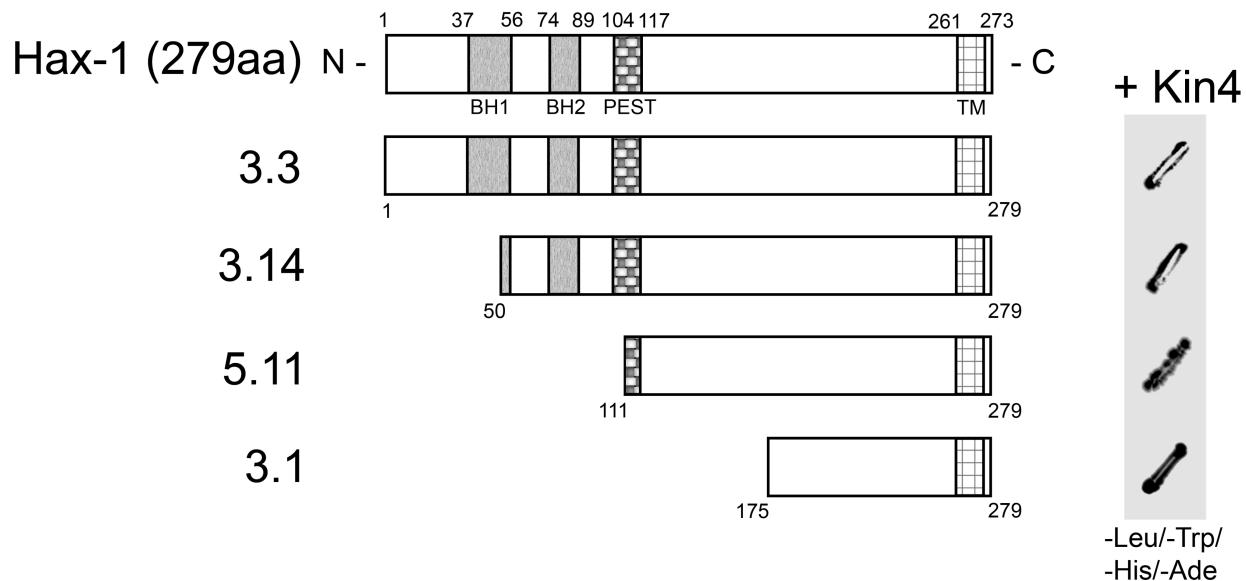


Figure 16. Yeast two-hybrid using ctKin4 identifies Hax-1 as a novel interacting partner. ctKin4 was fused to the Gal4 DNA-BD and was used to screen a HeLa cDNA library. 1.35×10^7 CFUs were screened on media lacking leucine, tryptophan, histidine, and adenine. 400 CFUs were isolated, and of those 30 were analyzed. 22 CFUs were one of four unique Hax-1 clones. Clone 3.3 contains the entire coding region of Hax-1 cDNA, whereas clone 3.14 encodes the C-terminal 249 amino acids, clone 5.11 encodes the C-terminal 168 amino acids, and clone 3.1, the smallest clone, encodes only the C-terminal 104 amino acids. The Hax-1 protein contains two Bcl-2 homology domains (BH1 & BH2), a PEST domain, and a putative transmembrane (TM) domain near its C-terminus.

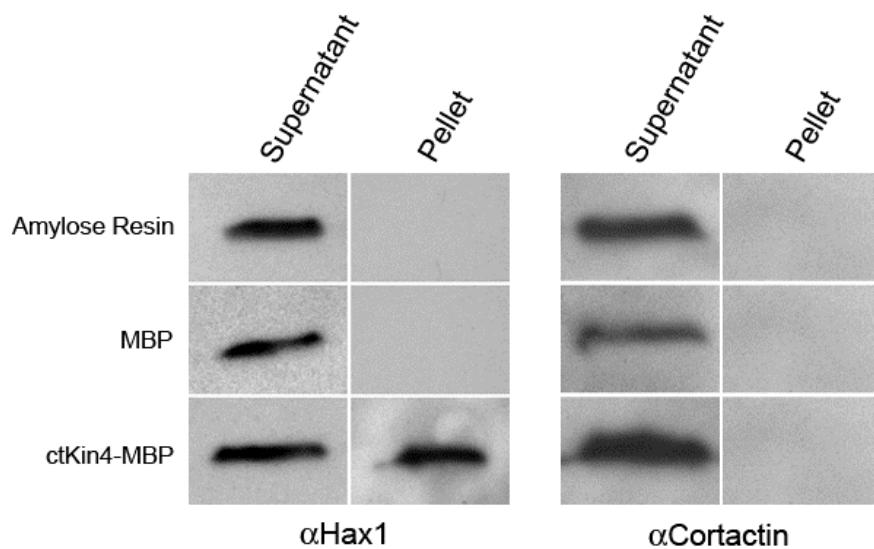


Figure 17. ctKin4-MBP specifically interacts with native HeLa cell Hax-1. ctKin4 was cloned to make a fusion protein with maltose binding protein (MBP). ctKin4-MBP was bound to amylose resin and purified. The soluble fraction from a HeLa cell extract was incubated for 1 hour at 4°C, washed five times, and fractionated on 15% SDS-PAGE. Proteins were blotted onto nitrocellulose and a Western blot was performed using anti-Hax-1 and anti-cortactin polyclonal antibodies (BD Biosciences). ctKin4-MBP specifically interacted with Hax-1 from the HeLa cell extract confirming our yeast two-hybrid interaction. Cortactin was not found to be detected as part of a complex through our experimental conditions suggesting that ctKin4 binding is competitive to Hax-1 with cortactin.

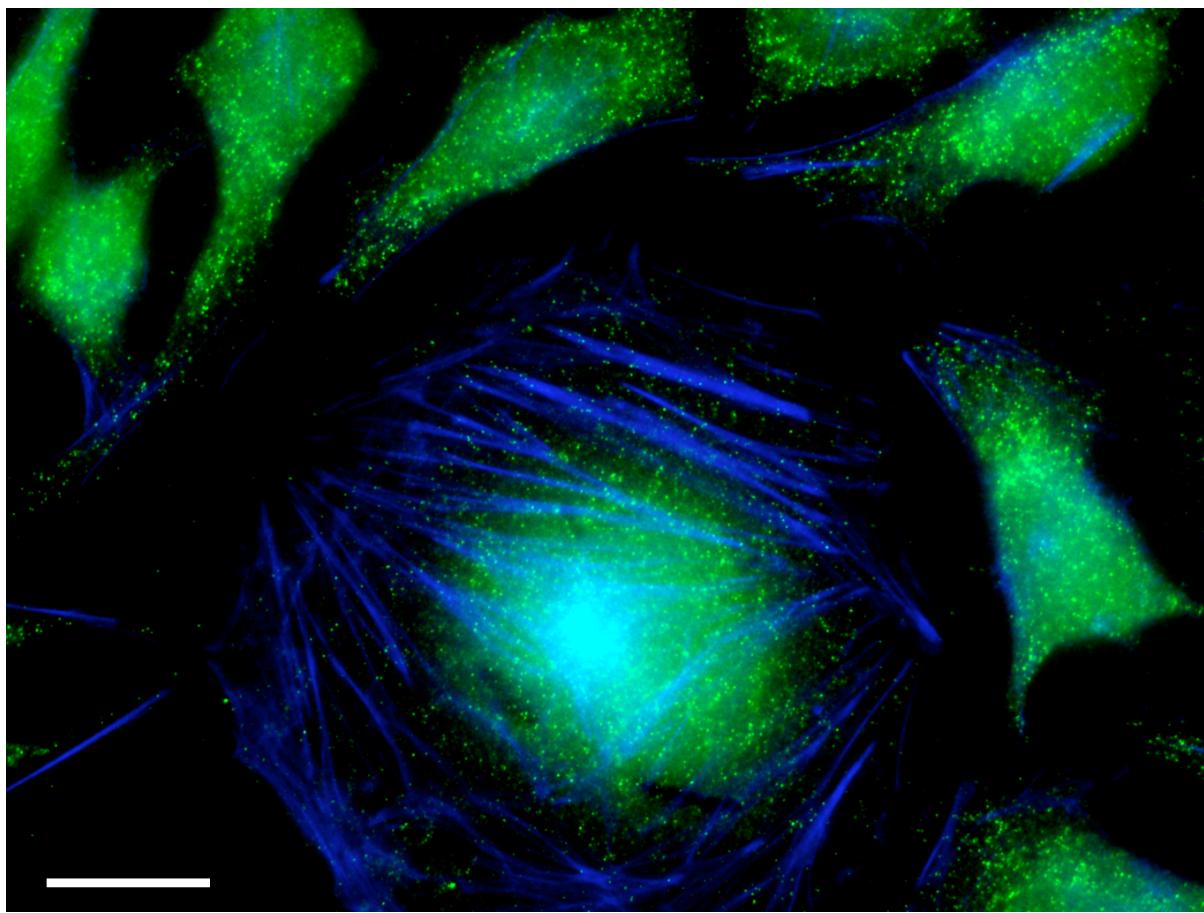


Figure 18. Hax-1 localization in HeLa CCL.2cells . HeLa cells were grown to a confluence of ~75%. The cells then were fixed and stained using an anti-Hax-1 antibody (Green) and Alexa Fluor 350 phalloidin (Blue). Hax-1 displays a punctate staining throughout the cytoplasm of the cell, however is not directly associated with the actin stress fibers (Blue). Scale bar=20 μ m.

CHAPTER 4

SUMMARY AND CONCLUSION

Nomuscle cells display a dynamic and highly organized array of stress fibers composed of alpha-actinin crosslinked anti-parallel actin filaments interdigitated with myosin-II bipolar filaments (Langanger et al., 1986). Highly similar to the skeletal and cardiac muscle sarcomere, stress fibers provide contractile force in processes such as cell migration, cell division, cell shape, and cytoskeletal reorganization. Some details of the mechanisms cells use to regulate stress fibers organization and dynamics remain to be determined, but it is clear that the family of Rho-GTPases regulate stress fiber formation through downstream effector molecules such as formins, which regulate actin filament formation, and ROCK, which regulates myosin-II filament organization (Hotulainen and Lappalainen, 2006). Previously, we discovered a high molecular weight protein ~4 MDa. in the extracts of chicken intestinal epithelial brush border and human platelets. This protein binds to both alpha-actinin and nonmuscle myosin-II and was named cellular titin, c-titin, due to its similarity in structure and function to skeletal muscle titin (Eilertsen et al., 1994; Eilertsen and Keller, III, 1992). In low-ionic strength conditions, c-titin can organize nonmuscle myosin-II bipolar filaments side-by-side and end-to-end into large coassemblies (Eilertsen et al., 1994). Based on the skeletal muscle titin paradigm, we hypothesize that c-titin is an important molecule in stress fiber formation and organization.

The work presented here further characterizes c-titin. In Chapter 2, we showed human c-titin is a novel titin isoform encoded by the titin gene on the long arm of chromosome 2. We have identified mRNAs for at least three isoforms of cellular titin in the extracts of human megakaryocytes; two full length isoforms, which are encoded with differential splicing in the PEVK region, and the truncated Novex-3 isoform. All three isoforms contain the differentially spliced alpha-actinin binding Z-repeat region that has

been reported in psoas muscle titin and in some fetal skeletal titin isoforms (Sorimachi et al., 1997).

Antibodies to the N-terminus and C-terminus of the titin molecule reacted specifically with the high molecular weight c-titin protein in platelet extracts, thus confirming cellular titin to be a novel isoform of titin. Moreover, immunolocalization showed c-titin is distributed in a punctate pattern with a periodic spacing of ~800nm-1.2 μ m along the stress fibers of fibroblast and epithelial cells. This is consistent with titin to be ~1 μ m in length. The N-terminal region of cellular titin localized in association with stress fiber actin filaments, most likely due to association of cellular titin with the actin-crosslinking protein alpha-actinin. Interestingly, the C-terminal region of titin, which contains the titin kinase domain, localized in a loose association with the stress fibers, but it appeared that this region of titin projects away from the stress fiber core into the cytosol. I hypothesize this projection away from the stress fiber core enables the titin kinase domain and adjacent domains to interact with, and in the case of the kinase domain to phosphorylate, proteins outside the tightly packed stress fiber structure. In order for titin kinase to bind to its substrate I believe its localization away from the highly proteinaceous actin based stress fiber fosters substrate interactions, and like the skeletal titin kinase domain may possibly be a site for a signaling complex.

Our sequence analyses showed that the c-titin A-band region contains the giant myosin filament binding exon 236. This single exon encodes for 5701 amino acids and is approximately 232nm in length. In the skeletal titin paradigm, the A-band region contains 7- and 11- domain super-repeats, which interact with the myosin tails in one-half, 800nm, of the skeletal muscle bipolar filament. The nonmuscle myosin-II bipolar filament, although similar in organization, is only 300-400 nm in length. Therefore, it is difficult to extrapolate how c-titin A-band could interact with one half (150-200 nm) of the nonmuscle myosin-II bipolar filament. Moreover, measurement studies suggest titin lies in a head-to-tail fashion along the stress fibers. The ultrastructure of cellular titin within the myosin filament binding region is still undetermined and single molecule double labeling experiments need to be completed in order to further shed light on this hypothesis.

We have determined the presence of mRNA encoding the truncated titin isoform Novex-3 in the total RNA extracts of megakaryocytes. We showed Novex-3 contains the same Z-repeat region found in the two full length cellular titin isoforms we discovered. When using antibodies raised to this Z-repeat region, however, we did not detect a truncated c-titin isoform in platelet extracts. Platelets are small subcellular fragments formed from bone marrow megakaryocytes. They are formed by pinching off as proplatelets from megakaryocyte cellular extensions that are filled with the contents of the platelet (Italiano, Jr. and Shivdasani, 2003; Hartwig and Italiano, Jr., 2003). Because we can detect Novex-3 transcripts in the megakaryocyte but cannot detect the protein in the platelet extracts, we hypothesize Novex-3 to be localized in the megakaryocyte and retained during platelet formation. This suggests a unique and novel role for the Novex-3 isoform in the megakaryocyte and suggests a possible novel role for Novex-3 in platelet production. The CHRF-288-11 megakaryocyte cell line we used does not adhere well to surfaces, thus making it difficult to do immunolocalization studies. Additionally, CHRF cells form platelets only poorly in culture. It would be interesting to analyze primary megakaryocytes that can produce platelets for how and where the three c-titin isoforms are localized within the proplatelets and the megakaryocyte.

Skeletal titin contains at its C-terminus a serine/threonine kinase domain in the myosin light chain kinase family. Although little is known about titin kinase function, it is evident that it plays a crucial role in sarcomere formation and organization (Mayans et al., 1998; Miller et al., 2003). Recently, the titin kinase region has been hypothesized to be a stretch sensor, because several signaling molecules bind to the titin kinase domain or regions close to the kinase domain (McElhinny et al., 2002; Lange et al., 2005). Our sequence analysis of c-titin shows both full length molecules contain the titin kinase domain. We hypothesize the cellular titin kinase domain to be fully functional and relevant within the nonmuscle cells and report here c-titin kinase is important in stress fiber organization and integrity. Cells expressing the c-titin kinase domain show a marked increase in breakdown of stress fiber structure and an overall rounding up phenotype compared to control cells. Live cell microscopy showed this is a rapid “contraction-like” event, which can take place even in the presence of a myosin inhibitor

Blebbistatin, suggesting this rounding up is due to a breakdown in stress fiber structure as opposed to myosin contraction stimulation. Expression of both a constitutively active and inactive kinase domain produced this same phenotype, suggesting the rounding up phenotype results from a protein-protein interaction similar to a dominant negative effect rather than a phosphorylation event. Our yeast two-hybrid analysis using the c-titin kinase domain revealed a novel interaction with HS1 associated protein X-1 (Hax-1).

Hax-1 is ubiquitously expressed in muscle and nonmuscle cells. Originally discovered to be a binding partner to HS1, a cortactin homologue in hemopoietic and lymphoid cells (Suzuki et al., 1997), Hax-1 is a small ~26 kDa. protein that contains two Bcl-2 homology domains, a PEST domain, and a putative transmembrane domain. Hax-1 is hypothesized to be anti-apoptotic (Cilenti et al., 2004;Lee et al., 2008;Han et al., 2006) and has also been shown to be important in cell motility and chemotaxis (Gallagher et al., 2000;Radhika et al., 2004;Mirmohammadsadegh et al., 2003;Klein et al., 2007). One of the more intriguing roles of Hax-1 is its mediation of the Rac/Rho pathways through the interaction to the G_α subunit of the G13 protein (Radhika et al., 2004). Stress fiber formation is primarily regulated through RhoGTP. When stimulated, G_α13 binds to RhoGEF that then activates Rho and its downstream effector proteins such as formins and ROCK. When Rac is activated stimulating cell migration, however, G_α13 is found to interact with a complex of Hax-1, cortactin, and Rac. It is unclear whether there is a specific order of binding to this complex; but it is interesting that Hax-1 is a mediator between Rac and cortactin. Cortactin in the Rho-stimulated spread cell is localized in the cytosol, where it remains in a punctate pattern, presumably bound to Hax-1 (Vafiadaki et al., 2007;Suzuki et al., 1997). Upon Rac activation, cortactin localizes with Rac and G_α13 on the plasma membrane of the cell, where it stimulates actin polymerization at the leading edge (Weed et al., 1998).

Based on its ability to interact with Hax-1, I hypothesize that the titin kinase domain is specifically involved in Hax-1-cortactin localization within the cell and ultimately plays a role in Rac-induced disassembly of stress fiber structures. We have previously shown the titin kinase domain has a punctate localization in a loose association with the actin based stress fibers in spread fibroblasts and epithelial cells. Localization of the titin kinase domain and Hax-1 in the same cells, however, showed

little colocalization. Moreover, the inability of the GFP expressed kinase domain to specifically pull down native Hax-1 in HeLa cell extracts suggests this interaction is not constitutive and that it may be regulated through an unknown mechanism. The spread epithelial and fibroblast cell lines we have used to investigate this work are well spread and most likely have active Rho pathways. We have not specifically looked at cells that clearly are undergoing Rac activation. Thus, it follows that the titin kinase-Hax-1 interaction and phenotypic result may not be evident unless Rac is activated. Future work will need to be done to specifically isolate Rac and Rho pathways to investigate localization and interactions of c-titin kinase with Hax-1 and perhaps cortactin. We currently are attempting to create a stable miRNA based c-titin knockdown cell line. It will be interesting to see how these cells react when the Rac and Rho pathways are activated.

The work presented in this dissertation has laid the groundwork for further characterization of c-titin and the specific role it plays in stress fiber organization and regulation. We have added to the repertoire of titin-interacting proteins and have strengthened the titin field by showing c-titin to be a novel titin isoform that is organized in a similar manner to muscle titin in the stress fiber structure. Through its interaction with Hax-1, c-titin has been shown to play a role in stress fiber signaling and regulation. This work has provided the basis for studying various regions of c-titin and investigating the role c-titin kinase has on overall cell shape and motility. It is my hypothesis that c-titin is a fundamental protein involved in nonmuscle cell cytoskeleton organization and regulation.

APPENDIX A
APOPTOSIS DETECTION IN CELLS EXPRESSING CTKIN4

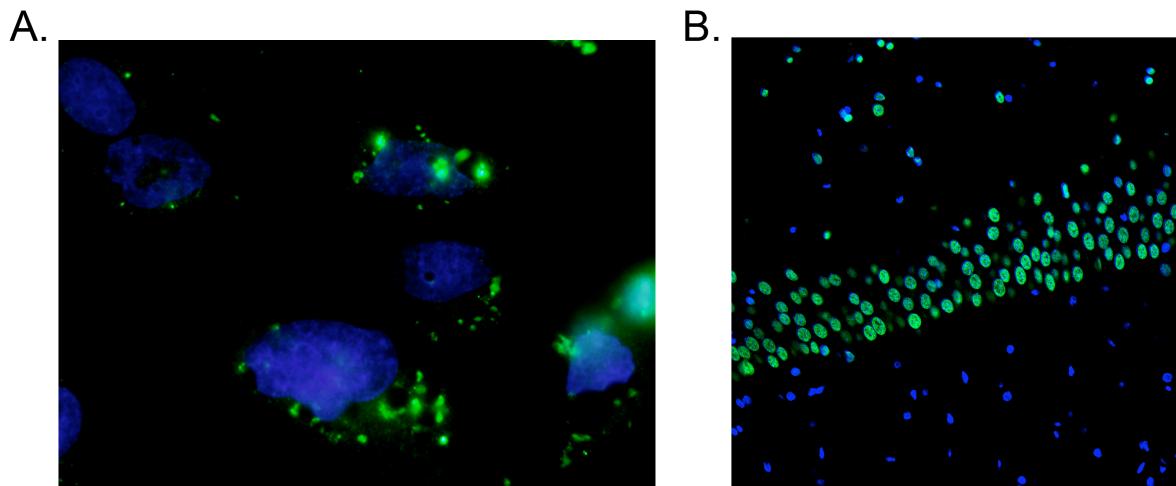


Figure 19. Apoptosis Detection of Cells Expressing ctKin4. (A) HeLa cells were grown to a 75% confluence and transfected with ctKin4-mCherry using DMRIE-C transfection reagent and allowed to express for 24 hours. Cells were gently fixed with paraformaldehyde and subjected to the TUNEL assay (Calbiochem) for apoptosis detection. Fixed slides were imaged at 63x and cells expressing ctKin4-mCherry were not shown to be apoptotic. (B) DNase I treated positive control of a P21 rat hippocampal slice imaged at 40x showing green stained nuclei indicative of a positive TUNEL assay reaction (Image courtesy of Mike Darcy, Charlie Ouimet Laboratory, FSU, Tallahassee, FL).

APPENDIX B
CELLULAR TITIN AND MYOSIN LOCALIZATION IN ACTIVATED PLATELETS

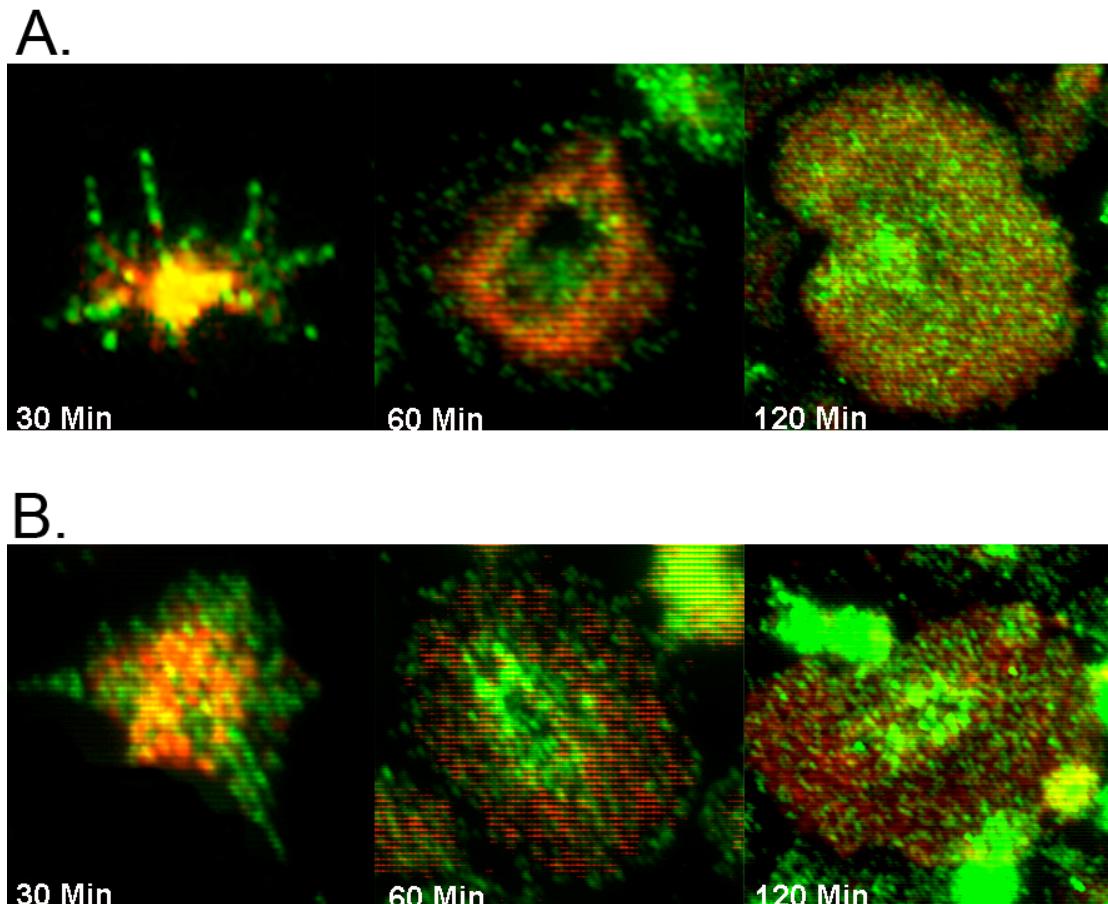


Figure 20. Cellular Titin and Myosin Localization in Activated Platelets. Human blood platelets were activated and spread on fibrinogen-coated cover slips and fixed after 30 minutes, 60 minutes, and 120 minutes. Cells were stained for cellular titin Z-repeat region (A, green) and myosin light chain (A, red), and for cellular titin kinase domain (B, green) and myosin light chain (B, red). After 30 minutes of activation, C-titin appears to be localized in a punctate pattern in the filopodia, whereas the majority of myosin remains in the cell body. After 60 minutes, C-titin is distributed throughout the platelet, and myosin is more evenly associated with C-titin. After 120 minutes, the platelet is fully spread, and C-titin and myosin are loosely colocalized. These data suggests cellular titin to be an early mediator in the myosin based contractility of platelet filopodia involved in wound healing.

APPENDIX C HUMAN SUBJECTS APPROVAL FORM



Office of the Vice President For Research
Human Subjects Committee
Tallahassee, Florida 32306-2742
(850) 644-8673 · FAX (850) 644-4392

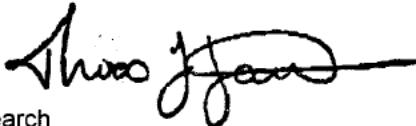
APPROVAL MEMORANDUM (for change in research protocol)

Date: 7/27/2006

To:
Tom Keller
MC: 4370

Dept: BIOLOGICAL SCIENCE

From: Thomas L. Jacobson, Chair



Re: Use of Human subjects in Research
Project entitled: Acquisition And Use Of Human Platelets For Investigation Of Structure And Adhesion

The memorandum that you submitted to this office in regard to the requested change in your research protocol for the above-referenced project have been reviewed and approved. Thank you for informing the Committee of this change.

A reminder that if the project has not been completed by 11/29/2006, you must request renewed approval for continuation of the project.

By copy of this memorandum, the chairman of your department and/or your major professor is reminded that he/she is responsible for being informed concerning research projects involving human subjects in the department, and should review protocols of such investigations as often as needed to insure that the project is being conducted in compliance with our institution and with DHHS regulations.

This institution has an Assurance on file with the Office for Protection from Research Risks. The Assurance Number is IRB00000446..

cc:
APPLICATION NO. 2005.952-R

INFORMED CONSENT FORM

I, _____, freely and voluntarily and without element of force or coercion, consent to be a donor of whole blood for the research project entitled "Acquisition and Use of Human Platelets for Investigation of Structure and Function".

The research laboratory of Dr. Tom Keller, Ph.D., who is an Associate Professor of Biological Science at Florida State University, is conducting this research. I understand the purpose of this research project is to better understand normal blood platelet function and that the platelets isolated from the blood I donate will be subjected to routine cell biological and biochemical tests. In addition to providing information about platelet function, the research also should yield insight into the activities of many other cells. I understand the benefits for participating in this research project are that researchers will learn more about the normal function of platelets, which may help elucidate defects that cause human platelet diseases.

I understand that if I participate in the project I will be asked to donate 20 milliliters of blood no more than twice a week and no more than 450 milliliters in any eight-week period. I understand I will receive no compensation for this donation.

I understand that the risks of blood donation are minimal but could include dizziness, fainting, nausea, bruising, vein damage, or nerve damage. I understand that the blood donation will be obtained by trained personnel in the FSU Thagard Health Center or the Southeastern Community Blood Center.

I understand my participation is voluntary, and I may stop participation at anytime. I also understand that my identity as donor of a specific batch of blood will be strictly confidential, that laboratory records of results obtained using my platelets will not list me as the donor, and that results will be reported with no identification of me as a donor.

I understand that this consent may be withdrawn at any time without prejudice, penalty, or loss of benefits to which I am otherwise entitled. I have been given the right to ask and have answered any inquiry concerning the study. Questions, if any, have been answered to my satisfaction.

I understand that I may contact Dr. Tom Keller, Florida State University, Department of Biological Science, at (805) 644-9813 and/or the FSU Institutional Review Board-Human Subjects Committee at (850) 644-7900, for answers to questions about this research or my rights. Published results of this research will be sent to me upon my request.

I have read and understand this consent form.

(Subject)

(Date)

(Witness)



REFERENCES

- Atkinson, R.A., C.Joseph, F.Dal Piaz, L.Birolo, G.Stier, P.Pucci, and A.Pastore. 2000. Binding of alpha-actinin to titin: implications for Z-disk assembly. *Biochemistry* 39:5255-5264.
- Bang, M.L., T.Centner, F.Fornoff, A.J.Geach, M.Gotthardt, M.McNabb, C.C.Witt, D.Labeit, C.C.Gregorio, H.Granzier, and S.Labeit. 2001. The complete gene sequence of titin, expression of an unusual approximately 700-kDa titin isoform, and its interaction with obscurin identify a novel Z-line to I-band linking system. *Circ. Res.* 89:1065-1072.
- Cavnar, P.J., S.G.Olenych, and T.C.Keller, III. 2007. Molecular identification and localization of cellular titin, a novel titin isoform in the fibroblast stress fiber. *Cell Motil. Cytoskeleton* 64:418-433.
- Centner, T., J.Yano, E.Kimura, A.S.McElhinny, K.Pelin, C.C.Witt, M.L.Bang, K.Trombitas, H.Granzier, C.C.Gregorio, H.Sorimachi, and S.Labeit. 2001. Identification of muscle specific ring finger proteins as potential regulators of the titin kinase domain. *J. Mol. Biol.* 306:717-726.
- Chi, R.J., S.G.Olenych, K.Kim, and T.C.Keller, III. 2005. Smooth muscle alpha-actinin interaction with smitin. *Int. J. Biochem. Cell Biol.* 37:1470-1482.
- Cilenti, L., M.M.Soundarapandian, G.A.Kyriazis, V.Stratico, S.Singh, S.Gupta, J.V.Bonventre, E.S.Alnemri, and A.S.Zervos. 2004. Regulation of HAX-1 anti-apoptotic protein by Omi/HtrA2 protease during cell death. *J. Biol. Chem.* 279:50295-50301.
- Coulis, G., M.A.Sentandreu, N.Bleimling, M.Gautel, Y.Benyamin, and A.Ouali. 2004. Myofibrillar tightly bound calcium in skeletal muscle fibers: a possible role of this cation in titin strands aggregation. *FEBS Lett.* 556:271-275.
- Eilertsen, K.J., S.T.Kazmierski, and T.C.Keller, III. 1994. Cellular titin localization in stress fibers and interaction with myosin II filaments in vitro. *J. Cell Biol.* 126:1201-1210.
- Eilertsen, K.J., S.T.Kazmierski, and T.C.Keller, III. 1997. Interaction of alpha-actinin with cellular titin. *Eur. J. Cell Biol.* 74:361-364.
- Eilertsen, K.J. and T.C.Keller, III. 1992. Identification and characterization of two huge protein components of the brush border cytoskeleton: evidence for a cellular isoform of titin. *J. Cell Biol.* 119:549-557.
- Elsner, J., J.Roesler, A.Emmendorffer, M.L.Lohmann-Matthes, and K.Welte. 1993. Abnormal regulation in the signal transduction in neutrophils from patients with severe congenital neutropenia: relation of impaired mobilization of cytosolic free calcium to

altered chemotaxis, superoxide anion generation and F-actin content. *Exp. Hematol.* 21:38-46.

Faulkner, G., G.Lanfranchi, and G.Valle. 2001. Telethonin and other new proteins of the Z-disc of skeletal muscle. *IUBMB. Life* 51:275-282.

Forbes, J.G., A.J.Jin, K.Ma, G.Gutierrez-Cruz, W.L.Tsai, and K.Wang. 2005. Titin PEVK segment: charge-driven elasticity of the open and flexible polyampholyte. *J. Muscle Res. Cell Motil.* 26:291-301.

Fugman, D.A., D.P.Witte, C.L.Jones, B.J.Aronow, and M.A.Lieberman. 1990. In vitro establishment and characterization of a human megakaryoblastic cell line. *Blood* 75:1252-1261.

Fukuda, N., Y.Wu, P.Nair, and H.L.Granzier. 2005. Phosphorylation of titin modulates passive stiffness of cardiac muscle in a titin isoform-dependent manner. *J. Gen. Physiol* 125:257-271.

Furst, D.O., U.Vinkemeier, and K.Weber. 1992. Mammalian skeletal muscle C-protein: purification from bovine muscle, binding to titin and the characterization of a full-length human cDNA. *J. Cell Sci.* 102 (Pt 4):769-778.

Gallagher, A.R., A.Cedzich, N.Gretz, S.Somlo, and R.Witzgall. 2000. The polycystic kidney disease protein PKD2 interacts with Hax-1, a protein associated with the actin cytoskeleton. *Proc. Natl. Acad. Sci. U. S. A* 97:4017-4022.

Gautel, M. 1996. The super-repeats of titin/connectin and their interactions: glimpses at sarcomeric assembly. *Adv. Biophys.* 33:27-37.

Gautel, M., D.Goulding, B.Bullard, K.Weber, and D.O.Furst. 1996. The central Z-disk region of titin is assembled from a novel repeat in variable copy numbers. *J. Cell Sci.* 109 (Pt 11):2747-2754.

Gotthardt, M., R.E.Hammer, N.Hubner, J.Monti, C.C.Witt, M.McNabb, J.A.Richardson, H.Granzier, S.Labeit, and J.Herz. 2003. Conditional expression of mutant M-line titins results in cardiomyopathy with altered sarcomere structure. *J. Biol. Chem.* 278:6059-6065.

Granzier, H., M.Helmes, O.Cazorla, M.McNabb, D.Labeit, Y.Wu, R.Yamasaki, A.Redkar, M.Kellermayer, S.Labeit, and K.Trombitas. 2000. Mechanical properties of titin isoforms. *Adv. Exp. Med. Biol.* 481:283-300.

Granzier, H. and S.Labeit. 2002. Cardiac titin: an adjustable multi-functional spring. *J. Physiol* 541:335-342.

Grater, F., J.Shen, H.Jiang, M.Gautel, and H.Grumbmuller. 2005. Mechanically induced titin kinase activation studied by force-probe molecular dynamics simulations. *Biophys. J.* 88:790-804.

- Greaser, M. 2001. Identification of new repeating motifs in titin. *Proteins* 43:145-149.
- Greene, D., T.Garcia, B.Sutton, K.M.Gernert, G.M.Benian, and A.F.Oberhauser. 2008. Single-molecule force spectroscopy reveals a stepwise unfolding of *C. elegans* giant protein kinase domains. *Biophys. J.*
- Gregorio, C.C., C.N.Perry, and A.S.McElhinny. 2005. Functional properties of the titin/connectin-associated proteins, the muscle-specific RING finger proteins (MURFs), in striated muscle. *J. Muscle Res. Cell Motil.* 26:389-400.
- Gregorio, C.C., K.Trombitas, T.Centner, B.Kolmerer, G.Stier, K.Kunke, K.Suzuki, F.Obermayr, B.Herrmann, H.Granzier, H.Sorimachi, and S.Labeit. 1998. The NH₂ terminus of titin spans the Z-disc: its interaction with a novel 19-kD ligand (T-cap) is required for sarcomeric integrity. *J. Cell Biol.* 143:1013-1027.
- Gutierrez-Cruz, G., A.H.Van Heerden, and K.Wang. 2001. Modular motif, structural folds and affinity profiles of the PEVK segment of human fetal skeletal muscle titin. *J. Biol. Chem.* 276:7442-7449.
- Han, Y., Y.S.Chen, Z.Liu, N.Bodyak, D.Rigor, E.Bisping, W.T.Pu, and P.M.Kang. 2006. Overexpression of HAX-1 protects cardiac myocytes from apoptosis through caspase-9 inhibition. *Circ. Res.* 99:415-423.
- Hartwig, J. and J.Italiano, Jr. 2003. The birth of the platelet. *J. Thromb. Haemost.* 1:1580-1586.
- Hayashi, C., Y.Ono, N.Do, F.Kitamura, M.Tagami, R.Mineki, T.Arai, H.Taguchi, M.Yanagida, S.Hirner, D.Labeit, S.Labeit, and H.Sorimachi. 2008. Multiple molecular interactions implicate connectin/titin N2A region as a modulating scaffold for p94/calpain 3 activity in skeletal muscle. *J. Biol. Chem.*
- Helmes, M., K.Trombitas, T.Centner, M.Kellermayer, S.Labeit, W.A.Linke, and H.Granzier. 1999. Mechanically driven contour-length adjustment in rat cardiac titin's unique N2B sequence: titin is an adjustable spring. *Circ. Res.* 84:1339-1352.
- Hoshijima, M. 2006. Mechanical stress-strain sensors embedded in cardiac cytoskeleton: Z disk, titin, and associated structures. *Am. J. Physiol Heart Circ. Physiol* 290:H1313-H1325.
- Hotulainen, P. and P.Lappalainen. 2006. Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. *J. Cell Biol.* 173:383-394.
- Houmeida, A., J.Holt, L.Tskhovrebova, and J.Trinick. 1995. Studies of the interaction between titin and myosin. *J. Cell Biol.* 131:1471-1481.
- Italiano, J.E., Jr. and R.A.Shivdasani. 2003. Megakaryocytes and beyond: the birth of platelets. *J. Thromb. Haemost.* 1:1174-1182.

Katoh, K., Y.Kano, M.Masuda, H.Onishi, and K.Fujiwara. 1998. Isolation and contraction of the stress fiber
5. *Mol. Biol. Cell* 9:1919-1938.

Keller, T.C., III, K.Eilertsen, M.Higginbotham, S.Kazmierski, K.T.Kim, and M.Velichkova. 2000. Role of titin in nonmuscle and smooth muscle cells. *Adv. Exp. Med. Biol.* 481:265-277.

Kim, K. and T.C.Keller, III. 2002. Smitin, a novel smooth muscle titin-like protein, interacts with myosin filaments in vivo and in vitro. *J. Cell Biol.* 156:101-111.

Klein, C., M.Grudzien, G.Appaswamy, M.Germeshausen, I.Sandrock, A.A.Schaffer, C.Rathinam, K.Boztug, B.Schwinzer, N.Rezaei, G.Bohn, M.Melin, G.Carlsson, B.Fadeel, N.Dahl, J.Palmblad, J.I.Henter, C.Zeidler, B.Grimbacher, and K.Welte. 2007. HAX1 deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease). *Nat. Genet.* 39:86-92.

Kontogianni-Konstantopoulos, A. and R.J.Bloch. 2005. Obscurin: a multitasking muscle giant
3. *J. Muscle Res. Cell Motil.* 26:419-426.

Kontogianni-Konstantopoulos, A., D.H.Catino, J.C.Strong, W.R.Randall, and R.J.Bloch. 2004. Obscurin regulates the organization of myosin into A bands. *Am. J. Physiol Cell Physiol* 287:C209-C217.

Kontogianni-Konstantopoulos, A., D.H.Catino, J.C.Strong, S.Sutter, A.B.Borisov, D.W.Pumplin, M.W.Russell, and R.J.Bloch. 2006. Obscurin modulates the assembly and organization of sarcomeres and the sarcoplasmic reticulum
1. *FASEB J.* 20:2102-2111.

Labeit, S., M.Gautel, A.Lakey, and J.Trinick. 1992. Towards a molecular understanding of titin. *EMBO J.* 11:1711-1716.

Labeit, S. and B.Kolmerer. 1995. Titins: giant proteins in charge of muscle ultrastructure and elasticity. *Science* 270:293-296.

Labeit, S., S.Lahmers, C.Burkart, C.Fong, M.McNabb, S.Witt, C.Witt, D.Labeit, and H.Granzier. 2006. Expression of distinct classes of titin isoforms in striated and smooth muscles by alternative splicing, and their conserved interaction with filamins. *J. Mol. Biol.* 362:664-681.

Langanger, G., M.Moeremans, G.Daneels, A.Sobieszek, M.De Brabander, and J.De Mey. 1986. The molecular organization of myosin in stress fibers of cultured cells. *J. Cell Biol.* 102:200-209.

Lange, S., F.Xiang, A.Yakovenko, A.Vihola, P.Hackman, E.Rostkova, J.Kristensen, B.Brandmeier, G.Franzen, B.Hedberg, L.G.Gunnarsson, S.M.Hughes, S.Marchand, T.Sejersen, I.Richard, L.Edstrom, E.Ehler, B.Udd, and M.Gautel. 2005. The kinase

domain of titin controls muscle gene expression and protein turnover. *Science* 308:1599-1603.

Lee, A.Y., Y.Lee, Y.K.Park, K.H.Bae, S.Cho, d.H.Lee, B.C.Park, S.Kang, and S.G.Park. 2008. HS 1-associated protein X-1 is cleaved by caspase-3 during apoptosis. *Mol. Cells* 25:86-90.

Lee, E.H., M.Gao, N.Pinotsis, M.Wilmanns, and K.Schulten. 2006. Mechanical strength of the titin Z1Z2-telethonin complex. *Structure*. 14:497-509.

Linke, W.A. 2008. Sense and stretchability: the role of titin and titin-associated proteins in myocardial stress-sensing and mechanical dysfunction. *Cardiovasc. Res.* 77:637-648.

Linke, W.A., M.Ivemeyer, S.Labeit, H.Hinssen, J.C.Ruegg, and M.Gautel. 1997. Actin-titin interaction in cardiac myofibrils: probing a physiological role
5. *Biophys. J.* 73:905-919.

Linke, W.A., M.Ivemeyer, N.Olivieri, B.Kolmerer, J.C.Ruegg, and S.Labeit. 1996. Towards a molecular understanding of the elasticity of titin. *J. Mol. Biol.* 261:62-71.

Linke, W.A., D.E.Rudy, T.Centner, M.Gautel, C.Witt, S.Labeit, and C.C.Gregorio. 1999. I-band titin in cardiac muscle is a three-element molecular spring and is critical for maintaining thin filament structure. *J. Cell Biol.* 146:631-644.

Ma, K., J.G.Forbes, G.Gutierrez-Cruz, and K.Wang. 2006. Titin as a giant scaffold for integrating stress and Src homology domain 3-mediated signaling pathways: the clustering of novel overlap ligand motifs in the elastic PEVK segment. *J. Biol. Chem.* 281:27539-27556.

Ma, K., L.Kan, and K.Wang. 2001. Polyproline II helix is a key structural motif of the elastic PEVK segment of titin. *Biochemistry* 40:3427-3438.

Ma, K. and K.Wang. 2003. Malleable conformation of the elastic PEVK segment of titin: non-co-operative interconversion of polyproline II helix, beta-turn and unordered structures
4. *Biochem. J.* 374:687-695.

Machado, C. and D.J.Andrew. 2000. D-Titin: a giant protein with dual roles in chromosomes and muscles. *J. Cell Biol.* 151:639-652.

Mayans, O., P.F.van der Ven, M.Wilm, A.Mues, P.Young, D.O.Furst, M.Wilmanns, and M.Gautel. 1998. Structural basis for activation of the titin kinase domain during myofibrillogenesis. *Nature* 395:863-869.

McElhinny, A.S., K.Kakinuma, H.Sorimachi, S.Labeit, and C.C.Gregorio. 2002. Muscle-specific RING finger-1 interacts with titin to regulate sarcomeric M-line and thick filament

structure and may have nuclear functions via its interaction with glucocorticoid modulatory element binding protein-1. *J. Cell Biol.* 157:125-136.

Miller, G., H.Musa, M.Gautel, and M.Peckham. 2003. A targeted deletion of the C-terminal end of titin, including the titin kinase domain, impairs myofibrillogenesis. *J. Cell Sci.* 116:4811-4819.

Mirmohammadsadegh, A., U.Tartler, G.Michel, A.Baer, M.Walz, R.Wolf, T.Ruzicka, and U.R.Hengge. 2003. HAX-1, identified by differential display reverse transcription polymerase chain reaction, is overexpressed in lesional psoriasis. *J. Invest Dermatol.* 120:1045-1051.

Muhle-Goll, C., M.Habeck, O.Cazorla, M.Nilges, S.Labeit, and H.Granzier. 2001. Structural and functional studies of titin's fn3 modules reveal conserved surface patterns and binding to myosin S1—a possible role in the Frank-Starling mechanism of the heart. *J. Mol. Biol.* 313:431-447.

Musa, H., S.Meek, M.Gautel, D.Peddie, A.J.Smith, and M.Peckham. 2006. Targeted homozygous deletion of M-band titin in cardiomyocytes prevents sarcomere formation. *J. Cell Sci.* 119:4322-4331.

Nicholas, G., M.Thomas, B.Langley, W.Somers, K.Patel, C.F.Kemp, M.Sharma, and R.Kambadur. 2002. Titin-cap associates with, and regulates secretion of, Myostatin 5. *J. Cell Physiol* 193:120-131.

Obermann, W.M., M.Gautel, K.Weber, and D.O.Furst. 1997. Molecular structure of the sarcomeric M band: mapping of titin and myosin binding domains in myomesin and the identification of a potential regulatory phosphorylation site in myomesin. *EMBO J.* 16:211-220.

Ojima, K., Y.Ono, S.Hata, S.Koyama, N.Do, and H.Sorimachi. 2005. Possible functions of p94 in connectin-mediated signaling pathways in skeletal muscle cells. *J. Muscle Res. Cell Motil.* 26:409-417.

Peterson, L.J., Z.Rajfur, A.S.Maddox, C.D.Freel, Y.Chen, M.Edlund, C.Otey, and K.Burridge. 2004. Simultaneous stretching and contraction of stress fibers in vivo. *Mol. Biol. Cell* 15:3497-3508.

Radhika, V., D.Onesime, J.H.Ha, and N.Dhanasekaran. 2004. Galphai3 stimulates cell migration through cortactin-interacting protein Hax-1. *J. Biol. Chem.* 279:49406-49413.

Ramsay, A.G., M.D.Keppler, M.Jazayeri, G.J.Thomas, M.Parsons, S.Violette, P.Weinreb, I.R.Hart, and J.F.Marshall. 2007. HS1-associated protein X-1 regulates carcinoma cell migration and invasion via clathrin-mediated endocytosis of integrin alphavbeta6. *Cancer Res.* 67:5275-5284.

Rezaei, N., M.Moin, Z.Pourpak, A.Ramyar, M.Izadyar, Z.Chavoshzadeh, R.Sherkat, A.Aghamohammadi, M.Yeganeh, M.Mahmoudi, F.Mahjoub, M.Germeshausen,

- M.Grudzien, M.S.Horwitz, C.Klein, and A.Farhoudi. 2007. The clinical, immunohematological, and molecular study of Iranian patients with severe congenital neutropenia. *J. Clin. Immunol.* 27:525-533.
- Sandquist, J.C., K.I.Swenson, K.A.Demali, K.Burridge, and A.R.Means. 2006. Rho kinase differentially regulates phosphorylation of nonmuscle myosin II isoforms A and B during cell rounding and migration. *J. Biol. Chem.* 281:35873-35883.
- Sanger, J.W., J.M.Sanger, and B.M.Jockusch. 1983. Differences in the stress fibers between fibroblasts and epithelial cells. *J. Cell Biol.* 96:961-969.
- Sharp, T.V., H.W.Wang, A.Koumi, D.Hollyman, Y.Endo, H.Ye, M.Q.Du, and C.Boshoff. 2002. K15 protein of Kaposi's sarcoma-associated herpesvirus is latently expressed and binds to HAX-1, a protein with antiapoptotic function. *J. Virol.* 76:802-816.
- Sorimachi, H., A.Freiburg, B.Kolmerer, S.Ishiura, G.Stier, C.C.Gregorio, D.Labeit, W.A.Linke, K.Suzuki, and S.Labeit. 1997. Tissue-specific expression and alpha-actinin binding properties of the Z-disc titin: implications for the nature of vertebrate Z-discs. *J. Mol. Biol.* 270:688-695.
- Suzuki, Y., C.Demoliere, D.Kitamura, H.Takeshita, U.Deuschle, and T.Watanabe. 1997. HAX-1, a novel intracellular protein, localized on mitochondria, directly associates with HS1, a substrate of Src family tyrosine kinases. *J. Immunol.* 158:2736-2744.
- Taylor, S.S., D.R.Knighton, J.Zheng, L.F.Ten Eyck, and J.M.Sowadski. 1992. cAMP-dependent protein kinase and the protein kinase family. *Faraday Discuss.* 143-152.
- Trinick, J. 1994. Titin and nebulin: protein rulers in muscle? *Trends Biochem. Sci.* 19:405-409.
- Tskhovrebova, L. and J.Trinick. 2003. Titin: properties and family relationships. *Nat. Rev. Mol. Cell Biol.* 4:679-689.
- Tskhovrebova, L. and J.Trinick. 2004. Properties of titin immunoglobulin and fibronectin-3 domains. *J. Biol. Chem.* 279:46351-46354.
- Vafiadaki, E., D.Sanoudou, D.A.Arvanitis, D.H.Catino, E.G.Kranias, and A.Kontogianni-Konstantopoulos. 2007. Phospholamban interacts with HAX-1, a mitochondrial protein with anti-apoptotic function. *J. Mol. Biol.* 367:65-79.
- Wang, S.M. and M.L.Greaser. 1985. Immunocytochemical studies using a monoclonal antibody to bovine cardiac titin on intact and extracted myofibrils. *J. Muscle Res. Cell Motil.* 6:293-312.
- Warren, C.M., P.R.Krzesinski, K.S.Campbell, R.L.Moss, and M.L.Greaser. 2004. Titin isoform changes in rat myocardium during development. *Mech. Dev.* 121:1301-1312.

Watanabe, N., P.Madaule, T.Reid, T.Ishizaki, G.Watanabe, A.Kakizuka, Y.Saito, K.Nakao, B.M.Jockusch, and S.Narumiya. 1997. p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *EMBO J.* 16:3044-3056.

Weed, S.A., Y.Du, and J.T.Parsons. 1998. Translocation of cortactin to the cell periphery is mediated by the small GTPase Rac1. *J. Cell Sci.* 111 (Pt 16):2433-2443.

Witt, C.C., N.Olivieri, T.Centner, B.Kolmerer, S.Millevoi, J.Morell, D.Labeit, S.Labeit, H.Jockusch, and A.Pastore. 1998. A survey of the primary structure and the interspecies conservation of I-band titin's elastic elements in vertebrates. *J. Struct. Biol.* 122:206-215.

Young, P., E.Ehler, and M.Gautel. 2001. Obscurin, a giant sarcomeric Rho guanine nucleotide exchange factor protein involved in sarcomere assembly 2. *J. Cell Biol.* 154:123-136.

Young, P., C.Ferguson, S.Banuelos, and M.Gautel. 1998. Molecular structure of the sarcomeric Z-disk: two types of titin interactions lead to an asymmetrical sorting of alpha-actinin. *EMBO J.* 17:1614-1624.

Young, P. and M.Gautel. 2000. The interaction of titin and alpha-actinin is controlled by a phospholipid-regulated intramolecular pseudoligand mechanism. *EMBO J.* 19:6331-6340.

Zastrow, M.S., D.B.Flaherty, G.M.Benian, and K.L.Wilson. 2006. Nuclear titin interacts with A- and B-type lamins in vitro and in vivo. *J. Cell Sci.* 119:239-249.

Zou, P., N.Pinotsis, S.Lange, Y.H.Song, A.Popov, I.Mavridis, O.M.Mayans, M.Gautel, and M.Wilmanns. 2006. Palindromic assembly of the giant muscle protein titin in the sarcomeric Z-disk. *Nature* 439:229-233.

BIOGRAPHICAL SKETCH

Education

- 2002-2008 Doctor of Philosophy, Florida State University, Tallahassee, FL
2006 Special Topics Course "Analytical and Quantitative Light Microscopy", Marine Biological Laboratory, Woods Hole, MA
1997-2001 Bachelor of Science, Florida State University, Tallahassee, FL
1993-1997 High School Diploma, Cardinal Gibbons, Ft. Lauderdale, FL

Teaching

- 2002-2006 Graduate Teaching Assistant, PCB3134, Cell Structure and Function
2003-2004 Graduate Teaching Assistant, MCB4403L, Prokaryotic Biology Lab
2003-2005 Graduate Teaching Assistant, MCB2004L, Microbiology for Health Sciences Lab

Research Experience

- 2002-2008 Graduate Research Assistant
Characterization of cellular titin
Dr. Thomas C.S. Keller III, Florida State University
- 2002 Research Technician
Canine olfactory systems
Dr. James Walker, Florida State University

Publications

Cavnar PJ, Olenych SG, Keller TC 3rd. (2007). Molecular identification and localization of cellular titin, a novel titin isoform in the fibroblast stress fiber. *Cell Motil Cytoskeleton* **64(6)**, 418-33.

Abstracts

P. J. Cavnar, S. Olenych, T.C. Keller. "Stress fiber organization and assembly disruption by cellular titin kinase". The American Society for Cell Biology 48th Annual Meeting. Washington, DC. December 2007.

P. J. Cavnar, S. Olenych, T.C. Keller. "Characterization of Cellular titin in Human Megakaryocyte, Mouse Fibroblast, and Deer Fibroblast Cell

Lines". The American Society for Cell Biology 46th Annual Meeting. San Francisco, CA. December 2006.

P. J. Cavnar, S. Olenych, T.C. Keller. "Characterization of Cellular titin in Human Megakaryocyte and Mouse Fibroblast Cell Lines". The American Society for Cell Biology 45th Annual Meeting. San Francisco, CA. December 2005.

Honors And Awards

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| 2006-2008 | Pre-Doctoral Fellowship, American Heart Association, FL-Puerto Rico affiliate |
| 2005 | The Brenda Weems memorial scholarship for expenses to attend an off campus educational course. |
| 2005 | The Robert B. Short Scholarship in Zoology for expenses to attend an off campus educational course. |

Professional Memberships

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| 2003-2008 | American Society for Cell Biology |
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