Generation of novel Zasp52 mutants and analyzing the associated phenotypes, and identifying the interaction domain of α-actinin with Zasp52 extended PDZ domain

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June 2022

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science Biology.

Table of Contents

List of Figures

Abstract

Striated muscles conduct many essential aspects of life such as moving and heart beating. Sarcomere structure and function as the smallest contractile unit of muscles, is highly conserved throughout evolution. It is composed of actin thin filaments and myosin thick filaments which are confined between two adjacent Z-discs. The Z-disc is a multi-protein complex that serves as an anchor-site for actin filaments. Different Z-disc cross-linker proteins such as α-actinin, filamin and Alp/Enigma proteins are involved in myofibril assembly and maintenance. The Alp/Enigma family is a class of Z-disc-associated proteins, whose mutations lead to myopathies and different muscle disorders. In *Drosophila*, Zasp52 is the major Alp/Enigma protein and composed of one PDZ domain, one Zasp-like motif (ZM), and four LIM domains. Zasp52 interacts with α-actinin and actin filaments at the Z-disc and contributes to myofibril formation and maintenance. In this thesis, using CRISPR mutagenesis and FLP-mediated recombination, we introduce three novel Zasp52 alleles and demonstrate how Zasp52 mutants disrupt the normal assembly of myofibrils. Moreover, we narrow down the interaction domain of Zasp52 with α-actinin, and show that the EF-hand domain of *Drosophila* α-actinin is sufficient to interact with the extended PDZ domain of Zasp52.

Résumé

Les muscles striés sont nécessaire pour de nombreux aspects essentiels de la vie tels que les mouvements et les battements cardiaques. La structure et la fonction du sarcomère en tant que plus petite unité contractile des muscles sont hautement conservées au cours de l'évolution des espèces. Le sarcomère est composé de filaments minces d'actine et de filaments épais de myosine confinés entre deux lignes Z adjacentes. La ligne Z est un complexe multi-protéique qui sert de site d'ancrage pour les filaments d'actine. Différentes protéines de réticulation de la ligne Z telles que l'α-actinine, la filamine et les protéines Alp/Enigma sont impliquées dans l'assemblage et la maintenance des myofibrilles. La famille Alp/Enigma est une classe de protéines associées aux lignes Z, dont les mutations entrainent des myopathies et différents troubles musculaires. Chez la drosophile, Zasp52 est une protéine Alp/Enigma majeure et composée d'un domaine PDZ, d'un motif de type Zasp et de quatre domaines LIM. Zasp52 interagit avec les filaments d'α-actinine et d'actine au niveau des lignes Z et contribue à la formation et au maintien des myofibrilles. Dans cette thèse, en utilisant le système CRISPR-Cas9 et la recombinaison médiée par FLP, nous introduisons trois séries alléliques de Zasp52 et démontrons comment les mutations de Zasp52 perturbent l'assemblage normal des myofibrilles. De plus, nous identifions le domaine d'interaction de Zasp52 avec l'α-actinine et montrons que le domaine EF-hand de l'α-actinine de la drosophile est suffisant pour interagir avec le domaine PDZ étendu de Zasp52.

Acknowledgments

I would like to thank my supervisor, Dr. Frieder Schoeck, for his continuous support and advice throughout my master's study which developed my technical skills and scientific thinking as a researcher. Especially, I would appreciate his kindness and supportive attitude that kept me motivated and optimistic at all times.

Also, I would like to thank Dr. Tuana Correis-Mesquita and Dr. Nicanor Morales-Gonzalez for sharing their scientific and technical expertise.

Then, I would like to thank the members of my supervisory committee Dr. Nam-Sung Moon and Dr. Stephanie Weber. Their scientific directions led me to a better understanding throughout my master's project.

I am grateful to my dear parents for their endless supports, encouragements and peaceful home environment they provided that allowed me stay focused during my studies.

During the pandemic, I encountered many restrictions on both lab works and social relationships. I would like to thank my dear wife, Bahar, who gave me moral support and stayed by me during these hard times.

Finally, I would like to dedicate my thesis to my father, who sadly passed away last September due to Corona virus infection.

1. Introduction

1.1 Overview

The cytoskeleton is a network of filamentous proteins that organizes the cytoplasm of eukaryotic cells. There are three types of cytoskeletal filaments in eukaryotes: microtubules, intermediate filaments, and actin filaments (microfilaments). These filaments are responsible for organization and mechanical properties of the cell and contribute to direct intracellular transport, signal transduction, endocytosis, and mitotic spindle formation during cell division by interacting with hundreds of accessory proteins. Actin filaments are primarily required for cell locomotion and determining the shape of the cells. In muscles, sliding of actin thin filaments across myosin thick filaments create mechanical force required for contraction. The accessory proteins are pivotal for cytoskeletal filament assembly and maintenance and provide an interesting model to study the basic principles of cytoskeleton regulation.

There are three types of muscle tissues in vertebrates; skeletal, heart, and smooth muscle. Skeletal and heart muscles are responsible for body movements and heart contraction, respectively. Because of their striped appearance under the microscope, skeletal and heart muscles are classified as striated muscles. On the other hand, smooth muscles show no striped appearance and can be found in lining hollow organs (stomach, intestine, uterus), as well as the vessel walls (arteries and veins), the skin and in ciliary muscles of the eye.

The sarcomere is the smallest repeating contractile unit of striated muscles which gives them the striped appearance because of their end-to-end alignment. Actin and myosin, the two main

filamentous proteins in sarcomeres, along with a network of cytoskeletal accessory proteins confer the main properties of striated muscles (Clark et al., 2002). Many sarcomeric proteins and their tight regulation have been discovered during the past decades; recent advances in microscopy and molecular techniques provided the opportunity to uncover the role of each component.

In the introduction chapter, I will focus on sarcomere components, their functions, and their role in sarcomere assembly and maintenance. Furthermore, I discuss Alp/Enigma as structural scaffolding proteins both in vertebrates and *D. melanogaster*.

1.2 Muscle structure

Skeletal muscles are composed of numerous long, cylindrical, and multinuclear fibers. The muscle fibers (myofiber) are 20 to 100 µm in diameter. Each myofiber is made of many 2 to 5 µm diameter myofibrils. Myofibrils consist of aligned sarcomeres. In vertebrates, the length of a sarcomere in resting muscles is approximately 2.2 to 2.3 µm. The sarcomere is flanked by two repeating Z-discs (Squire et al., 2005). (Figure 1.1). Z-discs are multi-protein complexes that serve as lateral boundaries of the sarcomere. The striated appearance of skeletal muscle is related to repeating dark and light bands in sarcomere structure. The dark band (A-band) mainly contains myosin thick filaments which are anchored to the M-line; the light band (I-band) which is shared between two adjacent sarcomeres contains actin thin filaments which are anchored to the Z-disc. In addition to actin and myosin as two main filamentous proteins in sarcomere structure, gigantic titin and nebulin filaments play an essential role in muscle contraction; the

gigantic titin polymer, the third most abundant protein after actin and myosin spans half of the sarcomere and nebulin, an actin-binding protein, spans the entire length of actin. There are many other different accessory proteins especially in the Z-disc and M-line which tightly regulate filament function; altogether protein complexes control many aspects of striated muscle function within the sarcomere (Mukund & Subramaniam, 2020).

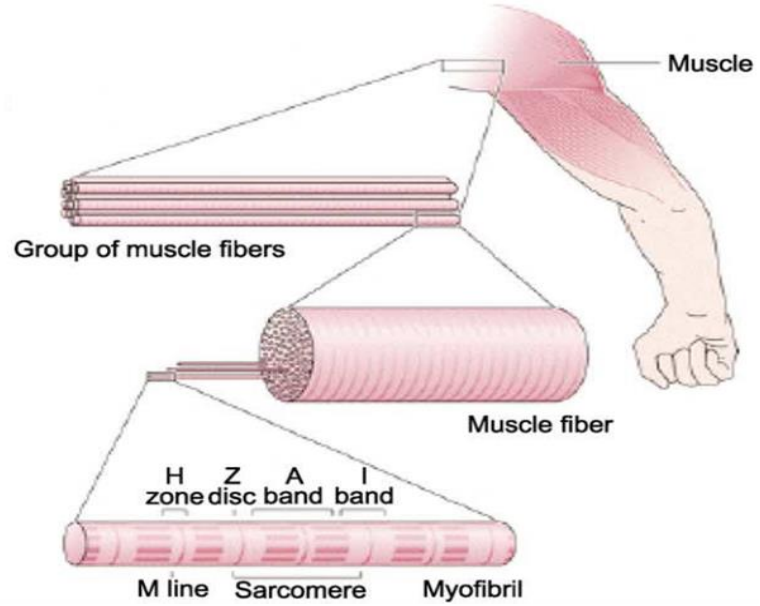


Figure 1. 1. Schematic figure illustrating the hierarchy of muscle organization (Squire et al., 2005).

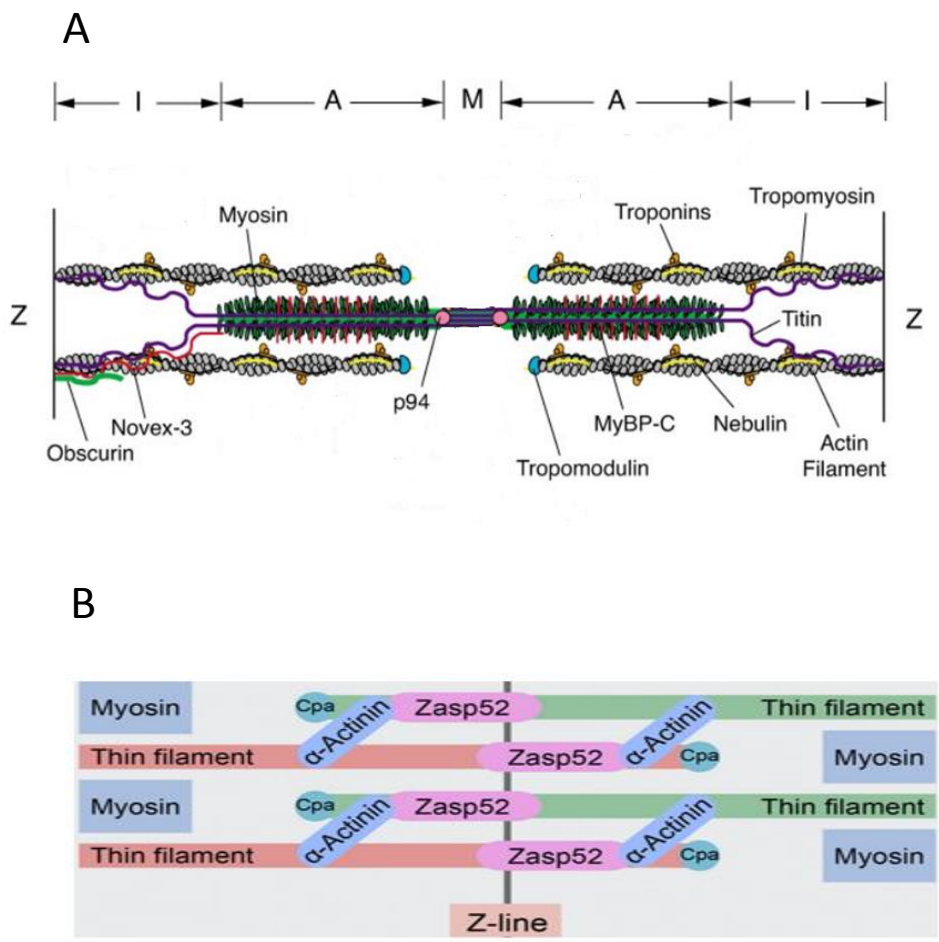


Figure 1. 2. Schematic representation of skeletal muscle sarcomere.

(A) Thick and thin filaments, as well as titin, tropomyosin, troponins, and obscurin are shown. The sarcomere is confined between two Z-discs; I-band, A-band, and M-line are shown (Clark et al., 2002). (B) Z-disc arrangement of CapZ, α-actinin and Zasp52 (a Zasp PDZ domain protein in *Drosophila*) (Szikora et al., 2020).

1.3 Molecular components of sarcomere structure:

1.3.1 Actin: thin filament

Actin is one of the most important cytoskeletal proteins in all eukaryotic cells. It drives several pivotal functions in the cell such as vesicle and organelle movement, cell signaling, and cytokinesis, maintenance of cell shape and polarity, and muscle contraction. The actin monomer (G-actin) is a 42 kD protein that generally consists of one large and one small domain separated by a cleft. Each domain further consists of two subdomains; the small domain composed of subdomains 1 and 2, and the large domain composed of subdomains 3 and 4. Actin monomer binds to an adenine nucleotide (ATP or ADP) and a divalent cation (calcium or magnesium) through the cleft between subdomains 2 and 4 which is called the pointed (-) end (head); the opposite side of the actin monomer, the exposed area between subdomains 1 and 3 is termed barbed (+) end (tail) (Kabsch et al., 1990; Pollard & Cooper, 1986). Under physiological conditions, actin monomers are assembled head-to-tail to form a right-handed helix called filamentous or F-actin. Actin polymerization occurs in three steps, nucleation (lag phase), elongation (growth phase), and steady-state (equilibrium phase). Because all monomers are in the same direction, the head-to-tail assembly of asymmetrical actin monomers gives polarity to the entire actin filament. This polarity is pivotal for the asymmetrical organization of the cell. During the polymerization, the two distinct ends grow at different rates, so that the barbed end grows faster than the pointed end (Fehon et al., 2010; Pollard & Cooper, 2009).

Actin is evolutionarily highly conserved among species. In vertebrates, there are six different actin isoforms, each encoded by separate genes. Both β- and γ- cytoplasmic actin isoforms as components of the non-muscle cytoskeleton are ubiquitously expressed. Four muscle isoforms, $\alpha_{skeletal}$-actin, $\alpha_{cardiac}$-actin, α_{smooth}-actin, and γ_{smooth}-actin, are expressed in skeletal, cardiac, and smooth muscle (Perrin & Ervasti, 2010). In *D. melanogaster*, six actin isoforms are encoded by six different genes. Actin 5C and Actin 42A are cytoplasmic isoforms; whereas the other four isoforms are differentially expressed in muscles; Actin 57B (body wall muscle), Actin 79B (thorax/ leg muscle), Actin 87E (body wall muscle), and Actin 88F (indirect flight muscle) (Röper et al., 2005). Despite a high similarity among different actin isoforms (93-99%) especially in muscles, they differ in function (Khaitlina, 2001). For example, *D. melanogaster* Act88F is expressed only in the indirect flight muscle (IFM), and differs in 15 amino acids from *H. sapiens* β-actin. The ectopic expression of *H. sapiens* β-actin in a *D. melanogaster* Act88F null mutant is unable to rescue sarcomere organization (Brault et al., 1999).

In striated muscle, CapZ is an important protein in Z-discs. The barbed end of actin filaments is anchored to the Z-disc and capped by CapZ, which prevents the disassembly of actin subunits. The pointed end of actin filaments is positioned toward the M-line and capped by tropomodulin. The myosin movement along the actin filament depends on actin polarity. Regulation of filament length by several important sarcomere proteins including titin, nebulin, CapZ, and tropomodulin is essential for the contractile function of the sarcomere.

1.3.2 Myosin: thick filament

Myosins are a large superfamily of motor proteins involved in different fundamental cellular functions of both non-muscle and muscle cells, like cytokinesis and contraction. Different myosin family proteins are expressed in eukaryotes, but only class ll myosin is expressed in skeletal and cardiac muscle cells (Sellers, 2000). A single myosin molecule is composed of two heavy chains interact with two pairs of myosin essential light chains and myosin regulatory light chains to form a hexamer (Weeds & Lowey, 1971). Each heavy chain consists of a globular head domain in the N-terminus and a very long rod domain. The head domain [subfragment-1 (S1)] or motor domain binds to the actin thin filament mediated by ATP hydrolysis. ATP hydrolysis provides the required energy for a conformational change in myosin head domain and finally leads to force generation in muscles. The rod domain is subdivided into subfragment-2 (S2) and light meromyosin (LMM). S2 with a coiled-coil structure serves as a linker between S1 and LMM. LMM positioned at the C-terminus of myosin heavy chain is important for myosin polymerization (Miller et al., 2009; Suggs et al., 2007).

In striated muscles, the bundling of many myosin ll molecules forms thick filaments. Each myosin ll molecule consists of a pair of myosin heavy chains (MHC), essential light chains, and regulatory light chains (MLC). There are multiple MHC and MLC isoforms, which can combine with each other to form different isomyosins with distinct functionality. Isomyosins are different isoforms of myosin. In mammals, there are eight MHC isoforms, each encoded by a separate

gene (Reggiani et al., 1997). In *D. melanogaster*, alternative splicing of a single MHC gene produces a diversity of specific MHC isoforms (George et al., 1989).

At the center of the thick filament, myosin filaments form an antiparallel bundle, while they form in a parallel fashion in the rest of the thick filament; therefore, a bipolar thick filament is formed, leaving a central bare zone in the middle (Ojima, 2019). The head domains point toward the Z-disc and interact with actin thin filaments, while the tails in the middle crosslink to several M-line proteins, such as the gigantic titin protein.

1.3.3 Titin

Titin is the largest protein known to date (M_r of a single polypeptide, 3-4 MDa) and the third most abundant muscle protein after actin and myosin. The N-terminal part of titin spans the entire Z-disc and overlaps the titin filament from the adjacent sarcomere within the Z-disc. A substantial proportion of titin (between 0.8 and 1.5 MDa, depending on the isoform) cross the I-band. The central part of I-band titin (known as elastic region) is composed of immunoglobulin-like (Ig) repeats which are divided into two separate proximal and distal Ig segments. Both Ig segments are separated by amino-acid residues rich in P (prolin), E (glutamate), V (valine), and K (lysine). This elastic region of I-band titin is known as the "PEVK domain". The C-terminal part of titin which binds to myosin thick filaments in the A-band is composed of regular patterns of Ig-like and fibronectin type lll (FN lll) repeats. The final C-terminal segment of titin that contains a kinase domain ends at the M-line. Titin filaments from adjacent half-sarcomeres fully overlap within the M-line. As a result, in vertebrates, the titin molecules form a continuous

filament system apart from actin and myosin filaments (Gregorio et al., 1998; Labeit & Kolmerer, 1995; Obermann et al., 1997). Titin shows a variety of functions depending on its sarcomeric localization. For instance, the titin N-terminus directly interacts with α-actinin (an actin cross-linker) within the Z-disc; and plays an important role in sarcomere maintenance. Furthermore, the interaction between titin and myosin thick filaments within the A-band contributes to thick filament assembly. Finally, the kinase domain of titin within the M-line is involved in the sensing of mechanical load (Clark et al., 2002; Sorimachi et al., 1997). In addition, the protein kinase domain interaction with the zinc-finger protein nbr1 through a mechanically inducible conformation leads to reduction of a nuclear transcription factor (SRF) and repression of transcription. A human mutation in the titin protein kinase domain disrupts this pathway and causes a hereditary muscle disease (Lange et al., 2005).

In vertebrates, there is only a single titin gene, but many distinct isoforms are generated by multiple splice pathways (Alexandra et al., 2000). In *D. melanogaster* titin proteins are encoded by three genes: *sallimus* (Sls), *bent* (projectin), and *stretchin-MLCK*. Kettin is the most abundant Sls isoform expressed in adult IFM. Kettin is a 527 kDa protein that spans from Z-disc to the I-band with the C-terminus attached to the end of thick filaments. Projectin spans the A-band and contains a kinase domain at the C-terminus. Stretchin-MLCK is also an A-band protein with a kinase domain (Alexandra et al., 2000; Champagne et al., 2000; Lakey et al., 1993).

1.3.4 α-actinin

α-Actinin, an actin-crosslinking protein, belongs to the spectrin superfamily along with spectrin and dystrophin. α-Actinin consists of an actin-binding domain (ABD) at the N-terminus, spectrin repeats (SR) in the middle forming a rod, and a C-terminal calmodulin-like domain (CaM). The ABD is composed of two consecutive calponin homology (CH1 and CH2) domains that are not functionally identical. Although the CH1 domain alone can loosely bind actin filaments, optimal binding affinity is only achieved by the tandem CH domains. The CH2 domain increases the binding affinity of the CH1 domain to the actin filaments (Gimona et al., 2002; Stradal et al., 1998). The central rod domain consists of four consecutive spectrin repeats and forms an antiparallel homodimer in α-actinin structure with an ABD at each end. As a result, this structure is suitable for cross-linking two adjacent actin filaments. The CaM-like domain of the dimer is composed of four EF-hand motifs involved in calcium binding. Also, EF-hands have a regulatory role on the actin-binding property of adjacent ABD and the other proteins (Ribeiro Jr et al., 2014; Tang et al., 2001) (Figure 1.3).

In mammalian cells, there are four α-actinin encoding genes that produce different isoforms. Based on expression patterns, biochemical characteristics, and tissue and subcellular location, these isoforms are categorized into two classes: muscle (calcium-insensitive) and non-muscle (calcium-sensitive) isoforms. Non-muscle isoforms (α-actinin 1 and 4) are expressed in different cell types and associated with focal adhesions and stress fibers. In contrast, the expression of muscle isoforms (α-actinin 2 and 3) is largely limited to muscles and localized in Z-discs. α-Actinin 2 is found in cardiac and oxidative skeletal muscles, while α-Actinin 3 is predominantly expressed in glycolytic skeletal muscles (Mills et al., 2001). During the evolution of α-actinin, different isoforms show distinct calcium sensitivity. Non-muscle isoforms contain calcium-sensitive EF-hands; upon calcium binding, the binding of non-muscle isoforms to actin filaments

is inhibited. Muscle isoforms (2 and 3) are insensitive to calcium, and their binding to actin filaments is regulated by the binding of EF-hand motifs to phospholipids (Sjöblom et al., 2008).

In *D. melanogaster*, there is a single α-actinin gene whose primary sequence is substantially similar to vertebrate muscle α-actinin. This gene encodes three isoforms by alternative splicing; two muscle isoforms (107 kD and 104 kD) and one non-muscle isoform (104 kD) (Roulier et al., 1992; Vigoreaux et al., 1991). *D. melanogaster* α-actinin is localized to Z-discs. Null mutations in the *D. melanogaster* α-actinin gene are lethal and exhibit severe defects in sarcomere assembly, but do not affect embryonic development and mutants survive to larval stages; it suggests that α-actinin is a dispensable component for initial sarcomere assembly, and instead, is required for stabilizing filament integrity when larval muscle contraction starts (Fyrberg et al., 1990). This can be explained by the presence of other actin filament cross-linkers than α-actinin which can fulfill the demands of α-actinin at the embryonic stage.

To date, many Z-disc components have been identified as α-actinin interacting proteins. One of the most important classes of these proteins are Alp/Enigma family proteins, which will be discussed next.

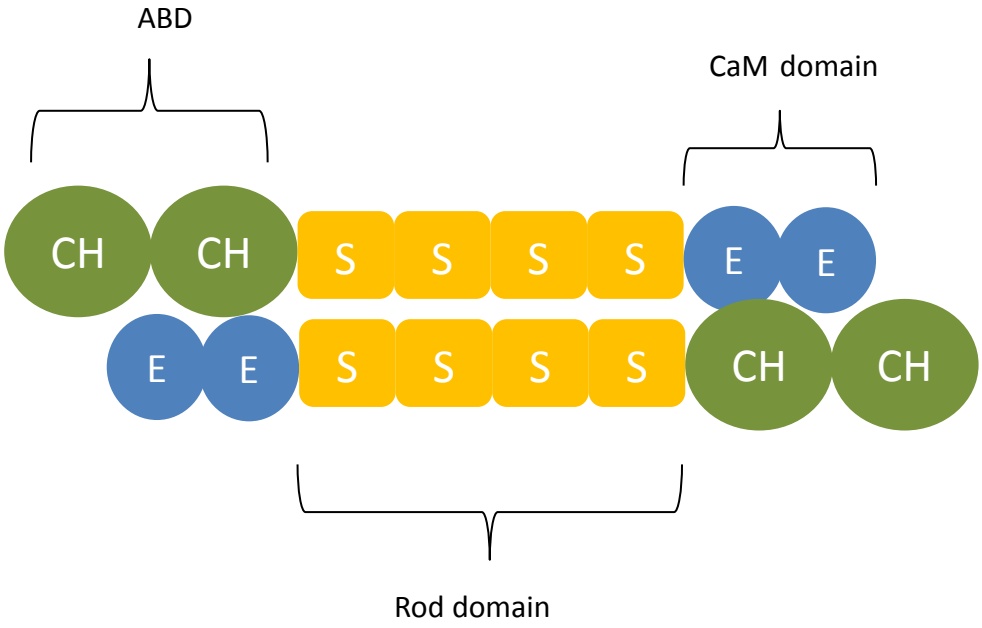


Figure 1. 3. The structure of α-actinin dimer

α-Actinin is an antiparallel homodimer consisting of an N-terminal ABD composed of two CH domains, four spectrin-like repeats that form a rod domain in the middle, and a CaM domain at the C-terminus with two pairs of EF-hand motifs.

1.4 Alp/ Enigma family proteins

The Alp/Enigma family is a new class of cytoskeletal proteins involved in a broad range of biological functions such as bone formation, platelet and epithelial cell motility, signal transduction, and tumor growth. Each Alp/Enigma family member has multiple splice variants and unique expression patterns; there are several muscle-specific Alp/Enigma family members that localize to the Z-discs and play key roles in sarcomere assembly and Z-disc maintenance (Jani & Schöck, 2007; Sheikh et al., 2007). In mammals, the Alp/Enigma family is divided into two subfamilies. The Alp subfamily includes Alp, CLP36, RIL, and Mystique. Each member contains one N-terminal PDZ domain and one C-terminal LIM domain. The Enigma subfamily consists of Enigma, ENH, and Cypher (mouse)/ZASP (human) which contains one N-terminal PDZ domain and three C-terminal LIM domains (Zheng et al., 2010). There is an internal conserved sequence between PDZ and LIM domains in Alp, CLP36, and Cypher/ZASP known as ZASP-like motif (ZM motif). The area surrounding the ZM motif interacts with spectrin repeats of α-actinin in some Alp/Enigma proteins (Klaavuniemi et al., 2004; Klaavuniemi & Ylänne, 2006; te Velthuis & Bagowski, 2007), but not in others (Liao et al., 2016). More recently, the ZM motif has been shown to interact with the LIM domains of Zasp52 (González-Morales, Xiao, et al., 2019). Although the structure of the ZM motif remains to be fully determined, both PDZ and LIM domains properly fold into a well-characterized structure.

1.4.1 PDZ domain

Postsynaptic density 95, discs large and zonula occludens-1 (PDZ) is one of the most common protein-binding motifs found in bacteria, yeast, and animals. The PDZ domain is composed of 80-90 amino acid residues with a highly conserved GLGF sequence at the N-terminus. It contains six β-strands and two α-helices forming a globular structure with a hydrophobic protein-binding interface for ligand binding. PDZ domains often bind to specific peptide motifs at the C-terminus of partner proteins, although PDZ domain-mediated recognition of internal motifs has been suggested (Jemth & Gianni, 2007; Ponting & Phillips, 1995). Since PDZ domains are often found in combination with other protein-binding domains (such as LIM) within the same polypeptide, the PDZ domain-containing proteins are able to form multi-protein complexes with a diversity of biological functions including the organization of postsynaptic density, maintenance of epithelial cell polarity and morphology, and organization of cell architecture (Harris & Lim, 2001; Ivarsson, 2012).

1.4.2 LIM domain

The LIM domain serves as a protein-binding interface (similar to the PDZ domain) in all eukaryotes. The name of the LIM domain comes from the first letters of three regulatory transcription factors: Lin-1 (a cell lineage protein), Isl1 (insulin enhancer-binding protein), and Mec3 (a mechanosensory neuron differentiation protein). LIM domains are approximately

composed of 55 amino acid residues and include highly conserved and spatially defined cysteine and histidine residues; these residues form a double-zinc finger motif that coordinates the binding of two zinc ions. The remaining sequence of LIM domains is highly variable and gives individual LIM domain functional specificity. LIM domains can be found in proteins that consist exclusively of LIM domains, or in conjunction with other domains. A single LIM domain can bind to the target protein; it can also interact with several targets simultaneously. Moreover, it has been shown that more than one LIM domain cooperate to bind to a single protein partner. There is a range of binding partners for the LIM domain regarding the localization of LIM domain-containing proteins. The majority of LIM domain-containing proteins are located in the cytoplasm and involved in signal transduction, actin organization, and integrin-dependent adhesion. The nuclear LIM domains bind to transcription factors and are involved in cell-fate determination. Finally, a small number with nuclear localization (NLS) or export (NES) sequences shuttle between nucleus and cytoplasm and their function remains to be exactly determined (Kadrmas & Beckerle, 2004; Krcmery et al., 2010; Li et al., 2012). It has been recently proposed that tandem LIM domains, for example in the Enigma subfamily, recognize an F-actin conformation that is enriched in the presence of mechanical stress, like actin filaments in stress fibers (Winkelman et al., 2020).

1.5 Alp/Enigma family in *D. melanogaster*

In *D. melanogaster*, the Alp/Enigma family consists of three members: Zasp52, Zasp66, and Zasp67. Zasp52 contains a PDZ domain, a weakly conserved Zasp-like motif (ZM), and four LIM domains; while Zasp66 and Zasp67 lack LIM domains (Figure 1.4). All Zasp members are required for adult Z-disc stability and maintenance. Zasp52 was identified in RNAi screening for genes that prevent integrin-dependent cell spreading in *D. melanogaster* cell lines (Jani & Schöck, 2007). The *Zasp52* gene locus contains 20 predicted exons, and more recently it has been shown that the *Zasp52* gene encodes 21 splice variant isoforms (Gramates et al., 2017). Zasp52 localizes to the Z-disc and contributes to Z-disc assembly by recruiting α-actinin. It also co-localizes with integrins at myotendinous junctions and is required for muscle attachment (Katzemich et al., 2011). Although primarily characterized in muscle tissues, Zasp52 is expressed in a variety of non-muscle tissues including salivary glands, central nervous system, somatic follicle cell layer, and epithelial tissues. In migrating cell populations, Zasp52 accumulates in the leading edge of the cell; also, in epithelial cells, Zasp52 localizes apically to adherens junctions and basally to the integrin adhesion sites. These expression patterns show that Zasp52 potentially plays important roles in cell-cell and cell-matrix adhesion as well as actomyosin contractility, reflecting its role in sarcomere assembly (Stronach, 2014). The vertebrate ortholog ZASP/Cypher localizes to Z-discs, interacts with α-actinin by its PDZ domain, and is involved in Z-disc maintenance. Defects in human ZASP induce myofibril myopathies and cardiomyopathies (Sheikh et al., 2007; Shieh, 2013).

Zasp66 was first identified with a GFP protein trap line expressing endogenous GFP-tagged Zasp66 (Hudson et al., 2008). The *Zasp66* gene encodes 12 splice variant isoforms. During a

duplication event, Zasp66 arose in primitive insects, which was identical to Zasp52 in terms of localization and expression; both Zasp52 and Zasp66 are co-expressed during embryonic, larval, and adult muscle development. Furthermore, similar to Zasp52, Zasp66 directly interacts with α-actinin and co-localizes with α-actinin to the Z-discs; in fact, they form a ternary structure essential for myofibril assembly and maintenance (González-Morales, Marsh, et al., 2019; Gramates et al., 2017; Katzemich et al., 2013). Zasp67 orthologs encode highly variable proteins. It has been formed from a more recent duplication often found in higher insects. Zasp67 function and expression are restricted to indirect flight muscles (IFMs), and along with Zasp52 co-localizes to the Z-discs. The expression of Zasp67 is mainly restricted to the late pupal stage when IFMs develop; suggesting the importance of Zasp67 in IFM maintenance rather than the sarcomere assembly during pupal development (González-Morales, Marsh, et al., 2019). It has recently been suggested that Zasp growing (with multiple LIM domains) and Zasp blocking isoforms (without LIM domains) together contribute to determine Z-disc and thereby myofibril diameter via protein oligomerization. The interaction of the ZM domain with LIM domains induces oligomerization, whereas the upregulation of blocking isoforms lacking LIM domains terminates the oligomerization at late developmental stages. An imbalance of growing and blocking Zasp isoforms results in formation of aggregates as a hallmark of genetically induced myofibrillar myopathies (González-Morales, Xiao, et al., 2019; Kley et al., 2016). More recently it has been shown that the extended PDZ domain and ZM motif of Zasp52 acts as an actin-binding site. Zasp52 binds to monomeric actin; also, a co-sedimentation assay showed that Zap52 bind to F-actin (Liao et al., 2020).

A


Drosophila Zasp52
PDZ
ZM
LIM1
LIM2
LIM3
LIM4
780 aa
Drosophila Zasp66
PDZ
ZM
298 aa
Drosophila Zasp67
PDZ
ZM
725 aa

B

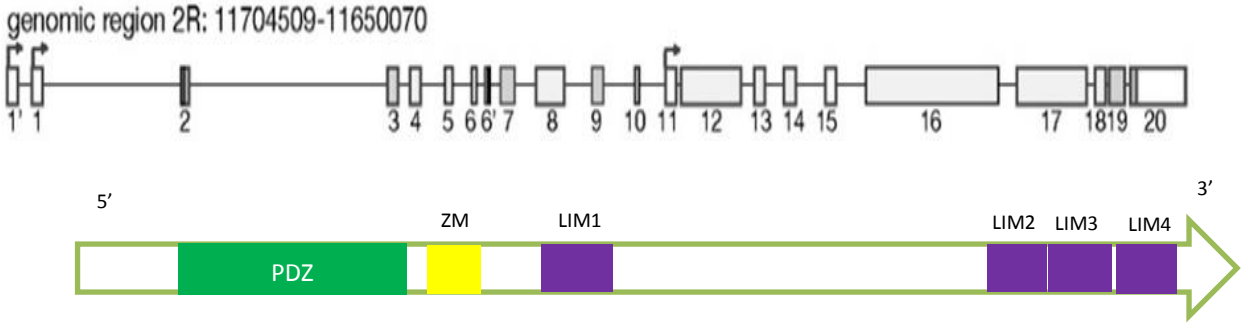

genomic region 2R: 11704509-11650070
1' 1 2 3 4 5 6 6' 7 8 9 10 11 12 13 14 15 16 17 18 19 20
5'
3'
PDZ
ZM
LIM1
LIM2
LIM3
LIM4

Figure 1. 4. Schematic representation of Zasp52, Zasp66, and Zasp67

(A) Zasp52 with a PDZ domain, a Zasp-like motif, and four LIM domains. Zasp66 mainly consists of a PDZ domain and a ZM motif. Finally, Zasp67 as the last member of the Alp/Enigma family proteins in *Drosophila* is composed of a PDZ domain and a weakly conserved ZM motif. The numbers represent the amino acid length of one representative protein isoform. (B) Zasp52 gene model. On top, genomic region of Zasp52. The exons are indicated by numbers. Arrows indicate alternative start sites. Below, protein domains of the most common isoform (Katzemich et al., 2011).

1.6 *D. melanogaster* IFM

The molecular components of sarcomere structure, as well as the cellular mechanisms involved in myofibril assembly, are highly conserved during the evolution of species (Steinmetz et al., 2012). Therefore, *D. melanogaster* is a suitable *in vivo* model system for studying muscle development and function, as it has a smaller genome and shorter muscle developmental time than mammals and other model systems. Both cardiac and skeletal muscles are categorized as striated muscles (composed of sarcomeres and similar protein components), and the contraction mechanisms of these two muscle types are very similar, and similarly both contract in response to an action potential on sarcolemmal membrane. Therefore, because of these and other similarities studying IFMs as a good model can give us useful perspective about both skeletal muscle disorders and cardiomyopathies. *D. melanogaster* indirect flight muscles (IFMs), corresponding to vertebrate skeletal muscles, are a very powerful model. IFMs are dispensable

for survival. Defects in the development and assembly of IFMs lead exclusively to flight defects. Either severe or mild defects can easily be detected using a variety of assays; for instance, stronger mutations in key sarcomere components lead to the emergence of flightless flies, or milder defects like an imbalanced expression of actin-crosslinking proteins leads to a difference in wing beat frequency (Liao et al., 2016). Furthermore, the presence of IFM-specific isoforms in some muscle structural proteins (like Zasp67), reveals the importance of IFMs to study the flight muscle function in the absence of these isoforms without affecting the viability of the fly (Nongthomba et al., 2004; Vigoreaux, 2001). IFM occupies the major volume of the thorax, and is the main powering source during flight. IFM consists of two sets of muscles: the dorsal-ventral muscles (DVMs) oriented in a dorsal-to-ventral manner, and dorsal-longitudinal muscles (DLMs) spanning the thorax from posterior to anterior. The DVMs are further divided into DVM I (3 fibers), DVM II (2 fibers), and DVM III (2 fibers). DLMs and DVMs develop differently; DVMs are formed *de novo* by fusion of myoblasts in the pupal stage, similar to vertebrate muscles. In contrast, in the larval stage, DLMs develop by fusion of myoblasts from pre-existing templates called larval oblique muscles (LOMs). Myoblasts migrate toward the three LOMs as a template, and the developing fibers split to generate 6 primitive DLM fibers (Farrell et al., 1996; Fernandes & Keshishian, 1996; Guruharsha et al., 2009).

1.7 Alp/Enigma family-related myopathies

Myopathy is a general term referring to a group of disorders that affect muscles; the main symptoms include muscle weakness and dysfunction. Myopathies are categorized into two

groups; acquired and inherited. The inherited myopathies consist of several types and mutations in a broad range of Z-disc proteins can lead to a diverse group of muscle abnormalities (Knöll et al., 2011). Defects in the Alp/Enigma family as a prominent class of Z-disc protein components cause several cardiomyopathies and myofibrillar myopathies (MFM). MFM is a disorder characterized by disintegration of Z-discs as well as myofibrils followed by abnormal accumulation of protein products. ALP knock-out mice show a pronounced right ventricular cardiomyopathy (Pashmforoush et al., 2001). Cypher knockout mice (mouse Zasp ortholog) die shortly after birth because of severe congenital myopathy with symptoms that include muscle weakness and cardiomyopathy. The skeletal and cardiac muscles of these mice were severely disorganized with discontinuous Z-discs (Zhou et al., 2001). Moreover, mutations in the ZASP gene (human ortholog) lead to dilated and/or hypertrophic cardiomyopathy (Arimura et al., 2004; Vatta et al., 2003).

1.8 Rationale for experiments

Zasp52 mutants die at the embryonic and larval stage, and, it has been suggested that, they do so because Zasp52 mutants fail to recruit α-actinin to the Z-disc leading to disorganized myofibril assembly. All Zasp genes have complex loci with multiple splice variants. Although double mutants have been studied in mammals (Mu et al., 2015), with seven family members there is still significant redundancy. In *Drosophila*, only hypomorphic mutants were made for the most important gene, Zasp52. Also, RNAi experiments and CRISPR mutants in indirect flight muscles

indicate that Zasp proteins play redundant roles. We have previously analyzed very useful alleles of Zasp52, which separately affect the PDZ domain or the LIM domains because Zasp52 has internal transcription start and stop sites. Our previously generated alleles are not null alleles. Therefore, generation and analysis of a *Zasp52* null mutant is important to evaluate the actual function of this protein and its relationship with α-actinin.

Diverse evidence implies the direct binding between Alp/Enigma family members and α-actinin. Human ZASP directly binds to α-actinin through the PDZ domain; also, the PDZ domain of mouse Cypher is responsible for the direct binding to α-actinin. It has been shown that Zasp52 binds to α-actinin via its PDZ domain. The PDZ domain of Zasp52 has a conserved motif (PWGFRLxGGxDFxxPL) crucial for α-actinin binding. However, the PDZ domain can only bind α-actinin weakly and an extended C-terminal sequence is necessary for optimal binding. Point mutations in the PWGFRL motif completely abolish α-actinin binding without affecting protein stability. Therefore, the C-terminal PDZ extension only enhances α-actinin binding affinity however does not provide any α-actinin binding aspects on its own. A 142 amino acid-long Zasp52 PDZ extended domain shows optimal binding affinity to α-actinin, but the shorter 111 amino acid-long protein very weakly binds to α-actinin. Previous work demonstrated the binding of the 142 amino acid-long extended PDZ domain of Zasp52 to full-length rabbit α-actinin (Liao et al., 2016). Here, we set out to narrow down the extent of the Zasp52 PDZ domain extension required for binding. We were seeking to test if binding also occurs to *Drosophila* α-actinin. Furthermore, we intended to test if the EF-Hand domain of *D. melanogaster* α-actinin is sufficient for binding to Zasp52.

This thesis investigates:

1. The generation and confirmation of a *Zasp52* null mutant using specific CRISPR mutants
2. The analysis of the IFM phenotype of a *Zasp52* null mutant as well as a *Zasp52* 5'3'FRT mutant
3. The biochemical interaction of different Zasp52 extended PDZ domains with the EF-Hand domain of *D. melanogaster* α-actinin

2. Results

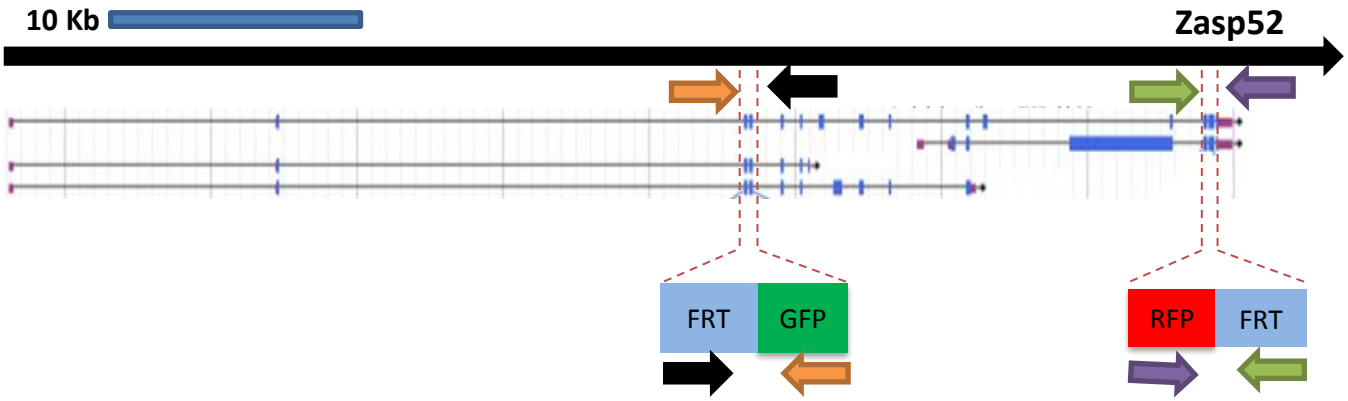
2.1 Generation of *Zasp52* null mutant from a novel CRISPR mutant (*Zasp52* 5'3'FRT)

We have previously analyzed IFM defects of Zasp52 using some useful alleles (Liao et al., 2016). The most important allele Zasp52^{MI02988} disrupts most splice isoforms by inserting a stop codon after exon number 2 and truncates Zasp52 inside the PDZ domain. However, because of

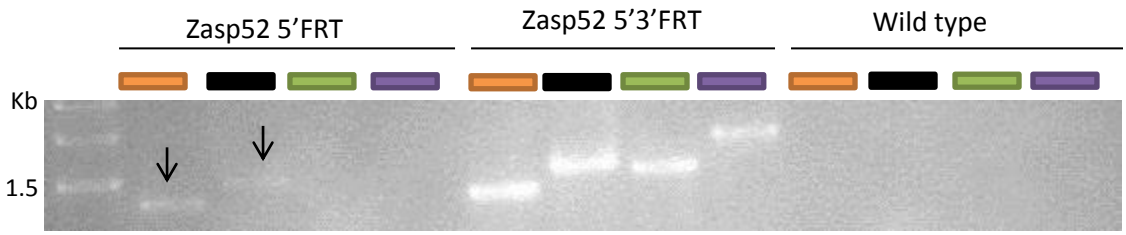
the presence of residual proteins and unaffected isoforms (like LIM-only isoforms), none of these alleles are close to a null. In order to further evaluate the actual function of Zasp52 as well as its relationship with α-actinin, we were aiming to generate a *Zasp52* null mutant. The *Zasp52* locus is very large (≈50 kb). Thus, to obtain a null mutant in Zasp52 we performed two rounds of CRISPR; the first one removing exons 3 and 4 which leads to PDZ domain deletion (Zasp52 5' FRT), and the second round disrupting exons 18 to 20 which removes the last three LIM domains (Zasp52 5'3'FRT) (exons numbered according to (Katzemich et al., 2011)). We simultaneously inserted Green Fluorescent Protein (GFP) at the 5'-side and Red Fluorescent Protein (RFP) at the 3'-side of the Zasp52 locus, each one flanked by Flippase Recognition Target (FRT) sequences at the deletion sites by homology-directed recombination (HDR) (Fig 2.1A). We used the fluorescence emission of both GFP and RFP to distinguish Zasp52 5'3'FRT flies from wild type, and Zasp52 5'FRT flies which only carry GFP (both GFP and RFP have their own promoters that are separate from Zasp52 gene promoter). To confirm the correct location of the cassette insertion genomic PCRs were performed (Fig 2.1B). Both Zasp52 5'FRT and Zasp52 5'3'FRT mutants show severe abnormalities in flying ability. Homozygous Zasp52 5'FRT is almost flightless; in fact the flies can barely beat their wings. On the other hand, Zasp52 5'3'FRT homozygotes are completely flightless, consistent with disruption of the majority of Zasp52 splice isoforms. Finally, with several steps of standard genetic crosses I used FLP/FRT mediated recombination to remove GFP, RFP and about 20 kb of intervening sequence (Fig 2.1C). I crossed Zasp52 5'3'FRT to flies with balancer chromosomes which carry fillipase (FLP) under control of a heat-shock promoter, then using heat-shock in 37◦C in water bath, I activated the FLP reaction. Finally, I screened the progeny for the absence of GFP and RFP. I confirmed the deletion by PCR as well as sequencing (Fig 2.1D, Fig 2.1E). Only a small number

of amino acids of the PDZ domain (the first 36 amino acids) and LIM4 domain (the last 6 amino acids) are remaining in this mutant. Therefore, I have generated a null mutant in Zasp52 for the first time. Although homozygous mutants cannot survive, they are still viable and flightless over a small deficiency (Zasp52 null/Df(2R)BSC427); one possible explanation is because of the presence of a lethal second-site mutation.

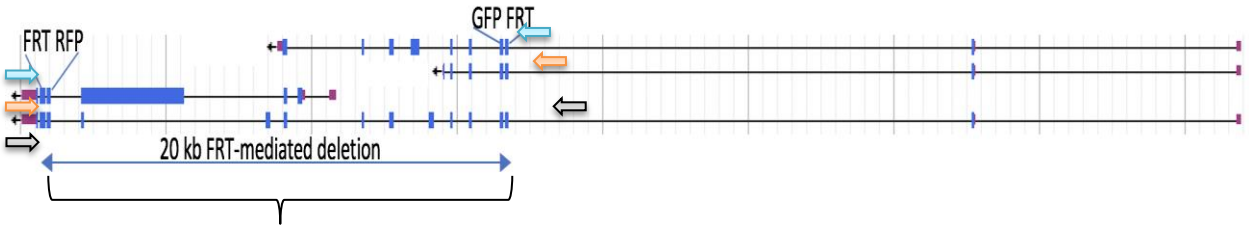
A



B

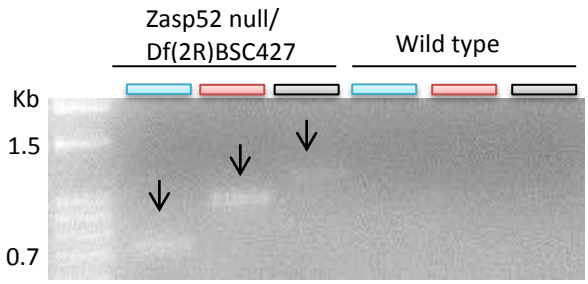


C



GFP, RFP, and 20kb intervening sequences are removed

D



E

aaataaatgatgaaaacaaacgcccaaaacgcacacaaagcagcgcctataaaactaaactttggccaacagttgcgggccacaaaaaacacagaaatatgcgtatatg

tatgtatatatctttatctgcatgtttcgatggacatgtgggatatacgtagtcggctaaagtcagcgcgagtttggaactcggagtgccgctcataatttttgcagttg

cagcataataaattttaaacccagttcggccaactaggcattttcagggtagtgagttatggccatggcaaatcgatgttgagttgagtaatggttact/GA

AGTTCCTATTCtctagaaaGtATAGGAACTTCgcaagaatcacgcgcgctaagccgcaactcagatgattttgttcgacttcagggat

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tgaataatgcgtaactattattatttctatttgtttttgaattgtgcacacaaatgttatcctaattttaactgNNNcgatcatcatcgattcgctcaaaatcaaa

Yellow_ 5’ side of the deletion

Green_ 3’side of the deletion

/_ break point

Purple_ FRT sequence

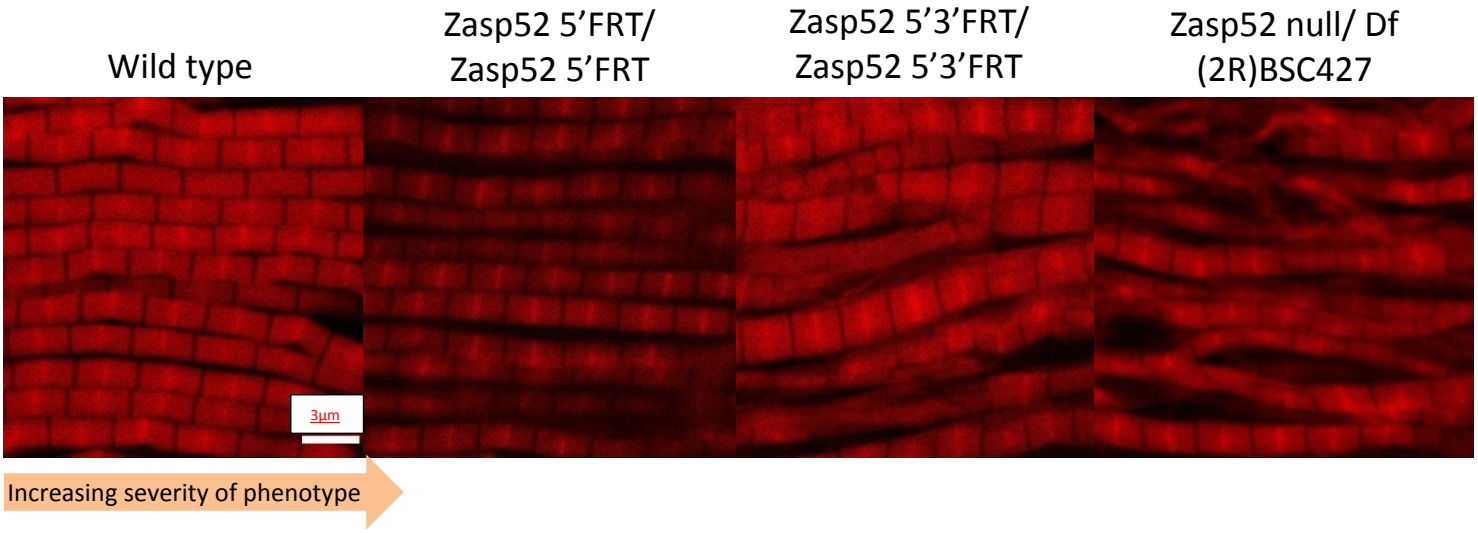
Figure 2. 1. Zasp52 null mutant is generated using Zasp52 5'3'FRT CRISPR mutant.

(A) Cartoon of Zasp52 genomic locus with selected transcripts. Both 5'-side and 3'-side gRNA-targeted break points are shown by red dotted lines. The two replacement cassettes are shown at the bottom. The primer sets used for validation of Zasp52 5'FRT and Zasp52 3'FRT are shown in different colors. (B) Ethidium bromide-stained agarose gel of PCR products from wild type flies as well as Zasp52 5'FRT and Zasp52 5'3'FRT homozygous CRISPR mutants. The colored lines correspond to the primer pairs drawn in (A). (C) Zasp52 genomic locus with four representative transcripts. N-GFP-FRT and C-FRT-RFP CRISPR mutations as well as deletion after FLP/FRT-mediated excision are shown. The primer sets used for validation of Zasp52 null mutant are shown in different colors. (D) Ethidium bromide-stained agarose gel of PCR products from wild type flies as well as Zasp52 null/Df(2R)BSC427 mutant. The colored lines correspond to the primer pairs drawn in (C). (E) The sequencing results of break point site of Zasp52 null mutant.

2.2 Zasp52 null mutant shows severe IFM defects

In order to obtain a better understanding about the structural defects of myofibrils, I analyzed the indirect flight muscles (IFMs) in our different Zasp52 mutants using confocal microscopy. Although the myofibrils of wild type IFMs are highly organized structures with similar sarcomere length and width, there are different levels of IFM abnormalities in Zasp52 mutants. Zasp52 null/Df(2R)BSC427 mutant shows the most severe defects in IFM structure, many myofibrils are split and the majority of the remaining myofibrils are wavy and frayed. The myofibrils of homozygous Zasp52 5'3'FRT are also intensively damaged; ranging from split and wavy myofibrils to extended Z-discs. Z-discs are sometimes bent and broken. In contrast, the IFM phenotype is milder in homozygous Zasp52 5'FRT. There are less obvious defects in myofibrils; Z-discs are sometimes absent (Fig 2.2A). I finally quantified the ratio of abnormal to total myofibrils in wild type as well as Zasp52 5'FRT, Zasp52 5'3'FRT, and Zasp52 null/Df(2R)BSC427, to confirm each Zasp52 mutant phenotype (Fig 2.2B).

A


Wild type
Zasp52 5'FRT/ Zasp52 5'FRT
Zasp52 5'3'FRT/ Zasp52 5'3'FRT
Zasp52 null/ Df (2R)BSC427
3µm
Increasing severity of phenotype

B

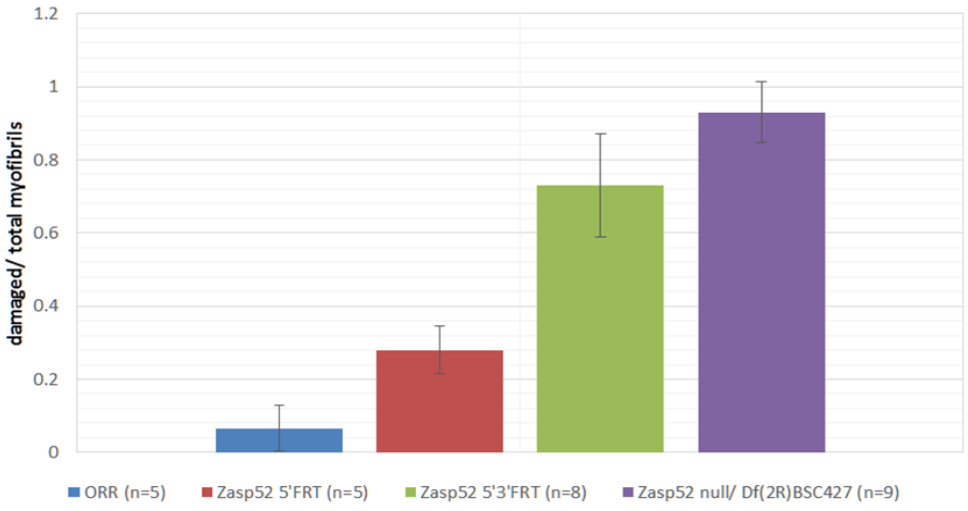

damaged/ total myofibrils
1.2
1
0.8
0.6
0.4
0.2
0
ORR (n=5)
Zasp52 5'FRT (n=5)
Zasp52 5'3'FRT (n=8)
Zasp52 null/ Df(2R)BSC427 (n=9)

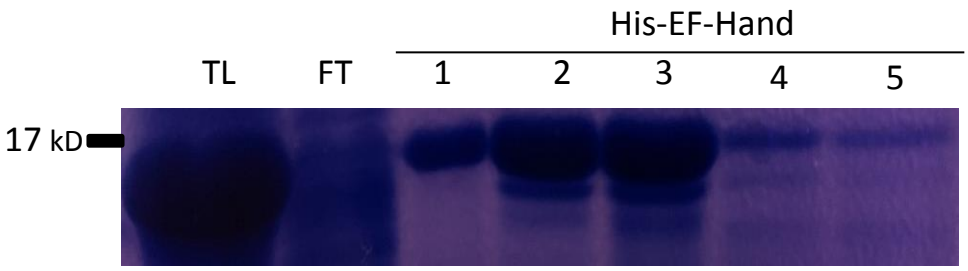
Figure 2. 2. Zasp52 null exhibit strong IFM defects

(A) Confocal microscopy of wild type (ORR), homozygous Zasp52 5'FRT and Zasp52 5'3'FRT, Zasp52null/ Df(2R)BSC427 IFM stained with phalloidin to visualize actin thin filaments in red. Homozygous Zasp52 5'3'FRT and Zasp52 null/Df(2R)BSC427 have severe defects, while homozygous Zasp52 5'FRT IFMs have mild defects. Scale bar, 3 μm. (B) Quantification of IFM defects. n corresponds to number of images analyzed. Each image is from a different myofiber from at least 10 different animals and contains 5-12 myofibrils. Ratio of damaged versus total myofibrils per image is given on the y-axis. Error bars represent the standard error of the mean.

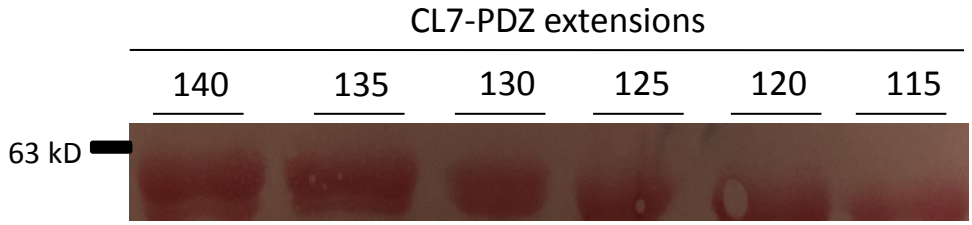
2.3 EF-Hand domain of *D. melanogaster* α-actinin is sufficient for binding to Zasp52 extended PDZ domain

We have previously shown that a C-terminal extended PDZ domain of Zasp52 is required for optimal binding to α-actinin. It has been revealed that a 142 amino acid-long extended PDZ domain of Zasp52 robustly interacts with full-length rabbit α-actinin (Liao et al., 2016); suggesting the PDZ-only domain is not sufficient for optimal binding to α-actinin and additional non-conserved amino acids are required for a strong interaction. Here, we set out to determine if *Drosophila* α-actinin also properly binds to an extended PDZ domain. Moreover, we were seeking to test if the EF-hand-only domain of *D. melanogaster* α-actinin is sufficient for binding to Zasp52. Finally, we narrow down the extent of Zasp52 PDZ domain extension required for binding. To this end, I first bacterially overexpressed and purified a His-tagged EF-Hand domain construct as well as six various constructs of CL7-tagged PDZ domain extensions. Because the the different CL7-tagged PDZ extensions are almost large proteins and they are very close in size, I used 8% SDS gel and lower voltage for running to distinguish between different extensions. (Fig 2.3A and 2.3B). Then, I tested the interaction of each PDZ domain extension with EF-Hand domain using pull-down assay (Fig 2.3C). Only 140 and 135 amino acid-long proteins interact with EF-Hand domain of α-actinin (Fig 2.3D, Fig S2.1). This shows that EF-Hand domain of *Drosophila* α-actinin is sufficient for an optimal interaction with Zasp52 extended PDZ domain. In addition, it shows that 135 amino acids of the extended PDZ domain is the minimal size required for optimal binding.

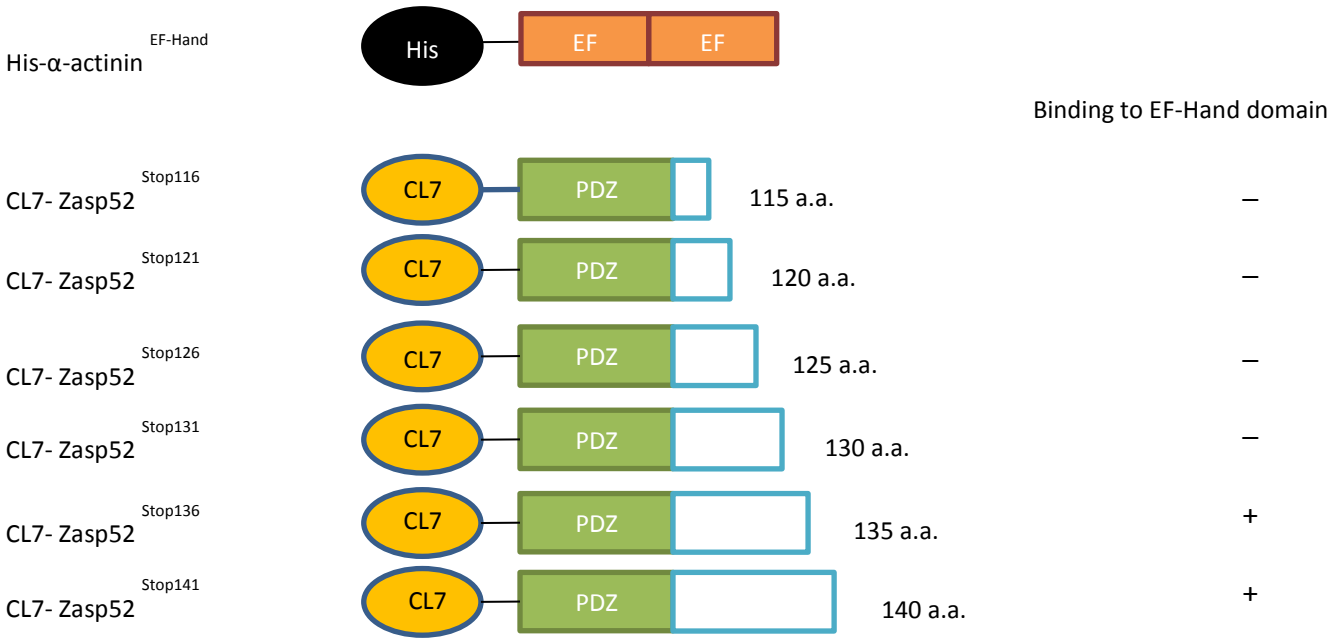
A


His-EF-Hand
TL
FT
1
2
3
4
5
17 kD

B


CL7-PDZ extensions
140
135
130
125
120
115
63 kD

C


His-α-actinin
EF-Hand
His
EF
EF
Binding to EF-Hand domain
CL7- Zasp52
Stop116
CL7
PDZ
115 a.a.
–
CL7- Zasp52
Stop121
CL7
PDZ
120 a.a.
–
CL7- Zasp52
Stop126
CL7
PDZ
125 a.a.
–
CL7- Zasp52
Stop131
CL7
PDZ
130 a.a.
–
CL7- Zasp52
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PDZ
135 a.a.
+
CL7- Zasp52
Stop141
CL7
PDZ
140 a.a.
+

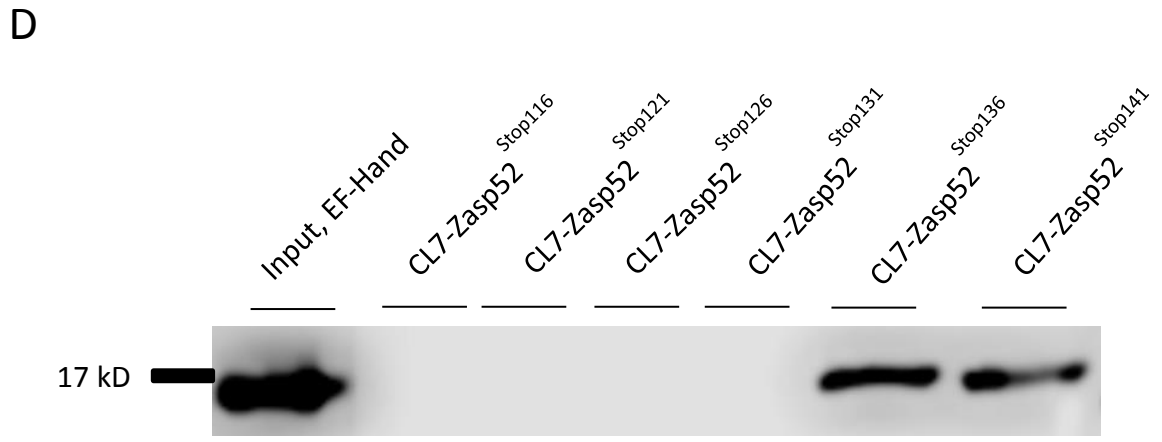

Figure 2. 3. EF-Hand domain of *D. melanogaster* α-actinin is sufficient for binding to Zasp52 extended PDZ domain

(A) His-EF-Hand domain of *Drosophila* α-actinin run on a SDS-PAGE gel after purification. TL – Total lysate; FT – Flowthrough; 1, 2, 3, 4, 5 – 500 µL eluted aliquots. (B) Ponceau S stained blot of a 8% SDS-PAGE gel to confirm different sizes of PDZ extensions (140 to 115 amino acid-long).(C) Schematic representation of CL7- and His-fusion constructs with amino acid length of Zasp52 part. Summary of EF-Hand domain binding ability is provided on the right: +, normal binding; -, no binding. (D) His pull-down assay with a series of truncated proteins. 140 and 135 amino acid-long Zasp52 proteins bind to EF-Hand domain of *Drosophila* α-actinin. However, 130, 125, 120, and 115 amino acid-long Zasp52 proteins cannot. 12% SDS gel was used. Primary antibody: 1:5000 Mouse anti-His tag; Secondary antibody: 1:10000 anti-Mouse ECL.

2.4 Supplementary figure

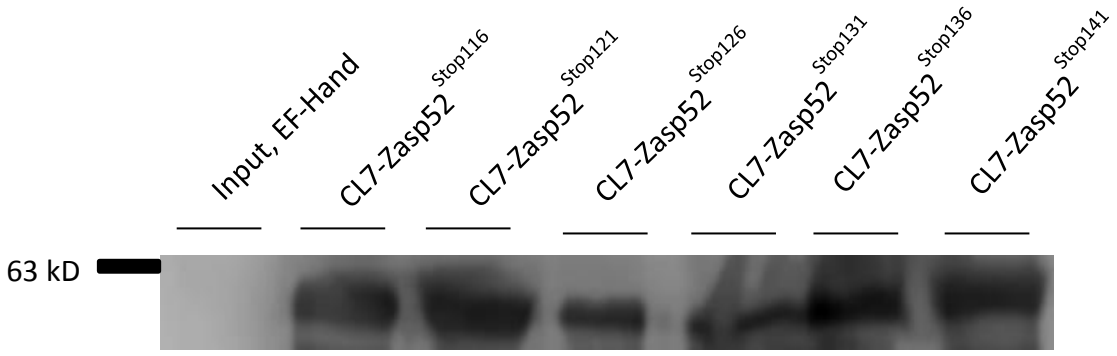

Figure S 1. Visualization of 140, 135, 130, 125, 120, and 115 amino acid-long Zasp52 proteins on the blot shown in figure 2.3D using different antibodies. 12% SDS gel was used. Primary antibody: 1:5000 Rabbit anti-N-terminal Zasp. Secondary antibody: 1:10000 anti-Rabbit ECL.

3. Material and methods

3.1 Fly Stocks and Genetics

The following fly stocks were used: Zasp52 5'3'FRT, Zasp52 5'FRT, Df(2R)BSC427, hs FLP y w, w; Tft/CyO. Zasp52 null stock was generated by standard genetic crosses; to this end, Zasp52 5'3'FRT/ CyO was crossed to hs FLP y w/Y; Sp/Sm6, then heat shocked in 37◦C water bath for one hour for three successive days. Next, the progeny were crossed to w; Tft/CyO; then Zasp52 null/CyO single males were crossed to w; Tft/CyO to provide a stable stock.

The CRISPR mutants were generated by injecting sequence-verified plasmids into y[1] M{w[+mC]=nos-Cas9.P}ZH-2Aw [*] (for Zasp52 5'FRT) and y[1] M{w[+mC]=nos-Cas9.P}ZH-2Aw [*]; Zasp52_5'FRT/CyO (for Zasp52 5'3'FRT) embryos by Genetivision. Landing sites were designed in cooperation with Genetivision, all follow-up work to generate the CRISPR mutant was performed by Genetivision.

3.2 Plasmids

Actinin EF-Hand as well as PDZ-140, PDZ-135, PDZ130, PDZ-125, PDZ-120, and PDZ-115 were synthesized by GenScript and cloned into PDV1-BUK-C2 to generate His and CL7 constructs, respectively.

3.3 α-actinin EF-Hand domain purification

E. coli strain BL-21 bacteria expressing His-tagged recombinant proteins were lysed by lysis buffer (20 mM Tris-HCL pH 8.0, 150 mM NaCl, and 10 mM imidazole) containing freshly added protease inhibitor tablet, DNAse I, RNAse A, and lysozyme and sonicated at 25% amplitude. The cell extract after centrifugation was filtered by a 0.45 µm filter to eliminate cell debris. Next, the total lysate was applied to the His-Trap column (1 hour at 4◦C). The column was washed with low-salt (20 mM Tris-HCL pH 8.0, 25 mM NaCl, and 40 mM Imidazole) and High-salt buffers (20 mM Tris-HCL pH 8.0, 250 mM NaCl, and 40 mM Imidazole), alternatively. Finally, His-EF-Hand domain was eluted from the column with elution buffer (20 mM Tris-HCL pH 8.0, 150 mM NaCl, and 250 mM Imidazole) to analyze by SDS-PAGE (Fig 2.3A).

3.4 His pull-down assay and immunoblotting

For His pull down assay, E. coli strain BL-21 bacteria expressing CL7-tagged recombinant proteins were lysed by lysis buffer (20 mM Tris-HCL pH 8.0, 500 mM NaCl, 5% glycerol, and 0.5 mM EDTA) containing freshly added protease inhibitor tablet, DNAse I, RNAse A, and lysozyme and sonicated at 25% amplitude. The cell extract after centrifugation was filtered by a 0.45 µm filter and applied to the Im7 column (1 hour at 4◦C), then the column retaining the CL7-tagged proteins was washed by low-salt (20 mM Tris-HCL pH8.0, and 25 mM NaCl) and high-salt buffers(20 mM Tris-HCL pH8.0, and 1.2 M NaCl), alternatively. Next, *Drosophila* α-actinin

EF-Hand was added to the column and incubated in a rotating platform at 4◦C overnight. Then, the Im7 column was washed by alternate low-salt and high-salt buffers to wash the unbound proteins. Finally, the column was washed by 6 M GuHCL to wash everything. Subsequently, 6 M GuHCL was substituted by lysis buffer using Hi-Trap column and final elution was analyzed by SDS-PAGE and immunoblotting.

3.5 Western analysis

The aforementioned protein samples were resolved by 12% SDS-PAGE and then detected by immunoblotting. Antibodies used were mouse anti-His (1: 5000, Sigma-Aldrich), and rabbit anti-N-terminal Zasp52 (1: 5000, (Katzemich et al., 2011)). Western analyses were performed at least three times and representative blots are shown.

3.6 PCR analysis and sequencing

Five flies were homogenized in squishing buffer (10 mM Tris-HCL, pH 8.2, 1 mM EDTA, 25 mM NaCl and 200 ng/µl freshly prepared Proteinase K), the lysates were incubated 30 minutes at 37◦C then heated 5 minutes at 95◦C. PCR products for cassette insertions were amplified by Taq polymerase using 4 different primer sets (two primer sets for 5’ inserted cassette, and the other two for 3’ inserted cassette). Among four primers for each inserted cassette, two of them

are cassette-specific primers and the other two are located outside of the left and right homologous arms through which the recombination occurred. The following primers were used for PCR reactions:

For Knock-in cassette using GFP as selection marker:

Set 1  Zasp52 forward 1: GCGCGTATAATTTTGGGGCACAC

Cassette reverse: AAGTCGCCATGTTGGATCGACT

Set 2  Cassette forward: CCTGGGCATGGATGAGCTGT

Zasp52 reverse: GCGGCCCAGACAATTGTTTG

For Knock-in cassette using RFP (DsRed) as selection marker:

Set 3  Zasp52 forward 2: GGTATCCTGAATAAGGCAGCCG

Cassette reverse: AAGTCGCCATGTTGGATCGACT

Set 4  DsRed forward: GATCCACAAGGCCCTGAAGC

Zasp52 reverse: CGCCAGGCCTCTAAAGTTTTGTATTC

To confirm Zasp52 null mutant, PCR products which all cross the junction after deletion were amplified by Taq polymerase using three different primer sets. The PCR products are different in size. Then, the PCR product was sequenced by Genome Quebec to confirm the deletion and reveal the DNA sequence close to the break point. The primers were designed using primer 3. The following primers were used for PCR reactions:

Set 1 ⇨ Forward primer: AACTAAACTTTGGCCAACAGTTG

Reverse primer: TTGAGCCTGAAAACTGTTGATCAA

Set 2 ⇨ Forward primer: CCGCTGGCCATATAGAAGAAT

Reverse primer: GCCTAGAATCGGGCTAATCA

Set 3 ⇨ Forward primer: CGACGCTTTGGCTTAGCAATAATAA

Revers primer: CTTAGTTGAGCCTGAAAACTGTTG

3.7 Confocal Microscopy

In order to remove mitochondria to make staining and imaging easier, half thoraces were transferred to Relaxing-Glycerol buffer (20 mM Na-Phosphate pH 7.2, 2 mM $MgCl_2$, 2 mM EGTA, 5 mM DTT, 0.5% Triton X-100, and 50% glycerol) at -20◦C overnight without agitation. 4% paraformaldehyde was added and incubated at room temperature for 45 minutes with slow agitation. IFMs were dissected and incubated with TRITC-phalloidin (1: 1000, ThermoFisher Scientific) for 2 hours at room temperature. Finally, a drop of mounting media was added to the samples and the glass slide was covered by a coverslip compatible with confocal microscopy (Xiao et al., 2017). Images were acquired by SP8 scanning confocal microscopy. IFMs were scanned at 9× magnification 1,024 × 1,024 pixels using 63×/ 1.4 NA oil immersion objective. In order to quantification of IFM defects, I used at least 10 different animals for each genotype; each image is from a different myofiber and contains 5-12 myofibrils.

4. Discussion

In this research we have extended our knowledge about the binding properties of α-actinin to Zasp52 by analyzing the interaction between the EF-Hand domain of *Drosophila* α-actinin and Zasp52 extended PDZ domain. In addition, we have obtained and analyzed three Zasp52 mutants with different levels of IFM abnormalities.

4.1 Protein domains involved in the α-actinin and Zasp52 interaction

Zasp PDZ proteins such as ALP, CLP36 and ZASP/Cypher have been revealed to bind directly to α-actinin through the PDZ domain (Bauer et al., 2000; Klaavuniemi et al., 2004). Moreover, mutational studies and inhibition experiments with synthetic peptides have demonstrated that the C-terminal EF-hands of α-actinin is responsible for direct binding to the PDZ domain of ALP (Klaavuniemi et al., 2004; Xia et al., 1997). On the other hand, in our lab the interaction between PDZ domain of Zasp52 and rabbit full-length α-actinin using GST pull-down assay has shown that a C-terminal extended PDZ domain is required for optimal interaction with α-actinin (Liao et al., 2016). We therefore set out to investigate the interaction between α-actinin and extended PDZ domain of Zasp52 using only EF-hand domain of Drosophila α-actinin for the first time. Also, in comparison to our previous studies (Liao et al., 2016), to further delineate the sequences

necessary for this interaction, we decided to narrow down the required amino acids of the extended PDZ domain to find the location of amino acid sequences which significantly affect the binding. Therefore, we used 115 to 140 amino acid-long extended PDZ domain. We first showed that only the EF-hand domain of *Drosophila* α-actinin is sufficient for binding to Zasp52 extended PDZ domain. Next, only 140 and 135 amino acid-long extended PDZ domain can bind to EF-hand domain and the smaller constructs cannot. Here, by focusing on the protein constructs which are very close in amino acid length (five amino acids difference); we could recognize the location of a few non-conserved amino acids which are pivotal to enhance the binding ability of PDZ domain to its target protein. Maybe using additional protein constructs (e.g between 135 to 130 amino acid-long) could further narrow down the minimal required domain, but this result will already greatly help in future structural studies determining the EF-hand extended PDZ domain protein structure. Interestingly, although it has been previously revealed Zasp52 111 amino acid-long extended PDZ domain as well as the PDZ-only domain (GST-tagged) weakly bind to the full-length α-actinin (Liao et al., 2016), the new results show that PDZ domain extensions smaller than 135 amino acids (e.g. 115 to 130 amino acid-long) cannot directly interact with EF-hand-only domain of α-actinin. In addition, it has been shown GST control extract cannot interact diretly with α-actinin (Liao et al., 2016). Therefore, it suggests that PDZ domain of Zasp52 and EF-hand domain of α-actinin are not the only involved domains for an optimal interaction between α-actinin and Zasp52. It means there is one possibility that the differences seen between two experiments are due to sequences outside the EF-hand domain; which is consistent with results obtained for ALP and α-Actinin-2, where the area surrounding the ZM motif interacts with spectrin repeats of α-actinin (Klaavuniemi et al., 2004, 2009; Xia et al., 1997). Furthermore, in spite of PWGFRL sequence (inside the PDZ

domain) that is required for interaction with α-actinin and strictly conserved among vertebrates (ZASP and ALP) and *Drosophila* (Zasp52) Zasp PDZ domain family members, the extended PDZ domain consists of mainly non-conserved amino acids; for example there is no obvious homology between sequences surrounding ALP's PDZ domain with Zasp52. Therefore, we cannot consider the extended PDZ domain as the second independent required binding-site and it just enhances the binding properties of PDZ domain to α-actinin; which it has been previously shown in our lab, where ZM motif and surrounding sequences (76 amino acids in total) cannot directly interact with α-actinin (Liao et al., 2016).

4.2 The impact of the novel Zasp52 allelic series on IFM organization

Here we characterized three novel Zasp52 alleles, and analyzed the corresponding IFM phenotype using confocal microscopy. We generated the first two mutants (Zasp52 5'FRT and Zasp52 5'3'FRT) using CRISPR mutagenesis followed by homology-directed repair leading to a modification of the encoded amino acids at the endogenous locus. Zasp52 5'FRT deletes the PDZ domain, and Zasp52 5'3'FRT removes the PDZ domain as well as most of the three C-

terminal LIM domains; both of them homozygously show IFM defects (Fig 2.2.A). Stronger myofibril defects of Zasp52 5'3'FRT is potentially because of the lack of LIM-only isoforms which carry some unique functions (Liao et al., 2016). Other studies have shown that LIM-only proteins play essential roles in the development of muscles (Pronovost et al., 2013; Tran et al., 2016). Finally, we used FLP-mediated recombination to generate the third Zasp52 mutant (Zasp52 null), which deletes all Zasp52 isoforms. It shows the strongest disruption of myofibrils over a deficiency deleting Zasp52 (Fig 2.2.A); demonstrating the importance of Zasp52 in myofibril assembly and maintenance. Being flightless and having similarly disrupted myofibrils (Fig. 2.2.B), suggests that Zasp52 5'3'FRT is close to a null mutant. In addition, Zasp52 5'3'FRT disrupts most functional isoforms e.g. Zasp52-PK, Zasp52-PP, and Zasp52-PQ. Altogether, we have created a Zasp52 allelic series (three mutants of increasing severity of phenotype) that will be very useful for future studies of Zasp52 function.

Summary and future directions:

The thesis is split in two sections. In the genetic section, I characterized a new Zasp52 allelic series including Zasp52 5'FRT, Zasp52 5'3'FRT, and Zasp52 null. Then, I analyzed the IFMs of these mutants using confocal microscopy. The IFMs were dramatically damaged, especially in Zasp52 null which removes Zasp52 completely and in Zasp52 5'3'FRT which is similar to a null. There were a broad range of myofibril defects, from extended Z-discs and wavy and frayed myofibrils to the complete absence of Z-discs. All of the aforementioned mutants will be useful

for future research; for example, generation the double and triple null mutants in the *Drosophila* Zasp family (Zasp66, Zasp67, and Zasp52) will lead to a deeper understanding of the true functions of these proteins and how they work together in muscles. In the second section of my thesis, I investigated the direct interaction of Zasp52 extended PDZ domain with α-actinin. Here, I used only the EF-hand domain of α-actinin. Elucidating the precise limit of interaction domains is of paramount importance for later structural studies. There are many complex proteins at the Z-disc enabling to bind different protein partners (sometimes more than two or three partners); therefore, finding the protein domains which are directly involved in binding to other proteins is essential. I demonstrated that the EF hand domain of *Drosophila* α-actinin is sufficient for interaction with the Zasp52 extended PDZ domain. In addition, I narrowed down the interaction domain of Zasp52 required for optimal binding to α-actinin.

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