ABSTRACT

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Directed By: Associate Professor, Shaojun Du, MEES

Myofibrillogenesis, the process of sarcomere formation, requires close interaction of sarcomeric proteins and molecular chaperones. Smyd1 is a lysine methyltransferase that plays important roles in myofibrillogenesis in both skeletal and cardiac muscles. Knockdown of *smyd1* results in complete disruption of sarcomere organization. The molecular mechanism by which Smyd1 controls myofibril assembly is not clear. In this study, we analyzed the sub-cellular localization of Smyd1, the effect of *smyd1* knockdown on protein methylation, and the effect of *myomesin* knockdown on sarcomere organization. We demonstrated that Smyd1b_tv1 is localized to the M-lines of skeletal muscles in zebrafish embryos. Knockdown of *myomesin-1b* or *myomesin-3* had no effect on the sarcomere organization. Western blot analysis revealed that knockdown of *smyd1* reduced the overall protein methylation in zebrafish embryos. Together, these studies indicate that Smyd1 is required for M-line organization and Smyd1 may play a role in protein methylation and is involved in sarcomere assembly.

FUNCTIONAL ANALYSIS OF SMYD1 AND MYOMESIN IN SARCOMERE ORGANIZATION IN ZEBRAFISH EMBRYOS

By

Jin Xu

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Advisory Committee:
Associate Professor Shaojun Du, Chair
Associate Professor Randall, William R
Associate Professor Shengyun Fang
Assistant Professor Kontrogianni-Konstantopoulos, A

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Chapter 1: Introduction and Background

1.1 Sarcomere

In vertebrates, striated muscles are the contractile tissues responsible for movement. Their contractile activity is regulated by a complex and interconnected network of muscle proteins (Clark et al., 2002). The basic contractile unit in skeletal and cardiac muscles is called the sarcomere, and is one of the most highly ordered macromolecular assemblies in cells. The highly ordered macromolecular complexes that make up the sarcomere can be divided into four major compartments: the Z-disc, actin-containing thin filaments, myosin II-containing thick filaments, and the M-line. The regions with myosin II-containing thick filaments are referred to as A-bands. Regions where actin-containing thin filaments do not overlap with myosin II-containing thick filaments are referred to as I-bands (Sparrow et al., 2009).

1.1.1 Z-disc

Z-discs form the boundaries of sarcomeres, and appear as dense lines under an electron microscope. The size of the Z-disc is fiber type-specific, and ranges from 30–50 nm in fast muscles and 100–140 nm in slow muscles (Knöll et al., 2011). Z-discs are localized at the center of thin filaments. Numerous proteins are present at a Z-disc site, indicating the diverse functions of the Z-disc. α-Actinin is a major component of Z-discs and plays an important role in Z-disc structure and function. Loss-of-function mutations in *α-actinin* cause severe muscle defects in Drosophila and *C. elegans* (Fyrberg et al., 1998; Moulder et al., 2010). It has been well-

documented that α-actinin monomers interact to form antiparallel dimers, which function as scaffolds linking sarcomeric contractile units in a series by anchoring the thin filaments of adjacent sarcomeres (Frank et al., 2006; Sanger et al., 2010). In addition to α-actinin, abundant intermediate filament proteins are localized at the periphery of Z-discs and form links between adjacent myofibrils (Clark et al., 2002). This linkage is critical for the passive transmission of tension, which can help maintain the stability of the sarcomeric structure (Luther et al., 2009). In addition, the Z-disc has been shown to play important roles in anchoring various proteins that are associated with signal transduction and sensing stretch (Epstein and Davis 2003; Pyle and Solaro 2004).

1.1.2 Thin filaments

Actin-containing thin filaments consist of double helix-polymerized actin monomers, which span the I-band and overlap myosin filaments at the A-band (Sparrow et al., 2009). Actin-containing thin filaments are anchored to Z-discs through the fibrous barbed ends of actin. Actin filaments are decorated with the regulatory proteins troponin and tropomyosin. Tropomyosin associates with actin filaments and blocks actin-myosin interaction in the absence of or at low concentrations of cytosolic Ca^{2+}. In the presence of cytosolic Ca^{2+}, troponin binds to Ca^{2+} and undergoes a conformational change that allows the interaction of myosin and actin. The interaction of myosin and actin in an ATP-dependent manner generates the force required for movement. Several actin isoforms have been identified in higher vertebrates and can be classified into tissue-specific muscle isoforms including cardiac, skeletal, vascular, and enteric isoforms and two ubiquitous non-muscle

isoforms (Vandekerckhove and Weber, 1979; Kumar et al., 1997). The amino acid sequences of different actin isoforms are highly conserved. Interestingly, the similarity of amino acid sequences does not translate into equivalent function. It has been shown that the substitution of the indirect flight muscle actin with human β-cytoplasmic actin in Drosophila impedes its ability to fly (Brault et al., 1999a; Brault et al., 1999b). Although smooth muscle actin can partially rescue the cardiac actin defect in cardiac α-actin-deficient mice, these mice are extremely hypertrophied, and their heart function is severely disturbed (Kumar et al., 1997).

1.1.3 Thick filaments

Thick filaments are composed of several hundred myosin molecules, including muscle myosin II. Myosin II molecules are grouped together to form the highly ordered bipolar thick filaments and are responsible for the generation of muscle contraction. Myosin II molecules can be characterized as a combination of two parts: the head and the rod. The head region contains the globular head domains of N-terminal myosin heavy chains as well as two pairs of light chains named the essential and regulatory light chains. It has been well documented that myosin heavy chains can form a catalytic motor and induce force (Sellers and Goodson, 1995). The essential and regulatory light chains, which play regulatory roles in contraction, bind to the heavy chains in the "neck" region between the head and tail. During contraction, force is generated by the attachment of the myosin head motors to actin molecules in an ATP-dependent manner (Boateng et al., 2008). Generally, the interaction of myosin-actin-ATP complexes can produce a power stroke, which provides the force for muscle movement. This process is regulated by the tropomyosin-troponin

complex (Ruegg et al., 2002). The rod region of myosin II is also known as light meromyosin (LMM). LMM contains long coiled-coil domains, which are responsible for myosin polymerization (Clark et al., 2002). All the regions of myosin II molecules are crucial for the proper functioning of thick filaments.

1.1.4 M-line

The M-line is localized to the middle of the A-band. The M-line plays a regulatory role in packaging thick filaments and stabilizing the sarcomeric structure (Knappeis et al., 1968). In the process of sarcomere alignment, the M-line is aligned earlier than myosin, indicating a vital role for the M-line in regulating myosin filament alignment (Yang et al., 2000). The interaction of the M-line and other sarcomeric proteins is critical for sarcomere organization in myofibrillogenesis. It has been reported that the M-line plays critical roles in cardiac myofibrillogenesis by affecting titin filament organization (Wang et al., 1998). Biomedical and physiological studies indicate that the M-line plays vital roles in transversing force during the activation of contraction. It has been proposed that the M-line structure might promote the symmetric shortening of the whole sarcomere (Agarkova et al., 2003). These findings reflect the multiple roles of the M-line in the sarcomere.

Multiple proteins are localized on the M-line, such as myomesin, C-titin, obscurin, and Muscle-specific RING finger proteins (MuRFs), which have diverse functions. Targeted deletion of M-line-localized titin results in a failure to differentiate at an early stage of myofibrillogenesis, indicating a critical role for M-line-localized titin in sarcomere assembly (Musa et al., 2006). Some of the M-line proteins link the sarcomere to other cellular structures and stabilize the myofiber. It

has been shown that the giant protein obscurin, which is predominantly localized to the M-line, helps stabilize the network of cross-linked myosin filaments by functioning as a linker to the sarcoplasmic reticulum (Kontrogianni-Konstantopoulos et al., 2009). In addition, M-line localized-proteins are involved in protein quality control in myofibers. For example, the M-line proteins MuRFs, which are muscle-specific RING-finger E3 ubiquitin ligases, are shown to play vital roles in regulating protein degradation in muscles (Lange et al., 2005).

1.1.5 Myomesin

Myomesins are the leading candidates thought to play the role of M-band bridges for regulating the packaging of thick filaments and distributing tension over the thick filament lattice in a uniform manner (Agarkova et al., 2003). Members of the myomesin family (myomesin 1, 2, and 3) are immunoglobulin (Ig)-like and fibronectin (Fn) type III domain-containing proteins. Myomesin-1 demonstrates a ubiquitous expression pattern and is found in all kinds of vertebrate striated muscles (Agarkova et al., 2000; Agarkova et al., 2004). In contrast to the ubiquitously expressed myomesin-1, M-protein (myomesin-2) and myomesin-3 are expressed in a muscle-type and fiber type-specific pattern. Myomesin-2 is primarily found in fast-type muscle fibers, and myomesin 3 is expressed in slow muscles (Schoenauer et al., 2007).

The patterns of expression of myomesin isoforms are highly correlated with developmental processes and are highly regulated by certain factors. It has been reported that myomesin-1 and -2 are direct targets of the MEF2C transcription factor in mice (Matthew et al., 2007).

Myomesin is capable of binding multiple partners and serves as a docking site for various proteins. Myomesin-1 binds to myosin in the central region of light meromyosin and interacts with a single titin Ig domain (Wolfgang et al., 1997). The muscle-specific creatine kinase interacts with the central domains of myomesin-1 and myomesin-2 (Hornemann et al., 2003). In addition, myomesin-1 and -3 can form homodimers using their C-terminal domains (Lange et al., 2005; Pinotsis et al., 2008).

In recent years, structural studies have revealed the importance of myomesin in force transduction and in maintaining the sarcomeric structure. Based on recently reported structural studies, myomesin is thought to act as a molecular spring with adaptable elasticity (Schoenauer et al., 2005; Pinotsis et al., 2012; Tskhovrebova and Trinick, 2012). In mature fibers, myomesin has high elasticity and can be stretched to about 2.5 times its original length by unfolding the α-helix linkers (Berkemeier et al., 2011; Pinotsis et al., 2012). This indicates that myomesin is a highly elastic structure that maintains sarcomere and M-line organization.

Mutations of certain proteins in the sarcomere can lead to severe defects in sarcomere organization. Recently, a myomesin mutant was found to be associated with hypertrophic cardiomyopathy (Siegert et al., 2011). In addition, the protein abundance of myomesin isoforms may change in disease conditions. The embryonic heart-specific isoform, EH-myomesin, was shown to be upregulated in some cardiomyopathies (Agarkova et al., 2000; Schoenauer et al., 2011). This raises the possibility that myomesin is critical for sarcomere maintenance, and that the isoforms' change in expression can act as a marker for some myopathies. Consistent with the importance of myomesin, siRNA-mediated knockdown of *myomesin* resulted in the

disruption of sarcomere organization (Fukuzawa et al., 2008). Although there has been much indirect evidence supporting the idea that myomesin is a pivotal factor in sarcomere assembly and organization, no knockout models have been reported to reveal the function of myomesin *in vivo*, presumably due to the early embryonic lethality of myomesin mutant mice.

1.1.6 Titin

Titin is the largest protein discovered to date. After myosin and actin, titin is the third most abundant protein in vertebrate striated muscles (Tskhovrebova and Trinick, 2003). A single titin molecule can stretch from the Z-disc to the M-line. Titin is largely composed of immunoglobulin-like and fibronectin domains. Fibronectin domain containing proteins usually have very highly elasticity (Pinotsis et al., 2008). Thus, one crucial function of titin is to transduce passive forces and stabilize the sarcomere (Granzier et al., 1997). In addition, titin is involved in some signal transduction pathways through its M-line localized C-terminal kinase region. Targeted deletion of M-band titin disrupts sarcomere formation in heart muscles (Musa et al., 2006).

Titin also has critical functions in sarcomere organization. It was proposed that titin acts as a molecular 'ruler' or blue print for the sarcomere, and that it interacts with dozens of proteins in the sarcomere from Z-disk proteins at the N-terminal end to M-band proteins at the C-terminal end (Sanger, J.W. and Sanger, J.M., 2001; Tskhovrebova and Trinick, 2003). To date, several theories have been proposed for the function of titin based on biochemical and domain-specific deletion studies;

however, the detailed mechanism by which titin contributes to sarcomere function remains to be understood.

1.2 Sarcomere disease

The sarcomere is the basic contractile unit of both skeletal and cardiac muscles. Defects in sarcomere protein function lead to various muscle diseases in both heart and skeletal muscles. By 2005, twenty different sarcomeric proteins had been associated with various skeletal muscle diseases (Laing and Nowak, 2005). Several types of cardiomyopathies that result from sarcomere protein dysfunction have been identified in recent clinical studies (Kamisago et al., 2000; Franz et al., 2001; Niimura et al., 2002). Sarcomere diseases exhibit different severities from mild to early lethality. Mutations in different proteins can lead to different effects. Some of these diseases, which affect newborn children, have been demonstrated to be caused by mutations in sarcomere proteins that are critical for muscle contraction (Laing and Nowak, 2005). Mutations associated with regulatory proteins in the sarcomere were shown to increase the probability of cardiac hypertrophy (Laing and Nowak, 2005). In addition, mutations in sarcomeric proteins that lead to protein misfolding have been shown to cause a severe disease defined by the failure to transverse force during the activation of contraction (Kiphuth et al., 2010).

Muscle contraction is generated by the interaction of actin and myosin. Thus, proper functioning of myosin and actin is critical for normal muscle movement. It has been shown that mutations in the cardiac isoform of α-actin (ACTC) can cause atrial septal defects (Matsson et al., 2008) and hypertrophic cardiomyopathy (Mogensen et al., 1999). Mutations in the skeletal muscle isoform of α-actin (ACTA1) are

associated with three different muscle diseases: (1) actin myopathy (AM), characterized by the accumulation of actin filaments in muscle fibers (Laing and Nowak, 2005), (2) nemaline myopathy (NM) characterized by sarcoplasmic nemaline bodies (rods) (Sparrow et al., 2003), and (3) intranuclear rod myopathy (IRM) characterized by the aggregation of actin and α-actinin-2 inside the nucleus of muscle cells (Domazetovska et al., 2007). In addition to actin-associated diseases, many myosin-associated sarcomere diseases have also been discovered, such as core-like lesions, hyaline body myopathy, early onset distal myopathy, dominant inclusion body myopathy, and distal arthrogryposis trismus-pseudocamptodactyly syndrome (Laing and Nowak, 2005).

Recently, additional mutations in sarcomeric proteins have been associated with various kinds of myopathies. However, the pathological mechanisms by which these mutations cause disease are largely unknown. A better understanding of the regulation of sarcomere protein organization during muscle cell differentiation will facilitate a better understanding of disease mechanisms, and is required for the development of new therapies for sarcomere protein-related myopathies.

1.3 Myofibrillogenesis

Muscle development is a multi-step process that starts with the commitment of multi-potent mesodermal precursor cells to myoblasts, thus giving rise to the muscle lineage. This is followed by the differentiation of myoblasts into myocytes and the fusion of myocytes into multi-nucleated myotubes. The final step of skeletal muscle formation is the maturation of differentiated myotubes into muscle fibers (McKinsey et al., 2002; Christ et al., 2002; Buckingham et al., 2003; Du et al., 2008a; Sparrow et

al., 2009). Muscle fibers are muscle cells that contain dozens of myofibrils. Myofibrils of skeletal muscles and cardiac muscles are characterized by groups of proteins arranged in contractile units called sarcomeres. The process of myofibril assembly into highly organized sarcomeres is called myofibrillogenesis.

1.3.1 Sarcomere assembly

The assembly of myofibril proteins into functional units is a rapid and well-coordinated process. Structural proteins are critical for the proper assembly of myosin thick filaments, actin thin filaments, and the organization of sarcomeres. Sarcomere assembly starts with the formation of small, membrane-associated aggregates called Z-bodies, which are premature forms of Z-discs (Sparrow et al., 2009). Then, the maturation of Z-discs leads to the formation of organized sarcomeric structures. Mature Z-discs are composed of various proteins including α-actinin, the N-terminal region of titin, and nebulin. Z-discs organize thin actin filaments to form "I-Z-I brushes". Similarly, the M-lines, which contain the C-terminal of titin, obscurin, and myomesin, are thought to play key roles in the integration of myosin thick filaments into the A-band.

Although the basic process of myofibrillogenesis is well characterized, details of the process of myofibrillogenesis are still open to debate. To date, four models have been proposed for the process of myofibrillogenesis: (1) the template model, (2) the independent assembly of thin and thick filaments model, both of which were proposed by Holtzer and colleagues (Dlugosz et al., 1984; Lu et al., 1992), (3) the premyofibril model proposed by Sanger and colleagues (Rhee et al., 1994), and (4) the direct assembly of myofibrils model proposed by Costa and colleagues (Costa et

al., 2002). The template model proposes that non-muscle proteins must serve as a temporary template for the formation of a single myofibril due to the inability of myofibril elements to assemble into mature myofibrils directly (Dlugosz et al., 1984). The independent assembly of thin and thick filaments model suggests that the I-Z-I bodies and the thick filaments are first assembled independently of each other, and then titin molecules join or stitch the scattered I-Z-I bodies and thick filaments together into elongating mature myofibrils (Lu et al., 1992; Holtzer et al., 1997; Sanger et al., 2005). The premyofibril model argues that the assembly of the sarcomere begins with premyofibrils that are composed of mini-sarcomeres whose boundaries are marked by Z-bodies containing muscle α-actinin. Non-muscle myosin II filaments are present in the mini-sarcomeres. Z-bodies in the adjacent myofibrils begin to align into nascent myofibrils. Then, M-band proteins are recruited when the Z-bodies become mature Z-discs. In the meanwhile, the thick filaments, which contain muscle specific myosin, align into an A-band (Sanger et al., 2005). The direct assembly of myofibrils model comes from a report that studied myofibrillogenesis in zebrafish embryos. The report demonstrated that myofibril assembly is a spontaneous process with no intermediate stages (Costa et al., 2002). The co-existence of various models indicates that sarcomere assembly might be a more complicated process than first predicted due to the structural complexity of the sarcomere.

1.3.2 Myosin assembly

Genetic studies and biochemical analyses have shown that myosin folding and assembly is an integral part of myofibrillogenesis during muscle development. Recent studies have indicated that molecular chaperones play important roles in myosin

folding and assembly. Unc45b and Hsp90α are key chaperones expressed in skeletal and cardiac muscles and are thought to play important roles in myosin folding and sarcomere assembly. The presence of point mutations in and knockdown of *unc45b* in zebrafish resulted in a complete lack of motility and myofibril disorganization of both skeletal and cardiac muscles (Wohlgemuth et al., 2007; Etard et al., 2007). Similarly, knockdown of *hsp90α* also exhibited defective motility and sarcomere disorganization (Hawkins et al., 2008; Du et al., 2008b). Other studies indicated that Unc45b may regulate myosin filaments by degrading misfolded myosin through a ubiquitin/proteasome system (Landsverk et al., 2007) or by helping the folding of the myosin motor domain (Liu et al., 2008). Recent studies indicate that Unc45b and Hsp90α may interact with each other to form a chaperone-co-chaperone pair that plays important roles in the assembly of the sarcomere (Etard et al., 2007; Liu et al., 2008; Srikakulam et al, 2008; Etard et al., 2008). Through powerful molecular and genetic approaches, additional factors involved in the process of myosin folding and sarcomere assembly are being identified. For example, our recent studies demonstrated that Smyd1, a newly identified methyltransferase expressed specially in skeletal and cardiac muscles, plays a vital role in sarcomere assembly in the skeletal and cardiac muscles of zebrafish embryos (Tan et al., 2006).

1.4 Smyd1

1.4.1 Smyd1 function in myofibrillogenesis

Smyd1 is a member of the Smyd family of proteins and plays an important role in muscle development (Gottlieb et al., 2002; Tan et al., 2006). Smyd1 is expressed

specifically in skeletal and cardiac muscles during embryogenesis and in adult muscle tissues. The regulation of *smyd1* expression in skeletal and cardiac muscles is well studied. Smyd1 is a direct target of the transcription factors Mef2c and Myod (Park et al., 2010; Phan et al., 2005). A recent report showed that *smyd1* expression is also regulated by serum response factor (SRF) and by myogenin through direct binding to the promoter region of *Smyd1* (Li et al., 2009). In addition, *Smyd1* gene expression can be repressed by Hepatoma-derived growth factor through interaction with a transcriptional co-repressor C-terminal binding protein (CtBP) (Yang et al., 2007).

The *in vivo* function of *Smyd1* has been uncovered in recent years. Loss-of-function studies demonstrated that *Smyd1* is essential for embryonic survival in mice and zebrafish. *Smyd1* knockout mouse embryos die around embryonic day 10.5 from cardiomyogenic defects (Gottlieb et al., 2002). Our recent functional studies in zebrafish showed that *smyd1* is required for myofibrillogenesis in skeletal and cardiac muscles in zebrafish embryos (Tan et al., 2006; Li et al., 2011). Knockdown of *smyd1b*, one of the two *smyd1* genes in zebrafish, resulted in myofibril disorganization in skeletal and cardiac muscles in zebrafish embryos (Tan et al., 2006; Li unpublished). This was confirmed recently in a zebrafish mutant carrying a mutation in the *smyd1* gene (Just et al., 2011).

At present, little is known about the mechanism by which Smyd1 functions in myofibrillogenesis. It has been shown that Smyd1 has histone methyltransferase activity that can methylate histone 3 lysine 4 *in vitro* (Hamamoto et al., 2004; Tan et al., 2006). Histone methylation plays a vital role in gene transcriptional regulation, supporting a potential role for Smyd1 in transcriptional regulation. Several pieces of

evidence support this view. These include the association of Smyd1 with the skeletal and heart muscle-specific transcription factor skNAC and the co-localization of Smyd1 and skNAC in the nucleus of muscle cells early in differentiation (Sims et al., 2002; Park et al., 2010). In addition, Smyd1 can function as a histone deacetylase (HDAC)-dependent transcriptional repressor by regulating chromatin modifications (Gottlieb et al., 2002).

However, arguing against its potential role in transcriptional regulation, Just and colleagues reported that the Smyd1 mutant lacking histone methyltransferase activity is biologically active in myofibril assembly (Just et al., 2011). Consistent with its potential role in the cytosol, little or no nuclear localization of Smyd1 could be detected during muscle development in zebrafish embryos. In fact, we showed that Smyd1 is primarily localized in the cytosol of myoblasts and myotubes, followed by sarcomeric localization (Li et al., 2011). The biological significance of this sarcomeric localization is currently under investigation. Recent studies have indicated that members of the Smyd family can also methylate non-histone proteins, raising the possibility that Smyd1 may have methylation targets in the cytosol of muscle cells.

Smyd1 has been shown to play a regulatory role in myosin assembly when associated with the Unc45b and Hsp90α complex. A recent report showed that GST-tagged Smyd1 is capable of specifically pulling down skeletal muscle-specific myosin heavy chain (Just et al., 2011). In addition, in *smyd1* mutant zebrafish embryos, muscle chaperones *hsp90α1* and *unc45b* were strongly upregulated, indicating a close relationship among Smyd1, Hsp90α1, and Unc45b (Just et al., 2011). Knockdown of *smyd1* causes an effect similar to that of loss-of-function of *hsp90α1* or *unc45b*,

resulting in sarcomere disorganization and the degradation of myosin (Landsverk et al., 2007; Hawkins, et al., 2008; Du et al., 2008b). It has been shown that Hsp90α and Unc45b shuttle from the Z-line to the A-band under conditions of stress. This indicates a regulatory role for Hsp90α and Unc45b when myosin homeostasis is disturbed (Etard et al., 2008). Recent studies by us and others have shown that Smyd1 is localized to the M-line (Li et al., 2011; Just et al., 2011) and that it performs a similar A-band shuttling (Just et al., 2011). This raises the possibility that the lysine methyltransferase Smyd1 may play a regulatory role in myosin folding or unfolding during myofibrillogenesis under conditions of stress. However, the physiological target protein(s) of Smyd1 have not been identified.

Some recent reports have shown that Smyd2, another member of the Smyd family, is capable of methylating Hsp90 (Abu-Farha et al., 2011; Donlin et al. 2012). Methylated Hsp90, Smyd2, and the sarcomere protein titin form a complex, which contributes to the stability of titin and the sarcomere (Donlin et al. 2012). This finding raises the question of whether Smyd1 can methylate Hsp90, which is involved in myosin folding and assembly. To date, several sarcomere proteins, such as myosin, actin, and creatine kinase have been shown to be methylated at their lysine residues (Tong and Elzinga, 1983; Iwabata et al., 2005). However, the function of the methylation of these proteins and the enzyme(s) that drive the methylation events are still unknown.

1.4.2 Smyd family lysine methyltransferases

1.4.2.1 Smyd family proteins and histone methylation

Smyd1, as well as other Smyd family proteins, contains the conserved **S**u(var) 3–9 (suppressor of position effect variegation), **E**nhancer of zeste, and **T**rx (trithorax) (SET) domain and **MY**eloid, **N**ervy, and **D**EAF-1 (MYND) zinc finger domains, which are involved in protein methylation and protein-protein interaction, respectively (Sirinupong et al., 2010). The Smyd subfamily consists of five members, Smyd1-Smyd5 (Fig. 1). Smyd1, Smyd2, and Smyd3 were shown to be capable of specifically methylating the lysine sites of histone 3 *in vitro* and to play important roles in regulating gene expression and cell proliferation (Hamamoto et al., 2004; Brown et al., 2006; Tan et al., 2006; Abu-Farha et al., 2008; Kim et al., 2009).

1.4.2.2 Smyd family proteins and non-histone methylation

In addition to histone methylation, it has been demonstrated that Smyd family proteins are capable of methylating non-histone proteins and play important regulatory roles. The methylation of non-histone proteins has distinct effects on protein stability, which can lead to either the stabilization or de-stabilization of the proteins (Eakin et al., 2007; Yang et al., 2009).

In recent years, several reports have given us a clearer view of the mechanism of SET domain methyltransferase-mediated non-histone protein methylation. It was a significant finding that the tumor suppressor p53 and its downstream genes are highly regulated by Smyd2-mediated methylation (Huang et al., 2006; Xie et al., 2008). Consistent with the critical functions of non-histone protein methylation in gene

regulation, myofilament organization has also been shown to be highly regulated by non-histone protein methylation (Donlin et al., 2012).

1.4.3 Structure of Smyd

Crystal structure studies reveal that Smyd1 is a member of the SET and MYND family of lysine methyltransferases (Fig. 2). The separated SET domain distinguishes Smyd proteins from other histone lysine methyltransferases. The SET domain is the catalytic motif, consisting of about 130 amino acids and sharing a conserved alpha/beta topology (Jiang et al., 2011). The SET domain is responsible for lysine methylation and does so by adding methyl groups to lysine residues of proteins using S-adenosylmethionine (AdoMet) as a donor substrate (Jiang et al., 2011). The MYND domain is a zinc finger motif that is involved in protein-protein interaction (Liu et al., 2007). Crystal structure studies reveal that Smyd family proteins also share a helix-turn-helix structure TPR motif-containing C-terminal domain (CTD) followed by the SET domain. It was proposed that the CTD domain plays a regulatory role in modulating the methyltransferase activity of Smyd proteins; however, the detailed mechanism by which this is achieved is still not well understood (Sirinupong et al., 2010; Jiang et al., 2011; Sirinupong et al., 2011).

A recent structural and biochemical analysis revealed that Smyd1 has lower enzymatic activity and weaker histone binding affinity as compared to other histone methyltransferases (Sirinupong et al., 2010), indicating that Smyd1 may not be involved in histone methylation. The SET and MYND domain protein family members (Smyd1-5) share relatively high sequence similarity but exhibit divergence in their substrate specificity (Xu et al., 2011). The methylation targets of Smyd family

proteins span various lysine sites on histone 3 and non-histone proteins. It has been reported that Smyd2 activity can be increased by the binding of Hsp90 (Abu-Farha et al., 2008), raising the possibility that the interaction of Smyd and Hsp90 may be involved in the regulation of substrate binding and the enzymatic activity of Smyd proteins.

1.5 Overview of background information and objectives

Smyd1 is a SET domain-containing protein methyltransferase that is specifically expressed in skeletal and cardiac muscles. Knockdown or mutation of *smyd1* results in defective myofibril assembly in skeletal and cardiac muscles in zebrafish embryos. Immunostaining studies reveal that sarcomere organization is completely disrupted in *smyd1b* knockdown embryos. Currently, little is known about the mechanism by which Smyd1 functions. Thus, to better understand the function of Smyd1 in myofibrillogenesis, the first objective of this work is to characterize the sub-cellular localization of the two alternatively spliced protein isoforms Smyd1b_tv1 and Smyd1b_tv2 of Smyd1 using GFP labeled fusion proteins. In addition, we aim to study the mechanism of Smyd1 function in myofibrillogenesis, specifically, to determine whether knockdown of *smyd1* affects the protein methylation associated with myofibrillogenesis.

Myomesin is thought to play a critical role in M-band formation and sarcomere organization. Knockdown of *smyd1* results in little or no detectable myomesin M-line organization. It has been shown that Smyd1 is required for myosin folding and thick filament assembly. It is not clear whether disruption of myosin thick filament organization by *smyd1* knockdown could indirectly affect M-line formation.

Alternatively, our preliminary data show that Smyd1 is localized to the M-line, arguing for a potential role for Smyd1 in M-line organization. Thus, the disruption of myosin thick filaments and sarcomere organization in *smyd1b* knockdown embryos might be due to the loss of M-line proteins, subsequently leading to the destabilization of sarcomeric organization. The second objective of this study is to test whether knockdown of myomesin expression results in defective M-line organization, and whether inhibiting myomesin expression results in defective thick filament organization.

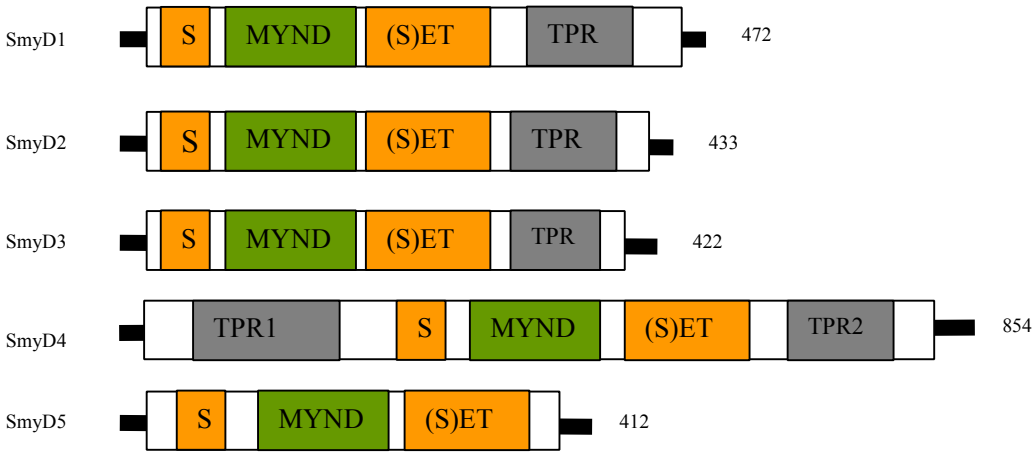


Fig. 1. Schematic of five mammalian Smyd proteins. Orange: Split SET domain, Green: MYND domain, Grey: TPR domain. Positions of amino acids are indicated behind each sequence.

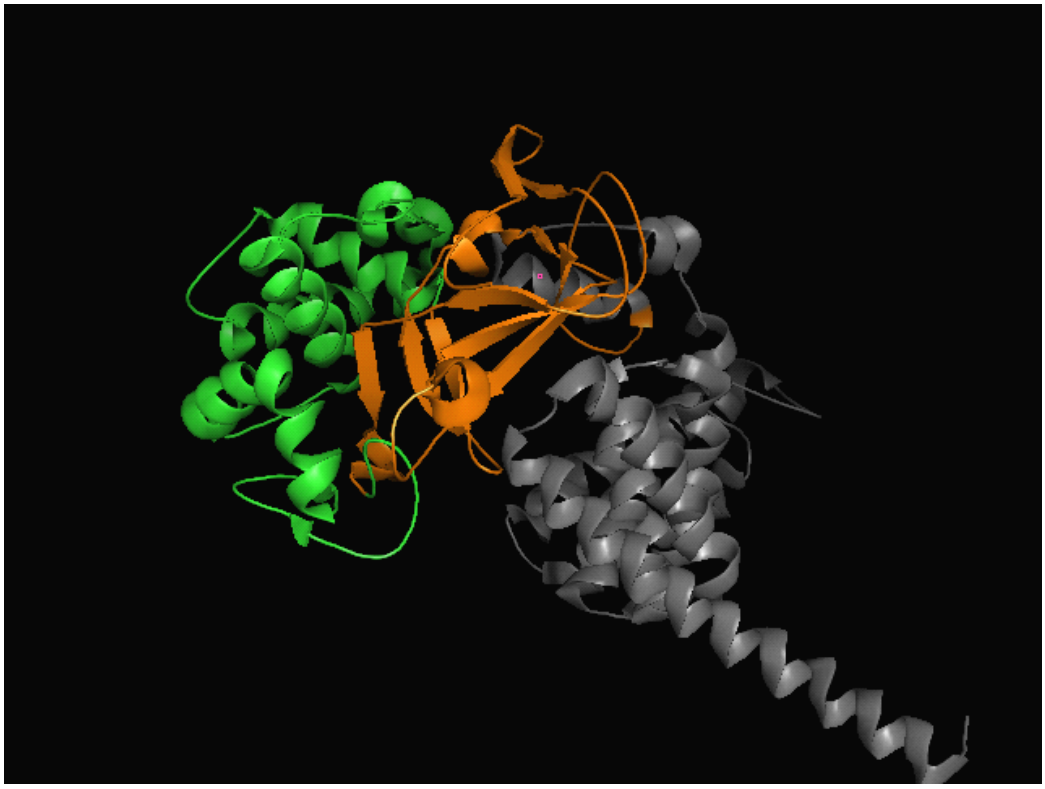


Fig. 2. Structure of Smyd1. The rough locations of the split SET domain, MYND domain, and C- terminal domain, which are represented by orange, green, and grey, respectively, are presented.

Chapter 2: Smyd1b_tv1 is localized to the M-line of skeletal muscle fibers

2.1 Abstract:

Smyd1b is a member of the Smyd family proteins and plays a key role in sarcomere assembly during myofibrillogenesis. *smyd1b* encodes two alternatively spliced protein isoforms, Smyd1b_tv1 and Smyd1b_tv2. To better understand the function of Smyd1b in myofibrillogenesis, we analyzed the sub-cellular localization of Smyd1b_tv1 and Smyd1b_tv2 in transgenic zebrafish by expressing Smyd1b_tv1-EGFP and Smyd1b_tv2-EGFP fusion proteins. Smyd1b_tv1 and Smyd1b_tv2 were primarily localized in the cytosol of myoblasts and myotubes in early stage zebrafish embryos. However, in mature myofibers of late stage embryos, Smyd1b_tv1, and to a small degree of Smyd1b_tv2, exhibited sarcomeric localization. Immunostaining with sarcomeric markers revealed that Smyd1b_tv1 was localized to the M-line. Smyd1b_tv1 differs from Smyd1b_tv2 by a 13-amino acid (aa) insertion encoded by the tv1-specific exon 5, suggesting that some residues within the 13 aa insertion may be critical for the strong sarcomeric localization of Smyd1b_tv1. Comparison of the zebrafish Smyd1b_tv1 sequence with that of orthologs from other vertebrates revealed several highly conserved residues (Phe223, His224, and Gln226) and two potential phosphorylation sites (Thr221 and Ser225) within the 13 aa insertion. To determine whether these residues are involved in the sarcomeric localization of Smyd1b_tv1, we mutated these residues individually into alanine. Substitution of Phe223 or Ser225 with an alanine residue significantly reduced the sarcomeric localization of Smyd1b_tv1. In contrast, other substitutions (His224 and Gln226) had

no effect. Moreover, replacing Ser225 with a threonine residue (S225T) retained the strong sarcomeric localization of Smyd1b_tv1. Together, these data indicate that Phe223 and Ser225 are required for the M-line localization of Smyd1b_tv1.

2.2 Introduction:

Smyd1, also known as m-Bop, represents a recently identified SET domain containing protein methyltransferase that is specifically expressed in skeletal and cardiac muscles. It plays critical roles in myofiber maturation and contraction (Tan et al., 2006). Targeted deletion of *Smyd1* in mice leads to embryonic lethality at embryonic day (ED) 10.5 (Gottlieb et al., 2002). Knockdown or mutation of *smyd1* in zebrafish leads to defective myofibrillogenesis and the disruption of the sarcomere (Tan et al., 2006; Just et al., 2011).

It has been suggested that Smyd1 functions as a transcriptional repressor that is dependant upon HDAC activity (Gottlieb et al., 2002). Further biomedical studies have revealed that Smyd1 can methylate histone 3 lysine 4 *in vitro* (Tan et al., 2006). Consistent with its potential roles in transcriptional regulation, Smyd1 colocalizes with a transcription factor called skNAC in the nucleus of C2C12 differentiated myoblast cells (Sims et al., 2002). However, our previous data using a myc-tagged protein showed the cytoplasmic localization of Smyd1b in the myofibers of zebrafish embryos, indicating potential roles for Smyd1b in the cytoplasm.

The *smyd1b* gene encodes two alternatively spliced protein isoforms in zebrafish that are named Smyd1b_tv1 and Smyd1b_tv2. Smyd1b_tv1 differs from Smyd1b_tv2 in that it contains a 13 amino acid insertion encoded by the tv1-specific exon 5. Functional analysis revealed that both *smyd1b_tv1* and *smyd1b_tv2* isoforms are able

to rescue the thick filament defect caused by *smyd1b* knockdown (Tan et al., 2006). It is not clear whether the two isoforms may have distinct localization and functions. A better characterization of the sub-cellular localization of Smyd1b_tv1 and Smyd1b_tv2 is critical for uncovering their potential functional specificity, and for gaining a mechanistic understanding of Smyd1b function in regulating muscle cell differentiation.

In this study, we generated transgenic zebrafish expressing EGFP tagged Smyd1b_tv1 and Smyd1b_tv2. We show that Smyd1b_tv1 and Smyd1b_tv2 are primarily localized in the cytosol of the myoblasts and myotubes of early stage zebrafish embryos. However, Smyd1b_tv1 exhibits sarcomeric localization in mature fibers in late stage embryos. Further studies reveal strong localization of Smyd1b_tv1 to the M-line. This strong M-line localization is dependant on the presence of the Phe223 and Ser225 residues unique to the additional 13 aa in Smyd1b_tv1.

2.3 Materials and Methods:

2.3.1 Construction of Tol2-Smyd1b_tv1-EGFP, Tol2-Smyd1b_tv2-EGFP

pTol2-Smyd1b_tv1-EGFP and pTol2-Smyd1b_tv2-EGFP constructs were generated by cloning the *smyd1b_tv1* and *smyd1b_tv2* coding sequences in-frame upstream of the EGFP coding sequence in the Tol2 vector. Briefly, the *smyd1b_tv1* and *smyd1b_tv2* coding sequences without the stop codon were amplified by PCR using Pfu DNA polymerase (Stratagene, Santa Clara, CA, USA) from DNA plasmids Smyd1-Smyd1b^{myc}_tv1 and Smyd1-Smyd1b^{myc}_tv2 using the following primers: Smyd1b-F (5'-CGGGATCCATGGAGTTTGTGGAAGTTTTTGA-3') and Smyd1b-R

(5'-CGGGATCCTTCCTGCGGAACAGGTTCTTGAT-3'). BamHI sites was introduced at the 5' and 3' ends of the *Smyd1b_tv1* or *Smyd1b_tv2* coding sequences via the PCR primers. The PCR products were digested with BamHI and then cloned into the BamHI site of the T2A200R150G vector (Urasaki et al., 2006). The DNA sequence at the *Smyd1b* and EGFP junction was confirmed by sequencing.

2.3.2 Mutagenesis

To generate Smyd1b_tv1-EGFP constructs with S217A, S225A, S225T, 225D, T221A, F223A, H224A or Q226A mutations, we carried out the mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene). The Smyd1-Smyd1b_tv1-EGFP plasmid was used as the DNA template. The following PCR primers were used:

S217A + T221A-f: 5'-AATCAGGCGGCCATCGATGCTGTGTTT-3'

S217A + T221A-r: 5'-AAACACAGCATCGATGGCCGCCTGATT-3'

S225A-f:5'-GTGTTTCACGCTCAGAAGAGG-3'

S225A-r:5'-CCTCTTCTGAGCGTGAAACAC-3'

A225T-f: 5' -GATACTGTGTTTCACACTCAGAAGAGGATTG-3'

A225T-r: 5'-CAATCCTCTTCTGAGTGTGAAACACAGTATC-3'

S225D-f: 5'-ATACTGTGTTTCACGATCAGAAGAGGATTGA-3'

S225D-r: 5'-TCAATCCTCTTCTGATCGTGAAACACAGTAT-3'

F223A-f: 5'-CATCGATACTGTGGCTCACTCTCAGAAG-3'

F223A-r:5'-CTTCTGAGAGTGAGCCACAGTATCGATG-3'

H224A-f: 5'-CGATACTGTGTTTGCCTCTCAGAAGAGG-3'

H224A-r:5'-CCTCTTCTGAGAGGCAAACACAGTATCG-3'

Q226A-f: 5'-TGTGTTTCACTCTGCGAAGAGGATTGAG-3'

Q226A-r:5'-CTCAATCCTCTTCGCAGAGTGAAACACA-3'

2.3.3 Generation and maintenance of transgenic zebrafish lines

Mature zebrafish were raised at the Zebrafish Facility of the Aquaculture Research Center, Institute of Marine and Environmental Technology. The fish were maintained at 28°C with a photoperiod of 14 h of light and 10 h in the dark in 8-gallon aquaria supplied with fresh water and aeration. For the generation of transgenic fish lines, ef1α-Smyd1b_tv1-EGFP and ef1α-Smyd1b_tv2-EGFP constructs were constructed by cloning the cDNA encoding the EGFP-tagged Smyd1b_tv1 or EGFP-tagged Smyd1b_tv2 downstream of the EF1a promoter in the Tol2 vector. The ef1α-Smyd1b_tv1-EGFP and ef1α-Smyd1b_tv2-EGFP constructs were microinjected into zebrafish embryos as described (Xu et al., 2012). Germ-line transgenic founders were identified from F1 embryos by screening with fluorescent microscopy (Zeiss, Oberkochen, Germany) at 24 hours post fertilization (hpf).

2.3.4 Immunostaining of whole-mount fish embryos

Immunostaining was carried out using whole-mount zebrafish embryos with the anti-MyHC antibody (F59 antibody) and anti-α-actinin antibody. Secondary antibodies were FITC or TRITC-conjugates (Sigma). The embryos were photographed under an upright microscope (Zeiss, Oberkochen, Germany) equipped with a confocal image analyzer (Bio-Rad Radiance 2100 Imaging Systems; Bio-Rad, Hercules, CA, USA).

2.4 Results:

2.4.1 Characterization of Smyd1b_tv1 and Smyd1b_tv2 sub-cellular localization in the myofibers of zebrafish embryos expressing the EGFP fusion protein

It has been reported that Smyd1 is first localized to the nucleus of C2C12 myoblasts, followed by strong localization in the cytoplasm of differentiated myotubes (Sims et al., 2002). In zebrafish, *smyd1b* encodes two alternatively spliced protein isoforms, Smyd1b_tv1 and Smyd1b_tv2, which are expressed in skeletal and cardiac muscles (Tan et al., 2006). Smyd1b_tv1 differs from Smyd1b_tv2 by the presence of a 13 amino acid insertion encoded by the *Smyd1_tv1*-specific exon 5 (Fig. 3). To better understand Smyd1b function in myofibril assembly, we analyzed the sub-cellular localization of Smyd1b_tv1 and Smyd1b_tv2 during muscle development in zebrafish embryos via transient expression of their EGFP fusion proteins by DNA injection. The DNA constructs pTol2-Smyd1b_tv1-EGFP and pTol2-Smyd1b_tv2-EGFP expressing the EGFP-tagged Smyd1b_tv1 and Smyd1b_tv2 fusion proteins, respectively, (Fig. 4A) was injected into fertilized zebrafish eggs at the 1–2 cell stage. In addition, the biological activities of these EGFP fusion proteins were confirmed by a rescue assay in zebrafish embryos. The rescue assay was performed by co-injecting *smyd1b* morpholino (MO) with the pTol2-Smyd1b_tv1-EGFP or pTol2-Smyd1_tv2-EGFP DNA constructs. The data revealed that Smyd1b_tv1-EGFP and Smyd1b_tv2-EGFP fusion proteins were biologically active (Fig. 4B–G). In the rescue experiment, a clear thick filament organization was detected in a mosaic pattern in the co-injected embryos, consistent with the mosaic pattern of Smyd1b_tv1-EGFP and Smyd1b_tv2-

EGFP expression. Moreover, clear sarcomeric localization was detected for Smyd1b_tv1-EGFP but not for Smyd1b_tv2-EGFP in 48 hpf embryos (Fig. 4B and C).

To further characterize the sub-cellular localization of Smyd1b_tv1 and Smyd1b_tv2 during development, the pTol2-Smyd1b_tv1-EGFP and pTol2-Smyd1b_tv2-EGFP DNA constructs were separately injected into zebrafish embryos at the 1–2 cell stage. The injected embryos were fixed at 48 hpf and 96 hpf. Smyd1b_tv1-EGFP exhibited clear sarcomeric localization at both 48 hpf and 96 hpf (Fig. 5A and D). However, Smyd1b_tv2-EGFP showed weaker sarcomeric localization as compared to Smyd1b_tv1-EGFP (Fig. 5B and E).

2.4.2 Characterization of the sub-cellular localization of Smyd1b_tv1 and Smyd1b_tv2 in transgenic zebrafish embryos

To further confirm the sub-cellular localization of Smyd1b_tv1 and Smyd1b_tv2, two transgenic zebrafish lines expressing EGFP-tagged Smyd1b_tv1 or EGFP-tagged Smyd1b_tv2 under the control of the EF-1α promoter were generated. Rescue studies revealed that expression of the *smyd1b_tv1-EGFP* and *smyd1b_tv2-EGFP* transgenes was sufficient to rescue the loss of endogenous Smyd1b expression induced by morpholino knockdown, indicating that the Smyd1b_tv1-EGFP and Smyd1b_tv2-EGFP fusion proteins from the transgenes were biological active (Fig. 6). We next examined the sub-cellular localization of Smyd1b_tv1-EGFP and Smyd1b_tv2-EGFP in zebrafish embryos at different stages of development. The results showed dynamic sub-cellular localization of Smyd1b_tv1-EGFP and Smyd1b_tv2-EGFP during muscle development. In the early developmental stage around 22 hpf, Smyd1b_tv1-

EGFP and Smyd1b_tv2-EGFP were primarily localized in the cytosol with little or no nuclear localization (Fig. 7A and B). As development proceeded, sarcomeric localization of Smyd1b_tv1-EGFP was observed in the first batch of mature myofibers in zebrafish embryos around 24–27 hpf (Fig. 7C and E). As more fibers matured during development, sarcomeric localization was observed in additional myofibers of late stage 48 and 72 hpf embryos (Fig.7G and I). In contrast, only weak sarcomeric localization could be detected for Symd1b_tv2-EGFP at 48 and 72 hpf (Fig. 7H and J).

2.4.3 Smyd1b_tv1 is localized to the M-line of sarcomeres

To better define the sarcomeric localization of Smyd1b_tv1 and Smyd1b_tv2, we analyzed the sub-cellular localization of Smyd1b_tv1-EGFP in zebrafish embryos expressing a myomesin-3-RFP fusion protein at the M-line. The myomesin-3-RFP expressing line was obtained from Ekker's lab (Clark et al., 2011). Myomesin-3 is the major component of the sarcomeric M-line structure in slow muscles. Thus, myomesin-3-RFP expression represents the M-line structure. The pTol2-Smyd1b_tv1-EGFP construct was microinjected into myomesin-3-RFP expressing zebrafish embryos at the 1–2 cell stage. The sarcomeric localization of Smyd1b_tv1-EGFP (green) and myomesin-3-RFP (red) was determined by confocal microscopy at 96 hpf. Clear co-localization of Smyd1b_tv1-EGFP and myomesin-3-RFP was observed (Fig. 8A, C, and E). Moreover, co-staining with anti-Myosin Heavy Chain (MHC) antibody revealed that Smyd1b_tv1-EGFP was localized in the middle of the A-bands (Fig. 8B, D, and F), consistent with M-line localization. Together, these data

indicate that Smyd1b_tv1 is localized to the M-line of skeletal muscles, and thus may be involved in M-line organization.

2.4.4 Phe223 and Ser225 are critical for the M-line localization of Smyd1b_tv1

Several conserved residues were identified within the 13 aa insertion specific to Symd1b_tv1. These include three potential phosphorylation sites at Ser217, Thr221, and Ser225 (Fig. 9A). To test directly whether these three residues are required for the sarcomeric localization of Smyd1b_tv1, substitutions were made in the DNA sequences encoding these residues by replacing the sequences with sequences encoding alanine residues in the pTol2-Smyd1b_tv1-EGFP expression construct. The mutant proteins were expressed in zebrafish embryos by DNA microinjection. The sub-cellular localization of these Smyd1b_tv1-EGFP mutant proteins was carefully examined in the injected zebrafish embryos under a confocal microscope. Previous studies in our lab have shown that the substitution of Ser217 and Thr221 with alanine has no effect on the sarcomeric localization of Smyd1b_tv1S217A+T221A (Li et al., 2011). However, the substitution of Ser225 with alanine abolished the sarcomeric localization of Smyd1b_tv1S225A-EGFP (Fig. 9C). Together, these results indicate that Ser225 is required for the sarcomeric localization of Smyd1b_tv1.

Sequence comparison revealed that Ser225 is replaced by threonine in the chick, mouse, and human orthologs of Smyd1b_tv1 (Fig. 9A). Serine and threonine are similar amino acids in that they are potential sites for post-translational modification by phosphorylation or glycosylation. To determine whether the substitution of Ser225 with threonine (S225T) has an effect on the sarcomeric localization of Smyd1b_tv1,

we generated the Smyd1b_tv1S225T-EGFP mutant and analyzed its sub-cellular localization in zebrafish embryos. The result showed that the S225T substitution had no effect on the sarcomeric localization of Smyd1b_tv1S225T-EGFP (Fig. 9D). To determine whether the potential phosphorylation of Ser225 could be involved in the sarcomeric localization of Smyd1b_tv1, we substituted Ser225 with aspartic acid (S225D). It has been reported that the substitution of serine residues with aspartic acid mimics serine phosphorylation (Leger et al., 1997; Saad et al., 2007). Our result showed that the S225D substitution abolished the sarcomeric localization of Smyd1b_tv1S225D-EGFP (Fig. 9E), indicating that post-translational modification by phosphorylation may not be involved in the sarcomeric localization of Smyd1b_tv1. The S225A substitution might lead to a change in protein structure, masking or changing the translocation signal required for its M-line localization. To evaluate the possibility that the highly conserved amino acids surrounding Ser225 might be involved in M-line localization, we mutated additional aa residues at F223, H224 and Q226. We observed that mutation of the conserved Phe223 (F223) residue dramatically diminished the M-line localization of the mutant protein (Fig. 9F). By contrast, mutating the two other conserved residues (H224, Q226) had no effect on protein localization (Fig. 9G and H). Collectively, these data indicate that Phe223 and Ser225 are required for the sarcomeric localization of Smyd1b_tv1. The S225A and F223A substitutions might lead to a change in protein structure that masks or changes the translocation signal or protein motif required for the M-line localization of the protein.

2.5 Discussion:

In this study, we analyzed the sub-cellular localization of Smyd1b_tv1 and Smyd1b_tv2 during muscle development in zebrafish embryos. We found that Smyd1b_tv1 and Smyd1b_tv2 were primarily localized in the cytosol in early stage embryos. However, they showed sarcomeric localization in differentiated myofibers. Compared with Smyd1b_tv2, Smyd1b_tv1 showed stronger sarcomeric localization, and the sarcomeric localization was restricted to the M-line. The Phe223 and Ser225 residues, which are located within the Smyd1b_tv1-specific 13 aa insertion, are necessary for the sarcomeric localization of Smyd1b_tv1.

2.5.1 Sarcomeric localization of Smyd1b_tv1 and Smyd1b_tv2 in myofibers

Recent studies have indicated potential roles for Smyd1 in regulating gene transcription in the nucleus. It has been reported that Smyd1 might act as an HDAC-dependent repressor *in vitro* (Gottlieb et al., 2002). Biochemical studies indicate that Smyd1 is capable of interacting with the transcription factor skNAC (Sims et al., 2002; Park et al., 2010). Although Smyd1 was found to be initially localized in the nucleus of C2C12 cells, it was later found to translocate to the cytoplasm (Sims et al., 2002). In this study, we found that both isoforms of Smyd1b are primarily localized in the cytosol at early developmental stages of zebrafish embryos. No nuclear localization of Smyd1b could be detected. Instead, we found that Smyd1b_tv1 exhibited strong sarcomeric localization. Co-staining with sarcomere specific markers revealed that Smyd1b_tv1 was localized to the M-line. Our findings are consistent

with a recent report by Just and colleagues showing the M-line localization of Smyd1b_tv1 in zebrafish skeletal muscles (Just et al., 2011). Due to the critical role of the M-line in sarcomere organization, the M-line localization of Smyd1b_tv1 might have important biological functions.

Despite the different extents of M-line localization of Smyd1b_tv1 and Smyd1b_tv2, both isoforms were able to rescue the loss of Smyd1b function induced by morpholino knockdown (Tan et al., 2006). Sequence analysis revealed that both isoforms contain a potential myosin binding region located at the C-terminal end of the protein. A biomedical study showed that Smyd1b-GST was capable of binding to myosin in a pull down assay (Just et al., 2011). Interestingly, an M-line to A-band shuttling event was observed for Smyd1b_tv1 under conditions of stress (Just et al., 2011). Strikingly, similar A-band shuttling events have been found for Hsp90α and Unc45b, which are molecular chaperones involved in myosin folding and assembly (Etard et al., 2008). Together, these data indicate that Smyd1b may play an important role in myofibrillogenesis by regulating myosin assembly.

Recent studies have also revealed that some non-histone proteins can be methylated by Smyd family members. The methylation of the non-histone proteins play important roles in protein stability (Huang et al., 2006; Donlin et al., 2012). We speculate that Smyd1b can methylate structural or regulatory proteins that are involved in sarcomere assembly and stability and are found at the M-line or A-band. However, the direct target(s) of Smyd1b remains to be determined.

2.5.2 Regulation of sarcomere localization by Phe223 and Ser225

In this study, we showed that Smyd1b_tv1 exhibits greater M-line localization than Smyd1b_tv2 in mature myofibers. The enhanced M-line localization of Smyd1b_tv1 requires Phe223 and Ser 225. Substitution of Phe223 or Ser225 with alanine significantly reduced the M-line localization of Smyd1b_tv1 to a level similar to that of Smyd1b_tv2. Sequence analysis indicated that Ser225 of Smyd1b_tv1 could be a potential phosphorylation site. Phosphorylation is a common post-translational modification involved in the regulation of protein sub-cellular localization. Consistent with the potential regulation of Smyd1b by phosphorylation of Ser225, substitution of serine with the amino acid threonine, which is also commonly phosphorylated, did not affect the M-line localization of Smyd1b_tv1^{S225T}. However, substitution of Ser225 with aspartic acid, which mimics phosphorylation, significantly reduced the M-line localization of Smyd1b_tv1^{S225D}. These data indicate that the M-line localization of Smyd1b_tv1 might not result from the phosphorylation of Ser225. An alternative explanation is that the Ser225 and Phe223 residues are critical amino acids for maintaining protein conformation. Structural analysis revealed that Ser225 and Phe223 are located in the core SET domain (Sirinupong et al., 2010). The substitution of Ser225 or Phe223 might lead to decreased enzyme activity or reduced binding affinity to target proteins.

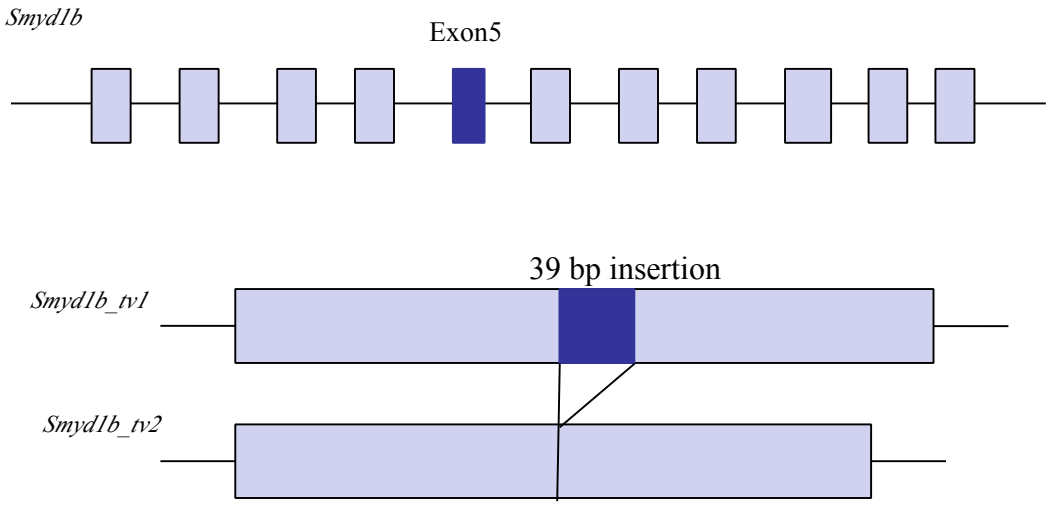


Fig. 3. Schematic representation of Smyd1b_tv1 and Smyd1b_tv2. *smyd1b_tv1* and *smyd1b_tv2* transcripts are generated by alternative splicing. Their cDNA sequences are identical, with the exception of a 39 bp insertion encoded by exon 5. Exon 5 translates into a 13 amino acid insertion in Smyd1b_tv1.

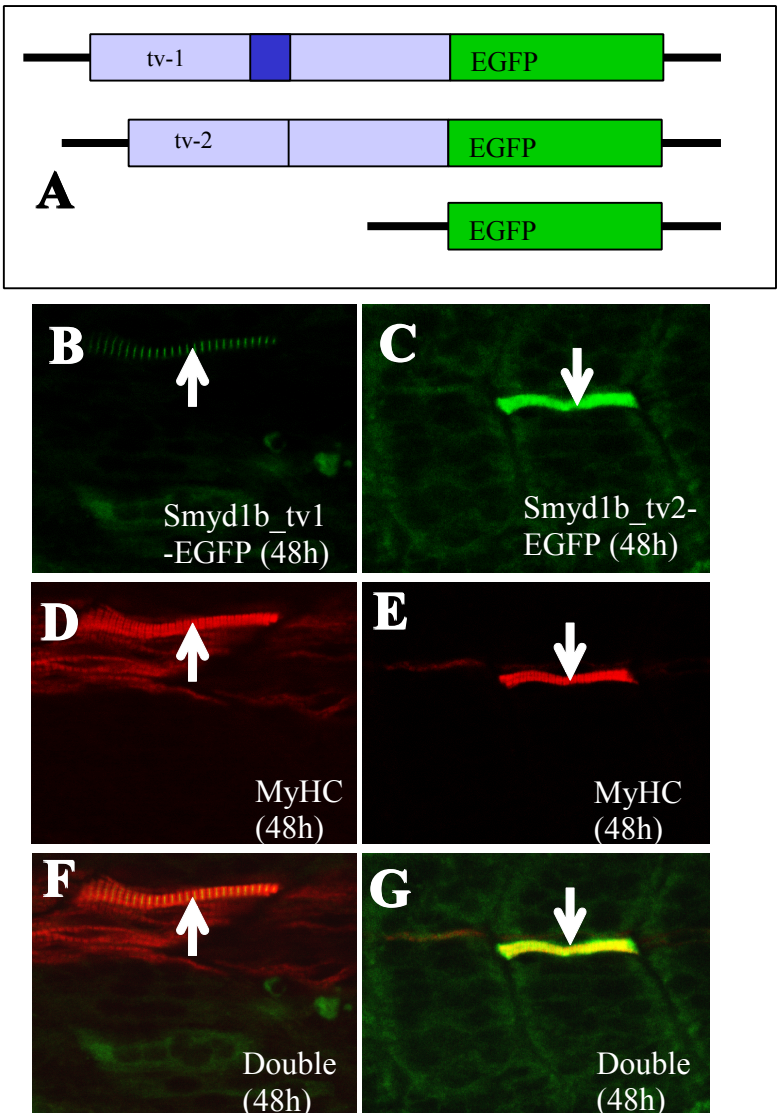


Fig. 4. Rescue of the myofibril organization defect in *smyd1b* knockdown embryos by the expression of Smyd1b_tv1-EGFP or Smyd1b_tv2-EGFP fusion proteins.

A. DNA constructs encoding Smyd1b_tv1-EGFP fusion protein, Smyd1b_tv2-EGFP fusion protein, or EGFP were generated and injected into zebrafish embryos. B and C. Myofibers expressing Smyd1_tv1-EGFP (B) or Smyd1_tv2-EGFP (C) were directly observed under fluorescence.

D and E. Myosin thick filament organization was determined by F59 antibody staining in Smyd1_tv1-EGFP (D) and Smyd1_tv2-EGFP (E) co-injected embryos.

F and G. Double staining showing the colocalization of normal fibers, which are indicated by F59 staining, with Smyd1_tv1-EGFP (F) or Smyd1_tv2-EGFP (G) expression.

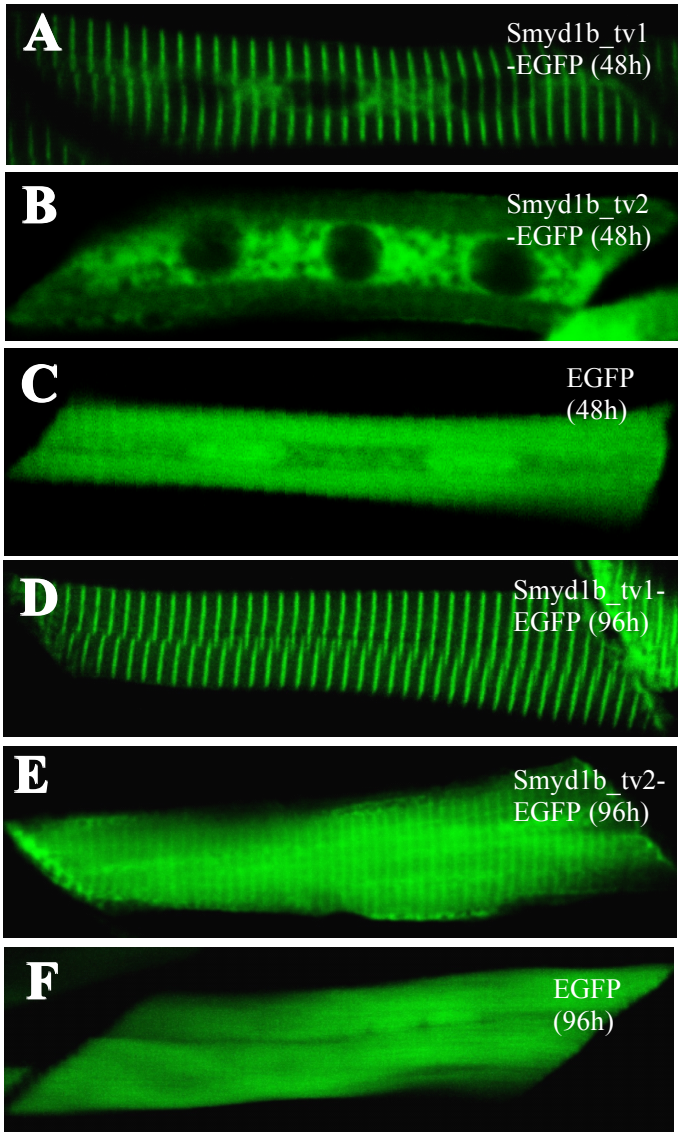


Fig. 5. Characterization of sarcomeric localization using Smyd1b_tv1-EGFP and Smyd1b_tv2-EGFP fusion proteins. DNA constructs encoding Smyd1b_tv1-EGFP fusion protein, Smyd1b_tv2-EGFP fusion protein, or EGFP were injected into zebrafish embryos. Protein expression and localization were determined in myofibers of the injected zebrafish embryos at 48 hpf (A–C) and 96 hpf (D–F).
A and D, Smyd1b_tv1-EGFP;
B and E, Smyd1b_tv2-EGFP;
C and F, EGFP control.

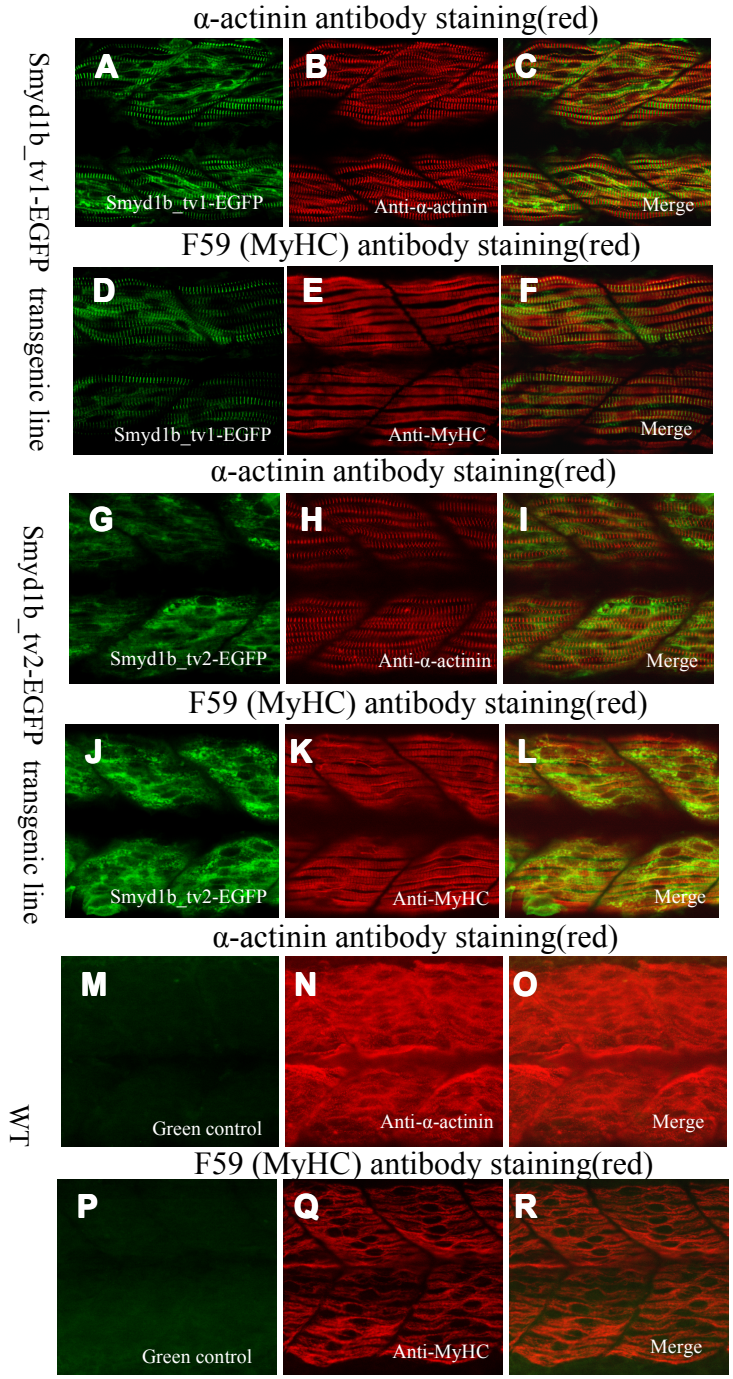


Fig. 6. Rescue of the myofibril organization defect caused by *smyd1b* knockdown in Smyd1b_tv1-EGFP or Smyd1b_tv2-EGFP transgenic fish.

A–F. Rescue of the myofibril organization defect caused by *smyd1b* knockdown in Smyd1b_tv1-EGFP transgenic fish. Sarcomere organization was examined by α-actinin antibody staining (A–C) and F59 (MyHC) staining (D–F).

G–L. Rescue of the myofibril organization defect caused by *smyd1b* knockdown in Smyd1b_tv2-EGFP transgenic fish. Sarcomere organization was examined by α-actinin antibody staining (G–I) and F59 (MyHC) staining (J–L).

M–R. Defective myofibril organization in WT fish embryos results from *smyd1b* knockdown. Sarcomere organization was examined by α-actinin antibody staining (M–O) and F59 (MyHC) staining (P–R).

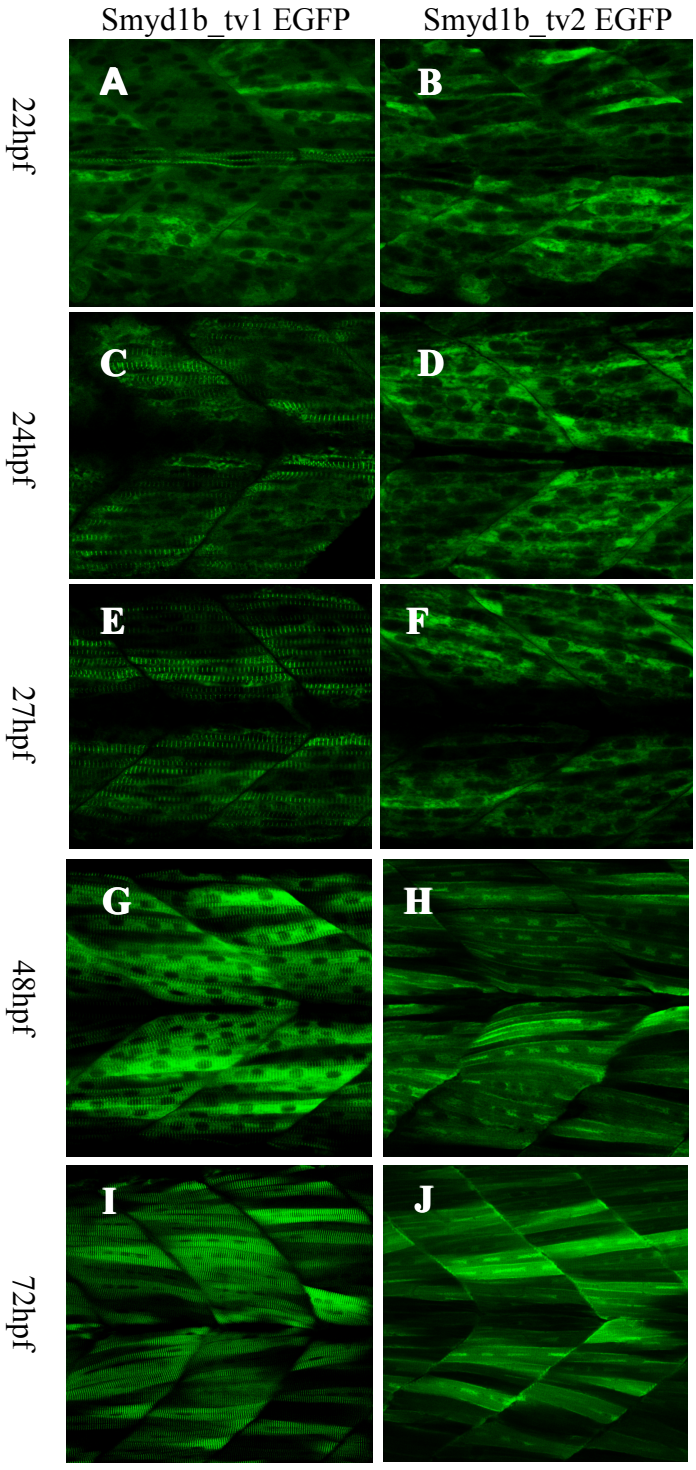


Fig. 7. Smyd1b_tv1-EGFP and Smyd1b_tv2-EGFP show dynamic localization during myofibrillogenesis in zebrafish embryos.
A and B. Cytosolic localization was exhibited by both Smyd1b_tv1-EGFP (A) and Smyd1b_tv2-EGFP (B) in transgenic zebrafish embryos at 22 hours post fertilization (hpf).
C–F. Sarcomeric localization of Smyd1b_tv1-EGFP was seen at 24 hpf (C) and was enhanced by 27 hpf (E). Sarcomeric localization of Smyd1b_tv2-EGFP could not be detected at 24 hpf (D) or 27 hpf (F).
G–K. Strong sarcomeric localization of Smyd1b_tv1-EGFP was exhibited in 48 hpf (G) and 72 hpf (I) embryos. No or weak sarcomeric localization of Smyd1b_tv2-EGFP could be detected by 48 hpf (H) and 72 hpf (J).

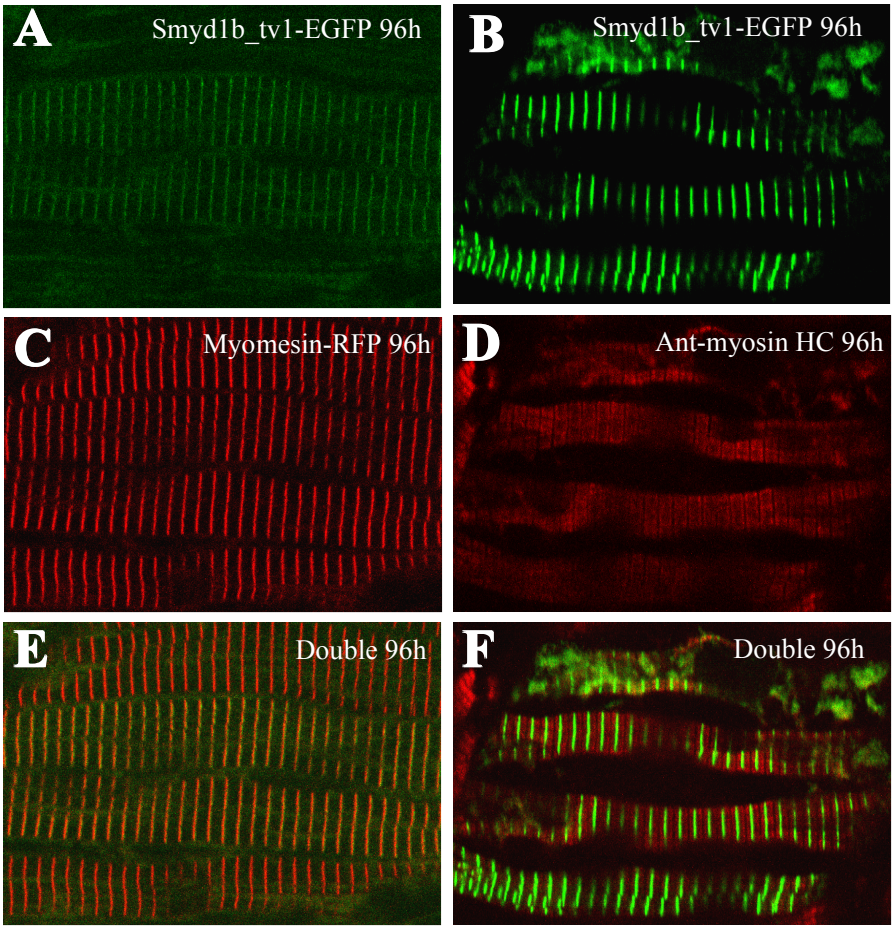


Fig. 8. Smyd1b_tv1-EGFP is localized to the M-line of zebrafish skeletal muscles.

The *smyd1b_tv1-EGFP* construct was injected into *myomesin-RFP* (A, C, and E) or wild type (B, D, and F) zebrafish embryos at the 1–2 cell stage. Smyd1b_tv1-EGFP localization was determined together with that of an M-line marker (myomesin-RFP) and an A-band marker (myosin heavy chain (MyHC)) at 96 hpf.

A, C, and E. Co-localization of Smyd1b_tv1-EGFP and myomesin-RFP was observed in the myofibers of the injected embryos.

B, D, and F. Immunostaining with anti-MyHC antibody (F59) showed the localization of Smyd1b_tv1-EGFP in the middle of the A-bands of myofibers in zebrafish embryos

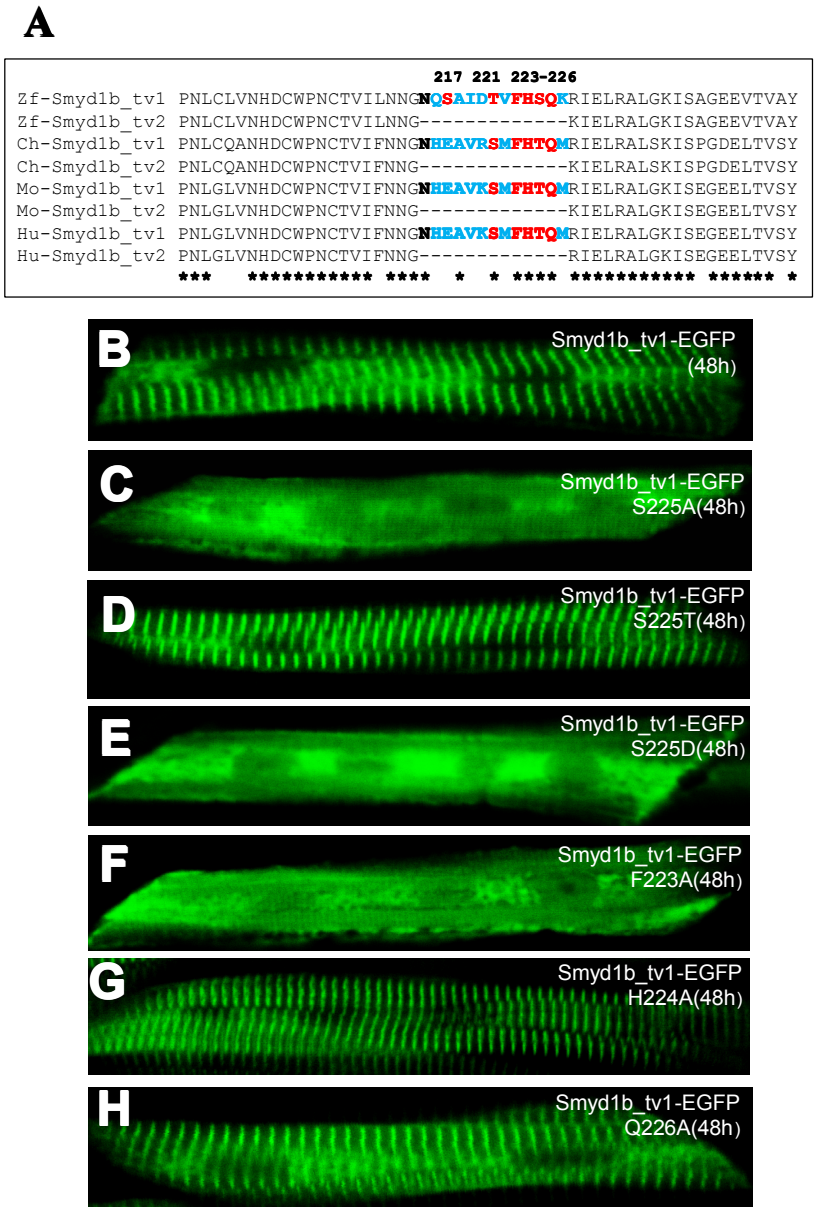


Fig. 9. Effect of S225A, S225T, S225D, F223A, H224A, and Q226A substitution on the sarcomeric localization of Smyd1b_tv1-EGFP in zebrafish embryos. DNA constructs expressing Smyd1b_tv1-EGFP or its derived mutants S225A, S225T, S225D, F223A, H224A, and Q226A were injected into zebrafish embryos. Their localization was analyzed in the myofibers of the injected embryos at 48 hpf. A. Sequence comparison shows the alternative splicing of *smyd1b* in various vertebrates and the conserved serine and threonine residues within the 13 aa insertion. B. Smyd1_tv1-EGFP; C, Smyd1_tv1-EGFP (S225A); D, Smyd1_tv1-EGFP (S225T); E, Smyd1_tv1-EGFP (S225D); F, Smyd1_tv1-EGFP (F223A); G, Smyd1_tv1-EGFP (H224A); and H, Smyd1_tv1-EGFP (Q226A).

Chapter 3: Expression and functional analysis of myomesins in myofibrillogenesis in zebrafish embryos

3.1 Abstract:

Myofibrillogenesis, the process of sarcomere formation, requires the close interaction and collaboration of sarcomeric proteins and various sarcomeric structures. The M-line structures are key components of sarcomeres and play a vital role in myofibrillogenesis and sarcomere organization. It has been suggested that myomesin, which is present on the M-line, interacts with myosin proteins and keeps the myosin filament in order (Obermann et al., 1997). However, the role of myomesin in myofibrillogenesis and sarcomere organization has not been elucidated *in vivo*. In this study, we classified the members of the myomesin gene family and analyzed the temporal and spatial expression pattern of each isoform. We carried out a loss-of-function analysis of the *myomesin* isoforms *myomesin-3*, which is specifically expressed in the slow muscles of zebrafish embryos, and *myomesin-1b*, which is highly expressed both in cardiac and skeletal muscles. We demonstrate that knockdown of neither isoform has any effect on the sarcomeric organization of M-lines or other sarcomere structures including the thick and thin filaments and the Z-disc. Together, these studies indicate that *myomesin-1b* and *myomesin-3* are dispensable for sarcomere organization and M-line formation.

3.2 Introduction:

The sarcomere, the basic contractile unit of skeletal and cardiac muscles, is one of the most highly ordered macromolecular assemblies in cells. The sarcomere can be divided into four major compartments: the Z-disc, M-lines, thick filaments, and thin filaments. The M-band, which is thought to crosslink the myosin filaments, plays pivotal roles in sarcomere organization and in the assembly of highly organized thick and thin filaments. The muscle-specific protein myomesin, which is one of the most abundant components of the M-line, is a major candidate thought to mediate the role of M-line bridges in regulating the packaging of thick filaments and in uniformly distributing tension over the myosin filament lattice in activated sarcomeres (Agarkova et al., 2003).

According to the current M-line model, neighboring myosin filaments are connected by myomesin molecules that bind with their N-terminal domains to the myosin rod (Obermmann et al., 1997; Auerbach et al., 1999), and dimerize in an antiparallel fashion via their C termini (Lange et al., 2005). In addition, myomesin can bind tightly to the C-terminal end of the titin string extending into the M-line.

As shown in Chapter 2, Smyd1b_tv1 localizes to the M-line. Consistent with its potential function in M-line organization, little or no organized myomesin could be detected in *smyd1b* knock-down embryos by immunostaining (Li et al., unpublished data). However, other key sarcomere structures, including the thick and thin filament as well as the Z-line were disrupted in the *smyd1b* knockdown embryos. This raises the question of whether the disruption of other sarcomere structures in *smyd1b* knockdown embryos is an indirect effect of the disruption of myosin thick filaments

leading to defective M-line organization, or whether it is due to the direct disruption of myomesin organization. Thus, a loss-of-function study could provide important insight into whether inhibiting *myomesin* expression directly, could result in myofibril defects similar to those seen upon *smyd1* knockdown. However, no knockout models have been generated as yet for *myomesin* due to potential early embryonic lethality. Although RNAi-mediated knockdown of *myomesin* disrupts the M-band structure leading to sarcomeric disorganization in cultured neonatal rat cardiomyocytes (Fukuzawa et al., 2008), the function of myomesin in sarcomere organization is yet to be analyzed *in vivo*.

In this study, we identified 5 *myomesin* genes in zebrafish and analyzed their patterns of expression during development. We specifically knocked down *myomesin-1b* and *myomesin-3* in zebrafish and analyzed the muscle phenotype in these fish in terms of sarcomere organization and M-line localization of Smyd1b_tv1. We demonstrate that the knockdown of neither *myomesin-1b* nor *myomesin-3* has any effect on sarcomere organization. On the contrary, the knockdown of *hsp90α1* and *unc45b*, which are thought to be involved in myosin folding, severely disrupts M-line organization.

3.3 Materials and Methods:

3.3.1 Time-course Reverse Transcriptase (RT)-PCR

Total RNA was extracted from zebrafish embryos at 0, 3, 6, 9, 12,14, 19, and 24 hpf and 2, 3, 4, 5, and 6 days post fertilization (dpf) using TRIZOL (Ambion, NY, USA) according to the manufacturer's instructions. Extracted RNA was converted to

cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, MD, USA). *myomesin1a/1b/2a/2b/3* were amplified using the primer pairs MyoM1a-FP1/MyoM1a-RP1, MyoM1b-FP1/MyoM1b-RP1, MyoM2a-FP1/MyoM2a-RP1, MyoM2b-FP1/MyoM2b-RP1, and MyoM3-FP1/MyoM3-RP1, respectively. *Elongation factor1α* (*ef-1α*) was amplified as a control.

ef-α1-P1, 5'-GCATACATCAAGAAGATCGGC- 3'

ef-1α-P2, 5'-GCAGCCTTCTGTGCAGACTTTG-3'

MyoM1a-FP1: 5'-TTAACAGATGACCAGTACACCTTCCAAA-3'

MyoM1a-RP1: 5'-ATTTAAGCTGCATGTACTGGTAATTCAA-3'

MyoM1b-FP1: 5'-GAAAGGAGGACTACGAAGAACTGTGGAG-3'

MyoM1b-RP1: 5'-CATAATTAAACAGCATAGTTGTGCTTGT-3'

MyoM2a-FP1: 5'-TCAAGAAGTAGAGCCCAGCGATCAGTAC-3'

MyoM2a-RP1: 5'-AAGGCAGTATTTATTTATACATTAAACC-3'

MyoM2b-FP1: 5'-ATATCATCAATGCCATCGCCAGTATT-3'

MyoM2b-RP1: 5'-TGTAGACACTGACTGTTATCTCCAC-3'

MyoM3-FP1: 5'-TCAGGACACACACAAGCGCTCGCTGGAT-3'

MyoM3-RP1: 5'-AGAGACTACATCTTTATTTATCAAGCAC-3'

3.3.2 Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was carried out using digoxigenin-labeled antisense probes. The fragments for probes were generated by PCR amplification using the primers discussed above (Time-course RT-PCR section). The PCR products were purified and cloned into pGEM-T Easy vectors (Promega, WI, USA) to generate the *pGEM-myomesin1a-probe, pGEM-myomesin1b-probe, pGEM-*

myomesin2a-probe, pGEM-myomesin2b-probe, and pGEM-myomesin3-probe plasmids. *pGEM-myomesin1a/1b/2b/3-probes* were linearized with NcoI, and *pGEM-myomesin2a-probe* was linearized with SphI. *pGEM-myomesin1a/1b/2b/3-probes* and *pGEM-myomesin2a-probe* were transcribed with SP6 and T7 RNA polymerases, respectively. These antisense probes hybridize with specific isoforms of *myomesin* mRNA transcripts because the sequences in the 3'UTR used for making antisense probes are highly diverse among the different isoforms of myomesin genes.

3.3.3 Immunostaining of whole-mount fish embryos

Immunostaining was carried out using whole-mount zebrafish embryos with the following antibodies: anti-actinin, anti-MyHC for slow muscles, anti-myomesin, and anti-actin. Secondary antibodies were FITC or TRITC-conjugates (Sigma, MO, USA). The embryos were photographed under an upright microscope (Zeiss) equipped with a confocal image analyzer (Bio-Rad Radiance 2100 Imaging Systems; Bio-Rad, CA, USA).

3.3.4 Synthesis of morpholino-modified antisense oligos

Morpholino (MO) antisense oligos were synthesized by Gene Tools (Carvalis, OR, USA). The translational blockers (Unc-45b-ATG-MO, Hsp90α1-ATG-MO, and Myomesin-3-ATG-MO) were devised based on the sequence near the ATG start site. The splice blocking morpholinos Myomesin-1b-MO1 and Smyd1b-E9I9-MO1 were devised based on the sequence at the exon-intron junctions exon15-intron15 and exon9-intron9, respectively.

Smyd1b-E9I9-MO: 5' -CGTCACCTCTAGGTCTTTAGTGATG-3'

Myomesin-1b-MO1: 5'-CTCTTCATGACAGAATCATACCCAA-3'

Myomesin-3-ATG-MO: 5'-CTTCTGCTCTTCATGCTCTCAGATA-3'

Unc-45b-ATG-MO: 5' -ATCTCCAATTTCTCCCATCGTCATT- 3'

Hsp90α1-ATG-MO: 5'-CGACTTCTCAGGCATCTTGCTGTGT-3'.

3.3.5 Microinjection in zebrafish embryos

Morpholino antisense oligos were dissolved in 1X Danieau buffer to a final concentration of 0.5 mmol/L or 1 mmol/L. DNA plasmids were dissolved in water at 50 ng/mL. Approximately 1–2 nL (5 ng or 10 ng) was injected into each zebrafish embryo at the 1–2 cell stage. For morpholino and DNA co-injection, morpholino (1 mM) and DNA (100 ng/mL) were mixed at a 1:1 ratio and 1–2 nL of the mixture was injected into zebrafish embryos at the 1–2 cell stage.

3.3.6 Morpholino efficiency analysis

To determine the effect of Myomesin-1b-MO1 on splicing, total RNA was extracted from WT and MO-injected embryos at 48 hpf using TRIZOL (Ambion) according to the manufacturer's instructions. Extracted RNA was converted to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). The *myomesin-1b* transcript was amplified by RT-PCR using the primers, MyoM1bRT-FP1 and MyoM1bRT-RP1. The transcripts were analyzed by sequencing.

MyoM1bRT-FP1: 5'-AGGAGGAGCCTGTGGAGGGAGTGG-3'

MyoM1bRT-RP1: 5'-GCCATAGGGTGGAGCAGGTGAAGC-3'

3.4 Results:

3.4.1 Identification of *myomesin* gene family members in zebrafish

In mammals, three isoforms of myomesin that exhibit distinct expression patterns in cardiac and skeletal muscles were identified. It is well known that the zebrafish genome contains duplicated genes. Zebrafish often have two copies of a gene that is present as a single copy in mammals. In order to identify the *myomesin* genes in zebrafish, *myomesin* orthologs were obtained from the National Center for Bioinformatics (NCBI) by blasting the coding sequences of human and mouse *myomesin* isoforms against zebrafish genome.

We showed that there are at least five *myomesin* genes expressed in the zebrafish. Zebrafish *Zfmyomesin-1a* (GeneID: 558671), together with *Zfmyomesin-1b* (GeneID: 100144770), which encodes two alternatively spliced mRNA isoforms named *Zfmyomesin1b_tv1*, *Zfmyomesin1b_tv2,* is an ortholog of the human and mouse *Myomesin-1*. Through EST and genome analyses, two predicted *Zfmyomesin-2-like* sequences named *Zfmyomesin-2-like-1* (GeneID: 100538326) and *Zfmyomesin-2-like-2* (GeneID: 568460) were shown to be part of the same gene and could be linked together by EST analysis. To confirm this result, we amplified a sequence using a forward primer that was located 3' of *Zfmyomesin-2-like-1* and a reverse primer located 5' of *Zfmyomesin-2-like-2*. Sequencing of the RT-PCR product showed that *Zfmyomesin 2-like-1* and *Zfmyomesin 2-like-2* yielded a single sequence as we predicted. The new *Zfmyomesin-2-like* sequence was named *Zfmyomesin-2a*. A newly identified gene *wu:fi38e05* (GeneID: 334269), which was named *Zfmyomesin-2b*, was confirmed as a homolog of *Zfmyomesin-2a*. Finally, the *Zfmyomesin-3* (GeneID:

559614) sequence was confirmed by RT-PCR. Multiple-alignment analysis showed that the five *myomesin* genes in zebrafish are highly conserved. A phylogenetic tree was constructed that revealed a clear relationship among the different orthologs when compared with the mouse and human *myomesin* genes (Fig. 10). Together, these data indicate that there are at least five *myomesin* genes in zebrafish: *Zfmyomesin-1a*, *Zfmyomesin-1b*, *Zfmyomesin-2a*, *Zfmyomesin-2b*, and *Zfmyomesin-3*.

3.4.2 Temporal and spatial expression patterns of *myomesin* genes in zebrafish embryos

The temporal expression pattern of the *myomesin* genes in zebrafish was determined by RT-PCR with gene specific primers derived from the less conserved 3' non-coding regions of the genes. Consistent with their potential roles in muscle development, strong expression of all five *myomesin* genes was detected in zebrafish embryos during myogenesis. However, the five *myomesin* genes exhibited distinct patterns of expression. *Myomesin-2b* was expressed the earliest and its expression levels increased gradually during myogenesis. *Myomesin-1b* and *myomesin-2a* also showed very high levels of expression after 24 hpf. *myomesin-1a* and *myomesin-3* expression levels were not as strong as that of the other three *myomesin* genes (Fig. 11). These data indicate that the temporal expression pattern of *myomesin* genes in zebrafish is highly regulated during development.

To determine the spatial patterns of expression of the various *myomesin* genes in zebrafish embryos, we analyzed their mRNA expression in 48 hpf zebrafish embryos by whole-mount *in situ* hybridization. Five gene-specific probes derived from the less conserved 3'UTR non-coding regions were generated. The results showed that all five

myomesin genes exhibited muscle-specific expression in zebrafish embryos (Fig. 12). The levels of expression were consistent with the RT-PCR data. However, the five *myomesin* genes exhibited different patterns of expression in cardiac and skeletal muscles. The data showed that *myomesin-1b* and *myomesin-2b* were expressed in both skeletal and cardiac muscles (Fig. 12F and L), whereas *myomesin-1a*, *-2a*, and *-3* were only expressed in skeletal muscles in zebrafish embryos and not in cardiac muscles (Fig. 12C, F, and O). Together, these data indicate that the five *myomesin* genes exhibit distinct patterns of muscle-specific expression and that their expression patterns are highly regulated.

3.4.3 Knockdown of *myomesin-1b* expression results in little or no effect on skeletal muscle development

To determine the function of myomesin in muscle cell development and sarcomere organization, we knocked down the expression of *myomesin-1b*, one of the most abundantly expressed *myomesin* genes in zebrafish embryos, by using a splicing MO (Fig. 13A). The effect on *myomesin-1b* splicing was determined by RT-PCR (Fig. 13B). The data showed that splicing of *myomesin-1b* transcripts was efficiently blocked. Compared with native transcripts, the transcripts in the MO injected embryos had a 16 bp deletion in exon 15. The deletion induces a frameshift in the open reading frame resulting in the disruption of proper translation of *myomesin-1b*. The splicing MO injected embryos were examined morphologically for 3 days after injection. The knockdown embryos appeared morphologically normal. No or little defects in muscle contraction or heart beat could be observed. To determine whether the splicing MO may have any effects on myofibril organization, the sarcomeric

organization of MO injected embryos was examined by immunostaining with anti-myomesin, anti-α-actin, and anti-α-actinin specific antibodies. Little or no difference in sarcomere organization could be detected in the MO injected embryos as compared to the WT embryos (Fig. 13C–H). Together, these data indicate that *myomesin-1b* is not required for myofibril organization during myofiber maturation.

3.4.4 Disruption of the *myomesin-3* locus by red fluorescent protein (RFP) trapping in zebrafish

Myomesin-3 is specifically expressed in the slow muscles of zebrafish embryos (Schoenauer et al., 2007). A zebrafish *myomesin-3* mutant was recently generated using a RFP trap (Clark et al., 2011). The protein trap integration site in *myom3* (mnGt0067) fish was recently mapped to an intron of the *myomesin-3* gene (Clark et al., 2011). It has been reported that the RFP protein trap integration results in over 99% reduction of *myomesin-3* gene expression (Clark et al., 2011). To better characterize the integration site and the effect of the RFP insertion on *myomesin-3* gene expression, we analyzed the expression of *myomesin-3-rfp* chimeric mRNA transcripts by RT-PCR (Fig. 14B). Sequence analysis revealed that the RFP integration site was located within intron 24 of *myomesin-3* (Fig. 14A). The RFP integration resulted in the production of a myomesin-3-RFP fusion protein that included the N-terminal 957 aa residues of myomesin-3 followed by 13 aa residues coded by part of intron 24 plus an additional 11 aa residues coded by the sequence upstream of the RFP coding sequence in the vector, and finally the RFP sequence (Fig. 14A). The myomesin-3-RFP fusion protein lacks the C-terminal sequence of 219 aa residues.

To determine the effect of RFP integration on *myomesin-3* mRNA expression, we carried out RT-PCR to amplify *myomesin-3* transcripts and *myomesin-3-rfp* fusion mRNA transcripts from total RNA from control and *myomesin-3-RFP* homozygous embryos with specific primers (Fig. 14B). We observed strong expression of *myomesin-3-rfp* fusion transcripts in *myom3* (mnGt0067) homozygous zebrafish embryos. However, no *myomesin-3* transcripts could be detected in *myom3* (mnGt0067) homozygous zebrafish embryos. This is consistent with the report that the integration of the RFP trap significantly reduces the expression of the *myomesin-3* mRNA transcripts to less than 1% of the normal levels (Clark et al., 2011).

3.4.5 Disruption of the *myomesin-3* locus by RFP trapping has no effect on sarcomere organization in slow muscles

To determine whether the disruption of *myomesin-3* expression by the RFP insertion could affect the M-line localization of *myomesin-3*, we analyzed myomesin-3-RFP localization in *myom3* (mnGt0067) homozygous fish embryos. Normal M-line localization of myomesin-3-RFP was seen in slow muscles (Fig. 15C), suggesting that the myomesin-3 N-terminal region contains the sequence required for the M-line localization of myomesin-3. To determine whether the disruption of *myomesin-3* expression by the RFP insertion could affect sarcomere organization in slow muscles, we characterized the organization of thick and thin filaments and Z-lines in *myom3* (mnGt0067) homozygous fish embryos. The results showed that blocking *myomesin-3* expression by RFP insertion had little or no effect on sarcomere organization (Fig. 16). Both thick and thin filaments as well as Z-lines appeared normal in the slow muscles of *myom3* (mnGt0067) homozygous fish embryos (Fig. 16B, E, and H).

Together, these data indicate that sarcomere organization is not affected by the RFP insertion in the *myomesin-3* gene locus. This is unexpected considering that the RFP insertion in *myomesin-3* resulted in the production of a truncated myomesin-3, and a 99% reduction in the expression of wild type *myomesin-3* mRNA transcripts (Clark et al., 2011).

3.4.6 Knockdown of *myomesin-3* has no effect on sarcomere organization and Smyd1b_tv1 M-line localization in slow muscles

The normal M-line organization of myomesin-3-RFP and the lack of sarcomeric defects in *myomesin-3-RFP* homozygous fish was unexpected. To rule out the possibility that myomesin-3-RFP may retain some myomesin-3 function involved in M-line organization, a knockdown experiment was performed to directly inhibit *myomesin-3* expression using a *myomesin-3* ATG-MO in zebrafish embryos. The *myomesin-3* ATG-MO was injected into *myomesin-3-RFP* embryos. Knockdown efficiency was monitored by examining Myomesin-3-RFP expression in the injected embryos at 48 hpf. The data showed that the *myomesin-3* ATG-MO was highly effective in knocking down the expression of *myomesin3-RFP* (Fig. 15D). To determine the effect of *myomesin-3* knockdown on sarcomere organization, we analyzed sarcomere organization by immunostaining the embryos with specific antibodies against myosin, α-actin, and α-actinin. The data showed that *myomesin-3* knockdown embryos had normal thick and thin filament formation and Z-line organization (Fig. 16C, F, and I). To test the effect of *myomesin-3* knockdown on M-line organization, we examined M-line organization using the EF-1α-Smyd1b_tv1-EGFP transgenic line, as mentioned above, which expresses the Smyd1b_tv1-EGFP

fusion protein on the M-line. The *myomesin-3* ATG-MO was injected into *Smyd1b_tv1-EGFP* zebrafish embryos. The M-line localization of Smyd1b_tv1-EGFP was determined. The data showed that M-line localization of Smyd1b_tv1-EGFP was normal in *myomesin-3* knockdown embryos (Fig. 15H), suggesting that the M-line structure was not disrupted by *myomesin-3* knockdown. Collectively, these data indicate that *myomesin-3* is dispensable for sarcomere formation in the slow muscles of zebrafish embryos.

3.4.7 Hsp90α1 and Unc45b are required for myomesin-3-RFP M-line organization in slow muscles

It has been shown that Hsp90α1 and Unc45b are myosin chaperones that play pivotal roles in myosin folding and sarcomere assembly (Du et al., 2008). Knockdown or mutation of either *hsp90α1* or *unc45b* leads to the complete disruption of the sarcomeric organization of thick and thin filaments in both slow and fast muscles (Du et al., 2008b; Bernick et al., 2010; Fig. 18A–C). The M-lines and Z-discs have also been shown to be disrupted in fast muscles (Du et al., 2008b). However, the effects of *hsp90α1* or *unc45b* knockdown or mutation on M-line organization have not been analyzed in the slow muscles of zebrafish embryos. To characterize the function of Hsp90α1 and Unc45b in M-line organization, we knocked down *hsp90α1* and *unc45b* expression in *smyd1b_tv1-EGFP(+/-)*myomesin-3-RFP(+/+)* fish embryos, and analyzed the localization of Smyd1b_tv1-EGFP and myomesin-3-RFP. The results showed that knockdown of *hsp90α1* and *unc45b* resulted in little or no sarcomeric localization of either Smyd1b_tv1-EGFP or myomesin-3-RFP in the slow muscles of zebrafish embryos (Fig. 17). Together, these data indicate that Hsp90α1

and Unc45b may play a direct role in M-line organization. Alternatively, because Hsp90α1 and Unc45b are required for myosin thick filament assembly, the disruption of thick filament formation by *hsp90α1* and *unc45b* knockdown could lead to M-line disorganization. Consistent with this idea, studies from our lab have shown that the knockdown of myosin expression in slow muscles results in defective myomesin-3-RFP localization (Xu et al., 2012).

It has been suggested that Unc45b and Hsp90α1 work as a complex and are involved in regulating myosin stability (Srikakulam et al., 2008). The binding of Unc45b to other proteins is dependent on the TPR domain, which is located at the N-terminal end of Unc45b (Chadli et al., 2008). However, it has been reported that the TPR domain of Unc45 is not essential for its chaperone function in *C. elegans* (Ni et al, 2011). To determine whether the TPR domain of Unc45 is critical for its function in zebrafish embryos, DNA constructs encoding Unc45b^{myc} or Unc45bΔTPR^{myc} were coinjected with *unc45b* MO into the zebrafish embryos. Double immunostaining analysis using the anti-MHC(F59) and anti-myc antibodies revealed that fibers expressing Unc45b^{myc} were rescued. Clear thick filament organization was detected in a mosaic pattern (Fig. 19D–F). However, those myofibers that expressed Unc45bΔTPR^{myc}, which is a truncated form of Unc45b lacking the TRP domain, showed severe defects without mosaic patterns (Fig. 18G–I). The loss-of-function of the TRP truncated form of Unc45 confirms the previous hypothesis that the TPR domain is required for Unc45b mediated protein-protein interaction, which plays important roles in myofibrillogenesis. Together, these data indicate that Hsp90α1 and

Unc45b are critical for sarcomeric organization of the M-line and that the TPR domain of Unc45b is required for Unc45b function in zebrafish embryos.

3.4.8 Knockdown of *smyd1b* has no effect on the mRNA levels of *myomesin*

Our previous studies have shown that the knockdown of *smyd1b* severely disrupts the sarcomeric localization of myomesin. Little or no myomesin can be detected by immunostaining in *smyd1b* knockdown embryos. This indicates a critical role for Smyd1 in the regulation of myomesin. However, whether the *myomesin* expression level is affected at the transcriptional level or at the protein level in *smyd1b* knockdown embryos is not clear. To determine the mechanism of regulation of myomesin by Smyd1b, we performed RT-PCR analysis to compare the expression levels of all five *myomesin* genes in *smyd1b* knockdown embryos. Our data showed that there were no differences in the transcript levels of any of the five *myomesin* genes in *smyd1b* knockdown embryos as compared to the WT control (Fig. 19). These data indicate that Smyd1b is not involved in the transcriptional regulation of *myomesin*. On the contrary, Smyd1b may be involved in the post-translational modification of myomesin and/or myosin and may control myofibril assembly.

3.5 Discussion:

In this study, we characterized the temporal and spatial expression patterns of five members of the *myomesin* gene family in zebrafish embryos. Functional analysis of *myomesin-1b* and slow muscle specific *myomesin-3* revealed that disruption of their expression had little or no effect on sarcomere organization of skeletal muscles in zebrafish embryos. On the contrary, knockdown of the molecular chaperones

hsp90α1 and *unc45b* severely disrupted the M-line localization of myomesin-3 and Smyd1b_tv1. Together, these data indicate that Hsp90α1 and Unc45b are required for M-line organization; however, myomesin-1b and myomesin-3 are dispensable for M-line formation in the sarcomere.

3.5.1 Characterization of *myomesin* genes in zebrafish

GeneBank and sequence analysis revealed that there are at least five *myomesin* genes in the zebrafish. All five *myomesin* genes showed muscle specific patterns of expression in zebrafish embryos. *myomesin-1b* and *myomesin-2b* exhibited both skeletal and cardiac muscle expression. *myomesin-1a*, *myomesin-2a*, and *myomesin-3* were only expressed in skeletal muscles. RT-PCR analysis revealed that *myomesin-1b* and *myomesin-2b* exhibited higher expression levels than *myomesin-2a* and *myomesin-3*. *myomesin-1a* only showed a mild expression level. These studies indicate that the expression patterns of the various *myomesin* genes is highly regulated in zebrafish embryos.

3.5.2 Loss of myomesin-3 and myomesin-1b has little effect on sarcomere organization

It has been suggested that myomesins play a key role in myofibrillogenesis. However, most of the suggested functions of myomesin are based on *in vitro* cellular and biochemical studies. The lack of knockout or mutant models has impeded a clear understanding of myomesin function in myofibrillogenesis. Moreover, no M-line-related sarcomere diseases had been discovered before 2005 (Laing and Nowak, 2005). Although recent reports show that cardiac hypertrophy might be associated

with a *myomesin* mutation in patients and that the knockdown of *myomesin* causes a sarcomere defect *in vitro* (Siegert et al., 2011; Fukuzawa et al., 2008), whether myomesin plays a critical role in sarcomere organization *in vivo* is still not well understood.

Data from our studies demonstrate that the knockdown of *myomesin-3* and *myomesin-1b* expression has little or no effect on myofibril organization in the skeletal muscles of zebrafish embryos. The lack of a muscle phenotype in the knockdown embryos is surprising. It may suggest that these genes are not essential for M-line organization. Alternatively, the highly conserved sequence of the five *myomesin* genes in zebrafish and their redundant patterns of expression in skeletal muscles suggest that there may be some functional redundancy between the different *myomesin* genes.

It has been reported that myomesin-3 can dimerize in an antiparallel fashion via the C-terminal end (Lange et al., 2005). This dimerization was suggested to have an important role in M-line organization. Interestingly, our data showed that myomesin-3-RFP lacking the C-terminal part of myomesin-3 is still localized to the M-line. These results indicate that the C-terminal dependent dimerization of myomesin-3 may not contribute to its M-line localization.

3.5.3 Hsp90α1 and Unc45b are required for M-line organization

Genetic and biochemical analyses have shown that chaperone-mediated myosin folding and assembly is an integral part of myofibrillogenesis during muscle development. It has been suggested that Hsp90α and Unc45b are required for sarcomere organization. Recent biochemical studies support the hypothesis that

Hsp90α and Unc45b, interact with Smyd1, forming a complex that is involved in regulating myosin folding (Tan et al., 2006; Liu et al., 2008; Srikakulam et al, 2008; Etard et al., 2008; Du et al., 2008). Consistent with their function in thick filament assembly and sarcomere organization, data from this study demonstrate that the knockdown of *unc45b* or *hsp90α1* expression abolishes the M-line localization of myomesin-3 and Smyd1b_tv1. The defective M-line organization in *hsp90α* and *unc45b* knockdown embryos can be explained by their function on M-line proteins.

Our previous studies showed that the knockdown of slow muscle myosin heavy chains disrupts sarcomere organization and the M-line localization of myomesin-3-RFP. This is similar in effect to *unc45b* or *hsp90α1* knockdown. This result suggests the alternative explanation that the disruption of the M-line in *unc45b* and *hsp90α1* knockdown embryos may indirectly result from the disruption of thick filament organization.

Our TPR domain deletion study showed that Unc45bΔTPR^{myc} could not rescue the myofibril defect caused by *unc45b* knockdown. This result is consistent with the previous hypothesis that the TPR domain of Unc45 is critical for protein-to-protein interaction with Hsp90, and is required for the formation of the regulatory complex (Chadli et al., 2008).

3.5.4 Smyd1b and myomesin:

Our previous studies indicated that Smyd1b was involved in myomesin M-line organization. Little or no myomesin could be detected in *smyd1b* knockdown embryos. In this study, we demonstrate that the mRNA levels of all five *myomesin* genes are unaffected by *smyd1b* knockdown. These data indicate that the loss of

sarcomeric localization of myomesin is not due to lower levels of *myomesin* mRNA expression in *smyd1b* knockdown embryos. Myomesin is thought to cross-link thick filaments and play critical roles in sarcomere organization. Our previous data demonstrated that Smyd1b_tv1 and myomesin-3 are co-localized on the M-line in slow muscles. These data suggest the possibility that Smyd1 may be involved in the modification or regulation of myomesin proteins. Alternatively, the loss of sarcomeric localization of myomesin could be affected by the disruption of thick filaments in *smyd1b* knockdown embryos. This theory is supported by our recent studies showing that the knockdown of *smyhc* completely disrupts the M-line localization of myomesin-3-RFP (Xu et al., 2012).

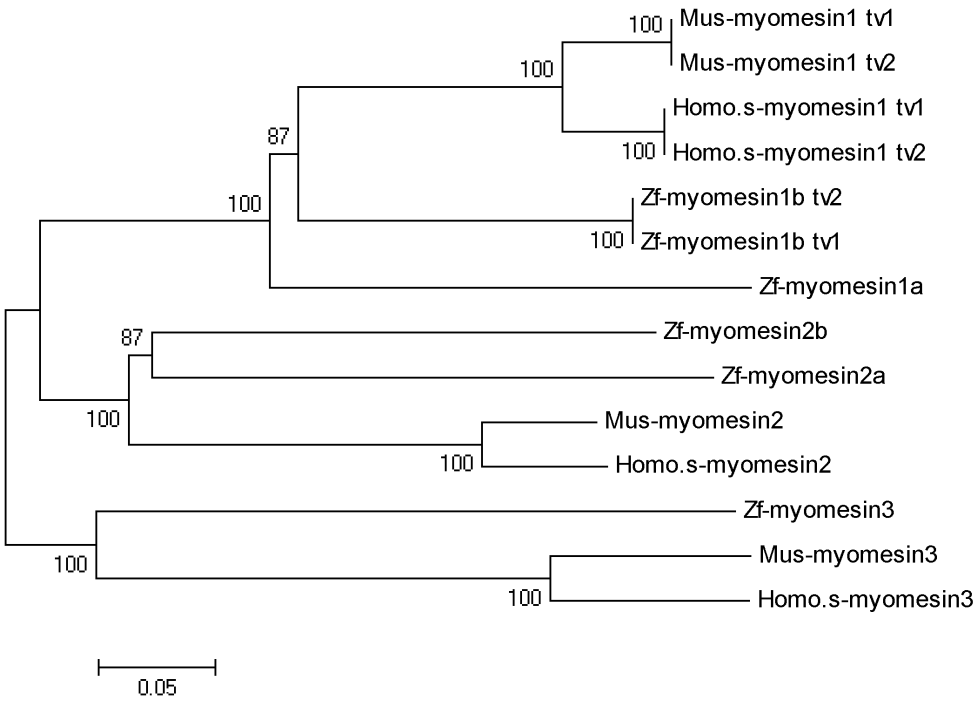


Fig. 10. The phylogenetic tree analysis of zebrafish myomesin-1a, myomesin-1b_tv1, myomesin-1b_tv2, myomesin-2a, myomesin-2b, and myomesin-3.
The phylogenetic tree analysis of the protein sequence of *myomesin* genes in zebrafish, mice, and humans. (Zf: zebrafish, Homo: human, Mus: mouse)

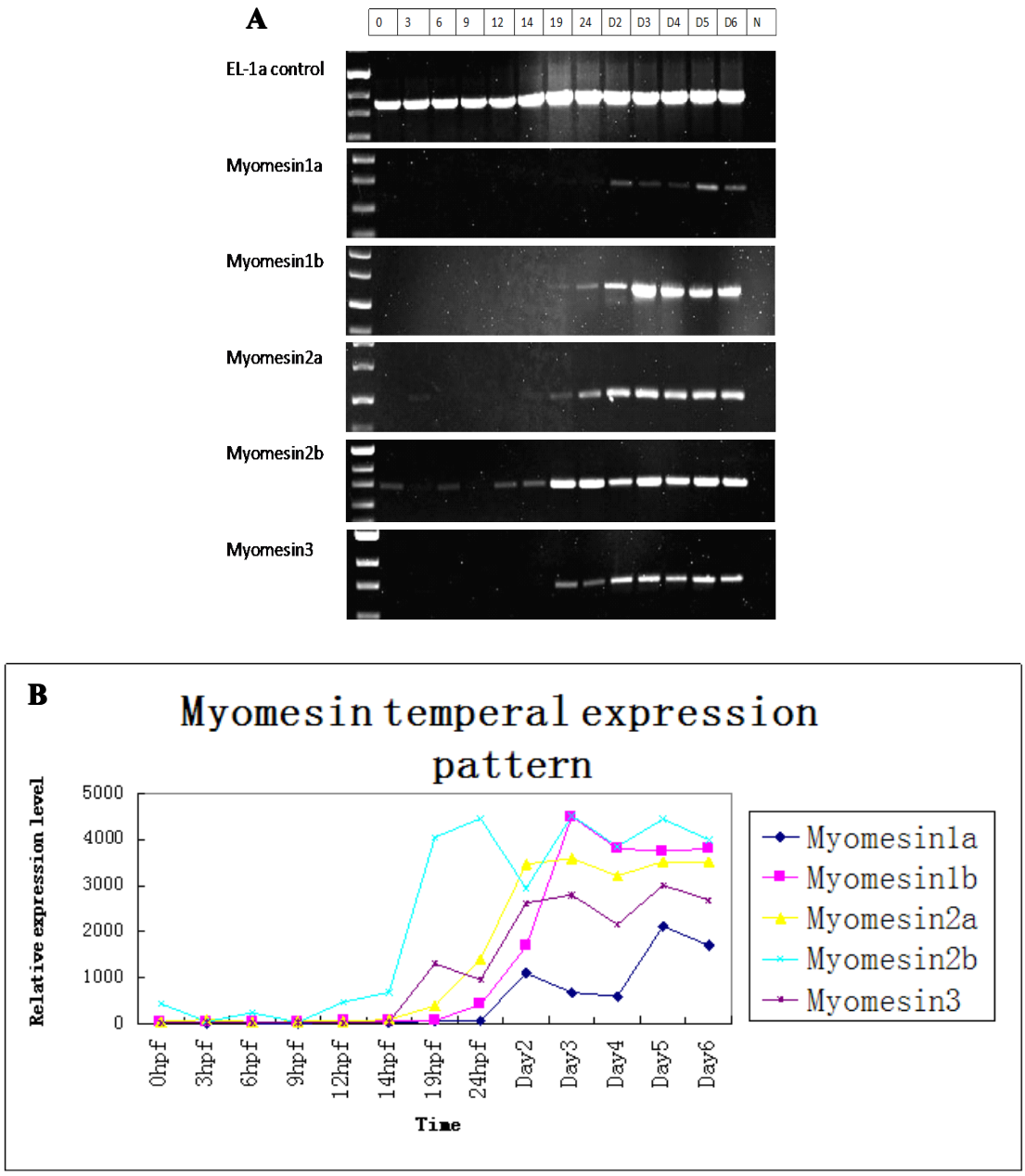


Fig. 11. Developmental stage specific expression of the *myomesin-1a, myomesin-1b* (tv1 and tv2 were detected using the same set of primers), *myomesin-2a*, *myomesin-2b*, and *myomesin-3* genes in zebrafish. Total RNA extracted from 0 hpf, 3 hpf, 6 hpf, 9 hpf, 12 hpf, 14 hpf, 19 hpf, 24 hpf, Day 2, Day 3, Day 4, Day 5, and Day 6 zebrafish embryos was used for reverse transcriptase (RT)-PCR analysis. Different sets of primers were used to detect *myomesin-1a, myomesin-1b, myomesin-2a, myomesin-2b,* and *myomesin-3*.
A. Time course RT-PCR analysis of the expression of *myomesin-1a, myomesin-1b, myomesin-2a, myomesin-2b,* and *myomesin-3*. EF-1α was used as a positive control. N stands for negative control without the cDNA template.
B. Densitometry roughly represents the expression levels of the different *myomesin* genes from 0 hpf to Day 6.

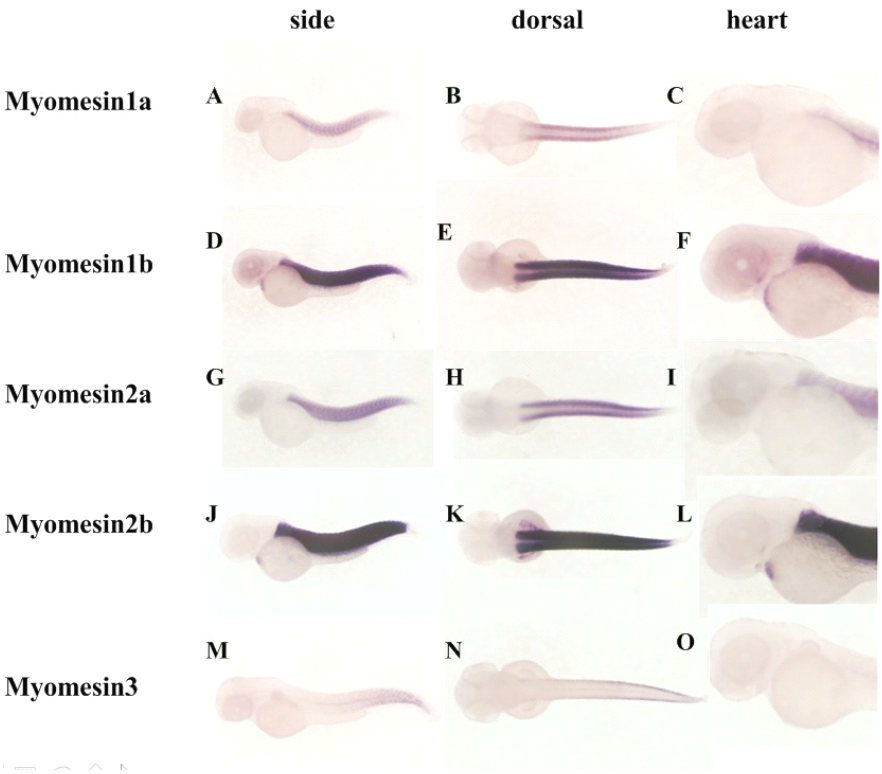


Fig. 12. Whole-mount *in situ* hybridization showing the expression patterns at 48 hours post fertilization of *myomesin-1a, myomesin-1b, myomesin-2a, myomesin-2b,* and *myomesin-3* using dig-labeled antisense probes. Lateral (A, D, G, J, and M), dorsal (B, E, H, K, and N), and heart areas enlarged (C, F, I., L, and O) views are shown. All five *myomesin* genes exhibited muscle specific expression. *Myomesin-1b* and *Myomesin-2b* were expressed in both skeletal and cardiac muscles. *Myomesin-1a, myomesin-2a,* and *myomesin-3* were only expressed in skeletal muscles.

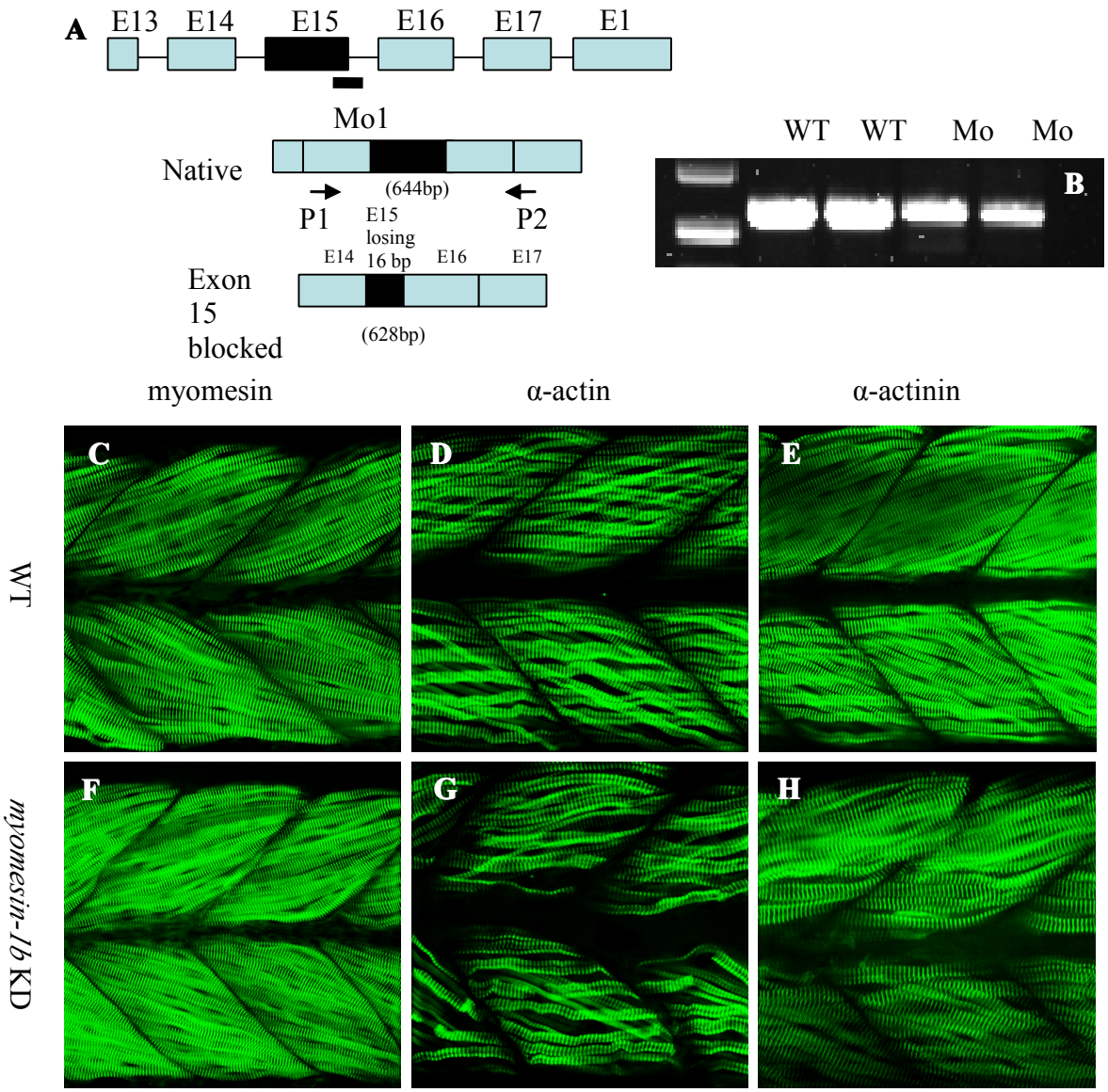


Fig. 13. Knockdown of *myomesin-1b* has little or no effect on skeletal muscle development.

A. Location of the morpholino splicing blocker MyoM1b-MO1 (E15I15). MyoM1b-MO1 was targeted to the exon15/intron15 junction and induced a 16 bp shift. This indicates the ORF of *myomesin-1b* shifted in MO injected embryos.

B. RT-PCR result showed the defective splicing induced by the E15I15 Mo. Compared with the native transcript, transcript from the E15I15 MO injected embryos had a 16 bp shift, which was verified by sequencing.

C–H: myomesin, α-actin, and α-actinin antibody staining showed little or no effect on sarcomere organization of knockdown embryos as compared to WT embryos at 48 hpf. (C) WT embryos, myomesin antibody staining. (D) WT embryos, α-actin antibody staining. (E) WT embryos, α-actinin antibody staining. (F) *myomesin-1b* knock-down embryos, myomesin antibody staining. (G) *myomesin-1b* knock-down embryos, α-actin antibody staining. (H) *myomesin-1b* knock-down embryos, α-actinin antibody staining.

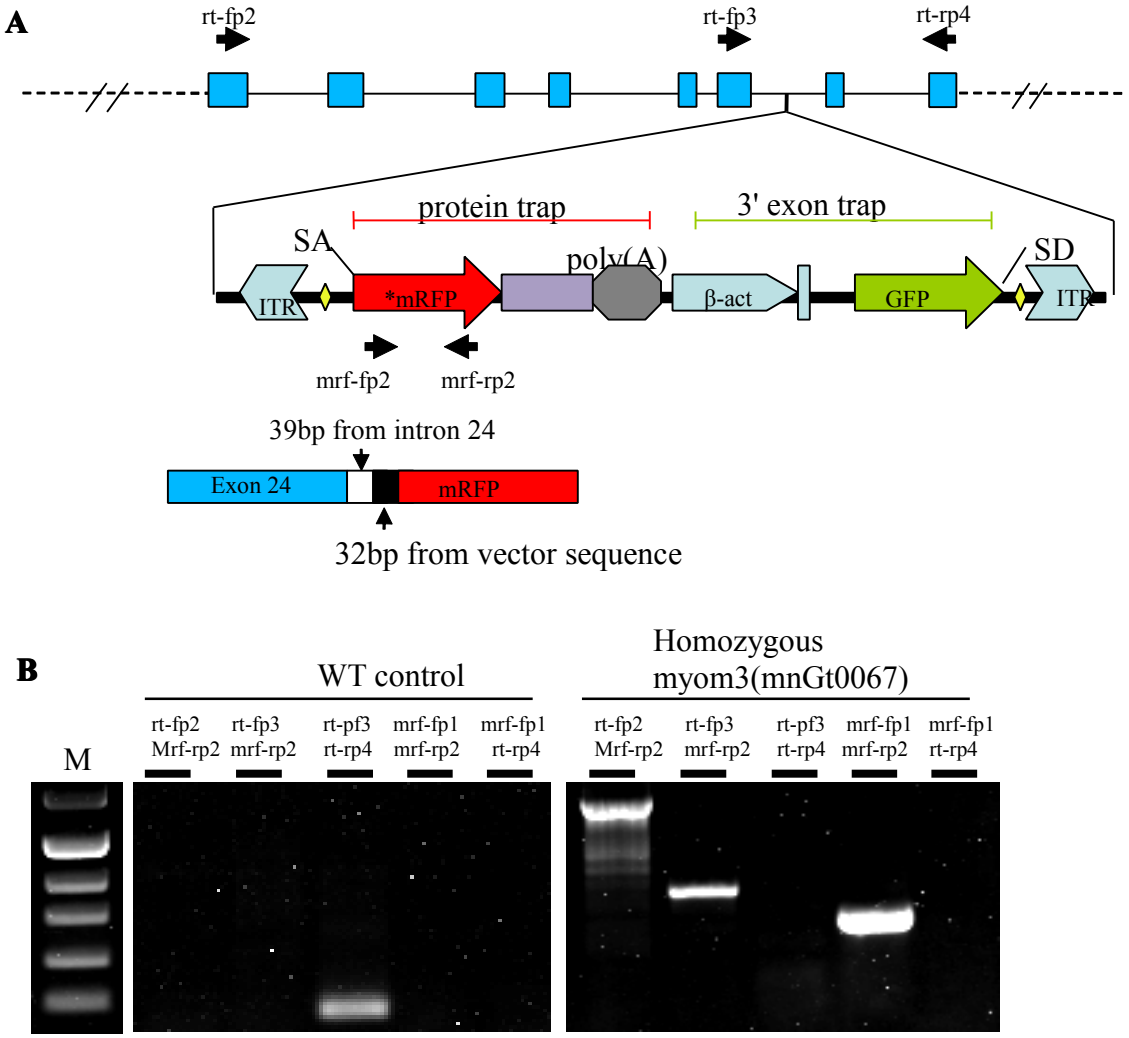


Fig. 14. Diagram showing the gene trap integration in *myomesin-3* gene and the PCR strategy used for analyzing the expression of *myomesin-3* and *myomesin-3-rfp* mRNA transcripts.

A: the RFP gene trap is integrated in intron 24 of *myomesin-3*. A cDNA fragment covering the junction site of *myomesin-3* and *rfp* fusion was amplified by RT-PCR. Additional sequence of 39 bp from intron 24 and 32 bp from the vector sequence upstream of *rfp* was found in the *myomesin-3-rfp* fusion transcript. *myomesin-3* GenBank accession no. XM_001921030.

B: RT-PCR results showed the expression of *myomesin-3* mRNA transcripts or *myomesin-3-rfp* fusion products in WT and homozygous *myom3* (mnGt0067) fish embryos.

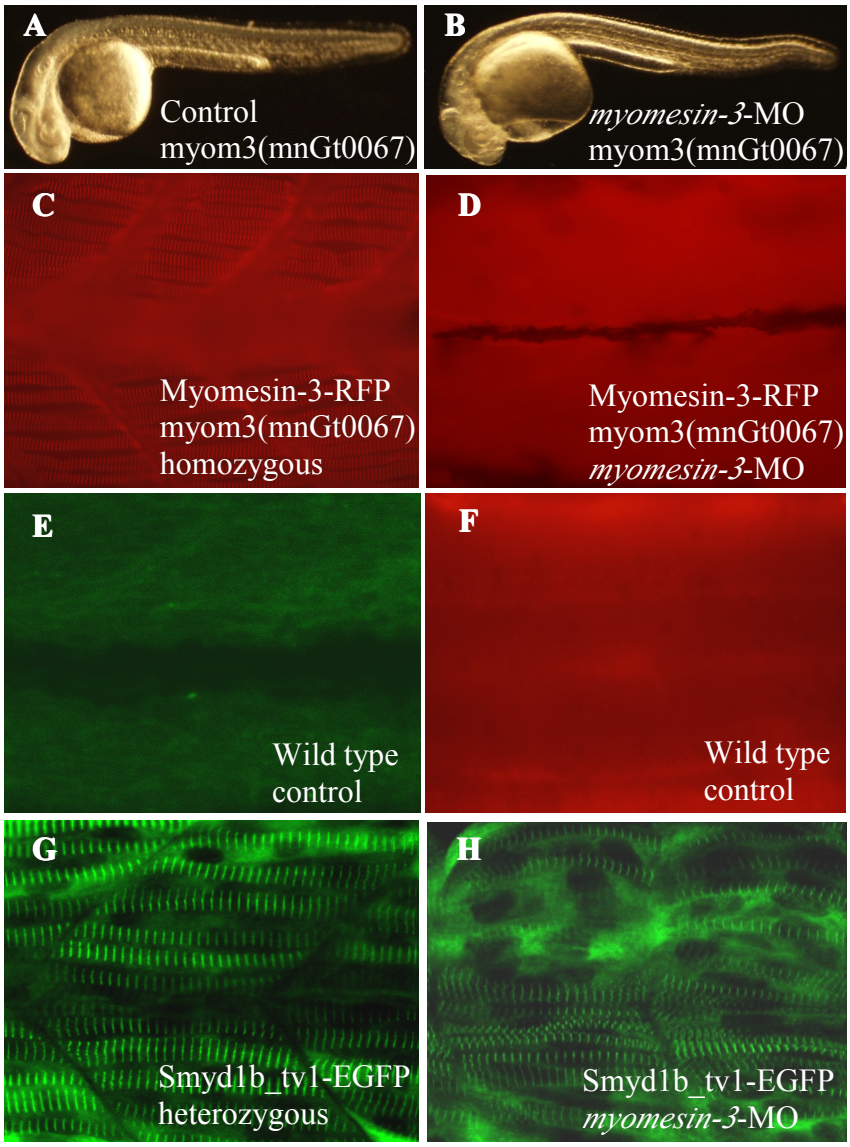


Fig. 15. The effect of RFP gene trap insertion and *myomesin-3* knockdown on M-line organization in the slow muscles of zebrafish embryos.
A and B: morphological comparison of the *myom3* (mnGt0067) homozygous control (A) and *myomesin-3* ATG-MO injected (B) embryos at 24 hpf.
C and D: the M-line localization of myomesin-3-RFP in *myom3* (mnGt0067) homozygous control (C) or *myomesin-3* knockdown (D) embryos at 48 hpf.
E and F: wild-type embryos for GFP (E) and RFP (F) at 28 hpf and 48 hpf, respectively.
G and H: sarcomeric localization of Smyd1b-EGFP at the M-lines in Smyd1b-EGFP heterozygous control (G) or *myomesin-3* knockdown (H) zebrafish embryos at 28 hpf.

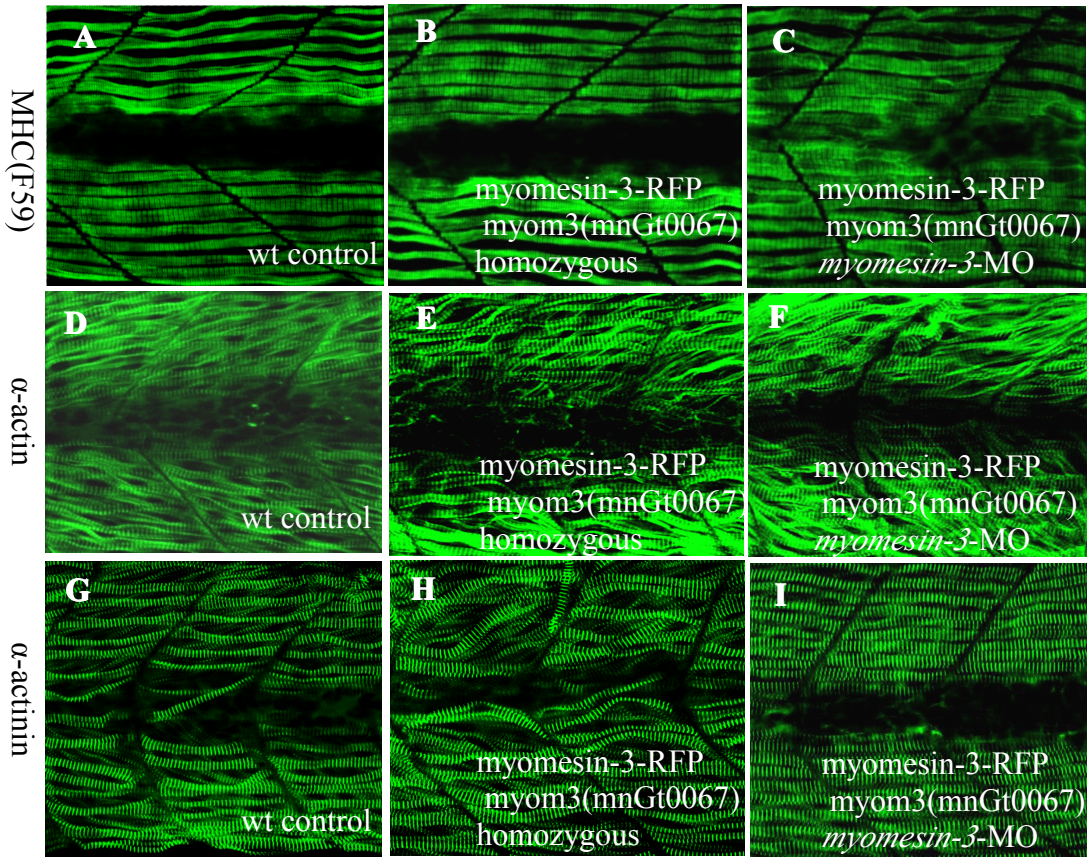


Fig. 16. Sarcomere organization in the slow muscles of homozygous myomesin-3-RFP and *myomesin-3* knockdown zebrafish embryos.

A–C: anti-MyHC antibody (F59) staining shows thick filament organization in the slow muscles of the control (A), homozygous *myom3* (mnGt0067) (B), and *myomesin-3* knockdown (C) embryos at 28 hpf.

D–F: α-actin immunostaining shows the organization of thin filaments in the slow muscles of the control (D), homozygous *myom3* (mnGt0067) (E), and *myomesin-3* knockdown (F) embryos at 28 hpf.

G–I: α-actinin immunostaining shows the organization of Z-lines in the slow muscles of the control (G), homozygous *myom3* (mnGt0067) (H), and *myomesin-3* knockdown (I) embryos at 28 hpf.

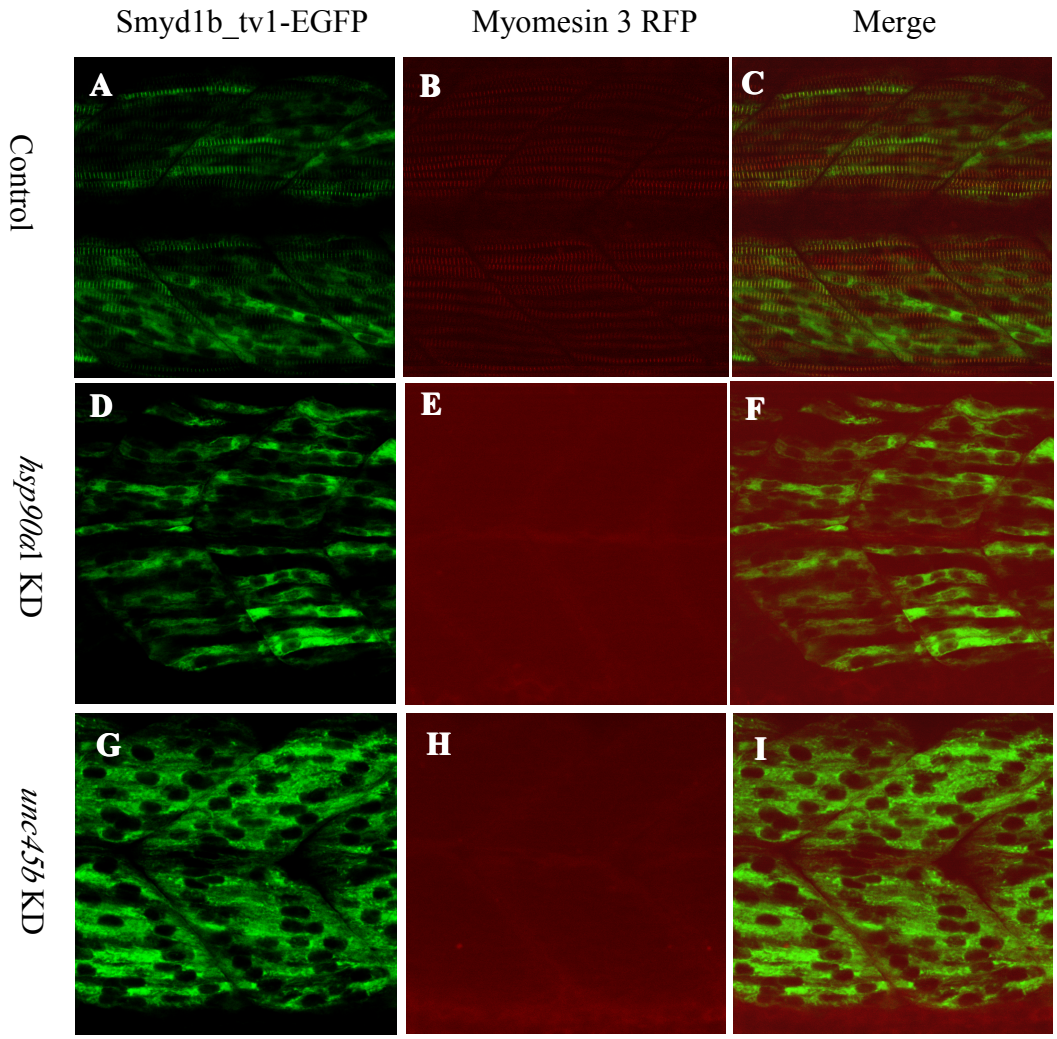


Fig. 17. Effects of *hsp90a1* and *unc45b* knockdown on the sarcomeric localization of Smyd1b_tv1-EGFP and myomesin-3-RFP in zebrafish embryos. Sarcomere localization of Smyd1b_tv1-EGFP (D: *hsp90a1* knockdown (KD) embryos, G: *unc45b* KD embryos) and myomesin-3-RFP (E: *hsp90a1* KD embryos, G: *unc45b* KD embryos) on *smyd1b_tv1-EGFP(+/-)*myomesin-3-RFP(+/+)* fish embryos at 27 hpf. A–C: Uninjected control.

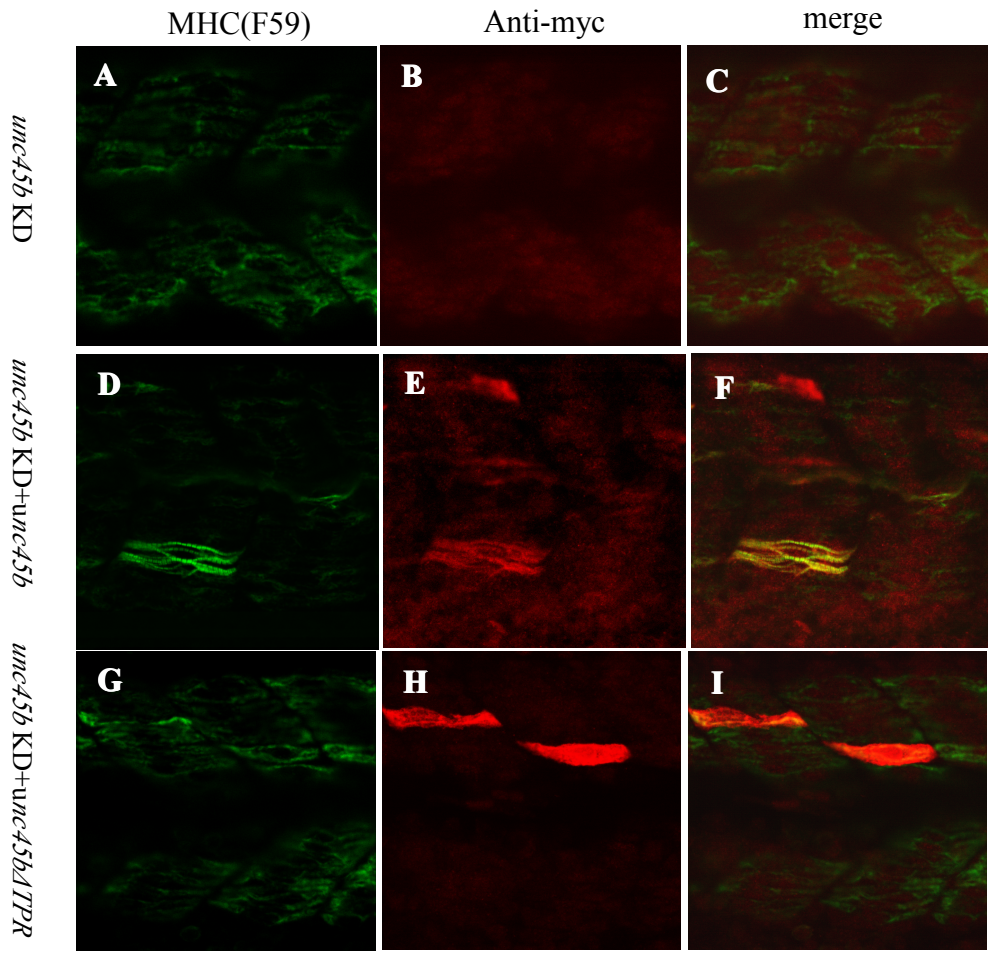
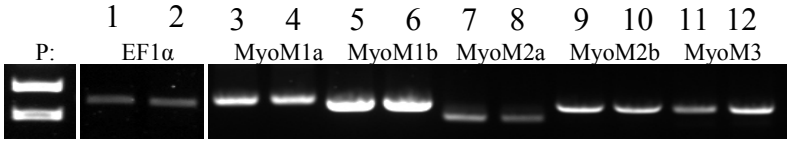


Fig. 18. Unc45bΔTPR protein cannot rescue the myofibril defect in *unc45b* knockdown embryos. Unc45b^{myc} and Unc45bΔTPR^{myc} were visualized by anti-myc antibody staining (red). Myosin thick filament organization was determined by F59 antibody staining (green) in *unc45b* knockdown embryos. A–C: antibody staining of *unc45b* knockdown control embryos at 27 hpf. (A) F59 antibody, (B) anti-myc antibody, and (C) merge.
D–F: DNA construct encoding Unc45b^{myc} was co-injected with *unc45b* MO into fish embryos and visualization was achieved by antibody staining at 27 hpf (D) F59 antibody, (E) anti-myc antibody, and (F) merge.
G–I: DNA construct encoding Unc45bΔTPR^{myc} was co-injected with *unc45b* MO into fish embryos and visualization was achieved by antibody staining at 27 hpf (G) F59 antibody, (H) anti-myc antibody, and (I) merge.



cDNA: odd: WT. even: SmyD1bKD.

Fig. 19. Analysis of the expression pattern of the five *myomesin* genes in *smyd1b* knockdown embryos by RT-PCR.

Total RNA was extracted form 48 hpf WT embryos (odd number) and *smyd1b* knockdown embryos (even number). RT-PCR expression analysis of the different *myomesin* genes by using different sets of primers. Primers: Lines 1–2: EF1α control. Lines 3–4: myomesin-1a primers. Lines 5–6: *myomesin-1b* primers. Lines 7–8: *myomesin-2a* primers. Line 9–10: *myomesin-2b* primers. Line: 11–12: *myomesin-3* primers.

Chapter 4: Effect of *smyd1* knockdown on protein methylation in zebrafish embryos

4.1 Abstract:

Myofibrillogenesis is a highly regulated process. Post-translational modification plays critical roles in protein regulation. Various sarcomere proteins have been shown to be methylated. However, the biological functions of such methylation and that of the methyltransferases involved are not well understood. Smyd1 is a lysine methyltransferase that is required for myofibril organization in zebrafish embryos. We hypothesize that Smyd1 may control sarcomere assembly via protein methylation. To better understand the mechanism of Smyd1 function in myofibrillogenesis, we analyzed the protein methylation state in *smyd1* knockdown embryos. The result shows that the general protein methylation level decreases in *smyd1* knockdown embryos.

4.2 Introduction:

Smyd1 is a methyltransferase that is capable of methylating histone 3 lysine 4 *in vitro* (Tan et al., 2006). We showed earlier that Smyd1b exhibits sarcomeric localization in mature myofibers without any nuclear localization. This finding indicates a potential role for Smyd1b in sarcomere organization by regulating non-histone sarcomere proteins. Although it has been reported recently that methyltransferase activity might not be required for the function of Smyd1 in sarcomere assembly (Just et al., 2011), previous studies in our lab have shown that

mutating the SET domain, which is required for the methyltransferase activity, completely abolishes the biological function of Smyd1 (Tan et al., 2006).

Protein methylation has been suggested to play important roles in protein stabilization/de-stabilization, activity, and protein-protein interaction (Yang et al., 2009; Egorova et al., 2010; Zhang et al., 2012). To date, several sarcomere proteins, such as myosin, actin, and creatine kinase, have been shown to be methylated at their lysine residues (Tong and Elzinga, 1983; Iwabata et al., 2005). However, the biological significance of such methylation and that of the methyltransferases involved in sarcomere protein methylation are not well understood.

In this study, we characterized the overall protein methylation in wild-type and *smyd1* knockdown embryos. Our data show that the overall protein methylation levels decrease in *smyd1b* knockdown embryos. Myosin protein levels also decrease upon knocking down *smyd1b*. These data indicate that Smyd1 might be involved in the regulation of sarcomere protein methylation and myosin protein quality control.

4.3 Materials and Methods:

4.3.1 Western blot analysis

Wild-type or *smyd1b* MO-injected zebrafish embryos at 72 hpf (50 embryos each) were dechorionated manually and crushed gently to remove the yolk by triturating with a glass pipet. The embryos were lysed in 100 μL lysis buffer containing DTT, PMSF, and protease inhibitor (P8340, Sigma) for 30 minutes and mixed with 100 μL 2X SDS loading buffer (0.125 M Tris-Cl, pH 6.8; 4% SDS; 20% glycerol; 0.2 M DTT; 0.02% bromophenol blue). Samples were boiled for 5 minutes,

and the proteins were separated on a 7.5% SDS-polyacrylamide gel. Following SDS-polyacrylamide gel electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Immobilon-FL, Millipore, MA, USA) using a standard electrophoresis transfer system (Hoefer, MA, USA). Membranes were treated for 10 minutes with Antigen Pretreatment Solution (SuperSignal Western Blot Enhancer Kit, Thermo Scientific, IL, USA) and were blocked with 5% nonfat milk in PBST (1X PBS, 0.1% Tween-20) for 1 hour at room temperature. After blocking, membranes were incubated with primary antibody for 1 hour in Primary Antibody Diluent (Super Signal Western Blot Enhancer Kit, Thermo Scientific) followed by three washes with PBST for 10 minutes each. Membranes were incubated with secondary antibody for 1 hour at room temperature in 5% nonfat milk in PBST followed by five washes with PBST for 10 minutes each. Blots were developed using the Pierce ECL Western Blotting Substrate (Thermo Scientific) according to the manufacturer's instructions.

4.3.2 MG132 proteasomal inhibitor treatment

24 hpf WT and *smyd1b* knockdown embryos were transferred in groups of 50 into 8-well plates containing 10 mL fish water. *smyd1b* knockdown embryos were treated with 2uM proteasomal inhibitor MG132 in DMSO for 48 hpf at 28°C. Control WT and *smyd1b* embryos were treated with DMSO. The final concentration of DMSO was 1% for both the treated and control wells (Winder et al., 2011).

4.4 Results:

4.4.1 Knockdown of *smyd1b* affects protein methylation in zebrafish embryos

To better understand the role of Smyd1b in protein methylation, we carried out Western blot analysis in *smyd1b* knockdown embryos using an anti-methyl lysine specific antibody. Compared with WT control embryos, the methylation levels of several proteins were significantly reduced in the *smyd1b* knockdown embryos although Coomassie blue staining showed that the general protein levels were unchanged (Fig. 20A and B). Among the proteins with reduced methylation, a protein with molecular weight 220 kD may represent myosin heavy chain.

The reduced levels of protein methylation could be due to decreased protein accumulation. To test this idea, we analyzed myosin expression in *smyd1* knockdown embryos. Consistent with previous results, we showed that the myosin protein levels were also significantly lower by Western blot using a myosin heavy chain specific antibody (Fig. 20C). It has been suggested that myosin can be ubiquitinated at lysine residues and that this increases its degradation rate in *C. elegans* (Landsverk et al., 2007). This finding indicates that myosin degradation could be regulated by the ubiquitin-proteasome pathway. Previous studies in our lab have demonstrated that knockdown of *smyd1* significantly reduces myosin protein accumulation in zebrafish embryos (Li et al., unpublished). It has been shown that treating worms with the proteasomal inhibitor MG132 efficiently blocks ATP-dependent myosin degradation (Landsverk et al., 2007). A new report in a zebrafish model demonstrated that the phenotype of muscular dystrophy could be significantly reduced in dystrophic

zebrafish larvae by treating with the proteasomal inhibitor MG132 (Winder et al., 2011). Thus, to determine whether inhibiting proteasome activity could slow down myosin protein degradation in *smyd1b* knockdown embryos, we treated the *smyd1b* knockdown embryos with MG132 for 48 hours. The results showed that myosin protein levels were not increased in MG132 treated *smyd1b* knockdown embryos when compared with untreated knockdown embryos (Fig. 21A). In addition, the general protein methylation levels showed no difference between *smyd1b* knockdown embryos and *smyd1b* knockdown embryos treated with MG132 (Fig. 21C).

4.4.2 Knockdown of *smyd1b* results in increased Hsp90α expression

It has been suggested that Hsp90α and Smyd1 may form a complex that is involved in regulating myofibrillogenesis (Tan et al., 2006). Consistent with a recent report showing that *hsp90α1* mRNA levels were higher in *smyd1* deficient (*fla* mutant) embryos (Just et al., 2011), our data revealed that Hsp90α protein levels were increased in *smyd1b* knockdown embryos (Fig. 21B). The increased Hsp90α levels may indicate a potential Smyd1b-mediated regulatory mechanism of Hsp90α expression or protein stability in zebrafish embryos. Together, these data indicate that the knockdown of *smyd1b* might affect protein methylation and alter Hsp90α expression levels in zebrafish embryos.

4.5 Discussion:

4.5.1 Smyd1b and protein methylation:

Smyd1 is a lysine methyltransferase required for sarcomere organization. It can methylate histone 3 lysine 4 *in vitro* (Tan et al., 2006). Some reports show that Smyd

may be also involved in non-histone protein methylation (Yang et al., 2009; Donlin et al., 2012). However, whether Smyd1 is involved in protein methylation *in vivo* is still not clear, as is the methylation target(s) of Smyd1. In this study, we analyzed overall protein methylation in zebrafish embryos, and showed that protein methylation levels were decreased in *smyd1b* knockdown embryos as compared to WT controls. In addition, we showed a significant reduction of myosin methylation in *smyd1* knockdown embryos. We speculate that methylation of myosin by Smyd1 is required for its folding and stability. Hence, the knockdown of *smyd1* expression may result in poor methylation of myosin proteins, thus leading to increased degradation.

It has been reported that proteasomal inhibitors efficiently block myosin degradation in *C. elegans* (Landsverk et al., 2007). However, our results show that the myosin protein levels were not different in *smyd1b* knockdown embryos with or without treatment with MG132. These data indicate either that myosin degradation in zebrafish does not occur through the ubiquitin-proteasome pathway or that the proteasomal inhibitor MG132 is not as effective in zebrafish as it is in worms.

4.5.2 Smyd1b and Hsp90α:

Hsp90α function relies on cofactors that provide additional binding specificities or enzymatic activities. Co-immunoprecipitation analysis revealed that Smyd is associated with Hsp90α and Unc45b, which are indicated to be molecular chaperones associated with myosin folding (Li et al, unpublished data). It has been shown that the knockdown of *smyd1* significantly upregulates *hsp* gene expression. In this study, we showed that the protein levels of Hsp90α were increased in *smyd1b* knockdown

embryos as compared to WT controls. These data indicate that Smyd1 is involved in the regulation of Hsp90α in zebrafish.

Non-histone methylation has been shown to play important roles in regulating protein stability (Yang et al., 2009). Recent studies have shown that Smyd2 is capable of methylating Hsp90α on lysine209 and lysine615 and that the methylation of Hsp90α is critical for its function in regulating sarcomere organization (Abu-Farha et al., 2011; Donlin et al. 2012). Lysine615 of Hsp90α can also be ubiquitinated (Kundrat and Regan, 2010), indicating that methylation and ubiquitination might compete to regulate this lysine residue and hence regulate Hsp90α protein stability. Sequence analysis showed that Smyd1 and Smyd2 are highly conserved, potentially indicating conserved function of Smyd1 and Smyd2. Thus, the regulation of Hsp90α by Smyd1b might be similar to its regulation by Smyd2, in that it is mediated by directly affecting the methylation status of Hsp90α. Alternatively, Smyd1 may affect the transcriptional levels of *hsp90α* by indirectly regulating other proteins.

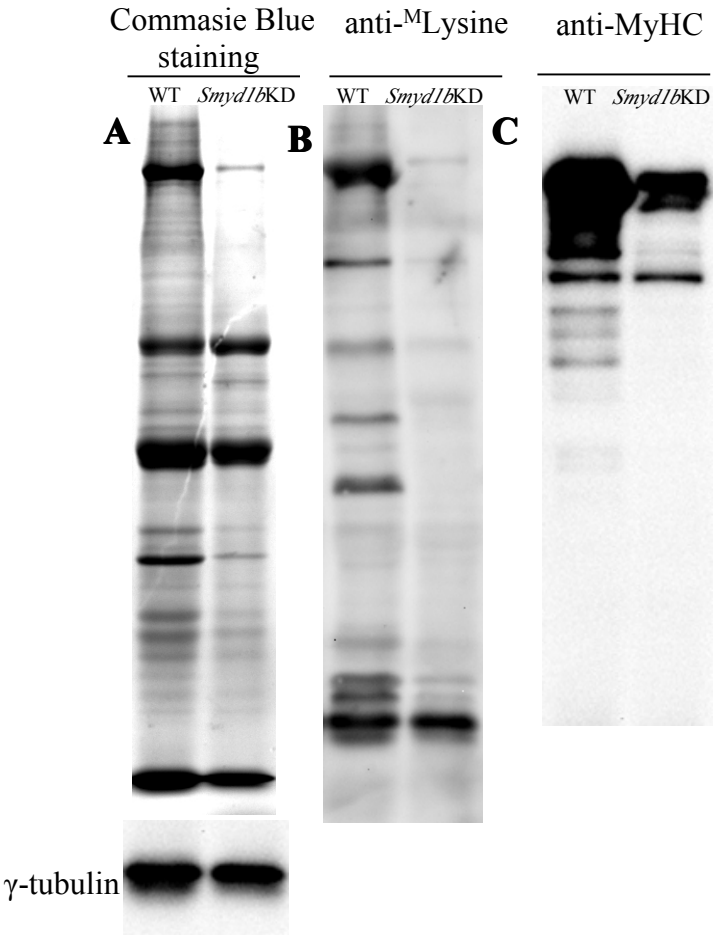


Fig. 20. Analysis of protein methylation in *smyd1b* knockdown 3 dpf embryos by Western blot.

A. Coomassie blue staining showing protein expression in WT control and *smyd1b* knockdown embryos.

B. Protein methylation in WT control and *smyd1b* knockdown embryos by Western blot using anti-methyl lysine antibody

C. Myosin heavy chain protein levels in WT control and *smyd1b* knockdown embryos by Western blot using MF-20 antibody.

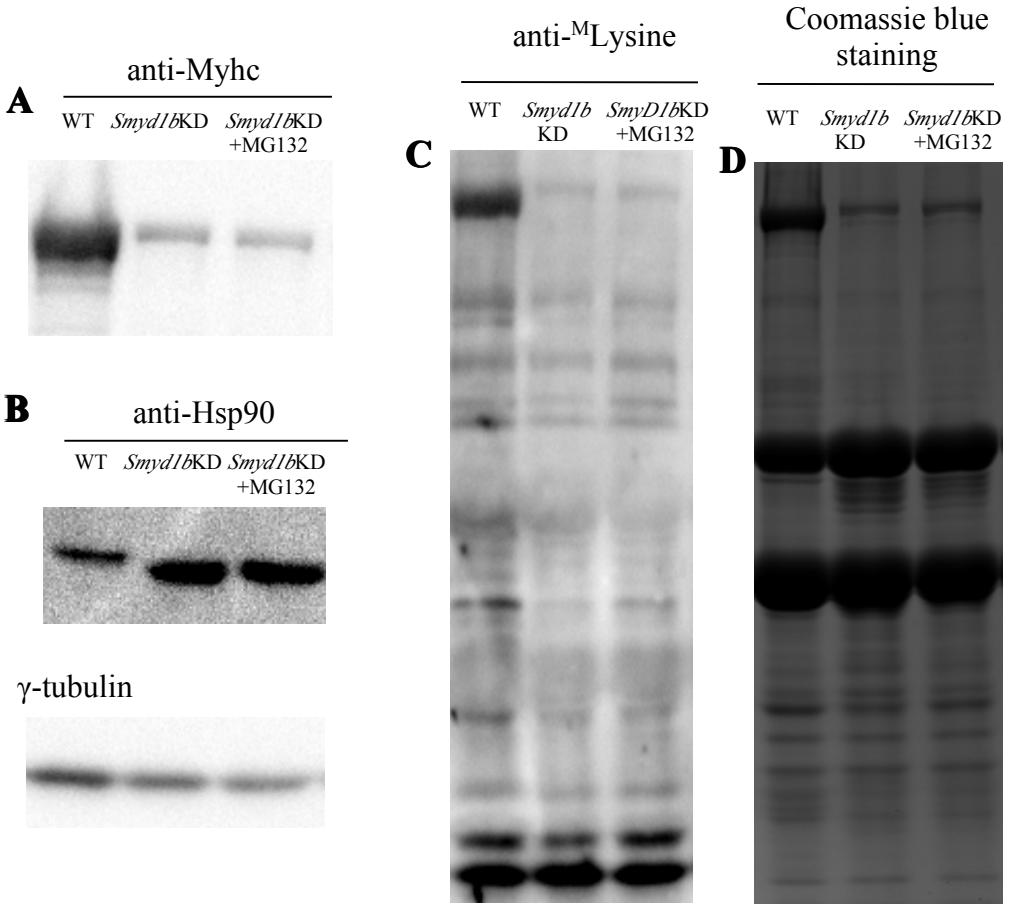


Fig. 21. Analysis of Hsp90 and Myhc protein levels in *smyd1b* knockdown embryos after treatment with the proteasomal inhibitor MG132.

A. Myhc protein levels significantly reduced in *smyd1b* knockdown embryos and were not rescued by MG132.

B. Hsp90α protein levels increased in *smyd1b* knockdown embryos with and without the treatment with MG132.

C. The general protein methylation levels in *smyd1b* knockdown embryos were not affected by MG132 treatment.

D. Coomassie blue staining showing protein expression in WT control, *smyd1b* knockdown embryos, and *smyd1b* knockdown embryos treated with the proteasomal inhibitor MG132.

Bibliography

Abu-Farha, M., Lambert, J. P., Al-Madhoun, A. S., Elisma, F., Skerjanc, I. S., and Figeys, D.. The tale of two domains: proteomics and genomics analysis of SMYD2, a new histone methyltransferase. *Mol Cell Proteomics.*2008; 7:560–572.

Abu-Farha, M., Lanouette, S., Elisma, F., Tremblay, V., Butson, J., Figeys, D., and Couture, J. F. Proteomic analyses of the SMYD family interactomes identify HSP90 as a novel target for SMYD2. *J. Mol. Cell Biol.* 2011; 3, 301– 308.

Agarkova, I., Auerbach, D., Ehler, E. and Perriard, J. C. A novel marker for vertebrate embryonic heart, the EH-myomesin isoform. *J. Biol. Chem.* 2000; 275, 10256-10264.

Agarkova, I., Ehler, E., Lange, S., Schoenauer, R. and Perriard, J. C. M-band: a safeguard for sarcomere stability? *J. Muscle Res. Cell Motil.* 2003; 24, 191-203.

Agarkova,I.,Schoenauer, R., Ehler, E., Carlsson, L., Carlsson, E., Thornell, L.E. & Perriard,J. C. The molecularcomposition ofthesarcomeric M-band correlates with musclefibertype. *Eur.J.Cell Biol.* 2004; 83 , 193 – 204.

Auerbach, D., Bantle, S., Keller, S., Hinderling, V., Leu, M., Ehler, E., Perriard,J.C., Different domains of the M-band protein myomesin are involved in myosin binding and M-band targeting. *Mol.Biol.Cell.* 1999; 10,1297-1308.

Berkemeier F, Bertz M, Xiao S,Pinotsis N, Wilmanns M, et al., Fast-folding alpha-helices as reversible strain absorbersin the muscle protein myomesin.*Proc Natl Acad Sci* 2011; 108:14139–14144.

Brault, V., Reedy, M. C., Sauder, U., Kammerer, R. A., Aebi, U. and Schoenenberger, C. Substitution of flight muscle-specific actin by human (beta)-cytoplasmic actin in the indirect flight muscle of Drosophila. *J. Cell Sci.* 1999a; 112, 3627-3639.

Brault, V., Sauder, U., Reedy, M. C., Aebi, U. and Schoenenberger, C. A. Differential epitope tagging of actin in transformed Drosophila produces distinct effects on myofibril assembly and function of the indirect flight muscle. *Mol. Biol. Cell*, 1999b; 135-149.

Brown MA, Sims RJ, 3rd, Gottlieb PD, Tucker PW. Identification and characterization of Smyd2: a split SET/MYND domain-containing histone H3 lysine 36-specific methyltransferase that interacts with the Sin3 histone deacetylase complex. *Mol Cancer.* 2006; 5:26.

Boateng SY, Goldspink PH. Assembly and maintenance of the sarcomere night and day. *Cardiovasc Res.* 2008; 77:667-675.

Buckingham, M., Bajard,L., Chang,T., Daubas,P., Hadchouel,J., Meilhac,S., Montarras,D., Rocancourt,D. and Relaix,F. The formation of skeletal muscle: from somite to limb. *J. Anat.,* 2003; 202, 59–68.

Clark KA, McElhinny AS, Beckerle MC, Gregorio CC. Striated muscle cytoarchitecture: an intricate web of form and function. Annual Review of Cell and Developmental Biology.2002; 18:637–706.

Christ,B. and Brand-Saberi,B. Limb muscle development. *Int. J. Dev. Biol.* 2002; 46, 905–914.

Chadli A, Bruinsma ES, Stensgard B, Toft D. Analysis of Hsp90 cochaperone interactions reveals a novel mechanism for TPR protein recognition. *Biochemistry* 2008; 47: 2850–2857.

Costa ML,Escaleira RC, Rodrigues VB, Manasfi M and Mermelstein CS. Some distinctive features of zebrafish myogenesis based on unexpected distributions of the muscle cytoskeletal proteins actin, myosin, desmin, alpha-actinin, troponin and titin. *Mech Dev.* 2002; 116: 95–104.

Dlugosz AA, Antin PB, Nachmias VT and Holtzer H. The relationship between stress fiber-likestructures and nascent myofibris in cultured cardiac myocytes. *J Cell Biol* 1984; 99: 2268–2278.

Donlin L.T., Andresen C., Just S., et al. Smyd2 controls cytoplasmic lysine methylation of Hsp90 and myofilament organization. *Genes Dev.* 2012; 26: 114-119.

Domazetovska A, Ilkovski B, Cooper ST, Ghoddusi M, Hardeman EC, Minamide LS, Gunning PW, Bamburg JR, North KN. Mechanisms underlying intranuclear rod formation. *Brain.* 2007; 130:3275–3284.

Du A, Sanger JM, Sanger JW. Cardiac myofibrillgenesis inside intact embryonic hearts. *Dev Biol.* 2008a; 318:236–246

Du SJ, Li H, Bian Y, Zhong Y. Heat-shock protein 90alpha1 is required for organized myofibril assembly in skeletal muscles of zebrafish embryos. *Proc Natl Acad Sci.* 2008b; 105:554–559.

Eakin CM, Maccoss MJ, Finney GL, Klevit RE. Estrogen receptor alpha is a putative substrate for the BRCA1 ubiquitin ligase. *Proc Natl Acad Sci USA* 2007; 104:5794-9.

Egorova, K.S.; Olenkina, O.M., & Olenina,L.V. Lysine methylation of nonhistone proteins is a way to regulate their stability and function. *Biochemistry (Mosc).* 2010; 75,535-548.

Etard C, Behra M, Fischer N, Hutcheson D, Geisler R, Strahle U. The UCS factor Steif/Unc-45b interacts with the heat shock protein Hsp90a during myofibrillogenesis. *Dev Biol.* 2007; 308:133-143.

Etard C, Roostalu U, Strahle U. Shuttling of the chaperones Unc45b and Hsp90a between the A band and the Z line of the myofibril. *J Cell Biol.* 2008; 180:1163-1175.

Epstein ND, Davis JS. Sensing stretch is fundamental. *Cell.*2003; 112:147–150.

Frank, D., Kuhn, C., Katus, H. A. & Frey, N. The sarcomeric Z-disk: a nodal point in signalling and disease. *J. Mol. Med.* 2006; 84 , 446–468.

Franz WM, Muller OJ, Katus HA. Cardiomyopathies: from genetics to the prospect of treatment. *Lancet.* 2001; 358:1627–1637.

Fukuzawa, A., Lange, S., Holt, M., Vihola, A., Carmignac, V., Ferreiro, A., Udd, B. and Gautel, M. Interactions with titin and myomesin target obscurin and obscurin-like 1 to the M-band: implications for hereditary myopathies. *J. Cell Sci.* 2008; 121, 1841-1851.

Fyrberg C, Ketchum A, Ball E, Fyrberg E. Characterization of lethal Drosophila melanogaster alpha-actinin mutants. *Biochem.Genet.*1998; 36:299–310.

Gottlieb, P. D., Pierce, S. A., Sims, R. J., Yamagishi, H., Weihe, E. K., Harriss, J. V., Maika, S. D., Kuziel, W. A., King, H. L., Olson, E. N. et al. Bop encodes a muscle-restricted protein containing MYND and SET domains and is essential for cardiac differentiation and morphogenesis. *Nat. Genet.* 2002; 31, 25-32.

Granzier, H, M Kellermayer, K Trombitas. Titin elasticity and mechanism of passive force development in rat cardiac myocytes probed by thin-filament extraction. *Biophys J* 1997 73:2043–2053.

Hamamoto, R., Furukawa, Y., Morita, M., Iimura, Y., Silva, F.P., Li,M., Yagyu, R., and Nakamura, Y. SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. *Nat.Cell Biol.* 2003; 6, 731–740.

Hawkins TA, Haramis AP, Etard C, Prodromou C, Vaughan CK, Ashworth R, et al. The ATPase-dependent chaperoning activity of Hsp90a regulates thick filament formation and integration during skeletal muscle myofibrillogenesis. *Development.* 2008; 135:1147-1156.

Holtzer H, Hijikata T, Lin ZX, Zhang ZQ, Holtzer S, Protasi F, Franzini-A rmstrong C and Sweeney HL. Independent assembly of 1.6 micron long bipolar MHC filaments and I-Z-I bodies. *Cell Struc Funct.* 1997; 22: 83–93.

Hornemann T, Kempa S, Himmel M, Hayess K, Furst DO, Walliman T. Muscle-type creatine kinase interacts with central domains of the M-band proteins myomesin and M-protein. *J Mol Biol.* 2003; 332:877-887.

Huang J, Perez-Burgos L, Placek BJ, Sengupta R, Richter M, Dorsey JA, Kubicek S, Opravil S, Jenuwein T, Berger SL. Repression of p53 activity by Smyd2-mediated methylation. *Nature*. 2006; 444:629–632.

Iwabata H, Yoshida M, Komatsu Y. Proteomic analysis of organ-specific post-translational lysine-acetylation and -methylation in mice by use of anti-acetyllysine and -methyllysine mouse monoclonal antibodies. *Proteomics.* 2005; 5:4653–4664.

Jiang Y., Sirinupong N., Brunzelle J., Yang Z. Crystal Structures of Histone and p53 Methyltransferase SmyD2 Reveal a Conformational Flexibility of the Auto inhibitory C-Terminal Domain. *PLoS One* 2011; 6, e21640.

Just S, Meder B, BergerIM, Etard C, Trano N,et al. The myosin-interacting protein SMYD1 is essential for sarcomere organization. *J Cell Sci.* 2011; 124 :3127–3136.

Kamisago M, Sharma SD, DePalma SR, Solomon S, Sharma P, McDonough B, Smoot L, Mullen MP, Woolf PK, Wigle ED, Seidman JG, Seidman CE. Mutations in sarcomere protein genes as a cause of dilated cardiomyopathy. *N Engl J Med.* 2000; 343:1688–1696.

Kiphuth IC, Neuen-Jacob E, Struffert T, Wehner M, Wallefeld W, Laing N, Schröder R. Myosin storage myopathy: a rare subtype of protein aggregate myopathies. *Fortschr Neurol Psychiatr.* 2010; 78(4):219-22.

Kim H, et al. Requirement of histone methyltransferase SMYD3 for estrogen receptor-mediated transcription. *J. Biol. Chem.* 2009; 284:19867–19877.

Knappeis, G.G.and Carlsen,F. The ultrastructure of the M line in skeletal muscle. *J. Cell Biol.* 1968; 38,202–211.

Knöll R, Buyandelger B, Lab M. The sarcomeric z-disc and z-discopathies. *J Biomed Biotechnol* 2011; 569628. doi:10.1155/2011/569628.

Kontrogianni-Konstantopoulos A, Ackermann MA, Bowman AL, Yap SV, Bloch RJ. Muscle giants: molecular scaffolds in sarcomerogenesis. *Physiol Rev.* 2009; 89:1217–1267.

Kundrat L, Regan L: Identification of residues on Hsp70 and Hsp90 ubiquitinated by the cochaperone CHIP. *J Mol Biol* 2010; 395(3):587-594.

Kumar, A., Crawford, K., Close, L., Madison, M., Lorenz, J., Doetschman, T., Pawlowski, S., Duffy, J., Neumann, J., Robbins, J., Rescue of cardiac α-actin-

deficient mice by enteric smooth muscle γ-actin. *Proc. Natl. Acad. Sci.* 1997; 94:4406–4411.

Laing, N.G., and K.J. Nowak. When contractile proteins go bad: the sarcomere and skeletal muscle disease. *Bioessays.* 2005; 27:809–822.

Lange, S., M. Himmel, D. Auerbach, I. Agarkova, K. Hayess, D.O. Fürst, J.C. Perriard, and E. Ehler. Dimerisation of myomesin: implications for the structure of the sarcomeric M-band. *J. Mol. Biol.* 2005; 345:289–298.

Landsverk, M.L., S. Li, A.H. Hutagalung, A. Najafov, T. Hoppe, J.M. Barral, and H.F. Epstein. The UNC-45 chaperone mediates sarcomere assembly through myosin degradation in Caenorhabditis elegans. *J. Cell Biol.* 2007; 177:205–210.

Leger J, Kempf M, Lee G, Brandt R. Conversion of serine to aspartate imitates phosphorylation-induced changes in the structure and function of microtubule-associated protein tau. *J Biol Chem.* 1997; 272: 8441–8446.

Liu Y, Chen W, Gaudet J, Cheney MD, Roudaia L, et al. Structural basis for recognition of SMRT/N-CoR by the MYND domain and its contribution to AML1/ETO's activity. *Cancer Cell.* 2007; 11:483–497.

Liu L, Srikakulam R, Winkelmann DA. Unc45 activates Hsp90-dependent folding of the myosin motor domain. *J Biol Chem.* 2008; 283:13185-13193.

Li D, Niu Z, Yu W, Qian Y, Wang Q, et al. SMYD1, the myogenic activator, is a direct target of serum response factor and myogenin. *Nucleic Acids Res.* 2009; 37: 7059–7071.

Li HQ, Xu J. Bian YH, Rotllant P, Shen T, Chu WY, Zhang JS, Schneider M, Du SJ. Smyd1b_tv1, a key regulator of sarcomere assembly, is localized on the M-line of skeletal muscle fibers. *Plos one.* 2011; 6(12): e28524. doi:10.1371/journal.pone. 0028524.

Lu MH, DiLullo C, Schulthei ss T, Holtzer S, Murray JM, Choi J, Fischman DA and Holtzer H. The vinculin/sarcomeric alpha-actinin/alpha-actin nexus in cultured cardiac myocytes. *J Cell Biol.* 1992; 117: 1007–10 22.

Luther PK. The vertebrate muscle z-disc: sarcomere anchor for structure and signalling. *J Muscle Res Cell Motil.* 2009; 30:171–185.

Matthew J. Potthoff, Michael A. Arnold, John McAnally, James A. Richardson, Rhonda Bassel-Duby, and Eric N. Olson. Regulation of Skeletal Muscle Sarcomere Integrity and Postnatal Muscle Function by Mef2c knockout. *Molecular and Cellular Biology,* 2007; Dec. 8143–8151.

Matsson H., Eason J., Bookwalter C.S., Klar J., Gustavsson P., Sunnegardh J., Enell H., Jonzon A., Vikkula M., Gutierrez I., et al. Alpha-cardiac actin mutations produce atrial septal defects. *Hum. Mol. Genet.* 2008;17:256-265.

McKinsey, T.A., Zhang,C.L. and Olson,E.N. Signaling chromatin to make muscle. *Curr. Opin. Cell Biol.* 2002; 14, 763–772.

Moulder GL, Cremona GH, Duerr J, Stirman JN, Fields SD, Martin W, Qadota H, Benian GM, Lu H, Barstead RJ. α-Actinin is required for the proper assembly of Z-disk/focal-adhesion-like structures and for efficient locomotion in Caenorhabditis elegans. *J Mol Biol*. 2010; 403:516–528.

Mogensen J, Klausen IC, Pedersen AK, Egeblad H, Bross P, Kruse TA, et al. Alpha-cardiac actin is a novel disease gene in familial hypertrophic cardiomyopathy. *J.Clin.Invest.* 1999; 103:R39–R43.

Niimura H, Patton KK, McKenna WJ, Maron BJ, Seidman JG, Seidman CE. Sarcomere protein gene mutations in hypertrophic cardiomyopathy of the elderly. *Circulation.* 2002; 105:446-451.

Ni W, Hutagalung AH, Li S, and Epstein HF. The myosin-binding UCS domain but not the Hsp90-binding TPR domain of the UNC-45 chaperone is essential for function in Caenorhabditis elegans. *Journal of Cell Science.* 2011; 3164–3173.

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

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

Park, C.Y., Pierce, S.A., von Drehle, M., Ivey, K.N., Morgan, J.A., Blau, H.M., and Srivastava, D. skNAC, a Smyd1-interacting transcription factor, is involved in cardiac development and skeletal muscle growth and regeneration. *Proc. Natl. Acad. Sci. U. S. A.* 2010; 107, 20750-20755.

Phan D, Rasmussen TL, Nakagawa O, McAnally J, Gottlieb PD, Tucker PW, Richardson JA, Bassel-Duby R, Olson EN. BOP, a regulator of right ventricular heart development, is a direct transcriptional target of MEF2C in the developing heart. *Development.* 2005; 132: 2669–2678.

Pinotsis, N., Lange, S., Perriard, J.C., Svergun, D., and Wilmanns, M. Molecular basis ofthe C-terminal tail-to-tail assembly of the sarcomeric filament protein myomesin. *The EMBO Journal* 2008; 27, 253–264.

Pinotsis N, Chatziefthimiou SD,Berkemeier F,Beuron F, Mavridis IM. Superhelical architecture of the myosin filament-linking protein myomesin with unusual elastic properties. PLoS Biol 2012; 10: 31001261. doi:10.1371/journal.pbio.1001261.

Pyle WG, Solaro RJ. At the crossroads of myocardial signaling: the role of Z-discs in intracellular signaling and cardiac function. *Circ Res.* 2004; 94:296–305.

Rhee D, Sanger JM. and Sanger JW. The premyofibril:evidence for its role in myofibrillogenesis. *Cell Motil Cytoskeleton* 1994; 28: 1–24.

Ruegg, C., C. Veigel, J. E. Molloy, S. Schmitz, J. C. Sparrow, and R. H. Fink. Molecular motors: force and movement generated by single myosin II molecules. *News Physiol. Sci.* 2002; 17:213-218.

Saad JS, Kim A, Ghanam RH, Dalton AK, Vogt VM, et al. Mutations that mimic phosphorylation of the HIV-1 matrix protein do not perturb the myristyl switch. *Protein Sci.* 2007; 16: 1793–1797.

Sanger, J.W.and Sanger,J.M. Fishing out proteins that bind to titin. *J. Cell Biol.* 2001; 154,21–24.

Sanger, J. W., Kang, S., Siebrands, C. C., Freeman, N., Du, A., Wang, J., Stout, A. L. and Sanger, J. M. How to build a myofibril. *J. Muscle Res. Cell Motil.* 2005; 26, 343-354.

Sanger J.W., Wang J., Fan Y., White J., and Sanger J.M. 2010. Assembly and Dynamics of Myofibrils. *Journal of Biomedicine and Biotechnology.* 2010; 858606,8 doi:10.1155/2010/858606.

Schoenauer, R., P. Bertoncini, G. Machaidze, U. Aebi, J.-C. Perriard, M. Hegner, and I. Agarkova. Myomesin is a molecular spring with adaptable elasticity. *J. Mol. Biol.* 2005; 349:367–379.

Schoenauer, R., Lange, S., Hirschy, A., Ehler, E., Perriard, J. C. and Agarkova, I. Myomesin 3, a novel structural component of the M-band in striated muscle. *J. Mol. Biol.* 2007; 376, 338-351.

Schoenauer R, Emmert MY, Felley A, Ehler E, Brokopp C, Weber B, Nemir M, Faggian GG, Pedrazzini T, Falk V, Hoerstrup SP, Agarkova I. EH-myomesin splice isoform is a novel marker for dilated cardiomyopathy. *Basic Res Cardiol.* 2011;106:233–247.

Sellers JR, Goodson HV. Motor proteins 2: myosin. *Protein Profile.* 1995; 2:1323–423.

Sims RJ 3rd, Nishioka K, Reinberg D: Histone lysine methylation: a signature for chromatin function. *Trends Genet.* 2003; 19: 629-639.

Sims RJ, Weihe EK, Zhu L, O'Malley S, Harriss JV, Gottlieb PD: m-Bop, a repressor protein essential for cardiogenesis, interacts with skNAC, a heart- and muscle-specific transcription factor. *J Biol Chem.* 2002; 277:26524-26529.

Siegert R, Perrot A, Keller S, Behlke J, Michalewska-Włudarczyk A, Wycisk A, Tendera M, Morano I, Özcelik C. A myomesin mutation associated with hypertrophic cardiomyopathy deteriorates dimerisation properties. *Biochemical and Biophysical Research Communications.* 2011; 405, 473–479.

Sirinupong N, Brunzelle J, Ye J, Pirzada A, Nico L, Yang Z. Crystal structure of cardiac-specific histone methyltransferase SmyD1 reveals unusual active site architecture. *J Biol Chem.* 2010; 285:40635–44.

Srikakulam R, Liu L, Winkelmann DA. Unc45b forms a cytosolic complex with Hsp90 and targets the unfolded myosin motor domain. *PLoS ONE.* 2008; 3:e2137.

Sirinupong N, Brunzelle J, Doko E, Yang Z. Structural insights into the autoinhibition and posttranslational activation of histone methyltransferase SmyD3. *J Mol Biol.* 2011; 406: 149–159.

Sparrow JC, Nowak KJ, Durling HJ, Beggs AH, Wallgren-Pettersson C, Romero N, Nonaka I, Laing NG. Muscle disease caused by mutations in the skeletal muscle alpha-actin gene (ACTA1). *Neuromuscul Disord.* 2003; 13:519–31.

Sparrow JC, Schock F. The initial steps of myofibril assembly: integrins pave the way. *Nat Rev Mol Cell Biol.* 2009; 10:293-8.

Tan X, RotllantJ, Li H, De Deyne P, Du SJ. SmyD1, a histone methyltransferase, is required for myofibril organization and muscle contraction in zebrafish embryos. *Proc Natl Acad Sci.* 2006; 103:2713–2718.

Tong SW, Elzinga M. The sequence of the NH_2-terminal 204-residue fragment of the heavy chain of rabbit skeletal muscle myosin. *J Biol Chem.* 1983; 258: 13100–13110.

Tskhovrebova, L.and Trinick,J. Titin: properties and family relationships. *Nat. Rev. Mol. Cell Biol.* 2003; 4,679–689.

Tskhovrebova L, Trinick J. Making Muscle Elastic: The Structural Basis of Myomesin Stretching. *PLoS Biol.* 2012; 10(2): e1001264. doi:10.1371/journal.pbio.1001264.

Urasaki A, Morvan G, Kawakami K. Functional dissection of the Tol2 transposable element identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. *Genetics.* 2006; 174: 639–649.

Vandekerckhove J, Weber K. The Complete Amino Acid Sequence of Actins from Bovine Aorta, Bovine Heart, Bovine Fast Skeletal Muscle, and Rabbit, Slow Skeletal Muscle. *Differentiation.* 1979; 14:123–133.

Wang, S. Lo, M., Shang, C., Kao, S., and Tseng, Y. Role of M-Line Proteins in Sarcomeric Titin Assembly During Cardiac Myofibrillogenesis. *J. Cell. Biochem.* 1998; 71 , 82– 95.

Winder, SJ, Lipscomb L, Parkin, CA, and Juusola M. The proteasomal inhibitor MG132 prevents muscular dystrophy in zebrafish. *Plos Curr.* 2011; doi: 10.1371/currents.RRN1286.

Wolfgang M.J. Obermann, Mathias Gautel, Klaus Weber and Dieter O. Fürst. Molecular structure of the sarcomeric M band: mapping of titin and myosin binding domains in myomesin and the identification of a potential regulatory phosphorylation site in myomesin *The EMBO Journal.* 1997; 16, 211 - 220.

Wohlgemuth, S.L., Crawford, B.D., Pilgrim, D.B. The myosin co-chaperone UNC-45 is required for skeletal and cardiac muscle function in zebrafish. *Dev. Biol.*2007; 15, 483 – 492.

Xie P, Tian C, An L, Nie J, Lu K, Xing G, *et al.*: Histone methyltransferase protein SETD2 interacts with p53 and selectively regulates its downstream genes. *Cell Signal.* 2008; 20(9):1671-8.

Xu S, Zhong C, Zhang T, Ding J. Structure of human lysine methyltransferase Smyd2 reveals insights into the substrate divergence in Smyd proteins. *J Mol Cell Biol.* 2011; 3:293-300.

Xu J,Gao J, Li JL. Xue LY, Ekker S.C., and Du Sj. Functional analysis of myosin-1 and myomesin-3 in sarcomere organization in zebrafish embryonic slow muscles. *J Genetics & Genomics.* 2012; doi:10.1016/j.jgg.2012.01.005.

Yang,Y .G., Obinata, T., and Shimada, Y.. Developmental relationship of myosin binding proteins (myomesin, connectin and C-protein) to myosin in chicken somites as studied by immunofluorescence microscopy. *Cell Struct. Funct.* 2000; 25,177–1 85.

Yang, J., and Everett, A. D. Hepatoma-derived growth factor represses SET and MYND domain containing 1 gene expression through interaction with C-terminal binding protein. *BMC Mol. Biol.* 2007; 8, 101.

Yang XD, Lamb A. and Chen LF. Methylation, a new epigenetic mark for protein stability. *Epigenetics.* Oct 2009; 4:7, 1-5.

Zhang X, Wen H, Shi X. Lysine methylation: beyond histones. *Acta Biochim Biophy Sin.* 2012, 44(1):14-27