In vitro Studies of Myofibers and Their Use in Analyzing the Differential Dynamics and Properties of α-Actinin Isoforms

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In vitro Studies of Myofibers and Their Use in Analyzing the Differential Dynamics and Properties of α-Actinin Isoforms

A dissertation presented

By

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to

The Division of Medical Sciences

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In vitro Studies of Myofibers and Their Use in Analyzing the Differential Dynamics and Properties of α-Actinin Isoforms

Abstract

Skeletal muscle is a highly organized tissue that requires cooperation of many different structures and components for proper function. We explored the use of a flexor digitorum brevis (FDB) myofiber culture system to better model highly differentiated aspects of skeletal muscle in an in vitro system. Indirect immunofluorescence of FDB myofibers allowed us to better determine the subcellular localization of KLHL41, a new nemaline myopathy (NM) gene product, to ER-like subdomains of the sarcoplasmic reticulum. By comparing FDB myofibers from wild type and myotubularin knockout mice with X-linked myotubular myopathy (XLMTM), we were also able to analyze satellite cell populations, showing that the knockout mice suffered a marked decrease in associated myogenic satellite cells. This supports concurrent data from our lab indicating a disease progression-related increase in apoptosis and a decrease in satellite cell proliferation in XLMTM.

We further used FDB cultures to study the α-actins, a highly conserved family of actin cross-linkers that mediate interactions between many proteins both in muscle and non-muscle cells. Non-sarcomeric α-actinin-1 and α-actinin-4 crosslink actin filaments in non-muscle cells, while sarcomeric α-actinin-2 and α-actinin-3 serve a crucial role in anchoring actin filaments to the Z-line in striated muscle. To assess the difference in protein exchange dynamics between these
isoforms at the sarcomeric Z-line we expressed each of the four α-actinins in FDB myofibers and used Fluorescence Recovery After Photobleaching (FRAP). We found that the recovery kinetics of these proteins follows three distinct patterns: α-actinin-2/α-actinin-3 were most stable, followed by α-actinin-1, then α-actinin-4. Interestingly, the isoforms’ pattern of recovery was reversed at adhesion plaques in fibroblasts. Domain-specific studies indicated that the three main domains of the protein contribute additively to their dynamics, and that the integrity of the linker between the actin binding domain and the spectrin-like repeats is essential for F-actin crosslinking.

In conclusion, by using a mature myofiber culture system with components reflective of in vivo tissue, but with the convenience of in vitro analysis, our studies add to current knowledge regarding the understanding of XLMTM and NM, as well as differences between α-actinin isoforms.
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Chapter 1

Introduction
Composition of Skeletal Muscle

Muscle tissue is composed of bundles of myofibers that contract and relax in synchronicity. These myofibers are highly organized, multinucleated cells that rely on a very specific, subcellular organization for correct function. Each fiber is filled with numerous myofibrils that run longitudinally along the cell from end to end, and their contractions are synchronized through their attachments via the sarcoplasmic reticulum. Within each myofibril lie tandemly-arranged contractile units called sarcomeres. The organization within each sarcomere is critical for its function as a contractile unit and is reflected in their appearance as 2-3 μm repeats of striations throughout the muscle.

Skeletal muscle fibers can be divided into two functional types: slow (Type I) and fast (Type II) twitch. Fast twitch fibers can be further classified as Type IIA and Type IIB, which differ in metabolism. Metabolically, Type I and IIA fibers use fatty acid oxidation and are referred to as oxidative fibers. They contain a high amount of myoglobin and subsequently have a reddish color. Type IIB fibers utilize glucose and are referred to as glycolytic fibers. They contain a lower amount of myoglobin, and are thus white in color. Due to their metabolic profiles, oxidative fibers are less prone to fatigue and can withstand sustained contractions, whereas glycolytic fibers fatigue easily but are suited for fast, rapid energy bursts. Despite these differences, both types of muscle fibers appear similar under a light microscope.

Healthy myofibers, which constantly undergo the wear and tear of contractile stresses, are regularly repaired by satellite cells, a resident pool of myogenic progenitor cells located between the sarcolemma and the basal lamina (1). These
are characterized by expression of the transcription factor Pax7 (2, 3) and can be activated to proliferate and fuse into injured muscle fibers.

The main function of skeletal muscle is to contract during voluntary movement. Proper organization of sarcomeres and triads (units for excitation-contraction coupling) are two components that are key for this function. Our interest is in better understanding the structure and function of muscle to ultimately understand the molecular mechanisms of various muscle diseases. This study is composed of two aspects: we are using dissociated FDB myofiber cultures to determine localization of novel proteins and analyze effects of myotubularin-deficiency on myofiber components, and we are taking a closer look at the different α-actinin isoforms, which are essential in functional integrity of the sarcomere.

**Structure of the Sarcomere**

Each 2-3 μm long sarcomere is bound on either end by narrow, electron-dense lines called Z-lines, which are shared between adjacent sarcomeric units. Surrounding each Z-line is a lighter zone, the ~1 μm long I-band, which extends from the Z-line towards the center of the sarcomere. At the middle of the sarcomere, the I-band extensions run into a dark 1.6 μm long A-band. Bisecting the A-band is a lighter H-zone and within the H-zone is the narrow, dark M-line. (Figure 1.1) (4)

The alternating light and dark regions within the sarcomere are a result of the precise arrangement of the sarcomeric proteins. Each sarcomere is
**Fig 1.1.** Organization of the sarcomere.

Muscle fibers are made up of small contractile units called sarcomeres, which contain a highly organized array of proteins for proper contraction. Within each sarcomere, the varying densities of these protein components show up as light and dark areas under the light microscope. Here, we show an electron micrograph image of an individual sarcomere, with electron-dense Z-lines bordering either end. The M-line bisects the unit transversely and is an area for stabilization through linking sarcomeric components to the sarcolemma and the extracellular matrix. The A-band reflects the area covered by the length of the myosin heavy chain, and the I-bands are areas at either end of the sarcomere where thin filaments do not overlap with myosin, and are thus lighter. The H-zone surrounding the M-line is a region where the lack of myosin heavy chain heads leads to lesser density. Figure adapted with permission from http://www.pradeepluther.com
symmetrical, and from the outside in, each unit is bound by the Z-lines, then the lighter H-bands, with the darker A-band in the center. The I-band is lighter than other regions because it reflects a region consisting solely of thin filaments, whereas the darker A-band is composed of the denser thick filaments. The Z-lines and M-lines (located at the center of the sarcomere and A-band) are electron-dense regions where thin filaments and thick filaments, respectively, are interconnected and tethered.

Although both fast and slow twitch muscle fibers have the same sarcomeric features under a light microscope, they have many differences on a molecular level. Most notably, they express different myosin heavy chain isoforms in their thick filaments (i.e., MHCI for slow fibers and MHCIIa and MHCIIb for fast fibers), which are commonly used for reliable muscle fiber typing. Other differences include isoforms of proteins that tend to segregate with specific fiber types (such as α-actinin and myozenins), differing amounts of mitochondria and myosin binding proteins, and varying M-line makeups. Additionally, the Z-line width is slightly different between fast and slow fibers.

Depending on the muscle group, the composition of fast vs. slow fibers can vary. For example, the soleus consists mainly of Type I fibers (~96%), while the extensor digitorum longus (EDL) is composed mostly of Type II fibers ~93%. These differences in fiber type composition leads to differences in muscle performance, as the muscle function is metabolically different. The best example of this is in α-actinin-3 knockout mouse models, where a lack of α-actinin-3 protein lead to
compensatory α-actinin-2 expression. Although mammals (mouse and humans) with this deficiency have no outward defects, analysis of murine tissue revealed that the metabolic profile of α-actinin-3 deficient muscle shift to a more oxidative state (5). Profiling of α-actinin-3-deficient human athletes show that a correlative result of this metabolic shift in muscles leads to superiority in endurance sports.

Molecularly, the major components of the sarcomere (thick and thin filaments, titin, and nebulin) are very regularly structured and well-characterized. (Figure 1.2) Thick filaments are made up primarily of myosin. Each myosin molecule is a hexamer and consists of one pair of myosin heavy chains (MHCs) and 2 pairs of myosin light chains (MLCs). Each MHC is made up of a globular head with an α-helical tail. The tail portions associate together in a parallel manner with both globular heads of the two molecules at one end. One pair of MLC proteins associates with each myosin heavy chain head. The tail portion of each myosin molecule aligns with other myosins in a staggered manner, which leads to a thick filament macromolecule with multiple MHC heads sticking outward in a “knobs in holes” fashion (4). These MHC heads are now able to interact with actin in the thin filaments along the A-band of the sarcomere.

Thin filaments are primarily made up of actin, which readily associate with each other to form polar, double-helix filamentous molecules called F-actin that have a pointed end and a barbed end. Normally, filamentous actin undergoes association and dissociation at its ends, which leads to polymerization at the barbed end and
**Fig 1.2**

**Fig 1.2. A molecular representation of the sarcomeric contractile apparatus.**

Within the sarcomere, important filamentous proteins are aligned longitudinally and are tethered to withstand the stresses of contraction. Here, we show how the main components are positioned in relation to one another. Myosin thick filaments are aligned in the center, where the tails associate together and multiple globular heads point outwards in both directions. These interact with F-actin in thin filaments. Filamentous proteins titin and nebulin associate with the thick and thin filaments, respectively, to provide structural stability. Capping proteins help tether the ends of filaments for stability: tropomodulin caps the pointed ends of actin filaments near the M-line, while CapZ caps and tethers it at the Z-line. α-Actinin also helps tether thin filaments and crosslink them to the neighboring sarcomere. Additional capping proteins telethonin and myopalladin tether titin and nebulin, respectively, to the Z-line.
depolymerization at the pointed end (4); these dynamics are typically important for the reorganization of cells. In sarcomeres, however, where the stability of the filaments is essential, the ends are capped to inhibit dynamic growth and retraction. Tropomodulin caps the pointed end of the thin filaments near the M-line, while CapZ caps the barbed end in the Z-line; another filamentous protein, tropomyosin, coils around the filaments throughout their lengths for extra support and contractile regulation. As a result, the thin filaments consistently extend from the Z-line to the edge of the H-zone.

Two additional filamentous proteins, titin (3000kD) and nebulin (600-800kD), extend through the sarcomere for flexibility and stability. Titin is a long, thin protein that stretches through half the length of the sarcomere; its N-terminus is located at the Z-line, where it is capped by telethonin, and its C-terminus overlaps and links to another titin molecule in an antiparallel manner at the M-line. In addition to interacting with myofibrillar proteins throughout its length, titin aids in keeping the thick filament centered in the sarcomere when there is instability and asymmetry (4). Nebulin is tethered at the Z-line via myopalladin and extends through the length of the thin filaments; it binds to the actin along the way, as well as to the F-actin capping protein, tropomodulin, at the edge of the H-zone. Nebulin was originally thought to act as a molecular ruler in determining the exact length of the thin filaments, but more recent studies indicate that it instead helps to determine the minimum thin filament length (6).
The Contractile Apparatus

Although sarcomeres are the individual contractile units of the muscle, contractions of the myofiber is achieved though synchronized excitation-contraction coupling throughout the myofibers at the triads, junctions where a transverse tubules (T-tubule) is paired with a terminal cisternae of the sarcoplasmic reticulum (SR) on either side. T-tubules are invaginations of the sarcolemmal membrane where dihydhropyridine receptors (DHPRs) (voltage gated calcium channels) are localized. Their role is to keep the large calcium stores in the SR under control of the DHPR (7). Various proteins (including caveolin 3, amphiphysin 2, and dysferlin) been shown to regulate triad formation and maintenance, a key one of which is myotubularin. A lack of this protein leads to a debilitating muscle disease with missing or mis-oriented T-tubules, while overexpression leads to abnormal sarcolemmal/T-tubule membrane structures (7). Therefore, formation of the triads is very sensitive to regulation and its function is essential in turning nerve impulses into mechanical action.

As a positive current is passed from the neuromuscular junction, DHPRs are activated, leading to a small calcium ion influx and activation of the ryanodine receptors (RyRs) on the SR. This in turn causes the SR to release stored calcium ions, which flood the myoplasm, leading to contractions. On a sarcomeric level, when the calcium concentration reaches high levels (i.e. $10^{-5}$ M), myosin and actin filaments slide along each other, regulated by calcium and ATP hydrolysis, in a concerted, repetitive manner.
When the sarcomere contracts, it requires a great deal of movement. Due to the thin filaments’ movements toward the M-line, a sarcomere can shorten by >1 μm (i.e., over 30% of its normal length). So that the unit is able to withstand the repeated stress of contractions, additional supports along the M-line and Z-line help lend stability. The M-line is a lattice structure, where myomesin links myosin and titin together. Its splice variant, skelemin, similarly tethers myosin to desmin intermediate filaments that surround the sarcomere, allowing for the dissipation of some of the stress from contractions.

To further dissipate contractile stress, the Z-line, also a lattice structure, anchors actin filaments, titin, and nebulin, and links them to the sarcolemma, neighboring fibers, and the extracellular matrix (4). The mode of transfer is done through connections to the sarcolemmal membrane through peripheral assemblies called costameres. Plaque proteins congregate at the costameres (including β1-integrins, α-actinin, talin, dystrophin and vinculin), where β1-integrins and dystrophin have been found to be essential in maintaining the integrity of the fibers by making proper adhesions to the sarcolemma and basement membrane in order to maximize the tensile resilience of each fiber (4).
Z-line Structure

The Z-lines appear as electron-dense, zigzag bands, linking opposing thin filaments from neighboring sarcomeres. When muscle fibers are viewed transversely under EM, Z-lines are found to have the appearance of “small square lattices” when in a relaxed state and a “basketweave”-like appearance under active contraction (8). Studies found that this difference in morphology is due to z-links (α-actinin that crosslinks neighboring anti-parallel thin filaments) forming acute bends in the relaxed state and angled spirals in the contracted state (9).

Longitudinal observations found that Z-lines can vary in width depending on muscle fiber type (fast vs. slow). The width of the Z-line is dependent on the number layers of α-actinin crosslinks on the thin filament overlaps, which have been found to exist in 2, 3, or 4 layers for fast fibers or 6 layers in slow fibers (9). This leads the widths to range from 30-50 nm for fast fibers to 100-140 nm for slow fibers. While multiple Z-line widths can be observed within a single healthy muscle fiber (believed to be due to fiber type switching), irregular and widened Z-line widths (a hallmark of nemaline myopathies) occurs in muscle fibers that have lost muscle LIM protein (MLP), or are sustaining injury and/or aging (8). It is currently unclear what are the mechanisms that lead to the structural change observed in Z-lines when a fiber undergoes contraction, or the mechanism which dictates the width of Z-lines, although differential splice isoforms of the filament protein titin is thought to contribute to the latter (10).

Within this region, α-actinin crosslinks F-actin and titin from adjacent sarcomeres, thus helping to transmit tension along the myofibril; α-actinin also
binds to many other Z-line proteins, which helps to complex all the sarcomeric components together. The Z-line is also where the N-termini of titin molecules from adjacent half sarcomeres overlap and link to each other, are capped and tethered by telethonin, and bind to α-actinin. Furthermore, nebulin is tethered via myopalladin to α-actinin. Other Z-line proteins include CapZ (which caps F-actin at the barbed end and helps anchor F-actin at the Z-line through α-actinin), myopodin (which assists in actin bundling), and other PDZ/LIM domain-containing proteins that aid in Z-line development and integrity.

The Z-line has long been studied from a structural perspective, where it has been found to anchor the thin filaments and provide structural support during contractions. More recent studies have turned the focus to the specific functions in individual proteins in its composition, revealing its additional roles in signaling, mechanosensation, and mechanotransduction. Desmin, the FHL proteins, and FAK are proteins at the Z-line that have been linked to mechanosensing, where more specifically, FAK is an integrin effector. In addition to mechanosensing, FHL proteins have also been found to mediate the ERK signaling pathway to alter transcriptional changes. This mechanotransduction is done alongside cypher (which mediates through PKC interaction), and telethonin (which mediates through calcineurin). More directly, the transcription factor NF-AT3 is partially localized at the Z-line, where it can be dephosphorylated by calcineurin to directly enter the nucleus and alter gene expression.

**Z-line Dynamics**
Z-lines are also likely an important location for monitoring protein exchange, as several E3 ubiquitin ligases have been found there. The E3 ubiquitin ligases TRIM32, TRIM63/MuRF1, and Fbox32/Atrogin1 localize to this location and have been found to participate in muscle remodeling, where TRIM32 has a role in maintaining muscle, structure, strengthening, and fiber type distribution, and TRIM63 and Fbox32 have roles in autophagy during muscle atrophy (11, 12). Some of their targets have been discovered but the extent of the Z-line's role in protein catabolism is still coming to light.

Moreover, while Z-lines are an important source of stability for striated muscle, the proteins within the Z-line are in constant flux. FRAP experiments of in vitro differentiated myotubes of avian Z-line proteins from Joseph Sanger's lab (13) show that the proteins vary greatly in their degree of exchange (amount of protein recovery) as well as exchange rate (slow vs. fast phase). Since this study, two labs have reported the utility of dissociated primary myofiber cultures from the mouse flexor digitorum brevis (14, 15). This is thought to be a better model for mature myofibers compared to striated myotubes differentiated from myoblast cultures. This system would be useful in additional studies of mammalian Z-line proteins, especially as a first step in parsing out differences between proteins that have multiple isoforms.

**Muscle Disorders**

Mutations in many of the components of striated muscle can lead to impaired function. Genetic disorders of muscle can generally be categorized into two groups:
congenital myopathies and dystrophies. Congenital myopathies are characterized by hypotonia and weakness, with static or slow progression, typically from birth (16). Dystrophies are characterized by muscle atrophy, accompanied by insufficient regeneration.

With the varied roles of the Z-line, and the interdependence of proteins in maintaining muscle function, it is of no surprise that mutations in Z-line proteins can lead to muscle disease. Mutations in α-actinin and MLP have been linked to cardiomyopathies (thickened myocardial walls that may lead to arrhythmias, and/or dilated ventricles with inadequate systolic function). Telethonin mutations have been linked to limb-girdle muscular dystrophy (LGMD), (weakening and deterioration of limb muscles), as well as cardiomyopathies, and Calsarcin1/FATZ2/Myozenin2 mutations have been linked to cardiomyopathy. Mutations in Cypher and bcl2-associated athanogene3 are linked to myofibrillar myopathy (muscle weakening throughout the body) and cardiomyopathies. And mutations in myotilin lead to LGMD as does mutations in desmin, which additionally leads to cardiomyopathies.

As our lab is also interested in muscle diseases, specifically myopathies, a current question is understanding the physiological effects of X-linked myotubular myopathy (XLMTM), a genetic disease that stems from loss of myotubularin, and is associated with severe muscle weakness and hypotonia, which can progress to respiratory failure (17).

In this study, we analyze the isoform-specific differences of human α-actinin proteins, in the context of differential exchange dynamics at the Z-line, as well as
any domain specific contributions to their exchange. We will also be further characterizing the XLMTM mouse models, both in determining the extent of excitation-contraction uncoupling in the knock-in model, as well as any disease-associated defects in myogenic potential.

**Methods in Studying Muscle Biology**

Over the years, many groups have delved into various methods in order to better understand skeletal muscle, both from the aspect of its basic biology in myofibrillogenesis and regeneration, as well as the effects from diseases. These methods included organ cultures, tissue explants, primary and derived cell lines, and dissociated adult fibers.

Organ cultures are an ideal way to study highly differentiated cell types within a complex tissue architecture and its associated influences, especially with constraints of cell number. Typical uses of these cultures include studies of the inner ear, cerebral tissue, or even whole embryos (18–20) to investigate morphogenesis *ex vivo* and pharmacological effects. Attempts at muscle organ cultures using mouse thorax (21) and strips of diaphragm (22) were complicated and tedious, and these types of studies fell by the wayside likely because other methods were found to be sufficient in studies of muscle biology.

Studies using muscle explants were developed for harvesting primary myogenic cells, which were then used in myoblast fusion studies (23). This allowed for studies of cells obtained from different ages and phenotypes (24), and harvest of primary cells by dissociation of muscle tissue is still used for satellite cell studies
(25), often in characterizing different populations of cells. However, for the purpose of obtaining myogenic cells to study myoblast fusion and effects of various manipulations on myoblasts, people have mostly turned to derived cell lines.

Derived rodent cell lines such as C2C12 and L6 show essential features of myogenesis, such as proliferation, migration, fusion, and contraction (Fig 1.3). While it is a useful and convenient system in studying muscle biology, it lacks certain aspects of an in vivo model such as lack of innervation, basal lamina, and myotendonous structures. Myotubes formed from C2C12 myoblasts have been found to express higher amounts of fetal proteins, as well as oscillating myogenic markers not representative of in vivo conditions (14). Additionally, muscle regeneration studies require analysis of satellite cells and enzyme-extraction of single fibers from various muscle types (including the FDB, TA, and EDL) have been used as a more representative source of adult satellite cells. Recently, FDB cultures have been characterized as a mature myofiber system that is more representative of myofibers differentiated in vivo, but has the convenience of in vitro studies (14).

**α-Actinin**

α-Actinin is essential within the Z-line and is involved in multiple interactions to link structural proteins, localize signaling proteins, and tether the Z-line to the extracellular matrix to relieve tension. The α-actinins are a highly conserved family of proteins that exist not only in skeletal muscle, but in cells throughout the body, where they crosslink filamentous actin, facilitating the structural stability and locomotion of various cell types (26–28). They belong to the
Fig 1.3. C2C12 myotubes show essential features of myogenesis.

Myotubes formed from C2C12 myoblasts show essential features of myogenesis, including proliferation, migration, fusion, and contraction. The above images show representative cultures of post-mitotic myotubes using phase-contrast microscopy (left) and fluorescence microscopy (right) stained against microtubules (green) and nuclei (blue).
spectrin superfamily of proteins, which also includes the spectrins and dystrophin (29). Together, the spectrin superfamily is a group of actin-bundling and membrane-anchoring proteins that are widely expressed from yeast to humans.

The actinins have been highly conserved throughout evolution: homologs of the mammalian $\alpha$-actinins have been identified in protists (e.g., $D. discoideum$), invertebrates (e.g., $D. melanogaster$ and $C. elegans$), and vertebrates, including birds, fish, rodents, and primates (26, 30, 31). Interestingly, protists and invertebrates have a single $\alpha$-actinin gene but utilize alternative splicing to produce distinct isoforms; for example, Drosophila $\alpha$-actinin is alternatively spliced to create non-muscle, larval muscle, and adult muscle isoforms (32). In contrast, mammals possess four $\alpha$-actinin genes that each produce a single isoform, which resulted from two gene duplication events (33). While the different $\alpha$-actinin isoforms exhibit high levels of identity and similarity in their structure, they regulate actin bundles and networks in different ways depending on tissue-specific modifications and protein-protein interactions in the cells. Mammalian $\alpha$-actinin-2 and $\alpha$-actinin-3 evolved to be sarcomeric-specific isoforms that localize to the Z-lines of striated muscle and crosslink actin filaments across sarcomeres. Mammalian $\alpha$-actinin-1 and $\alpha$-actinin-4 evolved to be non-muscle isoforms that crosslink actin at stress fibers and focal adhesions of non-muscle cells. Additional splicing of $\alpha$-actinin-1 also produces smooth muscle and brain-specific isoforms (34, 35). In all cases, the $\alpha$-actinins are crucial in maintaining the architecture and stability of the cytoskeleton, and compromises in their structure can lead to impaired cell function and various pathologies (36–39).
Structure of α-Actinin Isoforms

All α-actinin isoforms are composed of an N-terminal actin-binding domain (ABD), a C-terminal calmodulin-like EF hand domain, and a linker region with four spectrin-like repeats (SLR) (Fig 1.4). They form antiparallel homodimers via strong interactions between the SLRs, leading to rod-shaped structures with an actin-binding motif on either end (although heterodimers of skeletal muscle isoforms α-actinin-2 and α-actinin-3 have been found to be possible in vitro and in vivo (27), and heterodimers of non-muscle isoforms α-actinin-1 and α-actinin-4 have been found to be abundant in various cell lines (40). This structural design allows the resulting functional ~208 kD units to crosslink actin filaments and also serve as platforms for additional protein-protein interactions (26, 41).

The ABD consists of two tandem calponin homology repeats (i.e., CH1 and CH2), which contain three consecutive actin binding sites that work together synergistically to provide the strong interaction with F-actin (42). These interactions are regulated by conformational changes of the ABD via regulation from the opposing EF hand and phospholipids. Whereas the ABD of the non-muscle isoforms is subject to conformational changes that are induced by the binding of
The α-actinin protein is composed of three main functional domains: the N-terminal actin binding domain (ABD), the linker rod regions with spectrin-like repeats (SLR), and the C-terminal calmodulin-like EF hands (EF). The ABD is composed of two calponin homology repeats CH1 and CH2, within which sit three actin binding sites. The SLR is composed of four spectrin-like repeats (R1-R4), which drive anti-parallel dimerization, leading to a ~208kD dimer with a globular ABD on either end. The calmodulin-like domain consists of two tandem EF hands, each with a helix-loop-helix that can chelate calcium depending on a consensus at 16 amino acid residues. Calcium-binding to the EF domain can then regulate actin-binding activity of the opposing dimer partner. The conformation of this complex allows for F-actin crosslinking at various cell structures.
calcium to the C-terminal EF hands (43), the calcium-insensitive sarcomeric isoforms rely on the binding of phospholipids phosphatidylinositol 4,5-biphosphate (PiP2) and phosphatidylinositol 3,4,5-trisphosphate (PiP3) to the ABD, which results in the displacement of the CaM-like EF hands and a resulting diminishment of the ABD’s affinity for actin (44).

The C-terminal CaM-like domain consists of two EF hands, which are helix-loop-helix motifs that are involved in calcium binding (26, 45, 46). For the non-muscle α-actinin isoforms, calcium binding to the EF hands lowers the protein’s affinity for actin filaments (47, 48). Previous studies have shown that the non-muscle α-actins begin to lose their affinity for actin at calcium concentrations greater than $10^{-7}$ M (47). In contrast, the sarcomeric isoforms are not sensitive to calcium because their EF hand domains have several mutated residues that abolish their calcium binding ability (29, 49). This evolutionary change may have occurred to separate actin-binding from calcium regulation and thus allowing the maintenance of sarcomeric structural integrity during the calcium fluctuations of muscle contractions.

The central rod domain consists of four spectrin-like repeats (SLRs) and serves multiple functions, such as a mediator of protein self-dimerization, a linker and ruler for the functional ends of the dimer, and a platform for a wide variety of protein-protein interactions (Fig 1.5). Each repeat exhibits intrinsic elastic properties, and further elasticity is provided by the spacers between the adjacent repeats (50). Additionally, this domain has been shown to form a curved interface
Fig 1.5. α-Actinin isoforms can interact with a variety of proteins.

α-Actinin interactive studies have shown that the isoforms can interact with a variety of proteins, including adhesion proteins, contractile apparatus components, cytoskeletal proteins, ion channels, neuro-transmission proteins, PDZ-LIM adapter proteins, lipids, and signal transduction proteins. Most studies were done with single isoforms. Some interactive sites were narrowed down to a portion of the α-actinin protein, as indicated in the above diagram, while many currently have unknown binding locations.
by twisting approximately 90° from one end to the other (50). All of these characteristics allow for greater structural flexibility and mechanical stability, which is crucial for α-actinin function at focal adhesion points and sarcomeric Z-lines.

In addition, the spectrin repeat region has been shown to interact with a variety of proteins and molecules, including adhesion proteins (i.e., integrins, ICAMs, and L-selectin) (51–55), contractile apparatus components (i.e., nebulin, CapZ, and calsarcins) (51–55), cytoskeletal proteins (i.e., vinculin, a-catenin, and dystrophin) (56–58), ion channels (i.e., Kv1.5, Nav1.4, and Nav1.5) (59, 60), neurotransmission proteins (i.e., NMDA receptor, densin-180, and rabphilin-3A) (61–63), PDZ-LIM adapter proteins (i.e., hCLIM1, ALP, and ENH) (64–67), lipids (i.e., diacylglycerol and palmitic acid) (68), and signal transduction proteins (i.e., PI3-kinase, MEKK1, and PKN) (69–71). (Additional interacting partners are shown in Tables 1.1 and 1.2.) While these studies show that the α-actinins are involved in a wide range of interactions, these studies have, in large part, focused on the non-muscle and smooth muscle isoforms (i.e., α-actinin-1 and α-actinin-4). Furthermore, many studies have yet to identify the exact points of protein-protein interaction on the α-actinin proteins and the functional properties associated with each interaction.

Regulation of α-Actinin Function

α-Actinin is an important player in force generation in both muscle and non-muscle cells. In muscle cells, it tethers actin to the Z-line and allows the actomyosin cytoskeleton to generate the force needed for muscle contraction. In non-muscle
<table>
<thead>
<tr>
<th>Binding Partner</th>
<th>Interaction Domain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>ABD x SLR EF N/A</td>
<td>Mimura and Asano, 1987 (72)</td>
</tr>
<tr>
<td>Zyxin</td>
<td>x</td>
<td>Crawford et al, 1992 (73)</td>
</tr>
<tr>
<td>Phosphatidylinositol 4,5 bisphosphate</td>
<td>x</td>
<td>Fukami et al, 1992 (74)</td>
</tr>
<tr>
<td>Cysteine-Rich Protein 1</td>
<td>x</td>
<td>Pomies et al, 1997 (75)</td>
</tr>
<tr>
<td>Nav1.5 Channel</td>
<td>x</td>
<td>Ziane et al, 2010 (60)</td>
</tr>
<tr>
<td>α-actinin-2/3</td>
<td>x</td>
<td>Chan et al, 1998 (27)</td>
</tr>
<tr>
<td>NMDA Receptor</td>
<td>x</td>
<td>Wyszynski et al, 1997 (61)</td>
</tr>
<tr>
<td>Actin Associated LIM Protein</td>
<td>x</td>
<td>Xia et al, 1997 (66)</td>
</tr>
<tr>
<td>PKN</td>
<td>x</td>
<td>Mukai et al, 1997 (71)</td>
</tr>
<tr>
<td>Myotilin</td>
<td>x</td>
<td>Salmikangas et al, 1999 (76)</td>
</tr>
<tr>
<td>Cap-Z</td>
<td>x</td>
<td>Papa et al, 1999 (77)</td>
</tr>
<tr>
<td>Calsarcin 1/2</td>
<td>x</td>
<td>Frey et al, 2000 (78)</td>
</tr>
<tr>
<td>Myozenin</td>
<td>x</td>
<td>Takada et al, 2000 (79)</td>
</tr>
<tr>
<td>Vinculin</td>
<td>x</td>
<td>Belkin et al, 1987 (56)</td>
</tr>
<tr>
<td>Titin</td>
<td>x x</td>
<td>Young et al, 1998 (80)</td>
</tr>
<tr>
<td>Intercellular Adhesion Molecule 1/2</td>
<td>x x</td>
<td>Carpen et al, 1992 (53)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heiska et al, 1996 (55)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maruoka et al, 2000 (59)</td>
</tr>
<tr>
<td>Kv1.5 Channel</td>
<td>x x</td>
<td>Galliano et al, 2000 (81)</td>
</tr>
<tr>
<td>A Disintegrin and</td>
<td></td>
<td>Kotaka et al, 2000 (64)</td>
</tr>
<tr>
<td>Metalloprotease 12</td>
<td>x x</td>
<td>Faulkner et al, 1999 (82)</td>
</tr>
<tr>
<td>Human LIM Domain Protein</td>
<td>x</td>
<td>Zhou et al 1999 (83)</td>
</tr>
<tr>
<td>Cypher</td>
<td>x</td>
<td>Nakagawa et al, 2000 (67)</td>
</tr>
<tr>
<td>Enigma Homologue Protein</td>
<td>x</td>
<td>Shibasaki et al, 1994 (69)</td>
</tr>
<tr>
<td>PI3 Kinase</td>
<td>x</td>
<td>Shin et al, 2012 (84)</td>
</tr>
<tr>
<td>Syndecan-4</td>
<td>x</td>
<td>Mishra et al, 2011 (85)</td>
</tr>
<tr>
<td>Annexin A6</td>
<td>x</td>
<td>Park et al, 2000 (86)</td>
</tr>
<tr>
<td>Myocardial Phospholipase D</td>
<td>x</td>
<td>Pavalko and LaRoche,</td>
</tr>
<tr>
<td>Protein</td>
<td>Year</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
<td>------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Nebulin</td>
<td>1993</td>
<td>Nave et al 1990 (87)</td>
</tr>
<tr>
<td>Dystrophin</td>
<td></td>
<td>Hance et al, 1999 (58)</td>
</tr>
<tr>
<td>Synemin</td>
<td></td>
<td>Bellin et al, 1999 (88)</td>
</tr>
<tr>
<td>5-Lipoxygenase</td>
<td></td>
<td>Lepley and Fitzpatrick, 1994 (89)</td>
</tr>
</tbody>
</table>

*Table 1.1 (continued)*
Table 1.2. Binding Partners of Non-Muscle α-Actinin Isoforms

<table>
<thead>
<tr>
<th>Binding Partner</th>
<th>Interaction Domain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β1 Integrin</td>
<td>x</td>
<td>Otey et al, 1990 (51)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leinweber et al, 1999</td>
</tr>
<tr>
<td>ERK</td>
<td>x</td>
<td>(90)</td>
</tr>
<tr>
<td>Actin</td>
<td>x</td>
<td>Lazarides, 1976 (91)</td>
</tr>
<tr>
<td>α-actinin-1/4</td>
<td>x</td>
<td>Foley et al, 2013 (40)</td>
</tr>
<tr>
<td>Syndecan-4</td>
<td>x</td>
<td>Okina et al, 2012 (92)</td>
</tr>
<tr>
<td>α-Catenin</td>
<td>x</td>
<td>Nieset et al, 1997 (57)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>El-Husseini et al, 2000</td>
</tr>
<tr>
<td>Brain Expressed RING Finger Protein</td>
<td>x</td>
<td>(93)</td>
</tr>
<tr>
<td>PKN</td>
<td>x</td>
<td>Mukai et al, 1997 (71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Walikonis et al, 2001</td>
</tr>
<tr>
<td>Densin-180</td>
<td>x</td>
<td>Kato et al, 1996 (62)</td>
</tr>
<tr>
<td>Rabphilin 3A</td>
<td>x</td>
<td>Patrie et al, 2012 (94)</td>
</tr>
<tr>
<td>MAGI-1</td>
<td>x</td>
<td>Ronty et al, 2004 (95)</td>
</tr>
<tr>
<td>Palladin</td>
<td>x</td>
<td>Torii et al, 2012 (96)</td>
</tr>
<tr>
<td>Cytohesin-2</td>
<td>x</td>
<td>Hall et al, 2013 (97)</td>
</tr>
<tr>
<td>CaV1.2 Channel</td>
<td>x</td>
<td>Vallenius et al, 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(65)</td>
</tr>
<tr>
<td>Human LIM Domain Protein</td>
<td>x</td>
<td>Pavalko et al, 1995 (54)</td>
</tr>
<tr>
<td>L-Selectin</td>
<td>x</td>
<td>Merisko et al, 1988 (98)</td>
</tr>
<tr>
<td>Clathrin Heavy Chain</td>
<td>x</td>
<td>Burn et al, 1985 (68)</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>x</td>
<td>Christerson et al, 1999</td>
</tr>
<tr>
<td>Mekk1</td>
<td>x</td>
<td>(70)</td>
</tr>
</tbody>
</table>
cells, it tethers actin filaments to adhesion complexes and allows the cytoskeleton to generate the force necessary for the translocation of the cell body. Several modes of regulation affect the function of the α-actinins, specifically their actin-crosslinking capacity and stability at adhesion sites (i.e., focal adhesions and Z-lines). Known regulators include calcium, phospholipids, phosphorylation, and calpain cleavage. Some of these regulators have been studied in muscle and non-muscle cells, where differences in efficacy have been found, while others have not been studied at the isoform-specific level.

External Regulators

Calcium is a common secondary messenger whose stimulation is often coupled with exocytosis, contraction, and enzyme activation via binding to calcium-activated proteins. Calcium has also been established as an effective regulator of α-actinin function through its binding to the EF hands of α-actinins. Non-muscle α-actinin has been shown to be sensitive to calcium regulation, whereas muscle isoforms have been shown to be immune to calcium fluctuation. This is due to nucleotide differences in the sequences of their C-terminal EF domains. Through crystallography studies, Kretsinger and his colleagues found that sixteen of the twenty-nine amino acid residues of each EF domain contribute to the potential calcium sensitivity of a given EF hand (99), and at least twelve of the sixteen residues must match the consensus residue(s) for that position to confer calcium sensitivity. These characteristic positions are residues 1, 2, 5, 6, 9, 10, 12, 14, 15, 17, 18, 21, 22, 25, 26, and 29: residues 2, 5, 6, 9, 22, 25, 26, and 29.
provide hydrophobic side chains; 10,12,14,18, and 21 provide oxygen atoms for calcium binding; 15 and 17 comply with conformational constraints; and the first residue is conserved with unknown specific function (99).

Studies comparing chicken non-muscle vs. smooth muscle isoforms found sequence differences in the first EF hand, as well as in the five amino acid-long spacer between the two EF hands, which causes the non-muscle isoform to be sensitive to calcium while the smooth muscle isoform is not. It was also found that the second EF hand did not comply with the 12/16 consensus requirement described by Kretsinger, and therefore likely does not bind calcium even in the non-muscle isoform. Thus, these chicken α-actinin dimers would only bind two calcium ions (35). Additionally, studies comparing human skeletal muscle to non-muscle isoforms found that both α-actinin-2 and α-actinin-3 have only 11 residues matching out of the 16-residue consensus and thus probably do not bind calcium (49). An analysis of calcium sensitivity and its contribution to α-actinin’s role in actin-crosslinking showed that while the muscle isoform’s ability to crosslink actin is unaffected by changes in calcium concentration, the non-muscle isoforms are greatly affected. In low calcium environments (< 100 nM), non-muscle α-actinin isoforms are potent actin-crosslinkers; however, when free calcium exceeds 100 nM concentrations, there was a sharp decline in actin gelation (47). It is thought that muscle isoforms are insensitive to calcium due to their importance in maintaining stable, tethered actin filaments and Z-line integrity in a high calcium environment. This is supported by the fact that muscle fibers’ resting intracellular calcium levels are above 100 nM (e.g., mouse FDB levels are at 124 +/- 2 nM, and EDL and soleus
fibers are at 106 +/- 2 nM (100). Therefore, in muscle, while calcium helps to regulate the contractile apparatus via binding to Troponin C, calcium does not affect the actin-crosslinking ability of the α-actinin isoforms at the Z-line.

α-Actinin has been shown to respond to phospholipid regulation by binding to phospholipids through its ABD domain (101). Studies have found that when α-actinin binds PIP2 or PIP3 via PDGF activation, it relocates from the focal adhesions to membrane ruffles in fibroblasts (102, 103). Although technically the focal adhesion structure is still present (i.e., talin and paxillin localization are maintained), the actinin relocation makes the focal adhesion more malleable for cell morphological changes, such as motility, differentiation, and cytokinesis. This phospholipid-initiated change is believed to be due to a disruption of the α-actinin's interaction with β-integrin (102). Furthermore, PIP2/PIP3-induced inhibition of α-actinin-actin binding functions in a concentration-dependent manner (102, 104). Additional studies showed that mutant actinin that cannot bind PIP2 exhibits more stable dynamics in focal adhesions and stress fibers (44), which further supports the finding that phosphoinositide binding destabilizes α-actinin at adhesion sites.

Aside from phospholipids, phosphorylation has also been shown to regulate α-actinin binding to actin. α-Actinin-1 has been shown to be phosphorylated at tyrosine 12 by focal adhesion kinase, which results in a decreased affinity for actin (105). More recently, α-actinin-4 has been shown to have a lower association with actin when phosphorylated at tyrosine residues 4 and 31; this phosphorylation occurs as a result of EGF stimulation, but the specific kinase involved remains unclear (106). Furthermore, the actin binding domain on its own (i.e., without the
SLR and EF hands domains attached) with residues 4 and 31 phosphorylated led to stronger actin binding and an absence of EGF-stimulated phosphorylation. This suggests that the SLR and/or EF hands domains and/or the α-actinin dimerization has an effect on phosphorylation and actin binding. These findings indicate that tyrosyl-directed phosphorylation is an additional method by which adhesion stabilization by α-actinins can be regulated for cell migration/morphogenesis.

Calpain (CAPN) is also known to regulate α-actinin function by cleavage of the α-actinins at the C-terminus. Calpains are a family of sixteen proteins that selectively cleave substrates, which include transcription factors, transmembrane receptors, signaling enzymes, and cytoskeletal proteins, to regulate cell migration/spreading and adhesion complex formation/turnover (107). Using anti-CD3 monoclonal antibody stimulation, CAPN1 has been shown to translocate to the plasma membrane, where it becomes activated and cleaves α-actinin in T cells (108); this action results in cellular reorganization and new formation of pseudopodia. In skeletal muscle, tissue-specific CAPN3 is implicated in limb girdle muscular dystrophy type 2A, where the protease is thought to be essential in removing damaged sarcomeric proteins. CAPN3 substrates consist of the structural proteins nebulin, myosin light chain I, filamin C, and titin (109). Interestingly, however, sarcomeric α-actinin is not sensitive to calpain cleavage, which is another example of the functional differences between α-actinin isoforms. Instead, the sarcomeric α-actinins remain un-degraded and functional even after calpain treatment (110).
**Regulation through splicing**

Before α-actinin was duplicated through evolution, mutually exclusive splicing events were necessary to make functionally unique splice isoforms. For example, Drosophila can create separate splice isoforms to alter the actin binding domain for muscle, larval muscle, and non-muscle cells (111). α-Actinin-2 and α-actinin-4 still express splice isoforms with changes within the actin binding domain (exon8a and exon8b, which contains a cysteine to serine shift, as well as a charge change) (112). The former is expressed in their respective sarcomeric and non-sarcomeric cells, whereas the latter is expressed in the central nervous system (CNS). However, despite identifying these splice isoforms, it is currently unknown how the additional exon8b is functionally different from those proteins containing exon8a.

Splicing at exon19 was retained by the non-sarcomeric isoforms. This encodes part of the first EF hand and makes calcium sensitive and insensitive splice isoforms of non-sarcomeric α-actinin. α-Actinin-1 makes three splice isoforms: containing exon19a, exon19b, and exon19a+b (expressed in most cell types, smooth muscle, and the CNS, respectively) (34, 35). α-Actinin-4 also expresses proteins containing exon19a and exon19b (in most cell types and the CNS, respectively) (112, 113).

Although these splice isoforms have been identified by sequence, their unique functions (with the exception of calcium sensitivity) are still unknown. Moreover α-actinin-4 exon19b does not localize to smooth muscle as the same version of α-actinin-1 does. Rather, it localizes to the CNS, where α-actinin-1
exon19a+b and α-actinin-2 exon8b are found. Further studies would have to be done to determine the necessity of multiple isoforms and splice isoforms and what their unique functions might be at the dendritic spines in the CNS.

**α-Actinin: Isoform-Specific Differences**

Although the mammalian α-actinins have evolved into four separate isoforms, they are still very similar in function and structure. All the isoforms have the ABD, SLR, and EF domains, and their amino acid sequences are 80% identical and 90% similar. Additionally, they all form dimers and crosslink actin filaments while serving as a platform for protein-protein interactions, even though their subcellular localizations and expressions by cell type differ. The isoforms may have started diverging from one another through evolution due to the need for specialized functions, yet their primary function of crosslinking actin filaments was retained in all isoforms.

The dissimilarity between isoforms is most significant in the EF hands between the sarcomeric and non-muscle proteins, where the sarcomeric isoforms lack the conservation of calcium sensitivity (114). This uncoupling of actinin-actin interactions from calcium fluctuation in muscle cells adds to the stability of sarcomeres. The isoforms also differ from one another in the N-terminal region beyond the ABD. However, this region has not been found to contribute to actinin function, and thus, its lack of conservation throughout evolution is expected. Additionally, the SLR domain is the most dissimilar among the different isoforms.
based on amino acid sequence comparisons. Furthermore, within the four spectrin-like repeats, the similarity between the isoforms decreases from SLR1 to SLR4.

The localizations and interactions of the different α-actinins may differ based on tissue specificity, however, under the same environmental conditions, the isoforms could potentially function distinctly from each other, show complete redundancy, or exhibit partial redundancy, such as is seen with the tropomodulin (Tmod) isoforms, in which Tmod3 and Tmod4 can localize similarly to Tmod1 in muscle but cannot substitute for its function (115). Studies have started to parse out whether the isoforms are functionally redundant. For example, previous findings indicate that complete redundancy is unlikely even amongst the sarcomeric isoforms as a shift in muscle metabolic profiles was observed in mice where α-actinin-2 was substituted for α-actinin-3 (116). Furthermore, α-actinin-3 is not able to substitute for or rescue α-actinin-2 defects in zebrafish (117). Likewise, studies have shown α-actinin-1 and α-actinin-4 to be similarly non-redundant since α-actinin-1 is not able to rescue the loss of α-actinin-4 (106).

α-Actinins: Mutations and Disease

There are several disorders associated with α-actinin proteins (Table 1.3). Various groups have reported an association of α-actinin-4 with poor cancer prognosis (118–120). These groups found that aberrant α-actinin-4 expression (overexpression or nuclear translocation) can lead to increased cell motility. α-Actinin-4 mutations have also been associated with focal segmental glomerulosclerosis (FSGS), a disease that leads to declining kidney function. The
<table>
<thead>
<tr>
<th>α-Actinin Isoform</th>
<th>Domain Mutated</th>
<th>Hereditary Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Actinin-1</td>
<td>ABD, EF</td>
<td>Macrothrombocytopenia</td>
<td>Kunishima et al, 2013</td>
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<td></td>
<td></td>
<td></td>
<td>Gueguen et al, 2013</td>
</tr>
<tr>
<td>α-Actinin-2</td>
<td>ABD, SLR, EF</td>
<td>Cardiac myopathies</td>
<td>Mohapatra et al, 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chiu et al, 2010</td>
</tr>
<tr>
<td>α-Actinin-4</td>
<td>ABD, neck region</td>
<td>Focal segmental glomerulosclerosis</td>
<td>Kaplan et al, 2000</td>
</tr>
</tbody>
</table>
mutations have been narrowed down to the sequence between the ABD and SLR domains, which increased the protein's binding affinity for actin (122). α-Actinin-1 mutations have been associated with congenital macrothrombocytopenia, a disease where blood platelets are abnormally large and reduced in number. Mutation sites in the ABD and EF hands implicate altered actin binding as a cause (121, 123). α-Actinin-2 mutations have been linked to cardiac myopathies (Chiu et al., 2010; Mohapatra et al., 2003). Because these disorders are all caused by missense mutations, it seems likely that (with the exception of α-actinin-3) α-actinin proteins are essential in cellular function, and frame shifts, truncations, or null mutations would be detrimental to survival. This is supported by unpublished data from our lab showing that α-actinin-2 null mutation in mice leads to embryonic lethality, as well as a null mutation in Drosophila leading to myofibrillar disarray (30). α-Actinin-1 and α-actinin-4, likewise, are involved in essential cellular processes such as contractile ring formation and focal adhesion maturation, where knockdowns lead to severe cellular defects (failure of cytokinesis and detachment of focal adhesions, respectively) (124, 125).

Although α-actinin proteins have been studied for several decades, they have been revealed to be much more than structural proteins. They are carefully regulated in their actin binding ability and have unique isoform specific properties that have not been fully explored, as indicated by expression of splice isoforms from multiple α-actinin genes in the CNS. While some isoform specific interactive studies have already been done, studies imply that despite the high degree of similarity between isoforms, their isoform-specific binding affinities may be highly variable, as
α-actinin-2 splice isoforms have difference at only 2 residues, but the resultant proteins are expressed in different tissues. Therefore isoform-specific interactive studies are essential in future studies on isoform differences and possibilities of compensatory function.

**Myotubularin**

X-linked myotubular myopathy is a severe disorder characterized by profound muscle weakness and hypotonia, as well as external ophthalmoplegia and impaired respiratory function (17). More than 200 myotubularin mutations have been identified in patients with this disease. Myotubularin expresses phosphoinositide (PI) 3-phosphatase, which acts on important signaling lipids PIP2 and PIP3. Studies in myotubularin knockout mice show that disease symptoms appear at 3-4 weeks and life expectancy is reduced to 6-12 weeks. The protein was found to localize mostly to the triads of muscle fibers and excitation-contraction uncoupling was thought to be responsible for muscle weakness, as structural defects of t-tubules and impaired calcium release from the sarcoplasmic reticulum were observed (126).

Whether the severity of the triadic defects is a reflection of the severity of the disease is yet unknown. Analysis of the more recent knockin model expressing a missense mutation (p.R69C), and showing a less severe disease phenotype in patients and mice, would indicate whether the observed defects are dependent on the severity of the mutation. Furthermore, whether muscle weakness might be related to satellite cell defects was also unexplored. The aforementioned FDB
cultures would be helpful in answering both these questions as they offer the ability to test for membrane potential (126) as well as analysis of associated satellite cells (15).

Gene therapy for mice and dog models as well as enzyme replacement therapy for mice are already underway (127–129). Each of these studies has shown an alleviation of muscle weakness and muscle pathology. While such therapeutic methods for humans are still not immediate possibilities, these proof of concept studies are promising for potential clinical trials.

**Hypothesis and Overview of Dissertation**

Our lab is interested in understanding the structure and function of muscle proteins, in an effort to better understand the physiological bases of muscle diseases. For this study, I focused on developing the FDB myofiber culture system in our lab as a method for studying muscle biology and disease, more specifically in my studies of α-actinin isoform-specific protein dynamics.

In Chapter 2, I describe my contributions to the ongoing analysis of the myotubularin myopathy mouse model as well the novel protein KLHL41 by using the FDB culture system. The myotubularin knockout model was shown to have defects in its triads, indicating that excitation-contraction uncoupling is key in disease pathology. I sought to determine whether the knockin mouse, a less severe disease model, would likewise show a less severe phenotype at the triad. Additionally, I tested for changes in myogenic potential by analyzing myotubularin
deficient satellite cells. I also used FDB cultures to perform immunostain experiments to localize KLHL41 to subcellular domains.

In my study of α-actinins, I analyzed the differential protein exchange dynamics of the four human α-actinin isoforms (non-sarcomeric isoforms α-actinin-1,4 and sarcomeric α-actinin-2,3) at the adult myofiber Z-line. Findings of differential exchange would indicate that the isoforms have evolved after duplicating and branching off the ancestral gene to adapt to the functions of specific sarcomeric or non-sarcomeric cells. As sequence alignments between the isoforms have retained varying levels of identity/similarity, further domain-specific analyses would indicate any differences in isoform-specific adaptations to differential protein interactions. In Chapter 3, work on analyzing isoform-specific differences of α-actinins is described.

In Chapter 4, I discuss the impact and future directions for our findings.
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Chapter 2

FDB myofiber cultures are a mature muscle model for efficient analysis of novel protein localization and disease characterization
Myotubularin data from this chapter were published in The American Journal of Pathology on September 2012 (Lawlor et al, 2012), and KLHL41 data were published in the American Journal of Human Genetics on December 2013 (Gupta et al, 2013).

Attribution of Collaborator Contributions

All work in this chapter was performed by Cynthia P. Hsu except the following: Vandana A. Gupta performed the KLHL41 imaging and analysis.
**Introduction**

Skeletal muscle is essential for generating movement, and over the past few decades, many skeletal muscle diseases have been identified. As research has progressed to determine the molecular bases for the associated genetic mutations, *in vitro* models for muscle studies have become essential for identifying basic cellular mechanisms, as well as serving as models for disease studies and tests for therapeutic targets. Initial studies have depended on cultured primary chick or quail myoblasts and differentiating them *in vitro* (1), as they can be coerced to proliferate, differentiate, and form multinucleated myotubes with organized sarcomeres. Over the years, the avian *in vitro* model has remained a useful system for studying myogenesis due to their ease of harvest and efficiency in proliferation and differentiation (2–4).

Molecular studies in mammalian models have taken to the convenience of using C2C12 and L6 cell lines, myoblast lines developed from limb muscles of mouse and rat, respectively, that have been shown to be able to proliferate and fuse (5, 6). However, studies of differentiating C2C12 cells found that the line consists of a heterogeneous population, some of which do not differentiate and lack production of myogenesis markers Myf5 and MyoD, but can regain expression when returned to growth conditions (7), a characteristic not representative of *in vivo* cells. Similarly observed reduction in myotube formation from L6 cells suggest that the same might be occurring in both mammalian lines (8). Furthermore, all mammalian myotubes that have been differentiated *in vitro* (both from primary myoblasts and the C2C12 cell line) have been shown to predominantly express the immature isoform of
myosin heavy chain (9, 10), and are therefore not representative of an adult myofiber culture despite developing the signature striation pattern. Therefore, although usage of myoblast cell lines have aided studies such as molecular pathways of differentiation and myoblast stem cell potential (11–13), they are ultimately not entirely representative of in vivo models.

In 1986, Richard Bischoff developed a method to isolate dissociated single FDB myofibers from rats and culture them to study satellite cells that have retained its association with the mature myofiber (14). At the time, it was believed that FDB fibers were the only ones that could be dissociated and cultured in vitro due to its shorter length, however, this protocol was later adapted to isolate extensor digitorum longus (EDL), tibialis anterior (TA), and soleus myofibers and it was found that, as long as these longer fibers are cultured in isolation, they will not tangle and break (15). Adaptations of this protocol for different muscle tissues allowed for studies of myogenic satellite cells from various stages of maturity as well as from muscle that are predominantly slow versus fast twitch. Over time, the dissociated FDB protocol has been modified to be easily replicated in mice (16, 17). Studies using the FDB culture have included quiescence and activation of satellite cells (18), effects of culturing (14, 19), protein localization and signal transduction (20, 21), membrane potential (22), as well as molecular effects of dystrophic muscle (23, 24).

A recent study on FDB myofibers focused on its merits as an in vitro model for mature myofibers (25). It was found that, compared to myotubes differentiated from C2C12 cultures, FDB fibers had higher levels of mature sarcomeric proteins
such as MHC fast, tropomyosin, and troponin-T. Additionally, C2C12 myotubes expressed elevated cardiac α-actin and utrophin, the fetal forms of skeletal muscle α-actin and dystrophin, respectively (26), and had lower overall protein content (25). The deficiencies observed in C2C12 myotubes compared to myofibers are likely due to its being differentiated and fused in culture without stimulation from tissue innervation, as well as its development in a 2D culture dish rather than a 3D environment surrounded by muscle associated components such as vascularization and connective tissue/basal lamina. Based on these attributes, we believed FDB cultures were a suitable system for our experiments in characterizing protein expression (both exogenous and endogenous), as well as analyzing the effects of myotubularin deficiency on satellite cells, as it is, in essence, a replica of intact muscle in a tissue culture dish.

We are interested in comparing the recovery dynamics of various human isoforms of α-actinin to begin to analyze their differential interactions at the Z-line. As the Sanger group showed, it is not only possible to observe varying exchange dynamics of Z-line proteins, but efficient to do so by utilizing FRAP. In order to take advantage of this method, it is necessary to exogenously express fluorescence-tagged proteins, which necessitates confirmation of their localizations at the Z-line (endogenous location of muscle isoforms α-actinin-2 and α-actinin-3). This is due to two reasons: the non-muscle isoforms may localize to non-Z-line structures, and the GFP tag may additionally inhibit or acquire interactions and mislocalize the isoforms. Therefore, showing appropriate subcellular localization of both the
sarcomeric and non-sarcomeric isoforms will allow us to correctly interpret our data in comparing Z-line dynamics.

We also want to investigate the location of the Kelch-like family member 41 (KLHL41) protein, which is expressed by a newly-identified gene whose mutations cause typical to severe cases of congenital nemaline myopathy (27). Symptoms range from hypotonia, weakness, and feeding difficulties to reduced/absent spontaneous movements in utero, severe contractures, and respiratory insufficiency. Analysis of the muscle show nemaline bodies, which are rod-shaped, electron-dense structures (27). By identifying the endogenous localization of KLHL41, we would be able to better understand its function, how mutations in the gene might be affecting muscle structure and function, and be able to approach studies regarding therapeutic options.

Lastly, we are interested in using FDB cultures to characterize the effects of myotubularin deficiency, which leads to X-linked myotubularin myopathy (XLMTM). XLMTM is a severe disorder characterized by profound muscle weakness and hypotonia, as well as external ophthalmoplegia and impaired respiratory function (28). Myotubularin is a phosphoinositide (PI) 3-phosphatase, which acts on the important signaling lipids PIP2 and PIP3. Studies in myotubularin knockout mice show that disease symptoms appear at 3-4 weeks and life expectancy is reduced to 6-12 weeks. The protein was found to localize mostly to the triads of muscle fibers and excitation-contraction uncoupling was thought to be responsible for muscle weakness, as structural defects of t-tubules and impaired calcium release from the sarcoplasmic reticulum were observed in interosseus muscles (29).
A yet unanswered question is whether the severity of myotubularin-deficiency associated triadic defects reflect the severity of the disease. A recent knockin model expressing a missense mutation (p.R69C) is based on a patient mutation linked to a less severe disease phenotype. This amelioration is similarly presented in the knockin mouse model replicating the mutation. By analyzing the terminal cisternae of these mice, we aim to determine whether a lesser disease phenotype translates into a lessened triadic defect, as this may have therapeutic implications. Furthermore, histological studies showed that myotubularin deficiency led to diminished myofiber width, and whether this might be caused by satellite cell defects was unclear. Therefore, FDB cultures would be helpful in answering both these questions as they offer the ability to test for membrane potential as well as analysis of associated satellite cells. These studies in protein localization and disease defects would not only further our interests in protein dynamics and characterizing muscle diseases, they would also expand upon the usage of FDB cultures in showing incorporation of exogenous proteins into Z-line structures, efficient identification in localization of a novel protein, and new studies in the XLMTM model.

Rather than using dissociated FDB myofibers to investigate these questions, one can conceivably also use in vivo models. By sectioning FDB (or other muscle groups), immunohistochemical analysis can be done to determine endogenous protein localization and compare satellite cell abundance. In addition, it was also shown that analyses of exogenous protein expression and SR membrane integrity can be done by in vivo imaging of the FDB muscle after DNA transfection via
electroporation (30). However, by using dissociated myofibers, we can take advantage of batch culturing to analyze for satellite cells associated with individual myofibers, maximize imaging signal-to-noise ratio by removing non-myofiber components such as connective, vascular, and nervous tissue, and increase efficiency and sample size while keeping reproducibility amongst experiments.

Results

The mouse FDB yields abundant single myofibers for \textit{in vitro} primary cell culture studies.

The mouse FDB muscle runs along the bottoms of the paws, connecting the digits to the heel. Using enzymatic dissociation, the FDB muscles from the hind paws of each 1-3 month old mouse can yield thousands of single myofibers (Fig2.1). Each myofiber is around 30um in diameter and contains multiple peripherial nuclei as previously observed (14, 25). When plated on laminin-coated culture dishes, the myofibers are able to adhere within 3 hours, and, with daily media changes, survive for just under a week using our culturing conditions. They appear to be in a semi-contracted state, as their average sarcomere length is 1.8 um, as opposed to 2-3 um when sarcomeres are relaxed.
Fig 2.1. The mouse FDB muscle yields abundant single myofibers for *in vitro* primary cell culture studies.

Each mouse hind paw FDB yields thousands of single myofibers when enzymatically dissociated and cultured. When harvested from mice over 1 month old, the multinucleated cells show peripheral nuclei and are around 30um in diameter.
The FDB cultures indicate human α-actinin isoforms localize to the Z-line, and KLHL41 enrichment at SR-ER domains.

Despite other methods being available in analyzing skeletal muscle protein localization (e.g. immunohistochemistry of frozen sections or transfections of myoblasts followed by differentiation), the FDB culture system is ideal for our purposes as we were interested in determining protein function and exchange dynamics in adult muscle. By using cultured FDB myofibers, we were able to have a consistent, high throughput way of determining the subcellular localization of muscle proteins. Additionally, we can are able to do our analyses in a highly differentiated system with mature sarcomeric structures reflective of in vivo tissue. At the same time, we can easily transduce DNA into our samples via adenovirus (as has been shown to be effective in post-mitotic myofibers) and visualize and analyze the samples by standard microscopic methods such as FRAP.

For our studies of investigating differential dynamics of α-actinin isoforms at the Z-line, we needed to express various isoforms of human α-actinin-AcGFP in mouse myofibers. Due to the fact that the exogenously expressed protein is of a different species than the samples investigated, and also that two out of the four isoforms are non-sarcomeric and not typically expressed in striated muscle, it was essential that we confirm that the human isoforms are localizing to the Z-line. To do this we expressed the cultured myofibers with human AcGFP isoforms, then fixed and stained for either myosin heavy chain (fast isoform), or endogenous α-actinin-2, which alternate along the sarcomere. Confocal images and single-channel
densitometry tracings showed that the proteins (α-actinin-2-AcGFP and α-actinin-4-AcGFP) are localized to the Z-lines (data shown in Chapter 3).

In addition to confirming the correct localization of exogenously expressed α-actinin proteins, we were also interested in determining the endogenous localization of KLHL41. As immunohistochemistry results using frozen muscle sections were not optimal (data not shown), cultured FDB fibers were used in colocalization studies to determine KLHL41 subcellular expression pattern. Immunocytochemistry of KLHL41 costained against actin, α-actinin, MHC, protein disulfide isomerase, and ryanodine receptor (RyR1) along with their respective densitometry tracings indicated that KLHL41 generally co-localizes with PDI and RyR1, and is therefore localized to the SR-ER domain. (Fig 2.2)

**FDB myofiber cultures can robustly express exogenous proteins and withstand prolonged live cell imaging.**

In order to assess the differential dynamics of different human α-actinin isoforms in muscle, we needed a system where all four isoforms could be expressed at similar levels. We utilized the FDB culture system for its ability to be cultured in batches, its validity as an adult myofiber model, and its susceptibility to protein expression via viral infection.

To quantitatively assess the dynamics of various α-actinin isoforms at the Z-line, we used adenovirus to infect cultured FDB myofibers before FRAP analysis using confocal microscopy. After infection with adenovirus expressing expressing α-actinin isoforms tagged with AcGFP, protein expression was observable after 24
**Fig 2.2. Immunostaining analysis of KLHL41 against various myofiber markers**

**show localization at ER-like SR structures.** Cultured FDB myofibers from adult mice were co-stained for KLHL41 and markers for the I-band (actin and phalloidin), Z-line (α-actinin), thick filament (myosin heavy chain, fast isoform), endoplasmic reticulum (protein disulfide isomerase (PDI)), and triad (ryanodine receptor (Ryr1)). **A.** Z-stack of confocal images shows KLHL41 localizes to the perinuclear region and co-localizes with Ryr1. **B.** Single frame images show representative areas of myofibers in co-immunostain studies. Single channel densitometry tracings on the right show intensity overlap between KLHL41 and myofiber markers (colors reflect those shown in microscopy images). Comparisons of co-immunostains indicate KLHL41 localization at ER-SR domains.
Fig 2.2 (continued)
hours. However, initial protein expression is only around the myonuclear regions. By 48 hours post-infection, protein expression has spread throughout the myofibers, and samples could be bleached and imaged for FRAP analysis (data shown and discussed in Chapter 3). FRAP data with wild type and domain-exchanged α-actinin isoforms showed discernible differences in recovery dynamics from isoform to isoform, in some cases even when only a single domain (out of three) is exchanged. Viral MOI was not calculated, as titration assays showed that regardless of vial concentration, there was always a range of expression from myofiber to myofiber, ranging from lack of expression to high expression with protein aggregates (data not shown).

Comparisons of α-actinin isoform expression (α-actinin-2 vs. α-actinin-3) in in vivo versus in vitro cultured FDB fibers showed differences between the two methods. While analysis of frozen FDB sections indicated all fibers expressed α-actinin-2, with only 66% expressing α-actinin-3, the same analysis of FDB myofibers after three days of culturing indicated that all fibers expressed α-actinin-2 and α-actinin-3 (data shown and discussed in Chapter 3). While this did not affect the results of our quantitative imaging, this difference suggests that protein expression in myofibers might change during or after the culturing process.

**FDB myofiber culture is not an optimal system in showing myotubularin-deficiency related membrane potential defects.**

Previous studies of myotubularin showed that in addition to localization at triads, and its deficiency leading to T-tubule defects, lack of the protein leads to
compromised SR membrane integrity at the terminal cisternae \((29, 31)\). A recent knockin mouse model with a missense mutation (p.R69C) mimicking a less severe patient phenotype was developed and it was found to cause a similarly lessened myotubularin myopathy phenotype in mice \((32)\). By comparing the SR integrity of the R69C mice with wild type and myotubularin knockout mice, we would be able to determine whether a lesser XLMTM phenotype would be linked with less SR defects.

To begin this investigation, we sought to replicate the results found in knockout mice using the potentiometric dye di-8-ANEPPS \((29)\). However, comparisons of our results between wild type (WT) and myotubularin knockout \((MTMδ4)\) animals showed no difference in SR integrity (i.e. the terminal cisternae of myofibers from \(MTMδ4\) animals showed intact SR membranes). These results suggest that dissociated FDB myofibers might not be the optimal method in analyzing XLMTM-associated SR defects. (Fig 2.3)

**The FDB culture system indicates lowered myogenic potential from decreased numbers of satellite cells in myotubularin deficient mice.**

To determine the molecular effects of myotubularin myopathy on myogenic potential, our group initially used FACS to analyze the number of myogenic cells (defined as quadruple negative for SCA1, CD45, and PDGFRa cell surface markers, and propidium iodide) between WT and \(MTMδ4\) mice using pooled limb muscles. Results showed that \(MTMδ4\) mice consistently had more myogenic cells compared to wild type littermates despite lower tissue yields \((33)\). On the other hand, additional investigations evaluating Pax7 protein expression (a satellite cell marker)
Fig 2.3. Analysis of membrane integrity at the terminal cisternae show no discernible difference between wild type and myotubularin-deficient myofibers. FDB myofibers were dissociated and incubated with di-8-ANEPPS to visualize the membrane potential of the sarcoplasmic reticulum at its terminal cisternae. Representative images are shown here. Fluorescence represents voltage potential across intact SR membranes. All myofibers analyzed from both wild type and myotubularin-deficient mice showed intact SR structure, unlike previously described (29), indicating a contradictory result.
of these cells using Western blot analysis suggested a decreased expression in
MTMδ4 cells. As one method assays the number of cells while the other assays the
total Pax7 protein content, FDB myofiber culturing was used to elucidate the
number of satellite cells associated with WT vs. MTMδ4 mice.

By using dissociated FDB fibers in analyzing Pax7 positive cells, we are able
to quantitate the number of satellite cells associated with each fiber individually.
Immunocytochemistry of FDB fibers at 3 hours post-plating (to minimized satellite
cell migration away from myofibers) showed that the number of Pax7-expressing
cells was significantly lower in MTMδ4 mice (47.76 +/- 10.0 fibers containing Pax7+
cells per 100 fibers) compared to WT littermates (78.62 +/- 8.16 fibers containing
Pax7+ cells per 100 fibers) (Fig 2.4). These results suggest that despite the FACS
results indicating higher number of myogenic cells in MTMδ4 mice, they actually
have a decreased number of satellite cells.
Fig 2.4. Comparison of Pax7+ cells in FDB myofibers indicated less myogenicity.

Myotubularin-deficient muscle. A. Dissociated FDB fibers from 25-30 day old wild type and MTMδ4 mice were analyzed for Pax7+ cells using immunostaining. Representative images of wild type and myotubularin-deficient myofibers are shown here. Red arrows point to cells positive for Pax7 staining (green). Blue: DAPI staining. B. Quantitation of fibers show significant difference in myogenicity between wild type and knockout tissue. Per 100 fibers analyzed, wild type samples had 78.62 +/- 8.16 fibers with Pax7+ cells versus knockout samples, which had 47.76 +/- 10.0 Pax7+ cells. n > 300 myofibers per sample from 3 mice.
Discussion

Using dissociated FDB myofiber cultures as a mature muscle system for molecular studies was developed within the last decade. We recently utilized the FDB culture system in the process of characterizing KLHL41 and also in ensuring correct localization of exogenously expressed α-actinin isoforms. Expression of α-actinin isoforms in the myofibers allowed us to then systematically perform FRAP analysis to investigate comparative exchange dynamics. Additionally, we took advantage of the FDB culture system to elucidate the effects of the myotubularin deficiency on myogenic satellite cells.

Dissociated FDB myofibers appeared to be viable for up to 6 days with no obvious morphological changes. The cells were ~30 um in diameter and contain multiple peripheral nuclei, as previously described (14, 25). Analysis of sarcomere length indicates the myofibers are in a semi-contracted state, as they average at 1.8 um in length versus 2-3 um of relaxed sarcomeres. This is likely due to lack of tensile force exerted on the myofibers because they are no longer attached to tendons on either end, as they would be in vivo. The lack of tensile force may have long term effects on myofiber morphology and protein expression, as external physical forces have been known to affect cellular character (34, 35). However, we believe that, for the purposes of our short-term studies, this is unlikely to affect our results, as cellular organization appeared unchanged.

In investigating the subcellular localization of KLHL41, the standard method of doing immunohistochemistry on frozen muscle sections provided results that
were less clear, and by performing co-localization studies in FDB myofiber cultures, we were able to pinpoint it to the ER-like SR domains. Unlike frozen sections, analysis of dissociated FDB fibers ensures consistent longitudinal orientation of each sample. This is especially convenient in making Z-stacks on the confocal, as the myofiber Z-lines easily line up, and while samples consist of whole myofibers, we are able to maximize signal-to-noise ratio in ICC analysis due to the removal of other tissues and cell types that are typically surrounding the myofibers, which can contribute to non-specific staining. Additionally, it was more efficient, as dissociated FDB myofibers can be cultured in batches, allowing for many myofibers to be analyzed per sample, each of which contains all the myofiber structures, including sarcomeres, the triads, the SR and the surrounding sarcolemma. This enabled a fast, exhaustive way to localize proteins with unknown subcellular expression patterns.

The confirmation of Z-line localization of α-actinin isoforms was similarly advantageous in using the FDB cultures, as it allowed for analysis of multiple myofibers per sample. More importantly, the presence of the entire myofiber not only allowed us to confirm that the AcGFP tagged proteins are tethered to the Z-line, but also that they are not ectopically localized elsewhere. As we were exogenously expressing α-actinin proteins, ideally, we would have used additional Z-line markers to show localization specificity as endogenous protein expression may have been affected. However, the α-actinin marker gave a robust signal and co-localization of endogenous α-actinin-2 with α-actinin-4-AcGFP indicated that this possibility was unlikely.
We also showed that not only can cultured FDB myofibers easily express exogenous proteins from AV infection, but also that they can then be analyzed using FRAP. Although it does take up to 48 hours after infection for protein expression to extend throughout the myofiber, it can then withstand > 30 minutes of photobleaching and consecutive live cell imaging. Surprisingly, even in media containing calcium ions, the myofibers do not contract as long as typical live cell imaging protocols are followed (e.g. maintaining 37°C, 5% CO₂, minimal laser power). Moreover, this system is sensitive enough to show differential exchange dynamics of the various isoforms.

Using mature myofiber cultures rather than differentiated C2C12 cells was essential for this study, as fetal proteins have been found to be expressed at higher than normal levels in differentiated myoblast cell lines (26). Additionally, previous FRAP studies of Z-line proteins in differentiated quail myotubes showed that at various stages of myofibrillogenesis, dynamics of Z-line proteins can vary greatly (3). It was shown that at earlier stages of myofibrillogenesis, Z-disc associated proteins are significantly more dynamic compared to those in more mature myotubes. Therefore, by using mature myofibers differentiated in vivo, we hope to bypass the issues associated with myotubes expressing either fetal proteins and/or immature myofiber structures.

While this allowed us to gain information on comparisons of exchange rates of the α-actinin isoforms, whether any artifacts arose from culturing the myofibers is uncertain. Due to the need to wait for myofibers to produce sufficient levels of protein after viral infection, we were not able to perform FRAP analysis until 3 days
after dissociation and plating. This extended timing could potentially alter endogenous protein expression and metabolism, as the myofibers in culture are lacking innervation and vascularization, as well as some, if not all of the basal lamina. As we saw in our own experiments, culturing may have had an effect on endogenous α-actinin-3 expression in our samples, as analysis of frozen sections indicated only 66% of FDB myofibers expressed α-actinin-3, and analysis of 3-day-old cultures indicated 100%. As denervation has been shown in the past to alter gene expression \( t(36) \), it is conceivable that it, or the lack other endogenous structures of intact muscle, can lead to changes in the properties of dissociated cultured myofibers. Furthermore, studies of the structure of FDB fibers showed that after prolonged (2-3 weeks) culturing, FDB fibers showed dedifferentiation into myotube-like cells \( (14) \).

In addition to analyzing proteins within myofibers, we also utilized FDB cultures to analyze satellite cells in myotubularin deficient mice. Although satellite cell analyses in FDB cultures have been done before in determining differentiation rates in dystrophin-deficient mice \( (24) \), our use of the system allowed us to understand conflicting data from other experiments in understanding changes in the myogenic potential of myotubularin deficient satellite cells. While FACS analysis of dissociated muscle indicated that myotubularin deficient mice possessed more myogenic cells than wild type littermates, Western blot analysis for the satellite cell marker Pax7 indicated the opposite. By analyzing the immunocytochemistry results of satellite cells associated with FDB myofibers in culture, we were able to clarify that the number of satellite cells in myotubularin deficient mice were significantly
lower. This result was then confirmed by further FACS analysis, and gene expression (32).

While we have shown that FDB cultures are good systems for studies in protein localization and satellite cell abundance, unfortunately it is not ideal for investigations of XLMTM-associated SR defects. Our experiments in analyzing defects in the terminal cisternae of the SR in myotubulin-deficient cells compared to wild type, we saw no difference. The integrity of SR membranes looked unperturbed, unlike previously reported (29) (although it was unclear whether they analyzed the FDB or the interosseus fibers). Since the myotubulin protein has been found to localize to triads and myotubulin mutation-related T-tubule defects have been reported across species (29, 31), it is most likely that our inability to replicate previous finding is due to FDB cultures being a system that is not ideal for reproducible analysis of myotubulin-deficiency-related SR defects.

By using dissociated FDB cultures, we showed that they provide an efficient way to identify the endogenous localization of novel muscle proteins as well as confirm the localization of exogenously expressed proteins. They also allow for the molecular characterization of FDB-associated satellite cells. Additionally, these cultures are able to stably express proteins via viral infection and withstand > 30 minutes of laser imaging. However, the longer culture time required for the even distribution of expressed proteins might lead to yet unknown changes in endogenous protein expression and metabolism. Their ability to incorporate exogenous proteins into myofiber structures indicate that this system can additionally be used for analysis of protein binding partners as well as screens for
gene therapy, and the ease in batch processing indicates that they could also be useful in screening small molecules and their therapeutic effects in muscle diseases.

**Materials and Methods**

*FDB myofiber culture*

FDB muscles were isolated and cultured based on a technique previously described (25). 1-3 month old wild type C57BL/6J mice were used for α-actinin studies, 6-8 month old wild type C57BL/6J mice were used for KLHL41 studies, and 25-30 day old *Mtm1δ4* and wild type littermates were used for myotubularin studies. Myofibers were seeded on 20μg/mL laminin-coated Mattek P35G-1.5-20-C glass-bottom dishes for FRAP studies or Thermo Scientific Lab-Tek 8-well glass chamber slides for di-8-ANEPPS and immuno-studies.

*Di-8-ANEPPS staining*

5mg of di-8-ANEPPS was diluted to 10mM stock solution in DMSO and stored at at 4°C. 10μM working solution diluted in Tyrode was made fresh for each experiment. FDB fiber cultures were washed with Tyrode after 1 day *in vitro*, and media was replaced with 10μM di-8-ANEPPS and incubated for 15 minutes at room temperature. Solution was replaced with Tyrode after incubation and visualized immediately on Zeiss 510 LSM confocal at 60x magnification.

*Pax 7 staining and quantitation*
FDB myofiber cultures were fixed with 4% paraformaldehyde 6 hours after plating to minimize satellite cell migration. Standard ICC techniques were used for Pax7 immunostains using the antibody from Developmental Studies Hybridoma Bank (Lawlor et al, 2012). Cells were imaged using Nikon Eclipse 90i with Nikon Elements software. Myofibers from 3 wild type and 3 Mtm1δ4 mice were analyzed and > 100 myofibers were quantitated from each animal.

*KLHL41 co-localization staining*

FDB myofibers were fixed with 4% paraformaldehyde 1 day after plating and co-immunostained for KLHL41 (Sigma AV38732) alongside skeletal muscle markers actin (Sigma A22283), α-actinin (Sigma A7811), myosin fast (Sigma M1570), ryanodine receptor (Sigma R129), and protein disulfide isomerase (Abcam, ab2792). Imaging was done using Zeiss 700 LSM confocal. Image analysis and desitometry tracings were done using ImageJ software.
References


Chapter 3

Sarcomeric and non-muscle α-actinin isoforms exhibit differential dynamics at skeletal muscle Z-lines
Attribution of Collaborator Contributions

All work in this chapter was performed by Cynthia P. Hsu except the following: John H. Hartwig performed the rotary shadowing and negative staining.
Introduction

α-Actinin, the first actin crosslinking protein discovered by Ebashi in 1964 (1, 2), is now known to represent a family of different isoforms expressed by four genes (3–5). Skeletal muscle expresses two isoforms, α-actinin-2 and α-actinin-3, which are biologically similar but chemically distinct (6). Two additional isoforms, α-actinin-1 and α-actinin-4, are expressed in non-striated muscle cells (7, 8). Studies in the past decade have revealed that in addition to F-actin binding and crosslinking, α-actinins are platforms to which a plethora of proteins can interact and thus contribute to the organization and stability of actin-based cell structures as well as participating in biosensing and the regulation of cellular functions (9).

The four mammalian α-actinin genes resulted from two gene duplication events (10). Despite all retaining function as important players in force generation and organization of F-actin, and having high levels of identity and similarity in their structure, they regulate actin bundles and networks differently depending on tissue-specific cytoskeletal differences and protein profiles in the cells. Mammalian α-actinin-2 and α-actinin-3 evolved to be sarcomeric-specific isoforms that localize to the Z-lines of striated muscle and crosslink F-actin across neighboring sarcomeres, whereas α-actinin-1 and α-actinin-4 became non-muscle isoforms that crosslink F-actin in stress fibers and tether them at the focal adhesions of non-muscle cells. Additional splicing of α-actinin-1 also produces smooth muscle and brain-specific isoforms (11, 12). In all cases, the α-actinin proteins are crucial in maintaining the architecture and stability of the actin cytoskeleton, and compromises in their structure can lead to impaired cell function and various pathologies (13–16).
Although the mammalian α-actinins have evolved into four separate isoforms, they maintain a high degree of sequence conservation. All α-actinin isoforms are collinear and are composed of an N-terminal actin-binding domain (ABD), a C-terminal calmodulin-like EF hand domain, and a linker region with four spectrin-like repeats (SLR). Sequence identity amongst the isoforms is high, ranging from 87-94% in the ABD to 68-83% in the SLR, and 68-85% in the EF hands.

Functional α-actinin proteins exist as dimers through strong interactions between opposing SLRs, leading to dumbbell-shaped structures with an actin-binding motif on either end (17, 18). Most molecules are thought to be homodimers, although there is evidence that heterodimers between skeletal muscle isoforms α-actinin-2 and α-actinin-3 may exist (19). This structural design allows the resulting functional ~208 kD unit to crosslink F-actin and also to serve as a platforms for additional protein-protein interactions (20, 21).

The N-terminal ABD consists of two tandem calponin homology repeats (i.e., CH1 and CH2), which work synergistically to interact with F-actin (22). The C-terminal CaM-like domain consists of two EF hands, which are helix-loop-helix motifs that bind calcium (20). Dissimilarity between the sarcomeric and non-muscle isoforms is most significant in the EF hand domain as, in non-sarcomeric isoforms, calcium binding at this domain regulates the conformation of the ABD. Calcium binding to the EF hands lowers the affinity of these isoforms for F-actin (7, 8, 23) at calcium concentrations greater than $10^{-7}$ M (7). In contrast, the sarcomeric isoforms lack the conservation of calcium sensitivity (24) and have several mutated residues predicted to abolish their calcium-binding ability (4, 25).
The central rod domain consists of four tandem SLRs and serves multiple roles, such as a mediator of protein self-dimerization, a linker and ruler for the functional ends of the dimer, and a platform for a wide variety of protein-protein interactions, as well as a source for elasticity along the protein (26). These characteristics allow for greater structural flexibility and mechanical stability, which is crucial for α-actinin function at focal adhesion points and sarcomeric Z-lines. In addition, the spectrin repeat region has been shown to interact with a variety of proteins and molecules, ranging from adhesion proteins (i.e., integrins, ICAMs, and L-selectin) (27–31) to muscle contractile apparatus components (i.e., nebulin, CapZ, and calsarcins) (32–36) to ion channels and neuro-transmission proteins (i.e., Kv1.5, Nav1.4/5, NMDA receptor, densin-180, and rabphilin-3A) (37–41). Unfortunately, most studies have not yet identified isoform specificity, nor the partner protein interaction sites, of the α-actins; and neither have the functional properties imparted by each interaction been evaluated in detail.

Studies have demonstrated that α-actinin isoforms are functionally and structurally similar (19) and that expression of only the highly conserved ABD domain is sufficient to correctly localize avian sarcomeric α-actinin in muscle (42). Given that the localizations and interactions of the different isoforms differ based on tissue specificity, under the same environmental conditions, the isoforms could potentially function distinctly from each other, show complete redundancy, or exhibit partial redundancy. Some studies have started to parse out whether the α-actinin isoforms are functionally redundant. For example, previous findings indicate that complete redundancy is unlikely among the sarcomeric isoforms as a
shift in muscle metabolic profiles was observed in mice where α-actinin-2 was substituted for α-actinin-3 (43). Conversely, α-actinin-3 is not able to substitute for or rescue α-actinin-2 defects in Danio rerio (Gupta, Discenza, Guyon, Kunkel, & Beggs, 2012). Likewise, studies have shown α-actinin-1 and α-actinin-4 to be similarly non-redundant since α-actinin-1 is not able to rescue the loss of α-actinin-4 (44). Determining whether the different α-actinins exhibit different properties and which structural features contribute to those differences will help the understanding of their evolution and specific roles in cases of human disease.

To better understand the differences between the four mammalian α-actinin isoforms, we investigated their functional properties with regards to their ability to target to the skeletal muscle Z-lines and their dynamics in myofibers. More specifically, we examined whether there are differences in exchange dynamics between the sarcomeric isoforms (α-actinin-2 vs. α-actinin-3) as well as between the non-muscle and sarcomeric isoforms. To do this, we used fluorescence recovery after photobleaching (FRAP) in mouse myofibers to compare the differences in the rate and extent of protein recovery at the Z-line between α-actinin-1, α-actinin-2, α-actinin-3, and α-actinin-4. Domain-specific investigations between sarcomeric and non-muscle isoforms by using chimeric proteins demonstrated that differential exchange kinetics in FRAP experiments are determined by a complex interplay between multiple regions of the proteins.
Results

α-actinin proteins have differential exchange dynamics at the skeletal muscle Z-line.

As an initial step in determining the unique properties of muscle α-actinin proteins (α-actinin-2 and α-actinin-3) at the Z-line, their fluorescence recovery dynamics were compared with those of the non-muscle isoforms (α-actinin-1 19a and α-actinin-4 8a, 19a). To assess the differential exchange dynamics at the Z-line by each of the 4 α-actinin isoforms, each human α-actinin gene was tagged with AcGFP (an altered form of GFP that is less likely to aggregate) and expressed in adult dissociated flexor digitorum brevis (FDB) myofibers via adenoviral infection. Because the fiber type makeup of muscle may vary with age, only mice that were between 1 to 3 months old were used for sample consistency.

Immunocytochemistry of infected fibers comparing the relative localization of the GFP-tagged α-actinins with myosin heavy chain (MHC) and endogenous α-actinin-2 revealed that exogenously expressed, GFP-tagged α-actinins localize to the Z-line with endogenous muscle α-actinin (Fig 3.1). This result indicates that both sarcomeric and non-sarcomeric isoforms of α-actinin are similar enough to the skeletal muscle isoforms that they can localize to the Z-line and allow for the use of FRAP as a method to distinguish their differential dynamics.

FRAP results of the four α-actinin isoforms showed differential exchange dynamics at the Z-line (Fig 3.1. B-1D, Table 3.1). All isoforms were assessed for their fluorescence recovery over 30 minutes (Fig 3.1. C). As muscle proteins have
**Figure 3.1.** Exogenously expressed AcGFP-tagged α-actinin isoforms localize specifically to the myofiber Z-lines but have different exchange dynamics. (A) α-actinin-2-AcGFP and α-actinin-4-AcGFP were expressed in myofibers and samples were co-immunostained with either the α-actinin-2 or myosin heavy chain (MHC, fast-twitch; red) antibodies. Both α-actinin-2 and α-actinin-4 are analyzed for localization in relation to skeletal muscle markers. Confocal images (left) and fluorescence intensity plots (right) indicate that GFP-tagged α-actinin-2 and α-actinin-4 co-localized with endogenous α-actinin-2 but alternated with MHC. Scale bar = 20µm (B) Fluorescence recovery of wild type isoforms α-actinin-1, α-actinin-2, α-actinin-3, and α-actinin-4 in myofibers. Representative recovery images show (left to right) pre-bleach and 0, 10, 20, and 30 minutes after photobleaching. Scale bar = 10 µm (C) Average FRAP recovery curves for α-actinin-1 (green), α-actinin-2 (orange), α-actinin-3 (red) and α-actinin-4 (purple) over 30 minutes. Values are mean +/- standard error at each time point. (D) Comparison of fast (blue) vs. slow (red) mobile fractions of α-actinin-1, 2, 3, and 4. Values are means +/- standard error. * p<0.05 in total mobile fraction (M_{fast} + M_{slow}) difference.
Figure 3.1 (continued)
Table 3.1. Differential recovery phases, rates, and half-times of wild type α-actinin isoforms

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<tr>
<th></th>
<th>M1 (%)</th>
<th>k1 (min⁻¹)</th>
<th>t₁/₂₁ (min)</th>
<th>M2 (%)</th>
<th>k2 (min⁻¹)</th>
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<td>4.45 +/- 0.33</td>
<td>3.86 +/- 0.36</td>
<td>0.18 +/- 0.02</td>
<td>31.91 +/- 1.98</td>
<td>0.05 +/- 0.00</td>
<td>12.74 +/- 0.36</td>
</tr>
<tr>
<td>ACTN3</td>
<td>4.49 +/- 0.22</td>
<td>4.78 +/- 0.71</td>
<td>0.15 +/- 0.02</td>
<td>27.33 +/- 1.86</td>
<td>0.06 +/- 0.00</td>
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</tr>
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<td>ACTN4</td>
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<td>1.69 +/- 0.08</td>
<td>0.41 +/- 0.02</td>
<td>55.73 +/- 1.34</td>
<td>0.18 +/- 0.01</td>
<td>3.93 +/- 0.15</td>
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Values were derived by fitting fluorescence recovery of photobleached wild type α-actinin molecules in myofibers to \( R = M_1(1-e^{-k_1t}) + M_2(1-e^{-k_2t}) \). Data are shown as means +/- SE.
been found to have long half-lives, ranging from 3 to 9 days (45), and similar FRAP experiments done with protein synthesis inhibition by cyclohexamide showed no effect on Z-line protein recovery on the scale of minutes to hours (42), this observed recovery of over 30 minutes can be attributed to protein exchange that is independent of new protein synthesis.

Each isoform exhibited largely unique and reproducible recovery kinetics. The skeletal muscle isoforms, α-actinin-2 and α-actinin-3, had the least amount of recovery at the Z-line (both at 30%, and were not significantly different from each other), followed by α-actinin-1 (at 50%), and finally α-actinin-4 (at 90%) (Fig 3.1. C). These data indicate that the endogenous isoforms α-actinin-2 and α-actinin-3 are most stable at the Z-line, whereas α-actinin-4 is highly dynamic, while α-actinin-1 is intermediate in its rate of exchange.

By applying the formula $R = M_1(1 - e^{-k_1*t}) + M_2(1-e^{-k_2*t})(46)$, the recovery dynamics can be separated into two mobile fractions: a fast phase ($M_1$) and a slow phase ($M_2$) (Fig 3.1. D), where $M$ describes the portion of recovery attributed to each phase and $k$ describes the rate of recovery in each phase. The breakdown of α-actinin recovery dynamics into separate mobile fractions shows that while the amount of exchange differs between the isoforms, the ratios of fast to slow mobility are similar to each other and the majority of recovery for all 4 isoforms (60-90%) occurs in the slow binding-exchange phase (Figure 3.1. D, Table 3.1). These finding are consistent with previously reported recovery profiles for the avian sarcomeric
isoform (α-actinin-2), where Wang et al. found that different Z-line proteins recover with differing fast:slow phase ratios (42).

Many muscle groups in the mouse are composed predominantly of fast, type 2, glycolytic myofibers, including the FDB, that express both α-actinin-2 and α-actinin-3 (47). One factor that might contribute to variability of FRAP results could be heterogeneity of fiber types, in which different α-actinin isoforms are expressed, as Z-line proteins can bind preferentially to one isoform (48). To assess the fiber type composition of our experimental preparations, immunohistochemistry and immunocytochemistry were used to determine the composition of fast- versus slow-twitch fiber types as well as α-actinin-2- versus α-actinin-3-expressing fibers (Fig 3.2). The analysis for slow- versus fast-type fibers was done by myosin heavy chain (MHC) staining of frozen, sectioned muscle and cultured primary myofibers. The results showed that a large majority of fibers in the FDB are fast type II fibers (>90%), whereas a much smaller percentage are slow type I fibers (up to 20%) (Fig 3.2. A). These numbers exceed 100% because some fibers may express both types of MHC. The analysis of α-actinin-2 versus α-actinin-3 expression revealed that all of the FDB fibers are α-actinin-2-positive, and a subset of these (66%) also express α-actinin-3 (Fig 3.2. B).

**α-actinin 2/4 chimeric isoforms are able to dimerize and form stable structures similar to native isoforms.**

The wild type α-actinin protein exchange dynamics suggested that the skeletal muscle α-actinin isoforms are more stable at the Z-lines when directly
**Figure 3.2.** The mouse FDB muscle consists of a heterogeneous mix of myofibers. Immunological analysis of FDB sections and dissociated, culture FDB myofibers indicated heterogeneity in myofiber composition. (A) Analysis of fiber type using MHC Fast vs. Slow immunostaining showed 20% fast type fibers vs. 93% slow type fibers in sectioned tissue (upper panels) and 16% fast type fibers 98% slow type fibers in cultured samples (lower panels). 369 fibers were counted. (B) α-actinin-2-positive vs. α-actinin-3-positive immunological analysis of the FDB showed 100% of fibers to be α-actinin-2-expressing in sectioned tissue with 66% co-expressing α-actinin-3 (upper panels). Cultured myofiber samples (lower panels) were 100% positive for both α-actinin-2 and α-actinin-3. 190 fibers were counted. Scale bar = 25um
Figure 3.2 (continued)
compared to the non-muscle isoforms. To investigate the structural basis for the observed differences between Z-line exchange dynamics, we generated chimeric proteins with interchanged domains between muscle (α-actinin-2) and non-muscle (α-actinin-4) isoforms (Fig. 3.3). α-actinin-2 and α-actinin-4 were chosen for this experiment since α-actinin-2 is a consistent component in all the samples, and the α-actinin-4 Z-line dynamics are the least similar to α-actinin-2. Because the recovery of α-actinin-2 and α-actinin-4 are so dissimilar (p-value < 0.001, taking into account Bonferroni correction) from each other, changes in the recovery of α-actinin-2/α-actinin-4 chimeras were predicted to reflect whether the domain changes caused the proteins to shift toward α-actinin-2 or α-actinin-4 exchange rates.

Three domains, the ABD, the SLR, and the C-terminal EF hands, were interchanged using non-conserved areas in linker regions of α-actinin-2 and α-actinin-4. This lessened the possibility of altering the properties of any functional domain. To confirm that the resultant proteins dimerized with appropriate morphology, we rotary metal-shadowed and examined each isoform by transmission electron microscopy (Fig 3.4). The wild type α-actinin-2 and α-actinin-4, and each of the chimeric isoforms, exhibited the distinct α-actinin protein morphology: 40 nm long rods with slightly enlarged ends (18). Although there could still be unobservable anomalies, these results indicate that the chimeric proteins seem to have the expected tertiary structures and are able to dimerize.
Figure 3.3. α-Actinin-2 and α-actinin-4 isoform Z-line dynamics are determined by cooperation between the ABD, SLR, and EF domains. (A) Schematic showing cloning of chimeric α-actinin-2/4 proteins: α-actinin-2 domains, orange; α-actinin-4 domains, purple. (B) Fluorescence recovery of chimeric isoforms: α-actinin-422 (orange triangle), α-actinin-242 (orange square), α-actinin-224 (orange circle), α-actinin-244 (purple triangle), α-actinin-424 (purple square), and α-actinin-442 (purple circle) compared to α-actinin-2 (dark orange) and α-actinin-4 (dark purple). Values are mean +/- standard error at each time point. (C) Comparison of fast (blue) vs. slow (red) mobile fractions of chimeric isoforms to α-actinin-2 and α-actinin-4. Values are means +/- standard error. (D) Recovery comparisons of α-actinin-2/4 wild type and chimeric isoforms. Panels (left to right) show pairwise comparisons of percentage fluorescent protein recovery at 30min when the ABD is changed (Δ ABD), SLR is changed (Δ SLR) and EF hands are changed (Δ EF). Values are mean +/- standard error at each time point. * p < 0.05 using Bonferroni correction.
Figure 3.3 (continued)
Figure 3.4. Electron microscopy of rotary shadowed α-actinin-2/4 wild type and chimeric isoforms. α-Actinin proteins self assemble into rod-shaped dimers with 40x5 nm dimensions. All chimeric proteins assembled into identical rods. Scale bar = 200 nm. Insets show each isoform with additional 3x magnification.
**α-actinin protein exchange dynamics at the skeletal muscle Z-line is determined by cooperative composition of all three domains.**

We hypothesized that there existed two possibilities in the Z-line dynamics of chimeric α-actinin-2/α-actinin-4 proteins: 1) recovery could be tightly correlated with the change of a single domain or 2) recovery could reflect cooperative contributions from multiple domains. The FRAP results of the chimeric α-actinin-2/α-actinin-4 isoforms showed that Z-line dynamics of the proteins followed a trend (Fig. 3.3. B-D). Each isoform’s recovery was reflective of the proportion of α-actinin-2 or α-actinin-4 it contained rather than dictated by one single domain. As α-actinin-2 domains are increasingly exchanged to α-actinin-4 sequences (indicated by dark orange to light orange to light purple to dark purple), the chimeric proteins become more dynamic at the Z-line and recover more like α-actinin-4 (Fig 3.3. B).

For example, α-actinin-224 (1 domain change from wild type α-actinin-2) showed greater exchange than α-actinin-2, and α-actinin-244 (2 domain changes) showed greater dynamic exchange. By comparing the total percentage of protein exchange of each isoform after 30 minutes, we were able to do pairwise analyses of each functional domain’s effects on Z-line dynamics (Fig 3.3. D). In addition to confirming the contributions of the SLR and EF domains on protein exchange at the Z-line, these comparisons indicated that changing the ABD also had an effect on recovery, despite its high conservation between the isoforms. However, domain specific comparisons do show that the SLR and EF hand domains possess a greater contribution to differences in α-actinin-2 vs. α-actinin-4 dynamics than the ABD (24% and 26% mean difference in total recovery for the SLR and EF domains, respectively, vs. 11%
for the ABD). These contributions were observed in total protein exchange after 30 minutes, as well as in analyses of fast and slow phase constants M1, k1, M2, and k2 (Fig 3.5). The breakdown of the chimeric α-actinin-2/α-actinin-4 proteins’ recoveries showed similar ratios of slow to fast phases as before, which reflects the previously observed dynamics of α-actinin protein, in comparison to other Z-line proteins (42) (Fig 3.3. C). I.e. despite interchanging the domains, the chimeric proteins’ exchange profiles remained characteristic of those previously established for α-actinins. Further analysis of the half-times of both the slow and fast phases revealed that the chimeras that are mostly α-actinin-2 tended to have faster half-times for fast phases and slower half-times for slow phases (Table 3.2). This trend suggests that the sarcomeric and non-muscle isoforms have different dynamics through Z-line and non-Z-line interactions and dynamics.

We identified two exceptions to the observed trend, which are the chimeric isoforms α-actinin-424 and α-actinin-422. These proteins have one and two domains, respectively, from α-actinin-2, but show no characteristics of the binding dynamics of α-actinin-2. Instead, their dynamics mimic that of α-actinin-4 (almost 90% recovery after 30 minutes, p-value = 11.28 and 1.18, respectively), which suggests that a shared aspect within these chimeric proteins (i.e. the 4ABD-2SLR neck region) might cause unexpected tertiary changes that lessen the stability of association at the Z-line.
Figure 3.5. Pairwise comparisons of FRAP phase and rate constants of α-actinin-2/4 chimeric proteins at myofiber Z-lines. Values were derived by fitting recovery data to $R = M_1(1-e^{-k_1*t}) + M_2(1-e^{-k_2*t})$, and shown as mean +/- SE. Panels, top to bottom, show comparisons of $M_1$, $k_1$, $M_2$, and $k_2$; panels, left to right show comparisons of each value when the ABD, SLR, or EF domain is changed from that of α-actinin-2 to α-actinin-4. Note the higher number of pairwise significance when the SLR or EF domain is changed, versus the ABD. * $p<0.05$ using Bonferroni correction. ** $p<0.05$ using Bonferroni correction when sample does not include α-actinin-422 or α-actinin-424.
<table>
<thead>
<tr>
<th></th>
<th>M1 (%)</th>
<th>k1 (min⁻¹)</th>
<th>t₁/₂₁ (min)</th>
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<th>k2 (min⁻¹)</th>
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<td>4.45 +/- 0.33</td>
<td>3.86 +/- 0.36</td>
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<td>31.91 +/- 1.98</td>
<td>0.05 +/- 0.00</td>
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<td>1.69 +/- 0.08</td>
<td>0.41 +/- 0.02</td>
<td>55.73 +/- 1.34</td>
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Values were derived by fitting fluorescence recovery of photobleached wild type and chimeric α-actinin molecules in myofibers to \( R = M_1(1-e^{-kt_1}) + M_2(1-e^{-kt_2}) \). Data are shown as means +/- SE.
Structural integrity of the ABD-SLR linker region is critical for actin crosslinking function.

To investigate the potential changes in protein interaction of α-actinin-422 and α-actinin-424, we tested for actin crosslinking ability by using a miniature falling ball viscometry assay, which assesses how well each α-actinin isoform gels F-actin (49) under conditions with and without calcium. This assay would reveal whether each chimeric isoform is able to crosslink F-actin and how efficiently it does so compared to wild-type isoforms, as well as whether each isoform is calcium-sensitive in its actin-binding ability.

A viscometric analysis of actin-crosslinking at various actin: α-actinin ratios under calcium and calcium-free conditions confirmed that the wild-type isoforms of α-actinin-2 and α-actinin-4 exhibited their respective calcium-insensitive and sensitive properties (Fig 3.6. A, Table 3.3). In the absence of calcium, both isoforms were able to fully gel F-actin at an α-actinin: actin ratio of 1:5. With the addition of 0.5 mM calcium, only α-actinin-2 retained gelation activity at the 1:5 ratio. Viscosity of α-actinin-4-bundled actin dropped significantly (Fig 3.6. A, Table 3.3), requiring a >5-fold molar increase in α-actinin-4 to retain gelation in the presence of calcium. These results were comparable to previously published observations regarding the calcium sensitivity of non-muscle isoforms and the calcium insensitivity of muscle isoforms (Burridge and Feramisco, 1981).
**Figure 3.6.** Native structure of the α-actinin ABD-SLR neck region is essential for actin crosslinking and calcium sensitivity. **(A)** Viscometric assay comparing gel points of chimeric α-actinin-2/4 isoforms compared to α-actinin-2 and α-actinin-4 in the presence (circle) and absence (square) of 0.5mM calcium at varying α-actinin:actin molar ratios with actin fixed at 12.8 µM. Gel-to-solid transition is shown by ∞. Gel points of α-actinin-224 (upper row, second from left), α-actinin-242 (upper row, third from left), α-actinin-244 (upper row, right), and α-actinin-442 (bottom row, second from left) were comparable to those of α-actinin-2 and α-actinin-4 under calcium-free conditions with α-actinin-224, 244, and 442 exhibiting calcium sensitivity. α-actinin-424 (bottom row, third from left) and α-actinin-422 (bottom row, right) required a higher molar concentration of α-actinin protein to reach the gel point. **(B)** Electron microscopy of negatively stained actin filaments crosslinked by wild type and chimeric α-actinin-2/4 isoforms under calcium-free conditions. Note that parallel bundles were formed by all isoforms, indicating actin-crosslinking ability. Scale bar = 200 nm.
Fig 3.6 (continued)
Table 3.3. Comparative actin-crosslinking ability and calcium sensitivity of wild type and chimeric α-actinin-2/4 isoforms

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Viscosity at α-actinin: actin = 1:25 under calcium-free conditions were compared to each other, with high viscosity indicating full gelation of F-actin by α-actinin. Calcium sensitivity was determined by comparing gel points from viscometry results between calcium-added (0.5mM calcium) and calcium-free (0.5mM EGTA) conditions. Gelation fold shift between EGTA and calcium conditions were compared at α-actinin: actin = 1:25.
The analysis of chimeric α-actinin-2/α-actinin-4 proteins revealed that although most isoforms at a concentration of 0.5 µM formed gels with F-actin when calcium is absent, α-actinin-422 and α-actinin-424 were unable to do the same (Fig 3.6. A). However, these isoforms were able to form gels at higher concentrations. These results suggest that chimeric isoforms with the α-actinin-4ABD-α-actinin-2SLR linker had compromised actin-crosslinking ability but did not lose all functionality, which correlates with the FRAP findings (Fig 3.3. B). In addition to crosslinking, phenotypic analysis of the resultant polymers was performed by negative staining. These show that gels formed at high α-actinin:actin ratios, α-actinin not only crosslinks F-actin, but organize the filaments into bundles (Fig 3.6. B). Together, these results supporting our finding that all chimeric isoforms are able to dimerize and crosslink F-actin.

**α-Actinin protein domains are similarly cooperative in exchange dynamics at fibroblast adhesion plaques.**

To investigate whether the dynamics observed in the native and chimeric protein FRAP experiments were a result of differences in interactive stability with Z-line proteins via each the isoforms’ functional domains, the same analysis was performed in non-muscle cells. In fibroblasts, α-actinin-4 is endogenously expressed and can localize to focal adhesion plaques, which are analogous to, but distinctly different from, the highly structured muscle Z-lines (50). As with the FDB myofibers, the wild-type and chimeric isoforms of α-actinin-2 are similar enough to
α-actinin-4 to be able to localize to the focal adhesions of the 3T3 fibroblast cell line (Fig 3.7. A), allowing for investigation of their exchange dynamics. We hypothesized that chimeric α-actinin protein dynamics at fibroblast adhesion plaques would be converse to those from myofibers as α-actinin-4 should have more stable endogenous binding partners.

The fibroblast FRAP findings showed the opposite trend of that observed in the myofibers (Fig 3.7. B, C). α-Actinin-4 is more stable than α-actinin-2 at the focal adhesions, recovering to 50% in just over 4 minutes, whereas α-actinin-2 is more dynamic in fibroblasts and recovers to 80% in the same amount of time (Fig 3.7. B). In addition, as α-actinin-4 domains are interchanged for α-actinin-2 domains, the new protein becomes more dynamic at the cellular focal adhesions. Similar to FRAP findings in the myofibers (Fig 3.3), these recovery shifts seem to be due to cooperative contribution by all three functional domains rather than the effect of a single isolated domain. Analysis of the contribution of each domain and its effect on FRAP recovery points to a trend where as any single domain is changed from that of α-actinin-2 to α-actinin-4, it leads to a slight decrease in dynamic exchange at the focal adhesion (Fig 3.7. C). A notable outlier in the fibroblast FRAP results is α-actinin-442, which has more stable dynamics at focal adhesions compared to α-actinin-4. This finding is possibly due to the loss of calcium sensitivity in the EF domain, which is known to be a calcium-dependent source for destabilizing α-actinin interaction with actin (7).
**Figure 3.7.** Sarcomeric α-actinin-2 isoform recovers faster at fibroblast focal adhesions than the non-muscle isoform. (A) Fluorescence recovery of α-actinin-2 and α-actinin-4 in fibroblasts. Representative images of recovery show (left to right) pre-bleach and 0, 0.5, 1, and 4.25 minutes after photobleaching at focal adhesions (magnified from inset of image at far left). Scale bar = 10um (B) Fluorescence recovery at focal adhesions of chimeric isoforms α-actinin-422 (orange triangle), α-actinin-242 (orange square), α-actinin-224 (orange circle), α-actinin-244 (purple triangle), α-actinin-424 (purple square), and α-actinin-442 (purple circle) compared to α-actinin-2 (dark orange) and α-actinin-4 (dark purple). Values are mean +/- standard error at each time point. (C) Recovery comparisons of α-actinin-2/4 wild type and chimeric isoforms. Panels (top to bottom) show pairwise comparisons of isoforms when the ABD is changed (Δ ABD), SLR is changed (Δ SLR) and EF hands are changed (Δ EF). Values are means +/- standard error. No significance was observed between pairs.
Fig 3.7 (continued)
Discussion

α-Actinin proteins are highly conserved across species and isoforms. However, despite their high levels of similarity in structure and function, previous studies have shown that their isoforms are not able to fully compensate for one another. Studies of the non-muscle isoforms found that α-actinin-1 can functionally compensate for α-actinin-4 in some instances but not others (51, 52) and studies of the muscle isoforms found that α-actinin-2 can partially compensate for loss of α-actinin-3 but not vice versa (53, 54). These findings indicate that each isoform has evolved to have specific and optimal interactions within their endogenous environments. To investigate the basis for functional specificity of α-actinin isoforms, we undertook FRAP studies to analyze their differential dynamics at the skeletal muscle Z-line.

Previous studies using FRAP to analyze the exchange rates of α-actinin under various conditions, such as injecting in vitro generated proteins into cells and utilizing myofibers differentiated in vitro from myoblasts, showed significantly variable exchange rates, depending on the experimental system (42, 55, 56). In the present study, we expressed proteins in cultured adult myofibers differentiated in vivo, to better model endogenous conditions in mature skeletal muscle.

In these studies, we expressed human α-actinin isoforms in mouse myofibers. While this is not an ideal representation of α-actinin dynamics with Z-line proteins as these interactions are cross species, our choice in using human isoforms stems from the ability to use the same proteins in other studies we had
proposed at the same time. Due to high levels of conservation between mammalian isoforms of α-actinin, we think that, despite the use of human isoforms, our results should reflect α-actinin dynamics at the Z-line.

Our findings showed that the four human α-actinin isoforms have widely varying dynamics at muscle Z-lines, ranging from FRAP recoveries of 30% to 90% over a 30 minute time period. α-actinin-2 and α-actinin-3 were the most stably bound at the Z-line, only achieving 30% recovery, followed by α-actinin-1 (60%) and α-actinin-4 (90%). These results showed that muscle-specific isoforms are most stably associated at the Z-lines, suggesting that muscle isoforms have evolved specific interactions in striated muscle despite similarities in sequence and function with non-muscle isoforms. The differences observed indicate that as the muscle isoforms branched off from the ancestral gene (10), they evolved interactions with muscle-specific proteins and lost certain properties of the ancestral isoform, such as calcium sensitivity (retained by the non-muscle splice isoform of α-actinin-1 and α-actinin-4). These developments may have led α-actinin-2 and α-actinin-3 to be more strongly associated at the Z-line.

Although α-actinin proteins are commonly thought to form primarily homodimers, Chan et al (19) showed that α-actinin-2 and α-actinin-3 were able to form heterodimers at low concentrations in vitro. In our study, it was unclear whether α-actinin-2 and α-actinin-3 formed heterodimers in cultured myofibers due to their similarity in recovery, but the recovery patterns of the non-muscle isoforms indicated that heterodimers between muscle and non-muscle isoforms were unlikely. E.g. if α-actinin-4 were forming heterodimers with endogenous α-actinin-
2, its FRAP recovery profile should have reflected two distinct profiles, which was not observed. Since dimers are formed via anti-parallel interactions through the SLR domain, sequence variability between the muscle and non-muscle isoforms may have prevented heterodimer formation.

According to the FRAP models previously presented (57), the recovery curves we observed for α-actinin isoforms indicated dynamics of diffusion-uncoupled recovery (i.e. diffusion happens immediately after photobleaching, followed by a much longer recovery phase reflective of the exchange of binding partners). Therefore, a large part of the recovery curve is due to binding and unbinding events. A breakdown of the dynamics into fast versus slow phases revealed that, at the Z-line, muscle isoforms had faster half-times for fast phases and slower half-times for slow phases. As the fast phases are indicative of exchange with non-specific partners and the slow phases with specific interactors, our results suggest that muscle isoforms have faster exchange with non-specific interactors, and slower exchange with specific binding partners, thus suggesting more numerous and/or stable binding with Z-line proteins.

The calcium sensitivity of non-muscle isoforms may account for part of their decreased stability at the Z-line, as it was found that calcium concentrations at >100 nM leads to a decrease in non-muscle isoforms’ actin-binding ability (7), and the resting calcium concentration of FDB was found to be at 106 +/- 2 nM (58). Although calcium sensitivity is one main difference between muscle and non-muscle isoforms, the FRAP results indicate protein interactions as an additional source for the observed differences. Although α-actinin-1 is calcium-sensitive, it was over 30%
more stable than its fellow non-muscle isoform \( \alpha \)-actinin-4. A probable reason is that it is able to better interact with skeletal muscle proteins despite having calcium-sensitive actin binding, as a calcium-insensitive splice isoform of it is normally expressed in smooth muscle (3).

Our effort to delineate which portion of the \( \alpha \)-actinin protein dictated the observed recovery differences by analyzing the exchange of chimeric \( \alpha \)-actinin-2/\( \alpha \)-actinin-4 proteins showed that the ABD, SLR, and EF domains all contributed significantly to the differential dynamics of the muscle and non-muscle isoforms. Although the calcium sensitivity of the EF hands and the sequence diversity of the SLR domain suggested that these domains might contribute to differences in exchange dynamics, the nature of the ABD (the most highly conserved domain) surprisingly also significantly affected Z-line dynamics. This was unexpected as the function of this region is well-studied: it is the most highly conserved amongst all four isoforms, and there are no known muscle-specific interactions at the ABD. Our result indicated that interaction between \( \alpha \)-actinin and F-actin may be altered in an isoform-dependent manner, and that as an \( \alpha \)-actinin-2 isoform’s domains are replaced by those of \( \alpha \)-actinin-4, it incrementally becomes more dynamic at the Z-line.

Breakdown of the dynamics into fast and slow phases showed that overall, chimeric proteins that are predominantly \( \alpha \)-actinin-2 had faster half-times for fast phases and slower half-times for slow phases compared to those that are predominantly \( \alpha \)-actinin-4. The faster half-times for fast phases indicate that as an \( \alpha \)-actinin-4 isoform gains \( \alpha \)-actinin-2 domains, it loses interactions with non-Z-line
binding partners. Alternatively, its slower half-times for slow phases suggest that as α-actinin-4 domains are swapped for those of α-actinin-2, the resulting isoform becomes less dynamic at the Z-line due to more numerous and/or stable interactions with α-actinin-2-specific binding partners. Together, these results suggest that muscle isoforms are different from non-muscle isoforms because as they are transported through the myoplasm, each of the three domains help direct them to more efficiently bypass non-specific interactions and more stably bind at the Z-line.

Analysis of the chimeric isoforms at fibroblast focal adhesions showed the converse in exchange dynamics. In the non-muscle environment, the α-actinin-2 isoforms attained more stable dynamics at focal adhesions as its domains were swapped for those of α-actinin-4. This observed difference is unlikely to be due to differences in actin protein structure, as skeletal muscle alpha actin and fibroblast beta actin are 94% similar. Rather, this change indicates that not only did the muscle α-actinin isoforms evolve to gain specific interactions at muscle Z-lines, but also that they may have lost properties of the ancestral α-actinin protein that allowed non-muscle isoforms to maintain stability in non-muscle cells. An additional possibility is that α-actinin-1 and α-actinin-4 isoforms gained non-muscle specific properties after the α-actinin-2 and α-actinin-3 divergence.

For the fibroblast focal adhesions studies, we analyzed α-actinin-2 and α-actinin-4 and their chimeric isoforms. However, while the isoforms readily localized to the focal adhesions, it was previously shown that endogenously, α-actinin-1 predominantly localizes to the adhesion plaques, while α-actinin-4 predominantly
localizes to stress fibers. To ideally show localization of endogenous focal adhesion-associated-α-actinin and its changes in dynamics when domains are swapped for those of a sarcomeric isoform, we would have compared α-actinin-1 and α-actinin-2, but this would have dettracted from the continuity of our α-actinin2/4 comparisons from Z-line studies. Alternatively, we could have analyzed dynamics at the stress fibers, but our initial analysis showed that fibroblasts were highly dynamic and we were not able to see significantly different recovery rates between α-actinin-2 and α-actinin-4. Additionally, this would have been a structure that is not analogous to the myofiber Z-line. Ultimately, by analyzing dynamics of α-actinin-2 versus α-actinin-4, neither of which is typically localized to the focal adhesion, we might have seen a less dramatic difference in exchange dynamics, but our observations were sufficient in drawing conclusions complementing those we made in myofibers.

Although there was an apparent domain-dependent trend in our FRAP results, the chimeric proteins α-actinin-422 and α-actinin-424 both exchanged more dynamically than expected. Viscometry assessment showed that, compared to other isoforms, they have compromised gelation ability and required higher concentrations of α-actinin protein to form gels with F-actin, indicating a decreased ability to crosslink F-actin. As these isoforms share possession of the α-actinin-4ABD-α-actinin-2SLR linker, change in properties of this linker is a possible cause for the gelation defect. Rotary shadowing indicated that their dimerization ability is unaltered and their α-actinin-4-like dynamics suggest they are functional proteins, albeit with less stable binding at the Z-line. Although the linker is not part of a conserved domain, it closely neighbors both the ABD and SLR and interacts with the
opposing dimer’s EF domain. The altered linker region may have disrupted tertiary structure at and around the junction and affected interactions that may have lowered the isoforms’ ability to efficiently crosslink F-actin.

The viscometry assessment revealed unexpected calcium sensitivity of the chimeric isoform α-actinin-442, despite having a calcium-insensitive EF domain from α-actinin-2. The reason for this result is unclear, especially since its dynamics in fibroblast FRAP experiments indicated that the α-actinin-2 EF domain in this isoform may have contributed to calcium-related stability. In conjunction with the gelation results of α-actinin-422 and α-actinin-424, this indicates that the ABD domain is susceptible to functional changes as a result of changes in its neighboring linker region and/or the other two domains, perhaps through steric interactions.

Mutations of α-actinin-2 and α-actinin-4 have both been associated with human genetic diseases: α-actinin-2 with hypertrophic cardiomyopathy and dilated cardiomyopathy (13, 14) and α-actinin-4 with focal segmental glomerulosclerosis (FSGS) (15). Rather than localizing α-actinin-2-associated diseases to a specific functional domain, mutations obtained from patient data were found in all three domains. However, mutations for FSGS have been found to localize to the end of the α-actinin-4 ABD. These findings coincide with our results in supporting the sensitivity of the α-actinin-4 ABD-SLR regions and the cooperative contribution of all three domains in α-actinin function.

In this study, we determined that despite the conservation of α-actinin isoforms, they have highly variable dynamics in skeletal muscle. This variability could be attributed to muscle isoforms evolving muscle-specific interactions and
losing properties of non-muscle isoforms that allow them to be optimally stable at the Z-line. Moreover, we found that exchange differences result from cooperative contribution of all three domains and that the ABD-SLR linker region is essential for \(\alpha\)-actinin protein function. Further studies would be necessary to determine the specific protein interactions that account for differences found in \(\alpha\)-actinin dynamics. This in particular applies to studies of isoform-specific differences between \(\alpha\)-actinin-2 and \(\alpha\)-actinin-3, for while use of FRAP in isoform-specific analysis is sufficient in revealing differences between muscle and non-muscle isoforms, but it is not sensitive enough in determining differences between the two skeletal muscle isoforms.

In addition, although we have found the ABD, SLR, and EF domains to cooperatively contribute to dynamics, it is still unclear whether \(\alpha\)-actinin domains functionally regulate each other or their dimer partners. Together, our findings indicate that there are functional differences between isoforms of the \(\alpha\)-actinin family of proteins, which warrant isoform-specificity in future studies.
Materials and Methods

FDB myofiber culture

FDB muscles from 1-3 month old wild type C57BL/6J mice were isolated and cultured based on a technique previously described (59). Myofibers were seeded on 20µg/mL laminin-coated Mattek P35G-1.5-20-C glass-bottom dishes for FRAP studies or Thermo Scientific Lab-Tek 8-well glass chamber slides for immuno-studies.

Protein expression in myofibers

Full length human wild type α-actinin 1-4 (NM_001102.3, NM_001103.3, NM_001104.2, NM_004924.4) were tagged with AcGFP and cloned into pAd/CMV/V5-DEST using Gateway cloning. Chimeric α-actinin 2/4 were assembled using GENEART Seamless Cloning. Vectors were transfected into HEK293A cells using Lipofectamine 2000, (Invitrogen). Adenoviral production was performed as previously described (60) and purified according to http://www.imba.oeaw.ac.at/uploads/media/Adenovirus_purification_02.pdf. FDB myofibers were infected at 1 day in vitro (div) in DMEM with 10% FBS and 5µg/mL polybrene. Media was changed to DMEM with 20% FBS at 2 div and samples were used at 4 div.

Immuno-chemistry assays for fiber typing
Myofibers and frozen sections were fixed using 100% methanol. Myosin heavy chain, slow type (NOQ7.5.4D, Sigma) and fast type (MY-32, Sigma) monoclonal antibodies were used at 1:100 in 10% FBS in PBS. α-actinin-2 (4B3) and α-actinin-3 (5B4) rabbit polyclonal antibodies (described in Beggs et al 1992) were used at 1:100. Alexa Fluor 488 Goat anti-Mouse IgG and Alexa Fluor 488 Goat anti-Rabbit IgG antibodies were used at 1:200 for visualization. Samples were imaged at 25x using Nikon Elements on a Nikon Eclipse TE2000-S.

Immuno-cytochemistry for co-localization

Myofibers were infected for α-actinin-2-AcGFP and α-actinin-4-AcGFP expression and fixed at 4 div in 4% PFA and perforated using 3% Triton X-100. Myosin heavy chain, fast type (MY-32) and α-actinin-2 (4B3) antibodies were used at 1:100. All confocal images were collected with Zeiss LSM 700 using a 60x oil immersion objective. Protein co-localization analysis was done using ImageJ.

Fluorescence Recovery After Photobleaching

Myofibers expressing α-actinin-AcGFP were imaged for FRAP at 4 div in DMEM without phenol red and with 20% FBS. Samples were kept in a Tokai-Hit stage-top incubator set at 37°C with 5% CO₂. All images were collected using a Nikon A1R with the Perfect Focus System using a 60x oil immersion objective with Nikon-Elements. Areas containing 5-6 Z-lines were bleached with pinhole set to 1.5 AU (1.0um). Post-bleach images were collected every 10 seconds for the first minute, every 30 seconds for the subsequent 3 minutes, and every 1.5 minutes for the
remaining 25.5 minutes. Fibroblasts expressing α-actinin-AcGFP were similarly imaged and analyzed at 2 div in DMEM without phenol red and with 10% FBS. Samples were kept at 29°C during image collection to minimize cell movement. Bleach areas contained 1-2 focal adhesions. Image stacks of each series were aligned and fluorescence recovery was measured by averaging changes in fluorescence intensity of each Z-line in the bleach region. An unbleached area was recorded for each time point and background intensity was subtracted from both bleached and unbleached intensity values. The resulting values were corrected as a bleached:unbleached ratio. The initial post-bleached ratio (t=0) was subtracted from each ratio, and all values were divided by the pre-bleach value to set the pre-bleach value to 1 and initial post-bleach value to 0. Resultant values from 5 Z-lines or 1-2 focal adhesions were then averaged and analyzed using ImageJ and processed to fit $R = M_1(1 - e^{-(k_1*t)}) + M_2(1-e^{-(k_2*t)})$ using KaleidaGraph (Synergy Software) as previously described (42). Half-time of each recovery phase ($t_{1/2}$) was derived from $(\ln 2)/k$.

**Generation of recombinant proteins**

Ac-GFP-tagged human wild type and chimeric α-actinin-2/4 were cloned into pFastBac HT C. Bacmids were produced and transfected into Sf9 cells for baculovirus production according to the Bac-to-Bac System (Invitrogen). Cells were grown at 25°C and 5% CO² in spinner flasks and lysed using 1% Triton X-100. Proteins were purified from using Ni-NTA columns as described in Nakamura et al
(61). Purified proteins were concentrated using Amicon Ultra-15 100k filter units. Molecular weight was confirmed by SDS-PAGE and Coomassie Blue staining.

**Rotary shadowing**

Recombinant α-actinin, 0.5 µM, in 5 mM sodium phosphate and 50% glycerol, were sprayed onto freshly cleaved mica (62), dried under vacuum, and metal-coated with 1 nm of platinum at 6° with rotation and 3.0 nm of carbon at 90° without rotation. Replicas were floated in water, picked up on copper grids cleaned with 10% formic acid, and photographed in a JEOL 1200-EX electron microscope at 80 kV.

**Viscometry assay**

Gel-filtered rabbit skeletal muscle actin, stored at -70° C in 0.2 mM CaCl$_2$, 0.5 mM ATP, 0.2 mM MgCl$_2$, and 2 mM Tris, pH 7.0 was rapidly thawed, diluted to 40 µM in the same buffer, stored at 4° C for 12 hr, and clarified by centrifugation at 200,000 x g for 30 min. A final actin concentration of 12.8 µM was mixed with increasing amounts of the different recombinant α-actinin preparations and polymerization initiated with 0.1 M KCl and 2 mM MgCl$_2$ in the presence of a final concentration of either 0.5 mM EGTA or 0.5 mM CaCl$_2$. The solutions were loaded into 100 µl glass capillary tubes, the bottom sealed with hematocrit sealant, and incubated for 30 min at room temperature. The time required for small stainless steel balls to fall through the solution was measured at 90°. Experiments were repeated 2-3 times and gelation times were averaged.
Negative staining

F-actin and α-actinin solutions were removed from the 100 µl capillary tubes used for gel point determinations, diluted to 2 µM F-actin in actin polymerization buffer (100 mM KCl, 0.5 mM ATP, 2 mM MgCl₂, 2 mM Tris-HCl, pH 7.0 containing either 0.2 mM CaCl₂ or 0.5 mM EGTA), and adhered to the surface of carbon and formvar-coated copper grids. Samples were washed twice in polymerization buffer and negatively stained with 1% uranyl acetate.
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Chapter 4

Summary of Accomplishments and Significance of Findings
In these studies, we demonstrated how FDB myofiber cultures could be used as a highly differentiated system to determine subcellular localization of a novel protein, and to model and examine a disease process \textit{ex vivo}. Additionally, this mature \textit{in vitro} system is amenable to genetic manipulation and subsequent quantitative imaging to allow for comparisons of the association dynamics of a family of highly related sarcomeric and non-muscle proteins.

**Expanding the use of FDB myofiber cultures in muscle studies**

In our studies, we used previously-described FDB cultures to answer various muscle-specific questions and found it to be an efficient system in narrowing down the localization of previously uncharacterized proteins, as well as a good system for the satellite cell characterization of disease models.

In investigating the novel protein KLHL41, mutations of which are implicated in nemaline myopathy, we used FDB cultures to determine endogenous protein localization. Compared to similar \textit{in vivo} analysis by using frozen sections, our studies using FDB fibers were more definitive in showing localization at ER-like SR domains. The removal of tissues and cell types surrounding myofibers increased the signal-to-noise ratio in immunostaining studies, and the consistent orientation was convenient in whole-cell analysis. These results emphasize the efficiency of using dissociated myofiber cultures in analyzing protein localization and their benefits in studying the subcellular localization of proteins.

FDB fibers have been described as a mature muscle culture (I) compared to mammalian myotubes, which have been shown to differ from \textit{in vivo} myofibers.
Differences include expression of fetal versus adult protein isoforms, as well as over lower protein content (2). Analysis of protein dynamics using C2C12 myofubes would represent associations between mature and fetal proteins. Therefore, investigations using FDB fibers should be more representative of adult muscle dynamics than when using in vitro differentiated myotubes. To take advantage of this, we used the FDB system to express human isoforms of α-actinin proteins and demonstrated that FDB fibers can withstand > 30 minutes of photobleaching and live-cell imaging for FRAP analysis. And that, by using standard live-cell imaging techniques, we were able to use this method to determine the exchange dynamics of different isoforms.

We also showed that by individually assessing dissociated fibers, we were able to determine significant differences in the abundance of satellite cells in myotubularin-deficient mice compared to wild type mice. The alternative method would have been to quantitate overall number of satellite cells between wild type and myotubularin-deficient mice by analyzing frozen sections, in which case we would have had to account for different sizes of muscle (as myotubularin myopathy causes smaller muscle bundles), and we would not be able to determine satellite cell association with individual fibers. While use of myofiber cultures increases the risk of accidentally dislodging and losing satellite cells through the culturing process, we think that possibility is reduced by minimal handling and culturing time before analysis. Satellite cell analysis using FDBs have previously been done in dystrophin-deficient mice (3), and our results in these studies suggest that FDB cultures are useful in studies of various disease models.
Here, we used dissociated FDB muscles to analyze the myofibers and associated satellite cells for characterizing muscle diseases. Despite the convenience and utility of FDB cultures, therapeutics advances following characterization of disease phenotypes will heavily depend on the power of mononuclear myogenic cells. A currently expanding field in modeling diseases and analyzing therapeutic approaches is the use of induced pluripotent stem cells (iPS), which can be derived from adult fibroblasts by introducing certain stem cell factors (4). By transforming them to express missing or compromised elements and reintroducing them to patient muscle tissue, one could conceivably ameliorate disease symptoms while averting an immune response. Although use testing in humans is still not possible, this method has been shown to be promising in certain diseases in animal models (5, 6).

While the strength of FDBs is in its modeling of mature tissue, iPS or satellite cell studies could be used as a better way to study disease progression from a developmental/regenerative perspective. However, in order to properly study proliferation and fusion, better methods need to be developed for retaining the cells in a progenitor state, as well as providing conditions that better mimic those in vivo for cell differentiation and fusion.

Because in vitro differentiated myotubes differ from mature myofibers in that they retain fetal components (1, 2), the cause has been postulated to be the 2D tissue culture environment versus a 3D in vivo environment that includes interactions with surrounding tissues such as innervation, vascularization, and the basal lamina (1). Studies have shown that by adding back certain aspects of the in

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vivo environment, such as chronic stimulation and a fibroblast feeder layer, the resulting myotubes showed more mature characteristics (7, 8). Likewise, development of artificial 3D matrices with controlled stiffness have been underway to allow for chemical manipulations to mimic microenvironments, which could be used to replicate the ECM of muscle progenitor cells (9).

Together, our findings using FDB myofiber cultures indicate that FDB cultures are useful in different aspects of muscle studies. Although not the ideal method for all studies, this method is efficient at characterizing novel proteins and assessing disease phenotypes and is a good complement to more standard procedures currently being implemented such as analyzing frozen tissue sections and FACS analysis. As it can additionally be used for higher throughput analysis compared to other myofiber cultures (i.e. EDL and TA cultures), it also has utility in screens for gene and drug therapy alongside iPS studies.

**Further understanding of α-actinin proteins**

Although α-actinins are well-characterized proteins, many studies have not taken isoform-specific differences into account. Here, we used the FDB myofiber system to show that, at the myofiber Z-line, the sarcomeric isoforms α-actinin-2 and α-actinin-3 are most stably bound, followed by α-actinin-1, then α-actinin-4. This suggests that, due to their differential exchange dynamics at the Z-line structure, sarcomeric isoforms have evolved muscle-specific interactions that tether them more tightly at the Z-line, whereas non-muscle isoforms α-actinin-1 and α-actinin-4
either are missing Z-line interactions or have more non-specific interactions within the myoplasm.

As we further characterized the exchange dynamics between muscle isoform \( \alpha \)-actinin-2 and non-muscle isoform \( \alpha \)-actinin-4 by interchanging each of the 3 main domains (ABD, SLR and EF hands), we found that \( \alpha \)-actinin-4, which was loosely associated at the Z-line, incrementally gained stable interactions as each of the domains was changed for that of \( \alpha \)-actinin-2. This result indicated that each of the three domains contributes to the stabilizing dynamics of \( \alpha \)-actinin-2. This finding was surprising to us, as even the ABD domain, which was the most conserved domain among the four isoforms, showed differences in interactive affinity at the Z-line.

Converse results in dynamics at fibroblast focal adhesions supported our hypothesis that isoform-specific interactions at the structures of endogenous cell types lends to binding stability. These differences indicate that the muscle and non-muscle isoforms differ in more than just their cellular localization. Rather, it is unlikely they might be able to fully functionally substitute for one another as essential isoform-specific protein interactions might be missing. For instance, it could be that sarcomeric proteins such as CapZ, myotilin, and myozenin associate tightly with \( \alpha \)-actinin-2 but not at all with \( \alpha \)-actinin-4. Such isoform-specific interactions would lead to stable Z-line dynamics for \( \alpha \)-actinin-2 but leave \( \alpha \)-actinin-4 to interact only non-specifically. Alternatively, \( \alpha \)-actinin-4 could be more stably associate with fibroblast focal adhesions if non-sarcomeric proteins such as MAGI-1, palladin, and cytohesin-2 interact specifically with non-muscle isoforms of \( \alpha \)-actinin.
To test for the possibility of functional substitution, knockins and knockout studies can be done. Investigations for functional redundancy between the skeletal muscle isoforms have shown that α-actinin-2 can rescue for loss of α-actinin-3 (although causing a metabolic shift in the process) (10), however zebrafish studies from our lab showed that effects of morpholino knockdowns of α-actinin-2 could not be rescued by α-actinin-3 expression (11). Knockin studies are currently in progress to determine whether the same holds true for mice.

F-actin crosslinking experiments using α-actinin-2/ α-actinin-4 chimeric isoforms indicate that the structural integrity of the ABD-SLR linker region is essential for crosslinking activity. Rotary shadow experiments suggest that although the proteins are able to dimerize, chimeric isoforms that include the α-actinin-4 ABD linked to the α-actinin-2 SLR might be functionally altered as an effect of changes in steric interactions. The sensitivity of this region to changes is in congruence with previously identified α-actinin-4 mutations in focal segmental glomerulosclerosis (FSGS), where the mutations were localized to the ABD-SLR linker region and led to altered actin binding (12).

Our findings show that α-actinin proteins have isoform specific differences in each of the 3 domains. In addition, although linker regions between the domains are not established as being functionally important, changes here can lead to drastic functional differences, as was found with FSGS studies (12). By further studying these aspects of α-actinin, we can better understand the essential components that lead to functional integrity both in muscle and non-muscle cells.
**Future Directions**

Our studies have begun to show the extent of differences between α-actinin isoforms, most prominently between sarcomeric and non-sarcomeric proteins. Further interaction assays would be required to determine each isoform’s unique interactions within cells where they are endogenously expressed. Although our results indicate that most of the isoforms are unlikely to be functionally redundant for one another, understanding these differences would reveal the specialized interactions and functions that the isoforms have evolved to acquire. Moreover, as mutations in α-actinin-2 and α-actinin-4 have been identified in cardiac myopathies and FSGS, respectively, identifying the causes for compromised function would be a first step toward developing therapeutic options.

Although the linker regions between the functional domains of α-actinin proteins have not been well studied, it is apparent that they can be essential in affecting functional integrity. Protein interaction studies may indicate this region to have essential binding partners that were previously unknown. As mutations in α-actinin-4 that affecting binding have been identified (12), and in our studies chimeric proteins that altered this region led to similar functional defects, further studies of this region would be necessary to determine the structural/chemical properties of specific residues essential for regulating the ABD.

One of our original goals was to determine the differences between skeletal isoforms α-actinin-2 and α-actinin-3. We found that by FRAP analysis that there were no differences in their exchange dynamics. As previous studies have shown that α-actinin-2 can rescue for α-actinin-3, but not the other way around (10, 11),
determining measurable differences in their interactions would reveal essential isoform-specific functions. To further analyze the isoform specific differences revealed by our FRAP results, we originally proposed to perform immunoprecipitation studies using each of the isoforms to purify for their interacting partners. We would then use mass spectrometry to identify any candidates that seemed unique to specific α-actinin isoforms based on size differences on a gel. Subsequent knockdown experiments would reveal any functional relevance of each particular interaction. By looking at their respective unique binding partners in muscle, we would not only be able to better analyze interactive differences between α-actinin-2 and α-actinin-3, we would also be able to parse out the essential muscle specific interactions that to the observed binding stability of muscle isoforms at the Z-line. Furthermore, although we used the non-muscle isoform of α-actinin-1, it showed intermediate recovery in its exchange dynamics at the Z-line compared to skeletal muscle isoforms and α-actinin-4. As this is likely due to its ability to be spliced as a smooth muscle isoform, protein interaction studies would reveal which skeletal muscle components causes it to be better tethered at the Z-line compared to α-actinin-4, as well as which interactions it lacks compared to α-actinin-2 and α-actinin-3. Future studies in this area would allow better understanding of evolutionary adaptations of the four isoforms as well as their functional uniqueness.

In many of our experiments, we have taken advantage of the FDB culture as a method in answering our muscle related questions. While it is not an ideal system for all aspects of muscle analysis, it is a unique in vitro method that is representative of in vivo adult muscle. We have shown that it is efficient in the characterization of
novel protein localization, as well as analyzing disease phenotypes, and its adaptability for high throughput analysis indicate it can be a useful method in future studies of gene or drug therapy screens. Lastly, it enabled us to begin to analyze the isoform specific differences between the four α-actinin isoforms at the muscle Z-line.
References


